

ABSTRACT OF THESIS

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1. A series of transducing bacteriophages carrying different parts of the galactose operon was obtained, and those which were not already temperature sensitive were made so for ease of induction.

2. Hybridisation experiments using DNA from these bacteriophages and pulse labelled RNA from a fucose induced culture of a strain of *E.coli*, with a deletion of the galactose operon in the main chromonema but carrying the whole operon on an episome were performed. The results of these were used to determine the best method of measuring galactose messenger RNA contents. A linear formulation of the hybridisation reaction developed by Bishop et al (1969) was used to obtain the true saturation value for this RNA.

3. The method selected involved calculation of the difference between hybridisation to two λ dgs, one of which carried almost all three cistrons of the galactose operon, the other only the first cistron. This method, and the Bishop analysis, were used to estimate the galactose messenger RNA contents of F^- and F' strains under different conditions of induction and repression. The measurements obtained were related to the levels of the enzyme galactokinase assayed in the same cultures. The effects of induction by a gratuitous and a metabolised inducer were compared, and the effects of both upon the enzyme and messenger RNA contents of wild type and constitutive strains with different numbers of the galactose genes investigated. Messenger RNA and enzyme levels of a culture repressed with methyl- β -D-thiogalactoside were measured. The significance of the results is discussed, and hypotheses incorporating recent investigations into the mechanism of induction and repression are constructed.

4. The galactose messenger RNA and galactokinase levels of four polar mutants were measured by the same method, and the current hypotheses about polarity discussed in relation to the findings.

5. A genetic analysis of the episome and transducing bacteriophages used in these experiments was performed.

Galactose-specific messenger Ribonucleic acid
contents in Escherchia coli K12

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Introduction

The Operon

Hypotheses to explain the control of enzyme synthesis in bacteria have developed from the elegant simplicity of the model proposed by Jacob and Monod (1961 a and b) to the present multiplicity of interacting factors which regularly increases in complexity with the discovery of each new component.

Jacob and Monod proposed their hypothesis to make a unified scheme to explain several different sorts of phenomena.

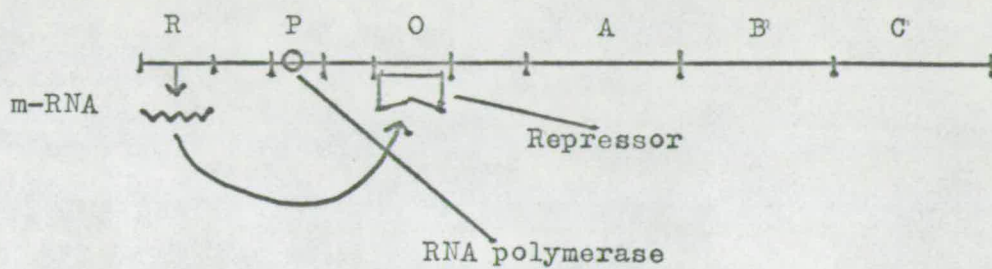
These were:-

a) The specific and rapid induction of the synthesis of metabolic enzymes in response to the addition of small molecules (inducers) to the growth medium.

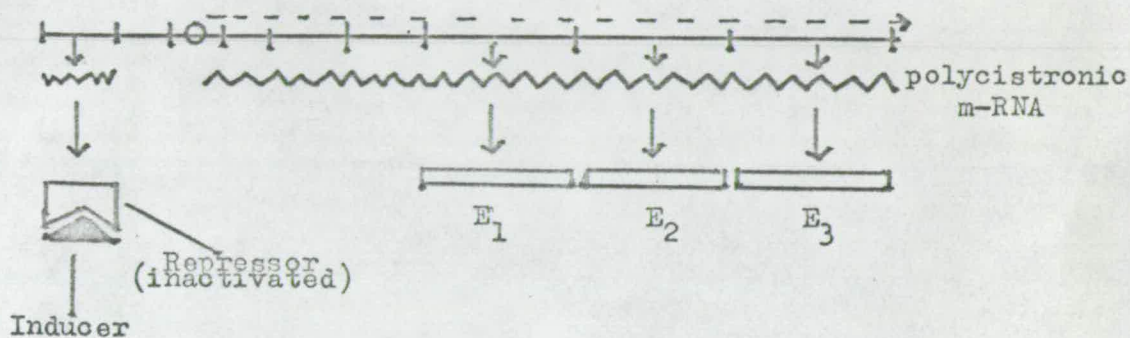
b) The coordinate synthesis and repression of functionally related enzymes (Vogel, 1957; Gorini and Maas, 1958; Pardee et al, 1959; Kalckar et al, 1959; Cohen and Jacob, 1959; Ames and Garry, 1959; Engelsberg et al, 1965; Power, 1967).

c) The genes for the different enzymes of a single biochemical pathway in the majority of cases lie in a cluster on the genetic map. Such a cluster of genes was named an operon by Jacob et al, 1960.

The operon was originally defined as a group of adjacent structural genes coding for one or more functionally related proteins, together with the operator. A structural gene contains only the code for the amino acids of a single polypeptide chain. The operator governs the function of the structural genes directly and coordinately.



A. Repression



B. Induction

Figure 1. Operon Regulation

- Legend:- R, regulator gene specifying m-RNA for repressor.
 P, promoter, recognition site for RNA polymerase.
 O, operator, attachment site for repressor.
 A,B,C, structural genes specifying m-RNA for enzymes.
 E₁, E₂, E₃, enzymes.

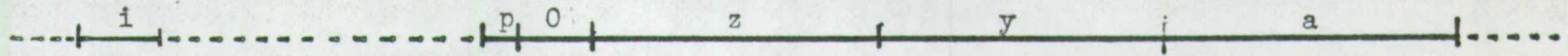
2.

That is, all the structural genes in an operon are functioning to the same extent at the same time. The operator can be "switched off", so that there is no protein synthesis, by a cytoplasmic "repressor" substance, the product of a regulator gene, which may or may not be closely linked with the operon. The mechanism for the regulation of the operon as proposed by Jacob and Monod is outlined in Figure 1.

Pardee et al (1959) first demonstrated the existence of the elements postulated by Jacob and Monod by genetic methods. The system studied was the lac operon and their conclusions are illustrated in Figure 2 (with the exception of the promotor, which was a later addition: see below).

Mutations of the regulator gene (i) were mapped at a distance from the operon and were found to be of two kinds. In the first, the gene products were synthesised constitutively in the absence of inducer. These mutations (i^{-}) were shown to be recessive to wild type in heterogenotes. The second type (i^s) showed only the repressed level of synthesis even in the presence of inducer. i^s mutations are dominant to wild type (Jacob and Monod, 1961a; Wilson et al, 1964).

Mutations of the operator were of one kind only, that is, constitutive (O^0). These mutations mapped at one end of the Z gene and were active only in the cis configuration (Jacob et al, 1960). A class of mutant originally thought to be in the operator, leading to non-inducibility and named O^0 has been shown to lie in the Z gene (Beckwith, 1963; Beckwith and Brenner, 1965) but to exert a polar effect on the whole operon. These pleiotropic mutations will be discussed later.



Symbols:-

i	regulator gene	
p	promoter	
O	operator	
z)	Structural genes	β - galactosidase
y)		galactoside permease
a)		galactoside transacetylase

Figure 2. Genetic Map of the lac Operon of E. coli

The operator was correctly distinguished from the Z gene by Reznikoff and Beckwith, 1969, using genetic methods; and, biochemically, by Bhorjee et al, 1969. Jacob et al, 1964, had arrived at this conclusion, but considered that the operator might still be translated as the start of the Z gene product.

The repressor (the product of the regulator gene i) was shown to be a cytoplasmic factor by the response of an operon to a regulator gene in the trans configuration. Suppressible nonsense mutations of the lac regulator were isolated in 1965 (Bourgeois et al), and the protein nature of the repressor was confirmed by its isolation and partial purification by Gilbert and Muller-Hill (1966). Gilbert and Muller-Hill (1967) and Riggs (1968) showed that the repressor specifically bound to double-stranded DNA containing the lac operator, and the addition of inducer inhibited this.

Ptashne (1967a) isolated a protein from cells infected with phage λ . He showed (1967b) that this protein bound to λ^+ DNA in vitro but not to a mutant λ_{imm}^{434} which carries the regulator gene of phage 434.

It seems that protein regulators may be universal among inducible operons. Reznikoff et al (1969) studied deletion strains in which the lac and trp operons were fused together, so that lac operon expression was increased by the derepression of trp. In these strains the transcription of lac, presumably initiated at the trp promoter, could be blocked by the attachment of lac repressor to the operator. This suggested that the repressor operates by blocking the progress of RNA polymerase.

In the original hypothesis the operator was considered to be

both the site of repressor action and of DNA dependent RNA polymerase attachment. Jacob et al, 1964 postulated another element, the promoter, for the site of polymerase attachment. In 1966 Scaife and Beckwith isolated mutants which gave a reduced level of maximal synthesis of all the enzymes, but were inducible or repressible. These are the properties expected of mutations in an element controlling the initiation of transcription. Ippen et al, 1968; and Miller et al, 1968, mapped these promoter mutations (p) between i and o.

Polar Mutations

Polarity describes the phenomenon in which a nonsense mutation in one gene of a bacterial operon may not only inactivate the protein which that gene specifies, but may also reduce the amounts synthesised of the (normal) proteins coded by other genes in the operon. This pleiotropic effect extends mainly to the genes on the operator distal side of the mutation, although antipolarity (reduction of expression of genes proximal to the mutation) has been observed (Ito and Crawford, 1965; Imamoto et al, 1966).

There is a gradient of polarity along the gene because the magnitude of the polar effect depends on the position of the mutation (Newton, 1966). The closer to the operator, the greater is the reduction in synthesis of the subsequent proteins.

Four types of mutations are known to cause polarity; these are deletions, insertions, frame shifts and nonsense mutations. In the case of the first three, the actual polar effect is due to the change in sequence generating a nonsense codon. Nonsense codons cause the termination of protein synthesis (Stretton and Brenner, 1965). Three nonsense codons have been identified, UAG (amber, Epstein et al, 1963),

UAA (ochre, Beckwith, 1963) and UGA (Brenner et al, 1967). Nonsense mutations are suppressible by the insertion of an amino acid by a transfer RNA (t-RNA) with a mutated anticodon which recognises the nonsense codon as coding for an amino acid (Engelhardt et al, 1965; Smith et al, 1966). The mechanism of polarity will be discussed in more detail in the section on transcription in nonsense mutants.

Transcription

a) Polycistronic messenger RNA

The fact that the activity of all the genes in an operon is regulated from a single terminally situated operator is in itself a good argument for the existence of polycistronic messenger (Martin, 1963).

The physical and biochemical evidence is good but not complete. Most of the investigations have involved the demonstration that messenger isolated from cells induced or derepressed for particular operons is enriched for a fraction of RNA larger than might be expected from the size of a single protein (Attardi et al, 1963b; Guttman and Novick, 1963).

Martin (1963) isolated m-RNA from the histidine operon of Salmonella and found that its sedimentation value was 34s, which agreed with the value of 38s calculated from the sum of the molecular weights of the proteins, converted into the equivalent size of RNA (Martin and Ames, 1961). Imamoto et al, (1965a and b) showed that RNA from the derepressed tryptophan operon of E. coli which hybridised to specific DNA had a sedimentation value 33s, while RNAs from trp deletion mutants were much smaller.

More recently (Morikawa and Imamoto, 1969; Morse et al 1969) it has been shown that degradation of m-RNA may be concurrent with

its synthesis. Morikawa and Imamoto found that trp m-RNA was degraded from the 5' end at about half the rate at which it was synthesised. However, fragments identified by hybridisation as complementary to the operator distal part of the operon were still present and of fairly large size when operator proximal messenger had disappeared. This evidence, therefore, in no way conflicts with the existence of polycistronic messenger nor with the rapid decay of such messenger. Morse et al found that the rate of degradation was greater in the presence of tryptophan than in its absence.

Imamoto (1968) and Baker and Yanofsky (1968) found that the initiation of transcription of the trp operon in derepressed cultures occurred at regular intervals of about 2.5 minutes, and that the rate of initiation was the same in partial deletion strains, i.e. it was not a function of the time taken to transcribe the whole operon. Morse et al (1968) produce evidence to show that the polycistronic messenger is translated by a single, tightly packed cluster of about 90 ribosomes, travelling immediately behind the RNA polymerase.

b) Fine control

The original concept of the operon involved switching on and off by the interaction of inducer molecules and regulator gene product. The RNA polymerase was considered to be equally active at all times. However, in 1969 Burgess et al found that RNA polymerase purified on a phosphocellulose column, though able to transcribe calf thymus DNA actively, was much less active than normal in the transcription of DNA from phage T4. They showed that the purification procedure

separated a protein component (the σ factor) from the core enzyme (consisting of two α , one β and one β' subunits) and that the normal activity could be restored by the addition of σ factor to the reaction mix. Travers and Burgess (1969) found that the σ factor was required for the initiation of RNA synthesis; after initiation it was released and could combine with a new polymerase core enzyme for a fresh initiation.

Further, Bautz et al (1969) showed that this σ factor selectively promoted transcription of T_4 bacteriophage pre-early genes, and Travers (1969) isolated a second factor from T_4 infected cells which directed transcription of a different part of the T_4 genome from that transcribed by core enzyme plus σ . In a later paper (Travers, 1970) the RNA transcribed in vivo two minutes after infection was found to compete with the RNA transcribed in vitro by the core enzyme plus σ^{T_4} .

Summers and Siegel (1969) thought that they had found a similar factor in cells infected with phage T_7 , but Chamberlin et al (1970) showed that this factor was really a new RNA polymerase, specified by the phage genome.

However, the "factor explosion" continued with the announcement by Davison et al (1969) of a factor M from the ribosomal wash of uninfected cells which enhanced the transcription of a wide range of DNAs, though the greatest increase was the transcription of T_4 and λ .

Another factor involved in the specificity of transcription by RNA polymerase was isolated by Roberts (1969b). He found that, in contrast to the normal in vitro situation, where molecules of RNA of various and often excessive lengths are synthesized (Richardson,

1966; Geiduschek and Haselkorn, 1969) and not released from the DNA-RNA polymerase complex, the addition of this factor (ρ for release) caused the synthesis on a bacteriophage DNA template of several discrete species of RNA, which were released on completion.

Schmidt et al (1970) attempted to explain the early synthesis of lysozyme in T_4 infection of E.coli by the existence of an anti-terminator factor, but this has not been confirmed.

Travers, Kamen and Schlieff (1970) identified a component in crude extracts of E.coli which specifically stimulated the transcription of ribosomal cistrons in vitro. This activity, termed ψ_r was also found to be a component of subunits III and IV of the RNA phage $Q\beta$ replicase. Travers et al suggested that there may be a relationship between ribosomal RNA (r-RNA) synthesis and phage replication because phages R17 and Z1K/1 inhibit r-RNA synthesis progressively after infection. Also, both RNA phage replication and r-RNA synthesis are subject to stringent control when rel^+ cells are starved for an amino acid (Cashel, 1969). One attractive explanation of these phenomena is that both RNA phage replicases and DNA dependant RNA polymerase compete for a protein which is necessary for both systems. Certainly subunits III and IV of the $Q\beta$ replicase, in the presence of the termination factor ρ , in 15 minutes synthesise an RNA of which 20% can be competed by r-RNA.

A consequence of rel gene function during amino acid starvation is the accumulation of guanosine tetraphosphate, ppGpp, (Cashel, 1969; Cashel and Gallant, 1969). Travers, Kamen and Cashel (1970) showed that ppGpp at a concentration of 0.4 mM inhibited the ψ_r stimulation of r-RNA synthesis by 50-60%, while synthesis by RNA polymerase

alone was only inhibited by 10%. The most attractive hypothesis is that ppGpp inhibits ψ_r directly, but it is possible that this compound only inhibits initiation of RNA chains with a 5' terminal guanine residue (Cashel, 1970). Evidence for the direct action on ppGpp on ψ_r is provided by the observation (Travers et al quoted in Travers, Kamen and Cashel, 1970) that ppGpp inhibits poly-G synthesis by $Q\beta$ replicase in a manner quantitatively similar to the inhibition of ψ_r stimulated r-RNA synthesis. Furthermore, the nucleotide acts by slowing the rate of initiation of $Q\beta$ replicase. It does not inhibit chain elongation nor does it compete with the initiating triphosphate. Guanosine tetraphosphate is stable in both the ψ_r system and the $Q\beta$ system, precluding the possibility of a breakdown product being the actual inhibitor.

c) Catabolite repression

Catabolite repression involves the inhibition of enzyme induction by glucose or biochemically related compounds such as glucose-6-phosphate, fructose or glycerol (Magasanik, 1961; Magasanik, 1970). The degree of inhibition is dependent on the bacterial strain, the source of the catabolite and the growth conditions. Enzymes which show the repression effect include those of glycerol metabolism, the lac operon, the gal operon, the arabinose operon, tryptophanase, D-serine deaminase thymidine phosphorylase, and histidase. Although observations on catabolite repression date back to the beginning of this century it is only recently that an understanding of the mechanism has been approached. The major breakthrough came when Makman and Sutherland (1965) showed that a rapid decrease in the intracellular level of cyclic 3':5' adenosine monophosphate (cAMP)

occurs in the presence of glucose. Perlman and Pastan (1968) showed that the repressing effect of glucose could be reversed by the addition of cAMP. De Crombrughe et al (1969) found that this effect could not be imitated or inhibited by a large number of cAMP analogues. Zubay et al (1970) thought that the nucleotide was unlikely to react directly with the operon, and looked for a protein which might mediate its effect. They isolated mutants unable to use lactose or arabinose as a carbon source (a characteristic of mutants lacking the mediating factor, because both lactose and arabinose are catabolite repressible operons). Using a DNA dependent β -galactosidase synthesising system, they found that this enzyme was not made in vitro if the supernatant components were derived from mutant cells, but was made when the extract made from wild type cells was used. They have purified a protein with a molecular weight of 45,000 which contains the activity. They call this protein the catabolite repressible gene activating protein (CAP).

Emmer et al (1970) approached the same problem from a biochemical point of view and isolated a protein which would bind cAMP. They too found a protein of molecular weight about 40,000. Mutants unable to respond to cAMP had an altered binding constant. The only significant difference in these proteins, apart from the name (Emmer et al call it the cAMP receptor protein, CRP) is a two orders of magnitude difference in affinity for cAMP.

This protein probably acts at the promoter, for mutations at this site affect the response of lactose to cAMP (Silverstone et al, 1970). Varmus et al (1970) have claimed that, by hybridising RNA to λ h80d lac DNA they found a direct proportionality between

the increase in lac m-RNA and that in β -galactosidase activity in response to the addition of cAMP to glucose repressed, IPTG induced cultures. They showed an increase in both, but it is impossible to quantify the m-RNA, and thus confirm the correlation, because of the faulty premises common to most attempts to measure m-RNA (see section on hybridisation in the Results). However, this increase implies that, as a result of its interaction with cAMP, the binding protein helps the initiation of transcription at the promoter of the lac and other operons.

De Crombrughe et al (1971a and b) have developed a system for studying the transcription and translation of the lac operon in vitro. They have shown that the system requires CAP and cAMP, ρ , lac repressor and an inducer, in addition to lac DNA and RNA polymerase. They consider Travers, Kamen and Schlieff, (1970) are wrong in assuming that ψ_r and CAP both act on the polymerase, but believe that CAP interacts with the DNA. They have shown that CAP binds to DNA containing the lac operon, and suggest that the promoter contains two elements, a CAP binding site and a RNA polymerase binding site. The formation of a DNA-CAP complex may be required for the binding of RNA polymerase. More recently the same group have studied the transcription and translation of the gal operon in vitro and have shown that cAMP has the same effects. This reinforces the conclusion that the cAMP control system stimulates the synthesis of m-RNA from all inducible operons in E.coli.

There are strong similarities between ψ_r and CAP. Both appear to be involved in the specific initiation of particular classes of RNA. They are approximately the same size, and both require sigma

function (Travers, Kamen and Schlieff, 1970). Further, they are both functionally involved in transcription processes regulated by a nucleotide, CAP being activated by cAMP, and ψ_r inhibited by ppGpp.

These parallels between ψ and CAP lead Travers et al to suggest that these two factors may represent a whole class of control elements for bacterial transcription which they propose to term factors. The existence of such a class would imply that a hierarchy of recognition elements for the initiation of RNA synthesis might operate in vivo. Sigma factors are the primary determinant for promoter recognition. The ψ factors would act as secondary specificity determinants allowing the RNA polymerase with sigma, but not the core enzyme alone, to transcribe general classes of transcription units, each ψ factor being specific for a given class. (Silverstone et al, 1969, have isolated a lac promoter mutant which no longer requires CAP). The regulation of ψ factors by low molecular weight effectors provides a system of control which is readily reversible and allows the bacterial cell to respond rapidly to changes in the environment.

De Crombrughe et al (1971a and b) contest the inclusion of CAP in the class of ψ factors, since they are unable to show it binding to RNA polymerase, as ψ_r does.

This proliferation of control mechanisms at the transcription level has not, however, succeeded in explaining the fact that most investigators, including myself, have found a greater increase in the amount of enzyme on induction than in the amount of specific m-RNA. Results show increases in enzyme level often several times that in messenger level (Lavalley and de Hauwer, 1970). This will

be discussed in a later section.

d) Transcription in nonsense mutants

Because polarity is caused only by nonsense codons, the most obvious explanation is that the premature termination of protein synthesis in one gene can affect the ability of ribosomes to translate the messenger RNA corresponding to the subsequent genes. This has led to complicated theories to explain the translation of polycistronic messenger, and although there is as yet no complete explanation (the number of factors implicated in translation is expanding almost as fast as those involved in transcriptional control) many polar effects can be explained at the level of translation.

More difficult to explain is the way in which polar mutations interfere with messenger RNA synthesis. Attardi et al (1963) hybridised RNA from "O⁰" mutants of the lac operon (now known to be nonsense mutations in the Z gene) to DNA from F'lac. They showed that the amount of hybridisation was comparable to that of non-induced wild-type, and this level was not increased by induction.

Contesse et al (1966) showed that more hybrid was formed by the more operator distal mutations. They concluded that RNA from beyond the mutant site was absent. Most of the polarity studies have been made on the tryptophan operon, using hybridisation to

80Od to measure the trp specific m-RNA produced. Imamoto et al (1966) found that some RNA from nonsense mutants was hybridised, but that suppression of the mutation enhanced the amount of hybridisation.

Imamoto and Yanofsky (1967a) found that most of the rapidly labelled RNA in polar mutants was smaller than in wild type. These shorter messengers contained only the RNA sequences corresponding

to the region of the operon before the mutant site (Imamoto and Yanofsky, 1967b). This is difficult to explain, because it implies that nonsense codons can affect transcription as well as translation. There are three possible mechanisms (Imamoto et al, 1966):-

a) Very little RNA is transcribed beyond the site of the nonsense mutation; i.e. the nonsense codon directly affects transcription.

b) The m-RNA is transcribed normally, but is never released from the DNA.

c) The m-RNA is transcribed normally, and released from the DNA, but because it is not translated it is rapidly degraded.

Lorse and Yanofsky (1969) and Lorse and Prinakoff (1970) have evidence for rapid degradation of post-mutation m-RNAs. They suggest that this could be caused by the dissociation of ribosomes from the messenger at the mutation, leaving it exposed to nucleases. This is supported by Webster and Zinder (1969) who find that translation of the genome of the RNA phage f_2 is terminated at a nonsense codon, with the rapid release of the ribosome.

However, Imamoto (1970) finds that, although polar mutants contain rapidly degraded RNA, this corresponds to the operator proximal part of the operon; m-RNA for the genes beyond the mutation does not appear to be synthesised at all.

The conflict between their findings is not resolved by the observation that polarity effects in the trp operon differ between repressed and de-repressed cultures, as there is an internal promoter between the D and C genes which leads to unequal transcription of proximal and distal cistrons in the repressed cultures, while in

derepressed cultures they are the same (Horse and Yanofsky, 1968). This internal promoter would re-initiate transcription and explain the differences found, but it might confuse the analysis of messenger RNA in polarity mutants.

The Galactose Operon

Three of the enzymes involved in the metabolism of galactose are coordinately inducible (Kalckar et al, 1959). The reactions which they catalyse are shown in Figure 3. The enzymes are galactokinase (kinase), galactose-1-phosphate uridyl transferase (transferase) and diphospho galactose-4-epimerase (epimerase).

They can be coordinately induced by D-galactose or D-fucose (6-deoxy-D-galactose) or repressed by glucose or thiomethyl- β -D-galactoside (TMG), (Buttin, 1961, 1963a). Fucose is a gratuitous inducer, that is, it is not phosphorylated by galactokinase, and does not support the growth of E.coli. Wilson and Hogness (1966) have shown that synthesis of the three enzymes is equimolar. Since fucose is a gratuitous inducer, induction of each enzyme is not dependent on the synthesis of the preceding one, although their appearance is sequential, with epimerase appearing first, followed by transferase and then kinase (Michaelis and Starlinger, 1967). Lederberg (1960) showed that these three enzymes could all be transduced by phage λ and were thus closely linked.

Genetic Map

The order of the gal structural genes has shown to be kinase-transferase-epimerase (Horse, 1962; Adler and Kaiser, 1963; Adler and Templeton, 1963; Buttin, 1963b; Shapiro, 1967; Davison et al 1967; Adhya and Shapiro, 1969; Pfeifer and St. Royspal, 1969).

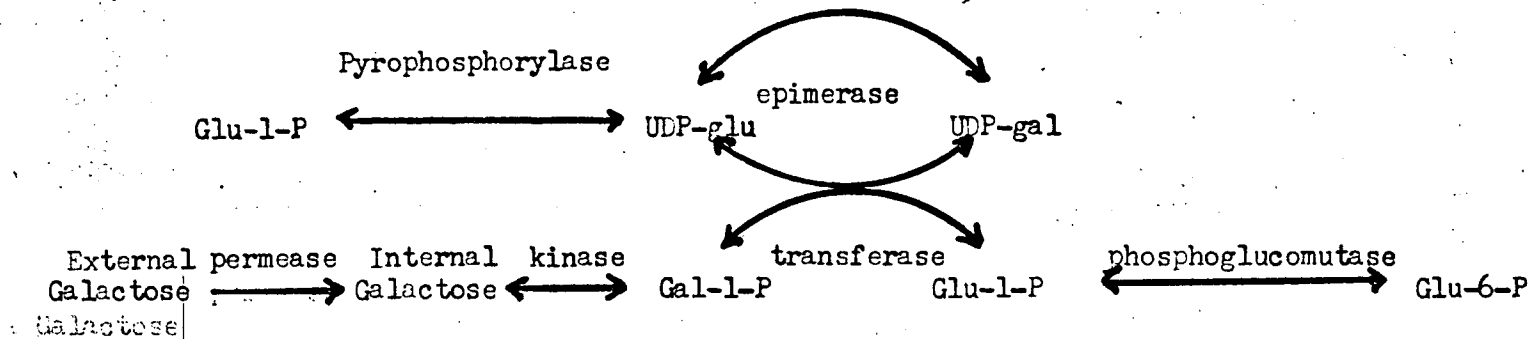


Figure 3. Utilisation of Galactose by E. coli

The kinase and pyrophosphorylase reactions take place with the participation of adenosine triphosphate (ATP) and uridine triphosphate (UTP) respectively. Abbreviations: Gal-1-P, galactose-1-phosphate; Glu-1-P, glucose-1-phosphate; UDP-gal, uridine diphosphate galactose; UDP-glu, uridine diphosphate glucose.

The orientation of the operon with respect to prophage was demonstrated to be kinase-transferase-epimerase- λ (Kayaanim and Campbell, 1966; Adhya and Shapiro, 1969). Shapiro and Adhya (1969) completed the mapping of these genes and the adjacent part of the genome. The details are shown in Figure 4.

That these genes form an operon as defined by Jacob and Monod (1961a and b) has been shown by Buttin (1961, 1962, 1963b). He placed the operator closest to the epimerase gene, farther from transferase, and farthest from kinase. Michaelis and Starlinger (1967) found that epimerase was the first gene to be translated. Guha et al, (1968) demonstrated that transcription of the gal operon proceeded from epimerase to kinase. Finally Jordan and Saedler (1967) and Jordan et al (1967) showed that polar mutations in epimerase reduced the synthesis of transferase and kinase, and polars in transferase reduced the level of kinase, but Shapiro and Adhya (1969) were unable to find mutations in the kinase gene which affected the other two enzymes.

Buttin, (1963b) found two classes of mutants which synthesised the galactose enzymes constitutively. One of these mapped close to the end of epimerase (O^0), the other was unlinked to the gal operon and mapped close to lysine. This locus was named Rgal and corresponds to the i gene of the lac operon. Saedler et al (1968) isolated non-inducible mutants (R^S) which mapped at the Rgal locus and confirmed it as the site of the regulator gene. By the construction of heterogenotes Saedler et al showed that the R^S mutants were dominant to wild type R^+ , which in turn was dominant to the R^- constitutives. This confirmed that, like the lac operon, gal is under the primary control of a repressor specified by the regulator

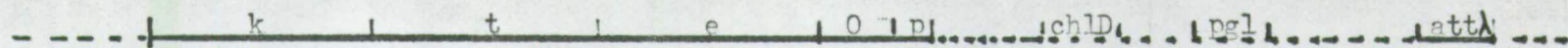


Figure 4. Orientation of the gal operon

Symbols:

k	)		kinase
t	)	Structural genes	transferase
e	)		
	)		
O			operator
p			promoter
attλ		site of integration of prophage λ	

gene Rgal, and that control is negative.

Transcription of the gal operon

A number of workers have demonstrated that induction of the galactose enzymes causes an increase in the amount of RNA which is hybridisable to DNA from λ dg (a defective phage λ carrying the gal operon) (Attardi et al, 1963; Hill and Echols, 1966; Guha et al, 1968).

Attardi et al took 8-10s RNA from sucrose gradients of RNA from pulse labelled induced and glucose repressed cultures. This was mixed with heat denatured DNA from λ^+ or λ dg phages and incubated. The hybrids were separated from free RNA by centrifugation in a CsCl density gradient, and the radioactivity in the λ dg hybrid, after correction for that in the λ^+ hybrid, was taken to be a measure of the gal specific RNA synthesised. They claimed to have shown a 6.5-fold increase on induction but, even if correct, this is much less than the increase in enzyme level, which they found to be about 30-fold.

Hill and Echols used a hybridisation technique based on that of Nygaard and Hall (1963, 1964) with the additional step of RNase treatment of the hybridisation mixture before collection on nitro-cellulose membranes. They found a 2.4-fold increase in the radioactivity of the hybrid on induction, compared with a 13-fold increase in kinase activity.

Guha et al separated the two strands of λ dg DNA and immobilised the purified single strands on membranes according to the method of Gillespie and Spiegelman (1965). They found that induction caused an increase of 5 times in the radioactivity of the hybrid with the lighter strand, and no increase in that with the heavier strand.

They did not investigate the level of the enzymes.

According to the classical hypothesis, control of the enzymes is brought about by control of the rate of m-RNA synthesis, and consequently of the amount of m-RNA available for translation. One would therefore expect the enzyme activity in an exponentially growing culture to reflect the m-RNA content. As the above examples show, results have not been consistent with this expectation. I therefore decided to make a further study of the relationship between enzyme and m-RNA contents in exponentially growing cells. In addition, Frame and Bishop (1971) have investigated the effect of gene dosage upon enzyme level. They found that, while with fucose induction the enzyme levels closely paralleled the calculated gene dosage, with galactose induction this correlation was not maintained.

I have therefore varied enzyme contents in two ways: by induction or repression, and by changing the gene dosage.

Previous attempts to measure the concentration of a specific messenger RNA in E.coli by DNA-RNA hybridisation (Imamoto et al, 1965; Attardi et al, 1963; Hill and Echols, 1966; Edlin et al, 1968; Stubbs and Hall, 1968; Lavallo and de Hauwer, 1968) have made a number of assumptions which are probably in error.

These are:-

a) That the DNA used (usually from a transducing bacteriophage or a substituted sex-factor) contains only the E.coli genes in which the investigator is interested. In most cases this is manifestly untrue. Probably the only case of an operon being purified from all other DNA is that of the lac operon (Shapiro et al, 1969).

b) That the proportion of total radioactivity in the hybridisable RNA is constant, and thus accurately reflects the amount of RNA in the hybrid. Our own results show how unlikely this is.

c) That all the specific m-RNA available becomes hybridised with the DNA. In one case (Lavalle and de Hauwer, 1968) the assumption made was that the reaction reaches a state of equilibrium. Both of these assumptions have been challenged and shown to be in error (Bishop, 1969c, 1970).

Using the hybridisation of E.coli ribosomal RNA with E.coli DNA as a model system, Bishop has investigated the parameters of the hybridisation reaction and developed a method of interpretation which enables one to measure the content of a specific messenger RNA (Bishop and Irving, 1971). The rationale of this method, which overcomes most of the objections to previous investigations will be described in the section dealing with the results.

Polar Mutations in the Gal operon

Polar mutations of the gal operon fall into two main classes. The first of these are sensitive to suppressors (Adhya and Shapiro, 1969) and are thus nonsense mutations. The second class are not suppressor sensitive, and are also insensitive to base analogues and frameshift mutagens. By comparison of buoyant densities of λ dgs carrying these mutations with λ dgal⁺, Jordan et al (1968) showed that three of these were due to an insertion of DNA. Shapiro (1969) demonstrated that four other extreme polar mutants were due to such insertions.

Among the mutants with the properties of an insertion gal⁻₃ (Lederberg, 1960) is of particular interest. The basal level of

kinase and transferase is only very slightly increased by fucose induction (Hill and Echols, 1966). Revertants may be constitutive (Hill and Echols, 1966), semi-constitutive (Morse, 1967) or normally inducible. Shapiro and Adhya (1969) mapped gal^{-}_3 at the operator end of the *gal* operon, and suggested that this mutation may involve the promoter. Other polar mutants with similar properties have been reported (Saedler and Starlinger, 1967a and b) and some of them mapped in the same region as gal^{-}_3 .

Three extreme polar mutations have been isolated in this laboratory. All three produce low levels of kinase and are inducible. One of them (NA121) has been shown to be an ochre nonsense mutant (Irving, 1970); the other two (NA119, NA156) have many of the characteristics of insertion mutants.

It was decided to examine the m-RNA and kinase contents in gal^{-}_3 , NA119, NA121 and NA156. To increase the gene dosage, and thus the level of m-RNA, all four were made into homogenotes.

A genetic analysis of the transducing bacteriophages and episome used in the hybridisation experiments was carried out.

Materials and Methods

A. Bacteria and bacteriophage strains

The bacteria and phages used are listed with their descriptions in Tables 1, 2, 3 and 4. Unless otherwise stated the strains were isolated in this laboratory by Drs. J.O. Bishop and J.N. Davison, Miss C.M. Ford, Mr. R. Frame and the writer.

All mutations were mapped in this laboratory. Their positions are shown in Figure 5. The lengths of *E.coli* DNA carried by transducing phages and the episome FR5, together with the extent of deletions are shown in Figure 6.

The hybrid episome FR5 was produced by Dr. J.O. Bishop at the suggestion of Dr. E. Meynell (Gosden et al, 1971) by transducing part of the resistance transfer factor (RTF) R1-19 (carrying resistance to streptomycin, S, sulphonamide, Su, kanamycin, K, Chloramphenicol, Cm, Ampicillin, Am, and tetracycline, T) from one strain of Salmonella typhimurium LT-2 to another, and then mobilising the drug resistance factors by introducing the substituted sex-factor F'8 (carrying *aro* G, *Gal*, *Chl* D, and *pgl*; isolated by Hirota and Sneath, 1961) and conjugation with *E.coli* 9-3t, selecting for both drug resistance and the ability to ferment galactose. The factor was initially very unstable, but was successfully stabilised by five cycles of conjugal transfer (from 9-3t to 9-3h and back) each followed by a period of relaxation and then re-selection for drug resistance and the ability to ferment galactose. FR5 now carries *aro* G, *gal*, *chl* D, and genes conferring resistance to Cm, Am, T and S.

Three of the four polar mutants studied (NA119, NA121, NA156) were produced by Dr. Bishop. Cultures of B1 were treated with

Table 1

Bacterial Strains: Male

Symbol	<u>General Genotype</u>		<u>Galactose Genotype</u>		Galactose Phenotype	Source
	Bacterial Genome	Episome	Bacterial Genome	Episome		
E1/FR5	his ⁻ , trp ⁻ , gal ⁺ , Sm ^r , Su ⁻ , λ ^s	gal ⁺ , Cm ^r , Kn ^r , Am ^r , T ^r	k ⁺ t ⁺ e ⁺	k ⁺ t ⁺ e ⁺	wildtype	
9-3h ⁺ /FR5	his ⁺ , trp ⁻ , gal ⁻ , Sm ^r , Su ⁻ , λ ^s	gal ⁺ , Cm ^r , Kn ^r , Am ^r , T ^r	Δ gal kte	k ⁺ t ⁺ e ⁺	wildtype	
TMG6/FR5	his ⁻ , trp ⁻ , gal ⁺ , Rgal ⁻ , Sm ^r , Su ⁻ , λ ^s	gal ⁺ , Cm ^r , Kn ^r , Am ^r , T ^r	Rgal, k ⁺ t ⁺ e ⁺	k ⁺ t ⁺ e ⁺	constitutive	
NA119/FR119	his ⁻ , trp ⁻ , gal ⁻ ₁₁₉ , Sm ^r , Su ⁻ , λ ^s	gal ⁻ ₁₁₉ , Cm ^r , Kn ^r , Am ^r , T ^r	k ⁺ t ⁺ e ⁻ ₁₁₉	k ⁺ t ⁺ e ⁻ ₁₁₉	polar	
NA121/FR121	his ⁻ , trp ⁻ , gal ⁻ ₁₂₁ , Sm ^r , Su ⁻ , λ ^s	gal ⁻ ₁₂₁ , Cm ^r , Kn ^r , Am ^r , T ^r	k ⁺ t ⁺ e ⁻ ₁₂₁	k ⁺ t ⁺ e ⁻ ₁₂₁	polar	
NA156/FR156	his ⁻ , trp ⁻ , gal ⁻ ₁₅₆ , Sm ^r , Su ⁻ , λ ^s	gal ⁻ ₁₅₆ , Sm ^r , Kn ^r , Am ⁻ , T ^r	k ⁺ t ⁺ e ⁻ ₁₅₆	k ⁺ t ⁺ e ⁻ ₁₅₆	polar	
F ¹ gal ⁻ ₃	his ⁺ , trp ⁺ , gal ⁻ ₃ , Sm ^s , Su ⁻ , λ ^s	gal ⁻ ₃	k ⁺ t ⁺ e ⁻ ₃	k ⁺ t ⁺ e ⁻ ₃	polar	Echols
138-1	gal ⁻ , B ₁ ⁻	Hfr	k ⁻ t ⁺ e ⁺	-	gal ⁻	Buttin
SA594	gal ⁻ , nic ⁻ , aroG ⁻ , B ₁ ⁻ , SuA, (λC _I 857)	Hfr	Δ gal kte	-	gal ⁻	Adhya
E1/F8	his ⁻ , trp ⁻ , gal ⁺ , Sm ^r , Su ⁻ , λ ^s	gal ⁺	k ⁺ t ⁺ e ⁺	k ⁺ t ⁺ e ⁺	wildtype	

Table 2

Bacterial Strains: Female

Symbol	General Genotype	Galactose Genotype	Galactose Phenotype	Source
E1	his ⁻ , trp ⁻ , Sm ^r , Su ⁻ , λ ^s	k ⁺ t ⁺ e ⁺	wildtype	
TMG6	his ⁻ , trp ⁻ , gal ⁺ , Rgal, Sm ^r , Su ⁻ , λ ^s	k ⁺ t ⁺ e ⁺ R ⁻ gal	Constitutive	
CF4	his ⁻ , trp ⁻ , gal ⁺ , O ^c gal, Sm ^r , Su ⁻ , λ ^s	k ⁺ t ⁺ e ⁺ O ^c gal	constitutive	
9-3	his ⁻ , trp ⁻ , gal ⁻ , Sm ^r , Su ⁻ , λ ^s	Δ kte	gal ⁻	
9-3h ⁺	his ⁺ , trp ⁻ , gal ⁻ , Sm ^r , Su ⁻ , λ ^s	Δ kte	gal ⁻	
9-3t ⁺	his ⁻ , trp ⁺ , gal ⁻ , Sm ^r , Su ⁻ , λ ^s	Δ kte	gal ⁻	
A22	his ⁺ , trp ⁺ , gal ⁻ ₂₂ , Sm ^s , Su ⁻ , λ ^s	k ⁺ t ⁺ e ⁻ ₂₂	gal ⁻	Adler
A24	his ⁺ , trp ⁺ , gal ⁻ ₂₄ , Sm ^s , Su ⁻ , λ ^s	k ⁻ ₂₄ t ⁺ e ⁺	gal ⁻	Adler
NR16	his ⁻ , trp ⁻ , gal ⁻ , Sm ^r , Su ⁻ , λ ^s	k ⁺ t ⁻ ₁₆ e ⁺	gal ⁻	
NR1	his ⁻ , trp ⁻ , gal ⁻ , Sm ^r , Su ⁻ , λ ^s	k ⁺ t ⁻ ₁ e ⁺	gal ⁻	
NA93	his ⁻ , trp ⁻ , gal ⁻ , Sm ^r , Su ⁻ , λ ^s	k ⁺ t ⁻ ₉₃ e ⁻	gal ⁻	
NR123	his ⁻ , trp ⁻ , gal ⁻ , Sm ^r , Su ⁻ , λ ^s	k ⁺ t ⁻ ₁₂₃ e ⁻	gal ⁻	

Table 3

Bacterial Strains: Lysogenic

E1(857) :	E1 lysogenic for temperature sensitive phage λ_{C_I857}
A22(17-8):	A22 doubly lysogenic for λ_{dg} 17-8 and λ_{C_I857}
A22(T166):	A22 doubly lysogenic for λ_{dg} T166 and λ^+
A22(JG1) :	A22 doubly lysogenic for λ_{dg} JG1 and λ_{C_I857}
A22(T328):	A22 doubly lysogenic for λ_{dg} T328 and λ^+
A22(JG3) :	A22 doubly lysogenic for λ_{dg} JG3 and λ_{C_I857}
A22(λ_{pg}):	A22 lysogenic for λ_{pg}

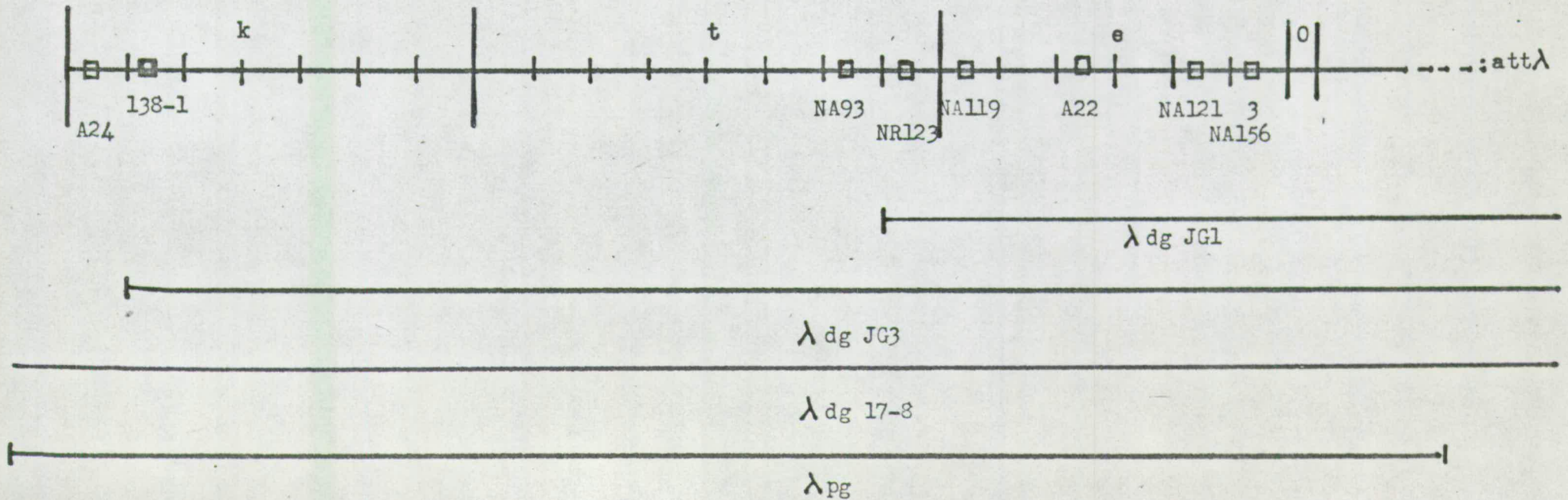
Table 4
Bacteriophage Strains

λ^+	wild type temperate phage
λ^V	virulent strain of λ
λ_{C_I857}	temperature sensitive strain of λ
$\lambda_{dg\ 17-8}$	temperature sensitive λ defective carrying complete gal operon
$\lambda_{dg\ T166}$	λ defective carrying epimerase cistron
$\lambda_{dg\ JG1}$	temperature sensitive recombinant of T166 and λ_{C_I857}
$\lambda_{dg\ T328}$	λ defective carrying complete gal operon except for distal end of kinase
$\lambda_{dg\ JG3}$	temperature sensitive recombinant of T328 and λ_{C_I857}
λ_{pg}	temperature sensitive λ perfect carrying complete gal operon
T_4	wild type virulent phage
$T_4\ r_{II}1272$	T_4 with complete deletion of r_{II} region
f_2	wild type, male specific RNA phage

The extent of the E. coli genome carried by transducing phages is illustrated in Figures 5 and 6.

Figure 5.

Point mutations and polar mutations in the gal operon, and the regions of the operon carried by transducing bacteriophages.



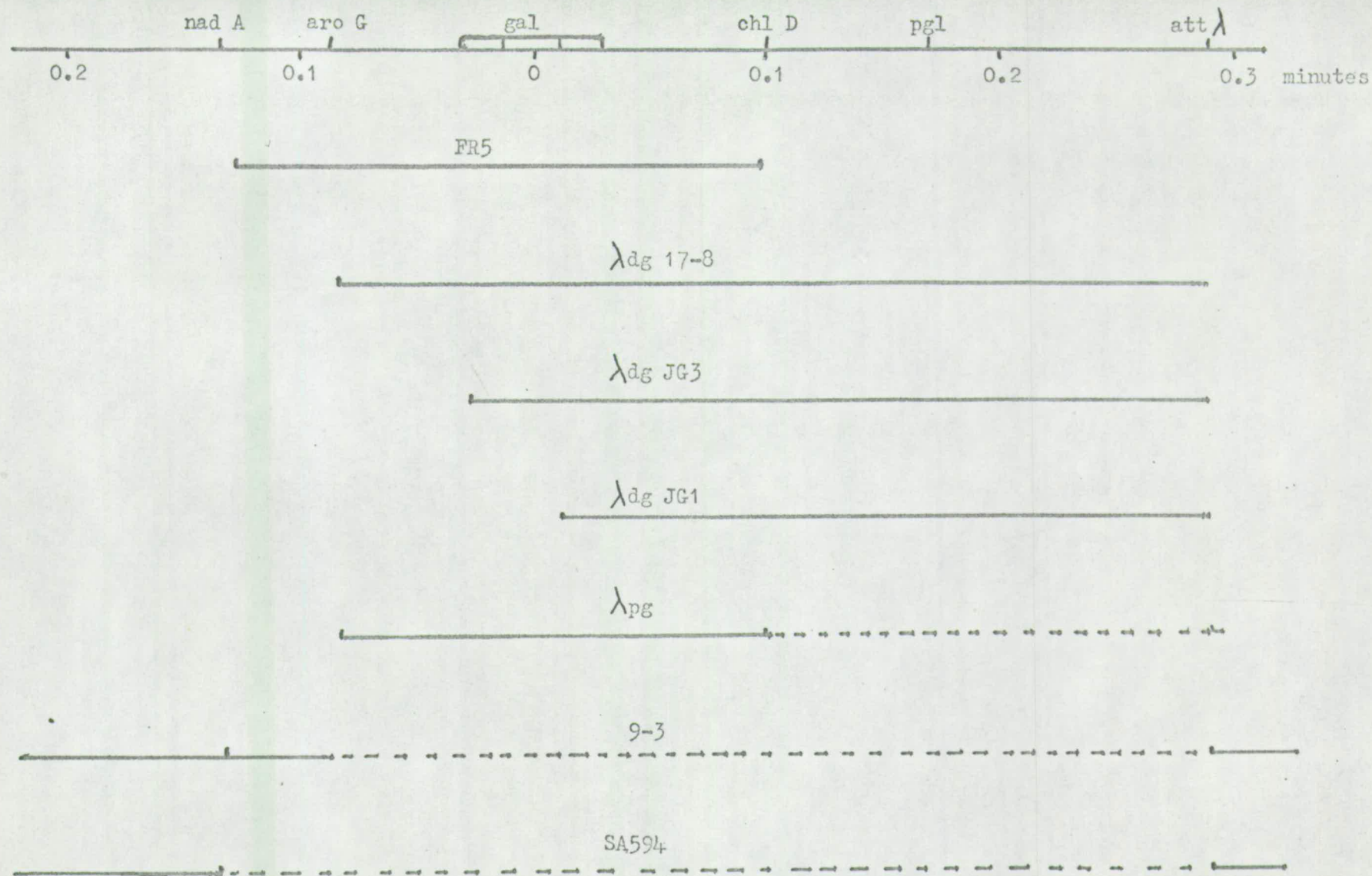


Figure 6.

The gal operon and neighbouring regions of the *E. coli* genome, together with the extent of the bacterial genome carried by FR5 and the transducing bacteriophages. Distances in minutes from Taylor, 1970.

nitrosoguanidine. Gal^- mutants were detected by replica plating from glucose to galactose. Polar mutations were identified from among the gal^- isolates by low transduction frequency in spot tests when transduction by λ_{dg} (incomplete) phage was compared with that by a λ_{dg} (complete) phage. The polar phenotype was confirmed by assaying all three enzymes, and proven by transduction with λ_{dg} T166 (carrying only epimerase) which more or less completely restored the activity of kinase, transferase and epimerase. The three mutations were transferred to F'8 and tested against 9-3 carrying amber or ochre suppressors. NA121 was shown to be an ochre mutant (Irving, 1970). The other two were not suppressible. Finally the three mutations were transferred to FR5 and homogenotes isolated.

B. Media

1) MS Broth (Davies and Sinsheimer, 1963) contains per litre 10g Oxoid Tryptone, 8g NaCl and 1g Difco Bacto Yeast extract. 10ml 20% glucose, 10ml 10% MgSO_4 and 2ml 1M CaCl_2 were autoclaved separately and added immediately before use.

2) 3XD (Fraser and Jerrel, 1953) contains per litre 10.5g Na_2HPO_4 , 4.5g KH_2PO_4 and 1g NH_4Cl . 100ml 15% casamino acids, 50ml 60% glycerol, 5ml 10% MgSO_4 and 0.3ml 1M CaCl_2 were autoclaved separately and added immediately before use, together with the appropriate supplements and antibiotics (see Table 5).

3) M9 (Clowes and Hayes, 1968) contains per litre 6g Na_2HPO_4 , 3g KH_2PO_4 , 0.5g NaCl, 1g NH_4Cl . 10ml 0.1M MgSO_4 , 10ml 20% glucose or galactose and 1ml 0.1M CaCl_2 were autoclaved separately and added immediately before use.

4) Minimal Medium (Clowes and Hayes, 1968) contains per litre 5g

Table 5

Supplements to basic media, and antibiotics

<u>Supplement</u>	<u>Abbreviation</u>	<u>Final concentration</u>
Galactose	Gal	2mg/ml
Galactose (Purified, Sigma)	G-Gal	2mg/ml
Glucose	Glu	2mg/ml
Histidino	His	200µg/ml
Phenylalanine	Phe	200µg/ml
Tyrosine	Tyr	200µg/ml
Tryptophan	Trp	1mg/ml
Nicotinamide	Nic	50µg/ml
Vitamin B ₁ (Thiamine)	B ₁	1µg/ml
<u>Antibiotic</u>		
Streptomycin	Sm	200µg/ml
Chloramphenicol	Cm	20µg/ml
Ampicillin	Am	50µg/ml

NH_4Cl , 1g NH_4NO_3 , 2g Na_2SO_4 , 1g K_2HPO_4 and 1.6g MgSO_4 . 10ml 20% glucose or galactose plus supplements and antibiotics were autoclaved separately and added immediately before use.

5) Nutrient Broth Agar contains 25g/l Oxoid Nutrient Broth No.2 and 15g/l Difco Bacto Agar.

6) Minimal Agar Minimal medium plus 15g/l Difco Bacto Agar.

7) MS Top Agar MS broth plus 8g/l Difco Bacto Agar.

All media, except those for the Microferm fermenter were autoclaved at 15lbs/sq.inch for 15 minutes. Media in the Microferm fermenter were autoclaved at 20lbs/sq.inch for two hours.

C. Buffers and Solutions

- a) Column buffer is 0.3M NaCl; 0.1M Na Acetate pH5.0
- b) Standard saline citrate (SSC) is 0.15M NaCl; 0.015M Tri-sodium citrate; the pH is adjusted to 7.5 with concentrated HCl.
- c) RNase is ribonuclease - A from bovine pancreas, 5 x crystallised. This is dissolved in 2 x SSC pH4.5 at 10mg/ml or 2mg/ml, boiled for 5 minutes to inactivate contaminating DNase, and the pH adjusted to 7.5 with 0.1N NaOH.
- d) DNase is deoxyribonuclease-1 from bovine pancreas, electrophoretically purified from RNase. This is dissolved in 0.1M K acetate pH 5.0 to a concentration of 2mg/ml.
- e) Counting fluid contains 12.5g PPO and 750mg dimethyl POPOP in 2.5 litres toluene.
- f) 0.12M Phosphate buffer is 0.6M NaH_2PO_4 ; 0.6M Na_2HPO_4 diluted 1:10 with distilled H_2O .
- g) Tris-acetate buffer is 3M tris adjusted to pH8.0 at 37°C with glacial acetic acid and diluted to 3 x volume.

D. Chemicals

Except for those detailed below, all chemicals were obtained from British Drug Houses Ltd. Analar grade was used wherever available.

Sigma: RNase, DNase, α - D(+) fucose (Fucose), Methyl- β -D-Thiogalactose (TMG), galactose for induction, lysozyme, sodium lauryl sulphate (SLS), adenosine 5' Triphosphate (ATP), Tris.

Calbiochem: Glutathione (reduced) crystalline, A grade.

Pharmacia: Dextran sulphate (DES) M.W. 2000 or 500. The DES2000 was used as a 20% solution. When this grade was unobtainable, DES500 was substituted, and found to give the best results at a 40% solution. Sephadex SPC-50 was equilibrated with column buffer.

Radiochemical Centre, Amersham: Uraoil-2-C14, Phosphorus-32, D-Galactose-1-C14. The ^{14}C -galactose was found to contain a positively charged contaminant which bound to DEAE paper with galactose-1-phosphate. It was necessary to pass the labelled galactose through a column of Dowex-1-chloride to remove this contaminant (Davison, 1967).

Packard: Dimethyl POPOP (1, 4 bis-2- (4-methyl-5-phenyloxazolyl)-Benzene and PPO (2, 5-Diphenyloxazole).

Sartorius: Nitrocellulose membrane filters SM 11306, pore size 0.45μ , diameter 30mm.

E. λ^+ assay

100-fold serial dilutions of the phage suspension were made in MS broth or phosphate buffer pH7.0. 0.1ml of these was mixed with 0.1ml of an overnight culture of A22, and incubated at 37°C for 15 minutes. To this was added 2.0mls of melted MS top agar

equilibrated at 45°C in a water bath. The mixture was poured onto a nutrient broth agar plate, allowed to set at room temperature and incubated overnight at 37°C. The plaques were scored the following day.

F. λ dg transduction assay

10-fold serial dilutions of the phage suspension were made in phosphate buffer pH7.0. 0.1ml of the dilution was mixed with 0.2ml of log phase bacteria (A22) and 0.1ml λ^+ at a multiplicity of infection (moi) of about 3.

The mixture was incubated at 35°C for 20 minutes, and 0.1ml pipetted onto gal plates and spread with a sterile glass spreader. Colonies were scored after incubation at 30°C for 3 days. Since 80% of the transductants may be expected to lyse, the score was multiplied by 5x to obtain the titre.

G. Bacteriophage recombination

λ dg was purified in a CsCl density gradient (see below) and dialysed against 0.1M NaCl; 0.01M Tris-HCl pH7.5 at 20°C. 100-fold serial dilutions of the λ dg were made in the same buffer. 0.1ml of these was added to 0.2ml of log phase A22 bacteria, and 0.2ml of $\lambda_{C_I}^{857}$ at a moi of 1-3, and the mixture incubated at 35°C for 20 minutes. 0.1ml was then spread on gal plates and incubated for 3 days at 30°C.

Colonies were picked and purified by two cycles of restreaking on gal plates. They were then incubated overnight in MS broth, subcultured and grown into log phase again (about 1 hour). 2ml aliquots of each culture were transferred to sterile plastic petri dishes at 37°C. The bacteria were irradiated with ultra-violet for

30 seconds at a lamp distance of 25cm with constant agitation of the culture. In order to minimise photoreactivation, the irradiation and subsequent incubation were carried out in a dark room. After 3 hours the contents of the petri dish were poured into sterile test tubes containing 0.5ml chloroform.

This treatment resulted in the formation of a low frequency transducing (LFT) lysate, which was used to transduce A22 with $\lambda_{C_I^{857}}$ helper as above. The plates were again incubated for 3 days at 30°C. They were then replicated to gal plates and grown at 42°C. The temperature sensitive colonies were restreaked to purify them before assaying to confirm their transducing properties. Lysogeny was confirmed by plating 0.1ml of an overnight culture with 2.0ml of MS Top agar on nutrient broth agar plates. After allowing to dry, phages λ^+ , λ^v , T_4 and $T_{4r_{II}^{1272}}$ were spotted on the plate, which was incubated overnight at 30°C. The plates were scored as follows (+ indicates lysis):

<u>λ^+</u>	<u>λ^v</u>	<u>T_4</u>	<u>$T_{4r_{II}^{1272}}$</u>	<u>Interpretation</u>
+	+	+	+	λ sensitive
-	+	+	-	λ lysogenic
-	-	+	+	λ resistant

The temperature sensitive transducing lysogenic strains were then capable of forming high frequency transducing (HFT) lysates in response to temperature induction.

H. F'- duction

Log phase cultures of male and female bacteria were mixed and diluted 1:10 together with controls. After incubation for one hour

the cultures were decanted into sterile tubes and centrifuged for 10 minutes at 10,000 rpm in the SS34 rotor of the Sorvall RB-2 centrifuge. Pellets were washed in minimal medium, resuspended in minimal medium and plated on selective plates. When colonies appeared (about 2 days at 37°C) they were re-streaked to purify them. Maleness was confirmed by plating 0.2ml of an overnight culture in MS top agar plus 0.1ml/50ml IMCaCl₂; 0.5ml/50ml 10% Glucose on nutrient broth agar plates. When the plates had dried a loopful of male-specific RNA bacteriophage f2 was spotted on them and they were incubated overnight at 37°C. Plaque formation confirmed the presence of F.

I. Mapping of Transducing Phages and Episome

One of the two deletions 9-3h⁺ or SA594 was used as the host for transduction or F'-duction with the phage or episome to be mapped. Markers were identified as follows:-

1. Nad A Growth in presence or absence of nicotinamide
2. Aro G (Wallace and Pittard, 1967). This involves the end product repression of iso-enzymes involved in the synthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan. Aro G is concerned with the synthesis of the phenylalanine - sensitive iso-enzyme, and aro G⁻ strains are unable to grow in the presence of tyrosine and tryptophan unless phenylalanine is also present. Strains were therefore scored for ability to grow on Glu Phe Tyr Trp or Glu Tyr Trp. Growth on the first but not the second indicates aro G⁻.
3. pgl (Kupar and Fraenkel, 1969). Normal cultures were grown on glucose plates and replicated to maltose plates. After 6 hours incubation the plates were flooded with 4ml 0.01M I₂; 0.03M KI.

Strains lacking 6-phosphogluconolactonase (pgl^-) immediately turned blue. Wild type strains remained white or became brown. The cause of this phenomenon is unknown, but it is a very quick and simple method of identification.

4. chlD (Hugh and Leifson, 1953). This locus is concerned with the sensitivity to chlorate under anaerobic conditions. It was mapped by Adhya et al, 1968. A colony was stabbed into tubes containing, per litre, Nutrient broth, 8g; NaCl, 5g; KH_2PO_4 , 0.3g; Difco Bacto agar, 3g; 1% Bromthymol blue (aqueous) 3ml; 20% glucose, 2ml; 5% KClO_3 , 4ml. Duplicate stabs were made, and one of each pair was covered with melted sterile petrolatum. The stabs were incubated at 30°C or 37°C for 2-3 days. Growth was indicated by an acid reaction. Only chlD^- strains were able to grow anaerobically in this medium.

The results of these experiments are included in Figure 6.

J. Growth of transducing phage for DNA preparation

All transducing phages which were not already temperature sensitive were made so as described in G. above.

1. A22, doubly lysogenic for a transducing λ_{dg} and for $\lambda_{\text{CI}}^{857}$ (with the exception of λ_{pg} which required no helper, and was thus able to lysogenise A22 by itself) was grown overnight in 400ml M9 plus galactose at 33°C . This was inoculated into 11 litres of MS broth prewarmed to 33°C in the Microferm fermentor, to give an initial optical density at 650 nm (OD_{650}) of 0.1 to 0.2.
2. The culture was grown at 33°C with stirring and aeration until the OD_{650} reached 0.6-0.8.
3. The thermostat was then raised to 42°C and 2 litres of MS broth

pre-heated to 80°C was added to raise the temperature quickly. 42°C was reached in less than 5 minutes.

4. After 20 minutes at 42°C the thermostat was lowered to 37°C and the culture incubated until lysis occurred. (OD_{650} drops rapidly to less than half maximum).

5. At lysis, 50ml of chloroform was added and the culture stirred without aeration for a few minutes. It was then allowed to stand overnight.

K. Purification of transducing phage preparations

The method is based upon that of Philipson et al, 1966, and Leberman, 1966. All steps were carried out in the cold.

1. The lysate was decanted into a large glass separator. Normally two fermenter runs were combined to give a volume of about 20 to 24 litres.

2. To this was added 23g/l NaCl. The lysate was stirred until the NaCl dissolved.

3. Continuing the stirring, 300ml/l original volume of 30% polyethylene glycol, MW 6000 (PEG) and 10ml/l original volume 20% Dextran sulphate 2000 or 40% Dextran sulphate 500 (DES500) were added. Stirring was continued for 1 hour, and the mixture allowed to stand overnight.

4. The lower phase was withdrawn and kept. The mixture was again stirred for one hour and allowed to stand overnight.

5. The second lower phase was withdrawn, together with some upper phase, and they were pooled with the first lower phase.

6. The withdrawn material was spun in the GSA rotor of the Sorvall centrifuge at 7,500 rpm for 40 minutes. The material separated

into three phases, of which the upper and lower were withdrawn and discarded.

7. The interphase was suspended in H_2O to about 300ml, $\frac{1}{4}$ volume of 3M KCl was added to precipitate the remaining DES. It was mixed well and left overnight.

8. The mixture was spun at 10,000 rpm for 30 minutes in the GSA rotor. The supernatant was removed and dialysed extensively against 0.01M NaCl; 0.01M Tris pH7.5 at 20°C.

9. To each 100mls of dialysate was added 2.3g NaCl; 30ml. 30% PEG; 15ml 40% DES500. It was stirred in the cold for one hour and then spun for 10 minutes at 10,000 rpm in the GSA rotor.

10. Most of the upper phase was removed, and the remaining material transferred to a 40ml polypropylene centrifuge tube and spun for 15 minutes at 15,000 rpm in the SS34 rotor.

11. The upper and lower phases were removed and discarded, and the interphase resuspended in water to about 30ml. $\frac{1}{4}$ volume of 3M KCl was added with stirring and the mixture left in ice for one hour.

12. It was then spun at 15,000 rpm for 5 minutes and the supernatant dialysed against 0.01M NaCl; 0.01M Tris pH7.5 at 20°C.

13. 1M $MgCl_2$ was added to 5mM, RNase to 5 μ g/ml and DNase to 10 μ g/ml. The mixture was incubated at 37°C for 1 hour.

14. It was spun for 1 hour at 28,000 rpm at 0°C in the 30 rotor of the Spinco Model L ultracentrifuge. The pellet was gently resuspended in 0.01M NaCl; 0.01M Tris.

15. CsCl was added to the partially purified and concentrated phages to make the final suspension 44.9% w/w, having a density of 1.49g/cm³ at 4°C (Vinograd and Hearst, 1962).

16. This was spun for 1 hour at 18,000 rpm in the SS34 rotor, and the material between the pellet and pellicle transferred to a fresh tube.
17. This was overlaid with paraffin and spun for 42 hours at 28,000 rpm at 15°C in the #30 rotor of the Spinco.
18. The gradient was fractionated by piercing the bottom of the tube with a needle. 5-drop fractions were collected. To each fraction was added 1.0ml of 0.1M NaCl; 0.01M Tris, and this was left overnight in the cold before mixing. The optical density of the diluted fractions was read at 260nm. Figures 7a and 7b show typical OD₂₆₀ profiles of the phages prepared from JG1 and JG3 lysogenic bacteria.
19. The transducing phage peak was confirmed by assay as above (F) and the fractions collected and dialysed against 0.1M NaCl; 0.01M Tris pH7.5 at 0°C.

20. Storage was over chloroform at 4°C.

L. ³²P - labelled λ C_I⁸⁵⁷ - Preparation and purification

1. An overnight culture of E1 (857) was inoculated into 200ml. MS broth to an OD₆₅₀ of about 0.1. Despite the comparatively high level of phosphate in MS broth, diluting the radioisotope, it was necessary to use this medium rather than a low phosphate medium as lysis was either very poor or totally inhibited in the low phosphate media tested.
2. The culture was grown in the NBSC Gyrotory shaker at 33°C to an OD₆₅₀ of 0.2. 4mC of ³²P as orthophosphate was added.
3. The OD₆₅₀ was followed to 0.6 to 0.8 when the culture was transferred to a water bath at 45°C for 20 minutes with aeration.

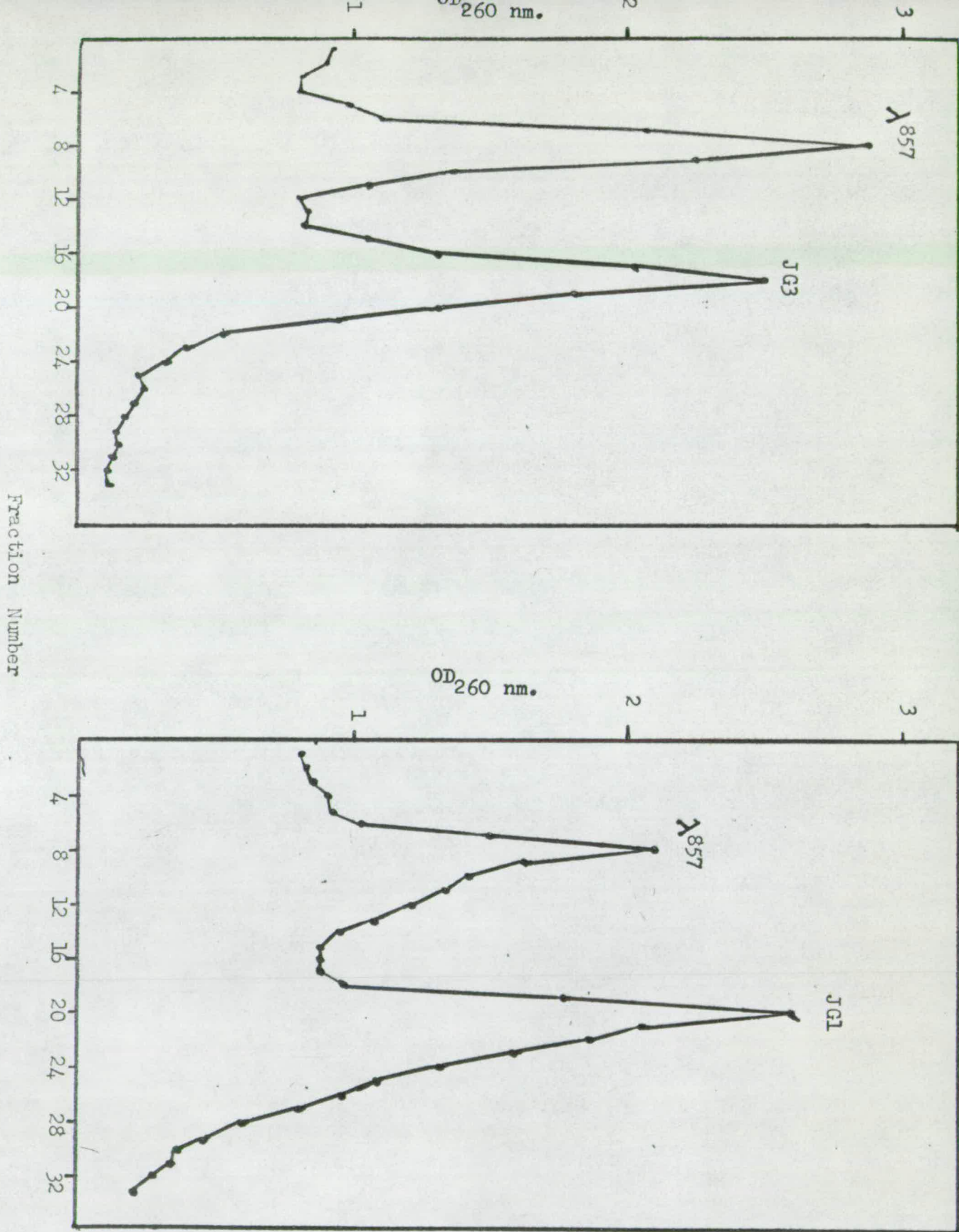


Figure 7.

Typical optical density profiles of caesium chloride gradients of phages prepared from bacteria doubly lysogenic for $\lambda_{C_I}^{857}$ and either dg JG3 or dg JG1.

4. The culture was returned to the shaker and grown at 37°C until lysis occurred. 5ml chloroform was added, and the culture shaken for 5 minutes. It was allowed to stand for 30 minutes at room temperature.
5. The lysate was decanted off the chloroform, and for each 100ml the following were added: 2.3g NaCl; 30ml 30% PEG and 15ml 40% DES. The mixture was stirred in ice for 1 hour.
6. The mixture was spun for 15 minutes at 10,000 rpm in the Sorvall SS34 rotor, the upper and lower phases removed and the interphase resuspended in about 4ml H_2O .
7. $\frac{1}{4}$ volume 3M KCl was added, and the mixture left in ice for 1 hour. It was then centrifuged for 5 minutes at 15,000 rpm and the supernatant dialysed against 0.01M NaCl; 0.01M Tris pH7.5 at 20°C .
8. MgCl_2 was added to 5mM, DNase to $10\mu\text{g/ml}$ and RNase to $10\mu\text{g/ml}$. The mixture was incubated for 30 minutes at 37°C .
9. The phages were then spun in the Spinco #50 rotor for 1 hour at 40,000 rpm, and the pellet gently resuspended in 0.1M KCl; 0.01M Tris.
10. CsCl was added to 46% w/w (density of 1.51g/cm^3 at 4°C) and the gradient overlaid with paraffin and spun for 18 hours in the Spinco #50 rotor at 45,000 rpm at 15°C .
11. The band was removed and dialysed against 0.1M NaCl; 0.01M Tris.

M. ^{32}P -labelled DNA preparation

1. Guha et al (1968) showed that cross hybridisation between λ DNA and E.coli RNA was very low under the conditions used in these experiments. We therefore used ^{32}P -labelled $\lambda_{\text{C}_1}^{857}$ phages, diluted more than 100-fold by cold transducing phages to give the

required specific activity. This mixture was diluted with 0.1M NaCl; 0.01M Tris to give the required phage concentration.

2. To this was added an equal volume of redistilled, H₂O - saturated phenol, and the mixture gently agitated for 5 minutes at room temperature.

3. It was then centrifuged for 10 minutes at 10,000 rpm at room temperature, and the phenol layer removed with a pasteur pipette.

4. The phenol extraction was repeated twice, and on the third occasion the aqueous phase was removed and dialysed against 0.1M NaCl; 0.01, Tris (2 changes) to remove phenol: 0.12M phosphate buffer (2 changes) to dilute out any free ³²Pi remaining: and finally 0.1M NaCl; 0.01M Tris.

5. The specific activity of the DNA was measured as follows:-

The concentration was determined by reading the UV absorption at 260 nm in the Beckman DB spectrophotometer. An OD₂₆₀ of 20 was taken as equivalent to a concentration of 1mg/ml of DNA. The radioactivity was measured by spotting 0.1ml of the DNA solution onto each of 3 mitrocellulose membranes (30mm diameter) which were dried and placed in scintillation vials. 15ml of counting fluid was added to each vial, and they were counted 3 times for 10 minutes in the Packard Tri-Carb Scintillation Spectrometer Model 3320.

N. Growth of bacteria for RNA preparation and galactokinase assay
(Gosden et al. 1971)

1. An overnight culture grown in 3 x D medium was inoculated into 400ml 3 x D to an OD₆₅₀ of about 0.1. The appropriate supplements and antibiotics were added to both cultures.

2. The culture was grown at 37°C in the NBSC Gyrotory shaker and

the OD₆₅₀ followed.

3. At OD₆₅₀ between 0.2 and 0.3 cultures to be induced had G-Gal to 0.2% or Fucose to 5mM added. Repressed cultures had glucose to 0.2% or TIG to 2.5mM added.

4. The generation time was calculated at 30 minute intervals from the relationship $G = \frac{(t - t_0)}{3.322 \log_{10}(A/A_0)}$ where G is generation time, t is time now, t₀ is time of previous reading (both in minutes), A is OD₆₅₀ now and A₀ is previous OD₆₅₀. At OD₆₅₀ 0.6 an aliquot was removed and chilled for the galactokinase enzyme assay.

5. At OD₆₅₀ 0.8, 50μCi of uracil 2 - ¹⁴C was added to each 400ml culture, and the culture shaken by hand at room temperature for 1% of the generation time. Growth was then stopped by pouring the culture onto 160ml of crushed frozen buffer (10mM Tris pH 7.5 at 0°C; 10mM MgCl₂; 10mM NaN₃).

6. The mixture was stirred to ensure a rapid and even drop in temperature (in 30 seconds the temperature was less than 5°C).

7. The cells were pelleted in the Sorvall GSA rotor (10,000 rpm for 10 minutes) and resuspended in 10mM NaN₃; 5mM EDTA; 20mM Na Acetate pH 5.0; 0.1M NaCl. At this point the preparation could be stored at -20°C.

0. Galactokinase assay

1. 40ml aliquots taken as described above were used. Since there is considerable variation in assays of the same strain on different occasions (Adhya and Echols, 1966), with each batch a culture of E1, fucose induced, was grown and assayed in parallel to act as a standard.

2. The OD₆₅₀ of the chilled samples was checked to ensure that growth had stopped. The bacteria were then collected by centrifugation at 10,000 rpm for 10 minutes in the Sorvall SS34 rotor.

3. The pellet was washed in 40ml 0.1M potassium phosphate buffer pH 7.2 by resuspension and centrifugation.
4. It was then resuspended in 4ml 0.1M potassium phosphate buffer pH 7.2 containing 5mM glutathione to preserve enzyme activity.
5. A 0.25ml portion was mixed with 0.75 ml of a solution containing 1mg/ml lysozyme; 5mM Tris-acetate buffer pH 8.0 at 37°C; 0.25mM EDTA; 1mM glutathione, and incubated for 5 minutes at 37°C.
6. The mixture was returned to an ice bath and lysis was checked by microscopic examination.
7. The assay was carried out by mixing three 0.025ml samples of the lysate with 0.025ml of a solution containing 0.05μmol of ¹⁴C-galactose (specific radioactivity 1 Ci/mol); 0.1μmol ATP; 0.0832μmol MgCl₂; 0.2μmol NaF; and 2.5μmol tris-HCl buffer, pH 7.5 at 37°C.
8. After 0, 5 and 10 minutes at 37°C the reaction in one tube was stopped by adding 0.95ml cold H₂O, and placing the tube in an ice-bath.
9. 0.1ml samples were spotted in duplicate on 2.3cm diameter DEAE paper discs (Whatman DE81), and the discs were washed four times in distilled H₂O to remove unconverted galactose. The discs, which retain galactose-1-phosphate quantitatively, were dried and placed in glass vials with 5ml counting fluid.
10. Two discs were left unwashed to check the amount of ¹⁴C-galactose in the reaction mix. The discs were counted in a Packard Tri-Carb Scintillation Spectrometer.
11. Enzyme activities were normalised by dividing by the optical density of the culture at 650nm, and by the activity/OD₆₅₀ ratio of the E1 fucose induced culture grown, harvested and assayed in parallel.

P. Preparation of RNA from bacteria

The method is based upon that of Nygaard and Hall, 1963, improved by Bishop and Robertson, 1969, and since further modified.

1. The cell suspension was thawed if necessary, and mixed well to disaggregate the cells.
2. $\frac{1}{20}$ volume of 10mg/ml lysozyme in 0.1M Tris was added, and the mixture left 5 minutes in ice. This caused the cells to form spheroplasts, with consequent rapid lysis.
3. The temperature of the mixture was raised to 10°C, and 10% sodium lauryl sulphate (SLS) added to 2%. The mixture was incubated at 45°C until lysis occurred (less than 2 minutes).
4. An equal volume of redistilled, H₂O - saturated phenol at 50°C was added, and the mixture shaken for 5 minutes at 50°C, then for 10 minutes at room temperature.
5. The mixture was centrifuged for 15 minutes at 12,000 rpm in the HB4 (swinging bucket) rotor of the Sorvall RB-2 centrifuge.
6. The aqueous phase was removed and kept, and the interphase and phenol phase re-extracted with an equal volume of 0.1M NaCl; 0.01M Tris, followed by centrifugation as above.
7. The aqueous phase was pooled with the first aqueous phase, and both were extracted with redistilled, H₂O - saturated phenol, followed by centrifugation.
8. The aqueous phase was removed, one volume of absolute alcohol added to it, and the precipitated nucleic acids spooled out with a glass rod. This was dissolved in 50mM Tris; 5mM MgCl₂.
9. To the remainder were added $\frac{1}{20}$ volume 2.0M Na Acetate pH5.0 and a second volume of absolute alcohol.

10. After 15 minutes at room temperature this was centrifuged for 10 minutes at 12,500 rpm in the HB4 rotor.
11. The pellet was dissolved in 50mM Tris; 5mM $MgCl_2$.
12. Both nucleic acid solutions were treated with DNase at a concentration of 50 μ g/ml. for 15 minutes at 37°C.
13. The two solutions were mixed, and an equal volume of redistilled, H_2O - saturated phenol at 50°C added. The mixture was shaken for 5 minutes at 50°C and 5 minutes at room temperature, followed by 10 minutes centrifugation.
14. The aqueous phase was re-extracted with one volume of phenol the mixture centrifuged and the aqueous phase kept.
15. The two phenol phases were pooled and extracted with one volume of 0.1M NaCl; 0.05M Tris. The mixture was centrifuged, the aqueous phase removed and pooled with the previous aqueous phases.
16. $\frac{1}{10}$ volume 2.0M Na acetate pH 5.0 and two volumes of absolute alcohol were added to the pooled aqueous phases, which were kept at 0°C for at least 30 minutes (usually overnight).
17. The mixture was centrifuged at 12,500 rpm for 10 minutes in the HB4 rotor. The pellet was resuspended in column buffer.
18. The resulting solution was passed through a column of Sephadex SP C-50 40cm long and 3cm in diameter.
19. 3 to 5ml fractions were eluted with column buffer, and those fractions containing more than 100 μ g/ml RNA with OD_{260}/OD_{280} ratio of 2.0 were pooled.
20. The pooled fractions were concentrated by precipitating with $\frac{1}{10}$ volume 2.0M Na acetate pH 5.0 and 2 volumes of absolute alcohol. The precipitate was pelleted by centrifugation and dissolved in

2 x SSC to a concentration of approximately 5mg/ml. The RNA was stored at -25°C .

21. The specific activity of the total RNA was measured as follows:-

The concentration was determined by making duplicates of a suitable dilution (usually 1:400) and reading the UV absorption at 260nm in the Beckman DB Spectrophotometer. An absorption of 24.0 OD₂₆₀ was taken as equivalent to 1mg/ml RNA. To measure the radioactivity, 0.1ml of the dilution was spotted onto each of 3 nitrocellulose membranes (30mm diameter) which were dried and placed in scintillation vials. 15ml of counting fluid was added to each, and they were counted 3 times for 10 minutes in the Packard Tri-Carb Scintillation Spectrometer.

Q. Preparation of cold rat liver RNA

1. Rats were starved overnight. The following morning they were killed and the livers removed.
2. The livers were homogenised with a Tri-R stirrer in 0.25M Sucrose; 0.05M Tris; 0.25M KCl; 3mM MgCl₂.
3. 10% SLS was added to a final concentration of 1%, and the mixture was shaken with an equal volume of chloroform; redistilled, H₂O - saturated phenol, 1:1.
4. The mixture was centrifuged for 10 minutes at 10,000 rpm in the GSA rotor, the aqueous phase removed and re-extracted with chloroform phenol, followed by centrifugation.
5. The aqueous phase was removed, $\frac{1}{10}$ volume 2.0M Na acetate pH 5.0 and 2 volumes absolute alcohol added to precipitate the nucleic acids, and the mixture chilled for at least 30 minutes.

6. It was centrifuged for 10 minutes at 10,000 rpm, and the pellet resuspended in 50mM Tris; 7mM MgCl_2 . 50 $\mu\text{g}/\text{ml}$ DNase was added, and the mixture incubated for 20 minutes at 37°C.
7. The DNase digestion was followed by two further extractions with chloroform phenol, and the final aqueous phase again mixed with $\frac{1}{10}$ volume 2.0M Na acetate pH 5.0, 2 volumes absolute alcohol.
8. After centrifugation the pellet was resuspended in column buffer and passed through a Sephadex SP C-50 column. The peak fractions were collected and precipitated with $\frac{1}{10}$ volume 2.0M Na acetate pH 5.0, 2 volumes absolute alcohol.
9. After a further centrifugation the pellet was dissolved in 2 x SSC at a concentration of 4mg/ml.

R. Hybridisation

The method is based upon that of Gillespie and Spiegelman (1965) in which denatured DNA is bound to nitrocellulose membranes, which are incubated in a solution of RNA.

a) Denaturing and Loading DNA

1. ^{32}P -labelled DNA prepared as described above was diluted with 0.1 x SSC to an appropriate concentration (About 10 $\mu\text{g}/\text{ml}$).
2. The DNA was denatured by raising the pH to 12.1 with 1N NaOH. The alkaline solution was left at room temperature for 10 minutes, the pH brought to 7.5 with 0.4N HCl, and the solution transferred to an ice-bath.
3. The concentration was adjusted to twice that required for loading, with 0.1 x SSC, and the DNA solution added to an equal volume of 12 x SSC to bring the salt to 6 x SSC.
4. Nitrocellulose membranes (Sartorius, 30mm diameter, 0.45 μ pore

size) were soaked in 2 x SSC for 5 minutes and then washed with 35ml of 2 x SSC under suction on the filtration apparatus.

5. The suction was released, and 1.0ml of the denatured DNA allowed to filter through each membrane under gravity. Between 1.0 and 2.5 μ g DNA was loaded on each membrane.

6. The membranes were then washed with 70ml of 2 x SSC under suction to remove any free DNA.

7. Blank membranes were washed in the same way.

8. Two loaded membranes and one blank membrane were rolled up together and placed in a 2" x 5/16" rimless test-tube. The tubes were placed in a dessicator under vacuum for 2 hours at room temperature, and then in a vacuum oven for 2 hours at 80°C.

b) Annealing and disaggregation

1. To each tube of three membranes, 1.0ml of hybridisation mix was added; this contained, in addition to increasing amounts of ^{14} C pulse-labelled bacterial RNA, 100 μ g cold rat liver RNA (to reduce non-specific binding). All reactants were in 2 x SSC, and 2 x SSC was added to make the volume to 1.0ml. Incubation was at 65°C in a liquid paraffin bath.

2. After incubation for 40 hours (determined as the time after which no increase in hybridisation was detected by Bishop, 1969b) the membranes were removed, separated, and placed in 20ml of 2 x SSC. The membranes were incubated for a further 1 hour at 65°C to allow unbound RNA to disaggregate from the membranes. The 2 x SSC was then decanted off and the beakers chilled in ice.

3. The membranes were placed on the filtration apparatus and washed under suction with 175ml 2 x SSC on each side.

4. After sucking dry the membranes were incubated in 20ml of RNase (20 μ g/ml) for 20 minutes at 37°C. At the end of this incubation the membranes were quickly rinsed in 2 x SSC and chilled in ice.

5. Both sides of each membrane were washed under suction with 175ml 2 x SSC. The washed membranes were sucked dry and placed in glass scintillation vials. They were dried in a vacuum oven at 80°C for 30 minutes. After cooling to room temperature, 15ml of counting fluid was added to each vial, and the vials counted three times for 10 minutes in the Packard Tri-Carb Scintillation Spectrometer Model 3320. The setting was adjusted so that cross-channel contamination from ^{14}C to ^{32}P was zero, and that from ^{32}P to ^{14}C was less than 5%.

Results

A. Mapping of loci carried by transducing bacteriophages and Episomes

There are two loci known between att λ and the galactose operon. These are pgl (activity of the enzyme 6-phosphogluconolactonase) and chlD (the inability to grow anaerobically in the presence of chlorate). The two loci nearest to the gal operon distal to kinase are aro G (the activity of one of a series of iso enzymes involved in the synthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan) and nad A (the ability to grow in the absence of nicotinamide).

In order to determine the extent of the E.coli genome carried by the transducing phages and episomes it was necessary to transduce or F'-duce them into strains in which these loci were deleted. If the transducing or F'-ducing particle was able to restore the activity of these loci, it was certain that they were carried by that particle. For pgl, chlD and aro G the deletion strain used was 9-3h⁺; for nad A, strain SA594 was used. Since SA594 was Hfr, it was necessary to mate it with E1 in order to obtain an F⁻ strain with the nad A - att λ deletion. These strains were made lysogenic for the transducing phages or mated with FR5 or F'8 as described in sections F and H of Materials and Methods. The individual results are given in Tables 6 to 9, and are incorporated in Figure 6.

Table 6Test for presence or absence of aro G⁺

<u>Strain</u>	<u>Glu Phe Tyr His Trp</u>	<u>Glu Tyr His Trp</u>	<u>Result</u>
9-3h ⁺	+	-	Absent
9-3h ⁺ /FR5	+	+	Present
9-3h ⁺ /F'8	+	+	"
9-3h ⁺ (17-8)	+	-	Absent
9-3h ⁺ (JG3)	+	-	"
9-3h ⁺ (JG1)	+	-	"
9-3h ⁺ (λpg)	+	-	"
E1	+	+	Present

+: Growth

-: No growth

Table 7Test for presence or absence of chld⁺

<u>Strain</u>	<u>Anaerobic growth</u>	<u>Aerobic growth</u>	<u>Result</u>
9-3h ⁺	+	+	Absent
9-3h ⁺ /FR5	+	+	"
9-3h ⁺ /F'8	-	+	Present
9-3h ⁺ (17-8)	-	+	"
9-3h ⁺ (JG3)	-	+	"
9-3h ⁺ (JG1)	-	+	"
9-3h ⁺ (λpg)	+	+	Absent
E1	-	+	Present

+ = acid reaction

- = no reaction

Table 8

Test for presence or absence of pgl^+

<u>Strain</u>	<u>Colour</u>	<u>Result</u>
9-3h ⁺	Blue	Absent
9-3h ⁺ /FR5	Blue	"
9-3h ⁺ /F'8	Blue	"
9-3h ⁺ (17-8)	Brown	Present
9-3h ⁺ (JG3)	Brown	"
9-3h ⁺ (JG4)	Brown	"
9-3h ⁺ (λ_{pg})	Blue	Absent
E1	Brown	Present

Table 9

Test for presence of absence of nad A⁺

<u>Strain</u>	<u>+nicotinamide</u>	<u>-nicotinamide</u>	<u>Result</u>
SA594	+	-	Absent
SA594/FR5	+	-	Absent
SA594/F'8	+	+	Present
9-3h ⁺	+	+	"
E1	+	+	"

+ = growth

- = no growth

B. Hybridisation Experimentsa) Analysis of hybridisation dataAbbreviations

- r = RNA hybridised in $\mu\text{g}/\mu\text{g}$ DNA
 r_s = RNA hybridised in $\mu\text{g}/\mu\text{g}$ DNA at infinite RNA concentration
 h = concentration of total RNA
 H = ratio of concentration of total RNA to that of DNA
 C = radioactivity of RNA in RNA-DNA hybrid in $\text{cpm}/\mu\text{g}$ DNA
 C_s = radioactivity of RNA in RNA-DNA hybrid in $\text{cpm}/\mu\text{g}$ DNA at infinite RNA concentration
 D = DNA in μg
 s = specific activity of RNA in RNA-DNA hybrid in $\text{cpm}/\mu\text{g}$ RNA
 S = specific activity of total RNA in $\text{cpm}/\mu\text{g}$ RNA
 α = proportion of total RNA which is gal m-RNA
 β = proportion of total radioactivity in the gal m-RNA
 m = number of molecules of gal m-RNA per gene copy
 K, K' = constants.

Most approaches to the analysis of hybridisation experiments involve the attempt to establish a saturation value, that is, the maximum amount of RNA which will hybridise to a given amount of DNA. This is calculated from the specific activities of the RNA and DNA. Such experiments, involving adding increasing amounts of RNA to a constant amount of DNA tend to reach a plateau at which further increases in the RNA concentration lead to no apparent increase in hybridisation. This saturation value is usually estimated visually

from the apparent plateau. There are two main objections to this approach. Nygaard and Hall (1964) and Bishop (1969b) produce evidence to show that the rate of the DNA-RNA hybridisation reaction is limited by the frequency of collision of complementary sequences. As the concentration of free complementary sites is decreased by hybridisation, the reaction rate becomes slower, so that hybridisation is only complete at infinite time or infinite RNA concentration. When the RNA contains only one or a few sequences, the saturation value may be accurately represented by the experimental plateau, because the concentration of each sequence will be very high, but when the RNA contains a complex mixture of sequences, and particularly when the complementary RNA is greatly diluted by ribosomal RNA (r-RNA), the true saturation value may never be approached. Secondly, the visual estimation of the saturation value is very subjective. Some points may be given undue weight, and others too little. The highest RNA concentration may appear to be on the plateau because the shape of the curve makes extrapolation difficult.

Bishop et al (1969) developed an alternative method of estimating the saturation value. Using RNA made in vitro on a T_4 DNA template, they found that, when the reciprocal of the RNA hybridised (r) was plotted against the reciprocal of the input RNA concentration (h) a linear relationship was obtained. This reciprocal plot can be described by

$$1/r = (k/h) + k_1 \quad (1)$$

When h is infinite $r = r_s$ and $k/h = 0$. Assuming that a linear extrapolation to this point is valid, $k_1 = 1/r$ at infinite RNA concentration, that is, $k_1 = 1/r_s$

$$\text{Thus } 1/r = k/h = 1/r_s \quad (2)$$

or

$$h/r = h/r_s + k \quad (3)$$

r_s is obtained as the reciprocal of the plot of h/r against h . Since this is a straight line, the data are subject to statistical analysis, and a standard error can be attached to the value for r_s .

Throughout the T_4 experiments RNA was in excess, and the time of incubation (16 hours) was less than that required for maximum hybridisation. Thus, the RNA concentration did not change significantly from the initial concentration.

In the experiments described here, the RNA was "in vivo" RNA, and the galactose messenger RNA was only a small proportion of the total. A greater proportion of DNA and a longer incubation time were used so that the reaction proceeded further towards completion. Under these conditions the amount of hybrid formed per unit DNA depended on the ratio of input RNA to DNA (H).

To obtain saturation curves r is plotted against H . The reciprocal plot then becomes H/r against H . However, the specific radioactivity of the hybridising sequences (s) is not known before analysis, so, instead of plotting the amount of RNA in the hybrid, r , one plots the radioactivity of the RNA in the hybrid in cpm/ μ g DNA, C . Since $C = r \times s$ equation (3) becomes

$$H/r \times s = H/r_s \times s + k/s \quad (4)$$

that is

$$H/C = H/C_s + k' \quad (5)$$

A second problem involves the relationship between the saturation value and the actual complementarity of the RNA and DNA. Some of

the DNA may fail to hybridise with complementary RNA even at infinite RNA concentration. Therefore, the saturation value is not necessarily an estimate of the proportion of the RNA which is complementary to sequences in the DNA. The ratio of true complementarity to the true saturation value is the hybridisation factor.

Bishop et al (1969) used the RNA-DNA hybrid formed when RNA is synthesised in vitro on a denatured DNA template (Chamberlin and Berg, 1964) as a source of RNA with a known homology with the DNA. After heat denaturation of the hybrid, the RNA was purified and hybridised to T_4 DNA. Although the ratio of RNA to DNA in the enzymic hybrid was 0.54, the saturation value was only 0.23 to 0.30. The hybridisation factor for this in vitro RNA is thus 1.8 to 2.4.

Landy et al (1967) and Andoh and Ozeki (1967) hybridised purified tyrosine transfer RNA (t-RNA) from E.coli with DNA from the transducing phage $\phi 80$ dsu_{III}. In both cases it was visually estimated from the saturation plot that one molecule of tyrosine t-RNA hybridised with each molecule of $\phi 80$ dsu_{III} DNA. Using the double reciprocal plot (equation 3) Bishop et al (1969) derived an estimate from the combined data of Landy et al and Andoh and Ozeki of 1.15 molecules of t-RNA hybridised per molecule of DNA. More recently (Russell et al, 1970) it has been shown that $\phi 80$ dsu_{III} contains two copies of the tyrosine t-RNA gene. This leads to an estimate of about 1.75 for the hybridisation factor. In vitro RNA contains at least 40% of self-complementary sequences. Calculations based on the rates of hybridisation and self-annealing of in vitro RNA show that the self complementarity will tend to increase the apparent value of the hybridisation factor. It was therefore

preferable to use the lower estimate of 1.75 for in vivo RNA.

In order to adapt this method of analysis to the measurement of a specific sequence in a mixture of sequences, as in in vivo RNA, it is necessary to determine the amount of RNA in μg which will hybridise with each μg of DNA at infinite RNA concentration by some other means. Once this can be done, the specific activity of the RNA of the specific sequence (e.g. a messenger RNA) can be calculated from:

$$s = C_s / r_s \quad (6)$$

Then, knowing the total RNA concentration in the hybridisation mix (H_0) it is possible to calculate the proportion of the total RNA formed by the m-RNA in question (α) from

$$\alpha = \frac{(\text{concentration of messenger radioactivity})}{H_0 \times s}$$

In the plot of H/C against H the intercept k' is the value to which H/C tends when $H = 0$, that is, when the ratio of DNA to RNA input tends to infinity. If the conditions allow the maximum hybridisation of complementary RNA, k' should approximate

$$\frac{H_0}{(\text{concentration of messenger radioactivity})}, \text{ since}$$

corresponding values of H and C are divided by the same value for D .

Thus:

$$\alpha = 1/k's \quad \text{and} \quad s = 1/k'\alpha$$

The condition most likely to produce this result is a combination of high DNA input and low RNA input with long annealing time. Bishop and Irving (1971) investigated this reaction using a system with known complementarity. The RNA was E.coli r-RNA and the DNA was also from E.coli. They found that, over a range

of DNA inputs, this method gave plots which approximated closely to linearity, and the values for r_s (0.392%) and α (0.92) were very close to expectation (0.4% and 1.0 respectively). The method was therefore adopted for the measurement of pulse-labelled galactose-specific messenger RNA.

In this case H/C is plotted against H (equation 5) to obtain estimates of C_s ($= 1/\text{slope}$) and sa ($= 1/k'$). To obtain a value for α it is necessary to use C_s to estimate s . This is only possible if the proportion of the DNA with which the RNA can hybridise is known independently. Here, the molecular weight of λ dg DNA is known, and the molecular weight of the sense strand DNA sequence of the galactose operon can be estimated approximately from the molecular weights of the polypeptide chains specified by the three cistrons of the operon. These are: kinase, 40,000 (Wilson and Hogness, 1966), transferase, 40,000 (Saito et al, 1967), epimerase, 40,000 (Wilson and Hogness, 1966). The sum of these (120,000) multiplied by 8.5 (the ratio of the molecular weight of an average trinucleotide, less one molecule of H_2O (935) to the molecular weight of an average E.coli amino acid (110)) gives a value for M_{gal} of 1.02×10^6 . $M\lambda$ is 30×10^6 . With a hybridisation factor of 1.75

$$r_s = \frac{M\lambda \times 1.75}{M_{gal}} \quad \text{and} \quad \frac{C_s}{r_s} =$$

$$s = 1.75 C_s M\lambda / M_{gal} = 53 C_s \quad (7)$$

Then the proportion of total RNA which is galactose messenger RNA is:

$$\alpha = 1/k'_s = M_{gal}/1.75 k'_C{}_s M\lambda \quad (8)$$

$$= 0.019 k'_C{}_s \quad (9)$$

and the proportion of total radioactivity contained in the galactose m-RNA is

$$\beta = 1/k'S \quad (10)$$

where S is the overall specific activity of the total RNA.

From these data it is possible to arrive at an estimate of the number of molecules of gal m-RNA per gal gene copy (m) in the growing culture. If the RNA/DNA ratio (Q) in the culture is known, the amount of gal m-RNA/unit DNA is αQ and the average number of copies per genome equivalent is $\alpha Q M_b / M_{gal}$ where M_b is the molecular weight of the bacterial genome. If the average number of genes per genome equivalent (o) is known:

$$m = \alpha Q M_b / o M_{gal} = Q M_b / 1.75 k'_C{}_s o M\lambda \quad (11)$$

Since M_{gal} occurs in the denominator of equation (11) and in the numerator of equation (8) the estimate of m is independent of the value assumed or calculated for M_{gal} . Taking the RNA/DNA ratio of cells with a generation time of 50 minutes to be 11.5 (Manor, Goodman and Stent, 1969), the molecular weight of the bacterial genome to be 2700×10^6 , and the average number of genes per genome equivalent to be 1, equation (11) becomes

$$m = \frac{\alpha 11.5 2700 10^6}{1.02 \times 10^6} = 31,000\alpha \quad (12)$$

(b) Experimental Results

This determination of gal m-RNA contents is complicated by the fact that RNA other than gal m-RNA hybridises with λ dg DNA. I made two approaches to this. In the first series of experiments (Series I and II) I used λ dgs 17-8 and JG1 (see figures 5 and 6). λ dg JG1 carries only the epimerase cistron, and a portion, probably very small, of the transferase cistron. λ dg 17-8 carries the whole gal operon and extends some distance beyond it. In these two experiments I assumed that the difference between the hybridisation to λ dg 17-8 DNA and that to λ dg JG1 DNA represented two-thirds of the gal m-RNA. This ignored any RNA which hybridised to 17-8 DNA beyond the kinase cistron, and any controlling elements prior to epimerase which are transcribed.

Later two more transducing phages were examined, λ dg JG3, carrying the whole gal operon with the exception of the extreme distal end of the kinase cistron, and λ pg, carrying the whole gal operon, but with the loci between λ att and the gal operator deleted (Figures 5 and 6). It seemed possible that λ pg might carry little or no E.coli DNA apart from the gal operon, and that hybridisation to DNA from this phage might in itself be sufficient to measure the galactose specific m-RNA.

The difference between the hybridisation to λ dg 17-8 DNA and λ dg JG1 DNA was capable of providing only a maximum estimate for the gal m-RNA content, as hybridisation of RNA to the non-gal DNA distal to kinase on 17-8 is likely to cause over-estimation of the gal m-RNA.

A minimum estimate could be obtained from the difference between the hybridisation to λ dg JG3 DNA and that to λ dg JG1 DNA.

Since JG3 carries almost the whole operon, and JG1 only the epimerase cistron, some factor of the difference between them should give a measurement of the gal m-RNA content. $1.5x (C_{JG3} - C_{JG1})$ would be the minimum estimate, since, if JG3 carried the whole galactose operon, and JG1 only the epimerase cistron, the difference between them would be two cistrons, and $1.5x$ the difference would be equivalent to the whole operon. Since JG3 carries slightly less than the whole operon, and JG1 slightly more than one cistron, the difference between them is slightly less than two cistrons, and therefore, to obtain the value for three cistrons, the factor might be more than $1.5x$. The maximum might be as much as $2x$, if the difference between them was only 1.5 cistrons.

To compare the value of these three methods of estimating gal m-RNA, I used RNA from 9-3h⁺/FR5. This strain has the advantage that all DNA homologous to the E.coli DNA of the transducing phages has been deleted, and the only homology is between the DNA of the episome and the phage E.coli DNA.

In the first of these experiments, RNA from 9-3h⁺/FR5 induced with fucose was hybridised with DNA from all four transducing phages (λ dgs 17-8, JG3, JG1 and λ pg). This experiment was repeated three times to minimise variation due to experimental error. In fact the three estimates agree quite well. The raw data of these and all other experiments are given in Appendix A, Tables 1 to 5. The saturation curves are shown in Figure 8a, and the plots of H/C against H, calculated from a regression analysis of the experimental values, in Figure 8b. The values of C_s , obtained from the reciprocal values of the slopes in Figure 8b, are given in Table 10, together with the repeats of

Legend to Figure 8

a) Saturation curves for RNA from 9-3h⁺/FR5 induced with fucose, hybridised with DNA from λ_{pg} and λ_{dgs} 17-8, JG3 and JG1

Each membrane was loaded with 1.5 μ g of DNA and two membranes with DNA from the same phage and one blank membrane were annealed for 40 hours with 1.0ml RNA (see Material and Methods Section R,b).

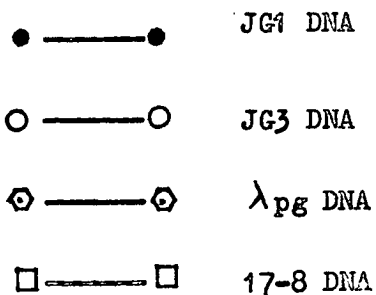
The number of counts per minute per μ g DNA in the hybrid (C) is plotted against μ g RNA per μ g DNA in the annealing mix (H).

Each point represents the mean of the values for the two membranes at each RNA concentration.

b) Calculated values of H/C for each value of H

The experimentally determined values of H and C (plotted in Figure 8a) were used to calculate H/C for each value of H.

Regression analyses were then performed upon these figures, and the slope and intercept determined for each DNA. The reciprocal of the slope is C_s , the number of counts in the hybrid at infinite RNA concentration, and the intercept is k' , the number of counts in the hybrid when the DNA/RNA input is infinity.



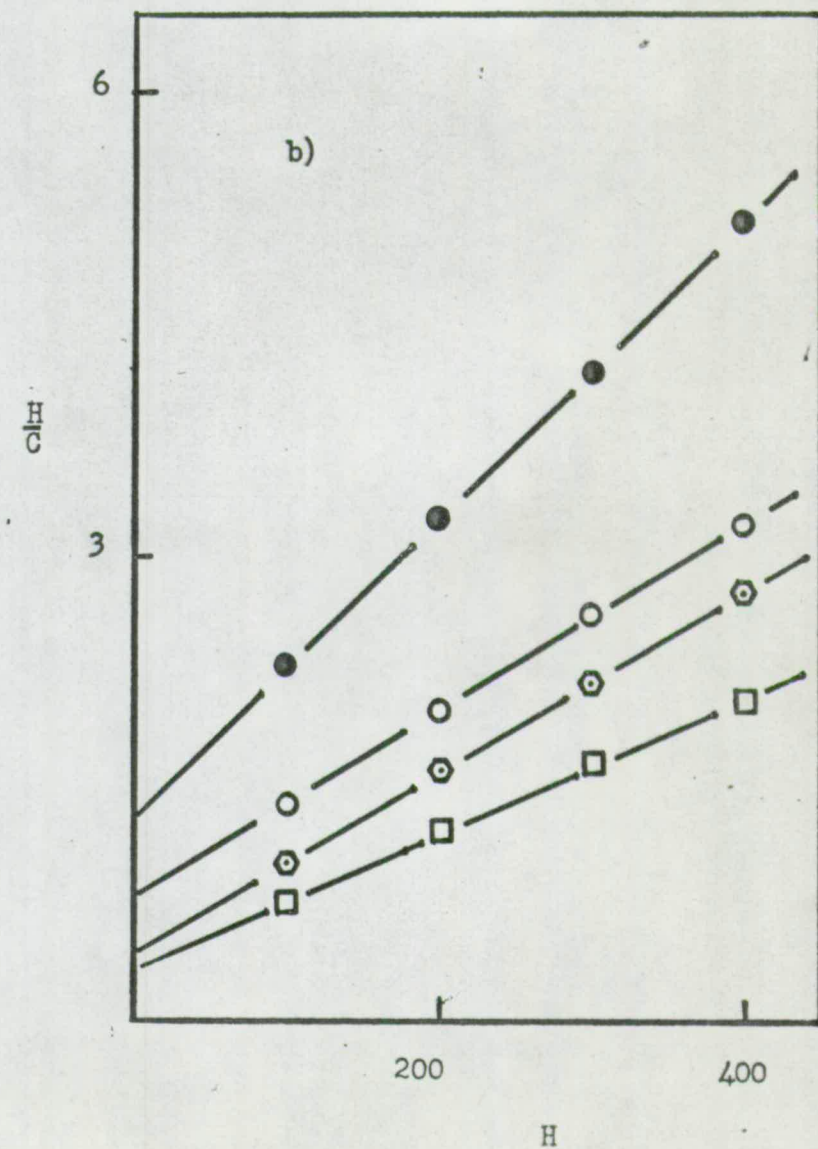
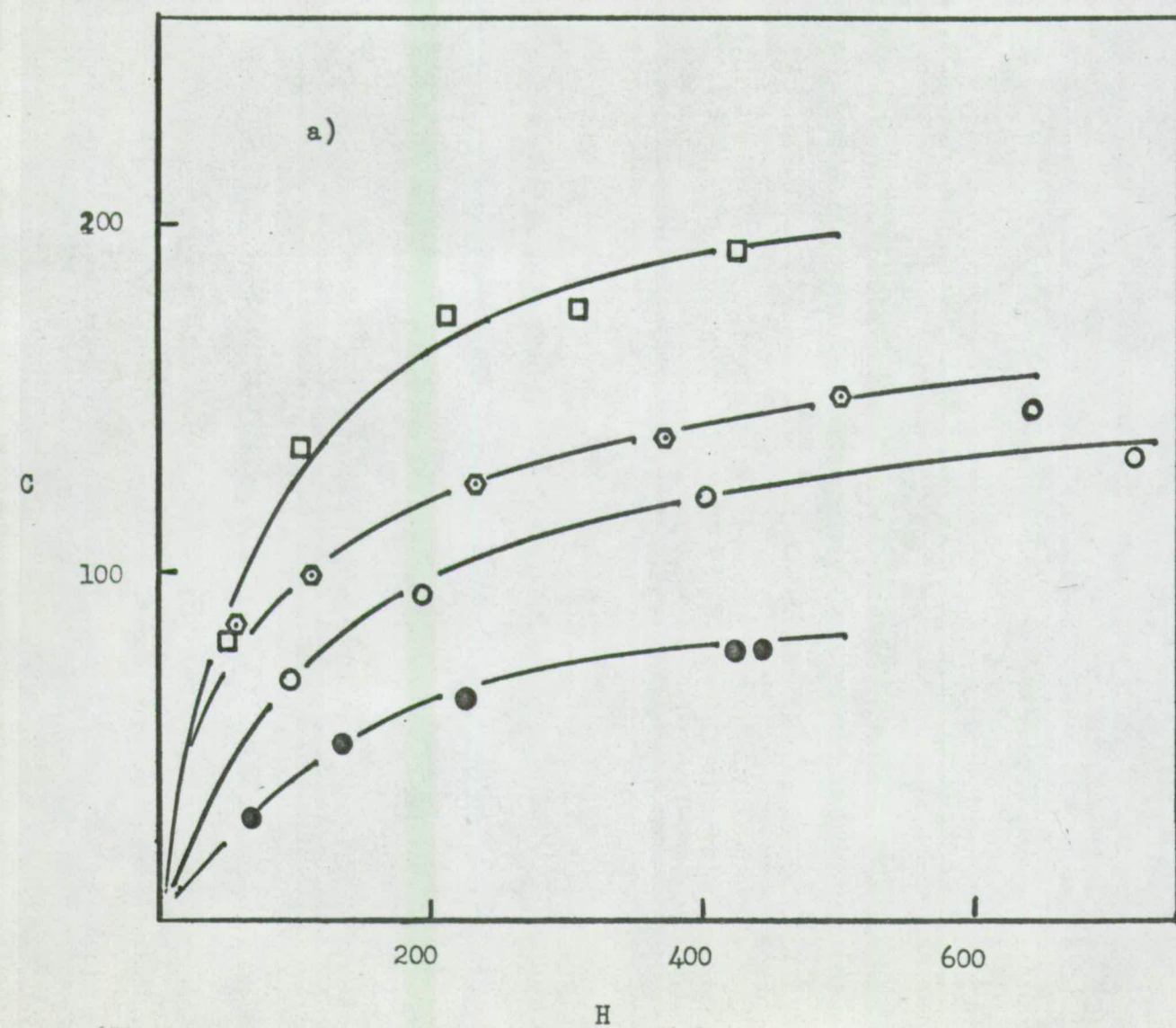


Figure 8.

Table 10

Saturation values (C_s) for each transducing bacteriophage DNA

Derived from three experiments using RNA from 9-3h⁺/FR5

<u>DNA</u>	<u>Expt.1</u>	<u>Expt.2</u>	<u>Expt.3</u>	<u>Pooled</u>
17-8	254	230	237	234
JG1	102	105	131	118
pg	141	172	171	163
JG3	184	169	190	178

the experiment, and the pooled values. From this Table,

$C_{sJG3} = 178$, and $C_{sJG1} = 118$, therefore

$$C_{sJG3} - C_{sJG1} = 60 \quad \text{and}$$

$$C_{s\text{gal}} = 1.5 (C_{sJG3} - C_{sJG1}) = 90$$

Three estimates for the gal m-RNA content were made from this data:

a) from $1.5 (C_{17-8} - C_{JG1})$

b) from C_{pg}

c) from $1.5 (C_{JG3} - C_{JG1})$

These are given in the first part of Table 11.

From the data obtained it is possible to determine empirically a maximum limit for the factor by which the difference between C_{JG3} and C_{JG1} should be multiplied to obtain C_{gal} . The values of C for each value of H are shown in Table 12. If $C_{gal} = 2x (C_{JG3} - C_{JG1})$ is used, the slope of $H/C_{non-gal}$ against H is non-linear, (Table 13). Since there is no reason why the hybridisation of non-gal RNA should not follow the same linear relationship in a reciprocal plot as other RNAs, it is possible to reject 2 as the factor. The limit is in fact reached at $1.75 (C_{JG3} - C_{JG1})$ where the slope is practically zero, (Table 13). For subsequent estimates the relationship used was

$$1.5x (C_{JG3} - C_{JG1}) = \text{Hybridisation due to gal m-RNA}$$

Using this relationship it is possible to determine the hybridisation of non-gal RNA to non-gal DNA in each of the phage DNAs used. These values are also given in Table 12. From the slopes of H/C against H for each of these determinations I obtained values for $K'Cs$ for both the gal and non-gal RNA hybridising to

Table 11

Calculated results of hybridisations from Series III experiments

RNA Source	$\alpha \times 10^5$	s	m	S	E	$\frac{m}{E}$	$\frac{\alpha \times 10^5}{E}$
<u>9-3h⁺/FR5 fucose induced</u>							
from 17-8 and JG1	29.03	9,842	9.00	496	1.376	6.54	21.10
from λ pg	29.07	8,579	9.01			6.55	21.13
from JG3 and JG1	22.20	5,158	6.88			5.00	16.13
<u>9-3h⁺/FR5 non-induced</u>	3.87	2,947	1.19	273	0.156	7.63	24.80
<u>9-3h⁺/FR5 galactose induced</u>	15.08	2,016	4.68	529	0.807	5.79	18.69
<u>9-3h⁺/FR5 TMG repressed</u>	8.09	2,403	2.51	203	0.232	10.80	34.87

Legend

α = proportion of total RNA which is gal m-RNA

s = specific radioactivity of gal m-RNA

m = number of gal m-RNA molecules per galactose gene copy

S = specific radioactivity of total RNA

E = galactokinase enzyme activity normalised to that of E1 fucose induced

Table 12

RNA Source:- 9-3h⁺/FR5 Fucose Induced Series III

Pooled data from three experiments

H	C _{JG3}	C _{JG1}	C _{JG3} -C _{JG1}	3Δ/2	JG3-(3Δ/2)	C _{pg}	C _{pg} -(3Δ/2)	C ₁₇₋₈	C ₁₇₋₈ -(3Δ/2)	C ₁₇₋₈ -C _{pg}
			= Δ	= C _{gal}	= C _{non-gal_{JG3}}		= C _{non-gal_{pg}}		= C _{non-gal₁₇₋₈}	
100	83	42	41	62	21	99	37	119	57	20
200	113	62	51	77	36	124	47	158	81	34
400	139	81	58	87	52	140	53	188	101	48
600	150	91	59	89	61	147	58	201	112	54

Table 13

H	Δ $= C_{JG3} - C_{JG1}$	2Δ	C_{JG3}	$C_{JG3} - 2 \Delta$	$\frac{H}{C_{JG3} - 2 \Delta}$	1.75Δ	$C_{JG3} - 1.75 \Delta$	$\frac{H}{C_{JG3} - 1.75 \Delta}$
100	41	82	83	1	100	72	11	9
200	51	102	113	11	18	89	24	8
400	58	116	139	23	17	102	37	11
600	59	118	150	32	19	103	47	13

Calculated values for $C_{\text{non-gal}}$ in JG3 if

$$C_{\text{gal}} = 2 (C_{JG3} - C_{JG1})$$

or

$$C_{\text{gal}} = 1.75 (C_{JG3} - C_{JG1})$$

each of these DNAs. These figures are given in Table 14 and are summarised in Table 15.

From the saturation figures (C_s) in Table 10 it is obvious that λ_{pg} must contain some non-gal DNA to which RNA will hybridise. Even if the correct estimate of C_{gal} were $2x(C_{JG3} - C_{JG1})$ the non-gal DNA of λ_{pg} would hybridise $163 - 120 = 43$ cpm at saturation, or 16% of its total counts. If $1.75x(C_{JG3} - C_{JG1})$ is the limit, then the figure is $163 - 105 = 58$ cpm or 35%, and at this limit, only 15 of the counts hybridised to 17-8 DNA and λ_{pg} DNA in excess of those hybridised to JG3 - JG1 would be due to gal. If the value I have used ($1.5x(JG3 - JG1)$) is correct, then $163 - 90 = 73$ or 45% of the counts hybridised to λ_{pg} DNA are due to non-gal RNA. These conclusions are illustrated pictorially in Figure 9.

The values of $k'C_s$ in Table 15 confirm this, besides providing additional information about the distribution of complementary sequences:

$$k'C_s \text{ JG3 minus } k'C_s \text{ JG1} = 60$$

$$k'C_s \text{ 17-8 minus } k'C_s \text{ JG3} = 66$$

Therefore the RNA hybridising to DNA distal to JG3 is either gal m-RNA or is at similar concentration to gal m-RNA.

$$k'C_s \text{ 17-8 minus } k'C_s \text{ pg} = 463$$

Therefore the RNA hybridising to 17-8 but not to pg is at very low concentration.

$$k'C_s (\text{pg-gal}) = 62$$

Therefore the non-gal RNA which is complementary to pg DNA is at high concentration. From the C_s values, up to 27% of this could be proximal to the gal operon.

Table 14

<u>H</u>	<u>$1.5\Delta(2C_{gal})$</u>	<u>H/C_{gal}</u>	<u>$JG3-C_{gal} = C_{non-gal}$</u>	<u>$H/C_{non-gal}$</u>
100	62	1.61	21	4.76
200	77	2.60	36	5.56
400	87	4.60	52	7.69
600	89	6.74	61	9.84

$$\therefore k'_{gal} = 0.60$$

$$k'_{non-gal} = 3.92$$

$$C_{s gal} = \frac{1}{0.010} = 100$$

$$C_{s non-gal} = 121$$

$$\therefore k'C_{s gal} = 60$$

$$\therefore k'C_{s non-gal} = 474$$

Table 14 Continued

<u>H</u>	<u>C_{pg}</u>	<u>H/C_{pg}</u>	<u>$C_{pg} - C_{gal}$</u>	<u>$H/(C_{pg} - C_{gal})$</u>
100	99	1.01	37	2.70
200	124	1.61	47	4.26
400	140	2.86	53	7.55
600	147	4.08	58	10.34

$$\therefore k'_{pg} = 0.42$$

$$\therefore k'_{pg non-gal} = 1$$

$$C_{spg} = 166$$

$$C_{spg non-gal} = 62$$

$$\therefore k'C_{spg} = 70$$

$$\therefore k'C_{spg non-gal} = 62$$

Table 14 Continued

H	$C_{17-8-pg}$	$\frac{H}{C_{17-8-pg}}$	$C_{17-8-gal}$	$\frac{H}{C_{17-8-gal}}$	$C_{17-8-JG3}$	$\frac{H}{C_{17-8-JG3}}$
100	20	5	57	1.75	36	2.78
200	34	5.88	81	2.47	45	4.44
400	48	8.33	101	3.96	49	8.16
600	54	11.11	112	5.36	51	11.76

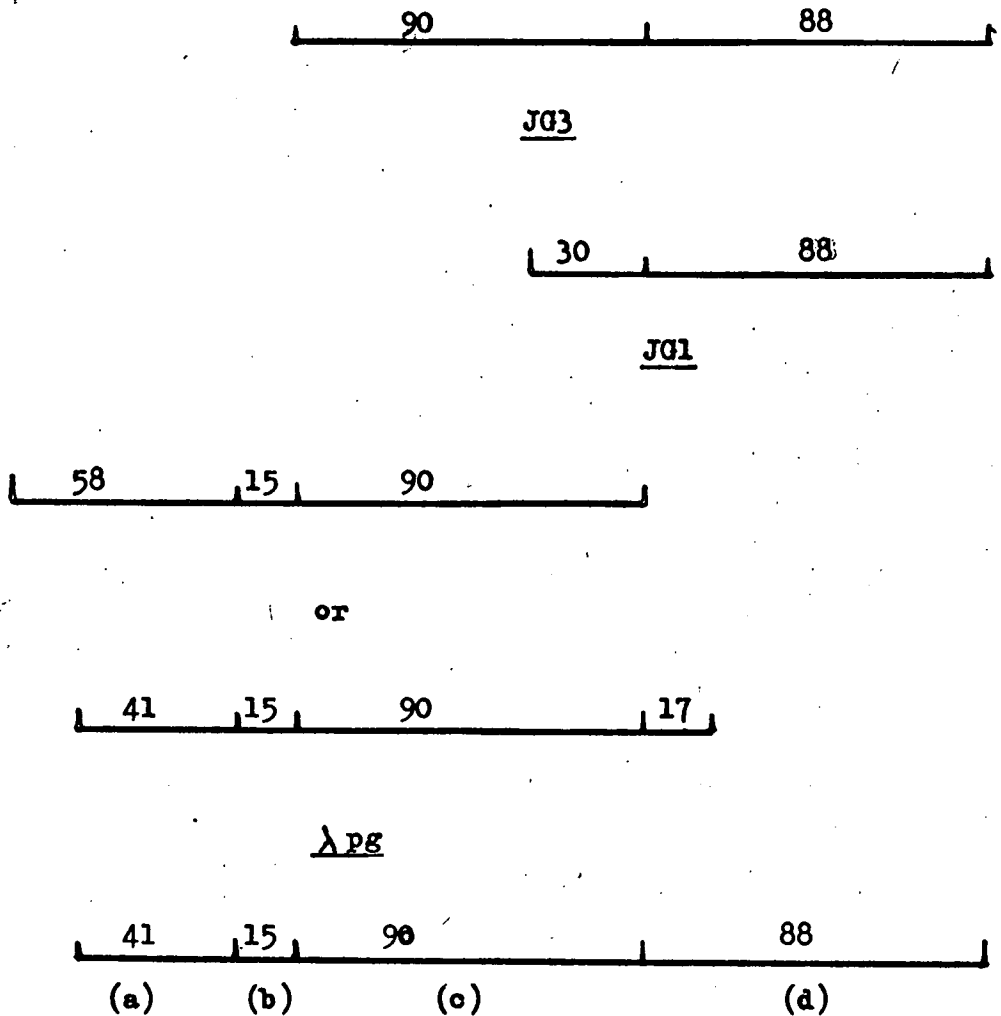
$$\begin{aligned}
 \therefore k'(17-8-pg) &= 4.1 & \therefore k'(17-8-gal) &= 1.05 & \therefore k'(17-8-JG3) &= 1.1 \\
 C_s(17-8-pg) &= 113 & C_s(17-8-gal) &= 139 & C_s(17-8-JG3) &= 60 \\
 \therefore k'C_s(17-8-pg) &= 463 & \therefore k'C_s(17-8-gal) &= 146 & \therefore k'C_s(17-8-JG3) &= 66
 \end{aligned}$$

Table 15

	$k'C_s$	
gal	60	- all gal m-RNA
pg	70	
pg - gal	62	
JG3 - gal	474	
17-8 - pg	463	- all non-gal
17-8 - JG3	66	
17-8 - gal	146	

Figure 9.

Calculated distribution of RNA counts hybridised to transducing bacteriophage DNA at saturation (C_s).



17-8

- (a) High concentration sequences, of the same order of concentration as gal m-RNA.
- (b) Possibly gal m-RNA.
- (c) Definitely gal m-RNA
- (d) Low concentration sequences.

$$k'C_g (JG3 - gal) = 474$$

This shows that it would be incorrect to subtract much more than $1.5 \times (C_{JG3} - C_{JG1})$ because the non-gal sequences complementary to JG3 are at low concentration.

$$k'C_g (17-8 - gal) = 146$$

This is consistent with the idea that the sequences distal to the gal operon are at high concentration, the proximal sequences at low concentration.

This experiment justified the assumptions made and indicated that the best estimate of C_{gal} available was from $1.5 \times (C_{JG3} - C_{JG1})$. In all subsequent experiments this was the system used.

From Table 11 it will be seen that the values for a and m derived from λ_{pg} and from the difference between 17-8 (and JG1) are very similar to each other but greater by some 30% than those derived from the difference between JG3 and JG1. While the first two methods of estimation may be invalid because of hybridisation of non-gal RNA, the third method must give a sure measure of the relative values, even though the absolute figures may be underestimated, because all hybridisation of non-gal RNA is eliminated.

The problems investigated were as follows:-

a) The effect of induction and repression on gal m-RNA and galactokinase. The inducers were fucose or galactose, and the repressors TMC and glucose. Repression with glucose gave levels of gal m-RNA that were too low to measure. The strain used in these experiments (series I and III) was 9-3h⁺/FR5. The data are given in Appendix A Tables 1 and 3. The results of series I have been published (Appendix B, Gordon et al, 1971) but are

Legend to Figure 10

a) Saturation curve for RNA from 9-3h⁺/FR5 induced with fucose,
hybridised to DNA from λ ds 17-8 and JG1

Each membrane was loaded with 1.5 μ g of DNA. Annealing etc. as described in the legend to Figure 8a.

b) Calculated values of H/C for each value of H

Regression analyses were carried out on the experimental values of H/C and H, and the slope ($1/C_s$) and intercept (k') determined for each DNA.

□ ——— □ 17-8 DNA
● ——— ● JG1 DNA

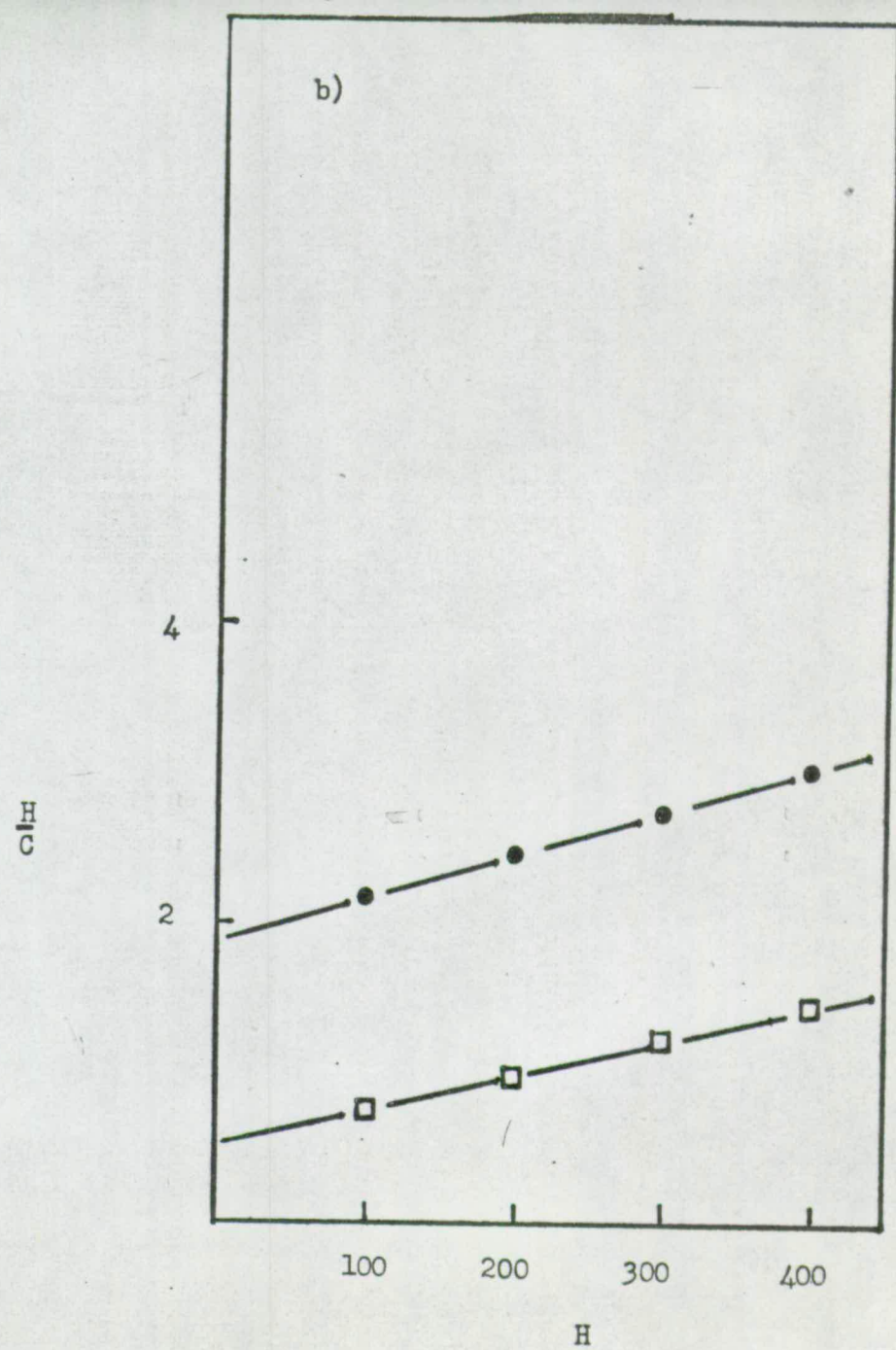
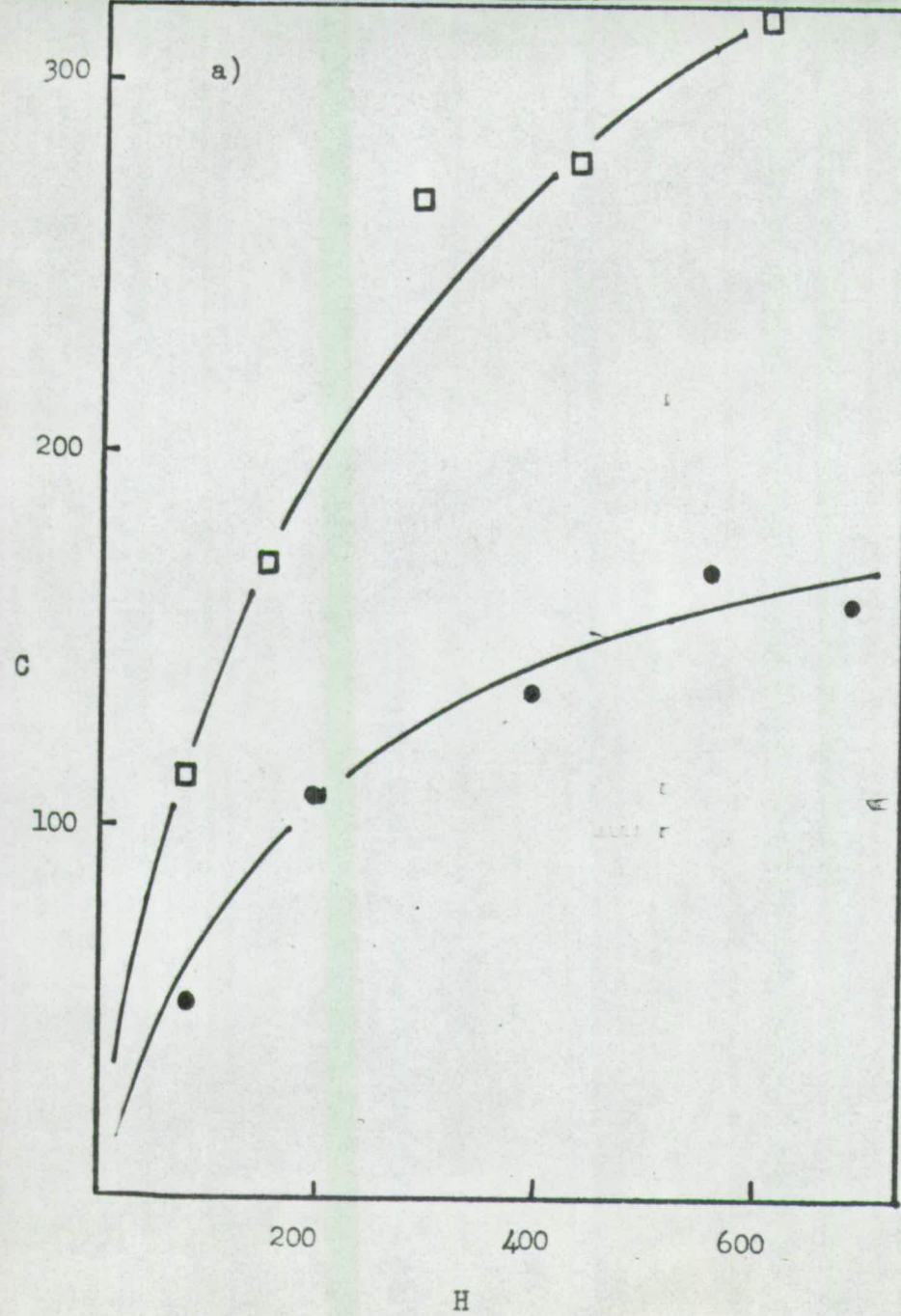


Figure 10.

Legend to Figure 11

- a) Saturation curves of RNA from 9-3h⁺/FR5 non induced hybridised with DNA from λ ds 17-8 and JG1

Each membrane was loaded with 1.5 μ g of DNA. Annealing etc. as described in the legend to Figure 8a.

- b) Calculated values of H/C for each value of H

Regression analyses were carried out on the experimental values of H/C and H, and the slope ($1/C_s$) and intercept (k') determined for each DNA.

□ ——— □ 17-8 DNA

● ——— ● JG1 DNA

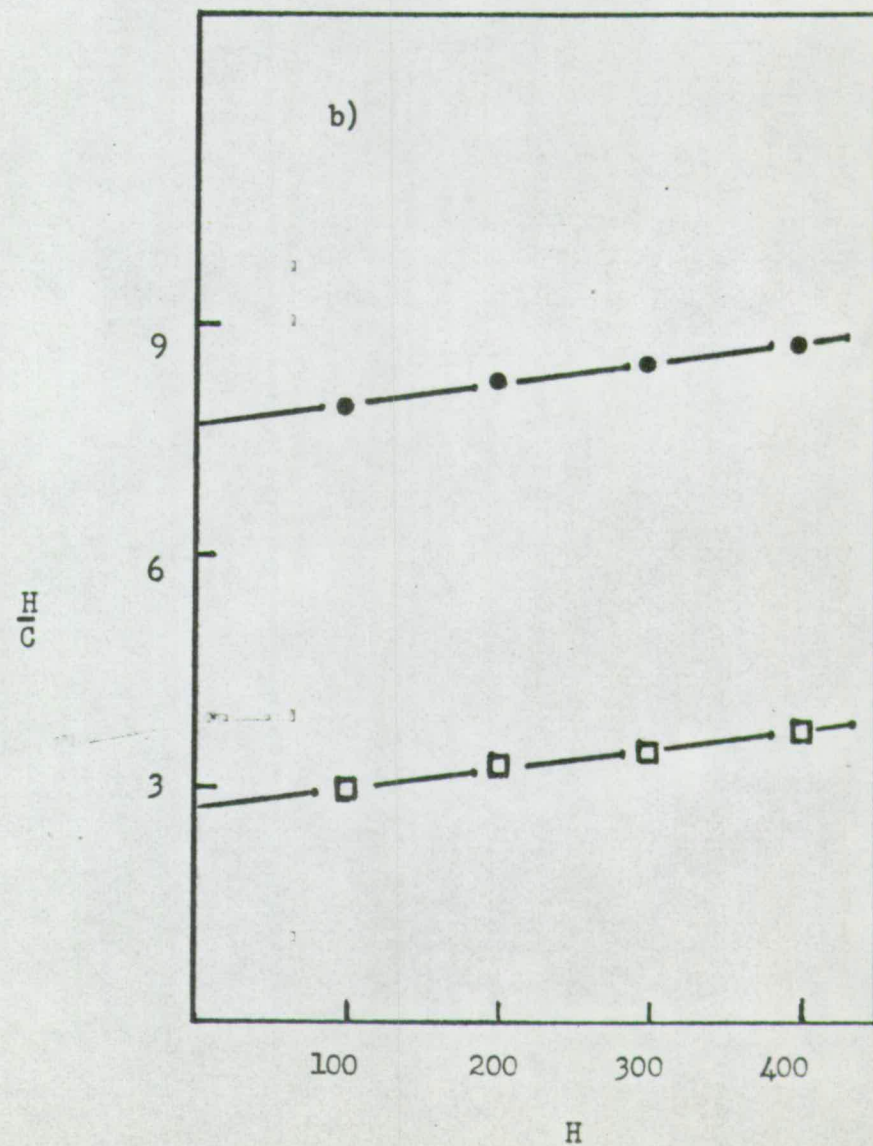
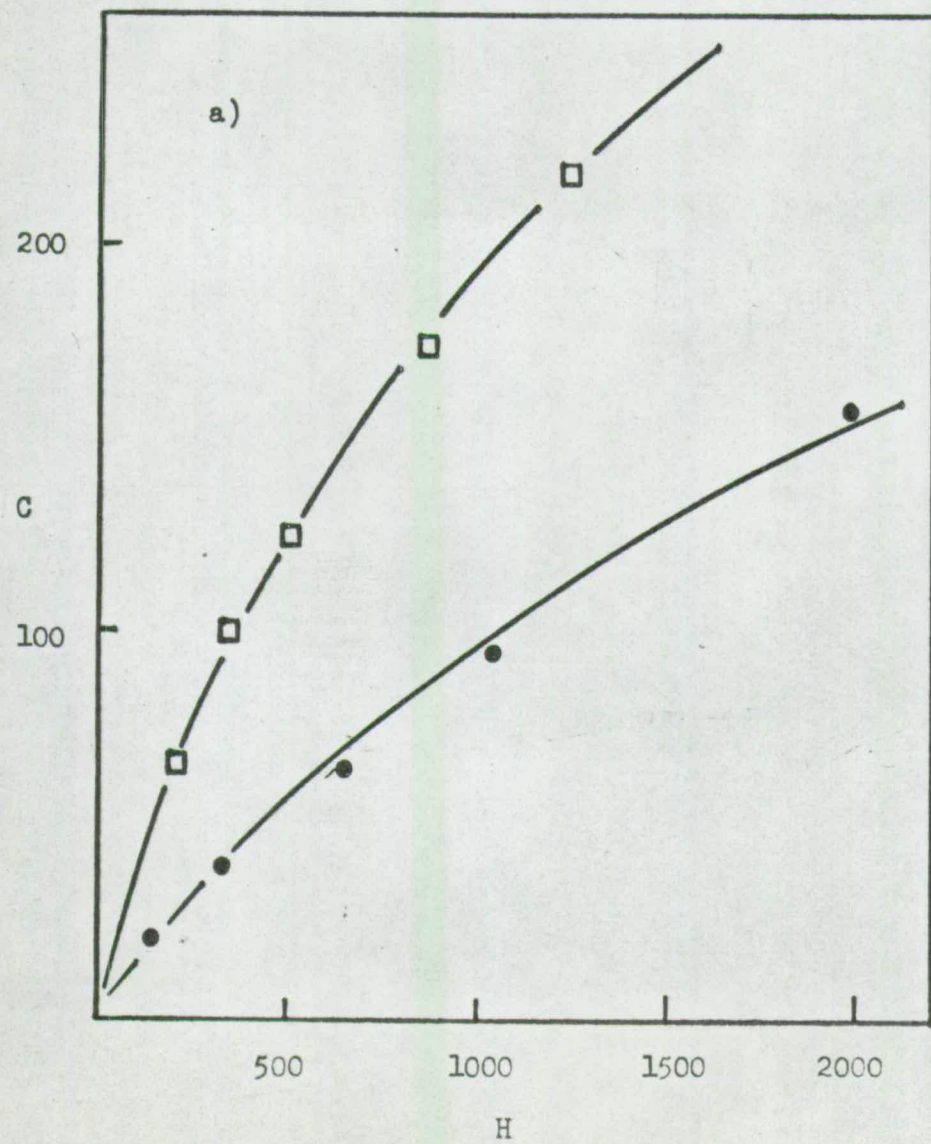


Figure 11.

Legend to Figure 12

- a) Saturation curves of RNA from 9-3h⁺/FR5 repressed with TMG, hybridised to DNA from λ dgs 17-8 and JG1

Each membrane was loaded with 1.5 μ g of DNA. Annealing etc. as described in the legend to Figure 8a.

- b) Calculated values of H/C for each value of H

Regression analyses were carried out on the experimental values of H/C and H, and the slope ($1/C_s$) and intercept (k') determined for each DNA.

□ ——— □ 17-8 DNA

● ——— ● JG1 DNA

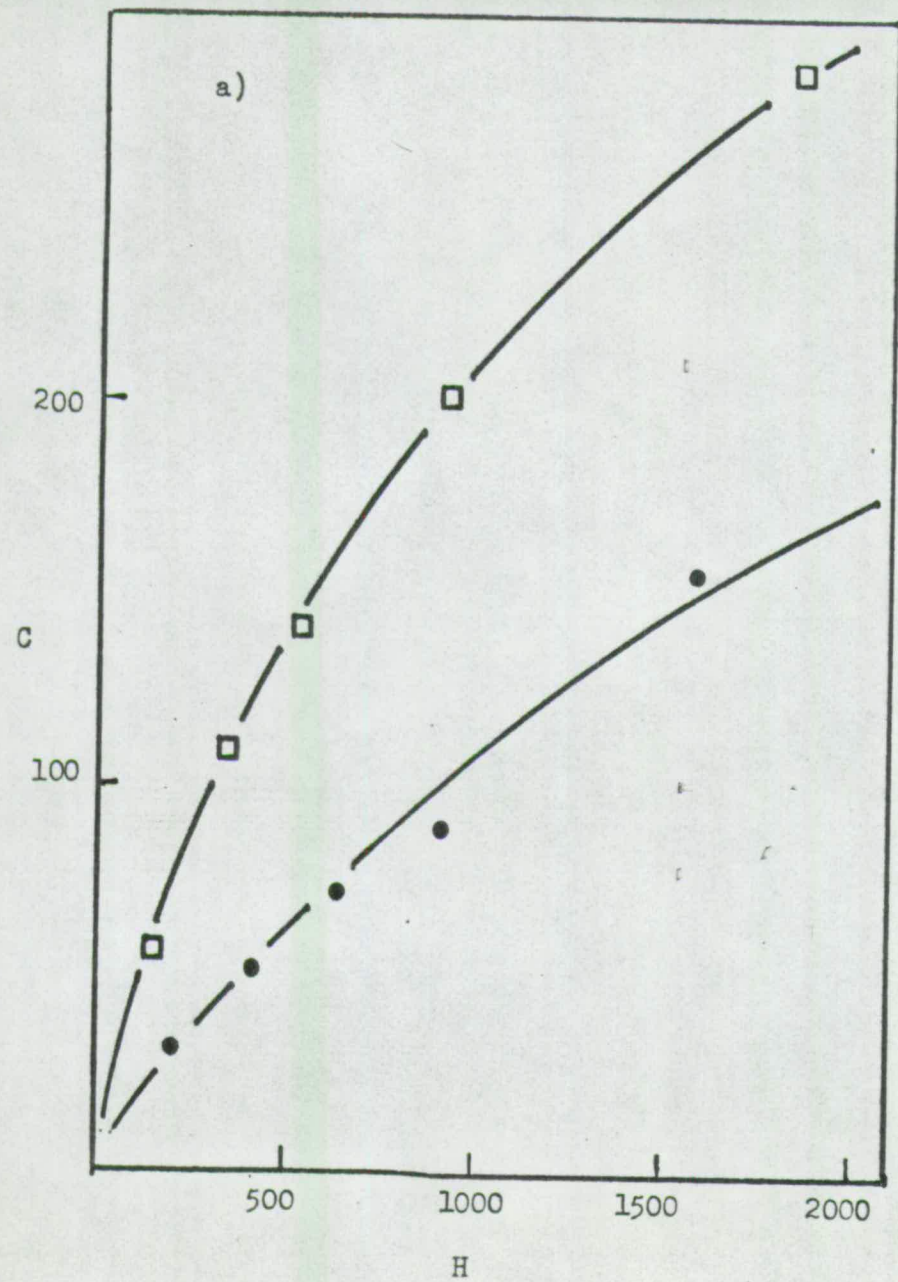


Figure 12.

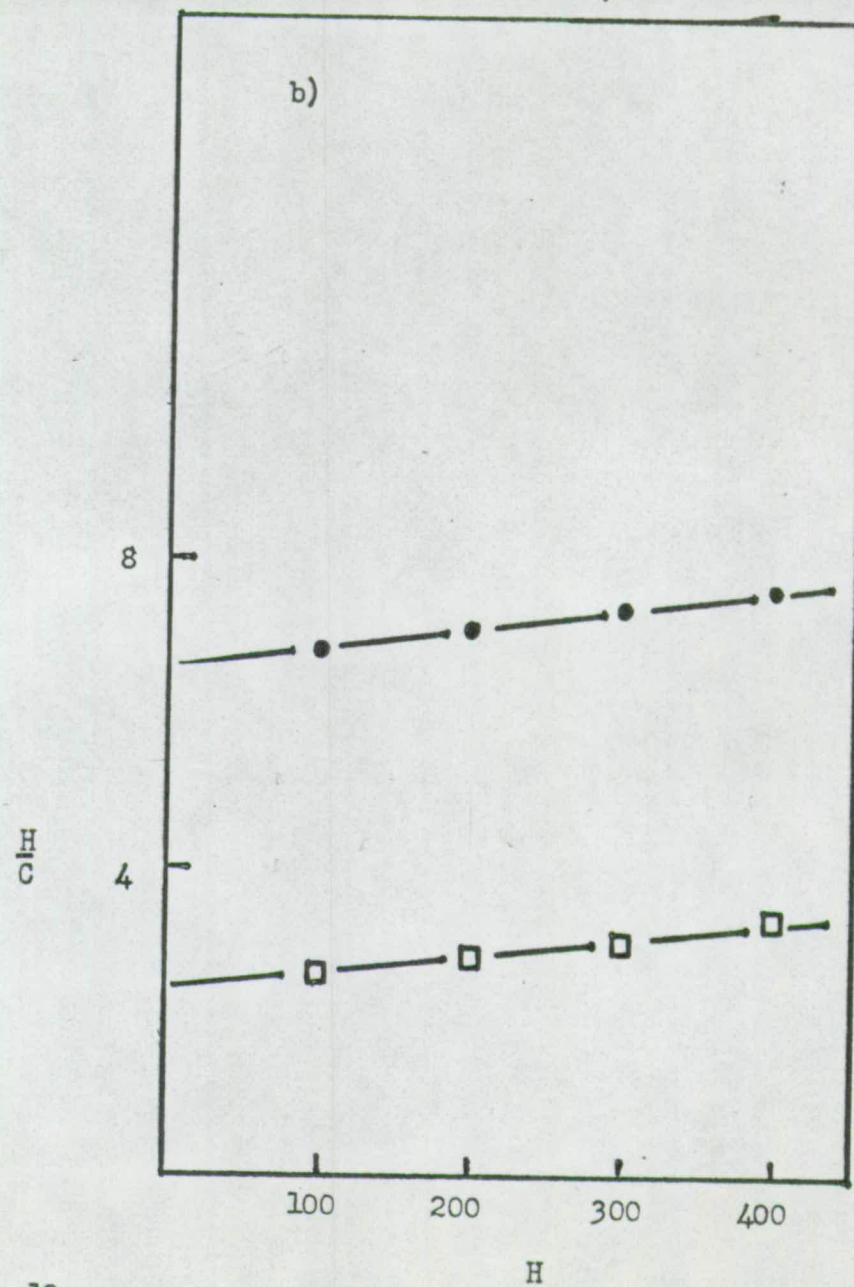


Table 16

Calculated results of hybridisation experiments Series I

RNA Source	$\alpha \times 10^5$	s	m	S	E	$\frac{m}{E}$	$\frac{\alpha \times 10^5}{E}$
<u>9-3h⁺/FR5 non-induced</u>	2.53	14,053	0.78	256	0.153	5.13	16.55
<u>9-3h⁺/FR5 fucose induced</u>	19.16	14,579	5.94	344	1.316	4.51	14.56
<u>9-3h⁺/FR5 TMG repressed</u>	2.54	15,474	0.79	277	0.078	10.06	32.44

Legend to Figure 13

a) Saturation curves of RNA from 9-3⁺/PR5 non induced hybridised with DNA from λ dgs JG3 and JG1

Each membrane was loaded with 1.5 μ g of DNA. One membrane with DNA from each phage, and one blank membrane were annealed for 40 hours with 1.0ml RNA (see Materials and Methods Section R,b).

The number of counts per minutes per μ g DNA in the hybrid (C) is plotted against μ g RNA per μ g DNA in the annealing mix (H). Each point represents the mean of the values for the two membranes at each RNA concentration.

b) Calculated values of H/C against H

The regression of H/C upon H was calculated from the experimental values from H and C, and the slope ($1/C_g$) and intercept (k') obtained.

○ ——— ○ JG3 DNA
● ——— ● JG1 DNA

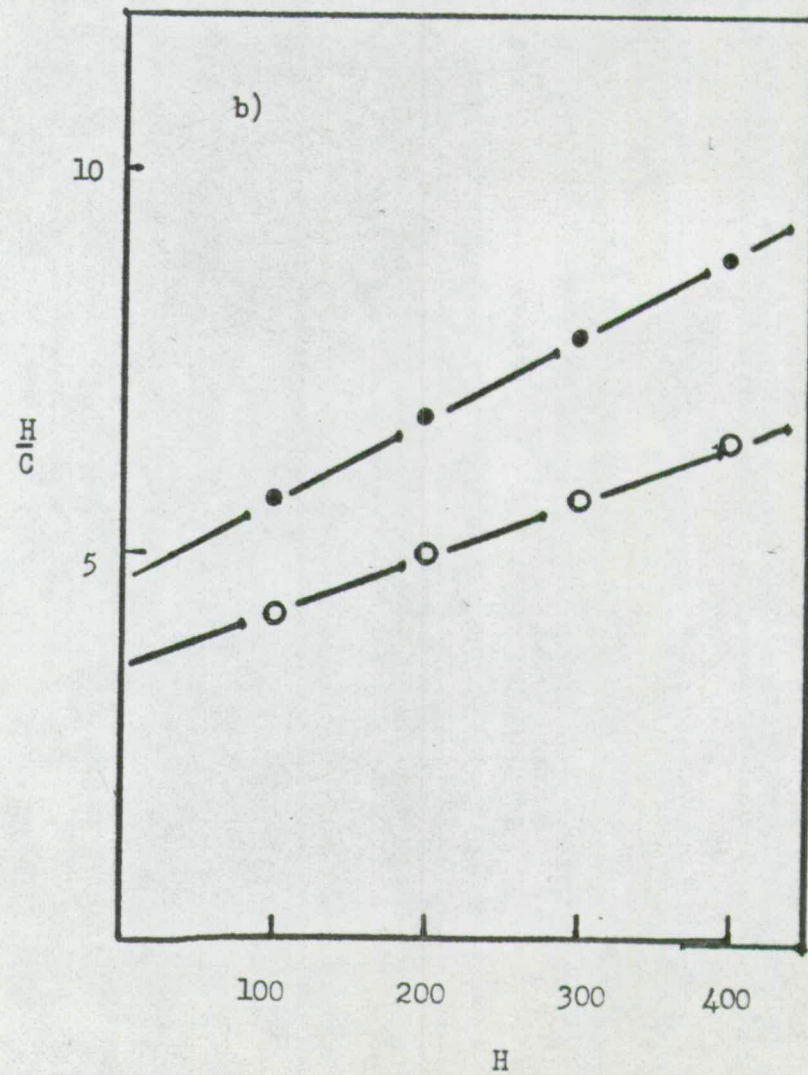
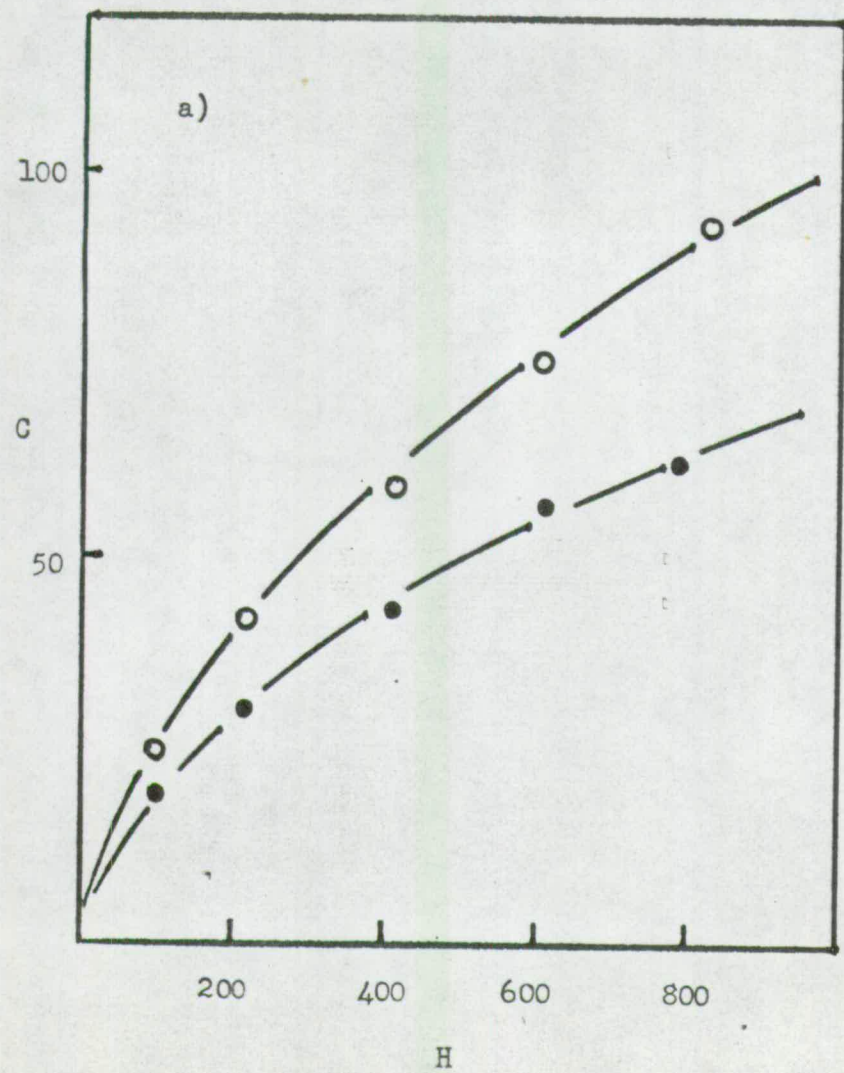


Figure 13.

Legend to Figure 14

a) Saturation curves of RNA from 9-3h⁺/FR5 induced with galactose, hybridised with DNA from λ dgs JG3 and JG1

Each membrane was loaded with 1.5 μ g DNA. Annealing etc. as described in the legend to Figure 13a.

b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was calculated, and the slope ($1/C_s$) and intercept (k') determined for each DNA

○ ——— ○ JG3 DNA

● ——— ● JG1 DNA

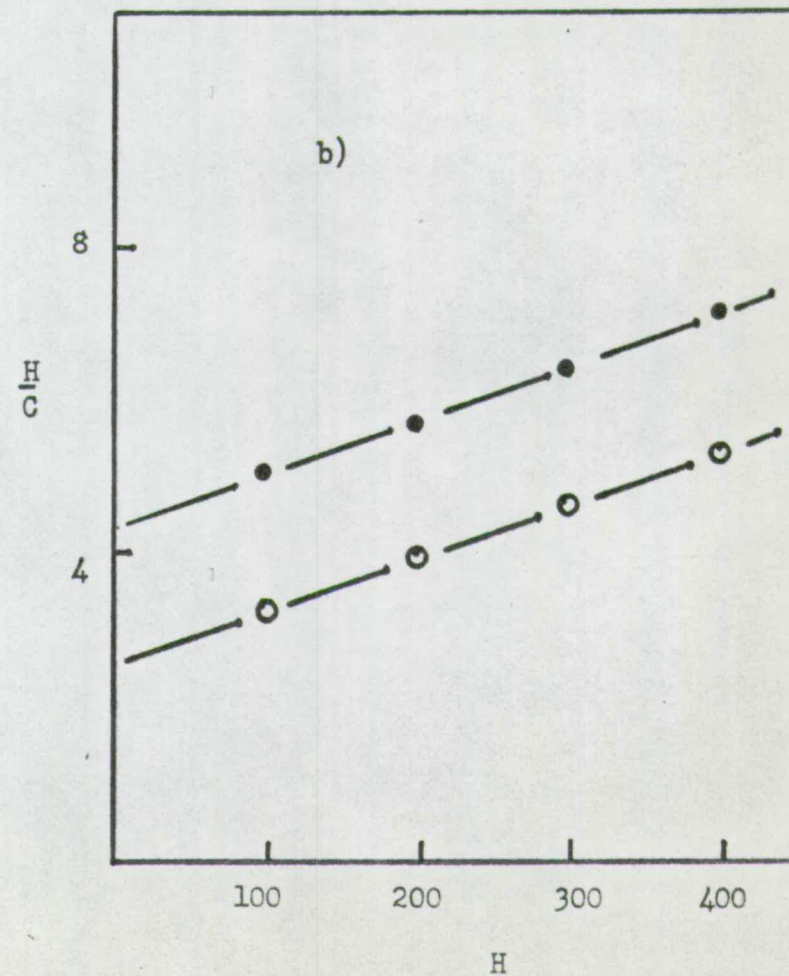
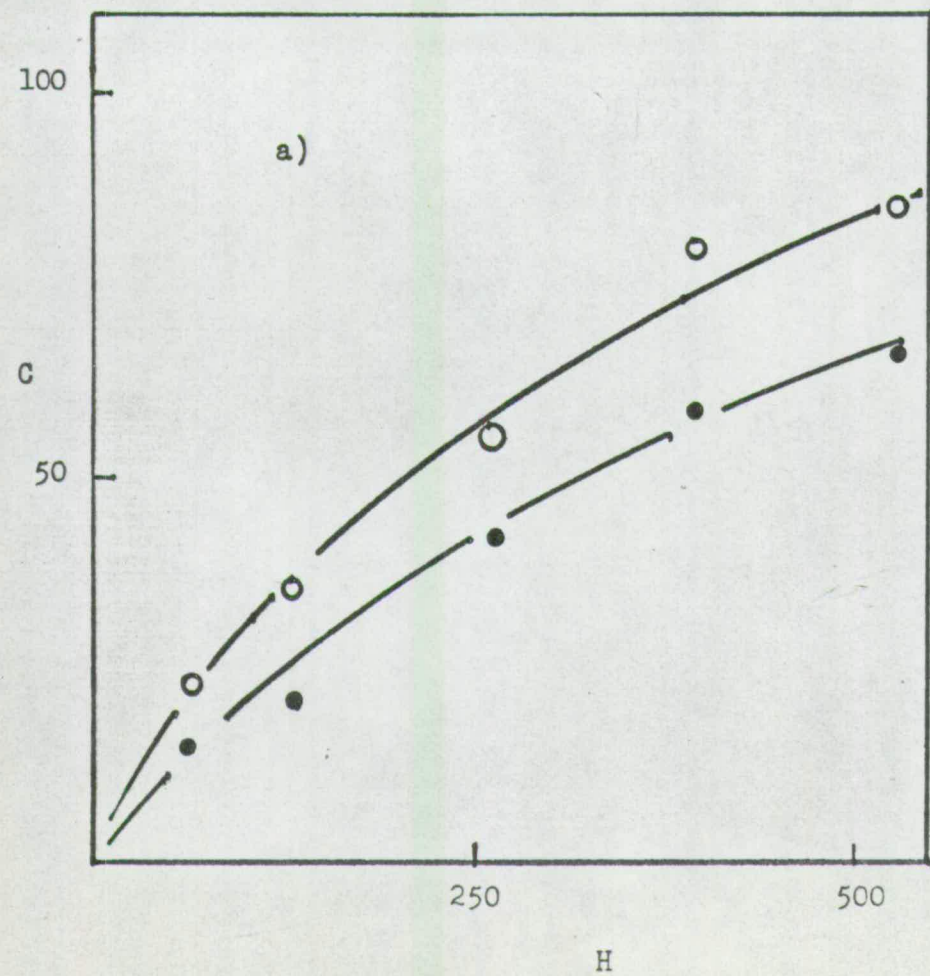


Figure 14.

Legend to Figure 15

- a) Saturation curves of RNA from 9-3h⁺/FR5 repressed with TMG, hybridised with DNA from λ dgs JG3 and JG1

Each membrane was loaded with 1.5 μ g DNA. Annealing etc as described in the legend to Figure 13a.

- b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was calculated, and the slope ($1/C_s$) and intercept (k') determined for each DNA.

○ ——— ○ JG3 DNA
● ——— ● JG1 DNA

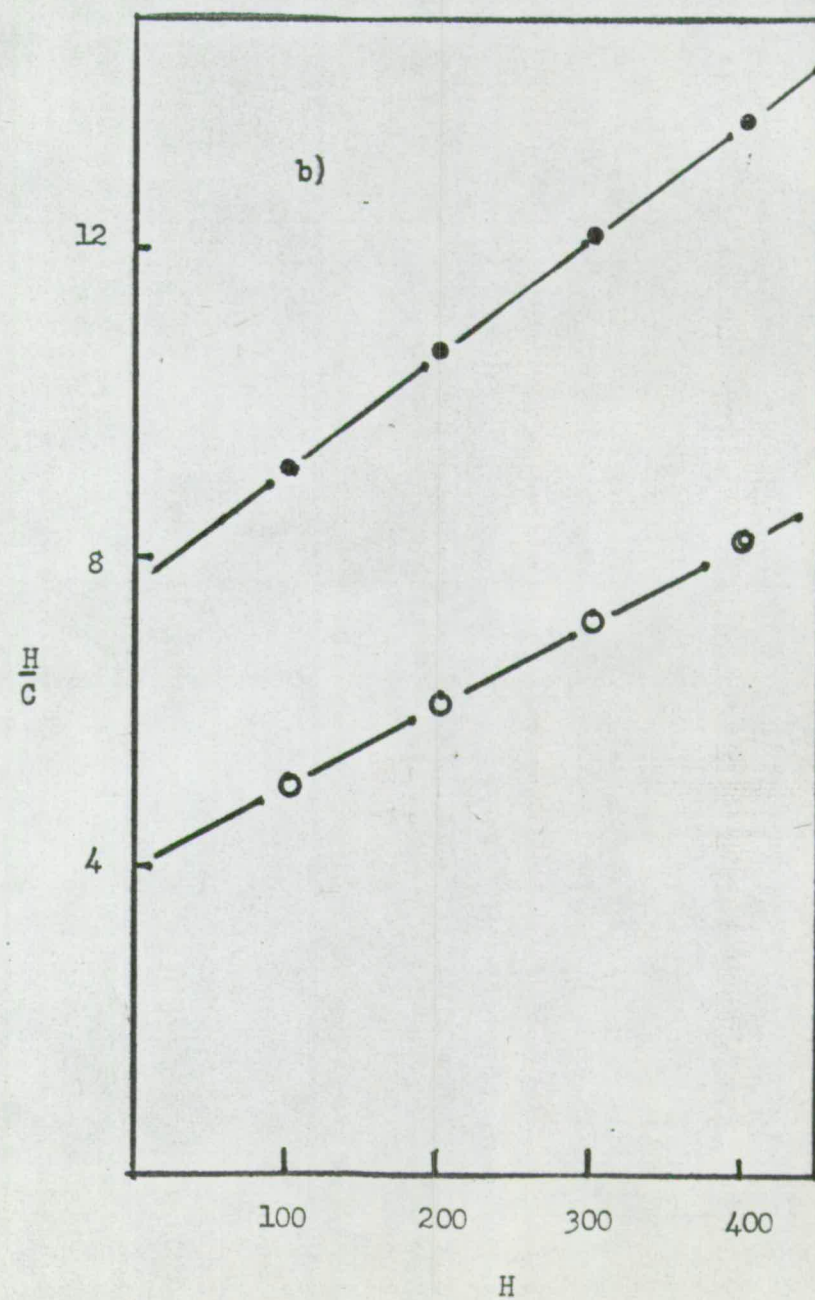
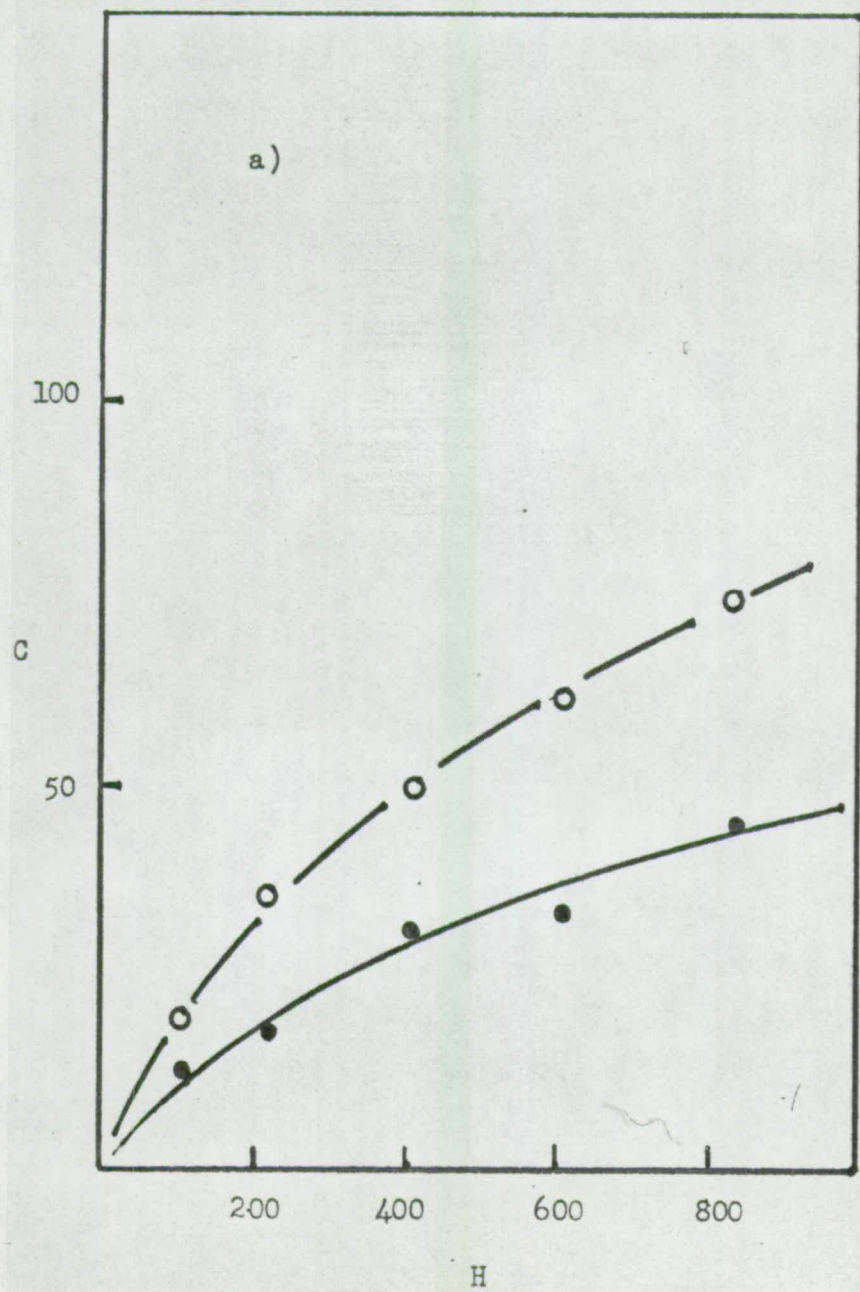


Figure 15.

summarised in Table 16 for convenience. They were derived from λ dg 17-8 and JG1 only. The saturation curves and calculated reciprocal plots are shown in Figures 10, 11, and 12. The saturation curves and calculated reciprocal plots of the experiments in series III, using JG3 and JG1 are shown in Figures 13, 14 and 15. The results are in Table 11.

In view of the possible errors in the absolute values of α and m it is not possible to make statements about the number of times each messenger is translated. However, the relationship between the values of m/E or $\frac{\alpha \times 10^5}{E}$ where E is the enzyme value normalised to that of E1 fucose induced, are definitely of significance. It will be noted from Tables 11 and 16 that there is most enzyme per unit messenger in the fucose induced culture, slightly less in the galactose induced, a greater drop in the non-induced culture (grown with glycerol as the only carbon source) and least of all in the culture repressed with TMG. I will discuss the significance of these and the following results later.

b) The effect of the genotype on the gal m-RNA and galactokinase contents. These experiments are described as Series II. The strains used were wild type (E1), fucose induced, a wild type male (E1/FR5) fucose induced, the male deletion strain, 9-3h⁺/FR5 fucose induced was repeated, and two non-induced constitutives were examined, an R⁻gal (TMG6) and an O^c gal (CF4). The phages used were 17-8 and JG1, and these results have also been published (Appendix B). The data are given in Appendix A, Table 2, and the saturation curves and calculated reciprocal plots are shown in Figures 16 to 20. The results are summarised in Table 17. Here it should

Legend to Figure 16

- a) Saturation curves of RNA from E1 induced with fucose,
hybridised with DNA from λ dgs 17-8 and JG1

Each membrane was loaded with 1.5 μ g of DNA. Annealing etc.
as described in the legend to Figure 8a.

- b) Calculated values of H/C for each value of H

Regression analyses were carried out on the experimental values
of H/C and H, and the slope ($1/C_s$) and intercept (k') determined
for each DNA.

□ — □ 17-8 DNA
● — ● JG1 DNA

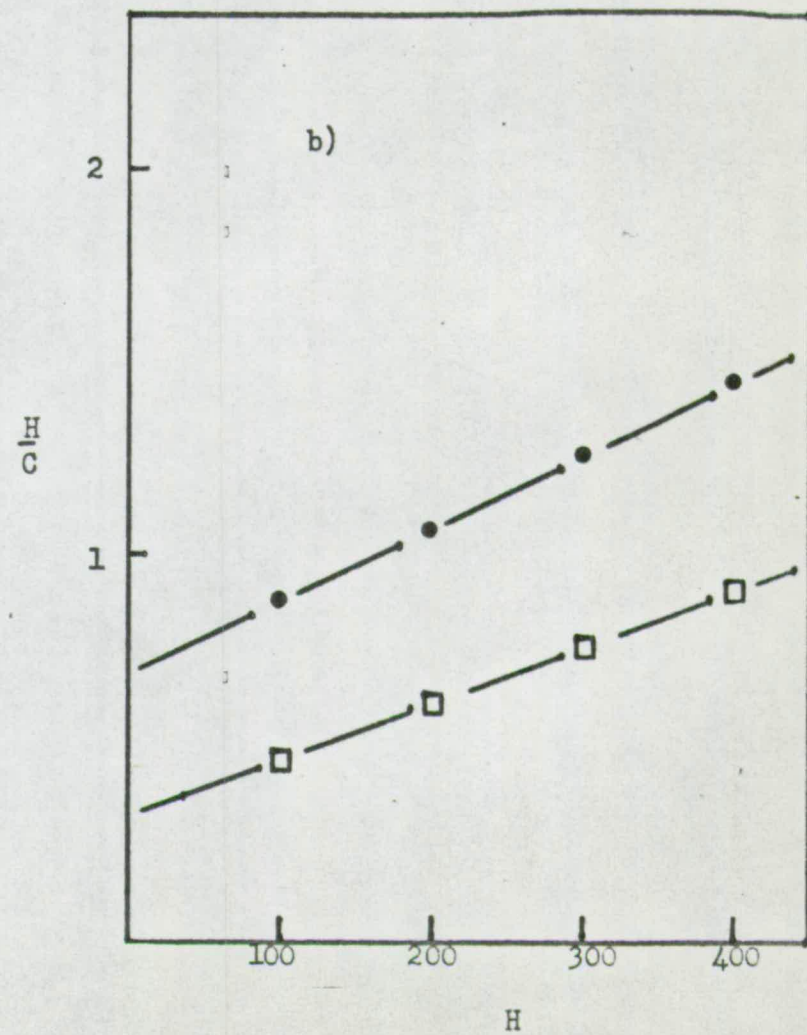
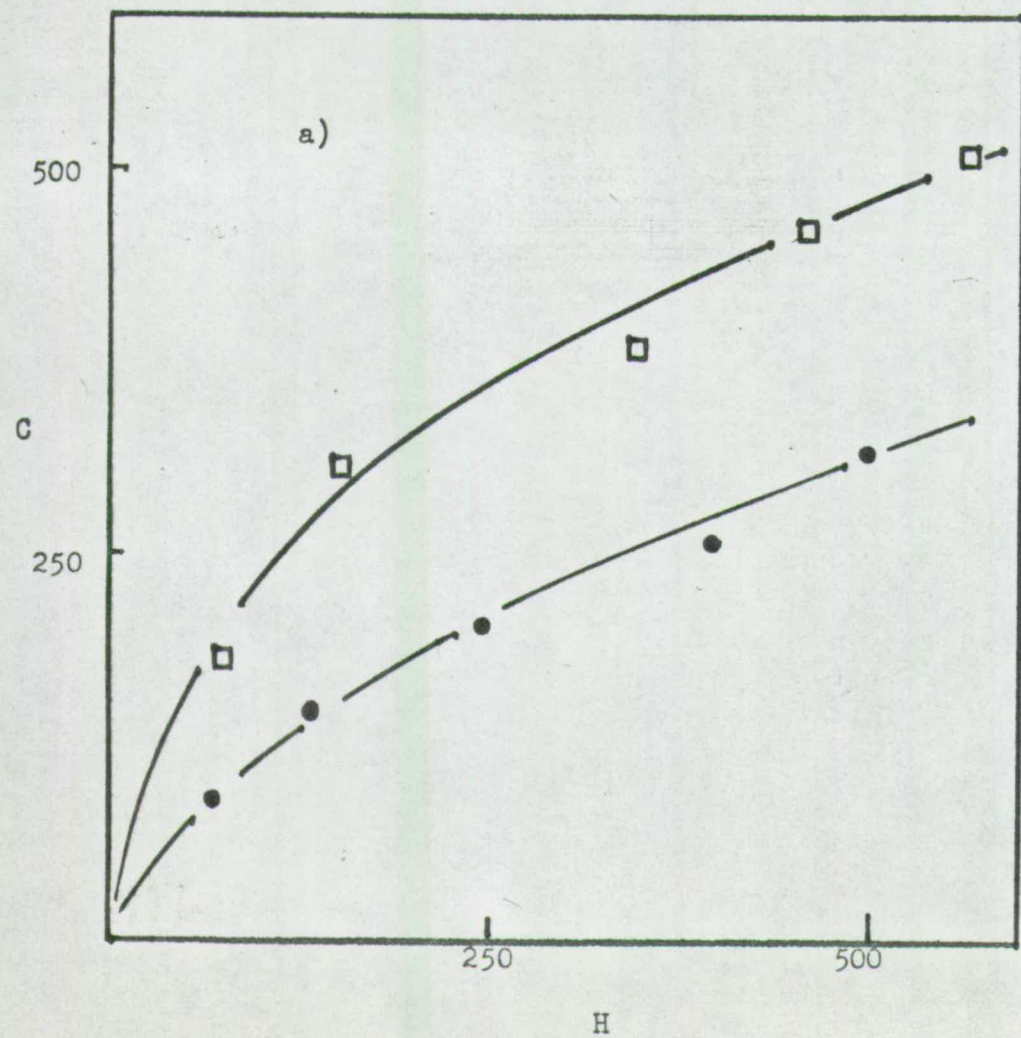


Figure 16.

Legend to Figure 17

a) Saturation curves of RNA from E1/FR5 induced with fucose,
hybridised with DNA from λ dgs 17-8 and JG1

Each membrane was loaded with 1.5 μ g of DNA. Annealing etc
as described in the legend to Figure 8a.

b) Calculated values of H/C for each value of H

Regression analyses were carried out on the experimental values
of H/C and H, and the slope ($1/C_s$) and intercept (k') determined
for each DNA.

□ ——— □ 17-8 DNA

● ——— ● JG1 DNA

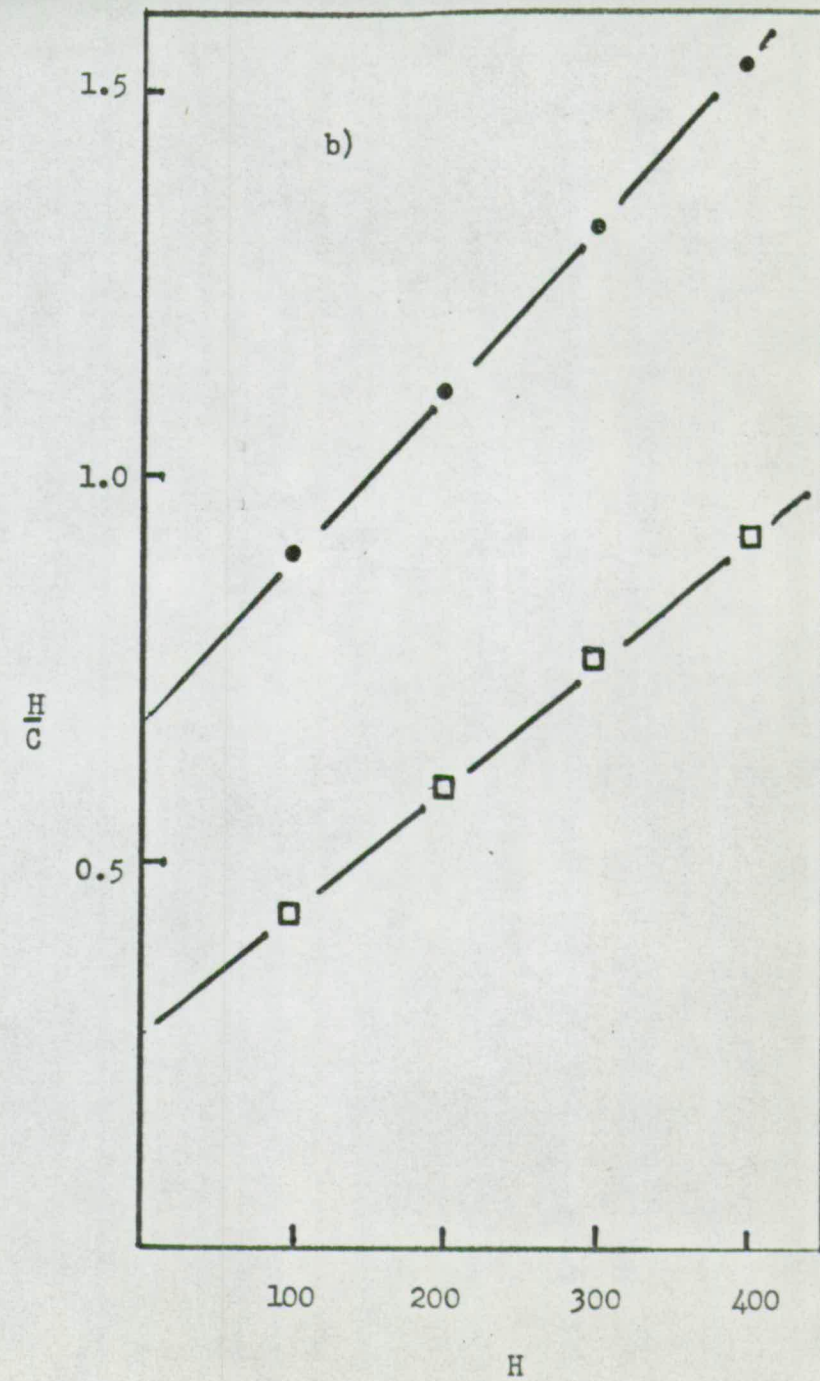
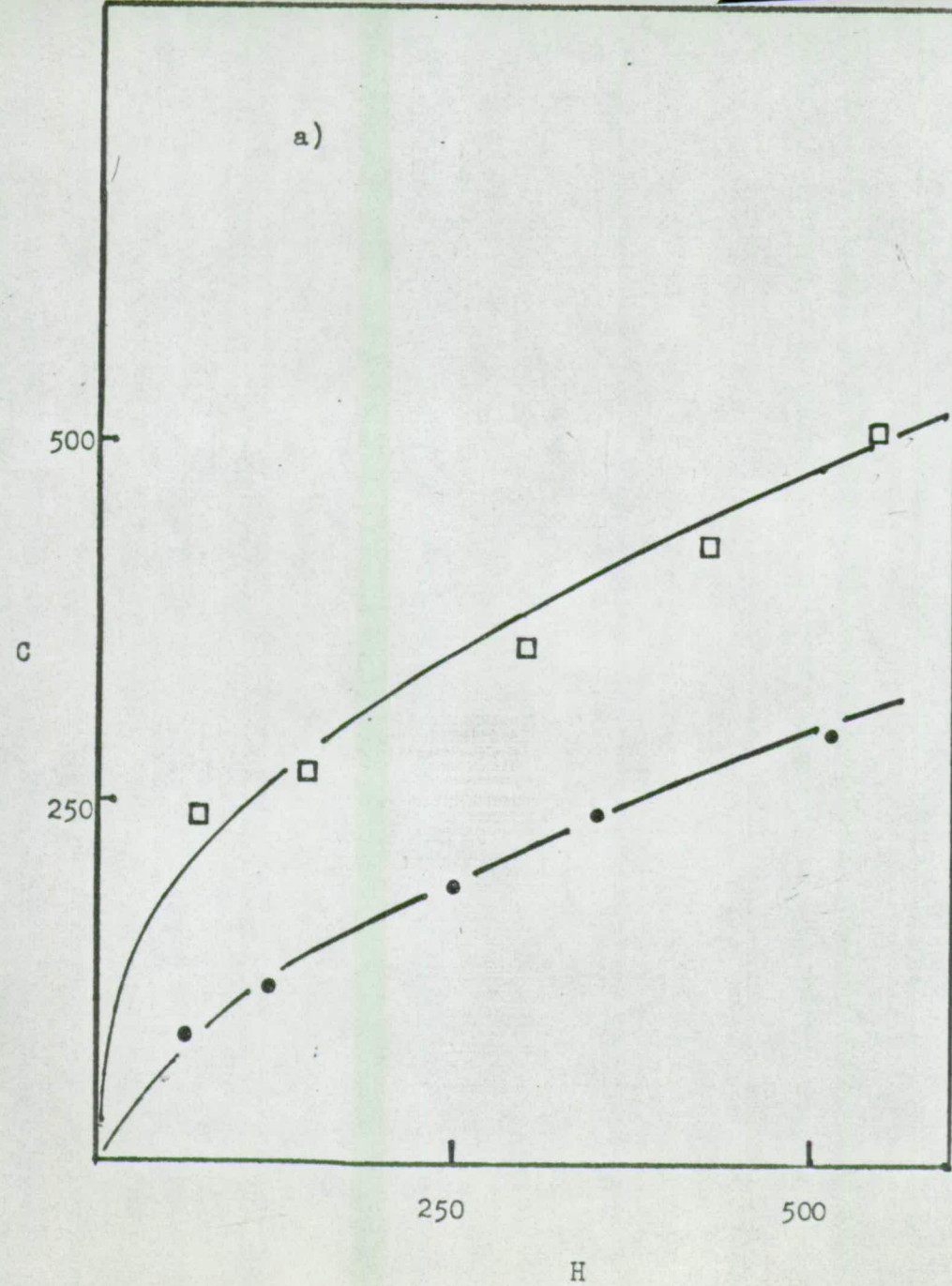


Figure 17.

Legend to Figure 18

- a) Saturation curves of RNA from 9-3h⁺/FR5 induced with fucose,
hybridised with λ dgs 17-8 and JG1

Each membrane was loaded with 1.5 μ g of DNA. Annealing etc.
as described in the legend to Figure 8a.

- b) Calculated values of H/C for each value of H

Regression analyses were carried out on the experimental
values of H/C and H, and the slope ($1/C_s$) and intercept (k')
determined for each DNA

□ ——— □ 17-8 DNA

● ——— ● JG1 DNA

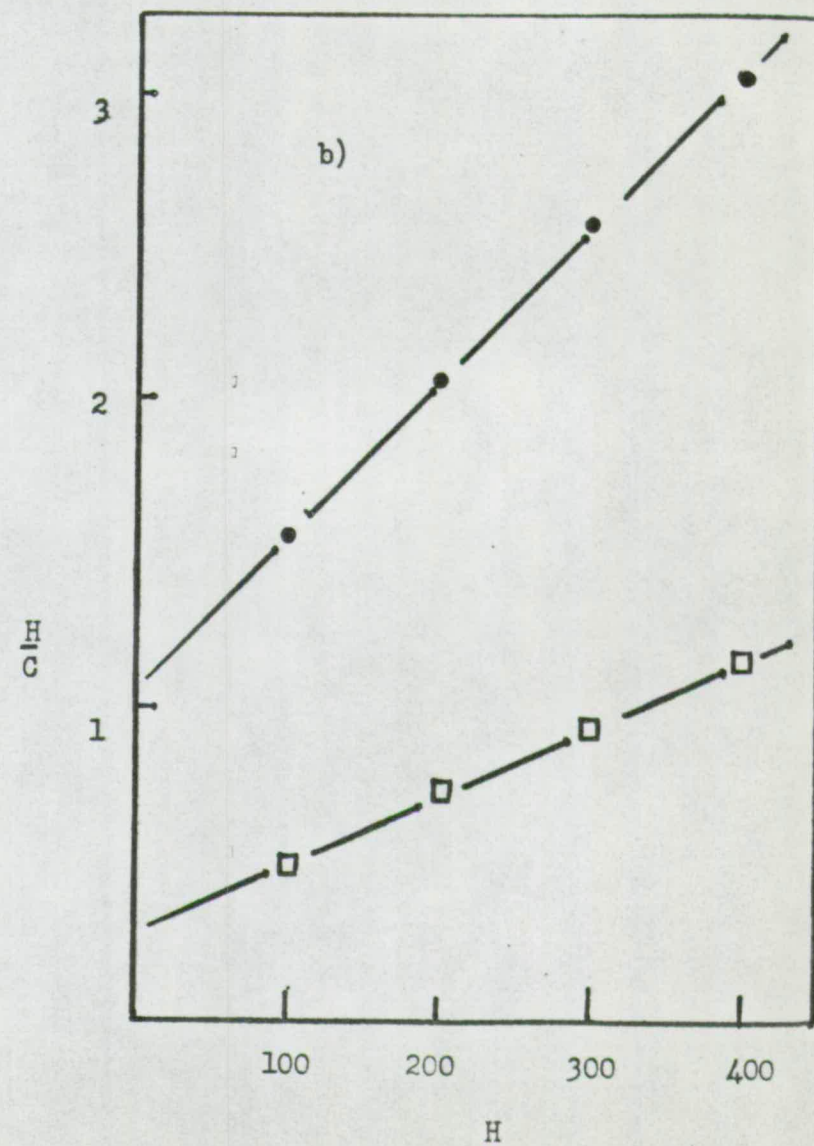
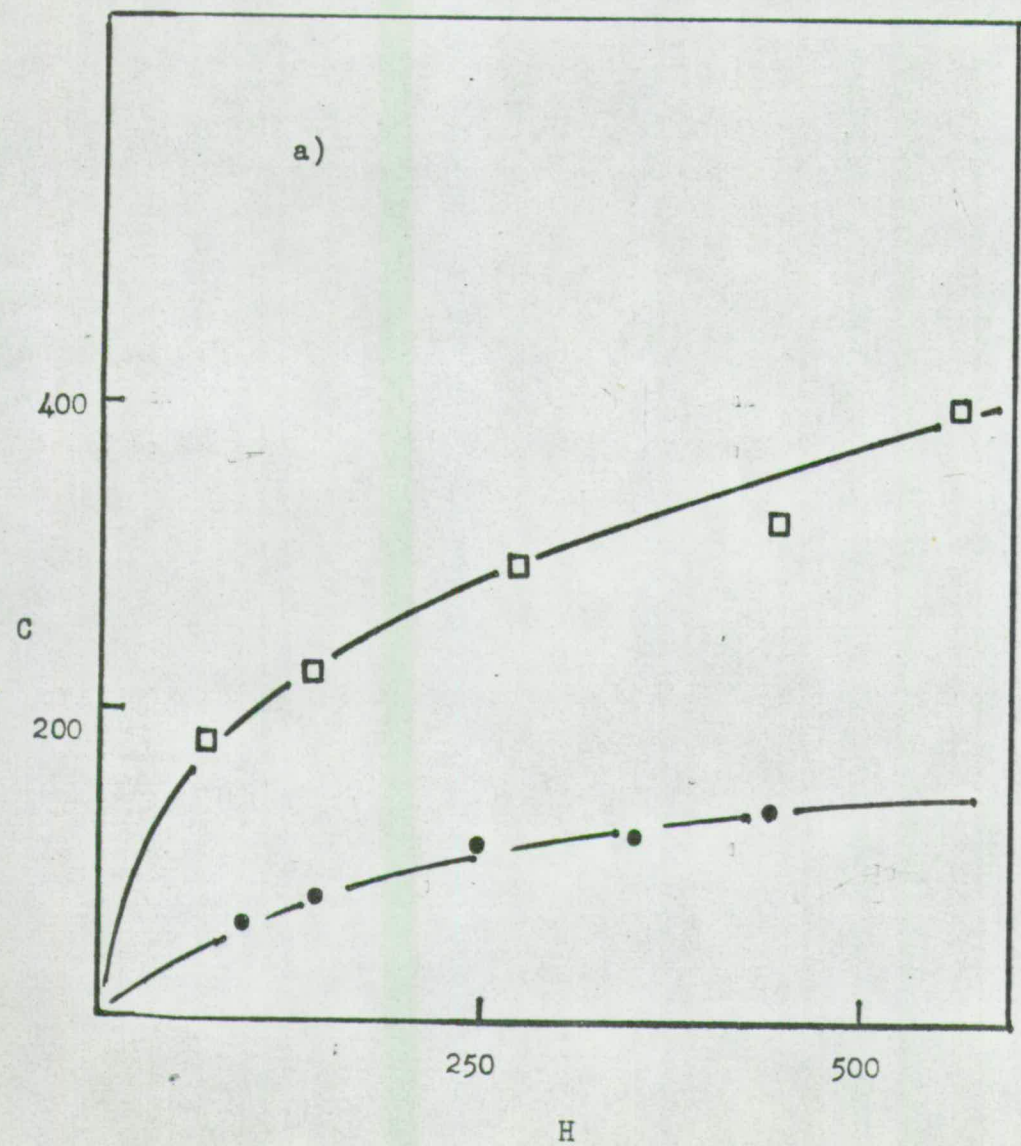


Figure 18.

Legend to Figure 19

a) Saturation curves for RNA from CF₄ non induced, hybridised with DNA from λ dgs 17-8 and JG1

Each membrane was loaded with 1.5 μ g of DNA. Annealing etc. as described in the legend to Figure 8a.

b) Calculated values of H/C for each value of H

Regression analyses were carried out on the experimental values of H/C and H, and the slope ($1/C_s$) and intercept (k') determined for each DNA.

□ ——— □ 17-8 DNA
● ——— ● JG1 DNA

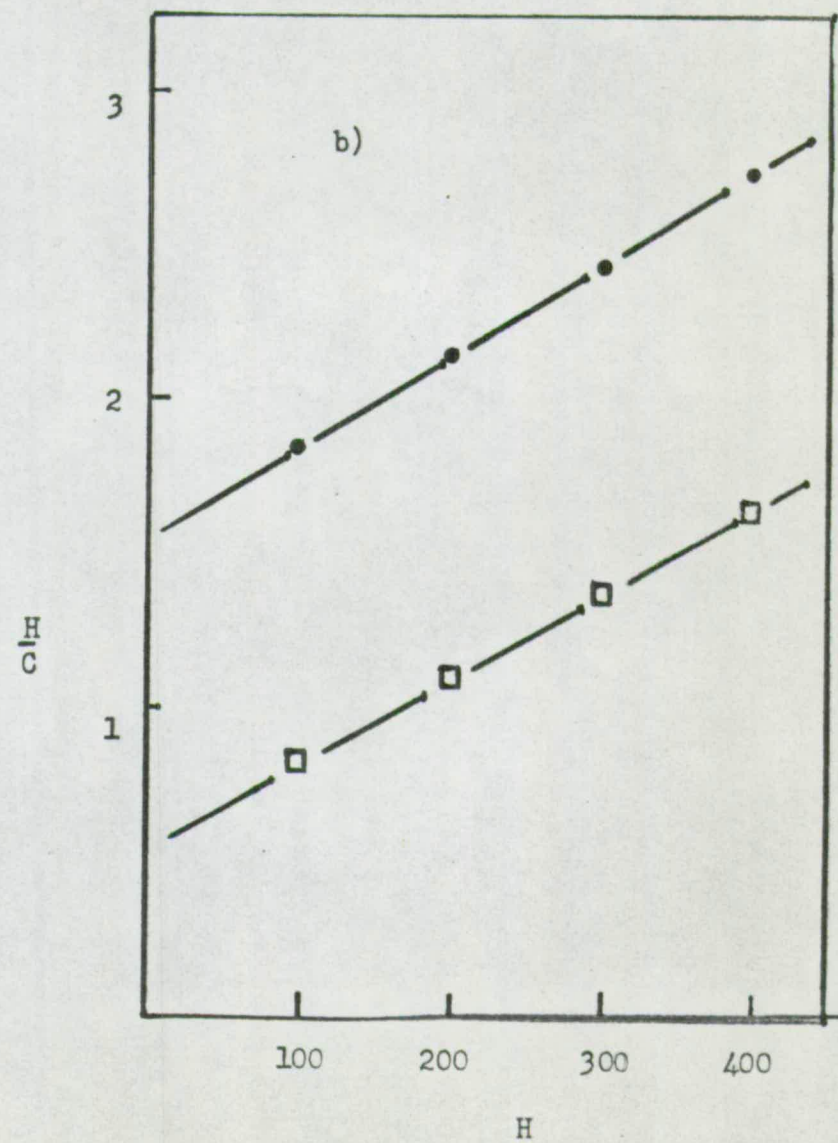
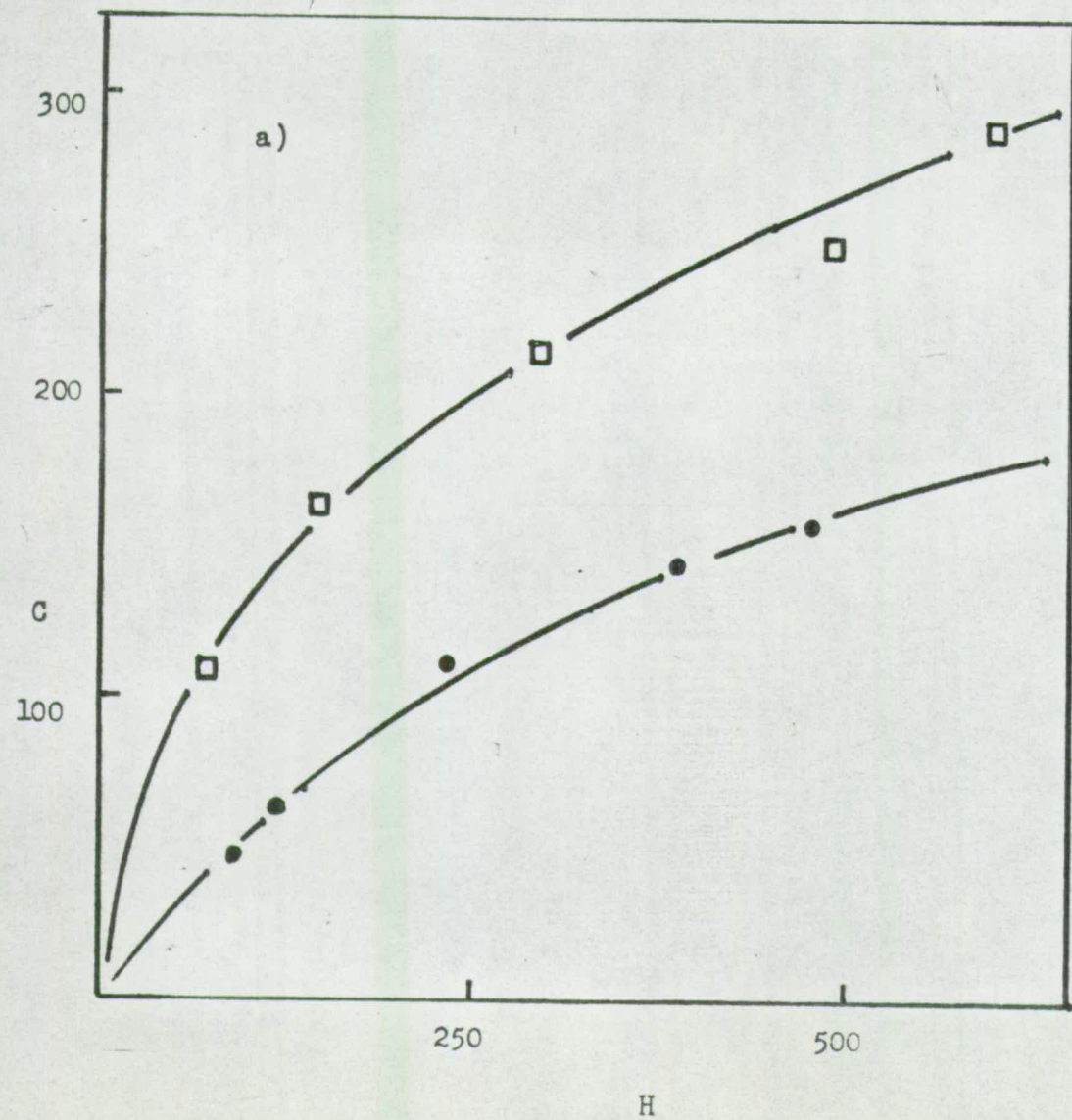


Figure 19.

Legend to Figure 20

a) Saturation curves for RNA from TMG6 non induced, hybridised with DNA from λ dgs 17-8 and JG1

Each membrane was loaded with 1.5 μ g of DNA. Annealing etc. as described in the legend to Figure 8a.

b) Calculated values of H/C for each value of H

Regression analyses were carried out on the experimental values of H/C and H, and the slope ($1/C_s$) and intercept (k') determined for each DNA.

□ ——— □ 17-8 DNA

● ——— ● JG1 DNA

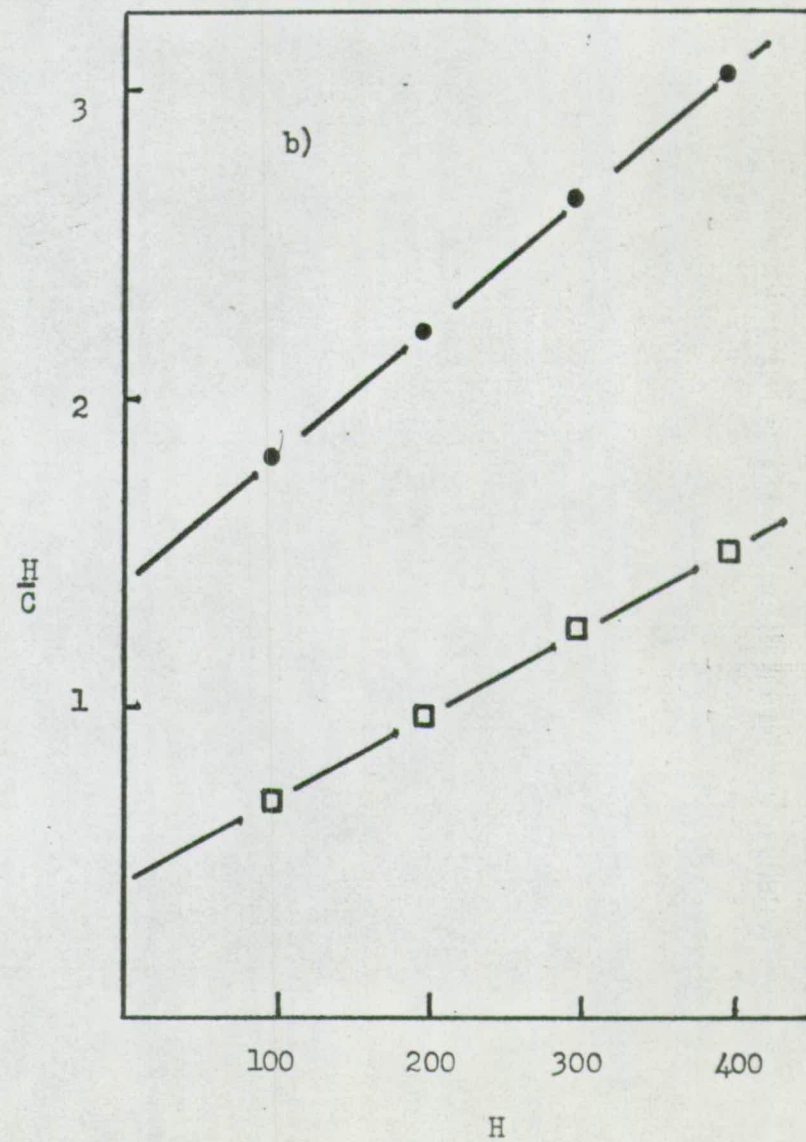
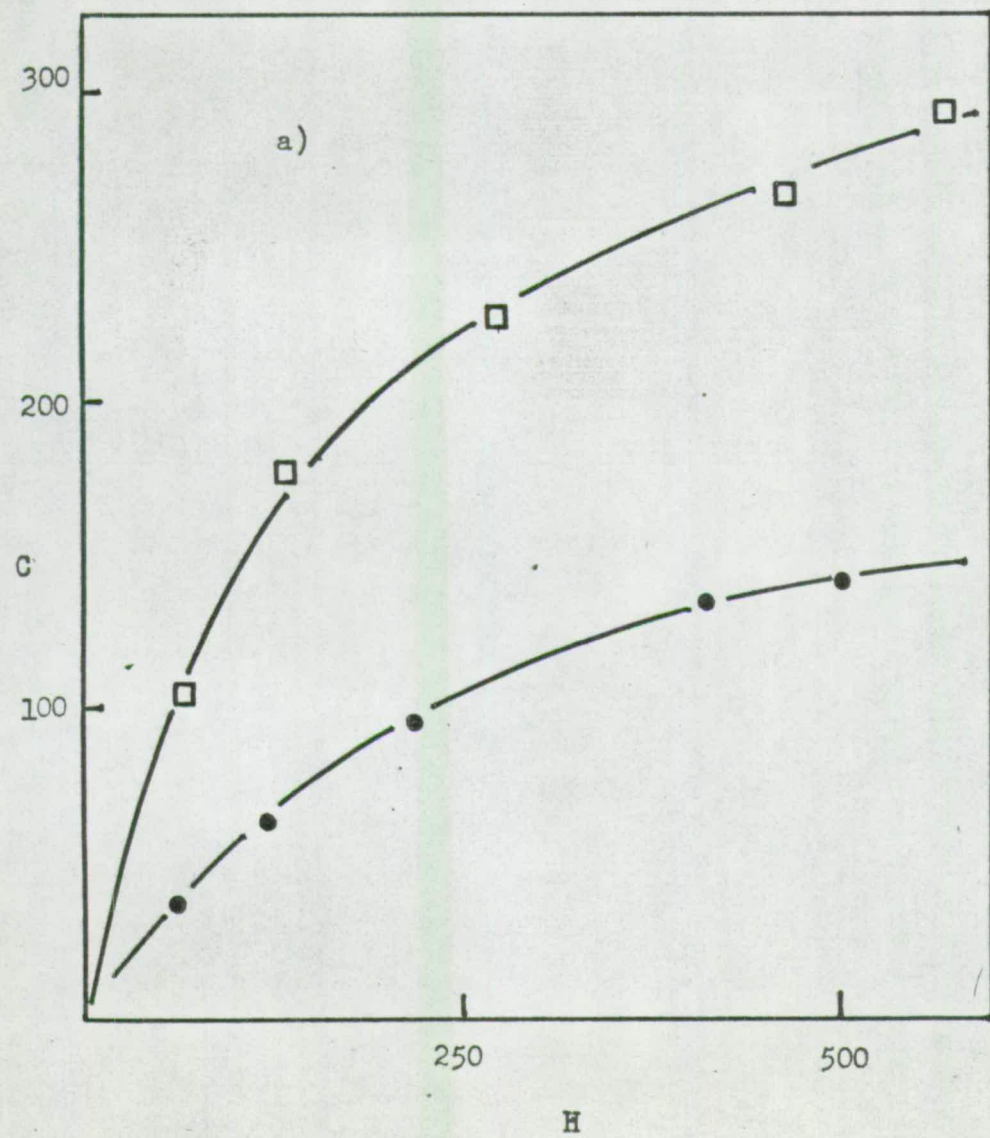


Figure 20.

Table 17

Calculated results of hybridisation experiments Series II

<u>RNA Source</u>	<u>$\alpha \times 10^5$</u>	<u>s</u>	<u>m</u>	<u>S</u>	<u>E</u>	<u>$\frac{m}{E}$</u>	<u>$\frac{\alpha \times 10^5}{E}$</u>
<u>E1 Fucose Induced</u>	22.69	15,737	7.03	611	1.00	7.03	22.69
<u>E1/FR5 Fucose Induced</u>	48.88	15,737	15.15	629	2.33	6.52	21.02
<u>9-3h⁺/FR5 Fucose Induced</u>	17.45	22,474	5.41	672	1.05	5.13	16.56
<u>CF4 Non-induced</u>	37.02	9,316	11.48	338	1.70	6.74	21.73
<u>TTC6 Non-induced</u>	27.41	12,579	8.50	507	1.26	6.77	21.84

Legend

α = proportion of total RNA which is gal m-RNA

s = specific radioactivity of gal m-RNA

m = number of molecules of gal m-RNA per galactose gene copy

S = specific activity of total RNA

E = galactokinase activity normalised to that of E1 fucose induced

be noted that all the values for $\frac{\alpha \times 10^5}{E}$ are very close, with the exception of that for 9-3h⁺/FR5, which is lower than the remainder. It seems possible that the other strains, which contain more DNA homologous with the E.coli DNA of the λ dgs, also contain more non-gal complementary RNA, and it is this which is increasing the apparent values of α and m . The values for the non-induced constitutives seem the same as those of the induced wild type.

c) The effect of changes in gene dosage, and type of inducer on the gal m-RNA and galactokinase levels in wild type and regulator constitutive mutants

In this, and the subsequent series of experiments, one membrane per annealing tube was loaded with JG3 DNA, one with JG1 DNA and there was one blank. Duplicate tubes were made for each concentration of DNA. The data from the experiments are given in Appendix A, Table 4, and the saturation curves and calculated reciprocal plots in Figures 24 to 29. The results calculated from these data are shown in Table 18.

From the values for the ratio of messenger content to enzyme, it can be seen that fucose induction of either E1 or E1/FR5 yields 1.2 times as much enzyme per unit messenger as galactose induction. For the constitutive strains, again there is no difference between TMG6 and TMG6/FR5, but the ratio this time is 1.6:1. The efficiency of translation of the messenger is thus independent of gene dosage and dependent on inducer and constitutivity versus wild type.

In the male strains the number of gene copies is 2.4 times that in the female (F⁻), assuming that, with a generation time of 50 minutes there is on average one replicating fork per chromosome,

Legend to Figure 21

a) Saturation curves for RNA from E1 induced with fucose,
hybridised with DNA from λ dgs JG3 and JG4

Each membrane was loaded with 1.5 μ g DNA. Annealing etc.
as described in the legend to Figure 13a.

b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was
calculated, and the slope ($1/C_g$) and intercept (k') determined
for each DNA.

○ ——— ○ JG3 DNA
● ——— ● JG4 DNA

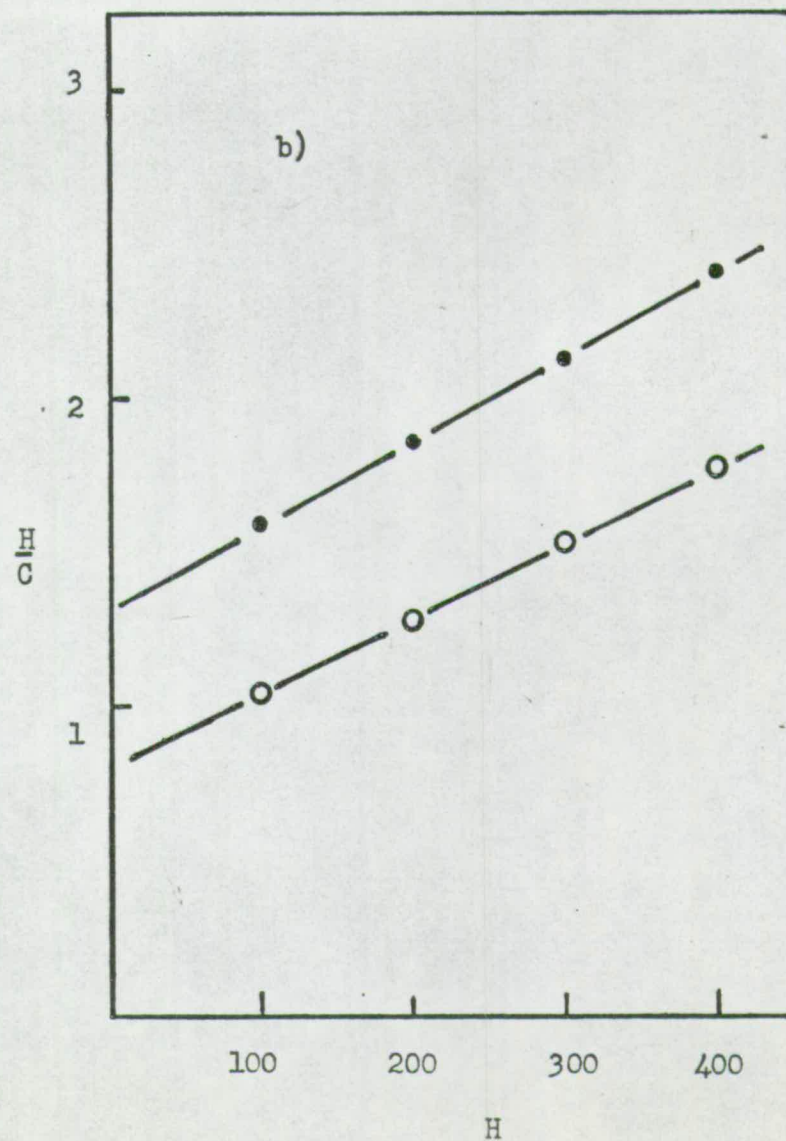
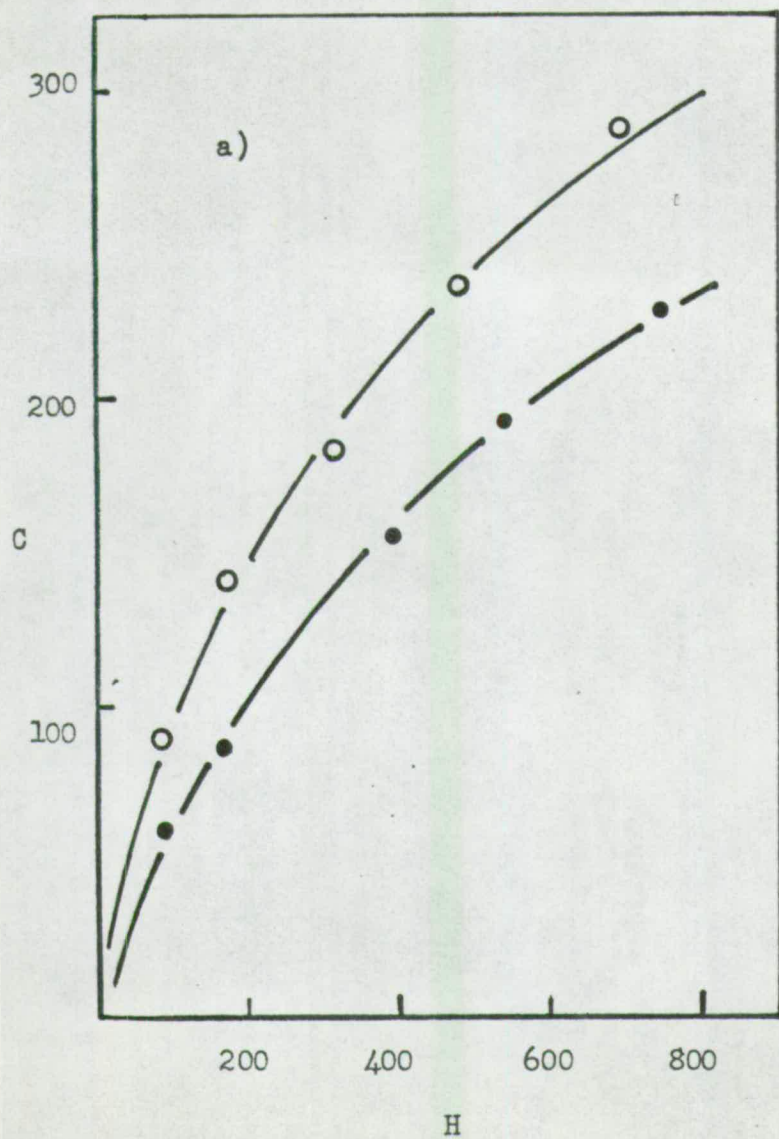


Figure 21.

Legend to Figure 22

- a) Saturation curves for RNA from E1 induced with galactose,
hybridised with DNA from λ dgs JG3 and JG1

Each membrane was loaded with 1.5 μ g DNA. Annealing etc.
as described in the legend to Figure 13a.

- b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was
calculated, and the slope ($1/C_s$) and intercept (k') determined for
each DNA.

○ ——— ○ JG3 DNA

● ——— ● JG1 DNA

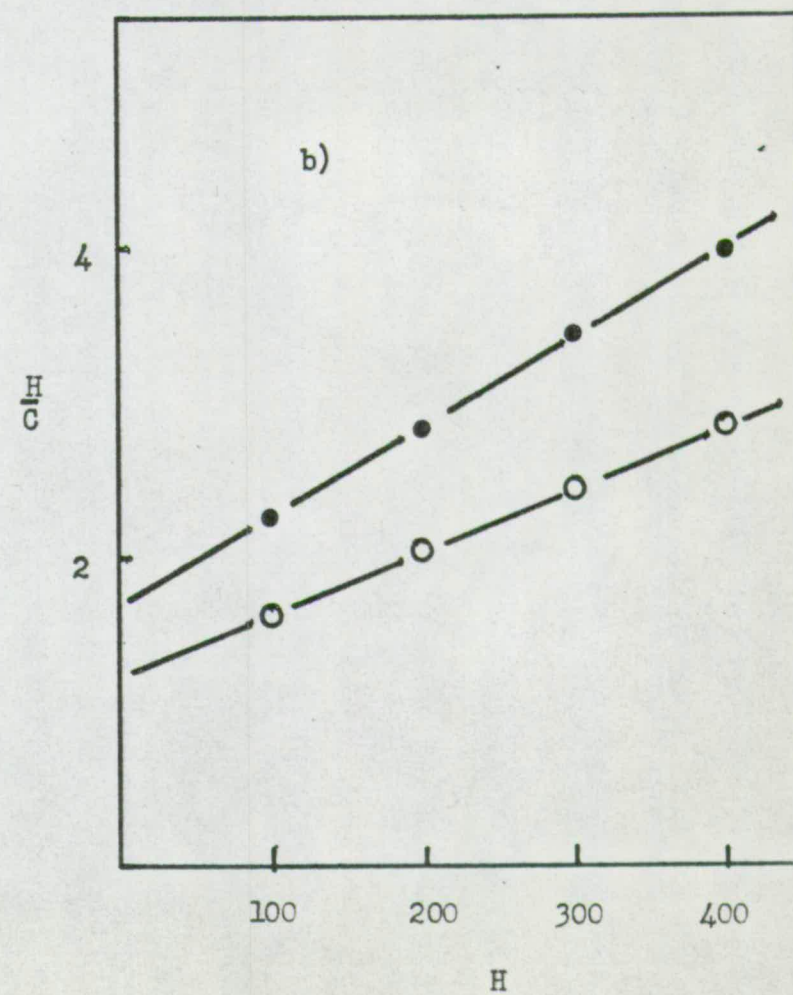
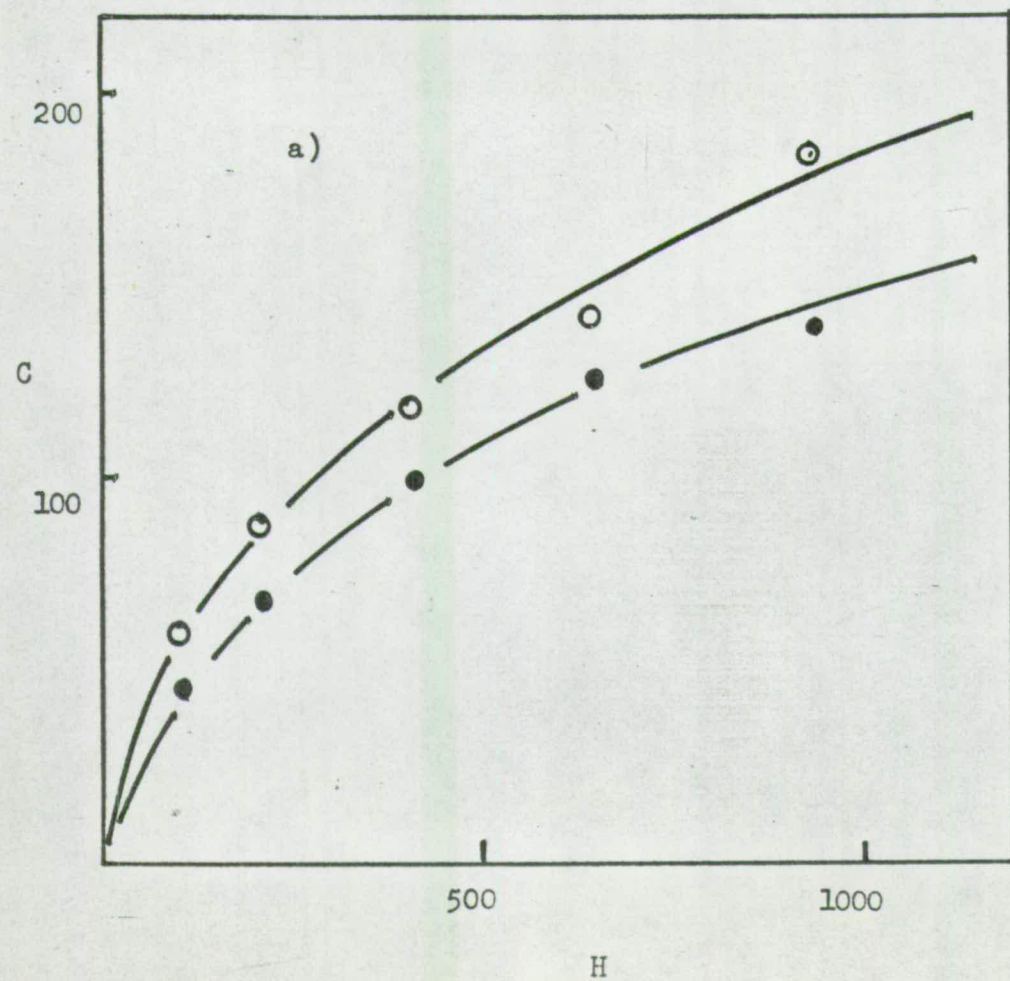


Figure 22.

Legend to Figure 23

- a) Saturation curve for RNA from E1/FR5 induced with fucose,
hybridised to DNA from λ ids JG3 and JG1

Each membrane was loaded with $1.5\mu\text{g}$ DNA. Annealing etc. as described in the legend to Figure 13a.

- b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was calculated, and the slope ($1/C_g$) and intercept (k') determined for each DNA.

○ ——— ○ JG3 DNA
● ——— ● JG1 DNA

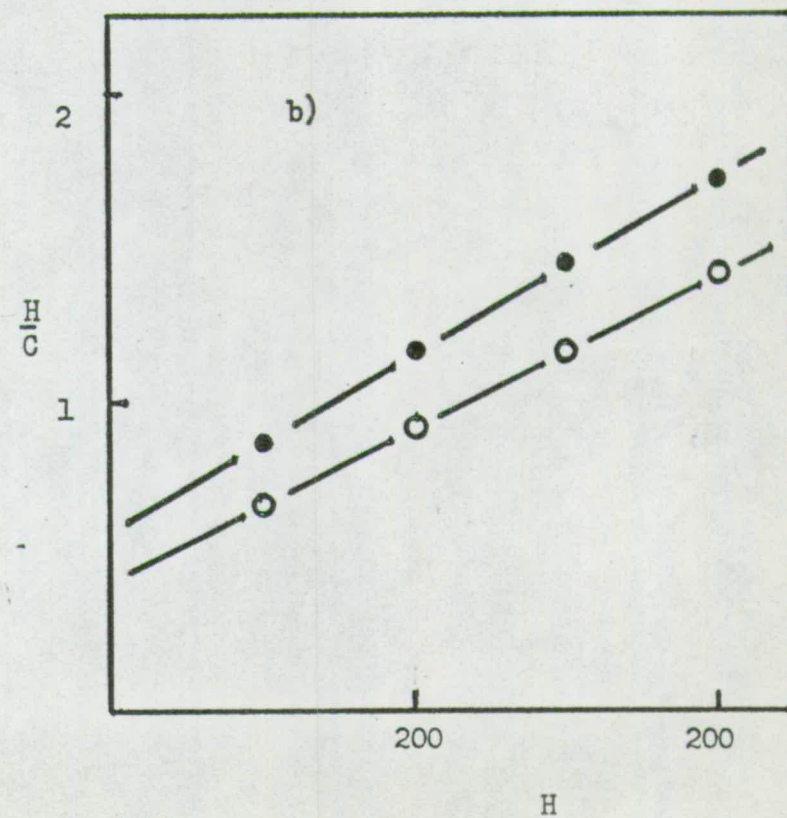
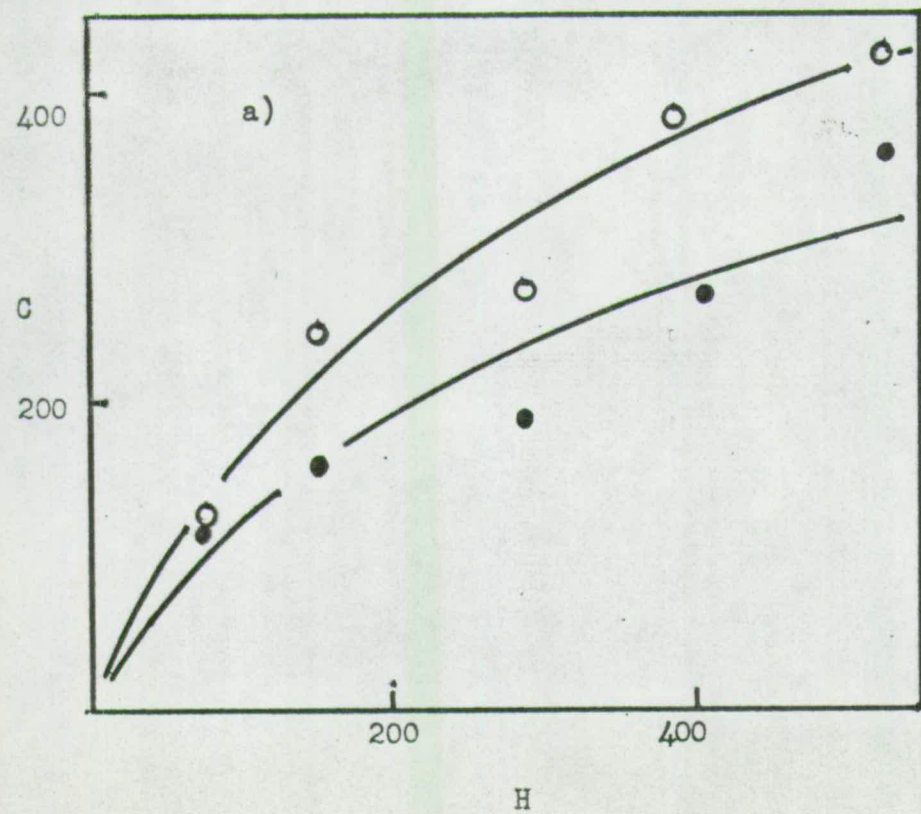


Figure 23.

Legend to Figure 24

a) Saturation curves of RNA from E1/FR5 induced with galactose,
hybridised with DNA from λ des JG3 and JG4

Each membrane was loaded with 1.5 μ g DNA. Annealing etc. as described in the legend to Figure 13a.

b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was calculated, and the slope ($1/C_g$) and intercept (k') determined for each DNA.

○ ——— ○ JG3 DNA

● ——— ● JG4 DNA

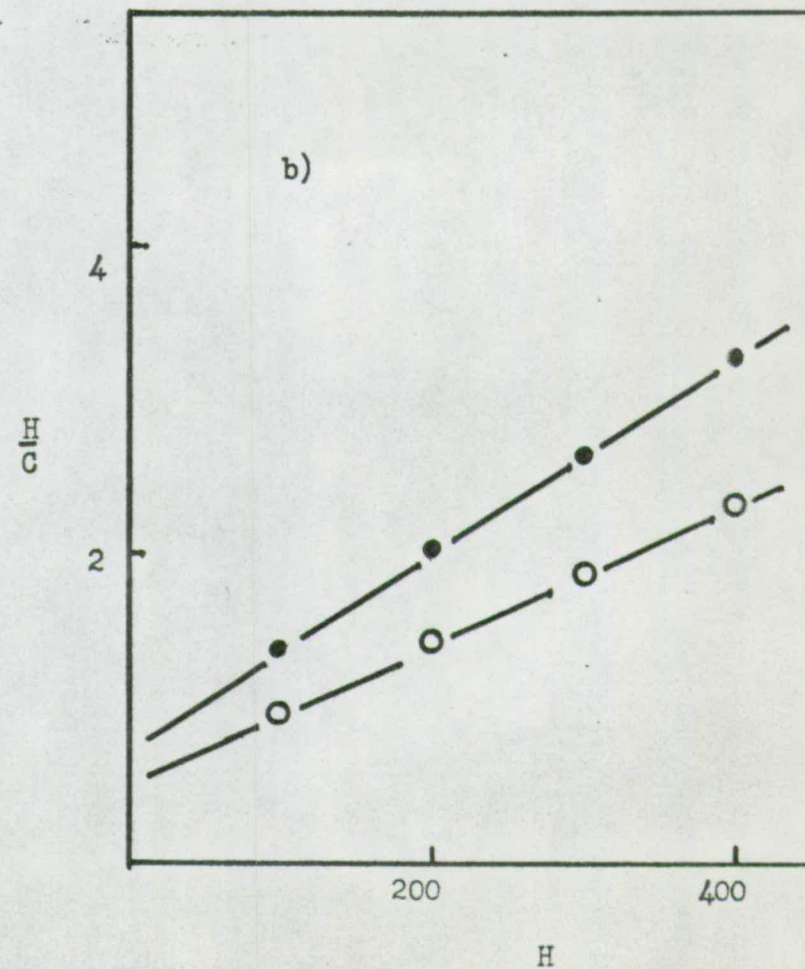
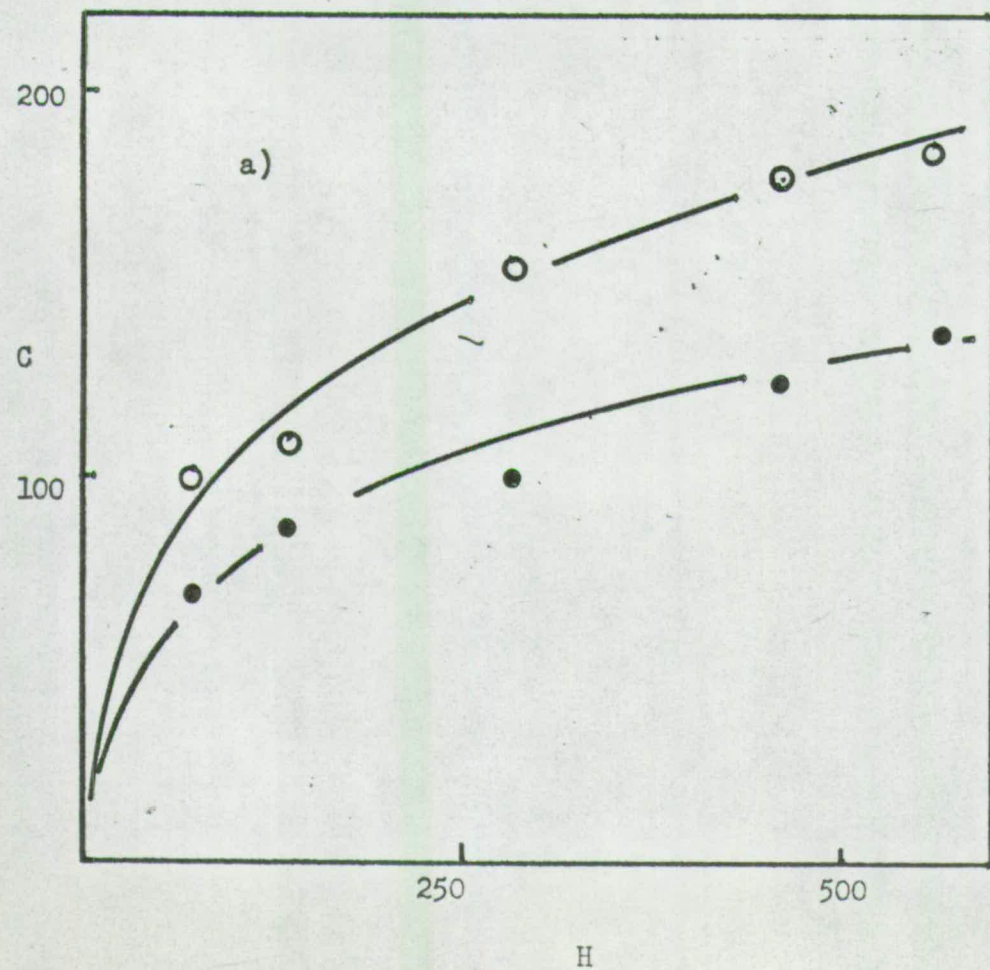


Figure 24.

Legend to Figure 25

a) Saturation curves for RNA from TMG6 non induced, hybridised with DNA from λ dgs JG3 and JG1

Each membrane was loaded with 1.5 μ g DNA. Annealing etc. as described in the legend to Figure 13a.

b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was calculated, and the slope ($1/C_s$) and intercept (k') determined for each DNA.

○ ——— ○ JG3 DNA

● ——— ● JG1 DNA

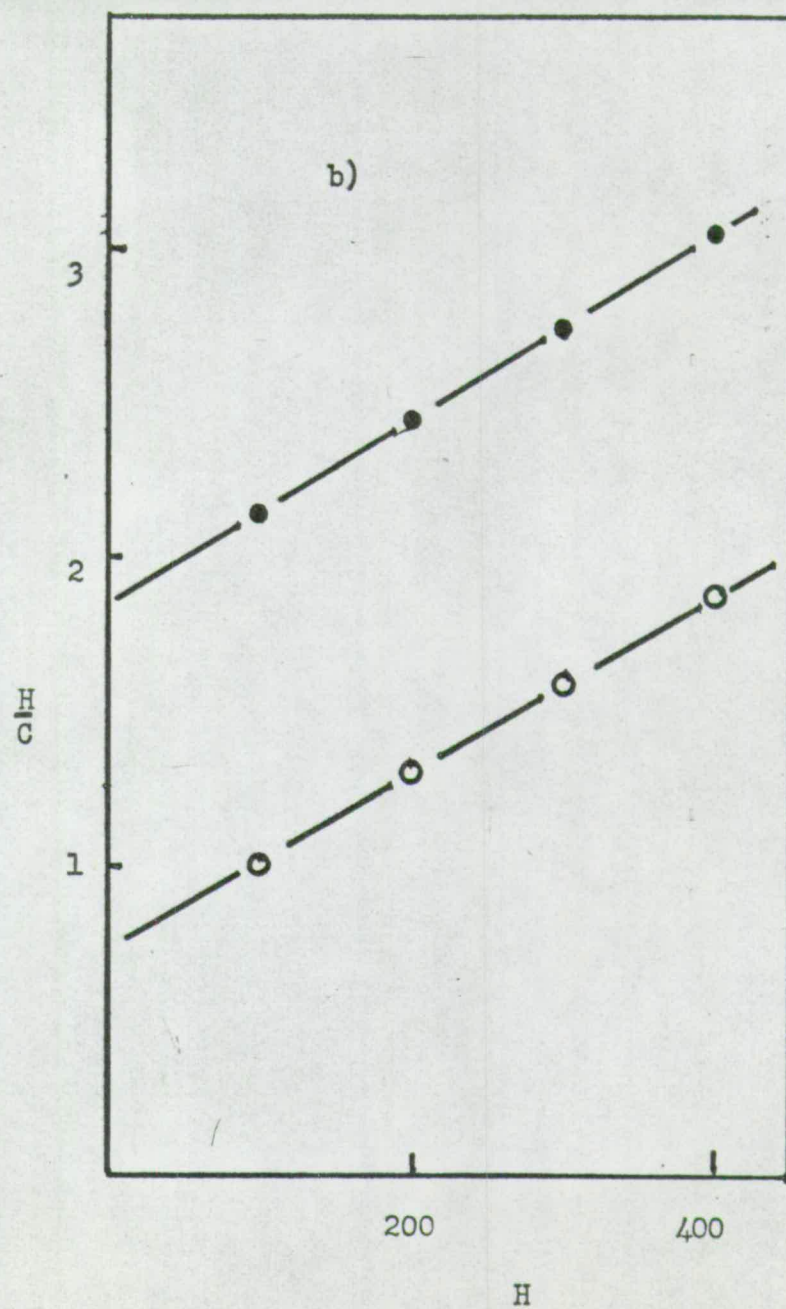
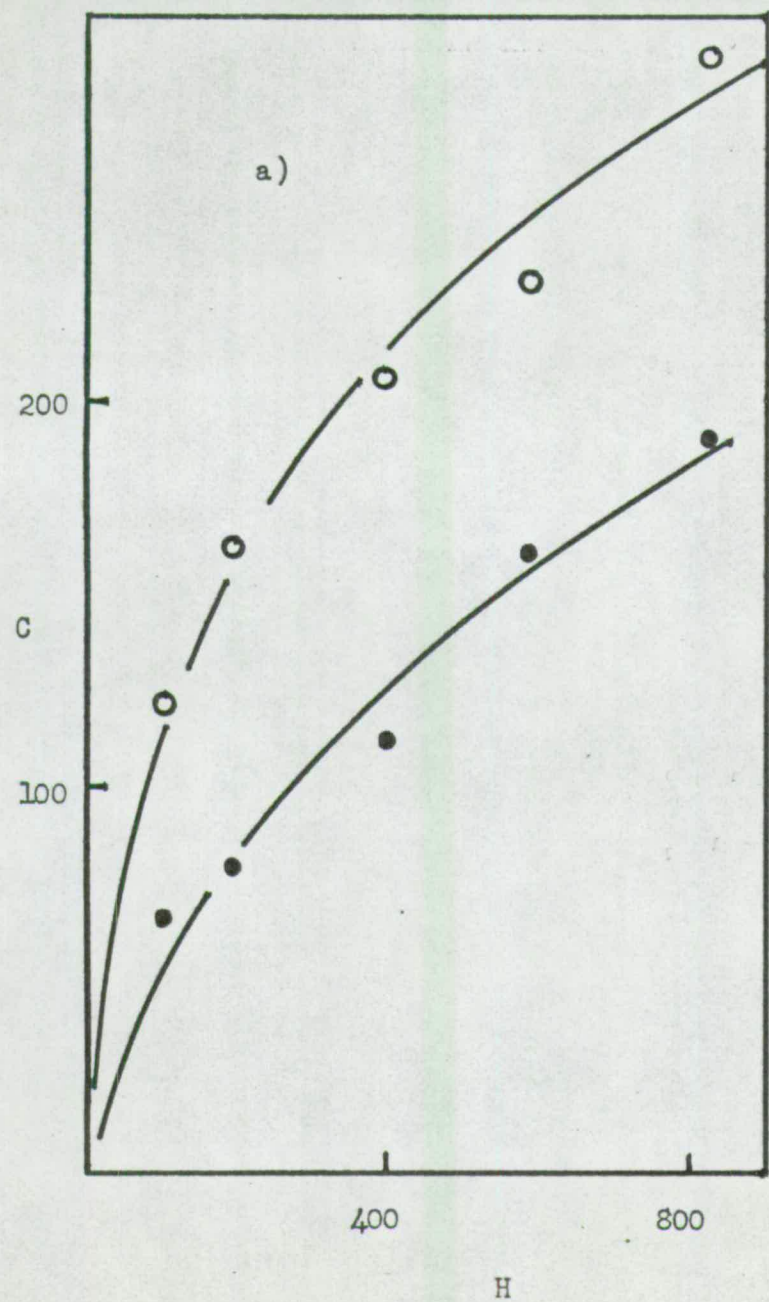


Figure 25.

Legend to Figure 26

- a) Saturation curves for RNA from TMC6 induced with fucose,
hybridised with DNA from λ den JC3 and JC4

Each membrane was loaded with 4.5 μ g DNA. Annealing etc.
as described in the legend to Figure 13a.

- b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H
was calculated, and the slope ($1/C_g$) and intercept (k') determined
for each DNA.

○ ——— ○ JC3 DNA

● ——— ● JC4 DNA

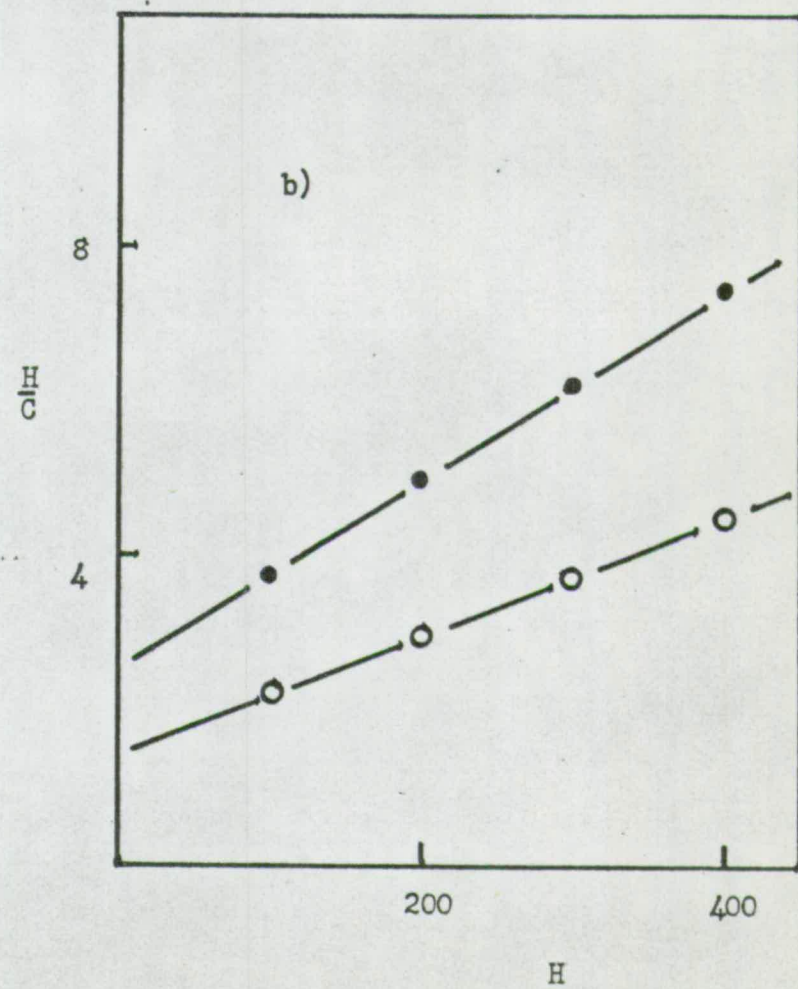
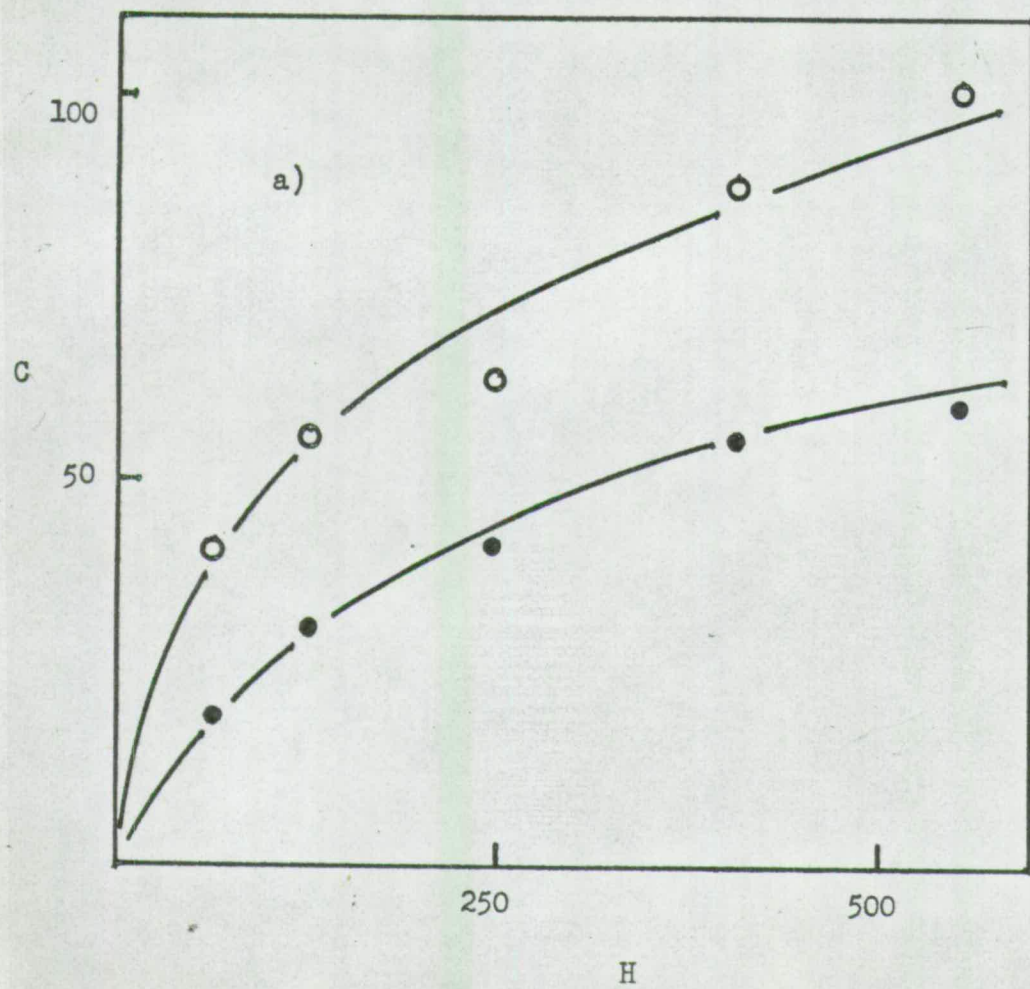


Figure 26.

Legend to Figure 27

a) Saturation curves for RNA from TMG6 induced with galactose,
hybridised with DNA from λ dgs JG3 and JG1

Each membrane was loaded with 1.5 μ g DNA. Annealing etc.
as described in the legend to Figure 13a.

b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H
was calculated, and the slope ($1/C_s$) and intercept (k') determined
for each DNA.

○ ——— ○ JG3 DNA

● ——— ● JG1 DNA

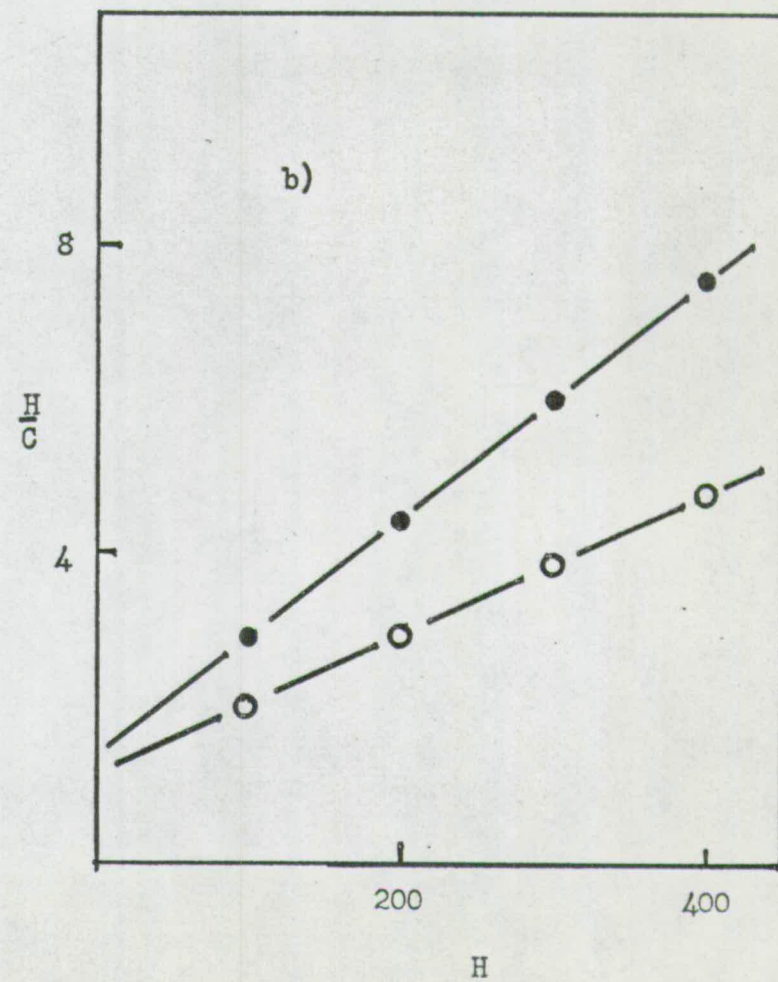
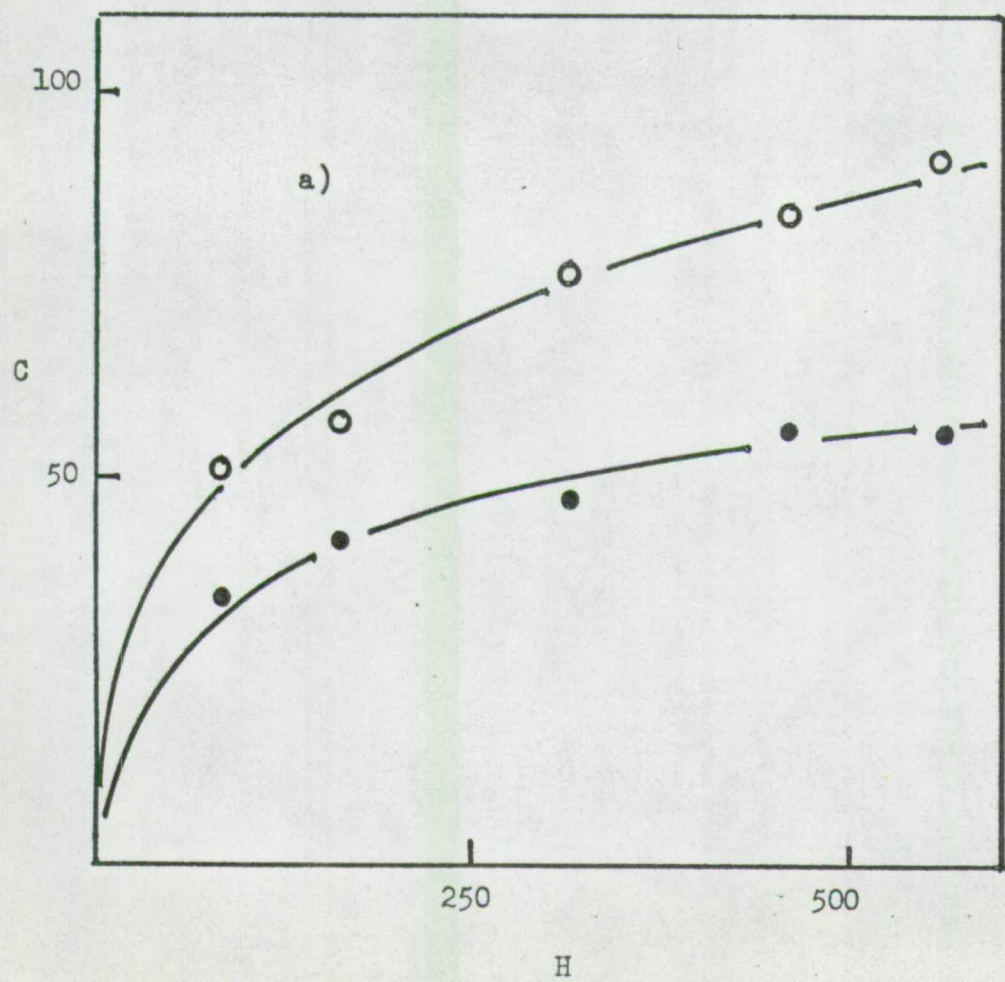


Figure 27.

Legend to Figure 28

a) Saturation curves for RNA from TMG6/FR5 induced with fucose, hybridised with DNA from λ dgs JG3 and JG4

Each membrane was loaded with 1.5 μ g DNA. Annealing etc. as described in the legend to Figure 13a.

b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was calculated, and the slope ($1/C_s$) and intercept (k') determined for each DNA.

○ ——— ○ JG3 DNA

● ——— ● JG4 DNA

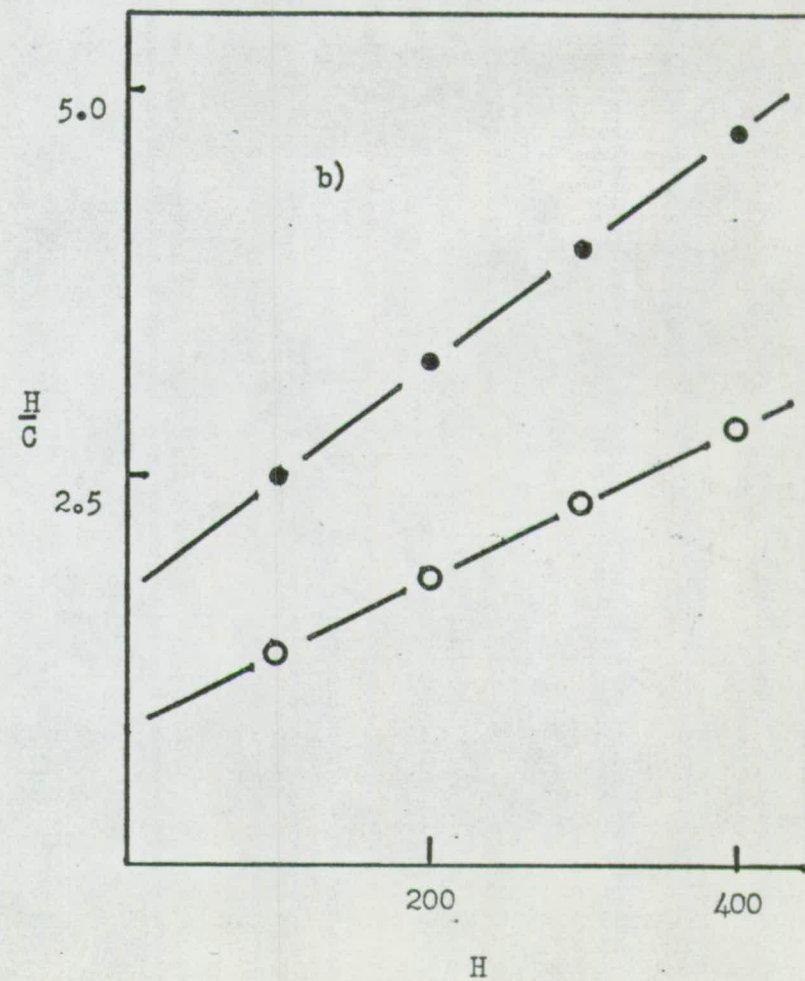
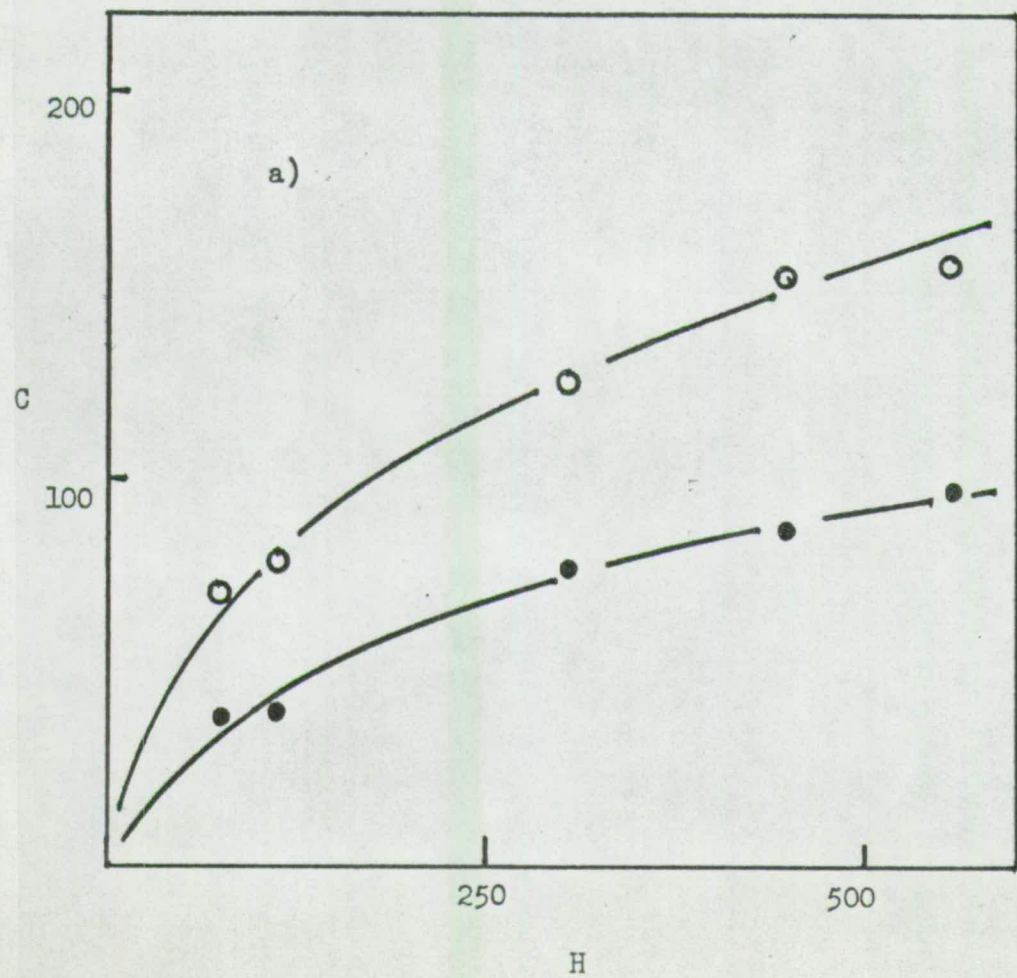


Figure 28.

Legend to Figure 29

a) Saturation curves for RNA from TMG6/FR5 induced with galactose, hybridised with DNA from λ dgs JG3 and JG4

Each membrane was loaded with 1.5 μ g DNA. Annealing etc. as described in the legend to Figure 13a.

b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was calculated, and the slope ($1/C_s$) and intercept (k') determined for each DNA.

○ ——— ○ JG3 DNA

● ——— ● JG4 DNA

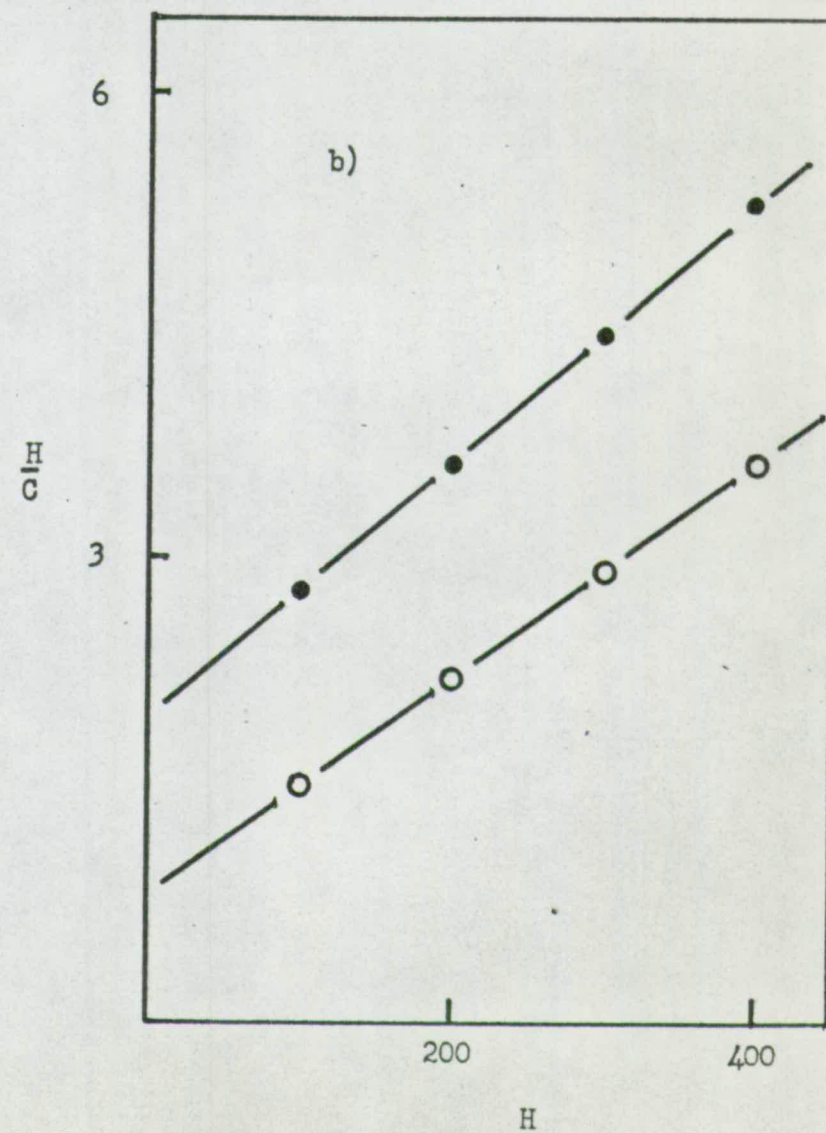
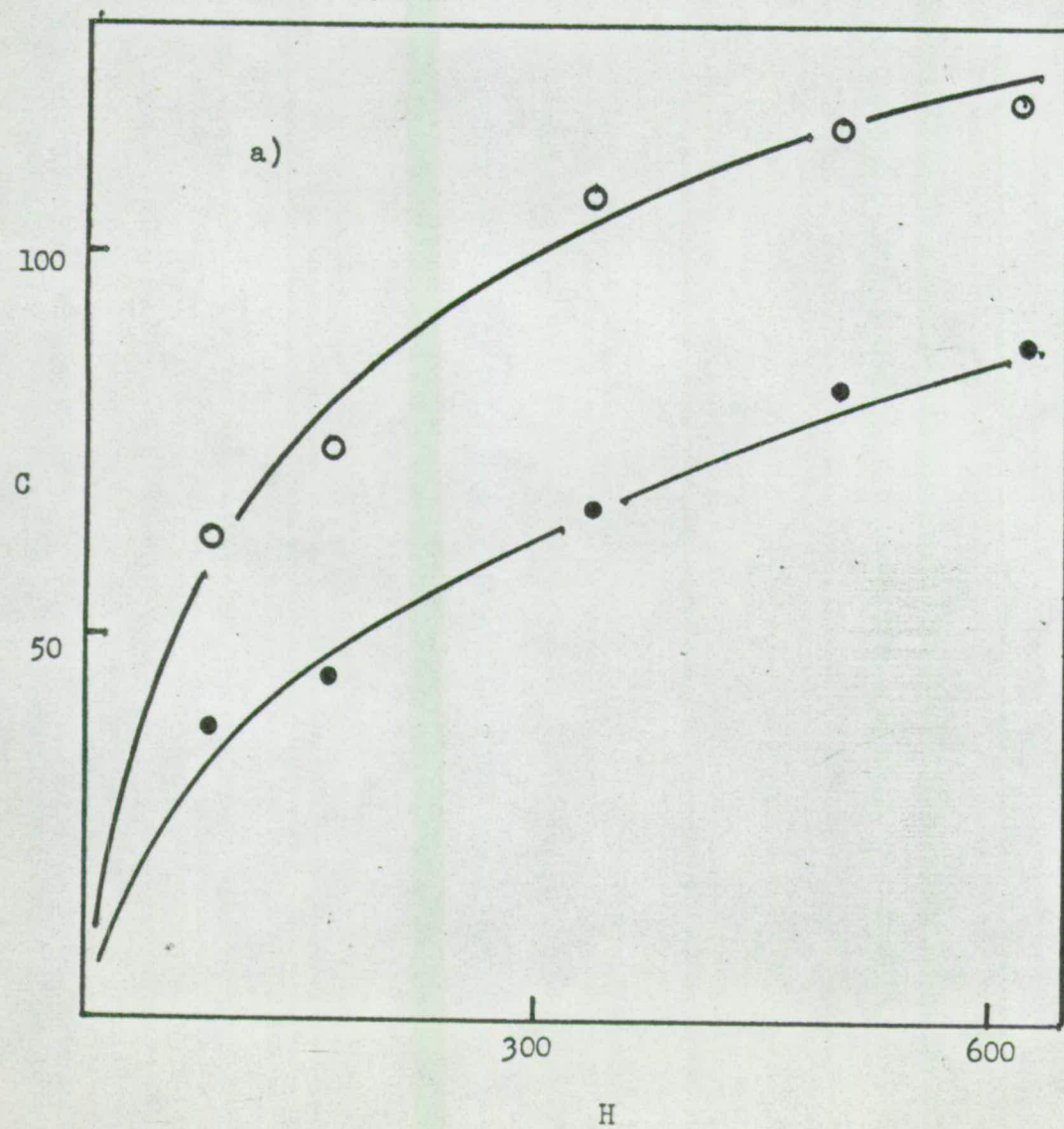


Figure 29.

Table 18

Calculated results of hybridisation experiments Series IV

<u>RNA Source</u>	<u>$\lambda \times 10^5$</u>	<u>s</u>	<u>m</u>	<u>S</u>	<u>E</u>	<u>$\frac{m}{E}$</u>	<u>$\frac{\lambda \times 10^5}{E}$</u>
<u>E1 Fucose Induced</u>	14.99	6,263	4.65	675	1.00	4.65	14.99
<u>E1 Galactose Induced</u>	12.05	5,842	3.74	403	0.66	5.71	18.40
<u>E1/FR5 Fucose Induced</u>	40.10	4,479	12.43	352	2.203	5.64	18.20
<u>E1/FR5 Galactose Induced</u>	15.08	5,158	4.67	538	0.708	6.59	21.30
<u>TMG6 Non-induced</u>	14.50	8,000	4.50	312	1.130	3.98	12.83
<u>TMG6 Fucose Induced</u>	11.80	3,784	3.66	386	2.480	1.48	4.76
<u>TMG6 Galactose Induced</u>	8.53	3,653	2.64	454	1.146	2.30	7.44
<u>TMG6/FR5 Fucose Induced</u>	16.40	5,895	5.08	581	3.055	1.66	5.37
<u>TMG6/FR5 Galactose Induced</u>	20.10	5,658	6.23	568	2.281	2.73	8.81

and two episomes per fork (Frame and Bishop, 1971).

Using this estimate I have prepared Table 19 from the data in Table 18. Table 19 relates the values of messenger RNA content per gene copy (m) and enzyme level as a proportion of that in E1 fucose induced (E) to the gene dosage. In the wild type with fucose induction the increase in both messenger and enzyme contents is closely proportional to the gene dosage. With galactose induction, however, the increase in messenger and enzyme levels is only half that with fucose induction, and there is slightly less enzyme per unit messenger (see above).

Examination of the results of the experiments using RNA from the constitutive mutants reveals a strikingly different picture. The non-induced constitutive TMG6 has m-RNA content and enzyme level not very different from those of fucose induced E1. Fucose induction, however, leads to a greater increase in the level of enzyme than in that of messenger, and the increase of both enzyme and m-RNA in TMG6/FR5 is little more than half the expected increase on the basis of gene dosage, when compared with the increase in TMG6 itself. With galactose induction, however, while the increases are smaller, they are more nearly proportional to the gene dosage.

d) The relationship between enzyme level and m-RNA content in polar mutants

The analysis of the m-RNA contents in polar mutants is complicated by the difficulty of distinguishing hybridisation of complete messenger molecules, capable of being translated, from that of fragments resulting from degradation or incomplete transcription.

The data from these experiments, involving the hybridisation of RNA from fucose induced homogenates of polar mutants to DNA from

Table 19

Variation of gal m-RNA with inducer and gene dosage

<u>Strain</u>	<u>Gene copies</u>	<u>M/ Gene copies</u>	<u>Ratio FR5:F⁻</u>	<u>E/ Gene copies</u>	<u>Ratio FR5:F⁻</u>
E1 Fucose induced	(1)	4.65	1.14	(1)	0.92
E1/FR5 Fucose induced	2.4	5.18		0.92	
E1 Galactose induced	(1)	3.74	0.52	0.65	0.46
E1/FR5 Galactose induced	2.4	1.95		0.30	
TMG6 Non-induced	(1)	4.50		1.13	
TMG6 Fucose induced	(1)	3.66	0.58	2.48	0.51
TMG6/FR5 Fucose induced	2.4	2.12		1.27	
TMG6 Galactose induced	(1)	2.64	0.98	1.15	0.83
TMG6/FR5 Galactose induced	2.4	2.60		0.95	

λ dgs JG3 and JG4 are given in Appendix A, Table 5. The saturation curves and calculated reciprocal plots are shown in Figures 30 to 33, and the results derived from these data are shown in Table 20. It can be seen that the RNA per unit enzyme increases with the gradient of polarity, even though the values for messenger content decrease with the polarity gradient. That is, the most extreme polar mutation F'gal₃ has one-tenth of the gal m-RNA of the least polar mutation, NA119/FR119, but only one-thirtieth of its enzyme. NA119/FR119 itself has slightly more gal m-RNA than does the wild type E1 (slightly less than half that of its genetic homologue E1/FR5) but only 24% of the wild type enzyme (10%). F'gal₃ however, with approximately 10% of the E1 messenger content has only 0.75% of the enzyme. The other two polar strains, NA121/FR121, and NA156/FR156, fall between these limits.

Legend to Figure 30

a) Saturation curve for RNA from NA119/FR119 induced with fucose,
hybridised with DNA from λ dgs JG3 and JG1

Each membrane was loaded with 1.5 μ g DNA. Annealing etc.
as described in the legend to Figure 13a.

b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H
was calculated, and the slope ($1/C_g$) and intercept (k') determined
for each DNA.

○ ——— ○ JG3 DNA
● ——— ● JG1 DNA

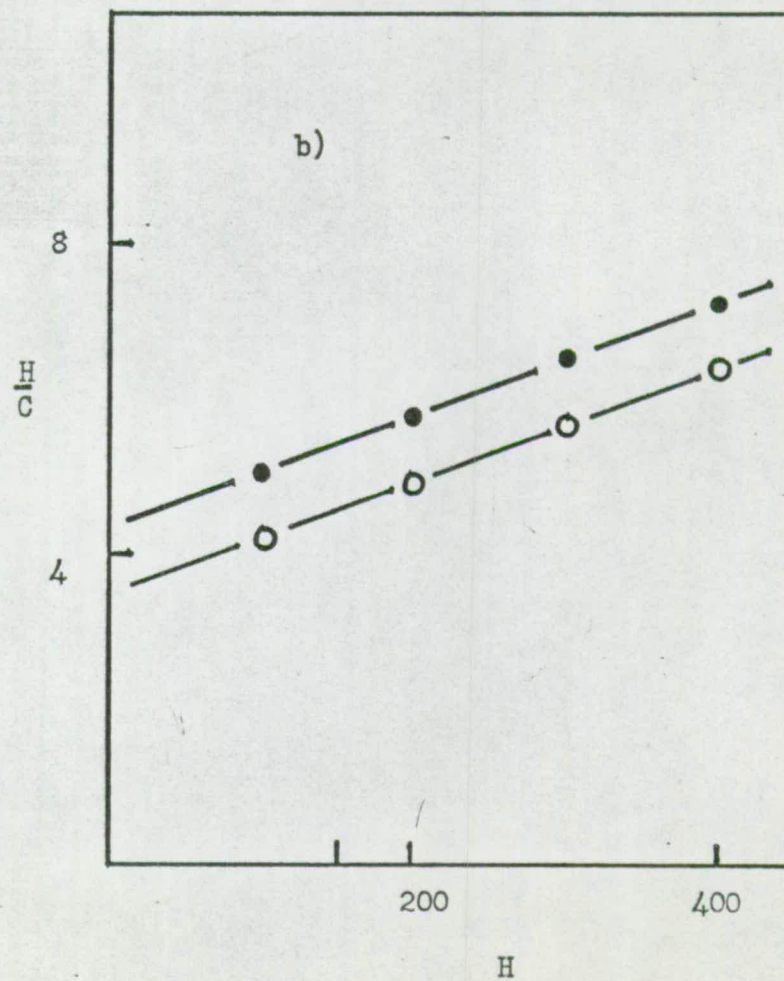
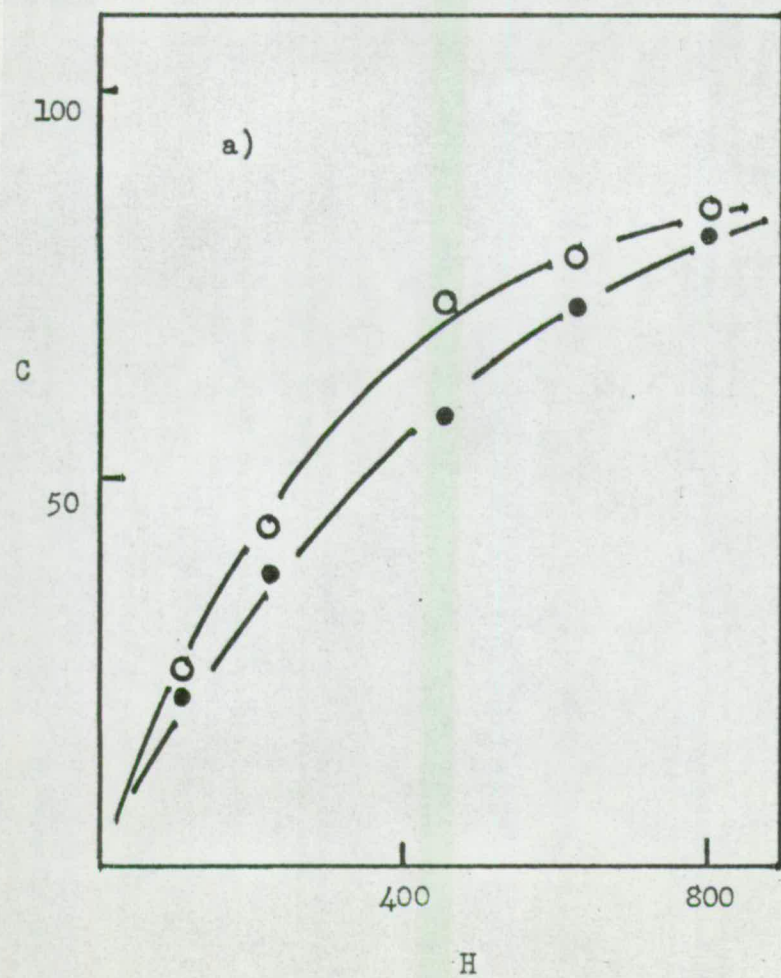


Figure 30.

Legend to Figure 31

- a) Saturation curves for RNA from NA121/FR121 induced with fucose, hybridised to DNA from λ dgs JG3 and JG1

Each membrane was loaded with 1.5 μ g DNA. Annealing etc. as described in the legend to Figure 13a.

- b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was calculated, and the slope ($1/C_s$) and intercept (k') determined for each DNA.

○ ——— ○ JG3 DNA

● ——— ● JG1 DNA

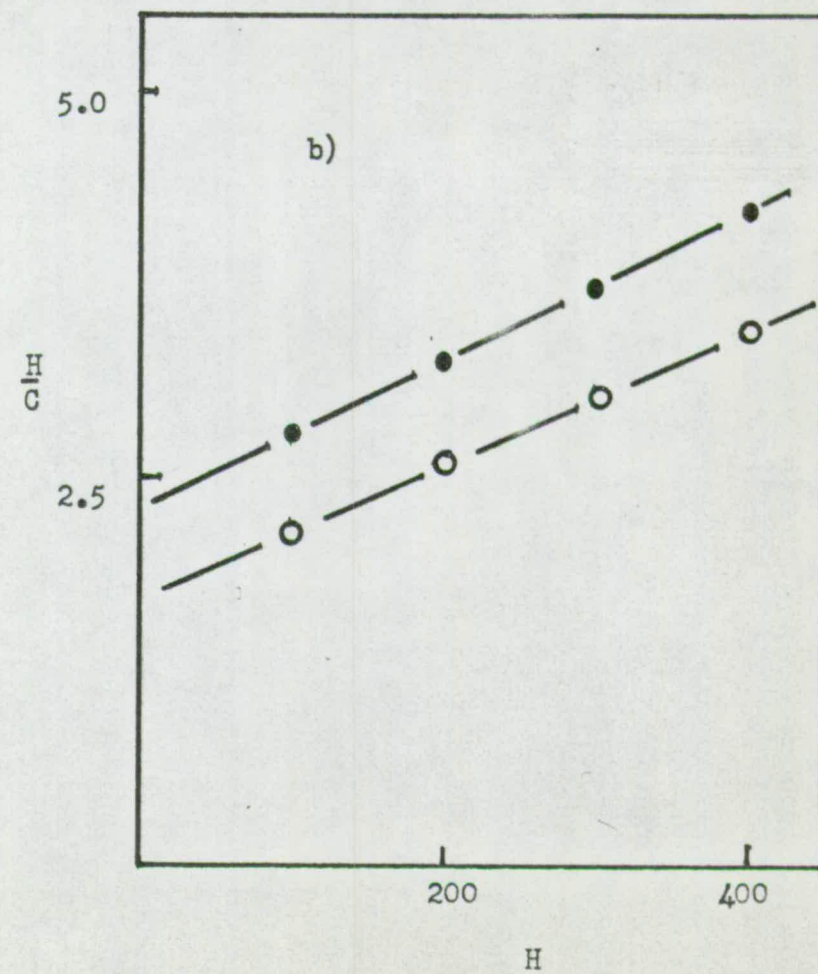
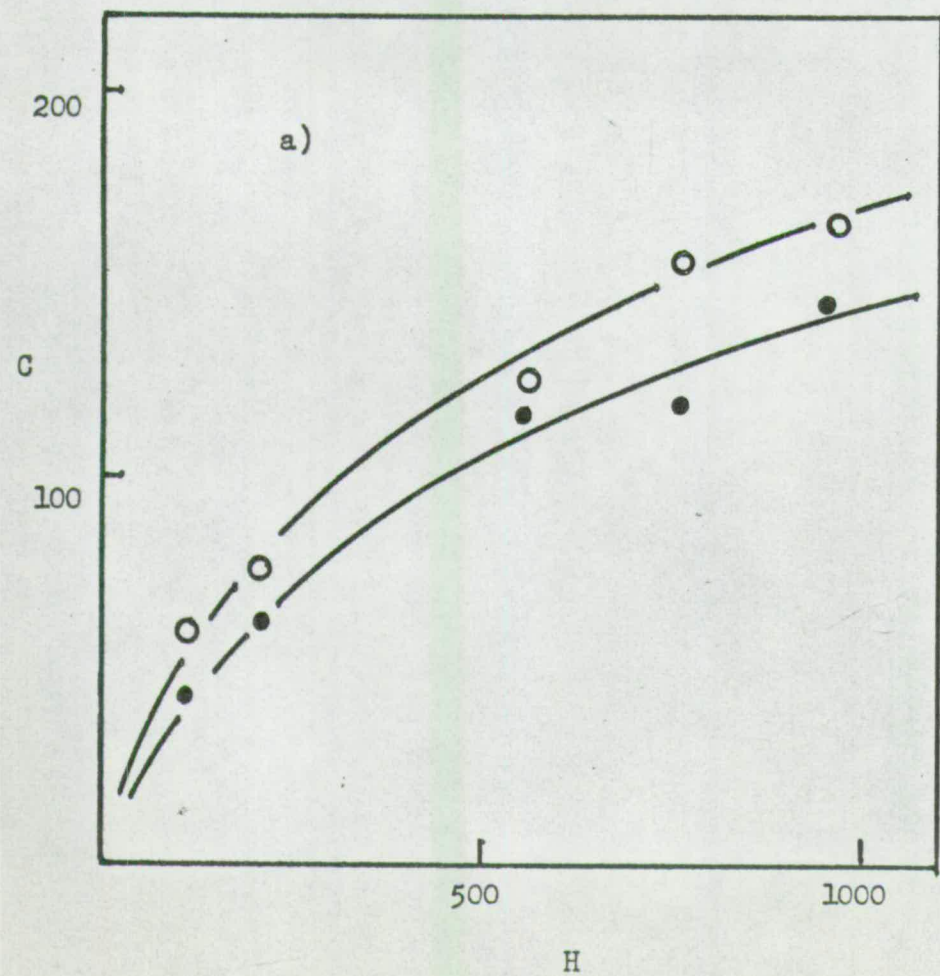


Figure 31.

Legend to Figure 32

a) Saturation curves for RNA from NA156/FR156 induced with fucose,
hybridised with DNA from λ ggs JG3 and JG1

Each membrane was loaded with 1.5 μ g DNA. Annealing etc.
as described in the legend to Figure 13a.

b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H was
calculated, and the slope ($1/C_s$) and intercept (k') determined
for each DNA.

○ ——— ○ JG3 DNA
● ——— ● JG1 DNA

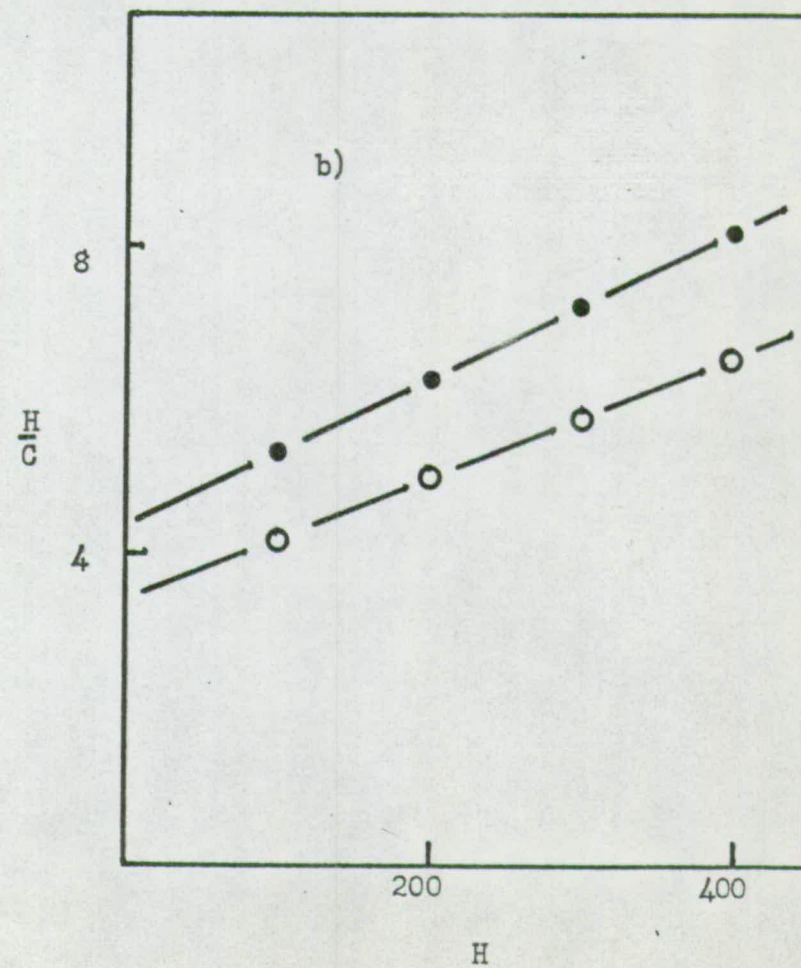
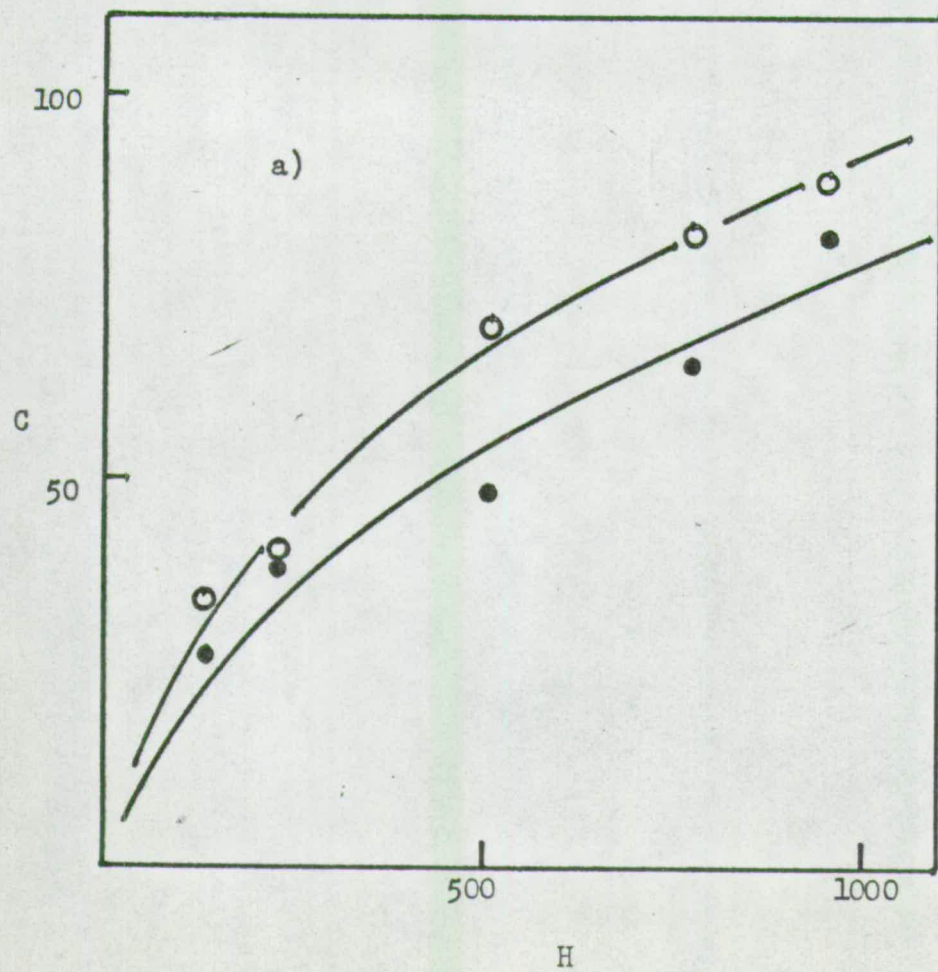


Figure 32.

Legend to Figure 33

a) Saturation curves of RNA from $F'gal^-$ induced with fucose,
hybridised with DNA from λ dgs JG3 and JG1

Each membrane was loaded with 1.5 μ g DNA. Annealing etc.
as described in the legend to Figure 13a.

b) Calculated values of H/C for each value of H

The regression of the experimental values of H/C upon H
was calculated, and the slope ($1/C_s$) and intercept (k') determined
for each DNA.

○ ——— ○ JG3 DNA
● ——— ● JG1 DNA

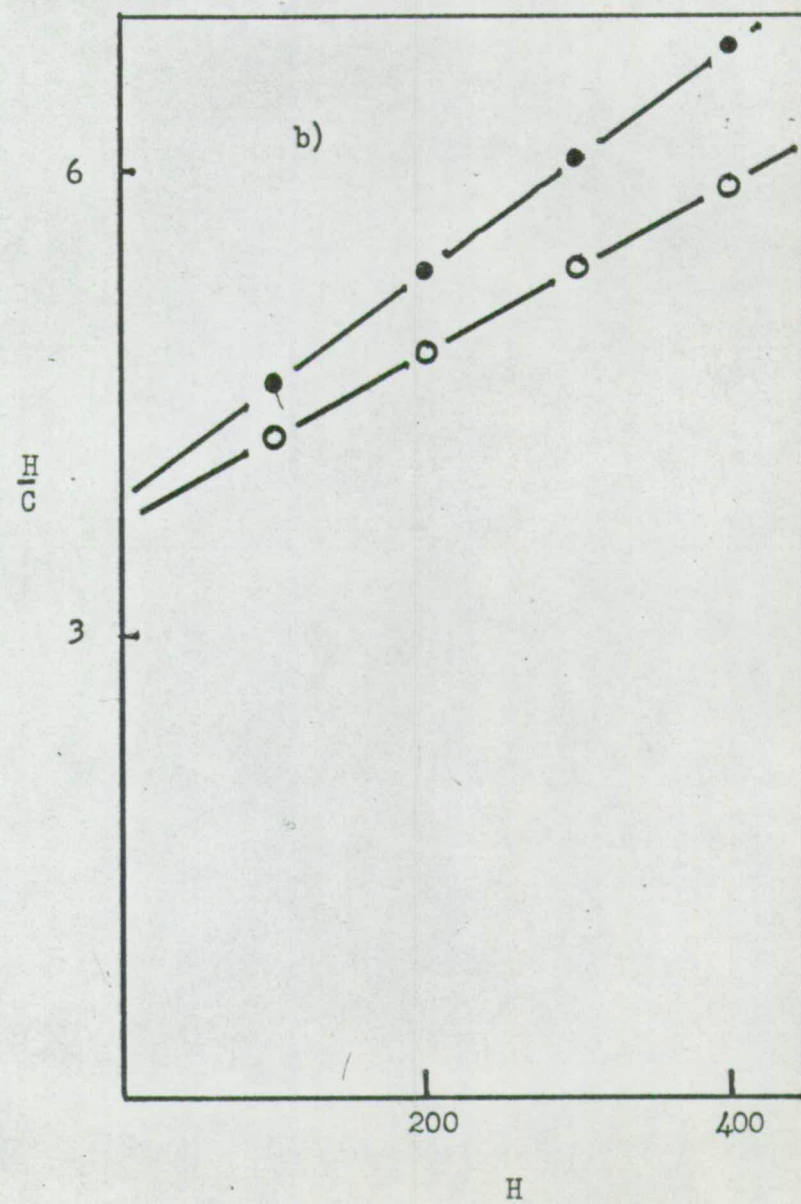
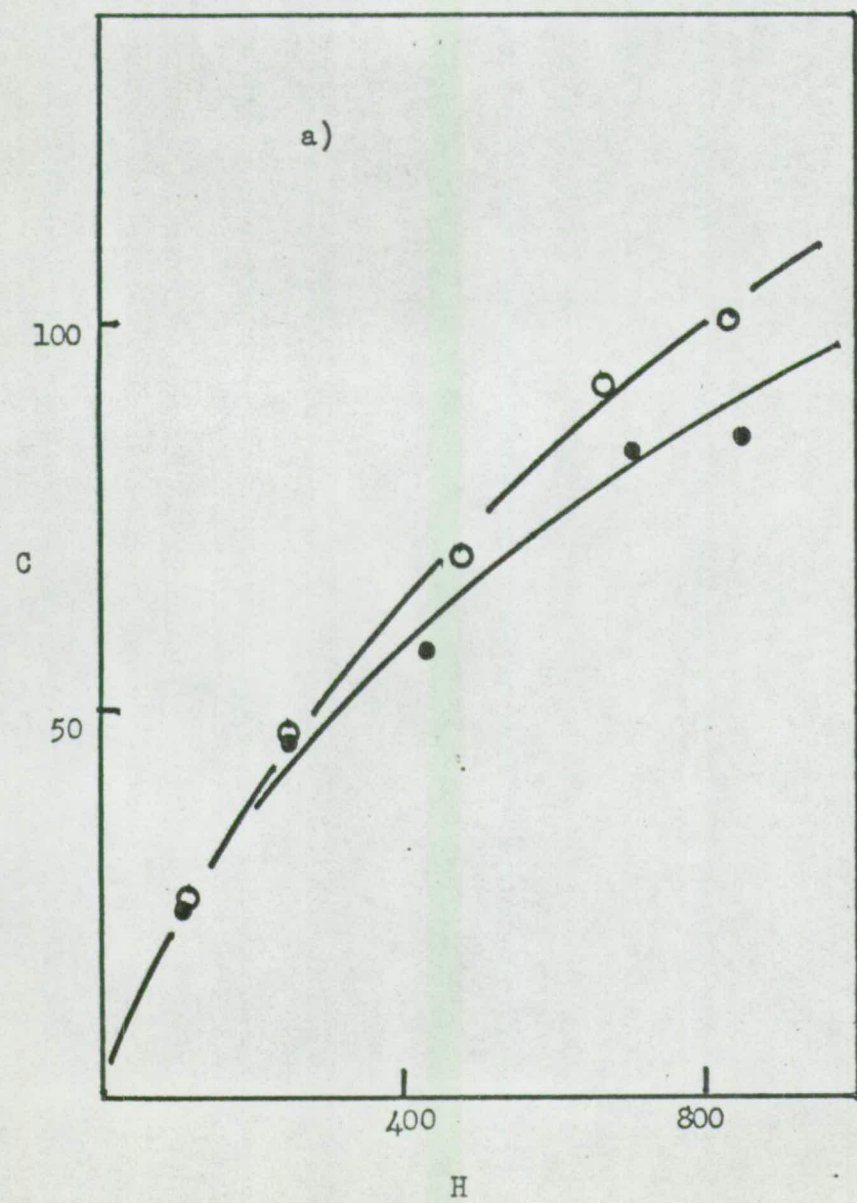


Figure 33.

Table 20

Calculated results of hybridisation experiments Series V

RNA Source	$\alpha \times 10^5$	s	m	S	E	$\frac{m}{E}$	$\frac{\alpha \times 10^5}{E}$
NA119/FR119 Fucose Induced	18.28	676	5.67	367	0.240	23.63	76.17
NA121/FR121 Fucose Induced	10.42	2,452	3.23	381	0.100	32.40	104.50
NA156/FR156 Fucose Induced	7.40	1,521	2.29	401	0.049	46.36	149.70
F'gal ₃ Fucose Induced	1.767	1,436	0.55	362	0.008	73.07	235.60

Legend

α = proportion of total RNA which is gal m-RNA

s = specific radioactivity of gal m-RNA

m = number of galactose m-RNA molecules per galactose gene copy

S = specific radioactivity of total RNA

E = galactokinase activity normalised to that of E1 fucose induced

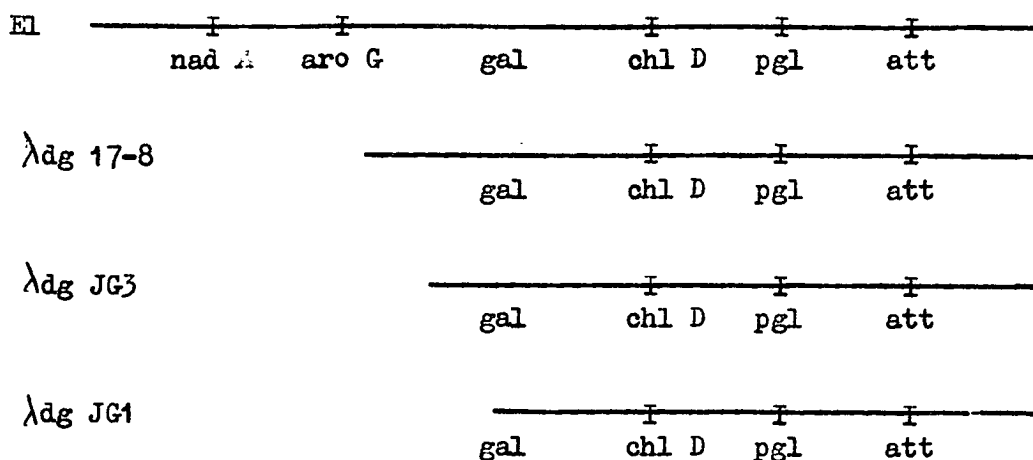
DISCUSSION

1. The Method

In the introduction attention was drawn to a number of fallacious assumptions which have been made in the majority of previous attempts to measure specific messenger RNAs.

Many of these used hybridisation in liquid medium (technique of Nygaard and Hall, 1963, 1964) in which both RNA and DNA are in solution. With this method, where complementary sequences are in approximately equal concentrations, renaturation of the DNA is in competition with formation of the RNA-DNA hybrid. This renders the results difficult to interpret, though it is possible, using the relationship between DNA size and renaturation rate (Wetmur and Davidson, 1968) and the ratio of the rate constants for the DNA-DNA and RNA-DNA reactions (Melli *et al.*, 1971) to estimate the differential between renaturation and hybridisation in these cases. The use of denatured DNA immobilised on nitrocellulose membranes (Gillespie and Spiegelman, 1965), however, completely eliminates DNA renaturation and allows the formation of RNA-DNA hybrid to be studied alone.

The results presented here support the assumption that estimation of gal m-RNA contents by calculation of the difference between hybridisation to DNA from λ dgs JG3 and JG1 would discount hybridisation of other RNAs.



9-3h⁺/FR5 has comparatively little homology with the non-*gal* bacterial DNA carried by the λdgs, since all the region between *att*λ and *nad A* is deleted in 9-3 and partially deleted in FR5. All this region is present in El, and some non-*gal* bacterial DNA is carried by λdg17-8 but not by λdg JG3. The estimates of *gal* m-RNA derived from the difference between hybridisation to 17-8 DNA and JG1 DNA (the maximum estimate), which includes hybridisation to non-*gal* DNA distal to the *gal* operon) are higher than those derived from the difference between hybridisation to JG3 DNA and JG1 DNA. However, the difference in the estimates for 9-3h⁺/FR5 fucose-induced is less than the difference between the estimates for El fucose-induced. It seems logical to conclude that this difference is in part, if not entirely, the result of the hybridisation of non-*gal* RNA to λdg 17-8. The fact that there is less non-*gal* DNA in 9-3h⁺/FR5 than in El accounts for the greater difference between the estimates for the latter. Thus although the value for *gal* m-RNA contents obtained from the difference in hybridisation to JG3 DNA and JG1 DNA may slightly under estimate the true value, the fact that no non-*gal* RNA is included in the estimate means that the ratios between the estimates for different cultures

are correct, and, unless one tries to discuss the number of molecules of m-RNA present, these values are an accurate measurement of the steady state level of gal messenger RNA. It is important to note that all measurements in this work are of steady state levels; no estimate of rates of synthesis or decay has been attempted.

The measurement of the specific radioactivity of the gal m-RNA (s) is, of course, dependent on the accuracy of the value calculated for the molecular weight of the gal DNA sense strand. This is, however, a constant in all the measurements, so that the ratio of the measurements should be accurate even if the absolute values are in error. The variation in the proportion of the total radioactivity which is in the gal m-RNA (β , from equation 10, page 49) shown in Table 21 indicates the inaccuracy of estimates of m-RNA contents based solely upon the proportion of total input radioactivity found in the hybrid.

Finally, the use of a linear formulation of the hybridisation reaction not only removes the subjective element from the estimation of the saturation plateau, but enables one to extrapolate to the true saturation value obtainable only at infinite time or with infinite input RNA/DNA ratio. This is only true in a case where one is able to eliminate hybridisation due to other species of RNA. Where there is a mixture of RNAs at different concentrations it would be necessary to calculate the differences between the saturation curves, and then make the double reciprocal plot.

Of the earlier attempts to measure specific n-RNAs, that of Stubbs and Hall (1968) is of particular interest. They attempted to isolate purified tryptophan m-RNA by hybridisation of total

Table 21

Proportion of total radioactivity found in gal m-RNA (β) $k' =$

Total RNA concentration

Concentration of messenger radioactivity

S = Specific Radioactive of total RNA

α = proportion of total RNA which is gal m-RNA

<u>Strain</u>	<u>k'</u>	<u>S</u>	<u>$1/k'S = \beta$</u>	<u>$\alpha \times 10^5$</u>
9-3h ⁺ /FR5, fucose-induced	0.87	496	0.00232	22.2
9-3h ⁺ /FR5, non-induced	8.77	532	0.00021	3.9
9-3h ⁺ /FR5, galactose-induced	3.29	529	0.00057	15.1
9-3h ⁺ /FR5, TMG repressed	5.14	203	0.00096	8.1
E1 Fucose-induced	1.065	675	0.00138	15.0
E1 galactose-induced	1.42	403	0.00175	12.1
E1/FR5 fucose-induced	0.56	352	0.00507	40.1
E1/FR5 galactose induced	1.29	538	0.00144	15.1
TMG6 non-induced	0.86	496	0.00232	14.5
TMG6 fucose-induced	2.24	386	0.00116	11.8
TMG6 galactose-induced	3.18	454	0.000693	8.5
TMG6/FR5 fucose-induced	1.04	581	0.00165	16.4
TMG6/FR5 galactose-induced	0.88	568	0.00200	20.1
NA119/FR119 fucose-induced	10.79	367	0.00025	18.3
NA121/FR121 fucose-induced	5.73	401	0.00043	10.4
NA156/FR156 fucose-induced	13.19	381	0.00020	7.4
F'gal ₃ fucose-induced	39.4	362	0.00007	1.8

³H-labelled RNA first to $\phi 80$, to remove phage-specific RNAs from the mixture, and then to $\phi 80$ trp (a $\phi 80$ phage carrying the complete trp operon). The RNA hybridised in the second reaction was recovered and used as a standard with which other total RNAs were competed in hybridisations with $\phi 80$ trp DNA. Stubbs and Hall found that only 54% to 60% of the "trp m-RNA" input was hybridised, and assumed that the inability to attain 100% hybridisation was the result of contamination by other bacterial RNAs, or to breakdown of the RNA. In fact these figures agree closely with Bishop et al (1969) who estimated the hybridisation factor (the total hybridisation attainable by completely complementary RNA and DNA) to be 57% (see above, page 46). It is probable that the RNA which Stubbs and Hall isolated was almost entirely that complementary to the non-phage DNA carried by $\phi 80$ trp. This does not, however, mean that it was pure trp m-RNA, as other bacterial sequences were almost certainly carried by the phage. In order to discount such sequences it is necessary to use deletion strains carrying a known proportion of the operon, as in the work described in this thesis. In addition they quantitative analysis of competition hybridisation experiments is complex (Irving, 1970) and the saturation hybridisation method used in this investigation is to be preferred. While it is not possible to set limits to the accuracy of the estimates presented here, because of the approximations involved, the methods and analysis used overcome most of the objections to earlier approaches.

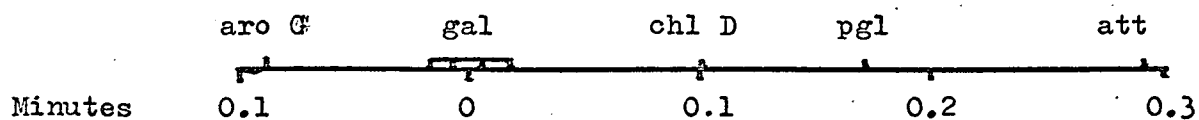
2. Correlation between hybridisation saturation values (C_s)

and the genetic map

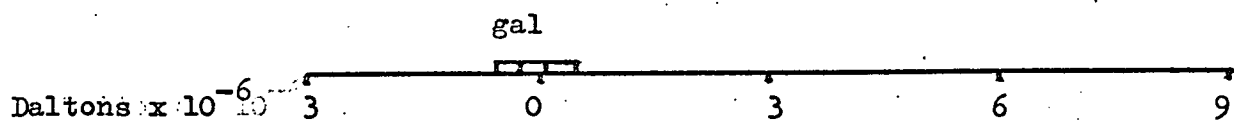
The analysis of the experiment described on pages 51 to 54 makes it possible to relate the saturation values shown in Table 10

very closely to the genetic map (Figure 6). In Figure 9 these C_s values are shown as gal m-RNA and non-gal RNA hybridised to the gal and non-gal DNA of the transducing phage used as the DNA source. However, a much more precise picture of the distribution of the hybridisation of these non-gal RNAs can be obtained. The genetic map of E.coli is normally described in terms of the time taken for two loci to be transferred from a donor (Hfr or F') to a recipient (F⁻) strain during conjugation. In these terms the total genome is 90 minutes long. It is reasonable to assume that, on average, all loci are transferred at the same rate, and therefore a length in minutes can be converted to a size in daltons. The molecular weight of the genome is 2.7×10^9 daltons. One minute of recombination time is therefore 3.0×10^7 daltons of DNA. The distance between loci whose recombination distance is known can thus be expressed in daltons, and the RNA hybridised be described in terms of counts per minute hybridised to a given amount of DNA in daltons. This has been done in Figure 34.

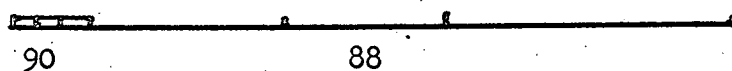
The gal operon is about 10^6 daltons of DNA. The molecular weight of the DNA between the gal operon and aro G is about 2×10^6 daltons. 56 cpm RNA hybridised to λ dg 17-8 DNA in this region, and between 56 and 73 cpm to λ pg DNA. The actual amount of non-gal DNA distal to the gal operon carried by these two phages is not known, but at maximum 15 cpm of this RNA is gal m-RNA. If 17 cpm of the non-gal RNA hybridised to λ pg is gal proximal, the remaining $(56-15) = 41$ cpm is hybridised to not more than 2.0×10^6 daltons of DNA, i.e. $20.5 \times \text{cpm/dalton} \times 10^6$. In the same way it is possible to tabulate the RNA hybridised in cpm/dalton $\times 10^6$ for the other regions of the bacterial DNA carried by the



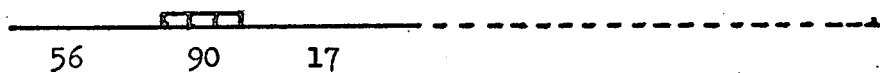
One minute = 3×10^7 daltons (See text).



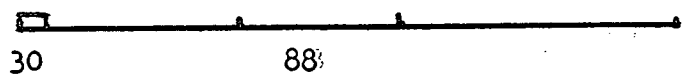
dg 17-8



dg JG3



pg



dg JG1

Figure 34.

Distribution of saturation hybridisation values (C_s) correlated with the genetic map.

phages. The distance from the gal operon to att λ is about 8×10^6 daltons, from gal to chl D 2.5×10^6 daltons and from chl D to att 5.5×10^6 daltons.

<u>Region</u>	<u>size in daltons</u>	<u>C_s</u>	<u>C_s/dalton</u>
gal distal	2.0×10^6	41-56	$20.5-28 \times 10^{-6}$
gal	1.0×10^6	90-105	$90-105 \times 10^{-6}$
gal proximal (all)	8.0×10^6	88	11×10^{-6}
gal proximal (λ pg)	2.5×10^6	17	6.8×10^{-6}
chl D - att λ	6.0×10^6	71	11.8×10^{-6}

The 17 cpm gal proximal on λ pg could be gal. If it is not gal, and is labelled equally with gal, only 0.2×10^6 daltons would be involved, i.e. a protein of molecular weight 10,000, which is quite feasible.

The 11.8×10^6 cpm/dalton between chl D and att λ would also be the same as gal if only 13% of the region were transcribed, i.e. 0.78×10^6 daltons. This figure is quite reasonable as an estimate for the size of these two loci.

The gal distal material which is not gal could also be the messenger from an unidentified locus. If labelled equally with gal this would correspond to a region of 0.25 to 0.3×10^6 daltons, which, if translated, would yield a protein of 10,000 to 15,000 molecular weight.

Such a direct correlation between hybridisation values and the genetic map is probably unique, but similar approaches could be made to the analysis of other regions of the bacterial genome.

3. Induction and repression

a) Induction of wild-type by fucose

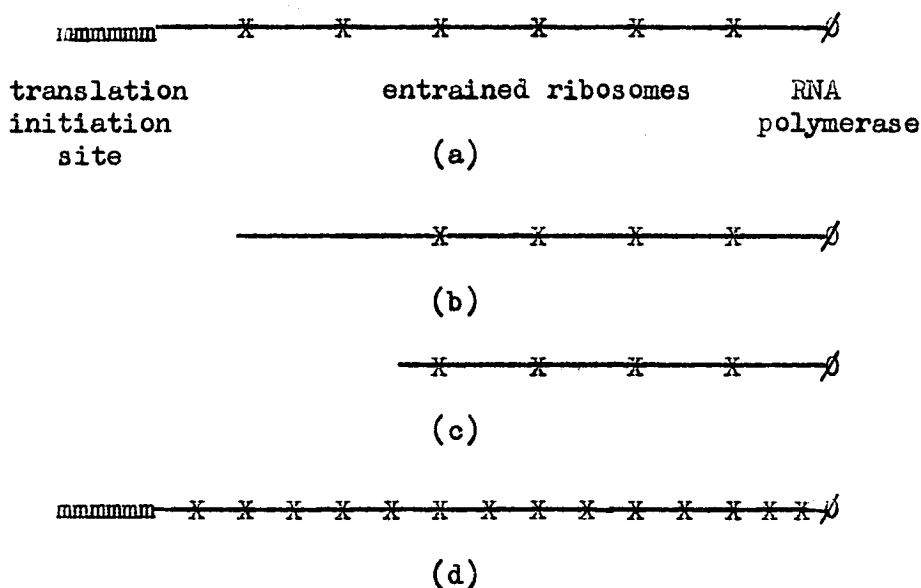
Despite the greater accuracy of the methods used in this investigation, complete correlation between the increase in messenger RNA content and that of galactokinase activity on induction has not been found. 9-3h⁺/FR5 shows a 6-fold increase in m-RNA content between non-induced and fucose-induced cultures, but a 9-fold increase in galactokinase activity. This result may be compared with those of Attardi et al., (1962, 1963a and b) and Hill and Echols (1966) who, using techniques less capable of accurate m-RNA measurement, showed much greater discrepancies between enzyme and m-RNA levels than have been found in this research. Their methods certainly overestimated the m-RNA content in non-induced cultures, and perhaps also underestimated it in induced cultures. The former is the more probable, since hybridisation by non-gal m-RNA would cause greater errors in the estimate of m-RNA from non-induced cultures, in which the non-gal m-RNA forms a greater proportion of the RNA in the LNA-DNA hybrid.

It is clear from the figures in the previous paragraph that fucose induction not only raises the gal m-RNA content, but also increases the amount of enzyme per unit messenger, i.e. the efficiency with which that RNA is translated. The figures for the ratio of the m-RNA content to the galactokinase activity, $\frac{a}{E} \times 10^5$, show this clearly. The value for 9-3h⁺/FR5 fucose induced is 16.13, that for the non-induced culture 24.8. That is, as well as increasing the level of m-RNA in the induced culture, the amount of enzyme synthesised per unit m-RNA has increased by 50%.

If control of the activity of an operon were solely at the level of transcription, one would expect to find a quantitative correlation between the level of induction of m-RNA and that of enzyme. The fact that this direct proportionality is not found makes the existence of some additional form of regulation indispensable to any hypothesis. It has been suggested that the additional method of control acts at the level of translation (Lavalley, 1970; Lavalley and de Hauwer, 1968; Goldberg and Chargaff, 1971; McClellan and Vogel, 1971). Two of these investigations (Lavalley, 1970; and McClellan and Vogel, 1971) have been made in the arginine synthetic system, which is unusual in consisting of a number of structural genes, each with an operator, widely distributed through the genome and controlled by a single regulator gene. McClellan and Vogel consider that the messengers are synthesised constitutively, but that in the presence of arginine, which represses the synthesis of the enzymes, the rate of degradation of the messengers is accelerated. Lavalley and de Hauwer examined the tryptophan operon. They could find no difference in the life times of the messengers between repressed and de-repressed cultures, and suggest that the control of translation may involve a new system of regulatory molecules. Their findings are open to doubt because of their method of investigation. This involved measurement of the loss of trichloroacetic acid (TCA) precipitable radioactivity from pulse-labelled cultures, and a negative result is not entirely convincing. Goldberg and Chargaff (1971) claim that, in synchronised cultures of IPTG-induced E.coli, the synthesis of β -galactosidase is cyclical and related to the generation time, while the level of

lac m-RNA is constant, and that this indicates translational control. There are obvious disagreements here, even if it is possible to accept the m-RNA measurements. It seems unnecessary to invoke a second control mechanism at the level of translation. An economical hypothesis is based upon the work of Ito and Imamoto (1968) and Morse et al (1968) whose data suggest that in a de-repressed culture each trp messenger RNA molecule is translated by a cluster of some 90 ribosomes entrained behind the RNA polymerase molecule. Synthesis of the m-RNA is thought to be accompanied by degradation from the 5'-end, following the entrained ribosomes. In terms of this model, fucose leads to an increase of 50% in the number of (x) ribosomes which translate each messenger, when compared with the non-induced culture.

The efficiency of translation could be altered in several different ways:



In (b) the initiation region has been degraded, so that no more ribosomes can attach to the messenger. This would appear as a

reduction in translation efficiency compared with (a). In (c) the number of ribosomes per unit m-RNA is the same as in (a), although the messenger is partially degraded. This would show an apparent loss of induction of messenger, although the rate of synthesis of messenger might be the same as in (a), with no change in the efficiency of translation, i.e. in the number of ribosomes per unit messenger. In (d) the number of ribosomes per unit messenger has been increased. If the rate of synthesis and degradation of messenger remained constant, or if they were altered proportionately, this would appear as an increase in the efficiency of translation only. If the rate of messenger synthesis were reduced, and that of degradation increased or remained constant, the efficiency of translation would appear to rise and the m-RNA content to fall. The results described here could thus be explained in a number of different ways, the most probable of which being an earlier onset of degradation of the m-RNA molecules in the non-induced culture, with each messenger being translated by fewer ribosomes. Morese et al (1969) showed that degradation of trp m-RNA was more rapid in the presence of tryptophan (which represses the operon) than in its absence, which agrees with this hypothesis.

b) Fucose induction and gene dosage in the wild type

As can be seen from Table 19, the difference between the m-RNA contents (a) of E1 and E1/FR5 when induced by fucose is almost exactly proportional to the gene dosage difference. This also correlates with the enzyme levels, and the slight difference in the ratio of messenger content to enzyme level is probably not significant.

c) Induction of the wild type by galactose

The effect of galactose induction as opposed to that of fucose must be interpreted at two levels. The initial observation was that there is less enzyme made with galactose induction than with fucose induction (Frame and Bishop, 1971). As the results presented here show, this is primarily due to less m-RNA being present. Parks et al (1971) have isolated and partially purified the gal repressor molecule. They found that galactose and fucose were equally effective in preventing the binding of gal repressor to λ pg DNA. At a concentration of 5×10^{-5} M of either inducer, binding was 50% inhibited, and at 5×10^{-4} M binding inhibition was complete. The basis for the lower efficiency of galactose induction must therefore lie in the fact that galactose is metabolised whereas fucose is not. The metabolism of galactose could result in the synthesis of a repressing catabolite, which must then repress the level of transcription of the operon. Fucose takes no part in metabolism, and the glycerol in the medium, which is the sole carbon source for the fucose induced cultures, does not cause catabolite repression.

There is also a slight secondary effect in that there is less galactokinase made per m-RNA molecule in the galactose induced cultures than in those induced with fucose. The difference is comparatively small (about 15% less enzyme per unit messenger) and might not be significant were it not that the differential is extremely consistent in each of the cultures studied. This can be explained if, as suggested above, the efficiency of translation is a function of the rate of decay of the messenger RNA. Since

galactose induction seems to involve partial repression, the rate of decay of the m-RNA would be expected to be greater than in fucose induced cultures, and each messenger might be translated by fewer ribosomes.

d) Galactose induction and gene dosage in the wild type

Much less m-RNA is found per gene copy in E1/FR5 than in E1 when the inducer is galactose. This is also explicable in terms of induction and repression. In E1/FR5 the higher gene dosage would mean an enhancement of the repression effect, because the increased enzyme level would result in an increase in the synthesis of the repressing catabolite, which in turn would inhibit the synthesis of m-RNA. In E1/FR5 as in E1 and 9-3h⁺/FR5, galactose induction leads to a 15% drop in the efficiency of translation when compared to fucose induction, and the same hypothesis is proposed to explain this.

e) Repression by methyl β -D-thiogalactoside (TMG)

TMG appears to have no effect on the m-RNA level of 9-3h⁺/FR5 since the m-RNA contents of the non-induced and TMG-repressed cultures are very similar. The efficiency of translation, however, is only half as great in the TMG-repressed culture as in the non-induced culture. This analogue of galactose thus appears to affect only translation, unlike galactose, which acts mainly on the process of transcription. Parks et al (1971) found that TMG had no effect on the binding of the gal repressor to λ pg DNA, which agrees with this conclusion.

Davison (pers. comm.) found that the repressive effects of glucose and TMG were additive. This suggests that the repressive

effect of TMG must also differ from that of glucose. Using glucose as the repressor, the m-RNA content was too low to measure, which agrees with this idea. The mechanism by which TMG reduces the efficiency of translation may again be related to the rate of degradation of the messenger. If, as in the arginine system (McClellan and Vogel, 1971) repression accelerates the rate of decay of the m-RNA, allowing fewer ribosomes to attach to each molecule this would be sufficient to explain the observations. Alternatively, TMG might act directly on the m-RNA by interacting in some way with the ribosome attachment site to reduce the frequency of attachment of ribosomes.

f) Induction of the constitutive mutants TMG6 and TMG6/FR5

Induction of these constitutive mutants has a completely different effect from induction of the wild-type. The m-RNA content is not increased (it is possible that it is decreased, but these differences may not be significant) and the major effect appears to be at the translational level. An increase in the m-RNA content might not be expected, since TMG6 is a regulator mutant (R^- gal) producing a mutant repressor protein. If the primary site of action of an inducer is on the repressor, preventing it from binding to the operator, the addition of inducer to a mutant lacking active repressor should have no effect, since there is no repressor blocking transcription. The results, however, show that there is a considerable increase in galactokinase activity on induction with fucose (2.2 times) while galactose induction has only a slight effect. The efficiency of translation appears to be greatly enhanced by both inducers, with fucose being approximately twice as

effective as galactose. The ratio of m-RNA content to galactokinase activity is lower in TMG6 and TMG6/FR5 induced than in any other strain studied. The non-induced cultures has an m-RNA content and a translation efficiency similar to L1 induced with fucose. The enhanced efficiency of translation, and depressed m-RNA contents of the induced cultures must therefore be the effect of the inducers. Myers and Sadler (1971) have identified a mutant of the regulator gene in the lac operon (i^{rc}) which is constitutive in the absence of inducers and repressed by the addition of IPTG. They suggest that this is due to a repressor with altered affinity for the lac operator, so that the repressor alone does not bind to the operator, but the repressor-IPTG complex will do so. They have not investigated the state of the m-RNA in this strain, all their conclusions being based on the levels of β -galactosidase. In the case of TMG6 it appears that we might have a somewhat similar mutant. In the absence of inducer both m-RNA content and enzyme activity are similar to those of the fucose-induced wild type. The inducers appear to reduce the level of m-RNA (which could be the result of a mutation like Myers and Sadler's i^{rc}) but stimulate the synthesis of galactokinase. Each messenger must therefore be translated by more ribosomes than in the wild type or non-induced constitutive cultures. The effect of increasing the gene dosage (i.e. TMG6/FR5) is less than in the wild type. With fucose induction the translational efficiency is similar to that of TMG6 and the increase in m-RNA content is not proportional to the gene dosage. The high rate of translation in both TMG6 and TMG6/FR5 might indicate that the m-RNA is more stable than in the wild type, and this hyperstability might

limit the rate of synthesis of new m-RNA molecules.

To summarise these findings, it appears that the basic mechanism for the regulation of enzyme synthesis is at the level of transcription. Inducers act primarily by binding to the repressor protein, preventing it from attaching to the operator. Fucose and galactose are equally effective in this derepression, but galactose-induction produces a lower level of m-RNA than fucose induction. This may be the result of a catabolite produced by the metabolism of galactose. The galactose operon is catabolite repressible, and although catabolite repression is not caused by any of the immediate products of the galactose enzymes (Paigen et al, 1967) the fact that these products enter the normal glycolytic pathway via glucose-1-phosphate (see Figure 3 in the introduction) indicates that the metabolism of galactose could lead to partial catabolite repression, with consequent depression of the level of cyclic AMP and reduction of the rate of transcription. This interpretation is open to experimental verification by assaying the levels of cyclic AMP in fucose-induced and galactose-induced cultures. A secondary mechanism of control appears to exist at the level of translation, which regulates the number of enzyme molecules translated from each messenger RNA molecule. This may be interpreted on the basis of the finding by Morse et al (1969) that m-RNA degradation is more rapid in repressed than in de-repressed cultures. In this model the number of ribosomes entrained behind the RNA polymerase molecule may be limited by the onset of degradation of the m-RNA. It is possible that regulation of translational efficiency requires a whole complex of new factors, as suggested by

Lavallé (1970) and Goldberg and Chargaff (1971), but there is as yet no experimental evidence for this. It is, however, certain that the regulation of bacterial metabolism is a great deal more complex than appeared likely to Jacob and Monod in 1961.

4. Polar Mutants

Bishop (pers. comm.) found that NA121 was a nonsense mutation of the ochre type, and that none of the other three polar mutants studied (NA119, NA156 and gal^-) were sensitive to nonsense suppressors. Irving (1970) was unable to obtain revertants of any of these by treatment with nitrosoguanidine or 2-amino purine (mutagens causing base changes) or with acridine compounds and their analogues, (which cause the insertion or deletion of one or a few bases and can revert reading frame shift mutations). She concluded that these three mutations could well be the result of insertions as described by Jordan et al (1968) and Shapiro (1969).

All four of the mutants show both transcriptional and translational effects. The translational effect, that of reduced efficiency (less enzyme synthesised per messenger RNA molecule) is proportional to the degree of polarity, i.e. the mutant with least enzyme also has least enzyme per messenger. This is compatible with the earliest explanation of the phenomenon of polarity, stated in 1966 by Malamy; Imamoto et al; and Martin et al. This explains the reduction in translational efficiency as follows:

Ribosomes can only attach to the messenger RNA at the operator end. At a nonsense codon peptide chain termination occurs (Sarabhai et al, 1964) and the ribosome slips along the rest of the messenger, in no particular phase, with a probability of being released from

the messenger which is proportional to the distance slipped.

If a ribosome encounters a chain initiation site, polypeptide synthesis may resume. The degree of polarity is then dependent both on the distance between the mutation and the initiation site and on the strength of the initiation signal. Webster and Zinder (1969) studied the effect of an amber mutation in the coat protein of the RNA phage ϕ_2 in vitro, and found that translation ceased at the mutation, and the ribosome was released shortly thereafter.

The results presented here also show a clear transcriptional effect in that there is a reduction in the amount of messenger found, this reduction being also proportional to the degree of polarity of the mutation. The shortage of m-RNA could mean

a) that transcription and translation are directly coupled (Stent, 1966; Lodish and Zinder, 1966), or

b) that the polar mutation has no effect on the rate of m-RNA synthesis, but that RNA synthesised distal to the mutation is rapidly degraded (Newton et al, 1965).

Lodish (1968) studied the effect of an amber polar mutation in ϕ_2 in vitro, and found that translation of the nascent viral RNA was independent of the synthesis of that RNA. He suggested that this might also be true of strongly polar bacterial mutations. This was consistent with the observation that translation of bacterial messenger is initiated before transcription is complete (Leive and Kollin, 1967; Das et al, 1967). Lodish suggested that translation might be required to protect the nascent m-RNA from degradation by nucleases.

Since the two hypotheses were stated in 1965 and 1966 both have

been refined, more evidence has been produced for each of them and there is no sign of their reconciliation. The majority of the studies have been made on the tryptophan operon of E.coli, where m-RNA molecules from polar mutants seem to be shorter than in the wild type because they lack the distal nucleotide sequences (Imamoto and Yanofsky, 1967a and b). Imamoto and Kano (1971), developing Stent's original (1966) hypothesis, suggest that nascent RNA chains not being translated might entangle the RNA polymerase and prevent it from proceeding, or that, in the absence of ribosome movement, the messenger cannot unfold from the DNA. Morse and Guertin (1971) supporting the alternative hypothesis, point out that E.coli has two methods of degrading m-RNA. One, common to all messengers, is a nucleolytic enzyme which may follow the last of a cluster of ribosomes as they translate a messenger. Polarity, they consider, results from internal scission and subsequent degradation of m-RNA left exposed by the release of ribosomes after translation of a nonsense codon. The *suA* mutation relieves polarity because it lacks the endonuclease present in the wild type.

It is impossible to reconcile these hypotheses. The data presented here certainly do not indicate that transcription ceases at the mutant site in the galactose operon, whatever the case may be in the tryptophan operon. Further, if transcription and translation are directly coupled as Stent and Imamoto suggest, one might expect to find that the amount of enzyme produced was directly proportional to the amount of messenger. There is certainly less gal m-RNA in the polar mutants than in the wild type, but the level is much higher than might be expected from the levels of galactokinase, and increasing the degree of polarity reduces the level of enzyme more

than it reduces the m-RNA content. This is not inconsistent with the Morse hypothesis, in that the excess m-RNA could be in the process of degradation from the 5' -end or from post-mutation scission, but still be large enough to be collected in the preparation and identified by hybridisation.

It is possible that the mechanism of polarity is not the same in all operons. The tryptophan operon, after all, differs from the galactose operon in a number of ways, not the least important of which is that the trp operon is end-product repressible, while the galactose operon is catabolite repressible. However, the *suA* mutation has been shown to relieve polarity in both the trp operon (Morse and Primakoff, 1970) and the lac operon (Beckwith, 1963; Scaife and Beckwith, 1966) as measured by m-RNA levels, without suppression of the original mutation. The lac and gal operons are very similar in regulatory mechanisms, and this suggests that all operons subject to polar mutations may be affected in the same way.

In summary, polar mutations have transcriptional and translational effects, both of which are proportional to the degree of polarity. The transcriptional effect may be the result of reduced synthesis of RNA or of increased degradation. These can not yet be distinguished from each other. The transcriptional and translational effects are each less than the total, and the polar phenotype is the sum of both effects.

5. Extensions of this research

In addition to the assays of cyclic AMP already suggested, the work described here is capable of further extension. The technique of separation of the DNA strands of λ dg (Hradecna and Szybalski, 1967;

Guha et al, 1968) can be used to purify part of the gal m-RNA. The sense strand may be identified by hybridisation, followed by hybridisation of RNA to the sense strand of λ dg JG3 in liquid (Nygaard and Hall, 1963, 1964). The hybrid can then be isolated, either by isopycnic buoyant density centrifugation or by fractionation on a hydroxylapatite column, and the RNA from the hybrid (including gal m-RNA and RNA species complementary to the non-gal DNA of the λ dg) recovered, purified and in turn hybridised to the sense strand of λ dg JG1. The RNA which did not hybridise to JG1, but had hybridised to JG3 could then be recovered, and should contain only the m-RNA sequences complementary to the transferase and kinase cistrons, i.e. that part of the gal operon carried by λ dg JG3 but not by JG1.

This purified gal m-RNA might then be used to confirm the saturation values, specific radioactivity etc. given here, and also for biochemical and biophysical analyses which could prove of value in further investigating the mechanism of bacterial enzyme regulation.

SUMMARY

1. A series of transducing bacteriophages carrying different parts of the galactose operon was obtained, and those which were not already temperature sensitive were made so for ease of induction.
2. Hybridisation experiments using DNA from these bacteriophages and pulse labelled RNA from a fucose induced culture of a strain of E.coli, with a deletion of the galactose operon in the main chromonema but carrying the whole operon on an episome were performed. The results of these were used to determine the best method of measuring galactose messenger RNA contents. A linear formulation of the hybridisation reaction developed by Bishop et al (1969) was used to obtain the true saturation value for this RNA.
3. The method selected involved calculation of the difference between hybridisation to two λ dgs, one of which carried almost all three cistrons of the galactose operon, the other only the first cistron. This method, and the Bishop analysis, were used to estimate the galactose messenger RNA contents of F^- and F' strains under different conditions of induction and repression. The measurements obtained were related to the levels of the enzyme galactokinase assayed in the same cultures. The effects of induction by a gratuitous and a metabolised inducer were compared, and the effects of both upon the enzyme and messenger RNA contents of wild type and constitutive strains with different numbers of the galactose genes investigated. Messenger RNA and enzyme levels of a culture repressed with methyl- β -D-thiogalactoside were measured. The significance of the results is discussed, and hypotheses incorporating recent investigations into the mechanism of induction and repression are constructed.

4. The galactose messenger RNA and galactokinase levels of four polar mutants were measured by the same method, and the current hypotheses about polarity discussed in relation to the findings.

5. A genetic analysis of the episome and transducing bacteriophages used in these experiments was performed.

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Appendix A

Experimental data tabulated in form: RNA input in μg per μg DNA
(H) against counts per minute hybridised per μg DNA (C).

Table A1

RNA Source:- 9-3h⁺/FR5 Fucose Induced Series I

17-8 DNA		JG1 DNA	
RNA input in μg/μg DNA (H)	Cpm hybridised per μg DNA (C)	RNA input in μg/μg DNA (H)	Cpm hybridised per μg DNA (C)
606	336) 298) 317	686	170) 149) 160
434	291) 264) 278	556	131) 206) 169
293	304) 230) 267	395	116) 156) 136
161	179) 158) 169	198	130) 86) 108
71	118) 103) 111	82	48) 56) 52

RNA Source:- 9-3h⁺/FR5 Non-induced Series I

17-8 DNA		JG1 DNA	
RNA input H	Cpm hybridised C	RNA input H	Cpm hybridised C
1244	219) 218) 219	2000	155) 160) 158
873	172) 175) 174	1049	103) 86) 95
513	122) 127) 125	659	70) 57) 64
352	95) 105) 100	338	40) 39) 40
212	64) 68) 66	155	19) 22) 21

Table A1 Continued

RNA Source:- 9-3h⁺/FR5 TMG Repressed Series I

17-8 DNA			JG1 DNA		
RNA input	Cpm hybridised		RNA input	Cpm hybridised	
H	C		H	C	
1844	271 } 300 }	286	1588	193 } 119 }	156
933	202 } 199 }	201	916	88 } 92 }	90
535	145 } 136 }	141	648	66 } 82 }	74
342	108 } 112 }	110	424	55 } 53 }	54
152	57 } 58 }	58	204	34 } 32 }	33

Table A2

RNA Source:- E1 Fucose Induced Series II

17-8 DNA		JG1 DNA	
RNA input H	Cpm hybridised C	RNA input H	Cpm hybridised C
568	480 } 504 528 }	501	307 } 319 330 }
460	500 } 460 419 }	399	198 } 257 315 }
348	375 } 382 388 }	248	195 } 203 211 }
153	298 } 304 309 }	135	156 } 145 133 }
76	174 } 178 181 }	69	85 } 86 86 }

RNA Source:- E1/FR5 Fucose Induced Series II

17-8 DNA		JG1 DNA	
RNA input H	Cpm hybridised C	RNA input H	Cpm hybridised C
547	477 } 503 528 }	517	278 } 297 316 }
429	424 } 424 424 }	351	258 } 241 224 }
302	359 } 355 351 }	251	209 } 190 171 }
149	268 } 269 270 }	124	123 } 122 120 }
73	217 } 237 256 }	63	102 } 90 78 }

Table A2 Continued

RNA Sources:- 9-3h⁺/FR5 Fucose Induced Series II

17-8 DNA		JG1 DNA	
RNA input H	Cpm hybridised C	RNA input H	Cpm hybridised C
564	437) 354) 396	441	140) 136) 138
445	327) 300) 319	351	115) 119) 117
273	265) 321) 293	247	107) 115) 111
138	214) 234) 224	144	92) 68) 80
68	164) 190) 177	95	63) 59) 61

RNA Source:- CF4 Non-induced Series II

17-8 DNA		JG1 DNA	
RNA input H	Cpm hybridised C	RNA input H	Cpm hybridised C
600	295) 280) 288	478	152) 162) 157
489	238) 259) 249	387	141) 146) 144
292	231) 197) 214	232	107) 116) 112
147	156) 141) 149	120	60) 65) 63
74	117) 100) 109	91	43) 51) 47

Table A2 Continued

RNA Source:- TMG 6 Non-induced

17-8 DNA			JG1 DNA		
RNA input	Cpm hybridised		RNA input	Cpm hybridised	
H	C		H	C	
568	303 } 284 }	294	502	127 } 158 }	143
461	258 } 272 }	265	413	146 } 120 }	133
274	231 } 222 }	227	222	100 } 91 }	96
133	174 } 176 }	175	124	68 } 58 }	63
69	118 } 90 }	104	61	36 } 37 }	37

Table A3.

RNA Source:- 9-3h⁺/FR5 Fucose Induced Series III (1)

17-8 DNA		JG1 DNA		λ pg DNA		JG3 DNA	
RNA Input	Cpm Hyb	RNA Input	Cpm Hyb	RNA Input	Cpm Hyb	RNA Input	Cpm Hyb
H	C	H	C	H	C	H	C
512	198) 199)198	463	79) 86)82	450	124) 129)127	614	149) 164)155
461	250) 208)229	329	75) 71)73	367	138) 114)125	473	157) 142)148
310	201) 191)195	220	58) 58)58	232	117) 136)126	257	119) 136)127
169	168) 159)163	129	54) 50)52	111	96) 97)97	125	110) 94)101
59	90) 90)90	54	34) 33)34	59	69) 88)78	63	66) 61)63

RNA Source:- 9-3h⁺/FR5 Fucose Induced Series III (2)

17-8 DNA		JG1 DNA		λ pg DNA		JG3 DNA	
RNA Input	Cpm Hyb	RNA Input	Cpm Hyb	RNA Input	Cpm Hyb	RNA Input	Cpm Hyb
H	C	H	C	H	C	H	C
423	200) 185)193	441	80) 78)79	501	169) 133)151	717	135) 132)134
309	179) 172)176	425	81) 76)79	372	130) 147)139	642	140) 156)148
212	173) 175)174	226	62) 66)64	235	121) 128)125	401	117) 129)123
105	134) 137)136	136	53) 49)51	114	96) 102)99	195	96) 92)94
52	85) 77)81	70	35) 35)35	67	82) 90)86	97	72) 67)70

Table A3 Continued

RNA Source:- 9-3h⁺/FR5 Non-induced Series III (1)

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
876	104	876	94
839	118	839	77
627	86	627	67
614	88	614	73
429	75	429	57
399	72	399	53
216	46	216	31
199	46	199	35
115	33	115	24
105	37	105	22

RNA Source:- 9-3h⁺/FR5 Non-induced Series III (2)

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
766	97	766	56
805	89	805	68
584	80	584	53
636	72	636	60
910	91		
412	59	412	43
210	42	210	33
227	42	227	27
99	26	99	19
108	24	108	20

Table A3 Continued

RNA Source:- 9-3h⁺/FR5 Fucose Induced Series III (3)

17-8 DNA		JG1 DNA		λ pg DNA		JG3 DNA	
RNA Input	Cpm Hyb	RNA Input	Cpm Hyb	RNA Input	Cpm Hyb	RNA Input	Cpm Hyb
H	C	H	C	H	C	H	C
732	212) 206) 209	739	113) 96) 104	558	167) 136) 151	772	183) 155) 168
467	162) 166) 164	477	89) 82) 85	324	137) 174) 159	566	160) 160) 160
333	174) 140) 157	308	67) 65) 66	252	125) 134) 130	350	152) 147) 150
160	138) 133) 136	198	61) 70) 65	130	110) 112) 111	165	119) 106) 112
76	102) 81) 91	79	41) 34) 38	71	93) 92) 93	97	104) 87) 96

Table A3 ContinuedRNA Source:- 9-3h⁺/FR5 Galactose Induced Series III

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
530	86	530	68
526	84	526	63
399	85	399	60
392	74	392	57
266	58	266	46
257	53	257	38
131	35	131	22
129	37	129	21
62	22	62	15
65	25	65	16

RNA Source:- 9-3h⁺/FR5 TMG Repressed Series III

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
847	74	847	40
833	71	833	49
597	61	597	29
625	60	625	37
386	48	386	30
431	50	431	32
214	37	214	18
220	33	220	18
102	20	102	11
103	19	103	15

Table A4RNA Source:- E1 Fucose Induced Series IV

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
614	296	614	207
790	282	790	233
505	222	505	159
716	277	716	227
311	182	311	153
465	248	465	181
171	138	171	116
161	146	161	71
82	91	82	68
90	89	90	54

RNA Source:- E1 Galactose Induced Series IV

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
798	160	798	118
1078	209	1078	156
608	139	608	114
684	145	684	139
375	123	375	91
443	114	443	109
199	91	199	61
228	87	228	75
92	54	92	44
110	62	110	48

Table A4 Continued

RNA Source:- E1/FR5 Fucose Induced Series IV

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
520	459	520	396
517	543	517	295
539	334	524	275
524	334	539	226
301	272	280	198
280	264	301	174
157	239	150	172
150	244	157	142
83	129	73	103
73	123	83	138

RNA Source:- E1/FR5 Galactose Induced Series IV

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
583	191	547	140
547	181	583	135
486	191	425	112
425	160	486	135
314	177	251	96
251	134	314	102
139	118	131	88
131	100	139	86
76	103	66	64
66	94	76	75

Table A4 Continued

RNA Source:- TMG6 Fucose Induced Series IV

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
539	94	568	61
568	107	539	58
432	90	382	56
382	86	432	55
269	64	224	39
224	62	269	44
124	53	129	31
129	48	124	31
60	40	66	18
66	42	60	22

RNA Source:- TMG6 Galactose Induced Series IV

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
567	87	553	57
553	95	567	55
450	83	474	60
474	85	450	52
310	71	322	47
322	81	310	47
181	57	146	38
146	57	181	45
89	56	84	37
84	46	89	32

Table A4 Continued

RNA Source:- TMG6/FR5 Fucose Induced Series IV

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
538	140	579	101
579	171	538	92
392	122	502	90
502	182	392	84
304	111	307	74
307	138	304	79
118	81	110	38
110	76	118	40
78	69	72	39
72	73	78	36

RNA Source:- TMG6/FR5 Galactose Induced Series IV

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
657	110	586	83
586	131	657	94
489	112	514	93
514	121	489	72
351	106	322	63
322	109	351	70
159	71	168	47
168	78	159	42
79	62	86	35
86	63	79	41

Table A4 Continued

RNA Source:- TMG6 Non-induced Series IV

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
785	270	860	225
860	310	785	178
525	225	645	181
645	233	525	143
368	181	425	130
425	230	368	90
223	175	169	63
169	148	223	96
99	117	115	72
115	127	99	59

Table A5

RNA Source:- F' gal-₃ Fucose Induced Series V

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
884	91	779	92
779	111	884	82
640	80	804	88
804	99	640	74
345	60	521	67
521	84	345	49
263	43	244	40
244	50	263	53
108	25	116	28
116	24	108	25

RNA Source:- NA119/FR119 Fucose Induced Series V

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
785	91	828	86
828	78	785	78
616	80	649	80
649	77	616	66
521	82	395	56
395	64	521	60
239	42	215	34
215	45	239	41
102	25	116	22
116	24	102	22

Table A5 Continued

RNA Source:- NA121/FR121 Fucose Induced Series V

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
895	144	1020	143
1020	181	895	148
701	158	820	130
820	163	701	107
486	95	631	119
631	150	486	117
199	74	231	72
231	90	199	51
118	57	111	40
111	63	118	46

RNA Source:- NA156/FR156 Fucose Induced Series V

JG3 DNA		JG1 DNA	
RNA Input	Cpm Hybridised	RNA Input	Cpm Hybridised
H	C	H	C
988	79	920	71
920	100	988	92
839	84	738	53
738	80	839	74
513	74	501	51
501	64	513	45
226	40	244	44
244	41	226	34
127	29	143	26
143	38	127	28

Appendix B

Galactose-specific messenger Ribonucleic Acid contents in
Escherichia Coli.

Gosden, J.R., Irving, M.I. and Bishop, J.O.

Biochem. J. (1971). 121, 109-116.

Galactose-Specific Messenger Ribonucleic Acid Contents in *Escherichia coli*

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A method is described for measuring the proportion of galactose-specific mRNA (gal-mRNA) in the total RNA extracted from pulse-labelled cells of *Escherichia coli* K12, by DNA-RNA hybridization with DNA prepared from bacteriophage λ dg. RNA from wild-type *E. coli* was compared with RNA from a homogenote carrying the *gal* operon both in the chromosome and in a substituted sex-factor, and with RNA from a deletion strain that carried the galactose operon only in the exogenote. In each case the cultures were induced with fucose. Under these conditions the amount of gal-mRNA was found to be proportional to the content of galactokinase in the different cultures, and to the gene frequency. The amounts of gal-mRNA in an O^c mutant and an R^- mutant were also proportional to the observed contents of galactokinase. In cultures repressed for the enzymes of the galactose operon with thiomethylgalactoside, the content of gal-mRNA was higher than expected from the content of galactokinase. Possible explanations of this finding are discussed.

The galactose operon of *Escherichia coli* K12 consists of structural genes that specify three enzymes of galactose metabolism, together with controlling elements (Buttin, 1963b). The three enzymes (kinase, transferase and epimerase) are co-ordinately and sequentially inducible (Buttin, 1963a; Michaelis & Starlinger, 1967). The effective inducers are the substrate D-galactose and the gratuitous inducer D-fucose. Control of induction is mediated by a regulator gene situated at a remote position on the *E. coli* chromosome (Buttin, 1963b), and control is of the negative type (Saedler, Gullon & Starlinger, 1968). The galactose operon is thus closely analogous to the lactose operon, and offers the advantage that the operator and regulator loci are widely separated.

Because of its similarity to the lactose operon, regulation of the galactose operon is generally interpreted in terms of the classical Jacob-Monod mechanism. Accordingly, control of the content of the enzymes is thought to be brought about by control of the rate of mRNA synthesis, and consequently of the amount of mRNA available for transcription. We therefore expect the amount of enzyme activity in an exponentially growing culture to reflect the mRNA content. Previous direct measurements of the amounts of enzyme and mRNA in induced and non-induced cultures have not always been consistent with this expectation, however.

In the present paper we report the results of a further study of the relationship between enzyme and mRNA contents in exponentially growing cells. Enzyme contents were varied in two ways: by induction and repression, and by varying gene dosage. mRNA was measured by the method described by Bishop & Irving (1971). The results show that enzyme and mRNA contents are strongly correlated, except when the galactose enzymes are repressed with thiomethyl galactoside.

MATERIALS AND METHODS

Bacteria. The strains of *E. coli* used were as follows. Strain W3805, F^-gal22 (epimerase-negative); strain E1 $F^-histrpsr^+$ (Frame & Bishop, 1971); strain 9-3, $F^-histrpsr^+gal$, a deletion of the entire galactose operon running from att^A to *aro-G* (Frame & Bishop, 1971; see Fig. 1); strains 9-3t and 9-3h, trp^+ and his^+ derivatives respectively of strain 9-3; strain TMG6, $F^-histrpsr^+Rgal$, a regulator gene mutant produced from strain E1 as described by Buttin (1963b); strain CN4, $F^-histrpsr^+O^cgal$, a spontaneous operator constitutive mutant of strain E1 isolated by Miss C. Ford; strains E1 (F'8), E1 (FR5) and 9-3h(FR5), derivatives carrying episomes (F'8 or FR5) which contain the galactose operon. F'8 is the substituted sex-factor isolated by Hirota & Sneath (1961), and contains both the *gal* and *aro-G* loci. FR5 is a sex-factor containing *gal*, *aro-G* and genes conferring resistance to chloramphenicol (Cm), ampicillin (Pn), tetracycline (T) and streptomycin (S). This was produced at the suggestion of Dr E. Meynell, by transducing part of the

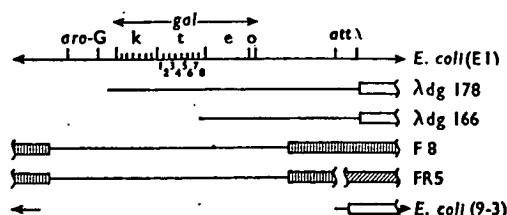


Fig. 1. Genetic map of the strains of *E. coli*, sex-factors and bacteriophage used. The distances between markers are arbitrary. The galactose operon (*gal*) is shown divided into kinase (*k*), transferase (*t*), epimerase (*e*) and operator (*o*) regions. The eight blocks of the transferase cistron were defined previously (Davison, Frame & Bishop, 1967). The left-hand end of λ dg166 defines blocks 7 and 8. The left-hand end of λ dg178 falls between *gal* and *aro-G*. Both F'8 and FR5 carry *gal* and *aro-G* and do not carry *att*⁺, but the extent of the segment of the *E. coli* chromosome is not more precisely known in either case. *E. coli* 9-3 carries *att*⁺ and lacks *aro-G* and *gal*. —, *E. coli*; ■, F; ▨, RTF; □, λ DNA.

RTF R1-19 (R;S,Su,K,Cm,Pn,T) from one strain of *Salmonella typhimurium* LT-2 to another, and then mobilizing the drug-resistance factors by introducing F'8 and conjugation with *E. coli* 9-3t, selecting both for drug resistance and for the ability to ferment galactose. The factor was initially very unstable, but was successfully stabilized by five cycles of conjugal transfer (from 9-3h to 9-3t or vice versa) each followed by a period of relaxation and then re-selection for drug-resistance and ability to ferment galactose.

Bacteriophage. The bacteriophages used were as follows: bacteriophage λ c₁⁸⁵⁷; bacteriophage λ dg 178, a derivative of bacteriophage λ c₁⁸⁵⁷ which carries the entire galactose operon, but does not carry the *aro-G* locus; bacteriophage λ dg 166, a derivative of wild-type bacteriophage λ that carries the epimerase cistron and a small piece of the transferase cistron, subsequently made heat-inducible by recombination with bacteriophage λ c₁⁸⁵⁷.

Growth and purification of transducing bacteriophage. Strain W3805, doubly lysogenic for the wild-type and transducing bacteriophage, was inoculated from minimal-galactose plates into minimal-galactose medium and grown overnight at 33°C with vigorous shaking. The culture was added to 12 litre of broth (1% tryptone, 0.1% yeast extract, 0.8% NaCl, 0.1% MgSO₄, 0.03% CaCl₂, 0.2% galactose) and grown with vigorous aeration to a density of about 10⁹ cells/ml (*E*₆₅₀ 0.8). The temperature was raised to 42°C, and after 20 min it was lowered again to 37°C. Aeration was continued until the cells were lysed. Chloroform was added and the culture was vigorously agitated, but not aerated. After being kept overnight at room temperature, the lysate was decanted, and transferred to the cold-room. To each litre of lysate were added 23 g of NaCl, 300 ml of 30% (w/v) polyethylene glycol 6000 and 15 ml of 40% (w/v) dextran sulphate 500. After 1–2 days at 4°C the lower phase and interphase were withdrawn and centrifuged at 10000 rev./min for 40 min in a Sorvall RC-2B centrifuge. The interphase was

recovered and dissolved in water to give a final volume of 20 ml/litre of initial lysate. One-quarter of the volume of 3M-KCl was added and after being kept overnight at 4°C insoluble material was removed by centrifugation and discarded. The supernatant was centrifuged at 30000 rev./min in the Spinco no. 30 rotor at 4°C for 120 min to sediment the bacteriophage, and the pellet was taken up in 2 ml of 0.1M-NaCl–7 mM-MgCl₂/litre of initial lysate and treated with 5 μ g of ribonuclease/ml and 10 μ g of deoxyribonuclease/ml for 30 min at 37°C. The bacteriophage was again sedimented by centrifuging for 45 min at 30000 rev./min in the no. 30 rotor, and the pellets were taken up in 0.1M-NaCl–0.01M-tris-HCl buffer, pH 7.5. Solid CsCl was added to 46% (w/w) and the solution was centrifuged for 1 h at 20000 rev./min in the Sorvall SS-34 rotor. The supernatant solution was then centrifuged (15 ml/tube overlaid with liquid paraffin) for 42 h in the Spinco no. 30 rotor at 27000 rev./min. Fractions were collected from the tubes, the transducing bacteriophages were identified and pooled fractions were dialysed against 0.1M-NaCl–0.01M-tris-HCl buffer, pH 7.5.

³²P-labelled wild-type bacteriophage λ was prepared by heat-inducing strain E1 (λ c₁⁸⁵⁷) grown in broth (as described above, but with glucose substituted for galactose) containing 1–2 mCi of [³²P]P_i/100 ml. The labelled bacteriophage was purified as described above, except that each overnight period at 4°C was replaced by stirring for 1 h in an ice-bath, and the final equilibrium centrifugation was carried out at 45000 rev./min in the Spinco no. 50 rotor.

DNA was prepared as required from suitable mixtures of labelled and unlabelled bacteriophage by extracting three times with water-saturated phenol. The final aqueous phase was dialysed against 10 mM-NaCl–10 mM-tris-HCl buffer, pH 7.5. The specific radioactivity of the DNA was measured by carefully measuring its extinction at 260 nm and its radioactivity when applied to membrane filters of the type used for DNA-RNA hybridization experiments. The extinction of a 1 mg/ml solution was taken to be 20. Less than 1% of labelled wild-type bacteriophage was added to unlabelled transducing bacteriophage preparations.

Growth of bacteria. Cells were grown in 400 ml batches in a rotary shaker at 37°C in glycerol-casamino acids (casein hydrolysate) medium containing 5 mM-D-fucose or 2.5 mM-thiomethyl galactoside in some cases. The cells were pulse-labelled at a cell density of about 10⁹/ml (*E*₆₅₀ 0.8) by adding 50 μ Ci of [¹⁴C]uracil/400 ml of culture, and harvested after precisely 1% of the doubling time by pouring each 400 ml of culture on to 160 ml of crushed frozen buffer [10 mM-tris-HCl (pH 7.5 at 0°C)–10 mM-MgCl₂–10 mM-Na₂EDTA]. The cells were collected by centrifugation and taken up in 10 mM-Na₂EDTA–0.1M-NaCl–20 mM-sodium acetate buffer, pH 5. At this point the preparation could be stored at –20°C.

RNA was prepared from freshly harvested or frozen and thawed cells as described by Bishop & Robertson (1969).

DNA-RNA hybridization. The method of Gillespie & Spiegelman (1965) was used, with the minor modifications described in the preceding paper. Annealing was for 40 h at 65°C in 0.3M-NaCl–30 mM-sodium citrate buffer, pH 7.

Assay of galactokinase. During growth in glycerol-casamino acids medium (see above) a 40 ml portion was

removed when the E_{650} of the culture was 0.6, and chilled in ice. After 30 min the E_{650} was again measured. The cells were collected by centrifugation, washed with 40 ml of 0.1M-sodium phosphate buffer, pH 7.2, recentrifuged and resuspended in 4 ml of phosphate buffer containing 5 mM-GSH. A 0.25 ml portion of the suspension was mixed with 0.75 ml of a solution containing 1 mg of lysozyme/ml, 5 mM-tris-acetate buffer, pH 8, 0.25 mM-EDTA, 1 mM-GSH and incubated for 5 min at 37°C. Lysis was checked by microscopic examination. The assay was carried out by mixing three 0.025 ml samples of lysate with 0.025 ml of a solution containing 0.05 μ mol of [^{14} C]-galactose (specific radioactivity 1 Ci/mol), 0.1 μ mol of ATP, 0.0832 μ mol of $MgCl_2$, 0.2 μ mol of NaF and 2.5 μ mol of tris-HCl buffer, pH 7.5 at 37°C. After 0, 5 and 10 min at 37°C the reaction in one tube was stopped by adding 0.95 ml of ice-cold water and placing the tube in an ice bath. Samples (0.1 ml) were spotted on 2.3 cm diameter DEAE-paper discs (Whatman DE81) and the discs were washed four times in water to remove galactose. The discs, which retain galactose 1-phosphate quantitatively, were dried and assayed in a liquid-scintillation counter. Enzyme activities were normalized by dividing by the extinction of the chilled culture, and by the activity/extinction ratio for a culture of *E. coli* strain E1 grown, harvested, and assayed in parallel.

Calculation of the proportion of galactose mRNA. The principle of the method is described by Bishop & Irving (1971). After annealing various amounts of total cellular RNA with a constant amount of DNA a plot is constructed of H/c against H , where H is the ratio of input RNA to DNA recovered (DNA recovered being the only estimate available of the DNA input) and c is the radioactivity in galactose mRNA hybridized/ μ g of DNA recovered. A straight line is fitted to the plot and values of c_s and K are obtained from the slope and intercept respectively according to the relationship:

$$H/c = H/c_s + K \quad (1)$$

In the present case we are faced with the difficulty that mRNA other than gal-mRNA hybridizes with bacteriophage λ dg DNA (Guha, Tabaczynski & Szybalski, 1968). We dealt with this in the following way. The polypeptide chains specified by the kinase, transferase and epimerase cistrons are approximately equal in size [kinase, 38000 daltons (Wilson & Hogness, 1964); transferase, 40000 daltons (Saito, Ozutsumi & Kurahashi, 1967); epimerase, 40000 daltons (Wilson & Hogness, 1966)]. The transducing bacteriophage λ dg 166 carries only the epimerase cistron and a portion, probably very small, of the transferase cistron. We assume that the gal-mRNA that hybridizes with λ dg 166 DNA is one-third of the total, ignoring the small amount of transferase RNA and any RNA that may be transcribed from the controlling elements of the operon. Secondly, we assume that the difference between hybridization to λ dg 178 DNA and λ dg 166 DNA represents two-thirds of all gal-mRNA, ignoring any RNA that may hybridize with λ dg 178 DNA to the left of the *gal* operon (see Fig. 1). Thus the radioactivity in gal-mRNA hybridizing at any given value of H is taken to be $3(c_{178} - c_{166})/2$, where c_{178} and c_{166} are the amounts of radioactivity in mRNA hybridized to λ dg 178 and λ dg 166 DNA respectively at a given value of H . In practice, it was impossible to arrange that the amounts

of the two types of DNA were precisely the same, owing to variable retention by the membrane filters. Values of $(c_{178} - c_{166})$ were therefore obtained by fitting straight lines to plots of H/c_{178} against H and H/c_{166} against H , reading off values for several values of H , and calculating $(c_{178} - c_{166})$ from these values.

The specific radioactivity of gal-mRNA (s , c.p.m./ μ g) was calculated from c_s according to eqn. 5 of the previous paper (Bishop & Irving, 1971), taking $M\lambda = 30 \times 10^6$. The value of $Mgal$ was calculated to be 1.02×10^6 by multiplying the sum of the molecular weights of the three polypeptide chains by 8.5 (the ratio of the molecular weight of an average trinucleotide, less one molecule of water, to the molecular weight of an average *E. coli* amino acid, less one molecule of water):

$$s = 1.75c_s M\lambda / Mgal = 53c_s \quad (2)$$

Then the proportion of the total RNA which is gal-mRNA is:

$$\alpha = 1/K's = 0.019/K's \quad (3)$$

and the proportion of the total radioactivity contained in gal-mRNA is:

$$\beta = 1/K'S \quad (4)$$

where S is the overall specific radioactivity of the total RNA.

RESULTS

Control experiments. (a) Time-course of hybridization. Fig. 2 shows the result of a time-course experiment with λ dg178 DNA and total pulse-labelled RNA from fucose-induced cells of the homogenote E1 (F'8). The amount of radioactivity becoming hybridized increased at least up to 40 h, which was adopted as the standard annealing time.

(b) Effect of varying the amount of DNA. The results for rRNA (Bishop & Irving, 1970) show that under the conditions used for these experiments, the specific hybridization (RNA hybridized/ μ g of DNA) is more directly related to the RNA/DNA ratio than to the RNA concentration. Fig. 3 shows that this is true also for the hybridization of *E. coli* pulse-labelled RNA with λ dg178 DNA. In Fig. 3(a) the hybridization (c , c.p.m./ μ g of DNA) is plotted against the RNA/DNA ratio (H). The curves generated by using 1.2 and 4.4 μ g of DNA per sample are virtually the same. In contrast, when c is plotted against the RNA concentration, the two curves are quite different (Fig. 3b).

(c) Hybridization to DNA of wild-type λ and hybridization of RNA from a deletion strain. RNA extracted from pulse-labelled cells of strain E1 (F'8) was compared with RNA from strain 9-3t, which carries a deletion from *att*⁺ to beyond *aro*-G. Both strains were grown in the presence of D-fucose. The RNA was annealed with DNA from wild-type λ and from λ dg178. When RNA from strain E1 (F'8) was used, about 20 times more radioactivity

hybridized to λ dg178 DNA than to wild-type DNA (Fig. 4). Thus at least 95% of the homology between pulse-labelled RNA and λ dg178 DNA is due to sequences not present in the wild-type. Very little hybridization was observed between RNA from the deletion strain and either type of DNA. Thus, when hybridization is observed, it is due to transcription from the region deleted, i.e. from *att* ^{λ} to beyond *aro*-G. This conclusion was tested further by hybridization-competition experiments between labelled E1 (F'8) RNA and unlabelled 9-3t RNA. In agreement with the results of the saturation experiments, very little competition was observed.

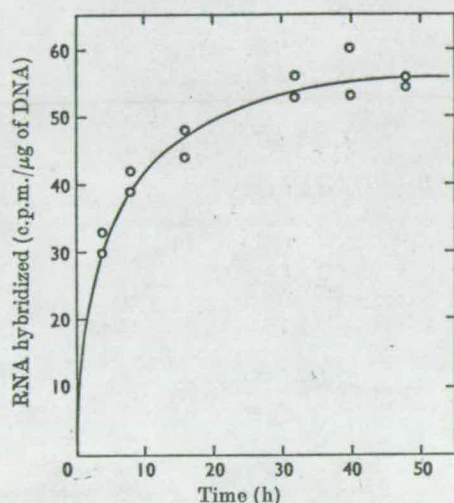


Fig. 2. Time-course of hybridization. The ¹⁴C-labelled RNA was extracted from a fucose-induced culture of E1 (F'8). ³²P-labelled λ dg178 DNA was prepared as described. The samples contained an average of 4.6 μ g of DNA (2.3 μ g/filter) and the RNA/DNA ratio was 96.

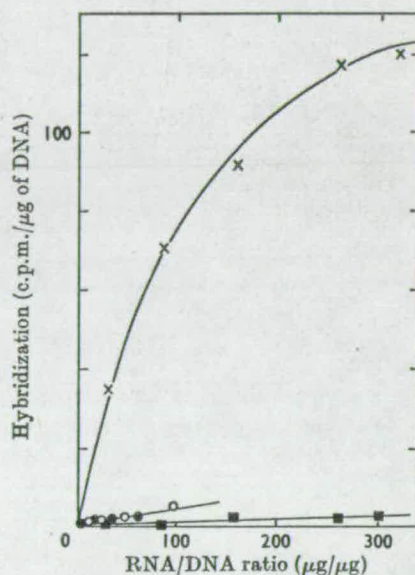


Fig. 4. Hybridization of RNA from a fucose-induced culture of E1 (F'8) and from the deletion strain 9-3h with DNA from λ dg178 and wild-type λ . $\times$, E1 (F'8) RNA $\times$ λ dg178 DNA; $\blacksquare$, E1 (F'8) RNA $\times$ λ DNA; $\circ$, 9-3h RNA $\times$ λ dg178 DNA; $\bullet$, 9-3h RNA $\times$ λ DNA.

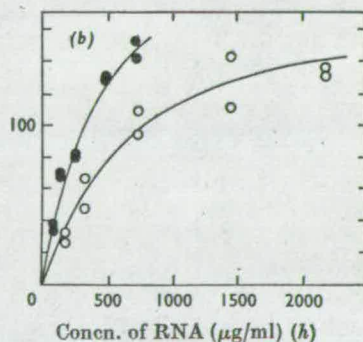
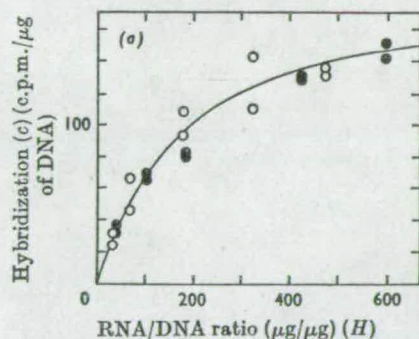


Fig. 3. Effect of DNA concentration on DNA-RNA hybridization. The samples contained an average of 1.2 ($\bullet$) and 4.4 ($\circ$) μ g of DNA (0.6 and 2.2 μ g per filter). The annealing time was 40 h. (a) Hybridization (c) plotted against DNA/RNA ratio (*H*). (b) Hybridization plotted against RNA concentration (*h*).

Measurement of galactose-specific mRNA. Fig. 5 shows the results of an experiment in which pulse-labelled RNA from a fucose-induced culture of strain E1 (F'8) was annealed in parallel with λ dg178 and λ dg166 DNA. Fig. 5(a) shows the hybridization observed as a function of RNA/DNA ratio (*H*). Fig. 5(b) shows the hybridization of gal-mRNA deduced from the data as described above, and Fig. 5(c) shows the reciprocal plot for gal-mRNA. This plot gives K' 1.08, c_s 42.6, from which *s* is

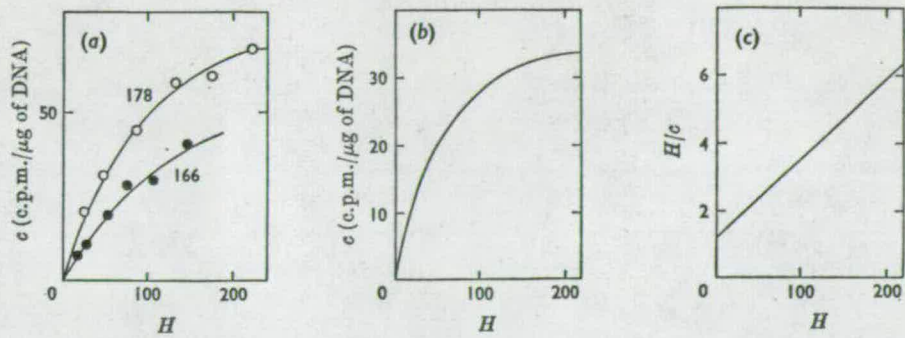


Fig. 5. Calculation of the hybridization of gal-mRNA. (a) Hybridization of RNA extracted from a fucose-induced culture of E1 (F'8) with λdg178 (○) and λdg166 (●) DNA. (b) Hybridization of gal-mRNA deduced from 5(a). (c) Reciprocal plot of the hybridization of gal-mRNA.

Table 1. Variation of gal-mRNA with gene dosage and enzyme content

All cultures were induced with D-fucose and pulse-labelled with [¹⁴C]uracil. The number of gene copies is given relative to wild-type assuming that in glycerol-casamino acids (generation time about 50 min) there is one replicating fork per chromosome on average, and two episomes per fork (Frame & Bishop, 1971). With one exception the amount of galactokinase was measured in a sample of the culture removed just before the pulse of uracil, and compared with a parallel sample of strain E1. In the exceptional case (*) the content of galactokinase commonly found in strains of E1 (F'8) was used.

Strain	Genotype	Gene copies	Kinase	Kinase	10 ⁵ × α	10 ⁵ α/Kinase
				Gene copies		
E1	gal ⁺	(1)	(1)	(1)	23	23
E1 (F'8)	gal ⁺ /Fgal ⁺	2.4	2.4*	1*	41.3	17
E1 (FR5)	gal ⁺ /Fgal ⁺	2.4	2.32	0.97	49.5	21
9-3h (FR5)	Δgal/Fgal ⁺	1.4	1.05	0.75	17.7	17
		1.4	1.31	0.94	24.8	19
					Average	19

Table 2. Variation of gal-mRNA content with enzyme induction and repression

Cultures were grown in glycerol-casamino acids medium and induced with D-fucose or repressed with thiomethyl galactoside. In most cases enzyme activities were measured on samples removed from the cultures just before pulse-labelling and compared with parallel samples of strain E1. In some cases (*) the enzyme activity characteristic of the strain and growth conditions was adopted. —, Indicates neither induced nor repressed, i.e. with glycerol alone.

Strain	Genotype	State of culture	Kinase	10 ⁵ × α	10 ⁵ α/Kinase
E1	gal ⁺	Induced	(1)	23	23
CN4	gal ⁺ 0 ^c	—	1.73	37.6	22
TMG 6	gal ⁺ R-	—	1.13	27.8	24
E1 (F'8)	gal ⁺ /Fgal ⁺	Induced	2.4*	41.3	17*
		Repressed	0.12*	6.2	52*
9-3h (FR5)	Δgal/Fgal ⁺	Induced	1.31	24.8	19
		—	0.15	3.9	26
		Repressed	0.08	3.2	40

Table 3. *Specific radioactivities of total and gal-mRNA*

The specific radioactivity of the total RNA was calculated directly. That of the gal-mRNA was obtained by using eqn. 2. [The overall specific radioactivities of the E1 (F⁸) preparations shown in Tables 1 and 2 are not available.]

Strain	State of culture	Specific radioactivity of total RNA (<i>S</i>) (c.p.m./ μ g)	Specific radioactivity of gal-mRNA (<i>s</i>) (c.p.m./ μ g)	<i>s/S</i> ratio
E1	Induced	693	21360	30.8
E1 (FR5)	Induced	654	21360	32.6
CN4	—	338	12640	37.4
TMG6	—	507	17070	33.6
9-3h (FR5)	Induced	533	30500	57.2
	Induced	344	19860	57.8
9-3h (FR5)	—	256	18640	73
	Repressed	277	26140	94

Table 4. *Proportion of total radioactive label contained in gal-mRNA*

Values of β were calculated by using eqn. 4. Values of α are taken from Tables 1 and 2.

Strain	State of culture	β (%)	β (normalized)	α (normalized)
E1	Induced	0.5	(1)	(1)
E1 (FR5)	Induced	1.2	2.4	2.2
CN4	—	1.0	2.0	1.6
TMG6	—	0.7	1.4	1.2
9-3h (FR5)	Induced	0.6	1.2	0.8
	Induced	0.5	1.0	1.1
9-3h (FR5)	—	0.13	0.26	0.17
	Repressed	0.15	0.30	0.14

found to be 2260 c.p.m./ μ g and $\alpha = 41.3 \times 10^{-4}$; i.e. in this case the gal-mRNA is estimated to make up 0.04% of the total cellular RNA.

(a) Variation of gal-mRNA with gene dosage. The results of a number of experiments are shown in Table 1. As previously reported, the galactokinase content in fucose-induced cells closely parallels the calculated gene dosage (Frame & Bishop, 1971). The values of α for the different genotypes are compared in Table 1 with the galactokinase contents observed in the same cultures. The ratios in the last column are relatively constant, showing that as gene dosage is varied in fucose-induced cultures the same proportional change occurs in both the mRNA and enzyme contents. The closeness of agreement here, where agreement is fully expected, is a partial justification of the method used to measure the mRNA content. Our estimate is that in fucose-induced wild-type cells, in the exponential phase of growth, galactose-specific mRNA makes up about 0.02% of the total mRNA.

(b) Variation of gal-mRNA with enzyme induction and repression. The contents of mRNA found in O⁺ and R⁻ strains were higher than that found

in the wild-type (Table 2). The increases, however, were exactly proportional to the higher enzyme contents in these strains. The mRNA contents found in repressed cultures were less than those in corresponding induced cultures, but the reduction was less than the fall in the galactokinase content. The results suggest that each gal-mRNA molecule is translated 2-3 times less often in the repressed culture. There is an indication that in the non-induced culture mRNA may also be less frequently translated than in fucose-induced cells. However, we consider the difference observed (Table 2, line 7) too small for confident interpretation at this time.

(c) Specific radioactivities of total RNA and gal-mRNA. The specific radioactivities of the total cellular RNA and specific radioactivities calculated for gal-mRNA are shown in Table 3. The highest gal-mRNA specific radioactivities are about half that expected (55×10^3 c.p.m./ μ g) if the [¹⁴C]uracil had been incorporated directly without dilution by endogenous uracil, and if all the pre-existing mRNA had been degraded during the pulse.

Because of variation in the specific radioactivity of the [¹⁴C]uracil and the difficulty of controlling precisely the timing of the pulse, the rate of cooling

of the pulsed culture, and other features of the labelling procedure, variations in the absolute specific radioactivities of the different RNA preparations are probably not meaningful. (An exception is the total RNA from strain CN4, which in repeated tests consistently had a specific radioactivity close to half that of RNA from a parallel culture of strain E1. The reason for this is unknown.) The ratio of specific radioactivities, s/S (Table 3), being internally controlled, provides a more valid basis for comparison. Two main effects are seen. (1) Strain 9-3h, with a deletion from *att^A* to *aro-G*, consistently shows a higher ratio (s/S) than the other strains. All of these strains, it should be noted, were derived from strain E1. The reason for this effect is not clear, but it may be related to the slightly longer generation time (10% longer) of strain 9-3h. (2) The ratio (s/S) is greater for the non-induced and repressed cultures of strain 9-3h (FR5) than for the induced culture. Since these cultures grow at the same rate, and the inducer and repressor are not metabolized, this suggests that gal-mRNA becomes labelled more rapidly in the induced culture, relative to the other species of RNA. The most direct explanation of this is that the lifetime of gal-mRNA is longer in the induced culture, so that newly synthesized gal-mRNA is diluted to a greater extent than it is in the non-induced and repressed cultures.

(d) Proportion of the total radioactivity contained in gal-mRNA. Values of β (eqn. 4) are shown in Table 4. The absolute wild-type (induced) value of β is rather higher than values reported by Hill & Echols (1966) and Guha *et al.* (1968). This may be due in part to differences in the growth conditions and labelling of the cells, and partly to our use of an extrapolation method. The ratio of induced to non-induced 9-3h (FR5) gal-mRNA is close to the ratios previously reported. Our ratio is 4. Hill & Echols (1966) who made no allowance for the hybridization of non-galactose-specific mRNA to λ dg DNA found a ratio of 3.4, and Guha *et al.* (1968) who measured hybridization to the separated strands of λ dg DNA found a ratio of 5. Since Guha *et al.* (1968) did not use an extrapolation method, they would tend to underestimate the non-induced content more seriously than the induced.

The normalized values for β are very similar to those for α except in the case of the non-induced and repressed cultures (Table 4). This is clearly related to the exceptionally high specific radioactivity of the gal-mRNA in these cases. These differences emphasize the importance of measuring the actual amount of mRNA rather than the radioactivity it contains.

For the fully-induced wild-type culture $\beta = 0.005$ and $\alpha = 0.00023$, i.e. there is a 21-fold difference in

the absolute values. The difference is due to the lability of mRNA relative to the stable types. If in our short labelling period incorporation into stable and unstable RNA is approximately equal (Mangiarotti & Schlessinger, 1967; Salser, Janin & Levinthal, 1968), gal-mRNA in the induced culture accounts for $(2 \times \beta)$ or 1% of the radioactivity in unstable RNA. If we assume that the rate of labelling of gal-mRNA is typical of all unstable RNA, we can calculate that unstable RNA makes up about (100×0.00023) or about 2% of the total bacterial RNA. This is in good agreement with previous more direct estimates (Lieve, 1965; Mangiarotti & Schlessinger, 1967; Salser *et al.* 1968; Mueller & Bremer, 1968).

DISCUSSION

It is not possible to set limits to the accuracy of the estimates we have made, because of the number of assumptions involved. The main support for the method is the close correlation observed between gal-mRNA content and enzyme activity when gene-dosage is varied. However, this corroborates the relative contents rather than the absolute determinations. The relative contents are set by the products $K'c$, (see eqn. 3) for the different mRNA preparations, and the normalized values of α (Table 4) which depend only on $K'c$, are independent of the value assumed for M_{gal} and the adjustment made for hybridization efficiency.

However, the number of gal-mRNA molecules per gene copy may be calculated from the data, and as pointed out before this calculation is independent of M_{gal} . We use eqn. (8) of the preceding paper:

$$m = \alpha QMb / \phi M_{gal} = 31\,000\alpha$$

taking the RNA/DNA ratio of cells with a generation time of 50 min (Q) to be 11.5 (Manor, Goodman & Stent, 1969), the amount of DNA per genome (Mb) to be 2700×10^6 daltons, and the average number of genes per genome equivalent (ϕ) to equal 1. For a fucose-induced wild-type cell (Table 1) we take a value of $\alpha = 2 \times 10^{-4}$ and $m = 6$ copies per gene. This, of course, is the steady-state content of gal-mRNA equivalents. If all mRNA molecules are in the process of simultaneous synthesis and degradation, the number of different molecules being concurrently synthesized will be greater than 6. From Table 2 we can calculate that a non-induced or repressed wild-type culture will contain just under 1 gal-mRNA equivalent per gene, and an O^c mutant 10 copies. These values are still subject to the adjustment made for hybridization efficiency (1.75) and would have to be modified if this should prove to be incorrect. If the average cell contains 1.4 genome equivalents of DNA, the

average number of gal-mRNA equivalents per cell is 8.4 and 1.2 for the induced and non-induced wild-type, and 14 for the O^c.

Our results show a lack of agreement between enzyme and mRNA contents in the case of the repressed culture, and possibly also the non-induced culture. This raises the question of whether there is a separate translational control mechanism. First, however, we must take account of the fact that when the proportion of gal-mRNA is very small, the possibility of obtaining an erroneous result increases. Failure to discount a small amount of hybridization due to RNA other than gal-mRNA might not significantly affect the calculations for the induced culture, but might be enough to increase the apparent gal-mRNA content in the repressed culture by the factor of 2-3 observed. This is a distinct possibility in the case of E1 (F8) but is much less likely in the case of 9-3h (FR5). In the latter case nearly all of the hybridization observed is attributed by our method to gal-mRNA, presumably because the remaining homology between the wild-type *E. coli* RNA and λ dg DNA is deleted both from the chromosome and from the hybrid sex-factor. Our calculated ratio of 3:1 for the gal DNA in λ dg 178 and λ dg 166 is directly supported by the results of Guha *et al.* (1968). Even if it is not strictly correct, it is very unlikely to be inaccurate by a factor of two.

The lower utilization of the gal-mRNA in the repressed cultures may be due to translational control. However, the existence of a second special mechanism to bring about a two- to three-fold reduction in the enzyme activity when transcriptional control has already brought about a seven- to eight-fold reduction in the content of mRNA seems at first sight unlikely. It is reasonable, therefore, to try to interpret the effect as an incidental result of transcriptional control. The work of Ito & Imamoto (1968) and of Morse, Baker & Yanofsky (1968) suggests that a package of ribosomes is entrained behind the polymerase molecule during the simultaneous translation and transcription of the *trp* operon. Synthesis of the mRNA molecule at the 3'-end of the growing chain is thought to be accompanied by degradation from the 5'-end, following the entrained ribosomes. The results we have obtained may be interpreted formally in terms of this model by supposing that in the repressed culture the number of entrained ribosomes is twofold less than in the induced

culture. This could be brought about by an earlier onset of RNA degradation from the 5'-end of the mRNA molecule, and we have shown that more rapid degradation of gal-mRNA may occur in the repressed culture. In terms of transcriptional control we expect the principal difference between induced and repressed cultures to be in the length of time during which repressor is associated with the operator region. To complete the formal model we must suppose that mRNA degradation is facilitated by the attachment of repressor to the operator.

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