THE REGULATION OF PROTEIN SYNTHESIS IN ANIMAL CELLS

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by

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The technique of DNA-RNA molecular hybridization was adopted for investigating the regulation of protein synthesis at the genome level in differentiated animal cells. In preliminary experiments, attempts were made to assay mammalian DNA-RNA complexes by means of methylated albumin-kieselguhr chromatography, Sephadex G-200 chromatography and by selective adsorption on nitrocellulose filters. The latter technique proved to be the most convenient for the present studies, especially when the DNA was immobilised to the nitrocellulose filter prior to hybridization with RNA.

Using this technique for the hybridization of *in vivo* labelled ^{14}C- rat kidney RNA to homologous DNA it was found that under conditions of maximum hybridization only 7% of the DNA formed hybrids with the RNA. This result was taken as evidence for the restriction of DNA template activity in animal cells. In an attempt to detect tissue specific RNAs in various rat organs, comparisons were made of the rapidly ^{32}P-labelled RNA fractions of rat liver, kidney and pancreas by competitive hybridization against unlabelled rat kidney RNA. Differences were observed in the hybridization kinetics of the RNAs from each organ. As it was not possible to determine the real specific activity of rapidly labelled RNA from *in vivo* labelling experiments, it was concluded that the observed differences in competitive hybridization kinetics of these RNAs could be due either to absolute differences in RNA types or due to differences in the specific activities of the RNAs isolated from each organ.

The difficulties associated with the use of *in vivo* labelled RNAs could be avoided by synthesising the RNAs *in vitro* from chromatin primers with the RNA polymerase of Micrococcus lysodeikticus. Experiments in which the RNA synthesised *in vitro* from calf thymus and rabbit thymus and bone marrow chromatins was hybridized to homologous DNA, showed that the template

activity of the isolated chromatin was restricted to 5-10% of the total DNA. Competitive hybridization experiments showed that the RNA synthesised *in vivo* from calf thymus chromatin was qualitatively identical to that synthesised in the *in vitro* system. This was also confirmed with the RNAs synthesised *in vivo* and *in vitro* from rabbit bone marrow chromatin. It was concluded that the restriction of DNA template activity found *in vivo* in animal cells is also a property of the isolated chromatin.

In further competitive hybridization experiments, the RNAs synthesised *in vivo* from rabbit thymus and bone marrow were compared separately with the RNAs synthesised *in vitro* from rabbit thymus and bone marrow chromatins. Evidence was provided for the existence of qualitative differences in the RNA populations of the two tissues. This suggests that the restriction of DNA template activity in differentiated cells is organ specific.

Investigations into the nature of the specific restriction of template activity in calf thymus chromatin suggested that protein ionically bound to DNA is responsible for the restriction. Under specified conditions it was found possible to reconstitute chromatin from crude DNA, histone and non-histone fractions. The nucleohistone obtained by reconstituting DNA with the histone fraction alone, could not direct the *in vitro* synthesis of any RNA capable of hybridising to calf thymus DNA. On the other hand, nucleohistone reconstituted from DNA in the presence of both histone and non-histone fractions resulted in a specific part of the DNA being available for transcription. The magnitude of this unrestricted portion was found to be the same as that of whole chromatin. These results suggest that there exists in calf thymus chromatin a non-histone fraction responsible for conferring the specific restriction of DNA template activity.

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1. INTRODUCTION.

It is currently thought that sequences of nucleotides in the DNA of chromosomes specify sequences of amino acids in proteins. However the question of how proteins are preferentially synthesized has recently attracted attention. This problem is particularly relevant in cytodifferentiation in higher animals, where certain cells are characterized by the specific proteins they synthesize.

The following pages are concerned with some aspects of the mechanism of protein synthesis and how specific protein synthesis might be achieved in animal tissues.

1.1 The working model for protein synthesis.

Detailed information concerning the individual reactions of protein synthesis has appeared in recent reviews (Moldave, 1965; Schweet and Heinz, 1966). Evidence has accumulated in support of the following model for protein synthesis.

Individual amino acids are activated in the presence of specific enzymes, aminoacyl t-RNA synthetases, to form enzyme bound aminoacyl adenylates (Hoagland, 1955; Hoagland *et al.*, 1957; Kingdon *et al.*, 1958; De Moss *et al.*,

1956 and Zachau et al., 1958). Each amino acid is thought to have a specific activating enzyme (Schweet et al., 1958; Berg and Ofengand, 1958; Herve and Chapeville, 1963).

Enzyme bound amino acids are transferred to specific acceptor RNA molecules, transfer RNA, where they are complexed to the adenosine moiety of the terminal cytidylyl-cytidylyl-adenylate sequence. The suggestion by Hoagland (1959) that transfer RNAs are responsible for the location of amino acids in their correct positions in the polypeptide chain as directed by messenger RNA, has been substantiated by Chapeville et al. (1962). Several workers have elucidated the base sequence analysis of specific transfer RNAs; however it has not yet been possible to account for the functional properties of the molecule in terms of its primary structure (Holley et al., 1965; Zachau, et al., 1966).

Aminoacyl transfer RNA complexes are aligned in specific positions determined by the nucleotide sequence of a template which is thought to be messenger RNA. During the formation of nascent protein, aminoacyl t-RNAs are specifically aligned in relation to the growing peptide chain, the C-terminal end of which is linked to t-RNA. The α-amino group of the incoming aminoacyl t-RNA reacts with the carboxyl carbon of the bound peptidyl t-RNA on the ribosome surface resulting in the formation of a new peptide bond and the release of the t-RNA molecule that was pre-

viously attached to the end of the nascent chain. The polypeptide chain which now contains an additional amino acid residue, is linked through the new amino acid to its corresponding t-RNA. The incorporation of aminoacyl moieties requires glutathione and two enzymes. By repetition of this reaction the polypeptide chain grows from its N-terminal to its C-terminal residue.

According to this model the sequence of amino acids is determined by the sequences of ribonucleotides in the messenger RNA which is in turn specified by the sequences of deoxyribonucleotides in the DNA. The transcription of complementary polyribonucleotide sequences from DNA is mediated by a DNA dependant RNA nucleotidyl transferase (E.C. 2.7.7.6). That DNA acts as a repository of information for protein synthesis has been suggested by a number of observations. Perhaps the strongest evidence comes from studies on bacterial transformation (Hotchkiss, 1955; Hotchkiss and Weiss, 1956; Chargaff et al., 1957, and Marmur and Hotchkiss, 1953). Further proof has come from studies in which the effects of mutations have been correlated with changes in protein structure. Yanofsky et al., (1964) examined mutations in a segment of the A gene and A protein of the enzyme tryptophan pyrrolase of E.coli and demonstrated a linear correspondence between the two structures.

Colinearity between gene and protein in animal tissues

has been suggested from investigations on the amino acid sequence in abnormal human haemoglobins. In patients with sickle-cell anaemia the amino acid valine replaces glutamic acid at one position in the α-globin chain of normal haemoglobin. This change is thought to correspond to a substitution of adenine for thymine at the appropriate point in the DNA chain (Ingram, 1961a, b).

The nature of the coding mechanism has been elucidated by Crick et al., (1961) from acridine induced mutations in rII locus of the phage T_4. This locus has not yet been associated with the production of a specific protein, however the findings suggest that the code is composed of non-overlapping base triplets and is degenerate.

It has also been argued that the metabolic stability of DNA as compared with other cellular constituents supports its genetic function. Experiments of Healy et al., (1956) and Thomson et al., (1958) with tissue cultured cells suggest that the DNA remains stable despite repeated cell division. The work of Meselson and Stahl (1958) on DNA replication in rapidly dividing E.coli cultures, provides a mechanism whereby the genetic material is passed on to daughter cells intact, by a "semi-conservative" mode of replication.

1.2 Messenger RNA.

The importance of messenger RNA.

The concept of messenger RNA (m-RNA) occupies a central role in protein synthesis. That RNA might act as a template for protein synthesis is not a new idea (Brachet, 1955); however it has yet to be demonstrated unequivocally that an RNA fraction acts as a messenger in eukaryotes. By definition m-RNA is a polyribonucleotide which determines the sequences of amino acids in a polypeptide chain. It acts as a carrier of information of the genome and when the genome is DNA the base sequence of the messenger duplicates the base sequence of the DNA. In certain viruses, however, RNA serves both as the carrier of genetic information and as messenger. Definite characterization of a messenger RNA would be achieved by isolating a unique cellular RNA that had been transcribed from a DNA template and the further demonstration that this RNA could direct the synthesis of a specific protein. This condition has not yet been satisfied with animal messengers and other criteria are often used. These are discussed in the following sections.

Detection of m-RNA.

The experiments of Cohen (1948), Volkin and Astrachan (1956), and Brenner (1961) on phage infected bacteria, demonstrated that on infection net synthesis of RNA was

stopped abruptly while a small fraction was found to turn over rapidly. The interpretation that this fraction represented phage specific messenger RNA developed largely from the consideration of enzyme induction and repression in bacteria by Jacob and Monod (1961). Thus the rapidly formed RNA in phage infection was not considered peculiar to phage infected cells but rather reflecting a more general sequence of events.

Assuming a rapid turnover, then, at any given time messenger RNA should represent a high proportion of the newly synthesized RNA, although it only constitutes a small proportion of the total cellular RNA. Brief exposure to radioactive RNA precursors should then preferentially label messenger RNA. This so-called pulse labelling technique has been used extensively in the detection of messenger in bacterial and animal cells. Gross *et al.*, (1961) and Risebrough *et al.*, (1962) detected such an RNA fraction in uninfected *E.coli* cells. The idea emerged that protein synthesis in the bacterial cell is controlled by the flow of short lived RNA molecules from the genes to the sites of protein synthesis, and prompted the search for a similarly unstable RNA fraction in animal cells. In early experiments, Siminovitch and Graham (1956) and Labaw *et al.*, (1960) reported that when cultured cells were exposed to radioactive phosphorus and subsequently trans-

ferred to non-isotopic medium, there appeared to be no loss of activity from the RNA, implying that in animals messenger RNA is stable. Watts and Harris (1959) demonstrated that in the mammalian macrophage some of the RNA was rather unstable and that breakdown products were rapidly re-incorporated if they were not displaced from precursor pools by unlabelled precursor. Since then, there have been numerous reports of rapidly labelled RNA in higher cells, e.g. in cultured cells by Scherrer and Darnell (1962), Tamaoki and Mueller (1962), Perry (1962), Paul and Struthers (1963) and Harris (1964a, b), in sheep thyroid by Seed and Goldberg (1963) and in rodent liver by Munro and Korner (1964) and Hoyer et al., (1963).

The stability of m-RNA.

The stability of RNA in general can be deduced by following the decay rate of pulse labelled material in cells after the addition of an inhibitor such as actinomycin D which specifically blocks further RNA synthesis. m-RNA stability can be studied by observing the effect such treatment has on the subsequent rate of protein synthesis.

Using B subtilis, Levinthal et al., (1962) found that pulse labelled RNA had a half life of about 2 minutes, and from a different approach Kepes (1963) determined a mean half life of 1 minute for β galactosidase specific m-RNA by following the induction of β galactosidase in E.coli cells

exposed to a pulse of inducer. Such results with bacterial systems are in accordance with the prediction of Jacob and Monod (1961) concerning the control of protein synthesis by short lived messengers. The occurrence of stable bacterial messengers has been reported by Harris (1964c) and Gause and Laiko (1962).

The fate of rapidly labelled nuclear RNA following actinomycin treatment has been studied in animal cell cultures by several workers (Harris, 1959; Watts and Harris, 1959; Harris, 1963a; Hiatt, 1962; Paul and Struthers, 1963; and Lieberman et al., 1963.) Using concentrations of actinomycin which completely inhibit RNA synthesis it is apparent that much of the rapidly labelled material undergoes breakdown to acid insoluble products within the nucleus. Harris (1964a) maintains that in HeLa cells and rat connective tissue cells, breakdown is not commensurate with the observed rate of protein synthesis within the cells, and concluded that the rapidly labelled RNA is devoid of template function. On the other hand, Hiatt et al., (1963_b) demonstrated rapid uptake of labelled orotic acid into the RNA of rat liver nucleus and cytoplasm and further showed that these fractions produced considerable stimulation of amino acid incorporation in an E.coli cell free protein synthesizing system. Treatment with Actinomycin D did not reduce the stimulatory effect of the cytoplasmic RNA,

suggesting that the template was also stable (Revel and Hiatt, (1964). In support of this view Reich et al. (1962) have shown that in mouse fibroblasts concentrations of actinomycin which inhibit RNA synthesis permit continued protein synthesis for at least 12 hours after administration. Goldstein et al., (1960) using cultured amnion cells and Hämmerling (1953) using the unicellular alga Acetabularia found continuing protein synthesis in the cytoplasm for a considerable length of time after removal of the nucleus. There are numerous other systems in which protein is synthesized in the absence of RNA synthesis, e.g. in reticulocytes (Marks et al., 1962); in sheep thyroid (Seed and Goldberg, 1963); and in fertilized sea urchin eggs (Gross et al., 1964).

Information about the stability of messenger RNA has also come from studies on polysomes in animal cells. In mammalian liver and reticulocytes and in bacterial systems, evidence suggests that protein synthesis is carried out by ribosomes linked together by messenger RNA to form polyribosomes; Warner et al., (1962); Marks et al., (1962); Wettstein et al., (1963); Gierer (1963); and Spyrides and Lipmann (1962). Electron micrograph studies on polyribosomes coupled with their ability to promote the incorporation of amino acids has also provided evidence for their participation in protein synthesis; Rifkind et al.,(1964); Staehelin et al.,(1963); Mathias et al., (1964); and Slayter et al.,(1963).

Several workers have shown that brief exposure of animal cells to radioactive RNA precursors results in the appearance of labelled RNA in the polysomes. In HeLa cells the rapidly labelled component of the polysomes has sedimentation characteristics distinct from those of the 28S and 16S ribosomal RNAs (Penman et al., 1963). After blocking further synthesis of RNA by actinomycin treatment and following the decay rate of polysomal protein synthesis it was concluded that the messenger component of the polysomes had an average half life of about 3 hours. Staehelin et al., (1963) arrived at a similar figure by following the disintegration of polysomes in rat liver after administration of sublethal doses of actinomycin to whole animals. Using the same technique, Trakatellis et al., (1964a) arrived at a half life of 10 hours for polysomal messenger in rat liver. However in a further experiment, under steady state labelling conditions, it was concluded that messenger RNA had a half life of about 4 hours (Trakatellis et al., 1964b). Staehelin et al., (1964) arrived at a mean half life of 2 hours for rat liver polysomal messenger by studying the specific activities of polysomes after administration of radioactive phosphorus to whole animals.

However, as mentioned previously, there are examples of much more stable messenger RNAs in animal systems.

Scott and Bell (1964) examined the effect of actinomycin D on the protein synthesizing capacity of polysomes from various chicken embryo organs. In specialized tissues like ocular lens, protein synthesis is largely unaffected even after 24 hours' exposure to actinomycin D, while in liver and smooth muscle complete cessation of protein synthesis occurred within several hours. The suggestion is made that in tissues which are committed to the synthesis of a restricted number of proteins, e.g. ocular lens (Reader and Bell, 1965), reticulocytes (Marks et al., 1962), and feathers (Humphreys et al., 1964), messenger RNAs may be essentially stable, while in other tissues such as liver shorter lived messengers are present.

As well as inter-tissue differences in messenger stability, evidence has also been found for a spectrum of messenger stabilities within the same cell by Seed and Goldberg (1963) with sheep thyroid, Scott and Bell (1964) with certain chicken organs, Reich et al., (1962) with mouse fibroblasts, and Pitot et al., (1965) with rat liver.

1.3 The function of rapidly labelled nuclear RNA in Animal Cells.

The fact that actinomycin D prevents the incorporation of precursor into both nuclear and cytoplasmic RNA in the intact cell has been considered by some authors as evidence that all cytoplasmic RNA originates in the nucleus (Merits,

1963; Tamaoki and Mueller, 1962). Thus in the preceding section it was tacitly assumed that the rapidly labelled polysomal material was messenger RNA derived from the nucleus.

The fate of rapidly labelled nuclear RNA is however complicated by the difficulty of demonstrating unequivocal transport of labelled material from the nucleus to the cytoplasm and also by the fact that some of the rapidly labelled RNA may function as precursors of ribosomal RNAs.

Transport of nuclear RNA to the cytoplasm.

The studies of Watts (1964b) in HeLa cells has shown that a considerable proportion of the rapidly labelled nuclear RNA is broken down in the nucleus with a half life of 15 to 20 minutes. Harris (1963, 1964a, b) maintains that in animal cells very little of the nuclear material is transferred to the cytoplasm but is degraded *in situ*. In contrast, Scherrer and Darnell (1962) and Scherrer *et al*., (1963) found that only 30 per cent of the rapidly labelled material is broken down. The fate of the remainder was followed after administration of actinomycin D and it was claimed that most of the label was transferred to the cytoplasm as 16S and 28S ribosomal RNAs via an unstable 45S intermediate. Girard *et al*., (1964) and Penman *et al*., (1966) obtained similar results using this approach.

However, Harris (1964b) disputes the interprstation

of these results and points out that if one omits to "chase" labelled material from precursor pools subsequent to actinomycin treatment then continuous incorporation of label occurs both in nucleus and cytoplasm. The result obtained simulates a precursor product relationship of the type found; however Harris maintains that if care is taken to "chase" then complete breakdown of labelled RNA occurs within the nucleus. Paul and Struthers (1963) also observed that if precursor pools are furnished with a continuous supply of labelled precursor then residual drug resistant incorporation occurs. Such results emphasize the difficulties associated with following the fate of rapidly labelled nuclear RNA subsequent to its synthesis.

Georgiev et al., (1963) on the other hand, found that with Ehrlich ascites and human amnion cells cultured in vitro, actinomycin not only inhibited further nuclear RNA synthesis but also prevented the appearance of labelled RNA in the cytoplasm. Furthermore, breakdown of nuclear RNA as described by Harris was not found. In rat liver nevertheless actinomycin treatment leads to a decrease in the activity of nuclear RNA with a corresponding increase in the activity of cytoplasmic RNA. These results were taken to demonstrate transport of RNA from nucleus to cytoplasm in rat liver, but Georgiev et al. also suggested that differential response of some cells to actinomycin D

treatment might also complicate the issue.

Relationship between nuclear and cytoplasmic RNA.

Although some of the rapidly labelled nuclear RNA appears in the "messenger" fraction of polysomes after a few minutes, evidence has been presented that a considerable proportion is also converted to cytoplasmic ribosomal RNA by a much slower process. The attempts of Scherrer and Darnell (1962), Scherrer et al., (1963, Girard (1964), Penman (1966), and Georgiev et al., (1963) to demonstrate this relationship have been mentioned in the previous section. More convincing evidence has come from the autoradiographic studies of Perry et al., (1961) and Perry (1962 and 1963). By treatment of animal cells with ultraviolet irradiation or minimal dosages of actinomycin D, nucleolar RNA synthesis was inhibited without affecting extranucleolar RNA synthesis. Under these conditions the synthesis of cytoplasmic ribosomal RNA is suppressed. In further studies Perry (1964) compared the nuclear RNA of actinomycin treated and untreated L cells by DNA molecular hybridisation. It was concluded that the poor hybridising capacity of nucleolar RNA as compared with total nuclear RNA was due to the presence of ribosomal sequences in nucleolar RNA. An attempt was made to establish the identity of the rapidly labelled 45S RNA component of the L cell by competitive hybridisation against the nucleolar and total

RNA preparations. The results were rather inconclusive as the partial actinomycin treatment did not inhibit nucleolar activity completely; however it would appear from competition data that at least part of the rapidly labelled nuclear RNA is converted to ribosomal RNA with preservation of base sequence and is not rapidly degraded and re-utilised.

Recent evidence suggests that the rapidly labelled nuclear RNA may actually associate within the nucleus to form precursor particles which then migrate to the cytoplasm and eventually form ribosomes. Thus Tamaoki (1966) described a 45S particle in the L cell nucleus comprising both RNA and protein. The formation of these particles preceded the formation of ribosomes, and it was shown by actinomycin administration that the disappearance of labelled 45S RNA was accompanied by a concomitant appearance of label in the ribosomes. Girard et al., (1965), McConkey and Hopkins (1965), Reetow and Köhler (1966), Latham and Darnell (1965b), Henshaw et al., (1965) and Joklik and Becker (1965a, b) have also described this particle in animal cells and in some instances particles of around 60S were observed. The available evidence suggests that 45S particles contain the 18S ribosomal RNA. Restow and Köhler have shown that, by treating KB cells with actinomycin D subsequent to a pulse of radioactive precursor

labelled 45S particles are incorporated directly into polysomes. The nature of the 60S particle is less clear. Several authors have found both 28S and 16S ribosomal RNA species present (McConkey and Hopkins, 1965; Reston and Köhler, 1966); however the similarity to mature 74S ribosomes makes isolation and characterization difficult.

McConkey and Hopkins (1965) isolated the rapidly labelled RNA of 45S particles from HeLa cell cytoplasm and provided evidence from sedimentation and hybridization data for the presence of an RNA species distinct from ribosomal RNA. These authors suggested that messenger RNA was also present in 45S particles in addition to 18S ribosomal RNA. The formation of such a complex as a first step in polysome assembly would provide a mechanism for the transport of messenger to the cytoplasm. Similar results were described by Okamoto and Takanami (1963) in bacteria where messenger RNA appears to complex with the smaller 30S ribosomal subunit prior to the formation of polysomes. Additional support for this scheme was provided by Henshaw et al., (1965) who demonstrated that ^{14}C-labelled polyuridylic acid is capable of binding to 45S particles of rat liver. Perhaps the strongest evidence comes from the studies of Joklik and Becker (1965b) on vaccinia infection of HeLa cells. Almost immediately after the synthesis of viral messenger RNA in the host

cytoplasm it attaches to 45S particles.

Spirin et al., (1964) and Spirin and Nemer (1965) have described similar particles in fish and urchin embryos which contain an RNA fraction capable of stimulating amino acid incorporation in a cell free system. These particles which are thought to contain the "maternal" messenger RNA in the unfertilized egg, become active in protein synthesis when fertilization takes place. Monroy et al., (1965) have shown that if polysomes taken from eggs prior to fertilization are treated with trypsin they are then capable of supporting high levels of protein synthesis.

The concept of a subribosomal particle containing messenger RNA is an attractive solution to the problems of messenger transport and protection but there is as yet no conclusive evidence to suggest that the non-ribosomal component is in fact messenger RNA. The implication that very little free messenger exists in the cytoplasm is supported by the work of Samarina (1964) who found that of the "DNA-like RNA" in rat liver cytoplasm only about 15 per cent existed free while the remainder was complexed in ribonucleoprotein particles. It is not certain whether the supposed messenger is complexed within the nucleus or the cytpplasm, however the possibility that such particles might be formed at the site of transcription on the DNA has been suggested by the findings of Byrne et al., (1964) who

showed that ribosomes could complex with T_2 DNA *in vitro* providing that RNA synthesis was taking place.

1.4 Other criteria for messenger RNA.

Molecular weight.

Numerous attempts have been made to estimate the molecular weight of the rapidly labelled RNA of animal polysomes by sucrose gradient sedimentation. In general a heterogeneous spectrum of molecular weights appears in this fraction. Munro and Korner (1964) found that the messenger RNA of rat liver microsomes had a sedimentation coefficient of between 4 and 18S. Similar observations have been reported by Staehelin *et al.*, (1964), Trakatellis *et al.*, (1964), and Ogata *et al.*, (1963) with rat liver; by Penman *et al.*, (1963) with HeLa cells, and by Kruh *et al.*, (1964_b) with rabbit reticulocytes.

Although a unique messenger RNA might well have a unique molecular weight (Burny and Marbaix, 1965) the above results emphasise the heterogeneity of total messenger RNA of the cell. The poly-disperse nature of the RNA renders molecular weight determinations a rather unsatisfactory criterion for messenger. Size distribution can also be affected by complications such as degradation (Higashi and Busch, 1965); association of messenger RNA with ribosomal RNA under certain conditions (Staehelin *et al.*, 1964); and with Polysaccharides (Martinez *et al.*, 1965).

Base composition.

The suggestion that the base composition of messenger RNA should reflect that of the cellular DNA has come mainly from work on bacterial systems (Hayashi and Spiegelman, 1961; Bolton and McCarthy, 1962). No conclusive proof of this complementarity has been offered in animal systems, although attempts have been made to establish such a relationship between the rapidly labelled RNA and homologous DNA by DNA-RNA hybridization (Scherrer et al., 1963; McCarthy and Hoyer, 1964; and Hoyer et al., 1963). From these experiments it is apparent that the messenger RNA represents a rather small proportion of the total DNA and might argue against equivalence of base composition.

In animal cells the base composition of ^{32}P rapidly labelled polysomal RNA has been determined by nearest neighbour frequency analysis. Penman et al., (1963) concluded that the messenger fraction of HeLa cell polysomes had a base composition more reminiscent of HeLa DNA than that of ribosomal RNA. However, Munro and Korner (1964) showed that there is a distinct dissimilarity between the polysomal messenger of rat liver and both rat DNA and ribosomal RNA. Ishikawa et al., (1964) and Brawerman (1963) arrived at similar conclusions from analysis of messenger fractions isolated from rat liver. Sibitani et al., (1962) also described a method for the isolation of rapidly labelled

RNA from calf thymus nuclei and demonstrated a considerable resemblance to the base composition of thymus DNA. The base composition of the isolated "messenger" appears to depend to some extent on the fractionation procedure employed. Where nuclei are taken as the source of rapidly labelled RNA, complications will also arise due to the presence of ribosomal precursor species.

Georgiev et al., (1963) attempted to circumvent these difficulties by injecting minimal concentration of actinomycin D into rats. Under these conditions it is maintained that a partial block of RNA synthesis occurs, mostly to the detriment of ribosomal species, while messenger RNA 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.

Hoyer et al., (1963) also attempted to fractionate the rapidly labelled RNA of mouse liver by DNA-RNA hybridization. Neither the hybridized nor unhybridized RNA resembled the overall DNA composition. Thus similarity to DNA is not necessarily a good criterion for complementarity. Furthermore, if only a limited region of the DNA in liver cells functions in producing complementary RNA the composition of this region may not reflect the average DNA composition. Other examples of disparity between base composition of the hybridized RNA and the DNA are known, and are typified by

the hybridization of ribosomal RNA (Yankofsky and Spiegelman, 1962a, b).

In vitro stimulation of protein synthesis.

There is a considerable body of evidence to implicate polysomes as the sites of in vivo and in vitro incorporation of amino acids into proteins (Rifkind et al., 1964; Staehelin et al., 1963; Mathias et al., 1964; Schaechter et al., 1963; Slayter et al., 1963; Marks et al., 1963; Henshaw et al., 1963; and Seed and Goldberg, 1963).

The discovery by Matthaei and Nirenberg (1961) that synthetic messenger RNAs can direct the in vitro incorporation of specific amino acids into protein provided a means for testing natural and synthetic messengers as templates for protein synthesis. In experiments with bacterial ribosomes it has been shown that the presence of polyuridylic acid gives rise to a spontaneous formation of polysome-like aggregates in which virtually all the activity for phenylalanine incorporation is concentrated (Barondes and Nirenberg, 1962; Spyrides and Lipmann, 1962). Ribosomes from Ehrlich ascites cells (Pedersen et al., 1963), rat liver (Henshaw et al., 1963) and rabbit reticulocytes (Weinstein et al., 1963), normally inert in in vitro incorporating systems, can be stimulated to incorporate phenylalanine in the presence of polyuridylic acid. The implication that synthetic messenger RNAs were assuming functions normally ascribed to in vivo messengers

led to the search in animal cells for RNA fractions capable of stimulating amino acid incorporation. Brawerman et al., (1963) described stimulation by an RNA fraction from rat liver nuclei and Hiatt et al., (1963b) obtained stimulation from cytoplasmic as well as nuclear fractions of liver. In the final analysis however the RNA in question must be shown to direct the synthesis of a specific protein. This criterion has been met in several investigations. Nathans et al., (1962) were able to demonstrate that coliphage f2 RNA is translated into f2 coat protein by the E.coli cell free system, and Schwartz et al., (1965) confirmed these data using a cell free system from Euglena gracilis. The messenger role of the RNA from coliphage MS2 (Nathans, 1965) and satellite tobacco necrosis virus (Clark et al., 1965) has been shown to act in a similar manner. Hall et al., (1965) isolated the messenger RNA for gramicidin S and demonstrated the synthesis of this antibiotic by adding the RNA to a B. brevis cell free system. On the other hand, Aach et al.(1964) found that the protein synthesised in the E.coli system under the direction of TMV RNA was not a TMV-specific protein.

In animal systems the quest for the in vitro synthesis of a specific protein has to a large extent concentrated on the enucleate mammalian reticulocyte because of the stability of the messenger RNA and because most of the protein synthesized in these cells in haemoglobin. Brawerman et al.,

(1965) showed that the protein synthesized upon adding reticulocyte RNA to the E.coli cell free system exhibited a valine to isoleucine ratio which approached that of globin; however in a similar study Schaeffer et al., (1964) reached a contrary conclusion. Drach and Lingrel (1966) presented evidence that the stimulation obtained when rabbit reticulocyte RNA was added to the E.coli system was due to the enhanced synthesis of coli-type protein rather than the synthesis of haemoglobin. The suggestion that the bacterial and mammalian components might be incompatible in the cell free system led some investigators to use homologous mammalian cell free systems. Arnstein et al., (1964) demonstrated the synthesis of haemoglobin in a rabbit reticulocyte cell free system in which RNA from polysomes was added to reticulocyte ribosomes. However the ribosomes alone were capable of incorporating amino acids; therefore it is not clear whether the messenger fraction was stimulating de novo synthesis or whether the in vivo synthesis of incomplete haemoglobin chains on the ribosomes was being completed during in vitro stimulation. Kruh et al., (1964a, b) carried out similar experiments with rabbit reticulocytes but failed to distinguish between stimulation and de novo synthesis. This drawback of homologous systems was demonstrated by Knopf and Dintzis (1965) who concluded that only about 1 per cent of the observed stimulation was attributable to de novo protein synthesis.

Additional complications in cell free systems from homologous or closely related species are seen from the results of Lamfrom (1961) who found that the type of haemoglobin formed corresponded in part to that of the ribosome donor and in part to that of the supernatant donor in a cell free system employing rabbit and sheep components. Kruh et al., (1961) and Shapira et al., (1966) present similar results mainly using rabbit and guinea pig.

Thus to date there are no conclusive experiments which demonstrate de novo synthesis of a specific animal protein in an in vitro system. Until this condition is satisfied the evidence for messenger RNA in animal cells will remain largely circumstantial.

1.5 The enzymic synthesis of RNA on DNA templates. RNA polymerase.

The messenger RNA hypothesis provides a specific model for the genetic control of protein synthesis, the central feature of which is the transcription of DNA nucleotide sequence into complementary sequences in the messenger RNA. Within recent years numerous systems of bacterial, plant and animal origin have been described that catalyse formation of polyribonucleotides from adenosine, guanosine, cytosine and uridine 5'-triphosphates. The enzyme catalysing this reaction, RNA nucleotidyl transferase (E.C. 2.7.7.6) or RNA polymerase has been isolated in soluble form from a number of bacterial

sources (Fuchs et al., 1964; Chamberlin and Berg, 1962; Furth et al., 1962; Nakamoto et al., 1964; Stevens and Henry, 1964). Till recently the only animal sources of soluble RNA polymerase were embryonic (Furth and Loh, 1963) neoplastic tissues (Furth and Loh, 1964) and rat testis (Ballard and Williams-Ashman, 1964). Most animal RNA polymerases appear to be inseparably bound to the cellular DNA giving insoluble aggregates (Weiss, 1960; Busch et al., 1962; Rho and Bonner, 1961; Hancock et al., 1962; and Gorski, 1964). In general, animal cells are rather poor sources of RNA polymerase, consequently most of our knowledge concerning the mechanism of transcription has come from studies with bacterial enzymes.

The transcription of DNA templates in vitro.

The reaction by which RNA polymerase catalyses the synthesis of RNA from a DNA template can be summarized by the following equation:

$$n_1ATP + n_2GTP + n_3CTP + n_4UTP \xrightarrow[DNA]{} (AMP_{n1}\text{-}GMP_{n2} - CMP_{n3} - UMP_{n4}) + (n_1 + n_2 + n_3 + n_4)\ PP.$$

The speculation that DNA might serve as a template for the polymerization of ribonucleotides into a complementary RNA was confirmed by studies from a number of different groups (Weiss and Nakamoto, 1961; Furth et al., 1961; Geiduschek et al., 1961; Hurwitz et al., 1962; Chamberlin and Berg, 1962). These results show that:

(a) When different DNAs are used as primers the base composition of the RNA product always resembles that of the primer used.

(b) The nearest neighbour frequencies for the 16 possible base pairs in the RNA formed are remarkably similar to the frequencies in the DNA primer.

(c) DNA-RNA hybrids can be formed only when the RNA product is hybridised with the DNA which served to direct its synthesis.

Further evidence has come from the use of synthetic polydeoxyribonucleotides as primers in *in vitro* systems. Chamberlin *et al.*, (1963) and Furth *et al.*,(1961) showed that when poly d.AT. is used as primer for the *E.coli* RNA polymerase the product of the reaction, poly r.AU, contains a regularly alternating sequence of AMP and UMP residues.

Native RNA has been shown to act catalytically in *in vitro* reactions (Stephens, 1961; Chamberlin and Berg, 1962). Also the primer remains unaltered after the reaction, as judged by buoyant density and transforming ability (Geuduschek *et al.*, 1961; Hurwitz *et al.*, 1962). Evidence suggests that the template sequences are copies in a fully conservative manner with the product existing as template free RNA (Geiduschek *et al.*, 1961; Hurwitz *et al.*, 1962; Chamberlin and Berg, 1964).

There is some uncertainty as to the mechanism of attach-

ment of the polymerase to DNA. It has been suggested that the enzyme interacts with a specific site or initiation point on the DNA and subsequently synthesizes RNA from that only. Bremer and Konrad (1964) reported that the T4 genome had at least 50 starting points for RNA transcription. The fact that heat denatured DNA is capable of acting as primer and that it binds very strongly to the polymerase (Wood and Berg, 1964; Maitra and Hurwitz, 1965) has led to the suggestion that impaired regions in the native primer may act as initiation points. However studies with synthetic homopolymer primers suggest that a specific initial sequence is not required (Fox et al., 1965) for binding. Nevertheless the lack of this requirement does not rule out the possibility that a specific sequence is involved. Indeed, recent evidence indicates that RNA synthesis with the E.coli polymerase starts preferentially with purine residues (Maitra and Hurwitz, 1965; Bremer et al., 1965).

Other enzymic syntheses of polyribonucleotides.

In addition to the DNA primed reaction requiring all four ribonucleotide triphosphates, several workers have described the formation of homopolymers where only a single triphosphate is present (Chamberlin and Berg, 1962; Goldberg et al., 1962; Fox and Weis, 1964; and Stevens, 1964). Also some RNA polymerases are capable of catalysing RNA synthesis in the presence of RNA primers (Fox et al., 1964; Krakow

and Ochoa, 1963). This reaction appears to be analogous to the DNA primed reaction, the product being complementary to the primer used. The presence of RNA dependent RNA polymerases has also been demonstrated during the infection of bacterial cells (Weissman et al., 1963; August et al., 1963, and Spiegelman and Doi, 1963) and animal cells (Baltimore and Franklin, 1963) with various RNA viruses. On infection the incoming viral RNA is thought to act as messenger for viral specific proteins including the polymerase, which then promotes the synthesis of more viral RNA.

The ~~catalysis~~ synthesis of polyribonucleotides from individual nucleoside 5' ~~mono~~di phosphates or from mixtures of the four nucleoside 5' ~~mono~~di phosphates has been demonstrated by the enzyme polynucleotide phosphorylase. Here the presence of primer is stimulatory but not obligatory (Grunberg-Manago, 1963).

1.6 Differentiation and the problem of specific protein synthesis.

Previous sections have discussed the phenomenon of protein synthesis in terms of information flow from the genetic material of the cell to the final protein molecule. Messenger RNA is thought to be an important intermediate in the transfer of information.

Although this scheme explains the mechanics of protein synthesis it is less clear how the synthesis of specific

proteins can be brought about in animals, where the development of an embryo from a fertilized egg is accompanied by the sequential appearance of cells with characteristic morphological and functional properties. As a result of this process, known as cytodifferentiation or cell differentiation, it can be shown that certain cells possess specific proteins which are not to be found in other cell types. For example, haemoglobin synthesis is a feature of reticulocytes as is insulin of pancreas and myosin of muscle tissue. The fact that different proteins arise in cells which were originally derived from a common precursor poses the problem of how this comes about. It is assumed that the synthesis of specific protein in certain cells is due to selective expression of the genetic material in these cells. The two most obvious mechanisms for effecting this would be:

(a) to destroy selectively that part of the genetic material which is redundant in the differentiated cell.

(b) to mask the redundant regions of the DNA so that they are incapable of expression.

Differentiation by chromosome deletion.

In certain invertebrates, namely the gall midge Mayetiola (White, 1950) and the horse nematode, Ascaris (Wilson, 1925) cytodifferentiation is achieved by the selective loss of genes. Only the germ cells of these species contain

the full chromosome complement. Another example of differentiation by this method is exemplified by the mammalian erythrocyte. In most species mature erythrocytes are formed by the extrusion of the reticulocyte nucleus. However it is generally believed that these examples are extreme forms of differentiation on the basis of evidence concerning the qualitative and quantitative characteristics of DNA in other differentiated animal tissues.

The qualitative and quantitative characteristics of DNA.

In general the chromosome set or karyotype is constant for the somatic cells of the individual and for all individuals of the species (Brachet, 1941). The nuclei of diploid somatic cells from tissues of a given species usually contain constant amounts of DNA, except for sperm cells which contain the haploid number of chromosomes and hence half the amount of DNA of somatic cells (Boivin et al., 1948; Mirsky and Ris, 1949, 1951; Davidson and Leslie, 1950; Vendrely, 1955). Other exceptions also exist; for example polyploid cells are found in rodent liver (Swift, 1950; Thomson et al., 1953) and in human liver (Swartz, 1956).

This evidence suggests that within the limits of chemical analysis the cellular DNA content of the tissues of a particular species is constant; however it reveals nothing to suggest that the genetic complement of these

tissues is also equivalent. Several workers have argued that the genetic complement is preserved throughout the differentiated tissues of an organism.

In the experiments of McCarthy and Hoyer (1964) DNA molecular hybridisation was used to compare the nucleotide sequences in DNA from a variety of mouse organs with those of whole mouse embryo DNA, which presumably contains sequences representative of every mouse cell. The results suggest that within the sensitivity limits of the method, the DNA molecules in all the mouse organs examined are identical.

More definitive results have been obtained in amphibia using the technique of nuclear transplantation as laid down by Briggs and King (1960). In these experiments the nucleus from a blastula or more advanced Rana embryo was transplanted into an egg the nucleus of which had been previously removed. The extent of subsequent development of the egg was then used as a measure of the morphogenic capacity of the nucleus. It was found that nuclei from early stages (up to blastula) could give rise to normal tadpoles; however if nuclei from later stages were taken (e.g. gastrula) most of the transplants showed abnormalities and ceased to develop. It was concluded that once the nuclei had progressed to a certain stage along a given developmental pathway they were incapable of initiating normal developmental processes. Hennen (1963)

on the other hand, found that karyotypic abnormalities account for the irreversible restriction of potency of nuclei transplanted into the egg cytoplasm of other species. He suggested that the result of Briggs and King may be a consequence of the experimental procedure rather than of normal differentiation.

Gurdon (1966) carried out similar experiments with Xenopus which are also contrary to those of Briggs and King (1960). In this case, nuclei which had been removed by microdissection were transplanted into eggs whose nuclei had been inactivated by ultraviolet irradiation. In some instances the eggs developed normally to produce embryos with the characteristics of the nucleus donor. In one such experiment the nucleus taken from an intestinal mucosal cell of a swimming tadpole eventually developed into a complete frog.

This evidence strongly suggests that in differentiated cells redundant genes are conserved and though they are inactive they can be made to function by exposure to the appropriate environment.

Differentiation by gene repression.

Jacob and Monod Theory. Consideration must therefore be given to mechanisms which regulate expression of genes without the genes being altered. The mechanism proposed by Jacob and Monod (1961) for the control of protein synthesis

in bacterial cells has attracted much attention and has been recently reviewed by several authors (Ames and Martin, 1964; Stent, 1964; Jacob and Monod, 1963).

In this scheme it is proposed that certain genes, regulating genes, make products that can act through the cytoplasm to prevent the functioning of other genes. These other genes are organized into operons, with operator genes behaving as acceptors for the repressor. Evidence for the existence of repressors has come from the isolation of R- mutants which are incapable of synthesizing repressor and which synthesize β galactosidase constitutively. On mating with an R+ or inducible strain the heterozygotes, in which R+ is dominant, show the normal (inducible) type of behaviour (Pardee et al., 1959). Recently Gilbert and Müller-Hill (1966) isolated the lactose repressor of E.coli by virtue of its affinity for a β galactoside inducer. It appears to be a protein of molecular weight around 200,000.

Although this theory accounts for many aspects of enzymic adaption in bacteria and animals it is less satisfactory in providing a solution to the problem of differentiation in animal cells. In enzyme induction the continual presence of inducer is necessary. Often the effect of induction is only to produce an increase in the concentration of an enzyme already present in very small amounts. Moreover, induction is generally a reversible phenomenon and

ceases on removal of inducer.

Cytodifferentiation on the other hand is generally a permanent change and may be initiated by brief exposure to stimuli at a specific developmental stage. Pollock (1963) has reported an exceptional case of pellicilinase induction in B.subtilis which is more reminiscent of cytodifferentiation. Here, a brief exposure of inducer was sufficient to propagate a continuous synthesis of enzyme. The most striking difference between the two phenomena is that enzyme induction is reversible whereas cytodifferentiation is generally an absolute static condition. Whereas low levels of enzyme may exist in the cell prior to induction, in the differentiated cell certain proteins are completely absent. It may be the case that a Jacob-Monod control system may operate in animals on those parts of the genome not subject to permanent repression. However, there are again certain differences between bacterial and animal systems. The efficacy of the control in bacteria relies on the short half life of mRNA; however in animal cells mRNA is considerably more stable. Also animal proteins are in general less stable than bacterial proteins. If this type of control does operate in animal cells the kinetics and details of the mechanism may differ substantially from those postulated for bacteria.

Cytodifferentiation therefore demands a permanent mode

of genetic repression, since some genes of the differentiated cell are destined to remain inactive for the remainder of its life cycle. McCarthy and Bolton (1964) found that very little of the E.coli genome is not transcribed in vivo, suggesting that this type of repression is not found in bacteria. For these reasons it has been proposed that differentiation in animal cells necessitates a special type of mechanism.

The masking hypothesis.

In earlier investigations Stedman and Stedman (1950) suggested that histones associated with DNA might function as gene regulators, although there was no evidence to support this at the time. Within recent years however some evidence has been obtained which suggests that masking may occur in animal cells.

Dipteran chromosomes. Probably the strongest evidence to support the masking theory has come from studies on the giant chromosomes found in larval Diptera. In the polytene chromosomes of Dipteran salivary glands, which have attracted most attention, repeated DNA replication in the absence of mitotic segregation results in a multi-stranded chromosome in which the individual chromosomes appear as transverse bands. These structures are subject to localized swelling or puffing at various loci along the chromosome length (Swift, 1962) and it has been shown that this is a highly

specific phenomenon, a given tissue being characterized by a unique pattern of puffing during the course of its development (Beerman, 1952; Breuer and Pavan, 1955; Kroeger 1960; Clever, 1962; and Pelling, 1964).

Beerman (1959) has argued that the time and tissue specific puffing of certain chromosome bands provides cytological evidence for differential gene activity. Evidence in support of this hypothesis has come from autoradiographic studies of Pelling (1959, 1964) which demonstrate that tritiated uridine was actively incorporated into puffed regions of the chromosome. Unpuffed regions were inactive.

Using elegant microanalytical methods, Edstrom and Beerman (1962) found that the base composition of RNA derived from puffs on the same Chironomus chromosome was characteristic, was highly asymmetrical and had a high adenine content. The suggestion was made that each puff synthesizes a specific messenger RNA. Beerman (1961) described a case in which the correlation between puffing and gene activity is quite clear. In Chironomus tentants the salivary secretion lacks a certain kind of granule which is found in other species. This deficiency is not found in crosses with normal species, and is accompanied by the appearance of a new puff not present in homologous C.tentans chromosomes. Further evidence of the specific nature of

puff formation has come from Clever (1966) who demonstrated that *in vitro* treatment of chromosomes with the Dipteran pupating hormone ecdysone produced the same puffing patterns observed in the intact larva.

Repressed and active chromatin. Although puffs have not been detected in the chromosomes of higher animals, evidence suggests that the chromatin exists in condensed (heterochromatic) and uncondensed (euchromatic) states within the nucleus.

Frenster *et al.*, (1963) described the occurrence of heterochromatin and euchromatin in the interphase nuclei of calf thymus lymphocytes. Previous studies (Allfrey and Mirsky, 1962; Allfrey et al., 1963) suggest that up to 80 per cent of the DNA is present as heterochromatin. Electron micrographs show heterochromatin as condensed agglomerates while euchromatin exists as extended fibrils. Morever it was found from isotope incorporation that the bulk of the rapidly labelled RNA of the nucleus was associated with the euchromatin while heterochromatin appeared to be less active in the RNA synthesis.

Support for this concept has come from investigations on the heterochromatic sex chromatin described by Barr (1949) in female mammalian cells. In normal females one of the two X chromosomes is heterochromatic. In humans with multiple X chromosomes only one of the X's is euchro-

matic while the remainder become heterochromatic. The evidence suggests that the heterochromatic chromosomes possess genes which are unexpressed in the cell phenotype (Lyon, 1961; Russel, 1961).

Additional evidence is provided from studies on the mealy bug. Here the paternal chromosome set becomes heterochromatic early in development and is discarded before sex cell formation (Brown and Nur, 1964). Only the euchromatic maternal chromosome set is transmitted by males to their offspring. Autoradiography has also shown that RNA transcription takes place from euchromatic and not heterochromatic chromosomes of the mealy bug (Berlowitz, 1965).

Suppression of RNA synthesis in vitro. It has been suggested that the behaviour of chromatin in in vitro RNA synthesizing systems provides evidence for the masking hypothesis. Huang and Bonner (1962) and Bonner et al., (1963) reported that pea embryo chromatin was a much less efficient primer for RNA synthesis than pea DNA and implied that this resulted from masking of DNA in the chromatin. Similar conclusions have been made by Dahmus and Bonner (1965) and Marushige and Bonner (1966) with liver chromatin, Frenster et al., (1963) with thymus lymphocytes, Barker and Warren (1966) with rat uterus, Flickinger et al., (1965) with Rana pipiens, and Kim and Cohen (1966) with tadpole

liver, using rates of RNA synthesis as a criterion for template activity.

Rationale behind the present approach.

The present work describes attempts to establish the existence and nature of masking in the DNA of animal tissues. It is assumed that the control of specific protein synthesis is exerted at the transcriptional level.

If the masking hypothesis is valid then the chromatin of animal cells should contain some DNA sequences which are active and some which are inactive in RNA synthesis. Several experiments were mentioned in the previous section in which comparative rates of RNA synthesis from DNA and chromatin primers were provided as evidence for masking. This conclusion was criticised by Sonnenberg and Zubay (1965) who pointed out that the rate of RNA synthesis from chromatin could be affected by the insolubility of the primer in the assay system. They strengthened their argument by demonstrating that if the chromatin was solubilised by sonication then its priming efficiency was markedly increased. Clearly rate kinetics are unsuitable criteria for masking. A more conclusive approach would be to compare the DNA sequences involved in RNA transcription and not the absolute amounts of RNA synthesized.

DNA molecular hybridization offers a direct method for testing the validity of the masking hypothesis. The aim

of preliminary work was to adopt a suitable hybridization technique and to develop a system for the synthesis of chromatin primed RNA.

MATERIALS AND METHODS.

2. Materials and Methods.

2.1 Materials.

Isotopes.
Nucleic acid precursors.
Enzymes.
Preparation of bentonite.
Preparation of UT^{32}P.

2.2 Methods.

Preparation of citric acid nuclei.
Preparation of RNA.
Isolation of *in vitro* synthesized RNA.
Preparation of *in vivo* labelled RNA.
Preparation of DNA.
Determination of radioactivity.
The quantitative estimation of protein.
The quantitative estimation of DNA.
The quantitative estimation of RNA.

2.1 Materials.

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

Isotopes.

^{14}C- and ^{3}H-nucleoside triphosphates were obtained from Schwartz Bioresearch Inc. (Orangeburg, N.Y.). ^{3}H uridine, carrier free ^{32}P-orthophosphate, ^{14}C-sodium formate and ^{32}P-cyanoethyl phosphate 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, (Colnbrook, England).

Highly polymerized yeast RNA was supplied by British Drug Houses Limited.

Enzymes.

Crystalline pancreatic ribonuclease (bovine) was obtained from Sigma Chemical Co. Stock solutions of 2 mg/ml in 0.01 M tris-HCl pH 7.5; 0.001 M $MgCl_2$ were first heated in boiling water for 10 minutes to destroy any deoxyribonuclease activity, and stored frozen.

Deoxyribonuclease I (E.C. ~~2.7.7.16~~ 3.1.4.5) was prepared electrophoretically by Worthington Biochemical Corp. (Free-

hold, N.J.). Solutions of 1 mg/ml in 0.01 M tris-HCl pH 7.5; 0.001 M $MgCl_2$ were stored frozen. Incubations always contained 0.001 M $MgCl_2$.

Pronase from Calbiochem. was supplied as a powder which was dissolved in water to a concentration of 500 µg/ml. Stock solutions were autodigested at 37°C for 2 hours prior to use to destroy any nuclease activity.

Preparation of bentonite.

Crude bentonite from British Drug Houses Ltd. was homogenised in 20 vols. (w/v) water and centrifuged at 15,000 g. for 25 minutes to give a pellet consisting of an upper yellow and lower brown layer. The former was removed and suspended in a volume of 0.1 M E.D.T.A. pH 7.5 equal to that of the original homogenate. After standing for 48 hours the material was centrifuged as before. The sediment was suspended by homogenization in a volume of 0.01 M sodium acetate equal to half the volume of the original suspension. The concentration of this suspension was determined by noting the wet weight of sediment obtained after centrifugation of a known volume at 15,000 g. for 25 minutes.

Preparation of UT^{32}P.

(^{32}P)UMP was prepared from ^{32}P cyanoethyl phosphate by the method of Tener (1961) and was phosphorylated by the method of Smith and Khorana (1958) to yield (α^{32}P)UTP.

^{32}P cyanoethyl phosphate was dissolved in 20 ml. 20 per

cent (v/v) acetic acid and passed through a Dowex-50-H^+ column (8 x 2 cm.). The effluent was taken to dryness and redissolved in 10 ml. anhydrous pyridine to which isopropylidine uridine had been added in the ratio of 1m mole isopropylidine uridine to 0.5 m moles cyanoethyl phosphate.

The solution was concentrated to an oil *in vacuo* at 40°C. 10 ml. anhydrous pyridine was added and the solution again concentrated to dryness. The process was repeated once more and the residue was dissolved in 5 ml. anhydrous pyridine and dicyclohexylcarbodiimide (DCC.) in the proportion 2.0 m mole DCC to 1 m mole isopropylidine uridine.

After 20 hours at room temperature the well stoppered flask was opened and the reaction terminated by the addition of 10 ml. of water. After 1 hour the mixture was concentrated to dryness *in vacuo*. The residue was hydrolysed for 90 minutes in 10 per cent (v/v) acetic acid (40 ml) at 100°C to remove the isopropylidine group and cleave phosphoamide bonds. The acetic acid was removed by evaporating the solution to dryness, the last traces being removed by adding 10 ml. of water and re-evaporating. The residue was heated at 60° for 90 minutes with 40 ml. 9 *N* ammonium hydroxide to remove cyanoethyl groups, and the ammonia removed by concentrating the mixture to dryness. 10 ml. of water was added to the residue and the insoluble DCU was removed by filtration under reduced pressure.

The precipitate was washed with a small volume of water and a sample of the filtrate taken for paper chromatography of the reaction products. The precipitate was then washed very thoroughly with 200 mls water. Descending chromatography was performed on Whatman No.1 chromatography paper using UMP, uridine and isopropylidine uridine as markers. The chromatogram was developed for 18 hours in an ammonium isobutyrate system containing 100 mls isobutyric acid, 55.8 mls. water, 4.2 mls. 0.88 NH_4OH and 1.6 mls 0.1 M versene. Ultraviolet absorbing spots were scanned for radioactivity.

The combined filtrates were diluted to about 250 mls then applied to a Dowex-1-Cl' column and washed with water until the extinction at 262 mμ was less than 0.05. The ^{32}P UMP was eluted with 500 mls. 0.05 N HCl, the total extinction determined and the yield of UMP calculated. The eluate was concentrated to an oil in vacuo at 45°C.

The reaction mixture for UTP synthesis contained the following components for each 100 μmoles of ^{32}P UMP;- 1.2 ml. tri-n-butylamine, 6 mls. pyridine, 0.2 ml. 85 per cent (v/v) phosphoric acid and 3 g. DCC.. The mixture was allowed to stand at room temperature for 48 hours. At the end of the reaction a thick precipitate of DCU had formed. The flask was shaken and left for 1 hour in ice. The DCU was filtered off under reduced pressure and washed

with water. The eluate was extracted with four 50 ml. portions of ether to remove pyridine and the ether washes extracted with two 10 ml. portions of water, these being added to the main aqueous phase.

The combined aqueous phase was concentrated at 40° in vacuo. The material was applied to a Dowex-50-Na^+ column (4 x 10 cm.) to remove tri-n-butylamine. The uridine derivatives were eluted by washing with water until the extinction of the eluate at 262 mμ was less than 0.05. The total extinction of the eluate at 262 mμ was determined and the yield of uridine derivatives calculated. The effluent was diluted to 1 litre and absorbed on Dowex-1-Cl' column (2 x 20 cm.). The column was washed with water until the extinction was less than 0.05. Gradient elution from the Dowex-1-Cl' column was then carried out with 1.3 litres of 0.01 N HCl in the mixing vessel and 2 litres of 0.3 M LiCl in 0.01 N HCl in the reservoir. The eluate was collected in 20 ml. fractions which were scanned automatically for radioactivity. The fractions containing $\alpha^{32}P$ UTP were pooled and the total yield determined. LiCl, HCl and inorganic phosphate were removed on an activated charcoal column which was prepared in the following manner. The charcoal column (1 x 10 cm.) was washed with ethanol ammonia (70 per cent v/v aqueous ethanol containing 10 mls. concentrated ammonia per litre) until the extinction at

262 mμ fell to less than 0.1. The column was then washed with water, 0.01 M $NaHCO_3$ and finally with 5 column volumes of 1 N HCl. The column was then resuspended in water, fine particles were decanted off and washing was continued until neutral. The column was then repacked.

The $\alpha^{32}P$ UTP fraction from the Dowex-1-Cl^- column was absorbed on to the charcoal column and water washing carried out until no chloride ions could be detected in the effluent. Washing was continued with small volumes of 0.01 M $NaHCO_3$ to remove inorganic phosphate until ultraviolet absorbing material began to appear in the eluate. The column was allowed to drain and then washed with 2 column volumes of water.

$\alpha^{32}P$ UTP was eluted with about 1 litre of ethanol ammonia and was concentrated in vacuo at 25°. The material was redissolved in water, passed through a small Dowex-50-Na^+ column and checked for purity by chromatography as above.

The amount of ($\alpha^{32}P$) UTP in the final eluate was estimated spectrophotometrically. Assuming a molar extinction coefficient at 262 mμ of 10^4, the concentration of the solution was adjusted to 10 μ moles/ml and stored at -15° in 2 mls. aliquots.

2.2 Methods.

Preparation of citric acid nuclei.

Nuclei were prepared by homogenizing chopped tissue in 20 volumes (w/v) 0.025 M citric acid at 0°C for 90 seconds.

The homogenate was strained through a layer of muslin, centrifuged at 2,000 g. for 10 minutes and the pellet homogenized in citric acid as above. Washing was continued until the supernatant was virtually clear. The purification of the final preparation was tested by staining with brilliant cresol blue and viewing under a microscope. The pellet was either used immediately or stored at -20°C until required.

Preparation of RNA.

Whole cell RNA was prepared from finely chopped tissue by homogenizing for 2 minutes at room temperature in 10 volumes (w/v) of a buffer containing 0.015 M tris-HCl pH 7.8; 1.5 per cent sodium dodecyl sulphate; 0.015 M sucrose; 0.015 per cent bentonite, and 0.75 per cent naphthalene disulphonate. Where nuclear RNA was required the above procedure was carried out on a nuclear pellet which had previously been neutralized with 0.5 M tris-HCl pH 7.5.

An equal volume of re-distilled 90 per cent phenol containing 0.1 per cent 8-hydroxyquinoline was added and the mixture shaken at room temperature for 5 minutes. After centrifuging the emulsion at 2,000 g. for 10 minutes, the aqueous phase was removed and re-treated with phenol until no protein interface was discernible. Three volumes of cold re-distilled ethanol were then added to the aqueous phase, mixed thoroughly and left at -20°C for 30 minutes.

The resulting precipitate was centrifuged at 600 g. for 5 minutes and the pellet dissolved in a convenient volume of 0.01 M tris-HCl pH 7.5; 0.001 M $MgSO_4$ and treated with 20 µg/ml deoxyribonuclease for 15 minutes at 30°C. The reaction was terminated by shaking with an equal volume of phenol as before. To the aqueous phase was added 0.25 volumes 6 M potassium acetate; 0.3 M NaCl with thorough mixing, followed by 0.25 total volumes 100 per cent ethanol. After mixing the solution was left at -20°C for 60 minutes. The precipitate was collected by centrifugation at 2,000 g. for 15 minutes and washed thoroughly in 100 per cent ethanol.

Finally the pellet was dissolved in 15 volumes 0.01 M sodium acetate; 0.1 M NaCl; 0.001 M $MgCl_2$ pH 5.0, mixed slowly with an equal volume of 100 per cent ethanol, 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 0.01 M tris-HCl pH 7.5; 0.001 M $MgCl_2$ and the concentration determined from the absorbancy at 260 mµ. An absorbancy of 22 was taken as being equivalent to 1 mg. RNA/ml.

Isolation of *in vitro* synthesized RNA.

A modified procedure, not employing precipitation steps, was devised for the isolation of RNA synthesized in a cell-

free system.

The RNA synthesis was terminated by centrifuging the incubation mixture at 0°C at 20,000 g. for 15 minutes on a Spinco Model L preparative centrifuge to remove fine particles of material. The supernatant was treated with 20 µg/ml deoxyribonuclease for 15 minutes at 30°C and the solution then shaken with an equal volume of phenol/hydroxyquinoline for 5 minutes. The aqueous phase was removed and phenol treatment repeated until no interfacial material was present. The aqueous phase was extracted twice with 2 volumes ether to remove traces of phenol, the ether then evaporated off by a stream of nitrogen gas and the solution dialysed overnight against 0.01 M tris-HCl pH 7.5; 0.001 M $MgCl_2$; and 0.3 per cent bentonite to remove unincorporated nucleotides. The remaining ~~dialysate~~ residue contained the in vitro synthesized RNA.

Preparation of in vivo labelled RNA.

(a) Preparation of ^{14}C-labelled whole cell RNA from rat kidneys. A 200 g. male rat was injected intraperitoneally with 300 µc ^{14}C- sodium formate in isotonic saline, and left for 18 hours without water in a fume cupboard. After this time, the animal was killed by cervical dislocation, the kidneys removed and immediately processed as described in Section 1.2. The final product had a specific activity of 220 disintegrations/minute/µg RNA.

(b) Labelling of Landschutz cells. ^{14}C- Landschutz ascites tumour RNA and DNA were prepared by injecting 300 microcuries of ^{14}C- sodium formate into the peritoneal cavity of a mouse suffering from a well-established Landschutz tumour. After 18 hours the mouse was killed, the cells harvested and RNA and DNA prepared as described in Sections 1.2 and 1.5 respectively.

Specific activities of the final product were 12,000 d.p.m/µg and 100,000 d.p.m/µg for DNA and RNA respectively.

(c) Preparation of rapidly labelled RNA from rat tissues. Three male rats (200 g. each) were injected intraperitoneally with 4 millicuries of neutralized ^{32}P-orthophosphate. After 1 hour the animals were killed and the livers, pancreases and kidneys immediately removed and homogenized in citric acid, as in Section 1.1. Nuclei were then frozen at -20°C and RNA later isolated according to Section 1.2. The specific activity of the isolated material, which also contained a predominance of unlabelled ribosomal RNA, was between 200 and 300 counts/minute/µg RNA.

Preparation of DNA.

DNA was prepared by a modification of the method described by Marmur (1961). Where individual tissues were used as starting material, citric acid nuclei were first prepared (Section 1.1).

After neutralization with 0.5 M tris-HCl pH 7.5,

10 volumes (v/v) 0.15 M NaCl; 0.1 M EDTA pH 8 were added and the purification continued as before. In some cases whole embryo DNA was prepared, by first decapitating the animals and homogenizing in 10 volumes (w/v) 0.15 M NaCl; 0.1 M EDTA for 2 minutes using a Waring Blender. The homogenate was then strained through muslin.

10 per cent sodium lauryl sulphate was added to the saline/EDTA suspensions to give a final concentration of 2 per cent, and stirred at room temperature for 60 minutes. 5 M sodium perchloriate was then added to 1 M final concentration and the mixture shaken with an equal volume of chloroform/octanol (24:1 v/v) for 30 minutes.

After centrifuging the emulsion at 10,000 g. for 10 minutes the aqueous phase was removed and the chloroform treatment repeated until no interface remained. The nucleic acids were precipitated by the addition of 2 volumes re-distilled ethanol. The precipitate was collected by spooling, re-dissolved in 0.01 x SSC, made up to 1 x SSC on solution. Ribonuclease was added to a concentration of 50 µg/ml and incubated at 37°C for 30 minutes. Following ribonuclease digestion, pronase was added to 50 µg/ml and incubated at 37°C for 2 hours.

Chloroform/octanol treatment was carried out until no

interface was present and the DNA precipitated and re-dissolved in 0.01 x SSC as before. A ninth volume of 3 M sodium acetate; 0.001 M EDTA was added with mixing followed by a half volume of isopropyl alcohol. The DNA was spooled, washed in 70 per cent and 95 per cent ethanol and finally re-dissolved in 0.01 x SSC. Solutions were stored frozen at -20°C.

Determination of radioactivity.

^{32}P was measured in a nuclear Chicago gas flow counter fitted with a micromil window. This gave an efficiency of almost 50 per cent with a background of 15-18 counts/minute. Precipitates containing radioactive RNA were dissolved in 1 ml 22 M formic acid and 0.5 ml. plated on steel planchettes. The samples were then dried under heating lamps and counted.

^{3}H, ^{14}C and very small amounts of ^{32}P were detected in a nuclear Chicago 725 liquid scintillation counter or a Packard Tricarb scintillation spectrometer. For material in aqueous solution, samples were made up to 0.5 ml. in water and 8 mls of dioxane based scintillator fluid added. This solution consisted of 0.7 per cent 2,5-diphenyl-oxazolyl (PPO); 0.03 per cent 1,4 bis (2-(5-(phenyl-oxazolyl)) benzene (POPOP) and 10 per cent naphthalene in 1,4 dioxane.

Radioactive RNA precipitates were counted by one of two methods. One drop of 22 M formic acid was added to the precipitate and left at 100°C for 30 minutes. When the

formic acid had almost evaporated, 0.5 ml. water was added and the sample counted in 8 mls. dioxane scintillator. Alternatively, after the initial precipitation with TCA, the precipitate was collected on a Millipore filter (pore diameter 0.45 μ) and washed with three successive portions of 5 per cent TCA saturated with sodium pyrophosphate. The filter was dried thoroughly at 50°C and placed in 4 ml. toluene scintillator fluid (0.42 per cent PPO; 0.021 per cent POPOP in Analar toluene). Corrections for counting efficiency were applied using standard quench data.

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. In cases of protein low in these amino acids (e.g. histones) the bromosulphalein method was used.

(a) The Lowry method. Reagents:

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

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

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

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

0.2 ml. aliquots of solution (25-250 µg protein/ sample) were added to 3 mls. Reagent C_4 followed 10 minutes later by 0.3 ml. Reagent E. After standing at room temperature for 1 hour the optical density at 750 mµ was measured on a Unicam SP 500 spectrophotometer. A calibration curve was also determined with 0-250 µg bovine serum albumin (Armour Pharmaceutical Co.Ltd.).

(b) The Bromosulphalein Method. The Bromosulphalein reagent is 1 ml. 5 per cent (w/v) sulphobromophenolphthalein Na salt (Koch-Light Ltd.) in 100 mls. 1 N HCl and 50 mls. 1 M citric acid in 250 mls. distilled water. 0.1 ml. samples of protein (15-20 OD units/ml at 260 mµ) were mixed with 0.1 ml. 0.5 N HCl and left for 1 hour at 0°C with occasional shaking. The supernatant was collected by centrifugation at 2,000 g. for 10 minutes and the precipitate washed with 0.2 ml. 0.25 N HCl. The supernatants which contained the histone component were pooled.

0.8 ml. of 1 M NaOH was added to the precipitate and 0.4 ml. 2N NaOH added to the supernatant. After incubation at 37°C for 1 hour 1 ml. of bromosulphalein reagent was added and the solutions left at 0°C for 10 minutes. The solutions were then centrifuged at 2,000 g. for 15 minutes and 0.5 ml. of the supernatants added to 3.5 mls. 0.2 M NaOH. The absorbancies were read at 580 mµ and calibrated against 0-400 µg bovine serum albumin.

The quantitative estimation of DNA.

This was 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 (British Drug Houses).

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

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

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

Procedure:

DNA was extracted from materials by heating for 15 minutes at 70°C in a total volume of 2 mls. 1 M PCA., removing the supernatant obtained on centrifugation at 850 g for 10 minutes, repeating the extraction procedure and combining the supernatants. Standard solutions contained up to 100 µg deoxyribose in a total volume of 4 mls. 1 M PCA.

2 mls. Reagent D were added and the tubes incubated at 30°C for 16 hours in the dark or at 70°C for 1 hour. The former procedure, unlike the latter, often gave cloudy solutions; therefore incubation at 70°C was generally employed. The absorbancies of the solutions were then read

at 600 mμ against reagent blanks using a Unicam SP 500 spectrophotometer. Standard curves were almost linear; 1 μg deoxyribose was taken to be equal to 6.2 μg of DNA.

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 deoxyribose solution (L. Light & Co.) in water at a concentration of 10 μg/ml.

Procedure:

To volumes of 3 mls. containing up to 130 μg of RNA, 3 mls. of A were added. Standards containing up to 30 μg of deoxyribose were similarly set up. 2 mls. of B were added and the tubes heated in a boiling water bath for 45 minutes. After quick cooling the absorbancies at 670 mμ were recorded using a Unicam SP 500 spectrophotometer. 1 μg of ribose was taken to be equivalent to 4.56 μg of RNA.

EXPERIMENTAL

3. Experimental.

3.1 Hybridisation techniques.

Methodology.
Fractionation of mammalian nucleic acids on MAK.
Isolation of hybrids on Sephadex G-200 columns.
Liquid hybridization using nitrocellulose filters.
Hybridisation with immobilised DNA.
An attempt to detect tissue specific RNAs in rat organs.

3.2 The DNA dependent RNA polymerase of Micrococcus lysodeikticus.

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3.1 Hybridization techniques.

Methodology.

The principles involved in DNA molecular hybridization can be summarised as follows. If native DNA is denatured by heat and allowed to cool slowly from 100°C, base pairing is re-established between complementary regions and most of the material is restored to its original native form. If this process is allowed to take place in the presence of homologous RNA, DNA-RNA hybrids are formed in addition to DNA re-naturation. In practice, DNA is first denatured either by heating to 100°C and cooling rapidly to prevent renaturation or, by the addition of alkali to pH 13 and subsequent neutralisation. Samples are then incubated in a solution of high ionic strength containing radioactive RNA and maintained at an intermediate temperature such that complementary sequences can associate and dissociate at random. At equilibrium the mixture is cooled rapidly and the hybrids separated out and estimated. Several methods have been devised for doing this.

Initially Hall and Spiegelman (1961) employed caesium chloride equilibrium gradient centrifugation to separate DNA-RNA hybrids from unhybridized material. Yankofsky and Spiegelman (1962a) exploited this technique to identify small complementary segments (less than 0.1 per cent of the DNA) by introducing ribonuclease treatment before centri-

fugation. This procedure removes adventitious RNA but leaves hybridised RNA unaffected. Less laborious techniques have been devised by Bautz and Hall (1962), Bolton and McCarthy (1962) and Britten (1963) in which denatured DNA is trapped in columns of inert material. After hybridization the unbound RNA is simply removed by washing the columns. Other chromatographic methods in which DNA is not immobilised were introduced by Attardi et al., (1965) using Sephadex and by Hayashi et al., (1965) using methylated albumin-keiselguhr (MAK).

Nygaard and Hall (1963) discovered that nitrocellulose membranes strongly bind single stranded DNA, alone or along with any hybridised RNA, but not unhybridised RNA. Gillespie and Spiegelman (1965) later modified this method by first irreversibly binding the denatured DNA to the membranes by heat. Hybridization is then carried out with DNA immobilised in situ.

In the present study MAK and Sephadex chromatography were explored. However, the advent of the membrane techniques provided more convenient methods for handling large numbers of samples.

Fractionation of mammalian nucleic acids on MAK.

Using columns of methylated albumin-kieselguhr, Mandel and Hershey (1960) and Sueoka and Cheng (1962) achieved separations of bacterial DNA and RNA on the basis of size

base composition and degree of intramolecular hydrogen bonding. In neutral solution nucleic acids form salt linkages with the charged groups on the albumin and can be eluted by increasing salt concentrations.

The preparation of methylated serum albumin. 50 g. bovine albumin (Armour Pharmaceuticals) were dissolved in methanol containing 4.2 mls. concentrated HCl. The mixture was left in the dark at 37°C for 5 days with intermittent shaking, after which time the precipitate was collected by centrifugation at 5,000 g. for 10 minutes, and washed twice with ether. The precipitate was dried in air and then *in vacuo* over solid KOH. The residue was powdered and stored over silica gel at -10°C.

The preparation of MAK. 20 g. kieselguhr (Hyflo Supercel, Koch-Light) was suspended in 100 mls. 0.1 M NaCl: 0.05 M $NaPO_4$ buffer pH 6.8, boiled to expel air and cooled. 5 mls. of a 1 per cent (w/v) aqueous solution of methylated albumin was added dropwise with stirring, followed by 20 mls. additional buffered saline. This stock suspension could be stored at 0°C for several wweks. On the day of use, 6 g. kieselguhr was suspended in 40 mls. buffered saline as above, and 10 mls. stock MAK was added.

Preparation and operation of MAK columns. A standard column (2 cm. diameter) was prepared by layering 4 mls. stock MAK suspension, expressing the excess buffer by gentle

air pressure, and packing the material by passing through another 4 mls. 0.1 M buffered saline. 4 mls. of the weaker suspension were then layered in the same fashion and finally the column was topped with 1 ml. of a 10 per cent (w/v) suspension of kieselguhr. The column was washed with 0.1 M buffered saline until the optical density of the effluent at 260 mμ was less than 0.05.

The nucleic acid sample was then diluted to 25 μg/ml. with 0.1 M buffered saline and passed through the column under pressure. The material was fractionated by passing an increasing gradient of buffered saline (0.1 M - 1.5 M) through the column at a rate of 1 ml/minute. Fractions were monitored at 260 mμ and salt concentrations determined from refractive indices using an Abbe refractometer.

The behaviour of mammalian DNA and RNA on MAK. Table 1 summarises the results obtained with various DNA and RNA preparations. In general, animal total RNA preparations gave two peaks; transfer RNA between 0.3 - 0.5 M NaCl, and ribosomal RNA between 0.6 and 0.8 M NaCl. In the case of Landschutz RNA partial separation of ribosomal species was obtained. These results are similar to those obtained with mammalian RNA by Yoshikawa-Fukada et al., (1965). Hershey and Burgi (1960) and Kano-Sueoka and Speigelman (1962) found similar results with E.coli RNA except that better resolution of ribosomal RNA is obtained with bacterial material.

Table 1.

THE BEHAVIOUR OF MAMMALIAN NUCLEIC ACIDS

ON MAK COLUMNS.

PREPARATION	PEAK	MOLARITY NaCl. Range	Peak Max.	RECOVERY
Rat liver RNA.	1. 2.	0.30-0.40 0.60-0.80	0.35 0.76	100%
Landschutz Ascites RNA.	1. 2. 3.	0.30-0.50 0.65-0.85	0.38 0.72 0.78	100%
Native calf thymus DNA.	1.	0.56-0.78	0.65	75%
Denatured calf thymus DNA.	-	-	-	0
Sonicated, denatured calf thymus DNA.	1.	0.30-0.60	0.43	82%
Denatured Landschutz DNA.	-	-	-	0
3 min. sheared and 10 min. sheared, denatured Landschutz DNA	1.	0.30-0.75	0.60	60%

Native DNA could be recovered from MAK with 75 per cent efficiency; however on denaturation the DNA was adsorbed very strongly to the column. Shearing for 3 minutes in a Waring blender improved the recovery of denatured material.

Since sheared DNA eluted between transfer and ribosomal RNAs, an attempt was made to hybridize whole cell Landschutz ^{14}C-RNA with sheared DNA and to separate the hybrids. 250 µg. of DNA and RNA were heated at 63°C for 60 minutes in 1 ml. of 0.5 M KCl, cooled, diluted to 25 µg/ml. and applied to a MAK column. The elution pattern obtained (Figure 1) gave the peaks expected for transfer and ribosomal RNAs and denatured DNA, with substantial quantities of radioactivity associated with the DNA peak. However the recovery of DNA was still only 60 per cent. and could not be improved by longer periods of shearing. With bacterial DNA however it is possible to obtain complete recoveries of denatured material by shearing (Hayashi et al., 1965; Mandel and Hershey, 1960; Hershey and Burgi, 1960).

As this approach did not lend itself so readily to the fractionation of mammalian material as it did to bacterial material, it was decided to investigate alternative methods of hybrid detection.

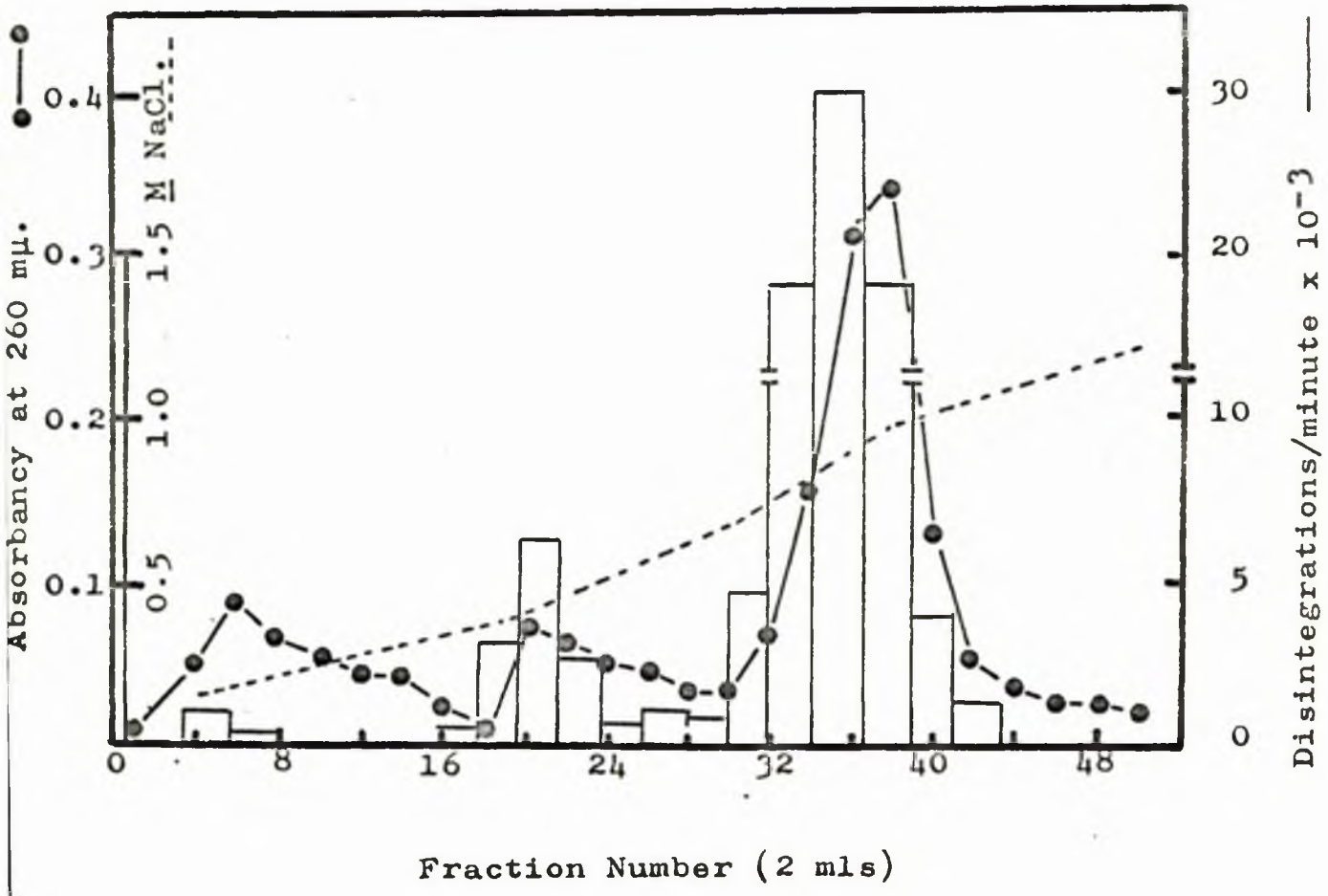
The isolation of hybrids on Sephadex G-200 columns.

A method of isolating hybrids using Sephadex chromato-

Figure 1.

The separation of ^{14}C-Landschutz RNA, Landschutz DNA and DNA-RNA hybrids on a column of methyiated albumin-kieselguhr. 250 μg of labelled RNA and 250 μg sheared DNA were hybridized for 60 minutes at 63°C and applied to the column in 0.17 M KCl. On elution with increasing concentrations of NaCl, three peaks were obtained: at 0.2 M, 0.3 M, and 1 M corresponding to t-RNA, DNA-RNA hybrids and ribosomal RNAs respectively. Only 60 % of the input DNA was recovered from the column.

Figure 1.



Fraction Number (2 mls)

graphy was described by Attardi et al., (1965) and by Scherrer (1965). Hybridization was carried out in 0.5 M KCl as before and the mixture treated with ribonuclease to reduce unhybridized RNA to small fragments. On chromatography the DNA, with associated RNA, appears at the solvent front while the degraded RNA is retarded in the gel.

A G-200 column (60 x 1.5 cm.) was equilibrated with 0.5 M KCl at a flow rate of 4 mls./hour. In preliminary experiments denatured rat kidney DNA (400 µg.) was mixed with ^{14}C-labelled whole rat kidney RNA (150 µg.) in 0.2 M KCl and incubated with 50 µg./ml. ribonuclease for 60 minutes without prior hybridization. The mixture was then restored to 0.5 M KCl and passed through the column. Every other fraction was collected, monitored at 260 mµ, and an equal volume of 10 per cent TCA added. After 30 minutes at 0°C the precipitates were collected and washed on Millipore filters. Figure 2 shows that under conditions where no hybridization takes place, no contamination of the DNA peak with RNA takes place. Figure 3 shows the result obtained when this procedure was applied to samples that had previously been incubated at 63°C for 60 minutes in 0.5 M KCl. A detectable amount of radioactivity was recovered under the DNA peak corresponding to 2.8 µg. RNA/400 µg. DNA. However before absolute

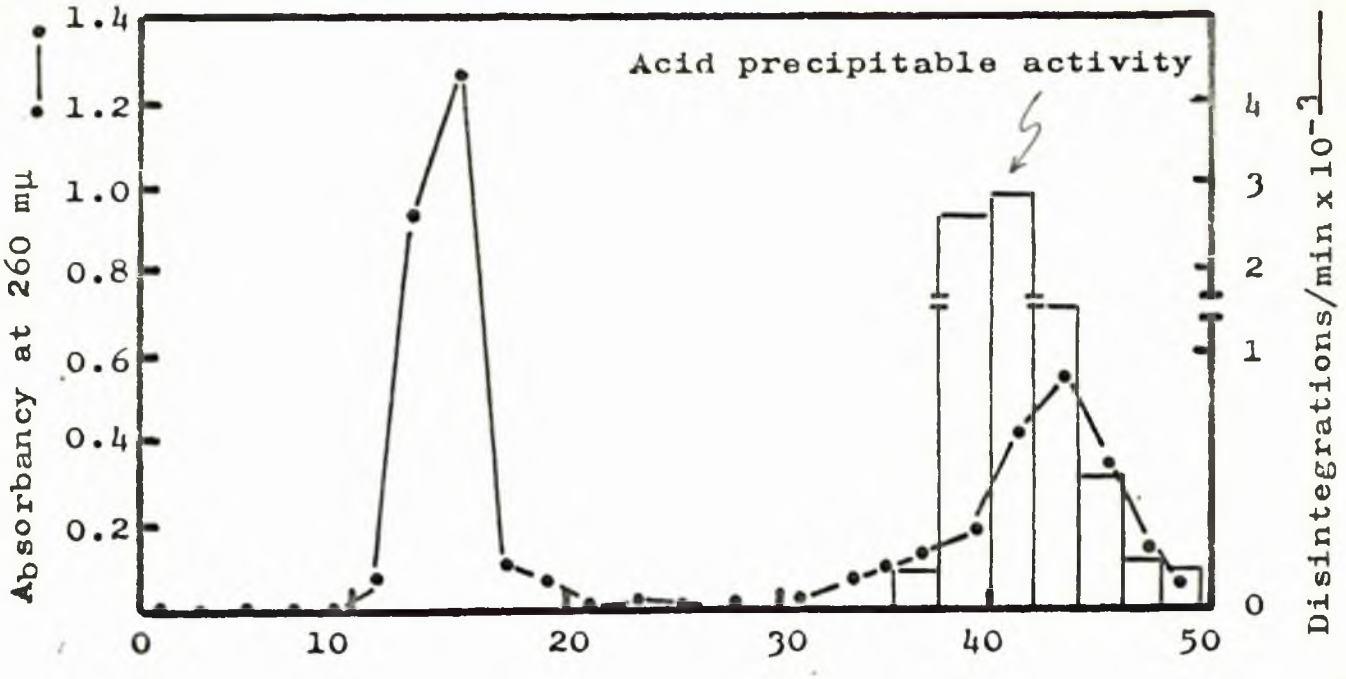
Figure 2.

The separation of 400 μg rat kidney DNA and 150 μg ^{14}C-rat kidney RNA on a Sephadex G-200 column, without prior hybridization. The mixture was incubated in 0.2 M KCl with 50 μg/ml. ribonuclease for 60 minutes at 30°C, applied to the column and eluted with 0.5 M KCl. The individual fractions were precipitated with 10% cold TCA, passed through MF 50 filters, dried and assayed for radioactivity.

Figure 3.

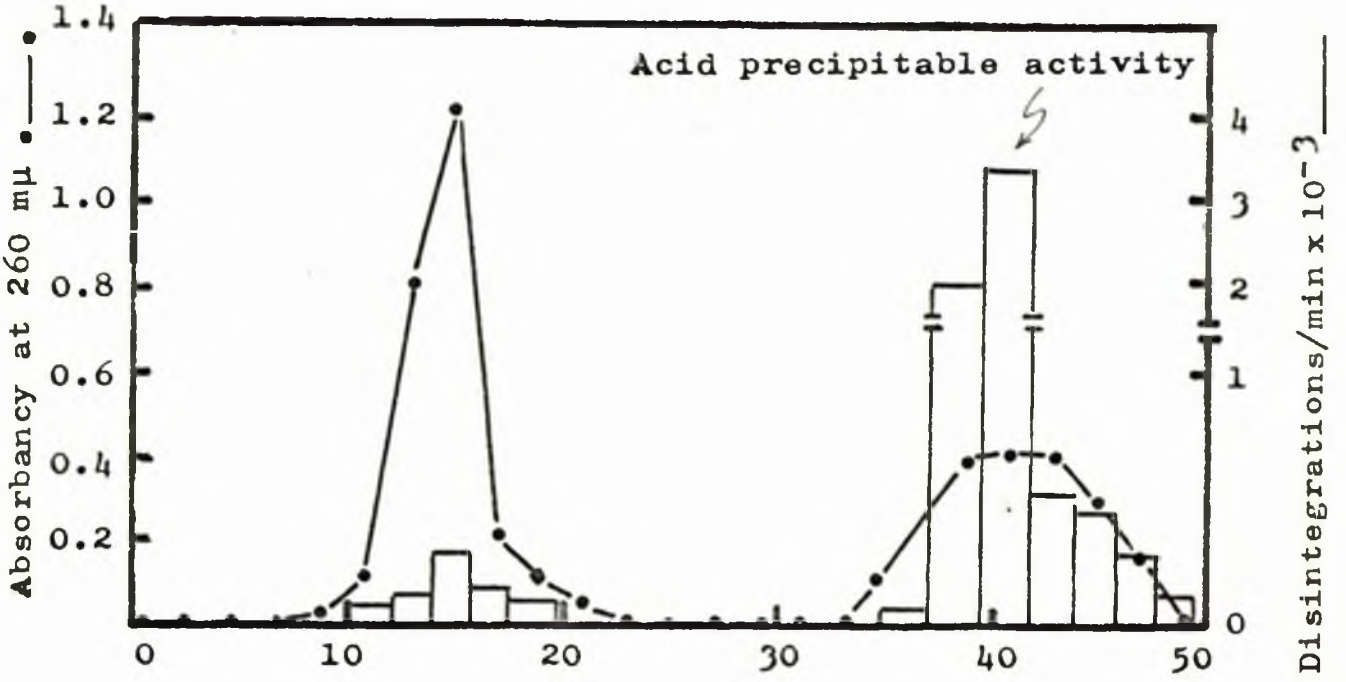
The separation of rat kidney DNA and ^{14}C-kidney RNA on Sephadex G-200. The conditions were identical to those of Figure 2, except that the mixture was hybridized at 63°C for 60 minutes prior to ribonuclease treatment.

Figure 2.



Fraction Number (2 mls)

Figure 3.



Fraction Number (2 mls)

estimations of percentage homology between RNA and DNA can be made, conditions must be such that the DNA is saturated with RNA. In the above trial experiment, this condition was probably not fulfilled. If a more rapid technique could be employed first to determine saturating conditions, then a precise determination of homology could be obtained by this method.

Liquid hybridization using nitrocellulose membranes.

This technique as described by Nygaard and Hall (1963) relies on the properties of nitrocellulose filters (Schleicher and Schuell, Keene, N.H. 27 mm., type B-6, or MF 50 filters from Sartorius Membranfilter, Gottingen) which in salt solutions can adsorb denatured DNA but not native DNA or RNA (Table 2).

The hybridization system was tested using DNA prepared from calf thymus and polyoma virus. Samples of DNA (0.2 - 2 mg./ml.) were denatured at 100°C for 15 minutes and then cooled rapidly in ice. ^{3}H-labelled RNA was prepared *in vitro* using M.lysodeikticus RNA polymerase with thymus or polyoma DNA primers. Hybridization mixtures containing DNA and RNA in a final volume of 0.15 ml. 0.5 M KCl were heated at 60°C for 1-4 hours and then cooled in ice. 3 mls. of 0.5 M KCl containing 15 µg. ribonuclease were added and incubated at 37°C. After 15 minutes the solutions were passed through nitrocellulose filters, pre-

Table 2.

THE ADSORPTION CHARACTERISTICS OF MAMMALIAN NUCLEIC ACIDS ON NITROCELLULOSE FILTERS.

AMOUNT AND TYPE OF PREPARATION	% RETAINED BY FILTER IN 0.5m KCl.
20 µg. native rat kidney DNA.	10.5
20 µg. denatured rat kidney DNA.	100.0
20 µg. rat kidney RNA.	0

viously 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 in toluene scintillator. Incubations containing RNA but no DNA were run as controls. Backgrounds of less than 1 per cent of the input activity were normally obtained.

The effect of incubation time on hybrid formation. Figure 4 shows the effect of increasing the time of incubation on the hybridization of 100, 10 and 1 µg. calf thymus DNA and constant amounts of ^{3}H-labelled RNA. For each DNA concentration the amount of RNA hybridized fell off with time. The possible reasons for this are discussed later.

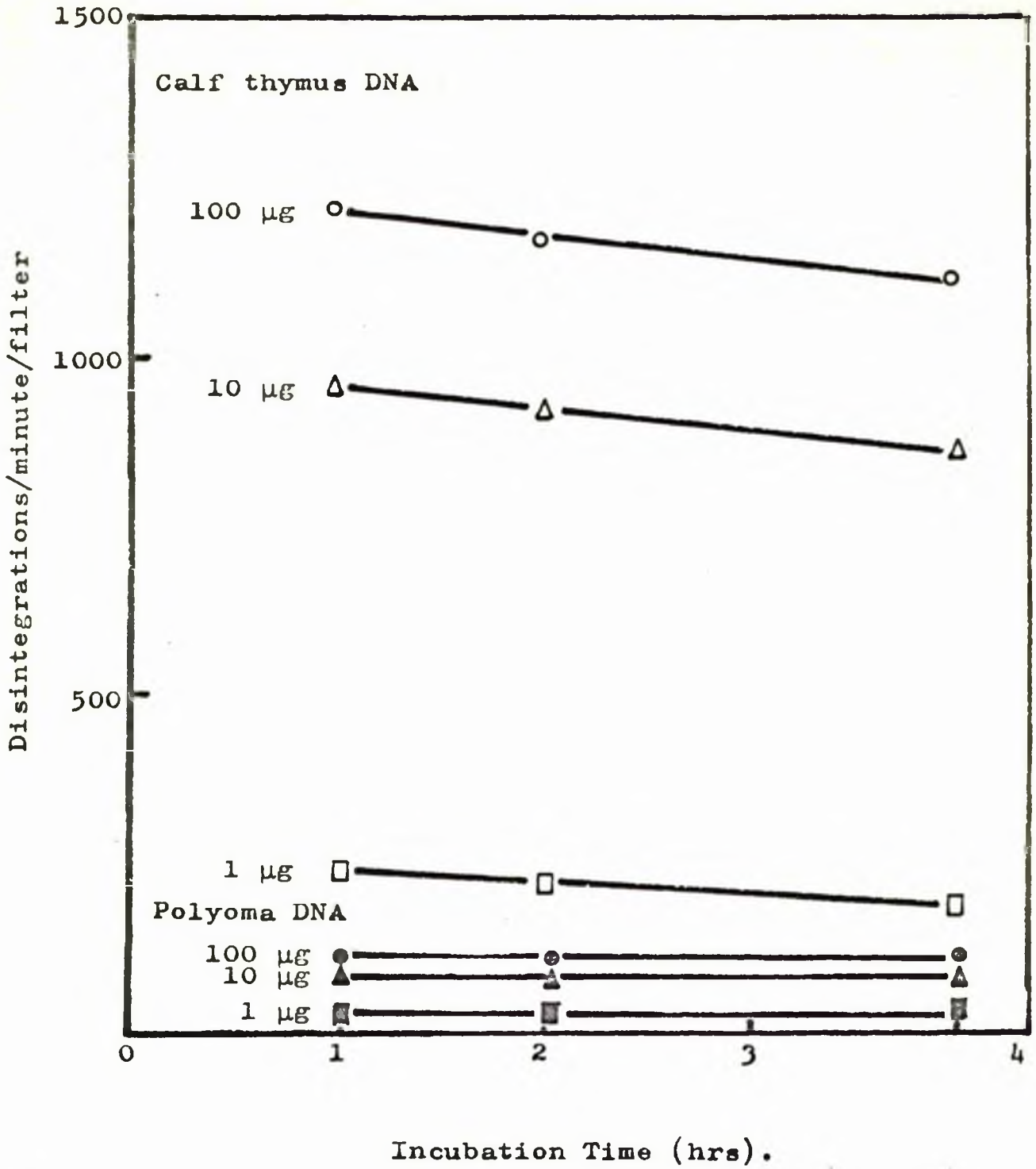
The specificity of hybrid formation. The specificity of the method was tested by incubating thymus RNA with samples of polyoma DNA. The results of Figure 4 indicate a small but detectable level of activity. Control samples which contain no DNA give considerably lower levels, suggesting that there is either a small degree of homology between the DNA or RNA or that mechanical trapping of RNA occurs when the DNA is adsorbed on the filter.

The RNA saturation curve. Saturation curves were determined by hybridizing a constant amount of DNA with increasing amounts of labelled RNA. Hybrid formation is then a function of RNA concentration and can be described graphically by an asymptotic curve. At infinite concen-

Figure 4.

The effect of increasing times of incubation on the hybridization of constant amounts of synthetic calf thymus RNA (13 μg/0.15 ml.) to 100, 10, and 1 μg of denatured calf thymus and polyoma DNAs by liquid hybridization technique.

Figure 4


1500
1000
500
Calf thymus DNA
100 µg
10 µg
1 µg
Polyoma DNA
100 µg
10 µg
1 µg
Disintegrations/minute/filter
0
1
2
3
4
Incubation Time (hrs).

tration, a maximum is obtained and the DNA is saturated with RNA. Although complete saturation is never obtained in practice it can be estimated from a double reciprocal plot of hybrid formation versus RNA input. This principle is shown in Figures 5 and 6. Increasing amounts of polyoma RNA were hybridized with 10 µg. polyoma DNA. The theoretical saturation value obtained from Figure 6 is equivalent to about 5 µg. polyoma RNA.

The DNA saturation curve. Hybridization of constant amounts of polyoma RNA (5 µg.) and variable amounts of DNA gives a response resembling a saturation curve (Figure 7). Over a restricted range of DNA concentrations, a constant amount of RNA hybridized with DNA, thereafter the plot deviates from linearity. It is possible that above a certain DNA concentration, hybrid formation substantially alters the concentration of free RNA in solution, and that this accounts for the pseudosaturation curve.

Hybridization with immobilised DNA.

One disadvantage of the previous method is that DNA renaturation can introduce quantitative errors when determining saturating values. Gillespie and Spiegelman (1965) described the following method where DNA is immobilised on the filter thereby reducing the risk of renaturation.

Denaturation of DNA. Pure DNA was dissolved in 0.01 x SSC (1 x SSC is 0.15 M NaCl: 0.015 M tri-sodium citrate)

Figure 5.

Kinetics of hybridization to 10 μg denatured polyoma DNA of increasing amounts of ^{3}H-RNA made *in vitro* from polyoma DNA by M.lysodiekticus polymerase.

Figure 6.

Double reciprocal plot of data shown in Figure 5. At infinite concentrations of RNA represented by the intercept on the ordinate, approximately 50% of the DNA exists as hybrid.

Figure 5.

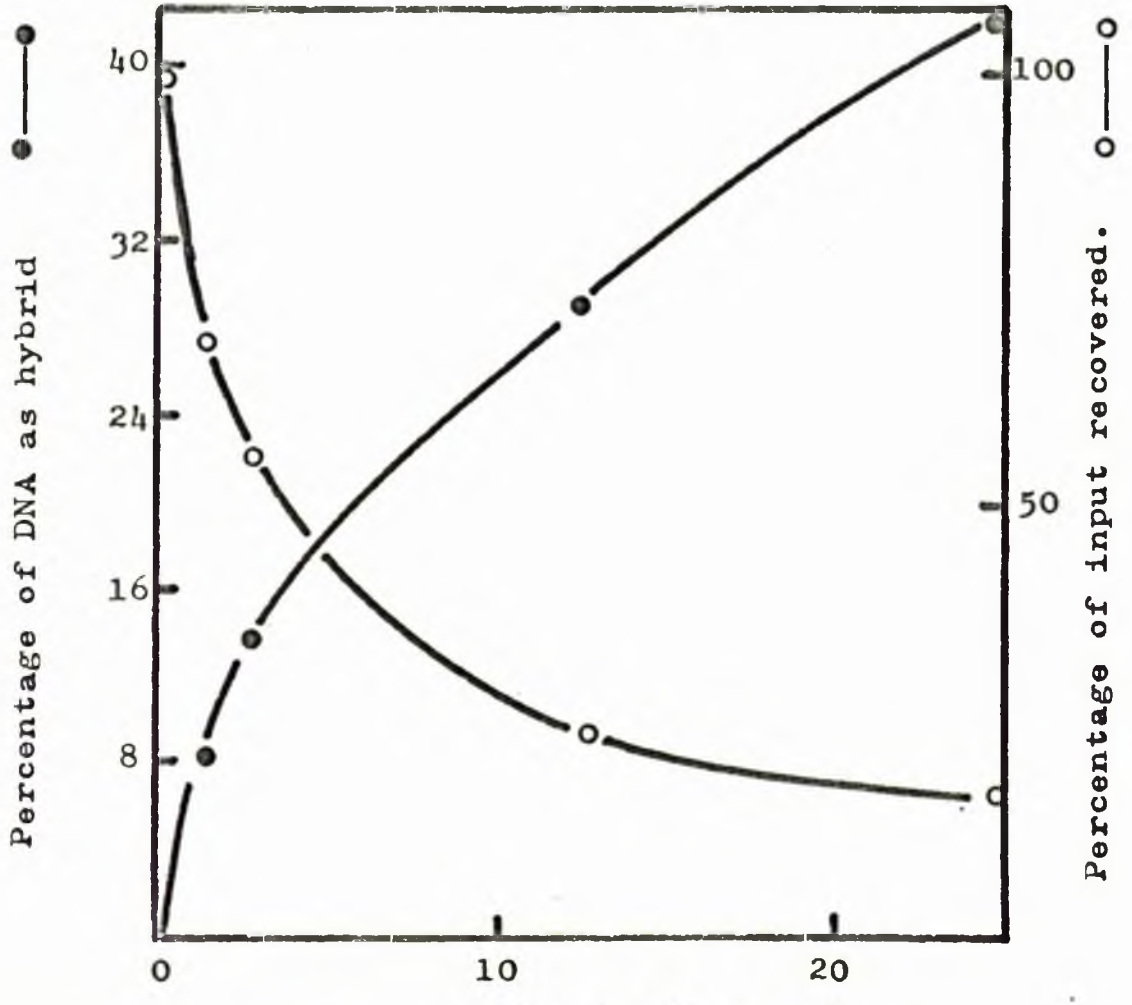


Figure 6.

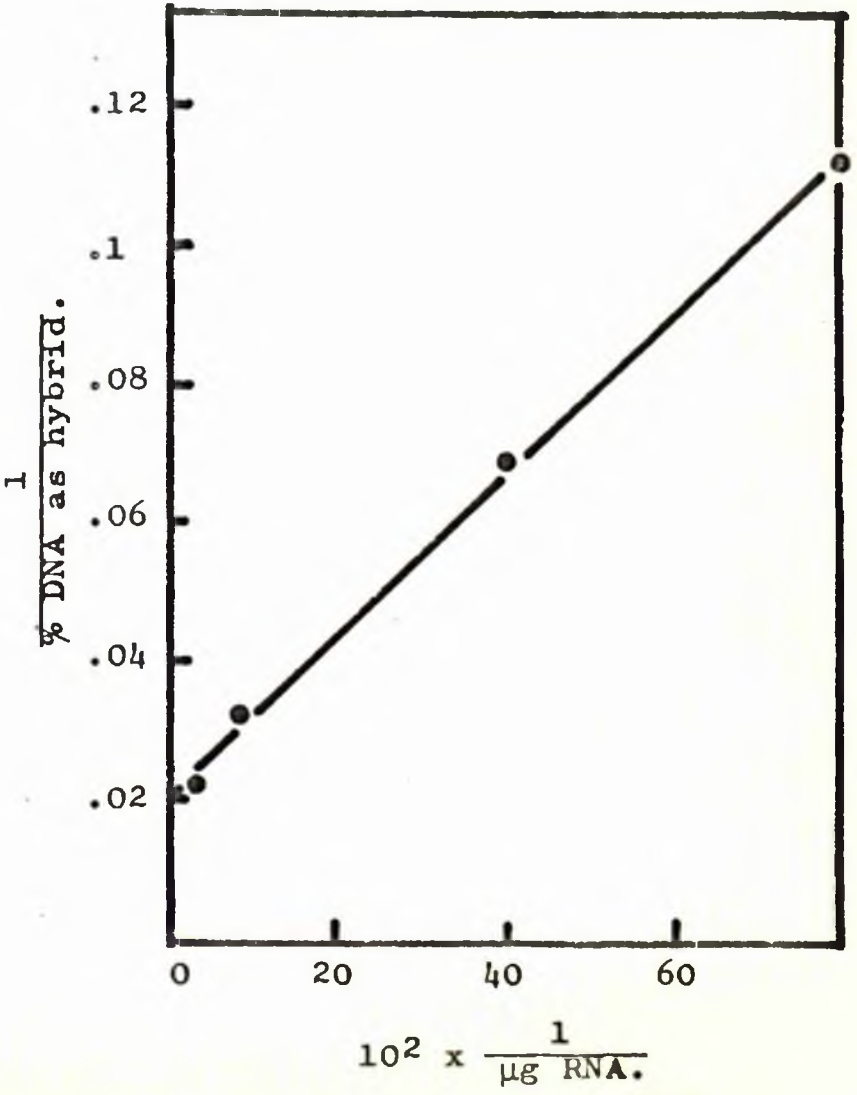


Figure 7.

Kinetics of hybridization of 5 μg *in vitro* synthesised polyoma RNA to increasing amounts of denatured polyoma DNA.

Figure 8.

Time course for the hybridization of 20 μg ^{14}C-rat kidney RNA with 100 μg denatured rat kidney DNA, immobilised on nitrocellulose filters by the method of Gillespie and Spiegelman.

Figure 7.

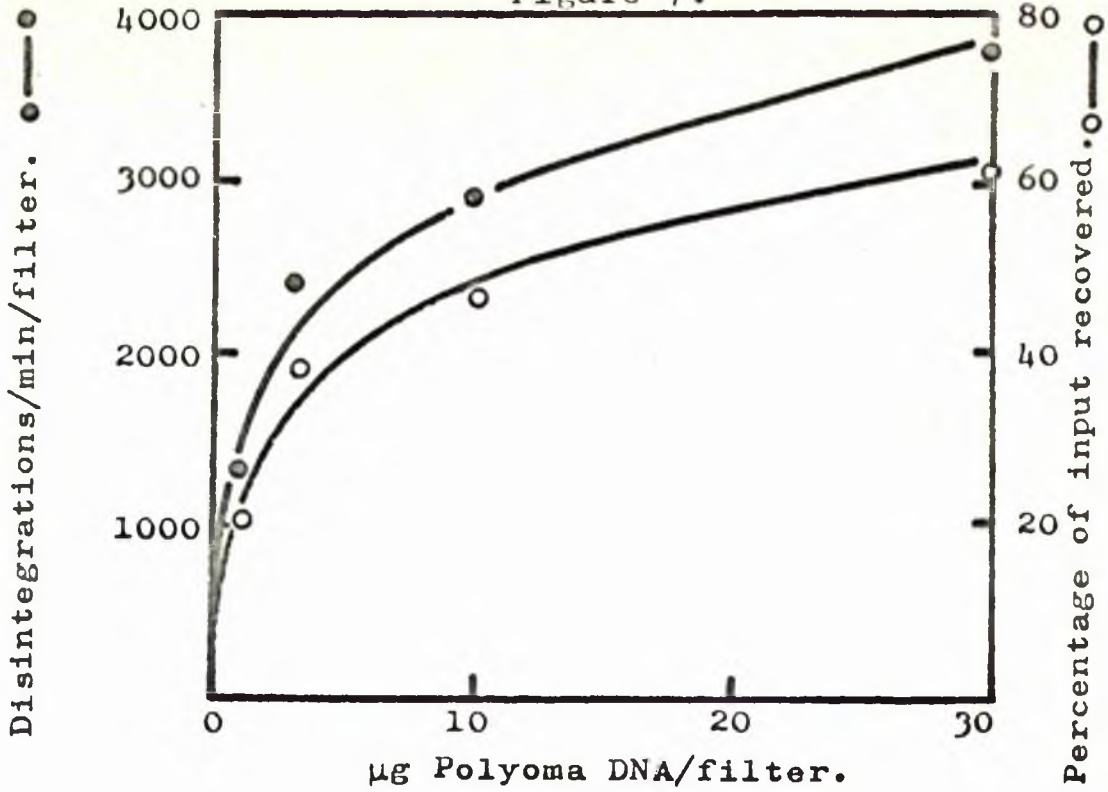
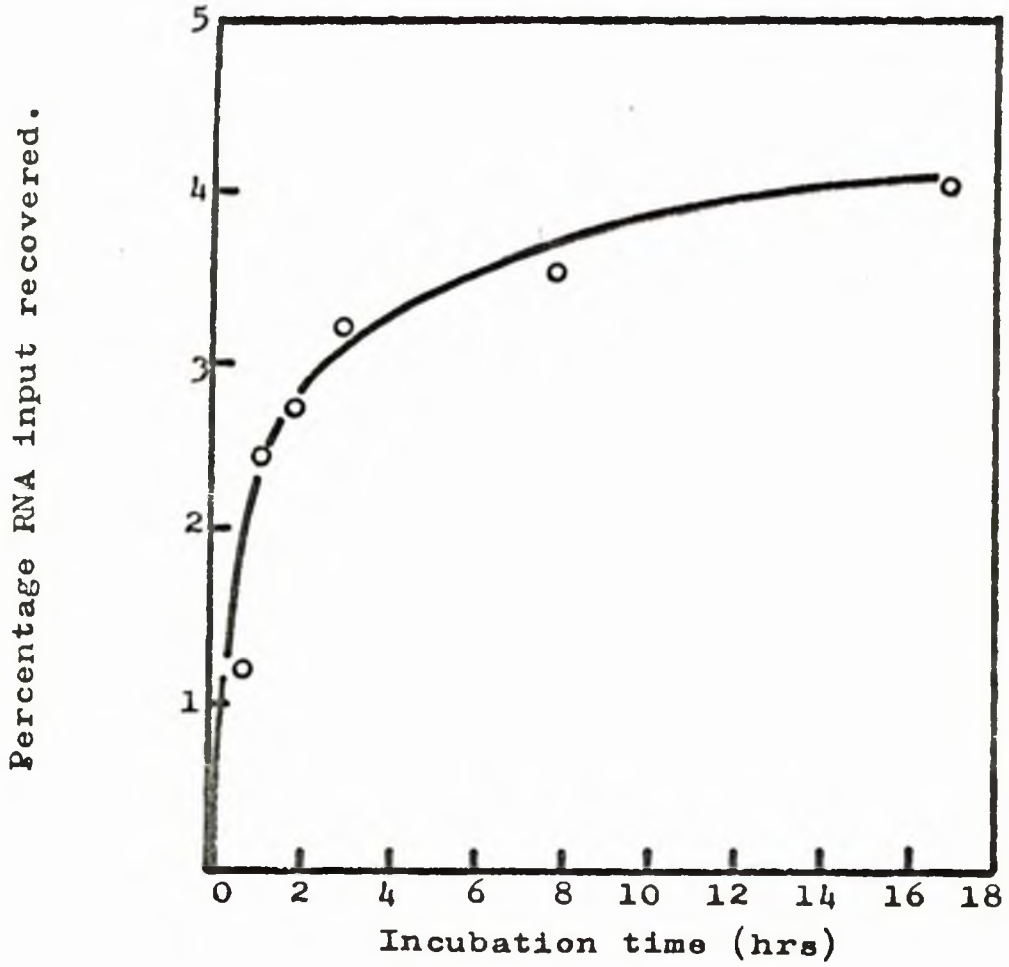


Figure 8.



at a concentration of 25 µg./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. Denaturation was also monitored by passing a sample of DNA in 6 x SSC through a filter and determining the amount retained. Any batch which did not exhibit 95-100 per cent retention was discarded.

The preparation of filters. Nitrocellulose filters were pre-soaked in 6 x SSC for 1 minute and washed in 20 mls. of the same solution. The required amount of denatured DNA in 5 mls. 6 x SSC were passed through the filters, which were then washed on both sides with 50 mls. 6 x SSC. After drying at room temperature for at least 4 hours the filters were vacuum desiccated for 2 hours at 80°C.

Hybridization technique. Hybrids were formed by immersing the filters in vials containing labelled RNA in 6 x SSC. Initially, volumes of 1-2 mls. were employed; however laterally this was reduced to economise on RNA. For this purpose small nitrocellulose filters (13 mm. diameter) were employed in a final volume of 0.2 ml. (semi-micro method) or 0.05 ml. (micro method).

Annealing was carried out at 65°C for 18 hours, after which time the vials were chilled in ice. The filters were removed, washed on both sides with 50 mls. 6 x SSC and

treated with 20 µg./ml. ribonuclease in 2 x SSC. After 1 hour at room temperature the filters were washed as before on both sides, dried and counted in toluene scintillator.

Retention of DNA on the filters. Labelled DNA (specific activity 12,500 cpm/µg) was prepared from Landschutz ascites cells using ^{14}C-sodium formate. 13 µg. samples were denatured and immobilised on nitrocellulose filters and one set subjected to the complete hybridization and washing procedures. Table 3 shows that immobilised DNA is retained on the filters throughout the procedure.

The effectiveness of washing and ribonuclease steps. The effectiveness of the working-up procedures was tested by incubating duplicate filters containing 10 µg. rat kidney DNA with 140 µg. ^{14}C-rat kidney RNA and subjecting them to complete or partial treatments. Table 4 shows that both washing and ribonuclease steps are necessary for the exclusion of adventitious label and for reproducibility. It is also of interest to note that ribonuclease treatment does not completely prevent hybridization.

Time course for hybridization. Figure 8 shows the time course of hybridisation of 100 µg. rat kidney DNA with 20 µg. ^{14}C-rat kidney RNA in 2 mls. At this low concentration of RNA at least 17 hours are required for maximum hybridization.

Table 3.

RETENTION OF IMMOBILISED DNA ON NITROCELLULOSE FILTERS.

TREATMENT OF DNA FILTERS	DISINTEGRATION/MINUTE/ FILTER
None	123, 335 132, 794
24 hr. incubation at 63°, washing, ribonuclease, washing.	136, 419 134, 680

Table 4.

THE EFFECT OF VARIOUS TREATMENTS ON THE LEVELS OF HYBRIDIZATION.

TREATMENT OF FILTERS	DISINTEGRATION/MINUTE/ FILTER
Complete: Wash, ribonuclease, wash.	140 140
Ribonuclease treatment, but no washing.	170 192
No ribonuclease treatment, but complete washing.	300 344
RNA treated with 40 µg. ribonuclease before hybridization, otherwise complete.	94 78

Saturation and competition kinetics of rat kidney RNA.

The effect of hybridising increasing amounts of ^{14}C-rat kidney RNA to 100 µg. rat kidney DNA is shown in Figures 9 and 10. From a double reciprocal plot, a saturation figure equivalent to 6.5 µg. RNA/100 µg. DNA was predicted. This suggests that of all the sequences present in kidney DNA only a restricted number are represented in the total RNA of the kidney cell.

In order to demonstrate that the activity associated with the DNA is specific, a competitive hybridization experiment was carried out. Here constant amounts of kidney DNA (100 µg.) and ^{14}C-kidney RNA (35 µg.) were hybridised as before but in the presence of increasing amounts of unlabelled RNA prepared from rat kidney. Figure 11 illustrates the displacement of labelled RNA from the DNA by the unlabelled RNA. Complete displacement of label (denoting 100 per cent homology between the two RNAs) can in theory only be obtained at infinite concentrations of the unlabelled material. However a prediction of the extent of homology can be made from a double reciprocal plot of diminution of radioactivity ($X_o - X$) versus the concentration of unlabelled RNA, where X_o is the activity in the presence of labelled RNA alone, and X the value obtained on the addition of unlabelled RNA. A linear plot is obtained (Figure 12) which cuts the ordinate at an infinite concentration of competing RNA. For complete competition, the value of X becomes zero and

Figure 9.

Kinetics of hybridization of increasing amounts of *in vivo* ^{14}C-labelled rat kidney RNA with 100 µg denatured rat kidney DNA/filter.

Figure 10.

Double reciprocal plot of the data of Figure 9. It is predicted that at saturating concentrations of RNA only 6.6% of the DNA exists as hybrid.

Figure 9.

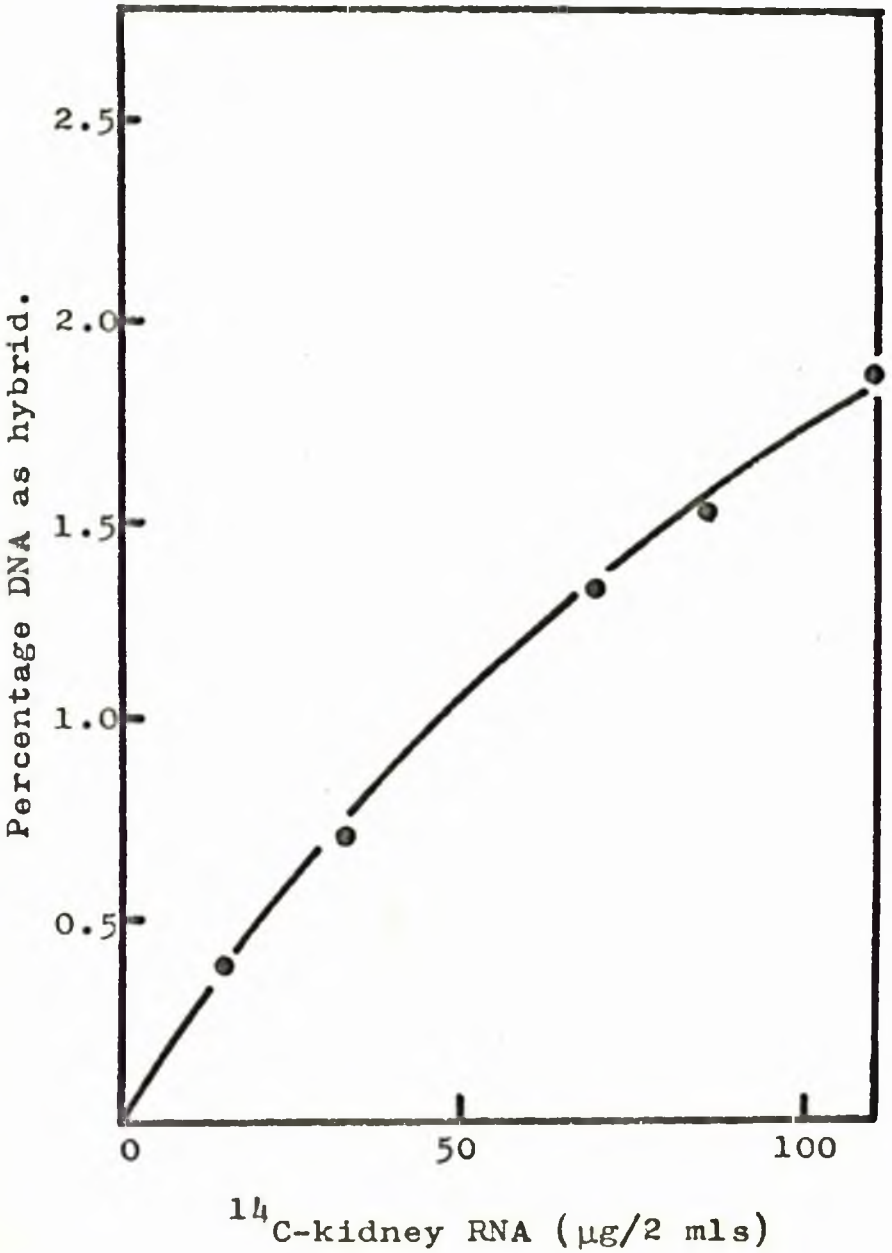


Figure 10

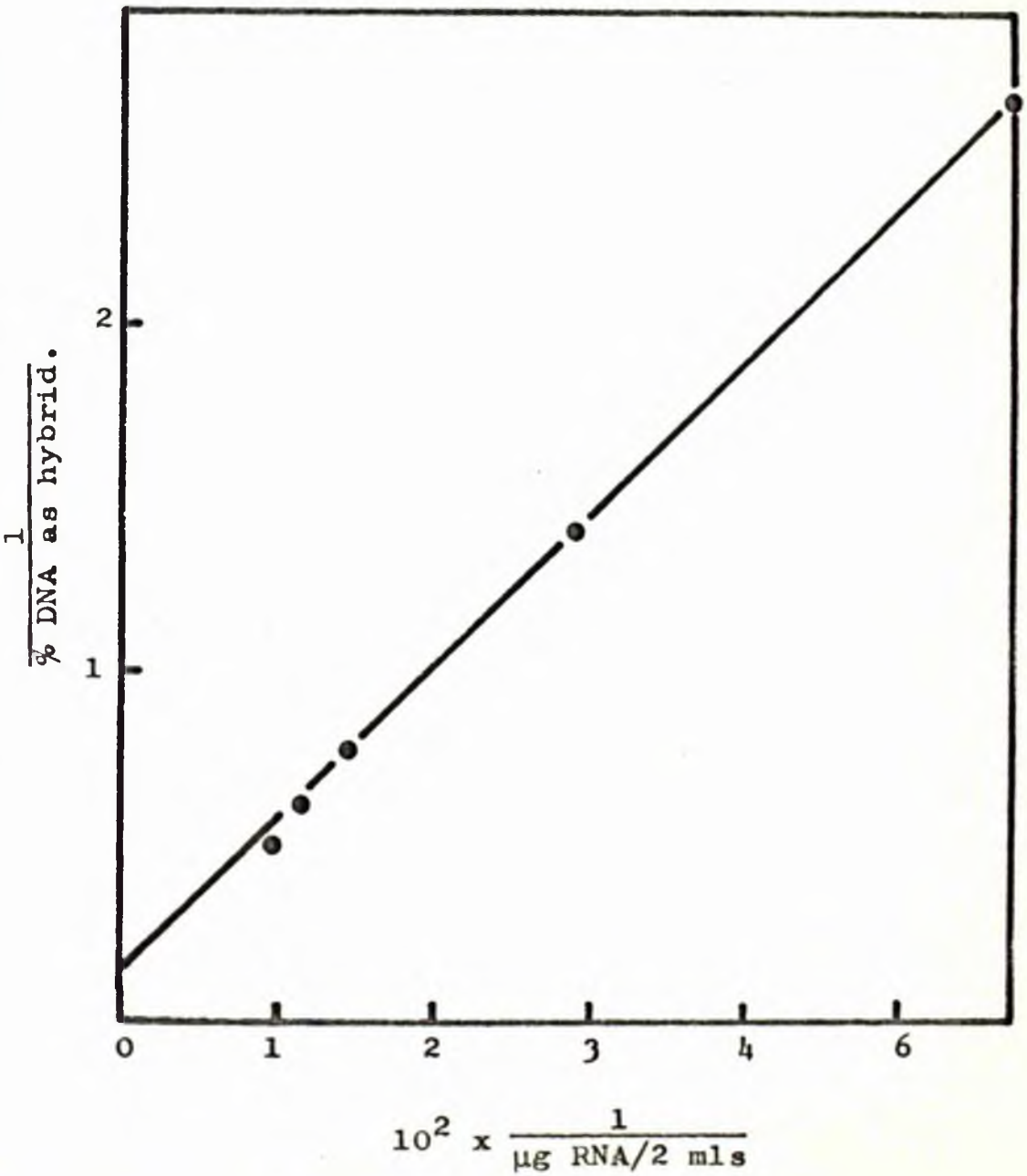


Figure 11.

Kinetics of the competitive hybridization to denatured rat kidney DNA of *in vivo* ^{14}C-labelled kidney RNA and unlabelled kidney RNA. Each filter contained 100 µg DNA and was hybridized to 35 µg labelled RNA in the presence of increasing concentrations of unlabelled RNA.

Figure 12.

Double reciprocal plot of the data of Figure 11. At infinite concentration of unlabelled RNA it is predicted that less than 10% of the hybrid would be labelled.

Figure 11.

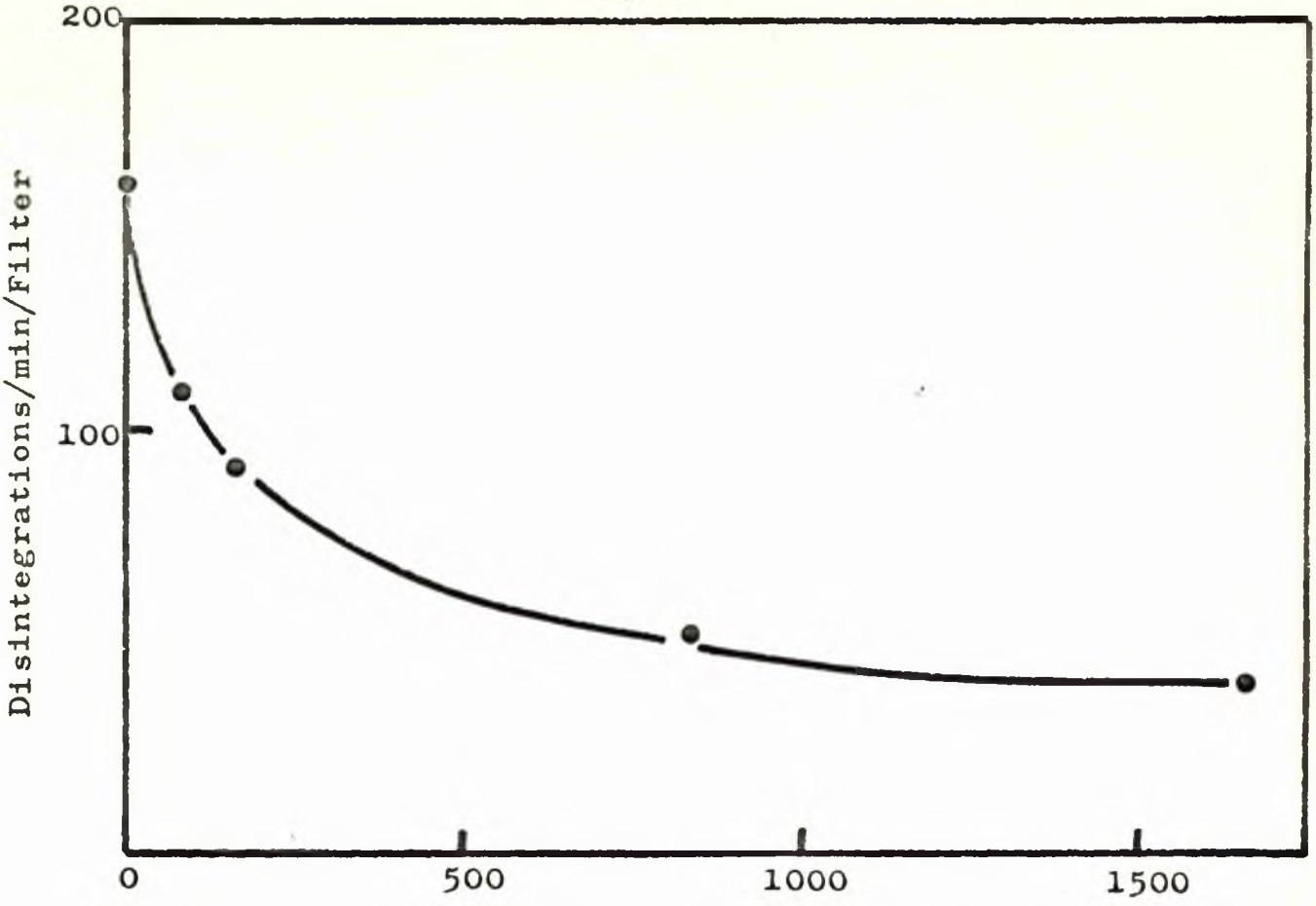
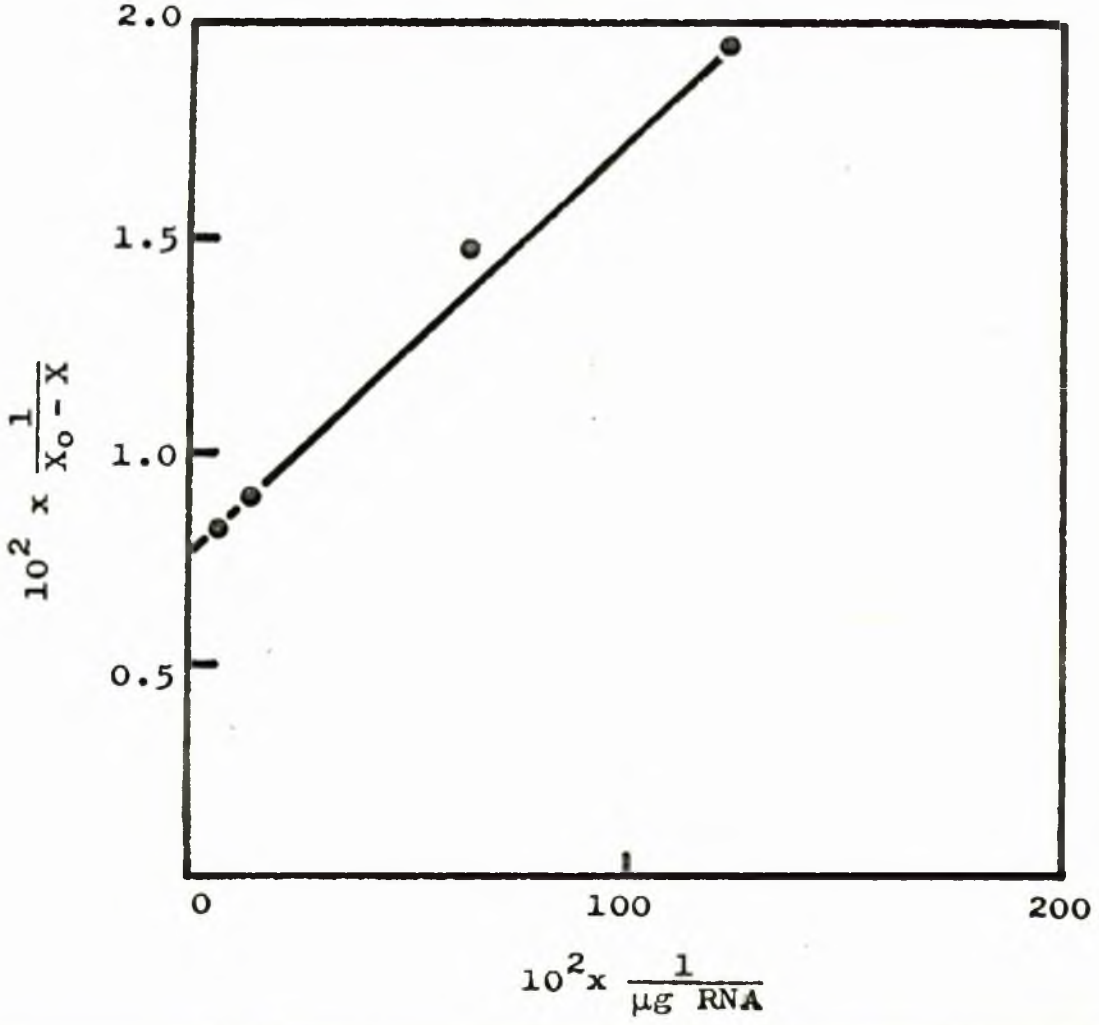


Figure 12.



$1/X_o - X$ equals the reciprocal of the activity in the absence of competing material. The theoretical value obtained from this experiment gave X_o = 143 dpm. compared with an experimentally determined value of 160 dpm. Within the limits of the method 90-100 per cent homology exists between the two RNA preparations.

An attempt to detect tissue specific RNAs in rat organs.

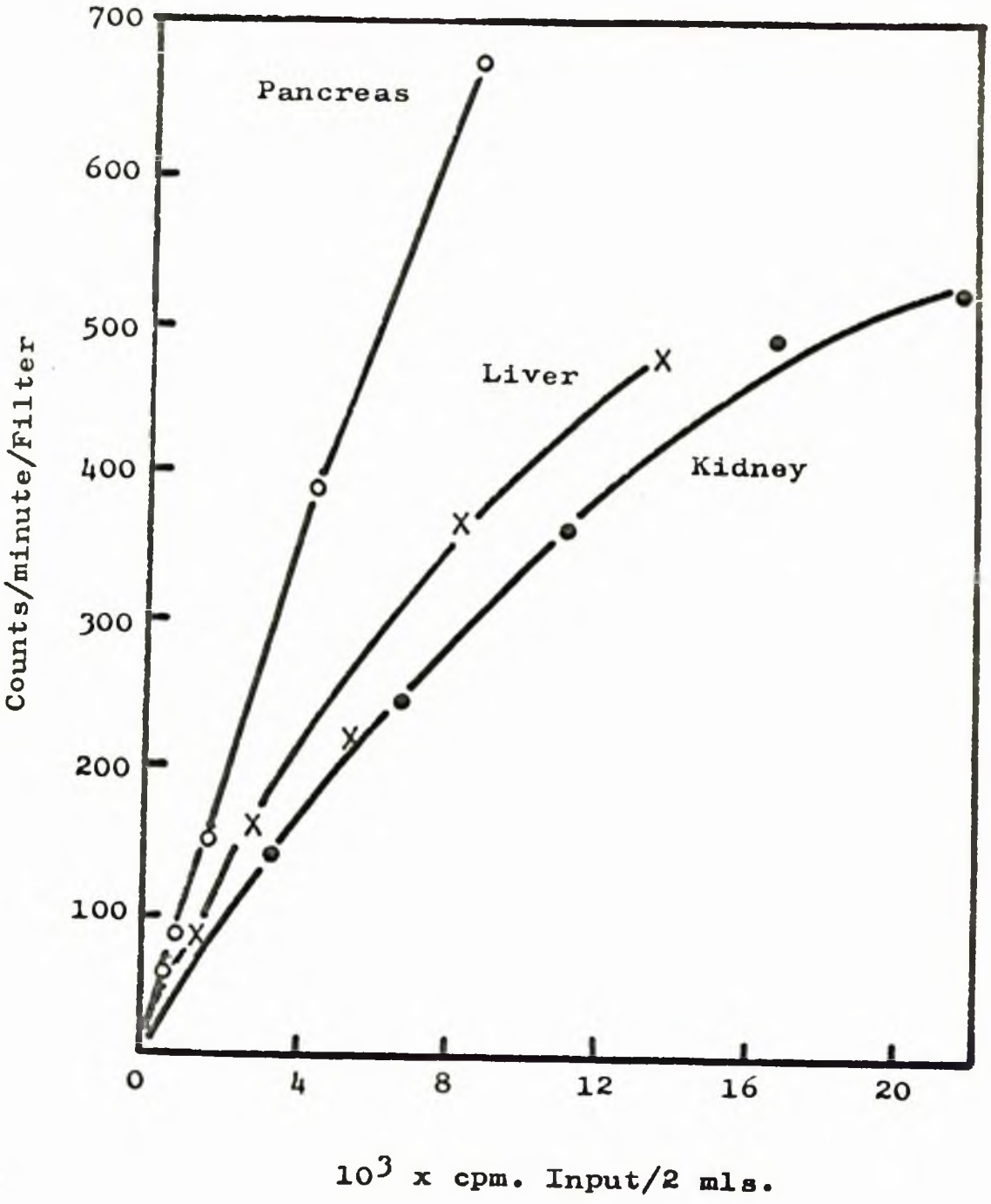
An attempt was made to detect tissue specific RNAs in rat kidney, liver and pancreas using this hybridization technique. Current theory suggests that such a difference might reside in the messenger RNA fraction of these tissues. After short term labelling of a rat with ^{32}P-orthophosphate as described in the methods, RNA was prepared from the nuclei of these organs. Because these preparations contain large amounts of unlabelled nuclear ribosomal RNA it was impossible to determine the true specific activity of the messenger fraction.

Figure 13 shows the saturation kinetics obtained when increasing concentrations of these RNAs were hybridized with 20 µg. rat kidney DNA. Even at high inputs of RNA saturation of the DNA is not achieved. In addition it was not found possible to express the saturating levels of radioactivity in terms of percentage homology since the true specific activity of the messenger fraction was unknown. The saturating levels of activity determined from a double

Figure 13.

Kinetics for the hybridization of increasing amounts of *in vivo*, ^{32}P-rapidly labelled RNAs from rat pancreas, liver and kidney with 20 μg denatured rat kidney DNA.

Figure 13


700
600
500
400
300
200
100
Pancreas
Liver
Kidney
Counts/minute/Filter
0
4
8
12
16
20
10³ x cpm. Input/2 mls.

reciprocal plot (Figure 14) shows differences in the saturation levels of pancreas RNA and kidney and liver RNA. This difference could be real or could be due to a difference in the specific activities of the RNAs synthesised in the organs. Neither possibility could be readily distinguished.

It was hoped that any differences in the messenger fractions of the RNAs would be detectable by competitive hybridization. Unlabelled reference RNA was prepared from rat kidney nuclei and added in varying concentrations to hybridization mixtures containing near saturating amounts of labelled RNA in the presence of 20 µg. kidney DNA. The displacement of pancreas, liver and kidney rapidly labelled RNAs from DNA by unlabelled kidney RNA is shown in Figure 15. The curves suggest that differences may exist. This is also shown in the double reciprocal plot of Figure 16. For complete homology the intercept on the ordinate is unity. As might be expected, kidney RNA shows the greatest homology while pancreas and liver RNAs are less homologous. Whereas the hybridization technique of Gillespie and Spiegelman was found to be the most convenient for the present studies, the use of <u>in vivo</u> labelled RNAs presented a number of difficulties, which will be discussed later along with the above results. It was therefore decided to set up an <u>in vitro</u> RNA synthesising

Figure 14.

Double reciprocal plot of the data shown in Figure 13.

Figure 14.

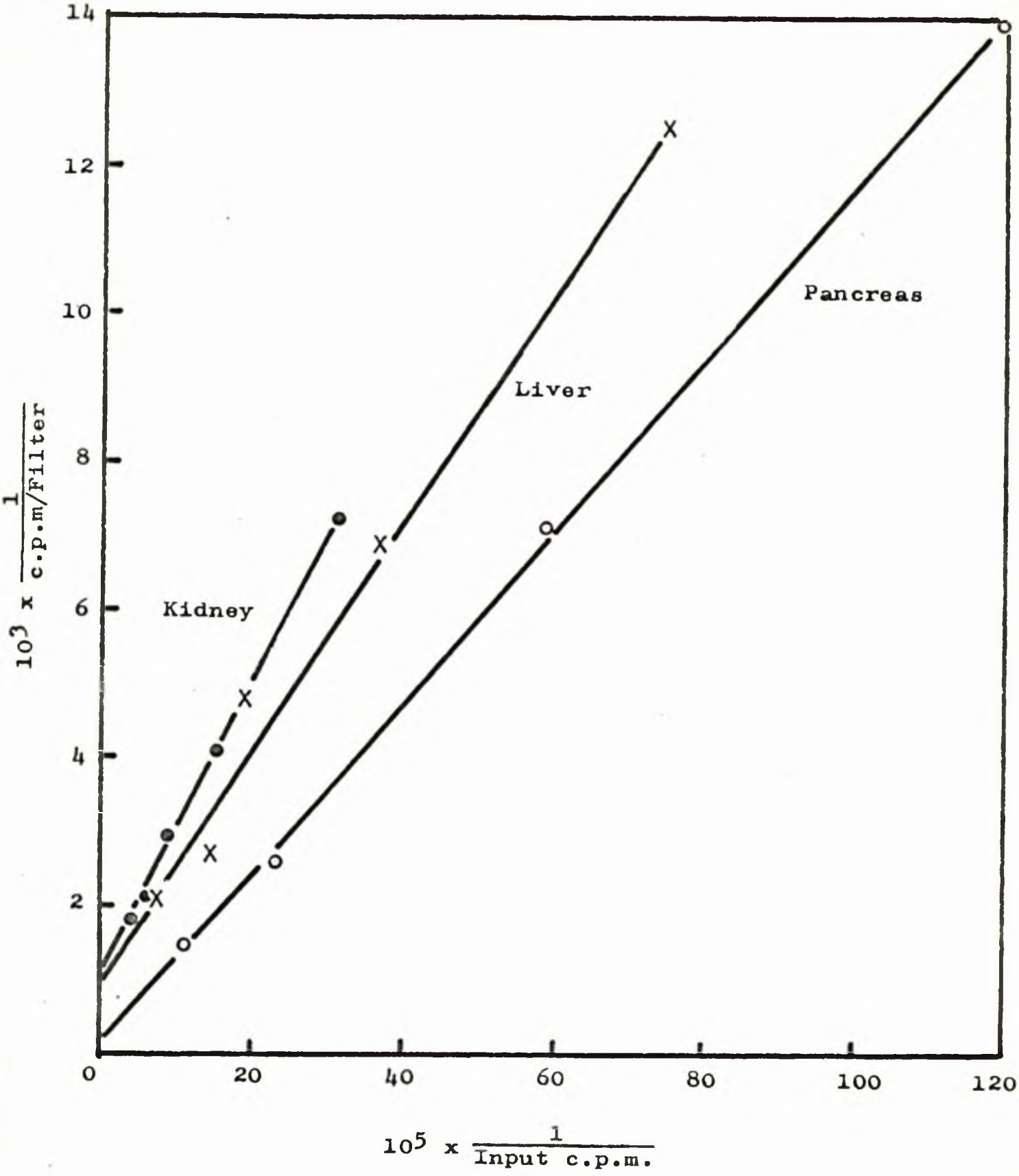

14
12
10
8
6
4
2
0
20
40
60
80
100
120
Kidney
Liver
Pancreas
$10^3 \times \frac{1}{\text{c.p.m/Filter}}$
$10^5 \times \frac{1}{\text{Input c.p.m.}}$

Figure 15.

Competition experiment between unlabelled rat kidney RNA and ^{32}P-rapidly labelled RNAs from rat pancreas (o —— o), liver (x —— x), and kidney (• —— •). Each filter, containing 20 μg denatured rat kidney DNA, was incubated with constant amounts of the labelled RNA under test in the presence of increasing concentrations of unlabelled kidney RNA.

Figure 16.

Double reciprocal plot of the competition data shown in Figure 16. For complete homology between labelled and unlabelled RNA the intercept on the ordinate at infinite concentrations of unlabelled RNA should be 1.

Figure 15.

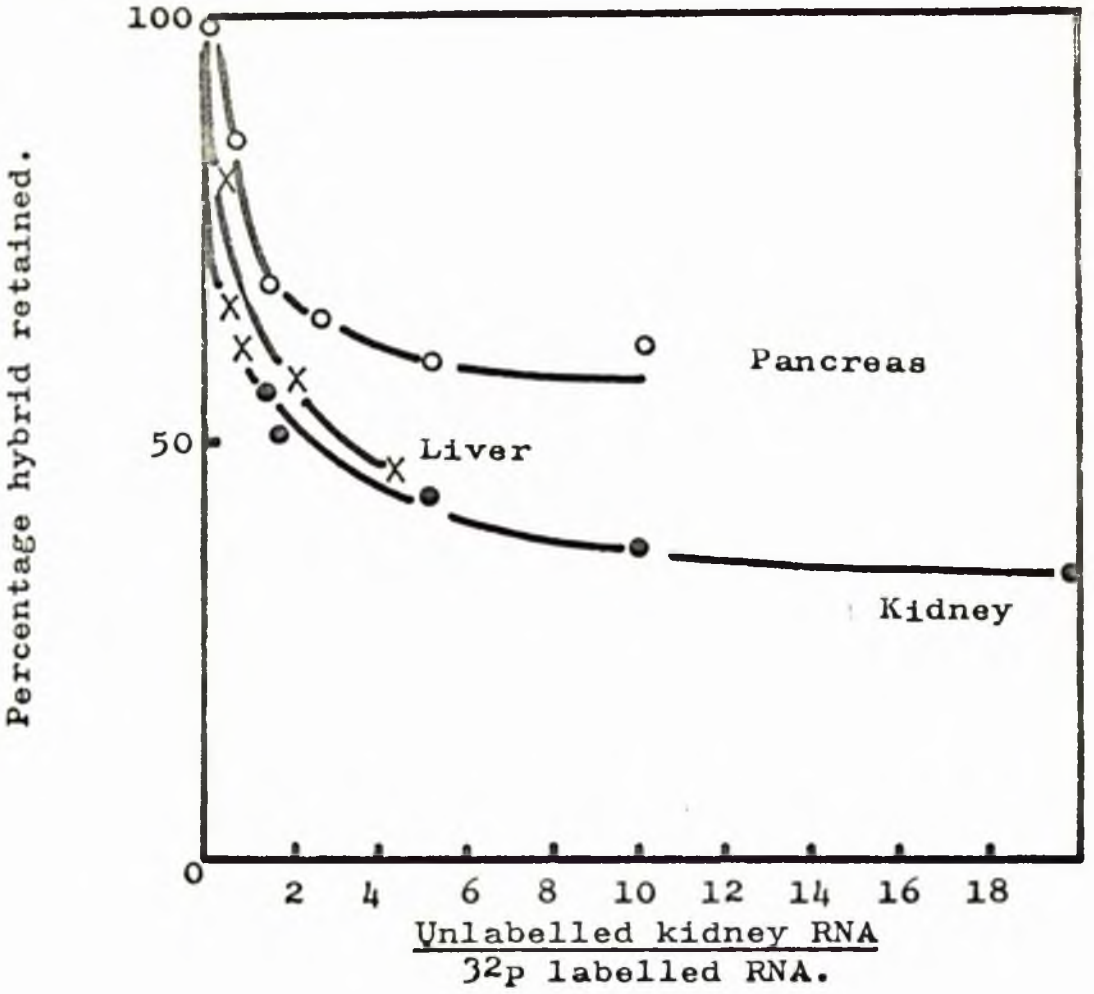
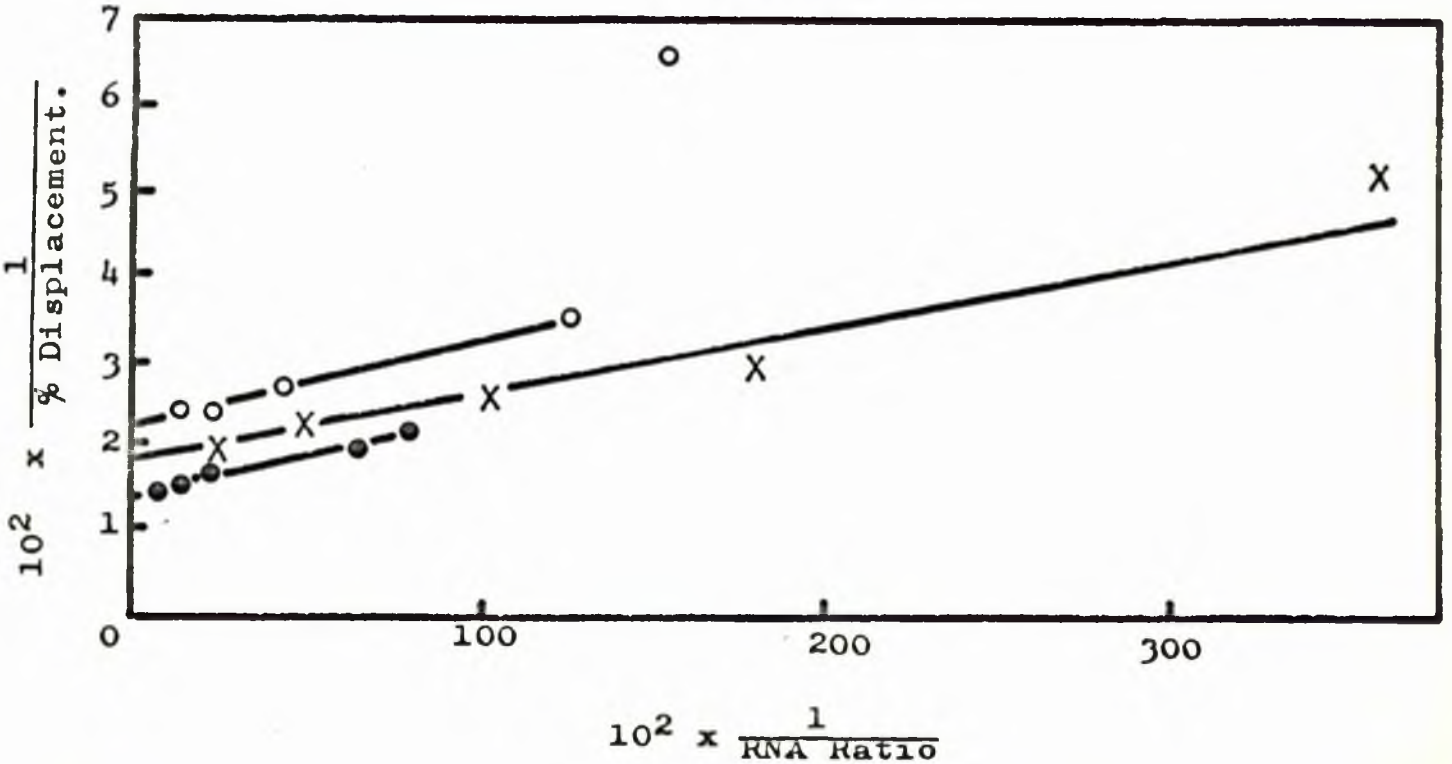


Figure 16.



system in which the chromatin from the tissues of interest acted as primer for the RNA polymerase of M.lysodeikticus. Aspects of this system are described in the following sections.

3.2 The DNA dependent RNA polymerase of Micrococcus lysodeikticus.

The purification and properties of RNA polymerase.

RNA polymerase was purified from M.lysodeikticus by the method of Nakamoto et al., (1964). A 30 g. batch of spray-dried cells from Cambrian Chemicals Ltd., London, was suspended by Waring blender in 300 mls. 0.01 M tris-HCl, pH 8, and centrifuged down at 20,000 g. for 10 minutes. The pellets were suspended in 600 mls. 0.2 M sucrose: 0.01 M tris-HCl, pH 8, at 30°C, 200 mg. of crystalline lysozyme added (L. Light and Co.) and the suspension incubated at 30°C for 45 minutes. After 15 minutes, 1.5 ml. 0.1 M $MgCl_2$ were added with vigorous stirring, and likewise after 45 minutes, 4.5 mls. 0.1 M $MgCl_2$ 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 steps were carried out at 0 - 4°C.

120 mls. 10 per cent (w/v) streptomycin sulphate (Glaxo Laboratories, England) 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 20,000 g. for 10 minutes. The supernatant was decanted and the pellets rinsed with 10 mls. distilled water. The pellets were washed by light homogenisation in 200 mls. 0.001 M tris-HCl, pH 8: 0.2 M sucrose : 0.1 per cent streptomycin sulphate : 0.25 M $MgCl_2$, mixed for 10 minutes and centrifuged at 20,000 g. for 10 minutes. The supernatant was discarded and the pellets rinsed with distilled water.

The pellets were suspended in 180 mls. of a solution containing 2 mls. 1 M potassium phosphate buffer pH 7.5 : 2 mls. 0.1 M $MgCl_2$: 20 mls. 2 M sucrose by homogenisation. After suspension 8 mls. of 1 M potassium phosphate buffer were added slowly with stirring. The solution became very viscous and was left for 10 minutes before slowly adding 10 mls. of 10 per cent streptomycin sulphate as before. After mixing for 10 minutes the supernatant was collected by centrifugation at 30,000 g. for 30 minutes and then further centrifugation in the Spinco 30 head at 80,000 g. 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 Co.) 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 M, enough 1 M phosphate buffer was added so that after the addition of protamine sulphate the final phosphate molarity was 0.14 M. The protamine sulphate was then added dropwise with continuous stirring and after 10 minutes the precipitate was collected by centrifugation at 20,000 g. for 10 minutes. The pellets were homogenised in 30 mls. 0.2 M sucrose : 0.30 M potassium phosphate, mixed for 10 minutes and centrifuged at 20,000 g. 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 extract was mixed and centrifuged as before. The supernatant was discarded and the pellet homogenised in 5 mls. 0.2 M sucrose : 0.3 M potassium phosphate pH 7.5. After 10 minutes, pellets were obtained by centrifugation at 20,000 g. for 10 minutes, and homogenised in 10 mls. 0.2 M sucrose : 0.14 M potassium phosphate buffer pH 7.5. After mixing for 10 minutes, the supernatant was collected by centrifugation at 20,000 g. for 10 minutes. The pellet was re-extracted with 5 mls. of the same buffer and combined with the previous extract.

The protein concentration of the extract was adjusted

to approximately 6 mg./ml. with extracting medium. 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 20,000 g. for 10 minutes. The pellet was dissolved in 0.1 M 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.

Table 5 shows a typical purification using the described method. The original lysate and the ammonium sulphate precipitate are the only stages free of protamine or streptomycin. Since these substances inhibit polymerase action by the precipitation of DNA primer, assays were only carried out on these stages. Spermidine phosphate, which produces a twofold stimulation of activity, was not added to the assay. This can account for the discrepancy in total units of enzyme recovered as compared with the purification data of Nakamoto et al., (1964). The usual specific activity of the enzyme at this stage was about 200 units/mg. protein.

The presence of ribonuclease and phosphodiesterase in the preparation was tested for by incubation with aliquots of E.coli transfer RNA in 2 mls. After 30 minutes at 30°C, 4 mls. of a solution containing 1 g. lanthanum acetate : 150 mls. ethanol : 50 mls. 5 N HCl were

Table 5.

THE PURIFICATION OF M.LYSODEIKTICUS RNA POLYMERASE USING THE METHOD OF NAKAMOTO ET AL. (1964).

	VOLUME ml.	TOTAL UNITS	TOTAL PROTEIN mg.	SPECIFIC ACTIVITY units/mg.
Cell lysate	1200	10,500	5520	1.9
Ammonium sulphate precipitate	6.5	10,432	40	261

Table 6.

AN EXAMINATION OF PURIFIED POLYMERASE FOR RIBONUCLEASE AND PHOSPHODIESTERASE.

DUPLICATE SAMPLES	µg POLYMERASE PREP/INCUBATION	INCUBATION TIME	ADDITIONS	OPTICAL DENSITY AT 260 mµ.
1.	None	0 mins.	0.02mKPO_4	0.20
2.	None	30 mins.	0.02mKPO_4	0.19
3.	None	0 mins.	None	0.20
4.	None	30 mins.	None	0.20
5.	350 µg.	0 mins.	0.02mKPO_4	0.21
6.	350 µg.	30 mins.	0.02mKPO_4	0.20
7.	350 µg.	0 mins.	Nons	0.22
8.	350 µg.	30 mins.	None	0.23

added, and the sample filtered through Whatman No. 1 filter paper. The eluates were monitored at 260 mμ and compared against a reagent blank. Assays for phosphodiesterase require the presence of 0.02 M potassium phosphate pH 7.5 which functions as an activator. Table 6 summarises the results obtained. Neither ribonuclease nor phosphodiesterase activity could be detected. The ammonium sulphate fraction was therefore used for *in vitro* studies without further purification.

The quantitative assay for polymerase activity.

Assay conditions were essentially those of Nakamoto *et al.*, (1964) except for the presence of spermidine. Incubations were carried out at 30°C for 10 minutes, in conical centrifuge tubes containing 50 μmoles tris-HCl, pH 7.5 ; 1.25 μmoles $MnCl_2$; 100 μg. calf thymus DNA ; 400 μmoles each of GTP, CTP, ATP, and labelled UTP (^{32}P - UTP or ^{3}H-UTP added to give 0.5 - 2 x 10^6 dpm. per μmole) and 0.1 - 0.5 mg. enzyme in a total volume of 0.5 ml.

The reaction was terminated by placing the tubes in ice and adding 0.1 ml. 50 per cent TCA, followed by 3 mls. 5 per cent TCA saturated with sodium pyrophosphate. Where small amounts of material were present, 0.5 mg. bovine serum albumin was added as carrier, before precipitation. After standing in ice for 10 minutes the precipitates were collected by centrifugation at 2,000 g. for 10 minutes.

The supernatants were discarded and the precipitates washed three times in 5 per cent TCA. Where ^{32}P-UTP was used, the precipitates were assayed in a gas-flow counter as described in the Methods. For ^{3}H-UTP, precipitates were trapped on Millipore filters and counted in a scintillation spectrometer.

A unit of activity is defined as the amount of enzyme which catalyses the incorporation of 1mμ mole of UTP into acid-insoluble material during 10 minutes' incubation at 30°C.

The properties of M.lysodeikticus polymerase.

The effect of omitting various components of the assay system was examined. The results are shown in Table 7. No significant activity was detected when polymerase, manganese or nucleoside triphosphates were omitted. Small amounts of activity were detected in the absence of DNA. Although putrescine was not found to be essential for activity, its presence caused a 2-3 fold increase in activity.

The requirement for a divalent cation. The activity of the polymerase in the presence of variable concentrations of manganese, magnesium, and calcium chlorides was determined. The results (Figure 17) show that manganese and magnesium are effective activators, manganese exhibiting a sharp optimum around 3 mM whereas magnesium appears to be effective over a wide concentration range above 3 mM, but at a lower

Table 7.

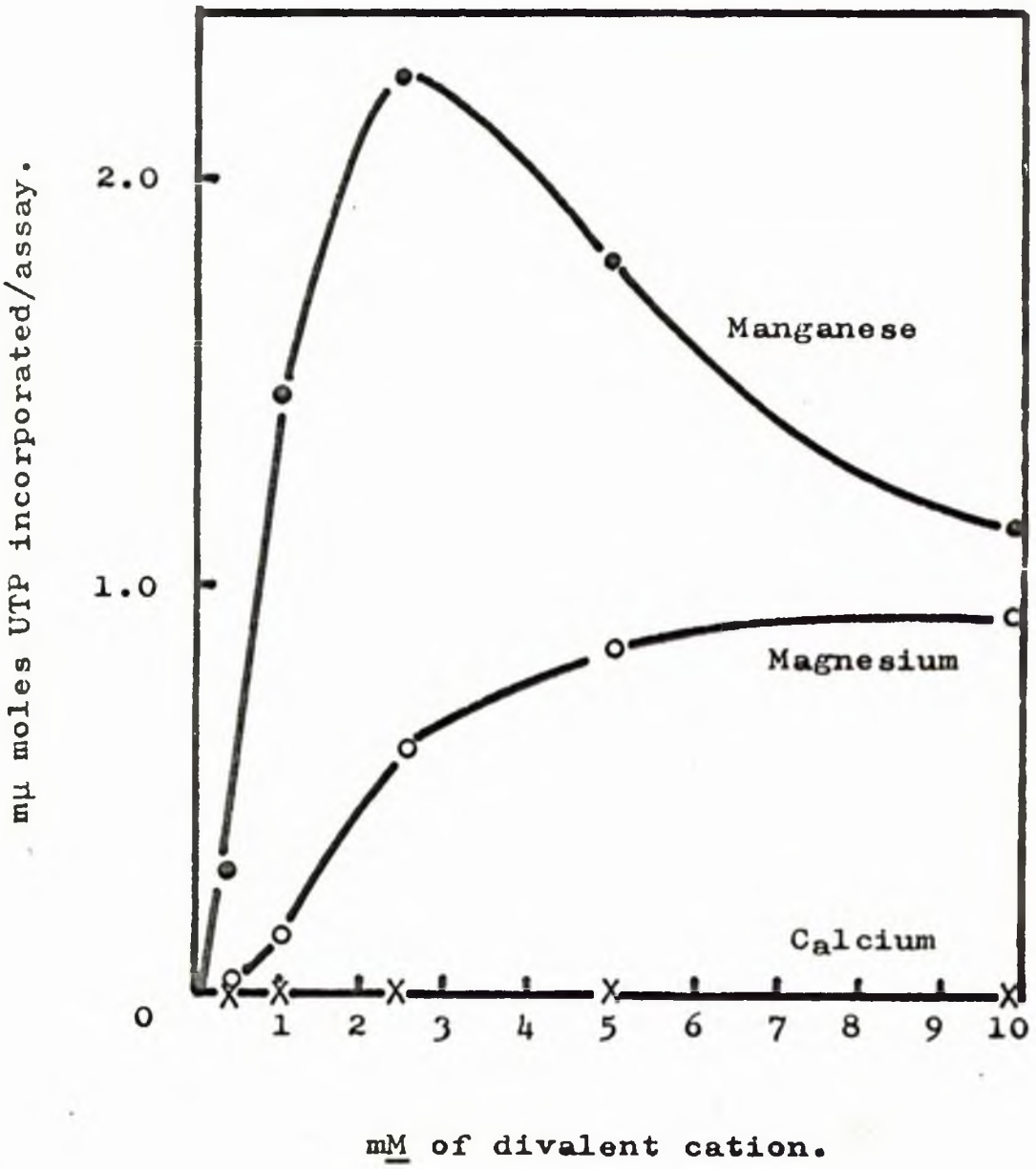
GENERAL CONDITIONS FOR POLYMERASE ACTIVITY.

ASSAY CONDITION	mμ MOLES UTP INCORPORATED/10 mins.
Complete with 16mM putrescine	4.48
Omit putrescine	1.75
Omit ATP, GTP and CTP.	0.22
Omit manganese	0.04
Omit DNA	0.12
Omit enzyme	0.025

Figure 17.

The effect of various divalent cations on the activity of M.lysodeikticus polymerase: manganese chloride (• —— •), magnesium chloride (o —— o), and calcium chloride (x —— x).

Figure 17.


mμ moles UTP incorporated/assay.
2.0
1.0
0
Manganese
Magnesium
Calcium
1
2
3
4
5
6
7
8
9
10
mM of divalent cation.

level. Calcium ions were found to be completely inhibitory.

The activity of the polymerase in mixtures of manganese and magnesium ions was also determined. With a basal concentration of 2.5 mM manganese chloride, the enzyme was assayed in the presence of additional magnesium chloride over a range of 0 - 50 mM final concentration. Figure 18 shows that apart from a small initial stimulation at very low magnesium concentrations, the combined effect of both cations is inhibitory. When a basal concentration of 10 mM magnesium chloride and variable concentrations of manganese chloride were employed (Figure 19), it was found that optimal activity occurred at 2.5 mM. No advantage could be found in using both cations together in the assay. In all subsequent reactions manganese chloride was present at a final concentration of 2.5 mM.

The requirement for DNA primer. The dependence of the polymerase on DNA primer is shown in Figure 20. 3.3 units of enzyme were added to assays containing calf thymus DNA over a range of 0 - 100 µg. Saturation of the enzyme was obtained at about 50 µg./0.5 ml. From a double reciprocal plot (Figure 21) a V_{max} of 3.3 mµmoles and a K_m of 5 µg. was predicted.

Time course for the polymerase reaction. The time course for the polymerase reaction was determined under

Figure 18.

The effect of a combination of magnesium and manganese ions on the activity of M.lysodeikticus polymerase. Each assay contained a final concentration of 2.5 mM manganese chloride, in the presence of varying concentrations (0 - 50 mM) magnesium chloride.

Figure 19.

The effect of a combination of magnesium and manganese ions on the activity of M.lysodeikticus polymerase. Each assay contained a final concentration of 10 mM magnesium chloride in the presence of varying concentrations of manganese chloride (0 - 8 mM).

Figure 18.

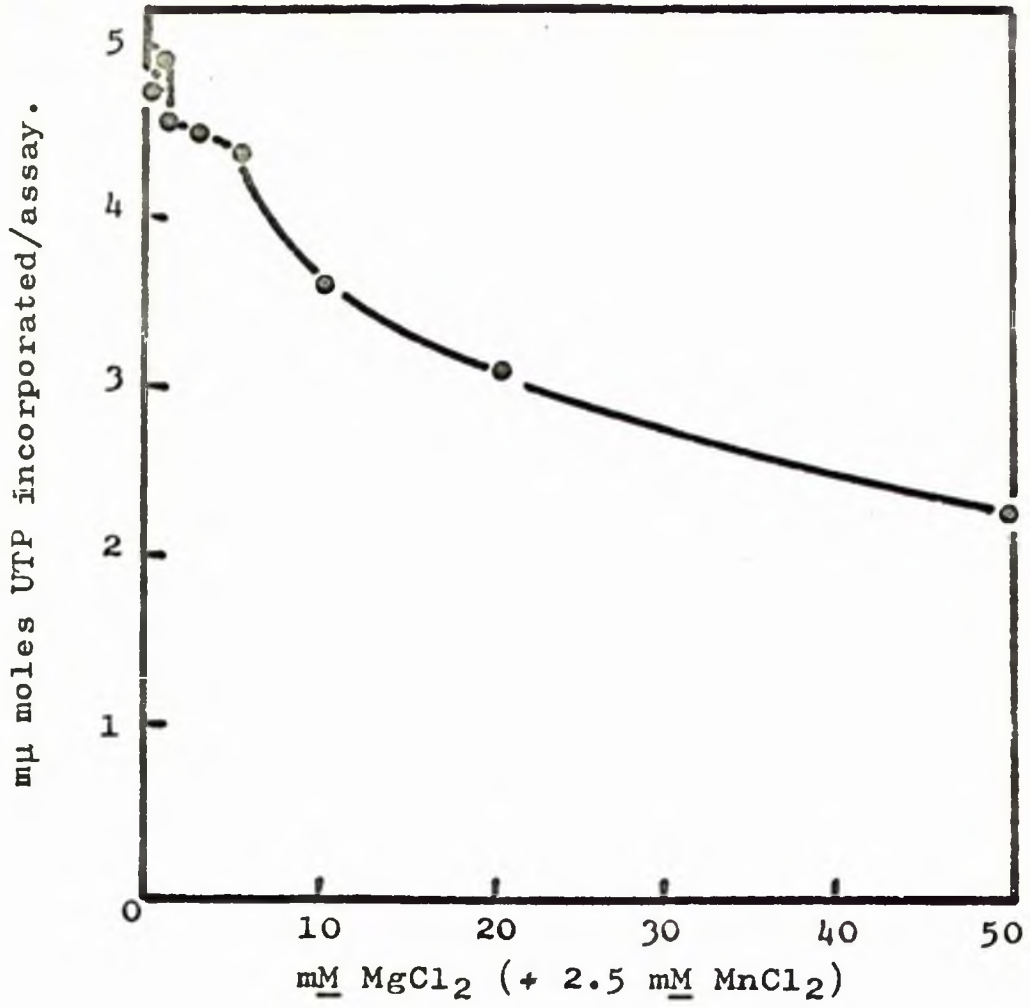


Figure 19.

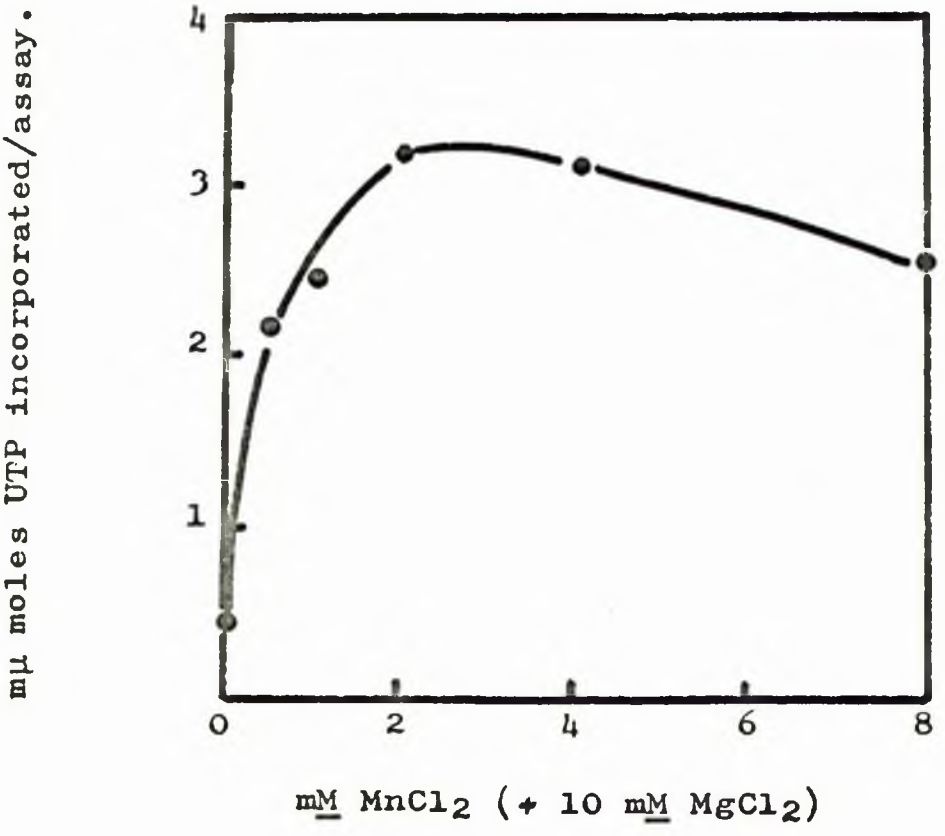


Figure 20.

The requirement of M.lysodeikticus polymerase for a DNA primer. Approximately 3 units of polymerase were incubated for 10 minutes in the presence of increasing amounts of calf thymus DNA. Maximum velocity was attained at about 50 μg DNA/0.5 ml.

Figure 21.

Double reciprocal plot of the data shown in Figure 20. A $V_{max.}$ = 3.3 mμ moles and a K_m = 5 μg was predicted.

Figure 20.

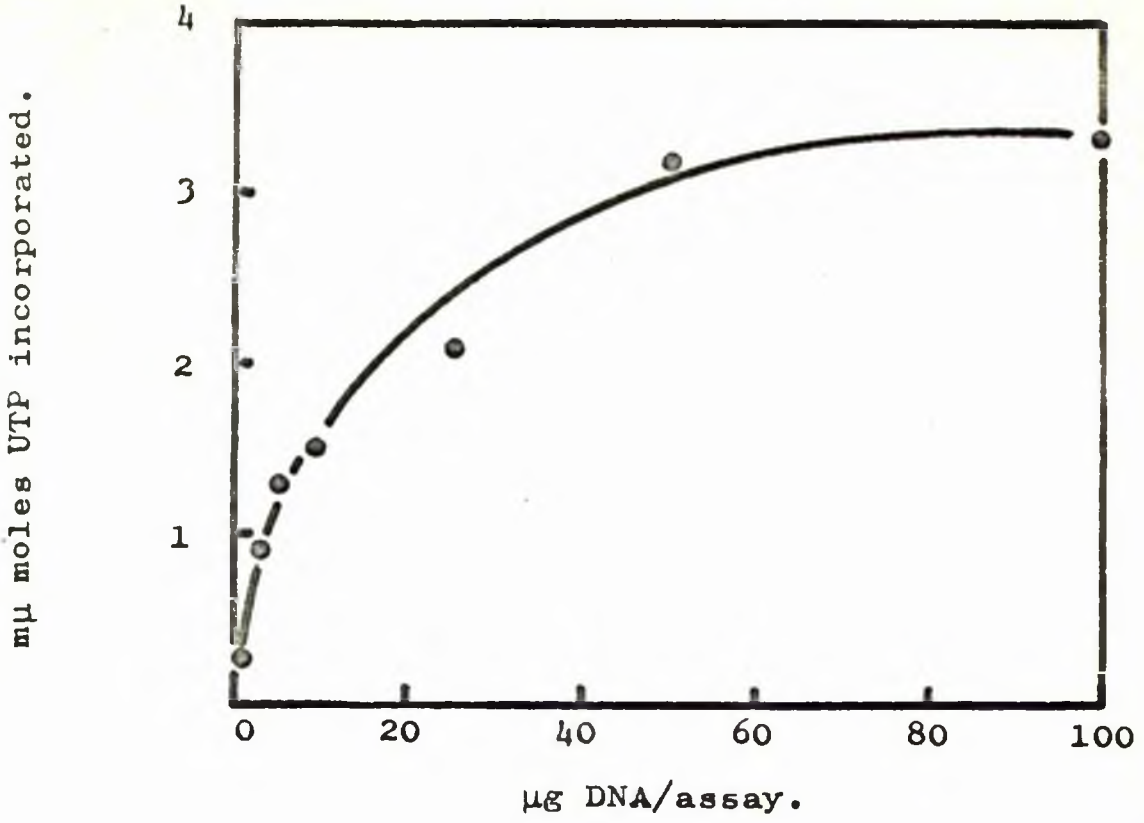
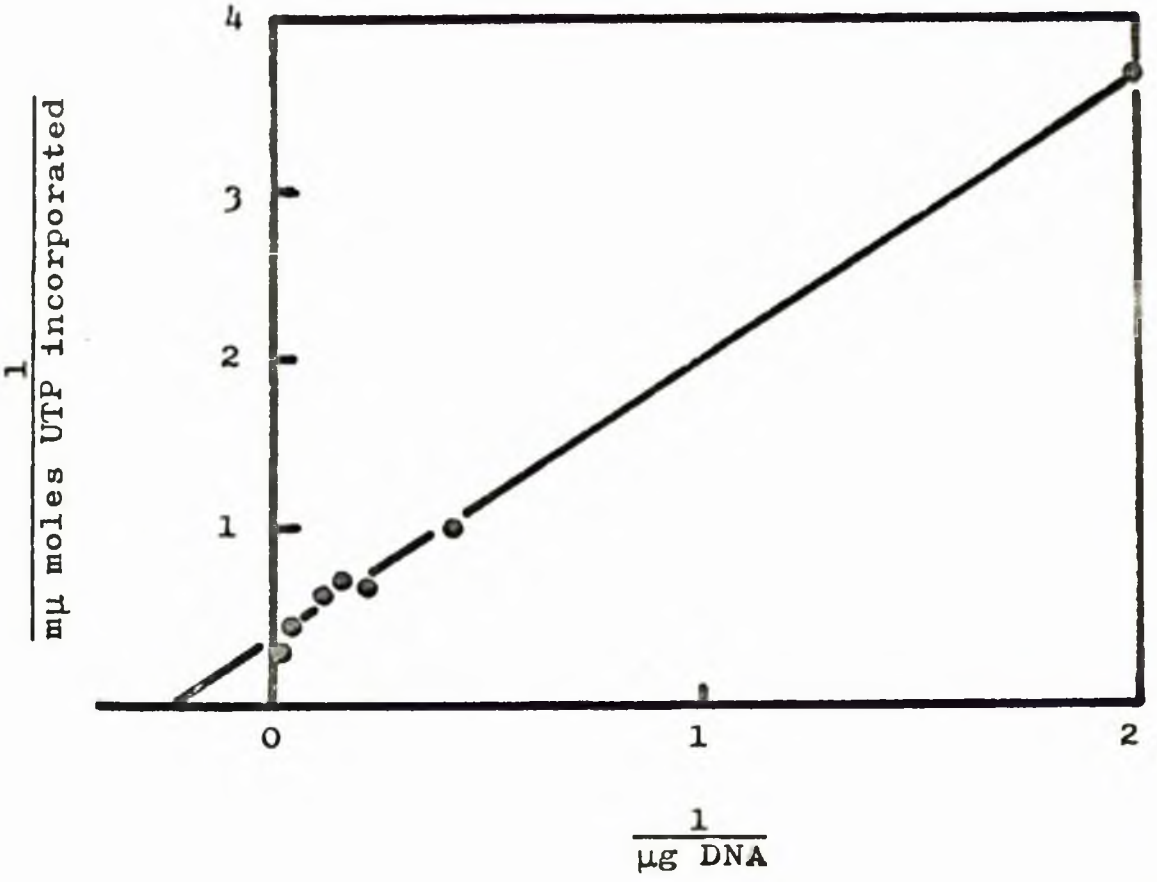


Figure 21.



standard conditions using a saturating concentration of DNA. Assays were incubated for varying times and the reaction terminated by the addition of 0.1 ml. 50 per cent TCA.

The reaction was found to be linear for the first 15 minutes (Figure 22), after which time a gradual decline in the rate of RNA synthesis occurred.

The effect of salts on polymerase action. It was found that polymerase activity is remarkably sensitive to the concentration of tris-HCl buffer used in the assay (Figure 23). This effect was also noted with lithium, potassium and sodium chlorides and also for arginine and lysine hydrochlorides. In all cases 0.1 M tris-HCl was present and the activities in the presence of the various salts expressed as a percentage of the activity obtained with 0.1 M tris-HCl alone. Marked inhibition occurred when the total ionic strength of the assay exceeded about 0.2 M.

3.3 Chromatin.

The preparation of chromatin.

Chromatin was prepared from mammalian tissues by one of the three methods described below. In essence the procedure adopted was first to isolate clean nuclei from cell homogenates and to remove soluble nuclear material with isotonic buffer. On further washing in a salt-free

Figure 22.

Time curve for the <u>in vitro</u> incorporation of ^{32}P-UTP into polyribonucleotides by M.lysodeikticus polymerase. 1 unit of polymerase was incubated with a saturating amount of calf thymus DNA for the various times indicated.

Figure 22.

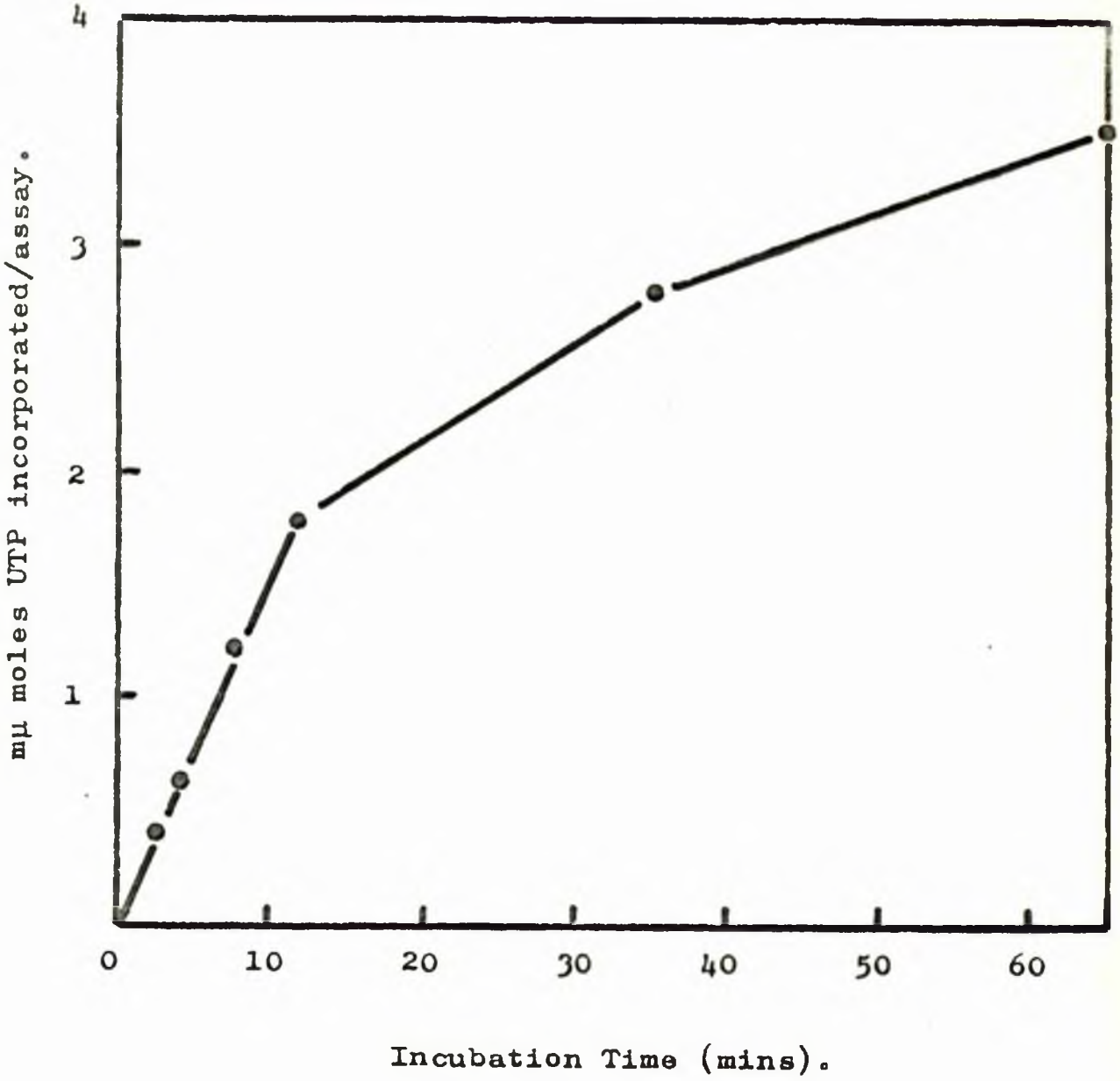

4
3
2
1
mμ moles UTP incorporated/assay.
0
10
20
30
40
50
60
Incubation Time (mins).

Figure 23.

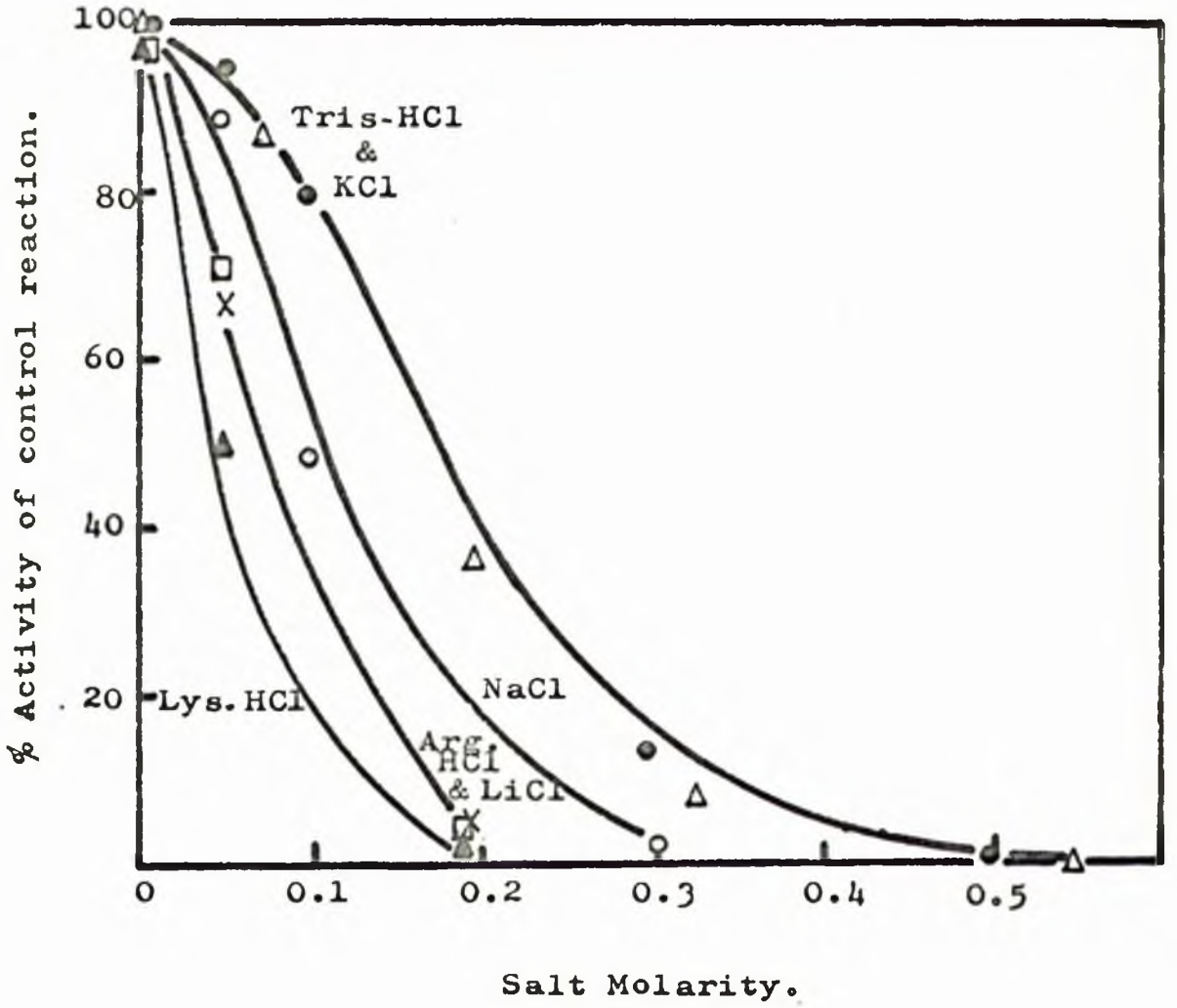
The effect of various salts on the activity of M.lysodeikticus polymerase. The activity of the polymerase in 0.1 M Tris/HCl, pH 7.5 is taken as 100% and the activities observed in the presence of additional salts expressed as a percentage of this figure.

Tris/HCl, pH 7.5, Δ—Δ ; KCl, • — •;

NaCl, o — o ; arginine/HCl, pH 7.5, □ — □ ;

LiCl, x — x ; lysine/HCl, pH 7.5, ▲—▲

Figure 23.


100
80
60
40
20
% Activity of control reaction.
Tris-HCl
&
KCl
X
NaCl
Lys. HCl
Arg. HCl
& LiCl
0
0.1
0.2
0.3
0.4
0.5
Salt Molarity.

medium the chromatin swelled to give a viscous gel. The success of a preparation was assessed by the ability of the material to gel during the final stages.

Method A. Nuclei were prepared by the citric acid procedure already described in the Methods Section. Washing was continued until a clear supernatant was obtained. The nuclei were then suspended in 0.2 M potassium phosphate buffer pH 7. After centrifugation at 2,000 g. for 10 minutes, the sediment was washed in 0.001 M EDTA. The gel which formed was centrifuged at 10,000 g. for 15 minutes. Finally the resulting pellet was dissolved in distilled water. This material constituted chromatin. Although this method worked well with calf thymus, considerable difficulty was encountered in preparing chromatin from other tissues such as rat liver and kidney. The following method was found to give reproducible results with all tissues examined.

Method B. Citric acid nuclei were suspended in 0.15 M NaCl : 0.1 M tris-HCl, pH 7.5 by light homogenisation. After 10 minutes in ice, the material was centrifuged at 2,000 g. for 10 minutes and the pellet taken up in distilled water. Water washing was continued until a viscous gel was formed.

Method C. A third method, avoiding the use of extreme pH values, was devised for comparative purposes. The tissue was homogenised in 20 volumes (w/v) distilled water

and left for 10 minutes at 0°C. The sediment obtained after centrifugation at 2,000 g. for 10 minutes was re-homogenised in water and filtered through muslin. The nuclear pellet was then suspended in 0.15 M NaCl for 10 minutes and centrifuged as before. Finally the material was washed in distilled water until gelation occurred.

Some chemical and physical properties of chromatin.

Chemical composition of chromatin. Determinations of DNA, RNA and protein were carried out according to the methods already outlined. For facility in pipetting, chromatin gels were first sonicated to abolish viscosity. 1 ml. aliquots containing 15 - 20 optical density units at 260 mμ were treated with 1 ml. of 0.6 N KOH for 2 hours at 37°C. 1 ml. of 1.5 N PCA was added at 0°C and the precipitate centrifuged down at 2,000 g. for 10 minutes. The hydrolysed RNA present in the supernatant was measured 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. 1 ml. aliquots were assayed for DNA by the diphenylamine method.

Estimation of basic and residual protein was carried out as follows. To 0.1 ml. aliquots of chromatin, 0.1 ml. 0.2 N HCl was added. After 1 hour at 0°C the supernatant was collected by centrifugation at 2,000 g. for 10 minutes and the pellet re-extracted with 0.2 ml. HCl. The super-

natants were combined and 0.4 ml. 2 N NaOH added. 0.8 ml. 1 N NaOH was added to each precipitate. After incubating the samples at 37°C for 1 hour protein was estimated by the bromosulphalein method as previously outlined. The results obtained for chromatin from various sources are shown in Table 8. While the RNA/DNA and acidic protein/DNA ratios are variable a fairly constant figure of around unity was found for basic protein/DNA irrespective of the chromatin source.

The solubility of chromatin in salt solutions. The effect of salt on the solubility of calf thymus chromatin was investigated by adding various amounts of concentrated ammonium sulphate solution to 1.3 mg. of chromatin in a final volume of 2 mls. After being thoroughly mixed and left in ice for 10 minutes the solutions were centrifuged at 2,000 g. for 10 minutes. The supernatants were then analysed for protein by the Lowry method and for DNA by the diphenylamine method. The results were expressed as percentage of the original material remaining in the supernatant. From the results shown in Figure 24, it would appear that chromatin is least soluble at physiological salt concentrations. As the ionic strength is increased the DNA-protein salt linkages dissociate and the DNA is solubilised while the protein tends to precipitate out. Some DNA may also co-precipitate with the protein.

Table 8.

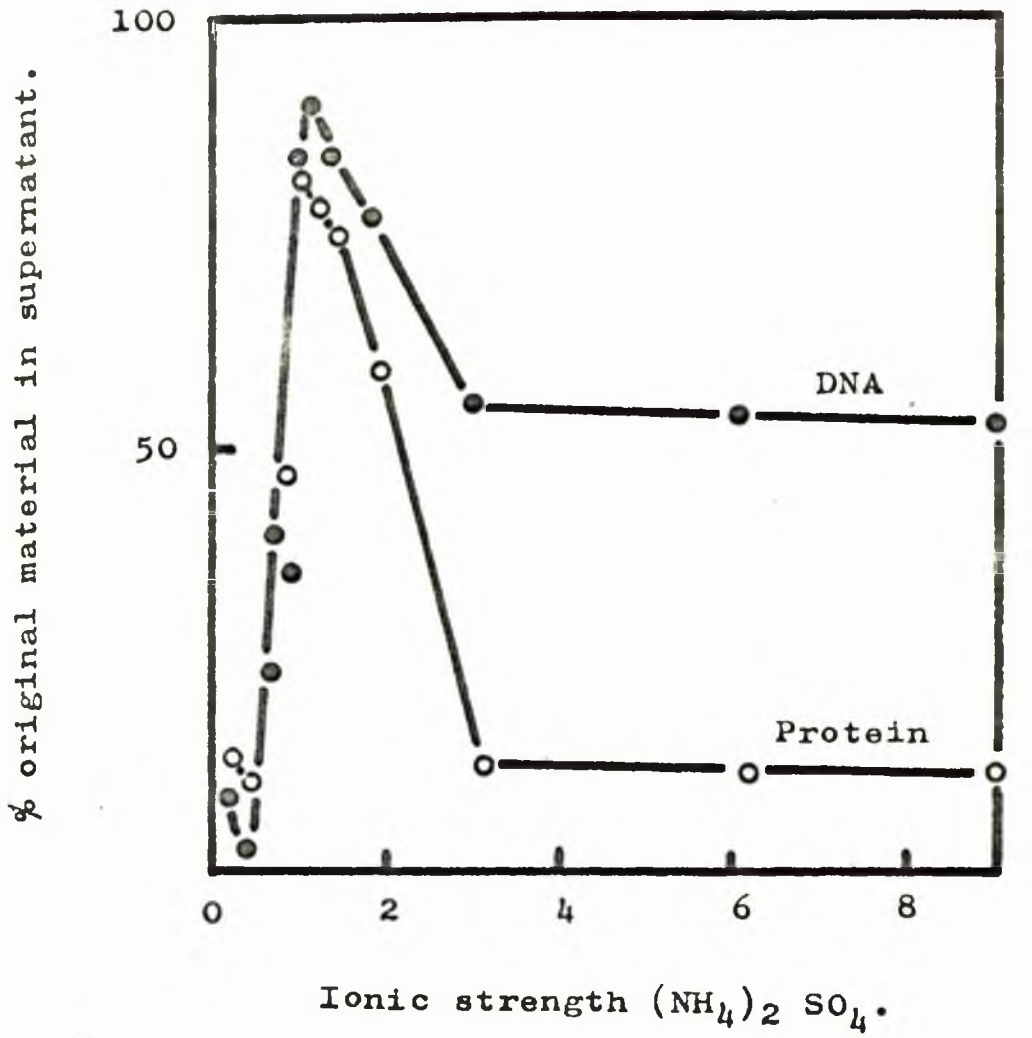
THE CHEMICAL COMPOSITION OF VARIOUS CHROMATIN PREPARATIONS.

CHROMATIN PREPARATION	RNA/DNA	BASIC PROTEIN/DNA	ACIDIC PROTEIN/DNA	ACIDIC/BASIC PROTEIN	ACIDIC PROTEIN/RNA
Rat liver	0.24	1.15	2.0	1.8	8.5
Rat thymus	0.08	1.00	0.5	0.5	7.1
Rat spleen	0.12	1.10	1.3	1.2	10.3
Rabbit thymus	0.04	1.15	0.5	0.4	13.5
Calf thymus	0.03	0.90	0.3	0.34	15.0

Figure 24.

The solubility of chromatin in increasing concentrations of ammonium sulphate. 1.3 mgs. of calf thymus chromatin was treated with ammonium sulphate solutions at the concentrations shown and the degree of solubility determined by analysing the supernatants for DNA and protein.

Figure 24.


100
50
% original material in supernatant.
DNA
Protein
0
2
4
6
8
Ionic strength $(NH_4)_2 SO_4$.

Melting profiles of chromatin. Melting profiles were carried out using a Unicam SP 800 spectrophotometer equipped with a cell block, the temperature of which could be raised to 100°C by an electric heater. DNA and chromatin solutions in 0.01 x SSC were diluted to 0.5 OD units at 260 mμ and readings taken for every 5° increment in temperature. Readings at 300 mμ were also taken to check for light scattering.

Typical melting profiles for calf thymus DNA and chromatin are shown in Figure 25. Both preparations gave 35 - 40 per cent hyperchromicity at 260 mμ with no detectable light scattering at higher wavelengths. In the absence of salt however, chromatin precipitates on heating, producing a spurious hyperchromicity by light scattering.

The melting temperature for calf thymus DNA and chromatin (i.e. the temperature at half maximum hyperchromicity) was found to be 61° and 85° respectively. The presence of the protein component in chromatin appears to stabilise the DNA double helix against thermal denaturation.

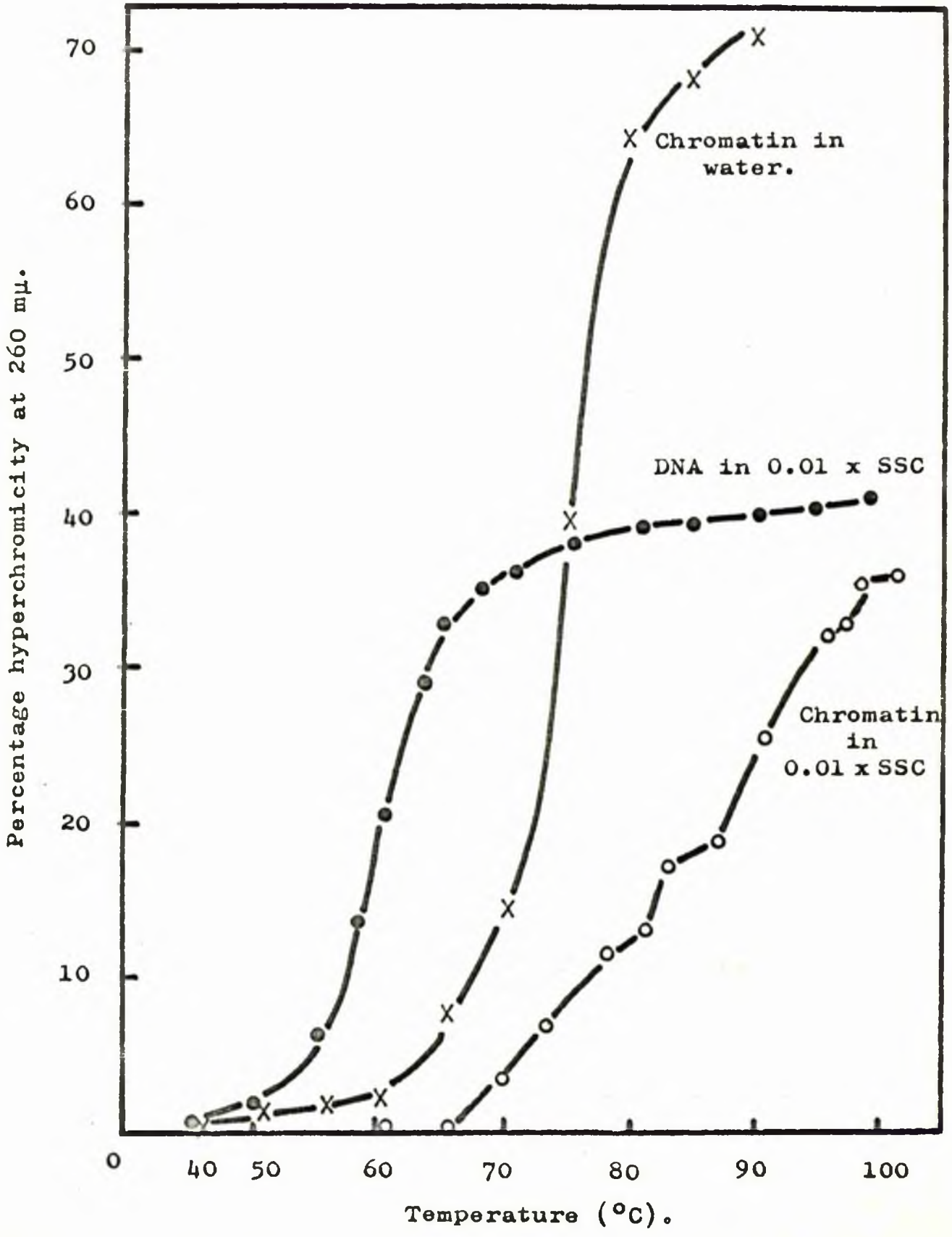
3.4 Chromatin primed RNA synthesis *in vitro*.

Experiments in which *in vivo* synthesised RNA is hybridised to homologous DNA suggest that in differentiated tissues only a small part of the genome is functional. Huang and Bonner (1962) suggested that this was achieved by the masking of non-functional parts of the genome and

Figure 25.

The melting profiles of calf thymus DNA and chromatin. Purified DNA in 0.01 x SSC, • —— • ; whole chromatin in 0.01 x SSC, o —— o ; whole chromatin in water, x —— x.

Figure 25.


Percentage hyperchromicity at 260 mμ.
70
60
50
40
30
20
10
0
40
50
60
70
80
90
100
Temperature (°C).
Chromatin in water.
DNA in 0.01 x SSC
Chromatin in 0.01 x SSC

that this control was preserved in the isolated chromatin. As already mentioned in the Introduction, several other workers have supported this claim; however in all cases the rates of *in vitro* RNA synthesis have been used as a criterion for restricted template activity. A more meaningful criterion is provided by molecular hybridization in which the sequence homology of the synthesised RNA can be determined directly. The following sections are devoted to testing this hypothesis.

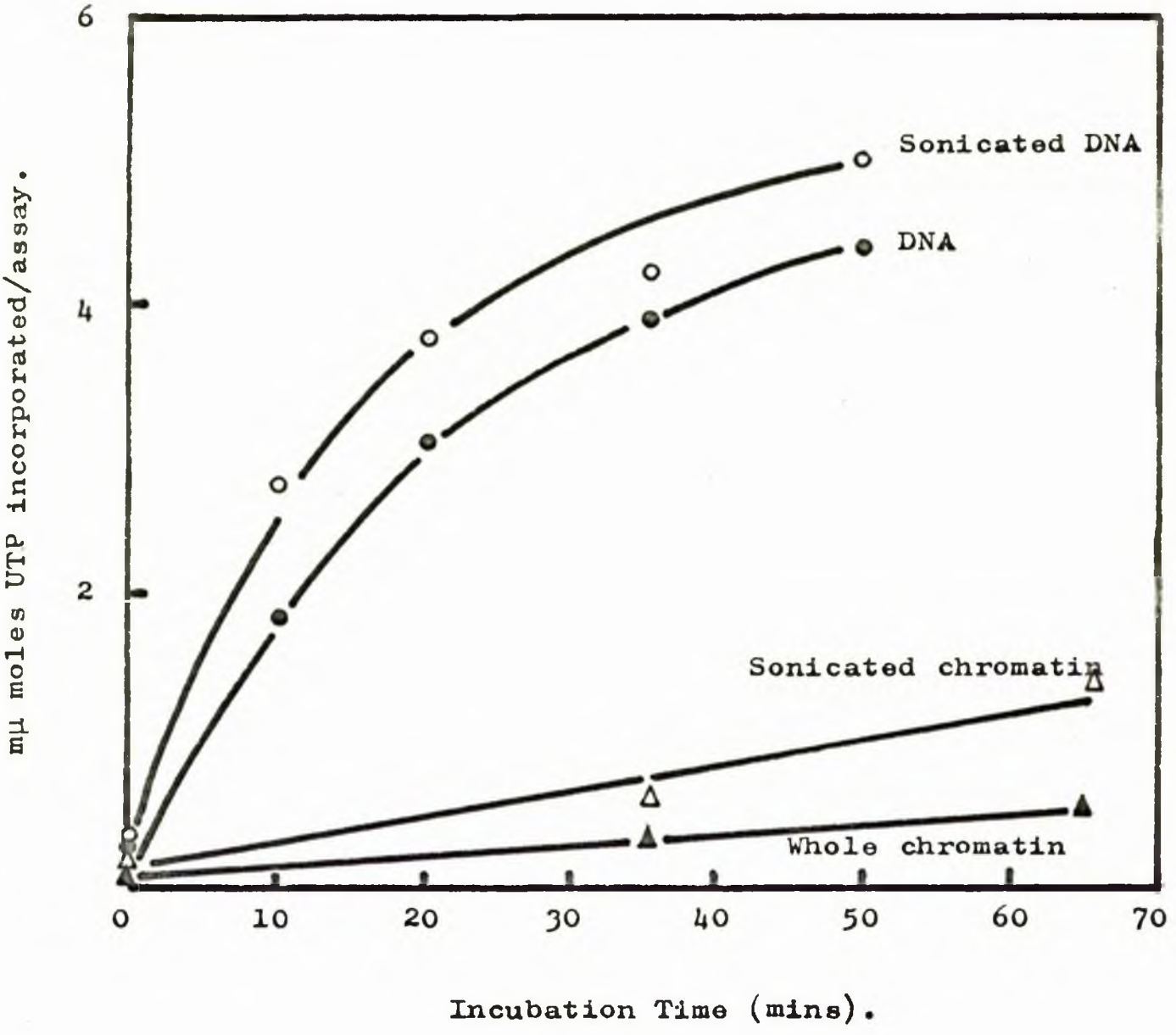
Rate studies on *in vitro* RNA synthesis.

Using conditions described, RNA polymerase was incubated with 100 µg. calf thymus DNA or chromatin for varying times. Figure 26 shows that the initial rate of RNA synthesis from DNA is much higher than that from chromatin. Sonnenberg and Zubay (1965) suggested that this difference might be due to the relative insolubility of chromatin under the incubation conditions. Solubilisation was achieved by sonication for 60 seconds in a MSE ultrasonic disintegrator. The effectiveness of chromatin as a primer was enhanced almost two-fold by this treatment; however it was still considerably less efficient than DNA. DNA is also rendered a more effective primer after sonication; this might result from a reduction in the viscosity in the incubation medium.

Figure 26.

The rates of RNA synthesis *in vitro* with M. lysodeikticus polymerase and as primers; calf thymus DNA, ● —— ● ; sonicated calf thymus DNA, o —— o ; whole chromatin, ▲——▲ ; sonicated chromatin, Δ——Δ . 10 μg DNA as DNA or chromatin was incubated with polymerase for the times shown.

Figure 26.


6
4
2
mμ moles UTP incorporated/assay.
Sonicated DNA
DNA
Sonicated chromatin
Whole chromatin
0
10
20
30
40
50
60
70
Incubation Time (mins).

Template activity of DNA in chromatin.

The previous results suggest that factors other than solubility are responsible for the diminished rate of transcription. Some authors have concluded that this is because the template activity of DNA in chromatin is restricted in some way; however it is not valid to draw such a conclusion purely from priming kinetics. To clarify this point, hybridization tests were employed to establish whether the RNA formed on chromatin templates is representative of all or only a few of the total available DNA sequences.

Chromatin was prepared from calf thymus by Method A and DNA also prepared from the same nuclear preparation as described previously. Samples were sonicated in 0.01 x SSC as before and 1 ml. aliquots containing 50 µg. DNA added to cell free incubation mixtures of 3 mls. Identical incubations containing unsonicated primers were also set up. After incubation at 30°C for 5 hours the reactions were terminated by freezing. The RNA was later extracted as outlined in the Methods Section. In each case the amount of RNA obtained was 2 - 4 times greater than the primer input.

Hybridization of the RNA products was carried out according to the method of Gillespie and Spiegelman (1965). Each 27 mm. filter containing 1 µg. of denatured calf thymus

DNA was incubated with varying amounts of synthetic RNA in a final volume of 2 mls. 6 x SSC. The extent of hybridization as a function of RNA input is shown in Figure 27. A double reciprocal plot of this data (Figure 28) permitted the prediction of saturation values. The regression obtained with whole and sonicated DNA preparations were identical and the results were combined to calculate the regression $y = 0.688 + 0.031x$. Knowing the specific activity of the ^{3}H-UTP in the incubation mixture, it was calculated that 0.4 - 0.5 µg. of RNA were bound to 1 µg. DNA at saturation. On the other hand, RNA transcribed from whole chromatin reached saturation at a level which corresponded to about 10 per cent of the saturation level for RNA transcribed from DNA. Using this criterion it was concluded that 90 - 95 per cent of each DNA molecule is "masked" in chromatin. It was also noted that sonication of chromatin increased the amount of DNA available for transcription.

These observations were later confirmed with rabbit tissues. Figures 29 and 30 show a similar experiment using chromatin primers from rabbit thymus and bone marrow and DNA from whole rabbit embryos. Again RNA prepared from embryo DNA saturates around 47 per cent of the DNA while thymus and marrow primed RNAs saturate 4.7 and 6.8 per cent respectively of the DNA.

Figure 27.

Kinetics of hybridisation to denatured calf thymus DNA of ^{3}H-RNA prepared <u>in vitro</u> with M.lysodeikticus polymerase and calf thymus DNA, • —— • ; sonicated DNA, o —— o ; whole chromatin, ▲——▲ ; and sonicated chromatin, Δ——Δ . Each filter containing 1 μg DNA was incubated with synthetic RNA at the concentrations shown.

Figure 27.

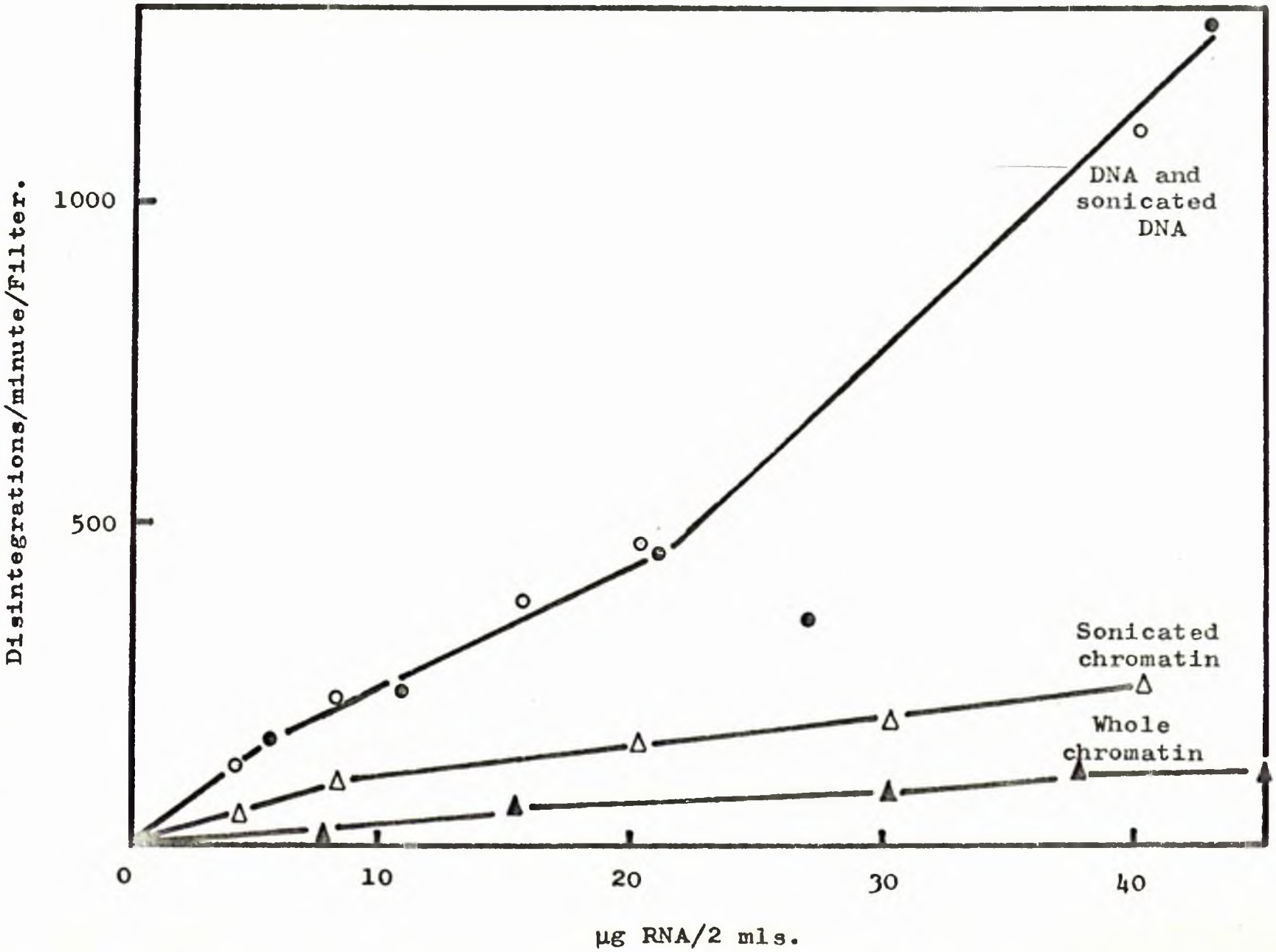

Disintegrations/minute/Filter.
1000
500
0
10
20
30
40
µg RNA/2 mls.
DNA and sonicated DNA
Sonicated chromatin
Whole chromatin

Figure 28.

Double reciprocal plot of the data shown in Figure 27. Both DNA and sonicated DNA results were combined to give the regression $y = 0.688 + 0.031\ x$. From this it was calculated that about 0.4 to 0.5 μg RNA would be bound to 1 μg DNA at saturation. RNA transcribed from whole chromatin reached saturation at a level corresponding to about 10% of that for RNA transcribed from DNA. RNA prepared from sonicated chromatin saturated about twice as much of the DNA.

Figure 28.

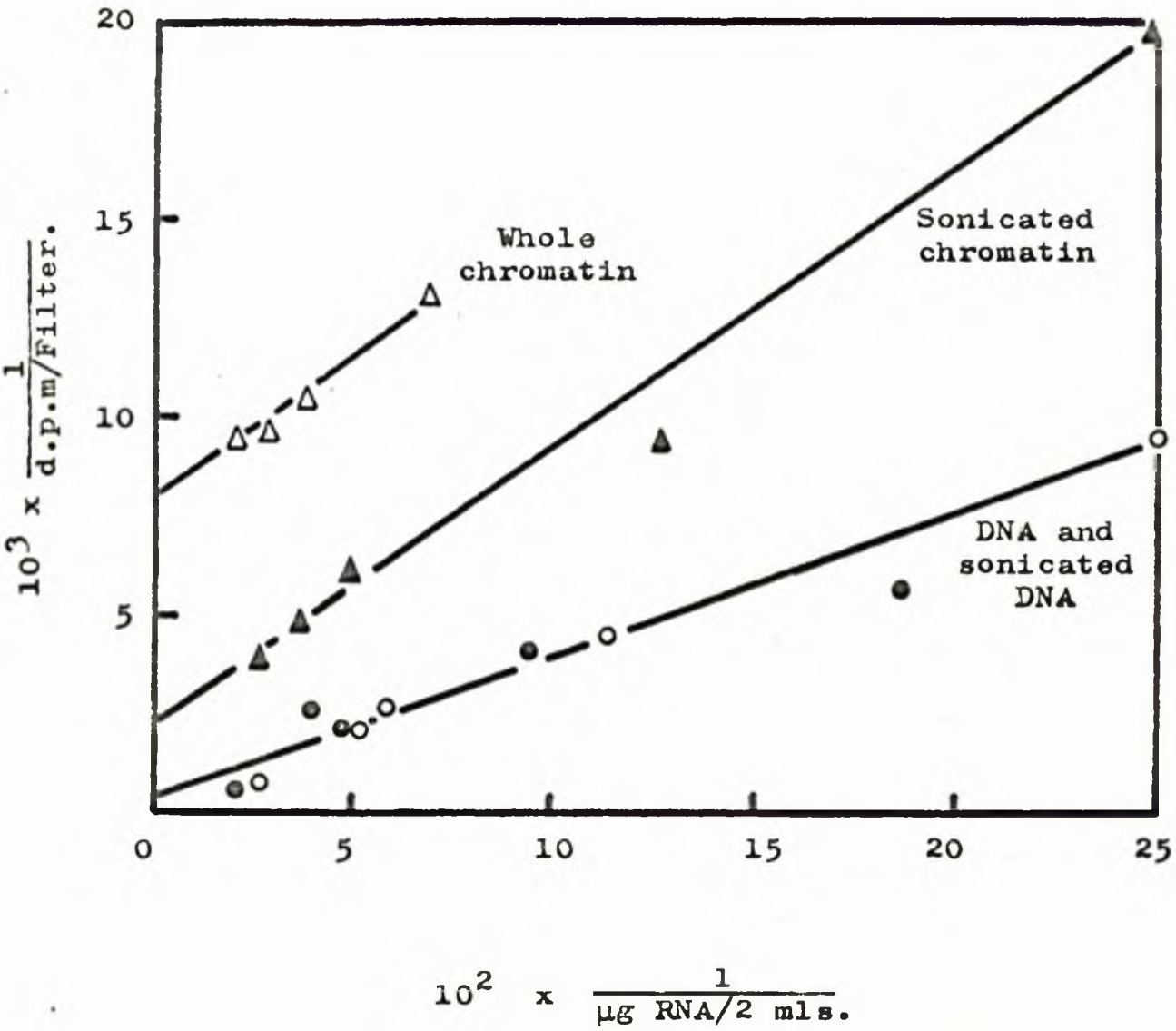

20
15
10
5
0
5
10
15
20
25
Whole chromatin
Sonicated chromatin
DNA and sonicated DNA
$10^3 \times \frac{1}{\text{d.p.m/Filter.}}$
$10^2 \times \frac{1}{\mu g \text{ RNA/2 mls.}}$

Figure 29.

Kinetics of hybridization to rabbit embryo DNA of ^{3}H-RNA made <u>in vitro</u> by M.lysodeikticus polymerase in the presence of rabbit thymus chromatin, x —— x ; rabbit bone marrow chromatin, • --- • ; and rabbit embryo DNA, o --- o. The filters were loaded with 1 μg denatured rabbit embryo DNA and the ^{3}H-RNA preparations were incubated with individual filters at the concentrations shown.

Figure 29.

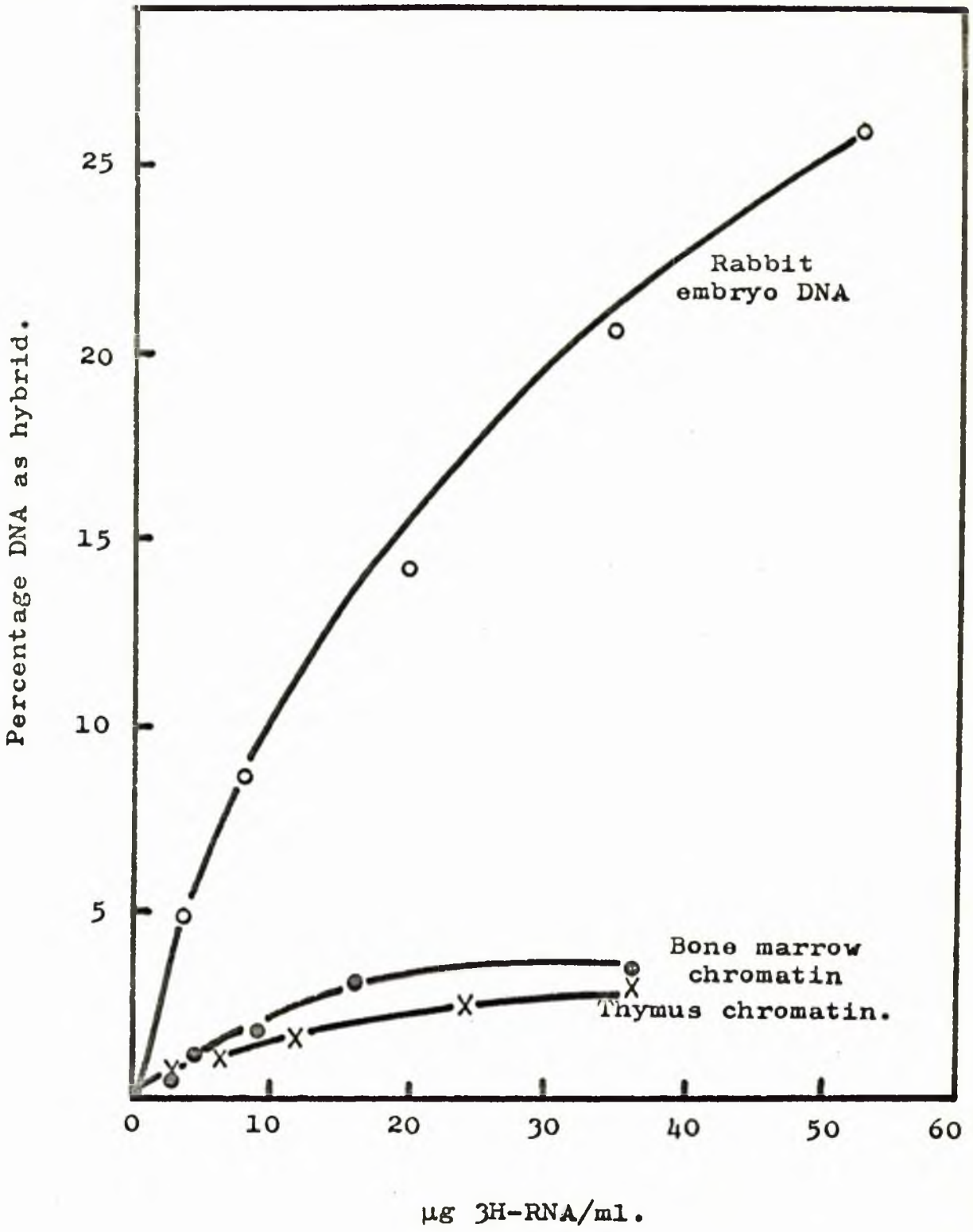
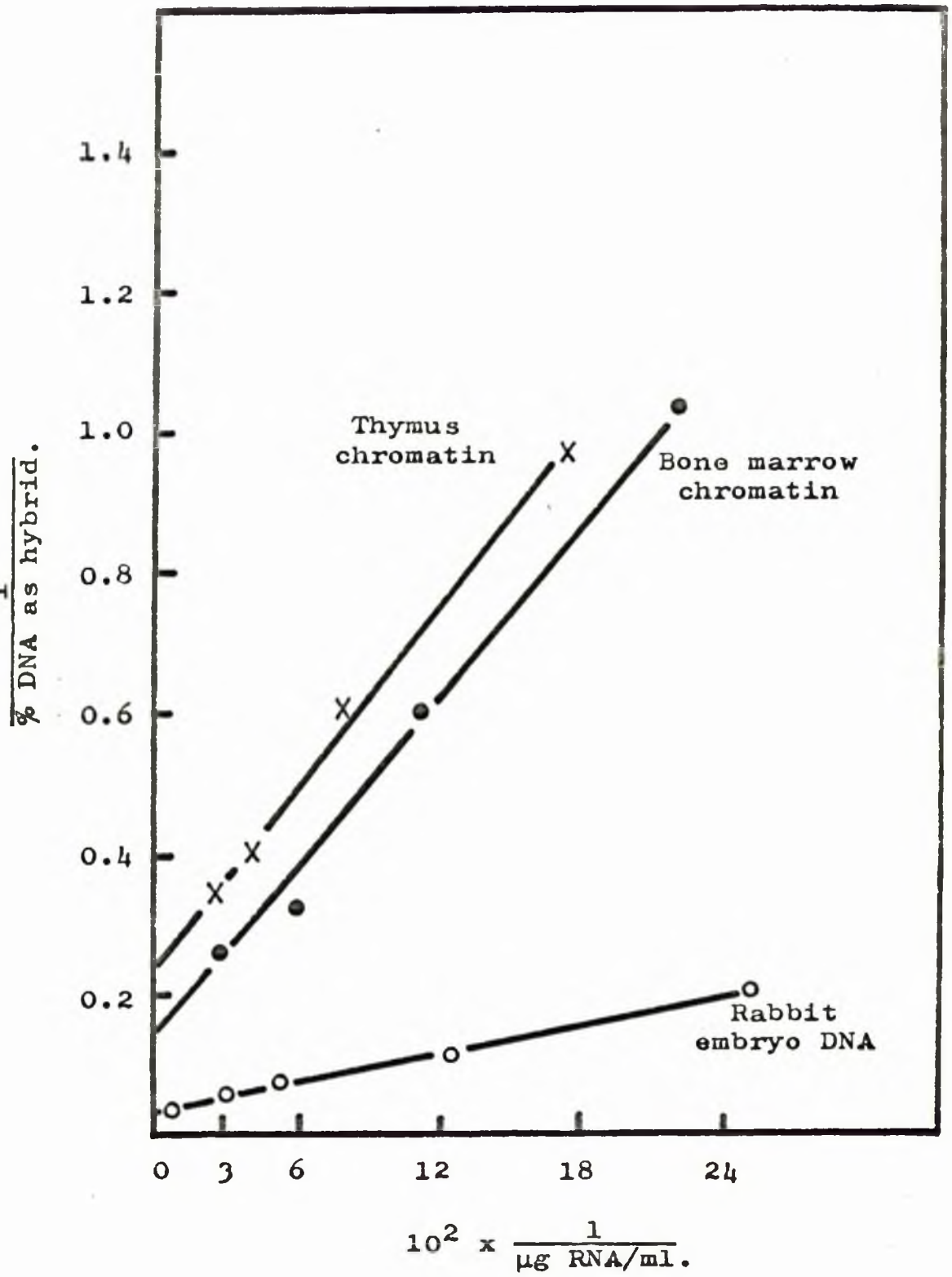

Percentage DNA as hybrid.
25
20
15
10
5
0
10
20
30
40
50
60
Rabbit
embryo DNA
Bone marrow
chromatin
Thymus chromatin.
µg 3H-RNA/ml.

Figure 30.

Double reciprocal plots of the data shown in Figure 29. The percentages of the DNA existing as hybrid when each RNA preparation saturates the DNA was predicted from the intercepts on the ordinate as follows: thymus chromatin, 4.7%; bone marrow chromatin, 6.8%; DNA, 47%.

Figure 30.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
1/% DNA as hybrid.
Thymus chromatin
Bone marrow chromatin
Rabbit embryo DNA
0
3
6
12
18
24
10² x 1/µg RNA/ml.

Comparison of *in vivo* and *in vitro* synthesised RNAs.

In order to determine whether the RNA formed *in vitro* from chromatin primers is the same as that formed by the tissue *in vivo* the following experiments were performed. Nuclei were first prepared from calf thymus using citric acid. A fraction was used to prepare chromatin; natural RNA was prepared from the remainder. The chromatin was used to prime the synthesis of RNA *in vitro*. Constant, near-saturating amounts were hybridized to 1 µg. of thymus DNA in the presence of increasing amounts of unlabelled natural RNA. Figure 31 shows the displacement of labelled hybrid from the DNA by the unlabelled RNA. By constructing a double reciprocal plot it can be shown (Figure 32) that at infinite concentrations of unlabelled RNA all the labelled material is excluded. To exclude artefacts, RNA from *E.coli* was also used for competition. As shown in Figure 31, it did compete with some of the labelled material; however analysis of the linear regression derived from the data indicate that only a small part of the RNA was affected. Figures 33 and 34 show the results from a similar experiment in which the RNA synthesised *in vitro* from rabbit bone marrow chromatin was compared with natural RNA from rabbit bone marrow nuclei. Again complete exclusion of labelled RNA was predicted at infinite concentrations of unlabelled RNA, suggesting that the *in vivo* and *in vitro* synthesised RNAs are qualitatively identical.

Figure 31.

Test for homology between calf thymus RNA and ^{3}H-RNA made *in vitro* with a calf thymus chromatin primer. Annealing mixtures contained 1 μg. denatured calf thymus DNA, a near saturating amount of synthetic chromatin-primed ^{3}H-RNA and varying amounts of natural thymus RNA.

Figure 31.

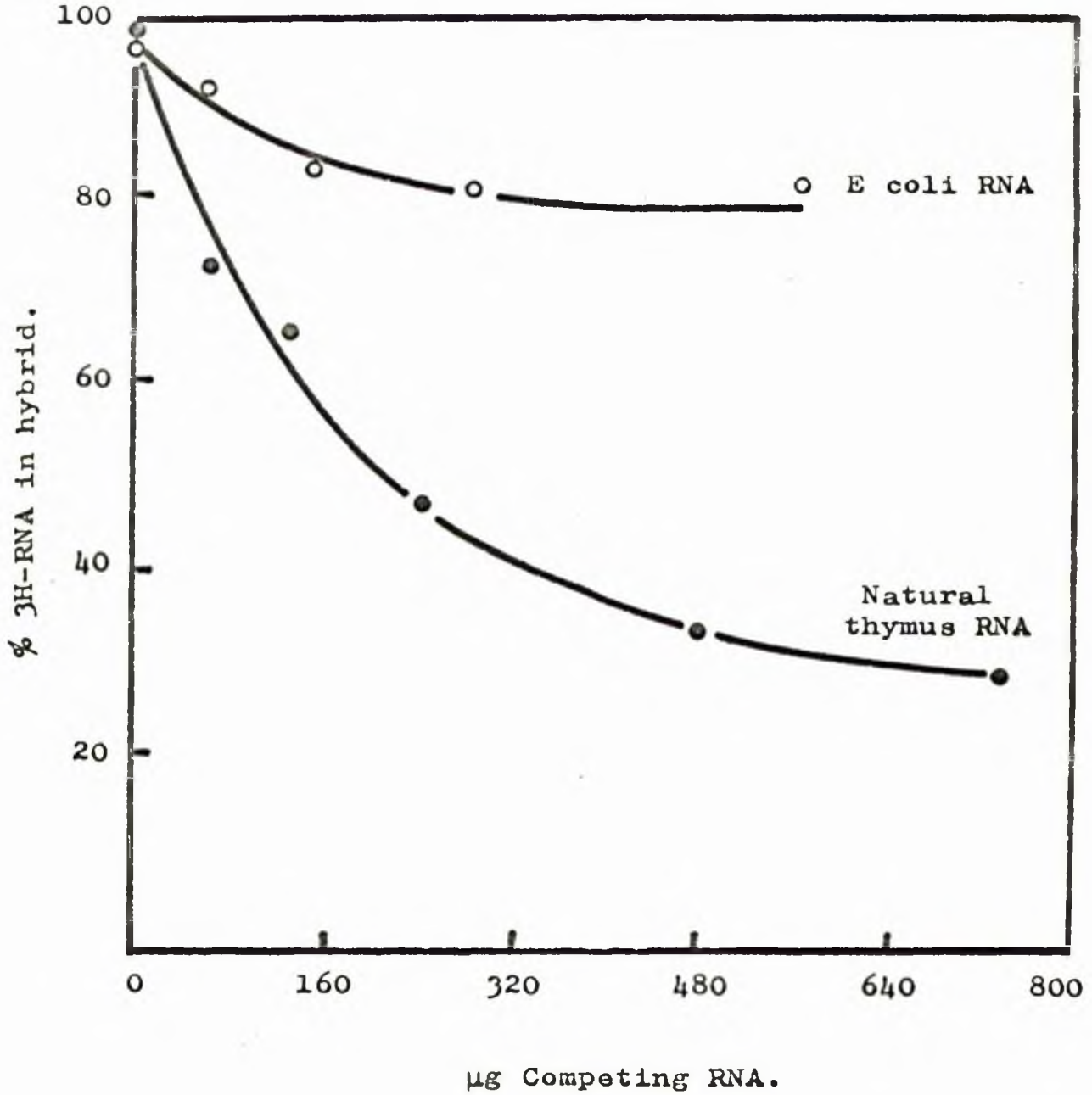

100
80
60
40
20
% 3H-RNA in hybrid.
E coli RNA
Natural
thymus RNA
0
160
320
480
640
800
µg Competing RNA.

Figure 32.

Double reciprocal plot of the data shown in Figure 31. Extrapolation to infinite concentrations of unlabelled RNAs gives $1/X_o - X$ values of 2670 d.p.m. for natural thymus RNA and 700 d.p.m. for E.coli RNA. The experimentally determined value for X_o was 2705 d.p.m.. From these figures it was concluded that natural calf thymus RNA and ^{3}H-RNA synthesized <u>in vitro</u> from calf thymus chromatin was at least 98% homologous, while homology between E.coli RNA and ^{3}H-RNA was only 25%.

Figure 32.

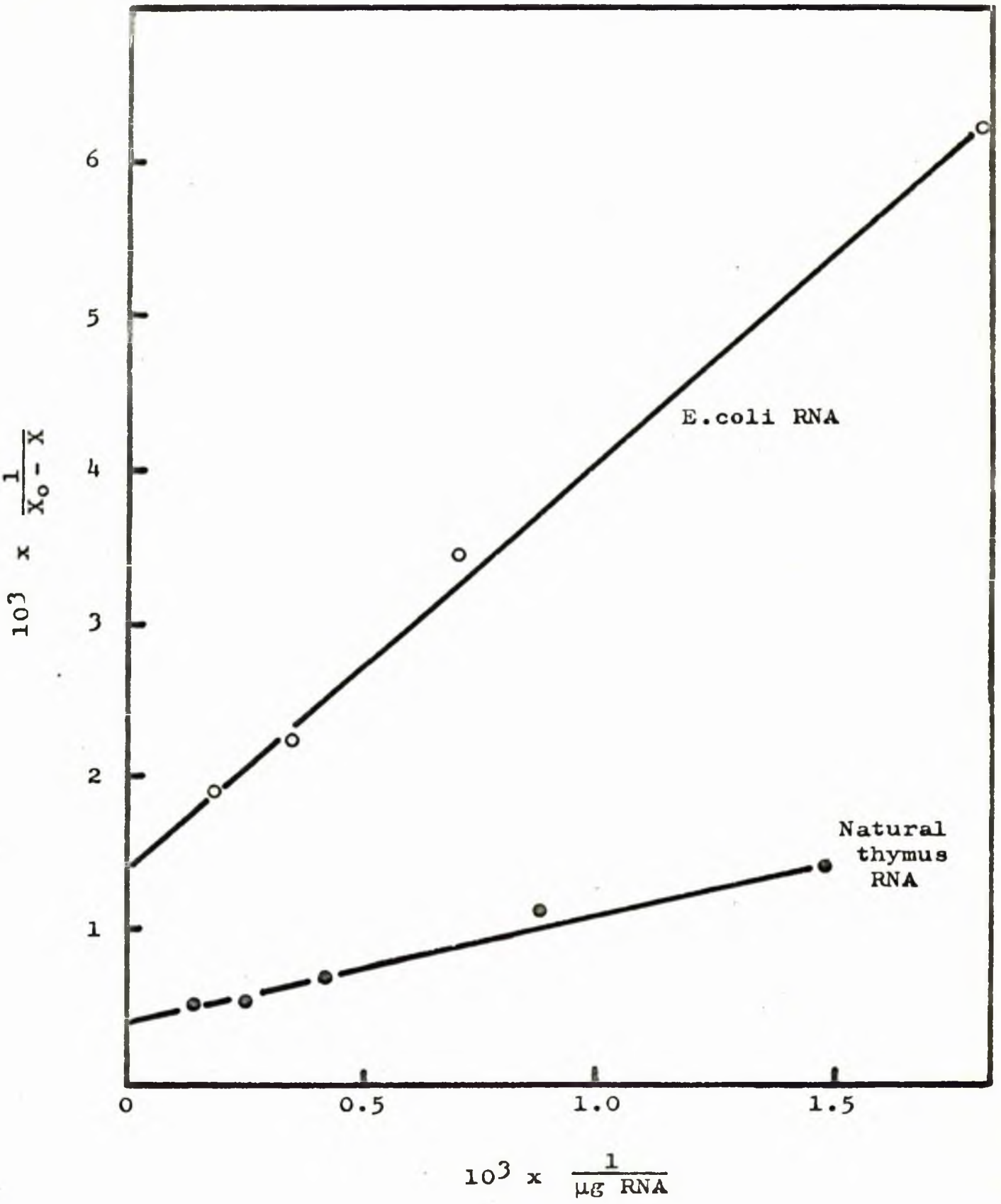

6
5
4
3
2
1
0
0.5
1.0
1.5
E.coli RNA
Natural thymus RNA
$10^3 \times \frac{1}{X_o - X}$
$10^3 \times \frac{1}{\mu g \text{ RNA}}$

Figure 33.

Competition experiment between natural rabbit bone marrow RNA and a synthetic ^{3}H-RNA made *in vitro* with rabbit bone marrow chromatin as primer. Filters containing 1 μg denatured rabbit embryo DNA were incubated with 25 μg synthetic ^{3}H-RNA in the presence of unlabelled natural RNA at the concentrations shown.

Figure 33.

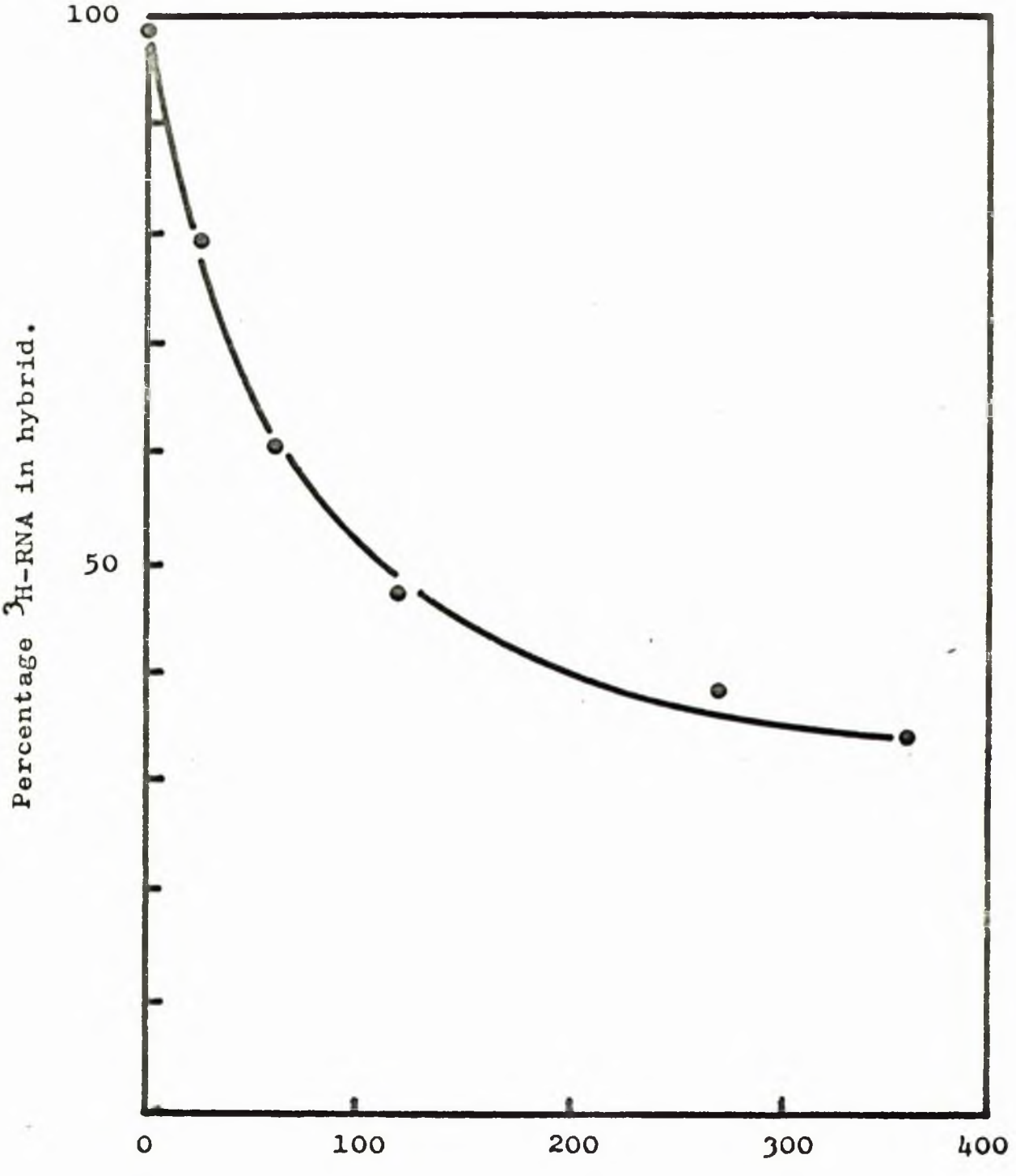
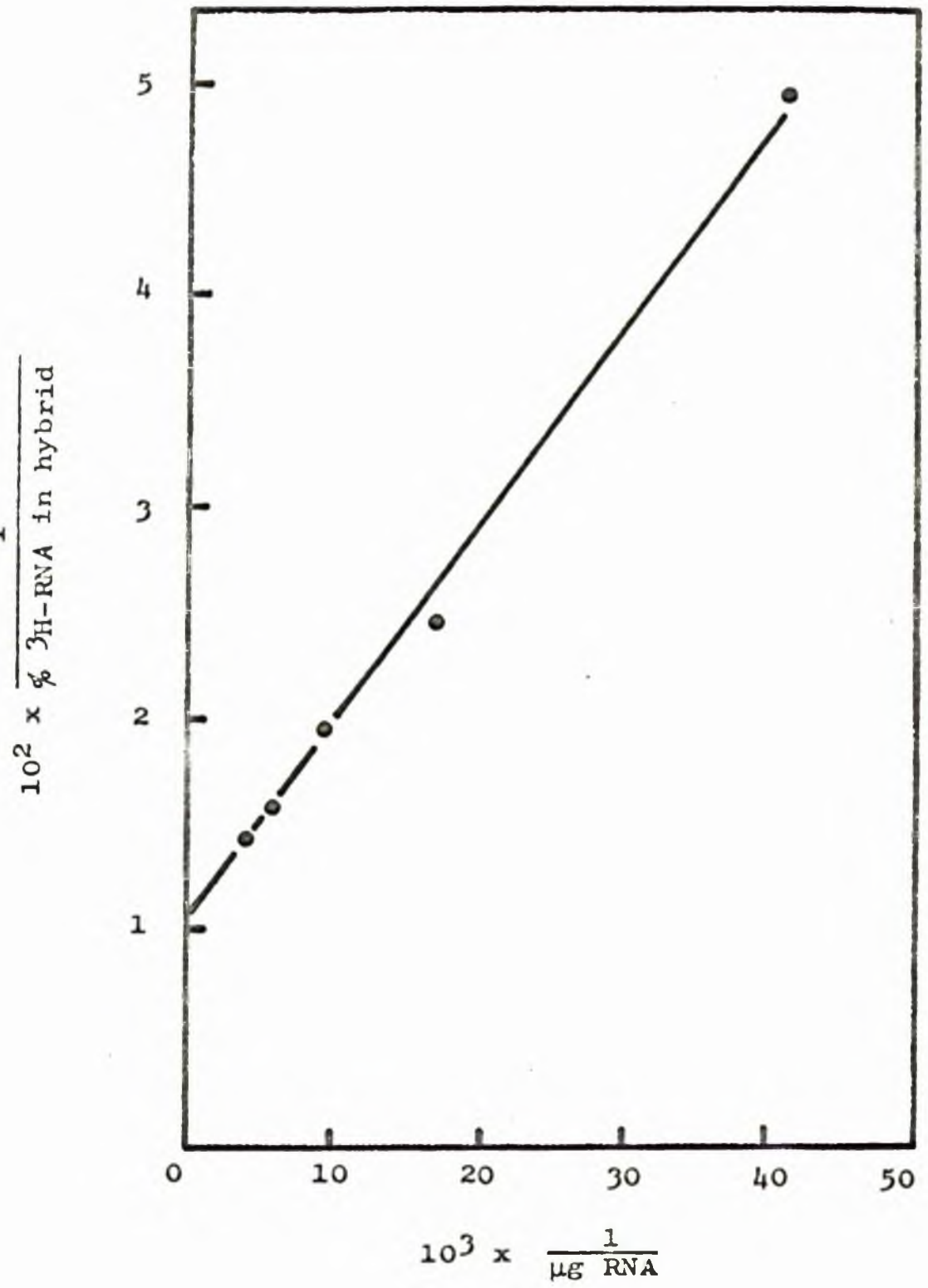

100
50
Percentage ^{3}H-RNA in hybrid.
0
100
200
300
400
µg Normal bone marrow RNA/ml.

Figure 34.

Double reciprocal plot of the data shown in Figure 33. At infinite concentration of unlabelled RNA it is predicted that not more than 5% of the hybridised RNA would be labelled.

Figure 34.


5
4
3
2
1
0
10
20
30
40
50
$\frac{1}{10^2 \times \%\ ^3H\text{-RNA in hybrid}}$
$10^3 \times \frac{1}{\mu g\ RNA}$

Other control experiments.

To exclude the possibility that the behaviour of chromatin *in vitro* is an artefact of the experimental procedure, the following control experiments were carried out.

The effect of the isolation procedure of chromatin on its template activity is shown in Figures 35 and 36. Thymus chromatin was prepared by Method B and also by Method C, which is more physiological in nature. Identical *in vitro* incubations were set up using these preparations and the saturation kinetics of the products determined. Although Method C gives a product which saturates the DNA more rapidly, it is clear from the double reciprocal plot (Figure 36) that at saturation both preparations give identical values. Method B. was used for all subsequent preparations.

It was found that chromatin could be readily dispersed in the incubation medium by very light homogenisation. It was of interest to determine whether this treatment altered the behaviour of the chromatin. Two incubations were set up; in one case the chromatin was dispersed by homogenisation, while in the other case partial dispersal was achieved by gentle swirling. Figures 37 and 38 show the saturation kinetics for the RNA products. Both preparations were found to give identical saturation values.

The significance of differences in the initial rates

Figure 35.

Hybridization to calf thymus DNA of ^{3}H-RNA synthesized <u>in vitro</u> from calf thymus chromatin prepared by Methods B and C. The RNA preparations were incubated with 3 μg denatured calf thymus DNA/filter in 2 mls. at the concentrations shown.

Figure 36.

Double reciprocal plot of the data shown in Figure 35. At saturation both RNA preparations hybridized with about 5% of the DNA.

Figure 35.

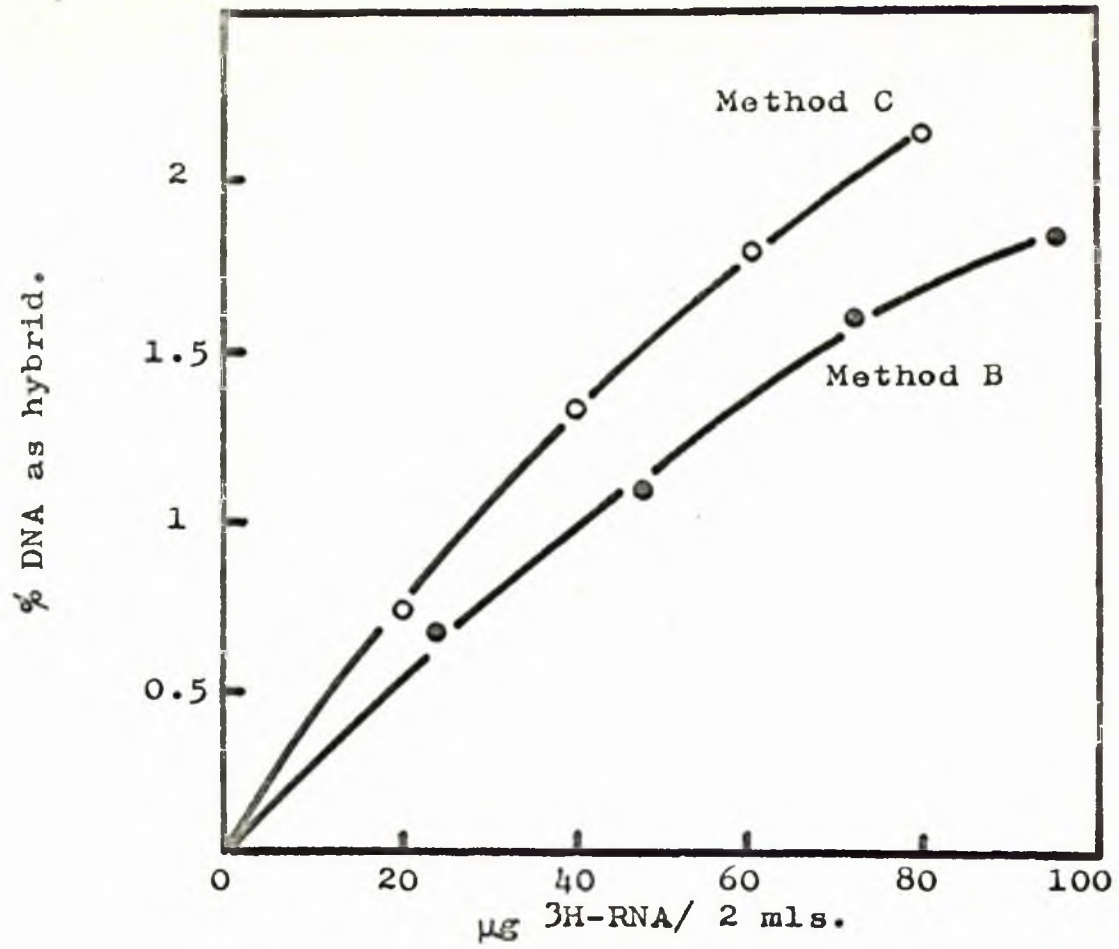


Figure 36.

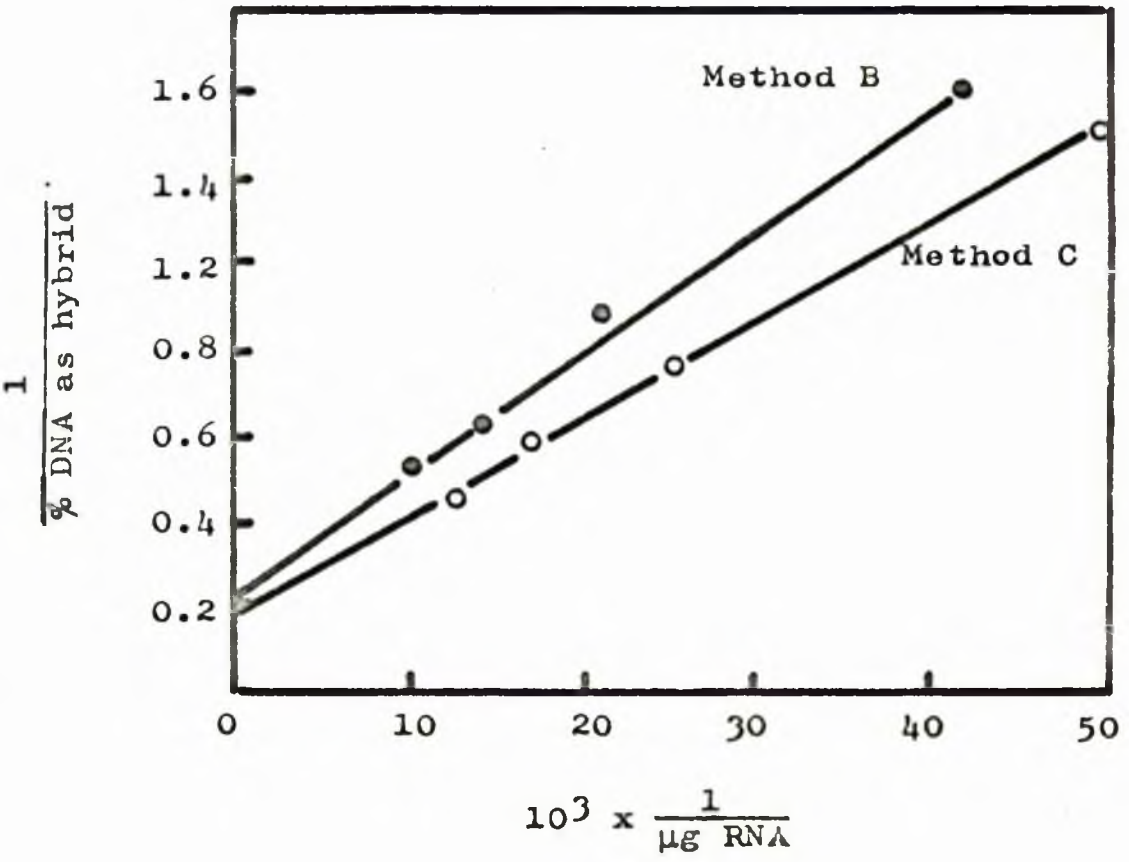


Figure 37.

Hybridization to calf thymus DNA of ^{3}H-RNA synthesized <u>in vitro</u> from calf thymus chromatin which had been dispersed in the incubation mix either by light homogenisation or by gentle swirling. The RNAs were hybridized to 3 µg denatured calf thymus DNA/filter in 0.2 ml. at the concentrations shown.

Figure 38.

Double reciprocal plot of the data shown in Figure 37. At saturation both RNAs hybridized with about 5% of the DNA.

Figure 37.

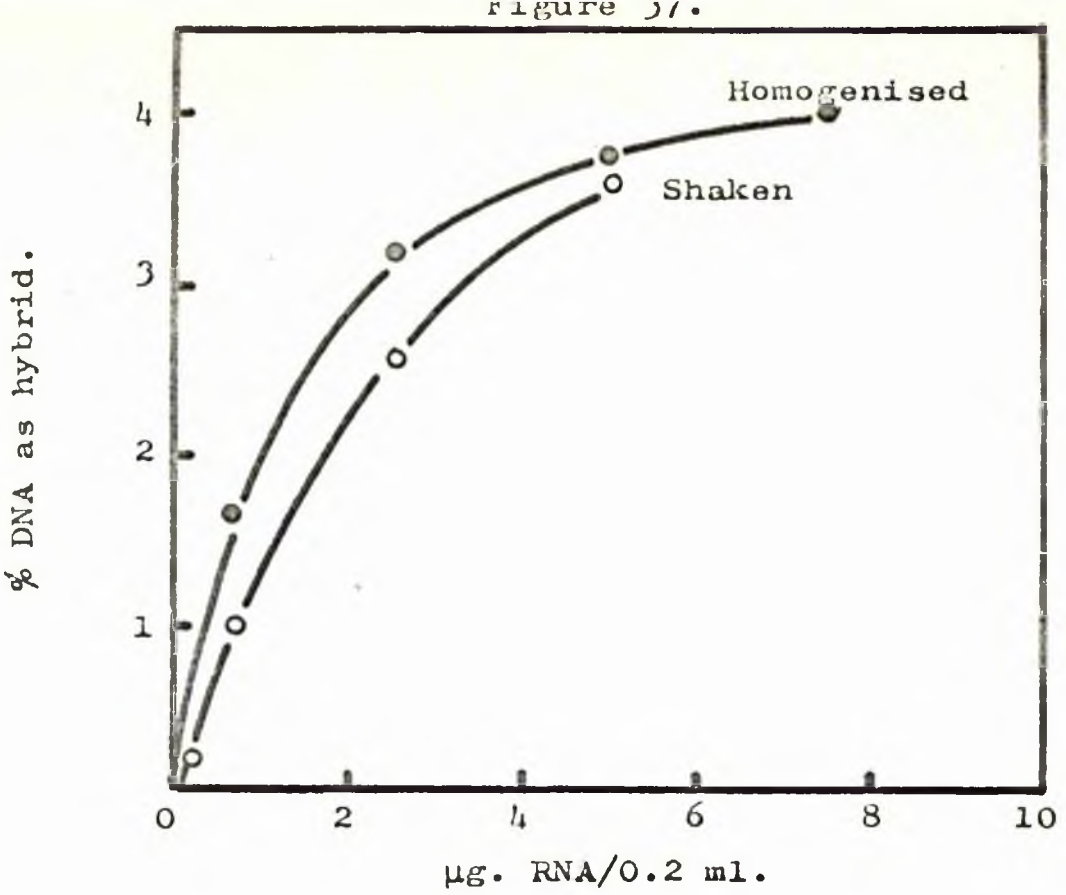
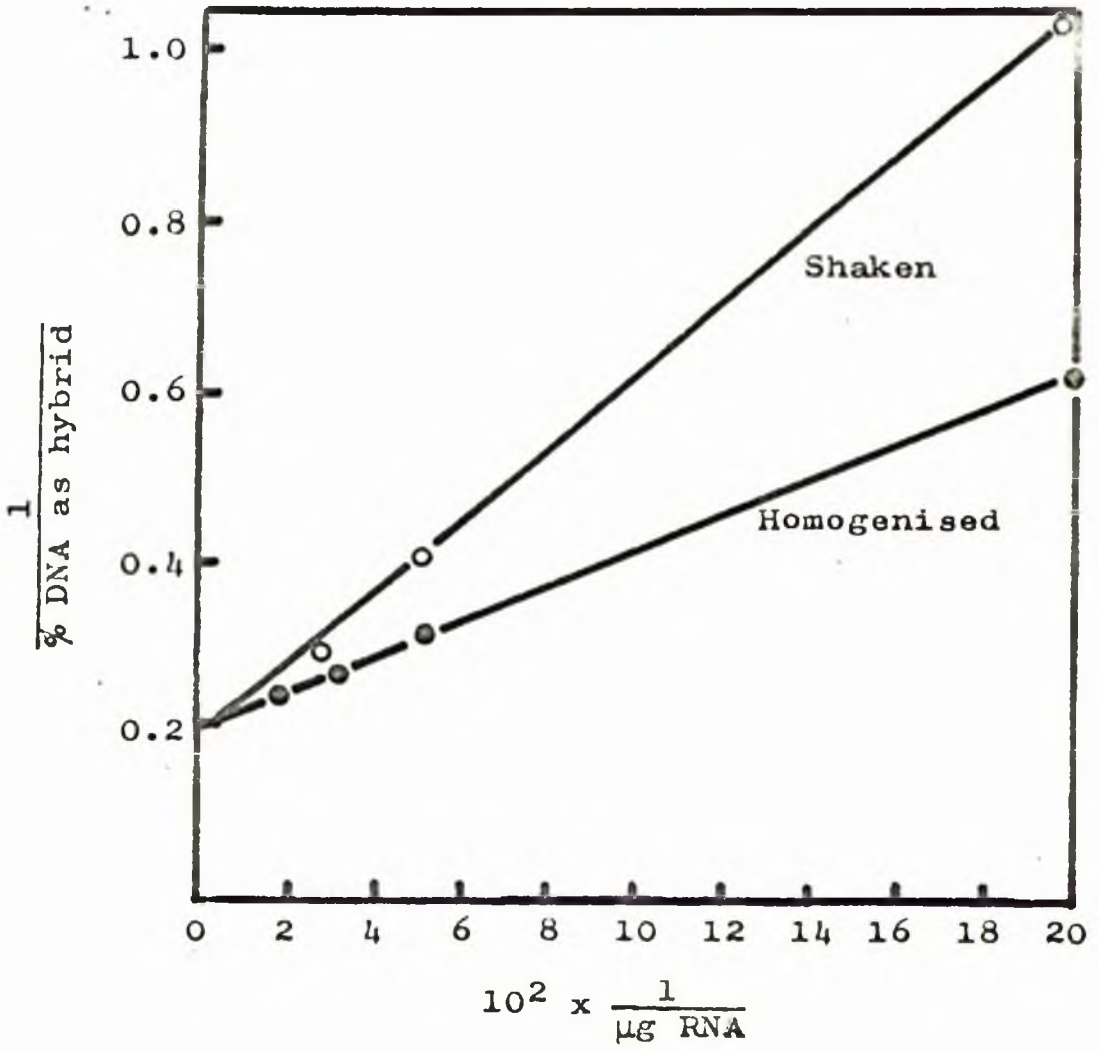


Figure 38.



of hybridization of identical RNA preparations will be discussed later.

The restriction of template activity in different organs.

Competitive hybridization was used to test whether RNAs prepared *in vitro* from rabbit thymus and bone marrow chromatins were homologous, first with natural RNAs from the homologous tissues and second with natural RNAs from heterologous tissues.

Nuclei were prepared from thymus glands and bone marrow of 4-6 month old rabbits. Chromatin and natural RNA was prepared from each, and RNA was synthesised *in vitro* from the chromatins. The saturation kinetics of these synthetic RNAs are shown in Figures 29 and 30.

Competitive hybridization experiments were set up in which saturating amounts of each chromatin primed RNA was hybridized with DNA in the presence of increasing amounts of homologous and heterologous natural RNAs. Figure 39 shows the competition effect when ^{3}H-RNA from marrow chromatin was challenged with natural RNA from marrow and thymus nuclei. Marked competition was obtained with the homologous RNA; the line extrapolated to zero indicating complete homology (Figure 41). On the other hand, when the same labelled marrow RNA was hybridized in the presence of natural thymus RNA the competition curve was less steep and did not extrapolate to zero. Figure 40 presents the results obtained when the

Figure 39.

Test for homology between natural rabbit bone-marrow or thymus RNA and ^{3}H-RNA made *in vitro* with a rabbit bone-marrow chromatin primer. Annealing mixtures contained 1.4 μg rabbit embryo DNA, 50 μg synthetic bone-marrow ^{3}H-RNA. To each annealing mixture was added varying amounts of competing RNA, either natural bone-marrow RNA or natural thymus RNA.

Figure 39.

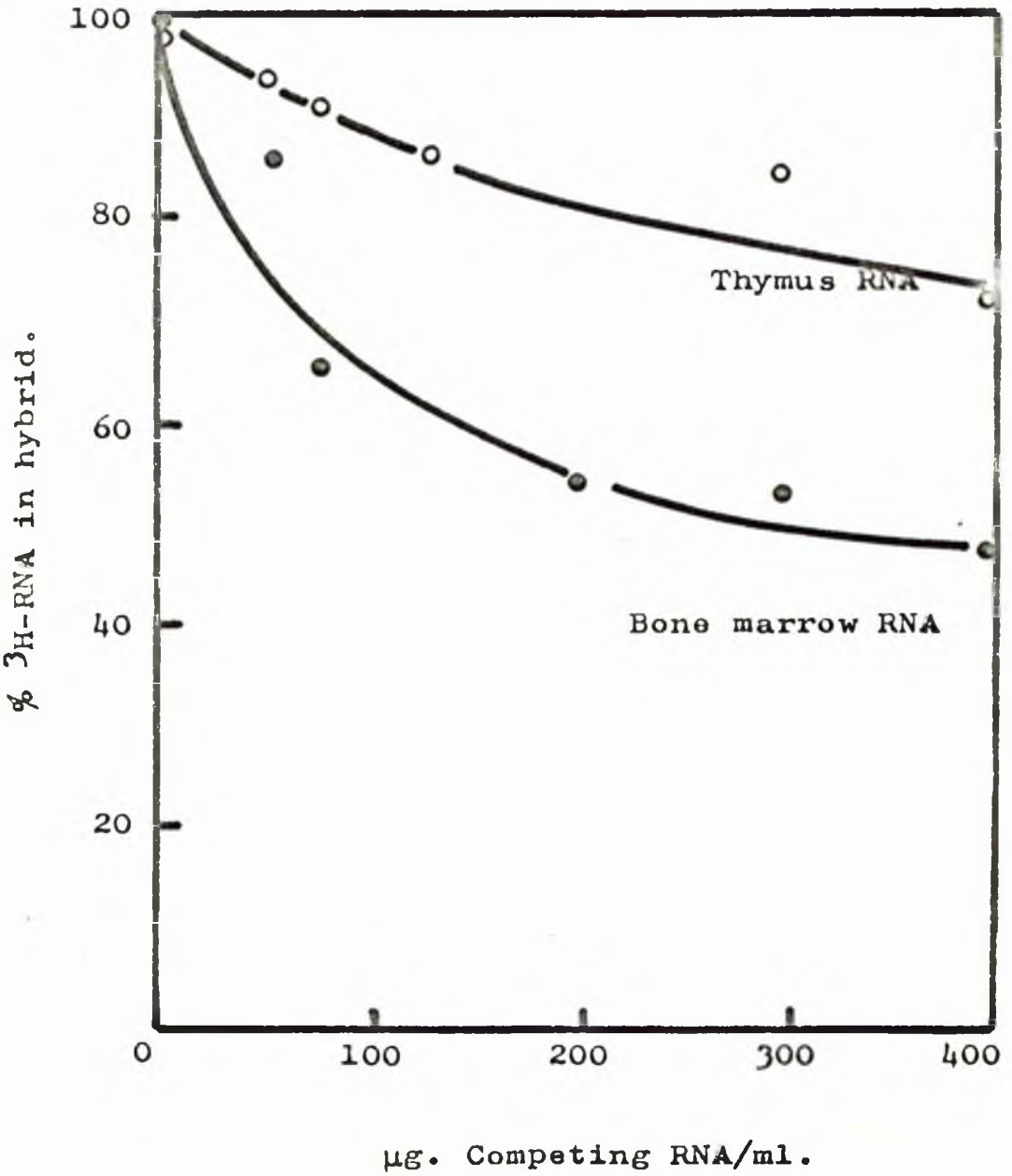

100
80
60
40
20
0
100
200
300
400
% 3H-RNA in hybrid.
Thymus RNA
Bone marrow RNA
µg. Competing RNA/ml.

Figure 40.

Test for homology between natural rabbit thymus or bone-marrow RNA and ^{3}H-RNA made *in vitro* with a rabbit thymus chromatin primer. This was a complementary experiment to that shown in Figure 39. The conditions were identical except that the synthetic ^{3}H-RNA was made with thymus chromatin instead of bone-marrow chromatin.

Figure 40.

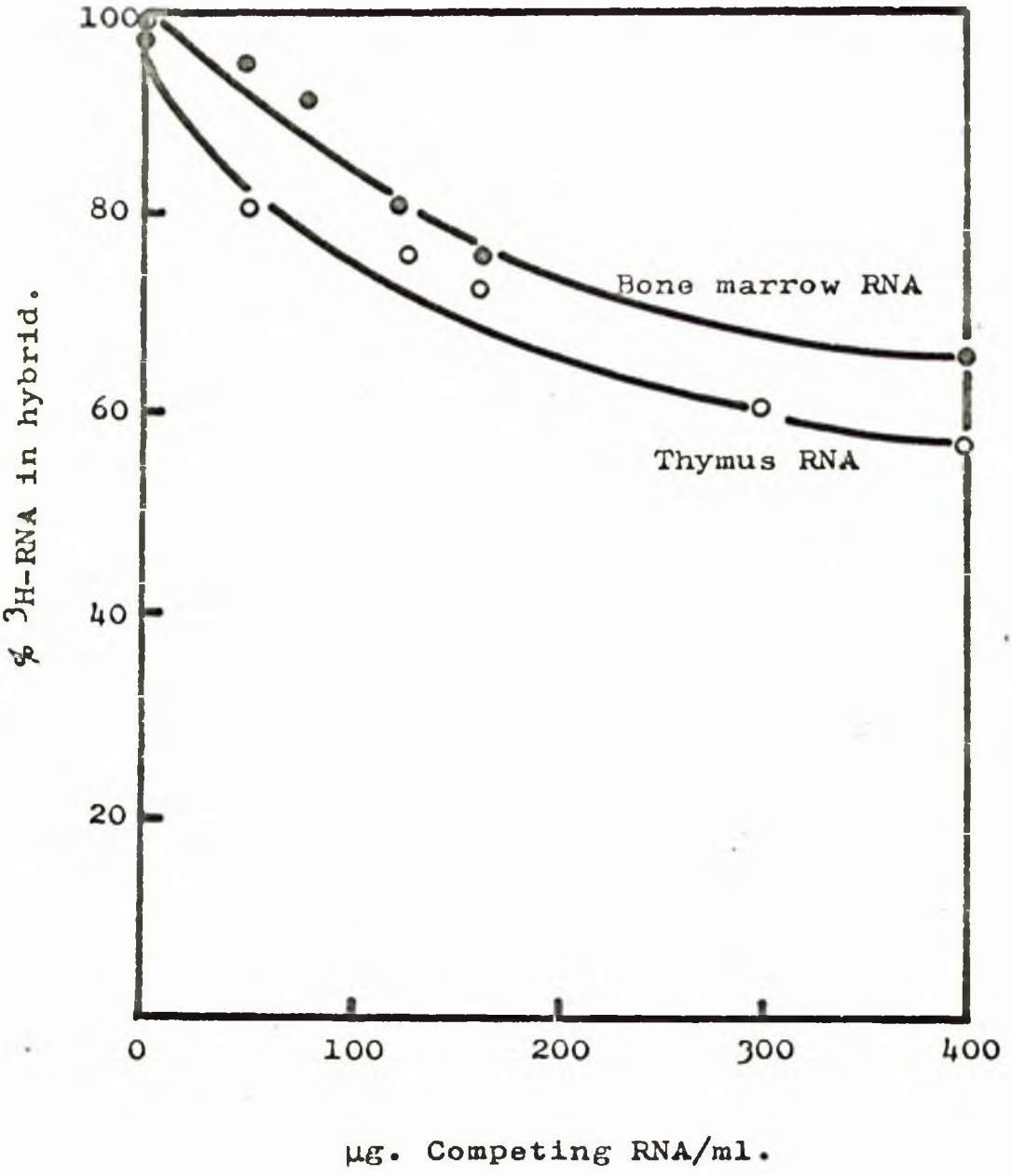

100
80
60
40
20
% 3H-RNA in hybrid.
Bone marrow RNA
Thymus RNA
0
100
200
300
400
µg. Competing RNA/ml.

Figure 41.

Double reciprocal plot for the competitive hybridization between ^{3}H-RNA from rabbit bone-marrow and natural RNAs from rabbit bone-marrow and thymus (data taken from Figure 39). By extrapolation to the ordinate it can be predicted that at infinite concentrations of bone-marrow and thymus RNA, 8% and 30% respectively of the original ^{3}H bone-marrow RNA remains as hybrid.

Figure 41.

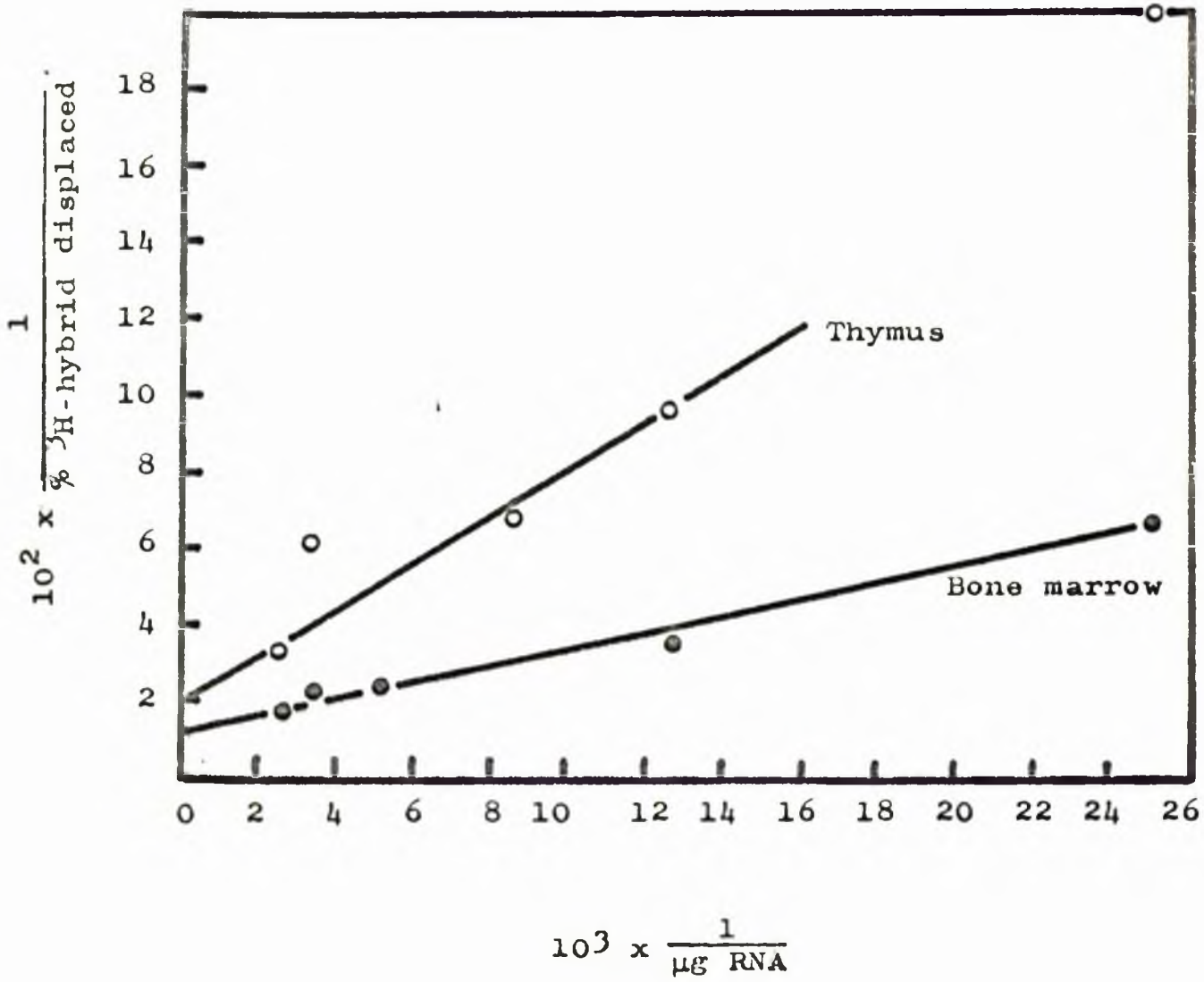
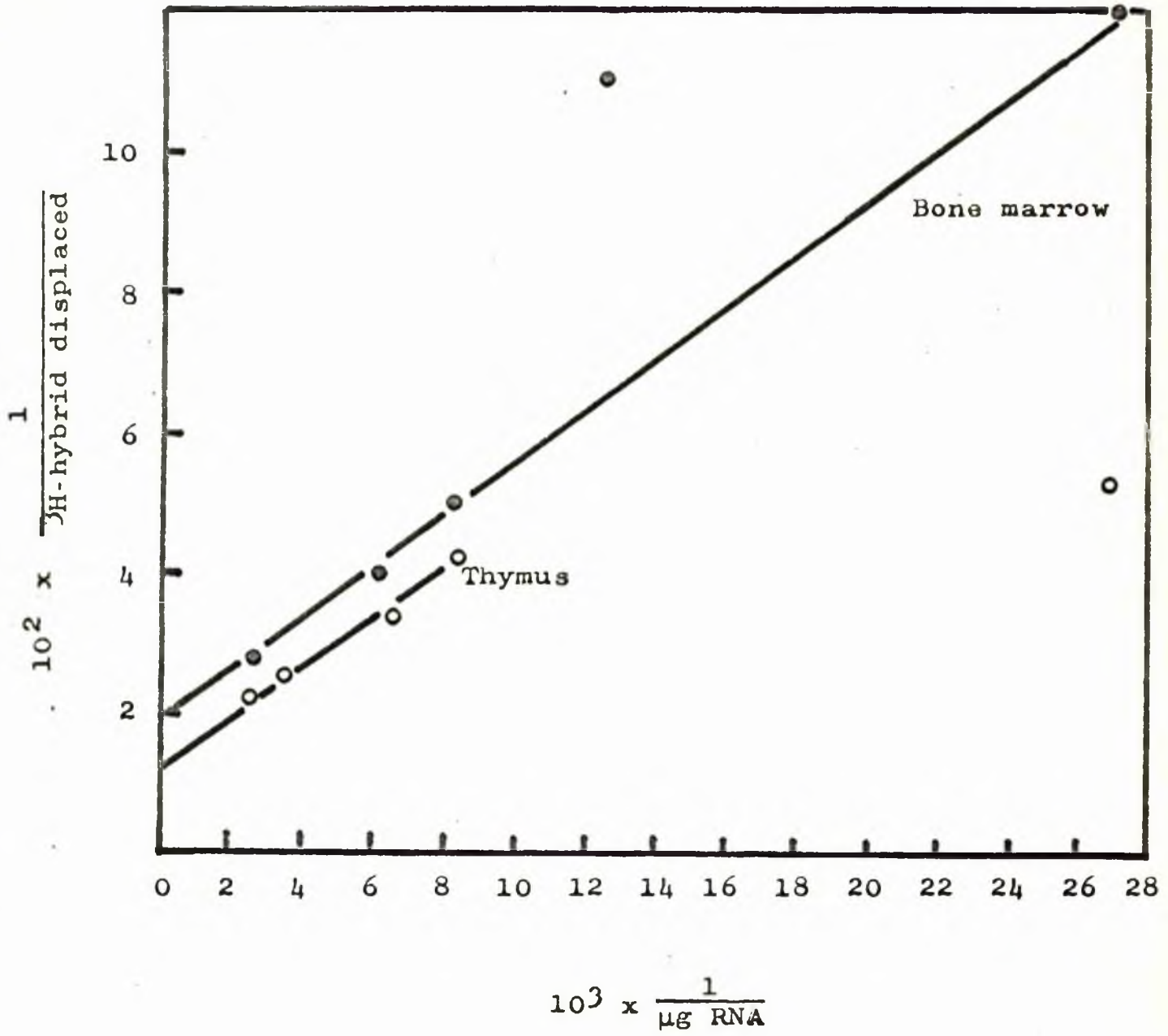

$10^2 \times \frac{1}{\%\ ^3H\text{-hybrid displaced}}$
18
16
14
12
10
8
6
4
2
Thymus
Bone marrow
0 2 4 6 8 10 12 14 16 18 20 22 24 26
$10^3 \times \frac{1}{\mu g\ RNA}$

Figure 42.

Double reciprocal plot of the competition data shown in Figure 40. At infinite concentrations of natural thymus and bone-marrow RNA, 10% and 38% respectively of the original ^{3}H-thymus RNA remains as hybrid.

Figure 42.


Bone marrow
Thymus
10
8
6
4
2
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
$10^2 \times \frac{1}{\%H\text{-hybrid displaced}}$
$10^3 \times \frac{1}{\mu g\ RNA}$

experimental system was reversed. In this case it could be demonstrated that natural thymus RNA and labelled RNA made in the presence of thymus chromatin were completely homologous. Natural marrow RNA on the other hand, competed less effectively (Figure 42).

It was concluded from those experiments that in rabbit thymus and bone marrow chromatin some common sequences are readily available for transcription by the Micrococcal polymerase; in addition in each type of chromatin unique sequences are available for transcription which are not available in the other.

3.5 The nature of the restriction mechanism in chromatin.

The results obtained with chromatin in the *in vitro* system suggest that the restriction of template activity is an inherent property of the isolated chromatin. The following sections describe experiments in which the nature of this restriction was investigated. Initially the effect of removing proteins from chromatin was investigated and the conditions required for reconstitution of chromatin were sought. In later experiments attempts were made to reconstitute chromatin from DNA, histone and residual fraction using this information. In all cases thymus chromatin was used. The degree to which modified or reconstituted chromatin corresponded to natural chromatin was determined from the hybridization kinetics of the labelled RNA synthe-

sised *in vitro* using these materials as primers.

The effect of degradation of chromatin on its template activity.

Calf thymus chromatin was subjected to a number of degradative procedures as follows:

(a) Chromatin (500 µg/ml.) was dissolved in 4 M caesium chloride, final concentration, and centrifuged in a MSE 50 SW 30 rotor at 170,000 g. for 4 hours. The protein component of chromatin dissociates from the DNA under these conditions and floats to the meniscus to form a pellicle. This was discarded and the DNA isolated from the caesium chloride solution by the addition of 1 volume water and 4 volumes absolute alcohol. This material constituted deproteinised chromatin.

(b) Chromatin (500 µg/ml.) was treated with 0.25 N HCl, final concentration, at 0°C for 1 hour. The suspension was centrifuged at 2,000 g. for 10 minutes. The supernatant which contained the histone component was dialysed against distilled water until free of acid and stored frozen for later use. The precipitate, which consisted of DNA combined with the residual or non-histone component, was washed until neutral in 0.15 M NaCl : 0.1 M Tris/HCl pH 7.5, centrifuged at 2,000 g. for 10 minutes and resuspended in distilled water. The resulting gel constituted dehistoned chromatin.

(c) A sample of chromatin was dialysed against 2 M NaCl for 16 hours, then against 0.6 M NaCl for 24 hours, and finally against water for 24 hours. The reconstituted gel, salt dissociated chromatin was pelleted by centrifugation at 2,000 g. for 10 minutes.

Labelled RNA was synthesised *in vitro* using 500 µg. of deproteinised chromatin, dehistoned chromatin, salt dissociated chromatin and natural chromatin as primers. The RNA products were hybridized with 5 µg. calf thymus DNA and saturation levels determined (Figures 43 and 44).

Removal of protein from chromatin results in a 4- to 5-fold increase in template activity; however removal of histones alone only produced a 2- to 3-fold increase over that of normal chromatin. The restriction of template activity in chromatin appears to be due to the presence of protein. Neither the histone nor the residual protein is wholly responsible. Dissociation of these proteins by 2 M NaCl and subsequent re-association by dialysis, gave a product which did not exhibit the same properties as the natural chromatin. This primer possessed about 50 per cent more template activity. It was concluded that, either the conditions for re-association were not correct or that the dissociated proteins did not possess the specificity necessary for faithful re-association. The first of these possibilities was investigated further by studying the

Figure 43.

Kinetics of hybridization to calf thymus DNA of ^{3}H-RNAs made *in vitro* with the following primers: whole calf thymus chromatin, X —— X; deproteinised chromatin, o —— o; dehistoned chromatin, Δ ——Δ ; salt dissociated chromatin, • —— •. Filters were loaded with 5 μg denatured calf thymus DNA and the ^{3}H-RNAs were incubated with individual filters at the concentrations shown.

Figure 43.

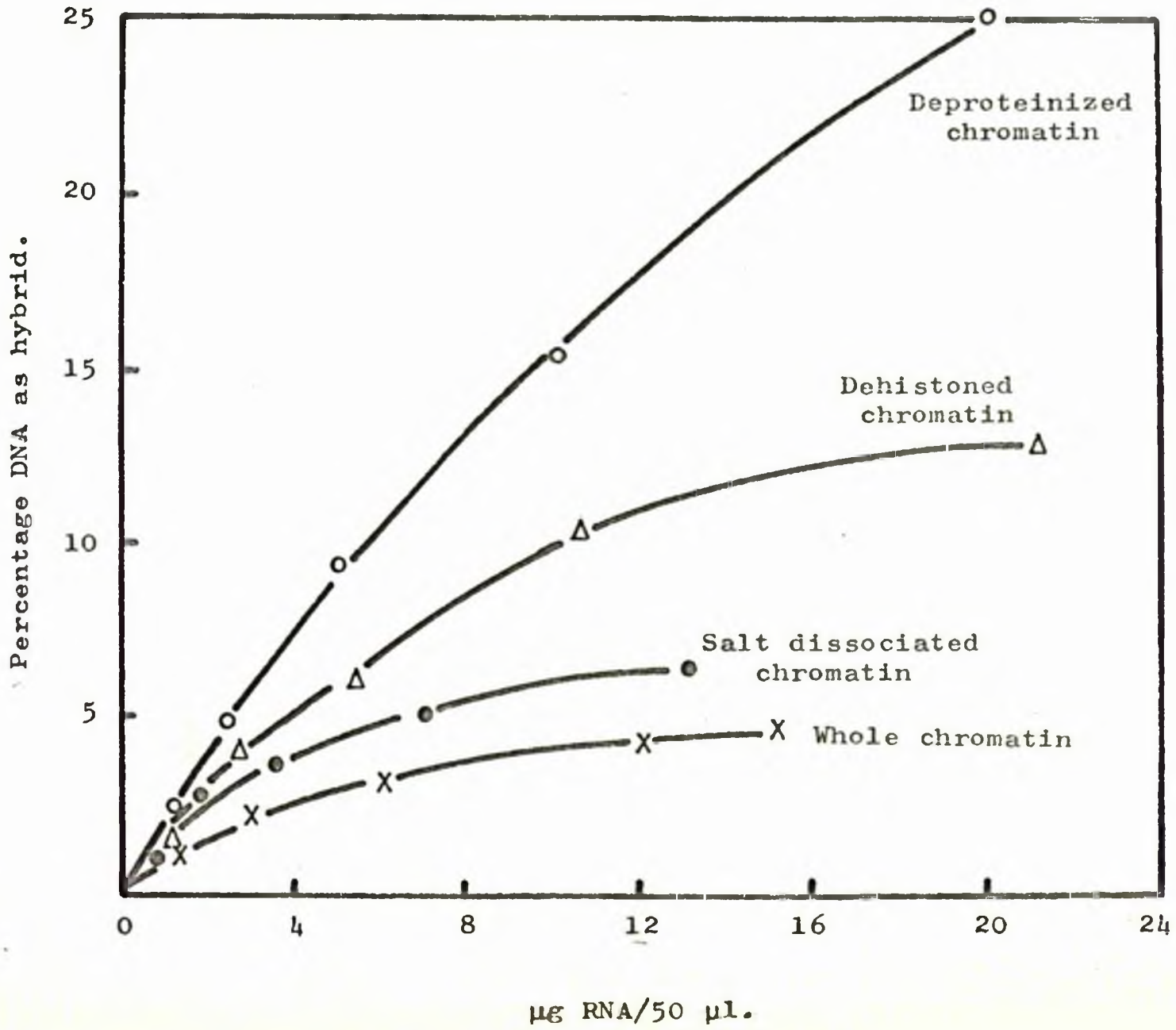

25
20
15
10
5
0
4
8
12
16
20
24
Percentage DNA as hybrid.
µg RNA/50 µl.
Deproteinized chromatin
Dehistoned chromatin
Salt dissociated chromatin
Whole chromatin

Figure 44.

Double reciprocal plots of the data shown in Figure 43. At infinite concentrations of the ^{3}H-RNAs the following percentages of the DNA existing as hybrid were predicted: whole chromatin, 5.8%; salt dissociated chromatin, 11%; dehistoned chromatin, 20%; deproteinised chromatin, 50%.

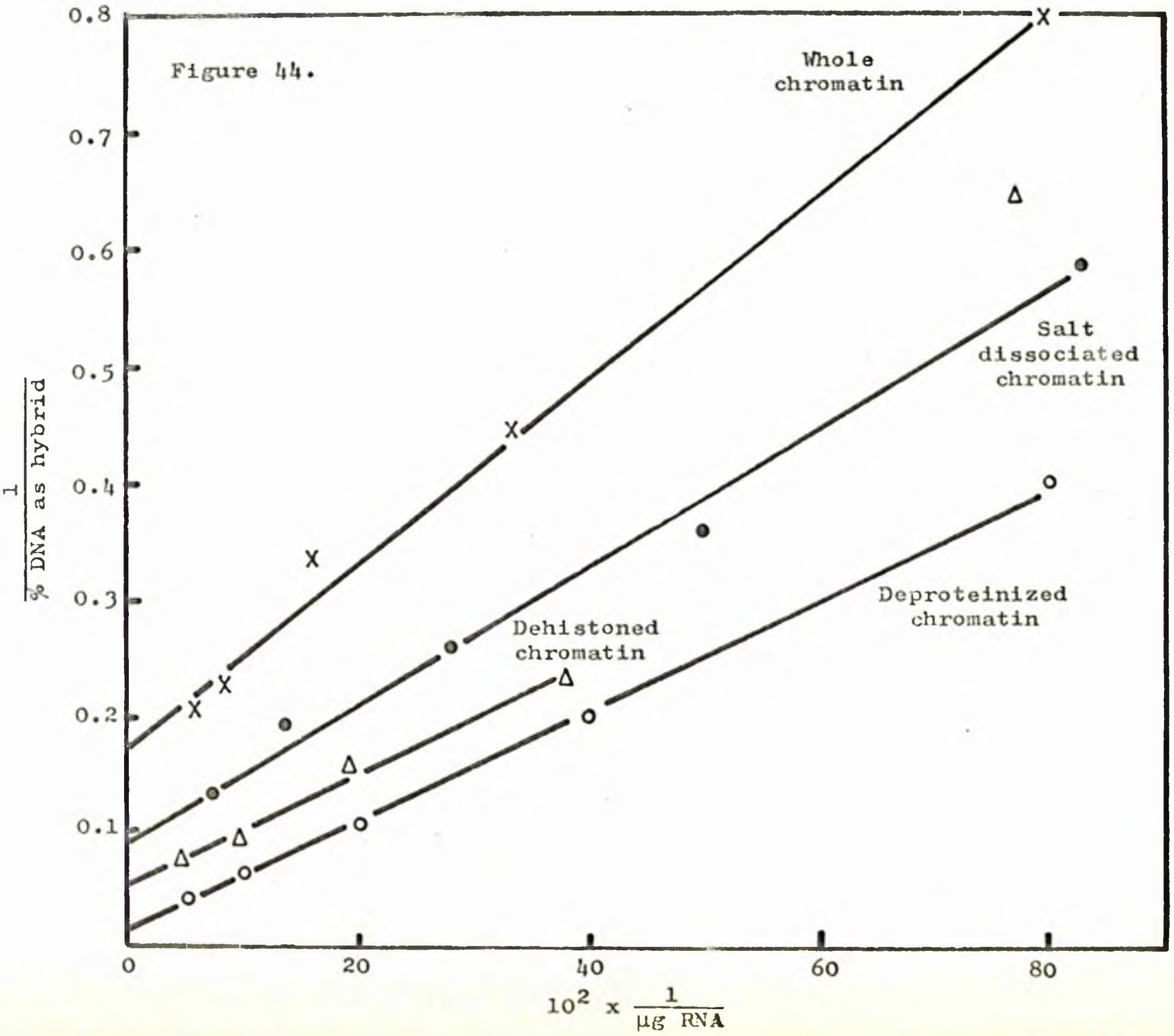


Figure 44.
Whole chromatin
Salt dissociated chromatin
Dehistoned chromatin
Deproteinized chromatin
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
20
40
60
80
1 / % DNA as hybrid
10² x 1 / µg RNA

reconstitution of chromatin with dehistoned chromatin and histone.

Conditions required for the reconstitution of chromatin.

Histone was reconstituted with dehistoned chromatin in two ways. In both cases a two-fold excess of histone over DNA was present. Firstly, an excess of histone was reconstituted with dehistoned chromatin in water. The resulting gel was collected by centrifugation and suspended in distilled water. Secondly, the two components were mixed in 2 M NaCl, dialysed against 0.6 M NaCl overnight at 0°C. and then against 0.4 M and 0.2 M NaCl for 3 hours each. The precipitate was collected by centrifugation at 2,000 g. for 10 minutes and washed with distilled water until gelation occurred.

Labelled RNA was synthesised with each chromatin as primer and the saturation kinetics of the products determined by hybridization with 5 µg. thymus DNA (Figure 45). The saturation value obtained with the RNA from the gradient dialysis method was identical to that of natural chromatin. Reconstitution in water however gave a product with a considerably higher template activity. When a sample of this chromatin was re-equilibriated overnight against 0.6 M NaCl and then through 0.4 and 0.2 M NaCl as before, the chromatin then behaved in the same fashion as natural chromatin (Figure 46).

Figure 45.

Kinetics of hybridization to calf thymus DNA of ^{3}H-RNAs made *in vitro* with the following primers: whole calf thymus chromatin, ● —— ●; salt reconstituted chromatin, □ —— □; water reconstituted chromatin, ○ —— ○; water reconstituted chromatin re-equilibrated in salt, Δ —— Δ; dehistoned chromatin + —— +. Conditions are the same as those described in Figure 43.

Figure 45.

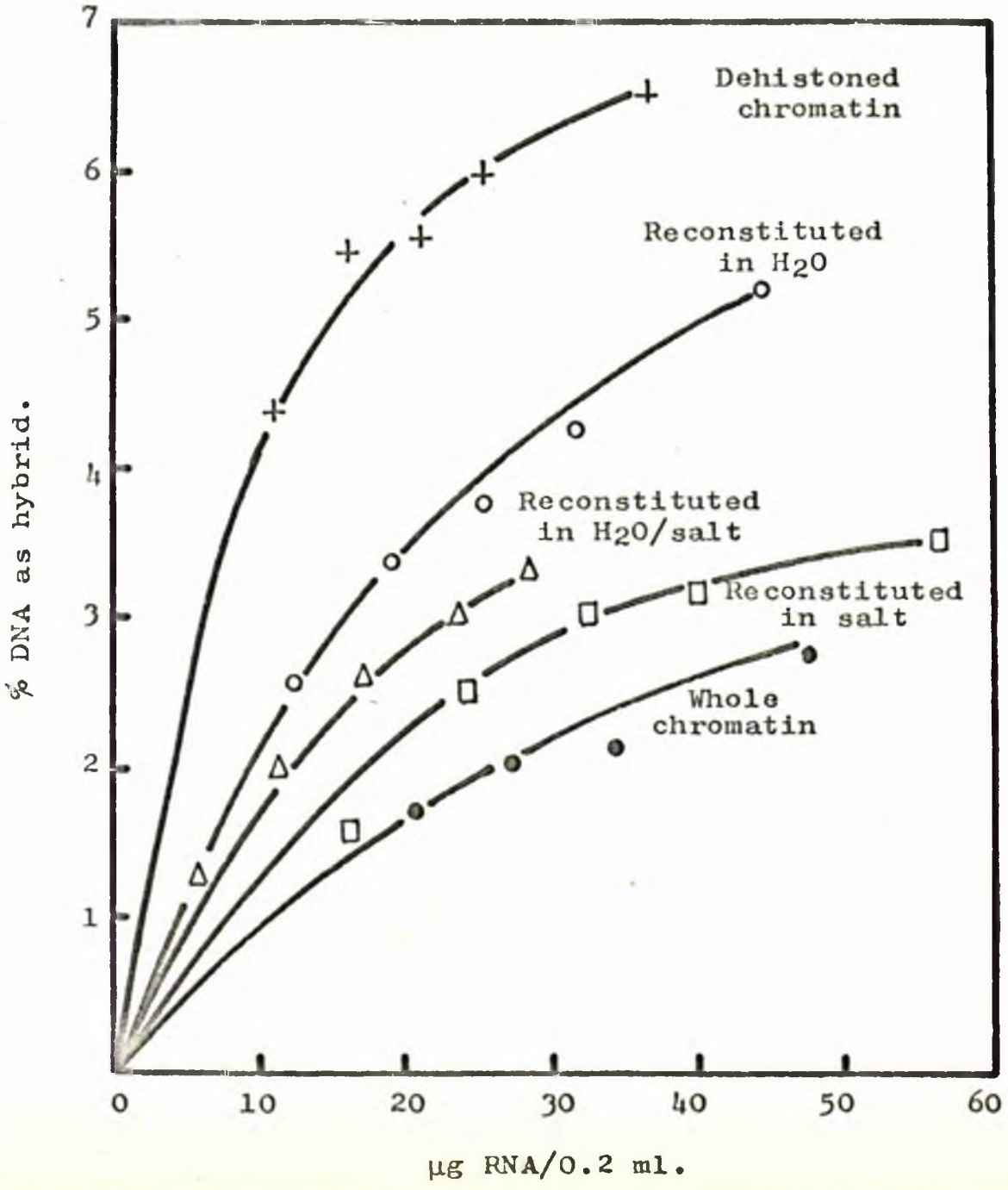

Dehistoned chromatin
Reconstituted in H_2O
Reconstituted in H_2O/salt
Reconstituted in salt
Whole chromatin
% DNA as hybrid.
µg RNA/0.2 ml.
0
10
20
30
40
50
60
1
2
3
4
5
6
7

Figure 46.

Double reciprocal plots of the data shown in Figure 45. Estimates of the percentage DNA as hybrid at saturation with each RNA are as follows: whole chromatin, salt reconstituted and salt re-equilibriated chromatins are all around 5%; water reconstituted chromatin, 8%; dehistoned chromatin, 10%.

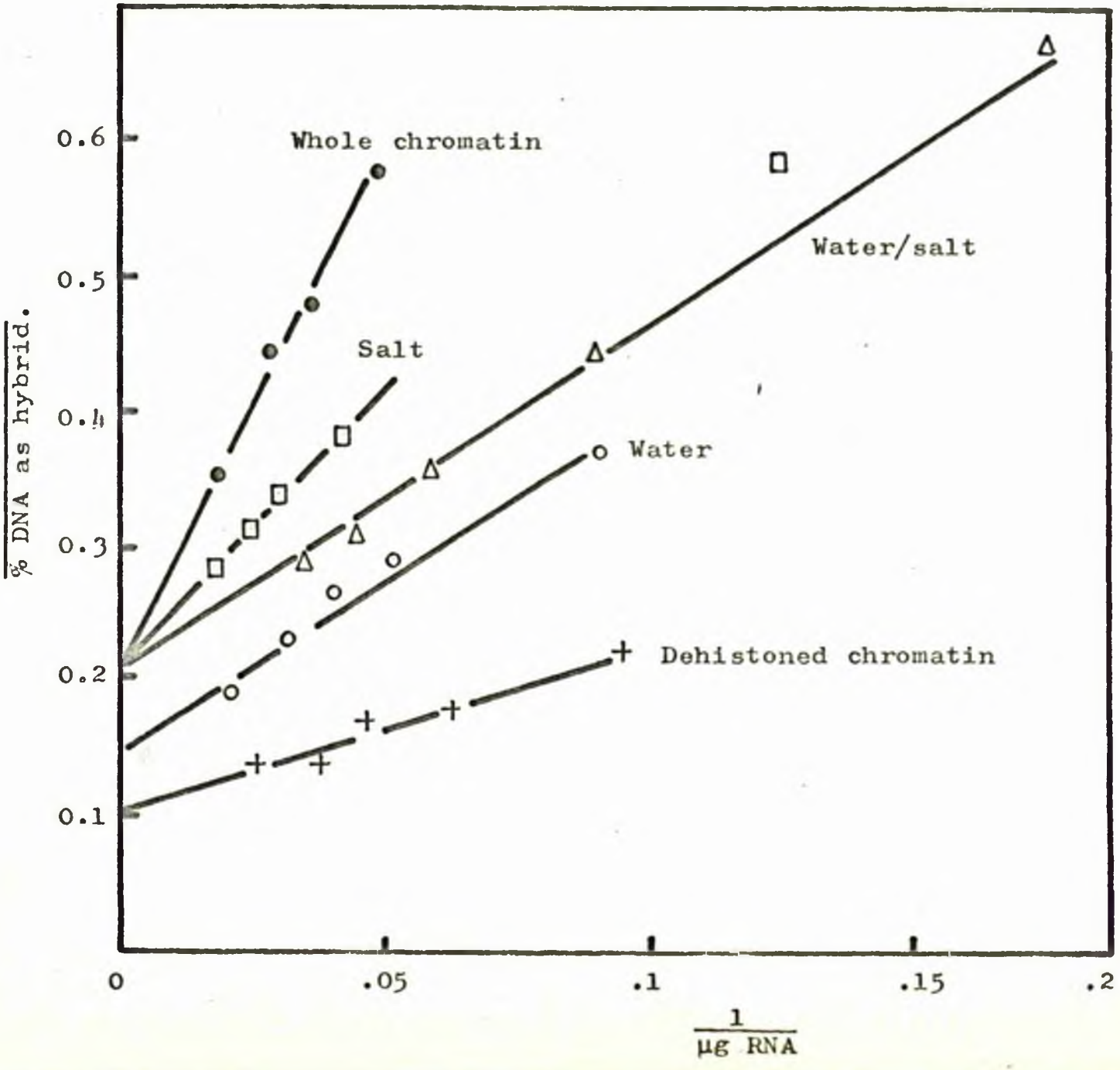

Whole chromatin
Water/salt
Salt
Water
Dehistoned chromatin
0.6
0.5
0.4
0.3
0.2
0.1
0
.05
.1
.15
.2
1 / % DNA as hybrid.
1 / µg RNA

Figure 46.

From Figure 45 it is seen that the initial rates of hybridization of the synthetic chromatins is higher than that of the natural chromatin. However a double reciprocal plot of these data (Figure 46) shows that the saturation levels are identical. Possible reasons for the initial difference in rates are discussed later.

From these experiments it was concluded that chromatin can be reconstituted from histone and dehistoned material if a suitable excess of histone is present and if the reconstitution takes place in a slowly decreasing salt gradient.

The fractionation and reconstitution of chromatin.

In further experiments, attempts were made to reconstitute chromatin from DNA and proteins from a crude fractionation of the caesium chloride pellicle. Chromatin was dissociated in caesium chloride as before and DNA recovered from the solution by alcohol precipitation. This time the pellicle was retained and dissolved by homogenisation in 1 M NaCl. About 70-80 per cent of the material remained in solution after centrifugation at 2,000 g. for 10 minutes. This supernatant (1 mg. protein/ml.) was then acidified with concentrated acetic acid to a final concentration of 0.25 M, left in ice for 10 minutes, and then centrifuged at 2,000 g. for 10 minutes. The pelleted material was dissolved in 1 M NaCl : 0.1 M tris-HCl pH 8 and constituted the "acidic"

fraction. The supernatant or "histone" fraction was adjusted to neutrality with 1 N NaOH and both fractions stored at 4°C.

Reconstituted chromatins were prepared by gradient dialysis of DNA with a two-fold excess of acidic and histone fractions either separately or together. A similar reconstitution was set up with DNA and the unfractionated pellicle. Synthetic RNAs were made *in vitro* using these primers and also natural chromatin, DNA and sonicated dehistoned chromatin. Hybridization of the RNA products to 5 µg. thymus DNA is shown in Figures 47 and 48. When the unfractionated pellicle is reconstituted with DNA, the chromatin obtained behaves in the same way as natural chromatin. When an excess of histone is reconstituted with DNA a complete restriction of template activity occurs. Even though polyribonucleotide was formed from this primer *in vitro*, none of the material hybridizes with calf thymus DNA. It is thought that this material RNA may represent homopolymer. On the other hand, when the acidic fraction is present with DNA and histone a specific part of the DNA remains available for transcription. The magnitude of this restricted region is comparable with that of natural chromatin. Although the initial rates of hybridization are slightly higher the saturation values for both RNAs are the same. Reconstitution with the acidic fraction alone pro-

Figure 47.

Kinetics of hybridization to calf thymus DNA of ^{3}H-RNA made <u>in vitro</u> with the following primers: whole calf thymus chromatin, + —— +; calf thymus DNA, □ —— □; nucleoprotein made by combining DNA with unfractionated pellicle proteins, • —— •; nucleoprotein made by combining DNA with the acidic fraction of the pellicle, o —— o; nucleoprotein made by combining DNA with the histone and acidic fractions of the pellicle, Δ —— Δ; nucleoprotein made by combining DNA with the histone fraction, X —— X; sonicated dehistoned chromatin, ■ —— ■. Conditions as in the legend of Figure 43.

Figure 47.

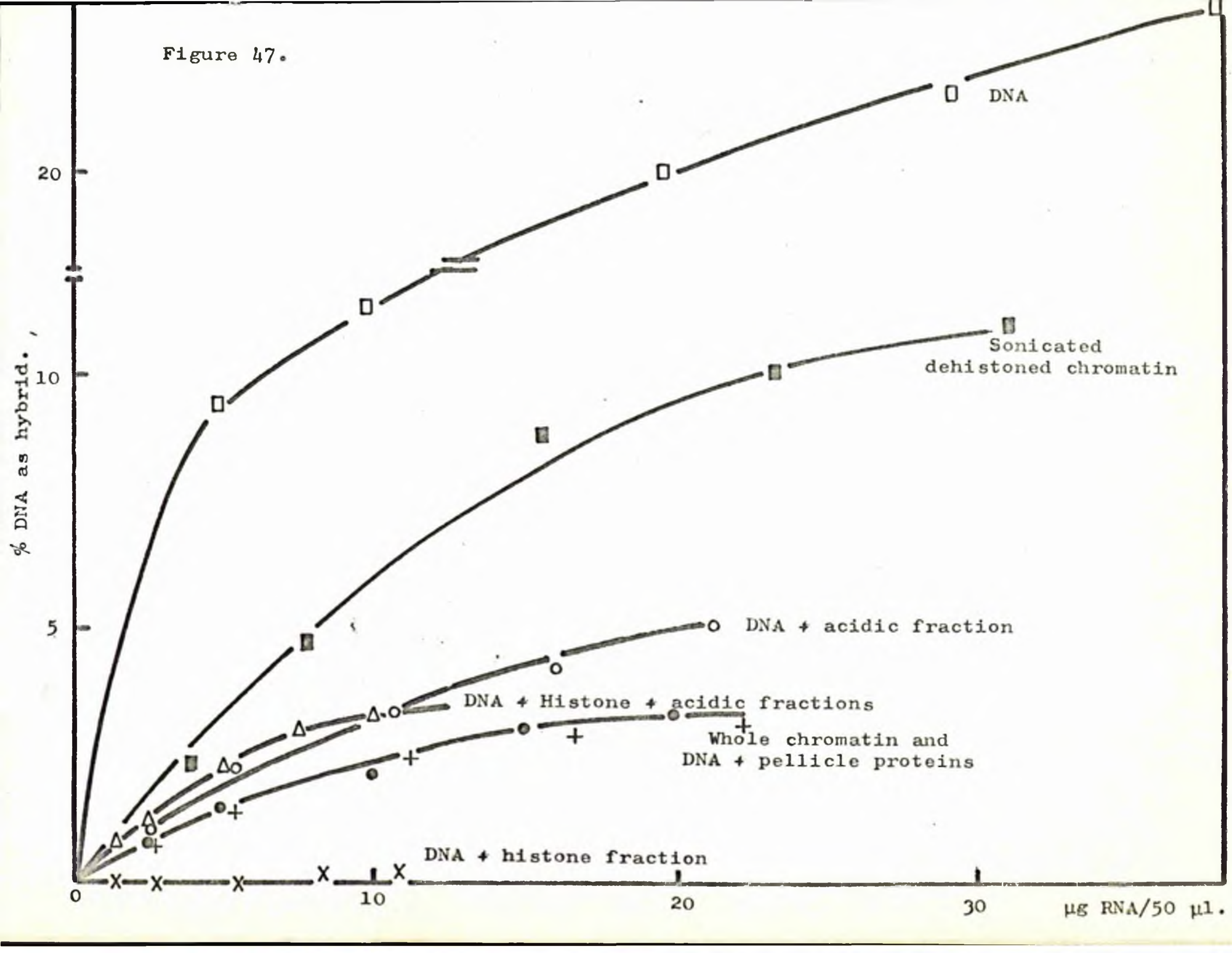
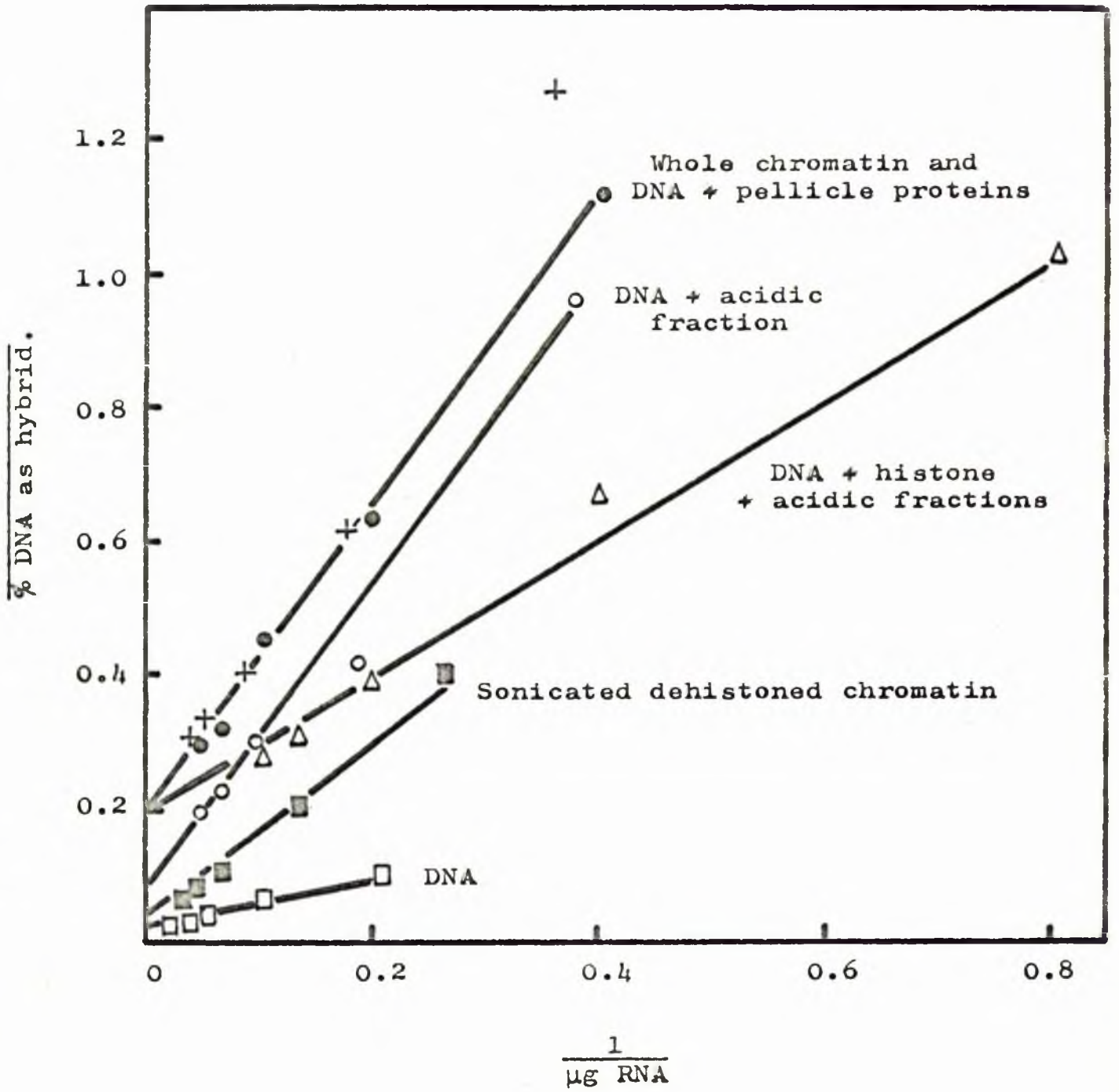

DNA
Sonicated dehistoned chromatin
DNA + acidic fraction
DNA + Histone + acidic fractions
Whole chromatin and DNA + pellicle proteins
DNA + histone fraction
% DNA as hybrid.
20
10
5
0
10
20
30
µg RNA/50 µl.

Figure 48.

Double reciprocal plots of the data shown in Figure 47. The following percentages of DNA as hybrid at saturation with each RNA were predicted: whole chromatin, DNA+ pellicle proteins and DNA + histone fraction + acidic fraction, 5%; DNA + acidic fraction, 12%; sonicated dehistoned chromatin, 25%; DNA, 40%.

Figure 48.


Whole chromatin and
DNA + pellicle proteins
DNA + acidic
fraction
DNA + histone
+ acidic fractions
Sonicated dehistoned chromatin
DNA
1.2
1.0
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1 / % DNA as hybrid.
1 / µg RNA

duces a chromatin with a substantially higher template activity. This result is similar to that obtained previously with dehistoned chromatin. It is interesting to note that if sonicated dehistoned chromatin is used as primer, the template activity is greater than that expected for the unsonicated material but still less than that of free DNA. This suggests that the restriction obtained with DNA in combination with the acidic fraction or with dehistoned chromatin may be real and not due to steric effects.

Finally, one important control experiment was carried out. The acidic fraction of the pellicle is nearly always contaminated with a small amount of DNA. The results might then be explained by inadequate fractionation of the DNA in such a way that the genes transcribed in the cell in question are concentrated in this fraction. To test this, DNA was isolated from the pellicle preparation and compared with whole cell DNA by annealing with labelled RNA synthesised from thymus chromatin. No difference between the two DNA preparations could be detected using this criterion, (Figures 49, 50).

Figure 49.

Kinetics of hybridization of ^{3}H-RNA synthesized *in vitro* from calf thymus chromatin, with DNA isolated from the pellicle proteins, ● --- ●; and from the caesium chloride solution, ○—○ , of a typical gradient run. Filters containing 5 μg of each DNA preparation were hybridized with varying concentrations of the ^{3}H-RNA preparation as shown.

Figure 50.

Double reciprocal plots of the data shown in Figure 49. The ^{3}H-RNA was found to hybridize with each DNA preparation to the same extent. At saturation with RNA it was predicted that 6% of both DNAs existed as hybrid.

Figure 49.

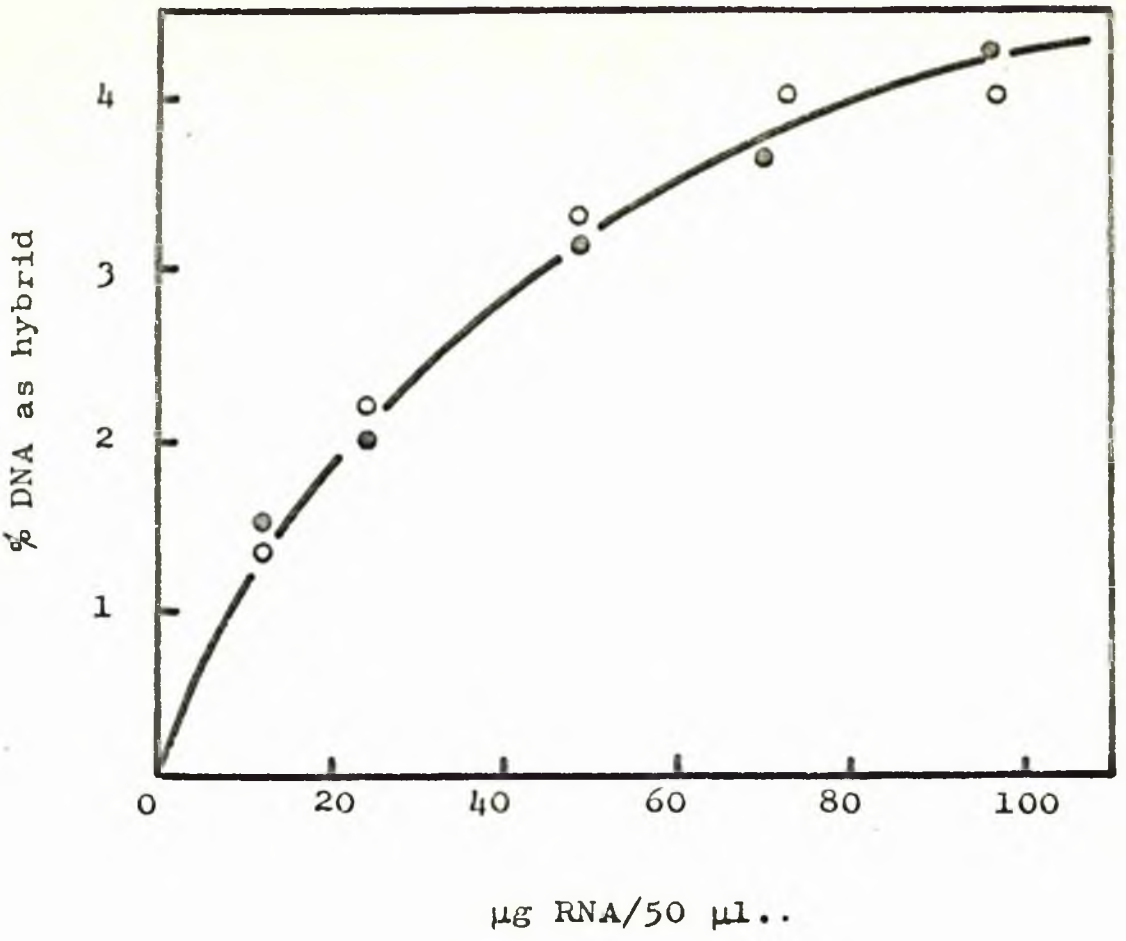
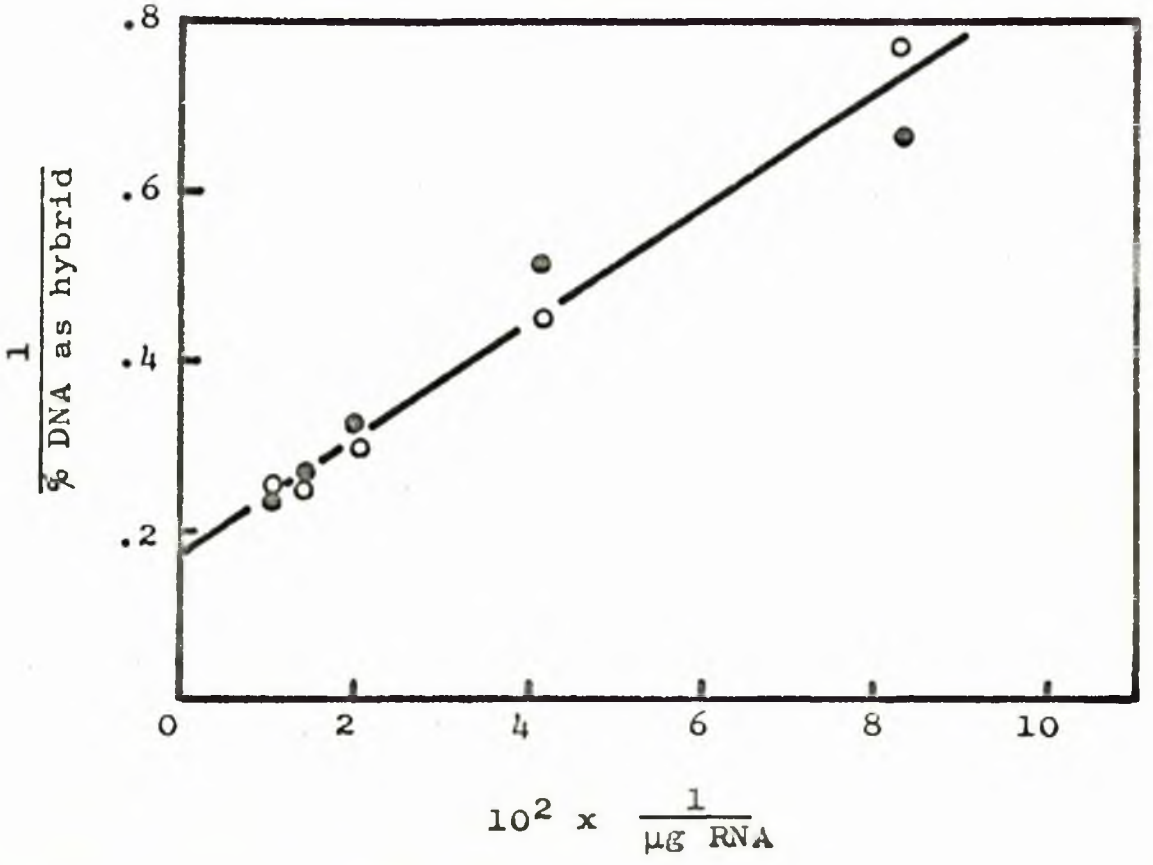


Figure 50.



DISCUSSION

4. Discussion.

4.1 Hybridization techniques.

MAK chromatography.
Sephadex chromatography.
Liquid hybridization using nitrocellulose filters.
Hybridization with immobilised DNA.

4.2 Assumptions and criteria.

4.3 The restriction of DNA template activity in animal cells.

4.4 The relationship between cellular RNA and DNA.

4.5 Symmetry of *in vivo* and *in vitro* transcription.

4.6 The nature of the masking mechanism.

4.1 Hybridization techniques.

Most of the available information on hybridization has come from work with bacterial and viral systems. In the present work a preliminary survey of some of these methods was carried out with a view to experimentation with mammalian materials. The relative merits and demerits of each are discussed in the following sections.

MAK chromatography.

Whereas mammalian RNA could be recovered quantitatively from MAK columns only partial recoveries were obtained with native and denatured animal DNAs. The observations of Hayashi et al. (1960), Mandel and Hershey (1960), and Hershey and Burgi (1960) that non-mammalian DNA could be quantitatively recovered if the material was first sheared, did not appear applicable to either calf thymus or Landschutz DNA. After shearing, 20 per cent and 40 per cent respectively of the material was irreversibly bound to the column. Where only partial recoveries are found it is difficult to ascertain whether all types of DNA molecule are affected or whether selective retention of certain species occurs. In view of this, it was decided to abandon MAK chromatography for the fractionation of hybrids and to examine other alternatives.

Sephadex chromatography.

Chromatography on Sephadex G-200 was found to give

a good separation of hybridised DNA from unhybridised material with quantitative recovery of DNA. The association of adventitious label was eliminated by ribonuclease treatment after hybridization and TCA precipitation of the individual fractions after chromatography. This procedure however was too laborious for kinetic studies requiring numerous point determinations.

Liquid hybridisation using nitrocellulose filters.

The preferential adsorption of denatured DNA and DNA-RNA hybrids by nitrocellulose filters offers a more convenient method for hybrid detection. Liquid hybridization using RNAs synthesised *in vitro* from DNA primers show that the hybridization reaction is second order, the rate being dependent on both DNA and RNA concentration (Figures 5 and 6). For a given set of conditions, maximum hybridization is attained within 1 hour; however the levels of hybridised RNA were found to fall off gradually with time. This was also observed by Gillespie and Spiegelman (1965). Nygaard and Hall (1964) found that when T_2 DNA was incubated in the absence of T_2 RNA it lost its capacity to hybridize with T_2 RNA in subsequent annealing tests. This suggests that DNA-DNA interaction also takes place during incubation. Under the incubation conditions used (60°C in 0.5 M KCl) any denatured DNA which renatures will give stable double helices. Over prolonged incubation times, the relative

concentration of denatured DNA would decrease. This possibility is not entirely supported by the results of Figure 4, as the rates of decline do not alter significantly with the concentration of DNA. Another explanation of this phenomenon is that the hybrids formed during short incubations are not entirely specific. Non-homologous regions of RNA would be rendered ribonuclease resistant. With longer equilibration times, these regions might be eliminated or rendered susceptible to ribonuclease. The possibility of DNA renaturation complicates the quantitative interpretation of results, since the exact concentration of denatured DNA may not be known.

Hybridization with immobilised DNA.

The problem discussed above was not found to occur if the denatured DNA was first immobilised on the filter. The time course shown in Figure 8 does not exhibit any tendency to fall off. With immobilised DNA however the time required to reach equilibrium is considerably longer. This may be due to the fact that immobilised DNA molecules are not capable of random collision within the medium. It is also possible that the equilibrium time for the liquid method is artificially short since DNA renaturation is constantly removing denatured DNA from the reaction.

Removal of adventitious label was achieved with ribonuclease. Some authors maintain that this gives low

estimates of hybrids; however omitting ribonuclease is equally likely to produce high estimates. Whether or not this is the case does not radically alter the conclusions of the experiments, since in most cases these are comparative. Caution must however be exercised when drawing conclusions from absolute figures.

4.2 Assumptions and criteria.

The quantitation of hybridization data depends largely on the mathematical treatment of the results. As an accurate determination of homology between DNA and RNA can only be drawn from saturating conditions, it was found necessary to predict these levels from non-saturating data by the use of double reciprocal plots. This treatment assumes that the hybridization reaction is analogous to adsorption and that it is freely reversible. On the other hand, it is possible that hybrid formation is only reversible when short sequences of homologous bases in the RNA are paired with those of the DNA. With longer sequences, the frequency with which all base pairs dissociate simultaneously may be so rare as to make the hybrid essentially stable. For the present purpose a reversible situation is assumed.

$$\text{DNA} + \text{RNA} \underset{k_2}{\overset{k_1}{\longleftrightarrow}} \text{DNA} : \text{RNA}$$

Application of the Law of Mass Action to this reversible reaction enables an expression to be derived for the velocity

of hybrid formation (v).

$$v = \frac{V \cdot RNA}{K + RNA}$$

where $K = \frac{k_1}{k_2}$

RNA = the concentration of RNA reacting with DNA.

V = the maximum velocity attained when the DNA is saturated with RNA.

It follows that from a plot of 1/v versus 1/RNA then

$$\frac{1}{v} = \frac{K}{V \cdot RNA} + \frac{1}{v}$$

The value of V can be estimated from the intercept of the straight line on the 1/v axis, i.e. when 1/RNA = 0 or RNA = ∞ In practice the rates are expressed as the extent of hybrid formation that takes place when varying concentrations of RNA react with a constant amount of DNA. In this way RNAs which hybridize at different rates can be compared directly by their values of V and theoretical estimates for percentage homology with DNA determined.

In the present work competitive hybridization is used extensively to determine sequence similarities and differences in two RNA populations. Competition is envisaged as follows. When hybridization takes place between a saturating amount of labelled RNA and homologous DNA, but in the presence of an equal amount of homologous unlabelled RNA, the DNA becomes saturated with RNA of half the specific activity of the original RNA. The presence of an equal amount of heterologous RNA should not affect the specific

activity of homologous sequences and no competition will take place. When the two RNAs contain identical complements of species, then at an infinite concentration of unlabelled RNA (where the specific activity = 0) all labelled RNA is excluded from hybridization. In practice this situation is never attained, but can be tested for as already described, from a double reciprocal plot of displacement of label versus concentration of unlabelled RNA.

The validity of competition as a criterion of homology may be questioned on the grounds that all RNAs whether homologous or not may compete to some extent. This suggestion might be supported by the findings of Figure 31, where whole cell E.coli RNA was found to compete with about 20 per cent of the labelled synthetic RNA formed from calf thymus chromatin. It is also possible that there are similarities in some of the proteins of E.coli and calf thymus, however as yet further experiments have not been carried out to test whether this is genuine homdogy or whether it is due to non-specific interaction. Perhaps the best evidence to suggest that non-specific interaction is very low in the system comes from the hybridization data with RNA from synthetic chromatin primers (Figure 47). When the RNA produced from chromatin comprising DNA and excess histone, no hybridizable RNA was detected even though the

system had synthesised considerable amounts of polyribonucleotide.

4.3 The restriction of DNA template activity in animal cells.

From the evidence that all tissues of a given organism contain the same DNA complement and that characteristic proteins are peculiar to certain organs and not others, it has been proposed that transcription of DNA in differentiated animal cells is restricted to specific regions of the genome. This hypothesis can be tested directly by the use of molecular hybridization. In the present work hybridization of ^{14}C-rat kidney RNA to rat kidney DNA (Figures 9 and 10) shows that only about 6 per cent of the DNA hybridizes at saturation.

Some authors have attempted to show that the restriction of template activity is an inherent property of the isolated chromatin by examining the kinetics of RNA transcription from chromatin *in vitro*. Figure 26 shows the type of result obtained. Experiments relying on this criterion should be interpreted with caution since a lower rate of transcription from chromatin as compared with DNA does not necessarily imply a restriction of template activity. In fact, Sonnenberg and Zubay (1965) suggested that the relative insolubility of chromatin in the *in vitro* conditions was responsible for the difference. However the

point in question is not whether less RNA is transcribed from chromatin but whether less of the specific nucleotide sequences of the DNA are transcribed. The hybridization kinetics of ^{3}H-RNA synthesised *in vitro* from calf thymus and rabbit thymus and bone marrow chromatins (Figures 28 to 30) show conclusively that the DNA template activity in chromatin is restricted. This conclusion is also supported by the hybridization studies of Georgiev *et al.*, (1966). Only 5 - 10 per cent of the total DNA is available for transcription in the chromatins so far examined. Solubilisation of the chromatin by sonication produced a specific increase in the template activity, however 80 per cent of the DNA in the chromatin was still not transcribed. This suggests that insolubility is not the reason why chromatin is a less efficient primer. The explanation of the specific increase in template activity produced by sonication is not clear.

Justification for the assumption that the RNA transcribed *in vitro* from chromatin is qualitatively the same as that formed *in vitro* is provided by competition experiments where synthetic RNAs primed by the chromatin of a given organ was tested against natural RNA from the same organ (Figures 31 to 34). Here it is possible to show complete homology between *in vivo* and *in vitro* RNAs from calf thymus and rabbit bone marrow as judged by this criterion.

Further experiments were carried out to test the hypothesis that if the restriction of template activity is related to the state of differentiation of a tissue, then the RNA synthesised from the chromatins of different organs of an animal will also reflect this difference. In this work two widely differing rabbit tissues were used. In both cases where the labelled RNA formed *in vitro* was challenged with natural RNA from the same tissue, the competition observed was greater than that obtained with natural RNA from the other tissue. This "crossing over" effect lends weight to the argument that the differences in the competing abilities of the RNAs are due to qualitative differences in the RNA populations. From Figures 39 and 40 it is seen that this difference is more marked when labelled marrow is used as the test material. This is interpreted as meaning that there are more unique species transcribed from marrow than from thymus chromatin and is consistent with the observed differences in the saturation levels of the two RNAs (Figure 39). The double reciprocal plot of these data also support the qualitative conclusions. With homologous labelled and unlabelled RNA competition is almost complete at infinite concentrations of unlabelled natural RNA, while with heterologous unlabelled RNA only partial competition is achieved. The scattered nature of some of the points makes accurate

extrapolation difficult. In some cases this might be due to the shallow bi-phasic nature of the competition curves. Such a response might be obtained from competition between RNAs which differed significantly in the relative proportion of the various RNA species present.

This type of system has certain advantages over that which uses *in vivo* labelled RNA. The RNA obtained is highly and uniformly labelled and of known specific activity. Attempts to detect inter-tissue differences with rapidly labelled RNA from rat tissues was hampered by the fact that the specific activity of the labelled fraction was not known. Differences in hybridization levels could either be due to absolute differences in the amounts of RNA hybridized or simply a reflection of differences in the specific activity of the precursor pools within the tissues (Figures 13 to 16). The *in vitro* system also has the advantage that all RNAs are probably transcribed at the same rate, and are not subject to differential turnover as is found in *in vivo* conditions (Harris, 1963, 1964a, b; Paul and Struthers, 1963). On the other hand, this *in vitro* system is highly heterologous with regard to the source of materials; however the evidence suggests that the Micrococcal polymerase does not show any preference for DNA of a particular source (Fox *et al.*, 1964). Competition experiments with natural RNA also support the validity of the system.

4.4 The relationship between cellular RNA and DNA.

The results of the previous section give rise to a number of questions which will be discussed further.

Hybridization of whole organ RNA or *in vitro* chromatin primed RNA consistently gave figures of 5-10 per cent of the available DNA being transcribed. It is presently thought that the synthesis of all RNA species in cells is a DNA dependent reaction involving RNA polymerase. It is possible that DNA independent amplification of individual species may take place subsequently; however, even if this is so, the DNA of the cell should contain sequences complementary to each RNA type. It should therefore be possible to account for the 5-10 per cent of transcribable DNA in terms of the individual RNA species within the cell.

Several groups of workers have examined this problem using DNA molecular hybridization. Yankofsky and Spiegelman (1962a, b; 1963) obtained evidence for sequences in *E.coli* and *B.megaterium* that were complementary to their respective 23S and 16S ribosomal RNAs and established that each is derived from unique DNA sequences. From saturation kinetics it was calculated that only 0.3 per cent of the total genome specifies ribosomal RNA. Similar studies have been carried out with pea plants (Chipchase and Birnstiel, 1963), insects (Vermuelen and Atwood, 1965) and with mammals (McConkey and Hopkins 1964; Perry et al., 1964).

Hybridisation of E.coli transfer RNA with its homologous DNA by Giacomoni and Spiegelman (1962) and Goodman and Rich (1962) revealed that only 0.02 per cent. of its sequences were specific for transfer RNA.

Hybrid formation has also been detected between supposed messenger RNA and homologous DNA in a number of bacterial and animal systems (Hoyer et al., 1963; McCarthy and Hoyer, 1964; Hayashi and Spiegelman, 1961; Bautz and Hall, 1962).

McCarthy and Bolton (1964) attempted to fractionate the rapidly labelled RNA of E.coli by hybridisation. The isolated messenger represented about 1.5 per cent of the total cellular RNA; however from hybridization data it appeared that nearly 50 per cent of the sequences present in the DNA were represented in this fraction. Figures are not available for the hybridization of supposed animal messenger with DNA. In the present work attempts to obtain saturation figures for rapidly labelled RNA from a number of rat tissues proved impossible as the true specific activity of the material could not be determined.

Obviously ribosomal and transfer RNA cistrons account for a very small proportion of the DNA in bacterial cells. If the same is true of animal cells then the nature of the bulk of the hybridizable RNA becomes obscure. There is as yet no evidence to suggest that this material represents animal "messenger".

4.5 Symmetry of *in vivo* and *in vitro* transcription.

One aspect of RNA polymerase action which is pertinent to the present work is the question of whether the asymmetry found *in vivo* also prevails *in vitro*.

Roth (1964) suggested that single stranded transcription was more likely than double stranded copying. Otherwise, complementary proteins would be found and the chance that two such proteins should possess discrete biological activities is somewhat remote. Single stranded copying would also be of survival value to an organism, as a single base pair mutation would affect only one protein. Evidence to support this concept has come mainly from *in vivo* studies with bacteriophage.

Bautz and Hall (1962) isolated T_2 specific messenger RNA and determined its base composition. The result suggested that transcription takes place from a single T2 DNA strand. The genetic studies of Champe and Benzer (1962) on rII mutants of T_4 phage provide additional evidence. It was found that mutant phenotypes could be partially reversed by treatment with 5-fluorouracil. Their explanation is consistent with single strand transcription in which the analogue is incorporated into the RNA at the point of mutation on the DNA. The modified messenger then functions in the same fashion as wild type messenger RNA.

Hayashi *et al*. (1963b) presented further evidence from

work on E.coli cells infected with ØX 174 phage. These workers made use of the fact that the mature form of ØX DNA is single stranded, whereas the replicating form which is an intermediate in the synthesis of mature DNA is double stranded. It was found that the phage specific RNA produced on infection was capable of forming hybrids with the replicative form but not with the mature DNA.

Other investigators have carried out similar experiments with certain phage which possess DNA strands of sufficiently differing base composition to permit their separation by physical means. Guild and Robison (1963) separated the strands of pneumococcal transforming DNA by centrifugation in caesium chloride. One of the isolated strands, the lighter of the two, contained the information for conferring resistance to the drug Novobiocin. When the two strands of DNA were assayed for transforming activity it was found that the lighter strand conferred resistance immediately on uptake, while the other strand required approximately one generation time before resistance was expressed. The inference is that the lighter strand is transcribed immediately as if it were native DNA, while the heavier strand required to go through replication before the relevant information for transcription was available.

Tocchini-Valentini et al. (1963) separated the strands of phage α by chromatography on methylated albumin-kieselguhr

and showed that the phage specific RNAsynthesised on infection of B. megaterium cells was capable of hybridizing with only one of the two strands. In an analogous experiment Greenspan and Marmur (1963) found that the RNA of SP8 bacteriophage was complementary to only one DNA strand.

Evidence that messenger RNA of E.coli is transcribed from only one strand of DNA has been presented by McCarthy and Bolton (1964). Purified messenger from pulse labelled cells was capable of hybridizing with almost 50 per cent of the available DNA. No evidence for complementarity within the population could be detected from thermal denaturation data.

The available evidence therefore indicates that in bacteria and bacteriophage infected cells, only one strand of the DNA serves for transcription. However, recent evidence from in vitro studies of DNA primed RNA synthesis are at variance with the in vivo results.

Geiduschek et al.(1962) found that the RNA formed in a cell-free system containing T_2 DNA and Micrococcus lysodiekticus polymerase possessed the same overall composition of the DNA primer and moreover showed a considerable degree of self-complementarity as judged by RNA-RNA hybridisation. Hayashi et al. (1963a) compared the synthetic RNA obtained when the vegetative and replicative forms of ØX 174 DNA were used as primers for E.coli polymerase. It was concluded that

when the replicative (double stranded) form was used, the vegetative strand was not the sole primer, but that both strands probably participated. Chamberlin and Berg (1962) arrived at a similar conclusion using the same system.

However several investigators claim to have demonstrated transcription from a single strand of native DNA primer in a cell-free system. Green (1963) and (1964) demonstrated that the product obtained from the action of E.coli polymerase on T_4 DNA possessed a high degree of homology with in vivo synthesised T_4 RNA. Moreover if the DNA primer was heat denatured or sonicated then this selectivity was lost and transcription took place from both strands. Using the same system Luria (1965) has shown almost complete homology between in vitro and in vivo synthesised T_4 RNAs.

The necessity for intact primer was further demonstrated by Hayashi et al. (1964) who isolated the circular replicating double stranded DNA of OX 174. Synthetic RNA was then made in vitro with E.coli polymerase using as primers intact DNA circles, sonicated circles and mature single stranded DNA. Nearest neighbour analysis of the products was consistent with single stranded transcription from intact circular DNA, however sonication led to double stranded copying. Also the RNAs from intact and ruptured DNA primers was capable of hybridizing with the replicating form of the DNA; however when the RNAs were challenged with

the mature single stranded DNA, only the RNA made by the symmetrical transcription of sonicated DNA showed any degree of competition. Hayashi et al. (1964) therefore suggested that symmetry is dependent on the physical integrity of the DNA primer, and that in the earlier studies of Geiduschek et al. (1962), Chamberlin and Berg (1962) and Hayashi et al. (1963a), this condition was not satisfied.

On the other hand, Geiduschek et al.(1964) examined the RNA formed from phage α DNA in the presence of crude B. megaterium polymerase and demonstrated that the product was complementary to the heavier DNA strand. In contrast to the results of Hayashi et al. (1964) with ΦX 174 DNA, when the primer was sheared by light mechanical stress, the product remained asymmetric. However if the primer was heat denatured then asymmetry was lost, but could be restored if the primer was subsequently renatured. It was concluded that single stranded copying was dependent upon the native conformation but not on the continuity of the DNA primer. The fact that the purified polymerase from Micrococcus lysodiekticus was incapable of asymmetrical transcription even in the presence of intact DNA, led these authors to suggest that the source and degree of purification of the enzyme might affect the symmetry of the product.

In further studies Colvill *et al.* (1965) described essentially asymmetrical RNA synthesis from phage α DNA using crude polymerase extracts from a variety of bacterial sources, thus showing that heterology of components was not a decisive factor. The effect of purification of *E. coli* polymerase on its ability to copy asymetrically was also determined. This property appears to be an intrinsic feature of the enzyme and is not altered by the purification procedure. However the fact that only partial asymmetry is shown when certain other DNA primers are used, raises the question of whether the enzyme is the only determinant.

In the present work using stage V RNA polymerase from M.lysodiekticus, hybridization of DNA primed RNA never gave saturation levels greater than 50 per cent. This would be the result expected for the hybridization of RNA transcribed from only one DNA strand. However more direct evidence is required since saturation values *per se* do not justify this conclusion. Indeed the available evidence suggests that this polymerase is not capable of asymmetrical transcription. The possibility that the experimentally determined saturation levels of 50 per cent are fortuitous artifacts of the working-up procedure must be considered. The use of ribonuclease treatment for the removal of adventitious RNA contamination could be responsible for low estimates of homology, such that the satur-

ation levels for DNA primed RNA are in reality nearer 100 per cent. Some workers (e.g. Shearer and McCarthy, 1967) have abandoned ribonuclease treatment because of this argument, however this could equally result in over-estimates of homology.

One piece of evidence which suggests that single stranded transcription takes place from chromatin primers using the Micrococcal polymerase, comes from competition experiments in which the in vitro synthesised RNA was challenged with natural RNA isolated from the same tissue. In these experiments complete homology between the two RNAs was predicted, whereas only 50 per cent homology would be found if both DNA strands were transcribed from chromatin in vitro. It is possible that asymmetrical transcription occurs when whole chromatin is used as primer but that this specificity is lost when purified DNA is used.

4.6 The nature of the masking mechanism.

Studies with histones.

Studies on the chromatin of animal tissues have shown that the DNA of the nucleus is associated with histone, residual proteins and small amounts of RNA. Dingman and Sporn (1964) characterised the chromatins from various chicken organs and concluded that while the ratio of total protein to DNA may vary widely from tissue to tissue, the

histone to DNA ratio is essentially constant. In many cases ~~the amounts of~~ histone and DNA are present in equal amounts. These results are in general agreement with the analyses of calf, rabbit and rat chromatins described in Section 3.3.

That the function of basic histones in chromatin is not simply a matter of neutralising the acidic phosphate residues in the DNA is suggested from examination of tryptic peptides of histones by Phillips (1964), who showed that the basic amino acids of histones are not equally spaced throughout the molecule but occur in an irregular fashion.

Since the original suggestion of Stedman and Stedman (1950), the possibility that histones might be responsible for masking has attracted considerable attention. Histones can readily be extracted from tissues and fractionated by a variety of physical techniques.

Such studies have shown that there are about 28 histones divisible into five main groups. The relative content of the basic amino acids arginine and lysine within each molecule is often used for classification purposes (Bonner and Tso, 1964). Some protagonists of the masking hypothesis maintain that there is a relationship between the chemical and biological characteristics of the histones. In the experiments of Allfrey et al. (1963) the effect of adding various histone fractions to thymus nuclei synthesising RNA in vitro was studied. It was

found that the arginine rich histones produced the greatest inhibition of RNA synthesis while the lysine rich histones were the least effective. Liau *et al*. (1965) carried out similar experiments in which histones were added to isolated nucleoli in a cell-free RNA synthesising system. In this case the lysine rich histones appeared to be the most inhibitory. Huang *et al*. (1964) came to a similar conclusion from examining the priming capacity of synthetic nucleohistones prepared by reconstituting specific histone fractions with DNA.

In addition Huang and Bonner (1962), Allfrey *et al*. (1963) and Liau *et al*. (1965) have shown that the removal of proteins from chromatin by chemical or enzymic means greatly enhances its priming capacity. Hindley (1963) described results in which increasing salt concentrations were used selectively to remove histones from chromatin. Removal of very lysine rich histones did not produce marked changes in priming capacity, however removal of slightly lysine rich and arginine rich histones resulted in a considerable increase.

From these *in vitro* studies it has been concluded that histones can exercise a regulatory effect on RNA synthesis and that some histones are more effective regulators than others. In many cases the differences in the rates of RNA synthesis have been equated with differences in the template

activity of the DNA. As already mentioned this may not be a valid assumption since the rates of RNA synthesis may be affected by a number of factors. The results of Section 2 show that the Micrococcal polymerase is extremely sensitive to the presence of salts. The amino acids arginine and lysine are most effective inhibitors of polymerase action and it is conceivable that histones might have a similar effect, distinct from that of complexing with the DNA primer.

In the present work the effect of removing proteins on the template activity of chromatin was ascertained by molecular hybridization. The results support the concept that masking of the DNA is due to the presence of proteins; however it was noted that neither the histones nor the residual material was wholly responsible for this. In the work of Georgiev et al. (1966) hybridization has also been used as a test for the template activity of Ehrlich ascites cell chromatin. Treatment of this chromatin with increasing concentrations of salt produces a regular increase in its template activity, while the RNA with the greatest hybridizability is produced from chromatin extracted with 0.4 to 0.6 M NaCl. The extracted material appears to consist of lysine rich histones and non-histone protein.

The specificity of the histones.

If the histones possess the necessary specificity to

interact with specific regions of the DNA then one might expect to find different types of histones in different tissues. However examination of histones from a wide variety of animal and plant sources reveals that the spectrum of histones found is qualitatively much the same (Hnilica et al., 1962; Neelin, 1961; Lindsay, 1964, Bonner and Tso, 1964). There are a few exceptions to this rule. Neelin (1964) showed that nucleated erythrocytes contain substantial amounts of a histone not present in other chicken tissues. Cruft (1966) described a species of sea cucumber whose somatic nuclei are devoid of histone. Sperm cells are also unusual in that histone is replaced by protamine.

Experiments in which chromatin is reconstituted by the interaction of histone and DNA suggest that, while there are marked differences in the affinities of some histones for DNA (Huang et al., 1964; Johns and Butler, 1964; Butler and Johns, 1964) there is no evidence for specific interaction between histones and parts of the DNA which are different in base composition (Johns and Butler, 1964).

Lack of specificity within the histones is also apparent from the in vitro studies of Huang and Bonner (1962) in which almost complete cessation of RNA synthesis was obtained by the addition of excessive amounts of histone

to DNA. This is in agreement with the results described in Section 5, where calf thymus combined with excess histone gave a chromatin from which no hybridizable RNA was transcribed. This would suggest that the histones do not possess the specificity to effect the type of restriction found in chromatin but that they act as general masking agents.

The specificity of masking.

Some workers have attempted to circumvent the problem of non-specificity of the histones by proposing that they are modified in such a way that they behave specifically. Huang and Bonner (1965) found that pea chromatin contains a peculiar type of RNA rich in dihydrourydilic acid. They suggested that it acts as a linker between histones to form specific complexes. Similar evidence has been obtained by Benjamin (1966); however Commonford and Delihas (1966) failed to detect any such RNA in liver. Another suggestion is that the histones are chemically altered by acetylation, methylation or phosphorylation, thereby conferring specificity (Pogo, 1966; Kleinsmith, 1966a, b; Allfrey et al., 1964).

Alternative theories propose that histones are general rather than specific masking agents and that the relevant parts of the DNA in the chromatin are kept unmasked by specific counter ions. Both the acidic or residual proteins

and the RNA components of chromatin have been assigned this function. Studies on Drosophila puffs have shown that whereas the DNA to histone ratio does not alter between puffed and unpuffed regions (Swift, 1964) there is an enrichment of acidic proteins in the puffs as compared with the rest of the chromosome (Clever, 1964). Although a good deal is known about the chemistry of the acidic proteins (Busch, 1965) relatively little is known about their biological function.

Frenster (1965a, b) has described a scheme whereby the DNA in chromatin is maintained in an unmasked state by hydrogen-bonding with specific RNA molecules. Here the base sequences in the RNA provide the necessary specificity.

In this work, evidence suggests that the histones are general, non-specific masking agents. Reconstitution experiments in which histone is recombined with dehistoned chromatin or with DNA and the acidic fraction, point to the non-histone component as the agent responsible for conferring specificity. The active factor in the acidic fraction is not known, however in this connection it is interesting to note the results of Ptashne (1967) who isolated the product of the repressor gene of phage λ. This repressor substance appears to be an acidic protein which combines specifically with a site on the DNA. In

this case the combination of repressor and DNA serves to block transcription. Results obtained with dehistoned chromatin and nucleohistone reconstituted from DNA + acidic fraction demonstrate that the non-histone fraction is capable of restricting template activity as well as conferring specificity for the masking reaction. These results also suggest that the molecules responsible for masking are combined to DNA by salt linkages.

As noted in previous experiments, some RNA preparations showed differences in the initial rates of hybridization although double reciprocal plots indicated the final saturation levels to be identical. This is thought to be due to varying degrees of homopolymer synthesis by the Micrococcal polymerase in the presence of nucleohistone primers.

Finally under the conditions employed in this last experiment, any protein bound covalently to the DNA would appear in the pellicle as an undissociated complex. The possibility that this material might represent the unrepressed parts of the DNA while the repressed DNA sedimented in the caesium chloride, was rejected by isolating DNA from both fractions and demonstrating that they gave identical saturation kinetics when hybridized with chromatin primed RNA.

5. SUMMARY.

1. A survey of some of the available hybridization techniques was carried out with a view to experimentation with mammalian materials. Methylated albumin - kieselguhr chromatography was found to be unsatisfactory, as it consistently gave poor recoveries of denatured DNA. Sephadex G-200 chromatography on the other hand gave a clean and quantitative fractionation of DNA and DNA-RNA hybrids from hybridized material, but was not convenient for numerous point determinations. The most satisfactory results were obtained with the nitrocellulose filter techniques of Nygaard and Hall (1963), and Gillespie and Spiegelman (1965). The latter was adopted for further studies. Some of the practical and theoretical aspects of hybridization are discussed.

2. In preliminary hybridization experiments, RNA from rat tissues was labelled <u>in vivo</u>. Using this material it was shown that only about 7 per cent of rat kidney DNA hybridized with labelled rat kidney RNA when the DNA was saturated with RNA. This was taken as evidence for the restriction of DNA template activity of rat kidney chromatin <u>in vivo</u>. In an attempt to detect differences in the so-called messenger RNA populations of rat kidney, liver and pancreas, the tissues were pulse labelled with ^{32}P-orthophosphate and RNA prepared from the nuclei. Hybridization

of these preparations to rat kidney DNA and also competitive hybridization against unlabelled kidney RNA revealed apparent differences in the hybridization kinetics of the rapidly labelled RNAs. It was not possible to ascertain whether this was due to genuine qualitative differences in the RNA species, or whether it merely reflected differences in the uptake of isotope by the precursor pools of each tissue.

3. The problems associated with the use of *in vivo* labelled RNA were resolved by synthesising the RNA *in* vitro from chromatin primers with the RNA polymerase of M.lysodiekticus. Investigations were carried out to characterise the components of the *in vitro* system.

4. Experiments in which the RNA synthesised *in vitro* from calf thymus and rabbit thymus and bone marrow chromatins, was hybridized to homologous DNA, showed that the DNA template activity of isolated chromatin was also restricted. Competitive hybridization experiments were employed to show that RNA synthesised *in vivo* and *in vitro* from calf thymus and rabbit bone marrow chromatin were qualitatively identical. Using this technique comparison of the RNAs synthesised *in vivo* and *in vitro* from rabbit thymus and bone marrow chromatins provided evidence that qualitative differences exist in the RNA populations of

the two tissues. The bearing of this result on current theories of differentiation is discussed.

5. The nature of the specific restriction of template activity in calf thymus chromatin was investigated. Preliminary results suggested that ionically bound protein in the chromatin was responsible for the restriction. Experimental conditions were sought for the reconstitution of calf thymus chromatin from crude DNA, histone and non-histone fractions. The degree to which the reconstituted chromatins resembled whole calf thymus chromatin was determined by comparing the hybridization kinetics of the RNAs synthesized from these primers in vitro. It was concluded from these experiments that the histone component of chromatin was capable of restricting template activity completely, in a non-specific manner. Reconstitution in the presence of the histone and the non-histone fraction (either as total chromatin proteins or as isolated fractions) results in a specific portion of the DNA being available for transcription. The magnitude of this unrestricted portion was found to be the same as that of whole chromatin. The nature of the factor present in the non-histone fraction is unknown, however it was noted that as well as conferring specificity for the masking mechanism, this material was also capable of restricting DNA template activity to some degree.

Abbreviations.

RNA	Ribonucleic acid.
DNA	Deoxyribonucleic acid.
AMP, CMP, GMP and UMP, ATP, CTP, GTP and UTP.	The 5'-phosphates and the 5'-pyrotriphosphates of adenosine, cytosine, guanosine and uridine respectively.
tRNA	Transfer RNA.
poly rN.	3'-5' polymer of ribonucleotide N.
poly dN	3'-5' polymer of deoxyribonucleotide N.
P_i, PP_i	Inorganic orthophosphate and pyrophosphate.
EDTA	Ethylenediamine tetra-acetic acid (versene).
Tris	2-amino-2-(hydroxymethyl)-propane-1:3-diol.
PCA	Perchloric acid.
TCA	Trichloroacetic acid.

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