STUDIES ON THE SHORT-TERM EFFECTS OF FEEDING AMINO ACID MIXTURES ON RNA AND PROTEIN METABOLISM IN RAT LIVER

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The investigations of Lagerstedt, Stowell and Stenram indicated that t rat liver nucleolus was extremely sensitive to starvation and to changes in protein content of the diet. One of the features of the nucleolar respons was the rapidity with which the nucleolus enlarged after feeding a high protein diet to a previously starved animal. Stenram demonstrated that th was due to an increase in dry matter and RNA content. Electron microscopic studies of the cytoplasm of rat liver have also shown that there is a rapid regeneration of endoplasmic reticulum and ribosomes when a fasted animal wa fed a high protein diet, and this response was somewhat reduced if the diet had a low protein content.

The use of a tube fed amino acid mixture made it possible to take the study of these events further. Fleck et al. showed that if the amino acid mixture lacked the essential amino acid tryptophan, then the cytoplasmic polyribosomes broke down to smaller, less efficient forms and that this breakdown could be reversed by feeding a complete amino acid mixture. They demonstrated that these cytoplasmic events could not be prevented by pretreatment of the animals with Actinomycin D and thus they must be indepen of the nucleus. Nonetheless the studies of Stenram and others had indicate that feeding induced nucleolar changes, and as feeding stimulates ribosome formation (which is now known to occur in the nucleolus) studies were made o

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nuclear function following the feeding of amino acid mixtures which were either nutritionally complete or which were lacking tryptophan.

The in vivo incorporation of radioactive precursors into subsequently isolated nuclear RNA was examined initially. Preliminary studies were ma on RNA separation by continuous agarase electrophoresis devices and by isokinetic sucrose density gradient analysis. During the course of the latter study a new isokinetic gradient maker was devised. Finally, the investigation was carried out by linear sucrose density gradient analysis a the results indicated that the absence of tryptophan from the amino acid mixture decreased the incorporation of isotope into nuclear RNA within 30 r of feeding. It was not possible to assume that the observed decrease indicated a decreased synthesis of ribosomal RNA because nuclear RNA is knc to consist of two main types - ribosomal type RNA (synthesised in the nucleolus) and heterogeneous RNA (synthesised elsewhere in the nuclear) and while there is considerably more ribosomal RNA present in RNA extracted fro nuclei, it cannot be stated with certainty that any observed changes are du only to changes in ribosomal type RNA. A second objection is that nothing known about any changes induced by diet in the RNA precursor pool size and therefore in any changes which would consequently occur in the precursor po specific activity.

Thus further evidence had to be obtained for the effect of tryptophan omission on ribosomal RNA synthesis. It is thought that the nuclear enzym DNA dependent Mg^{2+} activated RNA polymerase is involved in ribosomal RNA synthesis and it is largely located in the nucleolus. Thus, determination of the activity of this enzyme may indicate differences in ribosomal RNA sy

following the different diets. The assay of the enzyme was by an initial velocity technique which is theoretically more satisfactory than the more commonly used lapsed time method. It was shown that RNA polymerase was stimulated within 5-10 min. of feeding the complete diet, that the stimulation was maximal (twice normal level) one hour after feeding, and tha activity was still raised at two hours after feeding. In contrast the animals fed the tryptophan deficient mixture did not respond until about one hour after feeding and the response was small and present for less than one hour. The characteristics of the enzyme from the fasted animal and the animals fed both diets for one hour were examined for pH and Mg^{2+} optima substrate omissions and Actinomycin D sensitivity, and they appeared to be similar. It was also shown that pre-treatment of the animals with puromycin or cycloheximide lead to a lack of response by the RNA polymerase at 15 min. after feeding the complete diet, while control animals were unaffected. It was concluded that the RNA polymerase response to the diet was due to enzyme synthesis andthat this enzyme differed from the basal enzyme by its sensitiv to puromycin or cycloheximide and by its shorter life. This suggests that ribosomal RNA synthesis may be controlled by the synthesis of a short half-l enzyme which is stimulated by increases in the amino acid supply to the live

Finally, an effort was made to identify a protein synthesised in the li which was affected by feeding the incomplete amino acid mixture. Previousl it has been shown by Fleck et al. that there was a decreased in vitro incorporation of [^{14}C]leucine by microsomes isolated from these animals when compared to the incorporation following a complete amino acid mixture. The

synthesis of serum albumin was studied in slices of liver removed from animals fed the complete or tryptophan deficient diet for one or two hours, using an albumin immuno assay. The incomplete diet appeared to reduce albumin synthesis by the slices in the one hour group only, and thus it cou be concluded that albumin synthesis is sensitive to amino acid supply.

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Abbreviations

Abbreviations used without definition are those recommended by the IUPAC-IUB Combined Commission on Biochemical Nomenclature (Biochem.J., 1966, 101, 1).

Additional abbreviations used in the text are defined as follows:

BSA	Bovine serum albumin
PCA	Perchloric acid
POPOP	1,4-di-(5-phenyloxazolyl))-benzene
PPO	2,5-diphenyloxazole
SDS (SLS)	Sodium dodecyl sulphate (sodium lauryl sulphate)
TCA	Trichloracetic acid

No scientist is admired for failing in the attempt to solve problems that lie beyond his competence. The most he can hope for is the kindly contempt earned by the Utopian politician. If politics is the art of the possible, research is surely the art of the soluble. Both are immensely practical-minded affairs.

P.B. Medawar

Our highest truths are but half-truths,
Think not to settle down for ever in any truth.
Make use of it as a tent in which to pass a Summer's night,
But build no house of it, or it will be your tomb.
When you first have an inkling of its insufficiency
And begin to descry a dim counter-truth looming up beyond,
Then weep not, but give thanks:

A.J. Balfour

Here, work enough to watch
The Master work, and catch
Hints of the proper craft, tricks of the
tool's true play.

R. Browning

1. INTRODUCTION

1.1. HISTORICAL INTRODUCTION

1.1.1. DISCOVERY OF NITROGEN, OXYGEN AND CARBON DIOXIDE

In January 1747 the University of Glasgow appointed William Cullen as the first lecturer in Chemistry. "To William Cullen, Glasgow owes a deep debt of gratitude" (Guthrie, 1950) because he initiated the teaching of Chemistry in Glasgow and he was the founder of the Glasgow Medical School.

Before this appointment Cullen was a successful physician who for a few years practised with William Hunter in Hamilton, Lanarkshire. In 1740 this partnership was amicably dissolved - Hunter going to London and Cullen eventually going, in 1744, to Glasgow. There he started lecturing in Medicine and by 1746 he became convinced of the need for chemistry teaching at the "College" (as the University of Glasgow was and still is known). The circumstances surrounding the establishment of the lectureship are worth re-telling (Guthrie, 1950).

The newly appointed Professor of Oriental Languages, Alexander Dunlop, could not take up his duties immediately and he therefore proposed that the £30 saved by the University from his salary should be applied to equip a Chemical laboratory (Guthrie is tantalisingly vague about the reasons for this request). However the University agreed and added £22 to this sum and the laboratory and lectureship were established.

Cullen explained to his first class:

...if a young man delights to have his hands employed in experiment, Chemistry will furnish him sufficient exercise. If his imagination must be amused with

uncommon and curious appearances, Chemistry will constantly present them to him. If a man aims at gain and the improvement of the useful Arts, it is Chemistry that must feed his hopes and give him assistance. Or if, more liberal still, he aims at the study of causes, Chemistry will gratify him in explaining the most curious phenomena of the natural world (Guthrie, 1950).

Cullen's contribution to Chemistry was immense - not by what he discovered but by his example and philosophy. His advice to his son exemplifies this:

...study your trade eagerly, decline no labour, bear hardship with patience, be obliging to everybody above and below you, and hold your head up in a literal and figurative sense. (Guthrie, 1950).

In 1751 Cullen was promoted to the Chair of Medicine and in 1755 he left Glasgow to go to Edinburgh as the Professor of Chemistry and Physic . Cullen's lectureship in Chemistry was taken by his pupil Joseph Black who was to become "a morning star, a herald of that scientific dawn which ushered in the nine-:teenth century". (Read, 1950).

Black made remarkable contributions to science by his researches on latent heat and specific heat and by his work on "Fixed Air" (carbon dioxide). He set out on the latter project by searching for a more efficacious solvent for urinary calculi than the contemporary alkaline remedies - many of which bore a resemblance to caustic soda (Read, 1950). He attempted to obtain an alkaline solvent of a milder type by preparing basic magnesium carbonate (magnesia alba). This substance effervesced with acids and changed by ignitio into a white powder devoid of this property losing 7/12 of its weight in the ignition. Black showed that the property of effervescing with acids could also be restored to the white powder. He therefore concluded that magnesia alba is a compound of a peculiar earth and "fixed air".

His thesis for the Edinburgh M.D. contained the results of his observation on magnesia alba and it has been remarked that "there is, perhaps, no other

instance of a graduation thesis so weighted with significant novelty" (Dictionary of National Biography, 1886).

It was this work, carried out not a hundred yards from where I am writing this, which laid the foundations of Chemistry and eventually Biochemistry.

Black recognised the biological importance of "fixed air":

...I had discovered that this particular kind of air, attracted by alkaline substances, is deadly to all animals that breathe it by mouth (quoted by Robinson, 1803).

Rutherford (1772), a pupil of Black, who was then at the University of Edinburgh recognised that when fixed air was removed from air (which an animal had breathed until it died) the residual gas was still incapable of supporting respiration. This gas he called "aer malignus" and it is now thought that Rutherford was the first investigator to recognise nitrogen as an independent substance (McKie 1934). Nitrogen was also called phlogisticated air (Priestly) or vitiated air (Scheele). When chemical nomenclature was revised by the French School (1787) the inability of this air to support life was recognised by the name "azote".

In 1790 Lavoisier described experiments on the respiration of human subjects. He determined the quantity of O_2 absorbed by a resting man and he found that the O_2 consumption increased by 15% on reducing the environmental temperature from 26°C to 12°C. He also discovered the effect of work and food (specific dynamic action) on oxygen consumption and carbon dioxide production. Lavoisier's values are very close to presently accepted values for these various conditions (Lusk, 1922).

Lusk (1922) quotes Lavoisier:

...in the present advanced state of chemistry very expensive and complicated

instruments are becoming indispensably necessary for ascertaining the analysis and synthesis of bodies with the requisite precision as to quantity and proportion...

Lavoisier's instruments were clearly not simple - he possessed a balance which could weigh 600g. to within 5mg. and another which was sensitive to 0.1mg.

Lusk (p.29) quotes Lagrange, on Lavoisier's death, as saying:

...it took but an instant to cut off his head; a hundred years will not suffice to produce one like it...

1.1.2. MAGENDIE: IMPORTANCE OF NITROGENOUS COMPOUNDS

Magendie (1783-1855) recognised that foodstuffs containing nitrogen have important nutritional properties. Thus he showed that feeding a dog with sugar or fat only, resulted in death within a few weeks. He concluded that a nitrogen source was a nutritional essential.

In his textbook (1829) the distinction is drawn between azotised principle (albumin, gelatine, mucus, casein, etc.) and non-azotised principles such as sugar of milk, sugar of diabetic urine, lactate, etc. Later in his book (p.470) he states:

...Since chemical analysis has made known the nature of the different tissues of the animal economy, they have been all found to contain a considerable portion of azote. Our foodbeing also partly composed of this simple body, the azote of our organs likewise probably comes from them...

He also observed that dogs fed exclusively on cheese or eggs survived indefinitely although they were weak. His conclusion is "these facts make it very probable that the azote of the organs is produced by the food".

His views on the dynamic aspects of tissue structure are interesting (p.18

...daily observation teaches, that the organs of man, as well as those of all living beings, lose at each instant a certain quantity of that matter which composes them; nay, it is on the necessity of repairing these habitual losses

that the want of aliment is founded...we justly conclude that living bodies are by no means always composed of the same matter at every period of their existence.

Later on (p.418) he writes:

...Nutrition is more or less rapid according to the tissues. The glands, the muscles, skin, etc. change their volume, colour, consistence, with great quickness; the tendons, fibrous membranes, the bones, the cartilages, appear to have a much slower nutrition, for their physical properties change but slowly by the effect of age and disease.

1.1.3. IDENTIFICATION OF THE PROTEINS AS A CHEMICAL CLASS

The chemical analysis on which Magendie's workwas based was performed by Chevreul - a member of the great school of French chemistry. This school trained Justus von Liebig (in Gay-Lussac's laboratory) during 1823-24. Liebig then returned to Giessen where he created a vigorous school of organic chemistry. His study of organic chemistry naturally led into physiology:

...My object has been to direct attention to the points of intersection of chemistry with physiology, and to point out those parts in which the sciences become, as it were, mixed up together... In the hands of the physiologist organic chemistry must become an intellectual instrument, by means of which he will be enabled to trace the causes of phenomena invisible to the bodily sight. (Liebig 1842).

In his book on Animal Chemistry (1842) he stated his views on protein metabolism. He wrote (p.40):

...if we hold, that increase of mass in the animal body, the development of its organs, and the supply of waste, - that all this is dependent on the blood, that is, on the ingredients of the blood, then only those substances can properly be called nutrition or considered as food which are capable of conversion into blood. To determine therefore, what substances are capable of affording nourishment, it is only necessary to ascertain the composition of the food, and to compare it with that of the ingredients of the blood.

Two substances require special consideration as the chief ingredients of the blood; one of these separates immediately from the blood when withdrawn fro the circulation. It is well known that in this case blood coagulates, and separates into a yellowish liquid, the serum of the blood, and a gelatinous mas

which adheres to a rod or stick in soft elastic fibres, when coagulating blood is briskly stirred. This is the fibrine of the blood which is identical in all its properties with muscular fibre when the latter is purified from all foreign matters. The second principle ingredient of the blood is contained in the serum, and gives to this liquid all the properties of the white of eggs with which it is identical. When heated, it coagulates into a white elastic mass, and the coagulating substance is called albumin. Fibrine and albumin, the chief ingredients of blood, contain, in all seven chemical elements, among which nitrogen, phosphorus, and sulphur are found... Chemical analysis has led to the remarkable result, that fibrine and albumin contain the same organic elements united in the same proportion.

Liebig here reports the views of the Dutch chemist Mulder (1802-1880) who in 1838 coined the name "Protein" for a basic nitrogenous component present in nitrogen rich organic compounds. Mulder had examined fibrine, egg albumin and other proteins and he found the elements N,C,H,O,S and P to be present. He obtained the best empirical formula for protein $C_{48}H_{36}N_6O_{14}$ after finding that the relative proportions of H,O,C and N were constant and that they constituted the nucleus $\overline{Pr}$ of the protein. He found that P and S varied in the different proteins and he thus described albumin as $\overline{Pr}$ + P + S and fibrine as $\overline{Pr}$ + P + 2S. Mulder used this formulation of protein structure to answer the problem of how herbivora could increase in flesh without consuming meat in their food (Beach 1948).

However Liebig changed his opinion because in the five years between the English edition of "Animal Chemistry" (1842) and the English edition of "Chemistry of Food" (1847) much evidence had shown that Mulder's view was incorrect. Liebig (1847) wrote:

...the study of the products, which casein yields when acted on by concentrated hydrochloric acid, of which, as Bopp has found, tyrosine and leucine constitute the chief part, and the accurate determination of the products which the blood constituents, casein, and gelatine, yield when oxidised, among which the most remarkable are oil of bitter almonds, butyric acid, butyric aldehyde, valeronic acid, valeronitrile, and valeracetonitrile, have opened up a new and fertile

field of research into numberless relations of the food to the digestive processs and into the action of remedies in morbid conditions...

Liebig's views on protein metabolism seem almost less adventurous than those of Magendie. Liebig wrote (1842):

...the substances of which the food of man is composed may be divided into two classes: into nitrogenised and non-nitrogenised. The former are capable of conversion into blood: the latter incapable of this transformation. Out of those substances which are adapted to the formations of blood are formed all the organised tissues. The other class of substances, in the normal state of health, serve to support the process of respiration.

Munro (1964) remarks that although Liebig contributed little of value to the study of protein metabolism, he was responsible, by his application of the techniques of organic analysis to biological compounds, for many of the future developments in protein metabolism.

In "Chemistry of Food" (1847) p.10 Liebig wrote:

...the intermediary members of the almost infinite series of compounds which must connect urea and uric acid with the constituents of the food, are, with the exception of a few products derived from the bile, almost entirely unknown to us; and yet each individual member of this series, considered by itself, in as much as itsubserves certain vital purposes, must be of the utmost importance in regard to the explanation of the vital process or of the action of remedies.

In 1852 Liebig moved to Munich where he became Professor of Chemistry. In one of his classes was the young Carl Voit.

1.1.4. THE ERA OF CARL VOIT (1831-1908)

Carl Voit graduated in Medicine in Munich in 1854 and then:

...in order to prepare himself for a scientific career, he devoted the following year to attending lectures in physics, zoology, anatomy and chemistry... (Lusk, 1922, p.66).

In the laboratory of practical chemistry the teacher was Pettenkofer. With Pettenkofer Voit studied the urea output in cholera patients. This was the first project in a fruitful association between these men. After this period

Voit studied in Wohler's laboratory in Gottingen for a year then returned to Munich to work with Bischoff, who was the Professor of Anatomy and Physiology in the University of Munich.

In 1860 Voit and Bischoff published their findings of metabolic experiments with dogs. They had fed dogs with increasing amounts of lean meat and the urinary nitrogen output had been estimated. They reported (Lusk 1922, p.70):

> ...if we increase the food protein...the metabolism is constantly increased until we reach a point when loss from the body is equal to its repair. This is the moment when the metabolism of the protein parts of the organism has so increased as to acquire all the oxygen available, and the metabolism of fat ceases. If the amount of food be still further increased the metabolism scarcely increases, for the available oxygen, through union with metabolic products, has been reduced to a minimum. This is the moment when deposit, increase in mass, excess for reparation must and can ensue...sugar reduces the protein metabolism in the organs of the body and reduces the quantity of protein in the food needed for replacement purposes, and possesses these influences even more than fat, probably because it has a greater affinity for oxygen than either fat or baby fat.
> ...it is established for all time and is and must be correct that the nitrogen containing materials are the sources of physical powers...also it is equally incontrovertible that fat and carbohydrates can yield only heat and never motion.

Voit clearly echoes Liebig's (1842) view of protein metabolism. Later however, he produced evidence that muscular work did not increase the protein metabolism of a fasting dog or of one fed with meat. The tones of certainty are interesting. As he grew older Voit interpreted his experimental observations much more carefully and Lusk tells of workers in Voit's laboratory being warned to take care with their discussions of experiments as the "Chief" would not accept facile interpretations.

With Pettenkofer (who built a respiration apparatus) Voit determined the metabolic substrates utilised by a fasting man. It is of interest to note that this respiratory chamber was checked by burning candles. The carbon dioxide

production of the burning candles was measurable to 1% (Lusk 1922). Using this apparatus they demonstrated that (Lusk p.29):

...the quantity of oxygen needed in metabolism depends upon the chemical composition of the material that burns in the organism, and also the relation between the amount of oxygen absorbed and carbon dioxide excreted depends upon the same factor...

The technique of nitrogen balance used, but not originated by Voit, was exploited by his many pupils who came from all over the world. They included Lusk, Yandell Henderson and Attwater (USA), Rubner (Germany) and Cathcart (Britain).

1.1.5. NUTRITIONAL STUDIES SINCE THE TIME OF VOIT

1.1.5.1. RECOGNITION OF DIFFERENCES IN PROTEIN QUALITY

Munro (1964) in describing the early history of this subject observes that the one protein known to be nutritionally unsatisfactory from the earliest studies of dietary protein was gelatine. The Commission de la Gelatine (1841), of which Magendie was a member, reported unfavourably on its nutritive qualities.

In 1842 Liebig wrote:

...animals which were fed exclusively with gelatine, the most highly nitrogenised element of the food of carnivora died with the symptoms of starvation, in short the gelatinous tissues are incapable of conversion into blood...

Munro (1964) reports Bischoff and Voit (1860) observing that nitrogen equilibrium could not be attained by dogs fed on gelatine as the sole dietary protein.

As Rose (1938, p.110) wrote:

...although 13 amino acids had been discovered prior to 1900 very meagre information existed as to their quantitative distribution. Consequently there

prevailed little appreciation of the fact that the nutritive value of a protein depends upon the nature of the constituents. Thus there was undue emphasis on the quantity rather than quality of protein.

With the advent of the Kossel and Kutscher (1900) procedure for the isolation of the diamino acids, and of the Fischer (1901) ester method for the separation of the monoamino acids, reasonable tools were available for determining protein composition. Using these and other methods it soon became apparent that there were wide variations in the kinds and amounts of amino acids in protein. Thus gliadin was shown to be lysine deficient (Osborne and Guest, 1911), zein to be deficient in lysine and tryptophan (Osborne and Clapp, 1907) and gelatine almost entirely devoid of cysteine, valine, isoleucine, tyrosine and tryptophan (Dakin 1920). Osborne and Mendel (1914) could state:

...the current trend of the investigation of the chemistry of nutrition is emphasizing the significance of the amino acids as the fundamental factors in all problems in which hitherto the role of proteins has been involved...

It is of interest to examine the three methods of investigating amino acid requirements from 1900. They were:

1) Use of a purified protein known to be lacking amino acids.
2) Use of hydrolysed proteins from which amino acids had been removed.
3) Use of amino acid mixtures.

Using Method 1, Willock and Hopkins (1906) found that:

...a dietary containing zein as its only nitrogenous source is unable to maintain growth of young mice. The addition of tryptophane (an amino acid absent from the decomposition products of zein) to such a dietary does not make it capable of maintaining growth. On the other hand, this addition greatly prolongs the survival of animals fed upon zein, and materially adds to the well-being of such animals. The addition of tyrosine (which is already present in zein), in equivalent amounts, has not such effect. It is suggested that the tryptophane is directly utilised as the normal precursor of some specific hormone or other substance essential to the processes of the body...

It is interesting to note that before this work had been done and following the discovery of tryptophan by Hopkins and Cole (1901) Osborne and Harris (1903) applied the tryptophan colour reaction used by Hopkins (the Adamkiewicz test) to 35 proteins prepared by them. They were able to arrange these proteins in the order of intensity of the Adamkiewicz reaction and thus illustrate the diversity of proteins with respect to their tryptophan content.

Later Osborne and Mendel (1914) showed that zein lacked both tryptophan and lysine, and that the addition of these amino acids converted zein into a protein capable of sustaining the growth of young animals. They also drew a distinction between growth and maintenance by diet:

"growth sets a standard decidedly higher than that of maintenance"

Using method 2 St. Julian and Rose (1932) removed proline as completely as possible from hydrolysed proteins by forty extractions with hot absolute alcohol without preventing the growth-promoting value of the resulting material.

Rose concluded that three decades of endeavour using methods 1 and 2 could only show, with reasonable certainty, that tryptophan, lysine and histidine were indispensable.

Abderhalden had used Method 3 as early as 1912 using dogs as the experimental animal. He employed a diet of 16 amino acids but the dogs ex-:perienced vomiting, diarrhoea and anorexia. Hopkins (1916) found that rats which received cystine, tyrosine, lysine, tryptophan and histidine as the only sources of nitrogen manifested a "remarkably slow loss of weight and long maintenance of apparent health".

However it was not until 1930 that Rose carried out his extensive work on diets composed of purified amino acids. The first amino acid mixture was formulated from the composition of casein as it was known at that time. 19 amino acids were used and the mixture composed 20% of the diet which included carbohydrate fats, salts and vitamins. Rose (1931) found that this diet was totally incapable of meeting the demands of growth as well as causing a loss of appetite in the rats. He concluded that:

...experience...had taught us to expect a marked failure of appetite when the diet is completely devoid of an essential component.

The loss of appetite observed by Rose had also been noted by Aderhalden 20 years before. It is a mark of Rose's capability that he used this observation to advantage. Rose began to look for the missing component. He hydrolysed large quantities of casein and then separated the amino acids into groups, one of which contained the monoamino acids. Each of the fractions were added to the amino acid diet and it was found that the monoamino acid fraction manifested more activity than did whole casein supplements. Fractionation of the active fraction resulted in the isolation of threonine (Rose et al. 1935). Rose stated:

...this is in accordance with our conviction that growing animals lose the desire to eat when the food is not suitable for tissue synthesis.

Using pure threonine and the other 19 amino acids, Rose was able, by deletion of single amino acids, to determine that tryptophan, lysine, histidine, phenylalanine, leucine, isoleucine, threonine, methionine, valine and arginine were essential as indispensable amino acids for the rat. Rose defined an "essential amino acid" as one which is not synthesised by the animal organism out of the materials ordinarily available at a speed commensurate with normal growth.

The case of arginine illustrates the importance of carefully defining the concept of an essential amino acid. Scull and Rose (1930) showed that young rats grew on an arginine-free diet but that a growth response could be obtained by the addition of arginine (Borman et al. 1930). Thus synthesis of arginine is possible in the rat but the rate of synthesis is clearly limiting the rat's growth.

At the time Osborne was first investigating the amino acid content of proteins, Carl Thomas (1909) was developing an alternative technique for assessing the differences in protein quality. Thomas measured the "Biological Values" of proteins by nitrogen balance techniques. This method in the hands of Mitchell and his collaborators has been shown to be a precise method for evaluating protein quality. What is the relationship between the Biological Value (B.V.) and the chemical composition of the protein? In terms of Rose's work the Biological Value can be seen as a quantitative measure of the ability of a protein to fulfil the requirement for essential amino acids by an animal. It is known that the protein of whole egg has a B.V. = 100. By comparing the amino acid content of whole egg protein with the content of other proteins it is possible to construct a table of %age deficiency of the limiting essential amino acid for each protein. Black and Mitchell (1946) showed that there is a close relationship between the calculated deficit (Chemical Score) and the Biological Value.

Surprisingly a total lack of an essential amino acid produces an apparent B.V. of 30-40%. The basic assumption in this method is that the egg protein amino acid content is exactly equivalent to the animal's requirement for these amino acids.

1.1.5.2. GENERAL THEORIES OF PROTEIN METABOLISM

It was mentioned earlier that Liebig considered dietary protein as being transferred to blood and tissue protein which was then utilised as muscular fuel. The dietary non-nitrogenous constituents were thought to be the source of body heat.

This view of protein metabolism was shown to be untenable when several workers including Pettenkofer and Voit, showed that exercise was not associated with a significant increase in urea output. Voit (1867) proposed, on the basis of his experimental work, that there is a variable pool of labile protein in the body which is distinct from tissue protein. The amount of labile protein in the body is related to the level of dietary protein, and it is readily catabolised whereas tissue protein is not. Finally the tissue protein is slowly renewed from material drawn from labile protein.

Folin (1905) went further. He investigated the effect of nitrogen-rich and nitrogen-poor diets on the urinary composition. On a nitrogen-poor diet the urine contained creatinine, neutral sulphur, uric acid and ethereal sulphates. As the protein content of the diet increased so did the excretion of inorganic sulphate and urea. He therefore argued that protein metabolism could be divided into a constant tissue metabolism (endogenous metabolism - the urinary excretion on nitrogen poor diets) and a variable protein metabolism (exogenous metabolism the urinary excretion on nitrogen rich diets). Folin wrote:

...the two forms of protein catabolism are essentially independent and quite different...

Folin's concept of independent endogenous and exogenous metabolisms has received much criticism. Thus Borsook and Keighley (1935) concluded from studies on nitrogen and sulphur excretion:

...it is an open question whether the endogenous metabolism yielding urea, ammonia, and part of possibly all the uric acid has any physiological reality.

In 1942 Schoenheimer's book was published in which experiments with [^{15}N] amino acids fed to rats were described:

...According to the concept of independent exogenous and endogenous types of metabolism, most of the dietary nitrogen should have appeared directly in the urine. This was not the case. With leucine less than one third, with glycine less than one half was excreted: the balance remained in the body. Of the isotopic nitrogen retained, the non-protein nitrogen fraction contained only a small amount. The proteins must, therefore, have been involved in very rapid chemical reactions resulting in the fixation of at least half of the nitrogen of the added amino acids. As the weight of the animals had remained constant, the processes in question must have been so balanced as to avoid ultimate change in the amounts of the proteins. Different organs are not equally effective in the fixation of dietary nitrogen...The proteins of the internal organs, of serum, and of the intestinal tract are the most active; the proteins of muscles show less activity, but, as they constitute by far the largest part of the animal, the low concentration actually represents a high absolute amount of isotope. In fact, two thirds of the nitrogen deposited by the animal was recovered in the muscles, and only one third in the combined internal organs. As might be expected, the proteins of the skin show the least activity.

Mitchell (1962) remarks:

...but as far as end results are concerned, these isotope studies have not changed the Folin conception of the exogenous metabolism in the slightest. These reversible reactions revealed by isotope tracer studies between tissue and dietary constituents are not anarchistic in nature...

Moss and Schoenheimer (1940) comment thus:

...they seem to represent automatic and non-interruptable biochemical processes of synthesis as well as degradation, which are balanced by an unknown regulatory mechanism so that the total amount of the body material and its composition do not change.

...Hence (Mitchell concludes) for all practical purposes in the assessment of protein requirements and the utilisation of dietary protein, the tissue proteins may be considered to be in a static condition... These isotope studies of the intermediary protein metabolism render the term exogenous metabolism somewhat though not completely inappropriate. It is still the body's method of ridding itself of nitrogen consumed in amounts exceeding its current needs for endogenous replacement, growth and other purposes for which dietary protein is used by the body - nitrogen being the one element in the protein molecule that it cannot oxidise.

1.1.5.3. INTERMEDIATE STEPS IN PROTEIN METABOLISM

...the true starting point for all the tissues is consequently, albumin; all nitrogenised articles of food, whether derived from the animal or from the vegetable kingdom, are converted into albumin before they can take part in the process of nutrition. All the food consumed by an animal becomes in the stomach soluble, and capable of entering into the circulation...(Liebig, 1842, p.108).

Thus in Liebig's time the processes of digestion and protein synthesis were not clearly distinguished. The subsequent formulation of our basic ideas of digestion and absorption started with the discovery by Kuhne (1867) of the action of trypsin and ended with the demonstration by Van Slyke and Meyer (1912) that the concentration of free amino acids in the blood rises after a protein meal. The history of this era has been described in detail by Cathcart (1921) who made several contributions to this subject.

The second step in Liebig's scheme of protein metabolism "...converted into albumin..." proved a far harder problem. Even in 1953 Borsook could write:

...turning now to the chemical mechanisms of amino acid incorporation into proteins and of protein synthesis, it must be said at once that nothing specific is known of the processes involved. On the other hand, there is now a considerable body of peripheral information which defines the problem or problems...

However 15 years before Borsook wrote these words two investigators made important cytochemical observations which immensely influenced thought concerning protein biosynthesis. Caspersson (1939), using an ultra-violet micro-spectrophotometric technique, showed that the cytoplasm and nucleoli of cells possessed a strong U.V. absorption associated with Feulgen-negative areas thus suggesting that the absorbing material was RNA. Brachet (1942) using a histochemical technique (methyl green and pyronine strain) together with purified ribonuclease (which had just become available) showed that RNA is abundant in cells which synthesise large amounts of protein.

In addition Caspersson suggested a general theory of protein synthesis which involved nucleic acids. He proposed, on the basis of his observations, that the cellular heterochromatin, especially the nucleolar associated chromatin, controls the synthesis of histone-like proteins. From the nucleolus these basic proteins pass across the nuclear membrane and induce in the peri-nuclear cytoplasm an intensive production of RNA. The RNA in turn produces synthesis of cytoplasmic proteins (reviewed by Caspersson 1950). Clearly this theory has had to be modified but it does appear to have exerted a considerable influence on subsequent work.

Caspersson made another notable observation which has stimulated much nutritional research (reviewed in Section 1.2.).

...the increase in nucleolar masses is a most conspicuous phenomenon during cytoplasmic protein synthesis. During periods of intense growth, the nucleolus increases sometimes enormously... (Caspersson, 1950, p.106).

1.2. GENERAL INTRODUCTION

1.2.1. THE NUCLEOLAR RESPONSE TO DIET IN RAT LIVER: THE WORK OF LAGERSTEDT AND STENRAM

Following the observations of Caspersson on the prominent nucleolus found in cells actively synthesising protein, Lagerstedt in Lund, Sweden, commenced an investigation of the effects of starvation, and high and low protein diets on the size of the nucleolus.

He used 170-240g. male rats as experimental material and 4 groups of animals were treated as follows:

1) rats starved for 5 days (water ad libitum)

2) rats kept on a low protein diet (4%-brewer's yeast protein) for up to 18 days

3) starved rats (group 1) given a high protein diet (22%-casein and brewer's yeast protein) or a low protein diet

4) rats given a low protein diet (group 2) then given a high protein diet

All groups (except group 1) were supplied with adequate vitamins, minerals and carbohydrates.

In group 1 the nucleolar size decreased sharply (-50%) within 24 hours of starvation commencing and thereafter further starvation did not significantly decrease the nucleolar size. The basophilia of the cytoplasm was observed to decrease more slowly than the nucleolar changes. In group 2 the nucleolar changes were similar but the cytoplasmic basophilia disappeared more slowly than in the animals of group 1. In groups 3 and 4 the feeding of a high protein diet led to a very rapid increase (+100%) in the nucleolar size within 3 hours of feeding. Within 6 hours of feeding the basophilia of the cytoplasm increased around the nuclear membrane, and by 12 to 24 hours the cytoplasmic basophilia was observed to fill out the entire cytoplasm. Lagerstedt (1949) comments:

...the present investigations show that the nucleic acid containing proteins in the cytoplasm of the liver cell are built up closely to the nuclear membrane under the co-operation of the nucleolar apparatus and the nucleus. Thus the morphological features of these structures in the liver cell signify directly the intensity of the protein metabolism of the liver cell.

In the same year Stowell (1949), working in Caspersson's laboratory in Stockholm, published a short account of less extensive observations of the effect of high and low protein diets on nucleolar size. He used 200-300g. rats (sex not reported), a low-protein diet of 5% brewer's yeast (protein content not stated) and a high protein diet consisting of 5% brewer's yeast + 30% casein. Stowell reported (p.127):

...The size of individual nucleoli and of total nucleolar mass per nuclear section increased about three times during protein depletion and returned toward normal after a week on a high protein diet... The nucleolus comprised 0.4-0.7% of the nuclear volume in normal cells. After 21, 35, 54, and 92 days protein depletion, the nucleolar volume showed a definite increase by changing to 0.4, 0.7, 1.0 and 1.2% respectively. After $2\frac{1}{2}$, $4\frac{1}{2}$ and 8 days protein repletion, the nucleoli comprised 0.9, 0.5 and 0.5% of the nuclear volume.

Because of the discrepancy between Lagerstedt's and Stowell's results on the effects of the high and low.protein diets on nucleolar size, Stenram, then at Lund University, re-investigated the problem. He used 36g. male and female rats and he found that animals fed for 10 days with 0% or 3% casein diet had larger nucleoli than the animals fed the high protein diet of 25% casein for a similar period. All the diets used had adequate vitamins, minerals and carbohydrate (Stenram 1953). It is quite clear that this finding further confuses the effect of diet on nucleolar size and in a later paper Stenram (1956a) re-investigated the whole phenomenon very thoroughly indeed. He argued that the low protein diets used by Lagerstedt (1949), Stowell (1949) and Stenram (1953) were below the maintenance level for young rats and that this diet was likely to produce anomalo effects, which could be due to variations in the total caloric and vitamin conten of the diet, the dietary regimen prior to the experiment, the age and sex of the animal and the length of time the diet had been administered.

Stenram therefore omitted the low-protein diet completely and fed his animals on either a 25% casein diet or a non-protein diet with, in both cases, an adequate vitamin, mineral and carbohydrate content. Male 175g. rats were used, and they were starved for 5 days before commencing the experiment. Animals were killed at 6 hours, 3, 18 and 44 days while on the diets. He showed that the nucleoli of starved rats were smaller than normal and that feeding the high protein diet led to an increase in nucleolar size by 6 hours which increased until 3 days and by 18 days decreased to the 6-hour size. In the group fed on the

non-protein diet there were no changes, compared with the starved animals, at 6 hours but by 3 days onward the nucleolar size was larger than the starved animal group or any of the casein fed groups.

Stenram concluded (p.360):

...it appears... that...in agreement with Caspersson (1950), the nucleolar size.. of the starved and protein fed animals closely reflects the intensity of the presumed nucleoprotein synthesis of the cell.

He further investigated the nucleolar enlargement in the protein free group (1956b). He used similar rats starved for 5 days in the following groups:

1) 25% casein diet

2) Protein-free diet

3) Essential amino acid mixture optimal for rat growth

4-12) Essential amino acid mixture as above with one of the amino acids omitted.

All the diets contained adequate amounts of vitamins, minerals and carbo-:hydrate and they were fed for 3 days.

He found that the feeding of histidine, leucine or phenylalanine deficient amino acid mixtures did not produce differences in nucleolar size when compared to the groups fed casein or the complete amino acid mixture. However the groups fed amino acid mixtures deficient in lysine, isoleucine, methionine, threonine, tryptophan, valine, or all the essential amino acids had significantly larger nucleoli than in the casein fed group.

Stenram quotes other workers in support of his findings. Spector (1948) and Adamstone and Spector (1950) found that after two days of force-feeding a tryptophan-deficient casein diet, a single huge nucleolus developed in liver cells Dick et al.(1952) found that in threonine-deficient rats there tended to be one prominent nucleolus instead of several nucleoli and similar changes have been

found in lysine-deficient rats.

Thus by 1956 it was known that morphologically the nucleolus responded to starvation, high protein diets and to the absence of certain essential amino acids and that the cytoplasm responded to starvation and high and low protein diets.

Some of the biochemical aspects of this response have been described by Munro and his colleagues working in Glasgow.

1.2.2. THE RESPONSE OF RNA METABOLISM IN RAT LIVER TO DIET: THE WORK OF MUNRO

In section 1.2.1. the loss of liver cell cytoplasmic basophilia following the feeding of a protein deficient diet was described (Lagerstedt, 1949). About the same time Kosterlitz and his colleague, working in Aberdeen, described the chemical sequelae of a protein-deficient diet. They showed that the rat liver cell rapidly loses a part of its protein, RNA and phospholipid (Kosterlitz, 1947; Campbell and Kosterlitz, 1948 and 1952). Munro carried the observations on RNA metabolism further. He and his co-workers showed that when rats are transferred from a normal diet to a regimen free from protein the amount of RNA in the liver falls very rapidly during the first day but levels off around the third day onwards (Munro, Naismith and Wikramanayake, 1953). The uptake of [^{32}P]orthophosphate by liver RNA is considerably depressed in these animals du[^{32}P]orthophosphate of the protein-free diet and this has been explained by an increased pool of free nucleotides, created by the increase in RNA breakdown, diluting the pool of [^{32}P]nucleotides and thus reducing the incorporation of [^{32}P]orthophosphate into RNA (Munro *et al.* 1953). The feeding of protein to thes protein-depleted animals was found to decrease the acid-soluble pool of

nucleotides presumably because the increased RNA synthesis which occurred reduced the pool size. This interpretation was suggested by the marked rise in [^{32}P]orthophosphate uptake by RNA following the protein feed.

It is well known, since the studies of Berg (1914), that the amount of basophilia in the rat liver cytoplasm is diminished by starvation. This phenomenon has been extensively studied by Lagerstedt (1949) and the electron microscopic studies of Fawcett (1955) and Berhard & Rouiller (1953) have shown that the microsomes are the source of this "labile" RNA.

On feeding these starved animals with a protein rich meal there is a very rapid regeneration of the endoplasmic reticulum which is less marked if a low protein meal is administered (Fawcett, 1955).

Munro argued that the high-protein diet increased the microsome content of the cytoplasm because the diet was supplying all the amino acids necessary for protein synthesis to occur and this was the stimulus to maintain endoplasmic reticulum (ER) formation. If an essential amino acid was omitted from the diet then ER formation should be decreased. Munro and Clark (1959) showed that the uptake of [^{14}C]orotic acid and [^{14}C]glycine into rat liver RNA was decreased within the period of 1½–6hr. after feeding an amino acid mixture lacking tryptophan when compared with the corresponding uptake following the feeding of the complete amino acid mixture.

The amino acid mixture used in this work was Mixture 23 (Rose et al., 1948) which was known to be nutritionally adequate for the growing rat. The rats used were male 180–200g. albinos, maintained on a protein-free diet for the 5 days preceding the experiment.

This problem was laid aside in Glasgow for some 5 years. During this

interval great advances were made in our knowledge of the functionally active ribosome. Warner, Rich and Hall (1962) first showed, by electron microscopy that ribosomes appeared to clump together in a particular manner. They suggested that groups of ribosomes were linearly aligned along a strand of messenger RNA and one of their micrographs showed three ribosomes "joined" by a slender thread (10-15A thick). This observation was taken very much further by Hans Noll's group, then at Pittsburgh, who wrote, in 1963:

...We visualise the ergosome (later called the polysome) as a roughly spherical or dough-nut shape particle with a sedimentation constant of about 200S corresponding to a molecular weight of about 20 million. It is composed of five 73S particles whose 30S sub-units, oriented toward the centre of the aggregate, are adjacent to each other. The five 73S mononers are held together by one messenger - RNA molecule which may be pictured as running through the grove between the 30S and 50S sub-units. The spaces between the 73S monomers expose single stranded regions which are extremely sensitive to enzymatic cleavage. Single breaks in these regions result in a stepwise fragmentation of the ergosome into smaller oligosomes with a final liberation of free 73S particles. (Wettstein et al. 1963).

In 1965 Fleck, Shephard and Munro demonstrated that polysomes isolated from the liver of animals fed the incomplete amino acid mixture 1 hour before killing had an increased proportion of monosomes and disomes when compared with the animals fed the complete amino acid mixture. The rats were 150g. male albinos, starved overnight before feeding. The amino acid mixtures were similar to those used by Munro and Clark (1959). Polysomes were isolated by the method of Wettstein et al. (1963). It was also shown that microsomes isolated from the liver of animals fed the incomplete mixture showed a reduced capacity to incorporate [^{14}C]leucine in vitro. These findings were interpreted as indicating synthesis of new mRNA but pre-treating the rats with doses of Actinomycin D (75mg/100g. body-weight for 1.5hr. before feeding) known to be effective in preventing synthesis of RNA (Drysdale and Munro, 1965), did not prevent either th

decreased amino acid incorporation by microsomes *in vitro* or the polysome profile change. Thus they concluded that the response to amino acids does not require the formation of new mRNA.

Wunner, Bell and Munro (1966) showed that the tryptophan-deficient mixture produced an effect which lasted up to 7 hours and that feeding the complete mixture to an animal previously fed the incomplete mixture resulted in the polysome pattern returning to normal within 2 hours. They also showed that both polysome and oligosome fractions isolated from both groups of animals incorporated DL-[1-^{14}C]leucine and L-[Me-^{14}C]tryptophan *in vitro* in the molar ratio 4.5 : 1 which approximates to the relative requirements of these two amino acids by the rat (Hegsted, 1944), although the incorporation was less in the incomplete mixture. This finding suggests that both polysome and oligosome fractions were synthesising protein *in vitro* containing average proportions of these two amino acids.

The rats used in this study were 150g. male albinos starved overnight before feeding. The amino acid mixtures used were modelled on egg albumin which is known to have a Biological Value of 97 (Allison, 1914) in growing rats.

1.2.3. INTRODUCTION TO THE PRESENT WORK

The nucleolor response to diet described in Section 1.2.1. was observed several years before any firm biochemical knowledge had been obtained about the function of the nucleolus. Between 1955 and 1961 several investigators reported experiments which suggested that cytoplasmic RNA was synthesised in the nuclear region of the cell e.g. Goldstein and Micou (1959).

In 1961 Perry and his co-workers (Perry, Hell and Errera, 1961) reported experimental results which located the nucleolus as the site of synthesis of at

least 2/3 of the cytoplasmic RNA of the HeLa cells, and which suggested that the extra-nucleolar nuclear space was the site of synthesis of the remainder. These results were obtained by U.V. microbeam irradiation of the nucleolus together with auto-radiography and they can be considered as the mammalian cell equivalent to Brachet's enucleation studies on Amoeba proteus in which it was shown that there is a steady and marked decrease in the cytoplasmic RNA content following enucleation (Brachet, 1955).

Within the next few years it became clear that RNA was synthesised in the nucleolar space and by 1965 at an International Symposium on The Nucleolus two groups were able to give their scheme of synthesis for the ribosomal RNA sub-units Thus Penman et al. (1965) described the synthesis of a 45S RNA molecule in the HeLa cell nucleolus, followed by cleavage to a 16s RNA which emerges immediately into the cytoplasm, and a 35S RNA which is further broken down, in the nucleolus, to a 28S RNA. Busch et al. (1965) described a similar pathway for rat liver rRNA synthesis.

It therefore seemed likely that the enlarged nucleoli occurring in protein-deficiency or single essential amino acid deficiencies (described in Section 1.2.1.) could be due to the accumulation of precursor ribosomal molecules and RNA in the nucleolus.

Stenram was able to show:

1) that the increased nucleolar size was due to an increase in the dry matter and in the RNA content of the nucleoli (Stenram, 1958).
2) that the increased nucleolar size was accompanied by an increased rate of RNA synthesis estimated autoradiographically (Stenram, 1962).

In contrast to these observations of nucleolar enlargement following dietary protein or essential amino acid deficiences are the findings of Fleck et al. (1965) (described in Section 1.2.2.) on the effect of tryptophan deficiency on the cytoplasmic polysome pattern. It will be recalled that these authors showed that Actinomycin D did not alter the polysome response. Thus the nucleolus does not seem to be involved in this particular dietary response although the timely warning of Harris (1968) on the misinterpretation of Actimomycin D experiments should be borne in mind.

However Stenram was able to observe significant morphological changes in the rat liver nucleolus within 6 hours of feeding a high protein diet and as the amino acid mixture used by Fleck et al. (1965) and Wunner et al. (1966) were equivalent to such a diet it was decided to study the biochemical features of the immediate nuclear (and nucleolar) response to amino acid feeding.

2. MATERIALS AND METHODS

2.1. MATERIALS

Calf-thymus DNA, nor-harman HCl, puromycin diHCl, cycloheximide and all ribonucleoside triphosphates were obtained from the Sigma London Chemical Co. Ltd., London, S.W.6. All other chemicals, including the amino acids were of 'Analar' quality and were obtained from British Drug Houses Ltd., Poole, Dorset. Deoxyribonuclease I [EC. 3.1.4.5] from bovine pancreas (freed from ribonuclease activity) was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. Guanosine - 5' - triphosphate -[^{3}H]tetralithium was obtained from Schwarz Bio Research, Inc., Orangeburg, New York. All other isotopes were obtained from the Radiochemical Centre, Amersham, Bucks. Actinomycin D was a gift from Merck, Sharp and Dohme International, New York.

2.2. ANIMALS

2.2.1. RATS

Male albino rats from a strain bred in the Institute were used for all experimental work. The rats weighed between 140-160g. before fasting, except where indicated. These rats were about 80 days old and they were in the middle of their linear growth phase (Figures 1 and 2). They were maintained on Modified Diet 41B (Oxoid Ltd.,) which contained 15.9% crude protein and adequate vitamin and mineral supplements. All animals were handled daily for several weeks preceding feeding experiments.

For the 24hr. period preceding experiments the animals were removed from the animal house and housed in wire-bottomed cages to prevent coprophagia during

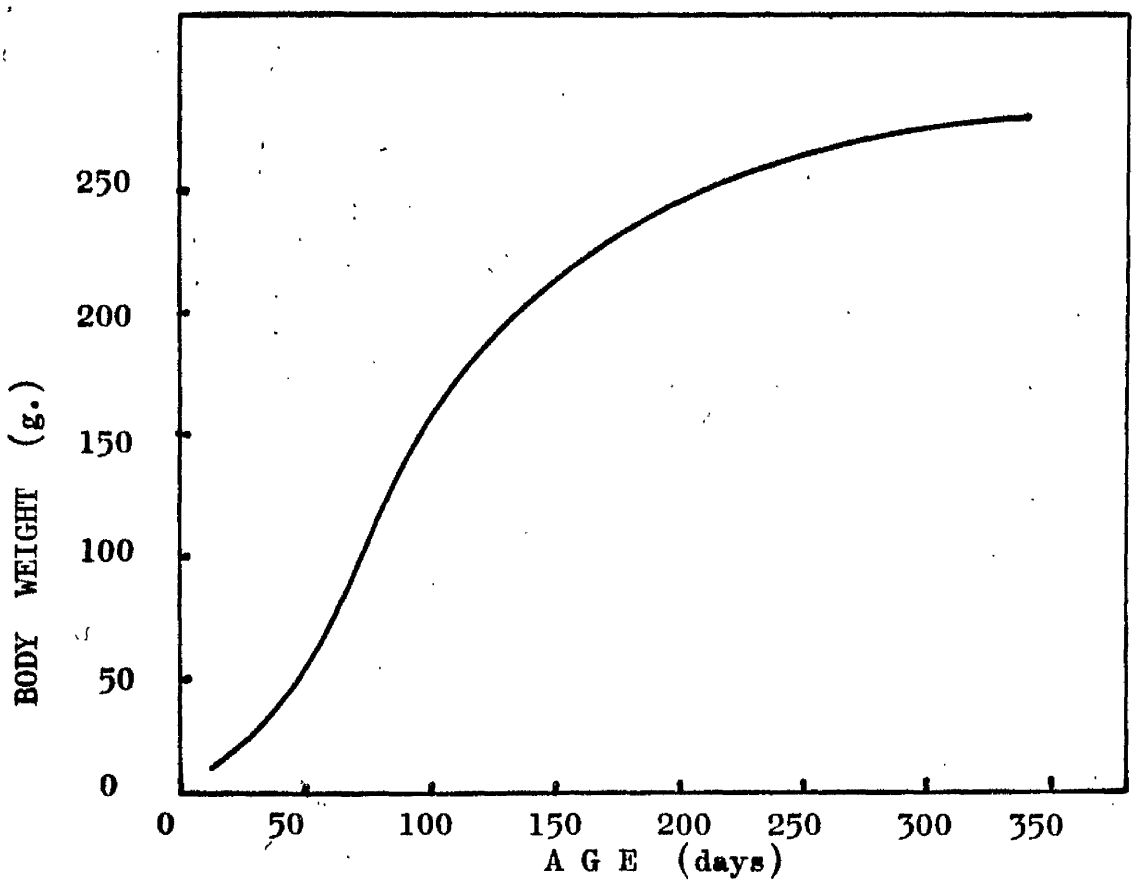


FIGURE 1: THE AGE - BODY WEIGHT GRAPH FOR MALE ALBINO RATS (DONALDSON, 1924).

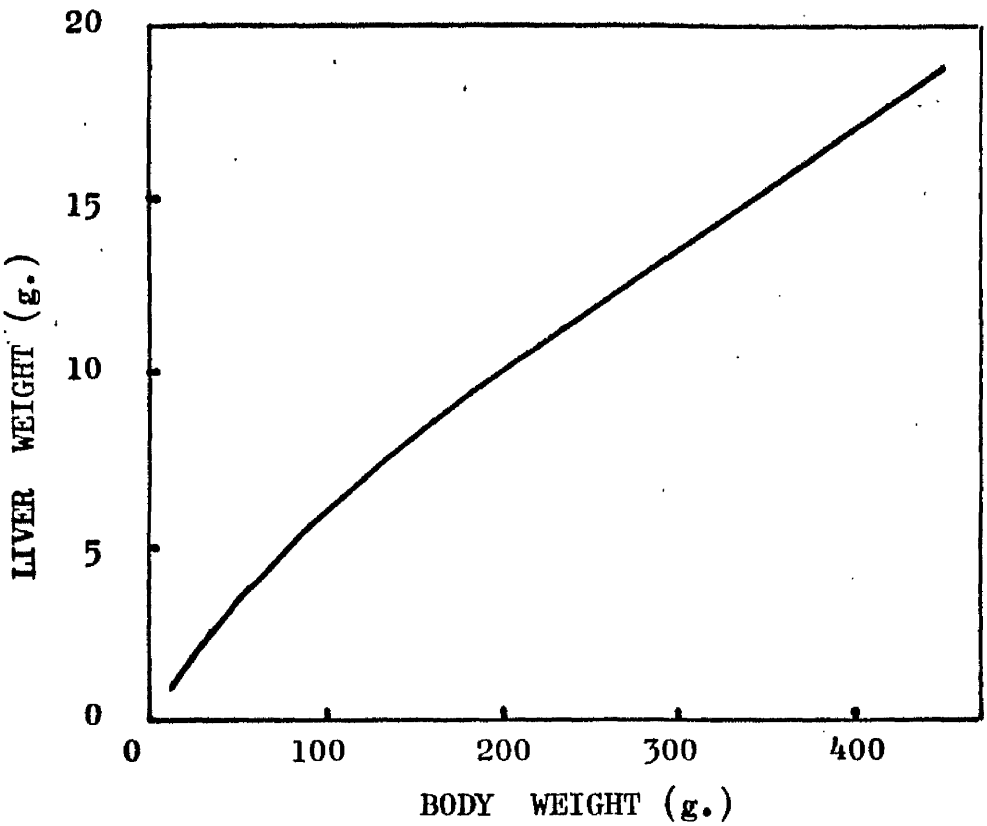


FIGURE 2: THE LIVER WEIGHT - BODY WEIGHT GRAPH FOR MALE ALBINO RATS (DONALDSON, 1924).

fasting. During this period the lighting was controlled by a 12hr. time switch (Sangamo Weston Ltd.,) giving fluorescent illumination from 0600 hours to 1800 hours and darkness from 1800 hours to 0600 hours, thus continuing the daylight-darkness cycle of the animal house.

"Colony fed" animals are animals allowed free access to the stock diet until death. "Starved" animals had the stock diet withdrawn at 1500 hours on the day preceding the experiments (18hr. starved) or at 1700 hours two days preceding the experiment (40hr. starved). All animals were allowed free access to tap water.

2.2.2. RABBITS

8-12 week old Californian or Dutch strain were used as a source of all immunological sera.

2.2.3. CHICKS

Poultry of 4-7 months age were used as a source of chick sera. They were starved for 18hr. preceding venesection.

2.3. AMINO ACID MIXTURES

The amino acid mixtures used (Table 1) for all experiments were similar to those used by Wunner *et al.* (1966) except for tryptophan, which was a quarter of the amount used by these authors. Two mixtures were used; the complete amino acid mixture (T + diet) and the incomplete mixture (T-diet) which contains no tryptophan but which was otherwise identical to the complete mixture.

Rats were fed by stomach tube (a 5ml. syringe with 40 x 2mm (internal diameter polyethylene tube attached). Each rat received 1.1gm of amino acids in 3 ml. of the mixture.

TABLE 1.

– COMPOSITION OF AMINO ACID MIXTURE

All amino acids were in the L-isomer form. The first nine amino acids listed were dissolved in 2.09 g. of $NaHCO_3$ per 50 ml. water; the remaining amino acids were slightly soluble or insoluble.

Amino Acid	Dose per rat (mg./3 ml.)
Glutamic acid	200
Lysine hydrochloride	120
Histidine hydrochloride	43
Arginine	80
Aspartic acid	20
Proline	20
Glycine	10
Alanine	20
Serine	10
Cystine	40
Methionine	56
Threonine	66
Phenylalanine	75
Tyrosine	45
Valine	100
Leucine	118
Isoleucine	86
Tryptophan	13

2.4. ESTIMATION OF RNA, DNA AND PROTEIN

2.4.1. GENERAL CONSIDERATIONS OF SPECTROPHOTOMETRIC ERROR

2.4.1.1. SINGLE BEAM NON-RECORDING SPECTROPHOTOMETERS

These instruments (e.g. Unicam SP500) produce a response directly proportional to the light falling on the detector. Thus the constant uncertainty in the measurement of transmittance (ΔT) results in a corresponding uncertainty (ΔC) in concentration. This uncertainty arises from unavoidable electrical and mechanical imperfections in the instrument (slidewire imperfections, non-linear meter scaling, non-linear response of detector, stray light variations, etc.). Thus an analysis should be conducted at a value of transmittance for which the value of ΔT is at a minimum. This value can be arrived at as follows:

Beer's Law $\quad C = -(\log T)/ab$

differentiating $\quad \frac{dC}{dT} = \frac{-0.4343}{T(ab)}$

$$= \frac{0.4343\ C}{T\ (\log T)}$$

re-arranging : $\quad \frac{\Delta C/C}{T} = \frac{0.4343}{T(\log T)}$

Thus the relative concentration error depends inversely on the product of transmittance and absorbance. The minimum transmittance error is found by differentiating this last expression i.e. when T = 0.368 or extinction = 0.4343. Thus the minimum error occurs at extinction values of 0.43 and the errors remain small in the range 0.2 - 0.8 (Lothian, 1969).

All estimations utilising the Unicam SP500 were adjusted so that all extinction values would lie within this range.

2.4.1.2. DOUBLE BEAM SPECTROPHOTOMETERS

When using this type of spectrophotometer (e.g. Unicam SP800 or Beckman DB) the noise generated by the photomultiplier tube is thought to be the principal source of error (Lothian, 1969) and by a difficult calculation it is possible to show that the minimum error occurs at extinction values of 0.87 with an optimum extinction range of 0.4 - 1.4 (Willard, Merritt and Dean, 1965). All estimations using double beam instruments were arranged to lie within this optimum range.

2.4.2. ESTIMATION OF RNA

Suitable volumes of homogenised tissue, isolated nuclei or tissue extracts were chosen so that the considerations of accuracy outlined above were observed.

The modified Schmidt-Thaunhauser method of Fleck and Munro (1962) was used for all RNA estimations. It is applicable, by suitable dilutions, to tissues, etc. containing RNA in the range 0.05mg - 0.35 mg.

2.4.2.1. PRECIPITATION OF RNA AND EXTRACTION OF LOW MOLECULAR WEIGHT NUCLEOTIDES ETC.

All solutions and tubes were kept ice-cold unless stated otherwise. A volume of material was pipetted into a test-tube and a half-volume of 0.6M-PCA added, mixed and stood in ice for 10 minutes.

The supernatant was discarded after centrifugation at $1000g_{av.}$/5 min./2° and the precipitate washed with 2 volumes of 0.2M-PCA, centrifuged and the supernatant discarded. This step was repeated and the PCA drained from the precipitate.

2.4.2.2. HYDROLYSIS OF RNA AND EXTRACTION OF THE RNA BREAKDOWN PRODUCTS

The acid insoluble precipitate is dissolved in a suitable volume (usually 3-5ml.) of 0.3M-KOH and incubated in a 37° water bath for 1hr. with constant agitation. After incubation the solution is cooled in ice and PCA is added (usually 1.2M) to make the final solution 0.2M-PCA. The tube is stood in ice for 10 minutes, centrifuged as described above and the supernatant collected. The precipitate is washed twice more with suitable volumes of 0.2M-PCA and the supernatants are made 0.1M-PCA by dilution with distilled water. The volumes of 1.2M-PCA and 0.2M-PCA are adjusted to give the necessary extinction values required by the considerations of accuracy outlined in an earlier section. The precipitate obtained from this procedure is used for DNA estimations (Section 2.4.3.)

2.4.2.3. ESTIMATION

The $E^{1cm.}_{260nm.}$ and $E^{1cm.}_{232nm.}$ are obtained and the RNA content determined from the formula RNA (μg./ml.) = $35.04\ E^{1cm.}_{260nm.} - 14.84\ E^{1cm.}_{232nm.}$

This two-wavelength method corrects for protein extracted by 0.2M-PCA and present in the acid-soluble material obtained from the alkaline hydrolysis (Fleck and Begg, 1965).

2.4.3. ESTIMATION OF DNA

The indole procedure of Ceriotti (1952, 1955) was used for all DNA estimations. It is applicable to tissues etc. containing DNA in the range 0.02mg. - 0.1mg. by suitable dilutions of the original material. The procedure is applied to the acid-insoluble precipitate obtained after alkaline digestion of the material (Section 2.4.2.2.). The precipitate is dissolved in a suitable

volume of 0.3M-KOH and diluted with distilled water to give a final solution of 0.1M-KOH. 2ml. of this solution is mixed with 1ml. of 0.04% (w/v) indole in distilled water and 1ml. of 1.19 S.G. HCl. The test tube is stoppered and heated at 100° for 15 minutes in a glycerol bath. The tube is cooled in ice, extracted three times with an equal volume of chloroform and the resulting aqueous solution $E^{1cm.}_{490nm.}$ determined. A 32µg. DNA standard (calf thymus DNA) and a distilled water blank are similarly treated. Blanks should have $E^{1cm.}_{490nm.} < 0.01$ and the unknown solution should have $E^{1cm.}_{490nm.} \leqslant 0.34 = E^{1cm.}_{490nm.}$ standard Therefore this estimation is best carried out on a single beam spectrophotometer for maximum accuracy. The calibration standard is the linear limit of the method and extinctions greater than 0.34indicate that the original solutions must be further diluted.

2.4.4. ESTIMATION OF PROTEIN

Protein was estimated by the Phenol-Biuret Method of Lowry et al. (1951). The modifications suggested by Munro and Fleck (1969) were used. The material to be estimated was diluted, or concentrated, to contain 25 - 500µg protein/ml. A standard solution of 100µg/ml. of bovine serum albumin was run with every test as was a blank.

Reagents:

1. 2% (w/v) Na_2CO_3 in 0.1M-NaOH.
2. 1% (w/v) $CuSO_4 . 5H_2O$ in water
3. 2% (w/v) Na or K tartarate in water
4. Copper reagent prepared by mixing equal volumes of reagent (2) and reagent (3) just before making up reagent (5).
5. Add 1ml. copper reagent (4) to 50ml. reagent (1).
6. The Folin-Ciocalteu phenol reagent is diluted 1:3 before use.

Procedure: Take 1ml. of sample, add 5ml. alkaline copper reagent (5) and allow to stand at room temperature for 10 minutes. Add 0.5ml. Folin-Ciocalteu reagent (6) rapidly with immediate shaking of the resultant solution. Stand for 30 minutes at room temperature and determine $E^{1cm.}_{750nm.}$ Note that extinctions exceeding the value of the standard (0.3) must be disregarded because of the non-linearity of the method above this value.

2.5. ESTIMATION OF TRYPTOPHAN

The fluorescent method of Denckla and Dewey (1967) was used for the determination of tryptophan in rat serum and liver. The method consists of the formation of the fluorophore nor-harman from tryptophan by condensation with formaldehyde and oxidation with $FeCl_3$. The Aminco-Bowman Spectrofluorimete was used with a 1cm. quartz cuvette. Excitation was at 373nm. and emission at 452nm. The sensitivity of the instrument was checked daily by setting the photomultiplier microphotometer to 100% (Meter Multiplier 0.1) with a 5nmde nor-harman solution. The sensitivity was about 28 in this procedure (this gave the instrument a sensitivity mid-way in its range). Recommended slit widths were chosen to give maximum sensitivity.

Glass distilled water was used for all solutions. It was stored in glass vessels as storage in plastic vessels lead to very high fluorimetric blanks. All plastic and glass tubes used were washed with 1M-nitric acid before use.

Reagents:

1. TCA/$FeCl_3$: 10% (w/v) TCA containing 0.3mM $FeCl_3$.
2. HCl/$FeCl_3$: 0.3mM $FeCl_3$ in 0.1mM HCl.
3. 75% (w/v)TCA.

4. 1.8% (w/v) formaldehyde.

5. Tryptophan standards in 0.1M-NH_4OH (5 and 2.5 mmoles per tube).

6. Nor-harman in 0.1-TCA (5 nmoles).

Procedure:

2.5.1. PLASMA

Blood was removed by tail section and collected in a heparanised tube. Plasma was collected by centrifugation. Duplicate samples of plasma (20µl.) were pipetted into a 2ml. plastic centrifuge tube, mixed with 1.8ml. of TCA/$FeCl_3$ and centrifuged at 20,000$g_{av.}$/10min./20°. The supernatant is decanted completely into a stoppered tube calibrated to 2ml., mixed with 0.2ml. formaldehyde solution and heated at 100° in a glycerol bath for 1hr. The Tube is cooled and the solution is made up to 2ml. with 10% TCA. The fluorescence is read as described above.

2.5.2. LIVER

Liver is rapidly removed in the cold laboratory, weighed and immersed in seven volumes of ice-cold 150mM-NaCl, homogenised rapidly in a teflon-glass homogeniser, made up to 50ml. with saline and sampled. It was found necessary to sample the homogenate immediately otherwise the tryptophan levels rose considerably. Duplicate 40µl. samples were taken, mixed with 1.8ml. of TCA/$FeCl_3$ in a 2ml. plastic centrifuge tube, further mixed with 0.2ml. of 75% TCA and centrifuged at 20,000$g_{av.}$/10min./20°. The supernatant was treated similarly to the plasma.

2.6. ISOLATION OF RAT LIVER NUCLEI

All homogenisations were carried out in the cold laboratory. All solutions and apparatus were cooled in ice before and during use.

2.6.1. CITRIC ACID PROCEDURE

2.6.1.1. THE USE OF 0.025 M-CITRIC ACID (DOUNCE, 1943)

Livers were removed, washed in citric acid, minced in 20 volumes of citric acid (with scissors) and homogenised by five full strokes of a glass-teflon 20ml. homogeniser supplied by A.H. Thomas Co., Philadelphia (clearance 0.006 in.). The homogenising motor was a 1/30 H.P. variable speed stirrer (Anderman and Co. Ltd.) run at half-speed.

The homogenate was filtered through 8 thicknesses of muslin cloth and centrifuged at $1500g_{av.}$/5 min./2°. The supernatant was discarded and the precipitate re-suspended in 10 volumes of citric acid and re-centrifuged. This procedure was carried out twice.

The resulting pellet was drained of citric acid and it was found, by DNA estimation, to contain about 50% of the total liver nuclei. The RNA/DNA ratio (mean $\pm$ standard deviation) was 0.178 $\pm$ 0.011 (n = 9). The nuclei appeared 'clean' by phase contrast microscopy and the electron micrographic appearance is shown in Plate 1. Note the single nuclear membrane (the cytoplasmic nuclear membrane having been stripped off during homogenisation), the cytoplasmic tags and the aggregation and condensation of the nuclear material. This type of preparation is known to have undergone considerable loss of nuclear protein (Dounce, 1955).

2.6.1.2. THE USE OF 5% CITRIC ACID (BUSCH, 1967)

Isolation of rat liver nuclei by 5% citric acid was found to yield larger quantities of 45s RNA, when the RNA preparation was examined by agarose gel electrophoresis (Section 2.9.1), than the 0.025 M-Citric Acid method. Accordingly 5% citric acid was used instead of 0.025M-citric acid in Section 2.6.1.1.

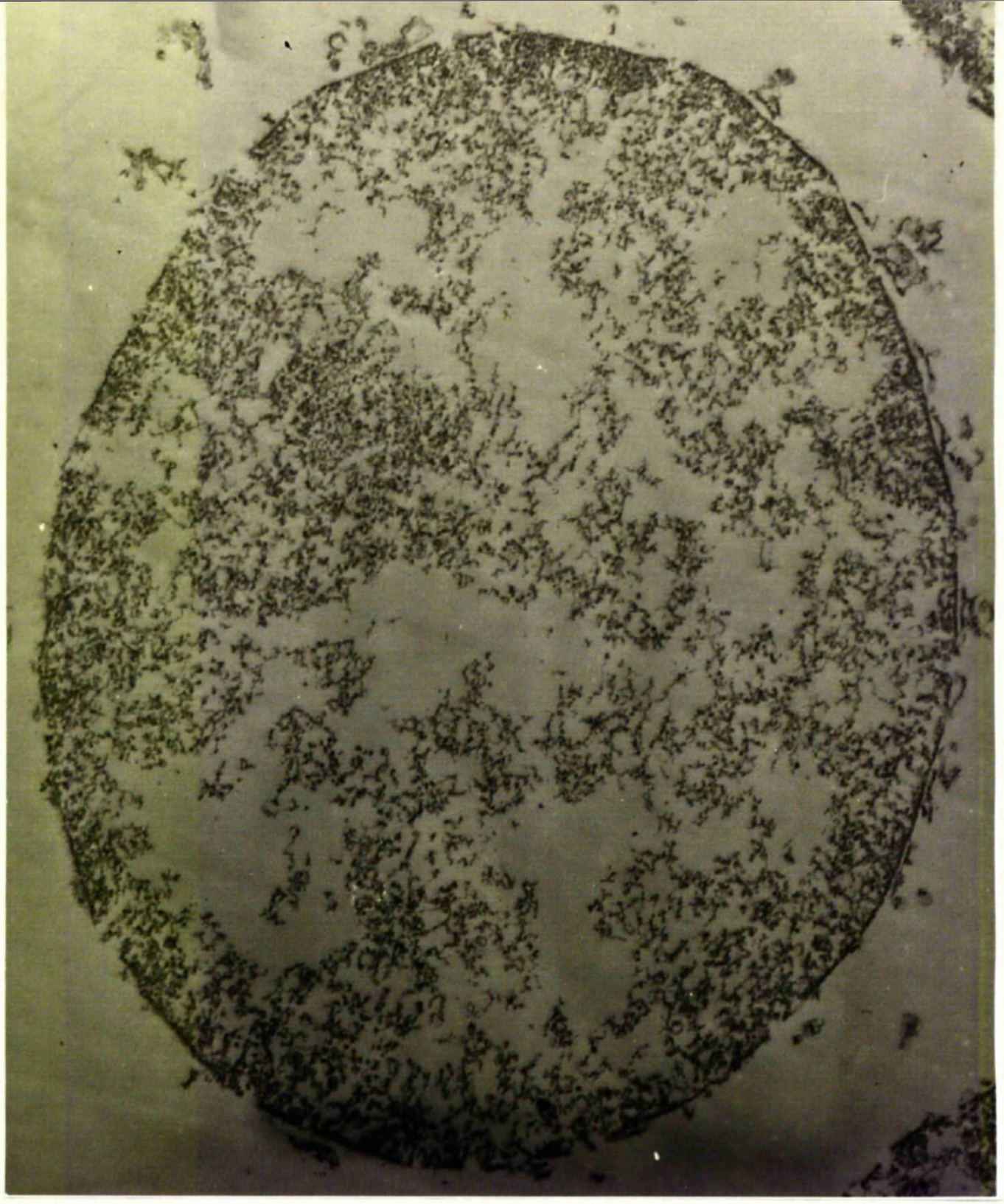


PLATE 1 : Rat Liver Nuclei isolated by the Citric Acid Procedure (Section 2.6.1.1.). Osmium fixation, lead staining. Magnification 50,000 x

2.6.2. THE MODIFIED CHAUVEAU PROCEDURE (MUNRO et al., 1965)

Livers were removed, washed in homogenisation medium and minced with scissors in 10 volumes of 0.25 M-sucrose containing 0.002 M-$CaCl_2$ and 0.001 M-$MgCl_2$. Homogenisation was carried out using the equipment described in Section 2.6.1.1. except that 10 full strokes were used at full motor speed.

The homogenate was filtered through 8 thicknesses of muslin cloth and layered over an equal volume of 0.34 M-Sucrose containing 0.002 M-$CaCl_2$ and 0.001 M-$MgCl_2$. After centrifugation at $600g_{av.}$/10min./2° the nuclear pellet was suspended in a small volume of homogenising medium and mixed with 19 volumes of 2.31 M-Sucrose containing 0.002 M-$CaCl_2$ and 0.001 M-$MgCl_2$ to give a final sucrose concentration of 2.2M-sucrose. After centrifugation at $30,000g_{av.}$/1hr./2° the pellet of nuclei was washed three times with 0.25 M-sucrose to wash out the $CaCl_2$ which is known to inhibit RNA nucleotidyl-transferase activity (Weiss, 1960).

The nuclei were found to be relatively pure, with a double nuclear membrane and tags of cytoplasmic material attached to it (Plate 2). The nucleoplasm was markedly less clumped and aggregated than were the citric acid isolated nuclei. The RNA/DNA ratio was 0.25 - 0.29 and the yield was about 25% when judged by DNA estimations.

2.6.3. THE BUSCH PROCEDURE (MURAMATSU et al., 1966)

When nuclei prepared by this procedure were used for RNA nucleotidyltrans-:ferase assays Ca^{2+}- salts were replaced by Mg^{2+}- salts in the concentrations contained in parentheses.

Livers were removed and washed in 0.25M-sucrose, transferred to 10 volumes of 2.4M-sucrose containing 3.3mM-$CaCl_2$ (5mM-$MgCl_2$) minced with scissors and

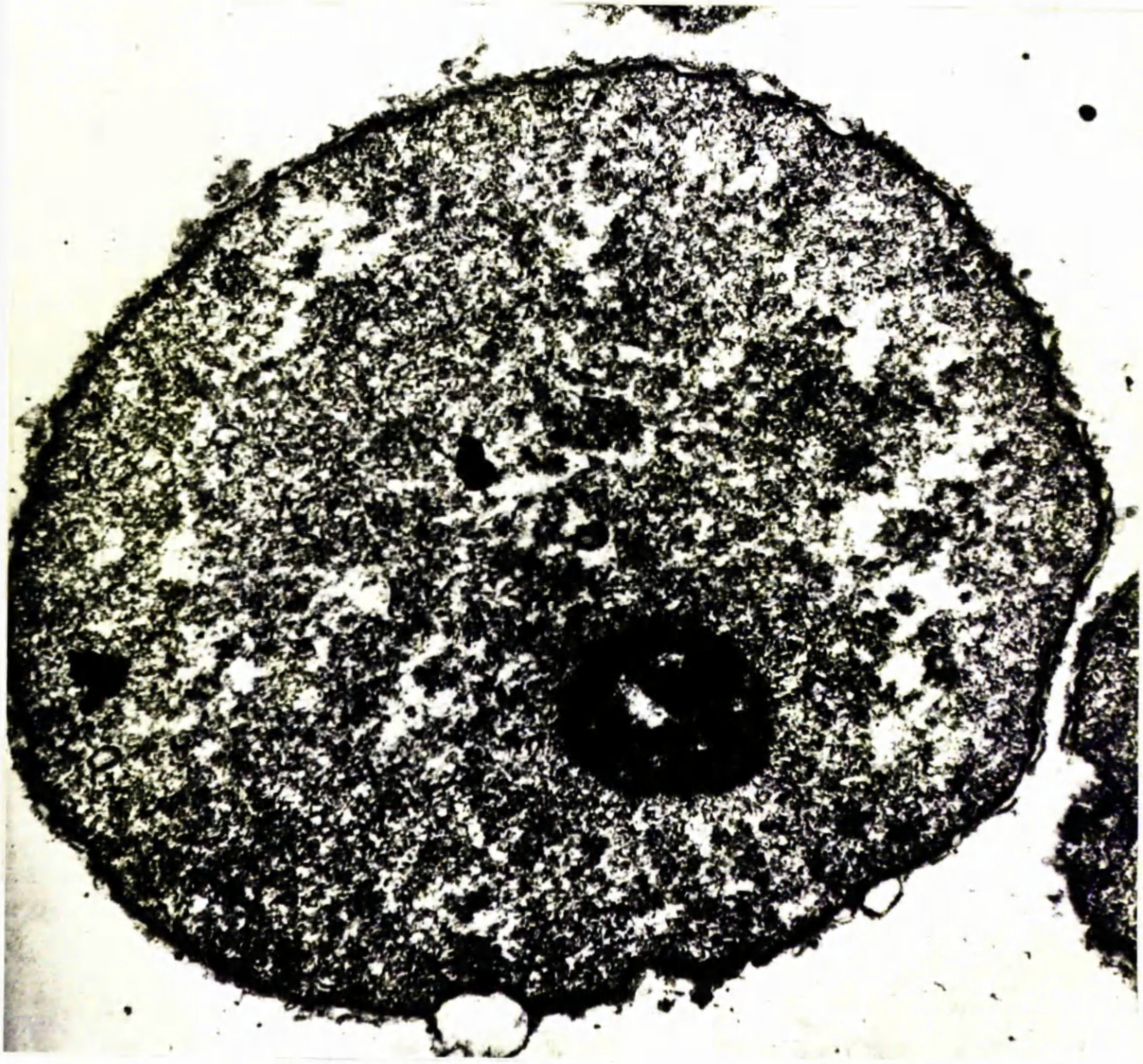


PLATE 2 : Rat Liver Nuclei isolated by the modified Chauveau Procedure (Section 2.6.2.).
Osmium fixation, lead staining. Magnification 50,000 x

homogenised in a 200ml. glass-teflon homogeniser supplied by Glass Engineering Co., Houston, Texas (clearance 0.005 in.). Six full strokes were used. The homogenising motor was a Black and Decker D900 $\frac{1}{2}$" drill, working at 900 revolutions per minute, and mounted in a vertical drilling stand clamped to the bench. This drill gives a sufficiently high torque to maintain the motor speed when homogenising in this very viscous medium. The homogenate was filtered through 8 thicknesses of white butter muslin (No. 106, supplied by Thomas Skinner and Hamilton, Glasgow) and centrifuged at $40,000g_{av.}$/1hr./2°. The nuclear pellets were suspended (1ml./gm. of original tissue) in 1M-sucrose containing 1mM-$CaCl_2$ (1mM-$MgCl_2$) and homogenised in a 5ml. glass-teflon homogeniser supplied by A.H. Thomas Co., Philadelphia (clearance 0.010 in.). Five full strokes were used and the pestle was rotated by hand. The homogenate was centrifuged at $800g_{av.}$/5 min./2°. The pellet of nuclei obtained was then used for RNA extraction or enzyme assay.

The quality of the nuclei judged by electron microscopy, was similar to the modified Chauveau procedure (Section 2.6.2.). The RNA/DNA was 0.25 and the yield was 25%. Busch (1967) claims that this procedure gives a yield of 60%. It is possible that this decreased yield is due to our using 10 volumes of homogenising media (necessitated by the available centrifuge rotor volume) instead of the 15 volumes suggested by him.

2.7. EXTRACTION OF RNA FROM NUCLEI

2.7.1. EXTRACTION PROCEDURE OF HIATT (1962)

The procedure of Hiatt was modified in use by using 'citric acid nuclei' and hot phenol extractions. Nuclei prepared by the Citric Acid Procedure (Section 2.6.1.) were suspended in 10ml. of 50mM-Tris buffer (final concentration -

adjusted to pH 7.5 with 1M-HCl) containing 1% (w/v) bentonite and 1% (w/v) sodium dodecyl sulphate, and homogenised in a 10ml. glass-teflon homogeniser by 5 full strokes at $\frac{1}{2}$ speed. The very viscous pale-coloured suspension was transferred to a 10ml. stoppered tube and shaken vigorously at 65° for 3$\frac{1}{2}$min. with an equal volume of 90% (v/v) phenol (containing 0.1% (w/v) 8-hydroxy-quinoline and equilibrated with 50mM-Tris buffer pH 7.5). The tubes were then chilled in ice and centrifuged at $1700g_{av.}$/30 min./2°. The aqueous layer and interface were removed and re-extracted with a half-volume of phenol as described above and centrifuged. This procedure was repeated once more and the aqueous layer only was removed and shaken three times with an equal volume of ether to remove traces of phenol. Ether was removed by bubbling nitrogen through the solution and gently warming the tube by hand.

The extracted RNA and DNA was precipitated from the aqueous layer by adding 2 volumes of 95% (v/v) ethanol containing 2% (w/v) potassium acetate (anhydrous) and leaving overnight at -10°. The precipitate was pelleted by centrifugation at $1500g_{av.}$/10 min./-10°, and dissolved in 3ml. of 50mM-tris (pH 7.5) containing 1% bentonite and 1mM-$MgCl_2$. 15µg. of deoxyribonuclease (EC 3.1.4.5) (from bovine pancreas, ribonuclease-free) was added and the incubation was allowed to proceed for 5 min. in air at room temperature although the solution was still cool at the end of the incubation. The reaction was stopped by the addition of 588mg. potassium acetate (anhydrous), making the solution 2M with respect to potassium acetate, and 1.1ml. of 95% (v/v) ethanol. Storing overnight at -10° and centrifuging at $1500g_{av.}$/10 min./ -10° pelleted the RNA which was contaminated by DNA oligonucleotides. These were removed by the dialysis of the pellet (dissolved in 0.5ml. of 10mM-sodium acetate buffer (final concentration - adjusted to pH 5.25 with glacial acetic acid) which

contained 50mM-NaCl against 1 litre of this buffer at 4° overnight to remove the contaminating oligodeoxy-ribunucleotides. The dialysis residue was centrifuged at $1500g_{av.}$/10 min./2° to remove bentonite and the supernatant was removed and retained for subsequent sucrose density gradient analysis.

2.7.2. EXTRACTION PROCEDURE OF PENMAN (1966)

Nuclei prepared by the Citric Acid Procedure (Section 2.6.1) were suspended in ice-cold 50mM - Tris buffer (final concentration - adjusted to pH 7.5 with 1M-HCl) containing 0.5M-NaCl and 50mM-$MgCl_2$, the final volume being 4ml. The solution was mixed until a highly viscous suspension of nuclei was obtained. 100ug. of Deoxyribonuclease [EC 3.1.4.5.] (from bovine pancreas, ribonuclease free was added and the solution was placed in a 20° water bath for 3 min. The reaction was stopped by the addition of SDS to 0.5% concentration and EDTA to a concentration of 0.1M.

An equal volume of 90% (v/v) phenol (equilibrated with 50mM-Tris buffer pH 7.5) containing 0.1% 8-hydroxy-quinoline was added and heated to 65° for 3½min. with regular shaking. A half-volume of chloroform containing 1% isoamyl alcohol was added and the solution was heated to 65° for 3½ min. with regular shaking. The mixture was then centrifuged at $1500g_{av.}$/10 min./20° and the phenol-chloroform phase was removed. The chloroform-isoamyl alcohol washes were repeated twice more and finally the aqueous layer containing the nuclear RNA was removed. The RNA was precipitated by the addition of alcohol and solid potassium acetate to give final concentrations of 25% (v/v) and 2M respectively and storing at -10° overnight. The RNA was pelleted by centrifugation ($1500g_{av.}$/10 min./-10°) and the pellet was dissolved in a small volume of 10mM-sodium acetate (pH 5.25) for subsequent sucrose density gradient analysis.

2.7.3. EXTRACTION PROCEDURE OF BUSCH (MURAMATSU et al., 1966)

Nuclear preparations were suspended (1 ml. per gm. original tissue) in 50mM-sodium acetate buffer (final concentration - adjusted to pH 5.1 with 1M-acetic acid) containing 0.3% SLS and 0.15M-NaCl. The mixture was homogenised in a 10ml. teflon-glass homogeniser for 1 min. then homogenised for a further minute after the addition of 5ml. 90% (v/v) phenol containing 0.1% 8-hydroxy-quinoline (saturated with the 50mM-sodium acetate buffer).

The mixture was then shaken at 65° for 10 min. and at room temperature for 15 min. and centrifuged at $2000g_{av.}$/15 min./20° to separate the phenol phase from the aqueous phase and interphase. The phenol phase was removed and the aqueous layer and interphase were shaken for 10 min. at room temperature with 5ml. of fresh phenol. After centrifugation, the clear aqueous layer was removed by careful pipetting and re-extracted with an equal volume of phenol. After centrifugation the aqueous layer was carefully removed and the RNA precipitated by the addition of 2 volumes of 90% alcohol containing 2% (w/v) potassium acetate. This procedure extracted about 70 - 80% of the nuclear RNA but DNA contamination was sometimes very high. This contamination was reduced to less than 5% of the total nucleic acid content by introducing a second 65° phenol extraction stage preceding the second room temperature extraction.

The precipitated RNA was kept overnight at -15°, washed once with 75% alcohol and dissolved in a small volume of 10mM-sodium acetate pH 5.1 for subsequent sucrose density gradient analysis.

2.8. SUCROSE DENSITY GRADIENTS

2.8.1. LINEAR GRADIENTS

Linear sucrose density gradients were prepared using the mixing device of Britten and Roberts (1960). Gradients so prepared were shown to be linear for 3ml. SW 39 and 30ml. SW 25 Centrifuge tubes. The 3ml. gradients were made and run according to Hiatt (1962) and the 30ml. according to Muramatsu et al. (1966).

2.8.2. ISOKINETIC GRADIENT

The basis for the computations were taken from Noll (1967). As there are several errors in this paper the derivation of the gradient is outlined in some detail.

At a given temperature the rate at which a particle of density ρ_p semiments through a medium of density ρ_m and viscosity η_m in a centrifugation field ωr^2 is

$$\frac{dr}{dt} = S_{20,w} \cdot \omega^2 \cdot r \cdot \frac{\rho_p - \rho_m}{\eta_m} \cdot \frac{\eta_{20,w}}{\rho_p - \rho_{20,w}} \quad \ldots(1)$$

If the particle moves at constant velocity then the RHS of equation (1) is constant, and it can be seen that r, ρ_m and η_m are the only variables. However, under the conditions of constant velocity at any point r in the centrifuge tube

$$r \cdot \frac{\rho_p - \rho_m}{\eta_m} = \text{constant}$$

Therefore for any radius of the tube

$$r \cdot \frac{\rho_p - \rho_m}{\eta_m} = r_t \cdot \frac{\rho_p - \rho_t}{\eta_t} \quad \ldots(2)$$

where the subscript t denotes the conditions at the top of the centrifuge tube. Re-arranging equation (2)

$$\eta_m = \frac{\eta_t}{\rho_p - \rho_t} \cdot r \cdot \frac{\rho_p - \rho_m}{r_t} \quad \ldots(3)$$

The values of the variables at the top of the gradient can readily be calculated once the top concentration of sucrose is decided. For the separation of nuclear RNA a top concentration of 5% (w/w) and a particle density of 1.7 were used. The values of η_m and ρ_m were calculated in steps of 3% (w/w) from 5% upwards. Viscosity was calculated from the empirical equation (Barber, 1966):

$$\eta = \exp\left\{ E_1 \frac{C}{100-C} + E_2 \left(\frac{C}{100}\right)^2 + \rho_m \eta_{water} \right.$$

where E_1 and E_2 are temperature-dependant constants and C is % (w/w) of sucrose. The temperature chosen was 2°.

Density was calculated from the empirical equation (Barber, 1966):

$$\rho = (B_1 + B_2 T + B_3 T^2) + (B_4 + B_5 T + B_6 T^2) Y + (B_7 + B_8 T + B_9 T^2) Y^2$$

where $B_1 - B_9$ are 8-figure constants, $Y = C/100$ and $T = 2$.

Thus by substituting values for ρ_m and η_m into equation (3) values of can be obtained. It is convenient to convert the values of Y into volume units and to plot sucrose concentration against this tube volume (Figure 3). This curve represents the constant velocity gradient (isokinetic gradient) for RNA molecules. It is known that a convex gradient of the type shown in Figure 3 can be produced by making the sucrose concentration, at any point of the gradient, a function of the volume. An equation describing this function can be derived as follows:-

let

C_R = sucrose concentration in the burette

C_t = initial sucrose concentration in the mixing chamber and thus the concentration at the top of the centrifuge tube

C = concentration at any volume V in the centrifuge tube

V_m = volume of mixing chamber

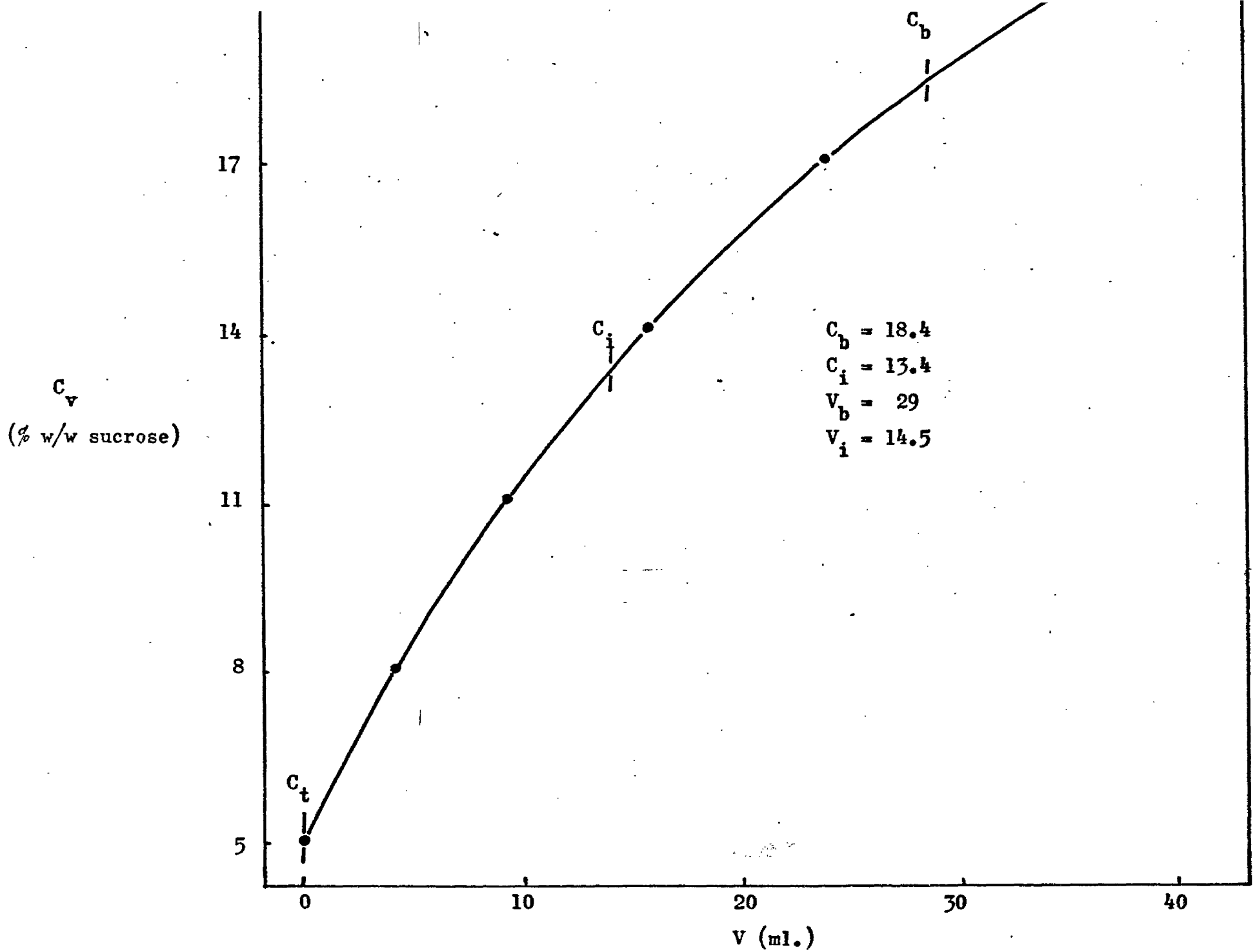
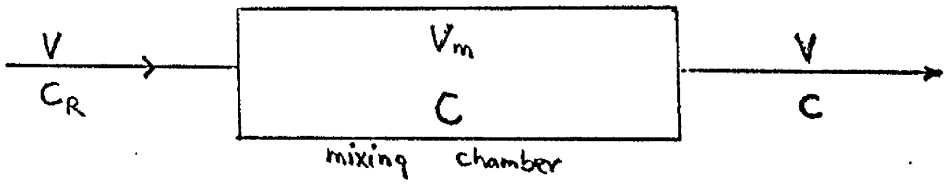


FIGURE 3 : THEORETICAL ISOKINETIC GRADIENT FOR RNA SEPARATION. SUCROSE CONCENTRATION AT TOP OF CENTRIFUGE TUBE IS 5 % (w/w).

This can be illustrated in the following flow diagram.



Now the rate of flow = $\frac{V}{t}$ = Q

rate of accumulation of sucrose = $Q(C_R - C)$

rate of change of concentration = $\frac{dC}{dt}$

total change of concentration = $V_m \cdot \frac{dC}{dt}$

$$\therefore \quad V_m \frac{dC}{dt} = Q(C_R - C)$$

$$\therefore \quad \frac{dC}{C_R - C} = \frac{Q \cdot dt}{V_m}$$

integrating : $$-\ln(C_R - C) = \frac{Qt}{V_m} + A$$

or $$C = C_R - A e^{-\frac{Qt}{V_m}}$$

initial conditions : $t = 0 \quad C = C_t$

$$\therefore \quad A = (C_R - C_t)$$

$$\therefore \quad C = C_R - (C_R - C_t) e^{-V/V_m}$$

$$\therefore \quad C = C_R - (C_R - C_t) e^{-V/V_m}$$

$$\text{or} \quad C = C_R(1 - e^{-V/V_m}) + C_t e^{-V/V_m} \qquad \ldots(4)$$

From this equation it can be seen that Vm and C_R are unknown and these parameters are required to construct the gradient. These values are obtained by the following technique.

Equation (4) is substituted for two sets of gradients $C_t \rightarrow C_i$ and $C_t - C_b$ (Figure 3). Thus

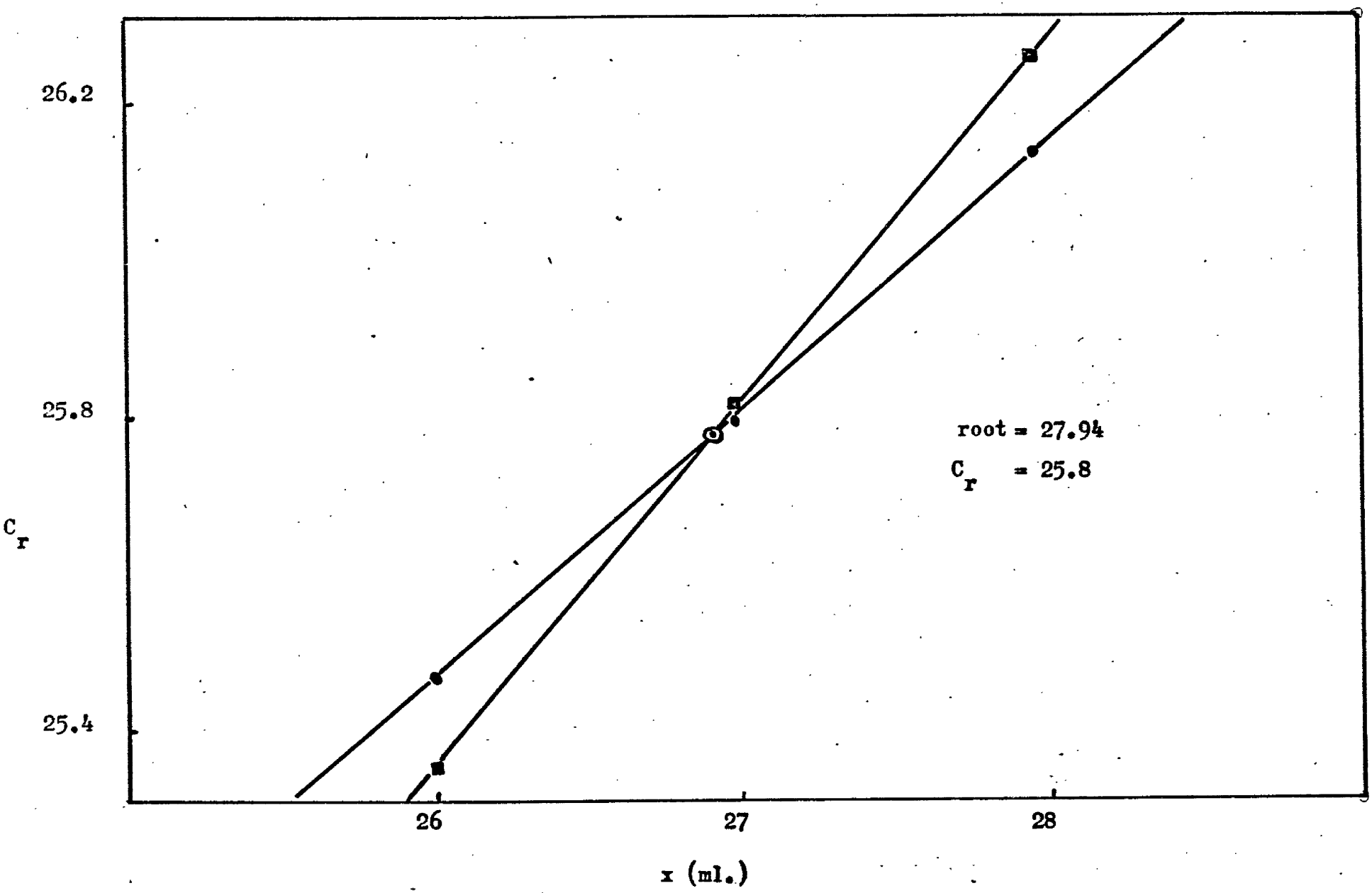


FIGURE 4 : ISOKINETIC GRADIENT - GRAPHICAL SOLUTION OF V_m .

$$C_i = C_R\left(1 - e^{-\frac{V_i}{V_m}}\right) + C_t\, e^{-\frac{V_i}{V_m}} \qquad \ldots(5)$$

and $$C_b = C_R\left(1 - e^{-\frac{V_b}{V_m}}\right) + C_t\, e^{-\frac{V_i}{V_m}} \qquad \ldots(6)$$

or after rearranging

$$C_R = \left(C_i - C_t\, e^{-\frac{V_i}{V_m}}\right)\left(1 - e^{-\frac{V_i}{V_m}}\right)^{-1}$$

and $$C_R = \left(C_b - C_t\, e^{-\frac{V_b}{V_m}}\right)\left(1 - e^{-\frac{V_b}{V_m}}\right)^{-1}$$ by

evaluating C_R at different values of V/V_m i.e.

$$C_{R_1} = \left(C_i - C_t\, e^{-x_1}\right)\left(1 - e^{-x_1}\right)^{-1}$$

and $$C_{R_2} = \left(C_b - C_t\, e^{-x_2}\right)\left(1 - e^{-x_2}\right)^{-1}$$

where $x_1 = \frac{V_i}{V_m}$ and $x_2 = \frac{V_b}{V_m}$.

A graph of C_R and can be obtained (Figure 4). The point of intersection is V_m. It should be noted that equations (5) and (6) are transcendental and they cannot be solved formally. This graphical technique and a numerical approximation technique are the most easily used numerical methods for obtaining a solution. From Figure 4 V_m = 27.9ml. and C_R can be calculated from equation (4) giving 25.8%

The apparatus of Noll (1967) was used for producing the gradients. It consists of a burette and mixing chamber. The burette contained a sucrose solution of a concentration C_R and it is connected through an air-tight rubber stopper to a mixing flask with a magnetic stirrer bar. A small thin tube connects this mixing flask to the bottom of the centrifuge tube.

The mixing flask is filled with a volume Vm of 'top' sucrose and the flow started by injecting a small volume of air into the flask. At the same time mixing is started and the burette is opened. After a volume V has been run in, the gradient maker is stopped and an isokinetic gradient for the parameters Vm and C_R will have been produced.

2.9. ELECTROPHORESIS

2.9.1. AGAROSE GEL ELECTROPHORESIS OF RNA (McINDOE AND MUNRO, 1967)

Agarose was obtained from L'Industrie Biologique Francaise S.A., Gennevilliers, France. This was found to be the most satisfactory form of agarose available for RNA separation. 2% gels were prepared in 0.02M-Tris (final concentration - adjusted to pH 7.9 with 1M-citric acid). A slurry of agarose was prepared in about 15ml. of buffer, washed into the remaining volume of buffer and heated to about 95°. When the agarose was completely dissolved it was cooled, with stirring, to 40° and poured into a Perspex gel slab former provided with a lid having projections which produced transverse sample channels in the setting gel.

About 60 µg. of RNA was applied to the sample slots. Electrophoresis was carried out using 0.2 M-tris (prepared as above - pH 7.9) in the electrode compartments. A current of 4mA./sq.cm. was applied for 90 min. and a sheet of polythene was placed over the gel after 10 min. to prevent evaporation from the surface of the gel. The gel was removed from its mould and stained for 1hr. in 0.05% toluidine blue which forms an insoluble salt with RNA, and then washed in several changes of water overnight.

2.9.2. POLYACRYLAMIDE GEL ELECTROPHORESIS OF PLASMA PROTEINS

5% gels with 5% cross-linking were used for all protein separation work. Two types of buffer systems were used:

1. Continuous System: Gel and electrode vessels contained 0.076 M-tris (final concentration - adjusted to pH 8.6 with 1M-citric acid).
2. Discontinuous System: Gel contained a similar buffer to above but the electrode vessels contained 0.3M-borate (Final concentration - adjusted to pH 8.6 with 2M-NaOH).

Gels were made up as follows:

25ml. of 25% (w/v) Cyanogum 41 (BDH) solution were added to 100ml. of the buffer solution and de-aerated. Three drops of TEMED (N,N,N',N'-tetramethylen-diamine) and 4 drops of 40% (w/v) ammonium persulphate were added, the mixture shaken quickly and then poured into the gel plates and allowed to polymerise with the air excluded.

Gels were run for 1 - 2 hr. at a current of 50 mA. in the cold laboratory. Voltages used ranged from 25 - 30 $V.cm^{-1}$. At the end of the electrophoretic run gels were stained by a Naphthalene Black stain for one min. then washed several times in 1% (v/v) acetic acid. The staining solution was made up as follows -

50 parts methanol
50 parts water
20 parts glycerol
1 part glacial acetic acid.

The mixture was saturated with Naphthalene Black.

2.10. RADIOACTIVE ASSAY

All radioactive assays were carried out on a Nuclear Chicago 725 Liquid Scintillation Spectrometer using the Channels-Ratio methods of quench correction. All counts were corrected for an adequately determined background and counts were continued for a time interval which was sufficient for a counting error of less than 1%

2.10.1. SUCROSE DENSITY GRADIENTS

2.10.1.1. 3ml. GRADIENTS

Drop counted fractions from the gradients (0.2ml.) were diluted with 0.4ml. water and mixed with 8ml. of Scinstant NE 572 (a dioxan based scintillator

supplied by Nuclear Enterprises Ltd., Edinburgh). Counting efficiencies were 10% (approx.) for tritiated compounds and 50% (approx.) for [^{14}C]-compounds. Radioactivity was expressed as disintegrations per minute (d.p.m.).

2.10.1.2. 30ml. GRADIENTS

Timed fractions (1ml.) from the density gradient fractionater (Instrumentation Specialties Co. Inc., Lincoln, Nebraska) were collected and mixed with 5ml. of 10% (w/v) TCA and 0.1ml. of 1% (w/v) DNA, allowed to stand in ice for 10 min. and the resulting suspension plated on a Millipore filter disc by suction. Each fraction tube and filter disc was washed with 10ml. of 5% TCA and the filter disc removed, placed in a scintillation vial and dried at 70° for 1hr. 10ml. of toluene solvent (containing 0.5% (w/v) PPO as a primary solute and 0.03% (w/v) POPOP as a secondary solute) was added and counting was carried out at efficiencies of 25% (approx.) for tritiated compounds and 60% (approx.) for [^{14}C]-compounds. Radioactivity was expressed as disintegrations per minute (d.p.m.).

2.10.2. INCORPORATION OF [^{3}H]OROTIC ACID INTO ISOLATED NUCLEI

Isolated citric acid nuclei (Section 2.6.1) were suspended in 50ml. distilled water and duplicate 1ml. aliquots were removed for radioactive assay. The nuclei were taken to dryness by infra-red illumination and 0.25ml. of 1M-Hyamine Hydroxide added, mixed and incubated in the dark for 6hr. at 37°. 10ml. of the toluene-PPO-POPOP mixture was added, mixed and left in the dark overnight at room temperature to reduce phosphorescence. Efficiency of tritium counting was approx. 30% with a background counting rate of 38 counts per minute. Radioactivity was expressed as pmole orotic acid incorporated.

2.10.3. INCORPORATION OF ISOTOPE BY RNA NUCLEOTIDYLTRANSFERASE

The acid-insoluble precipitate obtained after washing the polymerase assay mixture (Section 2.11) was dissolved in 0.5 ml. of Nuclear-Chicago Solubiliser (NCS) followed by suspension in the toluene-PPO-POPOP mixture described above. However, due to anomalous counting effects (see Results) the use of NCS was discontinued and IM-Hyamine Hydroxide dissolved in methanol was used instead. The precipitate was therefore suspended in 0.25 ml. of Hyamine Hydroxide and incubated, in the dark at 37° for 6 hr. mixed with the toluene-PPO-POPOP mixture, left at room temperature in the dark overnight and counted the following day. Efficiency of tritium counting was approx. 30% and the background counting rate was 38 counts per minute. Radioactivity was expressed as pmole GTP incorporated.

2.11. RNA NUCLEOTIDYLTRANSFERASE ASSAY IN WHOLE NUCLEI

2.11.1 ASSAY ACCORDING TO BEGG (PERSONAL COMMUNICATION)

This assay was a modification of the assays of Weiss (1960) Goldberg (1961) and Busch et al. (1962). The reaction mixture contained in a volume of 1 ml. :

250 µmole sucrose

100 µmole tris-HCl (Final concentration - adjusted to pH 8.4 with 1N-HCl)

3.75 µmole $MgCl_2$

3 µmole 2-mercaptoethanol

0.4 µmole each ATP, CTP and UTP

2.3 nmole [^{3}H -GTP] (Specific Activity 1.12 Ci/mmole)

0.1 ml. of nuclear suspension (containing approx. 0.1 mg. DNA)

The mixture, with the nuclear suspension omitted, was pre-incubated for 5 min. at 37° and the nuclear suspension (pre-incubated at 37° for 1-2 min.) was then added. The assay was carried out at 37°, in a shaking 37° incubator for variable times. Note that linearity with respect to time and [DNA], pH optima and $[Mg^{2+}]$ optima were all pre-determined.

The reaction was stopped by the addition of 1ml. of an ice-cold solution of 0.2M-tetrasodium pyrophosphate and 0.1% (w/v) BSA ice-cold (final concentrations - adjusted to pH 7.5 with 1M-HCl) followed by 1ml. of 0.6 M-PCA. The pyrophosphate solution diminishes the non-specific binding of nucleotides (Klemperer, 1963). The tubes were stood in ice for 10 min. and then centrifuged at $600g_{av.}$/10 min./2° and the supernatant discarded. The pellet was washed and centrifuged a further four times with 2ml. of ice-cold 0.2M-PCA which was necessary to remove all radioactive nucleotides (D.J. Begg, personal communication). The final acid - insoluble precipitate was allowed to drain overnight and the radioactivity of the pellet is determined as described in Section 2.10.3. For each assay several tests and two zero time estimations (Section 2.4.3.) were made for each nuclear preparation.

2.11.2. MODIFIED ASSAY ACCORDING TO WIDNELL AND TATA (1966a)

This assay system was modified to correspond to the Begg assay (Section 2.11.1) in respect of volume, triphosphate, sucrose and tris contents. Otherwise the assay mixture was similar to the original Widnell and Tata method. The reaction mixture contained, in a volume of 1ml.:

250 µmole sucrose
100 µmole tris-HCl (pH 8.5)
3.75 µmole $MgCl_2$
10 µmole L-cysteine hydrochloride

3 µmole NaF

0.4 µmole each ATP, CTP and UTP

2.3 nmole [^{3}H GTP] (Specific Activity 1.12 Ci/mmole)

0.1 ml. of nuclear suspension (containing approx. 0.2 mg. DNA)

The mixture, with the nuclear suspension omitted, was pre-incubated for 5 min. at 37° and the nuclear suspension (pre-incubated at 37° for 1 - 2 min.) was then added. The assay was carried out as 37°, in a shaking 37° incubator for 3 min. Note that linearity with respect to time and [DNA], pH optima and $[M]_{[Mg^{2+}]}^{2+}$ optima were all pre-determined.

The subsequent steps were similar to those outlined in Section 2.11.1. above.

2.12. ALBUMIN PREPARATION

2.12.1. RAT

200-250g. rats were bled by cardiac puncture under O_2/ether anaesthesia, then killed. The blood was allowed to clot at room temperature for 1 hr. then it was centrifuged at $800g_{av.}$/1hr./20°. The serum was collected and re-centrifuged at $800g_{av.}$/10min./20°, to remove residual red cells. The resulting serum was invariably haemolysed. About 3-4 ml. of serum was obtained from each rat.

The preparation of crude albumin was essentially that of Pederson (1945). The serum was diluted with an equal volume of 0.2 M-NaCl and stirred in the cold room at 4°. The cooled diluted serum was made 60% (w/v) by the addition of finely powdered $(NH_4)_2\ SO_4$ (38.7g. per 100 ml. of solution), each addition being allowed to dissolve before further additions. On completion the pH was adjusted to 6.8 with 1M-acetic acid. After stirring overnight the precipitate was

removed by centrifugation at $800g_{av.}$/30min./4°. The supernatant was made 65% (w/v) with respect to $(NH_4)_2\ SO_4$ concentration, by adding 3.5g per 100ml. of solution, and the pH was adjusted to 4.7 with 1M-acetic acid. The solution was stirred overnight in the cold room and centrifuged at $800g_{av.}$/1hr./4° to obtain a white precipitate which was dissolved in a small volume of distilled water and dialysed in the cold room with 5 daily changes of 5 litres of distilled water. The resulting material was freeze dried. Yields were about 7-10mg. crude albumin per ml. serum (Fraction RA_1).

The albumin was purified by column chromatography (Fleck et al. 1966) on DEAE-Sephadex A-50 using an exponential salt gradient. The procedure was as follows: 5g. of DEAE - Sephadex A-50 (medium grade) was suspended in 2 l. distilled water. It was stirred overnight then allowed to settle for 5 min. The 'fines' were poured off and the gel was re-mixed, allowed to settle for a short time and the 'fines' again poured off. This procedure was repeated a further four times. The gel was poured into a large Buchner Funnel and washed with gentle suction as follows:-

500ml. distilled water
500ml. 0.5M - HCl
1000ml. distilled water
500ml. 0.5M - NaOH
1000ml. distilled water
500ml. 0.5M - H_3PO_4
500ml. distilled water
1000ml. 0.02M - phosphate buffer (pH 6.6)

The gel was resuspended in phosphate buffer, de-aerated, then poured into a 2.5cm. diameter chromatography column (Whatman) and allowed to settle. A column of gel of about 40cm. was obtained and this was washed overnight with 0.02M - phosphate buffer (pH 6.6) with a hydrostatic pressure of 20-30cm. Albumin (1g.) was applied to the top of the column in a volume of 10-20 ml. of phosphate

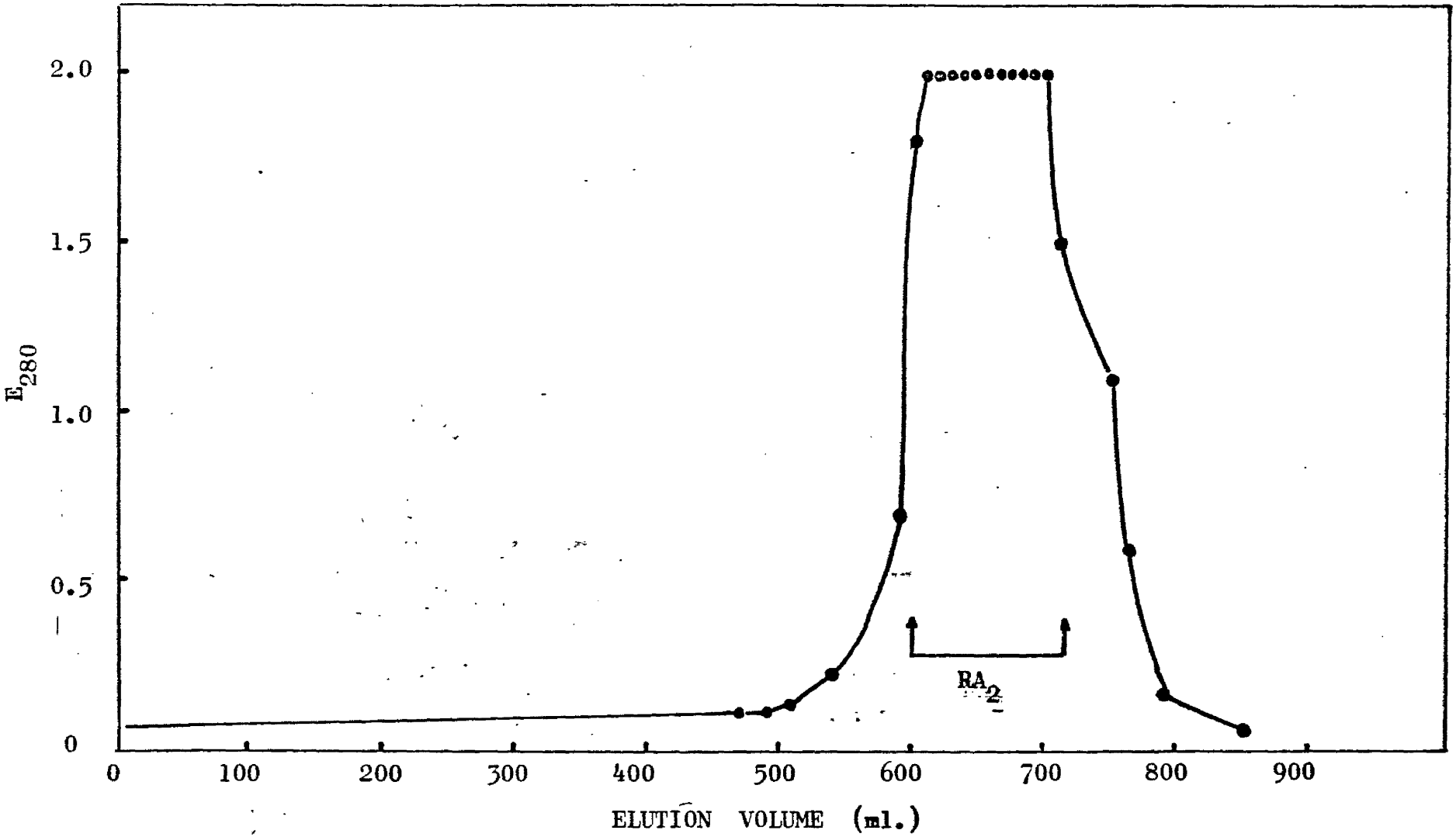
buffer and run in. Elution was achieved by an exponential salt gradient as follows. Three linked aspirator bottles were connected in series to the column. The bottle farthest from the column contained 566ml. of 3.93M - NaCl in 0.02M - phosphate buffer and the two others contained 650ml. each of phosphate buffer. Elution was commenced by starting the peristaltic pump (LKB Perspex Pump), connected to the outlet of the column, at a rate of flow of 16ml. per hr. and opening the taps between the aspirator bottles. Magnetic stirrers were used to obtain adequate mixing in all bottles. Fractions of approx. 8ml. were collected and the $E^{1cm.}_{280nm.}$ determined on each fraction. Figure 5 shows a typical crude albumin fractionation. The albumin fraction was selected by taking fractions containing albumin exclusively or predominantly with $E^{1cm.}_{280nm.}$ greater than 0.8.

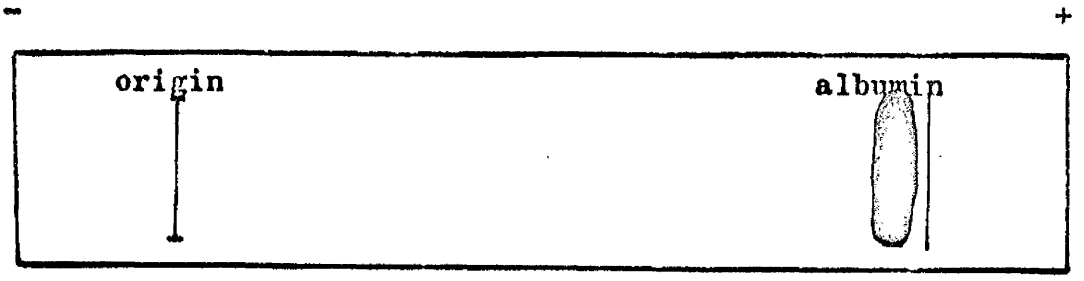
These fractions were checked by polyacryalamide electrophoresis (Section 2.9.2.). The selected fractions were pooled, dialysed and freeze dried (Fraction RA_2). lg. lots of Fraction RA_2 were re-run on the column (Fraction RA_3 and this fraction was re-run (after pooling, dialysing and freeze drying) producing a pure albumin fraction (Fraction RA_4) which was shown to be homogeneous by gel electrophoresis, agar immunoelectrophoresis and agar double diffusion (Figure 6).

2.12.2. CHICK

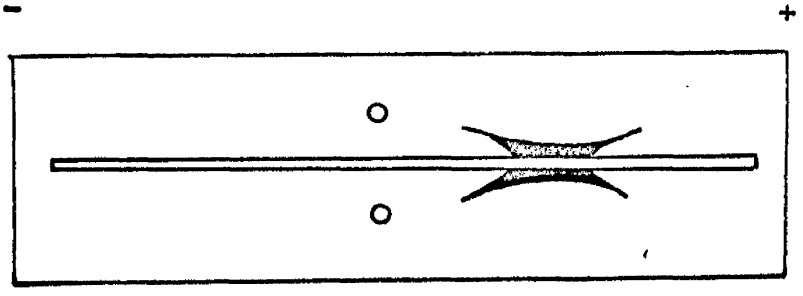
Birds were fasted overnight, then anaesthetised with chloroform and one jugular vein exposed and opened. After bleeding the animals were killed. Blood was allowed to clot at room temperature for 1hr. and serum was obtained as described above. Approx. 30-40ml. of serum was obtained from each bird. The

FIGURE 5: CHROMATOGRAPHY OF RA_1 ON DEAE - SEPHADEX A - 50 (medium grade). 1110 mg. APPLIED IN 15 ml. 0.02 M - PHOSPHATE BUFFER (pH 6.6). Column 40 x 2.5 cm. Flow rate 16 ml. per h.

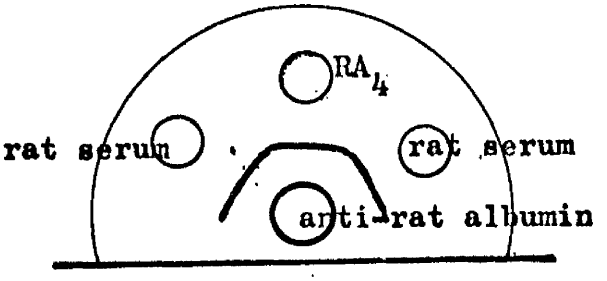




Polyacrylamide Gel Electrophoresis pH 8.6



Agar Immunoelectrophoresis pH 8.2



Agar Immunodiffusion

FIGURE 6 : RAT ALBUMIN FRACTION RA_4. GEL ELECTROPHORESIS, IMMUNO-ELECTROPHORESIS AND IMMUNODIFFUSION RESULTS.

subsequent preparation of crude albumin (Fraction CA_1) and a semi-pure Fraction CA_3 was similar to the procedure described above. The purity of the chick albumin was demonstrated by gel electrophoresis and agar immuno-electrophoresis (Figure 7).

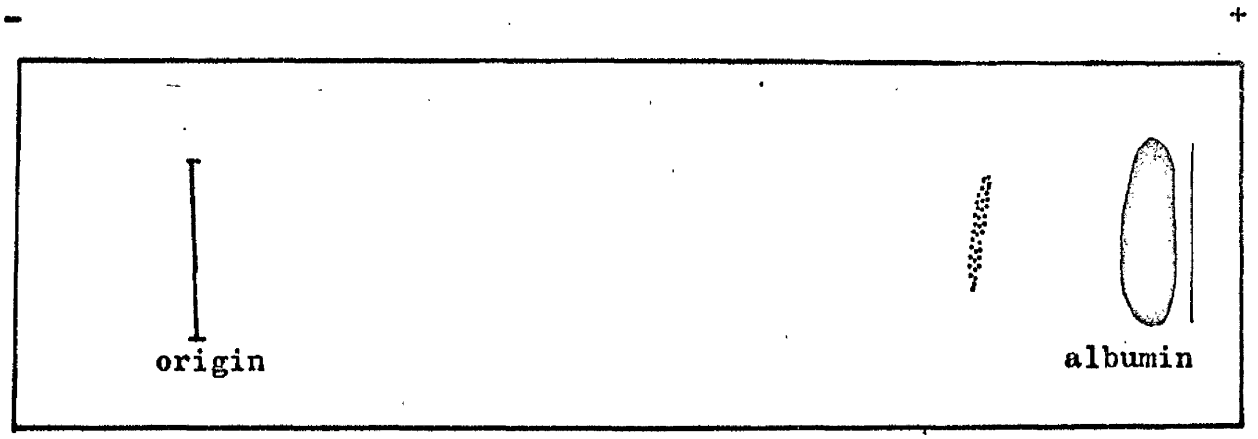
2.13. PREPARATION OF ALBUMIN AND SERA ANTIBODIES

2.13.1. PREPARATION OF RAT ALBUMIN AND CHICK ALBUMIN ANTIBODIES

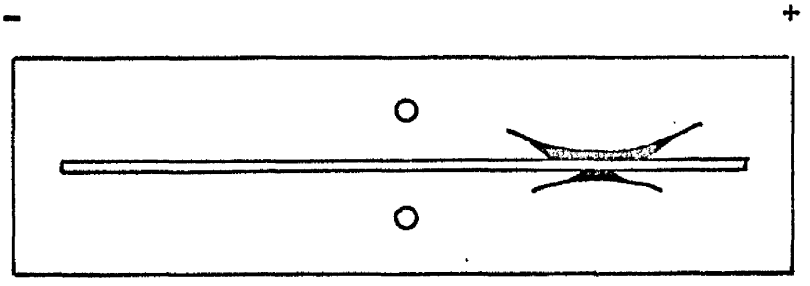
Rabbits were injected with the purified albumin by a modification of the method of Benjamin and Weimar (1963). The technique was as follows: 25mg. of pure albumin (Fractions RA_4 or CA_3) was dissolved in about 0.4ml. distilled water and emulsified with 2ml. of Freund's complete adjuvant (Difco) by constant suction and ejection for 10min. through a fine (No. 21) needle. The emulsion was considered satisfactory when a drop placed on water did not spread on the surface.

The emulsion was injected subcutaneously in the back (0.1ml. in each of 8 sites), intramuscularly in the hind quarters (0.4ml. in each site) and 0.2ml. to either side of the neck subcutaneously. Four weeks later 5mg. of albumin (dissolved in 1.5 ml. sterile 0.15M - NaCl) was administered intravenously. Five days later serum was obtained for immunodiffusion antibody demonstration. If a strong antigen-antibody precipitation line was obtained then the animal was bled by venesection from an ear vein. Usually about 30ml. of blood was obtained from each animal. This procedure was repeated 2-4 times at fortnightly intervals if the serum retained its antibody activity.

The blood was allowed to clot at room temperature for 1 hr. and it was then centrifuged at $800g_{av.}$/1hr./20°. The serum was collected and re-centrifuged at



Polyacrylamide Gel Electrophoresis pH 8.6



chick serum
anti-chick albumin
CA_3

Agar Immunoelectrophoresis pH 8.2

FIGURE 7 : CHICK ALBUMIN FRACTION CA_3. GEL ELECTROPHORESIS AND IMMUNO-ELECTROPHORESIS RESULTS

$800g_{av.}$/10min./20° to remove all red cells. The resulting serum was slightly haemolysed. The ɣ-globulins were purified by the method of Sargent (1967). Two volumes of 0.1M-phosphate buffer pH 7.8 containing 27% (w/v) Na_2SO_4 was added to the serum giving an 18% (w/v) solution. The precipitate was pelleted by centrifugation at $15,000g_{av.}$/10min./25° and dissolved in 0.15M - NaCl, and 27% (w/v) Na_2SO_4 added to give a final concentration of 16%. The precipitate was sedimented at $15,000g_{av.}$/10min./20°, washed once with 16% Na_2SO_4, dissolved in 0.15M - NaCl, dialysed against daily changes of 5 litres of distilled water for 3 days and freeze dried. Yields were of the order of 10-15mg. ɣ -globulin per ml. of sera.

2.13.2. PREPARATION OF RAT SERUM AND CHICK SERUM ANTIBODIES

An equal volume of sera was mixed with Freund's complete adjuvant emulsified and injected (2ml.) into rabbits as described in Section 2.13.1. above. Four weeks later 0.5ml. of serum was mixed with 2.5ml. sterile 0.15M-NaCl and administered by intraperitoneal injection. Within 3 days 0.2ml. of serum was mixed with 0.8ml. sterile 0.15M-NaCl and injected intravenously. The effectiveness of the antiserum was examined by agar double diffusion, and the antiserum was used without further purification.

2.14. PREPARATION AND INCUBATION OF LIVER SLICES

The rats were killed, following dietary treatment, by decapitation, allowed to bleed for about 1 min. with a jet of cold water washing the neck to prevent clotting, and the liver removed. 0.3mm. thick slices were prepared on a McIlwain tissue slicer (McIlwain and Buddle, 1953), the outer membrane-containing layers being discarded, and 4-5g. of the slices were washed in 100ml. of incubation media in order to reduce the amount of albumin initially present.

The slices were washed, with gentle agitation, for 45 min. at room temperature, the supernatant removed and 50ml. of fresh media added and washed for a further 15 min. The slices were drained on filter paper and weighed into 125ml. flasks containing 10ml. of medium at 37° in a humidified atmosphere of 95% O_2 and 5% CO_2. Each flask contained about 1g. of tissue.

The incubation media contained:
Na^+ 135, K^+ 10, Ca^{2+} 10, Cl 125 and HCO_3 40 mM/litre dissolved in distilled water. The slices were incubated for various periods up to 2hr. in the O_2/CO_2 atmosphere.

At the end of the incubation period the slices were separated from the medium (Slice Supernatant) by centrifugation at $10,000g_{av.}$/10min./2°. The slice supernatant was diluted with an equal volume of 0.15 M - NaCl containing 1:10,000 Thiomersalate and stored at -10°. For the estimation of intracellular albumin the precipitate was homogenised with a glass-teflon homogeniser (10 strokes at full speed in 5ml. of medium) and centrifuged at $15,000g_{av.}$/20min./2°. The supernatant was removed and stored at -10°.

2.15. ESTIMATION OF ALBUMIN CONTENT OF RAT LIVER AND SLICE SUPERNATANTS

2.15.1. SLICE SUPERNATANT (CAMPBELL AND STONE, 1957)

Before the estimation the slice supernatant was centrifuged at $15,000g_{av.}$/20min./17° to remove material precipitated by freezing and thawing. The cleared slice supernatant was then diluted to give levels of between 10-60µg. albumin per ml. and all estimations were made at two dilutions within this range. The average deviation between these duplicates was found to be 10% of the mean albumin content.

The antiserum solution used was 0.3ml. of a 1% (w/v) purified antiserum

(Section 2.13.1.) in 0.15M - NaCl. This was added to the slice supernatant and the volume made up to 1.5ml. with the saline solution. The tubes were incubated at 37° for 40min. and then left overnight at 2°. They were centrifuged at $300g_{av.}$/10min./2°, washed three times with 0.15M-NaCl at 2° and finally dissolved in 1.5ml. 0.1M - Na_2CO_3. After complete solution the tubes were centrifuged to precipitate any insoluble material. The $E^{1cm.}_{280nm.}$ was determined and the albumin content was calculated by reference to a series of albumin standards which were freshly prepared on the day of the assay. Figure 8 illustrates the albumin calibration graph. Results were expressed at ug. albumin per g. liver (wet weight).

In an attempt to find a simpler means of measuring the albumin concentration the fluorometric dye binding method of Rees et al. (1954) was examined. This method was shown to give a good correlation with the albumin content of standard solutions determined by immunoassay (Figures 9.1 and 9.2) but when applied to the slice supernatant the fluorescent method gave albumin levels some 2-3 times higher than the immunoassay method (Figure 9.3). It was concluded that the dye was not specific for serum albumin, due to dye binding by liver proteins, and the method was rejected.

2.15.2. SLICES

Campbell and Stone (1957) found that the tissue albumin levels were very variable when using the technique of Section 2.15.1 above. They showed that this resulted from substances co-precipitating with the albumin-antiserum precipitate which failed to dissolve in 0.1M-Na_2CO_3, and they demonstrated that this interference could be overcome by 'clearing' the solution before estimating the albumin content.

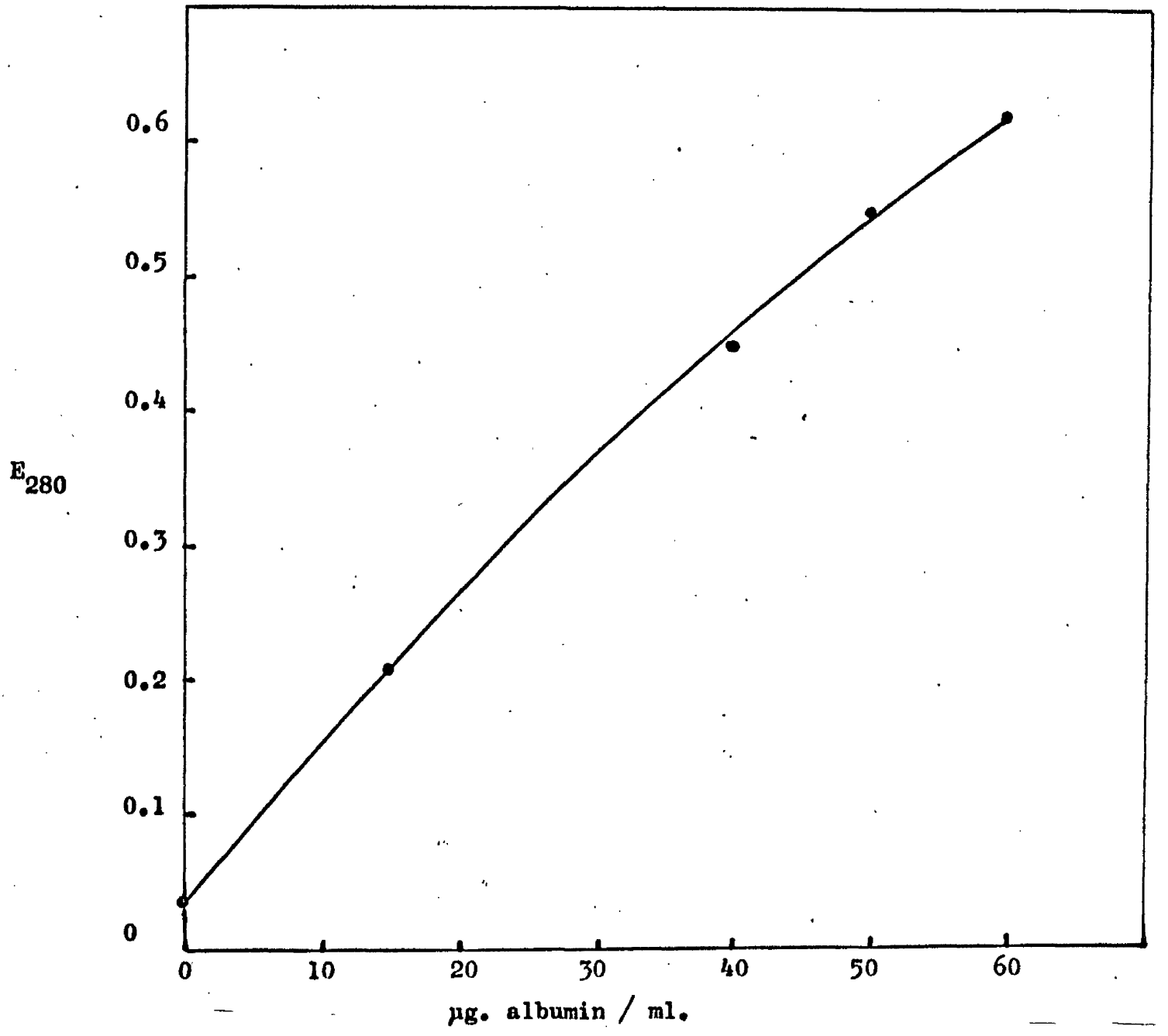


FIGURE 8 : STANDARD CURVE FOR THE ESTIMATION OF ALBUMIN BY THE IMMUNOPRECIPITIN ASSAY (SECTION 2.15.1).

Fig. 9.1 : Fluorescent assay calibration graph

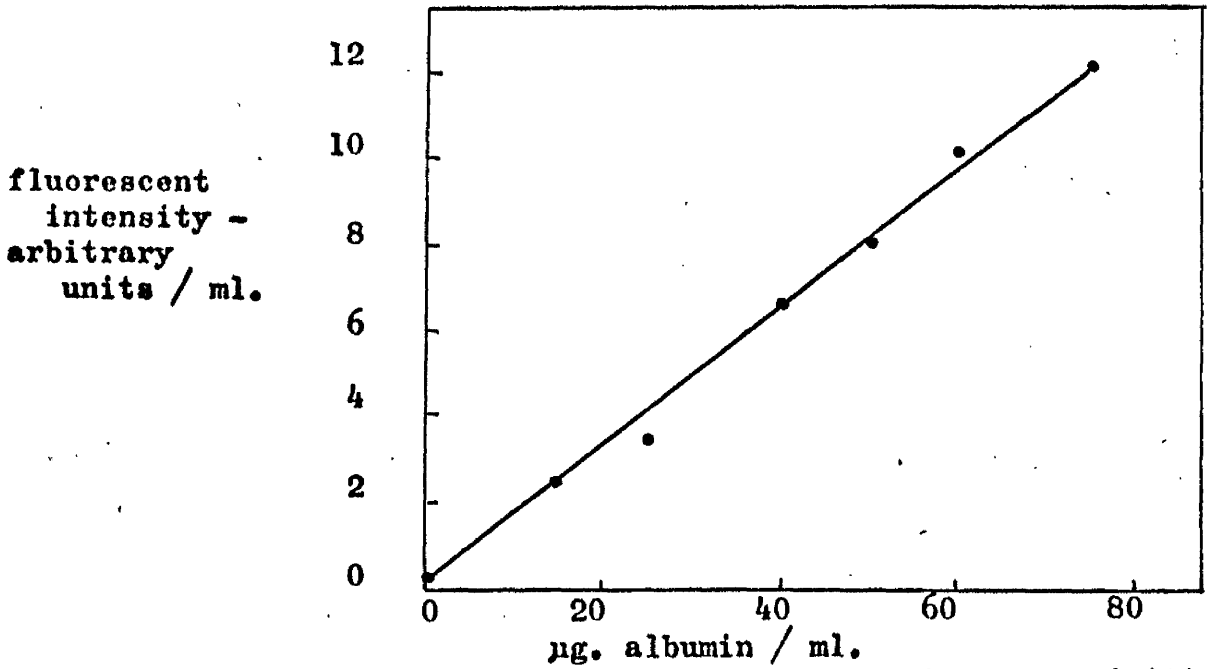


Fig. 9.2 : Correlation between fluorescent and immunoprecipitin albumin assays.

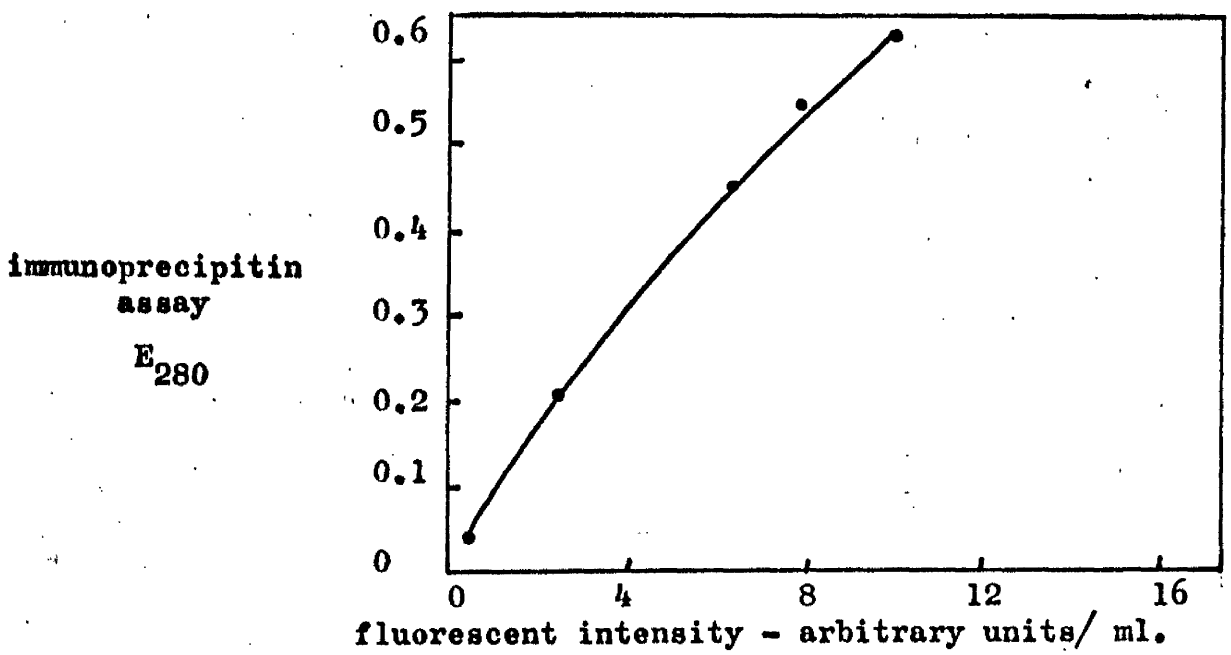
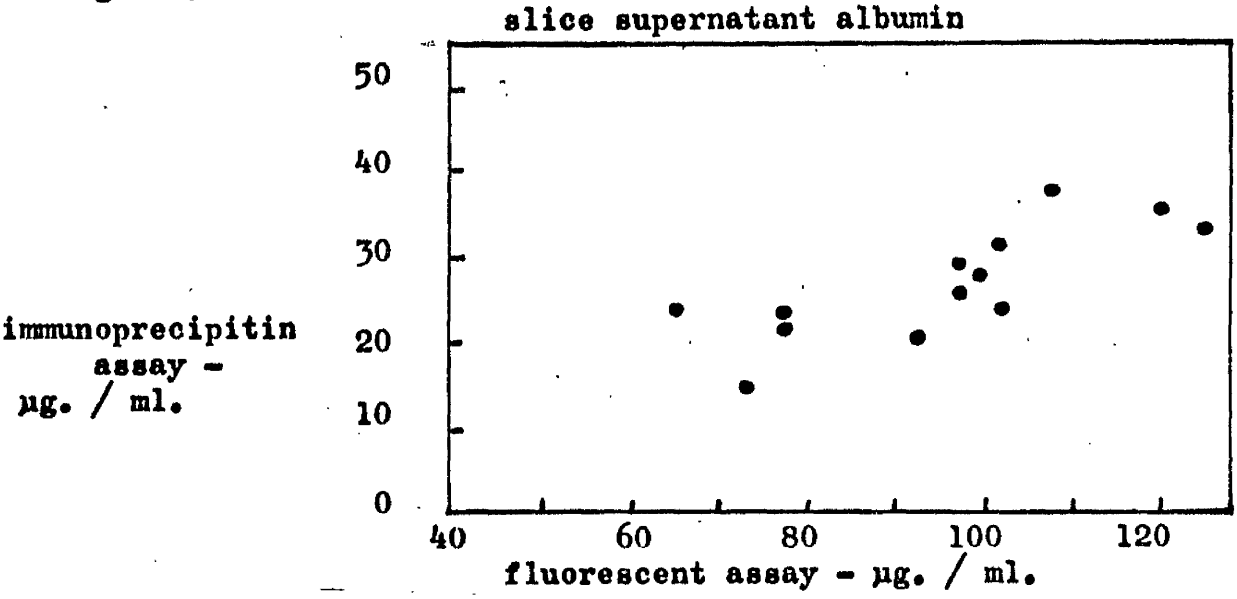


Fig. 9.3 : Correlation between fluorescent and immunoprecipitin assays of slice supernatant albumin



The method of clearing the tissue extract is as follows. 2ml. of extract was incubated for 30min. at 37° with 0.4ml. of 1% chick albumin antibody (w/v) and 0.6ml. of 0.1% chick serum albumin (w/v). After standing overnight at 2°, the precipitate was centrifuged at $15000g_{av.}$/20min./2° and the clear supernatant used for estimating albumin as described in Section 2.15.1. Results were expressed as ug. albumin per g. liver wet weight. Campbell and Stone (1957) showed that there was no loss of albumin by absorption onto the chick albumin-antibody precipitate.

3. RESULTS

3.1. THE EFFECT OF FEEDING WITH A TRYPTOPHAN-FREE AMINO ACID MIXTURE ON NUCLEAR RNA SYNTHESIS IN RAT LIVER

3.1.1. THE HIATT (1962) NUCLEAR RNA EXTRACTION PROCEDURE

The object of this work was to determine the nuclear response to the T+ and T- diets as described in the Introduction (Section 1.3). Several problems presented themselves at the outset. What nuclear preparations and nuclear RNA extract procedures should be used?

Roodyn (1969), in a masterly review of nuclear isolation techniques, described 11 basic methods and many modifications and adaptations of these methods. Because a rapid routine method which could be readily applied to the large numbers of animals involved in dietary work was required only two methods were considered:

1. The Citric Acid Procedure of Dounce (1943) and
2. The Chauveau Procedure (Chauveau et al., 1956)

The Citric Acid Procedure was originally applied by Dounce to frozen rat liver but in the present instance the method was applied to minced rat liver (Section 2.6.1). This procedure was used in preference to the Chauveau technique (which required homogenisation in high molarity sucrose) because the laboratory was not equipped with the necessary high-torque homogenising motors.

We later found that it was necessary to use the 5% Citric Acid Procedure of Busch (1967) because the extracted RNA contained more 45S RNA than could be obtained from nuclei isolated in 0.025M-Citric Acid. Although this type of preparation is known to cause nuclear protein loss (Dounce, 1955) the fact that citric acid lyses red cells and the method fulfils the requirements of rapidity coupled with a good yield of apparently 'clean' nuclei made the citric acid procedure first choice.

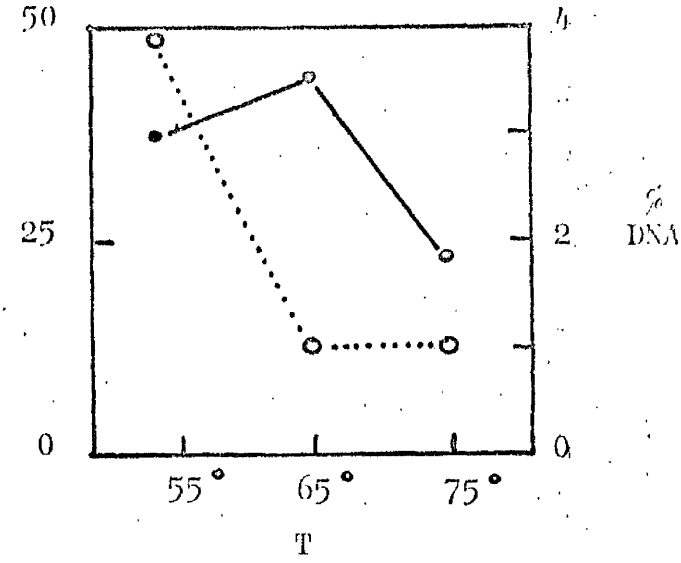
At the time this work was started (late 1965) there were two main methods for extracting RNA from rat liver. The low ionic strength cold phenol SLS method of Hiatt (1962) and the high ionic strength hot phenol SLS method of Steele (c.f. Muramatsu et al., 1966). While the gradient analysis produced by Steele's method was far superior to that produced by Hiatt, equipment limitations forced us to use the latter procedure initially.

Thus nuclei were isolated by the Citric Acid Procedure (Section 2.6.1.1) and RNA extracted by the cold phenol technique of Hiatt (Section 2.7.1). Initially the extraction procedure was found to produce only 25% of the expected yield of RNA and Figure 10 shows the effect of varying this extraction procedure by altering temperature, [NaCl] and [SLS].

Figure 10.1 shows the effect of varying the temperature of the initial phenol extraction. These temperature ranges were chosen because the bulk of published work suggested that temperatures around 65° aided RNA extraction (Rake and Graham, 1964; Samis, Wulff and Falzone, 1964; Scherrer and Darnell, 1962; Wecker, 1959). Each point represents the mean of 2-4 separate experiments. The optimum temperature appeared to be 65° as was expected, and this temperature was chosen for all subsequent extractions. The DNA contamination is also reduced considerably by temperatures of 65° and upwards (Rake and Graham, 1964) and this was a useful advantage in the Hiatt preparation which involves a DNase treatment stage to reduce DNA contamination.

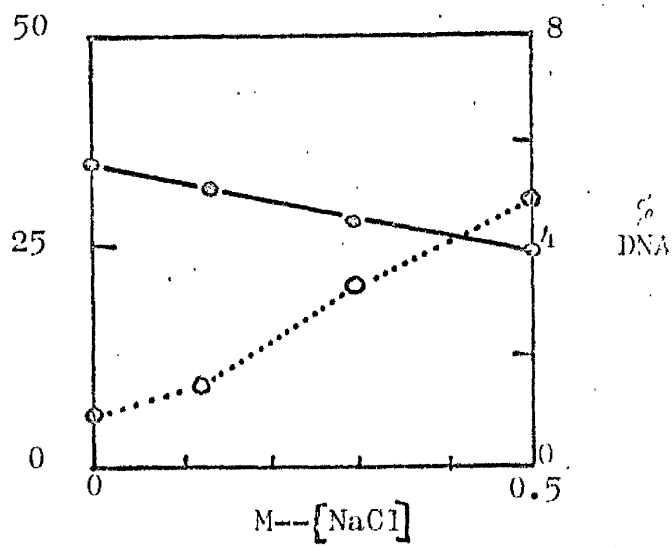
Next the ionic strength of the extracting buffer was altered by increasing the NaCl content over the range 0 to 0.5M-NaCl. Figure 10.2 shows the results when using a temperature of 65°. DNA extraction increased with ionic strength but the RNA extraction decreased. Stansly and Seese (1965) showed that Ehrlich ascites cells released about 85% of their RNA over the range

1. EFFECT OF TEMPERATURE



2. EFFECT OF [NaCl]

65°



3. EFFECT OF [SLS]

65°

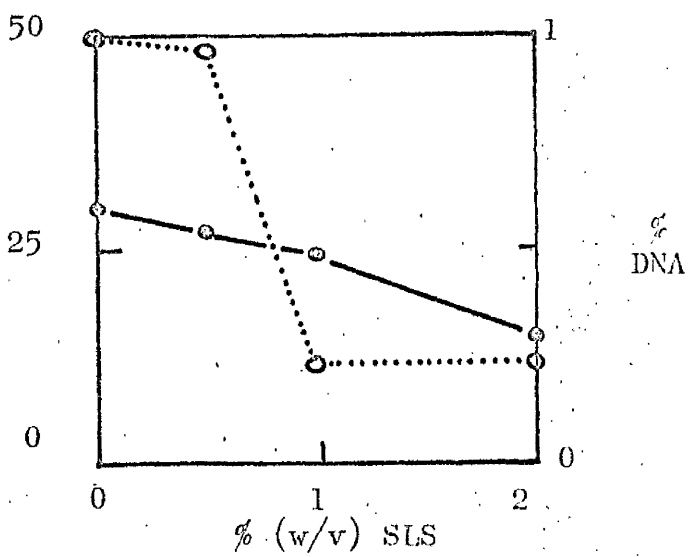


FIGURE 10 : THE EFFECTS OF VARYING THE EXTRACTION PROCEDURE

RNA o—o DNA o·····o

0.145 - 0.5M-NaCl but this was not the case with rat liver nuclei. These authors also showed that homogenisation of the cells in phenol led to an increase in the RNA yield but with a maximal release of DNA about 0.5M-NaCl. Clearly the hot phenol is reducing the DNA contamination in the case of rat liver but it is 400% times higher when salt is present at 0.5M. It is relevant to note that Steele used 0.3M-NaCl in his extraction procedure but this was associated with long phenol extraction times.

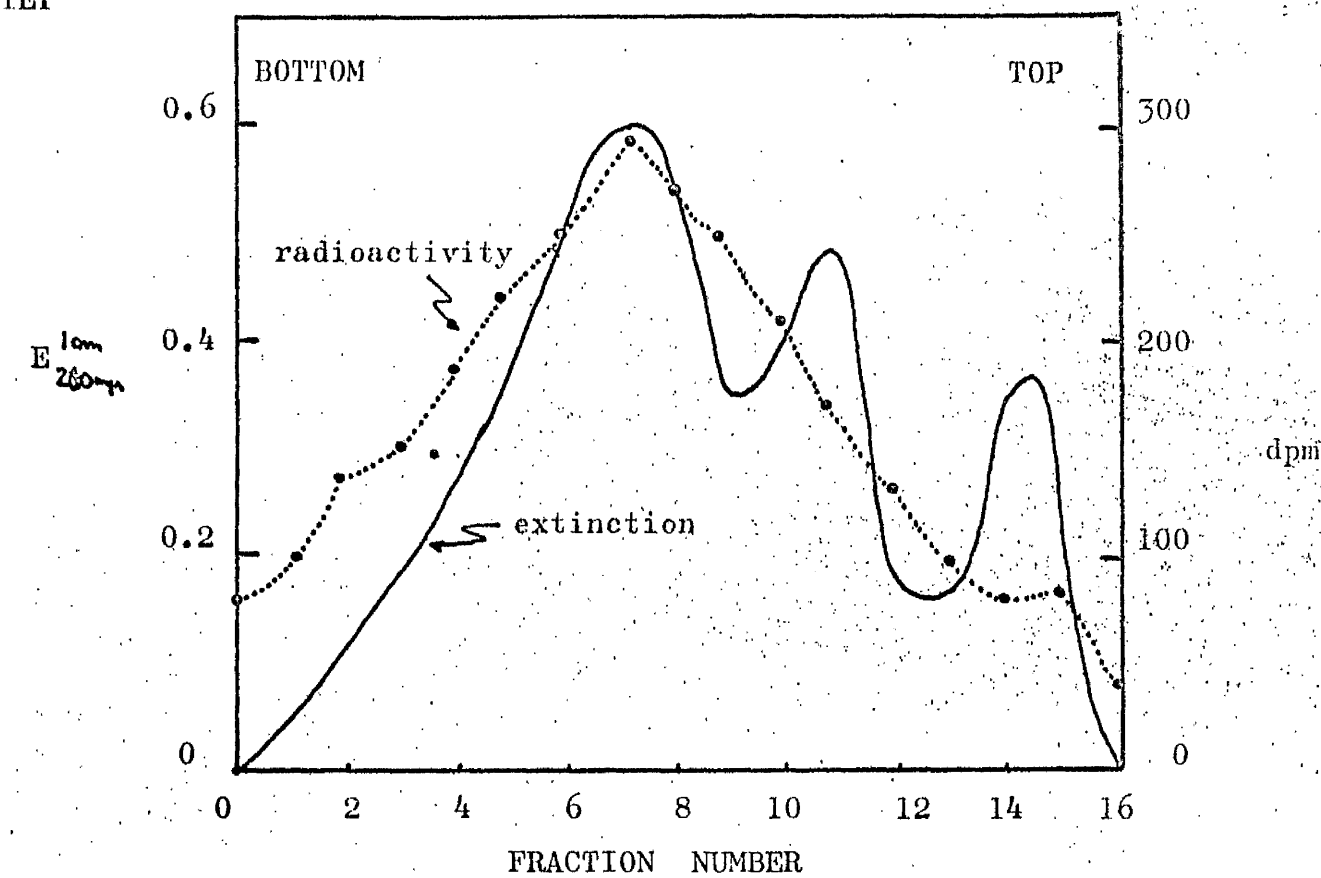
It was concluded that the presence of salt did not increase RNA extraction in this particular experimental procedure but this may be due to the absence of a homogenising step in phenol which Steele used. Unfortunately this step could not be done because there were no small volume homogenisers available.

In Figure 10.3 the effect of altering the SLS concentration is shown when using an extracting buffer at 65°. I am certain that the decreasing yield of RNA and DNA with increasing [SLS] is due again to the rapid gelling of the nuclei leading to poorer extractions. Again it is likely that the yield could have been increased by a homogenisation step. It should also be noted that the yields between groups of experiments were very variable (25 - 40%) even when using the final hot phenol preparation (Section 2.7.1).

3.1.2. THE EFFECT OF A TRYPTOPHAN-FREE AMINO ACID FEED ON NUCLEAR RNA SYNTHESIS EXAMINED BY THE HIATT PROCEDURE

The modified Hiatt procedure was used to examine the effect of feeding the T+ and T- diets on the synthesis of nuclear RNA as judged by the incorporation of radioactivity into isolated, sucrose density gradient separated, RNA. Rats were fed with the T+ and T- diets and killed 2hr. later. 5μCi of [8-^{14}C] adenine sulphate was administered by intraperitoneal injection and the rats were killed 30min. later. Figure 11 shows the results (mean of two separate experiments).

1. T+ DIET



2. T- DIET

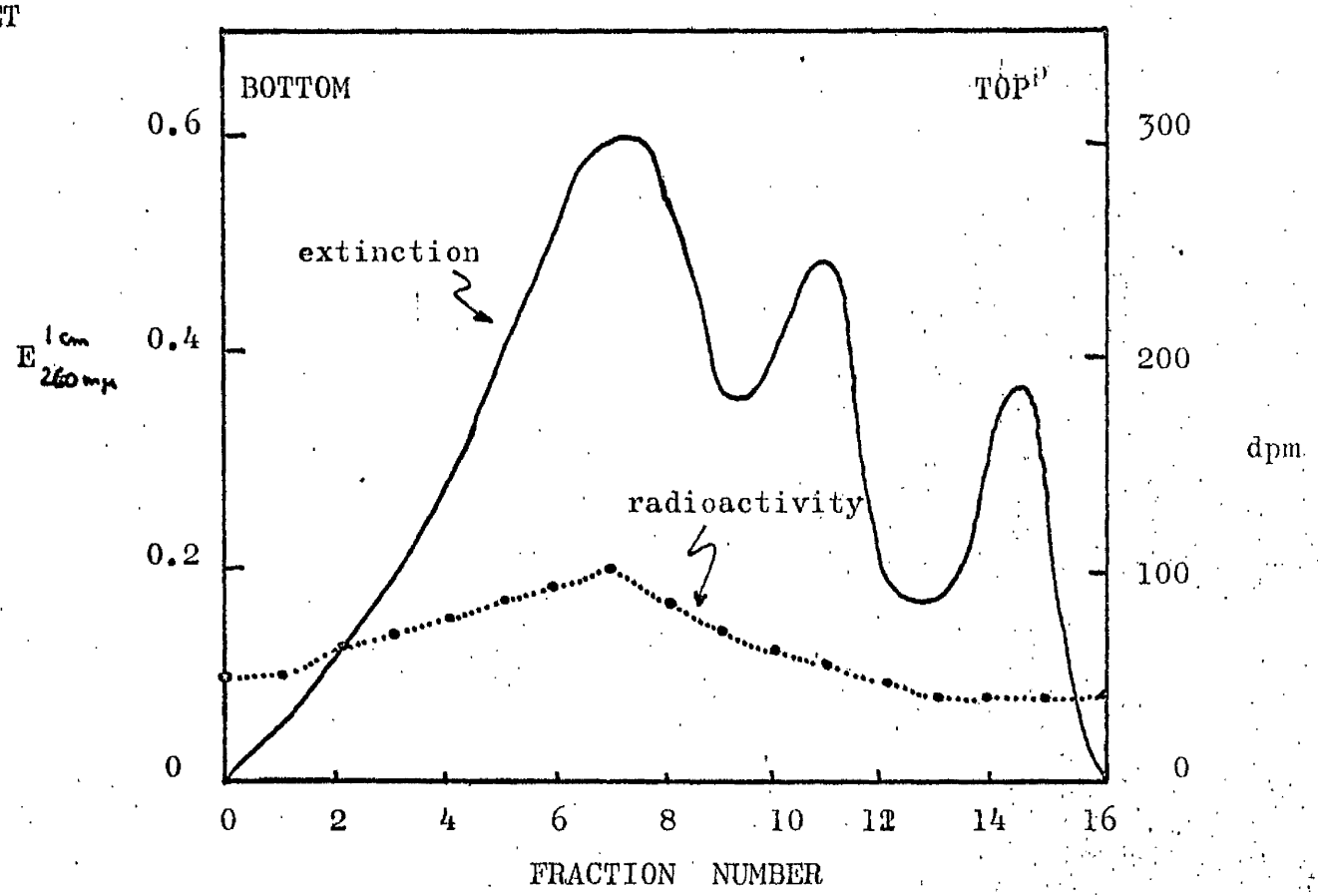


FIGURE 11 : THE EFFECT OF T+ AND T- DIETS FED 2 hr. PREVIOUSLY ON THE INCORPORATION OF [8-^{14}C]ADENINE SULPHATE INTO RAT LIVER nRNA.

Clearly there appears to be a greater incorporation of isotope into the nRNA of the T+ fed animal. A confirmatory experiment (similar conditions) was carried out using 200μCi of [5-^{3}H]orotic acid administered by intra-peritoneal injection (Figure 12). Here again the effect of the T+ diet is apparent as an increased incorporation of labelled compound into RNA.

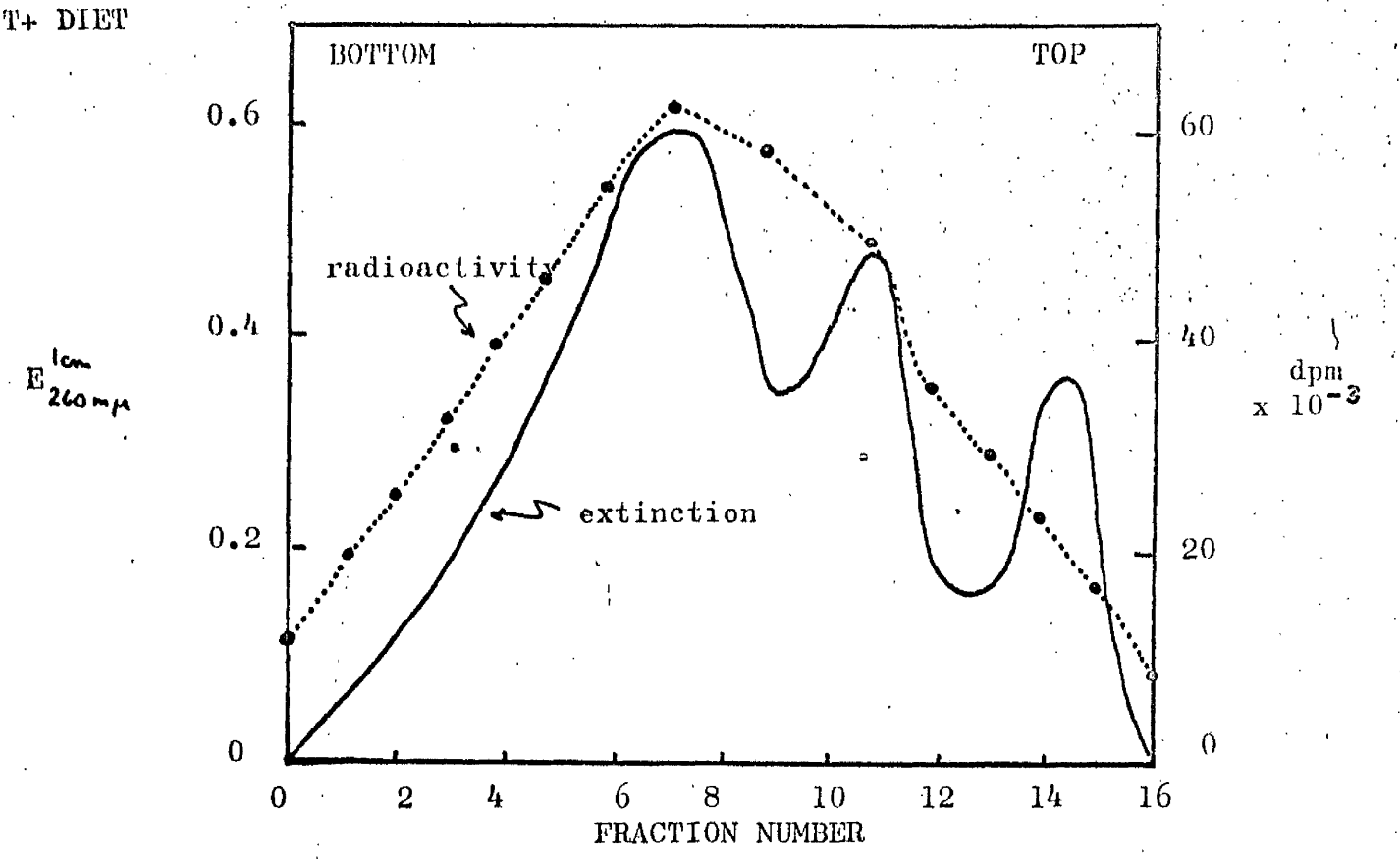
A comparison of Figures 11 and 12 suggests that there is a greater proportional incorporation of orotic acid into the T- nRNA than adenine sulphate. Before seeking a possible reason for this it is necessary to consider how these labelled materials are incorporated into RNA. Adenine sulphate is converted into AMP by adenine phosphoribosyltransferase [E.C. 2.4.2.7] in the presence of 5-phosphoribosylpyrophosphate. This enzyme is present in high activity in rat liver (Murray, 1966). A similar reaction converts orotic acid to orotidine-5'-monophosphate (orotate phosphoribosyltransferase [E.C. 2.4.2.10]) which can be decarboxylated to form UTP. All the nucleoside triphosphates can be formed from their monophosphates although CTP can be synthesised by CTP synthetase [E.C. 6.3.4.2.] from UTP.

The concentrations of ATP, CTP and UTP have been obtained in young male rats fasted for 12hr. (Bucher and Swaffield, 1966):

ATP	3 μmole/g. liver	(80% of adenosine phosphates)
UTP	0.3 μmole/g. liver	(70% of uridine phosphates)
CTP	0.07 μmole/g. liver	

These figures represent the total nucleoside triphosphate pool of the liver. They give no indication of the rapidity (or otherwise) of inter-pool exchange within the liver. Bucher and Swaffield (1966) were able to demonstrate that the i.v. injection of [6-^{14}C]orotic acid was followed by a maximum in UTP specific

1. T+ DIET



2. T- DIET

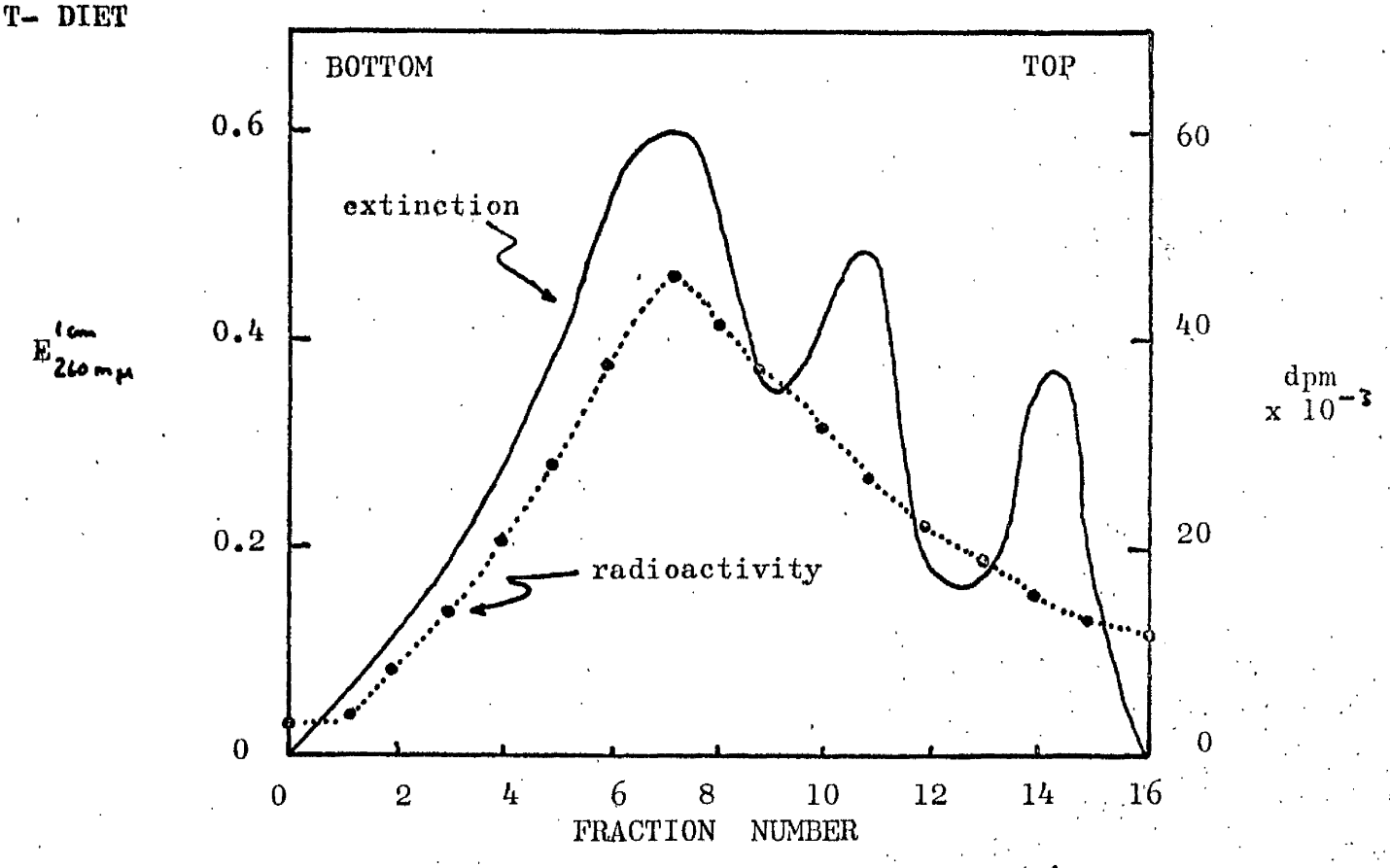


FIGURE 12 : THE EFFECT OF T+ AND T- DIETS FED 2hr. PREVIOUSLY ON THE INCORPORATION OF [5-^{3}H]OROTIC ACID INTO RAT LIVER nRNA.

activity, in the total liver pool, at 4 min. and this quickly dropped away as the UTP passed through the pool(s).

While it is likely that the ATP pool involved in RNA synthesis is smaller than the Bucher and Swaffield figures indicate (and their data is similar to other workers) it is probably bigger than the UTP or CTP pools. Thus the labelled adenine sulphate is liable to be diluted (as labelled ATP) to a greater extent than the orotic acid labelled UTP and CTP. In addition adenosine is present in ribosonal RNA to about 15 - 20% compared with the uridine + cytidine contribution of about 50% (Hirsch, 1966). Lastly, the rate of portal absorption and blood → liver uptake are likely to differ between orotic acid and adenine sulphate.

It is of interest to note that the radioactivity profile of the T+ experiments differ to a small extent between the 28S and 4S peaks, because of the greater relative radioactivity of the orotic acid injected group. Also, as previously noted, the activity of the T- RNA (Figure 12) is about 0.7 of the corresponding T+ RNA whereas the adenine sulphate group has a 1:0.3 proportion (Figure 11). One possible explanation may be animal variation but an alternative explanation may be the occurrence of a high G-C content RNA between the 28S and 4S peaks.

Finally an experiment was done using both [8-^{14}C] adenine sulphate and [5-^{3}H] orotic acid using the whole RNA pellet instead of the separated fractions. In retrospect this experiment should have been done in a different way but the results are nonetheless of interest. The experimental design is described in Table 2. All animals were killed 2.5hr. after they were first fed.

Table 2

The experimental design of the double labelling experiment described in the text.

Group	Fed 0 hr.	200 μCi [5-^{3}H] orotic acid 0 hr.	Fed 2 hr.	5 μCi [8-^{14}C] adenine sulphate 2 hr.
1	T-	Yes	No	Yes
2	T-	Yes	T-	Yes
3	T-	Yes	T+	Yes
4	T+	Yes	No	Yes
5	T+	Yes	T+	Yes

Before the second feed an attempt was made to aspirate the stomach contents although less than 1 ml. of aspirate was obtained. At death the stomachs of all animals were distended.

The experiments were done in duplicate and the results in Figure 13 represent the mean of 2 results. The [5-^{3}H]orotic acid was given 2.5 hr. before death and it is present in greater amounts than is the adenine sulphate which was given only 0.5 hr. before death. From the data of Bucher and Swaffield given above it would seem likely that no orotic acid should be present by 2 hr. after injection but there is clearly a marked increase in tritium labelling following the second T+ feed (group 5). This increase is also reflected in the ^{14}C labelling of the same group. The increase of ^{14}C labelling between the group 2 animals (T-, T-) and group 5 (T+, T+) is about 6x whereas the tritium increase is about 3x. This agrees well with the results shown in Figures 11 and 12, although the gradient analysis clearly gives more information.

It can be concluded, from this section, that the T+ diet appears to increase

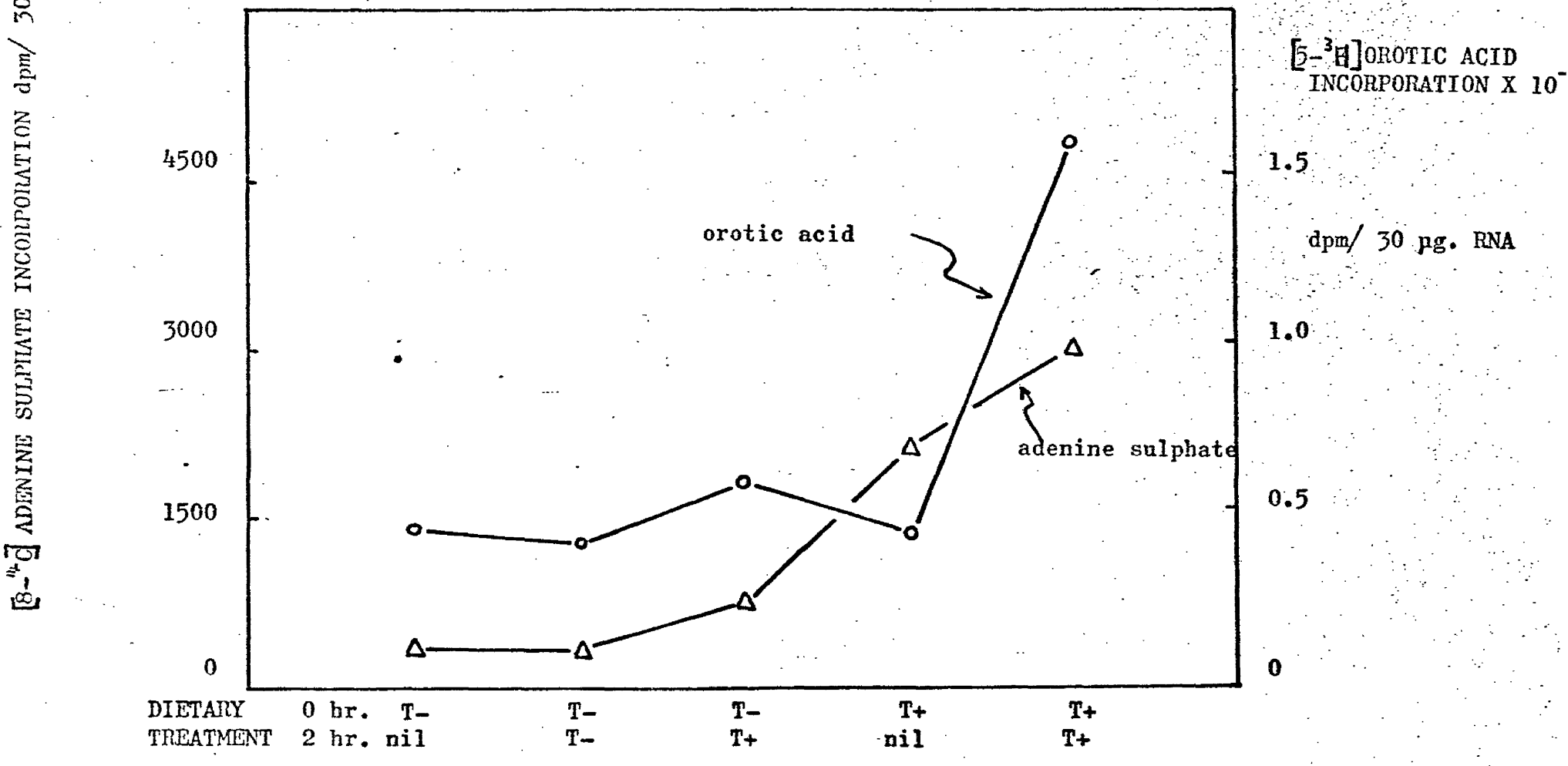


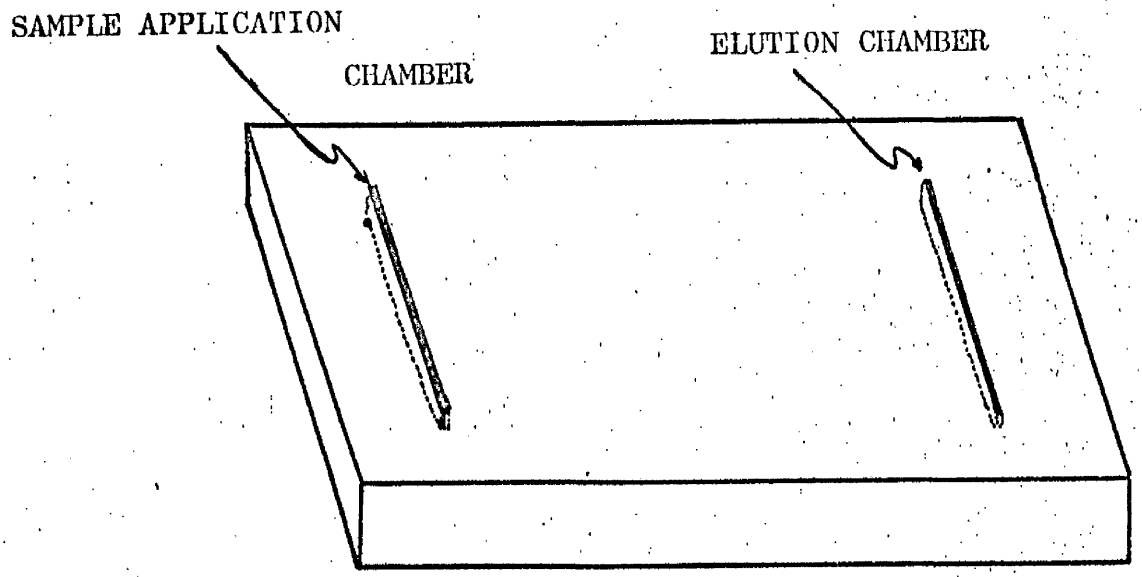
FIGURE 13: THE EFFECT OF THE T+ AND T- DIETS ON THE INCORPORATION OF [5-^{3}H] OROTIC ACID AND [8-^{14}C] ADENINE SULPHATE INTO RAT LIVER nRNA.

the incorporation of RNA precursors (adenine and orotic acid) into all RNA species except possibly around the 4S region.

3.1.3. AGAROSE GEL ELECTROPHORETIC SEPARATIONS OF NUCLEAR RNA

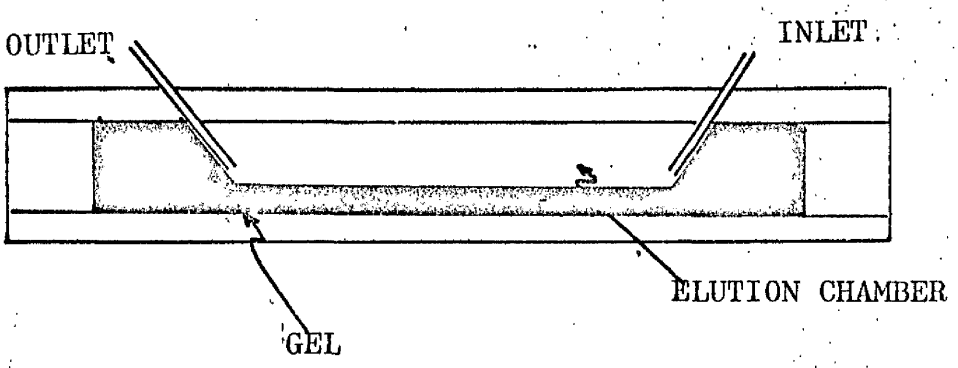
Currently the accepted technique for gel electrophoresis is the 2% polyacrylamide method devised by Loening (1967). Long before this technique had become available, and earlier than the agar technique (Tsanev, 1965), McIndoe, working in Glasgow, had devised a 2% agarose slab gel electrophoretic technique for the separation of rat liver RNA. The details of this technique were not in fact published until some years later (McIndoe and Munro, 1967) because after the method had been perfected McIndoe spent several years attempting to use the system as a continuous electrophoretic separative device. On joining the laboratory I collaborated with McIndoe in another attempt to devise a continuous electrophoretic separation of nRNA, because the potential of the agarose system was more promising than the current sucrose density gradient method which we were using.

The apparatus which we used was relatively simple (Figure 14), consisting of the ordinary slab with an elution chamber added and a single sample chamber which extended the whole width of the gel. A cover was placed on top of the elution chamber thus allowing perfusion by gel buffer. Initially we used rat liver tRNA for testing the system. Figure 15 shows the separation of 150 µg of tRNA achieved at 250v and 50mA. The elution chamber was perfused by gel buffer at 0.65ml/min. (details of buffer used are described in Section 2.9.1) and the total run time was 35min. The yield of RNA was 65% and this low yield was due in part to denatured RNA remaining at the origin where it could be stained after the separation had been completed.



GEL SLAB

WITH ELUTION CHAMBER COVER REMOVED



SECTION THROUGH ELUTION CHAMBER AND COVER

FIGURE 14 : CONTINUOUS ELECTROPHORESIS APPARATUS (MODIFIED GEL SLAB)

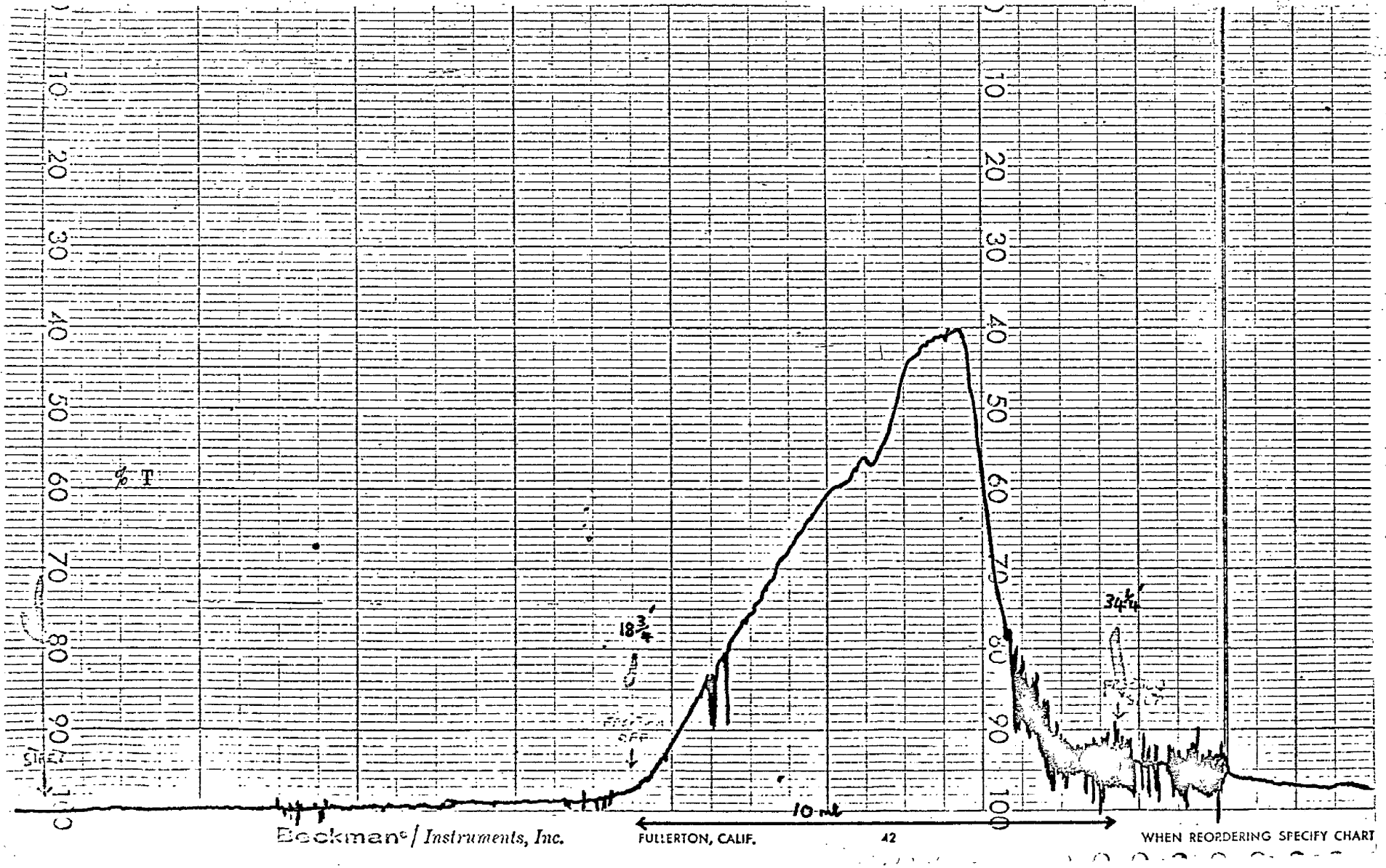


FIGURE 15: THE CONTINUOUS ELECTROPHORESIS PATTERN OF RAT LIVER tRNA OBTAINED BY GEL SLAB

The system appeared to work well and rat liver nRNA (obtained by the technique of Section 2.7.1) was examined. Figure 16 shows the separation of 300µg. of nRNA obtained over 2.5hr. at 250v and 50mA. The yield was 66%. The %T values are high but three definite peaks are present and the separation is certainly as good as the earlier sucrose density gradient work (Figures 11 and 12 Note, however, that the 28S peak is normally higher than the 18S peak but this is not so in Figure 16. The reason is due to the decreasing mobilities of the larger RNA molecules in relation to 4S. Thus each peak takes longer to be eluted and becomes therefore more dilute than the preceding peak. The dilution is exponential and it can only be corrected for by a reciprocal exponential decrease in the flow of the elution buffer. R.W.R. Baker (personal communication) has designed such a pump but this information was not available to me at the time we were working on this problem.

Despite the success of the nRNA separation shown in Figure 16 we were unable to repeat this work. The overheating of the gel due to the long exposure to a current of 50mA led to distortion of the gel around the elution chamber with consequent leaks and loss of RNA. After many unsuccessful modifications of the basic system it was decided to abandon this apparatus and to use one of the methods of continuous electrophoresis available and to use one of the methods of continuous electrophoresis available in the literature.

McIndoe (personal communication) had used the method of Racusen and Calvanico (1964) without success and the apparatus of Jovin, Chrambach and Naughton (1964) and Gordon and Louis (1967) were too difficult to build so we chose the system of Hjertén, Jerstedt and Tiselius (1965) which I had used in the FEBS Summer School in Uppsala in 1966. This apparatus was reasonably easy to

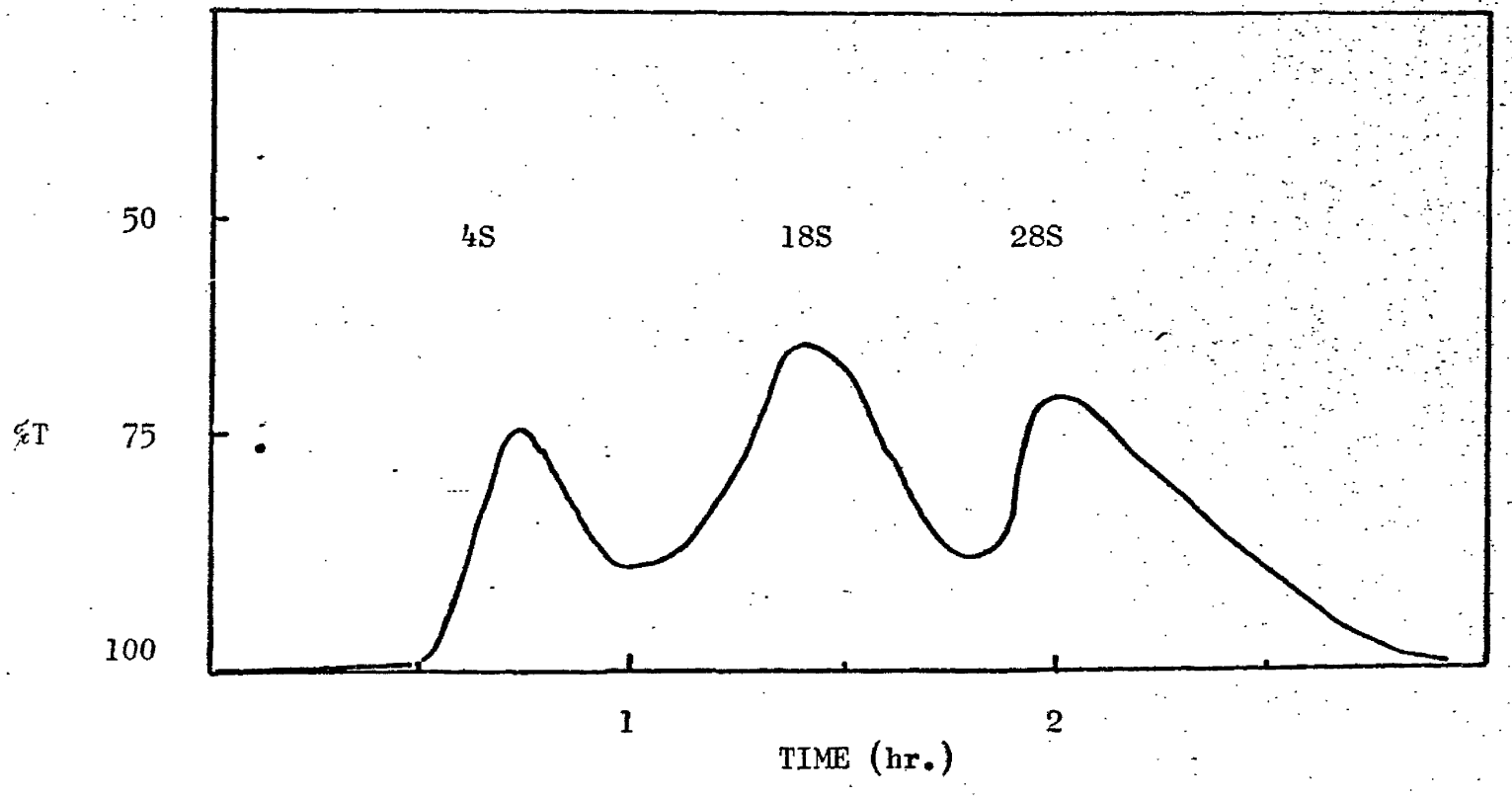


FIGURE 16 : THE CONTINUOUS ELECTROPHORESIS OF m-RNA ON AGAROSE GELS

build (Figure 17) and it consisted of an agarose gel column cooled by an outer water jacket. The side arm was filled with electrode buffer (similar to the buffer used in the McIndoe system) and the sample was applied, in a sucrose solution, to the top of the gel column. Current was applied by electrodes immersed in vessels into which both ends of the apparatus are inserted. Elution is obtained by applying gentle suction on the elution chamber outlet which draws buffer through the perforated wall of the chamber, through the Pevikon and out to a fraction collector. The results of two of the better separations are shown in Figures 18.1 and 18.2. It can be seen that they compare unfavourably with the slab technique which we had used.

In conclusion we were too ambitious. The results obtained by Weinberg, Loening, Willems and Penman (1967), using a simple polyacrylamide disc electrophoresis system, display a technical perfection which could never be equalled by a continuous electrophoresis system. While the agarose slab technique gave similar separations, the clear polyacrylamide gel allowed better u.v. scanning than the thickly opaque agarose gel and thus it would seem that we should have used the Loening system from the initial stages of our work. In retrospect this certainly seems true but at the time there were no u.v. scanning facilities available to us and this led us to attempt separations by continuous electrophoresis methods.

3.1.4. THE PENMAN (1966)-NUCLEAR RNA EXTRACTION PROCEDURE

The preparations of nRNA obtained by the Hiatt procedure (Section 2.7.1) were technically unsatisfactory because of the absence of 35S and 45S RNA profiles (cf. Muramatsu *et al.*, 1966) and the variable but low yield of nRNA obtained. The method of Penman (1966), described in Section 2.7.2, was attempted as an

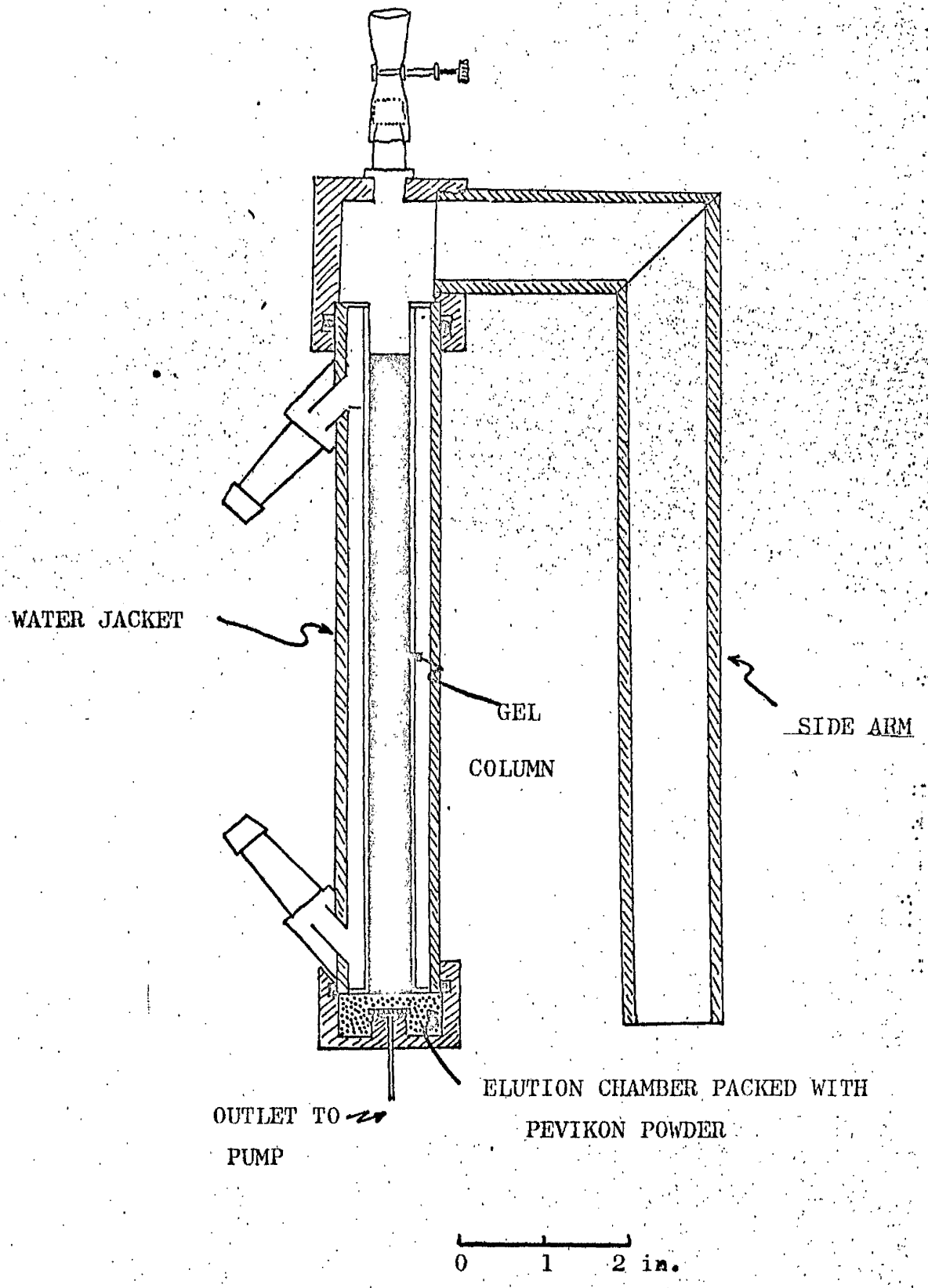


FIGURE 17 : CONTINUOUS FLOW ELECTROPHORESIS APPARATUS AFTER HJERTÉN et al. (1965)

FIGURE 18: nRNA SEPARATIONS ACHIEVED ON THE HJERTEN APPARATUS

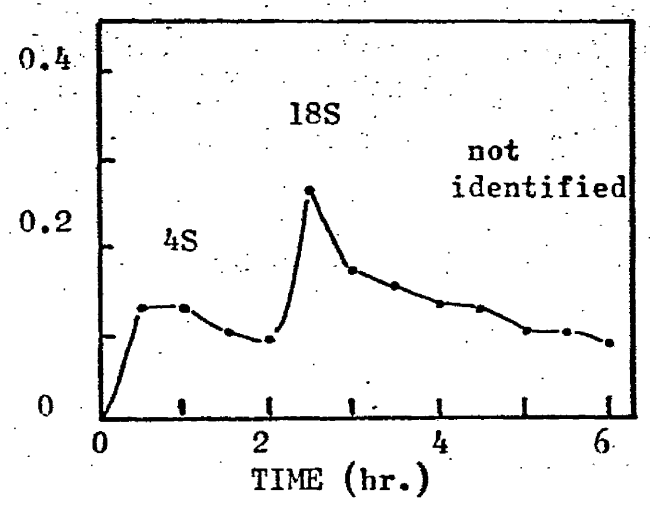


FIGURE 18.1: 0.4 mg. nRNA applied. 250v 15mA elution 6ml./hr. Yield 33%.

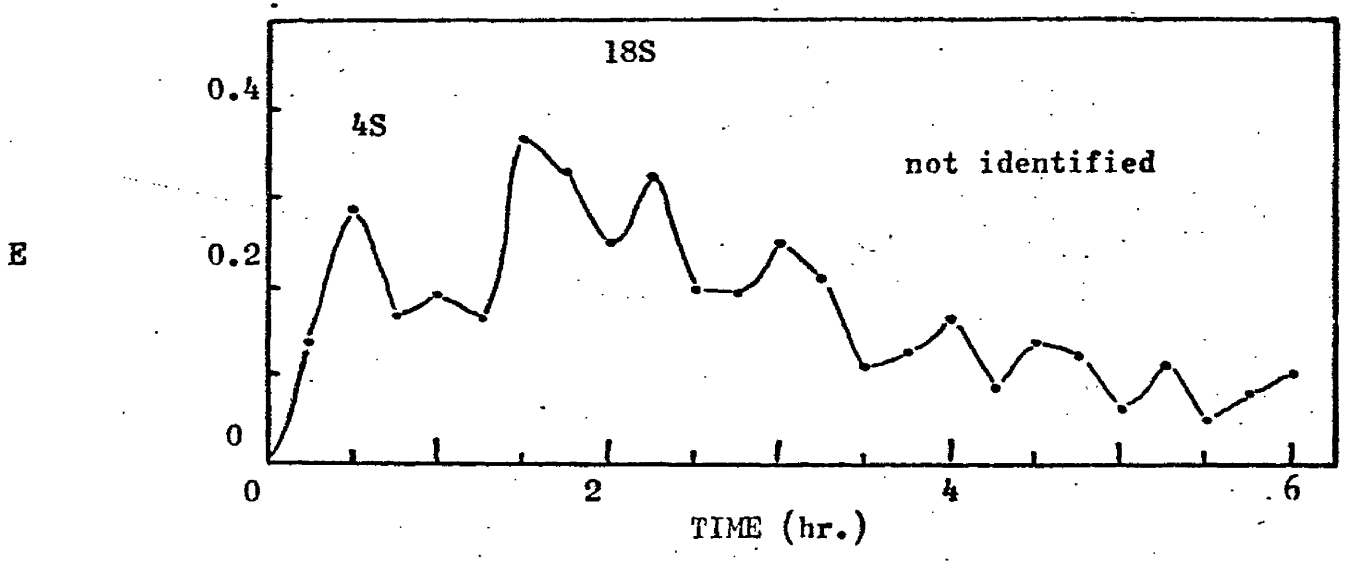


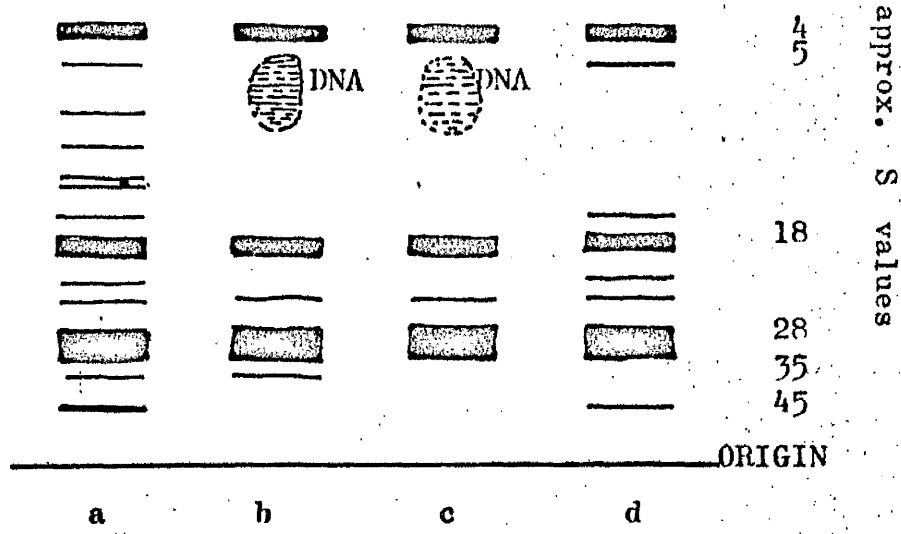
FIGURE 18.2 : 0.9 mg. nRNA applied. 250v 17mA elution 2.8 ml./hr. Yield 23%.

alternative nRNA extraction procedure because it was considerably quicker than the Hiatt procedure, and this in turn would mean less possibility of breakdown of high molecular weight RNA, and an additional advantage was the appreciable yield of 45S RNA from HeLa cell nuclei (Penman, 1966).

The method (Section 2.7.2) consists of the disruption of nuclei by suspension in a buffer containing 0.5M-NaCl and the digestion of the resulting thick gel by DNase [EC 3.1.4.5]. RNA is then extracted by a hot phenol-SLS technique and the phenol removed, together with protein contamination, by several chloroform-isoamyl washes. The agarose electrophoresis technique (Section 2.9.1) was used to examine the resulting RNA samples.

Figure 19(b) shows the electrophoretic pattern of nRNA obtained by this method. This should be compared with nRNA obtained by the Hiatt preparation (Figure 19a). It can be seen that the Penman procedure lacks 45S and 5S bands, as well as many of the minor bands, and that there is considerable DNA contamination of the RNA (also experienced by Penman) caused by DNase treatment of the nuclei. The DNA is readily differentiated from RNA by its metachromatic staining with toluidine blue. Clearly the 3min. DNase digestion of the nuclei (this time was found necessary to ensure complete digestion of the DNA gel) at 20° has allowed RNases to destroy the high molecular weight RNA species.

The DNase digestion of HeLa cell nuclei was found by Penman to leave a particulate fraction which was later recognised as nucleoli (Penman, Smith and Holtzman, 1966). This technique has been the basis of the vast amount of work on the synthesis of precursor rRNA in the nucleolus of HeLa cells (reviewed by Darnell, 1968). It seemed of interest to use this method to attempt the isolation of rat liver nucleoli as the technique is considerably simpler than the sonication method (Muramatsu, Hodnett, Steele and Busch, 1966).



a) nRNA obtained by Hiatt procedure

b) nRNA obtained by Penman procedure

c) "nucleoplasm" RNA

d) "nucleoli" RNA

b) and c) show DNA contamination behind the 5S position

FIGURE 19 : AGAROSE GEL ELECTROPHORETIC PATTERNS OF nRNA OBTAINED BY VARIOUS PROCEDURES.

'Nucleolar' and 'Nucleoplasm' fractions were obtained, the RNA extracted and examined by gel electrophoresis (Figure 19, c and d). It is interesting to note that a definite 5S and 45S band is present in the 'nucleolar' fraction which is not present in the 'nucleoplasm', and that 28S and 18S rRNA is present in both rat liver nuclear fractions. This indicated that the nucleolar preparation was contaminated, as Muramatsu et al. (1966) showed that 18S RNA was minimally present in rat liver nucleoli. Secondly we were unable to detect definite rat liver nucleoli in electron microscopic studies of the 'nucleolar' fraction.

Finally it should be noted that HeLa cell isolated nuclei are different, in several respects, from nuclei isolated from rat liver because:

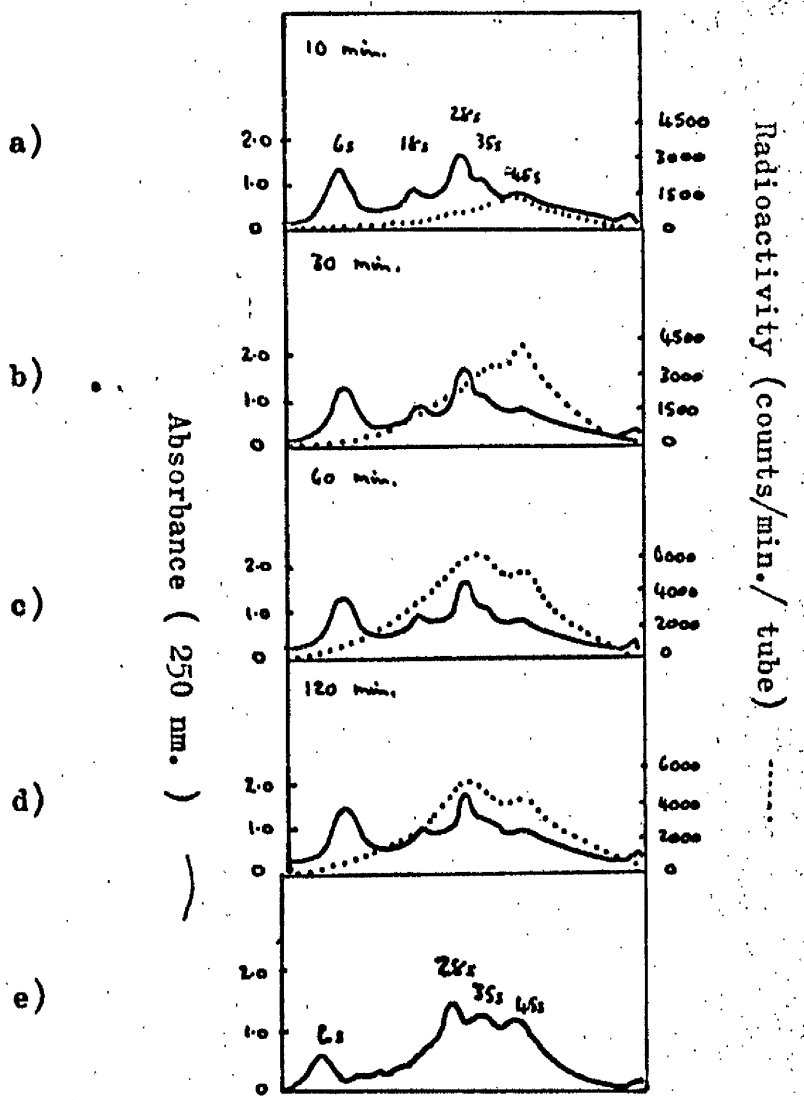
1) 45S RNA is not degraded to any great extent in HeLa cell nuclei during the Penman technique whereas there is considerable degradation in rat liver nuclei following this procedure.

2) Rat liver nucleoli contain appreciable amounts of 28S RNA (Muramatsu et al., 1966) while HeLa cell nucleoli contain very little.

In conclusion therefore it was found that the techniques developed by Penman for nRNA studies in HeLa cells were not applicable to rat liver nuclei. This may be related to the less active RNases present in the HeLa cell.

3.1.5. THE EFFECT OF A TRYPTOPHAN-FREE AMINO ACID FEED ON NUCLEAR RNA SYNTHESIS EXAMINED BY THE BUSCH PROCEDURE

The results of the nRNA extraction procedures described in Sections 3.1.2. and 3.1.4 were clearly not as technically satisfactory as the procedure used by Busch's group (Section 2.3.3). This can be seen from Figure 20 which shows the profiles of nRNA and nucleolar RNA (Muramatsu et al., 1966). A comparison with Figure 11 shows that definite 35S and 45S peaks are not apparent in this Hiatt



a) to d) : The Whole Nuclear Profile of Rat Liver RNA.
5 µCi of [^{14}C]orotic acid was injected i.v. into each rat which were killed at the times indicated.

e) : The Nucleolar Profile of Rat Liver RNA.

FIGURE 20 : THE NUCLEAR AND NUCLEOLAR RNA PROFILES OF RAT LIVER (from Figures 1 and 2 of Muramatsu et al., 1966)

procedure but that the 6S peak is higher in the Busch procedure than in the Hiatt procedure (our 5S peak).

The Busch nRNA extraction technique was next attempted on the Citric Acid nuclei obtained with 0.025M-Citric Acid (approx. 0.5% w/v). Using agarose gel electrophoresis to examine the RNA species obtained, it became clear that the 45S yield was lower than that obtained with the Hiatt technique. By varying the citric acid concentration we were able to show that 5% (w/v) was the optimum concentration (at least as far as 45S yield was concerned). This concentration of citric acid has also been used by other workers (see Busch, 1967).

The effect of the T- diet was repeated with these procedures. Animals were fed with the T+ or T- diets and simultaneously were given 5μCi of [8-^{14}C]adenine sulphate by intraperitoneal injection. One animal from each group was killed at 30, 60, 120 and 180 min., the nuclei were isolated (Section 2.6.1.2) and the nRNA extracted (Section 2.7.3). The nRNA was fractionated on 30ml. 10-45% (w/v) linear sucrose density gradients using the ISCO fractionator (Section 20.10.1.2) and the ^{14}C activity of the 1ml. fractions were determined by liquid scintillation spectrometry (Section 20.10. 1.2). The gradient profile and the radioactivity are shown in Figures 21 (T+ group) and 22 (T- group). Clearly both groups follow the general flow of radioactivity determined by Muramatsu et al. (1966) shown in Figure 20. It is interesting to note that neither the T+ nor the T- groups show a peaking of radioactivity in the 28S region and despite altering the timing of the killing of the animals we were unable to demonstrate this.

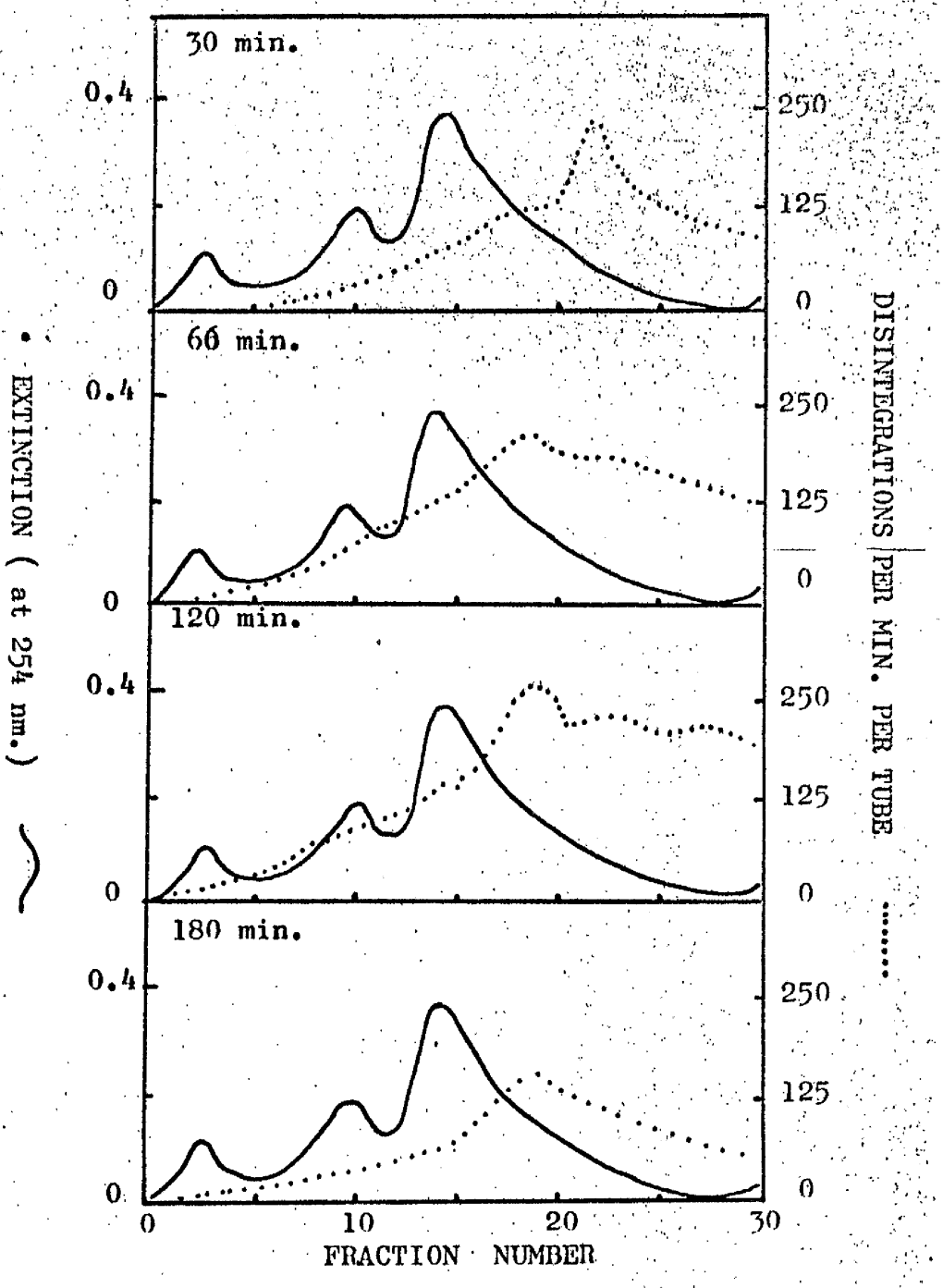


FIGURE 21 : SUCROSE DENSITY GRADIENT PROFILE OF NUCLEAR RNA. EACH ANIMAL WAS INJECTED WITH 5μCi OF [8-^{14}C]ADENINE SULPHATE, FED WITH THE T+ DIET AND KILLED AT THE TIMES INDICATED. GRADIENTS WERE CENTRIFUGED IN A SPINCO S.W. 25 ROTOR AT 25,000 REVOLUTIONS PER MIN. FOR 16 HR.

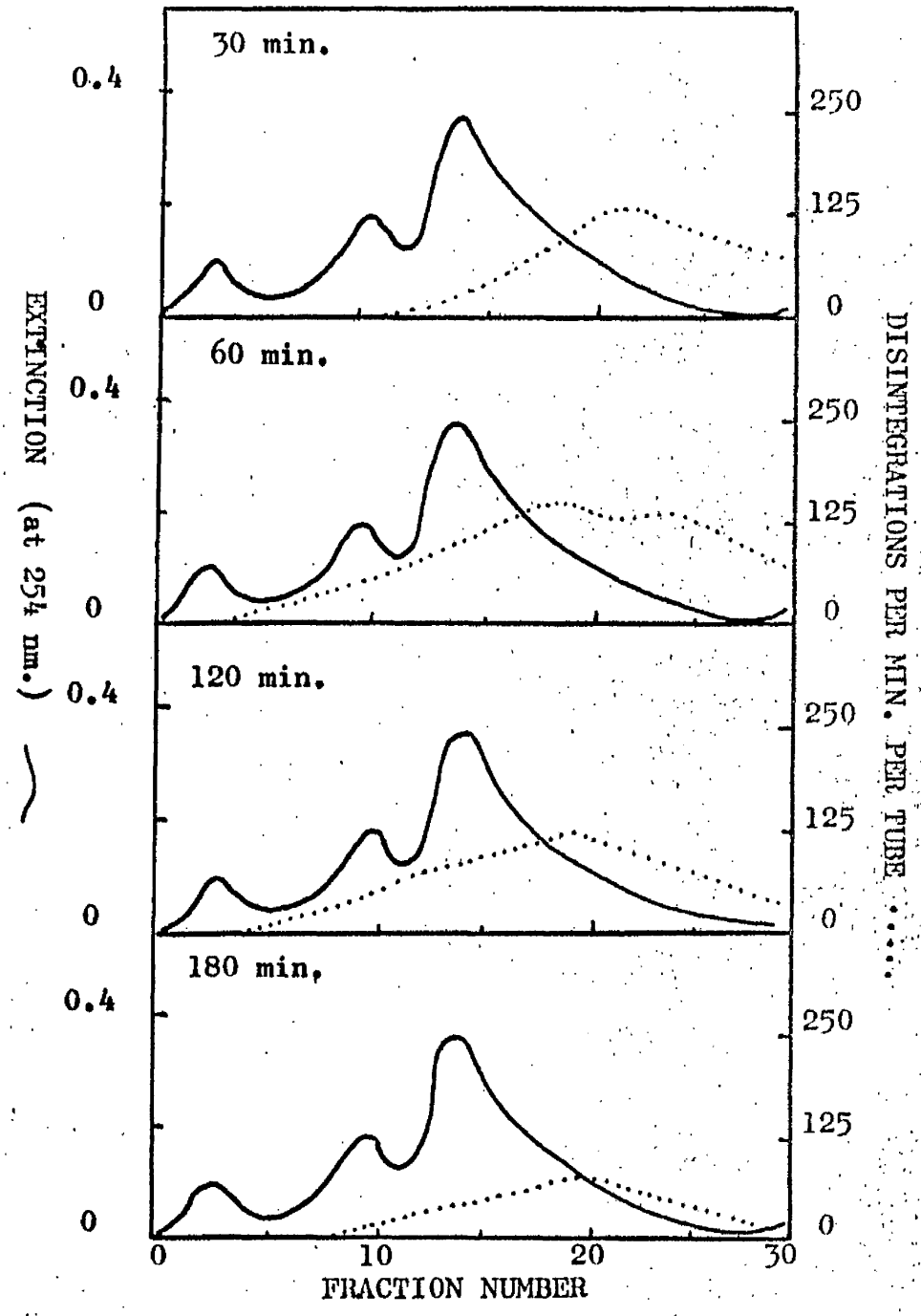


FIGURE 22 : SUCROSE DENSITY GRADIENT PROFILE OF NUCLEAR RNA. EACH ANIMAL WAS INJECTED WITH 5 μCi OF [8-^{14}C]ADENINE SULPHATE, FED WITH THE T- DIET AND KILLED AT THE TIMES INDICATED. GRADIENTS WERE CENTRIFUGED IN A SPINCO S.W. 25 ROTOR AT 25,000 REVOLUTIONS PER MIN. FOR 16 HR.

Comparison of the two groups shows that the activity of 45S in the T- animal at 30 min. is about 0.7 times that of the T+ animal. This activity ratio appears to hold over the first hour of labelling but by 2 and 3 hr. the activity is about 0.5 times that of the T+ animals.

What is the significance of these findings? They suggest that there is a greater, and longer continued, synthesis of nRNA in the T+ animals than in the T- group. This interpretation is difficult to establish however unless the pool size and specific activity of the labelled nucleoside triphosphate is known. Thus it could be argued that if the pool size of ATP in the T+ animal's liver (RNA precursor pool) was smaller than in the T- animal its specific activity would be greater and thus the newly synthesised RNA would have a higher activity. In retrospect, therefore, the experiments should have been conducted with dual labelling using [8-^{14}C]adenine sulphate and [5-^{3}H]orotic acid.

One aspect of the gradient analysis was unsatisfactory. Despite the demonstration of 45S RNA, by gel electrophoresis and by its labelling pattern, we were unable to demonstrate a 45S peak on the density gradient runs. One possible explanation for this could have been the poor resolution of the linear density gradients because this type of gradient tends to 'compress' any separations below the middle third of the tube. The reason is simply that linear increases in sucrose concentration are accompanied by polynomial increases in density and viscosity which means that the resistance to a sedimenting particl is increasing sharply (Barber, 1966).

The isokinetic gradient, devised by Noll (1967), differs from the linear gradient in that the RNA molecules move at constant velocity over the whole length of the tube. This implies superior separation of the RNA species and this increased resolution has in fact been demonstrated by Noll.

It therefore seemed likely that a better fractionation of nRNA could be obtained using this gradient. The theory and calculations involved in constructing this gradient have been described (Section 2.8.2) and originally the mixing device described by Noll was used for the preparation of the isolinetic gradients. On examining the fractionated gradient it became apparent that the concentration did not follow the theoretical gradient profile (Figure 23).

These deviations were thought to be due to the use of a semi-closed mixing device (described in Section 2.8.2), which allowed differences in the flow rate between the burette output and the mixing chamber output. Thus the centrifuge tube volume varied between 28.7ml. and 30ml. when 30ml. of heavy sucrose had been run in from the burette.

Noll's apparatus was therefore modified to a constant-volume device. It was constructed of Perspex and it consists of a piston and a cylinder (Figure 24.2). The cylinder sides are slightly cut out at the top to allow the piston to enter the cylinder despite a minimal piston clearance. A small concavity is present in the centre of the cylinder floor which engages with and stabilises the bar stirrer. The stirrer rotates at 700 revolutions per minute. The piston is fitted with a Neoprene O-ring which is lubricated with silicon grease, thus allowing the piston to move freely despite the small clearance allowed by the O-ring. This allows the gradient maker to be used to make isokinetic gradients over a wide range of vessel volumes. The piston assembly is drilled out along the midline to create an air outlet. This is closed by a nylon screw at the top and it is enlarged at the piston face so that air may easily enter the air inlet. The inlet and outlet tubes are 1mm. i.d. Teflon

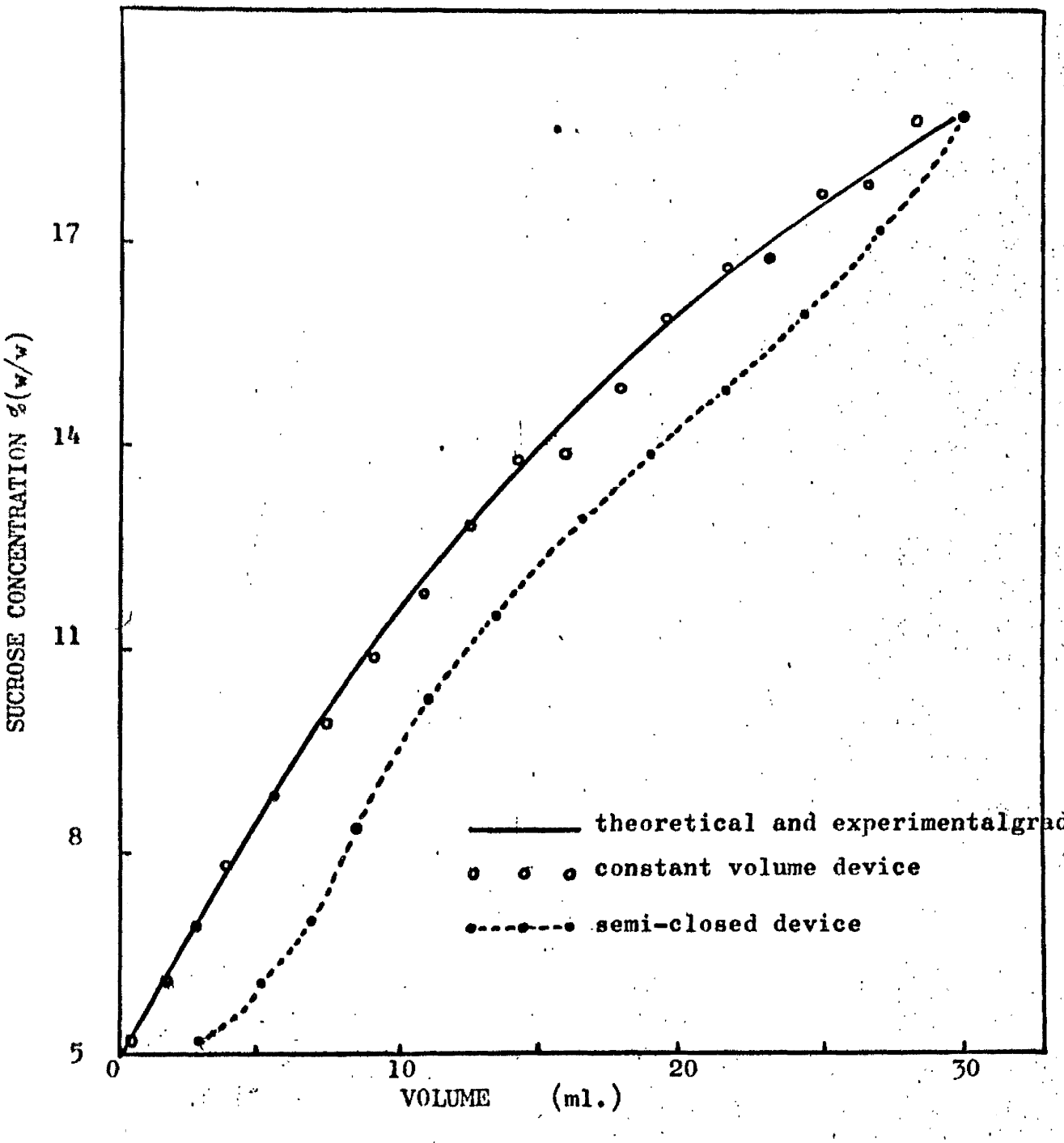


FIGURE 23 : ISOKINETIC SUCROSE DENSITY GRADIENT OBTAINED FROM DIFFERENT GRADIENT MAKERS.

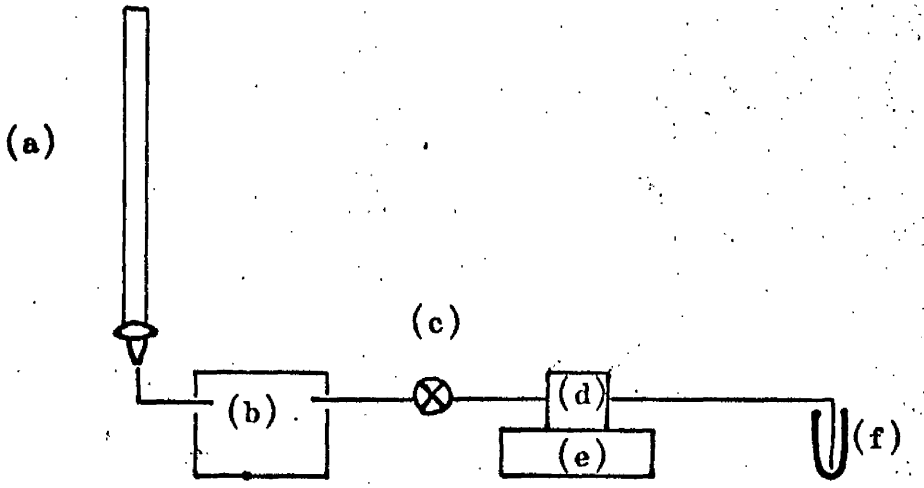


Figure 24.1 : Assembly for producing isokinetic gradients. (a) burette, (b) peristaltic pump, (c) valve, (d) gradient maker, (e) magnetic stirrer, (f) centrifuge tube.

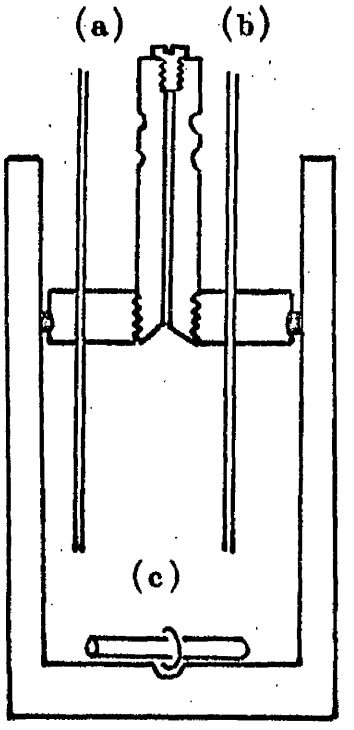


Figure 24.2 : Constant-volume isokinetic gradient maker. (a) inlet, (b) outlet, (c) magnetic stirrer. Cylinder dimensions are - internal height 6.5 cm., internal diameter 3 cm.

and they are cemented into the piston assembly. The other end of the inlet tube is connected to a Hamilton valve. The assembly for producing gradients is shown in Figure 24.1. It differs from Noll's apparatus by the addition of a pump (which controls the flow), valve and the constant-volume device.

Operation of the apparatus is as follows: The gradients are prepared at 2° by keeping all solutions on ice in the cold room. The burette and pump tubing are filled with the high-density sucrose, with the burette top open. The gradient maker is filled with a known volume of 5% (w/w) sucrose and the inlet tube is similarly filled; then the Hamilton valve is closed (this prevents the inlet tube emptying while fitting the piston). The total volume of the gradient maker and inlet tubing is 27.9ml. for this particular gradient. The piston is now fitted, with the air outlet open, so that all the air is displaced from the cylinder. The air outlet is closed, the pump tubing connected to the Hamilton valve which is then opened, the magnetic stirrer started, and the pump switched on (1.4ml/min). 30ml. of 25.6% (w/w) sucrose is run into the apparatus from the burette (this takes about 20 min); then the pump is stopped and the outlet tubing is carefully withdrawn from the centrifuge tube. Because the apparatus is a closed system the volume of sucrose in the centrifuge tube is 30ml.

We found that the device consistently produces isokinetic gradients (Figure 23). Slight deviations from the theoretical gradient are encountered at the bottom of the centrifuge tube because its curvature distorts the exponential form of the gradient.

Using this gradient for the separation of nRNA gave very similar results to the linear gradient except that the 28S RNA species were 'tailed' out further to the bottom of the tube. The increased resolution therefore showed that we were not extracting sufficient amounts of 35S and 45S nRNA to show on the gradient or

that the procedure of isolation and RNA extraction allowed this RNA to break down. We therefore concluded that the extraction procedure, in our hands, was less efficient than it has proved in other workers' hands.

3.2. IN VITRO DNA DEPENDENT RNA POLYMERASE ACTIVITY IN NUCLEI ISOLATED FROM RAT LIVER FOLLOWING DIETARY TREATMENTS

Caspersson (see Section 1.1.5.3) first suggested that the nucleolus was the site of RNA synthesis in animal cells. However, it was not until 1959 that Weiss and Gladstone obtained an enzyme preparation from rat liver nuclei which catalysed the transfer of labelled UMP from UTP into RNA. The incorporation required the presence of all four ribonucleotide triphosphates and it was found to be inhibited by pre-treatment of the preparation with deoxyribonuclease. The product of the reaction is a polyribonucleotide with 3', 5'-phosphodiester bonds and the RNA synthesised is complementary to the DNA present in the reaction mixture (Hurwitz and August, 1963). The enzyme catalysing this reaction was called a DNA dependent RNA polymerase but the recommended systematic name is Nucleoside triphosphate: RNA nucleotidyl transferase (DNA dependent) [EC 2.7.7.6] and the suggested trivial name is RNA nucleotidyl transferase. However, in this discussion the enzyme will be called DNA dependent RNA polymerase or RNA polymerase.

Huang and Bonner (1962) showed that in pea seedlings the RNA polymerase was tightly bound to the DNA fibrils. Early work on the polymerase work was carried out on this non-soluble enzyme preparation described as the "aggregate" enzyme (c.f. Weiss, 1960). In addition to this preparation a soluble enzyme ("soluble" enzyme) has been described in embryonic (Furth and Loh, 1963) and neoplastic tissues (Furth and Loh, 1964), in testes (Ballard and Williams-Ashman, 1964) and rat liver (Ramuz et al. 1965). These preparations do not contain DNA and addition of DNA is required to prime the enzyme. The relationship between the two enzymes is obscure and most physiological studies have been

carried out on "aggregate" enzyme (in the form of nuclei). Thus increased RNA polymerase activity has been observed in rat thyroid following the administration of thyroid hormone (Widnell and Tata, 1963), in rat uterus following oestrogen administration (Gorski, 1964), in rat liver following growth administration (Pegg and Korner, 1965), cortisol administration (Barnabei et al., 1966) and partial hepatectomy (Ro and Busch, 1967).

The soluble enzyme preparation has sometimes been studied as a procedure to obtain "pure" mammalian RNA polymerase, e.g. Cunningham and Steiner (1967), Liao, Sagher and Fang, (1968), and Jacob, Sajdel and Munro, (1968a). The requirements for both enzymes are similar. There is an absolute requirement for all four ribonucleotide triphosphates, Mg^{2+} or Mn^{2+} ions and DNA (in the case of the soluble enzyme only).

The studies which are reported here were carried out on RNA polymerase of whole rat liver nuclei. The object of the investigation was to obtain evidence for changes in the total activity of the enzyme following an acute dietary challenge because earlier work (Section 3.1) had suggested that there was an increased RNA synthesis in the T+ fed group of animals. It was thought essential to retain the structural integrity of the nuclei, in the initial investigation, in the hope that the 'control mechanisms' would remain intact and would perhaps be 'observable'.

Rat liver nuclei were prepared as described in Section 2.6.2. This procedure was known to give adequate nuclear preparations from rat liver (Begg D.J. - personal communication). The original Chauveau procedure had to be modified (Chauveau et al., 1956) because the available homogenising motor had insufficient torque to homogenise rat liver tissue in 2.2 M-sucrose. The

procedure was thus modified by Munro et al. (1965) so that the initial homogenisation was in 0.24M-Sucrose containing Ca^{2+}-salts to prevent clumping and aggregation of nuclei (Maggio et al., 1963) and finally centrifuging the crude nuclear preparation through 2.2M-sucrose (containing $CaCl_2$) as in the original Chauveau procedure. The nuclei are known to sediment in the media while all other cellular components float above the nuclear pellet. This procedure was used for the standard nuclear preparation until the laboratory was re-equipped with apparatus to prepare nuclei directly in high molarity sucrose (Method 2.6.3; Results 3.2.4).

3.2.1. - PROPERTIES OF DNA-DEPENDENT RNA POLYMERASE FROM RAT LIVER NUCLEI WHEN ASSAYED BY THE PROCEDURE OF BEGG (SECTION 2.11.1.)

The assay of nuclear RNA polymerase was carried out, in duplicate, at zero and other times. The radioactivity was expressed initially as disintegrations per minute and then converted into pmole of incorporated GTP per mg. of nuclear DNA. Initially each of the activities was plotted and the gradient (i.e. reaction velocity or tangent) was determined graphically but with the availability of the Programma 101 (British Olivetti Ltd.) the duplicate points were fitted by the Method of Least Squares and the gradient obtained directly by calculation. The enzyme activity was therefore expressed in terms of initial velocity (pmole of GTP incorporated/min./mg. DNA).

The optimal pH and Mg^{2+} concentrations were determined using the Widnell and Tata (1966a) data for initial concentrations. Figure 25 shows the optimal $[Mg^{2+}]$ of 3.75 µmole at pH 8.5 and an optimal pH of 8.4 at this Mg^{2+} concentration (Figure 26). Using these optimal conditions it was found that the nuclear content of the incubate (as judged by DNA estimations) gave a linear polymerase assay over the working range of 0.05 - 0.1 mg. DNA (Figure 27).

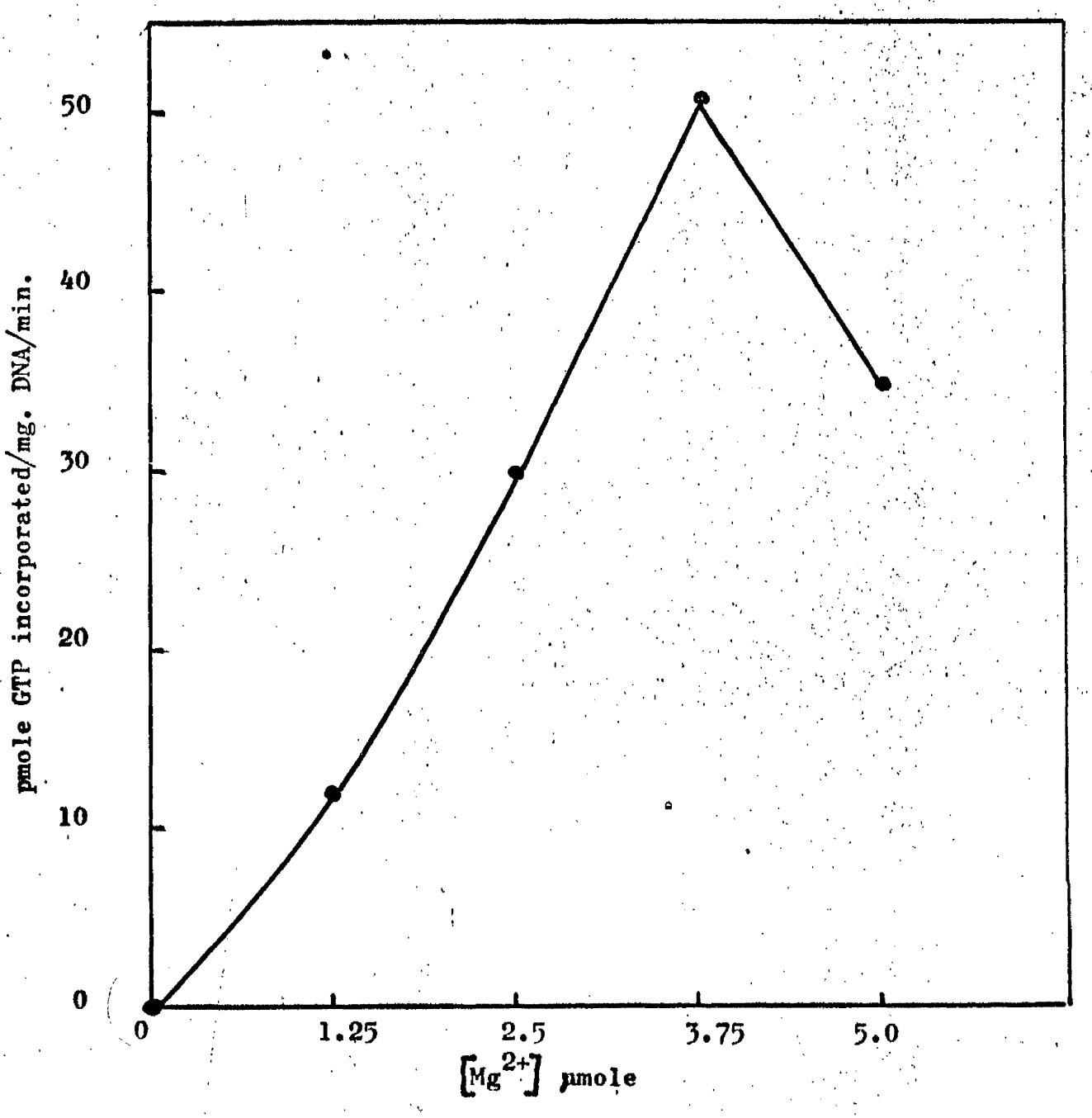


FIGURE 25: RNA POLYMERASE REACTION – THE EFFECT OF VARYING Mg^{2+} CONCENTRATION AT pH 8.5

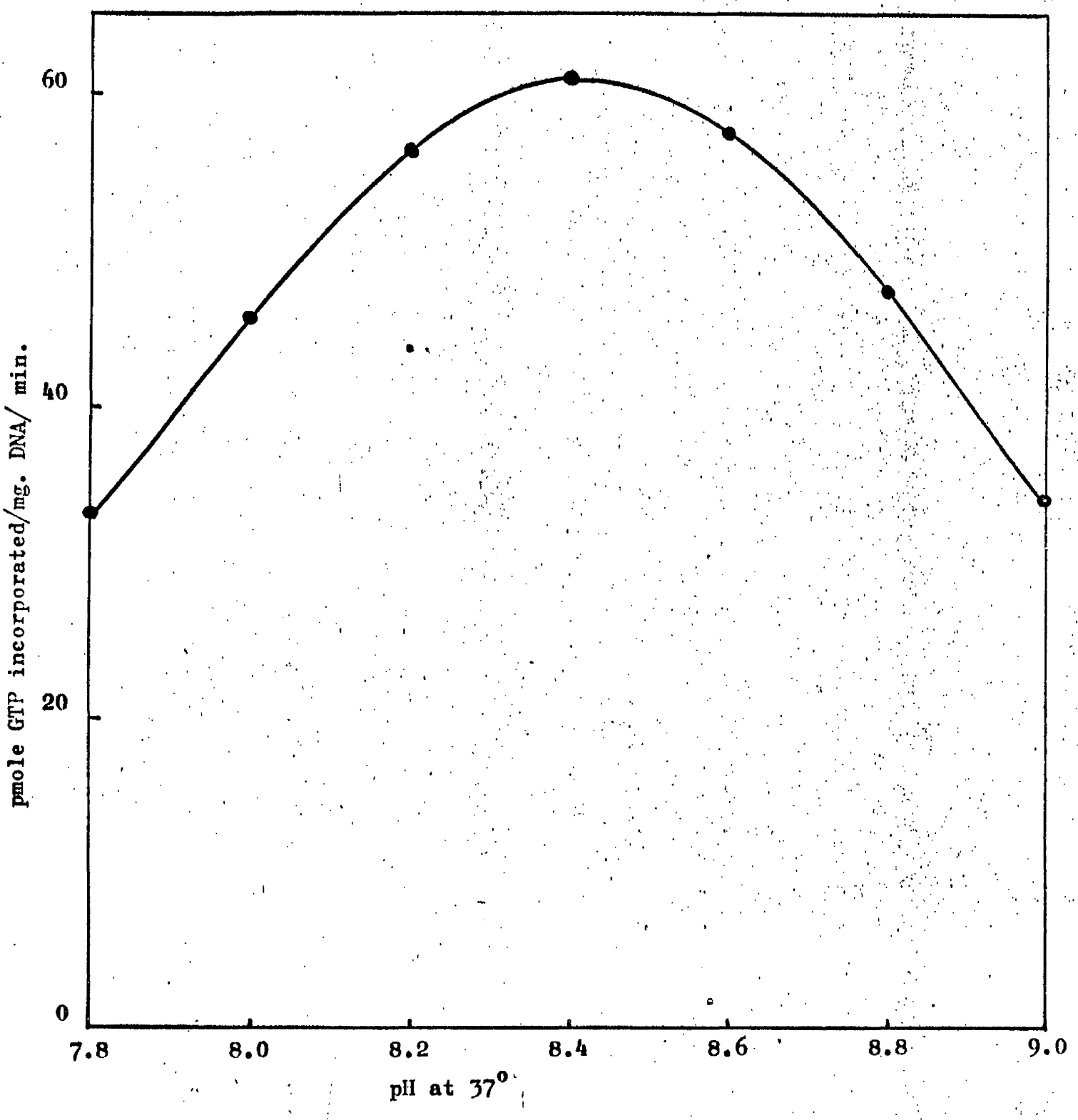


FIGURE 26: RNA POLYMERASE REACTION - THE EFFECT OF VARYING pH AT A $[Mg^{2+}]$ OF 3.75 µmole.

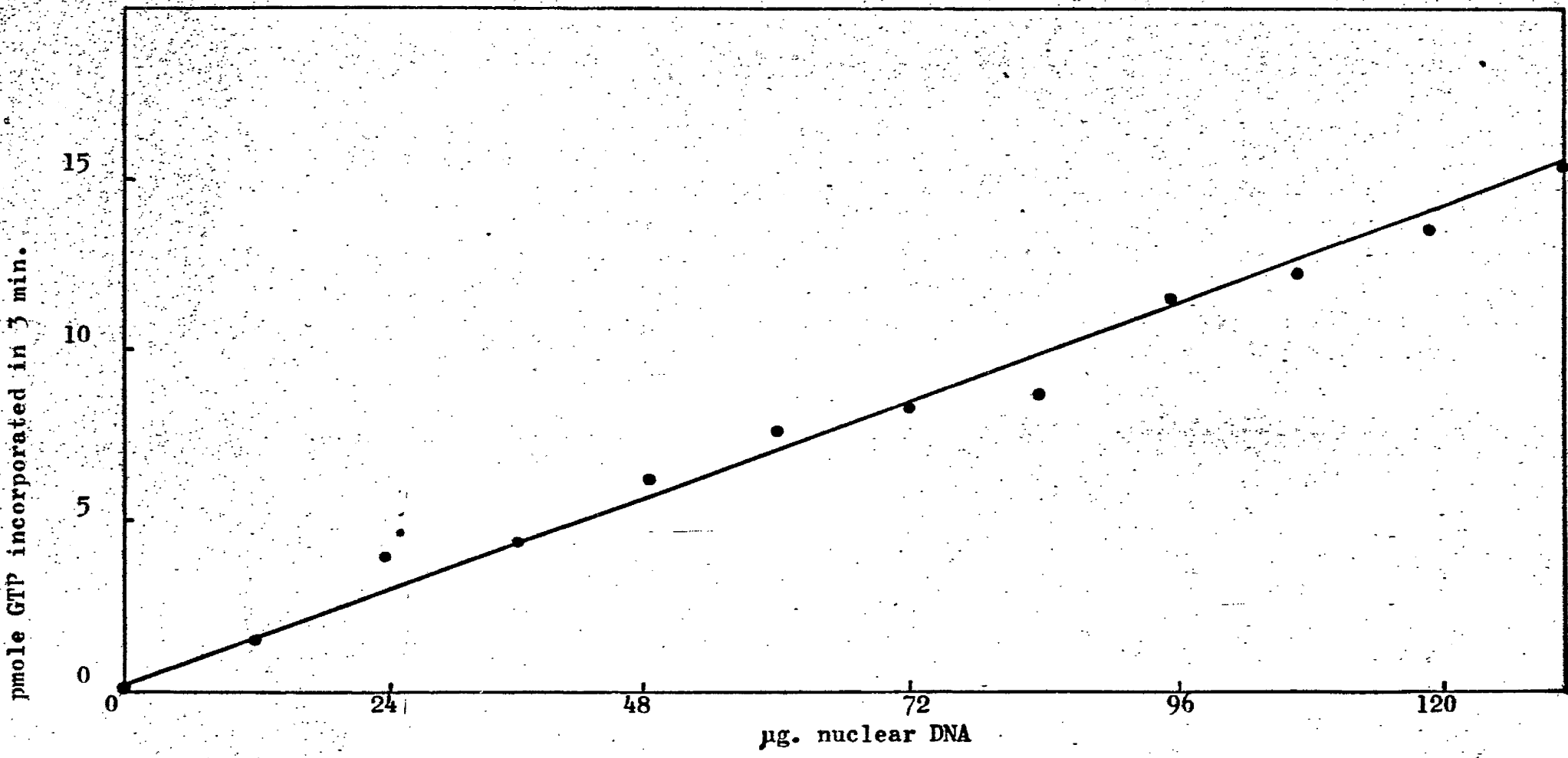


FIGURE 27 : RNA POLYMERASE REACTION - THE EFFECT OF VARYING THE NUCLEAR CONTENT OF THE INCUBATE.

The requirements for the incorporation of GTP were next studied (Table 3). Mg^{2+} and all four ribonucleoside triphosphates were necessary for activity. The reaction was inhibited by boiling the nuclei or by the presence of Actinomycin D (5 µg.). Finally it was shown that the radioactivity resided in the nuclear fraction which was alkali hydrolysable and subsequently acid soluble. Thus the incorporation of GTP was considered to reflect the activity of the DNA - dependent Mg^{2+} activated RNA polymerase of rat liver nuclei.

Initially the acid-insoluble pellet, obtained after washing the polymerase assay mixture (Section 2.11.1), was dissolved in Nuclear Chicago Solubiliser (NCS) before adding the PPO-POPOP-toluene scintillation mixture. However, several assays appeared to be heavily "quenched" despite the absence of colour or other obvious changes in the nuclear preparation. It was found, after repeated counting of these anomalously quenched samples, that the accumulated counts in the C channel (the lowest energy channel) decreased with time whereas the B channel counts remained constant. It was therefore concluded that the decrease in C channel counts (which usually took about 36 hr. to decay to a steady level but which sometimes took many days) was due to the decay of chemiluminescence of the sample. This is a weak emission which is readily detected in the lowest energy channel. Because of the long persistence of the chemiluminescence in some of the samples it was decided to use Hyamine Hydroxide as a digesting agent for the polymerase assay pellet.

This substance also produces chemiluminescence which decays much more rapidly than the NCS generated emission (Figure 28). The effect on the B/C ratio when using the channels ratio method of quench correction, could therefore lead to highly erroneous enzyme activities. Steinberg et al. (1958) have

TABLE 3

Characterisation of the DNA dependent Mg^{2+} - activated RNA polymerase of rat liver nuclei

Assay System	pmole GTP incorporated per mg. DNA per min. (total incubation 3 min.)
Complete	65
Mg^{2+} omitted	1
ATP omitted	14
CTP omitted	12
UTP omitted	22
ATP and CTP omitted	5
ATP and UTP omitted	10
CTP and UTP omitted	6
ATP, CTP and UTP omitted	7
Complete + Actinomycin D	3
Complete using pre-boiled nuclei	1

B/C RATIO	0.76	1.10	1.17	1.17

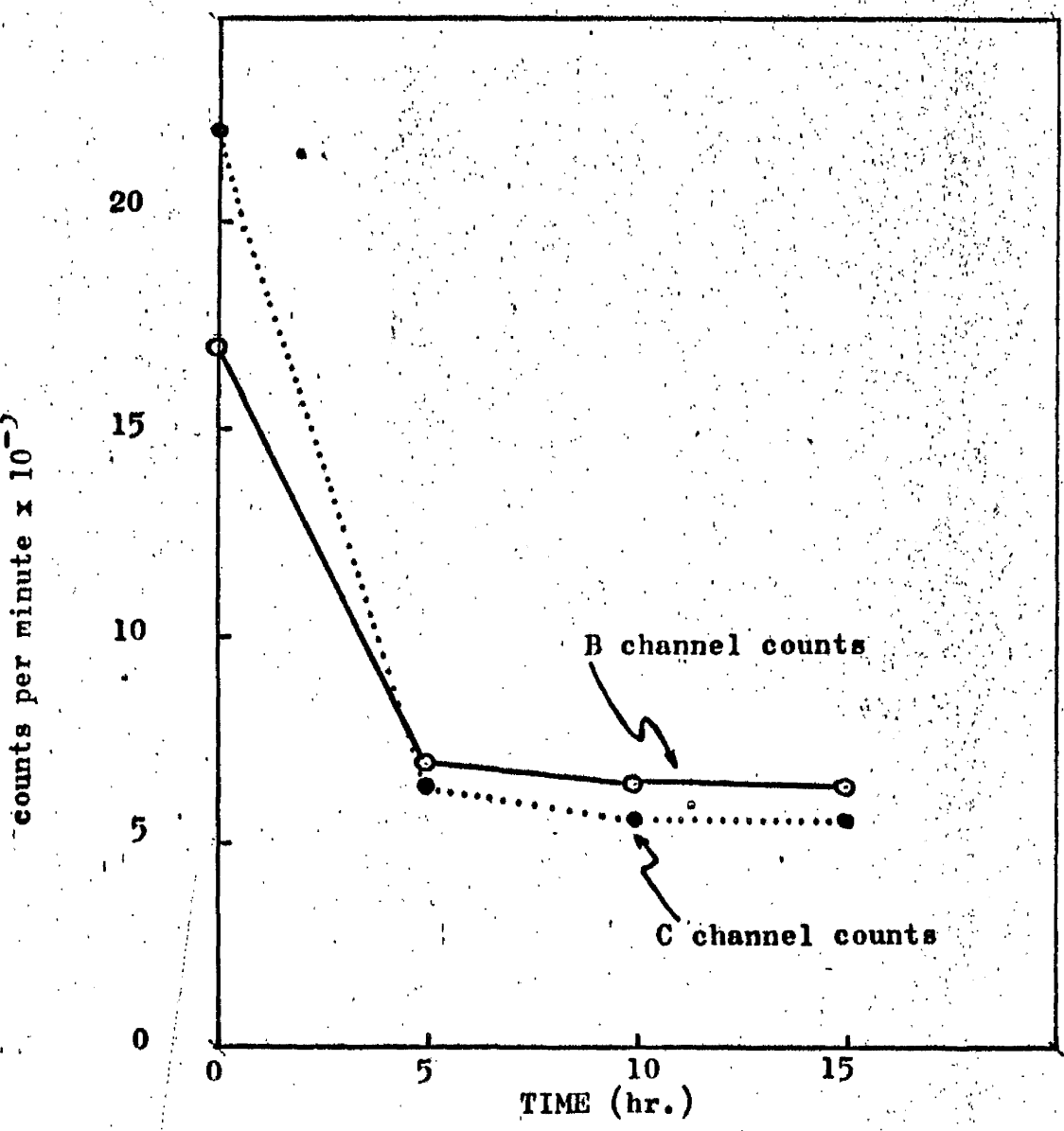


FIGURE 28 : RNA POLYMERASE ASSAY - THE DECAY OF CHEMILUMINESCENCE AND ITS EFFECT ON THE B/C RATIO. SAMPLES WERE TREATED AS DESCRIBED IN SECTION 2.10.3

described chemiluminescence which was thermally activated when using Hyamine Hydroxide to digest a protein solution. They also pointed out that impure Hyamine Hydroxide (caused by room temperature decomposition) added to non-radioactive protein produced high counting rates especially after dilution with toluene scintillation mixtures and this effect took a long time to decay. Purification of the Hyamine markedly reduced this effect and stable background counts could be obtained within a few hours. Another aspect of anomalous counts is the observation of Davidson and Feigelson (1957) that u.v. irradiation of empty vials just prior to their insertion into the spectrometer can give extremely high counting rates.

In light of these observations the procedure which was finally adopted was as follows:

The polymerase assay pellet was allowed to drain overnight and the tube wall was wiped dry the following morning. 0.25 ml. of Hyamine Hydroxide (which was stored at 4° to prevent decomposition) was added to the pellet and the tubes were covered in aluminium foil (to exclude light) and incubated at 37° for 6 hr. thus allowing complete digestion of the pellet. This procedure undoubtedly caused chemiluminescence (Figure 28, Zero time, Channel C). The dissolved pellets were clear and colourless and they were then dissolved in the scintillation mixture and transferred to glass scintillation vials which were then capped. This procedure was carried out with the fluorescent lights switched off as these lights have a slight U.V. emission (British Lighting Council 1966). The vials were then placed in the dark, at room temperature, for 18 hr. to allow chemiluminescence to decay as Udenfriend (1962) has pointed out that phosphorescence (defined as persistent luminescence) is markedly enhanced and

prolonged with the lowering of temperature.

From Figure 28 it can be seen that luminescent decay is complete within 10 hr. and a time period twice as long was chosen to prevent anomalous counting effects. Finally, the vials were transferred into the spectrometer for counting. At no time within the previous 18 hr. were the vials, or their contents, exposed to fluorescent lighting thus avoiding activation of luminescence in the samples.

Using the procedure outlined above required a new Quench Correction Factor and a series of [^{3}H]toluene standards (98610 disintegrations per min.) were dissolved in the toluene-PPO-POPOP mixture (total volume 10 ml.) and Hyamine Hydroxide was added over the range 0 - 0.9 ml., in steps of 0.1 ml. The quenching effect is shown in Figure 29. Note that the quenching curve is linear and it was found convenient to store the quench function (in the form Efficiency % = B/C ratio . gradient + intercept) in the Olivetti Programma 101 stores and directly calculate the disintegrations per minute by entering the accumulated counts in Channels B and C. This step omitted the usual graphical interpolation necessary for calculation of disintegrations per minute.

3.2.2. THE EFFECT OF FEEDING WITH COMPLETE AND TRYPTOPHAN-FREE AMINO ACID MIXTURES ON RAT LIVER NUCLEAR DNA DEPENDENT Mg^{2+} ACTIVATED RNA POLYMERASE (First Series)

Nuclei were prepared by the modified Chauveau Procedure (Section 2.6.2.), RNA polymerase activity was assayed by the Begg procedure (Section 2.11.1) and radioactivity determined by the procedure outlined in the preceding section. Figure 30 illustrates the method of determining the initial enzyme velocity. The Least Squares Method was used to determine the best fit, and the gradient of the line gives the initial enzyme velocity in pmole GTP incorporated per mg.

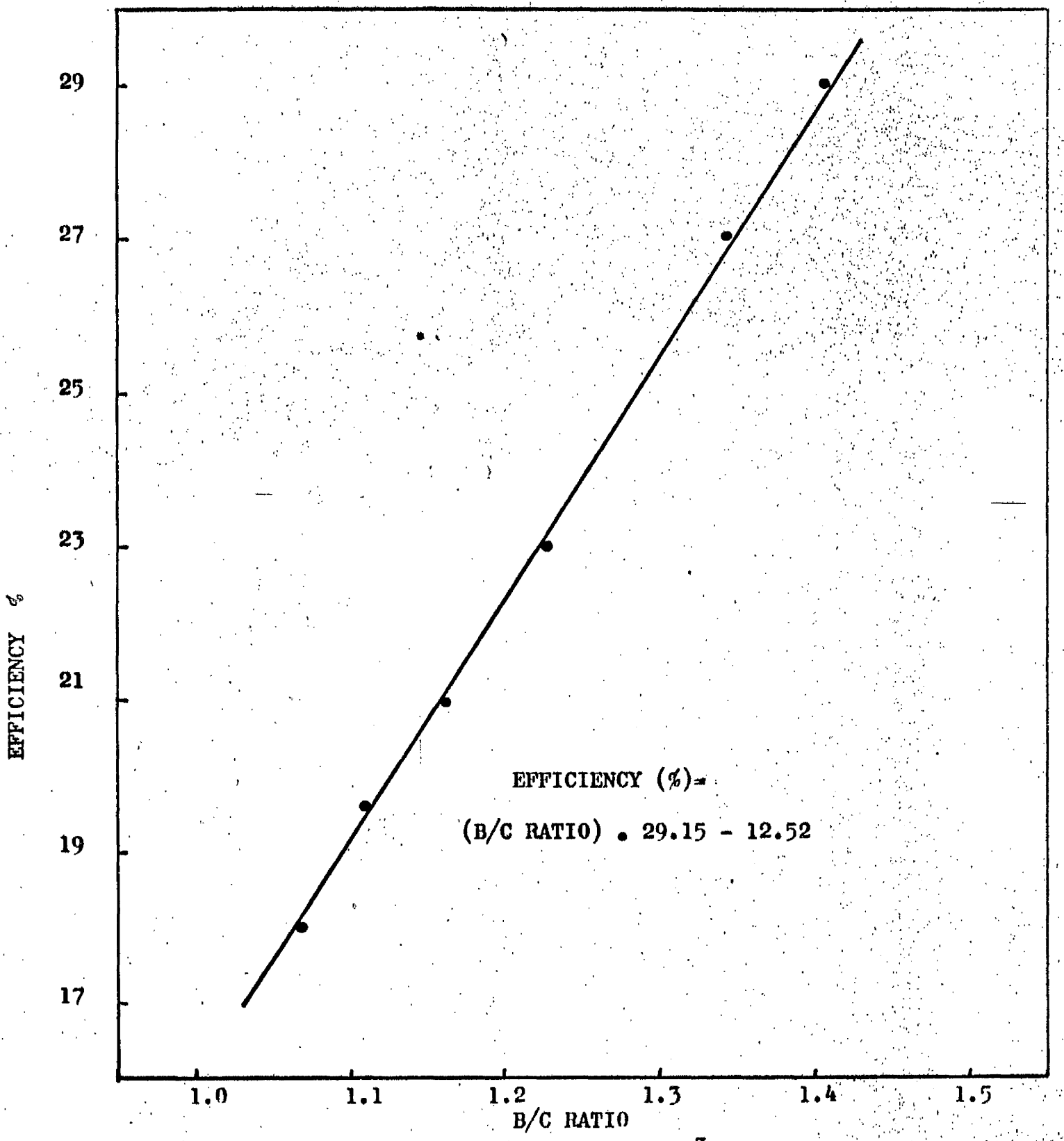


FIGURE 29 : RNA POLYMERASE ASSAY - THE QUENCHING OF [^{3}H]TOLUENE BY HYAMINE HYDROXIDE

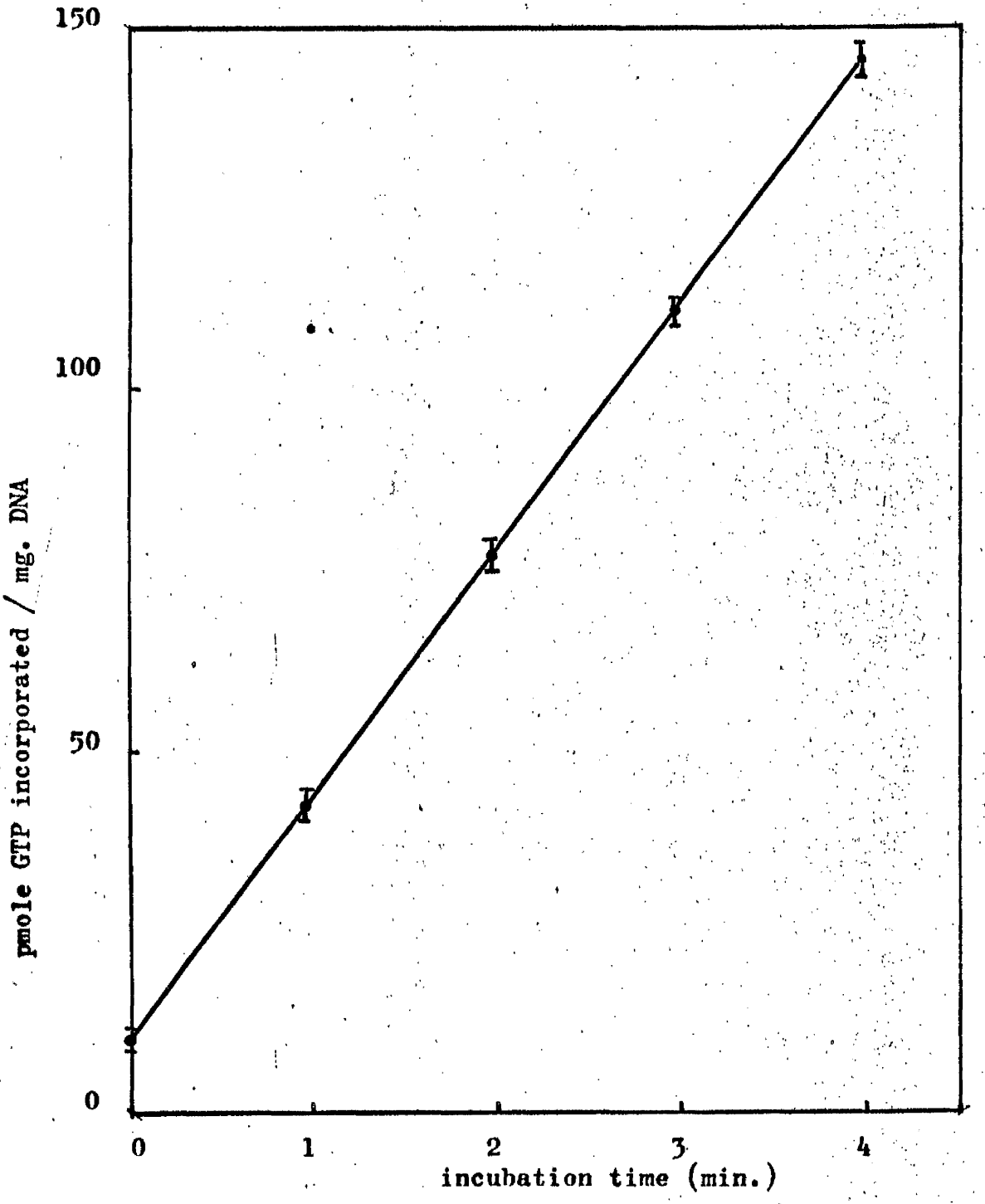


FIGURE 30 : RNA POLYMERASE ASSAY - THE EFFECT OF FEEDING WITH A TRYPTOPHAN FREE AMINO ACID MIXTURE 20 MIN. BEFORE KILLING. RESULTS ARE EXPRESSED AS MEAN ± S.E.M. (n=3).

DNA per min. It can be seen that the enzyme reaction is linear over the first 4 min. of incubation. The linearity of each enzyme determination was examined in the same manner. The RNA polymerase response to the T+ and T- diets was determined at 0, 10, 20, 30 and 50 min. after feeding. The results are shown in Figure 31. The number of estimations were 4 (2 animals - duplicate determinations) at all time intervals except 10 and 30 min. when n = 8 (4 animals - duplicate determinations)

I concluded

(1) that the T+ and T- diets produced similar responses (no significant difference by t-test).

(2) that the fasted animal had a lower enzyme activity than the 30 and 50 min. fed animal.

(3) that the colony-fed animal (no starving) had a similar activity to the 30 min. fed animal (no significant difference by t-test).

These results are at variance with the evidence from sucrose density gradient analysis (Section 3.1.) which appeared to suggest that the T+ fed animal responded, at all time intervals examined, by a greatly increased RNA synthesis compared to the T- fed animal.

I therefore decided to determine the effect of diet on the <u>in vivo</u> uptake of orotic acid by liver nuclei to confirm or refute the RNA polymerase assay results.

3.2.3. THE in vivo INCORPORATION OF [^{3}H]OROTIC ACID INTO RAT LIVER NUCLEI

Initial experiments established that linear uptakes of [^{3}H]orotic acid occurred up to 20 min. after intraperitoneal injection of the isotope, and that 200μCi of [5-^{3}H]orotic acid (specific activity 7 Ci/mole) in 1 ml. of steril

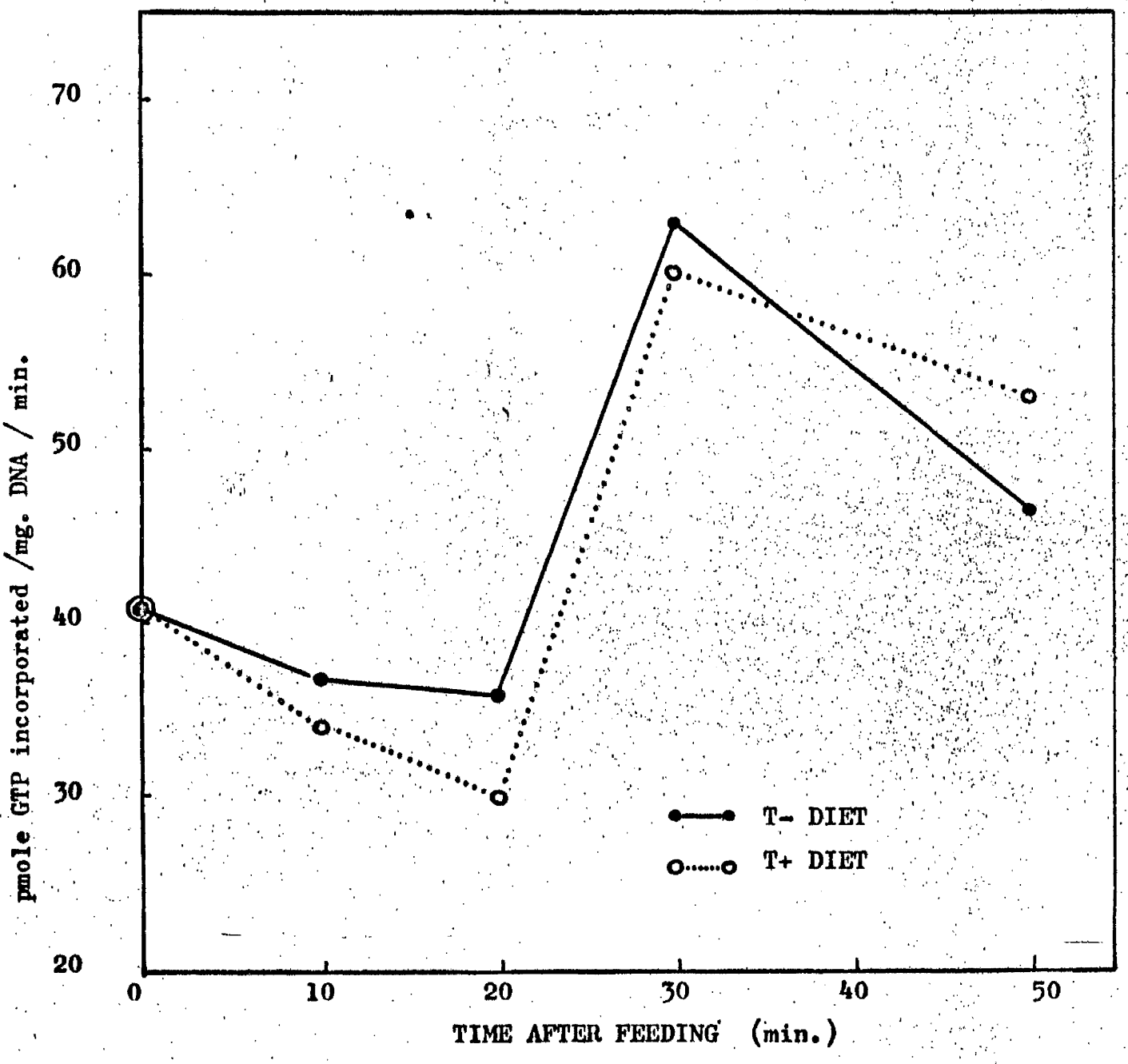


FIGURE 31 : RNA POLYMERASE ASSAY - THE EFFECT OF THE T+ AND T- DIETS ON THE RNA POLYMERASE ASSAY. ALL ANIMALS WERE STARVED FOR 18 HR. BEFORE FEEDING.

0.15M-NaCl gave adequate activity. Nuclei were isolated by the Citric Acid Procedure (Section 2.6.1) and the radioactivity determined by the procedure outlined in Section 2.10.2. Uptake was expressed as pmole orotic acid per mg. DNA. The uptake of isotope was determined in the 18 hr.-fasted animals and at two periods in the T+ and T- fed animals - directly following feeding and at 40 min. after feeding.

The results are shown in Figure 32 and my conclusions were:

1) The rate of uptake of isotope, directly following feeding of the T- diet, is similar to the fasted animals. The higher uptakes in the fasted group are probably due to a smaller pool of RNA precursors in the fasted liver causing less dilution of the labelled orotic acid.

2) Directly after feeding the rate of uptake of isotope by the T+ fed animal is approximately 1.5 times that of the T- fed animals.

3) 40 min. after feeding the rate of uptake of isotope by the T+ fed animals is 3 times that of the T- fed animal.

4) Both dietary groups have higher isotopic uptakes at 40 min. after feeding than directly after feeding.

Thus the orotic acid uptake results are at variance with the polymerase activities (except as noted in (1) above) and they are in broad agreement with the results of Section 3.1. I therefore concluded that the RNA polymerase activities did not reflect the same intranuclear events as the gradient analysis and isotopic uptake data.

The RNA polymerase assay used in this work differed in several respects from other assays used for rat liver RNA polymerase studies (Table 4). Thus the Begg assay contained mercaptoethanol but no NaF whereas other workers used cysteine or mercaptoethylamine with NaF.

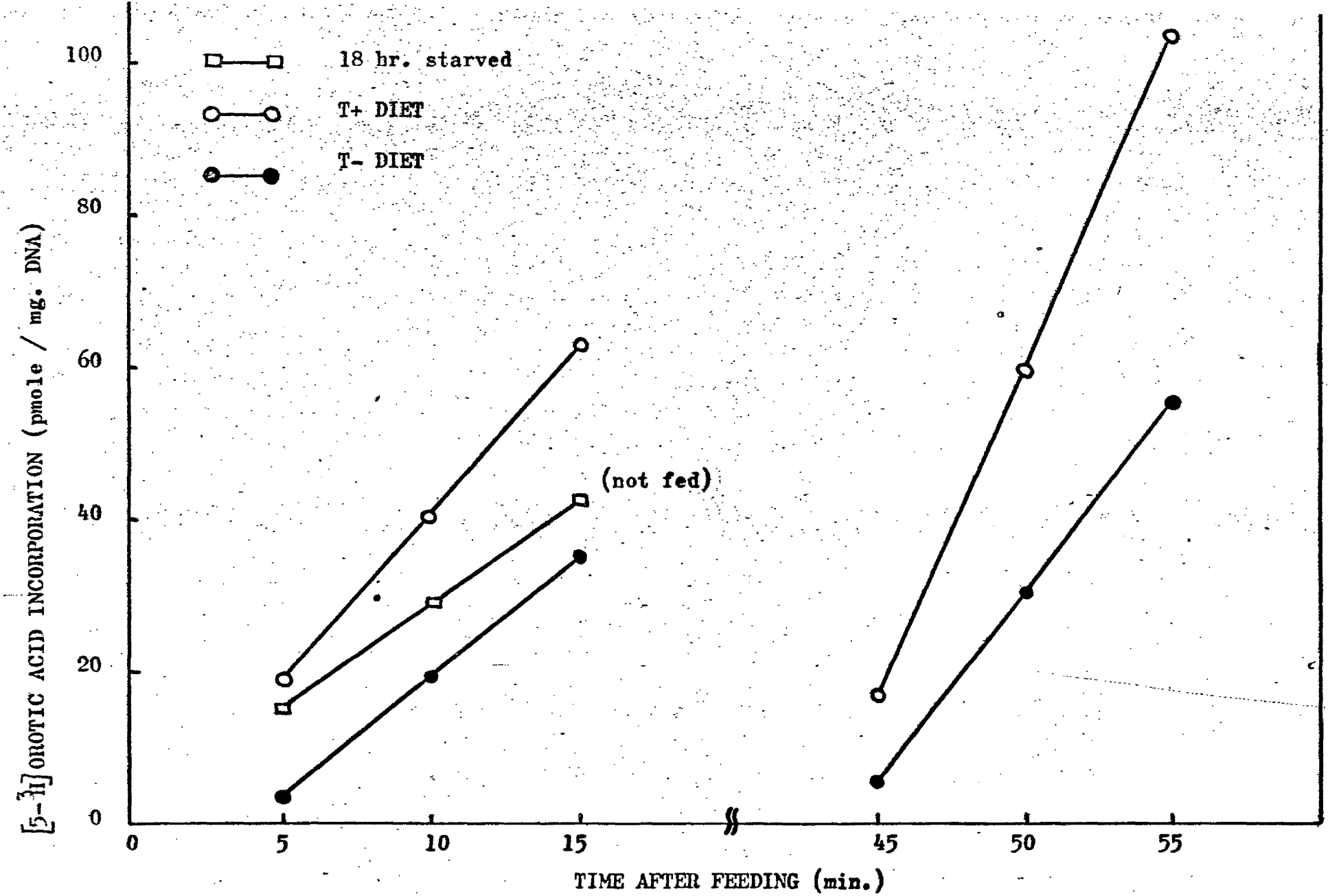


FIGURE 32 : THE <u>in vivo</u> INCORPORATION OF [^{3}H]OROTIC ACID INTO RAT LIVER NUCLEI AFTER OVERNIGHT FASTING AND SUBSEQUENT FEEDING WITH THE T+ OR T- DIETS. EACH POINT REPRESENTS DUPLICATE DETERMINATIONS ON ONE RAT.

TABLE 4

The components of the rat liver polymerase assay as used by several authors

Component (μ mole)	Weiss (1960)	Goldberg (1961)	S.Busch et al. (1962)	Widnell and Tata (1966)	Begg and present author
Volume (ml.)	1	0.5	0.25	0.5	1
pH	8.05	8.1	7.5	8.5	8.4
buffer composition	100 Tris	25 Tris-Phosphate	50 Tris	50 Tris-HCl	100 Tris-HCl
mercaptoethanol	NIL	NIL	NIL	NIL	2.5
mercaptoethylamine	NIL	NIL	0.25	NIL	NIL
cysteine	10	NIL	NIL	10	NIL
NaF	NIL	10	5	3	NIL
Mg^{2+}	5 (Cl)	NIL	7.5 (not stated)	2.5 (Cl)	3.7
Mn^{2+}	NIL	1.5 (Cl^{1})	NIL	not in Mg^{2+} dependent enzyme assay	NIL
ribonucleotide triphosphates	0.06 ATP GTP UTP	0.4 ATP CTP UTP	0.25 GTP UTP CTP	0.3 GTP CTP UTP 0.015 ATP	0.4 ATP CTP UTP
isotope	^{32}P - CTP 0.06	^{32}P - CTP 0.06	^{14}C - ATP 0.25	^{14}C - ATP 0.005	^{3}H - GTP 0.0023
Other salts etc.	NIL		KCL 5		250 sucrose

I therefore decided to continue the RNA polymerase work with a modified assay system. Mercaptoethanol was replaced by cysteine (10 µmole) and NaF (3 µmole) was added to the assay mixture. This new assay was therefore similar to but not identical with the well known Widnell and Tata (1966a) system. The full constitution is given in Section 2.11.2.

3.2.4. PROPERTIES OF DNA DEPENDENT RNA POLYMERASE OF RAT LIVER NUCLEI WHEN ASSAYED BY THE MODIFIED PROCEDURE OF WIDNELL AND TATA (Section 2.11.2.)

The assay of nuclear RNA polymerase followed the procedure described in Section 3.2.1. All zero and 3 min. assays were estimated in duplicate and radioactivity was expressed initially as pmoles of incorporated GTP for these times The zero time incorporation was 1pmole and the 3 min. incorporations were within 2% of each other. Assays exceeding these limits were rejected. The duplicate determinations were fitted by the Method of Least Squares and the gradient thus obtained was the initial enzyme velocity expressed as pmoles GTP incorporated per min. per mg. DNA. The IUB (1964) recommendations on Enzyme Units (Enzyme Nomenclature, 1965) suggested that the proteolytic enzyme unit should be defined as the amount of enzyme which will catalyse the transformation of a micro-equivalent of the group concerned per minute under standard conditions. I therefore define one micro-unit (µU) of DNA dependent RNA polymerase as the amount of enzyme which will catalyse the incorporation of 1 picomole of GTP per minute at 37°. Thus the initial enzyme velocity is expressed as µU/mg.DNA.

The optimal conditions for the assay were $[Mg^{2+}]$ 3.0 µmole at pH 8.5 and pH 8.6 at this optimal $[Mg^{2+}]$ for 18 hr. fasted rats. It will be noted from FIGURES 33 and 34 that the optimal conditions for the T+ and T- fed animals were slightly differen and the final pH and $[Mg^{2+}]$ chosen were 8.5 and 3.75 µmole respectively. The

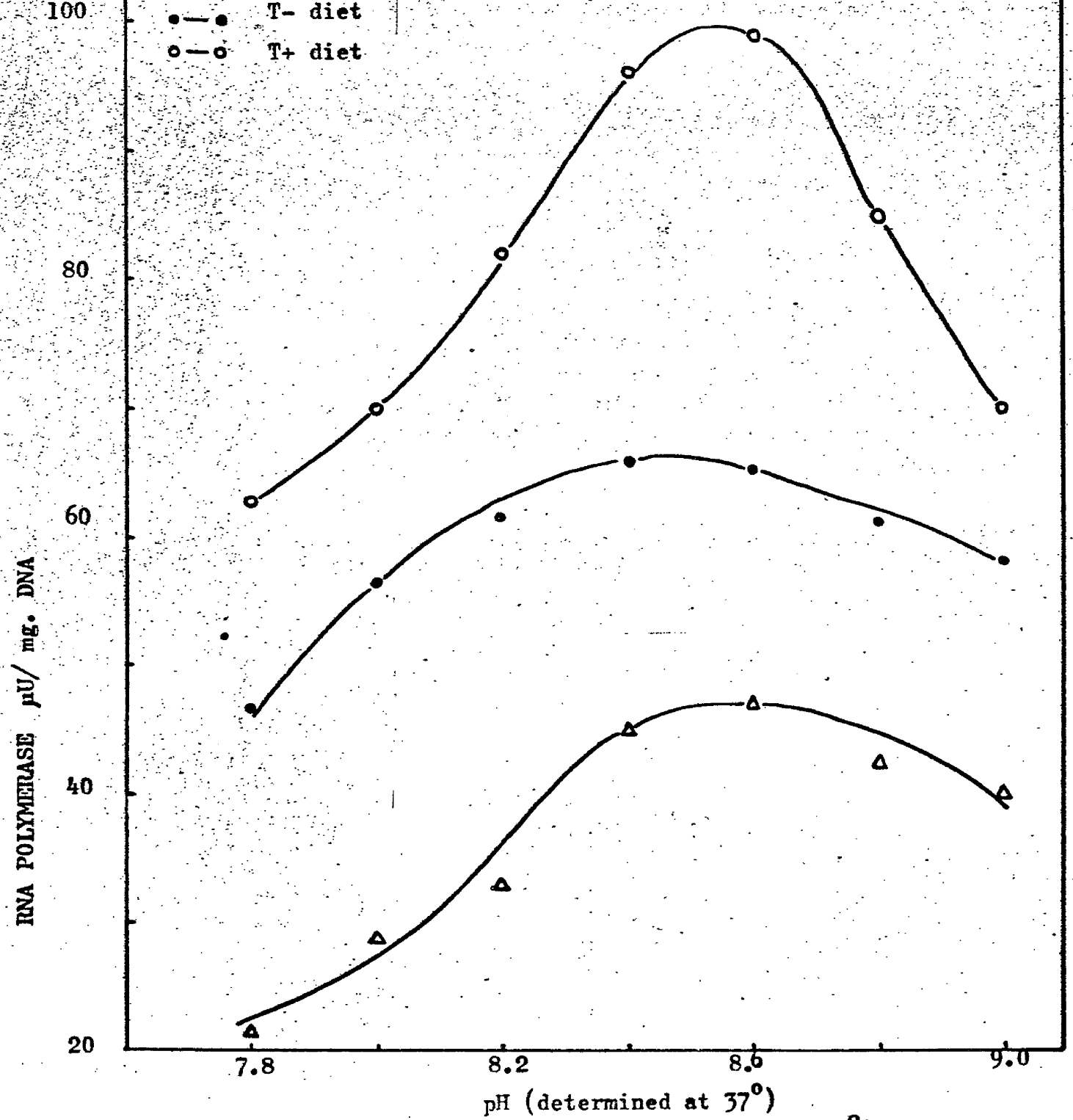


FIGURE 34 : RNA POLYMERASE ASSAY - THE EFFECT OF VARYING pH AT A Mg^{2+} LEVEL OF 3.0 µmole.

enzyme reaction was shown to be linear at least over the first 4 min. of incubation and there was a linear relation between the incorporation of GTP and the [DNA] over the range 0.1 - 0.5 mg.

The requirements for the incorporation of GTP are listed in Table 5. Mg^{2+} and all four ribonucleoside triphosphates were necessary for activity but note the appreciable formation of poly (G,A,U) and poly (G,A,C) in the 18 hr. starved animals. This effect was not observed by Widnell and Tata (1966a) using [^{14}C]ATP incorporation but it was observed in my earlier experiments (Section 3.2.1 - Table 3).

The effect of Actinomycin D was studied using the technique of Widnell and Tata (1966a). Results were expressed as % inhibition produced by a range of 0.1 µg. to 1 mg. Actinomycin D per mg. DNA present in the enzyme assay mixture. No prior pre-incubation period was used. The range of Actinomycin D used was 10 times greater than that used by Widnell and Tata because of the reduced incubation period (3 min. instead of 15 min.) but a similar sigmoid response was obtained (FIGURE 35).

3.2.5. THE EFFECT OF FEEDING WITH COMPLETE AND TRYPTOPHAN-FREE AMINO ACID MIXTURES ON RAT LIVER NUCLEAR DNA DEPENDENT Mg^{2+} ACTIVATED RNA POLYMERASE (SECOND SERIES)

Nuclei were prepared by the Busch procedure (Section 2.6.3), RNA polymerase assayed by the modified Widnell and Tata procedure (Section 2.11.2) and the initial enzyme velocity was expressed as µU/mg. DNA (Section 3.2.4). Each group of results were expressed as mean $\pm$ S.E.M. together with the number of observations (n) thus:

TABLE 5

Characteristics of the DNA dependent Mg^{2+} activated RNA polymerase of rat liver nuclei

ASSAY SYSTEM	18hr. FASTED	T+ ONE HR.	T- ONE HR.
		(μU/mg. DNA)	
Complete	53	120	98
Mg^{2+} omitted	1	2	1
ATP omitted	7	14	17
CTP omitted	12	17	20
UTP omitted	18	44	48
ATP and CTP omitted	4	5	4
ATP and UTP omitted	6	11	11
CTP and UTP omitted	2	2	2
ATP, CTP and UTP omitted	3	4	3

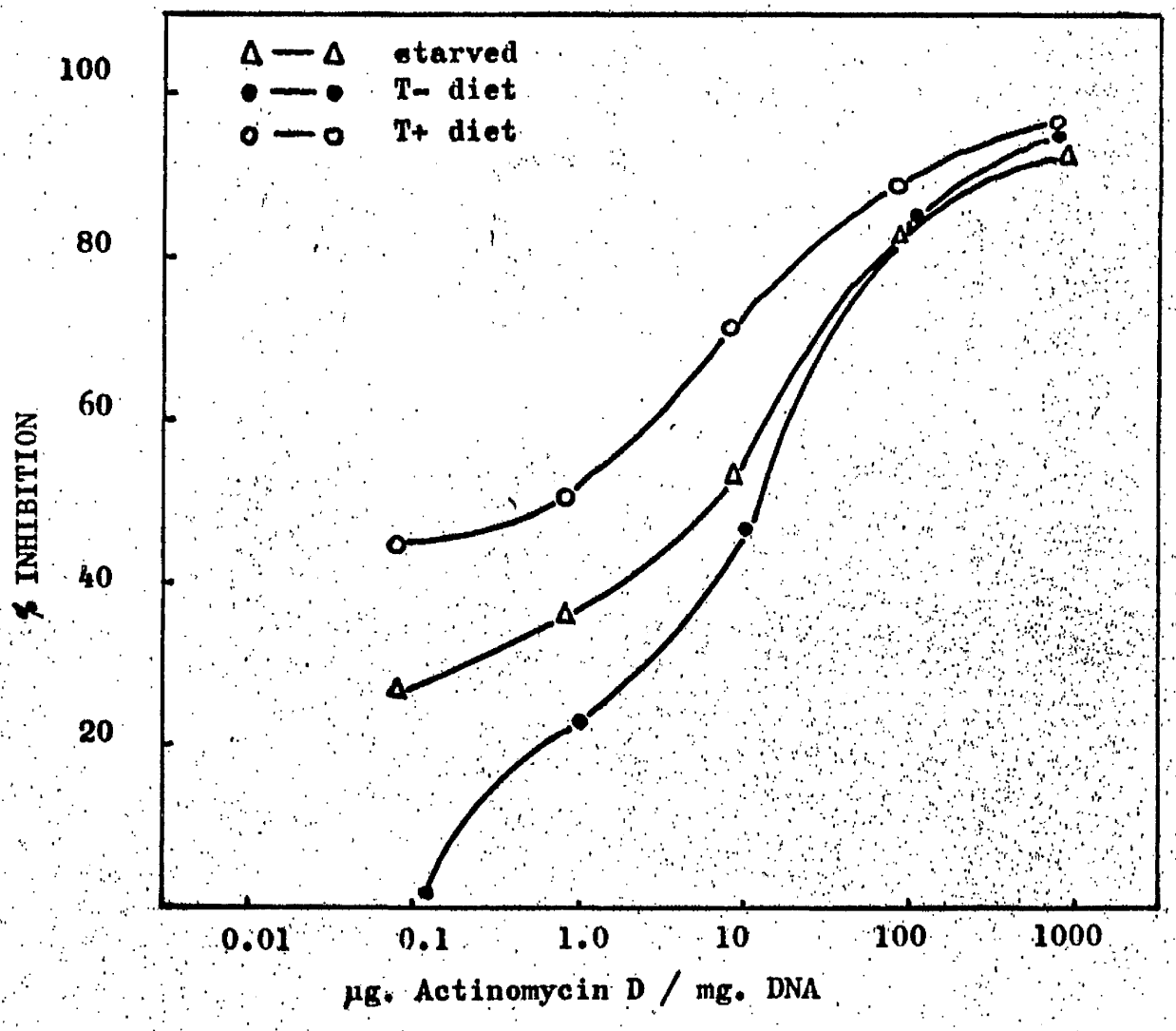


FIGURE 35 : THE EFFECT OF ACTINOMYCIN D ON RNA POLYMERASE in vitro.

Colony fed animals	65.9	±	2.5	(6)
18 hr. starved animals	53.2	±	1.2	(9)
40 hr. starved animals	49.2	±	2.2	(6)

Comparisons of these means by F-test and t-test or modified t-test for unequal variances showed that the colony fed animals' RNA polymerase activity was significantly different ($P < 0.001$) from the starved animals' activity and that there was no significant difference between the activity in the starved groups of animals.

The effect of the T+ and T- diets was then examined. The results are shown in Figure 36. Statistical analyses of these results showed that the 15, 30 and 45 min. T- groups were not significantly different from each other or from the 18 hr. starved group but that the 15 min. T+ group was significantly different from the 18 hr. starved animals ($P < 0.001$). Finally the T- and T+ 2 hr. groups were both significantly different from the colony fed group ($0.05 > P > 0.02$).

The rapidity of the RNA polymerase response to the T+ diet was shown by the response of 6 animals at 5 min. and 10 min. after feeding (Figure 37). There is a significant difference between the 18 hr. starved group and the T+ 5 min. group ($0.02 > P > 0.01$). The individual observations are shown in the graph and it is clear that some animals have responded in less than 5 min.

The enzyme characteristics of the 18 hr. fasted, the T+ fed 1 hr. and the T- fed 1 hr. groups were examined for Mg^{2+} optima (Figure 33), pH optima (Figure 34), ribonucleoside triphosphate requirements (Table 5) and Actinomycin D sensitivity (Figure 35). Apart from a different sensitivity to low concentration of Actinomycin D and small differences in the synthesis of poly (G,C,U), poly (G,A,U) the enzyme properties appeared to be similar. I therefore concluded

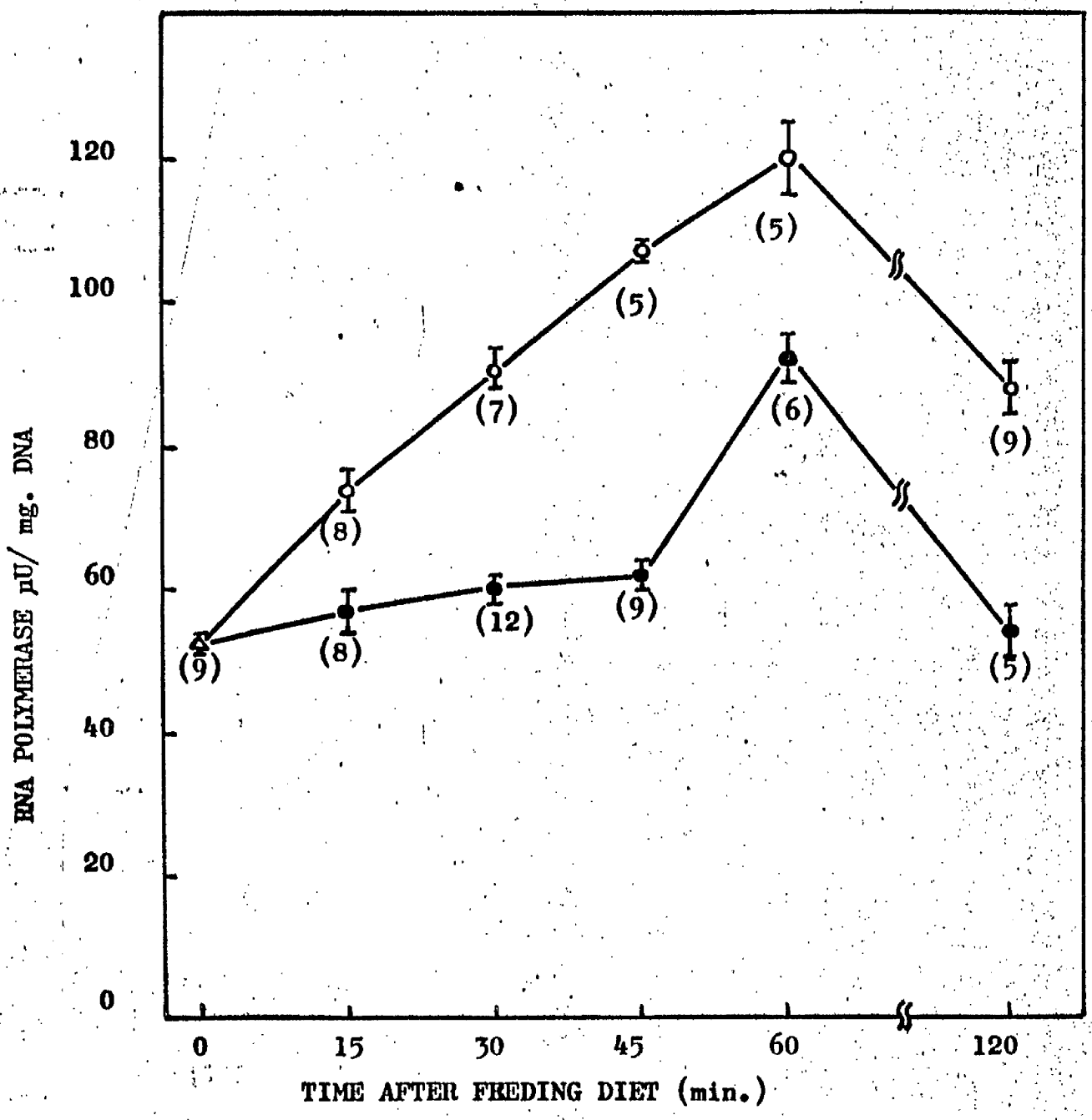


FIGURE 36 : RNA POLYMERASE ASSAY - THE EFFECT OF FEEDING WITH THE T+ AND T- DIETS. MEAN ± S.E.M. (number of observations are recorded below each determination)

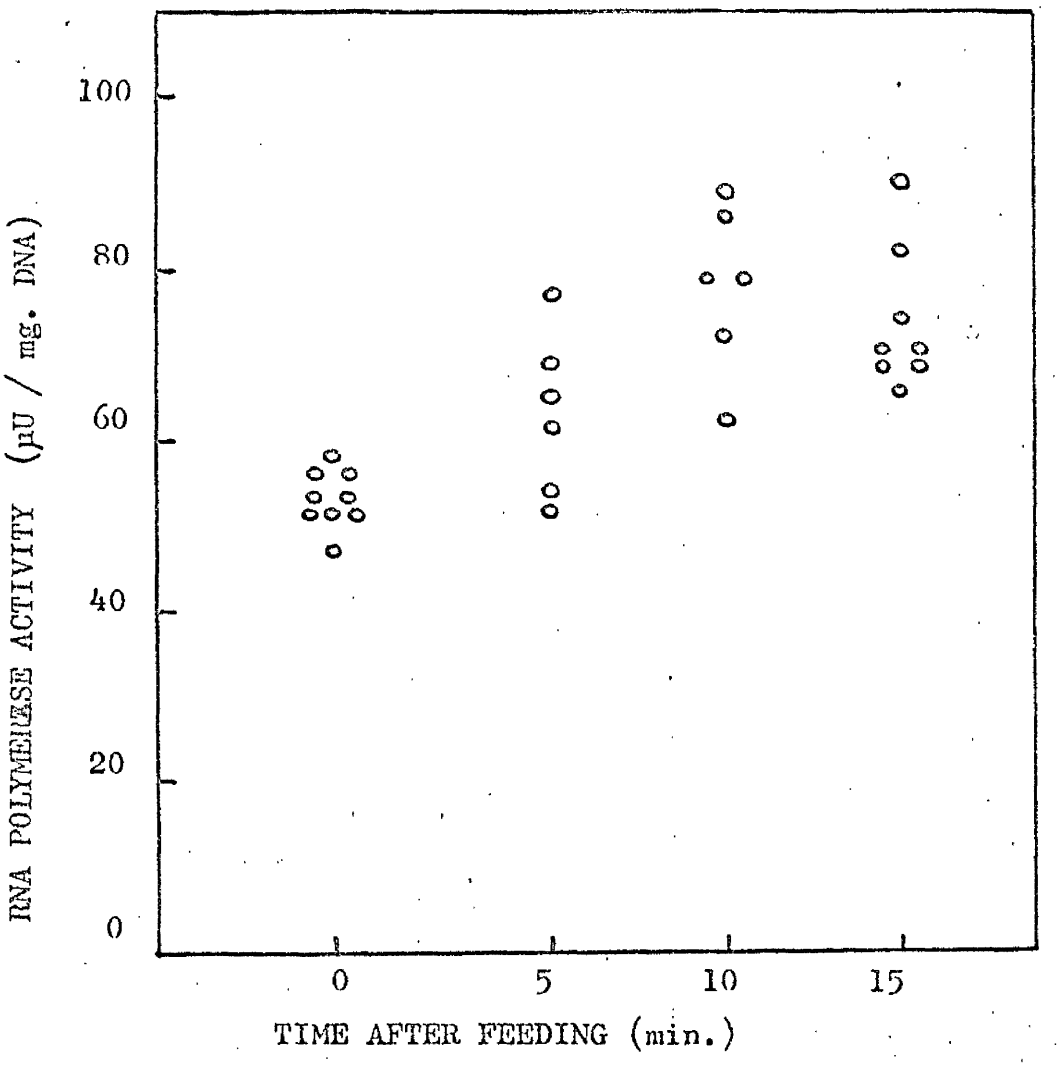


FIGURE 37 : RNA POLYMERASE ASSAY - THE RESPONSE TO THE T+ DIET OVER A SHORT TIME INTERVAL (individual observations)

that the increased enzyme activity following the dietary challenge appeared to be due to a similar enzyme as the 18 hr. starved activity.

This increased activity of the enzyme following the dietary challenge could be due to:

i) increased synthesis of the enzyme or its cofactors

ii) activation of the enzyme by dietary amino acids or their metabolites

iii) decreased breakdown of enzyme during the period after feeding

The first alternative seemed the most likely explanation because the increased activity of RNA polymerase observed after oestrogen administration in rat uterus (Noteboom and Gorski, 1963; Nicolette and Mueller, 1966) or in rat liver following growth hormone administration (Pegg and Korner, 1965) can be prevented by pre-treatment with puromycin or cycloheximide.

This possibility was tested by pretreating animals with cycloheximide or puromycin then feeding the T+ diet, and killing the animals 15 min. later. Puromycin diHCl was dissolved in 1 ml. of 150 mM-NaCl and the pH adjusted to 7.5 by the addition of 1M-KOH. This was done immediately before injection. The dose used was 100 mg./Kg. body weight (Villa-Trevino et al., 1964) and it was administered by intraperitoneal injection 45 min. before feeding. Cycloheximide was similarly dissolved in 1 ml. of 150mM-NaCl and injected in a dose of 50mg./Kg. body weight (Fallon, 1967) 30 min. before feeding. Controls consisted of 18 hr. starved animals injected with similar doses of cycloheximide or puromycin and killed after 45 min. or 60 min. exposure respectively.

Before assaying the RNA polymerase activity in these animals I showed that these compounds had no in vitro effect on the assay system in quantities up to 500 µg.

The effect of puromycin or cycloheximide on the RNA polymerase response to the T+ diet is shown in Figure 38. Statistical analysis showed that there was no significant difference between the 18 hr. fasted group, the puromycin or cycloheximide treated fasted group or the drug treated T+ 15 min. group.

Thus I concluded that these drugs had:

i) no effect on the fasted animals which were not fed

ii) prevented the synthesis of RNA polymerase or some necessary cofactor and thus abolished the dietary response to the T+ diet.

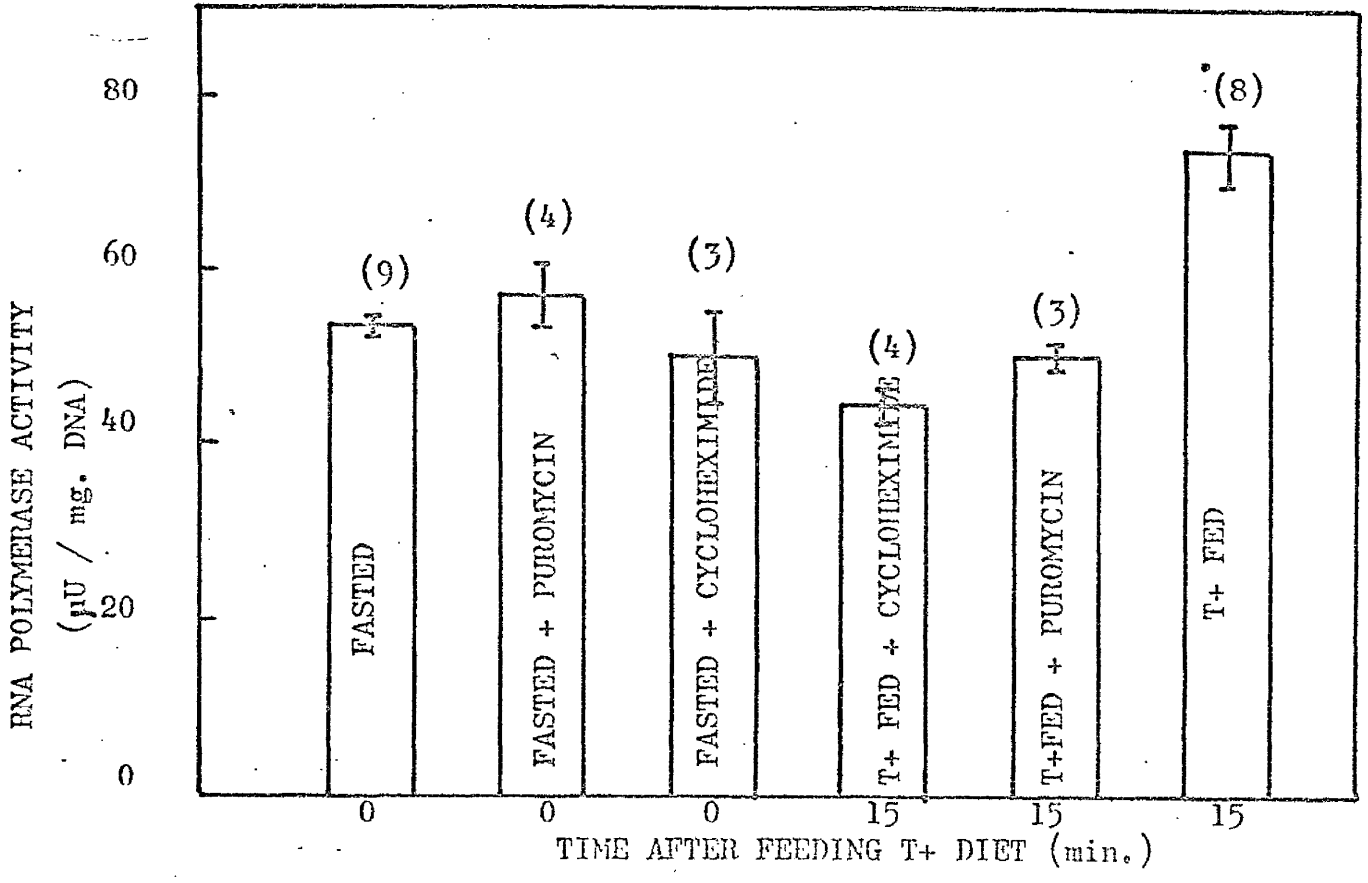
3.2.6. THE EFFECT OF FEEDING WITH COMPLETE AND TRYPTOPHAN-FREE AMINO ACID MIXTURES ON PLASMA AND LIVER-FREE TRYPTOPHAN LEVELS

The results described in the previous section were obtained by feeding diets differing only in the presence or absence of tryptophan. It was therefore of interest to measure the levels of tryptophan in blood and liver following the feeding of the diets.

Free tryptophan was determined by the procedure outlined in Section 2.5. The daily (in-batch) precision expressed as the coefficient of variation (C.V.) was 3% (n = 10) for plasma and 4.9% (n = 10) for liver. Between batch precision was 7% (n = 38) when using the 5 nmole tryptophan standard. Recovery of added tryptophan from plasma and liver was quantitative within the daily C.V. of the measurement.

Figure 39 shows the plasma levels of tryptophan following the feeding of th T+ and T- diets. As expected the T- diet causes no significant difference in the plasma levels whereas the T+ diet predictably causes a 3x increase in the plasma level within one hour of feeding. Figure 40 shows the intra-hepatic levels of free tryptophan in both dietary groups. Again, as expected, the T+

FIGURE 38 : RNA POLYMERASE ASSAY — THE EFFECT OF PUROMYCIN AND CYCLOHEXIMIDE ON THE T+ DIET RESPONSE. MEAN ± S.E.M. NUMBER OF OBSERVATIONS IN PARENTHESIS.



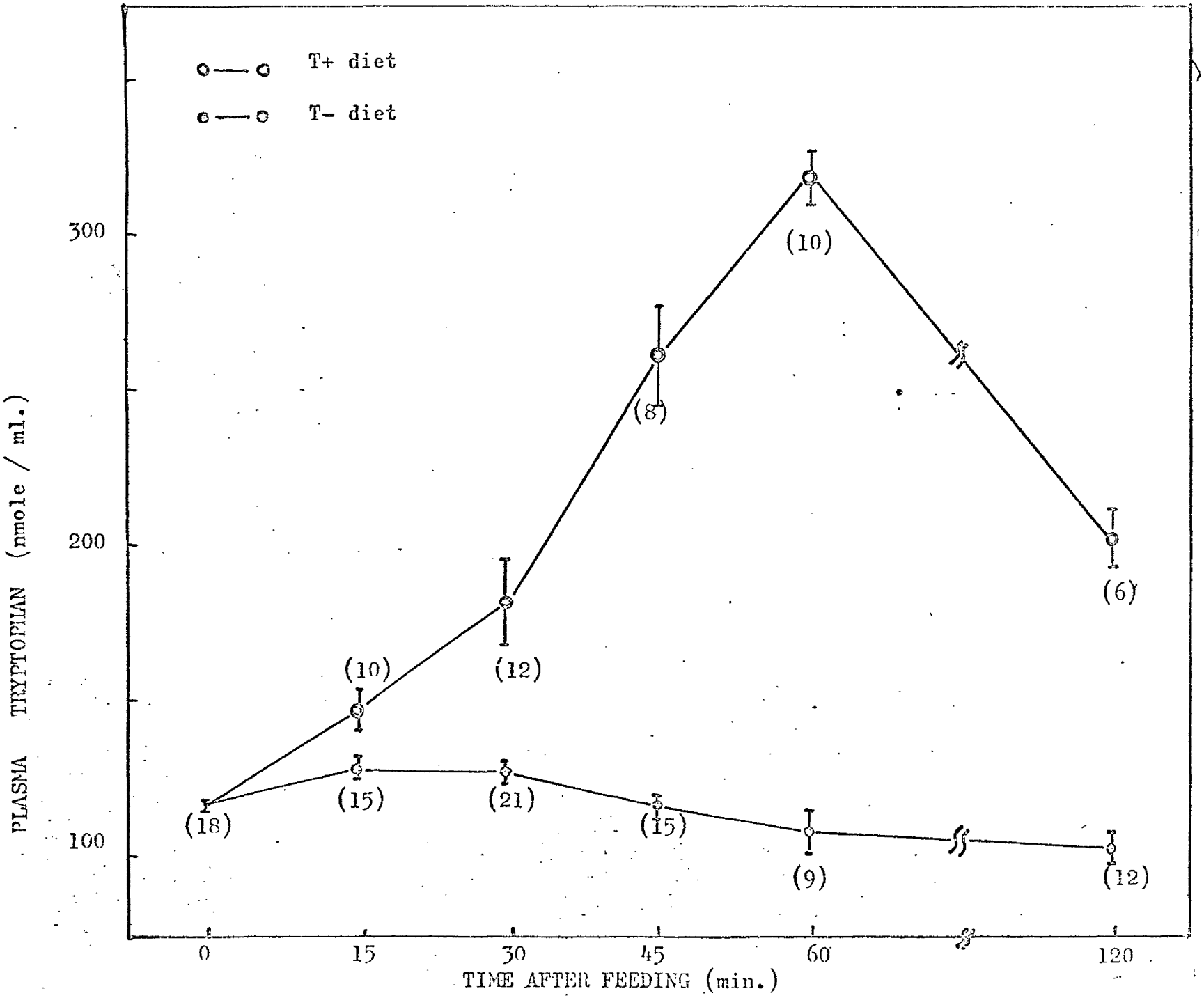


FIGURE 39 : THE EFFECT OF THE T+ AND T- DIETS ON RAT PLASMA TRYPTOPHAN. Mean ± S.E.M. Number of observations in parenthesis.

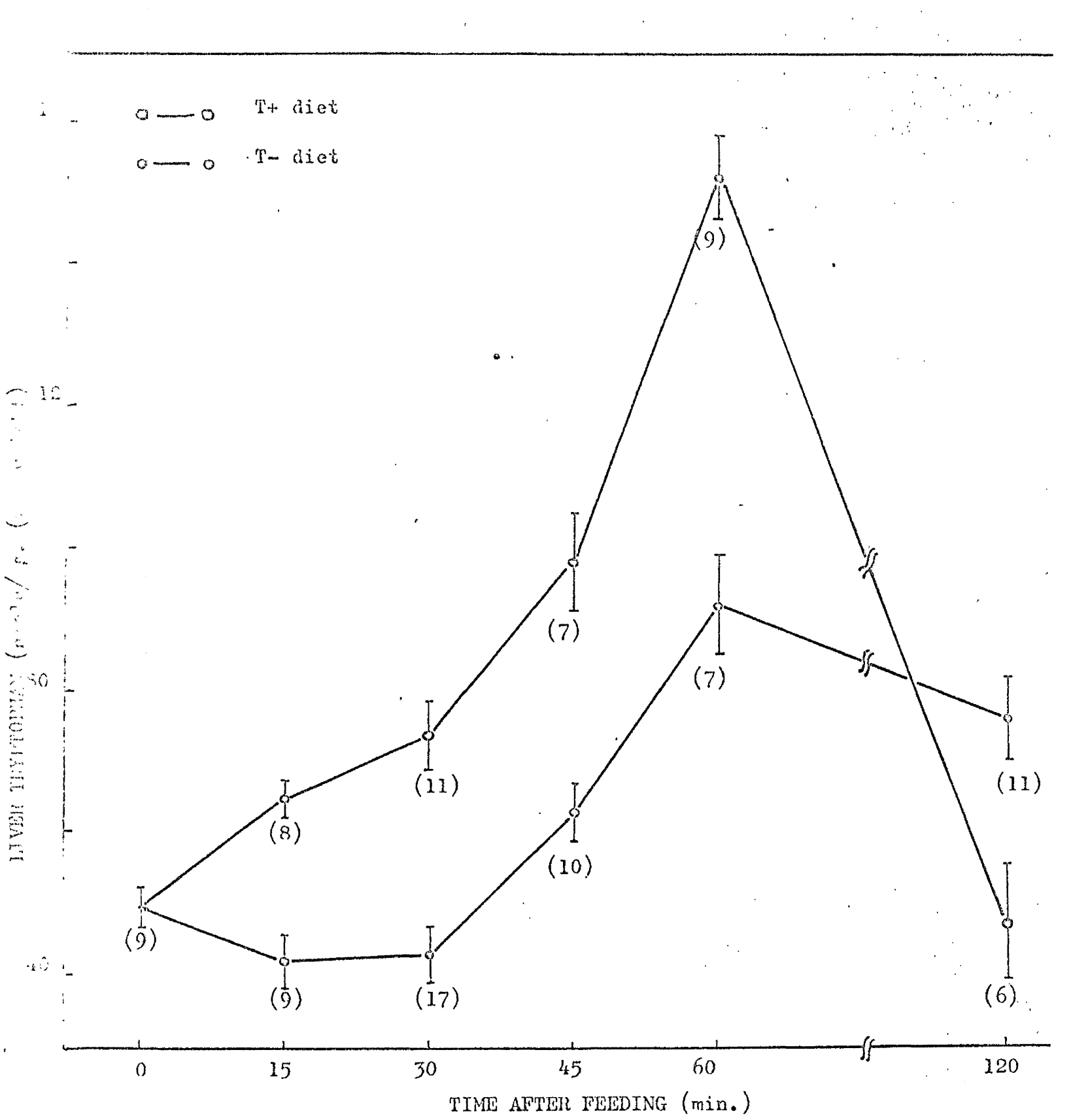


FIGURE 40 : THE EFFECT OF THE T+ AND T- DIETS ON RAT LIVER TRYPTOPHAN.

Mean ± S.E.M. Number of observations in parenthesis.

diet produces a rapid increase in free tryptophan which rises, by one hour, to a level 3x the fasting level. Note that within two hours after feeding, the liver tryptophan level is back to the fasting level. The T- diet produces unexpected results. Initially the tryptophan concentration does not change, but within 30-45 min. after feeding the tryptophan rapidly rises to a maximum of approx. 2x the fasting level by one hour and by two hours after feeding the hepatic tryptophan is still elevated compared with the T+ dietary group. It will be recalled that the plasma level remains constant throughout the two hour period studied, in the T- dietary group, and thus it could be argued that the increase in hepatic tryptophan could not be due to extra-hepatic tryptophan. However, an increased rate of flow of tryptophan out of, for instance, muscle accompanied by an increased uptake of tryptophan by the liver would result in a constant blood tryptophan level.

Several other explanations must be considered. Nasset and his co-workers have presented evidence that the exogenous protein of the gut contents (diet) is diluted by the endogenous protein derived from the gut (gastric, pancreatic and succus entericus digestive enzymes) (c.f. Nasset, 1956). Also Twombly and Meyer (1961) showed that rats fed whole egg protein had a 1 hour and an 8 hour peak of gut nitrogen content and that the 1 hour peak disappeared when a protein-free diet was fed. Gitler (1964) concludes from this and other evidence that only within 60 min. or less of feeding protein would amino acids released originate mainly from exogenous proteins. In the present series of experiments the exogenous protein is in the form of amino acids which are likely to be absorbed more rapidly than the comparable protein. It thus seems likely that the source of additional tryptophan is not due to the digestion of endogenous protein of the gut.

Recently Gan and Jeffay (1967) have shown that during early stages of fasting as much as 90% of the liver amino acid was derived from intra-hepatic protein degradation and as the animals used in the present study were starving for 18 hr. it would seem likely that the tryptophan was derived from this source However the timing of the increase is interesting and puzzling as it follows the influx of the dietary amino acids. Clearly this observation requires further investigation.

3.3. THE EFFECT OF FEEDING WITH A TRYPTOPHAN-FREE AMINO ACID MIXTURE ON ALBUMIN SYNTHESIS BY RAT LIVER SLICES

In 1965 Fleck and his colleagues described the effect of the T- diet on rat liver polysomes, and showed that microsomes isolated from the liver one hour after feeding the diet had a decreased ability to incorporate [^{14}C] leucine *in vitro* when compared to the microsomal activity of T+ fed animals.

Wunner *et al.* (1966) showed that a decreased ability to incorporate [^{14}C] leucine and [^{14}C] tryptophan was also found in polysomes and microsomes isolated from the liver of T- fed rats when compared with the incorporation obtained by similar particles in the T+ fed animals. They also observed that the molar incorporation ratio of leucine: tryptophan was 4.5 : 1 in both groups of animals. This ratio approximates to the relative requirement for these amino acids by the rat and it thus seemed likely that the observed incorporation reflected protein synthesis but neither group of workers characterised the proteins which were synthesised.

In 1968 Labrie and Korner, using adrenalectomised rats, found that tyrosine aminotransferase [EC 2.6.1.6] was synthesised following the feeding on a T+ diet or a T- diet. Thus tyrosine aminotransferase could not be considered as one of the proteins affected by the absence of tryptophan. Indeed tyrosine aminotransferase is known to be synthesised, in intact rats, following the injection of corticosterone (Lin and Knox, 1958), tyrosine, tryptophan and indoles related to tryptophan (Rosen and Milholland, 1963).

Labrie and Korner also studied tryptophan oxygenase [EC 1.13.1.12] which is more commonly known as tryptophan pyrrolase. They found, using adrenalectomised rats, that the T+ diet or tryptophan alone stimulated synthesis of tryptophan oxygenase, and although they stated that the T- diet effect was not significantly

different from the control group the activities quoted are intermediate between the control group and the T+ fed group and this does suggest that tryptophan oxygenase may be one of the proteins which are affected by the T- diet. This interpretation must be cautious however because this enzyme is known to exist in several forms (c.f. Knox, 1966):

i) inactive apoenzyme whose synthesis is stimulated in intact and adrenalectomized rats by hydrocortisone. This is the main form of enzyme in the liver.

ii) oxidised inactive holoenzyme formed from the enzyme in the presence of haematin (prosthetic group) and tryptophan.

iii) reduced active holoenzyme formed from the inactive holoenzyme in the presence of ascorbic acid and tryptophan.

Thus while tryptophan oxygenase may be affected by the T- diet the complexity of its synthesis makes it difficult to be certain. It is important to note that Labrie and Korner (1968) assayed the enzyme activity as holoenzyme + apoenzyme and they did not distinguish between the two.

It therefore seemed reasonable to further examine the effect of the T+ and T- diets on protein synthesis. The synthesis of albumin by liver slices has been a well established technique for some years since Peters and Anfinsen (1950) showed that incubated chick slices synthesised serum albumin for several hours and Campbell and Stone (1957) showed that rat liver slices behaved similarly.

The time intervals chosen were one and two hours after feeding because Wunner et al. (1966) had shown that the dimer concentration (compared to the 1 hr. T+ fed group) was 157% in the 1 hr. T- fed group and 285% in the 2 hr. T- fed group, and thus the functional activity of the polysomes might be expected to differ at these times.

Liver slices were prepared, incubated and their albumin contents estimated as described in Sections 2.14 and 2.15. The first experiment showed that the synthesis of albumin in the slices and slice supernatants had similar relationships in the 1 hr. T+ and T- animals (FIGURE 41). The liver slice albumin content dropped while the slice supernatant content increased in both animals. This preliminary experiment suggested that measuring the albumin content of the slice supernatant would be a satisfactory method of following albumin synthesis in the dietary groups.

A sample of slice supernatant was dialysed, freeze dried and examined by gel electrophoresis, gel immuno-electrophoresis and gel diffusion and it can be seen that albumin appears to be the principal protein synthesised (FIGURE 42).

Groups of animals were fed the diets and killed after 1 hr. (FIGURE 43) or 2 hr. (FIGURE 44). Tissues were incubated for 0, 30, 60 and 120 min. and the slice supernatant albumin content estimated and expressed as ug. albumin per g. wet weight of liver.

The results were compared by the one-tailed t-test with the following results:

Incubation Time (min.)	Significance of the Difference between the Means of Albumin Production
1 hour group	
0	$0.005 > P > 0.0005$
30	$0.05 > P > 0.025$
60	not significant
120	$0.05 > P > 0.025$
2 hour group	
all times	not significant

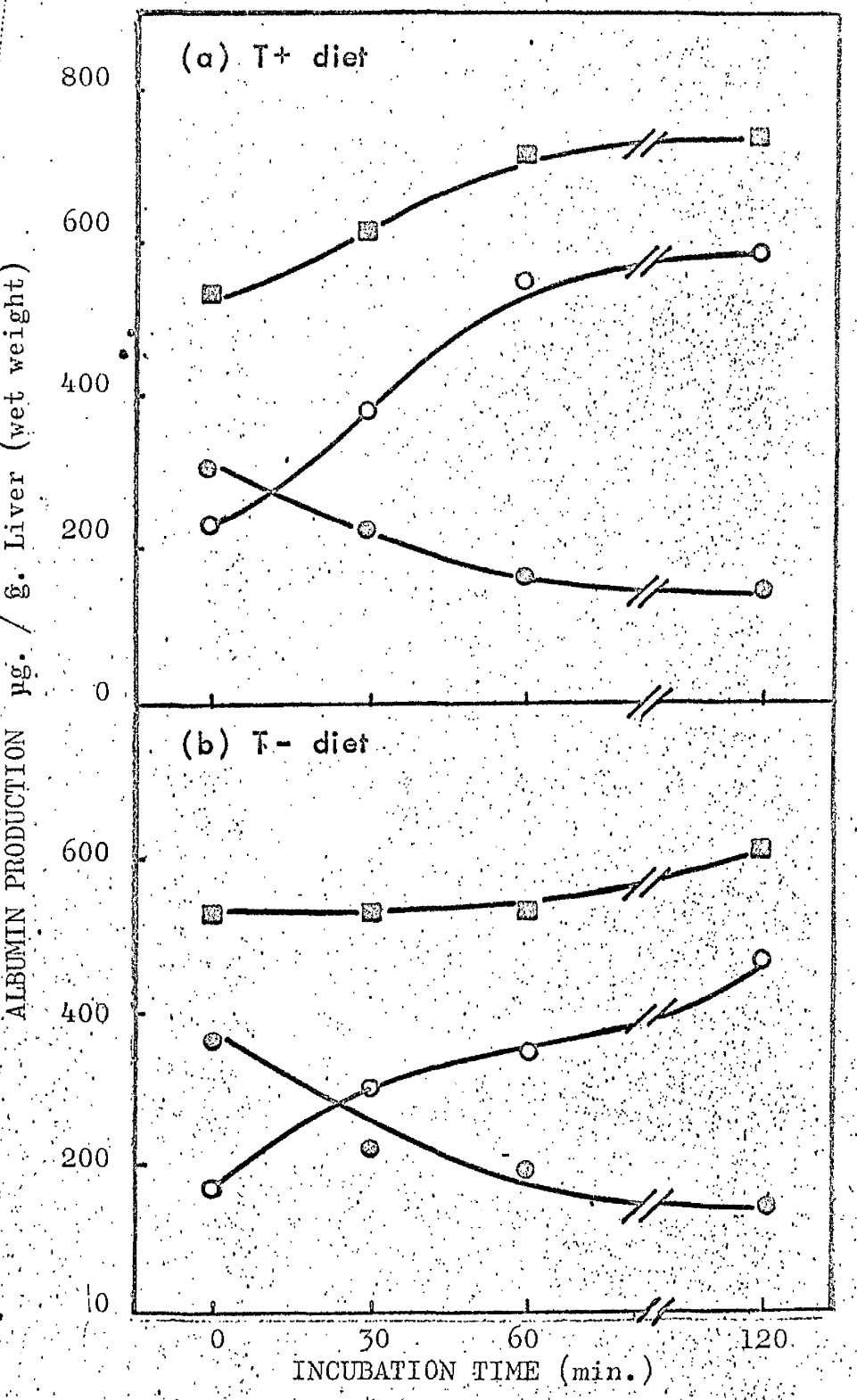
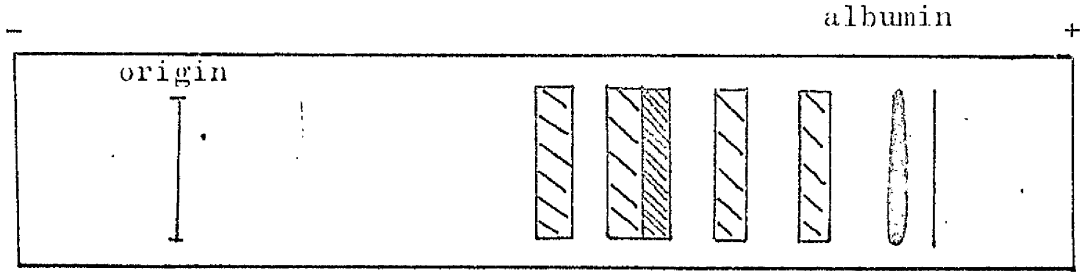


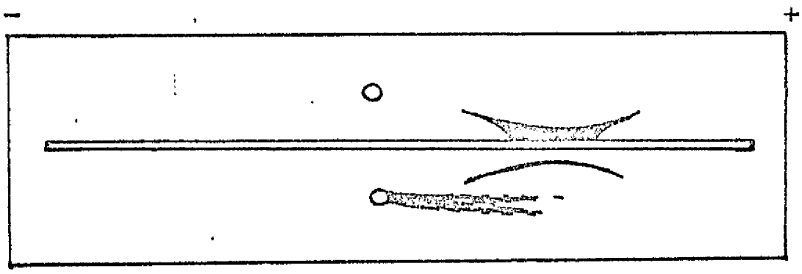
FIGURE 41 : ALBUMIN PRODUCTION BY RAT LIVER SLICES FROM ANIMALS FED AS INDICATED ONE HOUR BEFORE KILLING.

(▣ total content O supernatant production ⊙ slice content)



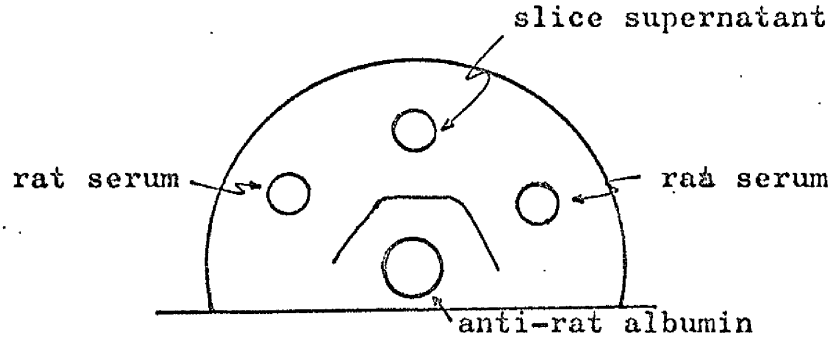
Polyacrylamide Gel Electrophoresis pH 8.6

5 minor bands are indicated by hatched areas - one being darker stained than the others.



rat serum
anti-rat albumin
slice supernatant

Agar immunoelectrophoresis pH 8.2



Agar Immunodiffusion

URE 42 : SLICE SUPERNATANT. EXAMINATION BY GEL ELECTROPHORESIS, IMMUNOELECTROPHORESIS AND IMMUNODIFFUSION

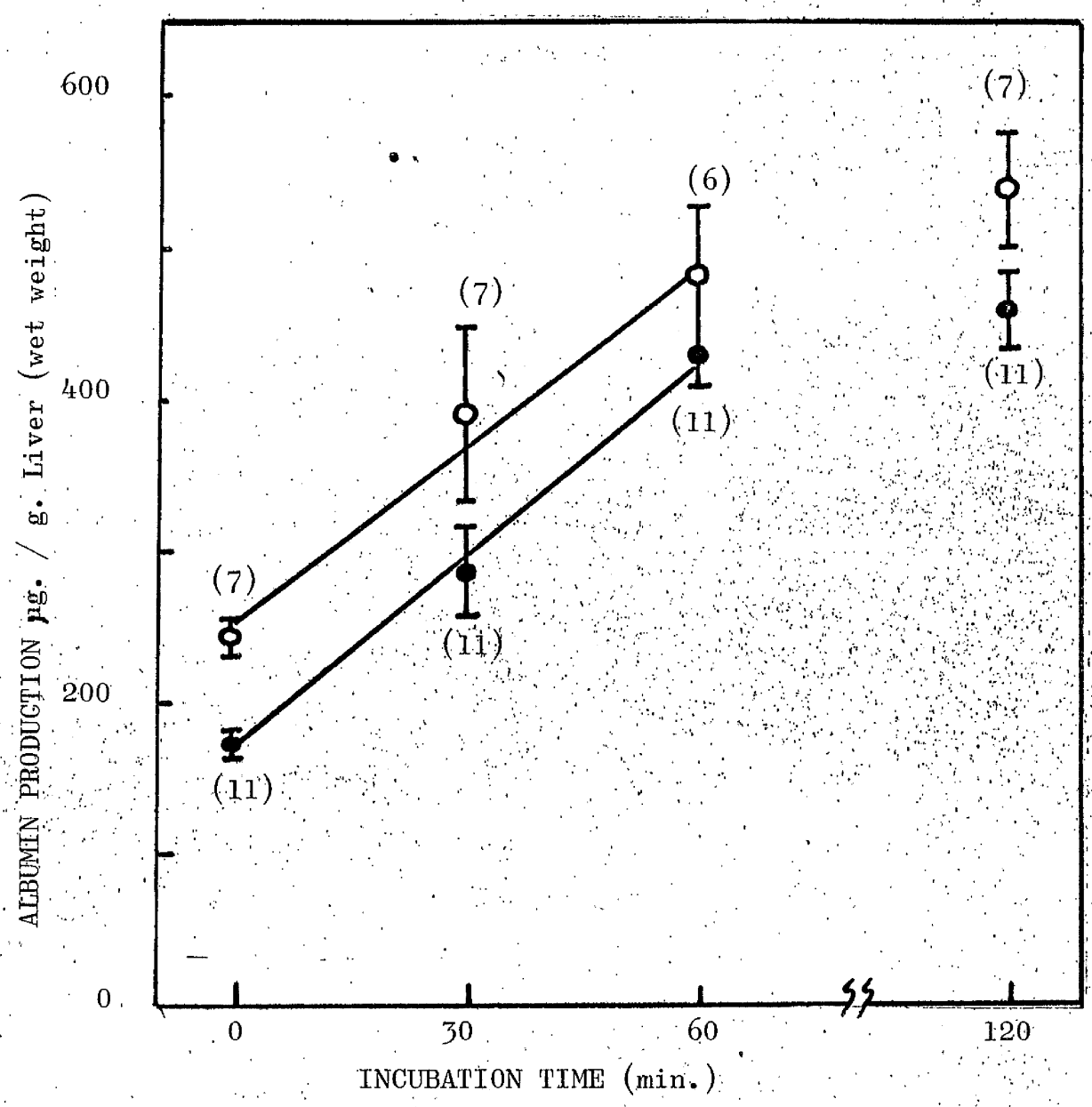


FIGURE 43 : ALBUMIN PRODUCTION BY RAT LIVER SLICES FROM ANIMALS KILLED 1 HR. AFTER FEEDING A T+ or T- Diet. Mean ± S.E.M. Numbers in each group are given in parenthesis. (o T+ ● T-)

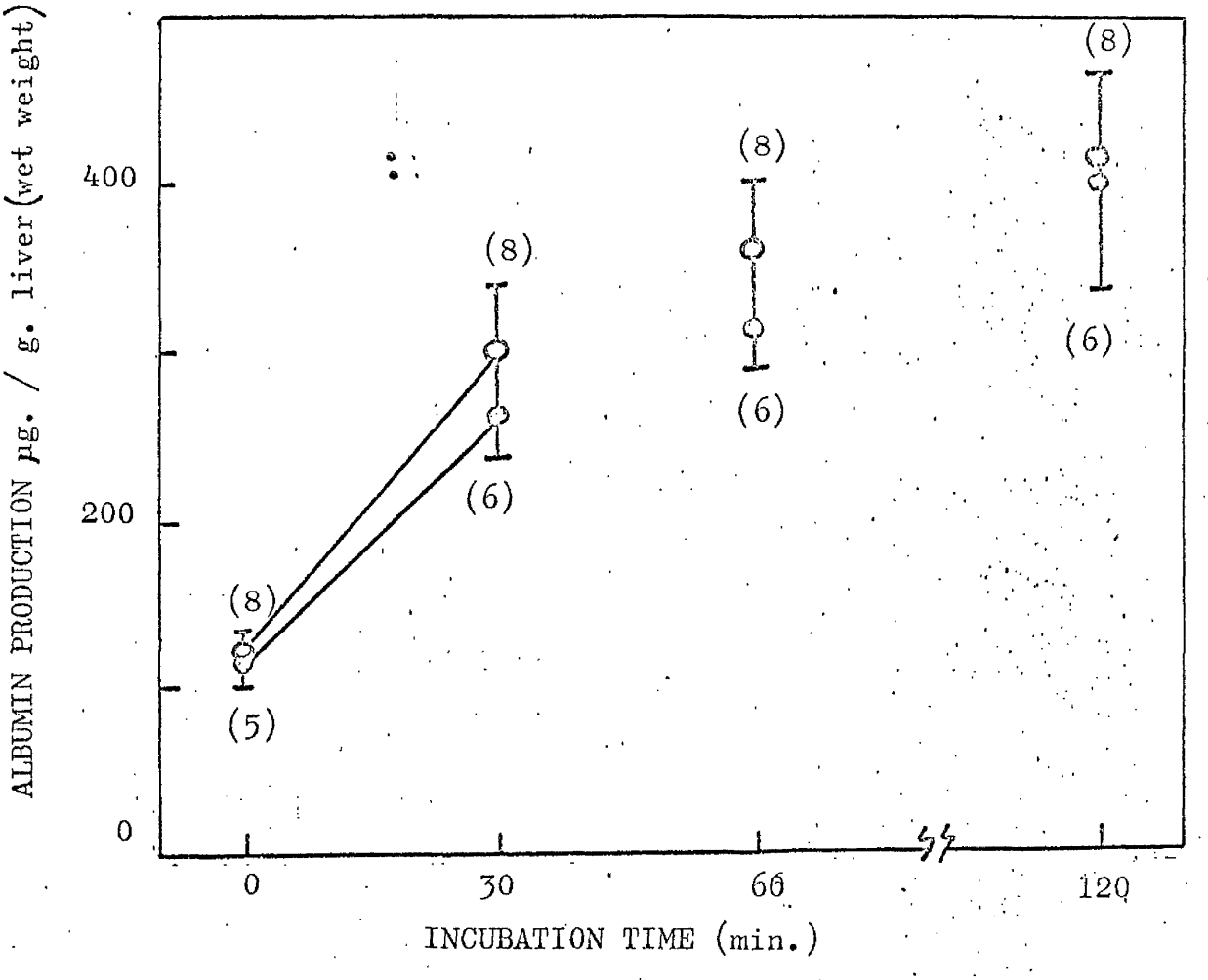


FIGURE 44 : ALBUMIN PRODUCTION BY RAT LIVER SLICES FROM ANIMALS KILLED 2 HR. AFTER FEEDING A T+ OR T- DIET. MEAN ± S.E.M. NUMBERS IN EACH GROUP ARE GIVEN IN PARENTHESIS. (o T+ ● T-)

In another group of experiments 13 mg. of tryptophan (the tryptophan content of the T+ diet) was fed by stomach tube to a group of rats and the liver slices were examined for albumin synthesis at 1 hr. after feeding. These results are shown in FIGURE 45. The group fed tryptophan alone were found to have a significantly greater albumin production than the 1 hr. T- group at 120 min. ($0.05 > P > 0.025$ by a one-tailed t-test) although the other incubation times were not significantly different from either T+ or T- groups because the tryptophan alone synthesises were midway between these groups.

Before considering these results further it is necessary to examine the validity of this experimental work. P.N. Campbell (personal communication) has criticised the work on the following grounds:

i) the intrahepatic estimation of albumin was invalid because the tissue homogenate was not treated with deoxycholate to release all the membrane-bound albumin.

ii) the specificity of albumin estimation is in doubt because of the findings of Schreiber et al. (1969) that intrahepatic albumin has to be considerably purified before immuno-assay is attempted otherwise the assay tends to give higher estimations - i.e. there is considerable cross reaction in the immuno-assay.

The first criticism is perfectly valid but unimportant because the intrahepatic estimation was carried out to compare the two dietary treatments. Our results show that "operationally" there appeared to be no gross difference between the dietary groups. It would seem likely that gross differences in intrahepatic albumin content detected by pretreatment with deoxycholate would also be apparent by the present method.

The second criticism is valid and important. The work of Schreiber

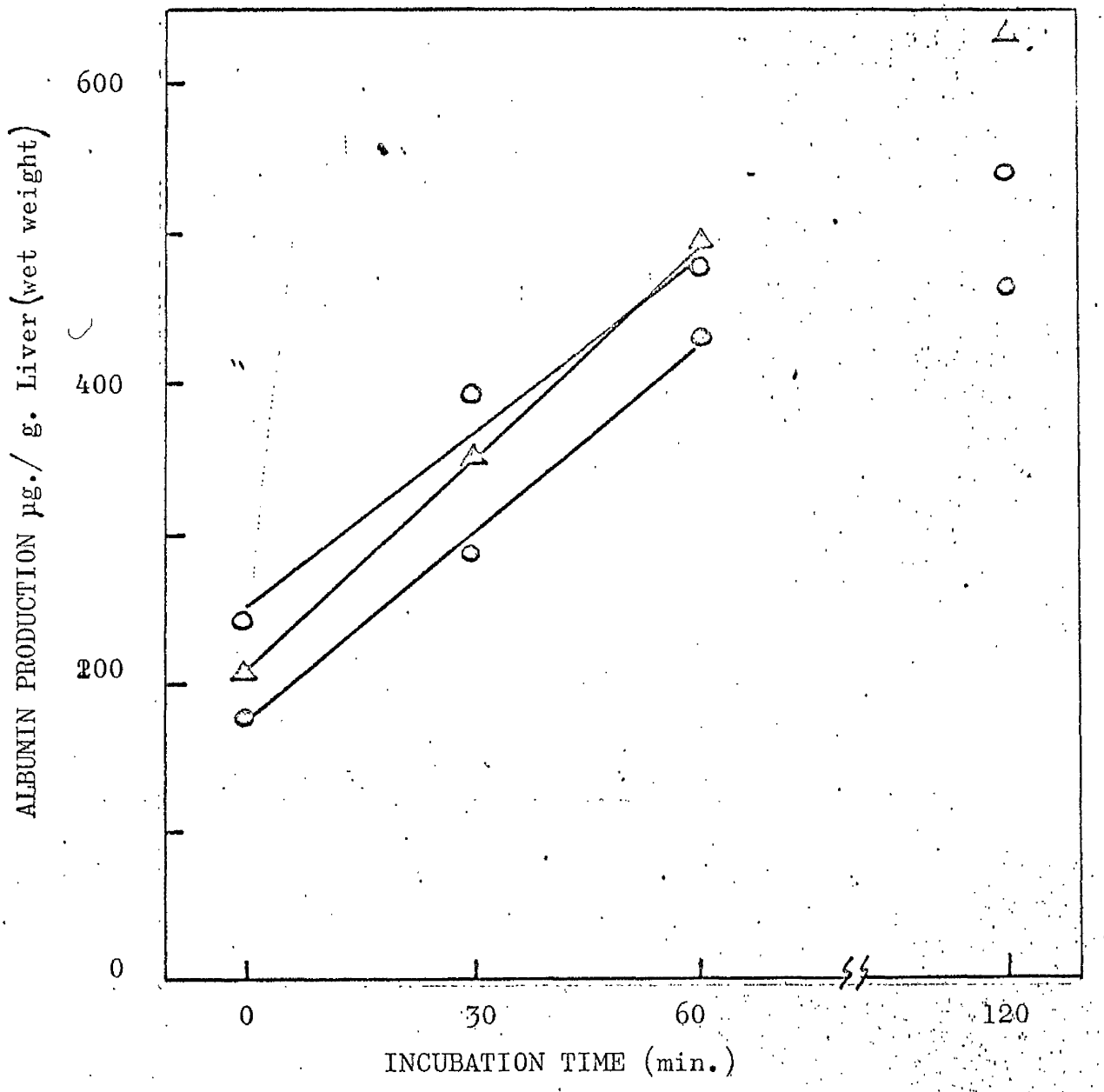


FIGURE 5 : ALBUMIN PRODUCTION BY RAT LIVER SLICES FROM ANIMALS KILLED 1 HR. AFTER FEEDING 13 mg. TRYPTOPHAN. EACH VALUE REPRESENTS THE MEAN OF FOUR ANIMALS. (△ T alone ○ T+ 1 hr. ◎ T- 1 hr.)

et al. (1969) demonstrated that radiochemically pure albumin cannot be obtained by immunological precipitation alone and thus an estimation of intrahepatic albumin had to be preceded by several purification stages. It will be recalled that the albumin content of the bath media is secreted from the slice during incubation - although the breakdown of the tissue slice allowing intracellular albumin to pass into the incubating medium is very likely - and this conclusion is supported by the gel electrophoresis results and the immuno-electrophoresis patterns obtained from the incubating bath protein (Figure 42). In this connection it is important to stress that Schreiber studied albumin from homogenised liver and not secreted albumin as in the present case. Clearly this objection is highly relevant and I intend to apply the purification technique of Schreiber to the albumin obtained by incubating liver slices when certain pieces of apparatus become available to me.

Finally comparison of the present work with earlier observations on albumin synthesis by rat liver slices is of interest. Campbell and Stone (1957) showed that their slice preparations produced albumin in the slice supernatant at a rate of 4.7 µg.albumin/g.wet weight/min. for 4 hours (estimated from their Figure 7). They do not state the age or nutritional state of the rats. Marsh and Drabkin (1958b) found that fed 100-175g. rats produced albumin from incubated slices at a rate of 6.1 µg./g.wet weight/min. The rates of production found in the present study were:

Dietary treatment	Rate of Synthesis µg.albumin/g.wet weight/min.	Linear Period
T+ 1 hr.	4.0	1 hr.
T- 1 hr.	4.2	1 hr.
T+ 2 hr.	5.9	30 mins. (?)
T- 2 hr.	5.0	30 mins. (?)
Tryptophan alone 1 hr.	4.8	1 hr.

Comparing the 2 hr. T+ rate with the Marsh and Drabkin results shows that the fed rats have a similar albumin synthesis rate which is greater than the 2 hr. T-group and that these results are greater in turn than the T+ or T- 1 hr. groups. It should be remembered that the significance tests carried out on the 1 hr. groups showed a significant difference between the 0, 30 and 120 min. synthesis whereas there was no significant difference between the 2 hr. groups. These results suggest that using tissue slices there is a demonstrable effect of omission of tryptophan for the diet on serum albumin synthesis one hour after feeding but that this difference is abolished two hours after feeding.

Finally it is important to remember that the liver slice <u>in vitro</u> is less effective than the liver <u>in vivo</u> at synthesising albumin. Thus Drabkin and Marsh (1958a) were able to calculate that a 75 g. rat synthesised albumin about 5 times faster than the liver slices. Thus it would be expected that the <u>in vivo</u> effects of the T+ and T- diets, which were demonstrated <u>in vitro</u>, would be clearly shown.

4. DISCUSSION

4.1. NUCLEAR RNA SYNTHESIS : AN ANALYSIS OF THE RESULTS OF THE SUCROSE DENSITY GRADIENT EXPERIMENTS

'.... It is obvious that sucrose-density gradient sedimentation is a poor method for determining the size, the homogeneity or the identity of RNA molecules: molecules that sediment in the same region may have widely different conformations or molecular weights, and molecules that sediment in different regions may, under other physical conditions, sediment together'. (Bramwell and Harris, 1967).

What then is the significance of the findings described in Section 3.1? Before they can be examined it is necessary to consider the different types of large RNA molecules which are synthesised in the nucleus. In a recent review Darnell (1968) distinguished between:

1) heterogeneous RNA (hnRNA) and
2) pre-ribosomal RNA (pre-rRNA).

As these molecules are synthesised in the nucleus (albeit in different parts of the nucleus) both RNA types would necessarily be extracted by the procedures which were used on isolated rat liver nuclei (Section 2.7).

4.1.1. HETEROGENEOUS RNA

In 1959 Harris found, using quantitative radioautographic techniques on cultured connective tissue cells (which multiply) and cultured macrophages (which do not), that both cells displayed a rapid turnover of nuclear RNA. A surprising finding, however, was the very small quantity of nuclear RNA which passed into the cytoplasm in both cells. He was also able to differentiate between the RNA synthesised in the nucleolus and in the rest of the nucleus

(Harris, 1959). Later Scherrer, Latham and Darnell (1963) and Harris (1964) produced evidence that when RNA synthesis was halted by Actinomycin D a large portion of the rapidly labelled RNA was very unstable.

Soon two other properties which this rapid-turnover RNA possessed were also described. Scherrer et al. (1966) discovered its rapid labelling and Scherrer and Marcaud (1965), Houssais and Attardi (1966), Warner et al. (1966) and Attardi et al. (1966) showed that it was rapidly sedimenting in a sucrose density gradient (20S to 80S) both in HeLa cells and immature duck erythrocytes. The results described from this latter cell type were most interesting as it was estimated that 60-70% of the total radioactivity in nuclear RNA was gradually lost. The hnRNA was found to have a GC content of 44% (similar to DNA) which is entirely different from the pre-rRNA (GC > 60%). Soeiro et al. (1966) were able to show that the hnRNA was associated with the non-nucleolar or nucleoplasm fraction of the nucleus whereas pre-rRNA was associated with the nucleolus. Similar findings were reported for rat liver (Steele and Busch, 1966) One other property of hnRNA is its relatively greater resistance to the effects of Actinomycin D compared to pre-rRNA in rat liver (Steele and Busch, 1966) and HeLa cells (Penman, Vesco and Penman, 1968).

Soeiro et al. (1968) were able to calculate that at least 90% of the hnRNA fraction in HeLa cells is rapidly synthesised and degraded within the nucleus - confirmation indeed of Harris's autoradiographic work (Harris, 1959).

Several suggestions have been made about a possible function for hnRNA. They include i) ribosomal precursor ii) messenger RNA iii) special intranuclear mRNA and iv) genetic controllers. Bramwell and Harris (1967) showed that HeLa cell hnRNA could sediment as a single 16S peak in low ionic strength media with Mg^{2+} absent. They suggested that this material might be a 16S rRNA precursor

but this assumption was incorrect (Section 4.1.2). This 16S hnRNA is however thought to have a similar size to 16S rRNA (Tamaoki and Lane, 1967; Riley, 1969).

These findings do support the mRNA function of hnRNA because this size of molecule is well within the range of mRNA isolated from rat liver polysomes which are 20S (Staehelin et al., 1964). Before Bramwell and Harris published their work it did seem very unlikely that the 'polydisperse' hnRNA had to be degraded to smaller mRNA molecules before leaving the nucleus because this meant that the messenger was subject to a series of random intranuclear events which is not reflected in the products of protein synthesis.

It is of interest to note that Attardi et al. (1966) did find a minor portion of RNA synthesised by duck erythrocytes to have mRNA properties and a 9S sedimentation value. It is known that 9 (or 10S) is the size of the haemoglobin mRNA (Chantrenne et al., 1967; Labrie, 1969). Attardi et al. did state, however, that no clear relationship existed between the hnRNA and mRNA. This was also the conclusion of Penman et al. (1968) in a study of the relationship between hnRNA and mRNA in HeLa cells.

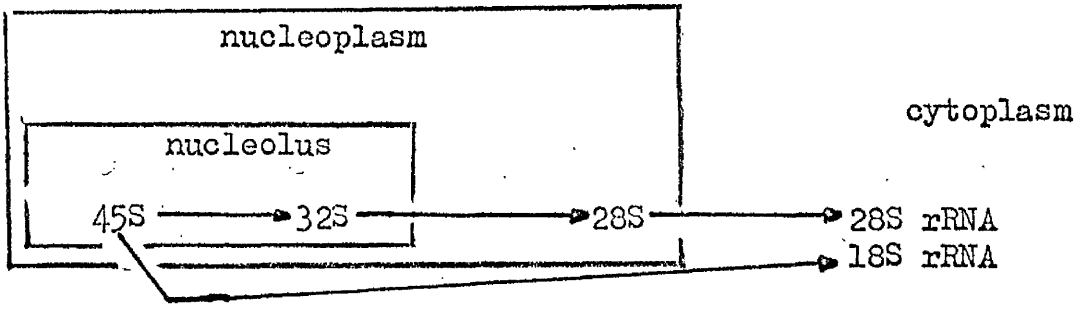
Recently it has been observed that the hnRNA of mice kidney nuclei rapidly disappears following unilateral nephrectomy. This reduction in hnRNA is thought to be related to the renal hypertrophy which occurs in the remaining kidney (Willems et al., 1969). In conclusion, therefore, the function of hnRNA still remains obscure.

4.1.2. PRE-RIBOSOMAL RNA

The second class of nRNA which can be extracted from nuclei is ribosomal RNA. The precursor to the two classes of rRNA has been well characterised in HeLa

cells and the properties of this pre-rRNA will be described together with the pathway of rRNA synthesis. There appear to be some differences in the pre-rRNA molecule and its subsequent processing in rat liver and this will be discussed after the HeLa system has been described.

Following the availability of isolated HeLa nucleoli (Penman, Smith and Holtzmann, 1966) and rat liver nucleoli (Maramatsu and Busch, 1964) it became clear that the pre-rRNA originated exclusively in the nucleolus. It was found to sediment at 45S, to have a molecular weight of 4.1×10^6 daltons and to contain the 18S and 28S rRNA sequences (reviewed by Darnell, 1968). Kinetic evidence for [^{14}C]uridine incorporation into nucleolar RNA of HeLa cells showed that the 45S RNA labelled within the first 10 min. and that within 30 min. of labelling radioactivity appeared in the 32S RNA (in the nucleolus) and 18S RNA in the cytoplasm (which appears to pass rapidly out of the nucleolus and nucleoplasm) and finally, after about 70 min. of labelling, the nucleoplasmic 28S RNA and cytoplasmic 28S rRNA are radioactive (Penman et al., 1966). Thus in 1966 the following scheme of rRNA synthesis was proposed by Penman:



Since this original scheme was outlined, many of the details have been filled in. Further knowledge has been gained by the discovery of:

1) 2'-O-ribose methylation of rRNA

2) Non-ribosomal sequences, and

3) Short-lived RNA intermediates between 45S and 28S + 18S in size.

4.1.2.1. METHYLATION

It was noticed that the addition of methyl labelled methionine to a HeLa cell culture resulted in a RNA labelling pattern similar to that obtained when using uridine (Greenberg and Penman, 1966). These workers also showed that methylation occurred in the nucleolus close to the growing point of the newly synthesised RNA because they discovered the existence of methylated nascent 45S RNA after very short periods of labelling (1.5 and 3 min.). From this data they calculated that a 45S RNA molecule is synthesised in 2.5 min. and this implies a chain growth rate of 80 nucleotides per second - slightly faster than 55 nucleotides per second found in bacterial systems (Bremer and Yuan, 1968).

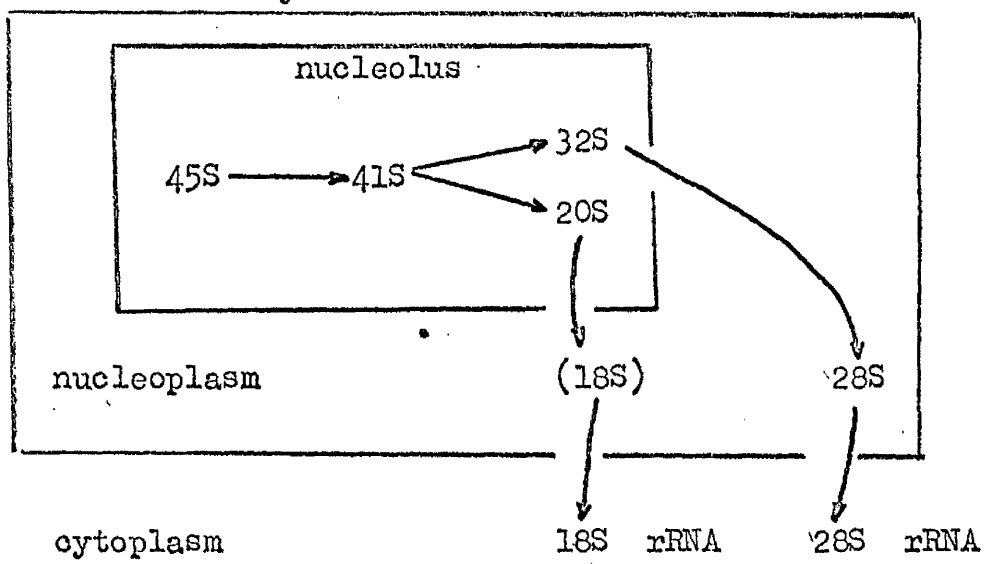
4.1.2.2. NON-RIBOSOMAL SEQUENCES

During the conversion of 45S RNA→28S + 18S the relative level of methylation increases twofold (Vaughan et al., 1967; Weinberg and Penman, (1970) and this implies the removal of a substantial amount of unmethylated polynucleotide. Jeanteur, Amaldi and Attardi (1968) showed that while 45S RNA has a GC content of 70%, 28S + 18S RNA has a GC content of 65%, and this implies that the material which is removed in the conversion process has a very high GC content. Molecular weight estimations have shown that 45S RNA is about three times larger than 28S rRNA and seven times larger than 18S rRNA (reviewed by Darnell, 1968).

4.1.2.3. SHORT-LIVED INTERMEDIATES IN 28S and 18S RNA PRODUCTION

The fine resolving power of polyacrylamide gel electrophoresis has enabled some short-lived intermediates to be recognised in the 45S RNA rRNA conversion process. Thus 41S RNA, 20S RNA and a small amount of 28S RNA have been detected in the nucleolus (Weinberg et al., 1967; Weinberg and Penman, 1970).

The scheme of rRNA synthesis is thus:



Recently some findings about the position of the 18S segment within the 45S molecule have been obtained from the observation that 3'-deoxyadenosine causes premature termination of transcription of 45S RNA and that its use leads to mature 18S rRNA but not 32S or 28S (Siev, Weinberg and Penman, 1969). This implies that the 18S sequence lies near to the point of growth of the 45S RNA molecule.

4.1.2.4. RAT LIVER RIBOSOME SYNTHESIS

The scheme outlined above for ribosome synthesis in HeLa cells seems, in general, to hold for all eukaryotes studied, but some findings from Busch's group, on the rat liver system, appear to differ from the HeLa cell results.

Thus a 55S nucleolar RNA has been recognised on sucrose density gradients from rat liver (Muramatsu et al., 1966; Steele and Busch, 1966), and isolated (Steele, 1968) and it has been shown to hybridise with nucleolar DNA (Steele, 1968). This RNA species is never visible on nucleolar RNA gradients of HeLa cells, and a possible explanation could be the degradation of 55S RNA during the DNase incubation of the nuclei. We certainly found that 45S RNA disappeared

from rat liver nuclei when the Penman technique was attempted (Section 3.1.4). It should be noted that rat liver nucleoli prepared by Busch's group were isolated by a sonication technique (Muramatsu and Busch, 1964).

A second difference was the suggestion by Muramatsu et al. (1966) that 18S rRNA did not arise in the nucleolus, but elsewhere in the nucleus, because that species was present in very low amounts in the isolated nucleus. As 18S rRNA has been shown to be complementary to nucleolar DNA (Steele, 1968) it seems likely that the 18S rRNA rapidly diffuses out of the nucleolus into the nucleoplasm (where it can be detected) - a situation analogous to that of the HeLa cell where 18S rRNA is not found in the nucleus because it rapidly diffuses out of both the nucleolus and nucleoplasm. Presumably polyacrylamide gel electrophoresis of isolated nucleolar RNA will answer this question as it has done in HeLa cells.

Finally an attempt has been made in rat liver to relate the ultrastructural components of the nucleolus to the RNA particles in the rRNA synthesis pathway (Muramatsu and Busch, 1967). Two main components can be observed by electron microscopy - a granular component which is approximately 100 - 200 A^{o} in diameter and a fibrillar component which is about 50 A^{o} in thickness. Both are RNase sensitive (Marinozzi, 1964). By utilising the effects of Actinomycin D (which stops pre-rRNA synthesis in the nucleolus) and thioacetamide (which increases nucleolar 45S and 35S RNA) (Steele, Okamura and Busch, 1965) it would appear that 6S RNA is found in the fibrillar components and 45S, 35S and 18S RNA in the granular components (Muramatsu and Busch, 1967).

4.1.3. THE EFFECT OF A TRYPTOPHAN-FREE AMINO ACID FEED ON NUCLEAR RNA SYNTHESIS EXAMINED BY SUCROSE DENSITY GRADIENT ANALYSIS

It has been known for over 50 years that feeding a starved animal leads to an increase in cytoplasmic basophilia of the liver cell (Berg, 1914). This basophilia was located around protein containing granules. The effect of diet on the distribution of these granules has been extensively investigated by many workers and a review of this early work is given by Lagerstedt (1949).

Examinations of rat liver by ultraviolet light showed that the Feulgen-negative, 260nm. absorbing cytoplasmic granules were RNase sensitive (Davidson and Waymouth, 1946), that they corresponded to the basophilic granules described by Berg (Lagerstedt, 1946) and that the nutritional changes in the cytoplasm, observed by earlier workers, corresponded to changes in the U.V. absorption of the cytoplasm (Davidson and Waymouth, 1946; Lagerstedt, 1966; Opie and Lavin, 1946).

Lagerstedt was able to show that the feeding of a high protein diet to a rat which had been starved firstly increased the nucleolar size in the liver cell and then basophilic inclusions appeared close to the nuclear membrant (Lagerstedt, 1949). Electron microscopy of the liver cell cytoplasm on feeding a starved rat with a high protein diet showed a rapid regeneration of the endoplasmic reticulum but in the early stages "..... they are found to consist of a compact network of small vesicles and short tubules. These differ from the elements of typical endoplasmic reticulum in their closer organisation and in that they generally lack the small dense particles on their surface. At longer intervals after the onset of protein feeding, dense reticular aggregations of this sort become increasingly prominent" (Fawcett, 1955). This process is much

less rapid if a low protein diet is fed to the starved animal. Munro and Clark (1959) showed that the uptake of [^{14}C]orotic acid and [^{14}C]glycine into rat liver RNA was decreased within the period 1½ hr. - 6 hr. after feeding an amino acid mixture lacking tryptophan when compared with the corresponding uptake following the feeding of the complete amino acid mixture.

The findings described in Section 3.1 appear to reflect the increased activity of RNA synthesis in the nucleus - the end result being increased basophilia in the cytoplasm - and they agree with earlier findings that the T- diet appears to produce a much less active stimulation of nRNA synthesis than the complete (T+) diet. However, there are two factors which must be considered before this conclusion can be reached. They are:

1) the nature of the extracted nRNA
2) the effect of the diets on the RNA precursor part of nucleoside triphosphates.

The extracted nRNA is now known to consist of ribosomal RNA and pre-rRNA which is synthesised in the nucleolus and heterogeneous nRNA (hnRNA) synthesised in the non-nucleolar part of the nucleus. Thus the gradient analysis results described in Section 3.1 will reflect both ribosomal RNA synthesis and changes in the hnRNA species. Steele and Busch (1966) showed that hnRNA had a number of components - 6S, 18S, 31S, 40S, 55S and 55+S. It is not possible to estimate the hnRNA contribution to the radioactivity, but the findings of Willems et al. (1969) on the changes in Kidney hnRNA following unilateral nephrectomy in mice suggest that there could be a differential response of hnRNA labelling to the diets. Clearly this requires investigation.

The nucleoside triphosphate pool size has already been referred to in Section 3.1.2. It is necessary to consider:

1) Intranuclear nucleoside triphosphate synthesis

2) The existence of an intranuclear (or nucleolar) nucleoside triphosphate pool.

Rat liver nuclei possess the enzymes (in high activity) necessary for glycolysis, and glycolytic metabolites have been found in nuclei (Siebert, 1961; Siebert et al., 1961). Some citric acid enzymes and some electron transport system components are present in rat liver nuclei but Conover (1967), in an extensive review of respiration in nuclei, could not come to a firm conclusion about rat liver nuclei. Thus we can conclude that some ATP may be synthesised, by glycolysis, but this may be the only source of intranuclear ATP production.

What of the existence of a nucleoside triphosphate pool in the nucleus? Siebert and Humphrey (1965) have divided the nucleus into three metabolic 'spaces' - the sodium, soluble and chromatin spaces. They suggested that the soluble space probably represents an extension of the cytoplasmic space, and this space presumably interfaces directly onto the chromatin space. There appears to be a lack of information on the ability of ATP to pass through the rat liver nuclear membrane although cell thymus nuclei are known to be impermeable to nucleotides (McEwan et al., 1963) but are freely permeable to nucleosides. Isolated rat liver nuclei cannot retain nucleotides and possess no demonstratable ATP synthesis (Conover, 1967) although this may be an artefact of isolation. Nuclei are known, however, to contain all the nucleoside triphosphates required for RNA synthesis (Hadjiolov, 1967) but their relationship to the cytoplasmic nucleoside triphosphate pool is not clear. Hadjiolov et al. (1965) showed, in Ehrlich ascites tumour cells, that there appeared to be a specific nucleoside triphosphate pool in nuclei.

Therefore before we can conclude that the response to feeding to T+ diet differs from the T- response we have to make the following assumptions:

1) there is no change in the synthesis or breakdown of hnRNA
2) there is no change in the nucleoside triphosphate pool or in its specific activity following the injection of a labelled precursor.

It would appear highly unlikely that these assumptions are justified, but nonetheless we can make a tentative conclusion that the synthesis of 45S pre-rRNA is increased when a complete (T+) diet is fed compared to the T- diet effects, i.e. the synthesis of pre-rRNA is sensitive to the absence of tryptophan in the diet. Possible reasons for the tryptophan effect will be discussed in the following Section (4.2).

It is of interest to compare the present findings with those of Munro's group who were investigating the effect of hydrocortisone on nucleolar RNA synthesis in adrenalectomised rats. They showed that hydrocortisone increased nucleolar 45S RNA synthesis by 60%, that extranucleolar hnRNA synthesis was only slightly increased, and that hnRNA breakdown was not affected (Jacob, Sajdel and Munro, 1969). However, their conclusion on the increased pre-rRNA synthesis was based on [^{14}C]orotic acid labelling and as they did not investigate the size or specific activity of the nucleoside triphosphate pool in the test and control animals it must be concluded that their figure of '60% increase' is unjustifiably definite. Technically, however, their work is excellent because they isolated a well defined 45S peak (although no 55S species was observed) which enabled them to give good estimations of the 45S radioactivity.

4.2. DNA DEPENDENT RNA POLYMERASE ACTIVITY IN NUCLEI ISOLATED FROM RAT LIVER FOLLOWING DIETARY TREATMENTS

4.2.1. SOME FACTORS AFFECTING THE ASSAY OF RNA POLYMERASE

4.2.1.1. THE DETERMINATION OF RNA POLYMERASE ACTIVITY

Dixon and Webb (1964) remark that enzyme reaction velocities usually fall with time. This is certainly true for the incorporation of radioactive nucleoside triphosphates into RNA in the RNA polymerase reaction. Amongst the reasons for this effect can be included (Dixon and Webb, 1964) :

a) products of the reaction inhibiting the enzyme,

b) degree of saturation of the enzyme falling with a decrease in substrate concentration as the reaction proceeds,

c) the reverse reaction becoming more important as the concentration of products increase,

d) the enzyme, carrier substrate or other cofactors being inactivated with time,

e) the product may be destroyed, or further utilised.

They then conclude " ..... it is only at the initial point, when the various causes just mentioned have not yet had time to operate that the conditions are accurately known, and it is a sound principle in enzyme work to determine the effect on the initial velocity of varying only one factor at a time while all the others are held constant."

Following this sound advice we have determined initial velocities in all RNA polymerase assays. It is interesting, however, to examine some of the published work on RNA polymerase assays to see if the principle outlined by Dixon and Webb has been followed.

Weiss (1960) showed that the incorporation of [^{32}P] CTP by rat liver enzyme was linear over a period of less than 2 min. but pH and cofactor optima were determined by a 15 min. incubation. S. Busch et al. (1962) showed that partial hepactectomy of rat liver produced an increase in RNA polymerase but they used a 10 min. incubation period without stating the linear velocity limits for the reaction. Gorski (1964) showed that oestradiol increased the RNA polymerase activity of rat uterus by using a 10 min. incubation period but again the initial velocity limits were not stated. Widnell and Tata (1964) showed that the RNA polymerase of rat liver nuclei had a linear incorporation of label over a period of less than 5 min. but their determination of cofactor and pH optima were done on 15 min. incubations. Tata and Widnell (1966) showed that an RNA polymerase from rat liver was linear in incorporation over about 3 min. but examined the effect of thyroidectomy by 15 min. incubations. Similarly Widnell and Tata (1966b) examined the additive effects of thyroid hormone, growth hormone and testosterone on rat liver nuclear RNA polymerase by 15 min. incubations. Pegg and Korner (1965) examined the effect of hypophysectomy and growth hormone treatment on rat liver RNA polymerase by using a 20 min. incubation period. It seems very unlikely that the polymerase reaction was linear over that time period. Barnabei et al. (1966) examined the effect of cortisone on RNA polymerase in rat liver nuclei using a 15 min. incubation, when they had shown that linear incorporation only occurred over the first 2 min. They then went on to examine the effects of many in vivo and in vitro factors using this 15 min. assay. Lukács and Sekeris (1967) examined the effect of cortisol in vitro on rat liver nuclear RNA polymerase by a method which might have been linear but they only show the 4, 8 and 12 min. incorporations.

They expressed enzyme activities as isotope incorporated/mg. DNA/min. unlike the previous authors mentioned above who used incorporations over the whole time interval. Ro and Busch (1967) examined the activity of RNA polymerase in rat liver nuclear fractions after hepactectomy by using 15 min. incubations when these authors had previously shown (Ro and Busch, 1964) that the reaction was only linear for less than 5 min. Jacob, Sajdel and Munro (1968b) described two RNA polymerase activities in rat liver nucleoli and stated that the activity of the Mg^{2+}-dependent polymerase was linear over the first 10 min. of incubation when the accompanying figure showed a linear period of 2 min. Later they used this assay system to examine the effect of hydrocortisone on nucleolar RNA polymerase in rat liver following adrenalectomy (Jacob et al., 1969) but 25 min. was used as the incubation time and results were expressed as total incorporation/mg. DNA.

It is, of course, difficult to establish what differences result from assaying by initial velocity instead of lapsed time methods but on the basis of technique alone it is surely reasonable to use a theoretically valid method. All RNA polymerase assays reported in Section 3.2 were carried out by initial velocity methods, and no results were obtained by lapsed time techniques. It is therefore impossible to state the degree of error which could occur by using the latter method in our own work.

4.2.1.2. SUBSTRATE CONCENTRATION

In the present work all assays were carried out at a nucleoside triphosphates level of 0.4 μmole except for the labelled compound (Section 2.11.2) Weiss (1960) examined the effect of increasing the triphosphates from 0.1 to

2.0 μmole/tube, and showed that the enzyme activity increased by 14 times. However, as the assays were not performed under initial velocity conditions it is difficult to be certain of optimal conditions. Novello and Stirpe (1969) examined the effect of nucleoside triphosphate concentration on the Widnell and Tata (1966a) assay. They had used a level of 0.3 μmole and Novello and Stirpe showed that the Mg^{2+}-dependent RNA polymerase assayed in the absence of ammonium sulphate was saturated at this level but unfortunately they did not use an initial velocity method. We omitted to check this ourselves (this is now being done) but as the work is internally controlled (T+ and T- diets) it seems likely that no serious errors would occur.

4.2.1.3. DIVALENT CATION REQUIREMENT AND IONIC STRENGTH OF ASSAY MEDIUM

The RNA polymerase assay used in this work is a low ionic strength Mg^{2+} optimised system but there also exists RNA polymerase activity in a high ionic strength (ammonium sulphate) Mn^{2+} optimised medium. This latter activity was first described by Goldberg (1961) who also showed that Mn^{2+} salts gave a greater activation than Mg^{2+} salts. This RNA polymerase activity has been carefully studied by Widnell and Tata (1966a) who showed that the base ratios of the RNA synthesised were unlike the RNA synthesised by the Mg^{2+} activated RNA polymerase (See Section 4.2.2.2).

The Mn^{2+} activated enzyme differs from the Mg^{2+} activated enzyme in that it is either not stimulated by hormones such as growth hormone and hydrocortisone (Widnell and Tata, 1966b; Jacob, Sajdel and Munro, 1969) or it is only slightly and sluggishly stimulated by hormones such as thyroid hormone and testosterone (Widnell and Tata, 1966b).

The nuclear site of this enzyme activity is uncertain - Jacob et al. (1968b) demonstrated that isolated nucleoli possessed this activity while Roeder and Rutter (1969) state that the extranucleolar region is greatly enriched with the Mn^{2+} activated RNA polymerase. It must be concluded that the function of this enzyme is uncertain; it most likely synthesises the hnRNA previously described (Section 4.1.1) and in my opinion its study has no place in the present work.

4.2.2. LOCALISATION AND FUNCTION OF THE ENZYME

4.2.2.1. LOCALISATION OF THE Mg^{2+} - DEPENDENT RNA POLYMERASE IN THE LIVER CELL

Weiss and Gladstone (1959) demonstrated that rat liver nuclei possessed an enzyme with the properties of an RNA polymerase (See Section 3.2). Ro, Muramatsu and Busch (1964) demonstrated that nucleoli isolated from rat liver also possessed this property. Siebert et al. (1966) showed that RNA polymerase was preferentially found in nucleoli. They fractionated nuclei by sonic disintegration, into a nucleolar pellet fraction, a fraction containing small nucleoli and chromatin, and a fraction containing chromatin and nuclear fragments. These fractions contained 45% and 14% of the original nuclear activity and it can be seen that the enzyme appears to be associated with nucleoli. Recently Roeder and Rutter (1969) extracted rat liver nuclei and obtained evidence for two distinct RNA polymerase activities which could be separated by DEAE - Sephadex chromatography, and they also reported that the Mg^{2+} - dependent enzyme was associated with the nucleolus and the Mn^{2+} dependent enzyme was found in the extranucleolar space.

4.2.2.2. PRODUCT OF THE Mg^{2+} -dependent RNA POLYMERASE

Ro and Busch (1964) examined the base ratios of RNA synthesised in the RNA polymerase reaction in isolated rat liver nucleoli. They found a GC content of about 50% but as they derived this estimate from a series of separate experiments for each base, and as a 15 min. incubation period was used, there is a strong possibility that the product may not have been similar in each reaction tube and that there may have been some degradation of the product. Later studies by Muramatsu and Busch (1964) using pulse labelling of RNA with ^{32}P, demonstrated the synthesis of a GC-rich RNA. Widnell and Tata (1966) determined the base ratios of the product formed by whole nuclei. The GC content was around 60% and this also suggests that the activity of the nuclear Mg^{2+} - dependent enzyme is located in the nucleolus. An interesting feature of the work described above is the apparent production of a ribosomal RNA rather than a pre-rRNA. This is presumably due to degradation of the product during the long incubation periods required for adequate labelling.

Thus it can be concluded that the nuclear Mg^{2+} - dependent RNA polymerase is located in the nucleolus and that the product of the reaction is a ribosomal like RNA. This conclusion is confirmed by the extensive work carried out on the pre-ribosomal RNA located in the nucleolus (Section 4.1.2.)

4.2.3. AN ANALYSIS OF THE RESULTS OF THE DNA DEPENDENT RNA POLYMERASE EXPERIMENTS

Using the Widnell and Tata (1966a) assay, results were obtained which showed a rapid increase in RNA polymerase activity, after feeding the complete (T+) diet, which reached a maximum around 1 hr. after feeding and which has not returned to basal levels (fasted animal) by 2 hr. The T- diet did not

alter the basal level until about 1 hr. after feeding and this activity had decayed to normal within 2 hr. of feeding.

The increased activity following the T+ diet noted within 15 min. of feeding could be totally prevented by suitable pretreatment of the animals with puromycin or cycloheximide. It should be noted that no inhibition of RNA polymerase occurred in the control animals, a finding similar to that of Nicolette and Mueller (1966) who were examining the effect of oestrogen on rat uterus, but in disagreement with the observations of Pegg and Korner (1966) on the effect of growth hormone on rat liver.

The second feature about the response of RNA polymerase to the T+ diet (and to greater extent in the T- animal) was the very short period of increased activity. This response is shorter than any reported in the literature (to my knowledge) but Tsukada and Lieberman (1965) demonstrated a response in rat liver nuclear RNA polymerase, following a 2 min. ligation of the portal vein, of 10 hr. duration.

The shortness of these responses and the puromycin or cycloheximide sensitivity of the "new" RNA polymerase tend to suggest that the enzyme is different from the basal enzyme. This is not borne out by the characteristics of the T+ 1 hr. or T- 1 hr. enzyme examined in Section 3.2.5 nor did Tsukada or Lieberman (1964) find any characteristics by which the stimulated RNA polymerase (following hepactectomy) differed from the basal enzyme. But Nicolette and Mueller (1966) noted an unusual temperature sensitivity to the oestrogen stimulation of RNA polymerase in rat uterus.

We can suggest, therefore, that the "new" enzyme is an isoenzyme of the basal RNA polymerase differing from it only by rapid synthesis and destruction,

and this isoenzyme acts as a regulator of rRNA synthesis. The requirement for such a regulator is obvious. Following the intake of a complete amino acid mixture the liver cell amino acid content will rise and there will be a stimulation of protein and ribosome formation (reviewed by Munro 1968). As the increased liver cell amino acid content is likely to be present for a few hours only (Christensen, 1964) some means of stopping the increased rRNA synthesis is required, and the short-lived isoenzyme of RNA polymerase would appear to fulfil this requirement. It will be remembered (Section 3.2.5.) that the 18 hr. and 40 hr. starved animals had RNA polymerase levels which was not significantly different from each other. Colony fed and tube fed animals had higher levels and this could be attributed to the presence of the isoenzyme.

Finally it is of interest to comment on the apparent close correlation between the liver tryptophan level and the RNA polymerase activity following the feeding of the T+ and T- diets. It is known that the T- diet produces a marked decrease in changing levels of tryptophanyl-$tRNA^{trp}$ (Allen, Raines and Regen, 1969) which in turn leads to a reduction in protein synthesis (Wunner et al., 1966) in the rat liver. The cytoplasmic response is well known (see Introduction) but the nuclear response is still an unsolved problem. Could it be that the lack of tryptophanyl-$tRNA^{trp}$ (or the increase in $tRNA^{trp}$) causes, not an inhibition of DNA dependent RNA polymerase, but merely no synthesis of RNA polymerase isoenzyme whereas the presence of all the charged tRNA species will cause synthesis of isoenzyme? In bacteria it has long been argued that the degree of charging of tRNA could regulate rRNA synthesis by controlling RNA polymerase (reviewed by Maaløe and Kjeldgaard, 1966). This suggestion seemed to have been finally disproved by the demonstration that there was only a small

difference in the inhibition of RNA polymerase caused by charged and uncharged species of tRNA (Bremer, Yegian and Konrad, 1966). It was therefore thought that this difference was insufficient to account for the differences in rRNA synthesis from the growth medium of growing bacteria. If however, the presence of all the charged tRNA species caused synthesis of new polymerase enzyme this could become an acceptable mechanism of control.

4.3. THE EFFECT OF FEEDING WITH A TRYPTOPHAN-FREE AMINO ACID MIXTURE ON ALBUMIN SYNTHESIS BY RAT LIVER SLICES

The *in vivo* effect of a short-term dietary deficiency of tryptophan on rat-liver polysome structure appears to be unique. Thus Wunner (1967) showed that the polysome breakdown obtained by feeding the T- diet could not be produced by the omission of any other single amino acid from the diet. This was confirmed by Pronczuk *et al.* (1968) and, in mice, by Sidransky *et al.* (1967). However, in tissue culture studies with Chang liver cells polysome breakdown occurs when arginine or glutamine is withdrawn from the medium (Eliasson, Bauer and Hultin, 1967).

The perfused liver behaves differently still. Thus Jefferson and Korner (1969) showed that optimal protein synthesis was produced by perfusing a rat liver system with amino acids at ten times their plasma concentrations. At this level the omission of asparagine, methionine or tryptophan decreased the incorporation of [^{14}C]phenylalanine by more than 40% of the level obtained when all amino acids were present.

The logical extension of this work is the *in vitro* demonstration of the tryptophan effect. This has been elegantly shown by Munro's group at the Massachusetts Institute of Technology. Using a cell-free protein synthesising system from rat liver together with an amino acid free cell preparation and uncharged tRNA Baliga, Pronczuk and Munro (1968) showed that [^{14}C]leucine incorporation proceeded for about 20 min. and then ceased together with polysome disaggregation. If to this exhausted system a complete amino acid mixture was added the polysomes reaggregated and [^{14}C]leucine incorporation increased. However, incorporation did not increase, and polysome re-aggregation did not occur if any of the following amino acids were omitted: arginine,

histidine, cysteine, lysine, methionine, phenylalanine, threonine, valine or tryptophan. Surprisingly, isoleucine did not appear to be necessary. Here then is the *in vitro* demonstration of the essential amino requirement by a protein synthesising system.

But why is tryptophan the only amino acid to produce the *in vivo* effect? Munro suggests that tryptophan is the least abundant amino acid in the free amino acid pool of the liver (Munro, 1970) and thus an increase in the size of the amino acid pool without a similar increase in the tryptophan pool (as would occur after feeding a T- diet) would make tryptophan rate limiting for protein synthesis if it was required. This is the probable explanation of the results of Rothschild *et al.* (1969) that the addition of tryptophan to the perfusing fluid of rabbit liver increased albumin synthesis by 140-175% over a $2\frac{1}{2}$ hr. period whereas the addition of methionine, lysine, leucine, valine or threonine had no effect. Isoleucine stimulated albumin synthesis by 90% - in contrast to its lack of *in vitro* effect noted above.

Recently Munro's group have been able to alter the free amino acid pool size of liver by feeding inbalanced amino acid mixtures and thus making isoleucine and threonine less abundant. Under these circumstances feeding an amino acid mixture lacking isoleucine or threonine produced the "T-" type of polysome pattern (Munro-personal communication).

Finally, what is the explanation of the tryptophan effect at the polysome level? The work of Hori, Fisher and Rabinovitz (1967) on haemoglobin synthesis by rabbit reticulocytes under conditions of tryptophan deficiency provides a basis for an explanation. They suggested that because tryptophan is located only at position 14 from the N-terminal end of the α-chain and at positions 15 and 37 on the β-chain (Diamond and Braunitzer, 1962) tryptophan deficiency would lead to a

hold-up of ribosomes moving along from the "N-terminal ends" of the messenger towards the tryptophan codons. Elsewhere the ribosomes can move freely towards the end of the messenger. Tryptophan deficiency would, therefore, result in a polysome with many N-terminal associated ribosomes but, given long enough, no others. The resulting polysomes would be considerably lighter than the "normal" haemoglobin polysomes and the free ribosomes would give the typical T- disaggregated pattern. This hypothesis has been confirmed by the work of Hunt, Hunter and A.J. Munro (1968).

Thus we can attempt an explanation of the effect of tryptophan deficiency on albumin synthesis as follows. Rat albumin contains one tryptophan residue per molecule (Peters, 1962), not located in the N-terminal 1-24 position (Bradshaw and Peters, 1969) and as tryptophan is the least abundant amino acid in the liver pool the rate of synthesis of albumin is likely to be limited by the tryptophan concentration. This suggestion receives support from our tissue slice studies on albumin synthesis.

4.4. CONCLUSION

The work of Spector and Adamstone (1950), Adamstone and Spector (1950) and Cole and Scott (1954) on long-term tryptophan deficiency and of Sidransky and his colleagues on threonine deficiency over periods of up to 7 days (Shinozuka, Verney and Sidransky, 1968; Sidransky, Verney and Shinozuka, 1969) have proved of great interest and value but they do describe rather artificial experimental systems. The use of very short term studies both of tryptophan deficiency and following a complete amino acid diet used by Munro and his colleagues appear to be superior experimental systems because the response is much more likely to be physiological in the sense that it may relate to the events which go on in the normal liver following a normal meal and thus these studies may give insight into the events which follow eating.

In the present studies we have shown that a reduction in rRNA synthesis occurs following the feeding of the T- diet by:

i) sucrose density gradient analysis

ii) orotic acid uptake by nuclei

iii) DNA dependent Mg^{2+} activated RNA polymerase assays.

A concomitant observation is the increase in rRNA synthesis which follows the feeding of the complete amino acid mixture. It is quite clear that the present study only outlines some of the complex events which follow feeding, but they do help to link the earlier extensive histological findings with our present knowledge of rRNA synthesis.

Evidence has also been presented that albumin synthesis may be affected by the feeding of the incomplete amino acid mixture and this finding raises the possibility that albumin synthesis may vary rapidly in response to dietary changes

5. SUMMARY

1. The conditions for optimum extraction of RNA from rat liver nuclei by the technique of Hiatt (1962) were examined. It was concluded that extraction at 65° was necessary to obtain minimal DNA contamination although the extraction of RNA was low and variable because of DNA gel formation in the phenol solution which trapped the nuclear RNA.

2. Using this modified Hiatt technique and linear sucrose density gradient analysis it was shown that both [8-^{14}C]adenine and [5-^{3}H]orotic acid were incorporated less rapidly into nuclear RNA one hour after an amino acid mixture lacking tryptophan was fed, compared with the effect of feeding a complete amino acid mixture.

3. Unsuccessful attempts to separate nuclear RNA by continuous flow agarose gel electrophoresis were made and it was concluded that the polyacrylamide technique of Loening (1967) was superior.

4. The Penman (1966) nuclear RNA preparation technique (which was devised for HeLa cells) was applied to rat liver nuclei. It was found to extract RNA which appeared to be degraded because very little 45S RNA could be obtained.

5. The Busch extraction technique for rat liver nuclear RNA was applied to animals (which had been fed with the T+ or T- diets) at 30, 60, 90 and 120 min. after feeding. The incorporation of [8-^{14}]adenine was followed and it was concluded that the absence of tryptophan reduced the incorporation of [^{14}C]adenine into nuclear RNA. As the pool of RNA precursor ATP, the specific activity of [^{14}C]ATP in it and the effect of the diets on this pool were unknown it was concluded that the differences in incorporation might not be due to changes in rRNA synthesis.

6. A fixed volume isokinetic gradient maker was constructed and shown to

be superior to the gradient maker used by Noll (1967).

7. The assay of DNA dependent Mg^{2+} activated RNA polymerase of isolated rat liver nuclei was studied using an assay system derived from that of Weiss (1960), Goldberg (1961) and S. Busch et al. (1962). Initial enzyme velocities were determined and expressed as pmole GTP incorporated /mg. DNA/min. It was shown that the T+ and T- diets produced similar responses which were not significantly different from each other (stimulation of activity between 20 and 50 min. after feeding) and that the peak of activity was similar to colony-fed animals.

8. As the RNA polymerase results were at variance with the sucrose density gradient analysis the uptake of [5-^{3}H]orotic acid into rat liver nuclei was investigated following the dietary challenge. It was shown that there was a difference, in uptake, between the T+ and T- groups, and that the T+ group uptake was higher 40 min. after feeding than directly after feeding.

9. The RNA polymerase activity after feeding was therefore re-examined using a modified Widnell and Tata (1966a) assay. Results were expressed as µU/mg. DNA. The following results were obtained (mean ± S.E.M., (n)):

colony fed animals	65.9 ± 2.5 (6)
18hr. starved animals	53.2 ± 1.2 (9)
40hr. starved animals	49.2 ± 2.2 (6)

The starved groups were not significantly different from each other but did differ from the fed group.

Feeding the T+ diet resulted, within 10 min. of feeding, in an increase in activity of RNA polymerase which peaked at 1 hour after feeding but which was still increased at 2 hours. This increase could be prevented by pretreatment

of the animals with puromycin or cycloheximide. Control animals were not affected. In contrast the T- diet did not give rise to an increase in RNA polymerase until 45 min. after feeding and the activity had returned to basal levels by 2 hours after feeding.

The hepatic levels of free tryptophan appeared to follow the RNA polymerase activities in both dietary groups. The characteristics of the fasted, T+ 1 hr. and T- 1hr. enzymes were determined and they did not appear to differ from each other, and it was concluded that the only difference between the basal enzyme activity (found in the starving animal) and the stimulated enzyme was its sensitivity to protein synthesis inhibitors and its short half life.

10. It was shown, using liver slice techniques, that there was a difference in albumin synthesis 1 hour after feeding the T+ and T- diets which was abolished in the 2 hour fed groups.

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