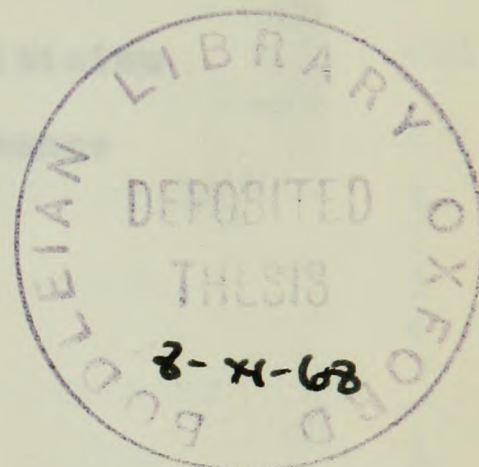


A STUDY OF THE INFLUENCE OF HORMONES
ON THE SYNTHESIS OF RIBONUCLEIC ACIDS AND PROTEINS
IN ANIMAL TISSUES



A thesis submitted for the Degree of
Doctor of Philosophy

by

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Trinity Term
1968

ACKNOWLEDGEMENT

I am deeply grateful to my supervisor,
Dr. J. M. Barry, for his advice, criticism
and encouragement during the performance
of this work and the preparation of
this thesis.

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ABSTRACT

Part One of this thesis describes studies on the counter-current distribution of rapidly-labelled RNA. Other workers had previously reported that several hormones, when injected into rats, altered the counter-current distribution profiles of the rapidly-labelled RNA isolated from their livers. It was hoped to use counter-current distribution, in this thesis, to study the RNA synthesised by mammary gland slices incubated with different hormones.

Initial experiments were performed to see if hormones influenced the counter-current distribution profiles of rapidly-labelled RNA formed in liver slices. Double-labelling was employed to eliminate differences in isolation and fractionation of control and experimental RNA: control slices were incubated with (^{14}C)adenine, and experimental slices with the hormone and (^3H)adenine. The slices were then combined for RNA isolation and fractionation and the ^{14}C and ^3H in each fraction measured simultaneously. However it was found that addition of corticosterone (50 $\mu\text{g./ml.}$), insulin (5 $\mu\text{g./ml.}$), oestradiol (5 $\mu\text{g./ml.}$) or triiodothyronine (5 $\mu\text{g./ml.}$) did not significantly alter the counter-current distribution profiles.

Attempts were then made to repeat some of the experiments in which hormones had been reported to produce marked profile changes in vivo. However it was found that intraperitoneal injections of hydrocortisone (5 mg.) for 50 min., insulin (1 mg.) for 35 min. or thyroxine (30 μ g.) for 35 min. did not alter the counter-current distribution profiles of liver RNA, labelled by a 20 min. pulse of radioactive orotic acid.

The rapidly-labelled RNA from liver slices, which was isolated mainly with ribosomal RNA (Method A), travelled more slowly on counter-current distribution than that from whole livers, which was isolated mainly with DNA (Method B). Since these large differences in profile resulted from different methods of isolation, it appeared possible that in previous experiments, where double-labelling was not used, differences between control and experimental profiles might have resulted from small and unavoidable differences in isolation.

Experiments were performed to see if the difference in profile between RNA isolated by Methods A and B was due to the rapidly-labelled RNA complexing with different nucleic acid species. However addition of DNA to material isolated by Method A, or ribosomal RNA to that isolated by Method B, did not alter the profiles, nor did digestion with deoxyribonuclease of material isolated by Method B.

Experiments were designed to test the suggestion that rapidly-labelled RNA was separated on counter-current distribution into fractions of differing base composition, those rich in adenine travelling faster than those rich in guanine. In the first experiment slices of rats' liver were incubated for 30 min. with (^3H)guanosine and (^{14}C)adenine. Despite differential labelling of the two bases, the (^3H) and ^{14}C profiles did not differ significantly. A second experiment used the fact that nuclei, isolated from rats' liver, synthesise RNA with a lower ratio of G+C:A+U in the presence of ammonium sulphate than in its absence. Rat liver nuclei were incubated for 30 min. with (^3H)ATP or with (^{14}C)ATP and ammonium sulphate (0.28 M) and the RNA isolated. In spite of different base compositions, the ^3H and ^{14}C counter-current distribution profiles were again superimposed. It was concluded that base composition is not the basis of fractionation of rapidly-labelled RNA.

When rapidly-labelled RNA, prepared by Method A, was stored at 2°C for 8 months, its speed of movement on counter-current distribution increased. Treatment of rapidly-labelled RNA with pancreatic ribonuclease also increased the speed of movement. It was concluded that the different fractionation of RNA prepared by Methods A and B was probably due to different degrees of degradation by an intrinsic ribonuclease.

In Part Two of this thesis, increases in the activity of mammary gland glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which occur in the living animal at parturition, have been reproduced in vitro for the first time. Some investigations of the biochemical basis of these changes have therefore been possible and the effects of various hormones and inhibitors of nucleic acid and protein synthesis on these enzyme increases are described.

Initial experiments showed that, as in rats, the activity of these enzymes in the mammary gland in vivo rose slowly during pregnancy but more sharply at parturition. The RNA and DNA contents of the glands also increased during pregnancy and lactation.

A culture method was developed in which 20 explants of mammary tissue were floated on the surface of defined medium "199" in sterile petri dishes. When explants from late-pregnant (day 19) mice were incubated with insulin (5 μ g./ml.) for 22 hr. the activity of glucose 6-phosphate dehydrogenase increased by 84% and that of 6-phosphogluconate dehydrogenase by 64%. Without insulin the activities of both enzymes fell.

In experiments in which the rate of appearance of glucose 6-phosphate dehydrogenase was followed, it was

found that there was no increase in activity during about the first 12 hr., but during the next 12 hr. the enzyme activity increased sharply.

If prolactin (10 $\mu\text{g.}/\text{ml.}$), corticosterone (1 $\mu\text{g.}/\text{ml.}$) or prolactin + corticosterone were added to 22 hr. cultures in addition to insulin, the glucose 6-phosphate dehydrogenase activity did not increase further. However in 45 hr. cultures with insulin or insulin + corticosterone the enzyme activity fell below the 22 hr. level, whereas with insulin + prolactin or with insulin, prolactin and corticosterone the 22 hr. level was approximately maintained. Change of medium or further addition of prolactin (100 $\mu\text{g.}/\text{ml.}$), growth hormone (10 $\mu\text{g.}/\text{ml.}$), triiodothyronine (5 $\mu\text{g.}/\text{ml.}$), oestradiol (0.05 $\mu\text{g.}/\text{ml.}$) or oxytocin (10^{-5} units/ml.) did not cause any further increase in enzyme activity.

In experiments to see if the increase in glucose 6-phosphate dehydrogenase activity in vitro was due to the removal of the tissue from inhibitory hormonal influences it was found that neither oestradiol (1 $\mu\text{g.}/\text{ml.}$), progesterone (1 $\mu\text{g.}/\text{ml.}$), oestradiol (0.01 $\mu\text{g.}/\text{ml.}$) + progesterone (5 $\mu\text{g.}/\text{ml.}$) nor an extract of mice placentae prevented the increase in enzyme activity.

The enzyme activities would continue to increase above the 22 hr. level during the second day of culture if serum from lactating mice was added to the medium. Insulin had to be included in the medium for this effect but, in the presence of serum, further addition of prolactin and corticosterone caused no further increase in activity. It was found that serum from virgin or pregnant mice was as good as that from lactating mice but that bovine serum albumin could not replace serum.

Some experiments were done with mice at other stages of development. When mammary tissue from mid-pregnant (day 12) mice was incubated for 22 hr. with insulin the activities of both enzymes again increased but there were certain differences from late-pregnant tissue. Thus glucose 6-phosphate dehydrogenase activity increased during both the first and second days of culture with insulin alone. If prolactin or prolactin + corticosterone were added to cultures in addition to insulin there was a further significant increase at both 22 hr. and 45 hr.. The implications of the greater effect of prolactin on the enzyme level in mid-pregnancy are discussed.

In some experiments virgin tissue behaved similarly to mid-pregnant tissue but in others there

was no increase in enzyme activity. No greater consistancy was obtained either by killing mice at oestrus or metoestrus or by adding oestradiol (0.03 $\mu\text{g./ml.}$) + progesterone (1 $\mu\text{g./ml.}$) to the medium.

The effect of different inhibitors on the increase in mammary glucose 6-phosphate dehydrogenase activity during 22 hr. culture was examined after determining their minimum effective concentrations. Actinomycin D (0.5 $\mu\text{g./ml.}$), cycloheximide (10 $\mu\text{g./ml.}$) or puromycin (10 $\mu\text{g./ml.}$), added at the start of culture, prevented the enzyme increase, but hydroxyurea (50 $\mu\text{g./ml.}$) had no effect. This suggested that RNA and protein synthesis, but not DNA synthesis, were required for the enzyme increase.

If actinomycin D was added after about $3\frac{1}{2}$ hr. it completely failed to prevent the enzyme rise, suggesting that RNA synthesis was only necessary during the first $3\frac{1}{2}$ hr..

Cycloheximide, added during the first 10 hr., completely inhibited the increase but, if added after 12 hr., had little effect. Since cycloheximide, added at 12 hr., still inhibited protein synthesis almost immediately, it appears that the increase in enzyme activity occurs in the absence of protein synthesis. This might be due to synthesis of the enzyme in an inactive form but preliminary experiments failed to detect inactive enzyme precursor in supernatant from explants incubated for 12 hr..

ABBREVIATIONS

Abbreviations adopted by the Biochemical Society (see Biochem. J. (1967) 102, 1) are used without definition. Other abbreviations used in this thesis are:

A	Adenine.
ACTH	Adrenocorticotrophic hormone.
C	Cytosine.
CCD	Counter-current distribution.
G	Guanine.
G6PD	Glucose 6-phosphate dehydrogenase.
MAK	Methylated albumin-kieselguhr.
N.S.	Not significant.
6PGD	6-phosphogluconate dehydrogenase.
U	Uracil.

Abbreviations of the names of journals are those given by Brown, P. and Stratton, G. B. (1963) (Ed.) World List of Scientific Periodicals, London: Butterworths.

1. INTRODUCTION

The purpose of the work described in this thesis was to study the way in which hormones act on the mammary gland to stimulate the synthesis of specific proteins.

1.1 The hormonal control of the mammary gland.

It is convenient in discussing the hormonal control of the mammary gland during pregnancy and lactation to consider three stages: mammary development, initiation of copious milk secretion and maintenance of lactation.

Mammary development.

From experiments in which various hormones were injected into animals from which different endocrine glands had been removed, it has been concluded that oestrogen, progesterone, glucocorticoids, growth hormone and prolactin are involved in the control of mammary development (see Lyons, 1958). Although these experiments did not show whether each hormone acts directly at the mammary gland, prolactin does appear to act in this way

since it can give a localised lactogenic response when injected into one galactophore of a rabbit (Lyons, 1942).

Experiments in which hormones have been added to mammary tissue in long term culture have helped to establish which other hormones act directly at the mammary gland (see Lasnitzki, 1965). Elias (1959) found that insulin is essential for the survival of small pieces of mid-pregnant mouse mammary tissue in culture and that the further addition of cortisol increases the size of the alveolar lumina. If prolactin is also added the alveoli enlarge further, the cells increase in size and a copious secretion appears in the lumina (Rivera and Bern, 1961). If cortisol is not included, and only insulin and prolactin are added, a full secretory response is not obtained although some secretion is observed (Stockdale, Juergens and Topper, 1966). Barnawell (1965) has obtained similar results with cultures of mammary tissue from other species. Rivera (1964a) showed that cortisol may be replaced by corticosterone or aldosterone, the predominant corticosteroids in the blood of mice, and it has also been found that prolactin may be replaced by human placental lactogen (Turkington and Topper, 1966) or growth hormone (Rivera and Bern, 1961). Growth hormone will only replace prolactin in some strains of mice and its effect does not appear to be due to contamination

by prolactin (Rivera, 1964b). Oestradiol and progesterone are not needed for the development of mid-pregnant mouse mammary tissue in culture (Elias and Rivera, 1959). However, when added to cultures of virgin mouse mammary tissue, these hormones cause duct growth and end-bud development, although strain differences are observed (Prop, 1960; Koziorowska, 1962).

Initiation of copious milk secretion.

It has generally been concluded that copious milk secretion at parturition is also initiated by the action of hormones at the mammary gland and the evidence for this has been reviewed (see Folley, 1956; Meites, 1961). The hormones involved are thought to be prolactin and glucocorticoids as secretion has been induced in some instances by the injection of either of these into suitably prepared animals (Lyons, 1942; Talwalker, Nicoll and Meites, 1961). It is also known that the pituitary prolactin content (Meites and Turner, 1942) and the plasma level of corticosterone (Gala and Westphal, 1965) increase at parturition. During pregnancy it is thought that the production of prolactin and ACTH by the pituitary is prevented by high levels of progesterone and oestrogens and that these might also inhibit pituitary hormone action directly at the mammary gland.

At parturition a fall in the levels of oestrogen and progesterone is thought to release the pituitary inhibition and also to allow an actual stimulation of pituitary function by low levels of oestrogens. There is evidence that oestradiol can stimulate the production of prolactin by pituitaries in culture (Nicoll and Meites, 1964) and the plasma level of progesterone in rats has been found to fall just before parturition (Grota and Eik-Nes, 1967).

Maintenance of lactation

There has long been indirect evidence that prolactin, and possibly other pituitary hormones, are also involved in the maintenance of lactation (see Meites, 1961). Seyle (1934) showed that suckling at only one set of nipples of a rat delayed the involution of the unsuckled set and he suggested that the suckling stimulus caused release of prolactin from the pituitary. Suckling has been found to cause a decrease in both the pituitary content of prolactin (Reece and Turner, 1937) and the granularity of the specific prolactin cells of the pituitary (Pasteels, 1963). It has also been shown that injection of ovine hypothalamic 'prolactin-inhibiting factor' reduces the lactational performance of rats (Grosvenor, Mena, Dhariwal and McCann, 1967).

Effects of hormones on the maintenance of lactation have not, however, been demonstrated in the perfused mammary gland. Hardwick and Linzell (1960) perfused a goat's mammary gland for a day and were able to achieve a milk yield of about 50% of that in the intact animal; however prolactin and other hormones did not affect the milk yield.

1.2 Some biochemical studies of the action of hormones on the mammary gland in vitro.

The development of the mammary gland under hormonal control is accompanied by great changes in its metabolic and biosynthetic activity. After parturition, especially, the gland contains increased amounts of proteins, nucleic acids and many enzymes (see Munford, 1964). Some of these changes have been studied by incubating mammary tissue with various hormones in vitro.

The effect of insulin on the metabolism of mammary slices has been studied during short incubations. Balmain, French and Folley (1950) showed that insulin rapidly increased the respiratory quotient of lactating rat mammary gland slices incubated with glucose or glucose plus acetate, and it was later found that this was associated with an increased incorporation of

the substrates into fatty acids (Balmain, Folley and Glascock, 1954). Insulin did not affect the respiratory quotient of mammary slices from pregnant rats or from lactating sheep (Balmain and Folley, 1961). Abraham, Cady and Chaikoff (1957) and McLean (1958) showed that in slices of lactating rat mammary gland insulin caused a greater increase in $^{14}\text{CO}_2$ production from (1- ^{14}C)glucose than from (6- ^{14}C)glucose, probably indicating an increase in the amount of glucose passing through the pentose phosphate pathway. McLean (1960) found that insulin also increased $^{14}\text{CO}_2$ production from (1- ^{14}C)glucose during pregnancy but the effect was smaller than in lactation.

In the above experiments, lactating tissue is more sensitive to insulin than pregnant tissue. In contrast, Mayne, Barry and Rivera (1966) found that in 3hr. incubations insulin stimulated the incorporation of (^{14}C)leucine into the protein of mammary gland slices of pregnant, but not of lactating, rats. They also found that insulin stimulated (^{14}C)adenine incorporation into the RNA of slices of the pregnant gland to a greater extent than in slices of the lactating gland.

Moretti and Abraham (1966) incubated mouse mammary tissue in culture for up to four days and found

that insulin affected the utilisation of (^{14}C)glucose in both pregnant and lactating tissue. Insulin was needed to maintain the uptake of glucose after about the first day and was shown to stimulate the formation of (^{14}C)fatty acids and $^{14}\text{CO}_2$ in both pregnant and lactating tissue and the formation of (^{14}C)lactic acid in pregnant tissue.

Biochemical effects of other hormones have been found with the culture system of Elias (1957). Juergens, Stockdale, Topper and Elias (1965) showed that in 48 hour cultures of explants of mid-pregnant mouse mammary tissue insulin, prolactin and hydrocortisone greatly stimulated the incorporation of (^{32}P)orthophosphate into casein over that with combinations of two of these hormones. Topper and coworkers have used colchicine, an inhibitor of cell division, to study this hormonal stimulation of casein synthesis (Stockdale and Topper, 1966; Lockwood, Stockdale and Topper, 1967). They conclude that the epithelial cells of the cultured tissue must divide before they can synthesise casein and that insulin initiates the mitosis. They find that while prolactin can act after mitosis, hydrocortisone cannot.

Lockwood, Turkington and Topper (1966) have shown that the incorporation of (^{14}C)amino acids into

the whey proteins, β -lactoglobulin and α -lactalbumin, is also greatest when all three hormones are present.

Stockdale, Juergens and Topper (1966) studied the effect of these three hormones on RNA and DNA synthesis. They found a greater incorporation of (^{14}C)uridine into RNA when all three hormones were present and this could first be seen at about 24 hours. No similar effect on the incorporation of (^3H)thymidine into DNA was seen until 48 hours. Workers in this department, however, have found a greater effect of the three hormones on the incorporation of (^{14}C)adenine into RNA by 9 hours of incubation (Mayne, Forsyth and Barry, 1967) and on the incorporation of (^3H)thymidine into DNA by 24 hours (Mayne and Barry, personal communication).

1.3 Some aspects of the hormonal control of enzyme activity and protein synthesis.

Some years ago it was suggested that hormones, like the water-soluble vitamins, might influence metabolism by acting as co-factors in enzymic reactions (Green, 1941). Although this analogy proved to be groundless, the possibility remained that hormones might be able to alter the activity of enzymes and the concept of allosteric proteins (Monod, Changeux

and Jacob, 1963) has suggested how this might be accomplished. It has in fact been shown that certain steroid hormones can exert an allosteric effect on the degree of dissociation of liver glutamic dehydrogenase, hence altering its activity (see Tomkins and Yielding, 1964). However the number of reports of similar effects is limited and their physiological significance is doubtful.

There is stronger evidence that, by altering the activity of adenyl cyclase, certain hormones can influence the activity of enzymes controlling major metabolic pathways. Adenosine 3'5'-monophosphate, added to tissues in vitro, has been found to mimic some of the actions of these hormones (see Sutherland, Øye and Butcher, 1965). The hormones may not act directly on adenyl cyclase and it has been suggested that their effects might be mediated through histamines, which are rapidly released from target tissues (Szego, 1965).

One feature of the action of some hormones involved in growth and development is augmentation of protein synthesis, often with a greater relative stimulation of the synthesis of specific proteins and enzymes. It appears likely that in many cases such stimulation of protein synthesis involves hormonal control of transcription or translation of RNA, and

is not merely a result of an increased metabolic rate caused by increased enzyme activities or, with insulin for example, increased substrate transport.

Some hormones may act by first stimulating RNA synthesis and hence cause an increase in protein synthesis. Evidence for this came from the observations that certain hormones stimulate RNA synthesis when injected into suitably prepared animals, that this effect precedes the stimulation of protein synthesis and that in many cases the stimulation of protein synthesis can be prevented by the administration of actinomycin D. Such studies have been performed with growth hormone (Korner, 1965), thyroxine (Tata, 1965), oestrogens (Mueller, 1965), testosterone (Williams-Ashman, 1965) and aldosterone (Sharp and Leaf, 1966).

The mechanism by which hormones stimulate this RNA synthesis is obscure. However Karlson (1963), by analogy with the model for the control of bacterial enzyme synthesis (Jacob and Monod, 1961), has suggested that hormones act directly at the gene and induce the synthesis of specific RNA templates. The main evidence for this theory is the effect of the insect hormone, ecdysone, on the chromosome puffs in the salivary gland of Chironomus tentans (Clever and Karlson, 1960; Karlson and Sekeris, 1966) and the report of a direct stimulation

of RNA synthesis when cortisol was added to rat liver nuclei (see Lukács and Sekeris, 1967). It has not been generally possible to obtain direct effects of other hormones on isolated nuclei, however, and this theory has recently been critically reviewed (Hechter and Halkerston, 1965; Tata, 1966; Korner, 1967).

Control of protein synthesis at the level of translation rather than transcription is also possible, especially for hormones such as insulin (Eboué-Bonis, Chambaut, Volfín and Clauser, 1963) and ACTH (Garren, Ney and Davis, 1965) whose stimulation of protein synthesis is independent of RNA synthesis. Thus Garren, Ney and Davis (1965) have presented evidence that the stimulation of corticosteroid biosynthesis by ACTH takes place on stable RNA templates and is mediated by a rapidly turning-over protein. A possible mode of translational control is suggested by the findings that the reduced protein synthesising ability of ribosomes from the heart muscle of diabetic rats (Rampersand and Wool, 1965) or the liver of hypophysectomised rats (Korner and Gumbley, 1966) may be due to a decreased ability of the ribosomes to attach to templates rather than a deficiency of available template RNA.

1.4 Effects of hormones on rapidly-labelled RNA.

The theory of Jacob and Monod (1961) implies that, in bacteria, template RNA has a shorter half-life than ribosomal RNA and this appears to be so (Levinthal, Keynan and Higa, 1962; Nakada and Magasanik, 1964; Meselson, Nomura, Brenner, Davern and Schlessinger, 1964). Thus the RNA labelled during a short pulse of radioactive precursor ("rapidly-labelled RNA") should be mainly template RNA.

In mammalian cells the half-life of template RNA may more nearly approach that of ribosomal RNA (Revel and Hiatt, 1964; Loeb, Howell and Tomkins, 1965) and there is evidence that RNA templates are transported from nucleus to cytoplasm in association with the 45s ribosomal subunit (Henshaw, Revel and Hiatt, 1965). Moreover much of the mammalian rapidly-labelled RNA appears to be rapidly broken down in the nucleus (Harris and Watts, 1962; Harris, Fisher, Rodgers, Spencer and Watts, 1963). Nevertheless it is reasonable to expect that at least part of the mammalian rapidly-labelled RNA is template RNA and it is for this reason that there have been numerous studies of it (see Singer and Leder, 1966).

If hormones stimulate the synthesis of specific RNA templates they might be expected to alter the fractionation profiles of rapidly-labelled RNA obtained by sucrose density gradient centrifugation. It is possible, however, that the lack of such alterations (see Karlson, 1965) is due to insufficient resolution by this method. MAK columns (Yoshikawa-Fukada, Fukada, and Kawada, 1965), electrophoresis (Tsanev, Markov and Dessev, 1966) and substituted DEAE-cellulose columns (Sedat, Kelly and Sinsheimer, 1967) have also been used to fractionate rapidly-labelled RNA but only achieve a similar degree of resolution.

Counter-current distribution has been used to fractionate soluble RNA (Holley, Apgar and Doctor, 1960) and Kirby and coworkers applied this method to other nucleic acids. They developed solvent systems with which they achieved good resolution of small molecular weight segments of (mainly ribosomal) RNA (Kirby, 1962) and of native DNA and DNA segments (Kidson and Kirby, 1963; Kidson and Kirby, 1964a). It was shown that while the basis of separation of the native DNA was its degree of denaturation, the segments of RNA and of DNA were separated according to base composition, segments with a high proportion of adenine moving more rapidly than those with a high proportion of guanine.

Kidson and Kirby (1964b) fractionated rat liver rapidly-labelled RNA by counter-current distribution and found it could be separated from much of the DNA and ribosomal and soluble RNA. The profile of the rapidly-labelled RNA had a number of peaks and different profiles were obtained with RNA from different tissues and with RNA from the livers of rats fed different diets. Altered profiles of rapidly-labelled RNA were also found in mouse hepatoma at different times after transplantation (Kidson and Kirby, 1964c) and in the livers of rats during carcinogenesis (Kidson and Kirby, 1965; Parish and Kirby, 1966a). It was suggested (Kidson and Kirby, 1964b) that the basis of the fractionation of rapidly-labelled RNA was base composition, by analogy with segments of DNA and ribosomal RNA. This would explain why resolution was greater than in methods whose basis was molecular size or conformation.

When rats were injected with various hormones there were changes in the profiles of rat liver rapidly-labelled RNA (Kidson and Kirby, 1964d). Effects were found with hydrocortisone, insulin, thyroxine, testosterone and stilboestrol and it was suggested that the hormones were promoting changes in the synthesis of specific RNA templates.

1.5 Aims.

Part One.

In Part One of this thesis it was hoped to study the effects of hormones on the RNA synthesised by the mammary gland during short incubations in vitro. Previous workers had not been able to detect hormonal stimulations of RNA synthesis before 3 hours in the case of insulin (Mayne, Barry and Rivera, 1966), and 9 hours in the case of prolactin (Mayne, Forsyth and Barry, 1967). Kidson and Kirby (1964d) found early effects of hormone injections on rat liver RNA, fractionated by counter-current distribution, and it was therefore hoped to apply this fractionation method to RNA from the mammary gland. However, as described in this thesis, it was not found possible to repeat the results of Kidson and Kirby (1964d) and evidence is presented that the fractionation of the RNA by counter-current distribution is probably due to an intrinsic ribonuclease.

Part Two.

In the absence of a satisfactory method for fractionating rapidly-labelled RNA, the study of the hormonal control of protein synthesis in the mammary gland was focussed on the enzyme, glucose 6-phosphate

dehydrogenase. This is the first enzyme of the pentose phosphate pathway, which is important in the lactating mammary gland in supplying NADPH for fatty acid synthesis. In Part Two of this thesis it was hoped to reproduce and study in vitro the increase in the activity of this enzyme that occurs in the living animal at parturition (Glock and McLean, 1954). A culture method was developed to achieve this and the effects of hormones and inhibitors on the enzyme rise are described.

2. EXPERIMENTAL

2.1 Materials

Carboxylic acids were obtained from the following sources. Vitamin-free insulin (lot 12-0501) and a gift from Dr. Otto Schreier, Eli Lilly Research Laboratories, Indianapolis, Ind., U.S.A.; Corticosterone (lot 120-10707), hydrocortisone (lot 120-10708) and 1,3,5-triacetoxy-2-vinylbenzene (lot 120-0700) were purchased from Sigma Chemical Co. Ltd., London, and thyroxine and oestrone from British Drug Houses Ltd., Poole, Dorset. For each experiment insulin was freshly dissolved in 3 mM NaCl (2 mg./ml.), thyroxine and hydrocortisone in 10 mM NaCl and all other hormones in 0.1M NaCl. The final concentration of alcohol in the incubation medium was 1%.

Deoxyribonuclease (EC 3.1.4.5, electrophoretically purified and free from ribonuclease) was purchased from Worthington Biochemical Corporation, Freehold, N.J., U.S.A.; pancreatic ribonuclease (EC 2.7.7.8) from Armour Pharmaceutical Co. Ltd., Eastbourne, UK; UTP, GTP and CTP from Sigma Ltd.; 2,5-dihydroxybenzoate (DHB) from

2. EXPERIMENTAL

2.1 Materials.

Hormones were obtained from the following sources. Glucagon-free insulin (lot PJ-4609) was a gift from Dr. Otto Behrens, Eli Lilly Research Laboratories, Indianapolis, Ind., U.S.A.. Corticosterone (lot 126-197C), hydrocortisone (lot H43B-288) and 3,3',5-triiodo-L-thyronine (lot 24B-0300) were purchased from Sigma Chemical Co. Ltd., London, and thyroxine and oestradiol from British Drug Houses Ltd., Poole, Dorset. For each experiment insulin was freshly dissolved in 3 mM HCl (2 mg./ml.), thyroxine and hydrocortisone in 10 mM NaOH and all other hormones in ethanol. The final concentration of ethanol in the incubation medium was 1%.

Deoxyribonuclease (EC 3.1.4.5, electrophoretically purified and free from ribonuclease) was purchased from Worthington Biochemical Corporation, Freehold, N.J., U.S.A.; pancreatic ribonuclease (EC 2.7.7.16) from Armour Pharmaceutical Co. Ltd., Eastbourne; ATP, UTP, GTP and CTP from Sigma Ltd.; 2,5-diphenyloxazole (P.P.O.),

α -naphthylphenyloxazole (P.O.P.O.P.) and naphthalene from Thorn Electronics, Tolworth, Surrey. All other reagents were purchased from British Drug Houses Ltd. and were, where possible, AnalaR grade. Glass-distilled water was used for all solutions.

(8-¹⁴C)adenine (specific activity 28.9 mc/m-mole), (2,8-³H)adenine (specific activity 3.3 c/m-mole), (6-¹⁴C)orotic acid (specific activity 44.5 mc/m-mole), (5-³H)orotic acid (specific activity 1.0 c/m-mole) and (G-³H)guanosine (specific activity 200 mc/m-mole) were purchased from the Radiochemical Centre, Amersham, Bucks.; and (8-¹⁴C)ATP (specific activity 21 mc/m-mole), (G-³H)ATP (specific activity 5 c/m-mole) and (2-¹⁴C)CTP (specific activity 20.6 mc/m-mole) from Schwartz Bioresearch Inc., Orangeburg, N.Y., U.S.A..

2.2 Isolation of rapidly-labelled RNA.

The following solutions were used:

Solution 1. Phenol (redistilled, 500 g.), m-cresol (redistilled, 70 ml.), 8-hydroxyquinoline (0.5 g.), water (55 ml.).

Solution 2. Sodium 4-aminosalicylate (6 g.) in water to 100 ml..

Solution 3. Sodium naphthalene 1,5-disulphonate (0.5 g.) in 100 ml. potassium phosphate buffer, pH 7.2 (0.05 M phosphate).

All operations were at 2°C.

Method A.

Method A was used with liver slices and was based on Method 2 of Kirby (1965).

Before isolating the RNA a crude nuclear fraction was prepared. This was done because it was later hoped to compare this RNA with that synthesised by isolated nuclei. Slices from control and hormone incubations were pooled and homogenised in a solution (20 ml.) of sucrose (0.32 M) and $MgCl_2$ (1.5 mM) in a Potter-Elvehjem type homogeniser. The homogenate was strained through muslin and centrifuged at 500g for 5 min.. The pellet was resuspended in fresh solution (15 ml.) and centrifuged at 450g for 3 min., and then resuspended in fresh solution (15 ml.) and centrifuged at 300g for 1 min.. The nuclear pellet obtained was contaminated with whole cells and had a RNA:DNA ratio of about 3.5 (compared with a ratio of 0.1 for nuclei prepared by the method of Widnell and Tata (1964)).

The nuclear pellet was shaken for 30 min. with 15 ml. each of solutions 1 and 2. After centrifuging for 30 min. at 4,000g, the upper aqueous phase was removed and the solid interphase was shaken once more with a similar mixture and centrifuged as before.

To the combined aqueous extracts, containing both the RNA and DNA, sodium chloride was added to give a 3% solution followed by 0.5 volume of Solution 1. After shaking and centrifuging as before, the nucleic acids were precipitated from the aqueous phase by adding 2 volumes of a mixture of ethanol and m-cresol (9 vol.:1 vol.). After standing at -40°C overnight, the precipitate was washed once with 75% (v/v) ethanol (containing 10 g./l. sodium chloride), once with 75% ethanol and twice with absolute ethanol. The precipitate was dried in a vacuum desiccator and stored in a desiccator at 2°C .

The total nucleic acid content of the final precipitate was 78% (mean value of 4 preparations, range 69-89%).

Method B.

Method B was used mainly for whole livers. It was based on the method of Kidson, Kirby and Ralph (1963), but included later improvements in extraction technique (Kirby, 1965). It was not used for nuclear preparations since the first extraction removed some of the rapidly-labelled RNA from the crude nuclear pellet.

Whole livers were dropped into liquid nitrogen immediately after excision. The livers from control and hormone-treated rats were blended together for 5 min. in a "Sunbeam" blender in 120 ml. each of Solutions 1 and 3. After shaking for 30 min., the mixture was centrifuged for 30 min. at 4,000g. The upper aqueous phase, which contained the bulk of the soluble and ribosomal RNA, was discarded. The solid interphase was removed and shaken for 30 min. with 180 ml. each of solutions 1 and 2. After centrifuging as before, the upper aqueous phase was collected and the interphase extracted once more in the same way. To the combined aqueous phases, sodium chloride was added to give a 3% solution, followed by 0.5 volume of Solution 1. The mixture was again shaken for 30 min. and centrifuged as before. DNA and rapidly-labelled RNA were precipitated from the aqueous phase with 2.5 volumes of ethanol. After standing at -40°C overnight, the precipitate was washed once each with 70% ethanol, 80% ethanol, 90% ethanol and, finally, absolute ethanol. The precipitate was dried in a vacuum desiccator and stored in a desiccator at 2°C .

The total nucleic acid content of the final precipitate was 33% (mean value of 5 preparations, range 15-42%).

2.3 Counter-current distribution.

The solvent system of Kidson and Kirby (1964d) was used for counter-current distribution of rapidly-labelled RNA. The following solutions were used:

'Organic Solution'. 2-methylbutan-2-ol (redistilled, 5 volumes), 2-butoxyethanol (redistilled, 4 volumes), 2-methoxyethanol (redistilled, 1 volume).

'Amine Solution'. Tri-n-amylamine (redistilled, 6 volumes), acetic acid (glacial, 1.08 volumes),

'Organic Solution' (100 volumes).

The '150/x' solvent systems, of different lithium contents, were made by mixing 'Organic Solution' (28 volumes), 'Amine Solution' (12 volumes), trilithium citrate (0.033 M, x volumes), water ((52-x) volumes). A two phase system resulted, the top phase being organic and the bottom phase aqueous.

The rapidly-labelled RNA was dissolved in the homogeneous solution formed at 2°C by 1.5 ml. of each phase of a 150/2 system. The amount of RNA used was usually 1 mg., although it was found that 0.25 to 1.5 mg. quantities of RNA gave similar counter-current distribution profiles. The RNA solution was placed,

at 22°C, in tube No.1 of a 50 tube, hand operated machine which had a capacity for 1.5 ml. of each phase (glass units made by the glassblower of the Department of Biochemistry, frame by the workshop of the Department of Agriculture). The next four tubes contained bottom phase (1.5 ml.) of 150/4, 150/6, 150/8 and 150/10 systems respectively. All remaining tubes contained bottom phase (1.5 ml.) of 150/12.5 system. The initial low lithium ion concentration was necessary because of the contribution made to the ionic strength by material associated with the rapidly-labelled RNA, the speed of movement of the rapidly-labelled RNA decreasing with increasing ionic strength. The top phase was passed along the series of tubes, tube No.1 being replenished with top phase of 150/12.5 system after each transfer. Fifty or 80 transfers were made, in the latter case fractions being collected from the end of the apparatus.

Ethanol (1 ml.) was then added to each tube to give one phase, the extinction at 260 m μ measured in a Zeiss PMQII spectrophotometer and the radioactivity measured as described below.

2.4 Measurement of the radioactivity of counter-current distribution fractions.

The single phase mixture from each tube was transferred to a counting vial and scintillation fluid (18 ml.) added. This latter was prepared by dissolving 2,5-diphenyloxazole (5 g.), α -naphthyloxazole (0.05 g.) and naphthalene (80 g.) in a solution of toluene (385 ml.), dioxan (385 ml.) and ethanol (230 ml.).

The ^{14}C and ^3H radioactivity of each fraction was measured simultaneously in a liquid scintillation counter and a correction made for the contribution of ^{14}C counts to the ^3H channel. Initially (Fig. 3.1 - 3.5) samples were counted by Mr. J.D.Eakins of A.E.R.E., Harwell, in a Packard TriCarb Liquid Scintillation Counter with efficiencies of 15% for ^{14}C and 7% for ^3H and a ^3H correction of about 100% of the ^{14}C counts. All other samples were counted in a Beckman Liquid Scintillation Spectrometer with efficiencies of 50% for ^{14}C and 20% for ^3H and a ^3H correction of about 25% of the ^{14}C counts. There was no significant variation in the quenching of different fractions as shown by the external standard channels ratio method.

Each sample was 'counted' for 20 or 40 min..

2.5 Formation of labelled RNA by isolated nuclei.

The basic medium used for incubating nuclei (DNA content about 4.5 mg., in 1.5 ml. of 0.25 M sucrose containing 1 mM MgCl_2) had the following composition: tris-HCl buffer pH 8.0 (450 μmole); MgCl_2 (45 μmole); KCl (540 μmole); NaF (60 μmole); CTP, GTP and UTP (3 μmole each); β -mercaptoethanol (90 μmole); in a total volume of 9ml.. Incubations without ammonium sulphate contained (^3H)ATP (15 μc ; 0.75 μmole). Incubations with ammonium sulphate (2.5 m-mole) contained MnCl_2 (30 μmole) instead of MgCl_2 and (^{14}C)ATP (1.5 μc ; 0.75 μmole). Incubation was for 30 min. at 37°C , after which the nuclei were precipitated by centrifuging at 42°C before combination for RNA isolation.

Pilot experiments to determine ratios of CTP:ATP incorporated into RNA under the two conditions were done with (^{14}C)ATP and (^{14}C)CTP in incubations of one-thirtieth the scale of the above. Synthesis was stopped by adding 10% trichloroacetic acid (2 ml.) and the precipitates were collected on membrane filters (type MF 50, Sartorius Co., Goettingen, W.Germany), washed with 10% trichloroacetic acid, glued to aluminium planchettes and counted in a Beckman Lowbeta II gas-flow counter.

3. RESULTS

3.1 Effect of hormones on the counter-current distribution of rapidly-labelled RNA.

In vitro experiments.

Kidson and Kirby (1964d) reported that several hormones, when injected into rats, caused rapid and specific alterations in the profiles obtained by counter-current distribution of rapidly-labelled RNA from the liver. It was therefore hoped to use counter-current distribution in this work to study the early effects of hormones on the RNA synthesised by mammary tissue in vitro. Since Kidson and Kirby (1964d) studied liver RNA, trial experiments were first performed to see if addition of hormones to liver slices affected the counter-current distribution of rapidly-labelled RNA.

Virgin, female, Wistar rats (about 200 g.), fed diet Oxo 41B, were killed by cervical dislocation and bled from the neck. The livers were then rapidly removed and sliced using a microtome (Stadie and Riggs, 1944). Portions of slices (1 g.) were incubated at 37°C

for 1 hr., with shaking, in Ringer-bicarbonate buffer (10 ml.) (Krebs and Henseleit, 1932) containing glucose (30 mg.) under an atmosphere of oxygen (95%) / carbon dioxide (5%). One flask contained the hormone from the start of incubation and received (^3H)adenine (200 μc ; 0.35 μmole) after 30 min., while a control flask was without hormone and received (^{14}C)adenine (10 μc ; 0.35 μmole) after 30 min.. After incubation the slices were combined and the RNA isolated by Method A (section 2.2). RNA (1 mg.) was fractionated by countercurrent distribution (section 2.3) and the radioactivity of each fraction determined (section 2.4).

The radioactivity profiles of RNA from slices incubated with various hormones are shown: corticosterone (50 $\mu\text{g.}/\text{ml.}$) - Fig. 3.1; insulin (5 $\mu\text{g.}/\text{ml.}$) - Fig. 3.2; oestradiol (5 $\mu\text{g.}/\text{ml.}$) - Fig. 3.3; 3,3',5-triiodothyronine (5 $\mu\text{g.}/\text{ml.}$) - Fig. 3.4. The ^3H and ^{14}C counts have been plotted on scales that give the same area beneath each profile to facilitate comparison. It can be seen that in these figures there is little difference between the profiles from control and hormone-treated slices.

To determine what degree of random variation between ^3H and ^{14}C profiles might be expected in a single experiment, a 'double control' experiment was performed. Slices were incubated with (^3H)adenine and

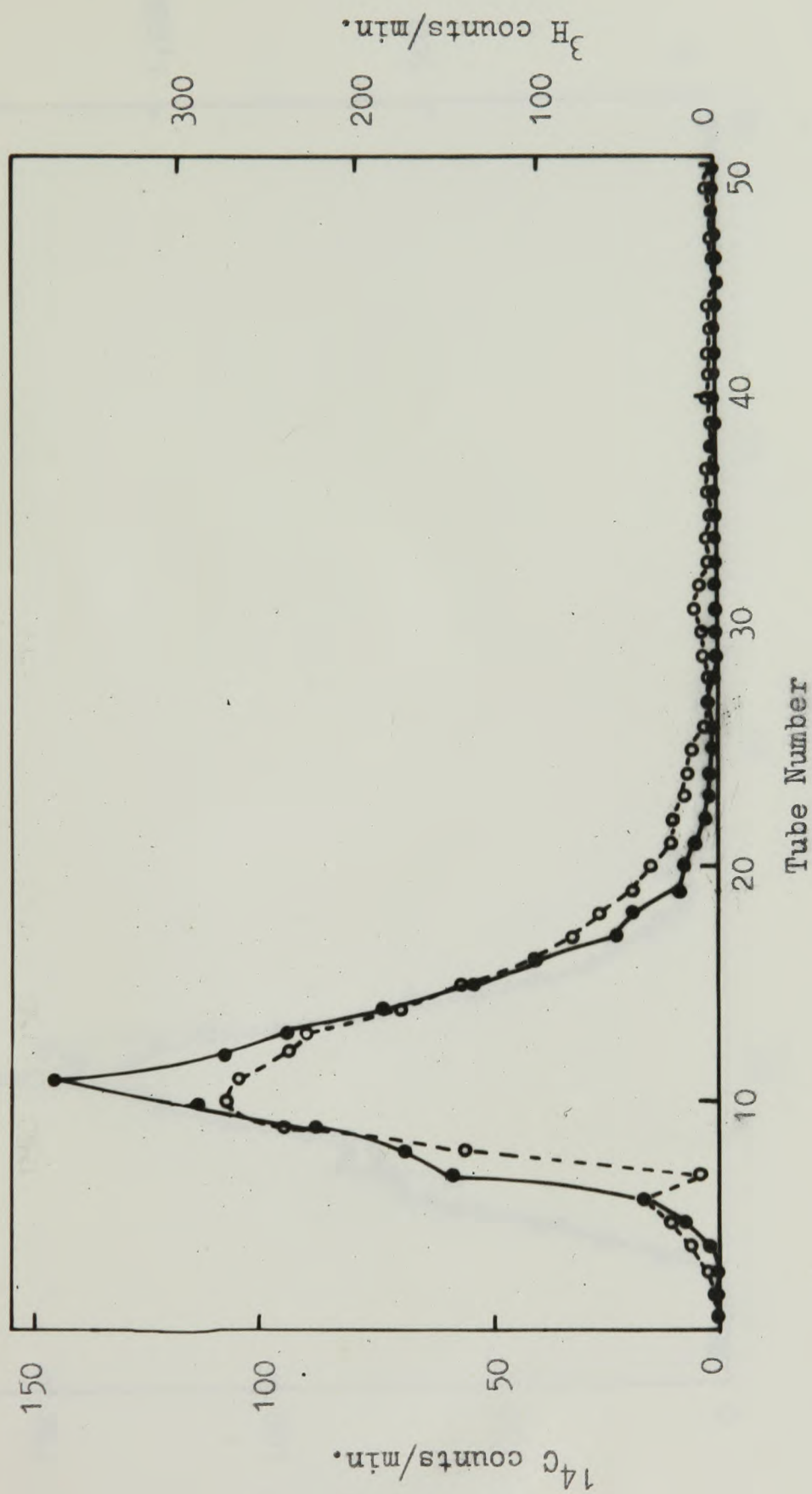


FIG. 3.1 CCD profiles of rapidly-labelled RNA from control slices of rat liver and slices incubated with corticosterone ($50 \mu\text{g./ml.}$).

●—● ^3H counts (Hormone treated slices); ○---○ ^{14}C counts (Control slices).

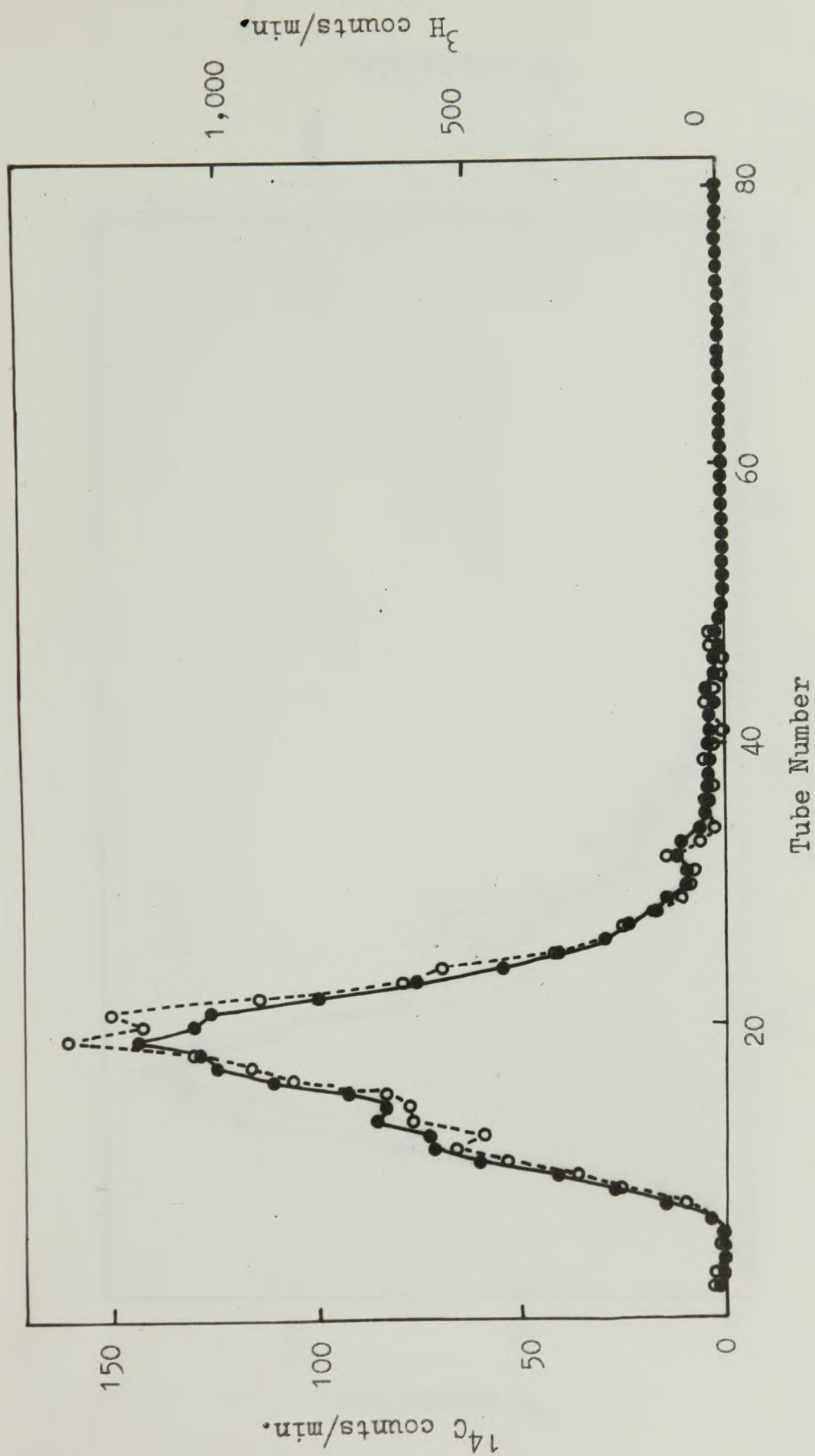


FIG. 3.2 CCD profiles of rapidly-labelled RNA from control slices of rat liver

and slices incubated with insulin ($5 \mu\text{g./ml.}$).

●—● ^3H counts (Hormone treated slices); ○---○ ^{14}C counts (Control slices).

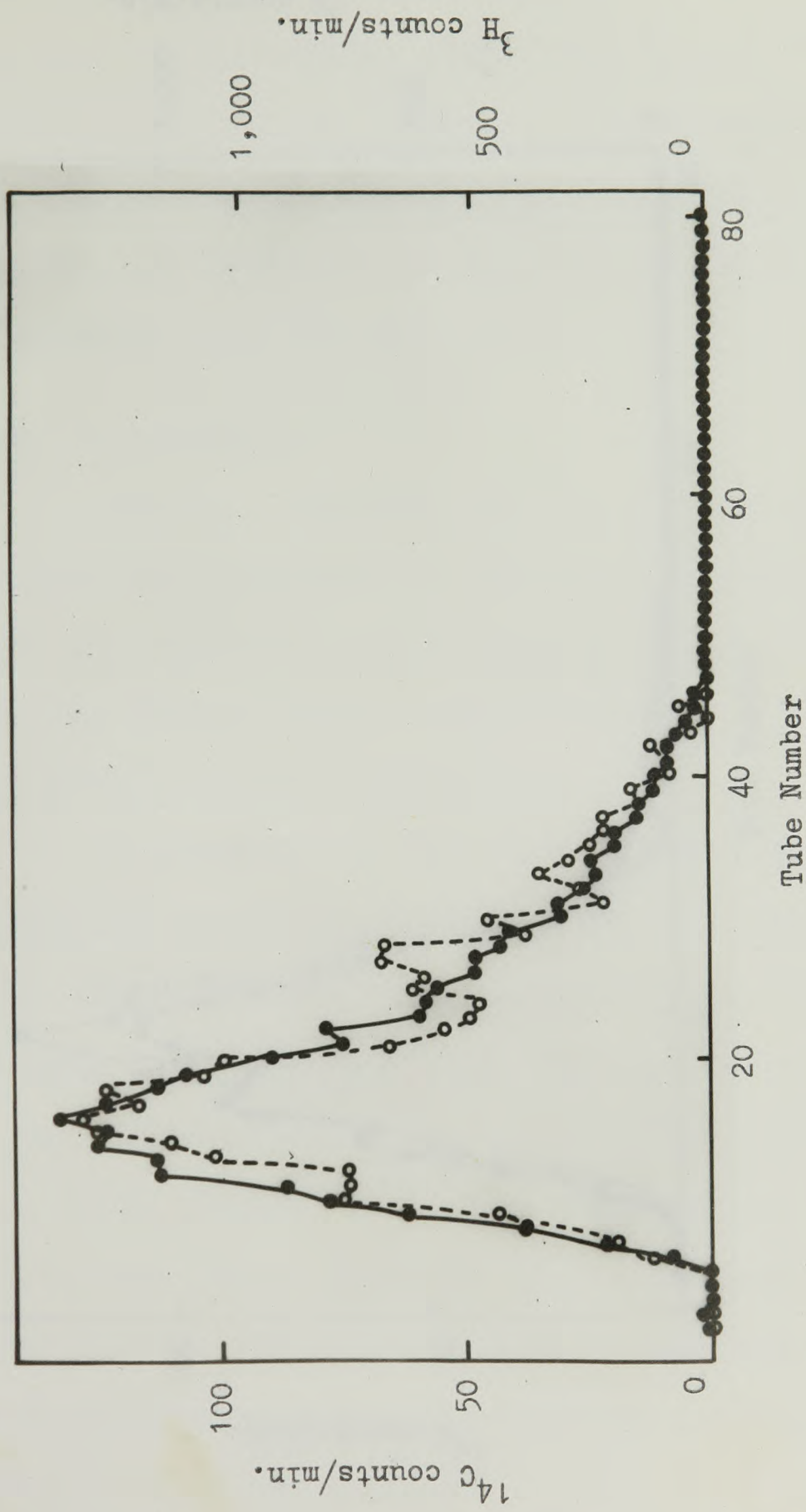


FIG. 3.3 CCD profiles of rapidly-labelled RNA from control slices of rat liver and slices incubated with oestradiol (5 $\mu\text{g./ml.}$).

●—● ^{14}C counts (Hormone treated slices); ○---○ ^3H counts (Control slices).

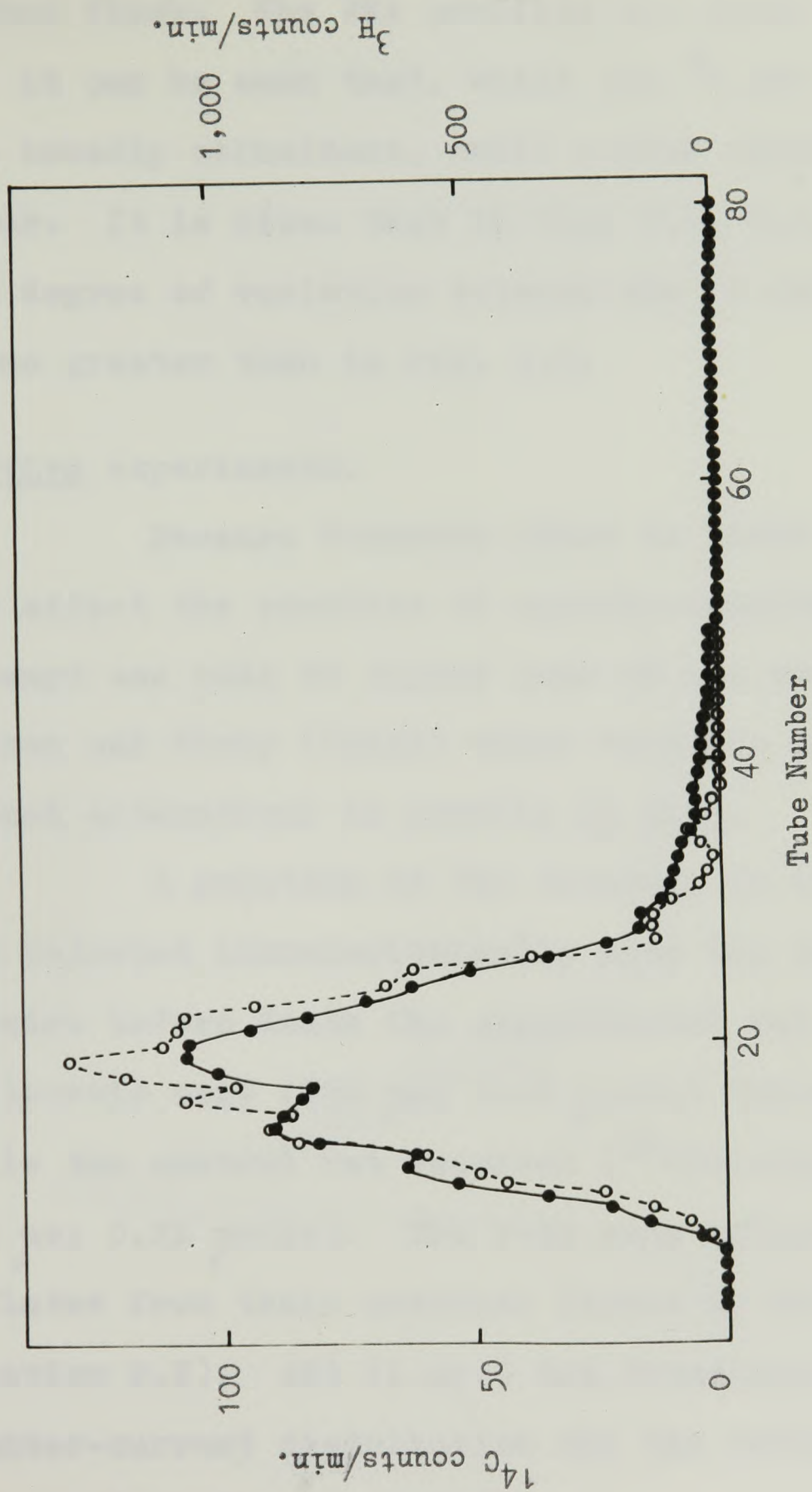


FIG. 3.4 CCD profiles of rapidly-labelled RNA from control slices of rat liver and slices incubated with 3,3',5-triiodothyronine (5 $\mu\text{g./ml.}$).

●—● ^3H counts (Hormone treated slices) ○---○ ^{14}C counts (Control slices).

(^{14}C)adenine as above, but without any hormone in either flask. The RNA profiles are shown in Fig. 3.5 and it can be seen that, while the ^3H and ^{14}C profiles are broadly coincident, small random variations do occur. It is clear that in Fig. 3.1, 3.2, 3.3 and 3.4 the degree of variation between the ^3H and ^{14}C profiles is no greater than in Fig. 3.5.

In vivo experiments.

Because hormones added to liver slices did not affect the profiles of rapidly-labelled RNA, an attempt was made to repeat some of the experiments of Kidson and Kirby (1964d) where hormones produced marked alterations in profile in vivo.

A solution of the hormone, or its solvent only, was injected intraperitoneally into the rats. Twenty minutes before death the experimental rat received (^3H)orotic acid (200 μc ; 0.22 μmole) intraperitoneally, while the control rat received (^{14}C)orotic acid (10 μc ; 0.22 μmole). The rats were killed and RNA isolated from their combined livers by Method B (section 2.2). RNA (1 mg.) was fractionated by counter-current distribution and the radioactivity of each fraction determined.

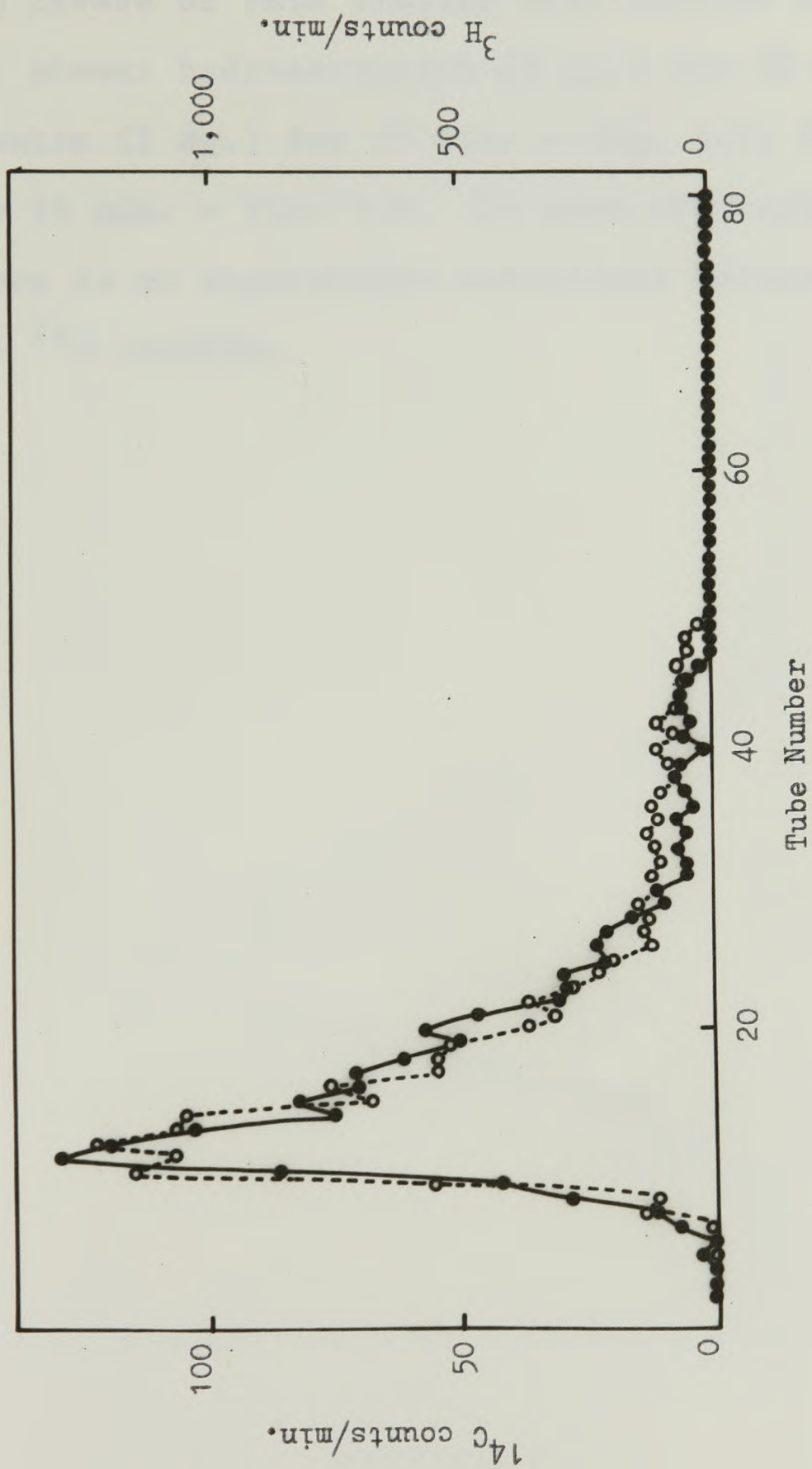


FIG. 3.5 CCD profiles of doubly-labelled RNA.

Neither batch of slices received hormone additions.

●—● ^3H counts; ○---○ ^{14}C counts.

The radioactivity profiles of RNA from the livers of rats treated with various hormones are shown: hydrocortisone (5 mg.) for 50 min. - Fig. 3.6; insulin (1 mg.) for 35 min. - Fig. 3.7; thyroxine (30 μ g.) for 35 min. - Fig. 3.8. In each of these figures there is no significant difference between the ^3H and ^{14}C profile.

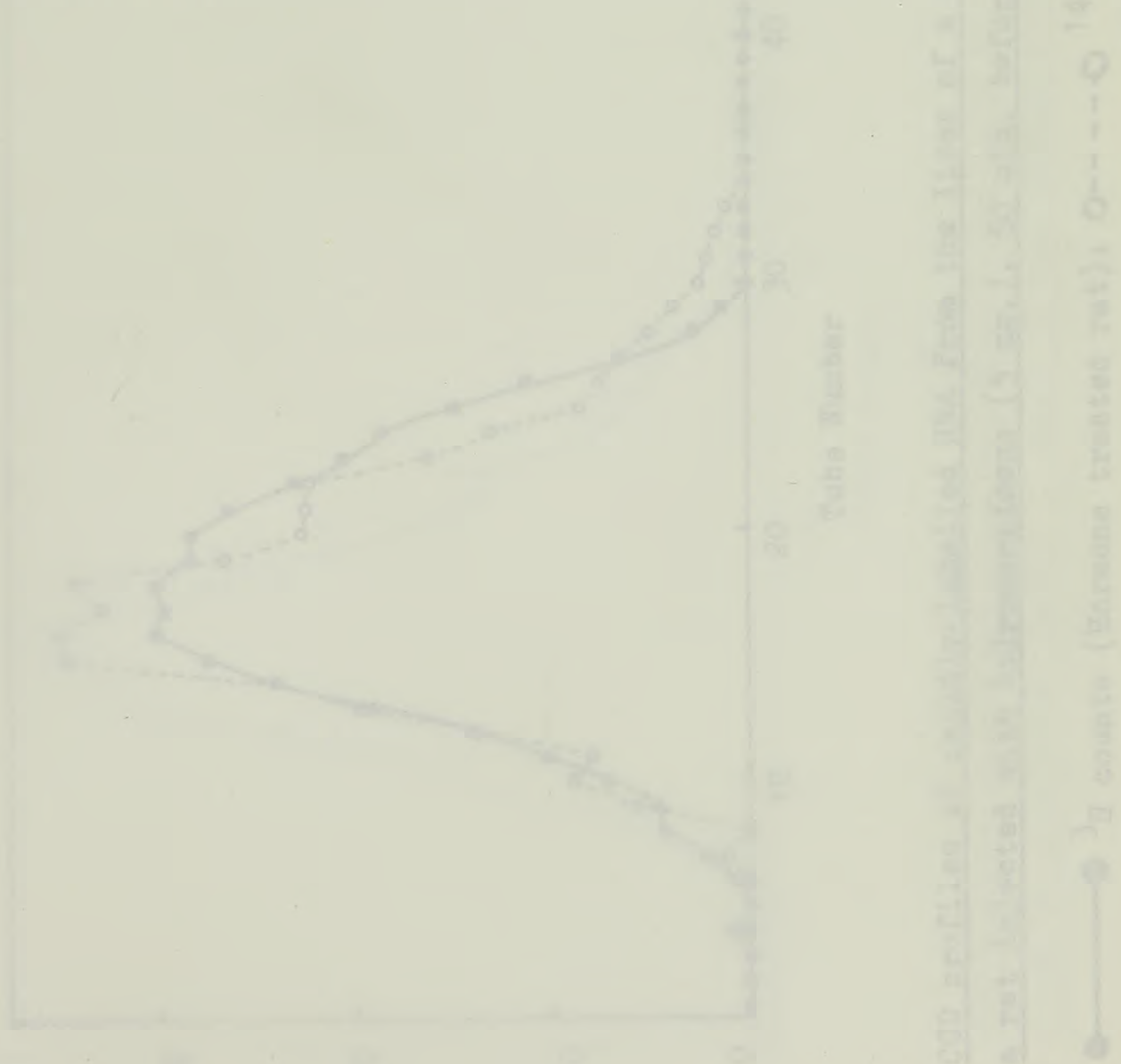


Fig. 3.6. Radioactivity profiles of RNA from the livers of a control rat and a rat collected with hydrocortisone (5 mg., 50 min. before death). $\bullet$ ^3H counts (hydrocortisone treated rat); $\circ$ ^{14}C counts (control rat).

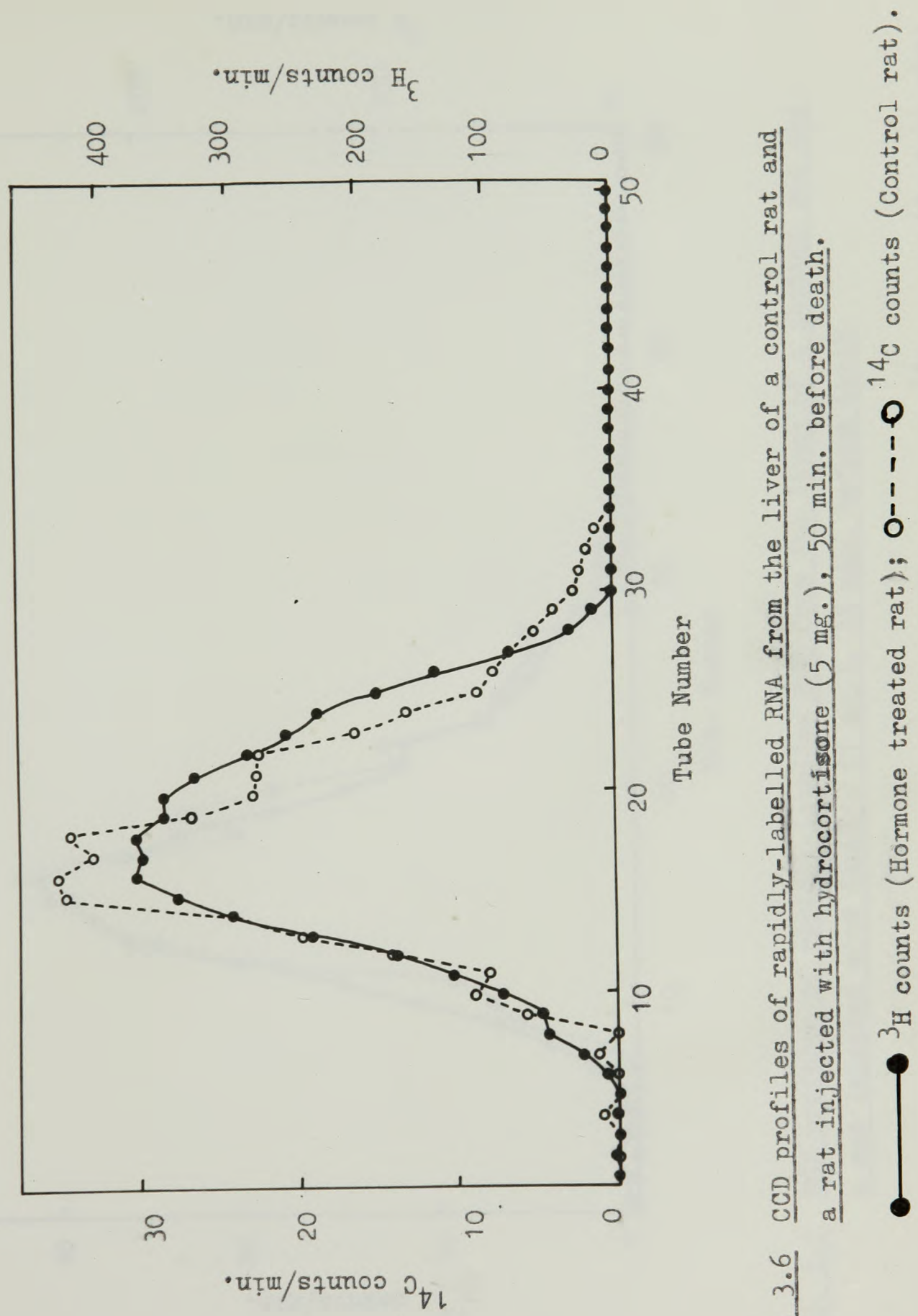


FIG. 3.6 CCD profiles of rapidly-labelled RNA from the liver of a control rat and a rat injected with hydrocortisone (5 mg.), 50 min. before death.

●—● ^3H counts (Hormone treated rat); ○---○ ^{14}C counts (Control rat).

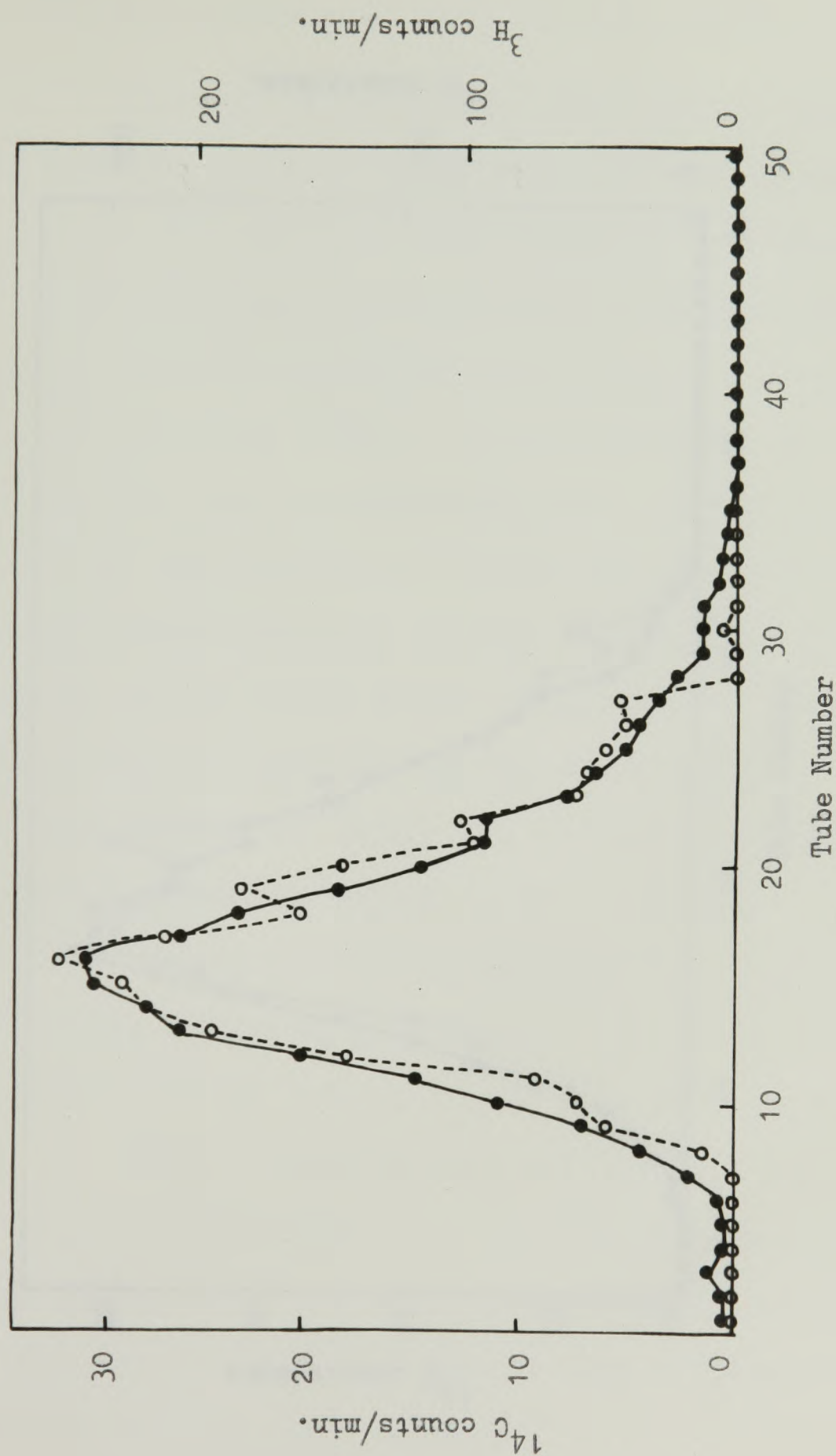


FIG. 3.7 CCD profiles of rapidly-labelled RNA from the liver of a control rat and a rat injected with insulin (1 mg.), 35 min. before death.

●—● ^3H counts (Hormone treated rat); ○---○ ^{14}C counts (Control rat).

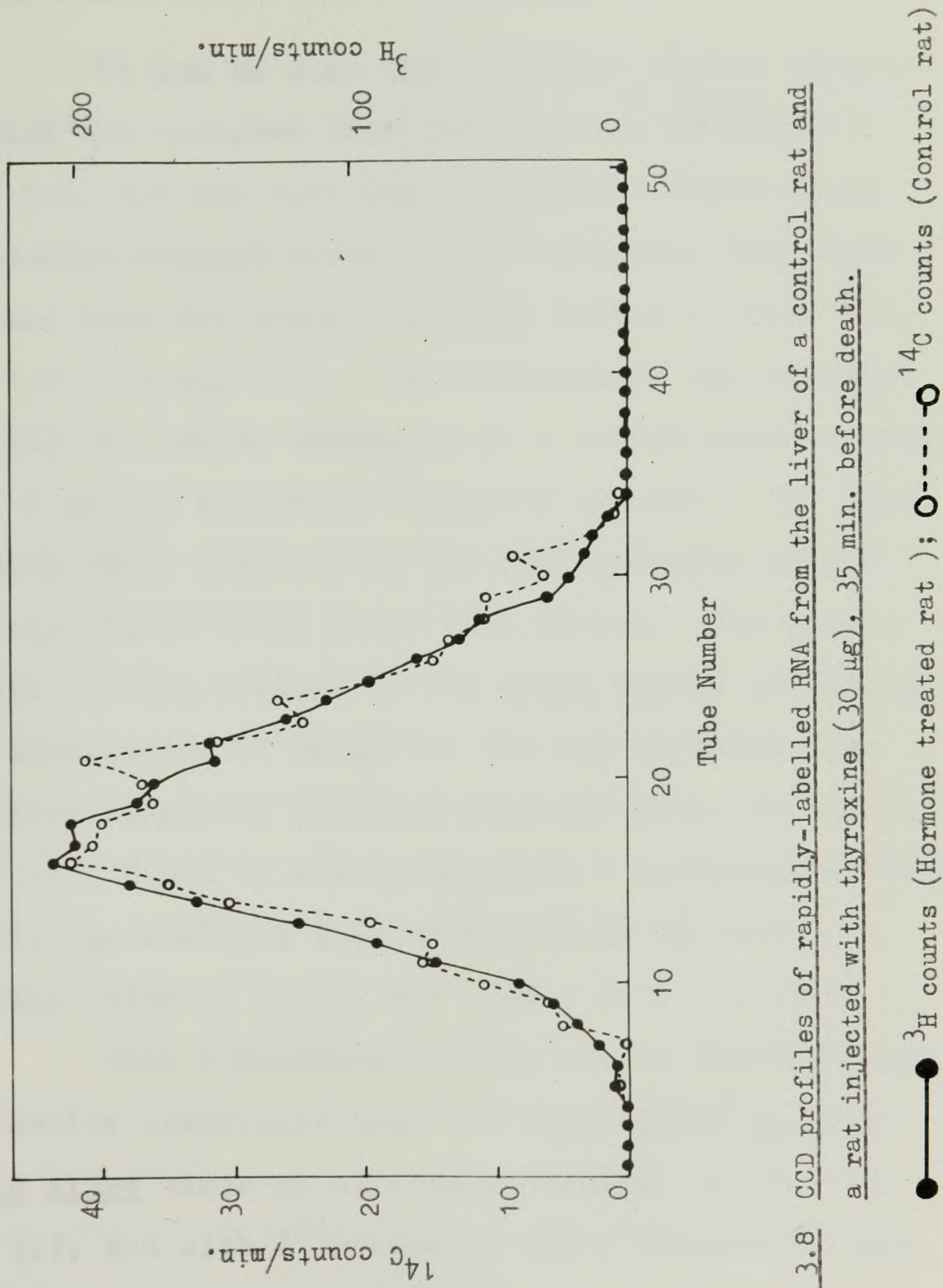


FIG. 3.8 CCD profiles of rapidly-labelled RNA from the liver of a control rat and a rat injected with thyroxine (30 μg), 35 min. before death.

3.2 Effect of associated DNA and ribosomal RNA on the counter-current distribution of rapidly-labelled RNA.

It can be seen that the bulk of the rapidly-labelled RNA isolated from whole livers by Method B (Fig. 3.6, 3.7 and 3.8) has travelled further along the counter-current distribution apparatus than that isolated from rat liver slices by Method A (Fig. 3.1, 3.2, 3.3, 3.4 and 3.5). The movement of the rapidly-labelled RNA can be expressed on a linear scale between 0 and 1 by the following "index of travel". The tube is found where one-half of the radioactivity in the apparatus is in tubes later than itself. The number of this tube is divided by the total number of exchanges. In 8 experiments in which the RNA was isolated from rat liver slices by Method A the mean index of travel was 0.22 ± 0.008 (S.E.M.), while in 6 experiments where the RNA was isolated from whole livers by Method B the mean index of travel was 0.36 ± 0.02 (S.E.M.).

This difference was not due to the different radioactive precursors used for experiments in vivo and in vitro since an experiment similar to that in Fig. 3.7, but with (^{14}C)adenine and (^3H)adenine, gave a profile in a similar position.

The rapidly-labelled RNA preparations isolated by the two different methods differed in their relative amounts of DNA and RNA. DNA was estimated by the diphenylamine method (Burton, 1956) and RNA by the orcinol method (Mejbaum, 1939), a correction being applied for the contribution of DNA. It was found that 4 samples of material prepared from slices by Method A had a mean DNA:RNA ratio of 1:3.5 (range 1:2.3 to 1:5.0) while 5 samples of material prepared from whole livers by Method B had a mean DNA:RNA ratio of 1:0.22 (range 1:0.1 to 1:0.4).

Association can affect the profile of rapidly-labelled RNA fractionated in sucrose density gradients (Hayes, Hayes and Guérin, 1966; Parish and Kirby, 1966b; Bramwell and Harris, 1967). It therefore seemed possible that the difference in profile positions of the RNA prepared by the two methods might be due to association with different nucleic acids.

Fig. 3.9 and 3.10 show the ultraviolet absorption profiles after counter-current distribution of the RNA isolated by the two different methods. In both the ultraviolet absorbing material is mainly found in a slow moving peak but the material of high DNA content (Method B) does appear to contain a greater proportion of fast-moving material. Although Fig. 3.11

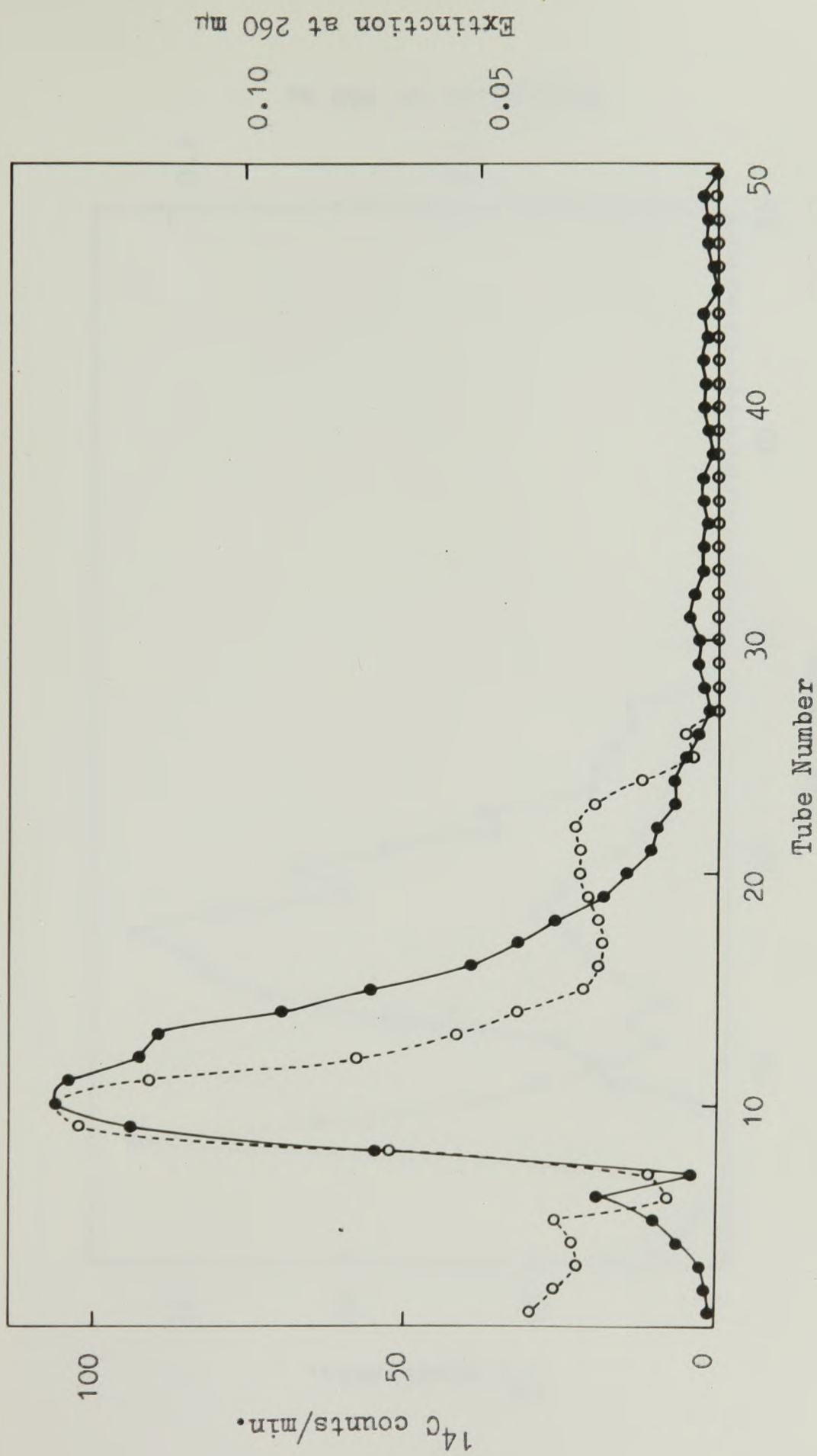


FIG. 3.9 CCD profiles of radioactivity and ultraviolet absorption of RNA isolated from slices of rat liver by Method A.

●—● ^{14}C counts; ○—○ Extinction at 260 mμ.

Slices of rats' liver were incubated for 30 min. with (^{14}C)adenine (10 μc; 0.35 μmole).

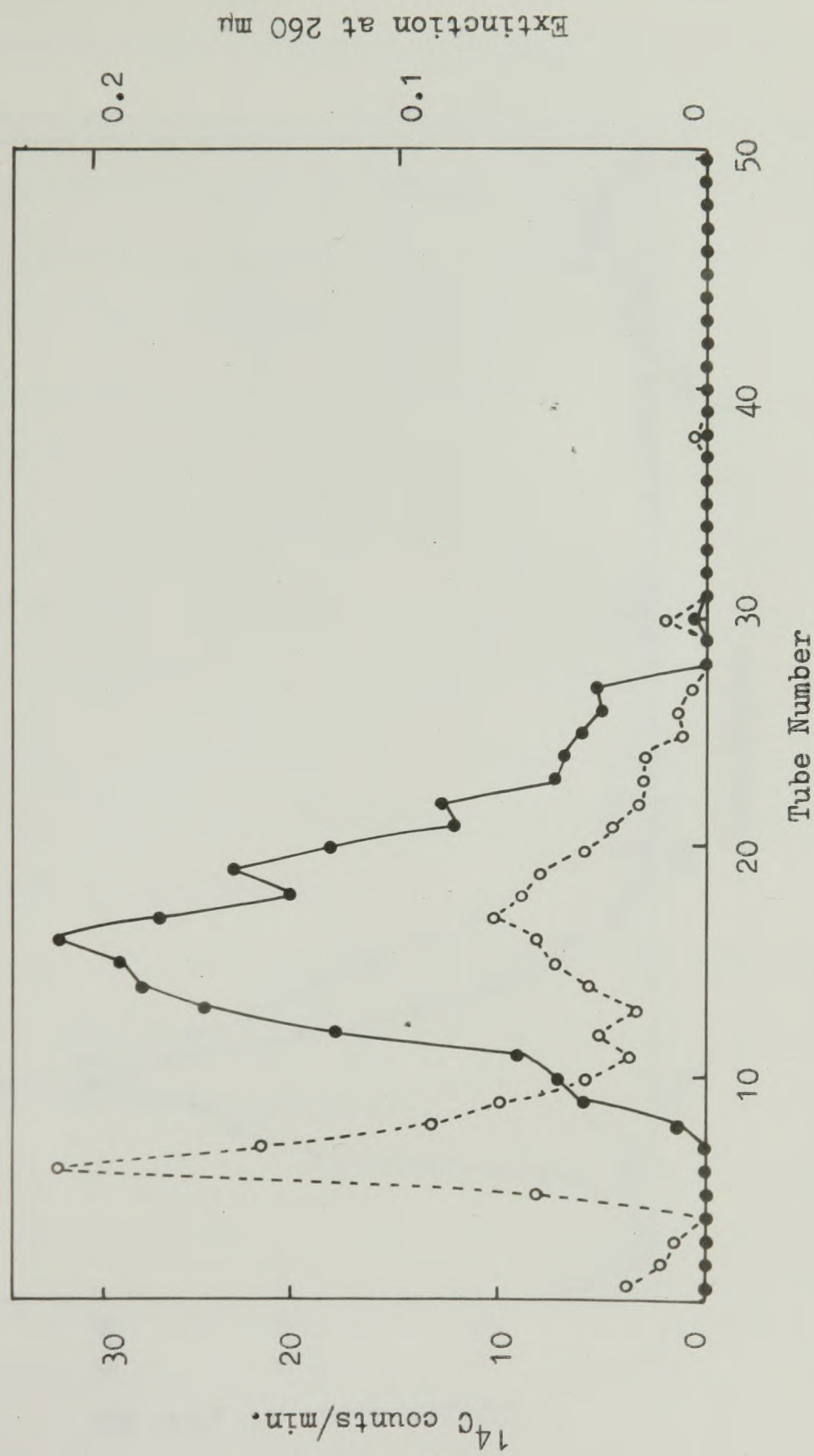


FIG. 3.10 CCD profile of radioactivity and ultraviolet absorption of RNA isolated from rat liver by Method B.

●—● ^{14}C counts; ○- - - ○ Extinction at 260 mμ.

Rats were injected with (^{14}C) orotic acid (10 μc; 0.22 μmole), 20 min. before death.

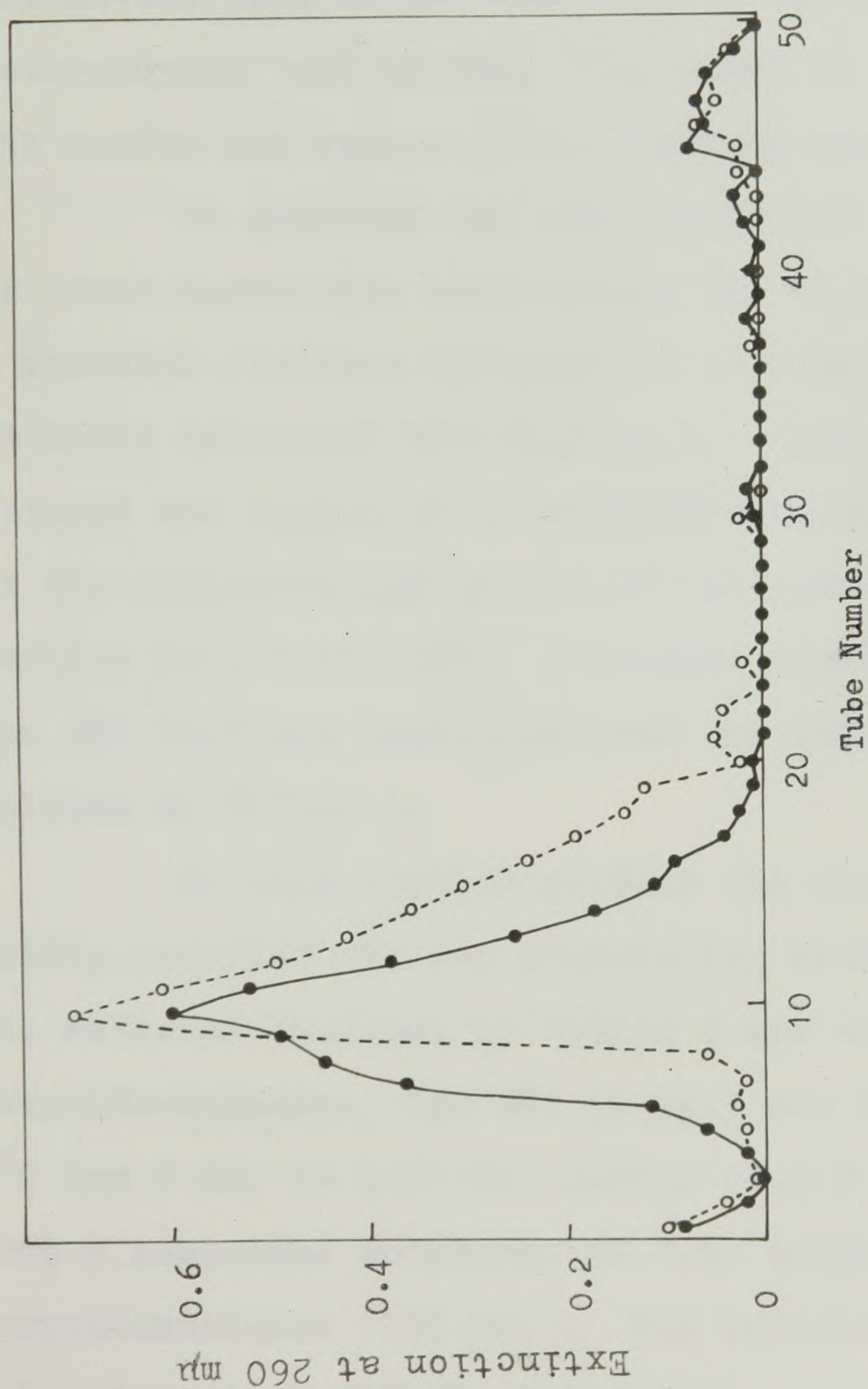


FIG. 3.11 CCD profiles of ultraviolet absorption of rat liver DNA (1 mg.) and rat liver ribosomal RNA (1 mg.).

●—● DNA; ○---○ Ribosomal RNA.

The profiles are from separate fractionations.

shows that both rat liver ribosomal RNA (prepared by Method 1 of Kirby, 1965) and native rat liver DNA (prepared by the method of Marmur, 1961) gave a single slow-moving peak on counter-current distribution, the faster-moving peak in Fig. 3.10 might be degraded DNA (see Kidson and Kirby, 1964a; Jackson and Sells, 1968).

To material isolated by Method A (0.2 mg.) was added commercial calf-thymus DNA (0.8 mg.), and to material isolated by Method B (0.2 mg.) was added rat liver ribosomal RNA (0.8 mg.). Each of these mixtures was subjected to counter-current distribution, but the positions and shapes of the radioactivity profiles were unchanged. A similar result was obtained when DNA that had been denatured was added to material isolated by Method A.

To test further whether the faster-moving rapidly-labelled RNA was associating with (degraded) DNA, material isolated by Method B was digested with deoxyribonuclease. The RNA (1 mg.) was incubated at 37°C for 2 hr. in 1.70 ml. buffer (0.1 M sodium acetate, 0.005 M magnesium sulphate, pH 7.6) containing deoxyribonuclease (150 µg.). The solution was then dialysed against 250 ml. trisodium citrate (0.00127 M) for 4 x 15 min., with shaking, and its composition made up to that of a 150/2 system by addition of

'Amine Solution' (0.39 ml.) and 'Organic Solution' (0.91 ml.). When the material was fractionated by counter-current distribution it was found that the position of the radioactivity profile was unchanged.

Hence these experiments lend no support to the idea that the different counter-current distribution profiles of rapidly-labelled RNA prepared by Methods A and B are due to physical association of the rapidly-labelled RNA with ribosomal RNA or with DNA.

3.3 Effect of base composition on the counter-current distribution of rapidly-labelled RNA.

Because changes in profile of rapidly-labelled RNA could not be obtained with hormones but differences in profile did result from different methods of preparation, the basis of the fractionation of rapidly-labelled RNA was investigated. Polynucleotide fragments from (mainly ribosomal) RNA and DNA are separated by counter-current distribution into fractions of differing base composition, those with a high proportion of adenine tending to move most rapidly and those with a high proportion of guanine most slowly (Kirby, 1962; Kidson and Kirby, 1963). It has been suggested that rapidly-labelled RNA is similarly separated into fractions of differing base composition (Kidson and Kirby, 1964b) and experiments were performed to see if this is so.

Differential labelling of adenine and guanine residues of RNA.

Slices of rats' liver (2 g.) were incubated for 30 min., as above (section 3.1), with (^3H)guanosine (200 μc ; 1 μmole) and (^{14}C)adenine (1 μc ; 0.035 μmole). The slices were then homogenised in a Potter-Elvehjem type homogeniser with Solution 3 (20 ml.), Solution 1 (20 ml) added and RNA isolated by Method B (sic), using

30 ml. each solution in subsequent extractions.

A portion of the RNA (0.5 mg.) plus carrier yeast RNA (0.5 mg.) was hydrolysed with 0.3 N KOH (0.5 ml.) at 37°C for 3 hr., DNA precipitated by adding 15% perchloric acid at 2°C, the ribonucleotides separated by the method of Katz and Comb (1963) and their ^3H and ^{14}C radioactivity determined. There was negligible incorporation into UMP or CMP and the distribution of radioactivity between GMP and AMP is shown in Table 3.1. It can be seen that approximately 90% of the (^3H)guanosine was incorporated into GMP. As expected, the (^{14}C)adenine incorporation was less specific but there was almost twice as much ^{14}C in AMP as in GMP. Hence the $^3\text{H}:$ ^{14}C ratios of the guanine and adenine residues of the labelled RNA differ by a factor of about 17.

The counter-current distribution profiles of this differentially labelled RNA are shown in Fig 3.12 and can be seen to be generally coincident. The radioactive material that travelled most rapidly cannot therefore have a higher than average adenine content, nor can that which travelled most slowly have a higher than average guanine content; for if this had been so the ^3H profile would have lagged behind the ^{14}C profile. There may be a local area of somewhat greater ^3H content

TABLE 3.1

Relative amounts of (^3H)guanosine and (^{14}C) adenine incorporated into individual ribonucleotides of rapidly-labelled RNA from rat liver slices.

Rat liver slices (2 g.) were incubated for 30 min. with (^3H)guanosine (200 μc ; 1 μmole) and (^{14}C)adenine (1 μc ; 0.035 μmole).

	Counts per min. incorporated into	
	GMP	AMP
^3H counts	60.2	7.0
(Percentage)	(89.7%)	(10.3%)
^{14}C counts	20.0	37.5
(Percentage)	(34.8%)	(65.2%)

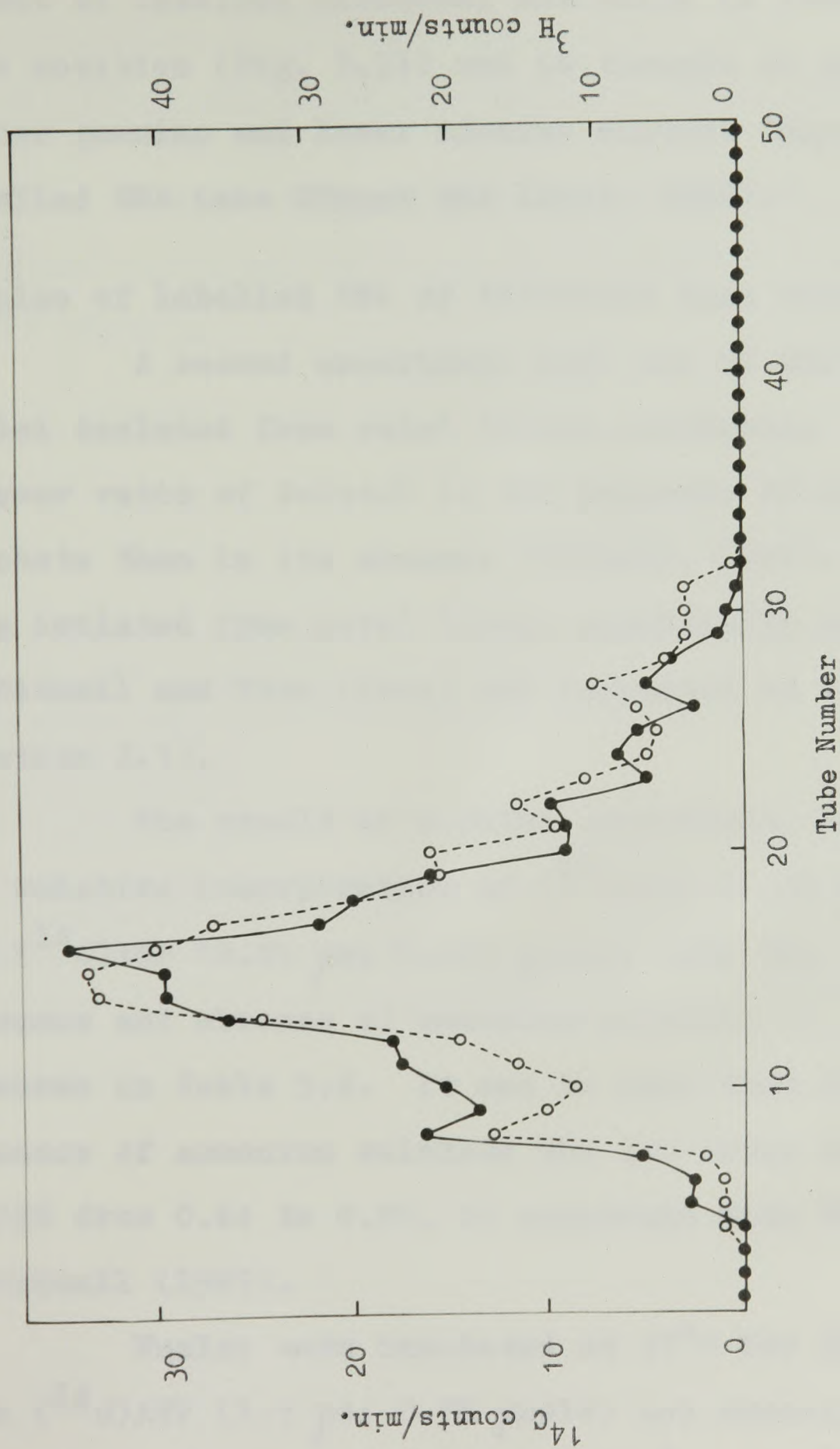


FIG. 3.12 CCD profile of rapidly-labelled RNA from rat liver slices incubated with (^3H) guanosine and (^{14}C) adenine.

●—● ^3H counts; ○---○ ^{14}C counts.

between tubes 8 and 12. This could be due to a small amount of labelled ribosomal RNA which is found in this position (Fig. 3.11) and is thought to have a higher guanine and lower adenine content than rapidly-labelled RNA (see Singer and Leder, 1966).

Samples of labelled RNA of different base compositions.

A second experiment made use of the fact that nuclei isolated from rats' livers synthesise RNA with a lower ratio of G+C:A+U in the presence of ammonium sulphate than in its absence (Widnell, 1965). Nuclei were isolated from rats' livers essentially according to Widnell and Tata (1964) and incubated as described (section 2.5).

The result of a pilot experiment, comparing the relative incorporation of (^{14}C)CTP (0.05 μc ; 0.025 μmole) and (^{14}C)ATP (0.05 μc ; 0.025 μmole) into RNA in the presence and absence of ammonium sulphate (0.28 M), is shown in Table 3.2. It can be seen that in the presence of ammonium sulphate the A:C ratio has increased by 33% from 0.64 to 0.85, in agreement with the findings of Widnell (1965).

Nuclei were incubated at 37°C for 30 min. with (^{14}C)ATP (1.5 μc ; 0.75 μmole) and ammonium sulphate (0.28 M) or in control medium with (^3H)ATP (15 μc ; 0.75 μmole).

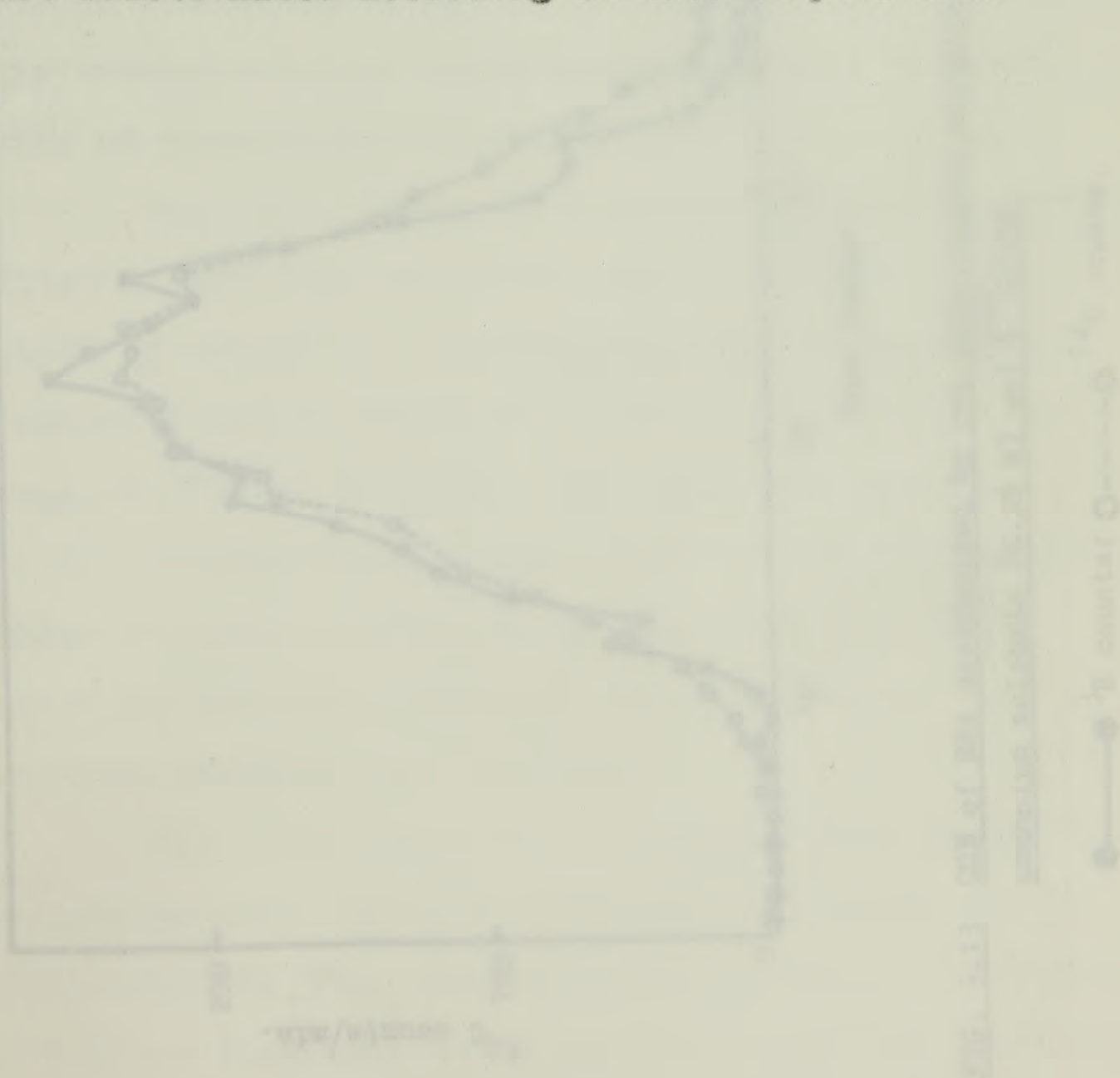
TABLE 3.2

Incorporation of (^{14}C)ATP and (^{14}C)CTP into RNA synthesised by rat liver nuclei at low and high ionic strength.

Nuclei were incubated at 37°C for 30 min. with (^{14}C)ATP (0.05 μc ; 0.025 μmole) or with (^{14}C)CTP (0.05 μc ; 0.025 μmole).

	<u>Ammonium sulphate concentration</u>	
	<u>0</u>	<u>0.28 M</u>
$\mu\text{mole } (^{14}\text{C})\text{ATP}$ incorporated/ mg. DNA/ min.	0.39	1.81
$\mu\text{mole } (^{14}\text{C})\text{CTP}$ incorporated/ mg. DNA/ min.	0.61	2.13
$\mu\text{mole ATP incorporated}$	0.64	0.85
$\mu\text{mole CTP incorporated}$		

The nuclei were combined, the RNA isolated by Method A and 1 mg. fractionated by counter-current distribution. If the RNA rich in adenine moved more rapidly along the apparatus than that rich in guanine, the ^{14}C profile would be expected to be ahead of the ^3H profile. However it can be seen from Fig. 3.13 that the profiles are coincident, again showing that rapidly-labelled RNA is not fractionated according to base composition.



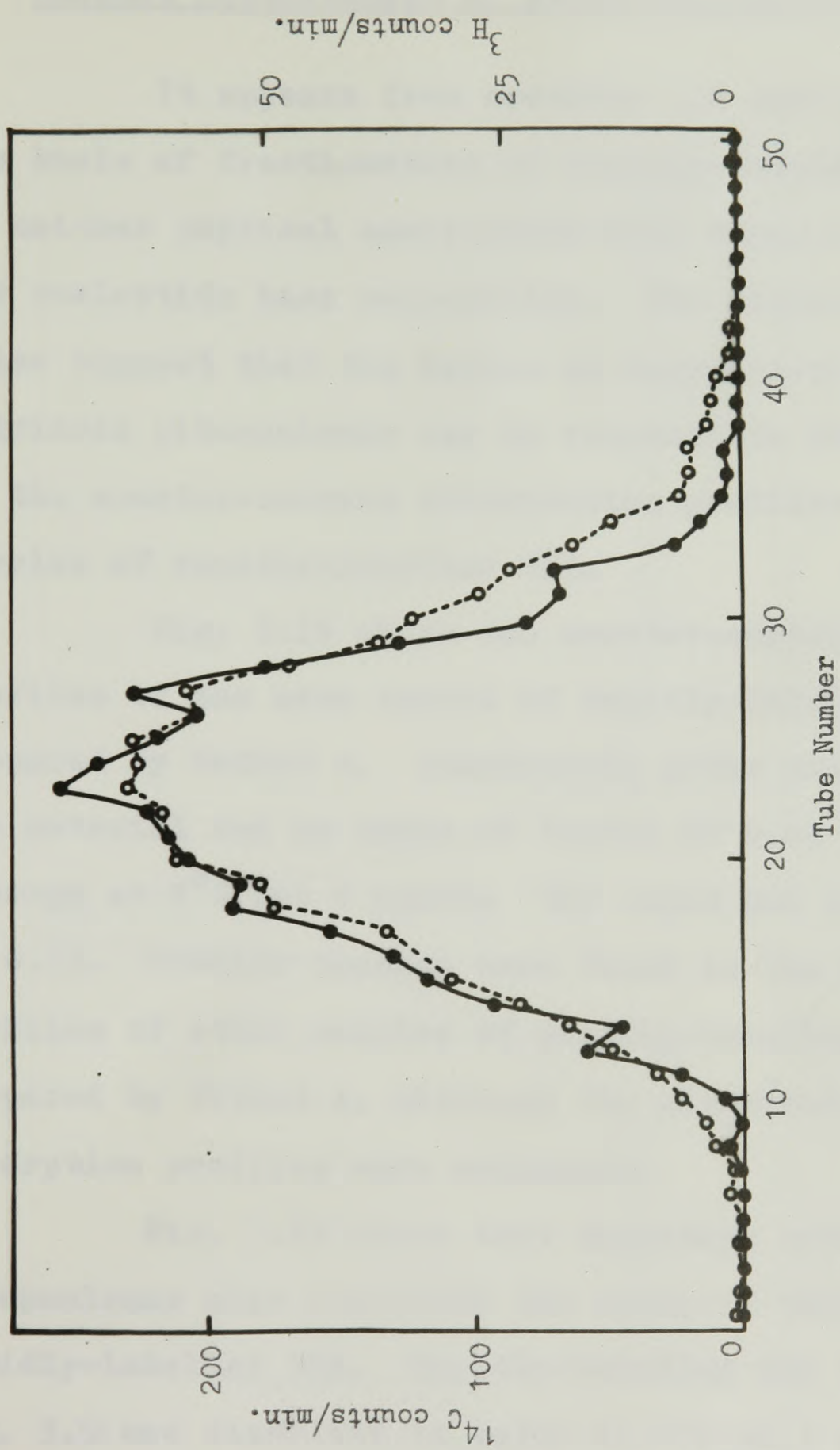


FIG. 3.13 CCD of RNA synthesised by rat liver nuclei incubated with (^3H)ATP or with ammonium sulphate (0.28 M) and (^{14}C)ATP.

●—● ^3H counts; ○---○ ^{14}C counts.

3.4 Effect of degradative procedures on the counter-current distribution of rapidly-labelled RNA.

It appears from sections 3.2 and 3.3 that the basis of fractionation of rapidly-labelled RNA is neither physical association with other nucleic acids nor nucleotide base composition. The results described below suggest that the degree of degradation by an intrinsic ribonuclease may be responsible for differences in the counter-current distribution profiles of different samples of rapidly-labelled RNA.

Fig. 3.14 shows two counter-current distribution profiles of the same sample of rapidly-labelled RNA prepared by Method A. Immediately after preparation the material had an index of travel of 0.24 but after storage at 2°C for 8 months the index had increased to 0.33. Similar changes were found in the radioactivity profiles of other samples of rapidly-labelled RNA prepared by Method A, although the ultraviolet absorption profiles were unchanged.

Fig. 3.15 shows that digestion with pancreatic ribonuclease also increased the index of travel of rapidly-labelled RNA. Rapidly-labelled RNA (1 mg.) from Fig. 3.12 was dissolved in water (1.635 ml.) and incubated for 1 hr. at 37°C with 10 µg. ribonuclease. Trilithium citrate (0.033 M; 0.065 ml.), 'Amine Solution' (0.39 ml.)

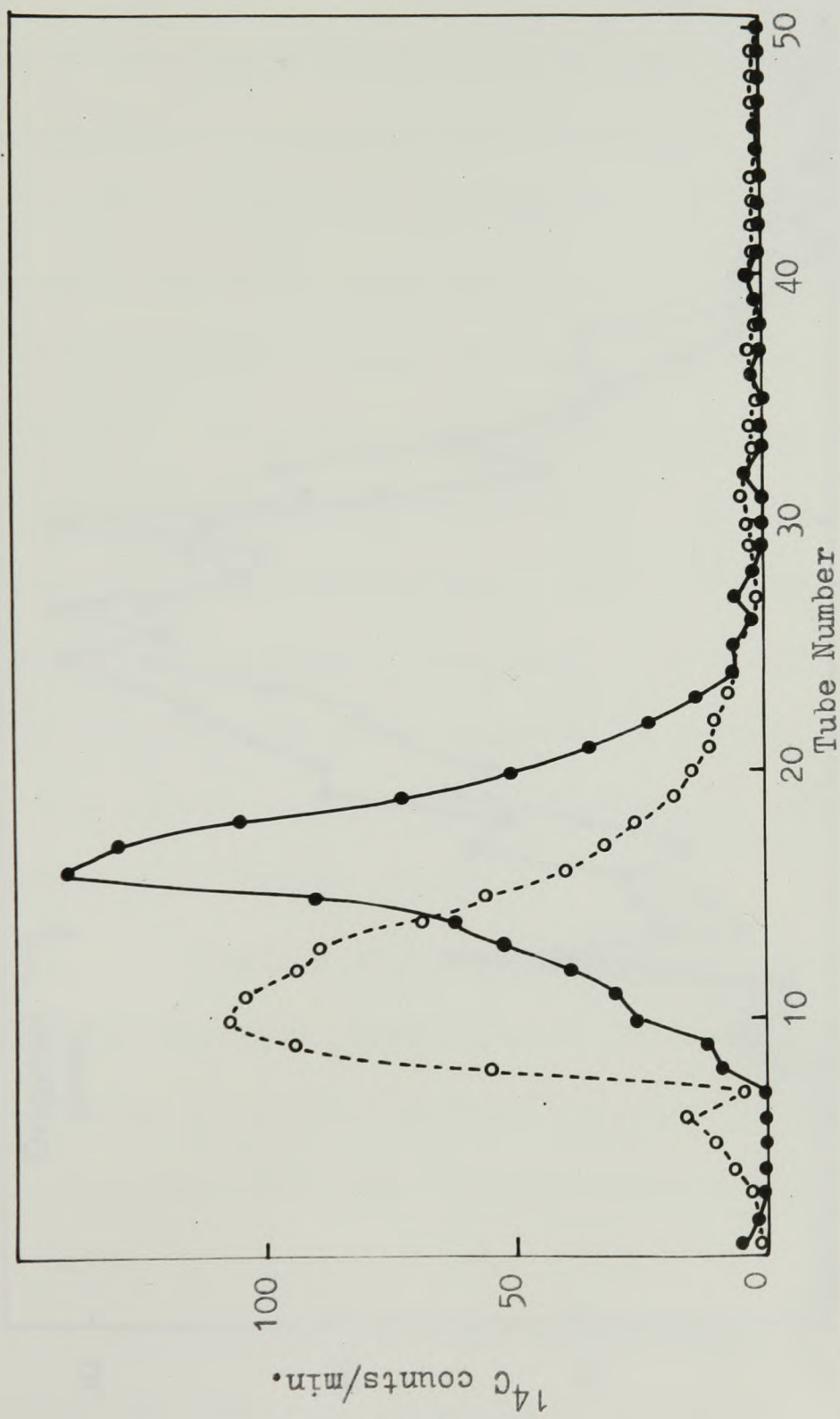


FIG. 3.14 CCD profiles of rapidly-labelled RNA immediately after preparation by Method A and after 8 months' storage at 2°C .

^{14}C counts: ○---○ immediately; ●—● after 8 months.

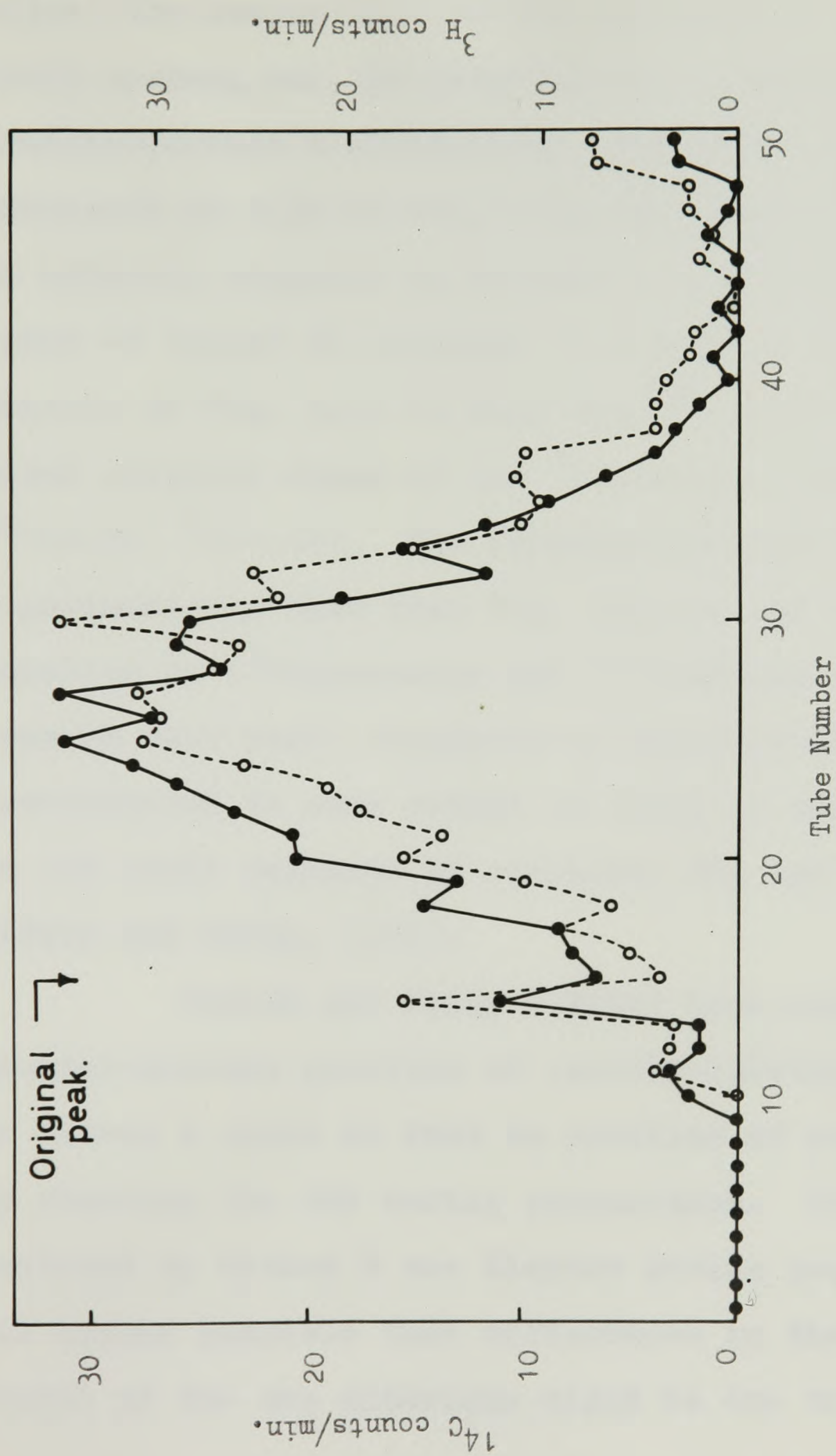


FIG. 3.15 CCD profiles of RNA of Fig. 3.12 after digestion with pancreatic ribonuclease (10 $\mu\text{g.}$) for 1 hr. at 37°C.

●—● ^3H counts (from (^3H) guanosine); ○---○ ^{14}C counts (from (^{14}C) adenine).

and 'Organic Solution' (0.91 ml.) were then added, to adjust the composition of the solution to that of a 150/2 system, and the material was fractionated by counter-current distribution. The index of travel has increased to 0.52 in Fig. 3.15 and ribonuclease digestion of material prepared by Methods A or B always caused the index of travel to increase to a similar value. Another feature of Fig. 3.15 is that the ^{14}C profile has now moved slightly ahead of the ^3H profile (indices of travel: $^{14}\text{C}=0.54$, $^3\text{H}=0.50$). The rapidly-labelled RNA in this experiment was that from Fig. 3.12 and had been differentially labelled by (^3H)guanosine and (^{14}C)adenine. It therefore appears that small fragments of rapidly-labelled RNA are fractionated to some extent in terms of base composition, as are small segments of ribosomal RNA and DNA (Kirby, 1962; Kidson and Kirby, 1963).

Parish and Kirby (1966b) have suggested that counter-current profiles of rapidly-labelled RNA isolated by Method B might in fact be profiles of segments produced by shearing the RNA during preparation. Since only RNA isolated by Method B was blended during preparation, it did appear possible that differences in the indices of travel of the two materials might be due to shearing.

RNA (1 mg.) isolated by Method A was therefore dissolved in 3 ml. solvent system 150/2 and blended for 5 min., at full speed, in a Vertis "45" homogeniser (The Virtis Co. Ltd., Yonkers, N.Y., U.S.A.) before counter-current distribution. However the index of travel was found to be unchanged.

That the presence of barbitone during the formation of rapidly-labelled RNA did not alter its counter-current distribution profile. The experiments with live cells were as similar as possible to those that have been reported to give marked changes in profile (Johnson and Kirby, 1964), except for certain improvements in the RNA extraction. These were the use of a mixed aminosalicylate extraction, a better ethanol precipitation and the inclusion of glycogen in the alcohol (see section 2.2). Whether unknown factors have prevented similar changes in this work cannot be certain. However there is clearly a large difference in the profiles of rapidly-labelled RNA isolated by Methods A and B (compare Figure 4 with Fig. 16-5); hence smaller differences may well result from small and unavoidable variations in isolation. In these experiments, but not in previous ones, variations in the isolation of control and experimental RNA were avoided by double-labelling.

4. DISCUSSION

It can be seen from Fig.3.1-4 and Fig.3.6-8 that the presence of hormones during the formation of rapidly-labelled RNA did not alter its counter-current distribution profile. The experiments with live rats were as similar as possible to those that have been reported to give marked changes in profile (Kidson and Kirby, 1964d), except for certain improvements in the RNA extraction. These were the use of a second aminosalicylate extraction, a sodium chloride deproteinisation and the inclusion of m-cresol in the phenol (see section 2.2). Whether unknown factors have prevented profile changes in this work cannot be certain. However there is clearly a large difference in the profiles of rapidly-labelled RNA isolated by Methods A and B (compare Fig.3.1-4 with Fig.3.6-8); hence smaller differences may well result from small and unavoidable variations in isolation. In these experiments, but not in previous ones, variations in the isolation of control and experimental RNA were avoided by double-labelling.

Since the publication of part of the work described in Part One of this thesis (Leader and Barry, 1967; Leader and Barry, 1968) somewhat similar studies have been reported by Jackson and Sells (1968). They were unable to detect changes in the counter-current distribution profiles of rapidly-labelled RNA from the livers of hypophysectomised rats after treatment with growth hormone, thyroxine or hydrocortisone. They also used double-labelling but isolated the rapidly-labelled RNA differently and used a 150/14 solvent system.

Storage (Fig. 3.14) and ribonuclease treatment (Fig. 3.15) caused an increase in the index of travel of rapidly-labelled RNA prepared by Method A. The different profiles of rapidly-labelled RNA isolated by Methods A and B most probably result from differing degrees of degradation by an intrinsic ribonuclease. Although it is known that ribonuclease does survive phenol extraction (see Huppert and Pelmont, 1962), Kidson and Kirby (1964b) claimed that their rapidly-labelled RNA preparations (isolated by a Method similar to Method B) were undegraded since these had high sedimentation coefficients on sucrose gradients. However Parish and Kirby (1966b) have since found that when certain rapidly-labelled RNA preparations were

examined on sulpholane/ethylurea gradients (in which complex formation is reduced) there was degraded material of low sedimentation coefficient which was not apparent on sucrose gradients. Indeed, because of their findings, Kirby and coworkers have altered their methods of RNA isolation and more recently have been using rapidly-labelled RNA prepared by a method similar to Method A (Parish and Kirby, 1966b; Wilkinson and Kirby, 1966). The results in Fig. 3.14, however, show that even RNA prepared by Method A is not free from ribonuclease.

It is not clear why rapidly-labelled RNA prepared by Method B should be more degraded than that prepared by Method A. While the material prepared by Method A may contain less ribonuclease, it is also possible that the large amount of ribosomal RNA protects this rapidly-labelled RNA from the ribonuclease.

It is necessary to consider whether other factors, which it has been suggested may influence counter-current distribution, could have caused the difference in profile between Methods A and B.

Kidson and Kirby (1964b) found differences between profiles of RNA labelled in vivo and in vitro and suggested that this might be due to different rates of uptake and utilisation of radioactive precursor.

Although, generally, Method A was used for experiments in vitro and Method B for experiments in vivo, this cannot be the cause of the different indices of travel for RNA isolated by the two methods, since RNA isolated by Method B from slices incubated in vitro had a high index of travel (Fig. 3.12).

Association of rapidly-labelled RNA with other species of nucleic acids might also influence its counter-current distribution profile (Kidson and Kirby, 1964b; Jackson and Sells, 1968). However the results described in section 3.2 provide no evidence that the difference between the RNA prepared by Methods A and B is due to association with different nucleic acid species.

Parish and Kirby (1966b) have shown that homogenisation by blending, which is used in Method B, may cause fragmentation of rapidly-labelled RNA. Though shearing of RNA isolated by Method A did not affect its counter-current distribution profile (p. 40), this could be conceivably due to the different experimental conditions employed. However when rapidly-labelled RNA was prepared from slices by Method B, homogenisation was done in a Potter-Elvehjem type homogeniser and not in a blender (see section 3.3). Since this RNA had

a high index of travel (Fig. 3.12), factors other than shearing must be involved.

It was originally suggested that the counter-current fractionation of rapidly-labelled RNA might depend on its base composition, as does the fractionation of segments of ribosomal RNA and of DNA (Kidson and Kirby, 1964b). However the two different experiments described in section 3.3 show that this is not so, although base composition may influence the fractionation when the RNA is extremely degraded.

It appears that any fractionation of rapidly-labelled RNA that does occur on counter-current distribution is probably on the basis of molecular size. However when the least degraded rapidly-labelled RNA was subjected to counter-current distribution in solvent system 150/12.5 little fractionation occurred and the rapidly-labelled RNA was not separated from other species of nucleic acids.

EXPERIMENTAL

5.1 Materials.

Specimens were obtained from the following sources:
Glucose-free insulin (Lot 23-440) was a gift from
Dr. Otto Lehmann, Eli Lilly Research Laboratories,
Indianapolis, Ind., U.S.A. Bovine prolactin (110-2-52)
and bovine growth hormone (110-2-52) were supplied by
the Endocrinology Study Series, National Institute of
Health, Bethesda, PART TWO ... Carbachol (Lot 1200-
1970) and 5,7,5'-trifluoro-L-tryptophan (Lot 110-2-52)
were purchased from the Sigma Chemical Co. Inc., Boston,
centralized and propagated from British Drug Houses Ltd.,
Poole, Dorset, and ergometrin 1" (Mylan), Batch No. 48007;
1 unit/ml. ethanol; from British Ltd., London, Switzerland.
For each experiment insulin was freshly dissolved in
0.1M NaCl (2 mg./ml.), prolactin and growth hormone in
0.1M NaCl (2 mg./0.5 ml.) and the other hormones in
ethanol. The final concentration of insulin in the culture
medium never exceeded 0.1%.

5. EXPERIMENTAL

5.1 Materials.

Hormones were obtained from the following sources. Glucagon-free insulin (lot PJ-4609) was a gift from Dr. Otto Behrens, Eli Lilly Research Laboratories, Indianapolis, Ind., U.S.A.. Bovine prolactin (NIH-P-B1) and bovine growth hormone (NIH-GH-B10) were donated by the Endocrinology Study Section, National Institute of Health, Bethesda, Md., U.S.A.. Corticosterone (lot 126B-197C) and 3,3',5-triiodo-L-thyronine (lot 24B-0300) were purchased from the Sigma Chemical Co. Ltd., London, oestradiol and progesterone from British Drug Houses Ltd., Poole, Dorset, and oxytocin ("Syntocin", Batch No.66083; 1 unit/ml. ethanol) from Sandoz Ltd., Basle, Switzerland. For each experiment insulin was freshly dissolved in 3mM HCl (2 mg./ml.), prolactin and growth hormone in medium"199" (2 mg./0.5 ml.) and the other hormones in ethanol. The final concentration of ethanol in the culture medium never exceeded 0.25%.

Actinomycin D was a gift from Dr. H. D. Brown, Merck, Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A.. Puromycin was purchased from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A., and hydroxyurea and cycloheximide from Sigma Ltd.. A stock solution of actinomycin D (1 mg./ml. H_2O) was stored at $-40^{\circ}C$ in the dark. Fresh aqueous solutions of other inhibitors were prepared for each experiment.

D-glucose 6-phosphate, dipotassium salt; 6-phosphogluconate, trisodium salt, and nicotinamide-adenine dinucleotide, oxidised form, monosodium salt, were purchased from Sigma Ltd.. Bovine serum albumin (fraction V) was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, and Tissue Culture Medium "199" and sera from Glaxo Laboratories Ltd., Greenford. All other reagents were purchased from British Drug Houses Ltd. and were, where possible, AnalaR grade. Glass-distilled water was used for all solutions.

L-(U- ^{14}C)lysine (specific activity 7.6 mc/m-mole), (2- ^{14}C)uridine (specific activity 58.1 mc/m-mole) and (6- $^3H(n)$)thymidine (specific activity 3.0 c/m-mole) were purchased from the Radiochemical Centre, Amersham, Bucks..

5.2 Animals.

All mice used were of the C3H strain derived from stock of the M.R.C. Laboratory Animal Centre, Carshalton, Surrey. They were housed and bred in the Department of Agriculture and were given diet FFG(M) (Dixon and Sons Ltd., Ware, Herts.) and tap water ad libitum.

Mice used for experiments were in their first pregnancy or lactation and approximately 3 months old. Pairs were mated in separate cages and examined each morning for the presence of vaginal plugs. The day on which the plug was detected was designated 'day 1' of pregnancy and pregnancy lasted about 19 days, young being born between midnight on 'day 19' and midday on 'day 20'. In all experiments 'mid-pregnant' refers to mice killed on the morning of day 12 of pregnancy and 'late-pregnant' to mice killed on the morning of day 19 of pregnancy. When mice were to be used in lactation, the males were removed after mating to prevent post partum coitus. The day on which young were first detected was designated 'day 1' of lactation.

In some experiments mice were used at a definite stage of the oestrous cycle. This was determined by examining vaginal smears under a phase-contrast microscope.

Oestrous mice were those in which the smears contained predominantly non-nucleated, cornified, cells.

Metoestrous mice were those in which the smears contained predominantly leucocytes.

5.3 Determination of DNA and RNA.

The DNA and RNA of mammary tissue was determined by the following procedure. All operations were performed in the cold room at 2°C.

Tissue was ground with acetone (3 ml.) in an all-glass homogenizer with a mechanical drive. The homogenate was allowed to stand for 20 min. before separating the residue by centrifugation (5 min. at 5,000g). The residue was washed 3 times with acetone (3 ml.), to remove all the fat, and twice with 0.2 N perchloric acid (3 ml.). The residue was then incubated with 0.3 N KOH (2 ml.) for 2 hours at 37°C, to hydrolyse the RNA. The solution was adjusted to pH 2 with 1.2 N perchloric acid and allowed to stand for 10 min., precipitating the DNA.

RNA was determined in a portion of the supernatant by the orcinol method (Mejbaum, 1939), with yeast RNA as a standard.

The residue was then washed twice with 0.2 N perchloric acid (1 ml.). The DNA was ^{extracted} ~~hydrolysed~~ by heating at 70°C for 25 min. with 0.5 N perchloric acid (4 ml.) and estimated by the diphenylamine method (Burton, 1956), using deoxyribose as a standard.

5.4 Preparation of tissue for microscopic examination.

Tissue for microscopic examination was fixed in Bouin's solution, sectioned serially at 7 μ and stained with haematoxylin and eosin (G. T. Gurr Ltd., London.).

5.5 Method of culture.

The watchglass organ culture method of Elias (1957) is suitable for histological or radioactive incorporation studies (Juergens, Stockdale, Topper and Elias, 1965). However to study changes in the enzyme activities of mammary tissue in culture it was necessary to develop a method of incubating greater amounts of tissue. This new method is described below.

All operations were performed under aseptic conditions, and instruments and apparatus were sterilised by autoclaving (15 min. at 15 lb./in.²). Solutions were sterilised by passing through a sterile filter, 0.22 μ

pore size, held in a "Swinny Adaptor" (Millipore Filter Corporation, Bedford, Mass., U.S.A.).

A single mouse was killed by cervical dislocation, immersed in 70% ethanol, dried, and the inguinal mammary glands removed and placed in medium "199" (5 ml.) in a petri dish. A large number of explants, of about 1.5 mm^3 size, were cut from the glands using iridectomy scissors and transferred with fine forceps to a second petri dish containing medium "199" (5 ml.). Groups of 20 explants, selected at random, were added to sterile plastic petri dishes, 5 cm. diameter, with lids specially designed to allow passage of gas into the dishes (Sterilin Ltd., Richmond, Surrey). The dishes contained medium "199" (5 ml.) with the relevant hormone additions and the explants floated on the surface of the medium. In each experiment two batches of explants were stored at -40°C for determination of the initial enzyme activity and the fresh weight of 20 explants was also determined, after blotting.

The composition of medium "199" is given by Morgan, Morton and Parker (1950). It contains 100 mg. glucose / 100 ml. and is supplied with added penicillin (200 units/ml.) and dihydrostreptomycin ($100 \mu\text{g./ml.}$). Before use an extra 132 mg. of NaHCO_3 / 100ml. was added to maintain the pH at 7.4 under the conditions of incubation.

The hormones, when added, were always at the following concentrations: 5 $\mu\text{g./ml.}$ insulin (8.7×10^{-7} M), 10 $\mu\text{g./ml.}$ prolactin (3.4×10^{-7} M) and 1 $\mu\text{g./ml.}$ corticosterone (2.9×10^{-6} M). The concentrations of other hormones are indicated in the text.

The culture apparatus, which was constructed in the Department of Agriculture, is shown in Fig. 5.1 and was sterilised before each experiment. The incubation vessel is a 3 litre glass beaker with a loose fitting lid inside which is a stainless steel stand on which about 20 petri dishes can be stacked. The gas mixture of oxygen (95%)/ carbon dioxide (5%) first passes through a water bubbler and then through a sterile filter tube containing cotton wool before entering the incubation vessel. The whole of the apparatus is housed in an incubator at 37°C and is not illuminated.

After incubation, the explants were removed and stored at -40°C . Their enzyme activities were determined when all the incubations had been completed (see section 5.6).

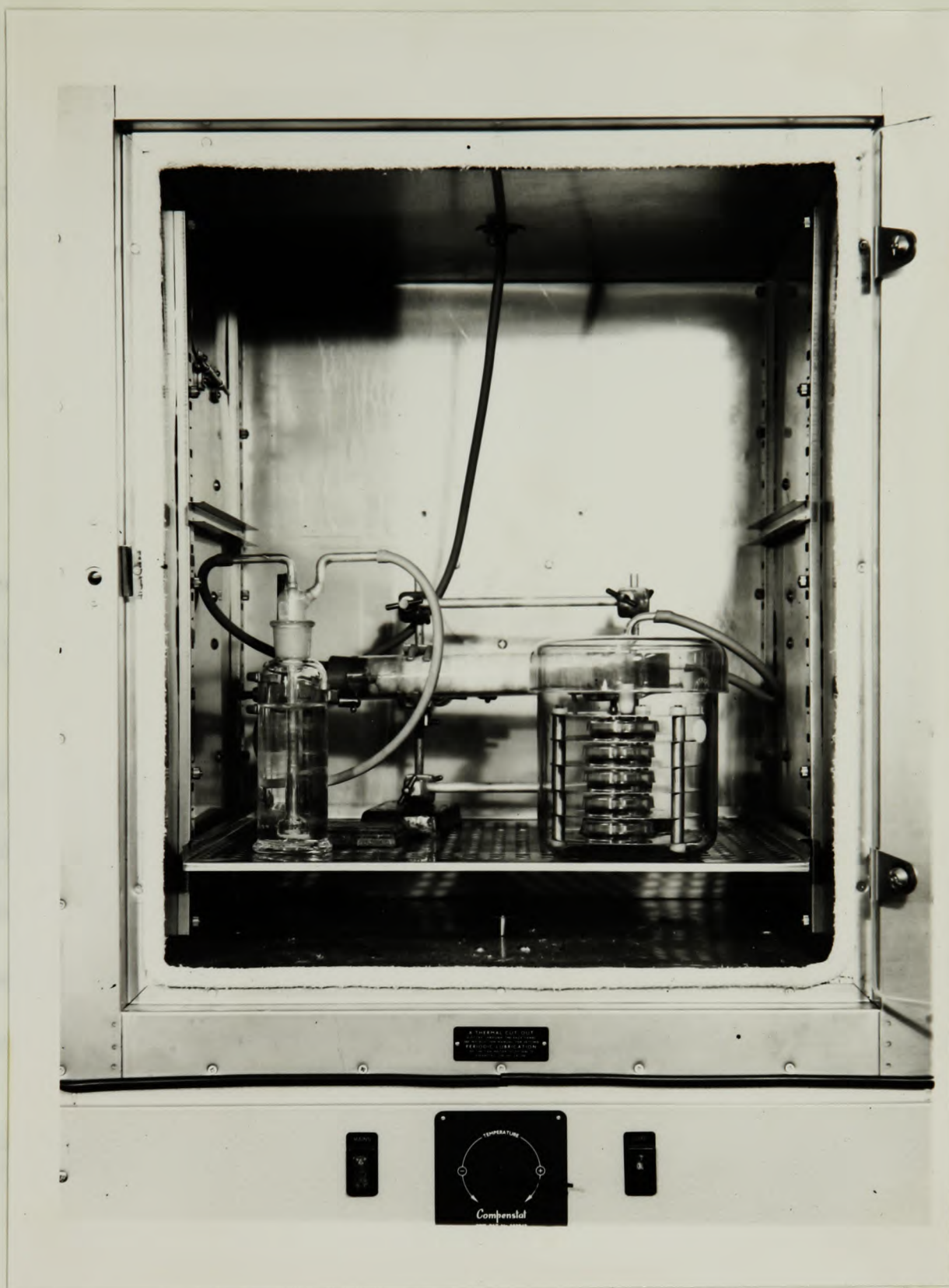
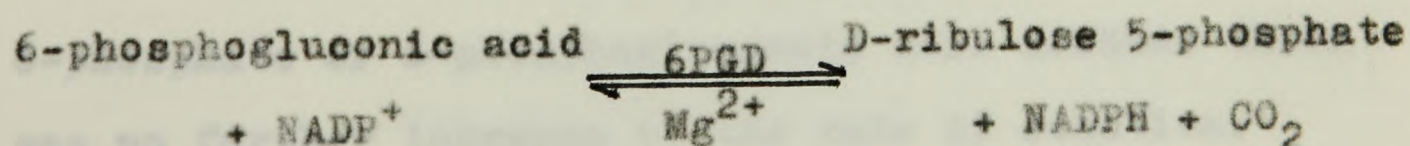
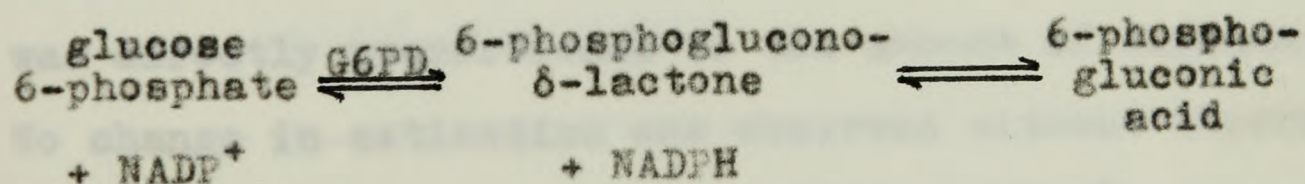


FIG. 5.1 Mammary explant culture apparatus.

5.6 Determination of enzyme activities.

A tissue supernatant for enzyme assay was prepared in the following manner. Each batch of 20 explants was ground at 2°C with 0.75 ml. of 0.15 M KCl containing 10 mM tris-HCl (pH 7.6) in an all-glass homogeniser with a mechanical drive. The homogenate was centrifuged at 100,000g for 45 min. at 2°C in an MSE "Superspeed 40" centrifuge. The supernatant was drawn off with a Pasteur pipette, care being taken to avoid the plug of fat which floated on the surface.

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP oxidoreductase, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate: NADP oxidoreductase (decarboxylating), EC 1.1.1.44) were determined according to Glock and McLean (1953). The reactions catalysed by these enzymes are shown below:



The enzymes were determined in the presence of excess substrates by following the appearance of NADPH spectrophotometrically at 340 mμ. In the presence of

glucose 6-phosphate and 6-phosphogluconate, NADPH formation is due to both enzymes, while with 6-phosphogluconate alone it is due only to 6-phosphogluconate dehydrogenase. The NADPH formation due to glucose 6-phosphate dehydrogenase can be found by difference. The complete reaction mixture contained: 40 mM glycyl glycine-HCl, pH 7.6 (1.5 ml.), 1 mM NADP^+ (0.3 ml.), 10 mM MgCl_2 (1 ml.), 30 mM 6-phosphogluconate (0.1 ml.), $\pm$ 20 mM glucose 6-phosphate (0.1 ml.) in 1 cm. quartz spectrophotometer cells. The reaction was started by adding supernatant (generally 0.1 ml.) and was followed at 20°C in a Zeiss PMQII spectrophotometer at 340 m μ . The increase in extinction was measured during the first 5 min. and was not allowed to exceed 0.03/min.. One unit of enzyme activity is equivalent to the reduction of 1 μ mole of NADP^+ /min..

Fig. 5.2 shows that the rate of reaction was linear over the first 5 min. and Fig. 5.3 shows that it was directly proportional to the amount of supernatant. No change in extinction was observed without supernatant or NADP^+ . When the concentrations of NADP^+ , glucose 6-phosphate or 6-phosphogluconate were doubled there was no further increase in the rate of reaction.

As unincubated explants were assayed after storage at -40°C for up to 24 hr., an experiment was performed to see if this affected the enzyme activities. Table 5.1 shows that it did not.

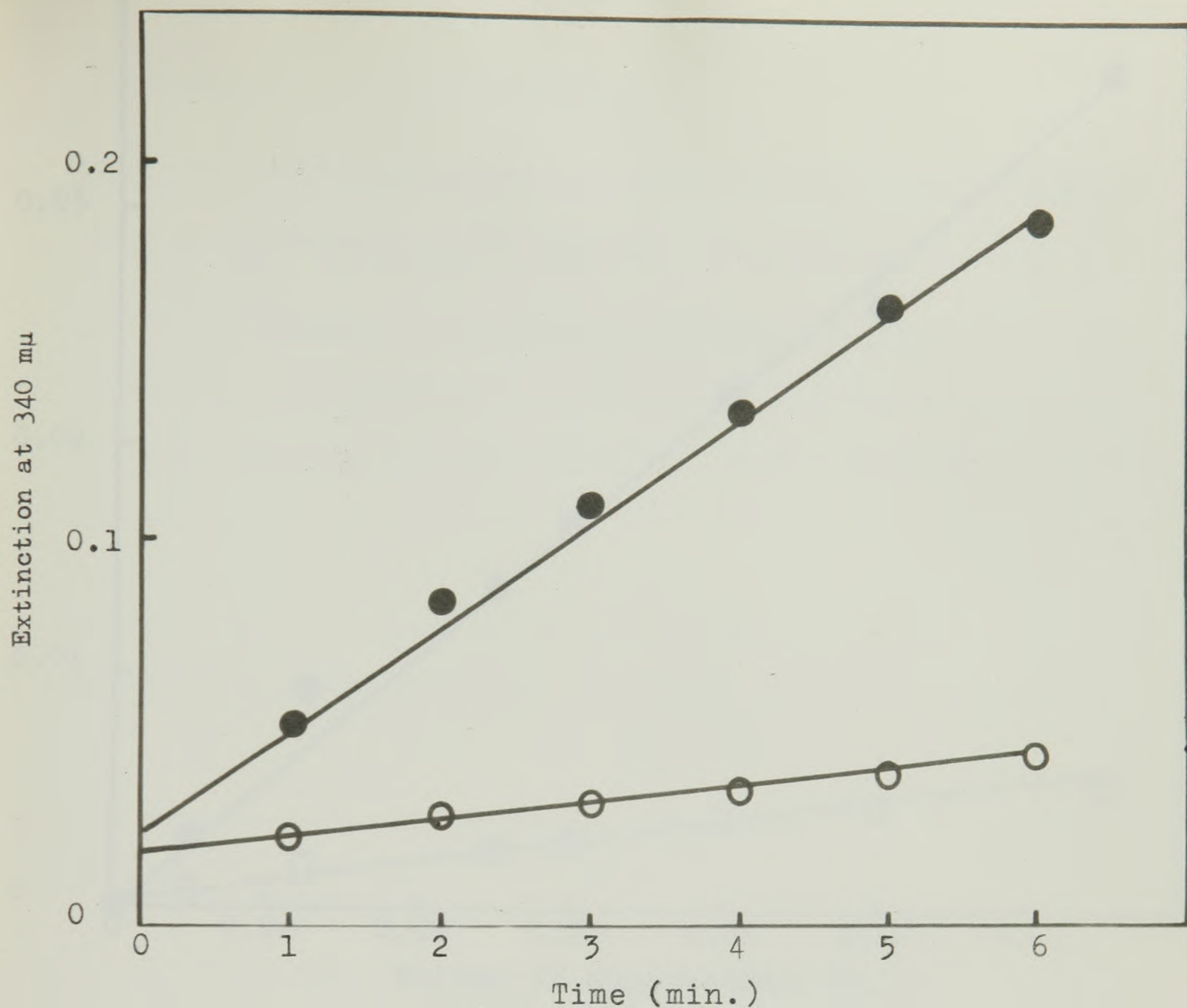


FIG. 5.2 Rate of reduction of NADP^+ by mouse mammary gland supernatant.

- — ○ 6-phosphogluconate in reaction mixture.
- — ● Glucose 6-phosphate and 6-phosphogluconate in reaction mixture.

Each reaction mixture contained 0.1 ml. supernatant (from 5 mg. tissue).

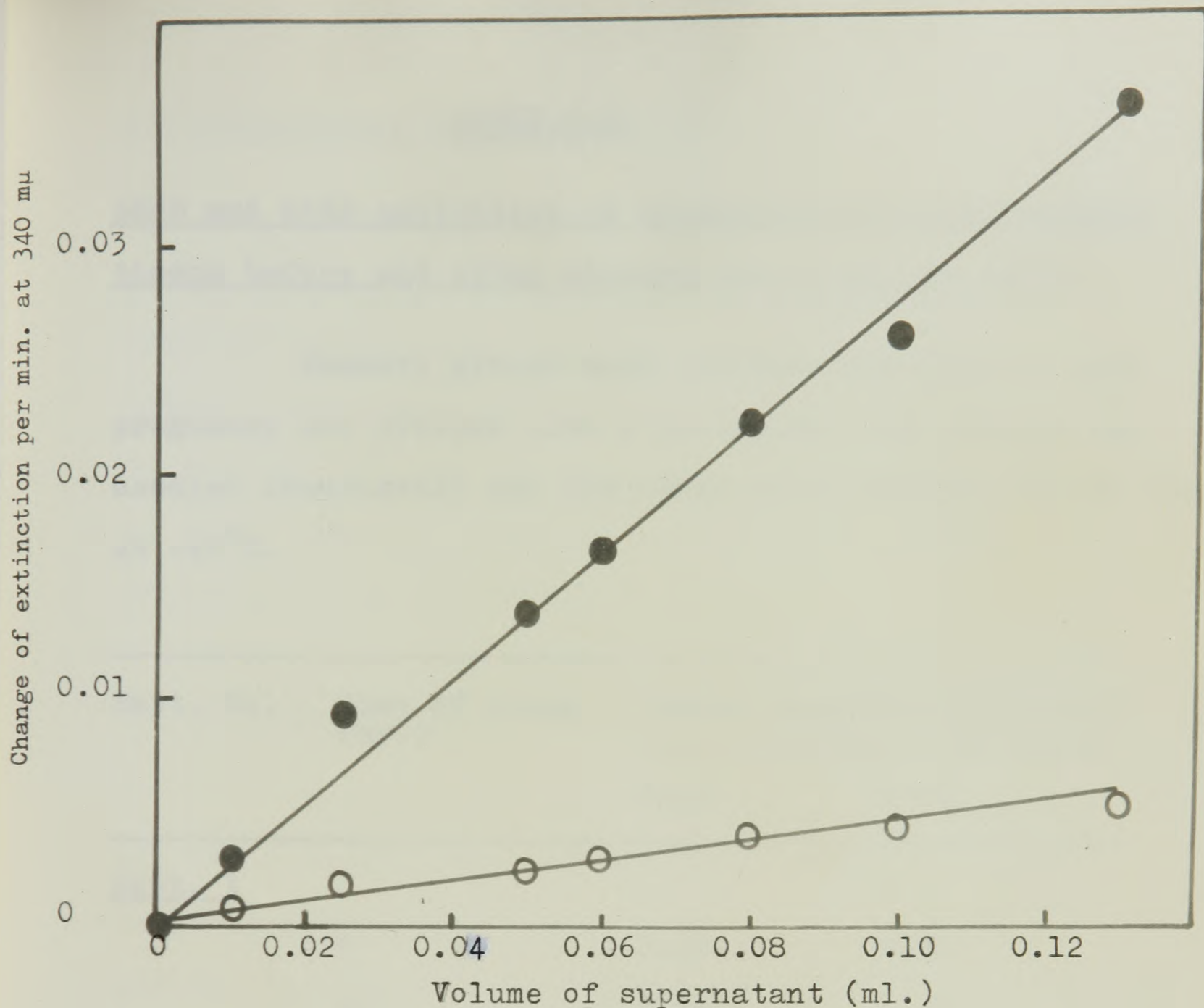


FIG. 5.3 Rate of reduction of NADP^+ by different amounts of mouse mammary gland supernatant.

○ — ○ 6-phosphogluconate in reaction mixture.

● — ● Glucose 6-phosphate and 6-phosphogluconate in reaction mixture.

The mammary gland was homogenised in 2 ml. medium / 100 mg. tissue. Each reaction mixture had a total volume of 3.1 ml..

TABLE 5.1

G6PD and 6PGD activities of late-pregnant mouse mammary tissue before and after storage for 24 hr. at -40°C.

Mammary glands were removed from mice in late pregnancy and divided into 2 portions. One portion was assayed immediately and the other after storage for 24 hr. at -40°C.

Expt. No.	Time of assay (hr.)	Enzyme activity (units/g.)	
		G6PD	6PGD
<u>Expt. 1</u>			
	0	2.40	0.36
	24	2.28	0.33
<u>Expt. 2</u>			
	0	1.98	0.44
	24	2.02	0.44

5.7 Preparation of mouse serum.

When serum was added to the culture medium it was freshly prepared for each experiment. It was obtained by bleeding mice from the right carotid artery under ether anaesthesia. The blood was allowed to clot at 37°C for 30 min., the clot precipitated by gentle centrifugation and the clear supernatant serum drawn off by Pasteur pipette.

5.8 Preparation of an extract of mouse placentae.

A mouse was killed on day 12 of pregnancy, the foeti removed and the placentae dissected out. Eight placentae were homogenised at 2°C in 1 ml. medium "199" in an all-glass homogeniser. The homogenate was centrifuged at 100,000g for 60 min. and the supernatant extract drawn off with a Pasteur pipette.

5.9 Determination of inhibition curves.

Inhibition curves were determined for the inhibitors of nucleic acid and protein synthesis to decide which concentrations were suitable for use.

Batches of 20 explants were prepared and incubated as described (section 5.5). Explants were

pre-incubated with the inhibitor for 30 min. in medium "199" containing insulin (5 μ g./ml.) before the addition of the particular radioactive nucleic acid or protein precursor (0.25 μ c of L-(U- 14 C)lysine, 0.25 μ c of (2- 14 C)uridine or 2 μ c of (6- 3 H(n))thymidine). The explants were incubated for a further 3 hr. and then stored at -40°C until analysed.

Protein was isolated by the method of Wool and Moyer (1964), dissolved in 90% (v/v) formic acid (0.7 ml.), the solution spread on an aluminium planchette and dried under an infrared lamp. The ^{14}C disintegrations were measured in a Beckman Lowbeta II gas-flow counter with an efficiency of approximately 25%.

DNA and RNA were isolated by the following procedure. Tissue was ground in (3:1, v/v) ethanol/ diethyl ether (3 ml.) and allowed to stand for 30 min. before separating the residue by centrifugation. The residue was extracted twice for 20 min. with 15% trichloroacetic acid (3 ml.) and then washed 3 times in (3:1, v/v) ethanol/ ether (3 ml.). The residue was then extracted twice at 100°C for 15 min. with 90% (v/v) formic acid (1 ml.), 0.5 ml. of the extract deposited on a glass-fibre disc (Whatman GF/A, 2.1 cm.), dried with an infrared lamp and placed in a counting bottle containing 4 ml. of scintillation fluid (toluene (1 l.), 2,5-diphenyloxazole (5 g.),

α -naphthylphenyloxazole (0.05 g.)). The ^{14}C or ^3H disintegrations were measured in a Beckman Liquid Scintillation Spectrometer with efficiencies of 45% and 5% respectively.

Fig. 5.4 shows the inhibition curve for cycloheximide (actidione), an inhibitor of protein synthesis. (Ennis and Lubin, 1964). At 1 $\mu\text{g.}/\text{ml.}$ it inhibited the incorporation of (^{14}C)lysine into protein by 93% and this concentration was chosen for use in culture experiments.

Fig. 5.5 shows the inhibition curve for puromycin, another inhibitor of protein synthesis (Yarmolinsky and de la Haba, 1959). At 10 $\mu\text{g.}/\text{ml.}$ it inhibited the incorporation of (^{14}C)lysine into protein by 90% and this concentration was chosen for use in culture experiments.

Fig. 5.6 shows the inhibition curve for actinomycin D, an inhibitor of RNA synthesis (Reich, Franklin, Shatkin and Tatum, 1961). At 0.5 $\mu\text{g.}/\text{ml.}$ it inhibited incorporation of (^{14}C)uridine into RNA by 87% and this concentration was chosen for use in culture experiments. Actinomycin D (0.5 $\mu\text{g.}/\text{ml.}$) inhibited incorporation of (^3H)thymidine into DNA by 16% and incorporation of (^{14}C)lysine into protein by 17%.

Fig. 5.7 shows the inhibition curve for hydroxyurea, an inhibitor of DNA synthesis (Young and Hodas, 1964). At 50 $\mu\text{g.}/\text{ml.}$ it inhibited incorporation

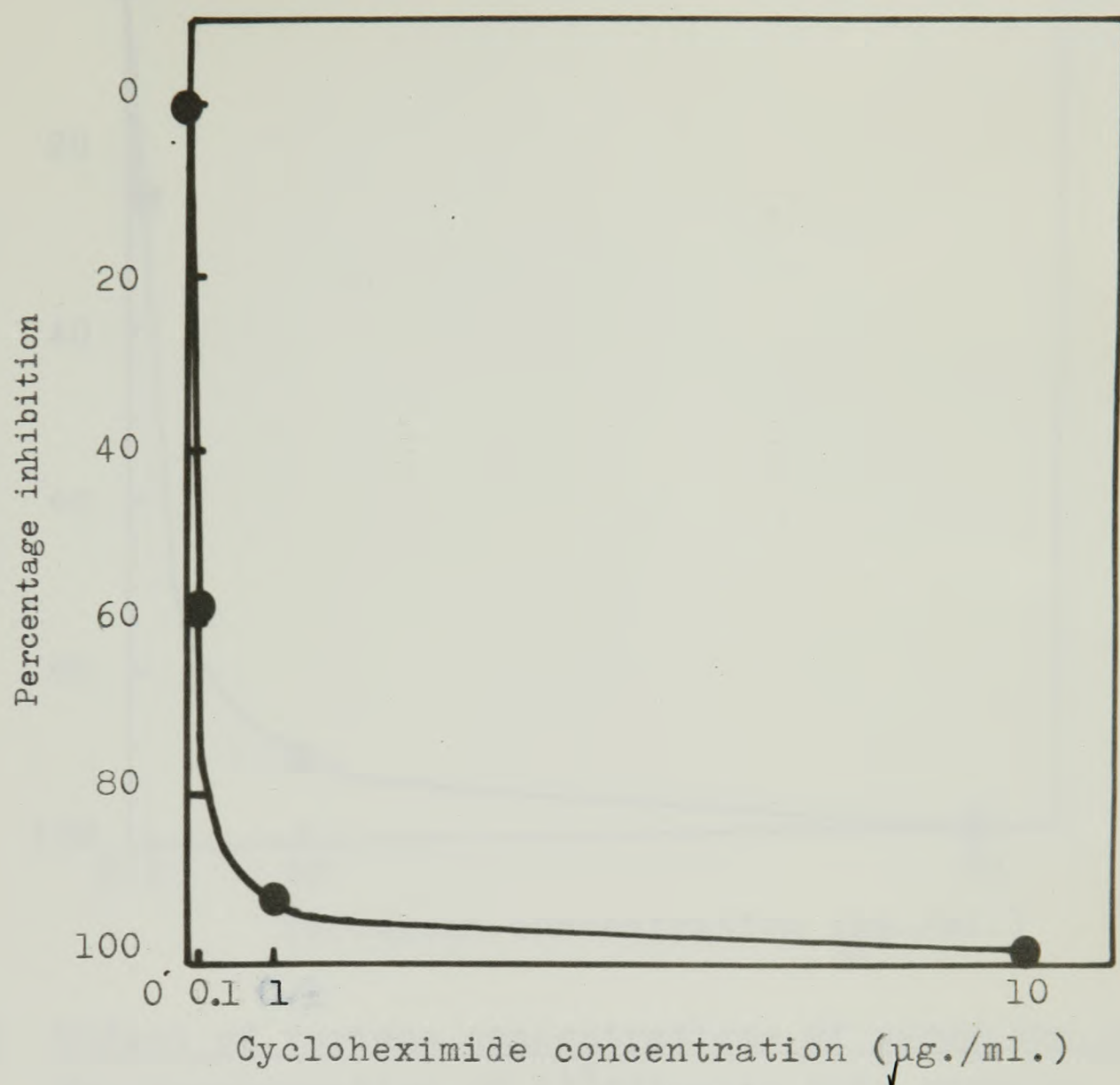


FIG. 5.4 Effect of various concentrations of cycloheximide on the incorporation of (^{14}C)lysine into protein.

The uninhibited level was 223 counts/min..
Each point is the average of duplicate determinations.

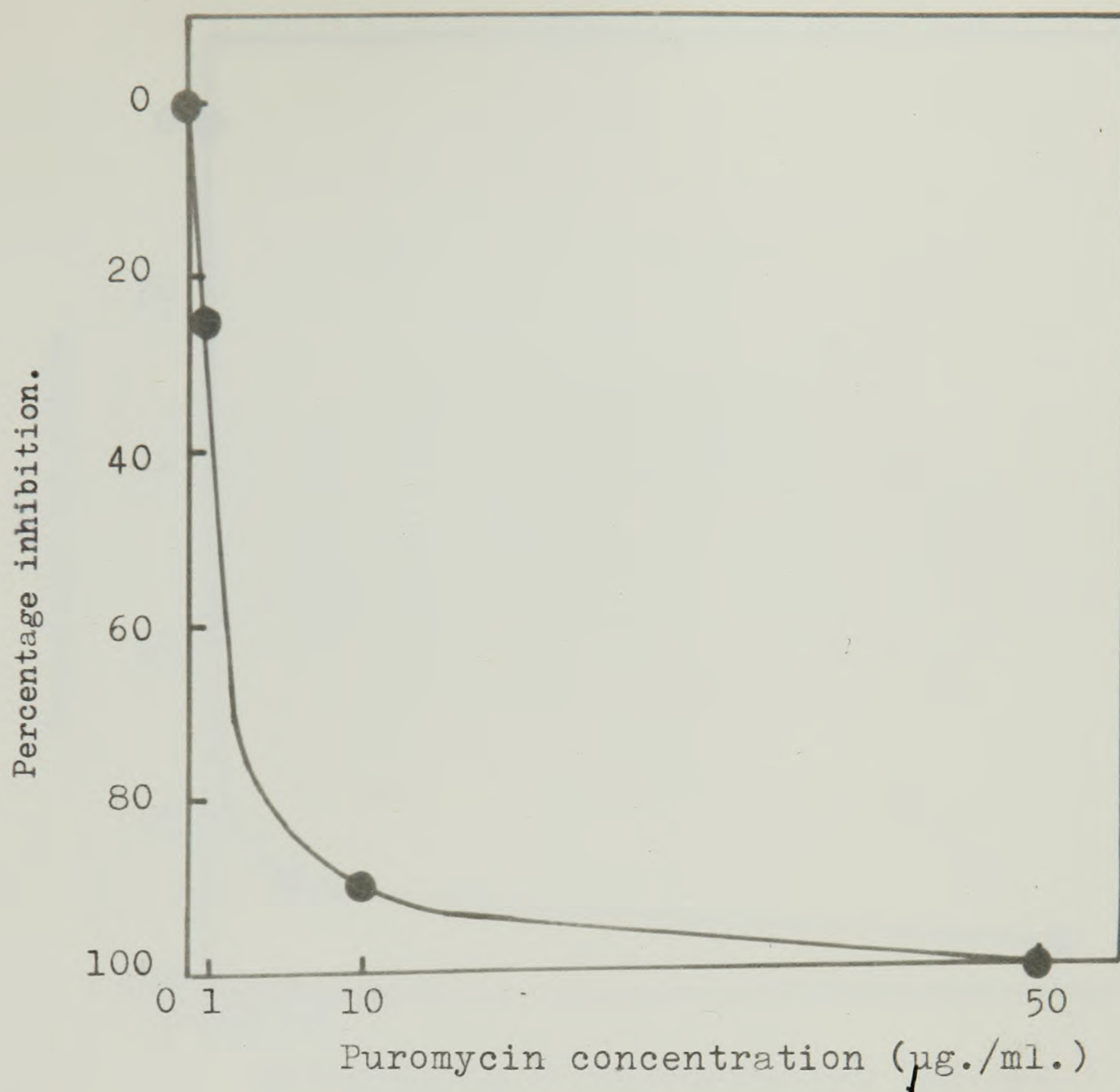


FIG. 5.5 Effect of various concentrations of puromycin on the incorporation of (^{14}C)lysine into protein.

The uninhibited level was 223 counts/min.
Each point is the average of duplicate determinations.

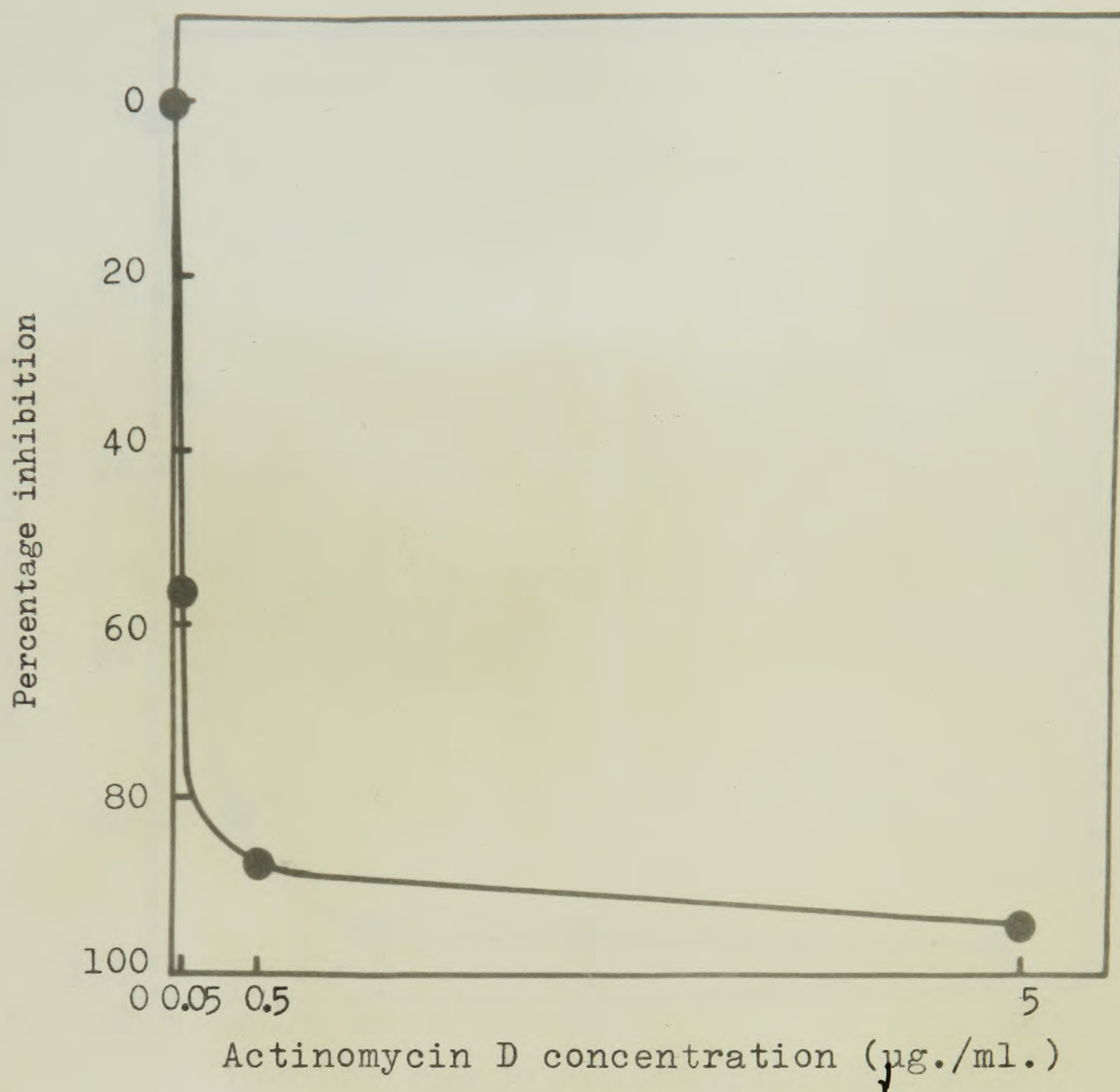


FIG. 5.6 Effect of various concentrations of actinomycin D on the incorporation of (^{14}C) uridine into RNA.

The uninhibited level was 967 counts/min.
Each point is the average of duplicate determinations.

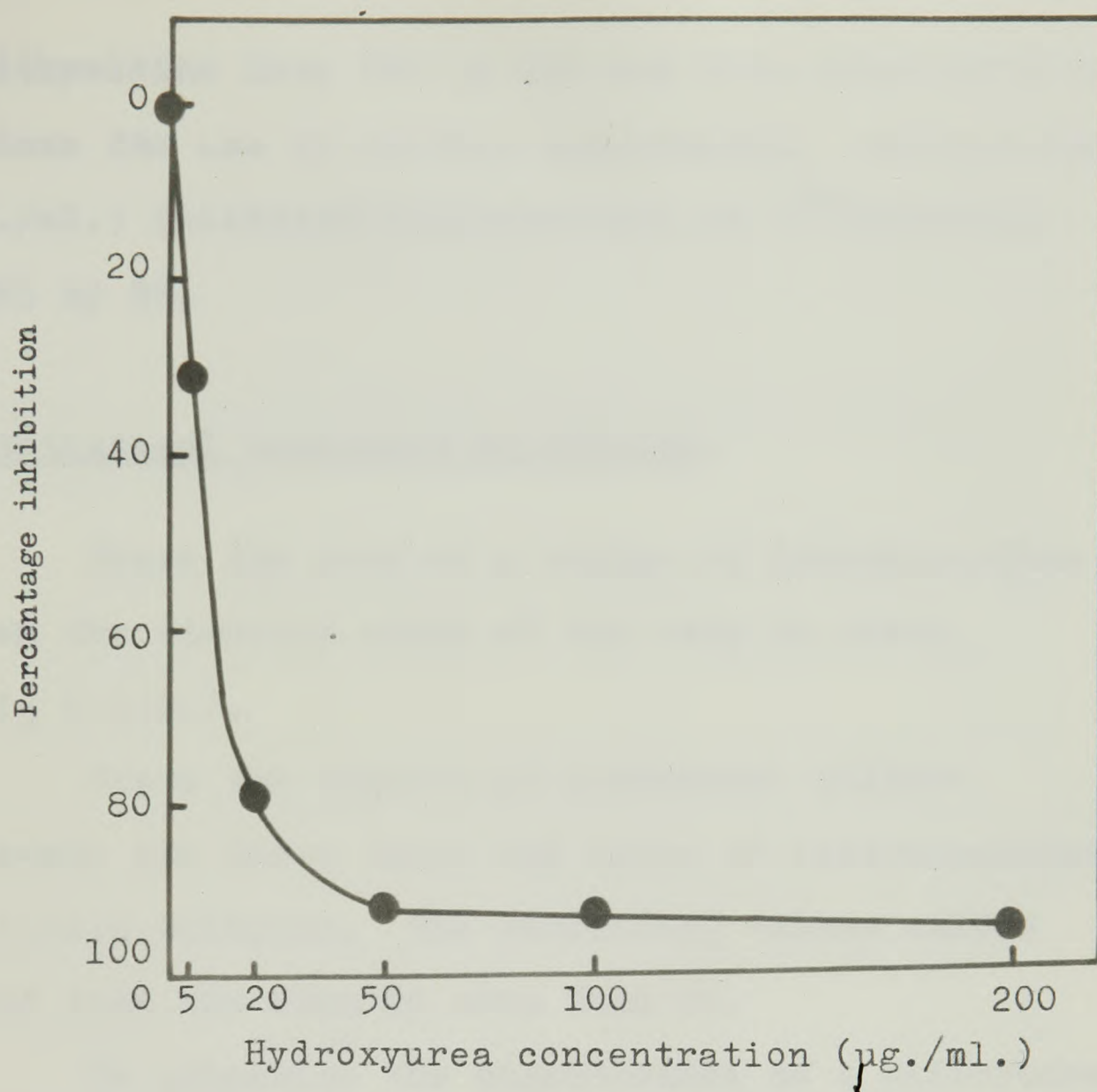


FIG. 5.7 Effect of various concentrations of hydroxyurea on the incorporation of (^3H)thymidine into DNA.

The uninhibited level was 1330 counts/min..
Each point is the average of duplicate determinations.

of (^3H)thymidine into DNA by 92% and this concentration was chosen for use in culture experiments. Hydroxyurea (50 $\mu\text{g./ml.}$) inhibited incorporation of (^{14}C)uridine into RNA by 8%.

5.10 Statistical treatment of results.

Where the mean of a number of determinations is shown the standard error of the mean is given thus: ($\pm$ S.E.M.).

Where the results of individual culture experiments are shown these are means of determinations on duplicate cultures. The individual values seldom differed from the mean by more than 5%.

To determine the significance of a difference between two conditions in a number of experiments it was necessary to take into account each duplicate value. The results were therefore subjected to an analysis of variance and values of P, the probability of the difference being due to chance alone, were obtained from Snedecor's F (variance ratio) distribution. It was regarded that differences which gave $P > 0.05$ were not significant, $P < 0.05$ were probably significant, $P < 0.01$ were significant and $P < 0.001$ were highly significant.

6. RESULTS

6.1 Changes in mouse mammary gland during pregnancy and lactation.

There have been many studies of changes in enzyme activities and nucleic acid contents of the mammary glands of rats and mice during pregnancy and lactation (see Munford, 1964; Baldwin and Milligan, 1966). It was decided to repeat the determinations of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase with the C3H mice used here so that any changes found with mammary tissue in vitro could be related to the changes in the living animal.

Three month old female mice were mated and the day of fertilisation determined (section 5.2). At different stages of pregnancy and lactation they were killed and the inguinal mammary glands removed and quickly weighed on a torsion balance. A portion of the gland was removed for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase determinations and the remainder rapidly frozen and stored at -40°C for later nucleic acid determinations.

Fig. 6.1 and 6.2 show the changes in total activities of these enzymes. As found by other workers, the activities increase only slowly during the second half of pregnancy but increase rapidly after parturition. Between day 19 of pregnancy and day 8 of lactation the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase increase by about eight and four times respectively.

Table 6.1 shows the change in the total weight of the inguinal mammary glands and also the changes in enzymes and nucleic acids per gram of tissue. It can be seen that the RNA content increases significantly during pregnancy and more rapidly after parturition. Most of the increase in DNA content is during pregnancy but there is a small increase in total DNA during early lactation.

Portions of tissue were prepared for microscopic examination (section 5.4). Tissue from virgin, 12 day pregnant, 19 day pregnant and 2 day lactating mice is shown in Fig. 6.3, 6.4, 6.5 and 6.6. It can be seen that the tissue changes as pregnancy proceeds. The ratio of parenchymal to fat cells increases and the alveoli and their lumina enlarge. At day 19 of pregnancy and day 2 of lactation secretion is present in the lumina.

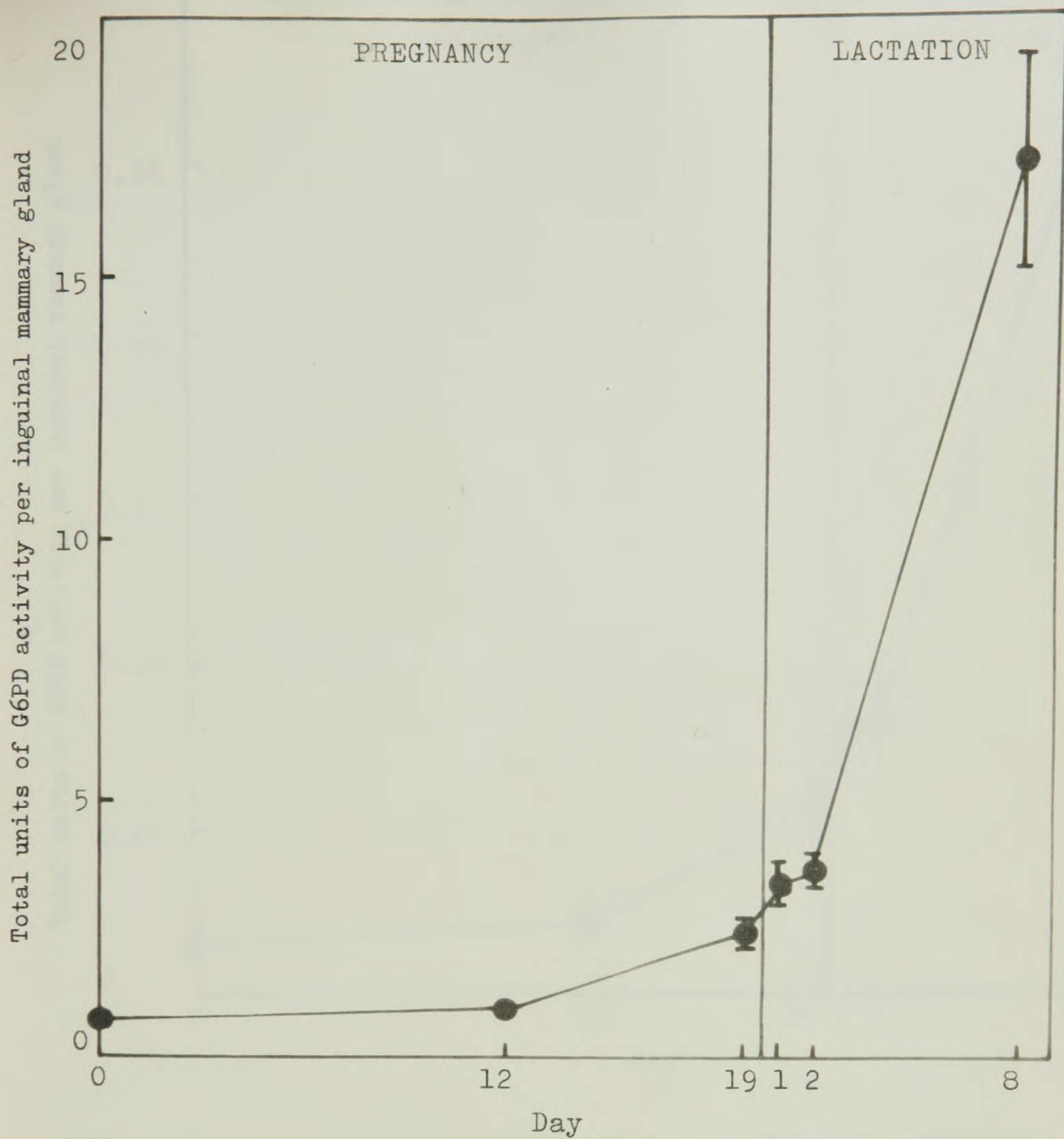


FIG. 6.1 Total G6PD activity of mouse inguinal mammary glands at various stages of pregnancy and lactation.

Each point is the mean $\pm$ S.E.M. of determinations on 6 mice.

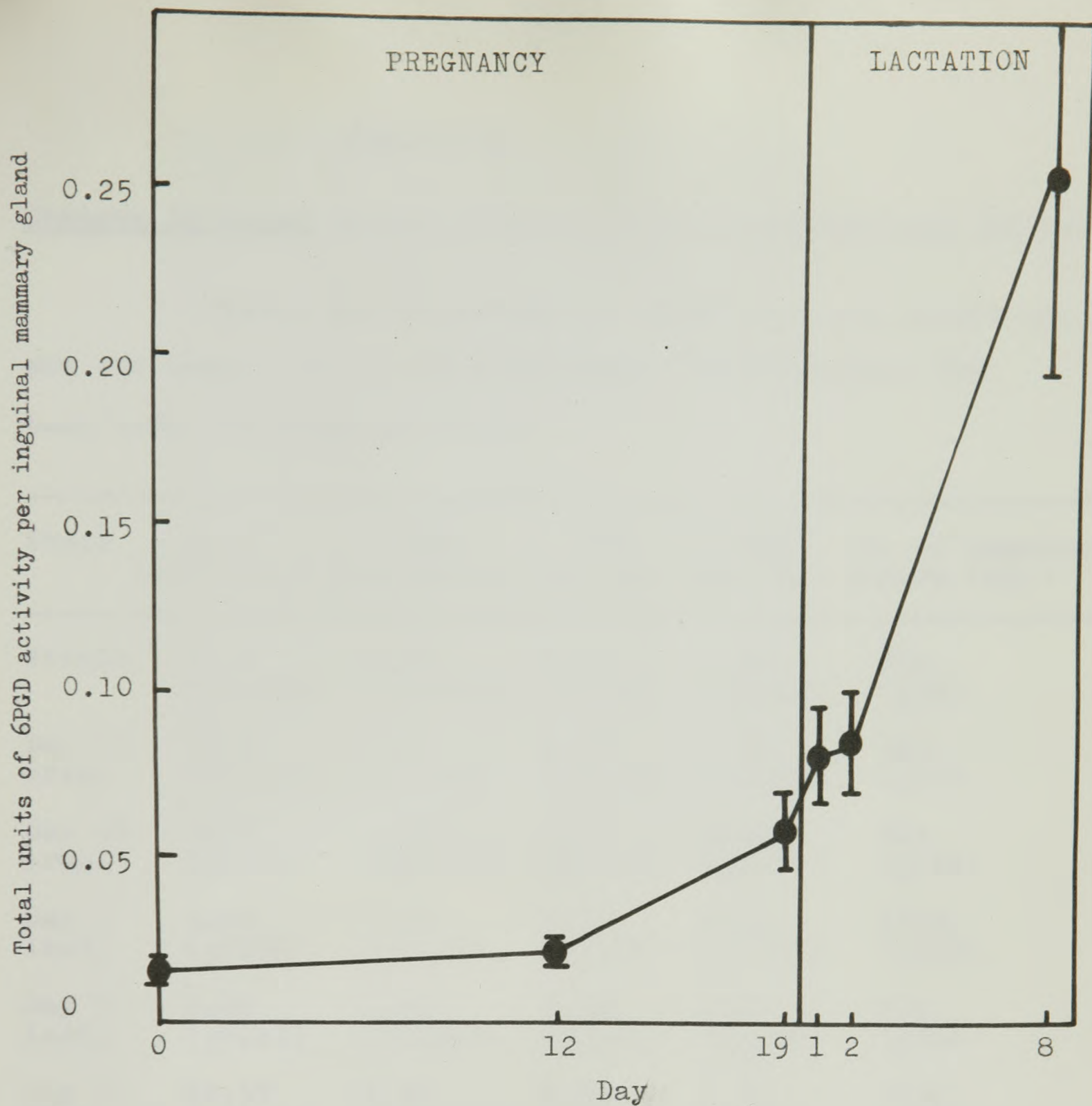


FIG. 6.2 Total 6PGD activity of mouse inguinal mammary glands at various stages of pregnancy and lactation.

Each point is the mean $\pm$ S.E.M. of determinations on 6 mice.

TABLE 6.1

Changes in mouse mammary glands during pregnancy and lactation.

Values are expressed in terms of fresh weight and are the mean $\pm$ S.E.M. of 6 animals. No correction has been made for retained milk.

Stage	G6PD (units/g.)	6PGD (units/g.)	RNA (mg./g.)	DNA (mg./g.)	Wt. of inguinal glands (mg.)
Virgin	3.37 (± 0.30)	0.75 (± 0.08)	1.11 (± 0.13)	1.12 (± 0.10)	214 (± 18)
Day 12 preg.	2.49 (± 0.13)	0.61 (± 0.09)	2.01 (± 0.28)	1.71 (± 0.16)	362 (± 18)
Day 19 preg.	2.50 (± 0.18)	0.61 (± 0.07)	2.92 (± 0.15)	2.49 (± 0.18)	974 (± 114)
Day 1 lact.	2.96 (± 0.28)	0.76 (± 0.12)	3.57 (± 0.27)	2.54 (± 0.22)	1122 (± 128)
Day 2 lact.	4.49 (± 0.40)	1.10 (± 0.19)	5.12 (± 0.20)	3.20 (± 0.08)	784 (± 36)
Day 8 lact.	10.37 (± 0.41)	1.55 (± 0.34)	7.07 (± 0.18)	2.31 (± 0.09)	1636 (± 132)

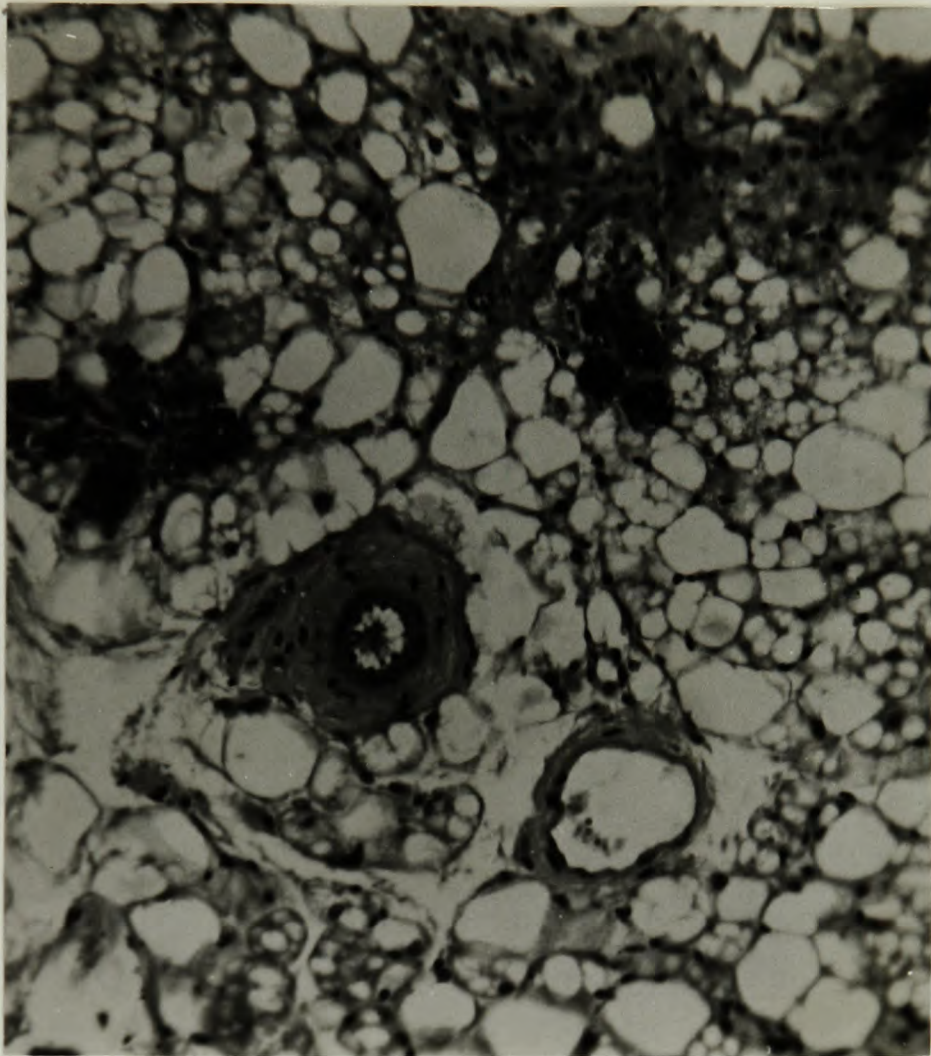


FIG. 6.3 Mammary tissue from a virgin mouse.

Magnification: x 150.

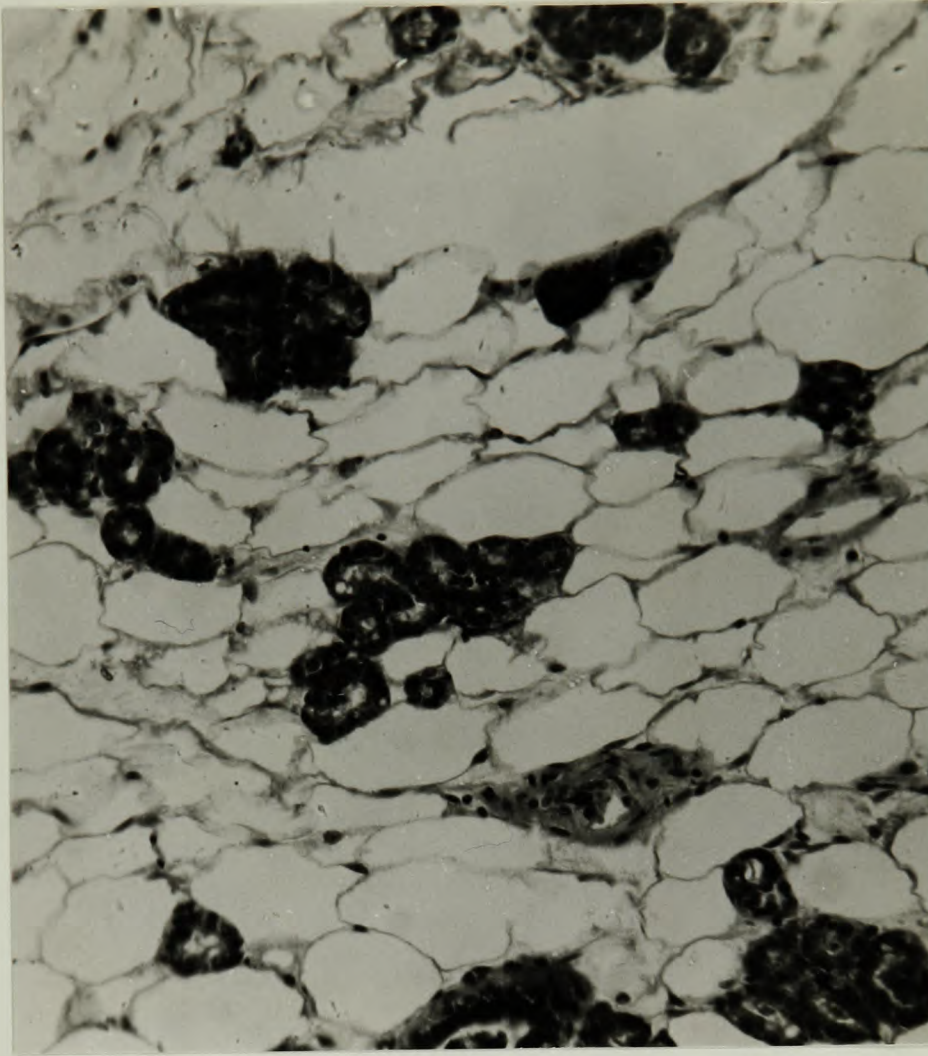


FIG. 6.4 Mammary tissue from a mouse on the 12th day
of pregnancy.

Magnification: x 150.

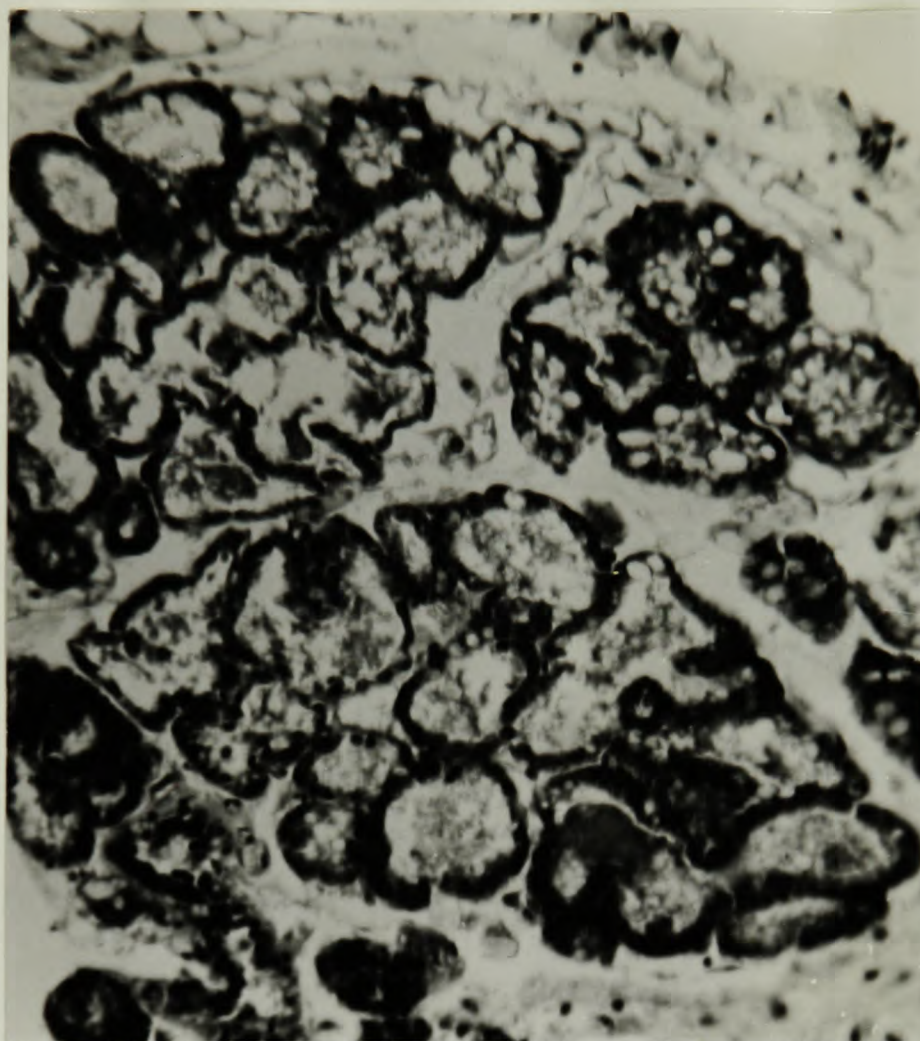


FIG. 6.5 Mammary tissue from a mouse on the 19th day
of pregnancy.

Magnification: x 150.

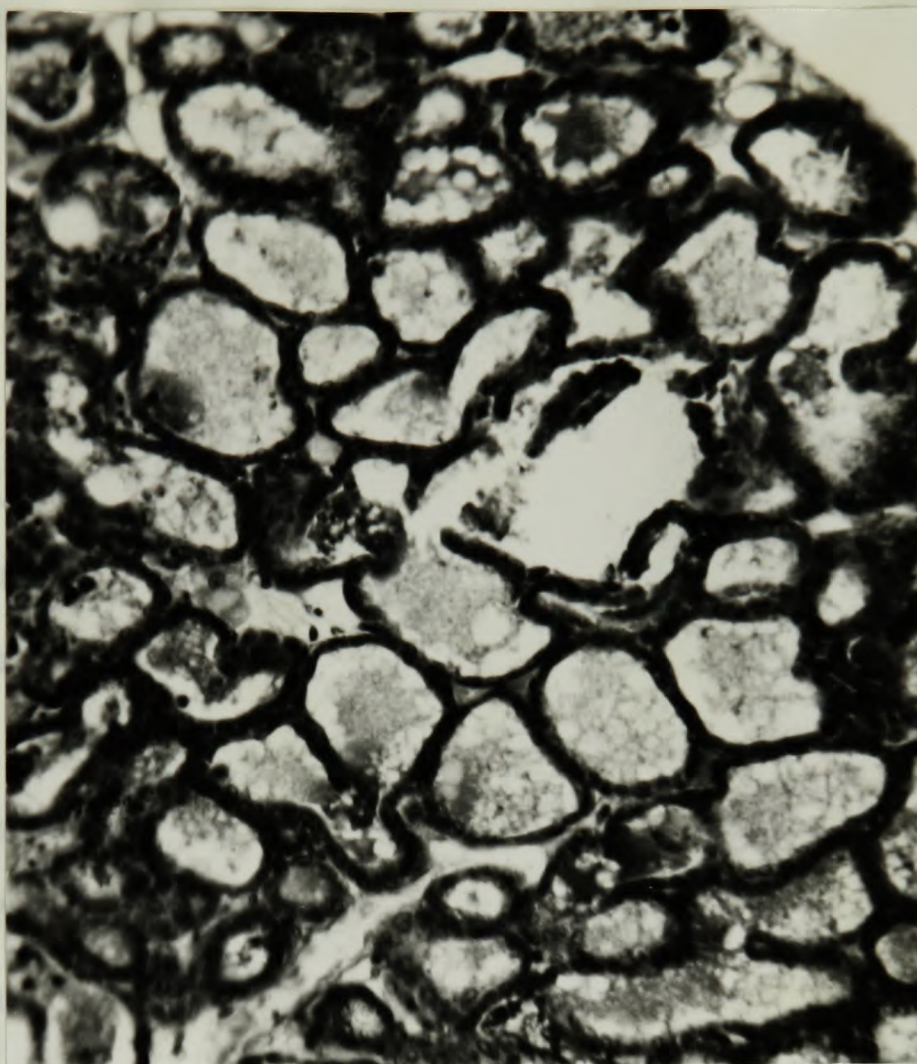


FIG. 6.6 Mammary tissue from a mouse on the 2nd day of lactation.

Magnification: x 150.

6.2 Action of hormones on cultures of mammary tissue
from mice in late pregnancy.

22 hour cultures with insulin.

Explants were cut from the mammary glands of mice in late pregnancy (day 19) and incubated for 22 hr. (section 5.5). They were then removed, homogenised, centrifuged and the enzymes in the supernatant assayed (section 5.6).

Table 6.2 shows that there was a highly significant increase in the activity of both enzymes after 22 hr. in culture with insulin (5 μ g./ml.). These increases are of the same order of magnitude as those which occur in early lactation over a similar time period (see Table 6.1). Moreover, as in the living animal, the percentage increase of glucose 6-phosphate dehydrogenase activity is somewhat greater than that of 6-phosphogluconate dehydrogenase. Because of the smaller optical density change with 6-phosphogluconate dehydrogenase, it was only possible to detect gross changes in the activity of this enzyme. Most of the results to be presented are, therefore, only for glucose 6-phosphate dehydrogenase.

The results of experiments with and without insulin are shown in Table 6.3 and it is clear that

TABLE 6.2

Increase in G6PD and 6PGD activity of late-pregnant mouse mammary explants after 22 hr. culture with insulin.

The figures are means $\pm$ S.E.M. of duplicate determinations on 19 mice.

	Enzyme activity (units/g.)	
	G6PD	6PGD
Mean initial value	1.82 (± 0.21)	0.56 (± 0.04)
Mean increase during 22 hr. incubation	1.53 (± 0.13)	0.36 (± 0.06)
Mean increase as percentage of initial value	84%	64%
P	< 0.001	< 0.001

insulin is necessary. TABLE 6.3

effect of insulin could be detected by assaying for G6PD and 6PGD activity of late-pregnant mouse mammary explants after 22 hr. culture with and without insulin.

The figures are averages of duplicate determinations.

Enzyme Expt. Enzyme activity of explants (units/g.)
No.

Not Incubated Incubated - insulin Incubated + insulin

G6PD

1	2.37	1.65	3.11
2	2.10	1.76	3.79
3	1.05	0.90	2.82

Mean change -20% +91%

P (- insulin v. + insulin) < 0.001

6PGD

1	0.49	0.36	0.55
2	1.43	1.11	2.01
3	0.42	0.43	0.78

Mean change -15% +46%

P (- insulin v. + insulin) < 0.01

insulin is necessary for the enzyme increases. The effect of insulin could be detected by at least 12 hr. as in trial incubations of 12 hr., although the enzyme activity did not increase with insulin, it fell without it.

Fig. 6.7 shows the time course of the increase in glucose 6-phosphate dehydrogenase activity. There is a lag of about 12 hr. before the activity starts to increase.

McKerns (1964) has suggested that some hormones alter glucose 6-phosphate dehydrogenase activity by direct activation or inactivation of the enzyme. To test whether insulin could directly affect the enzyme activity, supernatants from unincubated explants were incubated with insulin (5 $\mu\text{g./ml.}$) for 1 hr. at both 0°C and 37°C . As with rat liver (Glock and McLean, 1955), neither enzyme increased in activity.

FIG. 6.7 To test whether the increase in activity after 22 hr. culture with insulin could be due to the removal of an inhibitor, the experiment in Table 6.4 was performed. It can be seen that the activities in mixed supernatants from incubated and unincubated explants are the average of their components. If the supernatant from unincubated explants contained an inhibitor, a lower activity would be expected.

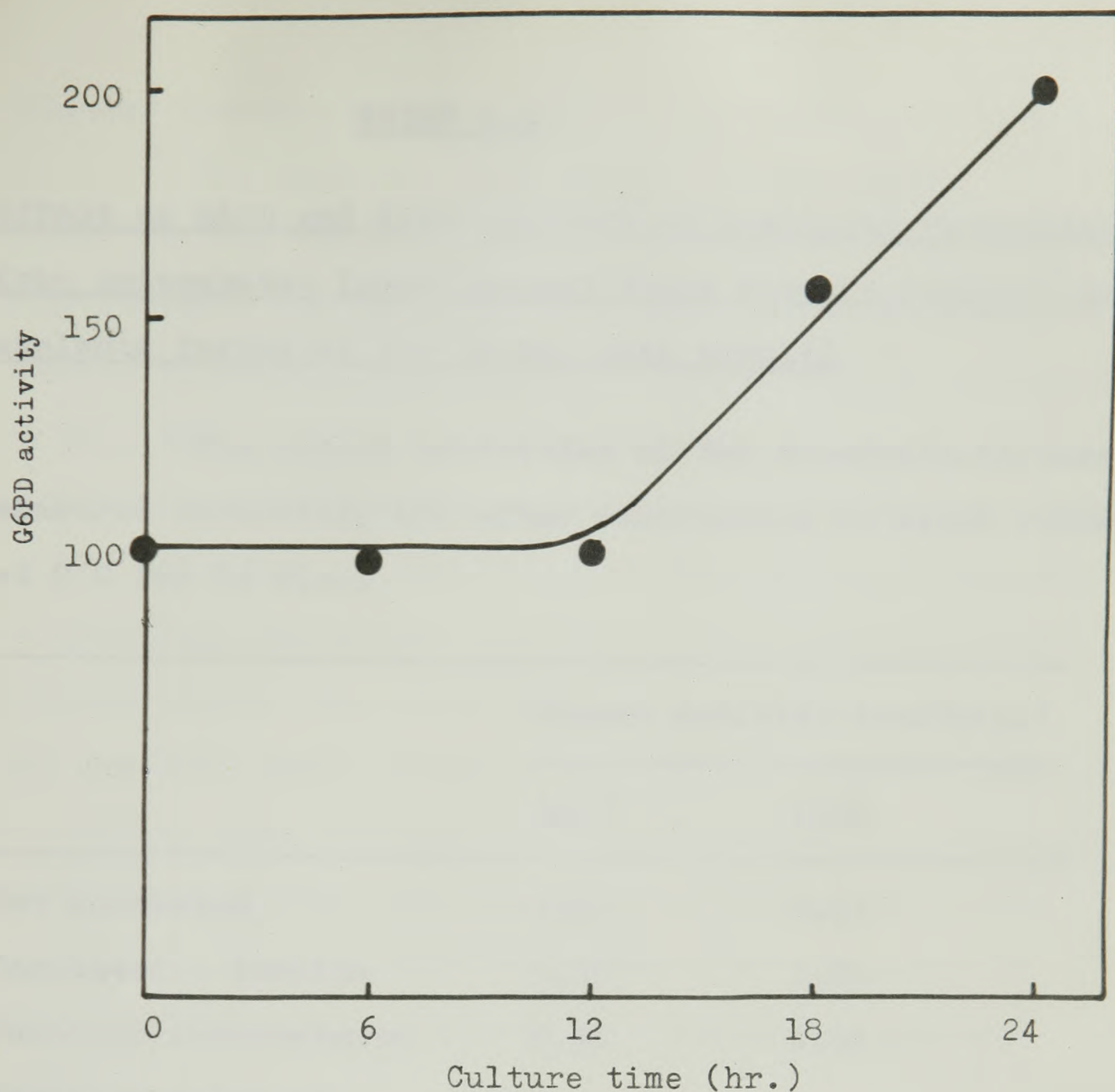


FIG. 6.7 G6PD activity of late-pregnant mouse mammary explants after culture with insulin for different times.

The figure is a combination of 2 experiments, each comprising duplicate determinations at the same times. In each experiment the initial and 24 hr. activities have been set at 100 and 200 respectively, to give each experiment equal weight.

Cellular location TABLE 6.4

Effect on G6PD and 6PGD activity of combining supernatants from unincubated late-pregnant mouse mammary explants and explants incubated for 22 hr. with insulin.

The enzyme activities of the supernatants were measured separately and after combination in equal volumes at 0°C for 15 min..

	Enzyme activity (units/g.)	
	G6PD	6PGD
Not incubated	1.45	0.53
Incubated + insulin	3.25	1.28
Combined supernatants	2.36	0.85
Expected value of combination	2.35	0.91

Cellular location of the increase in enzyme activities.

The mammary gland contains both mammary parenchyma and adipose tissue. As glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are present in adipose tissue, it was important to determine whether the increase in their activities occurred in the fat or parenchymal cells. Mayne, Barry and Rivera (1966), using facilities at the National Institute for Research in Dairying (Shinfield, Reading), were able to dissect adipose tissue free from mammary parenchyma. Here it has not been found possible to separate them so precisely but it has been possible, under a dissecting microscope, to separate tissue that contained a higher proportion of adipose tissue. This was taken from the edges of the gland and is referred to as 'peripheral' mammary tissue.

Table 6.5 shows the results of 22 hr. cultures of 'peripheral' mammary tissue, mammary tissue and also of adipose tissue. The initial enzyme activities of the 'peripheral' tissue are lower than those of the mammary tissue and during 22 hr. culture the enzyme activities of 'peripheral' tissue rose less than those of mammary tissue. It can be calculated from the figures in Table 6.5 that the increments of glucose 6-phosphate dehydrogenase and 6-phosphogluconate

TABLE 6.5

G6PD and 6PGD activity of explants of late-pregnant mouse mammary tissue, 'peripheral' mammary tissue and abdominal adipose tissue after 22 hr. culture with insulin.

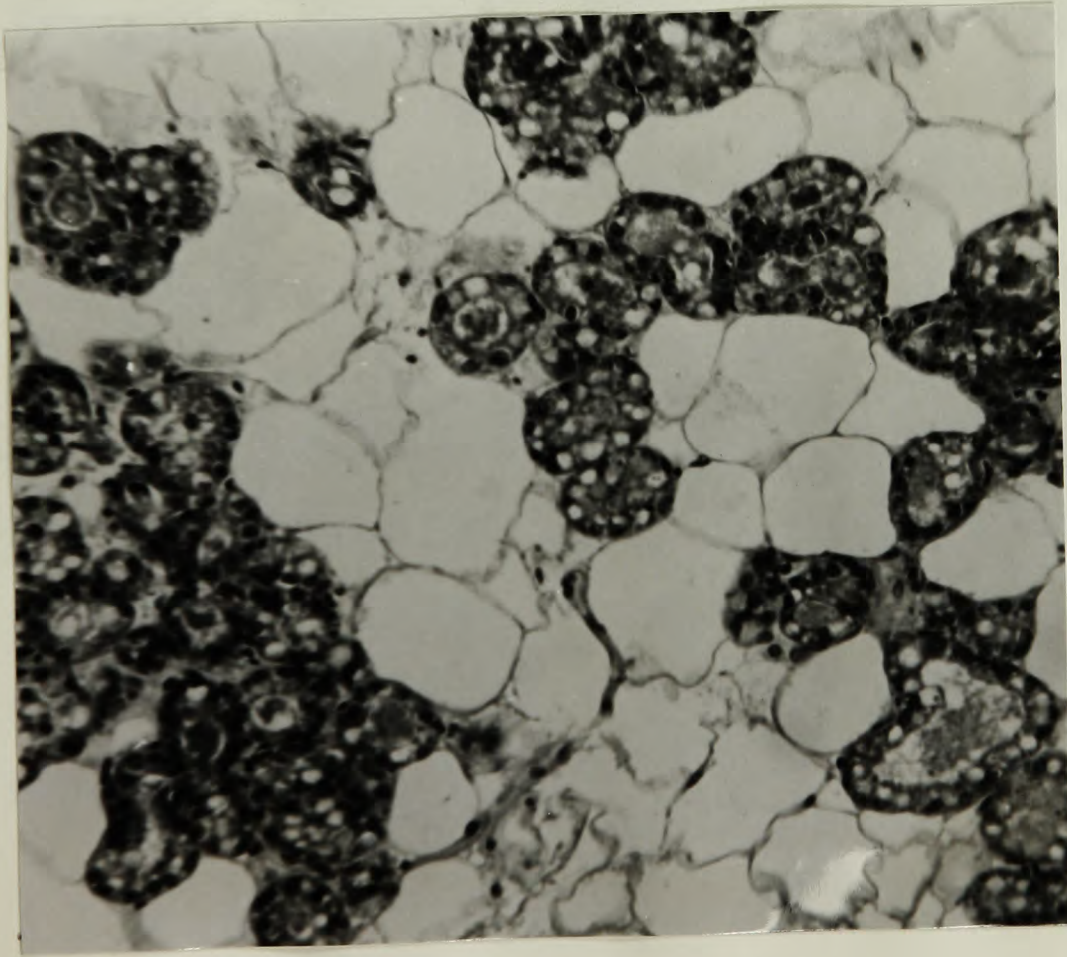
The figures are averages of duplicate determinations.

Enzyme	Enzyme activity of explants (units/g.)		
	Mammary tissue	'Peripheral' mammary tissue	Abdominal adipose tissue
<u>G6PD</u>			
Not incubated	1.05	0.56	0.23
Incubated + insulin	2.82	1.42	0.14
Change (Percentage)	+1.77 (+169%)	+0.86 (+154%)	-0.09 (-40%)
<u>6PGD</u>			
Not incubated	0.42	0.32	0.16
Incubated + insulin	0.78	0.52	0.09
Change (Percentage)	+0.36 (+86%)	+0.20 (+63%)	-0.07 (-44%)

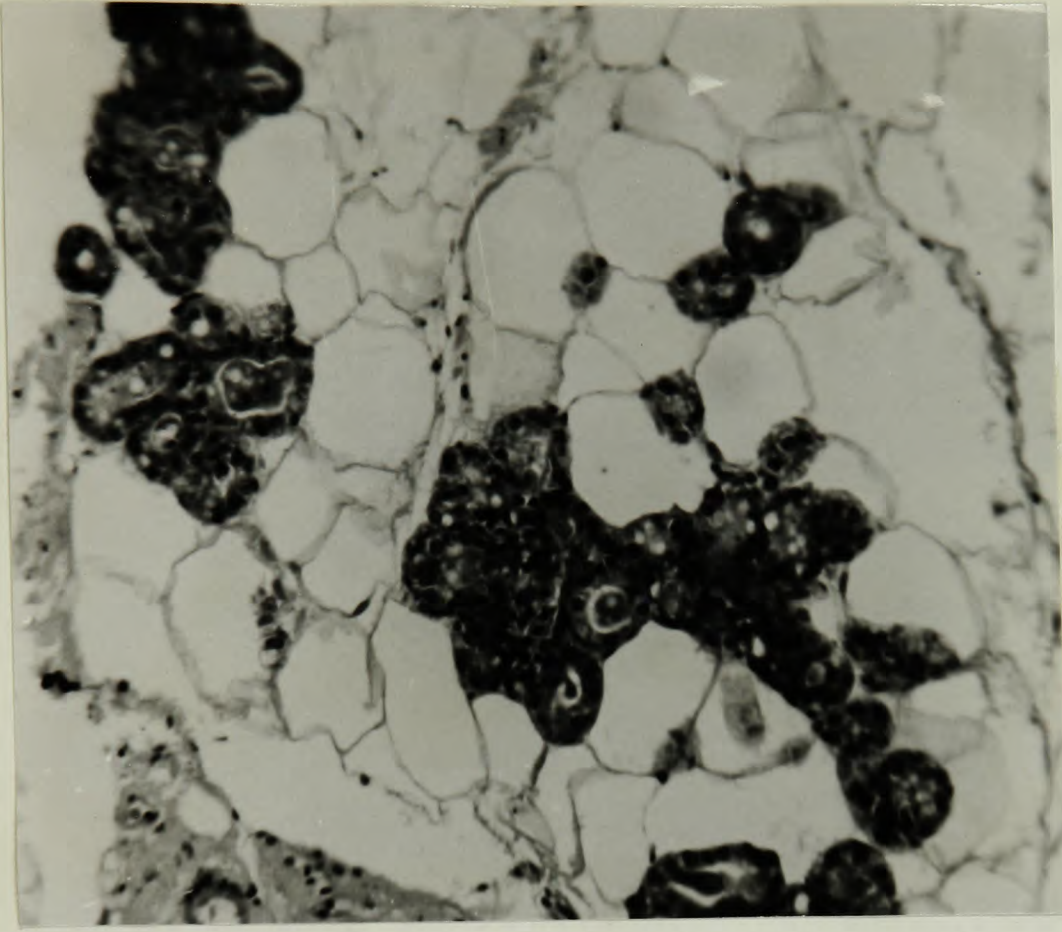
dehydrogenase activity in 'peripheral' tissue are 49% and 56% of those in mammary tissue. Hence the contribution of adipose tissue to the increases in enzyme activity could at the very most be about 50%. However Fig. 6.7P shows that the 'peripheral' mammary tissue still contained many parenchymal cells; and Table 6.5 shows that the percentage rise in activity of each enzyme in 'peripheral' tissue was similar to that in mammary tissue. These facts suggest that a large part of the increase in enzyme activities in 'peripheral' tissue is due to its parenchymal cells and hence that adipose cells contribute considerably less than 50% to the increases.

Table 6.5 also shows that the enzyme level in explants of abdominal fat fell during culture. This, though not proof, again does not lend any support to the idea of the enzyme rises being located in the adipose tissue of the mammary gland.

The conclusion that it is the enzymes of the parenchymal rather than the adipose cells which increase in vitro is consistent with results obtained by other workers in vivo. Thus Hershey, Lewis, Johnston and Mason (1963) dissected small pieces of mouse mammary parenchyma and measured glucose 6-phosphate dehydrogenase activity by a micro technique. They found that the enzyme activity rose between pregnancy and lactation.



Mammary tissue.



'Peripheral' mammary tissue.

FIG. 6.7P Microscopic appearance of mammary tissue and 'peripheral' mammary tissue from a late-pregnant mouse.

Magnification: x 150.

Bartley, Abraham and Chaikoff (1966) followed the activity of glucose 6-phosphate dehydrogenase in C3H mice in which parenchymal tissue from one of the inguinal mammary glands had been removed by the surgical procedure of DeOme, Faulkin, Bern and Blair (1959). They found that between pregnancy and lactation the activity in the intact gland rose but that in the operated gland and in abdominal adipose tissue fell.

22 hour cultures with various hormones.

Cultures of mammary explants from mice in mid-pregnancy incorporate (^{32}P)orthophosphate into casein more rapidly if prolactin and corticosterone are included in the medium in addition to insulin (Juergens, Stockdale, Topper and Elias, 1965).

Table 6.6 shows that in 22 hr. cultures the increase in enzyme activity when insulin (5 $\mu\text{g.}/\text{ml.}$), prolactin (10 $\mu\text{g.}/\text{ml.}$) and corticosterone (1 $\mu\text{g.}/\text{ml.}$) were included in the medium did not differ significantly from that with insulin alone. Table 6.7 shows that insulin + prolactin and insulin + corticosterone acted similarly to insulin, while neither prolactin nor corticosterone alone produced any increase in activity.

TABLE 6.6

Comparison of the effects of insulin and insulin + prolactin + corticosterone on the G6PD activity of late-pregnant mouse mammary explants in 22 hr. culture.

The figures are averages of duplicate determinations.

Expt. No.	G6PD activity (units/g.)			$\frac{B}{A} \times 100$
	Not incubated	Incubated +insulin	Incubated +insulin +prolactin +corticosterone	
	(A)	(B)		
1	2.37	3.11	3.15	101%
2	2.70	4.54	5.40	119%
3	2.62	4.01	4.01	100%
4	1.80	3.26	3.38	104%
		Mean		106%
		P		> 0.05

TABLE 6.7

Effects of prolactin and corticosterone on the G6PD activity of late-pregnant mouse mammary explants in 22 hr. culture with and without insulin.

The figures are averages of duplicate determinations. Percentages of the initial value are given in parentheses.

Expt. No.	Hormone additions	G6PD activity (units/g.)
1.	Unincubated	2.10 (100%)
	None	1.76 (84%)
	Insulin	3.79 (180%)
	Prolactin	1.58 (75%)
	Corticosterone	1.80 (86%)
2.	Unincubated	2.70 (100%)
	Insulin	4.54 (168%)
	Insulin + prolactin	4.99 (185%)
3.	Unincubated	1.45 (100%)
	Insulin	2.99 (206%)
	Insulin + corticosterone	3.03 (209%)

45 hour cultures with various hormones.

Fig. 6.8 shows the results of 22 and 45 hr. cultures with insulin alone or with insulin, prolactin and corticosterone. It can be seen that with insulin alone the glucose 6-phosphate dehydrogenase activity at 45 hr. has fallen below the 22 hr. level but that with insulin, prolactin and corticosterone the level has, on the average, been maintained. Table 6.8 shows that this difference at 45 hr. was highly significant. Table 6.9 shows that insulin + prolactin gave a similar effect to that of all three hormones while insulin + corticosterone was not significantly different from insulin alone.

It was seen in Fig. 6.8 that the increase in enzyme activity over the first day of culture did not continue over the second day, even with all three hormones in the medium. This contrasts with the living animal where the enzymes continue to rise for several days. This difference was not due to exhaustion of the medium for when the explants were transferred to fresh medium at 22 hr. there was no difference in the final enzyme activity.

Other hormones have been implicated in mammary development and the initiation of lactation (see section 1.1 or Cowie, 1961 for a review) and it was decided to test

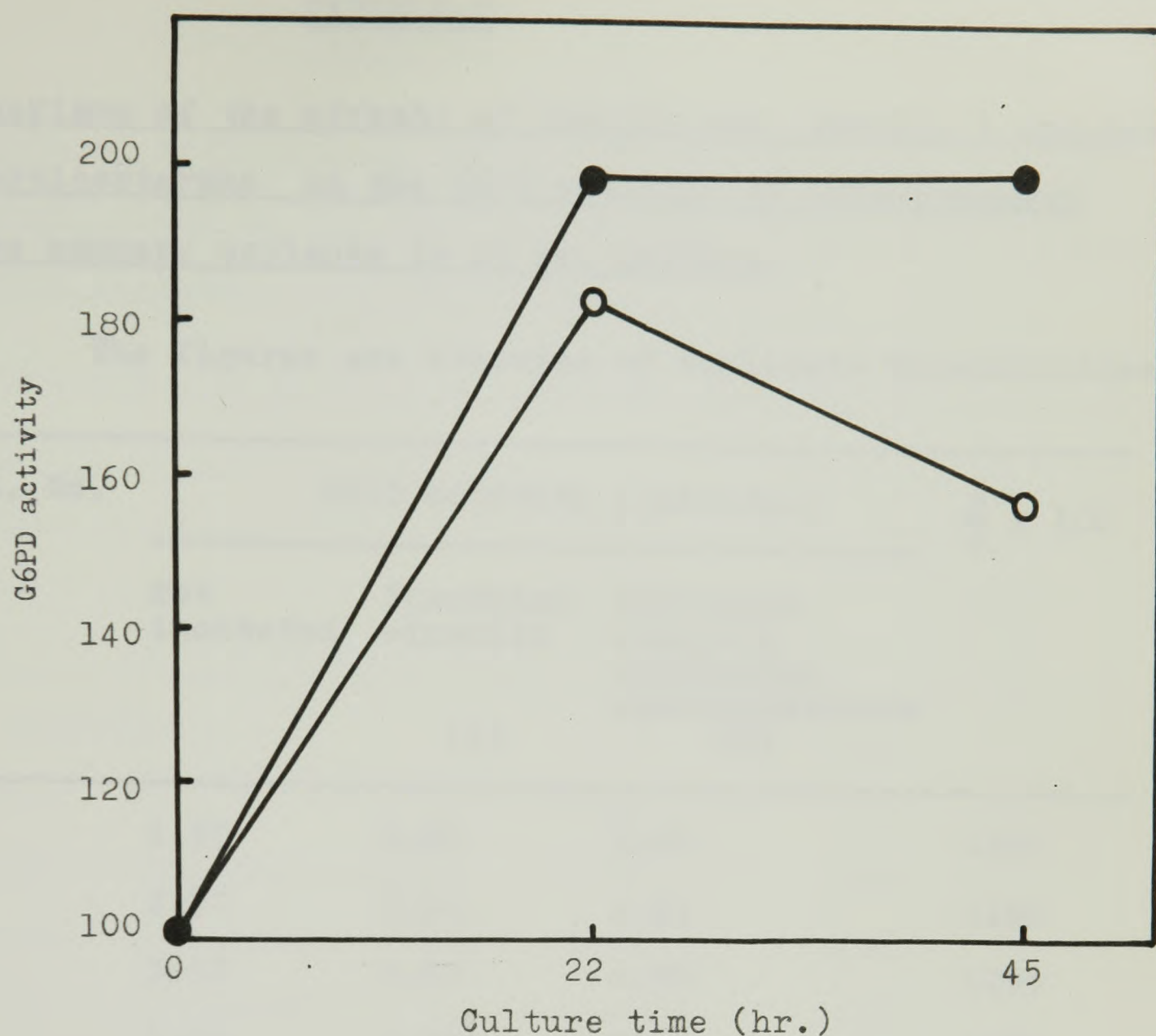


FIG. 6.8 Comparison of the effects of insulin and insulin + prolactin + corticosterone on the G6PD activity of late-pregnant mouse mammary explants in 22 and 45 hr. culture.

- Incubated with insulin.
- Incubated with insulin + prolactin + corticosterone.

The figure is a combination of 3 similar experiments, each comprising duplicate determinations at the same points. In each experiment the initial activity and that after 45 hr. culture with the three hormones have been set at 100 and 200 respectively.

TABLE 6.8

Comparison of the effects of insulin and insulin + prolactin + corticosterone on the G6PD activity of late-pregnant mouse mammary explants in 45 hr. culture.

The figures are averages of duplicate determinations.

Expt. No.	G6PD activity (units/g.)			$\frac{B}{A} \times 100$
	Not incubated	Incubated +insulin (A)	Incubated +insulin +prolactin +corticosterone (B)	
1	1.95	2.21	2.89	131%
2	2.70	3.96	4.72	119%
3	2.62	3.52	4.38	125%
4	1.80	2.92	3.68	126%
5	1.09	2.25	2.89	128%
6	1.45	2.28	2.95	129%
7	1.75	2.43	3.33	137%
8	1.04	2.35	3.28	140%
Mean				129%
P				< 0.001

whether growth hormone, placental, thyroid hormone, oxytocin or a higher level of prolactin affected the effects of various combinations of insulin, prolactin and corticosterone on the G6PD activity of late-pregnant mouse mammary explants in 45 hr. culture.

The figures are averages of duplicate determinations.

Hormone additions	G6PD activity (units/g.)	
	Expt. 1	Expt. 2
Not incubated	2.70	1.09
Insulin	3.96	2.25
Insulin + corticosterone	4.12	2.40
Insulin + prolactin	4.65	3.38
Insulin + prolactin + corticosterone	4.72	2.89

after 22 hr. of culture no difference was found between the enzyme activity of the explants incubated with and without the placental extract.

Cultures with added sera.

Although the additional hormones used in the above experiments did not affect the enzyme activity, it was possible that the correct hormone or concentrations had not been tested. Because of this possibility and

whether growth hormone, oestradiol, thyroid hormone, oxytocin or a higher level of prolactin affected the enzyme rise over the second day of culture. Table 6.10 shows that none of these hormones had any effect.

Some theories postulate that lactation is initiated by the removal of inhibitory oestrogens or progesterone (see section 1.1). If these act directly at the mammary gland they might possibly prevent the rise in enzyme activity. However Table 6.11 shows that none of the concentrations and combinations tried had any effect.

To test whether the enzyme activity in vivo might be restrained by some unknown factor secreted by the placentae, a placental extract was prepared (section 5.8) and 0.25 ml. (obtained from 2 placentae) added to culture medium containing insulin. However after 22 hr. of culture no difference was found between the enzyme activity of the explants incubated with and without the placental extract.

Cultures with added sera.

Although the additional hormones used in the above experiments did not affect the enzyme activities, it was possible that the correct hormones or concentrations had not been tested. Because of this possibility and

TABLE 6.10

Effect of various hormones on the G6PD activity of late-pregnant mouse mammary explants in 45 hr. culture.

The medium contained insulin, prolactin and corticosterone in addition to other hormones. The figures are averages of duplicate determinations.

Extra hormone additions	Concentration (µg./ml. medium)	G6PD activity (units/g.)
Initial value		3.00
None		4.86
Growth hormone	10	4.75
Prolactin	100	4.79
Oxytocin	10^{-5} units/ml.	4.65
Initial value		2.04
None		4.56
3,3',5-triiodothyronine	5	4.20
Oestradiol	0.05	4.64

the need for added TABLE 6.11

Effect of various hormones on the G6PD activity of late-pregnant mouse mammary explants in 22 hr. culture.

Fig. 6.9 shows that serum from lactating mice. The medium contained insulin in addition to other (section 5.7) had no effect at 22 hr. but by 48 hr. hormones. The figures are averages of duplicate determinations. had caused a further rise in glucose 6-phosphate

Extra hormone additions	Concentration (pg./ml. medium)	G6PD activity (units/g.)
Initial value		1.09
None		2.74
Oestradiol	1	2.89
Progesterone	1	2.81
Oestradiol + progesterone	0.01 5	2.68

for serum to increase the enzyme activity. In contrast to the stimulation in the absence of serum, further addition of prolactin + corticosterone had no effect.

The constituent of serum responsible for maintaining the rise in enzyme activity has not been identified. However it was found that a bovine serum albumin (Fraction V, dialyzed to pH 7.4 as described by Rodbell, 1964) in the medium did not replace serum. When various commercial sera were tested, fetal calf

the need for added serum in many tissue and cell cultures, sera from mice in different physiological conditions were tested.

Fig. 6.9 shows that serum from lactating mice (section 5.7) had no effect at 22 hr. but by 45 hr. had caused a further rise in glucose 6-phosphate dehydrogenase activity. Serum similarly affected the 6-phosphogluconate dehydrogenase activity.

Table 6.12 shows that this effect was not restricted to serum from lactating mice: sera from virgin and pregnant mice behaved similarly. Thus the effect of serum does not appear to be associated with changes in its hormone content at parturition.

Table 6.13 shows that insulin is required for serum to increase the enzyme activity. In contrast to the stimulation in the absence of serum, further addition of prolactin + corticosterone had no effect.

The constituent of serum responsible for maintaining the rise in enzyme activity has not been identified. However it was found that 4% bovine serum albumin (Fraction V, dialysed to pH 7.4 as described by Rodbell, 1964) in the medium did not replace serum. When various commercial sera were tested, foetal calf

corticosterone + lactating mouse serum
(10% of total volume).

Each point is the average of duplicate
determinations.

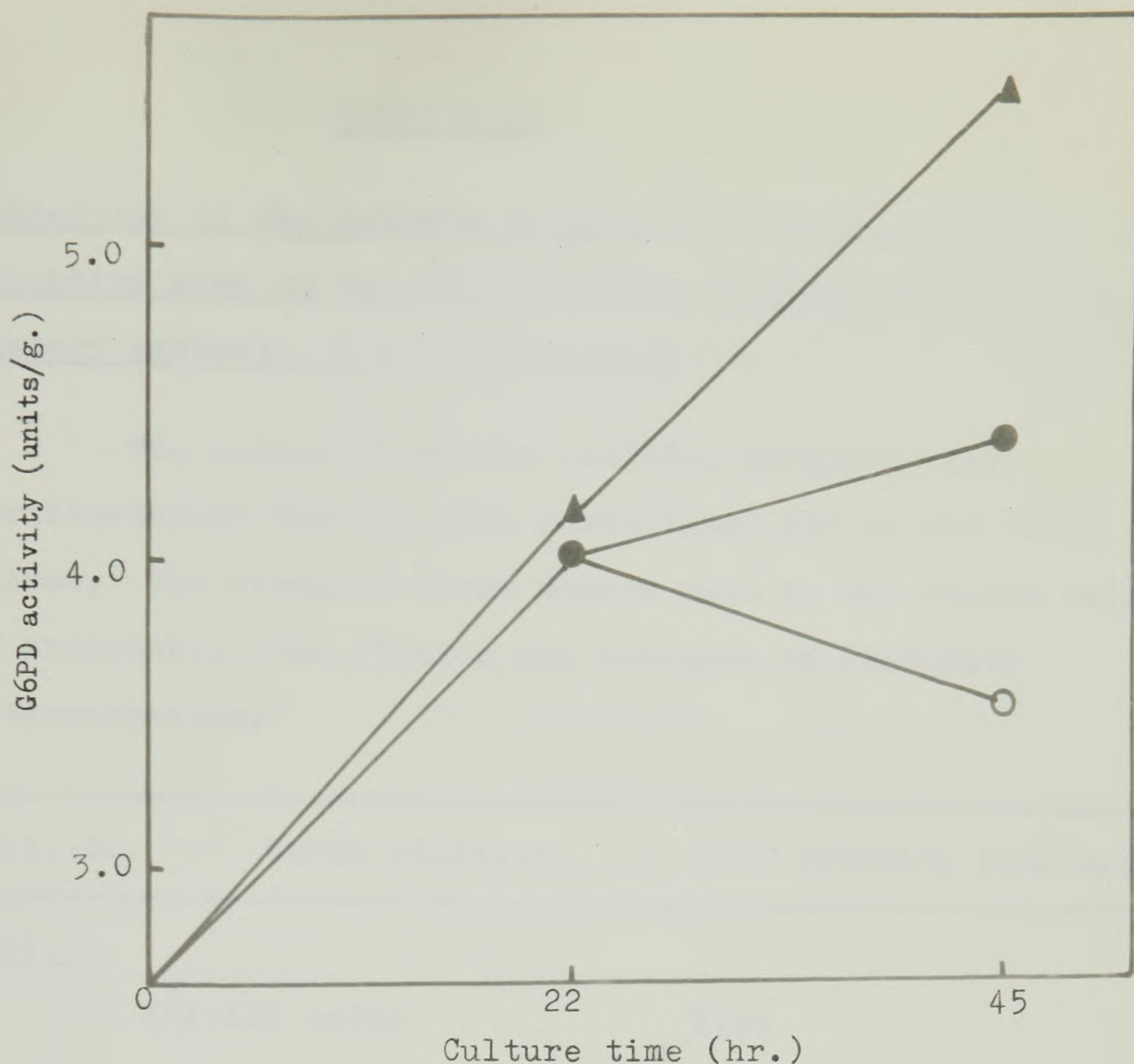


FIG. 6.9 Effect of serum from lactating mice on the G6PD activity of late-pregnant mouse mammary explants in 22 and 45 hr. culture with insulin + prolactin + corticosterone.

- Incubated with insulin.
- Incubated with insulin + prolactin + corticosterone.
- ▲—▲ Incubated with insulin + prolactin + corticosterone + lactating-mouse serum (10% of total volume).

Each point is the average of duplicate determinations.

TABLE 6.12

Comparison of the effects of sera from virgin, pregnant and lactating mice on the G6PD activity of late-pregnant mouse mammary explants in 45 hr. culture.

The medium contained insulin, prolactin and corticosterone and the sera constituted 10% of the total volume. The pregnant serum donors were in the second half of pregnancy. The figures are averages of duplicate determinations.

Expt. No.	Serum addition	G6PD activity (units/g.)
-----------	----------------	--------------------------

Expt. 1

Initial value	1.45
None	2.95
Lactating-mouse serum	4.37
Pregnant-mouse serum	5.05

Expt. 2

Initial value	2.10
None	2.78
Pregnant-mouse serum	3.75
Virgin-mouse serum	3.94

TABLE 6.13

Determination of the basic hormone requirements for the effect of serum from lactating mice on the G6PD activity of late-pregnant mouse mammary explants in 45 hr. culture.

The serum constituted 10% of the total volume of the incubation medium. The figures are averages of duplicate determinations.

Hormone addition	Serum addition	G6PD activity (units/g.)
Not incubated		1.80
Insulin	None	2.92
Insulin + prolactin + corticosterone	None	3.68
None	+ Serum	1.73
Insulin	+ Serum	4.57
Insulin + prolactin + corticosterone	+ Serum	4.28

serum was found to be about 90% as effective, horse serum about 50% as effective and calf serum about 35% as effective as serum from lactating mice.

Mid-pregnancy.

When tissue from mice in mid-pregnancy (Day 12) was incubated for 22 hr. with insulin, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase again rose in activity. This is shown in Table 4.11.

Comparison with Table 4.2 shows that these increases are somewhat smaller than those in late pregnancy.

Fig. 4.10 shows the results of 22 and 45 hr. cultures with insulin and with insulin, prolactin and corticosterone. It can be seen that even with insulin alone the glucose 6-phosphate dehydrogenase activity continued to rise during the second day of culture. In addition there was a stimulation by prolactin + corticosterone at 22 hr. which was almost as great as that at 45 hr.. In these respects mid-pregnant tissues differed from late-pregnant tissues (see Fig. 4.8).

Table 4.12 shows that the additional increase in enzyme activity due to prolactin + corticosterone was significant at 22 hr.. Table 4.13 compares different hormone combinations. It can be seen that insulin was again required for the rise in enzyme activity.

6.3 Action of hormones on cultures of mammary tissue from mid-pregnant and virgin mice.

Mice in mid-pregnancy.

When tissue from mice in mid-pregnancy (day 12) was incubated for 22 hr. with insulin, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase again rose in activity. This is shown in Table 6.14.

Comparison with Table 6.2 shows that these increases are somewhat smaller than those in late pregnancy.

Fig. 6.10 shows the results of 22 and 45 hr. cultures with insulin and with insulin, prolactin and corticosterone. It can be seen that even with insulin alone the glucose 6-phosphate dehydrogenase activity continued to rise during the second day of culture. In addition there was a stimulation by prolactin + corticosterone at 22 hr. which was almost as great as that at 45 hr.. In these respects mid-pregnant tissue differed from late-pregnant tissue (see Fig. 6.8).

Table 6.15 shows that the additional increase in enzyme activity due to prolactin + corticosterone was significant at 22 hr.. Table 6.16 compares different hormone combinations. It can be seen that insulin was again required for the rise in enzyme activity.

TABLE 6.14

Increase in G6PD and 6PGD activity of mid-pregnant mouse mammary explants after 22 hr. culture with insulin.

The figures are means $\pm$ S.E.M. of duplicate determinations on 7 mice.

	Enzyme activity (units/g.)	
	G6PD	6PGD
Mean initial value	1.50 (± 0.11)	0.54 (± 0.05)
Mean increase during 22 hr. incubation	1.03 (± 0.12)	0.29 (± 0.12)
Mean increase as percentage of initial value	68%	54%
P	< 0.001	< 0.001

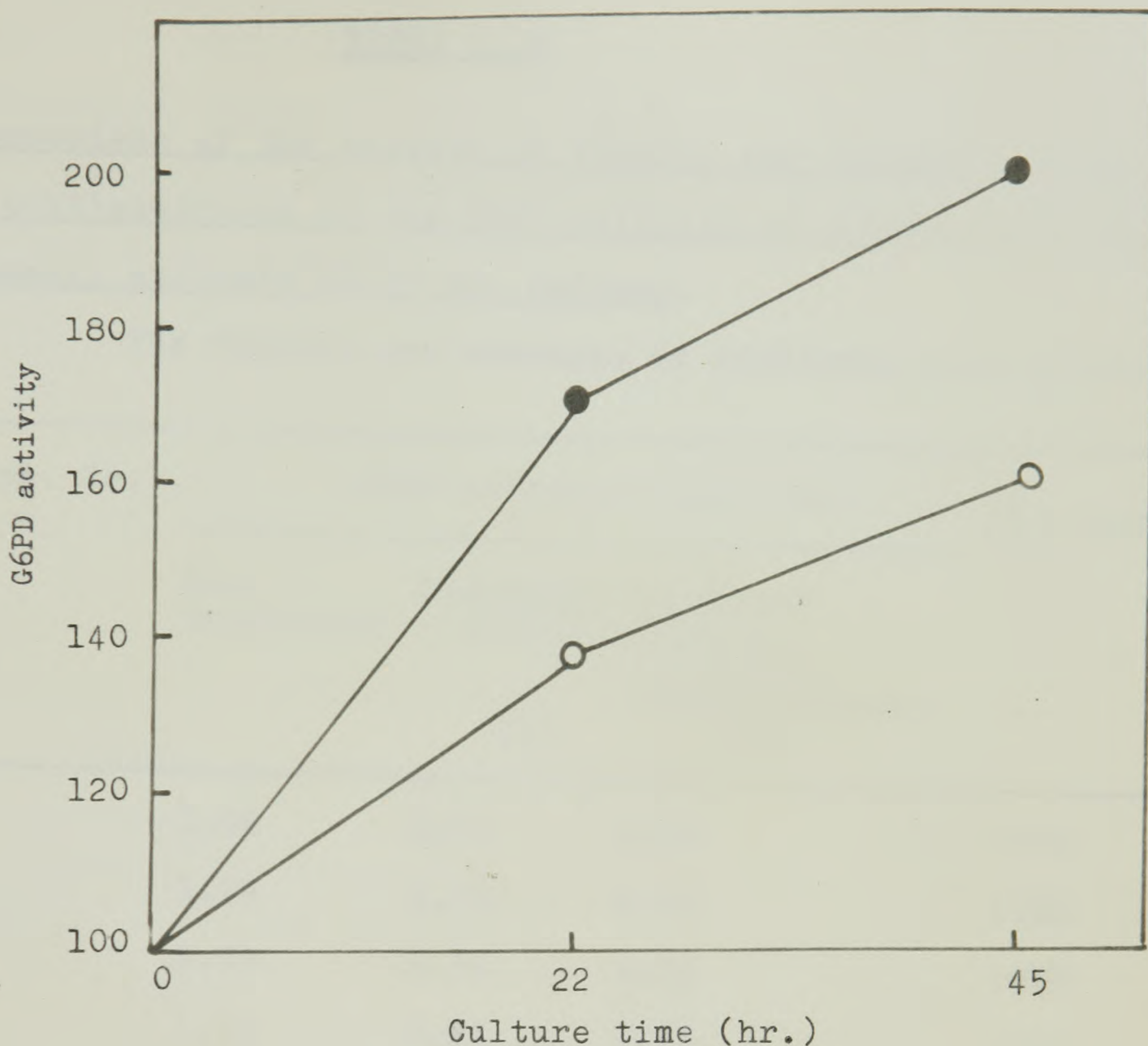


FIG. 6.10 Comparison of the effects of insulin and insulin + prolactin + corticosterone on the G6PD activity of mid-pregnant mouse mammary explants in 22 and 45 hr. culture.

- Incubated with insulin.
- Incubated with insulin + prolactin + corticosterone.

The figure is a combination of 4 similar experiments, each comprising duplicate determinations at the same points. In each experiment the initial activity and that after 45 hr. culture with the three hormones have been set at 100 and 200 respectively.

TABLE 6.15

Comparison of the effects of insulin and insulin + prolactin + corticosterone on the G6PD activity of mid-pregnant mouse mammary explants in 22 hr. culture.

The figures are averages of duplicate determinations.

Expt. No.	G6PD activity (units/g.)			$\frac{B}{A} \times 100$
	Not incubated	Incubated + insulin	Incubated + insulin + prolactin + corticosterone	
	(A)		(B)	
1	1.96	2.70	4.20	155%
2	1.75	2.39	2.77	116%
3	1.70	2.54	2.75	107%
4	1.36	2.64	3.43	130%
5	1.15	2.14	2.61	122%
6	1.30	2.90	3.45	118%
7	1.30	2.45	3.01	123%
		Mean		124%
		P		< 0.01

TABLE 6.16

Effects of prolactin and corticosterone on the G6PD activity of mid-pregnant mouse mammary explants in 22 hr. and 45 hr. culture with insulin.

The figures are averages of duplicate determinations.

Expt. No.	Hormone additions	G6PD activity (units/g.)	
		22 hr. culture	45 hr. culture
<u>Expt. 1</u>	Not incubated		1.96
	None	1.68	0.69
	Insulin	2.70	3.68
<u>Expt. 2</u>	Not incubated		1.75
	Insulin	2.39	3.10
	Insulin +corticosterone	1.89	2.32
	Insulin +prolactin	3.28	3.86
	Insulin +prolactin +corticosterone	2.77	3.54

Insulin + prolactin gave as great an increase in activity as insulin, prolactin and corticosterone; insulin + corticosterone, however, repeatedly gave a smaller increase than insulin alone.

Virgin mice.

Several experiments were performed with mammary tissue from virgin mice. Unlike late-pregnant or mid-pregnant tissue, virgin tissue gave inconsistent results. In 3 out of 8 experiments, results resembling those in Fig. 6.10 were obtained (e.g. Table 6.17); while in the remaining 5 no increase in activity occurred.

Mishkinsky, Dikstein, Ben-David, Azeroual and Sulman (1967) have reported conditions for the culture of mammary tissue from virgin rats. For full histological development they find that the rats must be killed in metoestrus and oestradiol and progesterone must be added to the culture medium. Table 6.18 shows that the reason for the inconsistent behaviour of virgin mouse tissue was neither the stage of the oestrous cycle nor the need for oestradiol and progesterone.

TABLE 6.17

Comparison of the effects of insulin and insulin + prolactin + corticosterone on the G6PD activity of virgin mouse mammary explants in 22 and 45 hr. culture.

The figures are averages of duplicate determinations and were typical of experiments in which the enzyme activity increased (see text).

Hormone additions	G6PD activity (units/g.)	
	22 hr.	45 hr.
Not incubated	2.11	3.09
Insulin	3.51	4.06
Insulin + prolactin + corticosterone	4.38	4.95
prolactin + corticosterone	2.98	3.87
Insulin + prolactin + corticosterone	4.11	4.67
corticosterone + progesterone		

TABLE 6.18

Effect of oestradiol + progesterone on the G6PD activity of 22 hr. cultures of mammary explants from mice in oestrus and metoestrus

Progesterone was added at a final concentration of 1 $\mu\text{g./ml.}$ and oestradiol at 0.03 $\mu\text{g./ml.}$ The figures are averages of duplicate determinations.

Hormone additions	G6PD activity (units/g.)	
	oestrus	metoestrus
Not incubated	2.11	2.35
Insulin	2.14	2.71
Insulin +oestradiol+progesterone	2.00	2.58
Insulin +prolactin+corticosterone	2.06	2.45
Insulin +prolactin+corticosterone +oestradiol+progesterone	2.11	2.41

6.4 Action of inhibitors on cultures of mammary tissue from mice in late pregnancy.

Effects of different inhibitors.

The increases in glucose 6-phosphate dehydrogenase activity and 6-phosphogluconate dehydrogenase activity in mammary cultures may be due to activation of pre-existing inactive enzymes, decreased degradation of the enzymes or increased enzyme synthesis. If there is an increase in synthesis of the enzymes, new RNA may also be required. It is also possible that new DNA synthesis is needed in view of the increase in total gland DNA at parturition (Table 6.1) and the suggestion that mitosis and new DNA synthesis must occur before mid-pregnant mammary tissue can synthesise casein (Stockdale and Topper, 1966).

Experiments were therefore performed with inhibitors of nucleic acid and protein synthesis to see if they affected the appearance of the enzymes in 22 hr. cultures of tissue from mice in late pregnancy. In all these experiments the culture medium contained insulin (5 μ g./ml.). To minimise possible toxic side-effects, the inhibitors were added at their lowest effective concentrations (see section 5.9).

The results of experiments in which different inhibitors were added at the start of culture are shown in Table 6.19. Both puromycin and cycloheximide prevented the increase in enzyme activities and in fact caused them to fall. This suggests that protein synthesis is required before the enzyme activities can rise. The large fall in activity with puromycin may be due to some of the side-effects of this inhibitor (Fain, 1964; Appleman and Kemp, 1966). The smaller fall with cycloheximide may perhaps represent enzyme turnover. In 4 experiments over 22 hr., cycloheximide reduced the initial glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities by averages of 33% and 34% respectively.

It can also be seen from Table 6.19 that the enzyme rises are inhibited by actinomycin D. This suggests that new RNA is required before the enzyme activities can rise.

When hydroxyurea (50 μ g./ml.) was added to the culture medium the rises in enzyme activity were unaffected. As this concentration of hydroxyurea inhibited the incorporation of (3 H)thymidine into DNA by 92% (section 5.9) it is clear that in the culture DNA synthesis is not required for the enzyme increase.

TABLE 6.19

Effects of inhibitors of RNA, DNA and protein synthesis
on the G6PD activity of late-pregnant mouse mammary explants
in 22 hr. culture with insulin.

The figures are averages of duplicate determinations.
Percentages of the initial value are given in parentheses.

Expt. No.	Inhibitor addition	Concentration (μ g./ml.)	Enzyme activity (units/g.)	
			G6PD	6PGD
<u>Expt. 1</u>				
	Not incubated		2.02(100%)	0.60(100%)
	None		4.20(208%)	1.20(200%)
	Puromycin	10	0.16(8%)	0.16(27%)
	Cycloheximide	1	1.50(74%)	0.30(50%)
<u>Expt. 2</u>				
	Not incubated		1.53(100%)	0.46(100%)
	None		2.91(190%)	1.06(230%)
	Hydroxyurea	50	2.80(183%)	0.82(178%)
	Actinomycin D	0.5	1.10(72%)	0.46(100%)

Peterkofsky and Tomkins (1967) have similarly shown that DNA synthesis is not required for the steroid-mediated increase in tyrosine aminotransferase activity in hepatoma cell cultures.

Studies with actinomycin D.

If new RNA is required for the enzyme rises, as is suggested by the result with actinomycin D, it is of interest to know whether this requirement is restricted to a particular stage of the incubation - possibly before the enzyme starts to appear (Fig. 6.7). Experiments were therefore performed to determine the period during which actinomycin D inhibited the enzyme rise.

Table 6.20 shows the results of a 22 hr. culture in which actinomycin D was added 0, 6 and 12 hr. after the start of incubation. It can be seen that actinomycin D, added at the start of incubation, again completely prevented the rise in glucose 6-phosphate dehydrogenase activity but, when added at 6 or 12 hr., the full increase in enzyme activity occurred. This was despite the fact that the activity had not increased by 12 hr..

Fig. 6.11 shows the results of an experiment in which actinomycin D was added at different times

TABLE 6.20

The G6PD activity of late-pregnant mouse mammary explants
in 22 hr. culture with insulin after addition of actinomycin D
at 6 or 12 hr..

Actinomycin D was added, as indicated, to a concentration of 0.5 $\mu\text{g./ml.}$ The figures are means of duplicate determinations.

Period of incubation (hr.)	Time of actinomycin D addition (hr.)	G6PD activity (units/g.)
0	-	1.28
12	-	1.20
22	-	2.78
22	0	1.08
22	6	3.38
22	12	2.86

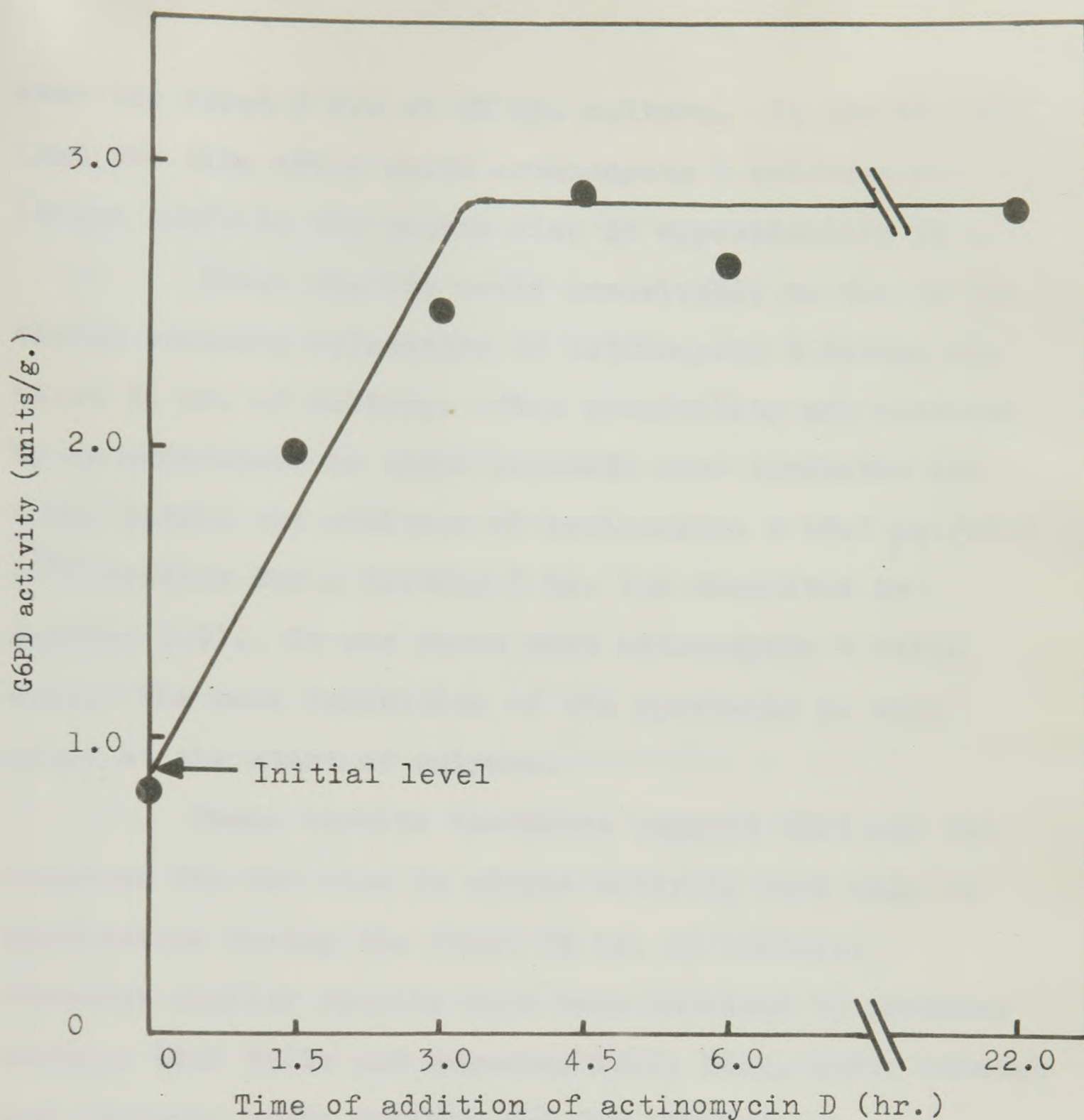


FIG. 6.11 Effect of addition of actinomycin D at various times on the G6PD activity of late-pregnant mouse mammary explants in 22 hr. culture with insulin.

Actinomycin D was added to a concentration of 0.5 $\mu\text{g.}/\text{ml.}$ at the times indicated and all the cultures terminated at 22 hr.. Each point is the average of duplicate determinations.

over the first 6 hr. of 22 hr. culture. It can be seen that the time after which actinomycin D addition no longer inhibits the enzyme rise is approximately $3\frac{1}{2}$ hr..

These results could conceivably be due to the tissue becoming refractory to actinomycin D during the first $3\frac{1}{2}$ hr. of culture. This possibility was excluded by an experiment in which explants were incubated for 4 hr. before the addition of actinomycin D (0.5 μ g./ml.) and (14 C)uridine for a further 3 hr. (as described in section 5.9). It was found that actinomycin D still caused the same inhibition of RNA synthesis as when added at the start of culture.

These results therefore suggest that new RNA required for the rise in enzyme activity need only be synthesised during the first $3\frac{1}{2}$ hr. of culture. Somewhat similar results have been obtained by previous workers (del Valle and Aronson, 1962; Kirk, 1965; Sussman and Sussman, 1965; Griffin and Cox, 1966a).

There have been reports of actinomycin stimulating the activities of certain enzymes, both in vivo and in vitro (Moog, 1964; Garren, Howell, Tomkins and Crocco, 1964; Eagle and Robinson, 1964; Thompson, Tomkins and Curran, 1966). No such stimulating effects were observed when actinomycin D (0.5 μ g./ml.) was added at 12 or 24 hr. to explants in 45 hr. culture with insulin.

Studies with cycloheximide.

Experiments were performed with cycloheximide to see whether inhibition of protein synthesis at different stages of the culture always prevented all further increase in enzyme activity. A preliminary experiment (Table 6.21) showed that this was not so. Cycloheximide, added at the start of culture, prevented the enzyme rise but, if added at 12 hr., the enzyme increased in activity almost as much as in the control without inhibitor. This result was confirmed in 2 more similar experiments.

This result could conceivably be due to the time taken by the inhibitor to penetrate the tissue. However Table 6.22 shows that after explants have been in culture for 12 hr., addition of cycloheximide inhibits 92% of protein synthesis within a few minutes.

Experiments were performed to find, more exactly, how cycloheximide affected the enzyme rise when added at various times. As it had been found that no further rise occurred if cycloheximide was added during the first 9 hr., an experiment was done with additions between 10 and 13 hr.. Fig. 6.12 shows that cycloheximide completely inhibited the increase in

TABLE 6.21

The G6PD activity of late-pregnant mouse mammary explants
in 22 hr. culture with insulin after addition of cycloheximide
at 12 hr..

Cycloheximide was added, as indicated, to a concentration of 1 μ g./ml.. The figures are means of duplicate determinations.

Period of incubation (hr.)	Time of cycloheximide addition (hr.)	G6PD activity (units/g.)
0	-	1.27
12	-	1.31
22	-	2.25
22	0	0.83
22	12	2.02

TABLE 6.22

Effect of cycloheximide pre-incubation period on the incorporation of (^{14}C)lysine into the protein of late-pregnant mouse mammary explants.

After 12 hr. incubation with insulin, (^{14}C)lysine (0.25 μc) was added to each batch of explants and these were then incubated for a further 3 hr.. Some batches had been pre-incubated with cycloheximide (1 $\mu\text{g./ml.}$), as indicated. The values are averages of duplicate determinations.

Cycloheximide pre-incubation time (min.)	Counts/min. in protein	Percentage of uninhibited level
No inhibitor addition	934	100%
0	77	8%
15	74	8%
30	74	8%
60	65	7%

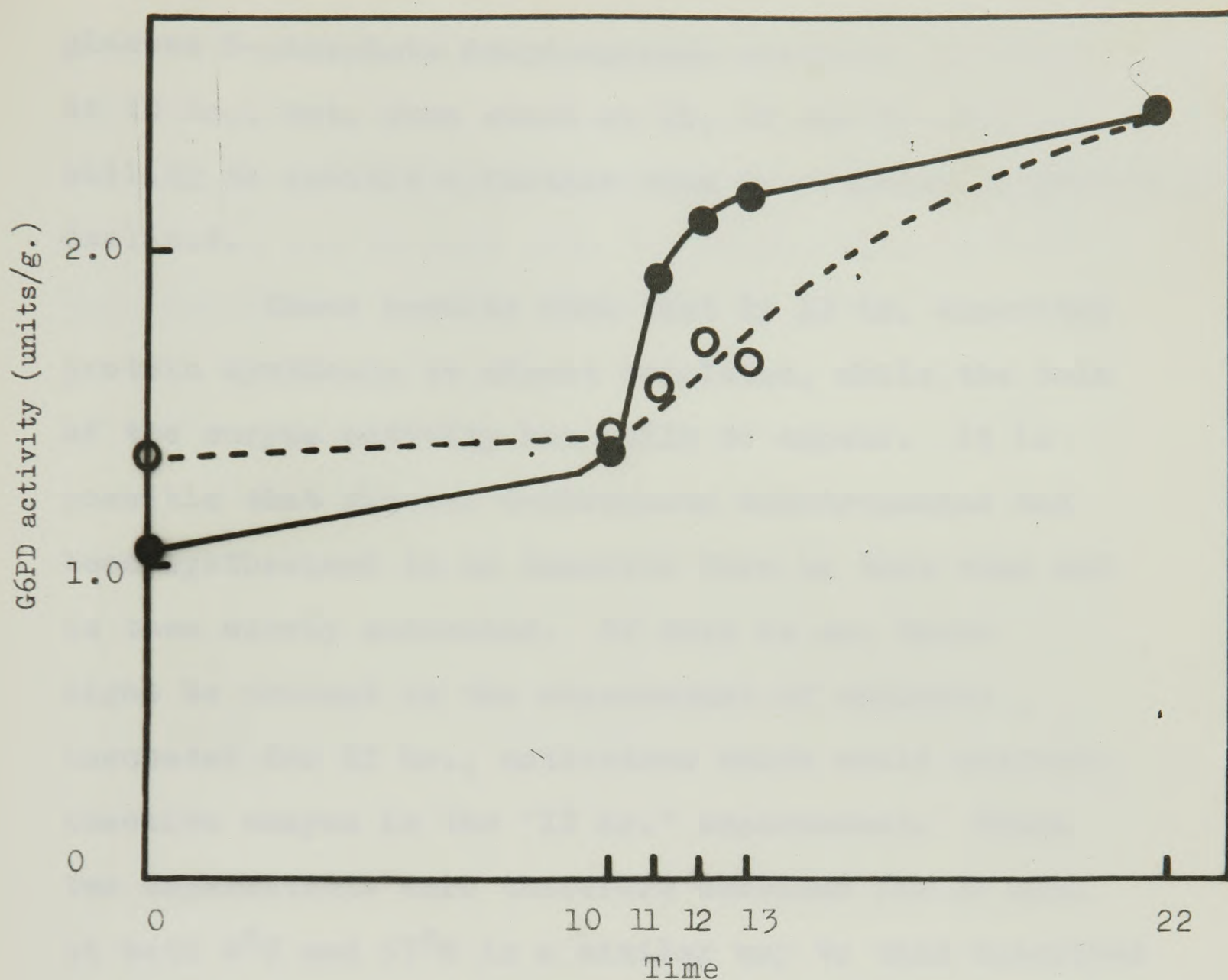


FIG. 6.12 Effect of the addition of cycloheximide at various times on the G6PD activity of late-pregnant mouse mammary explants in 22 hr. culture with insulin.

- G6PD activity after 22 hr. culture, cycloheximide (1 μ g./ml.) being added at the times indicated.
- G6PD activity after culture, in the absence of inhibitor, for the times indicated.

Each point is the average of duplicate determinations.

glucose 6-phosphate dehydrogenase activity, if added at 10 hr., but, when added at 11, 12 and 13 hr., its ability to inhibit a further rise in activity progressively declined.

These results show that by 12 hr. essential protein synthesis is almost completed, while the bulk of the enzyme activity has still to appear. It is possible that glucose 6-phosphate dehydrogenase has been synthesised in an inactive form by this time and is then slowly activated. If this is so, there might be present in the supernatant of explants, incubated for 22 hr., activators which would activate inactive enzyme in the '12 hr.' supernatant. These two supernatants were therefore combined for 20 min. at both 0°C and 37°C in a similar way to that described in Table 6.4. However it was found that the enzyme activities of the mixed supernatants were the average of the activities of their components.

Nevaldine and Levy (1965) have shown that mammary glucose 6-phosphate dehydrogenase may be dissociated into inactive subunits. These reassociate to active enzyme in buffer containing β -mercaptoethanol

and NADP^+ . Table 6.23 shows an experiment in which the supernatant from explants after 12 hr. culture was incubated in β -mercaptoethanol buffer containing NADP^+ . It can be seen that the enzyme activity did not increase.

The supernatant was incubated, as indicated, with an equal volume of a solution containing: β -mercaptoethanol (120 mM), Tris-HCl (100 mM), NADP^+ (0.14 mM), glucose 6-phosphate (3 mM), pH 7.4, for 2 hr. at 37°C.

Explant incubation time	Supernatant incubation	Glucose activity (units/g.)
0	No	2.75
12	Yes	2.77
12	Yes	2.65
22	No	2.44

TABLE 6.23

Effect of incubation with NADP⁺ and glucose 6-phosphate on the G6PD activity of the supernatant from late-pregnant mouse mammary explants incubated for 12 hr. with insulin.

The supernatant was incubated, as indicated, with an equal volume of a solution containing: β -mercaptoethanol (28 mM), tris-HCl (160 mM), NADP⁺ (0.14 mM), glucose 6-phosphate (5 mM), pH 7.6, for 2 hr. at 37°C.

Explant incubation time	Supernatant incubation	G6PD activity (units/g.)
0	No	1.35
12	No	1.73
12	Yes	1.65
22	No	2.44

7. DISCUSSION

7.1 Introduction.

The purpose of the work described in Part Two of this thesis was to develop a method of incubating mammary tissue which would allow some of the increases in enzyme activities that occur at the onset of lactation to be studied in vitro. A method is described (section 5.5) for the culture of sufficient tissue to allow accurate determination of changes in glucose 6-phosphate dehydrogenase activity, and less accurate determination of changes in 6-phosphogluconate dehydrogenase activity. With tissue from pregnant mice it has been possible to reproduce, in vitro, the increases in the activities of these enzymes that occur in the living animal after parturition. This is the first time such changes in vitro have been reported and it should be possible, by this method, to study changes in the activities of other enzymes in mammary tissue.

7.2 ~~Report~~ There are only a few previous reports of increases of enzyme activities in mammalian tissues in vitro. Firstly there are some examples of the induction of increased enzyme activities in HeLa cells (Cox and MacLeod, 1961) and hepatoma cells (Thompson, Tomkins and Curran, 1966; Potter, Watanabe, Becker and Pitot, 1967), when incubated with certain hormones. Perhaps more relevant to this thesis are reports of enzymes increasing in activity prematurely, when tissues are transferred from their natural environment to in vitro culture. These include glutaminotransferase activity of embryonic chick retina (Moscona and Hubby, 1963), UDP-glucuronyl transferase activity of embryonic chick liver (Ko, Dutton and Nemeth, 1967) and malate dehydrogenase activity of regenerative-phase human endometrium (Wilson, 1967).

7.2 Hormones and the stimulus for the increase in glucose 6-phosphate dehydrogenase activity.

The hormone requirements for the maximum increase in glucose 6-phosphate dehydrogenase activity in late-pregnant mammary tissue in vitro differ from those in mid-pregnant tissue. With late-pregnant tissue prolactin has no significant effect on the enzyme activity during the first day of culture (Table 6.6), whereas with mid-pregnant tissue the increase with insulin and prolactin is greater than that with insulin alone (Fig. 6.10, Table 6.15).

One possible explanation for this is as follows. The increase in enzyme activity will only occur in cells which have fully differentiated under the stimulus of prolactin (cf. Stockdale and Topper, 1966). In late pregnancy virtually all the parenchymal cells have already been fully influenced by prolactin but in mid-pregnancy only a proportion have and the remainder may, therefore, be stimulated by prolactin in the culture. In mid-pregnant tissue the enzyme rise that occurs with insulin alone would thus be taking place in cells which have already differentiated.

Against this idea is the fact that casein synthesis in mid-pregnant tissue is not stimulated by insulin alone but requires corticosterone and prolactin. In this respect casein synthesis more closely resembles microscopically observable differentiation (Stockdale, Juergens and Topper, 1966). Thus it seems unlikely that in mid-pregnant tissue there are many fully differentiated cells that can be stimulated by insulin alone. An alternative explanation might therefore be that the increase in enzyme activity can take place in cells which have not been influenced by prolactin. However because prolactin induces growth and differentiation in mid-pregnant tissue, it causes a general increase in many cell constituents, including glucose 6-phosphate dehydrogenase.

Over the second day of culture the enzyme activity continues to rise in mid-pregnant tissue (Fig. 6.10) but it only does so in late-pregnant tissue if the medium contains serum (Fig. 6.9). The reason for this difference is not clear. Though it is conceivable that the effect of serum was due to hormones contained in it, none of the hormones tested were able to replace serum (Table 6.10), although prolactin did prevent the enzyme decline (Fig. 6.8, Table 6.8). It seems more likely that the effect of serum is due to

some other components which prevent deterioration of the tissue (see Waymouth, 1965).

The lack of effects of other hormones on the increase in glucose 6-phosphate dehydrogenase activity in late-pregnant tissue in vitro (Table 6.7, 6.9 and 6.10), contrasts at first sight with the results of endocrine ablation and hormone replacement experiments in vivo. Thus in lactating rats, the mammary enzyme level is reduced by adrenalectomy (Willmer, 1960; Willmer and Foster, 1965), hypophysectomy (Jones, 1967; Baldwin and Martin, 1968), thyroidectomy (Walters and McLean, 1967) and ovariectomy (Barker and Ludwick, 1967). However these operations may alter the enzyme level in the mammary gland indirectly. Hypophysectomy, for example, causes reduced intestinal absorption of carbohydrates (Phillips and Robb, 1934) and it seems particularly relevant that, though hypophysectomy prevents the increase in liver glucose 6-phosphate dehydrogenase that accompanies a high carbohydrate diet, the operation has no effect if the rats are fed by stomach tube (Tepperman and Tepperman, 1962).

Although insulin is the only hormone needed to initiate the enzyme rise in vitro, it is difficult to envisage insulin as the initiator in vivo (Weber, Singhal, Stamm, Lea and Fisher, 1966) because of the large fluctuations in insulin level, and the fact that in women the level is higher pre partum than post partum (Spellacy and Goetz, 1963).

It seems more likely that the increases in vitro are caused by the removal of the tissue from an inhibitory influence which is present in the pregnant animal but disappears at parturition. However addition of oestradiol, progesterone or placental extract to the medium did not affect the enzyme rise (Table 6.11, p. 67) and they cannot, therefore, inhibit directly. The enzyme increase might instead be restrained by a limited intracellular concentration of one or more metabolic intermediates. These would rise both in vitro in the culture and in vivo at parturition when the foeti cease to exert a drain on the metabolites of the maternal blood and when the blood flow to the mammary gland increases (Linzell, 1960; Reynolds, 1965). The resulting increase in enzyme activities might be due to the metabolite combining with a specific repressor and thus inducing enzyme synthesis (Jacob and Monod, 1961), or it might merely be due to a more general increase in protein synthesis on pre-existing RNA templates when the rate of metabolism of the cell increases.

If the increase in mammary gland glucose 6-phosphate dehydrogenase after parturition is caused by an increased intracellular concentration of a metabolic intermediate, it would seem to be similar to the increase in liver and mammary gland glucose 6-phosphate dehydrogenase in (virgin) rats fed high carbohydrate diets (Fitch, Hill and Chaikoff,

1959; Hilf, Michel, Gibbs and Bell, 1966), and the increase in this enzyme in the liver when starved rats are refed (Tepperman and Tepperman, 1958). Other examples of effects of metabolite levels on enzymes and proteins have been reported. Katzen, Soderman and Nitowsky (1965) found an increased hexokinase activity when human liver cell cultures were transferred to a higher glucose medium; Schingoethe, Hageman and Larson (1967) found that the synthesis of milk proteins in mammary cell cultures was stimulated when the concentration of essential amino acids was increased above physiological and Jefferson and Korner (1967) found that growth hormone only stimulated protein synthesis in perfused rat liver when the amino acid concentration was raised above that in rat plasma.

It is interesting to speculate whether increased availability of metabolites rather than hormones could also be involved in the initiation, at parturition, of the synthesis of more specific mammary cell products such as lactose and casein. Studies of the hormonal requirements for the synthesis of these products in cultures of late-pregnant tissue should help to resolve this and experiments with a simplified medium might lead to the identification of the metabolite involved.

7.3 Biochemical events following the stimulus for the increase in glucose 6-phosphate dehydrogenase activity.

The experiments with inhibitors (section 6.4) were performed to elucidate the biochemical events involved in the in vitro increase in glucose 6-phosphate dehydrogenase activity. Care is needed in the interpretation of such experiments because of the possible side-effects of the inhibitors and their incomplete inhibition of nucleic acid and protein synthesis at the levels used. Though side-effects appear to be less important with cycloheximide, actinomycin D has been reported to have the following effects, independent of its inhibition of RNA synthesis: inhibition of protein synthesis (Revel, Hiatt and Revel, 1964; Honig and Rabinowitz, 1965), degradation of RNA (Wiesner, Acs, Reich and Shafiq, 1965), inhibition of respiration and glycolysis (Laszlo, Miller, McCarty and Hochstein, 1966) and inhibition of phospholipid synthesis (Pastan and Friedman, 1968). However, bearing in mind the minimal concentrations used (section 5.9) and the absence of any toxic effect when addition was suitably delayed (Fig. 6.11 and 6.12), it is probably justifiable to discuss the effects of actinomycin D and cycloheximide in terms of specific inhibition of RNA and protein synthesis.

If this conclusion is accepted, the results in Fig. 6.7, 6.11 and 6.12 and in Table 6.19 suggest that, for the increase in glucose 6-phosphate dehydrogenase activity in vitro, RNA must be synthesised between 0 and 3½ hr. and protein synthesised between 10 and 12 hr. (and possibly sometime before 10 hr.). Also the appearance of active enzyme between 10-12 and 22 hr. can occur in the absence of RNA or protein synthesis.

This last fact suggests that the enzyme is first synthesised in an inactive form. Although attempts to demonstrate the presence of an inactive enzyme precursor have been unsuccessful (p. 62, Table 6.4, p. 77, Table 6.23), a more extensive investigation is required. An alternative explanation is that active enzyme is transferred from a subcellular fraction to the supernatant. However this seems unlikely as glucose 6-phosphate dehydrogenase appears to be confined to the soluble fraction of mammalian tissues (e.g. Glock and McLean, 1953).

Perhaps the most obvious explanation of the RNA and protein requirements for the enzyme rise is that RNA templates are synthesised during the first 3½ hr. and inactive enzyme protein is synthesised on these templates between 10 and 12 hr., the enzyme then being slowly activated. The RNA templates would be

quite stable as the increase in enzyme activity was not affected by actinomycin D in the culture medium between 4½ and 22 hr. (Fig. 6.11). The reason for the lag between synthesis of template and protein is unclear. Such lags, which are a feature of many mammalian enzyme inductions, may represent the time needed for new ribosomes and RNA templates to be organised into new endoplasmic reticulum (Tata, 1967). To account for the increase in enzyme activity, an increase in RNA and protein synthesis seems most likely although a decreased rate of enzyme degradation could also be responsible (see Schimke, Sweeny and Berlin, 1964).

The requirements for RNA synthesis and even protein synthesis can, however, be explained in other ways. It is possible that the new RNA contains templates for the synthesis of another protein which in turn either activates a reserve of glucose 6-phosphate dehydrogenase precursor protein or else stimulates the synthesis of the precursor on pre-existing templates. This latter may seem rather unlikely but activation of pre-existing templates does occur on fertilisation of sea-urchin eggs (see Monroy, Maggio and Rinaldi, 1965).

Examples of enzyme activations catalysed by other enzymes are the formation of chymotrypsin (Kunitz and Northrop, 1935) and the interconversion of the a and b forms of α -glucan phosphorylase (Cori and Cori, 1945). A more indirect enzyme activation might occur if the newly synthesised protein were an enzyme catalysing the formation of a small molecular weight activator of glucose 6-phosphate dehydrogenase.

There is a precedent for enzyme activation which requires RNA and protein synthesis. When prednisolone is added to HeLa cell cultures there is an increase in alkaline phosphatase activity which is inhibited by low concentrations of puromycin and actinomycin D. There is no increase in radioactivity in (^{14}C)leucine labelled protein, precipitated by an antibody to the enzyme, but there are differences between the physical properties of the enzyme before and after induction. This suggests that the increased catalytic activity is due to a change in conformation of the enzyme and does not represent increased enzyme synthesis or decreased enzyme degradation (Griffin and Cox, 1966a and b).

Although the results of Emery and Baldwin (1967) suggest that there is an increase in the amount of glucose 6-phosphate dehydrogenase protein in the rat mammary gland during early lactation, a decision about the increase in vitro will have to await experiments with a specific antibody. It might also be possible to detect the presence of inactive enzyme precursor with an antibody to the active enzyme.

- Biochem. Biophys. Res. Commun. 21, 204.
- Baldwin, R. L. and Emery, S. J. (1968). J. Dairy Sci. 51, 740.
- Baldwin, R. L. and Williams, R. F. (1966).
J. Biol. Chem. 241, 2030.
- Baldwin, J. H. and Polley, S. J. (1961). Biochem. J. 42, 663.
- Baldwin, J. H., Polley, S. J. and Glasscock, R. F. (1954).
Biochem. J. 50, 274.
- Baldwin, J. H., French, L. G. and Polley, S. J. (1959).
Nature, Lond., 122, 607.
- Barker, E. L. and Ludwig, T. W. (1967). J. Dairy Sci. 50, 197.
- Barnwell, E. H. (1967). J. Dairy Sci. 50, 152, 157.
- Bartley, J. C., Abraham, S. and Chalkoff, I. L. (1966).
Proc. Soc. exp. Biol. Med. 121, 679.
- Barnwell, E. H. and Emery, S. (1967). Biochem. J. 103, 616.
- Barton, K. (1956). Biochem. J. 52, 315.

BIBLIOGRAPHY

- Abraham, S., Cady, P. and Chaikoff, I.L. (1957).
J. biol. Chem. 224, 955.
- Appleman, M.M. and Kemp, R. G. (1966).
Biochem. biophys. Res. Commun. 24, 564.
- Baldwin, R. L. and Martin, R. J. (1968). J. Dairy Sci. 51, 748.
- Baldwin, R. L. and Milligan, L. P. (1966).
J. biol. Chem. 241, 2058.
- Balmain, J. H. and Folley, S. J. (1951). Biochem. J. 49, 663.
- Balmain, J. H., Folley, S. J. and Glascock, R. F. (1954).
Biochem. J. 56, 234.
- Balmain, J. H., French, T. H. and Folley, S. J. (1950).
Nature, Lond., 165, 807.
- Barker, K. L. and Ludwick, T. M. (1967). J. Dairy Sci. 50, 1978.
- Barnawell, E. B. (1965). J. exp. Zool. 160, 189.
- Bartley, J. C., Abraham, S. and Chaikoff, I. L. (1966).
Proc. Soc. exp. Biol. Med. 123, 670.
- Bramwell, M. E. and Harris, H. (1967). Biochem. J. 103, 816.
- Burton, K. (1956). Biochem. J. 62, 315.

- Clever, U. and Karlson, P. (1960). Expl Cell Res. 20, 623.
- Cori, G. T. and Cori, C. F. (1945). J. biol. Chem. 158, 321.
- Cowie, A. T. (1961). In Milk: the Mammary Gland and Its Secretion, vol. 1, p. 163. Ed. by Kon, S. K. and Cowie, A. T. New York: Academic Press.
- Cox, R. P. and MacLeod, C. M. (1961). Nature, Lond., 190, 85.
- DeOme, K. B., Paulkin, L. J., Bern, H. A. and Blair, P. B. (1959). Cancer Res. 19, 515.
- Eagle, G. R. and Robinson, D. S. (1964). Biochem. J. 93, 100.
- Eboué-Bonis, D., Chambaut, A. M., Volfin, P. and Clauser, H. (1963). Nature, Lond., 199, 1183.
- Elias, J. J. (1957). Science, N.Y. 126, 842.
- Elias, J. J. (1959). Proc. Soc. exp. Biol. Med. 101, 500.
- Elias, J. J. and Rivera, E. M. (1959). Cancer Res. 19, 505.
- Emery, R. S. and Baldwin, R. L. (1967). Biochim. biophys. Acta, 136, 223.
- Ennis, H. L. and Lubin, M. (1964). Science, N. Y. 146, 1474.
- Fain, J. N. (1964). Biochim. biophys. Acta, 84, 636.
- Fitch, W. M., Hill, R. and Chaikoff, I. L. (1959). J. biol. Chem. 234, 1048.

- Folley, S. J. (1956). The Physiology and Biochemistry of Lactation. Edinburgh: Oliver and Boyd.
- Gala, R. R. and Westphal, U. (1965). Endocrinology, 76, 1079.
- Garren, L. D., Howell, R. R., Tomkins, G. M. and Crocco, R. M. (1964). Proc. natn. Acad. Sci. U.S.A. 52, 1121.
- Garren, L. D., Ney, R. L. and Davis, W. W. (1965). Proc. natn. Acad. Sci. U.S.A. 53, 1443.
- Glock, G. E. and McLean, P. (1953). Biochem. J. 55, 400.
- Glock, G. E. and McLean, P. (1954). Biochem. J. 56, 171.
- Glock, G. E. and McLean, P. (1955). Biochem. J. 61, 390.
- Green, D. E. (1941). Adv. Enzymol. 1, 177.
- Griffin, M. J. and Cox, R. P. (1966a). J. Cell Biol. 29, 1.
- Griffin, M. J. and Cox, R. P. (1966b). Proc. natn. Acad. Sci. U.S.A. 56, 946.
- Grosvenor, C. E., Mena, F., Dhariwal, A. P. S. and McCann, S. M. (1967). Endocrinology, 81, 1021.
- Grota, L. J. and Eik-Nes, K. B. (1967). J. Reprod. Fert. 13, 83.
- Hardwick, D. C. and Linzell, J. L. (1960). J. Physiol., Lond., 154, 547.
- Harris, H., Fisher, H. W., Rodgers, A., Spencer, T. and Watts, J. W. (1963). Proc. R. Soc. B, 157, 177.

- Harris, H. and Watts, J. W. (1962). Proc. R. Soc. B, 156, 109.
- Hayes, D. H., Hayes, F. and Guérin, M. F. (1966). J. molec. Biol. 18, 499.
- Hechter, O. and Halkerston, I. D. K. (1965). A. Rev. Physiol. 27, 133.
- Henshaw, E. C., Revel, M. and Hiatt, H. H. (1965). J. molec. Biol. 14, 241.
- Hershey, P. B., Lewis, C., Johnston, G. and Mason, S. (1963). J. Histochem. Cytochem. 11, 218.
- Hilf, R., Michel, I., Gibbs, C. C. and Bell, C. (1966). Biochim. biophys. Acta, 116, 589.
- Holley, R. W., Apgar, J. and Doctor, B. P. (1960). Ann. N.Y. Acad. Sci. 88, 745.
- Honig, G. R. and Rabinowitz, M. (1965). Science, N.Y. 149, 1504.
- Huppert, J. and Pelmont, J. (1962). Archs Biochem. Biophys. 98, 214.
- Jacob, F. and Monod, J. (1961). J. molec. Biol. 3, 318.
- Jackson, C. D. and Sells, B. H. (1968). Biochim. biophys. Acta, 155, 417.
- Jefferson, L. S. and Korner, A. (1967). Biochem. J. 104, 826.

- Jones, E. A. (1967). Biochem. J. 103, 420.
- Juergens, W. G., Stockdale, F. E., Topper, Y. J. and Elias, J.J.
(1965). Proc. natn. Acad. Sci. U.S.A. 54, 629.
- Karlson, P. (1963). Perspect. Biol. Med. 6, 203.
- Karlson, P. (1965) (ed.). Mechanisms of Hormone Action.
New York: Academic Press.
- Karlson, P. and Sekeris, C. E. (1966).
Recent Prog. Horm. Res. 22, 473.
- Katz, S. and Comb, D. G. (1963). J. biol. Chem. 238, 3065.
- Katzen, H. M., Soderman, D. D. and Witowsky, H. M. (1965).
Biochem. biophys. Res. Commun. 19, 377.
- Kidson, C. and Kirby, K. S. (1963).
Biochim. biophys. Acta, 76, 624.
- Kidson, C. and Kirby, K. S. (1964a).
Biochim. biophys. Acta, 91, 627.
- Kidson, C. and Kirby, K. S. (1964b).
J. molec. Biol. 10, 187.
- Kidson, C. and Kirby, K. S. (1964c).
Cancer Res. 24, 1604.
- Kidson, C. and Kirby, K. S. (1964d).
Nature, Lond., 203, 599.
- Kidson, C. and Kirby, K. S. (1965).
Cancer Res. 25, 472.

Kidson, C., Kirby, K. S. and Ralph, R. K. (1963).

J. molec. Biol. 7, 312.

Kirby, K. S. (1962). Biochim. biophys. Acta, 61, 506.

Kirby, K. S. (1965). Biochem. J. 96, 266.

Kirk, D. L. (1965). Proc. natn. Acad. Sci. U.S.A. 54, 1345.

Ko, V., Dutton, G. J. and Nemeth, A. M. (1967).

Biochem. J. 104, 991.

Korner, A. (1965). Recent Prog. Horm. Res. 21, 205.

Korner, A. (1967). Prog. Biophys. molec. Biol. 17, 63.

Korner, A. and Gumbley, J. M. (1966).

Nature, Lond., 209, 505.

Koziorowska, J. (1962). Acta Un. int. Cancer. 18, 211.

Krebs, H. A. and Henseleit, K. (1932).

Hoppe-Seyler's Z. physiol. Chem. 210, 33.

Kunitz, M. and Northrop, J. H. (1935).

J. gen. Physiol. 18, 433.

Lasnitzki, I. (1965). In Cells and Tissues in Culture,

vol. 1, p. 591. Ed. by Willmer, E. N.

New York: Academic Press.

Laszlo, J., Miller, D. S., McCarty, K. S. and Hochstein, P.

(1966). Science, N.Y., 151, 1007.

Leader, D. P. and Barry, J. M. (1967).

Nature, Lond., 215, 1374.

Leader, D. P. and Barry, J. M. (1968). Biochem. J. 106, 52P.

- Levinthal, C., Keynan, A. and Higa, A. (1962).
Proc. natn. Acad. Sci. U.S.A. 48, 1631.
- Linzell, J. L. (1960). J. Physiol., Lond., 153, 492.
- Lockwood, D. H., Stockdale, F. E. and Topper, Y. J. (1967).
Science, N.Y., 156, 945.
- Lockwood, D. H., Turkington, R. W. and Topper, Y. J. (1966).
Biochim. biophys. Acta, 130, 493.
- Loeb, J. N., Howell, R. R. and Tomkins, G. M. (1965).
Science, N.Y., 149, 1093.
- Lukács, I. and Sekeris, C. E. (1967).
Biochim. biophys. Acta, 134, 85.
- Lyons, W. R. (1942). Proc. Soc. exp. Biol. Med. 51, 308.
- Lyons, W. R. (1958). Proc. R. Soc. B, 149, 303.
- McKerns, K. W. (1964). Biochim. biophys. Acta, 90, 357.
- McLean, P. (1958). Biochim. biophys. Acta, 30, 303.
- McLean, P. (1960). Biochim. biophys. Acta, 37, 296.
- Marmur, J. (1961). J. molec. Biol. 3, 208.
- Mayne, R., Barry, J. M. and Rivera, E. M. (1966).
Biochem. J. 99, 688.
- Mayne, R., Forsyth, I. A. and Barry, J. M. (1967).
Biochem. J. 104, 41P.
- Meites, J. (1961). In Milk: the Mammary Gland and Its Secretion, vol. 1, p. 321. Ed. by Kon, S. K. and Cowie, A. T. New York: Academic Press.

- Meites, J. and Turner, C. W. (1942).
Endocrinology, 31, 340.
- Mejbaum, W. (1939). Hoppe-Seyler's Z. physiol. Chem. 258, 117.
- Meselson, M., Nomura, M., Brenner, S., Davern, C. and
Schlessinger, D. (1964). J. molec. Biol. 9, 696.
- Mishkinsky, J., Dikstein, S., Ben-David, M., Azeroual, J. and
Sulman, F. G. (1967).
Proc. Soc. exp. Biol. Med. 125, 360.
- Monod, J., Changeux, J-P. and Jacob, F. (1963).
J. molec. Biol. 6, 306.
- Monroy, A., Maggio, R. and Rinaldi, A. M. (1965).
Proc. natn. Acad. Sci. U.S.A. 54, 107.
- Moog, F. (1964). Science, N.Y., 144, 414.
- Moretti, R. L. and Abraham, S. (1966).
Biochim. biophys. Acta, 124, 280.
- Morgan, J. F., Morton, H. J. and Parker, R. C. (1950).
Proc. Soc. exp. Biol. Med. 73, 1.
- Moscona, A. A. and Hubby, J. L. (1963). Devel. Biol. 7, 192.
- Mueller, G. C. (1965). In Mechanisms of Hormone Action, p.228.
Ed. by Karlson, P. New York: Academic Press.
- Munford, R. E. (1964). Dairy Sci. Abstr. 26, 293.
- Nakada, D. and Magasanik, B. (1964).
J. molec. Biol. 8, 105.

Nevaldine, B. H. and Levy, H. R. (1965).

Biochem. biophys. Res. Commun. 21, 28.

Nicoll, C. S. and Meites, J. (1964).

Proc. Soc. exp. Biol. Med. 117, 579.

Parish, J. H. and Kirby, K. S. (1966a).

Biochim. biophys. Acta, 114, 198.

Parish, J. H. and Kirby, K. S. (1966b).

Biochim. biophys. Acta, 129, 554.

Pastan, I. and Friedman, R. M. (1968). Science, N.Y., 160, 316.

Pasteels, J. L. (1963). Archs Biol., Paris, 74, 439.

Peterkofsky, B. and Tomkins, G. M. (1967).

J. molec. Biol. 30, 49.

Phillips, R. A. and Robb, P. (1934).

Am. J. Physiol. 109, 82.

Potter, V. R., Watnabe, M., Becker, J. E. and Pitot, H. C.

(1967). Adv. Enzyme Reguln. 5, 303.

Prop, F. J. A. (1960). Expl Cell Res. 20, 256.

Rampersand, O. R. and Wool, I. G. (1965).

Science, N.Y., 149, 1102.

Reece, R. P. and Turner, C. W. (1937).

Proc. Soc. exp. Biol. Med. 35, 621.

Reich, E., Franklin, R. M., Shatkin, A. J. and Tatum, E. L.

(1961). Science, N.Y., 134, 556.

- Revel, M. and Hiatt, H. H. (1964). Proc. natn. Acad. Sci. U.S.A. 51, 810.
- Revel, M., Hiatt, H. H. and Revel, J-P. (1964). Science, N.Y., 146, 1311.
- Reynolds, M. (1965). Fedn Proc. Fedn Am. Socs exp. Biol. 24, 451.
- Rivera, E. M. (1964a) Proc. Soc. exp. Biol. Med. 116, 568.
- Rivera, E. M. (1964b). Endocrinology, 74, 853.
- Rivera, E. M. and Bern, H. A. (1961). Endocrinology, 69, 340.
- Rodbell, M. (1964). J. biol. Chem. 239, 375.
- Schimke, R. T., Sweeny, E. W. and Berlin, C. M. (1964). Biochem. biophys. Res. Commun. 15, 214.
- Schingoethe, D. J., Hageman, E. C. and Larson, B. L. (1967). Biochim. biophys. Acta, 148, 469.
- Sedat, J. W., Kelly, R. B. and Sinsheimer, R. L. (1967). J. molec. Biol. 26, 537.
- Seyle, H. (1934). Am. J. Physiol. 107, 535.
- Sharp, G. W. G. and Leaf, A. (1966). Recent Prog. Horm. Res. 22, 431.
- Singer, M. P. and Leder, P. (1966). A. Rev. Biochem. 35, 195.
- Spellacy, W. N. and Goetz, F. C. (1963). New Engl. J. Med. 268, 988.
- Stadie, W. C. and Riggs, B. C. (1944). J. biol. Chem. 154, 687.

- Stockdale, F. E., Juergens, W. G. and Topper, Y. J. (1966).
Devel. Biol. 13, 266.
- Stockdale, F. E. and Topper, Y. J. (1966).
Proc. natn. Acad. Sci. U.S.A. 56, 1283.
- Sussman, M. and Sussman, R. R. (1965).
Biochim. biophys. Acta, 108, 463.
- Sutherland, E. W., Øye, I. and Butcher, R. W. (1965).
Recent Prog. Horm. Res. 21, 623.
- Szego, C. M. (1965).
Fedn Proc. Fedn Am. Socs exp. Biol. 24, 1343.
- Talwalker, P. K., Nicoll, C. S. and Meites, J. (1961).
Endocrinology, 69, 802.
- Tata, J. R. (1965). In Mechanisms of Hormone Action, p. 173.
Ed. by Karlson, P. New York: Academic Press.
- Tata, J. R. (1966). Prog. nucleic Acid Res. 5, 191.
- Tata, J. R. (1967). Biochem. J. 104, 1.
- Tepperman, H. M. and Tepperman, J. (1962).
Am. J. Physiol. 202, 401.
- Tepperman, J. and Tepperman, H. M. (1958).
Am. J. Physiol. 193, 55.
- Thompson, E. B., Tomkins, G. M. and Curran, J. F. (1966).
Proc. natn. Acad. Sci. U.S.A. 56, 296.

- Tomkins, G. M. and Yielding, K. L. (1964).
In Actions of Hormones on Molecular Processes, p.209.
Ed. by Litwack, G. and Kritchevsky, D.
New York: J. Wiley and Sons.
- Tsanev, R. G., Markov, G. G. and Dessev, G. N. (1966).
Biochem. J. 100, 204.
- Turkington, R. W. and Topper, Y. J. (1966).
Endocrinology, 79, 175.
- del Valle, M. R. and Aronson, A. I. (1962).
Biochem. biophys. Res. Commun. 9, 421.
- Walters, E. and McLean, P. (1967). Biochem. J. 105, 615.
- Waymouth, C. (1965). In Cells and Tissues in Culture,
vol. 1, p. 99. Ed. by Willmer, E. N.
New York: Academic Press.
- Weber, G., Singhal, R. L., Stamm, N. B., Lea, M. A. and
Fisher, E. A. (1966). Adv. Enzyme Reguln. 4, 59.
- Widnell, C. C. (1965). Biochem. J. 95, 42P.
- Widnell, C. C. and Tata, J. R. (1964). Biochem. J. 92, 313.
- Wiesner, R., Acs, G., Reich, E. and Shafiq, A. (1965).
J. Cell Biol. 27, 47.
- Wilkinson, B. R. and Kirby, K. S. (1966).
Biochem. J. 99, 780.

- Williams-Ashman, H. G. (1965). In Mechanisms of Hormone Action,
p. 214. Ed. by Karlson, P. New York: Academic Press.
- Willmer, J. S. (1960). Can. J. Biochem. Physiol. 38, 1265.
- Willmer, J. S. and Foster, T. S. (1965).
Can. J. Physiol. Pharmacol. 43, 905.
- Wilson, E. (1967). Nature, Lond., 215, 758.
- Wool, I. G. and Moyer, A. N. (1964).
Biochim. biophys. Acta, 91, 248.
- Yarmolinsky, M. B. and de la Haba, G. L. (1959).
Proc. natn. Acad. Sci. U.S.A. 45, 1721.
- Yoshikawa-Fukada, M., Fukada, T. and Kawade, Y. (1965).
Biochim. biophys. Acta, 103, 383.
- Young, C. W. and Hodas, S. (1964).
Science, N.Y., 146, 1172.