GENOME EXPRESSION DURING GERMINATION AND EARLY DEVELOPMENT OF CUCUMBER (*CUCUMIS SATIVUS*)

by

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I declare that this thesis was composed by myself and that the work presented herein, unless otherwise stated, is my own.

January, 1980.

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Abbreviations and Definitions

A	-	Adenosine base
A_{260}	-	Absorbance log (Io/I) at 260 nm wavelnegth
AMPS	-	Ammonium persulphate
ATP	-	Adenosine-5'-triphosphate
av	-	average
bis-acrylamide	-	N,N' methylene bisacrylamide
ME	-	Beta mercapto ethanol
BSA	-	Bovine serum albumin
BPD	-	2-(4-tert-butylphenol)-5-(4 biphenyl)-1,3,4, oxadiazone
^{o}C	-	degrees centrigrade
CAT	-	Catalase (EC 1.11.1.6)
cDNA	-	Complementary DNA
Ci	-	Curie(s)
cm	-	centimeter(s)
CoA	-	Coenzyme A
c.p.m.	-	counts per minute
CTAB	-	Cetyltrimethylammonium bromide
DMOS	-	Dimethylsulphoxide (also Me_2SO)
DNA	-	Deoxyribonucleic acid
dT	-	2'-Deoxyribosylthymine
DTNB	-	5,5'-Dithiobis-(2-Nitrobenzoic Acid)
DTT	-	Dithiothreitol
EDTA	-	Ethylenediamine tetra - acetic acid
EGTA	-	Ethylene glycol bis (aminoethylether) tetra - acetic acid
fg	-	femto gram(s)
g	-	unit of gravitational force
g	-	gram(s)
GR	-	Glycolate reductase (EC 1.1.1.26)
GTP	-	Guanosine-5'-triphosphate
^{3}H	-	Tritium (radioactive isotope of hydrogen)
Hepes	-	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
H_2O_2	-	Hydrogen peroxide
8HQ	-	8 Hydroxyquinoline
HSB	-	High salt buffer (as defined p. 102)
ICL	-	Isocitrate lyase (EC 4.1.3.1.)

IEF	-	Isoelectric focusing
IgG	-	immunoglobulin G.
K^+	-	Potassium ion
LMW RNA	-	Low molecular weight RNA - includes 4S tRNA and 4·5S, 5S and 5·8S rRNAs
$\log_{10}$	-	Logarithm (base 10)
LSB	-	Low salt buffer (as defined p. 103)
LSU	-	Large subunit of RuBPC
mA	-	milliamp(s)
max	-	maximum
MDH	-	Malate dehydrogenase (EC 1.1.1.37)
mg	-	milligram(s)
Mg^{2+}	-	Magnesium ion
min	-	minute(s)
ml	-	millilitre(s)
mm	-	millimeter(s)
mM	-	millimolar
mmole	-	millimole(s)
mol. wt.	-	Molecular weight
mRNA	-	Messenger RNA
MS	-	Malate synthase (EC 4.1.3.2)
N	-	Normal (Normality)
NAD	-	Nicotinamide-adenine dinucleotide
$NADH_2$	-	Nicotinamide-adenine dinucleotide, reduced form
NaOCl	-	Sodium hypochlorite
ng	-	nanogram(s)
nm	-	nanometer(s)
no.	-	number
NP 40	-	Nonidet P40
OD	-	Optical density
^{32}p	-	radioactive isotope of phosphorous
/	-	per
%	-	per cent
PAS	-	4 amino-2-hydroxybenzoic acid (sodium salt)
pg	-	picogram(s)
pH	-	log hydrogen ion concentration
poly(A)	-	homopolyper of Adenosine
poly(A)$^+$ RNA	-	RNA containing sufficient poly(A) to be extractable by oligo dT-cellulose

poly(A)$^-$ RNA	–	RNA lacking sufficient poly(A) to be extractable by oligo dT-cellulose
PPO	–	2,5-Diphenyl oxazole
RNA	–	Ribonucleic acid
RNAase	–	Ribonuclease
rRNA	–	Ribosomal RNA
RuBPC	–	Ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39)
s	–	seconds(s)
S	–	Svedberg unit
^{35}S	–	radioactive isotope of sulphur
S.30	–	supnatant of a 30,000 g_{max} centrifugation
S.100	–	supernatant of a 105,400 g_{av} centrifugation
SAR antiserum	–	Sheep anti-rabbit antiserum
S. aureus	–	Staphylococcus aureus
SDS	–	Sodium dodecyl sulphate (also SLS)
'soluble' protein	–	protein not sedimented by a 10,000 g_{av} centrifugation
SSC	–	Saline sodium citrate
SSU	–	Small subunit of RuBPC
t	–	time
T	–	Thymidine base
TCA	–	Trichloracetic acid
TEMED	–	N,N,N',N' – tetramethylethylenediamine
TMV	–	Tobaco mosaic virus
TNS	–	Tri-iso-propylnaphthalene sulphonic acid (sodium salt)
TNT buffer	–	0·1 M tris-HCl pH 8·6; 0·5 M NaCl; 1% (w/v) Triton X-100
tris	–	Tris (hydroxymethyl) amino methane
Triton X-100	–	Octylphenoxypolethoxyethanol
tRNA	–	Transfer RNA
µCi	–	microcurie(s)
µg	–	microgram(s)
µl	–	microlitre(s)
µM	–	micromolar
µm	–	micrometer(s)
var	–	variety
v/v	–	volume per volume (as percent)
w/v	–	weight per volume (as percent)

ABSTRACT

Developmentally related changes in gene expression within the cotyledons of cucumber were studied during germination and early seedling development, in both light and dark-grown plants.

Profiles of heterotrophic and autotrophic proteins were correlated with changes in cotyledonary protein and RNA. The activity of the glyoxylate cycle enzymes, isocitrate lyase (ICL) and malate synthase (MS), increased markedly, from near zero in imbibed tissue, to peak levels during the period of maximum lipid mobilisation (day 4) and subsequently declined, though at a slower rate in the dark. A number of polypeptides with similar developmental profiles were identified by SDS gel electrophoresis of cotyledonary protein. The de novo synthesis of the autotrophic proteins, ribulose-1,5-bisphosphate carboxylase and glyoxylate reductase, at days 3-4 was found to be independent of light, while their continued accumulation was light stimulated. Other autotrophic indicators such as chlorophyll and certain polypeptides observed by SDS gel electrophoresis of cotyledonary protein were totally light dependent for their initiation. Utilisation of storage proteins was also observed from the electrophoretic profiles.

Cytoplasmic rRNA and low molecular weight RNA were found to increase markedly (10-fold and 8-fold respectively), particularly during the period of maximum glyoxylate cycle enzyme activities. Chloroplast rRNA was initially undetectable, synthesis being initiated at day 3. Light was found to affect the continued accumulation but not the initial synthesis and increase in these RNAs.

Messenger RNA was estimated by poly(A)$^+$ RNA extraction and

[^{3}H]-poly U hybridisation. Low levels of mRNA were present in the unimbibed and imbibed tissue. Marked increases subsequently occurred in both light and dark-grown tissue, to peak levels at days 3-4, followed by a decline as development proceeds. Light was found to stimulate accumulation of mRNA.

An optimised wheat-germ cell-free translation system was used to assay quantitative and qualitative changes in the translatable mRNA population. Tentative identification of glyoxysomal proteins among the in vitro translation products was carried out by immuno-precipitation techniques.

The developmental quantitative changes in translatable mRNA shown by in vitro assay were similar to those found by poly(A) estimation. There were marked changes in the spectrum of mRNAs associated with the increase and change in metabolic activity during this developmental sequence. It was found that the mRNAs for ICL and MS are present in the unimbibed and imbibed seed tissue, albeit at reduced levels. These mRNAs may be involved in the onset of de novo synthesis of these glyoxysomal enzymes. However, subsequent marked increases in the enzyme activities were associated with increases in translatable mRNA. After the normal heterotrophic growth phase there was a decline in these mRNAs and enzymes which was accelerated in light-grown tissue.

CHAPTER 1

INTRODUCTION

SECTION 1

GERMINATION AND EARLY SEEDLING DEVELOPMENT

Germination may be defined as the process of growth resulting from the breaking of dormancy of a seed and the resumption of growth and development. It occurs in response to favourable environmental conditions and brings the life cycle of the plant back into the growth phase from a period of quiescence or dormancy. By classical definition, germination is considered to start upon imbibition of the dry seed, ends with the protrusion of the radicle from the seed coat and involves all processes occurring in the intervening period (Köller *et al.*, 1962; Mayer and Shain, 1974).

Seed germination involves the resumption of active growth and metabolism from the quiescent state of the dormant tissue. In this study germination and early seedling development are taken to represent the period of dramatic developmental changes involving resumption of growth, differentiation and morphogenesis as the life cycle traverses from the dormant state to the heterotrophic growth phase and finally reaches the autotrophic growth phase.

This developmental period is characterised by a series of well-defined morphological and physiological changes. It is possible to obtain large amounts of material for biochemical studies since this growth sequence can be initiated relatively easily in response to appropriate environmental conditions. Thus, it is considered a suitable system for a study of the pattern for initiation of growth and development and also regulation of the underlying molecular events, particularly of gene expression (Dure, 1975).

One of the most striking features of this developmental

transition is the sudden, marked increase in activities of a wide variety of enzymes associated with the resumption of metabolism. Particularly notable are the enzymes involved in the utilisation and mobilisation of food reserves associated with the heterotrophic growth phase. Thereafter, changes of particular interest are those associated with the development of photosynthetic capacity of the seedling when the cotyledons or leaves emerge above ground and the chloroplasts develop.

Utilisation and mobilisation of storage reserves within the seed are essential for the rapid increase in growth of the apices observed during germination and heterotrophic growth. Thereafter the seedling depends upon its photosynthetic capacity for supply of materials for growth.

The main storage reserves consist of proteins, carbohydrates and lipids and are generally found in the endosperm of monocots and the cotyledons of dicots, though other organs may be involved. Although all three types of storage reserves occur within most seeds, the relative proportions of each varies considerably between species, e.g. legumes store predominantly proteins; wheat, barley, maize etc. predominantly carbohydrates; cucumber, sunflower, water melon, peanut etc. predominantly lipids.

In recent years considerable research effort has been devoted to the study of the synthesis and deposition of seed storage proteins (Millerd, 1975; Dure, 1975) and also to their mobilisation during germination and seedling growth (Ashton, 1976). Another area of interest has been the mobilisation of carbohydrate reserves, particularly the induction of α-amylase activity by

gibberellic acid (Varner, 1964; Mayer and Shain, 1975; Higgins et al., 1976).

Utilisation of these storage reserves requires the synthesis of hydrolytic enzymes which convert the stored polymers to suitable soluble compounds which can subsequently be transported and reutilised for growth of the developing seedling. In the case of storage reserves in the form of carbohydrates and proteins, hydrolysis results in the formation of sugars and amino acids respectively, which can then be mobilised and reutilised.

In seeds where lipid is the main storage reserve, stored protein is also often present but very little stored carbohydrate. In these cases the stored lipid is converted to carbohydrate prior to transport to the growing apices.

The ability to convert carbohydrate to fat is a common property of most organisms. This stored fat is then available as a source of energy which can be mobilised by β-oxidation and the tricarboxylic acid (TCA) cycle. In most plants and animals this degradation occurs within the tissue in which the fat is stored, for the purpose of energy production.

Plants which store lipids in the endosperm or cotyledons face a unique problem upon germination, since the lipid must first be coverted to sugars before it can be transported from the storage organs to the embryo. In most organisms the net conversion of fat to sugars is not possible because the oxidative decarboxylation of pyruvate to acetyl-CoA is essentially an irreversible step.

It is at this stage that micro-organisms which can grow on two-carbon compounds, and thus must effect this conversion (acetate ⟶ sugars) routinely, and fat storing seeds make use of the

glyoxylate cycle. This cycle enables the organism to effect net gluconeogenesis and thus by-pass a normally irreversible biochemical pathway (Beevers, 1961, 1969).

SECTION 2

THE GLYOXYLATE CYCLE

2.1. Function

The glyoxylate cycle, or by-pass, allows the organism in which it occurs to convert two-carbon acetyl-CoA units, the products of β-oxidation of fatty acids, into C_4-compounds (Kornberg and Beevers, 1957A, 1957B; Kornberg and Krebs), 1957) thus supplying precursors for cellular biosynthesis. It allows a by-pass of the two decarboxylation stages of the Krebs cycles and thus results in a net synthesis of the necessary intermediates for translocation of organic carbon from the storage tissue to the growing apices of the germinating embryo.

The discovery of this biochemical pathway followed reports that Pseudomonas KB and Pseudomonas aeroginosa could grow on acetate as a sole carbon source (Campbell et al., 1953; Olsen, 1954; Kornberg and Madson, 1957; Kornberg, 1957) and that labelled sucrose could be obtained from castor beans which had been fed radioactive acetate (Beevers, 1957). These observations could be explained if a by-pass of

FIGURE I The Glyoxylate Cycle

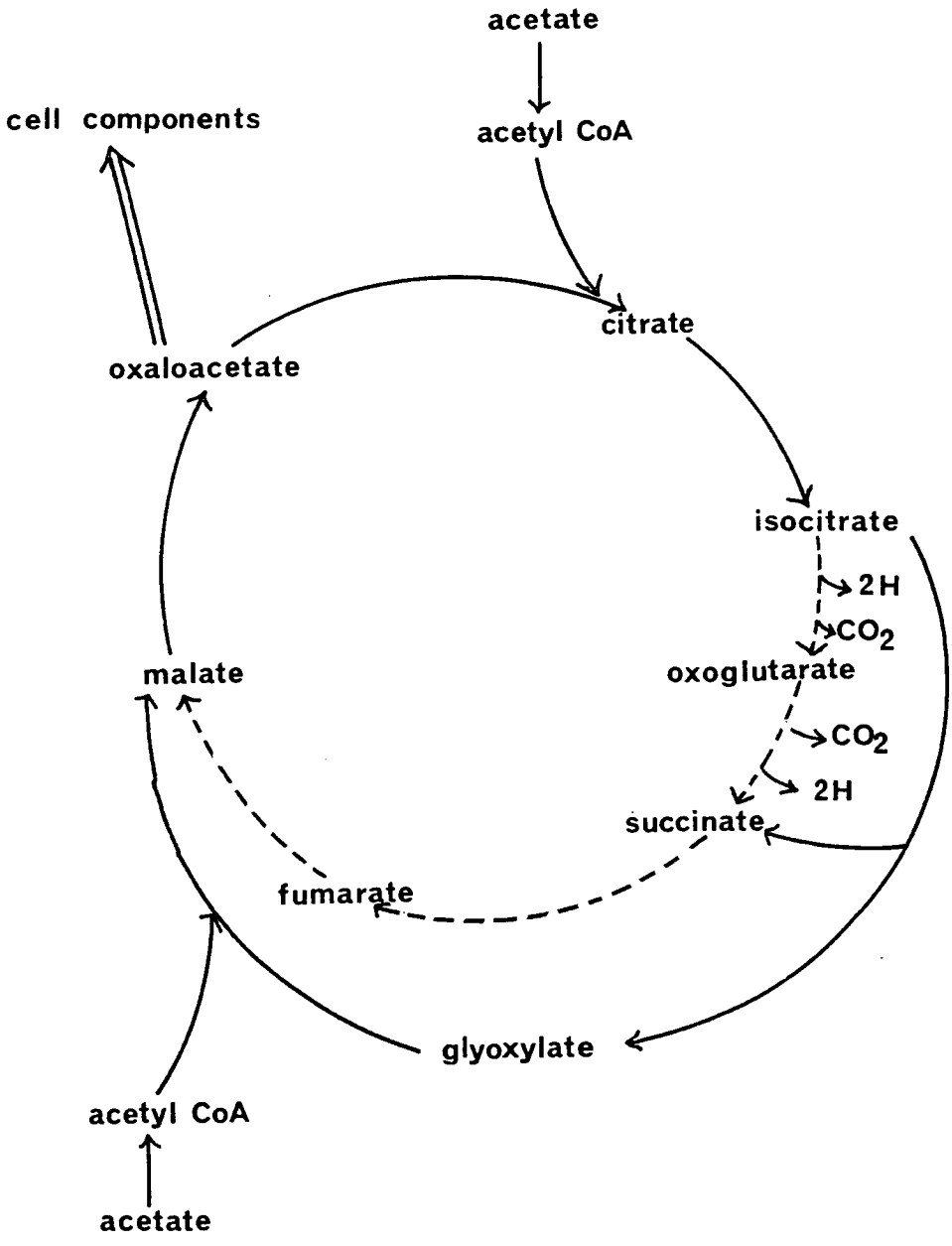
A. The relationship of the glyoxylate cycle (⟶) and the Krebs cycle (- - ->).

B. Reaction catalysed by isocitrate lyase (ICL).

C. Reaction catalysed by malate synthase (MS).

FIGURE I

A.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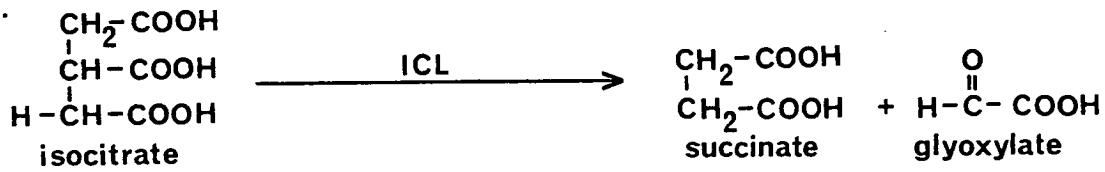
acetyl CoA
cell components
citrate
oxaloacetate
isocitrate
2H
CO_2
oxoglutarate
malate
CO_2
2H
succinate
fumarate
glyoxylate
acetyl CoA
acetate

B.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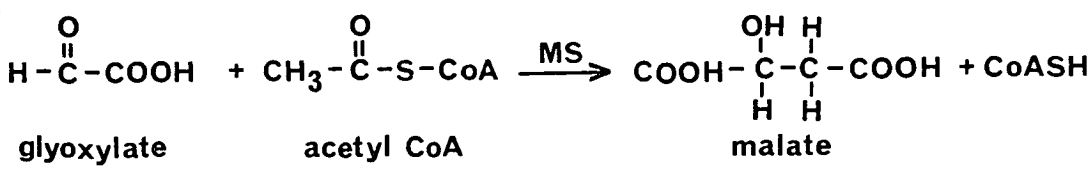
CH-COOH
H-CH-COOH
isocitrate
ICL
CH_2-COOH
CH_2-COOH
succinate
+
O
H-C-COOH
glyoxylate

C.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H-C-COOH
glyoxylate
+
O
CH_3-C-S-CoA
acetyl CoA
MS
OH H
COOH-C-C-COOH
H H
malate
+ CoASH

of this nature was in operation so that the acetate was not utilised as 'normal' in the Krebs cycle for energy production. The glyoxylate cycle has since been shown to be present in all organisms which utilise lipid as a source of material for growth (Hock and Beevers, 1966; Tolbert, 1971; Kornberg and Madson, 1958; Longo and Longo, 1970A; Cooper and Beevers, 1969A; Trelease et al., 1971A; Scragg et al., 1975; Woodcock and Merret, 1978).

A study of the relationship of the glyoxylate cycle and the Krebs cycle (Figure I) shows that most of the biochemical stages of the two systems utilise the same enzymes. However, there are two steps in the glyoxylate cycle which are unique to this cycle. These are firstly, the conversion of isocitrate to succinate and glyoxylate and secondly, the conversion of glyoxylate and acetyl-CoA into malate. These two steps are catalysed by two enzymes which are unique to this biochemical pathway - isocitrate lyase (ICL) and malate synthase (MS) respectively. Without these enzymes net gluconeogenesis could not be effected (see Figure I).

2.2. Temporal Activity and Subcellular Location of the Glyoxylate Cycle

Carpenter and Beevers (1958, 1959) studied the distribution of ICL within various plant tissues. They showed that ICL was confined to those tissues in which lipid was stored and utilised for glyconeogenesis. It has since been confirmed that the functional glyoxylate cycle is confined both spatially and temporally - spatially in that it is only present in those tissues in which lipid is stored and converted to carbohydrate, and temporally in that it is only

present during gluconeogenesis and not at any other time (Kornberg, 1966; Trelease et al., 1971A; Tolbert, 1971; Longo, 1968; Becker et al., 1978; Hock and Beevers, 1966). Most organisms do not contain this biochemical pathway and thus utilise lipid for energy production rather than carbohydrate synthesis.

Using castor bean endosperm, Breidenbach et al. (1967, 1968) showed that the glyoxylate cycle enzymes, ICL and MS, were localised within a subcellular organelle which Beevers (1969) termed the glyoxysome. Since then, various other enzymes have been shown to be present in glyoxysomes including those for β-oxidation (Cooper and Beevers, 1969A, 1969B; Cooper, 1971). Since their discovery in the endosperm of castor bean, glyoxysomes have also been shown to be present in fatty cotyledons of water melon (McGregor and Beevers, 1969; Kagawa et al., 1973B), peanut (Longo and Longo, 1970A), sunflower (Gruber et al., 1970; Schnarrenberger et al., 1971; Gerhardt, 1973) and cucumber (Trelease et al., 1971A & B; Köller and Kindl, 1977; Becker et al., 1978).

Glyoxysomes have been shown, by cytochemical detection of key marker enzymes (Vigil, 1969; Burke and Trelease, 1975) and by electron microscopy of isolated organelles (Trelease et al., 1971A; Vigil, 1970), to belong to a class of single membrane bound organelles described morphologically as microbodies (Tolbert, 1971; Beevers, 1979; Mollenhauser et al., 1966; Frederich et al., 1968).

SECTION 3

MICROBODIES

3.1. STRUCTURE AND FUNCTION

Microbodies are widely distributed in plant cells and invariably contain catalase, uricase and glycolate oxidase (Huang and Beevers, 1971; Beevers, 1979). Catalase is thus often used as a cytochemical marker of microbodies (Berger and Gerhardt, 1971; Frederick and Newcomb, 1969; Longo et al., 1972).

All plant microbodies are single membrane bound organelles, usually 0·2–1·5μm in diameter, which have an equilibrium density of approximately 1·25 g/ml in sucrose gradients and usually contain the enzymes previously mentioned. However, three types of plant microbody have so far been recognised depending upon their characteristic enzyme complement.

Glyoxysomes (Figure II) were the first plant microbodies isolated (Breidenbach et al., 1967, 1968; Cooper and Beevers, 1969). These highly specialised microbodies contain the enzymes of the glyoxylate cycle and all the necessary β-oxidation enzymes (Cooper, 1971; Hutton and Stumpf, 1969) in agreement with their major role in the conversion of stored lipid to sucrose in fatty seedling tissues.

A second form of microbody with a different function from the glyoxysomes is the peroxisome (Figure III). These organelles are found in green leaves and other photosynthetic tissue (Frederick and Newcomb, 1969; Tolbert and Yamazaka, 1969; Tolbert, 1971; Beevers, 1979). They contain enzymes

FIGURE II.

Electron Micrograph showing Association of a Glyoxysome with Lipid Bodies

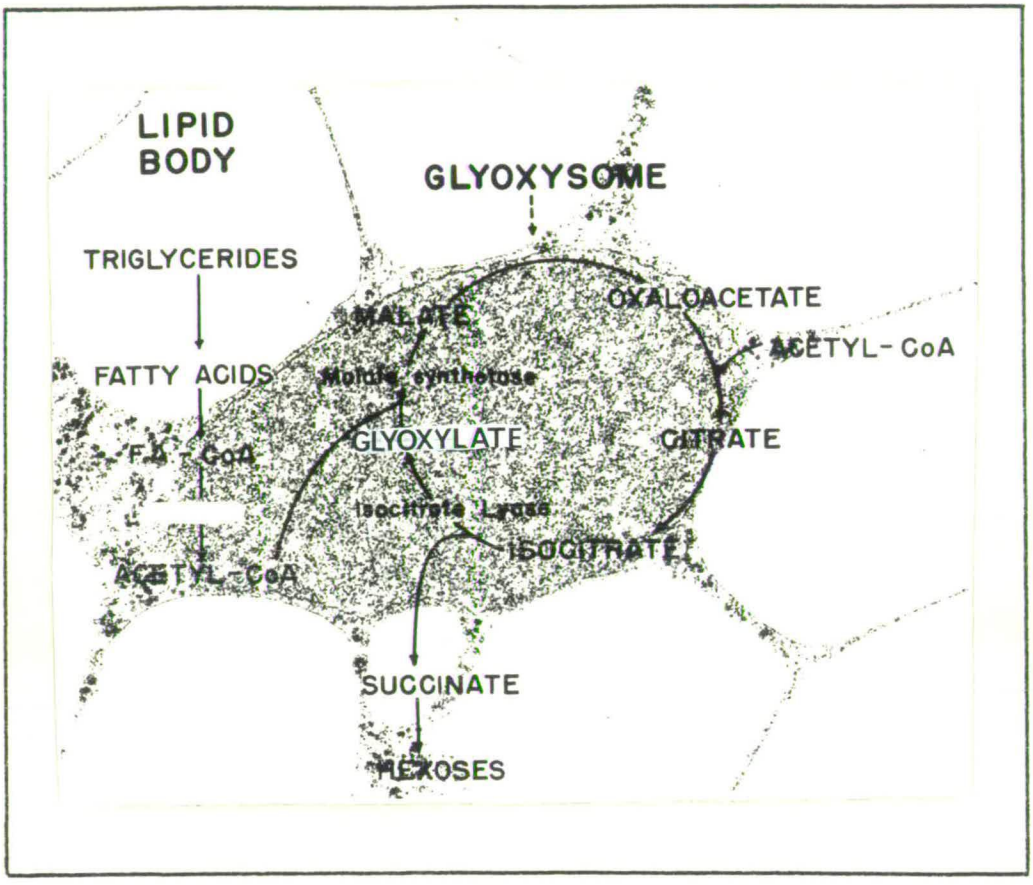
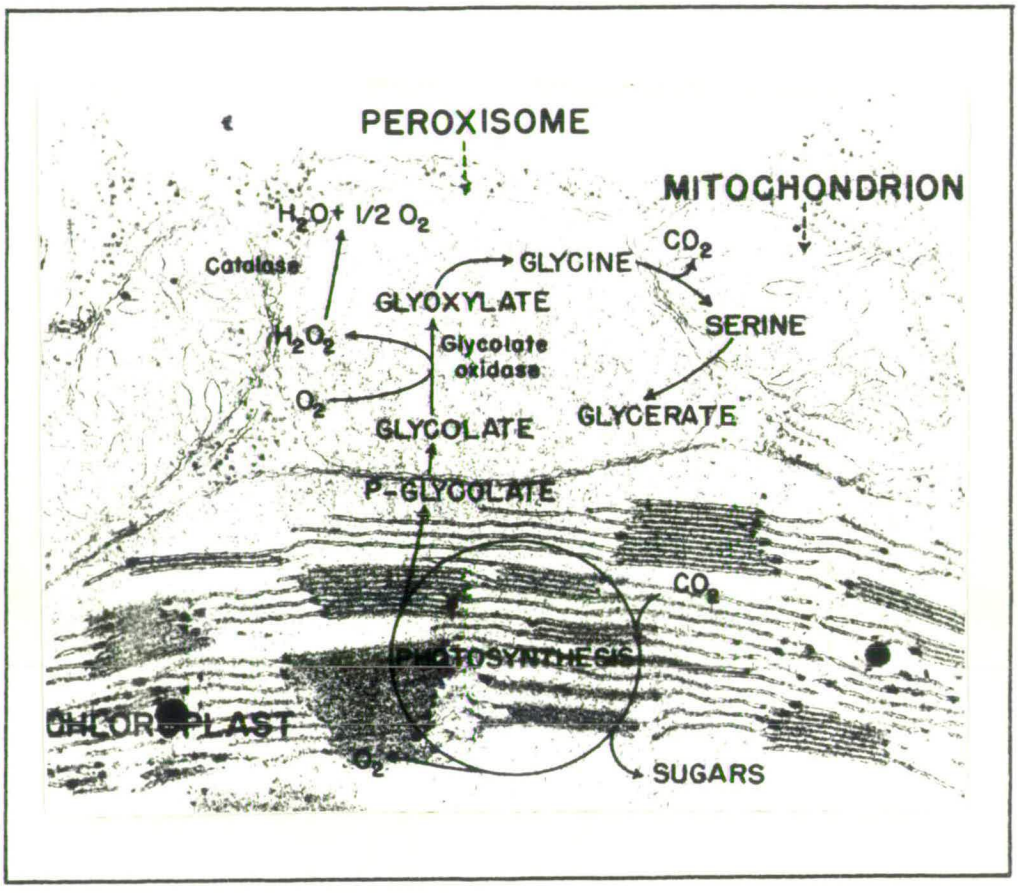


FIGURE III.

Electron Micrograph showing Association of a Peroxisome with a Chloroplast and Mitochondria



which are involved in photorespiration (Tolbert, 1971), particularly in the metabolism of glycolic acid, a product of the oxygenase activity of RuBPC. Functional differentiation of peroxisomes is associated with the aquisition of photosynthetic capacity.

Microbodies which cannot be classified as either glyoxysomes or peroxisomes have also been isolated from various plant tissues (Huang and Beevers, 1971). Apart from a role in H_2O_2 detoxication and purine catabolism the function of this class of microbody is unknown (Beevers, 1979). It should be noted that these three classes of microbody are structurally similar and indistinguishable by ultrastructure, bucyant density and catalase activity measurements.

Thus, during germination and early seedling development of fat storing dicots it would be expected that the cotyledons would initially contain functional glyoxysomes, associated with the heterotrophic growth period of the seedling. When the cotyledonary tissue becomes photosynthetic, microbodies which are metabolically defined as peroxisomes should be present.

Glyoxysomes in vacuolated cells are found to be intimately associated with the lipid bodies and cisternae (Frederick et al., 1968; Gruber et al., 1970; Trelease et al., 1971A; Vigil, 1970) and later show some association with mitochondria. The glyoxysomes are often contorted by pressure from surrounding organelles causing cytoplasmic invaginations to occur (Vigil, 1970; Gruber et al., 1970). Peroxisomes on the other hand are usually closely associated

with chloroplasts (Frederick and Newcomb, 1969). These associations often occur even when there is sufficient space within the cell for a more even distribution of the organelles, and may reflect the functional roles of the two classes of microbody (Tolbert, 1971).

The role of peroxisomes is emphasised by their distribution within the leaf tissues of grasses (C_4 plants). Numerous, large peroxisomes (1 mμ diameter) are present in the bundle sheath cells where the C_3 photosynthetic carbon cycle operates (Björhman and Gauhl, 1967; Hatch and Slack, 1970), while fewer and small peroxisomes (0·5 mμ diameter) are present in the mesophyll cells (Frederick and Newcomb, 1971; Laetsch, 1969A and 1969B). Frederick and Newcomb (1971) also showed that there were some ultrastructural differences between these two groups of peroxisomes.

By electron microscopy or detection of enzymes activities, microbodies have also been shown to be present in many other plant tissues such as tobacco stem and callus tissue (Frederick, 1968), oat coleoptiles (Frederick, 1968; Vigil, 1969; Thornton and Thiemann, 1964), mung bean hypocotyls (Plesnicar *et al.*, 1967), etiolated leaf tissue (Gruber *et al.*, 1972), root tissue (Breidenbach *et al.*, 1968; Mollenhauer *et al.*, 1966; Rocha and Ting, 1971; Frederick and Newcomb, 1971), aleurone and scutella of cereals (Doig *et al.*, 1975; Longo and Longo, 1970B), ripening pears (Miller and Romani, 1966), *Jojoba* bean (Moreau and Huang, 1977), and petals (Huang and Beevers, 1971). *Neurospora* has been shown to contain glyoxysomes

(Kobr et al., 1969) and uncellular green algae and yeast to contain peroxisomal enzymes activities (Szoba and Avers, 1969; Tolbert, 1963).

3.2. Enzyme Localisation with Microbodies

Glyoxysomes and peroxisomes, although containing some metabolic activities in common, have a significantly different enzyme complement related to their specific functions. Table I is a summary of the enzymes present in these two classes of microbody.

Most of the glyoxysomal enzymes have been shown to be readily soluble upon dilution from a sucrose gradient, although some membrane ghosts are not completely disrupted (Huang and Beevers, 1973). Many of the enzymes of the glyoxysomes, including ICL and CAT are soluble, are present in the matrix of the organelle but more than half the activity of MDH (malate dehydrogenase), fatty acyl-CoA dehydrogenase, and crotonase, and 90% of the MS and citrate synthase activities are attached to the organellar membrane. However, it has been shown that these are 'weak' attachments and therefore these enzymes are not apparently intrinsic membrane components since 0·05 M KCl treatment removes the first three from the membrane and 0·2 M KCl solubilised MS and citrate synthase (Brown et al., 1974; Huang and Beevers, 1973; Bieglmayer et al., 1973, 1974; Breidenbach et al., 1974; Wade et al., 1974; Koller and Kindle, 1977). After KCl treatment the remaining membrane proteins do not contain detectable levels of any of the glyoxysomal enzyme activities.

Similar studies of leaf peroxisomes indicated that

Table I

Some Enzymes Present in Plant Microbodies

	Glyoxysomes	Leaf Peroxisomes
Malate synthase	++	-
Isocitrate lyase	++	-
Citrate synthase	++	-
Aconitase	+	-
Fatty acyl-CoA synthetase	+	-
β-oxidation	+	-
Thiolase	++	-
Uricase	+	+
Allantoinase	+	-
Amino acid oxidase	+	-
Glyoxylate reductase	-	++
Glycolate oxidase	+	++
Hydroxypyruvate reductase	+	++
Glutamate-glyoxylate aminotransferase	-	++
Serine glyoxylate aminotransferase	-	++
Glutamate-oxalacetate aminotransferase	+	++
Catalase	++	+++
NAD-malate dehydrogenase	++	++
NADP-isocitrate dehydrogenase	+	+

References: for contents of glyoxysomes - Breidenbach and Beevers, 1967; Breidenbach, Kahn and Beevers, 1968; Beevers, 1969; Breidenbach, 1969; Cooper and Beevers, 1969A & 1969B; Hutton and Stumpf, 1969; St. Angelo and Ory, 1970; Theimer and Beevers, 1971.

for contents of peroxisomes - Bouck, 1965; Kisaki and Tolbert, 1969; Tolbert et al., 1968, 1969, 1970; Tolbert and Yamazaki, 1969; Rocha and Ting, 1970, 1971.

Symbols: +++ ++ = high activities

\+ = low activities

\- = no activities detectable

none of the enzymes were even loosely associated with the membrane (Huang and Beevers, 1973). Thus, in both types of microbody, the enzymes are not present as integral proteins of the organellar membrane and only a few of the glyoxysomal enzymes are loosely bound to the inner face of the limiting membrane. Of the enzymes studied in this thesis ICL and CAT are matrix enzymes while MS is loosely bound to the membrane.

Some of the enzymes present within these two classes of microbody can also be found in other cellular compartments. Malate dehydrogenase and the transaminases are found in both types of microbody and citrate synthase and aconitase are characteristic of glyoxysomes. Isozymes of these enzymes have also been shown to be present in other cellular compartments.

Three isozymes of MDH are characteristically found in the cytosol, mitochondria and microbodies (Breidenbach, 1969; Rocha and Ting, 1971; Longo et al., 1972; Huang et al., 1974; Wainwright and Ting, 1976A & B; Walk and Hock, 1976, 1977; Walk et al., 1977). Glyoxysomal and mitochondrial isozymes have been shown to be present for citrate synthase (Barbareshi et al., 1974) by chromatography and kinetic properties although they are immunologically indistinct (Huang et al., 1974), whereas the three MDH isozymes are distinguishable by immunological assays.

Isocitrate lyase has been shown to be present in some non-fatty tissues without the normal association with malate synthase (Godovari et al., 1973; Osmond and Avadhani,

1968; Hunt et al., 1978) and Hunt et al. (1978) showed that, in cultured rose cells ICL activity was located in the mitochonrial rather than the microbody fraction. It remains to be seen whether this mitochondrial form of ICL is an isozyme of the one normally present in glyoxysomes. However, work by Riezman et al. (1979A & B) and Kahn et al. (1971) has shown that within the glyoxysome itself there are two forms of ICL. These two forms of ICL can be distinguished by DEAE cellulose chromatography and purification on hydroxyabaptite followed by SDS-polyacrylamide gel electrophoresis. Recent evidence would suggest that the difference between the two forms of ICL is one of phosphorylation of the enzyme (H. Riezman and W. M. Becker - personal communication).

Similarly, for MS and citrate synthase there are apparently two forms, one small and acidic and the other large and basic (Riezman et al., 1979A; Köller and Kindl, 1977). It is also possible that catalase, being both glyoxysomal and peroxisomal, may have two isozyme forms related to the different locations, though this has not yet been shown.

3.3. Characterisation of Some Microbody Enzymes

Some of the enzymes of the glyoxysomes have now been purified and their kinetic properties and the molecular weights of the native enzyme protein and its subunits determined (Beevers, 1979).

Isocitrate lyase has been purified from a number of systems. The molecular weight of the native enzyme has been reported as 220,000 and 325,000 in cucumber (Kindl and Fervert, 1977; Lamb et al., 1977, 1978; respectively), 264,000 in

flax (Kahn et al., 1971), 170,000 for Chlorella (John and Syrett, 1967), 265,000 for Neurospora (Johanson et al., 1974) and 480,000 from the vinegar eelworm, Turbatrix aceti (Reiss and Rothstein, 1974). In flax the Km of ICL for isocitrate has been estimated at 0·29 mM (Kahn et al., 1971) while in cucumber it is 0·039 mM (Lamb et al., 1978). The subunit molecular weights quoted for ICL in glyoxysomes of cucumber cotyledons are 63,000 and 61,500 (Riezman et al., 1979A). Two forms of ICL have also been reported in flax though no molecular weights were estimated (Kahn et al., 1971). Kindl and Fervert (1977) have identified a single subunit of ICL as 63,000 mol. wt. Apart from cucumber ICL, this native enzyme apparently has a tetrameric structure (i.e. 4 subunits/active enzyme) (John and Syrett, 1967; Khan et al., 1977).

Native malate synthase (MS) has been reported to have a molecular weight of 500,000 in maize scutella (Servattaz et al., 1973), 540,000 in cucumber cotyledons (Köller and Kindl, 1977) and 575,000 in castor bean endosperm (Bowden and Lord, 1978). Subunit molecular weights have been quoted at 63,000-64,000 by Köller and Kindl (1977), Lord et al. (1978) and Bowden and Lord (1978) while Riezman et al. (1979A and 1979B) claim that it is closer to 57,000. They base their claim on the observation that the purified enzyme migrates at this molecular weight on SDS-polyacrylamide gel electrophoresis with the major salt-eluted protein from glyoxysomes (see p. 15).

Native catalase from cucumber has a molecular weight of 225,000 with subunit molecular weights of approximately

54,000 (Riezman et al., 1979A; Lamb et al., 1978). This subunit size agrees closely with values estimated in lentil (Schiefer et al., 1976) suggesting that catalase consists of four subunits of equal size.

Köller and Kindl (1977) have estimated the molecular weight of native MDH from cucumber to be approximately 225,000 with a subunit size of 34,000. Walk and Hock (1978) using water melon and Riezman et al. (1979) using cucumber have estimated the glyoxysomal MDH subunits to have a molecular weight of 33,000. Earlier estimations by Zschoche and Ting (1973) and Walk et al. (1977) had estimated peroxisomal and glyoxysomal MDH to have molecular weights of 70,000 and 67,000 respectively.

Other glyoxysomal enzyme subunit molecular weights have also been estimated from cucumber (Köller and Kindl, 1977) and these include citrate synthase (46,000), crotonase (75,000), thiolase (45,000).

3.4. Development of Microbodies

Of particular interest in this thesis are the developmental changes occurring within the microbody compartment of cucumber cotyledons associated with the characteristic appearance and decline of the glyoxylate cycle enzymes, ICL and MS.

Biochemical investigations have been carried out on microbody development in fatty seedling tissues in which the storage organ is either the endosperm or the cotyledons of the seed. In both seed types there has been shown to be a more or less synchronous, and marked increase in the

activities of the glyoxylate cycle enzymes from very low or undetectable amounts in the dry seed to peak activities during the first few days of growth (Carpenter and Beevers, 1959; Hock and Beevers, 1966; Longo and Longo, 1970A; Schnarrenberger et al., 1971; Trelease et al., 1971A; Kagawa et al., 1973B; Huang and Beevers, 1974; Theimer and Theimer, 1975; Becker et al., 1978). Gerhardt and Beevers (1970) and Kagawa and Beevers (1975) showed that, concomitant with this rapid increase in enzyme activities, there was a corresponding increase in the number of glyoxysomes within the storage tissue. Upon depletion of the lipid resources the glyoxylate cycle enzyme activities disappear rapidly and, in the case of endosperm tissue, this loss of activity is associated with a loss of this organelle (the glyoxysome) (Gerhardt and Beevers, 1970; Kagawa and Beevers, 1975).

Fatty cotyledons, which later function as photosynthatic organs for the young seedling contain peroxisomes in the autotrophic phase and these are indistinguishable from leaf peroxisomes (Gruber et al., 1970; Trelease et al., 1971A, 1971B; Schnarrenberger et al., 1971; Kagawa et al., 1973B). In light-grown cotyledonary tissue which is initially heterotrophic and later autotrophic, glyoxysomal enzyme activities are present during the initial stages and peroxisomal enzyme activities during the latter stages of development. Peroxisomal enzyme activities increase rapidly as the cotyledons appear above ground and become green, this increase coinciding with the loss in glyoxysomal enzyme activities (Trelease et al., 1971; Becker et al., 1978).

Inhibitor studies indicate that the post-imbibitional increase in activity of the glyoxysomal enzymes, ICL, MS and MDH, is a result of de novo synthesis rather than activation of pre-existing enzymes or precursors (Hock and Beevers, 1966; Gientka-Rychter and Cherry, 1968; Ihle and Dure, 1972; Smith et al., 1974; Doig et al., 1975; Tester, 1976; Walk and Hock, 1977). More substantial evidence has also been obtained using density labelling techniques (Gientka-Rychter and Cherry, 1968; Longo, 1968; Quail and Scandalios, 1971; Walk and Hock, 1977). Bowden and Lord (1976B, 1977) used pulse labelling techniques and the evidence suggests that the glyoxysomal enzymes and probably some of organelle membrane proteins are synthesised de novo upon germination. Sorenson and Scandalios (1976) reported the presence of an endogenous inhibitor of catalase which may be of developmental significance during maize germination. However, no other substantiating evidence of this nature has so far been established.

Studies have been carried out in which inhibitors of transcription and translation have been used in an attempt to elucidate at which level de novo synthesis of these enzymes is controlled. Ihle and Dure (1972) using cotton and Tester (1976) using soybean propose that mRNA preformed in the ripening seed is used for de novo synthesis of ICL. Tester (1976) claims that this preformed mRNA requires processing prior to translation. Hock and Beevers (1966) using actinomycin D found only partial inhibition of ICL and MS synthesis and this only when the inhibitor was applied within the first 1·5 days of growth. They proposed that these enzymes were

synthesised on relatively stable mRNA which was transcribed only during the first day of growth after imbibition. In contrast to these findings, other workers in this field propose that the synthesis of glyoxysomal enzymes is dependent upon postgerminative RNA synthesis in cotton (Smith *et al.*, 1974; Radin and Trelease, 1976; Trelease and Radin, 1976) and cucumber (Roberg and Becker, 1975). In the eukaryotic alga, *Chlorella fusca*, Scragg *et al.* (1975) found post-transcriptional control of ICL induction.

These conflicting results lead to no firm conclusion regarding the level of control of gene expression for these particular enzymes. They do however support the claim that inhibitor studies are often inconclusive, particularly when attempting to study the expression of particular genes. Thus, more sensitive assay techniques are required for a study of this nature.

The availability of more sensitive techniques including the isolation of messenger RNAs and their translation in cell-free systems, coupled with the use of antibodies to purified glyoxysomal enzymes, has allowed a more detailed investigation into the molecular events regulating glyoxysomal enzyme synthesis and packaging (Hock, 1974; Bowden and Lord, 1976, 1977, 1978; Leaver *et al.*, 1977; Walk and Hock, 1977, 1978; Lord and Bowdon, 1978). These methods will be discussed more fully at a later stage (see p. 37).

Another topic of interest regarding control of the glyoxysomal enzymes is the trigger or signal responsible for

the observed de novo synthesis and their subsequent rapid increase. Doig et al (1975) showed that the induction of glyoxylate cycle enzymes within the cereal aleurone layer was dependent upon the presence of the embryo but that application of gibberellic acid could replace the embryo effect. A similar regulatory role for the embryo has also been claimed in squash (Penner and Ashton, 1967), peanuts (Gientha-Rychter and Cherry, 1968), and castor bean (Marriot and Northcote, 1975B). Other evidence suggests that the glyoxysome development is independent of the embryo or embryonic axis (Huang and Beevers, 1974; Marriot and Northcote, 1975A; Tester, 1976). The claim of Penner and Ashton (1967) has been re-examined by Ford et al. (1976) who suggested that this and possibly other results may be a result of experimental technique. Slack et al. (1977) and Kerley and Becker (personal communication), working with cucumber, find no embryo effect upon enzyme levels.

It is apparent though, that exogenously applied hormones do affect the activities of glyoxysomal enzymes. Gibberellic acid (GA_3) has been shown to stimulate the development of glyoxylate cycle enzyme activities and the rate of lipid degradation (Marriot and Northcote, 1975B; Wrigley and Lord, 1977; Gonzalez, 1978) while Allfrey and Northcote (1977) found ABA inhibited ICL induction. Kinetin has also been implicated in regulation since removal of roots results in a premature or enhanced decline in microbody enzymes which can be alieviated by application of exogenous growth hormone (De Boer and Feierabend, 1974; Theimer et al.,

1976). Benzyladenine (BA), as well as influencing the levels of RNA in cucumber cotyledons (Kinoshita et al., 1979). has been shown by Servettaz et al. (1976) and Longo et al. (1979) to increase the level of glyoxysomal enzyme activity and accelerate their subsequent decline in sunflower and water melon cotyledons.

The presence of the axis is certainly essential for maximum lipid mobilisation since the degradation products are utilized for axis growth. Slack et al. (1977) suggest that the rate of mobilisation of sucrose to the axis may influence lipid degradation and utilization more than any hormonal control. It has been shown that microbody enzyme activities decline when a lipid utilising organism is supplied with sugars (Syrett, 1966; Lado et al., 1968; Bellion and Kim, 1978).

The decline in glyoxysomal enzyme activities is normally closely related to the depletion of stored lipid, and is stimulated by light in fatty cotyledons (Hock, 1969; Trelease et al., 1971B; Gerhardt, 1973; Kagawa et al., 1973B; Becker et al., 1978). Theimer (1976) claims the presence of a specific ICL inactivator in sunflower cotyledons which shows increased activity in illuminated cotyledons. Considering the reports of the effects of glucose on the glyoxysomal enzyme activities, it is more probable that the effect of light is indirect in that it results in the development of the photosynthetic acpacity of the cotyledons thus curtailing heterotrophic growth.

In fatty endosperm tissue, the decline in glyoxysomal enzyme activities is accompanied by a loss of total

glyoxysomal protein and thus a decrease in the number of these organelles (Gerhardt and Beevers, 1970; Brown et al., 1974). This degradation of the glyoxysomes has been claimed to be a result of digestion within a cellular compartment, probably the autophage vacuole (Gerhardt and Beevers, 1970; Vigil, 1970) in tissue such as the endosperm which itself degrades at this time.

In tissues which persist after fat utilization, such as maize scutelum, there is apparently a release of enzymes into the cytosol (Longo and Longo, 1970B). Theimer (1976) suggests that specific breakdown, involving proteinaceous inactivators, occurs for the individual glyoxysomal enzymes.

Another topic of developmental interest is the appearance of peroxisomal enzyme activities within fat-storing cotyledons and leaves as they become photosynthetic. Appearance of these enzyme activities is apparently phytochrome mediated (van Poucke and Barthe, 1970; Gerhardt, 1974; Feierbend, 1975; Frosch et al., 1977). However, this may not be total control of synthesis but rather of final enzyme levels since reduced enzyme levels have been detected in dark-grown tissue (Schnarrenberger et al., 1971; Gerhardt, 1973, 1974; Schopfer et al., 1976A, 1976B; Theimer et al., 1976). These reduced enzyme levels are present in leaf peroxisomal precursors which are smaller than the fully developed organelle (Feierbend and Beevers, 1972).

3.5. Microbody Biogenesis

Douglass et al. (1973) could not detect any 'genetic machinery' associated with the glyoxysomes. This observation

has been confirmed by ultrastructural studies which revealed no evidence for the presence of DNA and/or ribosomes in microbodies. Thus, synthesis of microbody proteins is dependent upon the nuclear genome and the cytoplasmic protein synthesising apparatus.

From electron micrographs it can be seen that microbodies are frequently closely associated with the endoplasmic reticulum (ER), and in some cases fuses with it (Gruber et al., 1970; Mollenhauser and Totten, 1970; Vigil, 1970; Newcomb and Frederick, 1971; Lopez-Perez et al., 1974). These results have led to the suggestion that microbodies may be derived from the ER (Lord et al., 1973; Bowden and Lord, 1976A, 1977) by vesiculation or budding.

Studies have been carried out to determine the relationships between the membranes of the ER and microbodies. Using castor bean endosperm it has been shown that the structural phospholipids of the glyoxysomal membrane are derived from the ER (Kagawa et al., 1973A, Lord et al., 1973; Moore et al., 1973; Bowden and Lord, 1975; Donaldson and Beevers; 1976, 1977). The distribution of lipids in the microbody membrane is much more closely related to the ER than to mitochondrial membranes (Donaldson and Beevers, 1976). Similarly, there is a marked resemblance between microbody and ER membranes in their protein components (Hock, 1974; Bowden and Lord, 1976A, 1977; Brown et al., 1976). Pulse labelling studies also suggest transfer of proteins from ER to glyoxysomal membranes (Bowden and Lord, 1976B).

If microbodies are derived from ER in this manner,

it is possible to speculate that synthesis of microbody matrix enzymes would occur on rough ER with immediate discharge into the cisternae which will form the microbody (Bowden and Lord, 1976A, 1976B). However, if microbodies were released from the ER without a full complement of matrix enzymes, subsequent insertion of these proteins would be necessary. Evidence from serological and pulse labelling experiments so far support the former mechanism (Gonzalez and Beevers, 1976; Bowden and Lord, 1977; Meller et al., 1978). Goodman and Blöbel (1978) on the other hand propose that only the membrane associated proteins are synthesised on rough ER while the matrix enzymes are synthesised on free ribosomes and transported post-translationally into the microbody. Walk and Hock (1978) have recently shown thatglyoxysomal MDH synthesised in vitro from poly(A)-containing mRNA is in the form of a precursor of 38,000 mol. wt. as opposed to the native subunit size of 33,000 mol. wt.

To date, there are two mechanisms proposed whereby cytoplasmically synthesised organellar or secretory proteins traverse membranes. These are the 'Signal Hypothesis' as described by Blöbel and Dobberstein (1975) and the 'Envelope Carrier Mechanism' described by Highfield and Ellis (1978).

The 'Signal Hypothesis' requires that a polypeptide destined to traverse a membrane contains a leader sequence (or signal) at its N-terminus. This sequence, of approximately 20-100 amino acids, binds the polysome translating the polypeptide to the membrane by a specific interaction with the membrane proteins and the polypeptide is trans-

ported co-translationally. Synthesis of such polypeptides would therefore occur on membrane-bound polysomes, since synthesis and transport are concomitant. Evidence substantiating this hypothesis has come from both animal and bacterial systems (see p. 43). In the case of immunoglobulin the NH_2-terminal extra sequence has been shown to be highly hydrophobic. It was suggested that the role of this sequence may be for direction of the polypeptide across the ER membrane (Schechter and Burnstein, 1976; Burnstein and Schechter, 1977). The signal sequence is often cleaved though its removal is not required for the hypothesis. Steiner (1979) showed that the signal sequence of ovalbumin is not cleaved although this protein is transported across the ER membrane co-translationally.

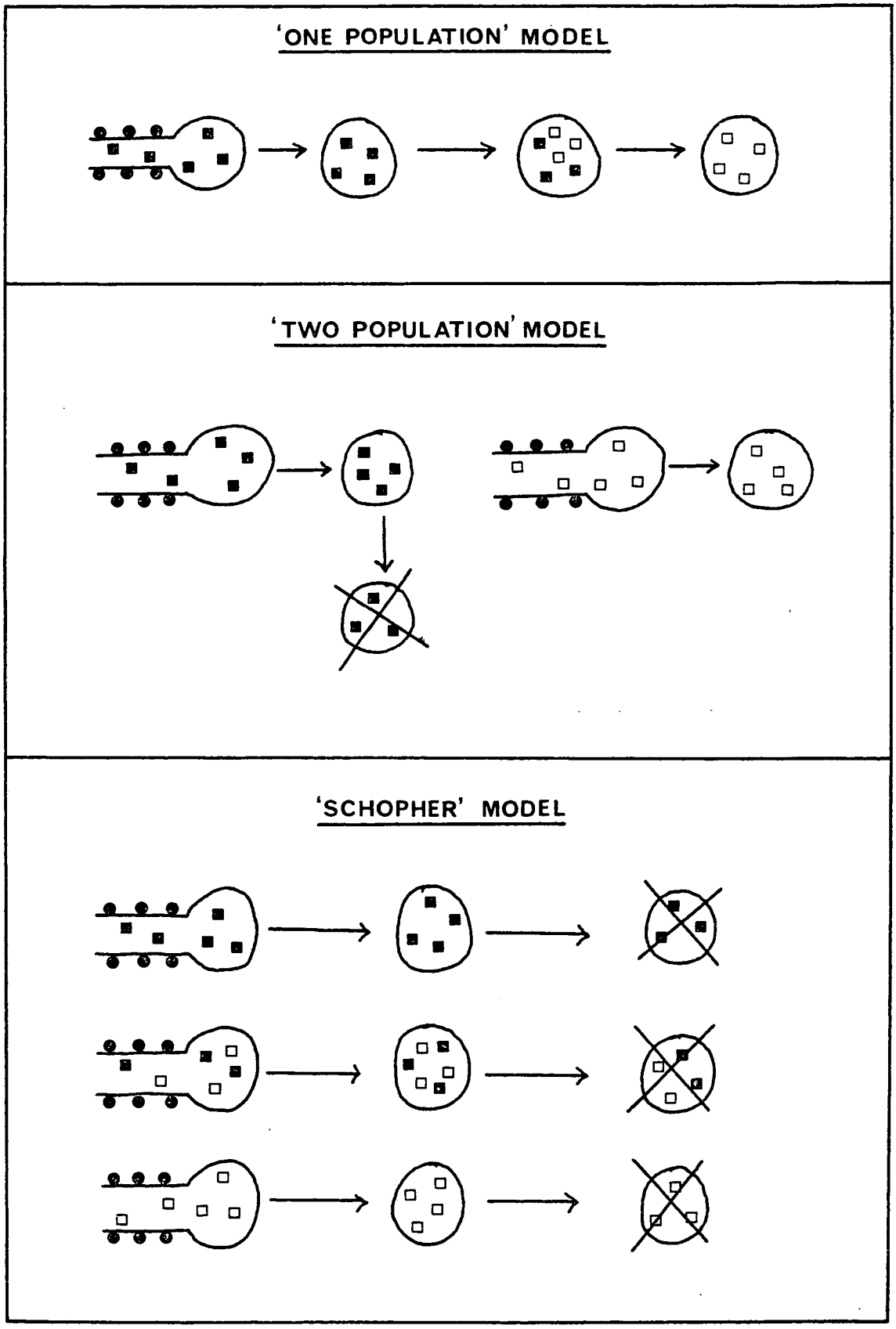
In the case of chloroplasts and mitochondria, a similar mechanism to that previously described would require the presence of ribosomes on the outer organelle membrane, but there is little evidence of this. Therefore Highfield and Ellis (1978) proposed the 'Envelope Carrier Hypothesis' which involves post-translational transport of the polypeptide. In this, the polypeptide is synthesised on free polysomes as a high molecular weight precursor and released prior to transport into the organelle. The polypeptide would assume a particular tertiary structure then bind by means of a recognition site on the polypeptide to specific carriers on the membrane. This binding would result in transport across the membrane and cleavage of the recognition sequence from the polypeptide. Evidence supporting this hypothesis has mainly

come from work on the small subunit of RuBPC, a chloroplast polypeptide coded in the nucleus and synthesised in the cytoplasm (Roy et al., 1977; Cashmore et al., 1978; Chua and Schmidt, 1978, 1979; Smith and Ellis, 1978). There is also recent evidence for such a mechanism involved in the transport of a polypeptide into the mitochondria (Maccecchini et al., 1979).

Fat storing cotyledons represent the situation where both glyoxysomes and peroxisomes are present in the tissue at separate stages of development with an intermediary stage when both types of functional microbody are present. There has been considerable speculation as to the method whereby one type of microbody (the glyoxysomes) gives way to the other (the peroxisomes) during this transition. The complication in such studies is the fact that the two classes of microbody are morphologically indistinguishable. Also, the standard method of purification of these organelles, by sucrose density gradient centrifugation, does not distinguish the two classes.

There have, so far, been three models proposed to explain the mechanism whereby this transition occurs (see Figure IV). The two-population model of Beevers' group proposes that glyoxysomes disappear and are replaced by peroxisomes during development, with each class of microbody representing an individual population (Kagawa et al., 1973A, 1973B; Kagawa and Beevers, 1975; Brown and Merret, 1977). They claim that in other organs the processes of complete degradation of glyoxysomes and de novo synthesis of leaf peroxisomes have been established and that in fatty cotyledons

FIGURE IV. Models of Microbody Biogenesis



■ glyoxysomal protein
□ peroxysomal protein
● ribosome

these processes occur as "normal" though overlap in time.

A "one-population" hypothesis was suggested by Newcomb's group and supported by data from other workers in this field (Trelease et al., 1971A; Gerhardt, 1973; Drumm and Schopfer, 1974; Theimer et al., 1975; Burke and Trelease, 1975). This proposes that at least some of the glyoxysomes undergo a change in enzymatic complement to become functional peroxisomes, with the possible existance of "glyoxyperoxisomes" which function as both types of microbody. Burke and Trlease (1975) used cytochemical techniques and could find no evidence for separate populations since the greater percentage of isolated microbodies stained for both malate synthase (glyoxysomal) and glycolate oxidase (peroxisomal).

Schopfer et al., (1976) recently proposed a third model which is an attractive alternative to the others and has been supported by work of Wainwright and Ting (1976) and Köller and Kindl (1978). This proposes that there is a continual turnover of microbodies within the cell, regardless of the class of microbody. During development this allows for production of intermediary glyoxyperoxisomes and eventually a homogenous population of peroxisomes. Results supporting both of the other models can be accommodated into the model of Schopfer et al. (1976). Gruber et al. (1973) concluded that leaf peroxisomes had a life time of 4 days, and, if this were true for microbodies in fatty cotyledons, this would further support this hypothesis as it would suggest a continual turnover of the microbody population, regardless of the developmental situation.

At present, all three models must still be considered until one or other can be proved unequivocably.

SECTION 4

REGULATION OF GENE EXPRESSION

As previously discussed, germination and early seedling development involves a dramatic developmental transition from the quiescent or dormant state to active growth and metabolism. Concomitant with this transition is a marked increase in the activities of numerous enzymes associated with the resumption of metabolism. Since many of these increases in enzyme activity have been shown to be a result of *de novo* synthesis, rather than activation of a previously inactive precursor protein, they undoubtedly involve gene expression. However, there are several stages at which gene expression may be regulated. Germinating tissue is considered ideal for a study of the biochemical and molecular events involved in gene expression and its regulation since the starting material is metabolically low in activity.

4.1. Levels of Control

An important feature of plant cells is their totipotency, which suggests that all cells contain the same genetic information. However, it is clear that differentiation occurs, producing cell types performing different functions as a result of different enzyme populations. Thus, specific regulatory mechanisms must exist to determine which genes are expressed within particular cell types. In eukaryotic cells, genetic information passes from the nucleus to the cytoplasm as RNA transcripts of DNA. Once in the cytoplasm these RNA transcripts become translatable mRNAs which code for the enzymes of the cell. There are basically five

stages at which gene expression may be regulated.

(a) Control at the level of the Genome

As well as nuclear DNA, extra-nuclear DNA is known to be present in eukaryotic cells. Mitochondria of animal cells and mitochondria and chloroplasts of plant cells are known to contain DNA. Variation in the amount of genetic information may occur by polyploidy, polyteney and selective gene amplification.

(b) Transcriptional Control

Control of gene expression at the transcriptional level is the main level of control found within prokaryotes, which contain short lived mRNAs. Jacob and Monod (1961) proposed the original model for transcriptional control within bacterial cells, in which a specific 'effector' molecule activates a gene, resulting in the synthesis of mRNA for a particular protein. Feedback repression or specific 'repressor' molecules would then act to control the levels of the specific protein by regulating the amount of mRNA transcribed. In eukaryotic cells, which contain longer lived mRNAs, transcriptional control has also been shown to be involved in the regulation of specific proteins, e.g. actin (Paterson et al., 1974); ovalbumin (Palmiter, 1975); tyrosine aminotransferase (Steinberg et al., 1975); α-amylase (Higgins et al., 1976).

(c) Post-transcriptional Control

After the synthesis of an RNA transcript, there are a number of events, prior to mRNA utilization in protein synthesis, at which regulation may occur.

(i) Processing of the mRNA precursors which includes nucleolytic cleavage, splicing, capping and methylation at the 5' end, internal methylation and addition of adenylic acid residues at the 3' end (Bawerman, 1974; Derman and Darnell, 1974; Shatkin, 1976; Goldberg et al., 1977).

(ii) Transport of the mRNA from the nucleus to the cytoplasm.

(d) Translational Control

(i) Availability of the mRNA at the site of protein synthesis.

(ii) Binding of the mRNA to the ribosomes, which involves recognition of a ribosome binding site at the 5' non-coding region preceding the initiator codon (AUG), and rates of initiation of protein synthesis (Lodish, 1976; Kozak and Shatkin, 1977; Revel and Groner, 1978).

(iii) Availability of ribosomes, tRNAs, amino acids and initiation and termination factors.

(iv) Stability of mRNA. The 3' poly(A) and 5' cap sequences have been implicated in conferring stability upon specific mRNAs (Blobel, 1973; Williamson et al., 1974; Furuichi et al., 1977) by protecting the mRNAs from nuclease degradation.

(e) Post-translational Control

Such regulation may involve any of the following mechanisms.

(i) Chemical modification of the polypeptide by phosphorylation, methylation, acetylation etc. or by addition of non protein moieties e.g. glycosylation (Blobel and Dobberstein, 1975).

(ii) Intracellular compartmentalisation. In particular those proteins functional in chloroplasts and mitochondria which are coded in the nucleus and synthesised in the cytoplasm.

(iii) With the advent of cell-free protein synthesising systems, it has been shown that numerous proteins are synthesised as precursors which are subsequently cleaved to produce the active protein (Chan et al., 1976; Roy et al., 1976; Sussman et al., 1976; Burr et al., 1978; Lingappa et al., 1978; Maurer and McKean, 1978).

(iv) Assembly of subunits to produce functional protein.

(v) Rates of protein turnover, i.e. synthesis, degradation, stability.

4.2. Methods Available for Studies on Gene Expression

In a study of the regulation of gene expression during development, including seed germination and early growth, there are various techniques which may be employed.

(a) Inhibitor Studies

This technique has previously been discussed in relation to microbody development (p. 22) so will only be outlined here.

Use of inhibitors which specifically block transcription (e.g. Actinomycin D) or translation (e.g. cycloheximide) used to be the classical method for developmental studies of this nature. However, results from such experiments are normally inconclusive, since it is difficult to determine what other side effects these inhibitors may have and how these affect the results obtained.

There are two classic examples of inhibitor studies in the study of seed germination. Yomo and Varner (1971) studied the induction of α-amylase by GA_3 in barley. They concluded that production of α-amylase was due to de novo synthesis on pre-existing mRNA and thus was controlled at the level of translation. However, this has now been disproved and convincing evidence presented that the control is at the level of transcription (Higgins et al., 1976; see also p. 45).

Again using inhibitors, Dure (1975) concluded that the early stages of cotton germination were dependent upon the use of preformed mRNAs which were present in the dormant tissue.

However, as previously shown in the case of micro-body development (p. 22), results from such experiments, utilising inhibitors, are often inconclusive and may in fact result in conflicting evidence.

(b) In Vitro Protein Synthesis

A number of cell-free systems capable of translating exogenous mRNA have been developed over the past few years including the ascites tumor system (Smith and Marcker, 1970), a reticulocyte lysate system (Pelham and Jackson, 1976), a what-germ system (Roberts and Paterson, 1973; Marcu and Dudock, 1974), a rye-embryo system (Carlier and Peumans, 1976A, 1976B) and an E. coli system (Ninenberg and Matthaei, 1961). The E. coli system, being bacterial in nature, is capable of translating mRNAs from procaryotic systems (including bacterial, and chloroplasts, mitochondria and some viruses). All the other cell-free systems contain 80S ribosomes and can

translate cytoplasmic and viral mRNAs. One other system also developed for assaying mRNAs is the cellular Xenopus oocyte system in which mRNA is injected into toad oocytes and translated in vivo (Gurdon et al., 1971).

These systems are used as a means of assaying for the presence of translatable mRNA, either within a total RNA or mRNA population or of a specific purified mRNA species. It is possible to analyse the types of mRNA present by fractionating the labelled, in vitro synthesised products by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and detecting them by autoradiography or fluorography (Bonner and Laskey, 1974; Laskey and Mills, 1975). The availability of purified proteins allows for the production of monospecific antibodies and subsequent identification of specific in vitro products (see p. 43).

It is unclear when using such system whether all mRNAs are 'translatable' in vitro regardless of their state in vivo. The assumption is often made that this is the case but it need not be so. In fact, on careful consideration, it would be more reasonable to assume that only mRNAs translatable in vivo would be capable of programming a cell-free system since the basic method of protein synthesis is the same in both situations. This would mean that post-transcriptional changes in mRNAs would need to have occurred before they were translated in vitro.

It has also been shown that detailed optimisation of cell-free systems (e.g. the wheat-germ system) is essential to assay for specific mRNAs. Tse and Taylor (1977) have shown

that changes in the K^+ and Mg^{2+} concentration in the *in vitro* translation system can markedly alter the size distribution of polypeptide products obtained and also the relative efficiencies of translation of individual mRNAs. Schwinghamer and Symons (1977), in studying translation of different RNA species of Cowpea mosaic virus in a wheat embryo system, showed that the individual mRNAs varied in the requirements for optimum translation. Thus, care should be taken in analysing results from cell-free translation systems. Another point to note from the work of Schwinghamer and Symons (1977) is that analysing a specific mRNA in different *in vitro* translation systems gave confusing results as different sized products were synthesised by a specific viral RNA in the different systems.

As well as studying the total translatable mRNA population in a tissue, it is also possible to study the mRNAs actively involved in protein synthesis at a designated stage of development. This may be achieved by preparing polysomes (Davies *et al.*, 1972) from the tissue and using these to programme a cell-free translation system (Boulter, 1970; Marcus, 1972).

Since the wheat-germ cell-free translation system was used in the research outlined in this thesis, the following discussion will be mainly concerned with this particular system.

In order to use any cell-free translation system to assay for translatable mRNAs, it is essential that the results are shown to be reproducible. The wheat-germ system has high activity and sensitivity, showing high

levels of incorporation of labelled amino acids into protein in response to low exogenous RNA concentrations. Since it apparently does not require special factors for translation of different mRNAs,it is non-selective. It is also useful in that it has low endogenous activity unlike the reticulocyte lysate system (Pelham and Jackson, 1976).

Fidelity of translation is essential so that discrete polypeptides are produced. Anderson et al. (1974) raised doubts about whether the wheat-germ system was capable of translating mRNAs for high molecular weight proteins. However, by altering incubation conditions, it has since been shown that efficient translation of high molecular weight polypeptides is possible. Polyamines apparently aid reproducibility of results (Hunter et al., 1977) by lowering the Mg^{2+} requirement and increasing the incorporation by stimulating the elongation rate. They also stimulate translation of high molecular weight polypeptides (Benveniste et al., 1976; Roewekamp et al., 1976). Increasing the K^{+} concentration above 100 mM has been shown to increase the fidelity of translation of polypeptides greater than 30,000 mol. wt. (Rosen, 1976; Shapiro et al., 1976).

As long as the wheat-germ system has been previously optimised, it should be an efficient assay system for mRNAs. Several studies using the wheat-germ cell-free translation system have shown that, with care, translation in this cell-free system is capable of reflecting changes in the mRNA levels. Such studies have been carried out in both animal and plant developmental systems, e.g. actin (Paterson et al.,

1974); growth hormone (Martial, 1977); phenylalanine ammonia-lyase and related enzymes (Halbrock *et al.*, 1979); it was found that the developmental changes in amounts of translatable mRNAs closely resembled the changes in the protein content of the tissue in each of these cases.

(c) Analysis of *In vitro* products

Levels of incorporation of a labelled amino acid into hot, TCA-precipitable material (Mans and Noveli, 1961) can be used as an approximate quantitation method to determine the amounts of mRNA in a sample used to programme an *in vitro* protein synthesising system.

To determine qualitative changes in *in vitro* 'translatable' mRNA it is necessary to analyse the products of translation by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) followed by autoradiography or fluorography to detect the *in vitro* synthesised products. This will show the range of polypeptides coded for by the mRNAs used to programme the system.

However, if specific products are to be determined several further stages of analysis should be attempted. It should be remembered that co-migration on SDS-polyacrylamide gels of two polypeptides does not necessarily imply that they are the same protein. This point is emphasised by 2-dimensional gel techniques (O'Farrell, 1975) which show that many different polypeptides may have the same molecular weight but vary by other criteria (e.g. isoelectric point).

Another important feature, which has become evident from use of *in vitro* translation systems, is that many polypeptides are initially translated as precursors of higher

molecular weight. This has been found to be the case for some proteins which are secreted or which are synthesised on cytoplasmic ribosomes but located within cell organelles, in both plant and animal cells. In animals, *in vitro* synthesis of precursor proteins has been recorded for insulin (Chan *et al.*, 1976; Shields and Blobel, 1977; Hew, 1978), corticotropin (Nakanishi *et al.*, 1976), growth hormone (Sussman *et al.*, 1976), lactin (Maurer and McKean, 1978), and immunoglobin (Schechter *et al.*, 1975, 1976). In plants, precursors of this nature have been shown to occur for the small subunit of RuBPC (Roy *et al.*, 1976, 1977; Dobberstein *et al.*, 1977; Cashmore *et al.*, 1978; Highfield and Ellis, 1978; Tobin, 1978), zein (Burr *et al.*, 1978), MDH (Walk and Hock, 1978) and a viral transmembrane glycoprotein (Lingappa *et al.*, 1978).

For these reasons it is essential that further analysis of the *in vitro* translation products is carried out in order to identify specific polypeptides. There are several methods which may be employed for more detailed analysis.

(i) Immunochemical Characterisation

This may be used if monospecific antisera to the proteins of interest are available. In order to efficiently immunoprecipitate the specific products from the *in vitro* translation mixture various methods have been employed by workers in this field including double antibody immunoprecipitation (Hunter, 1967; Higgins *et al.*, 1976; Nakanishi *et al.*, 1976; Shields and Blobel, 1977; Dobberstein *et al.*, 1977; Evans *et al.*, 1979), addition of carrier protein (Verma *et al.*, 1974; Cashmore *et al.*, 1978), use of heat-inactivated Staphyl-

ococcus aureus cells, containing Protein A, as an immunoabsorbant (Jonsson and Kronvall, 1974; Kessler, 1975; Goding, 1978; Ploegh et al., 1979) or immunoabsorption of the antigen-antibody complex on Protein A-sepharose (Hjelm et al., 1972; Goding, 1976, 1978; Mitchell et al., 1977A and 1977B). These immunoprecipitated in vitro synthesised products can then be analysed by fractionation on SDS-polyacrylamide gels followed by autoradiography or fluorography.

(ii) Sequence Homology

Comparison of the in vitro synthesised polypeptide and the native protein can be acheived by limited proteolytic digestion and analysis of the resultant peptides (Cleveland et al., 1977). Tryptic, or chymotryptic, peptide mapping may be used (e.g. Hall et al., 1979) with analysis of the digests by electrophoretic or chromatographic techniques (Ingram, 1963). Cyanogen bromide cleavage may also be used to compare in vitro synthesised and authentic polypeptides (e.g. Burr et al., 1978). Both these methods have been employed in comparing authentic and in vitro synthesised large subunit for RuBPC (Hartley et al., 1975; Bottomley and Whitfeld, 1979). In both cases the degree of sequence homology can be compared in order to establish if the two polypeptides (authentic and in vitro synthesised) are related.

Individually, immunodetection and sequence homology are useful in determining the relationship between authentic and in vitro synthesised products, and use of both gives added confidence to the results obtained.

4.3. Use of In Vitro Translation Systems in Plant Development Studies

In vitro translation systems have been used extensively as an assay procedure for specific mRNAs in plant tissues. Some examples of their use will be outlined in this section.

Higgins et al. (1976) used the wheat-germ cell-free system to study the regulation of α-amylase production by GA_3 in the barley grain upon germination. Yomo and Varner (1971), by using inhibitor studies, suggested that the level of control in this system was possibly at translation with utilisation of a stored mRNA present in the dormant tissue. However, by analysis of the mRNA present in the tissue under GA_3-plus and GA_3-minus conditions it was possible to show the absence of translatable mRNA for α-amylase in the latter. The regulatory effect of GA_3 was therefore apparently at the level of transcription of the specific mRNA. Identification of the in vitro synthesised polypeptide in these experiments was by immunoassay and co-migration with authentic α-amylase on SDS-polyacrylamide gels.

Various groups have also been involved in detection of the mRNA for the small subunit of RuBPC in pea (Roy et al., 1977; Highfield and Ellis, 1978; Cashmore et al., 1978, 1979), wheat (Roy et al., 1976), cucumber (Walden, 1979), Lemna gibba (Tobin, 1978), and Chlamydomonas (Dobberstein et al., 1977). In all cases, except cucumber, the direct translation product of this specific mRNA is 20,000 mol. wt., whereas in cucumber it is apparently 25,000 mol. wt..Cashmore et al. (1978) demonstrated that this 20,000 mol. wt. precursor

synthesised by pea mRNA showed extensive sequence homology with the authentic polypeptide. Highfield and Ellis (1978) showed that this precursor polypeptide (P20) could be processed to the size of authentic small subunit (14,000 mol. wt. in pea) by isolated, intact chloroplasts and that the processing enzyme was present as a stromal protein (Ellis et al., 1979). They also showed that the precursor could be transported into the intact, isolated chloroplast and assembled into RuBPC protein. Tobin (1978), working with Lemna gibba studied the translation products of the wheat-germ system when programmed with poly(A)$^+$ RNA from light and dark-grown tissue. Results indicated that white light had a dramatic effect on the steady state levels of specific polyadenylated mRNAs, including that for the small subunit of RuBPC.

Detection of the mRNA for the large subunit of RuBPC and also developmental studies on the regulation of the synthesis of this polypeptide have been carried out using cell-free translation. Unlike the small subunit of RuBPC which is coded in the nucleus, the large subunit is coded by the chloroplast genome. Since synthesis of this subunit is on 70S ribosomes, the E. coli cell-free system is used to detect this specific mRNA. Hartley et al. (1975) demonstrated that chloroplast RNA directed synthesis of the large subunit in the E. coli system. Bottomley et al. (1979) have shown by proteolytic digestion that the in vitro synthesised and authentic large subunit polypeptides show high sequence homology. Walden and Leaver (1978) studied the regulation of synthesis of the large subunit of RuBPC

during germination and early development of cucumber. They concluded that the initial rise in the level of translatable mRNA for this polypeptide is independent of light. However, increased levels of this specific mRNA and accumulation of higher levels of RuBPC protein were apparently light dependent.

The synthesis of seed storage proteins in various species has been studied by a number of laboratories, of which a few are mentioned here. Burr et al. (1978, 1979) and Larkins et al. (1979) have studied the synthesis of zein in maize. They have demonstrated, by use of in vitro translation in the wheat-germ system, that the mRNAs for zein are associated with membrane-bound polysomes. It was also found that in the wheat-germ system the zein polypeptides were synthesised as precursors. However, Larkins et al. (1979) showed that if these mRNAs were translated in Xenopus oocytes the zein polypeptides were the same size as the native protein. This was due to the fact that the oocytes, unlike the wheat-germ system, are capable of processing the signal sequence and compartmentalising the proteins within membrane vesicles (Blobel and Dobberstein, 1975). Since zein proteins are synthesised on membrane-bound polysomes, it was proposed that the precursor mediated sequestration into the protein bodies.

Hall and co-workers have studied the synthesis of bean seed globulin mRNA (Hall et al., 1979). They demonstrated that globulin-1 protein, which consists of 3 subunit polypeptides, was synthesised on membrane-bound polysomes (Sun et al., 1978). In the wheat-germ system these polysomes

directed the synthesis of 2 polypeptide bands that co-migrated on SDS-polyacrylamide gels with 2 of the native polypeptides, could be immunoprecipitated by antiserum to globulin-1 protein and showed high sequence homology with the native protein (Hall *et al.*, 1978). However it has been proposed from recent peptide mapping studies (Hall *et al.*, 1979) and from the fact that these native polypeptides are known to be glycosylated (Hall *et al.*, 1977), that the 2 polypeptide bands obtained *in vitro* do contain the three *in vitro* synthesised products.

Evans *et al.* (1979) have studied the synthesis of vicilin and legumin proteins in developing pea seeds. By use of polysomes to programme a wheat-germ cell-free system they demonstrated that the mRNAs for these proteins were preferentially associated with membrane-bound polysomes. Higgins and Spencer (1979) have shown that at least some of the storage proteins in peas are synthesised as precursors. They found that processing of these precursors increased the antigenic efficiency of the polypeptides. By comparison of *in vivo* and *in vitro* protein synthesis they concluded that the life span of these precursors *in vivo* may vary from being restricted to the period of translation to as much as a few days or even weeks. In the latter case processing apparently occurs after assembly of the subunit into the holoprotein.

These are just some of the examples of such studies on seed storage proteins. They indicate that use may be made of cell-free translation to identify specific mRNAs and also that seed storage proteins are probably synthesised as pre-

cursors for the purpose of mediating transport into the ER and subsequently the protein bodies of the storage tissue.

SECTION 5

EUKARYOTIC MESSENGER RNA

Since the existence of mRNA was first proposed by Jacob and Monod (1961), our understanding of the structure and function of this molecular species has greatly advanced. Messenger RNAs are responsible for direct expression of the structural genes, mediating the flow of genetic information from the DNA to the functional protein. Thus, an understanding of the mechanism whereby the synthesis and utilisation of specific mRNAs is controlled is of importance in determining how gene expression is regulated.

In eukaryotic cells there is compartmentalisation of the genetic information (DNA) within membrane-bound organelles. This results in the events of transcription and translation of eukaryotic cytoplasmic mRNAs being spatially isolated, whereas these two processes are closely coupled in chloroplasts, mitrochondria and prokaryotic cells.

It has been shown that approximately 5% of cellular RNA constitutes the cytoplasmic mRNA of a cell. This mRNA population may contain as many as 10^3–10^4 individual mRNA species each accounting for 0·001–0·2% of the total population (Perry, 1976; Dyer and Leaver, review, in press). As many as 15,000–30,000 different mRNAs have been estimated to be present on cytoplasmic ribosomes in a single tissue type (Goldberg *et al.*, 1978; Kiper, 1979). In studying gene complexity in tobacco, Goldberg (1979) has shown that a minimum number of approximately 8,000 mRNAs are common to all tissues of the plant. However, in the entire tobacco

plant he claims that as many as 60,000 structural genes are expressed. Goldberg (1979) has also shown that regulation of gene expression may be post-transcriptionally controlled. By hybridisation studies he showed that many mRNA sequences specific to the leaf tissue of tobacco could be detected in the nuclear RNA but not the polysomal RNA of the stem tissue.

Eukaryotic mRNAs are heterogeneous in size, containing approximately 400-4,000 nucleotides (c.a. $1{\cdot}2 \times 10^5$-$1{\cdot}2 \times 10^6$ mol. wt.). This comprises the protein coding region and also certain non-coding sequences. These non-coding sequences may be transcribed from the DNA or added during processing and will be discussed in more detail later in this section. Messenger RNAs in eukaryotes are monocistronic, containing a single functional site for initiation of protein synthesis (Swan et al., 1972; Palmiter, 1974) and coding for a single protein (Kozak, 1978) whereas polycistronic mRNAs are common in bacteria (Jacobson et al., 1970; Villa-Komaroff et al., 1975).

5.1. Biogenesis of Messenger RNA

The heterogeneous DNA-like RNA (hn RNA) which is synthesised in the nucleus has been proposed as the precursor of cytoplasmic mRNA since its base composition resembles mRNA, both are heterogeneous in size and both contain 3'-poly(A) sequences and 5'-terminal caps (Revel and Groner, 1978). Hybridisation studies have shown sequence homology between hnRNA and specific mRNAs, e.g. adenovirus (Parsons et al., 1971) and SV40 (Wall and Darnell, 1971) in transferred cells; haemoglobin (Melli and Pemberton, 1972); and globin (Bastos and Aviv, 1977; Stair et al., 1977).

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Heterogeneous nuclear RNA (hnRNA) ranges in size from 10^5 to 2×10^7 mol. wt. while mRNA in the cytoplasm has a size range up to $1{\cdot}5 \times 10^6$ mol. wt. (Georgiev and Lerman, 1964; Weinberg, 1973; Revel and Groner, 1978). Messenger RNA is transcribed and translated from the 5' end to the 3' end of the molecule and may also possibly be degraded in this manner. Processing involved in the conversion of hnRNA to mRNA occurs in the nucleus and includes association with protein, capping, addition of poly(A) sequences and splicing. Once processing has occurred the mRNAs enter the cytoplasm, through the nuclear membrane (Dyer and Leaver, in press). However, most of the hnRNA is degraded in the nucleus, with only about 10% exported to the cytoplasm (Soeiro et al., 1968).

5.2. Cleavage of HnRNA and RNA Splicing

One of the most recent discoveries in the field of mRNA maturation from hnRNA is that pre-mRNA may be spliced and rejoined to form the final mRNA capable of translation. This is due to the discovery that numerous eukaryotic mRNA genes contain intervening sequences which are not present in the mature cytoplasmic mRNA (Williamson, 1977). Split genes have so far been found to occur in eukaryotic nuclear, chloroplast and mitochondrial genomes and also viral genomes but not in bacteria, e.g. mitochondrial 24S rRNA of Neurospora crassa (Hahn et al., 1979); chloroplast 23S rRNA of Chlamydomonas (Rochaix and Malnoe, 1978); 28S rRNA in Drosophila (Wellaur and David, 1977; Barnett and Rae, 1979); 25S rRNA of Tetrahymena (Wild and Gall, 1979); tRNAs in yeast (Valenzuela et al., 1978; Ogden et al., 1979); imm-

unoglobulin in mouse (Brack and Tonegawa, 1977); β-globin of rabbit and mouse (Jeffreys and Flavell, 1977; Tilghman et al., 1978); ovalbumin of chicken (Breathnach, et al., 1977); Adenovirus late mRNAs (Chow et al., 1977; Goldenberg and Raskas, 1979); 19S and 16S mRNAs of simian virus 40 (Lavi and Groner, 1977; Khoury et al., 1979).

Considerable evidence suggests that these intervening sequences are present in the hnRNA transcripts as well as the DNA. It was proposed that looping of the hnRNA allows these coding sequences to be brought together by deletion of the inserts (Ohtsuki et al., 1977). Schmidt et al. (1978) showed that a nuclear extract from Xenopus oocytes was capable of accurately processing tRNA precursors, containing intervening sequences. The sizes and numbers of inserts varies widely between the different genes so far recognised to contain such sequences. The rRNA genes studied usually have a single insert. In Neurospora mitochondria this was 2·3 kilo bases (Hahn et al., 1979), in Chlamydomonas chloroplasts, 0·94 kilo bases (Rochaix and Malnoe, 1978) and in Drosophila, 9·6 kilo bases (Barnett and Rae, 1979). In yeast the gene for $tRNA^{Trp}$ contains a short 34 nucleotide insert (Ogden et al., 1979). The insert in the immunoglobulin light chain gene constitutes 1250 nucleotides in a single DNA fragment (Brack and Tonegawa, 1977). Ovalbumin (Tonegawa et al., 1978) and ovomucoid (Catterall et al., 1979) genes have been shown to each contain 7 intervening sequences.

Although many mRNA genes may contain inserts, it has been shown that those for the histones of sea urchin do not (Schaffner et al., 1978). The functional role of these sequences is not certain though they may be important in post-transcriptional control mechanisms. Barnett and Rae (1979) showed that these sequences in the 28S rRNA of different Diptera species varied in their degree of homology - the closer the species were taxonomically, the greater was the homology. They also point out that homologus sequences to the inserts were detectable elsewhere in the genome and conclude that they may be of significant value to have been conserved. However, in Tetrahymena mutants which lack the inserts in the 25S rRNA gene are metabolically functional (Wild and Gall, 1979).

At present, there is a relatively new subject to molecular biology and it waits to be seen what can be established as to the role and function of these inserts and also to their evolution.

5.3. 5' Capping and Methylation

Since transcription and translation of mRNAs occur in the 5' to the 3' direction, the structure of the 5' end of the molecular may be of importance for the control of gene expression. Many eukaryotic cellular and viral mRNAs have been shown to contain a 5' terminal capping structure, $m^7G^{5'}ppp^{5'}xmym$, in which the methylated guanosine residue (m^7G) is linked to the next base by a triphosphate bridge (Shatkin, 1976). This cap structure is also present in hnRNA. There have been a number of variations in cap

structure identified, this variation being in the degree of methylation of the two nucleotides after the triphosphate bridge (Shatkin, 1976; Dyer and Leaver, in press).

It was demonstrated by use of cell-free translation system, that the 5' cap was, in some cases, necessary for efficient translation of the mRNA (Both et al., 1975A; Muthukrishnan et al., 1975; Rose, 1975; Kemper, 1976). However, is capping necessary for translation in vivo? Certainly not all mRNAs are capped, e.g. polio virus from infected cells (Nomoto et al., 1976); and in some cases it was found to have a negligible effect on in vitro translation (Shih et al., 1976; Lodish and Rose, 1977). Revel and Groner (1978) suggest that some of the conflicting evidence on the requirement of the cap structure for efficient translation may be because, in some mRNAs, where the cap and initiation codon (AUG) are far apart, the cap structure is not so important. They also point out that the conditions of cell-free translation may alter the stringency of cap requirement.

There are at least two proposed functions of the 5' cap which may explain its role in translation. Firstly, the m^7G region may be a recognition site for formation of a stable initiation complex (Shatkin, 1976; Kozak, 1978). It has been shown that mRNAs containing m^7G are preferentially bound to the 40S ribosome subunit in wheat-germ (Both et al., 1975B) and that cap analogs can inhibit in vitro binding and translation (Hickey et al., 1976; Roman et al., 1976; Schafritz et al., 1976). The basic sequence adjacent to the cap

may also affect the affinity of the mRNA for ribosomes (Moss and Koczot, 1976; Muthukrishnan *et al.*, 1976). However, in eukaryotic mRNAs so far sequenced, the region between the 5' cap and the AUG codon is highly variable and thus, Kozak and Shatkin (1977) proposed that minimal recognition, involving the cap and initiation codon, but not the intervening sequence was involved. Kozak (1978) has recently proposed a mechanism whereby initiation in eukaryotes involves three stages - the 40S ribosomal subunit binds to the capping region then advances along the mRNA till it reaches the first AUG codon where the 60S subunit would then bind. Kozak (1978) suggested that base pairing of the met-tRNA, which is already attached to the 40S subunit (Hunter *et al.*, 1977B), with the AUG codon could act as the signal for attachment of the 60S subunit. In this model, the cap, rather than the AUG codon, would thus act as the ribosome recognition site.

Secondly the presence of the capping structure may confer stability on the mRNA since it protects the mRNA from 5' exonucleolytic degradation. Specific cap-hydrolysing enzymes which only cleave the cap and no other site on the mRNA have been identified (Nuss *et al.*, 1975; Kole *et al.*, 1976; Shinshi *et al.*, 1976). It has been suggested that removal of the cap *in vivo* may act as a negative control allowing degradation of a mRNA (Furuichi *et al.*, 1977; Revel and Groner, 1978).

Methylation of internal adenosine residues has also been reported in some viral and eukaryotic mRNAs (Wei *et al.*, 1975; Dimock and Stoltzfus, 1977) and also

hnRNA (Schibler et al., 1978). The functional significance and location of these altered residues is, as yet, unknown, though they are not present in the 3' poly(A) sequence (Parry et al., 1975).

5.4. Non-Coding Regions of mRNA

Size estimates of specific mRNAs have been carried out and it appears that these mRNAs are substantially larger than is required to code for the specific polypeptide (Gaskill and Kabat, 1971; Mach et al., 1973). Even allowing for the variable 3'-poly (A) sequence (see below) the size estimates indicate that other non-coding sequences are present on the mRNAs. Sequence analysis studies have shown that mRNAs contain non-coding sequences at both the 5' and 3' ends of the molecule. The 5' non-coding sequence lies between the cap and the initiator codon (AUG) and the 3' non-coding sequence between the terminator codon (UAA, UAG or UGA) and the 3'-poly(A) sequence which may be present. The latter have been shown to be relatively highly conserved for a particular mRNA e.g. human and rabbit -globin, while the former show marked heterogeneity in their base composition (Dyer and Leaver, in press). There is also a variation in the size of these sequences, particularly those at the 5' end of the molecular (Kozak, 1978) e.g. 6 to 200 nucleotides at the 5' end and more than 600 nucleotides at the 3' end (Dyer and Leaver, in press). Highly reiterated sequences have in some cases been shown to be present in the 5' non-coding segment (Dina

et al., 1973; Firtel and Lodish, 1973; Jelinek et al., 1974).

The function of these two non-coding sequences is as yet unknown though it has been suggested that the 5' sequence may give protection from nuclease digestion (Jelinek et al., 1974) while the 3' sequence may be involved in mRNA processing (Dyer and Leaver, in press). From their positions adjacent to the initiator and terminator codons it may also be thought that they are, in some say, involved in initiation and termination.

5.5. Poly(A) Sequences

Most eukaryotic mRNAs are now beieved to contain poly(A) sequences, of 50-200 nucleotides, at the 3' end of the molecule (Darnell et al., 1973; Brawerman, 1974; Dyer and Leaver, in press). Many higher plants have been shown to contain poly(A)-containing mRNA e.g. soybean (Verma et al., 1974), barley (Higgins et al., 1976); peas (Sieliwanowicz, 1978); castor bean (Roberts and Lord, 1979); tobacco (Goldberg et al., 1978). A few eukaryotic mRNAs have been shown to be devoid of poly(A), particularly the histone mRNAs (Adesnik and Darnell, 1972; Greenberg and Perry, 1972; Semanick, 1974; Nemer et al., 1975; Sonershein et al., 1976). Gray and Cashmore (1976) and Goldberg et al. (1978) have estimated that about 35-50% of the total cellular mRNAs contain a 3' poly(A) sequence. Poly(A) sequences of any significant length have not been detected in prokaryotic or chloroplast mRNAs and as yet it is uncertain whether these sequences are present in mitochondrial mRNAs (Dyer and Leaver, in press).

The poly(A) sequence is not transcribed but is synthesised by stepwise addition of adenylic acid residues to the 3' end of the hnRNA (Weinberg, 1973). This process is apparently essential for processing of the hnRNA to mRNA, as inhibition of poly(A) addition prevents the appearance of cytoplasmic mRNA (Weinberg, 1973) and also enzyme induction (Sarker et al., 1973). Poly(A) addition may occur before or after hnRNA processing and it has also been shown to occur in the cytoplasm (Derman and Darnell, 1974; Perry et al., Brawerman and Diez, 1975; Dyer and Leaver, in press). The latter process may be involved in gene expression at the post-transcriptional level by affecting the turnover rate of specific mRNAs.

The common occurrance of poly(A) sequences in eukaryotic mRNAs suggests that they have some functional significance, though as yet this is unknown. However, it has been proposed that it may be involved in transport of mRNAs to the cytoplasm, regulation of mRNA translation and also in the stability of the mRNA.

It has been shown that poly(A) sequences are not essential for correct translation of mRNAs in vitro. Histone mRNAs are known to lack poly(A) and yet they are translated with fidelity in vitro (Jacobs-Lorena et al., 1972; Gross et al., 1973). Munoz and Darnell (1974) hybridised the poly(A) segment to poly(U) prior to using the mRNA (from transformed rat embryo cells and mouse L cells) to programme in vitro translation and found no effect upon the ability to act as a template for protein synthesis. When

the poly(A) sequence was removed from rabbit globin mRNA by enzymatic digestion, this mRNA was still efficiently translated *in vitro* (Sippel *et al.*, 1974). However, these results are not conclusive since *in vivo* translation may have more stringent requirements for the presence of the poly(A) sequence.

Work by Soreq *et al.* (1974) and Williamson *et al.* (1974) suggested that the poly(A) sequence may have a role in mRNA stability. It was thought that binding of specific proteins to the poly(A) sequence may protect the mRNA from nuclease digestion (Kwan and Brawerman, 1972; Blobel, 1973; Schwartz and Darnell, 1976). *In vivo*, poly(A) sequences are known to shorten with the age of the mRNA (Singer and Penman, 1973; Sheiness *et al.*, 1975). Sheiness and Darnell (1973) showed that the mRNA 3' poly(A) sequence was reduced in length when it reached the cytoplasm. Deadenylation of globin has been shown to result in more rapid decay of the mRNA activity in Krebs axites cell-free system (Williamson *et al.*, 1974) or in oocytes (Marbaux *et al.*, 1975; Nudel *et al.*, 1976). It is suggested that a gradual decrease in the length of the poly(A) *in vivo* may determine the period of stability, and thus turnover rate, of an mRNA since the indication is that once the poly(A) is reduced nuclease degradation may affect the mRNA (Revel and Groner, 1978; Dyer and Leaver, in press).

5.6. Messenger RNP (mRNP)

Unlike prokaryotic mRNAs which occur free within the cell, eukaryotic mRNAs have been shown to be present in mRNP particles (Spirin, 1978). These are free messenger

ribonucleoprotein particles which are not associated with the cytoplasmic polyribosomes (Spirin, 1969; Jacobs-Lorena and Baglioni, 1972; Brawerman, 1974). Specific proteins have been shown to be associated with the mRNA in this particle (Revel and Groner, 1978). A specific 75,000 mol. wt. protein is associated with the poly(A) sequence (Kwan and Brawerman, 1972; Blobel, 1973; Schwartz and Darnell, 1976). It has been suggested that the purpose of this association was to protect the poly(A) sequence from nuclease digestion, thus possibly stabilising the messenger (Bergman and Brawerman, 1977). Nucleoplasmic RNA has also been shown to be associated with protein (Brawerman, 1974).

No definite functional role has so far been shown for either the nuclear or cytoplasmic RNP. Although neither has so far been fully characterised, it has been suggested that nuclear RNP may be involved in processing and transport of the mRNA to the cytoplasm (Georgiev and Samarine, 1971; Niessing and Sekers, 1973; Price et al., 1974). Cytoplasmic mRNP, on the other hand, has been suggested as a storage mechanism for mRNAs (masked mRNA) which are not immediately required for protein synthesis, or to allow modification of the secondary structure of the mRNA to increase ribosome binding (Dyer and Leaver, in press).

5.7. Isolation and Characterisation of mRNA

The presence of 3' poly(A) sequences in many eukaryotic mRNAs provides a useful criterion for identification and isolation. However, in all methods used for isolation and characterisation of mRNA, it should be remembered that not all

mRNAs contain this sequence and that the size of the poly(A) sequence in the total cell population may vary widely.

In order to optimise extraction of poly(A)-containing mRNA during phenol-detergent extraction of RNA, monovalent ions (e.g. Na^+ and K^+), must be avoided and alkaline and low concentration buffer used to reduce loss of the mRNA due to the poly(A) binding to denatured proteins (Brawerman et al., 1972; Brawerman, 1973; Taylor, 1979). Care should also be taken to minimise nuclease degradation (Leaver, 1973).

Since mRNA is apparently the only cytoplasmic RNA containing poly(A), certain procedures have been developed for its purification which utilise the presence of this 3' sequence. An RNA mixture may be adsorbed onto oligo dT-cellulose (Nakazato and Edmonds, 1972; Aviv and Leder, 1972) or poly(U) sepharose (Adesnik et al., 1972; Firtel and Lodish, 1973) in conditions which favour complementary base pairing with the poly(A). It has been shown that oligo dT-cellulose fails to bind poly(A) sequences of less than approximately 30 nucleotides (Groner et al., 1974; Cabada et al., 1977). However, poly(U) sepharose (or poly(U) agarose) is effective in binding mRNA with poly(A) sequences of more than approximately 10 nucleotides (Baulcombe, 1977; Taylor, 1979). These techniques are useful but do not result in extraction of the total cellular mRNA since some do not contain poly(A) or have only short sequences which will not efficiently bind to the columns.

Due to the heterodisperse size of mRNA and the fact that it constitutes a small proportion of the total

cellular RNA, it cannot be purified purely on the basis of its size, as can rRNA. However, size fractionation of mRNAs in conjunction with affinity chromatography or polysome preparation may be used (Taylor, 1979). Sucrose density gradient centrifugation and denaturing polyacrylamide gel electrophoresis have thus been used successfully for purification of specific mRNAs which are relatively abundant within tissues e.g. globin mRNA (Gaskill and Kabat, 1971; Nudel et al., 1977); histone mRNA (Gross et al., 1976); ovalbumin mRNA (Buell et al., 1978); vitellinogenin mRNA (Shapiro and Baker, 1977).

The techniques previously mentioned have been used for abundant mRNA purification. However, most mRNAs do not fall into this category and thus more sensitive techniques must be utilised. A more recently developed technique, initially used for abundant mRNAs but which may also prove useful for the less abundant classes, is the polysome immunoprecipitation procedure (Taylor, 1979; Dyer and Leaver, in press). This technique involves the immunoprecipitation of specific polysomes by reaction of the nascent polypeptide with a monospecific antibody prepared against the native protein, e.g. immunoglobulin light chain mRNAs (Schecter, 1973; Burnstein et al., 1976); ovalbumin mRNA (Groner et al., 1977).

The purity of a specific purified mRNA may be determined by whether it produces a single specific polypeptide upon in vitro translation and also a single RNA peak by sucrose density gradient or polyacrylamide gel fractionation.

Another useful technique which may be utilised in

the purification of mRNAs is the preparation of cDNA probes (Taylor, 1979; Dyer and Leaver, in press). These probes can be used by selective hybridisation to enrich particular mRNA species from a partially purified mRNA population. This requires preparation of the cDNA to partially purified mRNA by reverse transcription. Large amounts of cDNA may be prepared by cloning and particular clones of interest selected by hybridisation to the specific mRNA (Kurtz and Feigelson, 1977) or by their ability to arrest in vitro translation (Hastie and Held, 1978). Selective cDNA-cellulose may also be prepared (Venetianer and Leder, 1974) and used as an affinity chromatography technique for preparation of specific mRNAs from non-purified or partially purified mRNA populations e.g. globin mRNA (Levy and Aviv, 1976); ovalbumin mRNA (Anderson Schimke, 1976); tissue specific mRNAs (Hirsch et al., 1978).

Characterisation of purified mRNAs or their specific cDNA may be achieved by oligonucleotide fingerprinting (Cowan et al., 1976), partial digestion by restriction endonucleases or chemical cleavage (Simonscits et al., 1977; Maxam and Gilbert, 1977), or sequencing by transcription of the cloned DNA. Gel electrophoresis and in vitro translation will also characterise the size and polypeptide product of a purified mRNA (for reviews see Taylor, 1979, and Dyer and Leaver, in press).

SECTION 6

SYNTHESIS OF RNA DURING GERMINATION AND EARLY SEEDLING DEVELOPMENT

Upon germination a seed undergoes the transition from the quiescent state to one of active growth and development. Protein synthesis is a necessary prerequisite for this transition since many of the enzymes of the new metabolic state are synthesised *de novo* upon germination. This *de novo* synthesis may involve translation of preformed mRNA species (Ihle and Dure, 1972, and Harris and Dure, 1978) or require synthesis of new mRNA species (Hock and Beevers, 1966). As well as mRNAs, a sufficiency of other RNA species, including rRNAs and tRNAs, is necessary to support protein synthesis.

It has been shown that some dry seed (dormant) tissue does contain ribosomes, which are capable of protein synthesis, and also preformed mRNA species (Chen *et al.*, 1968; Walbot, 1971; Weeks and Marcus, 1971; Ihle and Dure, 1972). Reports from studies on germinating seeds of cotton (Dure and Waters, 1965; Waters and Dure, 1966), wheat (Marcus *et al.*, 1966; Chen *et al.*, 1968) and pea (Barker and Hollinshead, 1964) indicated that renewed protein synthesis occurred in the absence of concomitant RNA synthesis.

Messenger RNAs found in dry seeds have been shown to be almost exclusively present in the non-polysome fraction of the cell although some are associated with the cytoplasmic ribosomal or subribosomal fraction (Brooker *et al.*, 1978). It has been suggested that these mRNAs found in dry seed tissue may be involved in the resumption of metabolism at the start of germination. There has been shown to be a rapid increase

in polyribosome content of embryonic tissue during early germination (Walbot, 1971; Spiegel and Marcus, 1975) and that this increase in polyribosome formation is independent of transcription. Thus it has been suggested that the initial stages of resumption of metabolism are a result of the translation of stored (long-lived) mRNAs (Waters and Dure, 1966; Chen et al., 1968; Weeks and Marcus, 1971; Walbot, 1972; Gordon and Payne, 1976; Taneja and Sachar, 1976; Deltour, 1977; Cuming and Lane, 1978). There has also been a suggestion that polyadenylation of at least a part of the pre-existing mRNA in the dry seeds occurs prior to the initiation of protein biosynthesis (Sieliwanowicz et al., 1977; Harris and Dure, 1978). However, stored mRNAs have been shown to contain 3' polyA sequences (Delseny et al., 1977).

The concept of long-lived mRNAs and their involvement in early protein synthesis is, however, still questioned. Delseny et al. (1977) showed that, at the onset of germination, there was an initial, gradual decline in the poly(A)$^+$ RNA content and attributed this to the decay of stored mRNA. They claimed that although this stored mRNA was utilised at the onset of germination its role was soon taken over by newly synthesised mRNAs.

Ribosomal RNA synthesis is believed to resume early, and probably after about 2 hours of imbibition (Frankland et al., 1971; Sen et al., 1975). However, in both rye and corn embryos, hnRNA, the nuclear precursor of mRNA, is apparently synthesised earlier than the rRNAs (Van de Walle, 1971; Sen et al., 1975). Rejman and Buchowicz (1973) also indicate

that during germination of wheat seeds the mRNA is synthesised before the rRNAs and tRNAs. Longer periods prior to resumption of RNA synthesis have also been described (Mayer and Shain, 1974).

In a study of RNA metabolism in the endosperm of germinating castor beans, Roberts and Lord (1979) showed that low molecular weight, ribosomal and poly(A)-containing RNAs were all present in the dormant tissue but decreased slightly during imbibition. Thereafter, there was a marked increase in all classes of RNA to reach peak levels after 3-4 days of growth. They concluded that the metabolic activity of the endosperm during this period was dependent upon transcription and not preformed RNA species in the dry tissue. Srivastava et al. (1978) have shown that new RNA species, including mRNAs, are synthesised in Bengal grown cotyledons at early stages of germination.

The majority of studies on RNA synthesis in germination have involved the use of inhibitors (see p. 37) or labelling of the RNA with radioactive precursors. These techniques have resulted in some controversy about the role of RNA synthesis during this early developmental transition. However, it is anticipated that use of assay techniques for individual mRNAs such as cell-free translation systems (see p. 38) and cDNA probes (see p. 64) will improve our understanding of the level of control of gene expression during germination and early seedling development.

It is certainly apparent that both protein synthesis and RNA synthesis are early events in the germination process.

However, which actually starts first is still a matter of doubt. Brooker *et al.* (1978) suggest that preformed mRNAs are not involved in the temporal regulation of early development but function primarily to allow rapid resumption of growth. Work by Sen and Osborne (1977) would suggest that early synthesis of RNA has an important role in determining the role of germination. They found that loss of viability of rye embryos was associated with a reduction in the capacity for RNA and protein synthesis.

SECTION 7

AIMS AND APPROACH OF THIS RESEARCH

7.1. Aims

The aim of this research was to study the molecular and biochemical changes occuring within the cotyledons of cucumber, during germination and early seedling development, as an indication of developmentally related changes in gene expression. I have been particularly interested in the level of control of gene expression for the glyoxysomal enzymes, isocitrate lyase (ICL) and malate synthase (MS), which are known to be synthesised de novo upon germination and have a specific function in the early heterotrophic growth phase.

7.2. Aporoach

The specific developmental system chosen for this study of gene expression was the fat-storing cucurbit, Cucumis sativus. This was grown under controlled environmental conditions in a light/dark (12 hr/12 hr) or complete dark growth cycle. In seedlings, such as cucumber, which exhibit epigeal germination, the cotyledons, after acting as the storage organs during dormancy and heterotrophic growth, become the first photosynthetic organs of the young seedling. This developmental transition presents an interesting change in gene expression within a specific plant organ.

Initially, the developmental system was characterised in terms of biochemical changes within the cotyledons associated with the transition from quiescence to heterotrophic growth and then to autotrophic growth. Lipid and enzyme changes characreristic of the two developmental phases were

determined. Developmentally related, quantitative and qualitative changes in the protein content of the cotyledons were also studied.

As a measure of the protein synthetic capacity of the tissue, quantitative changes in the major RNA species within the cotyledons were estimated. Quantitative and qualitative changes in the translatable mRNA population of the cytoplasm were assayed in a wheat-germ cell-free translation system.

Since specific cDNA probes for the mRNAs for the glyoxysomal enzymes, ICL and MS, were not available, changes in the levels of translatable mRNAs for these enzymes were determined using the cell-free translation system followed by immunodetection of specific *in vitro* synthesised polypeptides.

The polypeptides synthesised in the cotyledons during germination and early seedling development were analysed by pulse labelling of the tissue followed by SDS-polyacrylamide gel electrophoresis and fluorography. This gave an indication of the mRNAs actively involved in protein synthesis at the different developmental stages. By immunodetection, it was possible to determine when the mRNAs for ICL and MS were being translated *in vivo* and relate this to the developmental profiles of these specific mRNAs determined from the *in vitro* studies and also to the profiles of enzyme activity during development.

CHAPTER 2

MATERIALS and METHODS

MATERIALS

SECTION 1

1.1. Plant Material

Seeds of Cucumis sativus L. var. Long Green Ridge were obtained from Lawson & Donaldson Ltd., Edinburgh, and stored dry at room temperature.

1.2. Chemicals

Acrylamide obtained from Eastman Kodak Co., was used for SDS-polyacrylamide gel electrophoresis. Specially purified acrylamide for electrophoresis, obtained from BDH Chemicals Ltd., was used for RNA gel electrophoresis and for two-dimensional gel electrophoresis.

Recrystalised bis-acrylamide (Loening, 1967) was prepared and used for gel electrophoresis.

Ampholines pH 3·5-10 were obtained as a 40% (w/v) sterile stock solution from L.K.B. Instruments Ltd.

2,5-Diphenyloxazole (PPO) was obtained from International Enzymes Ltd.

Oligo dT-cellulose, type T_3, was obtained from Collaborative Research Inc.

Ribonuclease A (pancreatic) was kindly supplied by Dr. P. Ford, University of Edinburgh.

Sephadex G-25 (course grade) and Sepharose 4B and 6B were purchased from Pharmacia Fine Chemicals.

Staphylococcus aureus Cowan I strain was a generous gift from Dr. R. Meagher, University of Georgia, U.S.A., and the methicillan-resistant strain A676 of the same organism

from Dr. G. Krönvall, University of Lund, Sweden.

Wheat Germ was obtained from General Mills Co..

The following chemicals were obtained from Sigma Chemical Co.:-

S-Acetyl Coenzyme A, sodium salt.
Agarose for electrophoresis, type 1, low EEO.
5,5'-Dithiobis-(2-Nitrobenzoic Acid), Ellman Reagent (DTNB).
Glyoxylic Acid (glyoxylate) monohydrate, sodium salt.
DL-Isocitric Acid, type 1, trisodium salt.
Micrococcal nuclease from S. aureus.
Nicotinamide-adenine dinucleotide, reduced form.
D-Ribulose-1,5-diphosphate carboxylase from spinach.
N,N,N',N'-Tetramethylethylenediamine (TEMED).

The following chemicals were obtained from BDH Chemicals Ltd.:-

Dimethyl sulphoxide (DMSO), Me_2SO, ANALAR Grade.
Nonidet P40 (NP 40).
Sodium dodecyl sulphate, specially purified for biochemical work.

All other chemicals were obtained from Sigma Chemical Co. or BDH Chemicals Ltd. as analytical reagent grade.

1.3. Radiochemicals

[^{35}S]-Methionine obtained in aqueous form from The Radiochemical Centre, Amersham, and stored in 2 mM DTT at -80°C. The specific activity varied from 550 to 1,380 Ci/mmole.

[^{3}H]-Poly U was a generous gift from Dr. P. Ford, University of Edinburgh.

^{32}P-labelled wheat polysomal RNA was kindly provided by Dr. A. Gatenby, University of Edinburgh.

1.4. X-Ray Film

Blue Brand (or Blue Brand Regulix) x-ray film,

obtained from Eastman Kodak Co., was used for autoradiography.

X-omatic H x-ray film, obtained from Eastman Kodak Co., was used for fluorography.

METHODS

SECTION 2

2.1. Culture Conditions

Seeds of Cucumis sativus L. var. Long Green Ridge were cold imbibed at 4°C for 16-17 hours in the dark, then planted at a depth of 1 cm in trays of vermiculite underlaid with soil. The trays were either kept in continuous darkness (dark-grown), or illuminated for 12 hours per day, with a mixture of fluorescent and incandescent lamps at an approximate intensity of 6,500 lux (light-grown). The temperature was maintained at 26-28°C for the 12 hour light period, followed by a night time temperature of 22°C for 12 hours, with the same alternating temperature sequence being maintained for the dark grown plants. Trays were watered regularly, with tap water, as required.

2.2. Harvesting Conditions

Germination time was measured in days from planting of the cold-imbibed seeds. Suitable numbers of cotyledons were harvested at daily intervals, from day 0 (cold-imbibed only) to day 7 in the light and day 8 in the dark. Seed coats were removed at all stages where they were still adhering. Cotyledons for cell counts and fresh and dry weights were processed immediately upon harvesting while cotyledons for all other assays were harvested onto dry ice and stored

at -80°C for later use.

Table 1. Number of cotyledons harvested

	Day	Cell nos.	Fresh & dry wts.	Chlorophyll determination	Protein & enzyme assays	Nucleic acid extraction	Polysome extractions
Light grown tissue	0	20	40	40	20	1,000	100
	1	20	40	40	20	800	100
	2	20	40	40	20	500	50
	3	20	40	40	20	300	30
	4	20	40	40	20	150	30
	5	20	40	40	20	150	20
	6	20	40	40	20	150	20
	7	20	40	40	20	150	20
Dark grown tissue	1	10	40	40	20	1,000	100
	2	10	40	40	20	600	50
	3	10	40	40	20	400	30
	4	10	40	40	20	300	30
	5	10	40	40	20	300	20
	6	10	40	40	20	200	20
	7	10	40	40	20	200	20
	8	10	40	40	20	200	20

2.3. Cell Number Determination

The number of cells in the cucumber cotyledons was determined by the method of Brown and Rickless (1949). Samples of light and dark-grown cotyledons were placed in 5% chromic acid and stored at 4°C for 48 hours, then macerated by rapidly passing in and out of a pasteur pipette. Aliquots of the supernatant were applied to a haemocytometer slide and the cells counted.

2.4. Fresh and Dry Weight Estimations

Fresh weights were determined on 40 cotyledons harvested directly into tared, stoppered glass bottles. Oven dry weights were determined after drying the samples for 24 hours at 90°C. Longer drying periods gave no further decrease in weight.

2.5. Lipid Determination

Total lipid content of the cotyledons was determined by a modification of the procedure of Radin (1969). 3 g of tissue, containing a known number of cotyledons, were chopped with razor blades then ground in 10 ml of methanol:chloroform (2:1, v/v). Following a low speed centrifugation, at 1,400 $g_{av.}$ for 10 min, the supernatant was collected and the pellet re-extracted by grinding in fresh methanol:chloroform. Pooled supernatants from both extractions were shaken with an equal volume of 2 M KCl. After phase separation had occurred, the lower organic phase was collected into a preweighed beaker and the aqueous phase was repeatedly washed with fresh methanol:chloroform. The pooled organic phases were evaporated to dryness, in a fume cupboard, and the amount of extracted lipid was determined by weight and expressed per cotyledon.

2.6. Chlorophyll Determination

Cotyledons were assayed for chlorophyll content by a modification of the procedure described by Arnon (1949), in which the chlorophyll is extracted by solubilisation in 80% acetone and is quantitated by determining the absorbance at the absorption maxima for chlorophylls a and b and applying the equation, noted below, which relates absorbance to the

amount of chlorophyll in the acetone extract.

$$\text{Chlorophyll concentration of acetone extract } (\mu g/ml) = 20{\cdot}2\ (A_{645}) + 8{\cdot}02\ (A_{663})$$

Forty cotyledons, from each developmental stage, were macerated to a paste in a pestle and mortar. When well macerated, 5 ml of 80% (v/v) acetone were added and grinding continued till a thin slurry was formed. This was centrifuged at 1,400 $g_{av.}$ for 5 min, together with a few ml of 80% acetone, which were used to rinse out the pestle and mortar. The supernatant was collected in a volumetric flask and the pellet re-extracted with 80% acetone until the supernatant was not detectably green. The pooled supernatant was made up to 25 ml with 80% acetone and its absorbance determined at 645 nm and 663 nm by scanning the spectrum between 660 and 750 nm, after zeroing against 80% acetone at 750 nm.

Chlorophyll content per cotyledon was determined using the above equation and any appropriate dilution factors.

2.7. Homogenate Preparation

A homogenate was prepared by an adaption of the method described by Trelease et al. (1971A). Twenty cotyledons, from each stage of development, were homogenised with a Polytron homogeniser (Northern Media Supply Ltd.) at top speed, for 10, 10 and 5 seconds, in 8 ml of buffer (50 mM Tris-HCl pH 8·5, 50 mM potassium acetate, 5 mM magnesium acetate, 2 mM dithiothreitol). The homogenate was collected in tared centrifuge tubes together with a further 2 ml of buffer, which was used to rinse the probe and grinding vessel, and the total volume of the homogenate was estimated by weight.

Aliquots of the homogenate were used immediately for quantitative protein, enzyme and RNA estimations, and samples were frozen, at -20°C, for electrophoretic analysis and for quantitation of RuBP Carboxylase.

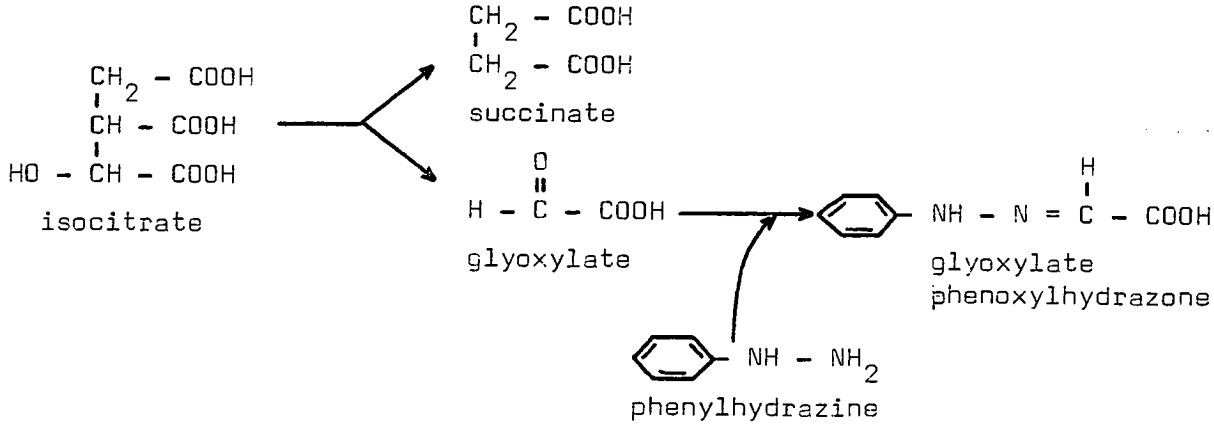
The remainder of the homogenate was centrifuged at 10,000 $g_{av.}$ for 10 min, at 4°C, in an MSE 18 centrifuge and samples of the resultant supernatant used for quantitative protein estimations or stored frozen for electrophoretic analysis.

2.8. Enzyme Assays

Samples of the homogenate previously prepared were used for the enzyme assays and dilutions of the homogenate were used where necessary.

a) Isocitrate Lyase (ICL) Assay

The assay procedure followed for this enzyme was an adaption of that described by Cooper and Beevers (1969) which determines the amount of active enzyme present by recording the change in absorbance at 324 nm due to glyoxylate, formed from cleavage of isocitrate by the enzyme, reacting with phenylhydrazine to form phenylhydrazone.



The reaction was monitored for the first 2 to 4 minutes under conditions of non-limiting isocitrate concentrations and the enzyme activity was expressed in enzyme units per cotyledon, where 1 enzyme unit is the amount of enzyme required to convert 1 nanomole of isocitrate to products per minute. A 1 ml-reaction volume was used for the assay, which was carried out in spectrophotometer cuvettes.

ICL stock solution	7·3 mM dithiothreitol
	14·0 mM $MgCl_2$
	16·0 mM phenylhydrazine
	0·1 M K-phosphate pH 6·9
ICL substrate	0·13 M DL-isocitrate, sodium salt.

(ICL stock solution is unstable and poisonous, and can be stored at the most for a few days if kept cold and dark, but should not be frozen).

Distilled water was used to zero the spectrophotometer, then 0·8 ml of the ICL stock solution was added to each cuvette. 0·1 ml of distilled water was added to the control cuvette and 0·1 ml of ICL substrate to the reaction cuvette. In rapid succession, 0·1 ml of the homogenate sample was added to each cuvette and mixed well before monitoring the absorbance values, at 324 nm, at 30 s intervals between 1 and 4 min after the start of the reaction. Using the following equation, the enzyme units of ICL present in the 0·1 ml sample was calculated.

$$\text{enzyme units/sample} = \Delta A \times 29{\cdot}4^{*}$$

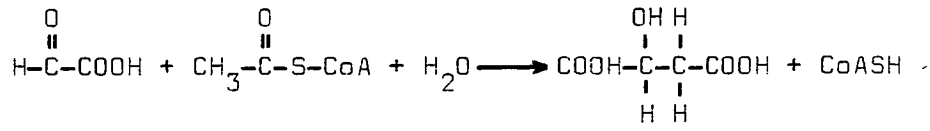
where $\Delta A = A_{4\ \text{min}} - A_{2\ \text{min}}$

b) Malate Synthase (MS) Assay

Malate synthase activity in the cucumber cotyledons during germination was measured by the adaption of Köller and

*see appendix

Kindl (1977) of the method described by Hock and Beevers (1966). This enzyme catalyses the following reaction:-



glyoxylate acetyl CoA malate

To determine the malate synthase activity, advantage was taken of the production of free sulphydryl groups due to the formation of CoA from acetyl CoA. Since DTNB (Ellman Reagent; Ellman, 1959) reacts with sulphydryl groups to produce a yellow complex, this reaction was used for a direct photometric assay of the malate synthase by determining the change in absorbance at 412 nm. Using total homogenate protein samples (p. 77), diluted if required, the assay was carried out in a total volume of 1 ml.

MS substrate solution A	1 M Tris-HCl pH 8
	1 mM DTNB (Ellman reagent)
	50 mM $MgCl_2$
	40 mM Na-glyoxylate
MS substrate B	0·5 mM acetyl CoA

The spectrophotometer was zeroed at 412 nm using distilled water in both cuvettes. These were emptied and 0·7 ml distilled water, 0·1 ml MS substrate solution A and 0·1 ml of the homogenate sample were added to each. To start the reaction, 0·1 ml of MS substrate solution B was added to the sample cuvette and 0·1 ml distilled water to the blank cuvette and these were mixed well. Absorbance readings were taken at 30 s intervals between 1 and 4 min after addition of the acetyl CoA. Using the following equation, the enzyme units of MS present in the 0·1 ml

sample were calculated

$$\text{enzyme units/sample} = \Delta A \times 37{\cdot}5 \text{ *}$$

where $\Delta A = A_{4\ min} - A_{2\ min}$

MS activity was expressed in enzyme units per cotyledon, where 1 enzyme unit is the amount of enzyme required to convert 1 nanomole of each of the substrates to malate per minute.

c) Catalase (CAT) Assay

Catalase activity during germination was assayed by the method of Lück (1963). The reaction, shown below, was monitored by direct observation of the decrease in absorbance at 240 nm, the absorption maximum of H_2O_2.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Total homogenate protein (p. 77) was used for the determination and a 3 ml-reaction volume was used for the assay which was carried out in spectrophotometer cuvettes. Because the reaction follows first order kinetics (non-linear) it was necessary that the cuvettes were carefully balanced to ensure that the absorbance reading of the sample would exactly equal that of the blank when all the H_2O_2 was consumed.

CAT buffer solution	0·006% (v/v) Triton X-100
	50 mM K-phosphate pH 7·2
CAT substrate solution	0·1% (v/v) H_2O_2
	0·0006% (v/v) Triton X-100
	50 mM K-phosphate pH 7·2

Catalase buffer was used in both cuvettes to zero the spectrophotometer. Catalase substrate was then placed in the sample cuvette in place of the CAT buffer, and the spectrophotometer reading, which should be in the range 0·5

*see appendix

to 0.7, determined and observed over several minutes to ensure that the absorbance remained constant.

Both cuvettes were then emptied and 2·9 ml CAT buffer added to the control cuvette and 2·9 ml CAT substrate to the sample cuvette, then 0·1 ml of the homogenate sample added to each cuvette and mixed well. The absorbance reading was observed and timed between 0·500 and 0·400 O.D. (t; to the nearest 0·1 s). If, by 2 minutes, the absorbance had decreased less than 0·100 O.D. the absorbance reading was noted. Using the following equation, the enzyme units of CAT present in the 0·1 ml sample were determined.

$$\text{enzyme units/sample} = \frac{\log_{10} R \times 332{\cdot}3}{t}^{*}$$

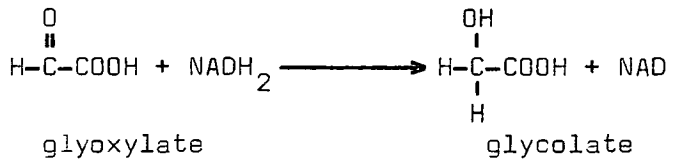
where $R = \frac{\text{initial absorbance}}{\text{final absorbance}}$

and t = time taken for change from initial to final absorbance

Since the assay reaction follows first order kinetics, one unit of CAT is defined as that amount of enzyme required to degrade 50% of the H_2O_2 to H_2O and O_2 in 100 seconds. Catalase activity in the cotyledons was calculated and expressed as enzyme units per cotyledon.

d) Glyoxylate Reductase (GR) Assay

Glyoxylate reductase activity during germination was measured by the procedure described by Tolbert (1971). Reduction of glyoxylate to glycolate is accompanied by the oxidation of $NADH_2$ to NAD, as shown.



*see appendix

Since the reduced but not the oxidised form of the coenzyme absorbs at 340 nm, the reaction was monitored by recording the decrease in absorbance at 340 nm, as function of time, during the first several minutes of the reaction, under non-limiting glyoxylate and $NADH_2$ concentrations. The assay procedure, using total homogenate protein samples (p.77), is described for a 1 ml-reaction volume.

GR stock solution	0·22 mM $NADH_2$
	0·2% (v/v) Triton X-100
	0·1 M K-phosphate pH 6·2
GR substrate	0·75 M sodium glyoxylate pH 6·2

In order to register the decrease in absorbance, due to $NADH_2$ consumption as the reaction proceeds, as an increase in readings, the sample and blank cuvettes were reversed from their normal positions during this assay. Distilled water was used to zero the spectrophotometer. Both cuvettes were emptied and 0·8 ml of GR stock solution added to each, then 0·1 ml of distilled water to the control cuvette and 0·1 ml of GR substrate to the sample cuvette. To start the reaction, 0·1 ml of the sample was added to each in rapid succession and mixed well. Absorbance readings were measured at 30 s intervals between 1 and 4 min after starting the reaction. Using the following equation, the enzyme units of GR present in the 0·1 ml sample were estimated.

$$\text{enzyme units/sample} = \Delta A \times 50{\cdot}0^{*}$$

where $\Delta A = A_{4\ \text{min}} - A_{2\ \text{min}}$

Glyoxylate reductase activity was expressed in enzyme units per cotyledon, where 1 enzyme unit is that

*see addpendix

amount of enzyme required to convert 1 nanomole of glyoxylate to glycolate per minute.

2.9. Quantitative Estimation of RuBP Carboxylase Protein

Ribulose bis-phosphate carboxylase was determined by electrophoretic fractionation of total protein under non-denaturing conditions. Recovery and quantitation of the stained protein, after excision of the appropriate gel band, was carried out.

Gels, 8 cm in length and 0·7 cm diameter, of 5% (w/v) acrylamide, 0·125% (w/v) bis acrylamide, 0·0875 M Tris-glycine pH 9·5, were polymerised with 0·034% (v/v) TEMED and 0·075% (w/v) AMPS. The gels were overlaid with water for 45 min until polymerisation had occurred, then were pre-run, from cathode to anode, for 30 min at 1-1·5 mA per gel with 50 mM Tris-glycine pH 9·5 electrophoresis buffer.

Total homogenate protein (p. 77), 100-200 µg per gel, was then loaded in 50 mM Tris-glycine pH 9·5 containing 10% (w/v) sucrose and bromophenol blue dye as marker. Electrophoresis was carried out for 2·5 hours, then the gels were removed and stained in 0·5% (w/v) amido black 10B in 20% (v/v) ethanol, 7% (v/v) glacial acetic acid for 20 hours followed by destaining in 20% ethanol, 7% glacial acetic acid. Identification of the carboxylase band was achieved by co-electrophoresis with purified enzyme, then the band was excised and incubated overnight at 25^{o}C in 1 ml of 1N NaOH in sealed tubes. The protein content of the NaOH extract was determined from the absorbance reading at 615 nm on an SP 8,000 spectrophotometer (Pye Unicam). Quantitation was

possible as a standard curve was prepared for electrophoresis using spinach D-ribulose-1,5,bisphosphate carboxylase (Sigma Chemical Co.).

2.10. Protein Estimation by Folin and Ciocalteus Phenol Reagent

The protein content of both the total homogenate (total protein) and the 10,000 $g_{av.}$ supernatant ('soluble protein') samples (p. 77) was determined by an adaption of the method described by Lowry *et al.* (1951), whereby production of a blue colour relies upon the reduction of the Folin reagent by the amino acids tyrosine, tryptophan and cysteine.

200 µl aliquots of the protein preparations were precipitated with an equal volume of 8% (w/v) TCA and kept on ice for 15-30 min. The protein precipitate was pelleted by centrifugation at 2,000 $g_{av.}$ for 10 min, in an MSE LR4 centrifuge, and washed twice with 4% (w/v) TCA, resuspending each time by vortex mixing. The final pellet was hydrolysed in a specific volume of 0·4 N NaOH, in sealed tubes, either overnight at room temperature or for 1 hour at 45°C.

Suitable aliquots of the protein hydrolysate were assayed in a final volume of 1 ml in 0·1 N NaOH. These samples were used directly for the protein estimations. The following stock solutions were prepared.

A. Fresh alkaline copper tartrate containing 2·5% (w/v) $CuSO_4 \cdot 5H_2O$, 6% (w/v) NaK tartrate, 6% (w/v) Na_2CO_3 in 0·05 N NaOH in a 1:1:100 ratio.

B. Commercial Folin and Ciocalteus phenol reagent (B.D.H.) diluted to 0·3 N (3 ml diluted to 24 ml with distilled

water).

C. A standard solution of Bovine Serum Albumin (BSA) in 0·4 N NaOH containing 1 mg/ml.

A standard curve of 10, 20, 40, 80 and 100 µg of BSA was prepared for each assay in a volume of 1 ml in 0·1 N NaOH.

To each 1 ml sample, 1 ml of the alkaline copper tartrate (A) was added, mixed well and allowed to stand for 30 min for the complete formation of the protein-copper complex. 1 ml of 0·3 N Folin's reagent (B) was added to each tube and mixed well immediately upon addition. After 30 min the blue colour which had developed was determined with a Corning Colourimeter 252 using a red (600 nm) filter and the protein content of the cucumber cotyledon samples was determined relative to the BSA calibration curve.

2.11. Electrophoretic Analysis of Cotyledonary Proteins

a) Discontinuous SDS-Polyacrylamide Slab Gel Electrophoresis

Both homogenate and 'soluble' protein changes occurring in the cotyledons were followed by SDS-polyacrylamide slab gel electrophoresis carried out by a modification of the method described by Laemmli (1970). A discontinuous gel system (Ornstein, 1964; Davis, 1964; Stewart and Barber, 1964) was routinely used with an approximate 7·2 ml stacking gel of 5% (w/v) acrylamide (1·3 mm x 35 mm x 158 mm) and a 34·5 ml separating gel of 15% (w/v) acrylamide (1·3 mm x 168 mm x 158 mm). On occasions a 10% (w/v) acrylamide separating gel was used. The following solutions were prepared and stored as indicated.

A. Acrylamide stock solution — 30% (w/v) acrylamide
0·2% (w/v) bis-acrylamide

This solution was filtered upon preparation and stored in the dark at 4°C for up to 4 weeks.

B. x5 Separating gel buffer — 1·875 M Tris-HCl pH 8·8
(stored at 4°C)

C. x10 Stacking gel buffer — 0·6 M Tris-HCl pH 6·8
(stored at 4°C)

D. Electrode buffer — 0·025 M Tris base
0·192 M glycine
0·1% (w/v) SDS
(freshly prepared)

E. Sample buffer — 60 mM Tris-HCl pH 6·8
<2% (w/v) SDS
<5% (w/v) BME
10% (w/v) sucrose

Sample buffer was prepared at double strength and stored at -20°C.

The final properties of the stacking gel and separating gel were:-

5% stacking gel

5% (w/v) acrylamide
0·033% (w/v) bis-acrylamide
0·06 M Tris-HCl pH 6·8
0·1% (w/v) SDS
0·1% (v/v) TEMED
0·05% (w/v) AMPS

15% separating gel

15% (w/v) acrylamide
0·1% (w/v) bis-acrylamide
0·375 M Tris-HCl pH 8.8
0.1% (w/v) SDS
0.05% (v/v) TEMED
0·05% (w/v) AMPS

The separating gel was poured into the prepared glass plates and overlaid with water to effect a flat surface and allow polymerisation to proceed in the absence of oxygen. After 45 min the water overlay was removed, the gel surface rinsed with 0·5 ml of unpolymerised stacking gel solution, then the stacking gel poured and either a 10 or 20 slot comb inserted. When polymerisation was completed, after 45 min, the comb was removed and the sample wells rinsed with electrode buffer to remove any non-polymerised gel solution. The bottom spacer was removed from between the glass plates to allow complete electrical contact between the upper and lower reservoir buffers via the gel, then the gel plates were set up in the electrophoresis tank and the sample wells filled with electrode buffer.

The protein samples, which had been previously prepared, were heated for 15 min at 70^{o}C then loaded onto the gel and complete electrical contact was achieved using a 3 MM chromatography paper wick (double thickness) between the top of the gel and the upper reservoir buffer. Electrophoresis, from cathode to anode, was carried out overnight, at room temperature, at a constant current between 10–15 mA per gel, until the bromophenol blue marker dye reached the bottom of the gel.

Fixing and staining were carried out in 0·2% (w/v) Coomassie blue (Brilliant R) in 45% (v/v) methanol, 7% (v/v) glacial acetic acid for at least 1 hour then the gel was destained in several changes of fresh destaining solution (45% methanol, 7% glacial acetic acid). When the gel was suffici-

ently destained it was dried down at 70°C under vacuum onto a piece of 3 MM chromatography paper (Whatman).

In order to determine molecular weights of the polypeptides being studied, standard molecular weight markers were used on each gel. These standard markers and their molecular weights (Weber and Osborn, 1975; Lambin, 1978) are shown below.

	Molecular Weights
Bovine serum albumin	68,000
Catalase (bovine liver)	60,000
Albolase	40,000
Carbonic anhydrase	29,000
Trypsin inhibitor (soybean)	21,000
Myoglobin	17,200
Lysogyme	14,300

Gels for total and 'soluble' proteins were loaded on a per cotyledon basis so that quantitative changes in cotyledonary protein content could be observed. On a 20 slot gel, 1/40 of a cotyledon of 'soluble' protein and 1/60 of a cotyledon of total protein were loaded.

b) IEF-SDS-Polyacrylamide Gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis of the 10,000 g 'soluble' proteins was carried out by the procedure described by O'Farrell (1975) with several modifications. This method gives high resolution and sensitivity by separating the proteins by two independent parameters. Proteins were separated according to their isoelectric point by isoelectric focusing in the first dimension and according to molecular weight by SDS-polyacrylamide gel electrophoresis in the second dimension.

i) Solutions prepared

A. Lysis buffer 9·5 M Urea
2% (w/v) Ampholines, pH 3·5–10
5% (w/v) BME

D. 30% acrylamide stock 28·38% (w/v) acrylamide
1·62% (w/v) bis-acrylamide

H. Gel overlay 8 M Urea

K. Sample overlay 9 M Urea
1% (w/v) Ampholines, pH 3·5–10

L. Lower gel buffer 1·5 M Tris-HCl, pH 8·8
0·4% (w/v) SDS

M. Upper gel buffer 0·5 M. Tris-HCl, pH 6.8
0.5% (w/v) SDS

N. 30% acrylamide stock 29·2% (w/v) acrylamide
0·8% (w/v) bis-acrylamide

O. SDS buffer 10% (w/v) glycerol
5% (w/v) BME
2% (w/v) SDS
0·0625 M Tris-HCl, pH 6·8

R. SDS Electrophoresis buffer 0·025 M Tris base
0·192 M glycine
0·1% (w/v) SDS

S. Sample buffer 10 M Urea
0·001 M EDTA, Na_2
0·01 M Na phosphate, pH 7
2% (w/v) BME
2% (w/v) Ampholines, pH 3·5–10

Solutions A, H, K and S were stored frozen at $-20^{o}C$ and D, L, M, N and O were stored at $4^{o}C$. Acrylamide stock solutions were filtered upon preparation and stored in the dark. Electrophoresis buffer (R) was freshly prepared each time.

ii) Sample preparation

Aliquots of the 10,000 g 'soluble' protein samples (prepared as previously described, p. 77) were acetone

precipitated and washed twice with 80% (v/v) acetone, with the pelleted protein being recovered by centrifugation at 2,000 $g_{av.}$ for 20 min, in an MSC LR4 Centrifuge. To ensure that all ions were washed out of the protein, the samples were kept overnight at $4^{o}C$, in one of the acetone washes. The final protein pellet was air dried, then resuspended in an appropriate volume of sample buffer(S) such that 100-200 µg of protein could be loaded on the gels in a maximum volume of 40 µl.

iii) 1st dimension - Isoelectric focusing (IEF)

IEF gels were prepared in silicone treated glass tubes, 2·5 mm internal diameter x 130 mm long, according to the method of O'Farrell (1975).

Gel mixture:-		
	10% (w/v) NP40	2 ml
	Urea	5·5 gm
	acrylamide stock (D)	1·33 ml
	water	1·97 ml
	Ampholines, pH 3·5-10	0·5 ml
	TEMED	7 µl
	10% (w/v) AMPS	10 µl

A final concentration of approximately 4% acrylamide provides sufficiently large pores to offer little restriction on the electrophoretic mobility, so that even high molecular weight proteins should achieve their isoelectric point on the pH gradient produced by the ampholines. The high level of urea (9·2 M), not only denatures and solubilises the the protein, but also confers some degree of mechanical stability to the weak acrylamide gel.

Polymerisation was allowed to proceed for 1-2 hours with the gel overlaid with 20 µl of solution H, then a further

1-2 hours with 20 µl lysis buffer (A) as the overlay. 20 µl of fresh lysis buffer (A) was overlaid, the gel tubes were filled with 0·2% (v/v) H_2SO_4 and the gels pre-run for 1·5-2 hours at 200 volts (constant) from anode to cathode using 0·2% (v/v) sulphuric acid (anode) and 0·5% (v/v) ethanolamine (cathode) as electrode buffers in place of those used by O'Farrell (1975). Electrophoresis was carried out in the opposite direction to that employed by O'Farrell (1975), but the same as that used by O'Farrell et al. (1977) when improving the resolution of basic proteins. Pre-running the gels, as well as starting to set up the pH gradient with the ampholines, removes the oxidising agent, ammonium persulphate.

At the end of the pre-run the overlay was removed, the samples loaded on the gels, overlaid with 10 µl sample overlay (K) and then the tubes were filled up with anode buffer. Fresh anode buffer was used for the electrophoresis which was for 18 hours at 200 volts (constant) at 20°C (regulated). Upon completion of electrophoresis the gels were each equilibrated in 5 ml of buffer O to remove much of the ampholines, replace the urea with SDS, and to move Cl^- into the gels. The gels were stored frozen, at - 80°C, after equilibration.

A blank IEF gel was used to determine the pH gradient. It was divided into 0·5 cm sections and each was incubated in 2 ml degassed water for 2 hours, the contents mixed and the pH of each determined with an E.I.L. pH meter, Model 38A.

iv) 2nd dimension - SDS-polyacrylamide gel electrophoresis

Focused polypeptides were further separated according to their molecular weight by electrophoresis on a discontinuous SDS slab gel (Laemmli, 1970; Davis, 1964; Ornstein, 1964; Stewart and Barber, 1964). The apparatus used was a Bio-Rad Model 220 Dual vertical slab gel electrophoresis cell with the glass plates modified, as described by O'Farrell (1975), to contain a notch. The gels were cooled by water contained in the internal chamber of the apparatus.

A 14·5 ml separating gel (0·8 mm x 154 mm x 105 mm) was prepared as a 10·5%-15% exponential gradient using the following acrylamide mixtures.

Weak acrylamide

buffer, L	3·75 ml
acrylamide stock, N	5·25 ml
water	6 ml
10% (w/v) AMPS	21·75 µl
TEMED	7·05 µl

Strong acrylamide

buffer, L	1·5 ml
acrylamide stock, N	3 ml
75% (w/v) glycerol	1·5 ml
10% (w/v) AMPS	8·7 µl
TEMED	3·0 µl

After the gradient separating gel was polymerised (c.a. 1 hour) it was left overnight, with an overlay of 4 x diluted buffer L, to ensure complete polymerisation. A 4 ml, 4·75% stacking gel was poured to a height of 2 cm, a flat surface effected with a perspex spacer, and allowed to polymerise for 45 min.

Stacking gel - 8 ml for 2 gels

buffer, M	2 ml
acrylamide stock, N	1·2 ml
water	4·8 ml
10% (w/v) AMPS	24 µl
TEMED	8 µl

Agarose was dissolved in buffer O, at a concentration of 1% (w/v), by heating to 90°C, then cooled to 50°C. After removing excess liquid from a thawed 1st dimension gel, the gel was attached to the top of the stacking gel by embedding in the agarose solution. Electrophoresis was carried out, from cathode to anode, at 20 mA (constant) per gel for approximately 3 hours, or until the bromophenol blue marker dye, added to the electrophoresis buffer (R) in the top reservoir, ran off the gel.

v) Staining and destaining of slab gels

After separation in the second dimension, the gels were fixed and stained for 30 min in 0·1% (w/v) Coomassie Blue (Brilliant R) in 50% (v/v) methanol, 7% (v/v) glacial acetic acid, destained in 3 washes of 25% (v/v) ethanol, 8% glacial acetic acid, for 40 min each wash, then in 5% methanol, 7% glacial acetic acid for at least 15 min to remove the ethanol. The gels were dried down at 70°C under vacuum onto 3 MM chromatography paper.

2.12. Quantitative RNA Estimation

Homogenate samples (p. 77) were assayed by the method of Schmidt and Thannhauser (1945) and the RNA content per cotyledon estimated for each of the developmental stages being studied.

Samples were precipitated with an equal volume of 1 N perchloric acid, left on ice for 60 min, then centrifuged at 3,000 $g_{av.}$ for 10 min. The pellets were washed twice by resuspension in 5 ml of 0·5 N perchloric acid, twice with ethanol:chloroform:ether (2:1:1, v/v/v) then drained dry and dissolved in 1·0 ml fresh 0·3 N KOH. After incubation for 18 hours at 37°C, in sealed tubes, to hydrolyse the RNA, the samples were cooled on ice then neutralised with cold 70% perchloric acid. The resulting precipitate was removed by centrifugation at 3,000 $g_{av.}$ for 10 min then the supernatant assayed for RNA content by determination of the A_{260} (assuming that an A_{260} of 10 corresponds to an RNA concentration of 31·7 µg/ml).

2.13. Extraction of Total Nucleic Acid

The cotyledons used for the nucleic acid preparation were stored frozen at -80°C until required. A range of 1,000 to 150 cotyledons (p. 75) were used for the extraction depending upon the stage of development of the tissue. Cotyledonary nucleic acid was extracted at 4°C (Leaver, 1973) by the phenol-detergent procedure of Leaver and Ingle (1971).

Homogenisation was achieved using a Polytron homogeniser operated for 20-30 seconds at top speed. 40 ml (c.a. 3 volumes) of extraction buffer (100 mM Tris-HCl pH 8·5, 6% (w/v) PAS, 1% (w/v) TNS) were used for the initial homogenisation, then a further 10 ml was used to rinse the Polytron probe and the grinding vessel. The homogenate was mixed well with an equal volume of phenol-cresol-8HQ (400g:40 ml:0·4g; saturated with 10 mM Tris-HCl pH 8·5), then centrifuged at 1,400 $g_{av.}$,

in an MSE Mistral 4L, for 10 min at 4°C, and the aqueous phase was further washed with fresh phenol-cresol until the interface was clean. Removal of the upper aqueous phase was carried out with care to obtain as quantitative a recovery of nucleic acid as possible.

The nucleic acid was precipitated from the final aqueous phase with 2·5 volumes of cold ethanol. After storing at -20°C overnight the nucleic acid was recovered by centrifugation at 1,400 $g_{av.}$ for 10 min, then washed twice with 80% ethanol and dried in a dessicator. The nucleic acid was then resuspended in a known volume of sterile distilled water and the concentration determined from the A_{260} reading in an SP8,000 spectrophotometer. Assuming 1·0 O.D. unit at 260 nm corresponds to 40 µg/ml of nucleic acid, the amount of phenol extractable nucleic acid per cotyledon could be estimated.

All steps of this preparation were carried out at 4°C in order to maintain molecular integrity of the chloroplast 23S rRNA (Leaver, 1973).

The nucleic acid extracted by this method was used for electrophoretic fractionation (p. 96), extraction of poly(A)$^+$ RNA (p. 102), direct poly(A) estimation (p. 104) and for programming a wheat germ cell free protein synthesising system (p. 105).

2.14. Fractionation and Quantitation of Nucleic Acid

Phenol-detergent extracted nucleic acid preparations were fractionated by electrophoresis on 2·4% cylindrical polyacrylamide gels by the method described by Loening (1967) as modified by Leaver (1973).

Acrylamide stock solution

15% (w/v) acrylamide

0·75% (w/v) bis-acrylamide

This solution was filtered upon preparation and stored in the dark at 4°C for up to 1 month.

Stock buffer (5 x E)

43·5 g Tris base

46·8 g $NaH_2PO_4.2H_2O$

3·7 g EDTA $Na_2 2H_2O$

The above were made up to 2 litres with distilled water. For use as electrophoresis buffer (E), dilute 5fold and make 0·05% (w/v) SDS.

Standard 2·4% gels

5 ml stock acrylamide

6·25 ml stock buffer (5 x E)

19·7 ml distilled water

25 µl TEMED

250 µl 10% (w/v) AMPS

Gels, 8 cm long x 0·7 diameter, were overlaid with water and allowed to polymerise for 30 min, then kept at 4°C for at least 2 hours. The gels were pre-run for 30 min at 50 volts (c.a. 6 mA/gel) at 4°C, from cathode to anode, to remove the catalysts and allow SDS to enter the gel. Samples of about 25 µg of nucleic acid were loaded onto the gels in electrophoresis buffer (E) made 5% (w/v) with sucrose. Electrophoresis was carried out for 3·5 hours at 50 volts at 4°C (Leaver, 1973).

After electrophoresis, the gels were removed and soaked for 1 hour in distilled water to remove any U.V. absorbing background material prior to scanning the gels at 265 nm using a Joyce Loebel U.V. Scanner.

These scans were used, together with the results

from the RNA assay (p. 94) for quantitative estimations of the changes in cytoplasmic and chloroplast RNAs, and low molecular weight RNA in the cotyledons during germination.

2.15. Extraction of Polysomes and Polysomal RNA

a) Total Polysome Extraction

Polysomes were extracted from frozen cotyledonary tissue (p. 75) by the adaption by Leaver and Dyer (1974) of the method of Davis et al. (1972), and polysomal RNA was subsequently prepared by phenol-detergent extraction (Leaver and Ingle, 1971). The following solutions were used for the extraction.

A. Polysome extraction buffer

100 mM Tris-acetate pH 8·5
0·25 mM sucrose
200 mM potassium acetate
20 mM magnesium acetate
1 mM dithiothreitol

B. 1M sucrose cushion

1 M sucrose
100 mM Tris-acetate pH 8·5
200 mM potassium acetate
20 mM magnesium acetate
1 mM dithiothreitol

C. Triton buffer solution

20% (v/v) Triton X-100
100 mM Tris-acetate pH 8·5
200 mM potassium acetate
20 mM magnesium acetate
1 mM dithiothreitol

D. Resuspension buffer

50 mM Hepes-KOH pH 7·6
50 mM potassium acetate

5 mM magnesium acetate

1 mM dithiothreitol

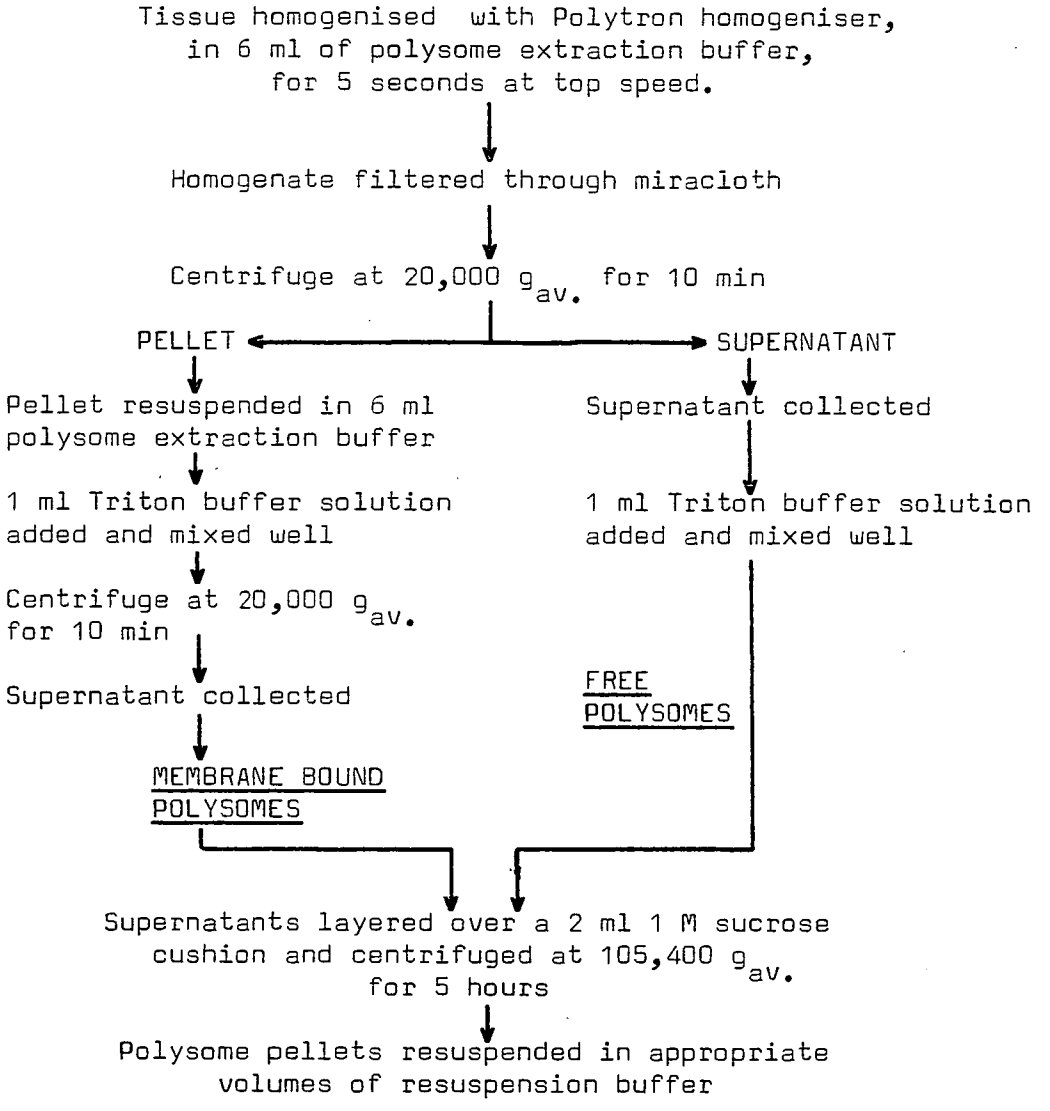
The tissue was homogenised, using a Polytron homogeniser, in 7·5 ml polysome extraction buffer (A), for 5 seconds at top speed. 1·25 ml Triton buffer solution (C) was added and the sample mixed well then filtered through miracloth and centrifuged at 20,000 $g_{av.}$ for 10 min, in an MSE 18 centrifuge, at 4°C. The resultant supernatant was layered onto a 2 ml 1 M sucrose cushion (B) and centrifuged for 5 hours at 105,400 $g_{av.}$ in a Spinco type 40 rotor. An appropriate volume of resuspension buffer (D) was used to resuspend the polysome pellet and samples removed for A_{260} determination and translation in a wheat germ in vitro system (p. 105).

b) Polysomal RNA Extraction

5 ml of nucleic acid extraction buffer (6% (w/v) PAS, 1% (w/v) TNS, 100 mM Tris-acetate pH 8·5) was added to the resuspended polysomes and the samples were well shaken before the RNA was prepared by phenol-detergent extraction and ethanol precipitation as previously described (p. 95).

c) Extraction of Free and Membrane Bound Polysomes

Preparation of free and membrane-bound polysomes in the cucumber cotyledons was carried out using light-grown tissue from days 0 to 5 of the developmental sequence (Larkins et al., 1976; Verma and MacLachlan, 1976). The solutions used were those described for the total polysome preparation and the procedure is outlined in the following flow diagram.



Both free and membrane polysomes were used for an A_{260} determination, to programme a wheat germ *in vitro* system (p. 105) and for polysomal RNA preparations (p. 99).

d) Measurement of Ribonuclease Activity Associated with Extracted Polysomes

This experiment was carried out in conjunction with Dr. J. M. Grienenberger. Free and total polysomes from spinach leaves and total polysomes from 3·5 day, light grown cucumber cotyledons were prepared as previously described (p. 99).

Samples of the three types of polysomes were added

to 13 µl ^{32}P-labelled polysomal RNA (100 µg/µl) in 2 x SSC buffer. Controls containing no added polysomes and two concentrations of RNAase A (pancreatic) in place of polysomes were carried out. The samples were incubated at 37°C for 30 min and 15 µl aliquots taken at 5, 10, 15, 20 and 30 min. Determination of radioactivity in TCA-precipitable RNA was carried out by adding the aliquots to 1 ml 10% (w/v) TCA containing carrier tRNA (30 µg) and standing on ice for 30 min then collecting the precipitate on 2·5 cm Schleicher and Schüll BA85 filters (pore size 0·45 µm). The filters were dried and counted in 5 ml scintillation fluid (8PD: Toluene; 4 g/l) in an SL30 Liquid scintillation spectrometer (Intertechnique).

e) Sucrose Density Centrifugation of Polysomes

This experiment was carried out in collaboration with Dr. J. M. Grienenberger. Free polysomes were prepared from 3 day light-grown tissue as previously described (p. 99) or by purification on a Sepharose 6B column (31 cm long x 3 cm internal diameter) instead of pelleting through a sucrose cushion. The column buffer was similar to the polysome extraction buffer (p. 98) except that the 0·25 M sucrose was omitted, and the fraction collected was the excluded volume. Polysomes prepared by both procedures were centrifuged on 5·1 ml, 15-60% (w/v) linear sucrose gradients at 149,000 $g_{av.}$ for 1·5 hours in a Spinco SW50 rotor and the polysome profile determined using an ISCO Model UA-4 absorbance monitor, and a Vitatron chart recorder. Both methods of polysome preparation were compared for the size range and distribution of monosomes

and polysomes.

2.16. Preparation of Poly(A)-containing RNA

RNA fractions containing poly(A) sequences on their 3' hydroxyl end were prepared by oligo dT-cellulose chromatography (Aviv and Leder, 1972; Bantle et al., 1976; Brawerman, 1974; Singer and Penman, 1973). Grade T_3 oligo dT-cellulose, which contains chains of up to 20 nucleotides (thymidine 5'-phosphate) in length, was used. This was stored as a wet slurry in 0·1 M NaOH at 4°C and regenerated for repeated use. As an antibacterial agent, 0·2% (w/v) sodium azide was used during storage.

Extraction of poly(A)-containing RNA was carried out by the column procedure of Aviv and Leder (1972).

A total loading volume of 10 ml was used on a 1·6 ml oligo dT-cellulose column (containing approximately 0·4 g oligo dT-cellulose) which had been equilibrated with high salt buffer (HSB-10 mM Tris-HCl pH 8·5, 0.4 M NaCl). Approximately 10 mg of total nucleic acid (p. 95) was placed in a pre-weighed tube and to this was added 0·1 ml 1 M Tris-HCl pH 8·5 and sterile distilled water to a volume of 9 ml. The sample volume was estimated by weight and duplicate A_{260} readings taken before the sample was heated at 60°C for 1·5 min then quick cooled on ice. NaCl was added to a final concentration of 0·4 M, then the sample loaded on the column, and the flow through repassed through the column twice. The column was then washed with 5 x 1 ml HSB and this was collected along with the unbound nucleic acid in preweighed tubes. This fraction, which contained all nucleic acid which did not

bind to the column, was hereafter called the poly(A)$^-$ fraction.

20 ml of HSB was used to further wash the column, then the bound fraction eluted with 6 x 1 ml of low salt buffer (LSB - 10 mM Tris-HCl pH 8·5). This eluted RNA was heated at 60^{o}C for 1·5 min, quick cooled on ice, made 0·4 M w.r.t. NaCl and reloaded on the column, which had been re-equilibrated with 10 ml HSB. 10 ml of HSB was used to wash the rebound material thoroughly then the poly(A)$^+$ fraction eluted with 4·5 ml LSB into preweighed tubes.

Volumes and nucleic acid contents of the poly(A)$^+$ and poly(A)$^-$ fractions were determined by weight and A_{260} estimations. The nucleic acid was ethanol precipitated overnight, after the poly(A)$^+$ fraction was made 2% (w/v) with potassium acetate to aid precipitation, and collected by centrifugation. Nucleic acid pellets were dried in a pre-cooled dessicator then resuspended in sterile distilled water and the nucleic acid content determined by estimation of the A_{260}. Extractable poly(A)$^+$ RNA per cotyledon was estimated for each stage of germination.

Sucrose density gradients of extracted poly(A)$^+$ RNA from light and dark-grown days 3 cotyledons were prepared (in conjunction with Dr. J. M. Grienenberger) on 5·1 ml, 5–35% (w/v) linear sucrose gradients in 100 mM Hepes-KOH pH 7·5, 0·1 M KCl buffer. Centrifugation was at 149,000 $g_{av.}$ for 6 hours in a Spinco SW50 rotor. The poly(A)$^+$ profile was determined using an ISCO Model UA-4 absorbance monitor and a Vitatron chart recorder.

2.17. Poly(A) Estimation by [^{3}H]-Poly U Hybridisation

Estimation of the poly A content of the total nucleic acid, poly(A)$^+$ RNA and poly (A)$^-$ RNA was carried out by the procedure described by Bishop *et al.* (1974). Suitable dilutions were prepared and the concentrations determined by estimation of the A_{260} prior to taking samples for the poly(A) estimation. The following samples were used in duplicate for each developmental stage.

i) 1 µg and 2 µg of total nucleic acid.
ii) 10 ng and 20 ng of poly(A)$^+$ RNA.
iii) 2 µg and 5 µg of poly(A)$^-$ RNA.

1 ml of ice cold 2 x SSC containing [^{3}H]-poly U (c.a. 5,000 c.p.m.) was added to each of the nucleic acid samples. After incubation for 30 min at 45^{o}C the samples were cooled on ice for 5 min then 20 µg of RNAase A (pancreatic) in 2 x SSC added and the samples kept on ice for 20 min to allow digestion of any non-hybridised [^{3}H]-poly U. 0·5 ml 4% (w/v) CTAB was added to precipitate the RNA and 0·5 ml of 1 M NaCl pH 5 containing 2 mg/ml yeast RNA, which acts as a carrier for the precipitation. Incubation for 2-5 min at 30^{o}C dissolved the CTAB and ensured the RNA was precipitated. Samples were filtered onto 2·5 cm GF/C Whatman filters, dried, and counted in 5 ml scintillation fluid for 10 min in an SL 30 liquid scintillation spectrometer.

Triplicate controls were also assayed together with the nucleic acid samples. These were:-

i) containing no RNA. This shows the amount of the [^{3}H]-poly U which had not hybridised but remains undigested by the ribonuclease.

ii) containing no RNA and no ribonuclease. This gives a measure of the amount of [^{3}H]-poly U added to the sample and allows a check to be carried out to ensure the amount of [^{3}H]-poly U was saturating.

The amounts of poly(A) per cotyledon in the total nucleic acid, poly(A)$^+$ RNA and poly(A)$^-$ RNA fractions were estimated using a standard curve which showed that [^{3}H]poly U containing 872 c.p.m hybridised to 1 ng poly(A).

2.18. Preparation and Use of Wheat-Germ Cell-Free Systems

a) Preparation of a Wheat-Germ S·30 System

A wheat-germ S·30 cell-free protein synthesising system was prepared by an adaption of the methods described by other workers (Marcu and Dudock, 1973; Roberts and Paterson, 1973; Rychlik and Zagorski, 1978; Zagorski, 1978). All steps of the procedure were carried out at 4^{o}C.

5 g of wheat-germ (General Mills Co.) was ground to a fine poweer, in a chilled pestle and mortar, with an equal weight of powdered glass. 13 ml of grinding buffer (20 mM Tris-acetate pH 7·6, 120 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol) was added and homogenisation continued till a fine paste was obtained. The homogenate was centrifuged at 30,000 $g_{max.}$ for 15 min at 4^{o}C, in an MSE 18 centrifuge and the resultant supernatant was passed through a Sephadex G-25 solumn (26 cm long x 1·5 cm internal diameter), which had been equilibrated with grinding buffer, at a flow rate of 3 ml/min. Fractions (1 ml) of the eluate were collected and those from the first peak, that had an A_{260} of >100 O.D./ml were combined. The peak fractions

were centrifuged at 30,000 $g_{max.}$ for 15 min and the supernatant dialysed overnight (Zagorski, 1978) against fresh grinding buffer. After dialysis against several changes of buffer, for approximately 17 hours, the preparation was centrifuged at 30,000 $g_{max.}$ for 15 min and the final supernatant was stored frozen in aliquots of 250 µl at -80°C.

A sample of wheat-germ preparation was analysed on a 5·1 ml 15-60% linear sucrose gradient with centrifugation at 149,000 $g_{av.}$ for 1·5 hours, in a Spinco SW 50 rotor, to determine the state of the ribosomes in the preparation.

b) Variations in Preparing the Wheat-Germ S·30 System

Four different methods of preparation of the cell-free system were compared. These different methods were:-

i) with dialysis

ii) without dialysis

iii) with micrococcal nuclease treatment and with dialysis

iv) with micrococcal nuclease treatment and without dialysis

The wheat-germ systems were prepared as previously described until the dialysis stage. Where no dialysis treatment was given (ii) the preparation was stored prior to this stage. For the dialysed wheat-germ system (i) the procedure was continued as normal (p.105), while for the other two cases (iii) and (iv) 960 µl of wheat-germ, 10 µl $CaCl_2$ (0·1M) and 20 µl micrococcal nuclease (1000 units/ml) were mixed and the sample was incubated at 20°C for 15 min. 20 µl 0·1M EGTA pH 7·4 was added per 800 µl of wheat-germ to halt the nuclease action. The nuclease only treatment (iv) was stored

at this stage while the remainder was given the usual dialysis and centrifugation treatment prior to storage.

All four systems were compared for incorporation of labelled amino acid ([^{35}S]-methionine) into TCA precipitable protein and for the products obtained.

c) Preparation of a Wheat-Germ S·100 System

A wheat-germ S·100 cell-free protein synthesising system (Marcus, 1972) was prepared in a similar manner to the S·30 system except that, prior to the dialysis step, the 30,000 g supernatant was centrifuged at 105,400 $g_{av.}$ for 3 hours, in a Spinco 40 rotor, to remove the ribosomes from the preparation. Dialysis and centrifugation were then carried out as for the S·30 system and the S·100 was stored in aliquots at -80°C. This was used as a 'run off' system for polysomes prepared from the cucumber tissue (p. 68).

d) Characterisation of the Wheat-Germ Translation System

The wheat-germ system was used as an assay for 'translatable' mRNAs in different RNA preparations. Incubations were routinely carried out in a total volume of 50 µl containing the constituents listed in Table 2.

Components A and B were contained in 20 µl of the incubation while the remaining 30 µl contained the nucleic acid and any sterile distilled water required to bring the volume to 50 µl. Incubations were carried out at 25°C for 90 min and a time course of incorporation

of [^{35}S]-methionine into protein was followed by taking 5 µl

Table 2. Contents of the wheat-germ cell-free system

Component	Final concentration per incubation
A.	
wheat-germ S·30	1–1·5 A_{260} units
or wheat-germ S·100	0·3–0·4 A_{260} units
B.	
Hepes-KOH pH 7·6	28 mM
potassium acetate	104 mM
magnesium acetate	2·25 mM
spermidine	0·25 mM
ATP	1 mM
GTP	50 µM
creatine phosphate	8 mM
creatine phosphokinase	5 µg
dithiothreitol	2 mM
amino acids (x19-methionine)	25 µM each
[^{35}S]-methionine	5–10 µCi
C.	
total nucleic acid	5–15 µg
or poly(A)$^+$ RNA	0·5–1 µg
or poly(A)$^-$ RNA	15–20 µg
or polysomes	30–50 µg

samples at 5, 30, 60 and 90 min. These samples were collected on 3 MM filter paper discs (Whatman) and were processed by the method of Mans and Novelli (1961) which determines the radioactivity in hot TCA precipitable material. The amount of radioactivity was determined by counting each disc in 5 ml scintillation fluid (BPD/Toluene) in an SL 30 liquid scintillation spectrometer. Duplicate samples of the original incubation mixture, prior to nucleic

acid addition, were taken and either treated by the Mans and Novelli procedure (1961) or counted directly without treatment. These showed, respectively, the amount of radioactivity which was not due to labelled protein but was not washed off the discs, and the amount of radioactivity supplied to each incubation. A control incubation was also carried out, in which no exogenous nucleic acid was added, so that the level of incorporation due solely to endogenous message could be determined.

Using the incorporation levels obtained for each sample (c.p.m./50 µl) and the amount of nucleic acid used in each incorporation, it was possible to calculate the c.p.m. incorporated per µg of exogenous nucleic acid (c.p.m./µg). This gives an indication of the amount of 'translatable' mRNA in each of the samples. In order to determine the relative amounts of 'translatable' mRNA in the cotyledons (c.p.m./cot) the c.p.m./µg was multiplied by the total µg of nucleic acid per cotyledon. Thus, it was possible to determine quantitative changes in the level of 'translatable' mRNA in the cucumber cotyledons during germination.

e) Fractionation and Autoradiography of In Vitro Translation Products

The products synthesised in the wheat-germ system were precipitated with 4 volumes of acetone and kept at $4^{o}C$ for at least 30 min. The precipitated protein was pelleted by centrifugation at 12,000 $g_{max.}$ for 1 min in a Micro-centrifuge (Quickfit Instruments) then resuspended in 50 µl of double strength gel sample buffer (p. 87) and

heated for 15 min at 70°C. These samples were then fractionated by electrophoresis on SDS-polyacrylamide gels as previously described (p. 86). Staining and destaining were carried out in the usual manner (p. 94) and the gels dried onto 3 MM chromatography paper (Whatman).

Radioactive polypeptides were detected on the gels by autoradiography. Dried gels were exposed for varying lengths of time, depending upon the amount of label incorporated into protein, to Blue Brand x-ray film (now called Blue Brand Regulix).

X-ray film was developed for 3 min in Ilford Phen-X developer, 20 second in 2% (v/v) glacial acetic acid to stop the action of the developer, then 30 min in fixative (Ilford IL-23) before washing thoroughly and drying.

f) Optimisation of the Wheat-Germ S·30 System

Prior to using the wheat-germ system to compare the translatable mRNA content of different tissues it was necessary to optimise the system so that high levels of incorporation and stimulation above the endogenous translation, and high molecular weight products were obtained.

i) Wheat-Germ and Ion Concentrations

From the results of other workers (Rychlik and Zagorshi, 1978; Hunter et al., 1977; Marcu and Dudock, 1974), it was decided that, in order to check the efficiency of the system, variations in the concentrations of wheat-germ, potassium and magnesium should be studied.

The following concentration ranges were tested:-

a) Wheat-germ - 0·5-3 O.D. units at 260 nm.

b) Potassium - 50-150 mM.

c) Magnesium - 1·25-3 mM.

ii) Total Nucleic Acid

In order to use the wheat-germ *in vitro* system under optimal conditions, the system was optimised using a range of concentrations (2·5-60 µg/50 µl incubation) of total nucleic acid from day 3, light-grown cotyledons.

As there was uncertainty that optimisation using only nucleic acid from one state of the developmental sequence gave a clear indication of the optimal concentration for the whole germination sequence, a similar optimisation was carried out with the total nucleic acid from each stage of the light-grown sequence.

iii) Poly(A)$^+$ RNA

Optimisation of the poly(A)$^+$ RNA concentration used in the wheat-germ system was also carried out since this was purer mRNA than the total nucleic acid and thus much more active in translational activity. A range of concentrations (0·05-1·7 µg/50 µl incubation) of poly(A)$^+$ RNA from day 2, light-grown tissue was used for the optimisation.

All optimisation experiments were tested for the levels of incorporation of [^{35}S]-methionine into hot, TCA precipitable protein and for the labelled polypeptides produced.

Nucleic acid concentrations used for developmental

studies were those where the system was not saturated with exogenous mRNA and where there was a linear relationship between exogenous nucleic acid concentration and the level of incorporation.

2.19. Immunological Estimation of Specific Products from In Vitro Translation

Monospecific antibodies to purified cucumber isocitrate lyase (ICL), malate synthase (MS) and catalase (CAT), raised in rabbits and mice, were prepared by H. Riezman, University of Wisconsin, U.S.A. (Lamb et al., 1978; Riezman et al., in press), and kindly provided as a means for identification of specific products from the wheat-germ system when it was programmed by cucumber cotyledonary mRNA.

Three methods were investigated to ensure quantitative immunoprecipitation of the in vitro synthesised antigens. These methods were:-

i) use of a double antibody technique (Hunter, 1967) in which the initial antibody-antigen complex was immunoprecipitated by a second antibody raised in sheep against the rabbit immunoglobulin.

ii) use of Staphylococcus aureus cells of the Cowan serotype 1 strain which contain Protein A in their cell wall (Jonsson and Kronvall, 1974; Kessler, 1975). Protein A binds immunoglobulins and can thus be used in a similar manner to the double antibody technique.

iii) use of a Protein A column in which Protein A, purified from S. aureus A676 strain (Kronvall, 1973), which secretes this protein, is attached to Sepharose 4B (H. Riezman et al., in press) and

the antibody-antigen complex is purified by attachment to the column via the Protein A.

The methods of preparation of the sheep anti-rabbit antiserum (SAR), S. aureus cells and Protein A column are described together with the method of using these to identify the specific wheat-germ translation products.

a) Preparation of Sheep anti-Rabbit (SAR) Antiserum

A crude immunoglobulin fraction was prepared from the sheep serum by precipitation with ammonium sulphate ($(NH_4)_2SO_4$, 0·196 g/ml, 35%), the pellet was washed with 40% saturated ammonium sulphate, resuspended in distilled water and dialysed overnight against running water at $4^{o}C$. Dialysis was continued for 2 x 6 hours against PBS buffer (50 mM Na-phosphate buffer pH 6·5, 0·15 M NaCl). The resulting antibody solution was used as a second antibody in the immunoassay experiments described later (p. 116).

Microprecipitin tests were carried out using the sheep anti-rabbit antisera against the rabbit anti-ICL and rabbit anti-MS antisera.

b) Preparation of Staphylococcus aureus for Immunoassays

Staphylococcus aureus Cowan I strain was grown and fixed as described by Kessler (1975) except that no vitamins were added to the culture medium. Cells were collected and centrifuged twice at 8,000 $g_{av.}$ for 10 min, in an MSE 18 centrifuge, in PBS-azide buffer (40 mM K-phosphate buffer pH 7·2, 0·15 M NaCl, 0·05 % (w/v) sodium

azide). After resuspension to approximately 10% (w/v) in PBS-azide, the cells were stirred at 23°C for 1·5 hours in the presence of 1·5 % (v/v) formalin, then centrifuged at 8,000 $g_{av.}$ for 10 min. Pelleted cells were resuspended to 10% (w/v) in PBS-azide, added to 1 litre flasks and killed by swirling at 80°C for 5 min, followed by rapid cooling to 4°C. The cells were washed twice more in PBS-azide, resuspended to 10% (w/v) and stored at 4°C.

c) Preparation of Protein A-Sepharose 4B Column

Activated Sepharose 4B beads were prepared by the procedure of March et al. (1974). The methicillan-resistant strain A676 of S. aureus was cultured according to Kessler (1975), again without added vitamins. Preparation of the Protein A and Protein A column was kindly carried out by H. Riezman (Riezman et al., in press) by the following procedure.

Protein A was purified from culture filtrates of of the methicillin-resistant strain A676 of S. aureus. After 18 hours growth, the cells were pelleted by centrifugation at 10,000 $g_{av.}$ for 10 min and the supernatant, containing the secreted Protein A was applied to a column of human IgG-Sepharose 4B and washed extensively with buffer A (50 mM Tris-HCl pH 7·5, 0·15 M NaCl, 0·1% NaN_3). The Protein A was eluted with 0·1 M glycine pH 3·6, neutralised with 1M Tris-HCl pH 8·8 and precipitated with 80% saturated $(NH_4)_2SO_4$. After dissolving the pellet in distilled water and dialysing extensively, the Protein A was analysed by ultraviolet scanning (Sjöquist et al.,

1972), SDS-polyacrylamide gel electrophoresis and immuno-electrophoresis, and was found to be homogeneous by all 3 criteria.

To 8 ml of activated Sepharose 4B beads was added 4 ml of a 4 mg/ml Protein A solution in 0·2 M Na H CO_3 pH 9·5. The beads were shaken at 4°C for 20 hours, collected by filtration and the filtrate was analysed for protein by the method of Kalb and Bernlohr (1977). Virtually all the Protein A was found to be coupled to the Sepharose. 1 M ethanolamine pH 9·0 was used to resuspend the beads, which were shaken at room temperature for 2 hours in order to mask any unreacted groups, then the beads were washed as descibed by March et al. (1974), packed into a glass column (20 mm long x 6 mm internal diameter) and washed extensively with buffer A, then 0·1 M glycine pH 3, and finally buffer A again. This Protein A-sepharose 4B column was then used to purify in vitro immunoprecipitates as described.

d) Immunoassay Methods

A wheat-germ incorporation was carried out as previously described (p.107) but using a larger incubation mixture depending upon the number of immunoprecipitations being carried out on each sample. The final incubation mixture was centrifuged at 105,400 $g_{av.}$ for 1 hour, in a Spinco 40 rotor, to remove the remaining polysomes and the resultant supernatant was used for the immunoprecipitations.

A 25-50 µl sample of supernatant was used for

each immunoassay, and to this was added an equal volume of double strength TNT buffer (0·1 M Tris-HCl pH 8·6, 0·5 M NaCl, 1% (w/v) Triton X-100) and the first antibody (rabbit anti-ICL, rabbit anti-MS or mouse anti-CAT) in a volume of 5 µl (diluted with TNT buffer). After mixing well the sample was incubated at 37°C for 30 min. Further treatment depended upon the method of purification of the antibody-antigen complex being used.

i) Double antibody method

20 µl of SAR antiserum was added in a total volume of 100 µl (diluted with TNT buffer) and incubation continued for 1 hour at 37°C then overnight at 4°C.

To collect and wash the immunoprecipitate, the sample was layered onto a 200 µl 15% (w/v) sucrose cushion (Strauss et al., 1975) in TNT buffer, in 1·5 ml Eppendorf tubes, and centrifuged at 2,000 $g_{av.}$ for 15 min in an MSE Mistral 4L centrifuge. The supernatant remaining above the sucrose was removed carefully prior to removing the sucrose above the pellet. Washing of the pellet was carried out once with TNT buffer, twice with TNT buffer containing 0·1% (w/v) SLS and once with 0·1 M Tris-HCl pH 8·6, recovering the pellet each time by centrifugation at 2,000 $g_{av.}$ for 15 min and taking care to resuspend the pellet thoroughly by vortexing. Upon removing the final wash solution, the samples were prepared for gel electrophoresis by addition of 50 µl 2x gel sample buffer (p.87) and heated at 70°C for 15 min before being fractionated by electrophoresis on SDS-polyacrylamide gels (p. 86).

ii) Immunoadsorption with S. aureus cells

20 µl of S. aureus Cowan I cells (washed in TNT buffer prior to use; 10% (w/v)) were added in a total volume of 100 µl, diluted with TNT buffer, and incubation continued on ice, for 10 min.

The cells were precipitated and washed as described in the previous method. Prior to loading the samples on an SDS-polyacrylamide gel they were centrifuged, for 6 min at 12,000 $g_{max.}$ in a Micro-Haematocrit centrifuge (Gelman Hawksley Ltd.), to pellet the dissociated S. aureus cells, and the resultant supernatant, containing the antibody and antigen, was fractionated by electrophoresis on the gel.

iii) Use of the Protein A-sepharose 4B column

After incubation with the first antibody, the samples were centrifuged at 12,000 $g_{max.}$ for 3 min, in a Micro-Haematocrit centrifuge, to remove debris and the supernatant applied to the column, which had been equilibrated with TNT buffer. Elution of the column was continued with TNT buffer until no more counts were detected in the eluate. The antibody-antigen complex, which was bound to the column, was eluted with 0·1 M glycine pH 2·3 and collected in 2 drop fractions. The samples were neutralised with 10 µl 1 M Tris-HCl pH 8·6 then an equal volume of double strength gel sample buffer (p. 87). Samples containing peak radioactivity, as determined by the method of Mans and Novelli (1961), were fractionated on SDS-polyacrylamide gels in the

usual manner (p. 86). Re-equilibration of the column was carried out with TNT buffer.

e) Fluorographic Detection of Immunoprecipitated In Vitro Products

As the radioactivity of the immunoprecipitated in vitro polypeptides was relatively low, they were detected by fluorography (Bonner and Laskey, 1974; Laskey and Mills, 1975). After the gel had been stained and destained in the usual manner (p. 94) it was dehydrated in 3 changes of 4 gel volumes (ca. 120 mls) of dimethyl sulphoxide (Me_2SO), for 30 min each wash, then washed in 3 gel volumes of 20% PPO-Me_2SO for 3 hours, with shaking. Rehydration was for 20 min in water, then the gel was dried onto 3 MM chromatography paper (p. 94). According to Bonner and Laskey (1974), the fact that rehydration of the gel causes precipitation of the PPO within the gel matrix, causing it to become opaque, does not apparently reduce the fluorographic efficiency.

To improve the sensitivity of the fluorographic technique, Kodak X-omatic H x-ray film was flashed from a height of 9 inches with a Sun-Pak GT 32 flash gun (flash duration 1/1000 second) through an Ilford S902 filter covered with Whatman No. 1 filter paper. The sensitized side of the film was placed against the gel and stored in a Kodak x-ray cassette at $-80^{o}C$ to expose. This procedure sensitizes the film by the production of a silver atom from a silver ion and the low temperature reduces the thermal reversion of this before it is stabilised by the formation of a second silver atom from the emission of

β-particles from the radioactive material in the gel.

Development of the exposed film was carried out as described for autoradiography (p. 109).

2.20. In Vivo Labelling of Cucumber Cotyledons

Qualitative developmental changes in protein synthesis occurring within the cucumber cotyledons were studied by *in vivo* pulse labelling of the tissue with [^{35}S]-methionine.

Cucumber seeds were surface sterilised with a 1:30 dilution of sodium hypochlorite (NaOCl) for 5 min, neutralised in 0·01 N HCl for 10 min (Abdul-Baki, 1974a & b), then washed thoroughly with sterile distilled water (8 washes of 20 volumes). They were then imbibed overnight (17 hours), at 4^{o}C, in sterile distilled water prior to planting in sterile vermiculite in the normal manner (p. 74). Watering was carried out when required with sterilised tap water. All glassware and water was sterilised in an autoclave at 15 lb/sq. inch for 20 min. Plastic containers were sterilised with ethanol then rinsed with sterile distilled water prior to use.

a) Method I - Labelling of Whole Seedling

Light-grown tissue was labelled by this method. Growth was allowed to continue until 24 hours prior to harvesting when 10 intact seedlings from each stage were removed from the vermiculite and placed in clear, sterile plastic containers (6 cm height, 4·5 cm diameter) between 3 MM chromatography paper and the side of the container, with any open cotyledons exposed above the top of the

container. Seed coats were retained where still adhering. 10 seedlings from each developmental stage were supplied with 24 ml of sterile tap water containing approximately 60 μCi of [^{35}S]-methionine (1005 Ci/mmole). For day 0, 10 seeds were cold imbibed in the same volume of [^{35}S]-methionine containing sterile tap water, for 24 hours at 4^{o}C in the dark.

Cotyledons (20 cots/day) were harvested 24 hours after the beginning of the labelling period. Samples of total homogenate and 'soluble' proteins were prepared as previously described (p. 77) and duplicate aliquots taken from each to determine:-

i) uptake of [^{35}S]-methionine into the cotyledons - 20 μl samples of the homogenate collected on filter paper discs and counted directly.

ii) incorporation of [^{35}S]-methionine into total protein - 20 μl samples of the homogenate were processed by the method of Mans and Novelli (1961).

iii) incorporation of [^{35}S]-methionine into 'soluble' protein - 20 μl samples of the 10,000 $g_{av.}$ supernatant were processed by the method of Mans and Novelli (1961).

Duplicate 20 μl samples of the [^{35}S]-methionine containing water applied to the seedlings were taken, to determine the amount of radioactivity applied, and were counted directly without processing. The discs were counted in 5 ml scintillant (BPD/Toluene), in an SL 30 liquid scintillation spectrometer, for 4 min each.

b) Method II - Labelling of Excised Cotyledons

A second labelling experiment was carried out in which sterilised cucumber seeds (p. 119) were grown as described, until 24 hours before harvesting, the cotyledons excised and then labelled. Both light- and dark-grown tissues, grown in the normal light/dark regime (p. 74), were labelled by this method. For the dark-grown tissue manipulations were carried out using a green safelight.

Four cotyledons (each from a different seedling) from each developmental stage were excised by cutting under sterile distilled water and placed in the same type of dishes as used for Method I, except that the chromatography paper wick was only 1·5 cm in depth, and the upper half of the cotyledons was exposed above this to allow for free gaseous exchange. Sterile tap water (2-3 ml) containing [^{35}S]-methionine (25-28 µCi/ml, ~1,000 Ci/mmole) was applied to the cotyledons from each developmental stage. Enough [^{35}S]-methionine containing water was used so that the chromatography paper wick remained saturated during the 24 hour labelling period. For day 0, 4 excised cotyledons were imbibed in 2 ml of labelling medium at 4^{o}C, in the dark, for 24 hours.

Cotyledons were harvested after 24 hours labelling, rinsed in sterile distilled water and dried on sterile tissue paper. Total homogenate protein was prepared, at 4^{o}C, by homogenisation in 2·5 ml of buffer (50 mM Tris-acetate pH 8·5, 50 mM potassium acetate, 5 mM magnesium acetate, 2 mM dithiothreitol) in a glass in glass

homogeniser. The homogenate volume was estimated by weight, 1·5 ml was centrifuged for 8 min at 12,000 $g_{max.}$ in a Micro-Haematocrit centrifuge (Gelman Hawksley Ltd.), which had been pre-cooled to 4°C, and the resultant supernatant was used as the 'soluble' protein fraction.

Uptake and incorporation of [^{35}S]-methionine were determined as described for Method I (p.119).

c) Determination of In Vivo Protein Changes

Samples of total homogenate and 10,000 g 'soluble' proteins were used for Lowry determinations (p. 85) of the protein content of the cotyledons (Lowry et al., 1951).

Fractionation of the polypeptides synthesised during the 24 hour labelling period was carried out by SDS-polyacrylamide gel electrophoresis (p. 86) and fluorography (p.118) as previously described.

d) Immunoprecipitation of In Vivo Labelled ICL and MS Polypeptides

Immunological detection of ICL and MS was carried out using in vivo labelled total homogenate protein samples from light- and dark-grown tissue (Method II, p.121).

400 µl of homogenate was used from each stage and to this was added 45 µl 2 M KCl. After mixing well, the samples were kept on ice for 1 hour. The KCl treatment allows dissociation of MS which is loosely bound to the inner membrane of glyoxysomes (Köller and Kindl, 1977). Centrifugation of the samples was carried out at 105,400 $g_{av.}$ for 1 hour, in a Spinco 40 rotor, and 100 µl of the resultant supernatant used for each immunoprecipitation. An

equal volume of 2x TNT buffer was added to each sample and 0·8 µl rabbit anti-ICL or 1 µl rabbit anti-MS used together with 20 µl sheep anti-rabbit antiserum in the double antibody procedure previously described (p.116). The immunoprecipitates were pelleted through a 260 µl 15% (w/v) sucrose cushion and washed as normal.

SDS-polyacrylamide gels and fluorography (p.118) were used to detect the immunoprecipitated, labelled polypeptides.

CHAPTER 3

RESULTS

SECTION 1

DEVELOPMENTALLY RELATED CHANGES IN CUCUMBER COTYLEDONS DURING GERMINATION AND EARLY DEVELOPMENT

1.1. Seedling Development

The characteristic appearance of both light and dark-grown cucumber seedlings is illustrated in Figure 1. To ensure maximum uniformity among the cotyledons used for analyses, plants were selected to correspond as closely as possible to the morphological stages illustrated.

The first two days of development are characterised by radical emergence. When the seedling first appears above the vermiculite at day 3 the cotyledons begin to emerge from the seed coat and greening starts from the proximal end. From day 3 there is a marked increase in hypocotyl growth and cotyledonary expansion. Greening is essentially completed by day 5 and by day 7 in the light the first leaves are just beginning to appear at the shoot apex. Dark-grown seedlings (Figure 1B) are characterised by marked hypocotyl elongation, retention of the hypocotyl hook, and lack of cotyledonary expansion and greening.

A study of the cell number of the cotyledons reveals that this remains constant during the period of study (Table 3). Cotyledons from ungerminated seeds contain about $6{\cdot}78 \times 10^5$ cells per cotyledon and it can be seen that no significant change in cell number occurs during the first 7 or 8 days of growth. This agrees with electron microscopic studies by Trelease *et al.* (1971) showing no cell division in cucumber cotyledons during seedling growth. Similar results have been

Figure 1

Morphological Changes during Germination and Early Seedling Development of Cucumber Cotyledons

A. Light-Grown - refers to plants grown under a 12/12 hour light/dark cycle.

B. Dark-Grown - refers to plants grown under complete darkness while the usual daytime/nighttime temperature sequence was maintained.

Plants were grown in a controlled environment chamber with a daytime temperature of 26-28^{o}C and nighttime depression to 22^{o}C. Light intensity was approximately 6,500 lux from a mixture of fluorescent and incandescent lamps.

FIGURE 1.

A.

B.

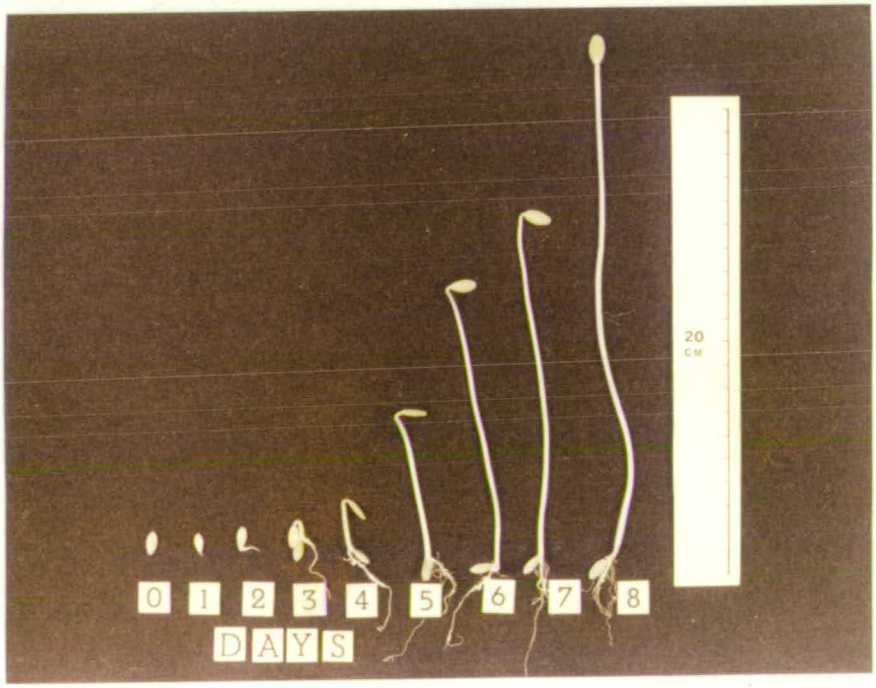


Table 3

Day	Cell no. per cotyledon x 10^{-5}		Average
	Light-grown	Dark-grown	
0	-	-	-
1	6·78	6·81	6·80
2	6·56	6·25	6·40
3	6·60	7·38	6·99
4	6·75	6·88	6·82
5	6·94	6·56	6·75
6	7·02	7·18	7·10
7	6·81	7·31	7·05
8	-	6·33	6·33
Average	6·78	6·84	6·81

obtained for cotyledons of squash (Lott, 1970) and mustard (Weidner, 1967) seedlings. The absence of cell division allows for results expressed on a per cotyledon basis to be directly related to the situation per cell in the tissue.

Changes in the fresh and dry weights of the cotyledons are shown in Figure 2. In light-grown cotyledons there is a rapid increase in fresh weight upon emergence from the seed coat resulting in a 10-fold increase by day 7. This increase corresponds to the marked cotyledonary expansion observed in Figure 1A. Dark-grown cotyledons show only a 2-fold increase in fresh weight.

It can be seen that the dry weight of both light and dark-grown cotyledonary tissue decreases during the first four days of seedling growth (Figure 2B). Subsequently there is an increase in the light but continued decrease in the dark. The decrease in dry weight of the tissue reflects the progressive depletion of both lipid and protein reserves of the cotyledons which are reuitilised to facilitate growth of the seedling until such time as the tissue becomes fully autotrophic. Dry weight measurements of the rest of the seedling axis (Becker et al., 1978) indicate that the depletion of food reserves observed in the cotyledons is balanced by an increase in dry weight in the remainder of the plant.

It is obvious that the dry weight increase towards the end of this developmental sequence does not account for the fresh weight increase observed in the light-grown tissue. Percentage hydration calculations (Figure 2C) show that the fresh weight increase during this period is due almost entirely to water uptake into the tissue. The increase in dry weight

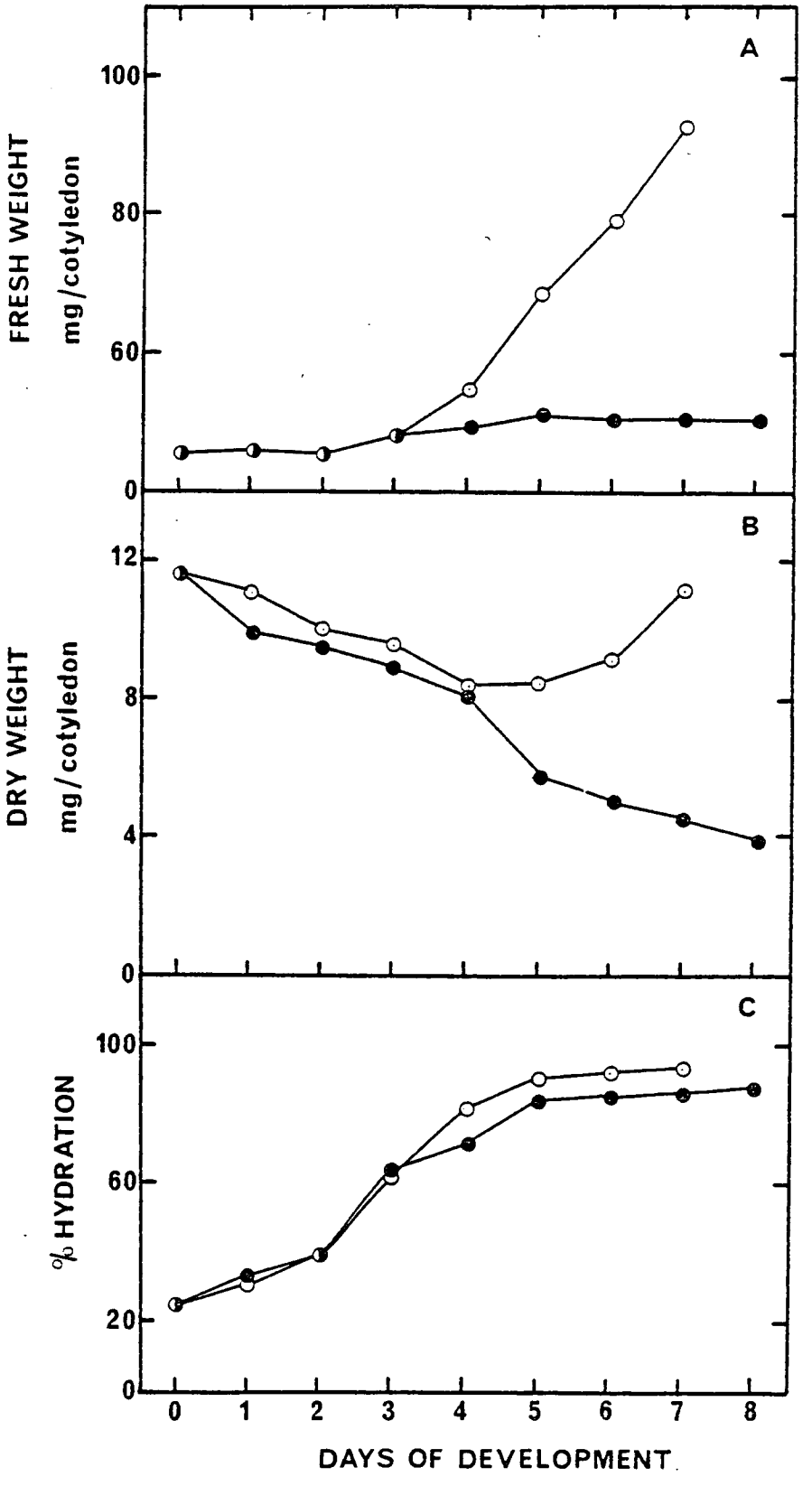
Figure 2

Developmental Changes in Fresh and Dry Weights of Cucumber Cotyledons

Fresh (A) and dry (B) weights were determined for each developmental stage from light (O—O) and dark-grown (●—●) cucumber cotyledons by the methods described (p. 76). Percentage hydration (C) was calculated from the fresh and dry weight determinations using the following equation:-

$$\% \text{ hydration} = \frac{\text{fresh weight} - \text{dry weight}}{\text{fresh weight}} \times 100$$

FIGURE 2.


A
FRESH WEIGHT
mg/cotyledon
100
80
60
0
B
DRY WEIGHT
mg/cotyledon
12
8
4
0
C
% HYDRATION
100
60
20
0
0
1
2
3
4
5
6
7
8
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observed for the light-grown tissue may be attributed to the fact that the tissue becomes green at this time and thus is due to accumulation of photosynthetically derived mass, whereas the dark-grown tissue remains dependent upon storage products of the cotyledons for continued growth.

1.2. Heterotrophic Indicators

Developmental changes in lipid content of light-grown cotyledons and the activity of several glyoxysomal enzymes are illustrated in Figure 3.

Mobilisation of stored lipid appears to be initiated within 24-48 hours after imbibition and is essentially completed by day 6 when the tissue is photosynthetic and no longer relies upon storage reserves.

The activities of isocitrate lyase (ICL) and malate synthase (MS), two enzymes unique to the glyoxylate cycle, are shown in Figures 3A and 3B.

Isocitrate lyase activity increases from barely detectable levels in the imbibed seed to reach a peak at day 4, then declines to undetectable levels by day 7 in the light. In the dark an essentially similar profile is obtained although the activity only decreases to 10% of peak values by day 8.

Malate synthase shows a similar activity profile to ICL in the light-grown tissue. In the dark-grown tissue there is a slightly different profile of MS activity after day 4 in that the loss of enzyme activity is slower than for ICL, only decreasing to ca. 45% of maximum activity by day 8.

As both ICL and MS are closely related in their function, it would be anticipated that they would show

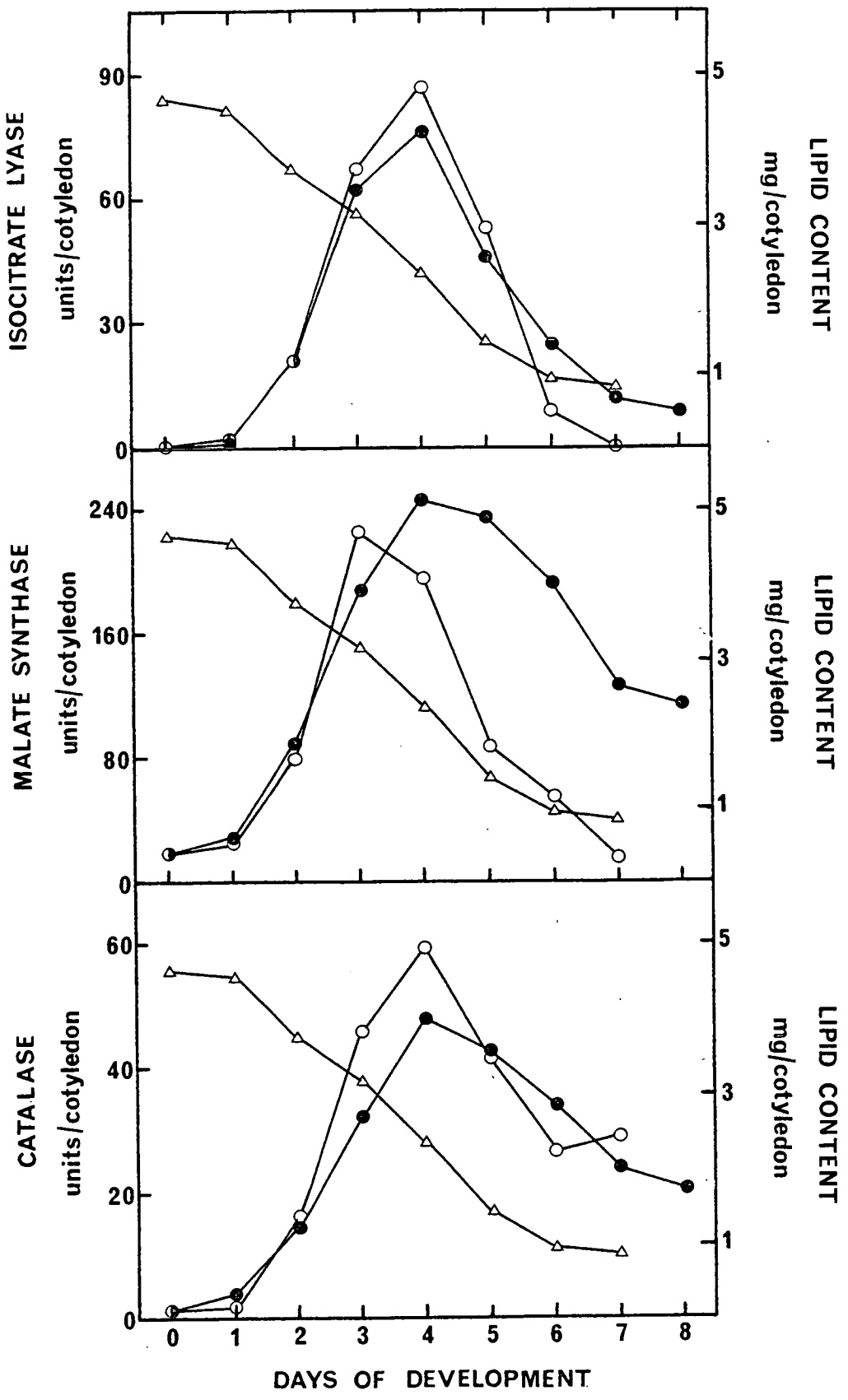
Figure 3

Developmental Changes in Heterotrophically Related Enzyme Activities

Isocitrate lyase (A), malate synthase (B) and catalase (C), enzyme activities in the cotyledons were estimated as described (p. 78) for each of the stages of cucumber germination and early seedling development in light (○——○) and dark-growth (●——●) conditions.

Lipid content (△——△) of the cotyledons was determined by the method of Radin (1969; p. 76) for each of the developmental stages.

FIGURE 3.


ISOCITRATE LYASE
units/cotyledon
90
60
30
0
LIPID CONTENT
mg/cotyledon
5
3
1
MALATE SYNTHASE
units/cotyledon
240
160
80
0
LIPID CONTENT
mg/cotyledon
5
3
1
CATALASE
units/cotyledon
60
40
20
0
LIPID CONTENT
mg/cotyledon
5
3
1
0 1 2 3 4 5 6 7 8
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similar activity profiles. The differences observed in the profiles and the absolute activities may be due to two factors. Firstly, the assay for MS was carried out on a different batch of seed to that used for ICL. Secondly, for MS, the later peak (c.a. 1 day) and slower decline in activity in the dark may be due, in part, to the fact that the two sets of seedlings (light and dark-grown) were grown in different growth chambers and so slight temperature differences may have affected the developmental profiles of the enzyme.

The enzyme activity profile shown in Figure 3C is that of catalase, which is known to be both a glyoxysomal and a peroxisomal enzyme. Catalase (CAT) resembles ICL and MS in its activity profile during the early stages of development, but then declines to a constant level at about half its maximum activity. As CAT is present in the peroxisomes of photosynthetic tissue it persists within the cotyledons as they age.

1.3. Autotrophic Indicators

Changes in several specific indicators of the autotrophic growth phase of the seedling, chlorophyll, RuBPC and glyoxylate reductase, are shown in Figure 4. Their appearance and development is closely related to the period of emergence of the cotyledons and thus, upon direct exposure to the light.

Accumulation of the photosynthetic pigment, chlorophyll, would appear to be light dependent as it is first detectable after day 3 in the light and does not appear in the dark (Figure 4A). Although the cotyledons do not accumulate chlorophyll in the dark it can be seen from the absorption spectrum in Figure 5B that carotenoids are present.

Figure 4

Developmental Changes in Some Autotrophic Indicators

Chlorophyll (A) content was determined by acetone extraction (p.76), RuBPC protein (B) was quantitatively estimated by electrophoretic fractionation of total protein under non-denaturing conditions (p. 84) and glyoxylate reductase (C) activity was estimated by the procedure of Tolbert (1971; p. 82). All estimations were carried out for each developmental stage of light (O——O) and dark-grown (●——●) cucumber cotyledons.

Lipid content (Δ——Δ) was estimated, as described (p. 76), for each developmental stage.

FIGURE 4.

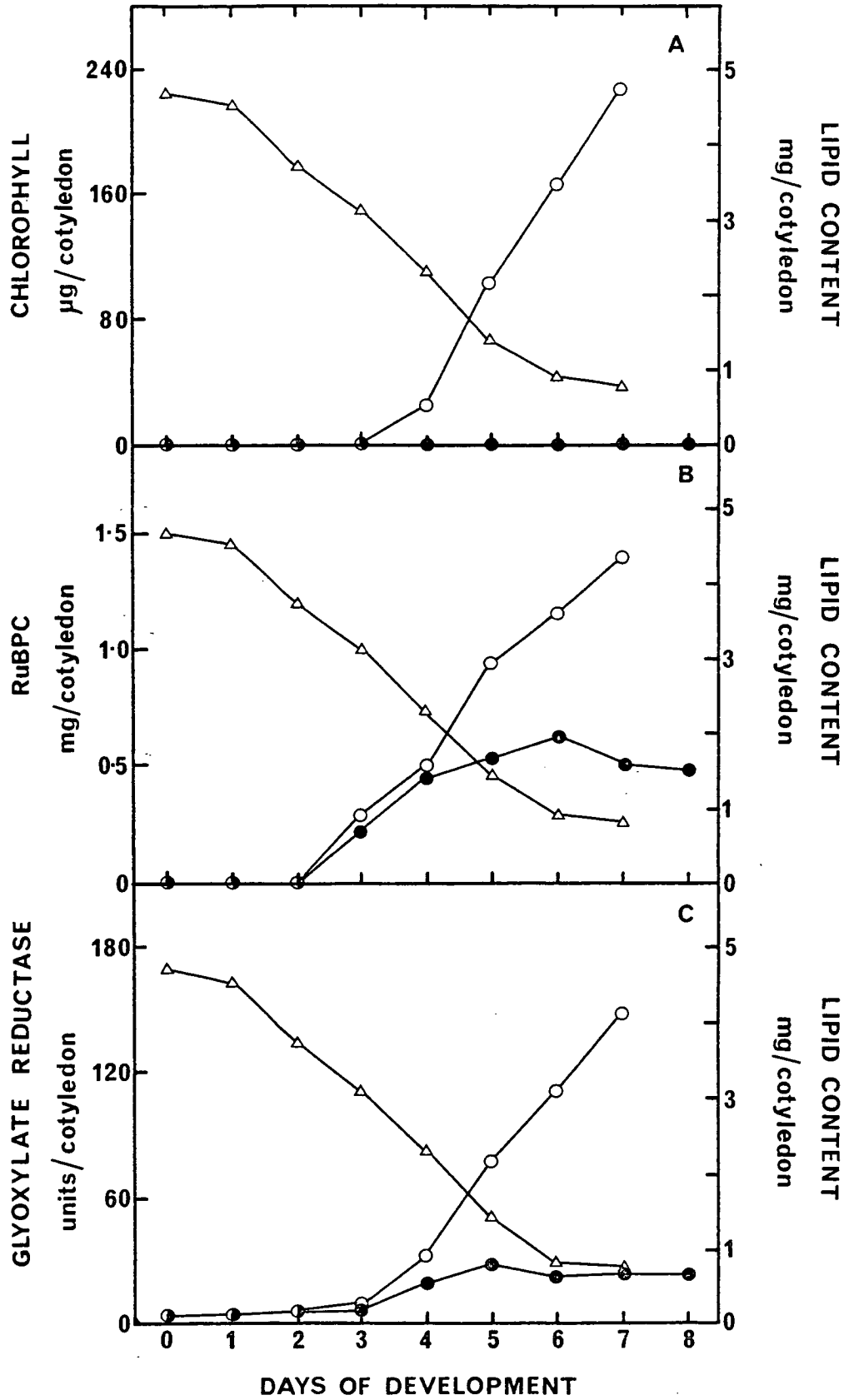
A
B
C
CHLOROPHYLL µg/cotyledon
RuBPC mg/cotyledon
GLYOXYLATE REDUCTASE units/cotyledon
LIPID CONTENT mg/cotyledon
240
160
80
0
1·5
1·0
0·5
180
120
60
5
3
1
0
0 1 2 3 4 5 6 7 8
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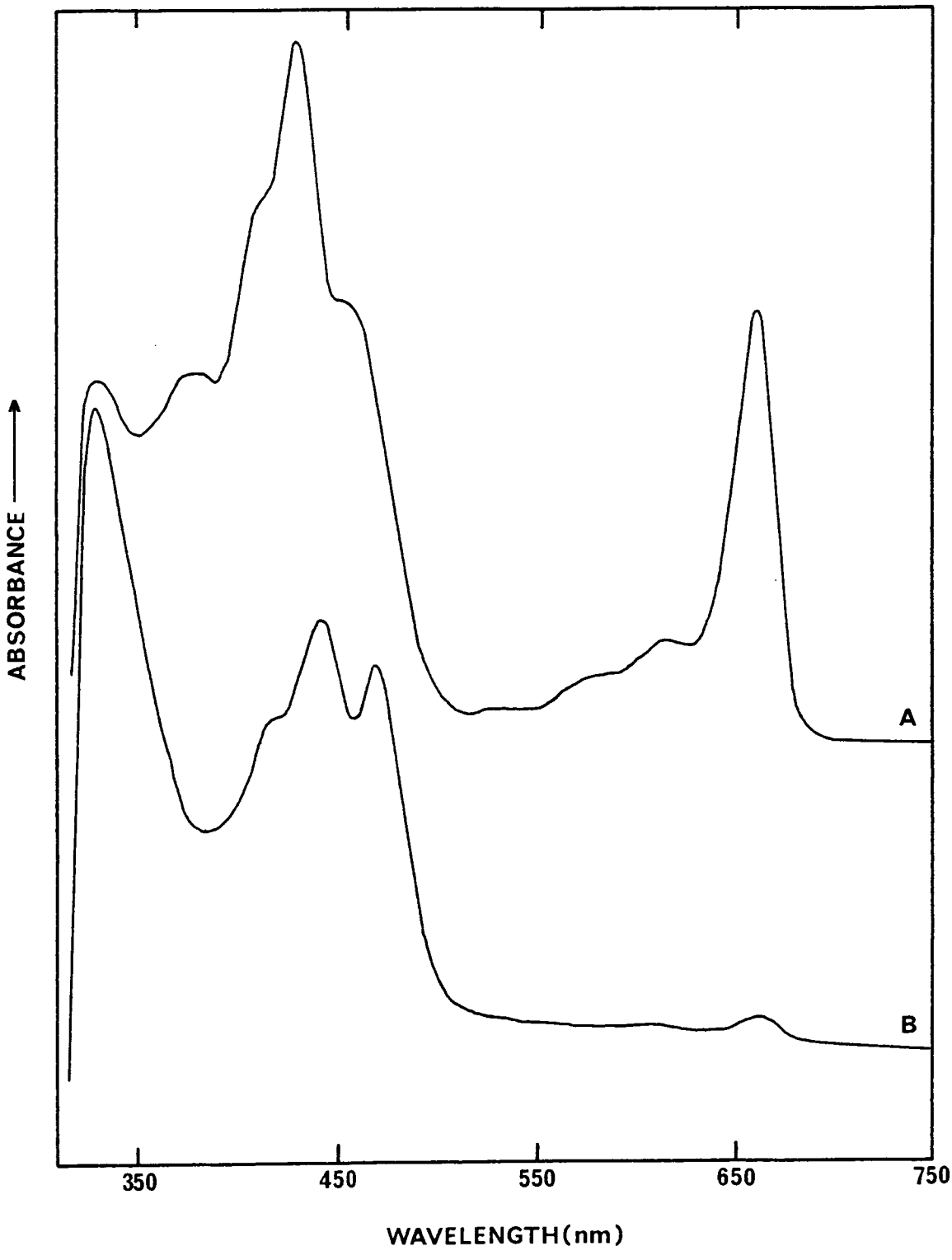
Figure 5A shows the absorption spectrum of the light-grown tissue during chlorophyll accumulation.

Changes in ribulose bisphosphate carboxylase (RuBPC) protein during seedling development were studied (Figure 4B). This enzyme, which is responsible for the initial step of CO_2 fixation, is initially undetectable in the cotyledons. RuBPC is first detectable at day 3 in amounts that indicate that its synthesis is initiated prior to chlorophyll development and thereafter this protein accumulates rapidly.

It is evident that light is not necessary for the initial synthesis and increase of RuBPC since it is synthesised in the dark as well as the light-grown tissue and also since its synthesis is apparently initiated prior to exposure of the cotyledons to the light at day 3. However, light is essential for maintained accumulation as after day 4 in the dark the content per cotyledon reaches a constant level which is maintained for the remainder of this developmental sequence.

Changes in activity of glyoxylate reductase (GR), a peroxisomal enzyme not present in glyoxysomes, was determined during germination and early seedling growth (Figure 4C). Synthesis of this enzyme is initiated after day 3 and thereafter it accumulates rapidly in the light. In dark-grown tissue GR is also synthesised but at very reduced levels compared with the content in the light. Thus, GR depends upon light for attainment of full activity but not for its initial increase from the barely detectable levels found during the early developmental stages. Other workers have reported that GR and also glycolate oxidase,

FIGURE 5.



another peroxisomal enzyme, can undergo small increases in activity in dark-grown cotyledons during the period when activity can be markedly enhanced by light (Docherty et al., 1977; Gerhardt, 1974; Schopfer et al., 1975, 1976; Schnarrenberger et al., 1971; Theimer et al., 1976).

Thus, of the 3 autotrophic indicators measured, only chlorophyll is totally dependent upon light for its synthesis and accumulation in the tissue.

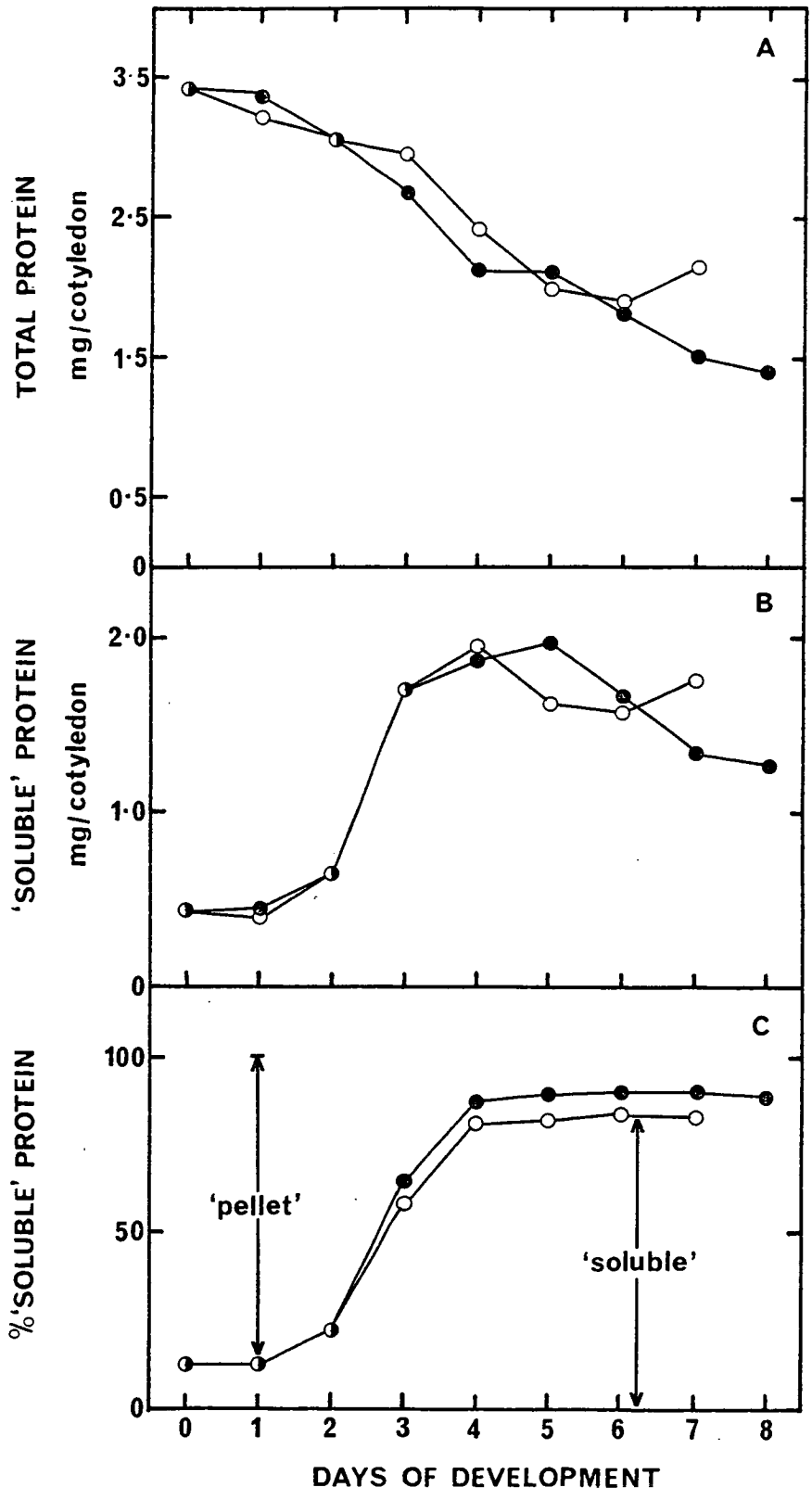
1.4. Cotyledonary Protein

Estimations were made of both the total cotyledonary protein and the amount of 'soluble' protein in the tissue by assaying the tissue homogenate and the 10,000 g_{av} supernatant, from which all membraneous and insoluble material had been removed (p. 77). Protein determinations were carried out on TCA precipitated samples of the total and 'soluble' protein fractions by an adaption of the Lowry procedure (1951) as described in the Methods (p. 85) and the results are shown in Figure 6.

It can be seen that during early seedling development, and particularly between days 3 and 5 when the most rapid growth is occurring, there is a significant decrease in the total protein of the cotyledons followed, in the last 24-48 hours in the light, by a levelling off and eventual slight increase in the protein content (Figure 6A). In the dark-grown tissue the total protein content continues to decline to the end of this developmental sequence.

Although cucurbits are essentially lipid storing seeds they also contain stored protein in organelles called protein bodies (Longo, 1968; Lott and Vollmer, 1973; Becker

FIGURE 6.


A
B
C
TOTAL PROTEIN
mg/cotyledon
'SOLUBLE' PROTEIN
mg/cotyledon
%'SOLUBLE' PROTEIN
3·5
2·5
1·5
0·5
0
2·0
1·0
100
50
'pellet'
'soluble'
0 1 2 3 4 5 6 7 8
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et al., 1978). During germination and heterotrophic growth, the seedling depends upon food reserves for growth, and the decrease in the total protein of the cotyledons is due to utilisation of storage protein.

Whereas the total protein content of the cotyledons decreases upon germination, the amount of 'soluble' protein increases after an initial lag during the first 2 days after imbibition (Figure 6B).

As can be seen from Figure 6C, the amount of sedimentable protein is 80-90% of the total protein at early stages of seedling development but that, after day 2, there is a striking decrease in this fraction such that, by day 4, over 80% of the total is present in the 'soluble' fraction. The changing proportion of 'soluble' protein observed is probably due to solubilization of stored protein reserves prior to their utilization for seedling growth. Increases in the 'soluble' protein fraction may also, in part, be attributed to an increase in metabolic proteins such as the glyoxylate cycle enzymes and autotrophically related proteins already mentioned (pps. 130 and 132).

In order to study changes in the protein content of the tissue in more detail, both total and 'soluble' proteins were fractionated on SDS-polyacrylamide gels. To facilitate direct comparison of the different developmental stages samples were loaded on a per cotyledon basis and the gels obtained are illustrated in Figure 7. There are a number of prominent developmentally related features which can be seen from these gels.

Firstly, there are a group of polypeptides in the

Figure 6

Quantitative Changes in Cotyledonary Protein during Development

Quantitative protein estimations of both total homogenate (A) and 'soluble' (B) (10,000 g_{av} x 10 min. supernatant) cotyledonary protein were carried out by an adaption of the method described by Lowry (1951; p. 85), for both light (O——O) and dark-grown (●——●) tissue.

Percentage non-sedimentable protein (C) was estimated using the following equation:-

$$\%\ \text{non-sedimentable protein} = \frac{\text{total protein} - \text{'soluble' protein}}{\text{total protein}} \times 100$$

Figure 7

SDS-Polyacrylamide Gel Fractionation of Cucumber Cotyledonary Proteins

Samples of 'total homogenate' and 'soluble' proteins from cotyledons, grown in both light and dark-grown conditions, were fractionated on one-dimensional, 15% SDS-polyacrylamide gels and stained for protein with Coomassie blue, as described (p. 86).

A. Total homogenate proteins from light-grown tissue.

B. Total homogenate proteins from dark-grown tissue.

C. 'Soluble' proteins from light-grown tissue.

D. 'Soluble' proteins from dark-grown tissue.

Total homogenate gels were loaded with 1/40th, and 'soluble' gels with 1/60th, of the protein content of a cotyledon, as estimated by the Lowry procedure (p. 85).

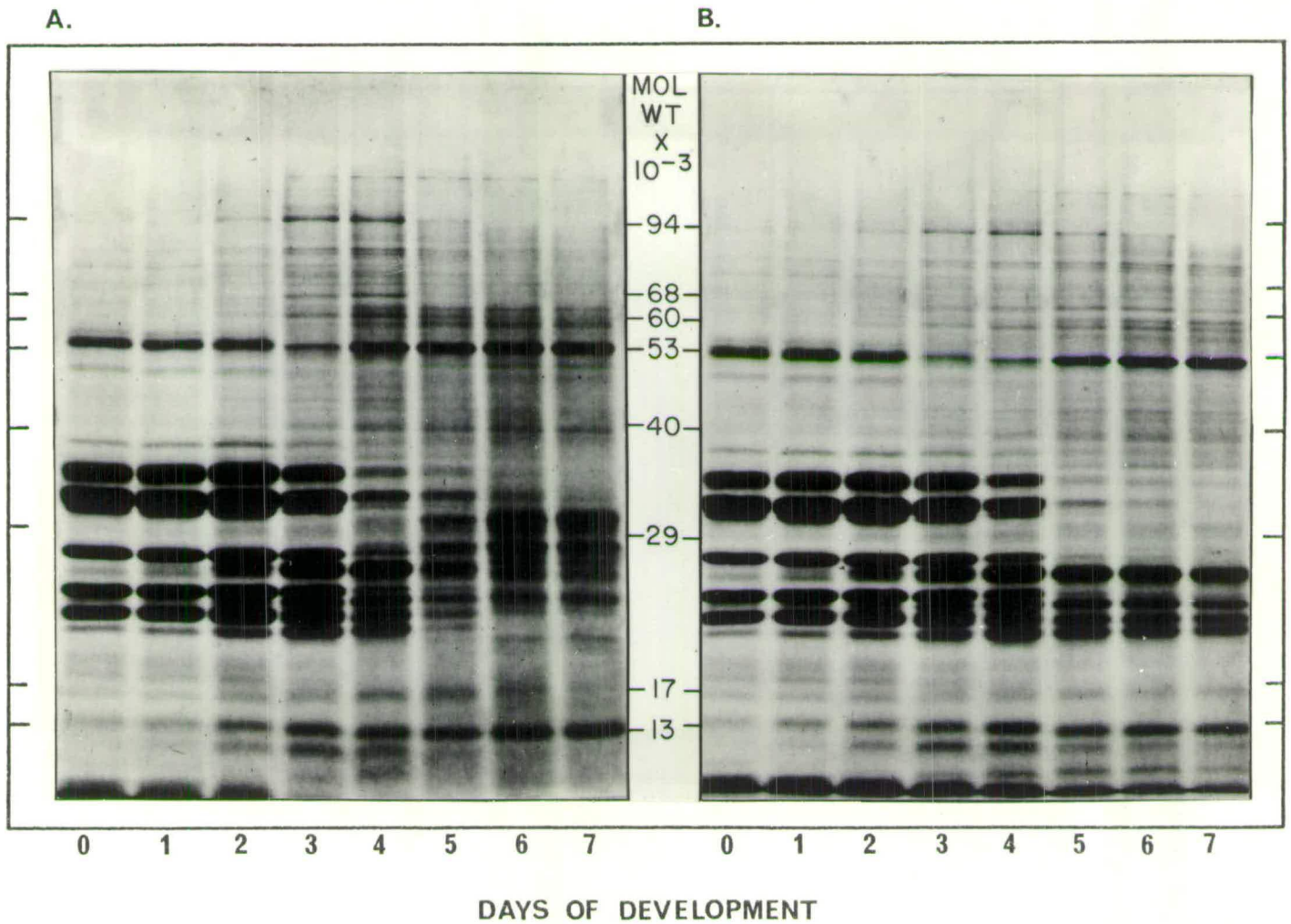
A.
B.
MOL WT X 10^{-3}
94
68
60
53
40
29
17
13
0 1 2 3 4 5 6 7
0 1 2 3 4 5 6 7
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FIGURE 7.

FIGURE 7 cont.

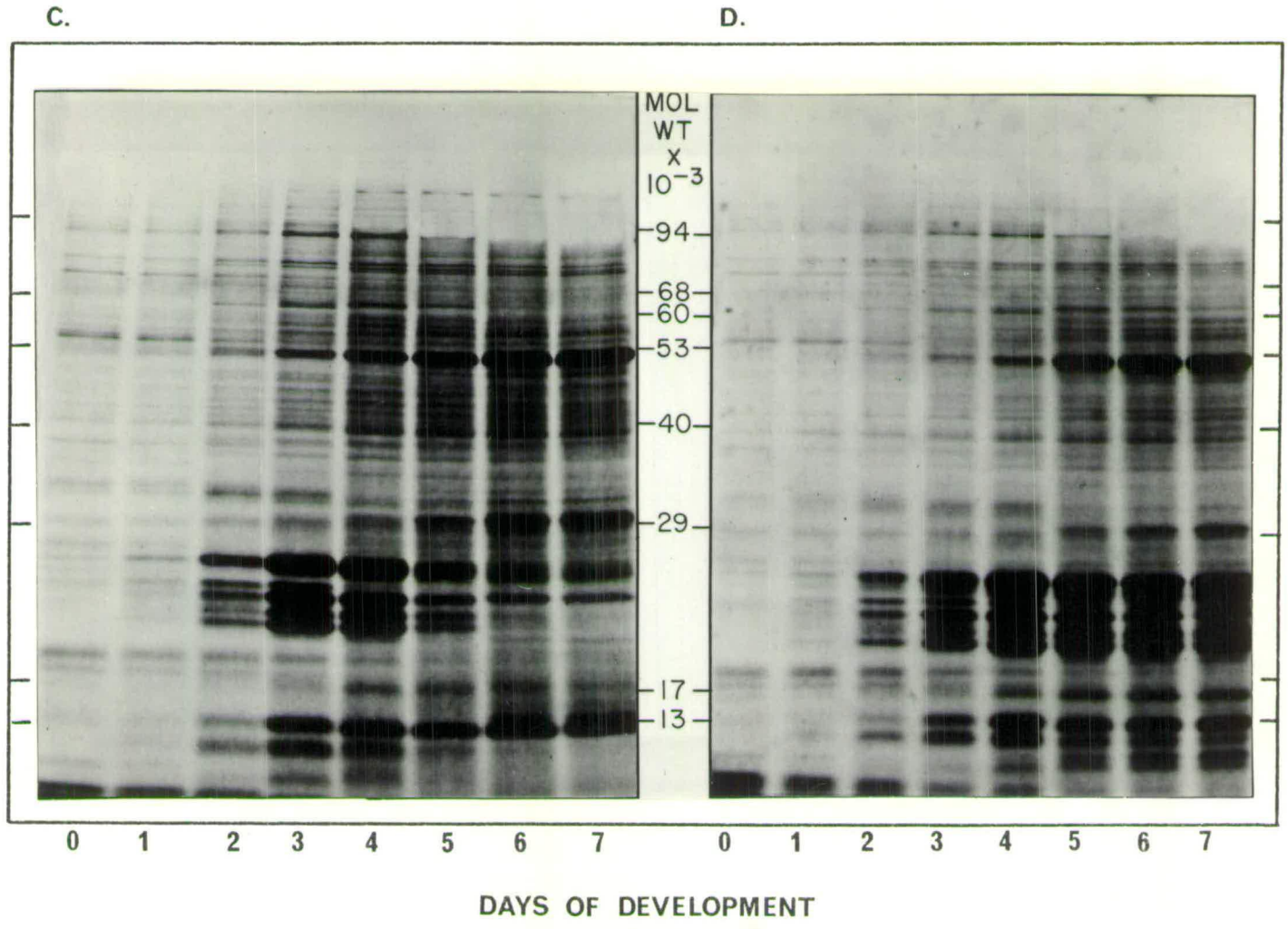


Figure 8

IEF-SDS Polyacrylamide Gel Electrophoresis of Cotyledonary Proteins

'Soluble' proteins from light-grown cucumber cotyledons were analysed by the two-dimensional gel electrophoresis procedure adapted from the method described by O'Farrell (1976; p. 89). Separation in the first dimension is dependent upon the isoelectric point, while the second dimension separates polypeptides according to their molecular weight. The gels were stained for protein with Coomassie blue. Each gel was loaded with 200 µg of cotyledonary protein.

20,000-35,000 mol. wt. range which are prominent at the onset of germination, decline as growth proceeds and have almost completely disappeared by day 4. These polypeptides would appear to be insoluble as they are only present in the total protein fraction (Figure 7A and 7B) and are almost certainly the storage proteins of the tissue. No attempt was made to identify these, either experimentally or from literature on seed storage proteins. Becker et al. (1978) confirm that these polypeptides are present in the tissue in particulate form as they co-migrate with the glyoxysomal fraction on sucrose density gradients. The fact that thay comprise such a large proportion of the total protein of the ungerminated seed and their insolubility point to their being the storage proteins.

Secondly, and most prominent in the 'soluble' protein gels (Figure 7C and 7D), the striking change in predominently sedimentable to 'soluble' proteins previously observed (Figure 6C) is mirrored by the appearance at day 2 of a series of polypeptides in the 20,000-25,000 mol. wt. region. These polypeptides correspond to intermediates in breakdown hydrolysis of the storage proteins. Although the initial solubilisation of the storage proteins occurs at the same time in light and dark-grown tissue, it can be seen by comparison of Figures 7C and 7D that these intermediary polypeptides persist significantly in the dark.

On both total homogenate and 'soluble' protein gels there are a number of high molecular weight polypeptides which, from their developmental profiles probably include the glyoxysomal enzymes. This is enforced by the microbody

profiles obtained by Becker et al. (1978) showing the developmental profiles of the glyoxysomes. Availability of subunit molecular weights for several glyoxysomal enzymes (Koller and Kindl, 1977; Lamb et al., 1978; Riezman et al., in press) including ICL (63,000 and 61,500), MS (57,000), citrate synthase (46,000), malate dehydrogenase (37,000), crotonase (75,000), thiolase (45,000) and CAT (54,000) facilitates identification of several of the polypeptides in this group.

Another group of polypeptides which are obvious from these gel profiles are those which develop during the later stages of this developmental sequence and whose function is related to the autotrophic growth of the plant. Of particular note are the large and small subunits of RuBPC, with molecular weights of 53,000 and 13,000 respectively, as this is one of the most prominent chloroplast proteins in photosynthetic tissue. These subunits first appear between days 2 and 3 and increase in amount in the cotyledon in agreement with the results obtained from estimating the complete protein on non-denaturing polyacrylamide gels (p.134).

There are also a number of other prominent polypeptides, both soluble and membrane in nature, which are obviously related to the autotraophic function of the cotyledons, particularly in the 25,000 to 32,000 mol. wt. range.

The 'soluble' proteins were also analysed by fractionation on two-dimensional IEF-SDS polyacrylamide gels by an adaption of the O'Farrell method (1976) (p. 89).

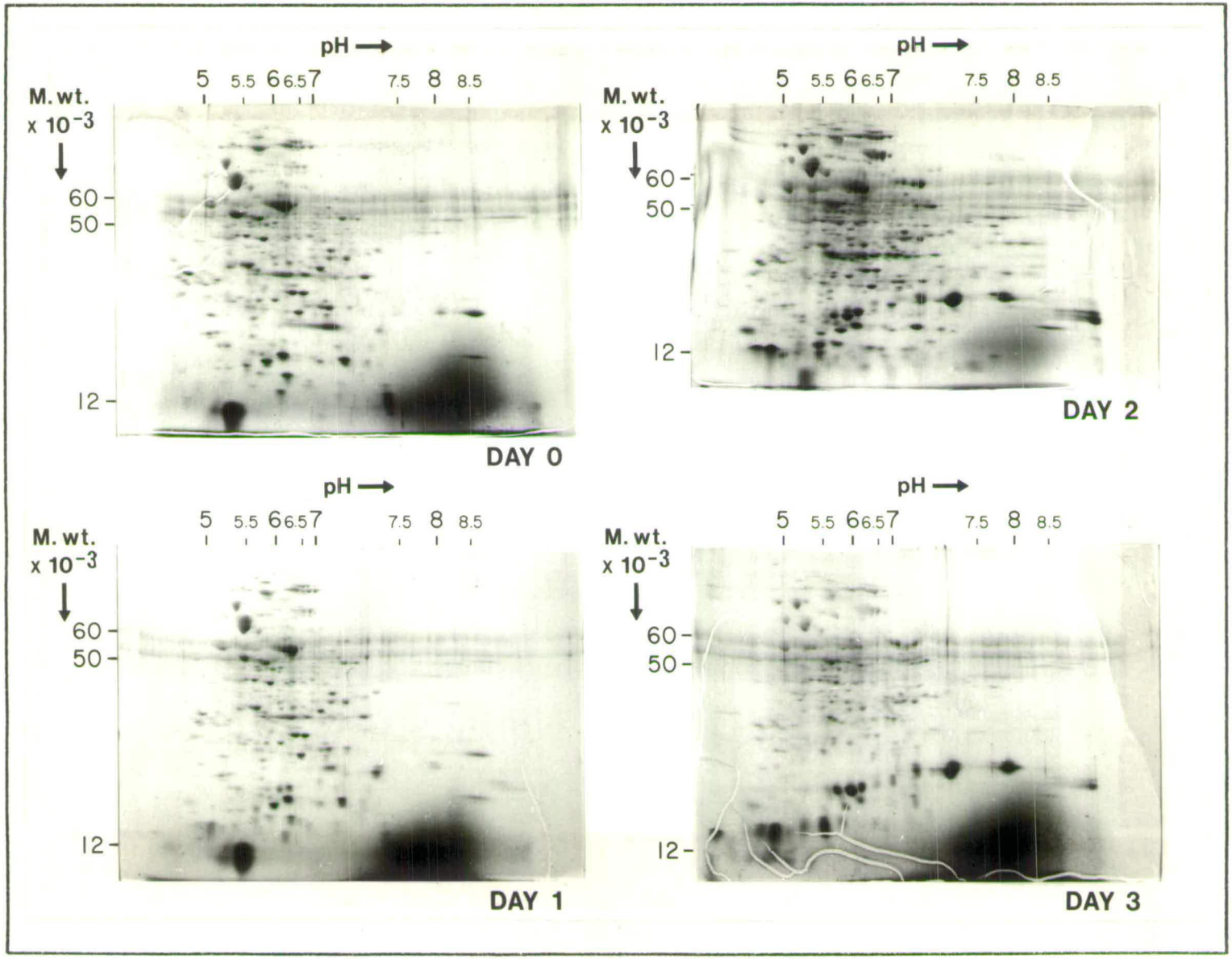


FIGURE 8.

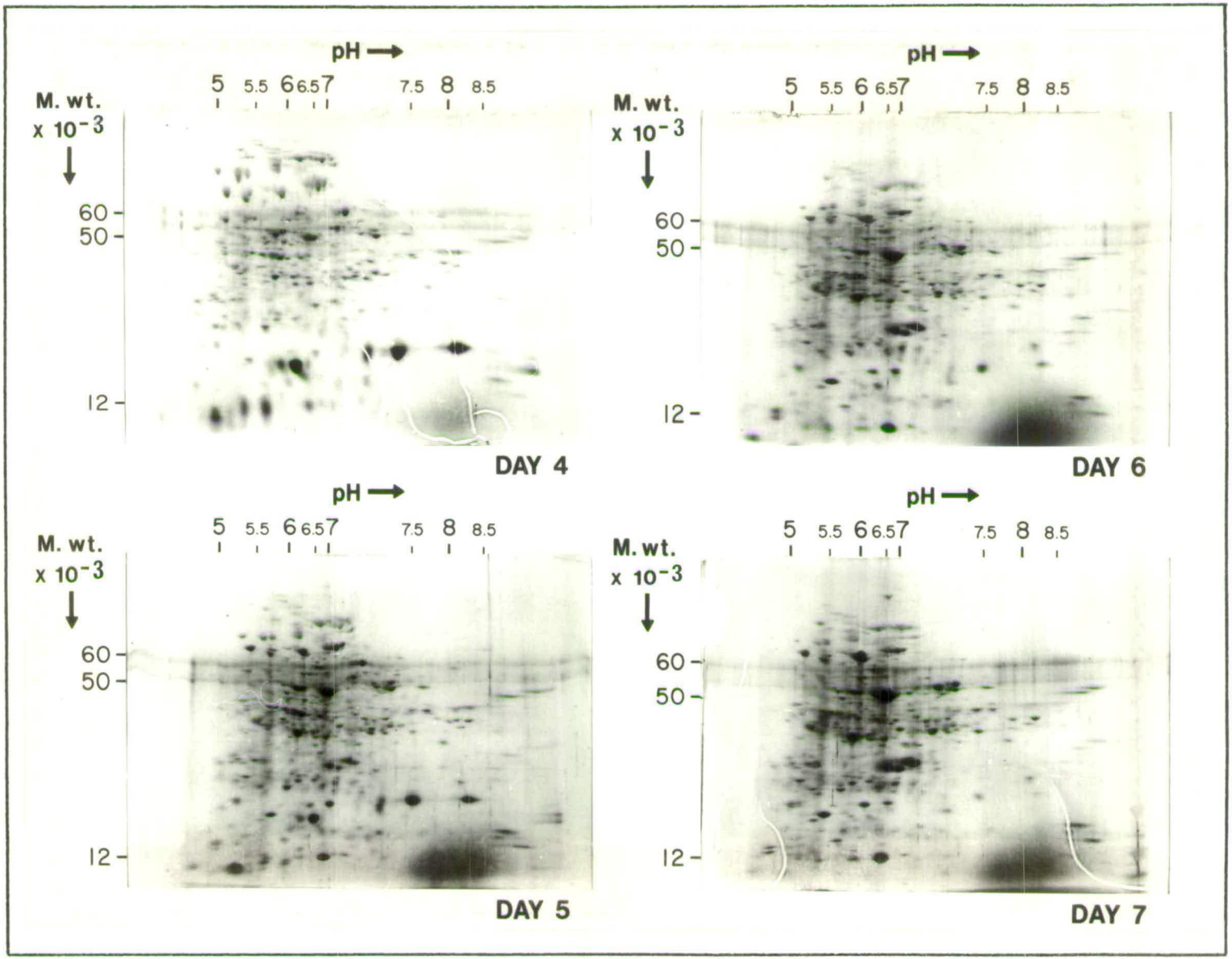


FIGURE 8 cont.

The gels of the light-grown developmental sequence are illustrated in Figure 8. Two-dimensional gels of this nature give a much higher degree of resolution by separating proteins by two independent parameters, their isoelectric points and their molecular weights.

As can be seen from these gels, and by comparison with the one-dimensional gels in Figure 7, this technique shows that there are many more polypeptides than are evident with the other method. The changes and types of polypeptides observed on the two-dimensional gels are more numerous, though similar to those previously identified.

Due to lack of purified proteins as a means of identifying the many polypeptides observed, this technique was only useful in this study for emphasising the fact that one-dimensional gels do not give a 'true' indication of the complexity of a protein sample.

1.5. Summary

During germination and early seedling growth changes in several parameters reflecting the metabolic state of the cotyledons have been measured and these have been used to characterise the stages of development under study.

Cell number has been shown to be constant, allowing all results to be expressed either on a per cotyledon or per cell basis.

Fresh and dry weight changes suggest that the function of the cotyledons in early seedling development is as a source of storage reserves which are mobilised and used to support growth of the remainder of the seedling.

Only in the later developmental stages in the light do the cotyledons increase in size and dry weight associated with the acquisition of their photosynthetic function.

The initial synthesis and attainment of peak levels of activity of the heterotrophically related proteins, ICL, MS and CAT, are independent of light although the later decrease in activity is significantly light dependent.

The synthesis and accumulation of chloroplyll is light dependent whereas for RuBPC and GR light is not necessary for the initial synthesis and increase but is essential for maintained accumulation.

The total protein content of the cotyledons decreases during early seedling growth as storage proteins are mobilised for growth of the plant apices. There is a shift from predominantly sedimentable to predominently 'soluble' protein, during the heterotrophic growth phase, associated with solubilisation of the storage proteins prior to re-utilisation.

SDS-polyacrylamide gel electrophoresis of cotyledonary proteins demonstrates characteristic changes in the spectrum of polypeptides associated with the changing metabolic state of the cotyledons.

SECTION 2

DEVELOPMENTAL CHANGES IN COTYLEDONARY RNA

The quantitative and qualitative changes in enzymes and protein which underly the metabolic transition described in the previous section, are dependent upon the synthesis of protein. It is therefore to be anticipated that there would be an associated increase in the cellular protein synthetic machinery to support this change in metabolic activity. There are reports in the literature of RNA being detectable in dormant seeds (Dure, 1975; Marriot and Northcote, 1976; Gordon and Payne, 1965; Giles et al., 1977), but that upon germination there is a marked increase in the RNA content of the tissue associated with the progression from the quiescent state to active growth and metabolism.

Quantitative and qualitative changes in the total nucleic acid, ribosomes and poly(A)$^+$ RNA as well as the poly(A) content of the RNA of the cotyledons were investigated during germination and early seedling growth.

2.1. Total Nucleic Acid

The total RNA content of the cotyledons was quantitatively estimated in the tissue homogenate (p.94) by the method described by Schmidt and Thannhauser (1945). Figure 9A shows the RNA content of the cotyledons of both light and dark-grown tissue.

At the onset of germination there would appear to be a low, though measurable, amount of RNA present in the cotyledons. In the light there is a marked increase after day 2, resulting in a 10-fold net increase over the content of the ungerminated

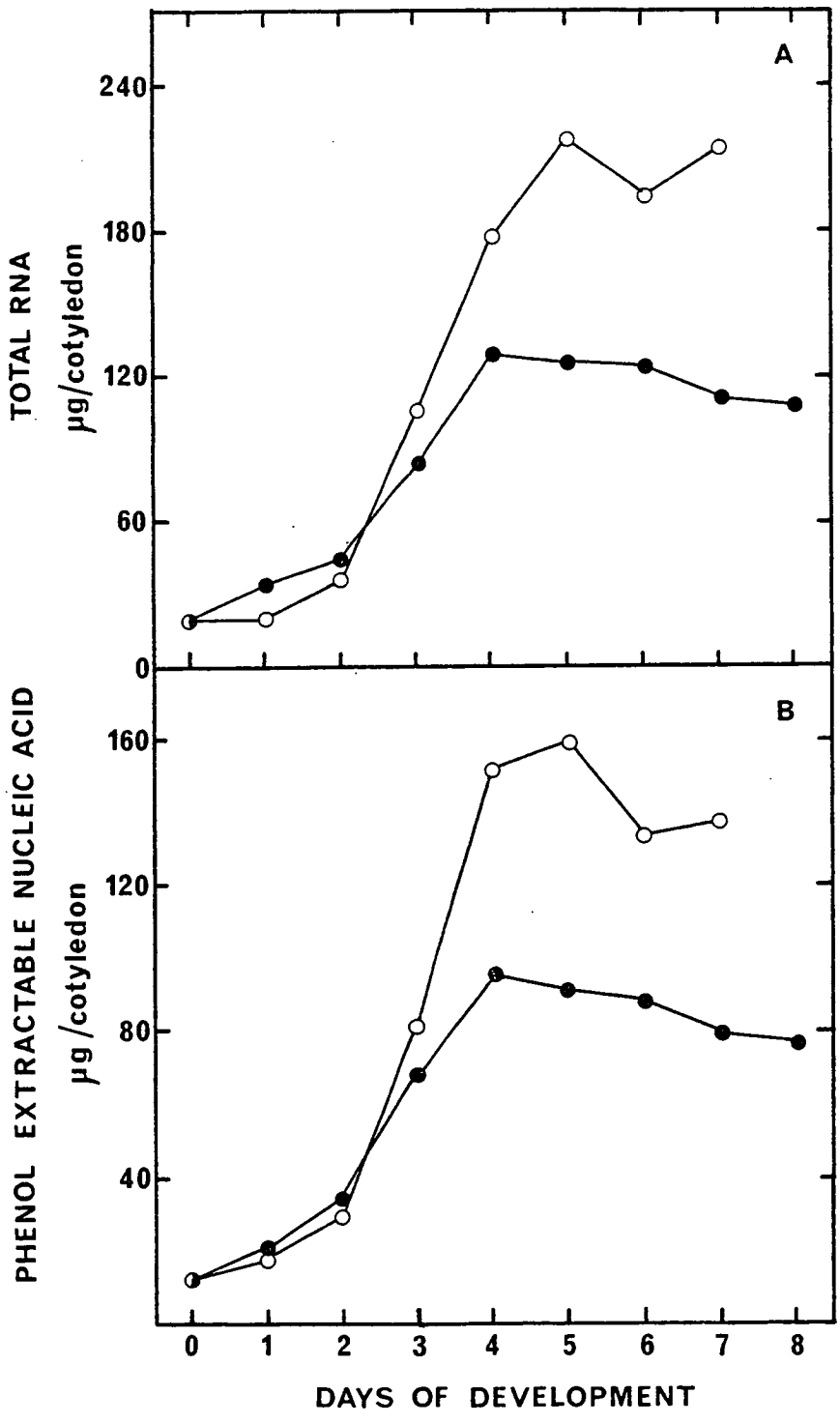
Figure 9

Developmental Changes in Cotyledonary Nucleic Acid

Quantitative estimation of total cotyledonary RNA (A) was carried out by the method of Schmidt and Thannhauser (1945; p. 94) for each developmental stage of light (○——○) and dark-grown (●——●) tissue.

Total nucleic acid (B) was extracted by the phenol-detergent procedure (p. 95) and by A_{260} determination the extractable nucleic acid of light (○——○) and dark-grown (●——●) cotyledons was estimated.

FIGURE 9.


A
B
TOTAL RNA
µg/cotyledon
PHENOL EXTRACTABLE NUCLEIC ACID
µg /cotyledon
240
180
120
60
0
160
120
80
40
0
1
2
3
4
5
6
7
8
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tissue. Dark-grown tissue shows a similar though not so dramatic change, resulting in a 5-fold net increase. For both light and dark-grown tissue, the most rapid accumulation of RNA occurs between days 2 and 4, coinciding with the period of maximum heterotrophic growth of the seedling.

In order to determine which species of the RNA were contributing towards these changes, total nucleic acid was prepared by the phenol-detergent extraction procedure (p. 95) and this was fractionated by polyacrylamide gel electrophoresis (p. 96) as described by Loening (1967).

From Figure 9B it can be seen that the phenol-detergent procedure is not strictly quantitative but does reflect the overall changes obtained with the Schmidt and Thannhauser method (Figure 9A). There is a similar or slightly higher RNA content in dry tissue than in the imbibed tissue, since the former yields 13·3 µg/cotyledon and the latter 12·6 µg/cotyledon of total nucleic acid by phenol-detergent extraction.

The gels were scanned using a Joyce Loebel UV Scanner and the resultant scans (Figure 10) were used to determine the proportions of cytoplasmic and chloroplast rRNAs and low molecular weight (LMW) RNAs by cutting and weighing the separate peaks. Using these weights, the proportions of the total RNA which comprised these different fractions were estimated and used, along with the quantitative RNA estimations (Figure 9A) to determine the changes in amount of these RNA species during germination and early seedling growth.

From a study of the gel scans illustrated in Figure 10 it can be seen that, at the onset of germination, the total nucleic acid is composed of DNA, 25S and 18S cytoplasmic rRNAs

Figure 10

Electrophoretic Fractionatin of Cotyledonary Nucleic Acid

Phenol-detergent extractable nucleic acid (p. 95) was fractionated on 2·4% cylindrical polyacrylamide gels (25 µg nucleic acid/gel) by the method of Loening (1967) as modified by Leaver (1973; p. 96). The gels were run for 3·5 hours at 50 volts, at 4°C. Densitometer scans of the gels were made using a Joyce Loebl U.V. Scanner.

A. Nucleic acid from light-grown tissue.

B. Nucleic acid from dark-grown tissue.

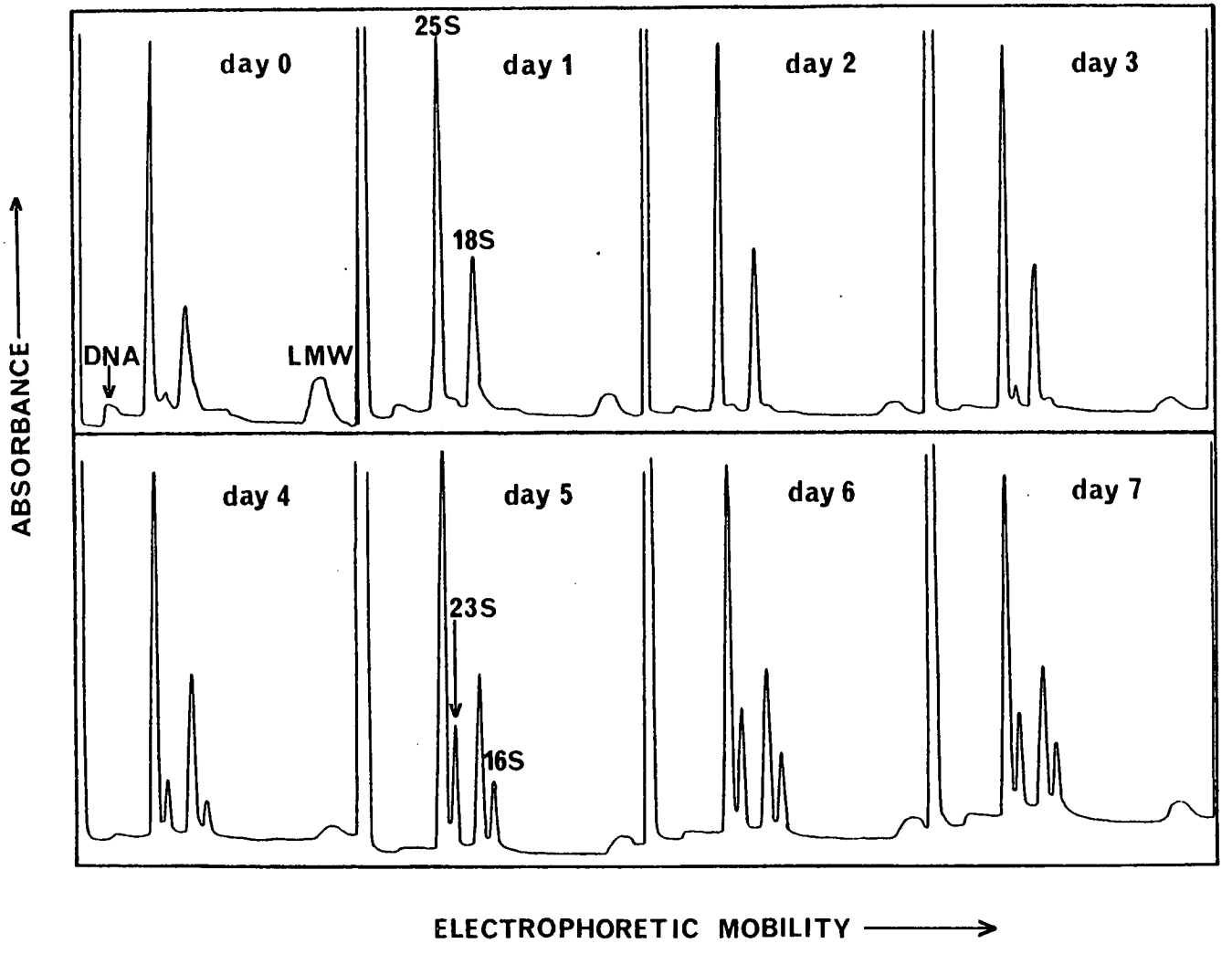

25S
day 0
day 1
day 2
day 3
18S
DNA
LMW
ABSORBANCE
day 4
day 5
day 6
day 7
23S
16S
ELECTROPHORETIC MOBILITY

FIGURE 10A.

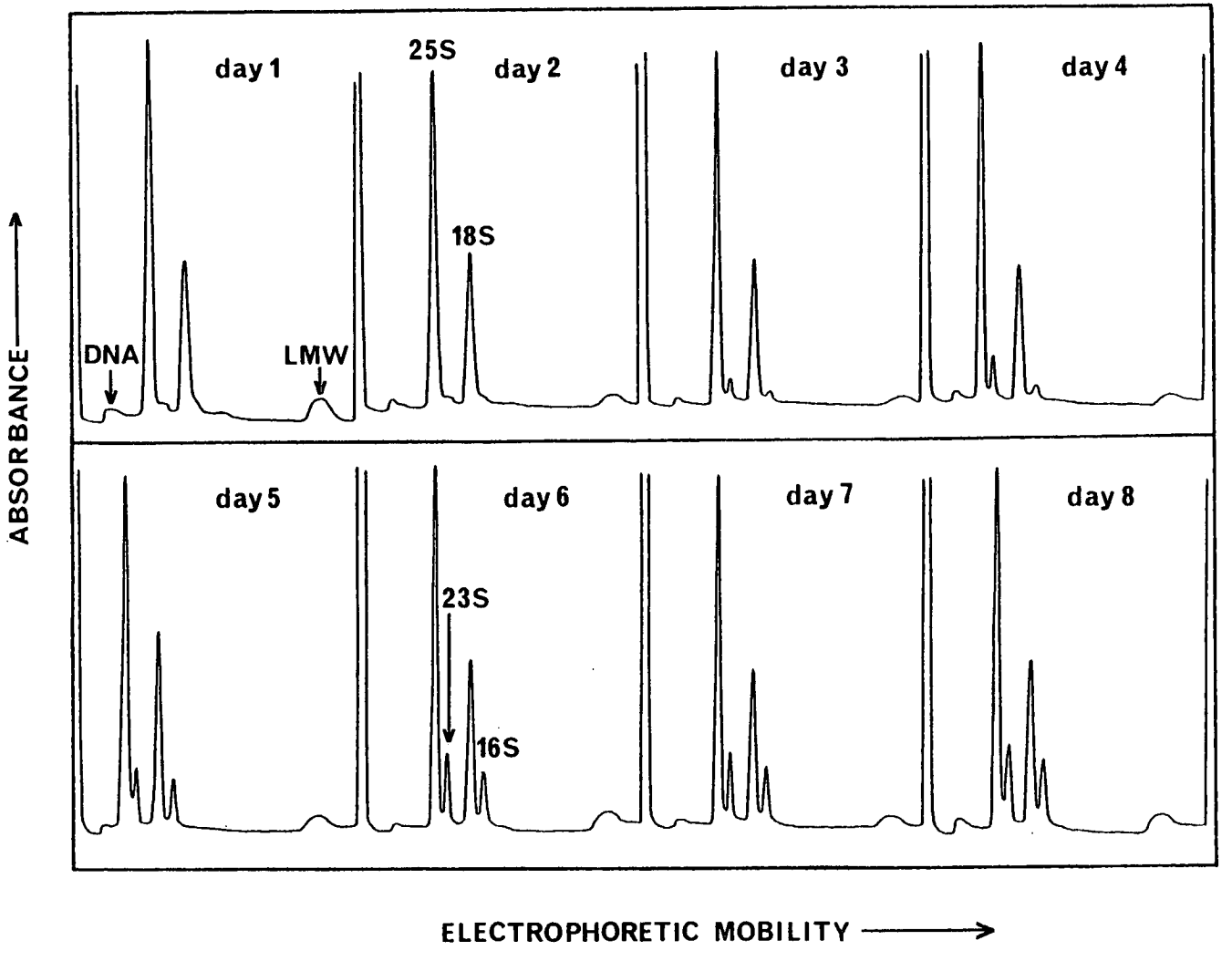

day 1
25S
day 2
day 3
day 4
18S
DNA
LMW
ABSORBANCE
day 5
day 6
day 7
day 8
23S
16S
ELECTROPHORETIC MOBILITY

FIGURE 10 B.

($1{\cdot}3 \times 10^6$ and $0{\cdot}7 \times 10^6$ mol. wt. respectively) and low molecular weight RNA, which includes the 4S tRNAs and 4·5S, 5S and 5·8S rRNAs. By carrying out the extraction and electrophoresis at 4^oC the integrity of the RNA is maintained as can be seen from the 2:1 ratio between the heavy and light rRNAs (Ingle, 1968A; Leaver, 1973). There is also a $0{\cdot}98 \times 10^6$ mol. wt. component present at early stages, which is characteristic of dry seed, forming a small proportion of the total and decreasing during growth (Ingle, 1968B). The 23S and 16S chloroplast rRNAs ($1{\cdot}1 \times 10^6$ and $0{\cdot}56 \times 10^6$ mol. wt. respectively) first become apparent at day 3 and thereafter increase as a proportion of the total. Synthesis of chloroplast rRNA is also initiated on day 3 and accumulates in the dark-grown tissue (Figure 10B).

Figure 11 shows the quantitative changes in cytoplasmic rRNA, chloroplast rRNA and LMW RNA in the cotyledons during germination and early seedling growth.

During this developmental period there would appear to be a 10-fold increase in cytoplasmic rRNA, in light-grown tissue, over the amount present in the ungerminated tissue, with most of the accumulation occurring between days 2 and 4. After day 5 there is a slight decrease in this amount. In the dark-grown tissue there is a 7-fold increase in the cytoplasmic rRNA followed by a decrease after day 4. It should be noted that the maximum accumulation occurs in both light and dark during the period of maximum heterotrophic growth.

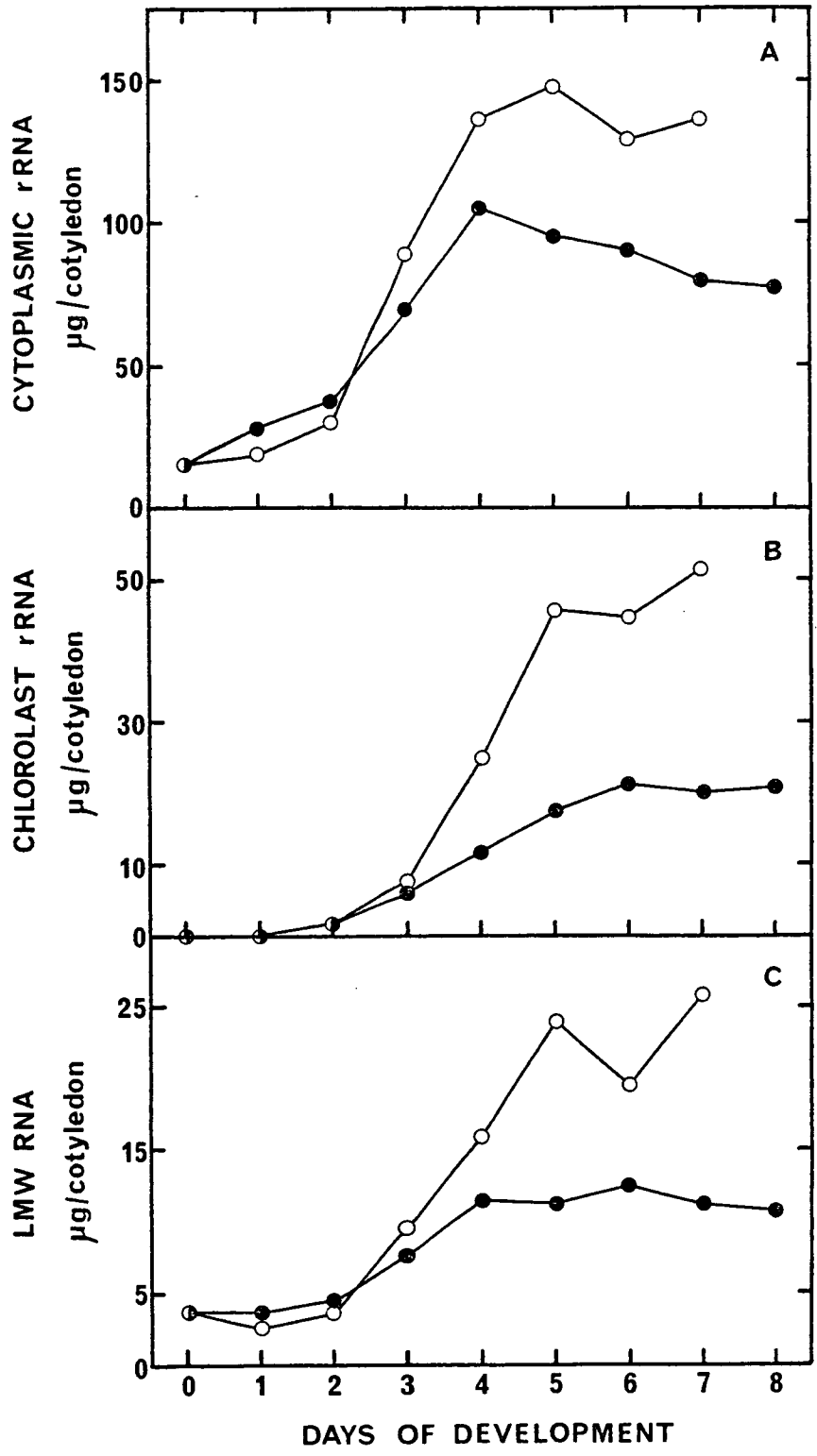
Chloroplast rRNAs (Figure 11B) are initially undetectable then appear and increase dramatically, particularly between days 2 and 5 in the light-grown tissue. There is a less marked increase in the dark. By the end of this

Figure 11

Developmental Changes in Cytoplasmic and Chloroplast rRNAs and LMW RNA

Using the densitometric scans (Figure 10) of the total nucleic acid, and the quantitative estimation of cotyledonary RNA (Figure 9A), quantitative changes in cytoplasmic (A) and chloroplast (B) rRNAs and LMW RNA (C) in the cotyledons, in light (○——○) and dark (●——●) growth conditions, were estimated. (LMW RNA refers to the cytoplasmic and chloroplast 4S, 4·5S, 5S and 5·8S RNAs not resolved on these percentage gels).

FIGURE 11.


A
CYTOPLASMIC rRNA
µg/cotyledon
150
100
50
0
B
CHLOROLAST rRNA
µg/cotyledon
50
30
10
0
C
LMW RNA
µg/cotyledon
25
15
5
0
0 1 2 3 4 5 6 7 8
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developmental period, chloroplast rRNA constitutes c.a. 24% of the total cellular RNA in the light and c.a. 19% in the dark. In the cucumber cotyledonary tissue, the maximum rate of chloroplast rRNA accumulation occurs between days 3 and 5, 24 hours after the period of maximum rate of accumulation of cytoplasmic rRNA. This pattern of development reflects the periods of maximum increase observed for RuBPC, chlorophyll and glyoxylate reductase already outlined (p.132) as well as the period of maximum expansion of the cotyledons. These changes in chloroplast rRNA indicate that the capacity for protein synthesis within the chloroplast is rapidly increasing during this period of development.

LMW RNAs (Figure 11C) are present in the ungerminated tissue and increase markedly after day 2. Accumulation continues until day 5 in the light. There is an overall increase of 7-fold in the light and 4-fold in the dark-grown tissue.

It would appear that the initial synthesis of all these major species of RNA is independent of light, while for continued accumulation, light is essential. The observed changes indicate that there is an increase in the protein synthetic capacity of the tissue, in both the cytoplasmic and chloroplast compartments, during progression from the quiescent state to heterotrophic and then autotrophic growth.

2.2. Polysomes

a) Total Polysomes

In order to show that the observed increases in RNA were associated with an increase in protein synthesis, the amount of extractable polysomes was determined for each stage of the

developmental sequence. The amount of polysomes present in the tissue is thought to be an indication of the proportion of RNA actively engaged in protein synthesis, thus this component was studied to see if the changes occurring mirrored the changes in the total RNA.

Polysomes, which included monosomes and polysomes (see Figure 42), were prepared by the method of Leaver and Dyer (1974) and the estimations of polysome content per cotyledon are shown in Figure 12A. After preparation of the polysomes, polysomal RNA was extracted by the phenol-detergent procedure (Leaver and Ingle, 1971) and the amount of extractable RNA from the polysomes is illustrated in Figure 12B.

At the onset of germination there is a low level of polysomes present in the cotyledons. In the light, there is then a dramatic increase in the polysome content of the tissue resulting in a 23-fold increase by day 5. Thereafter there would appear to be a slight drop in the amount of extractable polysomes. Again, the most rapid accumulation occurs between days 2 and 4 in the light. Polysome content also increases in the dark-grown tissue, yielding a 11·5-fold net increase over the content of the ungerminated tissue.

Figure 12B shows the amount of extractable polysomal RNA. A similar profile is seen for the RNA content of the polysome content of the tissue. The quantitative differences between the two values is due to the fact that, as previously mentioned, the phenol-detergent procedure is not a quantitative extraction method. The increase in RNA engaged in protein synthesis is most marked in the light during the heterotrophic and early autotrophic growth phases.

Figure 12

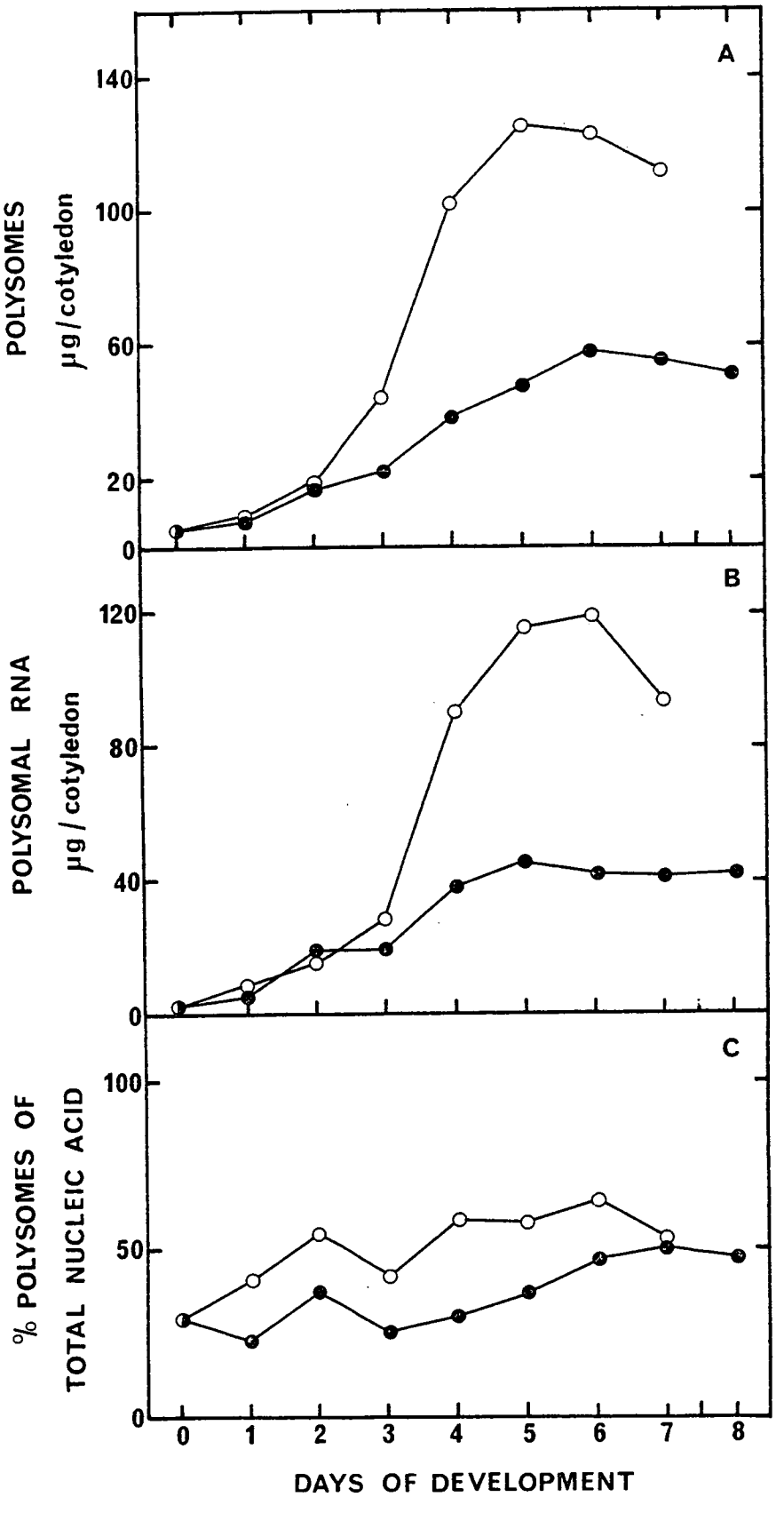
Developmental Changes in Polysomes and Polysomal RNA

Polysomes (A) and Polysomal RNA (B) were prepared as described (p. 98) and the cotyledonary content was determined by A_{260} estimation for each developmental stage of light (O———O) and dark-grown (●———●) tissues.

The amount of extractable polysomes per cotyledon was expressed as a percentage (C) of the amount of phenol-detergent extractable total nucleic acid (Figure 9B) by the following equation.

$$\text{extractable polysomes as a \% of total nucleic acid} = \frac{\text{amount of extractable polysomes}}{\text{amount of extractable total nucleic acid}} \times 100$$

FIGURE 12.


A
B
C
POLYSOMES µg/cotyledon
POLYSOMAL RNA µg/cotyledon
% POLYSOMES OF TOTAL NUCLEIC ACID
140
100
60
20
0
120
80
40
0
100
50
0
0 1 2 3 4 5 6 7 8
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By comparison of the polysome content (Figure 12A) and the total ribosomal RNA content (both cytoplasmic and chloroplast rRNA; Figure 11A and 11B) of the cotyledons, it was possible to estimate the percentage of the total rRNA that was present in extractable polysomes and thus actively involved in protein synthesis at the different developmental stages (Figure 12C). Any rRNA present in the tissue, but not included in the extractable polysomes would be present in ribosomal subunits and those monosomes which were not recovered by the extraction procedure.

After imbibition approximately 35% of the total rRNA is present in extractable polysomes compared with 70% by day 6 in the light. In the dark there is also an increase in the proportion of rRNA present in polysomes, but this is not quite so marked, only reaching 55% by day 7. There would appear to be two reproducible phases in the increase of the proportion of rRNA present in polyribosomes, the first occurring prior to day 2 and the second after day 3, with a slight decrease between the two.

Thus, not only is there an increase in the rRNA content of the cotyledons, during growth, but there is also an increase in the proportion of the rRNA actively involved in protein synthesis.

b) Free and Membrane Bound Polysomes

As there was obviously a dramatic increase in the polysome content of the cotyledons during growth, the subcellular fractionation of these into free and membrane-bound fractions was investigated during the first 5 days of growth

in the light. These two fractions were prepared as described (p. 99) and by spectrophotometric determination the content per cotyledon was estimated (Figure 13A).

Both free and membrane-bound polysomes showed pronounced increases, particularly between days 2 and 4, to yield a 16-fold and 25-fold increase, respectively, about the content of the imbibed tissue. There also appears to be a greater proportion of polysomes free in the cell than are bound to membranes.

The ratio of membrane-bound to free polysomes (Figure 13B) increases approximately 2-fold by day 3 then decreases again, indicating that membrane-bound polysomes increase more rapidly between days 2 and 3 than do the free polysomes. After day 3 the ratio of the two fractions decreases again.

2.3. Poly(A)$^+$ RNA

So far it has been shown that dramatic changes occur in the total nucleic acid and ribosomes of the cotyledons during germination and early seedling development. However, of specific interest are changes which may occur in the messenger RNA (mRNA) population of the tissue. When studying total RNA changes any changes in the mRNA fraction are masked by the predominance of ribosomal and LMW RNAs. Also, the polydisperse size of mRNAs does not allow easy identification of this fraction by gel electrophoresis.

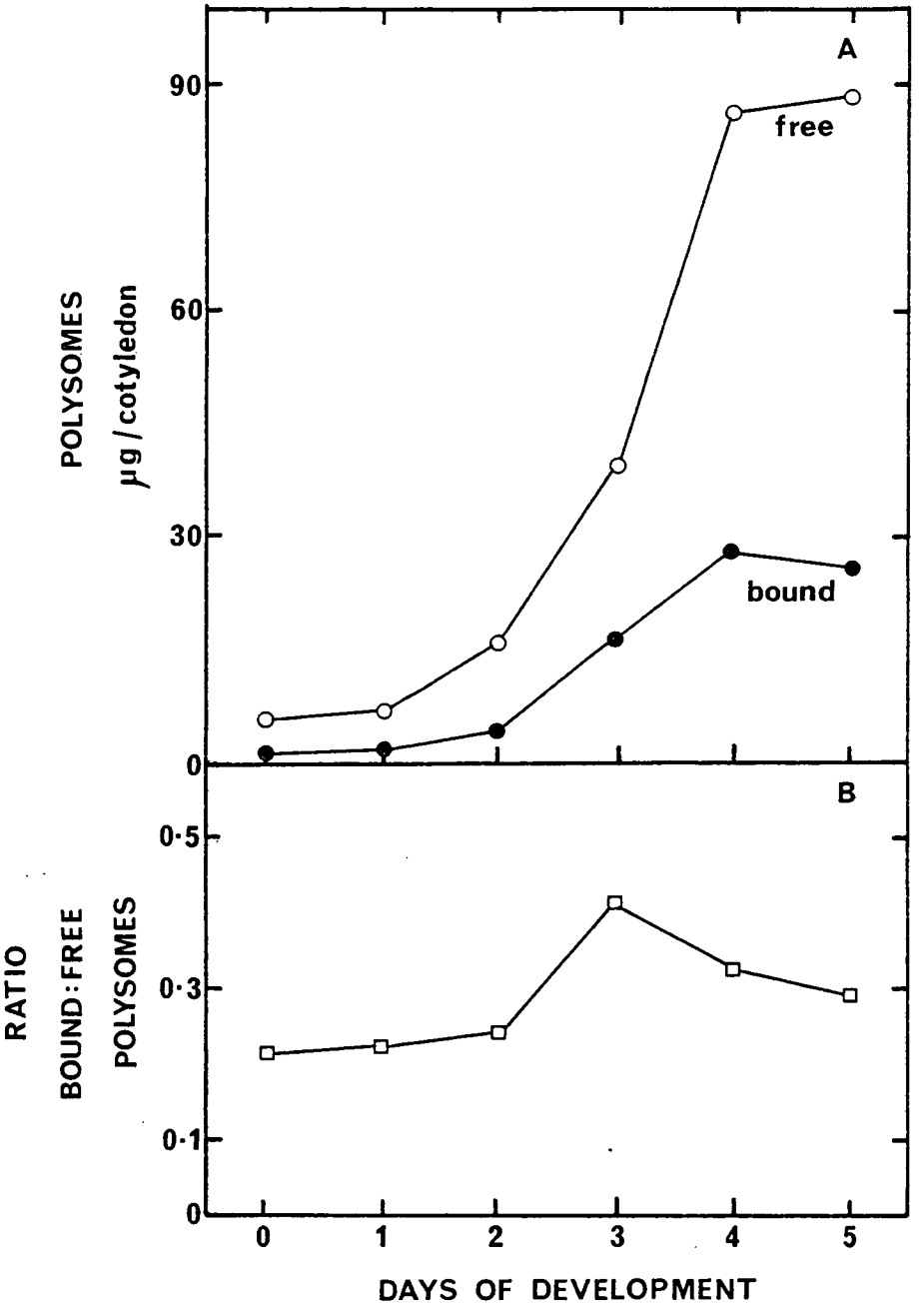
In recent years it has been shown that many, though not all, mRNAs contain a 3' hydroxyl attached polyadenylate sequence which can vary in length from 0 to 150-200 nucleotides depending upon the tissue and the particular mRNA (Kates, 1970;

Figure 13

Developmental Changes in Free and Membrane-Bound Polysomes

Free (○——○) and membrane-bound (●——●) polysomes (A) were prepared as described (p. 99) and the cotyledonary content determined for each developmental stage up to 5 days growth in the normal light conditions. The ratio (B. □——□) of bound to free polysomes was estimated.

FIGURE 13.


A
90
60
30
0
free
bound
POLYSOMES
µg / cotyledon
B
0·5
0·3
0·1
0
RATIO
BOUND:FREE
POLYSOMES
0
1
2
3
4
5
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Singer and Penman, 1973; Sarkar et al., 1978). To the investigator, the advantage of this poly(A) sequence is its capacity to form complementary base paired structures with oligothymidylic acid and thus affinity chromatography can be used as a method of separating mRNA from other RNA species. Two methods of affinity chromatography, on oligo dT-cellulose and poly U sepharose, utilize the ability of the poly(A) to bind reversibly to these specific ligands, which are covalently bound to a solid support, cellulose and sepharose respectively.

Use was made of oligo dT-cellulose chromatography to prepare poly(A) containing RNA, as described in the Methods (p.102), from phenol-detergent extractable total nucleic acid. The amount of extractable poly(A)$^+$ RNA per cotyledon was determined for dry seeds and each of the developmental stages (Figure 15).

Samples of poly(A)$^+$ RNA from day 3 light and dark-grown tissue were fractionated on sucrose density gradients (p.103). Figure 14 shows the poly(A)$^+$ RNA profiles from the gradients. As a comparison with the poly(A)$^+$ RNA, gradient C was loaded with day 2 total nucleic acid. The polydisperse size of the poly(A)$^+$ RNA is evident from the gradients (Figure 14A and 14B) and was also shown for other samples from the developmental sequence by fractionation on sucrose density gradients and polyacrylamide gels (Grienenberger - unpublished observations). It is apparent from the gradients that purifications of the poly(A)$^+$ RNA was reasonably, though not totally successful as there is a small amount of contaminant in the 18S rRNA region in both preparations.

Figure 15A illustrates the amounts of extractable

FIGURE 14.

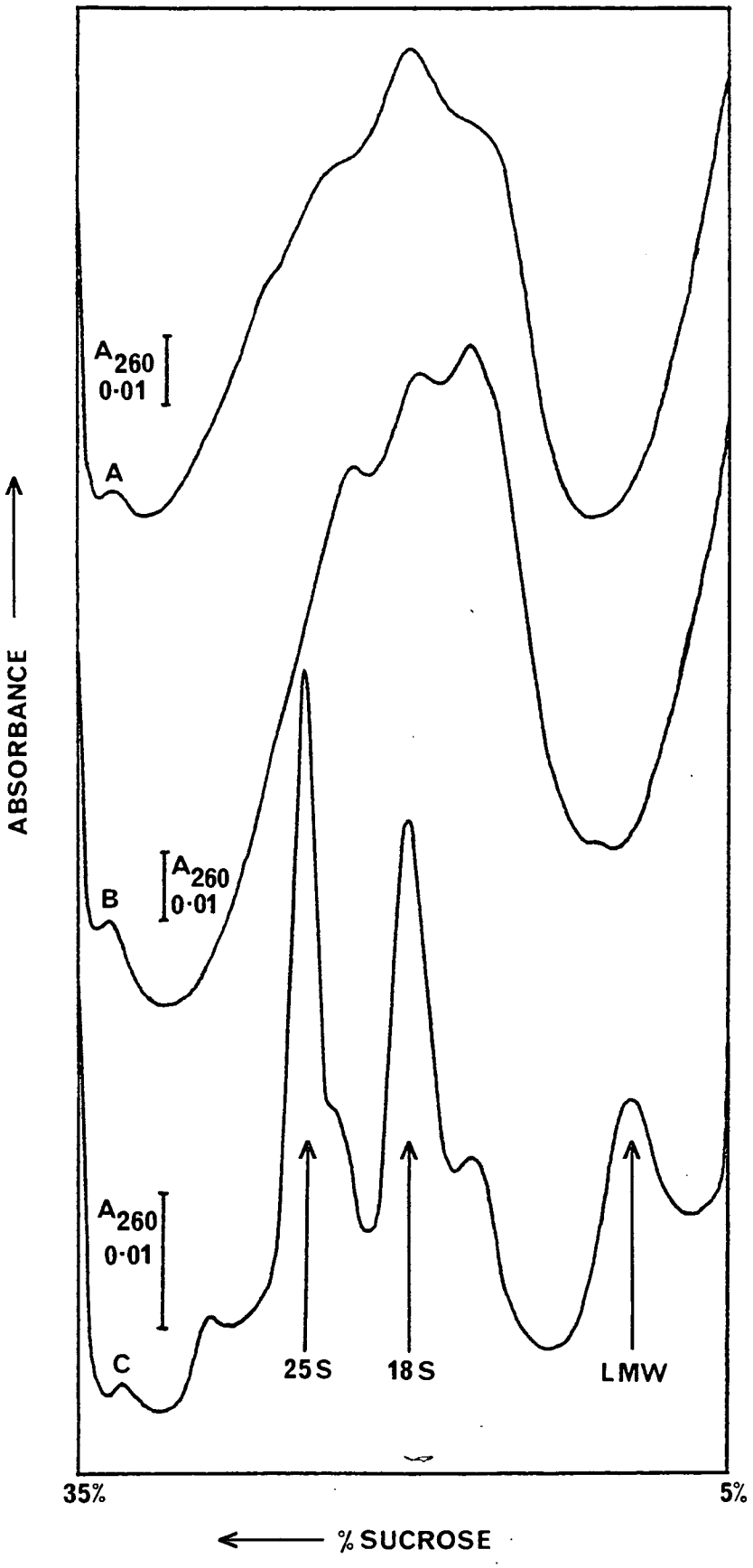

A260
0·01
A
B
A260
0·01
A260
0·01
C
25S
18S
LMW
ABSORBANCE
35%
5%
% SUCROSE

Figure 15

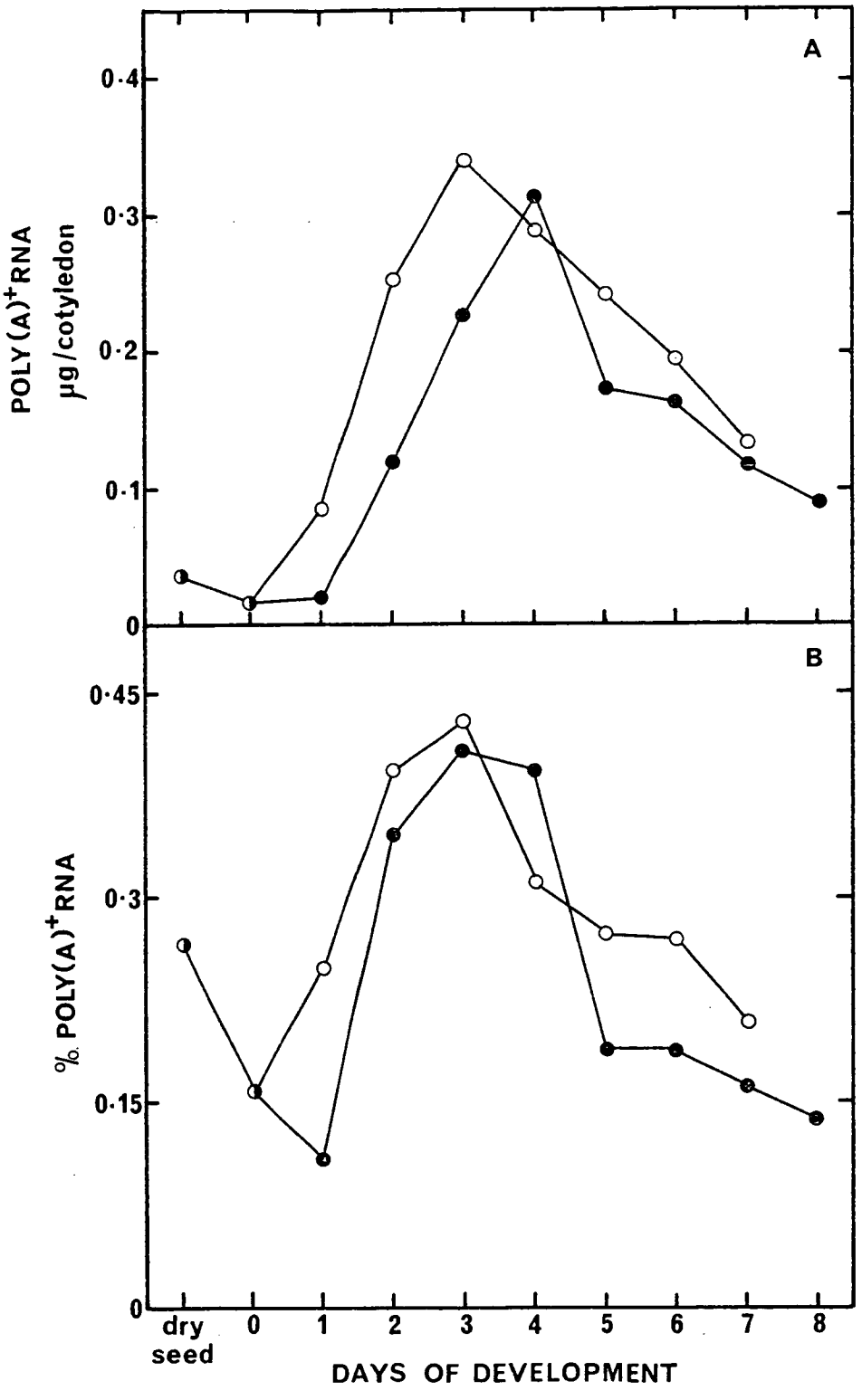
Developmental Changes in Cotyledonary Poly(A)$^+$ RNA

The amount of extractable poly(A)$^+$ RNA per cotyledon (A) was determined from each developmental stage of light (○———○) and dark-grown (●———●) cotyledons. The poly(A)$^+$ RNA was prepared by oligo-dT cellulose chromatography of phenol-detergent extractable total nucleic acid.

The extractable poly(A)$^+$ RNA was estimated as a percentage of the extractable total nucleic acid (Figure 9B) by the following equation.

$$\text{poly(A)}^+ \text{ RNA as \% of total nucleic acid} = \frac{\text{amount of extractable poly(A)}^+ \text{ RNA}}{\text{amount of extractable total nucleic acid}} \times 100$$

FIGURE 15.


A
B
POLY(A)+RNA µg/cotyledon
% POLY(A)+RNA
0·4
0·3
0·2
0·1
0
0·45
0·3
0·15
0
dry seed
0
1
2
3
4
5
6
7
8
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poly(A)$^+$ RNA per cotyledon. There would appear to be a small amount of extractable poly(A)$^+$ RNA present in the dry seed. During imbibition there is a drop in this fraction before it increases rapidly upon the onset of seedling growth, to yield a 21-fold increase by day 3 in the light. Both light and dark-grown tissue show similar increases though this is delayed by 24 hours in the dark. After peak levels have been reached by day 3 (or 4 in the dark), there is a rapid decrease in the poly(A)$^+$ RNA content of the cotyledons, as growth continues, to approximately 40% of peak values by the end of this developmental sequence.

The poly(A)$^+$ RNA content was expressed as a percentage of the total extractable nucleic acid and this is shown in Figure 15B. As can be seen, there is initially a decrease during imbibition followed by an increase to maximum levels at day 3 in both light and dark-grown tissue. After day 3 the poly(A)$^+$ RNA decreases as a proportion of the total nucleic acid, with a more rapid decrease in the dark after day 4.

The result obtained for day 1 dark-grown tissue indicates that this may be an inaccurate figure, even although it is the average of two experiments, since little difference would be expected between the two growth treatments at such an early stage of development.

2.4. Polyadenylate Sequences

The amount of poly(A) present in the total nucleic acid (p. 95), poly(A)$^+$ RNA and poly(A)$^-$ RNA fractions (p. 102) was determined using the [^{3}H]-poly U hybridisation procedure described by Bishop *et al.* (1974). It is known that the sucessful recovery of poly(A)$^+$ RNA by oligo dT-cellulose chromatography

depends upon the lengths of poly(A) being greater than c.a. 30 nucleotides (Cabada et al., 1977). Thus, it would be expected that not all poly(A) containing sequences would be recovered by this procedure and that, depending upon the type and developmental stage of tissue, a proportion of the poly(A) would remain in the poly(A)$^-$ RNA fraction.

[^{3}H]-labelled poly U which had a known capacity for hybridising to standard poly(A) was used and the poly(A) content per cotyledon from each fraction was estimated by comparison with results from standard experiments. Figure 16 illustrates the poly(A) contents of the three different fractions during germination and early seedling development.

The total poly(A) content per cotyledon (Figure 16A) decreases slightly during the 17 hour imbibition period. After imbibition there is a marked increase, producing a 9-fold net increase by day 4 in the light. Thereafter, the poly(A) content decreases to approximately 30% of the peak value. In the dark-grown tissue there is a similar profile for total poly(A) content but the peak value at day 3 is 44% of that in the light. Again, the period of maximum accumulation of this component coincides with the period of maximum heterotrophic activity of the tissue.

From the poly(A) content of the poly(A)$^-$ RNA fraction (Figure 16C) it is clear that, although this was the fraction which did not bind to the oligo dT-cellulose, it still contains a large proportion of poly(A). This indicates that either the chromatography has not been carried out successfully or, there are a large proportion of the mRNAs of the tissue which contain short poly(A) sequences (< c.a. 30 nucleotides) and thus do not

Figure 16

Developmental Changes in the Poly(A) Content of Cotyledonary RNA

The poly(A) content of the total nucleic acid (A), poly(A)$^+$ RNA (B) and poly(A)$^-$ RNA (C) from light (O——O) and dark-grown (●——●) cucumber cotyledons was estimated by ^{3}H-poly U hybridisation. The procedure used was that described by Bishop *et al*. (1974). 1 ng of standard poly(A) hybridised to 872 cpm of [^{3}H]-labelled poly U.

FIGURE 16.

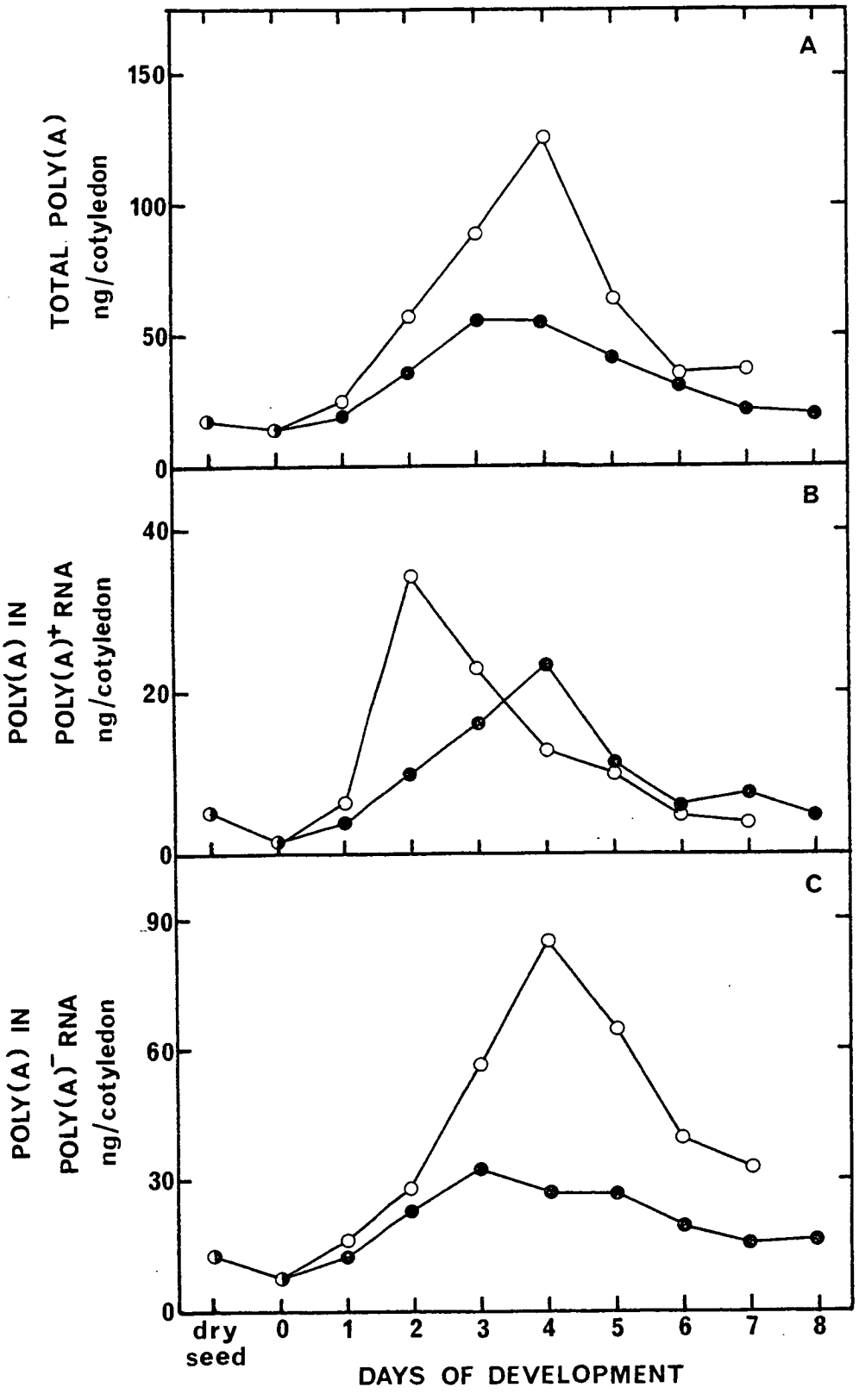
A
B
C
TOTAL POLY(A)
ng/cotyledon
POLY(A) IN
POLY(A)+ RNA
ng/cotyledon
POLY(A) IN
POLY(A)- RNA
ng/cotyledon
150
100
50
0
40
20
0
90
60
30
0
dry
seed
0
1
2
3
4
5
6
7
8
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Figure 17

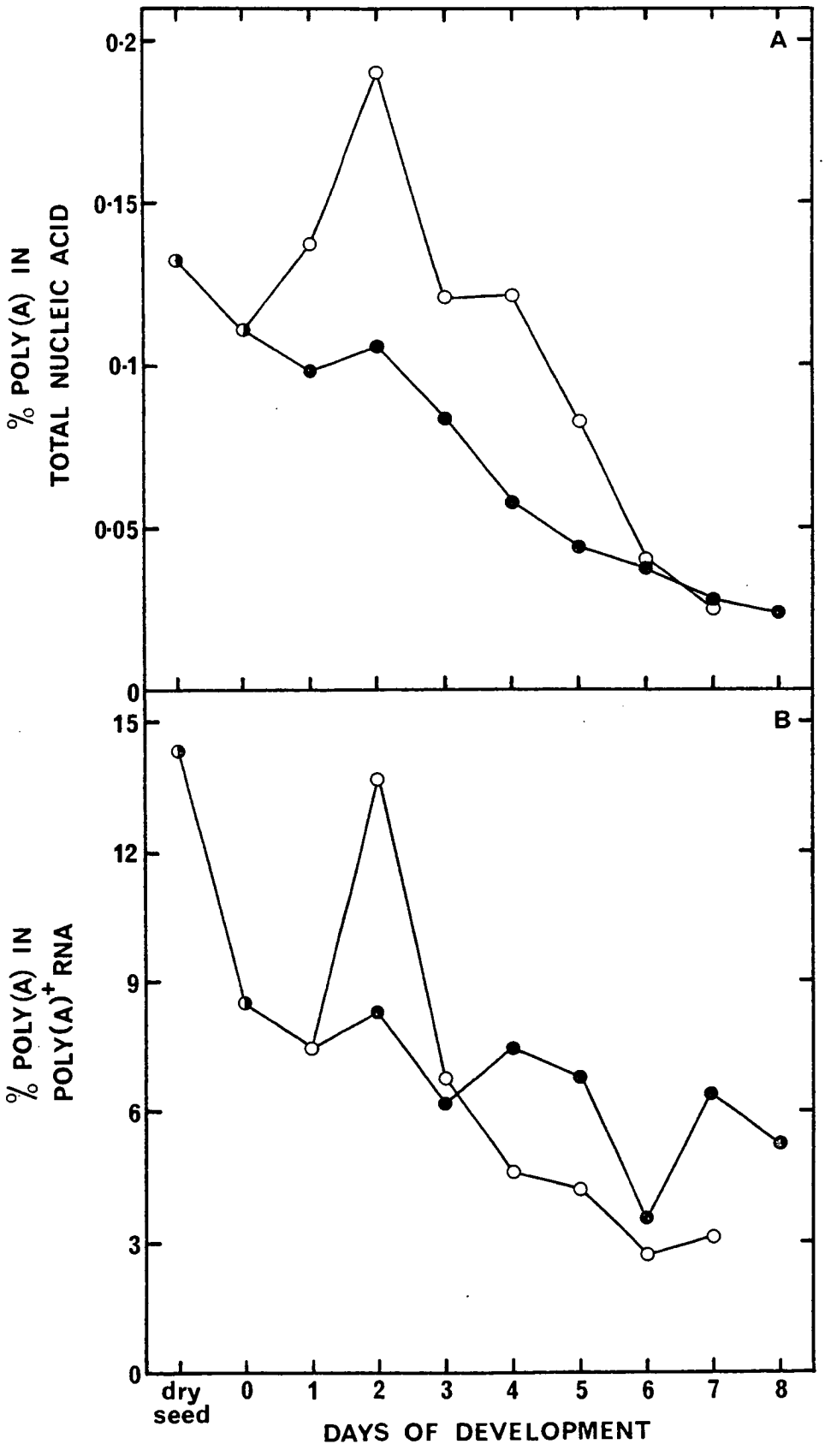
Changes in the Poly(A) Sequence as a Percentage of the Total Nucleic Acid and Poly(A)$^+$ RNA during Germination and Early Seedling Development

The poly(A) content of total nucleic acid (Figure 16A) and poly(A)$^+$ RNA (Figure 16B) from both light (o——o) and dark-grown (●——●) cotyledons was expressed as a percentage of the RNA from which it was estimated.

$$\text{total poly(A) as a \% of the total nucleic acid} = \frac{\text{amount of poly(A) in the total nucleic acid}}{\text{amount of total nucleic acid}} \times 100$$

$$\text{poly(A) in poly(A)}^+ \text{ RNA as a \% of the poly(A)}^+ \text{ RNA} = \frac{\text{amount of poly(A) in the poly(A)}^+ \text{ RNA fraction}}{\text{amount of poly(A)}^+ \text{ RNA}} \times 100$$

FIGURE 17.


A
B
0·2
0·15
0·1
0·05
0
% POLY(A) IN TOTAL NUCLEIC ACID
15
12
9
6
3
0
% POLY(A) IN POLY(A)+ RNA
dry seed
0
1
2
3
4
5
6
7
8
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bind to the oligo dT-cellulose. There is a similar profile for changes in the poly(A) content of this fraction as was observed for the total poly(A) content of the tissue (Figure 16A).

The poly(A) content of the poly(A)$^+$ RNA reveals a slightly different profile to those observed for the total nucleic acid and poly(A)$^-$ RNA fraction. Again a decrease occurs during imbibition and thereafter in both light and dark-grown tissue there is an increase in the poly(A) content then decline to sub-maximal levels by the end of this developmental period. However, there would appear to be a difference in the timing of peak poly(A) content of this fraction in the two growth conditions. In the light there is a very rapid increase to peak values at day 2 at a 25-fold net increase over the content of the imbibed tissue (day 0). The increase occurs at a slower rate in the dark-grown tissue to reach a peak of poly(A) at day 4 at approximately 67% of the maximum content in the light-grown tissue. By day 7 in the light the poly(A) content has declined to 12% of the peak value.

Figure 17 shows the poly(A) content expressed as a percentage of the nucleic acid fraction from which it was determined. This gives a very approximate indication of the average length of the poly(A) sequence, assuming the coding portion of the mRNA remains constant.

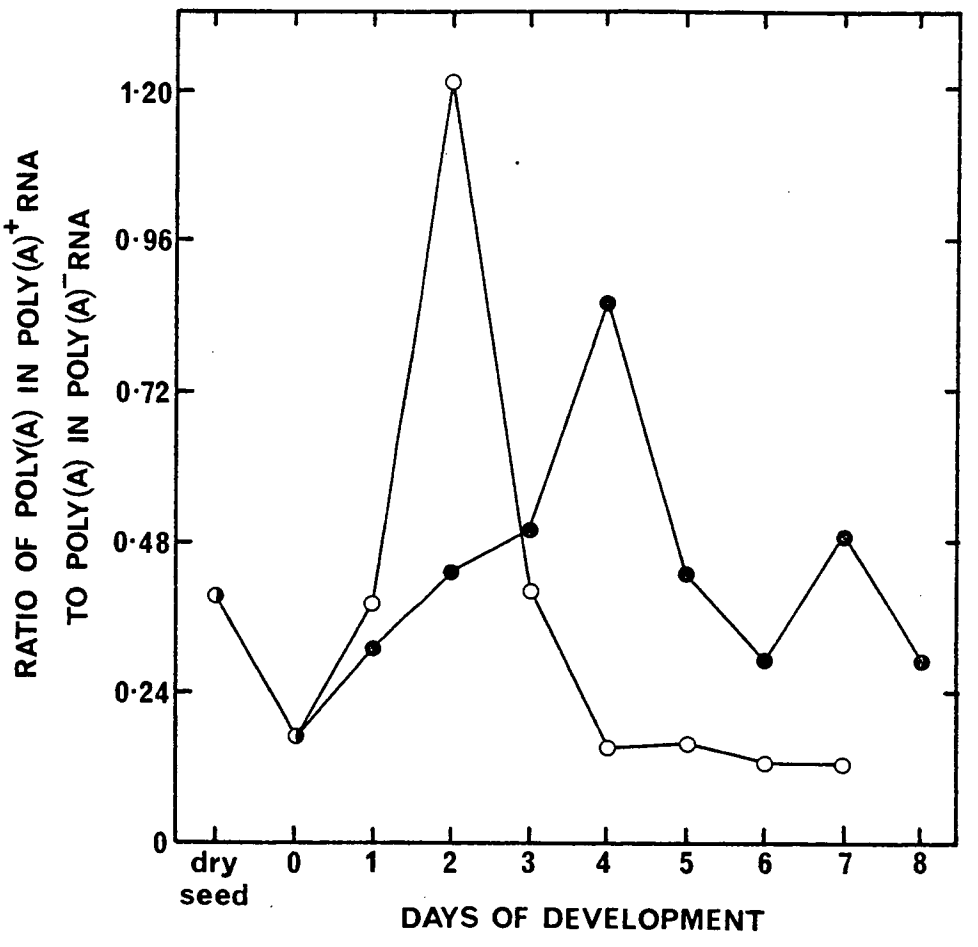
In the dry seed the total poly(A) content (Figure 17A) is approximately 0·13% of the total nucleic acid, then during imbibition this markedly decreases. After imbibition the poly(A) content increases to reach a peak at day 2 of 0·11% of the total nucleic acid, then declines so that, by day 7, it constitutes only 0·05% in the light and 0·03% in the dark-grown

Figure 18

Developmental Changes in the Ratio of Poly(A) in the Poly(A)$^+$ RNA to Poly(A) in the Poly(A)$^-$ RNA during Germination and Early Seedling Growth

The ratio of the poly(A) content of extractable poly(A)$^+$ RNA (Figure 16B) to that of poly(A)$^-$ RNA (Figure 16C) was estimated for both light (○——○) and dark-grown (●——●) tissue.

FIGURE 18.


RATIO OF POLY(A) IN POLY(A)+ RNA
TO POLY(A) IN POLY(A)- RNA
1·20
0·96
0·72
0·48
0·24
0
dry seed
0
1
2
3
4
5
6
7
8
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tissue.

The average poly(A) sequence of the poly(A)$^+$ RNA (Figure 17B) is approximately 14·5% in the dry seed and this decreases to 8·5% during imbibition. At day 2 in the light there would appear to be a marked, and short term increase in the average length of the poly(A). Thereafter there is a general decrease in the average poly(A) length until, by day 7 it only constitutes 3% of the poly(A)$^+$ RNA.

In the dark-grown tissue there is no increase at day 2 and the decrease in average poly(A) content of the poly(A)$^+$ RNA occurs at a slower rate than in the light. This suggests that, in some way, light affects the lengths of the poly(A) sequences on the mRNA molecules.

Figure 18 shows the ratio of poly(A) in the poly(A)$^+$ RNA and poly(A)$^-$ RNA fractions. This gives an approximate indication of the rate of turnover of poly(A) sequences in the tissue. During imbibition there was a decline in this ratio, indicating that net degradation of poly(A) was occurring. Subsequently there was a net synthesis of poly(A) which occurred earlier in the light than dark-grown tissue. Following peak periods of synthesis at day 2 in the light and day 4 in the dark there was an overall decline in the poly(A) content of the cotyledons which was more rapid in the light.

2.5. Summary

Changes in the nucleic acid content of the cotyledons have been studied during germination and early seedling development.

Total RNA, particularly the cytoplasmic rRNA and LMW

RNA species, have been shown to increase markedly during the heterotrophic growth phase. The synthesis and accumulation of chloroplast rRNA is more closely related to the onset of the autotrophic growth phase.

The polysome content increases dramatically during the heterotrophic growth phase and the proportion of rRNA actively involved in protein synthesis also increases.

Although light does not affect the initial synthesis and increase of these RNA species, it has been shown to be essential for attainment of the peak levels of the RNA species found in the light-grown tissue and for their more active involvement in protein synthesis.

Poly(A)$^+$ RNA, and the poly(A) content, taken as indicators of the mRNA content of the tissue, increase to peak values during the heterotrophic growth phase (days 2-4), then decline as development proceeds. There would appear to be a general decrease in the average length of the 3-terminal poly(A) sequences as the seedling develops with the exception that I have reproducibly measured an increase during the first 2 days of growth.

SECTION 3

DEVELOPMENTAL CHANGES IN COTYLEDONARY mRNA

From a study of the poly(A)$^+$ RNA and poly(A) contents of the cotyledons it has been shown that quantitative changes in the total mRNA population occur during germination and early seedling development. However, as well as these quantitative changes, any qualitative changes which occur in the mRNA population would be of interest. Changes in the relative amounts of mRNAs for individual polypeptides associated with the changing metabolic state of the tissue during this developmental period would be anticipated.

Over the past 10 years there has been an increase in the use of cell-free protein synthesising systems as assay systems for translatable mRNAs. Of the many cell-free protein synthesising systems available, I have made use of the wheat-germ *in vitro* system as a means of assaying both quantitative and qualitative changes in the cytoplasmic mRNA content of the cotyledons. In using the wheat-germ system, the assumption is made that it is capable of translating any cytoplasmic mRNA sequence with which it is programmed, and as yet, to my knowledge, no report has been made to the contrary.

3.1. Choice of Wheat-Germ System

At the onset of this study it was decided that, in order to obtain the best possible results from the wheat-germ *in vitro* system, it would be advisable to use a system which was as highly efficient as possible. Senger and Gross (1976) reported results which indicated that addition of exogenous RNA, even if this did not contribute significant template activity,

stimulates the endogenous protein synthesis of the wheat-germ system. If this were to occur, the resulting products of translation may be in part or *in toto* those of the wheat-germ extract itself. Therefore a wheat-germ system which had as little endogenous activity as possible was sought. Roberts and Paterson (1973) made use of a pre-incubation step to reduce the endogenous protein synthesising activity of the wheat-germ system.

Various different methods of preparing a wheat-germ S.30 protein synthesising system were tested and compared for stimulation above the endogenous activity and the products obtained upon addition of exogenous RNA. The methods of preparation are outlined in the Methods (p. 105) and the results obtained are shown in Tables 4 and 5 and Figures 19 and 20.

Zagórski (personal communication to Dr. C. J. Leaver; 1978) and Rychlik and Zagórski (1978) reported that dialysis of the wheat-germ system during preparation increased the efficiency of the system. I have compared wheat-germ preparations with and without such a dialysis treatment. From Table 4 it can be seen that the level of incorporation upon addition of exogenous RNA is higher in the dialysed wheat-germ preparation. The dialysed system also shows a lower endogenous activity than the non-dialysed wheat-germ, and thus is stimulated to a greater extent when programmed by exogenous RNA.

A study of the products obtained from these two *in vitro* protein synthesising systems (Figure 19) shows that, in agreement with the reduced level of endogenous activity already

Table 4

Effect of Dialysis of the Wheat-Germ S.30 during Preparation upon the Subsequent Efficiency of Cell-Free Translation

Wheat-germ cell-free synthesising systems were prepared with (A) or without (B) the inclusion of a dialysis step during the procedure (p. 105). Each system was programmed with 6 µg of total nucleic acid from day 3, light-grown cucumber cotyledons. Controls contained no exogenous RNA. The levels of incorporation (%) of [^{35}S]-methionine into hot, TCA-precipitable material and the stimulation of translational activity over the endogenous activity were estimated.

$$\% \text{ Incorporation} = \frac{\text{cpm into hot TCA-precipitable material}}{\text{cpm available in the incubation}} \times 100$$

$$\text{Fold Stimulation} = \frac{\text{cpm incorporated by primed system}}{\text{cpm incorporated by control (endogenous)}} - 1$$

Table 4

Exogenous RNA	Time (min)	Non-Dialysed Wheat-Germ S.30		Dialysed Wheat-Germ S.30	
		% Incorporation	Stimulation	% Incorporation	Stimulation
A. CUCUMBER COTYLEDONARY RNA	10	3•4	6•0	4.5	30•1
	30	12•5	5•8	17•8	13•2
	60	16•4	3•4	26•1	11•4
	90	16•9	3•3	27•6	10•1
B. - RNA	10	c.a. 0•5	-	c.a. 0•1	-
	30	1•8	-	1•2	-
	60	3•7	-	1•6	-
	90	3•9	-	2•5	-

Figure 19

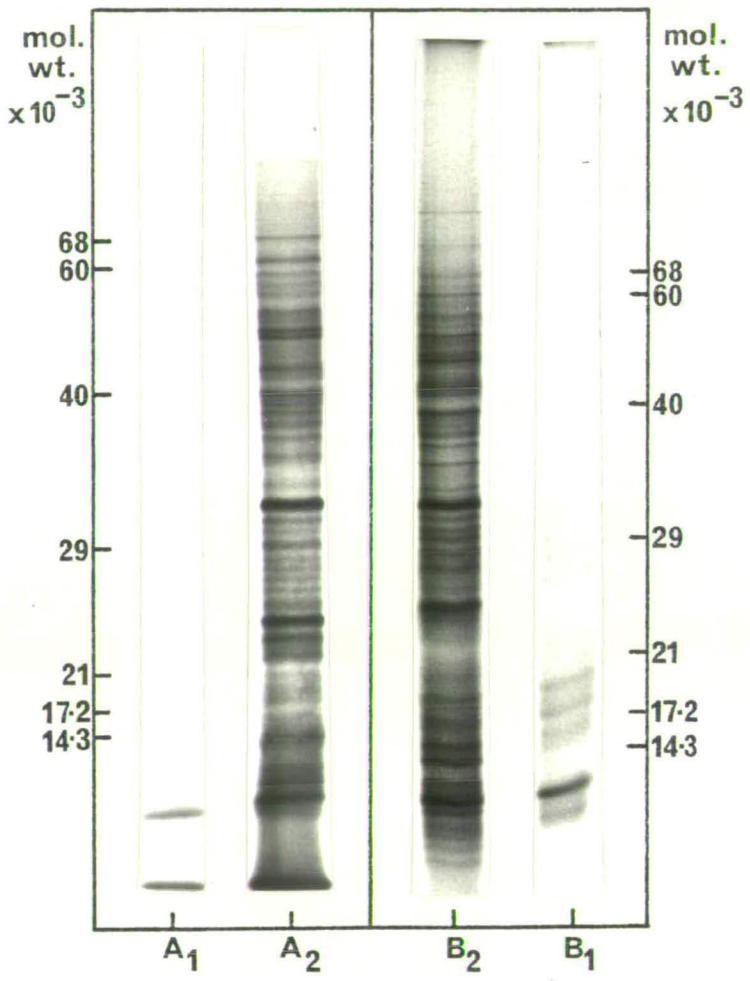
Translational Products of Wheat-Germ System with or without a Dialysis Step during Preparation

The *in vitro* labelled polypeptides from the wheat-germ system were analysed by SDS-polyacrylamide (15%) gel electrophoresis followed by autoradiography. Comparison of the products from two different wheat-germ preparations prepared with (A) or without (B) a dialysis step during preparation.

Lanes A_1 & B_1 - endogenous activity

Lanes A_2 & B_2 - polypeptides coded by day 3 cucumber cotyledonary total nucleic acid (6 µg)

FIGURE 19.


mol.
wt.
x10⁻³
68
60
40
29
21
17·2
14·3
A1
A2
B2
B1
mol.
wt.
x10⁻³
68
60
40
29
21
17·2
14·3

noted, there is a reduction in the endogenous products obtained with the dialysed compared with the non-dialysed system.

Thus, by dialysing the wheat-germ system during preparation, it would appear that the endogenous activity is reduced and the system becomes more responsive to exogenous messenger.

In reticulocyte lysate cell-free systems there is normally a greater endogenous template activity than is observed for the wheat-germ system. To reduce this activity, the lysate is treated with micrococcal nuclease (Pelham and Jackson, 1976). A wheat-germ system was prepared which was treated with micrococcal nuclease to remove the endogenous mRNA. A comparison of a dialysed and micrococcal nuclease treated wheat-germ system for levels of endogenous activity, stimulation upon addition of exogenous RNA and products obtained, was carried out. Table 5 and Figure 20 show the results from this experiment using cucumber and TMV RNA as exogenous templates.

From Table 5 it can be seen that the level of endogenous activity is lowest when the system is both dialysed and nuclease treated. However, this also reduces the level of incorporation due to exogenous RNA. The highest stimulation is obtained when the wheat-germ system is treated only with nuclease. The greatest difference between the various methods of preparation is obtained when viral RNA is used as template. In contrast, the differences are not so marked when cucumber cotyledonary RNA is used.

A study of the products (Figure 20) reveals that

Table 5

Comparison of the Incorporation Efficiency of Wheat-Germ Systems prepared with Nuclease and/or Dialysis Treatments

Wheat-germ cell-free protein synthesising systems were prepared, as described (p. 105) with either nuclease (A), dialysis (B) or nuclease and dialysis (C) treatments during their preparation (p. 106). Each system was programmed with TMV RNA (1 µg) or day 3, light-grown cucumber cotyledonary total nucleic acid (6 µg). Controls contained no exogenous RNA. Incorporations were carried out as described (p. 107). Incorporation (%) of [^{35}S]-methionine into hot, TCA-precipitable material and the stimulation by exogenous RNA were estimated.

$$\% \text{ Incorporation} = \frac{\text{cpm into hot TCA-precipitable material}}{\text{cpm available in the incubation}} \times 100$$

$$\text{Fold Stimulation} = \frac{\text{cpm incorporated by primed system}}{\text{cpm incorporated by control (exogenous)}} - 1$$

Table 5

Exogenous RNA	Time (min)	Nuclease Treated Wheat-Germ S.30		Dialysed Wheat-Germ S.30		Nuclease Treated & Dialysed Wheat-Germ S.30	
		% Incorporation	Stimulation	% Incorporation	Stimulation	% Incorporation	Stimulation
A. TMV RNA	10	6·4	37·7	2·1	2·5	2·9	2·5
	30	31·4	49·2	9·2	7·6	12·9	9·9
	60	39·1	30·1	15·5	8·6	18·7	28·3
	90	38·5	37·0	17·7	9·4	20·6	27·6
B. CUCUMBER COTYLEDONARY RNA	10	4·7	27·1	3·3	5·4	3·2	4·2
	30	18·1	28·0	13·8	11·6	11·3	8·5
	60	20·3	15·1	19·5	12·2	15·2	22·5
	90	26·5	25·1	24·4	13·9	19·3	25·8
C. - RNA	10	0·2	-	0·6	-	0·6	-
	30	0·6	-	1·0	-	1·2	-
	60	1·3	-	1·6	-	0·6	-
	90	1·0	-	1·7	-	0·7	-

Figure 20

Translation Products of Nuclease and/or Dialysis Treated Wheat-Germ Systems

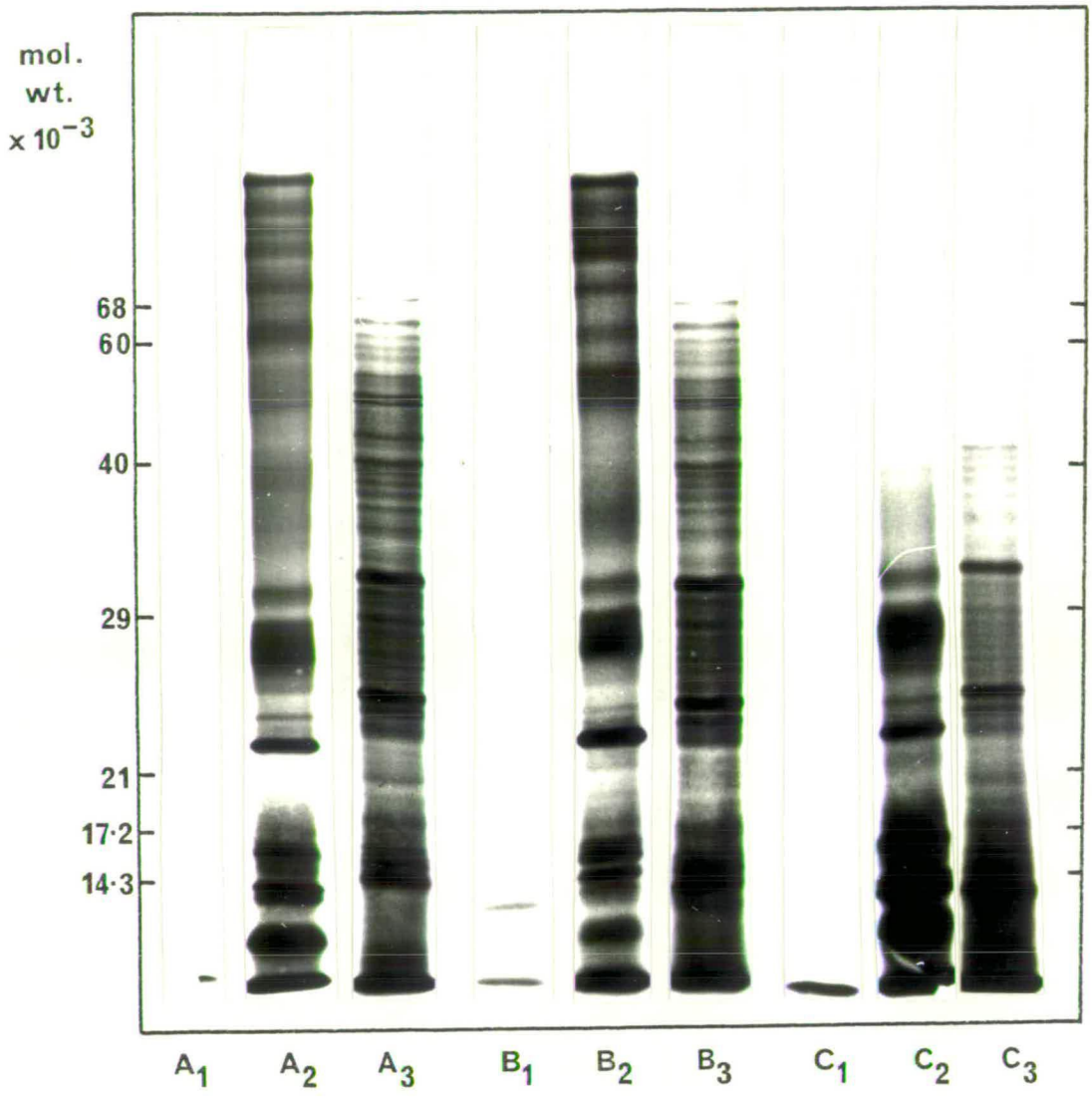
Translation products were compared from wheat-germ systems which were nuclease (A), dialysis (B) or nuclease and dialysis (C) treated during preparation (p.106). The *in vitro* labelled polypeptides were fractionated by SDS-polyacrylamide gel electrophoresis (15% w/v) and analysed by autoradiography.

Lane A_1, B_1, C_1 - endogenous activity

Lane A_2, B_2, C_2 - polypeptides coded by TMV RNA (1 µg)

Lane A_3, B_3, C_3 - polypeptides coded by day 3 cucumber cotyledonary total nucleic acid (6 µg)

FIGURE 20.



in all these systems there are few products due to translation of endogenous mRNA. Upon addition of exogenous RNA, translation of the endogenous message does not appear to be enhanced. High molecular weight products are synthesised by both the nuclease and dialysis treated wheat-germ systems. However, when these treatments are combined, there is a selective loss in ability of synthesise high molecular weight products.

The efficiency of the nuclease and dialysis treated wheat-germ systems, in terms of stimulation and products, was very similar when programmed with cucumber cotyledonary RNA. As this, rather than the viral RNA was to be used in further experiments, the dialysed system was chosen for all further in vitro translation experiments. Although the endogenous activity of the dialysed system was not reduced to the same extent as in the nuclease treated system, it was significantly reduced compared with the non-dialysed system.

Thus, for the study of developmental changes in the mRNA of the cotyledons, a wheat-germ in vitro protein synthesising system, which had been dialysed at $4^{o}C$ against the grinding buffer, was used.

In contrast to the report of Senger and Gross (1976), I found no direct evidence that exogenous RNA stimulates the translation of endogenous mRNA. In fact, Dr. J. M. Grienenberger (personal communication) has shown that the translational activity of poly(A)$^{+}$ RNA in the cell-free system is inhibited by added rRNA.

During the preparation of a wheat-germ S.100 polysome run-off system (p. 107) a dialysis step was also included to reduce the endogenous mRNA activity.

Figure 21

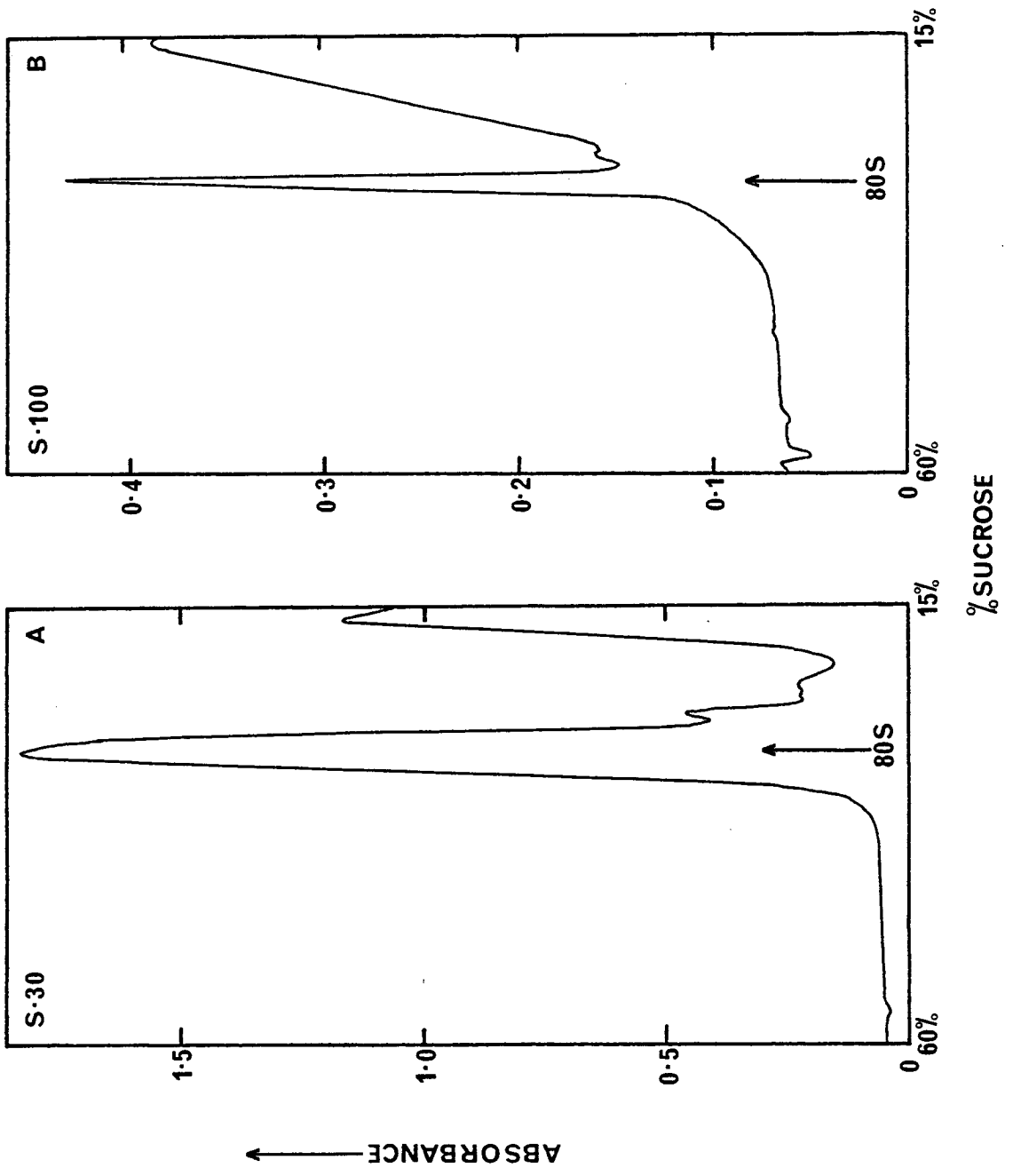
Sucrose Density Gradient Fractionation of S.30 and S.100 Wheat-Germ System

Samples of S.30 and S.100 wheat-germ cell-free systems were analysed by fractionation on 5·1 ml, 15-60% (w/v) linear sucrose gradients. Centrifugation was carried out at 149,000 g_{av} for 1·5 hours, in a Spinco SW50 rotor. The A_{265} profiles were determined using an ISCO Model UA-4 absorbance monitor and a Vitatron chart recorder. The position of the 80S monosome is indicated with an arrow.

A. S.30 system - 40 µl of the preparation were loaded on the gradient. (A_{260} = c.a. 150 O.D. units/ml).

B. S.100 system - 60 µl of the preparation were loaded on the gradient. (A_{260} = c.a. 0·35 O.D. units/ml).

FIGURE 21.


A
S·30
80S
15%
60%
0
0·5
1·0
1·5
B
S·100
80S
15%
60%
0
0·1
0·2
0·3
0·4
ABSORBANCE
% SUCROSE

Samples of both the S.30 and S.100 wheat-germ *in vitro* systems were fractionated on 15-60% continuous sucrose density gradients (Figure 21). It can be seen that the S.30 system contains a large amount of monoribosomes whereas, essentially all the ribosomes have been removed from the S.100 system. The S.30 system can be programmed by exogenous mRNA or polysomes while the S.100 system is used to translate the mRNA on added polysomes.

3.2. Optimisation of Wheat-Germ System

It is obvious from the reports of other workers in this field (Roberts and Paterson, 1973; Marcu and Dudock, 1974; Zagórski, 1975; Rychlik and Zagórski, 1978) that the wheat-germ system must be optimised to produce high efficiency and fidelity of translation of mRNA prior to its use as an assay system.

The dialysed wheat-germ system was optimised for 4 different factors. These were the concentration of wheat-germ used, the potassium and magnesium ion aconcentrations and finally the concentration of RNA added to the system. For each of these the optimum conditions in terms of the level of incorportion of a labelled amino acid into hot, TCA-precipitable protein, the stimulation when using exogenous mRNA as template and the products obtained were studied.

The optimum concentration of wheat-germ, based upon the levels of incorporation and stimulation above the endogenous activity (Figure 22A), would appear to be between 0·75 and 1·5 A_{260} units in a 50 µl incubation. A comparison of the products obtained from these incubations (Figure 23A), shows that, in terms of high molecular weight products, 1·5

Figure 22

Effect of Wheat-Germ, K^+ and Mg^{2+} Concentrations on In Vitro Translation

Wheat-germ *in vitro* incorporations were carried out using varying concentrations of wheat-germ (A), K^+ (B) and Mg^{2+} (C). The levels of incorporation (O——O) and stimulation (Δ- - -Δ) were calculated as described (Table 4). In each 6 µg of day 3, light-grown cucumber cotyledonary total nucleic acid was used to programme *in vitro* translation.

The normal concentrations used were 1⟶1·5 O.D. units/ml of wheat-germ S.30; 104 mM K^+; 2·25 mM Mg^{2+}.

FIGURE 22.

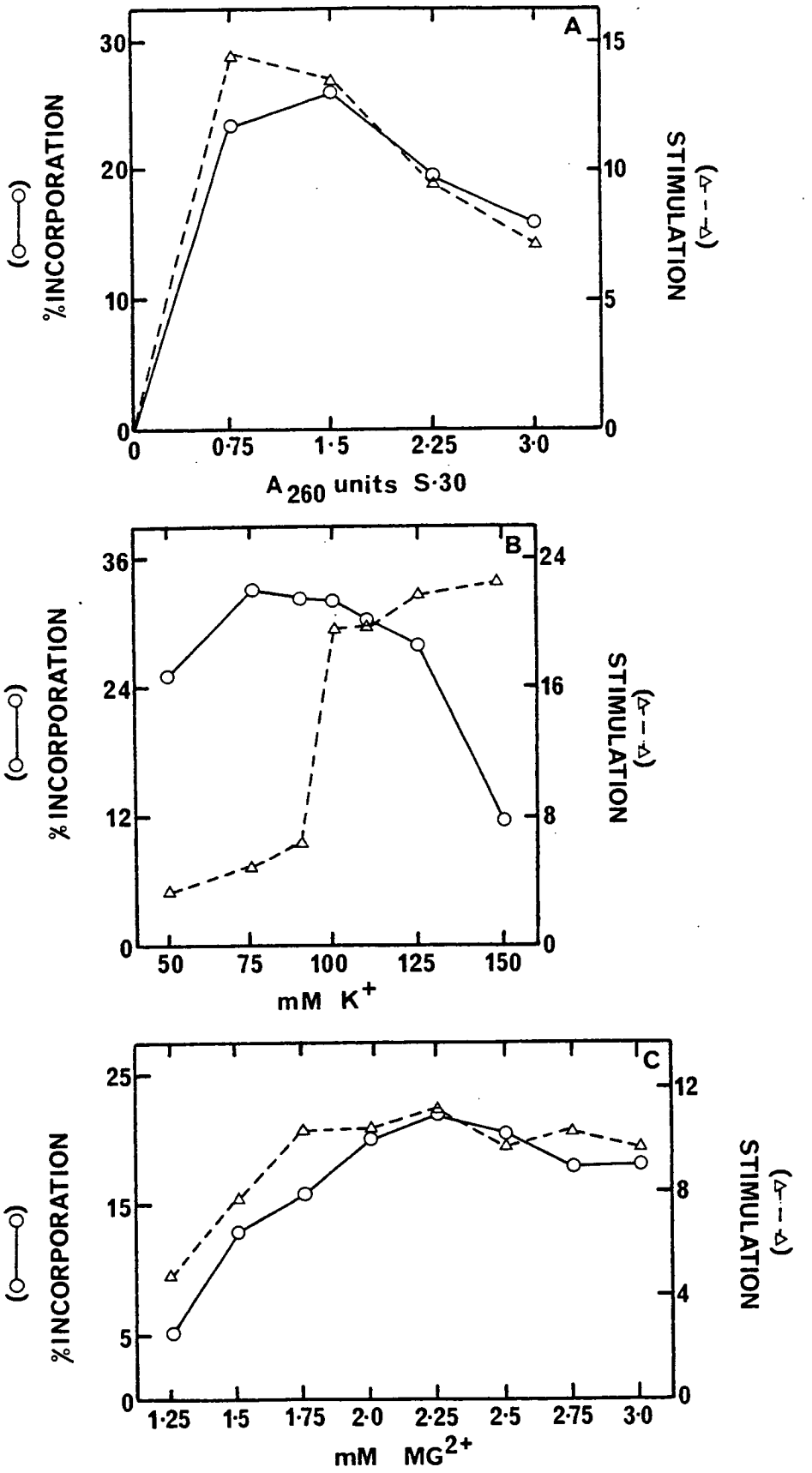

A
30
20
10
0
15
10
5
0
0
0·75
1·5
2·25
3·0
% INCORPORATION (o—o)
STIMULATION (Δ- -Δ)
A 260 units S·30
B
36
24
12
0
24
16
8
0
50
75
100
125
150
% INCORPORATION (o—o)
STIMULATION (Δ- -Δ)
mM K+
C
25
15
5
0
12
8
4
0
1·25
1·5
1·75
2·0
2·25
2·5
2·75
3·0
% INCORPORATION (o—o)
STIMULATION (Δ- -Δ)
mM MG2+

Figure 23

In vitro Labelled Products obtained with varying Wheat-Germ, K^+ and Mg^{2+} Concentrations

In vitro labelled products synthesised under varying buffer and ion concentrations were compared. Analysis of the products was by fractionation on SDS-polyacrylamide gels (15%, w/v) followed by autoradiography. In each incubation 6 µg of total nucleic acid from day 3 light-grown cotyledons were used to programme the cell-free system.

A. Varying S.30 concentration - Wheat-germ 0·75–3·0 A_{260} units. 104 mM K^+; 2·25 mM Mg^{2+}.

B. Varying K^+ concentration - 50–150 mM K^+; 2·25 mM Mg^{2+}. c.a. 1·5 A_{260} units wheat-germ.

C. Varying Mg^{2+} concentration - 1·25–3·0 mM Mg^{2+}; 104 mM K^+. c.a. 1·5 A_{260} units wheat-germ.

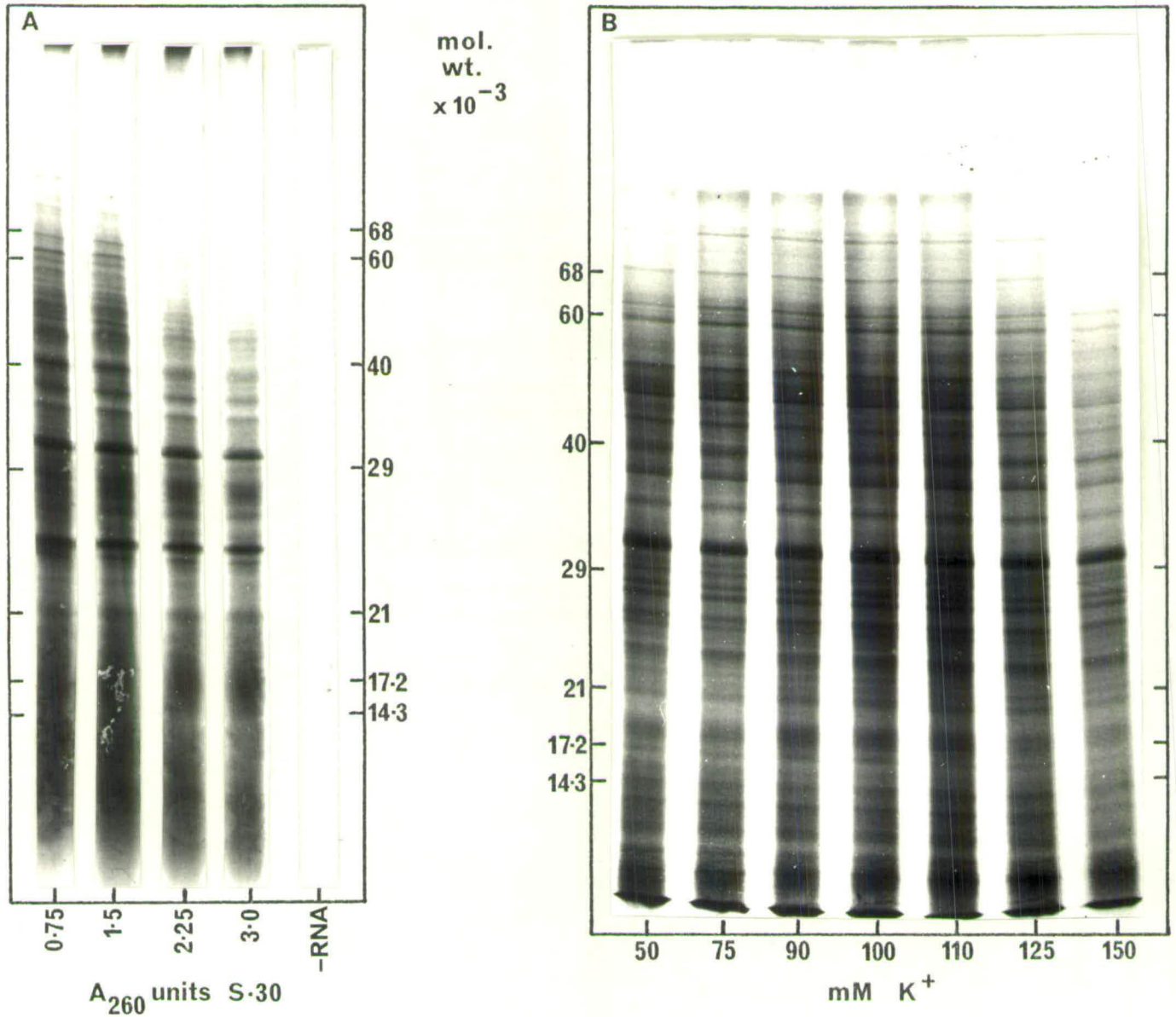
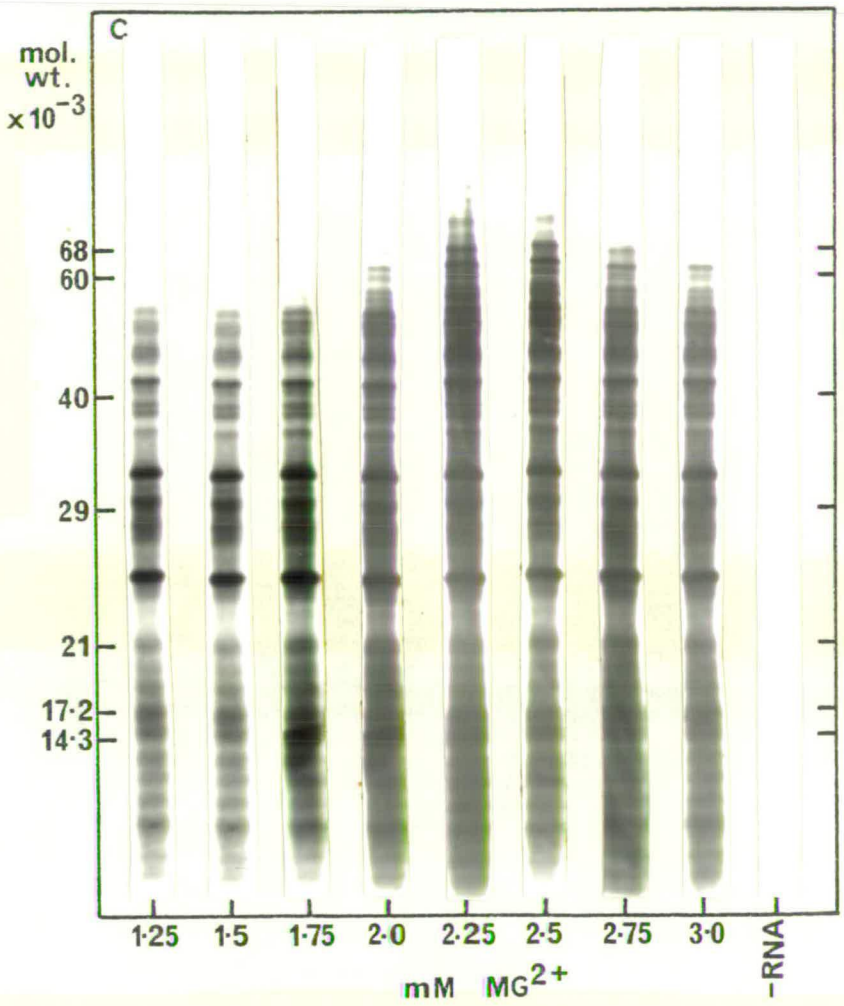

A
mol. wt. x 10⁻³
68
60
40
29
21
17·2
14·3
0·75
1·5
2·25
3·0
-RNA
A260 units S·30
B
68
60
40
29
21
17·2
14·3
50
75
90
100
110
125
150
mM K+

FIGURE 23.

FIGURE 23 cont.



A_{260} units/50 µl incubation would appear to be optimal.

Each new wheat-germ preparation was optimised and in any individual experiment a concentration of between 1 to 1·5 A_{260} units per incubation was used.

The potassium ion concentration was tested over the range of 50 - 150 mM (Figure 22B). In terms of incorporation, there is a peak of activity between 75 - 110 mM. Stimulation above the endogenous activity increases throughout the concentration range studied, since the efficiency of translation of the endogenous mRNA apparently decreases as the K^+ concentration is raised.

In terms of the products synthesised (Figure 23B), there is a loss of high molecular weight products at the lower K^+ concentrations. Since K^+ concentrations above 100 - 110 mM did not increase the types and amounts of high molecular weight products and also resulted in markedly decreased levels of incorporation, the optimum condition for efficient translation of the cucumber cotyledonary RNA was taken to be 100 - 110 mM.

Magnesium ion concentrations ranging from 1·25 - 3·0 mM were tested for their effect on the efficiency of translation in the wheat-germ system. From Figure 23C it can be seen that, in terms of both the level of incorporation and stimulation above the endogenous activity, 2·25 mM is the optimum concentration of this ion. Efficient translation of high molecular weight products was also obtained at this Mg^{2+} concentration (Figure 23C) and it was therefore used in all further experiments.

It should be noted that spermidine, at a concentration of 2·25 mM, was routinely included in the wheat-germ

incubation mixture. Hunter et al. (1977) have shown that polyamines, particularly spermine and spermidine, stimulate incorporation of amino acids into protein in the wheat-germ cell-free protein synthesising system. Addition of polyamines at optimum conditions apparently lowers the Mg^{2+} concentration required for optimum protein synthesis. Hunter et al. (1977) also suggest that the presence of polyamines in the wheat-germ system increases the efficiency of translation in terms of completion of full length translation products.

It was consistently observed that dialysis affected the K^{+} and Mg^{2+} concentrations for optimum in vitro protein synthesis in the wheat-germ system (personal observation). Presumably this was due to the endogenous ion concentrations of the wheat-germ extract being reduced by equilibration with the dialysis medium. This is consistent with the observation that other workers using non-dialysed preparations (e.g. Marcu and Dudock, 1974; Hunter et al., 1977A) use lower ion concentrations, while Rychlik and Zagórski (1978) who also use dialysis, show a requirement for the higher ion concentrations.

An important prerequisite to any study of developmentally related changes in an mRNA population using a wheat-germ cell-free system is to optimise the concentration of input RNA used to programme the system. Optimisation experiments were carried out using total nucleic acid from light-grown day 3 cotyledons and the translational activity and polypeptides synthesised examined over a range of 2·5 - 65 µg total nucleic acid per incubation.

Figure 24

The Effect of Increasing the Total Nucleic Acid Concentration upon the Efficiency of In Vitro Translation

A. The effect of varying the concentration of total nucleic acid from day 3, light-grown cucumber cotyledons upon the levels of incorporation (o——o) of [^{35}S]-methionine into hot, TCA-precipitable material in the wheat-germ S.30 in vitro protein synthesising system. The fold stimulation (●- - -●) above the endogenous activity was also determined. Minus RNA controls showed 1·8% incorporation. All determinations were carried out after 90 min. incubation.

Apart from varying the levels of exogenous nucleic acid, all other conditions were as described (p.107).

B. The levels of incorporation of [^{35}S]-methionine were expressed on a per µg basis.

FIGURE 24.

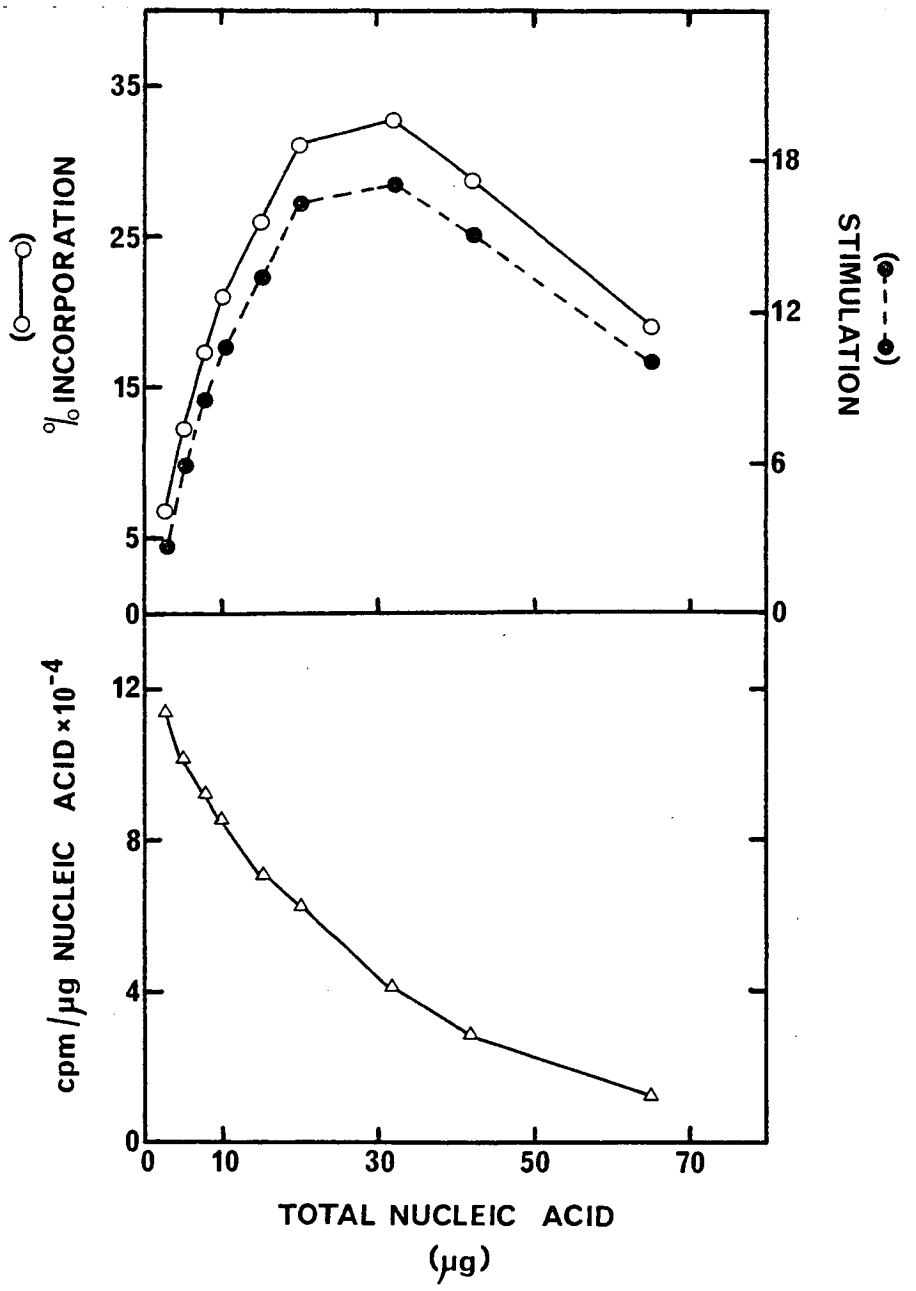
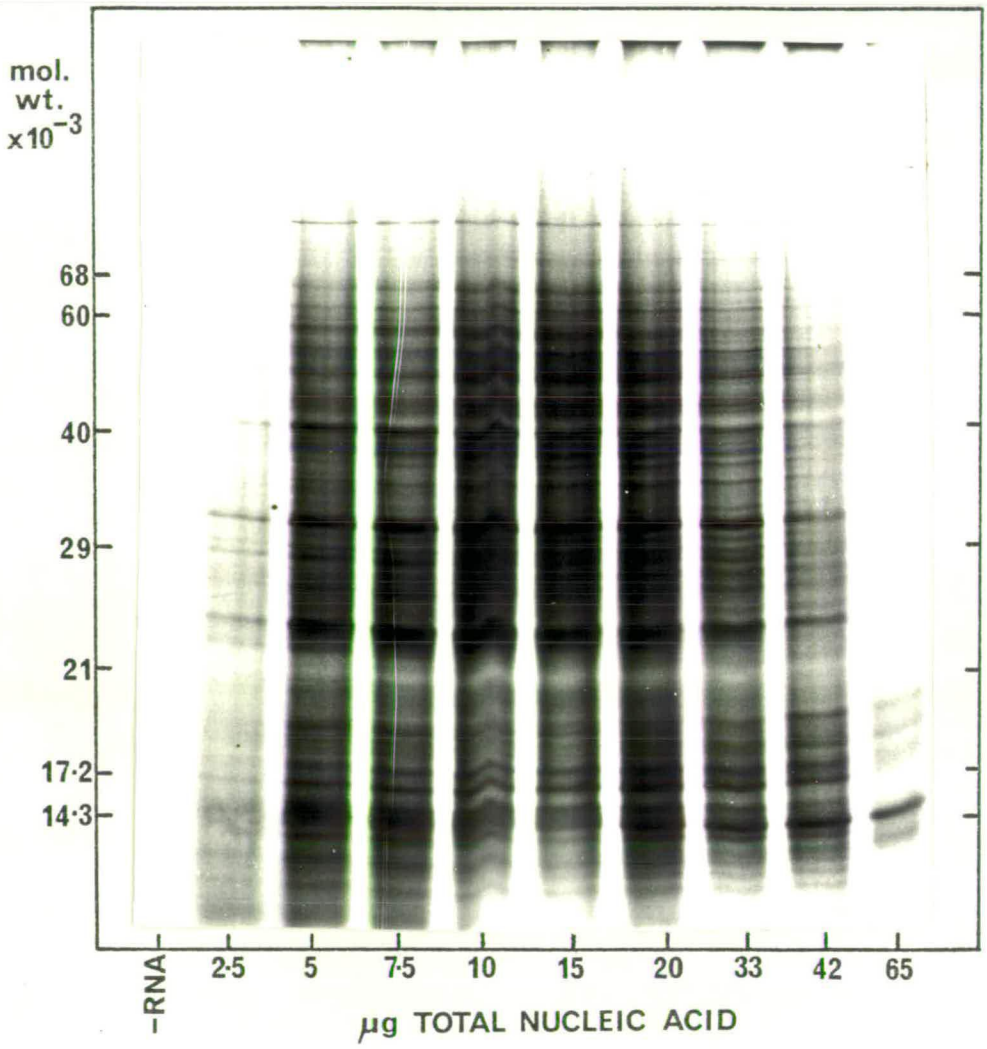

% INCORPORATION
STIMULATION
(●---●)
cpm/µg NUCLEIC ACID ×10⁻⁴
TOTAL NUCLEIC ACID
(µg)
0
5
15
25
35
6
12
18
4
8
10
30
50
70

Figure 25

In Vitro Labelled Polypeptides Synthesised by the Wheat-Germ System when Programmed with Increasing Concentrations of Total Nucleic Acid

The *in vitro* labelled polypeptides synthesised in the wheat-germ system, when programmed with varying concentrations of day 3, light grown cotyledonary total nucleic acid, were analysed by SDS-polyacrylamide gel (15% w/v) electrophoresis followed by autoradiography. The concentrations of total nucleic acid used to prime the system are as indicated.

FIGURE 25.


mol.
wt.
x10⁻³
68
60
40
29
21
17·2
14·3
-RNA
2·5
5
7·5
10
15
20
33
42
65
µg TOTAL NUCLEIC ACID

Both the incorporation and stimulation increase rapidly to peak somewhere between 20 - 30 µg of total nucleic acid, then decline as the RNA concentration is increased (Figure 24A). Thus, the system is saturated at a total RNA concentration of between 20 - 30 µg. A comparison of the labelled products obtained shows that there was a loss of high molecular weight products at either end of this concentration range (Figure 25).

In all developmental studies the nucleic acid concentration used to programme the wheat-germ system was in the range 5 - 15 µg where the amount of mRNA was limiting and high molecular weight products were efficiently synthesised.

A consistent observation, which is illustrated in Figure 24B, is that, although there is an approximate linear relationship between the amount of input RNA and the level of incorporation obtained at the lower concentration ranges, these two factors are not directly related on a per µg basis. This relationship was also shown for nucleic acid from other developmental stages.

To ensure that optimising for the amount of nucleic acid to be added to the wheat-germ system using nucleic acid from only one stage of development was sufficient, another experiment was carried out. In this, four different concentrations of nucleic acid from each of the stages of development of light-grown tissue were used to programme the wheat-germ system and the levels of incorporation were expressed as c.p.m./cotyledon (Figure 26). In addition, the products of translation were compared.

As can be seen, the developmental trends in incor-

Figure 26

The Effect of Total Nucleic Acid Concentration upon the Developmental Profile of Translatable mRNA Content of the Cotyledons as Determined by the Incorporation of [^{35}S]-Methionine into Protein in the Wheat-Germ System

Four different concentrations of total nucleic acid, from light-grown cucumber cotyledons from each day of development were used to programme the optimised wheat-germ S.30 system. The levels of incorporation of [^{35}S]-methionine into hot, TCA-precipitable material were expressed on a per cotyledon basis, by use of the following equation:-

$$\text{cpm/cotyledon} = \frac{\text{cpm/incubation}}{\mu\text{g total nucleic acid added}} \times \mu\text{g total nucleic acid/cotyledon (Figure 9B)}$$

Total nucleic acid concentrations used

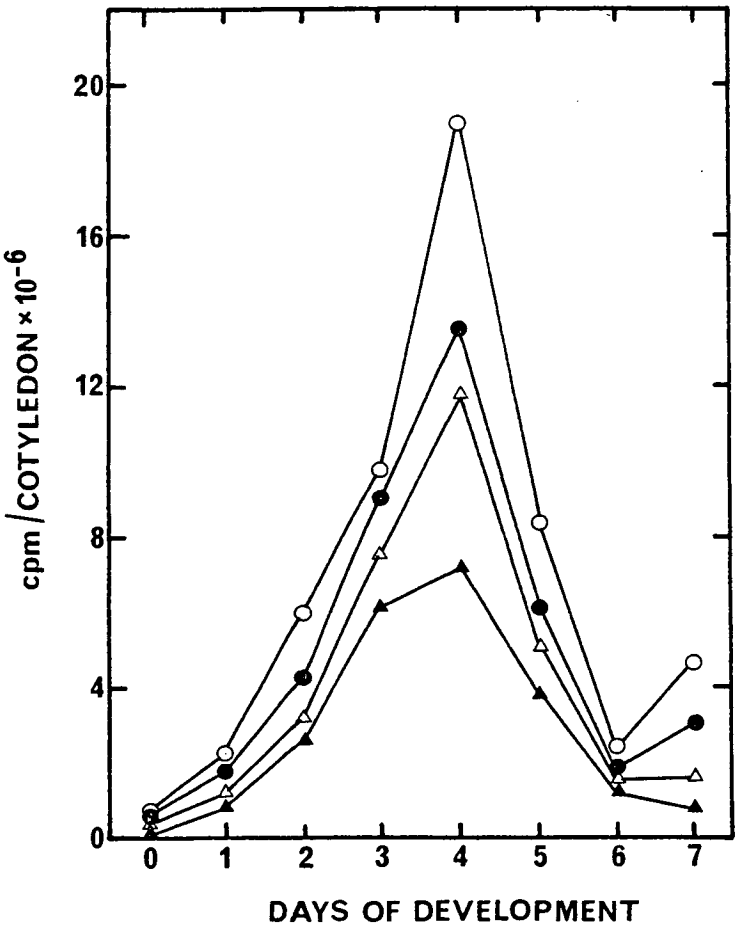
2·5 µg/incubation (○——○)

7·5 µg/incubation (◉——◉)

12·5 µg/incubation (△——△)

20·0 µg/incubation (▲——▲)

FIGURE 26.



poration were qualitatively identical using the different concentrations of nucleic acid. However, the absolute values varied depending on the source and concentration of nucleic acid used to programme the wheat-germ system.

A study of the products from the developmental sequence, over this concentration range, confirmed that high molecular weight polypeptides were efficiently translated at all but the lowest concentration tested (2·5 µg/50 µl incubation).

Thus, the system was routinely programmed with that amount of nucleic acid which gave the highest possible level of incorporation, yet was consistent with the limits already described.

As well as determining the optimum concentrations of nucleic acid, a similar experiment was carried out using day 2 poly(A)$^+$ RNA from light-grown cotyledons. In this experiment the concentration range studied was from 0·05 to 1·7 µg per incubation. Figure 27A shows the level of incorporation and stimulation and Figure 28 illustrates the polypeptides synthesised under these conditions. Since poly(A)$^+$ RNA is essentially pure messenger RNA as compared to the total nucleic acid, much lower amounts are required to programme the wheat-germ system.

From the levels of incorporation at the different concentrations, 1 µg of poly(A)$^+$ RNA apparently saturates the wheat-germ system. At the top end of the concentration range there can be seen to be a loss of high molecular weight polypeptides. Thus, 0·5 µg per incubation was chosen as a suitable concentration for experiments where the wheat-germ system

Figure 27

The Effect of Increasing Poly(A)$^+$ Concentration upon the Efficiency of In Vitro Translation

A. Increasing concentrations of poly(A)$^+$ RNA from day 2, dark-grown cucumber cotyledons, were used to programme the wheat-germ cell-free system. The level of incorporation (O——O) of [^{35}S]-methionine into hot, TCA-precipitable material and the fold stimulation (●- - -●) above the endogenous activity (2·3% incorporation) were estimated.

B. The levels of incorporation were expressed on a /µg basis (i.e. /µg added poly(A)$^+$ RNA for each concentration used). (Δ——Δ).

FIGURE 27.

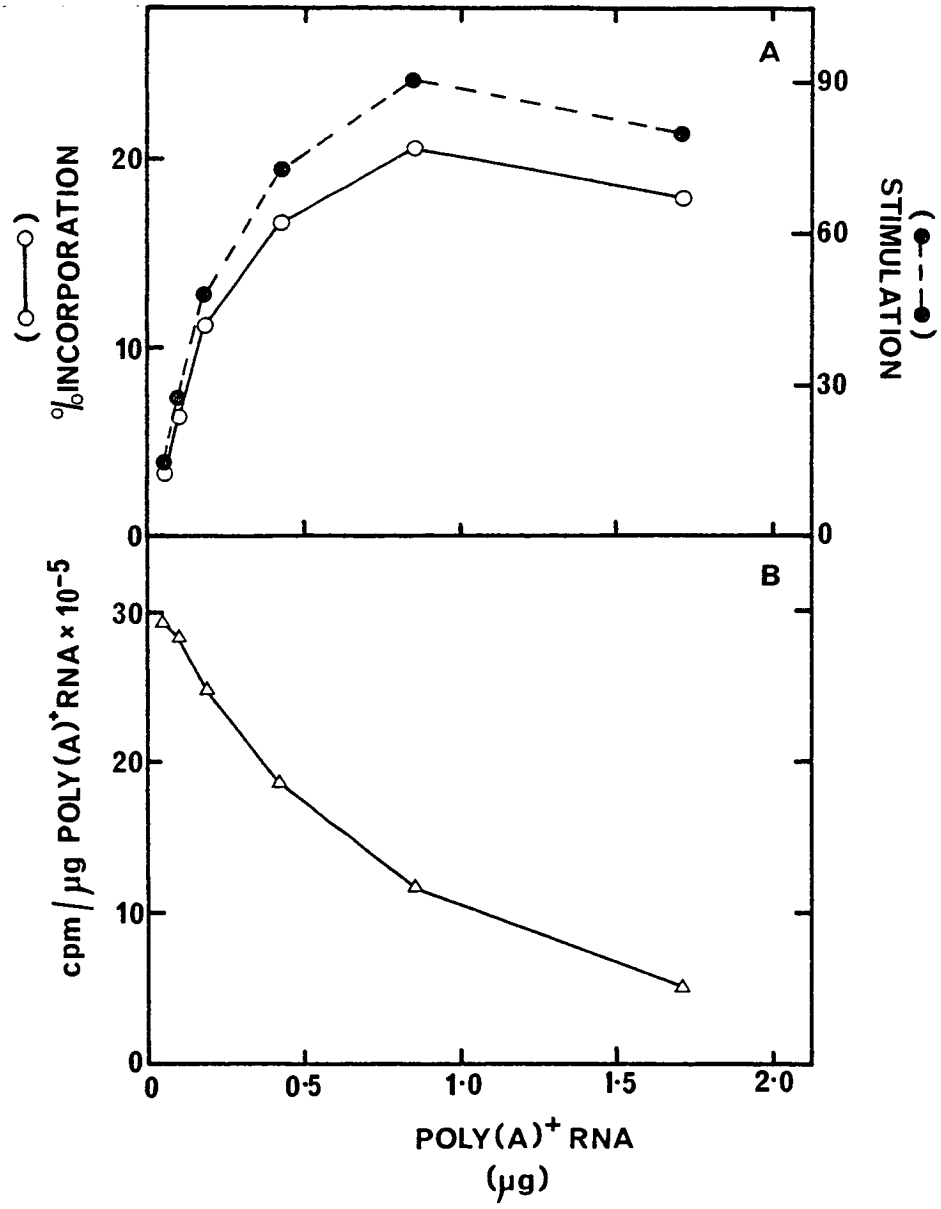
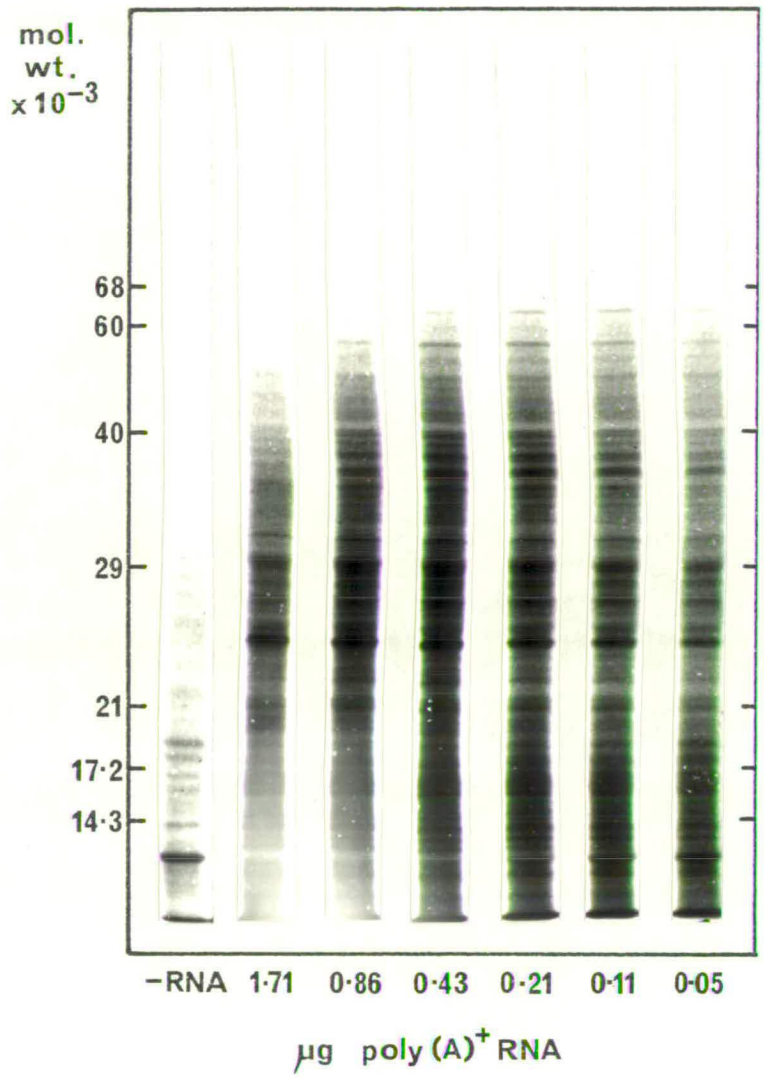

A
B
(○—○) %INCORPORATION
(●- - -●) STIMULATION
cpm / µg POLY(A)⁺RNA × 10⁻⁵
POLY(A)⁺ RNA (µg)
0
10
20
30
60
90
0·5
1·0
1·5
2·0

Figure 28

In Vitro Labelled Polypeptides Synthesised by the Wheat-Germ System Programmed with Increasing Concentrations of Poly(A)$^+$ RNA

The *in vitro* translation products of the wheat-germ system when programmed with varying concentrations of poly(A)$^+$ RNA from day 2, dark-grown cucumber cotyledons were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by autoradiography. The concentrations of poly(A)$^+$ RNA used to prime the system are as indicated.

FIGURE 28.


mol.
wt.
x 10⁻³
68
60
40
29
21
17·2
14·3
−RNA
1·71
0·86
0·43
0·21
0·11
0·05
μg poly (A)⁺ RNA

was programmed by poly(A)$^+$ RNA from all the developmental stages.

For the poly(A)$^+$ RNA, as for the total nucleic acid, there is initially an approximately linear, though not directly proportional relationship between the level of incorporation and the amount of input RNA, as can be seen in Figure 27B.

When using either total nucleic acid or poly(A)$^+$ RNA to programme the wheat-germ system, it was found that the rate of incorporation of [^{35}S]-methionine into hot, TCA-precipitable material was initially approximately linear then progressively declined although incorporation continued for at least 90 minutes (Figure 29). In all developmental studies described, the incubation was carried out for 90 minutes and the polypeptides synthesised during this time were analysed by SDS polyacrylamide gel electrophoresis and autoradiography.

3.3. Quantitative and Qualitative Changes in Cotyledonary mRNA

Using the optimised wheat-germ cell-free protein synthesising system, the total nucleic acid, poly(A)$^+$ RNA and poly(A)$^-$ RNA fractions were assayed for changes in the amounts and types of translatable mRNA in the cotyledons during germination and early seedling development.

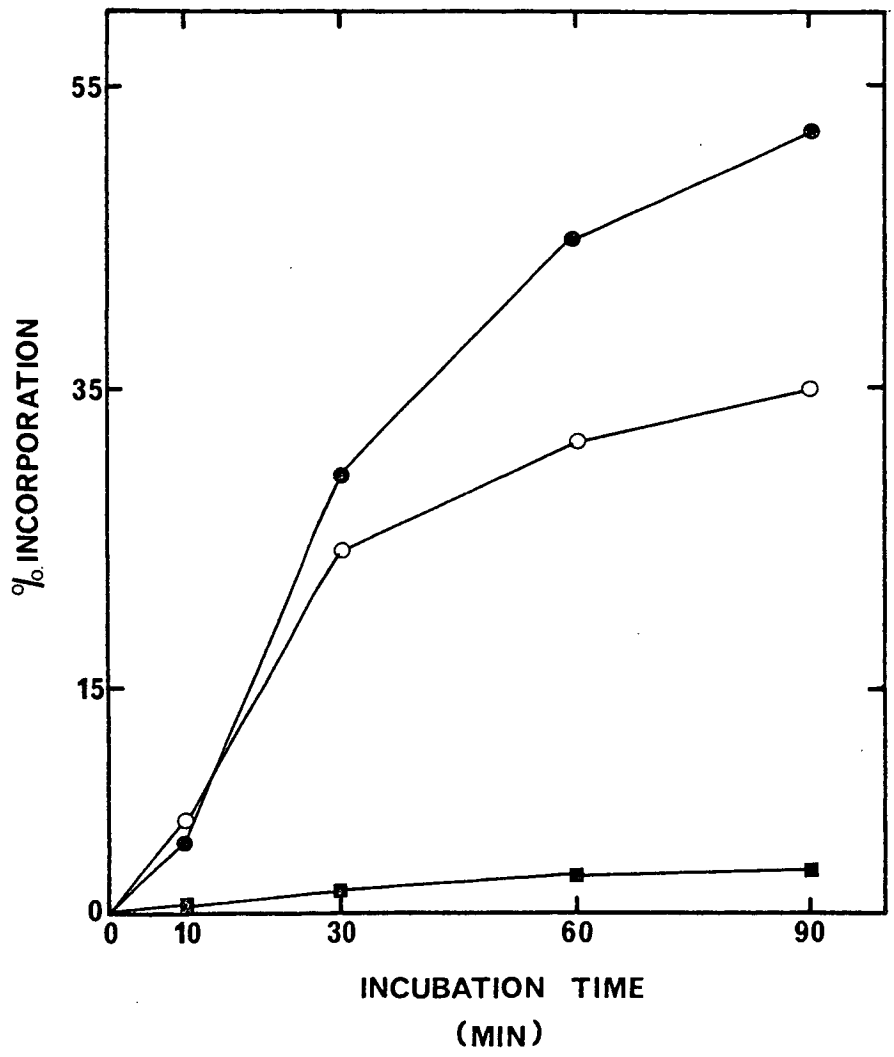
In all developmental studies the translational activity of the samples was expressed on a c.p.m./cotyledon basis. This was possible since the amount of nucleic acid used to programme the wheat-germ system and the nucleic acid content of the cotyledons (p.147) were known. Thus, assuming that the level of incorporation is an indication of the mRNA

Figure 29

Time Course of *In vitro* Protein Synthesis in the Wheat-Germ S.30 System

The optimised wheat-germ system was programmed with total nucleic acid (15 µg) from day 3, light-grown cotyledons (O——O) and poly(A)$^+$ RNA (0·5 µg) from day 2, dark-grown cotyledons (●——●). A control was carried out with no exogenous RNA (■——■). The levels of incorporation of [^{35}S]-methionine into hot, TCA-precipitable material were determined at 10, 30, 60 and 90 minutes of incubation, by the method of Mans and Novelli (1961), and expressed as a percentage of the radioactivity available in the incubation mixture (4·6 x 10^6 cpm).

FIGURE 29.



content, quantitative changes in the cotyledonary mRNA were studied. To determine qualitative changes occurring in the mRNA population of the cotyledons, the polypeptides synthesised *in vitro* were analysed using fraction on SDS-polyacrylamide gel electrophoresis followed by autoradiography (p.109).

Figure 30 shows the changes in translational activity of the total nucleic acid (A), poly(A)$^+$ RNA (B) and poly(A)$^-$ RNA (C).

From all three nucleic acid fractions there can be seen to be low, though measurable amounts of translatable mRNA present in the cotyledons of the dry seed. During imbibition, the amount of translatable mRNA decreases slightly. This is a repeatable result and is in agreement with the poly(A)$^+$ RNA and poly(A) contents of the cotyledons previously measured (pps.158 and 163).

Figure 30A shows that, after imbibition there is an increase in the mRNA content to peak values at day 4 in the light-grown tissue, with the most rapid increase occurring between days 2 and 4. By day 4 there is a 47-fold net increase in the mRNA content from the amount present after imbibition and thereafter, the mRNA content declines to approximately 35% of the peak value by day 7. In the dark-grown tissue the mRNA content only shows a net 14-fold increase by day 3 then subsequently decreases slowly. These results suggest that light is essential for maintained accumulation but is not required for the initial synthesis and increase of the mRNA of the cotyledons.

The translational activity of the poly(A)$^+$ RNA (Figure 30B) suggests that after imbibition there is a

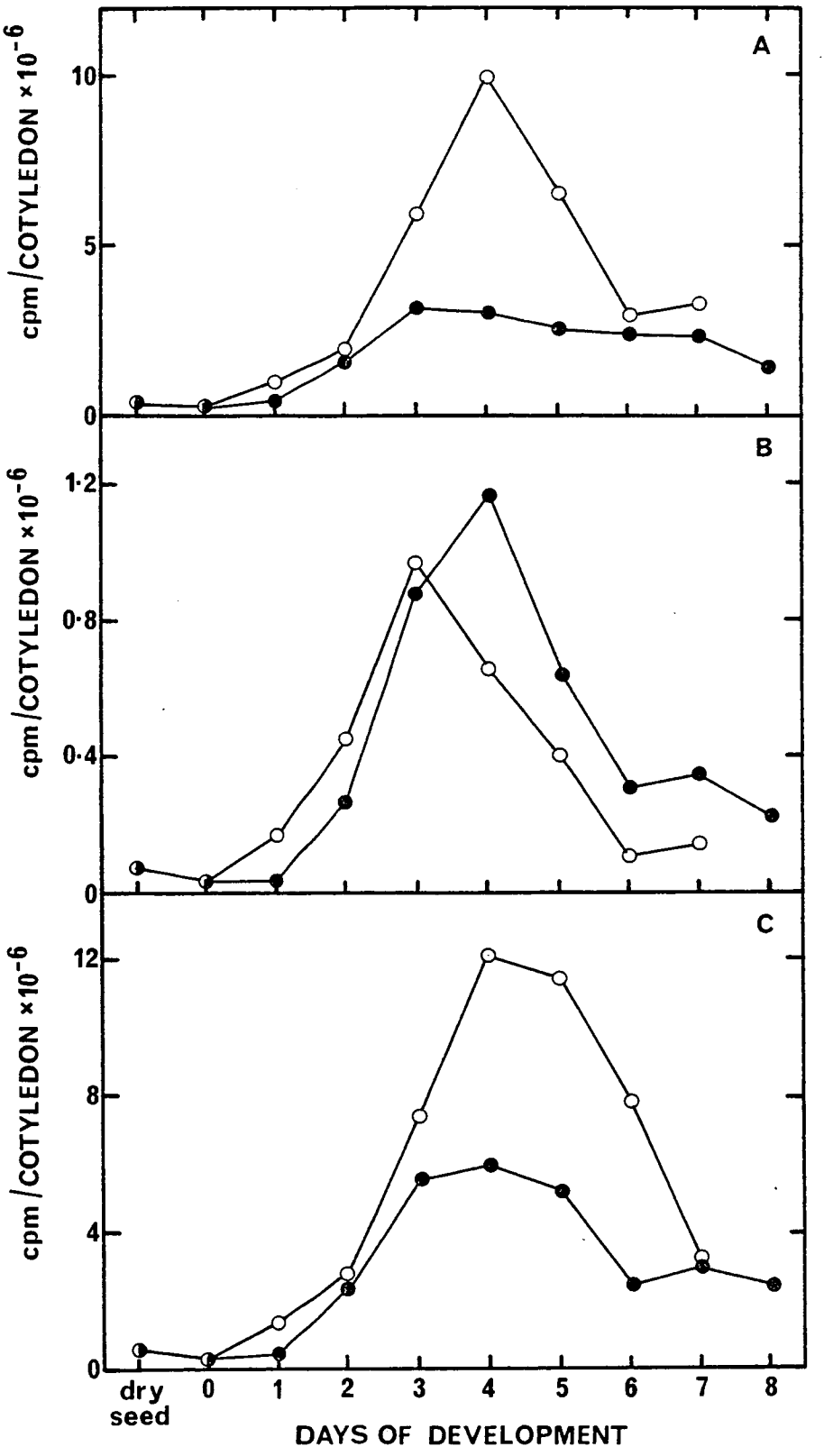
Figure 30

Quantitative Changes in the Translatable mRNA Content of the Cotyledons during Germination and Early Seedling Development, as determined by *In Vitro* Translation of Cotyledonary Nucleic Acid from the Different Days of Development

Total nucleic acid (15 µg; A), poly(A)$^+$ RNA (0·5 µg; B) and poly(A)$^-$ RNA (20 µg; C) from cotyledons at each day of development were used to programme the wheat-germ S.30 system. The levels of incorporation of [^{35}S]-methionine into hot, TCA-precipitable material after 90 min. incubation were determined. Incorporation was expressed on a /cotyledon basis and the developmental profiles for both light (O——O) and dark-grown (●——●) tissue are shown.

$$\text{cpm/cotyledon} = \frac{\text{cpm/incubation}}{\text{µg nucleic acid added}} \times \text{µg nucleic acid/cotyledon}$$

FIGURE 30.


A
B
C
cpm /COTYLEDON ×10⁻⁶
10
5
0
1·2
0·8
0·4
12
8
4
dry seed
0
1
2
3
4
5
6
7
8
DAYS OF DEVELOPMENT

marked increase in the amount of polyadenylated mRNA and that peak levels are reached by day 3 in the light and day 4 in the dark. Thereafter, the polyadenylated mRNA content of the cotyledons decreases to approximately 22% of peak values by day 7. This result is consistent with the measured poly(A)$^+$ RNA content of the cotyledons (p.162) which shows a similar profile.

When the poly(A)$^-$ RNA was used to programme the wheat-germ system, the profile of translational activity (Figure 30C) showed that there was a dramatic increase in the mRNA content of this fraction after imbibition. By day 4 in the light, the mRNA content reaches a peak then decreases to c.a. 30% of peak values by day 7. In the dark-grown tissue there is also an increase after imbibition, though this is less pronounced, giving peak values at day 4 of approximately 55% of the content of the light-grown tissue.

The levels of translational activity present in the poly(A)$^-$ RNA indicate that, although this was the fraction which did not bind to the oligo dT-cellulose, it still contained a significant amount of mRNA. These mRNAs must either lack or contain only short poly(A) sequences (< 30 nucleotides: Cabada *et al.*, 1977).

From these results there would appear to be low, though detectable amounts of mRNA present in the quiescent cotyledonary tissue with a slight decrease occurring during imbibition. After imbibition, there is a dramatic increase in the total mRNA and poly(A)$^-$ mRNA populations to reach peak values by day 4. There are reduced levels of these mRNA populations in the dark-grown tissue. Light is evidently

not required for the initial synthesis and increase but is essential for continued accumulation of these mRNAs prior to their decrease at the end of the developmental sequence under study.

Although light affects the accumulation of the total and poly(A)$^-$ mRNA populations it would appear to have very little effect upon the quantitative changes in the poly (A)$^+$ mRNA population except for a possible 24 hour delay in attaining peak values.

Analysis of the *in vitro* translation products of the total nucleic acid (Figure 31) shows that distinct changes occur in the types of translatable mRNA present at the different developmental stages.

There are specific mRNAs present in the total nucleic acid of the cotyledons in the quiescent seed. During imbibition, as well as the previously observed decrease in translational activity, there is a reduction in the number of polypeptides synthesised *in vitro*, particularly in the high molecular weight range.

Two types of developmentally related changes can be observed in the *in vitro* products. Firstly, there are a group of polypeptides, the mRNAs for which are present during the heterotrophic phase of growth. Particularly obvious are a number of polypeptides in the 45,000 - 70,000 mol. wt. range which are initially absent from the *in vitro* products, appear and are most prominent by days 2 and 3 and disappear soon after in the light-grown developmental sequence. The mRNAs for these polypeptides persist longer, though at reduced levels, in the dark-grown tissue. Since the

Figure 31

Qualitative Changes in the *In Vitro* Translation Products Programmed by Total Nucleic Acid from Cotyledons during Germination

The *in vitro* labelled products synthesised in the wheat-germ system programmed with total nucleic acid (15 μg) from cotyledons of light and dark-grown cucumbers were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by autoradiography. The molecular weights were determined using a standard protein mixture as described (p. 89).

FIGURE 31.

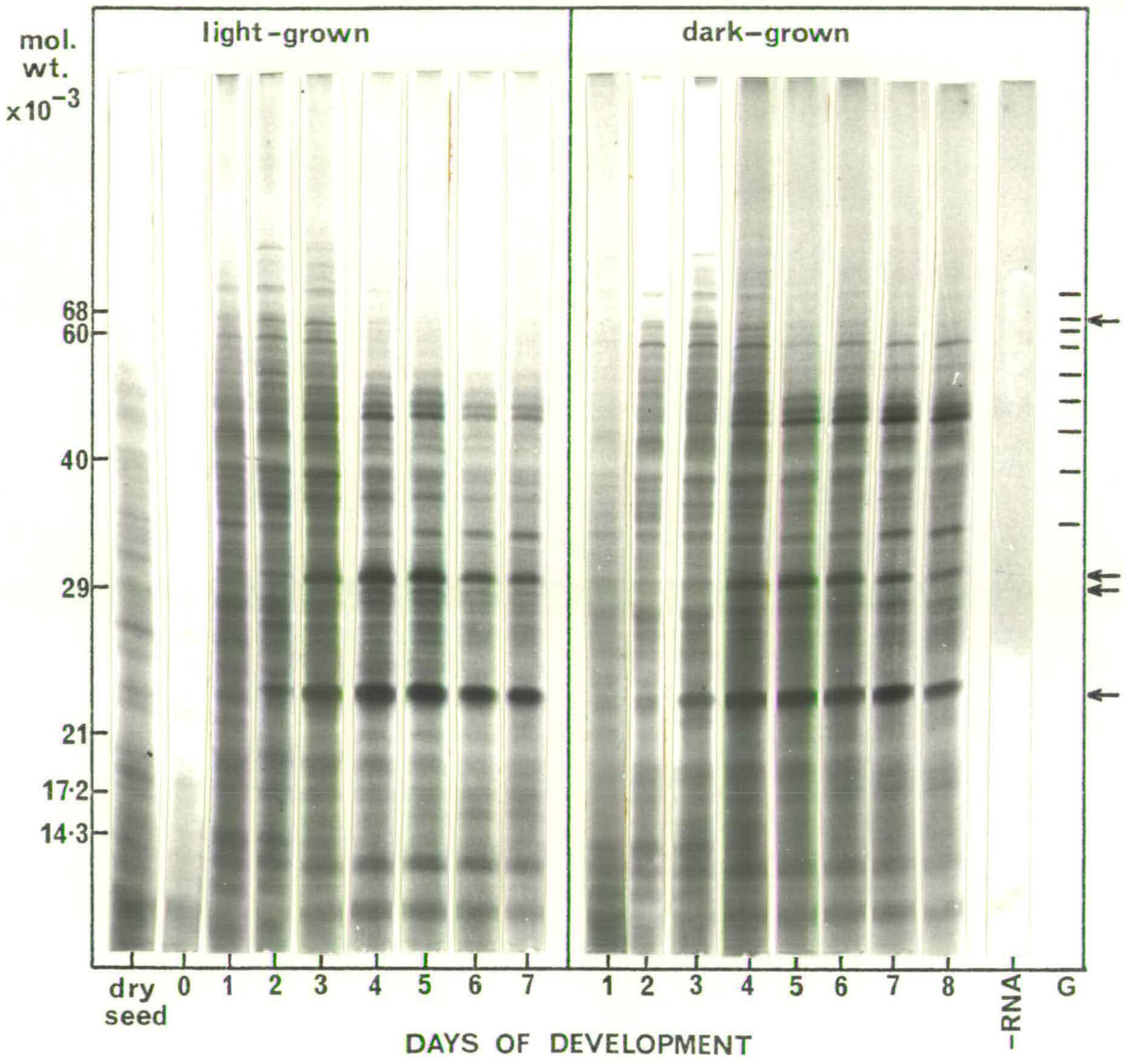

mol. wt. x10⁻³
light-grown
dark-grown
68
60
40
29
21
17·2
14·3
dry seed
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
-RNA
G
DAYS OF DEVELOPMENT

Figure 32

Qualitative Changes in the *In Vitro* Translation Products Programmed by Poly(A)$^+$ RNA from Cotyledons during Germination and Early Seedling Development

The *in vitro* labelled products synthesised in the wheat-germ system programmed with poly(A)$^+$ RNA (0·5 µg) from light and dark-grown cucumber cotyledons were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by autoradiography.

Figure 14

Sucrose Density Gradient Fractionation of Poly(A)$^+$ RNA and Total Nucleic Acid

Total nucleic acid and oligo-dT cellulose extracted poly(A)$^+$ samples were fractionated on 5-35% (w/v) 5·1 ml continuous sucrose density gradients in 100 mM Hepes-KOH pH 7·5, 0·1 M KCl buffer. Centrifugation was at 149,000 g_{av} for 6 hours at 4°C in a Spinco SW50 rotor. The A_{265} profile was determined using an ISCO Model UA-4 absorbance monitor and a Vitatron chart recorder.

A. Poly(A)$^+$ RNA from day 3 light-grown cotyledons - 27·3 g of poly(A)$^+$ RNA was loaded on the gradient.

B. Poly(A)$^+$ RNA from day 3 dark-grown cotyledons - 30·7 g of poly(A)$^+$ RNA was loaded on the gradient.

C. Total nucleic acid from day 2 dark-grown cotyledons -
37·4 g of phenol-detergent extracted total nucleic acid was loaded on the gradient. The positions of the 25S and 18S cytoplasmic rRNAs and the LMW RNAs are indicated by arrows.

FIGURE 32.

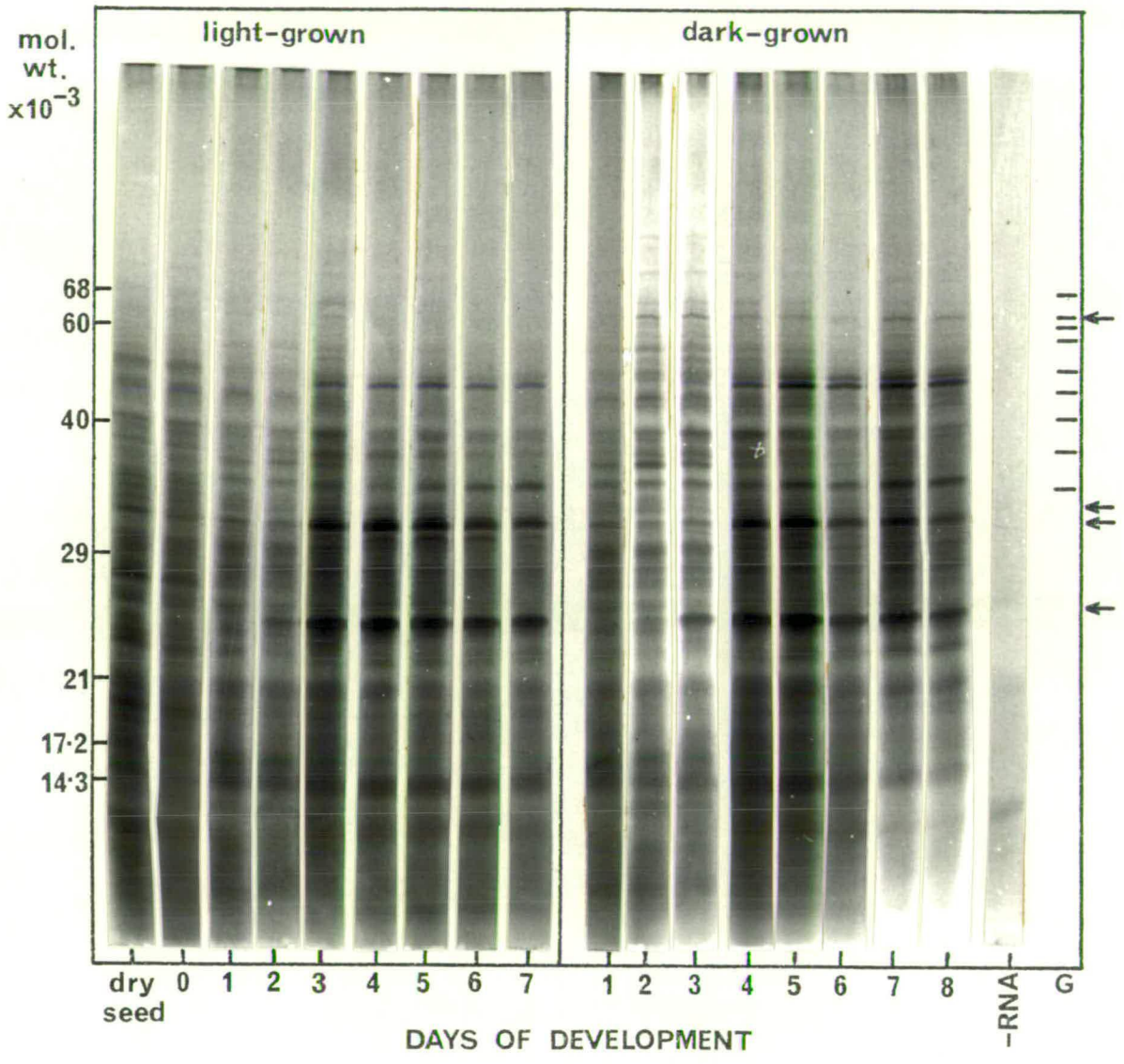
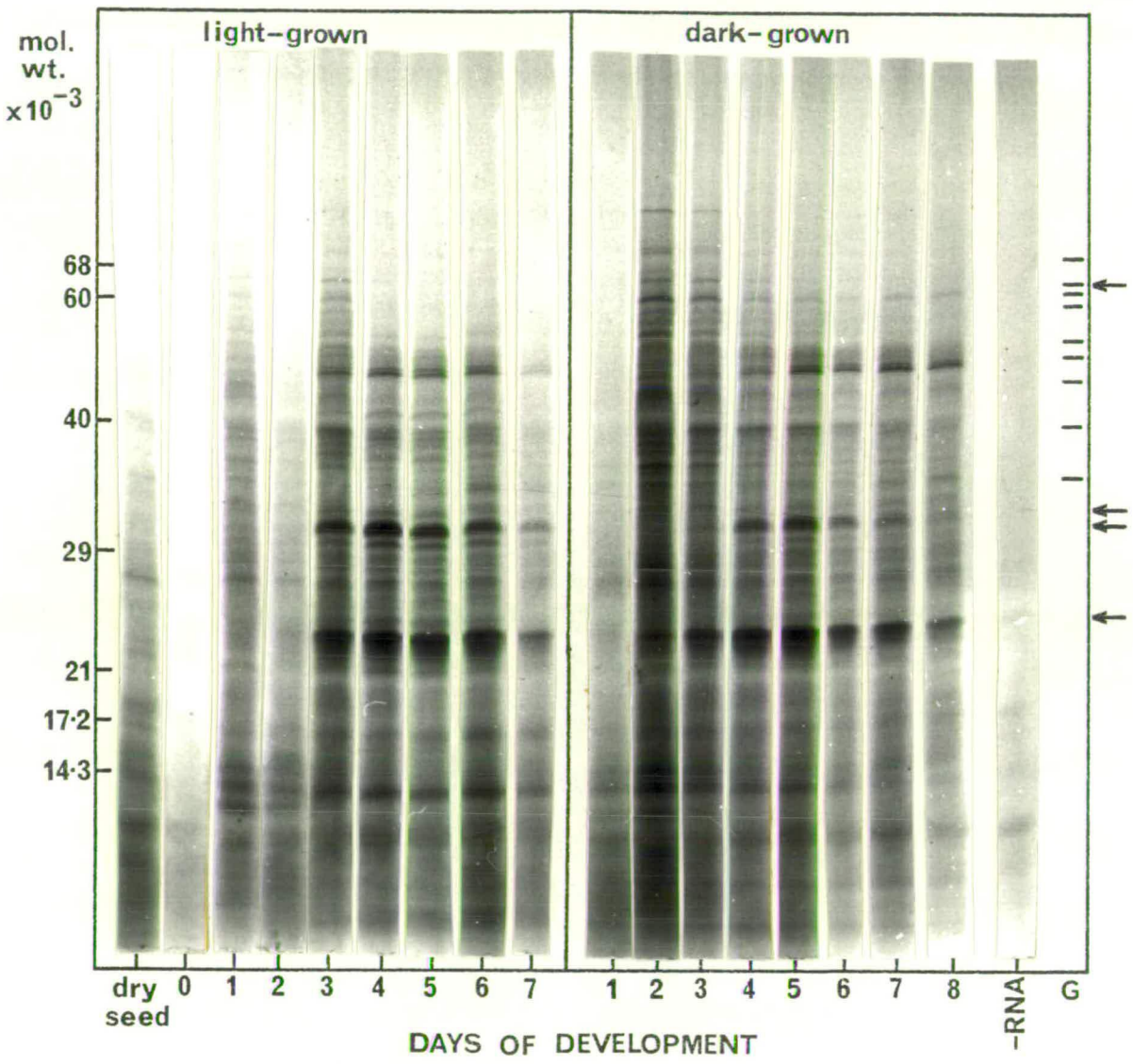

mol. wt. x10⁻³
light-grown
dark-grown
68
60
40
29
21
17·2
14·3
dry seed
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
-RNA
G
DAYS OF DEVELOPMENT

Figure 33

Qualitative Changes in the *In Vitro* Translation Products Programmed by Poly(A)$^-$ RNA from Cotyledons during Germination and Early Seedling Development

The *in vitro* labelled polypeptides synthesised in the wheat-germ system programmed with poly(A)$^-$ RNA (20 µg) from light and dark-grown cucumber cotyledons were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by autoradiography.

FIGURE 33.


mol. wt. x10⁻³
light-grown
dark-grown
68
60
40
29
21
17·2
14·3
dry seed
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
-RNA
G
DAYS OF DEVELOPMENT

glyoxysomal enzymes are known to have subunit molecular weights in this range (Becker *et al.*, 1978; Reizman *et al.*, in press), it is reasonable to expect that some of this group of polypeptides, which comprise a low proportion of the total *in vitro* products, may in fact be subunits of the glyoxysomal enzymes.

Secondly, there are a number of polypeptides that are first detectable about day 2 - 3 and persist until at least day 7. The timing of the synthesis of these mRNAs is consistent with a role in the autotrophic growth of the cotyledons. Of particular note is the 25,000 mol. wt. polypeptide which has been tentatively identified by Walden (1979) as the precursor of the small subunit of RuBPC. The relatively large amount of mRNA for this polypeptide present in the tissue is probably associated with the fact that RuBPC constitutes a large proportion of the total protein, as was seen from SDS-polyacrylamide gel electrophoresis of the total cotyledonary protein (p.139). Another prominent polypeptide in the group is present at approximately 32,000 mol. wt. This has not been identified in this study but is probably the precursor for the chlorophyll a/b binding protein (Schmidt *et al.*, 1979).

Figure 33 shows the *in vitro* products from the wheat-germ system when programmed by the poly(A)$^-$ RNA. The developmental profiles of qualitative changes in the mRNAs appears similar to those observed from the total nucleic acid fraction. This suggests that at least some copies of all mRNAs in the tissue contain short or no poly(A) sequences. It is possible that some minor differences are masked which

could be observed by two-dimensional gel electrophoresis, but these are probably due to less abundant mRNAs.

Figure 32 illustrates the poly(A)$^+$ RNA *in vitro* products. Again the qualitative changes are very similar to those previously observed. The most prominent difference here is in the products of the poly(A)$^+$ RNA from the imbibed tissue. Since this RNA fraction is 'purer' mRNA than the other two studied it is possible to show that many different mRNAs are still present in the imbibed tissue. The reason these products are not obvious in the other fractions could be either due to the low levels of the mRNAs as a proportion of the total or possibly due to some inhibitory effect upon translation of the rRNA and tRNA from this developmental stage.

The only other noticeable difference between these *in vitro* products and those from the other fractions is the possible absence of a product at approximately 53,000 mol. wt. from the poly(A)$^+$ RNA products (Figure 34). The identification of this polypeptide is uncertain but Walden (1979) suggests that it may be the large subunit of RuBPC. Since this is a minor product, this result would suggest that chloroplast mRNAs may be translated in the wheat-germ system, though not as efficiently as the cytoplasmic mRNAs.

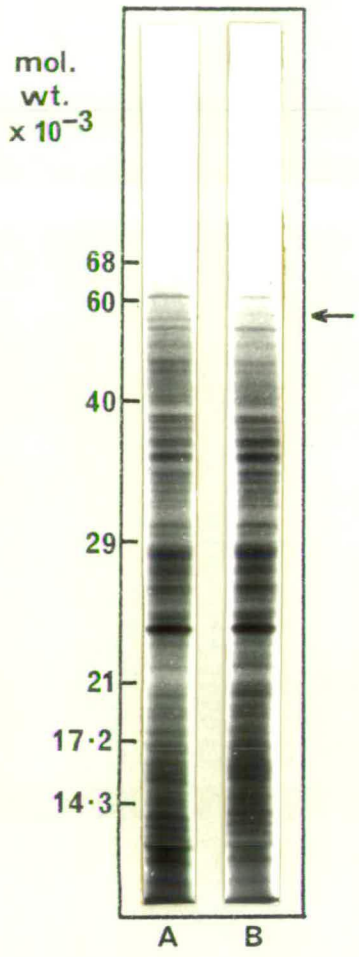
Thus, no distinct differences were observed in the *in vitro* products between these three nucleic acid fractions at any specific developmental stage. However, developmentally related changes in the types of mRNA have been shown to occur and some of these changes are apparently light dependent.

Fioure 34

A Comparison of the *In Vitro* Translation Products Programmed by Total Nucleic Acid and Poly(A)$^+$ RNA

The wheat-germ S.30 system was programmed with total nucleic acid (A) and poly(A)$^+$ RNA (B) from day 2, dark-grown cucumber cotyledons. *In vitro* translation products were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by autoradiography. The only clear difference in synthesised polypeptides is indicated with an arrow, and has an approximate molecular weight of 53,000.

FIGURE 34.



It should be noted that care should be taken in comparing the quantity of different *in vitro* products. Since different polypeptides contain different amounts of methionine it is not always significant to compare the degree of labelling of different products. It is however, appropriate to study quantitative changes in a single polypeptide.

3.4. Translation of Polysomes and Polysomal RNA

The earlier results discussed in this thesis showed that there were developmentally related changes in the mRNA population of the cotyledons during growth and early seedling development. In order to study quantitative and qualitative changes in the mRNAs actively involved in protein synthesis, polysomes and polysomal RNA were prepared and used to programme the wheat-germ cell-free protein synthesising system.

Polysomes from day 3 light-grown tissue were used to programme both an S.30 and an S.100 (i.e. without endogenous ribosomes) wheat-germ system. There is apparently no reinitiation in an S.100 cell-free system, which would explain the short time course of incorporation as compared with the S.30 system where reinitiation would be anticipated as normal (p.193) and thus higher levels of incorporation would be expected.

From Figure 35 it can be seen that incorporation of [^{35}S]-methionine into hot, TCA-precipitable material is essentially completed by 15 minutes of incubation in the S.100 system, whereas it apparently continues for slightly longer in the S.30 system. However, the time course of incorporation in the S.30 wheat-germ system is still a lot shorter than for the same system when programmed with RNA. As can

Figure 35

Time Course of In Vitro Protein Synthesis in the Wheat-Germ S.30 and S.100 Systems when Programmed with Polysomes from Cucumber Cotyledons

Polysomes were extracted from day 5, light-grown cucumber cotyledons (p. 98) and used to programme wheat-germ S.30 (○——○) and S.100 ('run off') (●——●) systems. The time course of incorporation of [^{35}S]-methionine into hot, TCA-precipitable material was determined by the method of Mans and Novelli (1961). Controls were carried out in which no exogenous mRNA source was added.

S.30 system – ○——○ 0·5 A_{260} units of polysomes

□——□ control

S.100 system – ●——● 0·5 A_{260} units of polysomes

▪——▪ control

FIGURE 35.

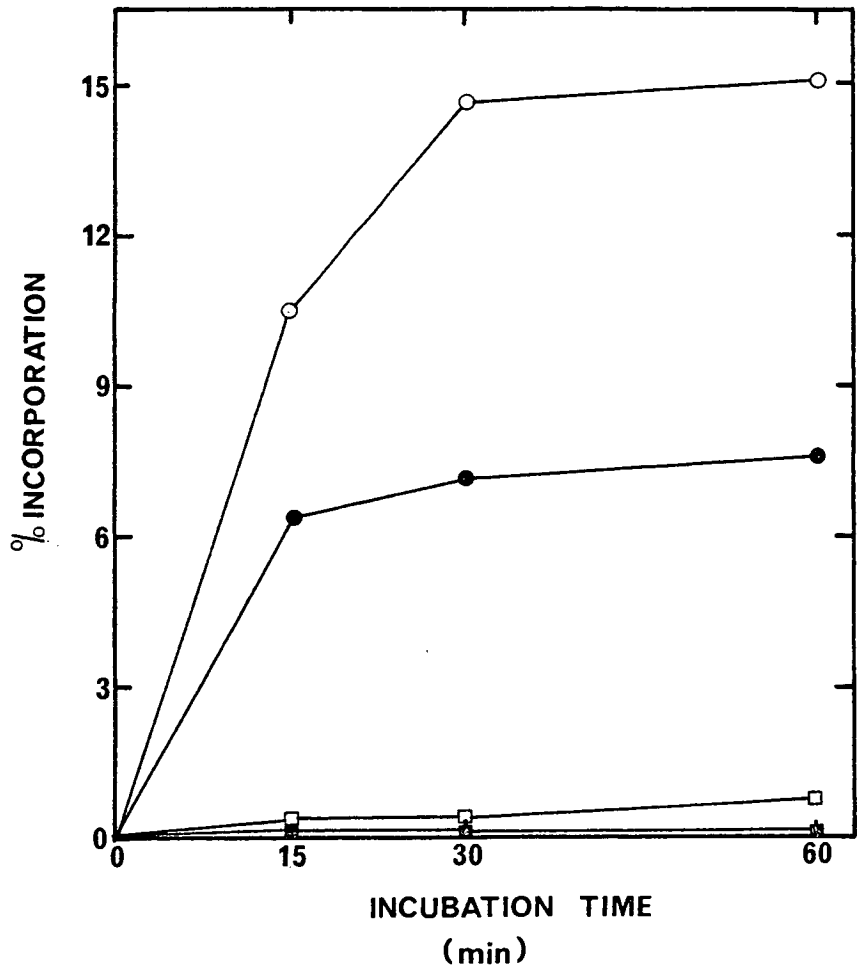


Figure 36

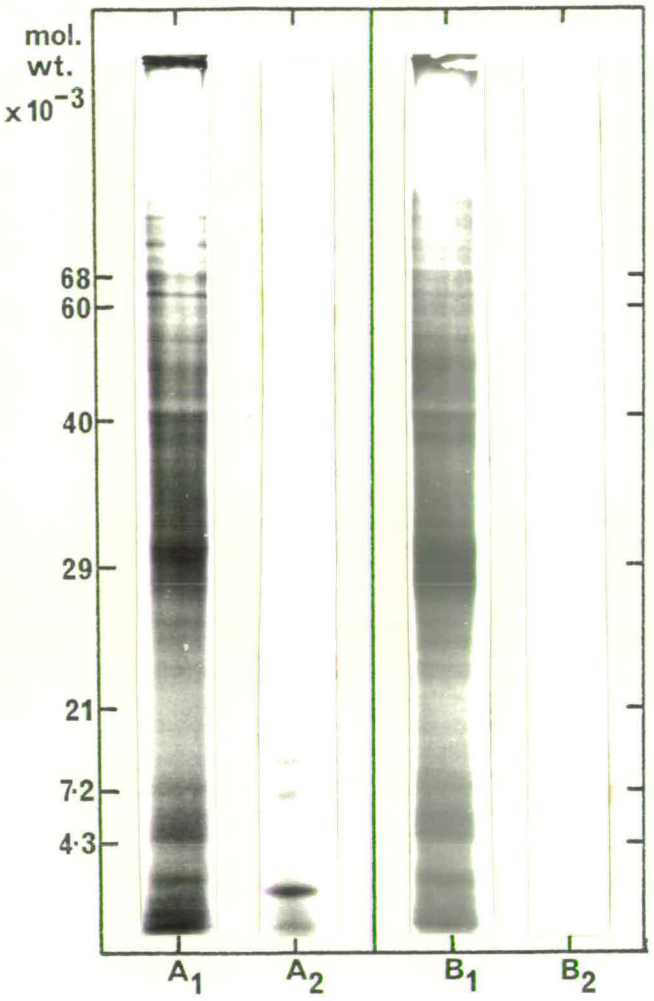
Translation Products of the Wheat-Germ S.30 and S.100 Systems Programmed with Polysomes from Cucumber Cotyledons

The *in vitro* labelled polypeptides synthesised in the experiment described in Figure 35 were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by autoradiography.

A. S.30 System
- A_1 - polypeptides programmed with cucumber polysomes
- A_2 - endogenous products

B. S.100 System
- B_1 - polypeptides programmed with cucumber polysomes
- B_2 - endogenous products

FIGURE 36.



be seen from the controls, the endogenous activity of the S.100 system is virtually zero.

The products synthesised in both these systems were analysed by SDS-polyacrylamide gel electrophoresis and autoradiography (Figure 36). It can be seen that there is a lack of distinct polypeptides synthesised in both systems, though there are some more easily detectable in the S.30 system. The higher incorporation levels in the S.30 system probably aid detection of the polypeptides.

Since the S.30 system gave higher incorporation levels and this was thought to aid rapid detection of the labelled products, this system was used for subsequent experiments using polysomes.

Total polysomes and polysomal RNA from cotyledons of light and dark-grown tissue and free and membrane-bound polysomes from light-grown tissue were used to programme the wheat-germ system. The levels of incorporation using the different templates were expressed on a per cotyledon basis and the developmental profiles are shown in Figure 37.

From these profiles it can be seen that there is initially an almost undetectable amount of mRNA associated with the polysomes (Figure 37A) and thus actively involved in protein synthesis. Between days 3 and 4 in the light-grown tissue there is a dramatic increase and thereafter a constant level of mRNA associated with the polysomes of the cotyledons. In the dark-grown tissue the amount of mRNA being translated is, at peak values, only 33% of that in the light-grown tissue.

The translational activity of the polysomal RNA

Figure 37

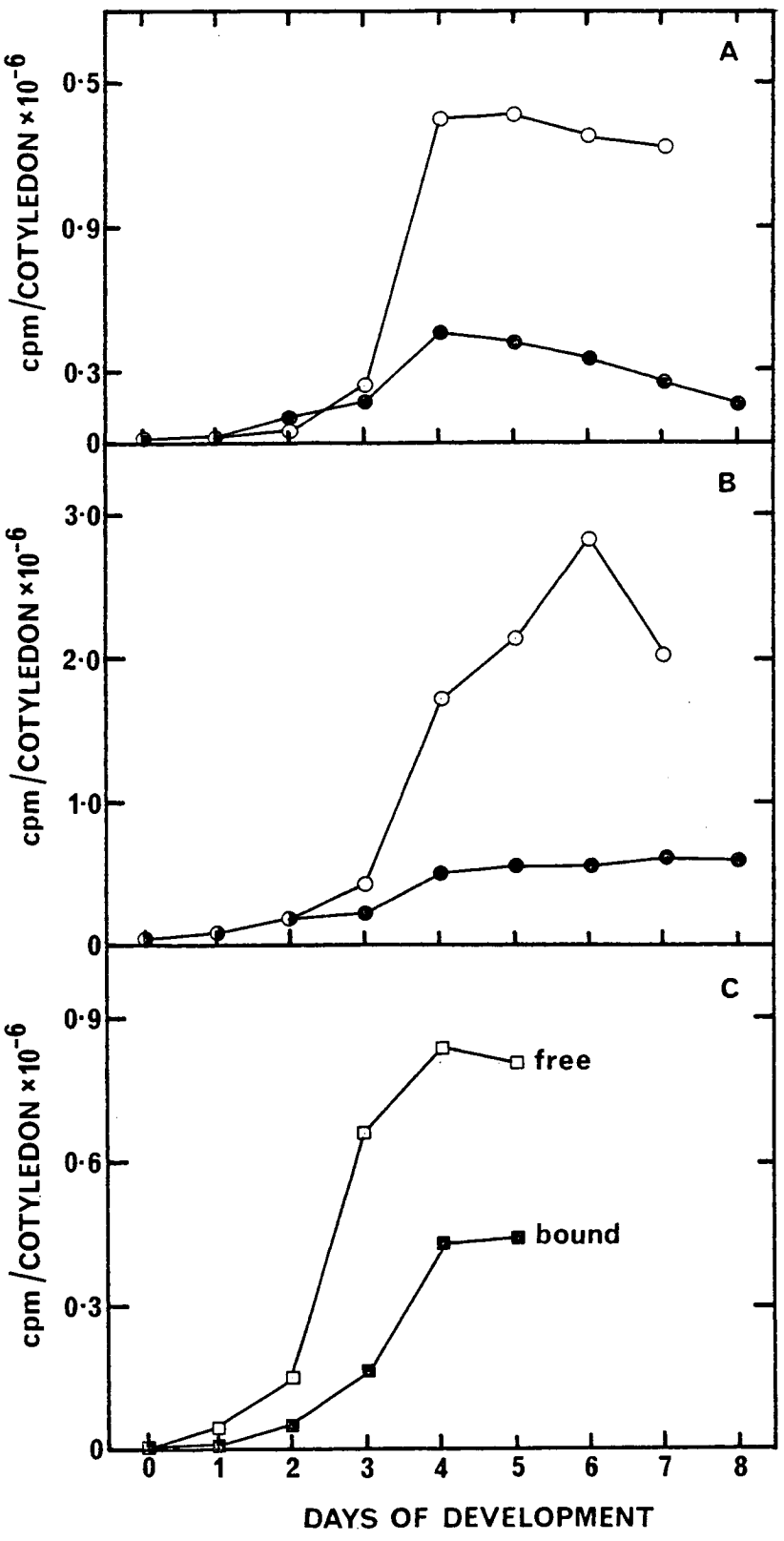
Developmental, Quantitative Changes in the Translatable mRNA Content of Total Polysomes, Polysomal RNA and Free Membrane-Bound Polysomes from Cucumber Cotyledons

Total polysomes (A) and polysomal RNA (B) from cotyledons of light (○——○) and dark-grown (●——●) tissue, and (C) free (□——□) and membrane-bound (■——■) polysomes from cotyledons of light-grown tissue were used to programme the wheat-germ S.30 system. The levels of incorporation of [^{35}S]-methionine into hot, TCA-precipitable material after 90 minutes incubation were determined. Incorporation was expressed on a /cotyledon basis.

$$\text{cpm/cotyledon} = \frac{\text{cpm/incubation}}{\mu\text{g polysomes or polysomal RNA added/incubation}} \times \mu\text{g polysomes or polysomal RNA/cotyledon}$$

Total polysomes	–	30–40 µg added/incubation
Polysomal RNA	–	15 µg added/incubation
Free polysomes	–	50–70 µg added/incubation
Membrane-bound polysomes	–	30–40 µg added/incubation

FIGURE 37.


A
B
C
cpm/COTYLEDON ×10⁻⁶
0·5
0·9
0·3
0
3·0
2·0
1·0
0·6
free
bound
0
1
2
3
4
5
6
7
8
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(Figure 37B) shows a slightly different profile to that of the polysomes. In the light, the activity increases until day 6 rather than maintaining a constant level after day 4. The dark-grown tissue shows only marginal increases in detectable mRNA as development proceeds.

A comparison of free and membrane-bound polysomes (Figure 37C) from imbibed tissue showed virtually no detectable translational activity although polysomes (presumed to be predominantly monosomes) were extractable. Thereafter there is an increase in the translational activity, this being most marked between days 2 and 3 for the free polysomes and days 2 and 4 for the membrane-bound polysomes. There is apparently more mRNA associated with free than membrane-bound polysomes as was indicated previously from the developmental profiles of these two types of polysomes (p.159).

The products obtained using polysomes, polysomal RNA and free and membrane-bound polysomes to programme the wheat-germ system were analysed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (Figures 38, 39 and 40 respectively).

I have consistently found that the products detectable when using polysomes or polysomal RNA in the wheat-germ system are very indistinct even although the levels of incorporation have been high. This can be seen from the autoradiographs illustrated in Figures 38, 39 and 40. Although there were few distinct products, there was a high background activity from each sample.

As already stated, the S.30 wheat-germ system is

Figure 38

In Vitro Translation Products Programmed by Total Polysomes from Cucumber Cotyledons

The *in vitro* labelled polypeptides synthesised in the wheat-germ S.30 system programmed with total polysomes from cotyledons of light and dark-grown tissue (Figure 37A) were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) and autoradiography.

FIGURE 38.

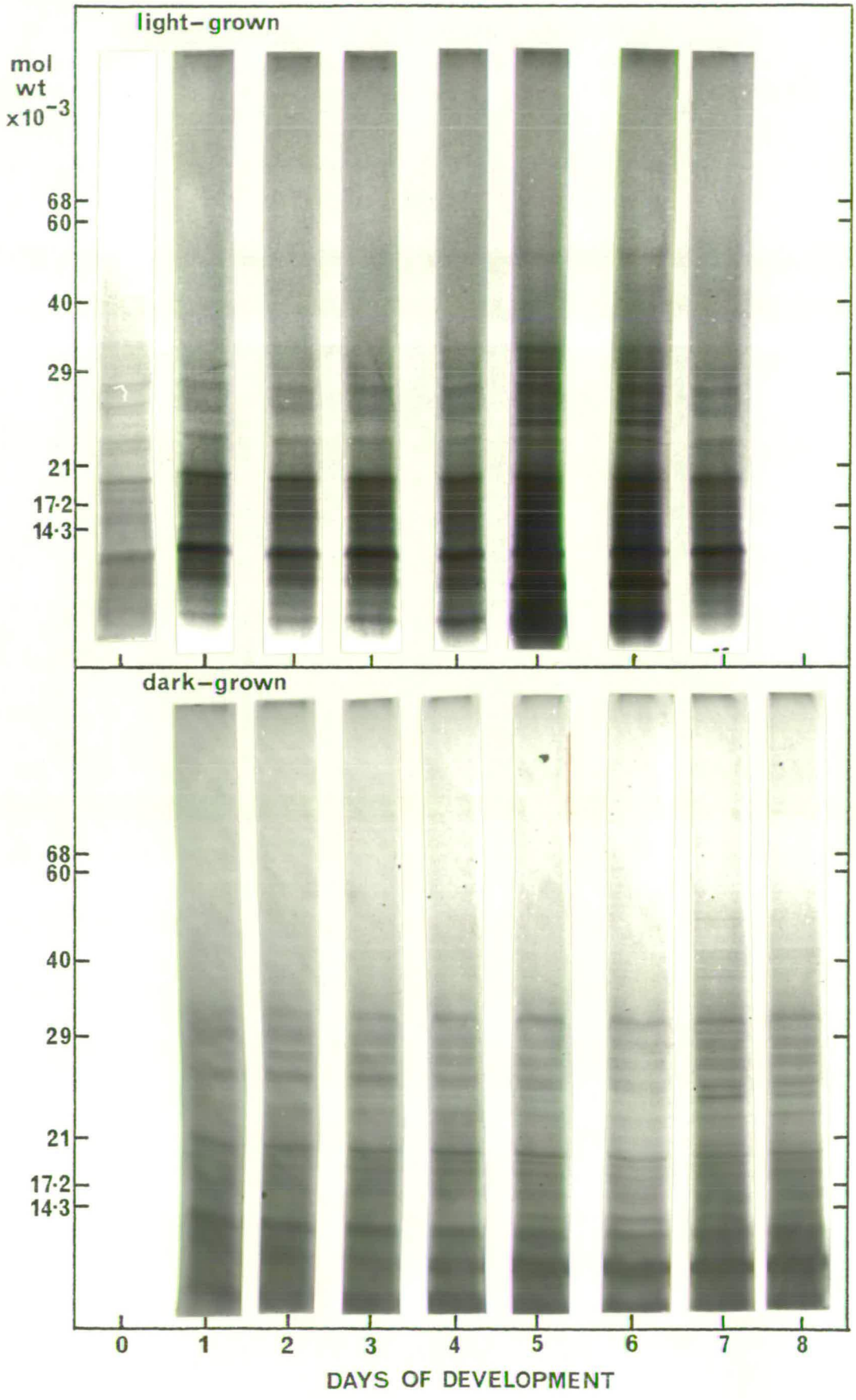

light-grown
mol wt x10⁻³
68
60
40
29
21
17·2
14·3
dark-grown
68
60
40
29
21
17·2
14·3
0
1
2
3
4
5
6
7
8
DAYS OF DEVELOPMENT

Figure 39

In Vitro Translation Products Coded by Polysomal RNA from Cucumber Cotyledons

The *in vitro* labelled polypeptides synthesised in the wheat-germ S.30 system programmed with polysomal RNA from cotyledons of light and dark-grown tissue (Figure 37B) were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) and autoradiography.

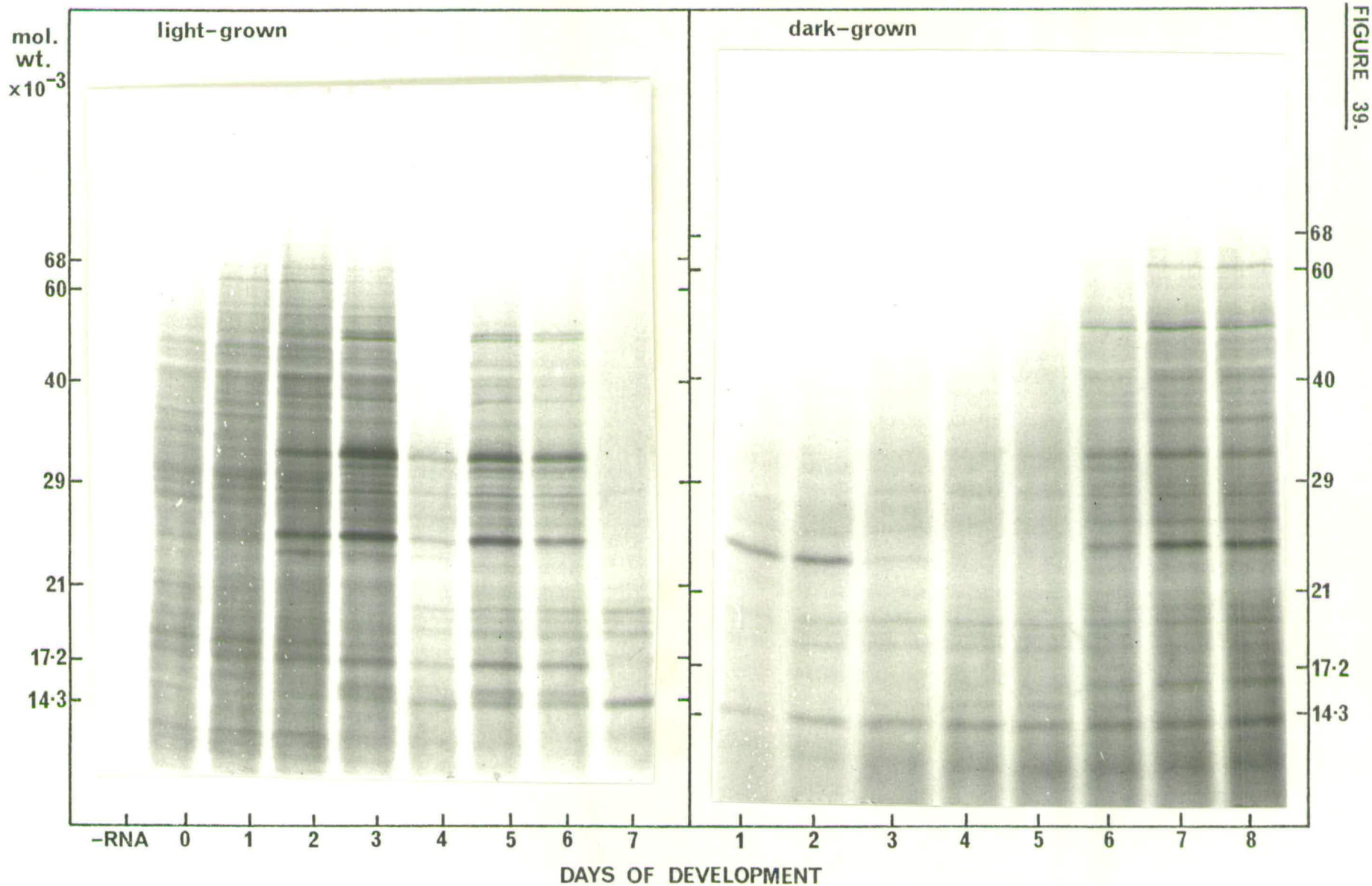

mol. wt. x10⁻³
light-grown
dark-grown
68
60
40
29
21
17·2
14·3
–RNA
0
1
2
3
4
5
6
7
8
DAYS OF DEVELOPMENT

FIGURE 39.

Figure 40

In Vitro Translation Products Coded by Free and Membrane-Bound Polysomes from Light-Grown Cucumber Cotyledons

The *in vitro* labelled polypeptides synthesised in the wheat-germ S.30 system programmed with free and membrane-bound polysomes from cotyledons of light-grown tissue (Figure 38C) were analysed by SDS-polyacrylamide electrophoresis (15% w/v) and autoradiography.

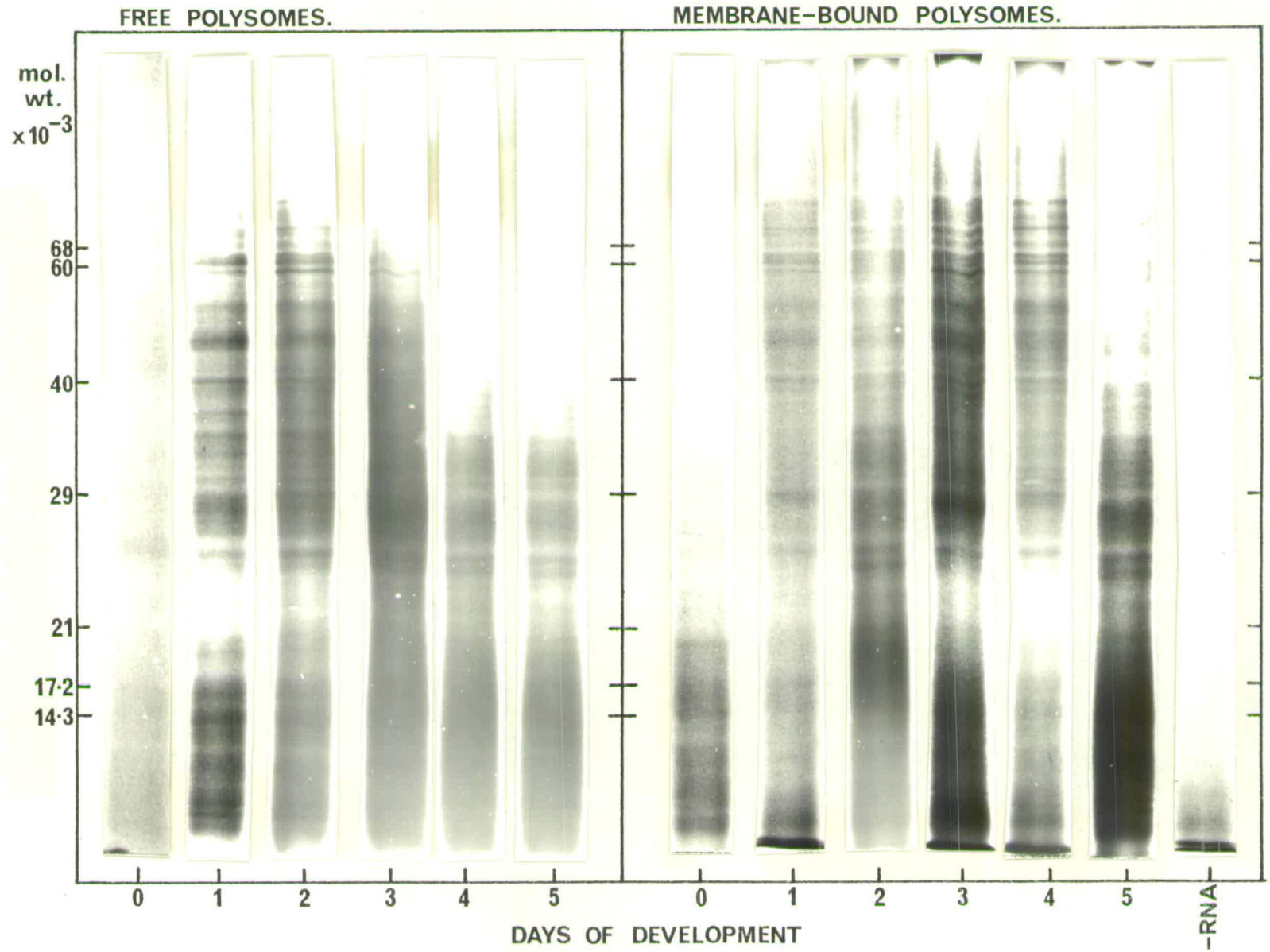

FREE POLYSOMES.
MEMBRANE-BOUND POLYSOMES.
mol. wt. x10⁻³
68
60
40
29
21
17·2
14·3
0
1
2
3
4
5
0
1
2
3
4
5
-RNA
DAYS OF DEVELOPMENT

FIGURE 40.

one in which reinitiation normally occurs, such that incorporation may continue for up to 90 minutes. However, in most experiments using polysomes to prime the system, protein synthesis essentially stopped after approximately 15 minutes of incubation.

Due to the lack of distinct products from the wheat-germ system and the early termination of protein synthesis occurring in a system which was otherwise highly efficient, it was decided that, in some way, the polysome preparations were inhibiting efficient translation. Several reasons for this poor translation were considered. Firstly, it was possible that some 'inhibitor' of translation, which could affect any or all of the stages of protein synthesis was present in the polysome preparations. Secondly, an RNAase may have been extracted from the tissue along with the polysomes.

If the first possibility was the case, virtually no incorporation of [^{35}S]-methionine would be expected to occur. However, it was shown that incorporation into hot, TCA-precipitable material did occur for approximately 15 minutes. Also, if an inhibitor was present, this should have been eliminated during preparation of the polysomal RNA, but it was shown that even with extracted polysomal RNA there was a lack of distinct products.

If the second possibility was applicable, it would be anticipated that the RNAase may have a gradual effect in degrading the RNA, particularly the mRNA, of the sample. Thus, it would be expected that a gradual loss of translational activity would occur and also that the extracted poly-

somal RNA may be affected. Since the presence of RNAase would best explain the observed results, this possibility was investigated in conjunction with Dr. Grienenberger.

Total and free polysomes from spinach leaves and total polysomes from day 3•5 light-grown cucumber cotyledons were prepared in the usual manner (p.98). Samples of each of these were added to ^{32}P-labelled polysomal RNA in 2 x SSC buffer and incubated for 30 minutes at 37^{o}C. As controls, two concentrations of RNAase A (pancreatic) were also added to ^{32}P-labelled polysomal RNA and one sample had no polysomes or RNAase added. During the incubation period, aliquots were removed from each sample and the radioactivity remaining in TCA-precipitable RNA was determined. Figure 41 shows the effect of the different polysomes and RNAase upon the labelled RNA.

Where RNAase was added to the labelled RNA there was a rapid decay in the amount of TCA-precipitable RNA to near zero by 5 minutes incubation. As can be seen, the changes in amount of TCA-precipitable RNA when the two spinach polysome samples were added are similar to the case where no polysomes or RNAase were added. When total cucumber cotyledon polysomes were added there is a marked degradation of the labelled RNA. This decay is most rapid in the first 10 minutes though it does continue throughout the 30 minute incubation. The observed decay of labelled RNA in the presence of the cucumber polysomes suggests that there is an RNAase present in the polysome preparation.

During the preparation of the polysomes, the buffer and ion conditions are such as to reduce RNAase

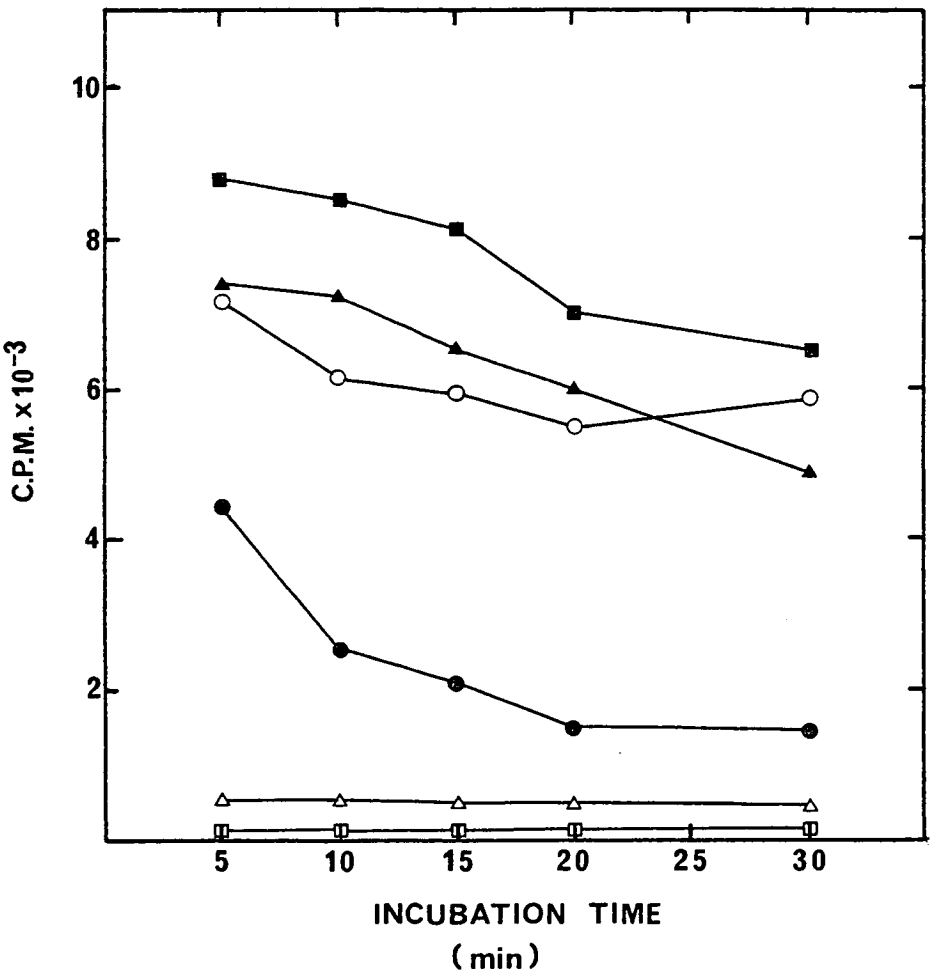
Figure 41

The Effect of Extracted Polysomes from Cucumber Cotyledons and Spinach Leaves upon the Breakdown of ^{32}P-Labelled Polysomal RNA as an Indication of the Presence of Ribonuclease Activity Associated with Extracted Cotyledonary Polysomes

Free and total polysomes from spinach leaves and total polysomes from day 3·5, light-grown cucumber cotyledons were prepared as described (p. 98). Samples of each were added to 13 µl ^{32}P-labelled polysomal RNA (14×10^3 cpm prior to TCA-precipitation). Controls were carried out with (i) no added polysomes and (ii) pancreatic RNAase A in place of polysomes. Incubations were carried out at 37°C for 30 min as described (p. 160) and the radioactivity remaining in TCA-precipitable RNA after 5, 10, 15, 20 and 30 min was determined.

- Spinach leaf free polysomes; 0·15 A_{260} units added.
- Spinach leaf total polysomes; 0·32 A_{200} units added.
- Cucumber cotyledons total polysomes; 0·23 A_{260} units added.
- Pancreatic RNAase A; 2·0 µg added.
- Pancreatic RNAase A; 0·5 µg added.
- Control blank; nothing added.

FIGURE 41.



activity. However, prior to adding the polysomes to the wheat-germ system, they were resuspended in low buffer and ion concentrations which will not limit the RNAase activity. It should also be noted that the polysomal RNA was extracted from polysomes which had first been prepared for use in the cell-free translation system. Thus, it is probable that degradation of the RNA, particularly the mRNA, of the polysomes had begun prior to addition to the wheat-germ system and this decay was accelerated when the samples were incubated at 25°C.

In order to eliminate this RNAase activity, inhibitors such as DEP are normally used (Solymosy et al., 1968). However, as DEP and other RNAase inhibitors act by inactivating enzymes, these would not be suitable for addition to polysomes to be used for in vitro translation as the enzymes involved in protein synthesis would also be inhibited.

Thus, a method of preparing polysomes but eliminating the RNAase activity was sought. If the RNAase was bound firmly to the polysomes it would be difficult to remove. However, if it was only loosely bound, it was thought that use of a chromatography procedure might facilitate removal of the RNAase.

Free polysomes were prepared from day 3 light-grown cotyledons by the usual procedure of pelleting through a 2M sucrose cushion or by chromatography on a Sepharose 6B column. Samples of both preparations were fractionated on 15 - 60% continuous sucrose density gradients. Figure 42 shows the gradients from both preparations.

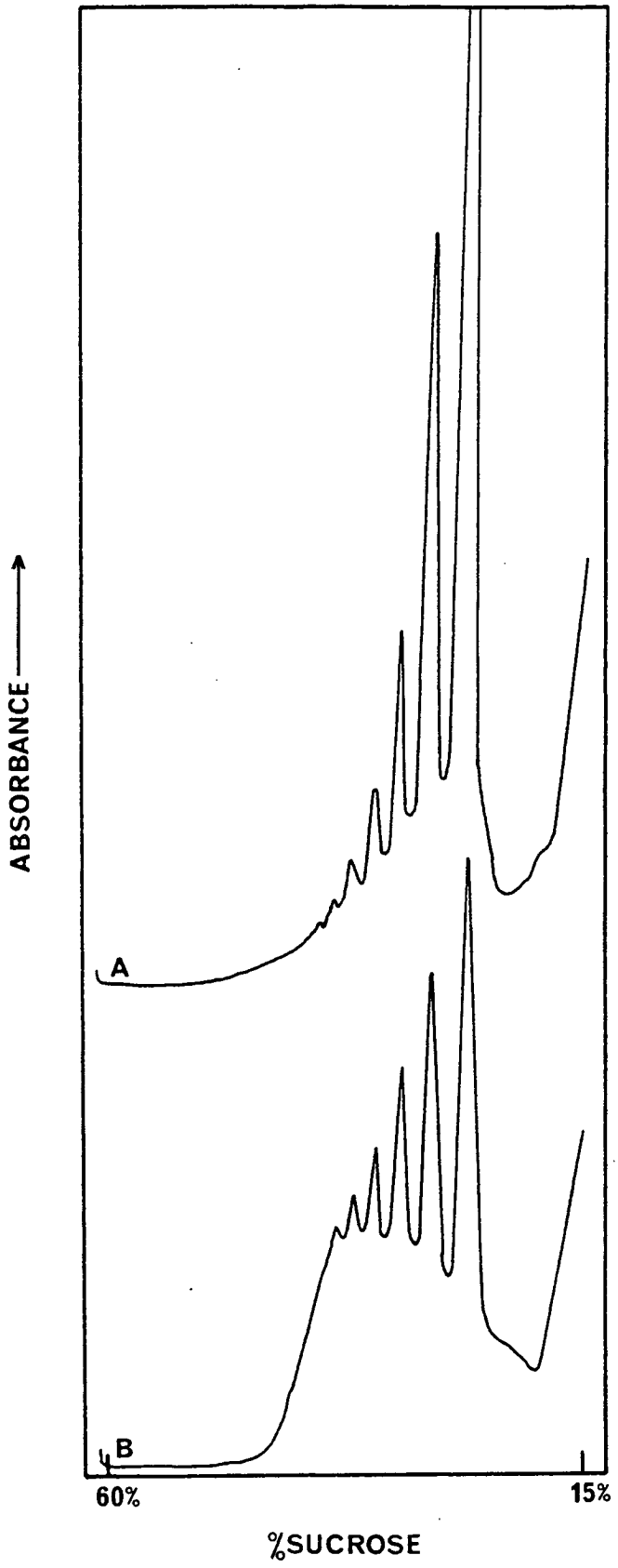
From the gradients it is clear that, when the

Figure 42

Sucrose Density Centrifugation of Free Polysomes Prepared from Cucumber Cotyledons by Two Different Procedures

Free polysomes were prepared from day 3, light-grown cucumber cotyledons either by pelletting through a sucrose (1M) cushion (A) or by purification on a Sepharose 6B column (B) as described (p.102). Polysomes prepared by both procedures were fractionated on 5·1 ml, 15-60% (w/v) linear sucrose gradients by centrifugation at 149,000g_{av} for 1·5 hours in a Spinco 50 rotor (4°C). The A_{265} profile was obtained by pumping the gradient through an ISCO Model VA-4 absorbance monitor with continuous recording on a Vitatron chart recorder. The position of the 80S monosome peak is indicated with an arrow.

FIGURE 42.



polysomes were prepared by chromatography, there was a greater proportion of polysomes present in the larger polysome sizes than when pelleting through the sucrose cushion. These results suggested that there was a reduction in the size of the polysomes during and after the pelleting procedure. This is probably due to the RNAase reducing the size of the mRNAs and thus reducing the average polysome size.

On the Sepharose 6B column all polysomes, RNA and high molecular weight components will pass through relatively quickly while all components with molecular weights of less than 100,000 (e.g. the RNAase) will be retarded.

Thus, it was shown that the polysomes in the wheat-germ probably produced poor products because the mRNAs had been degraded by RNAase. This would agree with the observations that although few distinct products were obtained there was usually a reasonable level of incorporation of [^{35}S]-methionine into hot, TCA-precipitable material and there was a high background activity on the autoradiographs.

Although it was not possible from these experiments to determine qualitative changes in the mRNAs actively involved in protein synthesis it was thought that the levels of incorporation could be taken as a reasonable representation of the amount of mRNA recruited into polysomes during this development sequence.

3.5. Summary

An optimised wheat-germ system, which showed

good efficiency and fidelity of translation of exogenous mRNA, was prepared and used to study developmentally related quantitative and qualitative changes in the mRNA population of the cotyledons.

It was shown that the total mRNA content of the cotyledons was initially low, increased markedly to peak values 3 - 4 days after imbibition, then declined as seedling growth continued. These increases in total mRNA were mirrored by similar changes in the poly(A)$^+$ and poly (A)$^-$ mRNA fractions. The quantitative changes in total and poly(A)$^-$ mRNA, but not the poly(A)$^+$ mRNA, were shown to be light regulated. Developmentally related qualitative changes in the mRNA population were shown to occur.

The amount of mRNA recruited into polysomes was shown to increase dramatically during the heterotrophic growth phase, but did not show the later decline that was detected for the total mRNA content of the cotyledons. Due to the presence of RNAase in the polysome preparations it was not possible to directly determine any qualitative changes in the mRNAs actively involved in protein synthesis.

SECTION 4

DEVELOPMENTAL CHANGES IN THE mRNAs FOR THE GLYOXYSOMAL ENZYMES, ICL AND MS

I have previously shown developmentally related quantitative and qualitative changes in the total mRNA population of cucumber cotyledons during germination and early seedling development. However, of specific interest in this study are any changes in the mRNAs coding for glyoxysomal enzymes.

The activities of ICL and MS, the two enzymes unique to the glyoxylate cycle, were shown to increase markedly, from vitually undetectable levels in the imbibed tissue, during the first 3 days of growth then decline as development continued (p.131). Catalase (CAT), a glyoxysomal and peroxisomal enzyme, has also been shown to increase markedly in activity at the onset of germination. Evidence suggests that the activity increases of these and other glyoxysomal enzymes is due to *de novo* synthesis (Hock and Beevers, 1966; Longo, 1968; Gientka-Rychter and Cherry, 1968; Ihle and Dure, 1972; Smith *et al.*, 1974; Doig *et al.*, 1975; Tester, 1976; Bowden and Lord, 1976A and 1977; Walk and Hock, 1977).

De novo synthesis of proteins may be attributed to synthesis from either preformed mRNA or *de novo* synthesised mRNA. The evidence available regarding the glyoxysomal enzymes is conflicting as it falls into both categories. Translation of preformed mRNA has been suggested to be responsible for the observed increases in ICL activity by Ihle and Dure (1972), for cotton seed, and Tester (1976) for soybean. However, these findings have been challenged by other studies which indicate

that postgerminative RNA synthesis is essential (Hock and Beevers, 1966; Smith et al., 1974; Roberg and Becker, 1975; Radin and Trelease, 1976). A more recent report by Choinski and Trelease (1978) showed that some glyoxysomal enzyme activities (including MS and CAT but not ICL) could be detected in cotton cotyledons during embryo maturation. Thus, it may be that the mRNAs for the glyoxysomal enzymes are present, albeit at reduced levels, in the dry seed. There is certainly some evidence that mRNAs exist in the dry seed tissue and that translation of at least some of these may be responsible for the initial changes in enzyme activities upon the onset of germination (Mayer and Shain, 1974).

The conflicting evidence concerning the level of control of synthesis of the glyoxysomal enzymes supports the conclusions that inhibitor studies are often inconclusive, particularly when attempting to assay for the synthesis of a specific mRNA.

In an attempt to clarify the situation, the translation products from a wheat-germ system primed with cucumber cotyledonary RNA were analysed for the presence of polypeptides related to the glyoxysomal enzymes, ICL, MS and CAT. As has already been shown, the total cucumber cotyledonary mRNA population codes for many different polypeptides and it is not possible to directly identify any specific in vitro translation product as being associated with these glyoxysomal enzymes.

In order to positively identify these specific products use was made of monospecific antibodies prepared against

ICL, MS and CAT purified from cucumber cotyledons (Lamb et al., 1978; Riezman et al., 1979). These antibodies were kindly provided by H. Riezman.

4.1. Selection of a Suitable Immunoprecipitation Technique

Using the monospecific antisera provided, an immunoprecipitation technique which would be efficient, reproducable and as quantitative as possible was sought. Due to the very low concentrations of the specific antigens in the translation mixture, it would not be possible to quantitatively immunoprecipitate the antigen-antibody complex and so some method to increase the efficiency of the immunoprecipitation was necessary.

Addition of a carrier protein would be a possible method to increase the efficiency of immunoprecipitation and has been used successfully by other authors (Verma et al., 1974; Chashmore et al., 1978). This would necessitate use of purified antigens and as these were not readily available this method was not used.

Another method which has been used successfully by other workers (Hunter, 1967; Higgins et al., 1976; Nakanishi et al., 1976; Shields and Bloebel, 1977; Dobberstein et al., 1977) is the double antibody technique. This method was tested using sheep anti-rabbit antisera where rabbit anti-ICL or MS was used as the first antibody. If carefully used this method is unaffected by variations in protein concentration (Hunter, 1967).

Another useful procedure developed recently has been the use of Protein A which is produced by Staphylococcus aureus cells and either incorporated into the cell surface or excreted.

The major feature of Protein A for immunologists is its ability to bind immunoglobulin, notably IgG. Use can be made of inactivated S. aureus cells to effectively bind an antigen-antibody complex. This can then be efficiently precipitated and washed while the complex remains bound to the cell surface. Mild SDS treatment will detach the complex from the cells, which can subsequently allow separation of the antigen-antibody complex from the cells. This method has been used successfully by several workers (Jonsson and Kronvall, 1974; Kessler, 1975; Gough and Adams, 1978; Ploegh et al., 1979; Goding, 1978) as an alternative to the double antibody technique.

S. aureus cells were prepared as described by Kessler (1975) and used in the immunoassay as described in the Materials (p.117).

Another possible method of utilising the properties of the Protein A is by affinity chromatography. Hjelm et al., (1972) showed that Protein A-Sepharose could be used as a means of purifying IgG. This method has been used successfully to prepare IgG fractions from human, rabbit (Goding, 1976; Riezman et al., 1979) and mouse (Mitchell et al., 1977a and b).

In this study chromatography on Protein A-Sepharose was used to purify the antibody-antigen complex from the in vitro translation mixture. The preparation of the affinity column and its use in the radioimmunoassay have been previously described (p.117).

To determine the most suitable method for the radioimmunoassay of the wheat-germ translation products, each was used in an experiment in which rabbit anti-ICL was the first

antibody. Using a microprecipitin test the appropriate concentration of second antibody to first antibody was determined (Figure 43). S. aureus cells were used at an original concentration of 10% (w/v) such that an excess of Protein-A was present as determined by its binding capacity for IgG (Jonsson and Kronvall, 1974; Goding, 1978). Controls using null serum or no antisera in place of the rabbit anti-ICL were carried out.

The immunoprecipitated in vitro products were detected by SDS-polyacrylamide gel electrophoresis followed by fluorography (p. 118) and are illustrated in Figure 44. Prior to loading the immunoprecipitated samples onto the gels, samples were taken to estimate the level of radioactivity immunoprecipitated and these figures are shown in Table 6.

From the fluorographs it can be seen that each of the immunoassay methods has been successful in purifying specific polypeptides from the in vitro products of the wheat-germ system when programmed with day 3 total nucleic acid from light-grown tissue. The double-band at slightly more than 60,000 mol. wt. is the immunoprecipitated polypeptides for ICL (see p.229).

If the results of the fluorograph (Figure 44) and the estimations of radioactive counts (Table 6) are compared, it can be seen that estimation of counts is not always very accurate. This is particularly noticeable in the higher antibody concentrations of the double antibody technique, where all the counts can be seen to constitute contamination and not the ICL related products.

The double antibody technique appeared to be most successful in the 0·25 µl (0·3 µg) range since this gave the

Figure 43

Microprecipitin Tests to Determine the Proportions of First and Second Antibodies for Efficient Immunoprecipitation

Rabbit anti-ICL (A) or rabbit anti-MS (B) were tested against sheep anti-rabbit antiserum, at varying concentrations of each, to find the relative proportions of each required for efficient immunoprecipitation. Reactions were allowed to continue overnight before scoring for the immunoprecipitation reaction.

Figure 43

A.

Rabbit anti-ICL antiserum

Sheep anti-rabbit antiserum

Dilutions	3	6	12	24	48	98	192	384
3	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+
12	-	±	+	+	+	+	+	±
24	-	-	-	-	+	+	-	-
48	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	-
192	-	-	-	-	-	-	-	-
384	-	-	-	-	-	-	-	-

B.

Rabbit anti-MS antiserum

Sheep anti-rabbit antiserum

Dilutions	5	10	20	40	80	60	320	640
3	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+
12	±	+	+	+	+	+	+	+
24	-	-	+	+	+	+	+	+
48	-	-	-	+	+	+	+	+
96	-	-	-	-	-	+	+	+
192	-	-	-	-	-	-	±	±
384	-	-	-	-	-	-	-	-

Figure 44

Immunoassay of *In Vitro* Synthesised ICL Polypeptides by Three Different Immunological Techniques

Total nucleic acid (15 µg/50 µl incubation) from day 3, light-grown cucumber cotyledons was used to programme *in vitro* protein synthesis in the wheat-germ S.30 system. After 90 minutes incubation, samples of the mixture (50 µl) were taken for immunoprecipitation of *in vitro* synthesised ICL polypeptides. Three methods of immunoassay were used - double antibody technique (A), *S. aureus* immunoadsorption (B) and Protein A-Sepharose affinity chromatography (C) - as previously described (p. 115). *In vitro* synthesised ICL polypeptides were immunoprecipitated using increasing concentrations of first antibody (rabbit anti-ICL), and second antibody (sheep anti-rabbit) or *S. aureus* as indicated in Table 6. Controls using non-immune serum or no serum carried out. The immunoprecipitated labelled products were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) and detected by fluorography (p. 118). Molecular weights were determined using a standard protein marker mixture, on the stained gel, and also a sample of purified glyoxysomal proteins (G).

FIGURE 44.

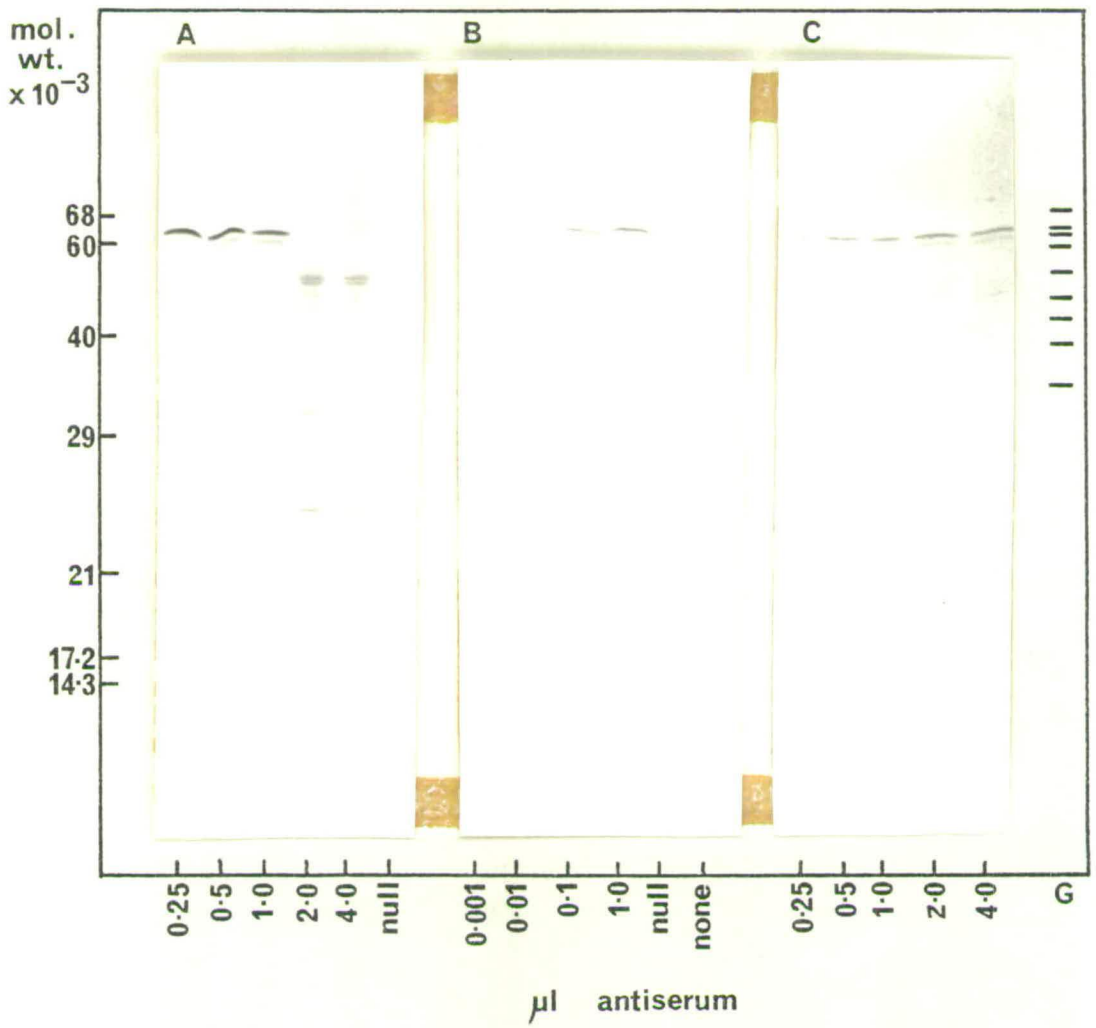

mol. wt. x 10⁻³
A
B
C
68
60
40
29
21
17·2
14·3
0·25
0·5
1·0
2·0
4·0
null
0·001
0·01
0·1
1·0
null
none
0·25
0·5
1·0
2·0
4·0
G
μl antiserum

Table 6

Estimation of Radioactivity present in the Immunoprecipitated *In Vitro* Synthesised ICL Polypeptides

Immunoassays were carried out by (A) the double antibody technique, (B) by *S. aureus* immunoadsorption or (C) Protein A-Sepharose affinity chromatography, in order to detect *in vitro* synthesised ICL polypeptides coded by day 3, light-grown cotyledonary total nucleic acid (15 µg/50 µl incubation) in the wheat-germ S.30 system (p. 115). Prior to analysing the samples by gel electrophoresis, aliquots were taken, after resuspension in gel sample buffer (p. 87), and the hot, TCA-precipitable radioactivity present in each determined as described (a) (p. 118). For the Protein A-Sepharose technique the total amount of radioactive protein bound to the column was also determined (b).

Table 6

A. Double Antibody Technique

µl rabbit anti-ICL antiserum	µl sheep anti-rabbit antiserum	c.p.m. immunoprecipitated and loaded on gel (a)
0·25	10	2,579
0·5	10	3,191
1·0	10	5,473
2·0	10	9,423
4·0	10	8,830
null serum	10	3,310

B. S. aureus Technique

µl rabbit anti-ICL antiserum	µl 10% (w/v) S. aureus cells	c.p.m. immunoprecipitated and loaded on gel (a)
0·001	0·02	213
0·01	0·2	283
0·1	2	638
1·0	20	2,173
null serum	20	2,950
no serum	20	1,867

C. Protein A – Sepharose Column Technique

µl rabbit anti-ICL antiserum	total c.p.m. eluted from column (b)	c.p.m. loaded on gel (a)
0·25	1,206	42
0·5	2,249	176
1·0	3,223	642
2·0	5,720	529
4·0	6,501	1,255

best immunoprecipitation and also the best non-specific reaction. At higher antibody concentrations and where there was no specific antibody-antigen reaction there is a relatively high level of contamination from non-specific reactions.

S. aureus cells were also used successfully to precipitate the antigen-antibody complex, but a comparison at the 1·0 µl (1·2 µg) antibody concentration with the double-antibody method, suggests the latter method is more quantitative. Another disadvantage of this technique was the fact that during washing the cells were found to be difficult to resuspend and thus wash efficiently.

Chromatography on the Protein-A-Sepharose column was also successful and yielded a relatively clean immunoprecipitate. However, this technique had the disadvantage that the antigen-antibody complex was eluted in a relatively large volume and, due to the low protein concentration, was difficult to concentrate. In order to be quantitative, total recovery of all the complex would be essential.

One other drawback in the S. aureus and Protein A-Sepharose techniques is that Protein A only binds to IgG and some IgM from rabbits (Goding, 1976, 1978). To overcome this problem, the antibody would first have to be purified on a Protein A-Sepharose column before being used for either method.

Thus, it was decided that for identification and quantitation of ICL and MS polypeptides synthesised in vitro, the double antibody technique would be most suitable as long as the precipitate was washed as thoroughly as possible. It was not possible to completely reduce non-specific binding as

the high pH and salt concentrations used, although conditions for quantitative immunoreaction with the specific polypeptides, also gave a low level of non-specific binding.

For the identification of the *in vitro* synthesised CAT polypeptide, the *S. aureus* procedure was used. The anti-CAT antisera was prepared in mice and no efficient second antibody was available.

4.2. Identification of *in vitro* Synthesised ICL, MS and CAT Subunits

The presence of polypeptides immunlogically related to ICL, MS and CAT in the *in vitro* labelled products from the wheat-germ system, programmed with total nucleic acid from day 3 light-grown cotyledons, was determined using the procedures outlined above.

Figure 45A shows the two polypeptides immunoprecipitated by rabbit anti-ICL antiserum. These polypeptides have moleculat weights of approximately 63,000 and 61,000 and correspond closely with two of the stained polypeptides from isolated glyoxysomes (indicated in Figure 45G) which co-migrate with purified cucumber ICL-A and ICL-B respectively (Riezman *et al.*, 1979).

Antiserum raised against MS immunoprecipitated a single polypeptide of molecular weight 57,000 which also corresponds to one of the stained polypeptides from isolated glyoxysomes which has been identified as native MS (Riezman *et al.*, 1979). Mouse anti-CAT antiserum immunoprecipitated a single polypeptide of approximately 55,000 mol. wt. which does not co-migrate with any native glyoxysomal polypeptide.

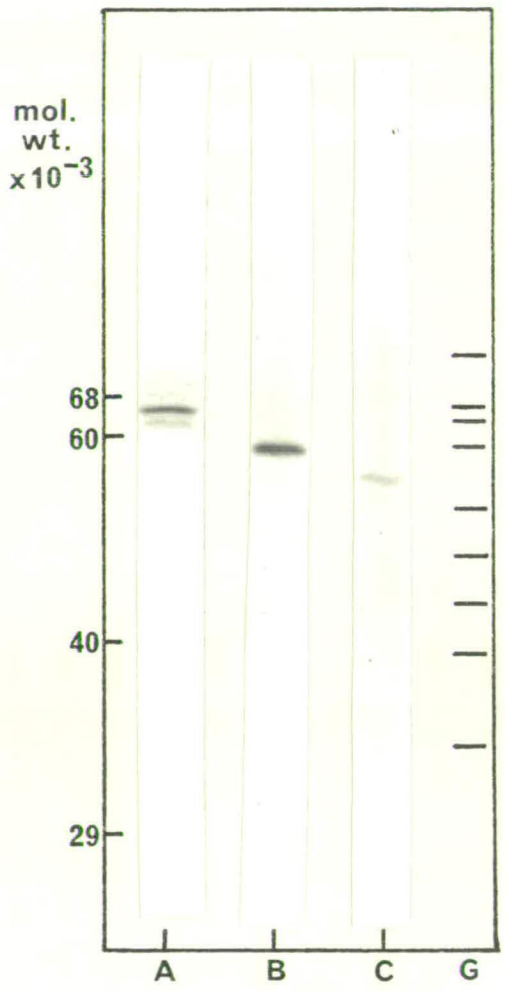
These three native enzymes have subunit molecular

Figure 45

Identification of ICL, MS and CAT polypeptides Synthesised In Vitro

Total nucleic acid (15 µg/50 µl incubation) from day 3, light-grown cucumber cotyledons was used to programme the wheat-germ S.30 cell-free system. Immunoprecipitations were carried out using a 50 µl sample of the incubation mixture. The double antibody technique was used to identify ICL (A) and MS (B) *in vitro* synthesised polypeptides. *S. aureus* immunoadsorption was used to identify *in vitro* synthesised CAT (C) polypeptides. The immunoprecipitated, labelled polypeptides were analysed by fluorography. Purified glyoxysomal proteins(G) were used as a reference and the positions of the stained polypeptides are indicated. The other molecular weights were determined using a standard protein marker mixture (p. 88).

FIGURE 45.



weights of 63,000 and 61,500 for ICL-A and ICL-B respectively, 57,000 for MS and 54,000 for CAT (Reizman et al., 1979). Thus, it would appear that the in vitro products of ICL mRNA may vary slightly compared to the corresponding native polypeptides (Reizman et al., 1979), native and in vitro synthesised MS has the same molecular weight and CAT is approximately 1,000 daltons larger synthesised in vitro than the corresponding native polypeptide.

4.3. Quantitative Changes in ICL and MS mRNAs

The monospecific antisera prepared against ICL and MS were then used to immunoprecipitate these specific polypeptides from the products of in vitro protein synthesis, programmed with the RNA from the different developmental stages, thus allowing an assay of these specific translatable mRNAs.

In vitro products from the total nucleic acid (Figure 46), poly(A)$^+$ RNA (Figure 47) and poly(A)$^-$ RNA (Figure 48) primed wheat-germ system were assayed for the presence of ICL and MS subunits. These immunoprecipitations were carried out under conditions where equal amounts of nucleic acid or RNA were used to programme the cell-free system.

It can be seen that, in all fractions, it was possible to detect translatable mRNA for ICL and MS in the dry seeds. However, of the RNA fractions prepared from the tissue after imbibition at 4^{o}C for 17 hours, only the poly(A)$^+$ RNA fraction gave immunodetectable polypeptides, thus indicating the presence of translatable mRNA.

At later stages the results obtained with the total nucleic acid fraction indicate that, in the light-grown coty-

Figure 46

Developmental Changes in the Translatable mRNAs for ICL and MS Polypeptides present in the Total Nucleic Acid Fraction during Germination and Early Seedling Development

Total nucleic acid (15 µg/50 µl incubation) from light and dark-grown cucumber cotyledons were used to programme the wheat-germ S.30 cell-free system. The *in vitro* translation products (50 µl) were analysed by double antibody immunoprecipitation (p. 116) for the present of ICL and MS *in vitro* labelled polypeptides. The immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by fluorography. Purified glyoxysomal proteins (G) were also fractionated on the gels and the positions of the stained polypeptides are indicated.

FIGURE 46.

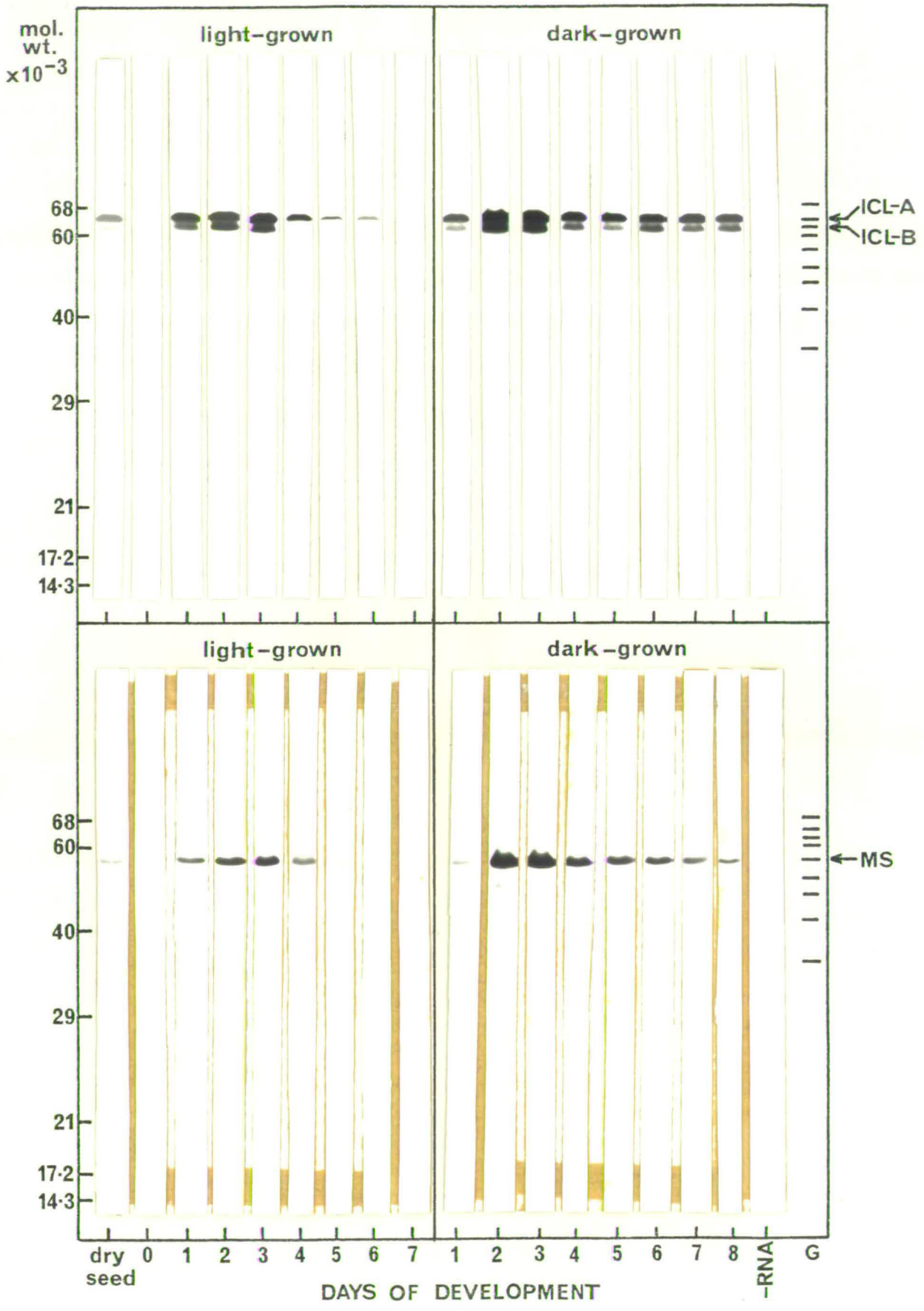

mol. wt. x10⁻³
light-grown
dark-grown
68
60
40
29
21
17·2
14·3
ICL-A
ICL-B
MS
dry seed
0
1
2
3
4
5
6
7
8
-RNA
G
DAYS OF DEVELOPMENT

Figure 47

Developmental Changes in the Translatable mRNAs for ICL and MS Polypeptides present in the Poly(A)$^+$ RNA Fraction during Germination and Early Seedling Development

Poly(A)$^+$ RNA (0·5 µg/50 µl incubation) from light and dark-grown cucumber cotyledons were used to programme the wheat-germ S.30 cell-free system. The *in vitro* translation products (50 µl) were analysed by double antibody immunoprecipitation (p. 116) for the presence of ICL and MS *in vitro* labelled polypeptides. The immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by fluorography. Purified glyoxysomal proteins (G) were also fractionated on the gels and the positions of the stained polypeptides are as indicated.

FIGURE 47.

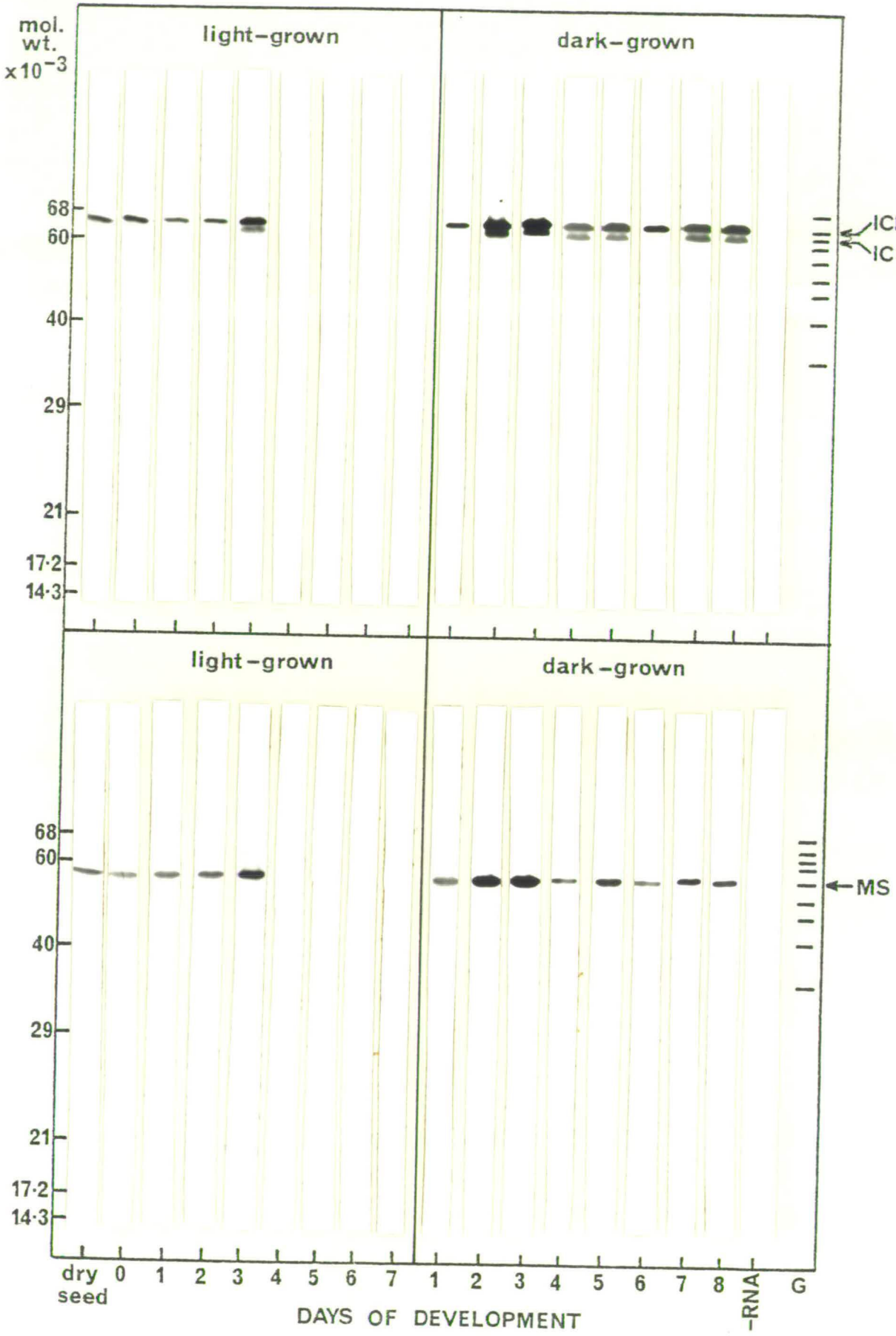
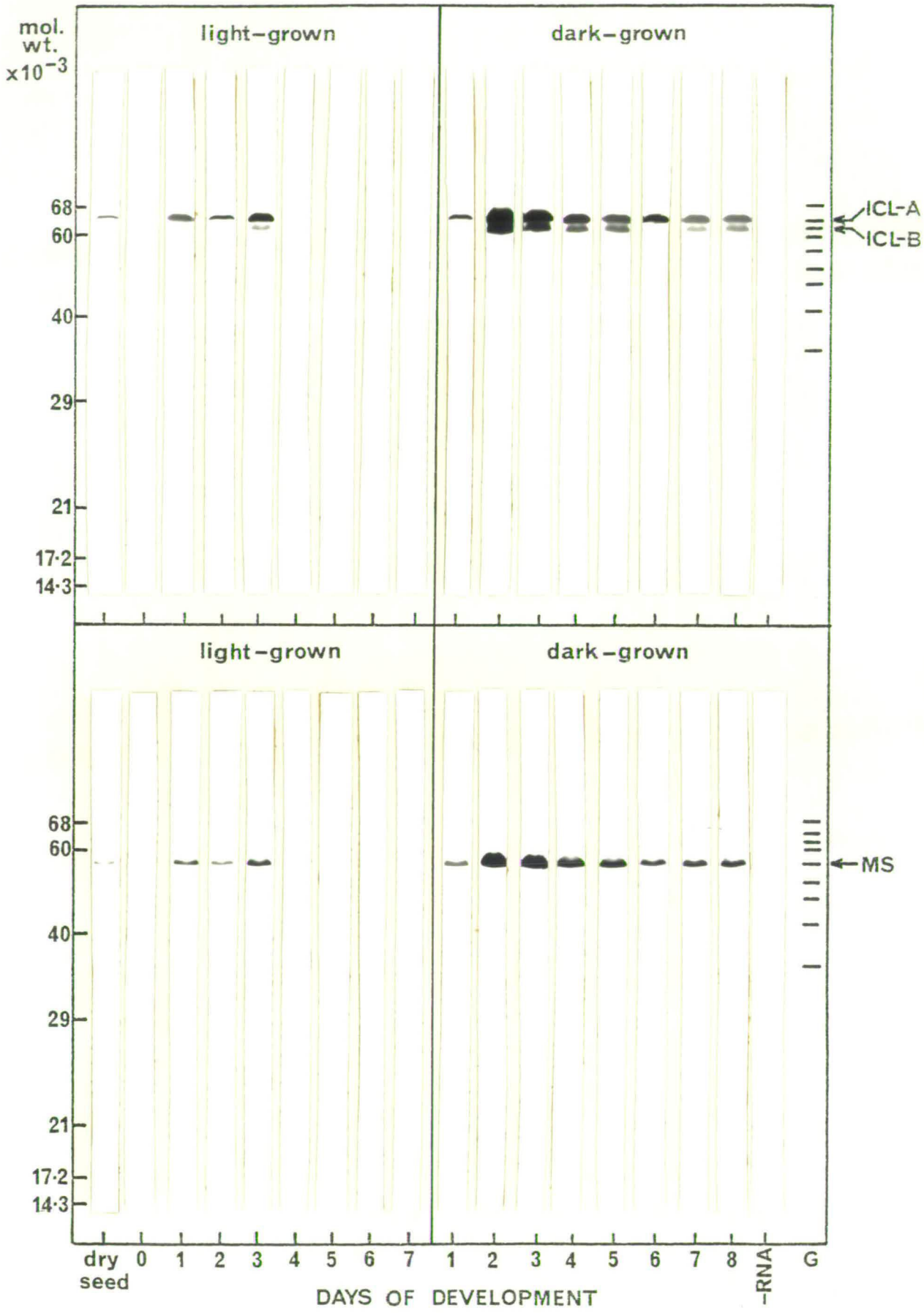

mol. wt. x10⁻³
light-grown
dark-grown
68
60
40
29
21
17·2
14·3
ICL-A
ICL-B
light-grown
dark-grown
68
60
40
29
21
17·2
14·3
MS
dry seed
0 1 2 3 4 5 6 7
1 2 3 4 5 6 7 8
-RNA
G
DAYS OF DEVELOPMENT

Figure 48

Developmental Changes in the Translatable mRNAs for ICL and MS Polypeptides present in the Poly(A)$^-$ RNA Fraction during Germination and Early Seedling Development

Poly(A)$^-$ RNA (20 µg/50 µl incubation) from light and dark-grown cucumber cotyledons were used to programme the wheat-germ S.30 cell-free system. Conditions for immunoprecipitation were as described in Figure 46 and 47. The immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by fluorography. Purified glyoxysomal proteins (G) were also fractionated on the gels and the positions of the stained polypeptides are as indicated.

FIGURE 48.


mol. wt. x10⁻³
light-grown
dark-grown
68
60
40
29
21
17·2
14·3
ICL-A
ICL-B
light-grown
dark-grown
68
60
40
29
21
17·2
14·3
MS
dry seed
0 1 2 3 4 5 6 7
1 2 3 4 5 6 7 8
-RNA
G
DAYS OF DEVELOPMENT

ledons, there is an increase in the amount of mRNA for both ICL and MS, as a proportion of the total message, to peak values at day 3. Thereafter there is a marked decrease in the mRNA for these enzymes though it does not completely disappear by day 7. In the dark-grown tissue there is also a peak at about day 3 but thereafter the decrease in these mRNAs is less marked than in the light.

Similar results were obtained for the poly(A)$^+$ and poly(A)$^-$ RNA fractions with peak translatable mRNA contents at day 3 in both the light and dark-grown tissue. The small amount of these mRNAs remaining in the light-grown tissue at later stages is not so clear in these fractions but their persistence in the dark is still obvious.

Fluorographs of the in vitro products programmed with total nucleic acid and poly(A)$^+$ RNA fractions were scanned to determine the relative amounts of labelled polypeptides at the different stages of development. Using these relative amounts and taking into account the total levels of incorporation in these experiments, and the translational activities of these RNA fractions on a per cotyledon basis (see p.192 and Figure 30), it was possible to show the relative amounts of these mRNAs during germination and early seedling development. Due to the poor quality of some of the incorporations and immunoprecipitations from the poly(A)$^-$ RNA this calculation was not carried out for this fraction. One unit of mRNA is defined as that amount of mRNA for either of the two enzymes (ICL and MS) which is present in day 4 dark-grown tissue.

Figure 49 shows the changes in the amount of total

Figure 49

Quantitative Estimation of the Amount of Translatable mRNAs for ICL and MS Polypeptides present in the Total Nucleic Acid Fraction during Germination and Early Seedling Development

The fluorographs (Figure 46) of *in vitro* synthesised ICL (A) and MS (B) polypeptides programmed with total nucleic acid from light and dark-grown cucumber cotyledons in the wheat-germ S.30 system were scanned on a Quick Scan R & D (Helena Laboratories) densitometer to determine the relative intensities of the labelled polypeptide seen on the fluorographic film. Using these relative intensities, the amounts of total nucleic acid added to each incubation and the amount of total nucleic acid per cotyledon (Figure 98), quantitative changes in the amount of translatable mRNAs for ICL and MS were estimated.

$$\text{relative amount of translatable mRNA in total nucleic acid} = \text{relative intensity of labelled immunoprecipitate} \times \frac{\mu g \text{ total nucleic acid/cotyledon}}{\mu g \text{ total nucleic acid added/incubation}}$$

1 unit of translatable mRNA was taken to be that amount of mRNA present in the cotyledons at day 4 (dark-grown).

FIGURE 49.

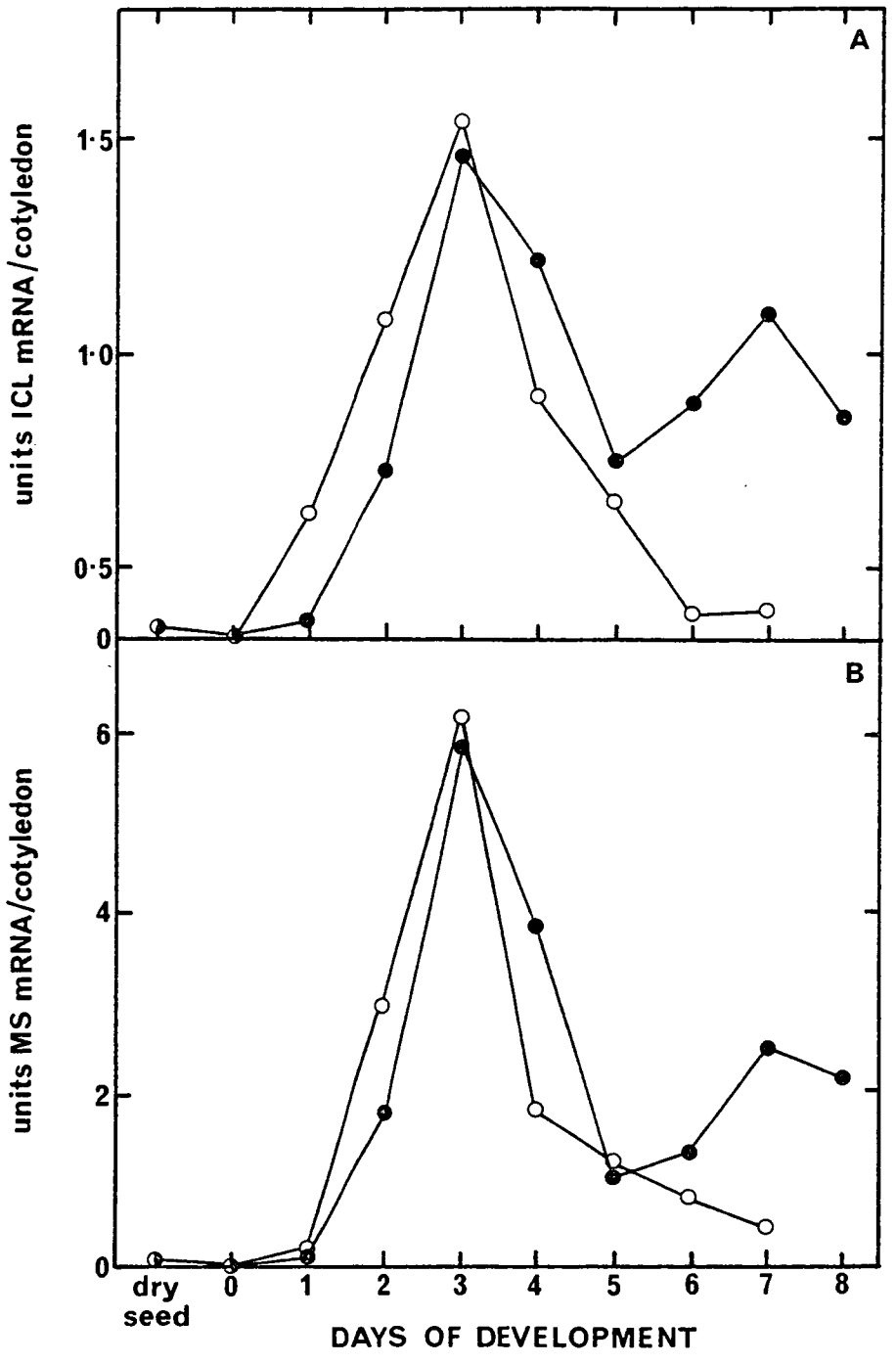

A
B
units ICL mRNA/cotyledon
units MS mRNA/cotyledon
1·5
1·0
0·5
0
6
4
2
0
dry seed
0
1
2
3
4
5
6
7
8
DAYS OF DEVELOPMENT

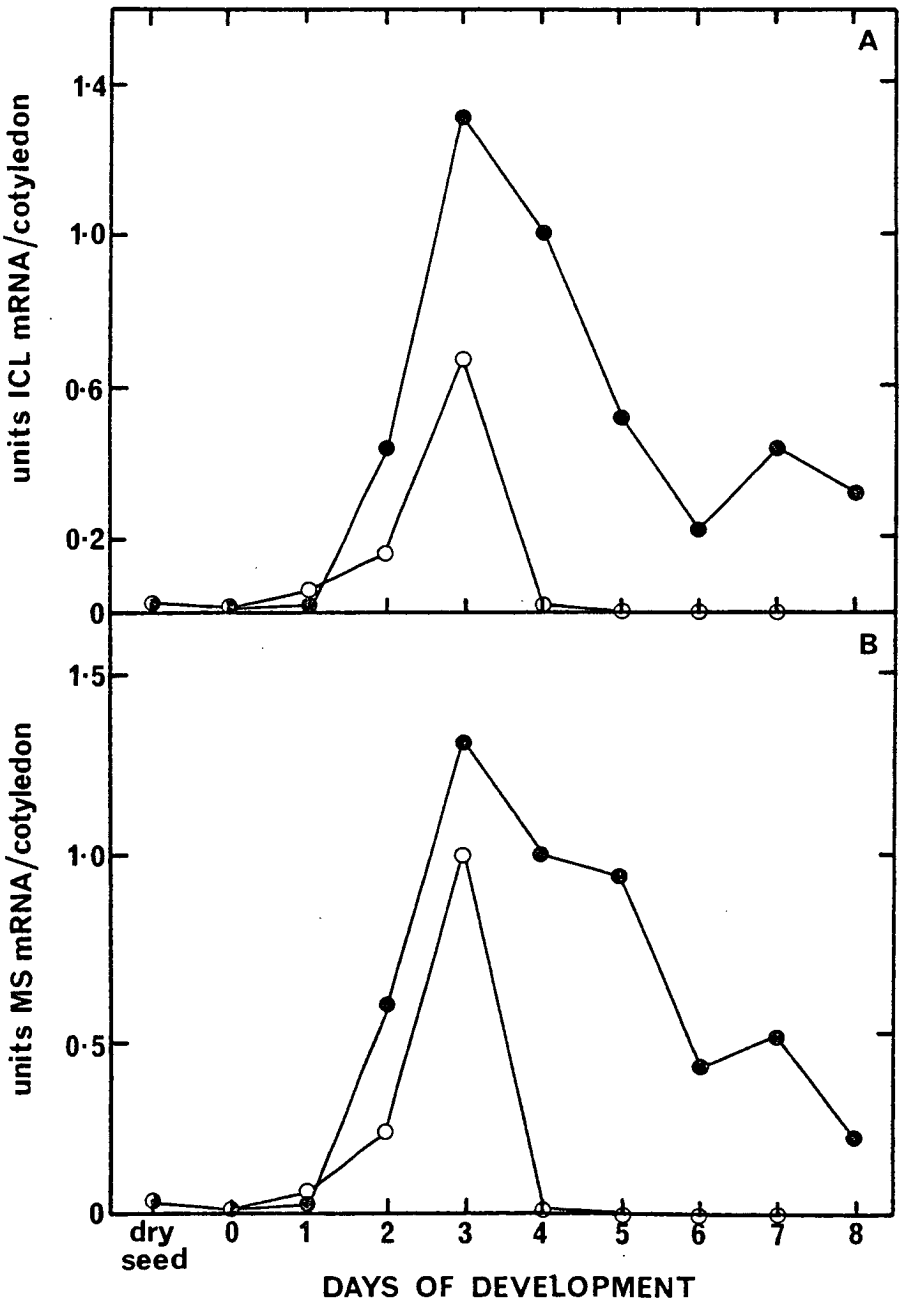
Figure 50

Quantitative Estimation of the Amount of Translatable mRNAs for ICL and MS Polypeptides present in the Poly(A)$^+$ RNA Fraction during Germination and Early Seedling Development

The quantitative changes in the amount of translatable mRNAs for ICL and MS present in the poly(A)$^+$ RNA fraction were estimated, from Figure 47, in a similar manner to that described in Figure 49.

$$\text{relative amount of translatable mRNA in poly(A)}^+\text{ RNA} = \text{relative intensity of labelled immunoprecipitate} \times \frac{\mu\text{g poly(A)}^+\text{ RNA/cotyledon (Fig. 15A)}}{\mu\text{g poly(A)}^+\text{ RNA added/incubation}}$$

FIGURE 50.


A
B
units ICL mRNA/cotyledon
units MS mRNA/cotyledon
1·4
1·0
0·6
0·2
0
1·5
1·0
0·5
0
dry seed
0
1
2
3
4
5
6
7
8
DAYS OF DEVELOPMENT

mRNA for ICL (A) and MS (B) during this developmental sequence, while Figure 50 shows the changes in the amounts of these two mRNAs present in the poly(A)$^+$ RNA fraction.

It can be seen from Figure 49 that there is a very low though measurable amount of mRNA for these two enzymes present in the dry seed tissue. No mRNA was detectable at day 0 when using the total nucleic acid to programme the wheat-germ system. Thereafter, in both light and dark-grown tissue there is a marked increase in these mRNAs to reach peak values by day 3. In the light-grown tissue these mRNAs then decrease as growth proceeds whereas, in the dark there is a marked decrease to day 5 followed by a slight increase in the amounts of these mRNAs.

The amounts of these mRNAs for the glyoxylate cycle enzymes present in the poly(A)$^+$ RNA fraction are initially low, though it can be seen from Figures 50 and 47 that there is in fact some (though not much) present after imbibition (day 0). There is then a marked increase to peak values by day 3 and thereafter in the light no translatable mRNA for these two enzymes was detectable in this fraction. However, in the dark-grown tissue there is apparently a much slower decrease in these mRNAs as growth continues. There would also appear to be higher levels of these mRNAs in the poly(A)$^+$ RNA fraction in the dark as compared to the light-grown tissue.

4.4. Estimation of Size of the ICL and MS mRNAs

In order to estimate the approximate sizes of the mRNAs for ICL and MS, a sample of poly(A)$^+$ RNA from day 2 dark-grown cotyledons was fractionated on a sucrose density gradient and fractions from the gradients were used to

programme the wheat-germ cell-free system. Figure 51 shows the separation of the poly(A)$^+$ RNA on the gradient and also which fractions were used in the cell-free system.

The total products synthesised by the different fractions are shown in Figure 52 - the numbers of the fractions corresponding to those in Figure 51. As a control, one incubation was carried out using unfractionated poly(A)$^+$ RNA from day 2 dark-grown tissue (lane 12). It can be seen that the fractions 1 and 2 (i.e. $<$16S) contained only mRNA which coded for low molecular weight polypeptides, while the higher molecular weight polypeptides were coded for by mRNAs which sedimented further along the gradient (i.e. $\geqslant$18S).

To determine which fractions contained the mRNAs for ICL and MS, samples of the <u>in vitro</u> labelled products were analysed by immunoprecipitation as previously described (p. 116) and the immunoprecipitates then detected by SDS-polyacrylamide gel electrophoresis and fluorography (Figure 53).

For both ICL and MS the corresponding <u>in vitro</u> synthesised polypeptides can be detected over the range 16→30·5S (fractions 4 to 10). However, the highest concentration of these mRNAs is located in fraction 7, which is in the 21→23·5S region.

Obviously, to obtain accurate molecular weight or S value estimates for an individual mRNA it is necessary to ensure that the secondary structure of the RNA is completely denatured by carrying out fractionation in the presence of, for example, formamide or DMSO (Strauss <u>et al.</u>, 1968). However, it is possible to obtain an approximate molecular weight using these non-denaturing conditions.

Figure 51

Sucrose Density Gradient Fractionation of Poly(A)$^+$ from Day 2 Dark-Grown Cucumber Cotyledons

31·9 µg of poly(A)$^+$ RNA from day 2 dark-grown cucumber cotyledons was loaded on a 5·1 ml, linear 5–35% (w/v) sucrose gradient and centrifuged at 149,000 g_{av} for 6 hours in a Spinco 50 rotor. The gradient buffer was 100 mM Hepes-KOH pH 7·5, 0·1 M KCl. The poly(A)$^+$ RNA profile was determined using an ISCO Model UA-4 absorbance monitor (A_{265}) and Vitatron chart recorder. The first 1·2 ml of the top of the gradient was discarded then 11 x 0·3 ml fractions collected. The RNA from these fractions was ethanol precipitated, dried and resuspended in sterile distilled water, then used to programme the Wheat-germ S.30 system as described (p. 107). The positions of 25S, 23S, 18S and 16S cytoplasmic and chloroplast rRNAs were determined by running total cucumber cotyledonary RNA on a parallel gradient.

FIGURE 51.

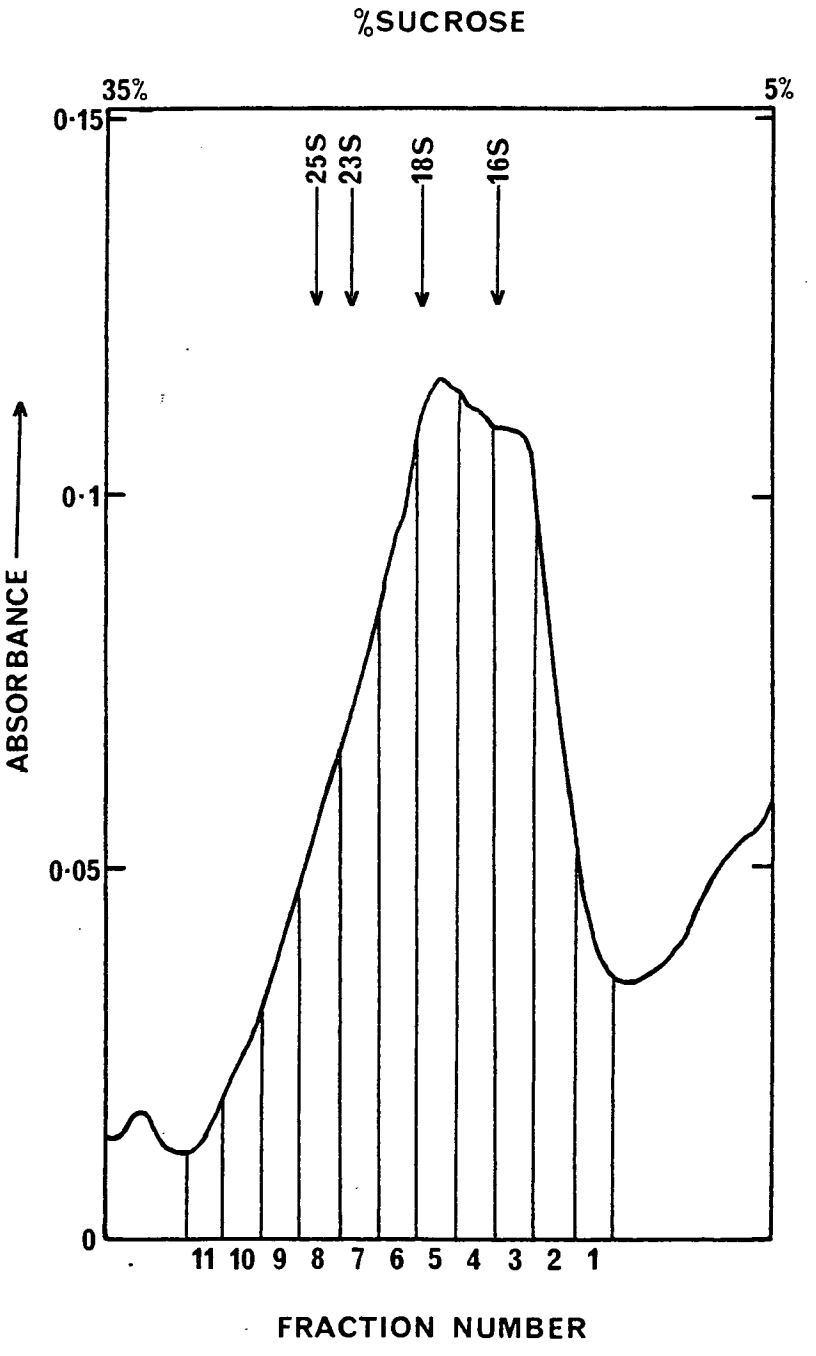

%SUCROSE
35%
5%
0·15
0·1
0·05
0
25S
23S
18S
16S
ABSORBANCE
11 10 9 8 7 6 5 4 3 2 1
FRACTION NUMBER

Figure 52

In Vitro Labelled Polypeptides Synthesised in the Wheat-Germ S.30 System Programmed with Different Size Classes of Poly(A)$^+$ RNA obtained by Sucrose Density Gradient Fractionation

Fractions (1⟶11; see Figure 51) from a poly (A)$^+$ RNA gradient and total poly(A)$^+$ RNA, from day 2 dark-grown cucumber cotyledons, were used to programme the wheat-germ S.30 system. Incubations were carried out in a total volume of 200 µl, 100 µl of which contained the poly(A)$^+$ RNA. The *in vitro* labelled polypeptides were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by autoradiography. The lane numbers correspond to the fraction numbers indicated in Figure 51 (all the RNA recovered from each fraction was used in the incubation); lane 12 was programmed with 1·59 µg of total poly(A)$^+$ RNA (i.e. non-fractionated).

FIGURE 52.

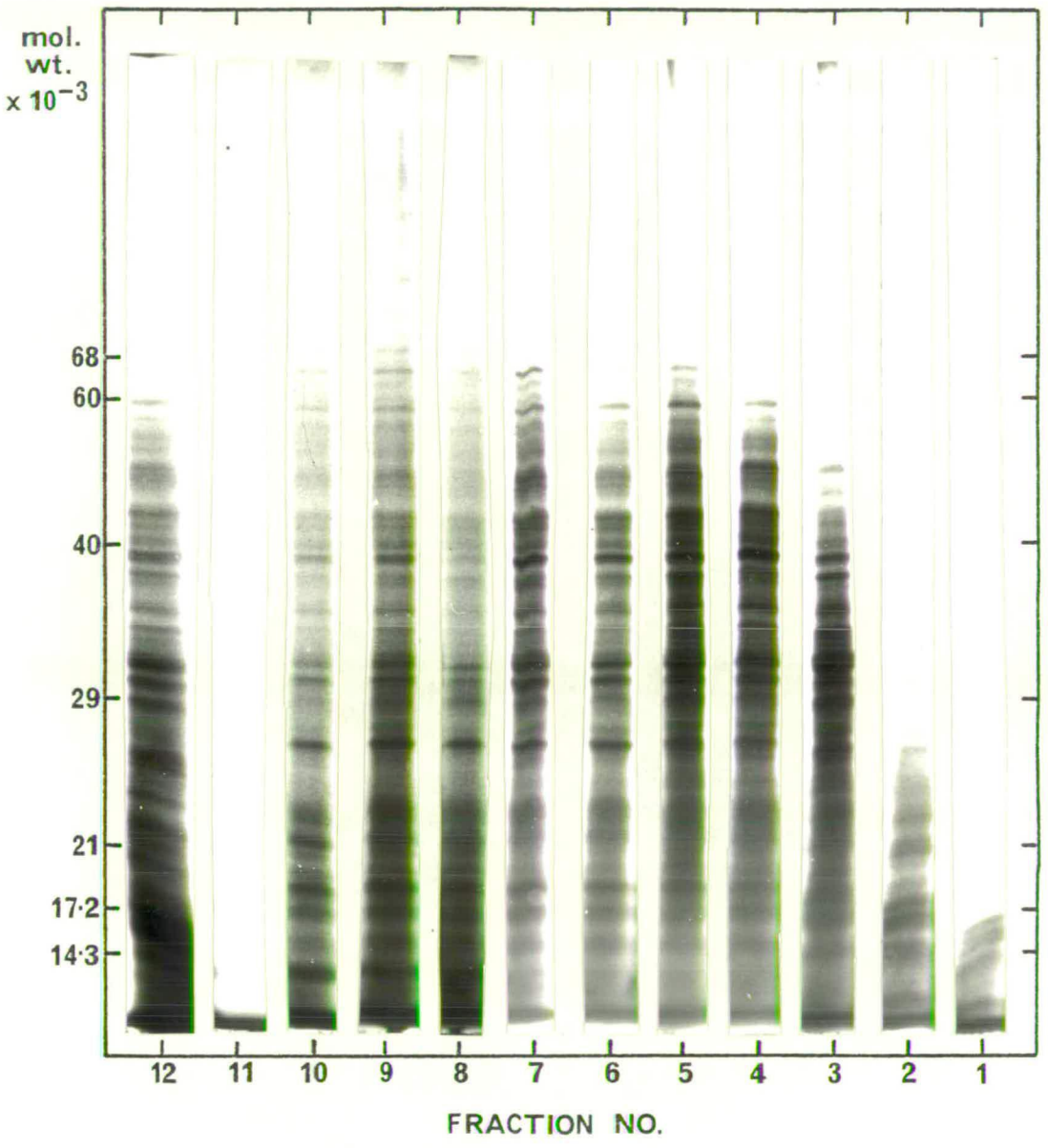
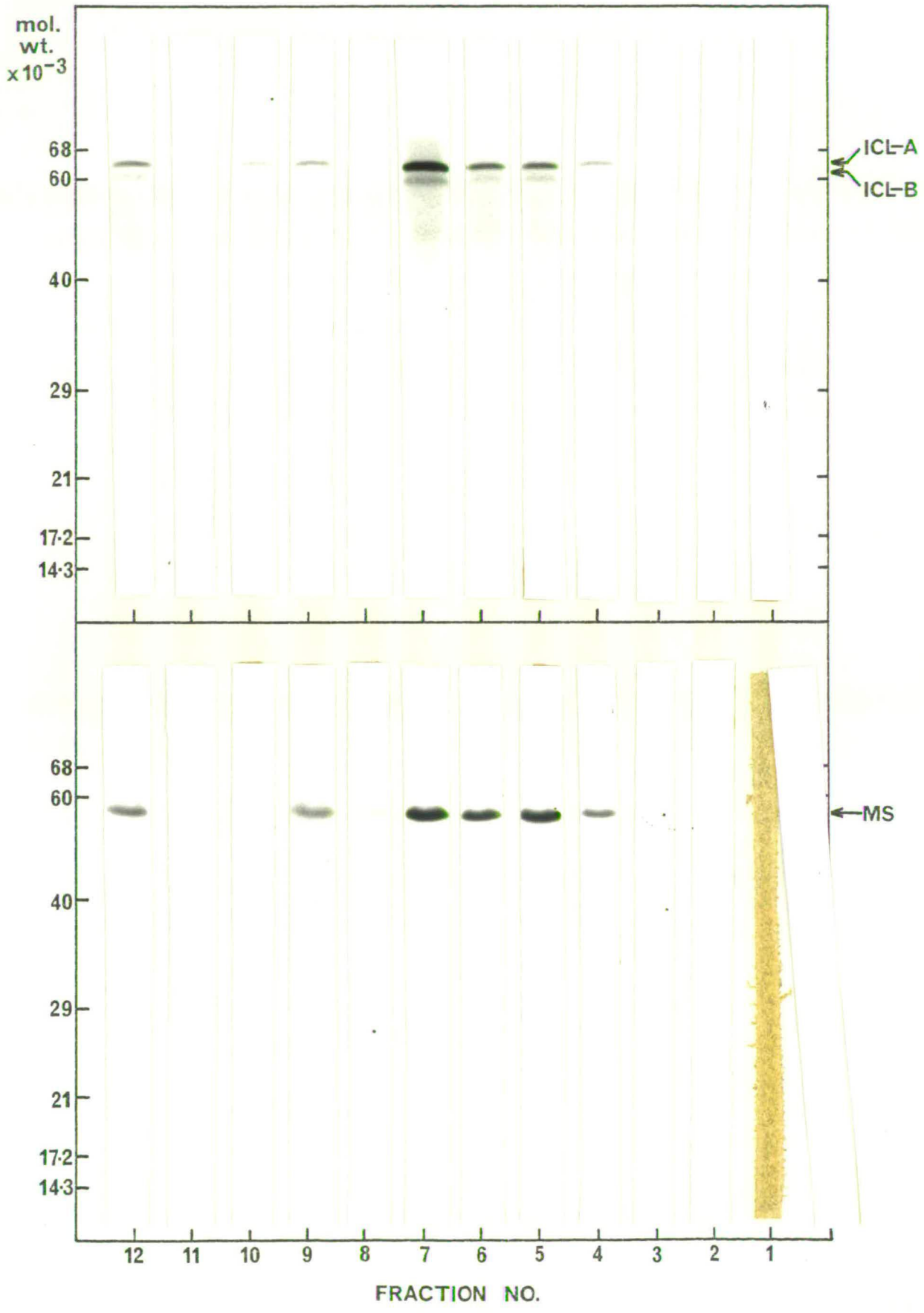

mol.
wt.
x 10⁻³
68
60
40
29
21
17·2
14·3
12
11
10
9
8
7
6
5
4
3
2
1
FRACTION NO.

Figure 53

Immunoprecipitation of *In Vitro* Synthesised ICL and MS Polypeptides Programmed with Different Size Classes of Poly(A)$^+$ RNA obtained by Sucrose Density Gradient Fractionation

The *in vitro* labelled products shown in Figure 52 were analysed for the presence of ICL (A) and MS (B) polypeptides by double antibody immunoprecipitation. 70 µl of the incubation mixture was used in each case and the antibody volumes adjusted as appropriate to maintain the standard concentrations. The immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis (15% w/v) followed by fluorography. The lane numbers correspond to the fraction numbers indicated in Figure 51; lane 12 was programmed with non-fractionated poly(A)$^+$ RNA.

FIGURE 53.


mol.
wt.
x10⁻³
68
60
40
29
21
17·2
14·3
ICL-A
ICL-B
68
60
40
29
21
17·2
14·3
MS
12
11
10
9
8
7
6
5
4
3
2
1
FRACTION NO.

Assuming that fraction 7 does contain the bulk of these mRNAs it is possible to estimate an approximate size for these mRNAs. By comparison with the total cytoplasmic and chloroplast rRNAs (25, 23, 18 and 16S) run on a parallel gradient in the same experiment, it is possible to estimate that the average molecular weight of an RNA molecule in this fraction would be $1{\cdot}025 \times 10^6$ ($0{\cdot}9 \longrightarrow 1{\cdot}15 \times 10^6$).

Since the sizes of the native ICL and MS subunits are known, it is possible to estimate the size of mRNA required to code for these subunits. For the ICL-A subunit of 63,000 mol. wt. an mRNA molecule of approximately 1,890 nucleotides and $0{\cdot}66 \times 10^6$ mol. wt. would be required assuming that the average mol. wt. for amino acids and nucleotides is 100 and 350 respectively. However, as this is only the minimum required coding capacity it is likely that this specific mRNA is actually larger. This estimation does not take into account the possibility of a 5' terminal non-translated sequence, a 3' non-translated sequence or a 3' poly(A) sequence. If the same calculations are carried out for ICL-B and MS polypeptides the minimum required coding capacity would be $0{\cdot}646 \times 10^6$ and $0{\cdot}599 \times 10^6$ mol. wt. respectively.

The first gradient fraction (fraction 4) in which these specific <u>in vitro</u> polypeptides can be detected has a mol. wt. range of $0{\cdot}56$ to $0{\cdot}64 \times 10^6$ and is thus the minimum size for an mRNA which could possibly code for these subunits.

It was previously shown that both these mRNA species could be detected in the poly(A)$^+$ and poly(A)$^-$ RNA fractions indicating that varying amounts of poly(A) are present on these

mRNAs. This may account for some of the variation in sizes of these mRNAs as estimated from the gradients.

4.5. Summary

Using immunoassay techniques it has been possible to identify the *in vitro* synthesised polypeptides immunologically related to native subunits for the glyoxysomal enzymes ICL, MS and CAT. These *in vitro* products have molecular weights of approximately 63,000 and 61,000 for ICL A and B, 54,000 for MS and 55,000 for CAT. Only the CAT *in vitro* product was significantly different in size from the native protein, being of the order of 1,000 molecular weight larger.

A developmental study of the changes in translatable mRNAs for ICL and MS shows that they are detectable in the dry and imbibed tissues albeit in very low amounts. The amounts of these mRNAs then increase dramatically to peak values at day 3 and thereafter decline rapidly in the light-grown tissue to only barely detectable lavels by day 7. In the dark-grown tissue the decrease in the mRNAs is less marked and there may be a second smaller peak of mRNA content towards the end of this developmental stage.

The mRNA species for both ICL and MS have been shown to be present in both the poly(A)$^+$ and poly(A)$^-$ RNA fractions indicating that they contain varying lengths of poly(A), presumably at the 3' end of the molecule.

Fractionation of the total poly(A)$^+$ RNA by sucrose density gradient centrifugation followed by translation of individual fractions in a cell-free system then immunoprecipitation allowed an estimate to be made of the molecular weights

of these mRNAs. The *in vitro* translatable mRNAs for these enzymes (ICL and MS) vary in size from an average of 0·6 x 10^6 to 1·64 x 10^6 mol. wt. with the majority at approximately 1·025 x 10^6 mol. wt. The minimum coding capacity required for mRNAs for these polypeptides is 0·66 and 0·646 x 10^6 for ICL-A and ICL-B, and 0·599 x 10^6 for MS.

SECTION 5

DEVELOPMENTALLY RELATED CHANGES IN IN VIVO PROTEIN SYNTHESIS

Quantitative changes in the mRNAs for ICL and MS, the two unique glyoxylate cycle enzymes, have been shown to occur during germination and early seedling development. However, in order to determine whether the synthesis is in some way controlled at the level of translation, it is necessary to establish when these mRNAs are actively involved in protein synthesis. One approach would have been to analyse the *in vitro* products of a wheat germ system programmed with polysomes or polysomal RNA extracted from different stages of development. Unfortunately, due to the presence of RNAase in the polysome preparations (see p.214) this was not possible.

Another method of approaching this problem is to study the proteins being synthesised *in vivo* at the different stages of this developmental sequence. This was carried out by pulse labelling the tissue with [^{35}S]-methionine for 24 hours followed by analyses of the labelled proteins by SDS-polyacrylamide gel electrophoresis and fluorography. This gave some indication of the polypeptides synthesised *in vivo* during this period.

5.1. Pulse Labelling of Whole Seedling

The first method used for the pulse labelling involved supplying [^{35}S]-methionine to the whole seedling via the root system. Prior to labelling, the seeds and all growth media and solutions were sterilised as described (p.119) in order to reduce bacterial contamination. The

results obtained for uptake of methionine into the cotyledons and incorporation of this into total homogenate and 'soluble' proteins are shown in Table 7.

It can be seen that the uptake of methionine into the cotyledons was no greater than 3·7% of the total supplied methionine and that the higher levels of uptake occurred in the later stages of this developmental sequence where almost all of the supplied labelled amino acid was absorbed by the seedling. In the earlier stages, (days 0⟶3) at the time when the glyoxysomal enzymes increase markedly in activity, the uptake of label into the cotyledons was less than 0·5%.

It is clear that there was good incorporation of label into total and 'soluble' protein. However, due to the low levels of uptake of radioactivity into the cotyledons, there was insufficient label in the protein to be able to study the qualitative changes in <u>in vivo</u> protein synthesis by SDS-polyacrylamide gel electrophoresis and fluorography.

Several factors were thought to have influenced the level of uptake of methionine into the cotyledons.

The main problem was possibly the fact that, in labelling the intact seedlings through the roots the majority of the label was going to the root and shoot apices. From Figure 1 it can be seen that the root system develops extensively during germination, particularly during the later stages. During germination and heterotrophic growth the cotyledons are acting as a source of nutrients for the growing apices and so most of the movement of material is out of the cotyledons, particularly during the earlier

Table 7

Uptake and Incorporation of [^{35}S]-Methionine into Cucumber Cotyledons during Pulse Labelling of Intact Seedlings

Cucumber seeds were sterilised as described (p. 119), then grown as normal (p. 74) until the start of the labelling period. Intact seedlings (10 for each day) were pulse labelled with approximately 60 μCi [^{35}S]-methionine (1005 Ci/mmole) for 24 hours, as described (p. 119). Cotyledons were harvested after labelling and total homogenate and 'soluble' (10,000 g_{av} supernatant) proteins prepared as described (p. 74). Duplicate aliquots of each were taken for the following measurements:

(i) Uptake of [^{35}S]-methionine into the cotyledons - 20 μl samples of the homogenate collected on filter paper discs and counted directly. Uptake was expressed as a % of supplied radioactivity.

(ii) Incorporation of [^{35}S]-methionine into total protein - 20 μl samples of the homogenate collected on filter paper discs and the radioactivity in hot, TCA-precipitable material determined (Mans and Novelli, 1961). Incorporation was expressed as a % of radioactivity taken up by the cotyledons.

(iii) Incorporation of [^{35}S]- methionine into 'soluble' protein - 20 μl samples of the 10,000 g_{av} supernatant collected on filter paper discs and the radioactivity in hot, TCA-precipitable material determined (Mans and Novelli, 1961). Incorporation was expressed as a % of radioactivity taken up by the cotyledons.

Table 7

Day	% uptake of [^{35}S]-methionine into the cotyledons	% Incorporation	
		total protein	'soluble' protein
0	0·5	7·7	6·5
1	0·3	13·5	10·0
2	0·3	34·9	17·7
3	0·4	32·7	13·9
4	1·8	61·0	25·0
5	3·3	69·0	36·6
6	2·5	76·4	42·9
7	3·7	64·2	35·5

stages of this developmental sequence. It should be noted that the higher levels of uptake of methionine into the cotyledons occurred when the cotyledons were undergoing rapid expansion and vacuolation and also becoming photosynthetic organs. Thus, the proportion of the supplied radioactivity which was reaching the cotyledons as opposed to the other parts of the plant was possibly very low.

Another factor which may have affected the uptake of label into the tissue is the age of the seeds. Walk and Hock (1977) observed that, with increasing storage time of dry seeds, the ability of the seedlings to incorporate supplied amino acids decreased. This may have been important here since the batch of seeds used was at least 18 months old.

A third factor which would have influenced the uptake of methionine at the early stages (days 0⟶3) was the presence of the seed coats.

5.2. Pulse Labelling of Excised Cotyledons

Having been unsuccessful in pulse labelling intact seedlings and obtaining sufficient label in the cotyledons for a study of the changes in protein synthesis, it was decided to attempt the labelling of isolated cotyledons. This was carried out as described in the Methods (p.121).

There is some controversy in the literature over the effect upon the sequence of developmental changes in the cotyledons of removing them from the seedling. In this experiment, the cotyledons were grown under 'normal' conditions up until the beginning of the labelling period so

that, if any effect of excision was occurring it would be reduced to the 24 hour labelling period.

Several workers in this field have claimed that the appearance and increase in glyoxysomal enzyme activities is dependent upon the presence of the embryo or embryonic axis (Penner and Ashton, 1967; Gientka-Rychter and Cherry, 1968; Bilderback, 1974; Doig et al., 1975; Gonzalez, 1978). However, from the reports of other workers there would appear to be no dependence upon the embryo or embryonic axis for these developmental changes (Huang and Beevers, 1974; Marriot and Northcote, 1975; Tester, 1976; Ford et al., 1976; Slack et al., 1977; Kerley and Becker - personal communication). Presence of the axis is, however, necessary for maximum lipid mobilisation and Slack et al. (1977) suggest that the close correlation between the axis and utilisation of the stored lipid in the cotyledons is attributed to the rate at which the axis can remove the major product of lipid utilisation (i.e. sucrose). It also seems questionable whether the glyoxysomal enzyme activities can be influenced by exogenously added hormones (Marriot and Northcote, 1975; Theimer et al., 1976; Wrigley and Lord, 1977; Allfrey and Northcote, 1977).

There are however, obvious differences between this growth system and the 'normal' system used which may affect the cotyledons though not necessarily the glyoxylate cycle development. Firstly, as pointed out in the last paragraph, excision of the cotyledons removes the axis which is the 'sink' for the products of lipid utilisation.

Secondly, by excising the cotyledons and observing their development for longer than 24 hours it was apparent that the cut surface became callused (personal observation). Thirdly, since excised cotyledons were used, the seed coat was removed in all cases where normally it was still present. Lastly, and of importance in the light-grown tissue, since the cotyledons were no longer under the growth medium (vermiculite) or protected by the seed coat they were exposed to the light at all developmental stages. It was observed that slight greening of the tissue occurred prior to day 3 whereas greening was normally only observed at the proximal end by this stage.

Taking all these drawbacks into consideration, the cotyledons were therefore excised just prior to the 24 hour labelling period, in order to reduce the effects of these differences to a minimum.

The uptake of the labelled amino acid and its incorporation into hot TCA-precipitatable protein are shown in Table 8. As can be seen by comparison with Table 7, much higher levels of uptake were achieved by this method. This would be due to direct application of the radioactive amino acid to the excised cotyledons via the cut surface, and also that the label was applied in more concentrated form, since less bathing solution was required for isolated cotyledons than for intact seedlings.

It can be seen that, apart from day 1, the levels of uptake of the label are very similar between the light-grown and dark-grown sequences. There is a

Table 8

Uptake and Incorporation of [^{35}S]-Methionine into Cucumber Cotyledons during Pulse Labelling of Excised Cotyledons

Cucumber seedlings were sterilised (p.119), then grown as normal (p. 74) until the start of the labelling period. Four cotyledons (each from a different seedling) from each day of development were excised and pulse labelled with [^{35}S]-methionine (50-80 Ci; c.a. 1,000 Ci/mmole) for 24 hours, as described (p.121). After labelling, the cotyledons were rinsed in sterile distilled water and dried on sterile tissue paper. Total homogenate and 'soluble' (12,000 g_{max} x 8 min supernatant) proteins were prepared (p. 121). Duplicate aliquots of each were taken for the following measurements:

(i) Uptake of [^{35}S]-methionine into the cotyledons - 20 µl samples of the homogenate collected on filter paper discs and counted directly. Uptake was expressed as a % of supplied radioactivity.

(ii) Incorporation of [^{35}S]-methionine into total protein - 20 µl samples of the homogenate collected on filter paper discs and the radioactivity in hot, TCA-precipitable material determined (Mans and Novelli, 1961). Incorporation was expressed as a % of radioactivity taken up by the cotyledons.

(iii) Incorporation of [^{35}S]-methionine into 'soluble' protein - 20 µl samples of the 12,000 g_{max} supernatant collected on filter paper discs and the radioactivity in hot, TCA-precipitable material determined (Mans and Novelli, 1961). Incorporation was expressed as a % of radioactivity taken up by the cotyledons.

Table 8

A.

Day	% uptake of [^{35}S]-methionine into the cotyledons	% Incorporation into I total protein	% Incorporation into II 'soluble' protein
0	1•0	15•0	19•4
1	43•6	85•4	51•2
2	23•4	63•2	44•0
3	11•9	73•2	52•8
4	7•8	90•2	48•1
5	11•6*	*	66•5*
6	16•0*	*	67•2*
7	18•8*	*	57•7*

B.

Day	% uptake of [^{35}S]-methionine into the cotyledons	% Incorporation into I total protein	% Incorporation into II 'soluble' protein
1	19•8	40•6	35•0
2	28•0	74•9	51•2
3	10•0	64•9	44•1
4	6•4	73•5	49•0
5	8•1	69•5	44•4
6	10•0*	79•4*	52•2*
7	17•0*	78•1*	49•0*
8	10•9*	*	65•3*

*inaccurate measurements due to quenching (see p. 253)

low level of uptake during imbibition. This agrees with the findings of Kerley and Becker (personal communication) that 80% of imbibition occurs within the first 12 minutes and this involves uptake of water into the seed coat rather than the seed, while the remaining imbibition occurs more slowly and is into the seed tissue.

There is then a peak in uptake at day 1 or 2 followed by a decline, then gradual increase again after day 4. The higher rates of uptake can be correlated with the periods of greatest transport into the cotyledons. The early peak in uptake occurs during rehydration of the tissue while the later, gradual increase in uptake is associated with cotyledonary expansion and vacuolation. Slower rates of uptake occurred during the period when maximum heterotrophic growth and presumably transport out of the cotyledons normally occur.

High levels of incorporation of methionine into total homogenate and 'soluble' protein occurred in both light and dark-grown tissue. It should be noted that for the last 3 days of the light-grown sequence and also the last few days of the dark-grown sequence it was not possible to accurately calculate the levels of uptake and incorporation. This was due to the fact that the tissue at these stages was highly pigmented (with chlorophyll in the light and carotenoids in the dark) and these pigments caused quenching of the radioactivity during the counting of the uptake samples. Unfortunately, no steps were taken to counteract this problem. Quenching was not a problem in

the measurement of incorporation since the washing procedure eliminated these pigments.

It is not possible to use these levels of incorporation as an indication of the rates of protein synthesis occurring since no estimation was made of the levels of free methionine or of the occurrence of different pools within the tissue. Rapid changes in the amounts of free methionine *in vivo* would be anticipated, particularly during the mobilisation of storage proteins, during heterotrophic growth, as this results in the production of free amino acids.

Samples of both total homogenate and 'soluble' cotyledonary protein were fractionated by SDS-polyacrylamide gel electrophoresis and then analysed by fluorography to detect the *in vivo* synthesised polypeptides. Gels were loaded with equivalent amounts of protein on a per cotyledon basis (Figure 54) or by loading equal counts (Figure 55). The level of radioactivity incorporated into protein at day 0 was very low and therefore no sample from this stage was loaded on the equal counts gel.

During imbibition there is apparently a low level of protein synthesis occurring in the tissue. These preliminary results suggest that, after imbibition, qualitative and most probably quantitative changes in the polypeptides synthesised *in vivo* occur.

A number of polypeptides can be seen to be synthesised predominantly during the heterotrophic growth phase, though their synthesis continues longer in the dark-grown tissue. The developmental profiles and molecular

Figure 54

Analysis of *In Vivo* Labelled Polypeptides by SDS-Polyacrylamide Gel Electrophoresis and Fluorography. I Gels Loaded with Equivalent Amounts of Protein on a Per Cotyledon Basis.

Cucumber cotyledons were labelled as described in Table 8. Total homogenate and 'soluble' proteins from light and dark-grown cucumber cotyledons were fractionated on SDS-polyacrylamide gels (15% w/v) as described (p. 86). The *in vivo* labelled polypeptides were visualised by fluorography (p. 118). Gels were loaded with protein as indicated.

A. Homogenate protein - light-grown; equivalent to 1·67% of cotyledonary protein.

B. Homogenate protein - dark-grown; equivalent to 1·67% of cotyledonary protein.

C. 'Soluble' protein - light-grown; equivalent to 2·5% of cotyledonary protein.

D. 'Soluble' protein - dark-grown; equivalent to 2·5% of cotyledonary protein.

FIGURE 54.

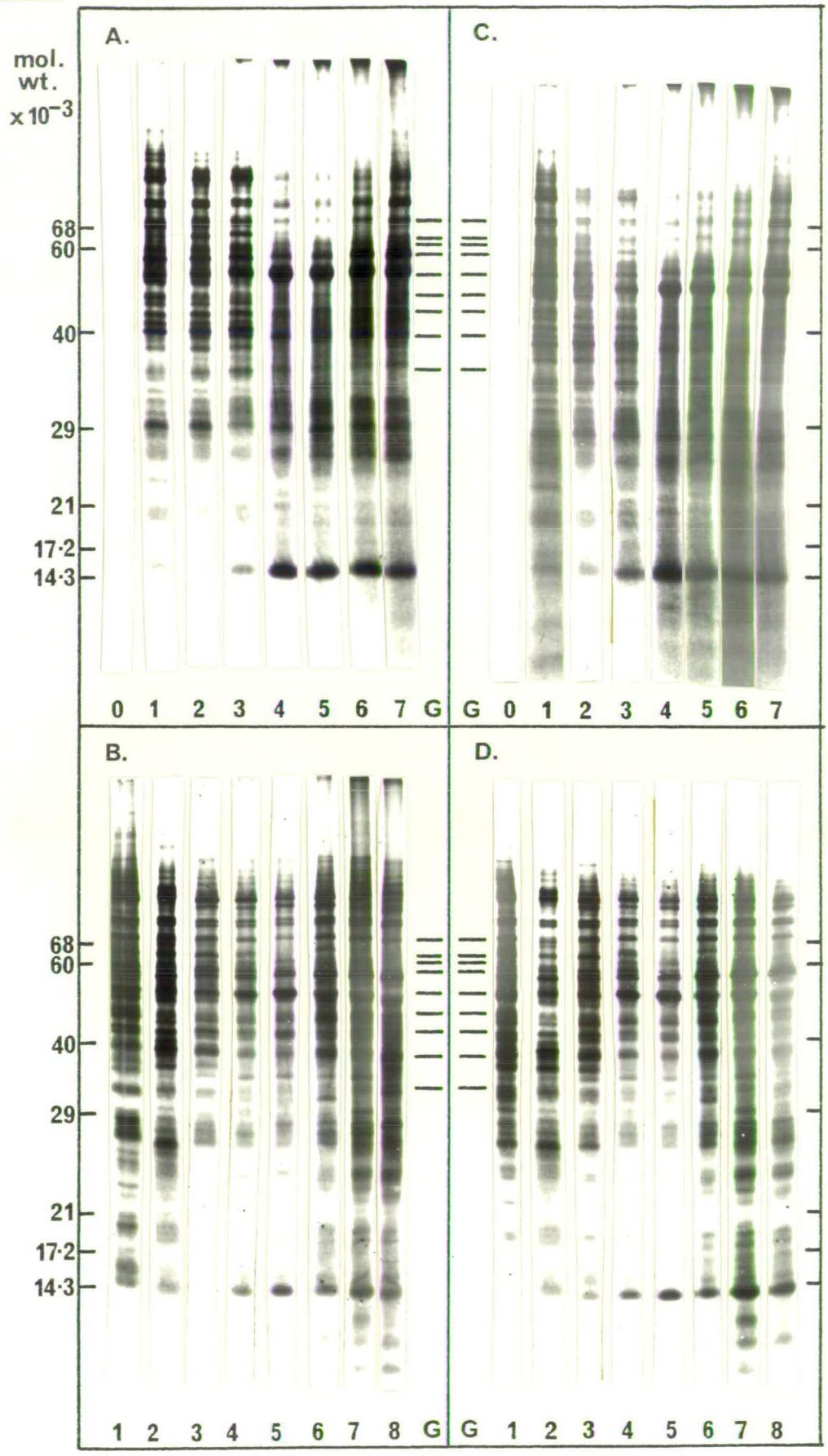

A.
C.
B.
D.
mol. wt. x10⁻³
68
60
40
29
21
17·2
14·3
0 1 2 3 4 5 6 7 G
G 0 1 2 3 4 5 6 7
1 2 3 4 5 6 7 8 G
G 1 2 3 4 5 6 7 8

Figure 55

Analysis of *In Vivo* Labelled Polypeptides by SDS-Polyacrylamide Gel Electrophoresis and Fluorography. II Gels Loaded with Equal Levels of Radioactivity.

Cucumber cotyledons were labelled as described in Table 8. Total homogenate and 'soluble' proteins from light and dark-grown cucumber cotyledons were fractionated on SDS-polyacrylamide gels (15% w/v) as described (p. 86). The *in vivo* labelled polypeptides were visualised by fluorography of the gels (p.118). Gels were loaded with approximately 19,000 cpm for total homogenate samples and 15,000 cpm for 'soluble' protein samples.

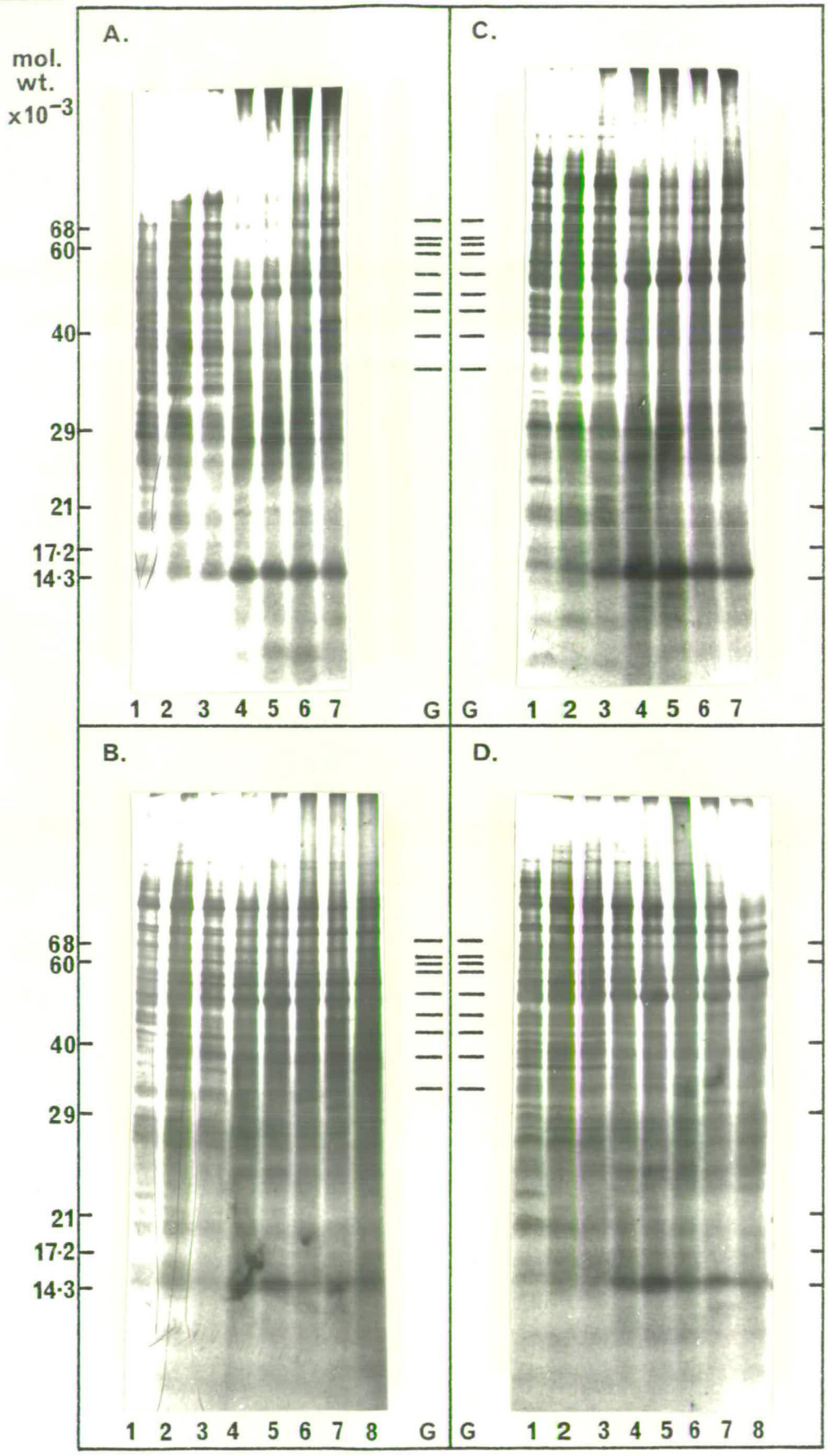
A. Homogenate protein - light-grown.

B. Homogenate protein - dark-grown.

C. 'Soluble' protein - light-grown.

D. 'Soluble' protein - dark-grown.

FIGURE 55.


A.
C.
B.
D.
mol. wt. x10⁻³
68
60
40
29
21
17·2
14·3
1 2 3 4 5 6 7 G
G 1 2 3 4 5 6 7
1 2 3 4 5 6 7 8 G
G 1 2 3 4 5 6 7 8
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weights (40,000 - >70,000 mol. wt.) of these polypeptides correspond to similar changes observed in the stained protein gels (see Figure 7). It was anticipated that this group may include the subunits of the glyoxysomal enzymes, particularly of ICL and MS.

Certain polypeptides, including the LSU and SSU of RuBPC, are synthesised during the period of aquisition of the autotrophic function of the cotyledons. It can be seen from Figure 54 that the accumulation of both the LSU (53,000 mol. wt.) and the SSU (13,000 mol. wt.) of RuBPC is apparently light regulated. The synthesis of a polypeptide of approximately 26,000 mol. wt. is completely light dependent, being synthesised only in the light-grown tissue. This polypeptide, which is a membrane component, is probably the chlorophyll a/b binding protein, the synthesis of which is known to be light dependent (Apel and Kloppstech, 1978).

5.3. In Vivo Synthesis of ICL and MS

Due to the large number of polypeptides synthesised *in vivo* during each 24 hour labelling period it was not possible to positively identify the subunits of ICL and MS by co-migration with glyoxysomal markers. Therefore, immunoprecipitations were carried out on samples of the *in vivo* labelled homogenates. The homogenates were incubated with 0·2 M KCl for 1 hour then the 'soluble' proteins obtained by at 105,400 $g_{av.}$ (p. 122). It was necessary to treat the samples with KCl to release the MS which is normally loosely bound to the inner membrane of the glyoxysomes (Bieglmayer *et al.*, 1974; Köller and Kindl, 1977). The resulting immuno-

precipitates (Figure 56) were analysed by SDS-polyacrylamide gel electrophoresis and fluorography.

In vivo synthesised ICL and MS subunits were not detected in the day 0 sample. Synthesis of these particular polypeptides increases markedly after day 0, with high levels of *in vivo* synthesised polypeptides detectable at days 1 and 2. By day 3 there is a decline in the amounts of detectable *in vivo* synthesised polypeptides and after day 4 in the light there is no detectable synthesis of these enzymes. In the dark-grown tissue there is also a decrease in the amount of synthesis of these polypeptides in the later developmental stages though this decrease was less marked than in the light.

It should be noted that only the 63,000 mol. wt. subunit of ICL was detectable in this experiment. This is due to the fact that the conditions of the experiment were such that an excess of antigen to antibody was used. H. Riezman (personal communication) has shown that the 61,500 mol. wt. subunit is not detected in antigen excess but only in antibody excess or at the equivalence point. The polypeptide seen at approximately 52,000 mol. wt. is a degradation product of ICL (Riezman - personal communication).

By scanning the fluorographs it was possible to determine the relative intensities of the immunoprecipitates at the different developmental stages. Thus, knowing the amount of protein present in the samples from which the immunoprecipitates were made, the amount of protein per

Figure 56

Developmental Changes in In Vivo Synthesis of ICL and MS Polypeptides

The in vivo labelled polypeptides shown in Figures 54 and 55 were analysed for the presence of ICL (A) and MS (B) polypeptides by double antibody immunoprecipitation (P. 116). The immunoprecipitated polypeptides were analysed by SDS-polyacrylamide gel electrophoresis and fluorography. Purified glyoxysomal proteins (G) were also fractionated on the gels and the positions of the stained proteins are as indicated.

FIGURE 56.

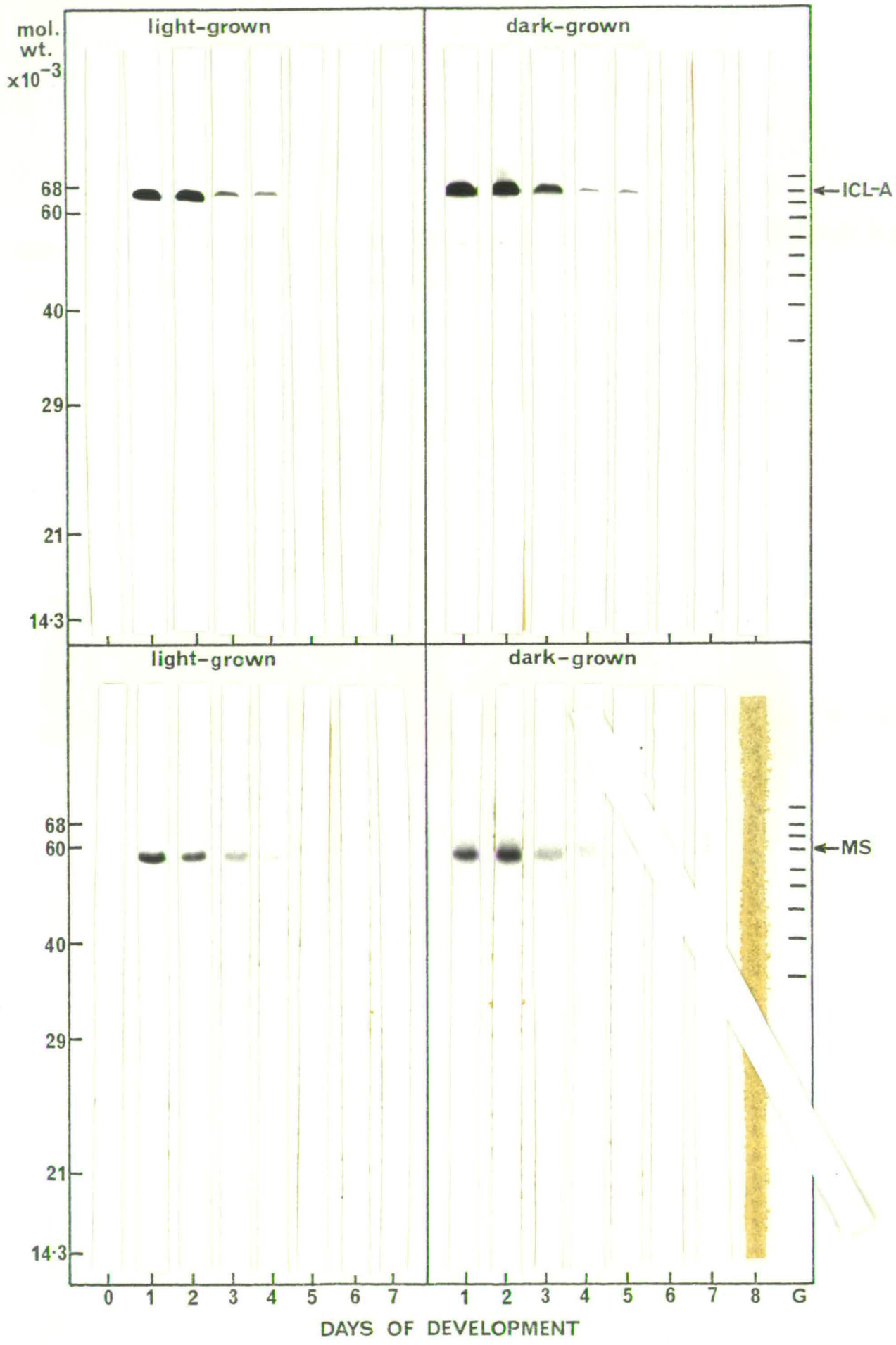

mol. wt. x10⁻³
light-grown
dark-grown
68
60
40
29
21
14·3
←ICL-A
light-grown
dark-grown
68
60
40
29
21
14·3
←MS
0 1 2 3 4 5 6 7
1 2 3 4 5 6 7 8 G
DAYS OF DEVELOPMENT

Figure 57

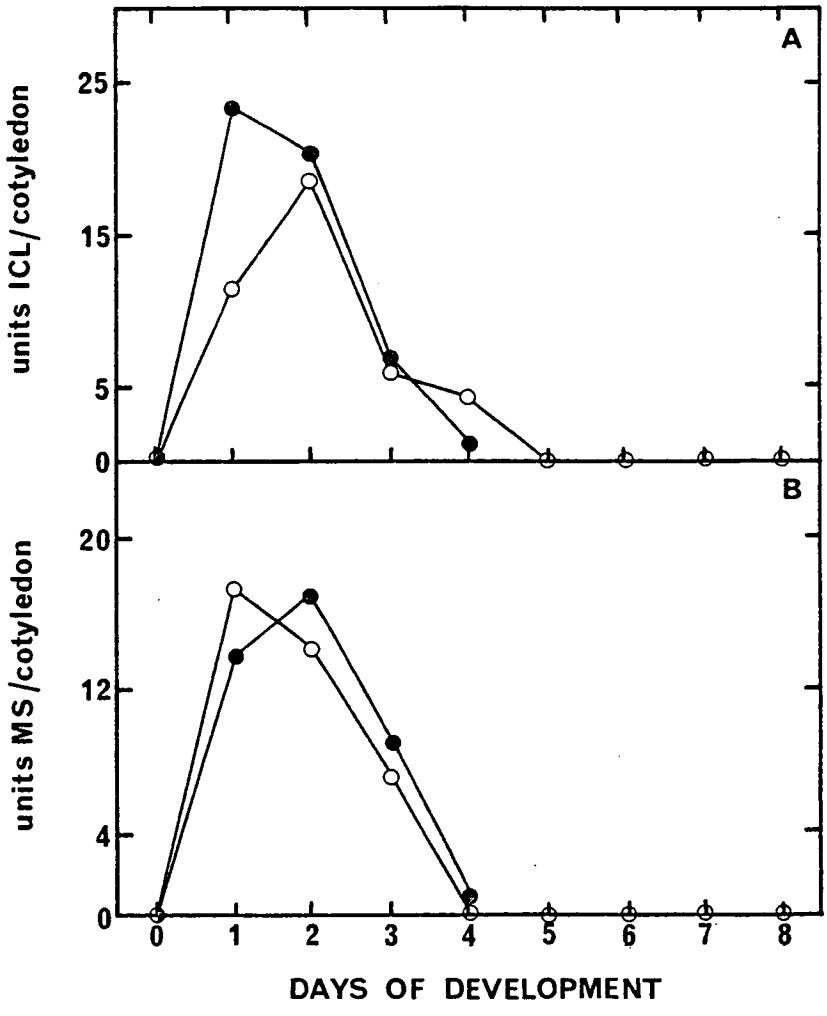
Preliminary Estimation of Quantitative Changes in In Vivo Synthesis of ICL and MS Polypeptides during Germination and Early Seedling Development

The relative intensities of the immunoprecipitates shown in Figure 56 were determined using a Quick Scan R & D (Helena Laboratories) densitometer. Using these relative intensities, the amount of total protein used for the immunoassay, the protein content per cotyledon (p. 137) and the differential uptake of [^{35}S]-methionine into the cotyledons, an estimate of the amounts of ICL and MS synthesised during the 24 hours labelling period was made.

$$\text{amount of protein synthesised} = \frac{\text{relative intensity of labelled immunoprecipitates}}{\text{amount of total protein used for immunoassay}} \times \frac{\text{amount of total protein/cotyledon}}{\text{\% uptake of label by cotyledons}}$$

1 unit of ICL or MS was taken to be the amount of labelled polypeptides recovered after the 24 hour labelling period between days 3 and 4.

FIGURE 57.


A
B
units ICL/cotyledon
units MS/cotyledon
25
15
5
0
20
12
4
0
0
1
2
3
4
5
6
7
8
DAYS OF DEVELOPMENT

cotyledon at each day of development and the different rates of uptake of label (Table 8), it was possible to make a very preliminary estimate of the relative amounts of these enzymes synthesised during the labelling period (Figure 57).

It should be noted that, since the pool size of free amino acids present in the cotyledons was not determined, these estimations contain a high 'error' factor when considering rates of synthesis. However, they do give an indication of some of the changes occurring.

It can be seen from these estimations that synthesis of these enzymes probably begins after imbibition. However, there may be low levels of synthesis which are undetectable by this technique. Certainly, after imbibition there is a marked increase in the rate of synthesis of ICL and MS with peak values probably between days 1 and 2. Thereafter, there is a decline in the rate of synthesis of these two enzymes. It was not possible to estimate the amounts of these polypeptides synthesised in the later stages of the dark-grown system as the darkening of the X-ray film by the labelled immunoprecipitates was too weak to be detected by the scanner. However, a study of the fluorographs indicates that low levels of synthesis are still occurring at this period (see Figure 56).

5.4. Summary

Pulse labelling experiments were used to study the changes in *in vivo* protein synthesis in the cucumber cotyledon during germination and early seedling development. Quantitative and qualitative changes in the proteins synthe-

sised were observed, and some of these changes could be correlated to the developmental changes in function of the cotyledons.

In vivo synthesis of ICL and MS was determined by immunoprecipitation of the total pulse labelled protein, followed by SDS-polyacrylamide gel electrophoresis and fluorography. Synthesis of these enzymes was shown to occur predominantly in the first 48 hours after imbibition then decline markedly as growth proceeds. Low rates of synthesis are believed to continue in the dark-grown tissue, but it was not possible to quantitate this.

CHAPTER 4

D I S C U S S I O N

SECTION 1

DEVELOPMENTAL CHANGES IN COTYLEDONARY PROTEIN AND ENZYME ACTIVITIES

1.1. Physiological Changes

The germination and early seedling development of cucumber seedlings was characterised in terms of the physiological and biochemical changes occurring within the cotyledons which could be used as indicators of the heterotrophic and autotrophic phases of this developmental transition.

Although the seeds were grown under controlled environment conditions, it should be noted that slight variations in the rates of germination and growth were observed. The reason for this variation was two-fold. Firstly, different growth chambers were used on occasion and so slight temperature differences may have affected the developmental profiles. Secondly, with different batches of seed, the rate of germination and growth was seen to vary slightly. In order to minimise the effect these differences may have had upon the molecular and biochemical changes occurring, seedlings were harvested to fit as closely as possible to the morphological stages illustrated (Figure 1). In retrospect, it may have been more applicable to designate developmental stages according to glyoxysomal enzyme activity within the tissue, since variations in the biochemical and molecular markers need not necessarily reflect morphological changes. However, variations appeared to be slight and it is assumed that they did not invalidate the basis of this thesis.

The morphological profiles of cucumber seedling devel-

opment (Figure 1) show that light is essential for inhibition of hypocotyl extension, loss of the hypocotyl hook and for cotyledonary expansion and greening.

Determination of the cell number of the cotyledons (Table 3) showed that no significant change in the cell number occurs during this period. This agrees with similar findings for cotyledons of squash and mustard seedlings (Weidner, 1967; Lott, 1970) and also with ultrastructural studies of cucumber cotyledons (Trelease et al., 1971A). The absence of cell division means that the developmental events involved in the transition from dormancy to heterotrophic and finally autotrophic growth are not complicated by superimposed cell division events. Since cell number remains constant, data were expressed on a per cotyledon basis and later used to give an indication of molecular and subcellular differentiation in an individual cell (p. 327).

Although cell number remains constant, it is clear that dramatic changes occur in the fresh and dry weights of the cotyledons (Figure 2). Decreases in dry weight during the early days of growth reflect utilisation of food reserves for growth of the axis while the later increase in the light-grown tissue is due to the ability of the tissue to accumulate mass by photosynthesis. It is clear that the increase in fresh weight of the cotyledons is due almost entirely to water uptake associated with vacuolation of the tissue. Vigil (1970) and Trelease et al. (1971A) have shown by ultrastructural studies that in the later stages of this developmental transition (i.e. day 5 onward), the cotyledonary cells

become highly vacuolated. Lott (1970), in a study of squash, observed similar changes in fresh and dry weights of the cotyledons to those observed in cucumber. He also found that by day 14, when the cotyledons were starting to senesce, the dry weight again started to decline.

1.2. Heterotrophic Metabolism

The early stages of this developmental sequence involve heterotrophic growth of the seedling. Gluconeogenic mobilisation of the lipids stored in the cotyledons is a prominent feature of this metabolism. It can be seen (Figure 3) that the lipid content of the cotyledons decreases markedly up until day 6. This observation agrees with the progressive loss of lipid bodies (spherosomes) reported in sunflower and cucumber cotyledons (Gruber et al., 1970; Trelease et al., 1971A) and castor bean endosperm (Vigil, 1970).

The activities of the glyoxylate cycle enzymes, ICL and MS (Figure 3A and B), were found to increase markedly to peak values at day 4, and subsequently decline, though in the dark-grown tissue a slower rate of decrease was observed. Peak activities of these enzymes corresponded closely to the period of maximum decline in stored lipid. Unfortunately, the lipid content of dark-grown tissue was not determined. However, it is possible that continued, though reduced, activities of ICL and MS relate to the fact that the tissue does not become autotrophic and therefore is dependent upon maintained utilisation of the cotyledonary reserves. Similar changes in the activities of these two enzymes have been observed in the endosperm of castor bean (Gerhardt and Beevers, 1970; Huang and Beevers, 1974) and

the cotyledons of watermelon (Hock and Beevers, 1966; Kagawa et al., 1973), sunflower (Schnarrenberger et al., 1971) and peanut (Longo and Longo, 1970A). According to recent work on Cucumis species (Kerley and Becker - personal communication; Ford et al., 1976; Slack et al., 1977), these developmental changes can be observed in cotyledons which have been excised from the embryonic axis , suggesting that the axis does not directly regulate these changes in enzyme activity. However, as was previously mentioned in the Introduction (p. 24), the axis is necessary for maximum lipid mobilisation since it acts as a sink for the hydrolysis products. The developmental profiles of ICL and MS reflect their function, as part of the glyoxylate cycle, in the mobilisation of stored lipid.

The activity profile of CAT in the cotyledons is, at the early stages of growth, similar to those for ICL and MS (Figure 3C). However, since CAT is present in peroxisomes as well as glyoxysomes (Huang and Beevers, 1971; Beevers, 1979), this enzyme activity persists, albeit at reduced levels, in the photosynthetic tissue. The role of CAT within the tissue is the detoxification of H_2O_2, a byproduct of β-oxidation in glyoxysomes (Cooper and Beevers, 1969) and a product of photorespiration in peroxisomes.

As well as lipid mobilisation during this phase, the protein reserves of the tissue are also degraded and mobilised for seedling growth. Protein bodies have been shown to be prominent ultrastructural features in squash (Lott and Vollmer, 1973) and cucumber (Trelease et al., 1971A) cotyledons during early stages of development and decline

markedly as growth proceeds. This decline in protein bodies is mirrored by a decrease in the total protein content of the cotyledonary tissue, which continues until day 6 (Figure 6A).

While the total protein of the tissue decreases during this developmental period, the amount of 'soluble' protein (Figure 6B) increases markedly, particularly between days 2 and 3. The increasing proportion of 'soluble' protein (Figure 6C) is due to solubilisation of stored protein reserves, prior to utilisation, and also to the increase in enzyme proteins.

Solubilisation of the protein reserves is further documented in the SDS-polyacrylamide gels of homogenate and 'soluble' proteins (Figure 7). Initially a large percentage of the protein is in particulate form, only being present in six major polypeptides bands in the total protein gels (20,000-35,000 mol. wt. and 55,000 mol wt.). These polypeptides decline at the same time as a cluster of four to five prominent polypeptides of lower molecular weight appear on the 'soluble' protein gels (20,000-25,000 mol. wt.) i.e. at day 2. The slower mobilisation of these soluble polypeptides in the dark-grown tissue is presumably due to the reduced growth rate of the seedling in the dark. Confirmation that this group of polypeptides arises from solubilisation of the storage proteins and not from *de novo* synthesis can be seen from the profiles of *in vivo* synthesised polypeptides illustrated in Figures 54 and 55. Since this group of polypeptides appear rapidly at days 2 and 3

and constitute a large proportion of the total protein (Figure 7), it would be anticipated that if they arose by de novo synthesis, they would be a prominent component of the in vivo labelled polypeptides. However, no such prominent polypeptides are synthesised between days 1 and 3. The disappearance of these hydrolysis intermediates is due to complete degradation of the proteins to amino acids, which are subsequently re-utilised for synthesis of new proteins, mainly after transport to the axis, but also possibly to a certain extent in the cotyledons.

The increased metabolism of the cotyledonary tissue upon germination and growth of the seedling is reflected by the appearance of new polypeptides in the tissue. A number of polypeptides in the 45,000-75,000 mol. wt. range shows developmental profiles similar to the profiles of enzyme activity previously observed for ICL and MS. They are detectable on the gels (particularly in the 'soluble' fraction) at day 2, peak shortly afterwards and thereafter decrease in prominence, though possibly persist longer in the dark-grown tissue. Such a profile would be anticipated for proteins solely involved in the heterotrophic growth phase.

It is known that several of the glyoxysomal enzymes have subunit molecular weights within this range (p. 142 ; Köller and Kindl, 1977; Lamb et al., 1978; Riezman et al., in press). In order to emphasise that this group of polypeptides possibly includes the glyoxysomal enzymes, an SDS-polyacrylamide gel of the peak microbody fractions (1·26

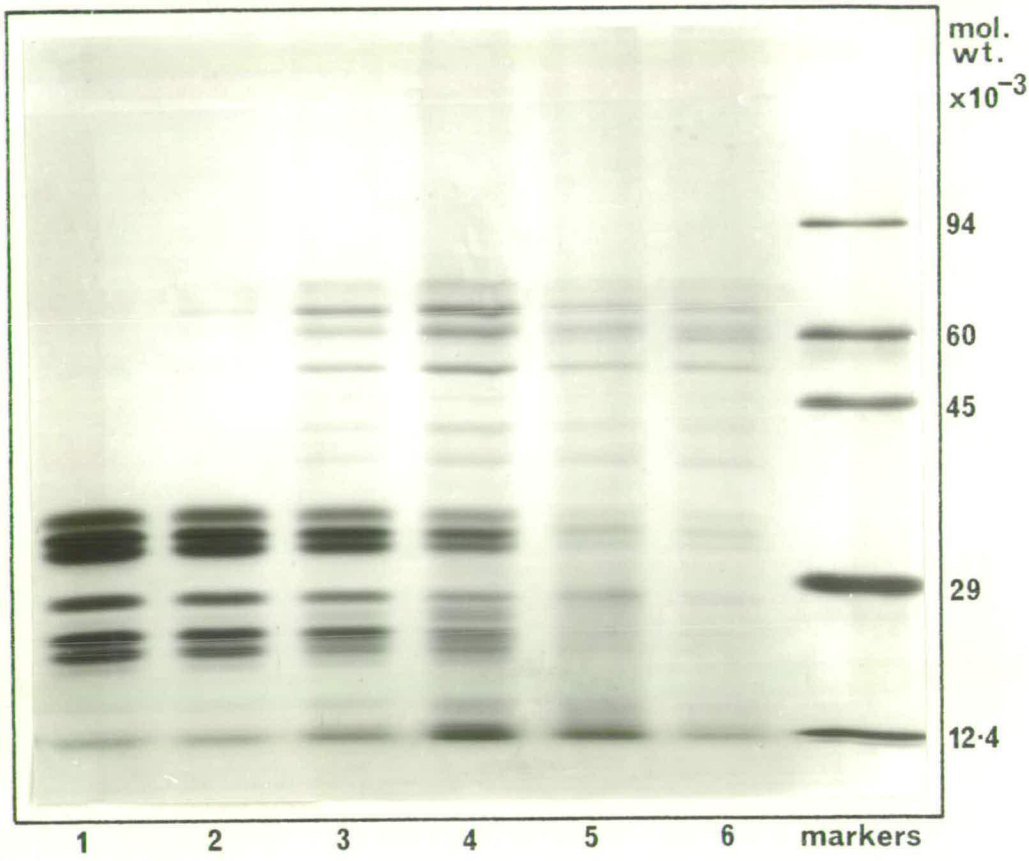
g/ml density) of sucrose density gradients for light-grown cucumber cotyledons is shown in Figure 58 (courtesy of H. Riezman). This gel was loaded on a per-cotyledon basis. Initially the most prominent polypeptides are detected in the 20,000-35,000 mol. wt. region. These are known to be the storage proteins which co-migrate with the microbody fraction on the gradients. Their recovery in this region confirms their presence in the tissue in particulate form at these early stages. Subsequent solubilisation and mobilisation of these storage proteins is reflected by their disappearance from the microbody fraction and the appearance of the solubilised proteins at the top of the gradient.

Of more particular interest to this discussion is the appearance of the higher molecular weight polypeptides of the glyoxysomes. The developmental profile of these polypeptides and their molecular weights correspond closely to the previously observed changes in the total and 'soluble' proteins (Figure 7) and also to the enzyme activities of ICL and MS (Figure 3). Similar changes in some polypeptides in this molecular weight range were observed on the two-dimensional gels of 'soluble' protein from light-grown tissue (Figure 8).

The extent to which the amino acids, produced by proteolytic degradation of the storage proteins, are transported to the growing apices or utilised within the cotyledons is unknown. However, from the results of the *in vivo* labelling of excised cotyledons (Table 8; Figures

FIGURE 58.

SDS-Polyacrylamide Gel of Protein from the Peak Microbody Fractions of Sucrose Density Gradients from Light-Grown Cucumber Cotyledons



54 and 55) it is obvious that considerable de novo protein synthesis occurs within the cotyledons and this probably involves utilisation of some of these hydrolysis products prior to the tissue becoming fully autotrophic.

During imbibition the level of in vivo protein synthesis was very low - as determined by the level of incorporation of [^{35}S]-methionine into hot, TCA-precipitable material (Table 8) and also by analysis of the polypeptides synthesised. However, it is clear that by day 1, and thereafter, protein synthesis increases markedly and that both membrane and 'soluble' proteins are synthesised. Numerous polypeptides are apparently synthesised de novo at the onset of the heterotrophic growth phase. In particular, a group in the 40,000-70,000 mol. wt. region show developmental profiles and molecular weights similar to the heterotrophically related polypeptides observed in the stained gels. This suggests that many of the proteins involved in heterotrophic growth may be synthesised de novo. Evidence supporting the proposal that the glyoxysomal enzyme activity increases are a result of de novo synthesis come from inhibitor studies (Hock and Beevers, 1966; Ihle and Dure, 1972; Smith et al., 1974; Tester, 1976) and density labelling (Gientka-Rychter and Cherry, 1968; Longo, 1968; Quail and Scandalios, 1971; Walk and Hock, 1977) and pulse labelling experiments (Bowden and Lord, 1976B and 1977). The results outlined above for cucumber cotyledons are in agreement with the proposal that de novo synthesis is responsible for the increases in glyoxysomal enzyme activities. Loss of enzyme activity is associated with loss of enzyme protein

(Figure 58) and this is apparently light dependent. The maintenance of enzyme activities in the dark-grown tissue may be due to continued synthesis or suppressed degradation of the enzyme proteins.

1.3. Autotrophic Metabolism

The synthesis and accumulation of chlorophyll and RuBPC protein and the increase in glyoxylate reductase activity, shown in Figure 4, are indicative of the transition to the autotrophic growth phase of the tissue. The synthesis of chlorophyll within the tissue is apparently light dependent and is closely associated with the emergence of the cotyledons from the seed coat and synthesis of chloroplast membrane proteins (see Figure 7 and p. 275).

Since the initial synthesis of RuBPC protein precedes that of chlorophyll by at least 24 hours, the initiation of its synthesis does not depend upon light as a trigger. However, after day 4, the continued accumulation of this enzyme protein is light dependent. This finding agrees well with the report of Docherty *et al.* (1977) that RuBPC development in castor bean endosperm and cotyledons is not light dependent.

The increase in glyoxylate reductase activity (Figure 4C) showed a similar light requirement to that found for RuBPC protein, though the initial synthesis occurs at the same time as chlorophyll synthesis is initiated and some 24 hours after RuBPC protein is first detectable. The level of activity is much lower in dark-grown than light-grown tissue. Glyoxylate reductase is a peroxisomal marker enzyme

and it has been shown by other workers that reduced increases in activity are detectable in dark-grown tissue (Schnarrenberger et al., 1971; Gerhardt, 1973, 1974; Schopfer et al., 1975, 1976; Theimer et al., 1976), although its activity is known to be ultimately under phytochrome control (Kagawa and Beevers, 1975).

Thus, during the period when the stored fat and protein of the cotyledons are being rapidly depleted and the activities of the glyoxylate cycle enzymes are decreasing, three specific markers of autotrophic metabolism become detectable. It would appear that the initial synthesis of RuBPC and GR, at characteristically different times, are already programmed into the tissue and thus do not require any environmental stimulus apart from hydration of the tissue and the onset of germination and seedling growth. On the other hand, chlorophyll synthesis is dependent upon light, which the cotyledons are exposed to when they emerge from the seed coat.

In terms of quantitative changes in the total protein of the tissue, the slight increase observed between days 6 and 7 (Figure 6A) is presumably due to the acquisition of photosynthetic function since no similar increase is detectable in the dark. The slower decrease in the 'soluble' protein fraction between days 4 and 6 may be attributed to a slower rate of utilisation of solubilised storage protein. This is also observed in the gel profiles shown in Figure 7. The reason for this is unknown, but it does indicate that light may play some role in the final utilisation of the storage products. Unfortunately, since no measurement of the lipid

content of the dark-grown tissue was made, I do not know if a similar reduced rate of utilisation occurs for this storage reserve.

Also observed on the denaturing protein gels (Figure 7) are numerous polypeptides, both 'soluble' and membrane associated, which appear and increase at the time when the autotrophic capacity of the tissue is developing. From the *in vivo* labelling study it is clear that many of these proteins are synthesised *de novo* during this period.

The most prominent of the autotrophically related polypeptides are the large and small subunits of RuBPC (53,000 and 13,000 mol. wt.) which show similar developmental profiles to the native protein (Figure 48). One other particularly prominent polypeptide is that of approximately 26,000 mol. wt. which is apparently a membrane protein. The synthesis and accumulation of this polypeptide is light dependent (Figures 7, 54 and 55) and it is probably the chlorophyll a/b binding protein (Apel and Kloppstech, 1978). Other less prominent and unidentified polypeptides also appear in the gel profiles associated with the onset of the photosynthetic function of the cotyledons and are probably components of the phososystems.

1.4. Summary

From this study of the biochemical changes occurring during germination and early seedling development, it is clear that the metabolic activity of the tissue increases markedly soon after imbibition and, thereafter, changes in the metabolic state are associated with the changing function

of the cotyledonary tissue. Protein synthesis is low during imbibition and thereafter increases markedly to support this increase in metabolic activity.

It is probable that the increases in activity of the glyoxysomal enzymes, ICL, MS and CAT, and possibly other hetrotrophically related proteins are a result of de novo synthesis. The developmental changes in the glyoxysomal enzymes apparently require no environmental stimulus until the latter stages of this transition, when light affects the final decline in activity of ICL and MS. This light effect is most likely to be due to a continued requirement for utilisation of storage reserves as a result of the lack in photosynthetic ability, rather than a direct effect.

From the developmental profiles of autotrophically related proteins (Figures 4 and 7) it would appear that the initial development of some of these (e.g. RuBPC and GR) only requires the onset of germination and growth for initiation while the initiation of synthesis of other components of this growth phase (e.g. chlorophyll and chlorophyll a/b binding protein) depend upon a light stimulus. However, full development of all autotrophic functions is light dependent.

SECTION 2

DEVELOPMENTAL CHANGES IN THE PROTEIN SYNTHETIC APPARATUS OF CUCUMBER COTYLEDONS

It has been shown that protein synthesis within the cucumber cotyledonary tissue increases markedly with the resumption of metabolic activity of the seed upon germination and early seedling growth. Many proteins are synthesised in the tissue during the hetrotrophic growth phase, particularly those of the glyoxysomes, which are probably synthesised *de novo*. As development proceeds, there is a change in gene expression associated with the transition from heterotrophic to autotrophic function of the cotyledons, resulting in a different spectrum of proteins being synthesised.

To accommodate first the increase and later the change in gene expression, it is anticipated that an increase in the cellular protein synthetic apparatus would occur. Thus, developmentally related changes in the RNA and polysomes were investigated.

2.1. RNA

At the onset of germination there was found to be a low, though measurable amount of RNA present in the cotyledons (Figure 9A). Analysis of this RNA by polyacrylamide gel electrophoresis showed this to consist of cytoplasmic rRNA and low molecular weight (LMW) RNA (Figures 10 and 11). During imbibition there was found to be a slight decline in the phenol-detergent extractable nucleic acid (p. 147), suggesting that degradation of RNA may occur in the tissue at this time.

However, it is possible that synthesis is also occurring, but at a slower rate than degradation, resulting in an overall decline in the amount of RNA in the tissue.

It is known that during maturation and dessication of seeds there is a decline in the RNA content of the tissues (Beevers and Poulson, 1972; Dure, 1975). However, low levels of RNA are detectable in dry seed tissues (Ingle, 1968B; Dure, 1975; Grierson and Covey, 1975; Marriot and Northcote, 1976; Gordon and Payne, 1976; Giles et al., 1977; Osborne, 1977; Roberts and Lord, 1979A and B). It has been shown that both protein and RNA synthesis are early events during germination (for reviews see Payne, 1976; Osborne, 1977) and that protein synthesis may be initiated first (review Dure, 1977). It has been difficult, though, to establish exactly when RNA synthesis is initiated. From a study of pea embryos (Robinson and Bryant, 1975), bean leaves (Grierson and Covey, 1975) and castor bean endosperm (Roberts and Lord, 1979A) during seed germination, it has been proposed that degradation of pre-existing RNA and synthesis of new RNA are early events occurring concurrently during imbibition and germination.

After imbibition (day 0) there was found to be an increase in the total cellular RNA of the cotyledons (Figure 9A), which was particularly marked between days 2 and 4 in the light. In the last 2 days of the developmental sequence there is a levelling off of the RNA content. However, in the dark-grown tissue, there is a slower rate of accumulation and lower final level of RNA than in light-

grown tissue. A similar profile of RNA changes was observed in radish cotyledons by Ingle (1968B). Roberts and Lord (1979A and B) have shown that in the non-greening castor bean endosperm there is a marked increase in the cellular RNA to peak levels at day 3 followed by a decline to day 6. This suggests that in the cucumber cotyledon, increases in the cellular RNA after day 3 may be due to a requirement for continued protein synthesis to support the change in gene expression necessary for acquisition of photosynthetic function by the tissue.

The increases observed in the cellular RNA of the cotyledons is due mainly to accumulation of rRNAs and LMW RNA (Figure 11). Both cytoplasmic rRNA and LMW RNA are present in the imbibed tissue and increase dramatically during the predominantly heterotrophic growth phase (days 2-5) - 21fold and 7fold, in light, respectively. For each of these RNA species the initial synthesis and accumulation appear to be independent of light. However, light does affect the subsequent accumulation, since higher contents of both species are attained in the light-grown than in the dark-grown tissue. In radish cotyledons (Ingle, 1968B) and bean leaves (Grierson and Covey, 1975) similar light dependent increases in cytoplasmic rRNA were observed during germination and early seedling growth. Thien and Schopfer (1975A and B) have reported that synthesis of rRNA precursors and accumulation of cytoplasmic rRNA in mustard seedlings are under the control of the phytochrome system.

Chloroplast rRNA is absent from the tissue during the

early stages of this developmental sequence (Figure 10 and 11B). The synthesis and accumulation of this RNA species occur in close association with the development of the autotrophic function. As in the case of the chloroplast protein, RuBPC, the synthesis is initiated prior to day 3, and thus does not require a 'light trigger'. Light is, however, essential for continued accumulation of chloroplast rRNA. It has been reported that chloroplat rRNA may accumulate in dark-grown seedling tissue - radish (Ingle, 1965B) and mustard (Thein and Schopfer, 1975B) cotyledons and bean leaves (Grierson and Covey, 1975) - although phytochrome has been shown to control the accumulation of this RNA species (Thein and Shopfer, 1975B). Walden (1979) has shown that synthesis and accumulation of the mRNAs for the LSU and SSU of RuBPC correspond closely to the developmental profiles observed for chloroplast rRNA, which suggests that the development of chloroplasts and synthesis of their cytoplasmically derived proteins are closely correlated.

The profiles of synthesis and accumulation of chloroplast rRNA shown in this thesis correspond well with the plastid development observed from ultrastructural studies of cucumber cotyledons carried out by Trelease et al. (1971A). In light-grown tissue they observed that at day 2 proplastids were distinguishable but contained little inner membrane differentiation. By day 5, differentiation of the inner membrane had occurred and the chloroplasts were found in the periphery of the cell in close association with peroxisomes. During development in the dark little internal differentiation

occurred and the plastids contained distinct prolamellar bodies although they were still found to form close associations with microbodies.

These results suggest that cytoplasmic rRNA and LMW RNA synthesis begin early in this developmental sequence with net accumulation being obvious between days 1 and 5. However, without labelling the RNA during growth of the tissue it is not possible to determine exactly when cytoplasmic RNA synthesis is initiated. On the other hand it is clear that chloroplast rRNA synthesis is initiated by day 2 and subsequently a marked accumulation of this organellar RNA occurs. Light stimulates the accumulation of these RNA species but shows a greater effect on the chloroplast rRNA (Figure 11) which is in agreement with the results shown by Ingle (1968B).

From the marked increases in cellular RNA it is clear that the majority of protein synthesis for heterotrophic growth and possibly all the protein synthesis for autotrophic growth is dependent upon transcription of ribosomal RNA. Sen and Osborne (1977) have proposed that stored RNA may be utilised to support the initial increase in metabolic activity but that subsequent protein synthesis is dependent upon newly synthesised RNA. A more detailed study of protein synthesis during imbibition and germination would be required for an understanding of the sequence of events occurring in the cucumber cotyledons during this phase.

Early RNA synthesis within germinating seed tissue is consistent with the finding that dry seed tissues contain abundant supplies of RNA polymerase (Jendrisak and Becker,

1974; Payne, 1976 - review).

2.2. Polysomes

The amounts of extractable cotyledonary polysomes, which includes both polyribosomes and monoribosomes, were determined for each of the days of the developmental sequence under study.

Polysomes have been shown to decline in maturing and dessicating seed tissue, yielding monosomes and ribosomal subunits which persist in the dry seed (Payne and Boulter, 1969; Beevers and Poulson, 1972; Dure, 1975). Small amounts of extractable polysomes were present in the cucumber cotyledons after imbibition (Figure 12). Dr. Grienenberger (personal communication) analysed these by sucrose density gradient centrifugation and showed that they contained a high percentage of monosomes. Thereafter, there is an increase in the polysome content of the tissue, which is particularly marked between days 2 and 5, during the peak heterotrophic and early autotrophic growth phases. Concomitant with this increase was an increase in the proportion of higher size classes of polysomes (see Figure 42).

It is known that early germination of seeds is characterised by a rapid formation of polysomes (Weeks and Marcus, 1971). It has been shown that polysome formation (Waters and Dure, 1966; Barker and Rieber, 1967) and protein synthesis (Stoddart et al., 1973) may occur during the initial phase of germination even if RNA transcription is inhibited. By analogy, it is probable that the initial polysome formation in cucumber cotyledons involves utilisation

of pre-existing ribosomes. However, the marked increase in extractable polysomes noted as development proceeds is clearly dependent upon synthesis of new ribosomes.

These observations suggest that there is an overall increase in the protein synthetic machinery of the tissue. This increase is partially light dependent since increases do occur in the dark-grown tissue but not to the same extent as in the light. Since the reduced content of extractable polysomes in the dark reflects that observed for the rRNAs this suggests that light controls accumulation of protein synthetic machinery by affecting rRNA transcription.

From Figure 12C it was seen that there was an increase in the proportion of ribosomes actively involved in protein synthesis, which can be interpreted to mean that the rate of protein synthesis during the earlier developmental stages may be limited by some other factor than the availability of ribosomal subunits. This is in agreement with the observation that protein synthesis may occur *in vivo* when RNA synthesis is inhibited e.g. in cotton (Waters and Dure, 1966) and pea seeds (Barker and Rieker, 1967). This interpretation is based on the assumption that the recovery of polysomes is proportionally the same at all development stages.

Such a marked increase in the protein synthetic machinery, as well as involving synthesis of RNA, also requires synthesis of the ribosomal proteins. To accommodate the marked increase in polysomes, part of the early protein synthesis must be involved in the accumulation of these ribosomal proteins. Cuming and Lane (1979) have shown that some of the mRNAs

present in imbibing wheat embryos code for the ribosomal proteins.

Polysomes from light-grown tissue were analysed for their distribution into free and membrane-bound fractions (Figure 13). It was found that initially almost all were present in the free fraction. This could be due to the fact that monosomes uninvolved in protein synthesis do not remain attached to membranes (Payne and Boulter, 1969). Both fractions showed slight increases after imbibition followed by a marked increase between days 2 and 4 associated with the synthesis of both 'soluble' and membrane proteins at this time (Figures 7, 54 and 55).

The ratio of membrane-bound to free polysomes increased between days 2 and 3 (Figure 13B). This may be associated with the biogenesis of glyoxysomes (Gerhardt and Beevers, 1970; Kagawa and Beevers, 1975) and also the start of chloroplast and peroxisome development (Trelease et al., 1971A) in light-grown tissue. Many chloroplast polypeptides are known to be synthesised in the cytoplasm and although it has been shown that the SSU of RuBPC is synthesised on free ribosomes (Ellis et al., 1979) it is possible that other chloroplast proteins, particularly those of the membrane, are synthesised on membrane-bound polysomes. It is also likely that the proteins of microbodies (glyoxysomes and peroxisomes) are synthesised on membrane-bound ribosomes (Bowden and Lord, 1976A and B).

The observation that polysomes extracted from cucumber cotyledons were inefficient in stimulating in vitro protein

synthesis (p.204) led to the proposal that they became associated with RNAase during the extraction procedure (p.213). Preliminary experiments were carried out, the results of which agree with this proposal though do not prove it. Further experimentation is necessary to establish with certainty that the inhibitory factor was RNAase. However, it is to be anticipated that there may be an increase in RNAase during the early stages of germination, as it is one of a group of hydrolytic enzymes which are known to increase during this period (Chrispeels and Varner, 1967A and B). There are marked changes in the mRNAs present in the tissue at this time which are reflected in the profiles of *in vivo* synthesised polypeptides (Figures 54 and 55), and was shown by the *in vitro* translation assay (see p.192; Figures 31-33). High levels of RNAase may therefore be present to facilitate the turnover of the mRNA population (see p.302).

2.3. Summary

These results show that at the onset of germination there are pre-existing ribosomes present in the cotyledonary tissue. During the first few days of this developmental sequence there is a mobilisation of ribosomes into polysomes associated with the onset of protein synthesis within the tissue, though it cannot be determined from these results whether this involves utilisation of pre-existing or newly synthesised ribosomes. Subsequently there is a dramatic increase in the protein synthetic machinery of the tissue which reflects accumulation of the constituent RNAs. This increase occurs at a time when the enzymes involved in

heterotrophic growth were seen to increase to peak activities and also when chloroplast development is initiated. The continued accumulation of the RNAs, particularly those of the chloroplast, is light dependent.

SECTION 3

DEVELOPMENTAL CHANGES IN THE mRNA POPULATION OF CUCUMBER COTYLEDONS

Since the mRNA population is at most a few percent of the total nucleic acid, it was not possible to determine from a study of the total cellular RNA by normal analytical methods, how this species was changing during development. Therefore, in an attempt to estimate quantitative changes in the mRNA population, three different assay procedures were used. These were (1) isolation of poly(A)$^+$ RNA (p.158), (2) estimation of the poly(A) content of the total nucleic acid, poly(A)$^+$ RNA and poly(A)$^-$ RNA fractions and (3) the ability of the cotyledonary RNA to stimulate protein synthesis in a cell-free translation system derived from wheat-germ (p.192). Qualitative changes in the mRNA population were followed by SDS-polyacrylamide gel analysis of the polypeptides from the wheat-germ system when programmed with cotyledonary RNA.

3.1. Poly(A)$^+$ RNA

The discovery that many eukaryotic mRNAs contain a 3' poly(A) sequence led to the development of affinity chromatography procedures for isolating the poly(A)$^+$ RNA (Kates, 1970; Aviv and Leder, 1972; Firtel and Lodish, 1973). However, these techniques do have limitations as a means of assaying mRNAs since not all mRNA species contain this sequence e.g. histone mRNA (Greenberg and Perry, 1972; Sonenshein *et al.*, 1976) and only poly(A)$^+$ RNA with a poly(A) sequence of more than 10–30 nucleotides may be isolated by

affinity chromatography (Baulcombe, 1977; Cabada et al., 1977; Taylor, 1979). Therefore, affinity chromatography is a useful means of isolating and quantitating eukaryotic mRNA as long as these limitations are realised. In this thesis, oligo dT-cellulose chromatography (Aviv and Leder, 1972) was used for the isolation of poly(A)$^+$ RNA and from phenol-detergent extracted total nucleic acid (p. 95).

It has previously been shown by other workers that dry seed tissue contains few ribosomes actively involved in protein synthesis (see p.282). However, there is substantial evidence to suggest that low amounts of mRNA are present in dry seed tissue (Marcus and Feeley, 1966; Waters and Dure, 1966; Chen et al., 1968; Weeks and Marcus, 1971; Schultz et al., 1972; Spiegel and Marcus, 1975; Payne, 1976; Dure, 1977).

A relatively small amount of poly(A)$^+$ RNA was found to be present in the unimbibed (dry) cucumber seed tissue (Figure 15). Poly(A)$^+$ RNA has also been detected in other dry seed tissues e.g. rye embryos, broad bean, pea, rape seed (Gordon and Payne, 1976), wheat embryos (Brooker et al., 1977, 1978; Cuming and Lane, 1978, 1979), radish embryos (Delseny et al., 1977) and castor bean endosperm (Roberts and Lord, 1979A and B). During imbibition there was found to be a decline in the amount of extractable poly(A)$^+$ RNA in the cucumber cotyledons. This decline may be due either to a degradation of the mRNA or a reduction of the poly(A) sequence such that it can no longer be isolated by oligo dT-cellulose chromatography. Roberts and Lord (1979A) have

observed a similar decline in the amount of poly(A)$^+$ RNA in castor bean endosperm during this period. They have shown that although there is an overall decline in the total amount of this mRNA, synthesis of poly(A)$^+$ RNA also occurs during imbibition.

After imbibition there is a dramatic increase (approximately 21-fold) in the poly(A)$^+$ RNA of the cucumber cotyledons, to reach peak values at day 3 in the light and day 4 in the dark-grown tissue. This increase was approximately 2-fold greater than that observed for the cytoplasmic rRNA. It was earlier suggested that, during early germination, the rate of protein synthesis was not limited by the availability of rRNA. Since the accumulation of mRNA is greater than that of rRNA it is possible that the availability of mRNA may have been a limiting factor determining the rate of protein synthesis during this period. Another factor favouring this proposal is that accumulation of poly(A)$^+$ RNA precedes rRNA accumulation (c.f. Figures 15A and 11A). This observation is in agreement with the results obtained in castor bean endosperm by Roberts and Lord (1979A). After attaining peak levels on days 3 or 4 there is a marked decline in the poly(A)$^+$ RNA as development proceeds.

It has been shown in several germination systems that mRNA synthesis is an early event and probably precedes rRNA synthesis (Dobrzanska *et al.*, 1973; Van de Walle and Deltour, 1974; Sen *et al.*, 1975; Spiegel *et al.*, 1975; Osborne, 1977). However, it is uncertain exactly when mRNA synthesis resumes in germinating tissue though Dure (1977) points out

that it can be detected within a few hours of the onset of germination in most embryo tissues. It has been suggested that early polysome formation involves association of ribosomes with stored mRNA in the dry seed (Weeks and Marcus, 1971; Filimonov et al., 1977; Brooker et al., 1977). Spiegel and Marcus (1975) and Dure and Harris (1976) inhibited mRNA synthesis with cordycepin, in wheat and cotton respectively, and found that polysome formation still occurred during early germination. They concluded that de novo mRNA synthesis is not essential for the onset of germination.

The results obtained for the poly(A)$^+$ RNA of cucumber cotyledons suggest that although preformed mRNAs may be involved in the initial resumption of protein synthesis, the majority of protein synthesis, associated with the increase and change in gene expression during this developmental transition, is a result of utilisation of newly synthesised mRNA. From Figure 15B it can be seen that there is a marked increase in the poly(A)$^+$ RNA as a percentage of the total RNA during the heterotrophic and early autotrophic growth phases. As development proceeds this again declines.

The early light effect on the level of extractable poly(A)$^+$ RNA noted in Figure 15A is perhaps rather surprising. I would suggest that there are 3 possible factors which may have contributed to this. Firstly, experimental error may have occurred during the extraction procedure even although these results are an average of two experiments. Secondly, it is possible that the vermiculite and seed coats covering the cotyledons prior to day 3 may not

have been a complete light barrier. Thirdly, the lights in the growth room may have had a heating effect on the seed trays thus effectively accelerating development of the light-grown tissue. However, it is probable that the more rapid loss of this RNA fraction in the last 4 days in the dark may be a result of the lack of a 'light trigger'.

3.2. Poly(A) Content

The poly(A) content of the total nucleic acid, poly(A)$^+$ RNA and poly(A)$^-$ RNA was determined by [^{3}H]-polyU hybridisation (Bishop et al., 1974) as a means of estimating quantitative changes in the mRNA population of the cotyledons. However, in using this technique for this purpose there are two limitations which should be realised. It is known that the lengths of poly(A) vary considerably between mRNA species and that this sequence may shorten with the age of mRNA (Singer and Penman, 1973; Sheiness et al., 1975). Thus it provides an estimate of changes only in the average poly(A)$^+$ RNA. Secondly, Bishop et al. (1974) have shown that the polyU may hybridise with DNA to form ribonuclease resistant complexes. Thus, the measurements of poly(A) content of the total nucleic acid and poly(A)$^-$ RNA presented here may be slight overestimations since both fractions contained DNA.

However, one experiment was carried out in which the total nucleic acid samples were treated with DNAase prior to carrying out the hybridisation assay. The results obtained were almost identical, with the profile of developmental changes remaining the same.

Figure 16 illustrates the estimated amounts of poly(A)

in each of the three fractions. It was found that there was a considerable amount of poly(A) in the poly(A)$^-$ fraction. Since this fraction was defined by its inability to bind to the oligo dT-cellulose, this suggests that a significant proportion of the mRNAs of the cotyledons contain poly(A) sequences of less than 30 nucleotides (Cabada et al., 1977). This may be due to mRNAs which normally contain short poly(A) sequences or to rapid rates of turnover of mRNAs reflected in degradation of the poly(A) sequences.

It is clear that there is poly(A) in the cotyledons of the dry seed. This is in agreement with the fact that poly (A)$^+$ RNA may be extracted from the cotyledons of cucumber and other dry seed tissues (Grierson and Covey, 1975; Spiegel and Marcus, 1975; Gordon and Payne, 1976; Cuming and Lane, 1978; Roberts and Lord, 1979). During imbibition there was a decline in the poly(A) content suggesting that there is a decrease in the average length of poly(A) or that an overall degradation of poly(A) containing mRNAs is occurring.

After imbibition the total poly(A) content of the tissue (Figure 16A) increases markedly during the heterotrophic and early autotrophic growth phases, to reach peak levels at day 4 in the light, then declines to low levels as development proceeds. Light apparently stimulates this increase in poly(A) content since in the dark the peak values are only 44% of those in the light.

The early peak of poly(A) in the poly(A)$^+$ RNA (Figure 16B) from light-grown tissue suggests that this may be partly due to changes in the average length of poly(A)

since the amount of extractable poly(A)$^+$ RNA (Figure 15A) did not show such a difference between light and dark-grown tissue.

Figure 17 shows the poly(A) content expressed as a percentage of the RNA fraction from which it was estimated and gives an indication of changes in the average length of the poly(A) sequence. The results suggest that there is a general decrease in the average length of poly(A) as development proceeds with the possible exception of an increase soon after imbibition, though only in the light-grown tissue. Since this increase was not observed in the dark-grown tissue it obviously requires further investigation. There may also be a slower decrease in the average poly(A) length of poly(A)$^+$ RNA in dark-grown tissue in the later stages of this developmental transition, though this too calls for further investigation.

As an indication of the turnover of the poly(A) sequence the ratio of poly(A) in the poly(A)$^+$ RNA to poly(A) in the poly(A)$^-$ RNA was estimated (Figure 18). Following degradation of poly(A) during imbibition there would appear to be a synthesis which is more rapid in the light-grown tissue, peaking at day 2 compared to day 4 in the dark. Thereafter there is a degradation of the poly(A) which occurs later and at a slower rate in the dark-grown tissue.

These results show that there is a period of poly(A) synthesis and accumulation between days 0 and 4. This accumulation coincides with the phase of rRNA and poly(A)$^+$ RNA accumulation and with the periods of heterotrophic

growth and initiation of development of the photosynthetic function of the cotyledons. Thereafter the poly(A) content of the cotyledons declines. The synthesis and accumulation of poly(A) is apparently stimulated by light. Taken as an indication of the mRNA population, these results are in agreement with the earlier observation that low levels of mRNA are present in the unimbibed tissue and that there is an overall decline in this species during imbibition. Synthesis and accumulation of mRNA then occurs during the heterotrophic and early autotrophic phase associated with the increase and change in gene expression previously noted. As the autotrophic function of the cotyledons develops there is a decline in the mRNA of the tissue.

A more accurate estimation of the sizes of the poly(A) sequences in each of these RNA fractions would give a clearer indication of the rates of synthesis and degradation of this sequence. This may be achieved by the method used by Cabada et al. (1977) when studying the mRNA population of Xenopus oocytes during oogenesis. They prepared poly(A) from RNA samples by digestion with DNAase and RNAase T, to remove any DNA and RNA sequences apart from poly(A) sequences. The remaining poly(A) was then fractionated by polyacrylamide gel electrophoresis and fractions eluted from the gels were identified for their poly(A) content by [^{3}H]-polyU hybridisation. The lengths of the poly(A) sequence in each fraction were determined by comparison with a standard curve by use of markers (5S and 4S RNA and bromophenol blue) fractionated on a parallel gel. A standard curve was prepared using [^{3}H]-

adenosine labelled poly(A)$^+$ RNA from HeLa cells. By hydrolysis of the eluted poly(A) and estimation of the [^{3}H]AMP:[^{3}H]-adenosine ratio by paper electrophoresis, poly(A) size classes were asigned to the markers - 5S RNA, 4S RNA and bromophenol blue.

3.3. In Vitro Translation

The wheat-germ cell-free protein synthesising system was used as a means of assaying translatable mRNA extracted from the cotyledons during germination and early seedling development. As discussed in the Introduction (p. 38), this technique may be used as an assay procedure to study both quantitative and qualitative changes in an mRNA population during development, providing its limitations are realised.

Optimisation of the wheat-germ system was carried out to determine under which conditions the cucumber mRNA was efficiently translated (p. 110). In choosing a particular wheat-germ cell-free system and optimising this for fidelity of translation it is of prime importance to study the in vitro labelled products obtained since these are not always correlated to the levels of radioactive amino acid incorporated into protein. This is illustrated in the optimisation experiments to determine the most suitable wheat-germ and ion concentrations (p.180) and also in the attempt to programme the in vitro translation system with polysomes (p. 204). In the former case under certain conditions which gave reasonable levels of incorporation, high molecular weight polypeptides were not efficiently synthesised. This was in agreement with the observation of other workers that, under certain conditions,

the wheat-germ system selectively synthesises low molecular weight polypeptides (e.g. Cuming and Lane, 1975). When polysomes were used to programme the system, high levels of incorporation were achieved although, due to the possible ribonuclease digestion (p.285), very few distinct polypeptides were synthesised. Thus, when optimising for suitable wheat-germ, K^+, Mg^{2+} and RNA concentrations, both the level of incorporation and the polypeptides synthesised were considered.

The dialysed wheat-germ system was chosen for this study since it showed a reduced level of endogenous activity (Figures 19 and 20; Tables 4 and 5). This decreased the possibility of stimulation of endogenous protein synthesis by addition of exogenous RNA which Senger and Gross (1974) reported could occur. Under optimised conditions, this system was capable of efficient translation of cucumber cotyledonary RNA and synthesised discrete polypeptides over the molecular weight range <10,000 - >70,000.

The selection of optimal K^+ and Mg^{2+} concentrations was dependent upon conditions which gave high levels of incorporation while allowing for efficient synthesis of high molecular weight polypeptides (Figures 22 and 23). It has been shown that K^+ concentrations over 100 mM increase the fidelity of translation of polypeptides larger than 30,000 mol. wt. (Rosen, 1976; Shapiro et al., 1976). Optimal concentrations of Mg^{2+} in conjunction with polyamines allow for reproducibility of results and stimulate the elongation rate thus permitting more efficient synthesis of high molecular weight polypeptides

(Hunter et al., 1977A).

The final conditions chosen to give optimal translation of cotyledonary mRNA were 1-1·5 A_{260} units of the wheat-germ extract in a 50 µl incubation volume, 104 mM K^+ and 2·25 mM Mg^{2+}. Each time a new wheat-germ system was prepared these conditions were tested. However, no significant variation was observed and thus these conditions were maintained throughout this study.

These optimisation experiments were carried out using the cucumber RNA on which further study was to be based. This is important since different mRNAs may have different requirements for efficient in vitro translation. Schwinghamer and Symons (1977) showed that 4 different mRNAs from cucumber mosaic virus had different ion requirements for efficient translation. It is also possible that mRNAs from different tissues or species may have different optimal requirements for efficient translation.

Suitable concentrations of total nucleic acid (Figures 24, 25 and 26) and poly(A)$^+$RNA (Figures 27 and 28) with which to programme the wheat-germ system were also determined. Again both the levels of incorporation and the polypeptides synthesised were studied. RNA concentrations chosen were those where the amount of RNA added was limiting in the range where polypeptide synthesis was approximately linear with respect to RNA concentration (i.e. 5-15 µg total nucleic acid and 0·5 µg poly(A)$^+$ RNA in a 50 µl incubation volume).

While optimising for the RNA concentrations required

for efficient *in vitro* translation, it was shown that the level of incorporation, although linear, was not directly proportional to the amount of mRNA added even below saturating concentrations (Figures 24B and 27B). The results suggest that, with increasing concentrations of mRNA, either each individual message is translated fewer times in this re-initiating system, or, there may be competition between individual mRNA species even although non-saturating concentrations were used. Lodish (1974) reported that both *in vivo* and *in vitro* there are differential rates of initiation of α- and β-globin mRNAs and that, in the reticulocyte lysate system (Pelham and Jackson, 1976), the translation of α-globin mRNA could be selectively inhibited by blocking the initiation reaction. However, an analysis of the polypeptides synthesised in the wheat-germ system with increasing concentrations of RNA (Figures 25 and 28) showed no obvious effects of competition as the same relative amounts of individual polypeptides were observed in all cases where high molecular weight polypeptides were being synthesised. This suggests that if the limiting factor is at the level of initiation, that it is non-selective in determining which mRNAs are translated. Since no study of this phenomenon was carried out, it is not possible to say what was causing it. However, it may be some limitation of the system at either initiation or elongation e.g. initiation factors; initiator tRNAs (Met-tRNAf); elongation factors. It is clear that this phenomenon occurs even though the *in vitro* system is unsaturated with mRNA and while incorporation of [^{35}S]-methionine

continues for 60-90 minutes (see Figure 29). It is also not due to the presence of RNAs other than mRNA in the nucleic acid sample since this phenomenon is also observed when purified poly(A)$^+$ RNA was used as template.

Grienenberger (personal communication) showed that addition of rRNA to poly(A)$^+$ RNA, which was normally highly efficient in the cell-free system, resulted in a marked inhibition of the translational activity of the mRNA. Personally, I feel that, particularly at day 0, when the mRNA content of the cotyledons is relatively low, this phenomenon may account for the lack of <u>in vitro</u> translation products observed with the total nucleic acid fraction (Figure 31) as compared with the poly(A)$^+$ RNA fraction (Figure 32). In determining the changes in the mRNA population using total nucleic acid, and poly(A)$^-$ RNA as templates, it was assumed that this effect was quantitatively the same for all the days of this developmental sequence. The results shown in Figure 26 of the developmental profiles of the mRNA population using different concentrations of total nucleic acid support this assumption. Thus, the results obtained probably give a good indication of the gross changes occurring in the mRNA population.

(a) <u>Quantitative Changes in the mRNA Population</u>

In all experiments where RNA from different days of development were used to programme the cell free system the level of incorporation was expressed as c.p.m./cotyledon. This allowed for quantitative changes in the translatable mRNA population of the cotyledons to be determined. Figure

30 shows the translational activity of the total nucleic acid, poly(A)$^+$ RNA and poly(A)$^-$ RNA fractions.

It was found that low levels of translatable mRNA were present in the unimbibed (dry) seed tissue. Similar findings have been reported for rye embryo, broad bean, pea, rapeseed (Gordon and Payne, 1976), wheat embryos (Caers et al., 1979; Cuming and Lane, 1978, 1979) and castor bean endosperm (Roberts and Lord, 19798). During imbibition there was a slight decline in the amount of translatable mRNA in the cotyledons. This is in agreement with the results shown for the amount of poly(A) and extractable poly(A)$^+$ RNA which indicated an overall decline in the mRNA population at this time. Thereafter there is an increase in the amount of translatable mRNA, to reach peak levels at day 4 in the light, followed by a marked decline as development proceeds. The most rapid accumulation of translatable mRNA occurs between days 2 and 4 in the light and coincides with the period of marked heterotrophic activity (p.130) and early development of the autotrophic function of the cotyledons.

Comparison of the activity profiles of the poly(A)$^+$ RNA (Figure 30B) and poly(A)$^-$ RNA (Figure 30C) fractions shows that the poly(A)$^+$ mRNAs are more prevalent in the earlier stages of this developmental sequence. This suggests that many of the 'heterotrophically related mRNAs' may be highly polyadenylated whereas those associated with the autotrophic growth phase may be polyadenylated to a lesser extent.

The results presented here suggest that light is essential for maintained accumulation but not for the

initial synthesis and increase of the total translatable mRNA population. However, it has relatively little effect on the poly(A)$^+$ mRNA fraction.

Roberts and Lord (1979B), studying the translational activity of the poly(A)$^+$ RNA of castor bean endosperm, found that there were low levels of translatable mRNA present in the dry seed and imbibed tissue. They found that as the heterotrophic activity of the endosperm increased there was an increase in the ability of the RNA to stimulate protein synthesis in the wheat-germ system.

In the cucumber cotyledons there is a marked accumulation of translatable mRNA during the heterotrophic and early autotrophic growth phases, which is probably a result of de novo transcription. Thus, although preformed mRNA may be utilised for the initial resumption of metabolic activity at the onset of germination, the majority of protein synthesis occurring during this developmental transition is a result of translation of newly transcibed mRNA.

Polysomes were also used to programme the wheat-germ system. Although there were problems involved due to the possible presence of RNAase in the polysome extracts (see pps.213 and 285), the profiles of in vitro translational activity of the polysomes were thought to give some indication of the amount of mRNA actively involved in protein synthesis.

The results shown in Figure 37 indicate that there are low levels of mRNA actively involved in protein synthesis

at the beginning of this developmental sequence. Following an increase, during the heterotrophic and early autotrophic growth phases, in the translational activity associated with the polysomes there is not the subsequent decline noted for the total mRNA population (Figure 30). This suggests that during the phase of accumulation of mRNA in the cotyledons there may be many mRNAs not actively involved in protein synthesis at any one time. This may be due to rapid turnover of the mRNAs being utilised for protein synthesis. There was found to be an increase in the mRNA associated with both free and membrane-bound polysomes as development proceeds, which closely parallels the changes in extractable polysomes already noted (p.282). In dark-grown tissues there was found to be lower levels of mRNA activity associated with polysomes. This suggests that the continued accumulation but not the initial formation of polysomes is light dependent.

(b) Qualitative Changes in the Total mRNA Population

Qualitative changes in the mRNA population during germination and early seedling development were studied by analysing the *in vitro* synthesised polypeptides by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The *in vitro* labelled polypeptides synthesised in the wheat-germ system when programmed with total nucleic acid, poly(A)$^+$ RNA and poly(A)$^-$ RNA are illustrated in Figures 31, 32 and 33, respectively. By *in vivo* labelling of the cucumber cotyledons with [^{35}S]-methionine (p.249) it was possible to determine which mRNAs present in the

tissue were actively involved in protein synthesis. The *in vivo* synthesised polypeptides are shown in Figures 54 and 55.

The mRNA population of unimbibed (dry) seed tissue was found to direct the *in vitro* synthesis of numerous discrete polypeptides with molecular weights ranging from <10,000 to >70,000. Other workers have also shown that poly(A)$^+$ RNA from dry seed tissue can programme the *in vitro* synthesis of discrete polypeptides, e.g. rye embryos, pea, rapeseed (Gordon and Payne, 1976; Cuming and Lane, 1978, 1979; Caers *et al.*, 1979). It has been proposed that preformed (stored) mRNA may be utilised during early germination (Spiegel and Marcus, 1975; Spiegel *et al.*, 1975; Payne, 1976). This proposal was based on the failure of inhibitors of mRNA synthesis to prevent polysome formation during early germination (Spiegel and Marcus, 1975). If this is the case, it is essential that at least some of the mRNAs present in the dry seed tissue code for proteins that are synthesised during the early resumption of protein synthesis. The fact that the mRNAs from cucumber cotyledons and other dry seed tissues do code for discrete polypeptides suggests that they are capable of being utilised for protein synthesis. However this does not prove that they are used. Cuming and Lane (1979) have shown that the polypeptides synthesised *in vitro* from poly(A)$^+$ RNA of dry wheat embryos are broadly similar to those synthesised *in vivo* during the first 40 minutes of imbibition. They also showed that a number of these polypeptides are ribosomal proteins. It was previously pointed out

that the marked accumulation of rRNA and polysomes within cucumber cotyledons would be dependent upon synthesis of ribosomal proteins. The polypeptides synthesised by the poly(A)$^+$ RNA from day 0 (17 hour imbibed) cucumber cotyledons (Figure 32) are qualitatively identical to those coded by the mRNA from the dry seed tissue.

From the evidence presented here it is possible that the resumption of protein synthesis involves utilisation of preformed mRNAs. However, the possibility still exists that the preformed mRNAs are rapidly degraded and that synthesis of new, though qualitatively similar mRNAs occurs during imbibition. Unfortunately, in the *in vivo* labelling experiments (p. 294) the level of incorporation of [^{35}S]-methionine into protein during imbibition was not sufficient for me to detect the polypeptides synthesised in the cotyledons during this period (Figure 54). Thus it is not possible to say if the *in vivo* synthesised polypeptides are similar to those detected from the *in vitro* experiments.

After imbibition there are numerous qualitative changes in the mRNA population of the cotyledons during this developmental sequence, as determined by the polypeptides synthesised *in vitro*. By day 1 it was found that a number of the mRNAs present in the dry and imbibed cotyledons were no longer detectable e.g. the mRNA coding for a 29,000 mol. wt. polypeptide. This is in agreement with the work by Trewavas (1979) which showed that there is a loss of several mRNAs in barley embryos during germination. These mRNAs may be those involved in embryogenesis which remain in the dry seed and

only decline at the onset of germination (Dure, 1977).

There are a group of mRNAs which, by their translation products, are identifiable predominantly during the heterotrophic growth phase. These polypeptides are mainly in the 45,000-70,000 mol.wt. range. They are initially undetectable in the products obtained when total nucleic acid is used to programme the wheat-germ system (Figure 31). However, from a study of the products derived from translation of poly(A)$^+$ RNA (Figure 32) it can be seen that these mRNAs may in fact be present in the unimbibed and imbibed cucumber tissue. The mRNAs for these polypeptides are most abundant by day 3 in the light then rapidly disappear. In dark-grown tissue these mRNAs persist throughout this developmental sequence albeit at reduced levels. The most prominent of these translation products is a polypeptide of approximately 63,000 mol.wt. (indicated by an arrow).

In the *in vivo* labelling experiment there was a group of polypeptides in this molecular weight range which were synthesised predominantly during the heterotrophic growth phase (Figures 54 and 55). These showed similar developmental profiles to the *in vitro* synthesised polypeptides in both light and dark-grown tissue. Their developmental profiles and molecular weights also corresponded closely to similar changes observed in the stained protein gels (Figure 7).

The developmental profiles of these *in vitro* and *in vivo* synthesised polypeptides are similar to the activity profiles for the glyoxysomal enzymes (Figure 3). Since

the glyoxysomal enzymes are known to have subunit molecular weights in this range it is anticipated that at least some of these *in vitro* and *in vivo* synthesised polypeptides are glyoxysomal in nature. Known molecular weights of glyoxysomal enzyme subunits include ICL - 63,000 and 61,500 mol. wt.; MS - 57,000 mol. wt.; citrate synthase - 46,000 mol. wt.; malate dehydrogenase - 33,000 mol.wt.; crotonase - 75,000 mol. wt.; thiolase - 45,000 mol. wt.; CAT - 54,000 mol. wt. (Köller and Kindl, 1977; Becker *et al.*, 1978). Certainly *in vivo* this group contains polypeptides with similar molecular weights to those of the glyoxysomal enzyme subunits. However, it is more difficult to identify specific *in vitro* synthesised polypeptides with these molecular weights. The most prominent of the *in vitro* products, of 63,000 mol. wt., by its developmental profile and co-migration with ICL-A may be the translation product of the mRNA for this enzyme subunit. By immunoprecipitation with anti-ICL antibody this was in fact identified as *in vitro* synthesised ICL-A (see p. 229). However, it is conceivable that since glyoxysomal enzymes are sequestered within membrane-bound organelles that at least some of the *in vitro* translation products of glyoxysomal enzyme mRNAs may be precursors of higher molecular weight (see p. 229; Blobel and Dobberstein, 1975; Highfield and Ellis, 1978). Walk and Hock (1977) and Riezman *et al.* (in press) have shown that glyoxysomal malate dehydrogenase is synthesised as a precursor of 38,000 mol. wt. Further identification of *in vitro* synthesised ICL, MS and CAT is outlined in more detail

in the following section (see pps.314 and 229).

From the developmental profiles of these *in vitro* and *in vivo* synthesised polypeptides it is possible that they are functional in the heterotrophic growth phase. Synthesis of these polypeptides in the cotyledons may initially result from translation of preformed mRNA. However, there is accumulation of these mRNAs during the first three days of this developmental sequence. These results, in conjunction with the results of quantitative changes in the mRNA population (see pps. 299 and 192) suggest that this increase in these mRNAs may be a result of transcription. However, the possibility of post-transcriptional control (see p. 34) cannot be totally ruled out unless it can be shown that the wheat-germ system is capable of translating precursor mRNAs (see p. 51). The decline in these mRNAs is apparently stimulated by light since their translation products can be identified, albeit at reduced levels, both *in vitro* and *in vivo* up to day 8 in dark-grown tissue.

Associated with the initiation and development of the photosynthetic function, there is a marked change in the mRNAs present in the cotyledons. The appearance of autotrophically related translatable mRNAs in the cucumber cotyledons is clearly mirrored by (1) the appearance of autotrophically related polypeptides in the stained protein gels (Figure 7), and during the *in vivo* labelling experiments and (Figures 54 and 55) (2) the developmental profiles of RuBPC, chlorophyll and GR (Figure 4).

This group of mRNAs are first detectable at day 2 and increase in prominence during the remainder of this devel-

opmental sequence. The *in vitro* translation products of these mRNAs include polypeptides of 50,000, 35,000, 32,000 and 25,000 mol. wt.. Walden (1979) has tentatively identified the 25,000 mol. wt. polypeptide as the precursor of the SSU (13,000 mol. wt.) of RuBPC. This is somewhat larger than the 20,000 mol. wt. precursors of the SSU reported by other workers for pea (Roy *et al.*, 1977; Highfield and Ellis, 1978; Cashmore *et al.*, 1978, 1979), wheat (Roy *et al.*, 1976), *Lemna gibba* (Tobin, 1978) and *Chlamydomonas* (Dobberstein *et al.*, 1977). The identification of the 50,000 and 35,000 mol. wt. polypeptides is unknown, but from the timing of the synthesis of their mRNAs and their preponderance, it is anticipated that they have a functional role in the autotrophic growth phase. These mRNAs are first detectable at days 2 or 3 and are synthesised in both light and dark-grown tissue. Their developmental profiles correspond closely to the changes observed for the small subunit of RuBPC synthesised *in vivo* (Figures 54 and 55) and detected on stained gels (Figure 7) and also to the developmental profile of native RuBPC protein (Figure 48). Walden (1979) has shown that maintained accumulation but not the initial synthesis and increase of SSU mRNA is light dependent. Similarly, the *de novo* synthesis of the mRNAs for the 50,000 and 35,000 mol. wt. polypeptides is independent of light.

The synthesis of other mRNAs associated with the autotrophic growth phase is light dependent. This includes the mRNA which codes for the 32,000 mol. wt. *in vitro* synthes-

ised polypeptide, which has not been identified in this thesis, but is probably the precursor for the chlorophyll a/b binding protein (Apel and Kloppstech, 1978; Schmidt et al., 1979). The native protein in cucumber cotyledons has a subunit molecular weight of 26,000 (J. Schouten - personal communication) which can be identified among the in vivo synthesised polypeptides (Figures 54 and 55) and on the stained protein gels (Figure 7). The timing of synthesis of this polypeptide in vivo corresponds closely to the developmental profile obtained by in vitro translation of the putative mRNA. Chlorophyll a/b binding protein is membrane associated and is coded in the nucleus and synthesised in the cytoplasm. Apel and Kloppstech (1978) have demonstrated that synthesis of the mRNA for this polypeptide is light dependent.

As well as cytoplasmic mRNAs for chloroplast proteins, it would be anticipated that mRNAs for peroxisomal enzymes would be synthesised in association with the transition from the heterotrophic to autotrophic function of the cotyledons. The peroxisomal enzymes constitute a low percentage of the total protein of the cotyledons and presumably respective mRNAs are similarly less abundant. In the absence of specific antibody probes for peroxisomal enzymes it has not been possible to identify the in vitro and in vivo synthesised peroxisomal enzymes.

It was found that the translation products obtained when total nucleic acid (Figure 31), poly(A)$^+$ RNA (Figure 32) and poly(A)$^-$ RNA were used to programme the cell-free

system were qualitatively the same, with the possible exception of a single, minor polypeptide of approximately 53,000 mol. wt. which was absent from the products of translation of poly(A)$^+$ RNA (Figure 34). The identification of this polypeptide was not further investigated. However, Walden (1979) suggests that the mRNA for the LSU of RuBPC may be translated in the wheat-germ system, though not as efficiently as cytoplasmic mRNA. He also showed that the poly(A)$^+$ RNA lacks LSU mRNA. Thus, it is possible that this polypeptide may be the product of translation of LSU mRNA. This would be in agreement with Sagher *et al.* (1976) who claim that LSU mRNA can be translated in the wheat-germ system.

The fact that the mRNAs in the poly(A)$^+$ RNA and poly (A)$^-$ RNA (Figures 32 and 33) are qualitatively identical is in agreement with the results obtained by Gray and Cashmore (1976) when studying the *in vitro* translation products of pea mRNA. This result suggests that there may be a rapid turnover of the mRNAs in cucumber cotyledons during this development sequence. It was estimated that the average length of poly(A) in the cotyledons showed a general decline (see p. 293) during germination and early seedling development. Singer and Penman (1973) and Sheiness *et al.* (1975) showed that a decline in the length of the poly(A) sequence may be associated with ageing of mRNA. Thus, if there is a rapid turnover of the mRNAs of the cucumber cotyledons it would be anticipated that these mRNAs may be detectable in both poly(A)$^+$ RNA and poly(A)$^-$ RNA.

Due to the presence of RNAase activity associated with the extracted polysomes (see p. 285) it was not possible to identify the mRNAs actively involved in protein synthesis by programming the wheat-germ system with polysomes. However, by comparison of the *in vitro* translation products of the RNA fractions (Figures 31, 32 and 33) with the *in vivo* synthesised polypeptides (Figures 54 and 55) it is seen that there is a close correlation between the mRNA species present in the tissue and those being translated. Certain polypeptides observed among the *in vivo* synthesised products are not seen in the *in vitro* labelled products. Firstly, it is clear that the wheat-germ system does not synthesis very high molecular weight polypeptides i.e. > 80,000 mol. wt.. This is in agreement with the findings of other workers (e.g. Cuming and Lane, 1978, 1979). Secondly, there are lower molecular weight polypeptides, such as the 53,000 mol. wt. LSU of RuBPC, which are not obvious in the *in vitro* products although they are prominent *in vivo*. This is presumably because the mRNAs for these are organellar and thus not efficiently translated in the wheat-germ system.

3.4. Summary

The results presented in this section show that there are low levels of mRNA present in the unimbibed seed tissue of cucumber. *In vitro* translation of these mRNAs resulted in the synthesis of numerous discrete polypeptides which were qualitatively similar to those synthesised when RNA from the imbibed tissue was used to programme the wheat-germ system.

Marked increases in the mRNA content of the cotyledons occurred during the heterotrophic and early autotrophic growth phases. As development proceeds the mRNA content subsequently declines.

Developmentally related qualitative changes in translatable mRNAs were observed, first in association with development of the heterotrophic function and later the autotrophic function of the cotyledons. Results suggested that the mRNAs for enzymes functional in the heterotrophic growth phase may be present in the unimbibed tissue. However, it was not possible to determine whether these were translated upon the onset of germination or whether the initial synthesis of these enzymes was dependent upon newly transcribed mRNAs. Certainly full development of the heterotrophic function was dependent upon an increase in the translatable mRNAs for these enzymes. Initiation and development of the photosynthetic function of the cotyledons was apparently dependent upon *de novo* synthesis of translatable mRNAs.

SECTION 4

IDENTIFICATION AND DEVELOPMENTAL CHANGES IN THE mRNAs FOR SOME GLYOXYSOMAL ENZYMES

It has been shown in the previous section of this thesis that there are marked quantitative and qualitative changes in the total mRNA population of cucumber cotyledons during germination and early seedling development. It has been proposed that some of these are associated with the heterotrophic growth phase while others are associated with the initiation and development of the photosynthetic function of the cotyledons. The main purpose of this thesis was to determine at which level the expression of genes responsible for the glyoxylate cycle was regulated i.e. transcription or translation. In the absence of specific cDNA probes with which to assay the available mRNAs for the glyoxysomal enzymes, use was made of monospecific antisera to identify specific *in vitro* labelled polypeptides synthesised in a wheat-germ system programmed with cotyledonary RNA.

In order to use this technique as a means of quantitating the mRNA present at different developmental stages, it was essential to choose a procedure which gave quantitative immunoprecipitation of the specific translation products. Therefore, three different methods for recovery of the antibody-antigen complex were tested for their efficiency and reproducability - (1) double antibody technique, (2) immunoadsorption with attenuated *S. aureus* cells, (3) protein A-sepharose 4B affinity chromatography. Such procedures have been used successfully by other workers

for identification and assay of specific mRNA translation products e.g. for α-amylase (Higgins et al., 1976); corticotropin (Nakanishi et al., 1976); SSU of RuBPC (Dobberstein et al., 1977; Walden, 1979); insulin (Shields and Blobel, 1977).

As discussed in the Results (p. 222), the most efficient and reproducible procedure for detection of ICL mRNA translation products was the double antibody technique. Therefore this procedure was used to identify the in vitro translation products of ICL and MS mRNAs and to follow the developmental changes in these specific mRNAs. Due to the unavailability of a second suitable antibody to the mouse anti-CAT antiserum the S. aureus immunoadsorption technique was used for identification of this specific in vitro translation product.

4.1. Identification of the Primary Translation Products of ICL, MS and CAT mRNAs

By use of monospecific antisera raised against purified ICL, MS and CAT from cucumber cotyledons (Riezman et al., in press) it was possible to show that the mRNAs for these three enzymes were translated in the wheat-germ system when programmed with total nucleic acid from day 3 light-grown cotyledons (Figure 45).

As discussed in the Introduction (p. 26), glyoxysomes do not contain any genetic machinery (Douglass et al., 1973) and are thus dependent upon the cytoplasmic protein synthetic apparatus for synthesis of their component enzymes. From studies on the relationships between ER and microbody membranes it has been proposed that microbodies (including

glyoxysomes) are derived from the ER by vesiculation (see p. 27). Since the glyoxysomes are probably derived from the ER it may be anticipated by analogy with the Signal Hypothesis (p. 28) that at least some of the enzyme complement of this microbody is synthesised on rough ER with vectoral discharge into the cisternae. However, by analogy with the 'Envelope Carrier Mechanism' (see p. 29) proposed by Highfield and Ellis (1978) for transport of the SSU of RuBPC into the chloroplast, it is equally possible that at least some of the glyoxysomal enzymes are synthesised on free polysomes and transported post-translationally into the organelle. In order for either mechanism to be operative it would be anticipated that the primary translation products for these enzymes may contain recognition sequences to facilitate their vectoral transport across the appropriate membrane.

It has been shown that ICL and CAT are present in the glyoxysomal matrix (Huang and Beevers, 1973) while MS is loosely associated with the inner surface of the limiting membrane (Huange and Beevers, 1973; Brown et al., 1974; Köller and Kindl, 1977). Goodman and Blobel (1978) have proposed that only membrane-associated microbody enzymes are synthesised on membrane-bound organelles, while those present in the matrix of the organelle are synthesised on free cytoplasmic polysomes. They based this proposal on the finding that CAT mRNA from rat liver was associated with free but not membrane-bound polysomes. Lord and Bowden (1978), using pulse labelling experiments, have

produced evidence to suggest that MS is synthesised on rough ER prior to sequestration into the glyoxysome. The same authors (Bowden and Lord, 1977), from other serological studies, have proposed that both membrane-associated and matrix enzymes are sequestered within the glyoxysomes via the ER.

The results presented in this thesis indicate that the molecular weights of the primary translation products for these enzymes are approximately 63,000 and 61,000 for ICL, 57,000 for MS and 55,000 for CAT (Figure 45). Native enzyme subunit molecular weights are, by comparison, approximately 63,000 and 61,500 for ICL, 57,000 for MS and 54,000 for CAT. Both ICL and MS _in vitro_ synthesised polypeptides co-migrate closely with the stained polypeptides form isolated glyoxysomes which have been identified as ICL and MS subunits (Riezman _et al._, in press). On the other hand, _in vitro_ synthesised CAT migrates more slowly than stained CAT subunits from isolated glyoxysomes on SDS-polyacrylamide gels.

Thus, of these glyoxysomal enzymes, only CAT is obviously synthesised _in vitro_ as a precursor. It is possible to detect such precursors since the wheat-germ system does not contain the membranes or the enzymes which are responsible for the processing. The size of the extra sequence (approximately 1,000 mol. wt.) on the _in vitro_ synthesised CAT is consistent with its role as a recognition sequence although it is in contrast to the report by Goodman and Blobel (1978) that rat liver peroxisomal catalase

is synthesised *in vitro* with the same molecular weight as the native polypeptide. In the case of the SSU of RuBPC, a stromal protein of the chloroplast, it has been shown that the precursor is synthesised on free cytoplasmic polysomes (Ellis, 1979) and transported post-translationally into the chloroplast. It is possible that CAT, being a glyoxysomal matrix enzyme is similarly synthesised on free polysomes and transported through the glyoxysomal membrane post-tranlationally.

Two *in vitro* translation products immunologically identifiable as ICL subunits were detectable, in agreement with the fact that there are two subunit sizes *in vivo*. It should be remembered though that the two subunit sizes are from different active forms of ICL (see p. 18 ; Riezman *et al.*, in press). The molecular weights of the *in vitro* and native subunits are approximately the same as far as I can determine - both from measurement of the molecular weight by comparison with marker proteins and by co-migration of the *in vitro* synthesised polypeptides with stained polypeptides of isolated glyoxysomes. Thus, it appears that ICL is not synthesised as a precursor. However, this does not necessarily mean that the ICL polypeptides do not contain a recognition sequence to mediate transport across the correct membrane system. It has been shown for ovalbumin that the signal sequence is present internally within the polypeptide and is not cleaved upon transport across the membrane (Steiner, 1979).

In vitro synthesised MS subunits were also found to

comigrate with the native polypeptide from isolated glyoxysomes. However, Lord and Bowden (1978) have shown that this enzyme is segregated by the ER into the glyoxysomes. There is also evidence to suggest that native MS is a glycoprotein (Mellor et al., 1978; Riezman et al., 1978, 1979) which is in agreement with the proposal that this enzyme is transported across ER membranes, since it is known the ER contains the glycosylation activity (Lingappa et al., 1978). Thus, it is possible that MS contains a signal sequence but that the glycosylation of the processed precursor results in the native protein migrating to the same position on SDS-polyacrylamide gels as the in vitro translation product.

From the sizes of the in vitro enzyme subunits shown here it is not possible to determine whether membrane-bound and matrix enzymes are compartmentalised by different mechanisms. In order to show which mechanism is responsible for segregation of the different enzymes it would be necessary to determine whether the mRNA for each is associated with free or membrane-bound polysomes. Further evidence could be obtained from the ability of ER or glyoxysomal membranes to process or compartmentalise the in vitro translation products. In the case of polypeptides which are normally transported co-translationally it would be necessary to supply the in vitro translation system with membranes during protein synthesis (Blobel et al., 1979) in order to obtain compartmentalisation and/or processing in vitro. On the other hand, those synthesised on free polysomes will readily be processed and/or compartmentalised

in the absence of concurrent synthesis (e.g. SSU of RuBPC - Highfield and Ellis, 1978). Thus, it should be possible to determine if ICL and CAT are synthesised on free or membrane-bound polysomes and to obtain further evidence that MS is synthesised on and sequestered by the ER.

It has been shown that MDH is synthesised *in vitro* as a precursor with a molecular weight of 38,000, some 5,000 larger than the native polypeptide (Walk and Hock, 1978; Riezman *et al.*, in press). This enzyme is also membrane-associated (see p. 15) and is thus possibly synthesised on the ER.

4.2. Developmental Changes in ICL and MS mRNAs

The ability to detect *in vitro* synthesised ICL and MS polypeptides makes it possible to assay for the presence of translatable mRNA for these enzymes during germination and early seedling development. This in turn allows one to determine whether the changes in enzyme activities previously shown (p. 130) are a result of control of gene expression at the level of transcription or translation.

There is substantial evidence and it is generally accepted that both ICL and MS are synthesised *de novo* at the onset of germination (Hock and Beevers, 1966; Longo, 1968; Gientka-Richter and Cherry, 1968; Ihle and Dure, 1972; Smith *et al.*, 1974; Doig *et al.*, 1975; Tester, 1976; Bowden and Lord, 1976A, 1977; Walk and Hock, 1977). The results shown earlier in this thesis (p. 266) are in agreement with these two enzymes being synthesised *de novo*. However, there is conflicting evidence concerning the level

of control of the synthesis of glyoxysomal enzymes. Certain workers have proposed that this de novo protein synthesis is a result of translation of mRNA newly synthesised upon germination (Hock and Beevers, 1966; Lado et al., 1968; Smith et al., 1974; Roberg and Becker, 1975; Radin and Trelease, 1976). On the other hand, Ihle and Dure (1969, 1972) and Tester (1976) have suggested that in fact the marked increases in glyoxysomal enzyme activities in cotton and soybean are a result of translation of preformed mRNA. These two conflicting groups of evidence were the result of studies carried out using inhibitors of RNA synthesis e.g. actinomycin D. Gientka-Rychter and Cherry (1968) also used actinomycin D and they found only partial inhibition of the increase in enzyme activity, which led them to propose that in fact both preformed and newly synthesised mRNA were used for synthesis of the enzymes.

It is clear that in this case, inhibitor studies are certainly non-conclusive. This may be due to the fact that actinomycin D has been shown to preferentially inhibit rRNA synthesis - though it may affect mRNA synthesis to a lesser extent (Fraser, 1975).

Using the monospecific antisera against purified ICL and MS it was possible to show the presence of these enzyme subunits among the in vitro translation products programmed by cucumber RNA from unimbibed (dry) seed tissue (Figures 46, 47, 48). Thus, it can be said that there is translatable mRNA for these enzymes present in the dry seed. These were also found to be present in the imbibed tissue (Figure 47).

However, when these mRNAs were quantitatively estimated, it was shown that only small amounts of each were present at these early stages (Figures 49 and 50). As germination and early seedling development proceed there is a marked increase to peak levels at day 3 in the amount of total translatable mRNA for these enzymes (Figure 49). The peak of translatable mRNA at day 3 is approximately 1 day before peak enzyme activities are attained. In light-grown tissue the amount of translatable mRNA subsequently declines until only barely detectable levels are present at days 6 and 7, while in the dark there is a decline till day 5 followed by a slight increase.

The translatable mRNAs, for these enzymes, present in the poly(A)$^+$ RNA are only detectable in light-grown tissue up to the peak at day 3 (Figure 50). This suggests that after day 3 there is a decline in the mRNAs firstly by a shortening of the poly(A) sequence, which is in agreement with the concept that shortening of the poly(A) sequence is associated with ageing of mRNAs (Singer and Penman, 1973; Sheiness *et al.*, 1975). In dark-grown tissue translatable mRNAs for ICL and MS remain detectable in the poly(A)$^+$ RNA till the end of this developmental sequence, suggesting that light stimulates the decline in the poly(A) sequence length and subsequently degradation of these mRNAs.

Of the two different forms of ICL, the high molecular weight one (63,000 - ICL-A) is more prominent *in vitro*, which is in agreement with the observation that isolated glyoxysomes contain more of this than the 61,500 polypeptide.

The absence of detectable ICL-B among the translation products at some stages of this developmental sequence when ICL-A was identified may reflect either the lack of translatable mRNA or the different proportions of the two polypeptides. Personally, I think that the latter is the case. However, longer exposure times for the autoradiographs would determine which of these possibilities was the case.

Immunoprecipitation of *in vivo* synthesised ICL and MS was carried out (see p. 257; Figures 56 and 57). Due to the very low level of incorporation of [^{35}S]-methionine into protein during imbibition it was not possible to determine if these two enzymes were synthesised within the tissue during this period. However, during the heterotrophic growth phase it was possible to detect *in vivo* synthesised ICL and MS.

Quantitative changes in the synthesis of these two glyoxysomal enzymes were estimated as described in the Results (p. 260; Figure 57). There appears to be maximum synthesis of these enzymes between days 1 and 2. However, the subsequent decline may reflect the increased availability of free amino acids within the tissue which was not taken into account in these calculations and thus may not occur as rapidly as suggested by the results obtained here. It is clear that no synthesis could be detected after day 4 in the light while in the dark synthesis continued albeit at a low level (Figure 56).

From the results presented here it is clear that low levels of translatable mRNA for ICL and MS are present in

the dry and imbibed seed tissue though it was not possible to determine whether these are translated *in vivo*. However, it was previously shown that protein synthesis did occur during the imbibition period (Table 8). It is therefore conceivable that low levels of these enzymes are synthesised at this time though the detection methods as utilised in this thesis were not sufficiently sensitive to detect these. Thus, it is possible that preformed mRNAs are used to initiate the synthesis of ICL and MS at the onset of germination. It is, however, obvious that the marked increases in enzyme activity (Figure 3) are a result of an increase in translatable mRNA within the tissue. Taken together with the observation that there is a marked accumulation of total mRNA during this time (see p. 311) this would suggest that the accumulation of ICL and MS proteins within the cotyledons is dependent upon transcription and accumulation of the respective mRNAs.

The decline in ICL and MS within the cotyledons is apparently a result of degradation of the mRNAs for these enzymes. In dark-grown tissue the maintenance of active glyoxysomal enzymes within the cotyledons (Figure 3) is due to continued availability of translatable mRNA.

4.3. Size Estimation of ICL and MS mRNAs

The sizes of the poly(A)$^+$ RNAs for ICL and MS were estimated as described in the Results (p. 238). It was found that the majority of these mRNAs were detectable on 5-35% (w/v) linear sucrose density gradients at a

position indicative of a molecular weight of $1{\cdot}025 \times 10^6$, by comparison with rRNA markers. The minimum coding capacities required for ICL-A, ICL-B and MS polypeptides are $0{\cdot}66 \times 10^6$, $0{\cdot}646 \times 10^6$ and $0{\cdot}599 \times 10^6$ respectively. However, they were also detectable at higher and lower sixe positions. It is known that aggregation of the mRNA may occur and also that any secondary structure of the molecule may affect its position on such gradients. Thus, to obtain accurate molecular weight estimations it will be necessary to carry out the fractionation under denaturing conditions in the presence of, for example, DMSO or formamide (Strauss et al., 1968; Taylor, 1979).

4.4. Summary

It has been shown that the presence of translatable mRNAs for ICL and MS could be detected by the procedures used in this thesis. The in vitro translation products for ICL and MS mRNAs were virtually indistinguishable from the native polypeptides though in vitro synthesised CAT was found to be 1,000 mol. wt. larger than the native polypeptide. From the results presented here it was not possible to determine the mechanism by which these polypeptides are compartmentalised. Further investigation of the mode of segregation would be necessary to determine whether all or only membrane-associated glyoxysomal enzymes are segregated via the ER.

It was possible to determine that the dry and imbibed cucumber tissues do contain translatable mRNAs for ICL and MS, albeit in very low amounts. It is possible that pre-

formed mRNAs for these enzymes are utilised at the onset of protein synthesis during germination. However, there then follows a marked increase in the amount of these translatable mRNAs during the heterotrophic growth phase. This increase occurs prior to the marked increase in enzyme activities previously observed suggesting that synthesis and accumulation of these enzymes is dependent upon accumulation of the respective mRNAs. Loss of active enzymes was associated with a reduction in the amount of the specific translatable mRNAs and light was found to stimulate the loss of these.

The majority of ICL and MS mRNAs was found to have a molecular weight of approximately $1{\cdot}025 \times 10^6$, which is larger than the minimum coding capacity required for the enzyme subunits.

SECTION 5

CONCLUSIONS

A study of cucumber cotyledons during germination and early seedling development showed that there are marked changes in the protein and enzyme complement of the tissue. It was possible to identify changes which were characteristic of the heterotrophic growth phase and later others associated with the transition to autotrophic growth. Peak heterotrophic metabolism typified by conversion of lipid to carbohydrate occurred between days 3 and 4. Certain characteristic markers associated with the photosynthetic function were initiated at this time though full development of autotrophic growth was dependent upon light. Underlying these metabolic changes was a dramatic increase in the cellular protein synthetic machinery. In addition, I have attempted to summarise the major molecular and biochemical changes in the cotyledons associated with germination and early growth - see Table 9.

It was shown that in the dry and imbibed cucumber seed tissue there is a low level of RNA, ribosomes and mRNA. Some of these mRNAs have been identified as coding for the glyoxylate cycle enzymes, ICL and MS. Protein synthesis is known to resume some time during the imbibition period and it is conceivable that this may involve utilisation of these preformed (stored) RNA species, including the mRNAs, in order to allow the onset of metabolic activity within the tissue. This would imply that genes expressed immediately upon the resumption of metabolic activity are

Table 9

Cellular Contents of Cucumber Cotyledons During Germination and Early Seedling Development

	units/cell	Days of Development				
		0	3 light	3 dark	7 light	7 dark
Fresh weight	ng	23	36	34	234	44
Dry weight	ng	17	14	13	16	7
Total protein	ng	50	4.3	3.9	3.2	2.2
'Soluble' protein	ng	0.6	2.5	2.5	2.6	2.0
Lipid	ng	6.9	4.6	-	1.2	-
ICL	e.u. $\times 10^{-4}$	~0	1.0	0.9	~0	0.2
MS	e.u. $\times 10^{-4}$	~0	3.3	2.8	0.2	2.2
CAT	e.u. $\times 10^{-4}$	~0	0.7	0.5	0.4	0.4
GR	e.u. $\times 10^{-4}$	~0	0.2	0.1	2.3	0.5
Chlorophyll	pg	~0	2	~0	334	~0
RuBPC	pg	~0	1.6	1.2	7.7	3.7
RNA - total	pg	28	155	139	316	164
- cyt. rRNA	pg	23	131	103	201	118
- chl. rRNA	pg	~0	11	1	76	29
- LMW RNA	pg	5	13	11	39	17
Ribosomes - cyt.	$\times 10^{6}$	7	39	31	61	36
- chl.	$\times 10^{6}$	~0	4	0.4	28	11
Extractable polysomes	pg	8.1	64.9	32.0	164.9	81.8
Poly(A)$^{+}$ RNA	fg	22	504	332	195	176
Poly(A)	fg		132	84	53	31

e.u. = enzyme units - designated as described in the Methods (p. 78)

This data was calculated assuming a constant cell number of 6.81×10^{6} per cotyledon.

regulated at the level of translation. However, it has been shown that the majority of protein synthesis during heterotrophic growth, including marked increases in the glyoxysomal enzymes, ICL and MS, is a result of an increase in the protein synthetic apparatus and synthesis of translatable mRNAs. Thus, although the initiation of protein synthesis may be regulated at the level of translation, accumulation of heterotrophically related enzymes is controlled at the level of transcription.

On the other hand, the results presented here suggest that the initiation and development of the photosynthetic function of the cotyledons are controlled at the level of transcription, being dependent both upon the synthesis of cytoplasmic and chloroplast ribosomes and mRNAs.

The decline in translatable mRNAs for the glyoxylate cycle enzymes after day 3 was shown to be stimulated by light. This stimulation may be indirect in that development of the photosynthetic capacity of the tissue reduces the requirement for heterotrophic growth. The initiation and synthesis of certain autotrophically related proteins and mRNAs were found to be light dependent e.g. chlorophyll a/b binding protein. However, other proteins and mRNAs associated with this growth phase were found to be synthesised in dark-grown tissue and only their continued accumulation was light dependent e.g. RuBPC, GR.

In the work described in this thesis quantitative and qualitative changes in the mRNA population were studied by

estimation of the poly(A) content of the tissue, isolation of poly(A)$^+$ RNA and the ability of the wheat-germ cell-free translation system to translate added cotyledonary mRNA into discrete polypeptides. Antisera raised against purified cucumber glyoxylate cycle enzymes were used in conjunction with *in vitro* translation and *in vivo* labelling experiments to determine the developmental changes in translatable mRNAs for, and *in vivo* synthesis of, these proteins. In order to confirm the results presented here it would be advisable to utilise other methods currently available to quantitatively assay for the presence of specific mRNAs within the cucumber cotyledons at different developmental stages.

The preparation of specific cDNA probes for ICL and MS mRNAs would facilitate a more discriminatory assay of these particular mRNAs. Preparation of a cDNA probe first requires isolation of the specific mRNA. This may be achieved by polysome immunoprecipitation (for review, see Taylor, 1979). In order to reduce non-specific reaction of the antibody with the polysomes, the antiserum can first be immunopurified by affinity chromatography using purified antigens bound to a solid matrix (e.g. Palacios *et al.*, 1973). This may be of greater importance when using this procedure for purification of mRNAs, such as those for ICL and MS, which constitute a small percentage of the total mRNA population. Since mRNAs for ICL and MS have been shown to be present in the poly(A)$^+$ RNA fraction, subsequent isolation of the mRNA may be achieved

by affinity chromatography techniques (Aviv and Leder, 1972; Firtel and Lodish, 1973). The purified poly(A)$^+$ mRNA can then be used to synthesise cDNA using reverse transcriptase and an oligo (dT) primer (Verma et al., 1972; Taylor, 1979). If sufficient cDNA can be prepared this may be used directly for the assay, otherwise it can be cloned and subsequently re-isolated. The cDNA of cloned DNA can then be used to assay for the presence of mRNA by hybridisation with RNA from different developmental stages. If the cotyledonary RNA is 'end labelled' with ^{32}P, then hybridisation can be carried out after immobilising the cDNA on nitrocellulose filters and the labelled hybrids detected by autoradiography (for review, see Dyer and Leaver, in press). Otherwise the hybridised mRNA can be released from the filter and used to programme the wheat-germ system (Smith et al., 1979).

If the cDNA or cloned DNA is bound to a solid matrix (e.g. cellulose) it could also be used for further purification of specific mRNAs (Levy and Aviv, 1976; Anderson and Schimke, 1976; Hirsh et al., 1978). It would then be possible to determine the sequence of the mRNA or cDNA by oligo nucleotide fingerprinting (Cowan et al., 1976) or partial digestion (Simoncsits et al., 1977; Mascam and Gilbert, 1977) procedures.

A large bodyof evidence has accumulated which shows that many viral and eukaryotic mRNAs are derived from structural genes which contain intervening sequences in the coding region of the molecule (Darnell, 1978). The

mRNAs are derived by spicing of the primary transcript in order to eliminate these intervening sequences. Thus, sequence analysis of cDNAs will not necessarily show the sequence of the respective gene. In order to overcome this problem, preparation of specific genes by cloning procedures may be used. Initially this was carried out by cloning of specific DNA sequences partially purified from the genomic DNA (Tonegawa et al., 1977; Tilghman et al., 1978). However, it has now been shown that such procedures can be adapted for unfractionated DNA with successful identification of specific cloned fragments.

Blattner et al. (1978) and Maniatis et al. (1978) prepared unfractionated eukaryotic genomes, fragmented to a suitable size to be efficiently inserted into the selected phage by in vitro packaging and the recombinants were subsequently amplified in bacterial hosts. Screening of the clones was achieved by adaption of the radioactive plaque screening technique described by Benton and Davis (1977). This procedure allows for the entire purification of a specific DNA fragment by clonal replication. It also has the added advantage that more than one gene can be purified at one time.

Identification of specific cloned genes can be achieved by various procedures. Availability of specific cDNA probes allows for cDNA:DNA hybridisation as a detection procedure. Similarly purified mRNAs which have been 'end labelled' (Dyer and Leaver, review, in press) may be

used to hybridise with the cloned DNA fragments. If cDNA probes or purified mRNAs are not available selection of specific fragments may be achieved by hybridisation with RNA from two different developmental stages, assuming expression of the gene of interest is known to be stage specific. This may, however, yield a group of stage specific genes. Further identification of specific genes in this case could be achieved by release and isolation of the mRNAs which hybridised to different clones and use of each of these in a cell-free translation system. The identification of specific cloned genes would be achieved by analysis of the translation product of the hybridised mRNA (Smith et al., 1979).

This procedure could be utilised for purification and isolation of genes for the glyoxylate cycle enzymes. These could then be characterised and sequenced to determine the presence or absence of intervening sequences. The purified genes could also be used in the same manner as cDNA probes to assay for the presence of specific mRNAs during cotyledon development and differentiation. Since it was found that low, though detectable levels of ICL and MS mRNAs were present in dry seed tissue, the procedures described above could also be used to determine when these mRNAs were first synthesised during embryogenesis and seed maturation.

Another problem in glyoxysome biogenesis which remains to be answered is the method by which the enzymes are

sequestered within these organelles. As previously discussed (p. 318) the answer to this requires a study of the ability of different membrane systems to process and/or sequester the polypeptides. Such a study could also be extended to determine if glyoxysomes and peroxisomes share a common membrane. The transport of polypeptides across membranes of different cell compartments requires that there is specificity in the recognition between polypeptide and membrane so that polypeptides are sequestered within the correct compartment (Blobel et al., 1979). If peroxisomes and glyoxysomes have different recognition sequences in their membranes it would be possible to show that polypeptides specific to one type of microbody cannot be transported across the membranes of the other or the ER membranes associated with the other. This would indicate that the transition from glyoxysomes to peroxisomes within the cotyledons is due to the synthesis of two discrete organelles and would thus substantiate the "two population" model (see p. 30 ; Beevers, 1979). However, the ability of both classes of microbody or their associated ER to compartmentalise the same polypeptides would indicate that the difference between them is only temporal.

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APPENDIX

Calculation of 'Factors' used in Enzyme Assays to Convert Absorbance Change to Enzyme Units

Beer-Lambert's Law $A = a_m bc$

where A = absorbance

a_m = molar extinction coefficient (litre.mole^{-1}.cm^{-1})

b = pathlength of light through solution (in cm)

c = concentration of solute (in moles.litre^{-1})

Example of Calculation for ICL Assay

Since the assay was carried out to determine absorbance change (ΔA), the element of time can be introduced into the Beer-Lambert's Law.

$$\Delta A = a_m . b . \Delta c$$

If, for the ICL assay a ΔA of 0·01/2 min was obtained, then, knowing that the $a_m = 1{\cdot}7 \times 10^4$ litre.mole^{-1}.cm^{-1} (for phenylhydrazone - see p. 78) and b = 1 cm, this equation now reads,

$$\Delta c = \frac{0{\cdot}01/2 \text{ min}}{(1{\cdot}7 \times 10^4 \text{ litre.mole}^{-1}.\text{cm}^{-1})\ (1{\cdot}0 \text{ cm})}$$

$$\Delta c = 2{\cdot}94 \times 10^{-7} \text{ mole.litre}^{-1}.\text{min}^{-1}$$

But, the reaction was carried out in a 1 ml volume.

$$\therefore \Delta c = 2{\cdot}94 \times 10^{-7} \text{ mole.litre}^{-1}.\text{min}^{-1} \times 0{\cdot}001 \text{ litre}$$
$$= 2{\cdot}94 \times 10^{-10} \text{ mole.min}^{-1}$$

However, I have defined 1 unit of ICL as that amount of enzyme required to convert 1 nanomole of substrate to products per minute.

$$\therefore \Delta c = 0{\cdot}294 \text{ nmole.min}^{-1}$$

Thus, a ΔA of 0·01/2 min was due to a Δc of 0·294 nmole.min^{-1}. Therefore, to shortcut the calculation, the ΔA may be multiplied by the 'factor' 29·4 in the ICL assay.

This 'factor' is specific for the reaction volume used (1 ml for

this assay).

Using this procedure it was possible to calculate conversion factors for all the enzyme assays and these are shown in the text. In these calculations the a_m for the appropriate substance being measured spectrophotometrically (e.g. phenylhydrazone for the ICL assay) and the volume of the reaction mixture were taken into account.

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"Regulation of Glyoxysomal Enzymes during Germination of Cucumber. 1. Developmental Changes in Cotyledonary Protein, RNA and Enzyme Activities during Germination."
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"Regulation of Glyoxysomal Enzymes during Germination of Cucumber. 3. In Vitro Translation and Characterisation of Four Glyoxylate-Cycle Enzymes."
Plant Physiology - in press.

E. M. WEIR, C. J. LEAVER, H. RIEZMAN and W. M. BECKER

"Regulation of Glyoxysomal Enzymes during Germination of Cucumber. 4. Developmental Changes in the Messenger RNAs for Isocitrate Lyase and Malate Synthase."
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Regulation of Glyoxysomal Enzymes during Germination of Cucumber

I. DEVELOPMENTAL CHANGES IN COTYLEDONARY PROTEIN, RNA, AND ENZYME ACTIVITIES DURING GERMINATION[1]

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ABSTRACT

Developmental patterns of glyoxylate cycle and photosynthetic activities have been correlated with electrophoretic profiles of cotyledonary RNA and protein in both light- and dark-grown cucumber seedlings (*Cucumis sativus* L.) Cytoplasmic rRNA increases 10-fold between days 0 and 5, and the steepest increase coincides with the most rapid rise in activities of the glyoxysomal enzymes, isocitrate lyase and malate synthase. Chloroplast rRNA and ribulose bisphosphate (RuBP) carboxylase begin rising at day 3, followed about a day later by increases in glyoxylate reductase activity and chlorophyll content. Of these phototrophic indicators, only chlorophyll requires light for its initial appearance. Sodium dodecyl sulfate gel electrophoresis of total and soluble cotyledonary protein showed several developmental patterns, including: (*a*) progressive disappearance of storage protein present initially in particulate form; (*b*) appearance and subsequent disappearance of a family of polypeptides identified by molecular weight, developmental profile, and density gradient centrifugation as subunits of glyoxysomal enzymes; and (*c*) appearance and progressive increase (in both light- and dark-grown cotyledons) of the large and small subunits of RuBP carboxylase, as well as other polypeptides presumably of chloroplast and peroxisomal origin.

Seed germination in fat-storing species requires a functional glyoxylate cycle to effect net gluconeogenesis from the acetyl-CoA derived by β oxidation of storage triglycerides (3). Although much is already known about the developmental physiology of the glyoxylate cycle enzymes and the glyoxysomal compartment in which they are localized (3, 8, 14, 15, 29, 35), little is understood about the regulatory mechanisms which underlie the developmentally orchestrated expression of this specific metabolic capability. We are interested both in the level(s) at which the activities of glyoxysomal enzymes are regulated during germination of cucumber (*Cucumis sativus*) and also in the control mechanisms involved in the subsequent transition from heterotrophy to autotrophy that takes place in the cotyledon upon emergence and greening.

This paper describes changes in size, cell number, metabolic function, and enzyme activities which occur in cotyledons of light- and dark-grown cucumber seedlings and attempts to relate these changes to developmental changes in cotyledonary protein and RNA, thus providing a characterization of the system necessary for further, more specific studies on the regulation of the glyoxysomal and peroxisomal enzymes. A preliminary report of this work has already been presented (2).

MATERIALS AND METHODS

Culture Conditions. Seeds of *C. sativus* L. var. Long Green Ridge or Improved Long Green were cold-imbibed (16 hr at 4 C) in distilled H_2O, then planted at a depth of about 1 cm in trays of Vermiculite underlaid with soil. Trays were either kept in continuous darkness (dark-grown) or illuminated for 12 hr/day with a mixture of fluorescent and incandescent lamps at an approximate intensity of 6,500 lux (light-grown). The temperature was in both cases maintained at 26 to 28 C for 12 hr, followed by a night depression to 22 C for 12 hr. Germination time was measured in days from planting of the cold-imbibed seeds.

Harvesting of Cotyledons. Cotyledons were harvested at daily intervals from day 0 (cold-imbibed only) to day 7, with harvesting initiated about 1 hr after the onset of illumination for the light-grown seedlings. The number of cells/cotyledon was determined by the method of Brown and Rickless (4). Fresh weight was determined on samples of 40 cotyledons harvested directly into tared, stoppered glass bottles. Oven dry weights were determined after drying the same samples for 24 hr at 90 C. Cotyledons for all other assays were harvested onto dry ice and stored at −80 C.

Homogenate Preparation. Cotyledons (20–100/sample) were first ground to a thick paste in a precooled mortar and pestle, without addition of medium. A volume of 0.05 M K-phosphate (pH 7.5) calculated to yield a supernatant protein concentration of about 5 to 10 mg/ml was then added and grinding was continued. Aliquots of the homogenate were removed for protein and RNA assays, for electrophoretic analysis, and for quantitation of RuBPC[4] protein. The remainder was then centrifuged at 10,000*g* for 10 min at 4 C, and samples of the resulting supernatant were taken for protein assay, gel electrophoresis, and enzyme assays.

Enzyme, Protein, and Chl Assays. Literature procedures were followed for the assay of isocitrate lyase (5), malate synthase (5),

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[4] Abbreviations: RuBPC: ribulose bisphosphate carboxylase; IL: isocitrate lyase; MS: malate synthase; Cat: catalase; GR: NADH-glyoxylate (hydroxypyruvate) reductase; Cyt Ox: cytochrome *c* oxidase; TEMED: N,N,N′,N′-tetramethylethylenediamine; EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N′-tetraacetic acid.

catalase (27), NADH-glyoxylate (hydroxypyruvate) reductase (34, with glyoxylate as substrate), and Cyt *c* oxidase (29). Results were expressed as enzyme units/cotyledon. For catalase, 1 unit is the amount of enzyme required to degrade 50% of the available H_2O_2 in 100 sec; for the other enzymes, 1 unit corresponds to the conversion of 1 nmol of substrate into product/min. Chl was determined by the method of Arnon (1). Protein was assayed by the procedure of Lowry *et al.* (26), after initial precipitation and washing of the protein with 5% (w/v) trichloroacetic acid.

Lipid Assay. Total lipid was determined by a modification of the methanol-chloroform extraction procedure of Radin (28). Cotyledons (3 g) were chopped with razor blades and ground with 10 ml of methanol-chloroform (2:1, v/v). Following low speed centrifugation, the supernatant was decanted and the pellet reextracted by grinding again with methanol-chloroform. Supernatants from both extractions were shaken with an equal volume of 2 M KCl. After phase separation, the organic phase was drained into a preweighed beaker, and the aqueous phase was then washed repeatedly with methanol-chloroform. The pooled organic phases were evaporated to dryness, and the amount of extracted lipid was determined by weight difference.

Electrophoretic Analysis of Cotyledonary Proteins. Proteins present in the total homogenate and in the 10,000*g* supernatant were analyzed by electrophoresis on 15% SDS-polyacrylamide slab gels, according to the method of Laemmli (17). Electrophoresis was carried out at room temperature with a current of 10 mamp (5 mamp during sample run-in) until the bromphenol blue marker reached the bottom of the gel. Gels were stained with 0.1% Coomassie blue in 50% methanol-5% acetic acid for 1 hr at 50 C, then destained by repeated changes of 40% methanol-7% acetic acid.

Electrophoretic Assay for RuBPC. RuBPC protein was determined by electrophoretic fractionation of total homogenate protein under denaturing conditions, followed by recovery and quantitation of the stained protein from the appropriate excised gel region. Gels of 5% acrylamide, 0.125% bis-acrylamide, and 0.0875 M Tris-glycine (pH 9.5) were polymerized with TEMED and NH_4HSO_4, prerun for 30 min at 1 to 1.5 mamp/tube, and loaded with 100 to 200 μg of homogenate protein in 50 mM Tris-glycine (pH 9.5) containing 10% sucrose and bromphenol blue as marker. Gels were run at 1 to 1.5 mamp/tube for 2.5 hr and then stained for 20 hr in 0.5% Amido black 10B in 20% ethanol-7% acetic acid, followed by destaining in 20% ethanol-7% acetic acid. The carboxylase band (identified by coelectrophoresis of the purified enzyme) was excised from the gel and incubated overnight at 25 C in 1 ml of 1 N NaOH. The protein content of the NaOH extracts was quantitated from the *A* at 615 nm, using a standard curve prepared by electrophoresis and NaOH extraction of graded amounts (10–100 μg) of spinach D-ribulose-1,5-diphosphate carboxylase (Sigma Chemical Co). Values obtained can therefore be regarded as relative approximations only.

Electrophoretic Analysis of Proteins from Microbodies Isolated by Density Centrifugation. For analysis of organellar proteins, light-grown cotyledons from days 1 through 6 (5–8 g/day) were harvested fresh and homogenized by chopping with razor blades and subsequent grinding in a mortar and pestle with 2.5 volumes of 0.40 M sucrose in TKME buffer (0.05 M Tris-HCl [pH 7.2] at 25 C, 50 mM potassium acetate, 10 mM magnesium acetate, and 1 mM EGTA). The homogenate was filtered through Miracloth and centrifuged at 600*g* for 10 min. Thereafter 12.5 ml of the supernatant was layered onto a 45-ml linear 16 to 60% (w/w) gradient of sucrose in TKME buffer. Gradients were centrifuged at 58,400*g* for 4 hr in a Spinco SW 25.2 rotor at 4 C. Fractions (1.5 ml) were assayed for sucrose density (determination of a refractive index at 20 C), and for isocitrate lyase, malate synthase, Cyt *c* oxidase, and protein as described above. For SDS-polyacrylamide gel electrophoresis, the protein in each gradient fraction was precipitated with 80% (v/v) acetone, redissolved in sample buffer, and loaded on a per-cotyledon basis onto 15% SDS-polyacrylamide slab gels. To facilitate direct comparison of proteins present in the microbody region of the gradient at each day, fractions corresponding to a buoyant density of 1.26 g/cm^3 (peak activities of isocitrate lyase and malate synthase; one fraction/gradient) were analyzed in adjacent lanes of the same slab gel, with loading again on a per-cotyledon basis.

RNA Assay. Homogenate samples to be assayed for RNA were precipitated with an equal volume of cold 1 N perchloric acid, allowed to stand on ice for 60 min, and then centrifuged at 3,000*g* for 10 min. The pellets were washed twice by resuspension in 5 ml of 0.5 N perchloric acid, then twice with 5 ml of ethanol/chloroform-ether, 2:1:1 (v/v/v). The pellets were allowed to drain and were then dissolved in 1 ml of fresh 0.3 N NaOH. After incubation for 18 hr at 37 C, tubes were cooled on ice and neutralized with cold 70% perchloric acid. The resulting precipitate was removed by centrifugation (3,000*g* for 10 min) and the supernatant was assayed for RNA content by determination of A_{260}, assuming an *A* of 1.0 to correspond to an RNA concentration of 31.7 μg/ml.

Electrophoretic Quantitation of RNA. Cotyledonary RNA was extracted at 0 to 4 C by the phenol procedure of Leaver and Ingle (20). Fractionation was achieved by electrophoresis on 2.4% polyacrylamide gels (25 μg of RNA/gel) as described by Loening (22), but at 5 C to maintain the integrity of the 23S chloroplast rRNA (19). Gels were run for 4 hr with a current of 3 mamp/gel. The 7.5-cm gels were scanned at 265 nm using a Joyce Loebl UV scanner. To quantitate the various species of nucleic acids (25S and 18S cytoplasmic rRNA, 23S and 16S chloroplast rRNA, and 4S + 5S RNA), individual peaks were cut from the scans, weighed, and expressed as a fraction of the total area under the curve for a particular scan. Each such fraction was then multiplied by the total RNA content (in μg/cotyledon) determined as described above for cotyledons of the same stage and growth conditions.

RESULTS

Seeding Development. The characteristic appearance of light-grown cucumber seedlings is illustrated in Figure 1. To ensure maximum uniformity among cotyledons used for analyses, plants were selected to correspond as closely as possible to the morphological stages shown in Figure 1. Dark-grown seedlings were similar to those shown in Figure 1 for days 0, 1, 2, and 3, but thereafter were characterized by marked hypocotyl elongation, retention of the hypocotyl hook, and lack of cotyledonary expansion or greening.

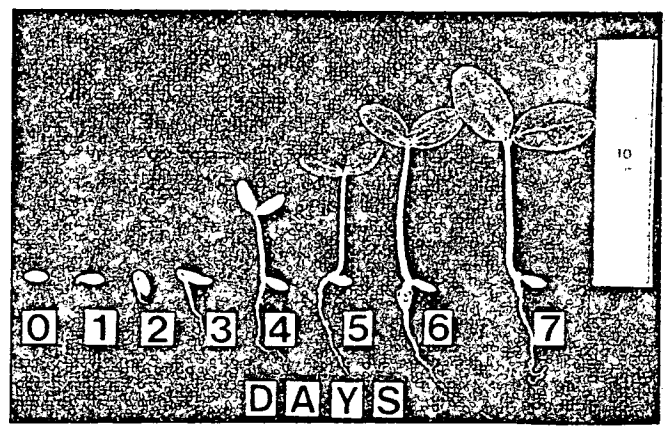

FIG. 1. Growth and development of light-grown cucumber seedlings. Seeds were cold-imbibed (16 hr at 4 C), then planted in Vermiculite and germinated under a 12/12 hr light/dark cycle, with time of germination measured in days from planting. Greening of cotyledons begins at the proximal end upon emergence from the seed coat at day 3 and is essentially complete by day 5.

Upon emergence from the seed coat, light-grown cotyledons undergo a rapid increase in size and fresh weight not seen in the dark (Fig. 2A); between days 1 and 7, fresh weight increases 10-fold in the light, but only 2-fold in the dark. This increase is due entirely to water uptake, as seen from the dry weight data (Fig. 2B), which actually decreases through day 4, reflecting the progressive depletion of both fat and protein stores. (The modest increase in weight seen in light-grown cotyledons after day 5 is presumably due to the accumulation of photosynthetically derived mass.) The decrease in cotyledonary dry weight is almost exactly balanced by an increase in the dry weight of the rest of the plant axis; the combined dry weight of axis plus two cotyledons is 22.6 mg at day 0, 22.1 mg at day 4 in the light, and 22.6 mg at either day 4 or day 7 in the dark.

Cell number remains constant through the time period under study. Ungerminated cucumber embryos contain about 6.78×10^5 cells/cotyledon, and this does not change significantly during the first 7 days of development in either light or dark. (Averaged over all stages and expressed as $\bar{x} \pm s_{\bar{x}}$, cell numbers for light- and dark-grown seedlings were, respectively, $6.78 \pm 0.08 \times 10^5$ and $6.84 \pm 0.15 \times 10^5$ cells/cotyledon.) This agrees well with electron microscopic studies (35) showing no cell division in cucumber cotyledons. Similar conclusions have been reached for cotyledons of other species (24, 36). Table I is a summary of the data for light-grown cotyledons, expressed on a per-cell basis (assuming 6.78×10^5 cells/cotyledon for all stages).

Heterotrophic Indicators. Developmental profiles for lipid utilization and for several glyoxysomal enzymes are shown in Figure 3. Lipid metabolism begins at day 1 and is essentially complete by day 6. This correlates well with the progressive loss of lipid bodies seen in ultrastructural studies (35). As expected (15, 29, 35), isocitrate lyase and malate synthase rise from near-zero levels in the ungerminated seed to peak activities at a time (day 4) which coincides with the period of most rapid lipid depletion. Activities then decrease rapidly and disappear within a week (slightly longer in the dark; about 10% of the peak activity of each enzyme is still left at day 8 in the dark). The same rise and fall of glyoxylate cycle activities are seen in half-cotyledons with and without the embryonic axis attached (D. G. Kerley and W. M. Becker, unpublished observations), confirming the findings of others (7, 32) that these enzymes are not under control of the axis. Catalase resembles isocitrate lyase and malate synthase in its activity profile during early stages, but retains about half of its peak activity at later stages, presumably because it is an enzyme common both to the glyoxysomes involved in heterotrophic metabolism during early stages and to the peroxisomes present at later stages.

Autotrophic Indicators. Several specific indicators of autotrophic function are presented in Figure 4. Their appearance coincides with the arrival of the seedling above ground and the emergence of the cotyledons from the seed coat. RuBPC precedes Chl by at least 24 hr in its initial appearance and, unlike Chl, does not depend upon light as a trigger, although illumination obviously affects the levels of RuBPC protein present at later stages (*i.e.* after day 4). This accords well with the report of Dockerty *et al.* (6) that light is not essential for RuBPC development in either the cotyledons or the endosperm of germinating castor bean. A similar pattern, though somewhat delayed in time, is seen for glyoxylate (hydroxypyruvate) reductase. Like RuBPC, this peroxisomal enzyme seems to depend upon light for attainment of full activity, but not for its initial appearance. Other workers have also reported that both this reductase and glycolate oxidase, a companion peroxisomal enzyme, can undergo at least some increase in activity in dark-grown cotyledons at a time in development when activity can be promoted by light (8, 9, 29–31, 33).

Cotyledonary Protein. The protein content of the cotyledonary homogenate and of the 10,000*g* × 10-min supernatant fraction is depicted as a function of germination time for light-grown seedlings in Figure 5A. In the dark (data not shown), the pattern is essentially the same, except that the decrease continues throughout the experiment, dropping by day 7 to 1.57 mg/cotyledon, com-

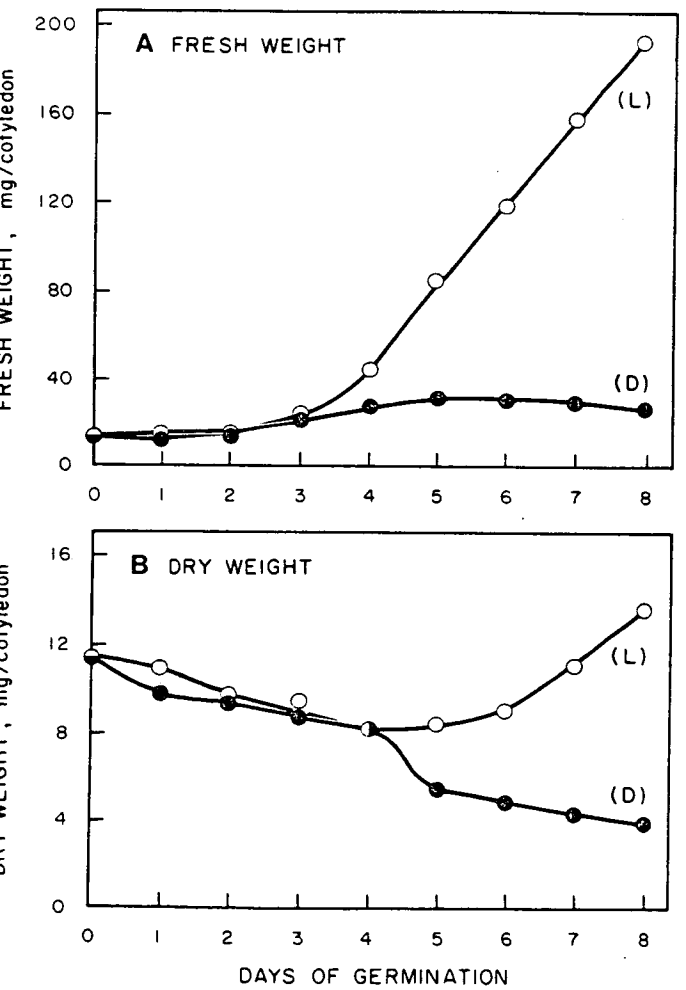

FIG. 2. Developmental changes in weight of cotyledons from cucumber seedlings grown in the light (○——○) or in the dark (●——●). A: fresh weight; B: dry weight.

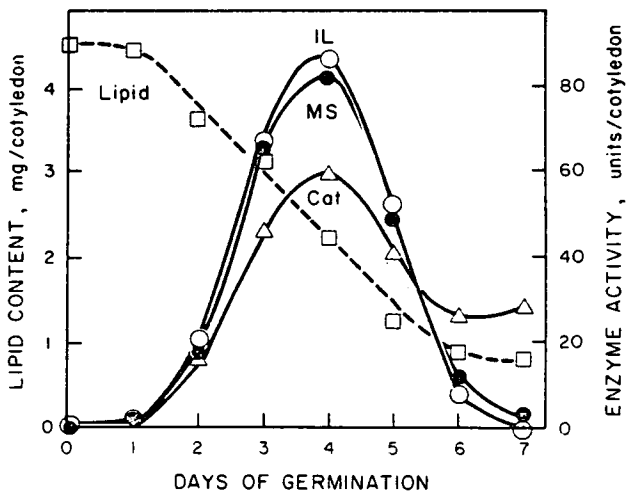

FIG. 3. Developmental changes in lipid content and glyoxysomal enzyme activities in cotyledons of light-grown cucumber seedlings. Homogenates were prepared from cotyledons of each seedling stage and were assayed as described in the text for isocitrate lyase (IL; ——○——), malate synthase (MS; ——●——), catalase (Cat:b—△——), and total lipid content (——□——), with all values expressed on a per-cotyledon basis.

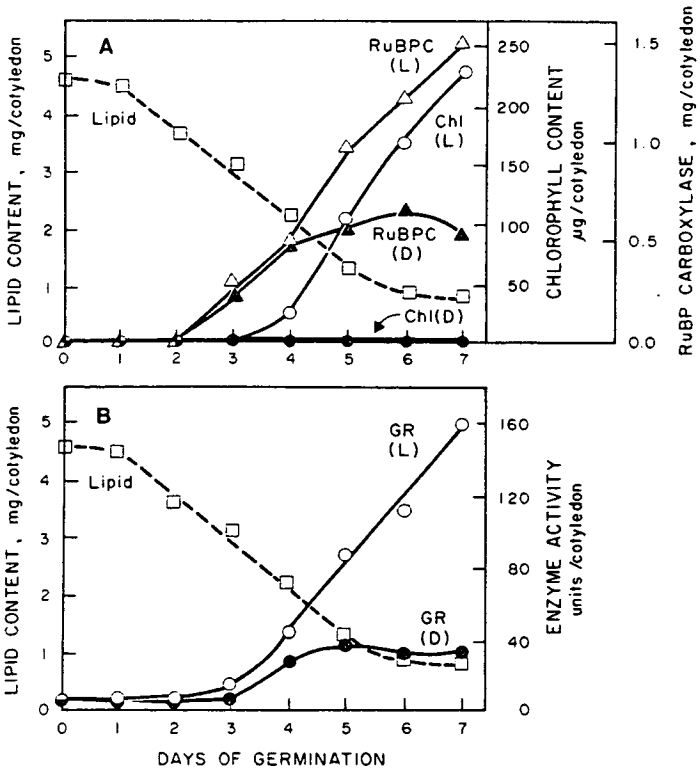

FIG. 4. Developmental changes in chloroplast and peroxisomal markers in cotyledons of light-grown (open symbols) and dark-grown (closed symbols) cucumber seedlings. A: amounts of RuBPC protein (△——△) and Chl (○——○) per cotyledon. B: activity of glyoxylate (hydroxypyruvate) reductase (GR; ○——○) per cotyledon. Lipid content (□——□) as in Figure 3.

pared with 2.23 mg/cotyledon in the light. Prior to day 2, most (75–85%) of the homogenate protein sediments upon centrifugation at 10,000*g* for 10 min, presumably because it is present in storage protein bodies. Protein bodies are prominent ultrastructural features of cucurbit cotyledons (25, 35) and are recovered with glyoxysomes at a density of 1.26 g/cm^3 upon sucrose density centrifugation of organellar preparations from early stages (see below). A striking decrease in sedimentable protein occurs between days 2 and 3, such that by day 4 more than 80% of the total protein is present in a form which no longer sediments at 10,000*g*. The same pattern of progressive mobilization of cotyledonary protein is seen in both the light and the dark, as shown in Figure 5B.

Shown in Figure 6 are SDS-polyacrylamide gel profiles of homogenate and supernatant protein for both light- and dark-grown cotyledons, loaded on a per-cotyledon basis to facilitate direct comparison of band intensities. The most prominent developmental feature of the homogenate gels (Fig. 6, A and B) at early stages is the progressive disappearance of a cluster of low mol wt polypeptides (mol wt 20,000–35,000), almost certainly representing storage proteins located initially in protein bodies. The striking change in sedimentable protein shown in Figure 5 is mirrored in the supernatant profiles of Figure 6, C and D by the sudden appearance at day 3 of a series of low mol wt bands, probably corresponding to solubilized but as yet incompletely digested storage polypeptides. Although the initial solubilization of particulate storage proteins occurs at the same rate in both light and dark, the resulting soluble polypeptides persist significantly longer in the dark (Fig. 6).

Also discernible on the gels of Figure 6 is a family of polypeptide bands which can by their mol wt range (45,000–75,000) and developmental pattern be tentatively identified as glyoxysomal enzyme subunits. This identification is facilitated by the subunit

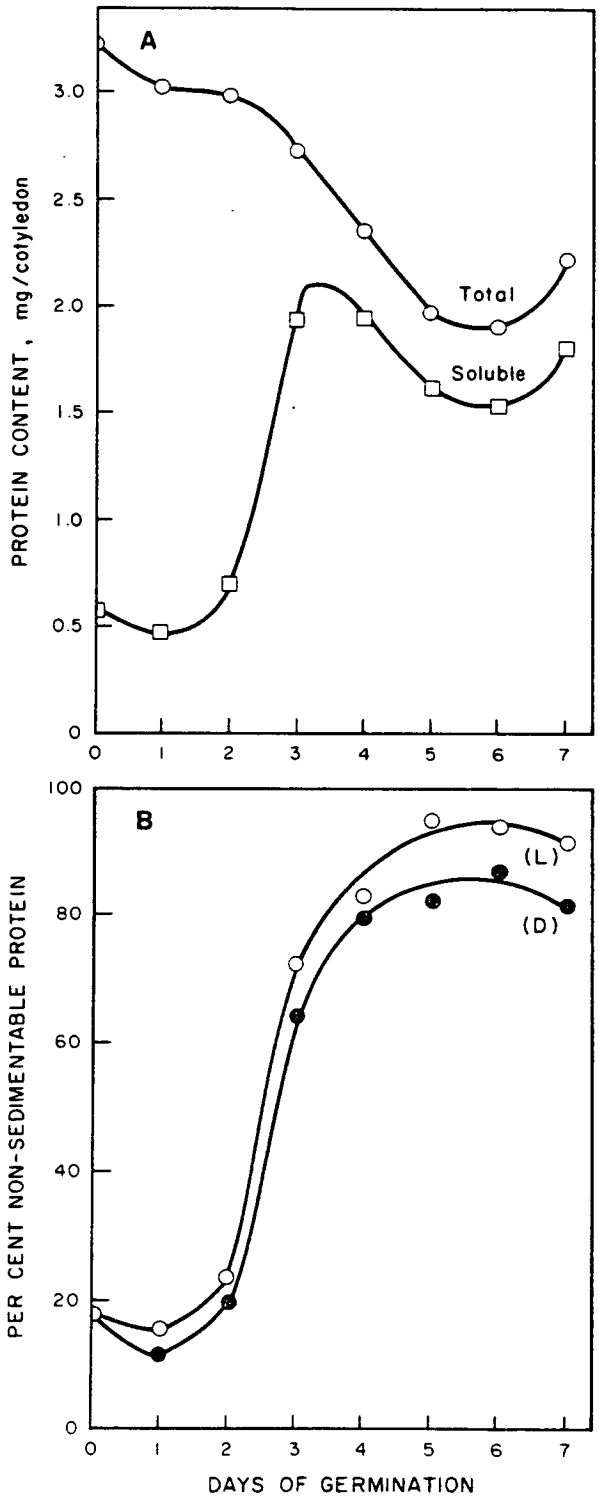

FIG. 5. Developmental changes in protein content of cucumber cotyledons during germination. A: amount of total (○——○) and soluble (□——□) protein per cotyledon. The difference between the two values at any given time point represents the amount of total homogenate protein removed by centrifugation at 10,000*g* for 10 min. B: nonsedimentable (10,000*g* supernatant) protein expressed as a per cent of total cotyledonary protein for light-grown (○——○) and dark-grown (●——●) seedlings.

mol wt now available (16) for several glyoxysomal enzymes from cucumber, including isocitrate lyase (63,000), malate synthase (63,000), citrate synthase (46,000), malate dehydrogenase (37,000), crotonase (75,000), thiolase (45,000), and catalase (54,000). Bands in this mol wt range become detectable by about day 2, peak

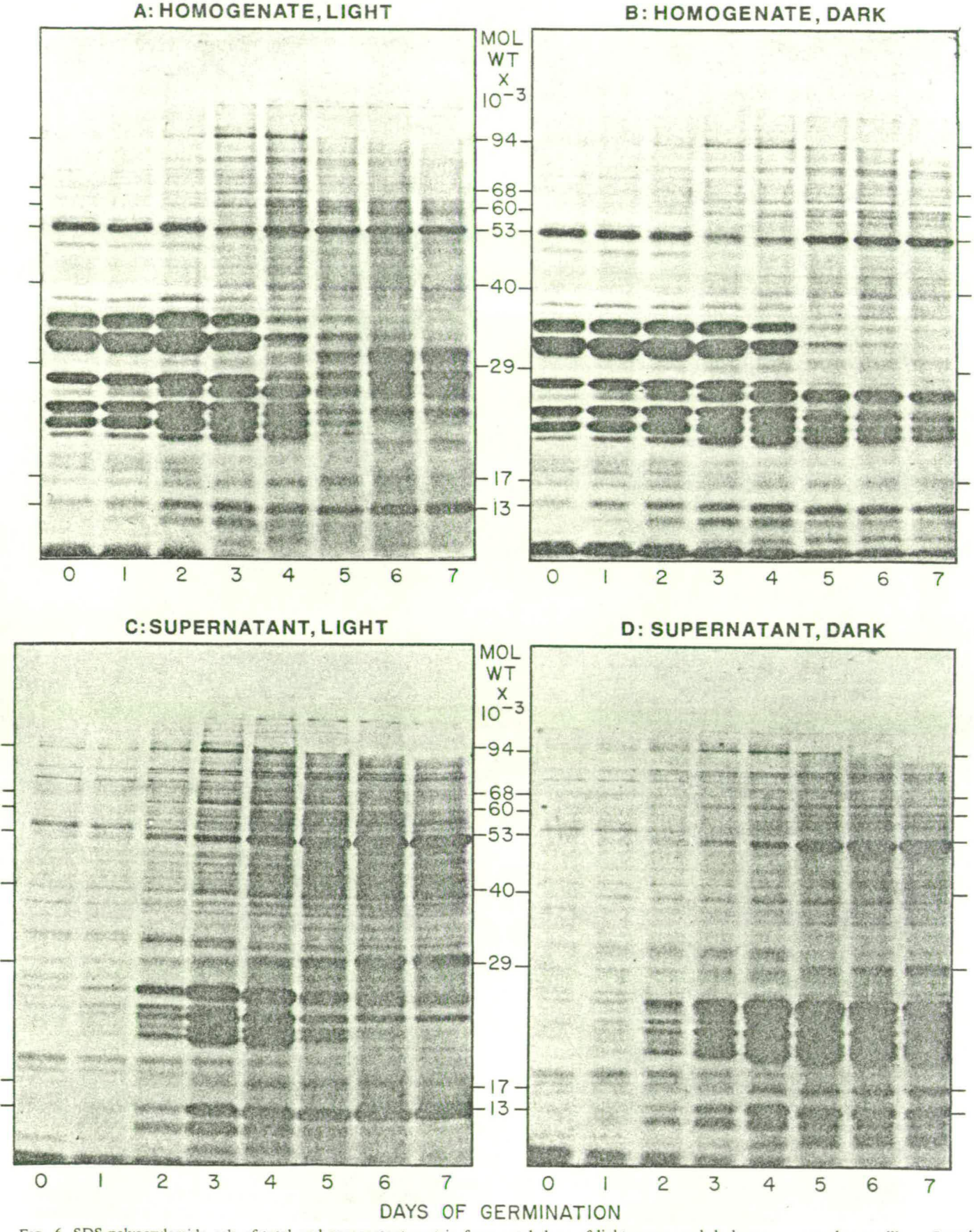

FIG. 6. SDS-polyacrylamide gels of total and supernatant protein from cotyledons of light-grown and dark-grown cucumber seedlings. Samples were loaded on a per cotyledon basis onto 15% polyacrylamide slab gels and subjected to electrophoresis as described in the text. Actual amounts of protein applied corresponded to 2.0% (homogenate) or 5.0% (supernatant) of the protein content of a single cotyledon at a given stage. Upper gels: homogenate proteins from light-grown (A) and dark-grown (B) seedlings. Lower gels: supernatant proteins from light-grown (C) and dark-grown (D) seedlings. Mol wt markers are, from top to bottom: phosphorylase (94,000), BSA (68,000), bovine catalase (60,000), carbonic anhydrase (29,000), and myoglobin (17,200). Positions of the large (53,000) and small (13,000) subunits of RuBPC are also indicated.

ntensities at day 4, and decrease in prominence thereafter, just as vould be expected for glyoxysomal enzymes, assuming a direct orrelation between activity and enzyme protein. Specifically, ands of the appropriate mol wt for the subunits of isocitrate yase, malate synthase, and catalase are readily identifiable and orrelate well in intensity across the gels of Figure 6 with the ctivity profiles for these enzymes in Figure 3. A further family of ands can be identified which increase in intensity after about day and appear to reflect the developmental patterns of the autorophic functions depicted in Figure 4. Especially prominent mong these polypeptides are the large (53,000) and small (13,000) ubunits of RuBPC.

Microbody Proteins. To identify specific polypeptides of Figure more directly with glyoxysomal function, cotyledonary homoenates were prepared in the presence of 0.4 M sucrose and ubjected to equilibrium density centrifugation on linear sucrose radients. As seen from the representative gradient profile (for ay 3) shown in Figure 7, glyoxysomal marker enzymes (isocitrate yase and malate synthase) band at a density of 1.26 g/cm^3, while he mitochondrial marker (Cyt *c* oxidase) peaks at about 1.185 /cm^3, with minimal cross-contamination. Figure 8 shows the gel rofiles for the peak microbody fraction (ρ = 1.26 g/cm) from radients corresponding to days 1 through 6 (light-grown), all oaded on a per-cotyledon basis. Initially (days 1–2), the most rominent polypeptides present in this region of the gradient are hose tentatively identified from Figure 6 as storage proteins. The ost prominent of these storage polypeptides have mol wt of 1,000, 23,000, 26,000, 30,500, 33,500, and 35,500. Their recovery n this region of the gradient confirms their presence in particulate orm at early stages. Their subsequent mobilization during gerination is substantiated by progressive disappearance of these olypeptides from this region of the gradient, accompanied by a eciprocal appearance at the top of the gradient of soluble polyeptides of the same or slightly lower mol wt (gels not shown). Iore relevant to our specific interest in the glyoxysomal enzymes s the family of higher mol wt polypeptides which appear at the .26 g/cm^3 region of the gradient at day 2, peak in intensity at ay 4, and disappear thereafter. This accords well not only with he over-all pattern expected of glyoxysomal enzymes (Fig. 3) but lso with the relative activities of isocitrate lyase and malate ynthase actually recovered in this region of the density gradient t each stage (*cf.* Fig. 7; other gradients not shown).

Cotyledonary RNA. One of the most striking features of cotyedonary development is the large increase in cellular RNA, due ainly to the accumulation of rRNA, as shown in Figure 9.

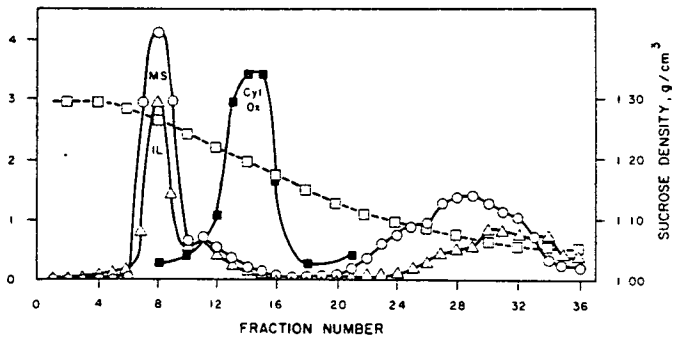

FIG. 7. Distribution of glyoxysomal and mitochondrial marker enymes on a sucrose density gradient. The 600*g* × 10 min supernatant from homogenate of 3-day light-grown cotyledons was applied to a 45-ml ucrose gradient (16–60%, w/w) and centrifuged to equilibrium (4 hr at 2,000 rpm in a Spinco SW 25.2 rotor). Fractions (1.5 ml) were assayed or isocitrate lyase (IL; △——△), malate synthase (MS; ○——○), Cyto idase (Cyt Ox; ■——■), and for sucrose density (□——□; determined om the refractive index at 20 C). To calculate actual enzyme activities in its/fraction, multiply ordinate values by 1,000 for isocitrate lyase and alate synthase and by 150 for Cyt *c* oxidase.

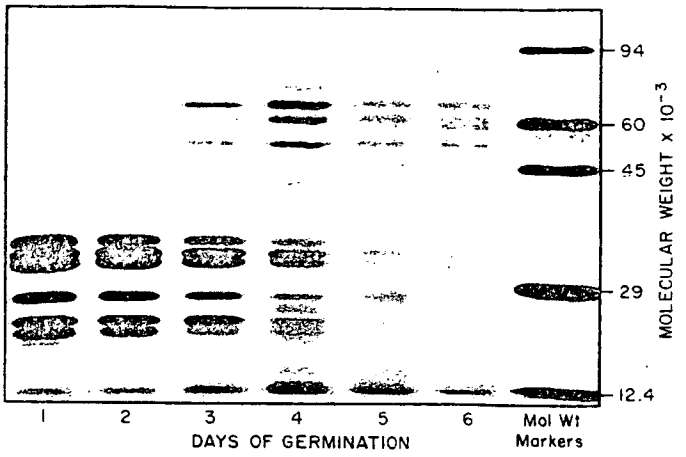

FIG. 8. SDS-polyacrylamide gels of protein from the peak microbody fractions of sucrose density gradients for light-grown cucumber cotyledons. Cotyledons from days 1 through 6 were ground in the presence of 0.4 M sucrose and the 600*g* × 10-min supernatant from each stage was centrifuged to equilibrium on a 45-ml sucrose gradient (16–60%, w/w). Conditions of centrifugation, collection, and assay were as in Figure 7. Protein was precipitated from each fraction with 80% acetone, redissolved in electrophoresis sample buffer, and loaded on a per cotyledon basis onto 15% polyacrylamide slab gels for electrophoresis as described in the text. The actual amount of protein applied corresponded to that portion of the protein from two cotyledons which was recovered from the sucrose gradient in the 1.5-ml fraction corresponding most closely to a density of 1.26 g/cm^3. Gels correspond, from left to right, to days 1, 2, 3, 4, 5, and 6. Mol wt markers in the right lane are, from top to bottom: phosphorylase (94,000), bovine catalase (60,000), ovalbumin (45,000), carbonic anhydrase (29,000) and Cyt *c* (12,400).

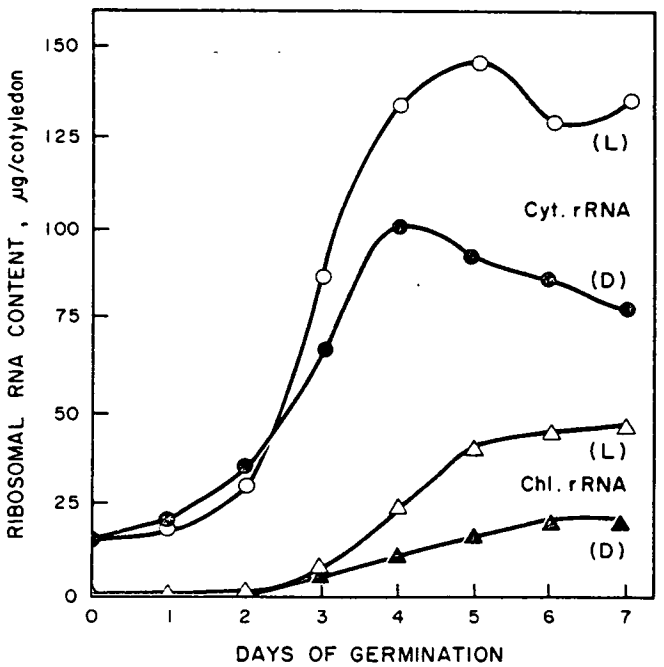

FIG. 9. Developmental changes in rRNA content of cucumber cotyledons during germination. Amounts of cytoplasmic (18S + 25S) rRNA (○——○) and chloroplast (16S + 23S) rRNA (△——△) per cotyledon were calculated for light-grown (open symbols) and dark-grown (closed symbols) seedlings by subjecting total phenol-extracted RNA from each stage to electrophoresis and scanning as described in Figure 10. The area under each rRNA peak was expressed as a fraction of the total area under the scan curve, and that fraction was then multiplied by the total RNA content (in μg/cotyledon) determined as described in the text.

Cytoplasmic rRNA (18S + 25S) increases almost 10-fold (from 15 to 148 μg/cotyledon) between days 0 and 5 in the light. The maximum rate of accumulation occurs between days 2 and 4, which correlates well with the most rapid increase in glyoxysomal enzyme activities (Fig. 3). Low mol wt RNA (4S + 5S) follows a similar pattern, increasing between days 0 and 5 from about 3 μg/cotyledon to a high of about 24 μg/cotyledon in the light and 12 μg/cotyledon in the dark (data not shown). Chloroplast rRNA (16S + 23S) also undergoes a dramatic increase during cotyledonary development in both the light and the dark, as seen both in the data of Figure 9 and on the gels of Figure 10 (which are not loaded on a per-cotyledon basis, however). Chloroplast rRNA is undetectable at days 0 or 1 but accounts for about 20% (dark-grown) to 25% (light-grown) of cellular RNA at day 7. Accumulation of chloroplast rRNA in etiolated cotyledons has also been reported for radish seedlings (13). For cucumber, the maximum rate of chloroplast rRNA accumulation occurs between days 3 and 5 in both light and dark, about 24 hr after the most rapid increase in cytoplasmic rRNA. This coincides with the most rapid increases in the several autotrophic indicators of Figure 4.

DISCUSSION

Much of the physiological data presented here have been reported previously for cucumber (35) or other (8, 14, 15, 29) fat-storing species, but few prior attempts have been made to correlate developmental changes in enzyme activities with electrophoretic protein and RNA patterns. In addition, it seems useful to have all of this information compiled for the same species as a data base for further studies on the molecular mechanisms underlying glyoxysomal enzyme appearance and organellar biogenesis.

Cotyledonary metabolism during cucumber germination is characterized initially by gluconeogenic utilization of stored fat via the glyoxylate cycle. By subjecting cotyledonary protein to SDS-polyacrylamide gel electrophoresis, specific bands can be identified which by size and developmental pattern appear to be subunits of glyoxylate cycle enzymes. This identification is strengthened by recovery of the same family of bands with the same developmental profile at a density of 1.26 g/cm^3 in sucrose gradients. Further confirmation is afforded by the observation that antiserum against either isocitrate lyase (18) or malate synthase from cucumber cotyledons reacts specifically with a single protein present in both the homogenate and a 1.26 g/cm^3 gradient fraction (J. E. Lamb and H. Riezman, unpublished observations). The good agreement in developmental profile between glyoxysomal enzyme activities and actual amounts of subunit protein detectable on gels is consistent with reports linking the appearance of glyoxysomal enzyme activities to *de novo* enzyme synthesis in a variety of species (10–12, 23), and suggests in addition that the subsequent disappearance of activity is a consequence of enzyme degradation.

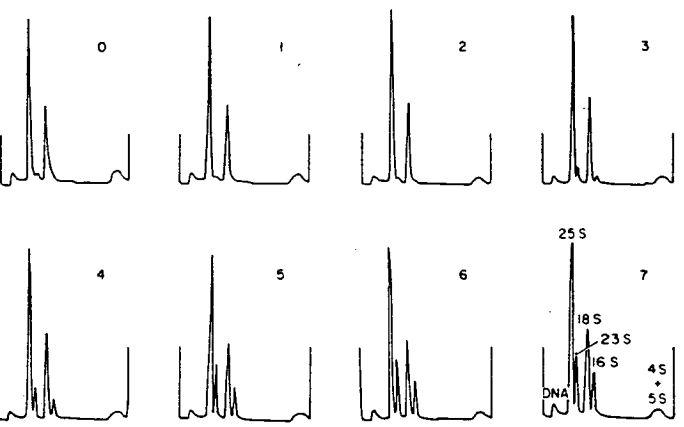

FIG. 10. Acrylamide gel profiles for RNA from cotyledons of light-grown cucumber seedlings. Each gel (10 cm; 2.4% polyacrylamide) was loaded with about 25 μg of phenol-extracted total nucleic acid, subjected to electrophoresis at 5 C for 4 hr with a current of 3 mamp/gel and then scanned at 265 nm.

The rapid increase in glyoxysomal enzyme activities between days 2 and 4 correlates well with the accumulation of cytoplasmic rRNA, and therefore presumably with the capacity of the cotyledonary cell for cytoplasmic protein synthesis. This is accompanied by increases in several indicators of messenger RNA availability, including a 9-fold enhancement in the level of poly(A)-containing RNA between days 0 and 5, a marked increase in the polysome content between days 1 and 5, and a dramatic increase, especially between days 2 and 3, in the ability of total cotyledonary RNA to stimulate amino acid incorporation in a cell-free protein-synthesizing system derived from wheat germ (21; E. M. Weir and C. J. Leaver, unpublished observations).

Concomitant with fat metabolism, protein reserves of the cotyledon are also mobilized and degraded. Initially, most of the protein of the cotyledon appears to be present as storage protein bodies which sediment rapidly at 10,000*g* and band in sucrose at or near the glyoxysomal density of 1.26 g/cm^3. The progressive mobilization of this particulate protein apparently involves initial solubilization to a nonsedimentable form, followed by gradual degradation of the solubilized polypeptides, probably to the amino acid level. The initial solubilization occurs at the same rate and to the same extent in both light- and dark-grown cotyledons, but the resulting polypeptides then disappear more rapidly in the light than in the dark. The significance of this observation is not yet clear, but it may suggest a light dependence of the proteolytic enzymes required for further digestion. Also unresolved is the extent to which the resulting amino acids are reutilized for protein synthesis within the cotyledon or are instead translocated to the growing axis. That substantial protein synthesis must occur within the cotyledon is clear, however, from the appearance of enzyme activities known to depend upon *de novo* protein synthesis (*e.g.* the glyoxysomal enzymes) and especially from the accumulation of large amounts of the single protein, RuBPC.

The accumulation of carboxylase protein and the appearance of chloroplast rRNA are among the most striking manifestations of the transition to autotrophy that occurs upon seedling emergence. From the rRNA data, it can be calculated (Table I) that the number of chloroplast ribosomes increases from essentially none at days 0, 1, and 2 to about 26 million/cell by day 7 (compared to about 60 million cytoplasmic ribosomes/cell at day 7). Most of this increase occurs between days 3 and 5, which

Table I. Postgerminative changes in cotyledonary cells of cucumber

Data of Figs. 2, 3, 4, 5 and 9 are expressed here on a per-cell basis, assuming 6.78 x 10^5 cells per cotyledon at all stages. Number of ribosomes per cell calculated by assuming both 70S and 80S ribosomes to be 50% rRNA (16S + 23S and 18S + 25S, respectively).

	Age of seedling (Light-grown)			
	day 1	day 3	day 5	day 7
Weight				
Total, ng/cell	23	36	125	233
Dry, ng/cell	16	14	12	16
Water, ng/cell	7	22	113	217
Protein				
Total, ng/cell	4.5	4.0	2.9	3.3
Sedimentable (10000g), ng/cell	3.8	1.1	0.5	0.6
Soluble, ng/cell	0.7	2.9	2.4	2.7
RuBP Carboxylase, ng/cell	~0	0.2	0.7	1.1
Lipid, ng/cell	6.6	4.6	1.9	1.2
Chlorophyll, pg/cell	~0	2	154	332
RNA				
Total, pg/cell	30	154	319	314
Cyt. rRNA (18S + 25S), pg/cell	26	130	217	201
Chl. rRNA (16S + 23S), pg/cell	~0	11	67	75
4S & 5S, pg/cell	4	13	35	37
Ribosomes				
Cytoplasmic (80S), millions/cell	8	39	65	60
Chloroplast (70S), millions/cell	~0	4	24	26

coincides well with the most rapid increases in the several autotrophic indicators investigated. RuBPC is quantitatively the most significant of these indicators; within a few days, its large (53,000) and small (13,000) subunits become the most prominent polypeptides on the gels. Closer examination of the gels reveals a family of polypeptides which appear and increase in synchrony with the carboxylase subunits, presumably because they are components of proteins involved in photosynthesis or related autotrophic functions.

We are at present interested in whether the developmental changes in the levels of specific cotyledonary proteins as documented here are accompanied by, and perhaps dependent upon, comparable changes in the levels of translatable mRNAs, and in the manner in which the synthesis of such proteins is coupled to enzyme packaging and glyoxysomal biogenesis.

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