ORGAN SPECIFICITY OF TEMPLATE ACTIVITY OF MAMMALIAN CHROMATIN

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Summary

Cell differentiation is correlated with the differential activity of genes in a given cell (and by implication in a functionally defined mixture of cell types, i.e. a tissue). One of the topics of major importance in differentiation is concerned with the proportion of the genome which is active in RNA synthesis, i.e. the relative amount of genomic information which brings about the morphological changes characteristic of adult tissues.

The template specificity of isolated chromatins in supporting RNA synthesis was studied in order to clarify the relationship between genomic expression and organ specificity. The molecular hybridization technique was used for RNAs transcribed *in vitro* from several mouse chromatins. This technique provides a sensitive method for distinguishing between various populations of RNA molecules. It was found first that different populations of RNA molecules examined are not only distinguishable but also show large differences among them and second, that only a part of the genome was active in transcription. By competition experiments it was found that the RNAs produced by chromatin in a cell-free RNA system were

similar to RNAs isolated from the whole homologous tissue. This provides evidence that the template specificity associated with chromosomes *in vivo* is retained by isolated chromatin. A particular feature of this study is the high degree of reproducibility of the hybridization values for each tissue and the dissimilarities displayed by the different tissues.

When DNA from mouse kidney was used as template in a cell free system and the RNA produced was annealed, then the amount of hybridization was almost three times greater than the hybridization which occurred when chromatin was used as template. From these results it appears that many of the genes in animal somatic cells, which are considered as being in a completely differentiated state, are in fact inactive in supporting RNA synthesis, the inactivation pattern of the genome in such cells being distinctive for each tissue.

The mechanisms which control cell division in mammalian organs are not understood. Various experimental models have been used to study this problem. The cases under investigation include *in vivo* models, i.e. induction by folic acid of RNA synthesis in mouse kidneys and the appearance of new species of RNA

in mouse kidneys after the ligature of one ureter. Studies have been made on the effect of folic acid administration in stimulating changes in the *in vitro* RNA synthesis such that new kinds of RNA molecules appear in mouse kidney at specific times after the treatment. The ability of kidney chromatin in supporting RNA synthesis has been studied in untreated (control) and treated animals. It was found that folic acid changes profoundly the template activity of the isolated chromatin from mouse kidneys. RNA transcribed from chromation at early stages hybridizes with twice as much DNA as RNA transcribed from normal chromatin. By competition experiments it was confirmed that the cell free system produces the same RNA species as are found *in vivo* which indicates that the integrity of the chromatin is maintained during isolation. Furthermore, the experiments showed that new kinds of RNA molecules are transcribed from kidney chromatin after the early hours of folate. The transcriptional activity of the genome eventually falls to the control level after two days.

Another method for stimulating the appearance of new species of RNA in mouse kidneys is to ligate one ureter. The template activity of chromatin from the ligated kidney has been investigated from zero hours

until 48 hours. The maximum hybridization efficiency (twice the control) is exhibited at 36 hours while RNA transcribed from chromatin isolated at early stages hybridizes with as much DNA as RNA transcribed from normal chromatin.

The purpose of these experiments has been to compare the template activity of chromatin isolated at various times after the ligature of one ureter with those obtained after folic acid treatment in mice and to determine if the same control mechanism operates in both phenomena. The relationship between these two systems is discussed.

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C O N T E N T S

INTRODUCTION

1. Messenger RNA

   Molecular weight
   Size of RNA
   Base composition
   Stability

2. Transcription of the genetic code

3. Evidence of the existence of mRNA in cytoplasm

4. Nature and function of rapidly labelled cellular RNA

   Transport of mRNA to the cytoplasm

5. Chromatin

   Composition
   Physical properties

6. Transcription of chromatin

   Repressed and active chromatin
   Specifically restricted priming
   efficiency of chromatin

7. Patterns of mRNA associated with changes in state of differentiation

8. Selective gene expression

   Enzyme adaptation
   Operon structure
   Deviations from the operon structure as described in the Jacob-Monod's model
   Control mechanisms

9. Evidence for the conservation of genetic material during differentiation

10. Characteristics of differentiation

11. Cell proliferation

12. Methodology of hybridization

INTRODUCTION

Cell differentiation is correlated with the differential activity of genes in a given cell, and by implication in a functionally defined combination of cell types, i.e. a tissue. The state of differentiation is characterised by the fact that only a distinct part of the information encoded in the genome of a given tissue is expressed. One of the major problems in cytodifferentiation, is how developing cells acquire specific biochemical characteristics and furthermore the mode in which these are linked to morphological changes and cellular functions.

Cytodifferentiation is manifested in the production of RNA molecules. We can therefore predict that the population of RNAs in a differentiated cell is characteristic of the functional operation of that cell. The validity of this prediction is supported by the findings of several investigators who have studied the pattern of RNA synthesised either in mammalian tissues or at different stages during the course of differentiation. It has also been proved that an altered pattern of gene expression is underlain by the state of de-differentiation and that this

alteration is displayed by cancer cells.

In the following pages some aspects of these problems will be discussed.

1. Messenger RNA

The ribosome and transfer RNA molecules have roles to play in the synthesis of every protein, but the actual dictation of amino acid sequence is accomplished by the mRNA. Many events of interest in animal cells are expressions of changing patterns of protein synthesis, e.g. differentiation, cell division and regeneration, and these must revolve around the availability of mRNA. Consequently to understand these processes in molecular terms, it clearly would be desirable to follow mRNA from its synthesis to its utilisation and ultimate destruction. Although much effort has gone into attempting to study mRNA in animal cells, very little concrete knowledge about this RNA has been uncovered. Various criteria, based on the properties predicted for mRNA, have been used in identifying an RNA fraction as mRNA. Unfortunately a problem is common to all these criteria, that not one

of them is unique for mRNA.

The definite characterisation of an RNA as mRNA would involve the isolation of a unique cellular RNA that had been made on a DNA template, and the demonstration that this RNA could direct the synthesis of a specific protein. Very recently, Laycock and Hunt (1969) and Labrie (1969) were able to isolate an RNA from rabbit reticulocytes with the properties of haemoglobin messenger, which was able to stimulate in a cell free system of Escherichia coli, the synthesis of rabbit globin.

Molecular Weight

Although a unique mRNA would clearly be expected to have a unique molecular weight, heterogeneity of molecular weight is expected for the total mRNA of a cell (Jacob and Monod, 1961). In general sucrose density gradient centrifugation has been used for size determination. Early work suggested that the sedimentation constants of bacterial mRNA range between 6S and 14S, but newer techniques designed to avoid extensive degradation, give values of up to 30S (Spiegelman and Hayashi, 1963). Similar results have been obtained with animal systems (Staehelin et al., 1964; Munro et al., 1964; Henshaw et al.,

1965). Additional technical problems arise from the interaction of mRNA with rRNA in presence of Mg^{++} (Monnier et al., 1962; Matus et al., 1964; Millar et al., 1965), from the binding of mRNA to ribosomes (Brenner et al., 1961; Gros et al., 1961) and from contamination with polysaccharides (Martinez et al., 1965). Furthermore, although rRNA and tRNA are readily distinguished by their characteristic molecular weight, the same is not true of precursors of rRNA (Girard et al., 1965; Stevens, 1965).

Size of RNA

Sedimentation velocity is the most common property determined on an isolated RNA sample. Although very accurate conclusions about the molecular weight of various RNA species cannot be obtained by sedimentation velocity experiments (eg. the sedimentation rates of different types of RNA respond differently to changes in ionic strength of the suspending medium) it is possible by zone sedimentation to assess the range of molecular weights and the homogeneity of an RNA sample. Sucrose gradient zone sedimentation analysis has therefore become a standard technique for analysing and preparing RNA (Britten et al., 1960).

A recent addition to the techniques which separate single stranded RNA molecules largely on the casis of chain length, is acrylamide gel electrophoresis of RNA (Loening, 1967). This technique offers technical advantages of speed and superior resolution between RNA species of very similar sizes, but for preparative purposes the density gradient techniques are still more widely employed.

Base Composition

The suggestion that the base composition of mRNA should reflect that of the cellular DNA has come mainly from work on bacterial systems (Hayashi et al., 1961; Bolton et al., 1962). Conclusive proof of this complementarity has not been obtained from animal tissues, in spite of the fact that many attempts have been made to establish such a relationship between the rapidly labelled RNA and homologous DNA by DNA-RNA hybridization (Hoyer et al., 1963; McCarthy et al., 1964; Scherrer et al., 1963). All these experiments point to the fact that the mRNA represents a rather small proportion of the total DNA in any given system, and that poses limitation against equivalence of base composition.

In animal cells, if the radioactive species is labelled with ^{32}P, the base composition can be determined by assaying the distribution of ^{32}P among the four ribonucleotides released by alkaline hydrolysis. The messenger fraction of HeLa cell polysomes (Penman et al., 1963) was found to have a base composition more similar to the corresponding DNA than that of ribosomal RNA. On the other hand, several workers (Munro et al., 1964; Ishikawa et al., 1964) claimed that a distinct dissimilarity was shown between the polysomal mRNA of rat liver and both rat DNA and rRNA. The base composition of the isolated "messenger" appears to depend to some extent on the fractionation procedure employed (Sibitani et al., 1962).

Georgiev et al., (1963) isolated RNA after injection of minimal concentration of actinomycin D in order to block mainly the synthesis of rRNA species while the mRNA synthesis is unaffected. The base composition of the rapidly labelled material from rat liver nucleus and cytoplasm revealed the presence of "DNA-like RNA" in both instances. Fractionation of the rapidly labelled RNA of mouse liver by DNA-RNA hybridization (Hoyer et al., 1963) showed that neither the hybridized nor the unhybridized RNA resembled the

overall DNA composition. Thus similarity to DNA is not necessarily a criterion for complementarity.

In the differentiated tissues of higher organisms, overall base ratio equivalence of total DNA and mRNA may not occur, for the reasons that a limited region of the DNA in higher organisms functions in producing complementary RNA. The composition of this region may not reflect the average DNA composition (McCarthy et al., 1964; Bolton et al., 1964).

Stability

The kinetics of induction and repression of bacterial enzymes indicate that the corresponding mRNAs are unstable relative to the life of a cell (Jacob and Monod, 1961). On the other hand, mammalian reticulocytes synthesise globin in the absence of RNA synthesis, suggesting that stable mRNA also exists (Moldave, 1965; Lipmann, 1963). Recent results support the view that all types of cells may produce both stable and unstable mRNA and, further, that other types of RNA may turn over rapidly.

Therefore, instability is not sufficient for the characterisation of an RNA fraction as mRNA. The pulse-labelling of RNA was used for the determination of mRNA stability. A rapid rate of turnover implies

that at any given time mRNA will represent a high proportion of newly synthesised RNA, although it is a small proportion of total cellular RNA. Thus, a brief pulse of radioactive precursor is expected to label mRNA preferentially (Brenner, 1961; Gros, 1961). However, two problems arise in the interpretation of such experiments: a) relatively stable mRNA will not be labelled during a brief pulse and may escape detection and b) all pulse-labelled RNA is not mRNA. In bacteria (Schaechter et al., 1965; Armstrong et al., 1965; Leive, 1965) and in animal tissues (Girard et al., 1965; Scherrer et al., 1963; Georgiev et al., 1964; Yoshikawa-Fukada et al., 1965) a large proportion of pulse-labelled RNA is relatively stable and appears to represent precursors of rRNA.

The portion of the pulse-labelled RNA that is rapidly degraded when labelling is stopped, is considered to be mRNA, although bacterial rRNA may be stable under certain experimental conditions (Levinthal et al., 1963) and may be unstable under others (Wade et al., 1964) and be degraded relatively rapidly. Similarly, liver rRNA has a half-life of only about five days which is not a long time in a relatively long-lived cell (Loeb et al., 1965).

The results of experiments dealing with mRNA stability come from several cases. The half-life of pulse-labelled RNA is of the order of 1 to 2 minutes in Bacillus subtilis (Hartwell et al., 1964; Levinthal et al., 1962) and in Escherichia coli (Leive, 1965). In these experiments actinomycin was used to halt additional RNA synthesis. In the Escherichia coli experiments, the half-life correlated well with loss of ability to support amino acid incorporation *in vivo* (Levinthal et al., 1962). The half-life of the mRNA of β-galactosidase of Escherichia coli has been estimated of about 2 minutes, using actinomycin D (Leive, 1965). Similarly, for the tryptophane in Escherichia coli, it is 2 - 3 minutes (Baker and Yanofsky, 1968).

In mammalian liver (Staehelin et al., 1964; Munro et al., 1964; Henshaw et al., 1965; Trakatellis et al., 1964) in HeLa cells (Girard et al., 1965) and in a mouse adenocarcinoma (Trakatellis et al., 1965) an RNA that can be partly distinguished from rRNA is indeed labelled rapidly, and is therefore tentatively designated mRNA. In mouse liver, the time required to reach a steady state level of label in material

between 5 and 20S indicates a half-life of two hours for mRNA (Trakatellis et al., 1964). Longer half-lives for liver mRNA are obtained if actinomycin D is used to stop RNA synthesis and mRNA is then assayed by the loss of polysomes, by the degradation of pulse-labelled polysomal RNA, or by the ability to support amino acid incorporation (Trakatellis et al., 1964; Revel and Hiatt, 1964); values from 12 hours (Trakatellis et al., 1964) to three days (Revel et al., 1964) have been reported. Similar discrepancies between the rate of labelling of mRNA and actinomycin D experiments were found with a mouse mammary adenocarcinoma (Trakatellis et al., 1965).

Estimates of the half-lives of the corresponding mRNAs have been obtained from studies of the rate of synthesis of specific proteins after treatment of animals with actinomycin D; values ranging from several hours (Pitot et al., 1965; Bloom et al., 1965; Bekhor et al., 1965) to a day or more have been reported (Spector and Kinoshita, 1965; Greengard et al., 1965).

2. Transcription of the genetic code

Transcription is the enzymatic synthesis of RNA by a process in which the bases in DNA specify the sequence of bases in the RNA. This process provides for the synthesis of mRNA as well as for the synthesis of rRNA and tRNA (Spiegelman et al., 1963; Robinson et al., 1964). The term "polymerases" has been used to denote enzymes capable of catalysing transcription. The synthesis of infectious viral RNA by a purified polymerase is conclusive proof that these enzymes can copy their respective templates faithfully (Spiegelman et al., 1965). The mechanism of transcription must account for the specificity of the "polymerases" and for the initiation, polarity and asymmetry of transcription, as well as for termination and release of the product.

Mechanism of transcription by RNA polymerase

A) Polymerase specificity: DNA dependent polymerases from bacteria are able to transcribe a wide variety of naturally occurring (Chamberlin and Berg, 1962; Fox et al., 1964) and synthetic (Chamberlin et al., 1963; Falaschi et al., 1963; Nishimura et al., 1964) DNA and RNA templates. Despite the lack of specificity for template exhibited by various

bacterial polymerases *in vitro*, other experiments suggest that polymerases may show a high degree of template specificity *in vivo* (Baltimore et al., 1963; Weissmann et al., 1963).

The strongest evidence favouring the highly specific nature of polymerases comes from the studies of Spiegelman and co-workers (Spiegelman et al., 1965; Haruna et al., 1963; Haruna and Spiegelman, 1965) on the RNA dependent polymerases which arise following infection of Escherichia coli with either of two different RNA phages. Under suitable conditions, each of the enzymes exhibits a template specificity for homologous phage RNA; heterologous RNA on the other hand is inactive as template.

A DNA-dependent polymerase differing from that usually present in the intact rat liver appears to be induced by partial hepatectomy (Tsukada et al., 1965). These experiments indicate that specificity may be shown by polymerases.

B) Initiation and formation of a ternary complex: The mRNA concept itself, as well as the existence of operons (Ames et al., 1964) suggest that transcription commences at particular points of initiation on the genome and proceeds to specified ends. An initiation site implies

the interaction of the polymerase with a specific site on the DNA and subsequent synthesis of RNA from that point only. DNA-dependent polymerases interact strongly with DNA. The irreversible formation of complexes between DNA and DNA-dependent polymerase has been demonstrated *in vitro* (Bremer et al., 1964; Berg et al., 1965; Fox et al., 1965).

Newly synthesised RNA is also bound to the DNA-polymerase complex *in vitro* and can be released by treatment with SDS (Bremer et al., 1964) or with polyamines (Fox et al., 1965). The binding of the RNA product to the DNA-polymerase complex occurs early in the course of synthesis, and thereby explains the very limited RNA synthesis generally found in such reactions (Bremer et al., 1964; Berg et al., 1965). Richardson (1967) has found that in T7 DNA the number of binding sites for the polymerase is approximately 50. Direct evidence for the formation of template-enzyme complex has been provided by electronmicrographs (Fuchs et al., 1964; Bladen et al., 1965).

When synthetic oligodeoxyribonucleotides are employed as templates then the interaction between polymerase and template is readily reversible

(Nishimura et al., 1964; Mehrotra and Khorana, 1965).

The chemical nature of the initiation sites on DNA is unknown. However, relevant experiments utilising Escherichia coli DNA-dependent polymerase have shown that the 3'-OH ends of the template may be involved in the binding of polymerase, because in the DNA-polymerase complex they are unavailable to other enzymes (Berg et al., 1965). However, the chain ends themselves are probably not required for initiation since the ring form of ØX-174 DNA is a template for the polymerase (Hayashi et al., 1964; Bassel et al., 1964; Sinsheimer et al., 1964). Denatured DNA contains more binding sites for polymerase than the equivalent amount of native material (Berg et al., 1965; Wood et al., 1964; Maitra et al., 1965). It has been suggested that unpaired regions in double-stranded templates may act as initiator sites for transcription (Maitra et al., 1965). Ends of chains may act as initiator sites as well.

The fact that polymerase binds to homopolymers was taken as indication that a specific sequence is not required for binding. However, recent evidence indicates that RNA synthesis with Escherichia coli polymerase starts preferentially with purine residues

(Bremer and Konrad, 1965).

C) Synthesis of RNA chains occurs in a fixed direction: In bacteria, genetic information can be expressed in a polarised manner starting at the operator end of an operon (Jacob and Monod, 1961; Ames et al., 1964). Evidence obtained with the tryptophane operon suggests that transcription also starts with the operator end and is polarised (Imamoto et al., 1965; Yanofsky, 1968). The chemical polarity of transcription with Escherichia coli polymerase is such that chain synthesis starts at the 5'-OH end of the new RNA and proceeds stepwise to the 3'-OH end (i.e. left to right) Goldstein et al., (1965). Assuming that copying proceeds by antiparallel, Watson-Crick type base pairing, chain synthesis starts by copying the 3'-OH end of the template.

D) Only one strand acts as a template: It appeared that transcription in vivo was asymmetric while purified polymerases copied both strands of the template in vitro (Stent, 1965). That was a paradox, but now asymmetric synthesis has been demonstrated in vitro as well. When intact molecules of T4 DNA are used as in vitro templates, the DNA strand which is copied is the same as that copied during in vivo viral replication. The general conclusion that purified DNA-dependent polymerases

carry out asymmetric transcription if the DNA template is in native conformation and if its length is not excessively short, has been confirmed (Luria, 1965; Green, 1964). Restriction of copying to a single chain of a double-stranded template is demonstrable in model systems. (Nishimura and Khorana, 1965).

It has to be emphasised that all the *in vivo* and *in vitro* work indicating asymmetric or so called single-stranded transcription has involved the use of viruses. If the genetic information is always on only one strand of the DNA, and if all the polymerases of the cell operons show the same polarity, then all the operons should be oriented in the same direction. However, in Salmonella typhimurium the histidine and tryptophane operons are oriented in one direction on the genome, but the leucine operon has the opposite polarity (Hartman, 1965; Sanderson, 1965; Margolin, 1965). Furthermore, transformation studies in B. subtilis show that two linked markers (histidine and tryptophane) are expressed together even if they are present on opposite strands of the transforming DNA (Bresler et al., 1964). It may be that in different regions of a bacterial chromosome, different strands serve as template for transcription.

E) Size of RNA molecule transcribed: The co-ordinated control of groups of related cistrons may be expressed by transcribing entire operons as single mRNA molecules, i.e. polycistronic messengers (Jacob et al., 1961; Ames et al., 1964.) Deletion mutants in the tryptophane operon of Escherichia coli produce mRNA that is smaller than mRNA from strains possessing the intact operon (Imamoto et al., 1965). This is further evidence for the hypothesis that the size of the mRNA produced, is determined by the size of the corresponding DNA unit.

F) Genetic signals for stopping the synthesis of RNA molecules: A specific termination mechanism must exist in order to explain the copying of DNA in RNA units of certain sizes as well as for the release of completed mRNA from the template-polymerase-RNA complex. One possibility is that the entire length of the mRNA may be involved in the ternary complex until transcription is complete, and then the whole molecule would be released. Another possibility is that the association with the complex may involve only a few bases close to the growing end of the mRNA chain (Chamberlin and Berg, 1964) leaving the completed portion of the mRNA free for other interactions. This

latter alternative is supported by the fact that in the complex between newly synthesised RNA and phage ØX174 DNA, the ribonuclease-resistant portion of the RNA corresponds to the growing end of the chain (Hayashi, 1965).

One favourite hypothesis envisages the ribosomes as an active participant in the separation of mRNA from the DNA template (Wood and Berg, 1964; Bremer, 1965; Stent, 1965; Byrne, 1964; Chamberlin, 1965).

G) Mechanism of in vitro DNA transcription by RNA polymerase: In vitro studies have shown that when the template is native DNA, RNA synthesis by DNA-dependent polymerase involves a conservative mechanism, thereby preserving the double helix of the template (Harwitz, 1963; Chamberlin et al., 1962). When single stranded DNA is used as the template, the product appears initially in a DNA - RNA hybrid (Bassel et al., 1964; Sinsheimer et al., 1964; Chamberlin and Berg, 1964).

The hybrid product may also serve as template and appears to function by a mixture of conservative and semiconservative replication (Sinsheimer et al., 1964; Chamberlin and Berg, 1964). In conservative replication on a double-stranded template, the strands of DNA may be

separated over conservative short regions to allow RNA synthesis by base pairing (Chamberlin and Berg, 1964; Hayashi, 1965; Chamberlin, 1965; Jehle, 1965).

The release of single-stranded RNA involves the competition between two polynucleotide strands, for complementary interaction with a third, but DNA - RNA hybrids formed from natural nucleic acids are generally less table than homologous double-stranded molecules (Chamberlin, 1965).

3. Evidence of the existence of mRNA in cytoplasm

The occurrence of mRNA in the cytoplasm was demonstrated soon after its observation in cell nuclei, when Hoagland and Asconas (1963) showed that the post-microsomal fraction of cytoplasm contains RNA capable of stimulating amino acid incorporation into isolated ribosomes. This RNA has sedimentation coefficients of about 6 - 14S. Stimulation of amino acid incorporation by cytoplasmic RNA was also observed by others (Barondes et al., 1962; Lang et al., 1964).

Penman et al., (1963) isolated pulse-labelled RNA from polysomes and found that this RNA sediments in the region of 6 - 14S and has a sedimentation behaviour different from that of ribosomal RNA. The

base composition of this RNA differed from that of ribosomal RNA, but did not correspond exactly to the base composition of DNA.

Several newly formed RNA components are also contained in the cytoplasm of early embryos with sedimentation constants between 6 - 35S which are not identical to ribosomal RNA species. These RNAs cannot be rRNA precursors since early embryos do not synthesise rRNA at all. In addition it had been shown by Spirin and Nemer (1965) that these RNA components can be hybridized with DNA and therefore may be regarded as cytoplasmic mRNA.

In order to detect cytoplasmic mRNA, Samarina (1964) used selective suppression of R-RNA synthesis by small doses of actinomycin. Under these conditions, practically all newly formed cellular RNA is DNA-like. This method permitted the demonstration of the presence of D-RNA not only in the nucleus but also in the cytoplasm of the animal cell. Identity of part of the chromosomal D-RNA and cytoplasmic mRNA is shown by competitive relations between them in hybridization experiments (Samarina and Georgiev, 1965). All these facts indicate that the cytoplasm of animal cells really

contains mRNA.

The finding of mRNA in cytoplasm together with its complementarity with DNA and the identity of its base sequences with those of newly formed chromosomal mRNA, proves the existence of the transport of mRNA from chromosomes to the cytoplasm.

In order to approach the problem of transport mechanism, the nature of mRNA containing nucleoprotein from various subcellular fractions has been studied. Hoagland and Asconas (1963) found that a part of the activity stimulating amino acid incorporation is present in a fraction of rat liver homogenate sedimented for 8 hours at 105,000 x g, i.e. in particles lighter than ribosomes.

Spirin (1965) described in embryonic cells a special class of particles containing mRNA. These particles were called "informosomes". Informosomes contain several components with discrete sedimentation coefficients, and each component contains an RNA with a definite molecular weight (Nemer et al., 1965). Ultracentrifugation in CsCl density gradients gives RNA contents in informosomes of about 25 - 40% and sedimentation constant about 45S. It has been suggested that informosomes are a form of conservation of mRNA

when it does not participate in protein synthesis. The fact that they contain mRNA is evidenced from their template activity and hybridizability with DNA (Henshaw et al., 1965; McConkey, 1965). Similar particles were found after infection of HeLa cells by vaccine virus. In this case these particles contained mRNA and possibly an 18S rRNA (Jocklin et al., 1965).

Thus in the cytoplasm of animal cells, mRNA is incorporated into special ribonucleoprotein particles of yet unknown nature. These particles may be a transport form of mRNA.

4. Function of rapidly labelled cellular RNA

Preparation of total cellular RNA including the nucleochromosomal have been studied, after pulse-labelling cells with radioactive RNA precursors, by means of ultracentrifugation in sucrose gradients (Scherrer and Darnell, 1962; Hiatt, 1962). Such newly formed pulse-labelled RNA which is localised in the nucleochromosomal complex has sedimentation properties differing from those of the pre-existing cellular RNA. The latter consists of three components: the ribosomal RNA peaks with sedimentation coefficients 28 - 30S and 16 - 18S and the tRNA with sedimentation

coefficients 4S. The newly formed RNA is concentrated in "heavier" regions. Most authors have observed two peaks with sedimentation coefficients 45 - 50S and 30 - 35S. A variable amount of RNA was found in the more slowly sedimenting region (25 - 30S).

After longer labelling times, the relative proportions of these peaks decrease and the label accumulates in the rRNA and tRNA regions. The details of this distribution pattern vary somewhat according to the source and method of isolation of RNA, but the general dynamics of radioactivity "flow" are similar (Rake and Graham, 1964; Tamaoki and Mueller, 1963). The base composition of the rapidly labelled cellular RNA is intermediate between that of DNA and rRNA but is usually closer to that of r-RNA. It follows that the rapidly labelled cellular RNA, synthesised in the nucleochromosomal apparatus, is a mixture of D-RNA and R-RNA molecules.

Experiments on Ehrlich ascites cells with subsequent gradient centrifugation and analysis of the fractions gave the following results (Georgiev et al., 1963). The total nucleochromosomal RNA shows the 18 and 28S peaks of the preformed RNA, and three peaks of newly formed RNA with sedimentation coefficients about 45,

35 - 40 and 25 - 30S. Hot phenol fractionation of nuclei separates these components. In a fraction extracted at 40°C containing R-RNA [1] only, only one peak of newly formed RNA is found (35 - 40S component). Its base composition corresponds exactly to that of rRNA. The preformed RNA of this fraction is found mainly in the 28S zone and to a lesser extent in the 18S zone.

The fraction solubilised at 65°C and containing only D-RNA [2] (both newly formed and preformed) contains heterogeneous labelled material with a maximum of label at 25 - 30S. On the other hand, the main part of the preformed D-RNA has the sedimentation coefficient of rRNA (approx. 18S); this is why the sedimentation pattern of bulk nucleohcromosomal RNA is similar to that of rRNA. Thus newly formed and preformed D-RNA also differ in sedimentation characteristics.

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1 R-RNA: an RNA having the base composition of ribosomal RNA and probably containing the same nucleotide sequences.

2 D-RNA: an RNA with the base composition of cellular DNA including mRNA (informational RNA) as well (Bolton and McCarthy, 1962).

The RNA fraction extracted at 55°C contains a mixture of D-RNA and R-RNA. Sedimentation analysis reveals two labelled components. These peaks are well separated and their analysis is feasible. The peak having a sedimentation coefficient of 45S is R-RNA, while the 25 - 30S material is D-RNA identical with the 25 - 30S material of the 65°C fraction.

The conclusions drawn from the base compositions of the fractions are confirmed by hybridization experiments (Perry et al., 1964). R-RNA has a very low ability to hybridize with DNA, and unlabelled purified D-RNA does not compete with them for binding sites, while purified rRNA does. On the other hand, 25 - 30S radioactive D-RNA from the above mentioned fractions effectively hybridizes with DNA and hybridization is suppressed by the addition of unlabelled D-RNA but not of rRNA.

Penman (1966) found similar results using labelled HeLa cell nuclei. Sedimentation analysis of the RNA extracted, shows a peak in optical density at 45S and a larger peak that sediments about 30S. The RNA in the 30S region is shown to be polydisperse and contains 35S and 28S RNA. It was shown that 45S is cleaved to form 16S RNA which emerges immediately into the cytoplasm, and 35S which remains in the nucleus. The 35S RNA undergoes a transition to 28S and then enters

the cytoplasm.

The evidence for DNA-like RNA in the nucleo-chromosomal complex of animal cells leads to the suggestion that this type of RNA corresponds to newly formed mRNA. Hybridizability of RNA from animal cells with the homologous DNA was first demonstrated by Hoyer et al., (1963) using DNA-agar columns. It has been shown that newly formed RNA binds to DNA much more effectively than does preformed RNA, and that nuclear RNA hybridizes better than cytoplasmic. Another characteristic of mRNA is its ability to stimulate protein synthesis on ribosomes (Nirenberg et al., 1961). Brawerman et al., (1963) prepared an RNA fraction enriched with D-RNA from rat liver nuclei and demonstrated that it has a greater stimulating activity than the rest of the nuclear RNA. It was also directly shown that D-RNA from rat liver and Ehrlich cell nuclei prepared by the hot phenol method is many times more effective in the stimulation of protein synthesis in the Nirenberg system, than is cytoplasmic RNA or nuclear R-RNA (Lang and Sekeris, 1964). All these characteristics are indicative of the fact that chromosomal D-RNA is, or at least contains, newly formed mRNA of animal cells (Samarina

et al., 1965).

A metabolic heterogeneity of chromosomal D-RNA means that its different components are synthesised at essentially different rates (Samarina et al., 1965). Consequently, at early times the specific activity of the heavier D-RNA components is considerably higher than the specific activity of the main 18S peak. Only at longer labelling times does the radioactivity curve begin to follow the curve of UV absorbance. Several explanations for this phenomenon have been proposed.

1. D-RNA is synthesised in chromosomes as a relatively long chain ($\geq$ 30S), which is later split, still in chromosomes, into shorter fragments (about 18S).

2. Chromosomal D-RNA is heterogeneous not only metabolically but also functionally. Besides the usual mRNA synthesised in chromosomes and migrating to the cytoplasm, there is a special class of D-RNA functioning in chromosomes themselves at the site of its synthesis. One may suggest that this RNA bears certain regulatory functions.

The following hypothetical scheme of interaction between different D-RNA classes was proposed by Georgiev:

heavy rapidly labelled D-RNA
- $D\text{-}RNA_1$ — chromosomal 18S D-RNA — mRNA of cytoplasm and nuclear sap
- $D\text{-}RNA_2$ — functioning and turning over in chromosomes

On the other hand Scherrer et al., (1963) suggested the following course of R-RNA conversions: $R\text{-}RNA_1$ (45S) $\longrightarrow$ $R\text{-}RNA_2$ (35 - 40S) $\longrightarrow$ $rRNA_A$ (28 - 30S) and $rRNA_B$ (16 - 18S).

Similar conclusions were drawn by Rake and Graham (1964).

Transport of n-RNA to the cytoplasm

A) m-RNA: The main form of mRNA in the cell nucleus is as a specific nucleoprotein particle with a sedimentation constant of 30S. These particles differ from ribosomal subunits in that they have a base composition of the AU type.

It has now been established (Samarina et al., 1968) that 30S particles are monomers of a more complex polysome-like structure. Such a structure is formed by a long D-RNA strand and a number of globular protein particles bound to it and tightly packed along the D-RNA strand. These complexes occupy a wide zone in sucrose gradients between 30S and 200S. In the electron microscope, one finds the 30S peak contains only single monomers; the 45S peak dimers; the 55S to

60S peak trimers and so on. Treatment with small amounts of RNase quantitatively converts large polyparticles into the 30S particles. The buoyant density and the protein composition of mono- and poly-particles are the same. They are very different from those of ribosomes. The sedimentation coefficient of RNA isolated from particles is related to the number of monomers in the complex.

It was calculated by the authors that one protein particle is bound to an RNA strand having a MW of about 2×10^5. The name "informofer" is suggested for monomeric protein particles. These studies explain how newly formed long chains of D-RNA with a very high MW of about several millions can be bound to a number of protein 30S particles located along the molecule. Such nuclear D-RNA-containing particles were obtained in the presence of RNase inhibitor.

B) rRNA: Data on the transfer of rRNA from the nucleus to the cytoplasm after pulse labelling, have been obtained by studying the RNA metabolism in the HeLa cell nucleus (Penman, 1966) as well as

the cytoplasm alone (Girard et al., 1965). Thus it was shown that about 30 minutes later, after the beginning of the incorporation of the radioactive precursor, the 16S ribosomal component begins to appear in the cytoplasm in the form of a 45S ribosomal subunit.

28S RNA first appears in the cytoplasm in a 60S ribosomal subunit about 60 minutes after the beginning of labelling. These observations have been interpreted to mean that, unlike messenger RNA, ribosomal RNA is delayed in the nucleus after synthesis, presumably because of the time required for its eventual incorporation into a functional ribosomal subunit. Part of the delay can be accounted for by the time required for the original 45S RNA to complete its transition to 16S and 28S components.

5. Chromatin

Composition: The genetic material of mammalian cells consists of DNA with which is associated RNA and protein, both histone and non-histone, the whole being known as chromatin.

Analysis of several chromatins from organs show

that they vary greatly with respect to their total protein : DNA ratios and RNA:DNA ratios (Dingman and Sporn, 1964). Highest values for both these measurements were obtained in chromatin from whole embryos, and the values declined with increasing age (of the embryos).

In view of the current interest in histones as possible regulators of genetic transcription, the histone:DNA ratio was determined for each type of chromatin. Within the error of experimental measurement, there were no significant differences in the histone:DNA ratio of the various types of chromatin; a mean value of 90 ± 8 µg of histone per 100 µg of DNA was found. Dingman and Sporn (1964) showed that a linear plot was obtained for all the different kinds of chromatin they analysed (brain, liver and erythrocytes from chicken) when the RNA:DNA ratio and the total protein:DNA ratio were plotted for each type of chromatin. As noted above, the histone:DNA ratio was a constant for all types of chromatin examined, while the total protein:DNA ratio varied markedly. Since the histone:DNA ratio was a constant, it is the non-histone-protein:DNA ratio which varies markedly with age and cell type and which is positively

correlated with the RNA:DNA ratio of the chromatin.

Cell differentiation in larval diptera is reflected in alterations in the functional activity of specific regions of the genetic apparatus (Dingman and Sporn, 1964; Beerman, 1963; Cooper, 1959; Clever, 1963; Schneiderman and Gilbert, 1964). These changes in functional activity can be observed cytochemically in specialised somatic cells having giant interphase chromosomes and are associated with distinct changes in the composition of the interface chromosome. Specifically, with induction of activity there is marked swelling or puffing of the chromosomes (Beerman, 1963), accumulation of RNA (Edstrom et al., 1962; Swift, 1962), an apparent increased synthesis of ribonucleic acid (Pelling, 1959), and accumulation of non-basic protein (Beerman, 1963; Swift, 1962). Moreover, the negligible amount of RNA and the low amount of non-histone protein present in the mature erythrocyte chromatin, are precisely what would be expected of this inactive interphase chromatin (Cameron, 1963). Furthermore, if both RNA and non-histone protein are characteristically located together in active regions of the vertebrate interphase chromosome, then one might expect to find a quantitative relationship between the

amount of RNA and the amount of non-histone protein in isolated interphase chromatin. According to Dingman and Sporn's data such a relationship exists.

Convincing evidence for this comes also from the work of Marushige (1967) on analysis of chemical composition of sea urchin embryo chromatin. Two kinds of chromatin were studied, namely blastula chromatin and pluteus chromatin. The pluteus chromatin contains: a) twice as much RNA as does blastula, b) less histones than does blastula and c) twice as much non-histone protein as does blastula chromatin. Furthermore, they found that chromatin isolated from pluteus is more active in support of RNA synthesis <u>in vitro</u> than chromatin from blastulae. Furthermore, Marushige and Dixon (1969) studying the template activity of chromatin from trout testis undergoing spermatogenesis, found that the decrease in template activity observed in later stages of maturation is accompanied by an increase of histone content and a decrease of non-histone protein content in chromatin.

Whether the RNA:DNA ratio, the non-histone protein: DNA ratio or both could be used as a quantitative

measure of the number of active loci in interphase chromatin remains to be determined. However, Paul and Gilmour (1968) have recently suggested as a result of the DNA-RNA hybridization studies, with RNA made on nucleoprotein templates reconstituted from various combinations of chromosomal components, that active regions of the genome may be determined by certain non-histone proteins.

Physical properties: The ultra-violet absorption spectra of chromatins shows an extinction maximum at 259mu and an extinction minimum at 237 to 240 mμ. The spectral ratios of any particular type of chromatin are a function of its total nucleic acid and total protein content. The E_{260}: E_{230} ratio is a particularly sensitive index of the relative proportions of the two constituents (Mirsky et al., 1946). The higher the E_{260}: E_{230} ratio, the greater is the nucleic acid content.

Another characteristic is the change in extinction as a function of temperature. The midpoint of the total extinction change (Tm) in 0.2mM trisodium EDTA according to Dingman and Sporn experiments, was 41 - 42°C for chicken DNA, 76 - 78°C for erythrocyte chromatin and 76 - 79°C for brain chromatin. The

reversibility of the extinction on quick cooling from 95°C, was much greater for chromatin than it was for DNA. This effect is presumably the result of protein which partially prevents irreversible separation of complementary DNA strands. The thermal profiles did not appear particularly sensitive to the different quantities of RNA and non-histone protein associated with different chromatins (Marushige, 1967). Quite probably the major determinant of the Tm in these preparations was the amount of highly basic proteins present (Mandel, 1962; Tabor, 1962). On the other hand, Bonner and Huang (1963) have described for the pea embryo chromatin a "two-step" melting profile.

Sedimentation velocity and analyses by zone centrifugation in a sucrose gradient were performed by Dingman and Sporn (1964) on brain, liver and erythrocyte chromatins. All three chromatin preparations sedimented in a single, but relatively broad, zone ranging from 10 - 40S. This low sedimentation velocity and polydisperse pattern probably is the result of the shear to which the preparations were subjected in the process of solubilisation.

6. Transcription of Chromatin

Repressed and active Chromatin

The presence of non-transcribing DNA regions can be demonstrated by electron microscope autoradiography. Chromosomal DNA of eukaryotic cells exists as a complex with proteins and perhaps with RNA. This complex is called chromatin and it has two states: condensed and extended. At metaphase of mitosis, all of the chromatin is condensed, and the chromosomes, as small and discrete as they can ever be, are moved about on the mitotic apparatus. This condensed chromatin is not transcribed, and metaphasic cells are deficient or totally lacking in RNA synthesis (Robbins and Scharff, 1966).

The interphase nucleus contains transcribing DNA, but it also contains some condensed chromatin ("heterochromatin") along with the extended, fibrillar "euchromatin". The inactive fraction of the genome is mainly present in condensed chromatin masses and the experiments of Littau et al., (1965) indicate that in such masses dense packing of the individual nucleoprotein fibres results from fibre cross-linking by lysine-rich histones. In the calf thymus nucleus

the lysine-rich histones amount to only 20% of the total histone content. If the lysine-rich histones are selectively extracted from the nucleus the condensed chromatin unravels and its characteristic, heavily clumped form opens up; by adding back to such extracted nuclei exogenous, lysine-rich histones the condensed form can be experimentally re-established.

Euchromatin is shown by accumulation of new, radioactive RNA thereon to be the site of RNA synthesis (Frenster 1965;1966). Heterochromatin immediately adjacent to labelled euchromatin is conspicuously lacking of radioactive RNA. Extensive studies of this kind have been done on isolated calf thymus lymphocytes (Littau et al., 1964). The ratio of euchromatin to heterochromatin varies greatly among the several somatic cell types and, in particular, the amount of condensed, apparently non-functional chromatin increases in the nuclei of cells which are known to be in the process of restricting their range of transcription. Such cells (e.g. erythroblasts) are also narrowing the range of proteins in synthesis, and are undergoing cytodifferentiation. This indicates that different cell types use different fractions of the genome. Evidence of this comes from the fact that differentiated

cells make some characteristic proteins not made by other differentiated cells.

Specifically restricted priming efficiency in chromatin

Support for the existence of a mechanism, which would explain the inactivation of genes during development, has been obtained from studies on the capacity of the puffs of the giant chromosomes of larval fruit flies to support RNA synthesis at a high rate. This high rate of RNA synthesis is restricted to the puffs, (Pelling, 1964) which constitute the euchromatin parts of these chromosomes. In areas of giant chromosomes in which there is no evidence for puffing there is no evidence for RNA synthesis. Moreover, Edstrom and Beerman (1962) found from base analysis studies of RNAs derived from puffs on the same Chironomus chromosome, that these RNAs have a DNA-like composition that is unique for each puff. The suggestion they made was that each puff synthesises a specific mRNA. Beerman (1961) had described a case in which the correlation between puffing and gene activity was quite clear. This speculation has led to the suggestion that a large part of the chromosome might be masked, and has led to attempts to isolate

chromosomal material (chromatin) for testing the masking hypothesis *in vitro*.

Several workers claimed on the basis of simple kinetics that the priming activity of chromatin was considerably lower than that of free DNA from the same source. For example Bonner et al., (1962;1963) following the time course of polynucleotide synthesis, found that the priming activity of chromatin from pea seedlings was considerably less than that of the corresponding free DNA. They proposed that most of the DNA in chromatin was unavailable for priming by virtue of masking, effected probably by histones.

Findings similar to those mentioned above have been reported by Frenster et al., (1963) in lymphocytes, Flickinger et al., (1965) in amphibian embryos and Marushige and Bonner (1966) for liver chromatin. A point that may render the validity of this finding questionable, is that animal chromatin preparations are, as shown by Sonneberg and Zubay (1965) insoluble in solutions of low ionic strength such as those used in reaction mixtures. Any kinetic study of a reaction in which even one of the reactants is insoluble is invalid unless this fact is taken into account in the analysis of the data. The

same authors found moreover that if chromatin was sonicated its solubility was enhanced. However, chromatin is undoubtedly a less efficient primer for RNA synthesis than the corresponding free DNA in a cell free system, its priming activity for calf thymus being approximately 10% of that shown by free DNA as found by Paul and Gilmour (1966).

The question arising consequently is whether the priming activity of the DNA in chromatin is attributed to a genomic part constituting a specifically distinct subset of genes present, or is due to the action of all the genes at a reduced rate.

The studies on the puffs of giant chromosomes from larval fruit flies, that were mentioned before, may be considered as providing evidence for the first alternative. This question, in the case where chromatin is 10% as effective as the corresponding free DNA, might otherwise be asked: whether only 10% of the genes present are transcribed with 100% efficiency or all kinds of genes present are transcribed with 10% efficiency.

The work of Paul and Gilmour (1966a) has provided a conclusive answer to this question. Using the molecular hybridization technique of Gillespie and Spiegelman (1965) for RNAs transcribed *in vitro* from

free DNA, intact chromatin and sonicated chromatin from calf thymus, they found that only about 10% of the DNA in chromatin was available for transcription. This provides direct evidence for a specific inactivation of 90% of the genes present in chromatin. They also showed that sonication increased the priming activity of the chromatin, this being presumably due to an alteration of its structure. By competition studies they found moreover that the RNA produced by chromatin in a defined cell free system was identical to RNA isolated from the whole calf thymus tissue. This provides evidence that the pattern of gene inactivation is faithfully conserved during isolation of the chromatin.

7. Pattern of mRNA associated with change in state of differentiation

It has been established as a generally valid concept that except for genes such as for rRNA and tRNA, the action of each gene or group of identical genes is expressed by an mRNA.

A question closely related to the absolute proportion of the genome active, is the relative amount of genomic information involved in changes in the pattern of gene activity, which are associated with

changes in the state of differentiation. An example is tissue-specific hormone response. Studies by Kidson and Kirby (1964) using countercurrent distribution demonstrate that the effect of cortisone on the pattern of mRNA synthesis in the liver nucleus is extremely variable. Cortisone treatment appears to result in a quantitatively enormous change in the spectrum of mRNA molecules in synthesis, a change which clearly affects a significant proportion of the activity taking place in the liver cell genome. The spectrum of RNAs changes markedly within 5 minutes from the time of hormone addition. In the same study, parallel results are obtained with various other hormones as well, i.e. testosterone (to which male and female liver react differently) and the non-steroids thyroxin and insulin. Means and Hamilton (1966) report that within 2 minutes of estrogen treatment the nuclear RNA of uterine cells increases in specific activity as much as 40%.

It is consequently predictable that the population of RNA messages in a differentiated cell is characteristic of the functions operating in that cell. The general validity of this conclusion is supported by the results of several authors who have attempted to

study the patterns of mRNA synthesis in mammalian tissues. Kidson and Kirby (1964) using counter current distribution profiles of RNAs extracted from mouse liver, kidney and spleen, after a single tritiated uridine pulse, found both considerable differences and similarities in the mRNA population of these tissues. These results suggest the existence of active genes that are unique, besides those in common, in differentiated animal tissues. But factors such as different rates of absorption (uptake) and utilisation of uridine by these different tissues may obscure the picture.

McCarthy and Hoyer (1964) used the DNA agar technique for the comparison between RNAs synthesised in mouse kidney and liver, which have received a pulse label. By competition studies they were able to indicate that the populations of RNA molecules examined were not only distinguishable but showed large differences among them. A point that is worth mentioning in connection with the above work, is that RNAs extracted from tissues which have been exposed to a pulse label contain labelled heavy rRNA precursors besides a large amount of unlabelled rRNA. Thus it becomes particularly difficult to determine the true specific activity of the messenger fraction. In order

to overcome this difficulty they prepared labelled "messenger" RNA from mouse L-cells in culture as well as from a primary culture of kidney cells. The L-cells were exposed to $^{32}PO_4$ for three days, the kidney cells to ^{14}C-adenine for 24 hours. When they competed out these RNAs with cold RNAs from the homologous tissues as well as from heterologous, in this case mouse spleen and liver, they found that the best competitor was the RNA from the homologous source, while spleen and liver RNA compete less well and to very different extents. Again this evidence was interpreted to point to large differences in the population of RNA molecules from these two tissues used as competitors.

In the case of the RNA isolated from culture of kidney cells, the fact that the in vivo RNA isolated from kidneys was the best competitor, suggests that the genomic control is not rapidly lost when the cells are transferred to culture conditions.

Paul and Gilmour (1966, 1968) using the molecular hybridization technique of Gillespie and Spiegelman (1965) for RNAs transcribed in vitro from chromatins from calf thymus, rabbit bone marrow and thymus, found that only a part of the genome was available for transcription. By competition studies under saturating RNA conditions, the same authors found moreover that the

RNA from the tissue homologous to the chromatin was the most effective competitor. RNA from heterologous tissue gave only partial competition. This was considered as suggesting that certain genes unique for each specific type of chromatin are active in RNA synthesis relevant to tissue function. The inactivation pattern of the genome is distinctive for each tissue exactly as the theory of variable gene activity necessitates. Ursprung et al., (1968) arrived at the same conclusion using mouse tissues. From the results of Paul and Gilmour as well as the results of Ursprung et al., it seems clear that differentiated animal tissues are characterised by a considerable degree of specificity that is reflected in the mRNA pattern.

The specificity is attained as a certain tissue approaches its final differentiated state and the number of active genes becomes more restricted.

Experiments by Church and McCarthy (1967) suggest that a very large fraction of the gene activity in a given cell type may undergo change in the course of differentiation. These experiments concern changes in gene function associated with liver regeneration and live embryogenesis in the mouse. Competition

curves were obtained using unlabelled RNAs extracted from embryonic liver of mice at 14, 15 and 17 days of gestation and from adult mice added to a hybridizing mixture of mouse DNA and pulse-labelled RNA extracted from 14, 15 and 17 day embryonic liver or from adult liver; the results demonstrate that remarkably large-scale alterations in the pattern of gene activity accompany liver differentiation. Thus within only one day of development, some 70% of the apparent homology in the liver RNA populations has disappeared. Changes of this magnitude also distinguish embryonic from adult liver RNA.

A further experiment by the same authors concerns liver regeneration. Here it is shown that the patterns of gene activity in regenerating liver remain constant for less than one hour after hepatectomy. As early as 1 hour after the operation the RNA in the surviving lobe appears to be many times more effective as a competitor to labelled 1 hour RNA than is RNA from the liver of sham-operated animals. By six hours, a significant portion of the informational RNAs synthesised during the first three hours as a result of hepatectomy have almost disappeared. As regeneration continues, the distribution of RNA

molecules in the liver continues to alter, just as it does in the developing embryonic liver. Some of the genes active in regeneration in fact appear to be the same as those which function in liver neogenesis.

In a general sense it is clear that a very large number of genes must be involved in differentiation. The rate of metabolic and cytological changes, underlying morphogenetic processes such as gastrulation, where cytodifferentiation is considered to be initiated, or embryonic induction, is so great that no other supposition is likely. Direct comparisons of the patterns of gene activity at successive stages of embryogenesis have demonstrated this to be the case.

Denis (1966) studying the embryogenesis of Xenopus has performed competition hybridization experiments and he found that the degree of competition increases as the newly synthesised labelled RNA and the unlabelled competing RNA preparation are drawn from embryos at closer stages of development. Thus labelled RNA from the swimming tadpole stage is not competed at all by RNA from blastulae, for example, while RNA from gastrulae, neurulae and tail-bud stage embryos competes with increasing success. Similarly,

the hybridization of RNA synthesised during gastrulation is not affected by the presence of blastular RNA, pointing to the novelty of the gene activity patterns which start at gastrulation. On the other hand, RNA from neurulae competes with the gastrular labelled RNA better than RNA from later stages. From these experiments it was concluded that some genes (or members of internally homologous families of genes) are functional throughout embryogenesis, from gastrulation on; that some are functional in gastrulation and neurulation but are not functional at later stages; and that in each later stage a certain group of genes (or families of genes) is functional for a while but is later repressed as development advances. One weak point about Denis' work is that his experiments were not carried out under saturation conditions.

Another study employing hybridization to investigate changes in the spectrum of genes functional during amphibian embryogenesis has been reported by Flickinger et al., (1966). Embryos are bisected at the blastula stage into animal and vegetal halves, and the RNA is extracted after 24 hours labelling period. Their results constitute a demonstration that partially different sets of genes are functional in the animal

and vegetal halves during the 24 hour labelling period, for in this case addition of the RNA preparation other than that used to presaturate the DNA results in an increase in total hybridization, while the controls show no increase. These data support the view that the basic regulatory phenomenon in early embryonic differentiation is the regulation of patterns of gene transcription, as Flickinger pointed out, since different genes are evidently active in embryonic tissues undergoing different courses of differentiation.

Conclusions drawn from Glisin et al., (1966) and Whiteley et al., (1966) from their studies on sea urchin embryogenesis are essentially similar to those from Denis' work with Xenopus. Glisin et al., carried out competition experiments under saturating RNA conditions. Their results indicate that as development progresses some of the early genes active in both oogenesis and cleavage are shut off and the RNA they have produced is degraded, since unlabelled gastrular and prism stage RNA fails to compete as efficiently with newly synthesised blastular RNA. The results obtained by Whiteley et al., correlate well with these conclusions, although their competition experiments are carried out in the presence of excess DNA, and are consequently more likely to measure changes in relative

frequency (i.e. number of copies) of the more common RNA species present, as well as qualitative changes in the spectrum of RNA's available.

8. Selective gene expression

Most of the information about selective gene action has been obtained from studies made in bacteria; similar studies in cells from multicellular organisms have encountered particular difficulties. Clarification of the mechanisms underlying this phenomenon in multicellular organisms is necessary for the understanding of cytodifferentiation, and this problem is usually approached in the following way.

In addition to direct investigation of possible mechanisms in cells of higher organisms, studies have been made with bacteria in order to define possible similarities that exist between them regarding differential gene expression and its control. Thus it can be found to what extent explanations accounting for gene control in bacteria might be valid for gene control in cells of higher organisms. Therefore, it is necessary to present an account of the existing evidence demonstrating peculiarities and points in common, between enzymic adaptation as occurs in micro-organisms, and cytodifferentiation which establishes

selective gene expression in cells of higher organisms.

Enzymic adaption

Studies in micro-organisms: According to the model established by Jacob and Monod on the induction of β-galactosidase in Escherichia coli, regulation continues only as long as the repressor is present, being associated reversibly, with the operator gene of the respective operon.

In enzymic adaptation the gene product may already exist in very small concentrations and is simply increased by induction. Differentiation however, is characterised by the appearance of a new protein which was previously absent from the cell. The gene control mechanism however, may be universal throughout the spectrum of biological organisms, being flexible enough so as to leave considerable freedom for deviations. Some data obtained from studies on bacterial pseudodifferentiation should be mentioned in this context, because they show similarities to cytodifferentiation. This phenomenon has been observed in the case of β-galactosidase induction in the wild type of Escherichia coli. An increased amount of the inducer is necessary for initiation of the induction which then continues even if the

concentration of inducer is extensively decreased. This is explained by the finding that β-galactosidase is induced simultaneously with permease whose presence facilitates the penetration of inducer into the cell. Furthermore, the presence of a permease renders the concentration of inducer within the cell independent of that in the medium where it may have been decreased; this results in continued induction of both the permease and β-galactosidase. In this situation the level of the permease is regulated by a kind of positive feedback. The point to be emphasised here is that β-galactosidase and the permease are under independent structural control.

Another instance where similarities with cytodifferentiation have been observed in micro-organisms is that reported by Pollock (1963) where induction of penicillinase in Bacillus cereus continues after removal of the inducer (penicillin) that had been briefly applied to the cells previously.

In the case of an inducible enzyme such as β-galactosidase of Escherichia, a mutant lacking the repressor will form the enzyme constitutively. For a repressible enzyme, the analogous mutant would be expected to be a recessive non-repressible strain.

Several examples of such mutants have, in fact, been obtained in Escherichia coli. For example, the formation of all the enzymes involved in the conversion of anthranilic acid to tryptophane is repressed by the addition of tryptophane to the growth medium, but Cohen and Jacob were able to isolate a mutant which formed these enzymes whether tryptophane was present or not. Again, Gorini (1961) has described a mutant on which the formation of all the enzymes concerned in arginine synthesis occur, irrespective of the presence of arginine in the medium; these enzymes are repressed by arginine in the original wild type strain. In both these examples the mutation supposed to cause loss of repressor is relatively distant from the genes controlling the structures of the enzymes whose formation is derepressed. A change from induction to repression depends on a change in the regulatory gene.

These findings provide support for the widely held assumption that induction and repression represent the operation of very similar regulatory mechanisms (Jacob and Monod, 1961a, 1961b; Cline and Bock, 1966; Vogel and Vogel, 1967).

The operon structure: In bacteria the enzymes that

determine a single metabolic pathway are often determined by a group of clustered genes that constitute a genetic unit of function. Such a unit of function has been called an "operon" by Jacob and Monod (1961). An operon will be defined as a group of contiguous structural genes showing co-ordinate expression and their closely associated controlling sites.

Controlling sites are elements which determine the expression of only those genes to which they are attached. This definition is similar to the original usage of the term (Jacob et al., 1960). It does not presume any particular mechanism of regulation, and it includes operons consisting of only a single gene as well as operons whose rate of expression does not appear to be subject to any specific regulatory process.

Relative locations within an operon have been described as either "proximal" or "distal", these terms referring respectively to whether a given gene or point within a gene is closer to or farther away from the controlling sites of an operon. Most of the well characterised operons are under the control of regulator molecules coded for by distinct regulatory

genes. Although some of these regulatory genes are closely linked to the operons they control, their mode of action does not require any such linkage and therefore they are not considered to be part of the operon. In cases where the operon is subject to control by regulatory genes, there is included amongst the controlling sites an operator region, which was defined as the site of action of the regulator. Again, this definition of an operator is a general one and includes no assumptions about the mechanism of regulator action. The controlling sites must include regions which are involved in the initiation of transcription and translation. The term "promoter" was used to refer to a site which serves to initiate transcription of an operon.

There is evidence that the promoter is separate from the operator region. Ippen et al., (1968) have reported that the operator in the lac operon in Escherichia coli has been mapped between the promoter and the first structural gene. Since present evidence does not suggest that any of the product controlling sites are translated into a protein molecule, these sites are not called genes. The term gene is reserved for a region which codes for a functional cytoplasmic

product.

The "effector" is the compound, usually a small molecule, which acts as the physiological signal to alter the rate of specific enzyme synthesis in response to changes in the environment of the cells. "Induction" and "repression" refer, respectively, to stimulation or inhibition of the synthesis of specific enzymes in response to the addition of a compound presumed to increase the effector concentration in the cell.

The initial process by which a gene is expressed as a protein, is the transcription of the gene into a single stranded molecule of mRNA complementary to the base sequence in one of the DNA strands of the gene (Hayashi et al., 1963; Marmur and Greenspan, 1963; Hall et al., 1963; Hayashi et al., 1964). This mRNA is synthesised in the 5' to 3' direction (Shigeura and Boxer, 1964; Bremer et al., 1965; Goldstein et al., 1965). Ribosomes, tRNA and a variety of enzymes co-operate to translate the unstable mRNA into protein, this translation also proceeding in the 5' to 3' direction (Trach et al., 1966; Wahba et al., 1966; Streisinger et al., 1966; Brammar et al., 1967). In operons in which both the location of the controlling sites and the direction of transcription or translation are known, the controlling sites are at the extremity

from which transcription and translation begin (Yanofsky et al., 1964; Imamoto and Yanofsky, 1967; Katze et al., 1966; Brown et al., 1967). This location suggests that specific regulation involves control of the initiation of either transcription or translation.

Deviations from the operon structure as described in the Jacob-Monod's model: While several examples are known where genes of related function are co-ordinately controlled in an operon, it is almost as easy to find examples of biosynthetic sequences to which the operon model seems not to apply.

The best known case is perhaps that of arginine synthesis in Escherichia coli. Arginine is synthesised through seven chemical steps starting with glutamic acid, each step being catalysed by a different enzyme. Gorini (1961) has shown that these enzymes are controlled by genes only two of which are closely linked, the remainder being scattered round most of the genetic map. In spite of this, the enzymes are, at least in some strains of Escherichia coli, subject to a common repression by arginine. The repression in this case does not, however, seem to be co-ordinated as between the different enzymes; it seems to act much more strongly on some enzymes of the arginine series than

on others.

In order to interpret this situation they proposed (Maas, 1961 and Vogel, 1961) that the easiest way of bringing the arginine case into line with the operon hypothesis, is to regard each isolated arg gene as an independent operon with its own operator. A common repressor might then act on all the operators but not necessarily to the same extent in each case. The idea of a common repressor is suggested by the existence of mutants in which at least several of the enzymes have become simultaneously non-repressible by arginine (Jacoby and Gorini, 1967; Gorini, 1961).

It is still somewhat difficult to see why some biosynthetic sequences should be regarded according to the operon pattern and others in a less co-ordinated way. It may be that when a set of enzymes is exclusively concerned with the synthesis of a single substance, so that there is no possibility of any member of the set having a function in the cell not shared by the others, then they are best controlled by a block of genes subject to strictly co-ordinated regulation. However, if some enzymes of the set have functions not shared by the others, perhaps through their being involved in breakdown as well as

biosynthetic reactions, then it may be to the advantage of the organism to be able to regulate their levels independently, and so a looser kind of co-ordination may have become established during evolution (Fincham, 1965).

Control mechanism: The co-ordinated expression of the genes in an operon can be explained by two postulates: a) that all of the proteins coded for by a particular operon are translated from a single polycistronic mRNA molecule; and b) that the regulation of operon expression occurs at the level of synthesis of this mRNA (Jacob and Monod, 1961a, 1961b; Martin, 1963). Thus the genes of an operon are either expressed, or not as a unit. Both of these postulates are strongly supported by studies on the synthesis and size of mRNA in a number of systems.

Biochemical evidence that operon mRNA is polycistronic, rests largely on the demonstration that in the three operons examined (his, lac, try) the size of the specific mRNA is about that necessary to carry the information from all of the genes of the operon (Guttman and Novic, 1963; Imamoto et al., 1965). Studies on the average size of lac polysomes are

consistent with the synthesis of a single polycistronic mRNA for the lac operon (Kiho and Rich, 1965). In addition, the genetic evidence that all of the controlling sites of an operon map at one end of the operon shows that independent initiation of mRNA synthesis at the beginning of each gene does not occur under normal conditions.

It has been found that under certain chemical conditions the initiation of transcription can start at internal sites of Escherichia coli (Imamoto, 1968). The regulation of the amounts of specific operon mRNAs in the bacterial cell has been examined by double-labelling techniques and by RNA-DNA hybridization experiments.

In four operons the amount of operon-specific mRNA rises when the operon is expressed, and for three of these operons there is a good correlation between the amount of specific mRNA and the degree of expression of the operon (Martin, 1963; Attardi et al., 1963; Hayashi et al., 1963; Imamoto, 1965).

In three operons lac, his and gal, the appearance of the enzymes after induction or derepression is sequential, the product of the more proximal genes appearing before that of the more distal ones (Alpers

and Tomkins, 1965; Leive and Kollin, 1967; Kepes, 1967). This sequence is entirely consistent with the evidence that both transcription and translation proceed from the proximal to the distal genes of an operon.

Which of these two processes is primarily responsible for giving the sequential appearance is not known. In the his operon, the sequential appearance is replaced by simultaneous appearance of all enzymes under some experimental conditions (Berberich, 1966, 1967). These alternate modes of appearance of the enzymes suggest that under some conditions initiation of translation of the regions of the mRNA corresponding to distal genes may occur independently of translation of the proximal genes.

The apparent sequential translation of operon mRNA does not, in general, result in decreasing amounts of distal gene products. In three operons (his, try, gal) there is an equimolar synthesis of monomeric protein sub-units from several of the genes including the most distal and the most proximal ones (Imamoto and Yanofsky, 1966; Wilson, 1966). The only operon for which this is not the case is lac, where the synthesis of the distal enzyme, acetylase, is ten-fold less than

that of β-galactosidase even though these two enzymes seem to be subjected to co-ordinate control (Zabin, 1963). Reduced expression of the acetylase may be related to the fact that this enzyme is not essential to the pathway determined by the lac operon (Fox et al., 1966).

9. Evidence for the conservation of genetic material during differentiation

The tissues of the adult organism differ essentially from one another in the proteins they contain. Information concerning the structure of each individual protein is contained in the DNA of each cell. In any one tissue, only a given fraction of the information enclosed in the DNA is transcribed and translated into protein; whereas in another tissue, a different portion of the genome is expressed. This is known as cytodifferentiation. A mechanism must, therefore, exist in each cell whereby a definite part of the genome is selected for translation into proteins; this could occur either by selectively destroying the unwanted genes in differentiated cells or by masking the unwanted genes so that they are conserved but incapable of expression.

Examples which follow the first kind of mechanism come from studies in certain invertebrates, namely,

the horse nematode, Ascaris (Wilson, 1925) and the gall midge, Mayetiola (White, 1950) in which differentiation is achieved by the selective loss of genes. Only the germ cells of these species contain the full chromosome complement. Another example which follows a similar mode of differentiation is provided by the mammalian erythrocyte. In most species mature erythrocytes are formed by the extrusion of the reticulocyte nucleus. However, these are thought to be extreme forms of differentiation on the basis of evidence concerning the qualitative and quantitative estimation and characterisation of DNA in other differentiated animal tissues. All of these examples share a common characteristic: they are irreversible as far as the fate of the cells involved is concerned.

The general rule in most animals is that the full chromosome complement as identified morphologically, is preserved in every tissue. Chemical analysis of the total DNA content of different tissues of a particular species indicated a constant DNA content in normal diploid nuclei of a variety of cell types, within the limits of accuracy of the method.

Studies of hybridization of DNA from different mouse organs with DNA from total mouse embryo by McCarthy

and Hoyer (1964) and in Xenopus by Denis (1967) shows that there are not any distinguishable differences in DNA polynucleotide sequences in all organs of the mouse. Inaccuracies of the method less than 1% would still permit great differences among the tissues to be undetected. These cases are not proof that alterations do not take place in the genes of the several tissues which have been studied; but in some cases there is strong evidence that the total genetic complement remains in differentiated cells.

The most convincing evidence is provided by some experiments done by Gurdon (1962 a, b; 1966). It was shown that transplantation of an intact nucleus from the intestinal epithelium of a tadpole to an egg in which the nucleus has been inactivated by radiation, can permit the egg to develop quite normally into an adult. This experiment provides strong evidence that some intestinal epithelial cells contain all the genetic material necessary for the normal development of a toad.

Another indication that cytodifferentiation in animals can proceed without any change in the genetic composition comes from the work of Hadorn (1965) on insect larvae. Furthermore, these results demonstrate

that differentiation need not involve irreversible changes in any significant part of the genome and irreversible gene inactivation mechanisms cannot be regarded as the fundamental cause of differentiation. A particularly relevant finding for plants is that of Braun (1959) who showed that a single somatic cell of tobacco is potentially able to give rise to an entire tobacco plant.

The experiments on nuclear transplantation in amphibia as well as the experiments from plant tissue provide conclusive evidence that differentiation may occur without any qualitative change in the DNA within the nucleus of the cell.

Given the dependence of functional cell character on the cell genome and the equivalence within any one organism of these genomes, one is led directly to the proposal that selective variation in gene activity lies at the root of the differentiation phenomenon. Thus the small fraction of the organism's (or cell's) genetically borne capabilities which actually materialise in any one cell type must indicate the restriction of gene expression to only the appropriate small fraction of genes needed to direct that cell's special behaviour. The rest of the genome in the cell is to be regarded as

repressed, i.e. inhibited from synthesising RNA. These two corollaries, that only a small portion of the genome is active and that in any differentiated cell most of the genome is repressed (reversibly), follow, necessarily from the variable gene activity theory of cell differentiation. The serious proposal that variable gene activity could underlie differentiation can be considered to date from the writings of Mirsky (1951; 1953) Stedman and Stedman (1950) and Sonneborn (1950).

10. Characteristics of differentiation

During cell division of an embryo, each daughter cell receives an identical set of chromosomes and presumably therefore an identical set of hereditary potentialities. Yet these daughter cells commonly follow their own independent pathways of development until finally at late stages of differentiation they exhibit remarkably different phenotypes (Bell, 1965; Locke, 1963). Thus identical genotypes give rise to considerably different phenotypes, as different as nerve and muscle and pigment cells. This process is known as cell differentiation.

One of the major problems of differentiation is how developing cells acquire specific biochemical

characteristics, and how these are linked to morphological development and cellular function (Harris, 1964; Mazia and Tyler, 1963). Though it is believed that the entire complement is contained in the differentiated cells (except for the extreme cases which have been mentioned before) each specialised cell contains characteristic amounts and kinds of proteins.

A classical example is the erythrocyte and its precursor the reticulocyte which produces principally a single protein, haemoglobin. It may be concluded that in the precursor cells of the erythrocytes, the genes for making haemoglobin are "turned on", to produce the mRNA for the production of haemoglobin. Contrarywise, in other specialised cells of the same organism, no haemoglobin is produced although other proteins are. With the new concept of knowledge this can be interpreted that in these other cells the genes for making haemoglobin are inert, and do not make their mRNA. The presence of partially diverse RNA populations specific to given differentiated cell types and to given states of differentiation represents a direct verification of the variable gene activity theory of

cell differentiation i.e. in liver regeneration (Thaler and Villee, 1967) and in hormone response (O'Malley et al., 1968.)

Thus in differentiating vertebrate cells, we find mechanisms responsible for the acquisition and loss of functional characteristics. However, unlike the bacterial cell, the stimuli that initiate and establish these alterations in developing vertebrate cells cannot be attributed to obvious environmental changes and are not as well understood as in bacterial cells.

However, several instances have been reported where animal cells demonstrate reversible enzymic induction in a mode similar to pseudo-differentiation. Such appears to be the case with glutamyltransferase in HeLa cells and strain L cells in continuous culture (De Mars, 1958; Paul and Fottrell, 1963).

Another example of mammalian enzyme which behaves in a similar way as the adaptive phenomena in bacteria is tryptophanepyrrolase. In rat and mouse liver the specific activity of this enzyme can be substantially increased either by cortisone treatment or by feeding excess tryptophane (Knox and Auerbach, 1955; Knox, 1956). The mechanism involved in the control of this enzyme is more complex than those postulated in micro-

organisms. It has emerged that different inducers operate at different stages of protein synthesis; the level of the enzyme results from several simultaneously operating factors.

Another example is the stimulation of tyrosine aminotransferase synthesis by dexamethasone phosphate (adrenocortical steroid) in HTC cells (Granner et al., 1968). Their result had suggested that the steroid-induced increase in tyrosine aminotransferase activity in these cells is due to a faster rate of enzyme synthesis. In this work the antigen-antibody precipitin reaction was used to study if the induction could be attributed either to an increased amount of enzyme protein or to an activation of pre-existing enzyme molecules. Immunotitration studies show that the increase in enzymic activity does not result from the conversion of an immunologically cross-reacting, catalytically inactive precursor to active enzyme. In addition, using the antibody, the rates of amino acid incorporation into tyrosine aminotransferase in induced and uninduced HTC cells were compared, and the results show that the dexamethasone increases the rate of tyrosine aminotransferase synthesis by a factor of 15 to 30 over the uninduced control.

Although differences between the two control mechanisms are undeniable, the existing similarities are considerable enough so that cautious extrapolations might be made from studies on micro-organisms to the situation in cells of higher organisms. One of the questions of major importance in differentiation, is concerned with the mechanisms by which differentiated cells maintain their characteristics in the mature organism. Adult cell types in animals do not ordinarily alter their functions except during degeneration or in dedifferentiation in consequence of tumorogenesis.

The masking hypothesis has been proposed as a possible mechanism for the explanation of inactivation of genes. Evidence for such a mechanism has been obtained from studies on the giant chromosomes of certain insects, where development is accompanied by intense RNA synthesis at localised specific regions of the chromosome (Beerman, 1959; Edstrom, 1962; Becker, 1959; Clever, 1966).

11. Cell proliferation

There are several populations of cells which under ordinary conditions, display a very slow DNA synthesis and proliferating activity, but which can become very active when exposed to the proper stimulus. If these

populations of cells are stimulated, first they enter a DNA synthesis phase which is followed by a wave of mitosis.

These situations are referred to as models of stimulated DNA synthesis and their interest lies in the fact that one can follow the steps structural or functional leading from a quiescent state to DNA synthesis and cell division. The cases which will be discussed include mainly in vivo models, i.e. a single application of stimulus in mammalian tissues or organs. All these models have in common a pre-replicative period, that is, a lag time varying from 12 hours to a few days, between the application of the stimulus and the onset of DNA synthesis.

There is evidence that a very early increase of RNA synthesis is predominant during the first hours after the application of the stimulus. Fukioka and Lieberman (1963) were the first to demonstrate an increase of RNA synthesis almost immediately after partial hepatectomy. This increase, reaches a maximum of about 80% above the control within the first 5 hours. The increase in RNA synthesis is accompanied by a doubling in RNA polymerase activity which reaches a maximum after 12 hours of partial hepatectomy

(Tsukada and Lieberman, 1964). A prompt increase in RNA synthesis has been shown in spleen cells after an injection of erythropoetin (Hodgson, 1967), in estrogen stimulated rat uterus (Hamilton, Widwell and Tata, 1965; Means and Hamilton, 1966) and in phytohemagglutinin-stimulated lymphocytes (Mueller and Le Mahieu, 1966).

Malt and Mueller (1967) showed that after nephrectomy the production of both nuclear precursors of rRNA and mRNA was observed. Estimates of mRNA were inversely related to the estimates of nuclear rRNA and to mitotic activity. Malt et al., (1969) reported the presence of labelled giant HnRNA (10S - 70S) during a pulse-label after uninephrectomy and its disappearance after 60 minutes. They suggested that the decrease within an hour after uninephrectomy is a consequence of faster metabolism of the HnRNA; this phenomenon is probably related to the "switch-on" of renal RNA synthesis that is also responsible for the compensatory hyperthrophy.

Very recently it has been observed that within 12 hours after a single injection of folic acid there is an increase of about 200% above the control of the nuclear RNA synthesis, in rat kidney (Threlfall et al., 1969). Basic nuclear proteins (histone-like) are also

increased after folic acid with a maximum after 26 hours of treatment. These early changes in RNA synthesis have been suggested by many authors as gene activation, an hypothesis which has not yet been rigorously proved. Perhaps good evidence in this respect is the finding of Lieberman et al., (1963) that the stimulation of DNA synthesis is inhibited by very small doses of Dactinomycin in regenerating liver and in primary explants of rabbit kidney cells.

More support for this hypothesis comes from the work of Church and McCarthy (1967) who investigated the appearance of new RNA species in the mouse liver after partial hepactectomy. They claimed that new species of RNA were synthesised as early as 1 hour after hepatectomy and that other species appeared at later times, while the species synthesised in the early hours were discontinued. They interpreted their results as indicating that regenerating liver RNA is the result of reactivation of genes repressed in adult liver.

It is reasonable to assume that part of the RNA synthesis during this period consists of ribosomal RNA, probably necessary for the growth of cells prior

to division and part of the RNA consists of template RNA for the synthesis of new enzymes necessary for DNA synthesis, such as thymidine kinase, thymidylic kinase, DNA polymerase, deoxycytidylate deaminase and others.

The first assumption is supported by the finding that the newly synthesised RNA is mostly ribosomal (Bloom, Torado and Green, 1966; Drew and Brawerman, 1967). The second assumption is supported by the observation that actinomycin D given at this early period blocks the appearance of DNA polymerase (Fausto and Van Laucher, 1965), thymidine kinase (Lieberman et al., 1963) deoxycytidylate deaminase (Holtzer et al., 1964).

However, other proteins must be synthesised at this time. Majumbare et al., (1967) have shown that partial hepatectomy increases the rates of synthesis of serum albumin and fibrinogen, proteins that are formed mainly or entirely by the liver. Mueller and Le Mahieu (1966) showed that phytohemagglutinin-stimulated lymphocytes synthesise γ-globulin. An accumulation of proteins in the lumen of kidney tubules stimulated by folic acid has been observed by Baserga and Thatcher (1965).

Changes in the RNA and protein synthesis seem to be the most prominent events in the first few hours of the prereplicative period after the application of a stimulus.

Any information of the fate of the stimulus can throw light on the process of DNA synthesis and especially to the regulatory mechanisms which precede it and control it. This of course is not possible with the regenerating liver but it is feasible in model systems stimulated by chemical means, especially when it is a chemically purified compound, such as folic acid, estrogen or isoproterenol. The stimulatory activity on DNA synthesis of folic acid (Threlfall, 1968) and isoproterenol has been recently discovered.

The prevalent hypothesis on the mechanism of stimulated-DNA synthesis is that of gene activation. It is mainly based on the theory that DNA synthesis depends on activation of a portion of the mammalian genome that directly controls the replication of the entire genome (Baserga, 1965). Consequently, the stimulus for DNA synthesis activates a segment of the genome, which responds by making RNA necessary for coding the various enzymes required for DNA synthesis and cell division.

12. Methodology of hybridization

Recently the DNA-RNA hybridization technique is applied to the analysis of differential gene transcription in simple and complex organisms. Several methods developed for the detection of hybrid formation will be described.

The first successful demonstration for hybrid formation between DNA-RNA was reported by Hall and Spiegelman (1961). They showed that the RNA is able to form a bybrid molecule with the DNA region on which it has been transcribed during a slow cooling of the mixture.

Volkin and Astrachan (1956) had shown that RNA formed after infection with bacteriophage T2 possessed an apparent base ratio analogous to that of T2-DNA. Nomura et al., (1960) confirmed this observation and in addition they offered evidence for the existence of a "T2-specific RNA" which was different from the bulk of the Escherichia coli RNA in electrophoretic mobility and average sedimentation coefficient.

Hall and Spiegelman (1961), in order to examine the complementarity of sequences between T2-specific RNA and T2-DNA, extracted RNA from Escherichia coli

after infection with T2, and that was hybridized with the homologous T2-DNA. They showed that the RNA is able to form a hybrid molecule with the DNA region on which it has been transcribed, during a slow cooling of the mixture in a solution of 0.03M sodium citrate and 0.3M NaCl at a pH of 7.8. When the temperature reached 26°C (starting temperature 65°C) the solution containing denatured DNA and RNA was removed and brought to a density of 1.74 g/ml by adding suitable amounts of CsCl and Water. Undenatured DNA was added to the solution as a reference marker. The solutions were centrifuged at 33,000 r.p.m. in the SW-39 rotor at 25°C for five days. The CsCl density gradient centrifugation permits a clear separation of ^{3}H-T2 DNA from ^{32}P-RNA and provides therefore a test for interactions leading to the formation of RNA-DNA hybrids. Comparison of the profiles of ^{3}H and ^{32}P obtained showed clearly that slow cooling of the single stranded DNA and RNA produces a new peak of ^{32}P which contains a DNA-RNA hybrid having approximately the same density as denatured T2-DNA. Thus the complex does in fact contain considerably more DNA than RNA, as it must be for complexes having a definite structure. The

DNA:RNA ≤ 5 in hybrid. That this interaction is unique to the homologous pair is shown by the virtual absence of such complexes when T2-specific RNA is slowly cooled with heterologous DNA.

In attempts to find out if the DNA contains sequences complementary to homologous ribosomal RNA Yankofsky and Spiegelman, (1962) used the technique described above, but they introduced a new technique, that is the dependence on the resistance of hybrids to ribonuclease for discrimination between chance pairing involving regions not completely complementary and specific hybridization.

Bautz and Hall (1962) used phospho-cellulose acetate to immobilise denatured T4 phage DNA. The mechanism in this attachment involves covalent bond formation between the glucosylic hydroxyls of the DNA and phosphate groups on the cellulose. The use of this method is thus limited, since only some bacteriophages contain glucosylated DNA.

Bolton and McCarthy (1962) used a new device for immobilising any high molecular weight DNA, by physical entrapment in cellulose acetate gels or in agar gels. Cellulose acetate or agar were mixed

with denatured T2-DNA and washed with 2 x SSC at 60°C. Trapping of DNA was between 50 - 100% of the input. Incubation of 50 µg labelled T2-specific RNA in 2 x SSC with DNA-cellulose acetate or DNA-agar containing 0.5mg DNA, were carried out at 60°C for 15 minutes either in a thick slurry in a screw top vial or in a chromatograph tube heated by circulating water. After incubation the preparations heated in vials were transferred to chromatograph tubes heated at 60°C. The column was washed with ten or more 5ml aliquots of 2 x SSC in order to wash away the unabsorbed material. The contamination was reduced to 0.01% of the total. The bound nucleic acid was then recovered by reducing the salt concentration to 0.01 x SSC, raising the temperature to 75°C and collecting the fractions.

Nygaard and Hall (1963; 1964) have described a technique for the formation of RNA-DNA complex. This method is based on the observation that the nitrocellulose filters in salt solutions can absorb denatured DNA but not native DNA or RNA. Reaction mixtures were prepared by mixing RNA, DNA which had been heat denatured, and KCl to a final concentration

of 0.5 M in a total volume less than 1ml. The mixture was heated at 67°C for 1 - 2 hours and then cooled in ice. 3mls of 0.2 M KCl containing 5mg/ml ribonuclease were added and incubated for 30 minutes at 37°C. The solutions were then passed through nitrocellulose filters, previously soaked in 0.5 M KCl for 30 minutes. The filters were then washed with 60 mls 0.5 M KCl, dried at room temperature and counted with scintillating solution and counted.

The above mentioned conditions were found as optimal conditions according to their kinetic experiments using T2-DNA and "T2-specific RNA" (RNA from Escherichia coli after infection with T2). The maximum amount bound was approximately 0.3 µg RNA/µg T2 DNA. This is consistent with the complex being one-strand RNA and one-strand DNA, provided that certain regions of the T2 DNA do not participate in the reaction. Hall et al., (1963) have shown that only 50% of the polynucleotide chains of denatured T2 DNA are capable of binding T2 RNA.

One disadvantage of the liquid hybridization technique has been pointed out, namely, the occurrence of DNA-RNA reannealing which reduces the opportunity for formation of RNA-DNA hybrids. As it has been

pointed out by Nygaard and Hall (1964) the DNA-DNA reannealing takes place under the same conditions as the formation of RNA-DNA hybrid.

Gillespie and Spiegelman (1965) described the following method where DNA is immobilised irreversibly on filters thereby reducing the risk of DNA-DNA re-association. DNA was denatured by the alkali method and immobilised on nitrocellulose filters by passing it through the filters. The DNA was previously diluted in 2 x SSC and the filters were presoaked in 2 x SSC for 1 minute. The filters were then washed from both sides with 100 ml of 2 x SSC and were subsequently dried at room temperature for at least four hours and at 80°C for another 2 hours in a vacuum oven. Hybrids were formed by immersing the filters in vials containing labelled RNA in 2 x SSC or 6 x SSC. Annealing was carried out at 67°C for 18 hours after which the vials were chilled in an ice bath. The filters were then washed on both sides with 50ml 2 x SSC. RNA not completely complexed is destroyed by immersing the filters for 1 hour at room temperature in 2 x SSC containing 20µg/ml of heated pancreatic RNase. After RNase treatment the filters were rewashed on

each side as described above. Finally, the filters were dried and counted.

Church and McCarthy (1967) claimed that the stability of the DNA-RNA hybrid is strongly dependent on ionic strength. They presented data in which thermal dissociation of the hybrid was studied at two different salt concentrations (2 x SSC and 0.5 x SSC) with or without RNase. From their results they suggested that whether or not RNase is used, the mean stability of the hybrid is the same and not higher when RNase was used.

Materials and Methods

1 Materials

Isotopes
Nucleic acid precursors
Enzymes
Preparation of macaloid

Methods

1.1 Preparation of nuclei
1.2 Preparation of chromatin

2 The DNA dependent RNA polymerase from M. lysodeikticus

2.1 The purification and properties of RNA polymerase
2.2 The quantitative assay for polymerase activity
2.3 A qualitative assay for RNase and polynucleotide phosphorylase activity

3 Preparation of RNA

3.1 Nuclear RNA
3.2 Preparation and isolation of *in vitro* synthesised RNA
3.3 Determination of radioactivity

4 Preparation of DNA

5 Quantitative estimations

5.1 The quantitative estimation of DNA
5.2 The quantitative estimation of RNA
5.3 The quantitative estimation of protein

6 Preparation of polyacrylamide gels

MATERIALS

Unless otherwise stated, British Drug Houses (Analar grade) supplied all reagents for chemical solutions.

Isotopes

^{3}H uridine carrier free ^{32}P-orthophosphate were supplied by the Radiochemical Centre (Amersham, England).

Nucleic acids and precursors

Nucleoside triphosphates, ATP, GTP, CTP and UTP were obtained from Koch Light Limited. Highly polymerised yeast RNA was supplied by British Drug Houses Ltd.

Enzymes

Crystalline pancreatic ribonuclease (bovine) was obtained from Sigma Chemical Company. Stock solutions of 2mg/ml in water pH 7.0 were first heated in boiling water for 10 minutes to destroy any deoxyribonuclease activity, and stored frozen.

Deoxyribonuclease I was prepared electrophoretically by Worthington Biochemical Corporation. Solutions of 1mg/ml in 0.02M glycine were stored frozen.

Pronase from Calbiochem. was supplied as a powder which was dissolved in water to a concentration of 500 g/ml. The solution was autodigested at 37°C for

2 hours prior to use to destroy any nuclease activity.

Preparation of macaloid

Macaloid, a purified bentonite (sodium magnesium lithofluorosilicate) is a potent inhibitor of ribonuclease activity (Stanley et al. 1965). It was a gift from the Baroid Division of the National Lead Company, Texas. Macaloid was further purified by blending a 5% (w/v) suspension in water and dialysing for two consecutive 24 hour periods against 25 x volumes of distilled water. It was stored at -15°C.

METHODS

1.1 Preparation of nuclei

1) Citric acid method:

The chopped tissue is homogenised for 1 minute in 20 volumes (w/v) of 0.025 citric acid using a Potter-Elvehjem type homogeniser fitted with a teflon pestle. The homogenate is filtered through two layers of terylene gause and centrifuged at 2500r.p.m. (approx. 1500g) for 10 minutes. The supernate is discarded and the nuclei are washed three or four times in 20 volumes of 0.025M citric acid until the supernate is clean. The entire procedure was carried out at 0 - 3°C. The purification of the final preparation was tested by staining with brilliant cresol and viewing under a microscope.

2) Sucrose method:

The tissue is homogenised in 10 volumes of 0.33M sucrose; 4mM $CaCl_2$ using the same type of homogeniser as in method I. The homogenate is filtered through two layers of terylene gause. It is centrifuged at 2000r.p.m. (900g) for 10 minutes. The pellet is resuspended in 0.25M sucrose; 3mM $CaCl_2$ and centrifuged at 2000r.p.m. (1000g) for 15 minutes. The pellet is suspended in 3ml of 0.25M sucrose; 3mM $CaCl_2$ and 15ml of 2.4M sucrose

3mM $CaCl_2$ is added. It is centrifuged for 1 hour at 3700r.p.m. in 8 x 25 rotor. The pellet is resuspended in 0.25 sucrose; 3mM $CaCl_2$.

3) The Triton X-100 method:

The tissue is homogenised in 9 volumes 0.25M sucrose; 2mM $MgCl_2$ using the same homogeniser as in method I. The homogenates were filtered through a single layer of coarse cheese cloth. After centrifugation of the filtrate at 2000r.p.m. (1000g) for 15 minutes the sediments were resuspended in a solution containing 0.5% (w/v) Triton X-100; 0.25M sucrose; 1mM $MgCl_2$. Two washings with the detergent solution were required to obtain a satisfactory nuclear preparation. The final nuclear pellet could be resuspended in 0.25 sucrose; 1mM $MgCl_2$. The entire procedure was carried out at 0 - 3°C.

1.2 Preparation of chromatin

The nuclei were suspended by slight homogenisation in 0.15M NaCl; 01M Tris-HCl pH 7.5. After 10 minutes in ice the material was pelleted at 2000g for 10 minutes. The pellet was suspended in distilled water. Water washing was continued until a viscous gel was formed. This material constitutes chromatin.

2. The DNA dependent RNA polymerase from Micrococcus lysodeikticus

2.1 The purification and properties of RNA polymerase

RNA polymerase was purified from M. lysodeikticus by the method of Nakamoto et al. (1964). A 30g batch of spray-dried cells from Cambrian Chemicals or Sigma Chemical Co. was suspended by Waring blender in 300mls 0.01M tris-HCl pH 8 and centrifuged down at 20,000g for 10 minutes. The pellets were suspended in 600mls, 0.2M sucrose; 0.01M tris-HCl pH 8 at 30°C, 200mg of crystalline lysosyme added and the suspension incubated at 30°C for 45 minutes. After 15 minutes, 1.5ml 0.1M $MgCl_2$ were added with rigorous stirring, and likewise after 45 minutes, 4.5mls 0.1M $MgCl_2$ added. The lysate was diluted with an equal volume of ice cold water and stirred thoroughly. A viscous gel formed and was allowed to stand for 10 minutes in ice. All subsequent were carried out at 0 - 4°C.

120mls 10% (w/v) streptomycin sulphate (Glaxo Labs.) were slowly added to the lysate with continual stirring. The whole procedure took 20 minutes and was accompanied by a noticeable drop in viscosity. After standing for 10 minutes the precipitate was collected by centrifugation at 20,000g for 10 minutes. The supernatant was decanted and the pellets rinsed with 10mls distilled water. The

pellets were washed by light homogenisation in 200mls 0.001M tris-Hcl, pH 8; 0.2M sucrose; 0.1 per cent streptomycin sulphate; 0.25M $MgCl_2$, mixed for 10 minutes and centrifuged at 20,000g for 10 minutes. The supernatant was discarded and the pellets rinsed with distilled water.

The pellets were suspended in 180mls of a solution containing 2mls 1M potassium phosphate buffer pH 7.5; 2mls 0.1M $MgCl_2$; 20mls 2M sucrose by homogenisation. After suspension 8mls of 1M potassium phosphate buffer were added slowly with stirring. The solution became very viscous and was left for 10 minutes before slowly adding 10mls of 10 per cent streptomycin sulphate as before. After mixing for 10 minutes the supernatant was collected by centrifugation at 30,000g for 30 minutes and then further centrifugation in the MSE 50 30 head at 80,000g for 120 minutes. The supernatant was decanted, carefully avoiding the loosely packed sediment, and its volume measured.

From a 2.5 per cent (w/v) unneutralised protamine sulphate (Sigma Chemical Company) solution, an amount equivalent to one-fifth the volume of the above supernatant was set aside. Assuming that the molarity of phosphate in the supernatant was 0.05, enough 1M phosphate buffer

was added so that after the addition of protamine sulphate the final phosphate molarity was 0.14M. The protamine sulphate was then added dropwise with continuous stirring and after 10 minutes the precipitate was collected by centrifugation at 20,000g for 10 minutes. The pellets were homogenised in 30mls 0.2M sucrose; 0.3M potassium phosphate, mixed for 10 minutes and centrifuged at 20,000g for 10 minutes. The supernatant was retained and the pellet re-extracted with 15 mls of the same buffer. To the combined extracts, 2 volumes of cold 0.1 per cent (w/v) protamine sulphate were added dropwise with stirring. The extracts were mixed and centrifuged as before. The supernatant was discarded and the pellet homogenised in 10mls 0.2M sucrose; 0.14M potassium phosphate pH 7.5. After 10 minutes, pellets were obtained by centrifugation at 20,000g for 10 minutes, and homogenised in 10mls 0.2M sucrose; 0.3M potassium phosphate buffer pH 7.5. After mixing for 10 minutes, the supernatant was collected by centrifugation at 20,000g for 10 minutes. The pellet was re-extracted with 5mls of the same buffer and combined with the previous extract.

The protein concentration of the extract was adjusted to approximately 6mg/ml by extracting medium

(by E260/E280 method). Saturated ammonium sulphate solution, saturated at 0°C, was added dropwise to 40 per cent saturation. After 30 minutes at 0°C the precipitate was collected at 20,000g for 10 minutes. The pellet was dissolved in 0.1M tris-HCl pH 7.5, an equal volume of glycerol added, and the solution stored at -20°C. The enzyme retained most of its original activity under these conditions.

The usual specific activity of the enzyme at this stage was about 200 units/mg protein.

2.2 The quantitative assay for polymerase activity

Assay conditions were essentially those of Nakamoto et al. (1964). Incubations were carried out at 37°C for 10 minutes in centrifuge tubes containing 50 μmoles tris-HCl, pH 7.5; 1.25μ moles $MnCl_2$; 100μg calf thymus DNA; 2.4μ moles each of GTP, CTP, ATP, and labelled UTP (^{3}H-UTP added to give 0.5 - 2 x 10^6dpm per μ mole) and 0.1 - 0.5mg enzyme in a total volume of 0.5ml. The reaction was terminated by placing the tubes in ice and adding 0.1ml 50 per cent TCA, followed by 3mls 5 per cent TCA. Where small amounts of material were present, 0.5mg bovine serum albumin was added as carrier, before precipitation. After standing in ice for 10 minutes the precipitate was collected by centrifugation

at 2,000g for 10 minutes. The supernatant were discarded and the precipitates washed three times in 5 per cent TCA. The precipitates were trapped on Millipore filters and counted in a scintillation counter.

A unit of activity is defined as the amount of enzyme which catalyses the incorporation of mμ mole of UTP into acid-insoluble material during 10 minutes incubation at 37°C.

2.3 A qualitative assay for ribonuclease and polynucleotide phosphorylase activities

Reagents:

1) Stock yeast RNA solution: A solution containing 5mg RNA/ml in 0.1M tris-HCl pH 7.5; 1mM magnesium sulphate.

2) Working RNA solution: dilution of 1 volume of the stock solution with 9 volumes of distilled water.

3) RNA precipitant solution: 2.5g of lanthanum nitrate in 125mls of 5M HCl and bringing the volume to 500ml with absolute ethanol.

4) Ribonuclease solution: 2mg ribonuclease/ml in 0.1M tris-HCl buffer.

5) 1.0M potassium phosphate buffer pH 7.4, required for the detection of the presence of polynucleotide phosphorylase.

Procedure:

Tubes were set up according to the following scheme:

Set of tubes	Sample to be tested	RNase soln	1M potassium phosphate pH 7.4	0.1M tris-HCl pH 7.4
Blanks	0.00	0.01	0.15	0.135
Samples	0.05	0.00	0.15	0.130
RNase standard	0.00	0.01	0.15	0.134

In each tube 1.5ml of RNA solution (0.5mg/ml) was added and were incubated at 37°C for 90 minutes. The incubation was terminated by placing the tubes in ice and adding 3.0ml of cold RNA precipitant solution. The content of each tube was mixed thoroughly and filtered through Whatman No. I filter paper. RNA is precipitated as the lanthanum salt but hydrolised nucleotides pass through the filter and contribute to the O.D 260 reading of the filtrates.

The reading for the blanks and for samples containing no detectable ribonucleolytic activity was about 0.2 O.D.

The absorbancy of ribonuclease standard solution is a measure of the ribonucleolytic activity of the samples under test.

3. Preparation of RNA

3.1 Nuclear RNA

Whole cell RNA was prepared from finely chopped tissue by homogenising for 2 minutes in a mixture of 6% (w/v) sodium 4 - aminosalicylate and phenol-m-cresol mixture (15 vol. of each solution). The phenol-cresol mixture: phenol (detached cyrstals) 500g, m-cresol 70ml, water 55ml and 8-hydroxyguinoline 0.5g. The mixture was stirred for 20 minutes at 20°C and then centrifuged at 6000g for 30 minutes at 5°C and if any emulsion remained in the upper phase this was removed and centrifuged again. Sodium chloride (3g) was added to each 100ml of the clear top layer, which was then re-extracted with 0.5 volumes of phenol-cresol mixture for 10 minutes at 20°C and then centrifuged at 8000g for 10 minutes at 5°C. The aqueous phase was removed and mixed with 2 volumes of ethanol-m-cresol (9:1 v/v) and the mixture allowed to stand for 30 - 60 minutes at 20°C. Where nuclear RNA was required the above procedure was carried out on a nuclear pellet.

The resulting precipitate was centrifuged at 600g for 15 minutes and the pellet dissolved in a convenient volume of 0.01M Tris-HCl pH 7.5; 0.001M $MgCl_2$ and treated with 20 µg/ml DNase for 15 minutes at 30°C. The reaction was terminated by shaking with an equal volume of phenolcresol as before. To the aqueous phase was added 0.25 volumes 6M potassium acetate; 0.3M NaCl with thorough mixing; followed by 0.25 total volumes 100% ethanol. After mixing the solution was left at -20°C for 60 minutes. The precipitate was collected by centrifugation at 2000g for 15 minutes and washed thoroughly in 100% ethanol.

Finally the pellet was dissolved in 15 volumes 0.01M sodium acetate; 0.01M NaCl; 0.001M $MgCl_2$ pH 5.0. An equal volume of 100% ethanol was added drop by drop with much stirring and the solution left at -20°C for at least 2 hours. The precipitate was centrifuged as before and the precipitation step repeated until a pinkish translucent pellet was obtained.

The final product was dissolved in 0.01M Tris-HCl pH 7.5; 0.001M $MgCl_2$ and the concentration determined from the absorbancy at 260mu. An absorbancy of 22 O.D. was taken being equivalent to 1mg RNA/ml.

3.2 Preparation and isolation of in vitro synthesised RNA

A modified procedure, not employing precipitation steps, was devised for the isolation of RNA synthesised in a cell-free system. Incubation mixtures were set up to contain the following components in a final volume of 3ml. 150μ moles Tris-HCl pH 7.5; 7.5μ moles $MnCl_2$; 50 to 100 Weiss units RNA polymerase; 2.4μ moles ATP; 2.4μ moles CTP; 2.4μ moles GTP; 2.4μ moles ^3H UTP (4 to 20×10^6 dpm/min/μ mole; 100 to 500 μg DNA, as whole DNA or chromatin. Chromatin does not dissolve in the reaction mixture but can be obtained as a fine suspension by mixing it rapidly with the other reagents. The reaction mixtures were incubated at 37°C for 1.5 hours, and the reaction was terminated by placing the tubes in ine. The mixtures were treated with 20μg DNase/ml at 37°C for 15 minutes. The solutions were shaken with phenol containing 0.1% 8 - hydroxyquinoline and centrifuged at 2000g for 10 minutes. This step was repeated until no interface remained. The extract was then dialysed overnight against 0.01M Tris-HCl pH 7.5; 0.001 M $MgCl_2$ and 0.05% bentonite or macaloid.

The dialysate which contains the in vitro synthesised RNA was lyophilised and the resulting powder was dissolved

in a convenient volume of 0.01M Tris-HCl pH 7.5; 0.001M $MgCl_2$.

3.3 Determination of radioactivity

Radioactive RNA precipitates were counted by one of the two methods 0.1ml of NCS (Nuclear Chicago Solubiliser) was added to the precipitate and left at room temperature. When the precipitate was completely dissolved 0.4ml water and 5.5mls dioxane scintillator was added and the sample was left in the cool and dark overnight and then counted. Alternatively, after the initial precipitation with TCA, the precipitate was collected on a Millipore filter (pore diameter 0.45μ) and washed with three successive portions of 5 per cent TCA. The filters were dried thoroughly at 50°C and placed in 5ml toluene scintillator fluid (0.42 per cent PPO; 0.021 per cent POPOP in toluene). Correction for counting efficiency were applied using standard quench data.

4. Preparation of DNA

DNA was prepared by a modification of the method described by Marmur (1961) and of Kay et al. (1952). Nuclei are prepared according to the citric acid method. The nuclear pellet was neutralised by adding a convenient

volume of 0.5M tris-HCl pH 7.4. 10 volumes (v/v) 0.15M NaCl; 0.1M EDTA pH 8 were added. In some cases whole embryo DNA was prepared, by first decapitating the animals and homogenising in 10 volumes 0.15M NaCl; 0.1M EDTA for 2 minutes using a Waring Blender. The homogenate was filtered through two layers gause.

10% sodium lauryl (or dodesyl) sulphate (w/v) was added to the suspension to give a final concentration of 2 per cent in 1 x SSC and stirred at room temperature for 60 minutes.

5M sodium perchlorate (or chloride) was added to a final concentration of 1M and the mixture was stirred for another 60 minutes at room temperature. The reaction was terminated by adding an equal volume of chloroform-octanol (24;1) and shaking the mixture gently for 15 minutes. After centrifugation for 20 minutes at 10,000g the aqueous phase was pippeted off. This step was repeated until no protein interface remained To the aqueous phase, 2 volumes re-distilled ethanol was added and the DNA precipitate was collected on a glass rod. The DNA was redissolved in 50mls 0.01 x SSC made up to 1 x SSC in solution Ribonuclease was added to a final concentration of 50μg/ml and incubated at 37°C for 30 minutes. Following ribonuclease

digestion pronase was added to 50μg/ml incubated at 37°C for 2 hours. The incubation was terminated by shaking the mixture with an equal volume of chloroform: octanol (24:1) and this treatment was repeated until no protein interface is present. The DNA from the aqueous phase was precipitated by the addition of 2 volumes ethanol as before, and dissolved in 45ml 0.01 x SSC. 5mls of 3M sodium acetate; 0.001M EDTA was added with mixing followed by a half volume isopropyl alcohol.

The precipitated DNA was spooled on a glass rod and then washed successively in 75% and 95% ethanol. Finally, this precipitate is dissolved in 0.01 x SSC; 0.15 NaCl and stored at -20°C.

5. Quantitative estimations

5.1 The quantitative estimation of DNA

This is based on the method of Burton (1956) which relies on the blue colour produced by the reaction of diphenylamine with deoxypentoses.

Reagents:

A. Re-distilled acetaldehyde (BDH)

B. 1.5g diphenylamine in 1.5ml concentrated sulphuric acid and 100mls glacial acetic acid.

C. Standard solution of deoxyribose in water at 100µg/ml.

D. Working diphenylamine reagent was made up on the day of use by addition of 1.0ml of a 1.6 per cent pollution A to 50mls of B.

Procedure:

DNA was extracted from materials by heating for 15 minutes at 70°C in a total volume of 2mls 1M PCA. The supernatant was removed by centrifugation at 850g for 10 minutes and the extraction procedure was repeated on the precipitate. The two supernatants were combined. Standard solutions contained up to 100ug deoxyribose in a total volume of 4mls 1 N PCA were made.

2mls of reagent D were added to one ml of the supernatant or the standard solution and the tubes were incubated at 70°C for 1 hour. The absorbancies were then read at 600mµ against reagent blanks using a Unican SP 800 spectrophotometer. Standard curves were linear.

1µg deoxyribose was taken to be equal to 6.2µg of DNA.

5.2 The quantitative estimation of RNA

This method was based on the orcinol procedure described by Mejbaum (1939) and modified by Slater (1956).

Reagents:

A. 0.1 per cent (w/v) solution of ferric chloride hexahydrate in concentrated hydrochloric acid.

B. 10 per cent (w/v) solution of orcinol (3,5, dihydroxytoluene) in re-distilled absolute ethanol.

C. Standard D-ribose solution (L. Light and Company) in water at a concentration of 10μg/ml.

Procedure:

Samples are diluted in KOH in final concentration 0.3 N KOH. The mixtures incubated at 35 - 37°C for 2 hours, cooled in ice and then equal volumes of 2N PCA were added. The pellet was removed by centrifugation at 850g for 10 minutes. To the 3mls of the supernatant containing up to 130μg of RNA, 3mls of A were added. Standards containing up to 30μg of D-ribose were similarly set up. 0.3mls of B were added and the samples heated

in a boiling water bath for 45 minutes. After quick cooling the absorbancies at 670mμ were recorded using a Unican SP 800 spectrophotometer.

1μg of D-ribase was taken to be equivalent to 4.56μg of RNA.

5.3 The quantitative estimation of protein

Protein content was estimated by the colorimetric method of Lowry et al. (1951) which is dependent on the presence of aromatic amino acids.

a) The Lowry Method

Reagent A: 2g of Analar sodium carbonate in 100mls of 0.1 N NaOH.

Reagent B: 0.5g Analar copper sulphate and 1.0g sodium citrate in 100mls distilled water.

Reagent C: was prepared by mixing 50mls of Reagent A with 1ml Reagent B just prior to use.

Reagent D: Folin Ciocalteau (BDH) reagent diluted with water to give a solution approximately 1M with respect to HCl.

In 1ml aliquots of solution (25 - 150μg protein/ml) were added, 5ml Reagent C followed 10 minutes later by 0.5ml Reagent D. After standing at room temperature for 1 hour the optical density at 750mμ was measured on a Unican SP 800 spectrophotometer. A calibration curve was also determined with 0 - 250μg bovine serum albumin.

b) The Bromosulphalein Method

The Bromosulphalein reagent is 1ml 5 per cent (w/v) sulphobromophenolphthalein Na Salt (Koch-Light) in 100ml 1N HCl and 50mls 1M Citric acid and distilled water to 250mls final volume. 0.1ml samples of protein (15-20 OD units/ml at 260mμ) were mixed with 0.1ml 0.5 N HCl and left for 1 hour at 0°C with shaking. The supernatant was collected by centrifugation at 2000g for 10 minutes and the precipitate was mixed with 0.2ml 0.25 N HCl for another hour. The supernatants which contained the histone component were pooled. 0.8ml of 0.5 N NaOH was added to the precipitate and 0.4ml 1N NaOH added to the supernatant. After incubation for 1 hour at 37°C 1ml of bromosulphalein reagent was added and the solutions left at 0°C for 10 minutes. The solutions were then centrifuged at 2000g for 15 minutes and 0.5ml of the supernatant was added to 3.5ml 0.2 N NaOH.

The absorbancies were read at 580mu and calibrated against 0 - 400ug bovine serum albumin.

6. Preparation of polyacrylamide gels

2.4g acrylamide was diluted in 10mls of a buffer containing 4.8% Tris; 2.1 per cent Sodium acetate and 0.74 per cent EDTA (pH 7.8) and 0.012g bisacrylamide was dissolved in the above solution. 0.008mls initiator of the polymerisation (N; N, N; N; tetramethylene diamine) was added followed by 0.08mls 10 per cent ammonium persulphate (catalyst). Using pasteur pipette the polyacrylamide solution was added to stoppered glass tubes and 0.1ml distilled water was layered on the top of each. Gels were left to polymerise at room temperature. When the polymerisation was over the stoppers were removed and adequate buffer was added to each reservoir. To remove catalysts etc. it is necessary to apply voltage to the gels for 1 hour (4 m A per tube). The samples were in 3 per cent sucrose in TKM buffer and were added on top of each gel. Voltage was applied for 45 - 60 minutes. Finally, the gels were removed from the tubes and put into tolnidine blue solution 0.2 per cent for one hour.

If it was desired to scan the gels in the chromoscan, then at the end of the run the gels were put at distilled water for 1 hour in order to remove the EDTA.

Experimental

1. Principles of hybridization technique

1.1 Liquid hybridization using nitrocellulose membranes
1.2 The RNA saturation curve
1.3 Hybridization with immobilised DNA
1.4 Competition hybridization experiments

2. Evidence that citric acid preparation of nuclei does not affect the integrity of nuclei

2.1 Nuclei isolated by sucrose method

3. In vitro RNA synthesis with chromatin as template

3.1 Chemical composition of chromatin
3.2 The properties of template activity of chromatin for RNA synthesis

4. Template acitivity of DNA in chromatin

4.1 Evidence for restriction of template activity in different tissues
4.2 Competition experiments by preincubation

5. Synthesis of RNA by chromatin isolated from folic acid-treatment mouse kidney

5.1 Hybridization of the various RNAs after folic acid treatment
5.2 Comparisons of RNA populations after folic acid administration
5.3 Relation between RNA synthesis in the *in vivo* and *in vitro* system
5.4 The effect of the presence of folic acid during isolation of nuclei
5.5 The effect on the template activity of exogenously added folic acid

6. The effect on the template activity of chromatin isolated from mouse kidney after ligation of one ureter

6.1 Hybridization of the various RNAs after ligation of one ureter

1. Principles of hybridization technique

The analysis of selective gene transcription may be conveniently undertaken by employing techniques of DNA-RNA hybridization. This method is based on the ability of RNA to form a hybrid molecule with the DNA region on which it has been transcribed (Hall and Spiegelman, 1961). In studies of viral and bacterial RNA hybridization, convincing evidence has been presented showing that β-galactosidase mRNA will combine specifically with the DNA of the lac operon (Hayashi et al., 1963) and galactokinase mRNA with DNA of the gal operon (Attardi et al., 1963) suggesting a locus-specificity.

Quite recently, similar approaches have been used for the analysis of transcription in higher organisms. There is however, a difficulty in demonstrating locus specificity due to the complexity of the genomes of higher organisms and to the existence of many partially related sequences in the DNA. These suggest that such specificity may be difficult to achieve.

At a given temperature, hybridization reactions

proceed more rapidly and to a greater extent at higher salt concentrations (Nygaard and Hall, 1964; Gillespie and Spiegelman, 1965). Thus it has been the practice, in recent studies of hybridization reactions with mammalian nucleic acids, to use salt concentrations in the range of 6 x SSC. It may be that increased reaction is to some extent attributable to decreased specificity. Using more stringent conditions, i.e. lower salt concentration or higher temperature it is possible to achieve locus specificity in homologous reaction.

Since the hybridization reaction is a collision phenomenon, it is advantageous to have the RNA molecules as concentrated as possible. Consequently, the volume of RNA solution in which the filters are soaked must be kept to a minimum but still large enough to wet effectively the filters and not to change by evaporation during the incubation.

The rates of reaction in the case of DNA-RNA duplex formation for mammalian DNA are too high to be consistent with locus specificity. Similar results are to be expected with DNA-RNA hybridization reactions (Church and McCarthy, 1967 a, b). Since the mammalian genome is much larger than that of a

bacterium in terms of nucleotide sequences, rates of reaction should be lower. However, this is not the case and reactions of bacterial and mammalian nucleic acids display similar rates.

McCarthy and McConaughy (1968) suggested that for the case of DNA-RNA duplexes, under some conditions, the high rate of reaction is a direct consequence of partial redundancy in nucleotide sequences in mammalian DNA. Thus under conditions where bacterial DNA undergoes true renaturation, mammalian DNA forms mispaired structures. Under more stringent conditions, these structures are selected against and the rate of reaction by mouse DNA is much lower than for bacterial DNA. Corresponding comparisons of DNA-RNA hybrid formation give analogous results. Data reported by Church and McCarthy (1968) show that between 60 and 75°C the reaction rate for B. subtilis RNA decreases 40% while in the case of mouse RNA the decrease is about 75%. The high rate of reaction by mouse RNA under conditions of moderate stringency may be explained by lack of locus specificity. This is the result of RNA molecules reacting with DNA of similar, but not identical, genetic regions. This explanation is borne out by the decrease in rate when reaction conditions

are made more stringent so that great base homology is necessary to form stable reaction products. Two basic methods which allow the RNA to anneal specifically with the DNA have been employed.

1) The annealing procedure which can be carried out by mixing RNA and DNA in liquid at an elevated temperature and a chosen salt concentration (Nygaard and Hall, 1963) and

2) The immobilisation of single stranded DNA to a membrane filter prior to exposure to RNA (Gillespie and Spiegelman, 1965).

1.1 Liquid hybridization using nitrocellulose membranes

This technique as described by Nygaard and Hall (1963) is based on the observation that the nitrocellulose filters (MF50 filters from Sartorius Membranfilter, Göttingen or Schleicher and Schuell, Keene N.H. 27mm, type B-6) in salt solutions can absorb denatured DNA but not native DNA or RNA.

The hybridization system was tested using DNA prepared from calf thymus, ascite cells and rat liver chromatin. Samples of DNA (0.2 - 0.5 mg/ml) were denatured at 100°C for 10 minutes and then cooled rapidly in ice. ^{3}H-labelled RNA was prepared *in vitro* using M. lysodeikticus RNA polymerase with calf thymus

DNA and rat liver chromatin as primers. Labelled RNA was extracted from Landschutz ascites cells using ^{3}H-uridine.

Hybridization mixtures containing denatured DNA, KCl in a final concentration 0.5M and RNA in a total volume of 0.15ml were heated at 60°C for 1 - 2 hours, and then cooled in ice. 3mls of 0.5M KCl containing 15ug ribonuclease were added and incubated at 37°C. After 15 minutes the solutions were passed through nitrocellulose filters, previously soaked in 0.5M KCl for 30 minutes. The filters were then washed with 60mls 0.5M Kcl dried at room temperature and counted in toluene scintillator.

Incubations containing RNA but no DNA were run as controls.

1.2 The RNA saturation curve

Saturation curves are determined by hybridizing exhaustively a constant amount of DNA with increasing amounts of labelled RNA. The objective of exhaustive hybridization is the complete uptake into hybrid complexes of all the RNA molecules which are complementary to specific groups of genes. Hybrid formation is then a function of RNA concentration and can be graphically expressed by an asymptotic curve. At infinite

concentration, a theoretical maximum is obtained and the DNA is saturated with RNA. Complete saturation is never obtained experimentally but it can be calculated if we used a plot which gives a straight line, i.e. a double reciprocal plot of hybrid formation versus RNA input. This is shown in figures 2 and 3.

As for the evaluation of data obtained from hybridization experiments it should be always taken into account that, as found by Britten and Kohne (1966), in higher organisms and under the conditions used for RNA-DNA hybridization experiments, this method is not sensitive enough to detect that part of the genome that occurs in only a few copies besides the so-called fast and intermediate genomic portions.

The theoretical saturation value obtained for the RNA, ^{3}H-labelled, synthesised *in vitro* on a DNA template isolated from calf thymus nuclei (figure 1), is equivalent to about 26%, ^{3}H-RNA synthesised *in vitro* using rat liver chromatin as template gives a 5% (figure 1) and 5.8% for RNA isolated from ascites tumour cells labelled with ^{3}H-uridine in culture (figure 4).

Figure 1

Kinetics of hybridisation to 5μg denatured calf thymus or rat DNA to increasing amounts of ^{3}H-RNA made *in vitro* from calf thymus DNA ●——● or rat liver chromatin x——x by M. lysodeiktieus polymerase.

Figure 1

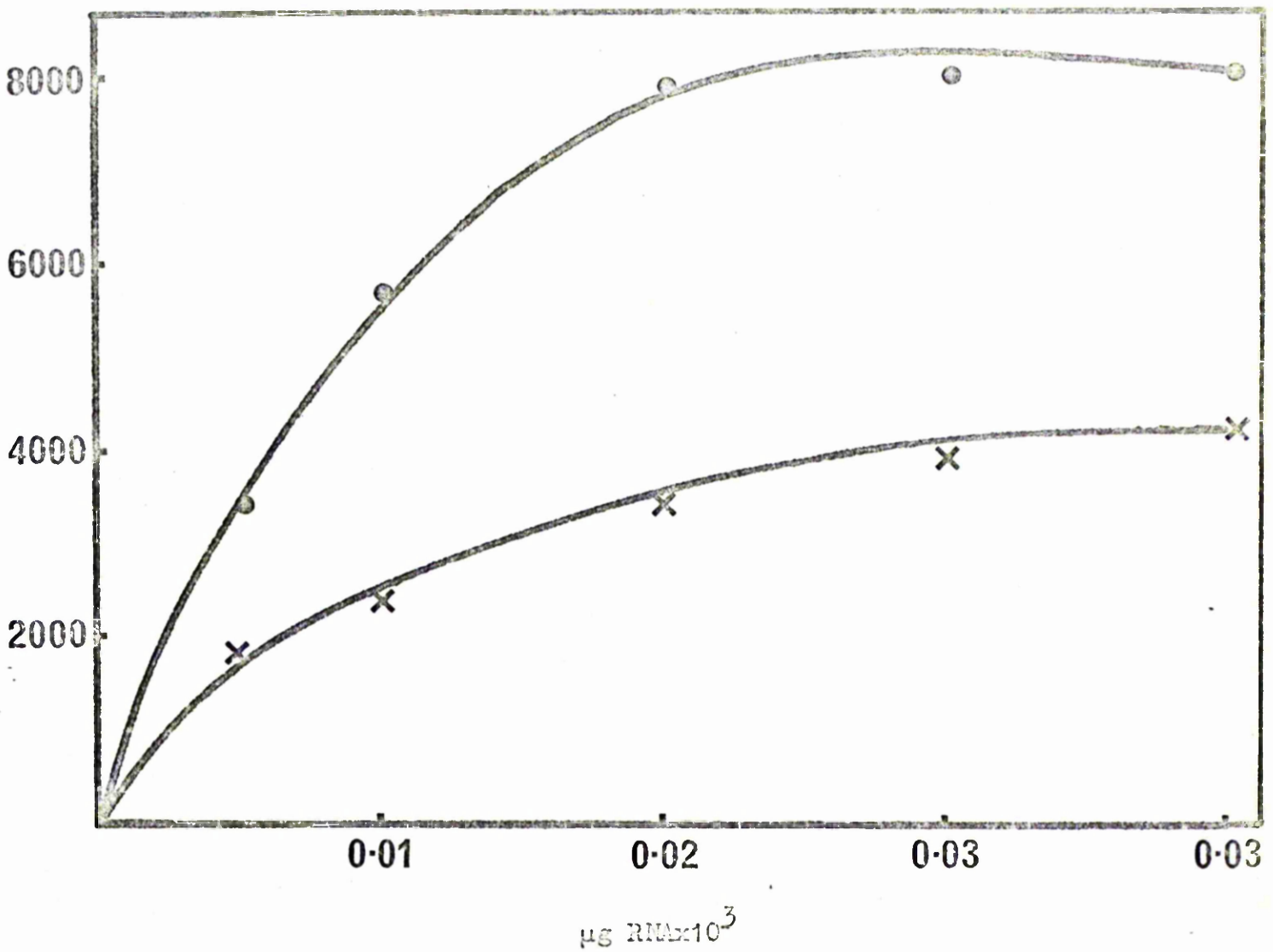


Figure 2

(a)

Double reciprocal plot of data of RNA made from calf thymus DNA. At infinite concentrations of RNA represented by the intercept on the ordinate, approximately 26% of the DNA exists as hybrid.

Figure 3

(b)

Double reciprocal plot of data of RNA made from rat liver chromatin. It is predicted that at saturating concentrations of RNA only 5% of the DNA exists as hybrid.

Figure 2

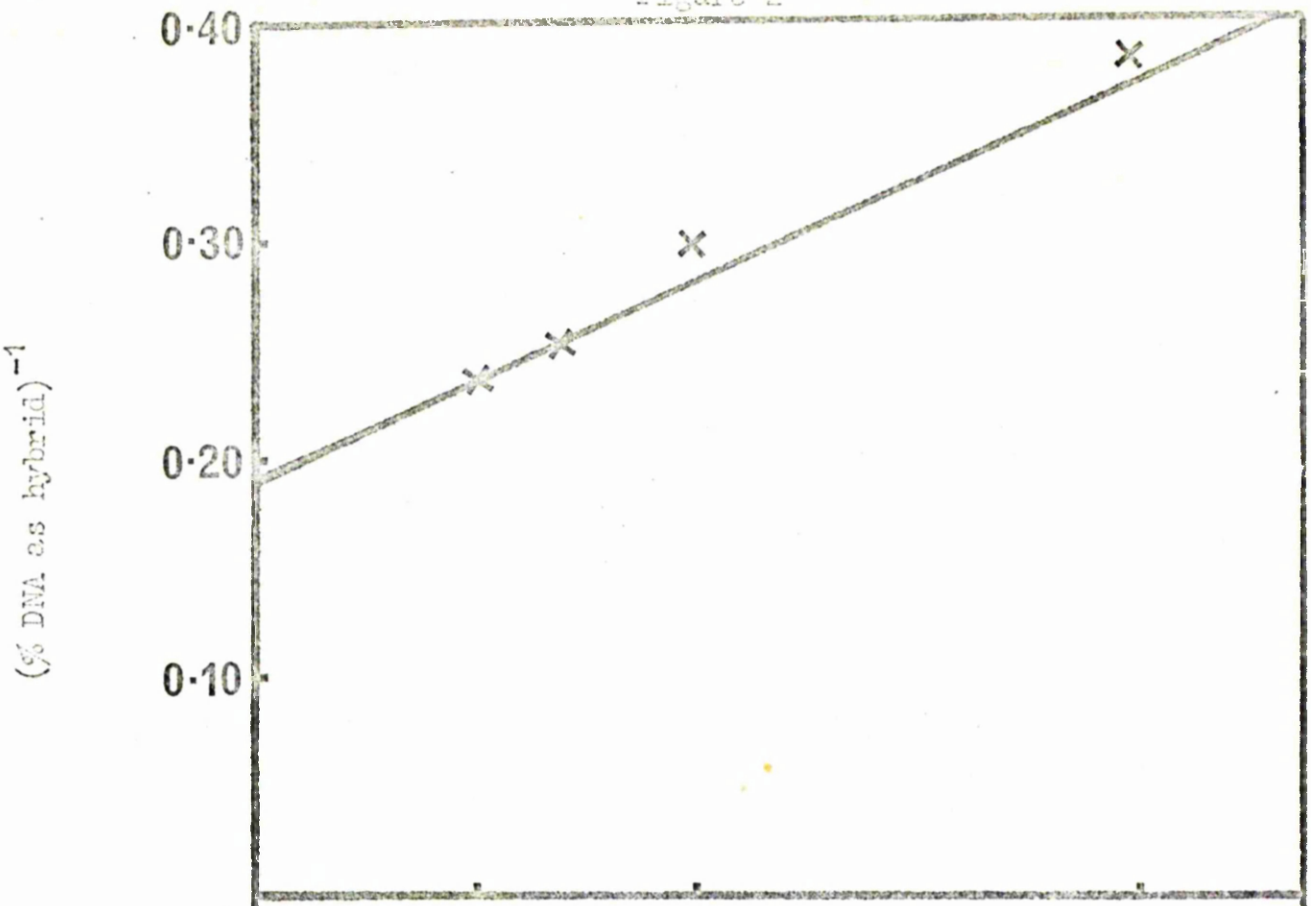


Figure 3

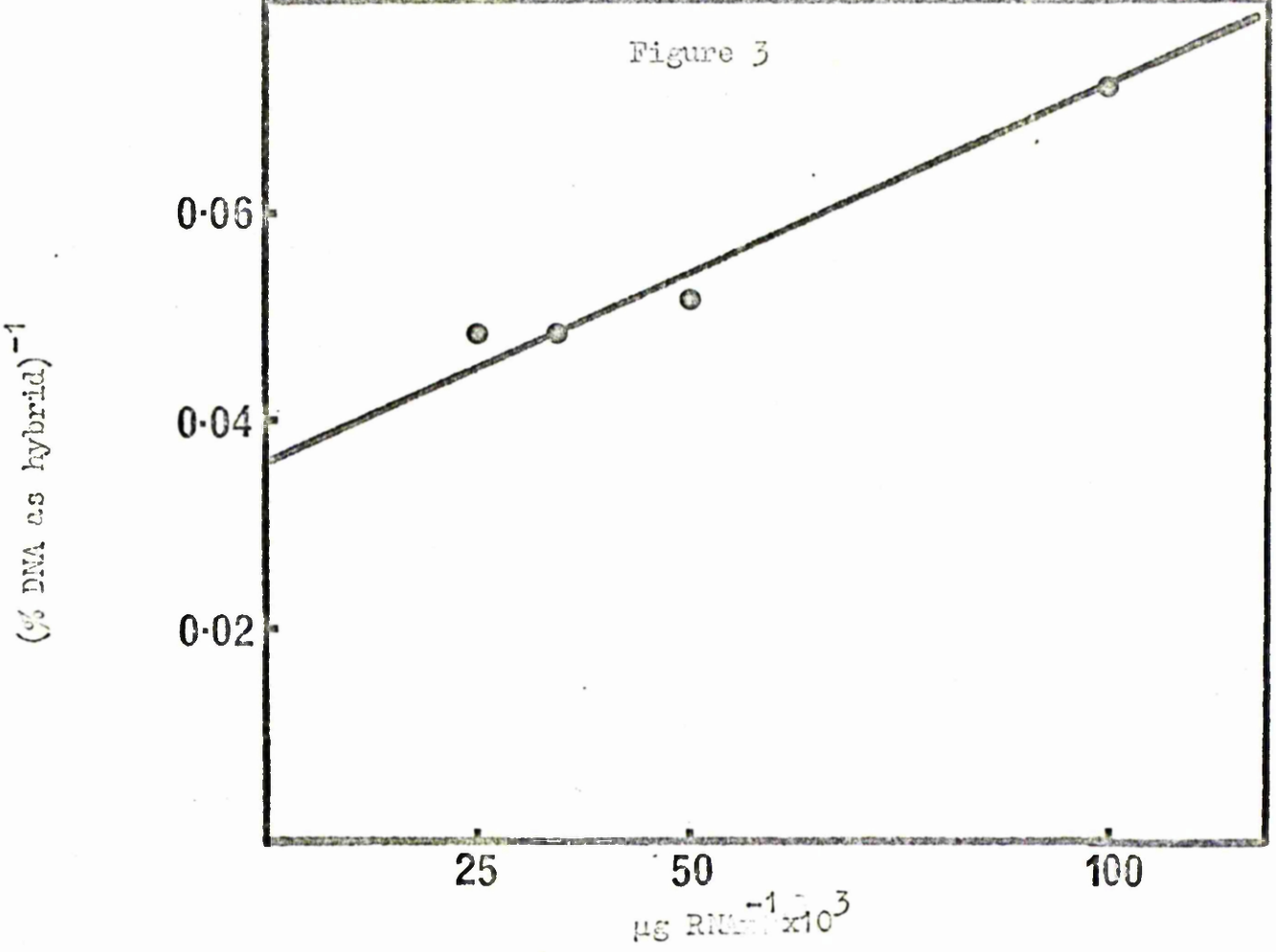


Figure 4

Kinetics of hybridisation of increasing amounts of *in vivo* ^{3}H-labelled Landschutz ascites tumor cells RNA with 5µg denatured ascites tumor cells DNA per filter.

Figure 4

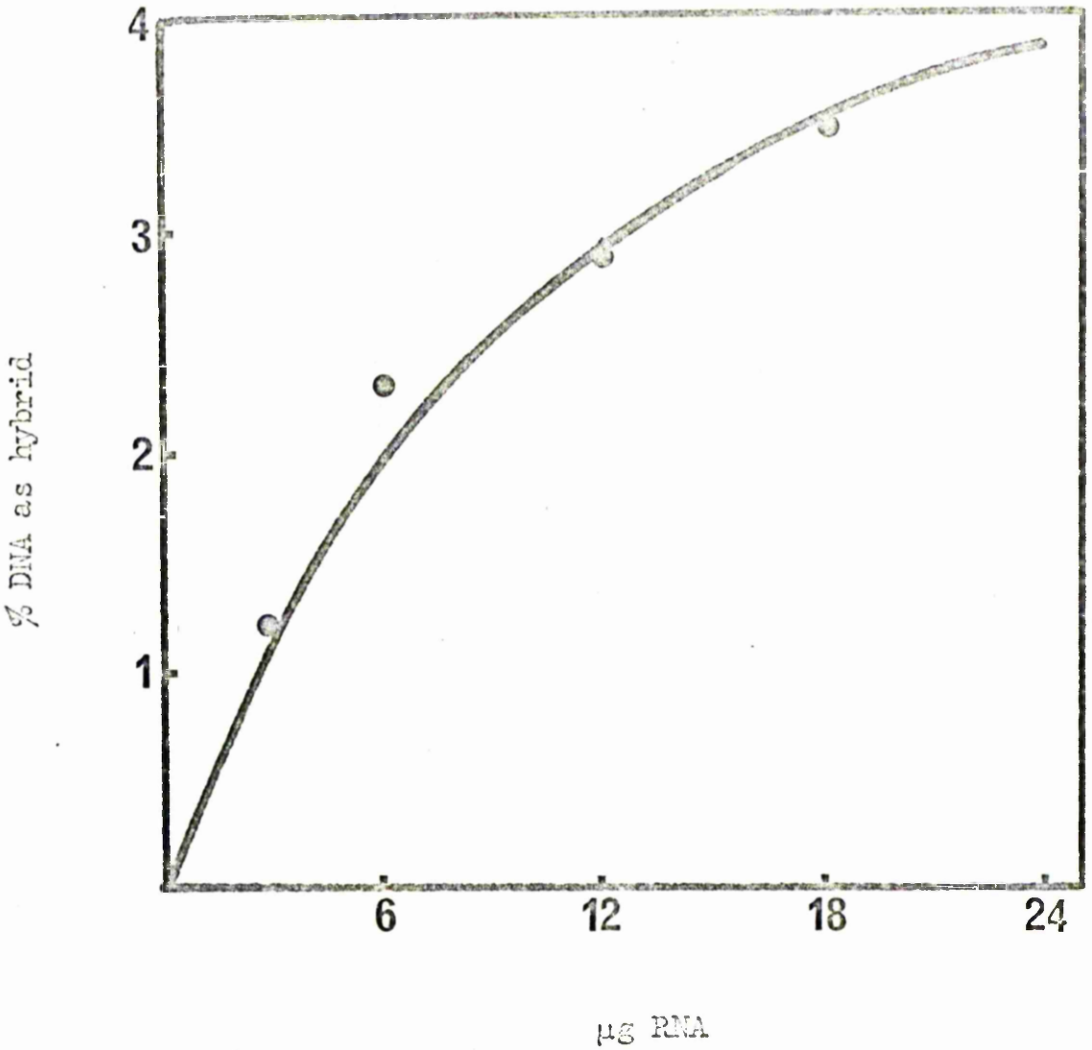
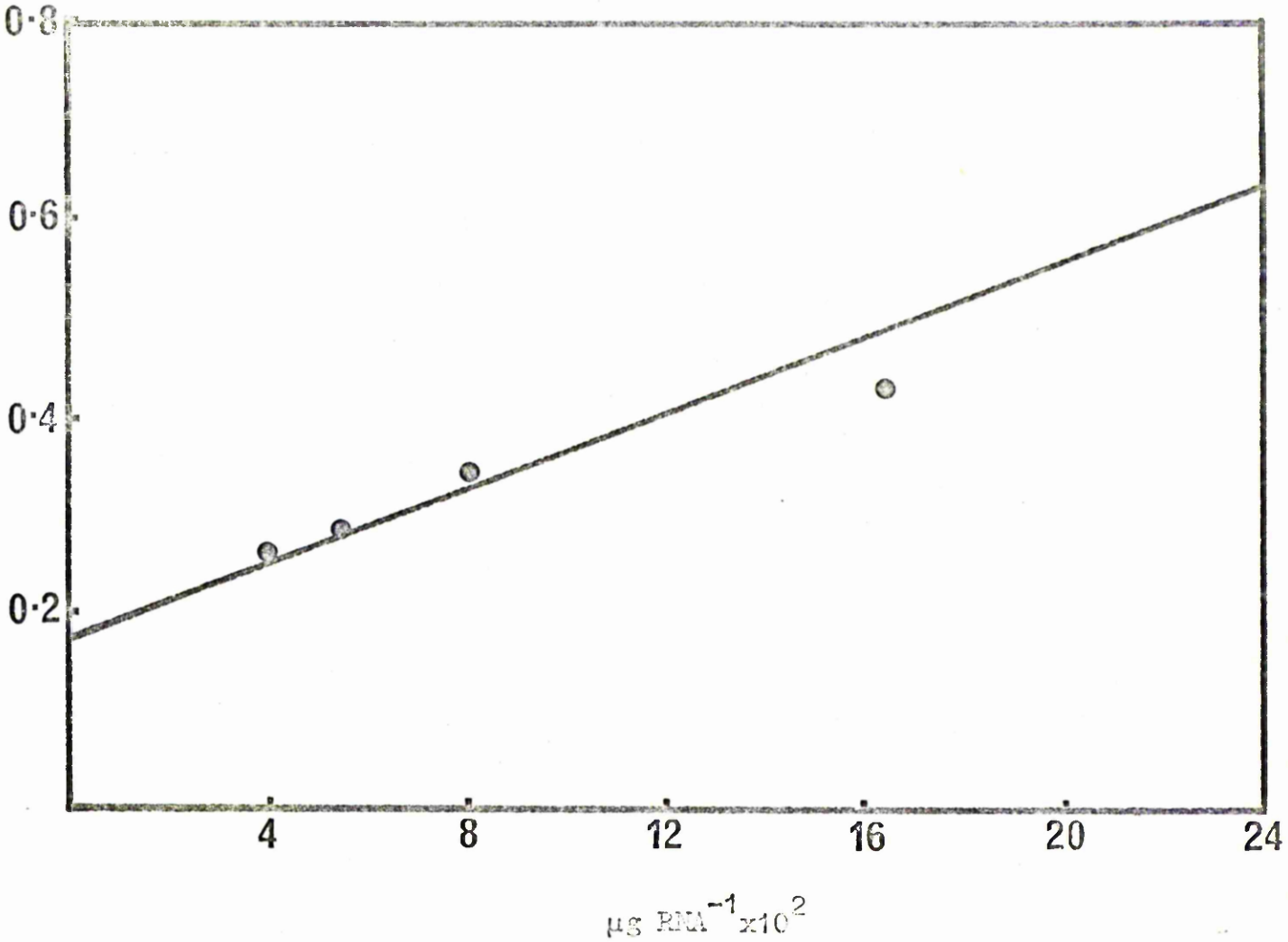


Figure 5

Double reciprocal plot of the data of figure 4. At saturating concentrations of RNA only 5.8% of the DNA exists as hybrid.

Figure 5



1.3 Hybridization with immobilised DNA

One disadvantage of the liquid hybridization technique has been pointed out, namely, the opportunity for DNA-DNA reannealing to proceed and thereby reduce the opportunity for formation of RNA-DNA hybrids. Gillespie and Spiegelman (1965) described the following method where DNA is immobilised on the filter thereby reducing the risk of renaturation.

Denaturation of DNA

Two procedures were usefully used.

a) Pure DNA was dissolved in distilled water at a concentration of 30 - 40µg/ml and adjusted to pH 12.5 with 10 N NaOH. After standing for 10 minutes, the solution was neutralised with HCl and the degree of hyperchromicity at 260 mµ determined.

b) Pure DNA was dissolved in distilled water at a concentration of 30 - 40 µg/ml and the sample heated in boiling water for 10 minutes.
After quick cooling in ice, the hyperchromicity at 260 mµ was recorded.

Preparation of filters

Nitrocellulose filters were pre-soaked in 4 x SSC for 2 - 3 hours and washed in 20mls of the same solution. The required amounts of denatured DNA were passed through the filters in 5mls. 4 x SSC. The filters were then washed on both sides with 20mls 4 x SSC. After drying at room temperature for at least 4 hours the filters were baked for two hours at 80°C.

Hybridization technique

Hybrids were formed by immersing the filters (13mm diameter) in vials containing labelled RNA in 4 x SSC in a final volume of 0.2ml. Later a micro-technique, using 60 - 90µl of hybridization mixture was used. The filters were allowed to soak up the mixtures and were then immersed in liquid paraffin during incubation. Identical results were obtained with the two methods. Annealing was carried out at 65°C for 18 hours, after which time the reaction mixtures were cooled in ice; the filters were then washed on both sides with 20mls 4 x SSC and treated with 20µg/ml ribonuclease in 2 x SSC. After 1 hour in room temperature the filters were washed as before

on both sides, dried and counted in toluene scintillator in a Nuclear Chicago counter.

Retention of DNA on the filters

Labelled DNA (specific activity 3420dpm/5µg) was prepared from Landschutz ascites cells using ^{3}H-uridine. Samples were denatured and immobilised on filters on a concentration of 5µg per filter. These filters were either subjected to complete hybridization and washing procedure or were immediately dried and counted. In one set non-radioactive RNA was included in the hybridization procedure. Table I shows that immobilised DNA is retained on the filters throughout the procedure.

The significance of time in the drying and baking procedure of filters

Filters in which a known amount of the above mentioned radioactive DNA was immobilised, were air dried and baked for different times in order to determine the best conditions for complete retention of the DNA. Table 2 shows that the 4 hours air drying and 2 hours baking at 80°C are as satisfactory as the overnight drying and 1 - 4 hours baking conditions.

Time course of hybridization

The time course of hybridization of 5ug calf

TABLE I

RETENTION OF IMMOBILISED DNA ON NITROCELLULOSE FILTERS

Treatment	Air Drying	Baking	Recovery
None	4 hours	2 hours	3352, 3250
Hybridization in 4 x SSC	over night	1 hour	3308, 3080
Hybridization in 4 x SSC	over night	2 hours	3238, 3185
Hybridization in 4 x SSC	4 hours	2 hours	3223, 3276
Hybridization in SSC + denatured DNA	4 hours	2 hours	3217, 3186
Hybridization in 4 x SSC + cold RNA	4 hours	2 hours	3323, 3186

TABLE 2

THE EFFECT OF TIME IN THE DRYING AND BAKING PROCEDURE OF FILTERS

Treatment	Air Drying	Baking	Recovery
None	4 hours	2 hours	2900, 3010
Hybridization in 4 x SSC	over night	1 hour	2780, 2860
Hybridization in 4 x SSC	over night	2 hours	2788, 2906
Hybridization in 4 x SSC	4 hours	2 hours	2817, 2837

thymus DNA with 20ug ^{3}H-labelled RNA made *in vitro* from calf thymus chromatin in 0.2mls is shown in figure 6. The maximum of hybridization is achieved in 16 hours and remains unaltered up to 24 hours.

1.4 Competition hybridization experiments

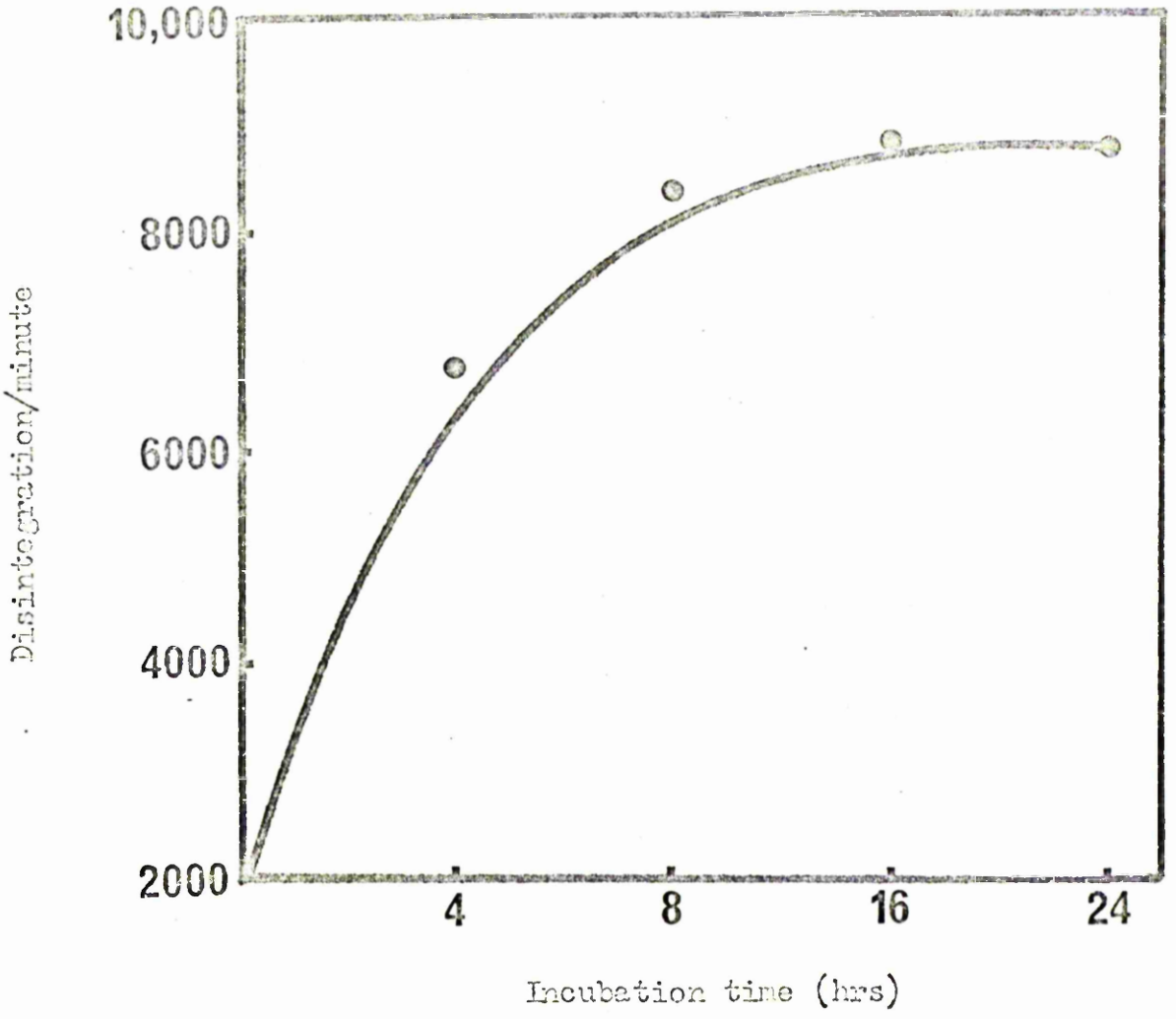
In order to demonstrate that the activity of an RNA associated with the DNA - (from a saturation curve) - is specific, competitive hybridization experiments were carried out. This consisted in hybridizing a constant amount of DNA and labelled RNA as before in the presence of increasing amounts of non-radioactive RNA (competitor). If the radioactive RNA and the competitor are the same, the latter will progressively replace the former in the hybrid and an apparent decrease in the percentage of binding will ensue. Complete displacement of labelled RNA can only in theory be obtained at infinite concentrations of the competitor. If the RNA's under comparison have no nucleotide sequence in common the addition of increasing amounts of competitor RNA will have no influence on the hybridization of the radioactive RNA with DNA.

The curve obtained by diluting a given amount of labelled RNA by increasing amounts of identical, non-labelled RNA should be linear in a double

Figure 6

Time course for the hybridisation of 30µg of ^{3}H-RNA made *in vitro* from calf thymus DNA with 5µg denatured calf thymus DNA, immobilised on nitrocellulose filters by the method of Gillespie and Spiegelman.

Figure 6


10,000
8000
6000
4000
2000
Disintegration/minute
4
8
16
24
Incubation time (hrs)

logarithmic plot and drop with a 45^{o} angle provided that all the complementary sites in DNA are saturated by the reference RNA (Denis, 1967). If the amount of labelled RNA used is insufficient to occupy all the complementary sites of DNA, more RNA becomes hybridized with DNA when increasing amounts of competitor RNA are added. In nonsaturating conditions, two contradictory factors are thus acting. On the one hand, the isotope dilution produced by the competitor RNA results in an apparent reduction in the percentage of hybridization; on the other hand, the addition of increasing amounts of RNA tends to increase the total amount of RNA (labelled and non-labelled) that binds to DNA. As a result, the slope of the dilution curve will be lower than 45^{o}, (Denis, 1967).

The displacement of labelled RNA from the DNA by the unlabelled RNA is expressed graphically by a second order reaction if the remaining amount of hybridized labelled RNA for a given input ratio (of cold over hot RNA) which is expressed as a fraction of the amount hybridized in the complete absence of unlabelled RNA is plotted vs. the input ratio.

A prediction of the extent of homology can be made, from a double reciprocal plot of diminution of radioactiviety (Xo - X) versus the concentration of

unlabelled RNA, where Xo is the activity in the presence of labelled RNA alone, and X the value obtained on the addition of unlabelled RNA. The expression of this empirical function yields a straight line and the Y intercept represents the percentage of competition.

2. Evidence that citric acid preparation of nuclei does not affect the integrity of nuclei

Liver nuclei were prepared with the three different methods, mentioned under materials, and EM pictures were taken from all the three different preparations. (Plate I). As it can be observed, the nuclei retained their characteristic shapes equally well when prepared by either citric acid or sucrose and the amount of cellular debris attached to nuclear membranes is negligible. On the other hand preparation of nuclei with Triton X-100 gave poor yields, large amounts of cellular debris attached to nuclear membranes and large amounts of debris lying free.

Another point that had to be investigated was, to find out if the citric acid had any effect on the integrity of the chromatin; in other words to see if the citric acid removes a part of the histones bound to the chromatin. In order to answer this

Plate 1, 2 and 3

1. EM photograph of mouse liver nuclei isolated by citric acid method (x 10,000). Double stained with uramyl-acetate and lead citrate.

2. EM photograph of mouse liver nuclei isolated by Chauveau's method (x 10,000). Double stained with uranyl-acetate and lead citrate.

3. EM photograph of mouse liver nuclei isolated by Triton x - 100 (x 17,000). Double stained with uranyl-acetate and lead citrate.

Plate 1.

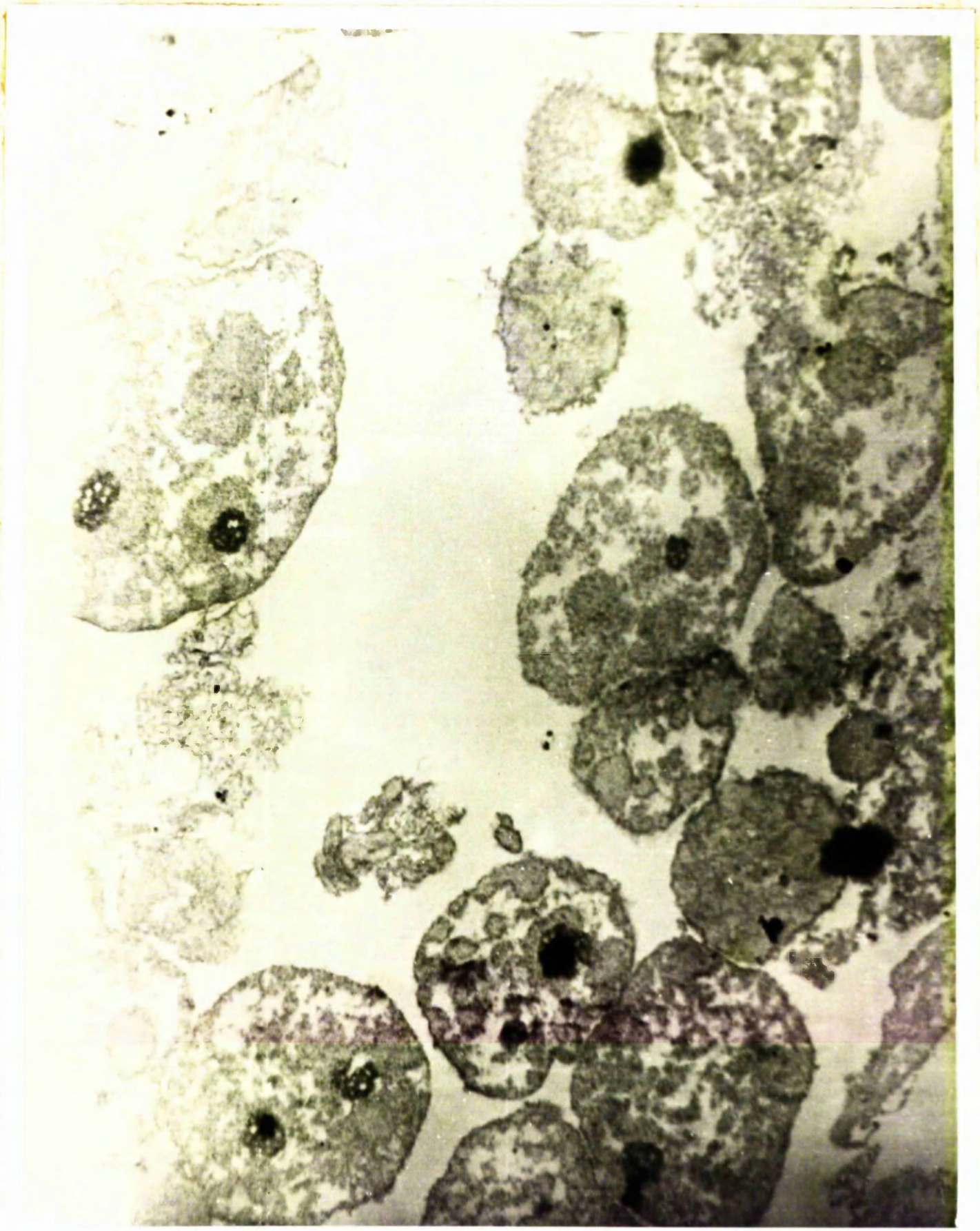


Plate 2.

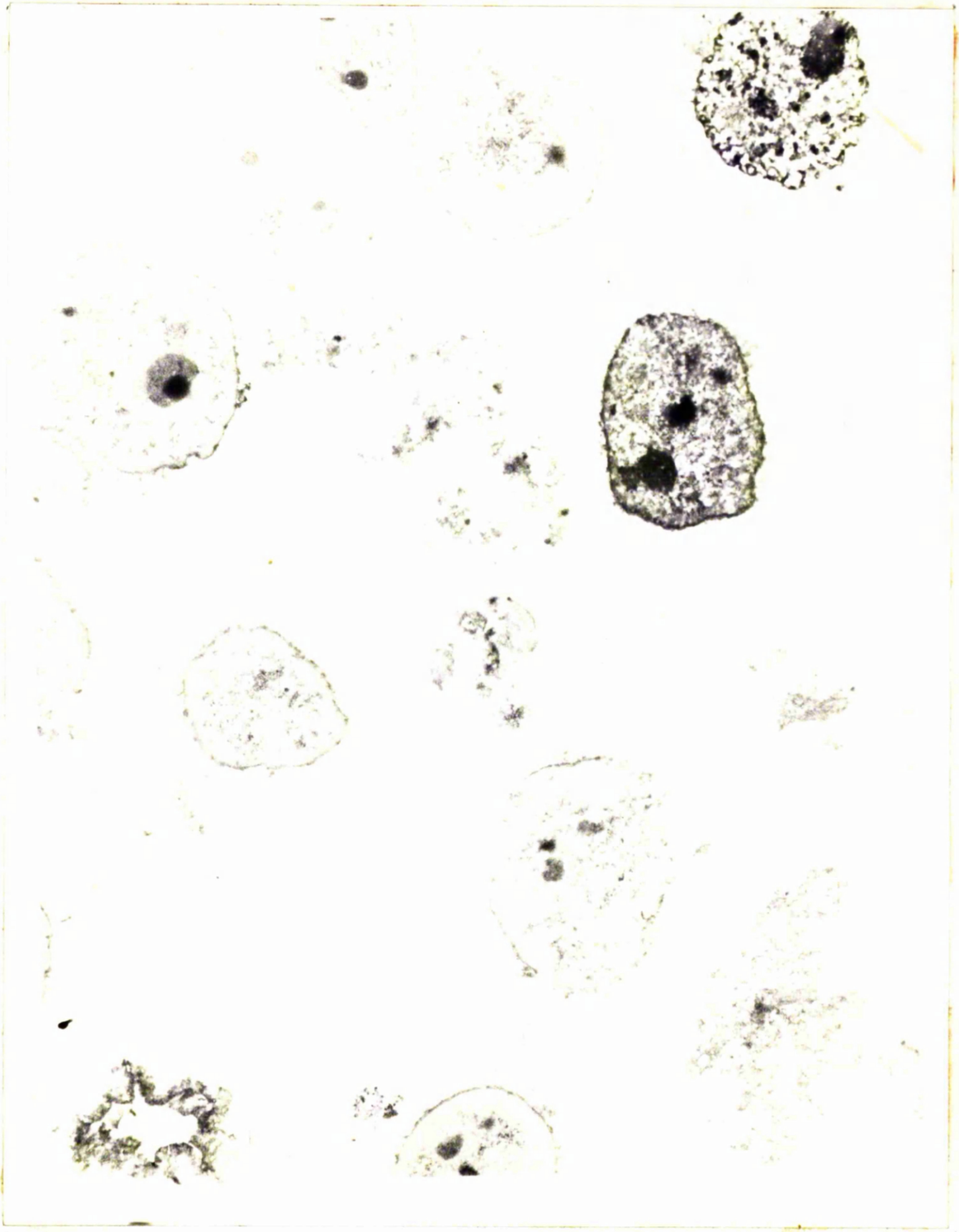


Plate 3.

problem, the following experiment was carried out. First, histones were extracted from liver chromatin with 0.25 N HCl as described under materials, then were dialysed over night against distilled water and lyophilised. 50µg of this sample dissolved in 5M urea was run in a 20% acrylamide gel as control. Second, liver tissue was homogenised in 0.025 M citric acid and the supernatant was kept. The pellet was rehomogenised in citric acid and the second and third supernatants were combined with the first. This mixture was centrifuged for 1½ hours in 105,000g and the supernatant was dialysed overnight against distilled water. The dialysate was lyophilised and a sample of 45µg was dissolved in 5M urea and run in a 20% acrylamide gel, as before.

Comparison of the two patterns shows that during citric acid preparation of nuclei there is no loss of histones. (Plate 4.)

2.1 Nuclei isolated by sucrose method

The effect of the procedure for isolation of nuclei and the possible consequences on the integrity of the chromatin prepared from those nuclei were tested. Kidney nuclei were prepared by Chauveau's sucrose method as well as by the citric acid method, and chromatin was

Plate 1

a) Gel electrophoresis of the first and second wash of mouse liver homogenised in 0.025M citric acid.

b) Gel electrophoresis of histones extracted with 0.25 N HCl from mouse liver chromatin. The nuclei have been isolated by citric acid method.

Plate 4.

a b

prepared as outlined under Methods. Identical *in vitro* incubations were set up using these two chromatin preparations as template for RNA synthesis. The RNAs yielded were hybridized with denatured mouse embryo DNA and the saturation levels were calculated. (Figure 7). It was found that at saturation both preparations give identical values. (Figure 8).

This was considered as evidence that the citric acid method does not interfere with the template activity of the chromatin by removing selectively part of the histones bound to chromatin. The citric acid was used for all subsequent preparations.

3. *In vitro* RNA synthesis with chromatin as template

From the results of several authors it appears quite clear that most of the genes in the nuclei of animal somatic cells which are considered as being in a complete differentiated state are inactive and unavailable for transcription.

In support of the more general validity of this conclusion drawn from the work of Paul and Gilmour (1966 a, b; 1968) come the results obtained from studies done on mouse tissues. The template activity of isolated chromatins in supporting RNA synthesis

Figure 7

Kinetics by hybridisation to whole mouse embryo DNA of ^{3}H-RNA made *in vitro* by M. lysodeiktious polymerase using mouse kidney chromatin as template.
Chromatin isolated from nuclei prepared by Chauveau's method ●——● ;
nuclei isolated by citric acid method x—x ;

Figure 8

Double reciprocal plot of the data of figure 7. At saturation concentrations of RNA 7.0% of the DNA exists as hybrid in both preparations.

Figure 7

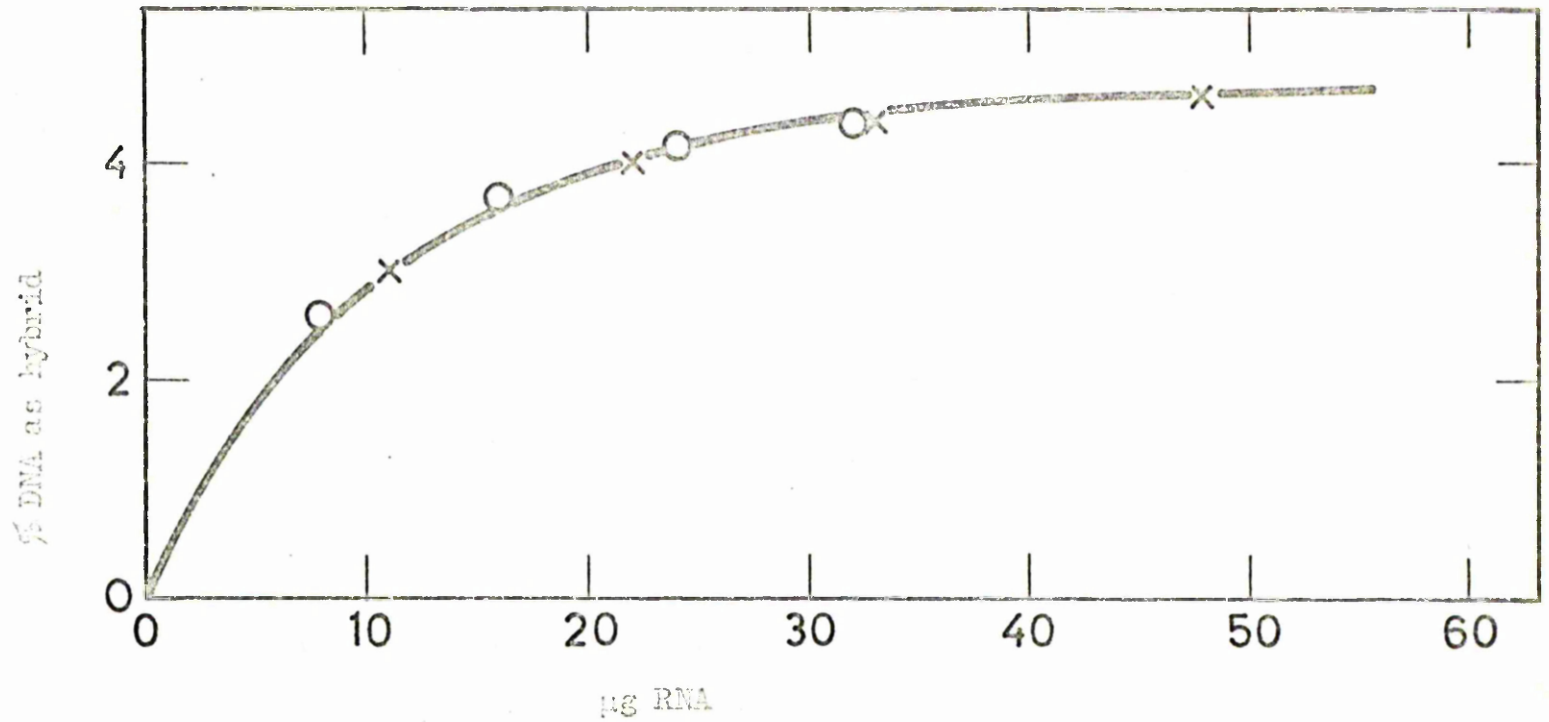
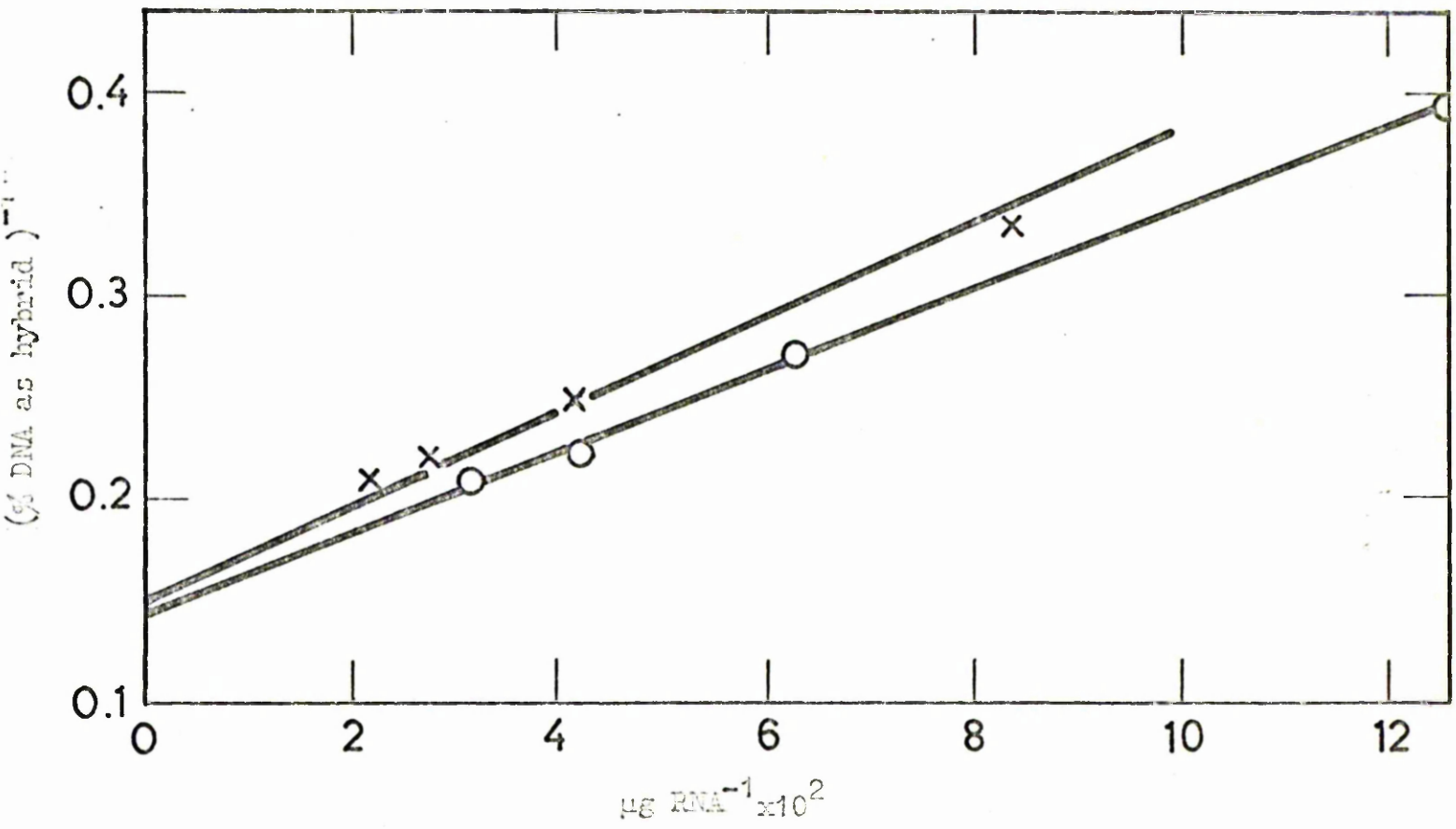


Figure 8



was studied in order to clarify the relationship between genomic expression and organ specificity.

3.1 Chemical composition of chromatin

Determinations of DNA, RNA and protein were carried out according to the methods already outlined. 1ml aliquots containing 15 - 20 optical density units at 260mμ were treated with 1ml at 0.6 N KOH for 4 hours at 37°C. 2mls of 2 N PCA were added at 0°C and the precipitate centrifuged down at 2000g for 10 minutes. The hydrolysed RNA present in the supernatant was estimated by the orcinol method. The precipitates were extracted twice with 1 N PCA at 70°C for 15 minutes. Residual material was pelleted and the supernatants combined. 1ml aliquots were assayed for DNA by the diphenylamine method.

Estimation of basic and acidic proteins was carried out as follows: To 0.75 ml aliquots of chromatin, 0.25ml 1 N HCl was added. After 1 hour at 0°C the supernatant was collected by centrifugation at 2000g for 10 minutes and the pellet re-extracted with 0.25 N HCl. The supernatants were combined. To the precipitate 1M PCA was added and the DNA was extracted as mentioned above. To the resulting

precipitate (free of DNA) 0.5 N NaOH was added. The proteins were estimated by the Lowry method as described under Methods.

The results obtained for chromatins from various sources are shown in Table 4. While the RNA/DNA and acidic protein/DNA ratios are variable a fairly constant figure of approximately unity was found for basic protein/DNA irrespective of the chromatin source.

3.2 The properties of template activity of chromatin for RNA synthesis

The consequence of omitting various components of the assay system was examined. The results are shown in Table 5. Although putrescine was not found to be essential for activity, its presence caused a 2 - 3 fold increase in activity.

The metal ion requirement

RNA synthesis in the presence of variable concentrations of manganese or magnesium was examined. Manganese ions are more effective than magnesium ions, the former exhibiting a sharp optimum around 3mM whereas the latter appears to be effective over a broad concentration range above 3mM but at a lower level. In all subsequent reactions manganese chloride was present at a final concentration of 2.5mM.

TABLE 4

ANALYSIS OF CHROMATINS FROM MOUSE TISSUES (PER ml OF CHROMATIN)

	DNA	RNA	RNA / DNA	histones	histones / DNA	histones / RNA	NHP	NHP / DNA	NHP / RNA	histones / NHP
Kidney	694	70	0,1	563	0,81	8,0	420	0.605	6,6	1,34
Kidney	282	30	0,1	232	0,82	7,7	205	0,73	6,7	1,131
Kidney	496	50	0,1	462	0,99	9,0	300	0,60	6	1,54
Kidney	600	74	0,123	437	0,70	6,0	558	0,93	7,5	0,783
Liver	694	114	0,164	565	0,80	5,00	990	1,42	8,0	0,57
Liver	496	110	0,23	412,5	0,90	3,75	520	1,05	4,73	0,79
Liver	682	138	0,20	660	0,97	4,78	800	1,17	5,7	0,825
Liver	930	200	0,21	990	1,06	4,95	1152	1,23	5,8	0,86
Spleen	496	43	0,086	381	0,77	8,8	250	0,50	5,8	1,524
Spleen	818	77	0,094	632	0,77	8,2	385	0,47	5,0	1,64
Spleen	864	68	0,078	696	0,8	10,0	336	0,39	5,0	2,07
Spleen	2256	205	0,091	1656	0,7	8,07	1320	0,51	6,0	1,254

TABLE 5

GENERAL CONDITIONS FOR RNA SYNTHESIS DIRECTED BY MOUSE KIDNEY CHROMATIN

Assay conditions	mμ Moles UTP incorporated/10 mins
Complete system with 16mM putrescine	4.5
Omit putrescine	1.7
Omit ATP, GTP and CTP	0.20
Omit manganese	0.04
Omit chromatin	0.03
Omit enzyme	0.45

Time course for RNA synthesis

The time course of RNA synthesis directed by mouse kidney chromatin was determined under the standard conditions used. Samples were incubated for varying times and the reaction terminated by the addition of 0.1ml of 50 per cent TCA. The reaction is linear for the first 15 minutes, but after this period of time the rate diverges from linearity and at about 90 minutes reaches a plateau. Further incubation results in a decline in the rate of RNA synthesis.

4. Template activity of DNA in chromatin

DNA was prepared from a whole mouse embryo, by the method described previously. 250 - 500µg DNA were added to a cell-free incubation mixture of 3mls. Identical incubation mixtures containing 500µg chromatin as template from mouse liver, kidney and spleen were also set up. After incubation at 37°C for 90 minutes the reaction was terminated by cooling. The RNA was extracted as described under Methods. An additional step was introduced into the procedure of its isolation.

In order to remove unincorporated ribonucleotides and unhybridizable oligonucleotides from hybridizable RNA, the aqueous supernate resulting from repeating phenol

treatments was applied to a 30cm x $1cm^2$ column of Biogel P30 (Calbiochem Limited, London), equilibrated in 2 x SSC at room temperature. The volume of the supernatant was reduced to 1ml by lyophylisation and applied to the column. It was found that three distinct fractions were eluted from the column by further elution with 2 x SSC. In order of elution these were: acid-precipitable polynucleotides, phenol and acid-soluble oligonucleotides. The amount of RNA synthesised under the used conditions was determined from the radioactivity of material precipitable with 5% w/v tri-chloroacetic acid. Oligonucleotide material as well as phenol were detected by their ultraviolet spectra. The measured radioactivity was indeed contained in RNA and this fraction was used in the hybridization mixture. The minimum sizes of RNA which would still hybridize with denatured DNA are 50 nucleotides at $67^{\circ}C$ (Gillespie and Spiegelman, 1966). The sizes of RNA produced by the cell-free RNA-synthesising system were much more greater than the minimum as it was estimated by the pattern they revealed in polyacrylamide gels.

Hybridization of the RNA product was carried out according to Gillespie and Spiegelman (1965). 13mm

filters containing 5µg of denatured whole mouse embryo DNA were incubated with increasing amounts of synthetic RNA in 4 x SSC. The ordinary used ratios of DNA:RNA was 1 : 7 - 10. As only 0.03 - 0.3% of the total DNA (Merits, Schulze; Overby, 1966) can serve as binding sites for ribosomal RNA interactions between DNA and rRNA were negligible at the ratios of DNA:RNA used in these experiments and any RNase-resistant radioactivity trapped on the nitrocellulose filters could safely be attributed to hybrid formation between the so-called mRNA and DNA.

The extent of hybridization as a function of RNA input is shown in Figure 9. A double reciprocal plot of this data permitted the evaluation of saturation value (figure 10). Knowing the specific activity of the synthetic RNA the percentage of the mouse genome that appears to have been used as template in this case has been found to closely approximate 20%.

When complementary RNA was synthesised on a chromatin template from the three tissues already mentioned, it was found that only a part of the genome restricted to approximately 3,5%, 5,5% and 7,5% for liver, spleen and kidney respectively was available for transcription. (Figure 11). These values may

Figure 9

Hybridization properties of RNA synthesized *in vitro* from mouse kidney DNA

Figure 10

Double reciprocal plot of the data shown in figure 9. At saturating concentrations of RNA 22% of the DNA exists as hybrid.

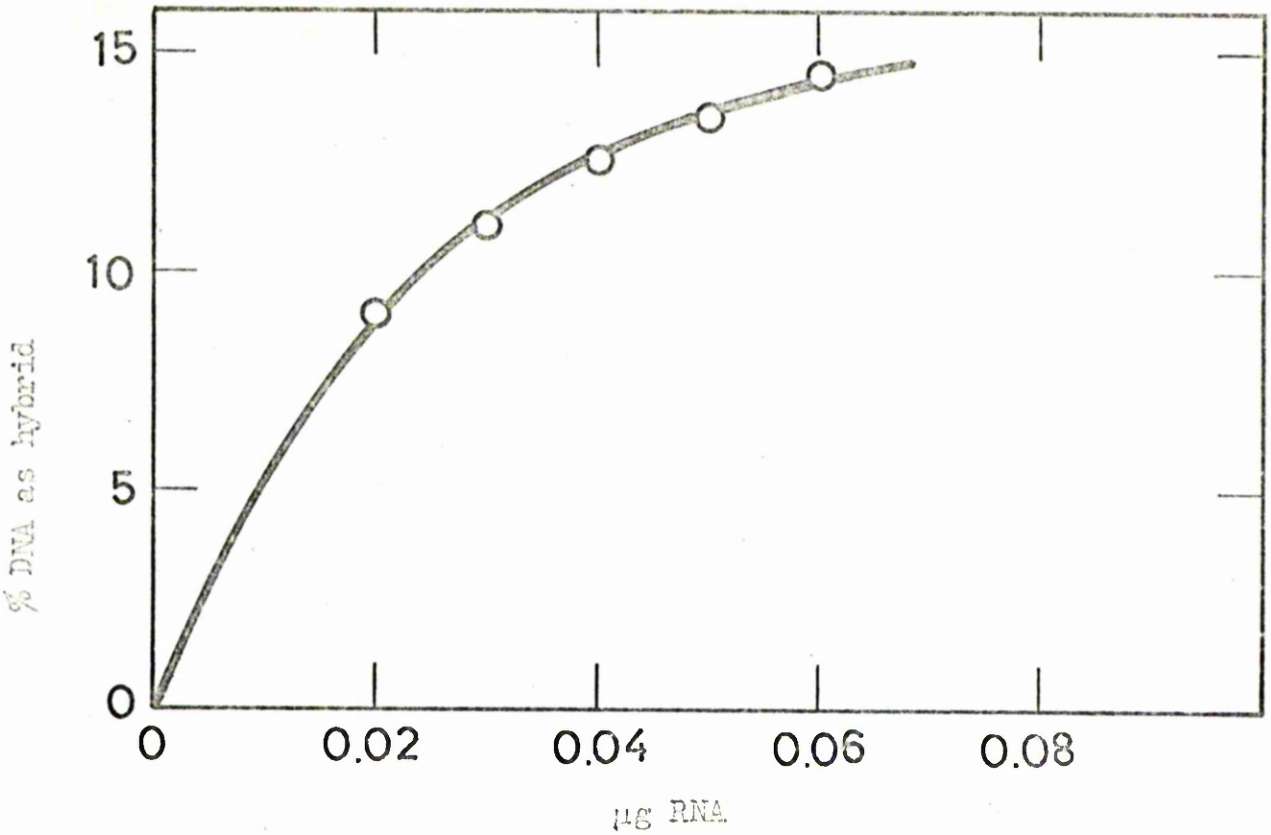

15
10
5
0
0
0.02
0.04
0.06
0.08
% DNA as hybrid
µg RNA

Figure 10

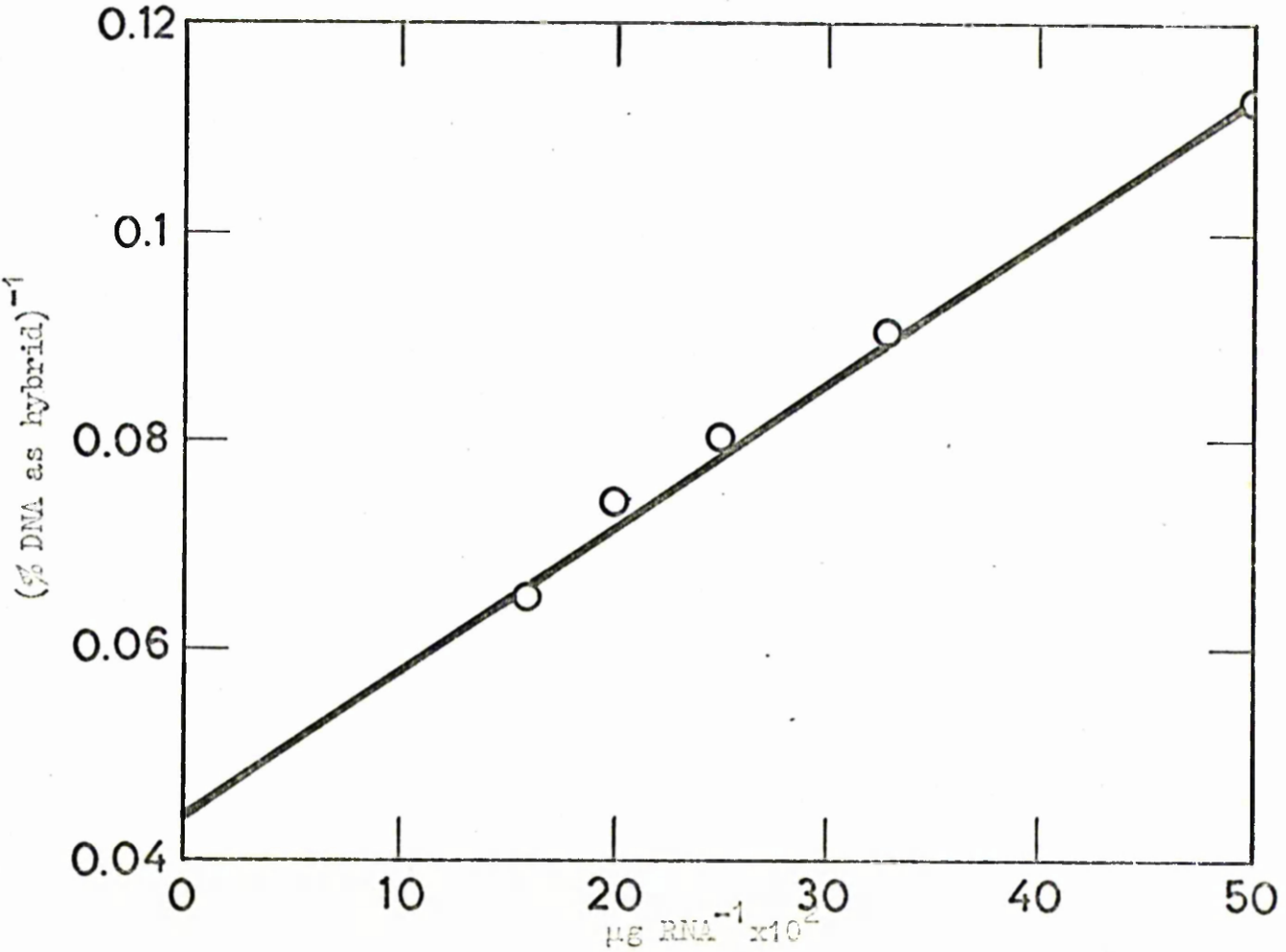

0.12
0.1
0.08
0.06
0.04
0
10
20
30
40
50
(% DNA as hybrid)$^{-1}$
µg RNA^{-1} x10^{2}

Figure 11

Kinetics of hybridisation to the whole mouse embryo DNA of ^{3}H-RNA made in vitro by M. lysodeikticus polymerase in the presence of whole embryo DNA, ○——○ ; mouse liver chromatin, ●——● ; mouse spleen chromatin, ▽——▽ ; mouse kidney chromatin, x——x ; The filters were loaded with 5µg denatured whole mouse embryo DNA and the ^{3}H-RNA preparations were incubated with individual filters at the concentration shown.

Figure 11

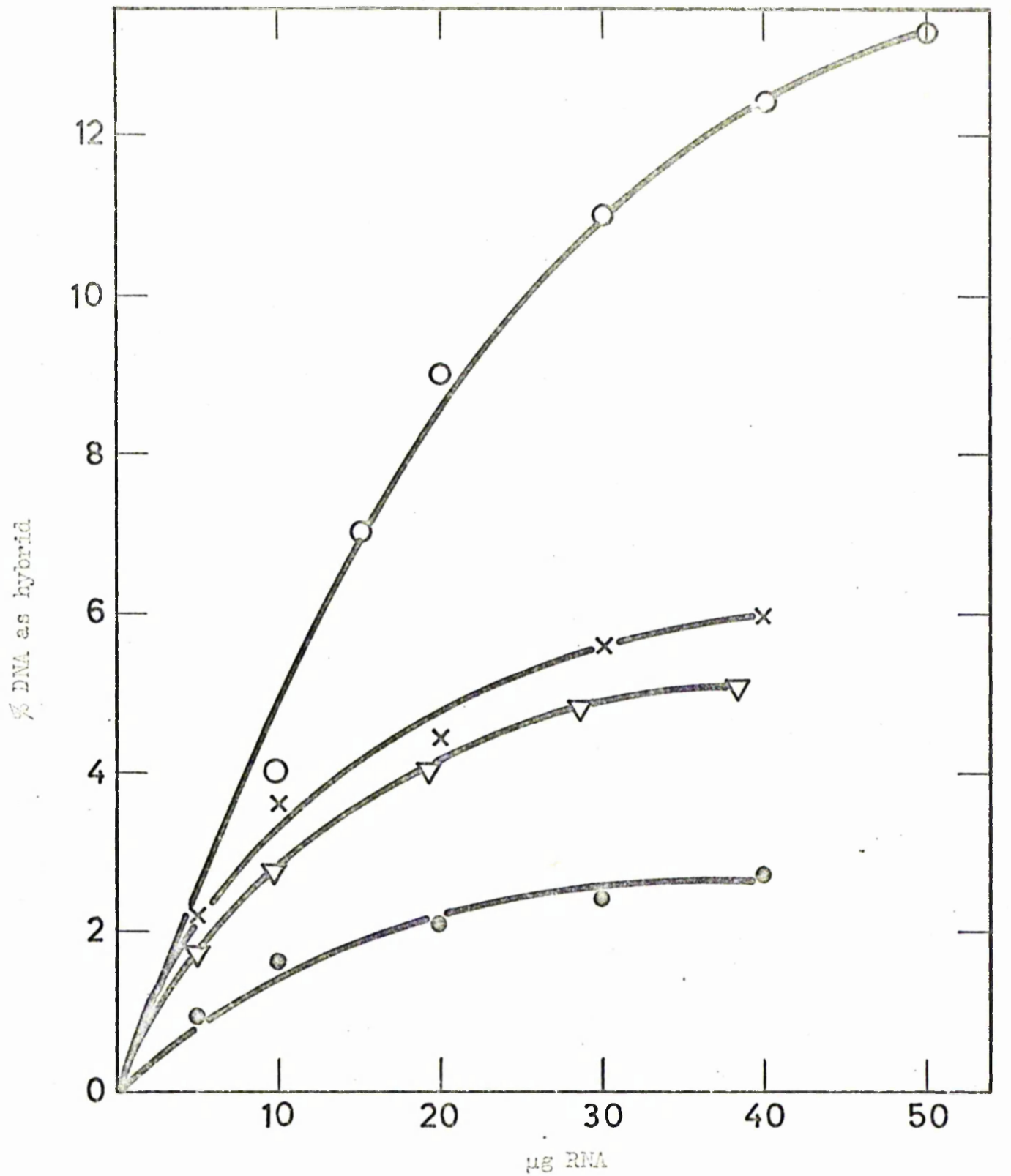


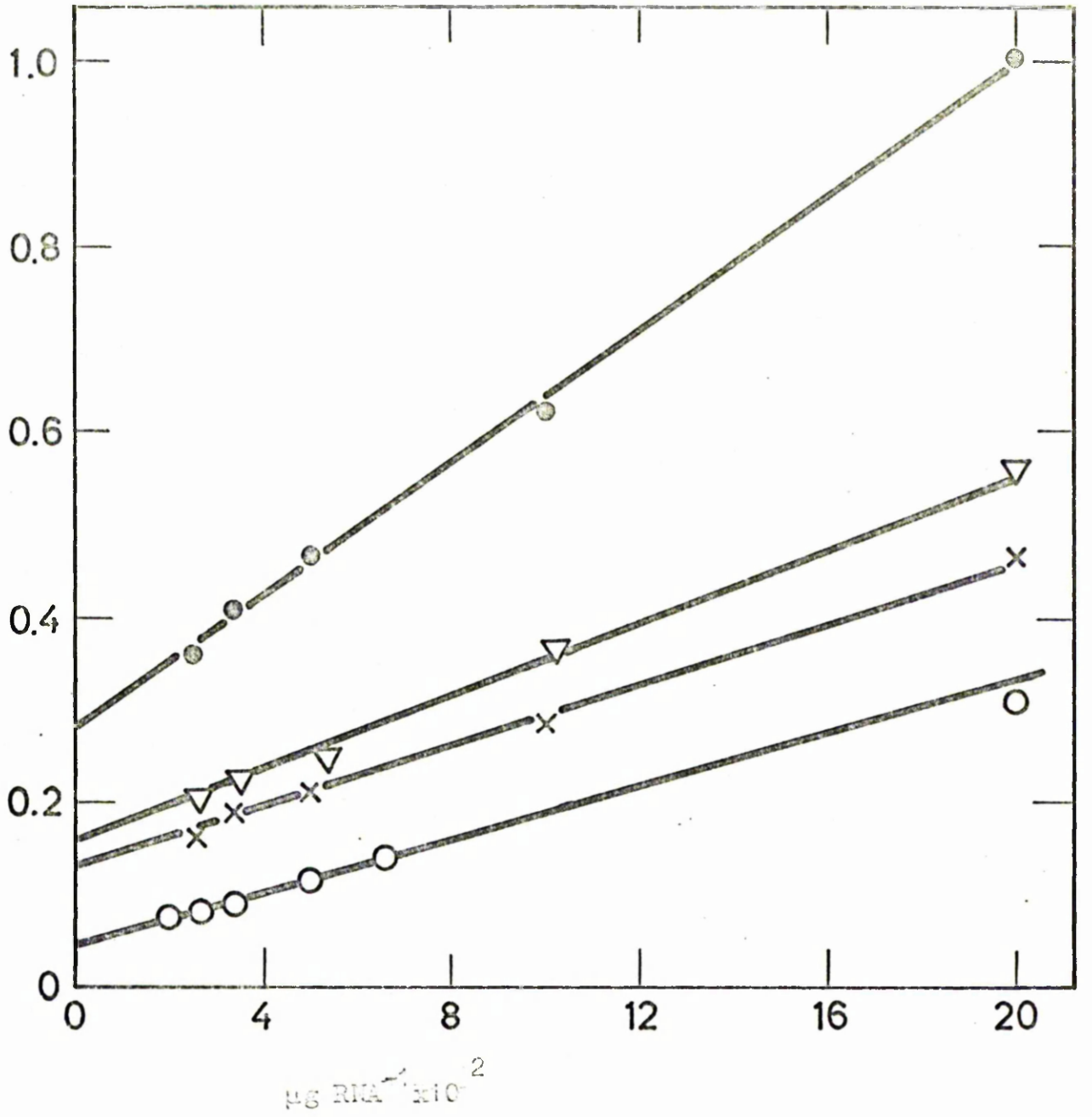
Figure 12

Double reciprocal plots of the data shown in figure 11. The percentages of the DNA existing as hybrid when each DNA preparation saturates the DNA was predicted from the intercept on the ordinate as follows:

DNA, 22%; liver chromatin, 3.5%;

kidney chromatin, 7.5%; spleen chromatin 5.5%

Figure 12



be considered as suggesting the possibility that certain genes unique for each specific type of chromatin are active in RNA synthesis relevant to tissue function.

A fact worth mentioning is the high degree of reproducibility of the hybridization values displayed by labelled RNAs, characteristic for each tissue, when hybridized to DNA. Populations of RNA molecules prepared from the same tissue but from different groups of animals gave the same percentage of hybrid with standard deviation of $\pm$ 0.2%. The results shown in Table 6 indicate the high reproducibility of the hybridization method employed.

For the evaluation of the data obtained from hybridization experiments it should be always taken into account that, as found by Britten and Kohne, in higher organisms and under the conditions used for RNA-DNA hybridization experiments, this method is not sensitive enough to detect that part of the genome that occurs in only a few copies besides the so-called fast and intermediate genomic portions. Furthermore, the relation between "genes" and "annealing sequences" is not known in this system.

TABLE 6

SATURATION DATA

No. of experiments	Liver	Kidney	Spleen
1	3,3	7	5,5
2	3,3	7,5	6,0
3	3,57	7,6	
4	3,4	7,6	
5	3,2	7,4	
6	3,5	7	
7		7,2	
8		7,2	
9		7,0	
10		7,6	
11		7,4	

4.1 Evidence for restriction of template activity in different tissues

Competitive hybridization was used to test whether RNAs prepared *in vitro* from mouse kidney, liver and spleen chromatins were homologous first with natural RNAs from the homologous tissues and second to show the degree of similarities and differences with natural RNAs from the heterologous tissues. Chromatin and natural RNA were prepared from the corresponding nuclei and ^{3}H-labelled RNA was synthesised *in vitro* using the chromatin as template. The saturation kinetics of these synthetic RNAs are shown in Figure 11.

Competitive hybridization experiments were carried out in which the competed (labelled) RNA is present at a constant and saturating level while the competitor's concentration - homologous or heterologous - is constantly increased. Figure 13 shows the competition effect when ^{3}H-RNA from kidney chromatin was challenged with natural nuclear RNAs from kidney, liver and spleen. The RNA from the tissue homologous to the chromatin was found to be the most effective competitor. By constructing a double reciprocal plot it can be shown (figure 14) that at excessive concentration of unlabelled RNA

Figure 13

Hybridisation competition experiments to determine specificity of RNA synthesised *in vitro* by mouse kidney chromatin. RNAs isolated from mouse kidney nuclei ×——×
liver nuclei ●——● ; and spleen nuclei ▽——▽ ; were used as competitors. For hybridization 5μg DNA, 30μg of labelled RNA and increasing amounts of unlabelled competitors were used.

Figure 13

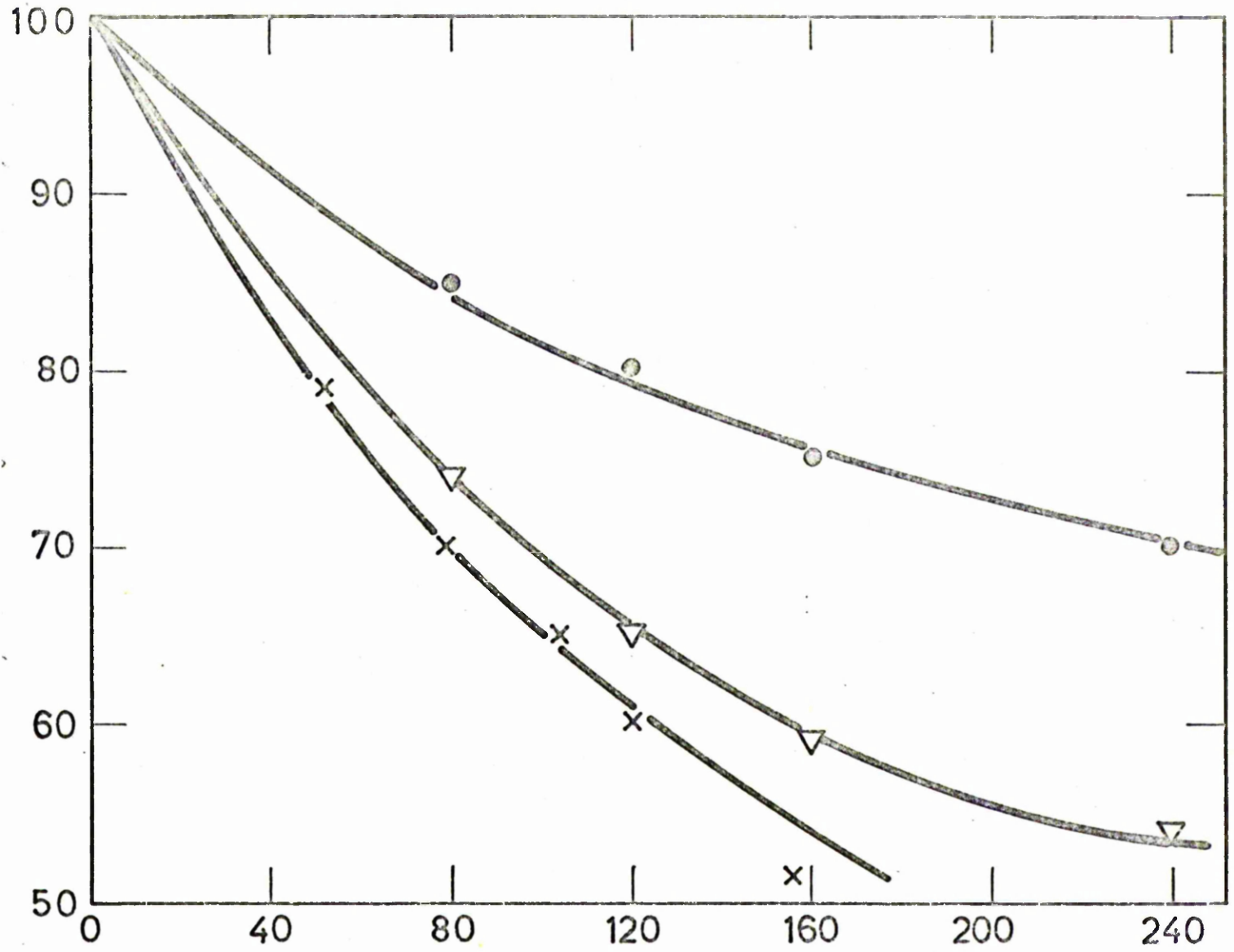
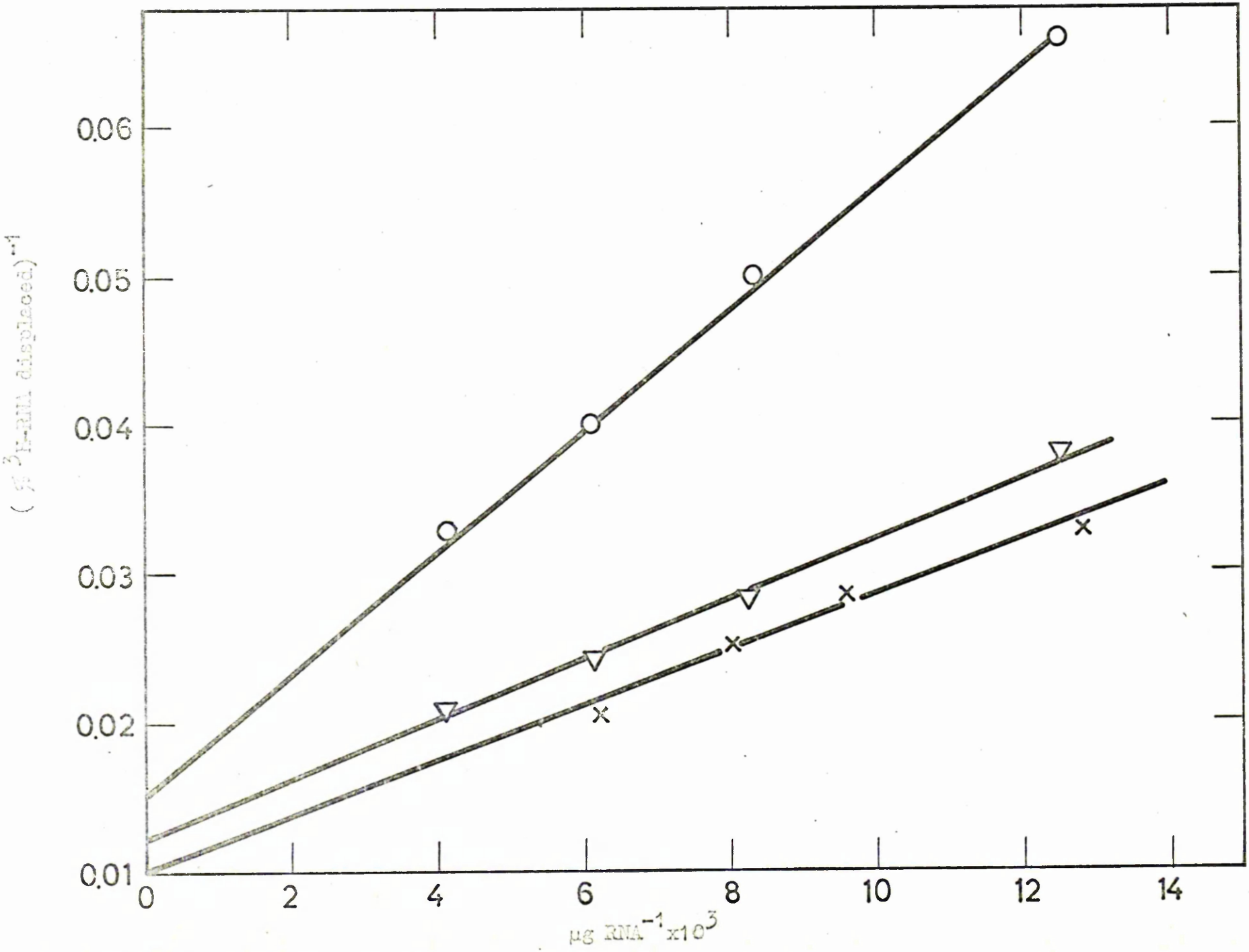


Figure 14

Double reciprocal plots of the competition data shown in figure 13.
By extrapolation to the ordinate it can be predicted that at infinite concentration of kidney, liver and spleen natural RNAs 0%, 34% and 17% respectively of the original ^{3}H kidney RNA remains as hybrid.

Figure 14


0.06
0.05
0.04
0.03
0.02
0.01
0
2
4
6
8
10
12
14
(% ^{3}H-RNA displaced)$^{-1}$
µg RNA^{-1} x10^{3}

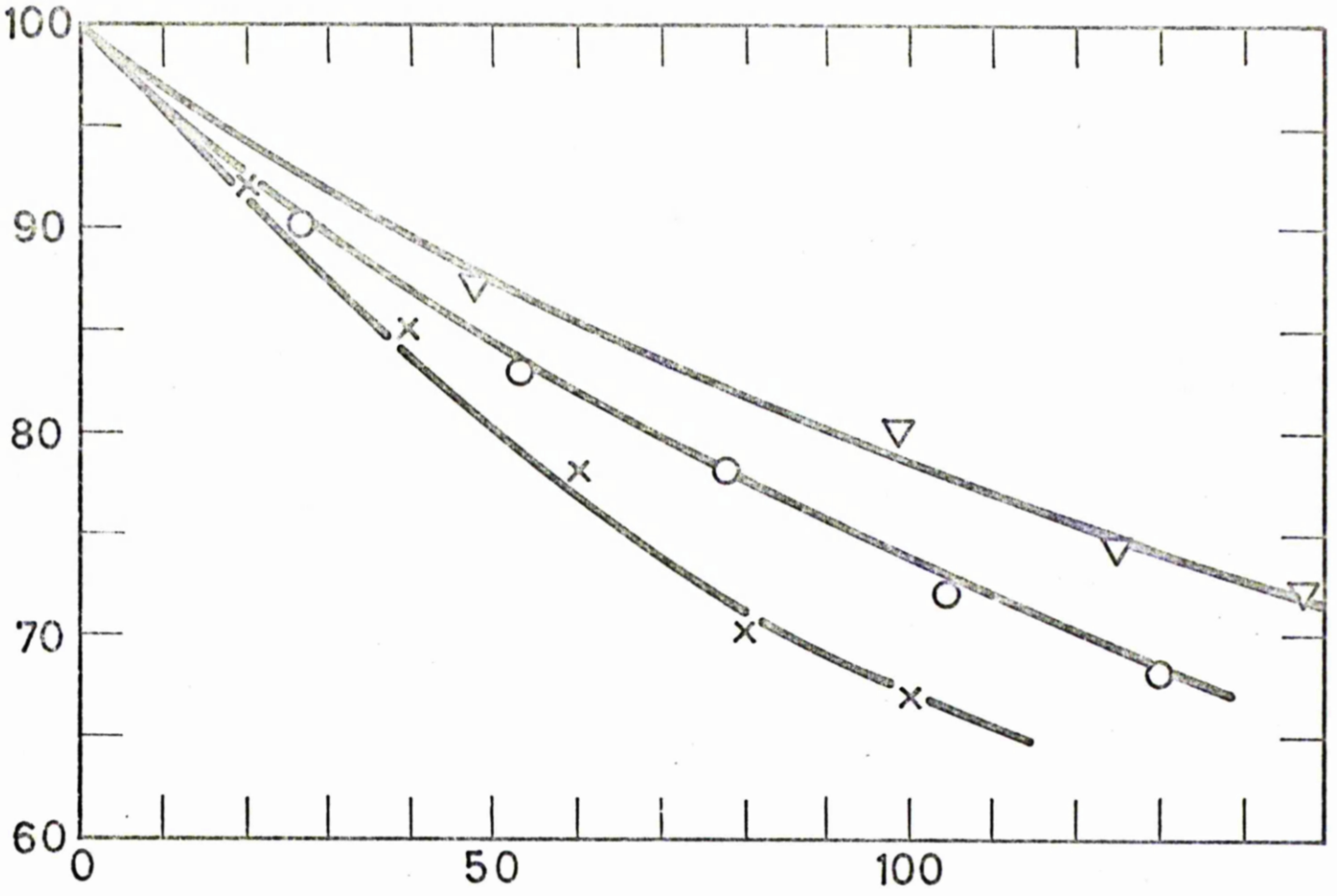
all the labelled material is excluded. Thus within the limits of the hybridization technique, the template specificity associated with the genome *in vivo* is retained by isolated chromatin. On the other hand, RNA from heterologous tissues is only partially competitive. This may suggest that a part of the labelled RNA molecules hybridizable under these conditions are essentially unrepresented in the heterologous mouse tissues. By these criteria both liver and spleen RNA are easily distinguishable from the RNA obtained from kidney and they must be different from one another.

Figures 15 and 17 present the results obtained when the same experimental system was applied for ^{3}H-RNA from spleen and liver and challenged with homologous and heterologous natural nuclear RNAs. Since the competition by the homologous natural RNA is essentially complete, the synthesising system *in vitro* has not generated any RNA molecules which are not normally present in the intact tissue. These results suggest the existence of active genes that are unique besides those in common in differentiated animal tissues. This may be considered as a further evidence in support of the

Figure 15

Hybridisation competition experiments to determine specificity of RNA synthesised *in vitro* by mouse spleen chromatin. RNAs isolated from mouse spleen nuclei x——x ; liver nuclei ▽——▽ ; and kidney nuclei ●——● ; were used as competitors. The conditions were identical as in figure 11.

Figure 15



µg Competing RNA

Figure 16

Double reciprocal plots for the competitive hybridisation of the data shown in figure 15. By extrapolation to the ordinate it can be predicted that at infinite concentration of spleen, liver and kidney natural RNAs 0%, 41% and 30% respectively of the original ^{3}H spleen RNA remains as hybrid.

Figure 16

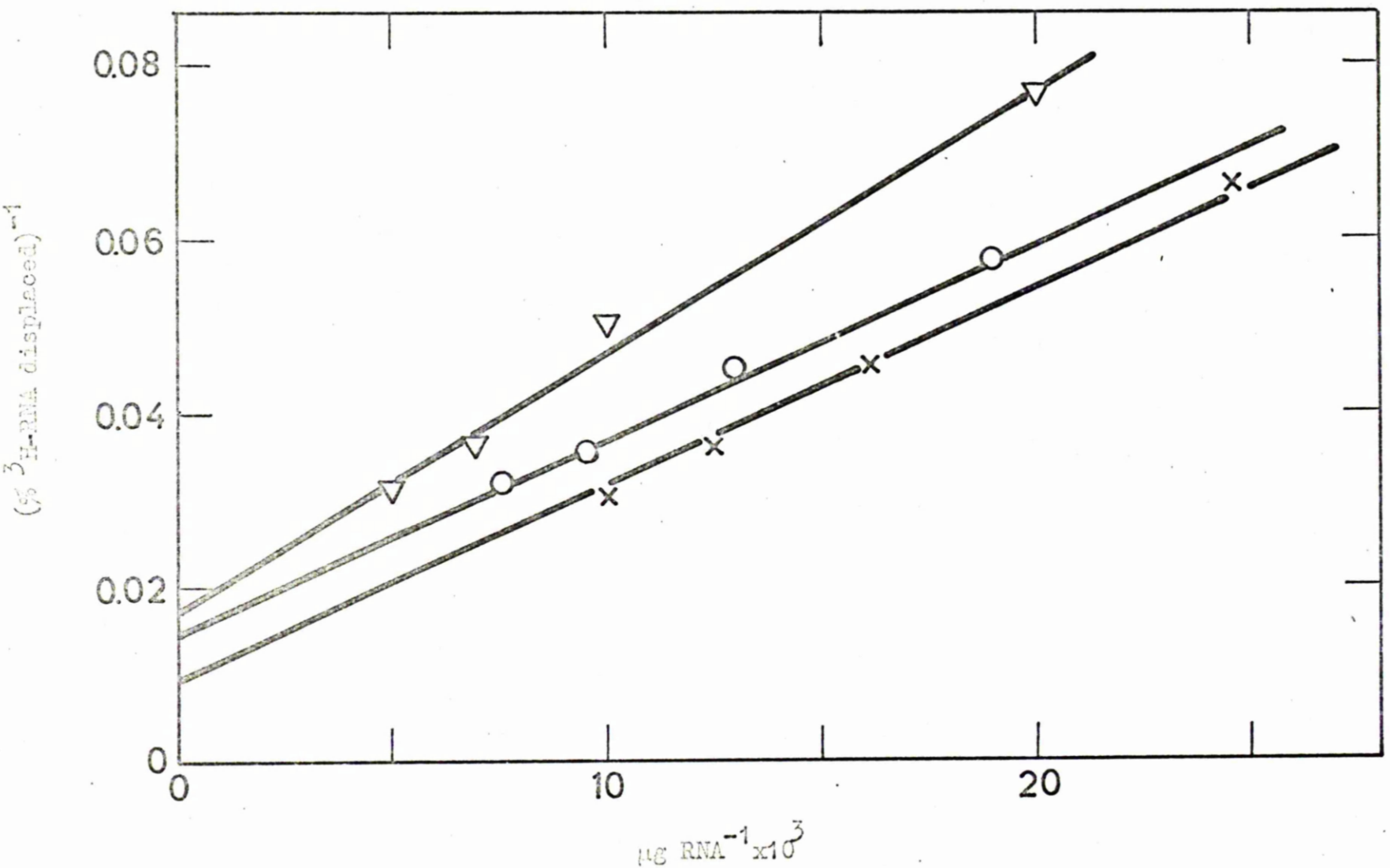


Figure 17

Hybridization competition experiments to determine specificity of RNA synthesised *in vitro* by mouse liver chromatin. RNAs isolated from mouse liver nuclei X—X ; kidney nuclei ●—● ; and spleen nuclei ▽—▽ were used as competitors in the hybridisation of *in vitro* synthesised RNA to DNA. Annealing mixtures contained 5μg whole mouse embryo DNA, 30μg synthetic RNA and increasing amounts of unlabelled competitor RNAs.

Figure 18

Double reciprocal plots for the competitive hybridization of the data shown in figure 17. By extrapolation to the ordinate it can be predicted that at infinite concentrations of liver, kidney and spleen natural RNAs 28%, 46% and 40% respectively of the original ^{3}H-liver RNA remains as hybrid.

Figure 17

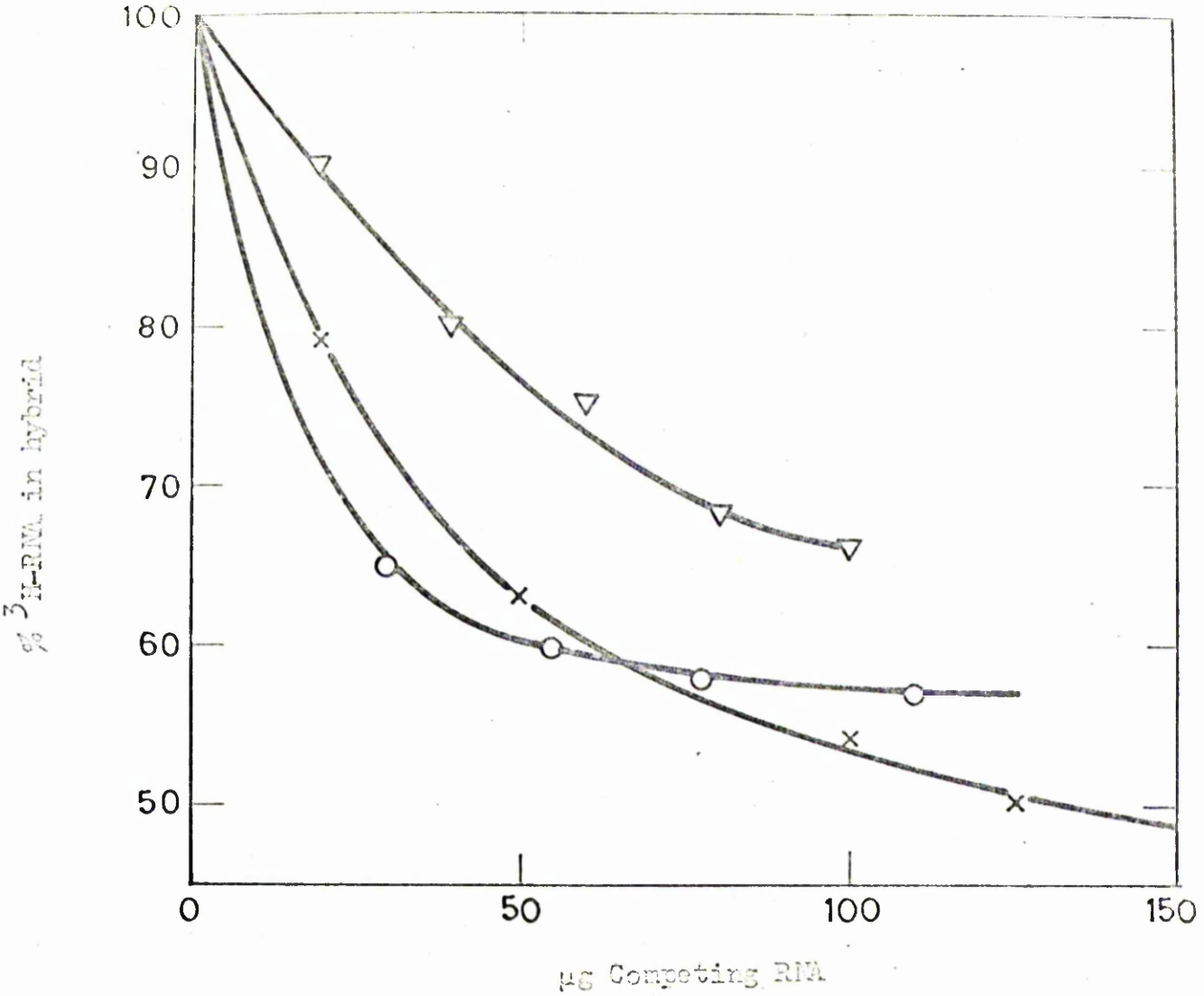
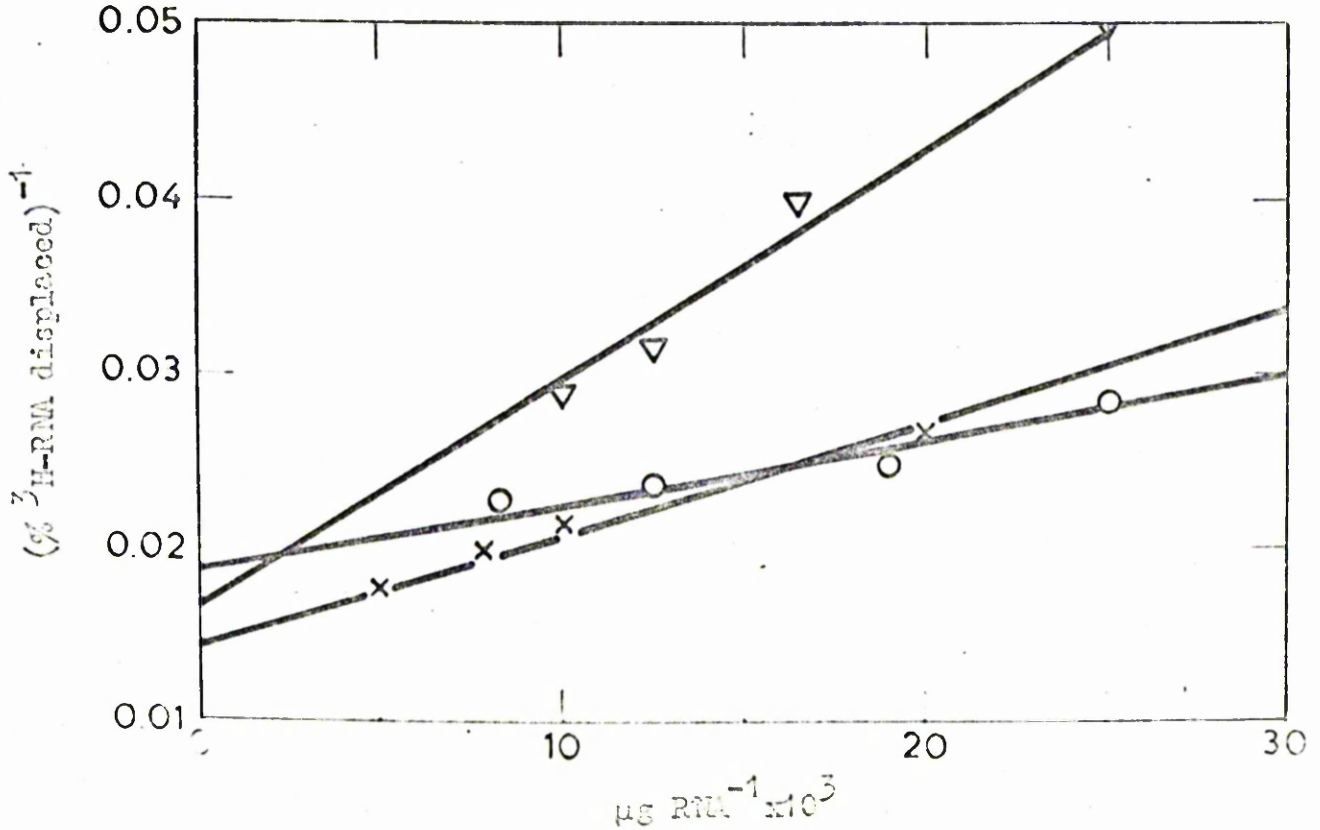


Figure 18



view that differentiated animal tissues are characterised by a considerable degree of specificity that is reflected in their RNA pattern.

Changes in the conditions (salt and temperature) can give quite different estimates of the similarity between different populations of RNA molecules. The more stringent the conditions the more differences appear (McCarthy and McConaughy, 1968). However, it is evident from the specificity of such reactions that competition experiments will always provide a minimum estimate of the extent of difference, since the existence of related sequences limits resolution. Two different RNA molecules present in the various tissues will, under most conditions, be able to compete for a DNA site which is sufficiently similar in base sequence, but which may not be concerned with their synthesis or be strictly complementary to either one. This effect is reduced at higher temperatures or lower salt concentrations, but will still be operative under most conditions normally used. Thus, even the estimate of approximately 70% difference between kidney and liver RNA at 67°C and 4 x SSC may be a minimal estimate for those RNA molecules actually hybridized; Table 7 presents data from the competition reactions performed under stringent conditions.

TABLE 7

COMPETITION EXPERIMENTS

* Liver - Liver	70%	66%	
* Liver - Kidney	55%	52%	
* Liver - Spleen	58%	56%	
* Kidney - Kidney	100%	100%	100%
* Kidney - Liver	66%	-	66%
* Kidney - Spleen	83%	72,5%	80%
* Spleen - Spleen	98%	100%	100%
* Spleen - Kidney	64,5%	71,4%	77%
* Spleen - Liver		62,5%	66%

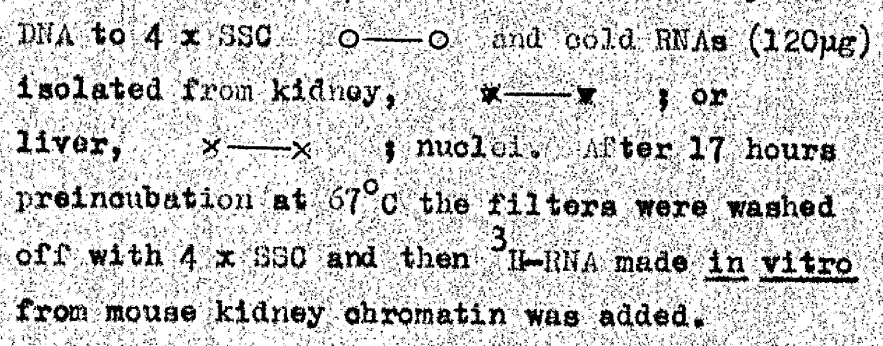
4.2 Competition experiments by preincubation

When hybridization competition experiments were carried out using the preincubation technique recommended by Riggsby and Merriam (1968) it was found that the preincubation with unlabelled RNA was unable to block DNA sites on nitrocellulose filters which might subsequently accept labelled RNA molecules. In order to clarify the situation the following experiments were performed.

First, mouse embryo DNA was immobilised on filters (5µg/ml) and preincubated in 4 x SSC for 17 hours at 67°C. The filters were washed with cold 4 x SSC and incubated for a further 17 hours with increasing amounts of *in vitro* labelled RNA from mouse kidney or liver in order to establish at what concentration saturation occurs under the conditions used. (Figures 19 and 20).

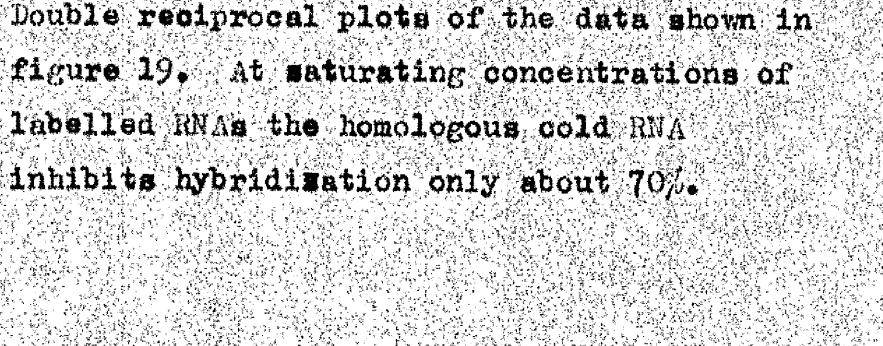
Second, fixed amounts of unlabelled RNA (120µg) from mouse kidney or liver were preincubated with the DNA filters in 4 x SSC for 17 hours at 67°C. At the end of the preincubation the filters were washed with cold 4 x SSC and incubated for another 17 hours at 67°C with increasing amounts of labelled RNA (up to 40µg) homologous or heterologous to the

Figure 19



Hybridisation of 5µg of whole mouse embryo DNA to 4 x SSC ○——○ and cold RNAs (120µg) isolated from kidney, ✱——✱ ; or liver, ×——× ; nuclei. After 17 hours preincubation at 67°C the filters were washed off with 4 x SSC and then ^{3}H-RNA made in vitro from mouse kidney chromatin was added.

Figure 20



Double reciprocal plots of the data shown in figure 19. At saturating concentrations of labelled RNAs the homologous cold RNA inhibits hybridisation only about 70%.

Figure 19

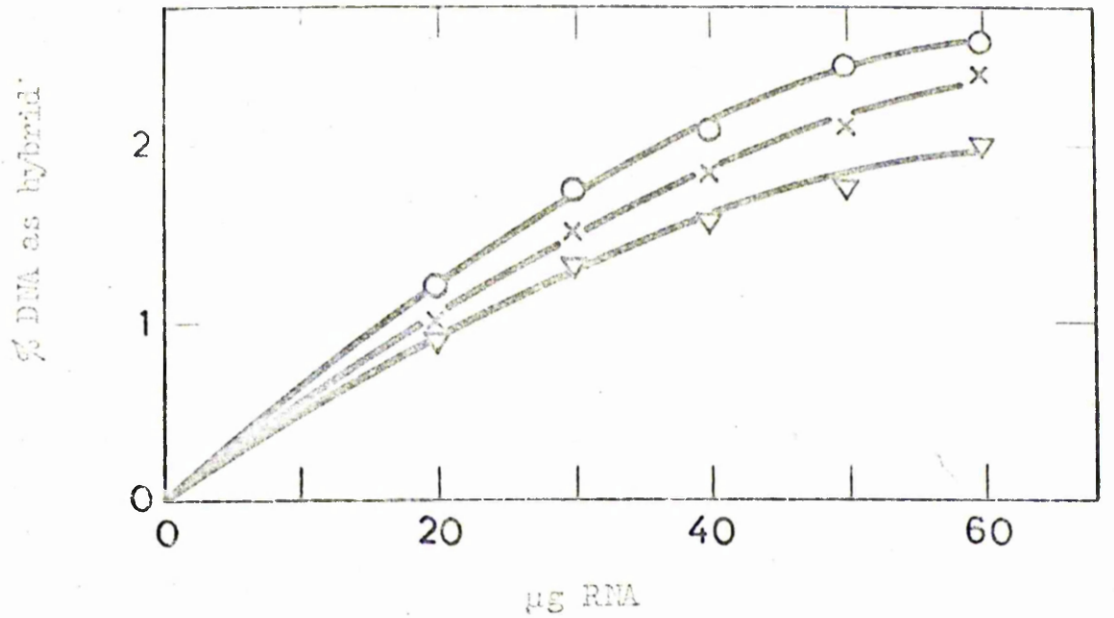
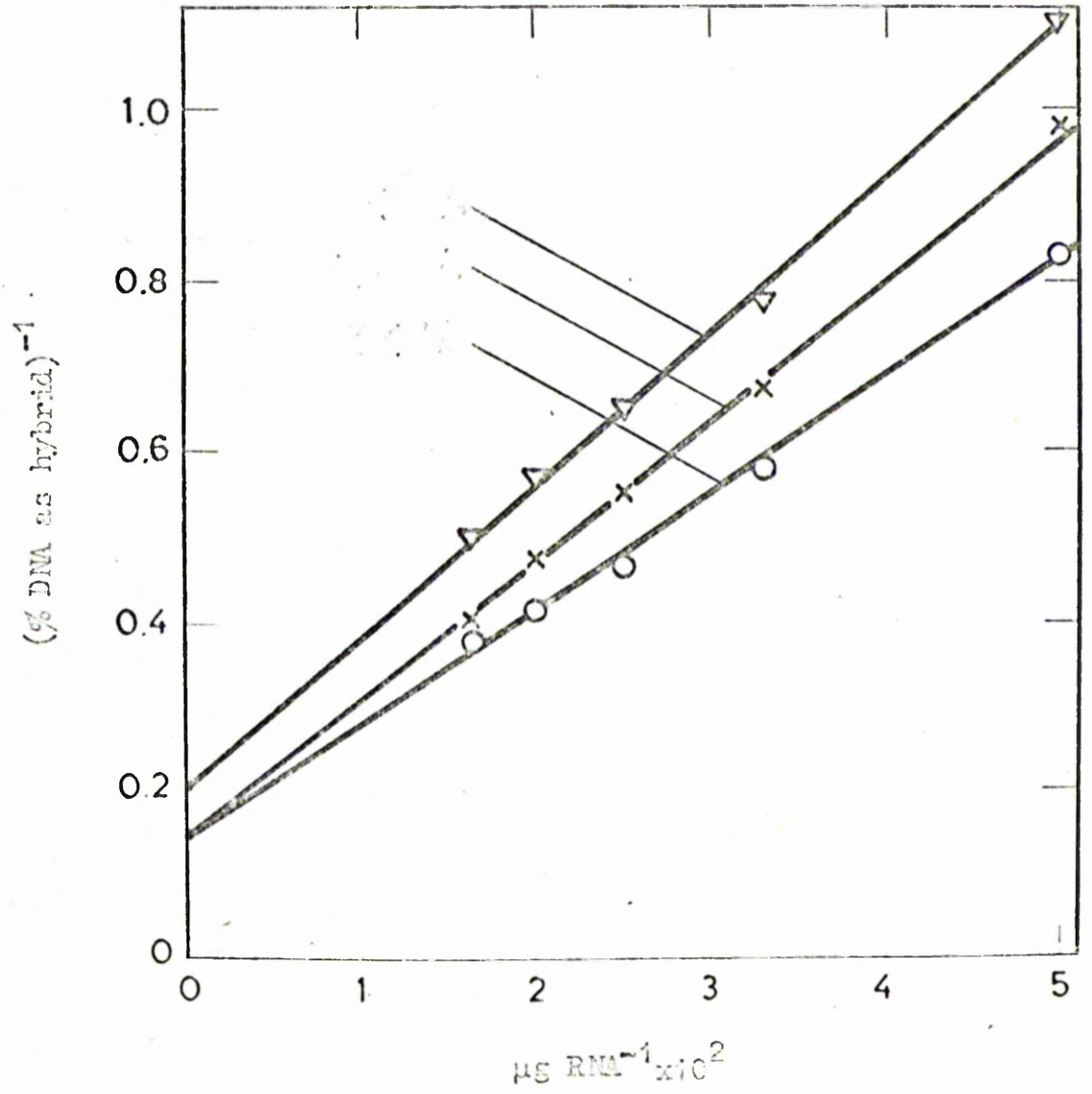


Figure 20



unlabelled RNA. In both cases, first and second, the incubation was terminated by cooling, the filters were washed on both sides and treated with 20µg/ml RNase in 2 x SSC. The following results were obtained:

Preincubation with homologous unlabelled kidney RNA inhibits hybridization between DNA and the corresponding types of labelled kidney RNA only about 70% (figure 19). On the other hand, competition was not demonstrated when pre-incubation with cold RNA was followed by subsequent incubation with heterologous labelled RNA. The values obtained were of the same order of magnitude as those observed when the labelled RNA was incubated either in the absence of unlabelled RNA during the preincubation or when no preincubation was applied. (Figures 21 and 22).

These results may be due to a dissociation of the unlabelled RNA/DNA complex during the subsequent annealing with labelled RNA. Brelka and Junghahn (1968) arrived to the same conclusions using rat liver D-RNA and R-RNA.

Figure 21

Hybridisation of 5μg of whole mouse embryo DNA in 4 x SSC, o——o ; and cold RNA (120μg) isolated from kidney nuclei, x——x
After 17 hours preincubation at 67°C the filters were washed off with 4 x SSC and then ^{3}H-RNA made *in vitro* from mouse liver chromatin was added.

Figure 22

Double reciprocal plot of the data shown in figure 21. At saturating concentrations of labelled RNA the heterologous cold RNA does not compete for binding sites on DNA.

Figure 21

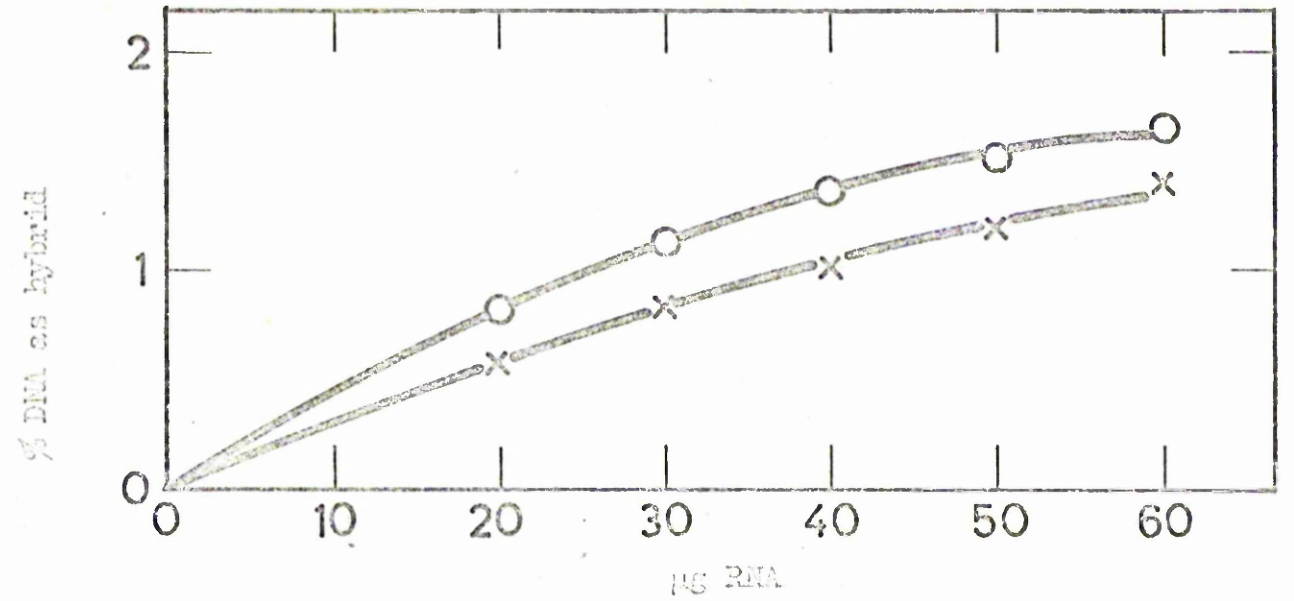
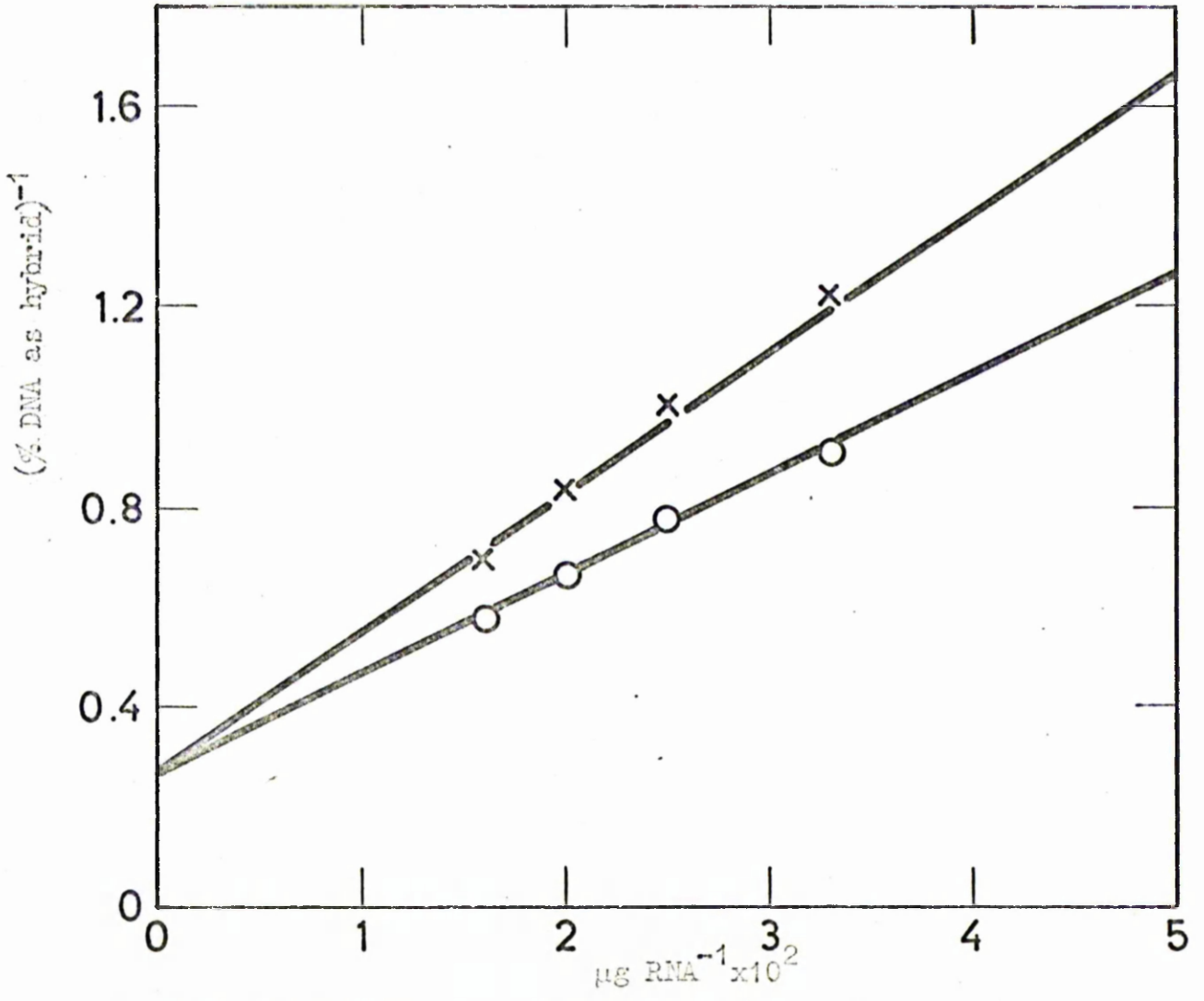


Figure 22



5. Synthesis of RNA by chromatin isolated from folic acid-treated mouse kidney

It has been found that a functionally defined population of cells under ordinary conditions, displays a very slow RNA-DNA synthesis and proliferating activity as well but which can become very active when exposed to the proper stimulus.

Recently it has been demonstrated that folic acid injected in rats increases the RNA, DNA and protein synthesis in the kidneys and a rapid cell proliferation follows (Threlfall et al., 1968). To establish the time of RNA synthesis in the strain of mice used studies were made after a single injection of folic acid. A rather similar pattern of response was revealed. ^{3}H-uridine was injected at a dose of 20µc/30g body wt. 60 minutes before removal of kidneys. The total amount of RNA was estimated by the orcinol method and the radioactivity in RNA was estimated by liquid scintillation counter. ^{3}H-thymidine was injected at a dose of 20µc/30g body wt. 60 minutes before removal of the kidneys and the total amount of DNA was measured by the diphenylamine method. The radioactivity in DNA was estimated by liquid scintillation counting.

RNA synthesis increases from 3 hours to reach a maximum at 9 hours before declining to normal levels at 48 hours. The increase in the rate of DNA synthesis commences at about 30 hours and reaches a maximum at 40 hours (Paul et al., in press). Thus the folate-stimulated kidney represents a potentially valuable system for the study of some of the factors involved in cell division. Up to now no attempts have been made to compare the populations of RNA molecules synthesised after treatment with chemical compounds with that of the normal tissue. Since RNA molecules are the phenotypic expression, alterations in gene function often involve the appearance of new species of RNA. For the investigation of the mechanism providing for the regulation of transcription the state of mouse kidney chromatin has been studied after folate administration.

Studies will be presented on the effect of folic acid in stimulating changes in the in vitro RNA synthesis such that new kinds of RNA molecules appear at various times after the treatment. The hybridization technique provides a sensitive method of distinguishing among various populations of RNA molecules.

Male ~~portion~~ Porton strain mice weighing up to 30g were used. Folic acid was dissolved in 0.3M sodium bicarbonate at a concentration of 25mg/ml. This solution was injected intraperitoneally at a dose of 5mg/30g body wt. 1 hour, 2, 3, 6, 12, 24 and 48 hours after folate injection the mice were sacrificed and the nuclei were isolated from the kidneys using the citric acid method. Nuclei from control animals were isolated as well. Chromatins were prepared from a part of nuclei and used as templates for the in vitro synthesis of ^{3}H-labelled RNAs. From the remainder of the nuclei, for some cases, natural RNA was extracted. Hybridization of the ^{3}H-RNAs with 5µg of denatured whole mouse embryo DNA was carried out by the Gillespie and Spiegelman (1965) molecular hybridization technique.

5.1 Hybridization of the various RNAs after folic acid treatment

The distribution of various classes of RNA molecules in the labelled populations were analysed by calculating the fraction of DNA hybridized as a function of the amount of RNA present. Folic acid changes profoundly the template activity of the isolated chromatin from mouse kidneys.

RNA from folic acid treated kidneys displayed considerably higher hybridization efficiency than RNA from normal kidney (Figures 23, 25 and 27). This was more pronounced with RNA preparations from earlier periods in the folate response. Thus the hybridization efficiency of RNA after 12 hours is intermediate between that of normal kidney and 3 hour preparation.

By constructing a double reciprocal plot of the data obtained from eight different in vitro labelled RNA preparations it is clear that the hybridization efficiency is highest at the early times after the folate treatment and declines steadily to approach that of normal kidney after 48 hours. (Figures 24, 26 and 28).

The RNAs produced from the different chromatin preparations may differ in the synthesis of a larger variety of different nuclear molecules which would increase the hybridization since more sites on the DNA would be saturated. Possibly the resulting differences in distribution may be attributed to a response to folic acid treatment which may well involve synthesis of a greater quantity and a wider diversity of RNA molecules.

Figure 23

Kinetics of hybridisation to whole mouse embryo DNA of ^{3}H-RNA made *in vitro* by mouse kidney chromatin isolated at zero hours ●——● ; half-hour X——X ; one hour ▽——▽ ; two hours □——□ ; and three hours ○——○ after folic acid treatment.

The filters were loaded with 5µg denatured whole mouse embryo DNA and the ^{3}H-RNA preparations were incubated in 4 x SSC for 17 hours at 67°C at the concentration shown.

Figure 24

Double reciprocal plots of the data shown in figure 23. The percentages of the DNA existing as hybrid when each RNA preparation saturates the DNA was predicted from the intercept on the ordinate as follows:

zero hours 7.2%, half-hour 7.2%, one hour 9.5%, two hours 11.0%, three hours 14.2%.

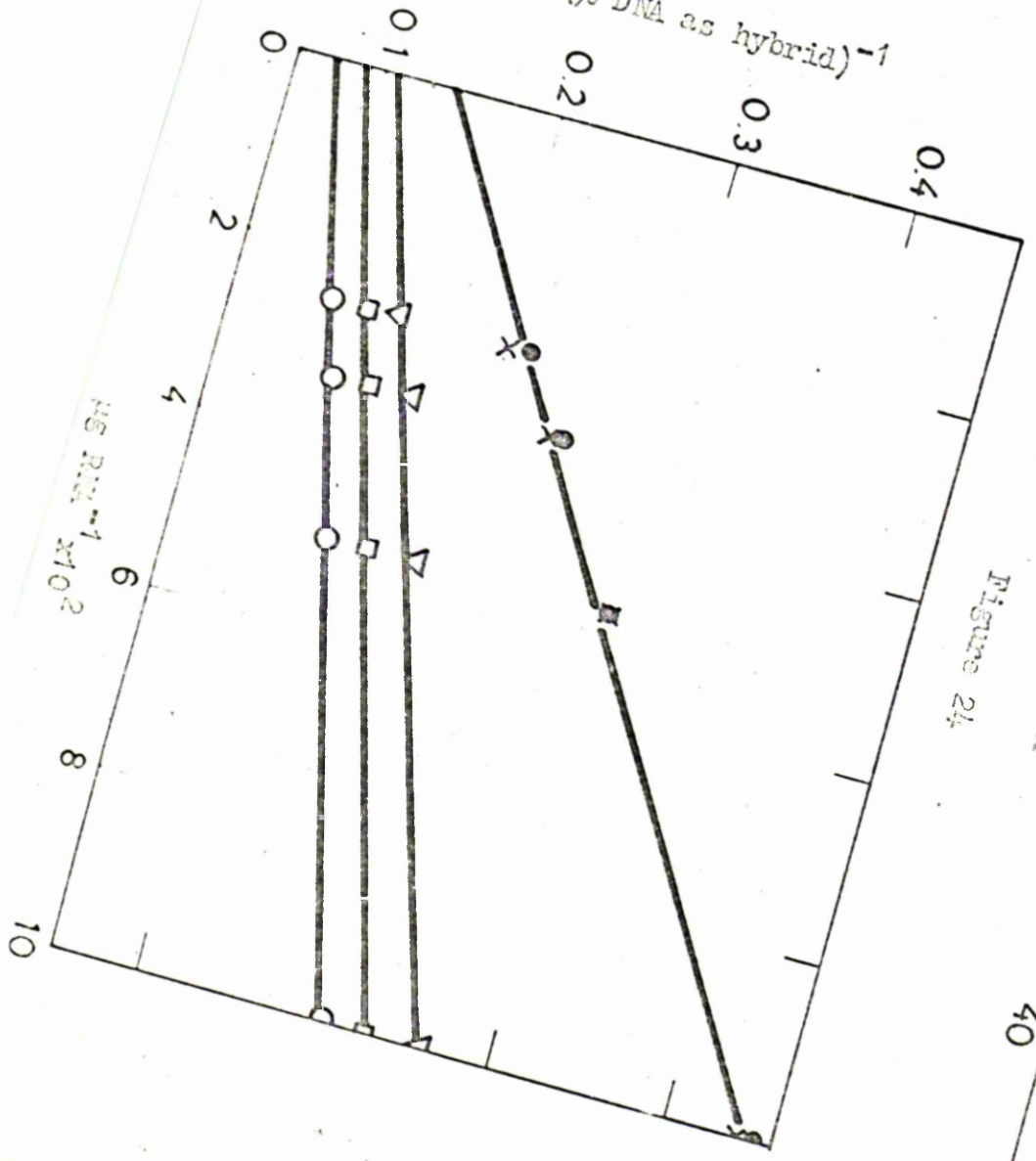


Figure 24

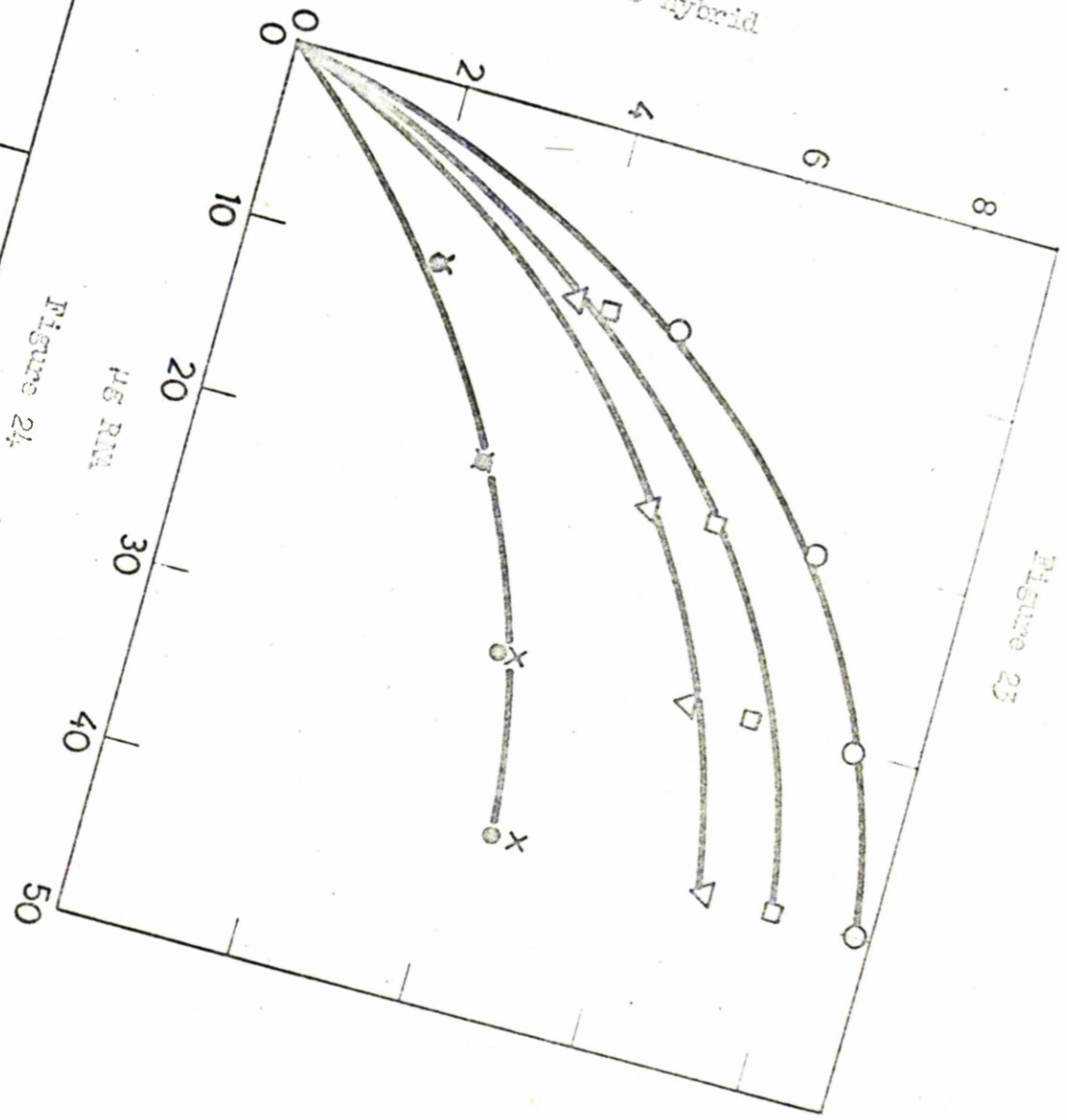


Figure 25

Figure 25

Kinetics of hybridisation to whole mouse embryo DNA of ^{3}H-RNA made *in vitro* by mouse kidney chromatin, isolated at zero hours, ●——● ; three hours, X——X ; six hours, ▽——▽ ; twelve hours, ○——○ ; after folate administration.

The conditions of hybridisation were identical as in figure 23.

Figure 26

Double reciprocal plots of the data shown in figure 25. The percentages of the DNA existing as hybrid when each RNA preparation saturates the DNA was predicted from the intercept on the ordinate as follows:

zero hours, 7.6%, three hours 14.1%, six hours 11.0%, twelve hours 9.5%.

Figure 25

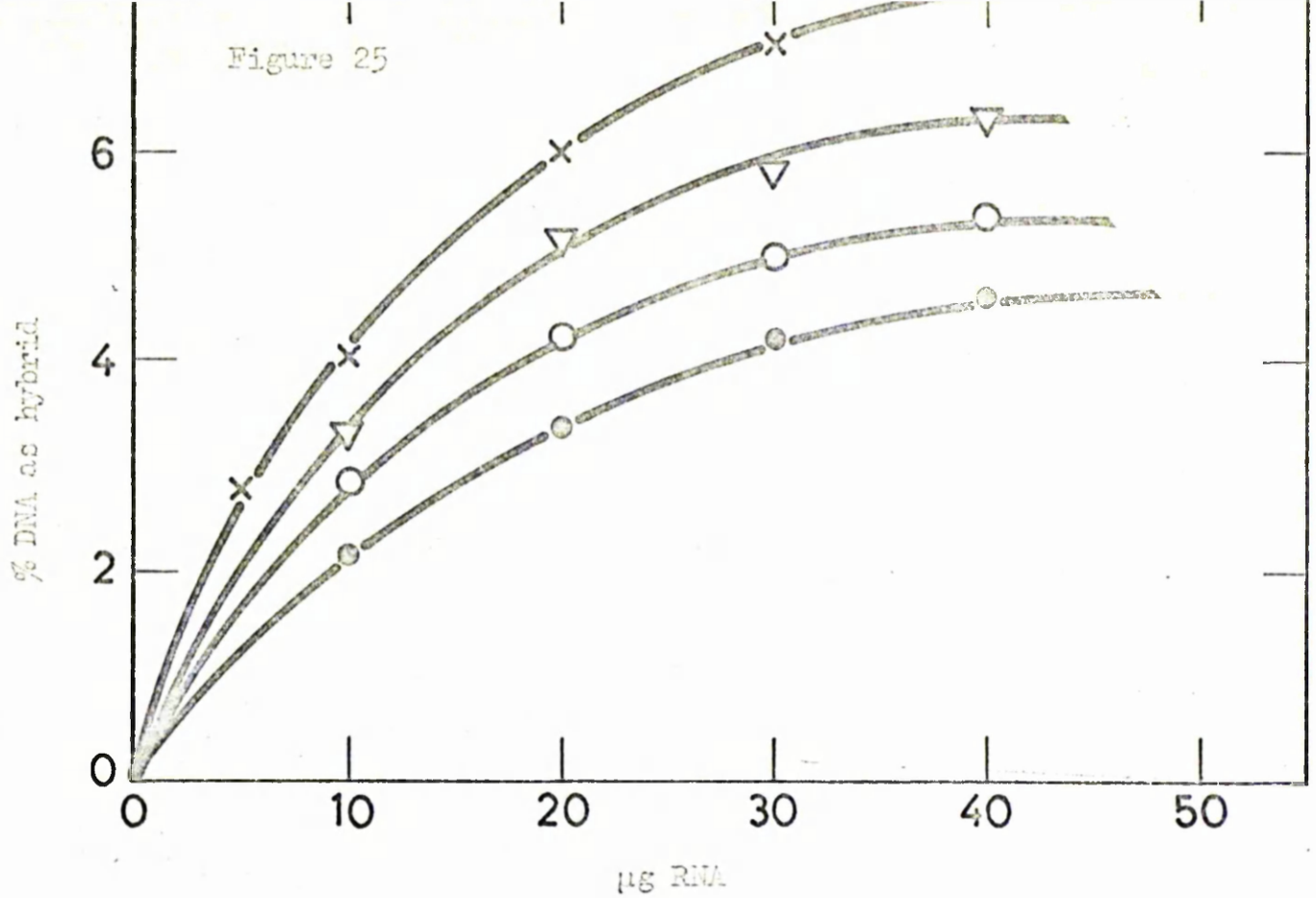


Figure 26

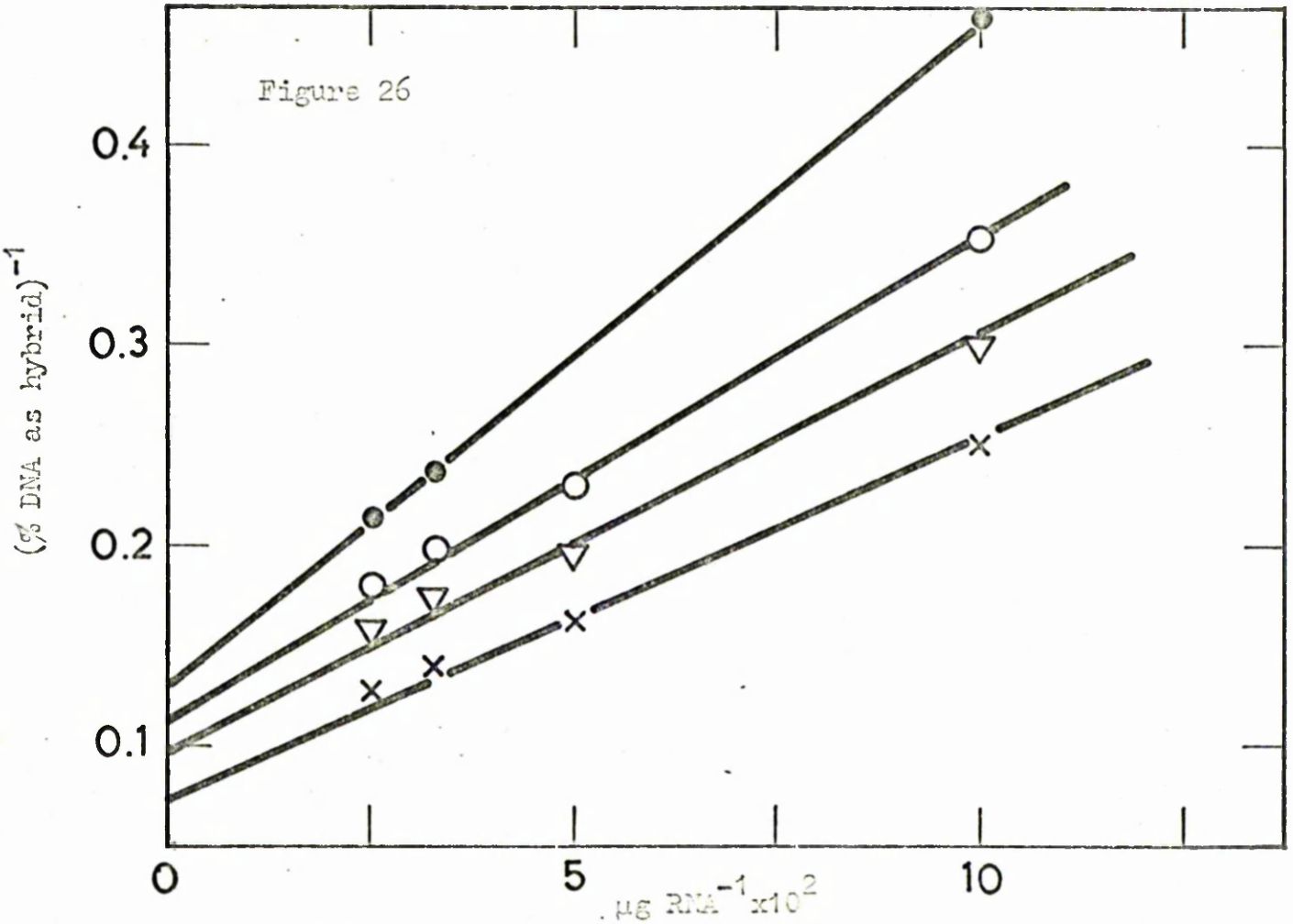


Figure 27

This is the continuation of figure 25. Kinetics of hybridisation to whole mouse embryo DNA of ^{3}H-RNA made *in vitro* by mouse kidney chromatin isolated at twenty-four hours X——X ; and forty eight hours O——O ; after folate administration.

Figure 28

Double reciprocal plots of the data shown in figure 27. The percentages of the DNA existing as hybrid when each RNA preparation saturates the DNA was predicted from the intercept on the ordinate as follows: twenty-four hours 9.0%, forty eight hours 7.2%.

Figure 27

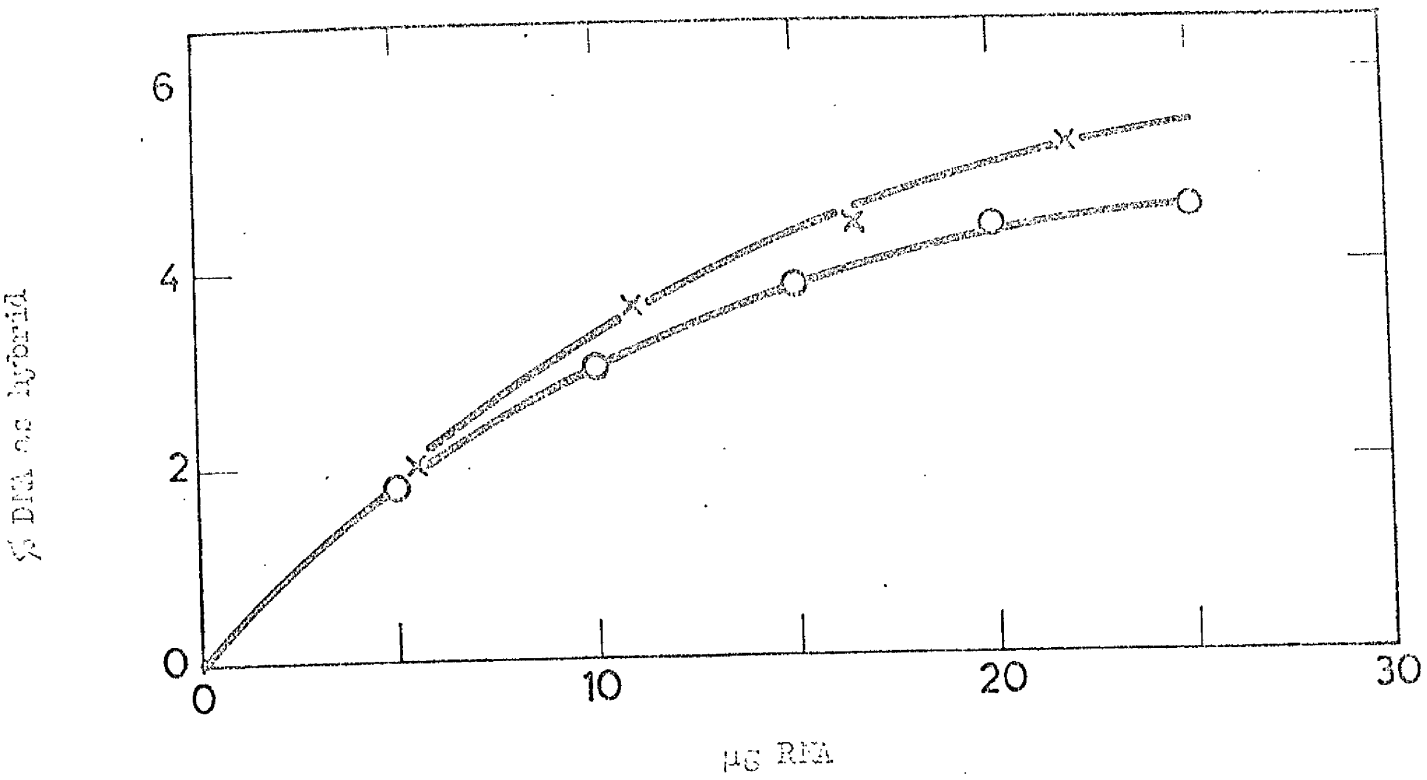
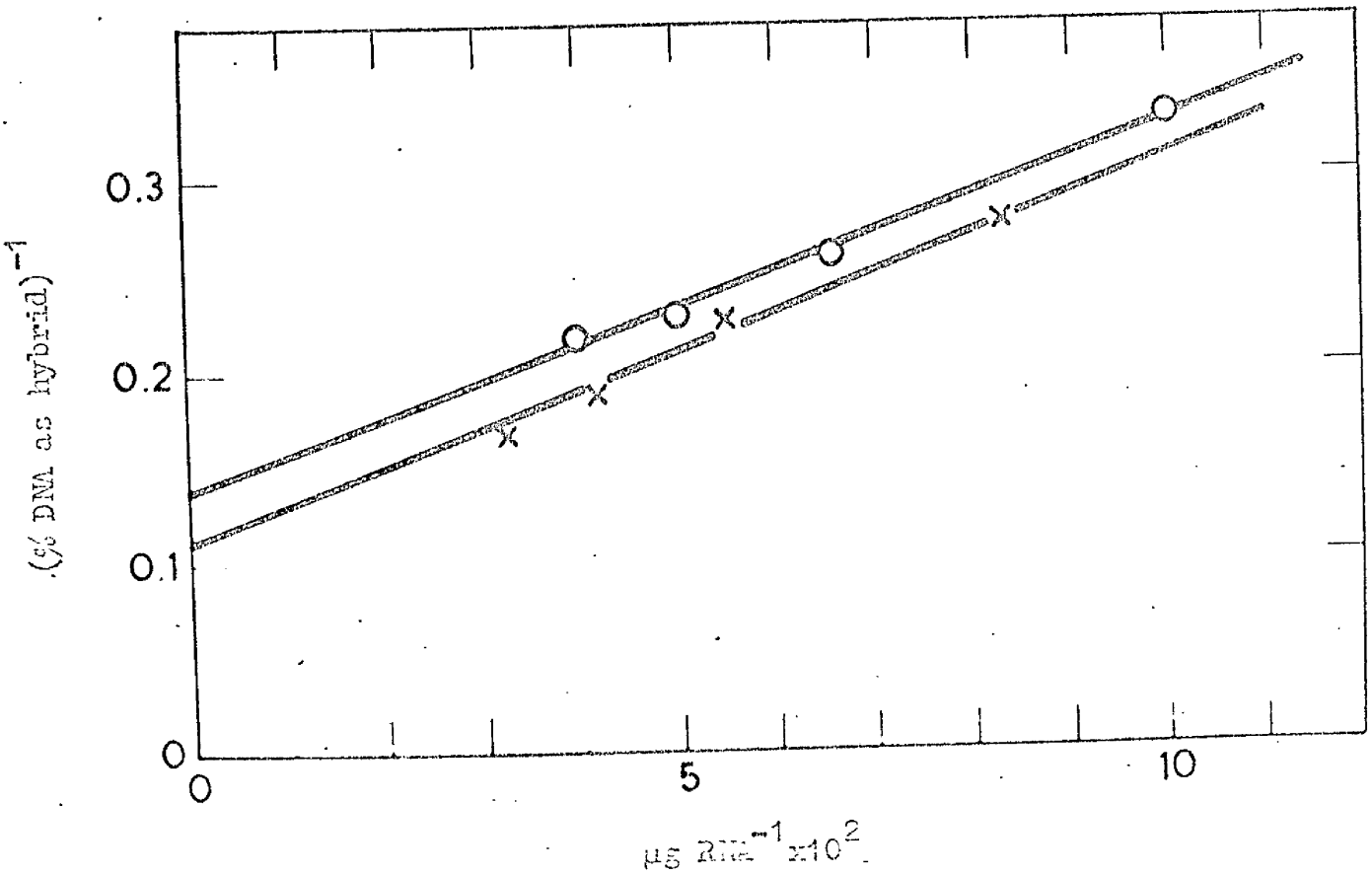


Figure 28



5.2 Comparisons of RNA populations after folic acid administration

Qualitative changes in the kinds of RNA present in normal kidney and during the folate period were examined by competition experiments between labelled and unlabelled RNA isolated at specific times. This allowed the identification of characteristic species of RNA present only at specific times. The curves at figure 29 and 31 illustrate these comparisons.

The competition curves presented at figure 29 show the reaction of ^{3}H-labelled RNAs from normal and different stages, in competition with increasing amounts of unlabelled competitor RNA from untreated tissue. As it is illustrated a small part of the population of molecules from normal RNA is also present in all stages of folate treated kidneys. On the other hand, the normal natural RNA does not contain representatives of all the types of RNA molecules synthesised in the treated animal.

Figure 31 shows the competitive activity of 3 hour cold RNA after folate with synthetic ^{3}H-RNA from normal and treated kidneys. The homologous RNA displaced most effectively while the RNA s from later stages are partially displaced. RNA made by six hours or later shows decreasing competition

Figure 29

Competition by increasing amounts of nuclear unlabelled RNA from untreated tissue in the hybridization reaction of approx. 30µg of ^{3}H-RNA made *in vitro* by mouse kidney chromatin isolated at specific times after folic acid administration.

The labelled RNAs are symbolised as follows:

Untreated kidney, ●——● ; three hours, X——X ; six hours, ▽——▽ ; twelve hours, ○——○ ; twenty four hours ▲——▲ ; forty eight hours, ●——● .

Figure 29

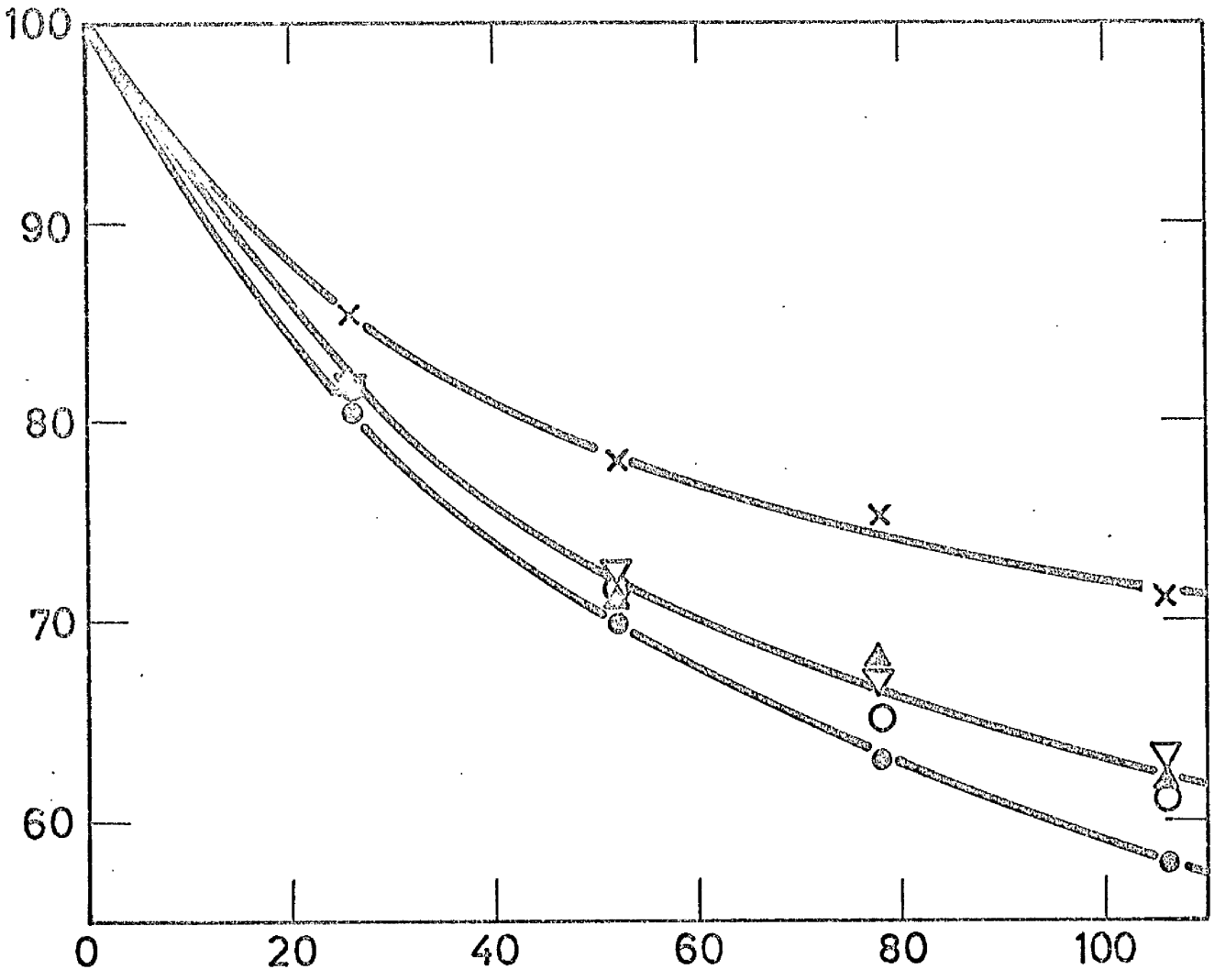
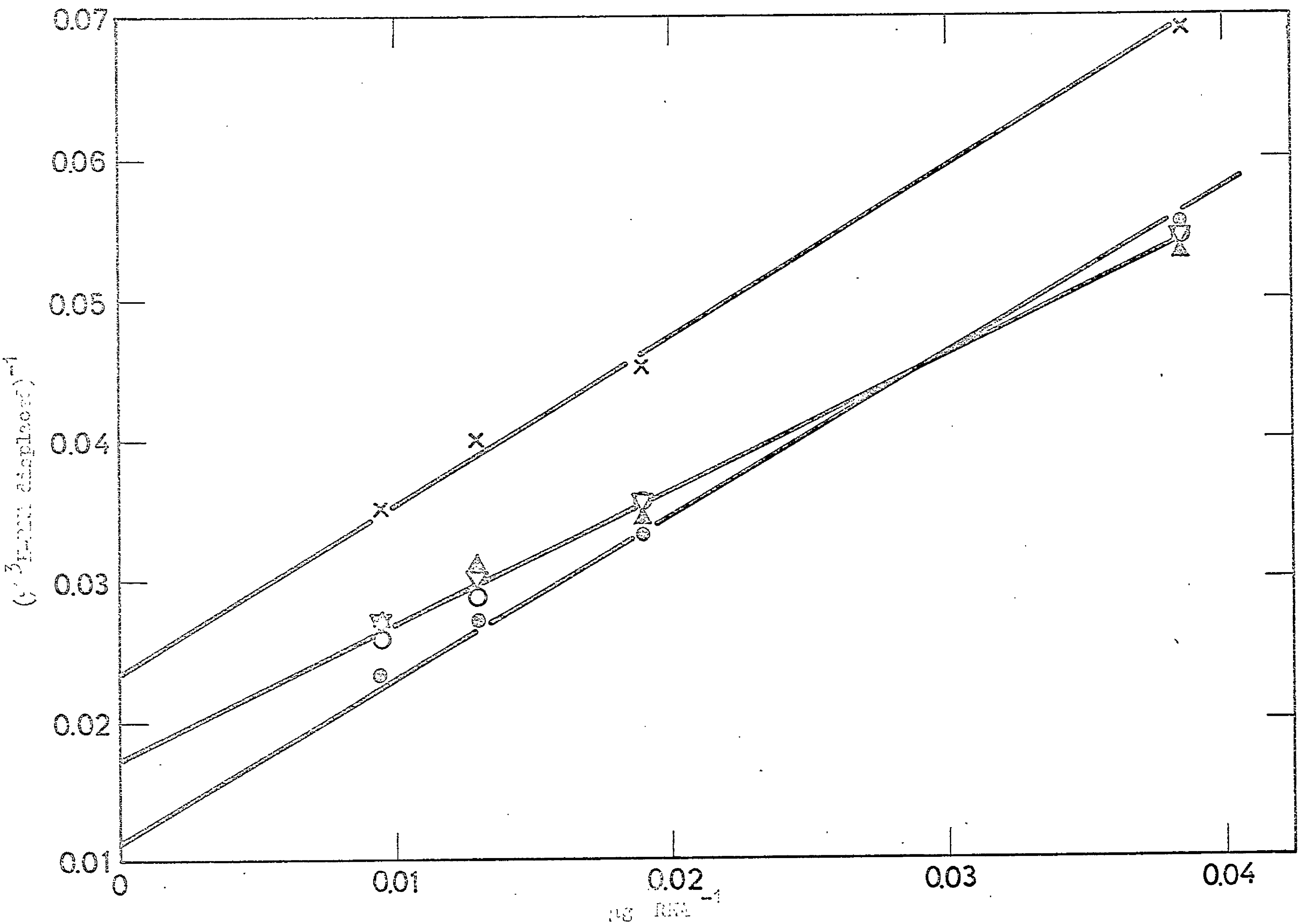


Figure 30

Double reciprocal plots of the data shown in figure 29. By extrapolation to the ordinate it can be predicted that at infinite concentration of natural RNA isolated from untreated tissue, the percentage of the homologous labelled RNA remaining as hybrid is 1%, of the three hours RNA 56% remains as hybrid and from all the other times 41%.


0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0.01
0.02
0.03
0.04

efficiency. This displacement is minimised by the 48-hour treated kidney which coincides with the control. RNA from various times after the administration becomes progressively less similar to the RNA synthesised by 3 hours. These results indicate that a significant proportion of the *in vitro* RNA transcribed during the early stages has disappeared by 48 hours.

5.3 Relation between RNA synthesis in the in vivo and in vitro system

Experiments using RNA extracted from kidney nuclei after folate treatment at the same specific times as the *in vitro* ones were performed by Kohl & Threlfall (in preparation). The nuclear RNAs were made radioactive by chemical introduction of ^{3}H-methyl groups and the formamide hybridization technique was applied. These RNAs were intercompared in order to establish the temporal relation of the appearance of new kinds of RNA molecules at various times. RNA saturation curves were obtained and the proportion of the genome synthesising RNA at a given time was established. Similar pattern of saturation values was obtained as the one *in vitro*; the only difference was that the maximum hybridization efficiency (of about 15%) was displayed by the 6 hour isolated

Figure 31

Competition by increasing amounts of nuclear unlabelled RNA isolated after 3 hours of folate treatment in the hybridisation reaction of 28 - 30µg of ^{3}H-RNA made *in vitro* by mouse kidney chromatin isolated at specific times after folic acid administration.

The labelled RNAs as symbolised as follows:

Untreated kidney, X——X ; three hour, ●——● ; six hour, ○——○ ; twelve hour, ▽——▽ ; twenty four hour, □——□ ; forty eight hour, ▲——▲ .

Figure 31

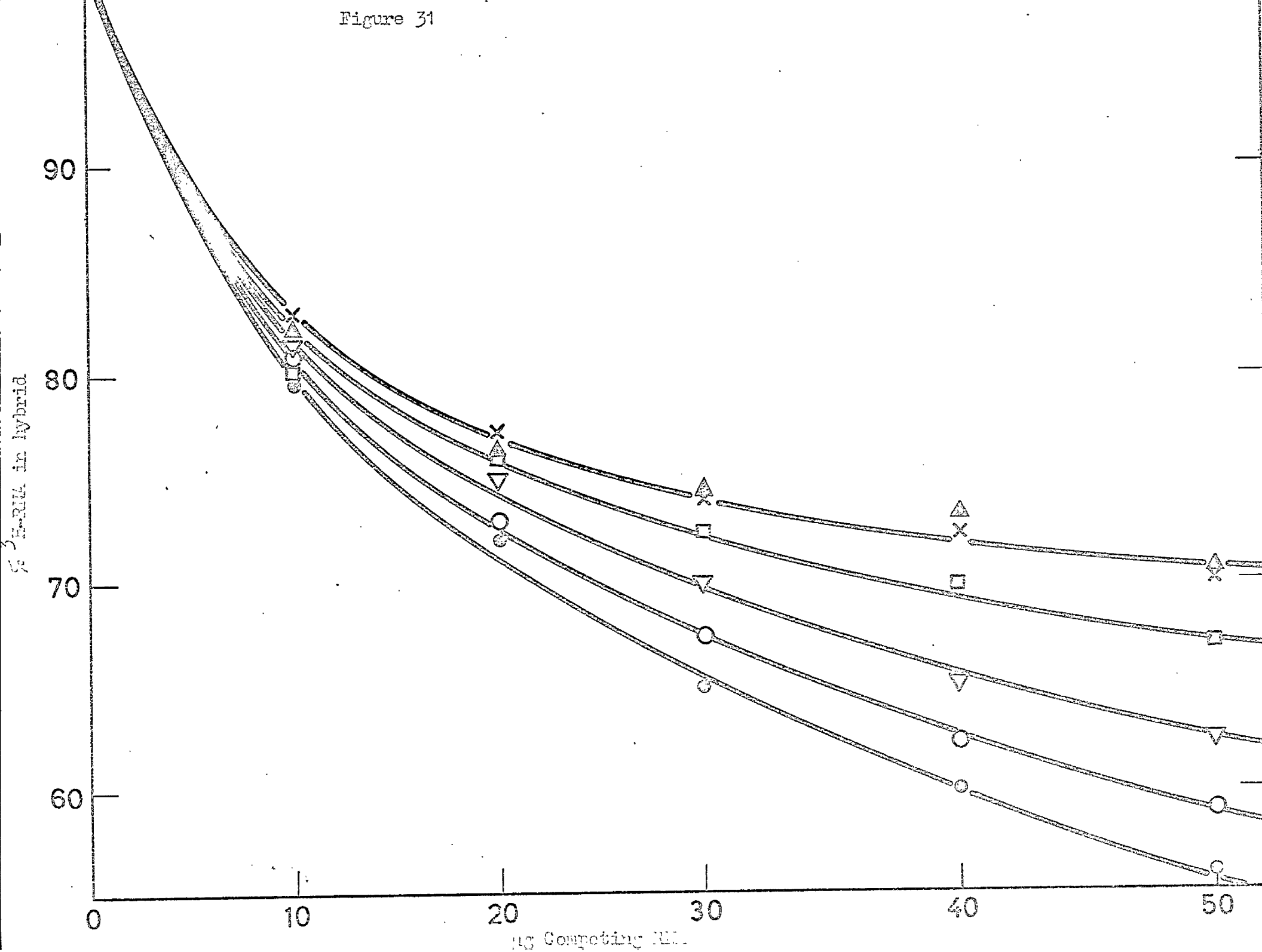


Figure 32

Conditions as described for figure 31.

Untreated kidney, ●——● ; three hour, x——x ; twelve hour, ▽——▽ .

Figure 32

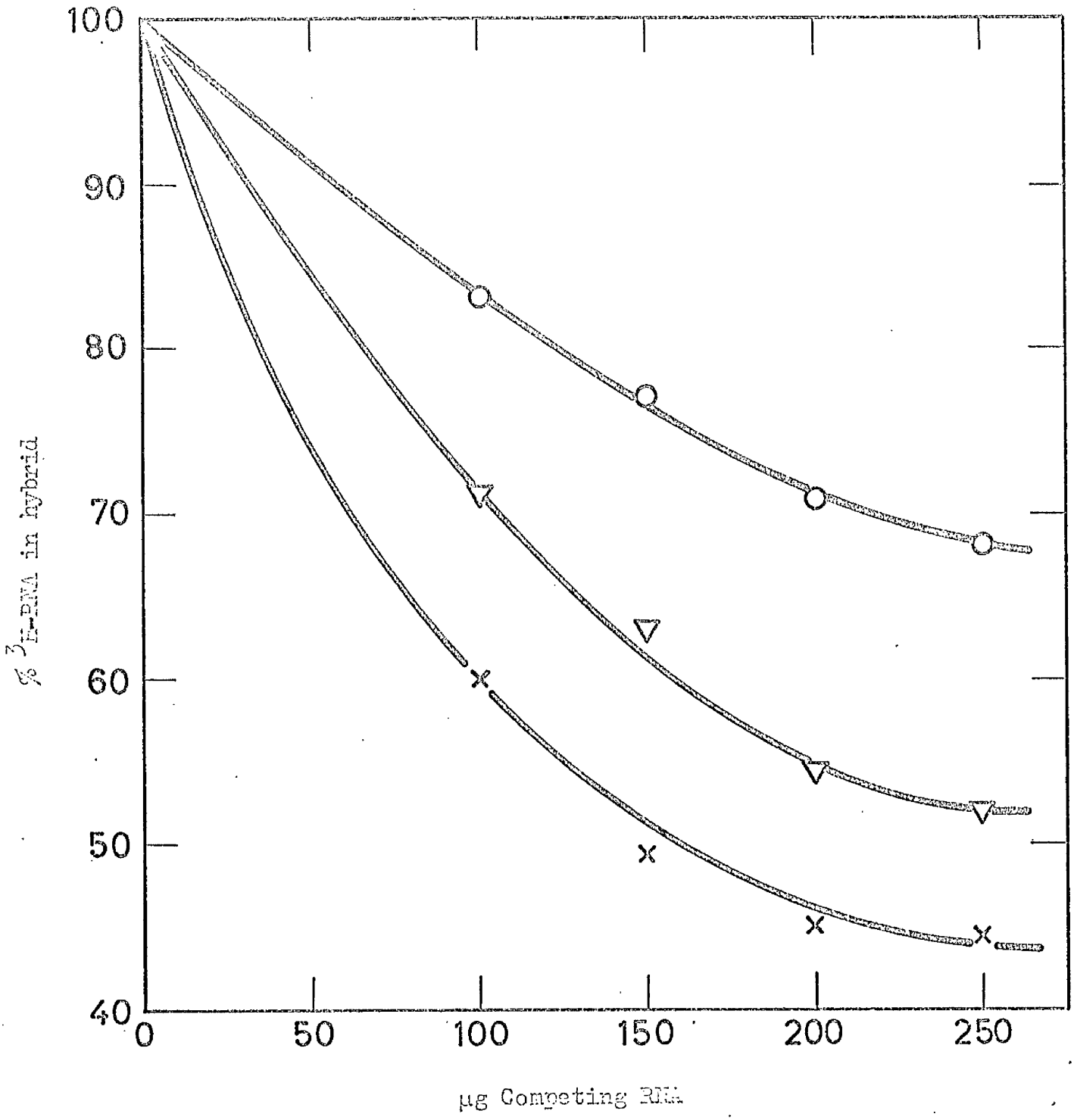
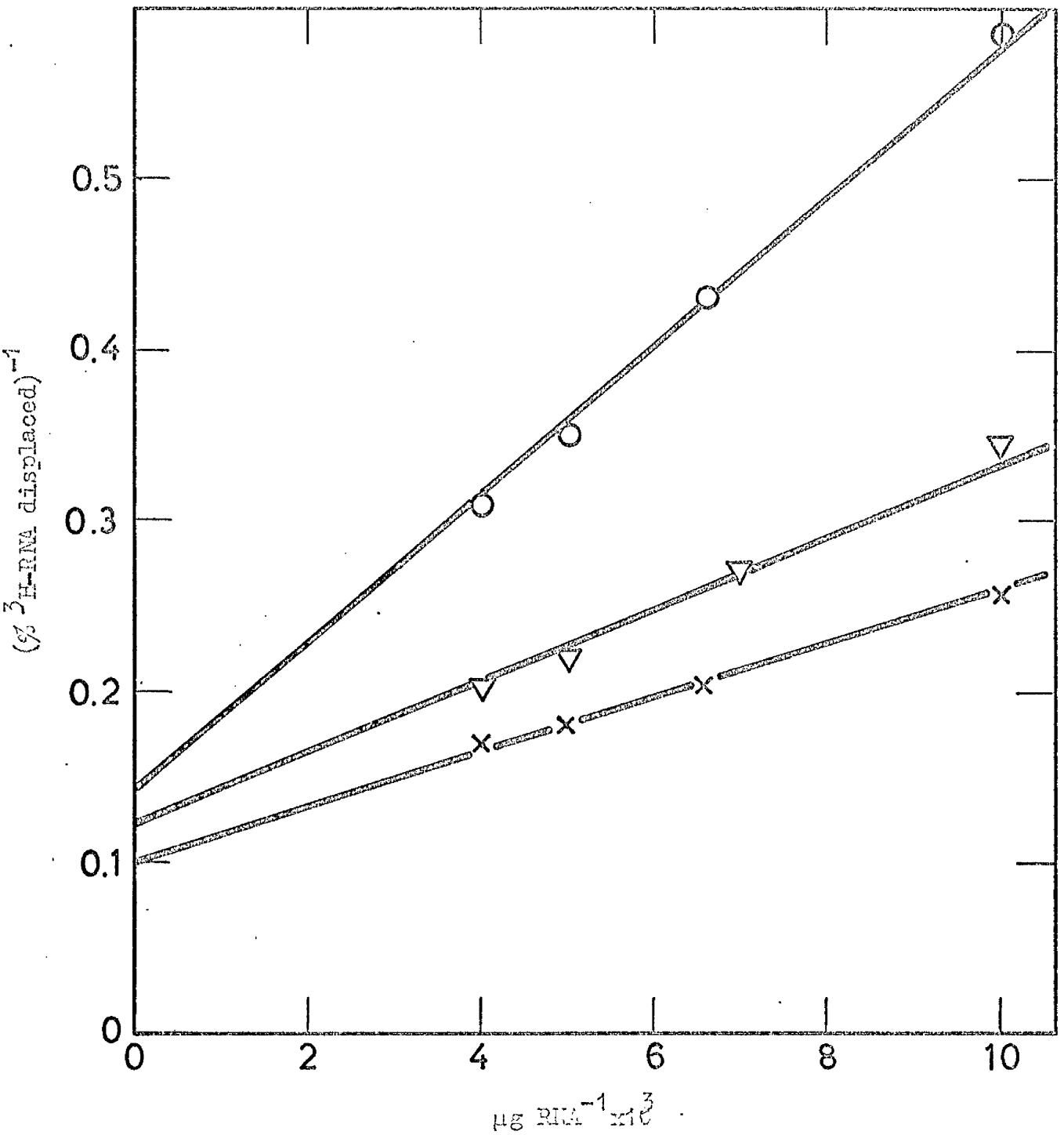

100
90
80
70
60
50
40
0
50
100
150
200
250
% ^{3}H-RNA in hybrid
µg Competing RNA

Figure 33

Double reciprocal plots of the data shown in figure 32. By extrapolation to the ordinate it can be predicted that at infinite concentration of natural RNA isolated after three hours of folate the percentage remaining of the homologous labelled RNA as hybrid is 0%, of the twelve hours RNA 17% remains as hybrid and of the three hours RNA 30%.

Figure 33



nuclear RNA while the same percentage was found to occur at the 3 hour preparation in the *in vitro* system.

One possible explanation for this discrepancy observed between the two systems may be that, in the *in vitro* system the part of the genome that is temporarily unmasked is transcribed by the excess of exogenuously added polymerase at a higher rate than by the corresponding engodenous enzyme alone, given that the tissue is not in the metabolic state characteristic of the adult kidney. As a result at 3 hours after folate administration the *in vivo* system shows a minor divergence suggesting that some of the RNA transcripts are still absent or are not present in such abundance at this stage of regenerating kidney. The accumulation of all the new kinds of RNA in the *in vivo* system characteristic for this stage would be manifested within a longer time period.

If this can be considered to be the case then the kinds of RNA molecules produced by the 3 hour cell free system must be more similar to the types of RNA molecules present at the 6 hour *in vivo* system than with the 3 hours one.

In order to establish the validity of this explanation the following experiment was performed.

^{3}H-RNA synthesised *in vitro* on a chromatin template isolated at 3 hours after folic acid treatment was competed out with increasing amounts of cold RNAs isolated from kidney nuclei at 0, 3, 6 and 24 hours after folate administration. It was found that the RNA isolated from kidney nuclei after 6 hours was the most efficient competitor for sites of the DNA in competition with the 3 hour *in vitro* synthesised RNA. (Figure 34).

The evidence provided by this competition experiment leads to the conclusion that a lag time exists between the appearance of the same kinds of RNA in the *in vitro* and *in vivo* system.

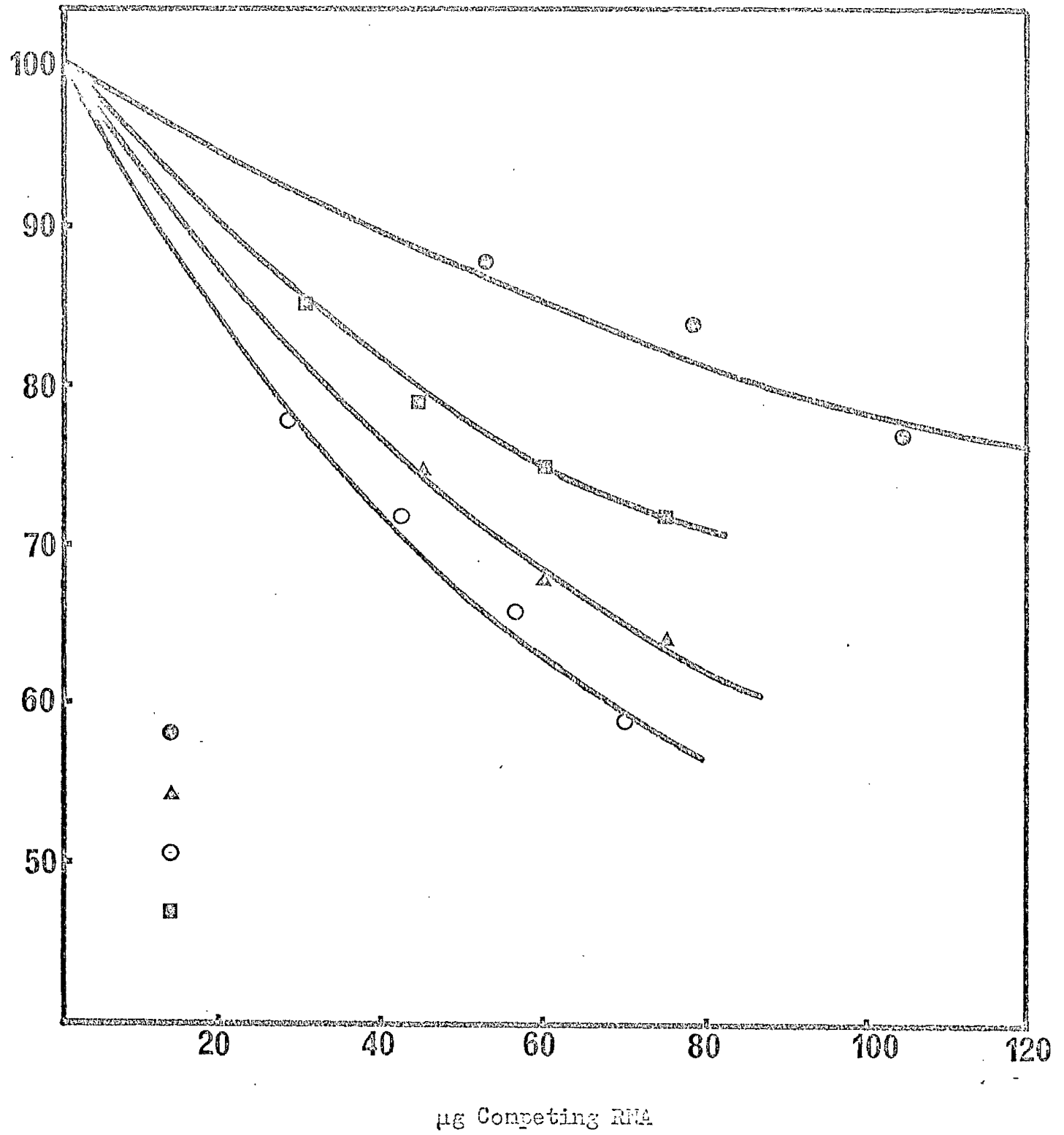
5.4 The effect of the presence of folic acid during the isolation of nuclei

Folic acid is precipitated along with nuclei isolated by the citric acid method. The possibility that it might have a direct effect on transcription from chromatin was therefore investigated. Three and six hours after folate administration the kidneys were removed and nuclei were prepared by the citric acid and Chauveau sucrose method. The second method yielded nuclei free of folic acid contamination. From both nuclei preparations chromatin was isolated

Figure 34

Competition of unlabelled RNAs in the hybridisation reaction of ^{3}H-RNA made *in vitro* from mouse kidney chromatin isolated at 3 hours after folic acid administration. The unlabelled RNA preparations used as competitors are symbolised as follows: Untreated kidney, ●——● ; three hours, ▽——▽ ; six hours, ○——○ ; twenty four hours, □——□ .

Figure 3A


100
90
80
70
60
50
% ^{3}H-RNA in hybrid
20
40
60
80
100
120
µg Competing RNA

and used as a template in the cell free system for the preparation of labelled RNA. The RNAs were hybridized to the same amount of denatured mouse embryo DNA and the results are shown in figure 35. It is apparent that the method of isolation did not affect the template activity of the chromatin. The RNAs both displayed the same hybridization efficiency and no discrimination between the two labelled preparations could be observed. That was true for both cases, i.e. three and six hour preparation (figure 36).

These results must be considered as supporting the view that during the washing, with 0.1M tris pH 7.5; 0.15M NaCl buffer, of the nuclei isolated by citric acid the folate is dissolved and completely removed from the nuclear pellet.

5.5 The effect on the template activity of exogenously added folic acid

In order to exclude the possibility that the results obtained from the folic acid experiments were due to an artefact, the effect of folate was examined under *in vitro* conditions. An amount of folic acid equivalent to that incorporated by the tissue *in vivo* as calculated spectrophotometrically, was added in the

Figure 35

Kinetics of hybridisation to whole mouse embryo DNA of ^{3}H-RNA made in vitro using as template mouse kidney chromatin isolated at three and six hours after folate treatment. Nuclei were prepared by two methods and the chromatin was isolated.

The labelled RNAs are symbolised as follows:

Three hours citric acid method, X——X ;
three hours Chauveau's method, O——O ;
six hours citric acid method, ▽——▽ ;
six hours Chauveau's method, □——□ .

Figure 36

Double reciprocal plot of the data shown in figure 35. At saturation concentrations of RNA the same hybridisation efficiency is displayed and no discrimination between the two labelled preparations can be observed for both cases.

Figure 35

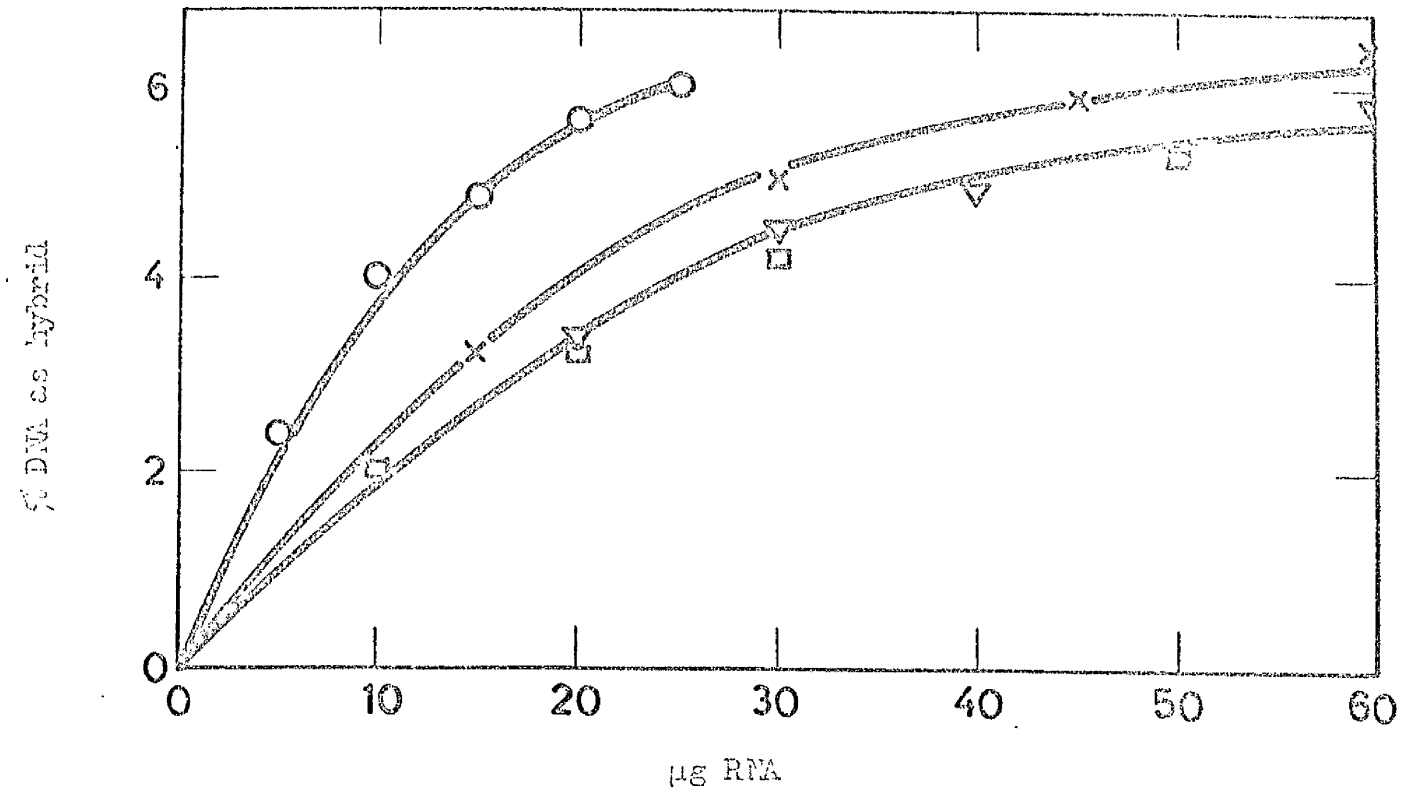


Figure 36

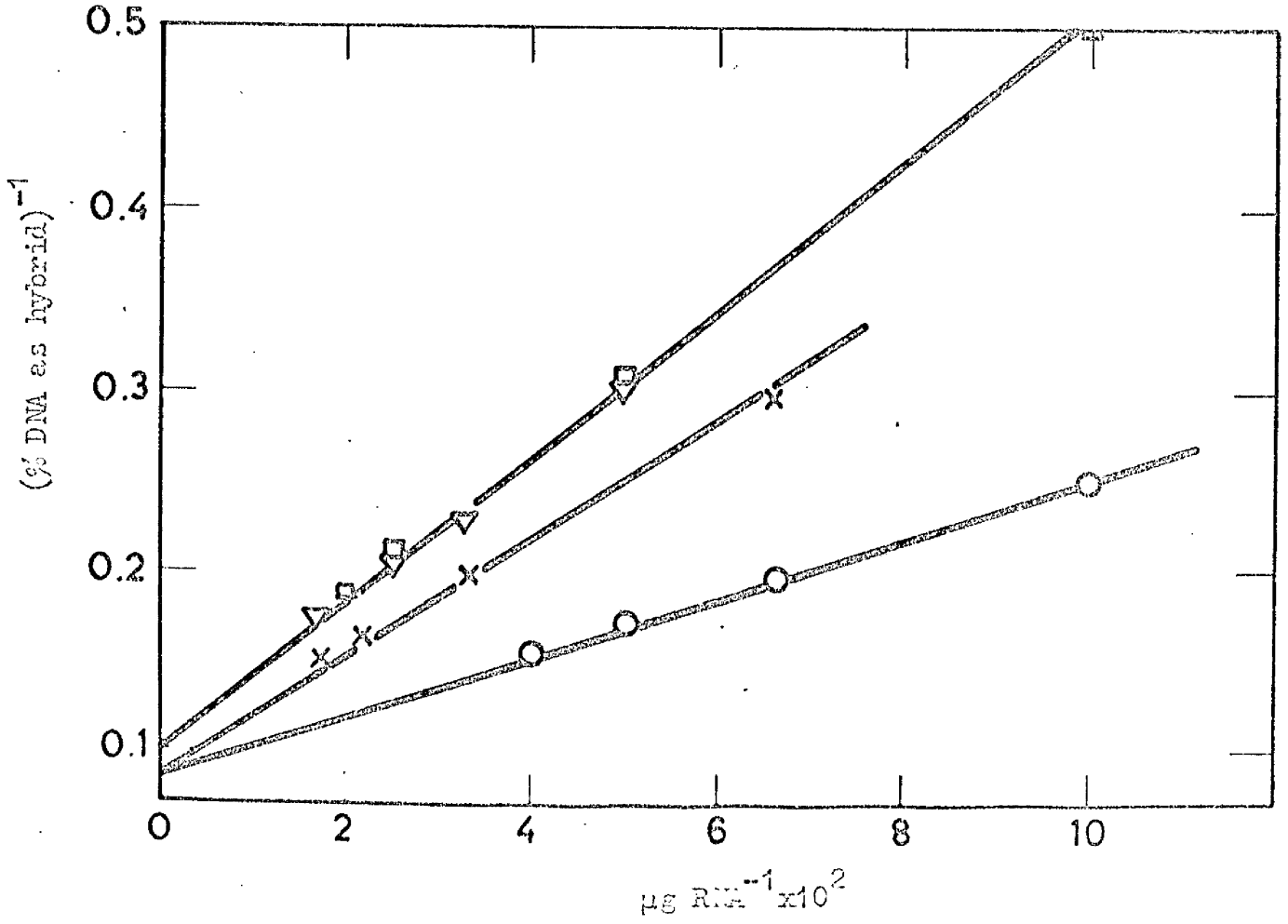


TABLE 8

ABILITY OF RNA-POLYMERASE IN INCORPORATING ^{3}H-UTP INTO RNA IN THE PRESENCE OF FOLIC ACID

Assay conditions		Percentage of the complete system
Complete system	1240	100
Complete system + 10µg folic acid	1210	97,6
Complete system + 20µg folic acid	1207	97,3
Complete system + 40µg folic acid	1217	98,1
Complete system + 80µg folic acid	1209	97,5

in vitro RNA synthesising cell free system.

It was found that whenever folate was exogenously added in the incubation mixture the RNA yielded had a lower ability for hybridization with the DNA. Two separate cases were investigated. In the first case chromatin was used as template and folic acid was added in a concentration of 0.08mg/ml of incubation mixture. In the second case DNA was used as template and an equivalent amount of folic acid was present.

It was found that in both cases the RNA product exhibited a restriction in hybridization of almost 40% (figure 37 and 39). The consequences of these findings are that the added folic acid interferes specifically either with the exogenously added RNA polymerase or with the template itself. In order to answer this question the following experiments were performed. Firstly, the ability of RNA polymerase in incorporating ^{3}H-UTP into RNA was examined in the presence of various amounts of folic acid. As it can be seen from Table 8 no apparent difference was detected between zero input of folic acid and 0.08mg/ml of incubation mixture. Secondly, an *in vitro* RNA synthesising system was set up with DNA as template and various amounts of folate

Figure 37

Kinetics of hybridization to whole mouse embryo DNA of ^{3}H-RNA made *in vitro* using as template mouse kidney chromatin ●——● ; and mouse kidney chromatin plus 0.08μg/ml folic acid. x——x

Figure 37

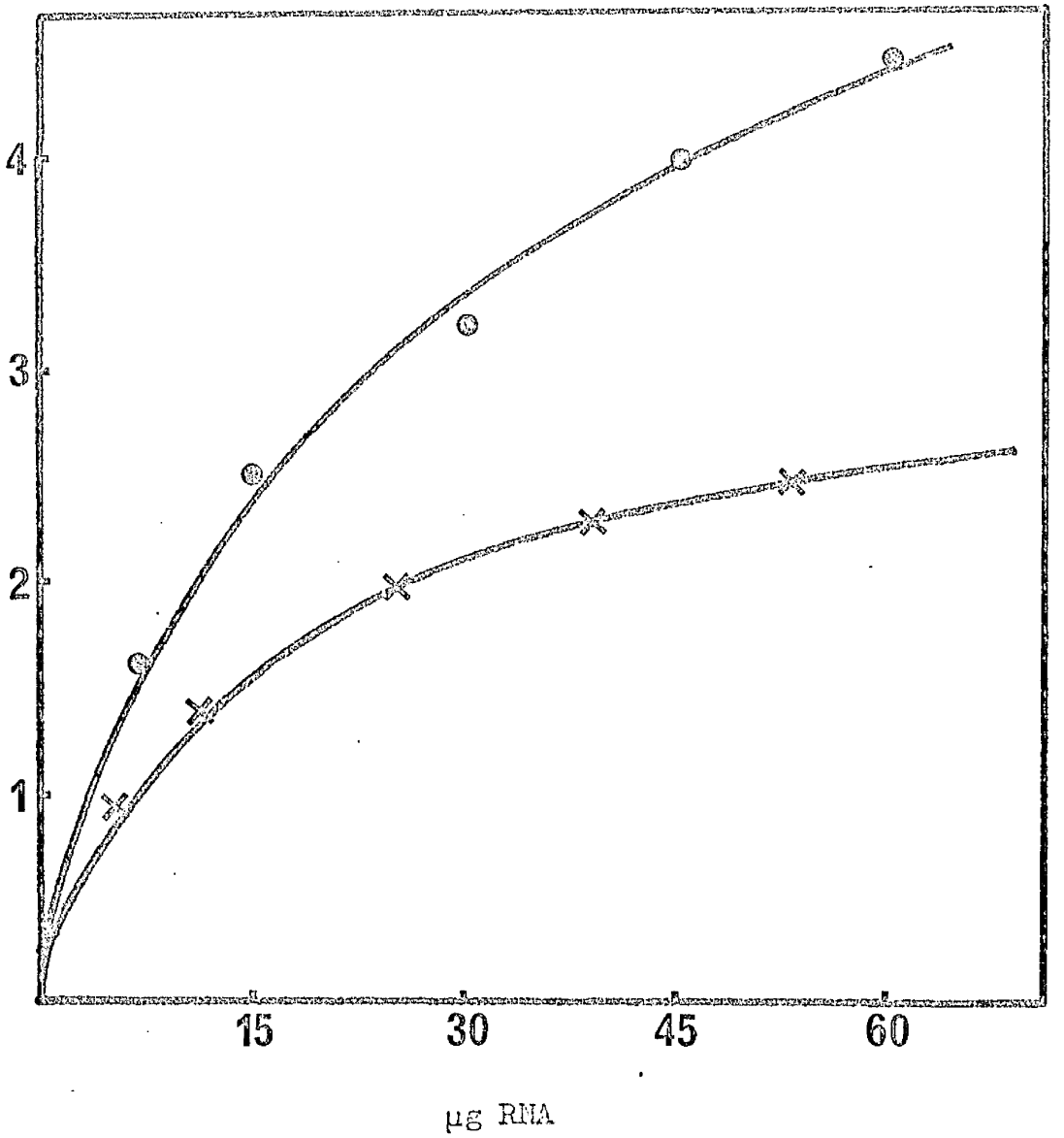


Figure 38

Double reciprocal plot of the data shown in figure 37. At saturation concentration of DNA a restriction in hybridisation of almost 40% is exhibited when folic acid is added exogenously.

Figure 38

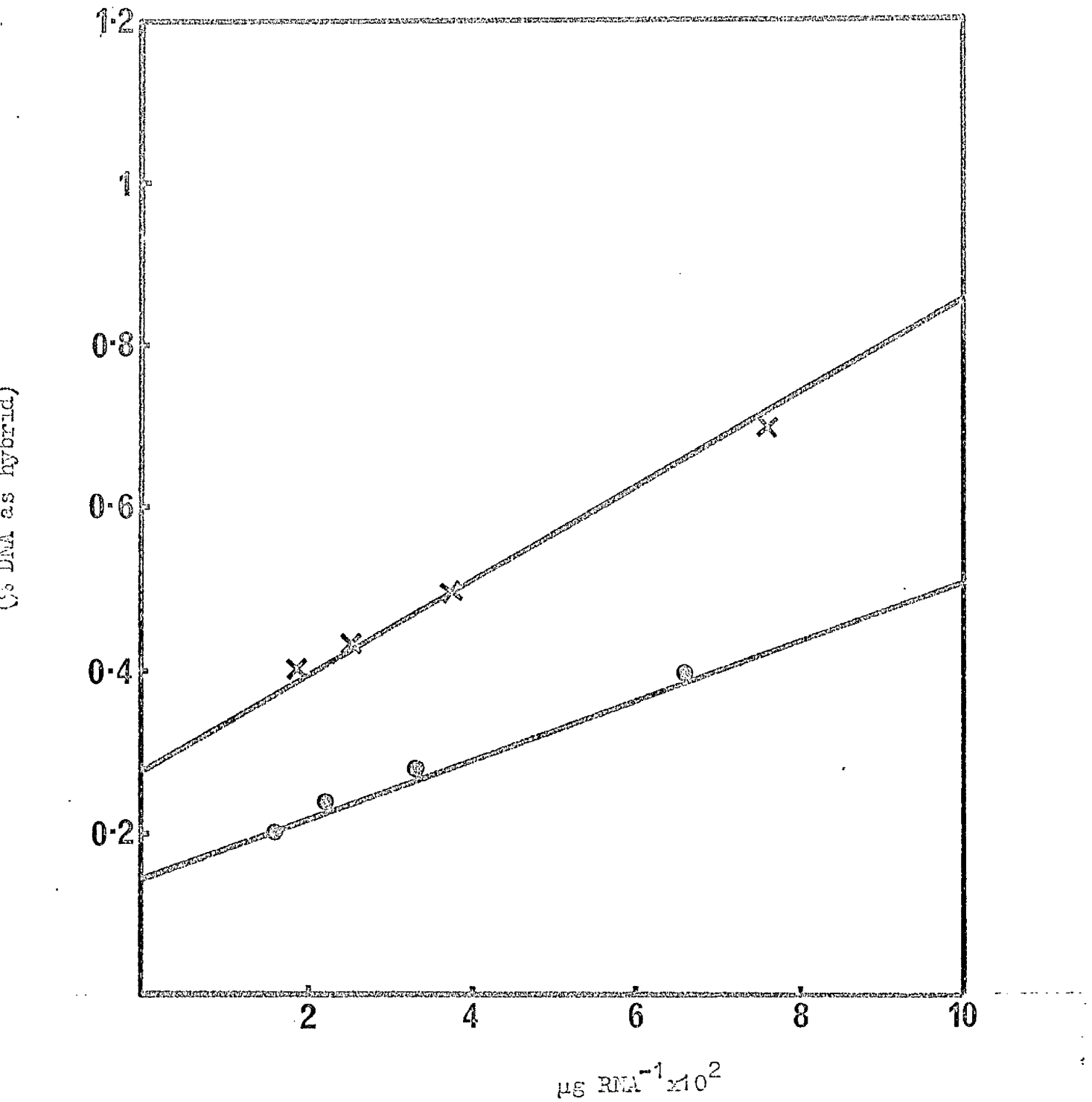


Figure 39

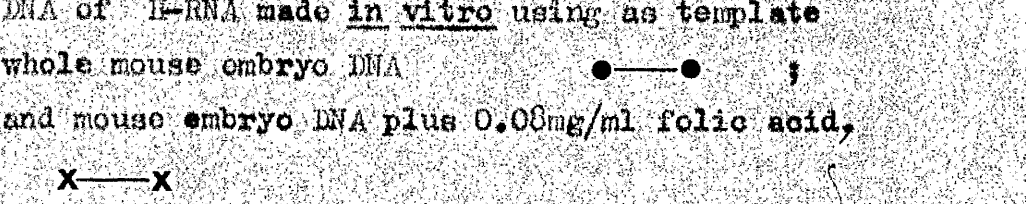

Kinetics of hybridization to mouse embryo
DNA of ^{3}H-RNA made *in vitro* using as template
whole mouse embryo DNA ●——●
and mouse embryo DNA plus 0.08mg/ml folic acid,
X——X

Figure 39

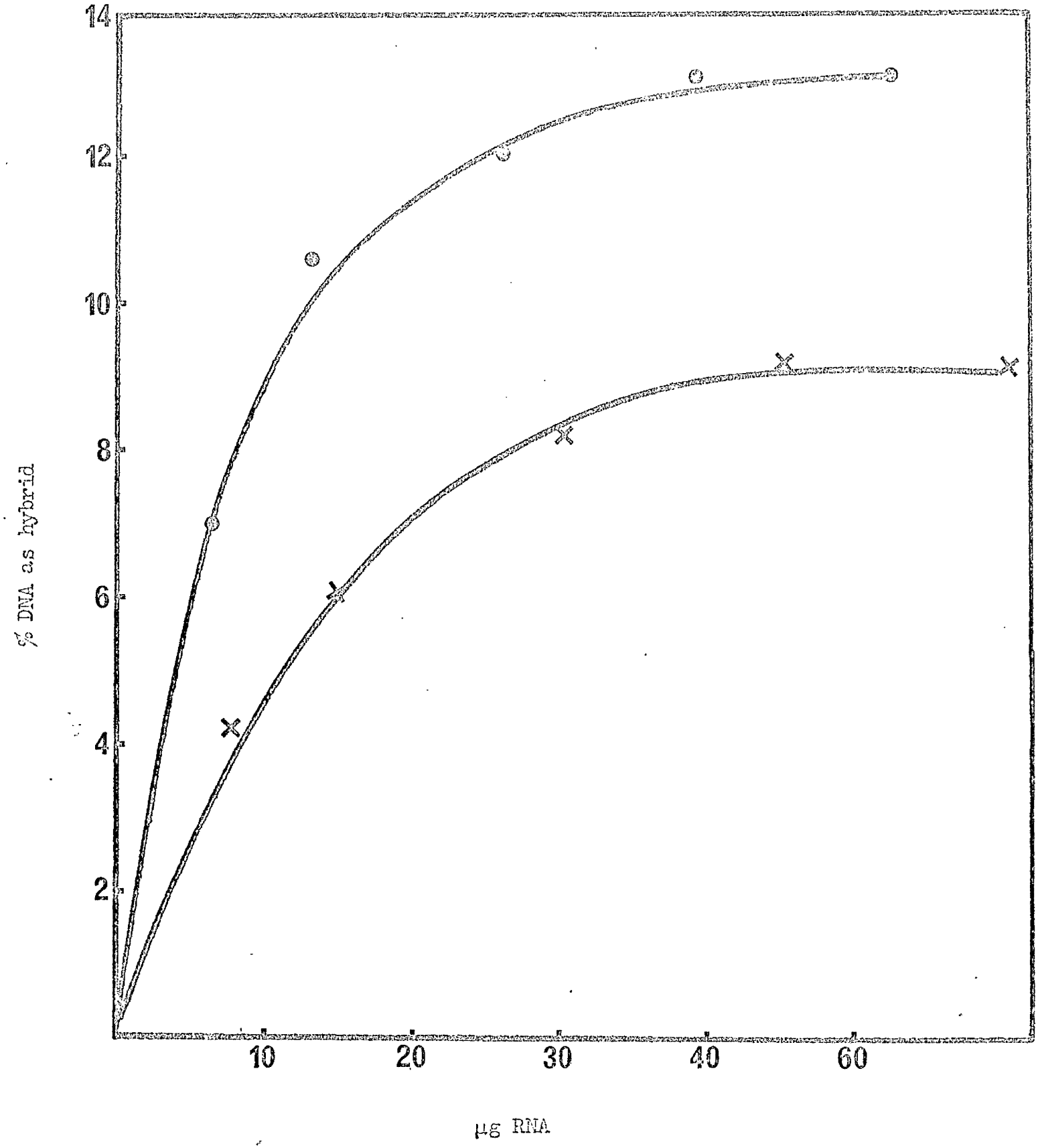
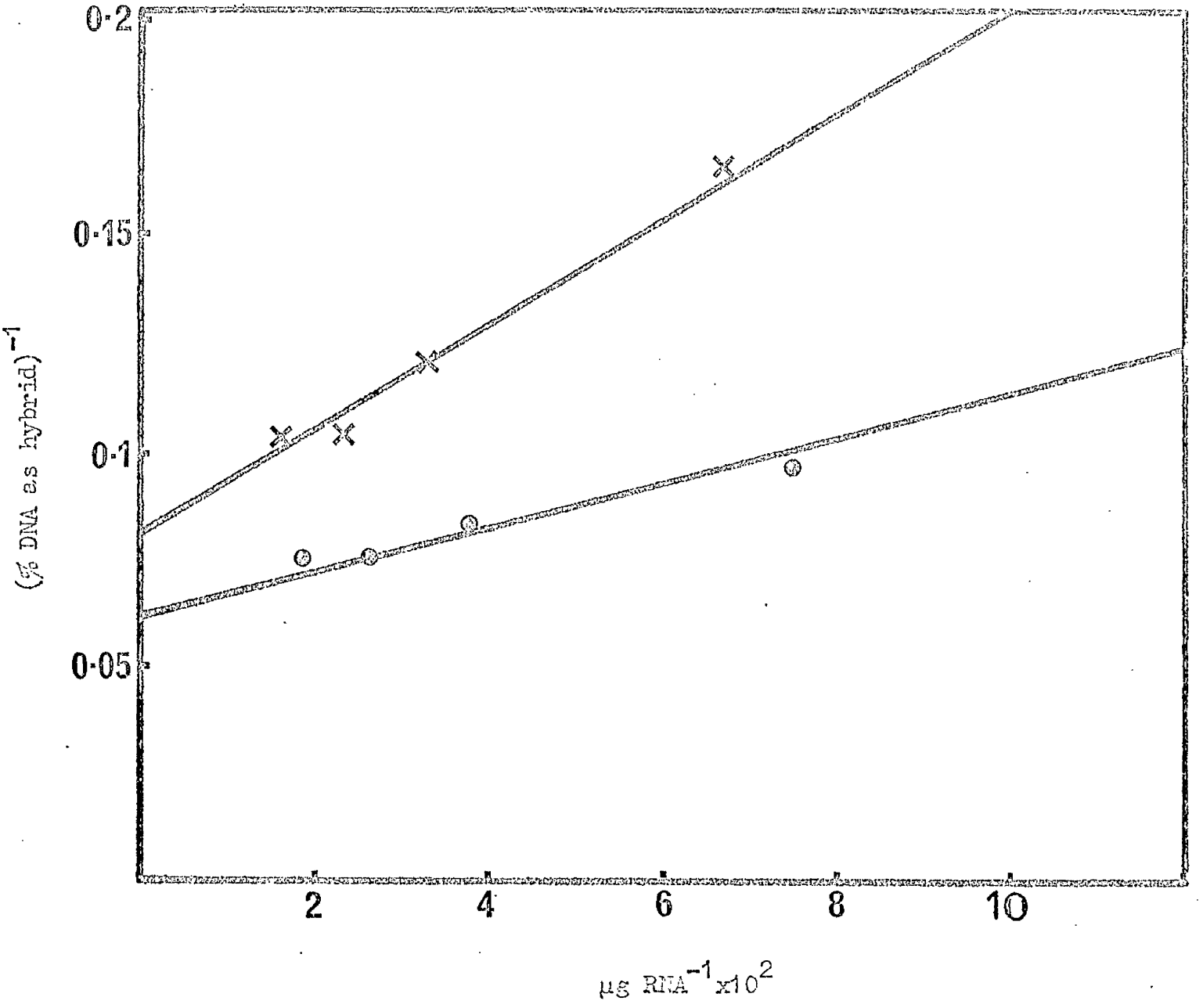


Figure 40

Double reciprocal plot of the data shown in figure 39. At saturation concentration of RNA a restriction in hybridization efficiency of almost 40% is exhibited when folic acid is added.

Figure 40



(0 - 0.08mg/ml). From figure 41 it is obvious that as the amount of added folic acid increases the *in vitro* synthesised RNA has a reduced ability for hybridization with denatured DNA. However, this restriction is not directly proportional to the folate input, a fact which suggests that the folate has a specific effect on DNA.

If that was not the case then the restriction would be directly proportional to the folic acid input; as a result a straight line would be obtained indicating that the folate has a non specific effect on the template.

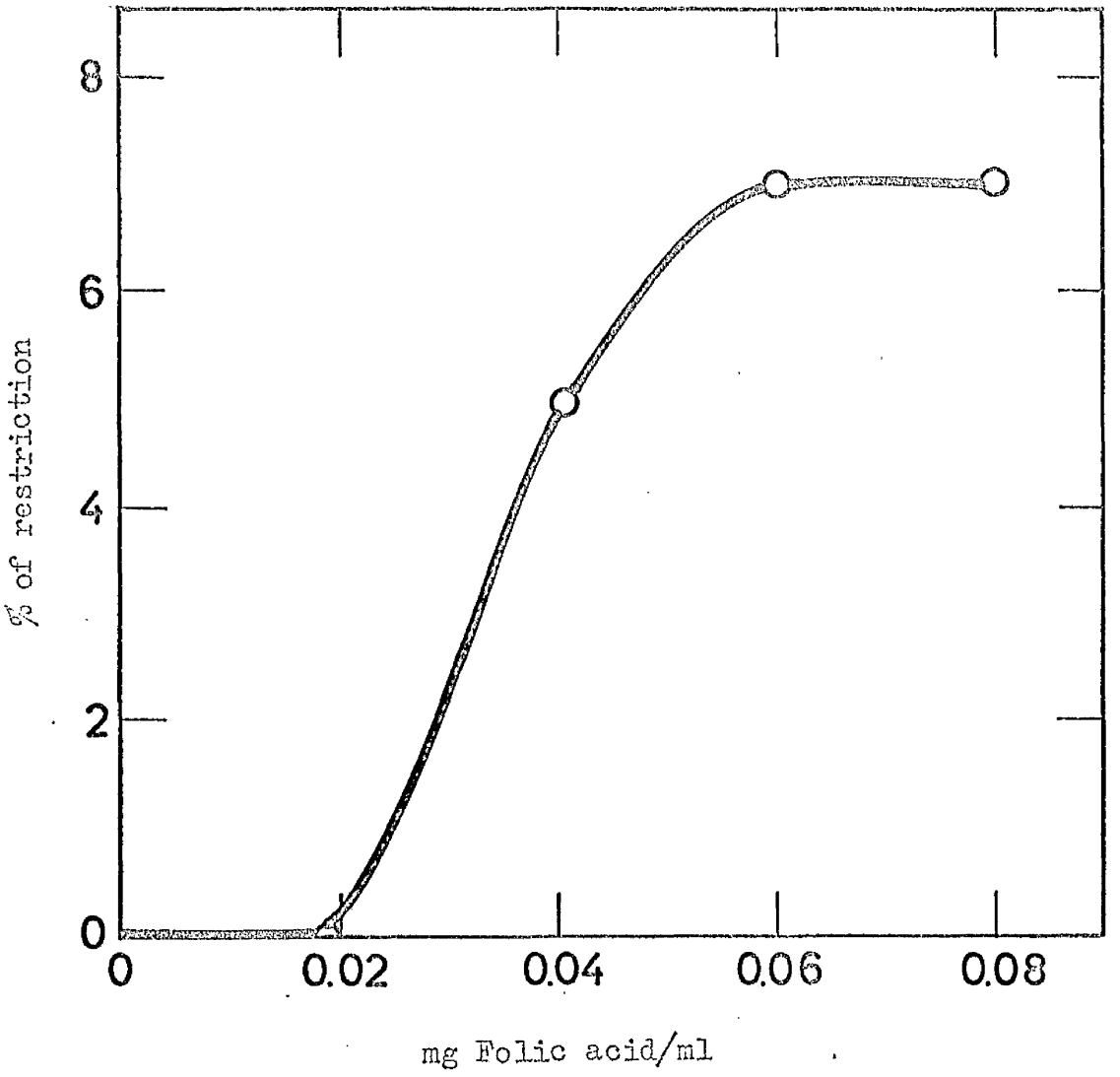
6. The effect on the template activity of chromatin isolated from mouse kidney after ligation of one ureter

The mechanisms which control cell division in mammalian organs are not understood. Various experimental models have been used to study this problem; among these has been investigation of the mitotic response in the kidney following surgical removal of the contralateral organ (compensatory hyperplasia) and investigation of the hyperplastic response to a single injection of folic acid in the animal.

Figure 41

The effect of exogenously added folic acid on the hybridization reaction of ^{3}H-RNA made *in vitro* using whole mouse embryo DNA as template.

Figure 41



Another method of stimulating cell proliferation in the kidney is to ligate one ureter. An increase in mitotic figures has been reported in the obstructed rat kidney 48 hours after ureteral ligation with a maximum of mitotic response at 72 hours (Benitez and Shaka, 1964). In this case both kidneys remained in situ. The rate of DNA synthesis in the ligated kidney reaches a maximum at 48 hours.

The purpose of the experiments reported here has been to compare the template activity of kidney chromatin isolated at various times after ligation of one ureter with those obtained after folic acid treatment in mice and to determine if the same control mechanism may operate as a result in both phenomena.

Male Porton strain mice weighing up to 30g were used. These animals were subjected to ligation of the left or right ureter. For each group four kidneys, two of which were ligated in the left and two in the right ureter, were pooled. Two kinds of controls were used. The first consisted from the nonobstructed kidneys of ligated animals; the second from kidneys of normal animals. Three, six, twelve, twenty four, thirty six and forty eight hours after the ligation the mice were sacrificed and the nuclei were isolated

from the kidneys using the citric acid method. Chromatins were prepared from nuclei and used as templates for the *in vitro* synthesis of ^{3}H-labelled RNA in the synthesising cell free system. Hybridization of the *in vitro* RNAs with 5µg of denatured whole mouse embryo DNA was carried out by the molecular hybridization technique (Gillespie and Spiegelman, 1965).

6.1 Hybridization of the various RNAs after ligation of one ureter

Direct comparisons were made between the labelled RNAs synthesised on chromatins isolated at specific times after the ligation. It was found that ligation changes the template activity of the isolated chromatin from mouse kidneys, but the change occurs several hours later than that exhibited by chromatin from folate-treated animals. As a result, RNAs from ligated kidneys displayed higher hybridization efficiency after 12 hours than RNA from normal kidney (Figure 42). The maximum was attained after 36 hours, while at the early times after ligation no response was detected (Figures 44 and 46). Numerical estimation of the actual percentage of hybridization efficiency was calculated by constructing a double reciprocal plot (Figures 43, 45 and 47).

The RNAs produced from the different chromatin preparations display considerably different hybridization efficiency which increases with the time after the ligature. This may be an indication that different kinds of nuclear RNA molecules are synthesized as a response to the ligation of one ureter.

Figure 42

Kinetics of hybridisation to mouse embryo DNA of ^{3}H-RNA made *in vitro* by mouse kidney chromatin isolated at zero hours, ●——● ; nonobstructed kidney ligated animals, X——X ; three hours, ▽——▽ ; six hours, ○——○ ; after the ligature of one uretor.
The filters were loaded with 5µg donatured whole mouse embryo DNA and the ^{3}H-RNA preparations were incubated in 4 x SSC for 17 hours at 67°C at the concentration shown.

Figure 43

Double reciprocal plots of the data shown in figure 42. The percentages of the DNA existing as hybrid when each RNA preparation saturates the DNA was predicted from the intercept on the ordinate as follows:

zero hours, 7.0%, nonobstructed kidney, 7.0%, three hours, 7.0% and six hours, 7.8%.

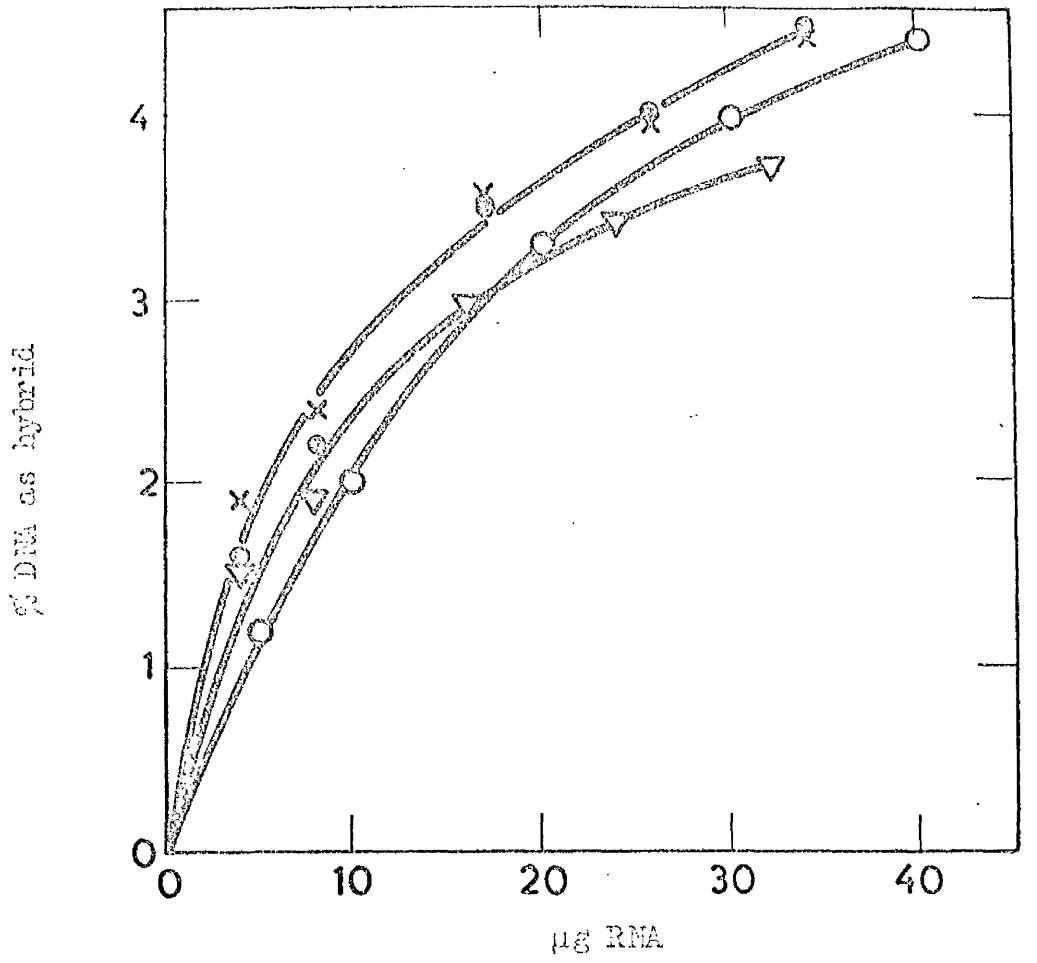

% DNA as hybrid
0
1
2
3
4
0
10
20
30
40
µg RNA

Figure 43

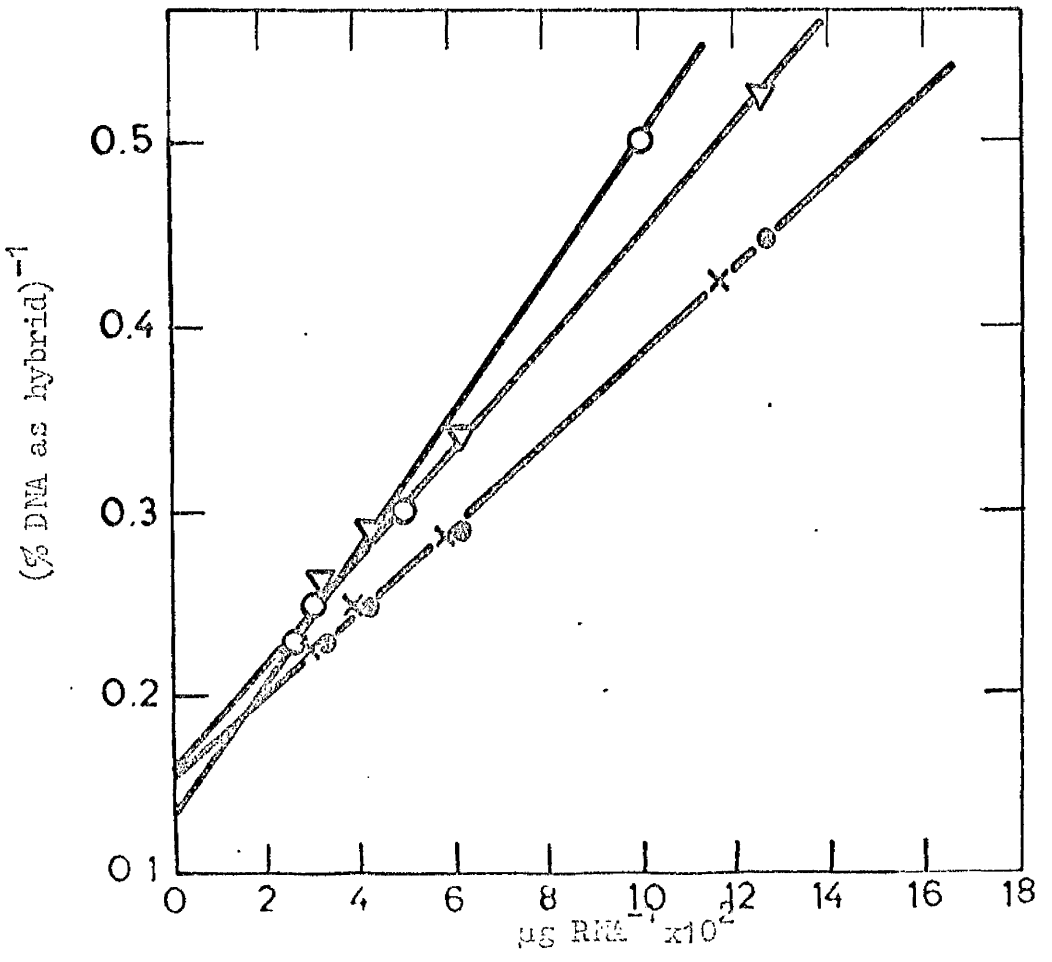

(% DNA as hybrid)$^{-1}$
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
12
14
16
18
µg RNA x10^{-2}

Figure 44

Kinetics of hybridisation to mouse embryo DNA of ^{3}H-RNA made *in vitro* by mouse kidney chromatin isolated at zero hours, o——o ; twelve hours, ×——× ; twenty four hours, ▽——▽ ; after the ligature of one ureter. Experimental conditions as described for figure 42.

Figure 45

Double reciprocal plots of the data shown in figure 44. The percentages of the DNA existing as hybrid when each RNA preparation saturates the DNA was predicted from the intercept of the ordinate as follows: zero hours, 7%, twelve hours, 9.0% twenty-four hours, 11.0%.

Figure 44

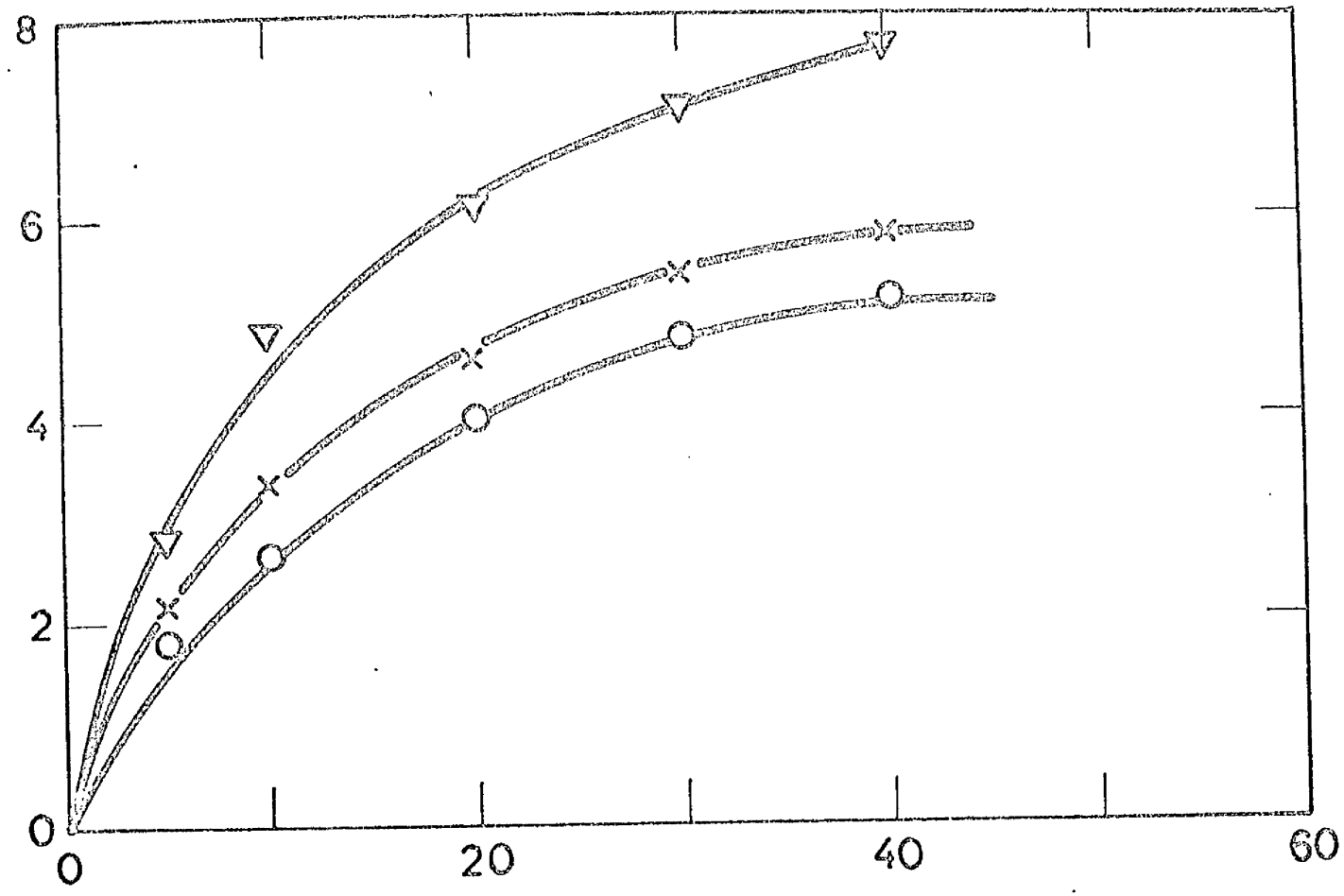


Figure 45

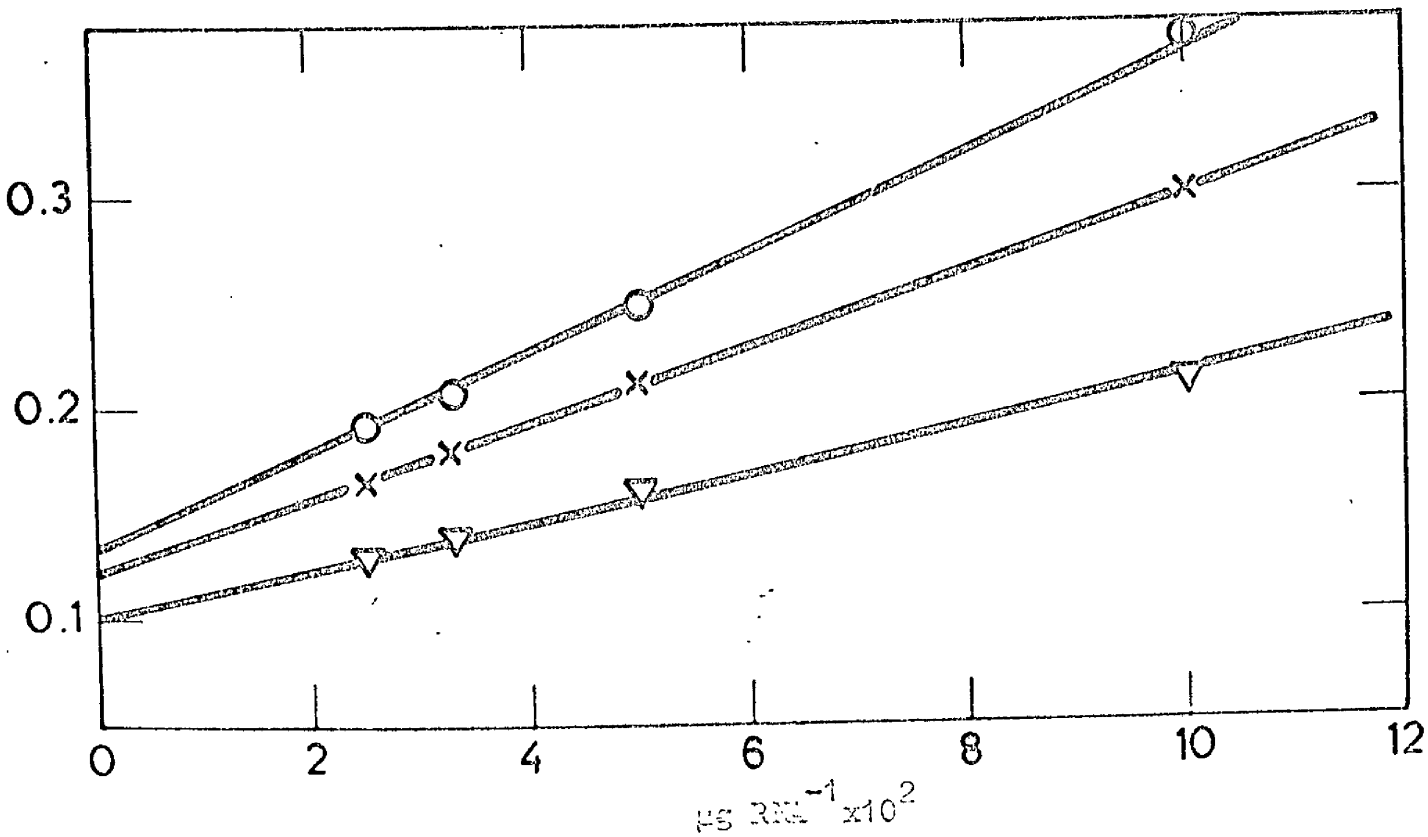


Figure 46

Kinetics of hybridization to mouse embryo DNA of ^{3}H-RNA made *in vitro* by mouse kidney chromatin isolated at zero hours, ●——● ; thirty six hours, ▲——▲ ; forty eight hours, □——□ .

Experimental conditions as described for figure 44.

Figure 46

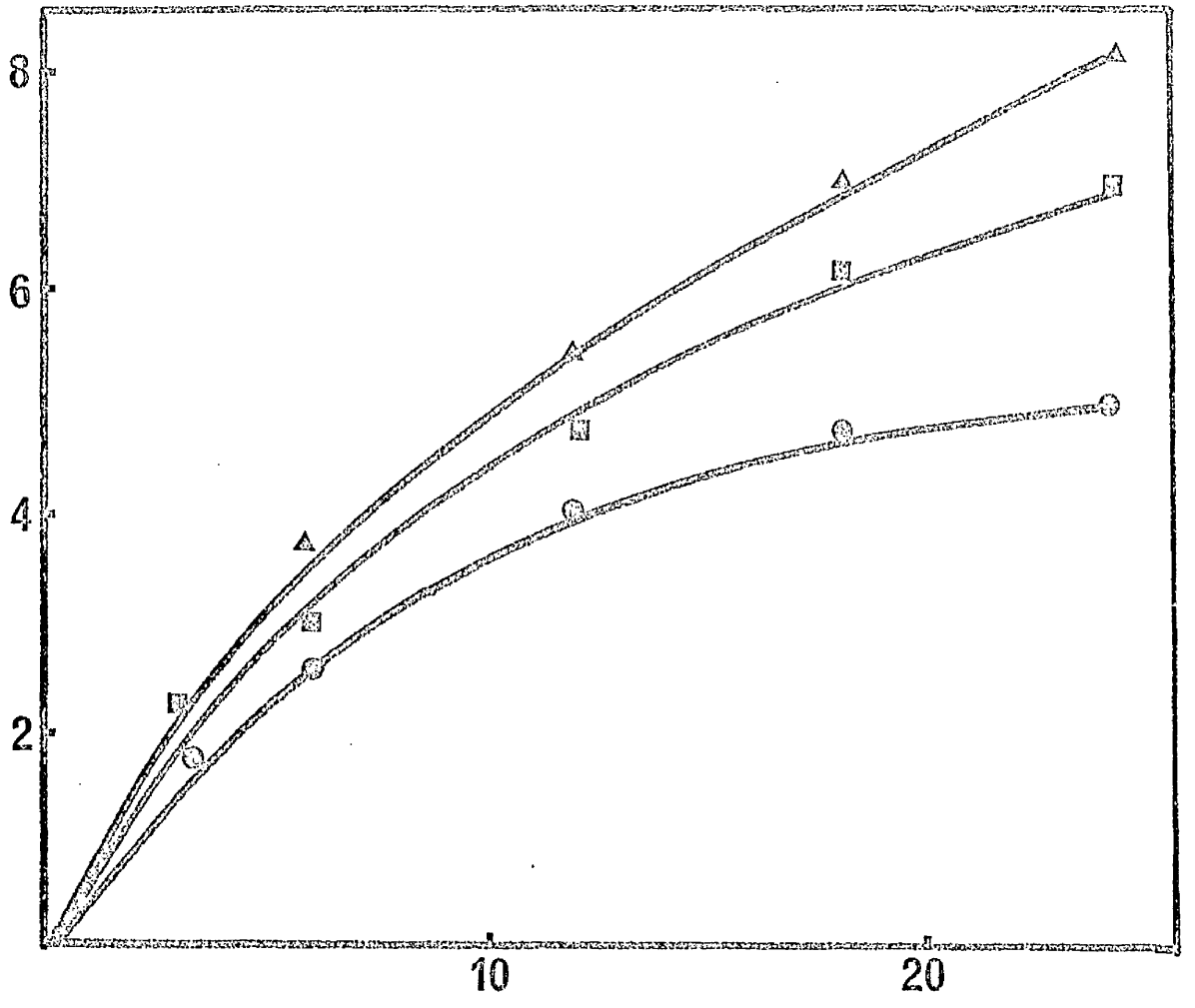
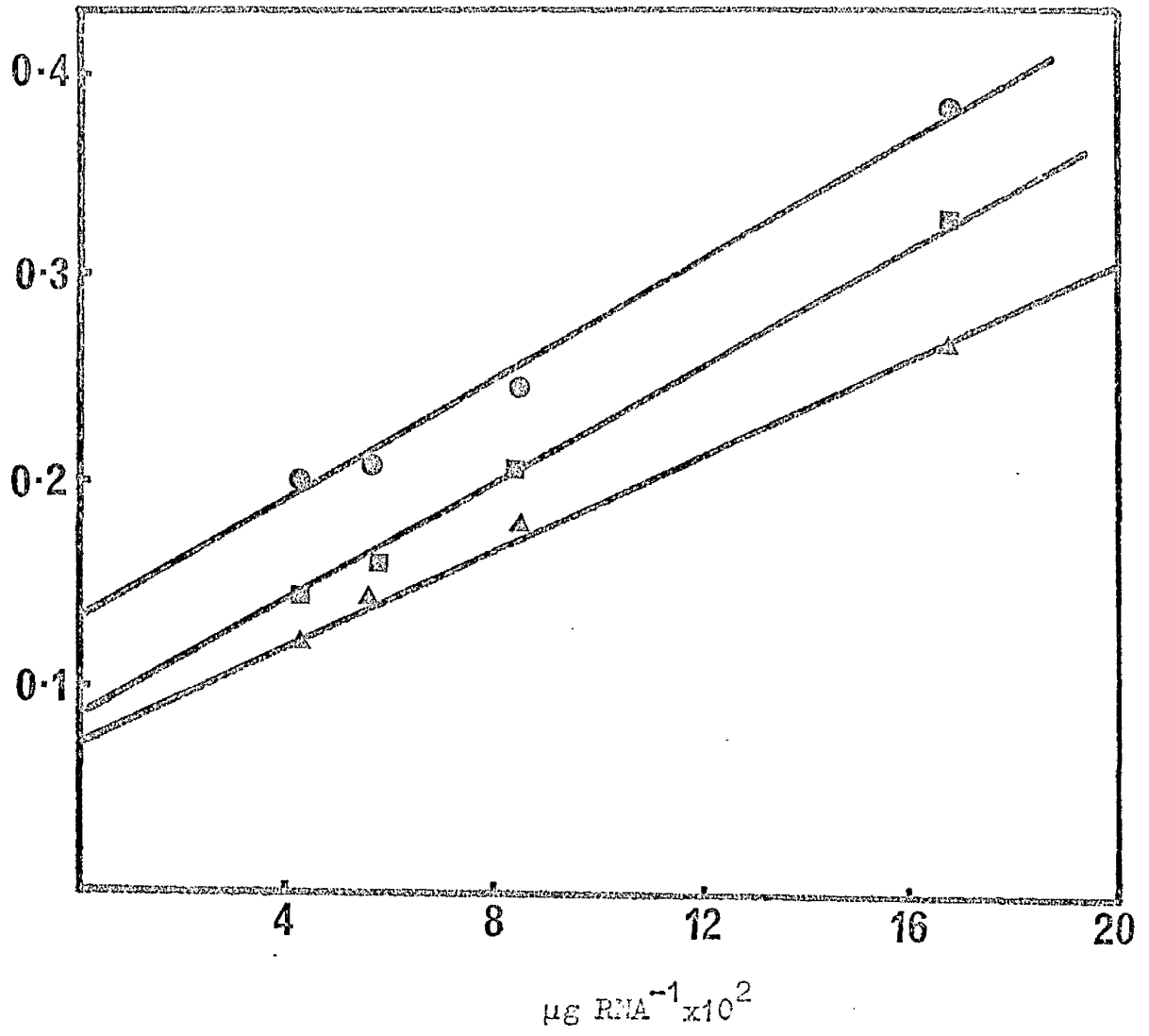


Figure 47

Double reciprocal plots of the data shown in figure 46. The percentage of the DNA existing as hybrid when each RNA preparation saturates the DNA was predicted from the intercept of the ordinate as follows:

zero hours, 7.65%, thirty six hours, 14.3%, forty eight hours, 12.4%.

Figure 47



DISCUSSION

1. Proposed mechanisms for achieving the differentiated state

2. DNA reiteration

3. Hybridization

3.1 Principles of thermal reassociation
3.2 Conditions for thermal reassociation
3.3 Rate of reassociation of DNA

4. Interpretation of DNA/RNA hybridization studies with mammalian nucleic acids

4.1 Saturation of DNA with RNA

5. Evidence for restriction of DNA template activity in animal cells

6. The effect of folic acid administration on the template activity of kidney chromatin

7. Changes in the template activity of kidney chromatin after ligation of one ureter

1. Proposed mechanisms for achieving the differentiated state

There is abundant evidence that most of the DNA in chromatin of somatic cells is not ordinarily transcribed (Paul and Gilmour, 1966 b; Marushige and Bonner, 1966; Marushige and Ozaki, 1967) and that the fraction that is transcribed differs from one cell type to another. The establishment of this concept depended on evidence indicating that the cells of an organism generally contain identical genomes (McCarthy and Hoyer 1964; Gurdon 1962). What then is the molecular mechanism of masking and how is the process by which the correct genes are selected to be inhibited (and/or activated)? At this point it is necessary to consider the question of how the functional state of the genome in the embryonic cell is achieved.

Three possible mechanisms will be discussed. First, the active portion of the DNA may be the result of selective derepression of the genome, the rest having

been wholly in a latent state i.e. cytodifferentiation proceeds by selective derepression of genes. Second, it represents the cistrons having remained active, the rest of them having been repressed during development i.e. cytodifferentiation proceeds by selective repression of genes. Thirdly, a combination of the two possibilities may exist according to which differentiation proceeds by selective derepression and repression of certain genes respectively.

Evidence for derepression (otherwise defined as activation) of specific genes is found in the work of McCarthy and Hoyer (1964) and Denis (1967). They compared the DNA s extracted from several tissues of the mouse and of Xenopus respectively and could not detect any difference in the nucleotide sequences that make up the various DNAs tested. If DNA is really in exactly the same state in all cells of the organism, the genetic information could be selected in the following way: only the region of the genome to be expressed would be accessible to the transcribing enzymes; the other regions would be covered or repressed.

Evidence for repression of specific genes comes from the work of Denis (1967). He reports that, as

a certain tissue approaches its final differentiated state, the number of active genes becomes more restricted. Support for the existence of such a mechanism comes from the work of Church and McCarthy (1967 a, b) with regenerating liver, which as they show behaves in a closely similar manner to developing liver with regard to genomic expression. In this case the RNAs synthesised during the first 3 hours are quantitatively greater and very different from the normal RNAs. As regeneration proceeds the RNA population alters significantly and the disappearance of previously present RNA molecules is accompanied by the appearance of new ones, just as happens in the developing embryonic liver. Nonetheless, some of the genes remain active throughout the regeneration process. This observation suggests that the whole phenomenon can proceed by selective derepression and repression of certain genes respectively. In support of this kind of mechanism comes the work of Denis (1965) on the evolution of genomic expression during embryonic development of Xenopus laevis. He has found that gene expression is of a generalised type at gastrula stage, where cytodifferentiation is considered to be initiated. Some of the genes appear to persist, remaining active in the adult organism

taken as a whole. Additional evidence in support of this mechanism comes from the work of Glisin et al., (1966) on gene activity during early stages of sea urchin development.

The only way to explain the results obtained from the studies on cytodifferentiation, is to assume the existence of at least two classes of mRNA with different decay rates (Denis, 1967). The differentiated embryo would synthesise both short-lived and long-lived mRNAs.

The DNA in chromatin is complexed, as has already been mentioned, with protein and also some RNA. One of the two kinds of proteins are the histones. Now it is very likely, that histones are the molecules responsible for the inhibition of RNA synthesis in most of the genome of higher cells, but serious difficulties arise in attempting to assign to histones the selective role of chemically specifying the genes they are to inhibit. Histones bind to any DNA (or to any other acid polyanion) and there is no evidence that histones possess the ability to recognise and selectively bind to any particular polydeoxyribonucleotide sequences (Johns and Butler, 1964). Furthermore, histones appear to include only

about five common varieties through phylogenetically different species (Bonner et al., 1968) as well as ontogenetically different tissues in a given organism (Beeson et al., 1967) in rat liver, kidney, brain, heart and lung. Similarly, histone fractions F1, F2a, F2b and F3 are completely identical in normal and in regenerating rat liver.

There have been several proposals for resolving this impasse. Allfrey et al., (1964) have suggested that chemical modifications of histone, in particular acelylation, could somehow confer enough diversity on the histone populations to permit chemical recognition by them of the required number of individual genetic sites. Histone acetylation takes place after the histone molecule is assembled, and it occurs in the chromatin, rather than elsewhere in the nucleus. Allfrey et al. have shown that acetylated arginine-rich histone in vitro is a less effective inhibitor of the RNA polymerase reaction than is nonacetylated histone. In subsequent studies Allfrey et al., (1966) have accumulated evidence indicating that change in the state of histone acetylation is correlated with change in the state of gene activation in a number of in vitro systems. These findings demonstrate a tight correlation between histone acetylation and

genomic activation, but the nature of the connection between these events is still unknown. The amount of possible increase in histone diversity resulting from histone acetylation seems too limited, given that the acetyl groups are present as N - terminal derivatives, to permit the hypothesis of selective gene recognition by a population of specific histones.

If it is concluded that histones in their various forms function as inhibitors of RNA synthesis, as essential structural elements of chromatin, and as facilitators of genomic activation, but not as the cistron-specifying selective agents in genomic regulation, then another class of molecule must be implicated in order to account for the latter aspect of regulation in higher cells. The molecules responsible for recognition of the genomic sites to be activated or repressed could be nonhistone chromatin proteins, or other proteins, or they could be non-protein macromolecules, the most likely of which is probably RNA. Furthermore, it would be necessary that these molecules present a considerable change in their turnover rate in those developmental steps involving switching "on" of genes. Such a finding has been demonstrated with the acidic proteins

by Dingman and Sporn (1964) in studying several developmental stages in chicken embryo; also Marushige found the same with developing sea urchins and spermatogenesis in trout.

Frenster (1966) proposed that the recognition is RNA mediated. This polyanion partially displaces histones from the DNA by forming polyanion-histone complexes within active euchromatin. The DNA then undergoes spontaneous strand separation and the one strand is stabilised by hybrid formation of derepressor RNA species specific for single gene loci with a single DNA strand at such a locus. The remaining DNA strand at the gene locus is then free to serve as a template for gene specific and strand specific mRNA synthesis.

The non-histone chromatin proteins bind to DNA and histone, turn over rapidly and may (or may not) be present in great diversity. Paul and Gilmour (1968, 1969) have recently presented experimental data, which suggest that the qualitative specification of that portion of the genome which is inhibited from transcription by histones, actually depends on the non-histone proteins present in the chromatin. Thus, according to hybridization experiments, a normal spectrum of RNA molecules is synthesised in reconstituted

chromatin, formed by adding the non-histone proteins of calf thymus nuclei plus calf thymus histone to DNA, while DNA plus histone alone results in a totally inhibited preparation. In addition when DNA was combined with pellicle residue, the RNA made from this nucleoprotein annealed with the DNA to the same extent as the RNA made when dehistoned chromatin was used as primer. It was then suggested that the pellicle residue contains a factor that restricts transcription in dehistoned chromatin.

A model based on the experimental findings was reported by the authors. According to this model the pellicle residue factor was thought to establish a stereospecific relationship with DNA in consequence of which a) it represses transcription of some sequences and b) it prevents the nonspecific transcription of some sequences caused by histones (Paul and Gilmour, 1968). The role of histones according to this model is quite passive. This situation has similarities with the bacterial repressors, since both the selective and inhibitory functions are performed at one and the same time.

The molecular species which must selectively recognise a particular genetic site must possess the

property of binding (reversibly) with given sequences of genetic DNA, and under normal conditions not with other DNA sequences. Various precedents exist for specific molecular recognition between proteins and polynucleotide sequences. These include a) the specific recognition and reversible binding to the appropriate tRNAs on the part of amino acid activating enzymes b) the binding of enzymes which alter certain nucleic acids but not others, e.g. the methylating enzymes affecting ribosomal RNA and c) the repressor proteins for the Escherichia coli lac operon (Gilbert and Müller-Hill, 1966) and the λ phage (Ptashne, 1967). These examples, particularly the last, prove that specific polynucleotide-protein recognition and binding are possible, and therefore a theory of selective gene recognition mediated by specific proteins is feasible. One problem which emerges, is that the acidic chromosomal proteins of animal cells are highly insoluble in neutral solutions of dilute salt and it is difficult to envisage free association with or dissociation from DNA in the nucleus of the living cell.

The non-histone fraction contains both acidic proteins and RNA. Recent claims have been made that some of this RNA is a very important factor in the

recognition process. Recently Bonner et al., (1969) reported their results on the control of specificity in pea cotyledon chromatin. Their conclusions were identical to the results of Paul and Gilmour (1968, 1969). The same authors have also shown that chromatin contains an associated RNA of a special class, which they call chromosomal RNA. The occurrence of this RNA in a number of animal and plant tissues has been reported. According to these authors the integrity of this RNA is of crucial importance for faithful reconstruction of specificity. They claim this on the basis of the findings that when this RNA was degraded, by ribonuclease or by zinc nitrate, hardly any of the RNA made from this chromatin resembled pea cotyledon RNA.

Huang and Huang (1969) describe almost identical experiments with chick embryo chromatin. They also degrade the chromosomal RNA and also concluded that its removal abolishes the ability of chromatin to reconstitute faithfully. Their argument is that specificity of masking still exists after removal of chromosomal RNA but this is not the appropriate chick embryo specificity.

Recent studies by Bonner et al., (1969) have been

made on the nature and properties of the chromosomal RNA. They found that it is a particular class of RNA distinguished by short chain length (40 - 60 nucleotides in length in different organisms, (Huang and Bonner, 1965; Dahmus and McConnell, 1969) that has a high (8 - 10%) content of dihydropyrimidine (dihydrouridylic acid in pea, cow and chick, dihydroribothymidylic acid in rat,)and is associated with DNA in the chromosomes of higher organisms. In such chromosomes, chromosomal RNA is on the one hand bound to DNA in RNase-resistant form (Bonner et al., 1961) and on the other hand is bound covalently to acidic proteins and by hydrogen bonding to histones. They have shown that this RNA is sequence-heterogeneous and hybridizes to the extent of 3 - 5 per cent denatured or native DNA, the exact percentage depending upon the organism involved (Dahmus et al., 1969; Bonner et al., 1967). The chromosomal RNA, unlike messenger RNA, possesses the ability to bind to native DNA, and from the experimental evidence available it was suggested that this interaction is in part at least by base pairing. Thus the interaction is considered by the authors as specific for homologous DNA, implying sequence specificity. Evidence for this comes from the data that homologous (rat) DNA

hybridizes with 3,2% as much chromosomal RNA while heterologous DNA (calf) retains only 1.2%, and Escherichia coli DNA retains 0.0%. The complex is formed only under the so-called hybridizing conditions (4M urea, 1 x SSC, for 24 hours at 1°C). The complex once formed can be destroyed by melting, as can duplex nucleic acid structures. The property of chromosomal RNA to form hybrid with native DNA was attributed in the uniformly high content of dihydropyrimidine in the chromosomal RNAs of all creatures, and of all organs of all creatures yet studied.

Since hybridization of chromosomal RNA is not influenced substantially by the presence of whole cytoplasmic RNA, the sequences characteristic of chromosomal RNA must be confined to the nucleus. Chromosomal RNA resembles in this respect the nuclear RNA of Harris et al., (1963), which turns over rapidly within, but does not appear to escape from, the nucleus. In L-cells Shearer and McCarthy (1967) have shown most of the gene activity (qualitatively speaking in terms of apparent diversity of genetic information) is in fact the synthesis of RNAs confined to the cell nucleus, and in addition hybridizes with ca. 4.4% of nuclear DNA. Furthermore, this RNA is not homologous to cytoplasmic RNAs. Shearer and McCarthy (1967) and

Attardi et al., (1966) have suggested that these RNAs serve a regulatory function.

If the nuclear RNAs do serve a regulatory function, their high turnover rate can be interpreted in terms of the responsiveness to the interaction between the outside environment and the regulatory machinery. The nature of the relations between environment and regulatory genomic elements is at present a most obscure matter. Equally obscure are the mechanisms by which the products of regulatory genes might function. We do not even know, for example, whether the RNAs confined to the cell nucleus are used *in vivo* as templates for protein synthesis.

A curious speculation suggests itself in this connection, viz., that the presence in higher cell genomes of a large amount of genetic material which is internally redundant, is related to the putative regulatory function of much of the animal cell genome. Thus the genome could be organised as a series of overlapping and interlocking groups of genes with partial complementarity among them serving as the primitive structural base for selective recognition among the regulative and structural elements, or among the various regulative elements (Britten and

Davidson, 1969). Other hypotheses as to the significance of the internally redundant sequences of higher cell genomes include the possibility that these sequences represent amplifications of the genetic potential to synthesise certain much needed RNAs. Irrespectively of whether or not there is any fundamental link between the presence of near-homologous gene families and the putative regulatory functions that have been attributed to the animal cell genome, it seems clear that both features exist and that both must affect the basic structure of the genome.

2. DNA reiteration

The complementary structure of DNA plays a fundamental role in the cell. Matching of complementary nucleotide sequences is probably involved in genetic recombination as well as in other events of cellular recognition and control.

Separated complementary strands of purified DNA recognise each other, and under appropriate conditions they specifically reassociate *in vitro* (Marmur et al., 1963; 1960; Warner, 1957). The reassociation of various DNAs follows the time course of a second order reaction. The rate of reassociation of the

complementary strands of DNA of viral and bacterial origin is inversely proportional to the DNA content per cell (Britten and Kohne, 1966). However, a large fraction of the DNA of higher organisms reassociates much more rapidly than would be predicted from the DNA content of each cell (Bolton, 1963; Waring and Britten, 1966). Another fraction appears to reassociate at the expected rate. It was concluded that certain segments of the DNA are repeated hundreds of thousands of times. A study of a number of species indicates that repeated sequences occur widely and probably universally in the DNA of higher organisms.

It now seems well established that the mammalian genome can generally be divided, with certain assumptions, into three categories based on the rate of DNA reassociation: first, the rapidly reannealing fractions which reassociate within minutes at ordinary concentrations, second the intermediate fraction, which takes hours to reassociate, and thirdly the slowly reannealing fraction which requires days or weeks to reanneal in vitro and necessitates high concentrations. In the mouse the first accounts for 10%. The rate of reassociation of this fraction is

very much faster than that calculated from the respective genome size. This fraction has the reannealing rate predicted by extrapolation of bacterial or viral rates. This fraction represents DNA molecules which are present in the genome in approx. 1×10^6 identical or nearly identical copies. The second fraction accounts for 20% of the genome (approx. 1,000 - 100,000 copies per genome) and the third for 70% which includes the unique DNA sequences.

The first class of DNA molecules (fast) which can reassociate with each other is called a family, since the similarity but not complete identity in sequences, implies a common origin. The nuclear RNA from all mouse strains (Kit, 1961; Flamm et al., 1966) and mouse tissues thus far investigated, exhibits two distinct DNA bands when centrifuged to equilibrium in CsCl density gradients. The principal band (main band) which comprises 90% of the nuclear DNA, is found at a buoyant density 0.01g cm^{-3} higher than the minor component, or satellite band, as it is generally called. From the reassociation kinetics of the fast fraction in the mouse it was calculated that the length of the repeating, closely similar, sequence is about 300 base pairs. Since the M.W. of the satellite DNA molecule in the mouse is 5×10^7

daltons, each molecule must consist of about 200 units of these repeating sequences.

The proportion of the genome of higher organisms represented by the intermediate DNA fraction varies quite widely, even among vertebrates. In the mouse, it makes up 20% of the genome, in the ox, 40% and in Salmon and Amphiuma 70%. It has been suggested, that this DNA consists of families of related sequences, and that there may be varying numbers of copies of a specific sequence which are exactly identical to each other. If this is the case, it would be necessary to postulate a mechanism to explain why identical copies of the same sequence are not allowed to diverge by mutation during evolution, and even more, how the products of such genes can display simple Mendelian inheritance, when there should be, theoretically, as many possible alleles as there are copies of the gene. Callan et al., (1967) have postulated several such mechanisms, all based on a Master/Slave hypothesis. According to this hypothesis there is a Master copy of the sequence against which all of the Slave copies are checked for agreement each cell cycle in the germ line at least. Mutations in the slave copies would be corrected to agree with the Master copy; any mutation in the Master

copy would be passed on to the Slaves. Evidence for this hypothesis derives mainly from cytological studies. RNA sequence work supports Master/Slave hypothesis for a limited number of RNA species (Williamson and Brownlee, 1969) but there could be a simple genetic reason.

These studies have revealed new properties of the DNA of higher organisms which must be attributed to the repetition of nucleotide sequences. In general more than one third of the DNA of higher organisms is made up of sequences which recur anywhere from a thousand to a million times per cell. Thus the genetic material is not entirely a collection of unique and unrelated genes. A minor degree of sequence repetition is to be expected from studies of protein sequences (Brew et al., 1967). The haemoglobin group shows similarities in sequences, and these point to a common origin of part or all of their structural genes. Trypsin and chymotrypsin also show similarities. There is evidence that, in some cases, different segments of the amino acid sequence of a given protein may have arisen by duplication and insertion of an earlier short segment.

In addition to genetic evidence, banding patterns in polytene chromosomes show that gene duplication occurs. Certain minor classes of DNA probably consist of many copies of a short segment. Such appears to be the case with ribosomal genes (Attardi et al., 1965; Ritossa et al., 1966) and in certain cells, at least, thousands of similar, if not identical, copies of mitochondrial DNA (Borst et al., 1967). Such classes of DNA do not make up more than a small percent of the total DNA and compared to the bulk of the repeated sequences, have a relatively low repetition frequency.

The families of repeated sequences range from groups of almost identical copies (e.g. mouse satellite DNA) to groups with sufficient diversity. When such strands reassociate the resulting pair is of low stability. It seems likely that this situation can only arise from manyfold duplication of an existing nucleotide sequence. With the passage of time and the occurrence of mutations and translocation of segments the number of a family diverge from one another in nucleotide sequence to produce a family of similar but not identical sequences.

The extensive studies of Hoyer et al., (1964; 1966) using the agar technique supply a measure of the repeated sequences held in common among different species. The reassociation conditions standard for the DNA-agar technique utilise low concentrations of DNA and relatively short time so only repetitious DNA fractions will reassociate appreciably under these conditions. These measurements were carried out at different temperatures (Hoyer et al., 1966) and the results were correlated with the period of time after divergence of the lines leading to the newer species (Hoyer et al., 1964). The melting temperature is low if strands of DNA from different species are reassociated. The reduction of thermal stability is proportional to the period after divergence of the species.

Britten and Kohne (1968) considered this as evidence that the members of families of repeated sequences in the DNA of a species slowly change in nucleotide sequences. The degree of divergence can be taken to be a measure of the age of the family, and it is of interest in relation to the history of evolution of the species (Britten and Kohne, 1968). Large, closely similar families are considered by

these authors as evolutionarily young, while greatly diverged families as evolutionarily old. Thus families are produced in a time short compared to the time required for their divergence (a few hundred million years). They suggested the term saltatory replication to describe this hypothetical event by which a family of hundreds of thousands of similar nucleotide sequences is produced in the DNA of an organism. There is a certain amount of evidence that at least some of these repeated sequences are genetically expressed.

Pulse labelled RNA (presumptive messenger) has been hybridized with the DNA of higher organisms (Church and McCarthy, 1967, a b; Brown and Gurdon, 1966; Whitely et al., 1966; Denis, 1966 a, b; Glisin et al., 1966). Since they were successful in detecting hybrids at all under the conditions they used, it maybe inferred that this hybridizing RNA is complementary to families of repeated RNA sequences. In the course of embryonic development and during liver regeneration (Church and McCarthy, 1967 a, b; Denis, 1966 a, b; Glisin et al., 1966; Whitely et al., 1966) changes occur in the patterns of types of hybridizable pulse-labelled RNA. These results suggest that during the course of differentiation

different families of repeated sequences are expressed at different stages.

Hybrids between RNA and non-repeated DNA sequences of higher organisms would not be expected to occur under such conditions for the reasons explained earlier in this section. A good working hypothesis is that repeated sequences commonly occur in structural genes. In any case, transcription as complementary RNA is direct evidence for the genetic function of at least some of the repeated sequences.

Studies on the capability of the first fraction to hybridize have revealed conflicting results. Harel et al., (1968) have shown that rapidly labelled RNA of high molecular weight from mouse liver, kidney and L cells is hybridizable with purified mouse satellite single DNA strands, and especially with the light one (rich in deoxyadenylic acid). They had assumed that the light strand seems to be replicative. On the other hand, Flamm et al., (1969) presented data supporting a completely different situation. They studied the ability of mouse labelled satellite single DNA strands (heavy and light) to reassociate with several unlabelled mouse tissues, namely liver, kidney and spleen. In none of these experiments is

there any evidence of RNA sequences complementary to mouse satellite. They confirmed these results by banding unlabelled single DNA strands with ^{32}P-labelled RNA from mouse L-cells.

It had been suggested that the RNA population made from repeated DNA sequences may have some role (perhaps regulatory) other than as messengers carrying structural information for protein synthesis. Walker (1968) proposed that the reiterated parts of the mouse satellite DNA have been preserved during evolution because it was functionally necessary and he discussed the hypothesis that, these similar sequences are "housekeeping" sequences concerned with chromosomal recognition and folding; a large number of blocks of similar sequences may be required for these purposes.

3. Hybridization

The technique widely used for the detection of genetic relationship between DNA and its transcription products, i.e. mRNA, rRNA and sRNA, is based on the amount of labelled RNA specifically bound to DNA under conditions of thermal renaturation of the hybrids. The amount bound under these conditions is considered to be a measure of the molecular homology between the

DNA and its transcription products.

At the present time, the only reasonable approach to the investigation of sequences of large RNA molecules, which are indistinguishable by the previously mentioned chemical tests, is to compare their ability to hybridize with DNA. This phenomenon enables us to decide whether the mRNA present in a given tissue is transcribed on the same or on different DNA sites compared with the mRNA present in another tissue. The hybridization technique between DNA and RNA was used in an attempt to describe the changes in gene activity that occur during cytodifferentiation.

The first demonstration of hybrid formation was reported by Warner (1957). Complementary ribopolymer pairs were shown to form a helical paired structure when mixed in solution. Marmur et al., (1960) showed that when DNA was dissociated into two strands, the physical properties and biological activity of double stranded DNA were restored by incubation under appropriate conditions. It was shown by Hall and Spiegelman (1961) that virus specific RNA, made by bacteria during viral infection was able to pair with the viral DNA.

Several techniques were developed for the immobilisation of single stranded DNA in cellulose (Bautz and Hall, 1962), in agar (Bolton and McCarthy, 1962) and on nitrocellulose filters (Nygaard and Hall, 1963, 1964; Gillespie and Spiegelman, 1965). It then became possible to assay the reassociation of radioactively labelled single-stranded fragments of DNA or RNA with the immobilised DNA. Another useful technique for measuring reassociation depends on the fact that double-stranded DNA or hybrid can be separated from single stranded DNA on a calcium phosphate (hydroxyapatite) column (Bernardi, 1965; Walker and McLaren, 1965; Miyazawa and Thomas, 1965). Reassociation can be followed by passing samples through hydroxyapatite and determining the amount adhering to the column. Reassociation of the DNA of vertebrates was observed by Bolton et al., (1963; 1964); Hoyer and McCarthy and Bolton (1964). The extent of reassociation between DNA strands derived from different species was shown to be a measure of the evolutionary relation between the species (Hoyer et al., 1964).

3.1. Principles of thermal reassociation

The principal conditions for renaturation of the hybrids are thermodynamically the same as those employed

for the reassociation between two complementary single DNA strands (Nygaard and Hall, 1964). To initiate renaturation, the complementary regions on the two strands have to collide in the proper steric orientation, forming a hydrogen-bonded "nucleation" centre. Once such an event occurs, the "zipping up" of the remaining portions of the DNA will proceed on either side of the nucleation centre.

Partially reassociated strands often implies either a) that a fragment of a complementary strand is lost or b) metastable states of the strands caused by a mismatching between the repetitive sequences on the strands with resulting loop formation (a) or the formation of duplexes with non base paired regions.

Both of the later forms can return to a thermodynamically more stable regular double helix when exposed for a sufficiently long time to reassociation conditions.

3.2 Conditions for thermal reassociation

The conditions for successful and rapid reassociation are mainly three (Szybalski, 1967). First, elevated

temperatures which will weaken intra-stranded secondary structure, increase the mobility of single strands and favour their unfolded conformation. The temperature must lie below the point of transition on the curve at which melting of the hybrid starts and this is about 25°C below the Tm. Second, an optimum concentration of the complementary strands is required, leading to frequent bimolecular collisions. Third, a moderately high salt concentration (e.g. 0.3 - 0.5M NcCl) which will neutralise the negative charge on the phosphate groups and decrease the electrostatic repulsion between the strands. Below 0.01M sodium ion the renaturation reaction is effectively blocked.

When DNA strands or DNA/RNA hybrids which are similar but not identical reassociate with each other, the resulting pair has reduced stability. At a given cation concentration it will dissociate at a lower temperature than a perfectly complementary or native double stranded molecule.

The temperature and salt concentration during a reassociation incubation establish a criterion of precision in that pairs are formed only if they are stableabove the incubation temperature. Thus the

incubation temperature determines which set of sequences will reassociate, and controls the resulting melting temperature.

3.3 Rate of reassociation of DNA

Much of the evidence for repeated sequences depends on measurements of the rate of reassociation. It has been estimated that these families or repeating sequences in mammalian DNA may comprise as much as 30% or more of the base sequences (Britten and Kohne, 1966, 1968). The remaining DNA sequences are presumably unique and RNA molecules synthesised on these sites will, under standard conditions, enter into DNA-RNA interaction only if their concentrations are sufficiently great.

The reassociation of a pair of complementary sequences results from their collision and therefore the rate depends on their concentration. The product of the DNA or RNA concentration and the time of incubation is the controlling parameter for estimating the completion of reaction. Britten and Kohne (1968) call this parameter Cot. Co: is the initial concentration of the reactants and t; is time of incubation in seconds. When they plotted their data with Cot in one axis and $\frac{C}{Co}$ in the other, which is the fraction remaining single stranded at

any time after the initiation of the reaction, they found that the progress of the reaction follows an ideal second order reaction. The formula they used in order to represent the progress of the reaction is the one applied when the initial concentrations of both reactants are the same, and this is:

$$\frac{C}{Co} = \frac{1}{1 + k\ Cot} \quad (1)$$

Where K is the rate constant of the reaction. The units of the k are litre x mole $^{-1}$ x sec $^{-1}$. The units can be found by rearrangement of the equation (1) as $C(1 + k\ Cot) = Co$, $C + C\ (kCot) = Co$, $Ck\ Cot = Co - C$ and $k = \frac{Co - C}{C\ Cot}$

Under conditions in which the reaction is observed to follow second-order kinetics, the rate limiting event must be a "nucleation" event, that is, the formation of one or few correct base pairs at some points along the strands. The subsequent zipping reaction in which the rest of the base pairs subsequently form must be fast, otherwise the overall rate of formation of base pairs as measured by optical absorbance would be kinetically first-order (Davidson, 1968).

The half life of a second-order reaction is found

from equation (1) by setting $C = \frac{Co}{2}$ when $\frac{t}{2} = T$ and $T = \frac{1}{kCo}$

The half life is thus inversely proportional to the initial concentration under fixed conditions for a particular DNA. Furthermore, one would expect for a given total DNA concentration that the time measured for the reassociation of one half of the DNA is just proportional to the number of different fragments originally present for a given DNA concentration. The DNA of a small virus (SV-40) cut to one tenth of its original length, yields 10 different fragments, each 1200 nucleotides long. The phage T4 has 35 times as much DNA and yields 350 different fragments of the same size. If the fragmented preparations of DNA are adjusted to the same total concentration under conditions suitable for reassociation, it is observed that the T4 viral DNA takes 35 times as long as the SV - 40 DNA for one-half of the pieces to become reassociated. From reassociation rate measurements, it is thus possible to determine the number of different nucleotide sequences present in the genome of a particular species by comparing its DNA reassociation rate to that of an appropriate standard DNA. It appears that the Cot required for half reassociation of DNA is proportional to the genome

size. This is only true, however, in the absence of repeated DNA sequences.

4. Interpretation of DNA/RNA hybridization studies with mammalian nucleic acids

Although the basic methodology for studies of mammalian DNA/RNA hybrid formation is similar to that used in studies of viral and bacterial nucleic acids, it is quite obvious that interpretation requires some careful consideration. Thus, where the salt concentrations are high, the nonspecies specific background binding is grossly magnified, and locus specificity must be minimal. Thus, if adquate precautions are taken, it seems that mammalian RNA will interact with specific base sequences in DNA, just as in the case for RNA derived from lower organisms.

From a consideration of the apparent complexity of mouse DNA for instance, it is obvious that the rates of reaction, similar to those with bacterial nucleic acids, are altogether too rapid to be associated with complete locus specificity. Again the fact that these rates fall rapidly with increasing temperature, suggests that a given RNA molecule may react with many DNA sites having base sequences

related to those of its parental gene. This selection of potential reaction sites will be reduced as more stringent criteria of hybrid formation, such as increased temperature or reduced ionic strength, are applied (Church and McCarthy, 1968).

In the face of the question as to the usefulness and appropriateness of this technique as a means of analysis of RNA synthesis in higher cells, it is perhaps best to consider the method as a chemical fractionation scheme. The immobilised DNA molecules may be regarded as a chromatographic material containing a large number of different binding sites, each possessing differential affinity for a different group of RNA molecules. The technique may then be seen as one of considerable flexibility and more sensitivity that any others currently available. As in all other fractionation methods, it is very difficult to prove identity since this may be confused with lack of resolution. From this point of view, it is apparent that differences among RNA populations may often be demonstrable, although the failure to detect differences does not establish identity (Church and McCarthy, 1968). On the other hand, it is justifiable to draw conclusions as to differences among populations

of RNA molecules, although again, as with chromatographic or molecular weight separations, these will be minimal estimates of the real extent of dissimilarity.

Thus the method has been shown to be a valuable one for the detection of systematic transcriptional changes in embryonic or regenerating systems (Glisin et al., 1966; Whitely et al., 1966; Church and McCarthy, 1967 a, b) or differential distribution of RNA within the cell (Shearer and McCarthy, 1967) and transcriptional differences among different tissues (Paul and Gilmour, 1966, 1968; Ursprung et al., 1968).

4.1 Saturation of DNA with RNA

Yankofsky and Spiegelman (1963) demonstrated that hybridization reactions may be used to measure the fraction of DNA concerned with a particular gene function. Thus, saturation of bacterial DNA by ribosomal RNA allows an estimate of the number of cistrons specifying this type of RNA. In cases where cells of higher organisms are involved, this kind of assay is potentially extremely useful for estimating the fraction of the genome functioning in a given type of differentiated cell or in an embryo at a certain stage of development.

In view of the question of locus specificity, Church and McCarthy (1968) have attempted to assess the validity of the hybridization method by comparing bacterial and mammalian reactions under similar conditions. They reported that the results obtained with B. subtilis are consistent with the general rule that in bacterial systems the same extent of saturation occurs over a wide range of temperature conditions (60 - 67°C). Presumably, in these cases, the predominant reaction is one between an mRNA molecule and one strand of the DNA of its parental gene (Hayashi et al., 1963; Attardi et al., 1963). The results with mouse RNA present a great contrast and are more consistent with an actual difference in the fraction of DNA which can be titrated at the various temperatures. As the temperature is increased, the demands on complementarity of base sequence are increased and thus the chances for cross-reaction among RNA and DNA molecules representing different genetic sites become more restricted. The thermal denaturation profiles of DNA/RNA hybrids have a broad range of thermal stability which is highly dependent on the reaction conditions used for their formation. Denis (1966) has demonstrated the variable stability

of amphibian DNA/RNA hybrids.

Church and McCarthy (1968) show that the stabilities of DNA/RNA hybrids are directly proportional to the different temperatures of incubation used during the hybrid formation. Moreover, they show that the thermal stabilities of the products do not tend to overlap with those of hybrids formed at a higher or a lower temperature. It is readily apparent from data presented in the studies obtained from competition reactions (Table 7) performed under stringent conditions, that the hybridized RNAs from mouse spleen and mouse kidney, for example, are different in at least 70% of their component molecules. The parameters of temperature and ionic strength both govern specificity.

5. Evidence for restriction of DNA template activity in animal cells

Cell differentiation is closely associated with the differential activity of genes in different tissues (Kidson and Kirby, 1964; McCarthy and Hoyer, 1964; Paul and Gilmour, 1966, 1968; Ursprung et al., 1968). The differentiation of cells is manifested in the production of RNA which acts as an intermediate between the genome and the protein-synthesising apparatus.

One of the topics of major importance in differentiation is concerned with the proportion of the genome active i.e. the relative amount of genomic information involved in changes in the pattern of gene activity which are associated with changes in the state of differentiation.

The template specificity of isolated chromatin in supporting RNA synthesis has been studied to answer that question. Paul and Gilmour, using rabbit bone marrow and thymus chromatin in a defined *in vitro* system, were able to show by the molecular hybridization technique that the inactivation patterns of the genome is distinctive for each of the two tissues, and only a part of the genome restricted to 7,5% and 5% of rabbit bone marrow and thymus respectively, was available for transcription. By competition studies the same authors also found that the RNA produced by chromatin in a cell-free RNA-synthesising system was identical to RNA isolated from the whole homologous tissue. This provides evidence that the template specificity associated with chromosomes *in vivo* is retained by isolated chromatin.

The present study is a further attempt to establish the applicability of molecular hybridization technique in the study of mechanisms of differential gene expression in animal tissues. In this study the chromatin from different mouse tissues has been isolated and used in a cell-free RNA synthesising system to study the effect of cell differentiation on the pattern of RNA synthesis. This type of system has certain advantages over that which uses *in vivo* labelled RNA. The RNA produced is highly and uniformly labelled and of known specific activity. On the other hand, RNAs extracted from tissues of animals which have received a pulse-labelling, contain labelled heavy rRNA precursors, besides a large amount of unlabelled rRNA. Thus it becomes particularly difficult to determine the true specific activity of the messenger fraction. The *in vitro* system also has the advantage that all RNAs are probably transcribed at the same rate, and are not subject to differential turnovers as is found in *in vivo* conditions (Harris, 1963; Paul and Struthers, 1963). Competition experiments with natural RNA also support the validity of the system.

The purpose of this work was to determine the kinds of RNA synthesised by chromatin using exogenous

RNA polymerase in the priming incubation mixture of different mouse tissues. It was found that different populations of RNA molecules examined are not only distinguishable but also show large differences between them. Mouse liver produced less hybridizable species of RNA than mouse kidney (Figure 11). When DNAs from mouse kidney or whole embryo were used as templates and the RNA produced was annealed, then these RNAs hybridized to the same extent and the amount of hybridization was almost three times greater than the hybridization which occurred when chromatin was used as template (Figures 9 and 11).

It appears that most of the genes in the nucleus of animal somatic cells, which are considered as being in a completely differentiated state, are in fact inactive in supporting RNA synthesis, the inactivation pattern of the genome in such cells being distinctive for each tissue. Furthermore, it seems clear from these results as well as from the results of Paul and Gilmour (1966 b) that the structural organisation of the chromosome accounts for the specificity of transcription associated with specific cell types. Moreover, these results may

be considered as suggesting the possibility that certain genes unique for each specific type of chromatin are active in RNA synthesis relevant to tissue function. It is therefore possible to estimate that fraction of the DNA which is responsible for the particular state of differentiation of any cell from those RNA molecules which are most common in that cell.

Evidence supporting this conclusion has also been obtained from competition experiments in which labelled RNAs synthesised *in vitro* on a chromatin template from mouse kidney, liver and spleen were competed by unlabelled RNA isolated from the respective tissue. In each case the RNA from the tissue homologous to the chromatin was the most effective competitor; RNA from the heterologous tissue gave only partial competition (Figures 13, 15 and 17). These findings make isolated chromatin an interesting assay system for the study of gene function. For they mean that chromatin retained its developmental integrity after isolation. From the competition experiments it seems clear that this system will provide an excellent assay for investigating the mechanism involved in

establishing and maintaining this specificity that is reflected in the RNA pattern.

6. The effect of folic acid administration on the template activity of kidney chromatin

A good deal of literature has accumulated on the sequence of metabolic events in the kidney which follow folic acid administration both in the rat and the mouse. Most research has concentrated on a search which can be related to cell proliferation for changes in nucleic acid metabolism.

Thus a new approach to an understanding of the control mechanism has involved studies of the rate and quality of RNA synthesis in the treated kidney. Threlfall et al., (1968) established that increased RNA labelling occurred after treatment in rats, and Paul et al. (in preparation) have studied the incorporation of ^{3}H-uridine into RNA for the first 24 hours after folic acid administration. The kidney control mechanisms cannot be fully understood from studies of the rate of RNA synthesis alone.

The present work is an attempt to clarify the qualitative changes in RNA synthesis by means of the molecular hybridization technique. By this method

it is possible to follow the sequential synthesis of new groups of RNA molecules after folate treatment. Within the first hours after treatment there is a profound change in the template activity of the treated kidney chromatin. Almost fifty per cent of the RNA molecules labelled at that time are essentially absent in the kidney of normal animals. On the other hand, as is shown by the competition experiments between RNA from treated kidney, and normal kidney RNA as competitor, all of the hybridizable RNA molecules present in normal kidney are not present in the treated tissue although some may still be synthesised but in small abundance. It should be emphasised that the greatest difference in the distribution of RNA molecules is observed at the early stages after folate administration. At later stages, there appears to be a sequential loss of various types of RNA molecules characteristic for the early times. RNAs isolated at later times are less effectively competed out by the RNA isolated at 3 hours. Thus many RNA molecules are actively synthesised during the first three hours but are not produced at later times. Their absence at later periods indicates that the process is mediated by short-lived RNA molecules. Most of the

RNA molecules synthesised during the early times must have a shorter life than those present in normal kidney. Since RNA isolated at 12 hours is already deficient in some molecules, this suggests that the 12 hour stage synthesises species of RNA that are either absent at the 3 hours or present in very low concentration. This indicates that their half-lives must be of only a few hours. When normal ^{3}H-RNA was competed by RNA isolated at 3 hours there was very little displacement (Paul et al., in preparation).

The overall effect of folic acid in stimulating changes in the in vitro RNA synthesis can be summarised as follows: kidney "regeneration" is associated with the appearance of short lived RNA molecules, the synthesis of which commences after the first hours of folic acid adminstration. The process implies the disappearance of some RNA species and the synthesis of new molecules which did not exist during early hours. Support for this aspect comes from results using in vivo RNAs isolated after folic acid treatment (Paul et al., in preparation).

At this point there is a discrepancy between the results obtained from folic acid treated kidneys and the results described by Church and McCarthy

(1967) who studied the regeneration process in mouse liver. Their results show that the population of RNA species characteristic of normal liver continues to be present in the hepatectomised tissue throughout regeneration. This observation is supported by their finding that, using the same RNA/DNA ratio, all the competing RNAs isolated from early stages up to 48 hours after hepatectomy compete out the labelled RNA isolated from normal liver equally well.

A thought that is worth mentioning, is the fact that the concentrations of RNA species characteristic of the normal liver are differently represented in the different stages, i.e. in the 1 and 3 hour preparations, these RNA species make up only a small fraction of the total RNA. The fact that all the different preparations compete out the labelled normal RNA at the same rate is a paradox.

Some points which are quite different from the procedure used in the folic acid experiments will be mentioned. First, Church and McCarthy used an RNA/DNA ratio of 0.33. This ratio is quite low and the probability of an appreciable proportion of ribosomal RNA being bound is not negligible, given that only at high levels of RNA the less abundant

classes of molecules account for the hybridized RNA. Second, the RNAs extracted from a whole tissue which have received a pulse label contain labelled heavy rRNA precursors besides a large amount of unlabelled rRNA. Thus it becomes particularly difficult to determine the true specific activity of a messenger fraction. Besides, factors such as different rates of uptake and utilisation of ^{32}P at these different stages may obscure the picture. Moreover, Church and McCarthy do not plot saturation curves for the different stages, so it is not clear if the amount of labelled RNA used in their competition experiments was at a concentration approaching that required to saturate the DNA sites. Under non-saturating conditions Denis (1967) claims, as was mentioned under Section III, the results of competition experiments are not clear cut. These are several reasons which may account for the discrepancy observed.

Another point that has also to be mentioned here, is that in competition experiments between labelled RNAs from folic acid treated animals and unlabelled competitor 3 hour RNA, reaction was not carried out to completion. There is a small possibility that if this was done, then the RNAs isolated at different times after folate treatment would be competed out by

the competitor RNA isolated at 3 hours to the same extent but at different rates. The ratio of RNA/DNA used is such that the hybridization of ribosomal RNA makes up a negligible amount of the total, while nuclear RNA would hybridize with high efficiency. The use of the term "messenger-RNA" is not a strict one since the role of the hybridized RNA molecules as templates in cytoplasmic protein synthesis has not been demonstrated. Conceivably other kinds of RNA molecules are present and synthesised in response to folate administration. Penman's experiments (1966) suggest the existence of rapidly labelled RNA molecules in HeLa cells which never pass from the nucleus to the cytoplasm. Thus, although some of the new RNA transcripts may be messengers for specific cytoplasmic protein synthesis, others may function within the cell nucleus. It is not possible to attribute cellular functions to the new RNA species although it is evident that many changes occur after folic acid treatment for which RNA synthesis may be necessary. Nuclear proteins are increased after folate (Threlfall et al., 1969) and these changes are related to the shift in metabolism for cell proliferation.

Nuclear RNA molecules for the specification of these proteins might be among those which appear more abundantly after the administration. Mitotic activity in the kidneys commences during the second day after folate administration in the mouse and reaches its maximum at 50 hours (Baserga et al., 1968). In the rat the mean maximum wave of DNA synthesis in the outer medulla was attained at 24 hours followed by DNA synthesis in the cortex with a maximum at 36 hours (Threlfall et al., 1968). The overall rate of DNA synthesis in the rat kidney reaches a maximum at 30 hours. Mitotic activity commences after the first day and reaches a maximum at 48 hours. In the mouse incorporation of ^{3}H-thymidine into DNA showed a peak at 40 - 42 hours after treatment, which is about 10 hours later than in the rat.

It is clear that a profound change in the metabolic activity of a tissue such as kidney after folic acid administration does involve gross changes in transcription. These new RNA molecules have a very limited liftime so they provide the means of control for a transitory response quite analogous to the synthesis of unstable mRNA for enzyme induction

in bacteria.

7. Changes in the template activity of kidney chromatin after ligation of one ureter

The mechanisms involved in normal cell division in mammalian organs are not well understood because studies in multicellular organisms have encountered particular difficulties. Nonetheless, clarification of the mechanism underlying this phenomenon in higher organisms is necessary for the understanding of cytodifferentiation; this problem is usually approached by studying various experimental model systems.

Several investigators have found that tissues with a very slow DNA, RNA and proliferating activity can display, if they are exposed to the proper stimulus, a high rate of DNA and RNA synthesis followed by an increase in mitotic activity. These situations provide model systems; their importance lies in the fact that one may follow the functional steps which lead from a quiescent state to RNA and DNA synthesis, and cell division. The case under investigation refers to the induction of hydronephrosis in mouse kidneys and the appearance of new kinds of RNA that follows.

Several studies have been made on the sequence of metabolic events which follow ligature of one ureter, particularly in the rat. It was found that DNA synthesis and mitotic activity in the cortex of the kidney with ureteral obstruction was significantly increased. The mechanism by which DNA synthesis and mitosis are stimulated following ureteral ligation is unknown. The proliferative response commences before there is any histological evidence of necrosis in the renal cortex and it is therefore unlikely that the initial response is due to cell loss (Benitez and Shaka, 1964). Local tension as a stimulus to local mitosis has been discussed by Abercrombie (1957), and Fox (1957) proposed that distention of the renal pelvis and tubules was the stimulus to cellular proliferation in the obstructed kidney. A good example is the proliferative response of the endometrium and myometrium following distention of the uterine lumen, a response independent of ovarian hormonal stimulation.

Benitez and Shaka (1964) found no increase in DNA synthesis or mitosis in the renal cortex of the unobstructed kidney following ligation of the

contralateral ureter. The failure of the kidney on the unobstructed side to respond in the early stages of unilateral hydronephoresis made it nighly improbable that a humoral factor was the stimulus for the cellular proliferation on the obstructed side. Though the stimulus is unknown, it is more likely that the stimulus is a local one, arising from alterations within the organ itself.

Most of the literature dealing with this phenomenon has concentrated mainly on a search for DNA synthesis and proliferation activity. Thus one approach to an understanding of the kidney control mechanism has involved studies regarding the appearance of new species of RNA in mouse kidney after the ligation of one ureter.

The purpose of these experiments has been to follow and compare the populations of RNA molecules synthesised during these stages with that of normal tissue. For the investigation of the mechanism providing for the regulation of transcription, the ability of mouse chromatin in supporting RNA synthesis has been studied in control and kidneys with ureteral obstruction. Quantitative changes in the RNA populations present in the ligated kidney during the

induction of hydronephrosis were examined by calculating the fraction of DNA hybridized as a function of the amount of RNA present. The efficiency of hybridization of RNA isolated at various stages after ligature increases steadily up to 36 hours. The percentage of hybridization at 36 hours coincides numerically with the one obtained from the 3 hour *in vitro* system after folate treatment. At 48 hours there appears to be a decrease in template activity of the chromatins isolated from ligated kidneys.

As it can be seen, the response of ureteral obstructed kidney is a slow one in contrast with the response of kidneys to folic acid administration. However, the numerical estimation of the part of the genome synthesising RNA, judged by the saturation values obtained, is the same in the two phenomena. This may be an indication that the two mechanisms are quite similar. To test accurately the similarity between the two mechanisms competition experiments are required between RNAs isolated from folate treated animals and RNAs from kidneys with ureteral obstruction. These studies will establish if the proportion of the information enclosed in the genome is identical in both cases.

Summary

Cell differentiation is correlated with the differential activity of genes in a given cell (and by implication in a functionally defined mixture of cell types, i.e. a tissue). One of the topics of major importance in differentiation is concerned with the proportion of the genome which is active in RNA synthesis, i.e. the relative amount of genomic information which brings about the morphological changes characteristic of adult tissues.

1) The template specificity of isolated chromatins in supporting RNA synthesis was studied in order to clarify the relationship between genomic expression and organ specificity. The molecular hybridization technique was used for RNAs transcribed *in vitro* from several mouse chromatins. This technique provides a sensitive method for distinguishing between various populations of RNA molecules. It was found first that different populations of RNA molecules examined are not only distinguishable but also show large differences among them and second, that only a part of the genome was active in transcription. By competition experiments it was found that the RNAs produced by chromatin in a cell-free RNA system were

similar to RNAs isolated from the whole homologous tissue. This provides evidence that the template specificity associated with chromosomes *in vivo* is retained by isolated chromatin. A particular feature of this study is the high degree of reproducibility of the hybridization values for each tissue and the dissimilarities displayed by the different tissues.

2) When DNA from mouse kidney was used as template in a cell free system and the RNA produced was annealed, then the amount of hybridization was almost three times greater than the hybridization which occurred when chromatin was used as template. From these results it appears that many of the genes in animal somatic cells, which are considered as being in a completely differentiated state, are in fact inactive in supporting RNA synthesis, the inactivation pattern of the genome in such cells being distinctive for each tissue.

3) The mechanisms which control cell division in mammalian organs are not understood. Various experimental models have been used to study this problem. The cases under investigation include *in vivo* models, i.e. induction by folic acid of RNA synthesis

in mouse kidneys and the appearance of new species of RNA in mouse kidneys after the ligature of one ureter. Studies have been made on the effect of folic acid administration in stimulating changes in the *in vitro* RNA synthesis such that new kinds of RNA molecules appear in mouse kidney at specific times after the treatment. The ability of kidney chromatin in supporting RNA synthesis has been studied in untreated (control) and treated animals. It was found that folic acid changes profoundly the template activity of the isolated chromatin from mouse kidneys. RNA transcribed from chromatin at early stages hybridizes with twice as much DNA as RNA transcribed from normal chromatin. By competition experiments it was confirmed that the cell free system produces the same RNA species as are found *in vivo* which indicates that the integrity of the chromatin is maintained during isolation. Furthermore, the experiments showed that new kinds of RNA molecules are transcribed from kidney chromatin after the early hours of folate. The transcriptional activity of the genome eventually falls to the control level after two days.

4) Another method for stimulating the appearance of new species of RNA in mouse kidneys is to ligate

one ureter. The template activity of chromatin from the ligated kidney has been investigated from zero hours until 48 hours. The maximum hybridization efficiency (twice the control) is exhibited at 36 hours while RNA transcribed from chromatin isolated at early stages bybridizes with as much DNA as RNA transcribed from normal chromatin.

The purpose of these experiments has been to compare the template activity of chromatin isolated at various times after the ligature of one ureter with those obtained after folic acid treatment in mice and to determine if the same control mechanism operates in both phenomena. The relationship between these two systems is discussed.

ABBREVIATIONS

RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
ATP, CTP, GTP, and UTP	Pyrotriphosphates of adenosine, cytosine, guanosine and uridine respectively
mRNA	Messenger RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
nRNA	Nuclear RNA
EDTA	Ethylenediamine tetra-acetic acid (versene)
Tris	2 - amino - 2 - (hydroxymethyl) - propane - 1:3-diol
PCA	Perchloric acid
TCA	Trichloroacetic acid
SSC	Saline Sodium Citrate

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REFERENCES.

Abercrombie, M. (1957). Symp.Soc.exper.Biol., 11, 235.

Allfrey, V.G., Faulkner, R., and Mirsky, A.E. (1964). Proc.Nat. Acad.Sci., 51, 786.

Allfrey, V.G., Pogo, B.G.T., Pogo, A.O., Kleinsmith, L.J., and Mirsky, A.E. (1966). "The metabolic behavior of chromatin". In Ciba Foundation Study Group No.24, p.42.

Alpers, D.H., and Tomkins, G.M. (1965). Proc.Nat.Acad.Sci., 53, 797.

Ames, B.N., and Martin, R.G. (1964). Ann.Rev.Biochem., 33, 235.

Armstrong, R.L., and Boezi, J.A. (1965). Biochim.biophys.Acta, 103, 60.

Attardi, G., Naono, S., Rouviere, J., Jacob, F., and Gross, F. (1963). Cold Spring Harbor Symp.quant.Biol., 28, 363.

Attardi, G., Parnas, H., Huang, M-I.H., and Attardi, B. (1966). J.mol.Biol., 20, 145.

Attardi, G.P., Huang, P.C. and Kabat, S. (1965). Proc.Nat.Acad. Sci., 53, 1490.

August, J.T., Shapiro, L., and Eoyang, L. (1965). J.mol.Biol., 11, 257.

Baker, R.F., and Yanofsky, C. (1968). Nature, Lond., 219, 26.

Baltimore, D., and Franklin, R.M. (1963). J.biol.Chem., 238, 3395.

Barondes, S.H., Dingman, C.W., and Sporn, M.B. (1962). Nature, Lond. 196, 145.

Baserga, R. (1965). Cancer Res., 25, 581.

Baserga, R. (1968). Cell and Tissue Kinetics, 2, 167.

Bassel, A., Hayashi, M., and Spiegelman, S. (1964). Proc.Nat.Acad. Sci., 52, 766.

Bautz, E.K.F., and Hall, B.D. (1962). Proc.Nat.Acad.Sci., 48, 400.

Becker, H.J. (1959). Chromosoma, 10, 654.

Beerman, W. (1959). "Developmental Cytology" p.83. Ronald Press, New York.

Beerman, W. (1963). Am.Zoologist, 3, 23.

Beeson, J.L., and Triplett, E.L. (1967). Exper.Cell Res., 48, 61.

Bekhor, I.J., Kung, G., and Bonner, J. (1969). J.mol.Biol., 39, 351.

Bekhor, I.J., Bonner, J., and Kung, Dahmus G. (1969). Proc.Nat.Acad. Sci., 62, 271.

Bekhor, I.J., Mohseni, Z., Nimni, M.E., and Baretta, L.A. (1965). Proc.Nat.Acad.Sci., 54, 615.

Bell, E. (1965). Ed. "Molecular and Cellular Aspects of Developmental Biology", p.525. Harper and Row, New York.

Benitez, L., Shaka, J.A. (1964). Am.J.Pathol., 44, 961.

Berberich, M.A., Venehamer, P., and Goldberger, R.F. (1966). J.biol. Chem., 241, 4426.

Berberich, M.A., Kovach, J.S., and Goldberger, R.F. (1967). Proc. Nat.Acad.Sci., 57, 1857.

Berg, P., Kornberg, R.D., Faucher, H., and Dieckmann, M. (1965). Biochem.biophys.Res.Comm., 18, 932.

Bernardi, G. (1965). Nature, Lond., 206, 779.

Bielka, H., and Junchalin, I. (1968). Biochim.Biophys.Acta, 166, 689.

Bladen, H.A., Byrne, R., Levin, J.G., and Niremberg, M.W. (1965). J.mol.Biol., 11, 78.

Bloom, S., Torado, G.J., and Green, H. (1966). Biochem.biophys.Res. Comm., 24, 412.

Boeji, J.A., Bolton, E.T., Britten, R.J., Cowie, D.B., McCarthy, B.J., Midgley, J.E., and Roberts, R.B. (1960). Ann.Yearbook Carnegie Institution.

Bolton, E.T., McCarthy, B.J. (1962). Proc.Nat.Acad.Sci., 48, 1390.

Bolton, E.T., Hoyer, B.H., and McCarthy, B.J. (1963). Yearbook, Carnegie Institution, 63, 366.

Bolton, E.T., and McCarthy, B.J. (1964). J.mol.Biol., 8, 201.

Bonner, J., Huang, R.C.C., and Maheshwari, N. (1961). Proc.Nat. Acad.Sci., 47, 1548.

Bonner, J., and Widholm, J. (1967). Proc.Nat.Acad.Sci., 57, 1379.

Bonner, J., and Huang, R.C. (1963). J.mol.Biol., 6, 169.

Borst, P., Ruttenberg, G., and Kroon, A.M. (1967). Biochim.biophys. Acta, 149, 140 and 156.

Brammar, W.J., Berger, H., and Yanofsky, C. (1967). Proc.Nat.Acad. Sci., 58, 1499.

Braverman, G., Cold, L., and Eisenstadt, J. (1963). Proc.Nat.Acad. Sci., 50, 630.

Bremer, H., and Konrad, H.W. (1964). Proc.Nat.Acad.Sci., 51, 801.

Bremer, H., Konrad, M.W., Gaines, K., and Stent, G.S. (1965). J.mol.Biol., 13, 540.

Brenner, S., Jacob, F., and Meselson, H. (1961). Nature, Lond., 190, 576.

Bresler, S.E., Krenera, R.A., Kusher, V.V., and Mosevitskii, M.I. (1964). J.mol.Biol., 8, 79.

Britten, R.J., and Kohne, D.E. (1966). Yearbook Carnegie Institution, 65, 73.

Britten, R.J., and Davidson, E.H. (1969). Science, 165, 349.

Britten, R.J., and Kohne, D.E. (1967). Yearbook Carnegie Institution, 78.

Britten, R.J., and Kohne, D.E. (1968). Science, 161, 529.

Brown, A.C. (1959). Proc.Nat.Acad.Sci., 45, 932.

Brown, J.L., Brown, D.M., and Zabin, I. (1967). Proc.Nat.Acad.Sci., 58, 1139.

Brew, K., Vaneman, T.C., and Hill, R.L. (1967). J.biol.Chem., 242, 3747.

Burton, K. (1956). Biochem.J., 62, 315.

Byrne, R., Levin, J.G., Bladen, H.A., and Niremberg, M.W. (1964). Proc.Nat.Acad.Sci., 52, 140.

Callan, H.G. (1967). J.Cell Sci., 2, 1.

Cameron, I.L., and Prescott, D.M. (1963). Exper.Cell Res., 30, 609.

Capecchi, M.R., and Gussin, G.N. (1965). Science, 149, 412.

Clever, U. (1963). Develop.Biol., 6, 73.

Clever, U. (1966). Am.Zoologist, 6, 33.

Chamberlin, M., and Berg, P. (1962). Proc.Nat.Acad.Sci., 48, 81.

Chamberlin, M., Baldwin, R.L., and Berg, P. (1963). J.mol.Biol., 7, 334.

Chamberlin, M., and Berg, P. (1964). J.mol.Biol., 8, 297.

Chamberlin, H. (1965). J.Federation Proc., 24, 1446.

Chauveau, J., Moulé, Y., and Rouiller, R. (1956). Exper.Cell Res., 11, 317.

Church, R.B., and McCarthy, B.J. (1967). J.mol.Biol., 23, 459.

Church, R.B., and McCarthy, B.J. (1967). J.mol.Biol., 23, 477.

Church, R.B., and McCarthy, B.J. (1968). Biochem.Gen., 2, 55.

Cline, A.L., and Bock, R.M. (1966). Cold Spring Harbor Symp.quant. Biol., 31, 321.

Cohen, G., and Jacob, F. (1959). Compt.Rend., 248, 3490.

Cooper, K.W. (1959). Chromosoma, 10, 535.

Dahmus, M., and McConnell, D. (1969). Biochemistry, 8, 1524.

De Lange, R.J., Fambrough, D.M., Smith, E.L., and Bonner, J. (1968). J.mol.Biol., 243, 5906.

De Mars, R. (1958). Biochim.biophys.Acta, 27, 435.

Dennis, H. (1966). J.mol.Biol., 22, 269 and 285.

Dennis, H. (1967). "L'achrite des gene, du cours du development embryonaire". Dosoer, Liege.

Dingman, C.W., and Sporn, M.B. (1964). J.biol.Chem., 239, 3483.

Doerfler, W., Zillig, W., Fuchs, E., and Albers, M. (1962). Z.Physiol.Chem., 330, 96.

Drews, J., and Brawerman, G. (1967). J.biol.Chem., 242, 801.

Edstrom, J.E., and Beerman, W. (1962). J.Cell Biol., 14, 371.

Falaohi, A., Adler, J., and Khorana, H.G. (1963). J.biol.Chem., 238, 3080.

Fausto, N., and van Lancker, J.L. (1965). J.biol.Chem., 240, 1247.

Fincham, J.R.S. (1965). Microbial and Molec.Genetics,

Flamm, W.G., Bond, H.E., and Burr, H.E. (1966). Biochim.biophys. Acta, 129, 310.

Flamm, W.G., Walker, P.M.B., and McCallum, M. (1969). J.mol.Biol., 40, 423.

Flickinger, R.A., Conrad, S.J., Miyagi, M., Moser, C., and Rollins,E. (1965). Proc.Nat.Acad.Sci., 53, 783.

Flickinger, R.A., Greene, R., Kohl, D.M., and Miyagi, M. (1966). Proc.Nat.Acad.Sci., 56, 1712.

Frenster, J.H., Allfrey, V.G., and Mirsky, A.E. (1963). Proc.Nat. Acad.Sci., 50, 1026.

Frenster, J.H. (1965). Nature, Lond. 206, 680.

Frenster, J.H. (1965). Nature, Lond. 208, 894.

Frenster, J.H. (1966). "The Cell nucleus - Metabolism and Radiosensitivity." p.27.

Fox, H. (1957). J.Embryol.exper.Morph., 5, 274.

Fox, C.F., Weiss, S.B. (1964). J.biol.Chem., 239, 175.

Fox, C.F., Beckwich, J.R., Epstein, W., and Singer, E.R. (1966). J.mol.Biol., 19, 576.

Fuchs, E., Zillig, W., Hofschneider, P.H., and Preuss, A. (1964). J.mol.Biol., 10, 546.

Fujioka, M., Koga, M., and Lieberman, I. (1963). J.biol.Chem., 238, 3401.

Georgiev, G.P., Samarina, O.P., Lerman, I., and Smirnov, M.N. (1963). Nature, Lond. 200, 1291.

Georgiev, G.P., and Lerman, M.I. (1964). Biochim.biophys.Acta, 91, 678.

Gilbert, W., and Muller-Hill, B. (1966). Proc.Nat.Acad.Sci., 56, 1891.

Gillespie, D. and Spiegelman, S. (1965). J.mol.Biol., 12, 829.

Girard, M., Latham, H., Penman, S., and Darnell, J.E. (1965). J.mol.Biol., 11, 187.

Glisin, V.R., Glisin, M.V., and Doty, P. (1966). Proc.Nat.Acad.Sci., 56, 285.

Goldstein, A., Kirschbaum, J.B., and Roman, A. (1965). Proc.Nat.Acad. Sci., 54, 1669.

Gorini, L., Gundersen, W., and Burger, M. (1961). Cold Spring Harbor Symp.quant.Biol., 26, 173.

Grammer, D.K., Hayashi, S.I., Thomson, E.B., and Tomkins, G.M. (1968). J.mol.Biol., 35, 291.

Green, M.H. (1964). Proc.Nat.Acad.Sci., 52, 1388.

Greengard, O., Sentenac, A., and Acs, G. (1965). J.biol.Chem., 240, 1687.

Gros, F., Hiatt, H., Gilbert, W., Kurland, C.G., Risebrough, R.W., and Watson, J.D. (1961). Nature, Lond. 190, 581.

Gurdon, J.B. (1962). Develop.Biol., 4, 256.

Gurdon, J.B. (1962). J.Embryol.exper.Morph., 10, 622.

Gurdon, J.B., and Uehlinger, V. (1966). Nature, Lond, 210, 1240.

Guttman, B.S., and Novick, A. (1963). Cold Spring Harbor Symp.quant. Biol., 28, 373.

Hadorn, E. (1965). Brookhaven Symp.Biol., 18, 148.

Hall, B.D., and Spiegelman, S. (1961). Proc.Nat.Acad.Sci., 47, 137.

Hall, B.D., Green, M., Nygaard, A.P., and Boeji, J. (1963). Cold Spring Harbor Symp.quant.Biology, 28, 201.

Hamilton, T.H., Widnell, C.C., and Tata, J.R. (1965). Biochim.biophys. Acta, 108, 168.

Harel, J., Hanania, N., Tapiero, H., and Harel, L. (1968). Biochem. biophys.Res.Comm., 33, 696.

Harris, M. (1964). Cell Culture and Somatic Variation. (Holt, Rinehart and Winston, New York), p.547.

Harris, H., Fisher, H.W., Rodgers, A., Spencer, T., and Watts, J.W. (1963). Proc.Roy.Soc., Ser.B., 157, 177.

Hartman, P.E., Rugis, C., and Stahl, R.C. (1965). Proc.Nat.Acad.Sci., 53, 1332.

Hartwell, L.H., and Magasanik, B. (1964). J.mol.Biol., 10, 105.

Haruna, I., Noju, K., Ohtaka, T., and Spiegelman, S. (1963). Proc. Nat.Acad.Sci., 50, 905.

Haruna, I., and Spiegelman, S. (1965). Proc.Nat.Acad.Sci., 54, 579.

Haruna, I., and Spiegelman, S. (1965). Science, 150, 884.

Hayashi, M., and Spiegelman, S. (1961). Proc.Nat.Acad.Sci., 47, 1564.

Hayashi, M., Hayashi, M.N., and Spiegelman, S. (1963). Proc.Nat.Acad. Sci., 50, 664.

Hayashi, M., Hayashi, M.N., and Spiegelman, S. (1964). Proc.Nat.Acad. Sci., 51, 351.

Hayashi, M., Spiegelman, S., Franklin, N.C., and Luria, S.E. (1963). Proc.Nat.Acad.Sci., 49, 729.

Hayashi, M. (1965). Proc.Nat.Acad.Sci., 54, 1736.

Henshaw, E.C., Revel, M., and Hiatt, H.H. (1965). J.mol.Biol., 14, 241.

Hiatt, H. (1962). J.mol.Biol., 5, 217.

Hoagland, M.B., and Askomas, B.A. (1963). Proc.Nat.Acad.Sci., 49, 130.

Hodgson, G. (1967). Proc.Soc.exp.Biol., 124, 1045.

Holtzer, R.L., Oda, A., and Chiga, M. (1964). Lab.Investig., 13, 1514.

Hoyer, B.H., McCarthy, B.J., and Bolton, E.J. (1963). Science, 140, 1408.

Hoyer, B.H., Bolton, E.T., McCarthy, B.J., and Roberts, R.B. (1964). Yearbook Carnegie Institution, 63, 394.

Huang, R.C.C., and Bonner, J. (1962). Proc.Nat.Acad.Sci., 48, 1216.

Huang, R.C.C., and Bonner, J. (1965). Proc.Nat.Acad.Sci., 54, 960.

Hurwitz, J., and August, J.T. (1963). Progress in Nucleic Acids, Vol.I, 59.

Hymer, W.C., and Kuff, E.L. (1964). J.Histochem.Cytochem., 12, 359.

Imamoto, F., Morikawa, N., Sato, K., Mashima, S., Nishimura, T., and Matsushiro, A. (1965). J.mol.Biol., 13, 157.

Imamoto, F., Morikawa, N., and Sato, K. (1965). J.mol.Biol., 13, 169.

Imamoto, F., Ito, J., and Yanofsky, C. (1966). Cold Spring Harbor Symp.quant.Biol., 31, 235.

Imamoto, F., and Yanofsky, C. (1967). J.mol.Biol., 28, 25.

Ippen, K., Miller, J.H., Scaife, J., and Beckwith, J. (1968). Nature, 217, 825.

Ishikawa, K., Ogato, K., and Sugano, H. (1964). Biochim.Biophys.Acta, 87, 522.

Jacob, F., Perrin, D., Sanchez, C., and Monod, J. (1960). Compt. Rend., 250, 727.

Jacob, F., and Monod, J. (1961). J.mol.Biol., 3, 318.

Jacob, F., and Monod, J. (1961). Cold Spring Harbor Symp.quant.Biol., 26, 193.

Jacoby, G.A., and Gorini, L. (1967). J.mol.Biol., 24, 41.

Jehle, H. (1965). Proc.Nat.Acad.Sci., 53, 1451.

Johns, E.W., and Butler, J.A.V. (1964). Nature, 204, 853.

Joklin, W.K., and Becker, Y. (1965). J.mol.Biol., 13, 496 and 511.

Katze, J., Sridhara, S., and Zabin, I. (1966). J.biol.Chem., 241, 5341.

Kay, E., Simmons, N., and Dounce, A.J. (1952). Am.Chem.Soc., 74, 1724.

Kepes, A. (1967). Biochim.biophys.Acta, 138, 107.

Kidson, C., and Kirby, K.S. (1964). Nature, 203, 599.

Kidson, C., and Kirby, K.S. (1964). J.mol.Biol., 10, 187.

Kiho, Y., and Rich, A. (1965). Proc.Nat.Acad.Sci., 54, 1751.

Kit, S. (1961). J.mol.Biol., 3, 711.

Knox, W.E., and Auerbach, V.R. (1955). J.biol.Chem., 214, 307.

Knox, W.E., Auerbach, V.H., and Lin, E.C.C. (1956). Physiol.Rev., 36, 164.

Labrie, F. (1969). Nature, 221, 1217.

Lang, N., and Sekeris, C.E. (1964). Life Science, 3, 161.

Laycock, D.G., and Hunt, J.A. (1969). Nature, 221, 1118.

Leive, L. (1965). Federation Proc., 24, 293.

Leive, L. (1965). Biochem.biophys.Res.Comm., 20, 321.

Leive, L., and Kollin, V. (1967). J.mol.Biol., 24, 247.

Levinthal, C., Keynan, A., and Higa, A. (1962). Proc.Nat.Acad.Sci., 48, 1631.

Levinthal, C., Fan, D.P., Higa, A., and Zimmerman, R.A. (1963). Cold Spring Harbor Symp.quant.Biol., 28, 183.

Levintow, L. (1965). Ann.Rev.Biochem., 34, 487.

Lieberman, I., Abrams, R., Hunt, N., and Ove, P. (1963). J.biol. Chem., 238, 3955.

Lipmann, F. (1963) in Progress in Nucleic Acids Res., Vol.I, 135.

Littau, V.C., Allfrey, V.G., Frenster, J.H., and Mirsky, A.E. (1964). Proc.Nat.Acad.Sci., 52, 93.

Littau, V.C., Burdick, C.J., Allfrey, V.G., and Mirsky, A.E. (1965). Proc.Nat.Acad.Sci., 54, 1204.

Locke, M. (1963). Ed., Cytodifferentiation and Macromolecular Synthesis. (Academic Press, New York).

Loeb, J.N., Howell, R.R., and Tomkins, G.M. (1965). Science, 149, 1093.

Loening, U. (1967). Biochem.J., 102, 251.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randel, R.J. (1951). J.biol.Chem., 193, 265.

Luria, S.E. (1965). Biochem.biophys.Res.Comm., 18, 735.

Maas, W.K. (1961). Cold Spring Harbor Symp.quant.Biol., 26, 183.

McCarthy, B.J., and Hoyer, B.H. (1964). Proc.Nat.Acad.Sci., 52, 915.

McConkey, E.H., and Hopkins, J.W. (1965). J.mol.Biol., 14, 257.

Maitra, U., and Hurwitz, J. (1965). Proc.Nat.Acad.Sci., 54, 815.

Majumbare, C., Tsukada, K., and Lieberman, I. (1967). J.biol.Chem., 242, 700.

Malt, R.A., and Miller, W.L. (1967). J.exper.Med., 126, 1.

Mandel, M. (1962). J.mol.Biol., 5, 435.

Margolin, P. (1965). Science, 147, 1456.

Marmur, J. (1961). J.mol.Biol., 3, 208.

Marmur, J., Lane, D. (1960). Proc.Nat.Acad.Sci., 46, 453.

Marmur, J., Lane, D. (1960). Proc.Nat.Acad.Sci., 46, 453.

Marmur, J., and Greenspan, C.M. (1963). Science, 142, 387.

Martin, R.G. (1963). Cold Spring Harbor Symp.quant.Biol., 28, 357.

Martin, M., and Hoyer, B. (1966). Biochemistry, 5, 2706.

Martinez, Segovia Z.M., Sokol, F., Graves, L.L., and Ackerman, W.W. (1965). Biochim.biophys.Acta, 95, 329.

Marushige, K., and Bonner, J. (1966). J.mol.Biol., 15, 160.

Marushige, K., and Ozaki, H. (1967). Develop.Biol., 16, 474.

Marushige, K., and Dixon, G.H. (1969). Develop.Biol., 19, 397.

Matus, A.I., Ralph, R.K., and Mandel, H.G. (1964). J.mol.Biol., 10, 295.

Mazia, D., and Tyler, A. (1963). Eds., The General Physiology of Cell Specialization. (McGraw-Hill, New York). p.434.

Means, A.R., and Hamilton, T.H. (1966). Proc.Nat.Acad.Sci., 56, 1594.

Mehrotra, B.N., and Khorana, H.G. (1965). J.mol.Biol., 240, 1750.

Mejbaum, W. (1939). Hoppe-Seyler's Z.physiol.Chem., 258, 117.

Merits, I., Schulze, W., and Overby, L.R. (1966). Biochim.biophys. Acta, 115, 197.

Millar, D.B.S., Cutier, R., and Niremberg, M. (1965). Biochemistry, 4, 976.

Mirsky, A.E., and Pollister, A.W. (1946). J.gen.Physiol., 30, 117.

Mirsky, A.E. (1953). Sci.Am., 188, 47.

Miyazara, Y., and Thomas, C.A.Jr. (1965). J.mol.Biol., 11, 223.

Moldave, K. (1965). Ann.Rev.Biochem., 34, 419.

Monier, R., Naomo, S., Hayes, D., Hayes, F., and Gros, F. (1962). J.mol.Biol., 5, 311.

Mueller, G.C., and Le Mahieu, M. (1966). Biochim.biophys.Acta, 114, 100.

Munro, A.J., Jackson, R.J., and Korner, A. (1964). Biochem.J., 92, 289.

Munro, A.J., and Korner, A. (1964). Nature, 201, 1194.

Nakada, D. (1965). Biochim.biophys.Acta, 103, 455.

Nakamoto, T., Fox, C.F., and Weiss, S.B. (1964). J.biol.Chem., 239, 167.

Nathans, D., Notani, G., Schwartz, J.H., and Zinder, N.D. (1962). Proc.Nat.Acad.Sci., 48, 1424.

Nathans, D. (1965). J.mol.Biol., 13, 521.

Nemer, M., and Infante, A.A. (1965). Science, 150, 217.

Nirenberg, H.W., and Matthaei, J.H. (1961). Proc.Nat.Acad.Sci., 47, 1588.

Nishimura, S., Jacob, T.M., and Khorana, H.G. (1964). Proc.Nat. Acad.Sci., 52, 1494.

Nomura, M., Hall, B.D., and Spiegelman, S. (1960). J.mol.Biol., 2, 306.

Nygaard, A.P., and Hall, B.D. (1963). Biochim.biophys.Res.Comm., 12, 98.

Nygaard, A.P., and Hall, B.D. (1964). J.mol.Biol., 9, 125.

O'Malley, B., McGuire, W.L., and Middleton, P.A. (1968). Nature, 218, 1249.

Paul, J., and Struthers, M.G. (1963). Biochem.biophys.Res.Comm., 11, 135.

Paul, J., and Fottrell, P.F. (1963). Biochim.biophys.Acta, 67, 1334.

Paul, J., and Gilmour, R.S. (1966). J.mol.Biol., 16, 242.

Paul, J., and Gilmour, R.S. (1966). Nature, 210, 992.

Paul, J., and Gilmour, R.S. (1968). J.mol.Biol., 34, 305.

Paul, J., and Gilmour, R.S. (1969). J.mol.Biol., 40, 137.

Paul, J., and Gilmour, R.S. (1968). Symp. Internat.Soc.Cell Biol., 7, 135.

Pelling, G. (1959). Nature, 184, 655.

Pelling, C. (1964). Chromosoma, 15, 71.

Penman, S., Scherrer, K., Becker, Y., and Darnell, J.E. (1963). Proc.Nat.Acad.Sci., 49, 654.

Penman, S. (1966). J.mol.Biol., 17, 117.

Perry, R.P., Srinirasan, P.R., and Kelley, D.E. (1964). Science, 145, 504.

Pitot, H.C., Peraino, C., Lamar, C.Jr., and Kennan, A.L. (1965). Proc.Nat.Acad.Sci., 54, 1845.

Pollock, M.R. (1963). Biochim.biophys.Acta, 76, 80.

Ptashne, M. (1967). Proc.Nat.Acad.Sci., 57, 306.

Rake, A.V., and Graham, A.F. (1964). Biophys.J., 4, 267.

Revel, M., and Hiatt, H.H. (1964). Proc.Nat.Acad.Sci., 51, 810.

Richardson, J.P. (1966). J.mol.Biol., 21, 83.

Riggsby, W.S., and Mevriam, V. (1968). Science, 161, 570.

Ritossa, F., Atwood, K., Lindsley, D., and Spiegelman, S. (1966). Nat.Cancer Inst.Monogr. 23, 449.

Robbins, E., and Scharff, M. (1966). Cell Synchrony, p.374.

Robinson, W.S., Hsu, W.T., Fox, C.F., and Weiss, S.B. (1964). J.biol.Chem., 239, 2944.

Samarina, O.P., Lucanidin, E.M., Molnar, J., and Georgiev, G.P. (1968). J.mol.Biol., 33, 251.

Samarina, O.P. (1964). Biochim.biophys.Acta, 91, 688.

Samarina, O.P., and Georgiev, G.P. (1965). 2nd Conf.Nucleic Acids.

Samarina, O.P., Lerman, M.I., Tumanjan, V.G., Ananieva, L.N., and Georgiev, G.P. (1965). Biokhimija, 30, 880.

Samarina, O.P., Kricherskaya, A.A., and Georgiev, G.P. (1966). Nature, 200, 1291.

Sanderson, K.E. (1965). Proc.Nat.Acad.Sci., 53, 1335.

Schaechter, M., Previc, E.P., and Gillespie, M.E. (1965). J.mol.Biol., 12, 119.

Schneiderman, H.A., and Gilbert, L.I. (1964). Science, 143, 325.

Schwartz, J.H., Eisenstadt, J.M., Brawerman, G., and Zinder, N.D. (1965). Proc.Nat.Acad.Sci., 53, 195.

Scherrer, K., and Darnell, J.E. (1962). Biochem.biophys.Res.Comm., 7, 486.

Scherrer, K., Latham, H., and Darnell, J.E. (1963). Proc.Nat.Acad. Sci., 49, 240.

Shearer, R., and McCarthy, B.J. (1967). Biochemistry, 6, 283.

Shigeura, H.T., and Boxer, G.E. (1964). Biochem.biophys.Res.Comm., 17, 758.

Sibitani, A., de Kloet, S.R., Allfrey, V.G., and Mirsky, A.E. (1962). Proc.Nat.Acad.Sci., 48, 471.

Sinsheimer, R.L., and Laurence, M. (1964). J.mol.Biol., 8, 289.

Slater, T.F. (1956). Ph.D. thesis, University of London.

Sonneborn, T.M. (1950). Heredity, 4, 11.

Sonnenberg, B.P., and Zubay, G. (1965). Proc.Nat.Acad.Sci., 54, 415.

Spector, A., and Kinoshita, J.H. (1965). Biochim.biophys.Acta, 95, 561.

Spiegelman, S., and Hayashi, M. (1963). Cold Spring Harbor Symp.quant. Biol., 28, 161.

Spiegelman, S., Haruna, I., Holland, I.B., Beaurdeau, G., and Mills, D. (1965). Proc.Nat.Acad.Sci., 54, 919.

Spirin, A.S., and Nemer, M. (1965). Science, 150, 214.

Spirin, A.S., and Belitsina, N.V. (1965). Usp.Sourem.Biol., 59, 187.

Staeholin, T., Wettstein, F.O., Oura, H., and Noll, H. (1964). Nature, 201, 264.

Stanley, W., and Book, R. (1965). Biochemistry, 4, 1302.

Stedman, E., and Stedman, E. (1950). Nature, 166, 780.

Stent, G.S. (1965). Genetic Transcription. Mendel Centennial Symp.Lecture, Royal Society, London, March.

Stevens, A. (1965). Federation Proc., 24, 655.

Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E., and Inouye, M. (1966). Cold Spring Harbor Symp. quant.Biol., 31, 77.

Swift, H (1962) in The Molecular Control of Cellular Activity. J.M.Allen, Ed. (McGraw-Hill, New York). p.73.

Szybalski, W. (1967). Thermobiology. A.H.Rose, Ed. (Acad.Press, London) p.73.

Tabor, H. (1962). Biochemistry, 1, 496.

Tamaoki, T., and Mueller, G.C. (1963). Biochem.biophys.Res.Comm., 11, 404.

Thaler, M.M., and Villee, C.A. (1967). Proc.Nat.Acad.Sci., 58, 2055.

Threlfall, G., Taylor, D.M., Mandel, P., and Ramuz, M. (1967). Nature, 215, 755.

Threlfall, G.(1968). Cell & Tissue Kinet., 1, 383.

Threlfall, G., and Taylor, D.M. (1969). Europ.J.Biochem., 8, 591.

Trakatellis, A.C., Axelrod, A.E., and Montjar, M. (1964). Nature, 203, 1134.

Trakatellis, A.C., Axelrod, A.E., and Montjar, M. (1964). J.biol. Chem., 239, 4237.

Trakatellis, A.C., Montjar, M., and Axelrod, A.E. (1965). Biochemistry, 4, 1678.

Traub, R.E., Sundararajan, T.A., Dewey, K.F., Brown, J.C., and Doty, P. (1966). Cold Spring Harbor Symp.quant.Biol., 31, 85.

Tsukada, K., and Lieberman, I. (1964). J.biol.Chem., 239, 1564.

Tsukada, K., and Lieberman, I. (1965). J.biol.Chem., 240, 1731.

Ursprung, H., Smith, K.D., Sofer, W.H., and Sullivan, D.T. (1968). Science, 160, 1075.

Vogel, H.J. (1961). Cold Spring Harbor.Symp.quant.Biol., 26, 163.

Vogel, H.J., and Voger, R.H. (1967). Ann.Rev.Biochem., 36, 519.

Volkin, E., and Astrachan, L. (1956). Virology, 2, 149.

Wade, H.E., Lovett, S., and Robinson, H.K. (1964). Biochem.J., 93, 121.

Wahba, A.J., Salas, M., and Stanley, W.M.Jr. (1966). Cold Spring Harbor Symp.quant.Biol., 31, 103.

Walker, P.M.B., and McLaren, A. (1965). Nature, 208, 1175.

Walker, P.M.B. (1968). Nature, 219, 228.

Waring, M., and Britten, R.J. (1966). Science, 154, 791.

Warner, R.C. (1957). J.biol.Chem., 229, 711.

Weissman, C., Simon, L., and Ochoa, S. (1963). Proc.Nat.Acad.Sci., 49, 407.

Wetmur, J.G., and Davidson, N. (1968). J.mol.Biol., 31, 349.

White, M.J.D. (1950). Texas Univ.Publ. 5007, 1.

Whiteley, A.H., McCarthy, B.J., and Whiteley, H.R. (1966). Proc. Nat.Acad.Sci., 55, 519.

Willems, M., Musilova, H.A., and Malt, R.A. Proc.Nat.Acad.Sci., (in press).

Williamson, R., and Brownlee, G.G. FEBS Letters (in press).

Wilson, E.B. (1925). The cell in development and heredity. (Macmillan, New York.)

Wilson, D.B. (1966). Doctoral thesis, Stanford University.

Yankofsky, S.A., and Spiegelman, S. (1962). Proc.Nat.Acad.Sci., 48, 1069.

Yanofsky, C., Carlton, B.C., Guest, J.R., Helinski, D.R., and Henning, U. (1964). Proc.Nat.Acad.Sci., 51, 266.

Yoshikawa-Fukada, M., Fukada, T., and Kowade, Y. (1965). Biochim. biophys.Acta, 103, 383.

Zabin, I. (1963). Cold Spring Harbor Symp.quant.Biol., 28, 431.