

Cytoplasmic polyadenylated RNA populations
in Drosophila melanogaster during development

BY

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Declaration:

I declare that the thesis has been composed by myself and that all the work described in the thesis is my own.

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SUMMARY

The number of different cytoplasmic poly(A)-containing RNA molecules present in a Drosophila cell line (L-3) and in embryo, larva, pupa and imago stages have been estimated. The study was carried out by means of DNA-RNA hybridization in RNA excess. It was found that about 5000 different polyadenylated sequences were present in the cytoplasm of the cell line; values of 4000, 3000, 5000 and 3000 are likely estimates for embryos, larvae, pupae and imagos respectively. The one chromosome, one protein coding gene relationship is discussed.

The similarities and differences between mRNA populations from different developmental stages have been studied by means of cross hybridization experiments.

Localization of imago mRNA sequences in the salivary gland polytene chromosomes has been attained by in situ hybridization. A single species of mRNA has been isolated from imagos of Drosophila melanogaster. Preparations with a high degree of purity have been obtained quite simply. The RNA is polyadenylated, has a sedimentation coefficient of about 14S and a molecular weight of 6×10^5 . This mRNA sequence is present at all stages of development. The possible origin and function of this mRNA is discussed.

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INTRODUCTION

Drosophila melanogaster has been the subject of intensive genetic and cytological analysis for many years and this makes Drosophila a particularly suitable organism for biochemical studies.

Genetic approaches to the question of how many genes higher organisms possess, and to differential gene expression during development have some limitations. This moved us to approach these very interesting questions from the biochemical point of view.

Two main events made our investigation possible: the establishment of Drosophila cell lines in culture (Schneider 1972) and the development of new methods for DNA-RNA hybridization (Kohne 1968, Davidson and Hough 1971; Bishop et al., 1974).

Cell lines can be manipulated in ways that are not possible using the whole organism. They provide homogeneous populations of cells in which nucleic acids can be easily labelled to high specific activity and the purification of nucleic acids is facilitated by their lower content of nucleases. Because cells cultivated in vitro grow in a constant and well defined environment, it is possible to study responses to specific agents or environmental changes without interference from other cell types or tissues. Lastly, comparing the results obtained in cell lines with those obtained in whole organisms, it is possible to measure how similar cells cultivated in vitro are to those from the organism from which they were isolated.

DNA-RNA hybridization has become a powerful method for the study of non-repetitive DNA sequences and their RNA products.

One method recently developed is the hybridization of trace amounts of labelled cDNA with an excess of unlabelled RNA. The rate

of the hybridization reaction is inversely proportional to the number of different complementary sequences present in the preparation and directly proportional to their relative abundance. I have used this method to estimate the complexity of populations of polyadenylated RNA isolated from a *Drosophila* cell line and from the different developmental stages of the organism. My aim was to determine how many protein coding genes an organism such as *Drosophila* possesses.

The number of genes in the *Drosophila* genome

The *Drosophila melanogaster* haploid genome contains about 10×10^{10} daltons of DNA, based on kinetic and cytochemical measurements. (Laird, 1971; Rasch et al., 1971). This is two or three times the DNA content of the slime mould *Dictyostelium discoideum*, 40 times that of *Escherichia coli* and only 5% of that of mammals.

Renaturation analysis has shown that 12% of the DNA in *Drosophila* is highly reiterated, 12% has a reiteration frequency of about 70 and the remainder is non-repetitive or unique DNA (Manning et al., 1975). The highly reiterated sequences seem to be clustered in very long blocks, while long stretches of moderately reiterated DNA (6Kb) are interspersed with even longer lengths of non-repetitive DNA (13Kb) (Manning et al., 1975). A very different pattern has been observed in both the amphibian *Xenopus laevis* and the echinoderm *Strongylocentrotus purpuratus* where short repetitive sequences (0.3Kb) are interspersed with not very much longer non-repetitive sequences (1Kb). Only a very small percentage of the repetitive sequences (about 25%) exist in clusters (Davidson et al., 1973). Not only is the organization of *Drosophila* DNA unusual, but also its chromosomal organization differs from that of many other eukaryotes. Polytenization

of the DNA results in the formation of giant chromosomes. The chromosomes reveal a pattern of alternating dark and light regions (bands and interbands), and this pattern may reflect an underlying gene organization. Evidence suggesting that this is the case comes from a genetic analysis of the region 3A1-3C2 of the X chromosome. (Judd, Shen and Kaufman, 1972, 1973): 16 loci were defined by complementation tests and 4 of these could be assigned with reasonable certainty to map^{to} the individual bands (Bishop, 1974). The total number of bands in the region, according to Berendes map (1970) is 16, i.e. the same as the number of complementation groups, and finally most of the mutants in each complementation group are reasonably homogeneous with respect to the time of death during development (Shannon et al., 1972). For these reasons, one band one function correspondence was suggested. Similar analysis exists for the right arm of *Drosophila* chromosome 4 (Hochman, 1971-1973) where 170 mutations were found to be confined to 40 complementation groups and about 50 bands are known to exist. A third study (Lifshytz and Falk, 1968, 1969) concerns 105 induced mutations in the proximal euchromatic region of the X chromosome. These fall into 34 complementation groups, again in agreement with the number of bands present in the region affected. In the study of Judd et al., as well as the one by Hochman, there is evidence to suggest that most of the mutations are point mutations. No chromosomal rearrangements or visible deletions were observed, and the chemical mutagens used to induce mutations, are known to produce point mutations. Their conclusions can not, therefore, be explained as a result of deletions covering more than one locus defined by complementation tests. Another

point open to discussion has been the question of whether the region studied by Judd et al. was really "mutationally saturated" (i.e. all mutants fall into one of the known complementation groups), and whether all the genes are capable of producing a lethal or gross morphological phenotype when mutated (O'Brien, 1973; Bishop, 1974). Theoretically, the genetic screen that Judd and coworkers used should have exposed all the functional units in the region under study. In practice, however, it is possible that some loci passed undetected.

Most of the evidence available thus suggests that the total number of genes in *Drosophila* is equal to the number of chromomeres and that the chromomere (band+ interband), is a unit of function with the assignment of each particular cistron to a particular band on essentially one band:one cistron basis. Nevertheless, cytogenetic localization of cistrons is difficult, and structural detail must be resolved in many cases beyond the powers of the light microscope. Sorsa (1973), attempting to determine the exact location of the gene white (w^+) by means of electron microscopy, discovered that all radiation induced changes in the function of w^+ were associated not with the band 3C2 as it was assumed but with a faint band area at the distal border of the 3C2 band in the X chromosome. This illustrates the difficulty of unambiguously correlating a particular band with its potential products and phenotypic effects.

Another relevant point related to the number of genes expressed in an organism is the extent of transcription and its correlation with the extent of translation.

The extent of transcription in *Drosophila* has been estimated by Turner and Laird (1973). Assuming asymmetrical transcription, RNA

complementary to at least 30% of the *Drosophila* genome is present during pupal metamorphosis; 28, 24 and 20% are the values for embryos, larvae and imagoes respectively. Thus it is clear that RNA transcription is not limited to minor chromosomal regions such as interbands (containing about 5% of the total DNA). There are several examples indicating that the extent of transcription can be much greater than the extent of translation. In mammals, the hnRNA is largely degraded in the nucleus, with at most 10% destined for export to the cytoplasm (Weinberg, 1973) and the size of the average primary transcript is considerably larger than that of mRNA (Darnell, 1968). But *Drosophila*, once more, seems to be different from mammals and the size of the nuclear RNA is not much larger than that of the cytoplasmic RNA (Lengyel, Spradling and Penman, 1975). One good example in insects where the size of the primary transcript (75S) seems to coincide reasonably well with that of the mRNA reaching the cytoplasm is the product of the Balbiani ring 2 of *Chironomus* (Daneholt, 1973). The complexity of the nuclear RNA could nonetheless, be much greater than the cytoplasmic mRNA. In insects, it is possible that the mRNA is derived from nuclear RNA directly, rather than through a process involving cleavage steps (Campo, 1973). If that is true, the numbers of different RNA molecules in the nucleus and cytoplasm (rather than their lengths) would correlate with the relative extent of transcription and translation, and the mechanism operating would select some whole molecules for transport to the cytoplasm while degrading others. It was shown for example that hnRNA is about 10-fold more complex than mRNA in the sea urchin gastrula (Smith *et al.*, 1974), the difference presumably represents sequences that are transcribed within the

nucleus and turnover there, without ever reaching the cytoplasm.

The percentage of the DNA complementary to mRNA sequences, has been estimated in a few cases, the sea urchin gastrula (Galau et al., 1974), HeLa cells (Bishop et al., 1974) and the *Xenopus* oocyte (Davidson and Hough, 1971), with saturation values of 1.3, 1.4 and 1% respectively. These values represent 8000, 33,000 and about 10,000 different mRNA sequences. The genetically-based estimates referred to above suggest that the *Drosophila* genome expresses about 5,000 functions. In what follows I will show that the number of mRNA sequences expressed by *Drosophila* is close to this.

Gene activity during development

The mechanisms by which a single cell gives rise to a more or less complex multi-cellular organism has been, and still is, one of the most interesting problems occupying the attention of biologists.

The process of cell differentiation is probably very closely related to the process of gene activation and repression during development. Developmental studies have been concentrated on echinoderms (see urchin), amphibians (frogs, salamanders) and insects (flies). The reason for such preference is that they are easy to manipulate during development. In contrast, birds and mammals are more difficult to work with, because all the embryological stages take place within the egg or the female parent. Echinoderm and amphibian eggs can be artificially fertilized and manipulated in very diverse ways, contributing much to our present knowledge about the extent to which differentiation is reversible and to positional information.

Differential gene activity has been approached in *Drosophila* in two different ways: (a) by studying the puffing activity in polytene chromosomes and (b) by observing the time of death in lethal

mutants, and the distortions of normal development which precede it.

(a) Gene activity in polytene chromosomes is manifested in the form of discrete swellings known as puffs. They are characterized by despiralization of DNA and local accumulation of RNA and proteins.

In *Drosophila* salivary glands, a temporal sequence of puffing activity takes place from about 10 hours before pupation to about 2 hours after. The number of bands involved is more than 125, and each one puffs at a definite time, for a characteristic period, before regressing (Ashburner, 1973).

That puffs represent the sequential activation and repression of coding genes for demanded products is suggested by the response of some specific puffs to ecdysone (Ashburner, 1973) and to temperature shocks (Berendes, 1968). In the latter case, newly synthesized mRNAs and proteins were identified after heat-shock treatment of a *Drosophila* cell line cultured in vitro. It was shown by in situ hybridization to polytene chromosomes that these specific mRNAs were complementary to the sites that puff after heat-shock (McKenzie, Henikoff and Meselson, 1975).

(b) Most lethal mutations are pleiotropic and act at a particular stage of development. A correlation can be postulated between time of death and activation of the particular mutant gene.

Analysis of the dead embryo larva or pupa will indicate which tissue or part of the body has been mainly affected. Although aphasic mutants (random distribution of death over the whole course of development among individuals of the same genotype) have never been observed, the existence of diphasic (2 lethal phases) and multiphasic mutants complicate the time-of-death-gene-action relationship. Besides, no

direct correlation between time of death and gene action can be convincingly drawn for post-embryonic lethals, where the mutant gene could have been activated much earlier than death takes place. Nevertheless it is interesting that the late embryonic phase and the beginning of pupation are undoubtedly the two phases in *Drosophila* development when death caused by lethal mutations is most likely to occur (Hadorn, 1948, 1951, 1955) (Shannon et al., 1972).

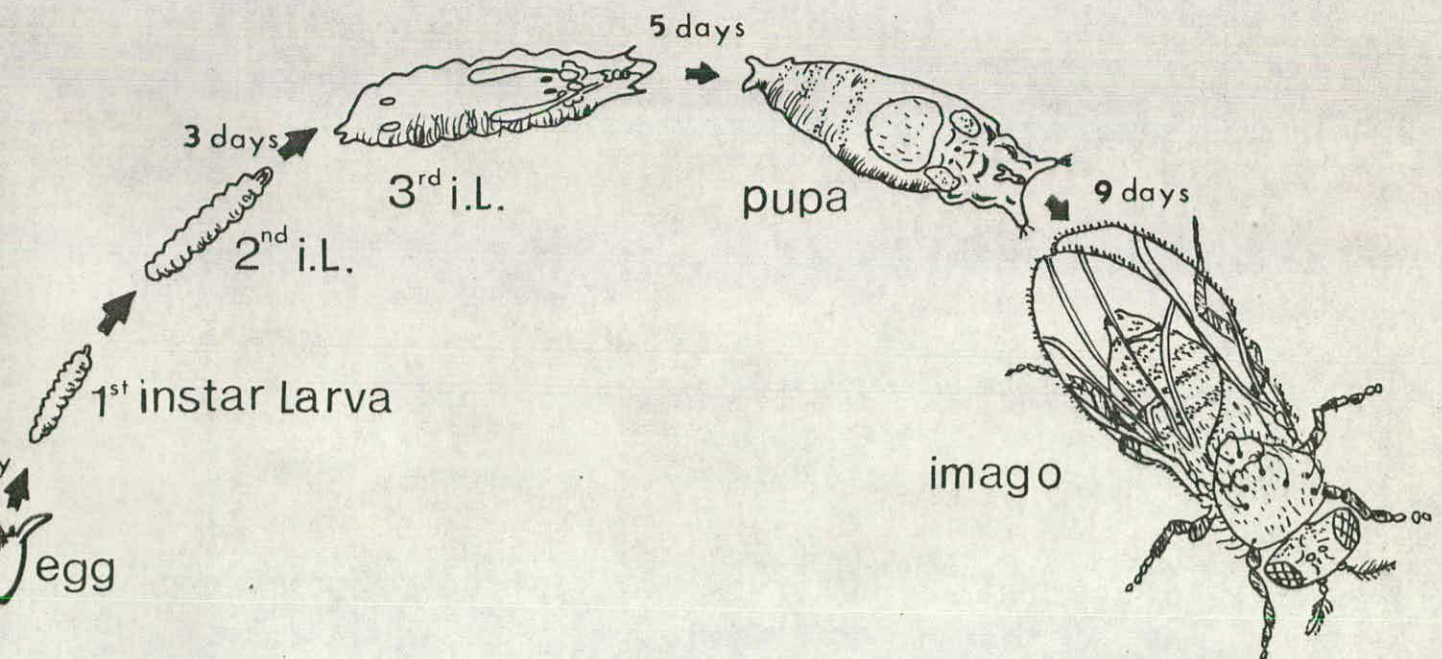
It seems likely that if many genes are activated during embryogenesis and early pupation, the mRNA population at those stages should be more complex than in other more quiescent periods such as the imago. Alternatively, differential gene activity may be due to regulation of concentrations. In this case the complexity of the mRNA would be the same throughout development but the abundance of some messengers would change according to the necessities of the developing organism.

A third possibility is the existence of both mechanisms, i.e. both qualitative and quantitative regulation.

The developmental biology of Drosophila

The mature unfertilized egg is arrested at metaphase of the first meiotic division until entrance of the sperm. It is then fertilized as it passes from the ovary through the uterus prior to being laid.

After fertilization, the nuclei undergo rapid and synchronous divisions to form a syncytium. Most of the nuclei then migrate to the peripheral cytoplasm. At this time, about 3 hours after egg deposition, the plasma membranes form simultaneously over the entire egg surface and about four thousand cells are formed all at once. The embryo is now called a blastoderm. The initiation of imaginal disc formation takes place during gastrulation and the discs are histologically evident in the late embryo. There are ten pairs of major imaginal discs and a genital disc, each characterized by its location in the organism, its size, shape, and by the part it forms in the imago.



Life cycle of Drosophila melanogaster

Ten to eleven hours after egg deposition, most of the larval organs are already formed and mitotic activity ceases in all parts of the embryo except in the nervous system, where certain neuroblasts continue to divide.

The result of this rapid cell, tissue and organ differentiation is a blind, actively feeding larva, in which all the organs, except the imaginal discs, grow by increase in cell size rather than cell number. At the end of larval life, the salivary gland chromosomes attain their maximum polytenization. Each DNA molecule is, at that point, about a thousand times laterally redundant.

During pupation, when the most radical metamorphosis of the insect takes place, many but not all of the larval tissues and organs undergo histolysis, and the new tissues and organs of the imago are formed from the imaginal discs. Even the larval muscles are discarded and the muscles of the imago are formed anew.

Six hours after emergence from the puparium (pupa case), the imago is fully mature and able to mate. This completes the life cycle.

ABBREVIATIONS

RNA	ribonucleic acid
DNA	deoxyribonucleic acid
cdNA	complementary DNA (DNA copied by the reverse transcriptase using RNA as template)
mRNA	messenger RNA
rRNA	ribosomal RNA
poly(A)	polyadenylic acid
poly(U)	polyuridylic acid
polyA(+) RNA	polyadenylated RNA
polyA(-) RNA	non-polyadenylated RNA
oligo(dT)	oligothymidylate
RNase	ribonuclease
DNase	deoxyribonuclease
hnRNA	heterogeneous nuclear RNA
Kb	kilobase (1000 bases or base pairs)
Cot	product of initial DNA concentration (Mols of nucleotides per litre) and time (seconds)
Rot	product of RNA concentration (Mols of nucleotides per litre) and time (seconds)
S ₁	single stranded specific nuclease
HAP	hydroxylapatite:hydrated calcium phosphate $\text{Ca}_5(\text{PO}_4)_3\text{OH}$
NaEDTA	ethylenediaminetetraacetic acid sodium salt
SLS	sodium lauryl sulphate
Tris	Trishydroxymethylaminomethane

TCA	trichloroacetic acid
BSA	bovine serum albumin
DEP	diethyl pyrocarbonate
NP-40	nonidet P.40 (detergent)
BIS	methylenebisacrylamide $\text{NH}_2 \text{COCH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CHCONH}_2$
TEMED	tetramethylethylenediamine $\text{Me}_2 \text{NCH}_2 \text{CH}_2 \text{NMe}_2$

EXPERIMENTAL PROCEDURESSolutions

Homogenization medium:	0.05M TRIS, pH 7.6
	0.025M KCl
	0.005M Mg acetate
	0.35M sucrose
Lysis medium:	0.1M NaCl
	0.01M $MgCl_2 \cdot 6H_2O$
	0.03M TRIS, pH 8.3
Parish-Kirby solution:	0.5% SLS
	1% Na-triisopropyl-naphthalene sulphonate
	6% Na-4-amino salicylic acid
	1% NaCl
	6.6% phenol
Binding buffer:	0.4M NaCl
	10mM Tris pH 7.5 at r.t.
	1mM EDTA
	0.1% SLS
Elution buffer:	10mM Tris pH 7.5 at r.t.
	1mM EDTA
	0.1% SLS
Column buffer:	0.3M NaCl
	0.01M $CH_3-COONa$ pH 5

Phosphate buffer (PB):	Equal parts of $\text{PO}_4\text{H Na}_2$ and $\text{PO}_4\text{H}_2\text{Na}$ pH = 6.8
Insect Ringer's solution:	0.75% w/v NaCl 0.035% " KCl 0.021% " CaCl_2
Standard saline citrate (SSC):	0.15M NaCl 0.015M trisodium citrate
PEB :	1.2M PB 5mM EDTA
KB.3:	0.15M $\text{CH}_3\text{-COONa}$ pH 4.5 0.003M Zn SO_4 0.1M NaCl 0.26M $\text{CH}_3\text{-COOH}$
Phenol solution:	500g. phenol = 550 ml water-saturated phenol 70g. m-cresol 0.5g. 8-hydroxyquinoline final volume 1 litre

Cell culture: DNA and RNA labelling procedures

Cells of Schneider cell line L-3 (Schneider, 1972) were kindly given to us by Prof. J. Kran of Rote Ste. Genese. The cells were grown in Shield's medium (Shields *et al.*, 1975) in spinner culture at 25°C and harvested at a cell density of 10^6 /ml. The generation time was close to 24h.

DNA labelling: cells at a density of 4×10^5 /ml were incubated for 48 hours in the presence of 25 μ Ci/ml of ^3H -thymidine and washed twice with Shields' medium (without serum) before DNA extraction.

RNA labelling: cells at a density of 10^6 /ml were incubated for 90min or 7 hours in the presence of $50\mu\text{Ci/ml}$ of ^3H -uridine.

Drosophila cells of the same density, were labelled for 30 min in the presence of $20\mu\text{Ci/ml}$ of ^3H -Adenosine or for 20 hours in the presence of $25\mu\text{Ci/ml}$ of ^3H -Adenine. The cells were washed twice before RNA extraction.

Culture of *Drosophila*: harvesting of eggs, larvae, pupae and imagos

The strain of *Drosophila melanogaster* used in this investigation was Oregon R. The organism was cultured in population cages to which food trays were added weekly. The food was on some occasions supplemented with a 50% suspension of dry yeast in water.

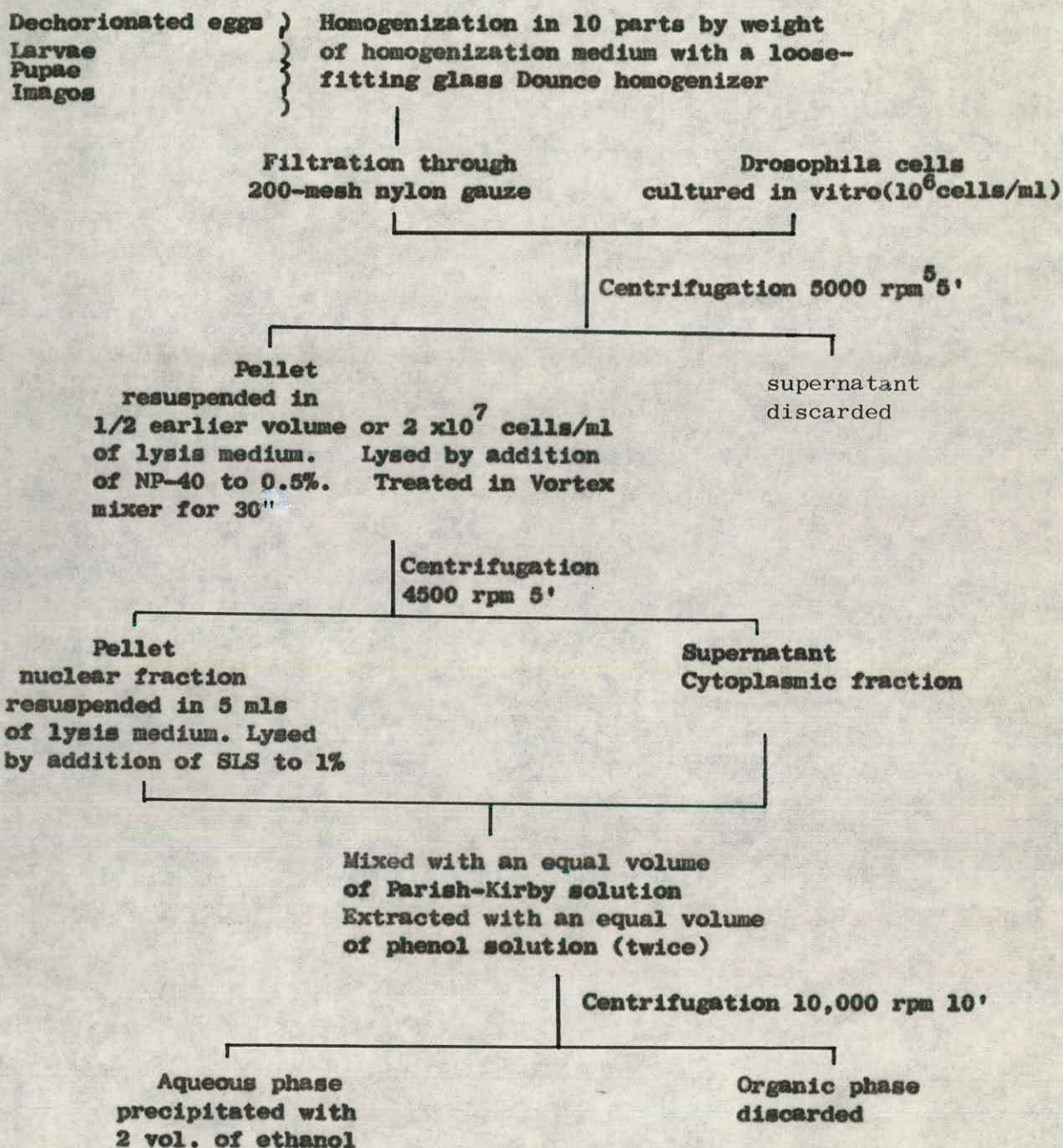
Eggs were collected, every $1\frac{1}{2}$ hours for 6 hours, in 3% agar trays doused with yeast solution. The eggs laid during the first 1/2 hour were discarded. Egg-trays were placed at 2°C until the 4th set of trays was removed from the population cage. The embryos were then incubated for 11 hours at 25°C , brushed off gently in 0.7% NaCl, 0.01% triton and retained on a 200 mesh sieve.

Larvae were removed from the medium by the addition of 60% sucrose in which they float, and then washed with water, in which they sink.

Pupae were collected from the sides of the food trays and placed in water in which they float, separating them from prepupae and contaminating larvae since these sink.

Imagos were harvested using a modified household vacuum cleaner and etherised at 2°C . All stages were thoroughly washed with warm tap water (25°C) and rinsed with distilled water.

Procedure to extract cytoplasmic RNA and nuclear DNA from the
Drosophila cell line and from different stages of development



Embryos were dechorionated by treatment for 2 min with a 3% Na hypochlorite solution and rinsed with distilled water.

The extraction procedure was carried out at 0°C.

The DNA was spooled, dissolved in 0.1 x SSC and treated with 200µg/ml of RNase (1 hour at 37°C), and 400µg/ml of pronase (2h at 37°C in the presence of 0.5% SLS) Deproteinization proceeded with the addition of 1/4 vol. of 5M NaClO₄, 1/10 vol. 3M tris pH 8.5; 3/10 vol. of phenol (at this point the mixture was gently shaken by hand 5') and 3/10 vol. of chloroform (shaken by hand 5' as before). The mixture was centrifuged at 10,000 rpm for 10' and the aqueous phase was collected and re-extracted once more with phenol and chloroform.

The DNA was then dialysed against column buffer, sonicated with a Dawe sonication to a size of about 400 nucleotides, and passed through a Sephadex SP50-chelex column developed with the same buffer (column buffer). The elution fractions were monitored by absorbance at 260nm and the peak fractions precipitated with 2 vols of alcohol. The precipitate was collected by centrifugation and dissolved in a convenient volume of 0.012M phosphate buffer (PB.).

Oligo (dT)-cellulose fractionation (Ross et al., 1972)

The RNA sample, once dissolved in a small volume of high salt buffer (binding buffer), was layered on a 500mg oligo(dT)-cellulose column equilibrated with the same buffer. After repeated washes, the poly(A)-containing RNA was eluted by addition of low salt buffer (eluting buffer). Either radioactivity or optical density was monitored.

Density gradients

Sucrose gradients were 15-30% sucrose in 5mM tris, pH 7.5,

100mM NaCl, 1mM EDTA, 0.5% SDS; run either in the 6 x 16 rotor of a MSE 65 centrifuge, or in the SW 27 rotor of a Spinco L-2 centrifuge. The running temperature was 25°C.

Alkaline sucrose gradients were 5-20% sucrose in 0.1N NaOH, 0.5% SLS, run at 25°C in the SW-41 rotor of a Spinco L-2 centrifuge. The gradients were collected with an Isco fraction collector; the absorbance at 254m μ monitored with an Isco flow analyser and recorded with a Bryans recorder. Radioactive profiles were obtained by counting each fraction with ten times the volume of Instagel.

Polyacrylamide gel electrophoresis

Acrylamide was purified by recrystallization from chloroform: a 5% solution was filtered at 50°C and slowly cooled to -20°C and the crystals were filtered off in the cold. Methylenebisacrylamide (BIS) was dissolved in the minimum volume of acetone at 50°C and recrystallized in the same way. Formamide was deionized before use with Dowex resin MB-3: a 3.3% suspension of resin in formamide was stirred for 2 to 3 hours and then filtered. Polyacrylamide gel electrophoresis utilised 2.85% acrylamide, 0.15% methylenebisacrylamide or 15% acrylamide, 0.37% methylenebisacrylamide in tris-acetate gel buffer (40mM tris, 20mM Na acetate, 2mM EDTA, 0.5% SDS, pH 7.4) to which 1 μ l/ml of the initiation TEMED, and 10 μ l/ml of a 10% solution of the catalyst ammonium persulphate were added. The gels were allowed to stand 2 hours or overnight at room temperature, and then pre-run for 30' at 5mA/gel (15 volt/cm). The RNA was dissolved in gel buffer plus 25% sucrose, and run for the appropriate length of time. The gels were scanned at 260nm in a Joyce-Leoble U.V. recorder and/or sliced with a Mickle gel slicer. In the case of radioactive

RNA the gel-slices were digested with NH_3 overnight at room temperature. The NH_3 was allowed to evaporate and 20 mls of Butyl PBD-ethoxyethanol were added to each vial before counting. Denaturing acrylamide gels were run according to a modification of the method of Gould and Hamlyn (1973) due to Dr. B.L. Paterson. The buffer was 20mM in barbitone, adjusted to pH 9 with NaOH. The gels were 3.5% acrylamide, 0.62% BIS in gel buffer (98% deionized formamide, 20mM barbitone). The buffer vessels contained no formamide. The samples, in gel buffer containing 10% sucrose, were layered under an overlay of gel buffer. The gels were run for 5 hours at 15V/cm, stained overnight with 0.5% glacial acetic acid, 0.1% pyronin B, 1mM citric acid and destained with several changes of 1% acetic acid.

In vitro synthesis of cDNA

We followed the procedure first described by Verma et al., 1972; Kacian et al., 1972, and successfully used in hybridization experiments by Bishop and Rosbash, 1973. The enzyme reverse transcriptase was incubated for 30' at 37°C with 2.5µg of mRNA in a volume of 0.2ml containing 50mM Tris-HCl, pH 8.3 at 20°C; 20mM dithiothreitol, 60mM NaCl, 6mM MgCl_2 , 0.5mM each dGTP, TTP, dATP and 0.05mCi of ^3H dCTP, specific activity 13.3 Ci/mM, 21µg of actinomycin-D and 0.1µg of oligothymidylic acid ((pT)₁₀). After incubation, the mixture was made 1% in SLS and incubated for 2 min. at 37°C. 0.3ml of 0.5N NaOH was added and the tube was placed for 5 min. in a boiling water bath and then chilled. 0.3 mls of 1M NaH_2PO_4 was added to neutralize the alkali and the solution was passed through a column (1.6 x 20cm) of "Sephadex SP-50" developed with 0.3M NaCl 10mM Na acetate pH 5. Collected fractions were made 0.5% in SLS and the radioactivity was monitored.

cdNA-RNA hybridization; estimation of the proportion of annealed
cdNA as a function of time

cdNA was annealed to an excess of poly(A)-containing RNA according to the procedure of Bishop et al. (1974). The reaction mixture, in 0.012M P.B., was heated for 3' in a boiling water bath and a control sample was taken and diluted at least 20-fold in ice-cold stop mixture (0.06M PEB, 100 μ g/ml duck denatured DNA). The reaction mixture was then transferred to a 70°C oil bath and after 30" the salt concentration was adjusted to 0.24M PB. Aliquots were withdrawn at different times and diluted in ice-cold stop mixture. The extent of cdNA annealing was measured by resistance to the enzyme nuclease S₁ which digests single-strand DNA (Sutton, 1971). 1/5 vol. of KB3 was added to the samples and each one was divided into two equal portions. One of the two aliquots was treated with nuclease S₁, and both were incubated at 45°C for 40'. The samples were placed in ice and 200 μ g of BSA and 2mls of 10% TCA were added. After 20', the samples were filtered on GF/C filters and washed with 5% TCA. The filters were dried in a vacuum oven at 80°C for 20', allowed to cool and then counted in toluene PPO-POPOP in a tricarb scintillation spectrophotometer; the ratio of the nuclease-treated sample to the untreated one was plotted as a function of Rot.

cdNA-DNA hybridization

cdNA from Drosophila cell line 3 was annealed to DNA from the same source in DNA excess (Bishop and Rosbash, 1973). The reaction mixture contained 1mg/ml of DNA and trace amounts of ³H-labelled cdNA. The sample was heated in a boiling water bath for 5 min. to denature the DNA. The subsequent procedure was as described for cdNA-RNA

hybrids except that the stop mixture was 0.06M PB.

DNA renaturation: isolation of non-repetitive DNA sequences

Drosophila cell line 3 ^3H labelled DNA was heated for 5' in a boiling water bath in order to achieve complete denaturation. A control sample was withdrawn and diluted with ice-cold water to 0.02M PB. The remaining solution was placed in a 70°C oil bath and after 30" (to allow the temperature to equilibrate) the salt concentration of the DNA solution was adjusted to 0.24M PB. Liquid paraffin was layered on the mixture to avoid evaporation. Samples were withdrawn at different times, and diluted to 0.02M PB. Each sample was loaded on a HAP column (2mg HAP per μg of DNA) at 65°C. After one wash with 1ml of 0.02M PB, single-stranded DNA was eluted with six 1ml washes of 0.12M PB and double-stranded DNA was eluted with four 1ml washes of 0.4M PB. 10 mls of NE-260 were added to each sample and radioactivity was measured. The percentage of annealed DNA was calculated as the ratio of the radioactivity released with 0.4M PB to the sum of the radioactivity in both fractions. This ratio was plotted against Cot. Non-repetitive DNA was purified by annealing to a Cot of 3 m.s.l.⁻¹ and isolation of the fraction eluting from hydroxyapatite at 0.12M PB. The eluate was extensively dialysed against column buffer and subsequently precipitated with 2 volumes of ethanol. The DNA was resuspended in a small volume of 0.012M PB and a further purification was obtained by annealing the DNA for a second time to a Cot of 3m.s.l.⁻¹ and isolating the 0.12M PB eluate as before.

Non-repetitive DNA-RNA hybridization

^3H -labelled non-repetitive DNA was hybridized to an excess of cytoplasmic polyadenylated RNA. The concentration of the RNA was

kept at about 70 times that of the DNA, the real excess of complementary sequences being of the order of 1000-fold. A control sample, with DNA but no RNA in the reaction mixture, was always hybridized in parallel in order to monitor the extent of DNA renaturation. After taking small volume samples at appropriate times, both control and experimental samples were applied to hydroxyapatite columns as previously described. The 0.4M eluates, representing the duplex structures, were dialysed exhaustively against 10mM tris, pH 7.5, and digested for 5 hours at 37°C with 25µg/ml⁻¹ of RNase. The samples were then adjusted to 0.02M PB and the hydroxyapatite fractionation step was repeated.

The proportion of single-stranded DNA eluting with 0.12M PB in the second HAP fractionation times the proportion of double-stranded material eluting at 0.4M PB salt in the first HAP fractionation is an estimation of the percentage of DNA complementary to the RNA. This value was plotted as a function of Rot.

Hybridization of ³H-labelled poly(U) along a sucrose gradient or a polyacrylamide gel

A small aliquot from each gradient fraction was hybridized with an excess of ³H-labelled poly(U) (3×10^5 cpm µg⁻¹) in 2 x SSC for 15 min. at 37°C. The samples were then chilled and diluted 20 times with cold 2 x SSC prior to treatment with 20µg ml⁻¹ of RNase in ice for 20 min. The samples were TCA precipitated, collected in millipore filters and counted as described.

Low molecular weight RNA was eluted from polyacrylamide gels in 2 x SSC at room temperature. Half the elution volume (1ml) was incubated with an excess of ³H-poly(U) at 37°C for 1 hour. After

incubation the samples were processed as above.

Estimation of the poly(A)-containing RNA concentration by titration with radioactive poly(U)

The poly(A) containing RNA concentration of each cytoplasmic RNA preparation was estimated by hybridization to poly(U) after oligo(dT) cellulose fractionation and sucrose gradient purification (Bishop et al., 1974b). A 10^{-1} and a 5×10^{-2} dilution of the RNA sample were hybridized with an excess of ^3H -labelled poly(U) (3×10^5 cpm μg^{-1}) at 37°C for 15'. In parallel, as calibration standards, a 10^{-1} and a 5×10^{-2} dilution of a poly(A) solution of known concentration were also hybridized with an excess of the ^3H -labelled poly(U). After incubation, all the samples were transferred to an ice bath and treated with RNase (20 $\mu\text{g}/\text{ml}$), TCA precipitated and the radioactivity measured as before.

In situ hybridization (Gall, J and Pardue, M.L., 1969)

The experimental procedure consists of several steps: fixation and squashing of tissue; denaturation; hybrid formation; removal of non-specifically bound nucleic acids and autoradiography. The method used for fixation and squashing of Drosophila melanogaster salivary glands was that described by Atherton and Gall, 1972. The glands were dissected from 3rd instar larvae in Ringer's solution, fixed for 2 min. in ethanol-acetic acid (3:1) and squashed in 45% acetic acid. The coverslips were removed by freezing the preparation in dry ice, flipping off the coverslip and plunging the slide into absolute ethanol. Slide preparations, sometimes pre-treated with RNase (20 $\mu\text{g ml}^{-1}$, 20' at 37°C), were denatured with sodium hydroxide, pH 12.5, made up in 2 x SSC to prevent chromosome swelling. The slides were

dehydrated after denaturation by passing them through 50, 70, 90 and 100% ethanol and air drying. During hybridization, the slides and coverslips were sealed using rubber solution diluted with petroleum ether (Jones, 1973); and incubation was carried out in 50% formamide, 4 x SSC at 36°C for 12 to 16 hours. After hybridization, the slides were exhaustively washed with 2 x SSC at room temperature before being incubated with pancreatic RNase (20 µg/ml in 2 x SSC) for 1 hour at 37°C. The slides were then rinsed several times in 2 x SSC and passed through 70 and 90% ethanol, before being air-dried. The dried slides were coated with Ilford K2 autoradiographic emulsion diluted 1:1 with distilled water; stored at 4°C for several weeks, developed for 3 min. with Ilford D-19 developer, rinsed with distilled water, fixed for 6' in Kodak fixer and finally stained for 10 min. with Giemsa stain.

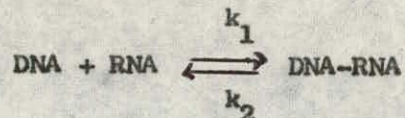
Electron microscopy of organs from the imago and tissue cultured cells

Gut, Malpighian tubes, trachea and thorax musculature were dissected from Drosophila melanogaster adult flies. Also 5×10^6 cells, from Drosophila cell line 3, were centrifuged to form a compact pellet. After dissection, the tissues and cell pellet were fixed with 2 to 3 mls of 1% osmium tetroxide (3 parts of insect Ringer solution and 1 part of 4% osmium tetroxide solution) for 30 min. (Jurand et al., 1967). Fixation was followed by dehydration with graded ethanol solutions. (35, 70, 95 and 100%). The material was passed through propylene oxide (1, 2 epoxy propane), then through a 1:1 mixture of propylene oxide and embedding mixture [10 g. of Epon. Araldite resin; 10 g. of DDSA (dodecenylsuccinic anhydride hardner 964); 0.7 g. of Di-n-Butyl phthalate to soften the mixture and

0.45g. of DMP-3 (2, 4, 6-tri dimethylaminomethyl phenol) to accelerate polymerization] and finally embedded in 100% embedding mixture (Jurand and Pavan, 1975) using a rotary shaker (Jurand et al., 1968). Golden-Silver color sections were obtained with a Porter-Blum MTL ultramicrotome collected in electron microscope grids and the grids stained with 1% potassium permanganate containing 2.5% uranyl acetate.

DNA-RNA HYBRIDIZATION: THEORETICAL CONSIDERATIONS

DNA-RNA hybridization follows the kinetics of a second order reaction.



If D_0 and R_0 are the initial reactant concentrations and DR is the concentration of the duplex:

$$\frac{dDR}{dt} = K_1 (D_0 - DR)(R_0 - DR) - K_2 DR$$

When the concentrations of DNA and RNA are equal ($D_0 = R_0$) and in hybridization conditions, where K_1 is much greater than K_2

$$\frac{dDR}{dt} = K_1 (R_0 - DR)^2$$

Integrating, with the condition $DR = 0$ at $t = 0$

$$\frac{DR}{R_0} = \frac{K_1 R_0 t}{K_1 R_0 t + 1}$$

In the experiments described here, the RNA is in such excess over the DNA that the concentration of the RNA in the hybrid form is negligible compared with the initial RNA concentration. Thus $R_0 - DR \approx R_0$.

Under these circumstances the reaction is pseudo first order:

$$\frac{dDR}{dt} = K_1 R_0 (D_0 - DR)$$

Integrating, with the same condition as before:

$$\frac{DR}{D_0} = 1 - \frac{1}{e^{K_1 R_0 t}}$$

In homogeneous RNA populations, it has been shown (Bishop *et al.*, 1975) that the complexity of the RNA (total length of unrepeated RNA sequences) is directly proportional to the $\text{Rot}\frac{1}{2}$ (product of the initial RNA concentration and time at which the reaction is half completed). It follows logically that the rate of the reaction is inversely proportional to the $\text{Rot}\frac{1}{2}$, and so is the abundance (concentration in terms of the RNA sequence) of the homogeneous DNA.

$$C \propto \text{Rot}\frac{1}{2}$$

$$A \propto \frac{1}{\text{Rot}\frac{1}{2}}$$

In a heterogeneous RNA population where not necessarily all the RNA families contain the same number of members, discrete abundance classes can be observed. In such cases the equation for the hybridization curve is given by:

$$\frac{DR}{D_0} = \sum P_n - \sum \frac{P_n}{K_n \text{Rot}\frac{1}{2}} \quad (\text{Bishop 1974})$$

Where P is the proportion of total hybridized molecules represented in each class; n denotes the abundance classes (1st, 2nd...n) and K_n is the rate constant for each class, given by $K = 0.69 \frac{1}{\text{Rot}\frac{1}{2}}$ (Bishop, 1972).

The complexity in each class is thus proportional to the product of the $\text{Rot}\frac{1}{2}$ and P

$$C \propto \text{Rot}\frac{1}{2} \times P$$

RESULTS

Sedimentation pattern of cytoplasmic RNA from Drosophila melanogaster cell line 3

Drosophila cells are rich in endogenous ribonucleases. Our early attempts to obtain undegraded cytoplasmic RNA, using methods described for HeLa cells (Singer and Penman, 1973), rat fibroblasts (Campo 1973) or the insect Aedes (Spradling et al., 1974) proved unsuccessful when applied to Drosophila cells. As a control for RNA degradation, ³H-Uridine labelled HeLa cells were mixed with Drosophila cells and the cytoplasmic RNA coextracted. The RNA pattern obtained was compared with the RNA profiles seen when both types of cells were lysed separately. An example of one of our early profiles is shown in Fig. 1. The HeLa cytoplasmic rRNA profile (a) is quite different from the one observed when RNA from the same cells is coextracted with Drosophila cells (b). We therefore concluded that the pattern we were observing in Drosophila cells (c) was that of degraded RNA.

After trying several extraction procedures we found that the use of a RNase inhibitor isolated from rat liver cytoplasm (Blobel and Potter, 1966) would prevent RNA degradation during the fractionation procedure (fig. 2a).

We observed, in agreement with Greenberg (1969), that Drosophila "28S" rRNA sediments 2 to 3 Svedberg units slower than the corresponding component of HeLa cell rRNA and the "18S" RNA of Drosophila sediments slightly faster than that of HeLa cells. For this reason it was thought best in the present work to use the values of 26 and 18S in referring to the large and small ribosomal RNA components of Drosophila.

Legend to Fig. 1

Sedimentation of partially degraded RNA in 15-30% sucrose gradients.

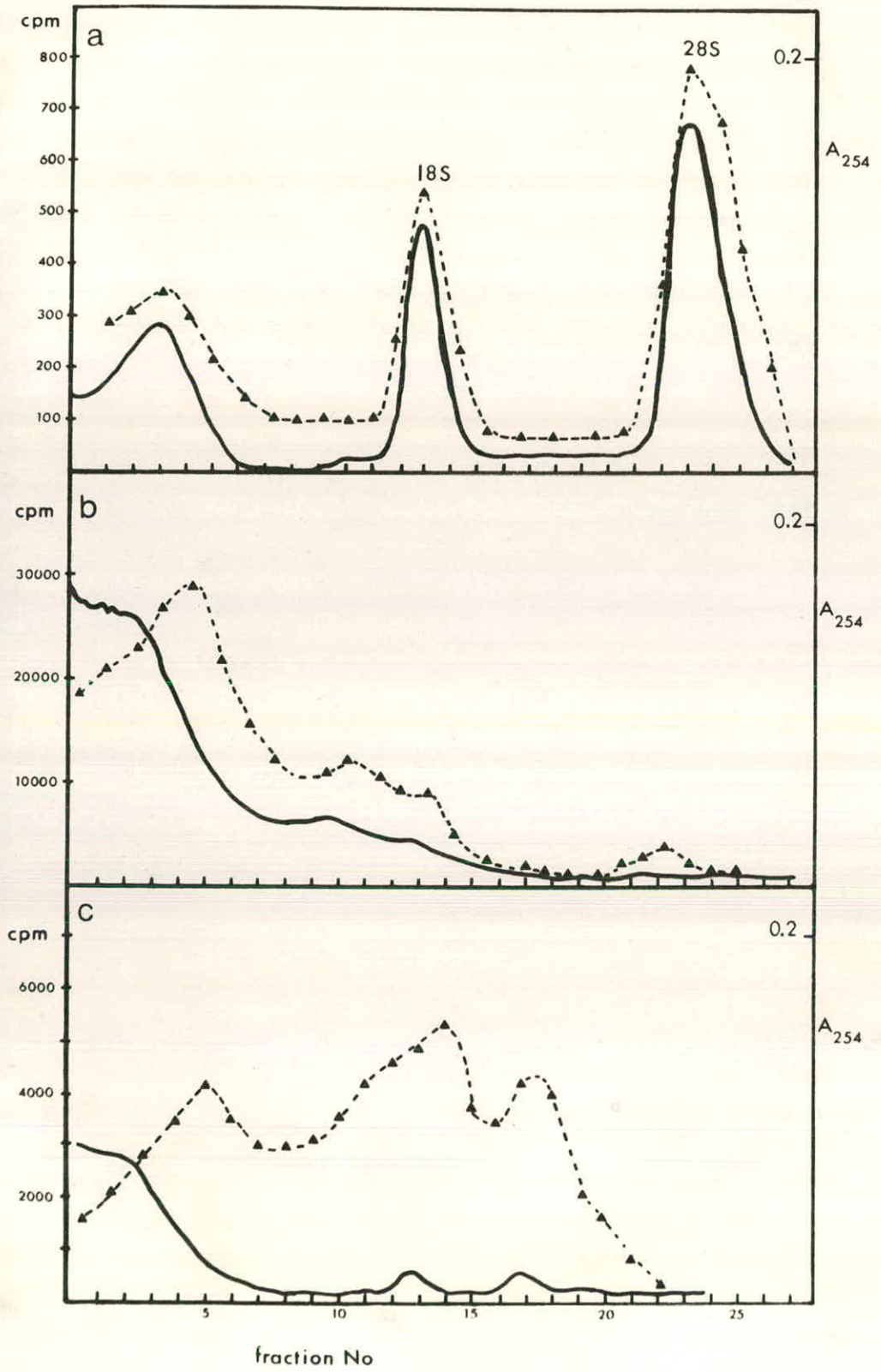
- (a) ^3H -labelled HeLa cells non-polyadenylated RNA.
- (b) ^3H -labelled HeLa cells RNA coextracted with unlabelled Drosophila RNA.
- (c) ^3H -labelled Drosophila RNA.

The gradients were centrifuged at 24K for 17 hours in the SW27 rotor of an L-2 ultracentrifuge. Sedimentation is from left to right.

-▲-▲ = radioactivity

— = absorbance (A_{254})

Fig. 1



Legend to Fig. 2

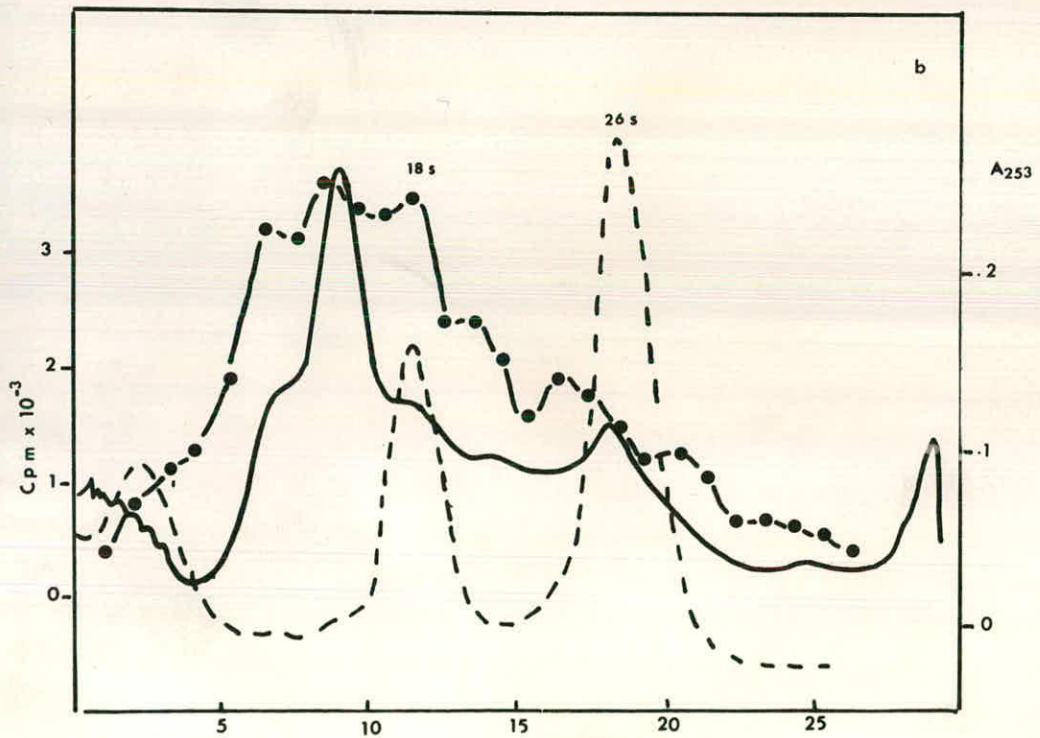
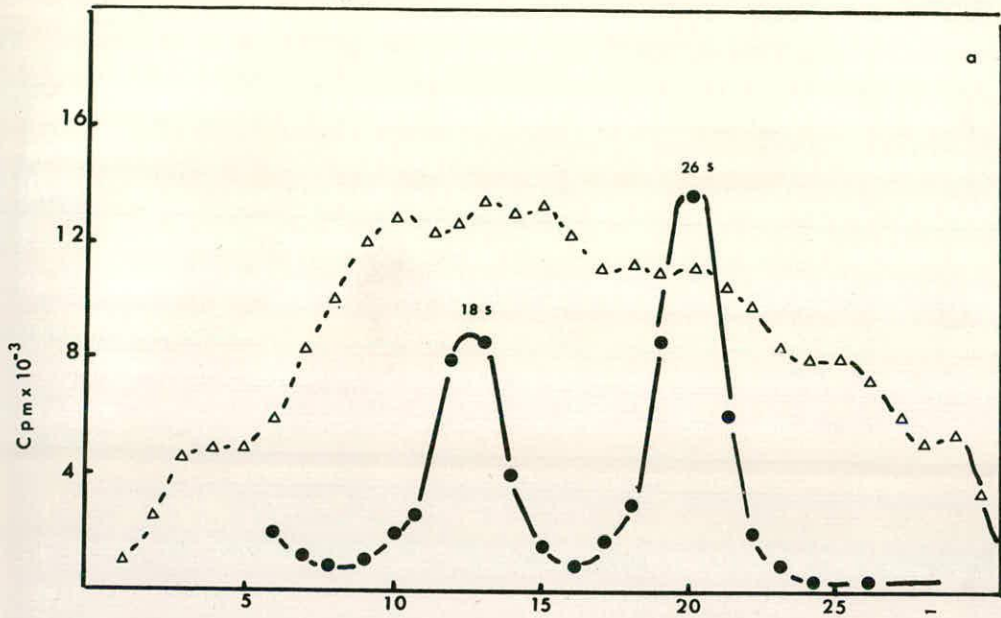
Sedimentation of *Drosophila* cell line RNA in 15-30% sucrose gradients.

(a) non-polyadenylated (● - ●) and polyadenylated (Δ - Δ) ³H-labelled RNA.

(b) Unlabelled non-polyadenylated (----) and polyadenylated (——) cell line RNA. The binding of ³H-poly(U) by fractions of gradient (● - ●) is also shown.

The gradients were centrifuged at 25K for 16 hours in the SW27 rotor of an L-2 ultracentrifuge. Sedimentation is from left to right.

Fig. 2



Drosophila cytoplasmic polyadenylated RNA has a size distribution similar to that of HeLa cells, ranging from 9 to 28S (fig. 2). Unlabelled mRNA was then extracted from the cytoplasmic fraction of Drosophila melanogaster cell line 3, purified through oligo(dT) cellulose and the size of the mRNA measured by hybridizing labelled poly(U) to each fraction of the gradient (fig. 2b). The result indicates that the size of the unlabelled RNA (fig. 2b) and the ³H-labelled mRNA (fig. 2a) are essentially the same. The number average S value is equal to $\Sigma O.D. / \Sigma O.D.S^{-1}$; from which we estimated an average S value for Drosophila mRNA close to 17S which corresponds to a molecular weight of about 1800 nucleotides.

By comparison with the ³H-labelled mRNA profile, the absorbance profile (fig. 2b) shows a very prominent peak at a sedimentation coefficient of about 14S. Its lower proportion in the profile from 90min. labelled cells (fig. 2a) suggests that the messengers sedimenting in this region have a half life greater than average.

Lengths of the poly(A) sequences in cytoplasmic RNA isolated from cell line 3

We observed that even after oligo(dT) cellulose fractionation, there is a certain contamination of rRNA in the polyadenylated RNA fraction. In order to determine the concentration of polyadenylated sequences, we normally hybridize an excess of ³H-labelled poly(U) to an aliquot of the RNA sample. The percentage of RNase-resistant material is compared to the amount of poly(U) hybridized to a sample of known poly(A) concentration. Knowing what part of the total RNA consists of poly(A) it is then possible to calculate the true concentration of the mRNA.

In order to measure the lengths of the poly(A) sequences present at the 3' ends of mRNA molecules, Drosophila cells were labelled with ³H-adenine for 20h. The cytoplasmic RNA was extracted and the fraction bound to oligo(dT) cellulose was digested with ribonuclease under conditions that preserve only the poly(A) sequences (Darnell, 1971). The ribonuclease-resistant residue was subjected to polyacrylamide gel electrophoresis, together with 4 and 5S markers. The result, shown in fig. 3(a), indicates that the poly(A) sequences are very heterogeneous. This is usually attributed to the change in the length of the poly(A) sequences as mRNA molecules age (Sheiness, D., Darnell, 1973). Alternatively, the poly(A) stretches of Drosophila may not be 100% adenine, in which case heterogeneity would be created by T₁ and pancreatic RNase treatment. If metabolic ageing is the cause of heterogeneity, we would expect uniformity in the size of the poly(A) of newly synthesized mRNA molecules reaching the cytoplasm. As fig. 3b shows, when Drosophila cells are labelled for 35' with ³H-adenosine, we find a single prominent species corresponding to the largest size in the "steady state" sample (170 nucleotides). We therefore concluded that the occurrence of short poly(A) sequences is due to its metabolic instability, and not to an artefact of experimental manipulation.

Poly(A)-homopolymers may not behave electrophoretically in the same way as the 4 and 5S markers, and their respective mobility may not be comparable. From data obtained by S. Perlman using thin layer chromatography to determine directly the size of poly(A) from HeLa cells, we considered that 5S and 4S RNA behave electrophoretically as poly(A) molecules 115 and 58 nucleotides long respectively.

Legend to Fig. 3

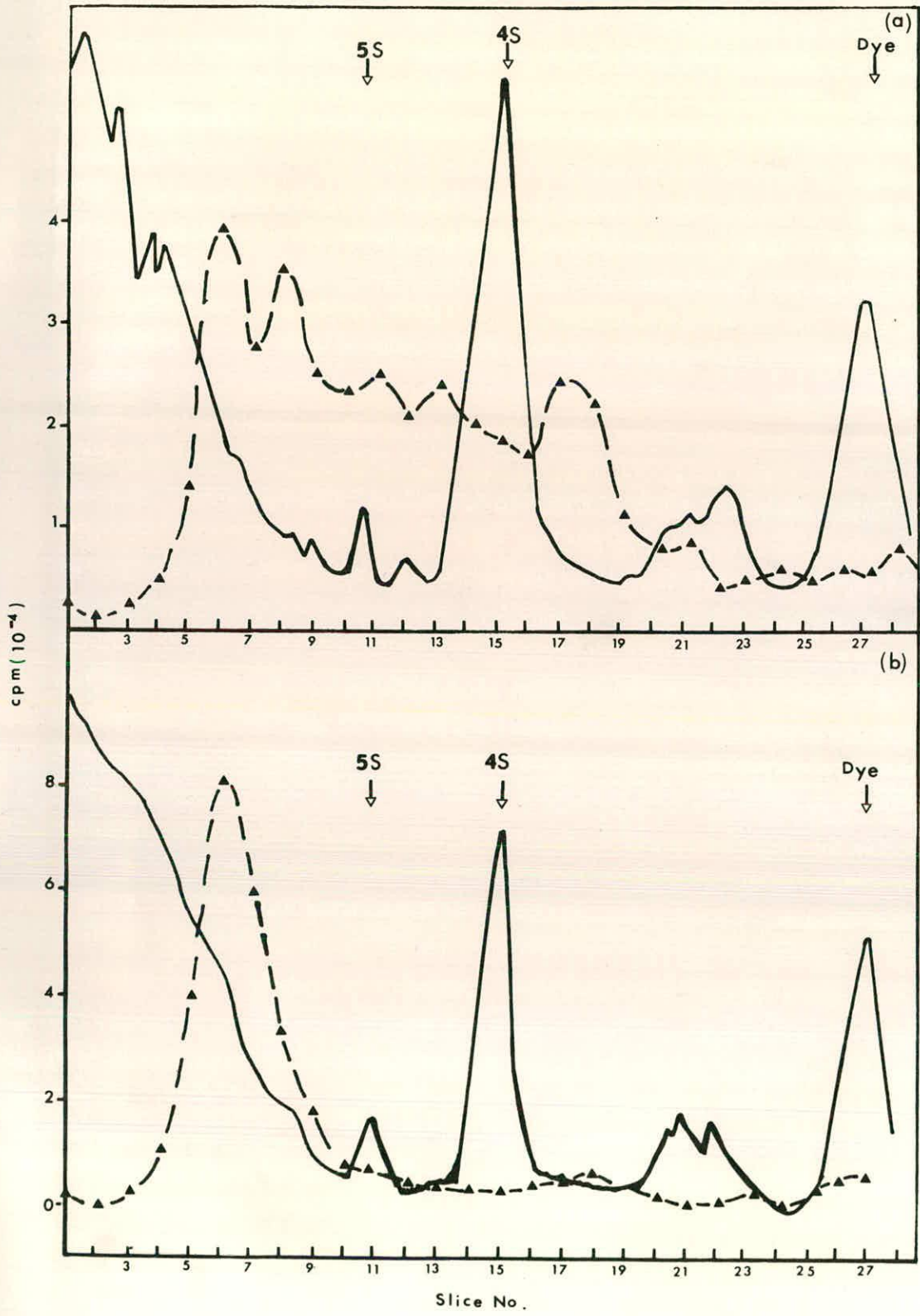
15% polyacrylamide gel electrophoresis of poly(A) isolated from (a) cells labelled for 20 hour in the presence of ^3H -Adenine or (b) for 30 min. with ^3H -Adenosine.

Electrophoresis was carried out for 5 hrs at 5mA/gel.

-▲- = radioactivity

— = absorbance.

Fig. 3



We calculated the number-average length of the poly(A) sequences in Drosophila cells to be about 70 nucleotides. This is 3.8% of the average mRNA sequence.

Length of the cDNA transcript

The reverse transcription of polyadenylated mRNAs by the RNA-dependent DNA-polymerase is a widely used method for generating probes valuable in a variety of molecular hybridization studies.

Unlabelled polyadenylated RNA from Drosophila cells was purified through a sucrose gradient. Fractions between 5 and 30S were precipitated and the mRNA used as template for cDNA synthesis by avian myeloblastosis virus reverse transcriptase. Under our experimental conditions (Bishop and Rosbash, 1973) the average size of the cDNA is smaller than that of the template. The average size of the cDNA transcript was measured by sedimentation in an alkaline sucrose gradient (fig. 4) using unlabelled Xenopus laevis DNA molecular weight 1.5×10^5 as an internal marker.

The cDNA has an average size of about 330 nucleotides. The size distribution appears rather broad, indicating the presence of transcripts longer and shorter than average. Synthesis of cDNA starts at the 3' end of the mRNA and is dependent upon priming by (pT)₁₀. Thus, about 20% of the 3' end of mRNA molecules is represented in the cDNA product. The poly(A) sequences represent 3.5% of the molecule but less of the transcript. Untranslatable regions at the 3' end of purified mRNAs are present in mammals and probably in all higher organisms (Lewin, 1975). The size of the non-coding region at the 3' end of mRNAs is known in the case of the immunoglobulin light chain mRNA (Milstein et al., 1974) and represents about 10% of the total mRNA size. We do

Legend to Fig. 4

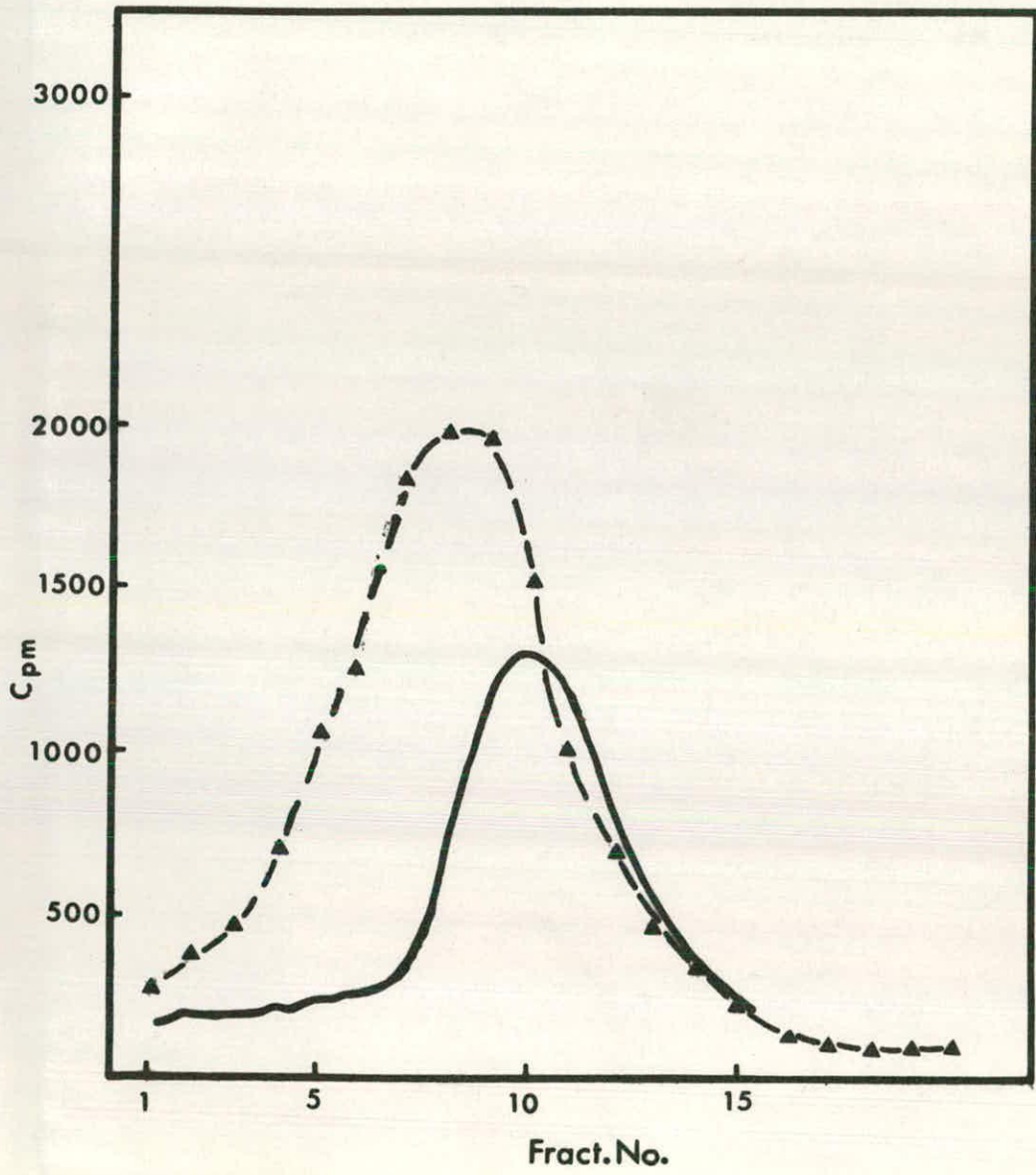
5-20% sucrose gradients in 0.1N NaOH, 0.5% SLS, run
at 33K for 18h. in the Spinco SW41 rotor.

Sedimentation is from left to right.

— = absorbance, Xenopus DNA marker

▲-▲ = radioactivity, cDNA

Fig.4



not know whether *Drosophila* mRNAs contain more nucleotides than required for coding but in any case it is very likely that the cDNA transcripts include at least 8% of DNA complementary to mRNA coding sequences.

Estimation of the complexity and abundance of cytoplasmic poly(A) mRNA populations in *Drosophila* cell-line 3 by means of cDNA-RNA hybridization

By hybridizing the cDNA prepared against *Drosophila* cell line 3 mRNA to the mRNA itself in substantial excess of the template, we are able to determine the complexity of the RNA by comparing the observed Rotl_2 with that of a kinetic standard. It is also possible to distinguish abundance classes, if any exist, because they react with different reaction rates. (Bishop et al., 1974, and see above)

Results obtained by annealing polyadenylated RNA obtained from *Drosophila* cell line cytoplasm with cDNA are shown in fig. 5. The reaction has a three-phase transition, showing that the mRNA population contains three distinguishable abundance classes (Table 1).

The Rotl_2 of rabbit globin mRNA, which is about 1320 nucleotides long, is 5.8×10^{-4} (Bishop et al., 1975)(b) We have taken 7.9×10^{-4} to be the Rotl_2 of an mRNA with a molecular weight of 1800 nucleotides. *Drosophila* has an analytical complexity (DNA content per haploid genome) of 10^{11} daltons. A total of about 4000 ± 1000 RNA sequences of average molecular weight 5.9×10^5 represents a total sequence length (total length of different sequences in a nucleic acid preparation) of about 2.3×10^9 , or 3% of the nonrepetitive DNA. Thus, assuming transcription from only one of the two complementary DNA strands, about 6% of the non-repetitive haploid genome will be represented in protein-coding genes.

Legend to Fig. 5

Hybridization reaction between mRNA and cDNA from Drosophila cell line 3 in mRNA excess. Annealing was at 70°C in 0.24M PEB containing 0.01% SDS. Percentage of hybrid is estimated as percentage of S₁ nuclease resistance. Rot is expressed in terms of moles nucleotide per litre x time of annealing in seconds.

The Rot values shown in this figure are based on the assumption that poly(A) forms 3.5% of Drosophila mRNA. Those in Table 1 are adjusted to the true value of 3.8%.

Fig. 5

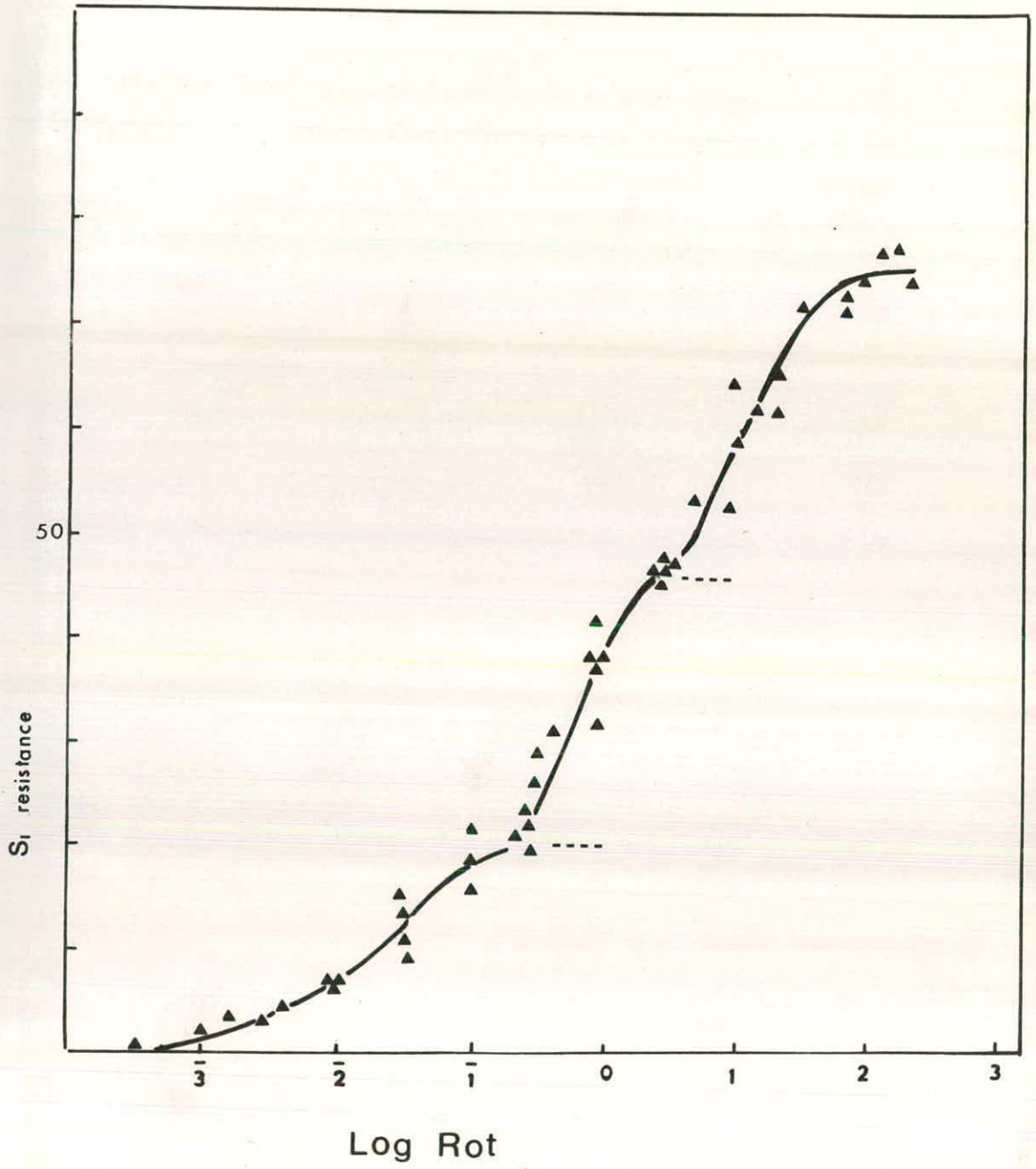


Table 1

Number of mRNA sequences in Drosophila melanogaster cell line 3
(poly(A) = 3.8% of mRNA)

Abundance classes	Observed $\frac{Rot1}{2}$ (M.S.l ⁻¹)	Proportion(P) of total	$\frac{Rot1 \times P}{2}$	K^h (l.M ⁻¹ S ⁻¹)	No. of different 1800 nucl. sequences
1	0.05	0.20(.26)	0.01	69	12(16)*
2	0.8	0.25(.33)	0.2	3.4	253(334)
3	9.2	0.30(.40)	2.76	0.25	<u>3493(4658)</u>
					<u>3758(5008)*</u>

(*) = inside brackets are shown values normalized to 100% hybridization

mRNA sequences transcribed from repetitive DNA

Eukaryotic messengers appear to be derived largely but not entirely from the non-repetitive component of the genome. About 20% of the polyadenylated mRNA from rat myoblasts hybridizes with repetitive DNA (Campo and Bishop, 1974) while less than 10% is found in the insect *Aedes* (Spradling et al., 1974). When *Drosophila* cell-line cDNA was annealed in a vast excess of unlabelled DNA, two transitions were seen (fig. 6). 10 to 15% of the cDNA reacted at low Cot, showing that it is complementary to repetitive DNA; the second major transition represents the renaturation of non-repetitive sequences (Laird and McCarthy, 1969).

Our conclusion is that 10 to 15% of the polyadenylated RNA in *Drosophila* cells is transcribed from repetitive DNA. This value agrees well with the 15% value reported very recently by Levy, W. and McCarthy (1975). These authors found that the repetitive DNA complementary to polyadenylated messenger is preferentially responsible for the most abundant molecules in the cytoplasmic population.

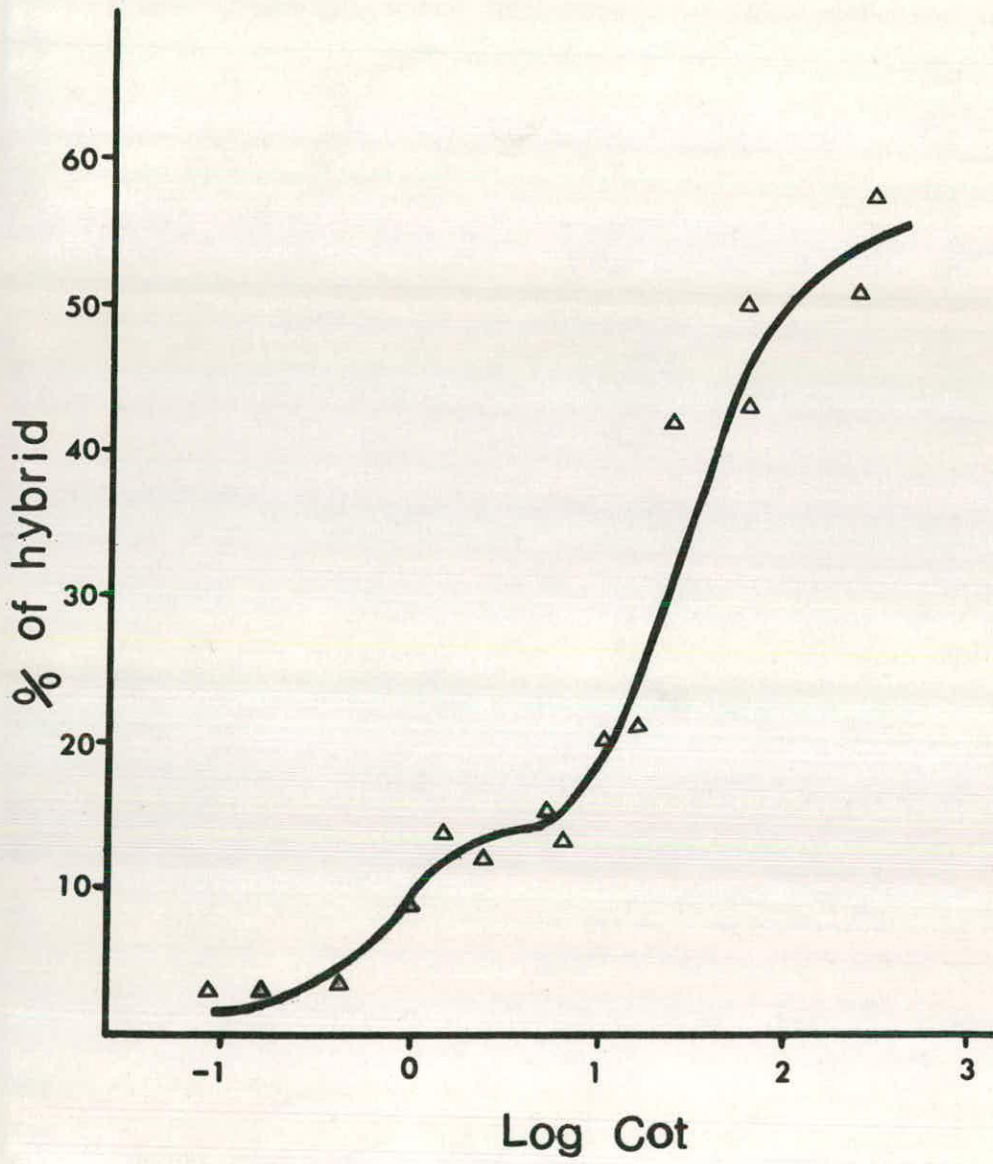
Estimation of the number of different cytoplasmic poly(A)-RNA molecules by hybridization between RNA and non-repetitive DNA

This method, successfully applied in previous studies (Davidson and Hough, 1971; Brown and Church, 1972; Bishop et al., 1974; Galau et al., 1974) involves annealing isolated non-repetitive DNA with an excess of unlabelled RNA. The duplex molecules are isolated by HAP fractionation. The RNA is exhaustively digested with ribonuclease (25µg/ml, in 10mM Tris for 5 hours at 37°C). The DNA molecules which are associated in hybrid duplexes with RNA are rendered single stranded by this treatment, which has little or no effect on DNA-DNA

Legend to Fig. 6

Renaturation of cDNA from Drosophila cell line 3 with an excess of unlabelled DNA. Annealing was at 70°C in 0.24M PEB. Percentage of double stranded is calculated as percentage of S₁ nuclease resistance. Cot is expressed in terms of moles nucleotide per litre x time of annealing in seconds.

Fig. 6



duplexes. The DNA is then fractionated on a second HAP column and the proportion of double and single stranded DNA are estimated. At saturation, the percentage (p) of DNA complementary to RNA is a measure of the complexity of the mRNA population (C), and from this we can readily estimate the number of mRNA sequences present (N)

$$N = \frac{C}{MW_A} = \frac{C \cdot p}{MW_A}$$

Where C* is the C-value of the DNA and MW_A is the average molecular weight of the mRNA.

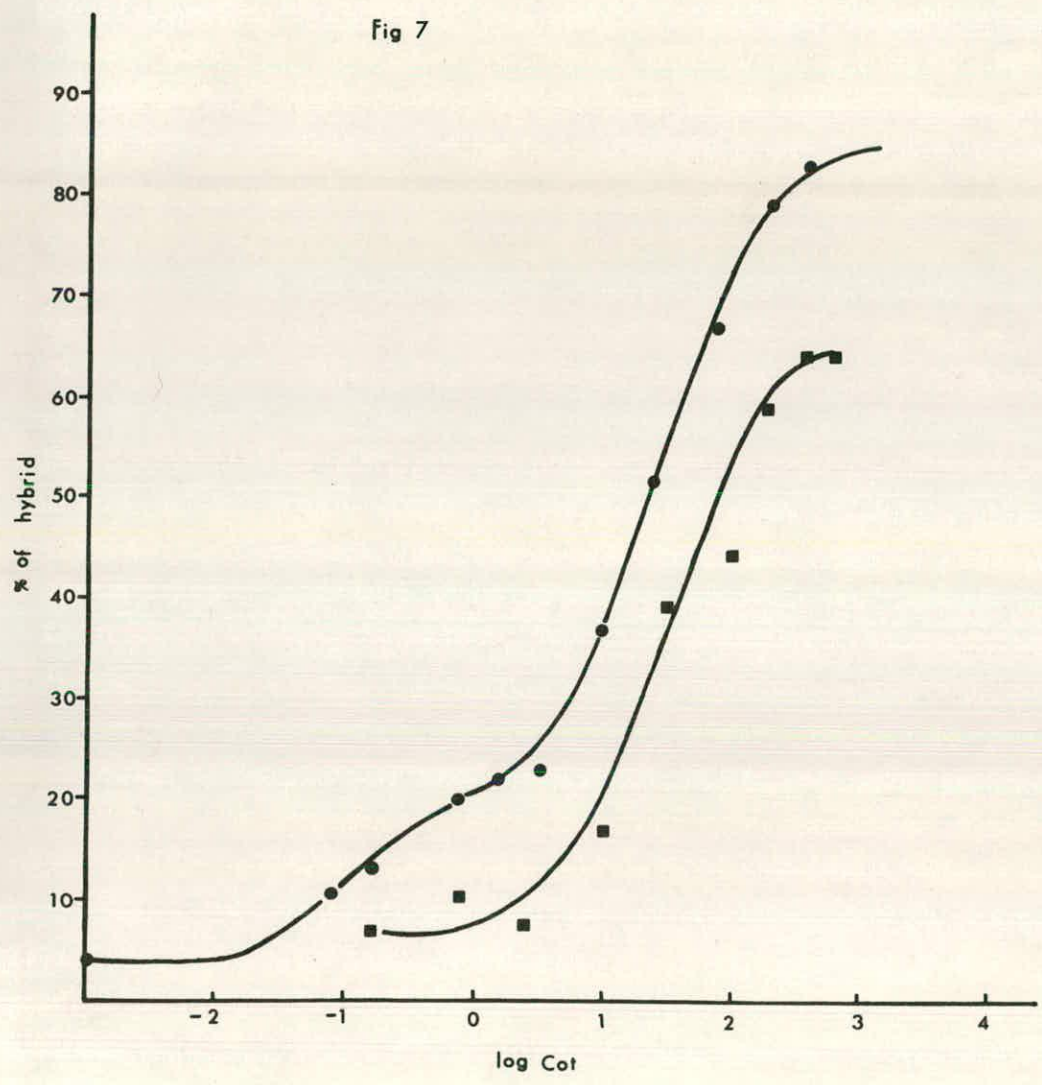
It is important for this type of analysis to obtain highly labelled DNA and to eliminate from it the repetitive fraction. Low specific activity DNA would require a very high input of RNA in order to attain a reasonable excess over the DNA molecules and so minimize DNA-DNA reassociation. Isolation of non-repetitive DNA avoids the predominant formation of imperfect hybrids with repetitive sequence transcripts.

Our first step was to isolate non-repetitive DNA from 3H -labelled nuclear DNA (2×10^5 cpm/ μg). The kinetics of DNA reassociation when duplex formation is monitored by HAP fractionation are shown in fig. 7. 20 to 25% of the DNA reassociates more rapidly than the non-repetitive fraction (Cot values lower than 3). These sequences must be present in higher concentration than the remainder. The conclusion that the slowly reassociating fraction is non-repetitive is based on its rate of reassociation relative to that of Escherichia coli DNA (Laird and McCarthy, 1969). After annealing twice to Cot 3, and each time reisolating the duplex fractions, the reassociation rate of the isolated non-repetitive DNA was examined. As shown in fig. 7,

Legend to Fig. 7

Reassociation of ^3H -labelled cell line DNA (●-●)
and isolated non-repetitive DNA component (■-■).
Annealing was at 70°C in 0.24M PEB. Cot is expressed
in terms of moles nucleotide per litre x time of
annealing in seconds. The percentage of double stranded
is expressed as percentage bound to hydroxyapatite in
0.12M PB.

Fig 7



most, if not all of the DNA that reassociates by a Cot value of 3 was eliminated.

The next step was to hybridize the labelled non-repetitive DNA with an excess of cytoplasmic poly(A)-RNA, and to estimate the proportion of DNA complementary to RNA. The results are shown in fig. 8. Two percentage points are plotted for each Rot value. One of them is generated by subtracting from the total duplex fraction (DNA-DNA and DNA-RNA) the DNA-DNA contribution estimated as the percentage renaturation in a parallel control tube with no RNA. The other point represents the proportion of DNA rendered single stranded by RNase treatment. These values have been corrected for the effect of RNase on DNA-DNA hybrids in parallel control experiments.

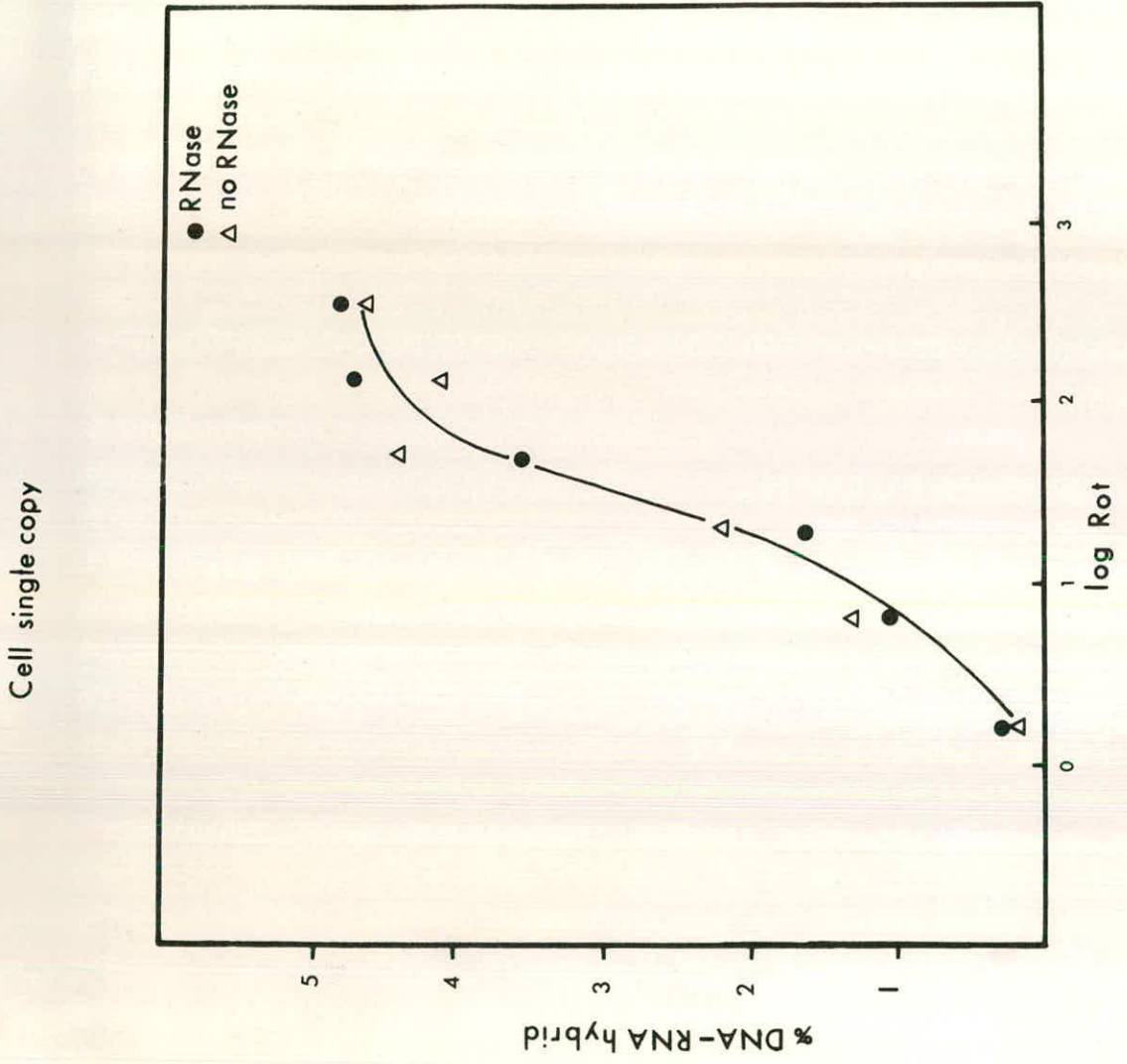
The results show that at saturation, 13% of the DNA is double stranded, but only 4.5% of the total DNA is present in RNA-DNA hybrid molecules. 4.5% of the non-repetitive DNA represents a sequence length of 3.3×10^9 daltons and this corresponds to about 5700 different sequences of average molecular weight. Both this value of about 5700 and that of 4000 ± 1000 obtained by cDNA-RNA hybridization are close to the number of bands that can be visualized in salivary gland polytene chromosomes. The agreement between the methods is good within limits. Very rare mRNA sequences might go undetected in cDNA-RNA experiments because of the small amount of cDNA complement they generate. Such sequences would be relatively better represented in non-repetitive DNA.

On the other hand, if our non-repetitive DNA fraction was slightly contaminated with repetitive DNA complementary to mRNA, the sequence length and therefore the complexity would be over-estimated.

Legend to Fig. 8

Hybridization between an excess of cell line mRNA and ^3H -labelled non-repetitive DNA. Annealing was carried out at 70°C in 0.24M PB. Percentage of hybrid is estimated as proportion of total, bound to HAP in 0.12M PB. Two values \bullet and Δ of DNA-RNA hybrid are given per Rot point (explanation in text).

Fig. 8



From these experiments we concluded that the number of different mRNA sequences present in the cytoplasm of Drosophila cell line 3 is close to 5000.

Sedimentation profiles of cytoplasmic RNA from embryos, larvae, pupae and imagos

The complexity of the mRNA population of a cell line may not represent the complexity of the living organism. Cell lines might be fully derepressed or they might on the other hand express only the minimum number of genes required to survive in vitro. The quantitative and qualitative study of mRNA populations during different stages of development will throw light upon the total number of sequences expressed at all stages of development and at the same time give information about gene expression during the life span of the organism.

Our first step was to isolate cytoplasmic RNA from embryos, larvae, pupae and adult flies. In most cases, RNA degradation was monitored by coextraction with ³H-labelled HeLa cells as previously described.

The results of coextracting embryo cytoplasmic RNA with ³H-uridine labelled HeLa cells are shown in fig. 9a and b. The absence of small molecular weight HeLa poly(A) RNA in fig. 9b suggest that no significant degradation of the embryo RNA is taking place during our isolation procedures. The sedimentation profile observed for embryo polyadenylated RNA in fig. 9c reveals once more a predominant peak in the 14S region and rRNA contamination in the polyadenylated fraction isolated by oligo dT cellulose fractionation.

In the case of pupal RNA (fig. 10), no low molecular weight

Legend to Fig. 9

Sedimentation on 15-30% sucrose gradients of :

- (a) Unlabelled Drosophila embryo RNA coextracted with ^3H -labelled HeLa cells RNA. Nonpolyadenylated fraction

— = absorbance

△-△ = radioactivity

- (b) Unlabelled Drosophila embryo RNA coextracted with ^3H -labelled HeLa cells RNA. Polyadenylated fraction

— = absorbance

●-● = radioactivity

□-□ = binding of ^3H poly(U) to gradient fractions

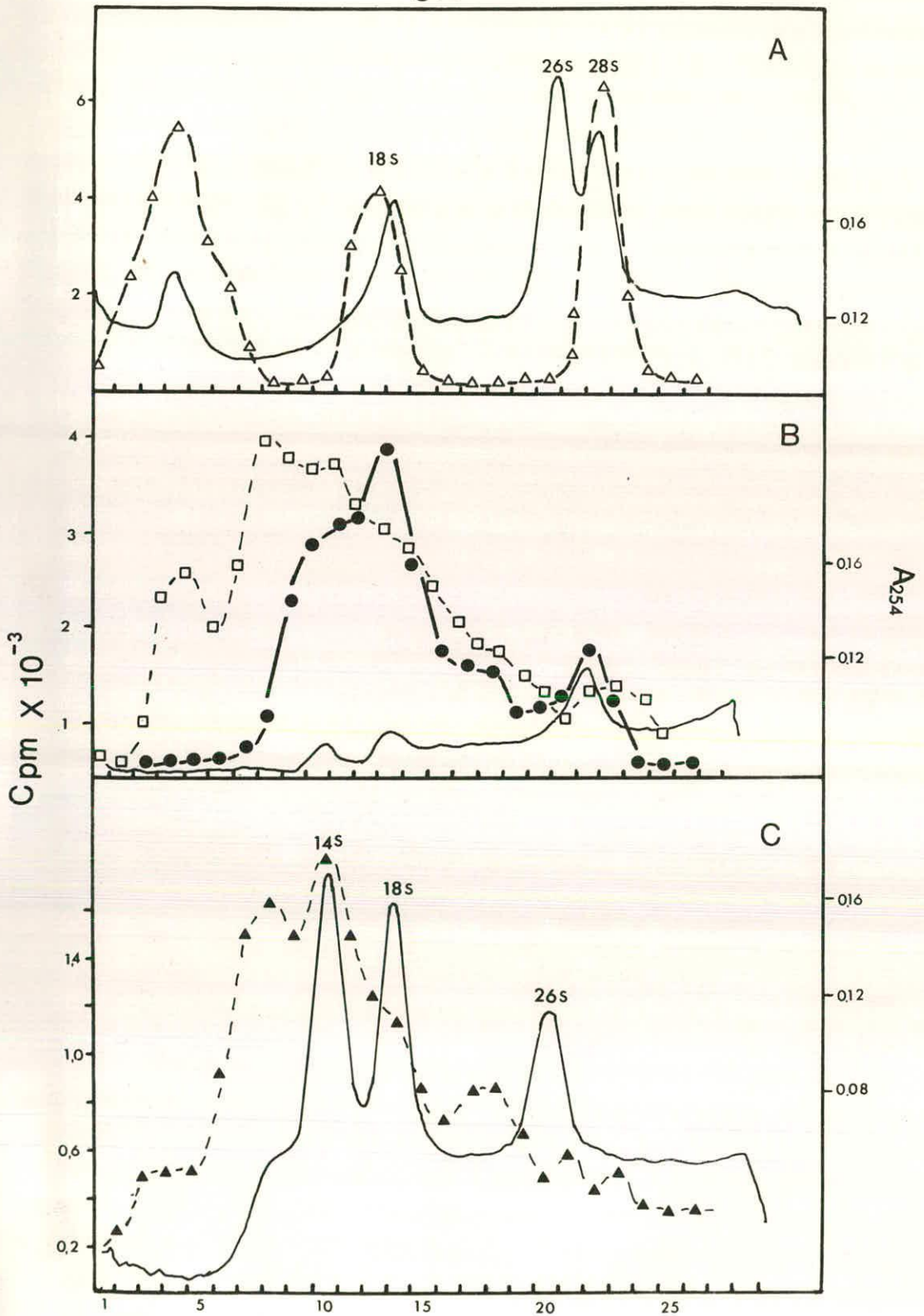
- (c) Unlabelled Drosophila embryo RNA. Polyadenylated fraction (oligo(dT)-cellulose bound).

— = absorbance

▲-▲ = binding of ^3H poly(U) to gradient fractions

Gradients were centrifuged for 16h at 25K in a SW27 rotor of an L-2 centrifuge. Sedimentation is from left to right.

Fig.9



Legend to Fig. 10

Sedimentation on 15-30% sucrose gradients of:

- (a) Unlabelled Drosophila pupa RNA coextracted with ^3H -labelled HeLa cells RNA.

— = absorbance

●-● = radioactivity in polyadenylated fraction

△-△ = radioactivity in non-polyadenylated fraction

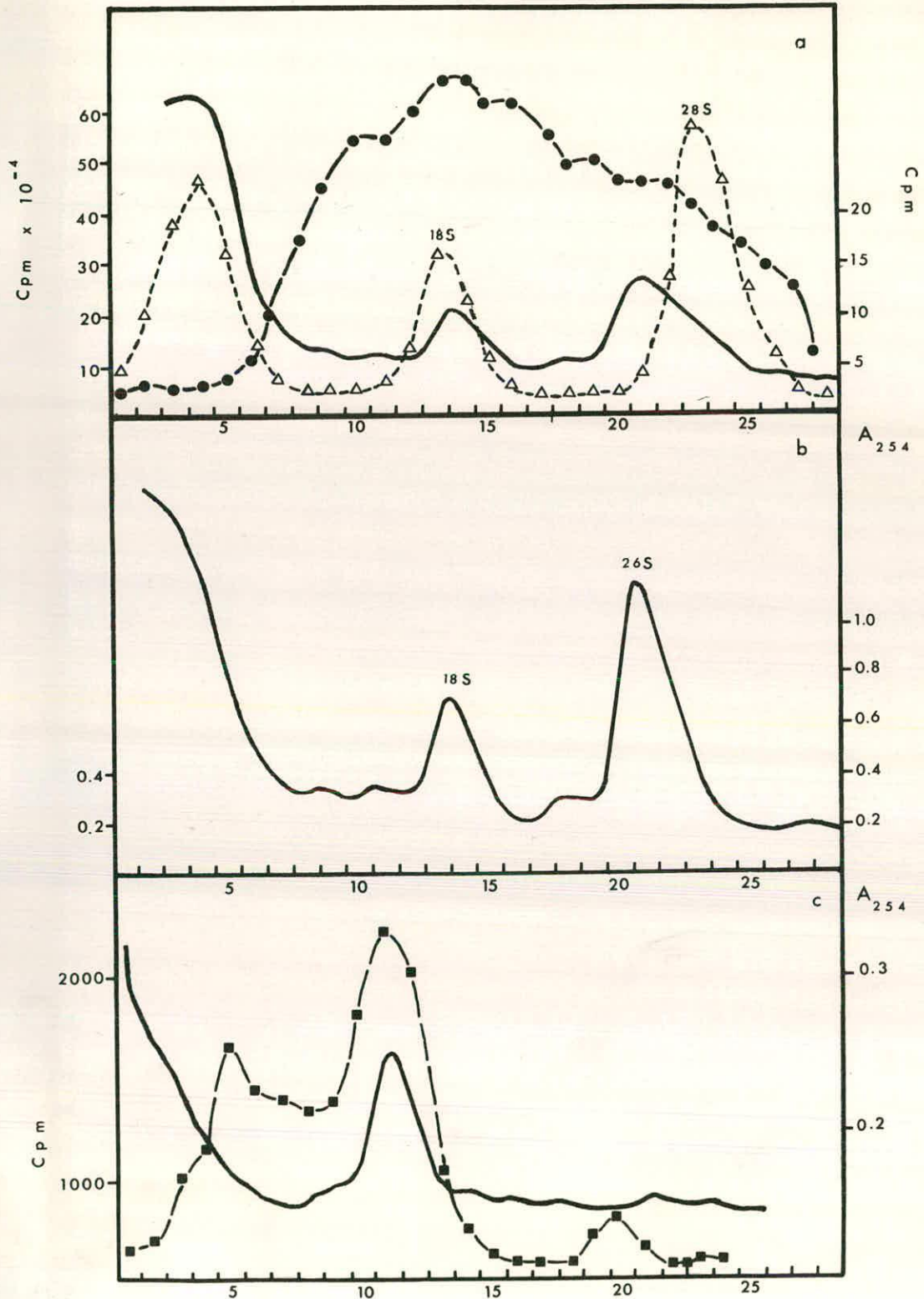
- (b) Unlabelled Drosophila pupa non-polyadenylated RNA

- (c) Unlabelled Drosophila pupa polyadenylated RNA.

The binding of ^3H -poly(U) by fractions is also shown (■-■)

Sedimentation is from left to right. Centrifugation conditions as in fig. 9.

Fig. 10



polyadenylated RNA is observed in the coextracted ^3H -HeLa labelled mRNA fig. 10a. Nevertheless when unlabelled pupal poly(A) RNA is hybridized in an excess of ^3H -poly(U) across the gradient (fig. 10c), a poly(A) peak in the slowly sedimenting region of the gradient becomes evident (top fractions). The existence of free poly(A) in the population of pupal messengers could be due to the massive tissue degradation that takes place during metamorphosis. Nonetheless, poly(U)-poly(A) hybridization in the slowly sedimenting region of sucrose gradients was also observed in the mRNA populations of larvae and imagoes.

Fig. 11 shows the sedimentation profile of imago mRNA co-extracted with ^3H -labelled HeLa mRNA(a), and the unlabelled polyadenylated fraction of imago mRNA when the cells are lysed on their own (b). The peak with a sedimentation coefficient of about 14S present in cells and other stages, is particularly prominent in the adult population of messengers. In all cases about 3% of the total cytoplasmic RNA was found to contain poly(A) as measured by oligo-(dT) cellulose fractionation.

The first few fractions of every gradient were discarded in order to avoid calibration errors when estimating the mRNA concentration by the ^3H -poly(U) saturation method. The rest of the fractions, showing absorbance at A254 were pooled, adjusted to 0.2M salt and precipitated with 2 volumes of ethanol. We have based calculations on the same average molecular weight of 1800 nucleotides, for mRNA of the cell line and all different stages of development.

Legend to Fig. 11

Sedimentation on 15-30% sucrose gradients of:

- (a) Unlabelled Drosophila imago RNA coextracted with ³H-labelled HeLa cells RNA.

— = absorbance

▲-▲ = radioactivity in oligo(dT)-
cellulose bound material

- (b) Unlabelled Drosophila imago RNA

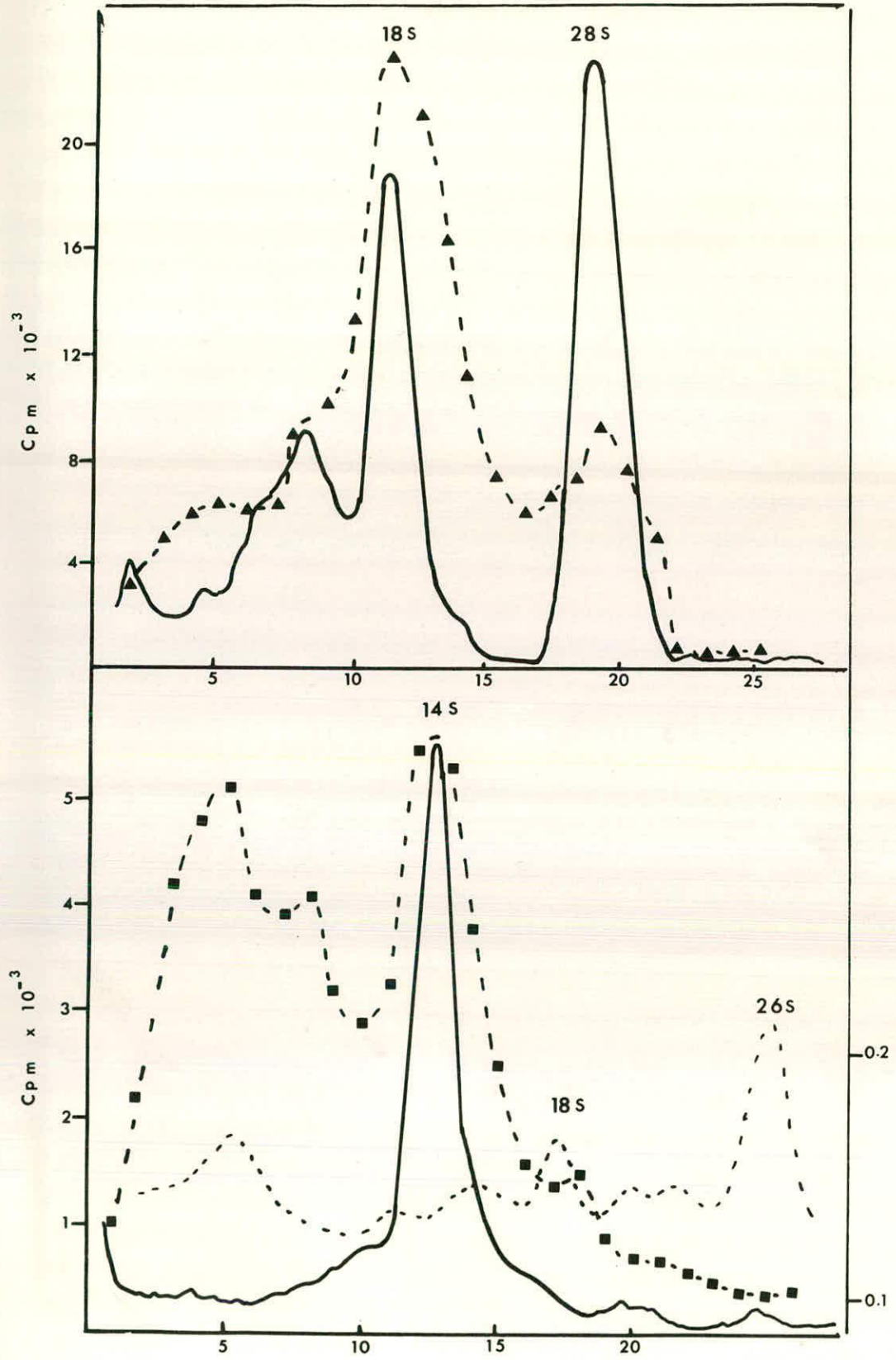
— = absorbance polyadenylated fraction

--- = superimposed non-polyadenylated
fraction from a parallel gradient

■-■ = binding of ³H poly(U) to gradient
fractions

Gradient (a) was centrifuged for 17h. Gradient (b) for 20 hours in the SW27 rotor of a Spinco L-2 centrifuge. Sedimentation is from left to right.

Fig. 11



Lengths of the poly(A) sequences in the cytoplasmic RNA isolated from embryos, larvae, pupae and imagos

We have seen that in tissue culture cells the polyadenylic acid residues correspond to about 3.8% of the mRNA. There is, however, no reason to assume that the amount of poly(A) remains constant throughout the different stages of development.

By comparison with tissue culture cells, it is difficult to label the RNA in the living organism to steady state. We surmounted this problem by studying the poly(A) sequences indirectly in the way described by Rosbash and Ford (1974). Unlabelled cytoplasmic RNA was digested with pancreatic and T_1 ribonuclease at moderate ionic strength (≥ 0.1 N Na^+). The resistant polyadenylic acid residues were subjected to 15% acrylamide gel electrophoresis, the gel was frozen, sliced and the poly(A) was eluted from sets of 4 adjacent 0.6mm slices in vials. These fractions were hybridized with an excess of 3H -labelled poly(U), and the amount of poly(A) observed was analysed as a function of distance migrated. Unlabelled RNA from tissue culture cells, embryos, larvae, pupae and imagos was examined in this way. All the patterns show a very similar heterogeneous profile (fig. 12). The tissue culture cell pattern obtained by this procedure should be the same as the one obtained by the labelling method (fig. 3). Indeed the average chain length was calculated to be 72 in perfect agreement with the value obtained previously. Average values of 66, 68, 71 and 64 were obtained for embryos larvae pupa and imago polyadenylated molecules. The difference is not significant and we have considered that poly(A) forms 3.8% of mRNA molecules in the cell line, and all through the different stages of development.

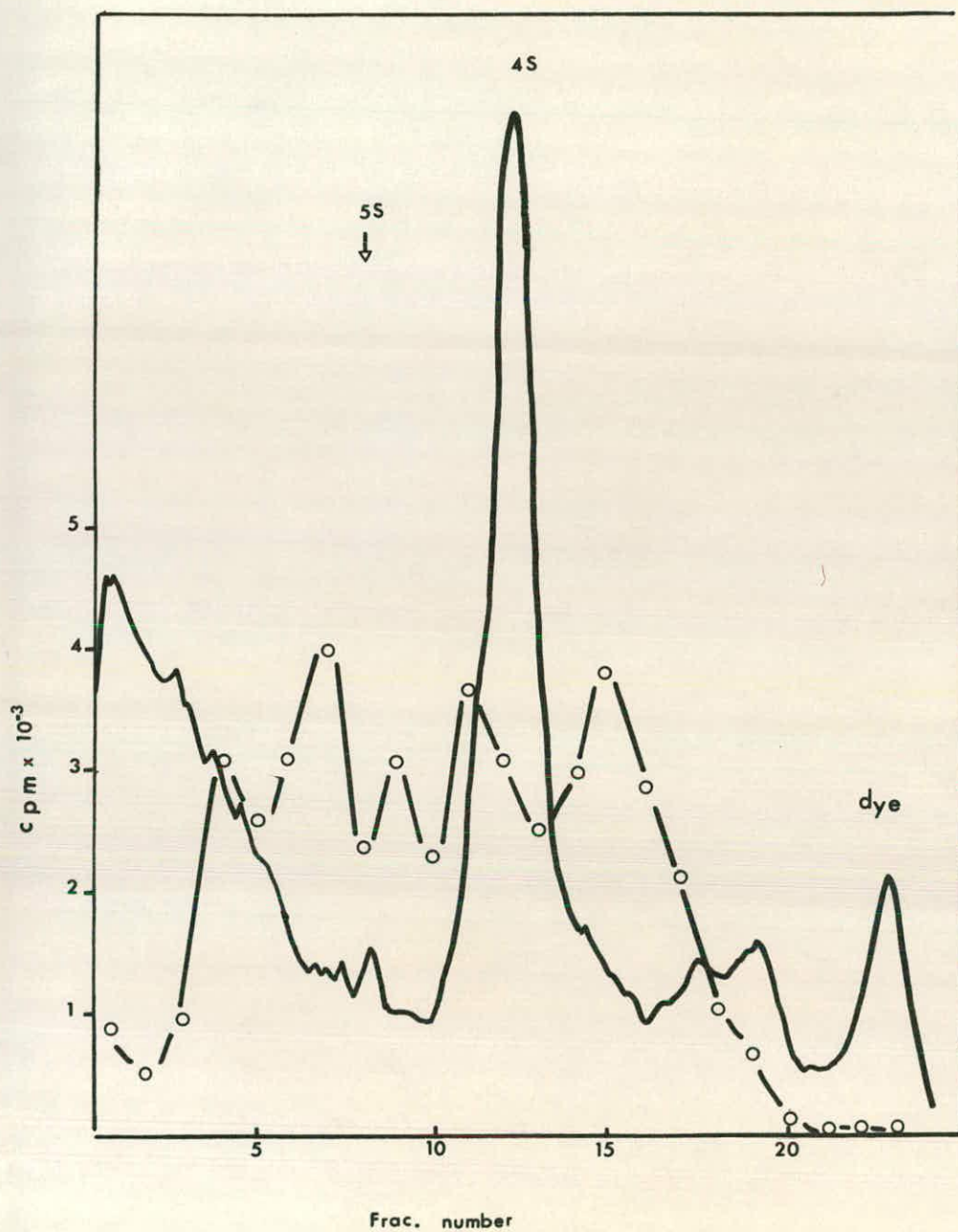
Legend to Fig. 12

15% polyacrylamide gel electrophoresis of embryo poly(A) molecules. Electrophoresis was for 5 hours at 3mA/gel. The RNA was eluted and ^3H -poly(U)-binding measured as described in experimental procedures.

— = absorbance

○-○ = radioactivity of poly(U) bound.

Fig. 12



Estimation of the complexity and abundance of poly(A)-RNA from embryo larva, pupa and imago

Total cytoplasmic RNA was extracted from embryos, larvae, pupae, and imagos. After oligo(dT)-cellulose fractionation, the polyadenylated fractions were purified through sucrose gradients and the material sedimenting between 9 and 30S used as template for the preparation of highly labelled cDNA. The cDNA was systematically tested for self-complementarity before being used in hybridization experiments.

The results of the homologous cDNA-RNA hybridization in RNA excess for each developmental stage are shown in fig. 13.

The three phase curve observed for embryos resembles that of the cell line whereas those for larvae, pupae and imagos appear substantially different from it.

Only two obvious transitions are seen in larva pupa and imago curves. The very fast first transitions of larva and imago have a Rot_1 very close to that expected for a single messenger, and in each case $\frac{70}{2}$ to 75% of the total cDNA seems to be involved. This means that, if the reverse transcriptase did not preferentially transcribe one sequence from the total RNA population, about three quarters of the poly(A)-RNA extracted from larvae or flies consists of a single species. This seems quite remarkable. The results are analysed in Table 2.

Pupation appears as the most complex stage of development with about 5000 different polyadenylated sequences. The first transition, containing about 8 very abundant messengers and contributing about 65% of the total cDNA, is rather broad (extended through 3 logarithmic

Legend to Fig. 13

Reactions between mRNA and mRNA-specific cDNA from:

△ embryos mRNA and homologous cDNA; X pupae mRNA and homologous cDNA; ● larvae mRNA and homologous cDNA and □ imago mRNA and homologous cDNA.

Annealing was at 70°C in 0.24M PEB. Percentage of hybrid molecules were estimated as percentage of S₁ nuclease resistance. Rot is expressed as moles nucleotide per litre x annealing time in seconds.

The Rot values shown in this figure are based on the assumption that poly(A) forms 3.5% of Drosophila mRNA. Those in Table 2 are adjusted to the true value of 3.8%.

Fig. 13.

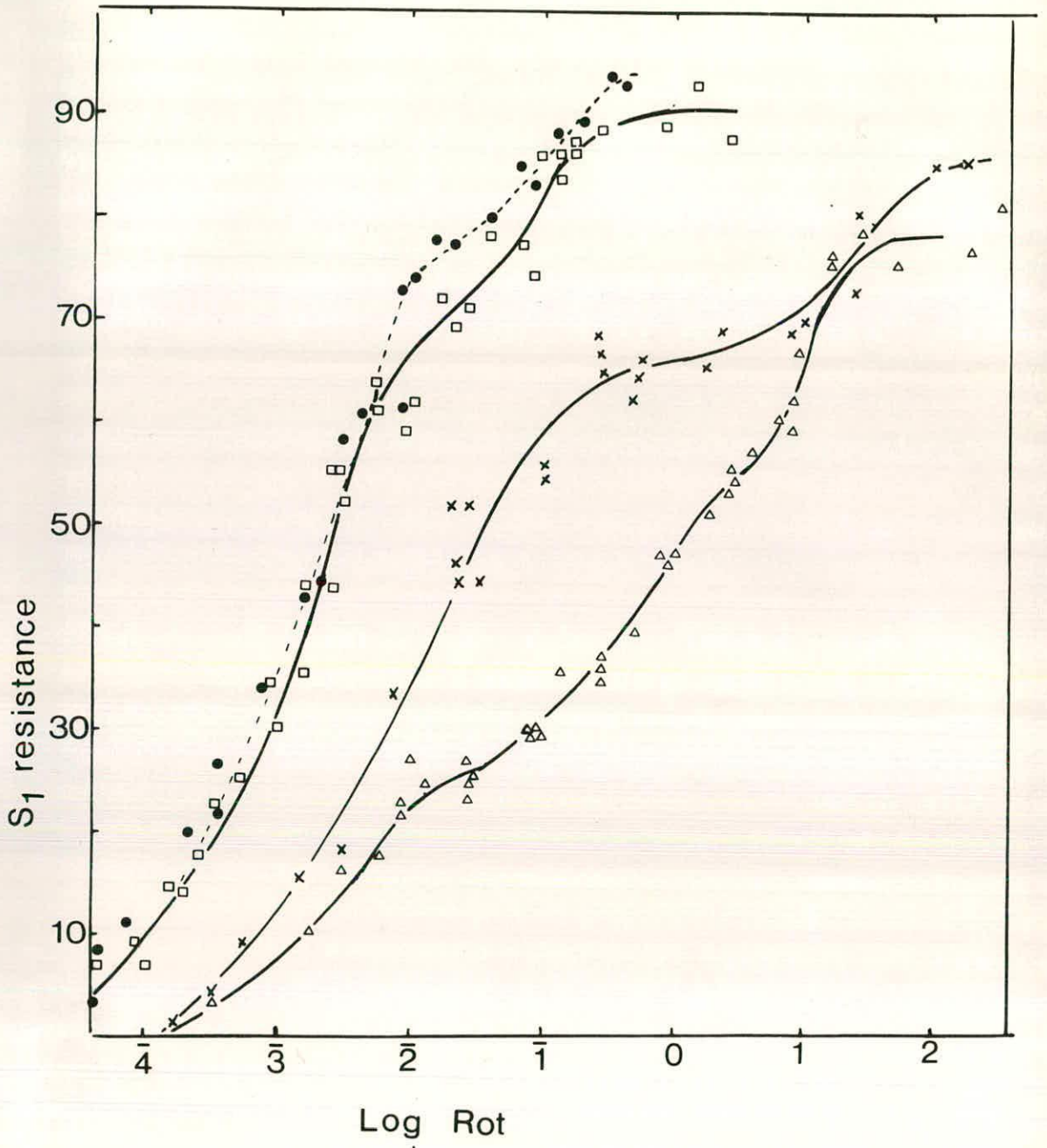


Table 2

Number of mRNA sequences in the different stages of development of
Drosophila melanogaster
(poly(A) = 3.8% of mRNA)

Stage of development	Abundance classes	Observed $\frac{Rot1}{2}$	(P)-proportion of total	$\frac{Rot1}{2} \times P$	K^h	No. of different 1800 nucl. sequences
Embryo	1	2.5×10^{-3}	26(33)*	6.5×10^{-4}	1000	1 (1)*
	2	0.47	28(36)	0.13	5.3	164(217)
	3	9.2	24(31)	2.2	0.3	<u>2784(3607)</u> 2948(3825)
Larva	1	8.2×10^{-4}	73(78)	6×10^{-4}	1150	1 (1)
	2	0.058	20(22)	0.01	69	<u>12 (15)</u> 13 (16)
Pupa	1	8.7×10^{-3}	65(76)	5.6×10^{-3}	123	7 (8)
	2	18.4	20(24)	3.68	0.18	<u>4658(5589)</u> 4659(5590)
Imago	1	1.1×10^{-3}	70(77)	7.7×10^{-4}	896	1 (1)
	2	0.087	20(23)	0.017	40	<u>21 (25)</u> 22 26

(*) = inside brackets are shown values normalized to 100%

hybridization.

units of Rot). It may include two different transitions, one of them comprising only 1 RNA sequence and 30% of the cDNA ($\text{Rot} \frac{1}{2} = 2.5 \times 10^{-3}$) and the other 14 different sequences and 35% of the cDNA ($\text{Rot} \frac{1}{2} = 3.2 \times 10^{-2}$). More experimental data and best fit analysis by a computer might resolve this question.

Because a slight modification in the $\text{Rot} \frac{1}{2}$ of the least abundance class will represent a considerable change in the number of mRNA sequences involved, and considering that the present data has not been analysed by a computer, the 5000 value given for pupa, should not be taken as an exact number but as an approximation to the real number of mRNA species present in the cytoplasm of pupae cells during metamorphosis.

The real complexities of larvae and imago on the other hand, may be hidden because of the magnitude of the first component. A late transition corresponding to about 10% or less of the cDNA would be difficult to detect. It is also possible that during the procedure of homogenizing whole larvae, pupae or imagos by hand in a loose-fitting homogeniser, we are preferentially disrupting soft tissues and therefore, underestimating the complexity of the whole organism. The cDNA-RNA embryo hybridization shows 3 abundance classes and a number of different polyadenylated sequences in between 3000 and 4000.

Our results suggest that the total number of mRNA sequences expressed within the life-cycle of the organism is very similar to the number of bands seen in the polytene chromosomes of the larval salivary glands. One possibility, nonetheless, is that the sequences expressed in two different stages of development are completely

unrelated. If that is the case, the total number of genes expressed within the life span of *Drosophila* would be much higher than the number of chromosomes. This moved us to study the similarities between mRNA populations from different stages of development.

Similarities and differences between mRNA populations from different stages of development

We have examined the homologies between different mRNA populations by a series of cross-hybridization experiments in which cDNA from one source is annealed with an excess of mRNA from another.

Fig. 14 shows the reaction of cell line mRNA with cDNA prepared against embryo mRNA. The three phase curve observed is essentially identical to that of embryo mRNA and homologous cDNA and both reach a maximum of 180% hybridization by a Rot value of 40.

The conclusion from this experiment is that all the polyadenylated sequences present in embryos are also included in the cytoplasm of the cell line cultured in vitro.

From the results shown in Table 3 we can conclude that there is no significant difference between the homologous and heterologous hybridization, and that therefore all the sequences present in the embryonic stage are also present in the cell line (once isolated from late embryonic tissues). The quantity and quality of their mRNA sequences seems to be the same.

Pupal cDNA was hybridized in an excess of cell line mRNA and vice versa (that is cell line cDNA in an excess of pupal mRNA). The results show that a minimum of 65% of the cell line cDNA hybridizes with pupal mRNA and 70% of the pupal cDNA does so with cell mRNA. This means that 82% of pupal cDNA is represented in cells

Legend to Fig. 14

Reactions between mRNA and mRNA-specific cDNA from *Drosophila* embryos and cell line 3. Reaction conditions as for Fig. 13.

X = embryo mRNA and homologous cDNA

●-● = cell line mRNA in excess over embryo cDNA

--- = cell line mRNA in excess over homologous cDNA

Rot values based on the assumption that poly(A) forms 3.5% of mRNA. Those in Table 3 are adjusted to the true value of 3.8%.

Fig. 14

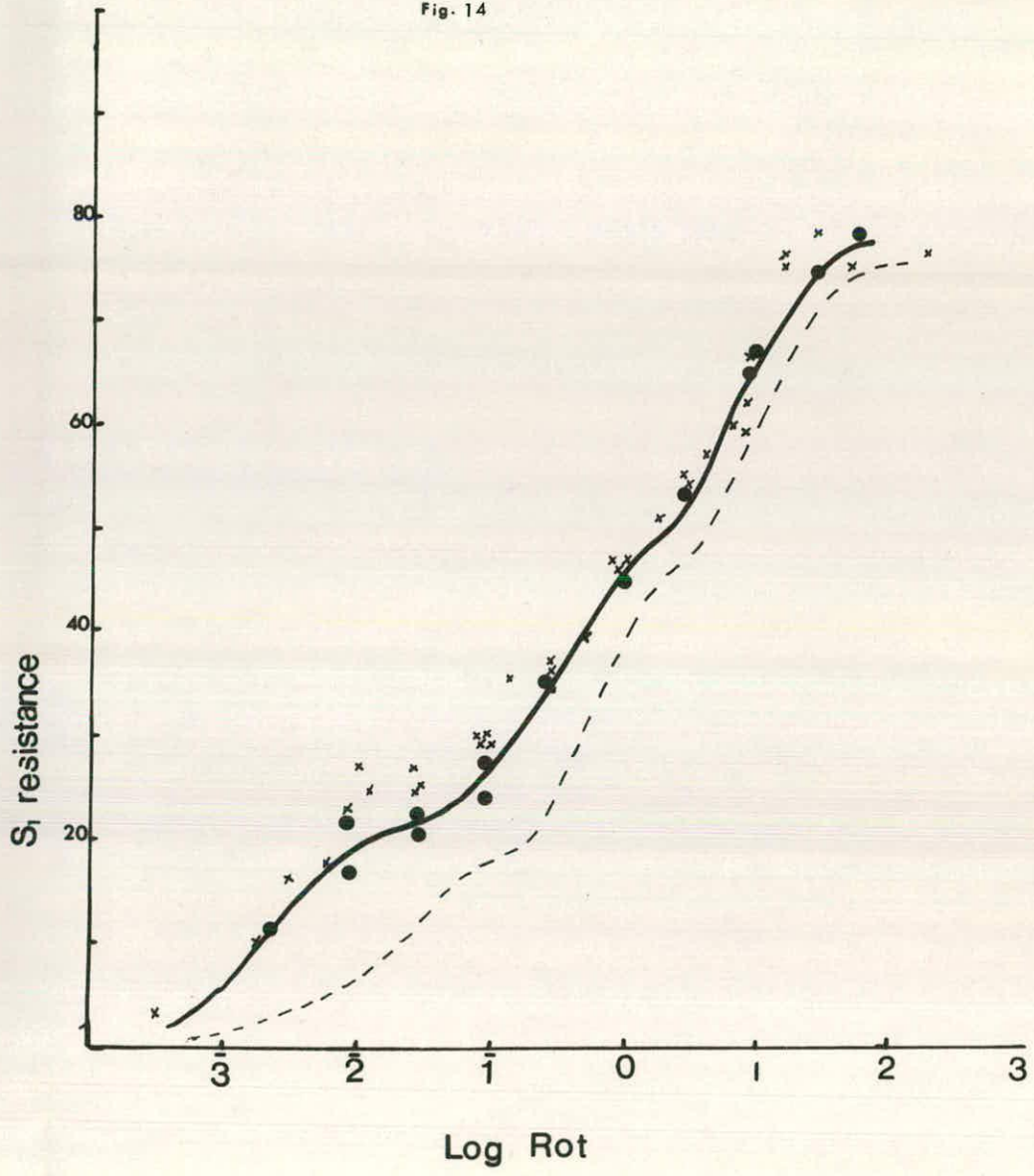


Table 3

Comparison between the number of mRNA sequences present
in embryos and the cell line

Hybridization reaction	Abundance classes	Observed $\frac{Rot1}{2}$	(P)	$\frac{Rot1 \times P}{2}$	No. of different sequences
embryo mRNA driving embryo cDNA	1	2.5×10^{-3}	26	6.5×10^{-4}	1
	2	0.47	28	0.13	164
	3	9.2	24	2.2	2784
cells mRNA driving cells cDNA	1	0.05	20	0.01	12
	2	0.8	25	0.2	253
	3	9.2	30	2.76	3493
cells mRNA driving embryo cDNA	1	2.3×10^{-3}	22	5.0×10^{-4}	1
	2	0.29	28	0.08	101
	3	8.28	28	2.3	2911

and 86% of cell cDNA is present in pupae (Table 4).

An interesting result was obtained when polyadenylated larval or imago mRNA was annealed with cDNA prepared against cell line mRNA (fig. 15). The observed hybridization reaction suggests that 65 to 70% of the mRNA population of the cell line is made up of sequences which are also present in flies and larvae. Considering that the cell line homologous reaction reaches a plateau at about 75% hybridization, 90% of the cell line cDNA seems to be complementary to larval and imago mRNA. A minimum value for the complexity of larvae and flies RNA can be estimated if it is assumed that 45% of the hybridized cDNA corresponds to the first and second abundant classes observed in the cell line. The remaining 25% at least, must belong to the more complex (less abundant) class containing 30% of the total cell line population of messengers. That is 83% of that cell line class. 83% of 3758 different sequences (considering the lowest value) is equivalent to 3119 sequences. This value thus represents the lower limit for the complexities of larva and imago populations of messengers.

We have hybridized non-repetitive ^3H -labelled cell line DNA with imago RNA in RNA excess. In these conditions the contribution of the very abundant messengers is negligible; instead, a small percentage of rare or less abundant sequences will be easily detected. By a Rot value of 160, 1% of the non-repetitive DNA was found in RNA-DNA duplexes. This represents a sequence length of 7.5×10^8 and about 1300 different mRNAs of average molecular weight.

Table 4

Cross hybridization reactions; % of hybrid molecules
at saturation values

cDNA

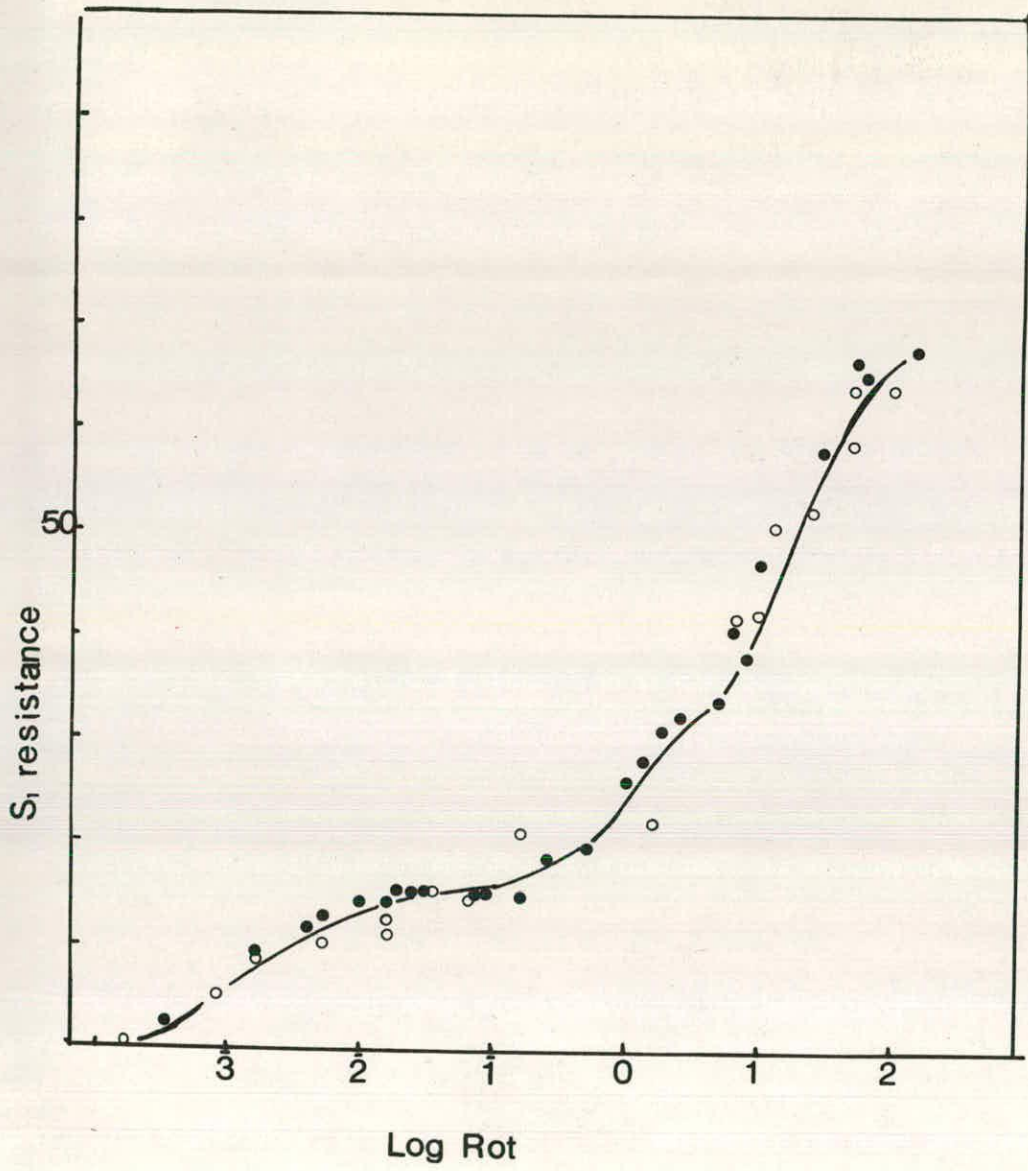
	Cell	Embryo	Pupa	Larva	Imago
Cell	75	80	70		
Embryo		80			
mRNA Pupa	65		85		
Larva	70			90	
Imago	70				90

Legend to Fig. 15

Reactions between cDNA prepared against cell line mRNA, with larvae mRNA (○) and with imago mRNA (●) in mRNA excess.

Reaction conditions as for fig. 13. Rot values based on the assumption that poly(A) forms 3.5% of mRNA. Those in the table are adjusted to the true value of 3.8%.

Fig. 15



Isolation and characterization of a single species of RNA which predominates in populations of imago polyadenylated RNA

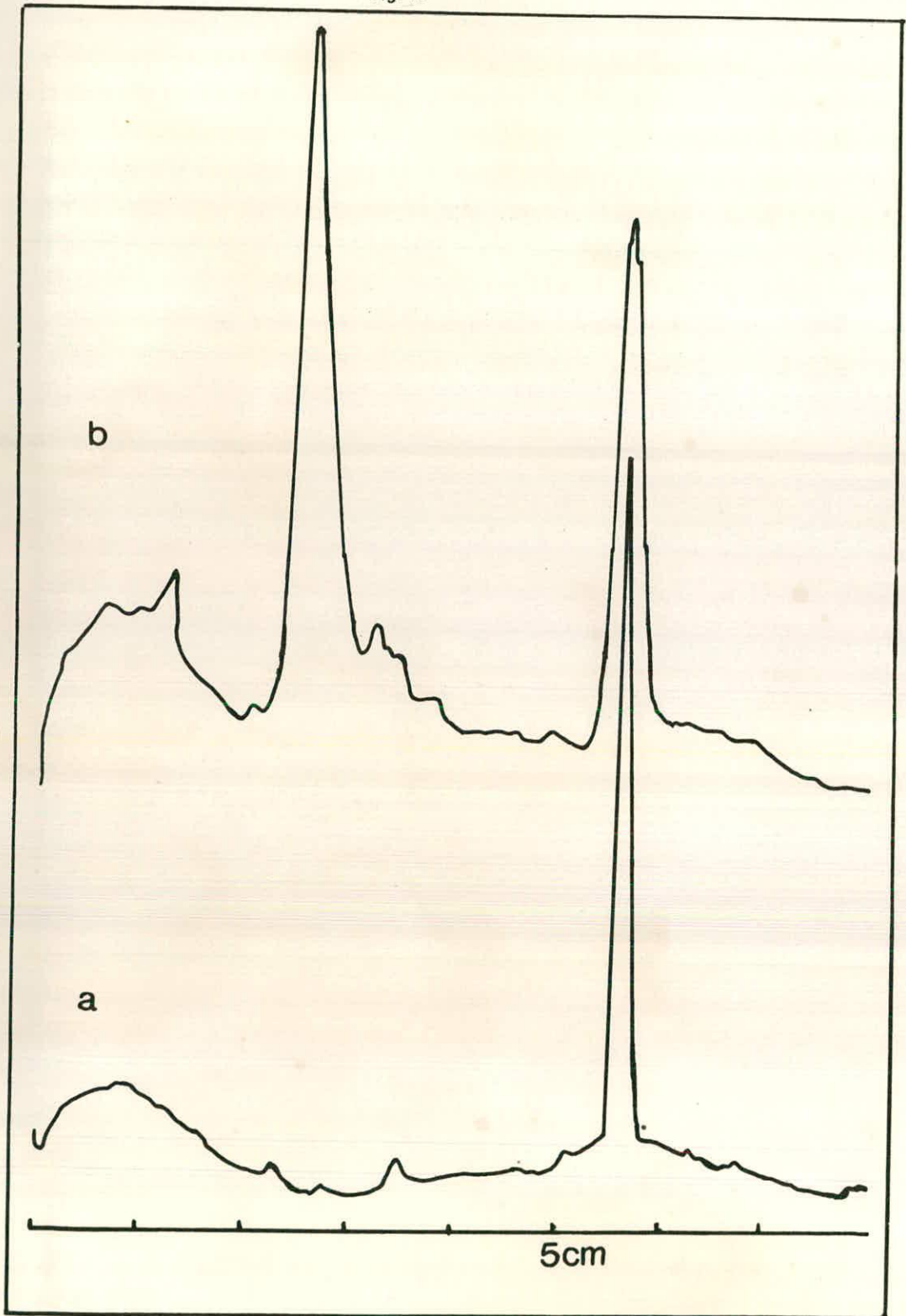
Analysis by sucrose density gradient sedimentation of the polyadenylated fraction of RNA from imagos showed a sharp peak of absorbance (A₂₅₄) and a sedimentation coefficient of about 14S units. We have also observed that about 65% of the flies' cDNA hybridizes with the kinetics expected for a single mRNA species. This suggested that the 14S component might constitute a single RNA sequence which it would be relatively easy to isolate. The peak fractions from a sucrose gradient were pooled and analysed by electrophoresis through a 3% polyacrylamide gel (fig. 16). The sharpness of the main peak when compared with the 18S and 28S ribosomal components from HeLa cells, suggests that the 14S component contains a single predominant homogeneous species of RNA. From the gel electrophoresis data we estimated a molecular weight for the 14S RNA of 6×10^5 . A more accurate measurement of the molecular weight was obtained by comparing the mobility of 14S RNA with the mobilities of known markers under denaturing conditions (98% formamide in 10mM barbitone buffer pH 9) (plate 1). The markers used were 4S, 5S, 18S, 28S and globin 9S mRNA from ducks (fig. 17). Relative to these we calculated the molecular weight of the Drosophila 14S RNA to be 5.9×10^5 daltons, corresponding to about 1800 nucleotides.

14S mRNA prepared by sucrose-gradient sedimentation was used as a template for cDNA synthesis by the reverse transcriptase. The kinetics of RNA-driven hybridization between mRNA and cDNA are shown in fig. 18. The reaction is close to ideal first order, with a $\text{Rot} \frac{1}{2}$ of 7.5×10^{-4} and a rate-constant of $1400 \text{ lM}^{-1} \text{ sec}^{-1}$. By

Legend to Fig. 16

3% polyacrylamide gel electrophoresis of (a) 14S RNA cut from a sucrose gradient and (b) 18 and 28S RNA from HeLa cells. The gels were run for 6h at 5mA/gel (15 volt/cm).

Fig. 16



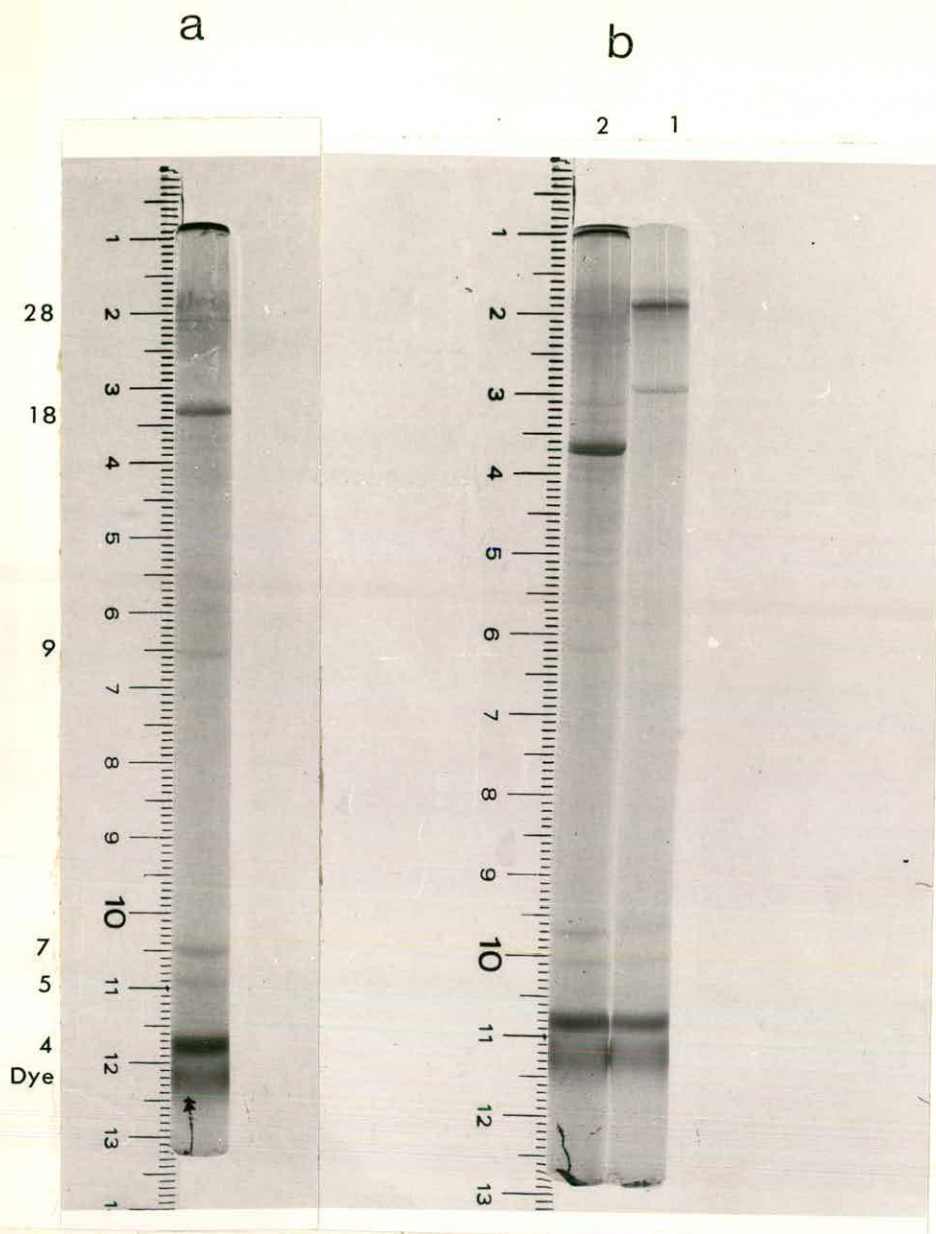


Plate I

Acrylamide gel electrophoresis in denaturing conditions (3.5% acrylamide, 97% formamide). Gels were run at 5mA/gel for 5 hours.

(a) 28S, 18S, 7S, 5S and 4S markers from HeLa cells and duck globin mRNA (9S).

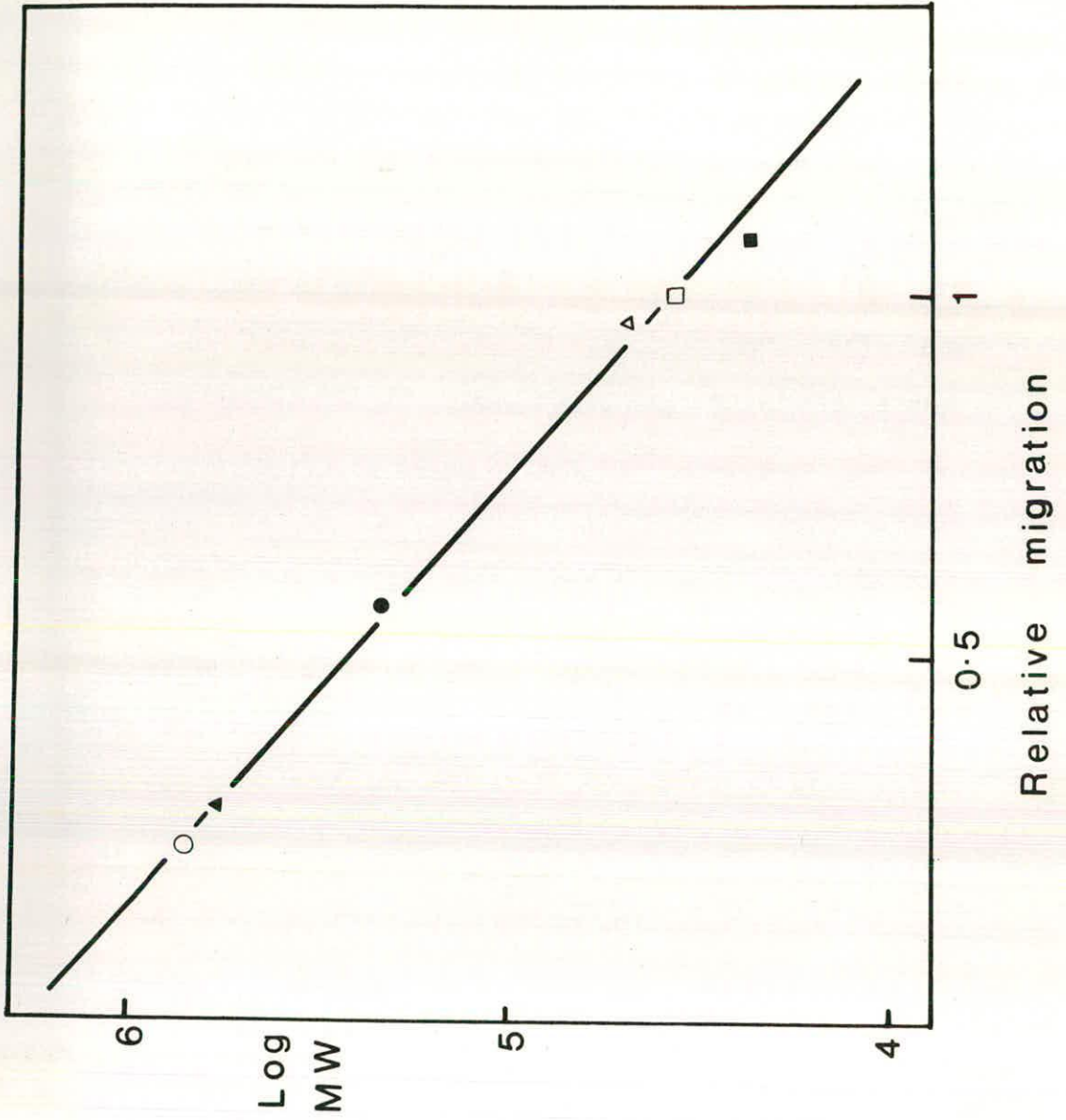
(b) 28S, 18S, 7S, 5S and tRNA (4S) markers from HeLa cells (1) and 14S mRNA from Drosophila imagos run with some 28S, 7S, 5S and 4S internal markers. (2).

Legend to Fig. 17

Acrylamide gel electrophoresis in denaturing conditions. The markers considered primary are 5S RNA (□) HeLa cell 18S rRNA (○) and duck globin mRNA (●). The positions of tRNA(■) and HeLa cell 5.8S rRNA(△) are also shown.

The position of 14S RNA is shown by (▲).

Fig. 17

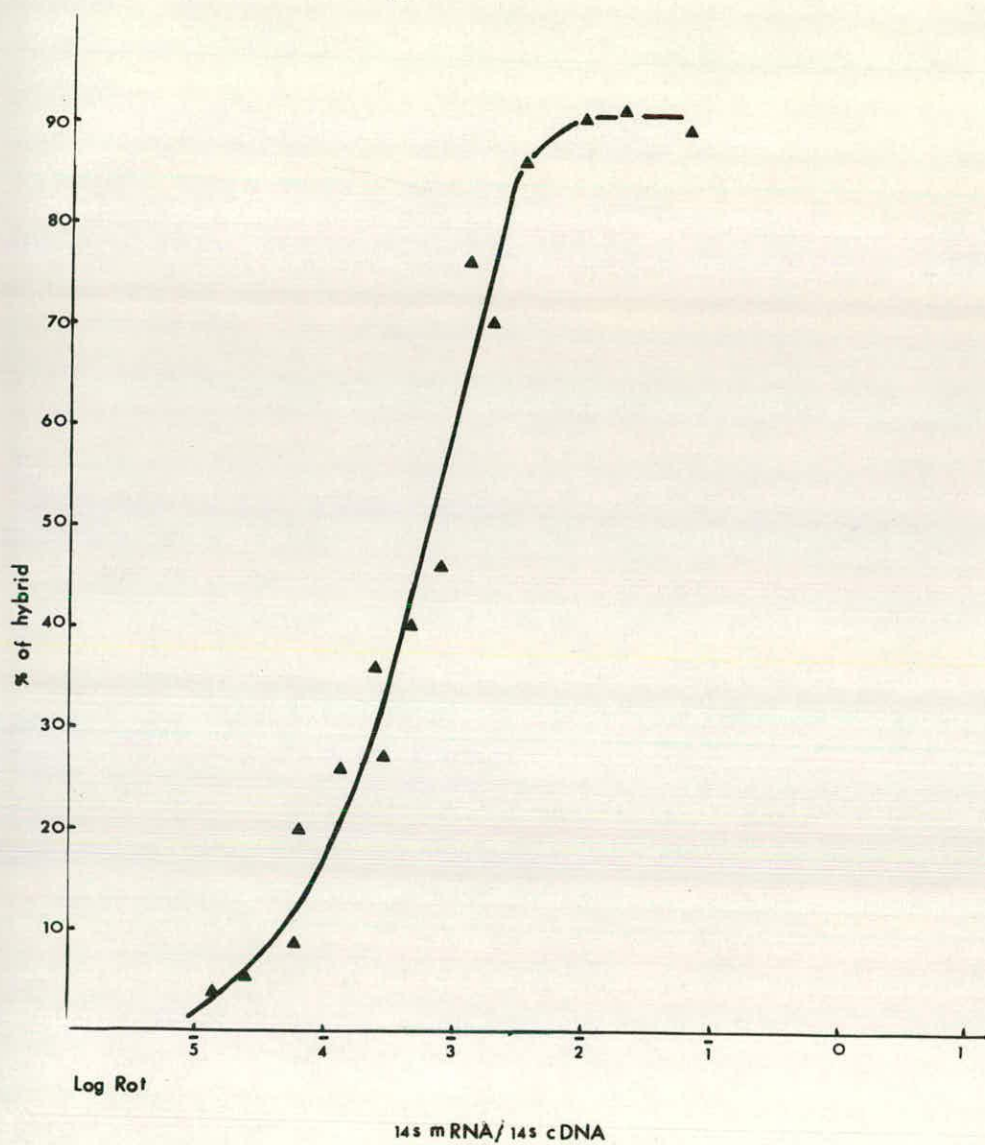


Legend to Fig. 18

Hybridization between 14S mRNA and cDNA prepared
against it.

Annealing conditions as for Fig. 13.

Fig. 18



comparison with kinetic standards (Bishop et al., 1975) a $\text{Rot}\frac{1}{2}$ of 7.5×10^{-4} corresponds to a driver RNA sequence complexity of 5.6×10^5 daltons. This is so close to our estimate of the molecular weight of the 14S RNA as to suggest strongly that it is a single RNA sequence.

An independent estimate of the purity of the 14S mRNA was obtained by using it to drive the hybridization of cDNA prepared against the total polyadenylated mRNA extracted from imagos (fig. 19). Only a single component was observed clearly, in which 70 to 80% of the cDNA reacted with a $\text{Rot}\frac{1}{2}$ of about 10^{-3} , very close to that observed in the reaction between 14S mRNA and its homologous cDNA complement. The curve, on the other hand is broader than the homologous indicating some heterogeneity. At most 80% of the total imago cDNA reacts with 14S mRNA while 90% of it reacts with total imago mRNA. We interpret these observations as showing that between 70 to 80% of imago mRNA is complementary to 14S-specific cDNA. The data earlier analyzed agree well with this conclusion.

Presence of D-14S mRNA in different developmental stages

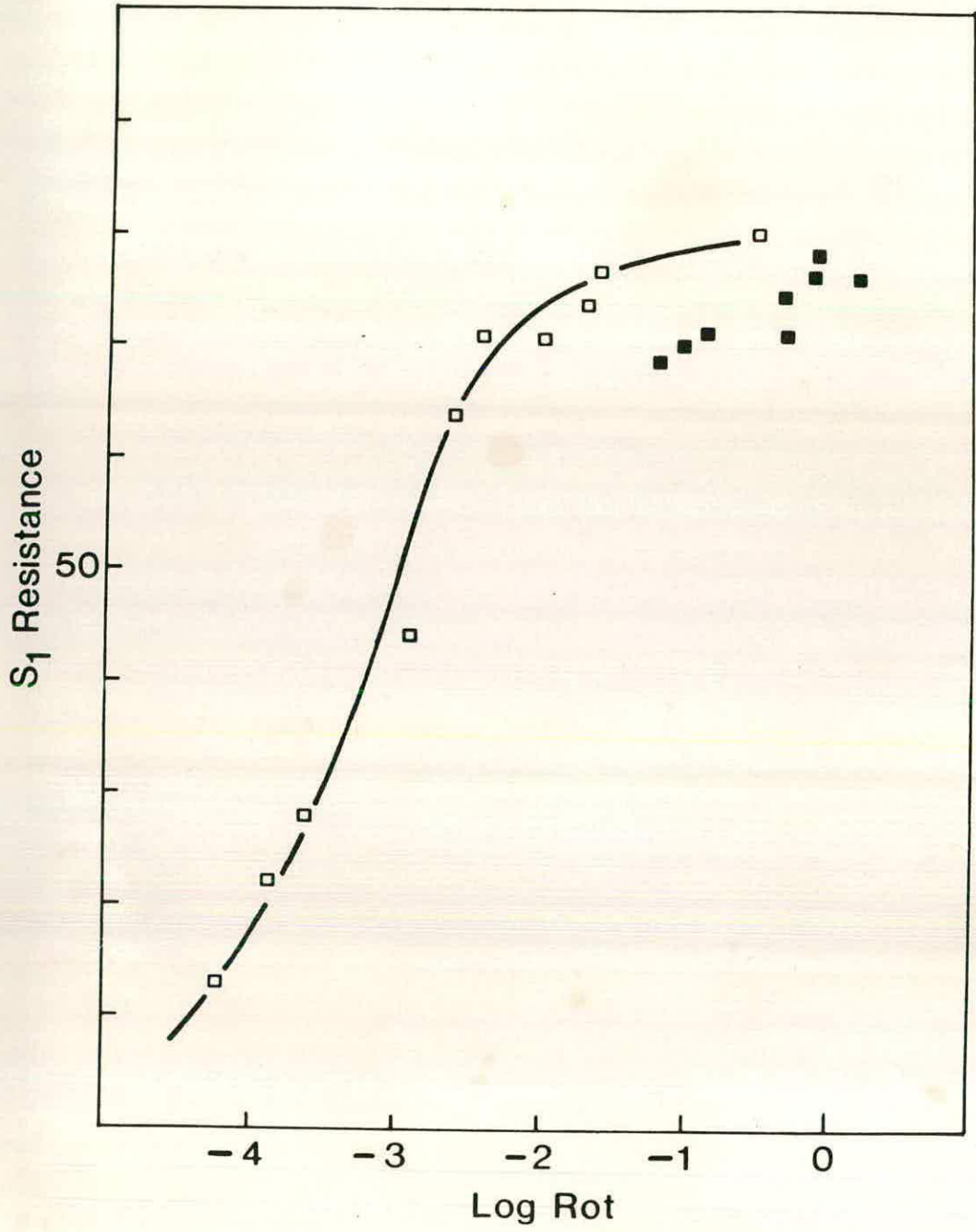
Total polyadenylated cytoplasmic RNA from embryos, larvae, pupae, imagos and from Schneider's cell line 3 were hybridized with cDNA complementary to 14S RNA in RNA excess. The results are shown in fig. 20 and summarised in Table 5. The clear conclusion is that RNA complementary to 14S cDNA is present in all stages of development but in different proportions.

The proportion of RNA complementary to 14S cDNA in each mRNA population is given by the ratio of the $\text{Rot}\frac{1}{2}$ of the homologous 14S reaction (14S cDNA driven by 14S mRNA) to the $\text{Rot}\frac{1}{2}$ of the cross-reaction.

Legend to Fig. 19

Hybridization between 14S RNA and cDNA prepared against the total poly(A)-RNA extracted from imago. 14S RNA was isolated by sucrose-gradient fractionation of oligo(dT)-cellulose-bound RNA. Two independent experiments are shown.

Fig. 19



Legend to Fig. 20

Hybridization between 14S-specific cDNA and mRNA from imagos (▲), larvae(■),embryos (●), pupae (□) and cell line 3 (○).

Fig. 20

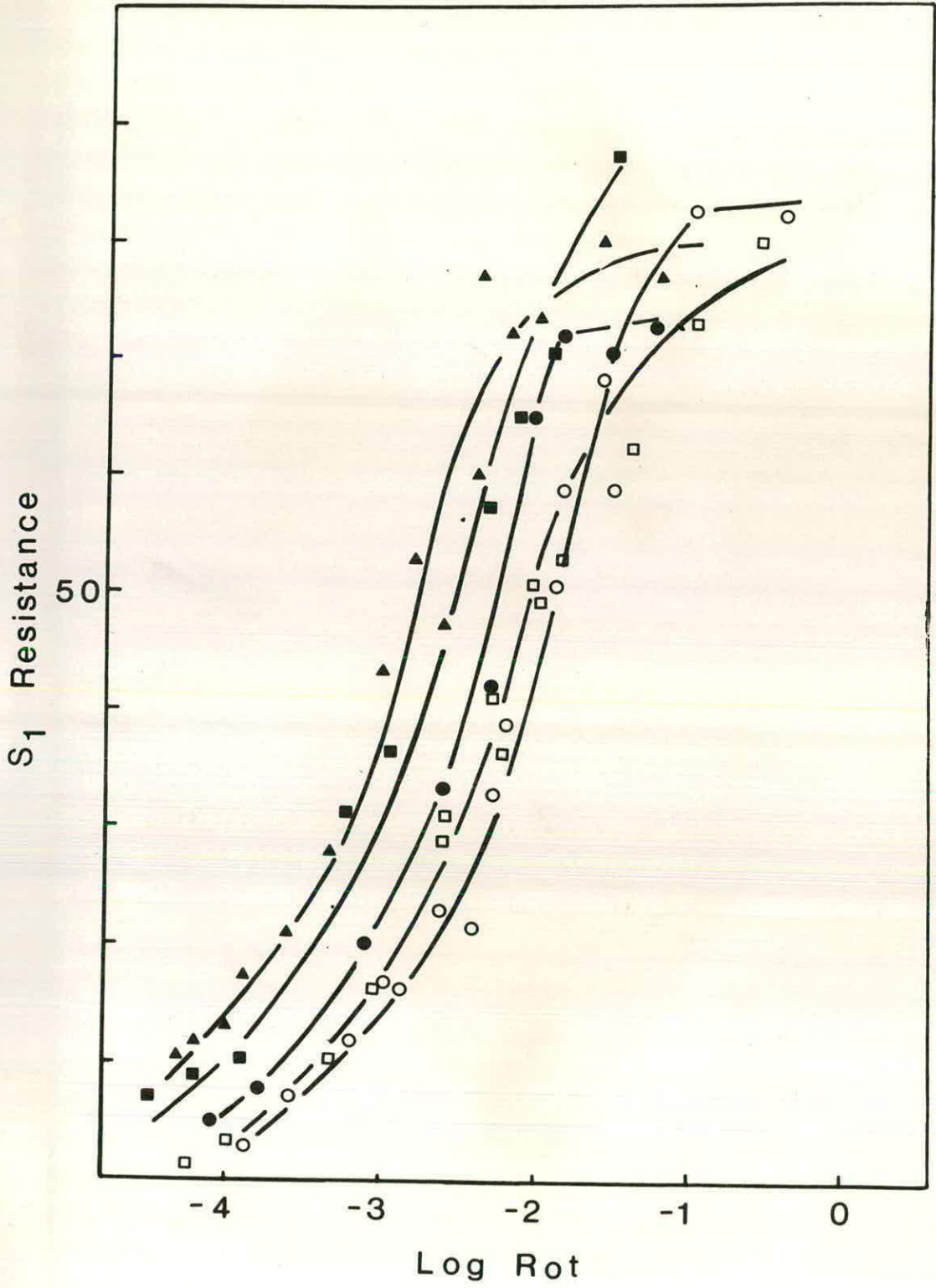


Table 5

Presence of 14S-mRNA in different developmental stages of Drosophila

Hybridization of 14S cDNA with an excess of cytoplasmic polyadenylated RNA from:	$\frac{Rot1}{2}$	Percentage of polyadenylated RNA complementary to 14S
Purified 14S	7.5×10^{-4}	90
Dros. cell line	9.1×10^{-3}	8
Embryo	3×10^{-3}	25
Larva	1.5×10^{-3}	50
Pupa	8.1×10^{-3}	9
Imago	1×10^{-3}	75

The sequence is very predominant in imagos and larva, making up 75% and 50% of the total poly(A)-RNA respectively.

Molecular hybridization between D-14S cDNA and male(♂) and female(♀) cytoplasmic polyadenylated mRNA

A female of Drosophila melanogaster, during her period of maximum egg production, will deposit daily a quantity of eggs equivalent to one-third her weight. Yolk proteins are very poorly characterized but their production is extremely high in the young Drosophila female. 14S RNA could be the messenger coding for one of them.

Polyadenylated RNA was extracted from males and females separately. cDNA prepared against 14S mRNA hybridized equally well and to the same extent to male as to female messengers (fig. 21). However, the reaction is about ten times slower than the hybridization between polyadenylated imago mRNA and 14S cDNA (fig. 20). The difference may be due to the omission of the sucrose gradient purification step in the male and female preparation. The free polyadenylic acid molecules will give a considerable overestimation of the mRNA concentration. From this experiment we concluded that the 14S RNA is not a messenger leading to a female specific product.

Virus-like particles in Drosophila melanogaster

Virus-like particles have been observed in tissues of imagos, in imaginal discs and in Schneider's cell lines L1, L2 and L3 (Kernaghan et al., 1964, Akai et al., 1967; Williamson and Kernaghan, 1972).

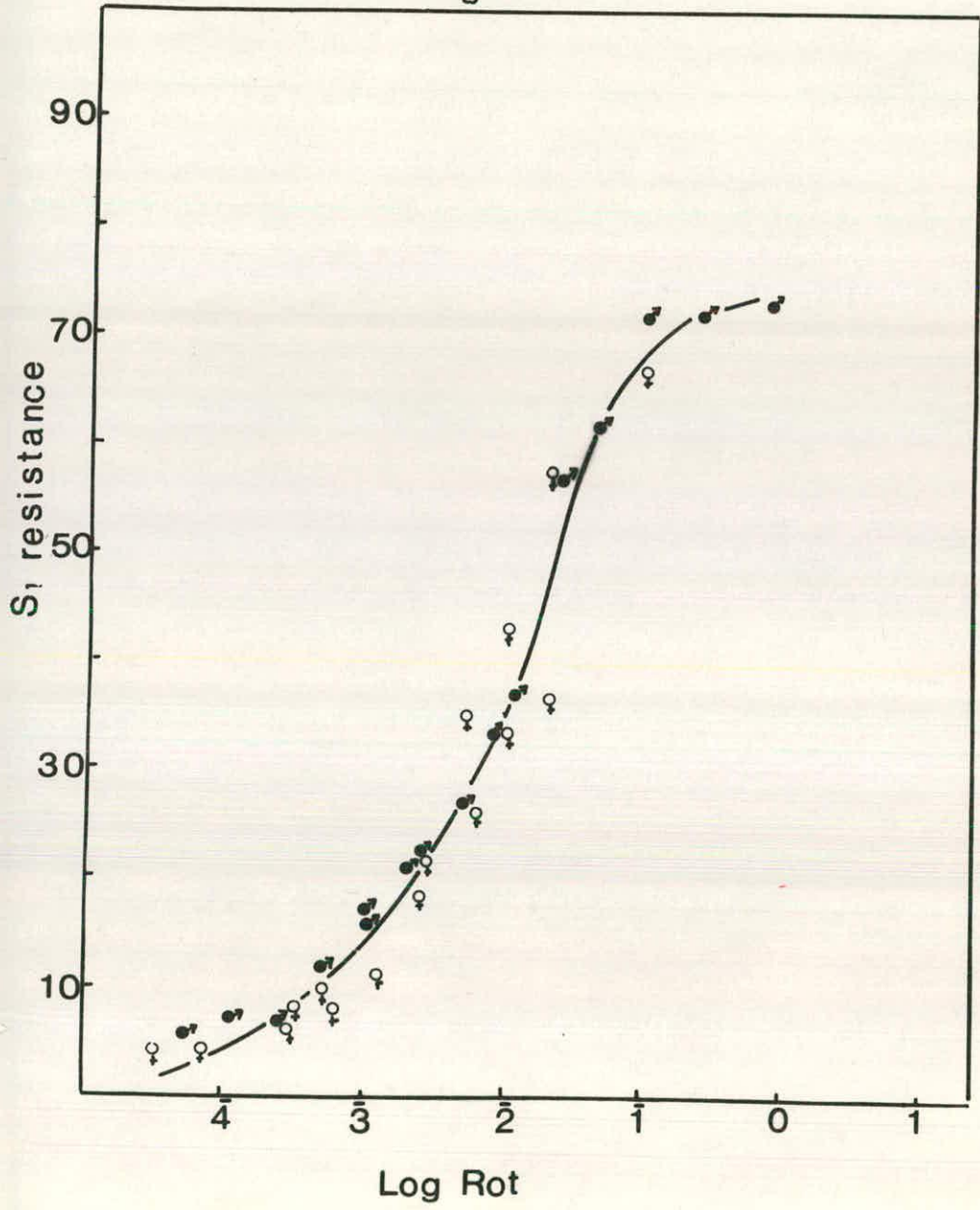
One possible explanation of the very large amount of a single mRNA found in whole organisms is that it is either the product of a viral genome or the viral genome itself.

We have examined our Schneider cell line L.3 for the presence of virus. A small number of virus-like particles was found mostly in

Legend to Fig. 21

Hybridization between 14S-specific cDNA and mRNA
from male (♂) or female (♀) imagos.

Fig.21



the nucleus, but occasionally in the cytoplasm of these cells (plate II). We have observed the presence of large cytoplasmic aggregates of particles about 60 μ in diameter (plate III). Each particle is made up of several subunits. We have identified this particulated component as glycogen (Giorgi, 1975; Perry, 1967, Drochmans 1962; Minio et al., 1966).

We have also examined gut, thorax, Malpighian tubules and trachea cells of our *Drosophila Oregon R* strain by electron microscopy, and have so far found only one cell containing virus-like particles in the nucleus and none with virus in the cytoplasm (plate 4). The particles have a diameter of about 37 μ .

These results suggest that the 14S RNA is not a viral genome. However, this does not exclude the possibility that 14S RNA is viral in origin.

In situ localization of poly(A)-containing sequences

³H-uridine-labelled mRNA from *Drosophila* L-3 cells was hybridized in situ to salivary gland chromosomes in 2 x SSC for 16 hours. After 6 months exposure, we observed the pattern shown in plate V. Grains are localized over the chromocenter and chromosomes. As in polytene chromosomes the DNA coding for rRNA is localized in the nucleolus (Pardue et al., 1970), the labelling in the chromocenter should correspond to mRNA sequences. This interpretation agrees with the results obtained by Spradling et al. (1975) and with the demonstration by Lakhota and Jacob (1974) that β -heterochromatin is active in RNA synthesis. We did not observe particular bands preferentially labelled and most radioactivity was scattered lightly throughout the chromosomes (plate V). The specific activity of the RNA was not very

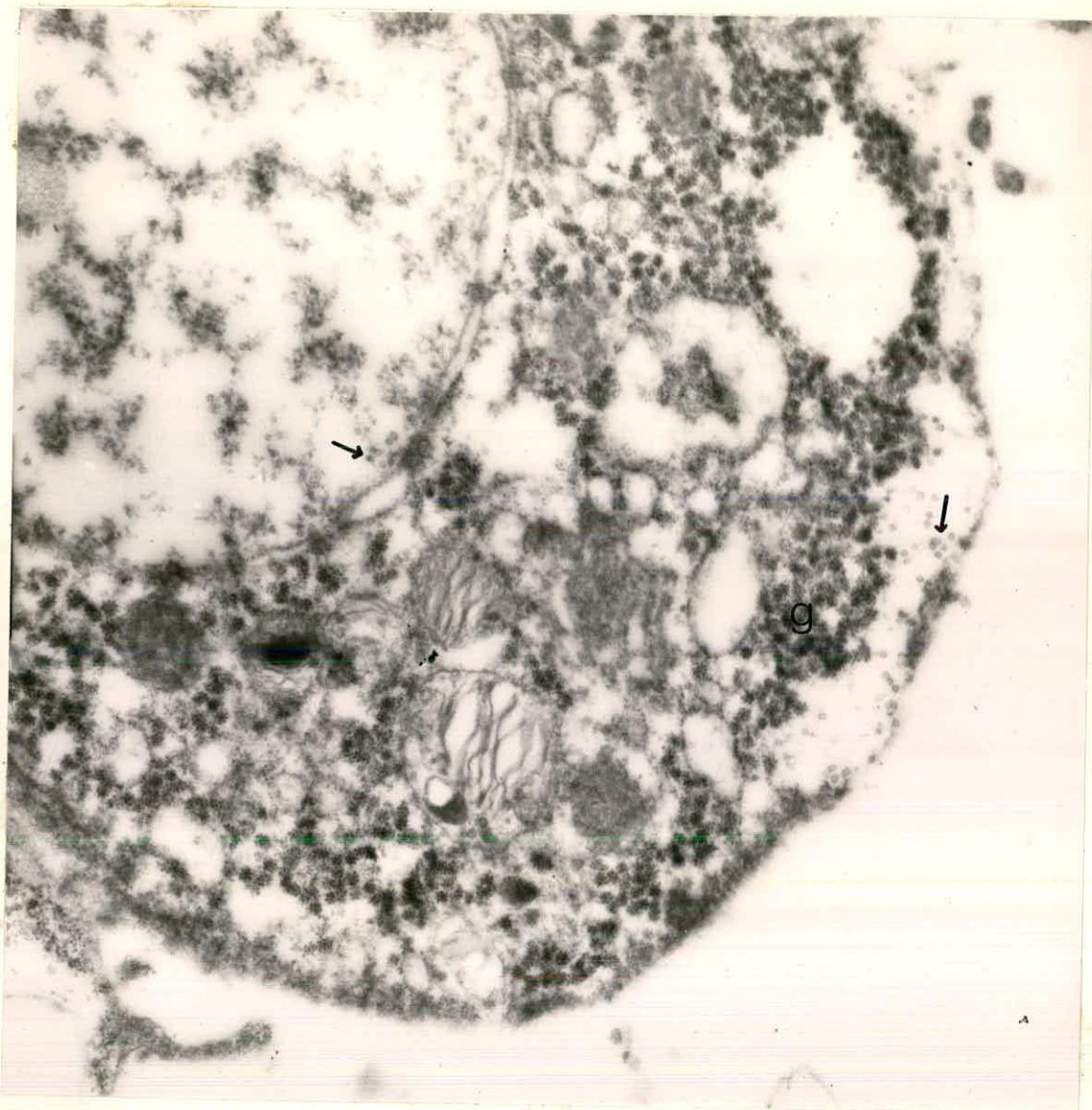


Plate II

Micrograph showing nuclear and cytoplasmic virus-like particles in Drosophila cell line, (arrows) and cytoplasmic glycogen (g). Magnification x 30000.



Plate III

Micrograph showing particulated aggregates of glycogen in the cytoplasm of Drosophila cell line 3 (g). No virus like particles can be observed in the cytoplasm and very few are seen in the nucleus (small arrows).

Magnification x 20000.

Plate IV

Micrograph showing nucleus (N) from a gut cell from
an imago containing virus-like particles (arrows).

Magnification x 40000.

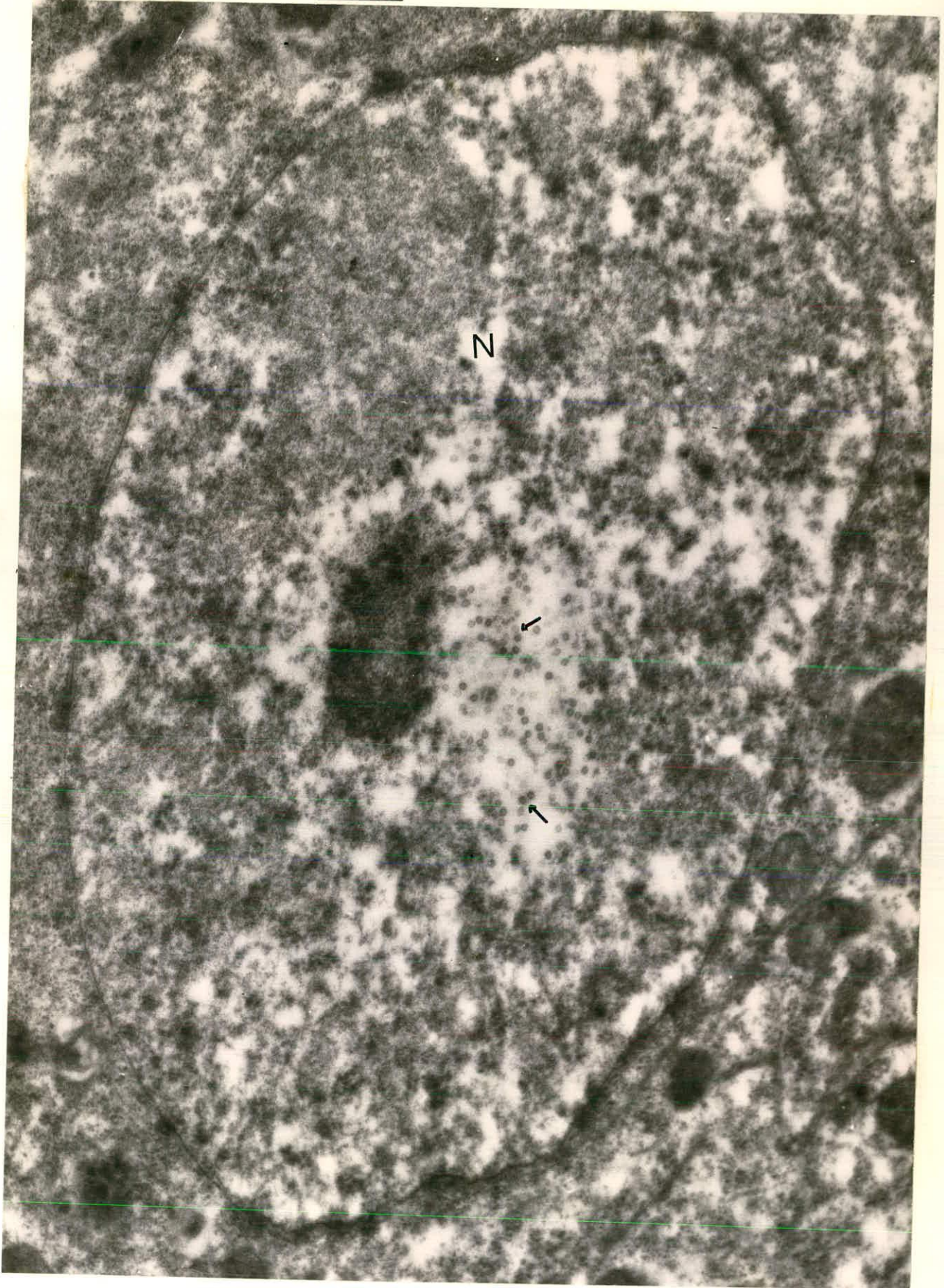


Plate V

Giant polytene chromosomes showing hybridization
"in situ" by ³H-labelled RNA from cells. Exposure
time, 6 months. Magnification x 3750.

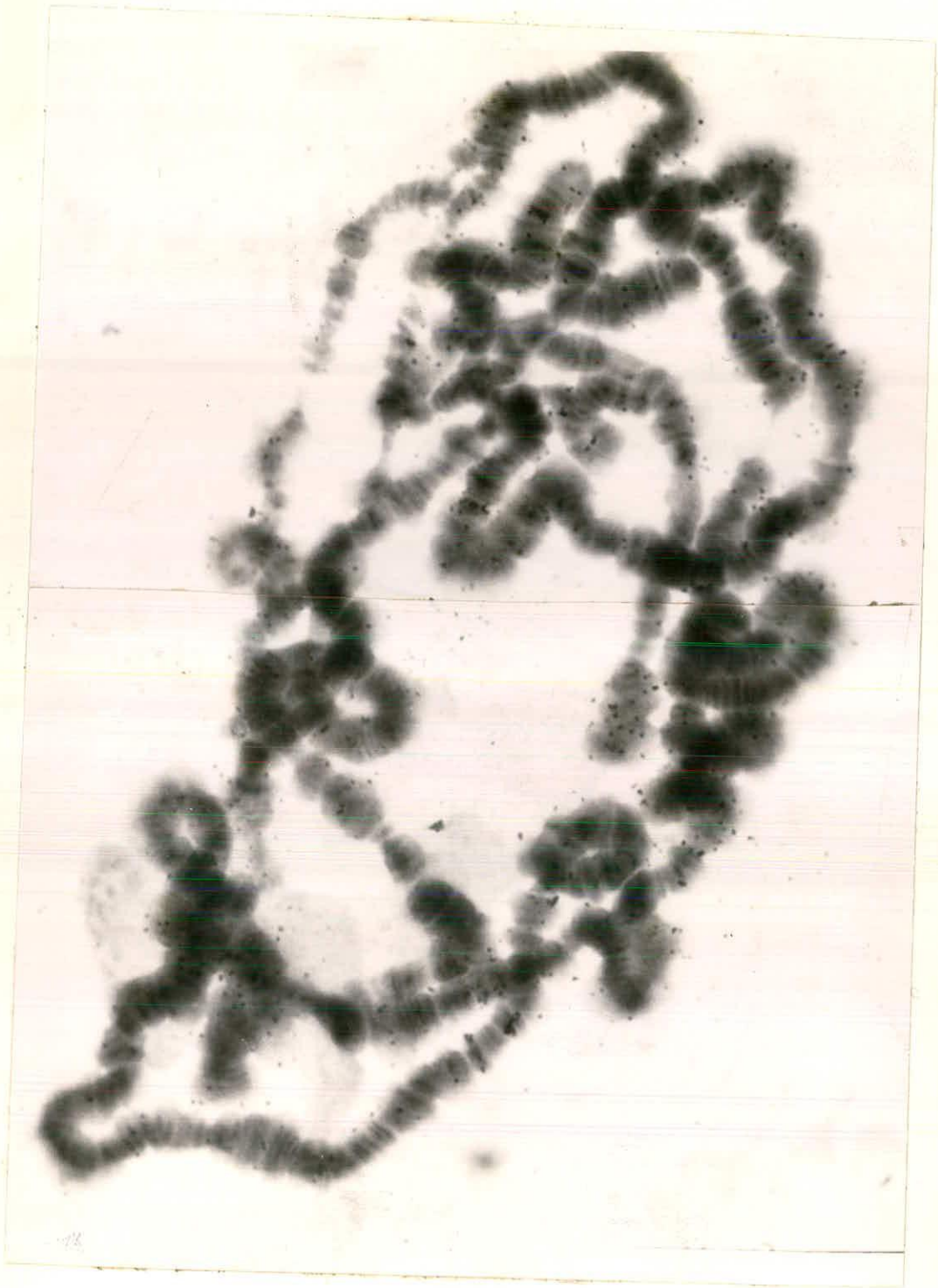


Plate V

high (7×10^5 dpm/ μ g) and extremely long autoradiographic exposure would be needed to obtain a reasonable number of grains over a particular site. The main difficulty in obtaining RNA of high specific activity in Drosophila cells is the presence of yeast extract in the culture medium which results in a high concentration of unlabelled purines and pyrimidines and therefore a dilution of the specific activity of added radioactive RNA precursor.

A much more convincing result was obtained when 125 I-labelled imago mRNA was hybridized in situ to already denatured salivary gland chromosomes in 4 x SSC. 50% formamide for 24h at 37°C (plates VI and VII).

The specific activity of the RNA was 2×10^7 dpm/ μ g and sufficient grains were produced after a reasonable autoradiographic exposure. About 30 sets of chromosomes were analyzed finding an average of 25 to 30 loci specifically labelled by the imago polyadenylated RNA. (plates VI and VII). This number coincides with the number of different mRNA sequences comprised by the first and second transition classes observed in the imago mRNA population by cDNA-RNA hybridization. We have also tried to hybridize 125 I-labelled 14S mRNA to salivary gland chromosomes. No particular site was preferentially labelled after a reasonable exposure time using the same conditions as before (plates VIII and IX). There are several possible explanations for this surprising result: the 14S RNA could be mitochondrial in origin in which case its complementary DNA will not be present in the polytene chromosomes of larvae. Alternatively the 14S DNA although of chromosomal origin, could be part of the centromeric heterochromatin (or other region) under-replicated during formation of Drosophila polytene chromosomes (Dickson et al., 1971). A third possibility

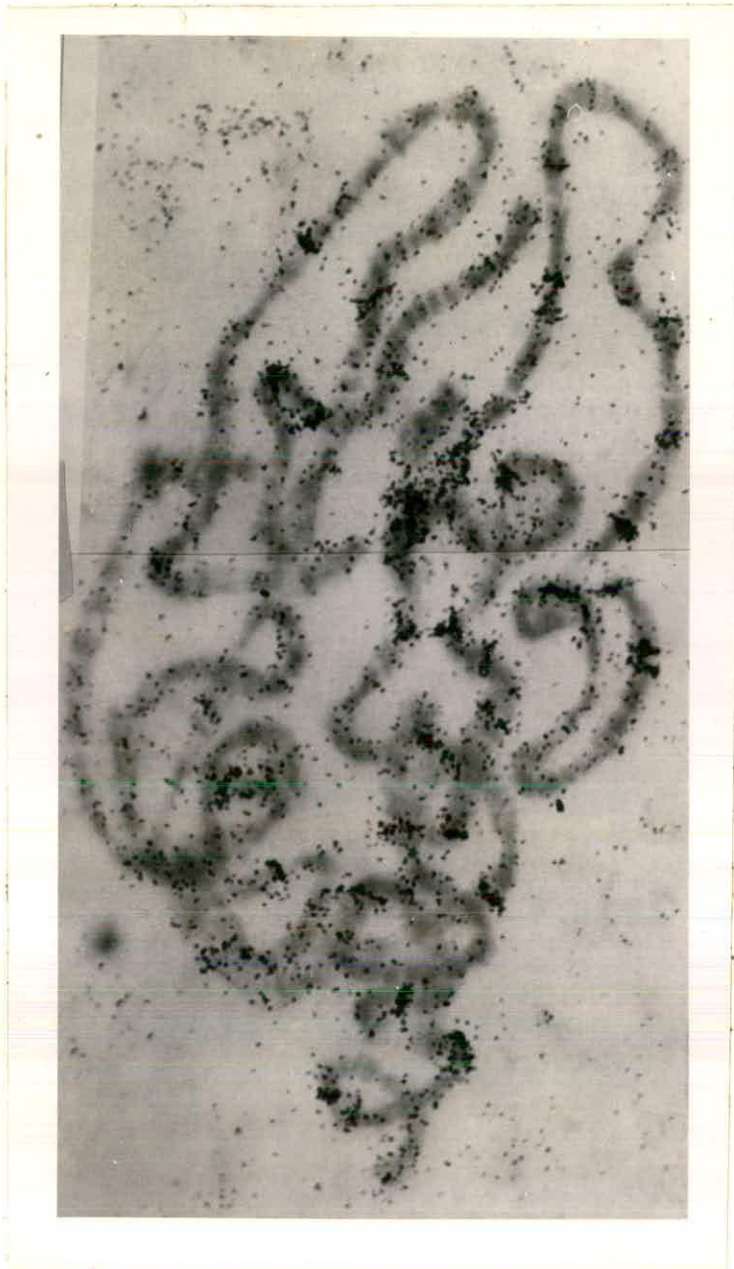


Plate VI

Giant polytene chromosomes showing hybridization
"in situ" by ^{125}I -labelled imago mRNA.

Exposure time, 4 weeks.

Hybridization was for 24h. at 37°C in 4 x SSC and
50% formamide.



Plate VII

Giant polytene chromosomes showing hybridization
"in situ" by ^{125}I -labelled imago mRNA.

Exposure time, 4 weeks.

Hybridization was for 24h. at 37°C in 4 x SSC and

50% formamide.

Plates VIII and IX

Giant polytene chromosomes showing hybridization
"in situ" by ¹²⁵I-labelled 14S mRNA.

Exposure time, 4 weeks.

Hybridization was for 24 hours at 37°C in 4 x SSC and
50% formamide.

Plate VIII

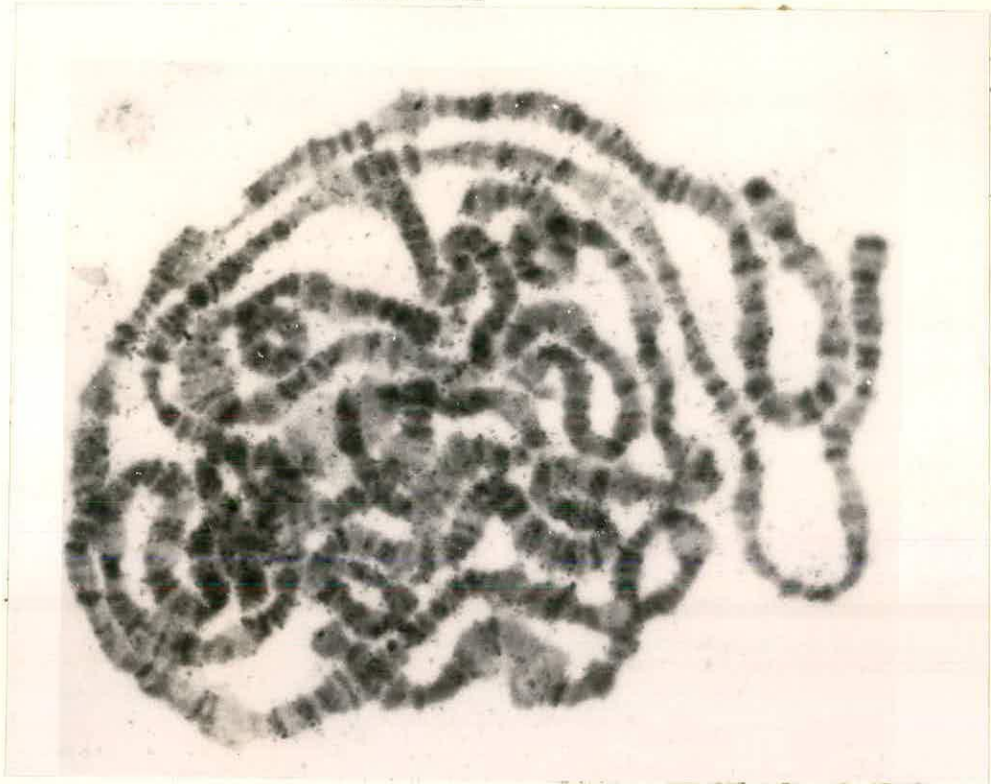


Plate IX



is simply an experimental failure in that particular experiment, in which case at least one of the labelled sites observed in the imago mRNA polytene chromosome DNA hybridization must correspond to that RNA so abundant in the imago population of messengers.

DISCUSSION

Analysis of the polyadenylated RNA population in Drosophila cell line 3 gives the estimate of about 5000 non-repetitive genes involved in the specification of most, if not all of the proteins present in these cells. Great confidence in this conclusion is given by the close agreement between the two different methods by which we approached the question of how much of the Drosophila genome serves as template for messenger RNA.

Our results obtained by cDNA-mRNA hybridization experiments are based upon two assumptions: first, that the average size of the mRNA is that shown by sucrose gradient sedimentation analysis, and second that the reverse transcriptase makes a faithful copy of the template without transcribing preferentially any given sequence. In relation to the first of the suppositions we have the data obtained for the D-14S mRNA. The size estimated for this messenger by sedimentation analysis (Spirin, 1963) is 4×10^5 . The gel electrophoresis data in both denaturing and not denaturing conditions suggest a molecular weight of 5.9×10^5 . The estimates argue against aggregation occurring under our sedimentation conditions, but we may be underestimating slightly the average size of the polyadenylated messengers. The molecular weight that corresponds to an average sedimentation coefficient of 17S using Spirin formula (1963) is 5.7×10^5 . We have used for our hybridization experiments an average value of 5.9×10^5 . Thus we may be still underestimating the average size of messengers by a factor of about 1.4. If that were the case, the proportion of poly(A) in the mRNA molecules will be smaller than estimated. This does not affect the number of mRNA

sequences estimated by cDNA-mRNA hybridization because the RNA concentration in these experiments was calculated from its poly(A) content. On the other hand, our estimates based on non-repetitive DNA-RNA hybridization would be lower by a factor of 0.7 (4000 instead of 5700 in the cell line).

The overall number of different sequences would still be close to 5000 (\pm 1000). A second uncertainty rises from the possibility of preferential transcription of some mRNA sequences by the reverse transcriptase enzyme. The good agreement between the kinetic data and the DNA saturation experiment virtually excludes this possibility, since the latter approach does not utilise a DNA copy of the mRNA.

The number of protein coding genes in the embryonic cell line Schneider L-3 is extremely close to the number of chromomeres (band + interband) observed in salivary gland polytene chromosomes. Some caution is warranted before the strict acceptance of this one to one correspondence. The gene numbers obtained using polyadenylated fractions should be regarded as a minimum because non-polyadenylated mRNAs other than histone mRNA may also be present in the mRNA population and they would not contribute to our measured complexity values. There are many technical problems in the estimation of the proportion of poly(A)(-) in the mRNA population. The presence of enormous amounts of rRNA is one of the main problems. The alternative is to take steps to selectively inhibit rRNA transcription with specific antibiotics or to isolate the mRNA from polysomes. In the latter case a heterogeneous sedimentation population of RNAs normally contaminates polysomal polyA(-) RNA fractions (Milcarek *et al.*, 1974). In the case of HeLa cells, 30% of the total mRNA population seems to lack poly-

adenylated segments affixed to the 3' end of the molecules (Milkarek et al., 1974). If Drosophila polyadenylated mRNA represented 70% of the total and the polyA(-) fraction were completely unrelated to the polyA(+), the number of different sequences in Drosophila cells would increase to about 6000. This number is still very close to the number of chromomeres. Nevertheless, the possibility that the number of bands is reasonably close to the number of genes by mere coincidence is still open.

Our experimental data suggests that the cell line L-3 initiated by Schneider 6 years ago from embryonic imaginal discs tissue, still transcribes and probably translates all the polyadenylated sequences expressed in the developing embryo.

By molecular hybridization of globin-specific cDNA to HeLa cells mRNA, it was shown by Bishop et al. (1975), that HeLa cells were not expressing the globin genes and therefore they were not completely derepressed. Considering our cDNA-mRNA hybridization results, pupal mRNA could be more complex than the cell line RNA, and this would be an indication that the cell line is not completely derepressed. Nevertheless, at saturation 85% of the cDNA from pupae is rendered resistant to single stranded nuclease while only 75% of the cell cDNA becomes resistant. In addition, the non-repetitive DNA-RNA hybridization experiment gave a complexity of 5700 different mRNAs for the cell line. This number is very close to the one determined by cDNA-RNA hybridization for pupae. The cross hybridization experiments between pupae and cells, again suggests that a minimum of 80% of their sequences are common. From our data, therefore, the possibility of a complete derepression in the cell line genome seems likely.



The results of the homologous hybridizations show that during metamorphosis the organism is expressing close to 5000 protein coding genes during embryogenesis close to 4000, and during larval or imago stage a minimum of about 3000 protein coding genes. All these values must be considered as minimal estimates for the following reasons:

- 1) The polyadenylated population of messengers represents most but not all mRNA sequences present in a given cytoplasm.
- 2) Rare or very infrequent sequences can go undetected in cDNA-mRNA hybridization experiments unless very high Rot values are obtained.
- 3) The reverse transcriptase may skip or transcribe preferentially some polyadenylated sequences.
- 4) Hard tissues from the organism may not be disrupted under our homogenization procedures and therefore will not contribute to our estimates.

From the cross-hybridization experiments we can temptingly suggest that about 80% of the total population of polyadenylated molecules could be common to cells and all the stages of development, and up to 20% of the sequences may be specific to each developmental phase.

From our data it becomes clear that the protein coding potential of the *Drosophila* genome is much greater than the actual number of different mRNA molecules the organism will translate during its life span. The percentage of non-repetitive DNA involved in transcription has been estimated by Turner and Laird, 1972, to be 15% in embryo, 14% in larva, 20% in pupa and 10% in adult. From our results we can calculate the percentage of DNA complementary to poly(A)-containing

molecules reaching the cytoplasm. The values we obtain are 3.2% in embryo, 4.5% in pupa and 2% in both larva and adult. There is a 5-fold difference between the percentage of DNA transcribed and the proportion of RNA selected to arrive to the cytoplasm, probably to be translated. Low percentages of non-repetitive DNA complementary to cytoplasmic mRNA have been obtained for other organisms: 2% for HeLa cells (Bishop et al., 1974), 2% also in sea urchin (Galau et al., 1974) and 1% in Xenopus oocyte (Davidson and Hough, 1971). The values obtained for Drosophila seem very reasonable taking into account the small genome size of the insect when compared to HeLa cells, Xenopus or sea urchin.

If the number of protein coding genes in Drosophila is insignificant in terms of the amount of DNA the organism has at its disposal, what other function for the DNA can be envisaged? Some loci in Drosophila may actually be control loci (similar to the operators and promoters in bacteria). These control sequences could constitute either a major or a very small part of the nuclear RNA which never reaches the cytoplasm. At present unfortunately, it is not known how much DNA in the organism is devoted to control function.

Our results could be consistent with a control mechanism for protein coding genes regulating the rate rather than the extent of transcription. All the protein coding sequences in the Drosophila genome could be transcribed throughout the lifetime of the organism. Prevention of the translation of unwanted mRNAs could take place in the nucleus by preventing them from entering the cytoplasm. The different abundant classes observed in cytoplasmic RNA could be the consequence of different transcription

rates. Inducers or repressor molecules would increase or decrease levels of transcription rather than switch "on" or "of" different protein coding transcriptional units.

With certainty, the most surprising result from the present work is the finding that 75% of the total polyadenylated imago population of messengers is a single sequence, and that this sequence is present in all developmental stages with proportions of 25%, 50%, 9% and 8% for embryo, larva, pupa, and cell line L-3 respectively. If we are preferentially disrupting soft tissues during our homogenization procedure, the selected cells will have the 14S in a proportion comparable to that of globin mRNA in reticulocytes and ovalbumin mRNA in the hen oviduct.

We have obtained D-14S RNA preparations with a high degree of purity and its molecular weight has been estimated to be 6×10^5 . Nevertheless we know very little yet about the origin of the 14S complementary DNA or the function of the 14S mRNA product.

There are three possibilities for the origin of the DNA coding for D-14S RNA.

- 1) The 14S DNA could be of viral origin. From our results we can only consider as very unlikely the possibility that the 14S RNA is the viral genome itself.
- 2) The 14S DNA could be mitochondrial origin. *Drosophila* possess large numbers of mitochondria and polyribosome clusters corresponding to RNA up to 8×10^5 daltons have been found inside *Drosophila* mitochondria (Choci and Laird, 1976).
- 3) The 14S DNA could be one of the 5000 protein coding genes that *Drosophila melanogaster* possesses.

In relation with the 14S RNA function, we know that the 14S RNA is a messenger RNA because 14S cDNA hybridizes with polyadenylated RNA obtained from polysomes. We concluded that the product of this messenger is not female specific thus eliminating yolk proteins as possible candidates. Another possibility is suggested by the fact that 14S mRNA is especially prevalent in mobile stages of development. 14S mRNA could specify a protein important for movement but at present we leave the 14S as a messenger with unknown origin and looking for a function.

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