# STUDIES ON THE TRANSCRIPTION OF MAMMALIAN DNA.

by

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The purpose of these studies was to establish the validity of a system for investigation of the manner in which transcription from mammalian DNA is regulated.

Mammalian chromatin was isolated; its chemical composition was determined in terms of relative quantities of DNA, histone, acidic chromosomal protein and chromosomal RNA. A procedure employing binding of the dye bromsulphalein to protein was developed for estimation of protein in chromatin. Correlation between the relativ quantities of DNA and histone and, to a lesser extent, between those of acidic chromosomal protein and chromosomal RNA, was noted.

RNA transcribed from mammalian DNA was also studied. The best available procedure for characterisation of such RNA was considered to be DNA-RNA molecular hybridisation. RNA was synthesised *in vitro* using purified bacterial DNA-dependent RNA polymerase. Procedures of purification from *Micrococcus luteus* (*lysodeikticus*) were compared with a view to obtaining both maximal yields and a product not contaminated by ribonuclease activity. Evidence that the enzyme catalysed the synthesis of RNA complementary to DNA template, that it activity was dependent on the presence of the latter and that a DNA-R hybrid might be formed during RNA synthesis was obtained. Synthet RNA was isolated by removal of all other components of RNA-synthesis mixtures. Particular care was required to remove acid-soluble ribo-

nucleotides. Otherwise, the fraction of synthetic RNA hybridizable to DNA appeared to be very small.

Procedures for isolation of native DNA from mammalian cells were established, as were procedures for denaturation of DNA and for separation of denatured Landschutz ascites tumour cell DNA into "fast", "intermediate" and "slow" fractions by virtue of their relative rates of renaturation. DNA which had renatured was separated from that which had not by passage through hydroxyapatite. Under certain controlled conditions, the former, but not the latter, was retained by hydroxyapatite. Conditions for optimal binding of denatured DNA to nitrocellulose and retention of it by nitrocellulose were established. DNA-nitrocellulose binding proved to be dependent on temperature, ionic strength and concentration of solvents which weaken hydrogen bonds, suggesting it is at least partly due to such bonding.

Procedures and conditions for carrying out DNA-RNA hybridisation between RNA in solution and DNA immobilised on nitrocellulose were established. RNA saturation curves and double reciprocal plots derived from them showed that RNA synthesized <u>in vitro</u> from native calf thymus DNA was hybridisable to approximately 20% of denatured calf thymus DNA under the conditions employed. This figure was the same after three different periods of incubation of an RNA-synthesizing mixture, but was greatly reduced when the materials used in such experiments were contaminated by ribonuclease activity. It was also

markedly reduced on incubation of hybridisation mixtures at room temperature in the presence of 30% v/v formamide instead of at 67°C in the absence of formamide. Incubation at 37°C in the presence of 50% v/v formamide was equivalent to incubation at 67°C, save that more prolonged incubation was practicable under the former conditions owing to lesser degradation of RNA or hybrid or both. The presence of sodium dodecyl sulphate proved advantageous; non-specific binding of RNA to nitrocellulose was reduced and a slightly greater extent of hybridisation was recorded. Studies on the action of ribonuclease on putative DNA-RNA hybrid indicated that DNA-RNA hybrid was completely resistant to degradation by it, while non-specifically bound RNA was eliminated. Treatment of nitrocellulose membrane filters with ribonuclease and washing them at room temperature, which was routinely included in the procedure of DNA-RNA hybridisation, was more effective in obtaining reproducible results than washing at 67°C or at room temperature. Some evidence that the DNA-RNA hybridisation reaction is at least partly reversible was also obtained. The solid-liquid procedure of DNA-RNA hybridisation was compared to the liquid-liquid procedure in which hybrids are formed in solution and then trapped on nitrocellulose. Identical RNA saturation curves for the calf thymus DNA system were obtained when the period of incubation in the latter procedure was carefully chosen.

RNA was synthesised <u>in vitro</u> from whole native Landschutz ascites tumour cell DNA and was hybridised with denatured whole homologous

DNA and "fast", "intermediate" and "slow" fractions thereof. To increase the chance of detecting hybridization of RNA with unique nucleotide sequences in "slow" DNA, incubation was carried out for 70 hours in 50% v/v formamide at 37°C. Hybridisation to all three fractions of DNA and to whole DNA was observed, though levels of saturation of separated fractions of DNA appeared to be much less than that of whole DNA

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# INTRODUCTION.

## 1. Determination of the Characteristics of Cells through Transcription of DNA.

The morphological characteristics of cells can be directly related to the protein molecules which they contain. These protein molecules have structures determined by the linear sequences of amino acid residues of which their polypeptide chains are composed (Campbell and Sargent, 1967).

Biosynthesis of polypeptide chains begins with activation of amino acids. Amino acyl-activating enzyme complexes are formed by reaction between amino acid, ATP and amino acid-RNA ligase enzymes (E.C. sub-group 6.1.1.). Each amino acid residue is then transferred to a tRNA molecule specific to it (Novelli, 1967). The amino acid-tRNA complexes then become attached to the nascent protein molecule at the ribosomal level. Ribosomes are composed of RNA and protein. Molecules of mRNA also become attached to ribosomes (Watson, 1964). The order of nucleotide base residues in mRNA molecules determines the order of incorporation of amino acid residues into polypeptide chains through the genetic code (Bretscher and Jones, 1967).

The genetic material of all living cells is DNA. It is also the genetic material of those viruses in which it is found. There is very good evidence that all types of RNA are formed on templates of DNA by the process of transcription. The base sequences of all RNA molecules are complementary to those of the DNA templates on which they are synthesized. mRNA therefore provides the functional link between transcription of DNA and expression of the genetic message in the structure of protein molecules.

## 2. Alteration in the Characteristics of Cells without Alteration of their DNA.

The DNA complements of somatic eucaryotic cells in any one species are, in general, identical. Some exceptions are found in such cases as the partial loss of DNA complement in differentiated cells of the gall midge (White, 1950) and of Ascaris (Wilson, 1925), and, of course, in the complete loss of DNA complement in almost all mammalian erythrocytes.

The studies of Hadorn (1965) probably provide the most convincing evidence that the complete genetic complement is preserved in differentiated cells. During development of some insect larvae some cells

remain in undifferentiated form in the imaginal disc. The remainder of the cells go on to form differentiated tissues. However, the undifferentiated cells are committed to definite lines of development. In Hadorn's experiments cells from different portions of imaginal disc were transplanted to adult insects. They were then serially transplanted in adult insects over many generations. Nevertheless, on transplantation to larvae they developed into mature organs. Over many transplants only one type of organ was formed. However, in certain cases changes in the type of organ formed occurred. This is termed "transdetermination".

Gurdon (1962a,b) and Gurdon and Uehlinger (1966) transplanted intact nuclei from the intestinal epithelium of swimming tadpoles to eggs in which nuclei had been inactivated by irradiation. This permitted development of the eggs into adults which could go on to reproduce. Steward, Mapes and Mears (1958) showed that isolated cells of carrot moristem can grow into complete carrot plants. These findings and those of Hadorn confirm that the complete genetic complement is preserved in differentiated cells.

Carcinogenesis does not necessarily involve alteration in the genetic complement. DiBerardino

and King (1965) showed that transplantation of frog Lucké tumour cells to enucleated eggs gives rise to the development of embryos which can grow quite normally for some time. Transplantation of nuclei from some normal tissues was shown to have a similar effect. McPherson (1965) found that viral-transformed baby hamster kidney cells, which produce highly-malignant tumours on inoculation into hamster brain, can revert to normal cells which do not exhibit this effect.

Bacterial cells synthesize different proteins at different times under different environmental conditions (Epstein and Beckwith, 1968). Such adaptation is also observed, though to a lesser extent, in eucaryotic cells. It involves alterations in transcription of DNA of the cells involved and not alteration of the DNA itself.

Examples of this phenomenon are also found in mammalian cells. Thomson, Tomkins and Curran (1966) showed that synthesis of tyrosine transaminase in tissue culture cells could be induced by steroid hormones. Both the normal and induced enzymes appeared to be identical. Induction was inhibited by inhibitors of RNA and protein synthesis in such a way that RNA synthesis appeared to be specifically required for

induction of enzyme synthesis. Peterkovsky and Tomkins (1967) also showed that the induction of synthesis of tyrosine transaminase by dexamethasone phosphate was associated with RNA synthesis, but not with DNA synthesis. Martin, Tomkins and Granner (1969) showed that dexamethasone phosphate, when present at concentrations in which synthesis of tyrosine transaminase is induced in this system, did not affect DNA synthesis and the cell cycle. However, synthesis of tyrosine transaminase could be induced only at certain stages of the cell cycle.

Labrie and Korner (1968) showed that administration of a mixture of amino acids to adrenalectomized rats stimulated synthesis of both tryptophan pyrrolase and tyrosine transaminase. Again, stimulation was dependent on RNA synthesis.

## 9. Relevant Features of DNA.

### Chemistry and Structure of DNA.

DNA is found in all procaryotic and eucaryotic cells (except almost all mammalian erythrocytes) and also in a large number of viruses. DNA and RNA have the same basic structure in that their polymeric molecules consist of nucleotide residues linked to one another through 3',5' phosphodiester bonds. The

nucleotide base composition of the DNA of all cells of an organism is the same and characteristic of that organism. Chargaff's rules, viz. A ≡ T, G ≡ C, A + G ≡ C + T and A + C ≡ G + T are observed in the DNA of all cells and all viruses which contain double-stranded DNA.

The physical structure of DNA molecules has been determined largely as a result of X-ray diffraction studies (Watson and Crick, 1953; Wilkins, Stokes and Wilson, 1953; Franklin and Gosling, 1953; Langridge, Wilson, Hooper and Wilkins, 1960; Langridge et al., 1960). DNA molecules consist of two right-handed helical polydeoxyribonucleotide chains. These are of opposite polarity and are plectonemically coiled around a common axis to form a double helix. There are ten base-pairs, A with T or C with G, to every turn of the helix. Their planes are perpendicular to that of the axis. The validity of this structure has been confirmed by alternative approaches such as electron microscopic autoradiography (Cairns, 1966), thermal denaturation studies (Szybalski, 1967) and ultracentrifugal sedimentation studies (Eigner and Doty, 1965).

Eigner and Doty (1965) have tabulated molecular weights of DNA. They are mostly in the region of

millions and tens of millions of daltons. Such estimations of the molecular weights of DNAs are almost certainly too low, since manipulations of DNA solutions may cause chain scission (Davison, 1959; Levinthal and Davison, 1961; Burgi and Hershey, 1961; Thomas and Berns, 1961). From electron microscopic autoradiographic studies it has been shown that molecular weights in the regions of $10^8$ and $2 \times 10^9$ are valid for DNA of, respectively, bacteriophage T2 and Escherichia coli (Cairns, 1963). Molecular weights of more complex DNA of eucaryotic cells are probably still greater.

Replication of DNA.

The validity of the concept of semi-conservative replication of DNA was confirmed by Meselson and Stahl (1958). They showed this occurred during growth of Escherichia coli. This was achieved by transfer of cells between media such that the densities of individual strands of DNA would be different. By similar approaches Meselson and Weigle (1961) and Chun and Littlefield (1961) showed that DNA of viruses and mammalian cells, respectively, underwent semi-conservative replication. Thus DNA is replicated in a manner which ensures that the DNA of parent cells is

identical to that of their progeny.

Evidence that DNA is the Genetic Material.

The manner in which DNA replicates is compatible with its being the genetic material. There is further convincing evidence for this. Bacteria which have lost a gene can, on treatment with isolated DNA of a wild-type organism, regain the lost activity corresponding to the lost gene (Avery, McLeod and McCarthy, 1944; Marmur and Hotchkiss, 1953; Hotchkiss and Weiss, 1956; Chargaff, Schulman and Shapiro, 1957).

A similar phenomenon occurs in bacterial transduction. A bacteriophage acts as carrier of DNA and genetic information between bacteria (Zinder, 1958).

In some mutations a change in amino acid sequence in protein can be correlated with changes in DNA. Through knowledge of the genetic code the difference in amino acid sequences found in sickle-cell and normal human haemoglobins can be correlated with replacement of T by A in DNA (Lehman and Huntsman, 1966).

Yanofsky et al. (1965) found that the positions of amino acid replacements in one segment of the A protein of the tryptophan synthetase system of Escherichia coli were in the same relative order as that of the mutationally altered sites of the A gene.

The metabolic stability of DNA (Levinthal, 1956; Siminovitch and Graham, 1956; Thomson, Paul and Davidson, 1958) is also compatible with its being the genetic material.

Nature of Forces holding together Complementary Strands in DNA Molecules.

There are two main types of force which hold together the complementary strands of DNA molecules. They are those associated with base-stacking and those of hydrogen bonding between complementary base residues. Bonding associated with base-stacking is largely due to interaction of electron clouds of adjacent base residues, simpler dipole-dipole interactions and dipole-dipole interactions of the type described as London dispersion forces; $\pi$ bonds within base residues ensure their planarity (Spenkman, 1959). The free energy of bonds associated with base-stacking considerably exceeds that of hydrogen bonding (Saybalski, 1967). It may be concluded that hydrogen bonds are largely responsible for the specificity of strand-strand interaction and that bonds associated with base-stacking are largely responsible for the stability of the DNA double helix.

Denaturation of DNA.

Isolated double-stranded DNA in solution is readily denatured (made single-stranded) by treatments which disrupt base-stacking and hydrogen-bonding. Among these are heating, raising the pH of the solution to an extreme value, decreasing the ionic strength and replacing water by an organic solvent.

Denaturation of DNA involves unwinding of the helix, rupture of hydrogen bonds of base-pairs, collapse of base-stacking and dissociation and complete separation of the complementary strands. Evidence for this has come from such studies as the X-ray scattering studies of Luzzati, Mathis, Masson and Witz (1964) and the circular dichroism studies of Brahms and Mommaerts (1964).

Assay of Denaturation and Renaturation of DNA.

Denaturation of DNA is most commonly assayed by following its ultraviolet hyperchromism at 260 mμ. Heterogeneity of DNA can be assessed by comparison of thermal denaturation profiles. The steepness of slope of these profiles increases with increasing homogenity (Kit, 1960). Other changes also accompany the denaturation of DNA. Its behaviour during ultra-centrifugal sedimentation and viscosity are affected

(Eigner and Doty, 1965); its retention on nitrocellulose membranes is altered (Nygaard and Hall, 1963); its susceptibility to certain deoxyribonucleolytic enzymes is altered, for example Exonuclease I of Escherichia coli (Lehman, 1960); its behaviour on polyacrylamide gel electrophoresis is changed (Loening and Williamson, 1969); its behaviour is also changed on column chromatography on pretreated silk fibroin (Huh and Helleiner, 1967) and on hydroxyapatite (Bernardi, 1969a,b; Bernardi, 1965; Miyazawa and Thomas, 1965; Walker and McLaren, 1965b).

The phenomena used to follow DNA denaturation can also be used to follow renaturation of DNA. By utilisation of one of the above differences between native and denatured DNA, DNA which has renatured can be separated from that which has not renatured. It is also possible to follow renaturation between DNA immobilised on such materials as agar (Walker and McLaren, 1965a) and nitrocellulose (Denhardt, 1966) and denatured DNA in solution.

Renaturation of DNA.

Separated strands of DNA molecules are brought back together in DNA renaturation. Wetmur and Davidson (1968) have summarised the well-established features of

this reaction. It is approximately second order with respect to DNA concentration; its rate is maximal at temperatures of 20°C to 30°C less than the Tm of the native DNA under the same conditions and its rate is very dependent on ionic strength at low ionic strengths, but almost independent of it at higher ionic strengths.

The complexity of DNA is the total quantity of DNA, in daltons, in a single genetic complement. Marmur and Doty (1961) found that bacteriophage DNA renatured faster than the more complex bacterial DNA, while they did not observe any renaturation of calf thymus DNA in their studies. From theoretical considerations and by consideration of actual results, Wetmur and Davidson (1968) have shown that the second order rate constant for DNA renaturation is inversely proportional to DNA complexity.

They took second order kinetics of DNA renaturation to imply that initial nucleation is a relatively slow, rate-limiting process. They assumed this reaction to be followed by a much faster "zippering" reaction. Ten to twenty base-pairings are probably necessary for initial stabilisation in the region of nucleation. Formation of short stretches of accidental base-pairing was considered unimportant at optimal temperature, but

much more common at lower temperatures. Intramolecular effects were considered to be largely responsible for this.

The rate constant for DNA renaturation was found to be proportional to the square root of the average number of nucleotide residues per single strand of the denatured DNA involved. On taking account of steric hindrance associated with strand interpenetration, this relationship was deduced theoretically. If all nucleation sites were equally available, the nucleation rate and hence reaction rate would have been proportional to molecular length. All nucleation sites cannot, therefore, have been equally available.

The Macroheterogeneity of Eucaryotic DNA.

Owing to the apparently great complexity of eucaryotic DNA, it was not generally expected that its renaturation would readily occur. It has, however, been possible to demonstrate renaturation of DNA from a wide variety of eucaryotic species (Britten and Kohne, 1968).

A hypothesis to account for the paradox was advanced by Britten and Waring (1965). They proposed that some nucleotide sequences were frequently repeated in DNA. They based their hypothesis on their observations that, in the ultracentrifugal density gradient

sedimentation of many eucaryotic DNAs, a satellite band of lesser density than that of the bulk of the DNA appeared. Satellite DNA was characterized by a very rapid rate of renaturation. Mouse satellite DNA rapidly renatured to form a double-stranded helical molecule at least very similar to that of native DNA.

Walker and McLaren (1965) separated mouse DNA which had been denatured into "labile" and "stable" fractions by chromatography on hydroxyapatite. The stable fraction consisted of rapidly, but imperfectly, renatured DNA. Rapidly-renaturing DNA prepared by either procedure was identical (Waring and Britten, 1966).

Britten and Kohne (1968) have reviewed much of the information available on renaturation and macro-heterogeneity of DNA. This has been collected largely by Britten and his colleagues and by Walker and his colleagues (Walker and McLaren, 1965a,b; McLaren and Walker, 1966; Flamm, McCallum and Walker, 1967; McLaren and Walker, 1968).

Repeated nucleotide sequences have been found in the DNA of all types of eucaryotic cell examined. It has not been possible to detect repetitious DNA in viruses nor in bacteria by DNA renaturation studies.

Nucleotide sequences of eucaryotic DNA may be divided into three types on the basis of degree of repetition. One type is that in which there are in the order of a million copies of each repeated sequence. This corresponds to satellite DNA. There are in the region of a thousand to a hundred thousand copies of each sequence in the second type. Sequences are unique or repeated only a very small number of times in the third type. In accordance with their relative rates of renaturation, these types of DNA are described as "fast", "intermediate" and "slow". They may be separated on this basis.

In mouse DNA, these fractions make up approximately 10%, 20% and 70%, respectively, of whole DNA. In calf thymus DNA, there is little or no fast fraction and the intermediate and slow fractions make up, respectively, approximately 40% and 60% of whole DNA. Slow DNA has not been found in salmon (Britten, 1969). All of salmon DNA may belong to the intermediate fraction. Human DNA contains a satellite fraction, but it makes up less than 1% of whole DNA (Comeo, Ginelli and Polli, 1968).

Thermal denaturation profiles of renatured DNA indicate imperfect matching of nucleotide sequences

(Britten and Kohne, 1968; McCarthy, 1967). Thus copies of repeated nucleotide sequences are probably not fully identical to one another. They would thus represent families of related genes. It has, however, been suggested that repetitious calf thymus DNA is composed of thousands of copies of one nucleotide sequence (Britten, 1969). Repeated nucleotide sequences are scattered throughout the length of the genome.

At present the functions of fractions of DNA and multiple copies of DNA nucleotide sequences are unknown. Species-specificity of repeated sequences is evident. It is the existence of families of related nucleotide sequences which has made possible studies on the renaturation of eucaryotic DNA. These may involve homologous or heterologous DNA strands. The extent of renaturation of heterologous DNA strands increases with the degree of evolutionary relationship of the species involved (Hoyer, Bolton, McCarthy and Roberts, 1965). However, McLaren and Walker (1968) have found that DNAs of very closely related species of laboratory mice and mouse tissue culture cells and of jumping mice and deer mice are distinguishable from one another in DNA renaturation studies. Two of these species were even sufficiently closely related to interbreed.

It appears that repeated nucleotide sequences have arisen from relatively recent times up to several hundred million years ago (Britten, 1969). Britten and Kohne (1968) have suggested that multiplication of nucleotide sequences of DNA occurs by sudden "saltatory" replication. To have been rapidly disseminated throughout a species, repeated nucleotide sequences of DNA must be associated with a favourable genetic element. They could provide for a more rapid rate of protein synthesis (Britten and Kohne, 1968). Owing to the degeneracy of the genetic code, divergence in nucleotide sequence in DNA need not be correlated with divergence in protein structure. Repeated nucleotide sequences in eucaryotic DNA may represent a situation akin to lysogeny in bacteria (Britten, 1969). If the virus or viruses made temperate were lethal, the rapid rate of dissemination of repeated nucleotide sequences of DNA would be expected. The maintenance of many similar species-specific nucleotide sequences in spite of the probability of random mutations suggests that they are functionally necessary.

More could be said about the possible functions of fractions of DNA if it were known which were transcribed. Unfortunately, a general conclusion on this cannot be reached at present. Flamm, Walker and McCallum (1969)

studied the effects of RNA of mouse liver and tumour cells on the rate of renaturation of mouse satellite DNA. They also attempted to follow DNA-RNA hybridization between separate strands of mouse satellite DNA and RNA of mouse liver, spleen and kidney. Their results indicated no, or very little, DNA-RNA homology. This suggests that it was unlikely that mouse satellite DNA had been transcribed as the chance of a transcribed RNA molecule finding a complementary DNA sequence would have been very high. However, Harel, Hamanda, Tapiero and Harel (1968) noted DNA-RNA hybridization between rapidly-labelled DNA of mouse liver, kidney and tissue culture cells and mouse satellite DNA. They also found that only the lighter dA strand of this DNA was involved in hybridization with tissue culture cell RNA. From their results, mouse satellite DNA appeared to be transcribed. Melli and Bishop (1969) saturated native and denatured rat DNA with RNA synthesized *in vitro* on homologous templates. Approximately 5% of DNA was involved in DNA-RNA hybridization. However, 50% of total denatured DNA was involved in formation of the enzymatic hybrid which was formed between denatured DNA and RNA during *in vitro* transcription. Probably less than 13% of rat DNA is composed of repeated sequences. Thus "slow"

DNA has probably been transcribed, but not hybridized to RNA complementary to it. The concentrations in and time of incubation of DNA-RNA hybridization mixtures were probably insufficient to permit significant hybridization between complementary DNA and RNA sequences. These would have been present at very slight concentrations in the total population of DNA and RNA molecules. DNA-RNA hybridization had apparently involved repetitions DNA.

Multiplication of genes and transcription from those may also be deduced from studies on closely similar polypeptide chains, as in the case of haemoglobin (Ingram, 1961).

Studies on hybridization between rRNA and homologous DNA have shown that the small fraction of whole DNA complementary to rRNA is composed of repeated nucleotide sequences. This fraction is very much smaller than those which repeated nucleotide sequences just described make up in whole DNA. There are multiple copies of genes for rRNA in DNA of bacteria and of eucaryotic cells.

In the original studies of Yankofsky and Spiegelman (1962b) it was concluded that there were several genes for rRNA in the genome of Escherichia coli. These could have been identical or similar and made up 0.2% of total

DNA. Midgley (1968) has concluded that 0.45% of the genome of Escherichia coli is responsible for transcription of rRNA. This corresponds to approximately 45 cistrons of molecular weight $5 \times 10^5$. 0.23% of the DNA is responsible for synthesis of most of each type of rRNA.

Brown and Weber (1968) have found that 18S and 28S rRNA of Xenopus laevis are transcribed from repeating units in DNA. There are about 450 of these in the haploid genome. They are composed of genes for 18S rRNA and 28S rRNA interspersed by DNA not complementary to either of these types of rRNA. Birnstiel (1968) has obtained results in general agreement with these. He states that there are in the region of 1200 to 1600 ribosomal 18S and 28S DNA complements in the genome of Xenopus laevis. rDNA makes up 0.2% of whole DNA. He has proposed a tandem arrangement for the genes for 18S and 28S rRNAs.

There is genetic evidence for amplification of genes for rRNA in Drosophila (Ritossa, 1968).

4. Regulation of Transcription of DNA in Bacteria.

It is generally believed that a mechanism for regulating transcription of genes into mRNA in accordance with environmental conditions has been evolved in bacteria (Epstein and Beckwith, 1968). Elucidation of the details

of control of the lac operon of Escherichia coli is largely due to the studies of Jacob and Monod (1961a,b). There are three main principles in the system postulated by them. Firstly, the rate of synthesis of each protein is directly controlled by the rate of synthesis of the mRNA for it. Secondly, several types of mRNA are transcribed from units of DNA called "operons". These are composed of several "structural" genes for a set of functionally-related mRNA molecules and an "operator" gene adjacent to one of these. Transcription of all the structural genes related to one of the operator genes depends on transcription of the latter. Thirdly, transcription at the operator locus is reversibly regulated by a product of another gene or genes described as "regulator" genes. These may be located elsewhere in the DNA of the cell. Their product, a "repressor", interacts with inducing or repressing substances such that its affinity for the operator locus is decreased or increased, respectively.

Operation of this system requires that the lifetimes of mRNA molecules be brief. Thus the rapid changes observed in rates of synthesis of enzymes in bacterial cells might occur. In fact, since the original studies of Levinthal, Keyman and Higor (1962) on turnover of

mRNA in Bacillus subtilis in the presence of actinomycin D, it has been confirmed that the half-lives of mRNA molecules in bacterial cells are in the region of a very few minutes (Golduschek and Hasselkorn, 1969). This still allows several polypeptide chains to be formed through utilisation of each mRNA molecule (Singer and Leder, 1966).

There is evidence that operator systems similar to that described for the lac operon of Escherichia coli by Jacob and Monod exist elsewhere in bacteria (Ames and Hartman, 1963; Büttin, 1963a,b; Novick and Richmond, 1965; Imamoto, Ito and Yanofsky, 1966; Cozzarelli, Freedberg and Lin, 1968).

Repressors appear to be proteins. The lac repressor of Escherichia coli has been shown to be an oligomeric protein molecule (Müller-Hill, 1966; Gilbert and Müller-Hill, 1966). Its monomeric molecular weight is in the range of 40,000 to 50,000. It binds to DNA at the operator site (Riggs and Bourgeois, 1968; Riggs, Bourgeois, Newby and Cohn, 1968). The repressor of the dormant bacteriophage $\lambda$ genome has been isolated and found to be an acidic protein with a monomeric molecular weight in the region of 30,000. It also binds to DNA (Ptashne, 1967a,b).

## 5. Regulation of Transcription of DNA in Eucaryotic Cells.

### Location of DNA in Chromatin.

It is first necessary to consider the nature of the material in which eucaryotic DNA is found. DNA of eucaryotic cells is almost entirely confined to chromatin. When cell division is imminent chromatin becomes condensed into definite chromosomes. Each chromosome is duplicated such that, on cell division, each daughter cell receives a chromosome complement identical to that of its parent cell. During interphase some of the chromatin is distributed throughout the nucleus (euchromatin). The rest remains in a condensed form (heterochromatin).

Chromatin is isolated from nuclei in the form of a viscous gel mainly composed of DNA, RNA and protein. Two major classes of protein are found in chromatin: histones and acidic chromosomal proteins.

### Histones.

Histones are readily extracted from chromatin in dilute mineral acid; strong acid can be used to achieve complete extraction (Butler, Davison, James and Shooter, 1954). Busch and Davis (1958) have defined histones as proteins of the nucleus that are basic and are characterized by a high content of basic amino acids, an

isoelectric point between 10 and 11, a nitrogen content of about 18%, insolubility in an excess of ammonia, a molecular weight large enough to prevent passage through dialysis membranes, low tryptophan and solubility in 0.3% M mercuric sulphate in 1.88 M sulphuric acid. Murray (1965) has simply defined histone as a basic protein that at some time is associated with DNA.

Histones have been found in cells of all higher plants and animals examined (Phillips, 1962). They are present in quantities approximately equal to that of DNA (Dingman and Sporn, 1964; Bonner et al., 1968). However, histones are present in very small quantities in or absent from many unicellular organisms (Lever, 1964). Available evidence suggests that histones at all similar to those of eucaryotic cells are not present in bacteria (Roth, 1965).

Johns and his colleagues (Johns, Phillips, Simson and Butler, 1960; Johns and Butler, 1962; Johns, 1964; Phillips and Johns, 1965) have studied the fractionation of calf thymus histone. They have divided it into five different fractions, viz. F1, F2a1, F2a2, F2b and F3. They appear to be substantially single components and each appears to account for approximately 20% of whole

calf thymus histone (Phillips and Johns, 1965). F1 is often described as "very lysine-rich", F2 as "lysine-rich" and F3 as "arginine-rich".

It is now apparent that similar histone fractions can be isolated from a variety of animals (Pallau and Butler, 1966; Butler, Johns and Phillips, 1968; MacGillivray, 1968) and from pea-bud (Bonner et al., 1968). It is also apparent that the histones of mammalian tumour cells are similar to those of normal cells (Ehilica, 1966; Laurence, Phillips and Butler, 1966). As even mild proteolytic degradation of histones can result in considerable heterogeneity (Billen and Ehilica, 1964), heterogeneity of histones is probably always less than it appears to be.

Histones are rather poor antigens (Ehilica, 1967). This is in accord with there being a very great degree of homology between histones of different tissues of one species and there being very considerable inter-species homology of histones. Noteworthy results of DeLange, Fambrough, Smith and Bonner (1968, 1969) have recently provided further evidence for the correctness of the concept of there being very great inter-species homology of histones. They have found that the amino acid sequence of calf thymus histone fraction F2a1 is almost

identical to that of the same histone fraction of pea-bud.

The functions of histones are believed to be structural and regulatory. Structural functions include stabilisation of the structure of chromatin and stabilisation of the structure of DNA therein.

Zubay (1964) proposed that the direction of histone molecules relative to those of DNA changes with changes in the state of hydration of chromatin. He proposed that histone molecules cause cross-linkage between DNA helices in chromatin gels but lie parallel to DNA molecules when chromatin is in solution. Similarly, Itzhaki (1966a,b) concluded that rat thymus chromatin consists of an array of laterally arranged molecules when in solution, but that its molecules make up an interlocked network when it is in gel form. These concepts are founded on the results of X-ray diffraction studies. Electron microscopic reconstitution and dissociation studies (Littau, Burdick, Allfrey and Mirsky, 1965) have indicated that the lysine-rich histone fraction F1 is that which causes cross-linkage in the structure of chromatin. Izawa, Allfrey and Mirsky (1963) found that addition of arginine-rich histones of calf thymus to lampbrush chromosomes isolated from Triturus viridescens

caused retraction of the loops of these chromosomes. Lysine-rich histones did not have a comparable effect. This suggests that arginine-rich histones participate in the association of individual DNA molecules and illustrates the very considerable lack of species-specificity of histones.

80% of the lysyl and arginyl residues in calf thymus chromatin are involved in electrostatic bonding to phosphate residues of DNA (Walker, 1965). Histones are known to stabilise DNA against thermal denaturation (Ohba, 1966). Various cationic homopolypeptides (Olins, Olins, Ada and von Hippel, 1967), polyamines and oxidised polyamines (Bachrach and Eilon, 1967) have a similar effect. Histones thus probably stabilise the structure of DNA in chromatin. The specificity required for this function is probably little.

Acidic Chromosomal Proteins.

Acidic chromosomal proteins make up a substantial part of the protein of chromatin. They appear to be firmly associated with nucleic acids (Wang, 1966; Dekker, Kung and Donner, 1969; Huang and Huang, 1969). Very little is known of their structure and the manner in which they are bound to the other components of chromatin owing to their insolubility. Attempts to dissociate

complexes of acidic chromosomal protein and nucleic acid often result in denaturation and precipitation of the former (Hnilica, 1967).

All the amino acid residues commonly found in protein are represented in the acidic chromosomal proteins of rat liver and Walker tumour cells. They have a high content of glutamate and aspartate residues (Steele and Busch, 1963). Their molecular weights are much greater than those of histone fractions (Busch, 1965). Studies on the effect of exposure of Ehrlich ascites tumour cells to bifunctional alkylating agents have shown that acidic chromosomal proteins are closely associated with DNA (Steele, 1962).

The enzymes DNA-dependent RNA polymerase (nucleoside triphosphate : RNA nucleotidyl transferase) and DNA-dependent DNA polymerase may both be classed as acidic chromosomal proteins (Huang and Bonner, 1962; Wang, 1967).

Chromosomal RNA.

Bonner and his colleagues (Huang and Bonner, 1965; Bonner and Widholm, 1967; Bonner et al., 1968; Bekhor, Kung and Bonner, 1969) have investigated pea chromosomal RNA. It is unlike other types of RNA in that it has a molecular nucleotide chain length of between 40 and 60

residues, a sedimentation coefficient of only 3.2S and a high content of dihydrouridylate residues. Its dihydrouridylate content is in the region of 8 to 10 moles per cent. It is not degraded by ribonuclease in isolated chromatin. It is covalently bound to acidic chromosomal protein, probably by means of an amide bond to a dihydrouracil residue, and is hydrogen-bonded to histone. Similar chromosomal RNA has been found in chick embryo (Huang and Huang, 1969), rat liver (Benjamin, Lenander, Colhorn and DeBellis, 1966), rat ascites tumour cells (Bekhor, Bonner and Kung Dahmus, 1969) and calf thymus (Shih and Bonner, 1969). Rat ascites tumour cell chromosomal RNA contains dihydrothymidylate residues instead of dihydrouridylate ones (Bekhor, Bonner and Kung Dahmus, 1969). DNA-RNA hybridization competition studies have indicated that chromosomal RNAs of pea cotyledon and pea bud are not identical, although they do contain some common nucleotide sequences (Bonner and Widholm, 1967). Bonner and Widholm (1967) were unable to detect homology of pea bud chromosomal RNA with DNA of cauliflower, rat or HeLa cells. It therefore appears that chromosomal RNAs may be species-specific and to some extent tissue-specific.

"Masking" of DNA in Eucaryotic Cells.

Some features of transcription of eucaryotic DNA are unlike those of bacterial DNA. There is almost permanent repression of many genes in any differentiated eucaryotic cell. However, during development of an organism brief stimuli to differentiate occur. These must involve temporary expression of genes otherwise permanently repressed. Such phenomena are not observed in bacteria.

The lifetimes of mRNAs of eucaryotic cells are much longer than those of bacterial cells. It has already been stated that the half lives of bacterial mRNAs are in the region of a very few minutes. The half lives of mRNAs of rat liver and HeLa cells have been estimated to be eight to twelve hours (Trakatellis, Axelrod and Montjar, 1964) and over three hours (Penman, Scherrer, Becker and Darnell, 1963), respectively. In some eucaryotic cells mRNA molecules appear to be essentially stable (Marks, Burke and Schlessinger, 1962; Humphreys, Penman and Bell, 1964).

It is hard to imagine how the system of Jacob and Monod (1961a, b) might generally operate in eucaryotic cells. Repressors would have to be continually present at suitable concentrations. Inducers would have to

appear at particular stages. Owing to the long lifetimes of mRNAs, protein synthesis could not be readily controlled unless by means of an inherently wasteful system of control at the ribosomal level.

In addition to this negative evidence, there is positive evidence for a system of control of transcription of eucaryotic DNA involving permanent repression ("masking") of many genes in any tissue. Giant chromosomes of cells of salivary glands and Malpighian tubules of larvae of dipterous insects represent exceptional cases of chromosomes being visible during interphase. At a few points they are swollen into "puffs". These are believed to be regions of local uncoiling and consequent outlooping of threads of DNA (Beerman and Clever, 1964). Puffing patterns are identical in all cells having the same function in insect larvae, but vary specifically from tissue to tissue (Clever, 1964). Use of tritiated uridine in autoradiographic studies has shown that chromosome puffs are regions of very active RNA synthesis; the remainder of the chromosome is inactive in RNA synthesis (Ficq and Pavan, 1957; Pelling, 1959, 1964; Sirlin, 1960). There is evidence that such RNA is mRNA. Edstrom and Beerman (1962) determined the base compositions of RNAs extracted from four separate puffs of chromosomes

I and IV of the salivary gland of Chironomus tentans. These were asymmetric and different in each case. This would be expected if the RNA were mRNA. The salivary secretion of Chironomus tentans lacks a type of granule found in that of Chironomus pallidivittatus and all other Chironomus species examined. Cytogenetical studies have shown that a chromosome puff of the salivary gland of Chironomus pallidivittatus is absent elsewhere in this organism and from salivary gland cells which do not produce this type of granule. They have also shown that it is absent from all of the salivary gland cells of Chironomus tentans (Beerman, 1961). The results of both these studies indicate that chromosome puffs are regions of mRNA synthesis. The remainder of such chromosomes contains masked DNA.

Comparative studies of euchromatin and heterochromatin also lead to the conclusion that much of the DNA of eucaryotic cells is masked. Electron microscopic autoradiographic studies have shown that, in calf thymocytes, RNA is actively synthesized in euchromatin but not in heterochromatin (Frenster, Allfrey and Mirsky, 1963; Littau, Allfrey, Frenster and Mirsky, 1964). Similar studies have shown that RNA synthesis occurs in euchromatin, but not in heterochromatin, of the mealy

bugs Planococcus obscurus and Planococcus citri (Berlowitz, 1965). Only euchromatic chromosomes of male mealy bugs are transmitted to their offspring. These are derived maternally; the heterochromatic chromosomes are derived paternally (Brown and Nur, 1964). Thus much of the DNA of male mealy bugs contains genes irrelevant to their offspring.

It can be concluded that much of the DNA of eucaryotic cells is not involved in RNA synthesis and is masked. Specific portions of DNA appear to be used in RNA synthesis in specific tissues.

Studies on the in vitro synthesis of RNA from chromatin and the properties of such synthetic RNA have led to confirmation of the concept of masking of DNA and to tentative establishment of the molecular mechanism involved.

Rates of in vitro RNA synthesis from chromatin and isolated DNA of plant (Bonner, Huang and Gilden, 1963), amphibian (Flickinger, Coward, Miyagi, Moser and Rollins, 1965; Kim and Cohen, 1966) and mammalian (Barker and Warren, 1966; Matsushige and Bonner, 1966) tissues have been compared. The rate of RNA synthesis from chromatin is much less than that from DNA. It has been suggested in such studies that this is due to gene repression.

Two main criticisms may be applied to this line of reasoning. Firstly, the rate of RNA synthesis from chromatin probably depends very considerably on the degree of solubility of the chromatin (Sonnenberg and Zubay, 1965). Secondly, even if a decreased rate of RNA synthesis from chromatin implies gene repression, it cannot be stated whether this is specific masking of certain portions of DNA or a more non-specific inhibition of RNA synthesis.

The technique of DNA-RNA hybridization has been used in attempts to obtain results not subject to these criticisms. Relative rates of *in vitro* RNA synthesis are irrelevant in DNA-RNA hybridization studies. Paul and Gilmour (1966a) found that RNA synthesized *in vitro* from isolated calf thymus DNA could be hybridized to a much larger fraction of homologous DNA than RNA synthesized from calf thymus chromatin. This showed that specific DNA nucleotide sequences are masked in chromatin. By hybridization competition studies they later showed that such synthetic RNA from chromatin is transcribed from the same portions of homologous DNA as is natural RNA of the same tissue. They were also able to show that the nature of masking of DNA is tissue-specific (Paul and Gilmour, 1966b; Paul and Gilmour, 1968). Although the

studies of Georgiev, Ananieva and Kozlov (1966) were much less complete than those of Paul and Gilmour, they were able to reach similar conclusions. They found that RNA synthesized from isolated DNA of Ehrlich ascites tumour cells could be hybridized to a much larger fraction of homologous DNA than RNA synthesized from chromatin prepared from these cells. They also obtained hybridization competition data suggesting that synthetic RNA from chromatin was transcribed from the same portions of homologous DNA as was natural mRNA. It may thus be concluded that much of the DNA of chromatin is masked in a tissue-specific manner and that this pattern of masking is retained in isolated chromatin.

Paul and Gilmour (1968) found that removal of histones from chromatin markedly increased the portion of DNA unmasked, but not to the extent of unmasking apparent in isolated DNA. Recombination of histones with such "dehistoned" chromatin resulted in restoration of the same degree of template restriction as was found in chromatin. Recombination of histones in twofold excess with DNA gave an almost completely masked template inactive in RNA synthesis. Combination of acidic chromosomal protein with DNA caused masking of the DNA template to the same extent as was apparent in dehistoned

chromatin. The technique of DNA-RNA hybridization between RNA synthesized *in vitro* on an appropriate template of calf thymus origin and homologous DNA was used throughout this study. Paul and Gilmour (1969) have also carried out DNA-RNA hybridization competition studies between RNAs synthesized *in vitro* from chromatin, "reconstituted" chromatin made by recombining DNA, histone and acidic chromosomal protein and natural RNA. Identical portions of DNA appeared to be involved in all three cases. These results show that much of the masking of DNA in chromatin is due to histones, but that their effect is exerted non-specifically. The great similarity of histones from different sources is in keeping with their having such a non-specific effect. Acidic chromosomal protein appears to have the effect of specifically activating certain portions of DNA molecules for transcription.

Bonner and his colleagues (Bonner *et al.*, 1968; Bekhor, Kung and Bonner, 1969) have come to similar conclusions from their studies on pea chromatin. However they have not studied the separate effects of removal and addition of histone and acidic chromosomal protein. They have found that chromosomal RNA is essential for specific reconstition of dissociated chromatin. Their

results have also been obtained by use of the technique of DNA-RNA hybridization. Huang and Huang (1969) have carried out studies similar to those of Bonner and his colleagues on chicken embryo chromatin. They have reached similar conclusions.

There is therefore good evidence from cytological studies and from DNA-RNA hybridization studies that many of the genes of eucaryotic cells are permanently masked. Specific genes are unmasked in specific tissues and the pattern of unmasking is preserved in isolated chromatin. Chromosomal polyanions appear to be essential for maintenance of this pattern.

Attempts have been made to use the technique of DNA-RNA hybridization and DNA-RNA hybridization competition to determine differences in masking of DNA in normal and tumour cells. Paul and Gilmour found that, within the limits of experimental error, they could not distinguish between RNAs synthesized <u>in vitro</u> from chromatin of normal baby hamster kidney cells and from chromatin of similar cells made tumorous by viral transformation (Paul, 1967). Neiman and Henry (1969) carried out DNA-RNA hybridization competition experiments between RNAs of normal human lymphocytes and of lymphocytes of patients suffering from chronic lymphocytic leukaemia and DNA of

normal cells. The results of this study indicated that the RNA of tumour cells contained molecules not or much more poorly represented in the RNA of normal cells. This suggests that genes unmasked in tumour cells may be masked in normal cells.

## 6. The Enzymatic Synthesis of RNA.

### DNA-dependent RNA Polymerase.

In 1955 Grunberg-Manago purified the enzyme polynucleotide phosphorylase from *Azotobacter vinelandii*. This enzyme catalyses the synthesis of polyribonucleotides from ribonucleoside diphosphates:

$$nXDP \rightleftharpoons (XMP)_n + nP_i.$$

It has been found to be widely distributed among aerobic and anaerobic bacteria. However, it catalyses the incorporation of ribonucleotide residues into polyribonucleotide in a relatively random manner. Its true function is still unknown and it has been concluded that it is not responsible for the synthesis of complementary RNA (Grunberg-Manago, 1963).

Some years later evidence for the existence of a polyribonucleotide-synthesizing enzyme utilising the four ribonucleoside triphosphates as substrates appeared

(Weiss, 1960; Weiss and Nakamoto, 1961; Ochoa, Burma, Krüger, and Weill, 1961). The activity of this enzyme has been observed in extracts from bacterial plant and animal cells. It has been isolated in soluble form from bacterial and animal cells (Mertelsmann and Matthaei, 1968; Liao, Sagher and Fang, 1968). The bacteria from which it is most commonly isolated are Escherichia coli (Chamberlin and Berg, 1962) and Micrococcus lutous (Nakamoto, Fox and Weiss, 1964). Its activity is largely dependent on the presence of a DNA template and it is described as nucleoside triphosphate: RNA nucleotidyl transferase (E.C. 2.7.7.6.) or simply RNA polymerase in accordance with its catalysing the transcriptive reaction:

$$\left.\begin{array}{l} n_1\text{ATP} \\ + \\ n_2\text{CTP} \\ + \\ n_3\text{GTP} \\ + \\ n_4\text{UTP} \end{array}\right\} + \text{DNA} \rightleftharpoons \left\{\begin{array}{l} \text{AMP}n_1 \\ \\ \text{CMP}n_2 \\ \\ \text{GMP}n_3 \\ \\ \text{UMP}n_4 \end{array}\right\} + \text{DNA} + (n_1 + n_2 + n_3 + n_4)\text{PP}_i.$$

Details of this reaction have recently been reviewed by Geiduschek and Haselkorn (1969).

Synthesis of RNA in vitro.

It is possible to synthesize useful quantities of RNA in vitro using the isolated enzyme in the presence of

suitable co-factors and a template of DNA, either free or complexed (Donner *et al.*, 1968; Georgiev, Ananieva, and Roslov, 1966; Paul and Gilmour, 1968.) That complementary RNA is thus transcribed is most clearly shown by hybridization of the RNA to the DNA which served as template. Additional evidence for this comes from the studies which first suggested complementarity of such synthetic RNA to DNA template (Furth, Hurwitz and Goldmann, 1961a). Also, when synthetic polydeoxyribonucleotides are used as templates, only the ribonucleotide residues which would base-pair with those of the template are incorporated into polyribonucleotide (Furth, Hurwitz and Goldmann, 1961b). In nearest-neighbour sequence analyses it is found that the relative frequency of occurrence of each of the sixteen possible nearest-neighbour pairs in the RNA product closely corresponds with that of the DNA template (Hurwitz, Furth, Anders and Evans, 1962). There is no evidence that the soluble RNA polymerase enzymes of *Escherichia coli* (Chamberlin and Berg, 1962) and *Micrococcus luteus* (Nakamoto, Fox and Weiss, 1964) catalyse different reactions.

Stages in Transcription.

The synthesis of RNA on a DNA template by RNA polymerase can be divided into four separate stages

(Chambon, 1968). These are, consecutively, binding between enzyme and DNA template, initiation of polymerization, propagation of polymerization along the DNA template and termination of polymerization. The last stage is presumed to involve liberation of newly-synthesized RNA and enzyme under suitable conditions.

Binding of Enzyme to DNA.

Several methods have been used to study the binding of enzyme to DNA. The substrate and enzyme saturation studies of Bremer, Konrad and Bruner, who used bacteriophage $T_4$ DNA and Escherichia coli RNA polymerase, have shown there are several hundred starting sites for RNA synthesis on the $T_4$ DNA molecule (Bremer, Konrad and Bruner, 1966). The technique of zonal centrifugation has been used by Richardson (1966) and others (Bremer and Konrad, 1964) to separate the enzyme-DNA complex from RNA-synthesizing mixture including, in the case of Richardson's work, Escherichia coli RNA polymerase and DNA from bacteriophages $T_7$ and F1. As a corollary to Richardson's work, electron micrographs showing T7 DNA molecules bearing Escherichia coli RNA polymerase molecules have been prepared by Slayter and Hall (1966). The selective retention of DNA-RNA polymerases complexes on Millipore membrane filters has been made use of

(Jones and Berg, 1966)

It is apparent, particularly from the work of Richardson (1966) and of Slayter and Hall (1966) that DNA molecules have a limited capacity for binding of those of RNA polymerase, that enzyme molecules are spaced out along those of DNA at wider intervals than can be due to juxtaposing, and that the secondary structure of DNA is important in influencing DNA-enzyme binding. That intact circular DNA of bacteriophage ØX 174 can act as a template for RNA synthesis catalysed by Escherichia coli RNA polymerase shows that free ends of DNA molecules are unnecessary for the commencement of transcription or other stages of it (Hayashi, Hayashi and Spiegelman, 1964).

Initial binding between enzyme and DNA is a reversible process (Walter, Zillig, Palm and Fuchs, 1967) but its equilibrium strongly favours the formation of the complex and this is a very rapid process (Richardson, 1966).

Initiation of RNA Synthesis.

Zillig and his colleagues (Walter, Zillig, Palm and Fuchs, 1967) have suggested that enzyme-DNA binding is followed by a reversible "melting reaction" in which an activated complex is formed:

Enzyme + DNA $\rightleftharpoons$ Enzyme-DNA ..... binding

Enzyme - DNA $\rightleftharpoons$ Enzyme - DNA* ..... activation.

The enzyme may thus affect a localized separation of the DNA double helix at the binding site. Many studies indicate that the commencement of transcription of native DNA is accompanied by a change in the nature of enzyme-DNA binding (Chamboh, 1968).

Recently Travers and Burgess (1969) have shown that RNA polymerase can be reversibly dissociated into two components. These they describe as "minimal enzyme" and the $\sigma$ factor. They have obtained evidence to suggest the following mechanism of involvement of the $\sigma$ factor in initiation of transcription.

The $\sigma$ factor initially forms a complex with the minimal enzyme. The equilibrium of this association probably highly favours complex formation in most conditions. The complex is able to bind to DNA and to cause initiation of transcription. During or after initiation, the $\sigma$ factor is released from the complex and becomes available for re-use by another minimal enzyme molecule. The $\sigma$ factor may be responsible for the recognition of specific initiation sites on the DNA template or it may be necessary for separation of DNA strands immediately before initiation.

Propagation and Termination of Transcription.

In vivo and in vitro, addition of ribonucleotide residues occurs at the 3' hydroxyl ends of growing RNA molecules (Maitra, Nakata and Hurwitz, 1967). By using γ-labelled ribonucleoside triphosphates, the number of RNA chains present at any time during transcription, their length and the nature of the ribonucleotide residues at the start of the growing RNA molecules can be estimated. On a native DNA template in vitro, these are generally purine ribonucleotide residues. Uridylate is very often the second ribonucleotide residue incorporated. It may very well be significant that the initiator codons for protein synthesis are AUG and GUG (Salas, Hille, Last, Wahba and Ochoa, 1967). Although synthetic and natural RNAs are, evidently, largely complementary to their DNA templates, there is evidence that forces acting between stacked bases can be important in determining the next ribonucleotide residue to be incorporated into growing RNA molecules (Slapikoff and Berg, 1967).

In another study using bacteriophage $T_4$ DNA and Escherichia coli RNA polymerase, Bremer and Konrad (1964) found that the number of nascent RNA molecules was constant throughout the course of in vitro RNA synthesis under conditions of low ionic strength. It was concluded

that each molecule of RNA polymerase catalyses the synthesis of only one RNA chain owing to the stability of the DNA-RNA-enzyme complex. However, Zillig and his colleagues (Fuchs, Millette, Zillig and Walter, 1967) have shown that RNA synthesis continues longer under conditions of high ionic strength than under those of low ionic strength and that RNA synthesis which has ceased under conditions of low ionic strength can be re-started by increasing the ionic strength of the in vitro RNA synthesizing mixture. They also suggest a process in which a newly-synthesized RNA molecule displaces the "non-codogenic" strand of the DNA template double-stranded molecule and forms a hybrid with a portion of the "codogenic" strand. The "non-codogenic" strand of the DNA template then displaces the strand of newly-synthesized RNA. Such a "strand exchange" reaction might well be the process accelerated by increasing ionic strength and would result in no nett change in DNA template and release of newly-synthesized RNA in agreement with observations. Bacteriophage $T_4$ DNA and RNA polymerase from Escherichia coli were used in these studies.

In studies in which poly dG:dC acted as template for the synthesis of poly rG from GTP catalysed by

RNA polymerase, Chamberlin showed that each RNA polymerase molecule can initiate the synthesis of more than one RNA molecule and that the enzyme is released from the template. *In vitro* DNA-dependent RNA synthesis has been observed to follow Michaelis-Menton kinetics with respect to DNA concentration (Chamberlin and Berg, 1962; Fox and Weiss, 1964). There is thus evidence that, under certain conditions, DNA-dependent RNA synthesis catalysed by RNA polymerase is a reversible process.

Symmetry and Asymmetry of Transcription *in vitro*.

Early studies suggested that both strands of double-stranded DNA molecules are transcribed *in vitro*. However, Hayashi, Hayashi and Spiegelman (1964) found that when intact DNA was used as template, only one strand was transcribed. In their studies RNA synthesized using *Escherichia coli* RNA polymerase and a template of intact, circular, double-stranded DNA from the replicative form of bacteriophage ØX 174 could be hybridized only to the strand of DNA peculiar to the replicative form. When damaged DNA was used as template, the RNA synthesized in this system could be hybridized to both strands of the native DNA of the replicative form. Also, RNA synthesized using *Micrococcus luteus* RNA polymerase and

bacteriophage α DNA as template could only be hybridized to the heavy strand of the DNA template (Geiduschek, Tocchini-Valentini and Sarnat 1964). It is currently thought that RNA is synthesized *in vitro* by asymmetric transcription if the DNA template is intact, but that symmetric transcription occurs from double-stranded DNA which has been damaged in the course of its isolation.

Transcription of DNA *in vivo*.

The complementary nature of RNA and the presence of DNA-dependent RNA polymerase enzymes in many different types of cell are taken to indicate that, *in vivo*, RNA is largely synthesized by the action of such enzymes acting on a DNA template, although the DNA is complexed.

There is evidence that transcription *in vivo* is similar to that observed *in vitro*. Paul and Gilmour (1966b) compared natural calf thymus nuclear and rabbit thymus RNAs with RNAs synthesized *in vitro* using chromatin prepared from the respective tissues as templates and RNA polymerase from *Micrococcus luteus* by competitive hybridisation. They found a high degree of identity between corresponding natural and synthetic RNAs. Similarly, Melli and Bishop (1969) found that RNA synthesized *in vitro* using *Micrococcus luteus* RNA

polymerase and a rat liver DNA template and rat liver nuclear RNA were completely cross-competable or almost so. Bonner et al. (1968) claim that RNA synthesized in vitro using pea cotyledon chromatin as template and Escherichia coli RNA polymerase can act as messenger in an in vitro protein-synthesizing mixture to cause the synthesis of pea-seed globulin. They claim that this shows the identity of natural RNAs and these synthesized in vitro using chromatin templates.

There is evidence that transcription occurs asymmetrically in vivo. RNA isolated from Escherichia coli infected by bacteriophage ØX 174 can be hybridized to DNA isolated from the replicative form of the virus, but there is virtually no hybridization with single-stranded DNA from the vegetative form of the virus (Hayashi, Hayashi and Spiegelman, 1963). The base composition of the RNA is found to be complementary to that of the strand of DNA peculiar to the replicative form. RNA isolated from Bacillus megatherium and Bacillus subtilis infected by bacteriophages α and SP8 (Tocchini-Valentini et al., 1963; Marmur and Greenspan, 1963) respectively can be made to form ribonuclease-resistant hybrids with only the heavy strands of the viral DNAs. The studies of Cohen and Hurwitz (1967)

and of Skalka, Butler and Echols (1967) indicate that in Escherichia coli infected by bacteriophage $\lambda$, RNA is complementary to portions of each strand of the viral double-stranded DNA, though not where the strands are base-paired to one another. There is also evidence from genetic studies that only one strand of double-stranded pneumococcal transforming DNA is transcribed (Wood and Berg, 1963). That the strands of DNA of the replicative form of bacteriophage $\phi$X 174 (Hayashi, Hayashi and Spiegelman, 1964) and of bacteriophage a DNA (Geiduschek, Tocchini-Valentini and Sarmat, 1964) which are transcribed in vitro are also the strands transcribed in vivo (Hayashi, Hayashi and Spiegelman, 1963; Tocchini-Valentini et al., 1963) is further good evidence that in vitro and in vivo transcriptive processes are very similar.

Such studies on the asymmetry of transcription have been possible because of the significantly different densities of the strands of the viral DNAs. At present it is thought that DNA from eucaryotic and procaryotic cells is transcribed asymmetrically in vivo and probably also in vitro. The DNA strand which is not transcribed is almost certainly not inactive in the process. Native DNA is a more efficient template for in vitro RNA

synthesis (Chamberlin and Berg, 1962; Fox and Weiss, 1964) than is denatured DNA. It has been shown by Wood and Berg (1963) that RNA synthesized by Escherichia coli RNA polymerase on viral DNA templates can stimulate significant in vitro polypeptide synthesis only when a double-stranded DNA template is used. The reasonable hypothesis of Fuchs, Millette, Zillig and Walter (1967) requires the DNA template to be double-stranded.

A possible difference between in vivo and in vitro transcriptive processes is the possible involvement of ribosomes in the former. In vivo these can become attached to messenger RNA molecules before their completion (Schaeter and McQuillan, 1966) and addition of ribosomes to an in vitro RNA-synthesizing mixture, in which an Escherichia coli deoxyribonucleoprotein complex provided both RNA polymerase activity and DNA template, stimulated RNA synthesis and release of newly-synthesized RNA in the form of an RNA-ribosome complex (Shin and Moldave, 1966).

## 7. The Technique of DNA-RNA Hybridization.

### Earlier Developments of Procedures of DNA-RNA Hybridization.

A group of RNA molecules can only be fully characterized once the complete nucleotide sequence of each of them has been established. Most RNA molecules contain at least 70 and often hundreds of nucleotide residues of only four main types. It is therefore not surprising that complete nucleotide sequences of only a few types of RNA molecules have been established.

Use of the technique of DNA-RNA molecular hybridization permits characterization of RNA molecules on the basis of their complementarity to DNA. Hall and Spiegelman (1961) reasoned that the closely analogous nucleotide base ratios found on analysis of DNA of bacteriophage $T_2$ and RNA isolated from cells of Escherichia coli infected by it might extend to a detailed correspondence of their nucleotide sequences. They were able to demonstrate formation of hybrids between these DNA and RNA species. Formation of hybrids was induced by slow cooling of denatured DNA and RNA in saline sodium citrate solution. On ultracentrifugal sedimentation of these solutions through caesium chloride solution, some

RNA was evidently associated with DNA. This was not seen when heterologous DNA was used. It was concluded that this RNA was part of a DNA-RNA hybrid.

Yankofsky and Spiegelman (1962a) introduced treating fractions of the density gradient with ribonuclease into this procedure. Some material of the DNA-RNA complex was resistant to degradation by this enzyme. Their evidence showed that the ribonuclease-resistant material was true DNA-RNA hybrid from which unpaired RNA had been eliminated. Thus the sensitivity of the technique was considerably increased.

Further developments in the technique have been largely associated with trapping hybrids in solid matrices and forming hybrids between DNA immobilised in a solid matrix and RNA in solution. Such procedures permit very much more rapid detection and measurement of the extent of DNA-RNA hybridization. Hayashi, Hayashi and Spiegelman (1965) and Attardi, Huang and Kabat (1965) developed procedures for isolation of DNA-RNA hybrids formed in solution on columns of methylated albumin coated on kieselguhr (MAK) and dextran molecular sieve material. Dautz and Hall (1962) and Bolton and McCarthy (1962) developed techniques employing immobilised DNA. Denatured DNA was

immobilised on columns of acetylated phospho-cellulose or of cellulose acetate or agar, respectively. RNA dissolved in saline sodium citrate solution was allowed to move down through these types of column. Thereafter free RNA was eluted in hot saline sodium citrate solution. Further elution in more dilute hot saline sodium citrate solution permitted separation of the RNA involved in formation of DNA-RNA hybrids.

Procedures suitable for detailed Kinetic Studies.

All of the procedures described are unsuitable for detailed kinetic studies on DNA-RNA hybridization. Procedures involving the use of cellulose and related compounds have permitted such studies. Britten (1963) demonstrated DNA-RNA hybridization between RNA in solution and denatured DNA immobilised in cellulose gels or on cotton threads by ultraviolet irradiation.

Nygaard and Hall (1963) introduced the use of nitrocellulose membrane filters into DNA-RNA hybridization studies. They found that these retained denatured DNA and DNA-RNA hybrid applied in salt solution of moderate concentration, but did not retain native DNA nor free RNA. They later extended their studies to a detailed investigation of the kinetics of the DNA-RNA hybridization reaction (Nygaard and Hall, 1964). Rate of

formation of hybrids between DNA and RNA of viral origins was found to be proportional to both DNA and RNA concentration over a wide range of DNA:RNA ratios. Second order kinetics were therefore indicated. The second order rate constants for DNA renaturation and DNA-RNA hybridization and their activation energies were found to be approximately equal under the conditions employed for DNA-RNA hybridization. Nygaard and Hall (1964) concluded that the initial rate of DNA-RNA hybridization could be measured without interference by DNA renaturation but that, on prolonged incubation of DNA-RNA hybridization incubation mixtures, it appeared that DNA renaturation led to reduction in the rate of DNA-RNA hybridization and gradual disappearance of DNA-RNA hybrid.

Increasing salt concentrations in the range of 0.2 M to 1.5 M with respect to potassium chloride concentrations increased the optimum temperature for DNA-RNA hybrid formation and the rate of hybrid formation at the optimum temperature. 0.5 M potassium chloride and 67°C, the optimum temperature in this salt concentration, were chosen by Nygaard and Hall (1964) as their standard conditions for incubation of DNA-RNA hybridization mixtures. Thus Nygaard and Hall (1963,

1964) described a quantitative, inexpensive and rapid procedure for routine formation and detection of DNA-RNA hybrids.

In the procedure described by Gillespie and Spiegelman (1965), DNA-RNA hybridization is carried out between RNA in saline sodium citrate solution and denatured DNA immobilised on nitrocellulose membrane filters. Thus the finding of Nygaard and Hall (1963) on retention of denatured DNA by nitrocellulose is utilised. Filters are treated with ribonuclease after incubation for hybrid formation.

Gillespie and Spiegelman made certain that DNA was not lost from filters during incubation for hybridization (in saline sodium citrate solution at 66°C) and subsequent routine treatments. Physical removal of DNA from incubation mixtures and subsequent washing of and treatment of the filters with ribonuclease was found to be highly effective in removing RNA not hybridized to DNA. It was found that denatured DNA quantitatively retained by nitrocellulose membrane filters during incubation for DNA-RNA hybridization was as freely available for hybridization with homologous rRNA as when hybridization was carried out by the procedure of Nygaard and Hall or by that of Yankofsky and Spiegelman (1963). The last

procedure involves separation of RNA hybridized to denatured DNA by ultracentrifugal sedimentation.

Gillespie and Spiegelman (1965) have obtained data which illustrate the advantages of kinetics of DNA-RNA hybridization in their procedure over those of the procedure of Nygaard and Hall (1964). In the former plateaux of fractions of DNA involved in DNA-RNA hybridization with respect to time were recorded; in the latter this fraction increased more rapidly but then began to become less as a consequence of competing DNA renaturation. Using the procedure of Gillespie and Spiegelman, it is possible to carry out hybridization between one sample of RNA and several samples of DNA immobilised on separate filters within one vial. It is also possible to include blank filters in the same RNA solution as that in which DNA-bearing filters are present. Finally, filters may be transferred from one RNA solution to another and, if desired, the quantity of RNA hybridized to DNA on such filters can be estimated during the course of such transfers. Thus the procedure of Gillespie and Spiegelman has distinct advantages over those of Nygaard and Hall (1963, 1964) and others. Accuracy and certainty of measurements of the extent of DNA-RNA hybridization are greater when it

is used. In addition, it permits useful practical manipulations which cannot be applied in the other procedures.

Some useful modifications of the procedure of Gillespie and Spiegelman (1965) deserve mention here. Paul and Gilmour (1968) described a procedure for effectively carrying out hybridization between RNA in volumes as small as 50 µl. and denatured DNA immobilised on small nitrocellulose membrane filter discs. The small volumes were maintained in droplet form on a flat base by overlaying them with liquid paraffin. Fujinaga and Green (1968) included sodium dodecyl sulphate in hybridization incubation mixtures to reduce background levels of binding of RNA to nitrocellulose membrane filters and to inhibit nuclease action. The extent of DNA-RNA hybridization was unchanged. Bonner, Kung and Bekhor (1967) have described a procedure in which inclusion of formamide in hybridization incubation mixtures is claimed to reduce the temperature at which they should be incubated. Curves of saturation of denatured pea DNA by RNA synthesized <u>in vitro</u> from pea cotyledon chromatin were constructed from data obtained on incubation under normal conditions at elevated temperature and at room temperature with formamide present.

They were almost identical. Bonner, Kung and Belchor have pointed out that incubation in the presence of formamide at lower temperatures has valuable advantages. They claim that there is very little breakdown of RNA and that there is increased retention of denatured DNA by nitrocellulose membrane filters and decreased non-specific adsorption of RNA by them.

Use of the technique of DNA-RNA hybridization between materials of eucaryotic origin has led to interesting hypotheses on the nature of masking of DNA in chromatin (Paul and Gilmour, 1966a,b; 1968; 1969; Georgiev, Anandeva and Kozlov, 1966; Bonner *et al.*, 1968; Belchor, Kung and Bonner, 1969; Huang and Huang, 1969; Neiman and Henry, 1969). To prove, refute or extend these hypotheses, as full an understanding as possible of the nature of the DNA-RNA hybridization reaction between materials of eucaryotic origin is required.

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# MATERIALS.

## Inorganic Reagents.

Unless otherwise stated, "Analar" grade reagents supplied by British Drug Houses Chemicals Ltd., Poole, England (B.D.H.) were used throughout. All aqueous solutions were made up using deionised, distilled water.

## Organic Chemicals.

Sources are detailed where these are first mentioned in the text.

## Tritiated Compounds.

Tritium was the only radioactive isotope used. All tritiated compounds were supplied by The Radiochemical Centre, Amersham, England.

## Enzymes.

Electrophoretically-purified, ribonuclease-free Deoxyribonuclease I (E.C. 3.1.4.5) was purchased from the Worthington Biochemical Corporation, New Jersey and was stored in powder form at 4°C as recommended by the suppliers. Solutions of 1 mg./ml. of 0.02 M glycine were stored at -15°C.

Crystalline bovine pancreatic ribonuclease A (E.C. 2.7.7.16) was purchased from the Sigma Chemical Company Ltd., London and was stored in powder form at

4°C. Stock solution of 2 mg./ml. in water (pH = 7) were heated at a temperature in the range of 90°C to 95°C on being made up in order to destroy any deoxyribonuclease activity. They were stored at 4°C.

Pronase was purchased in powder form from Calbiochem, Los Angeles and was stored at 4°C. Solutions of 1 mg./ml. in 0.005 M calcium chloride were incubated for 3 hr. at 37°C prior to use or storage at -15°C. Calcium chloride was included to ensure minimal loss of activity and, for the same purpose, solutions were used within 24 hr. of being made up ("Pronase").

Lysozyme (E.C. 3.2.1.17) (Grade 1) was purchased in crystalline form from Sigma Chemical Co.Ltd., London.

## Macaloid.

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

## METHODS AND RESULTS.

### 1. Establishment of Quench Correction Curves for Liquid Scintillation Counting of Tritium in Aqueous Solution and in Nuclear Chicago Solubilizer.

Dioxane-based scintillator (D.B.S.) was used in measuring the radioactivity of aqueous solutions. It was made up by dissolving 100 g. of naphthalene (B.D.H. microanalytical reagent), 7.0 g of 2,5-diphenyl oxazole (P.P.O.) and 0.3 g of 1,4-di(2-(5-phonyloxazolyl)benzene) (P.O.P.O.P.) to 1 litre using B.D.H. "Analar" grade dioxane. P.P.O. and P.O.P.O.P. were supplied by Nuclear Enterprises (G.B.) Ltd., Edinburgh, in scintillation grade.

The radioactivity of samples dissolved in Nuclear Chicago Solubilizer (N.C.S., Nuclear Chicago, Des Plaines, Illinois) was measured using toluene-based scintillator (T.B.S.). It was made up by dissolving 5.0 g of P.P.O. and 0.3 g of P.O.P.O.P. to 1 litre using B.D.H. "Analar" grade toluene.

n-Hexadecane-1,2-T was used as standard tritiated material and had been calibrated against standard material from the U.S. Board of Standards ("Radioactive Products", 1967/68). Its activity was corrected for radioactive decay during its storage at -70°C in a frozen condition.

A series of glass scintillation vials containing 5.0 ml. of D.B.S. including a known quantity of tritiated

hexadecane and up to 1.00 ml. of water was prepared. To obtain background count rates another series of vials containing 5.0 ml. of D.B.S. and up to 1.00 ml. of water was also prepared. All those vials were allowed to stand for 1 hr. at 2°C in a Model 6862 Nuclear Chicago Mark I Liquid Scintillation Counter, in which all count rates were determined, before count rates and external standard ratios were recorded (Instruction Manual, Mark I Liquid Scintillation Computer, 1967).

The contents of vials containing less than 0.10 ml. of water were observed to freeze and figures relating to these were excluded from standard curves relating counting efficiency to external standard ratio. Background count rates did not alter significantly with increasing amounts of water in the vials and the average background count rate was subtracted from each count rate. Standard curves were constructed every time that adjustments were made to the scintillation counter. The background count rate was always in the region of 30 counts per minute. Counting efficiencies were in the range of 12% to 30%.

By a similar procedure standard curves relating counting efficiency in 10 ml. volumes of T.B.S. with

up to 0.40 ml. volumes of N.C.S. added to external standard ratio were constructed. The background count rate was in the region of 25 counts per minute and, as in the addition of water to D.B.S., was unaffected by addition of N.C.S.. The contents of these vials did not freeze at 2°C. Counting efficiencies were in the region of 40%.

## 2. Studies on Chromatin.

### 2.1 Preparation of Chromatin.

A method described by Paul and Gilmour (1968, 1966a) and developed from previous studies (Slimming, 1966) was employed. Chromatin was prepared from all the tissues mentioned to which it was applied. These were thymus of two to three day-old calves, bone marrow, kidney, liver, spleen and thymus of six-month-old rabbits and liver, spleen and thymus of a four-week-old mouse.

The tissue was rapidly removed from the animal or carefully thawed. It was immediately finely chopped and homogenised at full speed for 1 minute using a Tri-R homogeniser in 10 volumes of 0,025 M citric acid at 0°C. The homogenate was filtered through a double layer of muslin. A drop of the

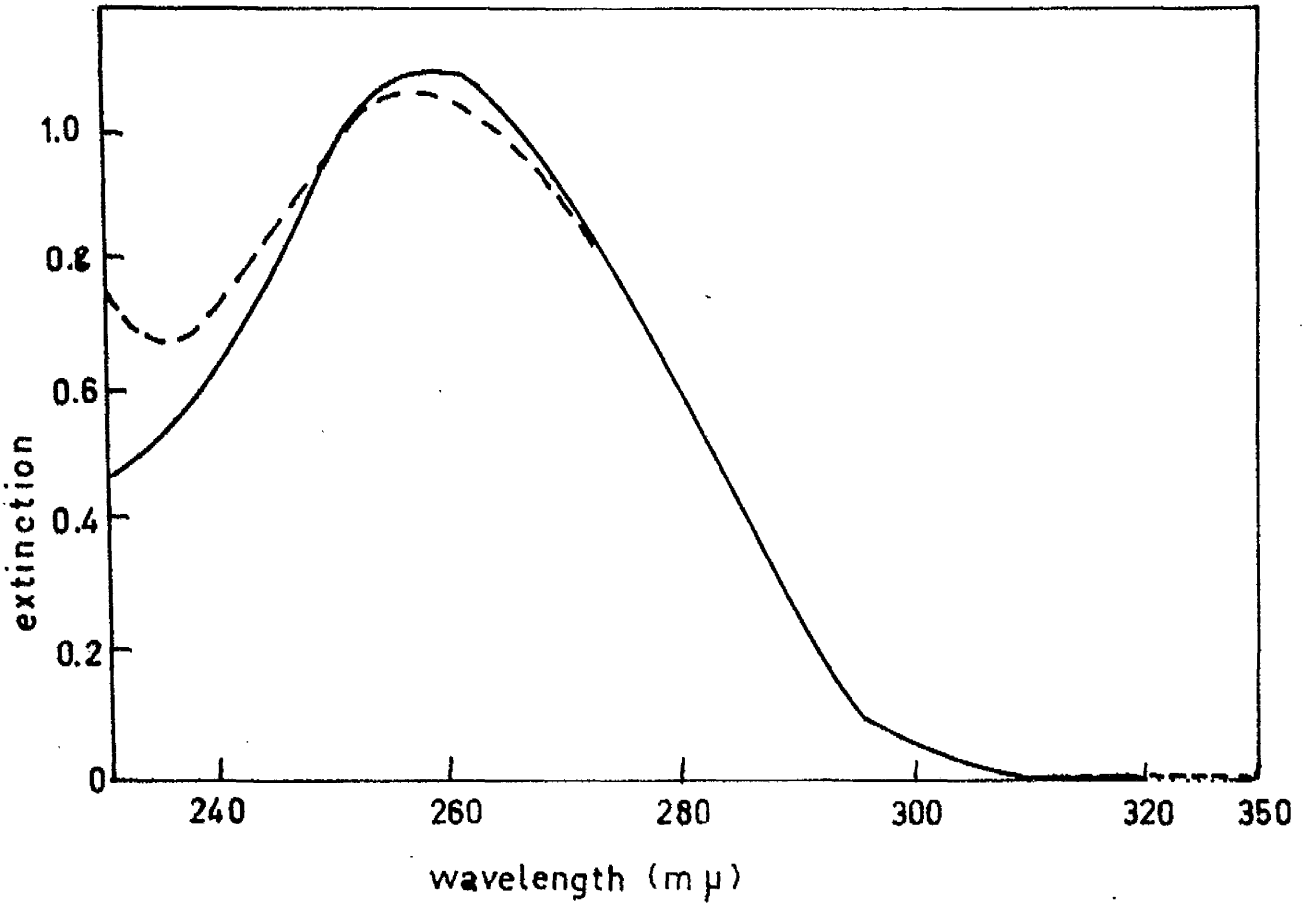
filtered homogenate was stained using a 1% solution of brilliant cresol blue in S.S.C., S.S.C. being 0.15 M sodium chloride, 0.015 M tri-sodium citrate. It was examined microscopically at a magnification of 400x to ensure that all cells had been ruptured. If necessary, the suspension was re-homogenised until this was achieved. The suspension was then centrifuged at 1,500 *g* for 20 minutes to obtain a crude preparation of nuclei. This and all subsequent steps were performed at 0°C.

The crude preparation of nuclei was washed twice in the same volume of 0.025 M citric acid, sedimentation being at 1,500 x *g* for 20 minutes in each case. The cleaned nuclei were then washed twice in the same volume of 0.15 M sodium chloride, 0.1 M tris/hydrochloric acid buffer solution, pH = 7.5, again sedimenting at 1,500 *g* for 20 minutes. The final pellet was resuspended in the same volume of deionised distilled water and was sedimented at 2,000 *g* for 20 minutes to form a gel. In some cases it was necessary to carry out a second water wash to obtain such a gel.

Gels thus formed whose absorption spectra over the wavelength range of 230 mμ to 260 mμ showed approximate maxima, minima and shapes typical of

spectra of nucleic acid solutions (Figure 1), were taken to be chromatin. Chromatin preparations were stored at 4°C for periods of up to 2 weeks.

Figure 1. Absorption Spectra of Calf Thymus DNA —— and Calf Thymus Chromatin ---- Solutions.



## 2.2. Quantitative Estimation of RNA and DNA in Chromatin.

Separation of RNA and DNA. 1 ml. volumes of chromatin or chromatin diluted with deionised distilled water containing approximately 1 mg. of combined nucleic acids were mixed with 1 ml. volumes of 0.6 M potassium hydroxide solution. The quantity of nucleic acids was roughly ostimated on the basis of both RNA and DNA having a specific extraction coefficient of 0.022 absorbancy units ml. $cm^{-1}$ $\mu g^{-1}$ at a wavelength of 260 mμ. The pH of the mixtures was checked to ensure it exceeded 13, which it did in all cases. To degrade RNA, but not DNA, to oligonucleotides soluble in ice-cold perchloric acid (P.C.A.) these mixtures were incubated with intermittent agitation from 2 hours to 48 hours until a clear solution was obtained. They were then placed in an ice bath and 1.0 ml. volumes of ice-cold 1.5 N P.C.A. were mixed with each of them. These mixtures were allowed to stand in the ice bath for 10 minutes and were then centrifuged at 1,500 g for 10 minutes at 0°c. Supernates were assumed to contain the RNA of chromatin as ribonucleotides and precipitates DNA in polymeric form.

Quantitative Estimation of RNA. Where possible the orcinol procedure for the estimation of ribose described by Mejbaum (1939) and modified by Slater (1956) was employed. 3 ml. volumes of a 0.1% w/v solution of ferric chloride hexahydrate in 10 M hydrochloric acid were added to the supernates and to 3 ml. volumes of standard solutions containing 0 to 30 µg. quantities of ribose (L.Light and Co.Ltd., Colnbrook, England). 0.3 ml. volumes of a 10% w/V solution of orcinol (3,5 dihydroxytoluene, B.D.H. reagent) in absolute redistilled ethanol were added to the solution. After thorough mixing, they were incubated in a boiling water bath for 45 minutes. They were then quickly cooled in cold water and their extinctions were recorded at a wavelength of 670 mµ. Direct proportionality of extinction at 670 mµ and quantity of ribose was observed. 1 µg. of ribose was taken to be equivalent to 4.56 µg. of RNA in chromatin.

Alternatively, the RNA content of chromatin was estimated by ultraviolet absorbance of tenfold dilutions of oligoribonucleotide solutions obtained as described above (Fleck and Munro, 1962). A solution of this degraded RNA was taken to have a specific extinction coefficient of 0.0285 absorbancy units ml.

$cm^{-1}$ $\mu g^{-1}$ at a wavelength of 260 mμ. The validity of this figure was confirmed by carrying out the above procedure on yeast RNA obtained from B.D.H..

Quantitative Estimation of DNA. The procedure employed was based on that of Burton (1956). It depends on the blue colour produced on reaction of diphenylamine with deoxyribose. "Diphenylamine reagent" was made up on the day of use by addition of 1 ml. of a 1.6% v/v solution of redistilled acetaldehyde (B.D.H.reagent) to 50 ml. of glacial acetic acid (B.D.H. "Analar" grade reagent) into which 0.75 g. of diphenylamine (B.D.H. reagent, "Analar" grade) had been dissolved and 0.75 ml. of 36 M sulphuric acid had been mixed.

The precipitates obtained on centrifugation after mixing P.C.A. into the alkaline digests were resuspended in 2 ml. volumes of 1 M P.C.A. and the suspensions were heated at 70°C for 15 minutes. They were then chilled in an ice bath and centrifuged at 1,500 g for 10 minutes at 0°C. The precipitates then obtained were similarly re-extracted using 1 M P.C.A.. 2 ml. volumes of diphenylamine reagent were added to 1 ml. volumes of the combined 1 M P.C.A. extracts made up to 4 ml. in 1 M P.C.A. and to 4 ml. volumes of 1 M P.C.A. containing 0 to 100 μg. of deoxyribose (B.D.H. reagent). All the

resulting solutions were incubated for 1 hour at 70°C. On cooling their extinctions were recorded at a wavelength of 600 mμ. Standard deoxyribose curves were near-linear and 1 μg. of deoxyribose was taken to be equivalent to 6.2 μg. of DNA.

## 2.3. Assay of Proteins in Chromatin and in other Materials.

Separation of Histones and Acidic Proteins of Chromatin. Histones were taken to be the protein fraction of chromatin soluble in 0.25 M hydrochloric acid at 0°C. Accordingly, in analysing chromatin preparations, quantities of chromatin containing between 30 μg. and 300 μg. of DNA were firstly incubated in 0.2 ml. volumes of 0.25 M hydrochloric acid for 2 hours in an ice bath with frequent agitation. The mixtures were then centrifuged at 1,500 g for 15 minutes at 0°C. The resulting precipitates were washed in further 0.2 ml. volumes of ice-cold 0.25 N hydrochloric acid and the mixtures centrifuged as before. Washed precipitates were taken to contain acidic proteins, RNA and DNA and combined supernatants histones.

The Bromsulphalein Method of Protein Estimation. This method was developed from those described by Nayyar and Glick (1954) and Paul (1965a).

The precipitates were thoroughly mixed into 0.8 ml. volumes of 0.5 M sodium hydroxide. 0.4 ml. volumes of 0.75 M sodium hydroxide were added to each of the histone solutions. Standard solutions containing 0 to 400 µg. quantities of bovine albumin (Fraction V, Armour Pharmaceutical Co., Eastbourne, England) were made up in 0.8 ml. volumes of 0.5 M sodium hydroxide. All these alkaline solutions were incubated for 1 hour at 37°C with intermittent agitation. Complete dissolution was thus achieved in all cases.

1.0 ml. volumes of bromsulphalein reagent (0.02% w/v bromsulphalein (sulphobromophthalein, sodium salt, Koch-Light Labs.Ltd., Colnbrook, England) in 0.4 M hydrochloric acid, 0.2 M citric acid) were added to each assay mixture with thorough mixing. The mixtures were allowed to stand for 15 minutes in an ice bath and were then centrifuged at 1,500 *g* for 20 minutes at 0°C. 0.5 ml. volumes of the resulting supernates were mixed with 3.5 ml. volumes of 0.2 M sodium hydroxide. Extinctions of the resulting solutions were maximal at approximately 580 mµ (Figure 2a) and were recorded at this wavelength. By comparison with the standard curve constructed in every assay (Figure 2b) quantities of histone and acidic proteins in the range of 30 µg. to 400 µg. in chromatin preparations

Figure 2a. Absorption Spectrum of No-Protein Control Solution used in a Typical Bromsulphalein Method Protein Estimation (for 30-400 ug. quantities of Protein).

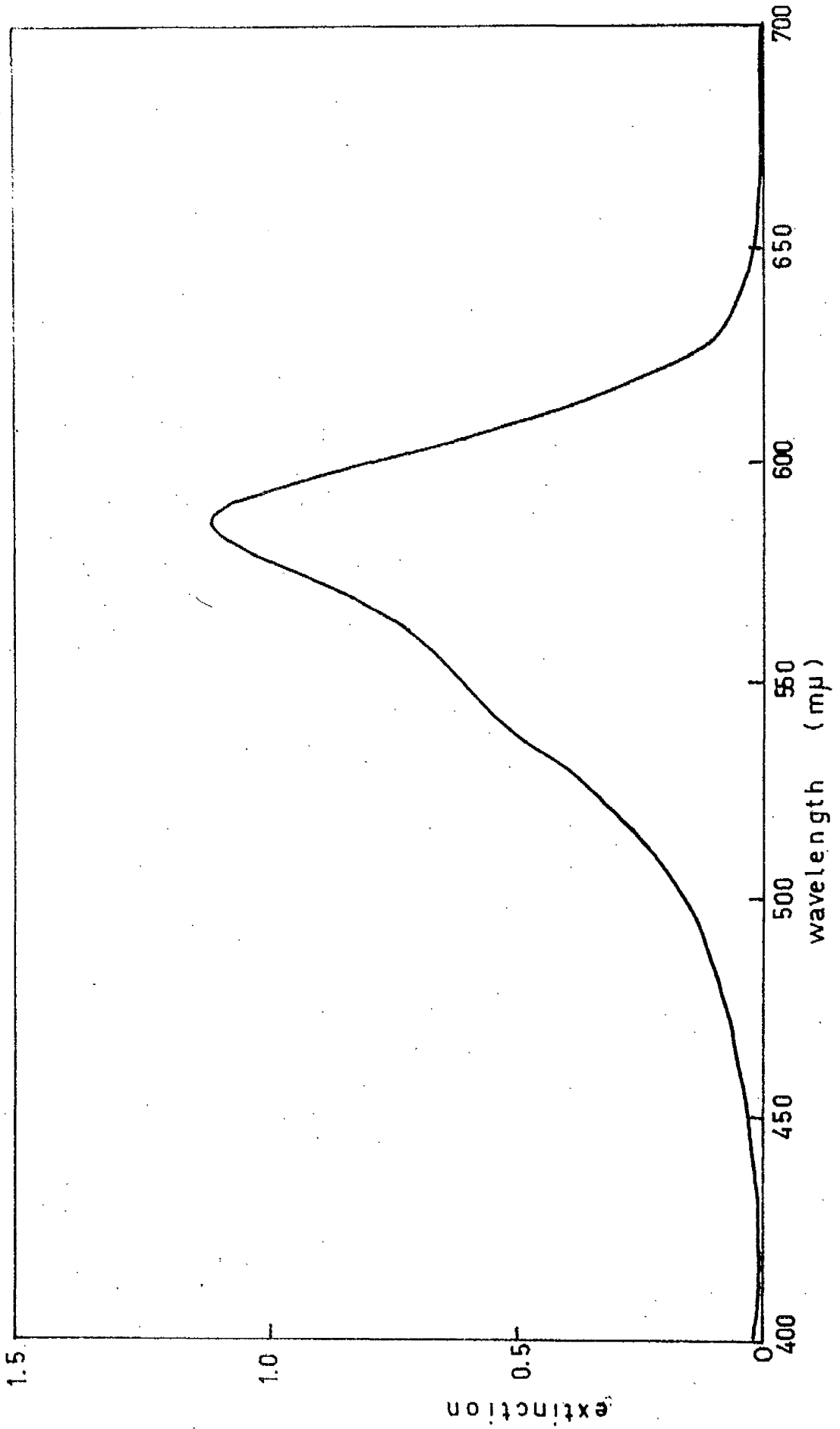
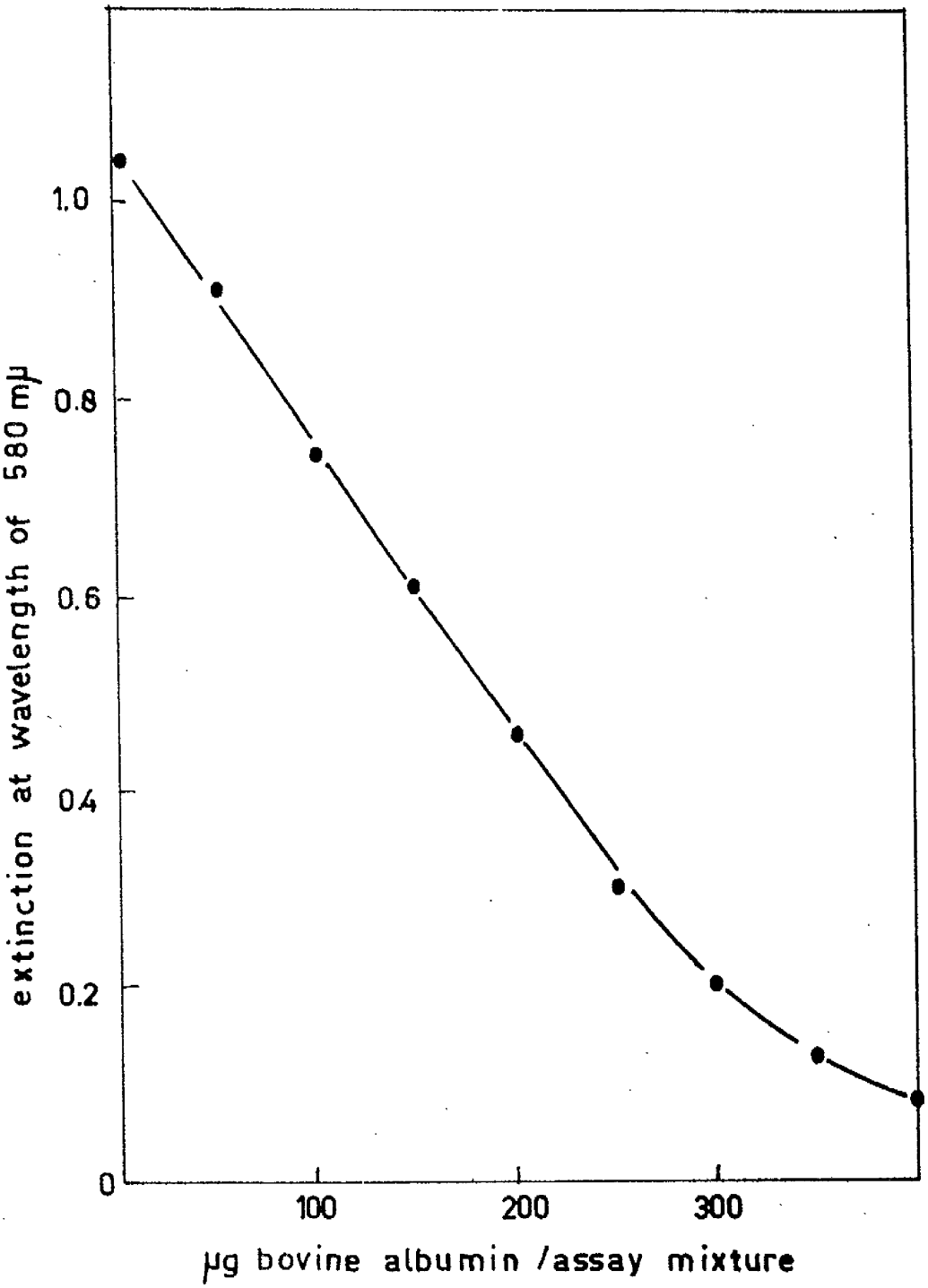


Figure 2b. Typical Standard Curve for the Estimation of 30-400 µg. quantities of Protein by the Bromsulphalein Method.



were estimated. When necessary, the scale of the method was reduced to allow estimation of 10 µg. to 150 µg. quantities of protein and a similar spectrum and standard curve were obtained.

The protein contents of other solutions were estimated by the same procedures as that described for standard solutions.

The protein contents of a typical preparation of calf thymus chromatin and its constituent histone and acidic proteins were estimated by this procedure. The sum of the latter was found to be equal to the protein content of the whole chromatin.

380 µg., 1600 µg. and 0.4 ml. quantities of calf thymus DNA, rabbit reticulocyte polysomal RNA and glycerol (B.D.H. "Analar" grade) respectively were included in typical assays. None of these gave a positive reaction for protein.

Agreement between estimations of the protein contents of calf thymus chromatin using the bromsulphalein method and total nitrogen estimation using Nessler's reagent was observed. As nucleic acids would give a positive reaction in a total nitrogen estimation, appropriate corrections based on nucleic acid estimations were applied to obtain accurate estimations of proteins. The nitrogen contents

of acidic proteins, histones and nucleic acids were taken to be 16%, 18% (Busch and Davis, 1958) and 17% respectively. Results of the comparisons of histone and acidic protein contents of calf thymus chromatin as determined by bromsulphalein and total nitrogen methods are shown in Table 1.

Estimation of Protein Contents of Calf Thymus Chromatin on a Total Nitrogen Basis.

The Nessler reagent was used for this purpose (Paul, 1965a). Solutions of histone and of acidic proteins of calf thymus chromatin were obtained in 0.5 N sodium hydroxide as already described. 1 ml. volumes of 0.1% w/v selenium dioxide (B.D.H. reagent) in 18 N sulphuric acid were mixed with 0.50 ml. and 0.25 ml. volumes of histone and acidic protein solutions respectively. These mixtures were allowed to simmer gently until clear. 25 ml. volumes of water were then added to each of them.

2 ml. volumes of Nessler's reagent (0.4% w/v potassium iodide, 0.4% w/v mercuric iodide, 0.35% w/v acacia (B.D.H. reagent)) were added to 2 ml. volumes of the diluted digests followed by 3 ml. volumes of 2 N sodium hydroxide. The extinctions of the resulting solutions were recorded between 15 and 45 minutes later at a wavelength of 450 mμ. The same procedure was also applied

Table 1.

Comparison of Protein Contents of Calf Thymus Chromatin determined by Bromsulphalein and Total Nitrogen Methods.

| Constituent of Chromatin | Assay Method | Result |
|---|---|---|
| RNA | orcinol procedure | 0.14 mg./ml. |
| DNA | diphenylamine procedure | 2.3 mg./ml. |
| Histone | total nitrogen estimation | 3.0 mg./ml. |
| | bromsulphalein method | 3.2 mg./ml. |
| Acidic Protein | total nitrogen estimation | 1.8 mg./ml. |
| | bromsulphalein method | 2.0 mg./ml. |

Details of the procedures of nucleic acid estimation will be found in Section 2.2.

to 2 ml. volumes of solutions containing 0 to 22 µg. of nitrogen as ammonium sulphate. A linear relationship between extinction at 450 mµ and total nitrogen present as ammonium ion was thus established and used in estimating protein in chromatin on a total nitrogen basis.

RNA and DNA were estimated in the acidic protein fraction of calf thymus chromatin by the orcinol and diphenylamine procedures (Section 2.2). Total protein nitrogen content of the acidic protein fraction of calf thymus chromatin was thus determined.

## 2.4. Analyses of Chromatin prepared from Rat and Rabbit Tissues.

Chromatin was prepared from the kidneys, liver, pancreas, spleen and thymus of an albino rat aged six weeks. Insufficient chromatin was obtained from kidneys and pancreas to permit estimations of DNA, RNA, histone and acidic protein contents. However, sufficient chromatin to permit this was obtained from liver, spleen and thymus. DNA was estimated by the diphenylamine procedure and it was possible to estimate RNA by the orcinol procedure. Proteins were estimated by the bromsulphalein method. Results of these analyses are shown in Table 2.

Table 2.

Analyses of Chromatin prepared from Tissues of a six-week-old Rat.

| Tissue | DNA content (ug/ml) | RNA content (ug/ml) | Histone content (ug/ml) | Acidic Protein content (ug/ml) | Histone / DNA | Acidic Protein / RNA | RNA / DNA | Acidic Protein / Histone | Histone / RNA | Acidic Protein / DNA |
|---|---|---|---|---|---|---|---|---|---|---|
| Liver | 1050 | 248 | 1200 | 2100 | 1.1 | 8.5 | 0.24 | 1.8 | 4.8 | 2.0 |
| Spleen | 830 | 102 | 900 | 1050 | 1.1 | 10.0 | 0.12 | 1.2 | 8.6 | 1.3 |
| Thymus | 1200 | 91 | 1200 | 650 | 1.0 | 7.2 | 0.08 | 0.54 | 13.0 | 0.54 |

In order to obtain a wider set of such data, chromatin was prepared from kidneys, liver, bone marrow, spleen and thymus of New Zealand white rabbits aged 4 months. These chromatin preparations were sonicated in ice baths for 30 seconds using an M.S.E. Ultrasonic Power Unit at 1.5 amperes before analysis to increase accuracy in volumetric manipulations. There was sufficient material to permit estimation of the DNA (by the diphenylamine procedure), RNA (by ultraviolet absorbance), histone and acidic protein (by the bromsulphalein method) contents of almost all of these chromatin preparations. The orcinol procedure proved unsuitable in estimating RNA in rabbit kidney chromatin owing to the appearance of a brown coloration during heating in a boiling water bath. A similar coloration is seen when it is applied to sucrose solutions. Results of these analyses are shown in Table 3.

## 3. Enzyme Assays.

### 3.1. Assay of RNA Polymerase prepared from Micrococcus luteus (lysodeikticus).

The incorporation of UTP-5-T into acid-insoluble material was followed. The procedure was based on that of Nakamoto, Fox and Weiss (1964). Assay mixtures routinely contained 0.1 M tris/hydrochloric acid buffer solution, pH = 7.4, 2.5 mM manganese chloride, 0.8 mM

Table 3.

Analyses of Chromatin prepared from Tissues of Three Four-month-old Rabbits.

| Rabbit | Tissue | DNA content (ug/ml) | RNA content (ug/ml) | Histone content (ug/ml) | Acidic Protein content (ug/ml) | Histone / DNA | Acidic Protein / RNA | RNA / DNA | Acidic Protein / Histone | Histone / RNA | Acidic Protein / DNA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Kidneys | 2300 | 122 | | 1380 | | 11.0 | 0.053 | | | 0.60 |
| 2 | | 1950 | 156 | 1530 | 1530 | 0.79 | 9.8 | 0.080 | 1.0 | 9.8 | 0.78 |
| 3 | | 2100 | 138 | 1530 | 1380 | 0.73 | 10.0 | 0.066 | 0.9 | 11.0 | 0.66 |
| 1 | Liver | 990 | 72 | 1530 | 830 | 1.5 | 11.0 | 0.073 | 0.54 | 21.0 | 0.34 |
| 2 | | 1760 | 96 | 1850 | 670 | 1.1 | 7.0 | 0.055 | 0.36 | 19.0 | 0.38 |
| 3 | | 1760 | 156 | 2430 | 720 | 1.4 | 4.3 | 0.089 | 0.30 | 15.6 | 0.41 |
| 1 | Bone Marrow | 435 | 38 | 360 | 250 | 0.83 | 6.6 | 0.088 | 0.70 | 9.4 | 0.58 |
| 2 | | 650 | 48 | 465 | 225 | 0.71 | 4.7 | 0.074 | 0.49 | 9.7 | 0.35 |
| 3 | | 580 | 50 | 475 | 225 | 0.82 | 4.5 | 0.086 | 0.48 | 9.5 | 0.39 |
| 1 | Spleen | 1520 | 160 | 2000 | 1120 | 1.3 | 7.0 | 0.11 | 0.56 | 13.0 | 0.74 |
| 2 | | 840 | 68 | 950 | 500 | 1.1 | 7.3 | 0.081 | 0.53 | 14.0 | 0.60 |
| 1 | Thymus | 3900 | 136 | 2800 | 1380 | 0.73 | 10.0 | 0.035 | 0.49 | 21.0 | 0.35 |
| 2 | | 1730 | 100 | | 870 | | 8.7 | 0.058 | | | 0.50 |
| 3 | | 4200 | 232 | 3450 | 2570 | 0.82 | 12.0 | 0.055 | 0.75 | 15.0 | 0.61 |

concentrations of ATP (Sigma Chemical Co.Ltd., London, England), CTP, GTP and UTP (all supplied by Koch-Light Labs. Ltd., Colnbrook, England), 0.03 M putrescine dihydrochloride (Sigma Chemical Co.Ltd.), 200 µg./ml. of purified native calf thymus DNA, 50 µg./ml. of purified Macaloid and enzyme. UTP-5-T was added to the unlabelled UTP so that the final specific activity of UTP in assay mixtures was in the range of $2 \times 10^3$ to $6 \times 10^3$ d.p.m./ mµmole.

Incubations at 37°C were started by addition of enzyme to otherwise complete assay mixtures pre-warmed for 5 minutes in a 37°C water bath. The normal incubation period was 10 minutes and the normal volume of incubation mixtures was 0.50 ml. Controls, in which enzyme was omitted, were included in every assay. Controls in which DNA was omitted were frequently included in assays. In following the time course of RNA polymerase action, volumes in the range of 0.05 to 0.5 ml. were removed at various times from incubation mixtures.

Incubations were terminated by addition of 0.10 ml. of ice-cold 50% w/v trichloroacetic acid (T.C.A., B.D.H. Analar grade reagent) to 0.50 ml. incubation mixtures with thorough mixing or by addition, again with thorough mixing, of a volume removed from the reaction mixture to 2.5 ml.

of ice-cold 5% w/v T.C.A.. Such mixtures were allowed to stand in ice. 2.5 ml. volumes of ice-cold 5% w/v T.C.A. were added to 0.50 ml. incubation mixtures. If there was no visible precipitate at this stage, 100 µg. of bovine albumin in 0.10 ml. of water were added to each mixture to ensure co-precipitation of all acid-insoluble material. This was necessary very infrequently.

After standing in an ice-bath for 5 minutes, the mixtures were centrifuged at 1,500 *g* for 20 minutes at 0°C. The resulting precipitates were re-suspended in further 2.5 ml. volumes of ice-cold 5% w/v trichloroacetic acid, allowed to stand in an ice-bath for 5 minutes and again centrifuged at 1,500 *g* for 20 minutes at 0°C. In all, three such washes of acid-insoluble material were carried out. Less thorough washing led to poor reproduction of incorporations in identical assay mixtures and higher, less reproducible count rates from control mixtures.

The washed precipitates were carefully drained of 5% w/v trichloroacetic acid and dissolved in 0.30 ml. volumes of Nuclear Chicago Solubilizer by agitation and, where necessary, by warming at temperatures of up to 67°C for times of up to 30 minutes. The resulting solutions were washed into scintillation vials with a total volume of 10.0 ml. of toluene-based scintillator in each case.

These vials were allowed to stand in the Nuclear Chicago Mark I Liquid Scintillation Counter for 1 hour at 2°C before counting in order to minimise the effects of fluorescence in producing unrealistic count rates. The number of mμ moles of UTP-5-T incorporated into acid-insoluble material was determined using appropriate quench correction curves.

This method was preferred to the collection of precipitates on filters owing to greater certainty of liquid scintillation counting efficiency, better reproduction of incorporations in identical assay mixtures and lower and more reproducible control count rates.

A unit of enzyme is defined as the quantity of enzyme catalysing the incorporation of 1 mμ mole of UTP into acid-insoluble material during 10 minutes' incubation at 37°C.

3.2. Qualitative Assay of Ribonuclease and Polynucleotide Phosphorylase Activities.

The release of ribonucleotides from yeast RNA was followed. The procedure was based on the results of Matthias, Williamson, Huxley and Page (1964), Edmonds and Roth (1960) and Roth and Wojnar (1961).

Torula yeast RNA was dissolved to a concentration of 4 mg./ml. in 0.10 M tris/hydrochloride buffer solution, pH = 7.4 and a quarter volume of 6 M potassium acetate/

acetic acid solution, pH = 7.3, was added. This was followed by two volumes of ethanol (British Petroleum Ltd., Hull, England) and the mixture was stored overnight at -15°C. On the following day the precipitate was collected by centrifugation at 15,000 g for 20 minutes at 0°C and was dissolved in 0.1 M tris/hydrochloric acid buffer solution, pH = 7.4, to a concentration of approximately 4 mg./ml.. The solution was stored at -15°C.

Assay tubes contained 1.50 ml. of a tenfold dilution of this stock yeast RNA solution in 0.1 M tris/hydrochloric acid buffer solution, pH 7.4, 0.15 ml. of a 1.0 M buffer solution of potassium phosphate, pH = 7.4, where it was desired to assay polynucleotide phosphorylase activity, a suitable volume, not exceeding 0.05 ml. of the solution or suspension under test, and 0.1 M tris/hydrochloric acid buffer solution, pH = 7.4, to bring the total volume to 3.0 ml.

The assay was initiated by addition of the material under test and was continued for 90 minutes at 37°C as these were normal conditions for <u>in vitro</u> RNA synthesis. Controls containing only RNA and buffer solutions and a bovine pancreatic ribonuclease standard were included in every assay. Incubations were terminated by addition of 0.1 ml. of a solution of bovine albumin in water of concentration 1 mg./ml. followed by 3.0 ml. of an ice-

cold solution of 0.5% w/v lanthanum nitrate ($La(NO_3)_3 \cdot 6H_2O$, B.D.H. reagent) in 1 M hydrochloric acid, 75% v/v ethanol. After standing for 30 minutes in an ice-bath, the mixtures were filtered through Whatman No.1 filter paper and the extinctions of the filtrates at 260 mμ relative to water were noted.

Extinction controls, from which test material was omitted, were in the region of 0.4. Bovine albumin was found to be necessary to ensure reproducibility of results obtained from identical incubations. The amount of ribonuclease activity measured was equivalent to that of between 0.5 and 3.0 mμg of bovine pancreatic ribonuclease, as shown in Figure 3.

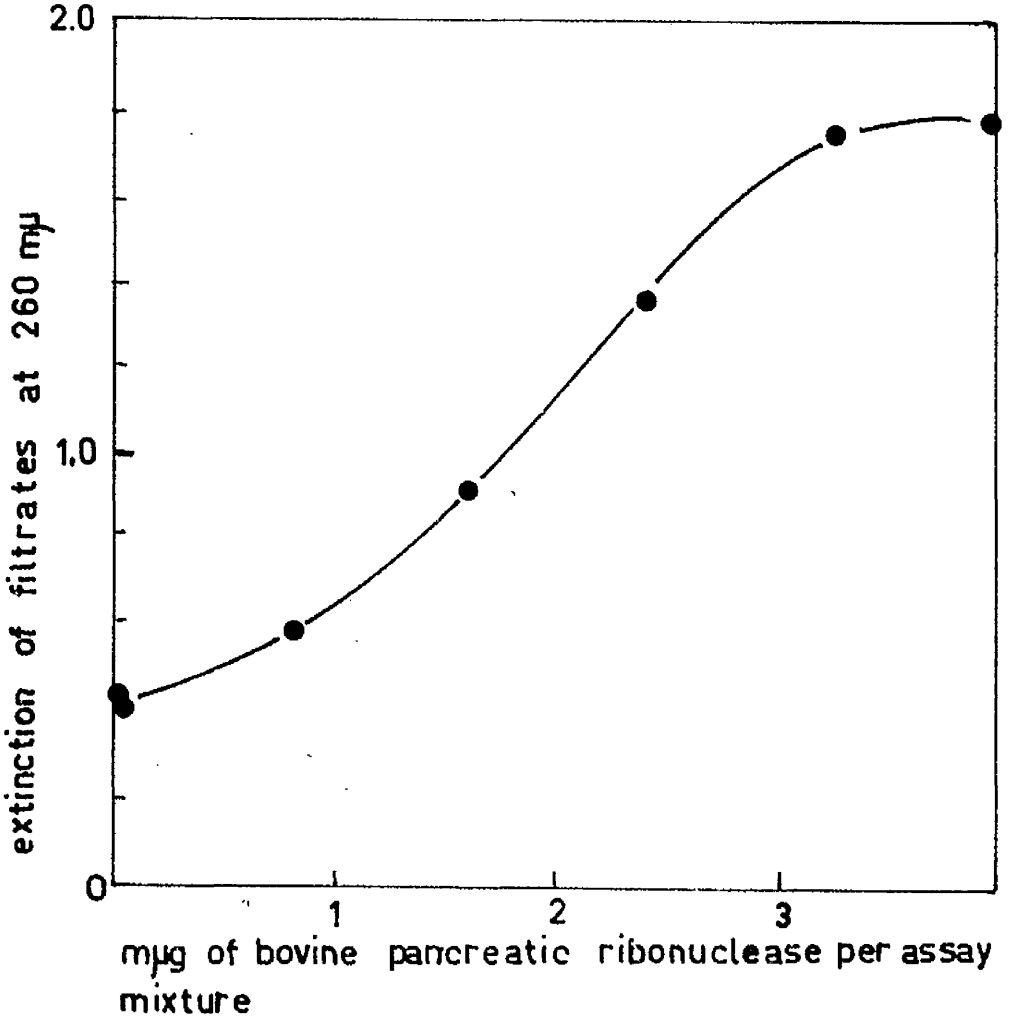
## 4. Purification of RNA Polymerase.

### 4.1. Procedure 1.

This procedure was closely based on that of Nakamoto, Fox and Weiss (1964) and incorporated modifications described by Gilmour (1967).

Washing of cells. 30 g. of spray dried cells of Micrococcus luteus (Cambrian Biochemicals Ltd., Croydon) were blended at room temperature into 0.01 M tris/hydrochloric acid buffer solution, pH 8.0, to a total volume of 300 ml. The suspension was centrifuged at

Figure 3. Release of ribonucleotides from yeast RNA by bovine pancreatic ribonuclease in the standard ribonuclease assay. Each assay mixture initially contained 600 µg. of RNA. Incubations were carried out as described in the text. Torula yeast RNA was purchased from Sigma Chemical Co.Ltd., London.



15,000 g for 10 minutes at 0°C and the washed cells were resuspended in 580 ml. of 0.01 M tris/hydrochloric acid buffer solution, pH = 8.0, 0.2 M sucrose (B.D.H. Analar grade reagent) at 37°C.

Lysis of cells. 600 mg. of crystalline lysozyme were dissolved in 20 ml. of 0.01 M tris/hydrochloric acid buffer solution, pH = 8.0, 0.2 M sucrose and this solution was mixed with the suspension of washed cells. After 15 minutes' incubation at 37°C, 1.5 ml. of 0.1 M magnesium chloride were added with mixing and, after a further 30 minutes' incubation, 4.5 ml. of 0.1 M magnesium chloride were mixed with the suspension. The mixture was then placed in an ice-bath and an equal volume of ice-cold deionised distilled water was added. After 10 minutes' standing in the ice-bath, the viscous mixture was said to be cell lysate. Throughout the lysing procedure, care was taken to ensure homogeneity of the mixtures described.

Crude separation of enzyme protein from DNA. A tenth volume of 10% w/v streptomycin sulphate (Glaxo Laboratories Ltd., Greenford, England) was added dropwise to the lysate and, after standing for 10 minutes, the suspension was centrifuged at 15,000 g for 10 minutes. The precipitate was resuspended in 200 ml. of 1 mM tris/

hydrochloric acid buffer solution, pH = 8.0, 0.2 M sucrose, 0.1% w/v streptomycin sulphate, 0.25mM magnesium chloride. It was then centrifuged at 15,000 g for 10 minutes. The resulting precipitate was rinsed with deionised distilled water and resuspended in 180 ml. of a solution containing 2.0 ml. of 1 M potassium dihydrogen orthophosphate/dipotassium hydrogen orthophosphate (B.D.H. reagent), pH = 7.4, 2.0 ml. of 0.1 M magnesium chloride and 20 ml. of 2 M sucrose. 8.0 ml. of the 1 M potassium phosphate buffer solution were then added. The viscosity of the solution increased markedly at this stage and care was taken to ensure its homogeneity. After it had stood for 10 minutes, 10 ml. of 10% w/v streptomycin sulphate were added dropwise. The resulting suspension was allowed to stand for 10 minutes and was then centrifuged for 150,000 g hours. The gelatinous precipitate was assumed to contain the bulk of the DNA.

Purification of enzyme protein from residual DNA and other proteins. The phosphate concentration of the supernate (assumed to be 0.05 M) was increased to 0.17 M by addition of the potassium phosphate buffer solution at 1 M concentration. A fifth volume of a 2.5% w/v solution of protamine sulphate (Grade I, Sigma Chemical Co.

Ltd.) at room temperature, was added dropwise to this solution, thereby reducing its phosphate concentration to 0.1$\frac{1}{4}$ M. After 10 minutes' standing, the mixture was centrifuged at 15,000 g for 10 minutes. The precipitate was resuspended in 30 ml. of 0.3 M potassium phosphate buffer solution, pH = 7.$\frac{1}{4}$, 0.2 M sucrose. After 10 minutes' standing, the suspension was centrifuged at 15,000 g for 10 minutes. The supernate was removed and the precipitate similarly re-extracted into 15 ml. of the same solution.

The two supernates were combined and 2 volumes of 0.1% w/v protamine sulphate were added dropwise with continual mixing. After 10 minutes' standing the mixture was centrifuged at 15,000 g for 10 minutes. The precipitate was extracted into 10 ml. of 0.1$\frac{1}{4}$ M potassium phosphate buffer solution, 0.2 M sucrose by gentle homogenisation, being allowed to stand for 5 minutes, repeating these steps and homogenising gently for a third time. The homogenate was centrifuged at 15,000 g for 10 minutes.

By the procedure already described, the precipitate was extracted into 0.3 M potassium phosphate buffer solution, 0.2 M sucrose, volumes being 10 ml. and 5 ml.

The protein concentration of the combined extracts was roughly adjusted to 6 mg./ml. of 0.3 M potassium phosphate buffer solution, 0.2 M sucrose, by application of the formula -

$$\text{protein concentration (mg./ml.)} = E_{280m\mu} \times 1.54 - E_{260m\mu} \times 0.76.$$

Saturated ammonium sulphate solution (neutralised by ammonia solution) was mixed gently with the combined extracts to 38% saturation. After 20 minutes' standing the mixture was centrifuged and the carefully drained precipitate dissolved in 0.1 M tris/hydrochloric acid buffer solution, pH = 7.4 of volume not exceeding 5 ml. An equal volume of glycerol was mixed with this and the solution was stored at -15°C.

Results of use of procedure 1 in the purification of RNA polymerase. Yields and specific activities of RNA polymerase purified by this procedure were much less than those described by Nakamoto, Fox and Weiss. They were, on average, 1300 units from 30 g. of spray dried cells of Micrococcus lutous and 45 units/mg. of protein.

4.2. Procedure 2.

The above procedure was therefore further modified

by incorporation of procedures employed by Dr. J.F. Szilagyi, The Institute of Virology, The University of Glasgow, in the isolation of RNA polymerase from Escherichia coli (Szilagyi, 1968).

Washing of cells. 30 g. of spray-dried cells of Micrococcus luteus (Sigma Chemical Co.Ltd., London) were blended at room temperature into 0.01 M tris/hydrochloric acid buffer solution, pH = 8.0 to a total volume of 200 ml. The suspension was centrifuged at 15,000 g for 10 minutes at 0°C and the washed cells were resuspended in 190 ml. of a solution of 0.01 M tris/hydrochloric acid buffer solution, pH = 8.0, 0.2 M sucrose, at 37°C.

Lysis of cells. 300 mg. of crystalline lysozyme were dissolved in 10 ml. of 0.01 M tris/hydrochloric acid buffer solution, pH = 8.0, 0.2 M sucrose and this solution was added to the suspension of washed cells. The suspension rapidly became very viscous and then less so within a period of 15 minutes' incubation at 37°C. 1.0 ml. of an 0.1 M solution of magnesium chloride was then added, followed, after a further 30 minutes' incubation of the solution at 37°C, by 3.0 ml. of the same solution. The mixture was then placed in an ice-bath and an equal volume of ice-cold deionised distilled water was added. After 10 minutes' standing in the ice-bath, the viscous mixture

was said to be the cell lysate. Throughout the lysing procedure, care was taken to ensure homogeneity of the mixtures described.

Crude separation of enzyme protein from DNA. All further steps in this procedure were carried out at a temperature as close as possible to 0°C. By addition of 1 M potassium dihydrogen orthophosphate/dipotassium hydrogen orthophosphate buffer solution, pH = 7.4, the lysate was made 0.14 M with respect to phosphate. Solid sodium benzoate (B.D.H. reagent) was then dissolved in the mixture to a concentration of 0.05 M. The mixture was gently stirred for 90 minutes and then a tenth volume of 10% w/v streptomycin sulphate solution was added to it dropwise with continual stirring. The mixture was allowed to stand for 10 minutes and was then centrifuged for 150,000 g. hours. The resulting supernate was dialysed overnight against 5 litres of the above 0.14 M phosphate buffer solution.

Purification of enzyme protein from residual DNA and other proteins. A fifth volume of 2.5% w/v protamine sulphate was mixed dropwise with the diffusate. After 10 minutes' standing, the mixture was centrifuged at 15,000 g. for 10 minutes and the resulting precipitate was treated as already described in Procedure 1.

Extraction of RNA polymerase from the streptomycin precipitate. Measured volumes of the streptomycin precipitate were diluted in 0.05 M tris/hydrochloric acid buffer solution, pH = 7.4 and sodium chloride solution such that the final concentrations of sodium chloride were nil, 0.1 M, 0.2 M and 0.3 M. The suspensions were thoroughly mixed for 1 minute, allowed to stand for 30 minutes and then thoroughly mixed again for 1 minute. They were then centrifuged at 10,000 g for 20 minutes. All operations were carried out at temperatures as close as possible to 0°C.

Results of this study indicated that, had all the streptomycin precipitate been thus extracted, yields of enzyme units could have been increased by factors of 22%, 41%, 54% and 47%, depending on the respective sodium chloride concentration employed.

Results of use of procedure 2 in the purification of RNA polymerase. Yields and specific activities of RNA polymerase purified by this procedure were considerably greater than those obtained by application of procedure 1. They were, on average, 2000 units from 30 g. of spray-dried cells of Micrococcus luteus and 80 units/mg. of protein. RNA polymerase purified by procedure 2 almost always contained no detectable

ribonuclease activity and never contained detectable polynucleotide phosphorylase activity between March, 1968 and November, 1968. It was therefore generally adopted during this period and, unless otherwise indicated, RNA polymerase purified in this way was used throughout. The results of a typical purification are shown in Table 4. Fractions obtained during the procedure were stored at 4°C for up to a few days.

After November, 1968, ribonuclease activity became detectable in RNA polymerase preparations obtained by this procedure. Procedure 1, using cells supplied by Sigma Chemical Co.Ltd., was, therefore, again employed. Lower yields of enzyme were obtained but it was found to contain no detectable ribonucleolytic activity.

## 5. Studies on the Activity of RNA Polymerase.

### 5.1. Time course of the RNA polymerase reaction and effects of Ribonuclease and Inhibitors of its activity.

When a preparation of RNA polymerase and a calf thymus DNA template exhibiting no detectable ribonuclease activity or, in the case of the enzyme, no detectable polynucleotide phosphorylase activity, were used in making up the normal RNA polymerase assay mixture,

## Table 4.

### Results of a typical Preparation of RNA Polymerase from 30 g. of Micrococcus luteus by Procedure 2.

| | Vol. (ml.) | Activity (units) | Protein (mg.) | Specific Activity (units/mg.) | Purification Factor. | RNase Test | P.Pase Test |
|---|---|---|---|---|---|---|---|
| Lysozyme lysate. | 220 | 7050 | 8600 | 0.82 | | +ve | +ve |
| 0.05 M in sodium benzoate (on addition). | 220 | 7400 | 9000 | 0.82 | | +ve | -ve |
| Streptomycin supernate. | 139 | 1520 | 2020 | | | +ve | -ve |
| Dialysate. | 180 | 1360 | 1880 | | | +ve | +ve |
| Ammonium sulphate precipitate. | 3 | 4600 | 47 | 98.0 | 120 | -ve | -ve |

RNase Test: Test for Ribonuclease Activity.
P.Pase Test: Test for Polynucleotide Phosphorylase Activity.

continued RNA synthesis was observed throughout the 90 minute incubation period (Figure 4a).

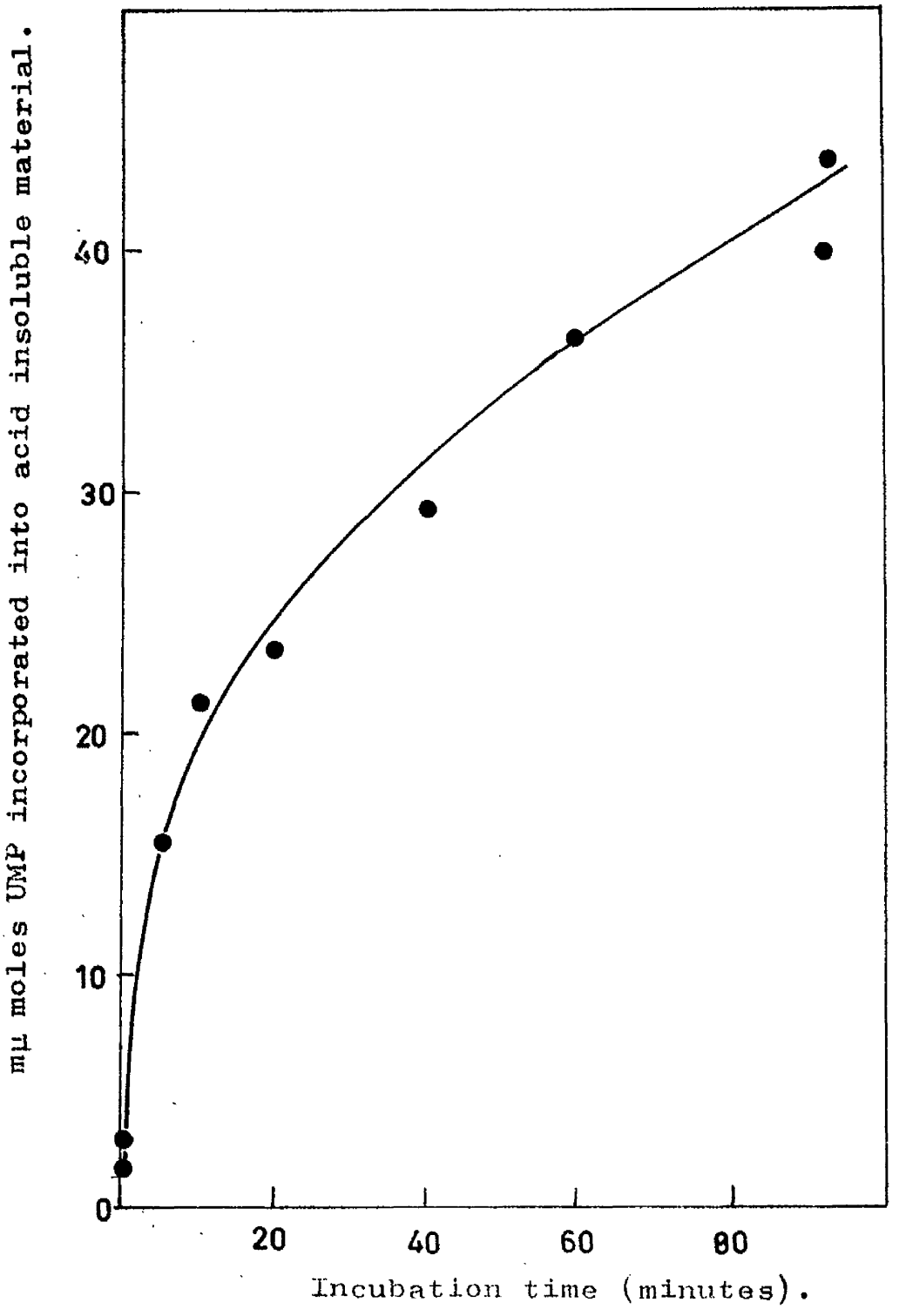
When the calf thymus DNA template, but not the RNA polymerase preparation, exhibited detectable ribonuclease activity (the approximate equivalent of 5.4 mµg. of bovine pancreatic ribonuclease per mg. of DNA), nett synthesis of RNA ceased after some time and a nett loss of RNA was observed thereafter. This effect was much more pronounced in the absence of macaloid (Figure 4b).

When calf thymus DNA template and RNA polymerase were pre-incubated together for 1 hour in 0.15 M sodium iodoacetate/iodoacetic acid (Sigma Chemical Co.Ltd., London), pH = 5.9 at 40°C and the usual assay mixtures then incubated throughout a 90 minute period, no RNA synthesis was observed.

5.2. Effect of DNA on the Activity of RNA Polymerase.

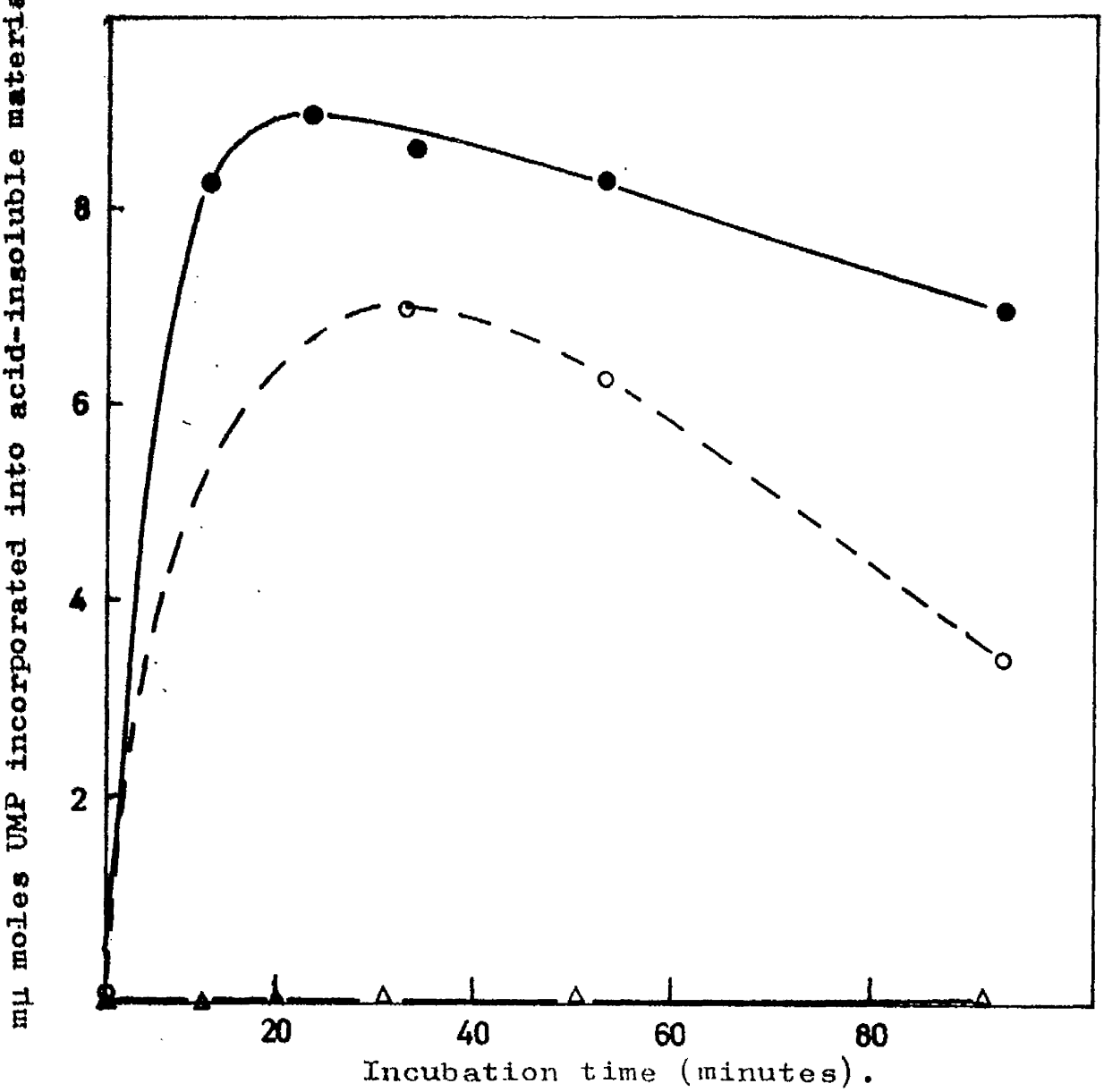
When RNA polymerase assay mixtures from which DNA had been omitted were incubated for periods of 10 minutes, very little RNA synthesis was observed. However, RNA synthesis was observed in control incubations containing calf thymus DNA. When an RNA polymerase assay mixture from which DNA had been omitted was incubated for 90 minutes, very little RNA synthesis was observed throughout the entire period. The calf thymus DNA used in

**Figure 4a.** Time Course of the RNA Polymerase Reaction where Ribonuclease Activity was not detectable in Calf Thymus DNA template nor in the enzyme.




The incubation mixture of original volume 5.0 ml. contained, initially, 20 units of RNA polymerase and 1.15 mg. of DNA. 0.50 ml. volumes were removed at the times indicated above.

**Figure 4b.** Time Course of the RNA Polymerase Reaction where Ribonuclease Activity was detectable in the Calf Thymus template but was not detectable in the enzyme.




●——● Macaloid included in incubation mixtures, (final concentration = 50 μg./ml.).

○- - -○ Macaloid omitted from incubation mixtures.

Δ——Δ Enzyme omitted from incubation mixtures but macaloid included.

The incubation mixture of original volume 2.5 ml. contained, initially, 20 units of RNA polymerase. 0.80 ml. volumes were removed at the times indicated above. Initially, incubation mixtures included 660 μg. of DNA containing the equivalent of 5.4 mμg. of bovine pancreatic ribonuclease per mg. of DNA.

the control incubation contained the equivalent of 5.4 mμg. of bovine pancreatic ribonuclease per mg., but the RNA polymerase used exhibited no detectable ribonuclease or polynucleotide phosphorylase activities (Figure 5a).

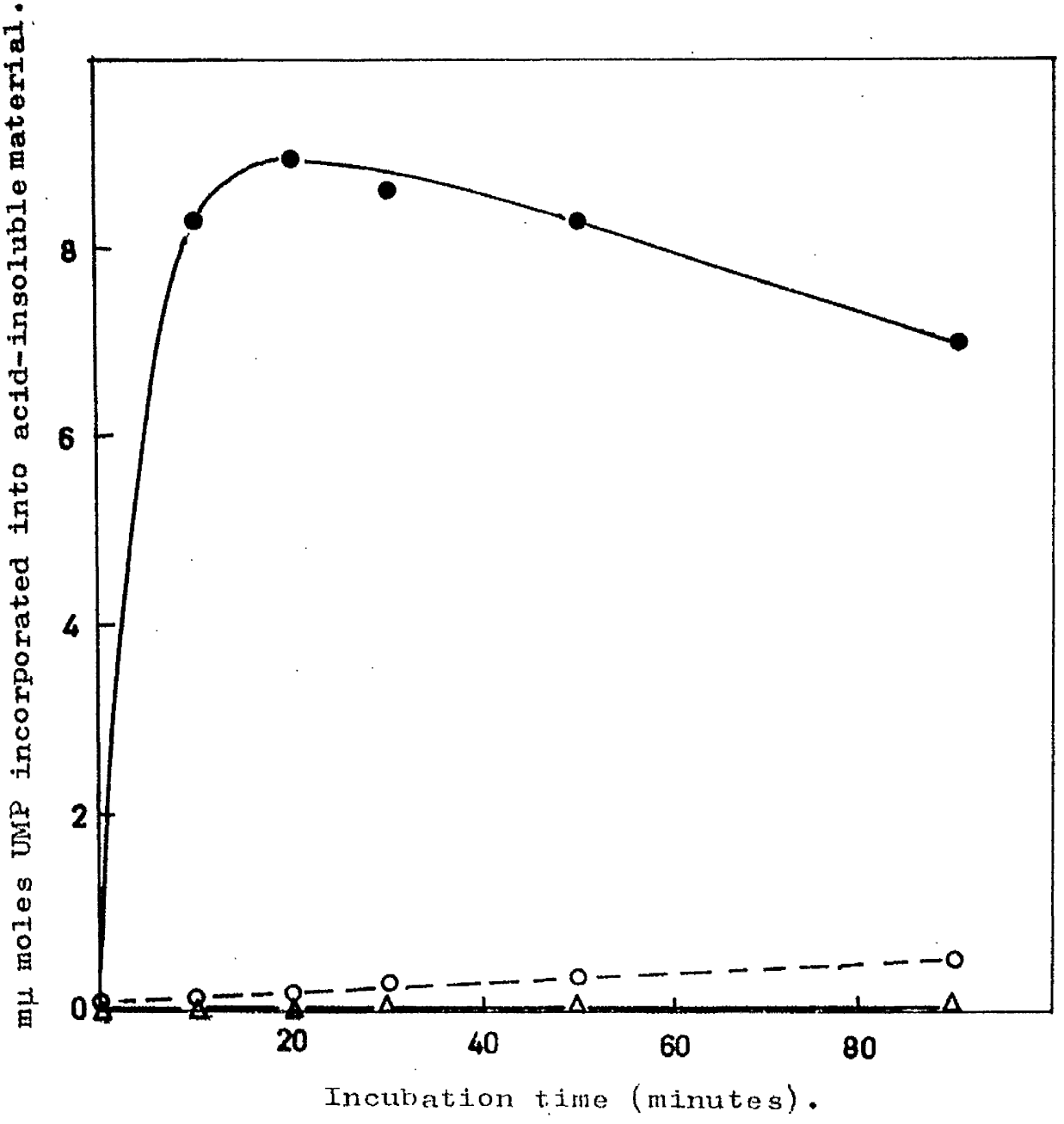
The effect of concentration of calf thymus DNA on the activity of RNA polymerase was studied. RNA polymerase assay mixtures containing 0 to 840 μg./ml. of DNA and 8.2 units/ml. of RNA polymerase were incubated for 90 minutes. Rate of RNA synthesis in mμ moles of UMP incorporated into acid-insoluble material per 90 minute incubation period was plotted against DNA concentrations. An asymptotic curve was obtained (Figure 5b). On plotting the reciprocals of these quantities against one another in the manner of Lineweaver and Burk, a straight line was obtained from which it was possible to deduce an enzyme-DNA binding constant of 28 μg. DNA /ml./unit of RNA polymerase (Figure 5c). This study was carried out using the same materials as that on the effect of putrescine dihydrochloride on RNA polymerase activity (Section 5.5).

5.3. Effect of RNA on the Activity of RNA Polymerase.

The RNA used in these studies was isolated from rabbit reticulocyte polysomes and was a gift by Miss

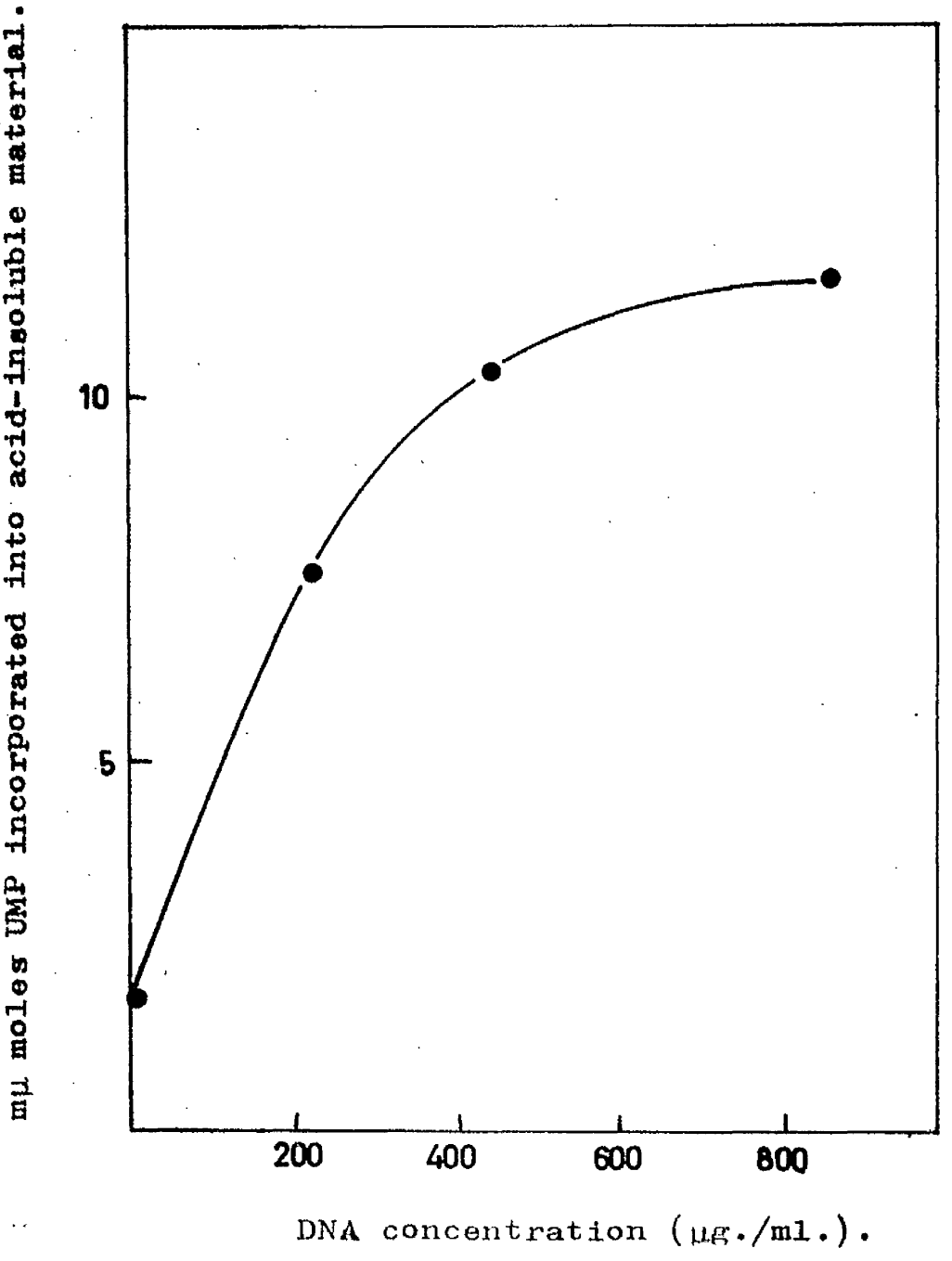
**Figure 5a.** Effect of Omission of DNA on the Time Course of the RNA Polymerase Reaction.

**The incubation mixtures of original volume 2.5 ml. contained, initially, 200 ug./ml. of calf thymus DNA ●——● or no DNA ○ - -○ and 4.0 units/ml. of RNA polymerase. 0.20 ml. volumes were removed at the times indicated.**



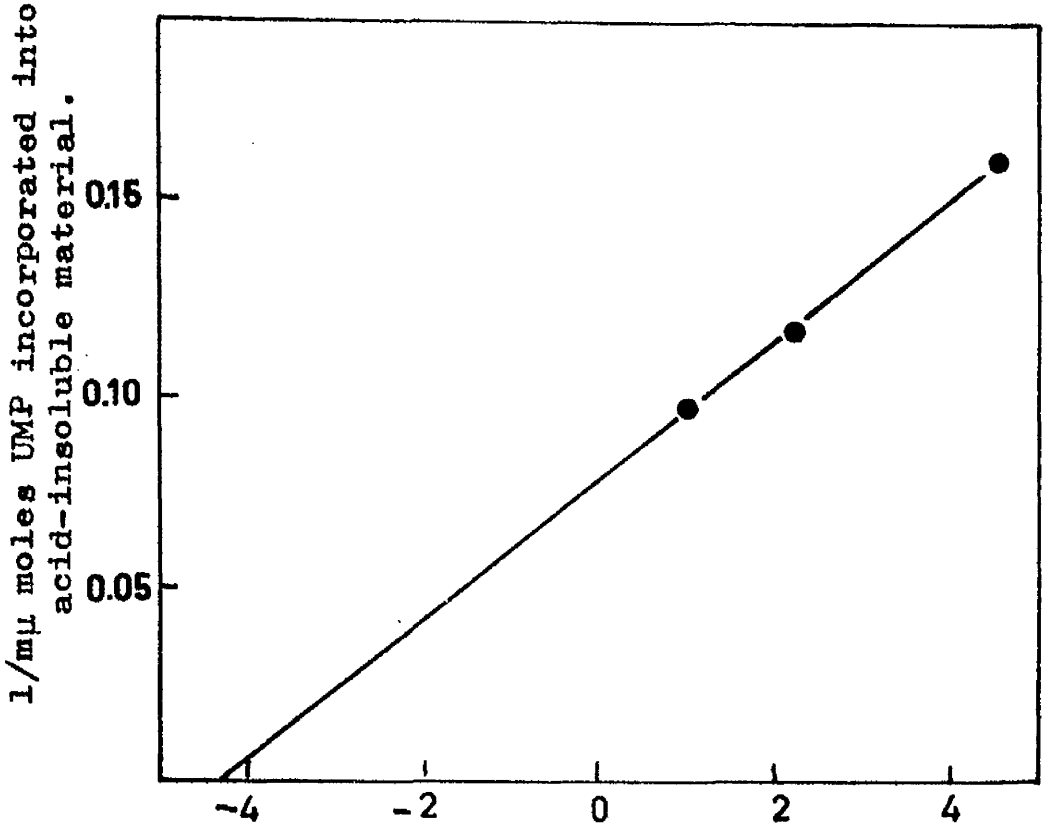

△——△ Enzyme omitted from incubation mixtures.

**Figure 5b.** Effect of Calf Thymus DNA Concentration on the Quantity of RNA synthesized in 90 minutes' Incubation with RNA Polymerase.




Enzyme concentration = 8.2 units/ml..

**Figure 5c.** Double Reciprocal Plot of the Data of Figure 5b.



Marcello Morrison. It was thermally denatured before use by heating at a temperature in the range of 90°C to 95°C for 10 minutes at a concentration of 700 µg./ml. in 1 mM tris/hydrochloric acid buffer solution, pH = 7.9 including 0.05 M potassium chloride and 1.5 mM magnesium chloride. It was then rapidly chilled in an ice-bath.

The quantity of RNA included in RNA-synthesizing mixtures was 55% of that of the calf thymus DNA included. In standard 10 minute incubations, the RNA polymerase activities observed on substitution of DNA by RNA and inclusions of both were, respectively, 1.5% and 25% of the RNA polymerase activity measured when the normal quantity of calf thymus DNA was included.

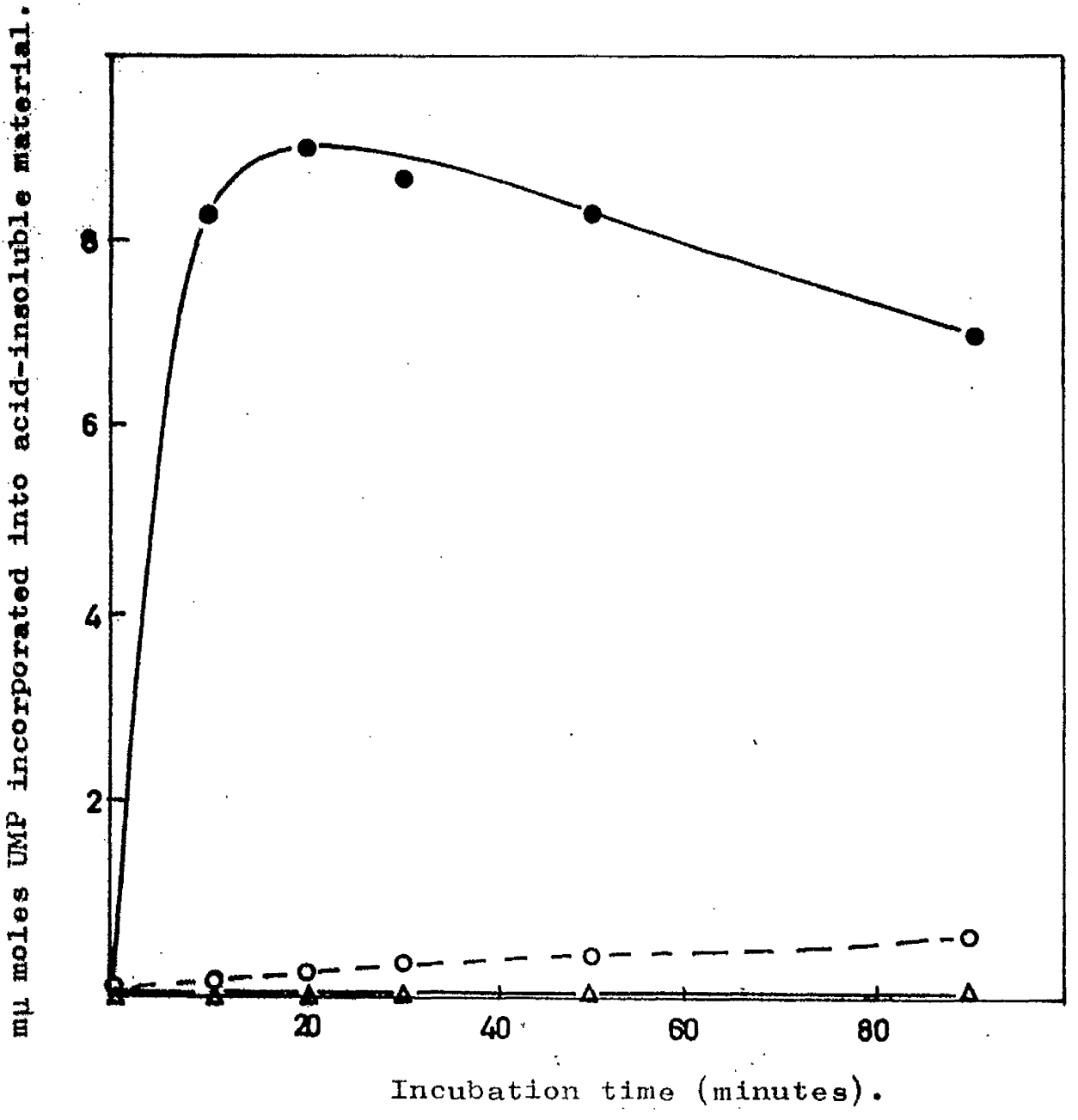
Little RNA synthesis was observed when an RNA-synthesizing mixture including RNA in place of DNA was incubated throughout a 90 minute period (Figure 6). The system used here was that used in investigating the effect of omission of DNA on the time course of the RNA polymerase reaction.

5.4. Effect of the Absence of one Ribonucleoside Triphosphate from an RNA-synthesizing Mixture.

The materials used here were, again, those used in investigating the effect of omission of DNA on the time course of RNA synthesis. On omissions of GTP, little

**Figure 6.** **Effect of Replacement of DNA by RNA on the Time Course of the RNA Polymerase Reaction.**

**The incubation mixtures of initial volume 2.5 ml. contained 260 μg./ml. of calf thymus DNA ●—● or 140 μg./ml. of denatured rabbit reticulocyte polysomal RNA O---O. 0.20 ml. volumes were removed at the times indicated.**




▲—▲ Enzyme omitted from incubation mixtures.

nett RNA polymerase activity was observed throughout a 90 minute incubation period. However, the level of all count rates was considerably higher than background (Figure 7).

5.5. Effect of Putrescine on the Time Course of the RNA Polymerase Reaction.

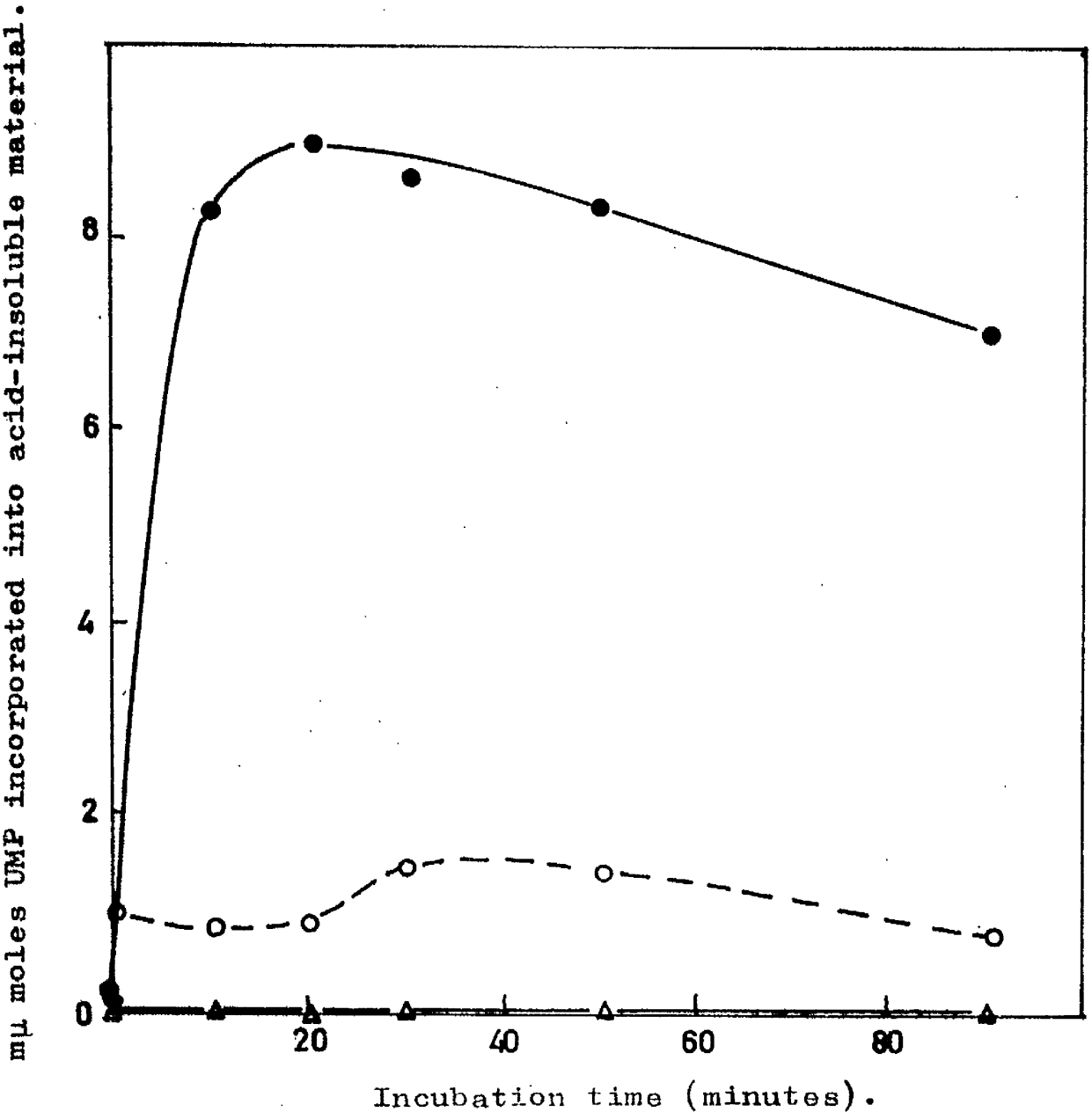
The activities of two preparations of RNA polymerase measured in standard assays involving 10 minute incubations were found to be equal on omission of putrescine dihydrochloride (Sigma Chemical Co.Ltd., London) and when it was included at 0.04 M concentration. However, when incubation of assay mixtures was maintained for 90 minutes, a stimulating effect of the presence of putrescine on RNA synthesis was observed (Figure 8). The calf thymus DNA template exhibited no detectable ribonuclease activity. The RNA polymerase used exhibited no detectable polynucleotide phosphorylase activity but contained the approximate equivalent of 0.12 mμg. of bovine pancreatic ribonuclease per unit. Concentrations of DNA and enzyme in this study were, respectively, 200 μg./ml. and 3.3 units /ml. of assay mixtures.

5.6. Comparison of Three Cations as Co-factors in the RNA Polymerase Reaction.

The effects of replacement of 2.5 mM manganous chloride

**Figure 7.** Effect of the Absence of GTP from an RNA-synthesizing Mixture.

**Incubation mixtures** of initial volume 2.5 ml. contained 260 µg./ml. of calf thymus DNA, 4.0 units/ml. of RNA **polymerase and either** all other reagents ●——● or all **others except** GTP o---o . 0.20 ml. volumes were re**moved at the times** indicated.

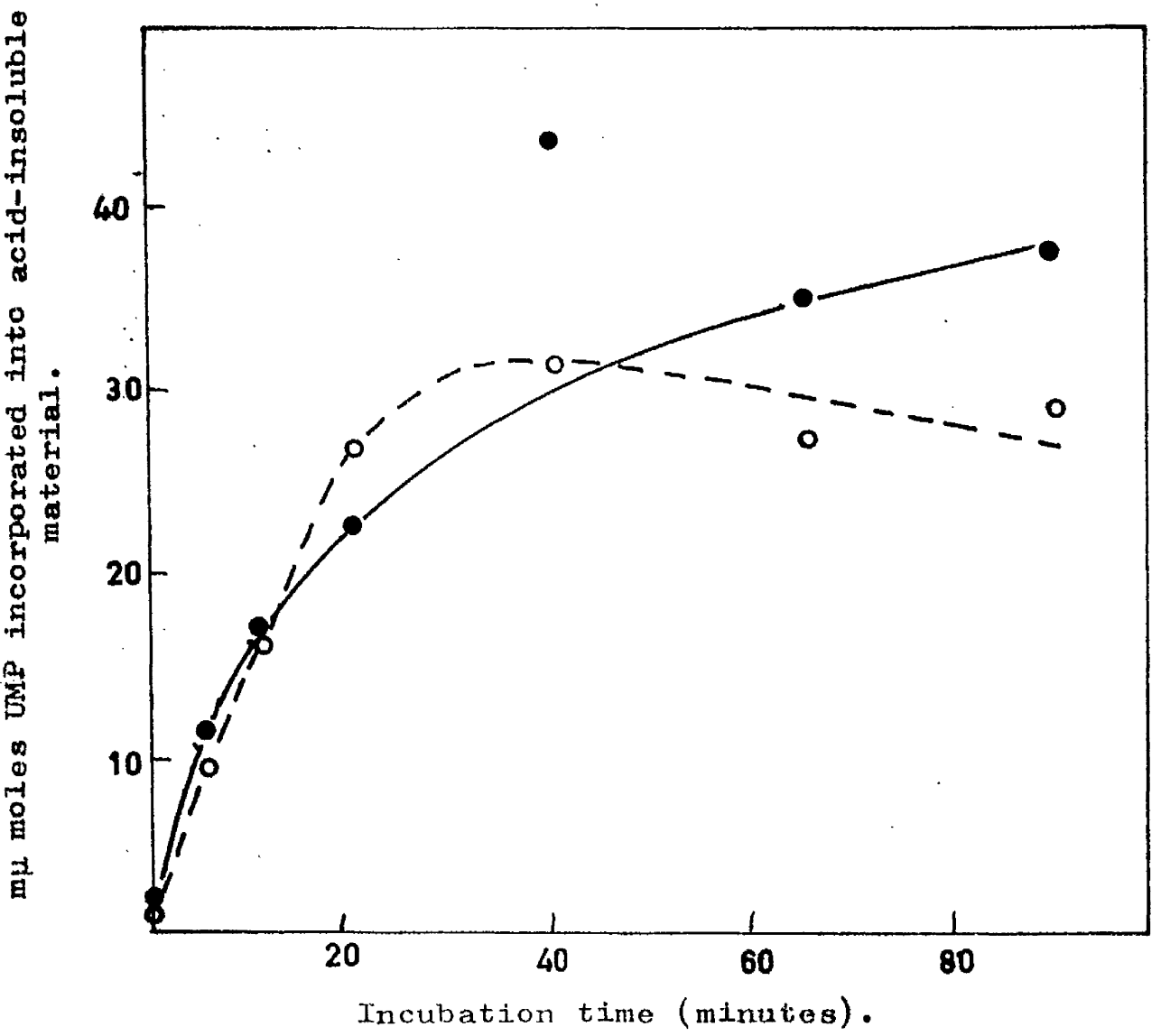



Δ——Δ Enzyme omitted from incubation mixtures.

**Figure 8.** Effect of Putrescine on the Time Course of the RNA Polymerase Reaction.

●——● Putrescine dihydrochloride was included in incubation mixtures at a concentration of 0.03 M.

o- - -o Putrescine dihydrochloride was omitted from these incubation mixtures.



in RNA polymerase assay mixtures by 2.5 mM cobaltous chloride and 2.5 mM magnesium chloride were investigated.

In the absence of DNA template from an RNA polymerase assay mixture incubated for 10 minutes there was no detectable RNA synthesis in the presence of either manganous or cobaltous chlorides. When calf thymus DNA was included in similar assay mixtures including the same materials, RNA polymerase activities measured in the presence of cobaltous and magnesium chlorides were, respectively, 55% and 3.5% of those measured in the presence of manganous chloride.

The time courses of *in vitro* RNA synthesis in the presence of manganous and cobaltous chlorides were compared. Throughout the 93 minute incubation period, approximately twice as much RNA synthesis was observed in the presence of manganous chloride as in the presence of cobaltous chloride (Figure 9).

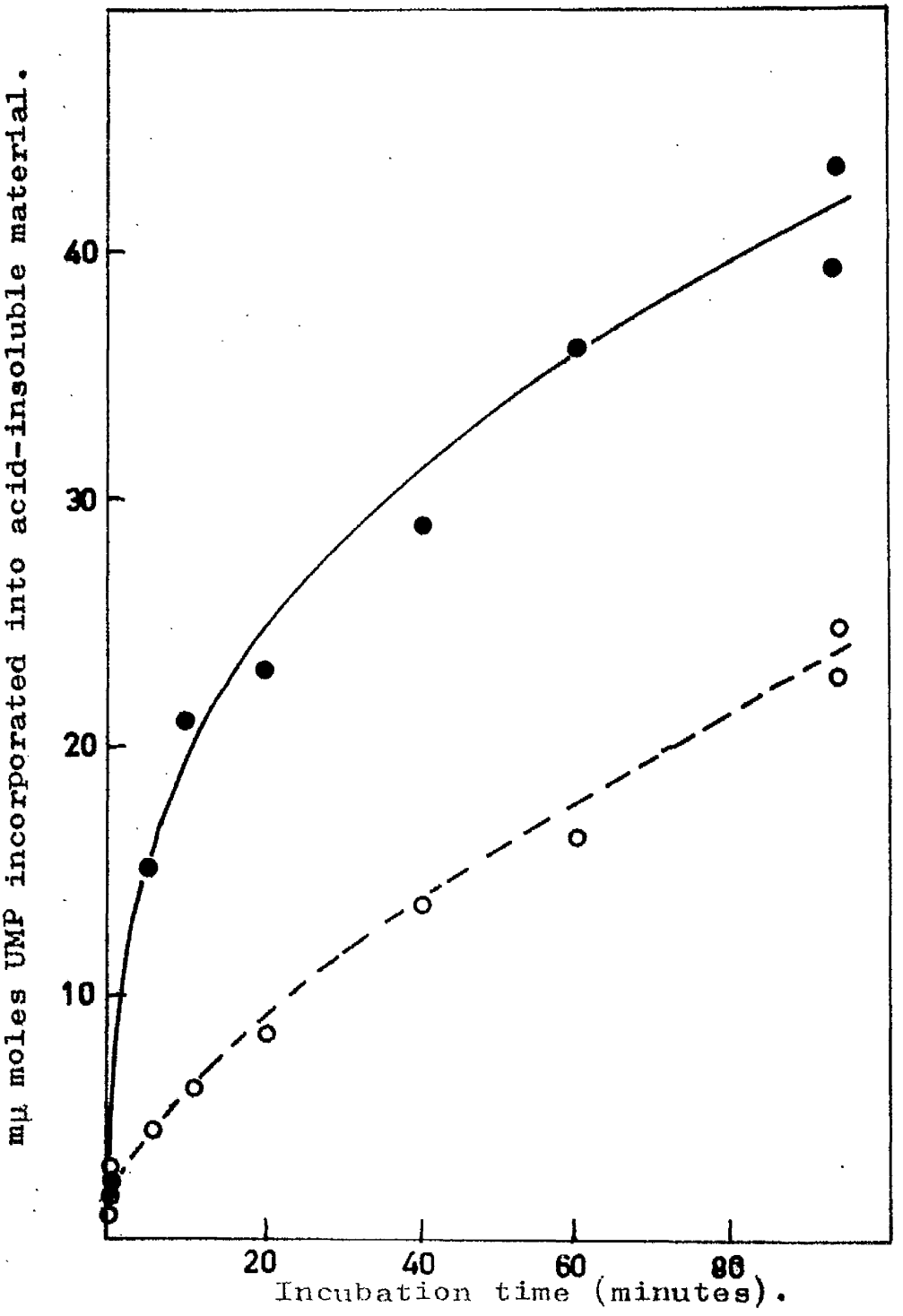
## 6. Studies on DNA.

### 6.1. Isolation of DNA.

DNA was isolated as sodium deoxyribonucleate by a procedure based on those of Kay, Simmons and Dounce (1952) and Marmur (1961).

Calf thymus and mouse embryo nuclei were obtained

**Figure 9.** Time Courses of *in vitro* RNA syntheses in the Presence of Manganous and Cobaltous Chlorides.




RNA-synthesizing mixtures of initial volume 5.0 ml. included μg./ml. of calf thymus DNA, 4.0 units/ml. of RNA polymerase, and 2.5 mM manganous ●——● or cobaltous o---o chlorides. 0.5 ml. volumes were removed at the times indicated.

as in the preparation of chromatin. They were not further washed in 0.025 M citric acid.

The following procedure was followed in obtaining nuclei from Landschutz ascites tumour cells. Seven-day tumour cells were obtained from the peritoneum of a male Porton white Swiss mouse suffering from a well-established Landschutz tumour. Similar mice were inoculated intraperitoneally with 0.5 ml. volumes of these. After a further ten-day period the mice were sacrificed and the tumour cells rapidly removed from their peritonea. The cells were washed twice in equal volumes of Hank's balanced salt solution (Paul, 1965b) at 0°C, the suspension being centrifuged at 250 g for 5 minutes at 0°C after each wash.

Care was taken to ensure complete lysis of these cells. They were allowed to stand suspended in 20 volumes of 5 mM citric acid for 10 minutes at 0°C and were homogenised in this solution at high speed for 1 minute. The presence of nuclei was confirmed microscopically using brilliant cresol blue in 1 x S.S.C. and a magnification of 400 X. The homogenate was centrifuged at 1,500 g for 15 minutes at 0°C to obtain a sediment of nuclei.

Tritiated DNA was also isolated from Landschutz ascites tumour cells. Cells obtained as described above

were incubated at a concentration of 0.5 x $10^6$/ml. in Eagle's balanced salt solution containing 5% v/v calf serum (Paul, 1965b) and including 200 μM thymidine (methyl-T, 3000 mC/mM) for 20 hours at 37°C. The cells were collected and washed and nuclei were obtained as described above.

To separate nucleic acids from proteins nuclei were suspended by gentle homogenisation and 1 hour's stirring at room temperature in as small a volume as possible of 0.025 M tris/hydrochloric acid buffer solution, pH = 7.4, 1 x S.S.C. and 2% w/v sodium dodecyl sulphate (S.D.S., Sigma Chemical Co.Ltd.). Unless otherwise stated, all subsequent operations were carried out at room temperature. Solid sodium chloride was dissolved in the suspension to a final concentration of 1 M and stirring was continued for a further 30 minutes.

Proteins were denatured by chloroform to render them readily precipitable. The suspension was gently shaken with an equal volume of chloroform (B.P., Macfarlan Smith Ltd., Edinburgh)/octan-2-ol (B.D.H. reagent, included to minimise foaming) 24:1 v/v mixture for 15 minutes. The resulting mixture was centrifuged for 15 minutes at 15,000 *g* at 0°C. The upper viscous aqueous phase was removed and treated similarly until no visible interface

was seen on centrifugation. Two further treatments with chloroform were normally required. Nucleic acids were precipitated by addition of 2 volumes of ethanol. The precipitate was spooled on to a glass rod and was re-dissolved by standing overnight at 4°C in 2 volumes of 1 x S.S.C. followed, if necessary, by gentle homogenisation.

In preliminary work ribonuclease was added to a concentration of 50 µg./ml. and the solution incubated for 30 minutes at 37°C to remove RNA from ethanol-precipitable material. In all isolations pronase was added to a concentration of 50 µg./ml. and the solution incubated for 90 minutes at 37°C to remove residual protein, including added ribonuclease, from ethanol-precipitable material. To remove pronase and any other residual protein from nucleic acids the solution was made 1 M in sodium chloride by dissolution of the solid and 2% w/v in S.D.S.. It was shaken gently for 15 minutes. Proteins were denatured and removed using chloroform as above. Two treatments with chloroform were normally required at this stage. Nucleic acids were again precipitated by addition of 2 volumes of ethanol, spooled and re-dissolved in as small a volume as possible of 1 x S.S.C. by allowing the suspension to stand for a period of hours at 4°C and, if necessary, gentle homogenisation. A ninth volume of

3 M sodium acetate (B.D.H., Analar grade reagent) 1 mM tetrasodium EDTA (B.D.H., Analar grade reagent) was added followed by 0.54 volumes of propan-2-ol (B.D.H., Analar grade reagent). The nucleic acids were thus precipitated and were spooled on to a glass rod, washed in 70% v/v ethanol, 90% v/v ethanol, squeezed to dryness and dissolved in 0.15 M sodium chloride, 0.15 mM trisodium citrate by allowing to stand at 4°C.

Yields of DNA were in the range of 0.3 to 2.3 mg. per g. or ml. of starting material. Highest yields were obtained from calf thymus. The specific extinction coefficient of native DNA was taken to be 0.022 absorbancy units. ml. $cm.^{-1}$ $\mu g.^{-1}$. Absorption spectra of DNA solutions were similar to those reported for purified DNA.

6.2. Denaturation of DNA.

Denaturation of DNA in solution was followed by recording hyperchromicities at a wavelength of 260 mμ. Complete spectra over the range of 230 mμ to 350 mμ were recorded so that any turbidity effects could be taken into account. None were, in fact, observed. Hyperchromicities in the range of 30% to 40% were assumed to indicate complete denaturation of native DNA.

Thermal denaturation. The solution of DNA in a

tenfold or greater dilution of 0.15 M sodium chloride, 0.15 mM trisodium citrate was heated for 5 minutes at a temperature in the range of 90°C to 95°C. It was then plunged into an ice-bath and allowed to stand in it for 5 minutes.

Alkaline denaturation. The solution of DNA was placed in an ice-bath and made 0.5 M with respect to sodium hydroxide so that its pH was in excess of 13. Its absorption spectrum was recorded at this stage. After 10 minutes it was neutralised at 0°C using hydrochloric acid.

Hyperchromicities obtained using both procedures were identical when that of alkaline-denatured DNA was recorded on addition of sodium hydroxide. On neutralisation of the alkaline solution, the hyperchromicity was considerably reduced.

Effects of Storage and Sonication. Calf thymus DNA stored in 0.01 x S.S.C. at 4°C was observed to denature significantly over a few days. The hyperchromicities recorded on denaturation of such stored samples by thermal and alkaline procedures were identical. On mixing thermally denatured calf thymus DNA with the native material, it was found that the hyperchromicity recorded on carrying out the procedure of thermal denaturation

Table 5.

Hyperchromicities observed in the Denaturation of DNA.

| Source of DNA | Percentage hyperchromicities | | |
|---|---|---|---|
| | On thermal denaturation | On alkaline denaturation | |
| | | on addition of sodium hydroxide. | on neutralisation. |
| Landschutz ascites tumour cells. | 35% | 35% | 14% |
| Calf thymus. | 31% | 31% | 13% |

expressed as a fraction of that recorded on thermal denaturation of the native material was approximately equal to the fraction of native DNA in the mixture. DNA dissolved in 0.15 M sodium chloride, 0.15 mM trisodium citrate remained native during storage at 4°C.

Ascites tumour cell DNA was sonicated at a concentration of 9 µg./ml. in 4 mM sodium chloride, 4 µM trisodium citrate. The solution was sonicated in an ice-bath for four 15-second periods interspersed by four 15-second periods' standing in the ice-bath. An M.S.E. Ultrasonic Power Unit was used at 1.5 amps. No hyperchromicity was recorded in this procedure and full hyperchromicity was recorded on thermal denaturation of the sonicated DNA solution.

Melting Curves of DNA. These were recorded using a Unicam SP 800 ultraviolet spectrophotometer in conjunction with the following accessories: constant temperature cell accessory (SP 870), electrical controller (SP 875), thermocouple (SP 872) and W.G.Pye "Scalamp" Thermocouple Galvanometer (SP 873). Solutions of native DNA in 4 x S.S.C. were heated from 40°C to temperatures of up to 98°C over a period of up to approximately 1 hour. Complete spectra over the wavelength range of 230 mµ to 350 mµ were frequently recorded and percentage hyper-

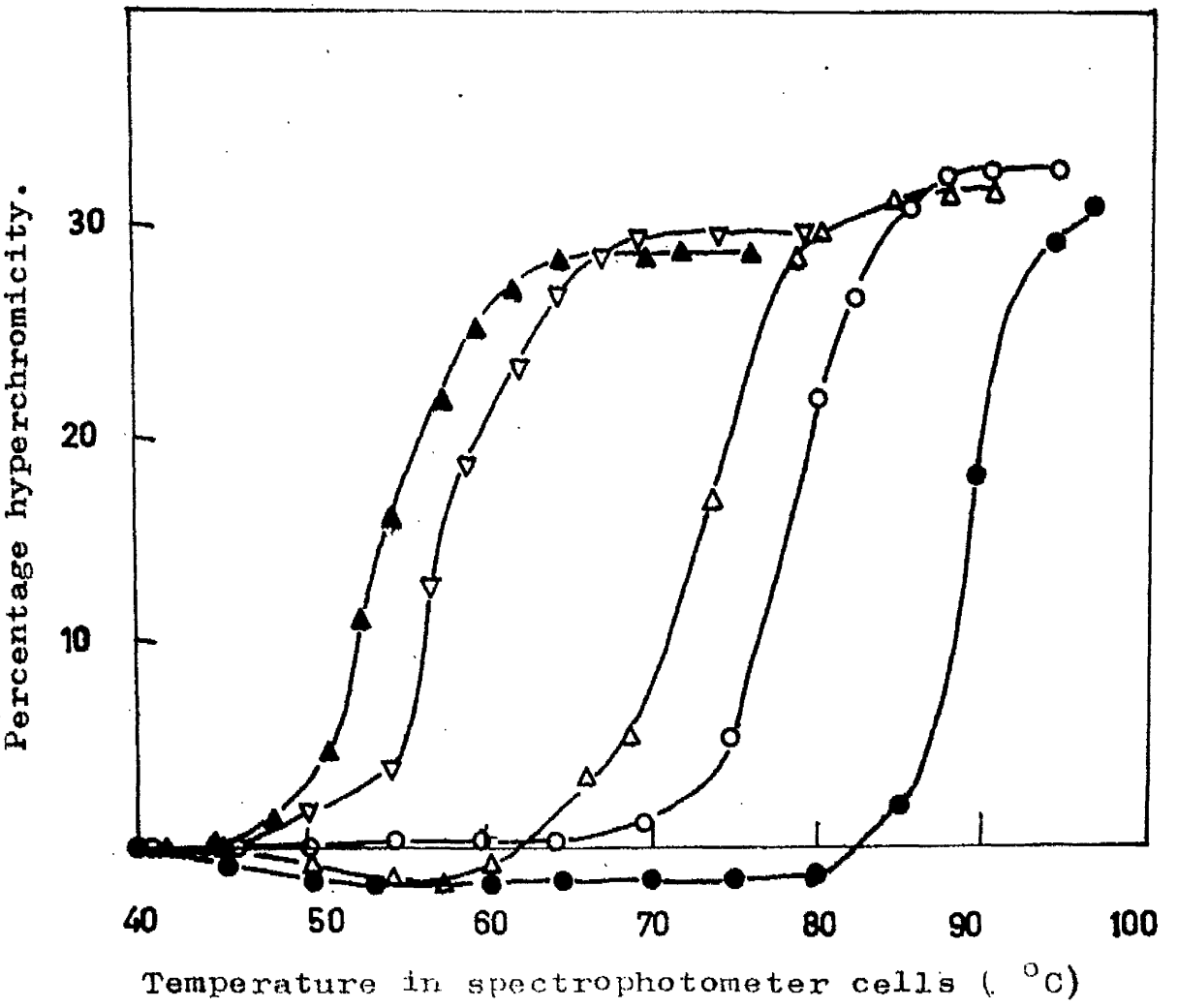
chromicities at the wavelength of maximal absorption in the range of 260 mμ to 265 mμ were plotted against the actual temperature of solutions in the spectrophotometer cells. These were determined from a previously constructed calibration curve. In no case was an increase in real absorbancy at 350 mμ observed. Absorbancies were recorded relative to solvent blanks. The effects of formamide (B.D.H. reagent) and dimethyl sulphoxide (D.M.S.O., B.D.H. reagent) on the melting of native DNA were investigated (Figures 10a, b, c, and 11). The melting temperature (Tm) of DNA under defined conditions was taken to be the temperature at which half of the final hyperchromicity was observed.

6.3. Application of Denatured DNA to Nitrocellulose Filters and Related Studies.

Application of denatured DNA to nitrocellulose Filters. A procedure based on that of Gillespie and Spiegelman (1965) was used. Nitrocellulose filters (Sartorius Membranfilter, format MF 50, catalogue No. 11006, 27 mm. diameter, supplied by V.A.Howe & Co.Ltd., London) were cut to circles of diameter 1.3 cm. One concentration of S.S.C. was used throughout the following steps. The filters were pre-soaked in S.S.C. (5 ml./filter) for 15 minutes to 1 hour. 10 ml. of

**Figure 10a.** Effect of Formamide on the Melting of Calf Thymus DNA dissolved in 4 x S.S.C.

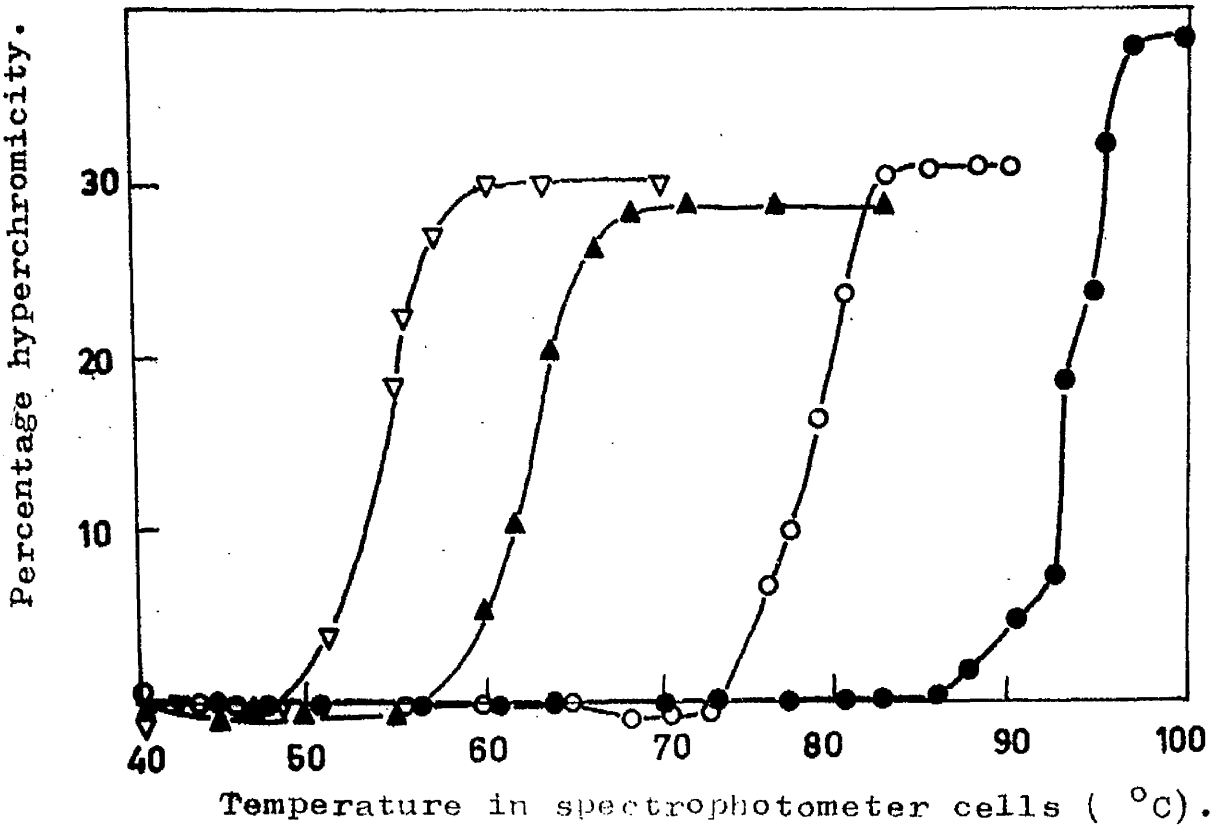
**Solutions of calf thymus DNA were heated over the temperature ranges shown in the constant temperature accessory in a Unicam SP 800 spectrophotometer. Extinctions at the wavelength of maximal absorption in the range 260 mμ - 265 mμ were recorded against solvent.**




●——● 0% v/v Formamide, Tm = 90°C.
○——○ 20% v/v " , Tm = 79°C.
△——△ 35% v/v " , Tm = 74°C.
▽——▽ 50% v/v " , Tm = 58°C.
▲——▲ 64% v/v " , Tm = 54°C.

**Figure 10b.** Effect of Formamide on the Melting of Landschutz Ascites Tumour Cell DNA dissolved in $\frac{1}{4}$ x S.S.C.

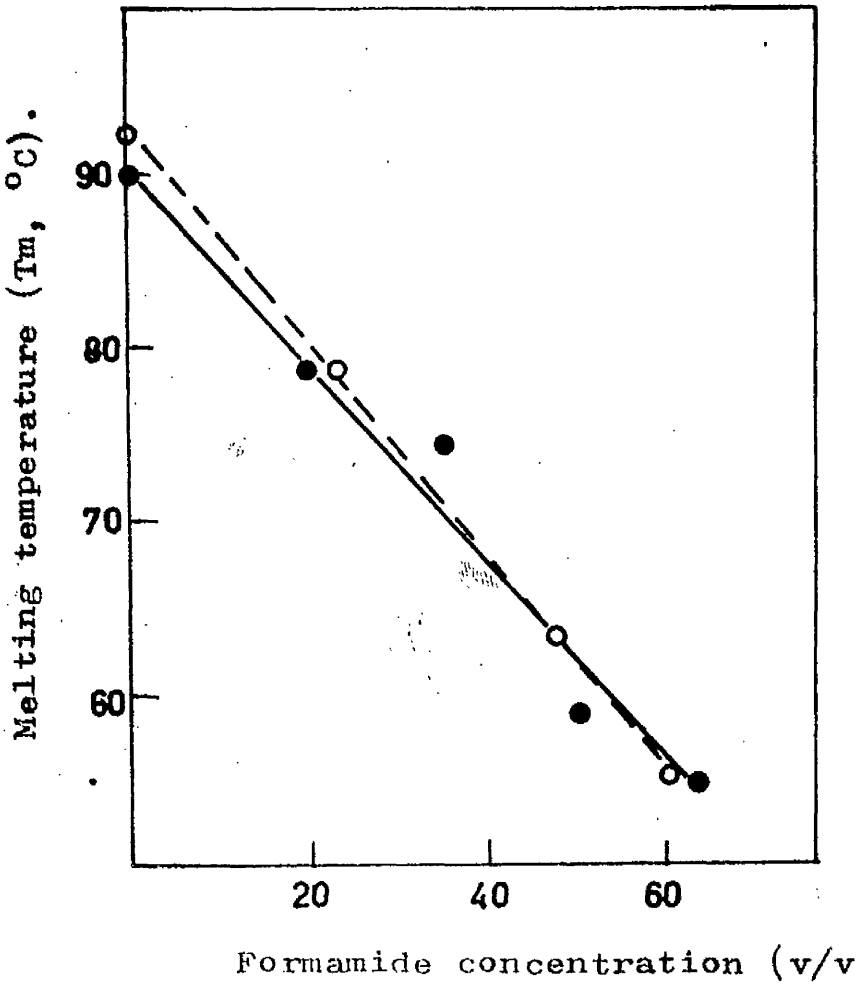
**Solutions** of Landschutz Ascites Tumour Cell DNA **were heated** over the temperature ranges shown in **the constant** temperature accessory in a Unicam SP 800 spectrophotometer. Extinctions at the wavelength of maximal absorption in the range of 260 mμ -265 mμ were recorded against solvent.




●——● 0% v/v Formamide, Tm = 93°C.
○——○ 23.5% v/v " , Tm = 79°C.
▲——▲ 47% v/v " , Tm = 63°C.
▽——▽ 59% v/v " , Tm = 55°C.

**Figure 10c.** Effect of Formamide on the Melting of Calf Thymus and Landschutz Ascites Tumour Cell DNA dissolved in 4 x S.S.C.

**Solutions of DNA in 4 x S.S.C. were heated in the constant temperature accessory in a Unicam SP 800 spectrophotometer. Extinctions at the wavelength of maximal absorption in the range of 260 mμ - 265 mμ were recorded against solvent blank.**

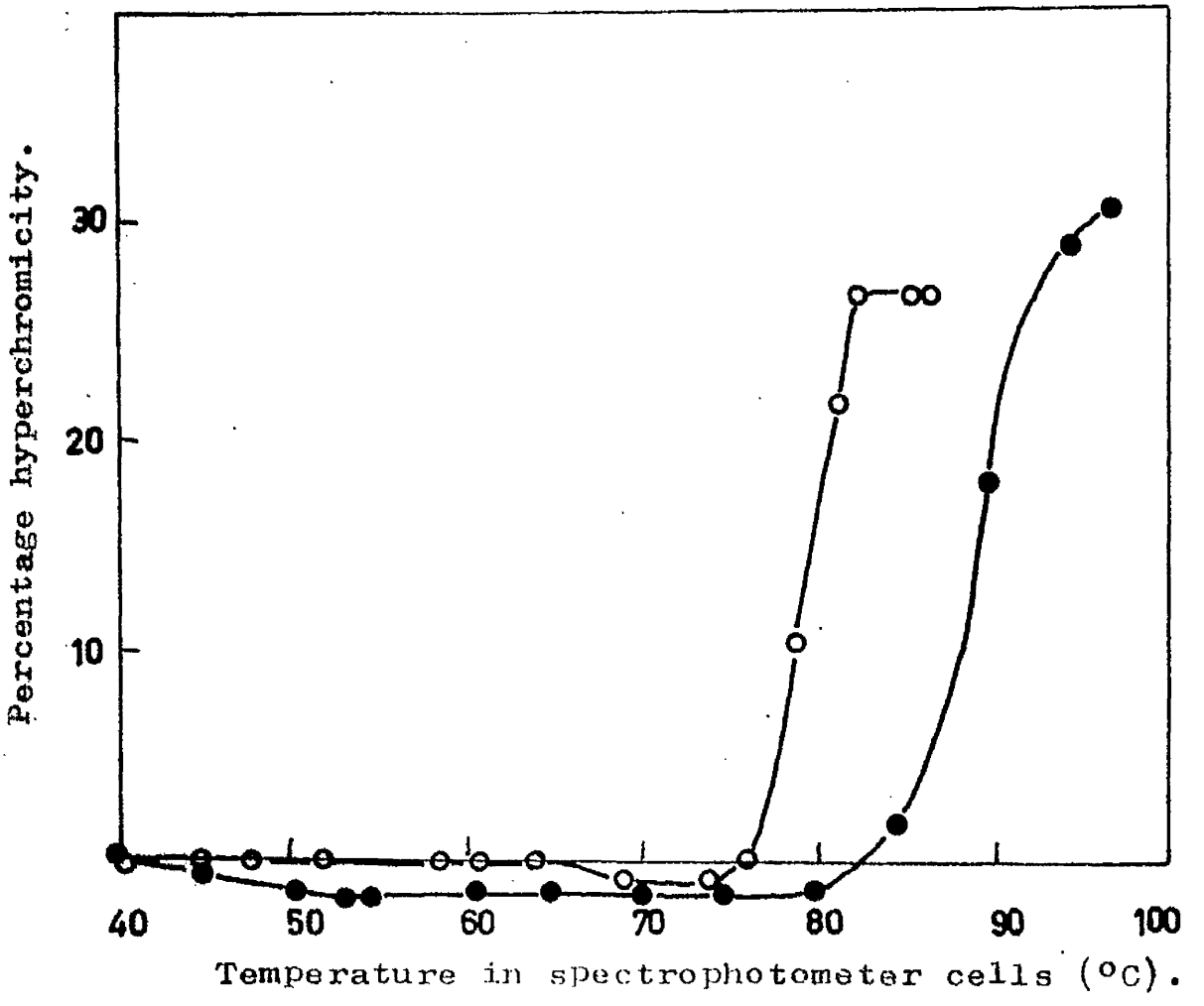



●—● Calf thymus DNA.

○—○ Landschutz Ascites Tumour Cell DNA.

**Figure 11.** Effect of Dimethyl Sulphoxide on the Melting of Calf Thymus DNA dissolved in ¼ x S.S.C.

**Solutions of calf thymus DNA were heated over the temperature ranges shown in the constant Temperature accessory in a Unicam SP 800 spectrophotometer. Extinctions at the wavelength of maximal absorption in the range 260 mμ - 265 mμ were recorded against solvent.**




●——● 0% v/v D.M.S.O., Tm = 90°C.

o——o 20% v/v " , Tm = 79°C.

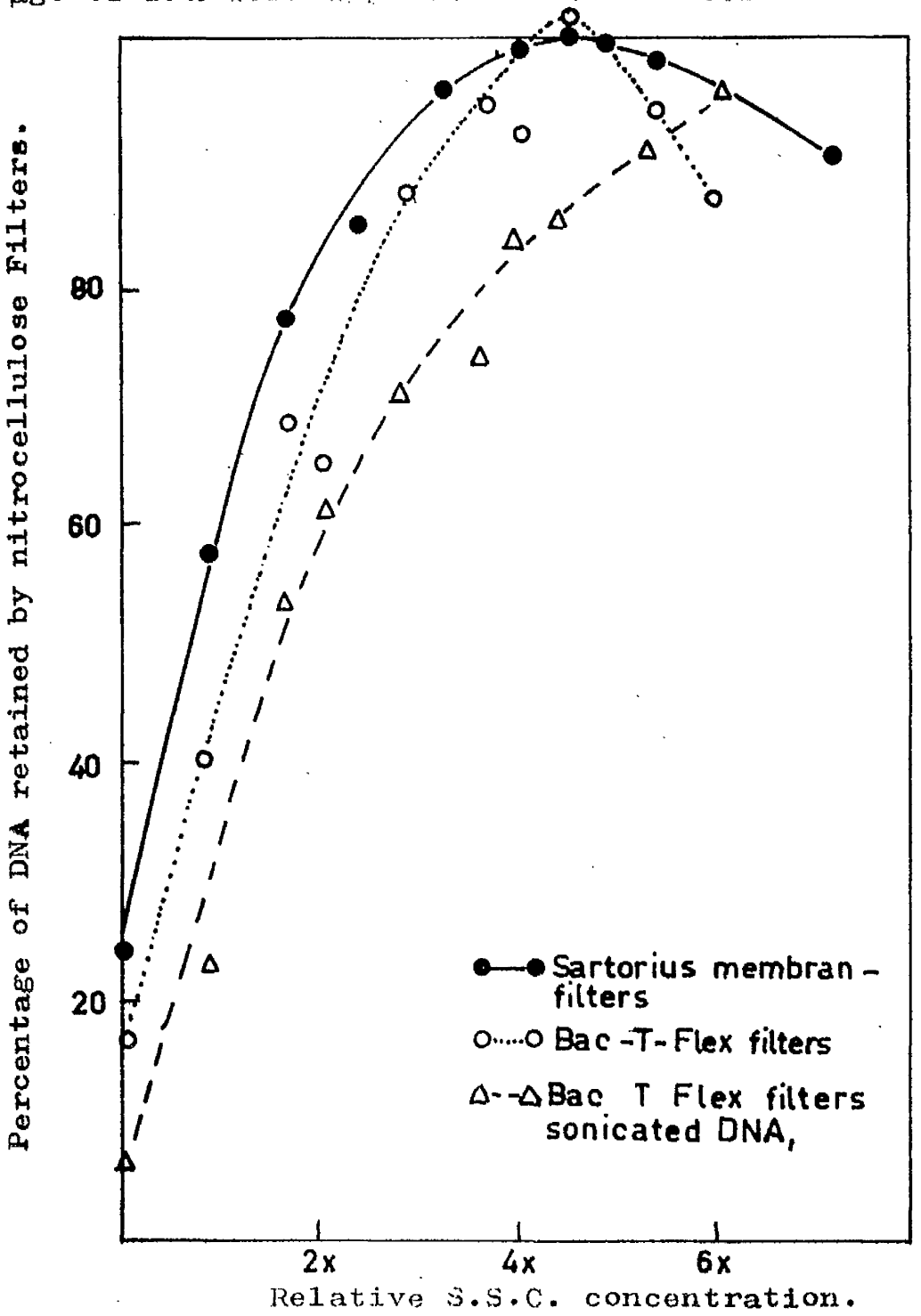
S.S.C. were then drawn through each filter, followed by 5 ml. of S.S.C. including approximately 5 µg. of denatured DNA and thereafter a further 10 ml. volume of S.S.C.. The filters were inverted and another 10 ml. volume of S.S.C. was drawn through each of them. The rate of suction filtration was in the range of 2 ml./minute to 20 ml./minute.

Effect of S.S.C. concentration on the retention of denatured DNA on application to nitrocellulose filters. Tritiated Landschutz ascites tumour cell DNA was mixed with similar unlabelled material to final specific activities of hundreds of d.p.m./µg.. Some of this DNA was sonicated as already described and all of it was then thermally denatured. It was applied to nitrocellulose filters using various S.S.C. concentrations. The final washing of inverted filters was omitted from the process.

In the case of unsonicated DNA, retention was maximal in 4 x S.S.C., although the effect of S.S.C. concentration on the retention of sonicated DNA was different (Figure 12). To check how complete retention of DNA in 4 x S.S.C. was, effluents from filters to which both alkaline and thermally-denatured Landschutz ascites tumour cell DNA had been applied

**Figure 12.** Effect of S.S.C.Concentration on the Retention of Tritiated thermally denatured Landschutz Ascites Tumour Cell DNA on its application to Nitrocellulose Filters.

**DNA was applied to 13 mm. diameter nitrocellulose filters in the S.S.C. concentrations indicated and by the procedure described in the text. Approximately 5 µg. of DNA were applied to each filter.**



were examined. The effluents were dialysed overnight against 55 x volumes of deionised distilled water to remove sodium chloride and trisodium citrate. The aim of this was to facilitate liquid scintillation counting in dioxane-based scintillator. The diffusates were lyophilized and redissolved in 0.3 ml. water and counted in dioxane-based scintillator. As no radioactivity was detected in these it was concluded that there was complete or almost complete retention of alkaline and thermally denatured DNA in 4 x S.S.C. by the nitrocellulose filters. In all further routine washing of and application of DNA to nitrocellulose filters 4 x S.S.C. was used.

Alkaline and thermally denatured DNA were applied to Bac-T-Flex nitrocellulose filters (type B-6, lot No.266/4427, 27 mm. diameter, supplied by Schleicher and Schuell, Keene, New Hampshire) and to Sartorius Membranfilters, respectively, in 4 x S.S.C. and in 1 x S.S.C. The effects of various ways of washing the filters on the fraction of DNA retained by them are shown in Table 6.

Efficiency of liquid scintillation counting of tritiated DNA applied to nitrocellulose filters. Nitrocellulose filters in which both alkaline and thermally

## Table 6.

### Effect of using various Concentrations of S.S.C. in washing Nitrocellulose Filters in the Application of DNA to them.

Approximately 5 µg. quantities of tritiated Landschutz ascites tumour cell DNA were applied to each filter as described in the text. Alkaline -denatured DNA was applied to Bac-T-Flex filters and thermally denatured DNA was applied to Sartorius membranfilters.

| Type of Filter | S.S.C. Concentrations in which DNA was applied: First wash | Second wash | Percentage retention of DNA |
|---|---|---|---|
| Sartorius membran-Filters. | 2x | 2 x — | 76% |
| | 2 x | 2 x 2 x | 83% |
| | 4 x | 4 x 1 x | 98% |
| | 4 x | 4 x 4 x | 102% |
| Bac-T-Flex Filters. | 1 x | 1 x 1 x | 45% |
| | 4 x | 4 x 1 x | 91% |
| | 4 x | 4 x — | 87% |
| | 4 x | 4 x 4 x | 100% |

denatured tritiated Landschutz ascites tumour cell DNA were known to be completely retained were dried by heating for 1 hour at 83°C in open scintillation vials. On addition of 6.0 ml. of toluene-based scintillator to each vial these were placed in the Nuclear Chicago Mark 1 Liquid Scintillation Counter and allowed to stand at 2°C for 1 hour. Their count rates were then recorded and, by comparison of these with rates of counting of DNA solution with dioxane-based scintillator, the efficiency of counting of DNA applied in 4 x S.S.C. to nitrocellulose filters was established. It was in the range of 17% to 22%, the precise value depending on the condition of the counter. The above drying and counting procedures for nitrocellulose filters were used throughout this work.

When 10 µl. volumes of tritiated native DNA in 0.15 M sodium chloride, 0.15 mM trisodium citrate and 10 µl. and 20 µl. volumes of uridine-5-T (2,220 d.p.m. and 4,440 d.p.m. respectively) were "spotted" on to nitrocellulose filters which were then dried and counted, counting efficiencies were found to be in the region of 12%.

Effects of various compounds on the retention of denatured DNA on application to Nitrocellulose Filters.

Approximately 5 µg. quantities of alkaline-denatured tritiated Landschutz ascites tumour cell DNA were applied to nitrocellulose filters by the normal procedure using 4 x S.S.C.. The effects of ranges of concentrations of various compounds included in the 4 x S.S.C. and of 0.5 M sodium hydroxide on retention of this DNA were observed by measurement of the count rates of the nitrocellulose filters. Results of these studies are shown in Figures 13a - 13e.

Two types of sodium carboxymethylcellulose were investigated. B.D.H. material gave almost unmanageably viscous solutions in the higher region of concentrations used, but solutions of I.C.I. "Edifas" B grade B10 (I.C.I.Ltd., Stevenston, Ayrshire) material were less viscous and more manageable.

To make a solution of concentrations of up to 1.5% w/v sodium dodecyl sulphate (S.D.S.) in 4 x S.S.C. it was necessary to warm the mixtures, for 30 minutes at 66°C. These solutions were then utilised simultaneously and as rapidly as possible.

Urea was supplied by B.D.H. in "Analar" grade. 8 M urea was passed through a column of AG-501 - x8 (D) 20-50 mesh analytical grade ion exchange resin (Bio-Rad Labs., Richmond, California) before use in order

Figure 13. Effects of Various Compounds on the Retention of Tritiated Alkaline denatured Landschutz Ascites Tumour Cell DNA on application to nitrocellulose Filters.

Figure 13a: Formamide.

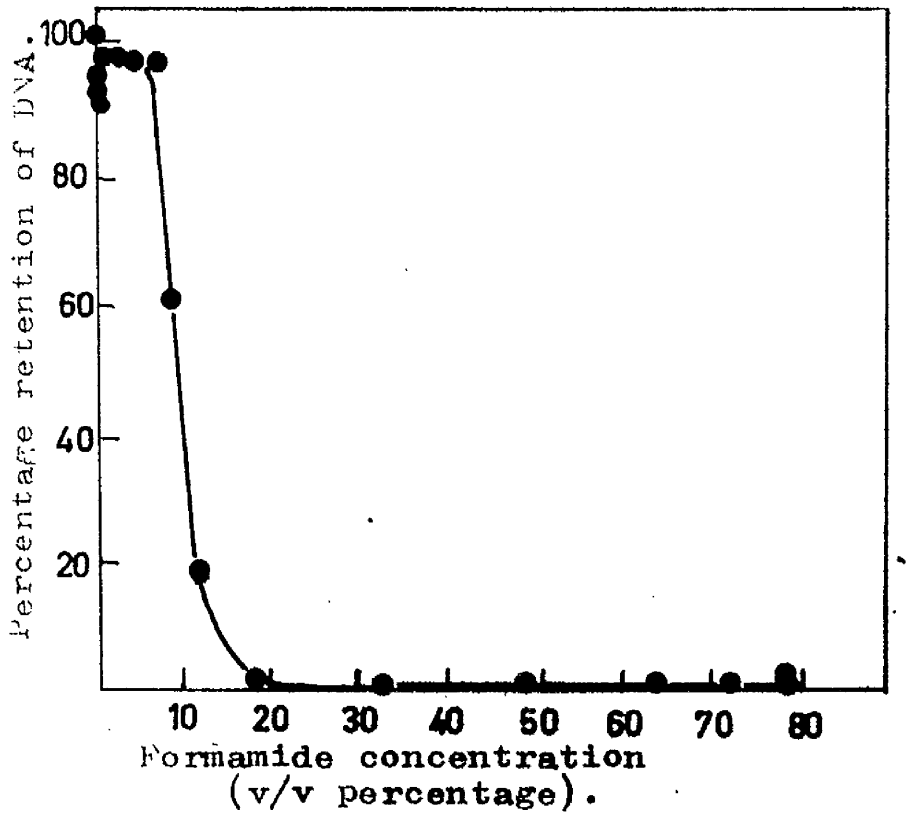



Figure 13b: Sodium carboxymethyl cellulose.

Two types of sodium carboxymethylcellulose were studied. B.D.H., which gave extremely viscous solutions, and I.C.I."Edifas" B, grade B10, which gave much less viscous solutions.

●——● I.C.I.

o---o B.D.H.

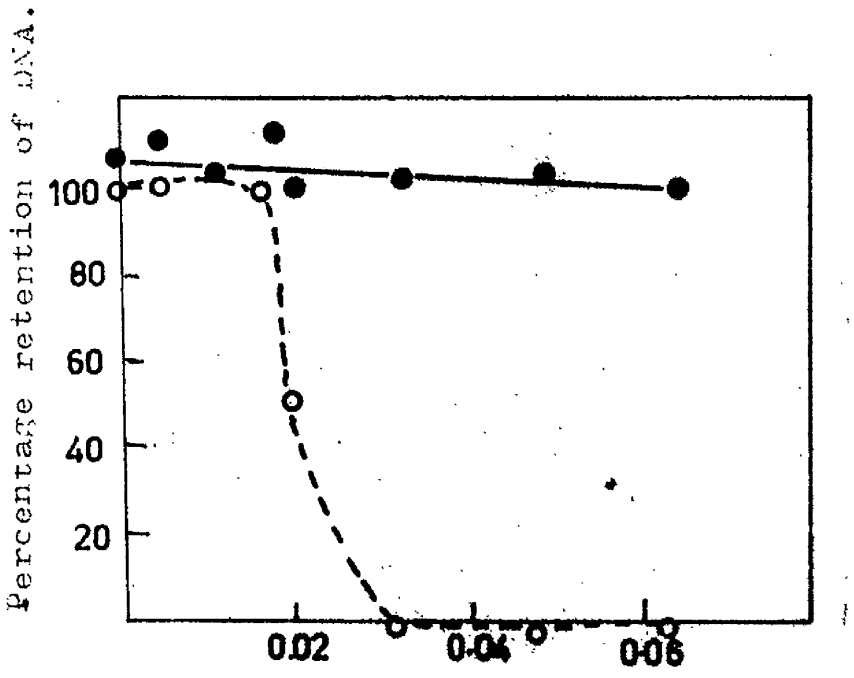


Figure 13c: S.D.S.

Mixtures were warmed at 65°C for 30 minutes. The resulting solutions were applied to the nitrocellulose Filters simultaneously and as rapidly as possible.

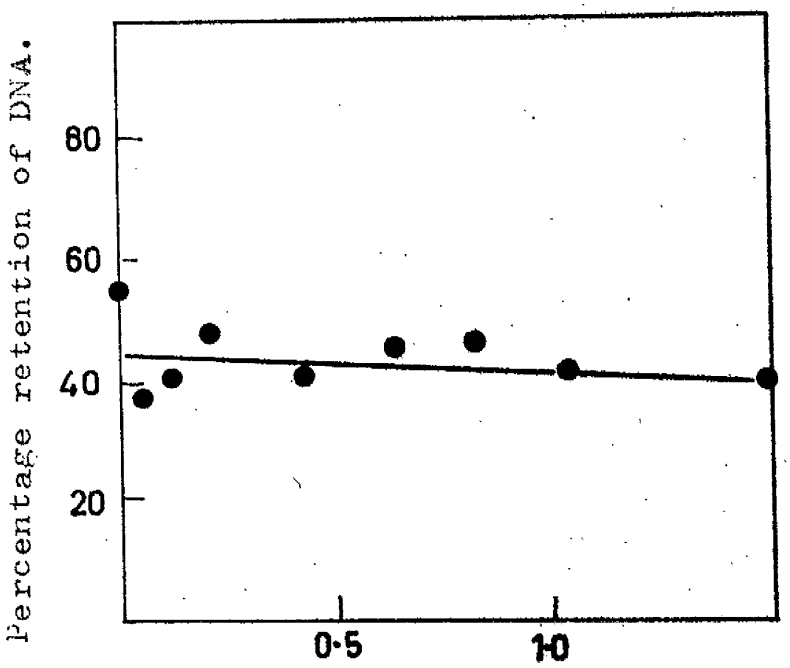



Figure 13d: Sucrose.

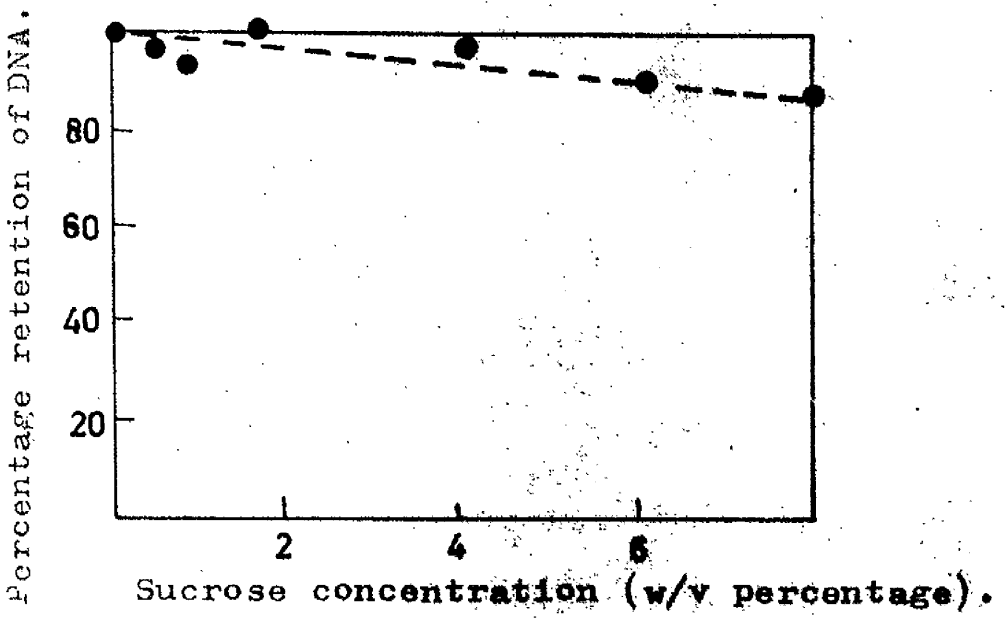
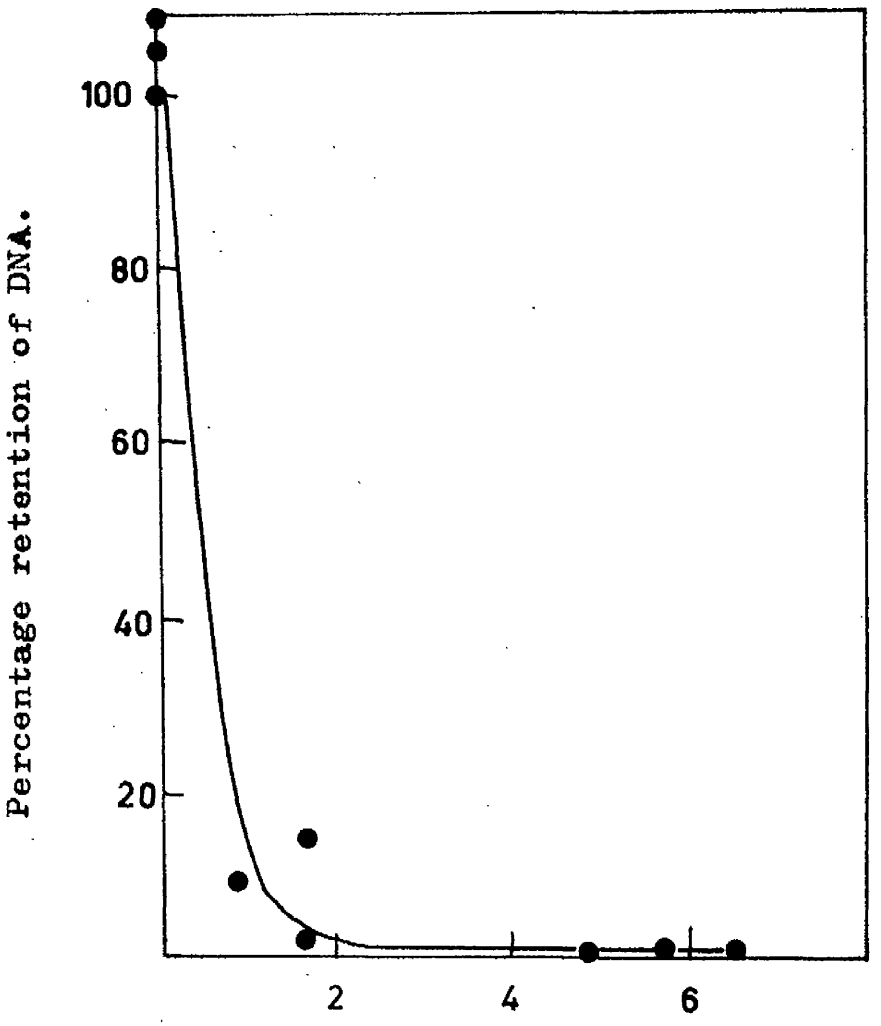


**Figure 13e:** Urea.



to remove any cyanide present.

This DNA was also applied to nitrocellulose filters in 0.5 M sodium hydroxide, as on alkaline denaturation without neutralisation of the DNA solution. The effect of making this solution to 4 x S.S.C. was also investigated. There was no retention of DNA applied to nitrocellulose filters in the alkaline solutions described (Table 7).

6.4. Attempted removal of ribonuclease activity from isolated calf thymus DNA.

Ribonuclease activity was detectable in calf thymus DNA isolated by the above procedure, including incubation with bovine pancreatic ribonuclease but omitting treatment with S.D.S. after incubation with pronase. Attempts were made to remove this ribonuclease activity from the DNA before its use as a template in the synthesis of RNA in vitro and in DNA-RNA hybridization studies.

50 ml. of calf thymus DNA of concentration 300 µg./ml. in 0.15 M sodium chloride, 0.05 M tris/hydrochloric acid buffer solution, pH = 7.4 were passed through a column of Sephadex C-25 Cation Exchange Resin (supplied by Pharmacia, Uppsala, Sweden) equilibrated with this solvent at room temperature. The DNA was quantitatively

Table 7.

Effect of Alkali on the Retention of Denatured DNA on application to Nitrocellulose Filters.

| Procedure of Application of Alkaline Denatured DNA to Nitrocellulose Filters. | Percentage Retention of DNA. |
|---|---|
| Normal procedure. | 99% |
| As normal with sodium hydroxide present at 0.5 N concentration. | 0% |
| Normal volumes but S.S.C. entirely omitted and sodium hydroxide present at 0.5 N concentration. | 0% |

recovered but ribonuclease activity was still readily detectable in it.

It was therefore mixed with and allowed to stand overnight at room temperature in 2% w/v S.D.S., 0.15 M sodium chloride, 0.05 M tris/hydrochloric acid buffer solution, pH = 7.4. On the following day solid sodium chloride was added to this solution to a final concentration of 1 M. The mixture was gently stirred to dissolve the sodium chloride and gentle stirring was continued for 30 minutes at room temperature. DNA was recovered from this by treatment with chloroform and other subsequent steps as in the last stages of the normal isolation procedure for DNA. There was no detectable ribonuclease activity in this DNA.

Calf thymus DNA was therefore routinely isolated by the procedure involving two treatments with S.D.S.. When incubation with bovine pancreatic ribonuclease was included in the procedure there was very little or no detectable ribonuclease activity (equivalent to 0 to 7 mµg. of bovine pancreatic ribonuclease/mg. of the DNA) in it and no polynucleotide phosphorylase activity.

When such DNA denatured by alkali containing small quantities of ribonuclease activity was incubated at 40°C for 45 minutes in 0.15 M sodium iodoacetate

pH = 5.9, iodoacetic acid, 0.005 x S.S.C. and subsequently recovered by precipitation with propan-2-ol and subsequent steps in the normal isolation procedure, it was found to contain approximately 60% of its previous quantity of ribonuclease (based on equivalent to bovine pancreatic ribonuclease activity).

Ribonuclease could not be detected in calf thymus DNA isolated by the normal procedure omitting incubation with bovine pancreatic ribonuclease. Polynucleotide phosphorylase activity was not detected in any sample of purified calf thymus DNA.

6.5. Ultracentrifugal Sedimentation of DNA.

A Beckman Spinco Model E Analytical Ultracentrifuge was used to study the sedimentation of DNA in solution. 1 ml. volumes of DNA solutions whose concentrations were in the range of 0.1 mg./ml. to 1.0 mg./ml. of 0.15 M sodium chloride, 0.15 mM trisodium citrate were placed in a cell with a 4°Kel-F centrepiece. This was inserted into an An-D rotor which was rotated at an angular velocity in the range of 42,000 r.p.m. to 51,000 r.p.m. (approximately 150,000 x g) for a period of 40 minutes. Schlieren photographs of the contents of the cell were taken at 8 minute intervals. Rotor temperatures were in the

region of 18°C during such sedimentations. Sedimentation coefficients were calculated from the formula

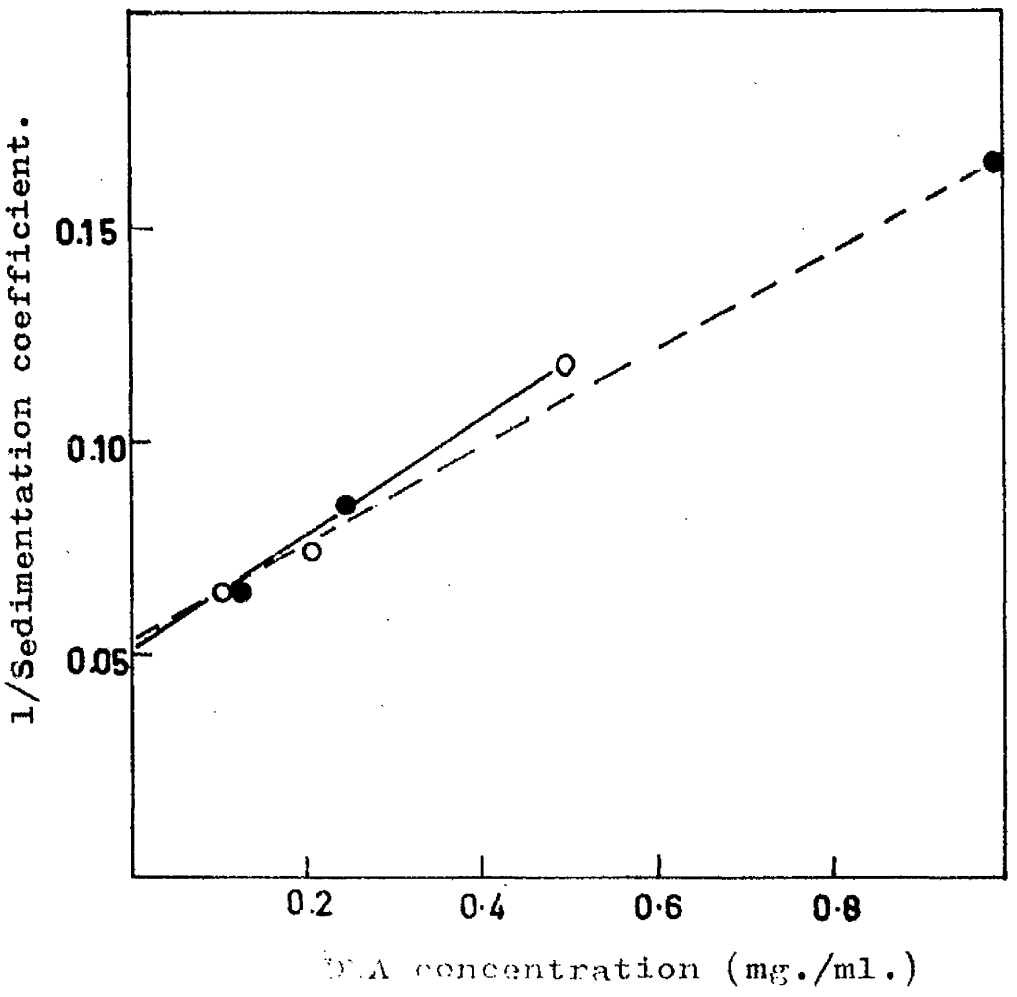
$$S = \frac{2.303}{\left(\frac{2\pi \times r.p.m.}{60}\right)^2} \cdot \frac{d \log r}{dt} \cdot 10^{13}$$

where S = sedimentation coefficient in Svedberg units, r = distance from centre of rotation to sedimenting boundary, and t = time for which sedimentation has been in progress.

Sedimentation Coefficients of Native Calf Thymus DNA. A single distinct peak was observed in Schlieren photographs taken during the sedimentation of native calf thymus DNA in solution. Sedimentation coefficients were recorded over a range of concentrations of one sample of DNA and their reciprocals were plotted against DNA concentration. The linearity of the relationship between these is shown in Figure 14, and this makes possible the estimation of the sedimentation coefficients at infinite dilution of samples of DNA.

The sedimentation characteristics and coefficients of typical samples of native calf thymus DNA in whose isolation incubation with ribonuclease was included and not included were not markedly different, being 18.5 and 19.5 S respectively on correction for solvent

**Figure 14.** Ultracentrifugal Sedimentation of Native Calf Thymus DNA.




o——o DNA from whose isolation procedure incubation with ribonuclease was omitted.

●– – –● DNA in whose isolation procedure incubation with ribonuclease was included.

viscosity and being adjusted to 20°C.

Ultracentrifugal determination of molecular weights of typical samples of native calf thymus DNA. The corrected sedimentation coefficients described above are denoted by $S^{o}_{20,w}$ and from them molecular weights can be determined by the formula

$$S^{o}_{20,w} = 0.034 \cdot M^{0.405} \quad \text{(Eigner and Doty, 1965)}$$

The molecular weights of the typical samples of native calf thymus DNA in whose isolation incubation with ribonuclease was included and not included were thus found to be $5.7 \times 10^6$ and $6.5 \times 10^6$ respectively.

Sedimentation of alkaline-denatured tritiated Landschutz ascites tumour cell DNA. This was sedimented at a concentration of 0.11 mg./ml. of 4 mM NaCl as described above. Again, one distinct peak was seen in Schlieren photographs taken during its sedimentation. Its sedimentation coefficient under these conditions was found to be 1.68 S. This DNA was that used in studies on retention of denatured DNA by nitrocellulose filters.

## 6.6. Separation of single-stranded and reannealed Mouse DNA fragments.

Preparation of DNA fragments for reannealing. DNA was isolated from Landschutz ascites tumour cells. Incubation with ribonuclease was omitted from the procedure and the final product was dissolved in 0.15 M sodium chloride. Citrate was omitted from all solutions used in the dissolution of ethanol-precipitated DNA when prepared and during its preparation. The DNA solution was sonicated using an M.S.E. Ultrasonic Power Unit at 1.5 amperes with the tip of the probe just touching the surface of the solution. The solution was sonicated in an ice-bath for a total period of 1 minute. This was composed of three 20-second sonication periods interspersed by 1-minute cooling periods. Traces of citrate and EDTA were removed from the solution by passing it through a 30 cm. x 1 sq.cm. column of Sephadex G-50 (fine) at room temperature equilibrated with 0.1 M potassium chloride. 0.1 M potassium chloride was passed through the column and fractions of volume 1.2 ml. were collected. The flow rate through the column was maintained at about 1 ml./10 minutes. Fractions containing more than 0.05 $E_{260\ m\mu}$ units were combined

and the excluded volume contained all the DNA in 0.1 M potassium chloride.

These DNA fragments were alkaline-denatured using potassium hydroxide in place of sodium hydroxide.

Conditions of incubation for reannealing of fragments of denatured DNA. These were derived from the studies of Britten and Kohne (1968). It was taken that fragments of whole mouse DNA consist of a rapidly-reannealing fraction, in which there are about $10^6$ copies of each nucleotide sequence, a fraction which can reanneal at an intermediate rate, in which there are $10^3$ to $10^5$ copies of each nucleotide sequence, and a slowly-reannealing fraction in which each nucleotide sequence is unique or is repeated only a very few times. These fractions were taken to represent approximately 10, 20 and 70 per cent of the total DNA.

$C_0t$ values in moles.sec./l required for complete reannealing of fractions capable of reannealing at fast and intermediate rates ("fast" and "intermediate" DNA) were taken to be $10^{-1}$ and 500 respectively with respect to whole DNA concentration (Britten and Kohne, 1968). Incubations were at 67°C in 0.5 M potassium chloride, 0.01 M tris/hydrochloric acid buffer solution, pH = 7.4, and were terminated by dilution with 4 volumes

of ice-cold deionised distilled water. The remaining non-renatured DNA is referred to as "slow" DNA.

Separation of single-stranded and reannealed fragments. A 2 cm. x 1 sq.cm. column of hydroxyapatite (Hypatite C, Clarkson Chemical Co., Williamsport, Pennsylvania) was equilibrated with 0.1 M potassium chloride at 70°C. Approximately 1 ml. of hydroxyapatite was used per 1.25 mg. DNA applied. Its temperature was maintained throughout the following procedure at 70°C. Great care was taken to prevent deformation of the column by air bubbles. This included bringing all salt and buffer solutions to boiling shortly before passing them through the column.

The sample containing DNA fragments was applied to the column in 0.1 M potassium chloride, which was passed through the column until the extinction at 260 mμ of the eluate was at baseline. This was continuously recorded using a Uvicord 4701A control unit in conjunction with a Y.E.W. Laboratory Recorder. To elute single-stranded DNA fragments from the hydroxyapatite, 0.1 M potassium chloride, 0.12 M potassium phosphate buffer solution, pH = 6.6, was passed through it (Hell, 1969; Flamm, McCallum and Walker, 1967; McCallum and Walker, 1967) until the absorbancy of the eluate at

260 mμ was again at baseline. 0.1 M potassium chloride, 0.25 M potassium phosphate buffer solution, pH = 6.6, was similarly used to elute reannealed DNA fragments from the column. Fractions of the eluate were regularly collected. Adjacent fractions containing peaks of material absorbing at 260 mμ were combined.

To remove phosphate from eluates which were to be fractionated again after further incubation to achieve reannealing, these were dialysed for two successive 24 hour periods against 100 X volumes of deionised distilled water at 4°C. Samples containing fractionated DNA fragments were stored at -70°C.

The percentages of input DNA which the "fast", "intermediate" and "slow" fractions, as judged by the above criteria, made up were, respectively, 8%, 15% and 77%.

6.7. <u>Alkaline "denaturation" of "fast", "intermediate" and "slow" fractions of DNA of Landschutz Ascites Tumour Cells and their application to nitrocellulose filters.</u>

The normal procedure of alkaline denaturation was followed and was carried out in all three cases in as like a manner as possible. Hyperchromicities observed were as in Table 8.

Table 8.

Hyperchromicities observed in the Denaturation of Fractions of Landschutz Ascites Tumour Cell DNA.

| Fraction of DNA | Percentage hyperchromicities | |
|---|---|---|
| | On addition of sodium hydroxide | On neutralisation |
| "fast" | 53% | 35% |
| "intermediate" | 18% | 10% |
| "slow" | 0% | 28% |

The procedure of application of denatured DNA to nitrocellulose filters was also the normal one. "Fast" DNA was applied to filters within 5 minutes of its neutralisation. On application of "fast" DNA at a concentration of 5 µg./ml. (so that ultraviolet extinction readings were readily made) 75% was estimated to be filter-retainable from the ultraviolet extinction of the filtrate.

## 7. Isolation of RNA.

### 7.1. Synthesis of RNA *in vitro* and its purification.

The components of RNA-synthesizing mixtures were essentially those of standard RNA polymerase assay mixtures. UTP-5-T was added such that the final specific activity of UTP in RNA-synthesizing mixtures was in the region of $10^4$ dpm/mμmole UTP, or entirely omitted when non-radioactive RNA was required. The mixtures contained 0.1 M tris/hydrochloric acid buffer solution, 2.5 mM manganese chloride, ATP, GTP, CTP and UTP (all 0.8 mM), 0.03 M putrescine dihydrochloride, approximately 250 μg./ml. of DNA template, present as purified DNA or in chromatin, 50 μg./ml. of purified macaloid and approximately 50 units/ml. of RNA polymerase prepared from *Micrococcus lutous*. They were routinely incubated for 90 minutes at 37°C and were terminated by chilling in an ice-bath or at -20°C.

The procedure of purification of any RNA thus synthesized was designed to remove all other materials from RNA-synthesizing mixtures. Mixtures containing chromatin were centrifuged at 1,500 g for 5 minutes at 0°C to remove insoluble material. All solutions were then incubated in the presence of 30 μg./ml. of deoxyribonuclease for 30 minutes at 37°C.

Equal volumes of 90% w/v phenol (B.D.H. "Analar" grade reagent, redistilled) solution were added to the incubation mixtures. The resulting mixtures were thoroughly mixed for 1 minute at room temperature, allowed to stand in ice for 1 minute and similarly mixed for a further 1 minute. They were then centrifuged at 1,500 *g* for 5 minutes at 0°C to precipitate denatured protein from the aqueous phases. These were withdrawn and shaken with further volumes of phenol at room temperature and again centrifuged at 1,500 *g* for 5 minutes at 0°C. This was continued until there was no visible interface of denatured protein on centrifugation. The original volume of the aqueous phase was maintained by addition of 0.01 M tris/hydrochloric acid buffer solution, pH = 7.4, 1 mM magnesium chloride.

To remove low molecular weight materials from synthetic RNA preparations they were then dialysed overnight at 4°C against 1000 X volumes of 0.1 mM tris/hydrochloric acid buffer solution, pH = 7.4, 1 mM magnesium chloride, 0.01% macaloid.

The dialysates were concentrated by complete lysophilization and dissolution in small volumes of 0.1 M tris/hydrochloric acid buffer solution, pH = 7.4. Resulting solutions were stored at -70°C to prevent

effectively the loss in ability of synthetic RNA to form DNA-RNA hybrids observed on its storage at -15°C. Their ultra-violet absorption spectra were typical of those of nucleic acids and, taking the specific extinction coefficient of RNA at a wavelength of 260 mμ to be 0.022, their specific activities were in the region of $10^4$ dpm/μg. RNA. Apparent yields of synthetic RNA were generally in the range of 30% to 50% of the quantity of DNA template present in the RNA synthesizing mixture.

Efficacies of manganous and magnesium ions as co-factors in the degradative action of DNase I. Solutions of calf thymus DNA of concentration 200 μg./ml. of 0.07 M tris/hydrochloric acid buffer solution, pH = 7.4 were incubated with DNase I at a concentration of 25 μg./ml. for 30 minutes at room temperature. Incubation mixtures including no cation, 2.5 mM magnesium chloride or 2.5 mM manganous chloride were set up. After incubation, the mixtures were chilled in an ice-bath and 2.0 ml. volumes of ice-cold 2 M P.C.A. were added to them. The resulting precipitates were sedimented by centrifugation at 1,500 g for 10 minutes at 0°C. The extinctions at 260 mμ of the resulting supernates were taken to represent the quantity of DNA degraded to oligo deoxyribonucleotides (acid-soluble extinction units).

The precipitates were extracted in 4.0 ml. volumes of 1 M P.C.A. by heating at 70°C for 30 minutes. The mixtures were then chilled in an ice-bath and centrifuged at 1,500 g for 10 minutes at 0°C. The extinctions at 260 mμ of the resulting supernates were taken to represent the quantity of less extensively degraded DNA remaining after incubation with DNase I (acid-insoluble extinction units).

Almost complete degradation of DNA into oligodeoxyribonucleotides was observed in the presence of 2.5 mM magnesium chloride or 2.5 mM manganous chloride (Table 9).

7.2. Preparation and purification of polysomal RNA from Escherichia coli.

Preparation of polysomes. Cells of Escherichia coli, strain MRE 600 (a ribonuclease-free mutant) supplied by The Microbiological Research Establishment, Porton, England, and stored at -70°C were blended into 2 volumes of ice-cold 1 mM magnesium chloride. The suspension was allowed to stand in an ice-bath for 10 minutes in order to effect their lysis. Lysed cell membranes and walls were sedimented from the suspension by centrifugation at 15,000 g for 10 minutes at 0°C. The supernate was centrifuged at 180,000 g for 1 hour at 0°C and the sediment obtained was taken to be polysomes.

Table 9.

Efficacies of Manganous and Magnesium Ions as Co-Factors in the Degradative Action of DNase I.

Incubation mixtures contained 200 µg./ml. of calf thymus DNA, 25 µg./ml. of DNase I and cations as indicated.

| Cation added. | Acid-soluble extinction units after incubation with DNase I. | Acid-insoluble extinction units after incubation with DNase I. | Total extinction units recovered after incubation with DNase I. | Percentages of Total Extinction Units recovered after incubation with DNase I in acid-soluble fraction. | Percentages of Total Extinction Units recovered after incubation with DNase I in acid-insoluble fraction |
|---|---|---|---|---|---|
| None | 1.68 | 10.7 | 12.4 | 13% | 87% |
| | 1.76 | 11.0 | 12.8 | 14% | 86% |
| 2.5 mM magnesium | 12.9 | 0.22 | 13.1 | 98% | 2% |
| | 13.2 | 0.20 | 13.4 | 98% | 2% |
| 2.5 mM manganous | 13.2 | 0.18 | 13.4 | 99% | 1% |
| | 13.2 | 0.18 | 13.4 | 99% | 1% |

Preparation of polysomal RNA. Polysomes were resuspended in a small volume of ice-cold 6% w/v sodium *p*-aminosalicylate (B.D.H. reagent). An equal volume of distilled phenol/m-cresol (B.D.H. reagent) /water/8-hydroxyquinoline (B.D.H. "Analar" grade reagent) 1000 : 150 : 100 : 1 w/w mixture was added to the suspension and the mixture was thoroughly shaken for 20 minutes at room temperature. It was then centrifuged at 15,000 *g* for 30 minutes at 0°C to sediment denatured protein. The aqueous phase was made 3% w/v with respect to sodium chloride and was thoroughly shaken with a half volume of the above phenol-containing mixture for 10 minutes at room temperature. The mixture was centrifuged at 15,000 *g* for 15 minutes at 0°C. 2 volumes of redistilled ethanol were mixed with the aqueous phase and this mixture was allowed to stand overnight at -20°C. The resulting precipitate was sedimented by centrifugation at 15,000 *g* for 20 minutes at 0°C and was allowed to drain. It was washed twice in the same volume of redistilled ethanol, centrifugation being at 15,000 *g* for 20 minutes in each case. It was then redissolved in 0.1 M tris/hydrochloric acid buffer solution, pH 7.4. Its absorption spectrum was typical of that of a nucleic acid solution and, from it, the yield of RNA was found

to be approximately 0.3 mg. per 1 g. of cells.

Purification of polysomal RNA. A solution of RNA in caesium chloride of specific gravity = 1.72 was centrifuged at 120,000 g for 50 hours at 25°C. Pellets thus obtained were washed with deionised distilled water and dissolved in 0.1 M tris/hydrochloric acid buffer solution, pH = 7.4. The absorption spectrum of the resulting solution was again typical of that of a nucleic acid solution and indicated approximately 50% recovery of RNA.

8. Demonstration of Enzymatic DNA-RNA Hybrid.

The procedure employed was based on that of Melli and Bishop (1969). Test and control *in vitro* RNA-synthesizing mixtures were set up. They contained 14 units/ml. of RNA polymerase and were incubated at 37°C for 90 minutes. Sonicated thermally-denatured Landschutz ascites tumour cell DNA was added to the test mixture before incubation and to the control mixture after incubation and on chilling in an ice-bath. In the former case it was added to a final concentration of 100 µg./ml, and in the latter to a concentration of 86 µg./ml., as the DNA made up 16% of the volumes of the incubation mixtures.

0.80 ml. and 0.40 ml. volumes of test and control

incubation mixtures, respectively, were diluted to 3.4 ml. in 0.2% w/v sodium dodecyl sulphate in plastic centrifuge tubes. These mixtures were incubated for 5 minutes at 32°C. 4.5 g. of caesium chloride were dissolved in each tube and they were centrifuged at 15,000 g for 15 minutes at 0°C to pack precipitated sodium dodecyl sulphate to the tops of the tubes.

The tubes were pierced and the resulting solutions were drained into ultracentrifuge tubes. By addition of not more than 0.25 ml. volumes of deionised distilled water the refractive indices of the solutions were reduced to 1.4015, equivalent to a density of 1.723 g. $cm^{-3}$. They were overlaid with thin layers of liquid paraffin and centrifuged for $10^7$ g hours at 15°C. The ultracentrifuge tubes were pierced and 10 drop fractions were collected by draining. Refractive indices of three fractions were determined in each case in order to determine the relationship between fraction number and buoyant density.

6.0 ml. of 2 x S.S.C. were mixed with each fraction derived from the test incubation and 3.0 ml. of 2 x S.S.C. were mixed with each fraction derived from the control incubation. The extinctions of the diluted fractions obtained from each were recorded at a wavelength of

260 mμ. The diluted fractions of volume 6.0 ml. were divided into two series of 3.0 ml. volumes. Ribonuclease was added to one set of diluted fractions derived from the test incubation to a concentration of 20 μg./ml. and the mixtures incubated at room temperature for 1 hour. Thereafter the solutions derived from all the fractions were applied to nitrocellulose filters as described in the procedure of DNA-RNA hybridization with both DNA and RNA in solution.

D.p.m. recorded on the filters, extinctions at 260 mμ of diluted fractions and buoyant density (derived from refractive indices) were plotted against fraction numbers (Figures 15a and 15b). The specific activity of UTP used in synthesizing RNA was 21,500 d.p.m. per mμmole.

## 9. DNA-RNA Hybridization Studies.

### 9.1. Procedure of DNA-RNA hybridization using DNA immobilised on nitrocellulose filters.

This procedure was based on that of Gillespie and Spiegelman (1965).

Approximately 5 μg. quantities of alkaline denatured DNA were applied to 13 mm. diameter nitrocellulose Sartorius Membranfilters of the type described in Section 6.3 and as described therein.

Figure 15a. Ultracentrifugal Sedimentation Profile of material of an RNA-synthesizing mixture which contained sonicated thermally denatured Landschutz Ascites Tumour Cell DNA as template.

o.....o D.p.m. recorded on filters not treated with ribonuclease.

●----● D.p.m. recorded on filters treated with ribonuclease.

Δ——Δ Absorbancy at 260 mμ.

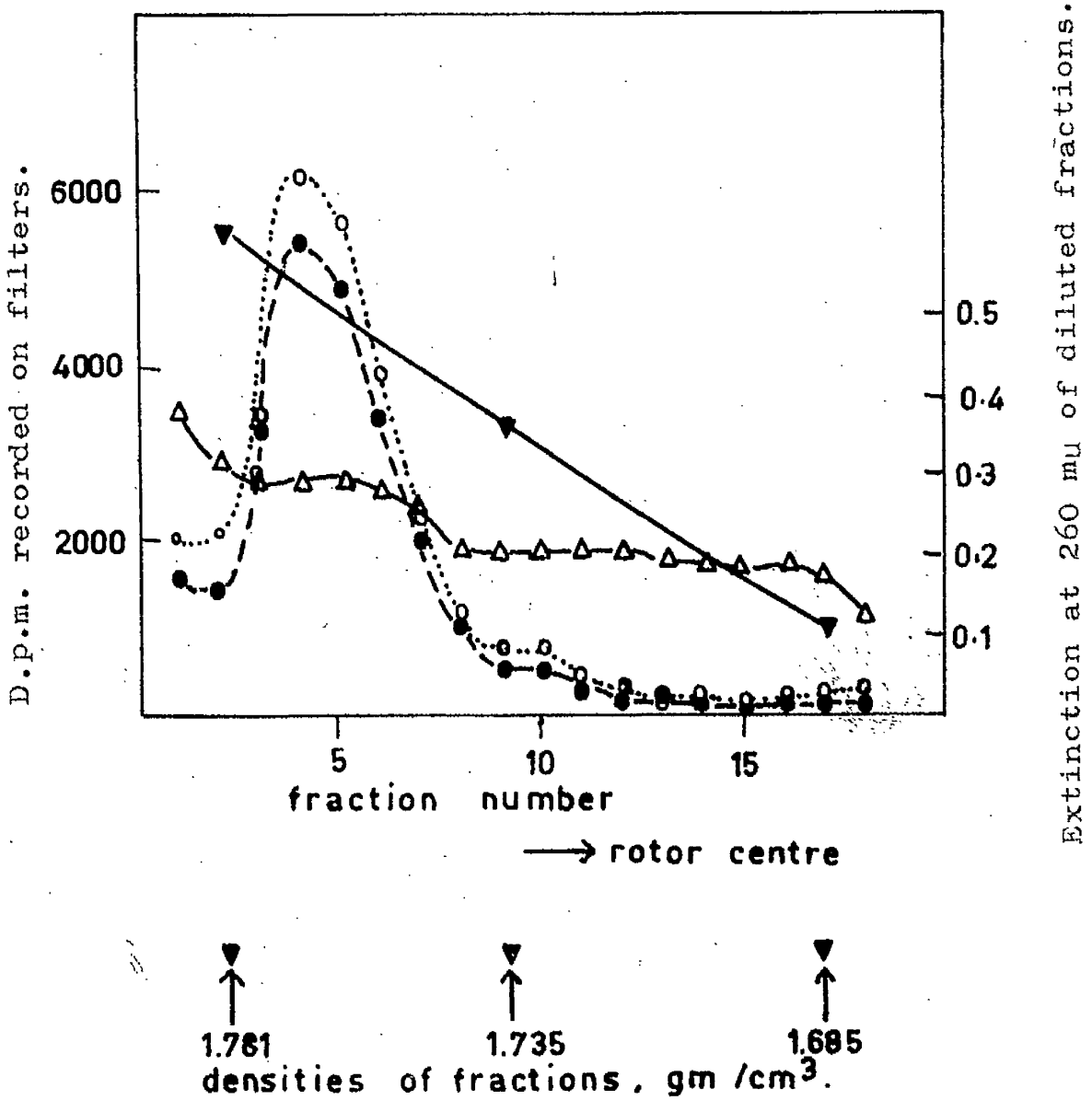
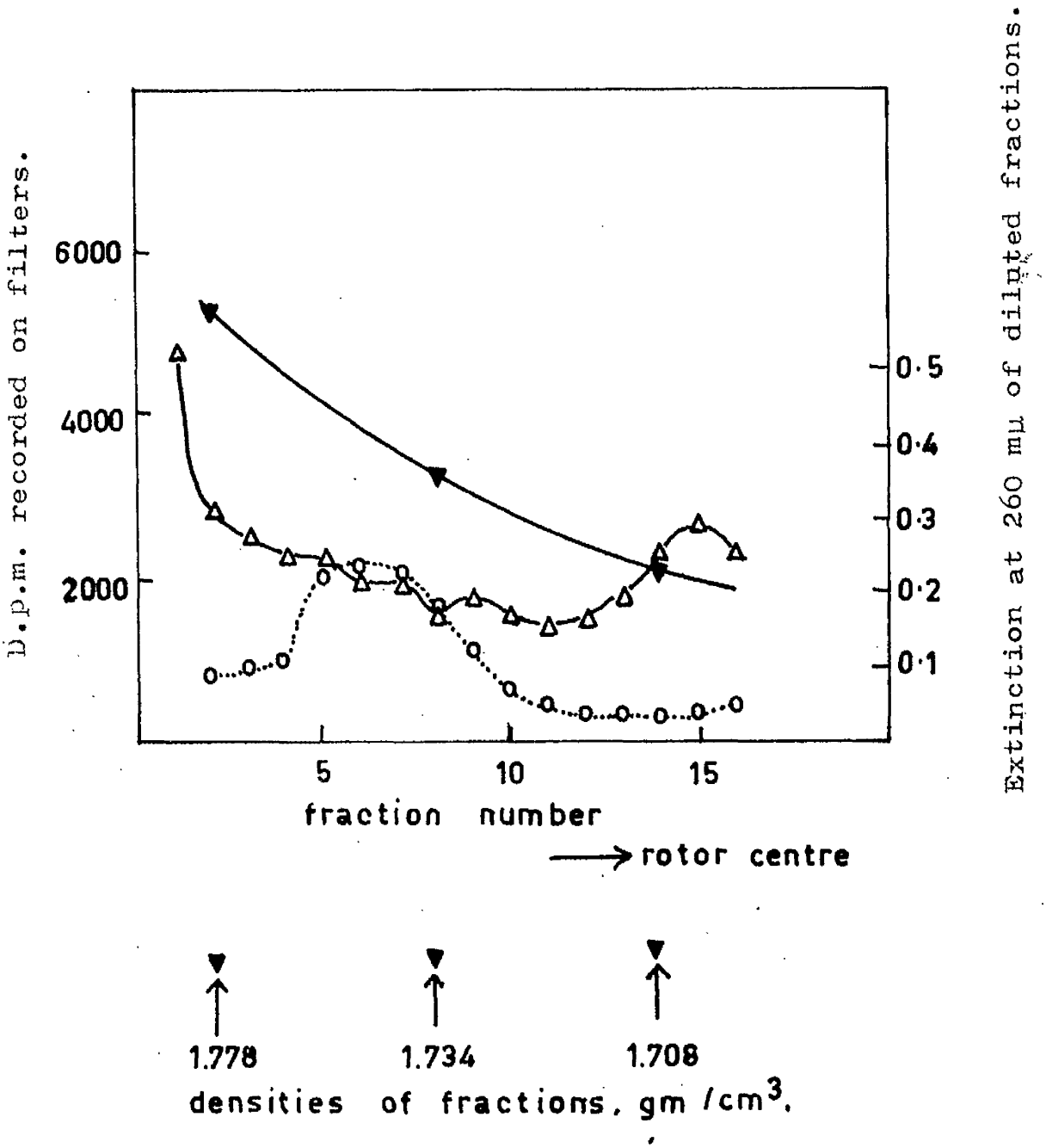


Figure 15b. Control to Figure 15a.

DNA was added after termination of the RNA-synthesizing incubation.

o·····o D.p.m. recorded on filters not treated with ribonuclease.

Δ——Δ Absorbancy at 260 mμ.



Those filters were pressed between Whatman No.1 filter papers and left overnight at room temperature and then baked at 83°C for 3 hours. They were kept pressed between these filter papers for periods of up to two weeks at room temperature. Filters bearing no DNA, but otherwise identical to these, were used as blanks. Filters were labelled on their edges (through which solutions were not drawn on suction filtration) using blue ink of a Bic fine point ball-point pen.

Drops of volume 75 µl. or 100 µl. containing tritiated DNA in ¼ x S.S.C. adjusted to pH = 7.0 were placed on the floor of plastic luncheon boxes. Two filters, one bearing DNA (the lower) and one blank (the upper) were placed within each drop, the upper being placed directly over the lower. The side of the filters to which a 5 ml. volume of ¼ x S.S.C. (containing DNA in the case of test filters) had been applied was the lower in all cases. As soon as every filter had been soaked in ¼ x S.S.C., the drops were covered by liquid paraffin (B.p., Evans Medical Ltd., Speke, Liverpool) and the luncheon boxes were placed in a 67°C oven and allowed to remain there for 17 hours.

On removing the luncheon boxes from the oven,

4 x S.S.C. was pipetted over the filters such that they could be removed through it. They were washed in successive volumes of 4 x S.S.C., usually four (approximately 2.5 ml./filter), until no paraffin appeared on the surface of the 4 x S.S.C. in which they had been washed.

Washed filters were immersed in a solution of ribonuclease, 20 µg./ml., in 2 x S.S.C. (approximately 5 ml./filter). They were allowed to remain in this solution at room temperature for 1 hour. During this period the solution was intermittently stirred.

On removal from this solution, the filters were washed in three successive volumes of 4 x S.S.C. (approximately 2.5 ml./filter) and were then washed in 4 x S.S.C. by suction filtration at a rate of between 2 ml. and 20 ml. per minute. Each side of every filter was washed by 20 ml. of 4 x S.S.C. in this procedure.

Filters were then dried for 1 hour at 83°C and their count rates determined using toluene-based scintillator, 6.0 ml./filter, as also described in Section 6.3. The efficiency of counting was assumed to be the same as that of tritiated denatured DNA applied to nitrocellulose filters as described in

that section (in the region of 20%).

The filters could be removed from toluene-based scintillator and made suitable for further DNA-RNA hybridization by washing in two successive volumes of toluene and two successive volumes of 4 x S.S.C. Approximately 10 ml. volumes of each were used per filter and each side was finally washed with 20 ml. of 4 x S.S.C. by suction filtration before being allowed to dry at room temperature.

Markings on filters almost always remained unchanged throughout all these procedures. When a filter was noticeably blue it was found to be contaminated by paraffin and to have an abnormally high count rate. Such filters were discarded.

9.2. Procedure of DNA-RNA Hybridization with DNA and RNA in Solution.

The procedure was based on the studies of Nygaard and Hall (1963, 1964).

Approximately 5 µg. quantities of DNA thermally denatured immediately before use and quantities of tritiated RNA were included in 100 µl. volumes of 4 x S.S.C. adjusted to pH = 7.0 in conical centrifuge tubes. Control solutions from which DNA was omitted were also set up and treated identically.

The tubes were sealed and incubated at 67°C for 2 hours to permit formation of DNA-RNA hybrids.

They were then placed in an ice-bath and, after standing for 2 minutes, 3.0 ml. volumes of 2 x S.S.C. at room temperature were added to each of them. Ribonuclease solution was mixed in to these solutions to a final concentration of 20 µg./ml. and they were allowed to stand for 1 hour at room temperature with intermittent agitation.

The S.S.C. concentration of the solutions was then increased from approximately 2 x to 4 x, by addition of 20 x S.S.C. They were then applied to 13 mm. diameter nitrocellulose Sartorius Membran filters in the same manner as that in which denatured DNA was applied to these filters in order to trap and estimate any DNA-RNA hybrid formed. The filters were dried and their count rates and hence the quantity of RNA bound to them were determined as in hybridization studies in which DNA was immobilised on filters. Filters were marked in the manner already described and markings remained completely intact throughout the procedure.

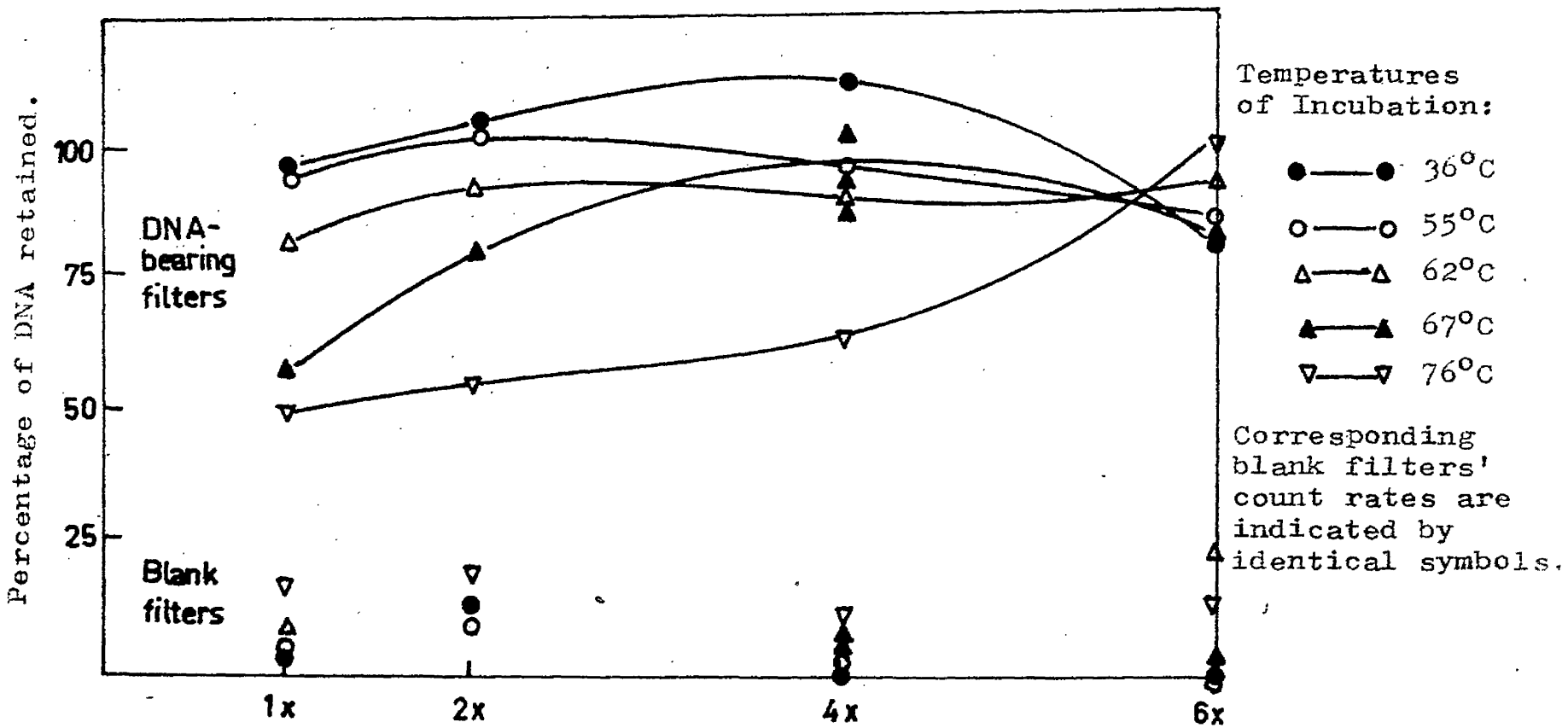
9.3. Effects of S.S.C. Concentration and Temperature of Incubation on the Retention of alkaline-denatured DNA applied to Nitrocellulose Filters during Hybridization.

4.4 µg. quantities of alkaline-denatured tritiated Landschutz ascites tumour cell DNA were applied in 4 x S.S.C. to the filters, by which they were completely retained. As in the normal hybridization procedure, the filters were allowed to stand pressed flat at room temperature overnight, baked, incubated in 100 µl. volumes of four S.S.C. concentrations adjusted to pH = 7.0 and including 0.1% w/v sodium dodecyl sulphate at five temperatures, washed in 4 x S.S.C., treated with ribonuclease in 2 x S.S.C., re-washed in 4 x S.S.C. and dried. Their count rates were then recorded in the normal manner. Percentage retentions of DNA under the different conditions employed were thus determined. Blank filters were included in every incubation. The results of this study are shown in Figures 16a and 16b.

9.4. RNA Saturation Curves.

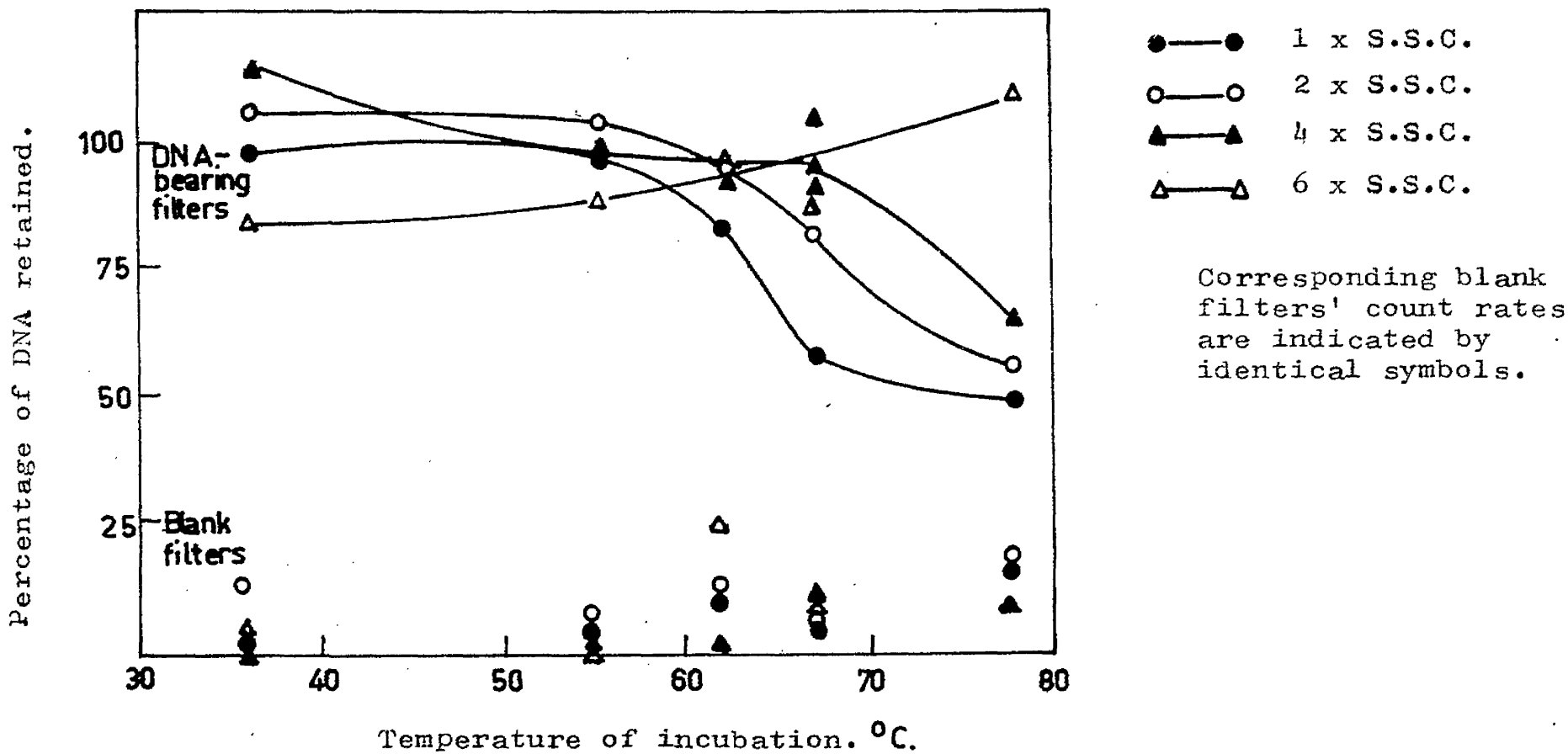
These were constructed by hybridizing fixed quantities of denatured DNA with a range of quantities of RNA under identical conditions. The percentages of DNA to which RNA had hybridized in each mixture were

Figure 16a. Effect of S.S.C. Concentration on the retention of DNA applied to Nitrocellulose Filters during incubation as in Hybridization.




Each DNA-bearing filter initially bore 4.4 µg. of tritiated alkaline-denatured Landschutz ascites tumour cell DNA. S.S.C. solutions were adjusted to pH = 7.0 and included 0.1% w/v sodium dodecyl sulphate.

Figure 16b. Effect of Temperature of Incubation on the retention of DNA applied to Nitrocellulose Filters during incubation as in Hybridization.




Each DNA-bearing filter initially bore 4.4 µg. of tritiated alkaline-denatured Landschutz ascites tumour cell DNA. S.S.C. solutions were adjusted to pH = 7.0 and included 0.1% w/v sodium dodecyl sulphate.

plotted against the concentrations of RNA present. Asymptotic curves were thus obtained as shown in some of the following figures. Although complete saturation of DNA by RNA was not usually observed, on plotting the reciprocals of these variables against one another a linear relationship was observed. It was thus considered possible to determine the percentages of DNA to which RNA would have hybridized at infinite RNA concentration, i.e. the saturation level of DNA by RNA.

Unless otherwise stated, the DNA-RNA hybridization procedure involving DNA immobilised on nitrocellulose filters was used throughout. Sodium dodecyl sulphate was not included in hybridization mixtures used in initial studies.

9.5. DNA Saturation Curves.

These were constructed in an analogous manner to RNA saturation curves by hybridizing fixed quantities of RNA with a range of quantities of template DNA. Asymptotic curves were obtained on plotting the percentages of RNA hybridized against the quantities of DNA present. On plotting the reciprocals of these quantities against one another, a linear relationship was observed. It was thus considered possible to

determine the percentage of RNA hybridizable to denatured template DNA.

The DNA-RNA hybridization procedure involving alkaline-denatured DNA immobilised on nitrocellulose filters was used in obtaining data for these studies. DNA was applied to nitrocellulose filters at a concentration in the region of 1 µg./ml. of 4 x S.S.C..

9.6. Saturation of Calf Thymus DNA by RNA synthesized *in vitro* on a Calf Thymus DNA Template.

Initially there was very little or no binding of such RNA to nitrocellulose filters bearing denatured calf thymus DNA. The DNA appeared to be contaminated by ribonuclease, as indicated by the test for ribonuclease activity, in which the level of contamination was just detectable, and by a markedly decreasing rate of RNA synthesis *in vitro* when it was used as template (Section 5.1).

Calf thymus DNA containing the approximate equivalent of 5.4 mµg. of bovine pancreatic ribonuclease per mg. was used as a template for *in vitro* RNA synthesis. As described in Section 6.4, some of it was treated with iodoacetic acid/sodium iodoacetate and found to contain the approximate equivalent of 3.2 mµg. of bovine pancreatic ribonuclease per mg.

of DNA. On alkaline denaturation it was applied to nitrocellulose filters and hybridized with RNA thus synthesized. The pH of the S.S.C. in these hybridization mixtures was not adjusted to 7.0. An RNA saturation curve was thus constructed.

A second RNA saturation curve was similarly constructed. Data shown in it were obtained using calf thymus DNA which had not been treated with ribonuclease during its isolation and had not been treated with sodium iodoacetate.

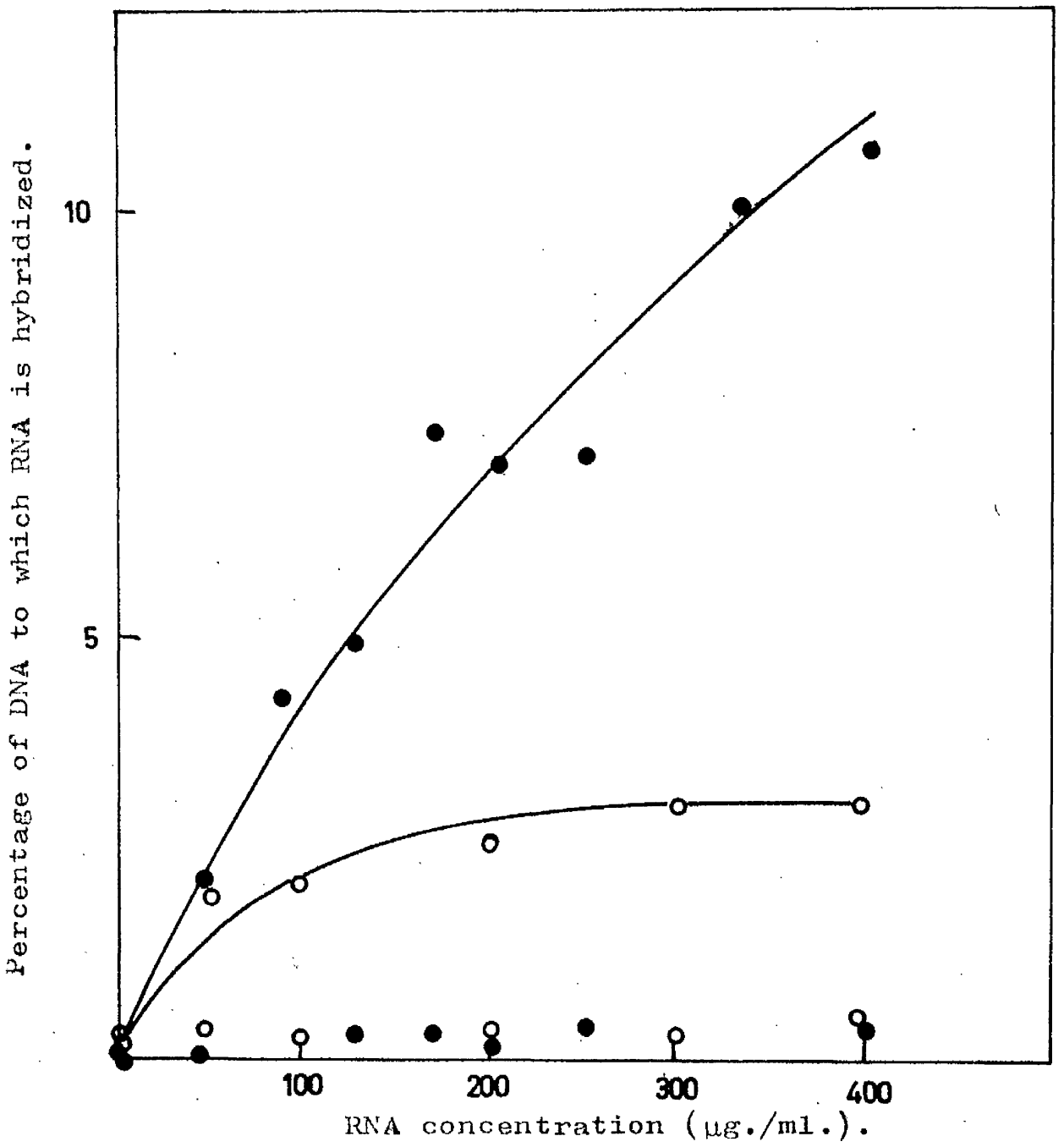
Results of these studies are shown in Figures 17a and 17b. Levels of saturation of DNA by RNA were 3.8% in the case of DNA in whose isolation procedure incubation with ribonuclease was included and 22% in the case of DNA from whose isolation procedure this was omitted.

DNA from whose isolation procedure incubation with ribonuclease was omitted was used in all further hybridizations.

9.7. Comparison of Three Procedures of DNA-RNA Hybridization.

RNA saturation curves were constructed using data obtained on hybridizing RNA synthesized *in vitro* on a calf thymus DNA template with alkaline denatured calf

Figure 17a. RNA saturation curves for the system in which RNA synthesized in vitro on a calf thymus DNA template was hybridized with alkaline-denatured calf thymus DNA immobilized on nitrocellulose filters.

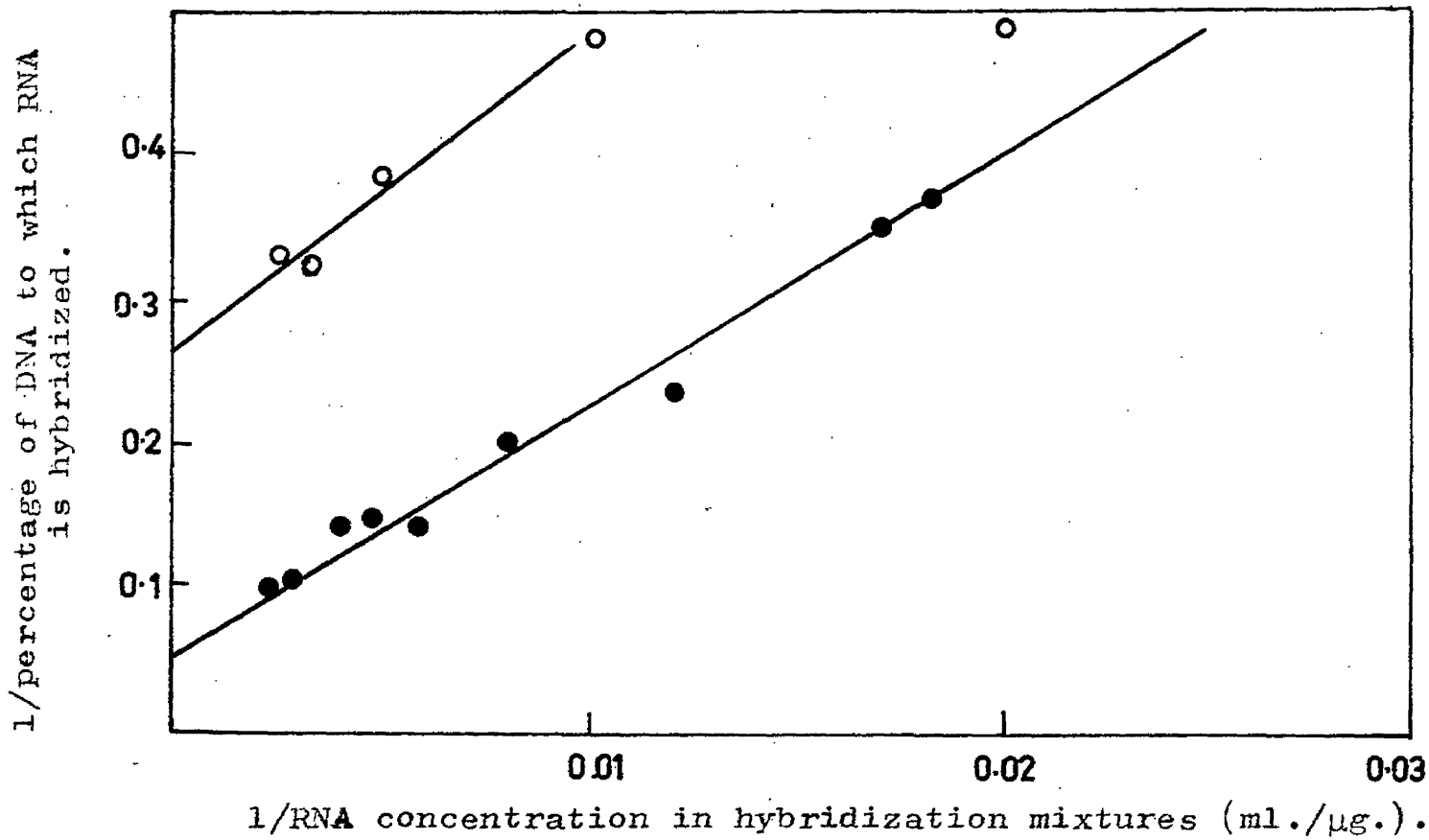



o—o Ribonuclease treatment was included in the isolation of DNA used in obtaining data for the construction of this curve.

●—● Ribonuclease treatment was omitted from the isolation of DNA used in obtaining data for the construction of this curve.

Blank filters' count rates are indicated by symbols identical to those of corresponding DNA-bearing filters.

Figure 17b. Double Reciprocal Plots of the data of Figure 17a.




o——o Ribonuclease treatment was included in the isolation of DNA used in obtaining data for constructing this line.

●——● Ribonuclease treatment was omitted from the isolation of DNA used in obtaining data for constructing this line.

thymus DNA. The three procedures were carried out using the same materials and within a 7-day period.

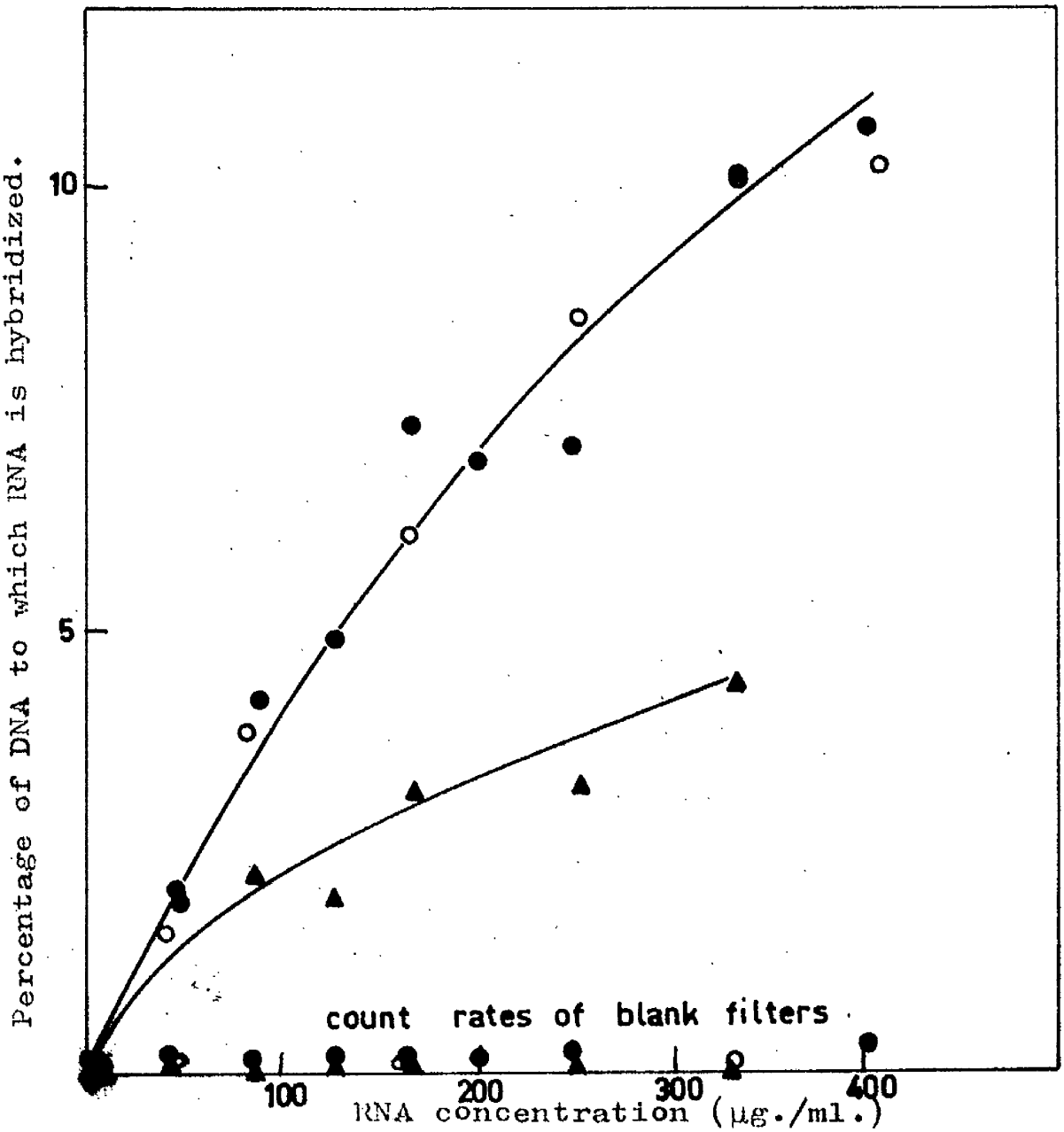
The first procedure was the normal one using DNA immobilised on nitrocellulose filters. In the second procedure, which was based on that of Bonner, Kung and Rekhor (1967), 30% v/v formamide was included in hybridization mixtures and these were incubated at room temperature. There were no other differences between this second procedure and the first. In the third procedure, hybridization was carried out with both DNA and RNA in solution.

Saturation curves and double reciprocal plots derived from them are shown in Figures 18a and 18b. Levels of saturation of DNA by RNA were found to be, respectively, 22%, 5.5% and 22%. The saturation curves and derived double reciprocal plots constructed from the use of the first and third procedures were found to overlap.

When nitrocellulose filters to which 4.8 µg. quantities of alkaline denatured tritiated Landschutz ascites tumour cell DNA were applied were treated as in the first and second procedures, the percentage retentions of DNA were found to be 90% in both cases. These filters were incubated for 17 hours in 4 x S.S.C.,

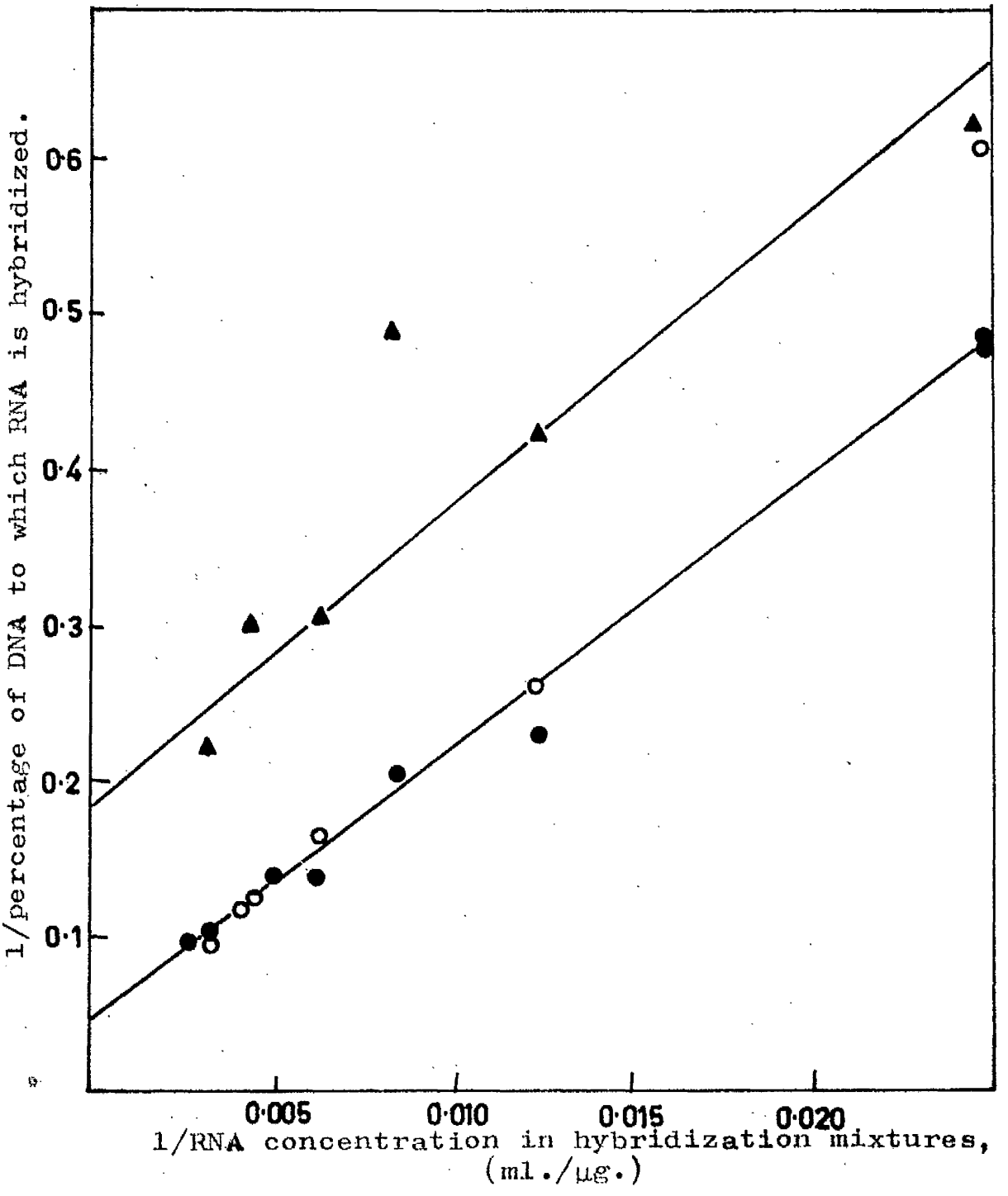
Figure 18a. RNA Saturation Curves for the system in which RNA synthesized in vitro on a calf thymus DNA template was hybridized with calf thymus DNA.

Three procedures were employed: ●——● normal procedure using DNA immobilised on nitrocellulose filters, o——o procedure in which both RNA and DNA are in solution, and ▲——▲ , as first procedure save that incubation in 30% v/v formamide at room temperature was substituted for incubation at 67°C.




Blank filters' count rates are indicated by symbols identical to those of corresponding DNA-bearing filters.

Figure 18b. Double Reciprocal Plots of the Data of Figure 18a.




●——● normal procedure using DNA immobilised on nitrocellulose filters.

o——o procedure in which both RNA and DNA are in solution.

▲——▲ as first procedure save that incubation in 30% v/v formamide at room temperature was substituted for incubation at 67°C.

0.1% w/v sodium dodecyl sulphate. The count rates of blank filters corresponding to them were background.

9.8. Effect of Inclusion of Sodium Dodecyl Sulphate in DNA-RNA Hybridization Mixtures.

Two RNA saturation curves were constructed using data obtained on hybridizing RNA synthesized *in vitro* on a calf thymus DNA immobilised on nitrocellulose filters. One RNA saturation curve was obtained with 0.1% w/v sodium dodecyl sulphate included in hybridization mixtures. The saturation curves and double reciprocal plots derived from them are shown in Figures 19a and 19b respectively.

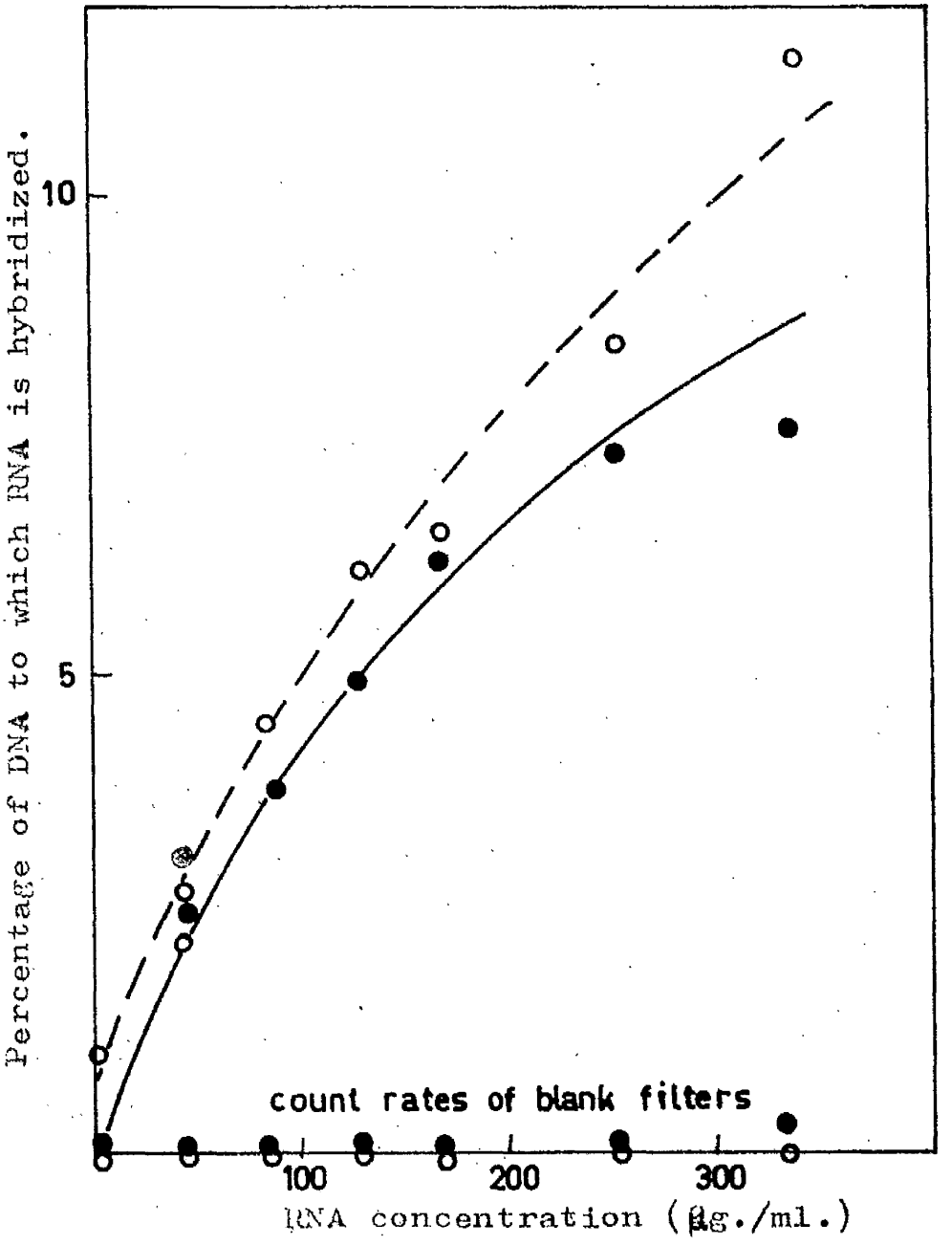
The presence of sodium dodecyl sulphate caused increased levels of hybridization of RNA to DNA, an increased level of saturation of DNA by RNA, saturation levels being 21% and 18% in the presence and absence of sodium dodecyl sulphate, respectively. It also caused decreased background count rates.

The RNA and DNA used were those used in the immediately preceding study (using DNA from whose isolation procedure incubation with ribonuclease was omitted). The RNA was stored between the times at which these studies were made, an interval of 7 days, at -15°C.

Figure 19a. Effect of Inclusion of Sodium dodecyl Sulphate in DNA-RNA Hybridization Mixtures.

o---o 0.1% w/v sodium dodecyl sulphate was included in hybridization mixtures.

●——● sodium dodecyl sulphate was omitted from hybridization mixtures.



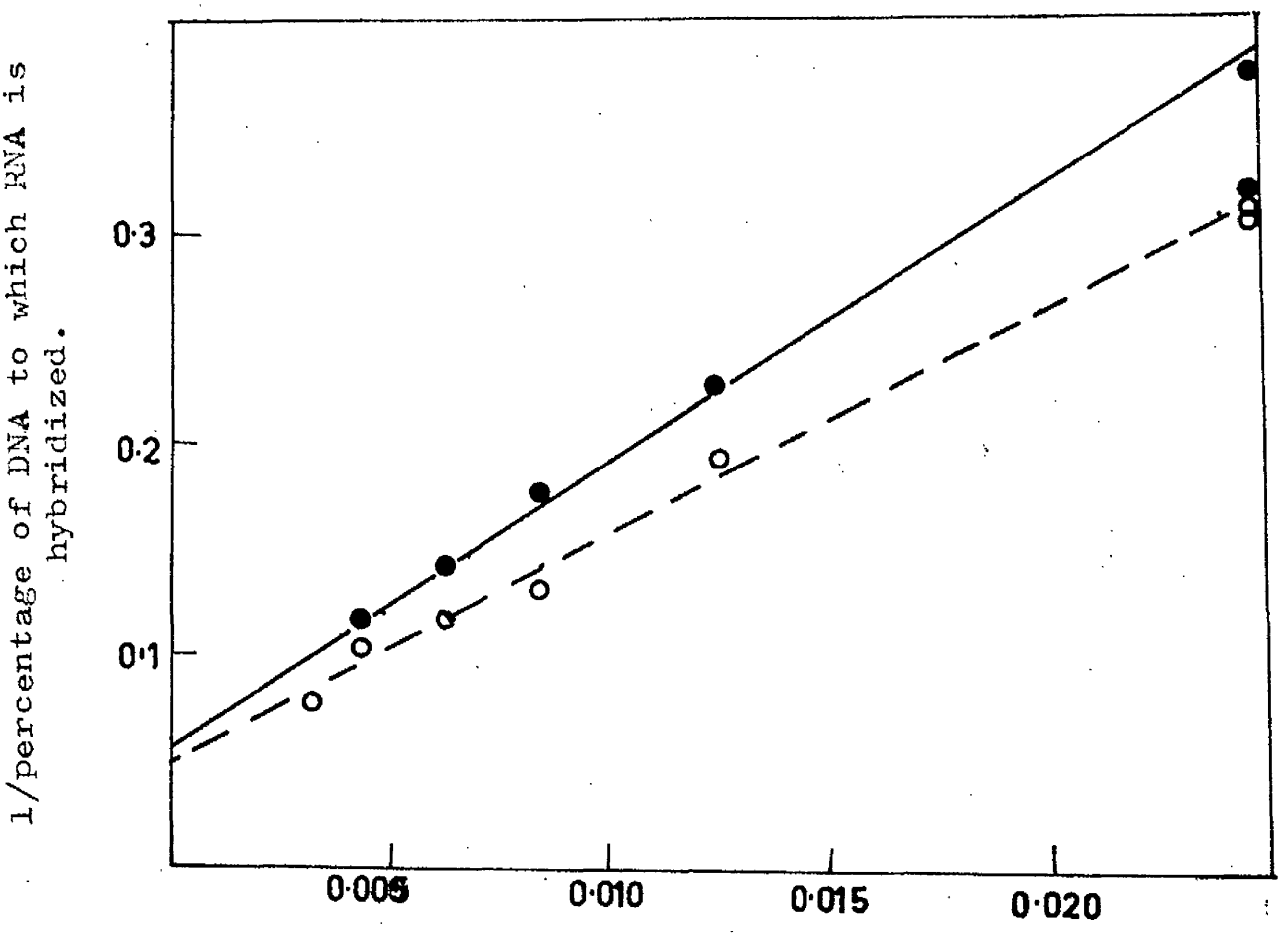

Alkaline-denatured calf thymus DNA was immobilised on nitrocellulose filters. Hybridization mixtures contained tritiated RNA synthesised *in vitro* on a calf thymus DNA template.

Blank filters' count rates are indicated by symbols identical to those of corresponding DNA-bearing filters.

Figure 19b. Double Reciprocal Plot of the Data of Figure 19a.

o— —o 0.1% w/v sodium dodecyl sulphate was included in hybridization mixtures.

●——● sodium dodecyl sulphate was omitted from hybridization mixtures.



9.9. Determination of the Percentage of RNA synthesized *in vitro* on a Calf Thymus DNA Template hybridizable with Alkaline-denatured Calf Thymus DNA.

DNA saturation curves and double reciprocal plots derived from them were constructed from data obtained using the same materials as were used in Sections 9.7 and 9.8. They are shown in Figures 20a and 20b, respectively. Hybridization mixtures of total volume 100 µl. contained 41.5 µg./ml. of RNA, but sodium dodecyl sulphate was omitted from them.

The percentage of RNA found to be hybridizable to DNA of the template was approximately 5%.

9.10. Hybridization of RNAs synthesized on a Calf Thymus DNA Template after 10, 30 and 90 minutes' Incubation of an RNA-synthesizing Mixture with Alkaline-denatured Calf Thymus DNA immobilized on Nitrocellulose Filters.

An RNA-synthesizing mixture of initial volume 10.0 ml. and containing 270 µg./ml. of calf thymus DNA and 56 units per ml. of RNA polymerase was incubated for 91 minutes. 0.05 ml. volumes were removed at various times and the quantity of radioactivity in them in acid-insoluble form was measured. The time course of the RNA polymerase reaction was thus established (Figure 21a).

Figure 20a. DNA Saturation Curve for the System in which RNA synthesized *in vitro* on a Calf Thymus DNA Template was hybridized with Alkaline-denatured Calf Thymus DNA immobilised on Nitrocellulose Filters.

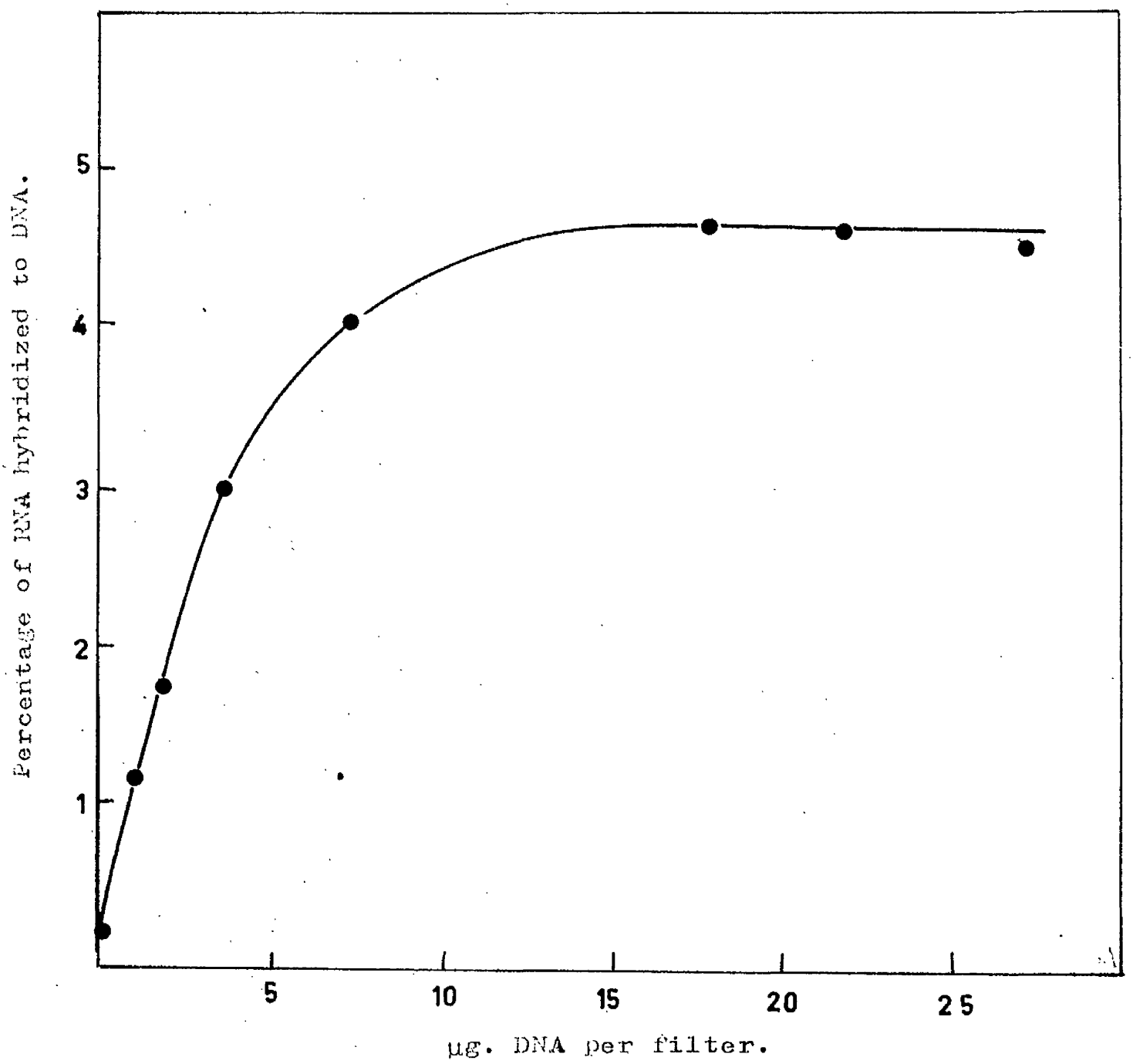
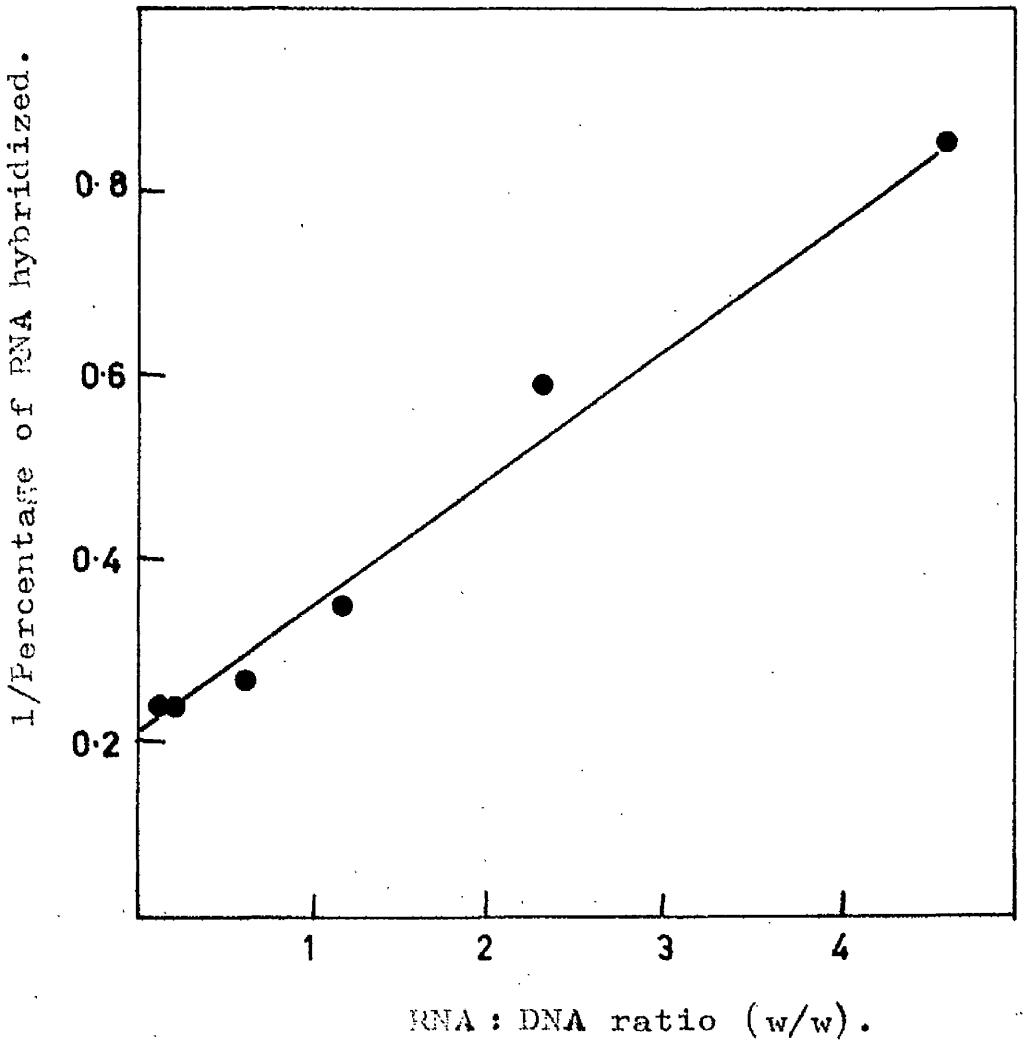


Figure 20b. Double Reciprocal Plot of the Data of Figure 20a.



After 10, 30 and 90 minutes' incubation of the RNA-synthesizing mixture, 2.0 ml. volumes were removed and RNA was purified from them. 200 µg., 270 µg. and 670 µg. quantities of RNA were thus obtained after these respective periods of incubation. Their specific activities were, respectively, 12,600 d.p.m./µg., 12,100 d.p.m./µg. and 11,900 d.p.m./µg..

RNA saturation curves and double reciprocal plots derived from them constructed by hybridizing RNA with 4.9 µg. quantities of alkaline-denatured calf thymus DNA are shown in Figures 21b and 21c, respectively. The saturation level of DNA by all three RNAs was approximately 20%. DNA saturation curves and double reciprocal plots derived from them were constructed for each type of RNA. Quantities in the region of 5 µg. of RNA were included in hybridization mixtures of volume 100 µl. which also included 0.1% w/v sodium dodecyl sulphate. The calf thymus DNA used was alkaline denatured and immobilised on nitrocellulose filters in 0 to 29 µg. quantities.

The percentages of RNA hybridizable to DNA were observed to increase with time of incubation of the RNA-synthesizing mixture being, approximately, 2%, 2.5% and 5% after, respectively, 10, 30 and 90 minutes' incubation

(Figures 21d and 21e).

The RNA polymerase used in this study was purified by Procedure 1.

9.11. Effects of S.S.C. Concentration and Temperature of Incubation on the Hybridization of RNA synthesized *in vitro* on a Calf Thymus DNA Template with Alkaline-denatured Calf Thymus DNA immobilised on Nitrocellulose Filters.

4.6 µg. quantities of alkaline-denatured calf thymus DNA were immobilised on nitrocellulose filters. These and corresponding blank filters were incubated for 17 hours at 67°C in solutions of total volume 100 µl., including S.S.C. at 1 x, 2 x, 4 x, or 6 x concentrations, 85 µg./ml. of synthetic RNA and 0.1% w/v sodium dodecyl sulphate. The filters were then washed in 4 x S.S.C. and incubated with ribonuclease in 2 x S.S.C. as in the normal procedure. The RNA used was synthesized *in vitro* on a calf thymus DNA template and its level of saturation of DNA was 20%. The results of this study are shown in Figure 22a.

4.7 µg. quantities of alkaline-denatured calf thymus DNA immobilised on nitrocellulose filters were similarly incubated in solutions containing 290 µg./ml. of RNA synthesized on a calf thymus DNA template. The

Figure 21a. Hybridization of RNAs synthesized on a Calf Thymus DNA Template after 10, 30 and 90 minutes' Incubation of an RNA-synthesizing mixture with Calf Thymus DNA immobilised on Nitrocellulose Filters. Time course of RNA synthesis.

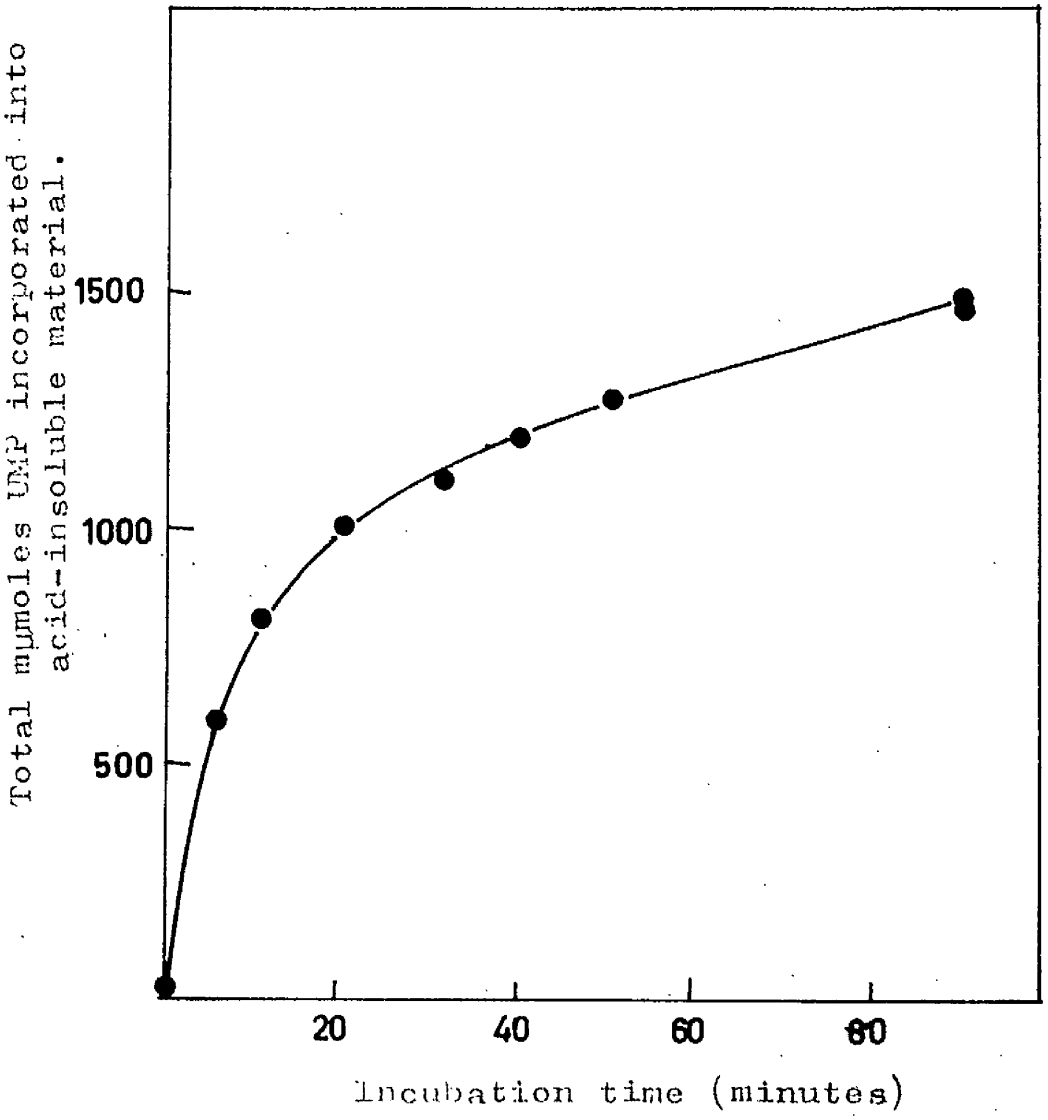


Figure 21b. Hybridization of RNAs synthesized on a Calf Thymus Template after 10, 30 and 90 minutes' Incubation of an RNA-synthesizing mixture with Alkaline-denatured Calf Thymus DNA immobilised on Nitrocellulose Filters: RNA saturation curves of RNA synthesized after 10 ●—● , 30 Δ--Δ , and 90 O·····O minutes' Incubation.

Alkaline-denatured DNA was applied to Nitrocellulose Filters and 0.1% w/v sodium dodecyl sulphate was included in hybridization mixtures.

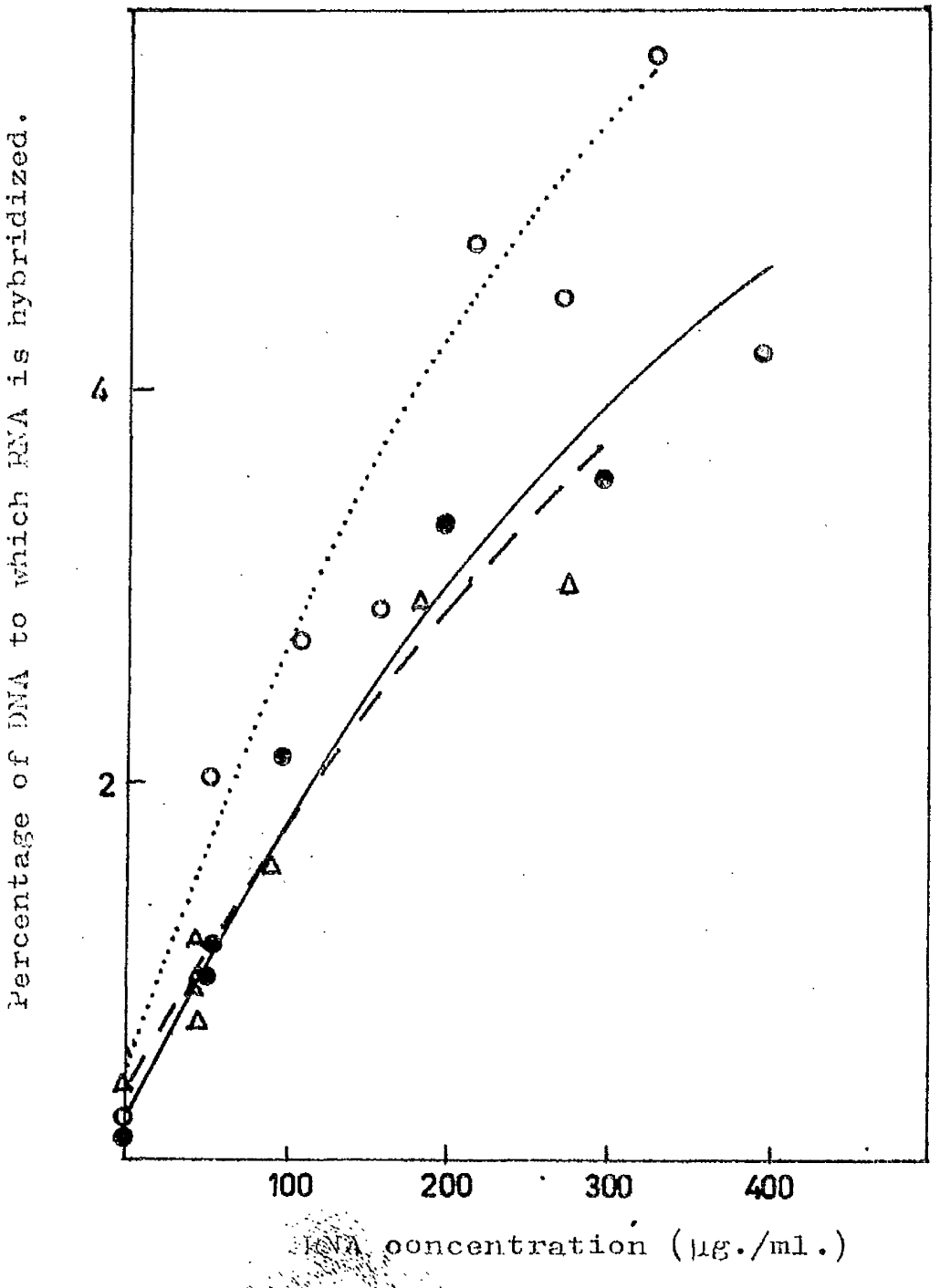


Figure 21c. Double Reciprocal Plot of the Data of Figure 21b.

RNA was purified from RNA-synthesizing mixtures after 10 ●——● , 30 Δ— —Δ , and 90 o·····o minutes' Incubation.

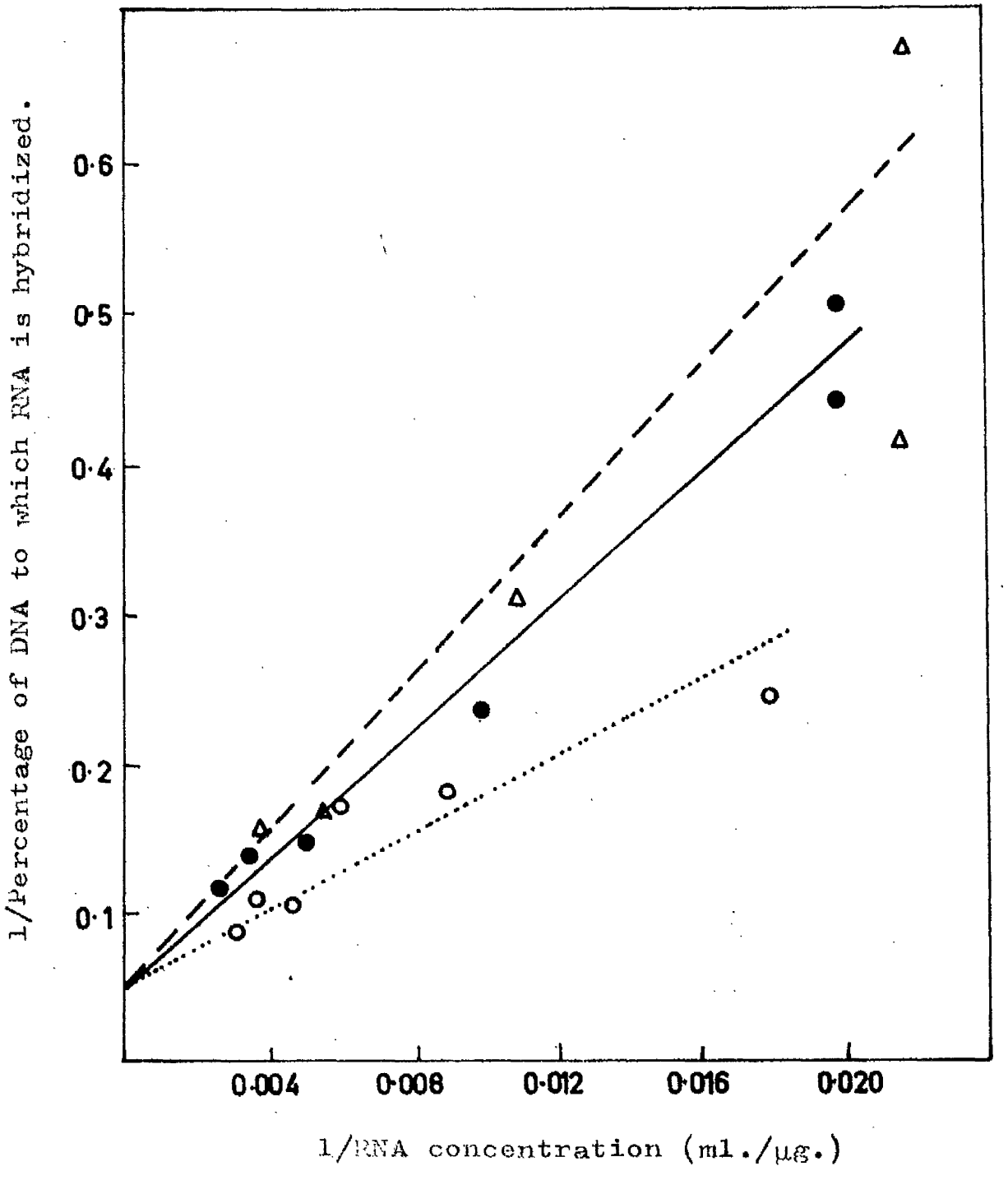


Figure 21d. DNA Saturation Curves for the System in which RNA synthesized *in vitro* on a Calf Thymus DNA Template is hybridized with Alkaline-denatured Calf Thymus DNA immobilised on Nitrocellulose Filters.

RNA was purified from the mixture after 10 ●—● , 30 Δ--Δ , and 90 O····O minutes' Incubation.

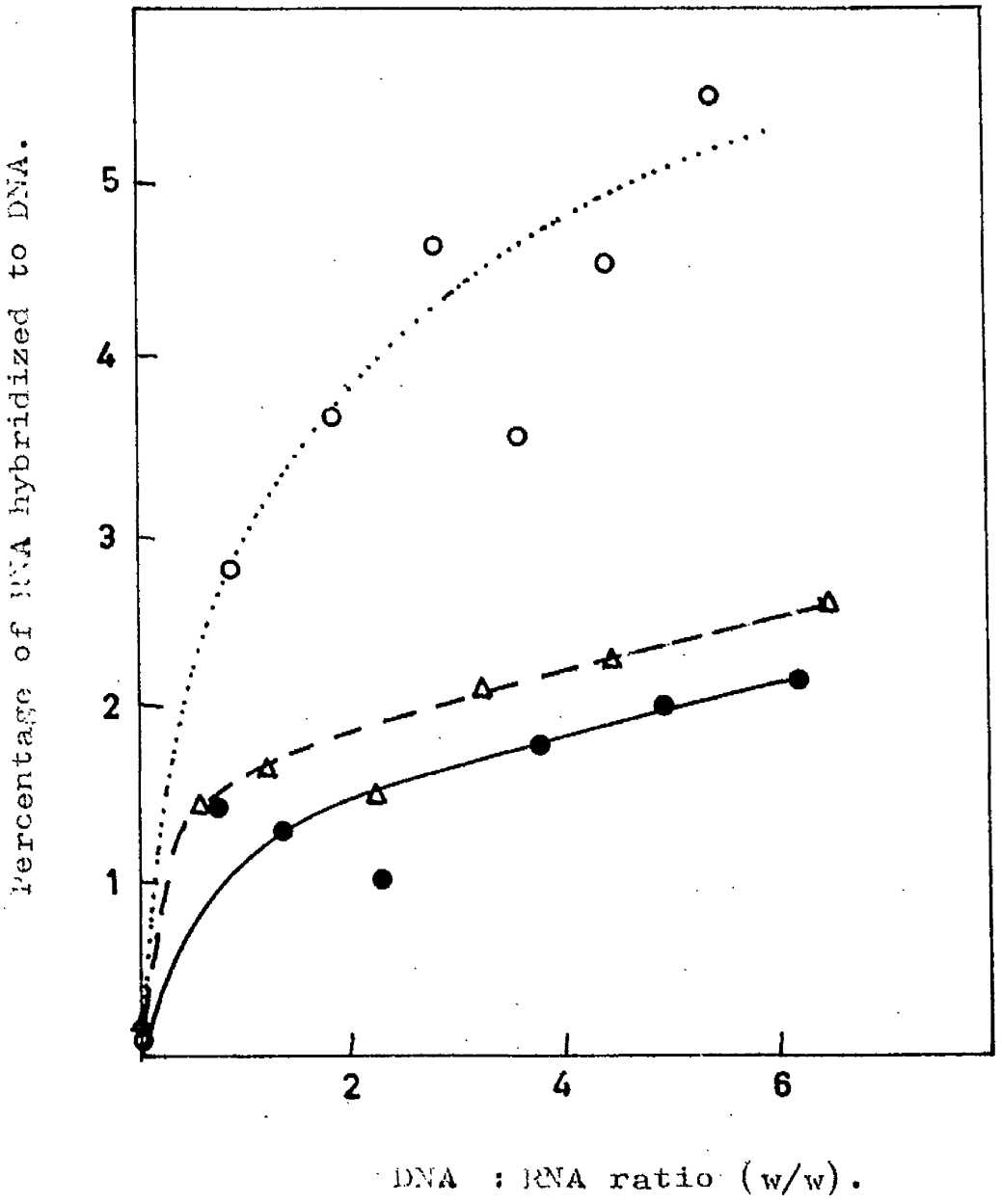
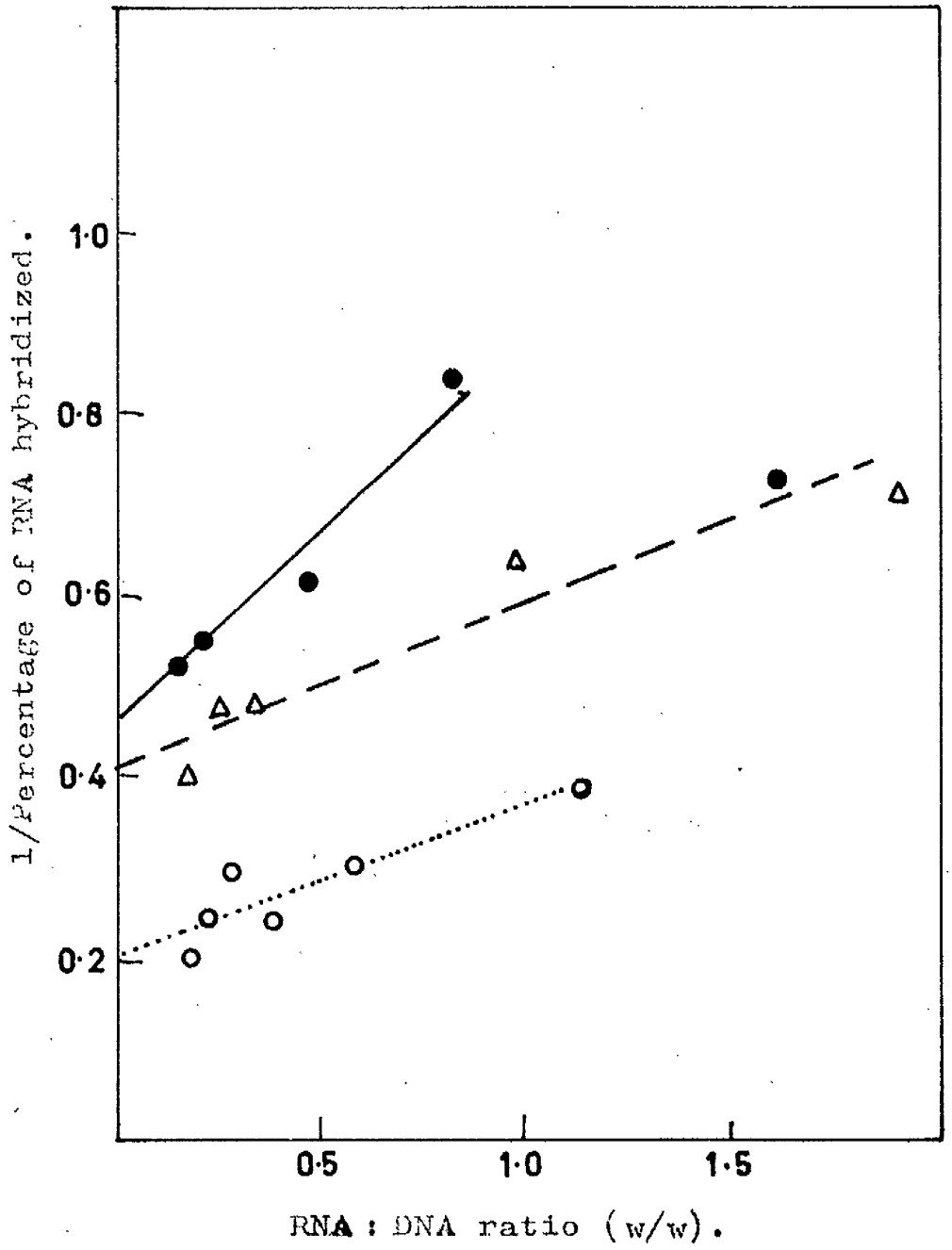


Figure 21e. Double Reciprocal Plot of the Data of Figure 21d.

RNA was purified from RNA-synthesizing mixtures after 10 ●——●, 30 Δ– – Δ, and 90 O·····O minutes' Incubation.

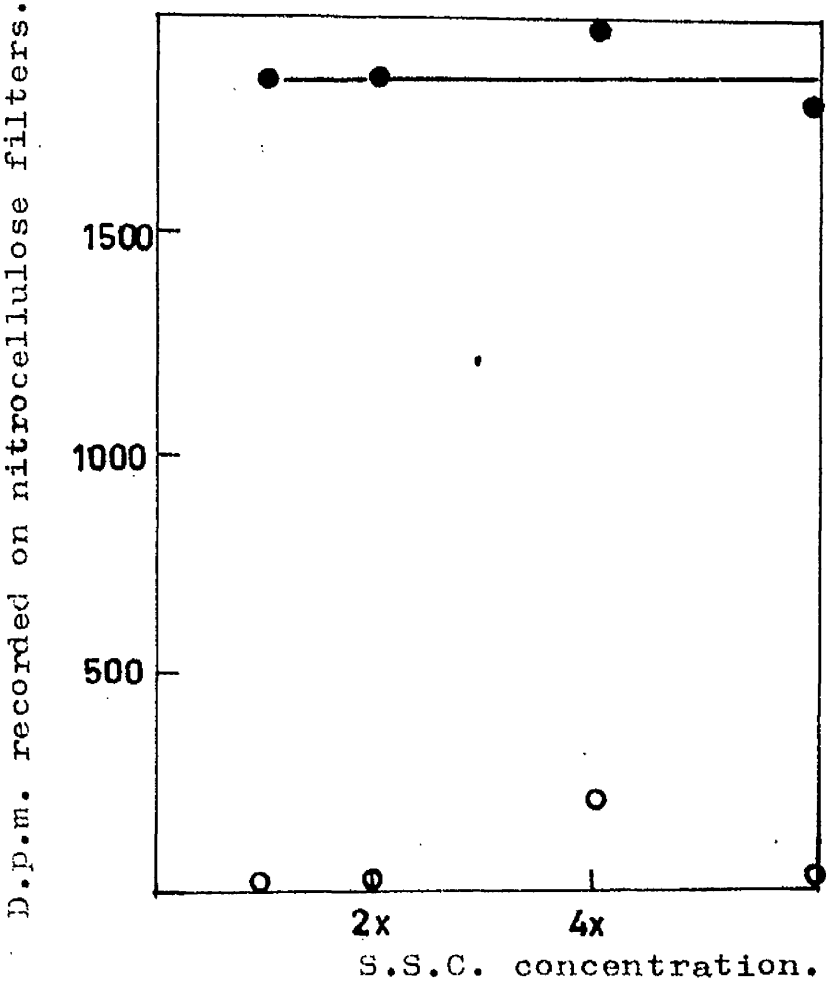


RNA used in this study saturated approximately 20% of the DNA, a more exact figure not being determined owing to shortage of this RNA. The hybridization mixtures contained 1 x, 2 x, 4 x, or 6 x concentrations of S.S. C. and were incubated at temperatures of 36°C, 55 °C, 62°C or 76°C. The filters and corresponding blank filters were then washed in 4 x S.S.C. and incubated with ribonuclease in 2 x S.S.C. as in the normal procedure. The results of this study are shown in Figures 22b and 22c. A glass dish was used in incubating at 76°C.

9.12. Effect of pH of Hybridization Mixtures on Hybridization of RNA in Solution with Alkaline-denatured DNA immobilised on Nitrocellulose Filters.

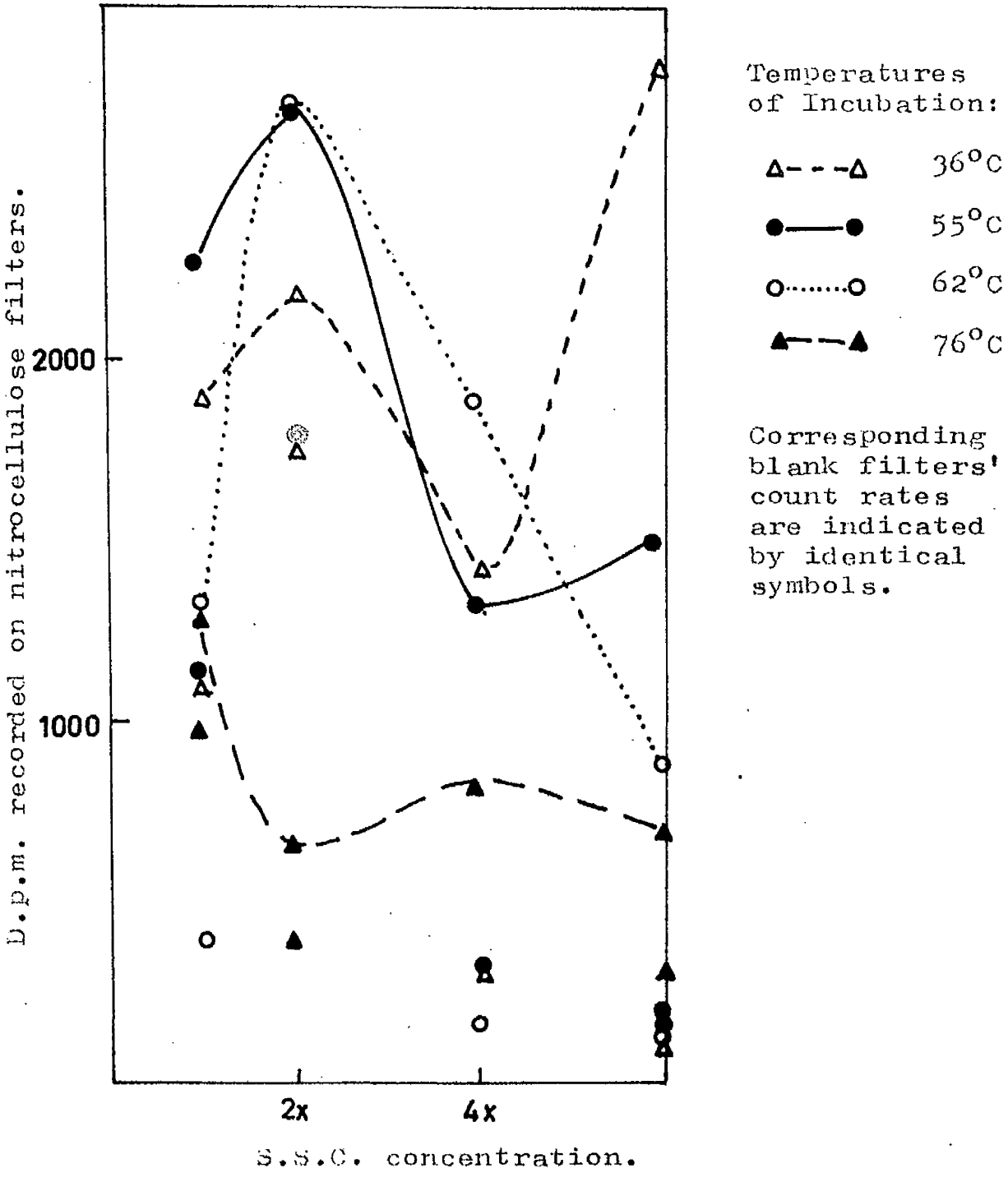
The DNA and RNA used in this study were the first described in Section 9.6. The pH of the S.S.C. included in hybridization mixtures of volume 100 µl. was not adjusted to 7.0. 4.1 µg. quantities of alkaline-denatured calf thymus DNA were applied to nitrocellulose filters. Hybridization with RNA synthesized on a calf thymus DNA template at a concentration 225 µg./ml. of 4 x S.S.C., 0.2 M tris/hydrochloric acid buffer solution was then carried out in the normal manner. 0.1% w/v

Figure 22a. Effect of S.S.C. Concentration on the Hybridization at 67°C of RNA synthesized *in vitro* on a Calf Thymus DNA Template with Alkaline-denatured Calf Thymus DNA immobilised on Nitrocellulose Filters.



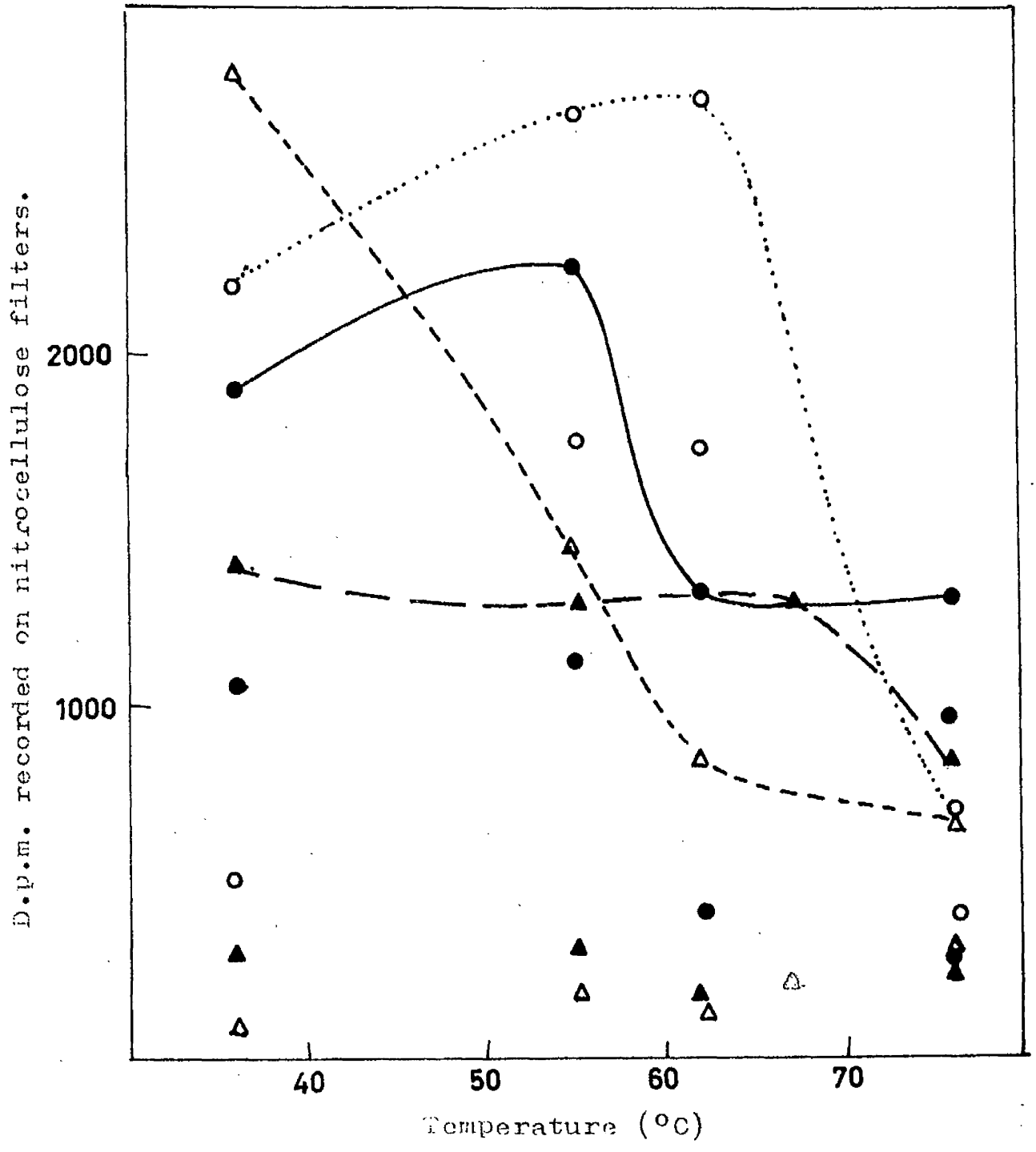

Each DNA-bearing filter ● bore 4.6 µg. of alkaline-denatured calf thymus DNA and corresponding blank filters were included with each of these o . Each hybridization mixture contained 0.1% w/v sodium dodecyl sulphate, 85 µg./ml. of synthetic RNA of specific activity 7,900 d.p.m./µg. and S.S.C. at the concentrations indicated in 100 µl.

Figure 22b. Effect of S.S.C. Concentration on the Hybridization at various temperatures of RNA synthesized *in vitro* on a Calf Thymus DNA Template with Alkaline-denatured Calf Thymus DNA immobilized on Nitrocellulose Filters.




Each DNA-bearing filter bore 4.7 µg. of alkaline-denatured calf thymus DNA and corresponding blank filters were included in hybridization mixtures. These contained 0.1% w/v sodium dodecyl sulphate, 290 µg./ml. of synthetic RNA of specific activity 15,300 d.p.m./µg. and S.S.C. at the concentrations indicated in 100 µl.

Figure 22c. Data of Figure 22b. Effect of Temperature on the Hybridization of RNA synthesized *in vitro* on a Calf Thymus DNA Template with Alkaline-denatured Calf Thymus DNA immobilised on Nitrocellulose Filters at various S.S.C.concentrations.




Corresponding blank Filters' count rates are indicated by identical symbols.

●——● 1 x S.S.C. ○·····○ 2 x S.S.C.

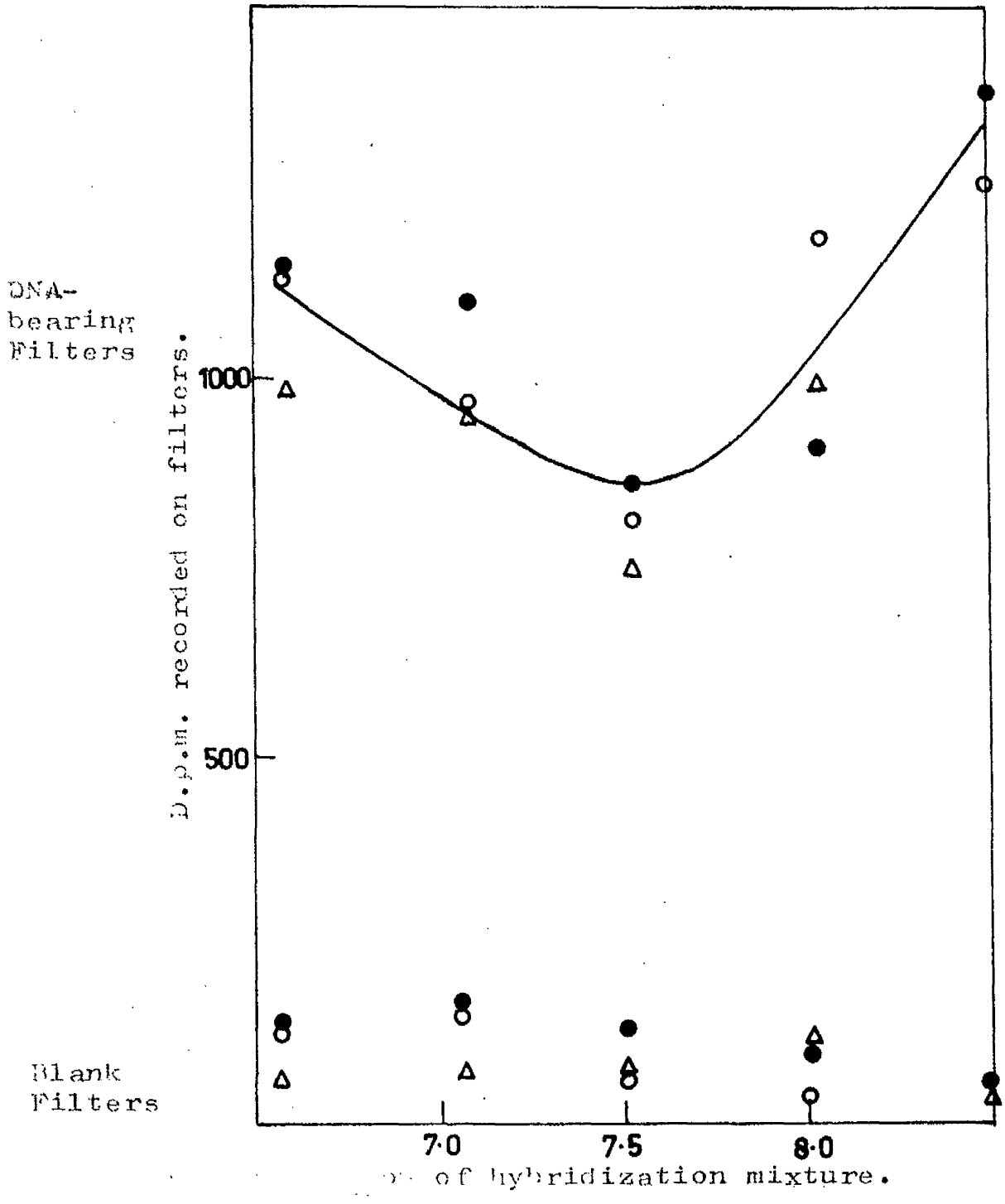
▲— —▲ 4 x S.S.C. △- - -△ 6 x S.S.C.

sodium dodecyl sulphate was omitted from hybridization mixtures. 1 M tris/hydrochloric acid buffer solutions of various pH values were used in making up hybridization mixtures. The same solutions were used in making up 50 ml. volumes of 0.2 M tris/hydrochloric acid buffer solutions, 4 x S.S.C.. The pH values of these solutions were recorded at room temperature before and after incubation in sealed containers for 17 hours at 67°C:

| Solution | pH Values | |
|---|---|---|
| | Before incubation | After incubation |
| A | 6.6 | 6.6 |
| B | 7.1 | 7.1 |
| C | 7.5 | 7.5 |
| D | 8.0 | 8.0 |
| 4 x S.S.C.only | 8.5 | 8.2 |

D.p.m. recorded on DNA-bearing and corresponding blank filters were then plotted against the appropriate pH values recorded before incubation (Figure 23).

Figure 23. Effect of pH of Hybridization Mixtures on Hybridization of RNA synthesized *in vitro* on a Calf Thymus DNA Template with Alkaline-denatured Calf Thymus DNA immobilised on Nitrocellulose Filters.




RNA concentration = 225 µg./ml. Specific Activity of RNA = 10,500 d.p.m./µg. Volume of hybridization mixtures = 100 µl. Quantity of DNA applied to each Filter = 4.1 µg. Different symbols refer to triplicate measure-measurements at each pH.

9.13. Comparison of the Effects of Hot and Room Temperature Washing and Treatment and Omission of Treatment with Ribonuclease after Hybridization.

28 µg. quantities of RNA of specific activity 11,900 d.p.m. per µg. synthesized *in vitro* on a calf thymus DNA template were hybridized with 4.25 µg. quantities of alkaline-denatured calf thymus DNA immobilised on nitrocellulose filters. Hybridization mixtures of volume 100 µl. contained 0.1% w/v sodium dodecyl sulphate. The level of saturation of DNA by this RNA was approximately 20%. It was prepared after 90 minutes' incubation of the RNA-synthesizing mixture described in Section 9.10.

Results of the use of different washing procedures and of incubation in ribonuclease under the conditions normally employed are shown in Table 10.

9.14. Effect of Time of Incubation with Ribonuclease on Count Rates of Nitrocellulose Filters bearing DNA to which RNA had been hybridized.

The normal procedure was used in hybridizing 16.5 µg. quantities of RNA synthesized *in vitro* on a calf thymus DNA template with 4.6 µg. quantities of alkaline-denatured calf thymus DNA immobilised on nitrocellulose

Table 10.

Comparison of the Effects of Hot and Room Temperature Washing and Treatment and Omission of Treatment with Ribonuclease after Hybridization.

| 1st treatment | D.p.m.on Filters. D | B | 2nd treatment | D.p.m.on Filters. D | B |
|---|---|---|---|---|---|
| Filters washed in 4 x S.S.C. at room temperature. | 8850 | 125 | None. | | |
| | 8350 | 170 | | | |
| | 8900 | 235 | Re-washed in 4 x S.S.C. at room temperature. | 4400 | 70 |
| | 8350 | 120 | | 4850 | 105 |
| | 7700 | 175 | Treated with ribonuclease and re-washed at room temperature. | 1860 | 10 |
| | 8600 | 155 | | 1840 | 45 |
| Washed in 4 x S.S.C. at 67°C. | 6470 | 85 | As above. | 1460 | 50 |
| | 6660 | 90 | | 2010 | 40 |

D = DNA-bearing Filters. B = blank Filters.

filters. 0.1% w/v sodium dodecyl sulphate was included in hybridization mixtures.

Throughout the period of incubation with ribonuclease, DNA-bearing filters and the corresponding blank filters were removed from the solution of ribonuclease in 2 x S.S.C, allowed to drain for a few seconds but not to dry, and were then immediately washed thoroughly in 4 x S.S.C..

They were washed by suction filtration, dried and their count rates were determined by the normal procedure.

The effect of time of incubation with ribonuclease on d.p.m. remaining on the filters is shown in Figure 24.

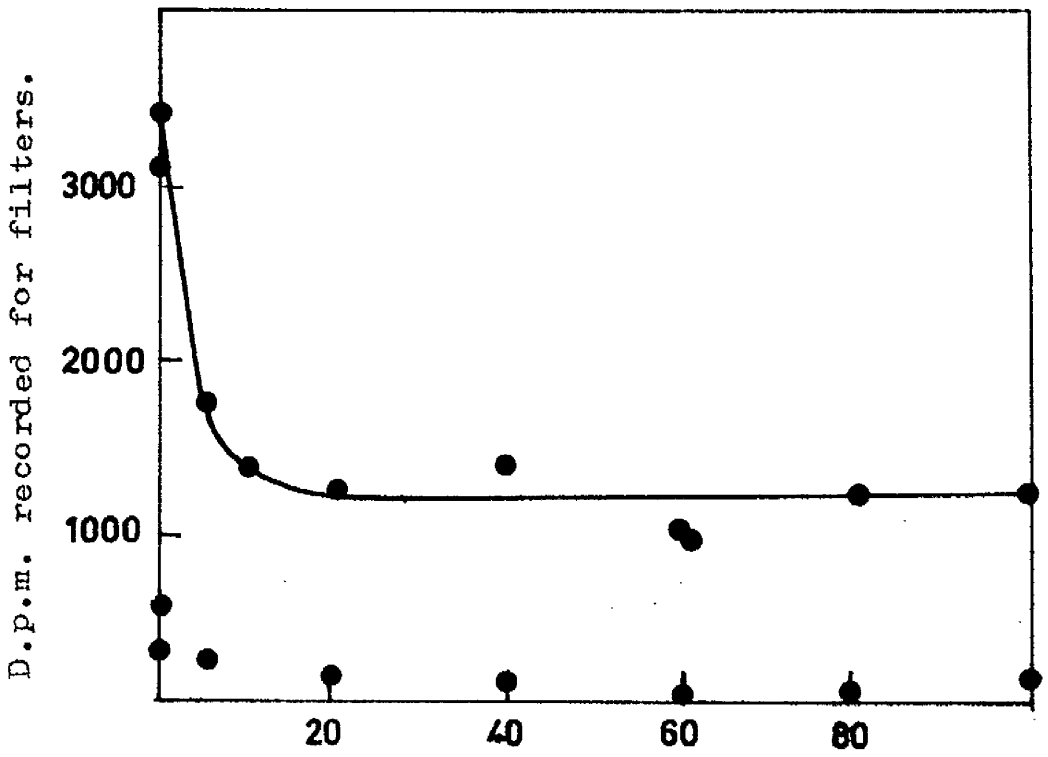
9.15. Studies on the Reversibility of and Effect of Additional Nucleic Acids in Solution on DNA-RNA Hybridization.

The RNA used in these studies was synthesized *in vitro* on a calf thymus DNA template. The level of saturation of calf thymus DNA by it was found to be 20%. This was determined using alkaline-denatured calf thymus DNA immobilised on nitrocellulose filters. RNA was hybridized in this determination and in the following with 4.6 µg. quantities of it.

Nitrocellulose filters bearing DNA and corresponding

Figure 24. Effect of Time of Incubation with Ribonuclease on Count Rates of Nitrocellulose Filters bearing DNA to which RNA had been hybridized.

Count rates of blank Filters are those of less than 1000 d.p.m. DNA-bearing Filters bore 4.6 µg. quantities of alkaline-denatured calf thymus DNA. 16.5 µg. quantities of RNA synthesized in vitro on a calf thymus DNA template were present in each hybridization mixture.




Conditions of incubation:

Ribonuclease concentration = 20 µg./ml.
S.S.C. concentration = 2 x
Temperature (room) = 23°C.

blank filters were incubated in up to three successive hybridization mixtures. The nucleic acid contents of the mixtures and the d.p.m. recovered on each filter are shown in Table 11. All hybridization mixtures were made up in 4 x S.S.C., 0.1% w/v sodium dodecyl sulphate, these being the only components of mixtures described as "blank". The specific activity of the RNA used was 7,900 d.p.m./µg.

9.16. Thermal Melting Profile of DNA-RNA Hybrids formed between Thermally-denatured Calf Thymus DNA and RNA synthesized in vitro on a Calf Thymus DNA Template.

Solutions of total volume 75 µl. containing final concentrations of 4 x S.S.C., 0.1% w/v S.D.S., 67 µg. DNA/ml. and 220 µg. RNA/ml. were incubated at 67°C for 2 hours as described in the Procedure of DNA-RNA Hybridization with DNA and RNA in Solution. 0.25 ml. volumes of 4 x S.S.C. at room temperature were then mixed with each solution and the mixtures were allowed to stand at room temperature for 15 minutes. 9.75 ml. volumes of water were then added to each mixture such that the final concentration of S.S.C. therein was 0.126 x.

The mixtures were warmed at a uniform rate of

## Table 11.

### Studies on the Reversibility of and Effect of Additional Nucleic Acids in Solution on DNA-RNA Hybridization.

| 1st Hybridization Mixture | | | 2nd Hybridization Mixture | | | 3rd Hybridization Mixture | | |
|---|---|---|---|---|---|---|---|---|
| Nucleic Acid Components | D.p.m. on each Filter | | Nucleic Acid Components | D.p.m. on each Filter | | Nucleic Acid Components | D.p.m. on each Filter | |
| | DNA-bearing | Blank | | DNA-bearing | Blank | | DNA-bearing | Blank |
| 6.8 µg. radioactive synthetic RNA. | 1710<br>1870 | 80<br>260 | Blank | 1375<br>1150 | 10<br>240 | Blank | 470<br>450 | 25<br>- |
| 6.8 µg. radioactive synthetic RNA + 7.0 µg. non-radioactive identical RNA. | 1070<br>1015<br>995 | 260<br>270<br>- | | | | | | |
| 6.8 µg. radioactive synthetic RNA + 6.7 µg. *E.coli* polysomal RNA. | 1780<br>1500 | 1700<br>1725 | | | | | | |
| 6.8 µg. radioactive synthetic RNA + 7.6 µg. purified *E.coli* polysomal RNA. | 1795<br>1715 | 20<br>25 | | | | | | |

Table 11 (continued).

| 1st Hybridization Mixture | | | 2nd Hybridization Mixture | | | 3rd Hybridization Mixture | | |
|---|---|---|---|---|---|---|---|---|
| Nucleic Acid Components | D.p.m. on each Filter | | Nucleic Acid Components | D.p.m. on each Filter | | Nucleic Acid Components | D.p.m. on each Filter | |
| | DNA-bearing | Blank | | DNA-bearing | Blank | | DNA-bearing | Blank |
| 6.8 µg radioactive synthetic RNA +6.6µg alkaline denatured calf thymus DNA. | 1885<br>1635 | 710<br>700 | | | | | | |
| 6.8 µg. radioactive synthetic RNA. | | | Blank | | | 7.0 µg. non-radioactive identical RNA | 600 | 30 |
| 6.8 µg. radioactive synthetic RNA. | | | Blank | | | 6.7 µg. E.coli polysomal RNA | 905<br>835 | -<br>90 |
| 6.8 µg. radioactive synthetic RNA. | | | Blank | | | 6.6 µg. alkaline denatured calf thymus DNA | 835<br>630 | 90<br>100 |

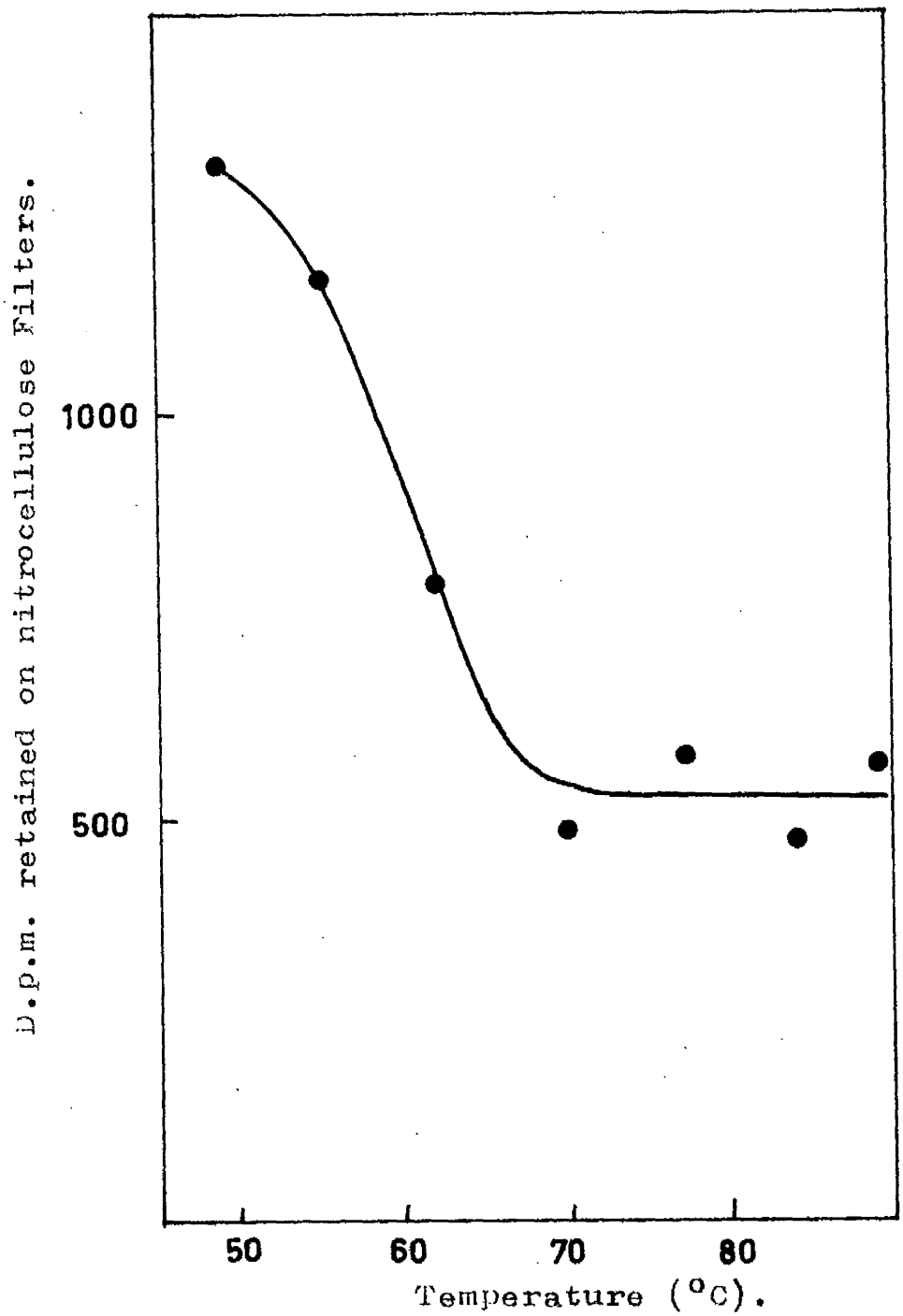
1.3C° per minute. They were successively removed and immediately placed in an ice-bath as their temperatures were increased. Their S.S.C. concentrations were then raised to 2 x S.S.C. and they were treated with ribonuclease and applied to nitrocellulose membrane filters, again as described in the Procedure of DNA-RNA Hybridization with DNA and RNA in Solution.

The specific activity of the RNA used was 11,200 d.p.m./µg. The melting profile of the DNA-RNA hybrids formed was plotted as d.p.m. retained by nitrocellulose membrane filters against temperature of incubation of DNA-RNA hybrid and is shown in Figure 25. A $T_m$ in the region of 58°C was indicated.

9.17. Procedure of DNA-RNA Hybridization in 50% v/v Formamide at 37°C using DNA immobilised on Nitrocellulose Filters.

In order to permit longer incubation periods to be employed in DNA-RNA hybridization experiments with minimal accompanying degradation of RNA, the use of formamide in place of elevated temperatures (Bonner, Kung and Bekhor, 1967) was investigated. As the $T_m$s of Landschutz ascites tumour cell DNA and calf thymus DNA in 50% v/v formamide, 4 x S.S.C. are both, approximately, 62°C, DNA-RNA hybridization under those conditions

Figure 25. Thermal Melting Profile of DNA-RNA Hybrids in 0.126 x S.S.C.




DNA-RNA Hybrids were formed in solution between thermally-denatured calf thymus DNA and RNA synthesized in vitro on a calf thymus DNA template. Specific activity of RNA = 11,200 d.p.m./µg.

was studied at 37°C ($(T_m - 25)°C$).

Firstly, retention of alkaline-denatured tritiated Landschutz ascites tumour cell DNA by nitrocellulose filters was studied under appropriate conditions. The normal procedures of application of DNA to filters and incubation and washing in DNA-RNA hybridisation studies were followed. Results of this study are shown in Table 12a. Only background count rates were recorded on blank filters incubated with those bearing DNA and each of the percentages quoted in Table 12a was a mean of three closely-agreeing figures.

Secondly, DNA-RNA hybridization between alkaline-denatured calf thymus DNA and RNA synthesized <u>in vitro</u> on a template of calf thymus DNA was carried out under the four sets of conditions of Table 12b. 4.8 µg. quantities of DNA and 1.5 µg. quantities of RNA of specific activity 5,600 d.p.m./µg. were included in each hybridization mixture. Results of this study are shown in Table 12b.

Table 12a.

Retention of Alkaline-denatured Landschutz ascites tumour cell DNA by Nitrocellulose Filters during Hybridization.

| Temperature of Incubation | Time of Incubation (hours) | Formamide Present | Percentage Retention of DNA |
|---|---|---|---|
| 67°C | 17 | No | 90 |
| 67°C | 70 | No | 69 |
| 37°C | 17 | Yes | 78 |
| 37°C | 70 | Yes | 72 |

Table 12b.

Effects of Incubation with Formamide at low temperature on DNA-RNA Hybridization between Alkaline-denatured Calf Thymus DNA and RNA synthesized *in vitro* on a Calf Thymus DNA Template.

| Temperature of Incubation | Time of Incubation (hours) | Formamide Present | D.p.m. recorded on Filters | |
|---|---|---|---|---|
| | | | DNA-bearing | Blank. |
| 67°C | 17 | No | 279 | 0 |
| 67°C | 70 | No | 105<br>155 | 5½<br>" |
| 37°C | 17 | Yes | 304<br>272 | 15<br>5 |
| 37°C | 70 | Yes | 305<br>267 | 0<br>0 |

9.18. Hybridization of RNA synthesized in vitro on a Landschutz Ascites Tumour Cell DNA Template with Alkaline-denatured Homologous DNA and Fast, Intermediate and Slow Fractions thereof immobilised on Nitrocellulose.

RNA was synthesized from Landschutz ascites tumour cell DNA by the normal procedure. RNA polymerase was purified by Procedure 1 from cells supplied by Sigma Chemical Co.Ltd., London. The specific activity of UTP used in the RNA synthesizing mixtures was $6.6 \times 10^4$ d.p.m. /mμ mole.

Two additional steps were incorporated into the procedure of isolation of synthetic RNA. To remove unincorporated ribonucleotides and unhybridizable oligoribonucleotides from hybridizable RNA, the aqueous supernate resulting from repeated phenol treatments was applied to a 30 cm. x 1 $cm^2$ column of Biogel P 30 (Calbiochem Ltd., London) equilibrated in 2 x S.S.C. at room temperature. Its volume was reduced to a maximum of 2 ml. by repeated treatment with phenol and was kept low. It was found that three distinct fractions were eluted from such columns by further 2 x S.S.C.. In order of elution these were: acid-precipitable polynucleotides, acid-soluble oligonucleotides and phenol. The acid used was 5% w/v

T.C.A. under ice-cold conditions; nucleotide material and phenol were detected by their ultraviolet absorption spectra. Before passage through the Biogel column, the solution of synthetic RNA was incubated in 0.15 M potassium iodoacetate/iodoacetic acid, pH = 5.5 for 45 minutes at 40°C. After passage through the Biogel column, the procedure of isolation of synthetic RNA was applied to the fraction first eluted.

Whole DNA and fractions of DNA were similarly treated with iodoacetate before the procedure of alkaline denaturation was applied to them. Iodoacetate was employed to reduce the level of any ribonuclease activity present in DNA, fractions of DNA or RNA. In fact, no ribonuclease activity was detected in DNA fractions nor in the RNA polymerase employed. It was tested for by incubating 20 µl. volumes of rabbit reticulocyte polysomal RNA containing 75 µg. of RNA with 10 µl. volumes containing 2.6 µg. fast or intermediate DNA, 5.4 µg. slow DNA or 3.5 units of RNA polymerase for 90 minutes at 37°C. Degradation of RNA was tested for by carrying out polyacrylamide gel electrophoresis of the incubated mixtures. The gels employed were 6 cm. in length, 0.6 cm. in diameter, and contained 2.4% w/v polyacrylamide. Electrophoresis was carried out at 60 volts and 3.3 mA/gel

for 90 minutes at 4°C. The gels were then examined at a wavelength of 265 mμ. in a Joyce-Loebl Chromoscan. Their ultraviolet absorbance profiles were identical to that obtained using RNA allowed to stand for 90 minutes at 37°C. The RNA was a gift from Dr. R. Williamson and the gel electrophoresis was carried out with the skilled technical assistance of Mrs. Elizabeth Hill.

DNA-RNA hybridization was carried out by the procedure incorporating incubation in 50% v/v formamide in 4 x S.S.C., 0.1% w/v S.D.S. at 37°C. Incubation mixtures were made up in the normal manner to a total volume of 100 μl. in sealable polyethylene vials (diameter 15 mm., height 40 mm.). Owing to increased ease of handling, their use was preferred to that of liquid paraffin. Incubation was continued for 70 hours to increase the probability of detection of hybridization of RNA to slow DNA. 1.5 μg. quantities of DNA were applied to each nitrocellulose membrane filter.

Results of this study are shown in Figures 26a and 26b. Extents of saturation of DNA or fractions thereof by RNA were: whole DNA 25%, fast fraction 2.0%, intermediate fraction, 3.6%. In the region of 1% of the slow fraction appeared to be saturated by RNA.

Figure 26a. RNA Saturation Curves for the system in which RNA synthesized *in vitro* from Landschutz Ascites Tumour Cell DNA is hybridized with fractionated homologous alkaline-denatured DNA.

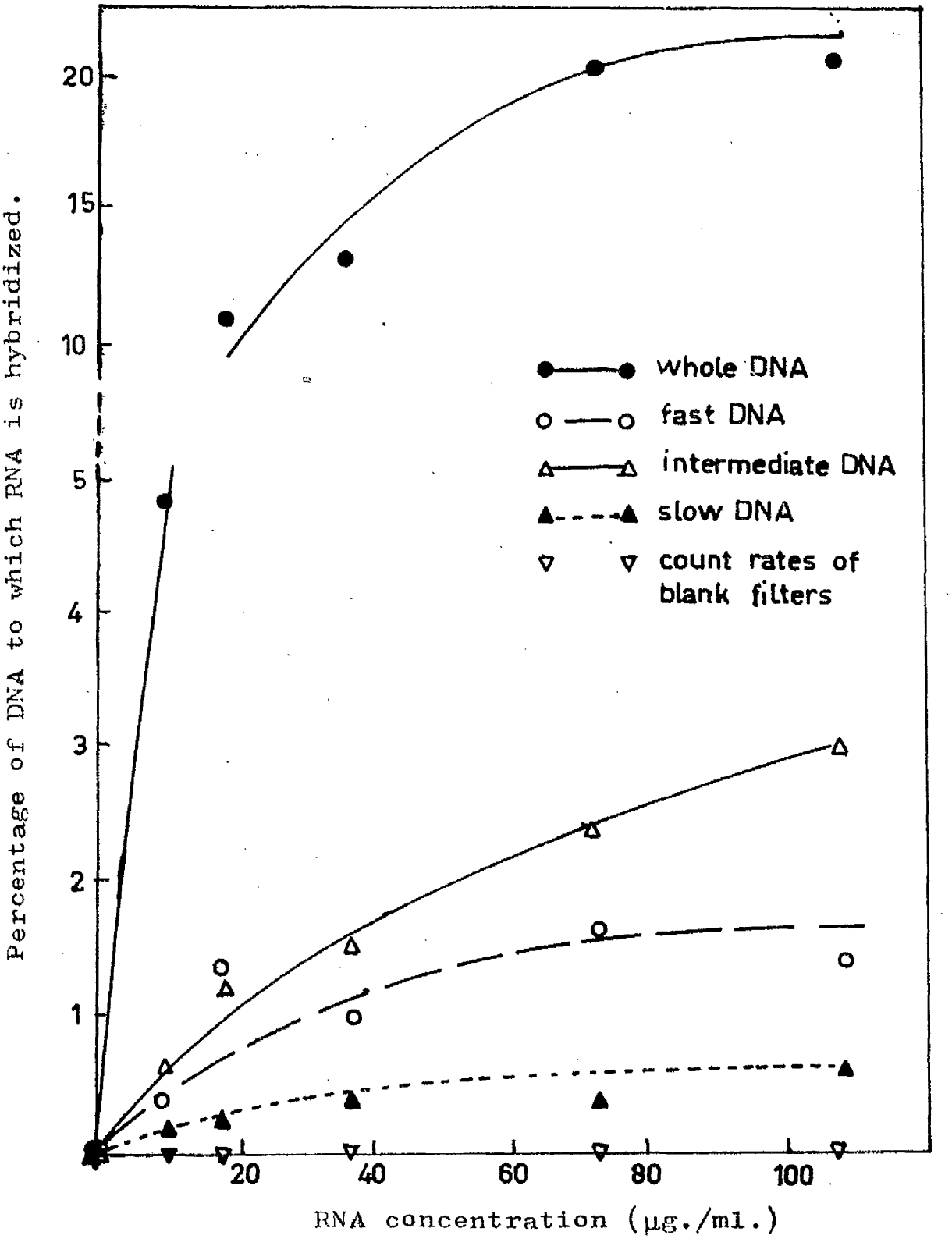
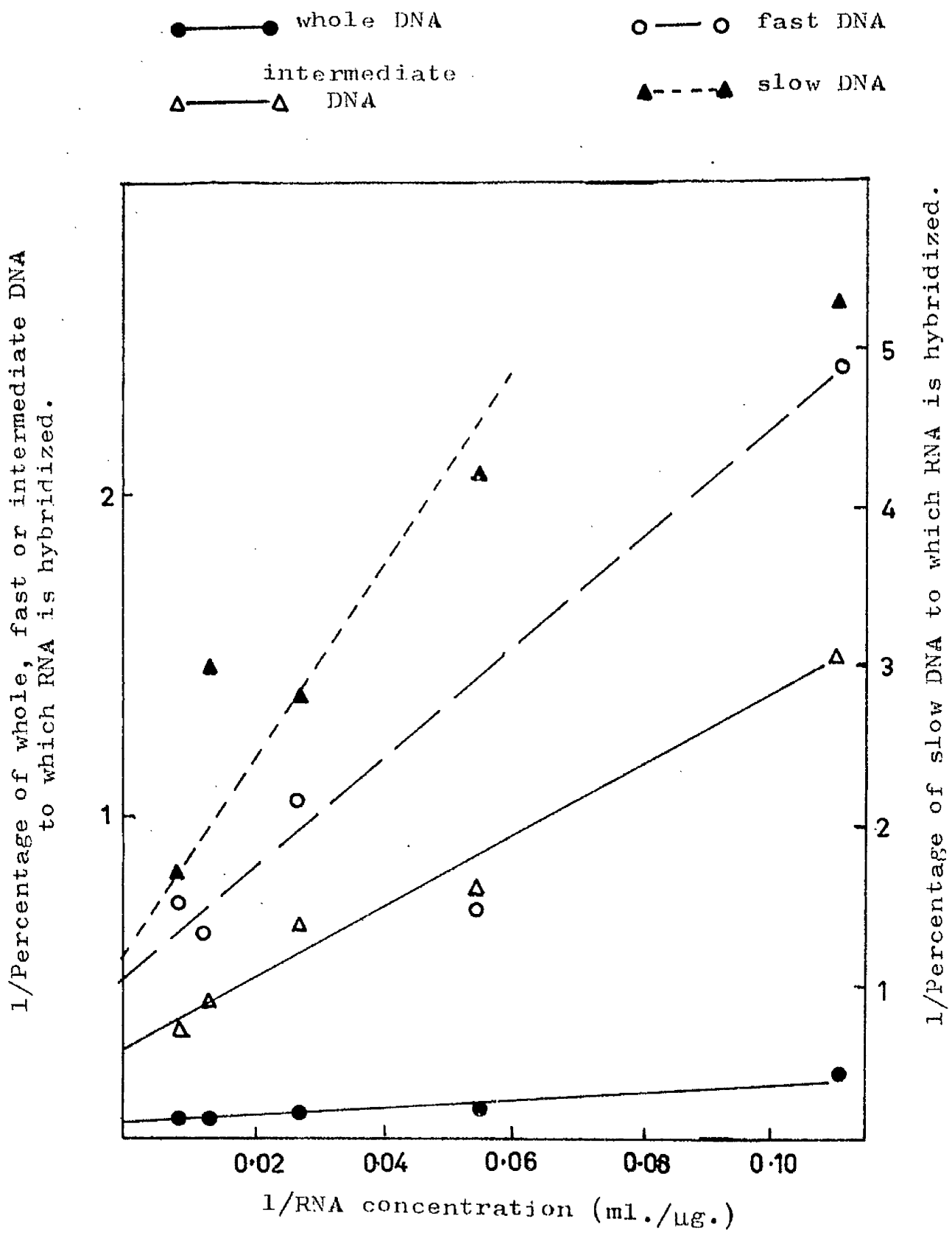


Figure 26b. Double Reciprocal Plots of the Data of Figure 26a.



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# DISCUSSION.

## 1. Chemical Analysis of Chromatin Preparations.

### Procedures of Analysis.

The procedures employed for quantitative estimation of DNA, RNA and total nitrogen by the Nessler reaction were standard ones. They were found to give consistent and satisfactory results over the range of experiments performed.

Two factors were considered to make it unsatisfactory to use the protein estimation procedure of Lowry, Rosebrough, Farr and Randall (1951). Firstly, the reaction involved depends on aromatic amino acid residues (Ruttor, 1967). These are atypically represented in histones (Busch, 1965; Butler, Johns and Phillips, 1968). Secondly, solubilisation of acidic chromosomal protein required incubation under harsh conditions. The possibility of degradation of amino acid and nucleotide residues to compounds interfering with the protein estimation procedure of Lowry *et al.* could not be excluded (Dingman and Sporn, 1964).

It was necessary to employ a procedure of protein estimation which would be unaffected by the presence of DNA and RNA and which would permit protein estimation on as firm a basis as that of total nitrogen estimation.

This itself was considered unsuitable owing to the expense in time involved in it.

The bromsulphalein procedure was found to satisfy the above criteria. It depends on binding of the dye bromsulphalein to protein. The complex was precipitated in acid conditions and removed by centrifugal sedimentation. The quantity of dye remaining in solution indicated how much protein had been present. This was estimated by use of the purple coloration of bromsulphalein in alkaline solution (Nayyar and Glick, 1954).

Results of Analysis.

Approximate equality of histone and DNA contents of rat, rabbit and calf thymus chromatin preparations was noted. Similar correspondence has been noted in chromatin of chicken tissues (Dingman and Sporn, 1964) and of a variety of plant and animal tissues (Bonner et al., 1968). This is in accord with three proposed functions of histone. Firstly, histones stabilise the structure of chromatin. Secondly, histones stabilise the structure of DNA within chromatin. Thirdly, there is good evidence that histone blocks transcription of DNA (Paul and Gilmour, 1968; Bekhor, Kung and Bonner, 1969; Huang and Huang, 1969). The fraction of total genetic information expressed at any one time in a eucaryotic

cell is probably very small. Therefore little difference between histone to DNA ratios would be expected in most tissues of most eucaryotic organisms.

Ratios of RNA to DNA and of histone to acidic chromosomal protein appeared not to follow any general pattern. However, the ratio of acidic chromosomal protein to RNA was relatively constant in all three rat chromatin preparations. A similar but less clear-cut tendency was observed in rabbit chromatin preparations. A more refined procedure of preparation of chromatin would perhaps permit definite establishment of whether there is such a constant ratio. The present procedure nevertheless yielded chromatin from which RNA similar to natural RNA was transcribed in vitro (Paul and Gilmour, 1968). Constancy of the relative quantities of acidic chromosomal protein and RNA in the preparations of chicken chromatin of Dingman and Sporn (1964) may be deduced from their results.

The nature of chromosomal RNA (Bonner et al., 1968; Beldhor, Kung and Bonner, 1969; Huang and Huang, 1969) is such that it can hardly be newly-transcribed RNA about to take part in protein synthesis. It cannot, therefore, be assumed that acidic chromosomal proteins modulate transcription of DNA in quantities proportional

to those of RNA being synthesized; nor can it be assumed that acidic chromosomal proteins are liberated with newly-transcribed RNA in constant relative quantities. Chromosomal RNA appears to be essential for establishment and maintenance of the structure and control of transcription of chromatin (Bekhor, Kung and Bonner, 1969; Huang and Huang, 1969). Acidic chromosomal proteins open specific portions of DNA in chromatin for transcription (Paul and Gilmour, 1968, 1969). Constant relative quantities of these may be necessary to ensure correct control of transcription through correct arrangement of the molecular structure of chromatin.

Chemical analysis of chromatin therefore yields results of interest. However, it appears that studies on transcribed RNA are more likely to allow conclusions to be reached on the nature of control of transcription of DNA in chromatin.

2. <u>Purification and Characterization of RNA Polymerase.</u>

<u>Purification.</u>

Yields of enzyme purified by Procedure 1 (similar to that of Nakamoto, Fox and Weiss (1964)) were, on average, fourteen times less than those reported by Nakamoto, Fox and Weiss (1964). Corresponding specific activities were twelve times less. Procedure 2 (using

benzoate) was adopted when its use was found to result in almost two-fold increases in yield and specific activity over Procedure 1. Inhibition of RNA polymerase by a reagent employed in this procedure would account for apparent loss of its activity during purification. No ribonuclease activity was detectable in RNA polymerase thus purified using cells obtained over a period of ten months. Then ribonuclease activity became detectable and Procedure 1 was again used. Yields were thus reduced but ribonuclease activity was not detectable.

Ribonuclease contamination of RNA polymerase purified by Procedure 2 occurred at the same time as a reduction in yield of enzyme purified by either procedure. This was probably due to differences in batches of cells. Yields of RNA polymerase from Micrococcus luteus vary greatly with phase of bacterial growth (Kohl, 1968).

Fewer stages involving precipitation and re-dissolution were involved in Procedure 2 than in Procedure 1. Superior yields and specific activities may, therefore, be due to lesser degradation. It was considered not worth while to routinely re-extract the streptomycin precipitates of Procedure 2. Purity of the final preparation might have been reduced and an extra day's delay would have been involved. The yield

of RNA polymerase might have been increased by 30%.

Evidence that the Enzyme is DNA-dependent RNA Polymerase.

The continued synthesis of RNA *in vitro* over 90 minutes was taken to indicate that incubation conditions were well-established. It is evidently essential that there should be no ribonuclease activity in the components of incubation mixtures.

Several lines of evidence point to the enzyme under study being DNA-dependent RNA polymerase. DNA was required for significant *in vitro* synthesis of RNA. It appeared to be a reactant in the enzymatic process. Specificity of the enzyme for DNA was indicated by its inability to use RNA as a template. This result also indicated that RNA synthesized *in vitro* did not act as a template for further RNA synthesis under the conditions employed here. However, it appears that it may well have inhibited it as reduced RNA synthesis was observed in the presence of DNA and RNA. This may explain why a linear relationship of quantity of RNA synthesized with time was never observed. Denaturation of the enzyme may also explain this observation.

Non-specific polyribonucleotide synthesis appeared unlikely as there was little or no incorporation of UTP into acid-insoluble material in the absence of GTP.

GTP was omitted because it was the least effective substrate in the "single-NTP" reaction described by Fox and Weiss (1964). The uniformly high count rates observed were probably due to co-precipitation of UTP with Macaloid in the abnormal mixture (Becker, 1968).

A DNA-RNA complex largely resistant to degradation by ribonuclease was formed during *in vitro* RNA synthesis when denatured DNA was used as template. The complex was thus almost certainly a hybrid of denatured DNA and nascent RNA. The smaller peak of radioactivity observed in the control to this experiment probably represented RNA synthesized on a template of DNA of *Micrococcus luteus*. This could have been present as a contaminant of RNA polymerase. This study provides additional evidence that the enzyme was DNA-dependent RNA polymerase.

Lastly, hybrids could be formed between denatured DNA and RNA synthesized *in vitro* by the action of RNA polymerase on a template of native DNA. This indicates complementarity of DNA template and RNA product.

Factors affecting the Synthesis of RNA *in vitro* and its Isolation.

Fox and Weiss (1964) studied *in vitro* RNA synthesis mediated by RNA polymerase prepared by the procedure of Nakamoto, Fox and Weiss (1964). They investigated the

effect of replacement of manganese by cobalt and magnesium and the effect of replacement of manganese by magnesium in the "single-NTP" reaction. The latter reaction, which presumably involves synthesis of non-specific polyribonucleotide, proceeded very slowly in the presence of magnesium. Replacement of manganese by cobalt and magnesium at near-optimal concentrations in in vitro RNA-synthesizing mixtures was therefore investigated with a view to further ensuring minimal synthesis of non-complementary RNA. Quantities of RNA thus synthesized were lower, especially when magnesium was used. Replacement of manganese by cobalt or magnesium was therefore not further investigated.

Manganese and Magnesium were fully effective co-factors in the degradative action of deoxyribonuclease I at the concentration of manganese routinely employed in RNA-synthesizing mixtures. It is therefore unlikely that synthetic RNA was significantly contaminated by DNA.

RNA synthesis was stimulated by putrescine, but only after incubation of an RNA-synthesizing mixture for some time. Similar stimulation by polyamines has been observed by Fox and Weiss (1964) and by Abraham (1968), who used RNA polymerase prepared from Escherichia

coli. Three possible reasons for such stimulation were suggested in his studies. Firstly, polyamine could prevent non-specific binding of polymerase molecules to those of DNA. Secondly, polyamine might bind to portions of RNA molecules already released from DNA. This could promote RNA synthesis by destabilization of a DNA-RNA hybrid region. Thirdly, polyamine could influence the structure of DNA to make it a more effective template for RNA synthesis. Polyamines and oxidised polyamines stabilise DNA against thermal denaturation (Dachrach and Dillon, 1967). There is a fourth possible explanation. Polyamine might render RNA less susceptible to degradation by ribonuclease, perhaps through its binding to RNA. The results of all these effects would be more evident after incubation of RNA-synthesizing mixtures for some time.

To increase yields of RNA putrescine was routinely included in RNA-synthesizing mixtures. It was omitted when the macro-heterogeneity of DNA was specifically under study. It may affect different fractions of DNA in different ways.

### 3. Characteristics of DNA.

Isolation and Storage.

DNA was not exposed to an aqueous solution of ionic

strength less than 0.15 M during its isolation and storage. This was because denaturation of DNA was noted on storage in solutions of very low ionic strength. Citrate concentration was kept very low in final solutions of DNA to be used as a template for *in vitro* RNA synthesis. Otherwise, a change in effective manganese concentration might have occurred through chelation in RNA-synthesizing mixtures. Separation of native and denatured DNA on hydroxyapatite could not be achieved in the presence of citrate. It was therefore not used in isolating nor in dissolving DNA to be used in such work.

Denaturation of DNA and its Application to and Retention by Nitrocellulose.

Hypochromicities of greater than 30% were taken to indicate that complete or almost-complete denaturation of DNA had been achieved. Hypochromicity of alkaline-denatured DNA on neutralisation is probably due to the establishment of some degree of base-stacking. Other than in the case of satellite DNA, significant renaturation under the conditions of temperature and DNA concentration involved is extremely unlikely.

The manner in which denatured DNA is immobilised on nitrocellulose remains uncertain. Conditions for

quantitative retention on application and maximal retention thereafter were established. The effects of salt concentration, formamide and urea on retention of denatured DNA on its application to nitrocellulose and of salt and formamide concentrations and temperature of incubation of DNA-RNA hybridization incubation mixtures suggest that the nature of DNA-nitrocellulose binding in some ways is similar to that of the binding between DNA strands. DNA renaturation is also dependent on salt concentration and temperature of incubation (Wetmur and Davidson, 1968) while formamide and urea (Hall, 1969) promote DNA denaturation and tend to prevent DNA-nitrocellulose binding. Graves (1968) has obtained evidence that hydrogen bonding can occur between natural and synthetic polysaccharides and denatured DNA.

It is therefore possible that DNA sites suitable for DNA-RNA hybridization may be blocked in DNA-nitrocellulose binding. DNA was applied to nitrocellulose in the presence of compounds which might have made it bind less tightly. Formamide was the only compound found to have the desired effect. The effects of sodium carboxymethylcellulose were probably mechanical.

Conditions in which DNA remained denatured and was also retained by nitrocellulose filters were not estab-

lished. Had such conditions been established, they would probably have been useful in the application of rapidly-renaturing mouse DNA to nitrocellulose. Reasonable retention of rapidly-renaturing DNA was achieved under more routine conditions.

The efficiency of liquid scintillation counting of tritiated DNA immobilised on nitrocellulose filters exceeded that of DNA spotted on to them by a factor of approximately 1.7 times. Kennel and Kotoulas (1968) compared the counting efficiency of tritiated RNA in hybrid form with that of acid-precipitated RNA. The RNA was on nitrocellulose filters in each case. The liquid scintillation counting efficiencies thus recorded also differed by a factor of 1.7 times in the same direction. However, corresponding factors for [$^{14}$C] RNA and [$^{32}$P] RNA were 1.2 and 1.06, respectively. Decrease in this ratio as isotopes of higher energy of emission were used indicates that DNA is immobilised on the surface of nitrocellulose filters and that DNA-RNA hybrids are formed there.

Fractionation of Mouse DNA.

$C_0t$ values used in renaturation of fractions of mouse DNA were deduced from the data of Britten and Kohne (1968). They were chosen such that renaturation

of the succeeding fraction was just about to commence on a significant scale. Estimations of the relative quantities of DNA represented by "fast", "intermediate" and "slow" fractions (approximately 8%, 15% and 77%) roughly agreed with those of Britten and Kohne (approximately 10%, 20% and 70%). Hyperchromicity was observed on attempted denaturation of supposedly renatured fractions of DNA, indicating that DNA had, in fact, renatured. No hyperchromicity was observed on attempted denaturation of "slow" DNA. Owing to the wide separation of $C_0t$ values required for half-renaturation of DNA fractions, contamination of "slower" DNA by "faster" DNA from this source must be slight. However, owing to its second order kinetics, the DNA renaturation reaction can never be complete. Denatured DNA can bind to hydroxyapatite to a small extent under conditions for retention of native DNA by virtue of its residual secondary structure (Britten, 1969). For these reasons, contamination of a "slower" fraction by a "faster" one is inevitable, even if very slight. This cannot be neglected in interpretation of results of experiments involving DNA fractionated on hydroxyapatite.

## 4. DNA-RNA Hybridization Studies.

### Choice of DNA and RNA.

Paul and Gilmour (1966a,b, 1968, 1969) have carried out many DNA-RNA hybridization studies using materials derived from calf thymus. DNA and chromatin are readily obtained from this source. Thus the use of calf thymus DNA was generally adopted.

There are, however, two disadvantages to its use. Firstly, it cannot readily be isotopically labelled. Thus it was necessary to use Landschutz ascites tumour cell DNA where labelled DNA was required. Secondly, calf DNA contains only two broad classes of nucleotide sequence (Britten and Kohne, 1968). Thus it was necessary to use Landschutz ascites tumour cell DNA in attempting to determine which fractions of DNA are transcribed to yield hybridizable RNA.

RNA was synthesized *in vitro* owing to the ease with which it could be isotopically labelled. Paul and Gilmour (1968) have shown that the hybridization characteristics of RNA transcribed *in vitro* from DNA in chromatin are very similar to those of natural RNA.

### Choice of a Procedure of DNA-RNA Hybridization.

Paul and Gilmour (1966a, 1966b, 1968, 1969) have used procedures based on that of Gillespie and Spiegelman

(1965). Adoption of such a procedure was therefore favoured. Two very desirable features of a DNA-RNA hybridization procedure are as follows. Firstly, formation of DNA-RNA hybrids should be the only reaction occurring. If this condition cannot be achieved, it should be approached as closely as possible. Secondly, the procedure should be as economical as possible in terms of time and materials so that many DNA-RNA reactions can be carried out simultaneously.

All of the earlier procedures involving use of agar or ultracentrifugal sedimentation were excluded through the second feature. Owing to concern over the effect of DNA renaturation on DNA-RNA hybridization when both DNA and RNA are in solution (Nygaard and Hall, 1964; Gillespie and Spiegelman, 1965; Kennel and Kotoulas, 1968), a liquid-liquid procedure of DNA-RNA hybridization was not generally employed. Nevertheless, very similar RNA saturation curves were obtained on hybridization of RNA synthesized in vitro from calf thymus DNA with denatured calf thymus DNA by procedures based on those of Nygaard and Hall (1963, 1964) and of Gillespie and Spiegelman (1965). The latter found that the extent of hybridization increased to a plateau in their bacterial solid-liquid system. Similar time courses

have been noted in mammalian systems (Church and McCarthy, 1967; Gilmour, 1967; Molli and Bishop, 1969). Maximal hybridization in a liquid-liquid system was observed by Gillespie and Spiegelman (1965) after 2 hours' incubation; thereafter the extent of hybridization decreased, presumably due to competition from DNA renaturation. Incubation for hybrid formation was, therefore, carried out for this time.

DNA immobilised on nitrocellulose is less available for reaction with poorly-represented species of RNA than is DNA in solution (Kennel and Kotoulas, 1968). Thus a liquid-liquid procedure might be more effective in statistically permitting hybridization between slow DNA and RNA transcribed from it owing to the low possibility of correct matching. However, the added complication of DNA renaturation was considered a serious hindrance to interpretation of liquid-liquid hybridization data. The solid-liquid procedure was thus considered more suitable for mammalian systems. Use of ribonuclease was included in it to ensure that non-specific DNA-RNA pairing would not be confused with DNA-RNA hybridization.

A disadvantage of this type of procedure is that the nitrocellulose membrane filters used are not uniform.

Each batch must be checked to ensure that they are satisfactory. Significantly high levels of binding of RNA to filters themselves can occur and must be guarded against.

That the extent of hybridization was minimal in the region of pH = 7.5 was taken to mean that the occurrence of poorly-paired hybrid was also minimal there. Synthetic RNA was, therefore, dissolved in tris/hydrochloric acid buffer solution pH = 7.4 or, where appropriate, in a solution of ¼ x S.S.C. in 50% v/v formamide adjusted to this pH.

Slightly higher levels of hybridization in the presence of sodium dodecyl sulphate were almost certainly due to its inhibition of ribonuclease activity (Fujinaga and Green, 1968). Lesser binding of RNA to blank filters is also in accord with the results of Fujinaga and Green. It was because of these two advantages of its use that sodium dodecyl sulphate was included in many incubation mixtures for DNA-RNA hybridization.

It is important to ensure that retention of DNA by nitrocellulose during hybridization is quantitative or that it is as close to this as possible. It cannot be assumed that equal fractions of all types of DNA nucleotide sequences will be lost from nitrocellulose

when loss of DNA is observed. 90% of the DNA applied to nitrocellulose membrane filters was retained during hybridization and treatment of the filters in the normal procedure. Unfortunately, there was greater loss of DNA from filters incubated in 50% v/v formamide. Account of this was taken in evaluation of fractions of DNA to which RNA had hybridized.

The extent of DNA-RNA hybridization was uniform on incubation at 67°C in S.S.C. solutions ranging in concentration from 1 X to 6 X. Binding of RNA to blank filters was uniformly low. The effect of ionic strength on the $T_m$ of DNA and hence the optimal temperature for DNA-RNA hybridization would probably be very slight over a sixfold range. At lower temperatures high levels of binding of RNA to blank filters were recorded at lower ionic strengths than that of 4 x S.S.C.. Extents of hybridization were erratic, which must have been a consequence of this, partly or entirely. Use of lower ionic strengths than that of 4 x S.S.C. and higher temperatures than that in the region of 25°C less than the $T_m$ of the DNA employed appeared disadvantageous for technical reasons. At such higher temperatures reduced levels of hybridization would be as likely to be due to loss of DNA from nitrocellulose as to increased

specificity of DNA-RNA hybridization (Church and McCarthy, 1968), while at lower ionic strengths than that of 4 x S.S.C. the advantages of increased specificity would be greatly outweighed by the disadvantage of high background count rates.

Thus conditions of 4 x S.S.C. and 67°C were routinely adopted for three main reasons: firstly, this temperature is approximately 25C° less than the $T_m$ of the DNA used under the same conditions; secondly, retention of DNA by nitrocellulose was optimal; thirdly, the ratio of quantity of RNA bound to DNA-bearing filters to that bound to blank filters was maximal.

Conditions for incubation in formamide were deduced from this. Their derivation agrees with that very recently described by McConnaughy, Laird and McCarthy (1969). In 50% v/v formamide and 4 x S.S.C. the Tm of DNA used appeared to be approximately 62°C. It was therefore appropriate to carry out incubation for DNA-RNA hybridization at 37°C. The $T_m$ of DNA is unlikely to be significantly affected by a two-fold change in ionic strength from 4 x to 2 x S.S.C. as it is proportional to the logarithm of ionic strength (MacGillivray and McMullen, 1966). The conditions of incubation derived from the work of Bonner, Kung and Bekhor (1967) involved

incubation at room temperature in 30% v/v formamide. These must give low specificity, as also suggested by McConaughy, Laird and McCarthy (1969). DNA-RNA hybrids of low specificity are sensitive to degradation by ribonuclease (Yankofsky and Spiegelman, 1962a). It is therefore not surprising that a low level of DNA-RNA hybrid formation was recorded between RNA synthesized *in vitro* from calf thymus DNA and alkaline-denatured calf thymus DNA incubated together in 30% v/v formamide in 4 x S.S.C. at room temperature after incubation of putative hybrids with ribonuclease. This finding has been confirmed by Kohl (1968). Under conditions such as these, incubation for hybridization was carried out at a temperature approximately 50C° less than the $T_m$ of the DNA involved under the same conditions.

As there appeared to be little or no degradation of DNA-RNA hybrids nor of RNA during 70 hours' incubation in 50% v/v formamide, its use was adopted where such longer periods of incubation for DNA-RNA hybridization were thought necessary. It appeared to be equivalent to standard conditions.

The time course of the action of ribonuclease on DNA-bearing and blank nitrocellulose filters after incubation for hybridization was of interest. It

suggested that the action of the enzyme resulted in loss of poorly-paired RNA and RNA adhering non-specifically to nitrocellulose. It also indicated that stable DNA-RNA hybrid insensitive to the action of ribonuclease, had been formed (Yankofsky and Spiegelman, 1962a). Specificity of pairing is almost certainly less in the present studies on eucaryotic materials than in the bacterial system of Yankofsky and Spiegelman. Nevertheless, there must be a high degree of correct base-pairing to result in such insensitivity to ribonuclease. Accuracy of the normal incubation time of 1 hour was evidently not of critical importance.

Alternative procedures of treatment of filters after DNA-RNA hybridization appeared less effective. Church and McCarthy (1967) reported washing of filters in 4 x S.S.C. at 67°C. They did not use ribonuclease owing to lack of evidence for complete specificity of its action and evidence that ribonuclease treatment of DNA-RNA hybrids alters the base composition of hybridized RNA and reduces its molecular weight by selective removal of pyrimidines. While such hot washing of filters reduced count rates much less than did the action of ribonuclease, the latter was evidently more effective in reducing poorly-agreeing count rates of

identical filters to closely-agreeing lower values.

The thermal melting profile of a DNA-RNA hybrid was obtained using hybrid formed and studied in solution (0.126 x S.S.C.). Attempts to obtain thermal melting profiles of DNA-RNA hybrids immobilised on nitrocellulose filters were unsuccessful. There was complete loss of RNA from hybrids on filters in 0.01 x S.S.C. including and excluding 0.1% w/v S.D.S. before heating. No RNA was lost from hybrid on filters in 1 x S.S.C. nor in 0.1 x S.S.C.

The shape of the thermal melting profile obtained was similar to that of DNA-RNA hybrid formed between pulse-labelled mouse liver RNA and mouse DNA described by Church and McCarthy (1967). They obtained DNA-RNA hybrids by the procedure of Gillespie and Spiegelman (1965), treated them with ribonuclease and eluted "melted" RNA in 0.5 x S.S.C. The $T_m$ reported in present studies is 19C° less than that reported by Church and McCarthy. Three possible reasons for this are as follows. Firstly, the $T_m$ of calf thymus DNA was found to be slightly less than that of murine DNA. Secondly, the salt concentration used was approximately four times less than that used by Church and McCarthy. Thirdly, precision of hybridization was probably

inferior owing to the greater proportion of calf thymus DNA in repetitious nucleotide sequences and use of RNA synthesized *in vitro* from whole DNA.

Significance of RNA Saturation Curves.

The time of incubation for DNA-RNA hybridization was such that its extent would attain a plateau level (Church and McCarthy, 1967; Gilmour, 1967; Molli and Bishop, 1969). Results of hybridization experiments were frequently plotted in the form of RNA saturation curves. While these appeared to represent the full extent of the reaction, the significance of the saturation values is open to discussion.

In viral and bacterial systems DNA-RNA hybridization is locus-specific. Three examples quoted by Church and McCarthy (1968) illustrate this. Bautz and Bautz (1967) isolated region-specific mRNA from cells of *Escherichia coli* infected by bacteriophage T4. This RNA hybridized to only the corresponding fraction of T4 DNA. Attardi, Naono, Rouviere, Jacob and Gros (1963) and Hayashi, Spiegelman, Franklin and Luria (1963) reached similar conclusions in their studies in bacterial systems. The former isolated mRNA from cells of *Escherichia coli* induced for galactose catabolism. The DNA of the *gal* operon was concentrated by transduction. Specific

DNA-RNA hybridization was observed between mRNA and such DNA. A similar result was recorded by the latter in their studies on the lac system of Escherichia coli.

Church and McCarthy (1968) studied saturation of DNA of the mouse and of Bacillus subtilis and Escherichia coli with natural homologous RNA. Plateaux of saturation of bacterial DNA by homologous RNA and melting characteristics of DNA-RNA hybrids tended to be independent of the temperature of incubation of DNA-RNA hybridization mixtures. Thus the predominant reaction in DNA-RNA hybridization between bacterial materials is probably one between an RNA molecule and its parental gene. It may, therefore, be possible to use the technique of DNA-RNA hybridization in bacterial and viral systems to estimate the fraction of total DNA specifying a particular group of RNA molecules.

A similar conclusion cannot be reached in eucaryotic systems. Church and McCarthy (1968) found that plateaux of saturation of mouse DNA by homologous RNA decreased with increasing temperature of incubation of hybridization mixtures. The stability of DNA-RNA hybrids against thermal denaturation also increased

in this direction. This indicates that in eucaryotic systems complete locus specificity is attained only at the highest temperatures of incubation. At these, reaction rates are vanishingly small.

Melli and Bishop (1969) carried out DNA-RNA hybridization between denatured rat DNA and RNA transcribed *in vitro* on a template of denatured rat DNA. The fraction of denatured DNA hybridizable with saturating quantities of RNA (approximately 5%) was only about 10% of that involved in DNA-RNA hybrid formation during *in vitro* transcription of denatured DNA (approximately 50%). Further results obtained by them point to isolated RNA being able to form DNA-RNA hybrid mainly or only with repetitious DNA.

From these considerations it can be concluded that DNA-RNA hybridization reactions between eucaryotic materials involve hybridization to repetitious DNA sequences predominantly. Nevertheless, McConaughy, Laird and McCarthy (1969) have reported very recently that Shenrer (1969) has obtained extensive hybridization of mouse RNA with unique sequences of mouse DNA. This was achieved through use of prolonged incubation periods made practicable by use of formamide in place of elevated temperatures. Similarly,

Davidson and Hough (Davidson, E.H., and Hough, Barbara, Proc.Natn.Acad.Sci. U.S.A., 63, 342 (1969)) have very recently reported hybridization between RNA of lampbrush-stage Xenopus oöcytes and the unique sequences of Xenopus DNA.

The effects of temperature of incubation on the quantity and nature of hybrid formed (Church and McCarthy, 1968) suggest that the precision of hybridization increases with increasing temperature of incubation. The studies of Church and McCarthy (1968) on competitive DNA-RNA hybridization show that the precision of hybridization increases with decreasing salt concentration of hybridization incubation mixtures. As more stringent conditions are approached, the fidelity of pairing of RNA sequences with DNA sequences increases. Under the conditions normally employed in DNA-RNA hybridization experiments, RNA sequences must be paired to DNA sequences not fully complementary to them but similar to those which are. Thus locus-specificity is not achieved and the absolute levels of saturation plateaux are of little significance. Church and McCarthy (1968) pointed out that reaction of RNA with partially complementary sequences will lead to overestimations of the fraction of DNA to which RNA

is complementary, while the failure of RNA derived from unique nucleotide sequences to react will lead to underestimation of this fraction. Nevertheless, they concluded that it is reasonable to suppose that saturation plateaux determined under similar conditions will be reasonable relative estimates of the derepression of the genome.

Construction of saturation curves and double reciprocal plots derived from them were purely empirical treatments of hybridization data. However, they bear some relation to Michaelis-Menten treatment of the kinetics of enzymatic reactions.

In similar DNA-RNA hybridization experiments the level of saturation of denatured calf thymus DNA by RNA synthesized in vitro on a template of native calf thymus DNA was always 20 ± 2% (when ribonuclease was shown to be absent). On repeating one experiment with sodium dodecyl sulphate included in hybridization mixtures (Fujinaga and Green, 1968), the saturation level rose from 18% to 21%. In another experiment it was also 20% after 10 and 30 minutes' incubation of the RNA-synthesizing mixture. This RNA saturation level therefore appears to be a reproducible measurement.

It is of interest that RNA synthesized after 10, 30 and 90 minutes' incubation of the RNA-synthesizing mixture could be hybridized to the same fraction of DNA. It cannot be stated that the same sequences in DNA are transcribed throughout the incubation period, although this would seem very likely. More certainty would be obtained on carrying out DNA-RNA hybridization competition experiments (Paul and Gilmour, 1968). The length of time of the 90 minute incubation period does not appear to be critically significant.

DNA Saturation Curves.

DNA saturation curves and corresponding double reciprocal plots were also derived empirically. Again, there is similarity to the Michaelis-Menten treatment of the kinetics of enzymatic reactions. The fraction of total RNA hybridizable to DNA was unexpectedly small. As Bekhor and Gilmour (1969) have found that ribonucleotides are not readily removed from RNA by dialysis between very dilute salt solutions, much of the material thought to be RNA was probably unincorporated ribonucleotides. Some of it may have been oligoribonucleotides whose length was less than that necessary for hybridization to DNA.

Molecular sieve chromatography was therefore introduced into the procedure of isolation of synthetic

RNA. The molecular weight operating range of the Biogel P-30 material employed was in the range of 20,000 to 50,000. A small fraction of purified RNA or ribonucleotide material was excluded indicating that RNA synthesized *in vitro* probably exceeded 60 nucleotide residues in molecular length.

Nature of the Materials involved in DNA-RNA Hybridization.

In present studies hybridization was achieved between denatured DNA and RNA molecules of considerable length. Denatured DNA has been used in all DNA-RNA hybridization studies reported except that of Bekhor, Bonner and Kung Dahmus (1969). They demonstrated complex formation between chromosomal RNA of rat Novikoff ascites tumour cells and homologous native DNA. They were unable to demonstrate complex formation between such native DNA and RNA synthesized *in vitro* from it. DNA and RNA were incubated together at a temperature in the region of 60C° less than that of the $T_m$ of the DNA. It is therefore unlikely that true DNA-RNA hybridization occurred. Complex formation is probably a property peculiar to chromosomal RNA.

Attention has been given to estimation of the minimal length of RNA molecules required for DNA-RNA

hybridization. This depends on the temperature and ionic environment in hybridization incubation mixtures and the nature of the RNA (Walker, 1969). Niyogi and Thomas (1967), Niyogi (1969) and Rüger and Bautz (1968) degraded RNA isolated from Escherichia coli infected by T bacteriophage to oligoribonucleotides. These were separated according to chain length by molecular sieve chromatography. They attempted to hybridize such RNA fragments with homologous and heterologous T bacteriophage DNA using a procedure based on that of Gillespie and Spiegelman (1965). The minimum chain length necessary for hybrid formation was found to decrease with decreasing temperature of incubation. This implies loss of specificity of hybridization (Church and McCarthy, 1968), which is evident from studies on the increase in hybridization between heterologous DNA and RNA on using shorter oligoribonucleotides. It was concluded that at least 12 perfectly complementary nucleotides must be involved in DNA-RNA hybrid formation for this to be species-specific. Molecular chain lengths of only slightly less than this were required for any hybridization to be observed. Gillespie and Spiegelman (1966) concluded, however, that the minimum length of rRNA molecules of Escherichia coli necessary

for stable hybrid formation with homologous denatured DNA was about 50 nucleotide units. This number was less at lower temperatures, again implying less specificity of hybrid formation. Gillespie and Spiegelman (1966) carried out incubations for hybrid formation at lesser salt concentrations than those used by Miyoqi and Thomas, Miyoqi, and Röger and Bautz. This would increase the specificity of DNA-RNA hybrid formation. Thus the length of RNA molecules used in hybridization studies described here is almost certainly considerably in excess of that required for species-specific formation of stable DNA-RNA hybrids.

It is of interest to compare these findings with those of McConaughy and McCarthy (1967) on the interaction of oligodeoxyribonucleotides with denatured DNA. They found that oligodeoxyribonucleotides interact with denatured DNA, albeit with little specificity, when their molecular chain length is 14 nucleotide residues. Species-specificity of interaction between mouse and Escherichia coli oligodeoxyribonucleotides and denatured DNA was demonstrated at a mean molecular chain length of 33 nucleotide residues. Specificity of interaction and stability of products were again observed to increase with increasing temperature of incubation.

Inclusion of denatured DNA in DNA-RNA hybridization incubation mixtures caused a marked increase in background binding levels of RNA. This may have been due to DNA and DNA-RNA hybrid formed with DNA in solution becoming attached to blank filters during incubation. Thus ineffective removal of DNA from RNA used in DNA-RNA hybridization experiments would probably result in high background levels of binding of RNA being recorded.

DNA-RNA hybrids were lost from nitrocellulose on successive incubations without any nucleic acid material present. This could be due to reversibility of the DNA-RNA hybridization reaction or breakdown and loss from nitrocellulose filters of DNA-RNA hybrids at 67°C. The apparent rate of decay of hybrid suggests that both of these effects have occurred. Degradation of hybrid is also suggested by loss of hybrid from nitrocellulose between 17 hours' and 70 hours' incubation at 67°C, but not at 37°C. The presence of other nucleic acids appeared to tend to prevent loss of hybrid from filters.

Competition between nonradioactive RNA and radioactive RNA otherwise identically synthesized and isolated indicates that the ability to interact

measurably with denatured DNA is not peculiar to radioactive RNA. It could otherwise be argued that isotope-exchange had been followed. Since saturating conditions were not employed and since different fractions of each apparent RNA may be small oligonucleotides or mononucleotides, a quantitative interpretation of this result is inappropriate.

Hybridization of RNA to Fast, Intermediate and Slow Fractions of Homologous DNA.

Hybridization of RNA synthesized *in vitro* from whole native Landschutz ascites tumour cell DNA to denatured whole DNA and fast, intermediate and slow fractions thereof was effectively demonstrated. However, the levels of saturation of fractions of DNA by RNA were much lower than that of whole DNA.

This was almost certainly not due to their being contaminated by ribonuclease: the steps involved in purification of fractions of DNA would reduce the level of any ribonuclease activity associated with the source of these materials; 25% saturation of whole DNA by RNA was observed simultaneously under identical conditions; both DNA and RNA included in hybridization incubation mixtures were treated

with iodoacetate; ribonuclease activity was not detectable in any of the three fractions of DNA, even before treatment with iodoacetate. It was also absent from the RNA polymerase employed.

The lesser extents of hybridization of RNA to fractions of DNA may have been associated with the native DNA from which the latter were derived having been sonicated. The length of molecules of this sonicated native Landschutz ascites tumour cell DNA was probably in the region of 1500 nucleotide pairs (Hell, 1969). Thus it greatly exceeded the minimal length of RNA molecules required for formation of stable DNA-RNA hybrids. It may be that sonicated DNA was lost from nitrocellulose, yet it was applied to the latter under conditions such that it was well retained. This could readily be tested, of course, on similarly obtaining tritiated Landschutz ascites tumour cell DNA fractions. It is of interest that Paul and Gilmour (1966a) obtained identical RNA saturation curves on hybridizing RNA synthesized *in vitro* from sonicated and non-sonicated calf thymus DNA with alkaline-denatured calf thymus DNA.

That no sure reason can be given for the apparent discrepancy in extents of hybridization is in keeping

with the conclusion of Church and McCarthy (1968) that the absolute levels of RNA saturation plateaux in eucaryotic systems are of little significance.

The levels of hybridization of RNA to each of the three fractions were sufficiently similar to one another to make it highly unlikely that they were entirely due to contamination of a non-hybridizable fraction by a hybridizable one. There may, however, have been some degree of cross-contamination. Determination of the time courses of the hybridization reactions with each of the three fractions would probably permit a definite conclusion on this possibility. To obtain these would be an appropriate next step.

While it can be concluded that all three fractions of whole native DNA were transcribed *in vitro*, this does not necessarily mean that they are transcribed *in vivo*. Another study which should soon be carried out is attempted hybridization of RNA transcribed from chromatin and natural RNA with separated fractions of DNA. Each fraction of DNA must have contained initiation sites for exogenous RNA polymerase in order that it may have been transcribed. There may, therefore, be initiation sites available for endogenous RNA

polymerase *in vivo*. It is not certain whether fast DNA is transcribed *in vivo* (Harel, Hanania, Tapiero and Harel, 1968; Flamm, Walker and McCallum, 1969). It may be transcribed to give only certain restricted types of RNA. There is evidence that natural RNA can be hybridized with slow DNA on prolonged incubation (McConaughy, Laird and McCarthy, 1969; Shearer, 1969; Davidson and Hough, 1969). The evidence of Davidson and Hough has been reported very recently (Davidson, E.H., and Hough, Barbara, Proc. natn.Acad.Sci.U.S.A., 63, 342 (1969)). This implies that unique nucleotide sequences of DNA are transcribed *in vivo*. As already described, there is evidence that almost all DNA-RNA hybridization studies involving eucaryotic materials must have predominantly involved rather non-specific hybridization to repetitious nucleotide sequences in DNA (Melli and Bishop, 1969; Church and McCarthy, 1968).

It therefore appears that all three fractions of eucaryotic DNA are transcribed, even if transcription of fast DNA *in vivo* is conditionally restricted. Britten and Davidson (1969) have recently proposed a scheme of regulation of transcription of eucaryotic DNA which involves transcription of RNA from repetitious

and unique nucleotide sequences of DNA. Unique "producer" genes are controlled by "receptor" genes, to which they are linked. Producer genes are analogous to the structural genes of bacteria (Jacob and Monod, 1961a,b). Receptor genes are activated by "activator" RNA, which is transcribed from "integrator" genes. Integrator and producer genes must match; there could be many copies of each of these such that they would correspond to the repetitious nucleotide sequences of eucaryotic DNA. This would permit formation of sequence-specific complexes between receptor genes and activator RNA molecules. Finally, many integrator genes may be linked to a sensor gene, which activates the former in response to an environmental stimulus. Thus transcription of a battery of producer genes could be induced by an environmental stimulus. The intervening activator RNA may correspond to chromosomal RNA described by Bonner and his colleagues (Bonner et al., 1968; Belcher, Kung and Bonner, 1969) and Huang and Huang (1969).

It is therefore important to know the mode of transcription of the different fractions of eucaryotic DNA. Further exploitation and refinement of the technique of DNA-RNA hybridization may well provide solutions to this.

Possible Mechanism of the DNA-RNA Hybridization Reaction.

The effects of temperature and salt concentration on the reactions of DNA renaturation and DNA-RNA hybridization are similar. Under similar conditions both reactions are second order, have similar rate constants and similar activation energies (Nygaard and Hall, 1964), and similar minimal nucleotide chain lengths are required for them. From studies such as those of Britten and Kohne (1968), Walker and his colleagues and McCarthy (1967), it appears that most experiments on the renaturation of eucaryotic DNA involve repeated nucleotide sequences predominantly. The same appears to be true for DNA-RNA hybridization between materials of eucaryotic origin (Church and McCarthy, 1968; Melli and Bishop 1969).

It therefore seems not unreasonable to propose that they involve a similar reaction mechanism. This implies that the initial step in DNA-RNA hybridization is nucleation between short complementary sequences, this step being second order and rate-limiting. This would be followed by a much faster zippering process obeying first order reaction kinetics (Wetmur and Davidson, 1968). As the minimal nucleotide chain lengths required for DNA-oligodeoxyribonucleotide interaction and DNA-RNA hybridization are roughly similar, studies on the minimum RNA or oligodeoxyribonucleotide chain length necessary for stable DNA-RNA hybrid formation or DNA renaturation may have indicated the minimum chain length necessary for specific nucleation.

# S U M M A R Y.

1. The purpose of these studies was to establish the validity of a system for investigation of the manner in which transcription from mammalian DNA is regulated.

2. Mammalian chromatin was isolated; its chemical composition was determined in terms of relative quantities of DNA, histone, acidic chromosomal protein and chromosomal RNA. A procedure employing binding of the dye bromsulphalein to protein was developed for estimation of protein in chromatin. Correlation between the relative quantities of DNA and histone and, to a lesser extent, between those of acidic chromosomal protein and chromosomal RNA was noted.

3. RNA transcribed from mammalian DNA was also studied. The best available procedure for characterization of such RNA was considered to be DNA-RNA molecular hybridization.

4. RNA of high specific activity was required. This was obtained by synthesizing RNA *in vitro* using purified bacterial DNA-dependent RNA polymerase.

Procedures of purification from Micrococcus luteus (lysodeiktieus) were compared with a view to obtaining both maximal yields and a product not contaminated by ribonuclease activity. Evidence that the enzyme catalysed the synthesis of RNA complementary to DNA template, that its activity was dependent on the presence of the latter and that a DNA-RNA hybrid might be formed during RNA synthesis was obtained. Conditions for optimal RNA synthesis and for isolation of synthetic RNA were established. Synthetic RNA was isolated by removal of all other components of RNA-synthesizing mixtures. Particular care was required to remove acid-soluble ribonucleotides. Otherwise, the fraction of synthetic RNA hybridizable to DNA appeared to be very small.

5. Procedures for isolation of native DNA from mammalian cells were established, as were procedures for denaturation of DNA and for separation of denatured Landschutz ascites tumour cell DNA into "fast", "intermediate" and "slow" fractions by virtue of their relative rates of renaturation. DNA which had renatured was separated from that which had not by passage through hydroxyapatite. Under certain

controlled conditions, the former, but not the latter, was retained by hydroxyapatite. Conditions for optimal binding of denatured DNA to nitrocellulose and retention of it by nitrocellulose were established. DNA-nitrocellulose binding proved to be dependent on temperature, ionic strength and concentration of solvents which weaken hydrogen bonds, suggesting it is at least partly due to such bonding.

6. Procedures and conditions for carrying out DNA-RNA hybridization between RNA in solution and DNA immobilised on nitrocellulose and procedures for plotting the results thereof were established, the latter empirically. RNA saturation curves and double reciprocal plots derived from them showed that RNA synthesized in vitro from native calf thymus DNA was hybridizable to approximately 20% of denatured calf thymus DNA under the conditions employed. This figure was the same after three different periods of incubation of an RNA-synthesizing mixture, but was greatly reduced when the materials used in such experiments were contaminated by ribonuclease activity. It was also markedly reduced

on incubation of hybridization mixtures at room temperature in the presence of 30% v/v formamide instead of at 67°C in the absence of formamide. Incubation at 37°C in the presence of 50% v/v formamide was equivalent to incubation at 67°C, save that more prolonged incubation was practicable under the former conditions owing to lesser degradation of RNA or hybrid or both. The presence of sodium dodecyl sulphate proved advantageous; non-specific binding of RNA to nitrocellulose was reduced and a slightly greater extent of hybridization was recorded. Studies on the action of ribonuclease on putative DNA-RNA hybrid indicated that DNA-RNA hybrid was completely resistant to degradation by it, while non-specifically bound RNA was eliminated. Treatment of nitrocellulose membrane filters with ribonuclease and washing them at room temperature, which was routinely included in the procedure of DNA-RNA hybridization, was more effective in obtaining reproducible results than washing at 67°C or at room temperature. Evidence that the highest possible purity of materials employed in DNA-RNA hybridization experiments is essential was obtained. Some evidence that the

DNA-RNA hybridization reaction is at least partly reversible was also obtained. The solid-liquid procedure of DNA-RNA hybridization was compared to the liquid-liquid procedure in which hybrids are formed in solution and then trapped on nitrocellulose. Identical RNA saturation curves for the calf thymus DNA system were obtained when the period of incubation in the latter procedure was carefully chosen.

7. RNA was synthesised in vitro from whole native Landschutz ascites tumour cell DNA and was hybridized with denatured whole homologous DNA and "fast", "intermediate" and "slow" fractions thereof. To increase the chance of detecting hybridization of RNA with unique nucleotide sequences in "slow" DNA, incubation was carried out for 70 hours in 50% v/v formamide at 37°C. Hybridization to all three fractions of DNA and to whole DNA was observed, though levels of saturation of separated fractions of DNA appeared to be much less than that of whole DNA.

8. The results of these studies are discussed in the light of information and speculation recorded in the literature.

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nucleotides. Otherwise, the fraction of synthetic RNA hybridizable to DNA appeared to be very small.

Procedures for isolation of native DNA from mammalian cells were established, as were procedures for denaturation of DNA and for separation of denatured Landschutz ascites tumour cell DNA into "fast", "intermediate" and "slow" fractions by virtue of their relative rates of renaturation. DNA which had renatured was separated from that which had not by passage through hydroxyapatite. Under certain controlled conditions, the former, but not the latter, was retained by hydroxyapatite. Conditions for optimal binding of denatured DNA to nitrocellulose and retention of it by nitrocellulose were established. DNA-nitrocellulose binding proved to be dependent on temperature, ionic strength and concentration of solvents which weaken hydrogen bonds, suggesting it is at least partly due to such bonding.

Procedures and conditions for carrying out DNA-RNA hybridization between RNA in solution and DNA immobilised on nitrocellulose were established. RNA saturation curves and double reciprocal plots derived from them showed that RNA synthesised <u>in vitro</u> from native calf thymus DNA was hybridisable to approximately 20% of denatured calf thymus DNA under the conditions employed. This figure was the same after three different periods of incubation of an RNA-synthesising mixture, but was greatly reduced when the materials used in such experiments were contaminated by ribonuclease activity. It was also