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## SUMMARY

### The Control of DNA Synthesis in L Cells

by J. Gordon Lindsay

Summary of the thesis presented for the degree of Doctor of  
Philosophy, University of Glasgow, October 1969.

Regenerating liver, rabbit kidney cortex cells cultured in vitro and PHA-stimulated lymphocytes have been widely employed to study the sequence of metabolic events which is required for the onset of DNA synthesis and cell division when resting cells are stimulated to renewed proliferation. A parallel resting cell system has been established with a permanent cell line by maintaining cultures of L 929 cells at high population density. Such a system is particularly suitable for study because of the ease of culture manipulations and the relatively high degree of synchrony obtained.

Released-stationary cells begin to synthesise DNA after a lag period of 14 hr. and by 20 hr. 70% of the cells are in S phase. Increases in cell number are observed by 25 hr. In contrast to primary resting systems no change in the rate of RNA synthesis is detected after release from stationary phase. RNA synthesis during the lag phase is required for subsequent DNA synthesis.

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Changes in the activity of DNA polymerase in nuclear and supernatant fractions of L cells have been investigated following release from stationary phase and particularly during S phase. The results of previous investigators have been confirmed and extended.

Nuclear preparations of L 929 cells show a 2-5-fold preference for native DNA primer while the supernatant DNA polymerase activity is correspondingly more active with denatured DNA. The general characteristics of the DNA polymerase(s) in these fractions have been investigated and methods for releasing the enzyme from isolated nuclei studied.

Preliminary purification of the enzyme was undertaken although difficulties were encountered because of the small amounts of material available from tissue culture cells. A 7-8-fold purification was achieved by pH 5 precipitation and Sephadex G200 chromatography and 95% of the DNase activity removed as judged by the relative capacity of the fractions to hydrolyse native or heat-denatured DNA to acid-soluble fragments. After purification DNA polymerase activity, primed by denatured DNA, was rapidly lost on freeze/thawing of the solution.

DNA polymerase activity in extracts of L 929 cells was found to be heterogeneous by fractionation on Sephadex G200, DEAE-cellulose and polyacrylamide gels. As similar results

have recently been reported for the rat liver enzyme the possible significance of this result in relation to in vivo replication is considered. The nuclear location of a fraction of the DNA polymerase activity and its preference for native DNA primer makes it a possible candidate in this respect.

Sephadex-purified nuclear and supernatant fractions have been used to synthesise [ $^3\text{H}$ ] DNA on their preferred primers and the characteristics of the DNA products examined. Both products were found to be resistant to degradation by exonuclease I suggesting that the newly-synthesised DNA was not present in a single-stranded form. Analysis of the DNA products on neutral and alkaline sucrose gradients reveals that the [ $^3\text{H}$ ] DNA is not covalently attached to the DNA primer. Our findings on the characteristics of the DNA products are compared to previous data on the DNA products formed by the calf thymus and Escherichia coli DNA polymerases.

CONTROL OF DNA SYNTHESIS IN L CELLS

by

J. Gordon Lindsay, B.Sc.

Thesis presented for the degree of

Doctor of Philosophy

at the University of Glasgow, October 1969.

## ACKNOWLEDGEMENTS

### ACKNOWLEDGEMENTS

I thank Professor J.N. Davidson, C.B.E., F.R.S. and Professor R.M.S. Smellie for providing the facilities for this research and I am particularly indebted to Dr. R.L.P. Adams for his guidance and friendly supervision during the course of this work.

I should like to express my thanks to Miss C.J. Adams, M.Sc. for providing a sample of DNase free, Landschütz ascites DNA polymerase and to Mr. D. Donnelly for preparing [ $^{32}\text{P}$ ] DNA. My thanks are also due to Miss J. McGill for the typing.

I acknowledge the receipt of a Medical Research Council grant during the first two years of this work.

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## ABBREVIATIONS

These are as laid down in the Biochemical Journal Instructions to Authors (revised, 1969) with the following additions:-

DNA polymerase	Deoxyribonucleosidetriphosphate :
	DNA nucleotidyltransferase, EC 2.7.7.7
Thymidine kinase	ATP : thymidine 5'-phosphotransferase, EC 2.7.1.21.
Pancreatic DNase (DNase I)	Deoxyribonucleate oligonucleotido- hydrolase, EC 3.1.4.5
DNase	Deoxyribonuclease
poly dA	Single-stranded polymer containing dAMP residues only
poly d(AT)	Double-stranded copolymer, each chain of which contains dAMP and dTMP residues
poly dA : dT	Double-stranded polymer with one strand of poly dA and one of poly dT
poly dA <sub>2</sub> : dT	Triple-stranded polymer containing two strands of poly dA and one of poly dT
poly d(ABu)	Double-stranded copolymer, each chain of which contains dAMP and 5-bromodeoxy- uridine 5'-monophosphate residues

poly dG : dC	Double-stranded polymer with one strand of poly dG and one of poly dC
tRNA	transfer RNA
BSA	Bovine serum albumin
BSS	Balanced salt solution
PPO	2,5-diphenyloxazole
POPOP	1,4 di-2-(5-phenyl oxazolyl)-benzene
TEMED	N,N,N',N'-Tetramethylethylene-diamine
SSC	Standard saline-citrate (0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0)
$\frac{1}{10}$ th SSC	0.015 M-NaCl, 0.0015 M-sodium citrate, pH 7.0
FPA	parafluorophenylalanine
MAK	Methylated albumin kieselguhr
*tris-sucrose, buffered sucrose	0.02 M-tris-HCl, pH 7.5 containing 0.25 M-sucrose
5-component buffer	0.02 M-tris-HCl, pH 7.5 containing 0.25 M-sucrose 1 mM-EDTA, 0.15 M-KCl and 5 mM-2-mercaptoethanol
BHK 21 (C13) cells	Baby hamster kidney cells, clone 13
HE <sub>p</sub> -2 cells	Human epithelioid carcinoma, no. 2
RK cells	Rabbit kidney cells
PPLO	Pleuropneumonia-like organisms

The terms DNase, endonuclease and exonuclease are used to describe uncharacterised activities in cell extracts which bring about degradation of added DNA.

Footnote - DNA primer

DNA polymerase activity in L cells functions in a manner which suggests that added DNA is a template for the enzyme. The term template is, however, very specific and implies that polymerisation is proceeding by specific base pairing with formation of new DNA strands complementary to the added DNA; it also implies the absence of non-specific addition to the termini of DNA chains. For this reason the more general term DNA primer has been normally adopted in this context to mean simply an initiator of polymerisation without defining exactly the type of polymerisation (non specific and/or template directed) which may be occurring.

INTRODUCTION

## CHAPTER I - INTRODUCTION

### 1. TISSUE CULTURE.

#### 1.1. Historical development

Tissue culture is a technique which has been increasingly employed in recent years in the study of a wide variety of biochemical problems. Isolated cell systems have proved particularly useful in the analysis of problems in carcinogenesis, viral reproduction, morphogenesis and cytogenetic variation and are now generally used as a basic research tool in many areas of biology and medicine.

Historically tissue culture developed as a natural extension to the techniques of embryology employed in the last century. An experiment by Harrison (1907) in which he explanted tissue from frog embryonic spinal chord into clots of frog lymph fluid and was subsequently able to show the development of nerve fibres in vitro is generally accepted as being the true beginning of tissue culture as we know it today.

Much of the pioneer investigation in this field was carried out by Carrel and his co-workers (Carrel, 1912, 1913a, b, 1924; Carrel and Ebeling, 1922; Carrel and Baker, 1926) who developed

rigorous aseptic techniques for cell cultivation and, by maintaining explants in plasma clots fortified with embryo extracts, were able to propagate cells for long periods of time. Indeed they were able to keep one strain of chick heart fibroblast cells in active proliferation for 34 years by these methods.

During this period most of the main animal cell types were cultured by a number of investigators (Fischer, 1930, 1946; Murray and Kopech, 1953; Willmer, 1958, 1960) and much insight was gained into cell morphology and the characteristics of isolated cell systems. Unfortunately, owing to the complexity and labouriousness of the traditional techniques of tissue culture, it came to be regarded more as an art than a scientific tool and consequently made little impact on the scientific world over the next 30 years. In addition, the complexity of the media and the difficulties of tissue manipulation made tissue culture unsuitable for quantitative analyses.

## 1.2. Modern techniques

The classical procedures of plasma clot cultures have now been superseded by the development of simpler systems for the serial propagation of mammalian cells. An important advance was made by Evans and Earle (1947) who found that, by growing a series

of tissue culture explants of mouse cells on a solid substrate such as glass or cellophane, a continuous monolayer of cells could be formed. These cells could then be scraped off, resuspended, and used to inoculate fresh cultures of the same kind. The advent of monolayer cultures allowed, for the first time, accurate determinations of cell number to be made and further permitted the setting up of duplicate plates for quantitative estimations.

Dulbecco (1952) and Moscona (1952) found that, by digesting fresh tissues with trypsin (EC 3.4.4.4) to form single cell suspensions, primary monolayer cultures could easily be established on glass surfaces. This technique was adapted by Scherer, Syverton and Gey (1953) for the routine subculture of all cell lines and is now widely used in this capacity. Other proteolytic enzymes e.g. elastase (pancreopeptidase E, EC 3.4.4.7) and collagenase (clostridiopeptidase A, EC 3.4.4.19) have proved effective in the dispersal of cells as have chelating agents such as citrate or EDTA (Rinaldini, 1958; Paul, 1960). This rapid evolution in technology greatly improved the usefulness of cell cultures as systems for experimental study. During this initial period methods for growing cells on defined or simplified media were developed as well as procedures for the cloning and long-term storage of cultures, thus bringing tissue culture on a par with microbiology.

### 1.3. Cell nutritional requirements

Establishment of monolayer cultures made possible for the first time accurate analysis of the nutritional factors for a given cell type. The principal contribution in this field has been by Eagle and his collaborators in an outstanding series of papers (Eagle, 1955, a, b, c, d, 1956, a, b, 1960 a, b; Eagle, Agranoff and Snell, 1960; Eagle, Barban, Levy and Schulze, 1958; Eagle, Freeman and Levy, 1958; Eagle, Oyama, Levy and Freeman, 1956, 1957; Eagle, Oyama, Levy, Horton and Fleischman, 1956; Eagle, Oyama and Piez, 1960; Eagle and Piez, 1960, 1962; Eagle, Piez and Fleischman, 1957; Eagle, Piez and Oyama, 1961; Eagle, Washington, Levy and Cohen, 1966). Their original observations were that primary cultures of established cell lines, for example, HeLa or L cells would grow on defined media containing the correct amounts of glucose, salts, amino acids and vitamins provided a small amount of dialysed serum was added. Since omission of a single essential nutrient caused cessation of growth and eventual death of the cells, it was possible to study the specific growth requirements of a given cell line.

Eagle has shown that strain L mouse cells require the presence of 13 amino acids for growth, namely Arg, His, Leu, Isoleu, Tyr, Glu, Met, Lys, Phe, Cys, Val, Trp and Thr. Most of these amino acids exhibit sharply defined optimal concentrations for growth and only the L-forms are effective (Eagle, 1955, a, b).

The pattern of amino acid requirements has been found to be remarkably similar for a wide variety of cell lines. While there may be some variation in the concentrations required for maximum response the same 13 amino acids are generally found to be essential for growth.

Initially some differences in amino acid requirements were noted. Cells of Walker rat carcinoma and rat leukaemia L-5178Y were reported to grow only on addition of L-asparagine to the culture (McCoy, Maxwell and Neuman, 1956; Haley, Fischer and Welch, 1961). Similarly a strain of rabbit fibroblasts was shown to need serine for growth (Haff and Swim, 1957a). The requirements for certain metabolites e.g. serine, glutamine and asparagine have been found to be dependent on the population density of the culture (Eagle and Piez, 1962; Eagle et al., 1966). This situation arises because in dilute cultures metabolites, synthesised by the cells, are lost into the medium and become limiting for growth. In this respect they resemble the "leaky" mutants of bacteria which exhibit changing nutritional requirements at different cell densities. Thus, in sparsely populated cultures, the cells often show increasing requirements for accessory growth factors.

Failure to realise this phenomenon resulted in initial difficulties in trying to establish colonies from the growth of single mammalian cells. L cells

were the first to be successfully cloned (Sanford, Earle and Likely, 1948). In this experiment individual cells were cultivated in capillary tubes embedded in plasma clots; thus nutrients leaking out of the cells were not diluted out and growth soon occurred although the cloning efficiencies obtained were low by modern standards.

Simpler methods of cloning are now commonly employed which have been adapted from the standard techniques of microbiology. These involve establishing the cells in suitable media stiffened with agar or methyl cellulose which allow "conditioning" of the media in the micro-environment of the cell (Puck, Marcus and Cieciura, 1956; Wallace and Hanks, 1958; Sanders and Burford, 1964; Macpherson and Montagnier, 1964; Stoker, O'Neill, Berryman and Waxman, 1968).

The use of "feeder layers" as a means of improving colony formation has been especially studied by Puck and his colleagues (Puck and Marcus, 1955; Puck et al., 1956). This technique involves subjecting plates of cells to X-irradiation and using these as a base for the proliferation of a few added viable cells. No requirement for "feeder layers" has been shown for permanent cell lines which will grow with high cloning efficiencies without previous conditioning of the media. This permits the isolation of specific mutants for genetic analysis, each one of which has arisen from a single cell.

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As with the amino acids, Eagle and his workers have demonstrated specific vitamin requirements for the continued growth of cells cultured in vitro (Eagle, 1955d; Eagle et al., 1960; Swim and Parker, 1958b). For the majority of cell strains 8 vitamins (pyridoxine, folic acid, choline, inositol, nicotinamide, riboflavin, thiamin and pantothenic acid) are needed for normal growth to proceed. Again exclusion of any vitamin ultimately leads to cell death although deficiencies may not become apparent for several days until cell reserves are depleted. No requirements for the fat-soluble vitamins i.e. vitamins A, D, E and K have ever been demonstrated in tissue culture populations. In cartilage organ cultures Fell, Dingle and Webb (1962) have shown a specific growth inhibition by vitamin A. This effect appears to be related to the release of proteolytic enzymes from the lysosomes of these cells.

A source of carbohydrate is also essential for the growth of all isolated cells and glucose, fructose and mannose can all be metabolised by chick heart cells or permanent cell strains (Chang and Geyer, 1957; Morgan and Morton, 1960; Eagle et al., 1958). Other hexose sugars appear to be relatively inert although maltose is able to promote growth, apparently because it is hydrolysed by specific maltases present in the serum or the

cells. Pentoses, apart from ribose, which can be utilised by a number of human cell strains (Eagle et al., 1958) are not effective in promoting growth.

While the roles of carbohydrates, vitamins and amino acids in the normal propagation of cultured cells have been clearly established, lipids do not appear essential for the growth of most cell lines. Triglycerides and phospholipids can, however, be extensively incorporated into cells in dividing cultures. Specific effects of lipids on the growth of one or two cell lines have been noted. Neuman and Tytell (1960c) observed that methyl oleate stimulated the growth of Walker 256 carcinoma in serum-free media while an apparent requirement for albumin during clonal growth in Chinese hamster cells can be eliminated by linoleic acid (Ham, 1963).

Although a number of isolated cell strains can now be cultivated in protein-free media, most cells also require the presence of added serum for normal growth in addition to the essential nutrients already discussed. The growth-stimulating properties of serum proteins have been the subject of considerable investigation but despite this, no single accepted role for proteins in cell nutrition has been elucidated. At the present time it is thought that serum fractions may serve as direct substrates, function in the attachment and spreading of cells,

act as carriers of essential nutrients or influence cell permeability. Recent evidence is reviewed by Harris (1964).

#### 1.4. Primary cultures and established cell strains

One of the principal aims of early investigators was the establishment of "pure" cell lines which could be propagated and studied as homogeneous cultures in the same manner as bacterial cells. The concept of homogeneous cell cultures was initially oversimplified before cloning procedures became available, although cells of a single histotype e.g. iris epithelium had been isolated.

With modern techniques of cytological analysis it has become apparent that most strains which multiply in vitro indefinitely are in fact modified or variant forms of the original strain. They are very often markedly aneuploid and exhibit other chromosomal abnormalities. In addition, they develop distinct morphological patterns and have, in the case of human diploid cells, for instance, been shown to exhibit differences in the patterns of RNA synthesis from the original primary cell line (Levine, Burleigh, Boone and Eagle, 1967). After approximately 20 generations in culture, a new rapidly labelled RNA species appears (14-20S) and coincidentally there is a marked decrease in the rate of synthesis of ribosomal RNA and rapidly labelled 35-45S RNA. RNA synthesis in early

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passage cells is also more readily inhibited by high population densities.

The early work of Carrel in which he maintained a line of chick heart fibroblasts by plasma culture for 34 years suggested that animal cells could proliferate for an indefinite period in vitro in an unmodified form. Carrel himself noted that these cells were morphologically similar from generation to generation. In view of the fact that early cell lines were not subjected to the rigorous techniques of cytological analysis available today, it becomes difficult to accept that the original cell line was maintained unaltered throughout this period. Very often there is a great simplification in morphology following outgrowth of primary explants (Kutsky and Harris, 1957) and the cells assume the morphology of 3 general categories, fibroblasts, epithelial cells or leucocytes.

Harris (1957) and Parker (1961) have further shown that monolayer cultures obtained from explants of chick tissue cannot be cultivated indefinitely in culture. Using chick skeletal muscle, Harris (1957) found that, after an initial period of vigorous growth lasting many generations, every culture entered a static phase where there was little or no growth; this was followed by gradual deterioration of the culture and eventual cell death.

The basis of these observations on the growth of primary cell populations is not well understood, although it is possible that death of a culture after a defined number of generations, characteristic of the individual cell line, may be related to the problems of senescence in vivo. Occasionally, however, during degeneration of the culture, modified or variant forms appear which become established as permanent cell lines.

#### 1.5. Specialised characteristics of cells in culture

The specialised characteristics of many animal cell types are often lost once the cells become adapted to cultivation in artificial media. For instance, Ebner, Hageman and Larson (1961) have shown the loss of 3 specialised functions of bovine mammary cells after growing in primary monolayer cultures. The ability of the cells to produce lactose was lost within 24 hours, while UDP glucose 4-epimerase (EC 5.1.3.2) activity disappeared 7-10 days after explantation.  $\beta$ -lactoglobulin synthesis declined slowly and reached a basal level after 2 weeks in culture. Lieberman and Ove (1958) examined enzyme patterns in rabbit kidney cortex cells in primary culture. Some, such as catalase ( $H_2O_2$ :  $H_2O_2$  oxidoreductase, EC 1.11.1.6) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1), decreased rapidly after isolation of the cells. The levels of acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2)

and  $\beta$ -glucuronidase ( $\beta$ -D-glucuronide glucuronohydrolase, EC 3.2.1.31), on the other hand, were unaltered while glucose-6-phosphate dehydrogenase activity (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49) actually increased.

Other regressive changes may occur in isolated systems involving loss of responsiveness of target cells to hormones or the loss of ability of cells to produce specific products e.g. pigments or hormones. Reusser, Smith and Smith (1962) were unable to detect somatotrophin synthesis in human anterior pituitary cells. Franks and Barton (1960) cultured mouse venal prostate as the intact organ. Marked morphological changes were observed in the outgrowing cell sheets which were unaffected by the addition of testosterone propionate to the medium. The same cells growing within the central mass, however, were markedly altered in appearance by addition of the hormone. Similarly Abbot and Holtzer (1966) found that chondrocytes from embryonic chick vertebrae, grown as monodisperse cells, rapidly ceased to make collagen or chondroitin sulphate and began to synthesise DNA and divide. On reaching a certain density the cells ceased to grow and resumed synthesis of collagen and chondroitin sulphate. The authors concluded that interaction between associated chondrocytes was important in maintaining the synthesis of the specific products

of these cells. Collagen synthesis in 3T6 mouse fibroblasts is also known to be greatly increased when the culture is at a high population density (Goldberg and Green, 1967). Thus at present it is not clear to what extent in vitro studies on many cell types can be related to their specialised in vivo metabolism; nor do we yet understand the importance of the environment for the accurate mediation of hormone action on its target cells.

In summary, it thus seems that cell cultures, grown as monolayers or in dissociated cell systems, often go through a process of dedifferentiation. By this, it is not implied that the cells return to their original embryonic state but only that certain regressive changes occur within the cells resulting in a loss of specific features of that cell type. It may be that the eventual loss of the control systems required for normal replication is one aspect of this process. In many cases, however, antigens, specific for the original organ, can be detected after many years in culture (Coombs, 1962; Stulberg, Simpson and Berman, 1961).

#### 1.6. Permanent cell lines

Cell lines which have been adapted to permanent passage in culture are now commonplace. The first of these was strain L, from mouse subcutaneous tissue (Earle, 1943) which after treatment with 20-methylcholanthrene acquired neoplastic properties

in vitro. Strain L was cloned by Sanford et al. (1948) and is now designated L 929 or NCTC 929 after the selected clone. HeLa cells are another familiar strain which originated from a cancer of the human cervix (Gey, Coffman and Kubicek, 1952). Like strain L, various sublimes have arisen from the recloning of cell populations in different laboratories. Not all cell lines, however, have arisen from cancerous tissue or acquired neoplastic properties in vitro, many strains having arisen directly from primary cell cultures with no evidence of neoplastic conversion.

In the United States an American Type Culture Cell Repository (12301 Parklawn Drive, Rockville Md.) has been established which supplies many cell lines of certified purity and free of pleuropneumonia-like organisms (PPLO). Full details on the history and general properties of each cell type are included. In Great Britain the British Tissue Culture Association publishes a list of all readily available cell strains and a list of members from whom they may be obtained.

#### 1.7. Contamination of cultures

Apart from the problems associated with our lack of knowledge on the nature of the changes occurring during establishment of a new cell line involving altered morphology, metabolism and chromosomal patterns, careful attention is needed on the presence of PPLO as contaminants in many cell lines.

These organisms live intracellularly without killing the host cells. As they often cause only minimal abnormalities in cell morphology or growth rate, their presence may go undetected for long periods of time. PPLO have been reported to cause altered morphology in established monolayer cultures (Pollock, Treadwell and Kenny, 1963) and can cause the breakdown of arginine in cell cultures (Schimke and Barile, 1963). In experiments involving incorporation of radioactive isotopes into growing cells, the results may be meaningless if PPLO are present.

Organisms of the PPLO group resemble very closely the L forms of certain bacteria and there is disagreement about the distinction between them. Penicillin is thought to induce the formation of L forms in some bacteria (Barile, Malizia and Riggs, 1962) and it is found that freshly isolated cells are rarely contaminated with PPLO as are cultures maintained with no antibiotics. In some cases, PPLO can be eliminated by heat treatment (Hayflick, 1960), or kanamycin (Kenny, Pollock and Syverton, 1960) but cultivation without antibiotics is preferable (Corriell, 1962). Techniques are now available for detecting PPLO in cell cultures (Rothblat and Morton, 1959; Barile, Malizia and Riggs, 1961; Barile and Schimke, 1963; Paul, 1960) and all cells supplied commercially are routinely tested before dispatch.

## 2. STUDIES ON ISOLATED CELL SYSTEMS

### 2.1. The cell cycle

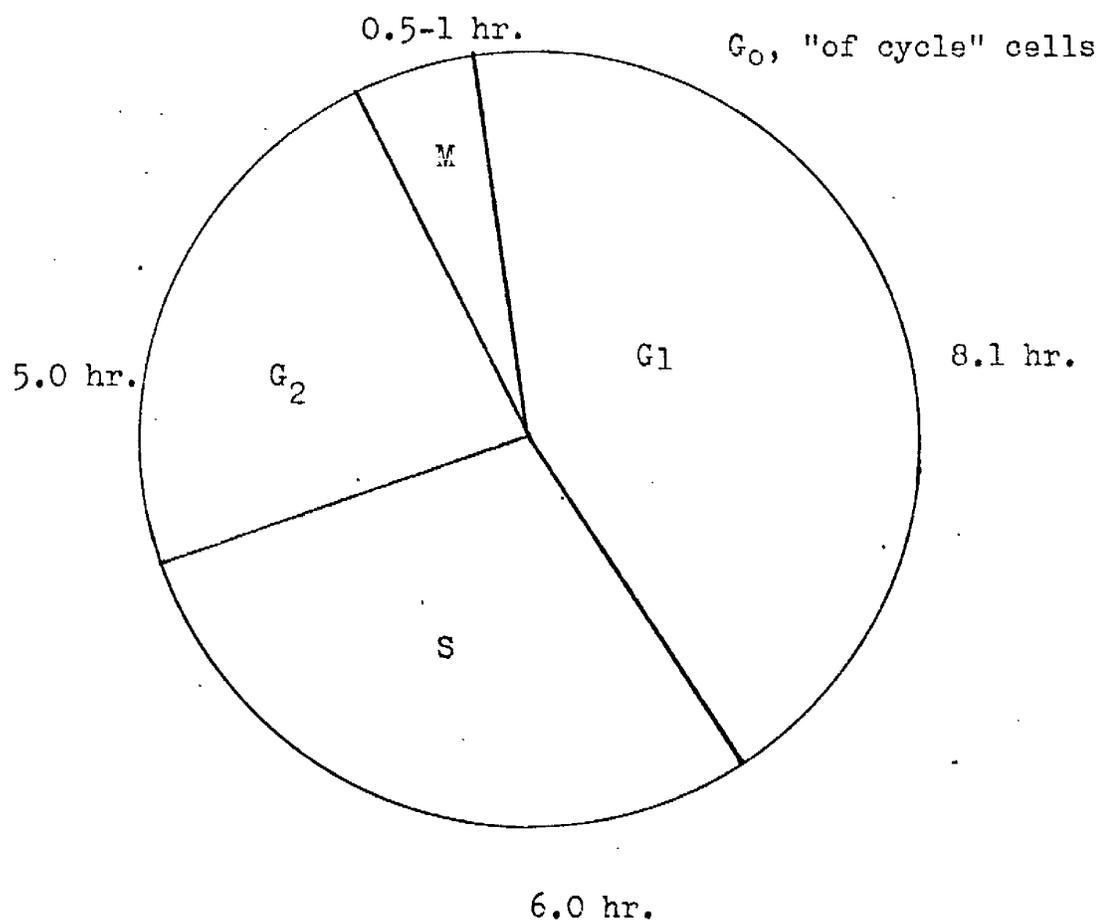
Under most conditions DNA synthesis in bacteria is almost continuous during interphase (Abbo and Pardee, 1960; Schaechter, Bentzon and Maaløe, 1959) and it has been shown in Escherichia coli that completion of a round of DNA replication is a sufficient condition for the initiation of cell division (Helmstetter and Pierucci, 1968). This situation does not hold for mammalian cells and it was initially shown by Howard and Pelc (1953), using radioactively-labelled DNA precursors, that DNA synthesis took place during a discrete period of the replication cycle. It is thus possible to divide the cycle arbitrarily into 4 phases:  $G_1$ , the period prior to DNA synthesis, S, the time of DNA replication and  $G_2$ , the period before the brief event of mitosis, M. Fig.1.1 illustrates this diagrammatically and shows the times of each period for L 929 cells.

The duration of the phases of the cell cycle has now been measured for many cell types (Cleaver, 1967) and certain general features have been noted.

(a) The length of the corresponding phases of the cell cycle vary with cell type. Moreover, no phase of the cycle represents a constant proportion of the total cycle duration.

Fig. 1.1.

Cell cycle for L 929 cells



(b) There is a greater range of variation for  $G_1$  compared to S and  $G_2$  phases. In tissue culture, for instance,  $G_1$  can last between 1.5 and 60 hr., S between 4.1 and 13.5 hr. and  $G_2$  from 1 to 7.5 hr. Corresponding in vivo studies, where the cells are not dividing at their maximal rate, have found that  $G_1$  may range from 1 hr. to several days in length, while S and  $G_2$  have similar lengths to their in vitro values.

(c) No clear and consistent difference has been shown between the phase durations in normal and malignant cells.

The relative constancy of S and  $G_2$  phases as compared to  $G_1$  applies to a large number of cell types. The DNA content of most somatic cells corresponds to that of  $G_1$  cells and the stimulus to growth results, first in the initiation of DNA synthesis, which eventually leads to mitosis and cell division. Thus the control of growth may operate through the control of initiation of DNA replication (Baserga, 1965).

## 2.2. Cell synchrony

Cell synchrony can be achieved by two basic procedures, induction or selection. Using the former method, the cells are induced to grow in a synchronous manner by the influence of some external agent e.g. inhibitors, temperature shocks or nutritional limitations while, with selection synchrony, cells at a specific stage in the cycle are separated from the rest of the culture.

Induction techniques are subject to the criticism that artificial methods using external agents may bring about distortions in the normal metabolic patterns of the cell. A limitation of selection synchrony is the relatively low yields of cells which are obtained. The introduction of automated procedures for the efficient separation of cells at a given stage is, however, serving to alleviate this problem.

The first cells to be deliberately synchronised in culture were *Chlorella* (Tamiya, Iwamura, Shibata, Hase and Nihei, 1953), *Amoeba* (James, 1954, 1959) and bacteria (Hotchkiss, 1954; Maaløe and Lark, 1954; Barner and Cohen, 1955). Original attempts at synchrony were aimed at gaining further insight into the events required for cell division. The advent of radioactive precursors of DNA, RNA and proteins, however, allowed the study of specific metabolic events at every stage of the cell cycle. For a review of present techniques in cell synchrony see Cameron and Padilla (1966).

One of the techniques commonly employed in tissue culture is the use of inhibitors of DNA synthesis. These include the folic acid analogues aminopterin and amethopterin, 5-fluorodeoxyuridine, excess thymidine and hydroxyurea.

Aminopterin has been utilised to synchronise HeLa cells at the beginning of S phase (Mueller, Kajiwara, Stubblefield and Rueckert, 1962; Stubblefield and Mueller, 1962; Mueller and Kajiwara, 1965). These investigators have extensively studied chromosome labelling during DNA replication as well as the requirements for RNA and protein synthesis during this time for the normal completion of S phase and eventual cell division. Kishimoto and Lieberman (1965), using aminopterin-synchronised L cells and rabbit kidney cortex cells cultured in vitro, have compared changes in the electrophoretic mobilities of the nuclear membranes during the cell cycle in these 2 systems. Aminopterin and 5-fluorodeoxyuridine have been employed in studies with L cells, examining the changes in the activity of DNA polymerase (deoxynucleoside triphosphate: DNA nucleotidyltransferase, EC 2.7.7.7) during S phase (Littlefield, McGovern and Margeson, 1963; Gold and Helleiner, 1964; Adams and Lindsay, 1969).

Bootsma, Budke and Vos (1964), investigating the potential of high levels of thymidine as a synchronising agent for a line of human kidney cells, found that 80-90% of the cells were in S at 3 hr. after the release of inhibition. A mitotic peak occurs at 8-10 hr. Moreover, microscopic observations and cloning studies indicated that no cytotoxic effects resulted from

this procedure. The inhibitory action of thymidine on DNA synthesis was previously attributed to a blockage in the synthesis of dCTP (Xeros, 1962). High levels of deoxyguanosine similarly prevent DNA replication (Kajiwara and Mueller, 1962) and addition of deoxyadenosine to the culture relieves the inhibition.

The temporal relationship of DNA and histone biosynthesis has been investigated in thymidine-synchronised HeLa cells (Spalding, Kajiwara and Mueller, 1966; Robbins and Borun, 1967) and both groups conclude that a large fraction of basic nuclear proteins is synthesised during S phase. Simultaneous synthesis of histone and DNA is also reported in Tetrahymena Pyriformis (Hardin, Einem and Lindsay, 1967) using cells synchronised by heat treatment (Scherbaum and Zeuthen, 1954).

Adams (1969a) has employed aminopterin and thymidine-synchronised L cells to examine the phosphorylation of exogenously supplied thymidine at different stages of the cell cycle. Evidence is presented to show that only cells in S and early G<sub>2</sub> phases carry out this process. It is believed that this effect is due, in part, to increased levels of thymidine kinase (ATP: thymidine 5'-phosphotransferase, EC 2.7.1.21) during S phase, but primarily to a feedback mechanism whereby the dTTP which accumulates in early G<sub>2</sub> inhibits this enzyme. Increased levels of thymidylate may also repress synthesis of thymidine kinase.

In similar studies Adams (1969) has examined the effect of endogenous pools of thymidylate on the apparent rate of DNA synthesis in L 929 cells. Cells synchronised with excess thymidine show a constant rate of DNA synthesis throughout S phase while aminopterin-synchronised cells have a slower initial rate of replication. A 2-3-fold increase in the rate of DNA synthesis after 2 hr. in S phase using aminopterin-synchronised HeLa cells has been previously reported (Mueller et al., 1962).

Hydroxyurea, an antineoplastic agent, inhibits DNA synthesis and cell division without affecting RNA or protein synthesis (Gale, 1964; Young and Hodas, 1964; Yarbro, Kennedy and Barnum, 1965; Rosenkranz and Levy, 1965). The drug inhibits the formation of deoxyribonucleoside diphosphates from the corresponding ribonucleoside diphosphates, a critical step in DNA synthesis (Turner, Abrams and Lieberman, 1966; Krakoff, Brown and Reichard, 1968; Elford, 1968). Its potential as a cell-synchronising agent has been investigated (Lindsay and Adams, 1967).

Terasima and Tolmach (1963a) have developed a purely selective procedure for obtaining metaphase cells and have shown that the lengths of the different phases of the cycle are not significantly altered compared to randomly-growing cultures. This

system has been employed to investigate changes in the sensitivity of HeLa and L cells to X-irradiation at various stages of the cycle (Terasima and Tolmach, 1963b, c; Djordjevic and Tolmach, 1967; Weiss and Tolmach, 1967). Mitotic cell populations are relatively sensitive but early in the  $G_1$  period they become more resistant to treatment. Progress through  $G_1$  phase is marked by an increasing sensitivity to X-irradiation which again decreases during the subsequent S(DNA-synthetic) phase. Thus the region of maximal interphase sensitivity has been identified at the  $G_1 \rightarrow S$  transition. These results are consistent with other reports for a number of cell lines, employing various techniques for obtaining synchronous populations (Sinclair and Morton, 1965; Erikson and Szybalski, 1963).

Colchicine and colcemid have been shown to be specific inhibitors of mitosis in mammalian cells (Puck and Steffen, 1963). The accumulation of metaphase cells in the presence of the drug has provided the basis for a technique designed to elucidate biochemical events occurring at specific stages in the replication cycle using randomly-growing cultures (Puck and Steffen, 1963; Puck, Sanders and Petersen, 1964). In addition, mitotic inhibitors are important where large amounts of chromosomes are required for in vitro studies (Maio and Schildkraut, 1967; Mendelsohn, Moore

and Salzman, 1968). Unfortunately, under most conditions the effects of the drug are irreversible and it is therefore not suitable as a synchronising agent. In one case, however, Stubblefield and Murphree (1967) have been able to follow the activity of thymidine kinase through an entire division cycle using colcemid-synchronised Chinese hamster cells.

### 2.3. Resting systems

#### 2.3.1. Regenerating liver

Cell strains in continuous culture are constantly moving round the replication cycle. Most tissues, on the other hand, such as liver or kidney are not actively dividing and consequently their mitotic index is extremely low. Furthermore, these tissues contain only low levels of the enzymes involved in DNA synthesis and are consequently considered not to be proceeding round the cycle but to be in a resting or  $G_0$  phase. After partial hepatectomy, in which the medium and left lateral lobes of the liver are removed, the remaining portion exhibits the phenomenon known as regeneration. Within 36 hr. of the operation a large increase in mitotic index is noted and the liver gradually increases in weight until at around 28 days it reaches its original size.

Investigators have closely studied the metabolic changes occurring immediately after operation with a view to establishing the sequence of events required for the onset of DNA synthesis and cell division. Earlier workers had noted that extracts from regenerating liver contained all the enzymes required for the conversion of ribonucleosides and deoxyribonucleosides into RNA and DNA respectively (Mantsavinos and Canellakis, 1959; Bollum and Potter, 1959; Weissman, Smellie and Paul, 1960).

Evans, Holbrook and Irvin (1962), studying the timing of histone biosynthesis in regenerating rat liver, showed that a peak of mitotic division occurred around 32 hr. after operation, while incorporation of labelled adenine into DNA was maximal at 26 hr. The initial event following partial hepatectomy appeared to be increased biosynthesis of RNA (Holbrook, Evans and Irvin, 1962). Fujioka, Koga and Lieberman (1963) found that the rate of incorporation of [ $^{14}\text{C}$ ] orotic acid into rat liver RNA begins to rise immediately, reaching a maximum at about twice the initial rate about 5 hr. post-operatively. Enhancement of RNA synthesis is related to the amount of liver removed as 10% removal causes little or no alteration in RNA metabolism. Levels of p-fluorophenylalanine (FPA) and actinomycin D, which do not affect normal RNA turnover, prevent the rise in the rate of RNA

synthesis although their effects are readily reversible. DNA synthesis is correspondingly delayed. No increase in the rate of DNA synthesis was detected by this group (Fujioka et al., 1963) until 16 hr. after operation. Increases (3-10 fold) in the levels of thymidine kinase and DNA polymerase also occurred about the time of DNA synthesis (Bollum and Potter, 1959).

The RNA polymerase (nucleosidetriphosphate: RNA nucleotidyltransferase, EC 2.7.7.6) activity and the RNA content of isolated nuclei and nucleoli are also elevated during regeneration (Tsukada and Lieberman, 1965). A 2-fold increase in RNA polymerase activity occurs over the first 12 hr. No significant changes in the levels of other enzymes during this early phase have been described.

### 2.3.2. Rabbit kidney cortex cells cultured in vitro

Similar studies have been carried out on rabbit kidney cortex cells cultured in vitro (Lieberman and Ove, 1962; Lieberman, Abrams and Ove, 1963; Lieberman, Abrams Hunt and Ove, 1963; Adams, Abrams and Lieberman, 1965). These cells are not rapidly dividing in the rabbit but after removal and dispersal in culture, there is rapid growth after an initial lag period. DNA synthesis is not initiated until about 32 hr.

after removal but profound changes in RNA metabolism have been observed before this time.

A doubling in the rate of RNA synthesis occurs between 12 and 22 hr. after which RNA synthesis continues at its new high level. Addition of low levels of actinomycin D or FPA before 12 hr. completely abolish this rise and also prevent subsequent initiation of DNA synthesis. Normal RNA turnover continues at its initial rate in the presence of these inhibitors (Lieberman et al., 1963). After 22 hr. the cells became increasingly resistant to the effects of FPA and actinomycin D. Zinc ions are also required during the period 12-22 hr. as these overcome inhibition caused by EDTA during this time. X-irradiation of the cells, however, prevents DNA synthesis if the cells are treated at any time up to the beginning of S phase. Thereafter they become relatively insensitive to its effects (Lieberman et al., 1963).

The activities of thymidine kinase and DNA polymerase again do not rise in this system until about the time of DNA synthesis. Other enzymes e.g. lactate dehydrogenase (L-lactate: NAD oxidoreductase. EC 1.1.1.27) and hexokinase (ATP: D-hexose 6-phospho-transferase. EC 2.7.1.1) begin to increase in specific activity immediately when the cells are cultured in vitro (Lieberman et al., 1963).

### 2.3.3. The lymphocyte-phytohaemagglutinin system

A comparable system to come under intensive study in recent years is the lymphocyte-phytohaemagglutinin system. Although lymphocytes from a wide variety of animal species such as human, monkey, rabbit and horse will not normally proliferate in vitro, addition of phytohaemagglutinin (PHA), an extract of the red kidney bean Phaseolus vulgaris brings about a striking transformation; about 90% of the cells in culture enlarge, eventually synthesise DNA and divide (Nowell, 1960). Early changes in the patterns of RNA and protein metabolism as well as in histone acetylation have been documented (Mueller and Mahieu, 1966; Cooper and Rubin, 1965a, b, 1966; Cooper, 1968; Pogo, Allfrey and Mirsky, 1966). Recently Loeb, Agarwal and Woodside (1968) have shown that the DNA polymerase activity of human lymphocytes increases 30-100-fold in the presence of PHA. Maximal activity is detected on the third day after stimulation when DNA synthesis is also at a peak and there is a close correlation between the DNA polymerase activity of disrupted cell preparations and the ability of the cells to incorporate thymidine into DNA.

Lymphocytes in culture can respond to a variety of mitogenic agents although to varying extents. These include specific antigens (Dutton and Eady, 1964; Dutton and Bulman,

1964), streptolysins O and S. (Hirschhorn, Schreibman, Verbo and Gruskin, 1964) and various products of micro-organisms e.g. tetanus toxoid and polio virus (Elves, Roath and Israels, 1963), yeast extract (Gandini and Gartler, 1964) and endotoxins of Gram negative organisms (Oppenheim and Perry, 1965). The mechanism of action of these agents and the nature and function of receptor sites on the lymphocytes are under active investigation but the picture to date is incomplete. Several reviews on lymphocyte stimulation have appeared in recent years (Robbins, 1964; Mellman, 1965; Cooper and Arniel, 1965; Gowans, 1966). A comprehensive survey on the whole field of lymphocyte metabolism is made by Ling (1966).

#### 2.3.4. Importance of resting systems

Resting systems ( $G_0$ ) which can be stimulated to growth and cell division are important in allowing a close study of the sequence of events required for renewed proliferation. There is the added advantage that these systems exhibit a degree of natural synchrony, free from abnormalities of metabolism possibly arising through the use of artificial methods of synchronisation with established cell lines. Permanent strains also differ from resting systems in that the cells are continually progressing round the cycle and consequently never enter  $G_0$  phase. However,

the high degrees of synchrony obtainable by artificial agents and the ease of manipulation of cell lines in culture makes them useful in defining the series of metabolic events required for DNA replication and mitosis.

#### 2.3.5. Differences between $G_0$ and $G_1$ phase

The differences between  $G_0$  and  $G_1$  are not well defined. Regenerating rat liver and primary rabbit kidney cortex cells are sensitive to X-irradiation in that treatment of these cells at any time before they enter S phase, results in marked inhibition of DNA synthesis (Kelly, 1957; Holmes and Mee, 1956; Lieberman et al., 1963). It is of interest that hydroxyurea-synchronised rabbit kidney cortex cells are no longer as sensitive to X-irradiation and consequently must have passed the radiation-sensitive event which occurs at the onset of S phase (Adams et al., 1966). In contrast the  $G_1$  to S transition by cells in continuous proliferation is more resistant to X-ray treatment (Adams, personal communication).

Analysis of cell proliferation after partial hepatectomy has led to the idea that the majority of cells are in a state of "no cell cycle" i.e.  $G_0$  until stimulated to divide (Lajtha, Oliver and Gurney, 1962). Post and Hoffman (1965) have confirmed this model in rat liver, showing that there is a massive increase in

the proportion of cells in DNA synthesis (the growth fraction) following partial hepatectomy.

Recently Brown (1968), however, using standard autoradiographic procedures, concluded that hamster cheek pouch cells in vivo have a mean cell cycle time of 140 hr. with approximately 90% of this time occupied by  $G_1$  phase. The growth fraction has also been shown to be equal to unity for the epithelial cells of rat oesophagus and mouse tongue (Leblond, Greulich and Pereira, 1964) and hamster cheek pouch (Brown and Oliver, 1968).

Thus, the model of a variable growth fraction to account for the entrance of  $G_0$  cells into the cycle after stimulation, does not appear to be applicable to all tissues. It still remains to be proved whether there is any absolute difference between  $G_0$  and  $G_1$  cells in terms of their responses to external stimuli.

### 3. DNA REPLICATION

#### 3.1. DNA synthesis in bacterial systems

The twin-stranded helical structure for DNA proposed by Watson and Crick (1953) has revolutionised present-day research

in molecular biology and provided the basis for many of the spectacular advances of the past 15 years. Indirectly, for instance, it has led to the development of cell-free protein synthesising systems (see Davidson, 1969), elucidation of the genetic code (see Crick, 1963; Woese, 1967) and the in vitro synthesis of biologically active DNA (Goulian, Kornberg and Sinsheimer, 1967).

One of the most exciting aspects of the proposed DNA structure was that it immediately suggested a method for its own replication. Impressive support for this scheme was forthcoming with the classical experiments of Meselson and Stahl (1958) who were able to show conclusively that replication of the Esch. coli chromosome occurred in a semi-conservative manner, implying that strand separation took place during replication with subsequent distribution of one parent and one newly-synthesised strand to each daughter cell.

Autoradiographic studies of the replicating Esch. coli chromosome (Cairns, 1963a, b) showed the presence of Y-shaped replicating forks and confirmed that the process took place semi-conservatively and unidirectionally. The average rate of replication of each strand can be expressed as 12.5 nucleotide units per sec. per strand, assuming the average length of two nucleotide

units to be  $34 \text{ \AA}$  (Watson and Crick, 1953); as the 2 parent strands remain attached to the starting point until replication is complete Cairns also postulated the presence of a swivel mechanism at this region to allow unwinding of the double-helical, circular DNA during DNA synthesis (Cairns, 1963b).

Several factors appear to be involved in the initiation of replication. Models of chromosome replication have postulated that separate processes are required in the attachment of the chromosome to the cell surface and in the initiation of replication (Jacob, Brenner and Cuzin, 1963; Lark, 1966). Use of inhibitors such as chloramphenicol and phenethyl alcohol combined with amino acid starvation supports the view that more than one process is required for initiation. Recently Lark and Renger (1969) have distinguished 3 physiological processes required for initiating DNA synthesis in Esch. coli. Rapidly-growing bacteria are known to initiate new rounds of replication before the completion of previous rounds (Yoshikawa, O'Sullivan and Sueoka, 1964; Maaløe and Kjeldgaard, 1966; Helmstetter and Cooper, 1968; Bird and Lark, 1968).

### 3.2. In vivo studies on DNA replication in higher organisms

In higher organisms the problem is complicated by our lack of knowledge on the precise details of chromosome structure and

the manner in which DNA is located within this unit. Although much is known about the chemical components of the chromosome only scant evidence is available on its structural organisation. Several models of chromosomal structure have been presented (Freese, 1958; Taylor, 1963; Du Praw, 1965; Pelling, 1966) and the presence of a repeating structural unit has been suggested by Davies and Small (1968).

Chromosome replication in plants and animals has also been shown to take place via a semi-conservative mechanism (Forro and Wertheimer, 1960; Prescott and Bender, 1963; Simon, 1961; Taylor, Woods and Hughes, 1957). In addition, pulse-labelling techniques on cultures from human peripheral blood have shown that DNA synthesis is asynchronous in individual chromosomes and that sex chromosomes are unusual in terminating replication later than the others (Mukherjee and Sinha, 1965; Bianchi and Bianchi, 1965; Lima-de-Faria, 1964). Painter (1961) has shown that while replication begins almost simultaneously in HeLa  $S_3$  cells, synthesis was terminated at times varying up to 2 hr. amongst different chromosomes.

Using synchronised cultures of HeLa cells Stubblefield and Mueller (1962) have obtained evidence for the non-random and focalised nature of DNA replication in various chromosomes

and shown that reproducible patterns of chromosome labelling occurred on segments of a particular chromosome. Pulse-treatment of these cells during various periods of S phase with 5-bromodeoxyuridine showed that cells treated during the first 2 hr. were especially sensitive and rapidly became non-viable, suggesting that early-replicating DNA is that which is actively transcribed in the cell. There is now considerable evidence to suggest that late-labelled chromosomes contain the heterochromatin material of interphase nuclei (Lima-de-Faria, 1959; Evans, 1964).

#### 4. IN VITRO STUDIES ON DNA REPLICATION

##### 4.1. Introduction

Kornberg, Lehman and Simms (1956) were the first to isolate an enzyme from Esch. coli capable of catalysing incorporation of the 4 deoxyribonucleoside triphosphates into DNA in the presence of  $Mg^{2+}$  and a DNA primer. Since then similar enzymes have been reported in a large number of bacterial, viral (Aposhian and Kornberg, 1962) and mammalian sources (Bollum and Potter, 1957).

##### 4.2. DNA polymerase activity in vitro

###### 4.2.1. Bacterial DNA polymerases

The most intensively studied DNA polymerase is the Esch. coli enzyme which has been investigated since 1956 mainly by

Kornberg and his co-workers. Their investigations culminated recently with the employment of this enzyme to synthesise biologically active DNA of the virus  $\phi$ X 174 (Goulian et al., 1967).

The enzyme can catalyse the polymerisation of deoxyribonucleoside triphosphates, in the presence of  $Mg^{2+}$  and DNA primer, into acid-insoluble material with the stoichiometric release of PPI (Bessman, Lehman, Simms and Kornberg, 1958b). Either native or heat-denatured DNA will serve as primer. (Richardson, Schildkraut, Aposhian and Kornberg, 1964). Nearest neighbour frequency analysis of the product suggests that the enzyme only incorporates by specific base pairing in a manner determined by the DNA template (Josse, Kaiser and Kornberg, 1961). Esch. coli DNA polymerase is also active to some extent in the presence of  $Mn^{2+}$  ions but under these conditions will incorporate ribonucleoside triphosphates into DNA (Berg, Fancker and Chamberlain, 1963). This property of the enzyme has been utilised in determining the structure of the DNA product (Richardson, Inman and Kornberg, 1964).

The enzyme has now been purified to homogeneity and has been shown to consist of a single polypeptide chain of mol. wt. 109,000, having an N terminal methionine residue and containing one SH group and one S.S. bond (Englund et al., 1968). Physical

and chemical studies on the homogeneous DNA polymerase have been recently carried out (Jovin, Englund and Bertsch, 1969; Jovin, Englund and Kornberg, 1969).

Binding studies using the active  $^{203}\text{Hg}$  derivative of the purified enzyme have shown that there is only one DNA binding site per molecule as well as a single site for the deoxyribonucleoside triphosphates (Englund *et al.*, 1968; Englund, Huberman, Jovin and Kornberg, 1969). The influence of DNA structure on the binding of the enzyme has also been investigated (Englund, Kelly and Kornberg, 1969). With single-stranded  $\phi\text{X 174}$  DNA ( $1.7 \times 10^6$  daltons) approximately 20 molecules of enzyme are bound per molecule of DNA under conditions promoting maximum binding. No attachment of DNA polymerase to the circular duplex of plasmid DNA could be observed, however, unless the template was nicked either with DNase I (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5) introducing 3'-hydroxyl groups or micrococcal nuclease (EC 3.1.4.7) producing 3'-phosphate groups. In each case the number of enzyme molecules bound was very close to the number of nicks produced although only DNase I treatment converted plasmid DNA into an active template for the enzyme. Linear double-stranded DNA from T7 phage provided 2 enzyme binding

sites per molecule indicating the Esch. coli DNA polymerase can bind at the ends of DNA duplexes.

Partial degradation of the double-stranded primer by an endonuclease which produces 3'-hydroxyl ends converts the DNA into an efficient template for the enzyme (Kornberg, 1957). Poly d(AT) is also a most effective primer and, in the absence of added DNA, the enzyme will initiate de novo synthesis of poly d(AT) after a lag period of 2 - 5 hr. in the presence of the appropriate deoxyribonucleoside triphosphates (Schachman, Adler, Radding, Lehman and Kornberg, 1960). In this respect it is interesting that Lezius, Hennig, Menzel and Metz (1967) have separated 2 DNA polymerases from Esch. coli differing only in their ability to initiate de novo synthesis of poly d(AT).

When the product of the Esch. coli DNA polymerase with native DNA as primer is examined by electron microscopy it is found to have a highly branched structure (Schildkraut, Richardson and Kornberg, 1964). In addition an abnormal degree of renaturation occurs after treatments designed to bring about complete strand separation. It appears that this unusual "pleated" or hairpin structure of the DNA product can be explained by the failure of the enzyme in vitro to catalyze simultaneous replication of both strands.

By contrast, if double-stranded DNA is treated with exo-

nuclease III to produce a partially single-stranded molecule by the release of mononucleotides from the 3'-hydroxyl ends of each chain, the Esch. coli enzyme can accurately repair the degraded DNA to form a product which is identical to the original DNA in denaturability, in appearance by electron microscopy and in biological activity (Richardson et al., 1964).

The most critical test of fidelity of replication is to determine whether the enzyme can catalyse the synthesis of biologically active DNA. This experiment has recently been performed using the infectious, circular, single-stranded DNA (+ strand) of bacteriophage  $\phi$ X 174 as template (Goulian et al., 1967). In this manner they were able to synthesise the complementary (-) strand. Bromodeoxyuridine triphosphate was substituted for d-TTP in the assay, so that the newly-synthesised strand could be separated by CsCl centrifugation. This (-) strand was fully infectious and could be utilised as template to form (+) strands with the same specific activity as the original  $\phi$ X 174 DNA. In both cases the enzyme polynucleotide ligase was required to link the ends of the DNA chain to form closed, infectious circles. Goulian (1968) has shown that the priming ability of circular  $\phi$ X 174 DNA is slow and uncertain unless a boiled extract from Esch. coli, which can be shown to contain

oligonucleotides, is added. Thus there is still considerable doubt as to the ability of the Esch. coli polymerase to initiate de novo synthesis of DNA strands (Englund et al., 1968).

The purified Esch. coli DNA polymerase has a number of associated activities which remain in constant ratio to the polymerising abilities of the enzyme throughout the purification (Englund et al., 1968). Thus the enzyme supports exchange of the  $\beta$ ,  $\gamma$  groups of deoxyribonucleoside triphosphates with PPI and can also catalyse degradation of DNA by hydrolysis or pyrophosphorolysis.

Pyrophosphate exchange is identical with the polymerisation reaction in its requirements for a template strand, strict specificity in base pairing and a 3'-hydroxyl-terminated primer. In contrast to polymerisation, however, appreciable exchange is detected in the absence of a full complement of triphosphates (Deutscher and Kornberg, 1969a). During pyrophosphorolysis the enzyme catalyses an attack by PPI on the 3'-terminal nucleotide and progressively removes mononucleotides from the end of the DNA chain. Since the enzyme can bring about both synthesis and degradation of DNA from the 3'-hydroxyl end, it is capable of moving along the DNA in both directions.

The presence of exonuclease II activity in purified polymerase preparations was shown by Lehman and Richardson (1964). This activity hydrolyses polydeoxyribonucleotides from the 5'

as well as the 3' -terminus (Klett, Cerami and Reich, 1968; Deutscher and Kornberg, 1969b). The rates of hydrolysis from either end are similar using native DNA as substrate. Beyersmann and Schramm (1968), from kinetic data, have provided evidence for a common site for hydrolysis and pyrophosphorysis.

Association of exonuclease activity with purified T4 phage DNA polymerase (Goulian, Lucas and Kornberg, 1968) and the DNA polymerases of herpes simplex virus (Paton and Morrison, 1969) and Ehrlich ascites tumour cells (Roychoudhury and Bloch, 1969) has also been noted. The relationship of the various activities of isolated DNA polymerases is clearly important to a proper understanding of the in vivo role of the enzymes. It is of interest, however, that the purified Bacillus subtilis DNA polymerase, which exhibits similar properties to the Esch. coli enzyme has no detectable associated nuclease activity (Okazaki and Kornberg, 1964).

While the Esch. coli DNA polymerase has been successfully employed in the production of infectious viral DNA, its in vitro characteristics do not serve to clarify its role in the semi-conservative replication of the bacterial chromosome. Cairns (1963a) has shown that replication of the bacterial chromosome occurs by

unidirectional, simultaneous synthesis of both DNA strands. The in vitro Esch. coli enzyme is only capable of synthesising a single DNA chain in the 5' - 3' direction. Failure of the enzyme to replicate double-stranded DNA in a semi-conservative manner led to speculation that the enzymic activity concerned in in vivo DNA synthesis remained undetected in cell extracts. Several lines of evidence, however, now argue against this point of view:

(a) The mutagenic action of  $Mn^{2+}$  and the altered specificity of the Esch. coli DNA polymerase in the presence of  $Mn^{2+}$  argues for the participation of the enzyme in the replication process (Berg et al., 1963).

(b) De Waard, Paul and Lehman (1965) have shown that certain mutants of T4 and T5 phages which are defective in DNA synthesis also fail to induce normal DNA polymerases.

(c) Subsequent work on temperature-sensitive T4 mutants has shown that the fidelity of replication is decreased in phages inducing DNA polymerase with altered characteristics (Speyer, 1965).

Evidence for participation by the enzyme in selecting the correct incoming deoxyribonucleoside triphosphate has also been obtained (Speyer, Karam and Lenny, 1966; Freese and Freese, 1967). A report that Cairns (unpublished results) has recently isolated a mutant of Esch. coli which exhibits no DNA polymerase activity

in cell extracts awaits further clarification.

A more likely possibility is that the enzyme or some part of the replication machinery is damaged during isolation so that the enzyme is unable to fulfil its proper catalytic function in vitro. In this connection, the question of a multi-unit structure for the enzyme has been raised, although the results of Englund et al. (1968), showing the Esch. coli DNA polymerase to consist of a single polypeptide chain, would appear to preclude this idea. Cavalieri and Carroll (1968), however, have evidence that this enzyme exists as a multiple molecular species with mol. wts. of 120,000-140,000, 60,000-78,000 and 24,000-30,000 implying a tetramer-dimer-monomer relationship. Since the Kornberg enzyme has a mol. wt. of 109,000 a satisfactory explanation for these results has still to be obtained. Hori, Fujiki and Takagi (1966) have separated 2 DNA polymerases from Alcaligenes faecalis which can be distinguished by their differing preferences for native or heat-denatured DNA primer. The relationship of these activities to DNA replication is as yet unknown.

Further evidence comes from the Kornberg group (Jovin et al., 1969) who have shown that, under certain conditions, active

dimers of purified DNA polymerase can be formed containing one Hg atom; thus it may be that during purification there is breakdown of a more complex replication unit which has the capacity to synthesise DNA in a semi-conservative fashion on a native template.

Isolation of the enzyme polynucleotide ligase from Esch. coli (Gellert, 1967; Olivera and Lehman, 1967a; Zimmerman, Little, Oshinsky and Gellert, 1967; Gefter, Becker and Hurwitz, 1967), phage-infected cells (Weiss and Richardson, 1967a; Cozzarelli, Melechen, Jovin and Kornberg, 1967; Becker, Lyn, Gefter and Hurwitz, 1967) and mammalian sources (Lindahl and Edelman, 1968) has led to the extension of our ideas on possible mechanisms of DNA replication and these are discussed in section 5.1.

#### 4.2.2 Mammalian DNA polymerases

Research into DNA polymerases of higher organisms has lagged behind studies in bacterial systems largely owing to difficulties in obtaining highly purified preparations. Englund et al. (1968) have now been able to obtain 600 mg. of Esch. coli enzyme, purified to homogeneity, starting from 90 Kg. of cell paste.

The most extensively studied mammalian DNA polymerase to

date is the calf thymus enzyme (Bollum, 1960). It has been purified about 250-fold from crude extracts and exhibits the same general properties as the bacterial enzymes. Magnesium ions are essential for activity as are the 4 deoxyribonucleoside triphosphates and a DNA primer. From gel filtration studies the enzyme has a mol. wt. of 110,000 and is free of endonuclease activity as judged by prolonged incubation with biologically active DNA. Ultracentrifugation of the purified enzyme shows that the enzyme still only represents a small proportion of the total protein (Yoneda and Bollum, 1965).

Heat-denatured DNA is a much better primer for the enzyme than double-stranded DNA and in nuclease-free fractions there is little or no activity with native primer. The product after 100% replication of added single-stranded DNA has been shown to be native DNA by MAK column chromatography and counter-current distribution in aqueous polymer systems (Bollum, 1963). With poly dA as primer, however, while the final product is found to be poly dA:dT, at intermediate stages in the replication process, material is formed which has a higher buoyant density than either poly dA or poly dA:dT in CsCl gradients. Bollum (1966) attributes this to the formation of a 3-stranded complex, poly dA<sub>2</sub>:dT.

In recent years Bollum has been especially concerned in defining the template requirements of the calf thymus DNA polymerase. (Bollum, 1966). Using synthetic homopolymers as templates for the enzyme, it was found that these were essentially inactive unless a small amount of complementary oligodeoxyribonucleotide was added. Synthesis on poly dA, for instance, can be initiated by addition of hexathymidylate,  $d(pT)^6$ , but not by  $d(pA)^6$ . Self-initiation can take place after a lag period if the deoxyribonucleoside triphosphate complementary to the primer is present in the reaction mixture. In the presence of an initiating oligonucleotide the newly-synthesised DNA plus initiator can be separated from the primer while in the absence of initiator the reaction is slower and the product is a "hairpin-like" structure with the new strand covalently bound to the primer DNA.

#### 4.2.3. Other mammalian polymerases

Many DNA polymerases have been detected in extracts of mammalian tissues and as might be expected their activities are often high in rapidly proliferating tissues. These enzymes, in general, exhibit similar properties to the calf thymus enzyme. Amongst those sources investigated are ascites tumour cells (see Keir, 1965; Shepherd and Keir, 1966), rat

thymus (Walwick and Main, 1962) and a number of other mammalian tissues including, mouse spleen, Walker 256 carcinoma, rat spleen, kidney, brain, lung, testes, skeletal muscle and pancreas, rabbit liver, bone marrow, thymus and spleen (Keir, 1965). Activity has also been reported in various tissue culture lines, HeLa cells (Harford and Kornberg, 1958; Bach, 1962; Magee, 1962), BHK 21 (C13) cells (Keir and Gold, 1963), HEp2 cells (Keir, Hay and Subak-Sharpe, 1964), RK cells (Nohara and Kaplan, 1963) and L cells (Littlefield et al., 1963; Gold and Helleiner, 1964).

All these DNA polymerases resemble the calf thymus enzyme in being more active with heat-denatured DNA as primer. In view of the double-stranded helical structure of DNA in vivo, the relationship of these activities to in vivo replication is not understood at present.

#### 4.2.4. Mammalian DNA polymerases preferring native DNA primer

In recent years an increasing number of DNA polymerases have been reported in higher organisms which exhibit a marked preference for native DNA templates. Rat liver has received much of the attention in this respect. Mantsavinos and Munson (1966) partially purified an enzyme from soluble ex-

tracts of regenerating rat liver which was more active with native DNA and resembled the Esch. coli enzyme in its capacity to utilise poly d(AT) as primer. Previously Mukundan, Devi and Sarkar (1963) had measured the activity of rat liver DNA polymerase in crude extracts as a function of the age of the rat. The ratio of activity on native to denatured DNA fell markedly with increasing age while after partial hepatectomy there was a marked stimulation in activity primed by native DNA. De Recondo (1967) has observed a factor in supernatant extracts of rat liver which, when pre-incubated with native DNA, caused an 8-fold increase in the priming ability of the DNA with rat liver polymerase. On the basis of comparative experiments in which double-stranded DNA was treated with DNase I or DNase II (De Recondo, 1966), she suggests that the stimulating factor is not either of these endonucleases but may be similar to exonuclease III (Richardson, Schildkraut and Kornberg, 1964). DNA polymerase has also been purified 200-fold from the non-histone component of rat liver nuclei (Patel, Howk and Wang, 1967) and has been shown to prefer native DNA. The properties of this enzyme appear similar to those of the enzyme isolated by Mantsavinos and Munson (1966).

A very active DNA polymerase has been isolated from the nuclei of developing sea urchin embryos (Loeb, Mazia and Ruby,

1967). The enzyme markedly prefers native DNA and exhibits a  $Mg^{2+}$  optimum of 16 mM compared to the values of 4 - 8 mM reported for most of the DNA polymerases in higher organisms. Isolated nuclei are active without exogenous primer but are stimulated 10-fold on the addition of native DNA. During early cleavage divisions the DNA per nucleus ( $1.8 \times 10^{-12}$  g..) can double every 12 - 15 min. at  $15^{\circ}C$  in this system. At this stage all the DNA polymerase activity is located in the nucleus. Loeb (1969) has purified this activity 300-fold from isolated nuclei and shown the enzyme to have a mol. wt. of approximately 150,000. Birnie and Fox (1966) have compared nuclear and cytoplasmic extracts of mouse embryo cells and found differences in the DNA polymerase activity of the 2 fractions with respect to  $Mg^{2+}$ ,  $Mn^{2+}$  and pH optima and activity with double- and single-stranded primers. Whereas the cytoplasmic extract is stimulated both by native and denatured DNA, with  $Mg^{2+}$  the nuclear extract is inhibited slightly by denatured DNA.

Recently partial purifications of DNA polymerase from rat liver mitochondria have been reported by 2 groups (Meyer and Simpson, 1968; Kalf and Ch'ih, 1968). The former group find that the crude enzyme has a 5-fold preference for denatured DNA but, after  $(NH_4)_2SO_4$  precipitation and DEAE-cellulose

chromatography, it is equally active on double- or single-stranded DNA. Separation from the nuclear enzyme is also effected at this stage. The mitochondrial polymerase is markedly stimulated (8-fold) by 60 mM  $K^+$ . Kalf and Ch'ih (1968) have purified the enzyme 22-fold from crude extracts and find that it is most active with native DNA. The remarkable feature of their data is that mitochondrial DNA polymerase is markedly more active with native, circular rat liver mitochondrial DNA than with double-stranded DNA from other sources. In this manner they have been able to achieve a 3.5-fold net synthesis of product only previously carried out using bacterial enzymes.

#### 4.3. Localisation of mammalian DNA polymerases in the cell

Early successes in the detection of DNA polymerase in eucaryotic cells overcame initial doubts concerning the presence of the enzyme in supernatant extracts of these cells. Some light was thrown on the problem by Canellakis and his colleagues (Krakow, Coutsogeorgopoulos and Canellakis, 1962; Krakow, Kammen and Canellakis, 1961) who found that calf thymus nuclei retained a much higher degree of activity if 3 mM- $CaCl_2$  was present in the homogenising medium. Calcium ions are known to be required for isolation of nuclei in a morphologically undamaged state (Hogeboom, Schneider and Striebich, 1952). This

initial observation was extended by Main and Cole (1964) by measuring DNA polymerase activity after repeated extractions of rabbit thymus nuclei in the presence and absence of  $\text{Ca}^{2+}$ . These ions were found to greatly reduce the rate at which enzymic activity was lost from the nuclei.

As the problem appeared to be the leaching of enzyme from the nucleus during isolation, extraction techniques were developed using non-aqueous solvents. Keir, Smellie and Siebert (1962) prepared nuclear and cytoplasmic powders from regenerating rat liver and showed that nuclear extracts contained substantial activity while cytoplasmic fractions had lower activity. Similar results were obtained by Behki and Schneider (1963) using non-aqueous preparations from regenerating rat liver and Novikoff hepatoma.

It now seems probable that the enzyme may exist as a cytoplasmic or nuclear component depending on the mitotic index and rate of proliferation of the tissue concerned. Mazia and Hinegardner (1963) and Mazia (1963), for instance, have shown that DNA polymerase activity is high in sea urchin embryo nuclei during the early stages of cleavage division when cell multiplication is rapid but that this activity decreases markedly by the time of gastrulation when the rate of new cell production is low.

Two reports have appeared attempting to correlate DNA polymerase levels in cytoplasmic and nuclear extracts of L cells during the DNA-synthetic (S) period (Gold and Helleiner, 1964; Littlefield et al., 1963). Both groups observe a slight drop in the activity of the supernatant enzyme during S which subsequently rises again at the cells finish replication and enter mitosis. A small increase in the activity of the "particulate" fraction is also detected during the S period. While these observations appear contradictory to the findings from regenerating liver (Bollum and Potter, 1959), rabbit kidney cortex cells (Adams et al., 1965) and human lymphocytes (Loeb et al., 1968) where a generalised elevation in the levels of DNA polymerase occurs during S, it seems likely that the high degree of synchrony obtained with established cell lines allows observations on transient changes in enzymic activity not detectable in more asynchronous systems.

Recently Friedman and Mueller (1968) have described a nuclear preparation from HeLa cells which can incorporate deoxyribonucleoside triphosphates into DNA. The general properties of these nuclei are:

- (a) Synthesis occurs without addition of exogenous template and no increase in activity is observed on addition of native DNA.

(b) Their activity closely follows the DNA-synthetic capacity of intact cells in synchronised cultures.

(c) The product is DNase I sensitive and characteristic of replicating DNA as most of the product is found at the interphase during phenol extraction (Friedman and Mueller, 1969).

It also behaves as HeLa cell DNA on sucrose or CsCl gradients.

(d) For maximal activity these nuclei require ATP, Na<sup>+</sup> ions and a heat-labile factor from the cytoplasm which has different properties to the DNA polymerase activity found in this fraction. Moreover, cytoplasmic extracts from S phase cells do not stimulate DNA synthesis in nuclei from randomly-growing cultures to any great extent, suggesting the presence of a controlling factor in the nucleus.

## 5. THEORIES ON THE MECHANISM OF DNA REPLICATION

### 5.1. Bacterial systems

A major discrepancy between in vivo and in vitro studies on DNA replication is that the Esch. coli enzyme is only capable of initiating synthesis in the 5<sup>1</sup> - 3<sup>1</sup> direction whereas in vivo evidence suggests that the 2 strands of the bacterial chromosome are replicated simultaneously from a single growing fork. A mechanistic difficulty arises, because of the anti-parallel

nature of the 2 DNA strands, in that a different reaction is required for the insertion of the incoming deoxyribonucleoside triphosphates onto the ends of growing DNA chains. On the one strand synthesis can proceed by addition of a deoxyribonucleoside 5'-triphosphate to the 3'-hydroxyl terminus of the polynucleotide chain. Polymerisation on the complementary strand requires the attack of the 3'-hydroxyl of incoming triphosphates on the 5'-phosphate end of the DNA chain.

Canellakis, Kammen and Morales (1965) have described an enzyme from B. subtilis which can form thymidine 3'-triphosphate from the corresponding diphosphate. This finding has led to speculation that the deoxyribonucleoside 3'-triphosphates may be involved as precursors in the replication of the 3'-5' strand. No successful incorporation of these precursors into DNA has been reported to date. Moreover, there is evidence on the incorporation of  $[^{32}\text{P}]$  Pi into DNA in Esch. coli which suggests that only the 5'-triphosphates function in this capacity (Price, Darmstadt, Hinds and Zamenhof, 1967).

The discovery of polynucleotide ligase in Esch. coli (Gellert, 1967; Olivera and Lehman, 1967a; Zimmerman et al., 1967; Gefter et al., 1967) has led to the development of fresh ideas on the problems of the replication process. Polynucleotide

ligase can repair single-stranded nicks in double-stranded DNA by joining a free 5'-phosphate terminal to an adjacent 3'-hydroxyl group to form a 3'-5' phosphodiester bond. The enzyme has also been detected after T4 and T7 infection of Esch. coli (Weiss and Richardson, 1967a; Cozzarelli et al., 1967; Becker et al., 1967) and has been purified 200-fold from rabbit bone marrow (Lindahl and Edelman, 1968). While the Esch. coli polynucleotide ligase requires  $\text{NAD}^+$  as co-factor, the others utilise ATP. In all cases, however, an intermediate adenylate-enzyme complex is formed (Little, Zimmerman, Oshinsky and Gellert, 1967; Weiss and Richardson, 1967b). Olivera, Hall and Lehman (1968) have also detected a DNA-adenylate intermediate using the Esch. coli enzyme. It seems likely that DNA ligases have a role in DNA repair and recombination processes although no evidence is available on this point at present.

These enzymes have recently been implicated in the mechanism of DNA replication (see Okazaki et al., 1968; Newman and Hanawalt, 1968; Mitra, Reichard, Inman, Bertsch and Kornberg, 1967; Oishi, 1968). Short, single-stranded pieces of DNA have been found during in vivo replication in Esch. coli, B. subtilis and phage-infected cells. This nascent DNA is shown to sediment more slowly than bulk cell DNA in alkaline sucrose gradients and to

be susceptible to the action of exonuclease I (Okazaki et al., 1968). Moreover, this low mol. wt. DNA accumulates in large amounts in Esch. coli infected with T4 mutants having a temperature-sensitive ligase.

On the basis of these findings Okazaki et al. (1968) and Mitra et al. (1967) have suggested that while synthesis of the  $5' \rightarrow 3'$  chain occurs as in vitro, short segments are synthesised on the opposite chain also in the  $5' \rightarrow 3'$  direction, which are subsequently joined to the remainder of the chromosome by polynucleotide ligase. One disadvantage of this mechanism is that it implies a requirement for multiple initiation during replication of the  $5'$ -phosphate strand. This has led Englund et al. (1968) to suggest a modified mechanism which involves covalent extension of primer strands rather than fresh starts.

(a) replication would begin as usual on the  $3' - 5'$  template strand and at some point switch to the complementary strand and continue reading in the  $3' - 5'$  direction until this segment of the DNA is copied.

(b) the V-shaped product could be cleaved by a specific endonuclease at its apex.

(c) DNA synthesis could then be re-initiated on the  $3'$ -hydroxyl terminal created by the cleavage. By repetition of the above process, the complete chromosome could be replicated using poly-

nucleotide ligase to join up the short pieces along the 5' - parental chain.

In theory, such mechanisms, which postulate that native DNA is replicated in a discontinuous fashion, overcome many of the problems arising from apparent differences in in vitro and in vivo replication studies. Thus

(1) Cairns (1963a) observed that synthesis was uni-directional and occurred simultaneously on both strands of the Esch. coli chromosome but the degree of resolution from autoradiographs would not enable him to detect discontinuities in replication over short segments of DNA.

(2) These mechanisms overcome the difficulty that Esch. coli DNA polymerase only catalyses synthesis of a 5' - 3' strand and

(3) They provide an explanation for the greater than 200 molecules of enzyme present per cell which had previously led to speculation that the Esch. coli DNA polymerase was only involved in the repair of DNA.

The critical experiments have still to be performed, however, and the precise control which may be required for the operation of such a replication scheme may be difficult to achieve at present in in vitro systems.

## 5.2. Replication mechanisms in higher organisms

Whether the mechanisms of replication postulated in bacterial systems will be acceptable for mammalian cells is as yet a matter for speculation. As previously discussed our lack of knowledge about the precise organisation of DNA within the chromosome is as yet a great difficulty in this respect.

Tsukada, Moriyama, Lynch and Lieberman (1968) have detected polydeoxyribonucleotide intermediates during DNA replication in regenerating rat liver. After pulse-labelling of the DNA this intermediate is present as a slowly sedimenting fraction in alkaline sucrose gradients. It differs from the corresponding bacterial intermediate in being resistant to the action of exonuclease I. This result has been criticised on the grounds that the separation between bulk cell DNA and pulse-labelled DNA is due to an artefact in the measurement of the mol. wt. of pulse-labelled DNA (Lehmann and Ormerod, 1969). However, short pieces of DNA have been detected during DNA synthesis in isolated HeLa cell nuclei (Kidwell and Mueller, 1969). Addition of a cytoplasmic fraction which stimulates the nuclei 3-4-fold also promotes formation of larger DNA units.

Efforts to determine the mode of DNA synthesis in chromosomes have largely been carried out by autoradiographic

techniques. Gentle lysis of the cells has shown the DNA to be arranged in the form of long fibres. Sasaki and Norman (1966) have isolated fibres more than 2 cm. long from human lymphocytes while Cairns (1966) and Huberman and Riggs (1968) have detected fibres 500  $\mu\text{m}$ . and 1800  $\mu\text{m}$ . long respectively in HeLa and Chinese hamster cells. From the results of pulse-labelling experiments Cairns (1966) reports that these fibres contain many separately-replicated, tandemly-joined sections. Huberman and Riggs (1968) have confirmed this result and also detect the presence of fork-like growing points as in the Esch. coli chromosome (Cairns, 1963a). Earlier ideas that these long strands of DNA are divided into segments joined by protein linkers (Taylor, 1958) now seem improbable (Taylor, 1963) and has led to the hypothesis that most, if not all, chromosomes contain a single DNA molecule.

Estimations of the size of independently replicating sections (replicons) in mammalian cells suggest that these units are much smaller than the Esch. coli chromosome. By  $5^3$ -bromodeoxyuridine labelling of the DNA in L-5178 Y mouse leukaemic cells, Okada (1968) concludes that the upper limit is  $3.4 \times 10^7$  daltons and that the number of replicons per cell lies between 1.6 and  $4.1 \times 10^5$ . Electron microscopic studies show small

circular DNA-containing structures in pig sperm and wheat germ of similar size (Hotta and Bassel, 1965). Cairns (1966) suggests the presence of more than 100 replicating units per cell in HeLa cells; a separate estimate by Painter, Germany and Rasmussen (1966) indicates a figure of  $10^3 - 10^4$  replicons per cell.

It is interesting that, despite the fact that mammalian replicating sections appear to be much smaller than bacterial chromosomes, the rate of bacterial DNA replication ( $30\mu\text{m}$  per min., Cairns, 1963a) is much faster than the rate of mammalian DNA replication. Cairns (1966), from autoradiography on HeLa cells, concludes that the rate of DNA replication is less than  $0.5\mu\text{m}$ . per min. Similar measurements (Huberman and Riggs, 1966) suggest replication rates from  $0.5 - 1.2\mu\text{m}$ . per min.

Thus it may be that during the course of evolution, higher organisms may have subdivided large DNA molecules into smaller units to allow more efficient replication and to allow limited transcription of a small number of active replicating units (Littau, Allfrey, Frenster and Mirsky, 1964; Paul and Gilmour, 1966). Perhaps also the necessity for synthesising and organising the many chromosomal components of mammalian cells requires a lower rate of DNA replication.

MATERIALS AND METHODS

CHAPTER II - MATERIALS AND METHODS1. MATERIALS1.1. Chemicals

$(\text{NH}_4)_2\text{SO}_4$  ("Enzyme grade") was obtained from Mann Research Laboratories, 136, Liberty Street, New York and  $\text{Cs}_2\text{SO}_4$  (optical grade) from the Harshaw Chemical Company, Cleveland, Ohio.

Hyflo Super Cel was purchased from Koch-Light Laboratories, Colnbrook, Bucks. Other inorganic chemicals were, wherever possible, ANALAR grade or equivalent and normally obtained from B.D.H. Biochemicals, Poole, Dorset.

1.2. Organic chemicals1.2.1. Polyacrylamide gel reagents

Acrylamide was purchased from Koch-Light Laboratories, Colnbrook, Bucks., N'N'-Methylene-Bis-Acrylamide from the British Drug Houses Limited, B.D.H. Laboratory Chemicals Division, Poole, Dorset, N, N, N', N'-Tetramethylethylenediamine (TEMED) from Eastman Organic Chemicals, Rochester 3, New York and riboflavin from Mann Research Laboratories, 136, Liberty Street, New York.

1.2.2. Buffers

Tris base ("Trizma") was purchased from the Sigma Chemical Company, 3,500 DeKalb Street, St. Louis, Mo. as was glycine and glycyL-glycine. These buffers were dissolved in distilled  $\text{H}_2\text{O}$

and the pH of the solution adjusted with HCl or NaOH as required. Buffer stocks were normally made up in 5 or 10 x concentrated solutions and the pH checked, and altered if necessary, after dilution.

### 1.2.3. Inhibitors

Actinomycin D was the generous gift of Merck, Sharp and Dohme, Rahway, New Jersey. Hydroxyurea was purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio and colcemid from CIBA Laboratories Limited, Horsham, Sussex.

### 1.2.4. Dyes

Acridine orange was obtained from George T. Gurr Limited, London, S.V.6., as were methyl green and naphthalene black. Bromophenol blue was the product of B.D.H. Laboratory Chemicals Division, Poole, Dorset.

### 1.2.5. Materials for autoradiography

Nuclear track emulsion (type L 4) was supplied by Ilford Limited, Ilford, Essex, as was the ID, 19 developer. Amfix was obtained from May & Baker Limited, Dagenham.

## 1.3. Biochemicals

### 1.3.1. Nucleic acids and proteins

Salmon testes DNA and Escherichia coli DNA were purchased

from the Worthington Biochemical Corporation, Freehold, New Jersey. Poly d(AT) and poly dG : dC were obtained from Miles Laboratories Inc., Elkhart, Indiana. The soluble RNA preparation employed was from Esch.coli, strain K-12 as supplied by Calbiochem, 10 Wyndham Place, London, W.1. Cytochrome c was the product of the Sigma Chemical Company, 3500 DeKalb Street, St. Louis, Mo., and bovine serum albumin (BSA) of the Armour Pharmaceutical Company Limited, London.

### 1.3.2. Enzymes

Enzymes were obtained from the following suppliers:- spleen phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1), micrococcal nuclease, pancreatic DNase and pancreatic RNase (polyribonucleotide 2-oligonucleotide transferase (cyclising), EC 2.7.7.16) from the Worthington Biochemical Corporation, Freehold, New Jersey.

Esch. coli alkaline phosphatase from the Nutritional Biochemicals Corporation, Cleveland, Ohio. Snake venom 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) from the Sigma Chemical Company, St. Louis, Mo.

Exonuclease I was prepared from 100 g. of Esch. coli strain B supplied by the Sigma Chemical Company, St. Louis, Mo. (see section 2.3.5.).

### 1.3.3. DNA and RNA precursors

Non-radioactive deoxyribonucleoside 5'-triphosphates were

purchased from Calbiochem, Los Angeles, California and P-L Biochemicals, Milwaukee, Wisconsin.  $[Me-^3H]$  dTTP and  $[5-^3H]$  dCTP were the products of Schwarz BioResearch Inc., Orangeburg, New York and  $[\alpha-^{32}P]$  dTTP of the International Chemical and Nuclear Corporation, City of Industry, California,  $[6-^3H]$  thymidine and  $[6-^3H]$  uridine were purchased from the Radiochemical Centre, Amersham, England.

#### 1.4. Biological Materials

L 929 mouse cells (Sanford *et al.*, 1948) were maintained in minimal essential Eagle's medium (MEM: see Paul, 1960) supplemented with 10% ( $\frac{V}{V}$ ) calf serum in the absence of antibiotics. Cells and culture media were supplied by Flow Laboratories Limited, Heatherhouse Road, Irvine, Scotland. Cultures were routinely tested for contamination by PPLO.

#### 1.5. Materials for chromatography

Whatman filter paper No. 1, 3 MM and DE 81 (DEAE-paper) were purchased from H. Reeve Angel and Company Limited, London as was the ion exchange cellulose, DE 52.

Sephadex G200 and blue dextran 2000 were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

#### 1.6. Materials for liquid scintillation counting

1, 4 di-2-(5-phenyl oxazolyl)-benzene, (POPOP), and 2, 5-diphenyl oxazole (PPO) were purchased from Koch-Light Laboratories,

Colnbrook, Bucks. and "Hyamine" Hydroxide, 1 M in methanol, from Nuclear Enterprises (G.B.) Limited, Edinburgh, Scotland.

## 2. METHODS

### 2.1. General techniques

#### 2.1.1. Protein and DNA estimations

Protein was measured by the method of Lowry, Rosebrough, Farr and Randall (1951). Standard curves were constructed in the presence and absence of KCl and 2-mercaptoethanol which were found to interfere with the assay. A standard solution of BSA (1 mg. per ml.) was always measured as a control.

DNA was determined by the method of Burton (1956) or directly by ultraviolet absorption using the relationship that a solution of DNA at 42.5  $\mu$ g. per ml. has an  $E_{260} = 1.0$ .

#### 2.1.2. Isobutyric acid paper chromatography

The purity of deoxyribonucleoside triphosphate preparations was checked by chromatography on Whatman No. 1 filter paper using the conditions described by Keir and Smellie (1959). The ascending chromatogram was developed overnight in isobutyric acid, ammonia (sp. gr. 0.88), 0.1 M-EDTA and H<sub>2</sub>O (100 : 4.2 : 1.6 : 55.8, by vol.) as solvent. Rf values of the mono-, di- and triphosphates have been measured by Grav (1967).

2.1.3. Measurements of radioactivity(a)  $^{32}\text{P}$  material

This isotope was prepared for counting by (1) precipitation of 0.05 ml. of DNA polymerase assay solution onto discs of Whatman No. 1 filter paper (2.5 cm. diam.) with 5% ( $\frac{W}{V}$ ) trichloroacetic acid and BSA as co-precipitant, followed by 3 washes in 5% ( $\frac{W}{V}$ ) trichloroacetic acid (15 ml. per disc), 2 washes in absolute ethanol and final drying in a small vol. of ether or (2) drying the supernatant solution obtained by acid precipitation directly onto stainless steel planchettes, after neutralisation with 7 M-KOH if necessary. Radioactivity was estimated on a Nuclear-Chicago gas-flow counter (98.7% Helium and 1.3% butane) with a background of 1-2 counts per min. (efficiency, approximately 50%).

(b)  $^3\text{H}$  and  $^{32}\text{P}$  material

DNA containing these isotopes was precipitated with 2 ml. of 5% ( $\frac{W}{V}$ ) trichloroacetic acid containing Hyflo Super Cel at 20 g. per litre. A further 2 ml. of this solution was added to a Whatman No. 1 filter paper disc (2.5 cm. diam.) in a Millipore microanalysis filter holder (No. XX 1002500). Samples were then washed twice with 5 ml. of cold 5% ( $\frac{W}{V}$ ) trichloroacetic acid containing 0.05 M- $\text{Na}_4\text{P}_2\text{O}_7$  by suspension and recentrifugation

of the acid-insoluble material at 800 g for 10 min. The acid-insoluble suspension was transferred to the filter with washings using 5% ( $\frac{W}{V}$ ) trichloroacetic acid, 0.05 M- $\text{Na}_4\text{P}_2\text{O}_7$ . The pad of Hyflo Super Cel containing the dispersed precipitate was then washed with portions of the following:-

- (i) 3 times with 10 ml. of 5% ( $\frac{W}{V}$ ) trichloroacetic acid containing 0.05 M- $\text{Na}_4\text{P}_2\text{O}_7$ .
- (ii) 2 times with 10 ml. of absolute ethanol
- (iii) 2 times with 2 ml. of ether

The DNA in the precipitate was dissolved by heating 0.5 ml. of 1 M-"Hyamine" hydroxide solution for 20 min. at 60° in a counting vial. After addition of 10 ml. of toluene-based scintillator (0.5% ( $\frac{W}{V}$ ) PPO and 0.03% ( $\frac{W}{V}$ ) POPOP), estimation of the radioactivity in the doubly-labelled samples was performed with reference to standard counting efficiency curves using a Nuclear-Chicago (model 725) liquid scintillation spectrometer. Acid-soluble  $^{32}\text{P}$  counts could be detected independently of any  $^3\text{H}$  counts present as described in the previous section (2.1.3.a).

(c)  $^3\text{H}$  material

(i) DNA

DNA polymerase activity in cell extracts was routinely assayed by measuring the incorporation of [ $^3\text{H}$ ] dTTP into acid-insoluble, alkali-stable material. Samples were prepared for counting exactly as described in section 2.1.3.b.

In vivo DNA synthesis was followed by estimating the incorporation of [ $^3\text{H}$ ] thymidine into acid-insoluble material. In this case samples were transferred directly to Millipore filter holders without prior washing.  $0.05 \text{ M-Na}_4\text{O}_2\text{P}_7$  was also omitted from the washing solutions; otherwise the procedure was identical to that already described (section 2.1.3. b).

(ii) RNA

Incorporation of radioactivity into RNA was carried out using [ $^3\text{H}$ ] uridine (see section 2.2.2.). Labelled RNA samples (1 ml.) were incubated overnight at  $37^\circ$  in  $0.3 \text{ M-NaOH}$  to hydrolyse the RNA. After cooling, 5 drops of  $50\%$  ( $\frac{\text{W}}{\text{V}}$ ) trichloroacetic acid were added to acidify the solution and the DNA was precipitated by addition of 2 ml. of cold  $5\%$  ( $\frac{\text{W}}{\text{V}}$ ) trichloroacetic acid containing 20 g. per litre Hyflo Super Cel. The acid-insoluble material was removed by centrifugation at 800 g for 10 min. and an aliquot of the supernatant fraction measured for radioactivity in 10 ml. of dioxane-based scintillator ( $10\%$  ( $\frac{\text{W}}{\text{V}}$ ) naphthalene,  $0.7\%$  ( $\frac{\text{W}}{\text{V}}$ ) PPO and  $0.03\%$  ( $\frac{\text{W}}{\text{V}}$ ) POPOP). Estimations were carried out on a Packard Tri-carb (model 4322) scintillation spectrometer with efficiencies of 15-20%.

2.2. Techniques associated with in vivo studies on L 929 cells

2.2.1. Maintenance of cell cultures

L 929 cells were routinely propagated as monolayer cultures

in flat-sided Roux flasks or in rotating Winchester bottles.

Every 3-4 days the cells were harvested by trypsinisation, diluted in fresh medium to approximately 200,000 per ml. and inoculated into sterile bottles. If the cells were required for labelling experiments they were normally inoculated into 6 cm. plastic Petri dishes (3 ml. of cell suspension per dish). Petri dish cultures were incubated in an atmosphere of 5% ( $\frac{V}{V}$ ) CO<sub>2</sub> in air.

### 2.2.2. Labelling of cells with DNA and RNA precursors

Labelling of L 929 cells was carried out for various periods of time by addition to the medium of 2  $\mu$ c. of the radioactive precursors together with the appropriate ribo- or deoxyribonucleoside at a final concentration of 5  $\mu$ M.

After labelling, the cells were washed twice in warm, non-radioactive balanced salt solution (BSS: see Paul, 1960) and the acid-soluble pool extracted by washing 4 times with 3 ml. of 5% ( $\frac{W}{V}$ ) trichloroacetic acid. The cells were washed with 1 ml. of absolute ethanol and then dissolved in 0.5 ml. of 0.3 M-NaOH. The dissolved cell material was transferred with washings to a 15 ml. glass, conical centrifuge tube. The final sample (1 ml.) was assayed for radioactivity in DNA and/or RNA as previously described (section 2.1.3. c, i and ii).

### 2.2.3. Autoradiography

For autoradiography, the cells were labelled with 5  $\mu$ c. of [6-<sup>3</sup>H] thymidine in the presence of 1  $\mu$ M non-radioactive thymidine. After a 1 hr. incubation the cells were rapidly washed twice with 3 ml. of warm, non-radioactive BSS and fixed in 3 ml. of absolute alcohol: acetic acid (9 : 1,  $\frac{V}{V}$ ) for 15 min. The cells were then rinsed in this solution for a further minute and kept in 70% ( $\frac{V}{V}$ ) ethanol until required.

Immediately before use, the dishes were left in running tap water for about 20 min. to remove water-soluble radioactivity from the cells. Nuclear track emulsion (type L 4) was spread evenly over the dishes in a very thin layer. After drying, they were exposed for 7 days before development in ID, 19 developer for 2 min., followed by 2 min. in a 1 in 5 dilution of Amfix. Dishes were immediately rinsed in tap water and stained in 0.1% ( $\frac{W}{V}$ ) aqueous methyl green. Microscopic examination showed background grains to be almost zero and nuclei with 2 grains or more over them were considered to be labelled. Counts were made from 2 fields on each dish and the average result for % nuclei labelled was calculated.

### 2.3. Techniques associated with studies of enzymic activity in L 929 cell extracts

#### 2.3.1. Harvesting of cells and preparation of cell extracts

For isolation of nuclei, 2-3 day old, rapidly growing cultures were normally used. The cells were scraped off into culture

medium and centrifuged at 400 g for 10 min. at 3°. All subsequent operations during the isolation were carried out at 0-3°. To remove any remaining medium from the cell pellet the cells were successively washed with 5 ml. portions of 0.9% ( $\frac{w}{v}$ ) NaCl and 0.02 M-tris-HCl, pH 7.5 containing 0.25 M-sucrose (buffered sucrose) and centrifuged as before. Disruption of the cells, suspended at  $1-2 \times 10^7$  per ml. in buffered sucrose, was carried out using a Potter-Elvehjem homogeniser (Teflon pestle, 0.308 in. diam.; tube, 2 ml. capacity; both obtained from Sireica, Jamaica, New York, U.S.A.; clearance between tube and pestle 0.004-0.006 in.).

The homogenate was centrifuged at 800 g for 10 min. and the supernatant fraction used as a source of soluble enzyme after spinning at 105,000 g for 60 min. (Spinco model L ultracentrifuge, rotor no. 40). Nuclei were washed twice more in the same vol. of buffered sucrose before final suspension in the same medium as a source of nuclear DNA polymerase. The integrity and purity of nuclei were checked by fluorescent microscopy after staining with 1% ( $\frac{w}{v}$ ) acridine orange.

### 2.3.2. Disruption of nuclei

#### (a) Preparation of sonicated extracts of nuclei

Sonication of nuclear preparations ( $1-2 \times 10^7$  per ml.) in buffered sucrose was carried out in 4-6 ml. batches using a Dawe

Soniprobe fitted with a brass sample holder. Treatment was for 40 sec. at 3 amps (setting 6) in 2 x 20 sec. bursts. Sonicated extracts were observed by fluorescent microscopy to check that nuclear disruption was complete. After centrifugation of sonicated preparations at 105,000 g. for 45 min. the supernatant was retained as a source of nuclear enzyme.

(b) Preparation of KCl extracts of nuclei

This procedure was carried out essentially as described by Patel et al. (1967). Nuclear preparations were suspended in 1.5 M-KCl in a Waring blender (setting 3) to disperse the gelatinous material. The solution was diluted to 0.15 M-KCl by the addition of 0.02 M-tris-HCl, pH 7.5 at which concentration DNA and histones recombine and precipitate out. The insoluble material was pelleted by centrifugation at 800 g for 10 min. leaving the non-histone material in the supernatant fraction. This fraction was retained as a source of DNA polymerase.

2.3.3. DNA preparations

(a) Isolation of  $[^{32}\text{P}]$  DNA from Esch. coli

$[^{32}\text{P}]$  DNA from Esch. coli was routinely prepared in the department using a modification of the method of Lehman (1960). Approximately 3 g. of cells, grown in 200 ml. of glycerol-lactate medium containing  $[^{32}\text{P}]$  orthophosphate (0.2  $\mu\text{g.}$  at 50 c. per  $\mu\text{g.}$ )

were sedimented by centrifugation for 20 min. at 12,000 g in the 6 x 250 ml. rotor of the M.S.E. 18 centrifuge. They were then washed in standard saline-citrate (SSC: 0.015 M-sodium citrate, 0.15 M-NaCl, pH 7.0), recentrifuged and resuspended in 100 ml. of SSC. 1 g. of sodium dodecyl sulphate was dissolved in the solution which was then incubated at 60° for 10 min.

NaClO<sub>4</sub> was added to 1 M and the DNA extracted with an equal vol.

of chloroform-isoamyl alcohol (25 : 1,  $\frac{V}{V}$ ). The interphase NaClO<sub>4</sub> was added to 1 M and the DNA extracted with an equal vol

of chloroform-isoamyl alcohol (25 : 1,  $\frac{V}{V}$ ). The interphase in SSC and treating with sodium dodecyl sulphate and chloroform-isoamyl alcohol as previously. The process was repeated until a negligible interphase remained. The DNA was precipitated from the pooled top layers of each extraction by 2 vol. of absolute ethanol and redissolved in  $\frac{1}{10}$ th SSC. Pancreatic ribonuclease was added at 20 µg. per ml. and the solution incubated at 37° for 30 min. The DNA was then extracted twice by addition of 1 vol. of 90% ( $\frac{W}{V}$ ) redistilled phenol in SSC. Two ethanol precipitations were performed and the DNA was dissolved in 0.02 M-KCl and dialysed against 0.02 M-KCl for 16 hr. Acid-insoluble backgrounds were in the region of 2%.

(b) Denaturation of DNA

[<sup>32</sup>P] DNA and commercially obtained salmon testes and

Esch. coli DNA (dissolved in 0.05 M-KCl at 2 mg. per ml.) were heated at 100° for 10 min. and then rapidly cooled to 0° in ice water.

On occasions Esch. coli DNA was alkali-denatured. The DNA solution was made 0.3 M with respect to NaOH and left for 10 min. at room temperature. The pH was readjusted to pH 7.5 by addition of 1 N-HCl and a few drops of 0.8 M-tris-HCl, pH 7.5. This solution was then dialysed for 4 hr. against 100 vol. of 0.05 M-KCl.

(c) Activation of DNA

Treatment of DNA with small amounts of pancreatic DNase increases the priming efficiency of the DNA in DNA polymerase assays. Activation of salmon testes DNA was carried out by a modification of the method of Aposhian and Kornberg (1962).

The DNA (2 mg.) was exposed to a variety of concentrations of pancreatic DNase (100 µg. to 10<sup>-5</sup> µg.) in a solution of 1 ml. containing 0.5 mg. of BSA, 5 µmoles of MgCl<sub>2</sub> and 50 µ moles of tris-HCl, pH 7.5. After 15 min. at 37°, the DNA solutions were rapidly cooled and frozen for later use in DNA polymerase assays.

2.3.4. Enzyme assay procedures

(a) Assay of DNA polymerase activity

The basic assay system was that of Shepherd and Keir (1966).

Nuclear and supernatant fractions containing DNA polymerase activity were incubated at 37° for 60 min. in a total vol. of 0.25 ml. with 5 $\mu$  moles of tris-HCl buffer, pH 7.5, 1  $\mu$  mole of MgCl<sub>2</sub>, 15  $\mu$  moles of KCl, 0.1  $\mu$  mole of EDTA, 1.5  $\mu$  moles of 2-mercaptoethanol, 100  $\mu$ g. of DNA and 50  $\mu$  moles each of dATP, dGTP, dCTP and [Me-<sup>3</sup>H] or [ $\alpha$ -<sup>32</sup>P] dTTP. On occasions [5-<sup>3</sup>H] dCTP was also employed. The reaction was terminated by the addition of 0.05 ml. of 2 N-NaOH and samples were left overnight at 37° before being prepared for radioactive counting as described in sections 2.1.3. a or c.

DNA polymerase activity is expressed as  $\mu$  moles dTMP or dCTP incorporated into acid-insoluble, alkali-stable material per hr. per mg. of protein at 37°.

(b) Assay of DNase activity

DNase activity in nuclear and supernatant preparations was estimated under DNA polymerase assay conditions by measurement of the degradation of native or heat-denatured DNA (radioactive DNA) to acid-soluble fragments.

The reaction vol., components and time of incubation were similar to that for DNA polymerase but the deoxyribonucleoside triphosphates were omitted. In addition 10  $\mu$ g. of Esch. coli

[<sup>32</sup>P] DNA was substituted for the DNA primer used in polymerase assays. The incubation was terminated by cooling the reaction mixture to 0° in iced water. 0.2 ml. of BSA at 10 µg. per ml. and 0.6 ml. of 1 N-perchloric acid were then added. After 10 min. at 0° the assay tubes were centrifuged at 800 g for 10 min. and the <sup>32</sup>P material in an aliquot of the supernatant fraction was measured as described in section

DNase activity was expressed as µg. of radioactive DNA rendered acid-soluble per hr.

(c) Assay of cytochrome oxidase

Cytochrome oxidase activity (cytochrome c : O<sub>2</sub> oxidoreductase, EC 1.9.3.1) was measured in nuclear fractions and cell homogenates exactly as described by Cooperstein and Lazarow (1951).

The activity of cytochrome oxidase was expressed as  $\Delta$  log (ferrocytochrome c) per min. at room temperature.

(d) Assays of micrococcal nuclease, calf spleen phosphodiesterase and alkaline phosphatase

[<sup>32</sup>P] DNA, synthesised by nuclear and supernatant extracts of L 929 cells, was degraded to the 3'-monophosphates as reported by Josse and Swartz (1963). The conditions of assay of micrococcal nuclease and calf spleen phosphodiesterase were exactly as described in the above paper.

The assay for completeness of digestion of the radioactive DNA employing Esch. coli alkaline phosphatase was also performed according to the procedures layed down by Josse and Swartz (1963).

### 2.3.5. Preparation of exonuclease I

#### (a) Purification

Exonuclease I was purified from sonicated extracts of Esch. coli, strain B as described by Lehman (1960). The purification (approximately 140-fold) involves a protamine sulphate precipitation and elution, an  $(\text{NH}_4)_2\text{SO}_4$  concentration and fractionation step followed by chromatography on a DEAE-cellulose column. The enzyme at this stage of the purification has only slight activity on native DNA but attacks single-stranded DNA from the 3'-hydroxyl end with the liberation of 5'-mononucleotides in a stepwise manner until the terminal dinucleotide is reached.

#### (b) Assay of exonuclease I

The assay system is similar to that employed for measuring DNase activity in crude extracts and measures the conversion of  $[\text{}^{32}\text{P}]$  DNA to acid-soluble fragments. The incubation mixture was essentially as described by Lehman and Nussbaum (1964) and contains 20  $\mu$  moles of glycine buffer, pH 9.2, 2  $\mu$  moles of  $\text{MgCl}_2$ , 0.5  $\mu$  moles of 2-mercaptoethanol and 1-5  $\mu$ g. of  $[\text{}^{32}\text{P}]$  DNA. Incubations were usually carried out in a vol. of 0.3 ml. for 15

min. at 37°; then 0.2 ml. of cold "carrier" DNA was added (salmon testes DNA, 2 mg. per ml.) and 0.5 ml. of cold 0.35 N-perchloric acid. After 5 min. at 0° the resulting precipitate is pelleted by centrifugation at 12,000 g for 3 min. and a sample of the supernatant fraction measured for  $^{32}\text{P}$  acid-soluble material (section 2.1.3. a).

(c) Characteristics of DEAE-cellulose fraction

To test that our purified preparation from Esch. coli B (DEAE-cellulose fraction) had the properties associated with exonuclease I, its activity on native and heat-denatured Esch. coli  $^{32}\text{P}$  DNA was compared under conditions in which the total DNA would not be rendered acid-soluble. Fig. 2.1. shows that a 20% degradation of native DNA occurs during the incubation while 60% of the denatured DNA is rendered acid-soluble. Addition of more enzyme at 60 min. results in a further 25% breakdown of single-stranded DNA in the next 30 min. while no further hydrolysis of native DNA takes place.

In Fig. 2.2. the capacity of exonuclease I to completely degrade single-stranded DNA is seen under conditions in which only a 20% degradation of native DNA is observed.

(d) Cs<sub>2</sub>SO<sub>4</sub> gradient analysis of Esch. coli  $^{32}\text{P}$  DNA

The kinetics of hydrolysis of a fraction of the native

Fig. 11.1.

Time course of activity of exonuclease I (DEAE-cellulose fraction)  
on native and heat-denatured *Esch. coli*  $^{32}\text{P}$  DNA.

A preparation of exonuclease I (see section 2.3.5. a) was assayed for activity on native and heat-denatured primer using the standard assay (see section 2.3.5. b). Enzymic activity is expressed as the % DNA rendered acid-soluble in a given time. There were 5  $\mu\text{g.}$  of  $^{32}\text{P}$  DNA (1,150 c.p.m. per  $\mu\text{g.}$ ) and 20  $\mu\text{g.}$  of protein per assay.

- denatured DNA rendered acid-soluble
- native DNA rendered acid-soluble

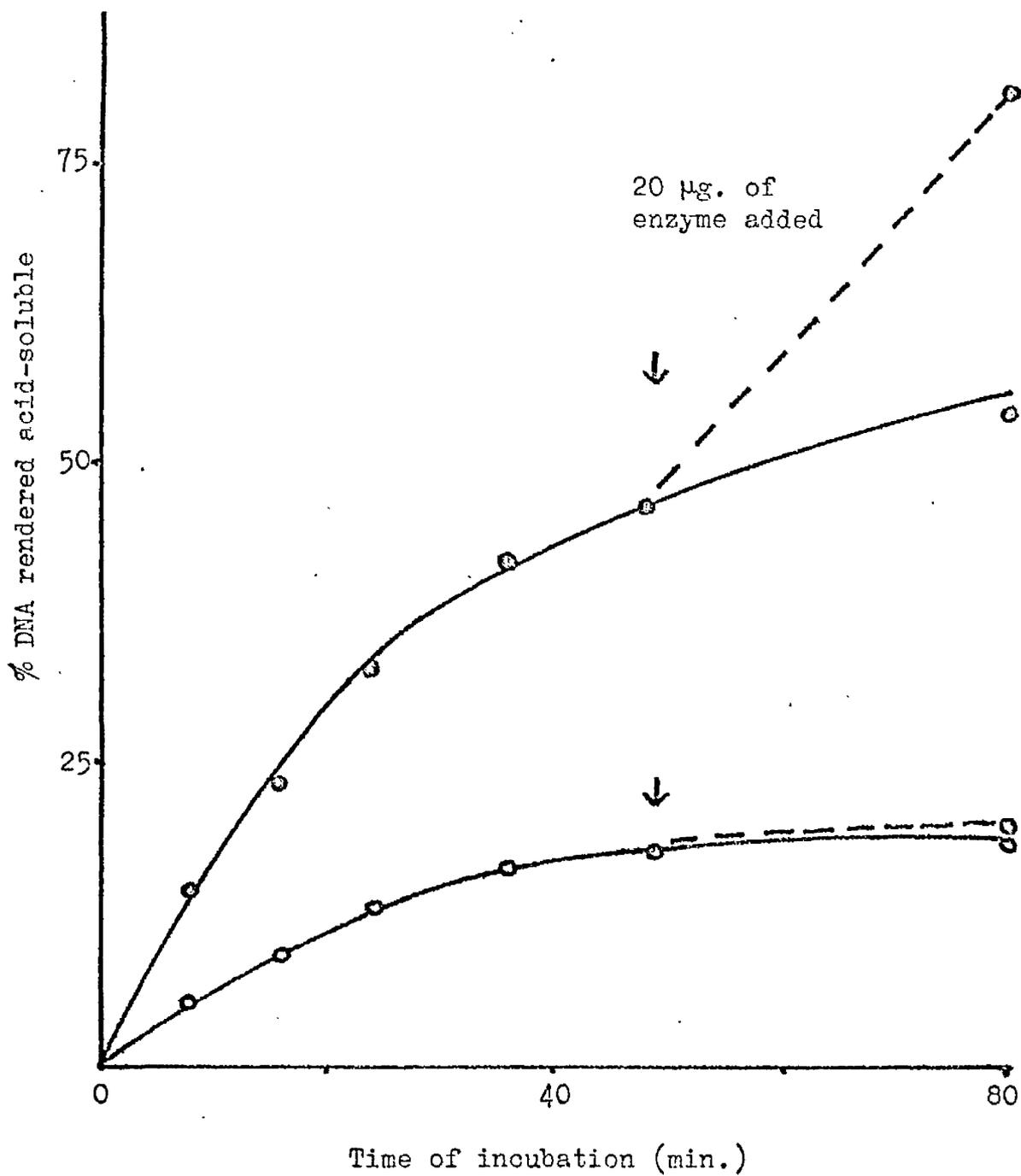
Fig. 11.1.

Fig. 11.2.

Variation in activity of exonuclease I with protein concentration.

Using the standard assay varying amounts of exonuclease I (DEAE-cellulose fraction) were added and tested for their activity on native or heat-denatured  $[^{32}\text{P}]$  DNA (see Fig. 11.1 for details). The specific activity of the  $[^{32}\text{P}]$  DNA was 1560 c.p.m. per  $\mu\text{g}$ . (5  $\mu\text{g}$ . per assay).

●—● denatured DNA rendered acid-soluble

●—● native DNA rendered acid-soluble

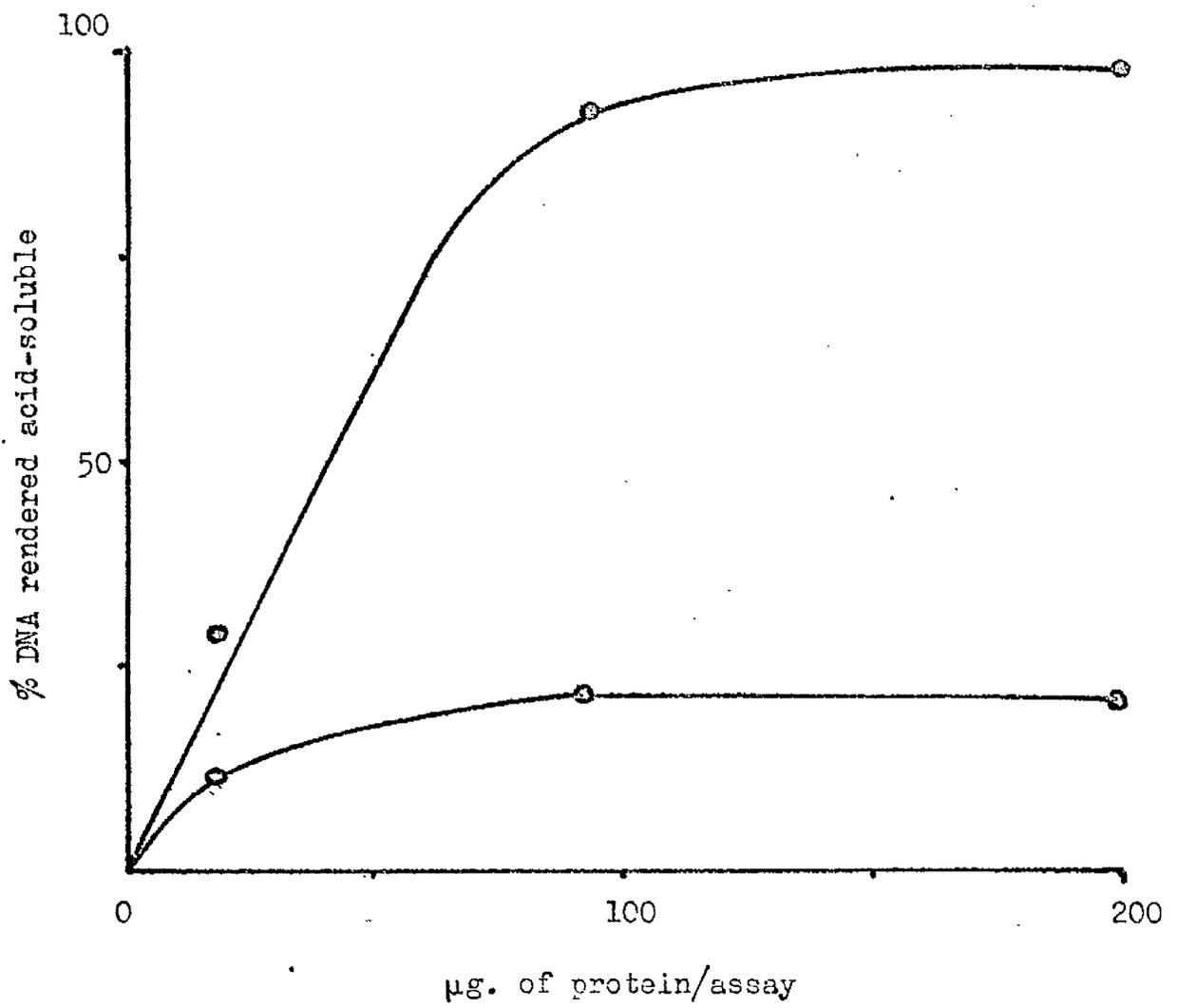
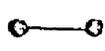
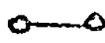
Fig. 11.2.

Fig. 11.3.

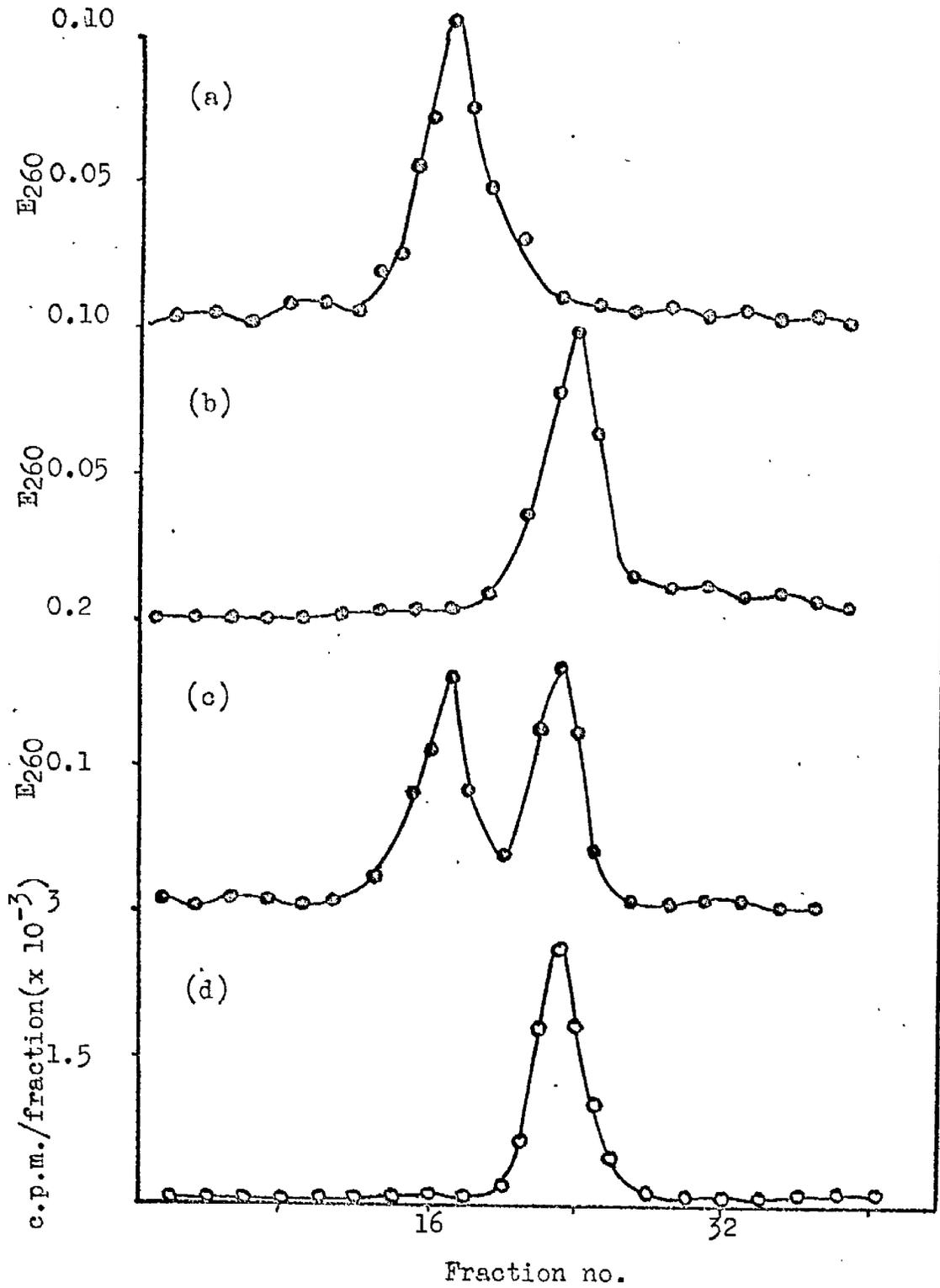
Analysis of Esch. coli  $[^{32}\text{P}]$  DNA on  $\text{Cs}_2\text{SO}_4$  gradients.

Native and alkali-denatured (see section 2.3.3. b) Esch. coli non-radioactive and  $[^{32}\text{P}]$  DNA was subjected to analysis as described in section 2.3.6. g.

  $\text{E}_{260}$   
 acid-precipitable  $[^{32}\text{P}]$  DNA

- (b) native Esch. coli DNA
- (a) alkali-denatured Esch. coli DNA
- (c) native + alkali-denatured Esch. coli DNA (equal amounts)
- (d) native Esch. coli  $[^{32}\text{P}]$  DNA

Fig. 11.3.



DNA by exonuclease I suggested that this activity might represent the presence of single-stranded DNA fragments in the native Esch. coli DNA preparation. Fig. 2.3. shows the optical density profiles of native and alkali-denatured Esch. coli DNA on  $\text{Cs}_2\text{SO}_4$  gradients spun to equilibrium. The  $^{32}\text{P}$  counts of our isolated DNA sample correspond closely to peaks of native Esch. coli DNA with less than 2% of the total counts running in the region of single-stranded DNA. It may be, however, that the native DNA sample contains "frayed" ends and partially single-stranded regions which are susceptible to exonuclease I attack.

(e) The presence of endonuclease I activity in exonuclease I preparations

Lehman and Nussbaum (1964) have purified exonuclease I (DEAE-cellulose fraction) a further 10-fold on hydroxylapatite columns and have shown that the enzyme now exhibits no significant activity for double-stranded DNA. This increased specificity is attributed to the removal of traces of endonuclease I activity during the final purification stage.

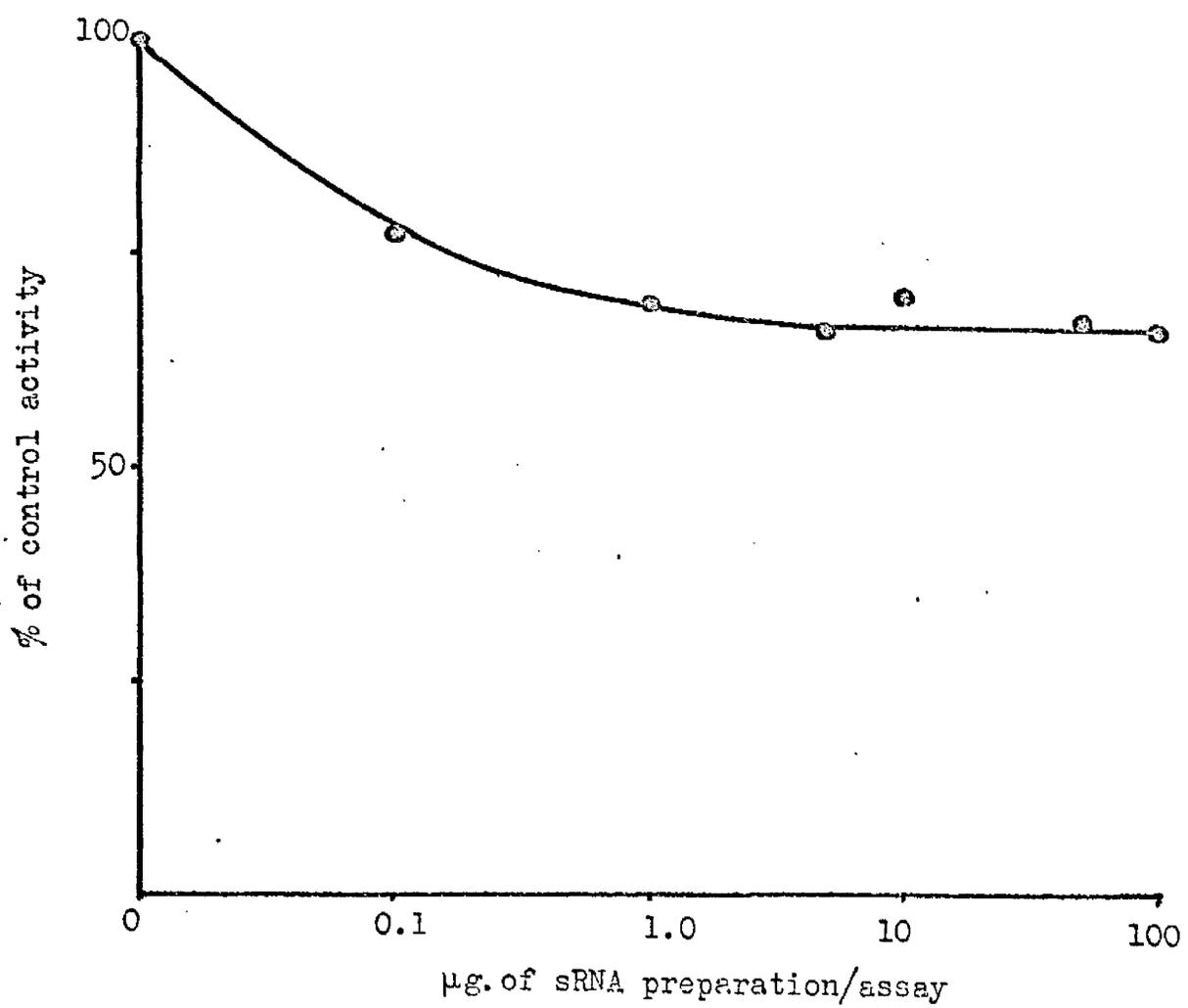
To examine whether the activity on native DNA in our preparations might be caused by endonuclease I contamination, the rate of hydrolysis of heat-denatured DNA was followed in the

Fig. 11.4.

The presence of endonuclease I activity in exonuclease I preparations.

Exonuclease I activity (DEAE-cellulose fraction) was assayed under routine conditions (see Fig. 11.1) in the presence of increasing amounts a commercial preparation of soluble RNA (see section 1.3.1.). There were 40  $\mu\text{g.}$  of protein per assay and 5  $\mu\text{g.}$  of heat-denatured  $[\text{}^{32}\text{P}]$  DNA (1010 c.p.m. per  $\mu\text{g.}$ ). Results are expressed as a percentage of the acid-soluble radioactivity in control assays containing no soluble RNA.

●—● % control activity

Fig. 11.4.

presence of increasing amounts of a soluble RNA preparation from Esch. coli (see section 1.3.1.). RNA, especially tRNA is known to be a potent inhibitor of endonuclease I action (Lehman and Nussbaum, 1964).

Indication of the presence of endonuclease I comes from the 30-40% decrease in the rate of hydrolysis of DNA, even with only 0.1 µg. of soluble RNA present per assay. Similar results are reported by Lehman and Nussbaum (1964).

(f) DEAE-paper chromatography of the products of exonuclease I digestion.

The mode of action of the purified exonuclease I preparation was examined by DEAE-paper chromatography of the products of reaction.

High mol. wt. DNA and oligonucleotides of chain length greater than 30 remain at the origin (Furlong, 1966). Smaller oligonucleotides run with Rf values 0-0.2 while purine and pyrimidine nucleotides have Rf values 0.5 and 0.7 respectively (Morrison, J., Ph.D. Thesis, University of Glasgow, 1967). There is also some broadening of the purine mononucleotide peak as there is a partial separation of dAMP and dGMP.

Fig. 2.5. shows that the purified enzyme is an exonuclease

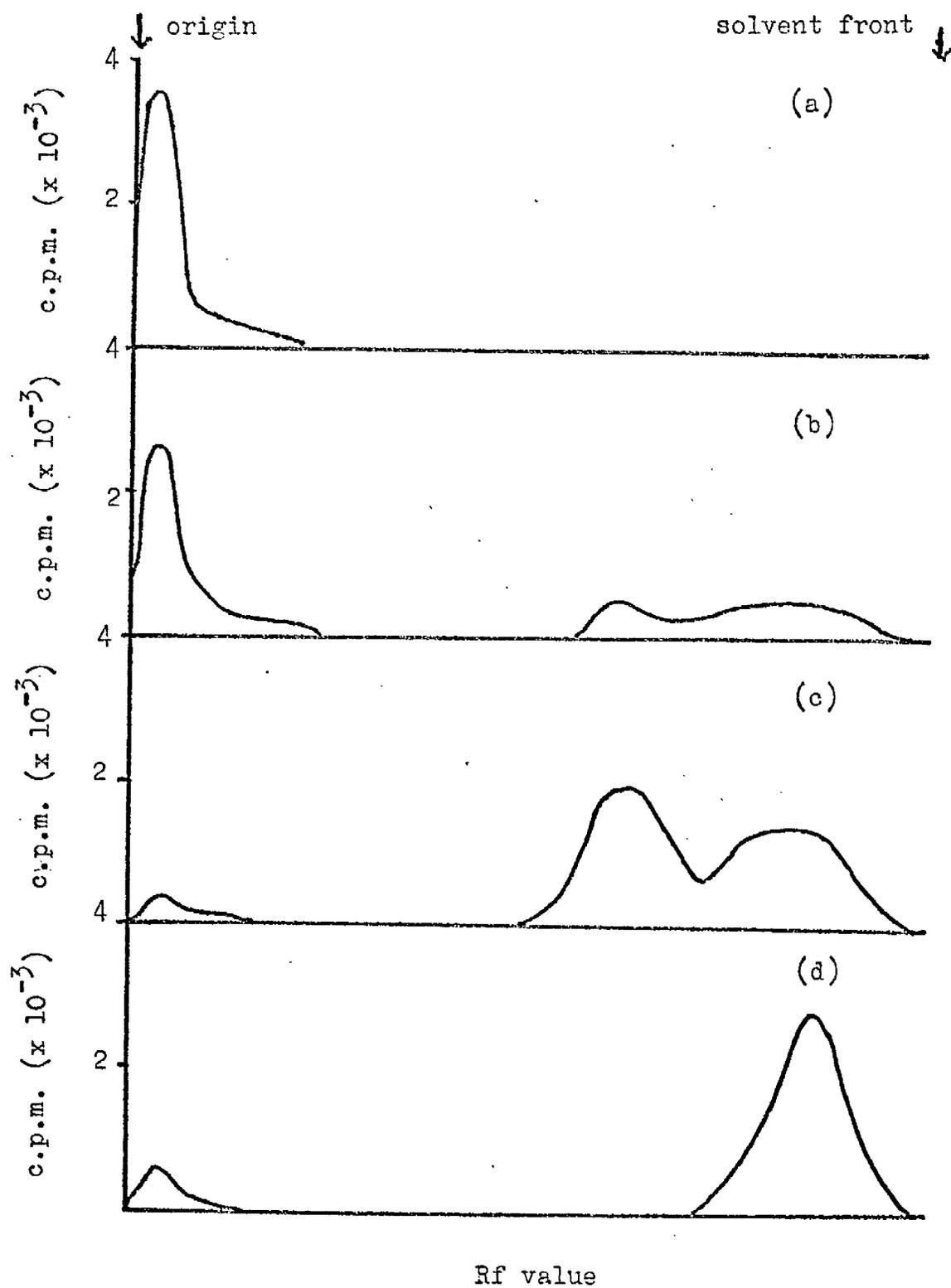
Fig. 11.5.

DEAE-paper chromatography of the products of exonuclease I digestion.

Exonuclease I hydrolysis of heat-denatured  $[^{32}\text{P}]$  DNA was carried out as in Fig. 11 .1. and the products of digestion analysed by chromatography on DEAE-paper as described in section 2.3.6. d. There were 50  $\mu\text{g.}$  of protein and 5  $\mu\text{g.}$  of DNA (750 c.p.m. per  $\mu\text{g.}$ ) per assay. In some cases, 10  $\mu\text{g.}$  of 5' - nucleotidase (see section 1.3.2.) was added for a 15 min. incubation after exonuclease I treatment.

- (a) unincubated sample
- (b) 5 min. digestion with exonuclease I
- (c) 20 min. digestion with exonuclease I
- (d) 20 min. digestion with exonuclease I + further 15 min. with 5' - nucleotidase

Fig. 11.5.



as the primary products of reaction are purine and pyrimidine mononucleotides having Rf values of 0.54 and 0.74 respectively. No endonuclease activity is observed by this method, although it should be remembered that the products of endonuclease action would be good substrates for exonuclease I.

Treatment of the products of enzymic hydrolysis with 5'-nucleotidase converts the 2 peaks of radioactivity corresponding to purine and pyrimidine monophosphates to a single peak with Rf 0.8 which corresponds to inorganic phosphate. As exonuclease I degrades single-stranded DNA sequentially from the 3'-hydroxyl terminus with the release of deoxyribonucleoside 5'-monophosphates, our enzymic preparation thus exhibits all the characteristics associated with this enzyme. It should be noted that in these experiments the pyrimidine mononucleotide peak was broader than the purine mononucleotide peak, contrary to that reported by Morrison. The presence of phosphatase activity in the exonuclease I preparation as reported by Lehman's group would account for this discrepancy.

### 2.3.6. Purification and fractionation procedures

#### (a) pH 5 precipitation

Sonicated nuclear and supernatant preparations were maintained at 0-3° and 0.1 N-acetic acid added dropwise with constant

stirring. The pH of the solutions was constantly monitored until pH 5 was reached. Solutions were kept at 0° for 5 min. before centrifuging at 800 g for 10 min. at 0-3°. The resulting supernatant was discarded and the pellet redissolved in buffered sucrose and a few drops of 0.8 M-tris-HCl, pH 7.5 added if required.

(b) Gel filtration on Sephadex G200

Sephadex G200 was pre-swollen, equilibrated and packed into 24 x 1.4 cm. columns according to the supplier's instructions. Void volume determinations were carried out using blue dextran 2000. The buffer employed was 0.02 M-tris-HCl, pH 7.5 containing 5 mM-2-mercaptoethanol. If fractionation of DNA polymerase activity was required 0.15 M-KCl was included in the eluting buffer. Flow rates were 10-15 ml. per hr. and 1.0-1.2 ml. fractions were collected.

(c) DEAE-cellulose columns

The micro-granular DEAE-cellulose (DE 52) was prepared as indicated in the supplier's instructions. The standard buffer was 0.02 M-tris-HCl, pH 7.5 containing 5 mM-2-mercaptoethanol and gradient elution was performed using KCl solutions in standard buffer. Samples were applied to a 1 x 15 cm. column in standard buffer, the column washed with buffer to remove non-adsorbed material and elution commenced by applying

a linear gradient of KCl (50 ml. of standard buffer in the mixing beaker and 50 ml. of buffer containing 0.35 M-KCl in the reservoir beaker). Sample vols. were approximately 1.5 ml. and flow rate was 10-15 ml. per hr.

(d) Chromatography on DEAE-paper

The products of exonuclease I and DNase action could be applied directly onto sheets of DEAE-cellulose (DE 81), 28 x 11 cm. (Furlong, 1966). 0.1 ml. samples were spotted 5 cm. from the top of the chromatogram which was developed for 1-2 hr. in the descending direction using 0.75 M-ammonium bicarbonate, pH 8.6, as solvent. After drying the chromatogram was cut into strips, 4 cm. in width, and scanned for  $^{32}\text{P}$  using the Nuclear-Chicago Model III Actigraph. Rf values of the mono-, di- and triphosphates are described by Furlong (1966).

(e) Polyacrylamide gel electrophoresis

The details of gel preparation and electrophoretic separation and gel staining are exactly as described by Davis (1964). Electrophoresis was carried out at  $3-8^{\circ}$  for  $2-2\frac{1}{2}$  hr. using bromophenol blue marker. The riboflavin-TEMED system was employed to promote polymerisation as it was thought that ammonium persulphate, a strong oxidising agent, would cause denaturation of proteins.

After electrophoresis gels were removed quickly and excess buffer adsorbed on blotting paper. The gel was sliced into 4 mm. fractions with a razor blade by placing the gel between 2 steel combs held apart and perfectly aligned by means of a perspex block  $7\frac{1}{8}$  in. by  $\frac{3}{8}$  in. by  $\frac{1}{2}$  in. The cylindrical sections obtained were again halved to give a final vol. of approximately 0.1 ml. and the gel slices immersed in DNA polymerase assay mixture at  $0-3^{\circ}$  for 30 min. to allow time for diffusion. Samples were then incubated at  $37^{\circ}$  for 60 min. in the usual manner. After incubation the gel slices were disrupted by homogenisation and the samples prepared for liquid scintillation counting as described in section 2.1.3. c.

(f) Nearest neighbour frequency analysis

The distribution of  $[\alpha\text{-}^{32}\text{P}]$  dTMP in the DNA product was examined by the technique described by Josse and Swartz (1963). The following modifications were employed:-

(i) The deoxyribonucleoside 3'-monophosphates resulting from the digestion of the DNA product were separated by high voltage electrophoresis on Whatman 3 MM paper. The voltage was 3,000-4,000 (48-64 volts per cm.). Electrophoresis was carried out for  $2-2\frac{1}{2}$  hr. in 0.05 M-ammonium formate, pH 3.5.

(ii) Radioactivity in the spots was determined by immersing each spot in 10 ml. of toluene-base scintillator (0.5% PPO and 0.03% PCPOP) and counting in a Nuclear-Chicago

liquid scintillation spectrometer. The areas between the spots and the area corresponding to inorganic phosphate were counted and shown to contain negligible radioactivity.

(g) Cs<sub>2</sub>SO<sub>4</sub> gradients.

This technique was performed to check for the presence of single-stranded DNA in isolated Esch. coli [<sup>32</sup>P] DNA preparations. Cs<sub>2</sub>SO<sub>4</sub> gradients give better resolution of double- and single-stranded DNA species than do CsCl gradients (Szybalski, 1968). To 1-2 O.D. units of native or alkali-denatured Esch. coli DNA in 1.3 ml. of 0.05 M-KCl was added 0.7 ml. of a saturated Cs<sub>2</sub>SO<sub>4</sub> solution and the initial density adjusted to 1.42 gm. per cm<sup>3</sup>. Centrifugation of the Cs<sub>2</sub>SO<sub>4</sub> solutions (under paraffin) was carried out in the Spinco SW 50 rotor at 45,000 rev. per min. (165,000 g) for 18 hr. at 20°. The tubes were removed and 2-drop fractions collected by puncturing the bottom of the tube. The fractions were diluted with 0.5 ml. of H<sub>2</sub>O and E<sub>260</sub> or radioactivity measured (section 2.1.3. c).

(h) Neutral and alkaline sucrose gradients

This technique was performed to examine whether the newly-synthesised DNA formed by partially purified DNA polymerase preparations from L 929 cells was covalently attached to the primer DNA.

(i) Neutral sucrose gradients

0.15 ml. of [ $^3\text{H}$ ] DNA solution (assay mixture dialysed for 48 hr. against 4 x 100 vol. of 0.02 M-tris-HCl, pH 7.5 to remove deoxyribonucleoside triphosphates) was layered on top of a 5 ml. sucrose gradient (5-20% sucrose in 1 M-NaCl, 1 mM-EDTA and 0.02 M-tris-HCl, pH 7.5) and centrifuged for 10 hr. at 24,000 rev. per min. (44,000 g) at 4° in a Spinco SW 39L rotor. Two-drop fractions were collected, diluted to 0.5 ml. with distilled H<sub>2</sub>O, and assayed at E<sub>260</sub> and for radioactivity (section 2.1.3. c).

(ii) Alkaline sucrose gradients

The procedure was similar to that for neutral sucrose gradients with the following modifications:-

- (a) [ $^3\text{H}$ ] DNA was alkali-denatured before layering on the gradient.
- (b) The gradient contained 0.1 M-NaOH.
- (c) Centrifugation was for 16 hr. at 32,000 rev. per min. (84,000 g).

RESULTS

## CHAPTER III - RESULTS

### 1. STUDIES ON L 929 CELLS IN STATIONARY PHASE

#### 1.1. Introduction

Regenerating liver (Fujioka et al., 1963; Lieberman and Kane, 1963), primary cultures of rabbit kidney cortex cells (Lieberman et al., 1963; Adams et al., 1965) and the lymphocyte-PHA system (see Ling, 1968) have been widely employed to investigate the sequence of events during the transition from the resting ( $G_0$ ) state to one of rapid cell proliferation. All 3 systems suffer from several disadvantages.

(a) The degree of synchrony obtained in all cases is relatively poor.

(b) The work on regenerating liver is subject to variation between animals. In addition, it is difficult to take account of all the parameters in such a complex system. For instance, Bucher and Swaffield (1965), using starved rats, find that the UTP pool increases significantly after partial hepatectomy. If the rats are fed ad libitum, however, the 2-fold rise in the rate of RNA synthesis is accompanied by only a 20-30% increase in the specific activity of the uridylylate pool (Ove, Adams, Abrams and Lieberman (1966).

(c) All 3 systems require the use of difficult manipulations to make accurate quantitative analyses.

(d) Regenerating liver and kidney cortex cells contain a mixture of cell types which may respond differently to external stimuli.

The present work has been concerned with establishing a parallel system, using cultures of L 929 cells with a view to elucidating some of the biochemical events required for the initiation of DNA replication and cell division. If "out of cycle" ( $G_0$ ) cells could be obtained in culture, ease of manipulation and the use of a more simplified system would enable more precise studies to be made during the changeover from  $G_0$  to S phase than those with whole animals or primary systems.

1.2. The rate of DNA synthesis in cultures of L 929 cells at high population density

Weissman et al. (1960) have followed the induction of thymidine and dTMP kinases (ATP: thymidinemonophosphate phosphotransferase, EC 2.7.4.c) in L cells which had been brought to resting phase by exhaustion of the medium. In order to overcome the criticism that stationary cells, obtained by this procedure, cease to synthesise DNA because of starvation or cytotoxic effects, fresh medium is substituted on every alternate day during the experiment. Fig. 111.1 shows the rate of DNA

synthesis in L cells as a function of the age of the culture. The rate of DNA synthesis per cell on day 1 is low and there is little increase in cell number during this period. This initial lag represents the time required for the cells to become adapted to their new environment (see Chapter 1, section 1.3). Cell proliferation and the rate of DNA synthesis are at a maximum on day 3, after which there is a gradual decline in both these parameters as the cultures become more densely-populated. After 9 days, the rate of DNA synthesis is only 5% of that observed during maximum growth, while no increase in cell number is observed after 7 days, by which time the cells have increased 12-fold over the initial value.

Some stimulation (approximately 2-fold) in the rate of DNA synthesis is observed if the medium is replaced 6 hr. before labelling the cells with [ $^3\text{H}$ ] thymidine. This effect may be caused by the presence of a number of growth-promoting factors in the serum (see Harris, 1964) or by the removal of an inhibitor of cell multiplication. Bürk (1966) has described an inhibitor produced by BHK 21 (C13) cells in culture which is not produced by a polyoma-transformed derivative, Py Y cells. Even in fresh medium, however, the capacity of stationary L 929 cultures to synthesise DNA is markedly reduced, being only 5-10% of the values obtained during logarithmic growth. L 929 cells could be maintained for 20 days or more with little or no detachment

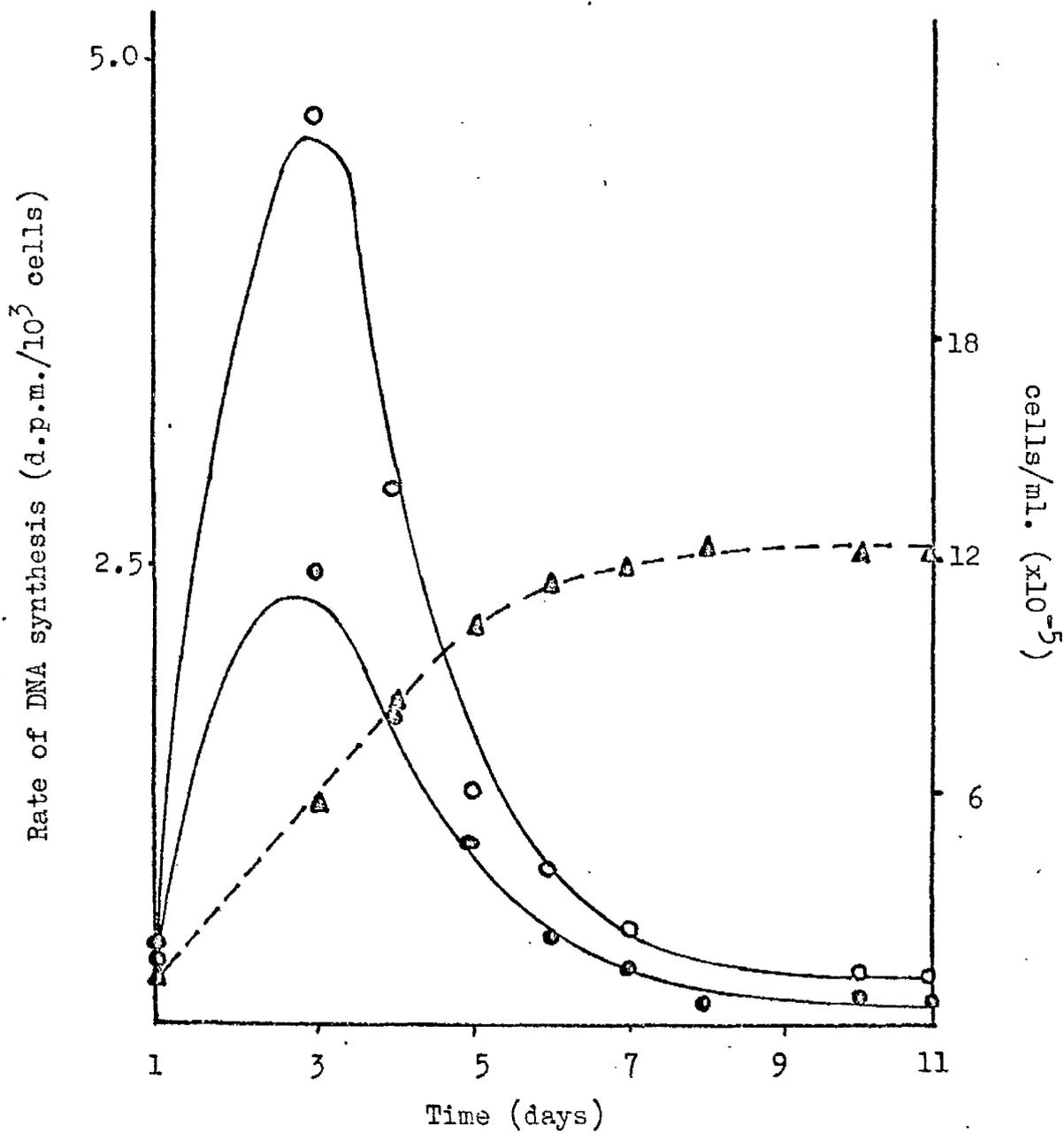
Fig. 111.1.

The rate of DNA synthesis in cultures of L 929 cells grown to high population density

Cultures of L 929 cells were established at approximately 100,000 cells per ml. in 2 oz., flat-sided, glass medicine bottles (5 ml. per bottle). The medium was replaced every second day throughout the experiment. The rate of DNA synthesis during growth of the cultures was followed by pulse-labelling the cells for 60 min. with 2  $\mu$ c. of  $[6-^3\text{H}]$  thymidine (80  $\mu$ c. per  $\mu$  mole). The final concentration of thymidine in the medium was 5  $\mu$ M and incorporation of radioactivity into DNA was determined as in Methods, section 2.1.3. c.i. Cell number was estimated by counting aliquots of the cell suspension on a Coulter counter, model D.

- incorporation into DNA (24 hr. following medium replacement)
- incorporation into DNA (6 hr. following medium replacement)
- △---△ cell number

Fig. 111.1.



of cells from the glass. A good recovery of cells occurs when they are eventually subcultured in warm, fresh medium.

1.3. The release of L 929 cells from stationary phase

Ten day old cultures of stationary L 929 cells are harvested by trypsinisation and diluted in fresh medium to approximately 200,000 cells per ml. A synchronised burst of DNA synthesis is observed after a lag period of 16 hr. (Fig. 111.2). During the period from 12-20 hr. there is a 10-20-fold increase in the incorporation of [ $^3\text{H}$ ] thymidine into DNA with a peak at around 20 hr. when 70% of the population are in S phase. A second broader peak of incorporation is detected at around 39 hr. after release.

Control cells, set up from rapidly growing cultures also exhibit some degree of synchrony. This suggests that the trypsinisation procedures or cooling of the cells during initiation of the cultures makes some contribution towards the synchronisation process. While the rate of DNA synthesis is initially quite high in control cells, there is a gradual decline with a minimum rate of DNA replication occurring at 8-10 hr. Such results suggest that  $G_1$  cells are prevented, at least temporarily, from entering S phase. Newton and Wildy (1959) have shown that brief cooling of HeLa cells to  $4^\circ$  for 60 min. delays their entry

Fig. 111.2.

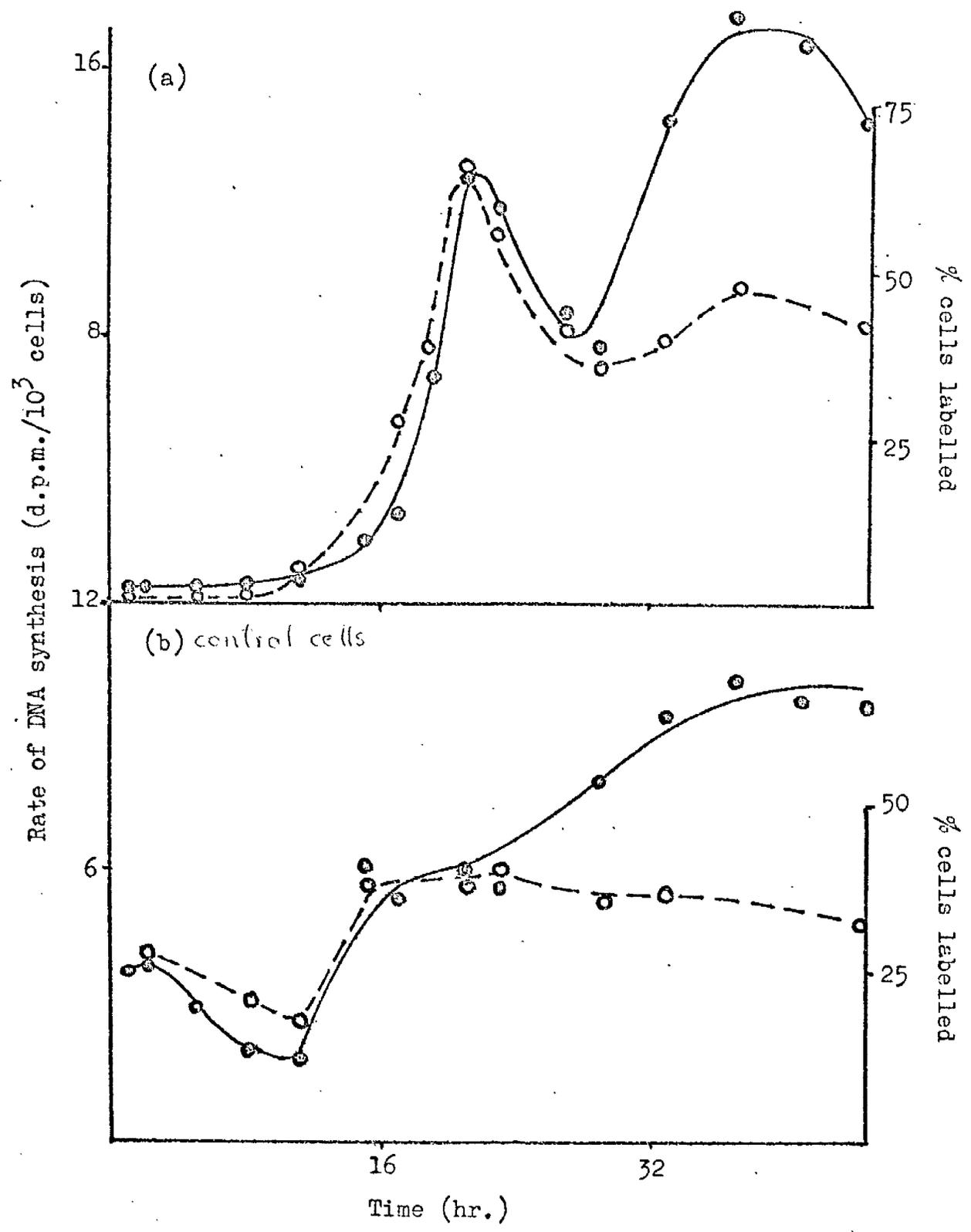
The release of L 929 cells from stationary phase.

Ten day old cultures of L 929<sub>s</sub> were diluted to approximately 200,000 per ml. (3 ml. per dish). Control dishes were also set up from rapidly growing cultures. The rate of DNA synthesis at various times thereafter was estimated as described in the legend to Fig. 111.1. Specific activity of [6-<sup>3</sup>H] thymidine was 133  $\mu$ c. per  $\mu$  mole. For autoradiography, the cells were labelled with 5  $\mu$ c. [6-<sup>3</sup>H] thymidine (333  $\mu$ c. per  $\mu$  mole) for 60 min. and subsequently treated as described in Methods, section 2.2.3.

●—● incorporation into DNA

●---● % cells labelled

Fig. 111.2.



into S phase.

1.4. Effect of colcemid on the rate of DNA synthesis in released-stationary L 929 cells

To examine whether the second broad peak of DNA synthesis at around 39 hr. is caused by cells re-entering S after one replication cycle, the previous experiment is repeated in the presence of the mitotic inhibitor, colcemid. Fig. 111.3 shows the rate of DNA synthesis and % S cells at various times after release from stationary phase. On this occasion colcemid at 0.25  $\mu$ g. per ml. is added at zero time.

No second peak of DNA synthesis is observed by 36 hr. strongly suggesting that this secondary increase in the rate of DNA synthesis is due to cells entering S for a second round of replication. As a normal first peak of DNA synthesis is found in the presence of the drug, it appears that the stationary cells are initially in  $G_0$  or  $G_1$  phase and can therefore proceed normally through the S and  $G_2$  periods before being blocked at mitosis.

The usual result of treatment with colcemid at 0.25  $\mu$ g. per ml. is to promote the progressive appearance of large numbers of metaphase-blocked cells (Puck and Steffen, 1963). However, in our experiments with L cells, we have failed to observe

Fig. 111.3.

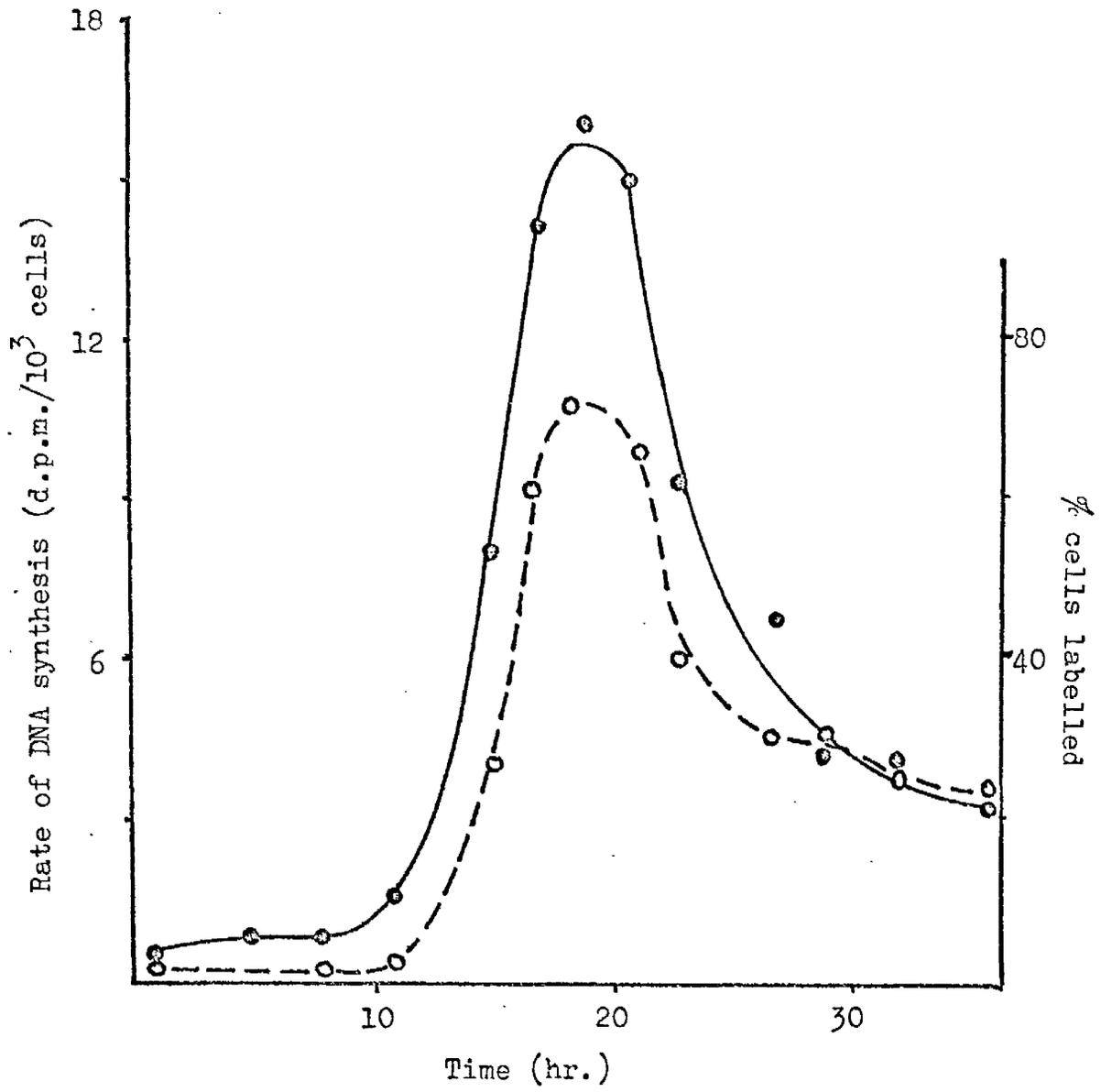
The effect of colcemid on the rate of DNA synthesis in released-stationary cells.

The procedure was identical to that used in Fig. 111.2. except that colcemid (0.25  $\mu$ g. per ml.) was added at zero time.

●—● incorporation into DNA in released-stationary cells

○—○ % cells labelled

Fig. 111.3.



an accumulation of cells in metaphase. On the other hand, after 20 hr., a significant proportion of the cells, treated with colcemid, appear to be multi-nucleate. Rao and Engelberg (1964) report that L cells, treated with colcemid, are only arrested at metaphase for 10-12 hr. during which time they form multi-nucleate cells and subsequently re-enter interphase. The time scale of our experiments is not sufficiently long to detect any of these colcemid-blocked L cells should they eventually re-enter S phase.

1.5. The rates of RNA synthesis in L 929 cells released from stationary phase

The  $G_0$ -S phase transition in regenerating liver and PHA-stimulated lymphocytes is characterised by early increases in the rate of RNA synthesis (Fujioka et al., 1963; see Ling, 1968). In rabbit kidney cortex cells a doubling in the rate of RNA synthesis occurs between 12 and 22 hr. after establishment in culture. These elevated rates of RNA synthesis are required for subsequent DNA replication and low levels of actinomycin D or FPA completely abolish this rise while not preventing the normal rates of RNA turnover (Lieberman et al., 1963).

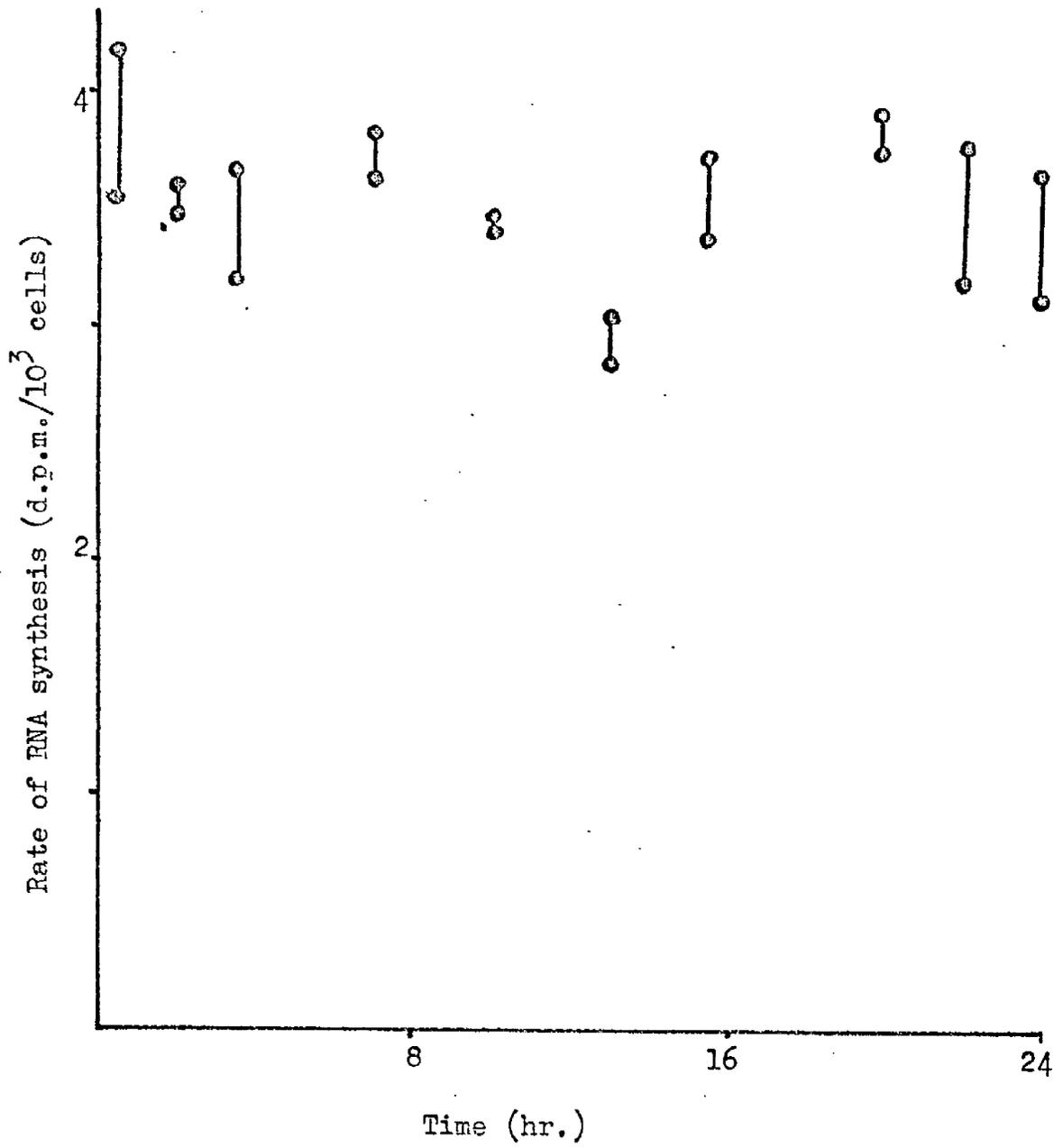
Changes in the rate of RNA synthesis in L 929 cells are examined after release from stationary phase by pulse-labelling

Fig. 111.4.

The rate of RNA synthesis in cultures of L 929 cells released from stationary phase.

10 day old cultures of L 929 cells were subcultured into fresh medium as in Fig. 111.2. The cells were pulse-labelled for 60 min. at various times thereafter with 1  $\mu$ c. of  $[6-^3\text{H}]$  uridine (67  $\mu$ c. per  $\mu$  mole) at a final concentration of 5  $\mu$ M. Incorporation of radioactivity into RNA was determined as described in Methods, section 2.1.3. c.ii.

●—● incorporation into RNA

Fig. 111.4.

at various times with [ $^3\text{H}$ ] uridine. It can be seen that, in contrast to the work on other resting cell systems, no marked changes in the rates of RNA synthesis, preceding the onset of DNA synthesis, are observed after release from stationary phase (Fig. 111.4).

1.6. The rate of RNA synthesis in cultures of L 929 cells at high population density

The apparent constancy in the rate of RNA synthesis following subculture of stationary cells may reflect a high rate of RNA turnover in densely populated cultures. Alternatively, a rapid increase in the rate of RNA synthesis in released-stationary cells may have escaped detection. To distinguish between these 2 possibilities, the rate of RNA synthesis is measured in cultures grown to stationary phase (Fig. 111.5).

Only a slight drop (10-20%) in the rate of RNA synthesis in 10 day old cells is observed as compared to rapidly growing control cultures. After the third day the DNA-synthetic capacity of these cells declines rapidly. Levine et al. (1967) have shown that primary cultures of diploid human fibroblasts develop lasting changes in their patterns of RNA synthesis after 20 generations in culture. In addition the rate of RNA synthesis in

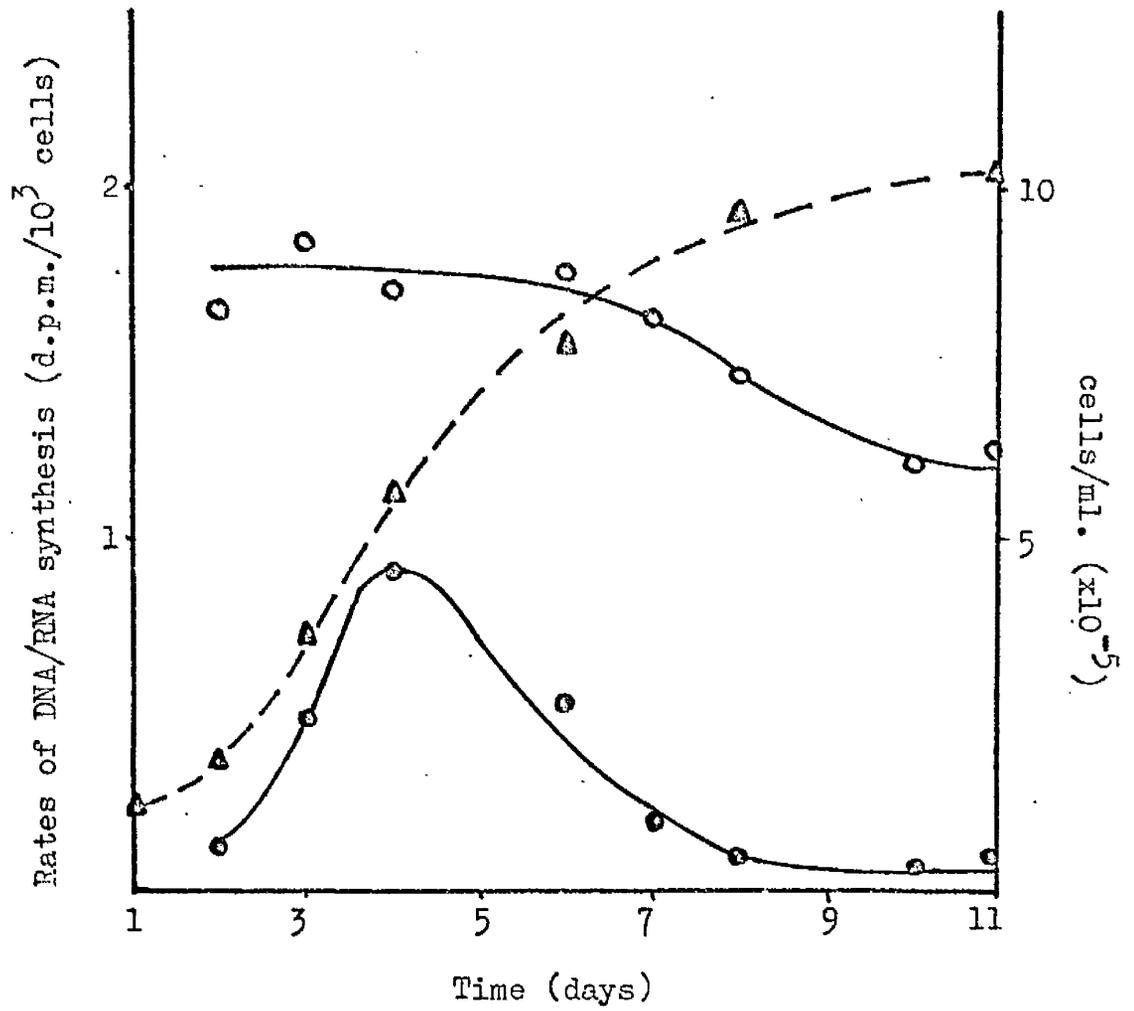
Fig. 111.5.

The rate of RNA synthesis in cultures of L.929 cells at high population density.

L 929 cells were inoculated into flat-sided, 2 oz. medicine bottles at approximately 100,000 per ml. and the rates of incorporation of  $[6-^3\text{H}]$  uridine into DNA and RNA during growth to stationary phase determined as described in Fig. 111.1. and 111.4. The medium was replaced on every second day during the course of the experiment.

- incorporation into RNA
- incorporation into DNA
- △—△ cell number

Fig. 111.5:



early passage cells is markedly inhibited in confluent cultures but this is not the case in late passage cultures. Thus the possibility exists that the high rate of turnover in resting cultures of L 929 cells may represent a difference between permanent cell lines and primary systems.

1.7. The effect of actinomycin D on RNA synthesis and subsequent DNA synthesis in released-stationary cells

Lieberman et al. (1963) can abolish the 2-3-fold rise in the rate of RNA synthesis which occurs between 12 and 22 hr. in rabbit kidney cortex cells in vitro by the addition of low levels of actinomycin D (0.0075  $\mu$ g. per ml.) at any time up to 12 hr. The original rate of RNA synthesis is maintained under these conditions but subsequent initiation of DNA synthesis is prevented.

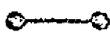
It is of interest to determine whether DNA synthesis can be inhibited in released-stationary L cells by levels of actinomycin D which have no effect on the rates of RNA synthesis. Accordingly, different concentrations of the drug are added at zero time along with [ $^3\text{H}$ ] uridine or [ $^3\text{H}$ ] thymidine and incorporation into RNA or DNA estimated after labelling for 25 hr.

Fig. 111.6 shows that both DNA and RNA synthesis are completely inhibited by actinomycin D at 0.1  $\mu$ g. per ml. In

Fig. 111.6.

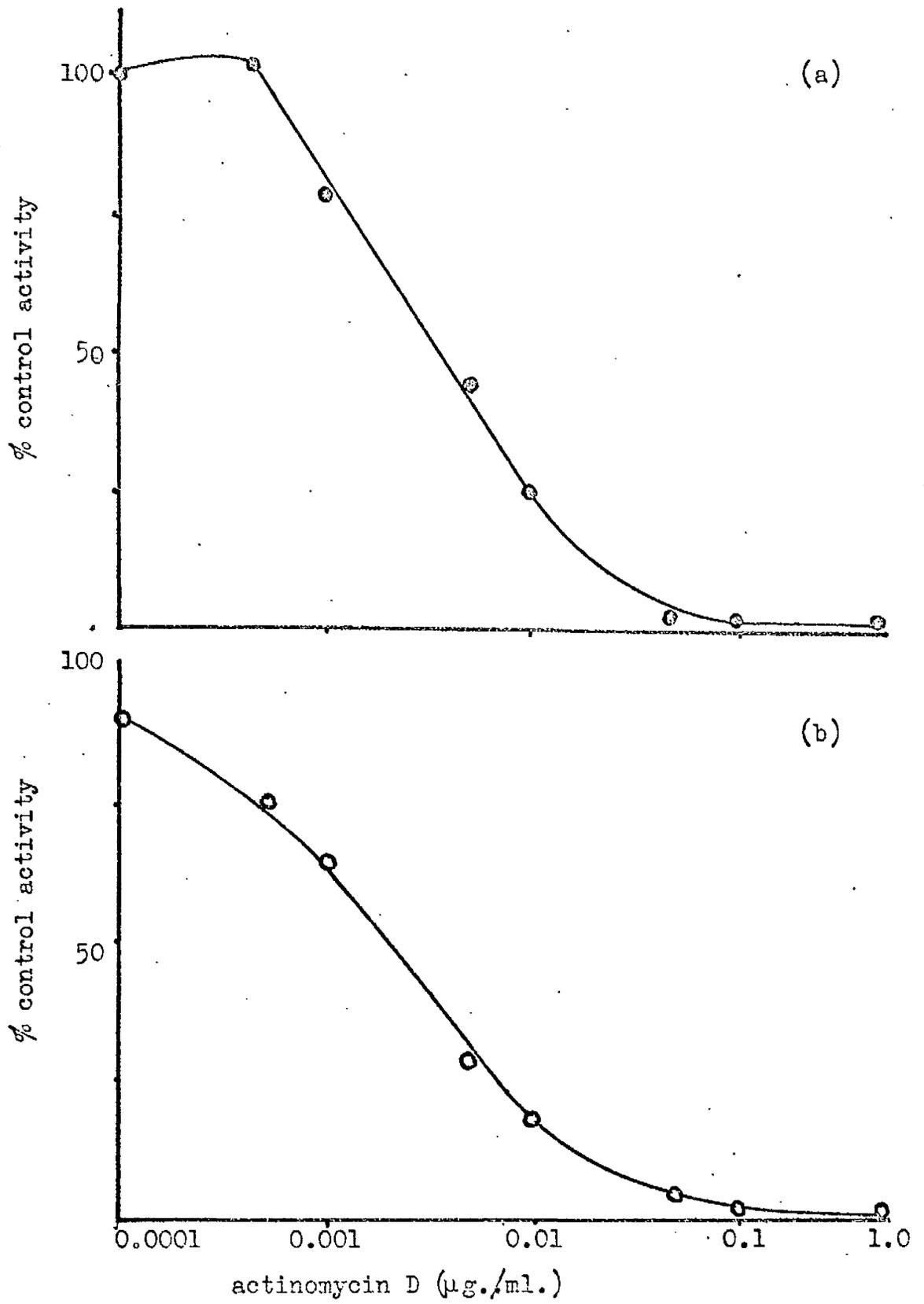
The effect of actinomycin D on the rates of RNA synthesis and subsequent DNA synthesis in released-stationary cells.

Cultures of 10 day old stationary cells were established as in Fig. 111.2. and various concentrations of actinomycin D added at zero time as indicated. 2  $\mu$ c. of  $[6-^3\text{H}]$  thymidine (133  $\mu$ c. per  $\mu$  mole) or 1  $\mu$ c. of  $[6-^3\text{H}]$  uridine (67  $\mu$ c. per  $\mu$  mole) was also added at zero time and the incorporation into RNA or DNA after 25 hr. estimated as described in Methods, section 2.1.3. c.

- (a)  incorporation into DNA
- (b)  incorporation into RNA

Results are expressed as  $\%$  of the incorporation into DNA or RNA in the absence of actinomycin D.

Fig. 111.6.



contrast to the rabbit kidney cortex and regenerating rat liver systems, no concentration of drug can be found which prevents DNA replication without affecting the normal rates of RNA turnover in these cells.

Actinomycin D is known to be primarily an inhibitor of RNA synthesis. When added at zero time to released-stationary cells, however, it is equally inhibitory to subsequent DNA synthesis indicating the RNA synthesis during the lag period is essential for the initiation of DNA replication.

1.8. The effect of the time of actinomycin D addition on the subsequent rates of DNA and RNA synthesis

The final stages of the lag period in rabbit kidney cortex cells cultured in vitro are characterised by an increasing resistance to the effects of actinomycin D and FPA on DNA synthesis. The effect of adding actinomycin D (0.1  $\mu$ g. per ml.) at various times during the lag period in L 929 cells is shown in Fig. 111.7.

DNA synthesis (as judged by [ $^3$ H] thymidine incorporation into DNA from 20-21 hr.) is completely abolished if the drug is added at any time before 12 hr. After this time, however, the cells become progressively more resistant to the drug. RNA synthesis is always greatly inhibited by the presence of actinomycin D. Even 0.1  $\mu$ g. per ml. added at 20 hr. along with [ $^3$ H]

Fig. 111.7.

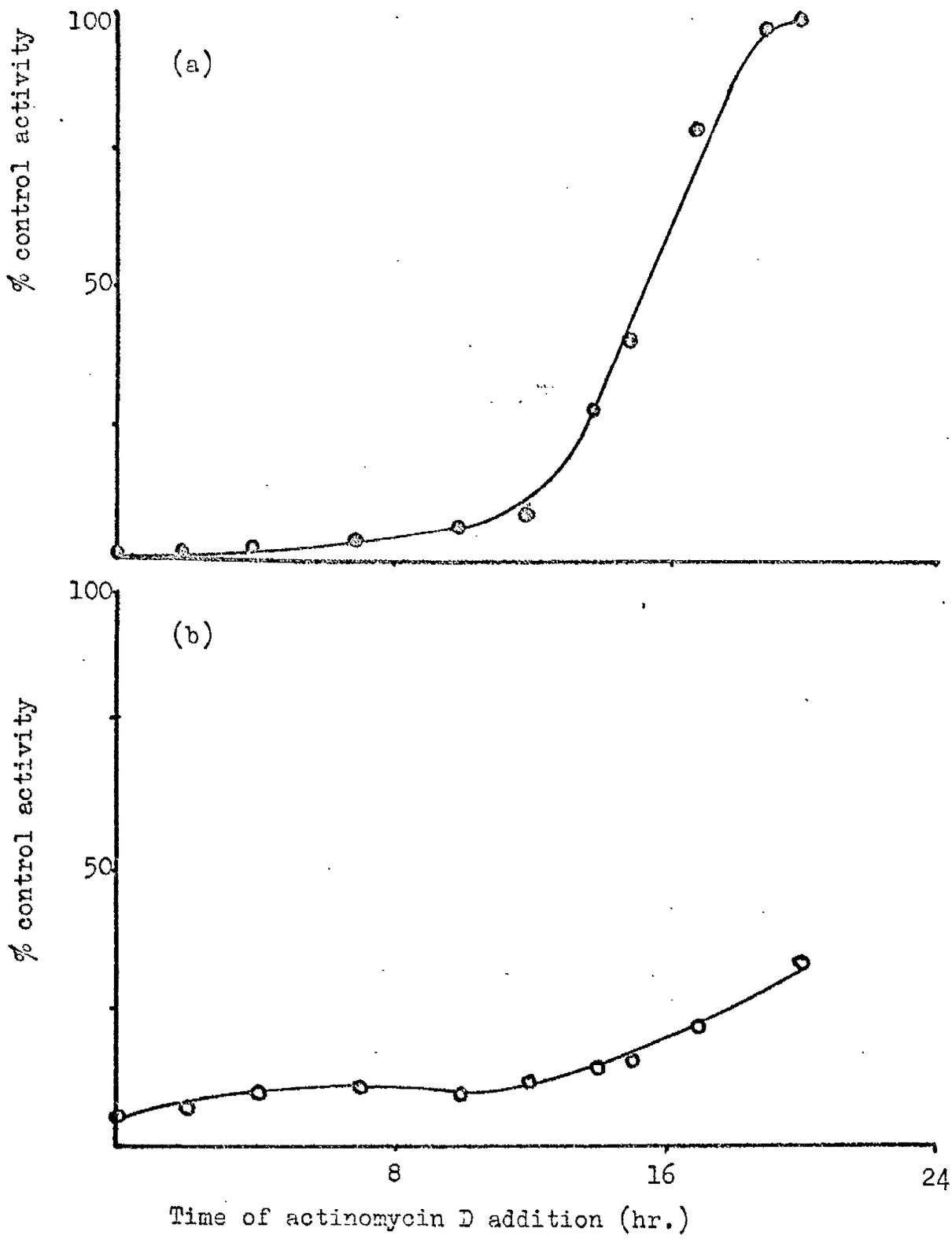
The effect of the time of addition of actinomycin D on the subsequent rates of DNA and RNA synthesis in released-stationary cells.

Actinomycin D (0.1  $\mu$ g. per ml.) was added to cultures at various times up to 20 hr. and the rates of RNA and DNA synthesis between 20 and 21 hr. determined by pulse-labelling as in Fig. 111.1. and 111.4.

- (a)      ○—○      incorporation into DNA
- (b)      ○—○      incorporation into RNA

Results are expressed as % of the incorporation into DNA or RNA in the absence of actinomycin D.

Fig. 111.7.



uridine inhibits incorporation into RNA during the next hr. by 70%.

These results confirm the studies of Mueller and Kajiwara (1966) and support the conclusion that an actinomycin D-sensitive process, presumably RNA synthesis, is required at the onset of S phase for the cell to replicate its full complement of DNA. Using the drug at 0.1  $\mu$ g. per ml. in synchronised HeLa cells the above authors have shown that only 50% of the DNA is replicated when the drug is present at the beginning of S but addition 2 hr. after reversal of the thymidine-less state permits the cells to synthesise a full complement of DNA. The increased resistance to the drug after 12 hr. in L 929 cells presumably reflects the number of cells which are entering S phase during this period.

1.9. Variations in the DNA polymerase activity of nuclear and supernatant fractions of L cells after release from stationary phase

Resting tissues are characterised by having low activities of enzymes involved in nucleic acid biosynthesis. In regenerating rat liver (Bollum and Potter, 1959), rabbit kidney cortex cells cultured in vitro (Adams et al., 1965) and PHA-stimulated human lymphocytes (Loeb et al., 1968), increases in DNA polymerase activity occur about the time of DNA replication. The greater degree of synchrony obtained in our system enables a more precise correlation to be made between the levels of DNA poly-

merase in released-stationary cells and the period of DNA synthesis.

Figs. 111.8 and 111.9 show the results of such an experiment. A normal peak of DNA synthesis is observed at 18-19 hr. after release. The activity of DNA polymerase, primed with native or heat-denatured DNA, is followed in nuclear (Nl) and high speed supernatant (Sl) fractions of the cells. Preparation of these fractions and the importance of the physical state of the DNA primer has been previously described (Lindsay and Adams, 1968). The nuclear fraction shows no large changes in activity with native DNA as primer during the experiment although a 50% increase occurs between 10 and 13 hr. at about the onset of DNA synthesis. With denatured DNA primer, on the other hand, a 5-6-fold increase in activity is detected. The denatured DNA-primed activity of the nuclear fraction closely parallels the rate of DNA synthesis in the cells.

The most dramatic changes in DNA polymerase activity are noted in high speed supernatant extracts where by 22 hr. a 10-20-fold rise in activity occurs when the primer is single-stranded DNA. In this case there is an initial increase in activity at the start of DNA synthesis. During the middle of the S period, however, no further changes take place, but as the cells begin

Fig. 111.8.

Variations in the DNA polymerase activity of nuclear (N1)  
fractions of L cells after release from stationary phase.

10 day old stationary cultures were established in 9 cm. (10 ml. per dish) or 6 cm. Petri dishes (3 ml. per dish) at a concentration of 500,000 per ml. Actinomycin D (0.1  $\mu$ g. per ml.) or hydroxyurea (2 mM) was also added to some cultures after 11 hr. DNA synthesis was followed as in Fig. 111.1. Nuclear (N1) fractions were prepared as described in Methods, section 2.3.1. (see Fig. 111.10) and DNA polymerase activity was measured under standard assay conditions (Methods, 2.3.4. a) using native and heat-denatured DNA as primer. The specific activity of [ $^{3}\text{H}$ ] dTTP (2.5  $\mu$ c. per assay) was 0.05  $\mu$ c. per  $\mu$  mole. DNA polymerase activity was expressed as  $\mu$  moles of [ $^{3}\text{H}$ ] dTTP incorporated into alkali-stable, acid-insoluble material per mg. of protein per hr. at 37 $^{\circ}$ .

- (a)  incorporation of [ $^{3}\text{H}$ ] thymidine into DNA  
 incorporation into DNA in actinomycin D-treated cultures  
 incorporation into DNA in hydroxyurea-treated cultures
- (b)  DNA polymerase activity of N1 nuclei with native DNA primer  
 activity in actinomycin D-treated cultures  
 activity in hydroxyurea-treated cultures
- (c)  DNA polymerase activity of N1 nuclei with denatured DNA primer  
 activity in actinomycin D-treated cultures  
 activity in hydroxyurea-treated cultures

Fig. 111.8.

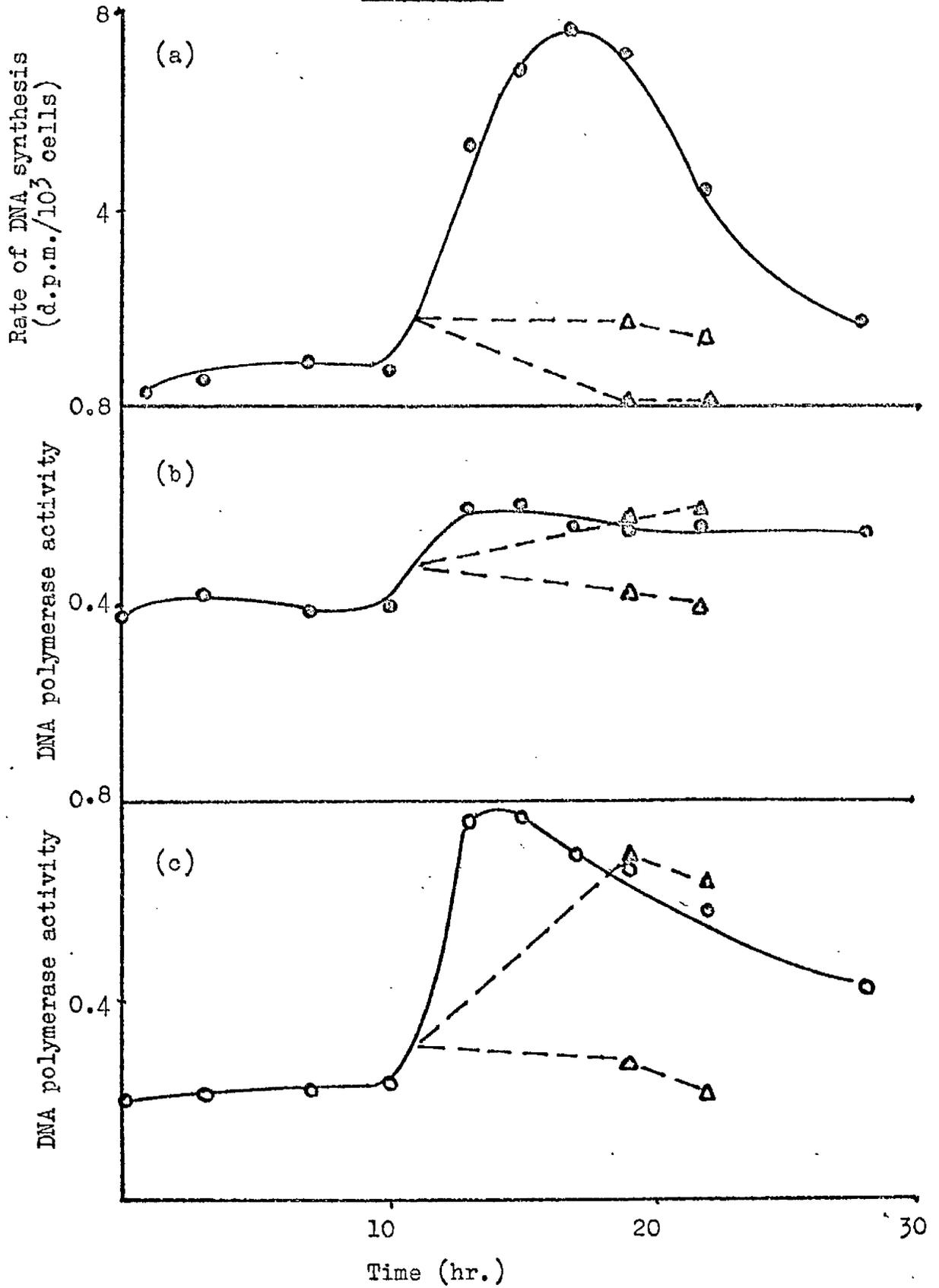


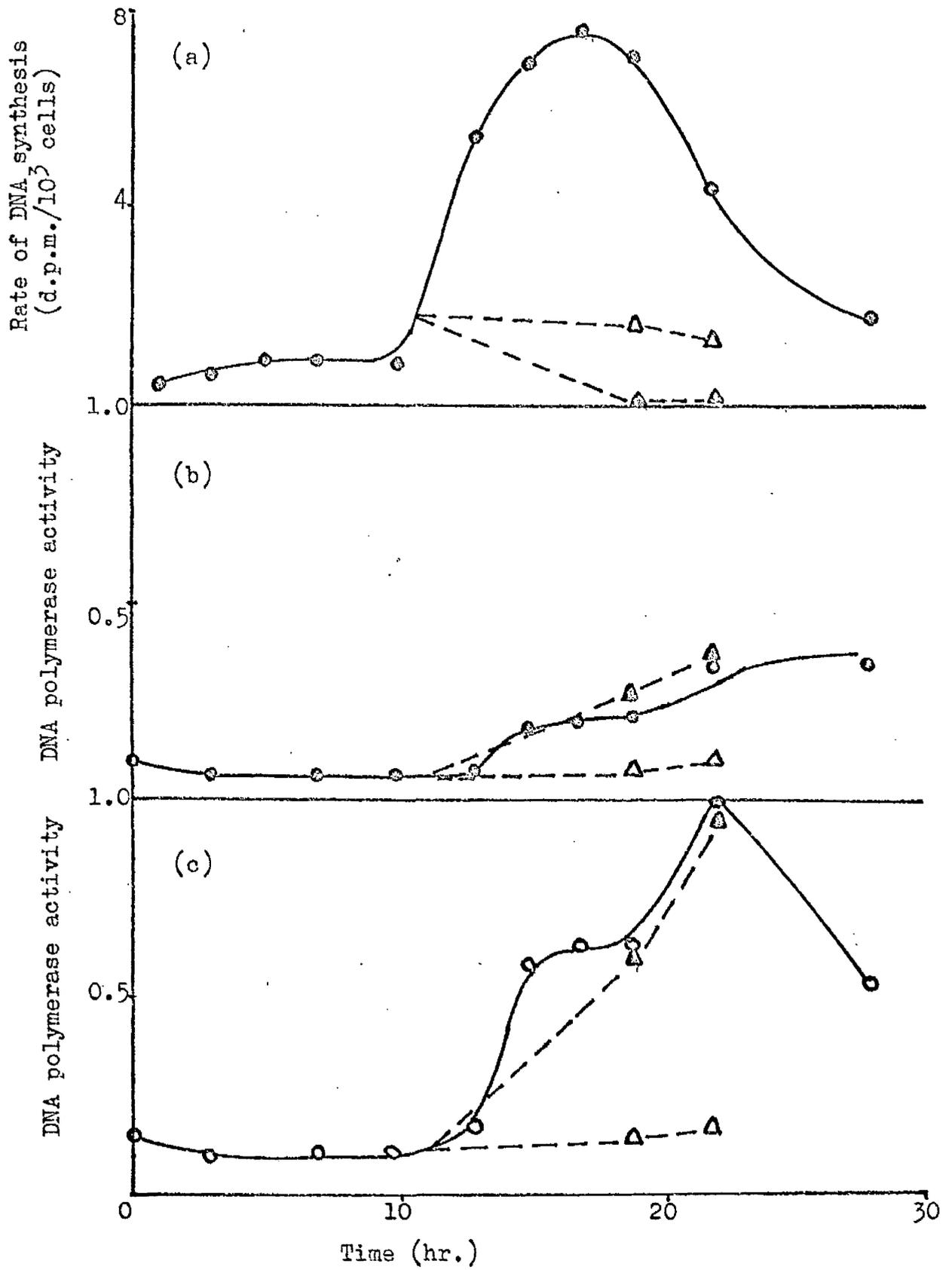
Fig. 111.9.

Variation in the DNA polymerase activity of high speed supernatant (S1) fractions after release of L 929 cells from stationary phase.

For details of method, see Fig. 111.8. In this experiment Fig. 111.8a is included for purposes of comparison.

- (a)      ○—○      incorporation of  $[6-^3\text{H}]$  thymidine into DNA  
          △---△      incorporation into DNA in actinomycin D-treated cultures  
          ▲---▲      incorporation into DNA in hydroxyurea-treated cultures
- (b)      ○—○      DNA polymerase activity of S1 fractions with native DNA primer  
          △---△      activity in actinomycin D-treated cultures  
          ▲---▲      activity in hydroxyurea-treated cultures
- (c)      ○—○      DNA polymerase activity of S1 fractions with denatured DNA primer  
          △---△      activity in actinomycin D-treated cultures  
          ▲---▲      activity in hydroxyurea-treated cultures

Fig. 111.9.



to leave S this activity sharply increases once more. By 28 hr., a 50% increase in cell number has occurred and the levels of enzymic activity have declined. As in the nuclear fraction DNA polymerase activity in supernatant preparations, primed by native DNA, increases gradually (3-4-fold) during the course of the experiment. No correlation between this activity and the rate of DNA synthesis in the culture is apparent.

The addition of actinomycin D (0.1 µg. per ml.) at 11 hr. causes an 80% reduction in the rate of DNA synthesis and also prevents the rise in DNA polymerase activity with either native or heat-denatured DNA. On the other hand, hydroxyurea (2 mM) completely inhibits DNA synthesis but does not abolish the increase in DNA polymerase activity in nuclear or supernatant preparations. This confirms the results of Adams et al. (1966) who show that DNA synthesis is not required to obtain increased levels of DNA polymerase in cultured rabbit kidney cortex cells.

Previously 2 groups (Littlefield et al., 1963; Gold and Helleiner, 1964), studying the changes in DNA polymerase activity during S phase in 5-fluorodeoxyuridine-synchronised L cells, had detected a slight drop in activity in the supernatant fraction during this period. A corresponding increase in the activity of the particulate enzyme was also noted. Only activity primed

by denatured DNA was detected in these earlier studies. Adams and Lindsay (1969); in a re-investigation of this problem, have confirmed and extended these original observations.

## 2. DNA POLYMERASE ACTIVITY OF L 929 CELLS

### 2.1. General properties

#### 2.1.1. Introduction

The discovery of substantial amounts of DNA polymerase activity in the nuclei of L 929 cells is of considerable interest and has led us to carry out a more detailed investigation of the general properties of the enzyme. The intra-nuclear location of this activity and its preference for native DNA makes it a suitable candidate for a role in the in vivo replication process, perhaps corresponding to the "intact" form of DNA polymerase proposed by Keir (1965). Previous studies on sea urchin embryos (Loeb et al., 1967) and normal rat liver (Patel et al., 1967) have also detected nuclear DNA polymerases showing a requirement for native DNA.

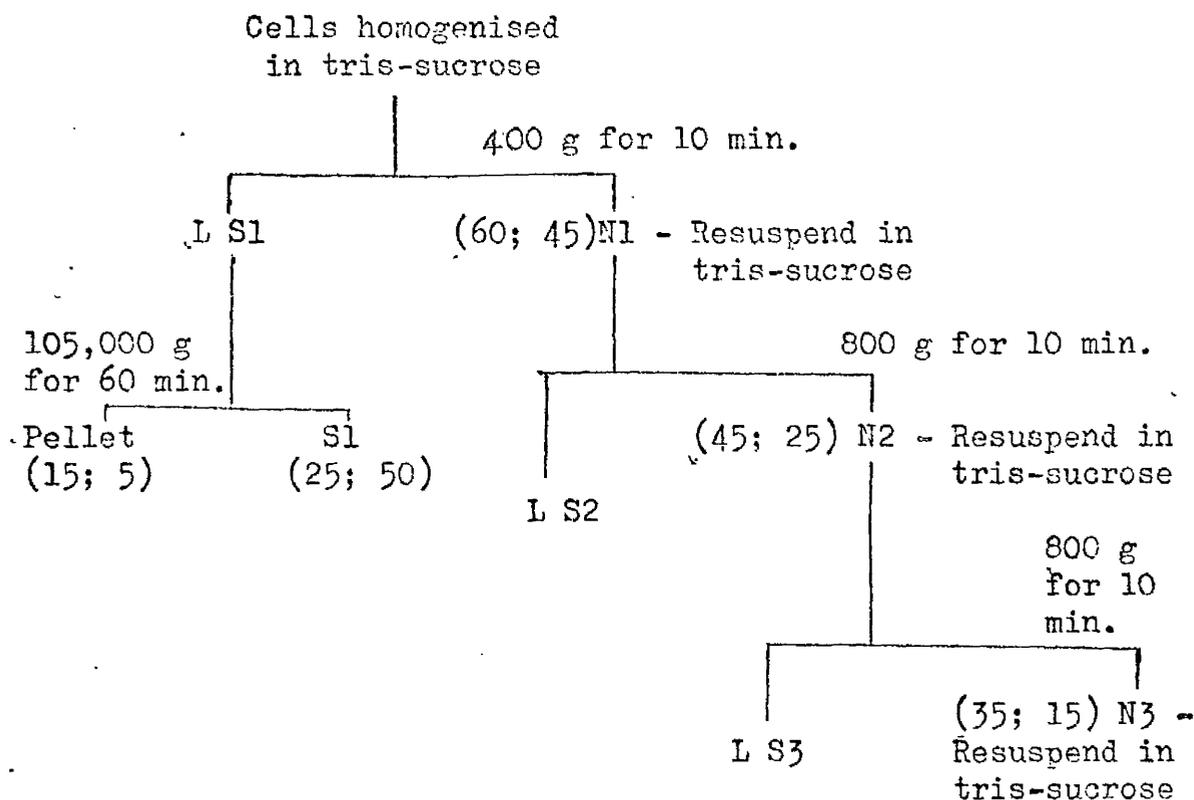
#### 2.1.2. Preparation of nuclear and supernatant fractions of L 929 cells

The isolation procedures employed and the characteristics of the DNA polymerase activity in crude extracts have been previously described (Lindsay and Adams, 1968). A fractionation diagram together with the enzymic activity associated with each

Fig. 111.10.

Preparation of nuclear and supernatant fractions of

L 929 cells from a cell homogenate



N1, N2 and N3 are successively washed nuclear fractions.

L S1, L S2 and L S3 and S1 are low and high speed supernatant fractions obtained as shown. Figures in brackets (;) represent the average activity in each fraction with native or denatured DNA expressed as % of activity found in the homogenate.

preparation is shown in Fig. 111.10. It is of interest that nuclei are able to catalyze incorporation of the deoxyribonucleoside triphosphates in the absence of added DNA although their activity is stimulated 5-10-fold by the addition of exogenous double-stranded primer.

### 2.1.3. Purity of the nuclear preparations

The integrity and purity of the nuclei are routinely checked by fluorescence microscopy after staining with acridine orange. To test for the contamination of nuclear preparations (N3) by mitochondrial material, cytochrome oxidase activity is compared in cell homogenates and N3 nuclei by the method of Cooperstein and Lazarow (1951). Final nuclear preparations have in the region of 2% of the cytochrome oxidase activity found in whole cell homogenates (Fig. 111.11), while retaining 20-60% of the total DNA polymerase activity primed by native DNA.

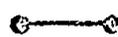
### 2.1.4. Time course of DNA polymerase activity in nuclear and supernatant preparations

Isolated nuclear (N3) and high speed supernatant (S1) fractions of L 929 cells are assayed for DNA polymerase activity using either double- or single-stranded DNA as primer. DNA

Fig. 111.11.

Contamination of nuclear preparations (N3) by mitochondrial material.

Cytochrome oxidase activity was compared in cell homogenates and N3 nuclear fractions (see Fig. 111.10) using the standard assay procedure (Methods, section 2.3.4. c). Enzymic activity is expressed as  $\Delta \log [\text{ferrocyanochrome c}]$  per min. at room temperature.

 activity in cell homogenate.

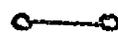
 activity in N3 nuclear fraction

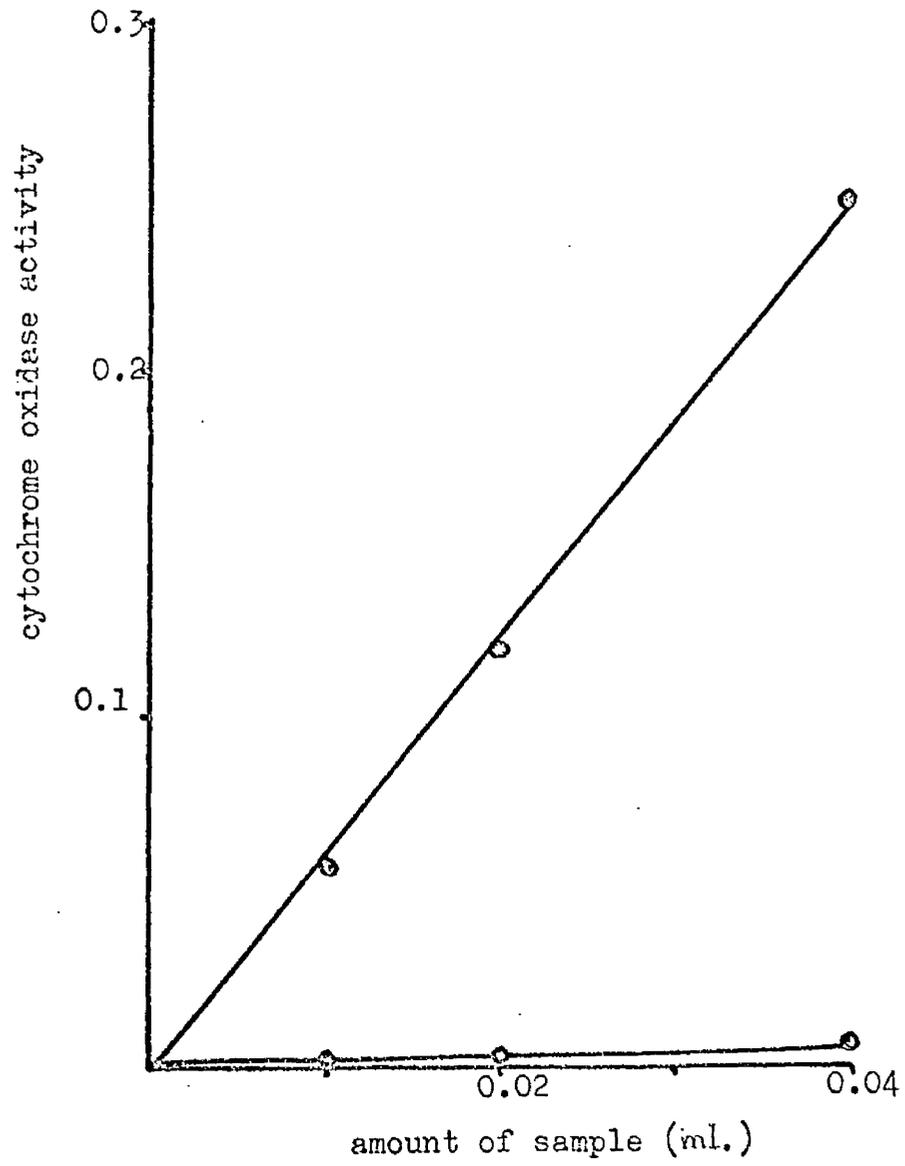
Fig. 111.11.

Fig. 111.12.

The time course of DNA polymerase activity in nuclear and supernatant preparations.

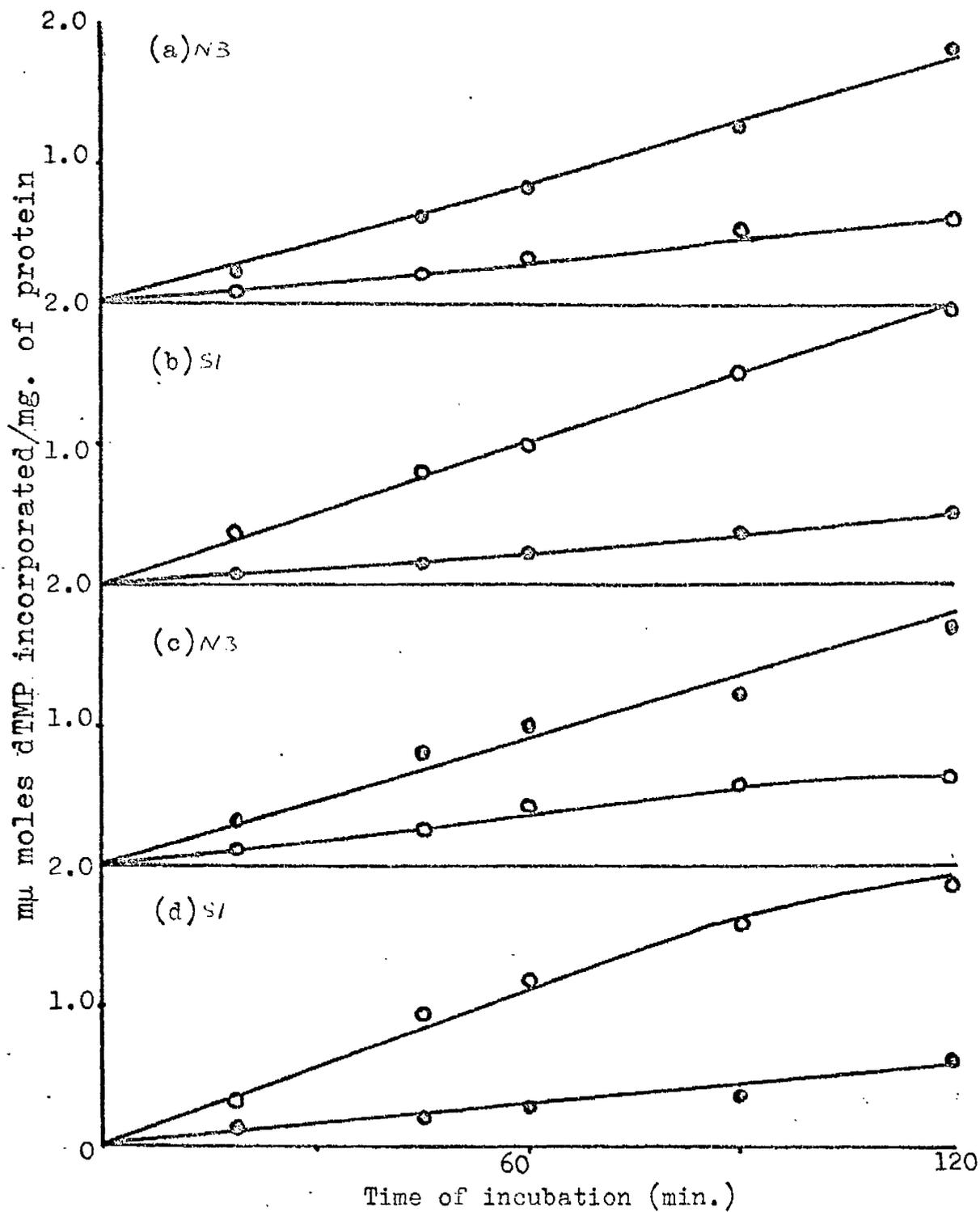
Isolated nuclear (N3) and high speed supernatant (S1) fractions (see Methods, section 2.3.1; Fig. 111.10) were assayed for activity with native and heat-denatured DNA as primer. The standard assay procedures were employed (Methods, section 2.3.4. a). Both  $[Me-^3H]$  dTTP (2.5  $\mu$ c. per assay; 0.05  $\mu$ c. per  $\mu$  mole) and  $[\alpha-^{32}P]$  dTTP ( $14.85 \times 10^6$  c.p.m. per  $\mu$  mole) were used on this occasion although only the former was employed routinely. There were 72  $\mu$ g. of nuclear and 148  $\mu$ g. of supernatant protein per assay.

- activity of N3 nuclei with native DNA primer
- activity of N3 nuclei with denatured DNA primer
- activity of S1 fractions with native DNA primer
- activity of S1 fractions with denatured DNA primer

(a) and (b)  $[Me-^3H]$  dTTP incorporation into DNA

(c) and (d)  $[\alpha-^{32}P]$  dTTP incorporation into DNA

Fig. 111.12.



synt  $[^3\text{H}]$  dTTP or  $[\alpha\text{-}^{32}\text{P}]$  dTTP into acid-insoluble material (Fig. 111.12). The nuclei are more active when the primer is native DNA while high speed supernatant extracts prefer single-stranded DNA. In general, the DNA polymerase activity of nuclei is 2-5-fold higher when the primer is native DNA while the supernatant shows an equivalent preference for denatured primer. In all cases, using crude preparations, the reaction is linear with time for at least 60 min. at  $37^\circ$ .

2.1.5. The DNA polymerase activity of nuclear and supernatant preparations of L 929 cells as a function of protein concentration

Enzymic activity is found to be directly proportional to protein concentration up to at least 130  $\mu\text{g}$ . nuclear protein and 160  $\mu\text{g}$ . supernatant protein in a total reaction vol. of 0.25 ml. (Fig. 111.13). Normally experiments are performed using between 80 and 150  $\mu\text{g}$ . protein per assay. On occasions, when larger amounts of protein are used, the linearity is tested by assaying at several protein concentrations.

2.1.6. The effect of pH on the activity of DNA polymerase in nuclear and supernatant extracts of L 929 cells

Nuclear and supernatant fractions respond similarly to

Fig. 111.13.

Variation in DNA polymerase activity of nuclear and super-  
natant fractions of L 929 cells with protein concentration.

The standard assay was employed and various amounts of N3 or S1 fractions added as indicated opposite (see Fig. 111.12).

- (a)      ●—●      activity of N3 nuclei with native DNA primer  
          ●—●      activity with denatured DNA primer
- (b)      ●—●      activity of S1 fractions with native DNA  
                                 primer  
          ●—●      activity with denatured DNA primer

Fig. 111.13.

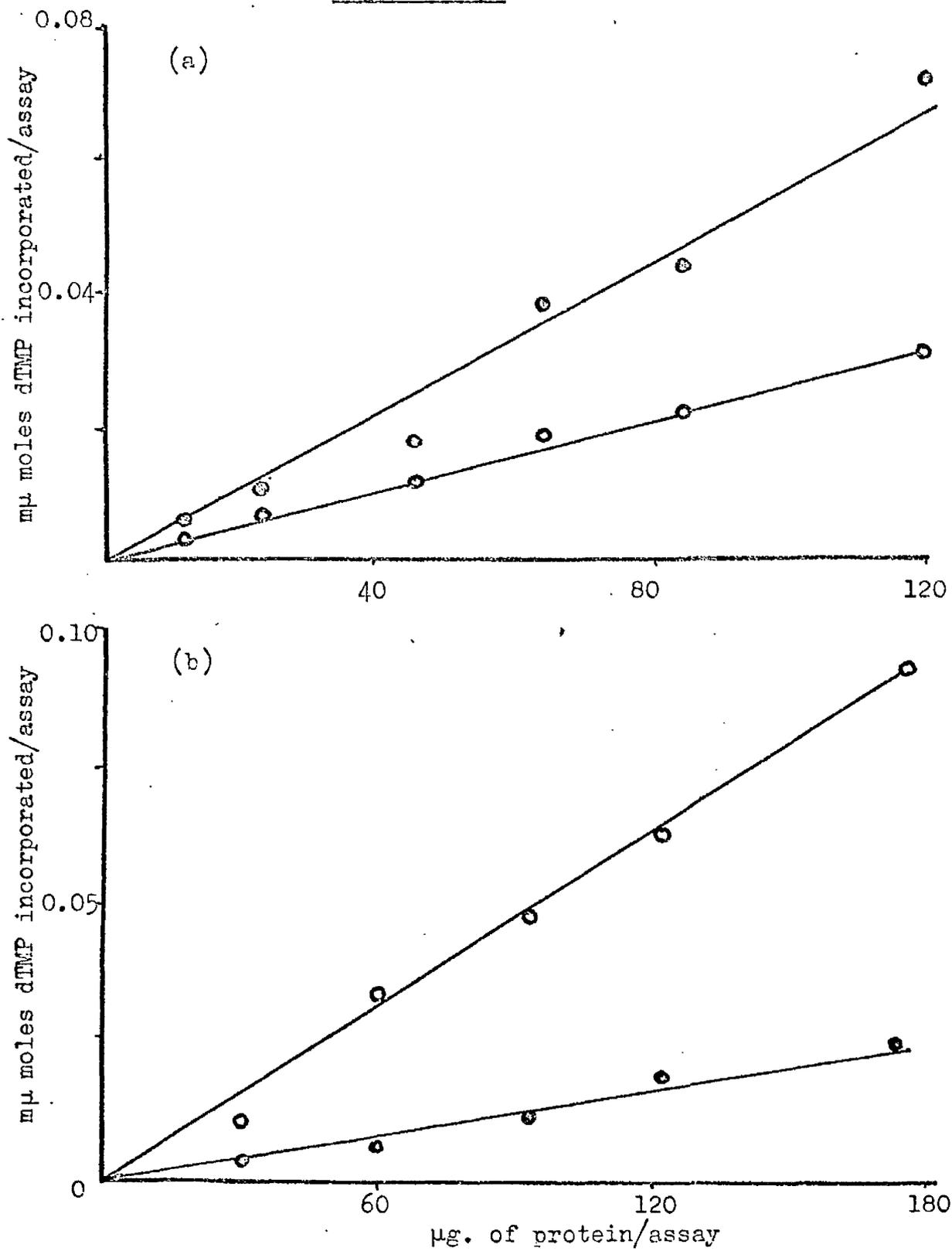


Fig. 111.14.

The effect of pH on the activity of DNA polymerase in nuclear and supernatant fractions of L 929 cells.

N3 and S1 fractions were isolated in the usual manner except that 0.02 M-tris-HCl, pH 7.5 was omitted. The standard assay was employed (see Fig. 111.12) and 5  $\mu$ moles of tris buffer at various pH's added per assay as shown opposite. There were 84  $\mu$ g. of nuclear and 132  $\mu$ g. of supernatant protein present per assay.

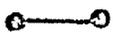
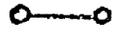
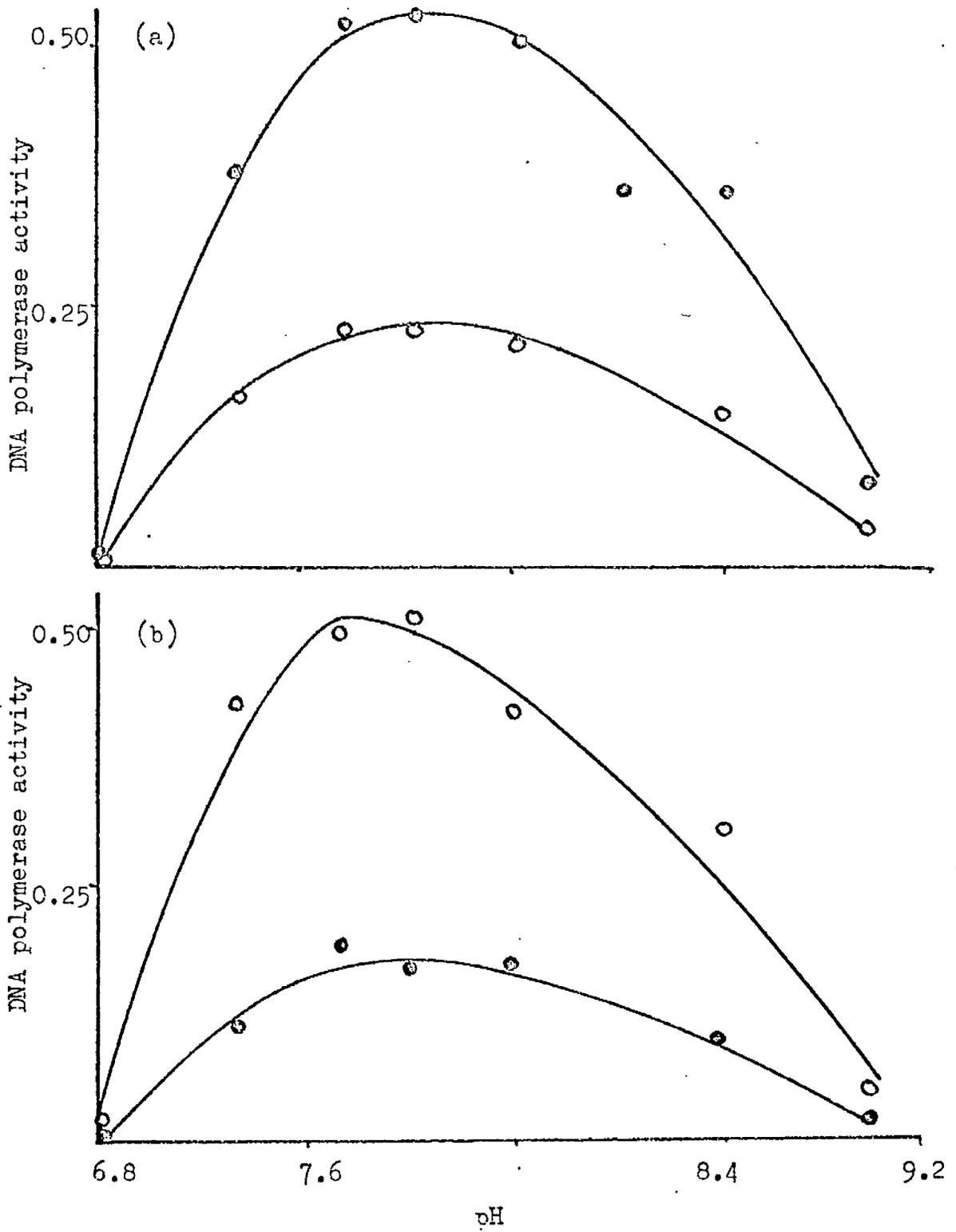
- (a)       activity of N3 nuclei with native DNA primer  
          activity with denatured DNA primer
- (b)       activity of S1 fractions with native DNA primer  
          activity with denatured DNA primer

Fig. 111.14.



changes in the pH of the assay mixture. Both preparations are active in the pH range 7.0 - 9.0 and exhibit identical optima at pH 7.5 - 8.0. Changes in pH do not alter the relative ability of either fraction to utilise native or heat-denatured primer (Fig. 111.14). Birnie and Fox (1966), in a study of DNA polymerase in crude nuclear and supernatant extracts of primary mouse embryo cells, find significant differences between the 2 preparations. Both preparations are more active with  $Mn^{2+}$  than with  $Mg^{2+}$ . However, with  $Mg^{2+}$ , nuclear extracts are more active at pH 7.5 than 6.5 while the reverse is true for the supernatant extract.

#### 2.1.7. The effect of $Mg^{2+}$

Magnesium ions are essential for DNA polymerase activity. The optimal concentration with either native or heat-denatured primer is 6-8 mM- $Mg^{2+}$  (Fig. 111.15). This value is identical to that obtained for L cell DNA polymerase by Gold and Helleiner (1964) who, however, only detected activity with denatured DNA. No second peak of activity at higher  $Mg^{2+}$  concentrations corresponding to the "intact" form of the enzyme suggested by Keir (1965) could be observed. In this connection, it is of interest that several DNA polymerases, found to prefer native DNA, have rather higher  $Mg^{2+}$  optima than those reported here e.g. rat liver

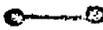
Fig. 111.15.

Comparison of the effect of  $Mg^{2+}$  on the DNA polymerase activity of nuclear and supernatant fractions of L 929 cells.

N3 and S1 fractions were assayed for activity under standard conditions (Fig. 111.12). The  $Mg^{2+}$  concentration was varied as indicated opposite. There were 124  $\mu$ g. of nuclear and 154  $\mu$ g. of supernatant protein per assay.

(a)  activity of N3 nuclei with native DNA primer

 activity with denatured DNA primer

(b)  activity of S1 fractions with native DNA primer

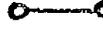
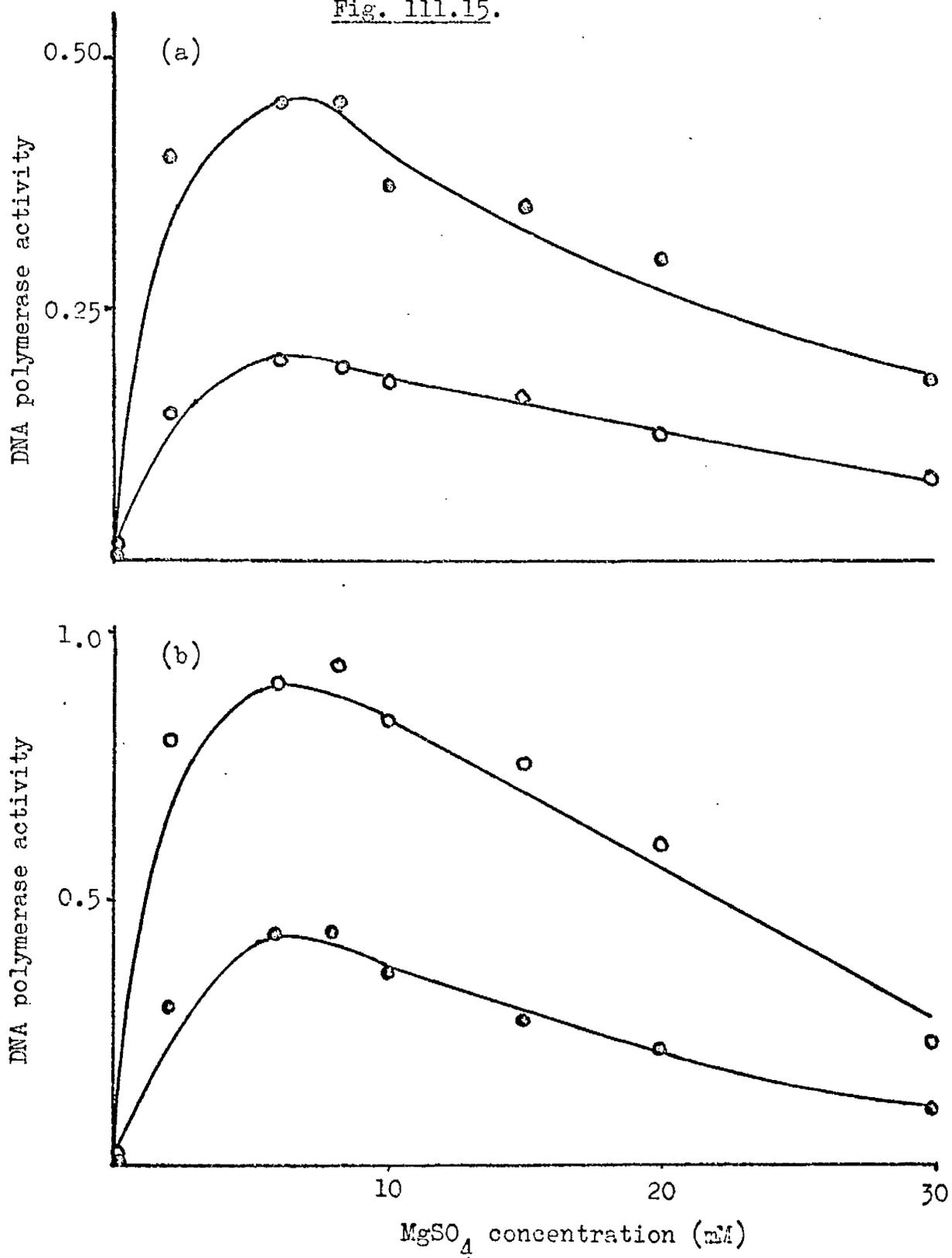
 activity with denatured DNA primer

Fig. 111.15.



nuclear enzyme (10 mM) and sea urchin nuclear enzyme (16 mM).

#### 2.1.8. The effect of $K^+$

Potassium ions are found to have a stimulatory effect on L cell DNA polymerase activity. Both fractions are increased approximately 2-fold in activity by 60 mM- $K^+$  (Fig. 111.16). Similar results have been obtained for the calf thymus enzyme (Keir, 1965) and the Landschütz ascites tumour enzyme (Keir and Shepherd, 1965). Recently Kalf and Ch'ih (1969) have shown that the nuclear enzyme from rat liver is stimulated 2-fold by 60 mM- $K^+$  while the mitochondrial enzyme, which also utilises native DNA is increased 7-fold under equivalent conditions.

#### 2.1.9. Homopolymer formation

To eliminate the possibility that the acid-insoluble material forming under DNA polymerase assay conditions may be largely homopolymer, the distribution of  $[\alpha\text{-}^{32}\text{P}]$  dTTP in the DNA product is investigated using the partial nearest neighbour frequency analysis technique of Josse and Swartz (1963). It is found that  $[\alpha\text{-}^{32}\text{P}]$  dTTP is incorporated extensively next to all 4 bases in the DNA product (Table 111.17), indicating that, under these conditions, little if any, homopolymer synthesis is occurring.

Fig. 111.16.

Comparison of the effect of  $K^+$  on the DNA polymerase activity of nuclear and supernatant fractions of L 929 cells.

N3 and S1 fractions were assayed under standard conditions (see Fig. 111.12). The  $K^+$  concentration was varied as indicated opposite. There were 70  $\mu$ g. of nuclear and 158  $\mu$ g. of supernatant protein per assay.

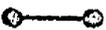
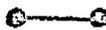
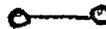
- (a)       activity of N3 nuclei with native DNA primer
-  activity with denatured DNA primer
- (b)       activity of S1 fractions with native DNA primer
-  activity with denatured DNA primer

Fig. 111.16.

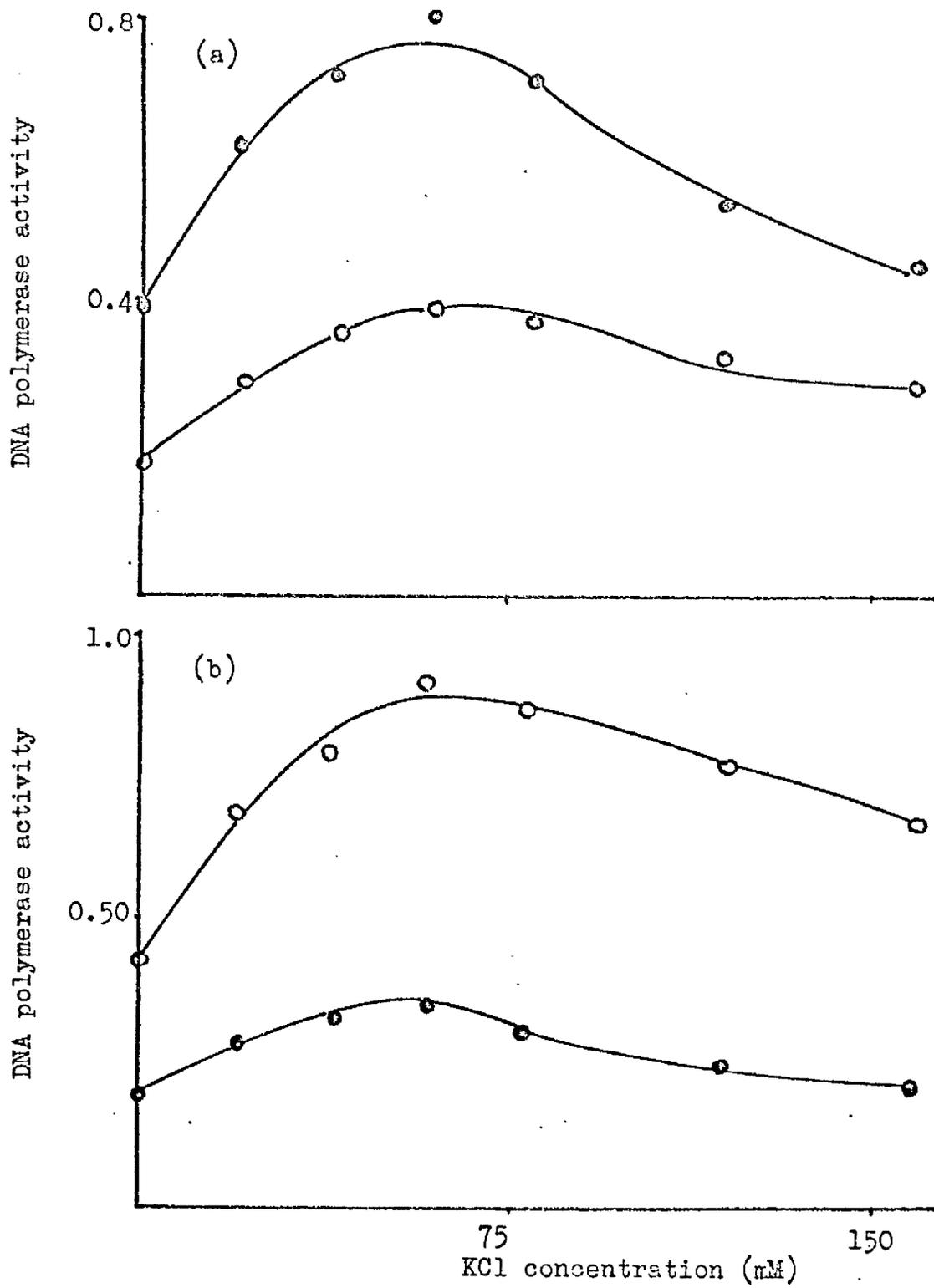


TABLE 111.17.

The distribution of [<sup>32</sup>P] dTMP in the DNA product

<u>Deoxyribonucleoside</u> <u>3'-phosphate</u>	<u>Nuclear fraction</u>		<u>Supernatant fraction</u>	
	<u>Native</u> <u>DNA</u>	<u>Denatured</u> <u>DNA</u>	<u>Native</u> <u>DNA</u>	<u>Denatured</u> <u>DNA</u>
dGp	26.0	18.5	21.3	20.0
dAp	29.2	18.5	23.7	24.5
dGp	28.6	28.9	28.0	27.7
dTTP	16.4	34.1	27.0	27.8

[<sup>32</sup>P] DNA product was synthesised under standard assay conditions using N3 nuclear and S1 supernatant fractions. The DNA was subsequently degraded to deoxyribonucleoside 3' - monophosphates and analysed as described by Josse and Swartz (1965). See Chapter II, section 2.3.6. f. for further details. Results are expressed as % of <sup>32</sup>P counts present in each deoxyribonucleoside 3' -phosphate.

### 2.1.10 End terminal addition activity

In common with other nuclear preparations (Patel et al., 1967; Smith and Keir, 1963; Keir and Smith, 1963), nuclear DNA polymerase, primed by native DNA, exhibits a high degree of activity in the absence of one or more deoxyribonucleoside triphosphates. Using native DNA as primer, incorporation of [ $^3\text{H}$ ] dTTP in the presence of dTTP alone is 40% of that obtained in the complete assay while with dTTP, dATP and dGTP present incorporation is 80% of the control values. The supernatant fraction, primed by denatured DNA, has 15% of the control activity with dTTP present, rising to 39% when only dCTP is omitted from the assay (Fig. 111.18).

The extent of end terminal addition activity is markedly reduced in partially purified DNA polymerase preparations (Sephadex fraction, see section 2.5.1). In addition, little or no incorporation of [ $^3\text{H}$ ] dCTP is detected using poly d(AT) as primer but with [ $^3\text{H}$ ] dTTP as precursor, poly d(AT) is found to be a more efficient primer than native salmon testes DNA (Fig. 111.18). Poly dG : dC is a relatively poor primer for the enzyme. Such results indicate that, under complete assay conditions, incorporation into the DNA product is governed by the nucleotide sequence of the DNA primer i.e. replicative synthesis is occurring.

Fig. 111.18. (a)

The presence of end terminal addition activity in crude and Sephadex-purified (see Table 111.28) nuclear and supernatant fractions of L 929 cells.

Samples were incubated with their preferred DNA primer in complete assay mixture or in the absence of one or more deoxyribonucleoside triphosphates as indicated opposite; otherwise routine assay conditions were employed (Fig. 111.12).

- (i) nuclear fractions with native DNA primer
- (ii) supernatant fractions with denatured DNA primer



crude enzyme fractions



Sephadex-purified fraction

1. DNA polymerase activity in complete assay
2. activity with dGTP, dATP and dTTP
3. activity with dTTP alone

Results are expressed as % of DNA polymerase activity under complete assay conditions.

Fig. 111.18(a)

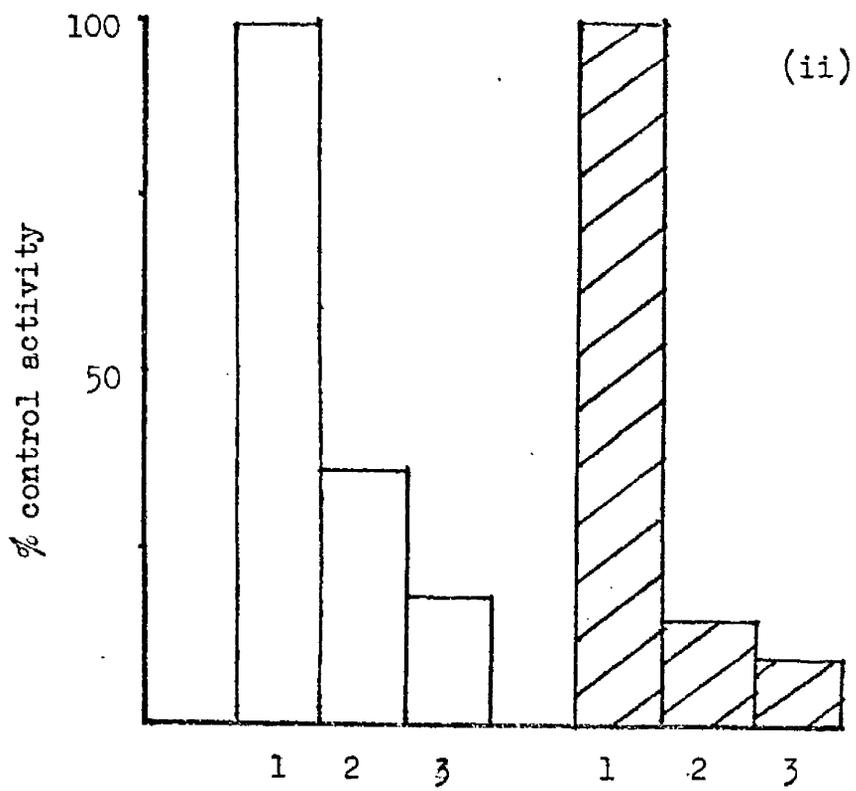
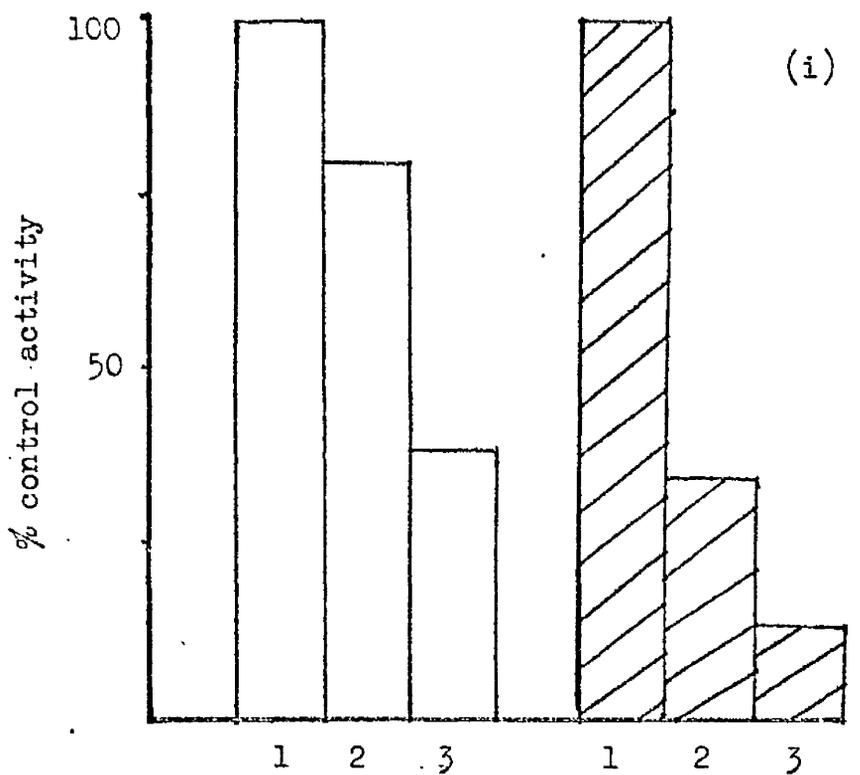


Fig. 111.18. (b)

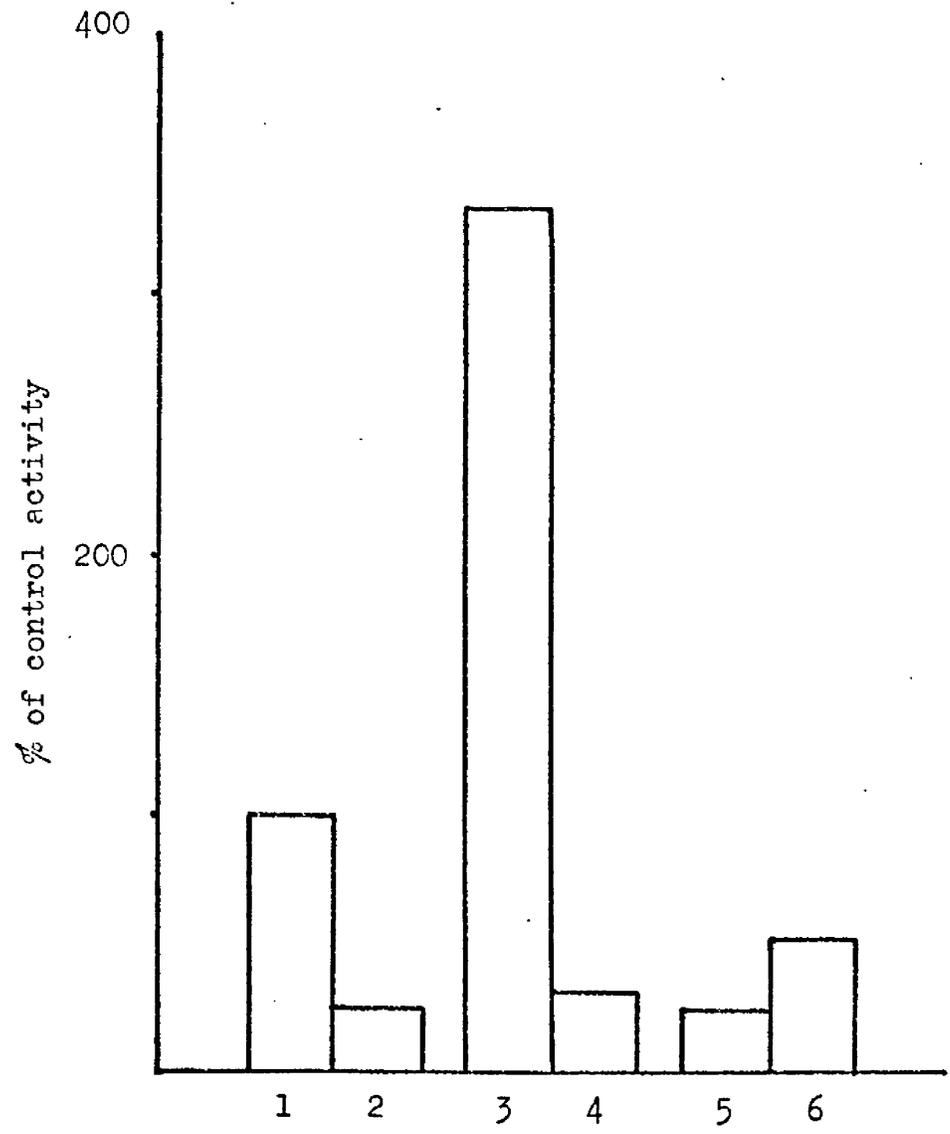
The priming efficiencies of poly d(AT) and poly dG: dC compared to native salmon testes DNA.

A Sephadex-purified nuclear fraction (see Table 111.28) was tested for its ability to utilise various primers in DNA polymerase assays. Assays were performed under standard conditions except that only 1  $\mu$ g. of DNA primer was added. DNA polymerase activity was measured by the incorporation of  $[Me-^3H]$  dTTP or  $[5-^3H]$  dCTP (both 2.5  $\mu$ c. per assay; 0.05  $\mu$ c. per  $\mu$  mole) into acid-insoluble material as described in Fig. 111.12. There were 120  $\mu$ g. of protein per assay.

1. DNA polymerase activity on salmon testes DNA with dTTP as precursor
2. no DNA primer added
3. activity on poly d(AT) with  $[Me-^3H]$  dTTP as precursor
4. activity with  $[5-^3H]$  dCTP as precursor
5. activity on poly dG : dC with  $[Me-^3H]$  dTTP as precursor
6. activity with  $[5-^3H]$  dCTP as precursor

Results are expressed as % of DNA polymerase activity obtained in 1.

Fig. 111.18(b)



2.1.11 The effect of DNA concentration and sonication of DNA on the activity of DNA polymerase in nuclear and supernatant extracts of L 929 cells

The effect of increasing DNA concentration on the activity of the crude enzyme fractions is tested using native and heat-denatured DNA. In each case saturation occurs at around 50 µg. DNA per assay (Fig. 111.19). Sonication of the DNA decreases the maximum velocity of the reaction with both native and denatured primers.

Degradation of DNA molecules by sonic waves occurs by double-strand scissions leaving the double-helical structure intact (Doty, McGill and Rice, 1958). Freifelder and Davison (1962) have shown that this is a non-random process and that sonication proceeds by means of shearing which results preferentially in successive halving of the polymer. The lowered maximum velocity on sonicated DNA may be due to the production of 3'-phosphate termini during degradation, which do not support DNA polymerase activity (section 2.3).

2.1.12 The effect of having double- and single-stranded primer in the same assay

Fig. 111.20 shows the effect of adding increasing amounts of native and heat-denatured DNA to nuclear and high speed super-

Fig. 111.19.

The effect of DNA concentration and sonication of DNA on the activity of DNA polymerase.

N3 and S1 fractions were incubated under standard conditions (Fig. 111.12) with varying amounts of native or heat-denatured primer. Sheared DNA was prepared by sonicating 5 ml. of salmon testes DNA (2 mg. per ml.) for 30 sec. at full power using a Dawe soniprobe. There were 74  $\mu$ g. of nuclear and 110  $\mu$ g. of supernatant protein per assay.

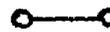
- (a)       DNA polymerase activity in N3 nuclei (native DNA primer)
-  activity in N3 nuclei (sonicated, native DNA primer)
- (b)       DNA polymerase activity in S1 fractions (denatured DNA primer)
-  activity in S1 fractions (sonicated, denatured DNA primer)

Fig. 111.19.

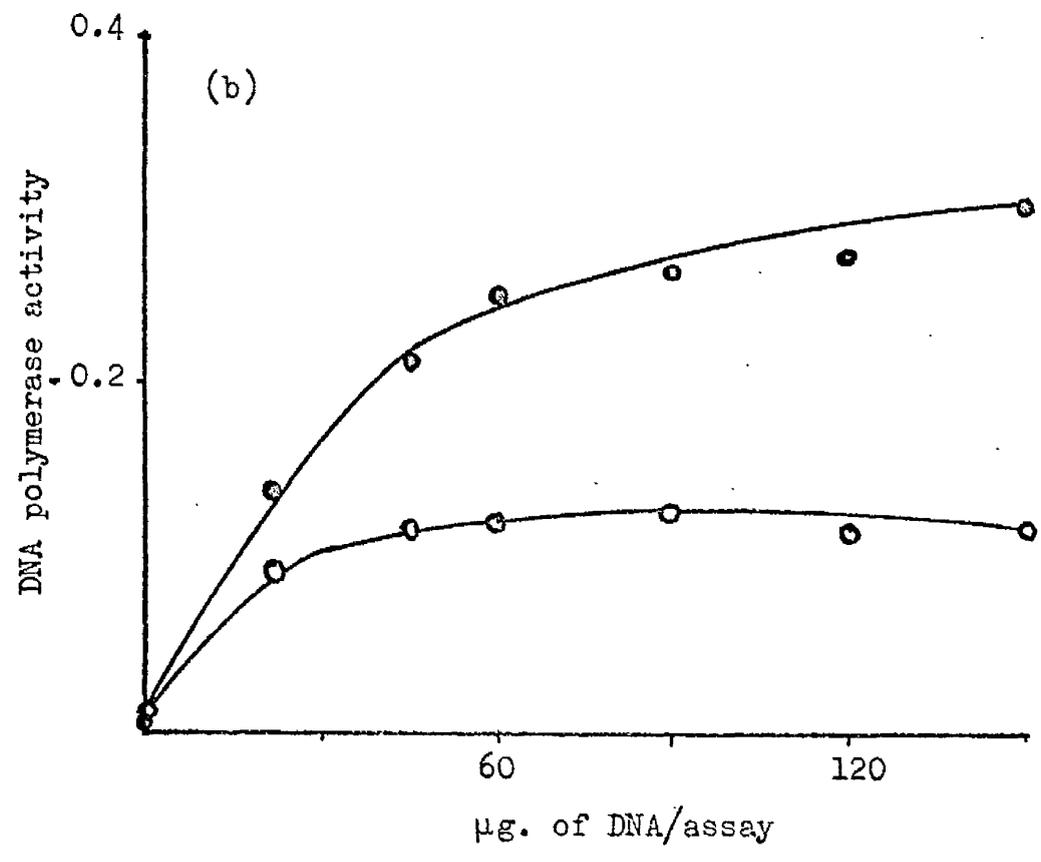
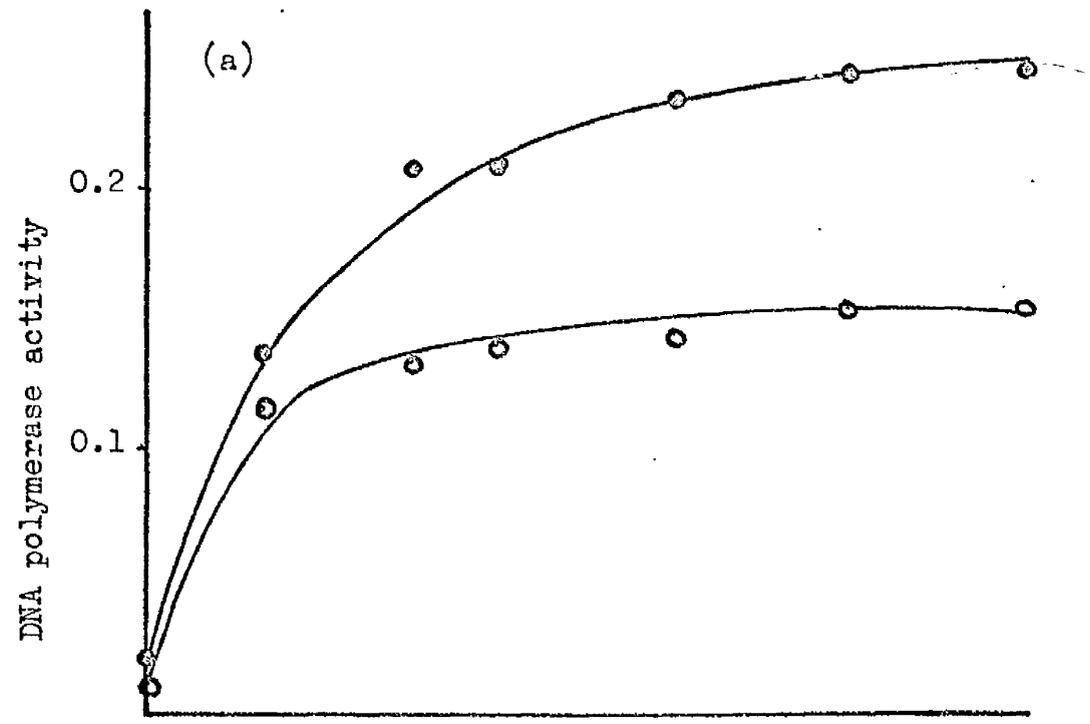


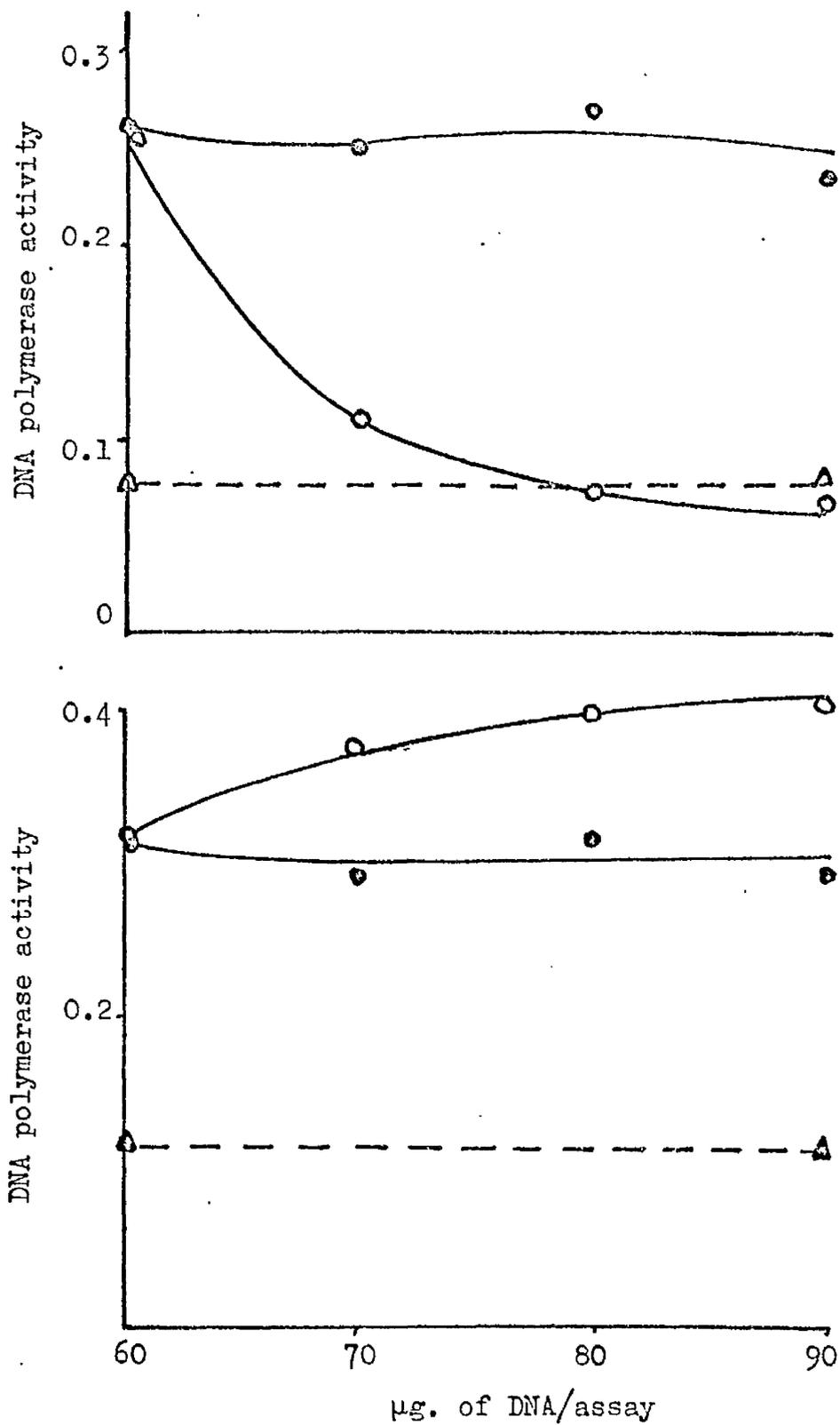
Fig. 111.20.

The effect of having double- and single-stranded DNA primer  
in the same assay.

N3 and S1 fractions were incubated with fixed amounts of their preferred primer (60  $\mu\text{g.}$  per assay) in the presence of increasing amounts of heat-denatured and native DNA respectively. Routine assay procedures were otherwise employed (Fig. 111.12). There were 128  $\mu\text{g.}$  of nuclear and 178  $\mu\text{g.}$  of supernatant protein per assay.

- (a)      ●—●      DNA polymerase activity of N3 nuclei (native DNA primer)
- activity of N3 nuclei (native + denatured DNA primer)
- ▲---▲      activity of N3 nuclei (denatured DNA primer)
- (b)      ●—●      DNA polymerase activity of S1 fractions (denatured DNA primer)
- activity of S1 fractions (native + denatured DNA primer)
- ▲---▲      activity of S1 fractions (native DNA primer)

Fig. 111.20.



natant fractions respectively in the presence of saturating amounts of preferred DNA primer. Addition of small amounts of denatured DNA to nuclear preparations markedly inhibits their activity on native primer. In contrast, the addition of native DNA to supernatant fractions does not alter their activity with denatured DNA.

Multiple binding of Escherichia coli DNA polymerase to single-stranded DNA occurs while, on linear DNA duplexes, binding is limited to nicks and the ends of DNA chains (Englund et al., 1968). A similar situation may exist with enzymic preparations from L 929 cells although the possibility that there is more than one enzymic species which bind preferentially to denatured DNA cannot be excluded.

2.1.13. Stability of DNA polymerase activity in L 929 cells

The storage characteristics of the enzyme in crude extracts as well as those of partially purified preparations are investigated (Fig. 111.21). Crude extracts loose 80-100% of their activity with native or denatured DNA after 8 days storage at 0-3°. In contrast to the Landschültz ascites tumour DNA polymerase (Keir and Shepherd, 1965) addition of EDTA or 2-mercapto-ethanol does not help to protect the enzyme but denaturation is markedly inhibited by the presence of DNA (Adams and

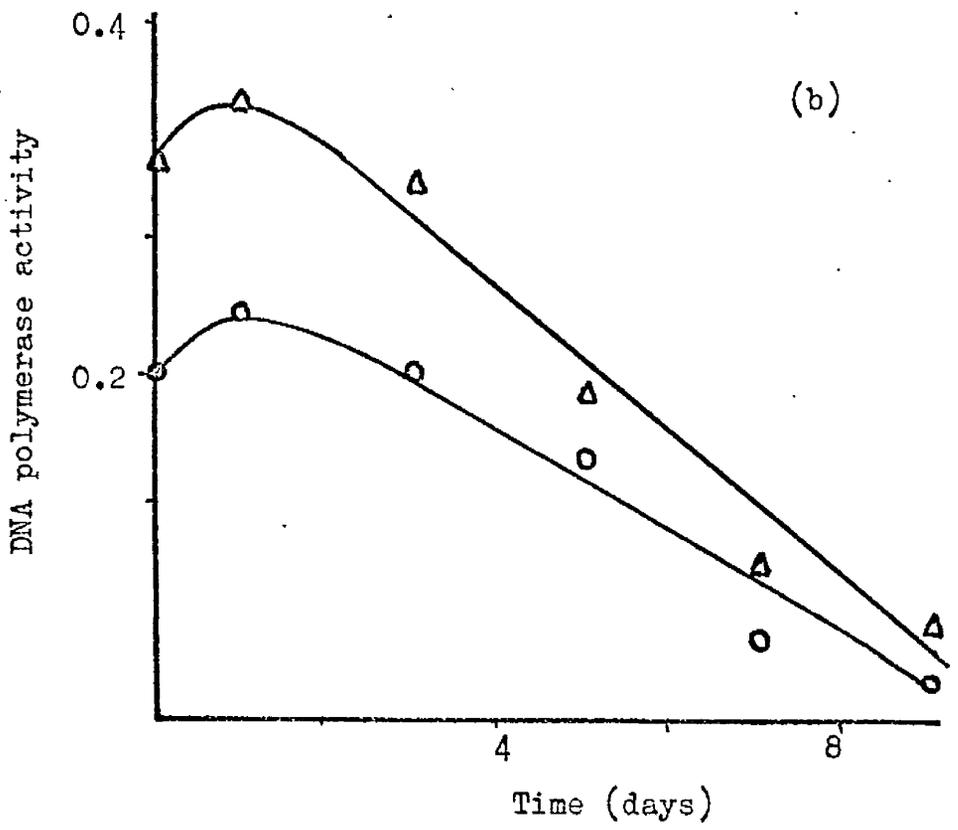
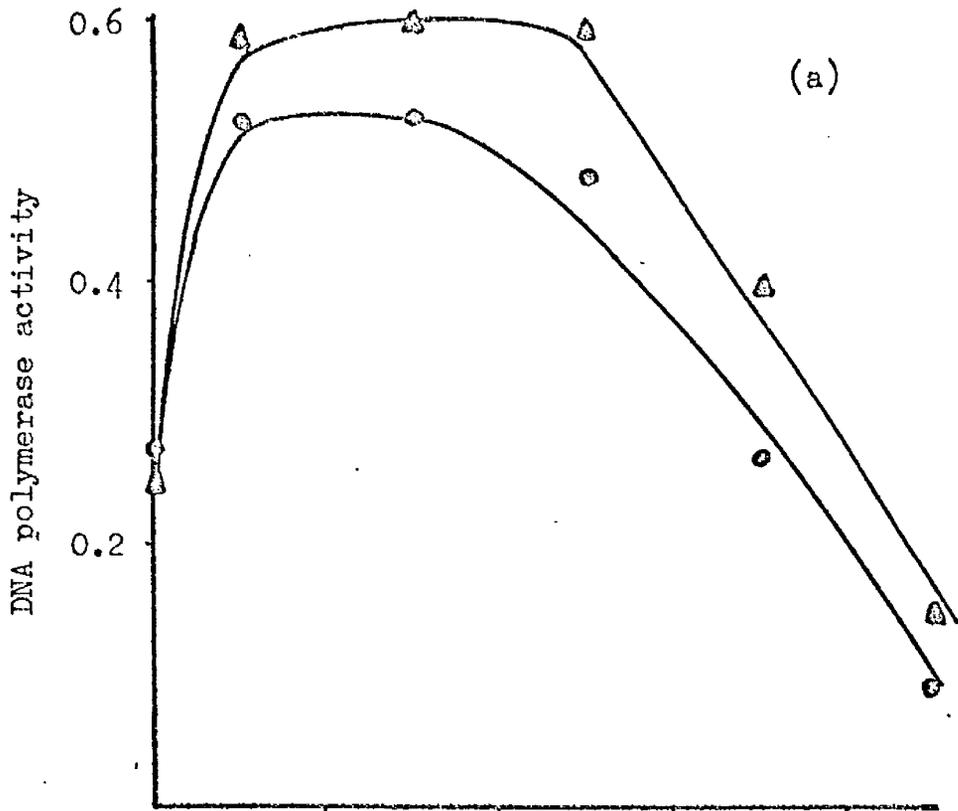
Fig. 111.21.

Stability of DNA polymerase in crude extracts of L 929 cells.

N3 and S1 fractions were isolated by the normal procedures in buffered sucrose or in 5 component buffer. Samples were stored at 0-3° and their activity with native or heat-denatured DNA as primer measured at various times thereafter under routine assay conditions. There were 84 and 92 μg. of nuclear and 125 and 140 μg. of supernatant protein respectively present per assay.

- (a)      ●—●      activity of nuclear DNA polymerase stored in buffered sucrose (native DNA primer)
- ▲—▲      activity stored in 5-component buffer.
- (b)      ●—●      activity of S1 fraction stored in buffered sucrose (denatured DNA primer)
- ▲—▲      activity stored in 5-component buffer.

Fig. 111.21.



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Lindsay, unpublished results). An initial stimulation of activity is also observed during storage at 0-3°. The cause of this activation has not been investigated.

Crude or partially purified preparations can be stored for a minimum of 6-9 months at -70° without loss of activity although freeze/thawing may cause some inactivation (section 2.5.1). Storage in 40% glycerol containing 0.02 M-tris-HCl, pH 7.5 and 5 mM-2-mercapto-ethanol has also proved satisfactory. No significant loss of activity occurs after 2-3 months at -20°.

## 2.2. DNase activity in nuclear and supernatant fractions of L 929 cells

The primer specificity of DNA polymerases is known to be influenced to some extent by contaminating nucleases. Thus Shepherd and Keir (1965) observe that the ability of the Land-schütz ascites tumour enzyme to accept native DNA decreases during the purification procedure. In contrast the crude DNA polymerase from rat liver mitochondria exhibits a 5-fold preference for denatured DNA in crude extracts but is equally able to accept native primer after partial purification (Meyer and Simpson, 1968).

Measurement of the nuclease activity in nuclear and supernatant extracts is followed by the hydrolysis of  $[^{32}\text{P}]$  DNA

Fig. 111.22.

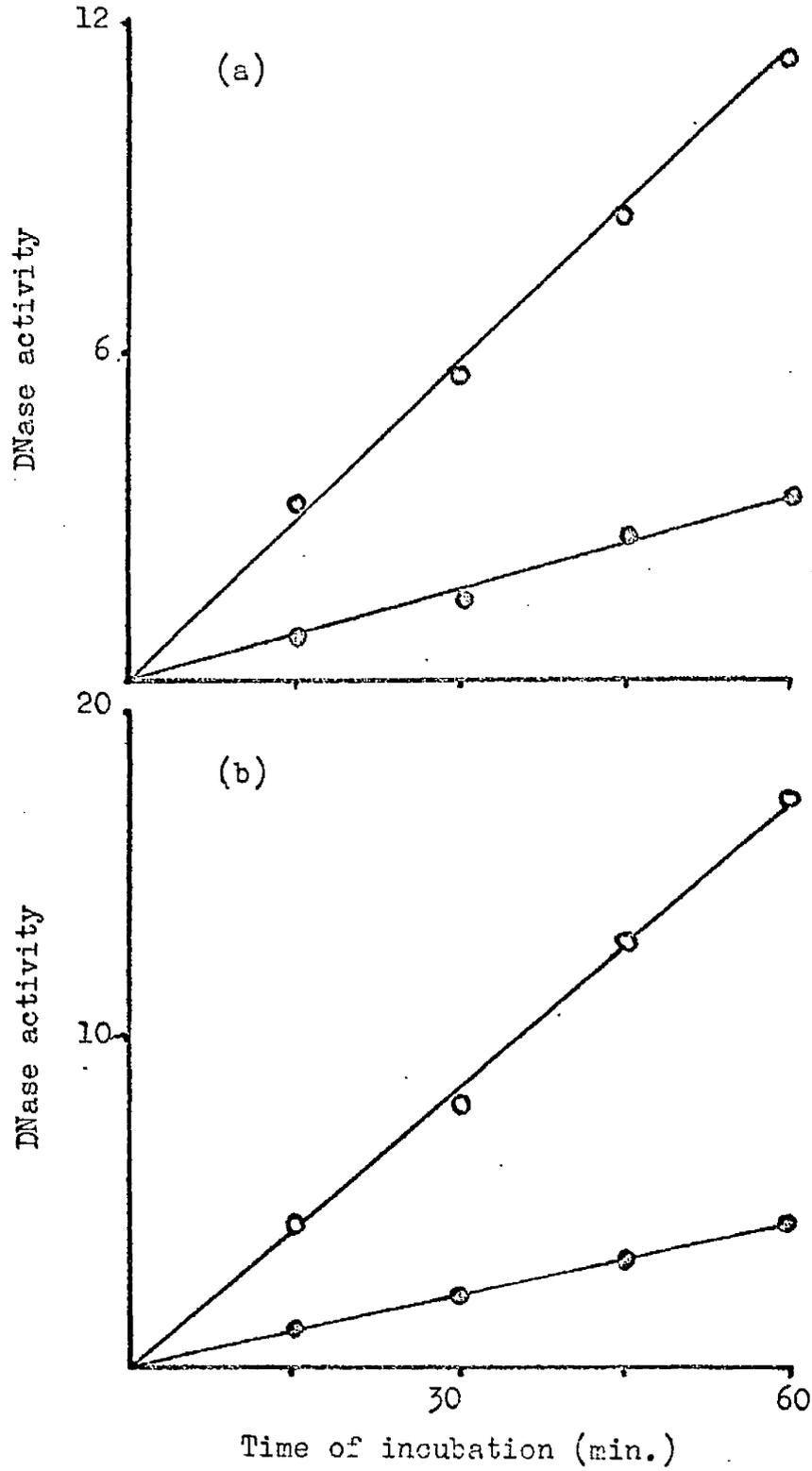
DNase activity of nuclear and supernatant fractions of L 929 cells.

N3 and S1 fractions were incubated with 10  $\mu$ g. of native or denatured Esch. coli  $^{32}\text{P}$  DNA (950 c.p.m. per  $\mu$ g.) under standard assay conditions (see Methods, section 2.3.4. b). There were 105  $\mu$ g. of nuclear and 152  $\mu$ g. of supernatant protein per assay. Activity is expressed as  $\mu$ g. of  $^{32}\text{P}$  DNA rendered acid-soluble per mg. of protein at 37 $^{\circ}$ .

●—● DNase activity on native DNA  
○—○ activity on heat-denatured DNA

- (a) N3 nuclear fraction  
(b) S1 supernatant fraction

Fig. 111.22.



(native or denatured). In both fractions activity on denatured DNA is 3-4-fold greater than on native primer (Fig. 111.22). Partial purification of these extracts (7-8-fold), removes 95% of this nuclease activity, without affecting the primer specificities of the two preparations. Moreover, additive results are obtained on combination of crude nuclear and supernatant extracts, indicating the absence of any factor which could affect the relative priming abilities of the 2 enzymes.

2.3. The effect of modifying the DNA primer by DNase I treatment

To examine the ability of a characterised endonuclease to alter the capacity of L 929 cell DNA polymerase to utilise native or denatured DNA, these primers are treated with minute amounts of DNase I. Such treatment greatly improves the efficiency of native DNA as primer for L 929 nuclear extracts using limiting concentrations of DNA. Similar treatment of heat-denatured DNA, however, does not significantly enhance its priming efficiency for high speed supernatant fractions (Fig. 111.23). This result probably reflects the ability of pancreatic DNase to degrade native DNA more rapidly than denatured DNA. Modification of either primer with micrococcal nuclease,

Fig. 111.23.

The effect of modifying the DNA primer with pancreatic DNase.

Native and heat-denatured DNA was pretreated with pancreatic DNase (Methods, section 2.3.3. c) and used as primer in DNA polymerase assays. Only 5  $\mu$ g. of DNA were added per assay; otherwise routine assay conditions were employed (Fig. 111.12). There were 96  $\mu$ g. of nuclear and 106  $\mu$ g. of supernatant protein per assay.

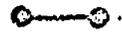
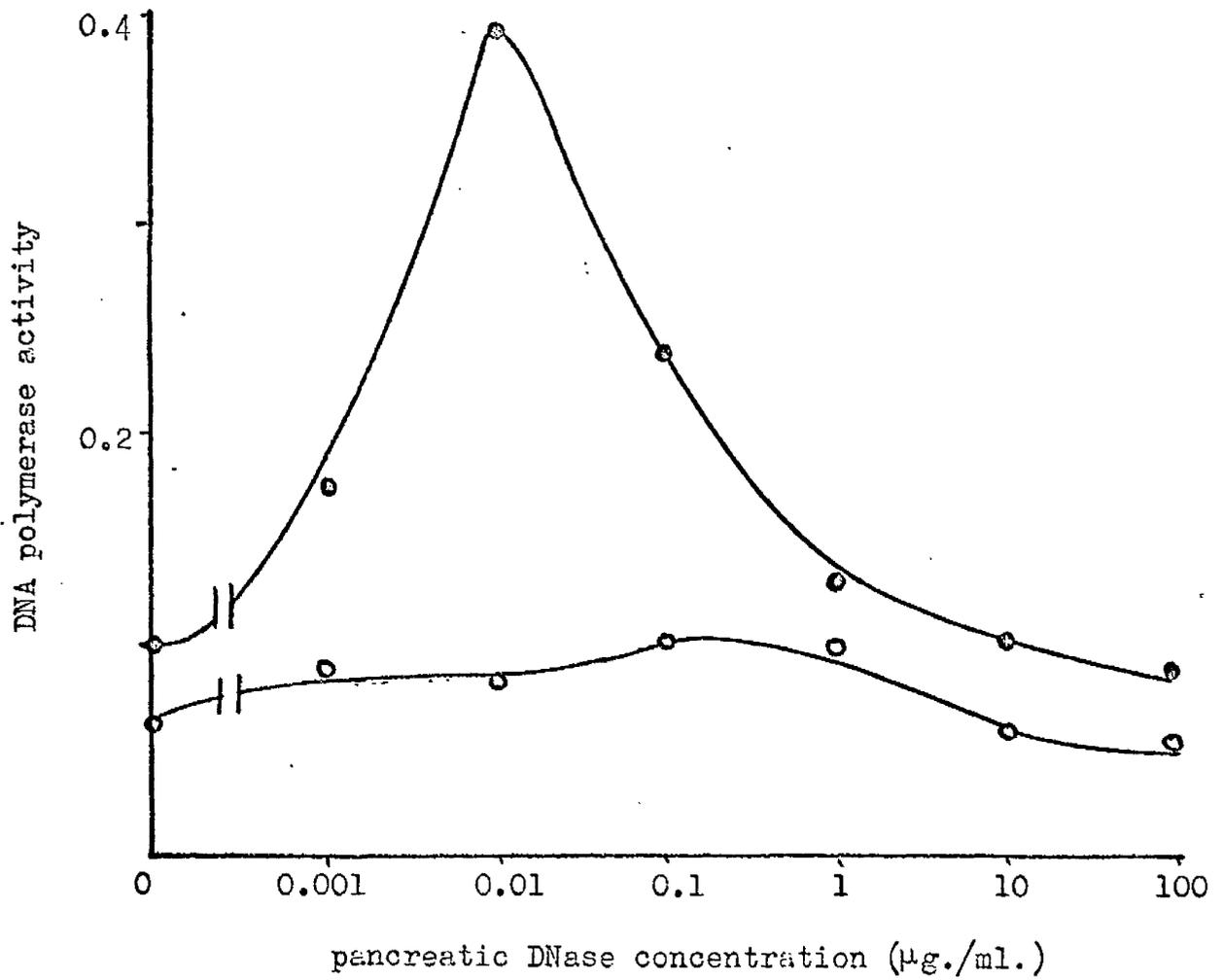
-  DNA polymerase activity of N3 nuclei (native DNA primer)
  
-  activity of S1 fractions (denatured DNA primer)

Fig. 111.23.



which produces 3'-phosphate termini, inhibits DNA polymerase action.

Thus, in contrast to the majority of DNA polymerases from mammalian sources, the L 929 nuclear enzyme resembles the Esch. coli enzyme in its ability to utilise poly d(AT) and in its preference for "activated" native DNA primer. Such an experiment also clearly demonstrates that contaminating nuclease activity may alter the relative capacity of DNA polymerase to use native or heat-denatured DNA, under conditions when DNA is rate limiting.

2.4. Location of enzymic activity in the nuclear fraction and procedures for release of the enzyme

2.4.1. Effect of various isolation treatments and washing procedures on the DNA polymerase activity of L 929 cell nuclei

Nuclei are isolated in 0.02 M-tris-HCl, pH 7.5 containing 0.25 M-sucrose (buffered sucrose) in the presence of Ca<sup>2+</sup> and in 0.02 M-tris-HCl, pH 7.5 containing 0.25 M-sucrose, 0.02 M-EDTA, 5 mM-2-mercapto-ethanol and 0.15 M-KCl (5-component buffer). Calcium and control nuclei are dialysed 2 x 2 hr. against 100 vols. buffered sucrose before assay. A comparison of the activity of N1 and N3 nuclei isolated in tris-sucrose solutions or in 5-component buffer shows that addition of 0.15 M-KCl, 5 mM-2-mercapto-ethanol and 1 mM-EDTA to the medium does not

prevent loss of activity during washing (Table 111.24). The activity of  $\text{Ca}^{2+}$  nuclei is low, apparently caused by the extreme sensitivity of the enzyme to dialysis. However, nuclei isolated in the absence of  $\text{Ca}^{2+}$  lose 50% of their activity during 2 washes (N1 - N3). Little or no difference is observed between N1 and N3 nuclei isolated in  $\text{Ca}^{2+}$ -containing media with respect to their ability to use native DNA. The presence of  $\text{Ca}^{2+}$  does not prevent loss of activity towards heat-denatured DNA. In all cases, whether  $\text{Ca}^{2+}$  is present or not, there is a relatively greater loss of activity, primed by denatured DNA, resulting in an increase in the ratio of activity on native to denatured DNA from N1 to N3.

Table 111.25 shows a similar experiment in which nuclei (N3) are prepared by isolating and washing in buffered sucrose containing 3 mM- $\text{Ca}^{2+}$ . In this case, however, the nuclei are finally resuspended in buffered sucrose only. The presence of  $\text{Ca}^{2+}$  again largely prevents the loss of activity with native DNA during washing.  $\text{Ca}^{2+}$  nuclei, however, are less active than control nuclei isolated in tris-sucrose solutions. This may reflect the presence of traces of  $\text{Ca}^{2+}$  which are not removed before suspension of the nuclei in tris-sucrose alone. Washing

TABLE 111.24.

Effect of various isolation treatments on the DNA polymerase activity of L 929 cell nuclei

<u>Nuclear preparation</u>	<u>Isolation medium</u>	<u>DNA primer</u>	<u>protein/assay (μg.)</u>	<u>μmoles dTMP incorporated/hr. at 37°</u>	<u>Ratio of activity on native and denatured DNA</u>
N1		native denatured	92	37.0 16.1	2:3
N3	buffered	native denatured	50	19.8 5.2	3.8
N3 (dialysed)	sucrose	native denatured	48	8.2 2.5	3.3
N1	5-component	native denatured	84	31.1 13.0	2.4
N3	buffer	native denatured	55	16.5 5.2	3.2
N1	buffered sucrose	native denatured	85	17.4 6.0	2.9
N3	+ 3 mM- CaCl <sub>2</sub>	native denatured	78	14.1 3.1	4.5

Nuclear fractions were assayed under standard conditions (F.g 111.12). See text for further details.

TABLE 111.25.

Effect of washing L 929 cell nuclei in the presence or absence of Ca<sup>2+</sup> ions

<u>Nuclear preparation</u>	<u>Isolation medium</u>	<u>DNA primer</u>	<u>protein/assay (μg.)</u>	<u>μm moles dTMP incorporated/hr. at 37°</u>	<u>Ratio of activity on native and denatured DNA</u>
N1	buffered	native denatured	150	98.8 53.4	1.7
N3	sucrose	native denatured	84	50.4 16.6	3.0
N1	buffered sucrose	native denatured	133	74.8 48.4	1.5
N3	3 mM-CaCl <sub>2</sub>	native denatured	110	64.8 16.4	4.0

Nuclear fractions were assayed under standard conditions. See text (section 2.4.1.) for further details.

of nuclei in the absence of  $\text{Ca}^{2+}$  (N1  $\longrightarrow$  N3) generally results in a 20-60% loss of activity with native DNA primer and is paralleled by the loss of protein from the nuclear fraction.

2.4.2. Location of the nuclear enzymic activity and the DNA product during assay

It is of interest to determine whether the DNA polymerase of activity of L 929 cell nuclei remains in the nucleus under normal polymerase assay conditions. Accordingly N2 nuclei are incubated for various times in the presence of complete assay mixture minus  $[^3\text{H}]$  dTTP. Nuclei are removed by low speed centrifugation and the DNA polymerase activity in the supernatant fraction and resuspended nuclei is assayed.

Fig. 111.26 indicates that even at zero time 60% of the activity of N2 nuclei with native DNA primer is now present in the supernatant fraction and little further loss occurs by pre-incubation of the nuclei at  $37^\circ$ . Similarly 90% of the DNA product is located in the supernatant, almost negligible activity being associated with the pelleted nuclei (Fig. 111.26). The ready extractability of the enzyme from N2 nuclei in assay mixtures has been attributed to the presence of exogenous DNA. Recently Adams and Lindsay (unpublished results) have employed

Fig. 111.26.

Location of the nuclear DNA polymerase activity and DNA product during assay.

2 ml. of nuclei (N2) were suspended in complete assay mixture (3 ml.) minus  $[6-^3\text{H}]$  dTTP and incubated for various periods of time at  $37^\circ$  as shown. Samples (0.7 ml.) were withdrawn at intervals, rapidly cooled and centrifuged at 800 g for 10 min. at  $0-3^\circ$ . Supernatant solutions were decanted off and retained. Pelleted nuclei were resuspended at their original concentration in buffered sucrose. DNA polymerase activity in the supernatant fraction and resuspended nuclei was assayed in the normal manner (Fig. 111.12). Incubation period was 30 min. Results are expressed as % of total activity and protein present in the supernatant fraction.

N2 nuclei (1 ml.) were also suspended in complete assay mixture (1.5 ml.) and incubated at  $37^\circ$  under standard conditions.

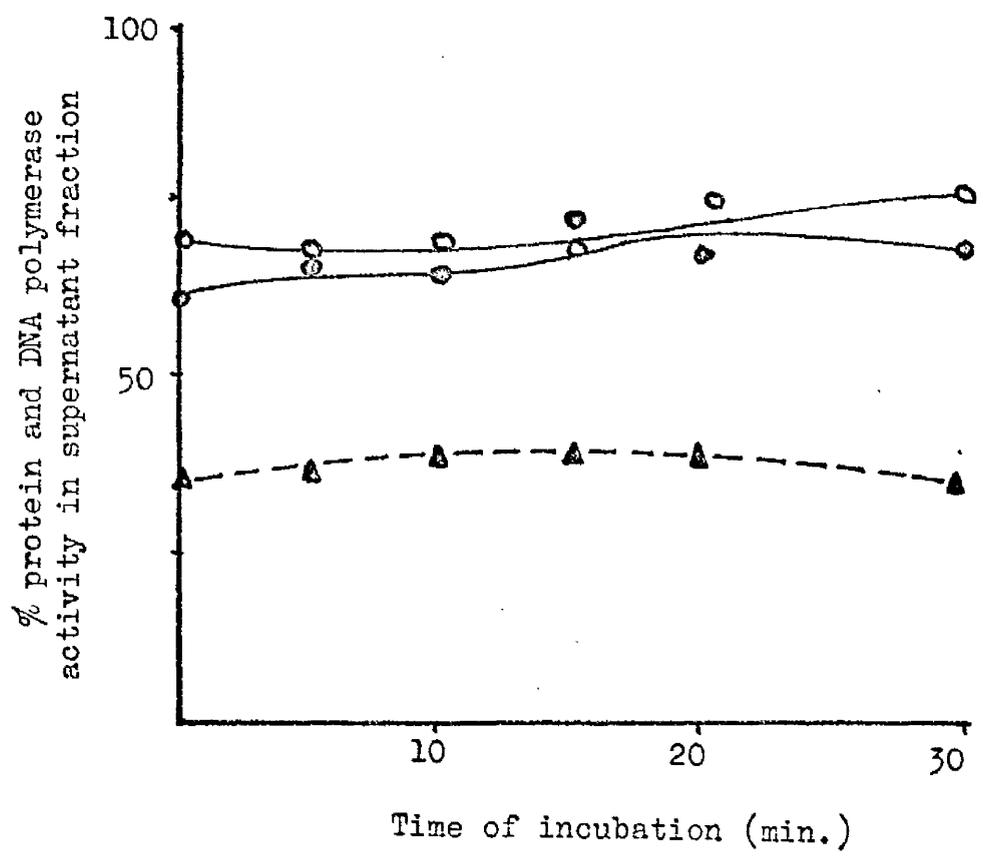
Samples (0.5 ml.) were withdrawn at intervals and the supernatant fraction and pelleted nuclei assayed for the presence of  $[^3\text{H}]$  DNA product (see insert).

●—● activity on native DNA primer  
○—○ activity on denatured DNA primer  
▲---▲ protein

Fig. 111.26.

(insert)

<u>Sample</u>	<u>DNA primer</u>	<u>Incubation period (min.)</u>	<u>% of DNA product in supernatant fraction</u>
N2	native	15	84
	denatured	15	78
nuclei	native	30	92
	denatured	30	85



the washing of L 929 nuclei with solutions of salmon testes DNA to obtain a differential extraction of the enzyme resulting in a 2-3-fold purification.

2.4.3. Release of the DNA polymerase activity from  
L 929 nuclei

Sonication of nuclei, according to the method of Frenster, Allfrey and Mirsky (1963), brings about release of the nuclear DNA polymerase activity into the supernatant fraction after centrifugation of the sonicated nuclei at 105,000g for 60 min. Using calf thymus lymphocyte nuclei these workers are able to remove up to 80% of the nuclear DNA in the condensed heterochromatin fraction by centrifugation at 1,000g for 10 min. but, in our hands, 50% of the nuclear DNA remains in the supernatant fraction after centrifugation at 105,000g for 45 min. Maximum recovery of enzymic activity (approximately 70%) is achieved by sonication for 20-50 sec. after which time activity begins to decline, presumably owing to denaturation of the enzyme (Fig. 111.27). As in the earlier washing experiments (section 2.4.1) nuclear activity with denatured DNA is found to be less firmly associated with the nuclear fraction. In view of the marked preference of DNA polymerase activity in supernatant extracts for single-stranded DNA, the corresponding activity in nuclear fractions may represent cytoplasmic contamination of the nuclei.

Fig. 111.27.

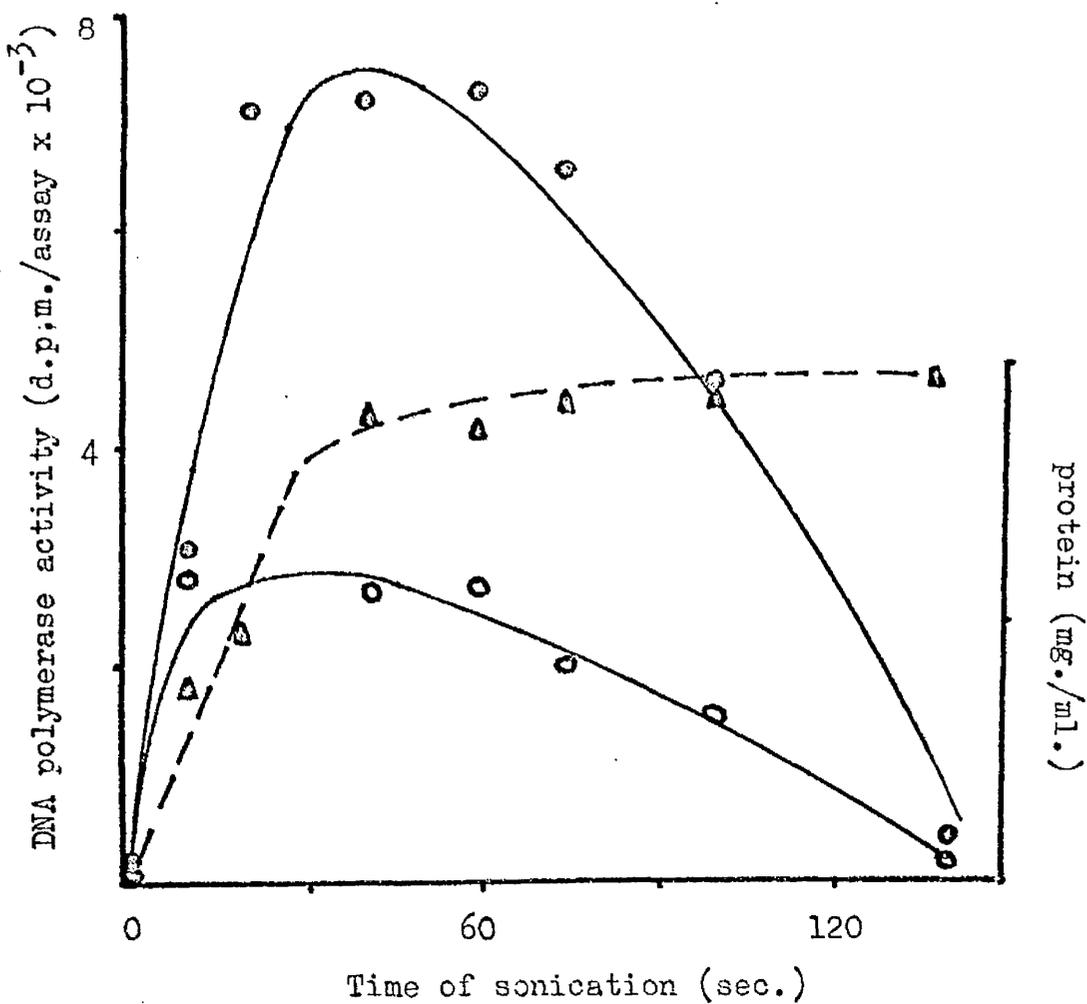
The release of DNA polymerase activity from L 929 cell nuclei.

N3 nuclei were disrupted for various periods of time by sonication (Methods, section 2.3.2. a). Sonicated extracts were centrifuged at 105,000 g in the no. 40 rotor of a Spinco model L ultracentrifuge for 45 min. (0-4°). Sonicated extracts and N3 nuclei were assayed for activity using the routine procedure (Fig. 111.12).

- DNA polymerase activity with native DNA primer
- DNA polymerase activity with denatured DNA primer
- ▲---▲ protein

Fig. 111.27.

	Native DNA	Denatured DNA
Whole nuclei	9630	4370
% recoveries	74	67



Satisfactory extraction of the DNA polymerase activity from nuclei is achieved using the technique of Patel et al. (1967). Nuclei are disrupted in 1.5 M-KCl and the solution is subsequently diluted to 0.15 M. At this ionic strength DNA and histones recombine and precipitate out leaving chromosomal "acidic proteins" in solution. By this method we have been able to obtain 50-70% recoveries of the enzymic activity from L 929 nuclei and remove 98% of the nuclear DNA.

2.5. Preliminary purification and fractionation of the DNA polymerase activities in nuclear and supernatant extracts of L 929 cells

2.5.1. Purification procedures

Large scale purification of the DNA polymerase activity in L 929 cells is difficult owing to the small amounts of material available from tissue culture cells. A 7-8-fold purification is achieved, however, by pH 5 precipitation and gel filtration on Sephadex G200. In buffer of low ionic strength the DNA polymerase activity in sonicated nuclear and supernatant extracts is eluted at or near the void volume on G200 and 80-100% recoveries of the enzyme are routinely achieved after these 2 purification steps (Table 111.28).

The Sephadex-purified enzyme contains less than 5% of

TABLE 111.28.

Preliminary purification of DNA polymerase activity in nuclear and supernatant extracts of L cells

<u>Fraction</u>	<u>DNA primer</u>	<u>protein/ml. (mg.)</u>	<u>total protein (mg.)</u>	<u>Specific activity</u> <u>μμ moles dTMP</u> <u>incorporated/hr.</u> <u>mg. of protein</u>	<u>purification</u>	<u>% recovery</u>
N <sub>3</sub> sonicate	native denatured	2.24	57.2	0.31 0.17		
pH 5 ppte.	native denatured	5.24	26.2	0.79 0.44	2.5 2.6	117 119
Sephadex- pooled fractions	native denatured	1.15	6.7	2.20 0.25	7.0 1.5	83 17
S1 supernatant fraction	native denatured	5.3(8.4)	137(126)	0.17(0.15) 0.49(0.50)		
pH 5 ppte.	native denatured	9.8	57.4	0.51 1.03	3.0 2.1	126 88
Sephadex- pooled fractions	native denatured	2.4(2.6)	15.5(16.0)	1.48(1.10) 0.77(4.03)	8.7(7.3) 1.6(8.0)	97(94) 18(102)

DNA polymerase was assayed by the standard procedures (see Fig. 111.12). All fractions, except those in brackets ( ), were frozen after elution from the Sephadex G200 column and assayed on the following day. See text (section 2.5) for further details.

the nuclease activity of crude cell extracts as judged by the relative ability of the 2 fractions to degrade double- or single-stranded [<sup>32</sup>P] Esch. coli DNA to acid-soluble fragments. As Furlong (1966) has shown that oligonucleotides longer than 7-9 units are trichloroacetic acid-precipitable, the amount of DNA rendered acid-soluble is chiefly a reflection of the exonuclease activity of the preparations. Using a similar procedure in the purification of DNA polymerase activity from Landschütz ascites tumour cells Keir (1965) has shown that pH 5 precipitation removes the bulk of the DNase I activity and a partial separation of DNA polymerase and the remaining nuclease activity is obtained on Sephadex G150 columns.

On several occasions when Sephadex G200 fractions of L 929 cell extracts are frozen before assay, low recovery of activity primed by denatured DNA is obtained. A typical example is shown in Table 111.28. The crude nuclear enzyme, originally exhibiting a 2-fold preference for native DNA, is 9 times more active with this primer after 2 freeze/thaws. Similarly the ratio of activity on native and denatured primer of the supernatant enzyme rises from  $\frac{1}{3}$  to  $\frac{2}{1}$  during the purification.

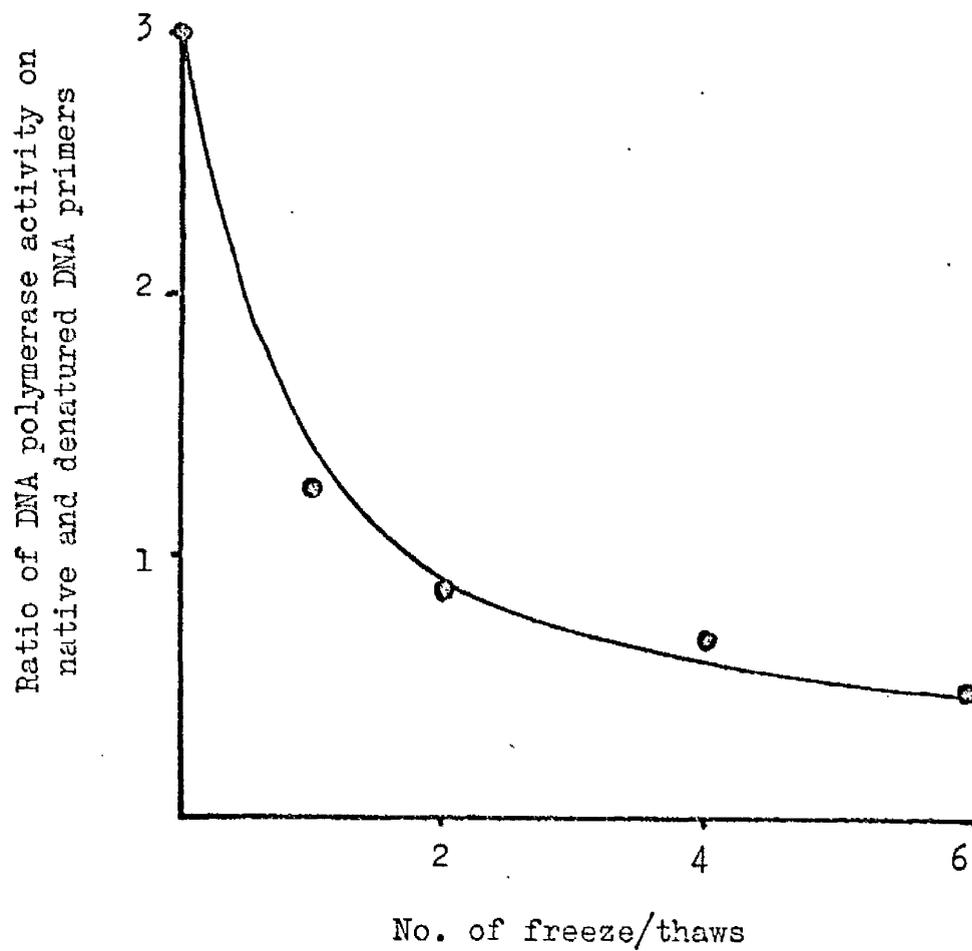
Confirmation of this result is obtained when Sephadex-

Fig. 111.29.

The effect of freeze/thawing on the ability of L 929 cell DNA polymerase to utilise native or heat-denatured DNA as primer.

A 4 ml. sample of a Sephadex-purified supernatant fraction (1.2 mg. per ml.) was subjected to freeze/thawing. The sample was frozen at  $-20^{\circ}$  and rapidly thawed by vigorously shaking in a water bath at  $37^{\circ}$ . 0.6 ml. aliquots were removed and maintained at  $0-3^{\circ}$  until required. The DNA polymerase activity of the various fractions was assayed in the usual manner (Fig. 111.12).

●——● ratio of DNA polymerase activity on native and heat-denatured DNA.

Fig. 111.29.

purified enzyme is subjected to slow freeze/thawing. Rapid inactivation of activity with denatured DNA is again observed (Fig. 111.29). This effect has been repeatedly detected in partially purified preparations although the degree of inactivation is rather variable.

2.5.2. Time course and DNA concentration dependence of DNA polymerase activity using Sephadex-purified nuclear and supernatant fractions

To obtain the most suitable conditions for maximal replication of the DNA primers, the time course and the effect of DNA concentration on the DNA polymerase activity of Sephadex-purified extracts is examined. Fig. 111.30 shows that incorporation of [ $^3\text{H}$ ] dTTP into acid-insoluble material is linear in both cases for 3-4 hr. at  $37^\circ$ . With the native DNA-primed enzyme, activity is still detected between 7 and 8 hr. No breakdown of the newly-synthesised DNA is observed after 9 hr. incubation.

As with crude extracts, DNA ceases to become rate-limiting at about 50  $\mu\text{g}$ . per assay (Fig. 111.31). Contamination of Sephadex-purified fractions with DNA leads to some activity in the absence of added primer. DEAE-cellulose fractions, which contain little or no DNA, show a complete dependence on exogenous primer.

Fig. 111.30.

Time course of DNA polymerase activity in Sephadex-purified  
nuclear and supernatant extracts.

Partially purified nuclear and supernatant extracts were incubated under standard assay conditions (Fig. 111.12) for various times as indicated opposite. There were 120  $\mu$ g. of nuclear and 150  $\mu$ g. of supernatant protein per assay.

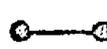
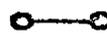
-  DNA polymerase activity with native DNA primer
  
-  activity with denatured DNA primer

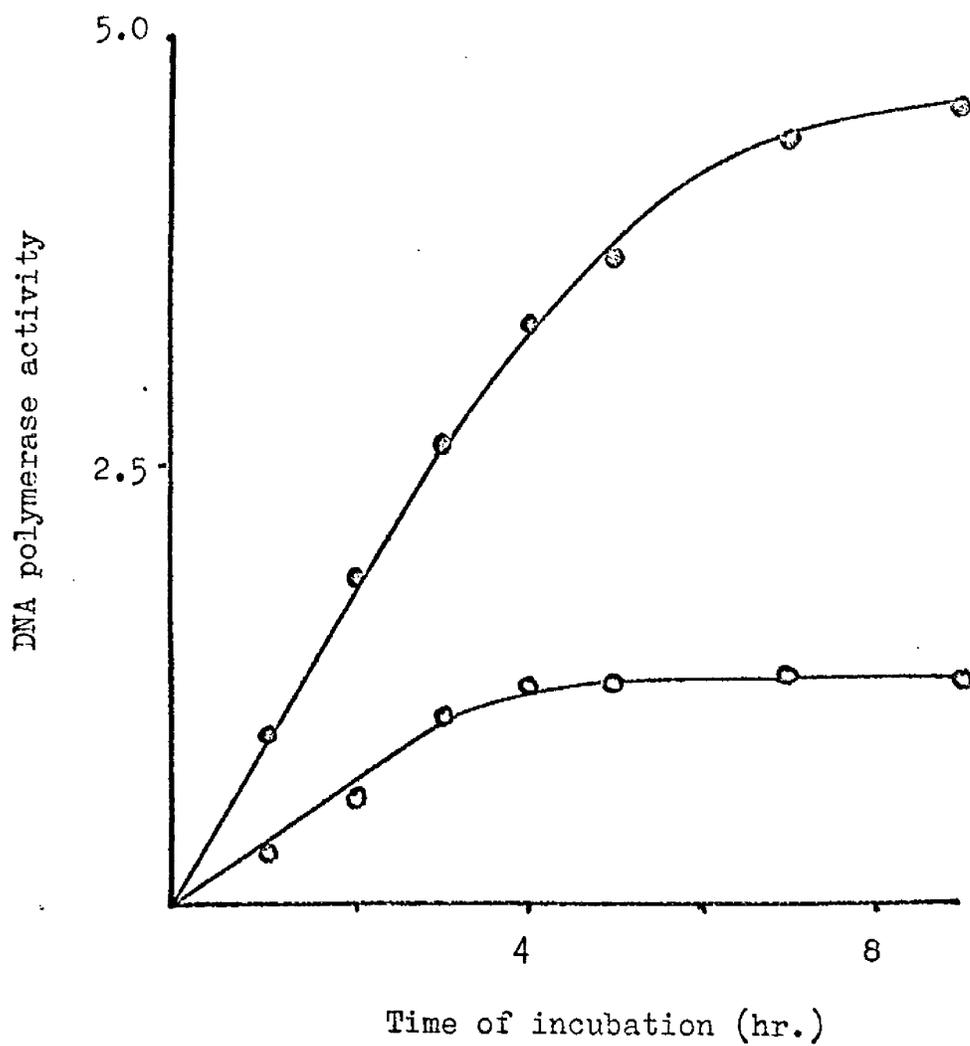
Fig. 111.30.

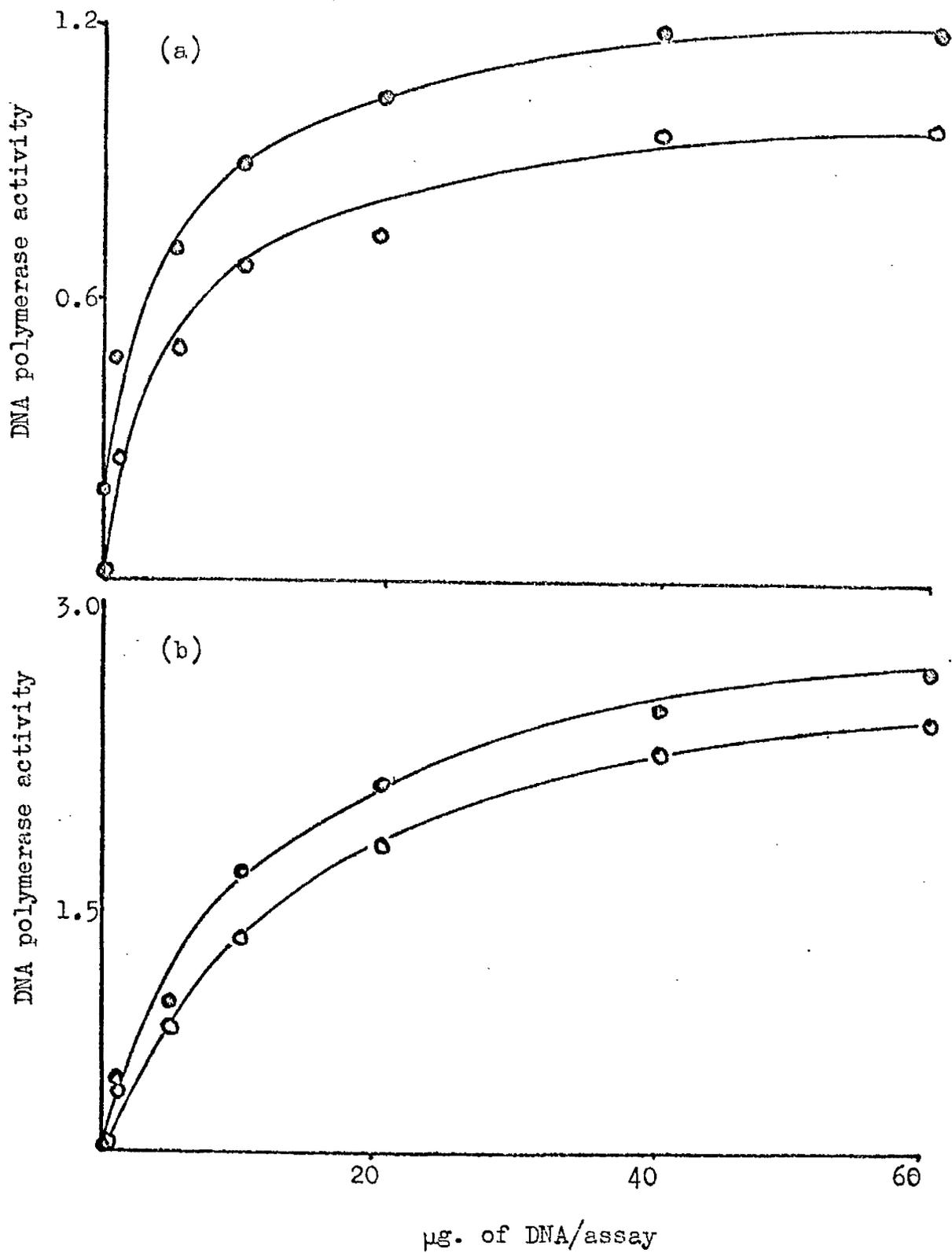
Fig. 111.31.

DNA concentration curves using Sephadex-purified nuclear and supernatant fractions and a DEAE-cellulose column fraction (see Fig. 111.34).

These preparations were assayed with different concentrations of native and heat-denatured DNA primers under standard conditions (Fig. 111.12). There were 120  $\mu$ g. of Sephadex-purified nuclear and 150  $\mu$ g. of supernatant protein per assay. 110  $\mu$ g. of DEAE-cellulose fraction were added per assay.

- (a)                    DNA polymerase activity with native DNA primer (Sephadex nuclear fraction)
-         activity with denatured DNA primer (Sephadex supernatant fraction)
- (b)                    DNA polymerase activity with native DNA primer (DEAE-cellulose fraction)
-         activity with denatured DNA primer (DEAE-cellulose fraction)

Fig. 111.31.



2.5.3. The apparent heterogeneity of DNA polymerase activity in L cells

The differing primer requirements of the nuclear and supernatant DNA polymerase activities in L 929 cells leads us to consider the possibility that there may be more than one species of enzyme present in these extracts. Previously 2 DNA polymerases, having differing priming capacities and separable on DEAE-cellulose chromatography, had been reported in Alcaligenes faecalis. Until recently no similar activities in a single organism had been detected in mammalian tissues. Two groups of workers (Bellair, 1968; Iwamura, Ono and Morris, 1968) have now reported that normal rat liver DNA polymerase is heterogeneous on Sephadex G200 columns.

2.5.4. Fractionation of DNA polymerase activity from sonicated nuclear and high speed supernatant extracts of L 929 cells

The elution profile of a mixture of high speed supernatant and sonicated nuclear extracts is affected by the ionic strength of the eluting buffer (Fig. 111.32). If extracts are run in 0.02 M-tris-HCl, pH 7.5 containing 5 mM-2-mercaptoethanol, all the DNA polymerase activity is eluted at the void volume directly coincident with the main protein peak. In these conditions a

Fig. 111.32.

Fractionation of DNA polymerase activity in extracts of L 929 cells on Sephadex G200 columns.

Sephadex G200 gel filtration was carried out as described in Methods, section 2.3.6. b.

- (a) a combined extract of sonicated nuclei (see Methods, 2.3.2. a) and S1 fraction (2 ml.) was run in 0.02 M-tris-HCl, pH 7.5 containing 5 mM-2-mercaptoethanol.
- (b) a similar preparation was subjected to fractionation. On this occasion the eluting buffer also contained 0.15 M-KCl. DNA polymerase assays were performed using the standard assay.

○—○ DNA polymerase activity with native DNA primer  
○—○ activity with denatured DNA primer  
△--△ protein concentration  
△--△ DNA concentration

Fig. 111.32(a)

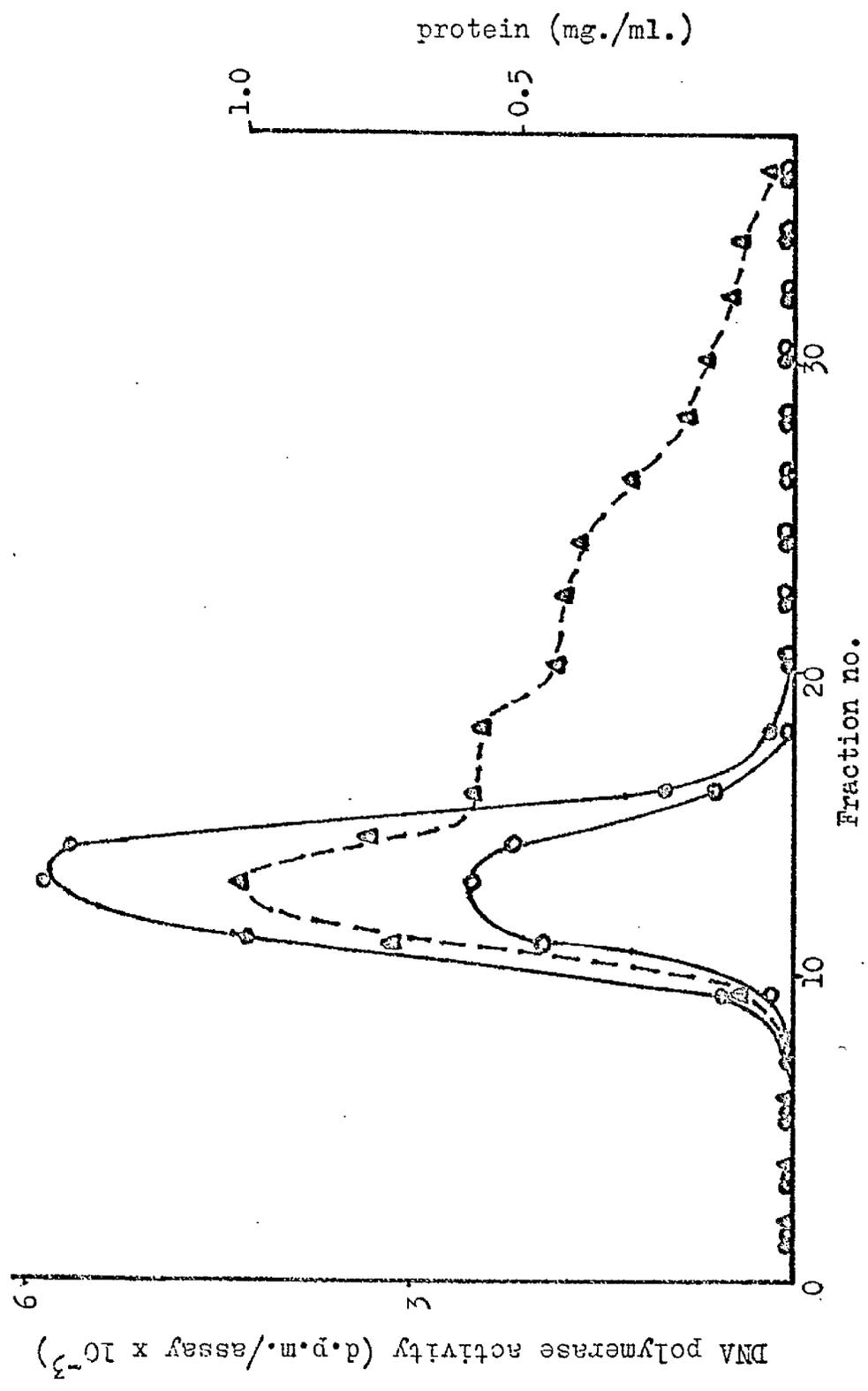
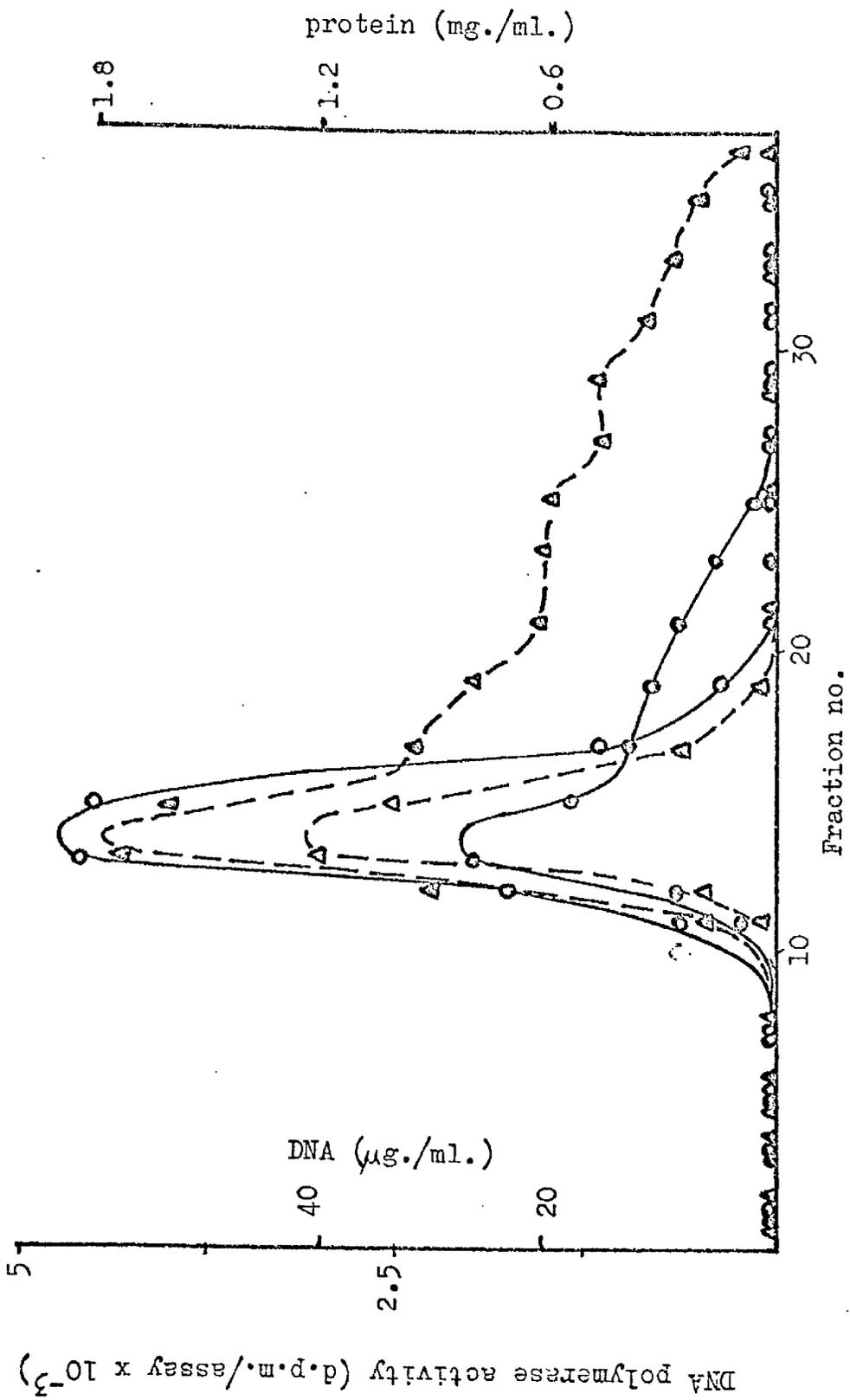


Fig. 111.32(b)



3-5-fold purification of the enzyme(s) is achieved with 80-100% recovery of activity. If similar runs are carried out using buffer containing 0.15 M-KCl, a pronounced trailing in the peak of activity with native DNA is observed. There is no change in the elution profile of the DNA polymerase activity primed by heat-denatured DNA.

2.5.5. Fractionation of DNA polymerase activity from KCl extracts of nuclei and high speed supernatant extracts of L 929 cells

Contaminating DNA in sonicated nuclear extracts is eluted at the void volume on Sephadex G200 (Fig. 111.32). To examine the possibility that binding of DNA polymerase to DNA is preventing a possible separation, nuclear extracts containing low amounts of contaminating DNA are prepared as described by Patel et al. (1967). The patterns of elution of individual and combined nuclear and supernatant extracts are illustrated in Fig. 111.33. Two peaks of activity with native DNA are observed in mixed preparations. Peak I elutes at the void volume along with all the activity primed by denatured DNA while peak II is slightly retarded on the column although not completely separated from peak I. Running of nuclear extracts indicates that peak II

Fig. 111.33.

Fractionation of DNA polymerase activity in extracts of L 929 cells by gel filtration on Sephadex G200.

KCl extracts of nuclei (Methods, section 2.3.2. b) and S1 fractions were prepared in the normal manner and fractionated on G200 columns as follows:-

- (a) 2 ml. of combined KCl nuclear and S1 extract
- (b) 2 ml. of KCl extract alone
- (c) 2 ml. of S1 fraction alone

The eluting buffer, in all cases, was 0.02 M-tris-HCl, pH 7.5 containing 5 mM-2-mercaptoethanol and 0.15 M-KCl. DNA polymerase activity was measured by the standard procedures.

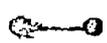
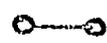
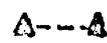
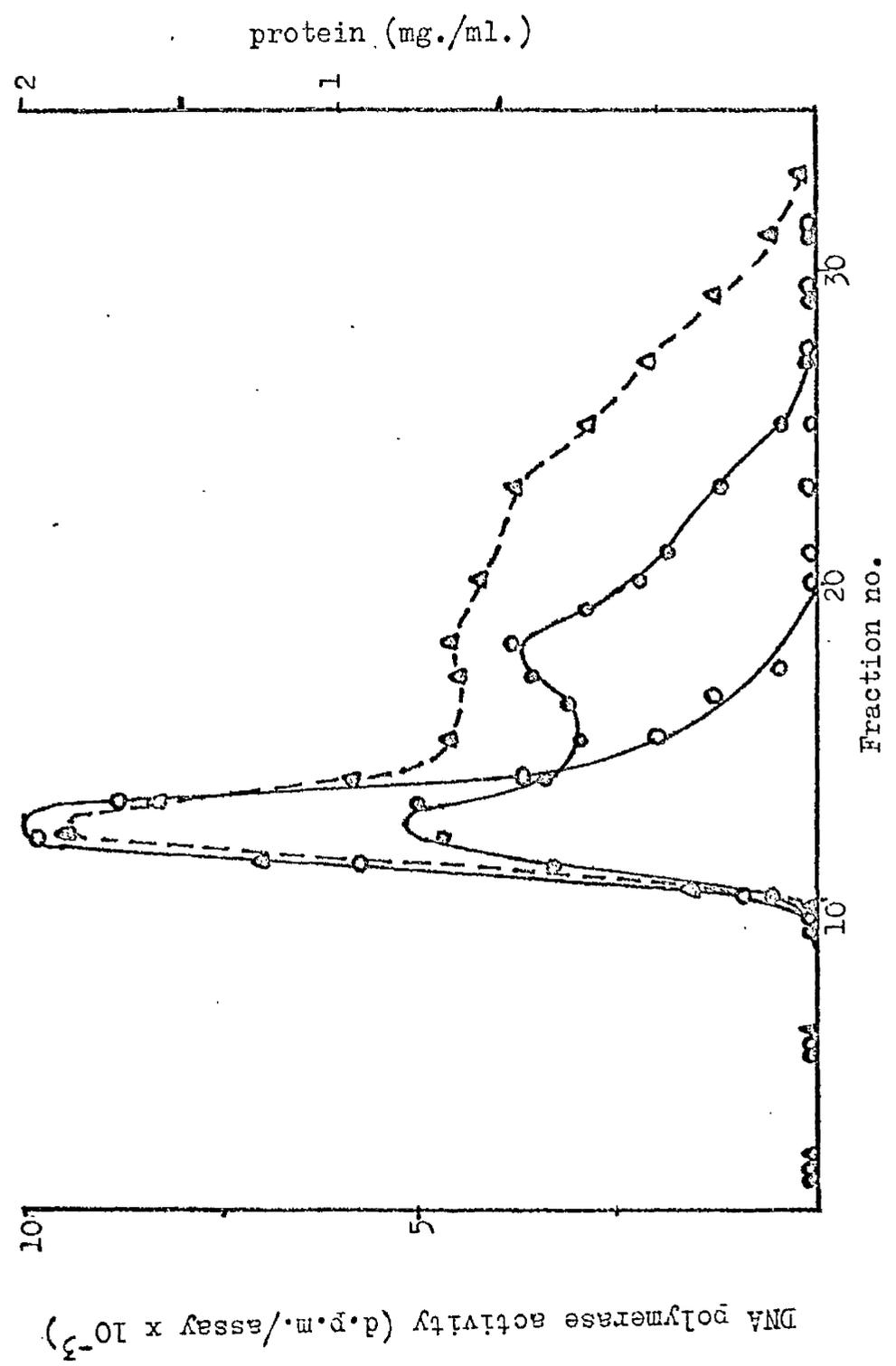
-  DNA polymerase activity with native DNA primer
-  activity with denatured DNA primer
-  protein concentration

Fig. 111.33(a)



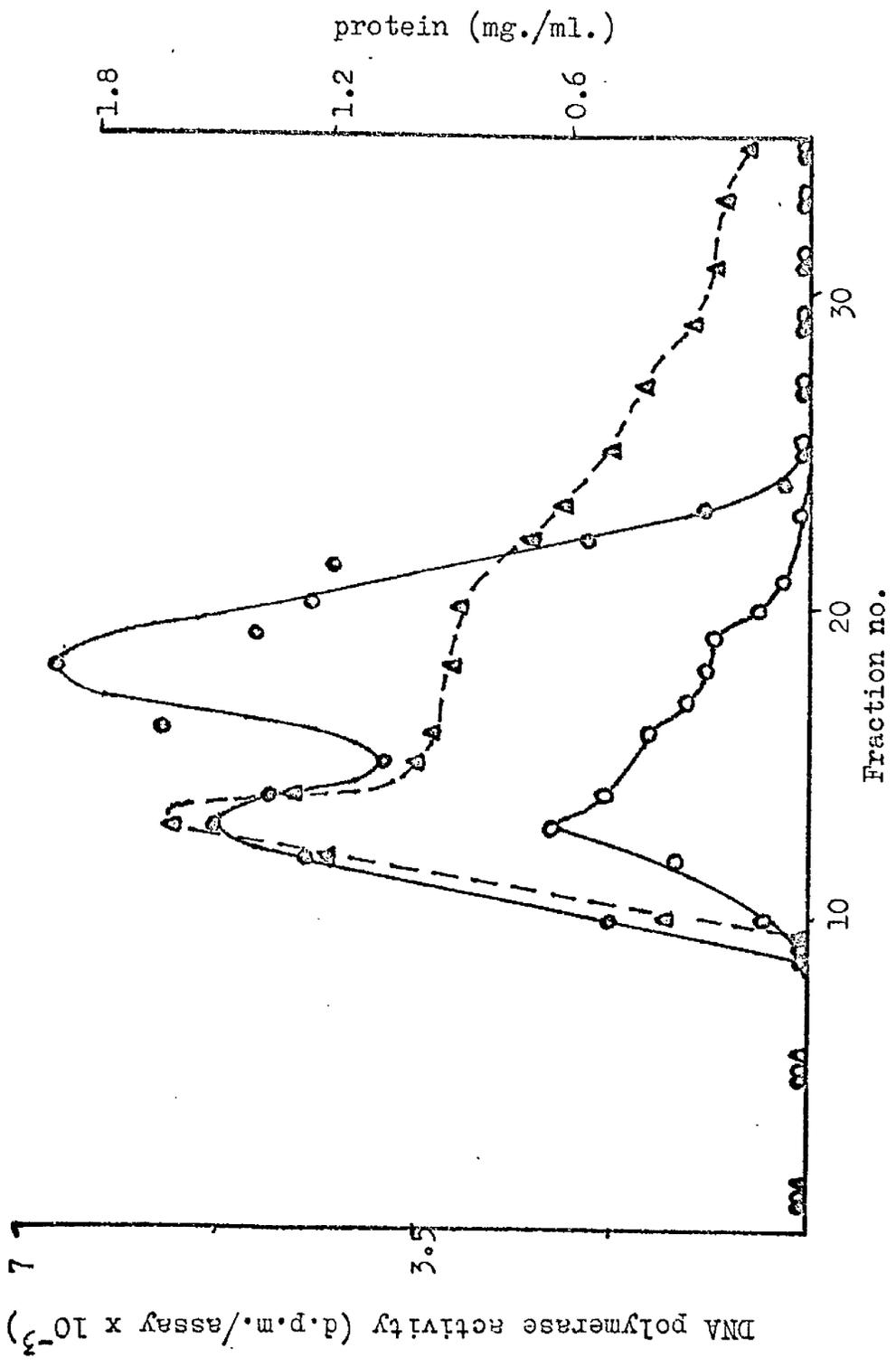


Fig. 111.33(b)

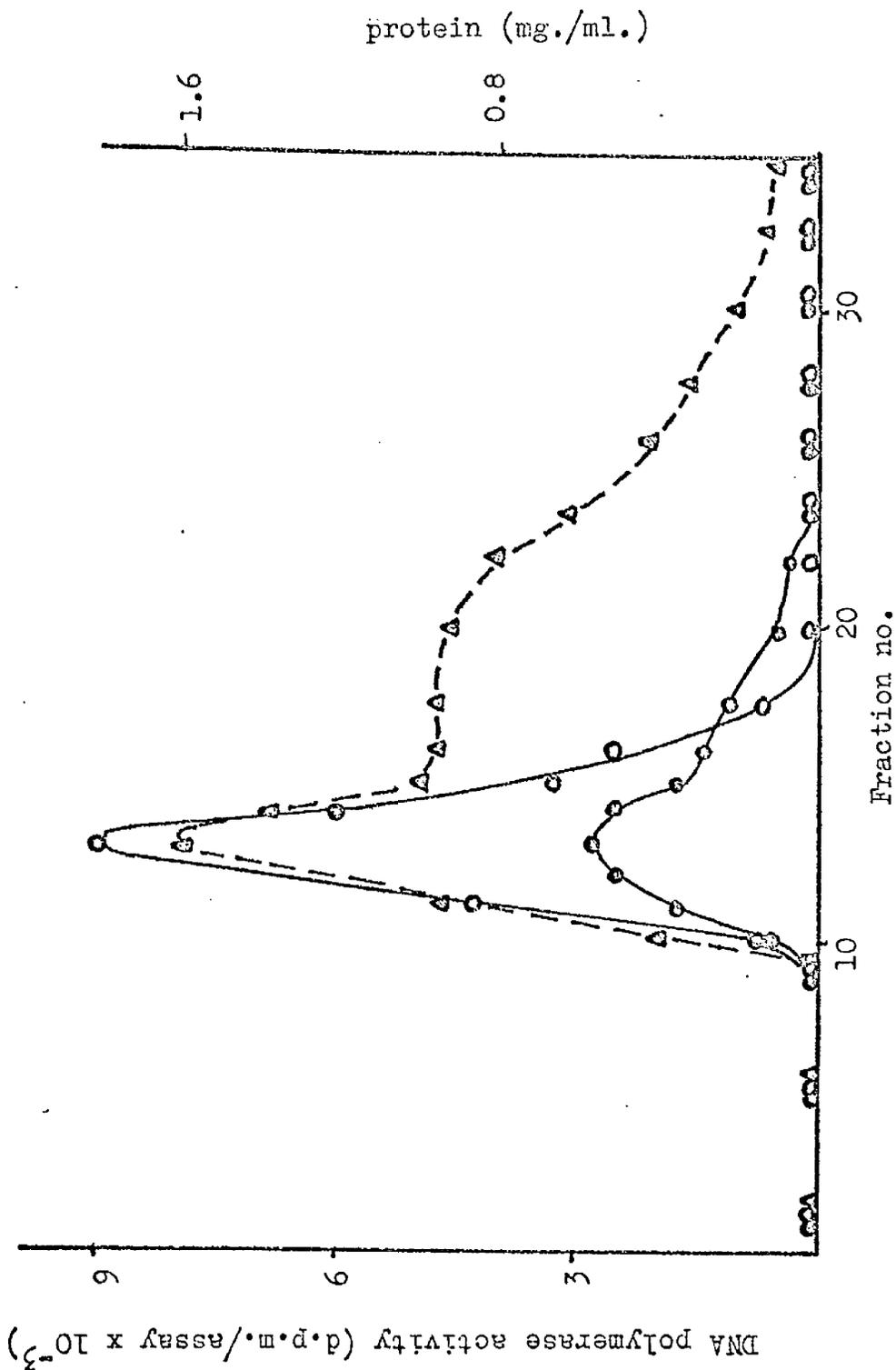


Fig. 111.33(c)

activity is exclusively associated with the nuclear fraction. In supernatant preparations activity with either primer elutes together at or near the void volume (peak I).

It was thought possible that extraction of the nuclei in 1.5 M-KCl had caused the partial dissociation of an enzyme complex resulting in the appearance of 2 active sub-unit fractions of different mol. wts. Gel filtration of nuclear extracts in 1 M-KCl, however, does not alter the relative activities of the 2 peaks.

2.5.6. DEAE-cellulose chromatography and polyacrylamide gel electrophoresis of sonicated nuclear and supernatant extracts of L 929 cells

To obtain further evidence on the heterogeneity of the DNA polymerase activity in L 929 cells, nuclear and supernatant preparations are subjected to fractionation by ion exchange on DEAE-cellulose and electrophoresis on polyacrylamide gels.

On DEAE-cellulose chromatography, eluting with a 0-0.35 M-KCl gradient, the bulk of the DNA polymerase activity comes out in 0.11 M-KCl; two smaller peaks of activity, markedly preferring native DNA are also detected (Fig. 111.34). The first is not adsorbed to the column and is eluted in the washing buffer while a second, more active fraction appears at 0.03 M-KCl. The

Fig. 111.34.

DEAE-cellulose chromatography of DNA polymerase in extracts of  
L 929 cells.

10 ml. of combined sonicated nuclear and S1 fraction (1.5 mg. per ml.) were adsorbed onto a 1 x 15 cm. DEAE-cellulose column in 0.02 M-tris-HCl, pH 7.5 containing 5 mM-2-mercapto-ethanol. Elution was performed as described in Methods, section 2.3.6. c. Samples were assayed for DNA polymerase using the routine assay (see Fig. 111.12) although fractions were not corrected for the changing KCl concentration.

- DNA polymerase activity with native DNA primer
- activity with denatured DNA primer
- △---△ protein concentration
- △---△ DNA concentration

Fig. 111.34

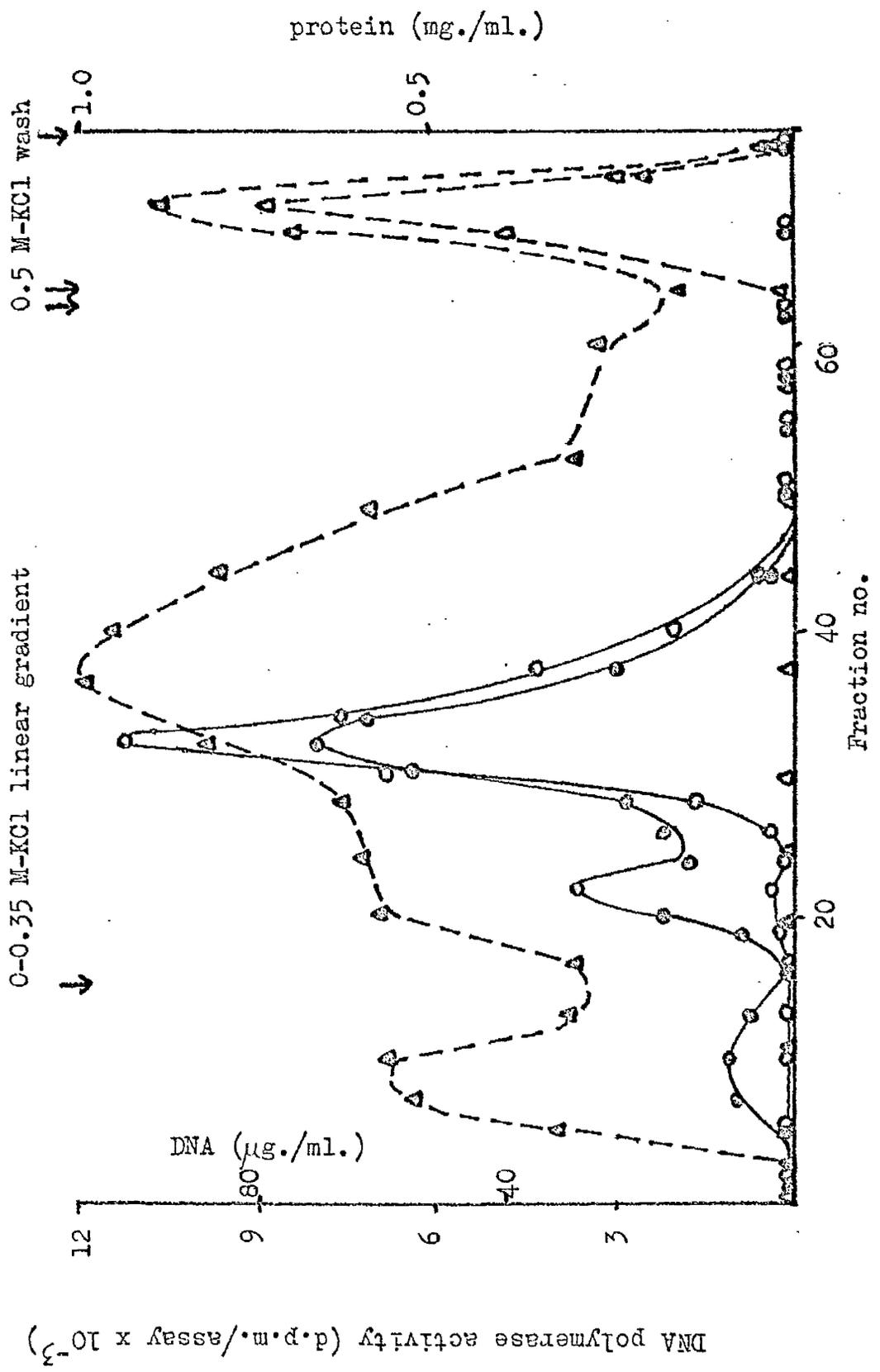


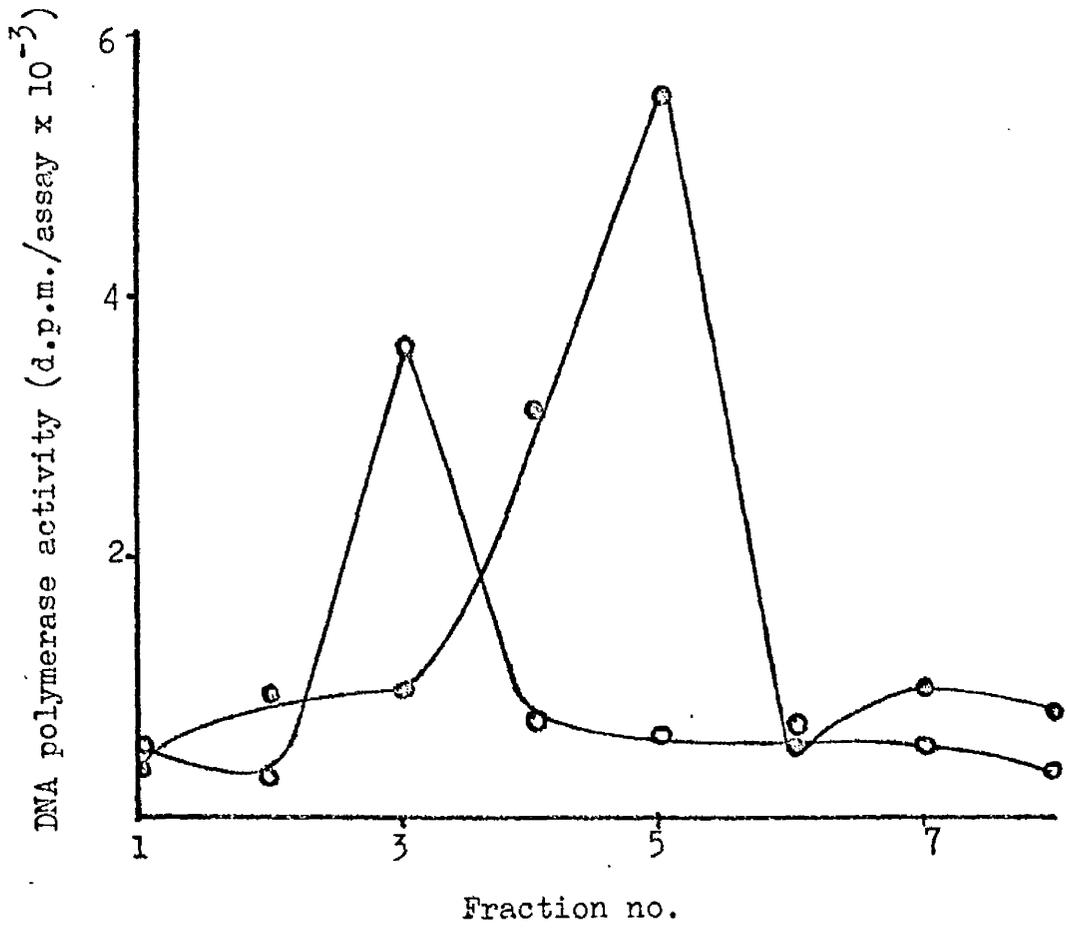
Fig. 111.35.

Polyacrylamide gel electrophoresis of nuclear and supernatant  
extracts of L 929 cells.

100-200  $\mu$ g. of sonicated nuclear or S1 extracts were layered onto 7 $\frac{1}{2}$ % polyacrylamide gels, subjected to electrophoresis, and assayed for DNA polymerase using the normal assay mixture (Fig. 111.12) as described in Methods, section 2.3.6. e.

- DNA polymerase activity with native DNA primer
- activity with denatured DNA primer

Fig. 111.35.



possibility that these 2 minor peaks represent one form of the

enzyme which is poorly retained by the column has not yet been

investigated. The relationship of these activities to those

obtained on Sephadex G200 gel filtration also requires further

analysis.

Further proof of the presence of separable DNA polymer-

ase activities is found by fractionation of nuclear and super-

natant extracts on polyacrylamide gel electrophoresis where 2

peaks of activity with differing primer specificities are re-

solved (Fig. 11.35). Electrophoresis is carried out at pH

8.3 in 0.05 M-tris-glycine buffer (Davis, 1964) and, under these

conditions, the bulk of the activity primed by native DNA migrates

further towards the anode than the corresponding activity with

denatured DNA primer.

Polymerisation of the gels is catalysed using riboflavin

rather than ammonium persulphate (Oster, Oster and Prati, 1957;

Oster, Bellin and Holstrom, 1962). Difficulty is still experienced,

however, in recovering enzymic activity from the gels. Overnight

pre-running of the gels to remove impurities is recommended by

Cavallieri and Carroll (1968). Removal of acid-soluble radio-

activity from gel slices after assay also presents technical

problems and results in the occurrence of high background radio-

activities in control samples. In view of these difficulties fractionation of DNA polymerase activities by this method is not extensively employed. There exists the possibility that minor peaks of enzymic activity are obscured by the high blank values.

### 3. CHARACTERISATION OF THE DNA PRODUCTS OF SEPHADEX-PURIFIED NUCLEAR AND SUPERNATANT DNA POLYMERASE ACTIVITIES USING NATIVE AND HEAT-DENATURED DNA AS PRIMER

#### 3.1. Introduction

In mammalian systems only the product of the calf thymus DNA polymerase has been extensively investigated (Bollum, 1966). This enzyme requires single-stranded DNA as primer and will catalyse synthesis of native DNA in the presence of initiating oligonucleotides. If no initiator is present the product is a "hairpin-like" structure in which the newly-synthesised strand is covalently bonded to the primer DNA. The Esch. coli enzyme is similarly able to catalyse repair synthesis of native DNA partially degraded by exonuclease III (Richardson et al., 1964) and can form the complementary (-) strand using  $\phi$ X 174 DNA (+ strand) as template (Goulian et al., 1967). The product of replication on native DNA is highly branched, however, and has anomalous physical properties (Schildkraut et al., 1964).

The finding of DNA polymerases having different primer

specificities in L 929 cells has prompted us to investigate some of the characteristics of the DNA products formed by these enzymes on native and alkali-denatured templates.

3.2. Susceptibility of the DNA products to the action of exonuclease I

3.2.1. The DNA product of the partially purified Land-schütz ascites tumour enzyme

To examine the DNA products of L 929 cell DNA polymerase activities [ $^3\text{H}$ ] DNA is synthesised using Sephadex-purified nuclear and supernatant fractions with native or alkali-denatured Esch. coli DNA as primer. Incubations are carried out for 3 hr. at  $37^\circ$  and the reaction is terminated by the addition of 0.1 N-NaOH to adjust the pH to 9.2 ready for use in the assay of exonuclease I. In all cases the amount of DNA synthesised represents only 3-7% of the original primer DNA.

The validity of the method is initially tested using a purified, DNase free, ascites tumour DNA polymerase (Adams, C.J., 1969, M.Sc. Thesis, University of Glasgow) which displays similar characteristics to the Bollum enzyme and has an absolute requirement for denatured DNA primer. The [ $^3\text{H}$ ] DNA product of this enzyme is shown to be resistant to attack by exonuclease I while [ $^{32}\text{P}$ ] DNA (heat-denatured) added as control is rapidly

Fig. 111.36.

Susceptibility of the DNA product formed by Landschütz ascites  
tumour DNA polymerase to hydrolysis by exonuclease I.

$[^3\text{H}]$  DNA was synthesised by using a modification of the conventional assay procedure (see Fig. 111.12; scaled up 10 fold) and a DNase free preparation of Landschütz ascites DNA polymerase. The incubation period was 3 hr. and 100  $\mu\text{g.}$  of alkali-denatured Esch. coli DNA was used as primer. 7% replication of the added DNA was achieved. The reaction was terminated by altering the pH of the assay mixture to 9.2 with 0.1 M-NaOH. 0.1 ml. samples containing 3  $\mu\text{g.}$  of  $[^3\text{H}]$  DNA were withdrawn and tested for their susceptibility to exonuclease action as described in Methods, section 2.3.5. b. 2  $\mu\text{g.}$  of alkali-denatured Esch. coli  $[^{32}\text{P}]$  DNA were also present as a control.

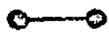
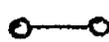
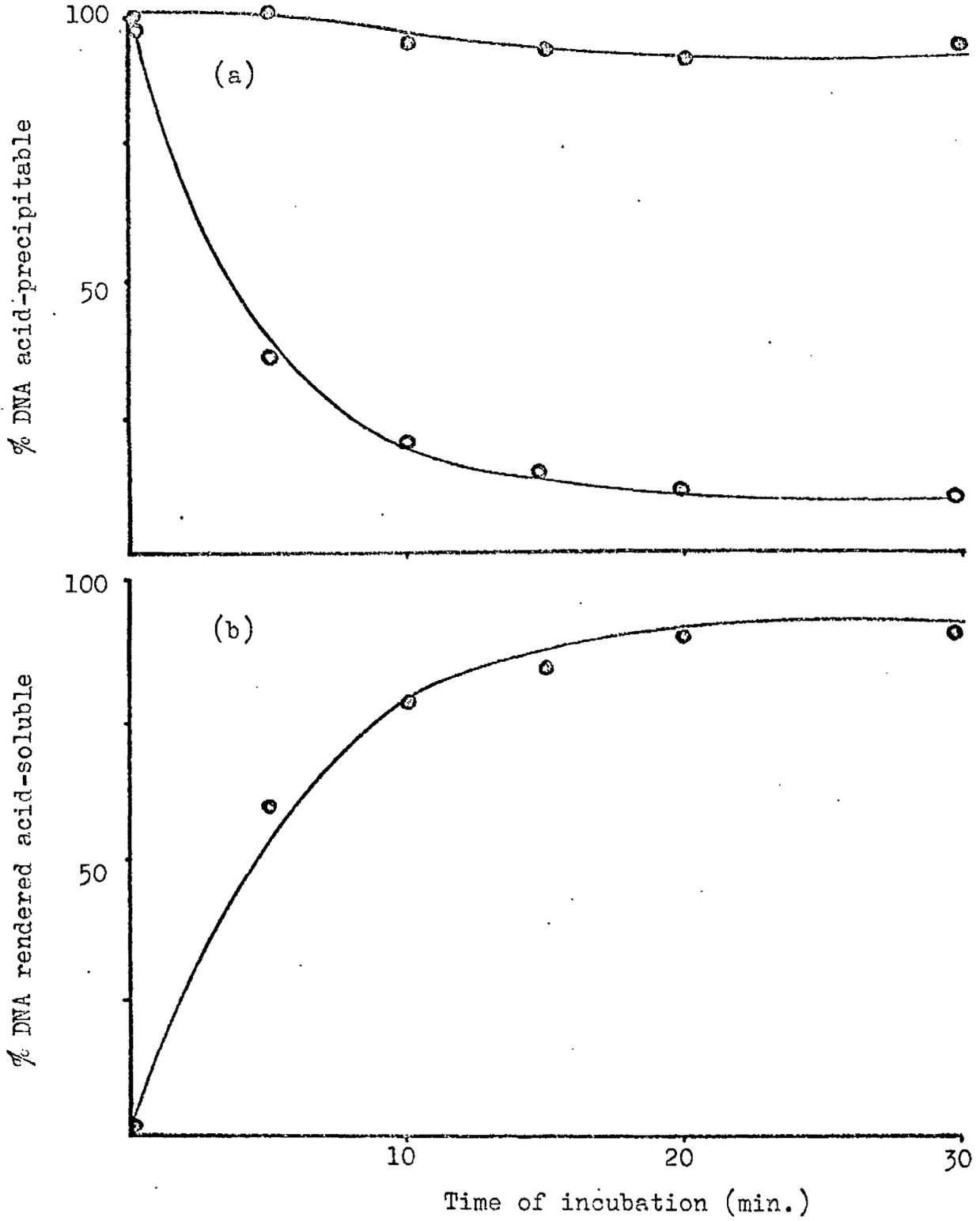
- (a)       acid precipitable  $[^3\text{H}]$  DNA  
          acid precipitable  $[^{32}\text{P}]$  DNA
- (b)       acid soluble  $^{32}\text{P}$  counts

Fig. 111.36.



rendered acid-soluble (Fig. 111.36). Acid precipitable  $^3\text{H}$  and  $^{32}\text{P}$  counts are measured simultaneously on a Nuclear Chicago scintillation counter. The release of  $^{32}\text{P}$  radioactivity into the acid-soluble fraction is also followed using a Nuclear Chicago gas flow counter. Although only 7% replication of the primer is achieved using single-stranded DNA template, the product is not susceptible to exonuclease I. A partially double-stranded structure as proposed under the Bollum replication scheme would exhibit this property.

3.2.2. DNA product of the supernatant enzyme from  
L 929 cells

A similar experiment is performed on the DNA product of the partially purified supernatant enzyme using alkali-denatured Esch. coli DNA as primer. As previously found for the ascites tumour enzyme, no degradation of the  $[^3\text{H}]$  DNA product occurs although single-stranded  $[^{32}\text{P}]$  DNA present in the assay is extensively degraded (Fig. 111.37).

3.2.3. DNA product of the nuclear enzyme from L 929  
cells

The product formed by the nuclear enzyme utilising native DNA as primer is also tested in this system (Fig. 111.38). An

Fig. 111.37.

<sup>3</sup>H DNA product formed by the Sephadex-purified supernatant DNA polymerase from L cells.

$[^3\text{H}]$  DNA was synthesised and tested for its susceptibility to exonuclease I action as in Fig. 111.36. 6% replication of the alkali-denatured primer DNA was achieved.

- (a)       acid precipitable  $[^3\text{H}]$  DNA  
          acid precipitable  $[^{32}\text{P}]$  DNA
- (b)       acid soluble  $^{32}\text{P}$  counts

Fig. 111.37.

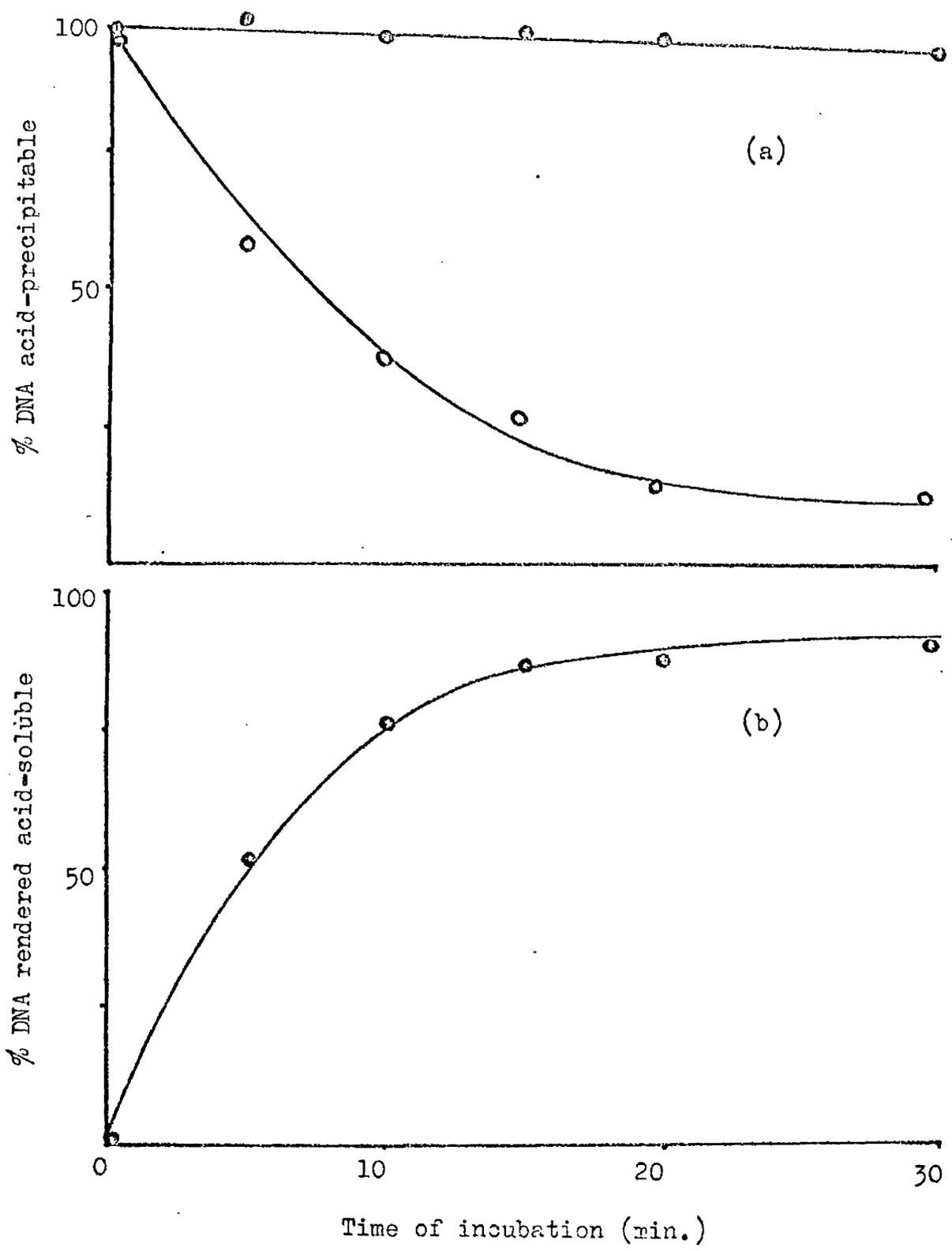


Fig. 111.38.

DNA product of the Sephadex-purified nuclear DNA polymerase from  
L cells.

With native DNA as primer 4% replication was achieved using the techniques employed in previous experiments. The susceptibility of the product to hydrolysis by exonuclease I was tested as in Fig. 111.36.

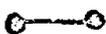
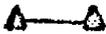
- (a)       acid precipitable  $[^3\text{H}]$  DNA  
           $[^3\text{H}]$  DNA control (exonuclease I omitted)
- (b)       acid precipitable  $[^{32}\text{P}]$  DNA  
          acid soluble  $^{32}\text{P}$  counts  
           $[^{32}\text{P}]$  DNA control (exonuclease I omitted)

Fig. 111.38.

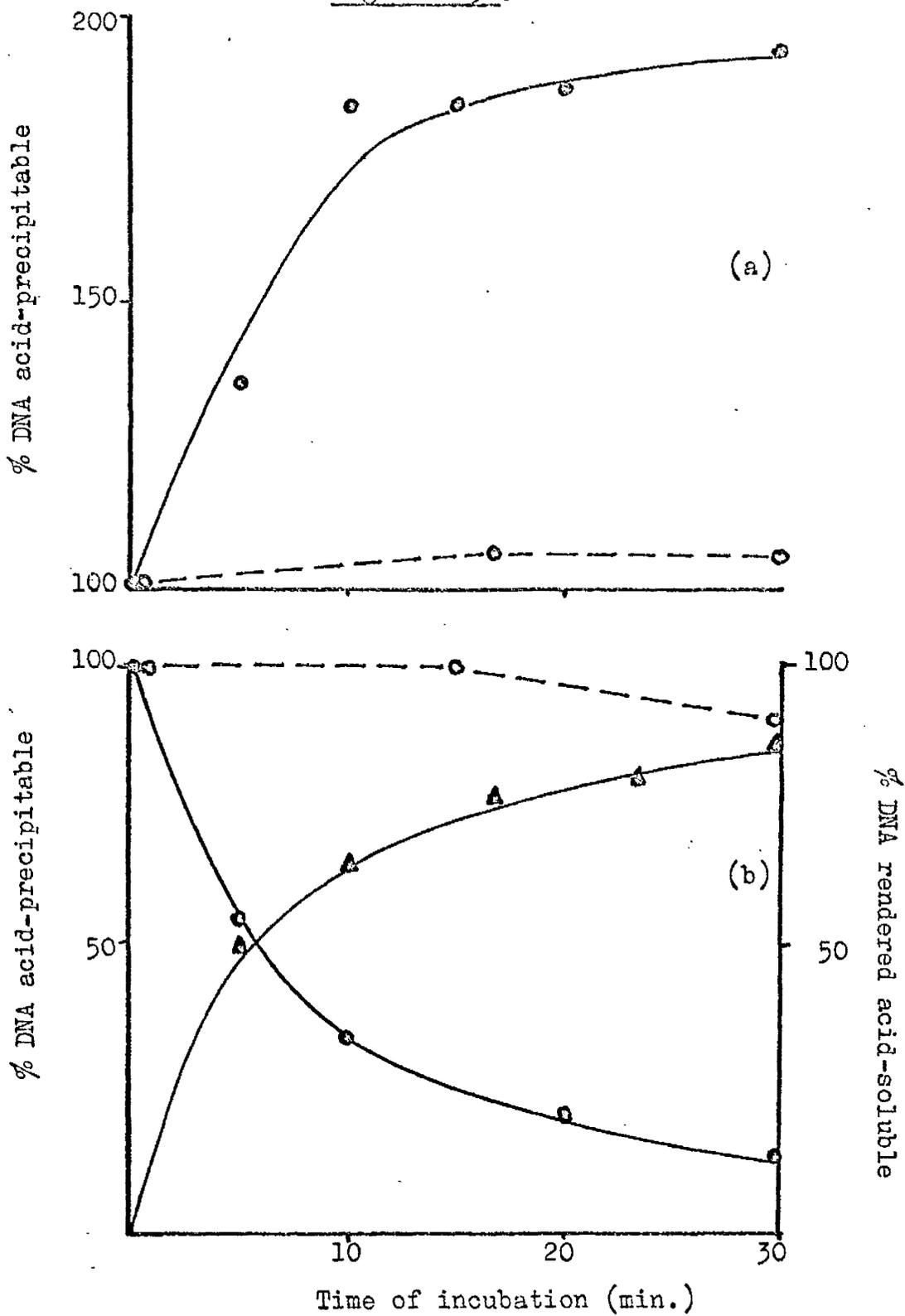


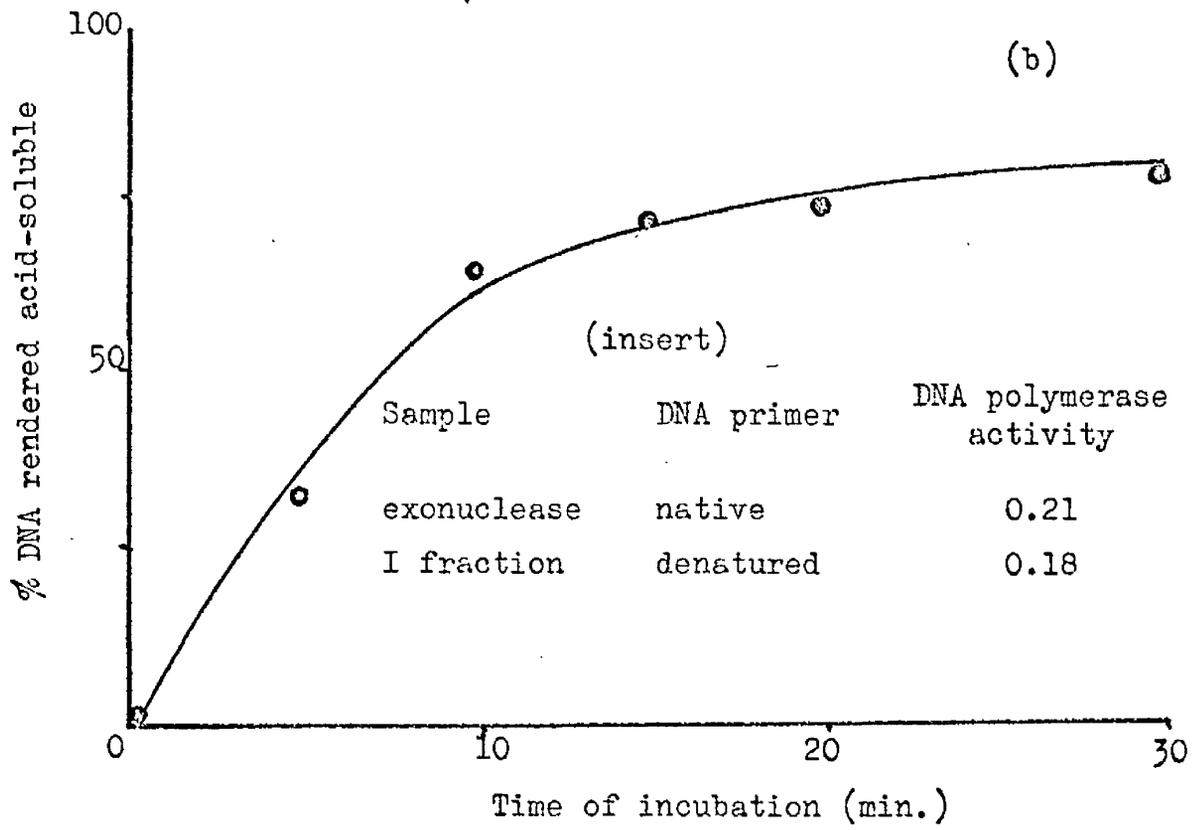
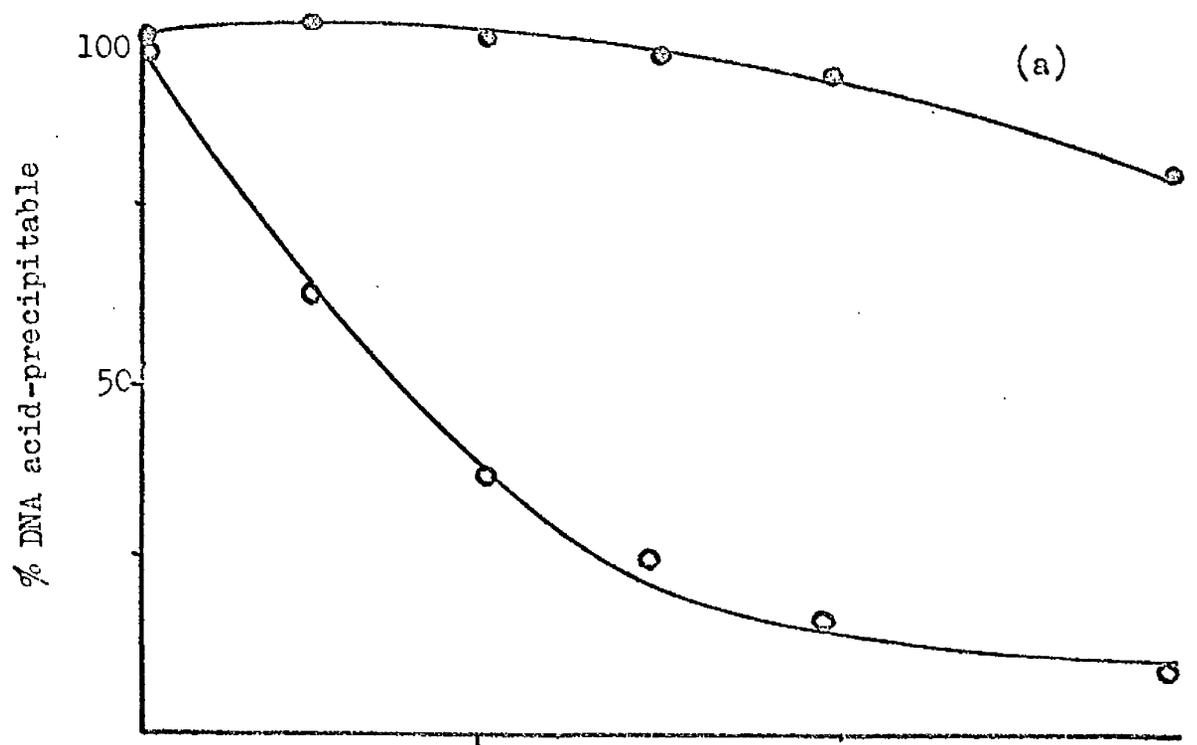
Fig. 111.39.

DNA product of the Sephadex-purified nuclear DNA polymerase from  
3L cells.

The previous experiment was repeated after exhaustive dialysis of the [ $^3\text{H}$ ] DNA product (dialysed for 48 hr. against 4 x 100 vol. of 0.02 M-tris-HCl, pH 7.5) to remove deoxyribonucleoside triphosphates. DNA polymerase activity in the exonuclease I preparation was measured under standard assay conditions. The incubation period was 30 min.

- (a)       acid precipitable [ $^3\text{H}$ ] DNA  
          acid precipitable [ $^{32}\text{P}$ ] DNA
- (b)       acid soluble  $^{32}\text{P}$  counts

Fig. 111.39.



increase in acid-precipitable [ $^3\text{H}$ ] DNA of 90% over control values is noted during the course of the incubation while denatured Esch. coli [ $^{32}\text{P}$ ] DNA is rendered acid-soluble as before. As the [ $^3\text{H}$ ] counts in controls, incubated in the absence of exonuclease I, do not rise it seems that this effect does not reflect the presence of residual L 929 cell DNA polymerase activity. Moreover, L cell DNA polymerase is inactive at pH 9.2. Slight contamination of the exonuclease I preparation with Esch. coli DNA polymerase is found to be the cause of this problem. This activity is equally effective on native or heat-denatured primer (insert, Fig. 111.39).

To overcome the criticism that degradation of the DNA product may be obscured due to simultaneous synthesis by the contaminating DNA polymerase activity, the DNA product is exhaustively dialysed to remove deoxyribonucleoside triphosphates. Treatment of the dialysed preparation with exonuclease I shows that the product synthesised on native DNA is also resistant to hydrolysis by this enzyme (Fig. 111.39). The product of the Esch. coli enzyme on native DNA templates is similarly resistant to exonuclease I (Schildkraut et al., 1964).

#### 3.2.4. Susceptibility of the DNA products to exonuclease I after alkaline-denaturation

After alkali-denaturation of the DNA products of nuclear

Fig. 111.40.

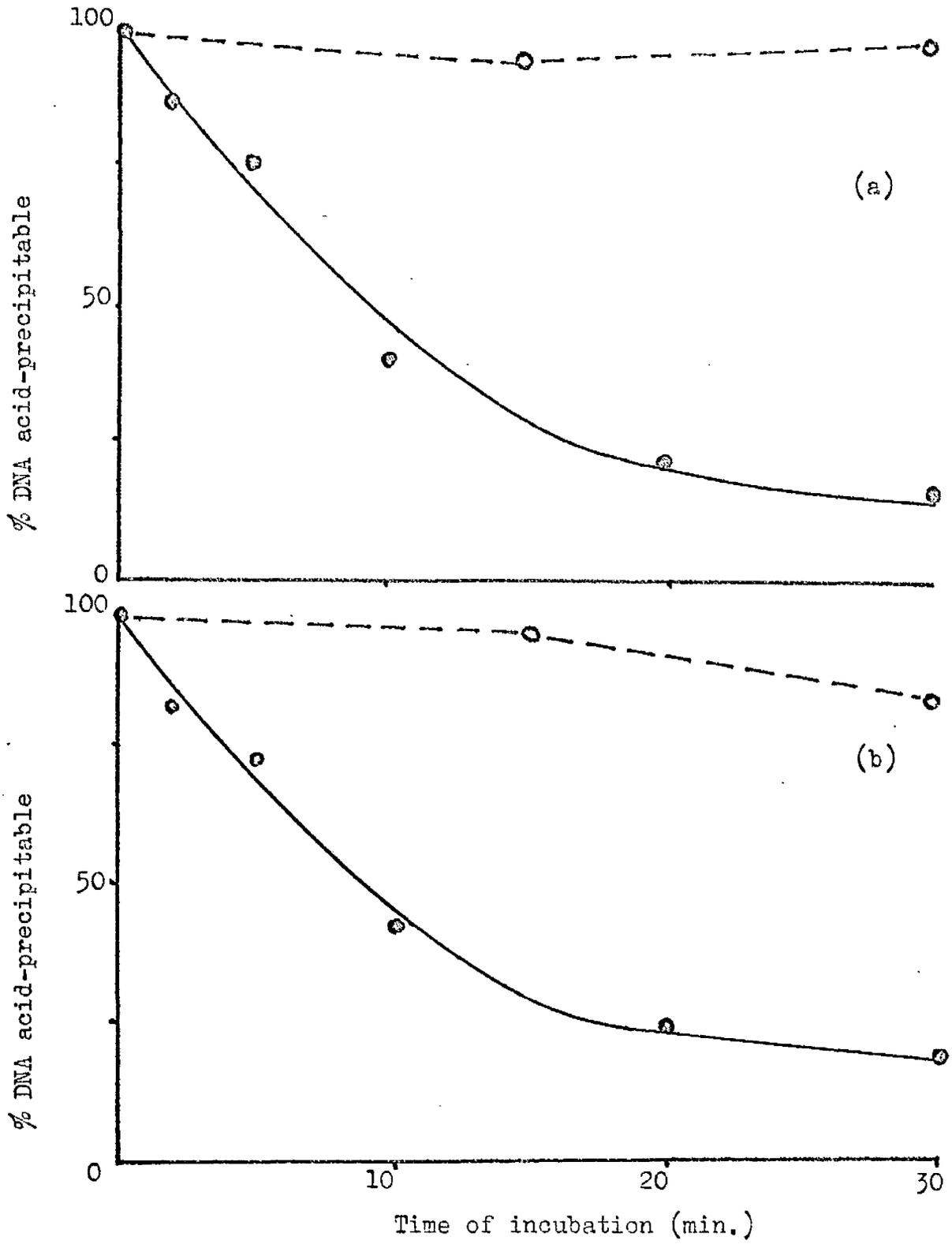
Susceptibility of the DNA products to exonuclease I after alkali-  
denaturation.

The DNA products, synthesised by Sephadex-purified nuclear and supernatant extracts of L cells were alkali-denatured as described in Methods, section 2.3.3. b. Their susceptibility to degradation by exonuclease I was assayed as described in the legend to Fig. 111.36.

●—● acid-precipitable [<sup>3</sup>H] DNA  
●---● [<sup>3</sup>H] DNA control (exonuclease I omitted)

- (a) DNA product of the nuclear DNA polymerase
- (b) DNA product of the supernatant DNA polymerase

Fig. 111.40.



170

and supernatant fractions, both products become susceptible to exonuclease I attack (Fig. 111.40). This eliminates the possibility that exonuclease I is inhibited from acting on the native DNA products because the newly-synthesised DNA is 3'-phosphate rather than 3'-hydroxyl terminated. A situation where the 3'-hydroxyl groups are occupied by DNA polymerase molecules which prevent hydrolysis of the DNA products cannot be ruled out.

3.2.5. Comparison of the rate of release of  $^3\text{H}$  and  $^{32}\text{P}$  counts from the DNA products

As the extent of replication achieved in all experiments is only in the order of 5% of the added primer, an experiment is designed to test for the location of the newly-synthesised  $^3\text{H}$  DNA with respect to the DNA primer.  $^3\text{H}$  DNA is synthesised using limiting amounts of native or single-stranded Esch. coli  $^{32}\text{P}$  DNA as primer, the products denatured by alkali treatment and the kinetics of release of  $^3\text{H}$  and  $^{32}\text{P}$  counts followed during degradation by exonuclease I. In both cases  $^3\text{H}$  counts are released 2-3 times more rapidly than  $^{32}\text{P}$  counts from the DNA product (Fig. 111.41), indicating that the newly-synthesised DNA is situated towards the 3'-termini of the primer molecules.

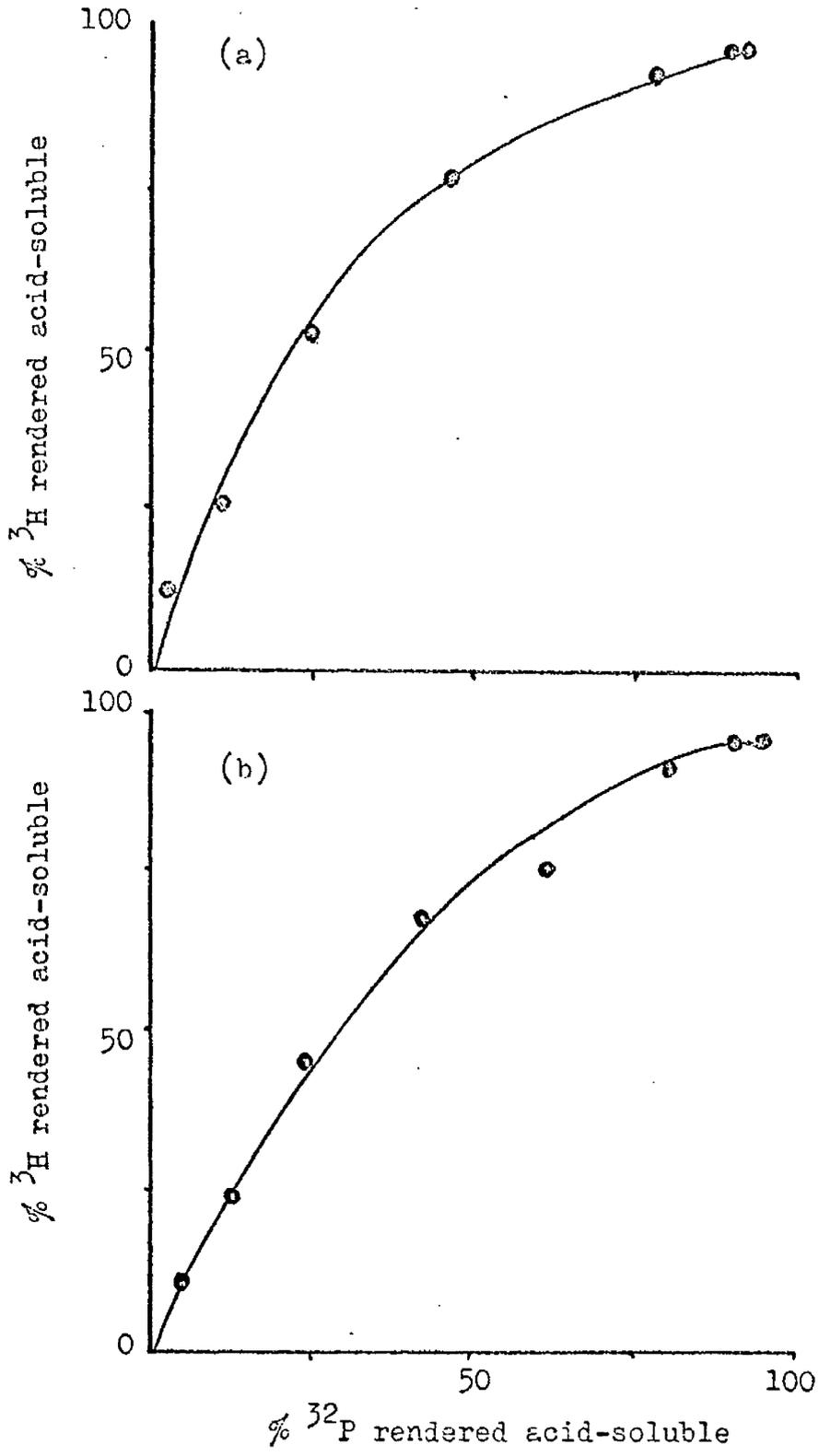
Fig. 111.41.

Comparison of the rates of release of  $^3\text{H}$  and  $^{32}\text{P}$  counts from the DNA products.

Sephadex-purified nuclear and supernatant fractions were used to form  $^3\text{H}$  DNA product from their preferred DNA primer as in the legend to Fig. 111.36. The extent of replication was 3 and 4% respectively. On this occasion Esch. coli  $^{32}\text{P}$  DNA was used as primer. The products were alkali-denatured and the relative rates of release of  $^3\text{H}$  and  $^{32}\text{P}$  counts into acid-soluble material determined during hydrolysis by exonuclease I.

- (a)  DNA product of nuclear DNA polymerase
- (b)  DNA product of supernatant DNA polymerase

Fig. 111.41.



Theoretically, if the  $[^3\text{H}]$  DNA is present at the 3'-termini of the DNA strands, it should be released before any  $^{32}\text{P}$  counts are rendered acid-soluble. However, Okazaki *et al.* (1968) have shown in studies to determine the direction of synthesis of short, single-stranded DNA intermediates that the size of the terminal label should be very small (less than 1% of the total length of the DNA chain) in order to obtain clear-cut kinetics. This phenomenon may reflect asynchrony of action by exonuclease III and DNA polymerase during degradation and repair synthesis of the original native DNA.

A further possibility is that contaminating endonuclease activity may influence the kinetics of release. Exonuclease I (DEAE-cellulose fraction) is known to contain endonuclease I activity but the presence of sRNA in the assay inhibits its action (Chapter 2, section 2.3.5. e).

A similar situation may prevail if the newly-synthesised DNA is not covalently attached to the DNA primer. In this case the rates of release of  $^3\text{H}$  and  $^{32}\text{P}$  would depend on the relative lengths of the primer and the newly-synthesised DNA strands.

### 3.3. Analysis of the DNA products on sucrose gradients

#### 3.3.1. Introduction

A partially-degraded DNA, prepared by limit digestion with

exonuclease III, can be restored to its original double-stranded structure by Esch. coli DNA polymerase (Richardson et al., 1964). The newly-synthesised DNA is covalently attached to the primer. DNA synthesis which follows the repair phase produces a structure, in which the newly-replicated DNA is not covalently attached to primer and which exhibits a highly-branched appearance in the electron microscope. Studies by Wake and Baldwin (1962) show that poly d(A Bu) produced in the replication of poly d(AT) is physically separable from the original primer.

Sucrose gradient analysis of the product of L 929 nuclear DNA polymerase on native templates is undertaken to observe whether a similar situation exists in these cells. The product of the supernatant enzyme, using denatured DNA, is also examined for comparison with previous data obtained with the calf thymus enzyme (Bollum, 1966).

### 3.3.2. Neutral and alkaline sucrose gradients

The DNA products of the Sephadex-purified nuclear and supernatant enzymes using their preferred primer are examined by high speed centrifugation in 5-20% sucrose gradients according to the method of Oishi (1968). In both cases the  $^3\text{H}$  counts are found directly under the optical density peak (Fig. 111.42).

Fig. 111.42.

Neutral sucrose gradient analysis of the DNA products of  
Sephadex-purified nuclear and supernatant L cell DNA poly-  
merases.

The products were synthesised as in Fig. 111.36 except that 600  $\mu$ g. of heat-denatured DNA primer was added per assay. 0.15 ml. of the above solutions (dialysed for 48 hr. against 4 x 100 vol. of 0.02 M-tris-HCl, pH 7.5) were layered on top of 5-20% sucrose gradients (5 ml., containing 0.02 M-tris-HCl, pH 7.5, 1 M-NaCl and 1 mM-EDTA) and centrifuged for 10 hr. at 24,000 rev. per min. (44,000 g) in the SW 39L rotor of a Spinco model L ultracentrifuge at 0-4<sup>o</sup>. Two-drop fractions were collected and analysed as described in Methods, section 2.1.3. b.



E<sub>260</sub>



acid precipitable [<sup>3</sup>H] DNA

- (a) DNA product of nuclear DNA polymerase
- (b) DNA product of supernatant DNA polymerase

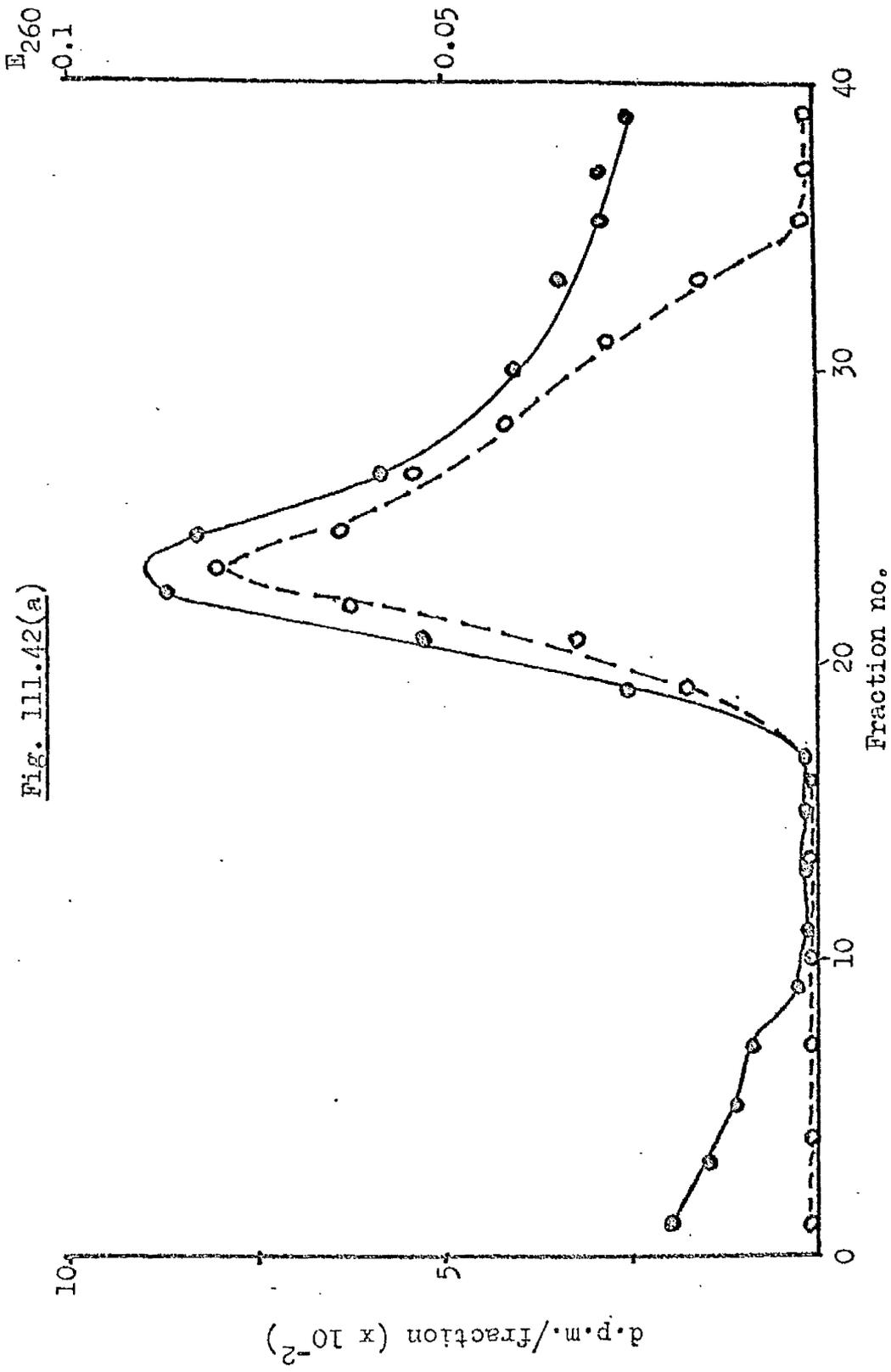


Fig. 111.42(a)

Fig. 111.42(b)

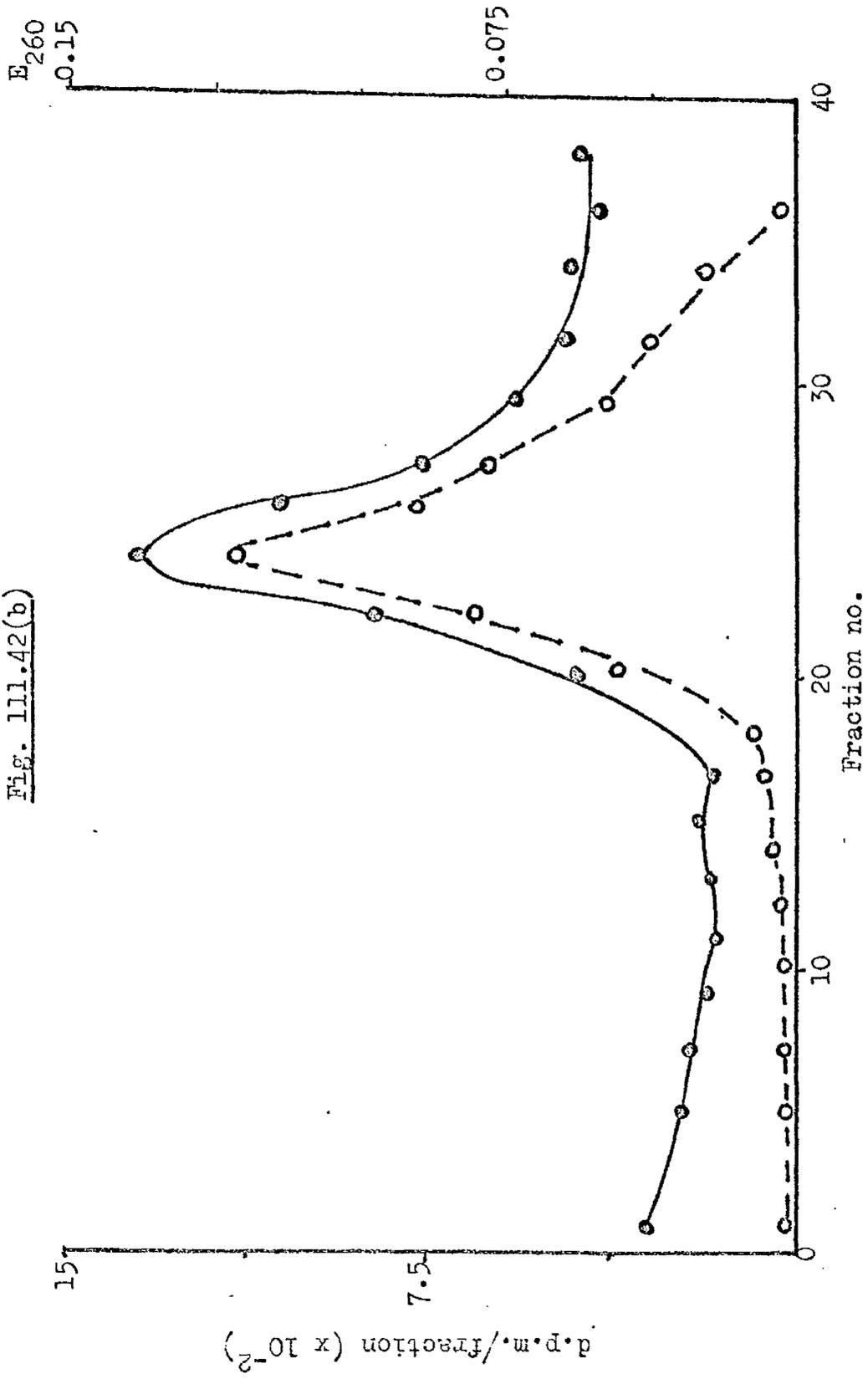


Fig. 111.43.

Alkaline sucrose gradient analysis of the DNA products of  
Sehadex-purified nuclear and supernatant L cell DNA poly-  
merases.

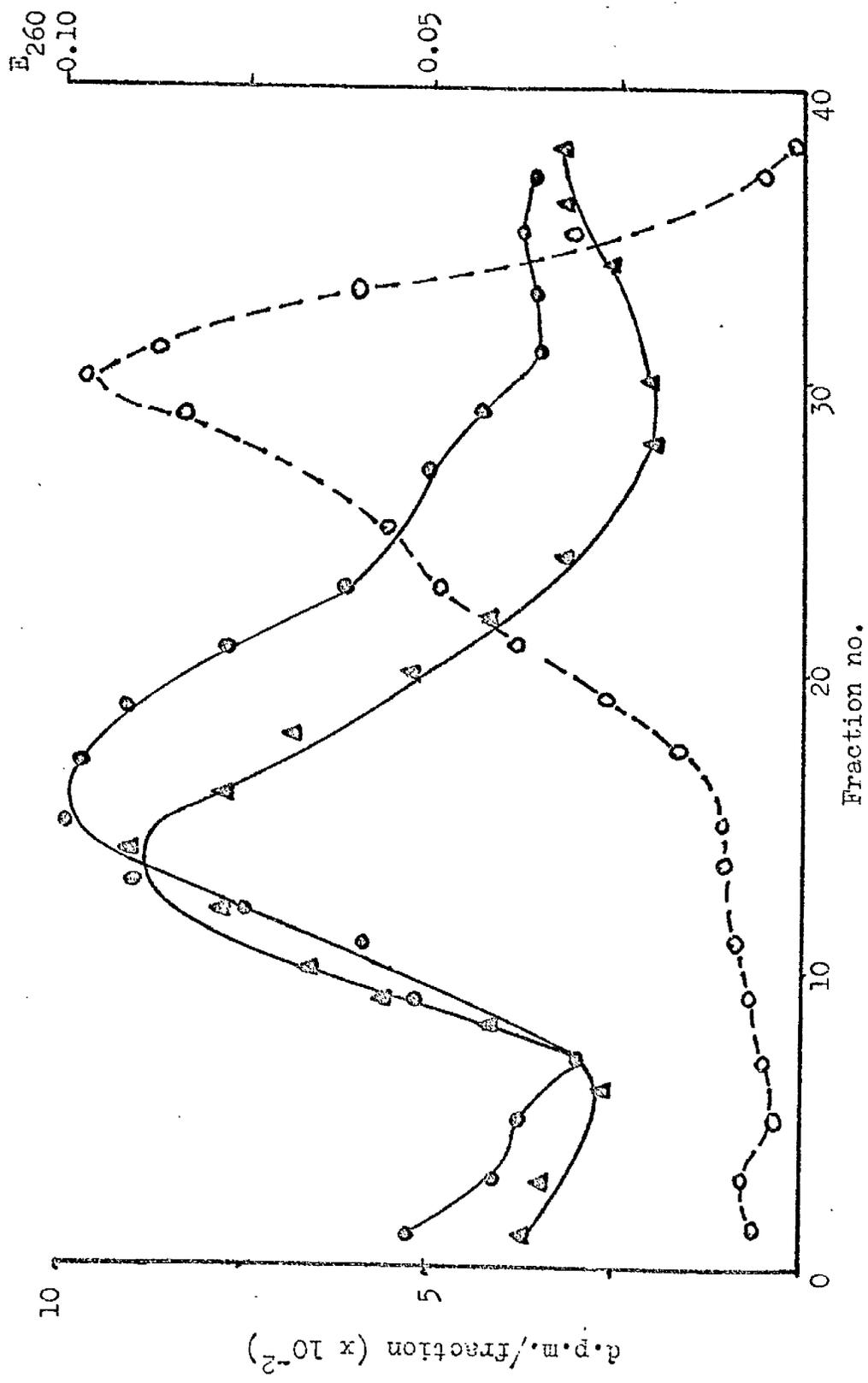
A sample of unincubated Esch. coli DNA was also used as control. The procedure was similar to that employed in the previous experiment with the following modifications:-

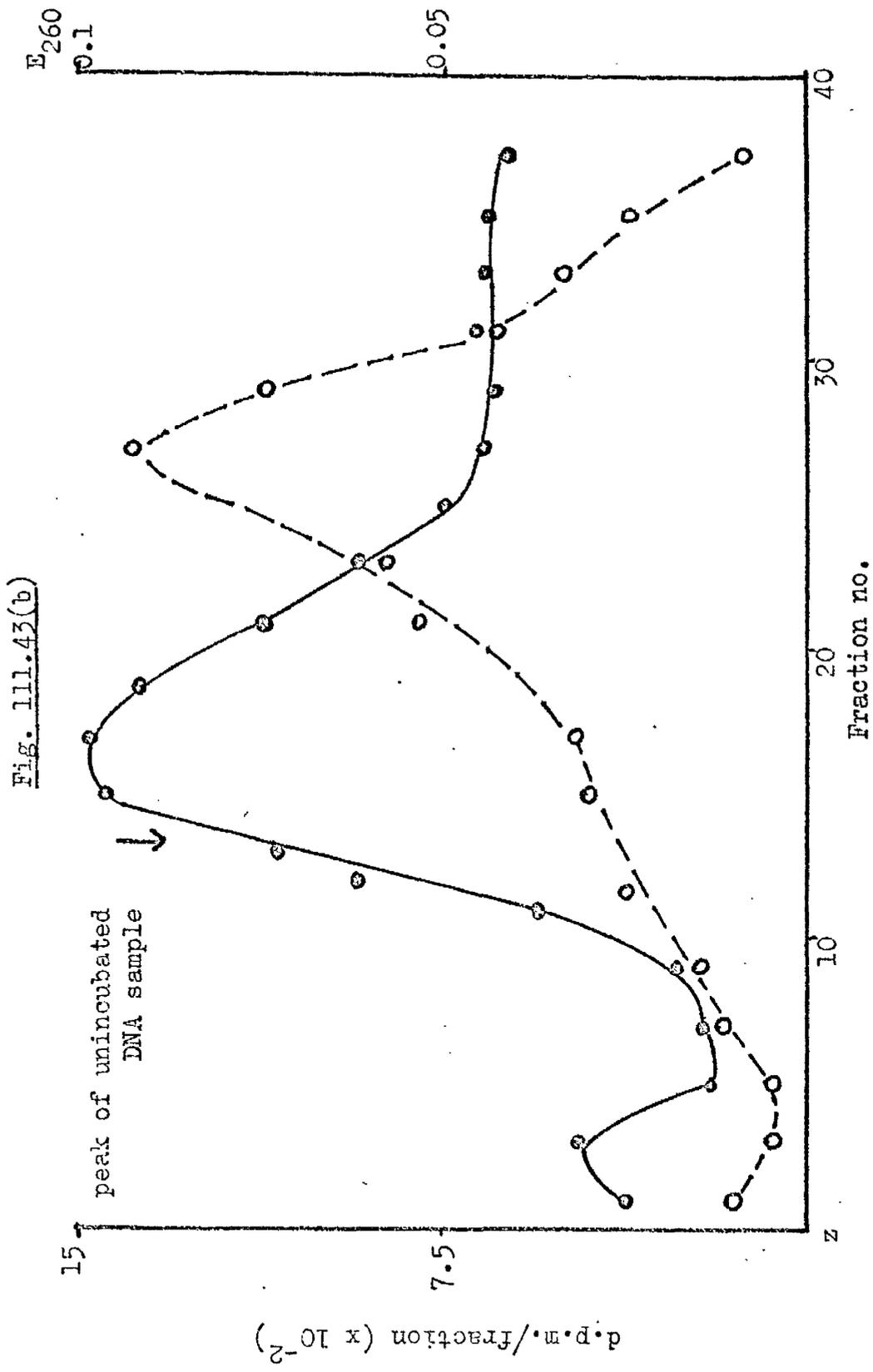
- (i) DNA samples were alkali-denatured before gradient analysis
- (ii) gradient contained 0.1 M-NaOH
- (iii) centrifugation was for 16 hr. at 32,000 rev. per min. (84,000 g)

●—● E<sub>260</sub> of DNA product  
▲—▲ E<sub>260</sub> of unincubated DNA  
○---○ acid precipitable [<sup>3</sup>H] DNA

- (a) DNA product of nuclear DNA polymerase
- (b) DNA product of supernatant DNA polymerase

Fig. 111.43(a)





With alkaline sucrose gradients, however, the bulk of the radioactivity sediments more slowly than the optical density profile, suggesting that the product of synthesis is not covalently attached to the primer DNA (Fig. 111.43).

Separation of the newly-synthesised DNA on single-stranded DNA is not surprising as the Sephadex-purified enzyme is known to contain contaminating oligonucleotide material (section 2.5.4.). In the case of native primed synthesis similar results are obtained to those on the Esch.coli enzyme. It thus seems unlikely that the activity on native templates is due to repair synthesis of single-stranded regions in the native DNA molecule. Preferential cleavage of the newly-synthesised DNA by contaminating endonuclease cannot be excluded in this case.

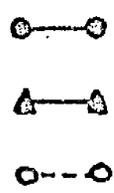
3.3.3. Sucrose gradient analysis of DNA products formed using L cell DNA polymerase (DEAE-cellulose column fraction)

Incubation of the DNA primer with Sephadex-purified DNA polymerase under polymerase assay conditions appears to cause little or no decrease in the mol. wt. of the DNA as judged by its position in sucrose gradients compared to a control DNA sample. Using a DEAE-cellulose fraction, however, extensive degradation

Fig. 111.44.

Alkaline sucrose gradient analysis of the DNA products formed by  
L cell DNA polymerase (DEAE-cellulose fraction).

$[^3\text{H}]$  DNA was synthesised as in Fig. 111.36 on both native and alkali-denatured DNA primers. Gradients were performed as described in the legend to Fig. 111.43.

  $E_{260}$  of DNA product  
 $E_{260}$  of unincubated DNA  
acid precipitable  $[^3\text{H}]$  DNA

- (a) DNA product using native DNA primer  
(b) DNA product using denatured DNA primer

Fig. 111.44(a)

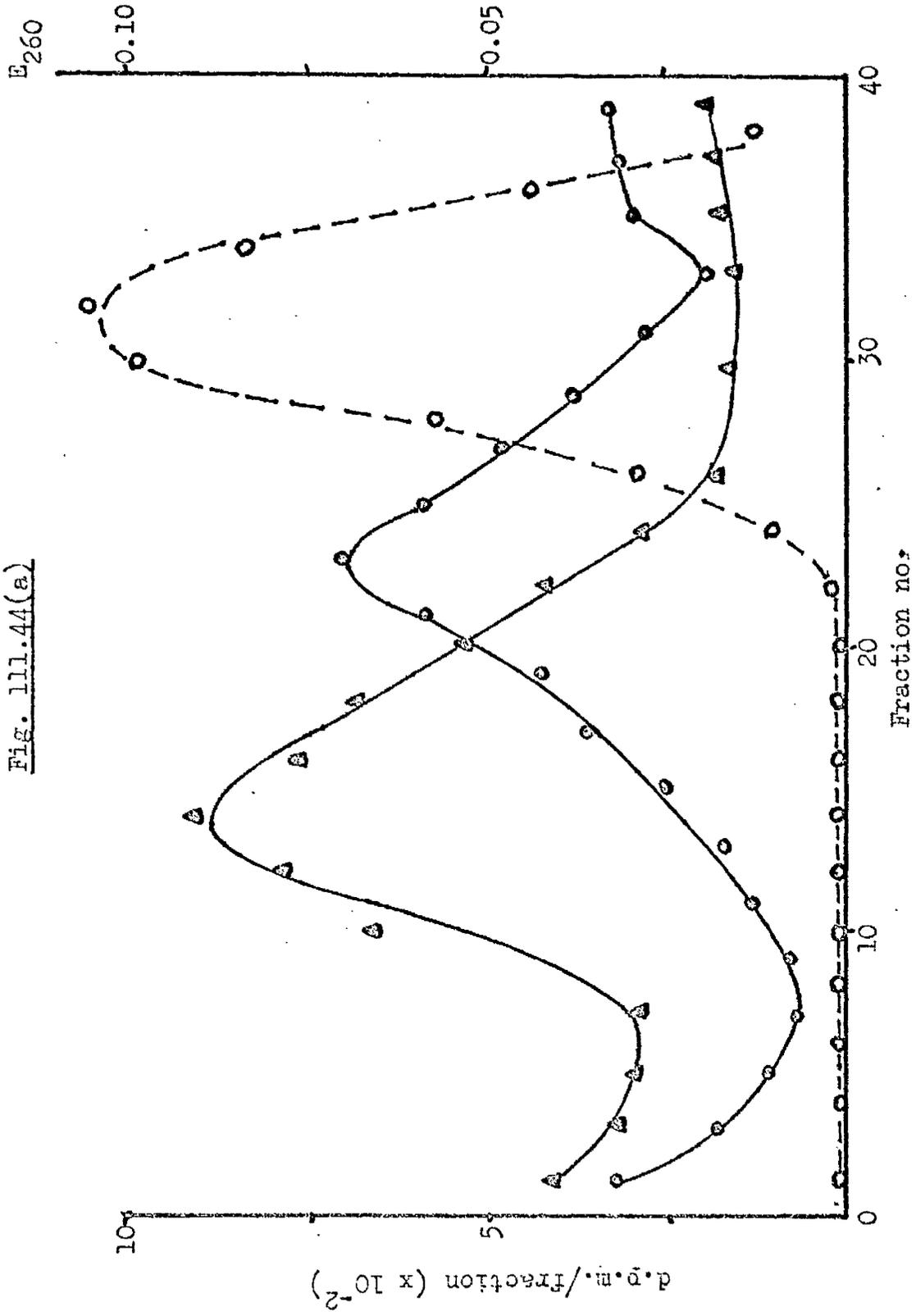
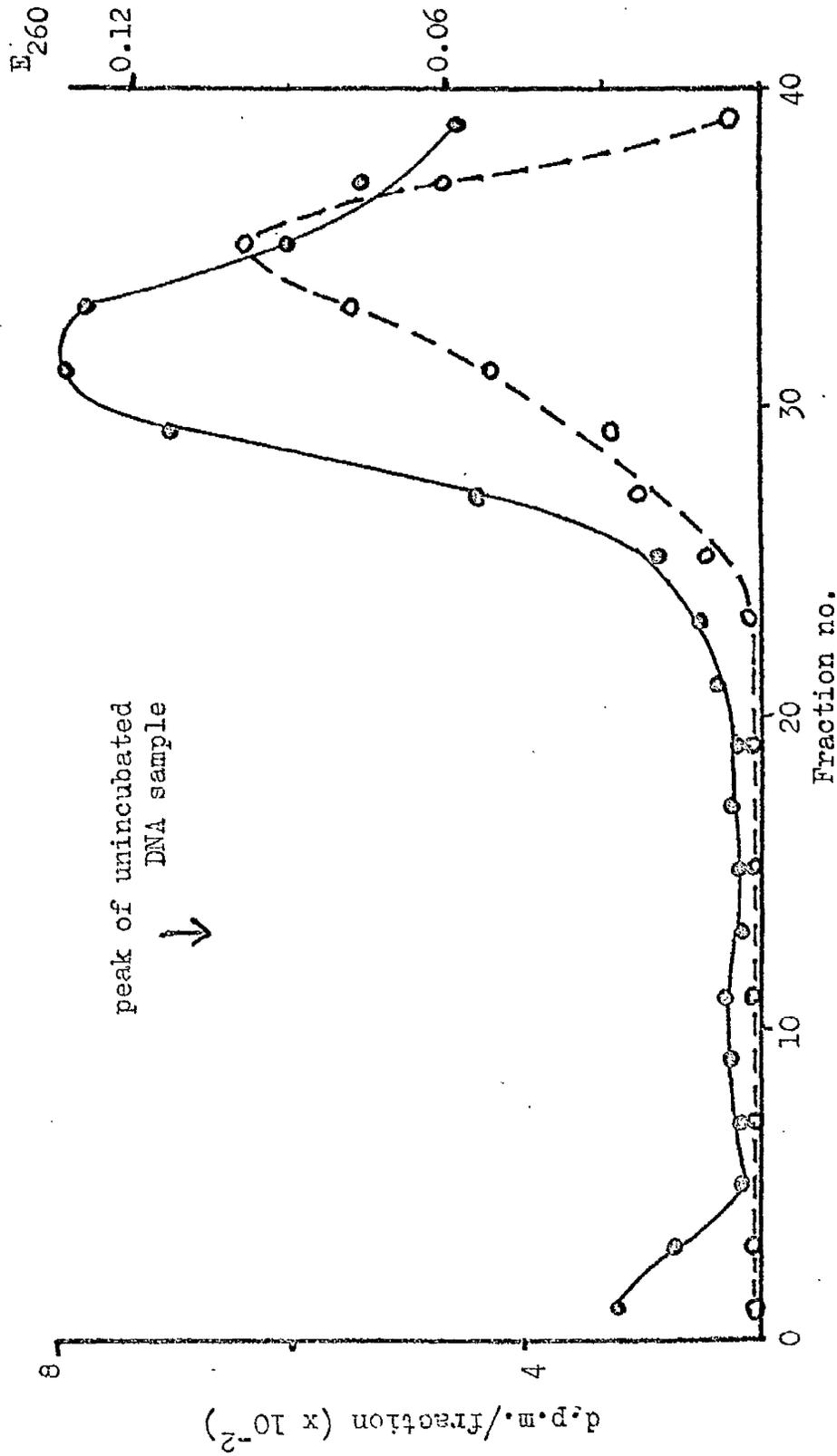


Fig. 111.44(b)



of the primer DNA is seen to occur although separation of the newly-synthesised DNA from the original native or alkali-denatured DNA primer is again evident (Fig. 111.44). It appears, therefore, that purification of the enzyme by pH 5 precipitation and gel filtration on Sephadex G200 gives a preparation which is relatively endonuclease free as found previously for the Landschütz ascites tumour DNA polymerase (Keir, 1965).

DISCUSSION

CHAPTER 4. DISCUSSION1. INTRODUCTION

The purpose of this section is to discuss various aspects of the processes involved in initiation and control of DNA synthesis with special reference to resting cell systems. Our findings on the changes of activity and apparent heterogeneity of DNA polymerase in L 929 cells are also reviewed in relation to our present ideas on the mechanism of DNA replication and our knowledge of the in vivo process.

1.1. Comparison of stationary L 929 cultures with primary resting cell systems

The original aim of this work was to establish a suitable system in tissue culture, as a parallel to the regenerating liver system, in which to define more closely the sequence of metabolic events required for eventual DNA synthesis in cells stimulated to renewed proliferation. Consequently several of the major features of resting L 929 cell populations were established.

(a) Stationary L 929 cultures have a greatly reduced capacity to synthesise DNA in comparison to rapidly dividing cells.

(b) Stationary populations, subcultured in fresh medium, begin to enter S phase after a lag phase of 16 hr. The degree of

synchrony obtained (approximately 70%) is comparable to that obtained with chemically-synchronised cultures.

(c) A high rate of RNA turnover is found in resting cells and no elevation in the rate of RNA synthesis occurs in released-stationary cells.

(d) RNA synthesis during the lag period is required for eventual DNA synthesis.

(e) Stationary cells have low DNA polymerase activity, primed by denatured DNA. Increases in these activities in nuclear and supernatant fractions are observed about the time of DNA synthesis.

(f) The amounts of nuclear, native DNA-primed activity are similar to those found in rapidly-growing cultures.

In several respects, therefore, stationary cultures of L 929 cells resemble the primary cell systems. However, important differences are also noted especially in relation to the rates of RNA synthesis during the lag period (c) and the levels of DNA polymerase activity (f) in stationary populations.

#### 1.2. RNA synthesis during the lag period

Although actinomycin D is primarily an inhibitor of DNA-dependent RNA synthesis, addition of low concentrations during early lag phase in regenerating liver or rabbit kidney cortex cells in vitro inhibits DNA synthesis while allowing normal RNA

turnover. This apparent contradiction is explained by the observation that actinomycin D prevents the rise in the rate of RNA synthesis which appears essential for subsequent DNA replication. In both cases once the new elevated rates of RNA synthesis are established, the cells become increasingly resistant to the effects of actinomycin D.

The effectiveness of the antibiotic in suppressing changes in RNA metabolism, without apparently affecting normal RNA turnover, suggests that certain genetic sites, at least some of which are required for eventual DNA synthesis, are exceptionally sensitive to the drug. Thus, it may be that induction of new species of messenger RNA molecules is this actinomycin D-sensitive process. Church and McCarthy (1967a, b), using DNA-RNA hybridisation techniques, have detected changes in the types of RNA formed during regeneration in mouse liver.

In contrast released-stationary L 929 cells show no increase in the rate of RNA synthesis during the lag phase. Moreover, no concentration of actinomycin D can be found which abolishes DNA synthesis while allowing continuation of the original rate of RNA turnover. However, RNA synthesis at this period is still required for the entry of cells into S phase.

Addition of actinomycin D also suppresses the rise in DNA polymerase activity in these cells which takes place about the time of DNA synthesis. Thus in stationary L 929 cultures, the induction of new RNA species required for DNA replication, may not be uniquely sensitive to the effects of the antibiotic.

High rates of RNA turnover have also been found in late passage confluent cultures of human diploid cells (more than 20 generations old). RNA synthesis in early passage cells, however, is markedly inhibited (> 90%) in high density populations (Levine et al., 1967). This phenomenon may reflect, therefore, a basic difference between cells in continuous culture and primary cell systems.

Contradictory results, however, have been reported for 3T3 mouse fibroblasts in culture which, under normal conditions, cease growing at low cell densities and are thought to be extremely sensitive to contact inhibition (Todaro, Lazar, and Green, 1965). After dilution of the cells in fresh medium there is a 10-fold rise in the rate of RNA synthesis within 30 min., followed at 2 hr. by increased protein synthesis. A small fraction of the cells divide some 28 hr. later. The sequence of events suggests that regulation of RNA synthesis is the means by which contact inhibition controls cell division. Evidence is presented by Holley and Kiernan (1968), however,

to show that exhaustion of essential growth factor(s) in the serum is responsible for the cessation of growth in confluent 3T3 mouse fibroblast cultures.

The differing results obtained may reflect the differing requirements of primary and established cell systems for growth-stimulating serum fractions. No primary cell systems have yet been observed to grow in protein-free synthetic media (Levintow and Eagle, 1961). Indeed some of these grow very poorly on synthetic media supplemented with serum protein alone and are markedly stimulated by extracts from adult or embryonic tissues. Moreover, Short, Zemel, Kanta and Lieberman (1969) have recently been able to enhance the rate of DNA synthesis in normal rat liver by the injection of bovine serum fractions (III and IV,  $\beta$ -globulins and  $\alpha$ -globulins. Cohn *et al.*, 1946). The stimulus for cell division after partial hepatectomy is known to be carried in the circulating blood (Moolten and Bucher, 1967; Lieberman, 1969).

Established cell lines, on the other hand, can be propagated in the complete absence of serum proteins (Rappaport, Poole, Bishop and Rappaport, 1960). Viral-transformed cells and late passage human diploid cells also show a reduced requirement for serum proteins to allow normal cell growth (Holley and

Kiernan, 1968; Levine et al., 1967). Thus the regulation of RNA synthesis, DNA replication and cell division in resting systems appears to be intimately related to the presence of stimulatory factors in the serum proteins. Further analysis is necessary to understand the manner in which these factors are utilised by the cell and solve the problem as to why established cell lines appear to show a reduced requirement for these proteins. Present evidence favours the interpretation that tissue proteins inhibit or compensate for metabolite loss in primary cell systems which are not yet well adapted to proliferation in vitro. However, the exact mechanism remains to be determined and awaits a more complete knowledge of the whole field of growth-promoting effects by serum proteins (see Harris, 1964).

1.3. DNA polymerase activity in released-stationary L 929 cells

Stationary cells were also remarkable in retaining high activities of nuclear DNA polymerase, primed by native DNA, while the supernatant denatured DNA-primed activity was only 5% of that found in rapidly-dividing cultures. The increase in activity in released-stationary cells and the changes in aminopterin-

or thymidine-synchronised cells apart from being due to de novo synthesis of the enzyme may be influenced by a number of other factors. Evidence for and against the role of these factors in determining the activity of DNA polymerase in crude extracts will now be discussed.

(a) External agents

- (i) removal of an inhibitor
- (ii) appearance of an activator

(b) Alteration of enzymic structure or association with other cellular components

- (i) changes in tertiary or quaternary structure
- (ii) masking of enzymic activity when in association with particulate cell components e.g. nuclear membrane

(c) Influence of nuclease activity

- (i) modification of the DNA primer
- (ii) degradation of the DNA product

The activation of existing DNA polymerase molecules by the presence of an activator or removal of an inhibitor appears to be ruled out by the requirement for RNA synthesis unless the activator is a protein which has to be synthesised de novo. This possibility is difficult to eliminate as such a regulatory process would be susceptible to the same inhibitors as enzyme synthesis.

Mixing experiments, however, fail to provide evidence for any stimulation caused by the presence of such a protein.

Similar reasoning tends to eliminate increases in activity brought about by alterations in the tertiary or quaternary structure of previously-formed enzyme (b, i). Experiments in which nuclear and supernatant extracts from cells in S phase were combined with similar preparations from stationary cells, in general gave super-additive results, although no greater than a 2-fold stimulation was observed. At first sight, this data appears to favour either a, i or a, ii; it is possible, however, that such effects may be explained in terms of a changing nuclease content in rapidly-growing cells (c, i or c, ii).

On the question of the masking of enzymic activity (b, ii), there is some evidence to suggest that DNA polymerase is less active in in vitro assays when it is present in a particulate fraction. A corollary of the investigations of Littlefield et al. (1963) on the changes of DNA polymerase activity in L cells during S phase is that the enzyme is only  $\frac{1}{10}$ <sup>2</sup>th as active when bound in particulate material.

In our nuclear preparations from L 929 cells, release of the enzyme into the high speed supernatant by sonication or extraction in 1.5 M-KCl gave 50-80% recoveries of the activity

measured in intact nuclei. Such results do not support the view that DNA polymerase is less active in a particulate state. Moreover, Adams and Lindsay (unpublished results) have recently shown that DNA facilitates the extraction of the enzyme from intact nuclei and that a large proportion of the activity of intact nuclei is released into the supernatant fraction on suspension of the nuclei in assay mixture.

Contaminating nucleases may influence the apparent activity of DNA polymerase by modification of the DNA primer (c,i) or hydrolysis of the DNA product to acid-soluble fragments (c, ii).

#### Exonuclease action

Furlong (1966) has shown that polydeoxyribonucleotides of chain length 7-9 units or greater are trichloroacetic acid-precipitable. Consequently measurement of the acid-soluble material formed from  $[^{32}\text{P}]$  DNA gives predominantly an estimate of the exonuclease activity of the preparation. In nuclear and supernatant fractions of L 929 cells, single-stranded DNA is preferentially rendered acid-soluble. Little or no release of acid-soluble material from native DNA occurs using these preparations. The differing primer requirements of nuclear and supernatant fractions in DNA polymerase assays would suggest

that there is no significant degradation of the DNA product. In support of this view, the products of L 929 cell DNA polymerase have been shown to be resistant to the action of exonuclease I (i.e. are not single-stranded) and would therefore be less susceptible to hydrolysis by exonucleases. In addition, partially-purified enzyme preparations, from which 90-95% of the contaminating exonuclease activity has been removed, exhibit similar primer specificities to the crude extracts.

#### Endonuclease action

Pretreatment of native DNA with pancreatic DNase markedly increases its effectiveness as a primer for nuclear extracts of L 929 cells when the primer is the limiting factor. With excess primer no increase in activity is observed. All assays were performed using saturating levels of DNA to prevent endonuclease action providing more initiation sites for the enzyme.

Measurement of DNase activity in aminopterin-, thymidine-synchronised and released-stationary cells has indicated that no significant variation in these activities occurs during S phase using either native or heat-denatured DNA as substrate. Mixing experiments, however, give evidence of some degree of activation (2-fold) of DNA polymerase. The cause of this activation is not clear at present. A similar effect has been observed after

infection of BHK 21 (C 13) cells by herpes simplex virus (Morrison, Ph.D. Thesis, University of Glasgow, 1967).

The above author has also shown that the main products of hydrolysis of native [ $^{32}\text{P}$ ] DNA by extracts of HEp-2 and BHK 21 (C 13) cells are oligonucleotides. We have confirmed this result in L 929 cells and shown that the Sephadex-purified enzyme is relatively free of endonuclease activity compared to crude extracts and to enzyme purified on DEAE-cellulose. Such preparations exhibit similar primer requirements to crude enzymic fractions.

Although there is no doubt that a variety of factors may influence the activity of DNA polymerase, there is no evidence to attribute the large changes of activity observed in synchronised cell populations to the operation of any of the above-mentioned processes. The results are most consistent with the view that de novo synthesis of DNA polymerase occurs about the time of DNA synthesis. Direct evidence for de novo formation will require purification of the enzyme to homogeneity in order to demonstrate its time of synthesis by pulse-labelling techniques.

#### 1.4. The relevance of changes in DNA polymerase activity during S phase to the control of DNA replication

Evidence has accumulated in recent years for the presence of DNA polymerase in both nuclear and cytoplasmic fractions of rapidly-growing cells. Such results have suggested the possibility

of a multi-unit structure for the enzyme, the native form of which is present in the nucleus during DNA replication. Several models for the structure of DNA polymerase as well as for the unwinding of the DNA helix have been proposed (Atwood, 1960; Fong, 1964; Sibatani and Hiai, 1964).

Recently Erhan (1968) has proposed a scheme incorporating many of the ideas of previous investigators. It is suggested that the key for the commencement of DNA synthesis is the formation of an initiator or "wedge" protein which is the last component of the replication complex to be synthesised during  $G_1$  phase. This protein would be responsible for locating the initiation site on the chromosome and initiating the unwinding of the DNA helix. After attachment of the initiator, the active subunits of DNA polymerase, formed previously in the cytoplasm, are added to complete the replication complex. When DNA synthesis is terminated, the complex dissociates and the enzymic subunits diffuse out into the cytoplasm where they are active on denatured DNA as primer using conventional assay procedures.

#### Evidence to support the above scheme

(a) Both nuclear and cytoplasmic extracts of mammalian cells contain DNA polymerase activity when the nuclei are isolated in non-aqueous media or in the presence of  $Ca^{2+}$  to prevent loss

of activity.

(b) The DNA polymerase activities of nuclear and supernatant fractions in mouse embryo and L 929 cells have differing primer requirements.

(c) During early cleavage division in sea urchin embryos, when DNA synthesis is extremely rapid, all the DNA polymerase activity is located in the nucleus.

(d) BHK 21 (C 13) cells, after infection with herpes simplex virus, show a 600% increase in the DNA polymerase activity of the nuclear fraction, the site of viral replication.

(e) In synchronised L cells, a marked decrease in the activity of the supernatant enzyme is observed during S phase while an increase in the activity of the nuclear fraction is also noted at this time.

Situation in L 929 cells

DNA polymerase activity, primed by native DNA, is high even in 10 day old stationary cultures of L 929 cells. Under these conditions the bulk of detectable enzymic activity is in the nucleus as the levels of incorporation in cytoplasmic extracts with denatured DNA primer are only 5-10% of the specific activity found in randomly-growing cultures. The presence of normal amounts of native DNA-primed DNA polymerase in stationary cells does not support the concept that the "intact" enzyme is

only situated in the nucleus during DNA replication. In the model of Sibatani and Hiai (1964) 2 forms of the enzyme are postulated to carry out replication of the DNA duplex in a co-ordinated fashion. The DNA strands of non-replicating chromosomes are bridged at the origin by a DNA polymerase molecule of either form which serves as the starting point for DNA synthesis.

Both the native and denatured DNA-primed enzymes have similar stability as judged by heat inactivation studies (Adams and Lindsay, unpublished results) and are markedly stabilised by the presence of DNA. It may be that the intranuclear location of the enzyme, primed by native DNA, and its possible attachment to the nuclear DNA confers great stability on the in vivo activity. Patel et al. (1967) have found a similar enzyme in the non-histone fraction of normal rat liver nuclei which exhibits a requirement for native DNA and we have confirmed this result.

The time course of events following partial hepatectomy and the release of stationary L 929 cells is very similar. In cultured mouse cells major increases in the activity of DNA polymerase with denatured DNA primer occur during this period of rapid growth. Recently Iwamura et al. (1968)

have shown that DNA polymerase activity in regenerating rat liver primed by single-stranded DNA, closely parallels the in vivo rate of DNA synthesis. The opposite result to this was obtained by Mukundan et al. (1963) who observed an increased activity with double-stranded primer in rat liver after partial hepatectomy. The reason for this discrepancy is not known at present.

As the activity of the native DNA-primed enzyme does not alter markedly in either regenerating liver or L 929 cells in response to increased DNA synthesis, this might argue against its participation in the in vivo process; however, its location in the nucleus, the site of DNA replication, and its preference for double-stranded DNA makes it a likely candidate for this role. Thus its constant activity during S phase may represent a balance between the breakdown of used enzyme and its replacement with newly-synthesised enzyme. The decrease in supernatant activity during DNA synthesis may reflect an increased demand by the nucleus for a reserve of enzyme. Indeed S phase nuclei are found to exhibit higher DNA polymerase activity with denatured DNA as primer. The mode of binding of this activity to the nucleus and its cytoplasmic origin have not been proved.

It is tempting to speculate at this stage, in agreement with the model of Erhan (1968), that the active subunits of DNA

polymerase are formed in the cytoplasm (where the preferred primer is single-stranded DNA) and are subsequently transported to the nucleus where they function as a precursor of the native DNA-primed enzyme. This may then represent the intact DNA-synthesising complex.

In stationary cell experiments, a fall in the supernatant activity was not observed during S phase although no further increase occurred at this time. While this difference might be explained in terms of the different techniques employed to obtain synchronous cultures, it seems likely that this discrepancy is a reflection of the better synchrony obtained using aminopterin- or thymidine-blocked cells, thus allowing greater definition of the transient changes in the levels of the enzyme during S phase.

Using chemically-synchronised cells, addition of puromycin (25  $\mu\text{g}$ . per ml.) at the time of reversal does not prevent the fall in the activity of the supernatant fraction. The activity does not fall to zero, however, as might be expected if the supernatant were the source of DNA polymerase required for DNA synthesis. Adams and Lindsay (unpublished results) have also shown that puromycin is equally inhibitory to DNA synthesis when added at the beginning of S. Thus the impairment of DNA synthesis may prevent the normal uptake of enzyme into the nucleus at this time.

The equivocal results of this experiment do not permit us to define the supernatant enzyme as the source of the increased levels of nuclear activity with denatured DNA during S phase.

Addition of puromycin 2 hr. after the beginning of S also inhibits the subsequent rise in the activity of supernatant enzyme as the rate of DNA synthesis declines. Hence, it appears that this rise represents de novo synthesis of DNA polymerase during the latter half of S phase, although the possibility that dissociation of a replication complex and transport of the enzyme subunits requires the aid of a puromycin-sensitive event cannot be excluded.

### Conclusions

In most cell types, micro-organisms as well as mammalian systems, variations in the activity of thymidine kinase, DNA polymerase and a host of other enzymes are closely linked with events in the life cycle of the organism. On the basis of the findings presented in this section and elsewhere it is clear that a close temporal relationship exists between the formation of enzymes involved in DNA synthesis and the period of DNA replication. The mere fact that these events take place at closely related times does not prove that one exercises control

over the other. Indeed it is known that increases of activity of DNA polymerase occur in the absence of DNA synthesis.

On the other hand, it seems likely that the initiation and control of DNA replication is dependent on a whole series of factors apart from the activity of the enzymes which are concerned in its synthesis. To quote Stone and Prescott (1965) "The appearance of these enzymes, required for the synthesis of DNA, only at the beginning of S serves as an example of the type of controlled derepression which must be operating in continuous sequence to maintain the orderly progress of biosynthetic events which make up the cell life cycle."

1.5. The apparent heterogeneity of DNA polymerase activity in L cell extracts

The above proposed multi-unit structure for DNA polymerase implies the existence of more than one enzymic species which has catalytic activity. The DNA polymerase activity in extracts of L 929 cells was submitted to fractionation by several methods. Of these, gel filtration on Sephadex G200 was the most extensively studied; the enzyme was also found to be heterogeneous by DEAE-cellulose chromatography and polyacrylamide gel electrophoresis. Several possibilities will now be considered concerning the significance of these separations.

Possible interpretations of the apparent heterogeneity L 929  
cell DNA polymerase

- (a) Differential binding of DNA polymerase to contaminating DNA fragments.
- (b) Differential binding to proteins or nuclear material e.g. nuclear membrane.
- (c) Contamination by mitochondrial DNA polymerase.
- (d) Contamination by end terminal addition enzymes.
- (e) Effects of nuclease activity.

(a) As KCl extracts of nuclei gave better resolution of the enzymic activities on Sephadex G200 than extracts prepared by sonication, it was thought that this difference might be attributable to the higher DNA content of the sonicated preparations.

In support of this idea, omission of KCl from the column buffer caused all the enzymic activity in sonicated extracts to be eluted at the void volume along with the contaminating DNA.

In conditions of low ionic strength a large fraction of the ascites tumour DNA polymerase is bound to DNA but in the presence of 0.15 M-KCl no association with DNA is detectable on sucrose gradients (Adams, C.J., M.Sc. Thesis, University of Glasgow, 1969).

These results are in agreement with those of Kornberg's group who find that the binding of Escherichia coli DNA polymerase to

poly d(AT) is strongly inhibited by 0.2 M-potassium phosphate.

Further analysis of sonicated preparations from L 929 cells on Sepharose 4B columns (exclusion limit,  $3 \times 10^6$ ) gave only one peak of enzymic activity in fractions 38-45. As the bulk of the DNA present eluted at the void volume (fraction 17), there was no evidence for the formation of a DNA-DNA polymerase complex in the presence of 0.15 M-KCl. Thus, while there is no doubt that the enzyme can bind to DNA in solutions of low ionic strength, our data would suggest that the presence of DNA is not influencing the pattern of elution under the conditions employed.

(b) The better resolution of activity obtained using KCl extracts of nuclei compared to sonicated preparations may also be explained in terms of the different procedures used for extraction of the enzyme. The relative difficulty of extraction of the native DNA-primed activity from L 929 cell nuclei implies its close association with other cell components e.g. the nuclear membrane. Treatment of the nuclei with 1.5 M-KCl may bring about release of the enzyme from its binding site or complex in the nucleus while sonication of nuclei produces fragments of various sizes without causing efficient breakdown of the bound DNA polymerase complex.

The presence of 2 peaks of activity with native DNA on Sephadex G200 after KCl extraction of nuclei may also represent the incomplete dissociation of the enzyme from its binding with other nuclear components. This argument is weakened because the elution patterns were unaltered when the nuclear extracts were run in 1 M-KCl. The possibility still exists, therefore, that peak II is a subunit of peak I which is normally present in the cell and has a functioned role in DNA replication.

(c) Although the rat liver mitochondrial DNA polymerase has recently been partially purified and shown to prefer native DNA primer, it seems improbable that the activity in either peak I or peak II could be caused by the presence of a similar enzyme in extracts of L 929 cells.

(i) Contamination of the nuclear fraction by mitochondrial material has been found to be extremely low.

(ii) The reported activities for the mitochondrial enzyme in crude extracts are much lower than for the corresponding nuclear DNA polymerase.

(iii) The rat liver mitochondrial DNA polymerase is unusual in being markedly stimulated (8-fold) by the presence of  $K^+$ . The nuclear enzyme from L 929 cells resembles the rat liver nuclear DNA polymerase activity which is only enhanced 2-fold by optimal concentrations of  $K^+$ .

(d) Yoneda and Bollum (1965) have separated the calf thymus DNA polymerase from a terminal addition enzyme on Sephadex G150 and shown the latter activity is of lower mol. wt. This raises the possibility that peak II may represent contaminating end terminal addition activity in L 929 cell extracts. Several lines of evidence, however, weaken this point of view:-

(i) Other terminal addition enzymes investigated (Krakow et al., 1962; Yoneda and Bollum, 1965; Keir and Smith, 1963; Wang, 1968; and Rothschild, Halpern and Smith, 1968) are found to require heat-denatured DNA as primer while peak II is only active with native DNA.

(ii) The firm association of peak II activity with the nuclear fraction agrees with the results of Rothschild et al. (1968) who find the nuclear fraction of Walker 256 carcinoma cells contains mainly replicative activity while most of the end terminal addition enzyme is located in the supernatant fraction.

(ii) Two other groups of investigators (Bellair, 1968; Iwamura et al., 1968) have recently achieved similar separations of DNA polymerase activity from normal rat liver. The latter workers were also unable to detect any peak II activity in extracts of calf thymus cells which contain the end terminal addition enzyme.

(e) Influence of nuclease activity

It is quite conceivable that endo- or exonuclease activity in our preparations may influence the activity as well as the preference for DNA primer of the enzyme by modification of the primer DNA. However, experiments in which fractions containing peaks of activity with double- or single-stranded DNA were combined with other fractions from the same column did not provide evidence for the presence of external agents which could alter the primer specificity or the activity of the purified enzyme(s). In addition, no correlation between the distribution of nuclease and DNA polymerase on the columns was observed.

Conclusions

The available evidence thus points to the possibility of a multi-unit structure for DNA polymerase in rat liver and L 929 cells. It remains to be proved, however, if these activities represent individual enzymic species each having a special function in the replication process. Recently Ono and Umehara (1968) have reported the resolution of peak I in rat liver into two active fractions on phosphocellulose columns. Both these peaks are more active on single-stranded DNA but one peak appears to be located only in the nucleus. In peak I from L 929 cells we have been able to show that the activity, primed by denatured

DNA, is more sensitive to freeze/thawing than the native-DNA primed activity. No further fractionation of peak I has yet been achieved. Alternatively these individual species may represent artefacts of isolation formed by the breakdown of a supra-molecular, DNA-synthesising unit.

#### 1.5. Possible mechanisms of DNA replication

The inability of in vitro DNA polymerases to replicate native DNA has led to the development of several hypotheses in an attempt to overcome the problem.

(a) The finding in Bacillus subtilis of an enzyme fraction which catalysed the formation of deoxythymidine 3'-triphosphate from deoxythymidine 3'-monophosphate caused speculation on the possible role of the 3'-triphosphates as precursors in replication. No reports on the successful incorporation of 3'-triphosphates into DNA have appeared to date.

(b) Recent evidence implies that the process of DNA replication in bacteria takes place in a discontinuous manner as short, single-stranded pieces have been detected after pulse-labelling of growing cells. Hypotheses invoking the involvement of polynucleotide ligase in joining these DNA segments to the main body of the chromosome have been proposed (Okazaki et al., 1968; Mitra et al., 1967). These theories suggest that both DNA

strands are synthesised in the 5'-3' direction and the former group have shown this to be the case. Such a process would, of course, nicely bridge the gap between in vivo evidence on DNA replication in bacteria and the in vitro characteristics of DNA polymerase.

Although polynucleotide ligase activity has also been detected in mammalian cells, no substantial evidence is available to suggest that discontinuous DNA replication occurs in higher organisms. Short-stranded intermediates have been found during DNA synthesis in regenerating liver. However, these DNA segments are resistant to exonuclease I attack. These results have been criticised on the grounds that pulse-labelled DNA will always appear to have a lower mol. wt. than fully-labelled DNA when shearing produces a population of DNA molecules of different lengths. Furthermore high resolution electron microscopy of replicating DNA from L-5178Y murine lymphoma cells has revealed the presence of characteristic branch points. No localised strand separation or other structural alteration was discernible at these points (Coleman and Okada, 1968).

(c) The failure of isolated DNA polymerases to catalyse simultaneous synthesis of both DNA strands may reflect the disruption

of the structural organisation of the replication complex during isolation such that its components are still able to fulfil some aspects of their in vivo function. A parallel situation exists in mitochondria where electron transport particles can be dissociated into 4 active complexes which can be reconstituted to carry out the complete processes of oxidative phosphorylation.

Proof of such a concept requires the purification and characterisation of the various activities fractionated on Sephadex G200 as it is important to establish whether these represent separate species of enzyme or whether they are components of a partially-dissociated multi-enzyme system, each concerned with a particular aspect of the replication process. The high mol. wt. of the DNA polymerase activity (it elutes ahead of apoferritin, mol. wt. 480,000, Gaba and Adams unpublished results) and the similar responses of the two activities to changes in the assay conditions suggest that they may be part of a single DNA-synthesising unit. Preliminary attempts at interconverting these activities by treatment with urea, guanidinium chloride, amylase and phospholipases have been unsuccessful.

Apart from maintaining the structural integrity of the replicating enzyme system, it is probable that the organisation of the genetic material within the nuclear mass in bacteria or

the chromosomal framework in eucaryotic cells is important in the unwinding of the double helix, in the process of replication and in the eventual segregation of the 2 daughter molecules.

In bacterial systems the enzyme system responsible for replication is attached to a point on the inside of the cell membrane and the mode of replication appears to be semi-conservative (see Ganesan, 1968). Moreover, nascent DNA also appears to be bound to the cell membrane (Ganesan and Lederberg, 1965) as proposed by Jacob et al. (1963). Lark et al. (1967) have also shown that the replicating Esch. coli chromosome is attached to the cell membrane and suggests that this mechanism is required in the ordered segregation of the 2 daughter chromosomes. It is difficult to envisage that all these elements of control are retained in in vitro systems.

The DNA in mammalian cells is reported to be attached to many points on the interior of the nuclear membrane (Du Praw, 1965) although it has not yet been possible to identify the sites of replication with any specific location within the nucleus. Friedman and Mueller (1968) have observed that nascent DNA from HeLa cells appears at the interface during phenol extraction and attribute this anomaly to the presence of a cellular component, containing lipo-polysacchride material, at or near the

site of replication. Recently Comings and Kakefuda (1968), in a heteroploid line of human amnion cells, have observed by electron microscope studies, that in short pulse-labelling of the cells with [ $^3\text{H}$ ] thymidine at the beginning of S, all the grains are located in the area of the nuclear membrane.

Duplication of its genetic material is probably, therefore, the most complex biosynthetic function that the cell has to fulfil. Thus it is conceivable that simultaneous replication of both strands of the DNA duplex cannot be achieved in vitro because of loss of organisation in the DNA-synthesising complex itself or in the accessory mechanisms required for the unwinding of the helix and separation of the newly-formed strands.

The theories of discontinuous DNA replication would also appear to demand a high degree of organisation for the machinery of the replication process, involving DNA polymerase, polynucleotide ligase and a specific endonuclease. With the discovery of the heterogeneity of DNA polymerase activity in mammalian cells, having different locations in the cell and with different primer specificities, the most promising approach for the present seems to be in the study and characterisation of these species with the eventual aim of relating their activities to the overall mechanism of DNA synthesis.

SUMMARY

SUMMARYThe Control of DNA Synthesis in L Cellsby J. Gordon Lindsay

Summary of the thesis presented for the degree of Doctor of  
Philosophy, University of Glasgow, October 1969.

Regenerating liver, rabbit kidney cortex cells cultured in vitro and PHA-stimulated lymphocytes have been widely employed to study the sequence of metabolic events which is required for the onset of DNA synthesis and cell division when resting cells are stimulated to renewed proliferation. A parallel resting cell system has been established with a permanent cell line by maintaining cultures of L 929 cells at high population density. Such a system is particularly suitable for study because of the ease of culture manipulations and the relatively high degree of synchrony obtained.

Released-stationary cells begin to synthesise DNA after a lag period of 14 hr. and by 20 hr. 70% of the cells are in S phase. Increases in cell number are observed by 25 hr. In contrast to primary resting systems no change in the rate

of RNA synthesis is detected after release from stationary phase. RNA synthesis during the lag phase is required for subsequent DNA synthesis.

Changes in the activity of DNA polymerase in nuclear and supernatant fractions of L cells have been investigated following release from stationary phase and particularly during S phase. The results of previous investigators have been confirmed and extended.

Nuclear preparations of L 929 cells show a 2-5-fold preference for native DNA primer while the supernatant DNA polymerase activity is correspondingly more active with denatured DNA. The general characteristics of the DNA polymerase(s) in these fractions have been investigated and methods for releasing the enzyme from isolated nuclei studied.

Preliminary purification of the enzyme was undertaken although difficulties were encountered because of the small amounts of material available from tissue culture cells. A 7-8-fold purification was achieved by pH 5 precipitation and Sephadex G200 chromatography and 95% of the DNase activity removed as judged by the relative capacity of the fractions to hydrolyse native or heat-denatured DNA to acid-soluble fragments. After purification DNA polymerase activity, primed by denatured

DNA, was rapidly lost on freeze/thawing of the solution.

DNA polymerase activity in extracts of L 929 cells was found to be heterogeneous by fractionation on Sephadex G200, DEAE-cellulose and polyacrylamide gels. As similar results have recently been reported for the rat liver enzyme the possible significance of this result in relation to in vivo replication is considered. The nuclear location of a fraction of the DNA polymerase activity and its preference for native DNA primer makes it a possible candidate in this respect.

Sephadex-purified nuclear and supernatant fractions have been used to synthesise [ $^3\text{H}$ ] DNA on their preferred primers and the characteristics of the DNA products examined. Both products were found to be resistant to degradation by exonuclease I suggesting that the newly-synthesised DNA was not present in a single-stranded form. Analysis of the DNA products on neutral and alkaline sucrose gradients reveals that the [ $^3\text{H}$ ] DNA is not covalently attached to the DNA primer. Our findings on the characteristics of the DNA products are compared to previous data on the DNA products formed by the calf thymus and Escherichia coli DNA polymerases.

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