THE INTERRELATIONSHIP OF RIBONUCLEIC ACID AND PROTEIN SYNTHESIS.

A study of protein synthesis with special reference to the rôle of ribonucleic acid.

by

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GENERAL INTRODUCTION.

INTRODUCTION.

The nucleic acids have long been recognised as playing a fundamental rôle in living processes. Deoxyribonucleic acid (DNA), which is found only in the nucleus, is now accepted as being an intimate part of the genetical mechanism of the cell. In contrast to DNA, ribonucleic acid (RNA) is found in all fractions of the cell - nucleus, mitochondria, microsomes and cell sap. The exact functions of RNA have not yet been definitely established, but the possibility that it may in some way be connected with the synthesis of proteins has attracted many workers since this rôle of RNA was originally proposed by Brachet and Caspersson in 1941. The following is an account of the main pathways of investigation of this problem which have been followed in the last fifteen years.

Localisation of RNA in the Tissues.

The earliest work in this field was carried out using histochemical and ultra-violet microspectrophotometric techniques by Brachet and Caspersson respectively, but later chemical studies have served to confirm the conclusions derived from this earlier qualitative evidence. The general picture so obtained shows that cells actively synthesising protein contain large amounts of RNA.

Caspersson and Schultz (1938) demonstrated that RNA was abundant in rapidly growing cells of onion root-tips and imaginal discs of Drosophila larvae; in secretory tissue, the exocrine part of the pancreas, the peptic cells of the gastric mucosa (Caspersson, 1947) and the salivary glands (Caspersson & Schultz, 1939). High concentrations of RNA were also demonstrated in cells actively synthesising protein such as liver cells (Davidson & Waymouth, 1946; Davidson, 1947), developing red and white blood cells (Thorell, 1944; Davidson, Leslie & White, 1948) and in nerve cells after stimulation of the corresponding nerve (Hydén, 1943). In addition, Brachet (1941) and later Denucé (1952) have shown that the silk gland of the silk worm has a high concentration of RNA, the only known function of this gland being the production of silk, which is a protein.

Rapidly dividing cells such as oocytes (Brachet, 1950) and bacteria during the logarithmic phase of growth (Malmgren & Heden, 1947; Boivin, 1947) also exhibit high concentrations of RNA. In chick and mouse embryos, a coincidence of high RNA and DNA concentrations with high concentrations of protein has been reported (Novikoff & Potter, 1948; Reddy, Lombardy & Cerecedo, 1952).

Histochemical and chemical evidence indicate therefore that RNA is abundant in tissues characterised as sites of active protein synthesis. As added evidence it might be

pointed out that tissues which have a high physiological activity but do not synthesise protein exhibit a low concentration of RNA; for example, heart and skeletal muscle (Davidson, 1950), and oxyntic cells of the stomach (Caspersson, 1947) do not contain much RNA.

Even on a subcellular basis, active protein synthesis is associated with a high concentration of RNA. The microsomal particles derived from endoplasmic reticulum in the intact cell have been found to be the subcellular fraction richest in RNA and also to exhibit the highest rate of incorporation of labelled amino acids. Thus Keller, Zamecnik and Loftfield (1954) have shown that 70% or more of the initial incorporation of ^{14}C-leucine and ^{14}C-valine into rat liver *in vivo* occurs in the protein of the microsome fraction, which contains most of the cytoplasmic RNA. *In vitro* work on a cell free system shows that the microsome fraction and the 'soluble fraction' of the liver cell are both essential for incorporation. This incorporation occurs almost entirely in the protein of the microsome fraction. Littlefield, Keller, Gross and Zamecnik (1955), in a further study of the incorporation of ^{14}C labelled amino acids into ribonucleoprotein particles of rat liver microsomes, *in vivo*, concluded that cytoplasmic ribonucleoprotein particles are the site of the initial incorporation of free amino acids into proteins, the bulk of the microsomal protein being more slowly and progressive-

ly labelled. Similarly Allfrey, Daly and Mirsky (1953), working with mouse pancreas microsomes, suggested from the results obtained that the rôle of microsomal nucleoprotein was as the precursor of the secretory enzymes of the supernatant. However, Daly, Allfrey and Mirsky (1955) found that, when they studied the uptake of glycine -N^{15} by trypsinogen and chymotrypsinogen of mouse pancreas, isolated in a highly purified form, the uptake into these enzymes is much higher than into any ribonucleoprotein component of the pancreas they had so far investigated. This is not in agreement with their previous finding that these proteins serve as precursor material for the digestive enzymes synthesised by mouse pancreas. They concluded, however, that the original hypothesis could not be rejected, since the uptake of labelled amino acids into proteins of ribonucleoprotein was increased when synthesis was stimulated by secretion and that for a better understanding of the rôle of ribonucleoprotein in protein synthesis it might be significant to investigate the incorporation of labelled amino acids into that fraction of the digestive enzymes most closely associated with ribonucleoprotein, namely, amylase and protease.

Changes in Amount of RNA Accompanying Changes in Protein Synthesi

There is some evidence to show that, in a single organ or tissue, changes in the rate of protein synthesis lead to

changes in RNA content. Alterations in the protein content of the diet have a pronounced effect on the protein and RNA content of the liver. Thus Davidson (1947) found that fasting or administration of a protein-free diet produced a decrease in the RNA content of the liver. Mandel, Jacob and Mandel (1950) again demonstrated this fact and also showed that the amount of DNA remained unchanged. Campbell and Kosterlitz (1947; 1952) studied the effect of protein intake on the amount of protein, ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) in rat liver and found that protein and RNAP per unit of liver cells was dependent on the protein content of the diet, whereas DNAP was independent of this.

The relationship between the growth of tissues and their RNA content has been studied. In developing chick and mouse embryos a coincidence of high protein concentrations with high concentrations of RNA and DNA has been reported. (Novikoff & Potter, 1948; Reddy et al., 1952). Davidson and Leslie (1951) found that the percentage increases in DNAP, RNAP and protein nitrogen (N) content of chick heart explants over a 10 day period were very similar.

The protein and RNA contents of regenerating tissue have also been studied. Thus after partial hepatectomy the RNAP content per unit weight in the regenerating lobes is at

its highest 1½ - 6 days after the operation, which coincides with the period in which liver growth (i.e. weight increment) is most rapid (Price & Laird, 1950; Thomson, Heagy, Hutchison & Davidson, 1953; Lombardo, Cerecedo & Reddy, 1953; Novikoff & Potter, 1948). That this rise in RNAP is not part of a general increase in cellular constituents was shown by Novikoff and Potter (1948). They found that there was no corresponding increase in lactic acid, ATP or ADP contents per unit weight and that the activities of several enzymes of the tricarboxylic acid cycle remained unaltered. A similar situation obtains for renal hypertrophy. After unilateral nephrectomy, the remaining kidney increases in size and at seven days the cell size, RNA and protein content increase between 40 - 50%, but little change in DNA content occurs (Mandel, Mandel & Jacob, 1950).

Thorell (1947) has shown that during blood cell formation the amount of RNA and the size of the nucleolus increase until the erythroblast stage, when the growth of the cell ceases, and from this point there is a decrease in the size of the cell nucleolar mass and RNA content, partly associated with the endocellular formation of haemoglobin.

In micro-organisms, considerable evidence indicates a close relationship between the total amount of RNA and the rate of protein synthesis. Di Carlo and Schultz (1948) found that the concentrations of RNA and DNA increased in rapidly

growing yeast cells. From a comparison of mutants of a yeast with different rates of growth or of cells from the same strain cultivated under different conditions, it has been shown that a linear relationship exists between RNA concentration per mg. organic N and the reciprocal of the mean generation time, i.e., "growth rate" (Caldwell, Macker & Hinshelwood, 1950). Similar findings with bacteria were reported by Northrop (1953), Wade (1952) and Price (1952). Morse and Carter (1949) demonstrated in E. coli during the lag phase that large amounts of RNA were formed and a maximum was reached just before the onset of cell multiplication. Similar findings have been demonstrated for several other micro-organisms (Leonardi, 1949). Jeener (1952) however, found that a comparison of the cells of the flagellate, Polytomela coeca, during the various stages of growth of a culture shows that there is no linear relationship between the quantity of RNA per mg. protein N and the rate of protein synthesis. When he (Jeener, 1953) used continuous cultures under conditions providing a constant growth rate, however, a strict relationship existed between the quantity of RNA present and the rate of protein synthesis. He concluded that a relationship between the amount of RNA present and protein synthesis exists only for systems in the steady state and if the physiological conditions of the cell are changing rapidly, as at the end of both lag and logarithmic phases of growth, no such correlation can be established.

Gale and Folkes (1953a) report that there is a strong correlation between the nucleic acid content of the cells of Staphylococcus aureus and the rate of protein synthesis. Staph. aureus will synthesise protein in the presence of amino acids and glucose. If purines and pyrimidines are added to this medium, nucleic acids are also synthesised and protein synthesis enhanced. However, if the medium contains no amino acids, nucleic acid synthesis does not occur.

In the giant unicellular alga Acetabularia mediterranea, the RNA content of the nucleolus markedly decreased when the organism stopped growing after it had been left in the dark for some weeks (Stich, 1951).

The amount of RNA would appear to change in cancerous growth also. In leukemic mice there is a very considerable increase in the RNA content of the spleen compared to that of normal animals. The increase involved mainly the nuclei and submicroscopic particles of the cytoplasm, whereas the larger granules and the mitochondria remained maffected. No increase in DNA content was observed (Peterman, Alfin-Slater & Larace, 1949).

In tissues which synthesise specific proteins, e.g. hormones and enzymes, there is a close correlation between RNA content and changes in the rate of synthesis of the specific protein. Desclin (1940) and Herlant (1943) showed that stimulation of hormonal secretion in the pituitary produced a

marked increase in its RNA content. Abolins (1952) also stated that the amount of RNA in the cells of the anterior pituitary is related to hormone synthesis in that gland. In the salivary gland of Drosophila larvae, RNA concentration is directly related to the intensity of secretion, both of which increase and decrease together (Lesher, 1952).

Certain hormones have a stimulatory effect on particular tissues and these effects appear to be accompanied by changes in nucleic acid content. In the seminal vesicles of rats injected with testosterone propionate, there is a positive correlation between the RNA content and protein synthesis (Rabinovitch, 1951). Drasher (1953) has shown that the RNA/DNA ratio (i.e. an estimate of relative RNA per cell) at the stimulated stages of the oestrus cycle is maximal at the time of maximal uterine growth. Cellular RNA content can be significantly correlated, under the conditions studied here, with the amount of protein N synthesised. The injection of oestradiol into pigeons was followed by significant increases of the amounts of DNA and by strong increases of the amounts of RNA and phosphoprotein in the liver (Mandel & Mandel, 1948). Similar observations on the nucleic acid fractions of the livers of pregnant rats were made by Campbell and Kosterlitz (1948). Cavallero, Di Marco, Fuoco and Sala (1952), studying the effect of cortisone on the nucleic acid and protein content of chick embryo, found that cortisone inhibited the growth and

concurrently reduced the protein, DNA and RNA content of whole chick embryo; when the results were referred to initial weight, however, only protein and RNA showed a significant decrease. The authors suggested that the growth inhibiting effect of the hormone is closely connected with a decrease in protein and RNA synthesis. Hypophysectomy in the female rat produces a concomitant decrease in RNAP, zymogen granules and amylase activity of the pancreas but not in DNA (di Sesso, Tramezzani, Valeri & Migliorini, 1955). Administration of growth hormone to rats after hypophysectomy, which had produced a marked fall in nuclear and cytoplasmic RNA and protein of the liver cells, caused protein N of the liver to return to normal and cytoplasmic and nuclear RNA to increase to a level above that of the control (Di Stefano, Bass, Diermeier & Tepperman, 1952).

It would appear from the literature cited that variations produced in the protein synthesis of widely varying tissues by different factors cause alterations in the concentration of RNA in these tissues and it may be concluded that the amount of RNA in a tissue is dependent on the intensity of protein synthesis in that tissue.

Changes in Protein Synthesis following Addition or Removal of RNA from a System.

It has been shown by several groups of workers that

variations in the RNA content of different systems affect protein synthesis. Gale and Folkes (1954), using disintegrated Staph. aureus preparations which do not respire nor multiply, found that the system can still incorporate amino acids into its proteins provided adenosine tri-phosphate (ATP) and hexose di-phosphate (HDP) are present. Removal of the nucleic acid by nucleases or sodium chloride treatment strongly reduced the incorporation. Addition of the nucleic acid fraction restored to a large extent the capacity to incorporate several amino acids into the proteins. These workers also studied the effect of removal and addition of nucleic acids on the actual synthesis of several enzymes, namely, catalase, 'glucozymase' and β-galactosidase. The addition of bacterial RNA to the disrupted cells from which the nucleic acids were removed was most active in enhancing the synthesis of catalase while DNA, especially if isolated from adapted cells, was best for the stimulation of the synthesis of β-galactosidase. Lester (1953), de Nisman, Hirsch, Marmur and Cousin (1955), and Beljanski (1954), using Micrococcus lysodeikticus lysates, found the ability to incorporate amino acids into proteins was inhibited by ribonuclease. de Nisman, Hirsch and Marmur (1955) studied the synthesis of proteins in cellular fragments of E. coli and found that treatment with ribonuclease and deoxyribonuclease inhibited the incorporation of amino acids into proteins considerably. More recently Groth (1956) has

reported an inhibition in the uptake of ^{14}C-glycine into the bacterial protein of Bacillus megatherium "mutilat" following treatment with ribonuclease. He did not observe any effect on the incorporation of glycine into nucleic acid purines.

Ribonuclease has been shown to inhibit the incorporation of glutamate into pea-root protein, by Webster and Johnson (1955). The inhibition could be reversed by the addition of RNA. Brachet (1954) has shown that ribonuclease inhibits the incorporation of labelled amino acids into the proteins of the living cells of onion-root tips without affecting their respiration. The same worker (Brachet, 1955a) also showed that ribonuclease inhibits the incorporation of amino acids into the proteins of live amoeba.

Treatment of microsomal preparations of liver and pancreas with ribonuclease has also been shown to inhibit amino-acid incorporation into protein (Zamecnik & Keller, 1954; Allfrey, Daly & Mirsky, 1953; Novikoff, Ryan & Podber, 1954). The effect of ribonuclease on the protein forming systems of liver microsomes may be specific, since Novikoff et al. (1954) showed that ribonuclease treatment had no effect on the activities of microsomal glucose-6-phosphatase, esterase, tri-acetic acid lactonase, adenosine-5'-phosphatase, acid-phosphatase, adenosine triphosphatase and succinoxidase.

From thesestudies on changes in the amount of RNA in

cell-free systems, it is apparent that there is support for the hypothesis of a rôle of RNA in protein synthesis.

Comparison of the Rates of Synthesis of RNA and of Protein.

The data presented above support the hypothesis that the rate of protein synthesis is correlated to the amount of RNA present. If such were the case, it might be expected that the synthesis of RNA and of protein would show coincidental changes under varying conditions. This aspect of the problem has been investigated by many workers and two conflicting conclusions have been obtained, one supporting a relationship between the two phenomena and the other suggesting a dissociation. A wide diversity of biological systems have been used for a study of this problem and while the conflicting results obtained may in some measure be due to the experimental conditions chosen, it is apparent that they are not explicable as genuine differences in the rôle of RNA in these different types of biological systems. The following is an account of the evidence leading to the two viewpoints:

(a) RNA synthesis in tissues with a high rate of protein synthesis. Davidson (1950) has demonstrated a high rate of turnover of RNA in sites of active protein synthesis such as intestine, liver and spleen. In liver regenerating after partial hepatectomy, the radio-activity of RNA is 3 - 4 times that in resting liver (Volkin & Carter, 1951; Brues, Tracy & Cohn, 1944; Smellie, McIndoe, Logan, Davidson & Dawson, 1953).

(b) Hormonal Studies. Hormonal agents can affect both RNA and protein synthesis in some tissues. Fraenkel-Conrat and Li (1949) have shown that cessation of growth of hypophysectomised rats is parallelled by a decrease in the turnover rates of thymus and liver nucleic acids. Albert, Johnson and Cohan (1951) have shown that the incorporation of ^{32}P into the RNA of pregnancy-stimulated mammary gland or mammary carcinoma is appreciably higher than in the normal gland. Schrader and Leuchtenberger (1950) found that, during spermatogenesis, protein and RNA synthesis are closely related, while DNA synthesis is independent of protein synthesis. In the mucosa of the gizzards of pigeons treated with prolactin, Jeener (1948) has shown an increased synthesis of RNA accompanied by an increased protein synthesis. Lu and Winnick (1954) studied the incorporation of radio-active metabolites into the nucleic acids of embryonic chick heart cultures. A combination of insulin, cortisone and growth hormone stimulated the synthesis of both RNA and DNA and increased the rates of uptake of ^{14}C-adenine and ^{14}C-thymidine into these fractions. A correlation between this incorporation and the growth of the cultures was observed.

Thus it would appear that hormonal action on various tissues produces concomitant changes in both RNA and protein synthesis.

(c) Effects of X-irradiation. Studies on the effect of irradiation on various tissues are not in favour of an interdependence

of RNA and protein synthesis. For example, Abrams (1951) showed that X-irradiation decreased the total amount and turnover of RNA without affecting the uptake of ^{14}C-glycine into protein in rabbit bone marrow and intestine and rat intestine. Similarly Holmes (1951) could find no relationship between the effects of irradiation on protein turnover and nucleic acid formation in Jensen sarcoma. Without knowledge of the effects of the treatment on precursor pool activities these data are difficult to evaluate.

(d) The Effect of Inhibitors on RNA and Protein Synthesis. If there is a correlation between RNA turnover and protein synthesis it would be expected that an inhibition of the one system would produce a similar effect in the other.

Studies on the inhibitory effect of ultra-violet light on the synthesis of enzymes in bacteria suggest a relationship between the synthesis of these specific proteins and RNA turnover. Swenson (1950) demonstrated that when ultra-violet light inhibited the adaptive synthesis of galactozymase in yeast the action spectrum was very similar to the nucleic acid absorption spectrum and bore no relationship to that of unconjugated protein. He concluded that nucleic acid is probably the cellular constituent affected by ultra-violet light. Halvorson and Jackson (1956) have shown that the synthesis of DNA in yeast can be abolished by dosages of ultra-violet light which permit RNA and protein synthesis to continue. Dosages of ultra-violet light which

inhibit α-glucosidase synthesis not only prevent net utilisation of the free amino acid pool but also inhibit the incorporation of glycine into the proteins and decrease to a minimal value ^{14}C-glycine and ^{32}P incorporation into RNA.

Where the synthesis of the nucleic acids is inhibited by deprivation of precursors from the medium a positive correlation between protein and RNA synthesis also seems to exist. Thus Jeener and Jeener (1952) found that by the removal of uracil or DNA from the culture medium they could interfere with RNA and DNA synthesis in Thermobacterium acidophilus. In the absence of DNA the cells still grew as elongated filamentous forms but the number of bacterial nuclei remained small. Deprivation of uracil caused inhibition of growth and both nuclei and cytoplasm were affected. They concluded that protein synthesis is dependent on RNA synthesis and is much less directly related to DNA synthesis. Studies of a similar nature have been reported by Schmidt and his co-workers (Schmidt, Seraidarian, Greenbaum, Hickey & Thannhauser, 1956). Baker's yeast incubated in the absence of phosphate more than doubled its content of protein N and that of its organic non-protein N, but no increase in its content of purine and RNA was observed. However, if the phosphate starved cells were transferred to a phosphate-containing medium, purine and nucleic acid synthesis is resumed at rates which are approximately four times larger than those of simultaneous protein and amino acid biosynthesis. If the protein

synthesis were suppressed by omitting sulphate from the medium or by the presence of ethionine, no RNA was formed. The authors suggest that it is RNA synthesis which is dependent on a preceding or simultaneous protein synthesis.

The action of various other inhibitors of RNA synthesis has been investigated by a number of workers, yielding conflicting results. Thus Jeener (cited by Brachet, 1955b) found that when tobacco leaves infected with tobacco mosaic virus are treated with thiouracil a considerable and parallel decrease in the turnover of both RNA and protein of the virus and leaves occurs. On the other hand, uranyl chloride has been shown to inhibit the incorporation of radio-active glycine into the nucleic acids but not into the protein of protoplasts of B. megatherium (McQuillin, 1955). Further evidence suggesting a dissociation of RNA and protein synthesis is provided by the work of Levy, Skutch and Schade (1949), who showed that when Proteus vulgaris was grown in a medium containing ^{32}P and aliquots then inoculated into media containing no ^{32}P the turnover rate of RNAP was greater if the cells were prevented from growing by the presence of cobaltous sulphate, thus showing that RNA is actually more rapidly metabolised in the absence of protein synthesis.

The inhibitory action of certain antibiotics on bacterial systems has been widely studied. Gale and Folkes (1953b), in an extensive study of the effects of various antibiotics on

protein and RNA synthesis in Staph. aureus, found that chloromycetin, aureomycin and terramycin increased nucleic acid synthesis but inhibited protein synthesis. Similarly Wisseman and his co-workers (1954) found that chloramphenicol inhibited protein synthesis in a rapidly growing culture of E. coli, strain B, although RNA and DNA continued to be synthesised at an unaltered rate. The action of the antibiotic usnic acid on bacteria gave similar results (Miura, Nakamura, 1951; Miura, Nakamura & Matsudaira, 1951).

The general conclusion reached from these antibiotic studies is that there is no obligatory relationship between RNA turnover and protein synthesis. This conclusion is not necessarily at variance with the findings of others using inhibitors under different circumstances. In all inhibitor studies the specificity of the action of the inhibitor requires careful consideration since it may not act as a specific inhibitor of the systems under examination but as a general metabolic inhibitor of all the cellular processes and erroneous conclusions concerning a relationship between the systems being considered may result. This interpretation of inhibitor mechanisms will be considered in more detail when we come to our own data on thienylalanine inhibition of RNA and protein synthesis in liver slices.

(c) RNA Synthesis and Enzyme Formation. The pancreas is a system in which synthesis of a specific protein can be studied

free from any accompanying cell division. Fernandes and Junquiera (1955) showed that in pigeon pancreas *in vivo* the uptake of glycine by RNA and protein was roughly parallel. Guberniev and Il'ina (cited by Hokin, 1952) found that an *in vivo* stimulation of enzyme secretion of the parotid, liver and pancreas resulted in an increase in the rate of incorporation of ^{32}P into the ribonucleoproteins. Hokin (1952) reported evidence which confirmed this finding in the pancreas but he later showed (Hokin & Hokin, 1954) that stimulation of enzyme secretion *in vitro* or *in vivo* was not accompanied by an increased rate of RNA synthesis or turnover, his earlier findings being due to the contamination of his RNA preparations with highly active phosphoprotein. Pilocarpine which stimulated enzyme secretion in mouse pancreas was found to cause a 50% decrease in the specific activity of RNA relative to that of the acid-soluble phosphate ester fraction. Studies by Rabinovitch, Valeri, Rothschild, Camara, Sesso and Junquiera (1952) and De Deken-Grenson (1953) also failed to demonstrate a connection between the turnover of nucleic acid P and enzyme secretion. The latter (Grenson, 1952) also found a dissociation between protein synthesis and the uptake of ^{32}P by RNA in the secreting oviduct of the laying hen. Thus we must conclude from these studies of secretory activity that there is no concomitant changes in RNA turnover with changes in protein synthesis.

Adaptive enzymes have been extensively studied in bacteria and the findings are fully dealt with by Spiegelman and Campbell (1956). From a study of adaptive enzymes in various micro-organisms it has been concluded by several workers that continued RNA synthesis is essential to induced enzyme formation but once the RNA is formed it cannot effect a further synthesis of enzyme. Thus Gale and Folkes (1955) found as mentioned previously that while the ability to develop 'glucozymase' or catalase in nucleic acid depleted staphylococci could be restored by the addition of staphylococcal RNA, no such restoration occurred in the case of the adaptive enzyme β-galactosidase. A mixture of purines and pyrimidines or of ribonucleotides from which RNA can be synthesised was however found to enable β-galactosidase formation to occur. Marmur, Nisman and Hirsch (1955) with E. coli found the synthesis of succinic dehydrogenase in cellular fragments was inhibited by treatment with ribonuclease and was restored by a mixture of ribonucleotides but not by nucleic acid preparations of the organism. Pardee (1954) found certain uracil-requiring mutants of E. coli could not synthesise β-galactosidase unless adequate amounts of uracil were present in the medium. The synthesis of α-glucosidase in yeasts has been shown by Spiegelman, Halvorson and Ben-Ishai (1955) to be dependent upon the size of the purine pool within the cells. In addition several workers have shown that purine and pyrimidine analogues inhibit induced enzyme formation in

various micro-organisms (Spiegelman et al., 1955; Pardee, 1955; Creaser, 1955(a), 1955(b)). The above data on adaptive enzyme formation would indicate that for induced enzyme formation to occur a concomitant synthesis of RNA must take place.

It is apparent from a consideration of all the evidence relating RNA synthesis to protein synthesis that concomitant changes have been observed in animals subjected to hormonal stimuli, that there is evidence of a dissociation in some inhibitor and X-irradiation studies, and that enzyme formation can occur in mammals without involving co-synthesis of RNA. In an attempt to rationalise the conflicting data, De Deken-Grenson (1953) has suggested that a correlation between the turnover of RNAP and protein synthesis only exists where there is cellular multiplication and that where there is no cellular multiplication, RNAP turnover behaves in two different ways, (1) a very rapid turnover as in adult liver, and (2) a very slow turnover as in pancreas and oviduct. She also suggests that the correlation between RNA turnover and protein synthesis in cell division may be fortuitous and can be explained by the fact that all the cell constituents are reproducing at the same rate.

Purpose of Present Experiments.

The general picture presented by this survey of the literature would appear to have established with reasonable certitude that the amount of RNA in any given cell is closely correlated with its capacity to fabricate proteins. The literature on RNA metabolism however, has provided conflicting evidence of a relationship of RNA synthesis and protein synthesis. The large number of exceptions to such a relationship must carry considerable weight in deciding whether RNA synthesis is an obligatory component in the mechanism of protein synthesis. With this uncertainty in mind, we therefore set out to explore the relationship of RNA metabolism to protein synthesis in the liver. Our choice of this system was dictated by the fact that the amount of protein in the liver is readily influenced by dietary conditions and it is a fair inference that protein synthesis in this organ is thus under dietary control to a considerable extent. The experiments are divided into five sections, as follows: Section I deals with the influence of protein intake on ^{32}P and ^{14}C-glycine incorporation into rat liver RNA in the whole animal. These experiments indicated that the supply of amino acids from the diet affected RNA metabolism through alterations in the rate of RNA breakdown. Section II extends these observations to the effect of a meal of protein on animals depleted of protein by previous feeding of a low-protein diet. Section III describes the response of

nucleic acid metabolism in whole animals to amino acid mixtures deficient in one essential component. Positive effects on the metabolism of the purine and pyrimidine bases were obtained. As another approach to the effects of deficiency of an essential amino acid, Section IV briefly records some experiments in which amino acid analogues were used to produce deficiency of an essential amino acid. Uptake of labelled precursors into proteins and RNA were examined. In Section V, a few preliminary studies on the rate of RNA breakdown *in vitro* are described. The intention is to explore by *in vitro* procedures the mechanism through which protein intake affects RNA stability in the liver, but these investigations are still in an embryonic state. The general bearing of these findings on the function of RNA in protein synthesis is considered in a general discussion at the end of the thesis. Finally, in a short appendix, some pertinent observations on the use of trichloracetic acid and perchloric acid in studies of RNA metabolism are presented.

SECTION I.

THE INFLUENCE OF DIETARY PROTEIN ON THE INCORPORATION OF ^{14}C-GLYCINE AND ^{32}P INTO THE RIBONUCLEIC ACID OF RAT LIVER.

INTRODUCTION.

The significant effect of dietary protein on the RNA content of rat liver has been established by a number of workers. Davidson (1947) found that fasting or administration of a protein-free diet was followed by a decrease in basophilia and a parallel drop in the actual RNA content. Mandel et al. (1950) confirmed this result, and Campbell and Kosterlitz (1952) stated that the RNAP content of a unit of liver cells was determined mainly by the protein content of the diet. In an extensive study of the effect of both energy and protein intake on the total amount and turnover of RNAP in the livers of rats, Munro, Naismith and Wikramanayake (1953) found that while the amount of RNAP in the liver is influenced by protein intake the rate of incorporation of ^{32}P into RNAP is determined by energy intake. Thus feeding of a protein-free diet caused a large reduction in the amount of RNA without significantly affecting the absolute rate of uptake of ^{32}P by RNA. This would suggest that the influence which protein intake exerts over the amount of RNA in the liver does not involve changes in the rate of RNA formation. The alternative way in which the dietary supply of protein could influence the amount of RNA in the liver is by regulating its rate of breakdown and it was decided to study this aspect of the problem. The animals in these experiments were trained to eat protein-containing or

protein-free diets at fixed times each day; in consequence, at the time of isotope injection we arranged to have each group of animals in one of three nutritional conditions, namely, (a) in the post-absorptive state after a protein-free diet, (b) in the post-absorptive state after a protein-containing diet, and (c) in the absorptive state, i.e., actively absorbing amino acids from dietary protein. In addition the effect of energy intake in each of these nutritional conditions was studied.

The uptake of ^{14}C-glycine by protein and ^{14}C-glycine and ^{32}P by RNA in each of the above nutritional groups was studied and it was concluded that the protein content of the diet does in fact regulate RNA breakdown. We then proceeded to provide further evidence for this conclusion by a study of the uptake of ^{14}C-glycine by the acid-soluble purines, the total amount of RNA in the liver, the uptake of ^{32}P by the RNA of the different cellular components and allantoin excretion in the urine under the different nutritional conditions described.

EXPERIMENTAL.

Animals and Diets. Young adult male albino rats weighing 180-200 g. in the fasting state were used in all experiments. The rats were housed individually in metabolism cages and maintained under thermostatic conditions.

The rats were fed for eleven days on a diet either containing adequate amounts of protein or free from protein. Tables 1 and 2 give the composition of these diets and the amounts fed. During the first seven days the energy level of the diet was maintained at 1,200 cals./sq.m. body surface area/day in both groups. Thereafter during the remaining four days, the carbohydrate intake of the diet was varied to provide two energy levels in each group, either 800 cals./sq.m./day or 1,600 cal./sq.m./day. The rats also received a vitamin-mineral-roughage supplement (V.M.R.). The composition of this mixture and its various constituents is given in Tables 3, 4 and 5. The rats received 1.0 g. of this mixture daily.

The diets were given in two portions, the V.M.R. mixture and variable energy source (glucose) at 9 a.m. and the rest of the diet, including any protein present, at 4 p.m. The food was weighed carefully into heavy ointment jars and moistened with water to prevent scattering. The rats consumed the meals rapidly and dishes were empty an hour after feeding. Thus the rats on the morning after the last day of feeding were all in

Table 1.

Composition of Protein-containing Diet.

Starch (potato)	69 g.
Glucose	69 g.
Margarine	42 g.
Casein	240 g.

4.2 g. given daily.

Table 2.

Composition of Protein-free Diet.

Starch (potato)	189 g.
Glucose	189 g.
Margarine	42 g.

4.2 g. given daily.

Table 3.

Vitamin Mixture.

Pyridoxine Hydrochloride	25 mg.
Riboflavin	25 mg.
Thiamine Hydrochloride	25 mg.
Nicotinic Acid	100 mg.
Menaphthene	5 mg.
Biotin	5 mg.
Calcium Pantothenate	200 mg.
p-Aminobenzoic Acid	500 mg.
Inositol	1 g.
Choline Chloride	10 g.
Folic Acid	Trace
Potato Starch	to 500 g.

Table 4.

Salt Mixture "446".

Sodium Chloride	243.2 g.
Potassium Citrate	533.0 g.
Di-potassium Phosphate	174.0 g.
Di-calcium Phosphate. H_2O	800.0 g.
Calcium Carbonate	368.0 g.
Ferric Citrate. $3H_2O$	360.0 g.
Copper Sulphate. $5H_2O$	0.4 g.
Potassium Aluminium Sulphate. $24H_2O$	0.2 g.
Magnesium Carbonate	92.0 g.
Manganese Sulphate	2.8 g.
Potassium Iodide	0.1 g.
Zinc Carbonate	0.1 g.
Cobalt Chloride. $6H_2O$	0.2 g.
Sodium Fluoride	0.002 g.

Table 5.

The Vitamin-mineral-roughage Mixture (V.M.R.).

Sodium Chloride	32.5 g.
"446" Salt Mixture	130.0 g.
Vitamin Mixture	250.0 g.
Agar Powder	62.5 g.
Margarine	77.5 g.

1 g. α-tocopherol acetate was mixed with 14 ml. radiostoleum (B.D.H.).

0.8 ml. of this was mixed with above mixture.

the post-absorptive state. At this stage all the rats on the protein-free diets and some of the rats on the protein-containing diets were injected with ^{14}C-glycine and ^{32}P and remained fasting until killed. In addition, some of the rats on the protein-containing diet were fed 2.5 g. casein solubilised with 0.15 g. $NaHCO_3$ one hour prior to injection of isotopes. In this way rats actively absorbing amino acids from the gut could be compared with animals in the post-absorptive state.

Administration of isotope and excision of liver. The rats were injected at 9 a.m. with ^{32}P (10 μc./100 g. body weight), one injection into each thigh. They were killed at 3, 6 and 9 hours thereafter by exsanguination under ether anaesthesia. It was noted that the stomachs of the casein-fed rats contained food 3, 6 and even 9 hours after isotope injection, consequently ensuring a continuous absorption of amino acids from the gut during most of the labelling period.

After perfusion with 0.9% ($^{W}/_{V}$) saline, the liver was removed, weighed and homogenised in a Nelco blendor (ice-jacketed) with 5 volumes ice-cold 10% ($^{W}/_{V}$) trichloracetic acid (TCA) for 3 minutes. This was then subjected to a modified Schmidt and Thannhauser (1945) procedure as described by Davidson and Smellie (1952). The homogenate was centrifuged at 0° in the M.S.E. Major refrigerated centrifuge and the supernatant fluid filtered and set aside for the determinations

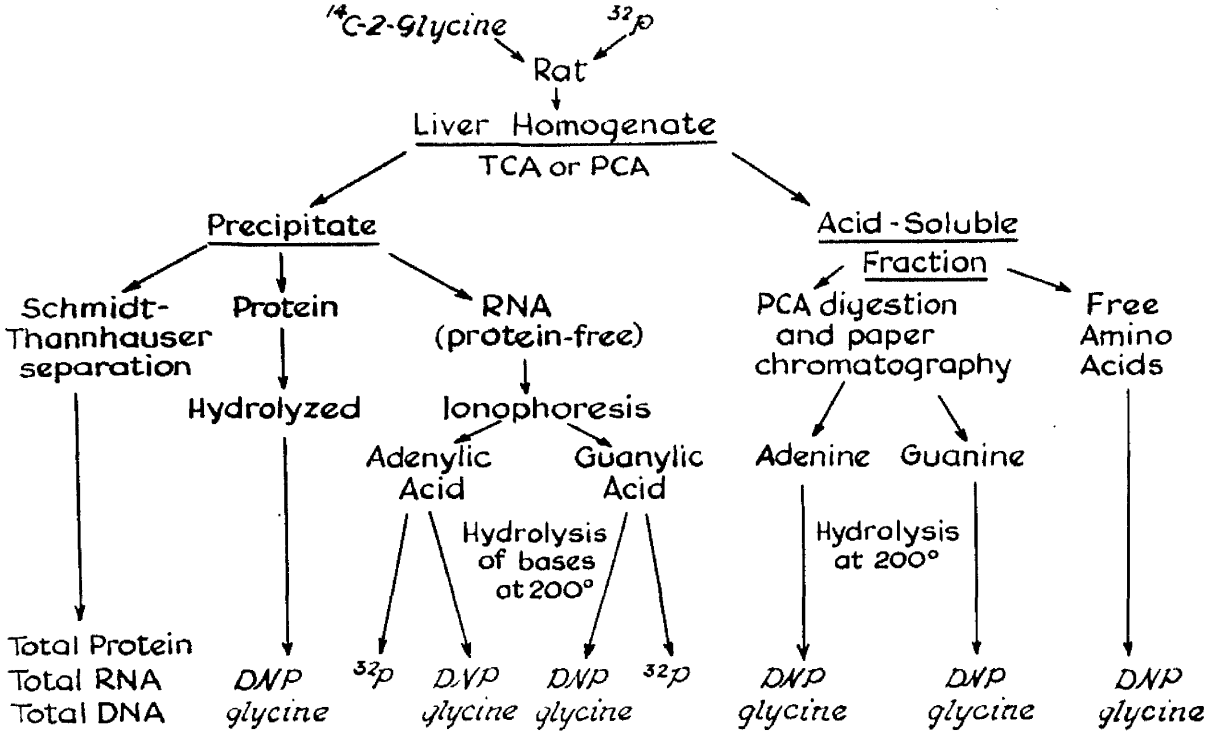


Fig.1. - Schematic outline of liver analysis.

of specific activities (S.A.) of inorganic P and free glycine.

The residue was washed twice with ice-cold 10% TCA and then with 20 ml. portions of the lipid solvents in the following order:- absolute ethanol (twice), ethanol:chloroform (3:1), ethanol:ether (3:1) (twice) and ether - and allowed to dry in air. In a few experiments in which acid-soluble purines were examined, perchloric acid (PCA) was used as a protein precipitant. Appendix I discusses in detail the merits and demerits of TCA and PCA in such experiments.

The TCA precipitate was used for the quantitative determination of protein-N, RNAP and DNAP and the determination of the S.A. of protein glycine, S.A. of ^{32}P of RNA nucleotides and S.A. of ^{14}C-glycine from RNA purines. These procedures are outlined diagrammatically in Fig.1 and are described in detail in the succeeding pages.

Total amount of protein N, RNAP and DNAP per liver. This was carried out using the method of Schmidt and Thannhauser (1945). A known weight of the dry lipid-extracted powder was digested with N-NaOH at 37° for 15-18 hours. The digest was made up to a known volume and an aliquot taken for an estimation of total N by the micro-kjeldahl technique (Ma & Zuazaga, 1942), using the distillation apparatus of Markham (1942). A portion of the digest was neutralised with 2.5N HCl and the DNA and protein precipitated by the addition of ice-cold 30% TCA to

give a final concentration of 10%. The precipitate was centrifuged down at 0° and washed twice with ice-cold 5% TCA. The supernatant and washings were pooled and RNAP estimated by the method of Allen (1940). The precipitate was dissolved in 1 ml. N NaOH and DNAP estimated by the same method. The protein N was obtained by subtracting the N of RNA and DNA ([RNAP + DNAP] x 1.69) from the total N of the alkaline digest.

The difficulties of measuring nucleic acids quantitatively after using PCA as a protein precipitant are discussed in Appendix I.

Radioactivity of Phosphorus Compounds.

1) S.A. of inorganic P. The S.A. of the inorganic P in the TCA-soluble fraction was estimated by the method of Davidson, Frazer and Hutchison (1951). Mathison's reagent (1909) was added (1 ml. to 10 ml. extract) to the acid solution and the mixture made alkaline to phenolphthalein with NH_4OH. This was allowed to stand overnight at 0° and the precipitate of $Mg(NH_4)PO_4$ separated by centrifugation and filtration, washed twice with 10% NH_4OH and then dissolved in N HCl. An aliquot of this was taken for the estimation of P by the method of Allen (1940) and the radioactivity measured in a Veall liquid counter.

11) S.A. of RNAP. The method described by Davidson and Smellie (1952) was used for the isolation of the nucleotides of RNA. This method affords the isolation of the four nucleotides free from contamination of inorganic P and relatively free from other radioactive contaminants such as phosphoprotein.

The procedure is as follows: the dry lipid extracted residue was incubated with N KOH for 18 hours at 37°. The digest was then cooled in ice and the pH adjusted to 1 by the addition of a drop of ice-cold 60% ($^{W}/_{V}$) PCA. The precipitate of DNA and $KClO_4$ was centrifuged down at 0° and the supernatant containing the nucleotides of RNA was removed. The precipitate was washed twice with a small volume of N $HClO_4$ and the supernatant and washings pooled and the pH adjusted to 3 in the cold to minimise hydrolysis of nucleotides. A suitable aliquot of this fraction containing 100-120 µg. P was applied to a spot 6 cm. from one end of a strip of Whatman 3 MM filter-paper 7 cm. broad and 72 cm. long and paper ionophoresis carried out in buffer solution (0.02 M citric acid-trisodium citrate, pH 3.5) for 18 hours at a potential gradient of approximately 11 V/cm. length. The separated nucleotides were then eluted from paper with water and P estimations and radio-activity determinations carried out.

Radioactivity of Carbon Compounds.

Glycine was isolated from liver protein, RNA adenine

and guanine, acid soluble adenine and guanine and the free amino-acid pool (Fig.1) and the dinitrophenyl-(DNP) derivative formed, using a procedure based on that of Campbell and Work (1952). In this method the material is reacted with an excess of 1-fluoro-2:4-dinitrobenzene (FDNB), dissolved in a mixed organic solvent and the reaction mixture fractionated on a buffered celite column. The isolated DNP-glycine is subsequently purified on celite columns developed with ether. The radioactivity of this pure sample is then determined and the amount present estimated colorimetrically.

An amount of the sample containing about 200 ug. glycine was dissolved in 1.5 ml. water and the solution made alkaline by the addition of a knife-point of $NaHCO_3$ and shaken with a 20-fold excess of a 10% solution of FDNB in methanol for 4 hours. At the end of this period the reaction mixture was diluted with 5 ml. water, shaken with 20 ml. ether to remove excess FDNB. This ether solution was then shaken with 5 ml. water three times, the washings being added to the original aqueous layer. The latter was then acidified with 3 ml. of 2.5 N HCl and extracted 5-8 times with 20 ml. portions of ether. The ether extracts containing the DNP-glycine were combined and evaporated to dryness with a current of cold air. Any moisture in the residue was removed by desiccation over P_2O_5.

The dry residue was dissolved in a mixture of chloroform: n-butanol (93:7) prepared by the method of Krol (1952). This solution was applied to a celite column, 1 cm. internal

diameter, 15 cm. long, buffered at pH 6.6, packed in ether, and then washed with chloroform:n-butanol as described by Krol (1952). The column was developed with chloroform:n-butanol, the DNP-glycine band collected and the organic solvents removed by evaporation in a current of air. The residue was dissolved in ether (0.5 ml.) and applied to a celite column prepared as above but using ether saturated with water as the developing solvent. The DNP-glycine band was collected and the ether removed. The dry residue was dissolved in the minimum of ether and transferred to a counting planchette on which it dried as an even film in air. The sample was counted using an end-window counter. The DNP-glycine was then dissolved from the planchette with chloroform:n-butanol (20 ml.) and then extracted from the latter with 10 ml. 1% $NaHCO_3$. The amount of DNP-glycine present in the $NaHCO_3$ was estimated colorimetrically with a 'Spekker' absorptiometer, using Ilford 601 violet filters. The S.A. of the glycine was expressed as counts/minute/100 µg. glycine.

The reliability of this method of determining the S.A. of DNP-glycine was checked by Dr. D.J. Naismith of this laboratory. He found that the DNP-glycine fraction isolated from the celite column by elution with chloroform:n-butanol required to be further purified by adsorbing it on another celite column and eluting with ether. The S.A. of the sample was found to increase by 20-50% as a result of this step.

Table 6.

Correction Factors for self-absorption of β-rays by different thicknesses of DNP-glycine labelled with ^{14}C. The factors correct the observed count to a thickness of 100 µg. per planchette of a standard size.

Amount of Glycine	Factor (to be divided into observed count.)
µg./planchette	
10	1.16
20	1.13
40	1.09
60	1.06
80	1.03
100	1.00
120	0.98
140	0.97
160	0.95
180	0.94
200	0.92
250	0.90
300	0.88

Further purification was proved unnecessary since no increase in S.A. was observed if the sample was now submitted to paper-chromatography (Blackburn & Lowther, 1951) and, after elution, run through a celite column using ether as the eluting solvent.

Self-absorption curves were obtained by plating out various amounts of a single sample of ^{14}C-labelled DNP-glycine and Table 6 shows the combined data obtained from seven such series of determinations carried out by Dr. Naismith. The factors here are somewhat larger than those obtained by Calvin (1949) for $Ba^{14}CO_3$. The purity of samples of DNP-glycine obtained was checked periodically by paper-chromatography using the solvent of Blackburn and Lowther (1951). Spots eluted from these chromatograms and counted gave S.A.s similar to those obtained before chromatography.

Since ^{32}P was also administered, the DNP-glycine obtained from all sources was checked periodically for contamination with ^{32}P. This was achieved by interposing a thin plate of copper foil between the sample and the end-window counter. This was of such thickness that it excluded all the weak β-emission of the ^{14}C but permitted the passage of 66% of any radiation from ^{32}P if it were present. In no case was any contamination detected.

(a) <u>S.A. of free glycine in liver.</u> About $^1/_3$ of the TCA extract of the liver was taken and the bulk of the TCA removed

by repeated ether extractions until the aqueous phase was pH 4-5. The latter was then evaporated to dryness and the residue dissolved in a suitable volume of water. An aliquot of this solution was reacted with FDNB and the DNP-glycine isolated.

(b) S.A. of liver protein glycine. About 100 mg. of the lipid-extracted Schmidt-Thannhauser residue was refluxed with 20 ml. 6N HCl (Analar) for 14 hours on an oil-bath. The HCl was removed from the hydrolysate by vacuum distillation and the dry residue dissolved in 10 ml. water. 1.5 ml. of this solution was reacted with FDNB and the DNP-glycine isolated.

(c) S.A. of purine glycine. Since the activity of protein-glycine was likely to be so much greater than that of purine-glycine and since the conditions for degradation of the purine nucleus to glycine would also bring about the hydrolysis of proteins or peptides to free amino acids, the presence of protein in the RNA sample would invalidate measurements of the S.A. of ^{14}C in RNA. It was therefore essential to ensure the complete removal of protein from the RNA preparation and this was achieved using a modification of the method of Dounce and Kay (1953) for the preparation of sodium ribonucleate. This method involves the use of sodium dodecyl sulphate (SDDS) as a protein denaturant. SDDS was first purified by crystallisation (twice) from hot ethanol.

The dry, lipid-extracted residue of the liver was suspended in 30 ml. 0.9% NaCl and 3 ml. SDDS (5% purified SDDS in 45% ethanol) added. The pH of the mixture was adjusted to 7 with a few drops of 10% NaOH and the solution stirred for 1 hour at room temperature. NaCl was then added to make the solution molar with respect to NaCl and the cloudy solution centrifuged at 18,000 g. for 15 minutes. The supernatant was removed and the nucleic acids present precipitated by the addition of 2 volumes ethanol. The precipitate was allowed to settle in the cold, then centrifuged down, washed with ethanol and acetone and finally allowed to dry in the air. This dry powder was dissolved in 10 ml. water and 0.9 ml. 5% SDDS in 45% ethanol added. The solution was stirred for 1 hour at room temperature and the above procedure again repeated. The dried powder was finally dissolved in 5 ml. 0.14% NaCl, cooled to 0^{o} and the pH adjusted to 4.5 with 0.1 N HCl. This was centrifuged at 18,000 g. for 20 minutes at 0^{o}. The supernatant was made molar with respect to NaCl and the pH adjusted to 7.0 with 0.1 N NaOH. The nucleic acids were precipitated by the addition of 2 volumes ethanol, washed twice in succession with 50% ethanol, ethanol and finally ether.

The dried precipitate was incubated with 0.3 ml. 0.5 N KOH for 18 hours at 37^{o}. The digest, containing ribonucleotides and DNA, was cooled to 0^{o} and the pH adjusted to 1 with 60% PCA. The precipitated DNA and $KClO_4$ were centrifuged down and the

supernatant removed and its pH adjusted to 3. This was applied to filter paper and the nucleotides separated by ionophoresis (Davidson & Smellie, 1952).

Ionophoretic separation of the nucleotides was carried out on Whatman 3 MM paper. This paper was shown to be contaminated with peptides containing glycine, aspartic acid, glutamic acid and alanine. Before use, therefore, the papers were washed free of peptides using the procedure of Hanes, Hird and Isherwood (1951) which consists of agitating the strips of paper in the following sequence of solutions: N-Na_2CO_3, distilled water, 2N acetic acid, distilled water, 0.1% ($^{w}/_{v}$) calcium acetate, distilled water and lastly 50% ($^{v}/_{v}$) aq. ethanol. The efficacy of this treatment was proved by submitting washed strips of paper to ionophoresis, eluting the areas which would correspond to the positions of adenylic and guanylic acids and after hydrolysis in 6N HCl for 18 hours at 110° examining for amino acids by two dimensional paper chromatography.

After ionophoresis, adenylic and guanylic acids were eluted separately from the paper and the respective bases of these acids isolated by the method of Tyner, Heidelberger and Le Page (1953) which yields bases free from contamination with ^{32}P. This procedure consists of hydrolysing the ribonucleotide for 1 hour at 100° with N HCl, adding a 10 molar excess of carrier phosphate and separating the base from the phosphate

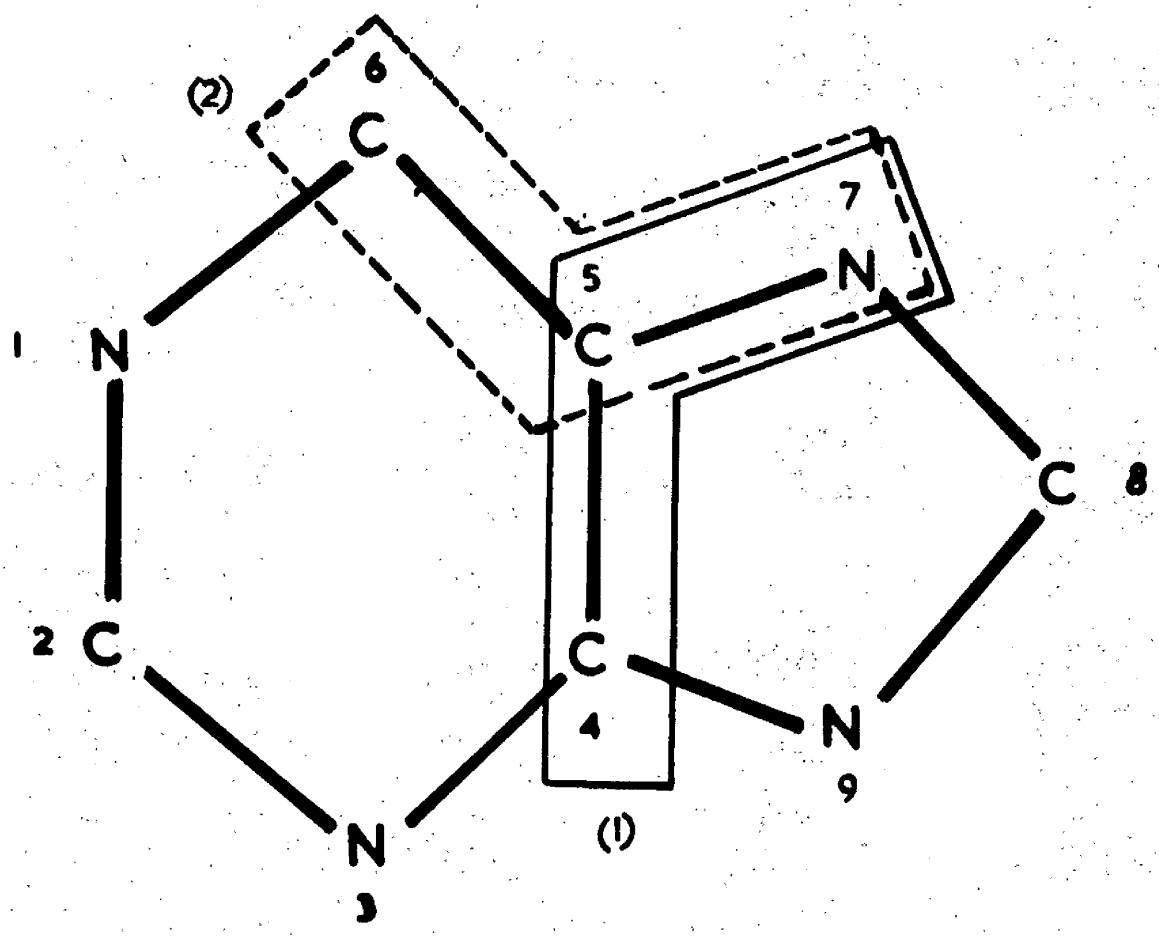


Fig.2. - Possible sources of glycine produced by chemical degradation of purine ring.

on columns of Dowex 50 resin. To ensure that the RNA thus prepared was free from protein, the eluates from the Dowex 50 resin columns were hydrolysed for 18 hours in 6 N HCl at 110° and the product submitted to two dimensional paper chromatography. Only glycine, derived from partial degradation of the purines, was found. A test for peptides (Rydon & Smith, 1952) carried out on the ionophoretic strips after development was negative.

The bases eluted from the Dowex resin columns were then degraded to glycine by the method of Tinker, Cavalieri and Brown (1949). Each base was dissolved in 11 N HCl and heated in a sealed tube for 18 hours at 200°. This treatment causes the breakdown of the purine nucleus to give glycine. The HCl was removed *in vacuo* and the S.A. of the glycine determined in the usual way. By degrading the bases to glycine, any difficulties in the interpretation of the results due to the contribution made to the activity of the base by C atoms 2 and 8 of the purine ring were avoided. (See Fig.2). While it is known biologically that glycine is the source of only C atoms 4 and 5 and N atom 7 of the purine nucleus, chemically degradation of the purine nucleus could theoretically occur in two ways to give glycine, viz. from (1) C atoms 4 and 5 and N atom 7 and (2) C atoms 6 and 5 and N atom 7. In the case of uric acid, this occurs at random (Dalgleish & Neuberger, 1954).

However, it has been shown by Tinker et al. (1949) that adenine degradation under the above conditions gives rise to glycine from C atoms 4 and 5 and N atom 7 only. Guanine has not been examined in this way. However, even if randomization gave a 50% contribution from C_6 to the glycine, this would only influence the results obtained through the presence of radioactive carbon in C_6, representing CO_2 fixation. Calculation of the likely contribution of $^{14}CO_2$ from 2-^{14}C-glycine indicates that this source of error would be negligible in a 9 hour period.

This method of preparation of RNA free from protein was used in experiments 1 and 2. However, the yields of RNA-glycine were very low and this was thought to be due to incomplete extraction of the RNA during the first treatment of the liver powder with SDDS. Accordingly the preparation was carried out by the method of Dorner and Knight (1953). This method was used by these authors to remove RNA from tobacco mosaic virus and also involves the use of SDDS. The dry liver was suspended in 30 ml. M-NaCl and 3 ml. of the SDDS solution and treated at 100° for 10 minutes with constant stirring. The supernatant was then filtered and the residue again extracted. The nucleic acids were precipitated from the combined filtrates by the addition of 2 volumes ethanol and acetone. The dry powder was then subjected to the same process of purification as before. When the resultant product was tested for the presence of

protein, as described above, the result was negative.

(d) S.A. of glycine in acid-soluble purines. The acid-soluble purines were isolated using a modification of the method of Hurlbert and Potter (1952).

The proteins and nucleic acids of the liver were precipitated by the addition of 2 volumes of ice-cold N PCA and the precipitate washed twice with ice-cold 0.7 N PCA as rapidly as possible at 0° for reasons given in Appendix I. The combined supernatant and washings were filtered and the pH of the filtrate was adjusted to 7 with 5 N KOH in the cold. The precipitated $KClO_4$ was spun down and the supernatant fluid removed and filtered. The filtrate was evaporated to dryness and the residue digested with 72% PCA at 100° for 1 hour. The digest was cooled in ice and the pH adjusted to 7 by the addition of KOH. The precipitate of $KClO_4$ and carbon was removed by centrifugation and the supernatant extracted 8 times with 2 volumes n-butanol/water (86:14) to separate the bases from brown degradation products of digestion. This extraction procedure gives a quantitative yield of the purine bases. The butanol extracts were evaporated to dryness and the residue taken up in water and applied to Whatman 3 MM chromatography paper and submitted to a two-dimensional separation. The first solvent was 5% Na_2HPO_4 - isoamyl alcohol (Light & Co., Technical grade) devised by Carter (1950) and the second solvent system was the n-butanol:formic acid:water (77:10:13) mixture

described by Markham and Smith (1949). The R_F values obtained agreed with these authors' findings except in the case of guanine which moved in the first solvent with an R_F value of 0.45 as compared with Carter's reported R_F value of 0.02. The adenine spots were eluted in 0.1 N HCl at 37° for 18 hours and the guanine spots in 1.6 N HCl. The eluates were evaporated to dryness and the residual bases hydrolysed to glycine in 11 N HCl and the DNP-glycine prepared from the product as described above.

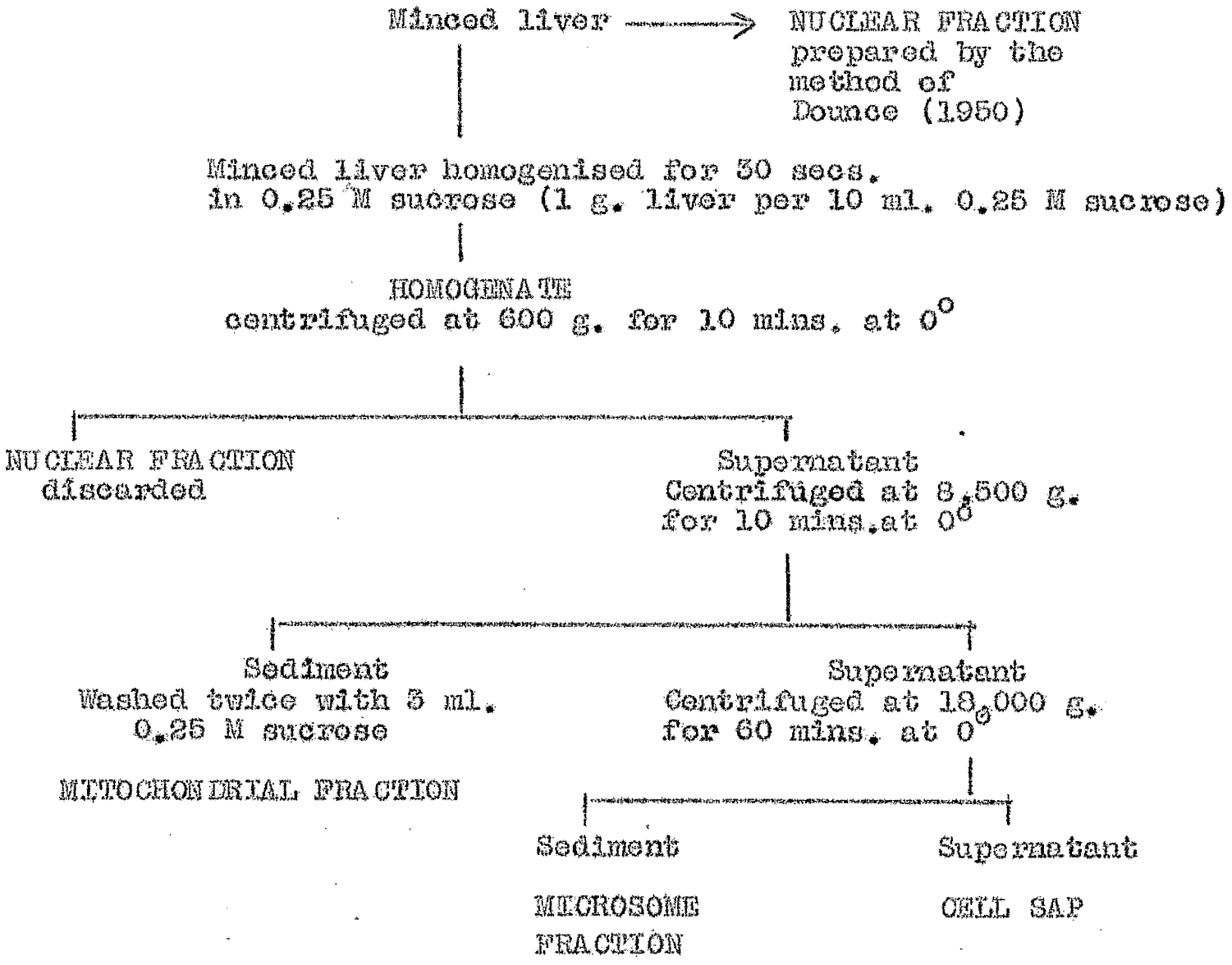
The possibility that free glycine in the PCA-soluble fraction of liver might travel with the purines during chromatography was also considered. Areas of paper around the adenine and guanine spots from 8 papers were therefore cut out and eluted; the eluates were combined and then put through the procedure for DNP-glycine preparation. Only about 1 µg. glycine was obtained from the paper around each spot, and it was devoid of radioactivity. This amount of glycine is insignificant.

Differential fractionation of liver.

In some experiments the specific activities of the ribonucleotides of RNA and lipid P were measured in different fractions of the liver cell, 6 hours after the injection of ^{32}P. The technique used for the separation of the different cell fractions was that of Schneider (1948). The nuclei however

Fig. 3.

Fractionation of the liver cell in 0.25 M sucrose.



were prepared independently by the citric acid method of Dounce (1950). Fig.3 gives a schematic outline of the method.

The livers of 1 or 2 rats in each dietary group were pooled and minced finely with scissors and a portion taken for the isolation of nuclei. A portion was homogenized for 30 seconds in ice-cold 0.25 M-sucrose solution (1 g. liver/ 10 ml. sucrose), using a Potter-Elvejhem (1936) type of homogeniser with a plastic pestle. The homogenate was sampled for analysis of RNAP, phospholipid-P and inorganic-P and the remainder mixed with a knife-point of NaF to prevent enzymic degradation of RNA during the differential separation. The homogenate was centrifuged at 600 g. for 10 minutes at 0° in the M.S.E. 'Major' refrigerated centrifuge and the sediment of whole cells and nuclei discarded. The supernatant was centrifuged at 8,500 g. for 10 minutes and the sediment, the mitochondrial fraction (MT), washed twice with 5 ml. 0.25 M-sucrose and suspended in a small volume of 0.25 M sucrose. The supernatant was centrifuged at 18,000 g. for 60 minutes and the sediment, the microsomal fraction (MS) suspended in 0.25 M-sucrose. The supernatant, the non-particulate cytoplasmic fraction (S) was removed. The nuclei, as already stated, were prepared independently by the method of Dounce.

Each fraction was treated with 0.5 volumes ice-cold 30% ($^{W}/_{V}$) TCA and was then analysed for S.A. of inorganic ^{32}P, phospholipid ^{32}P and RNA ^{32}P by the methods previously

described. The phospholipid fraction was prepared by washing the TCA-precipitated material with 20 ml. portions of the lipid solvents in the following order: absolute ethanol (twice); ethanol:chloroform (3:1); ethanol:ether (3:1) (twice); ether. The washings were pooled and a suitable volume evaporated to dryness and the P estimated by the method of Allen (1940) and the solution counted.

Collection of Urine and Estimation of Allantoin and Creatinine.

In some experiments urine was collected for 24 hours using the method of separating urine and faeces described by Thompson and Munro (1955) except that chloroform was used as a preservative. The method initially used for the estimation of allantoin was that of Young and Conway (1942) and the procedure is as follows: 5 ml. of the solution to be estimated and 1.5 ml. 0.5 N NaOH were placed in a boiling water-bath for 7 minutes, cooled for 3 minutes in a water-bath at 20° and 1.0 ml. 0.64 N HCl added (to make mixture 0.02 N with respect to acid). The tubes were then placed in a boiling water-bath for exactly 2 minutes, immediately thereafter transferred to a bath at -10° for 3 minutes. 1.0 ml. ice-cold 0.33% phenylhydrazine hydrochloride solution (prepared fresh each day) was added and the reaction allowed to proceed for 15 minutes in a bath maintained at 30°. The tubes were then immersed in a bath at -10° for 3 minutes and 3 ml. concentrated HCl at -10°

added followed by 1.0 ml. 1.67% $K_3Fe(CN)_6$ cooled to 0°. The solutions were mixed, allowed to stand at room temperature for 30 minutes and then diluted to 25 ml. with water and the optical density measured at 525 mμ on the Unicam SP 600 within 15 minutes of dilution.

Briefly this method can be considered to consist of four stages:-

1. Alkaline hydrolysis,

Allantoin $\xrightarrow{0.08\ N\ NaOH\ at\ 100^{\circ},\ 7\ min.}$ Allantoic acid

2. Acid hydrolysis,

Allantoic acid $\xrightarrow{0.02\ N\ HCl\ at\ 100^{\circ},\ 2\ min.}$ Glyoxylic acid + Urea

3. Phenylhydrazone formation,

Glyoxylic acid $\xrightarrow[30^{\circ}\ for\ 15\ min.]{phenylhydrazine\ HCl}$ Glyoxylate phenylhydrazone

4. Chromophore formation,

Glyoxylate phenylhydrazone $\xrightarrow{K_3Fe(CN)_6 + HCl}$ Red colour.

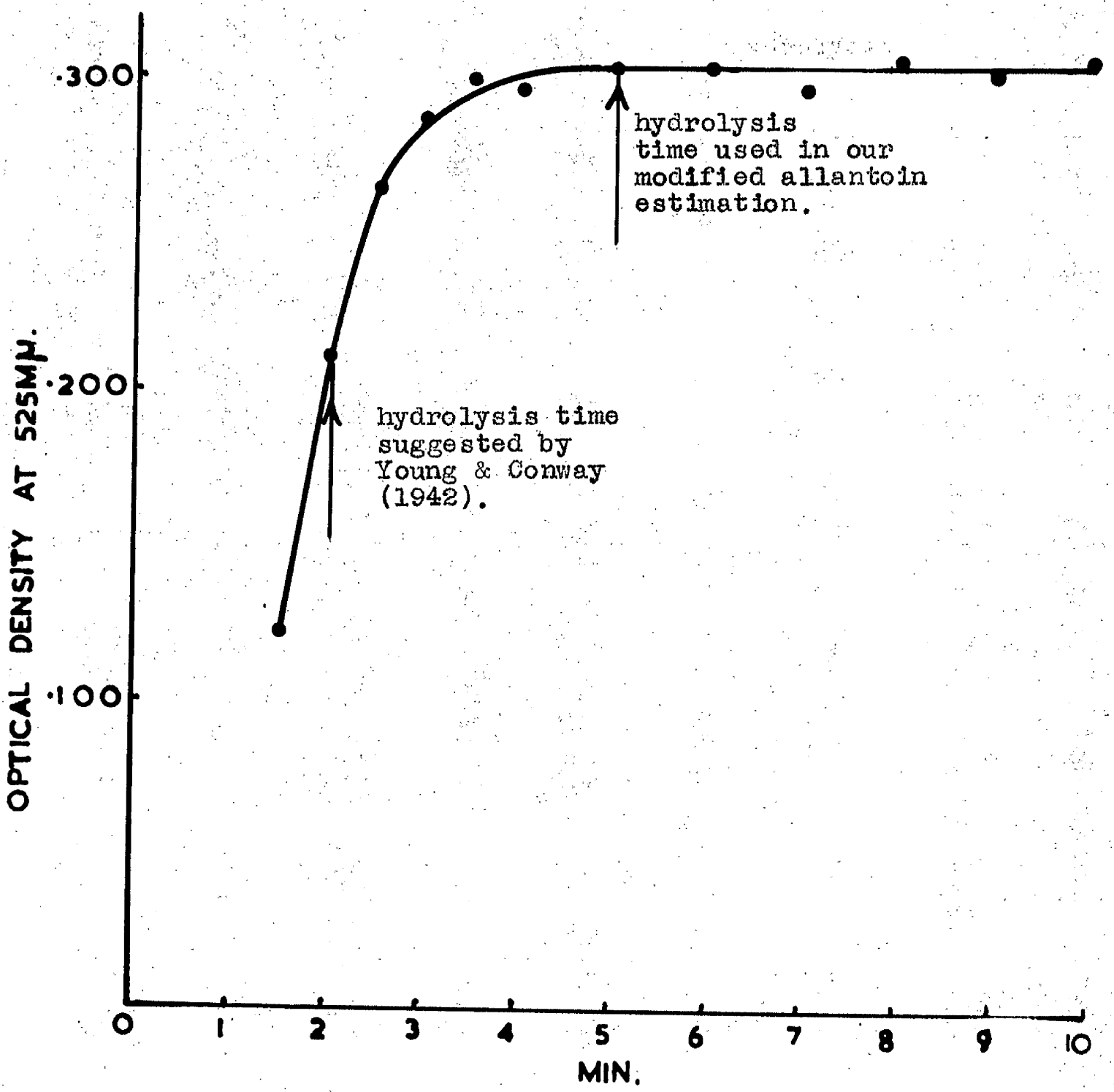
Satisfactory duplicates could not be obtained using this method, even when done simultaneously, and it was decided therefore to investigate each stage of the reaction sequence in an attempt to find the cause of the variation. Accordingly the factors responsible for uncontrolled variations were investigated in the reverse order of the estimation. This technique may conveniently be named retrograde sequential analysis of the

Table 7.

Examination of method of allantoin estimation of Young and Conway (1942) by retrogade sequential analysis.

Step No.	Stage at which duplicates removed and treated separately	Optical Density at 525 mμ.			
		Sample 1		Sample 2	
			Mean		Mean
4	Chromophore Formation	0.246 0.247	0.247	0.166 0.167	0.167
3	Phenylhydrazine Incubation	0.219 0.219	0.219	0.232 0.235	0.233
2	Acid Hydrolysis	0.168 0.190	0.179	0.195 0.180	0.188

Fig.4. - Effect of varying the time of hydrolysis of allantoic acid with 0.02 N HCl on the final optical density produced in the allantoin estimation.



reaction. Two samples of the same solution were subjected to the first three steps of the procedure and each was then divided into two portions, both of which were submitted to the fourth step, i.e., chromophore formation. If this fourth step were not the origin of the variability, the two aliquots of each sample would agree internally, but the mean result for each sample carried through separately would probably not agree because an earlier step was the source of variability. This was indeed found to be the case (Table 7). The procedure was then repeated on a further two samples containing allantoin, but this time each sample was split into two equal parts at step 3. Again the replicates agreed internally but the means for each sample were not in accord. When step 2 was used as the point of sub-division into two samples, however, the duplicates no longer agreed among themselves (Table 7). This would suggest that stage 2 (acid hydrolysis) is the cause of the variable results found with this estimation. We therefore proceeded to investigate this stage by carrying out estimations on duplicate samples which had been heated at 100° with 0.02 N HCl for varying degrees of time. Figure 4 indicates that after 2 minutes in the water-bath at 100°, only 70% hydrolysis is achieved and 100% hydrolysis is only reached after 3½ minutes. In view of these findings, hydrolysis at stage 2 was prolonged to five minutes. There is apparently no destruction of the hydrolysis products, as suggested by Young and Conway, if the

Table 8.

Allantoin Estimation - variation of optical density of final solution with time.

Time of reading after colour development	Optical density at 525 mμ.		
	0.20 mg. Allantoin	0.16 mg. Allantoin	0.10 mg. Allantoin
(min.)			
3	1.33	1.15	0.785
5	1.30	1.125	0.785
10	1.30	1.125	0.790
20	1.26	1.085	0.771
30	1.22	1.060	0.750
40	1.18	1.020	0.724
50	1.14	1.000	0.711
60	1.10	0.979	0.695

time in the water-bath is prolonged to 10 minutes. Each point on the graph represents the average of two duplicate estimations which did not differ by more than 10%. Although Young and Conway recommended that certain stages of the estimation should be carried out at low temperatures, it may be pointed out that all the above operations were carried out at room temperature and this in no way affected the intensity of the colour produced.

A calibration curve was obtained for the range 0.02 mg. - 0.14 mg. allantoin and gave a linear relationship between optical density and amount of allantoin. However it was thought advisable to carry out the estimation of a standard solution of allantoin and a blank with each set of tubes and calculate the absolute values of allantoin by proportion. It was found that the intensity of the colour obtained in our modified reaction fades on standing: Table 8 shows the results obtained from a study of the relationship of time to optical density. All readings were therefore made within 15 minutes of dilution.

The method finally adopted for the estimation of allantoin was as follows:-

5 ml. of the sample to be estimated and 1.0 ml. 0.5 N NaOH were heated on a boiling water-bath for 7 minutes. The tubes were then allowed to stand in a water-bath at 20° for 2 minutes, 1 ml. 0.64 N HCl added and the tubes again heated in a boiling water-bath for 5 minutes. The tubes were then transferred to

Table 9.

Creatinine Estimation - variation of optical density of final solution with time.

Time of Reading after Colour Development	Optical Density at 500 mμ.	
	0.12 mg. Creatinine	0.36 mg. Creatinine
(min.)		
5	0.168	0.477
10	0.210	0.487
20	0.223	0.632
30	0.227	0.642
40	0.226	0.635
50	0.226	0.631
60	0.223	0.628
70	0.225	0.630

a water-bath at 30° and left for 3 minutes. 1.0 ml. 0.33% phenylhydrazine hydrochloride was added and the tubes incubated at 30° for 15 minutes, after which time they were transferred to a water-bath at 20° and allowed to stand for 2 minutes before the addition of 3 ml. conc. HCl followed by 1.0 ml. 1.67% $K_3Fe(CN)_6$. The tubes were shaken and allowed to stand for 30 minutes at room temperature. They were then diluted to 25 ml. with distilled water and the optical density measured at 525 mμ in the Unicam SP 600 within 15 minutes of dilution.

Creatine estimations were carried out by the method of Varley (1954). 3 ml. of the solution to be estimated were placed in a test-tube and 1.0 ml. saturated picric acid solution added followed by 1 ml. 0.75 N NaOH. The solutions were allowed to stand for 20 minutes and read in the Unicam SP 600 at 500 mμ within the next 15 minutes.

A standard curve was obtained for creatinine and was found to be linear within the range 0.01-0.06 mg. creatinine and all subsequent estimations were carried out within this range. The practice however was to do standard estimations and blanks with each set of estimations. The optical density of the standard altered only if a new solution of picric acid were used. The relationship between time of colour development and optical density was also studied and Table 9 indicates the results obtained. It can be seen that optical density reaches

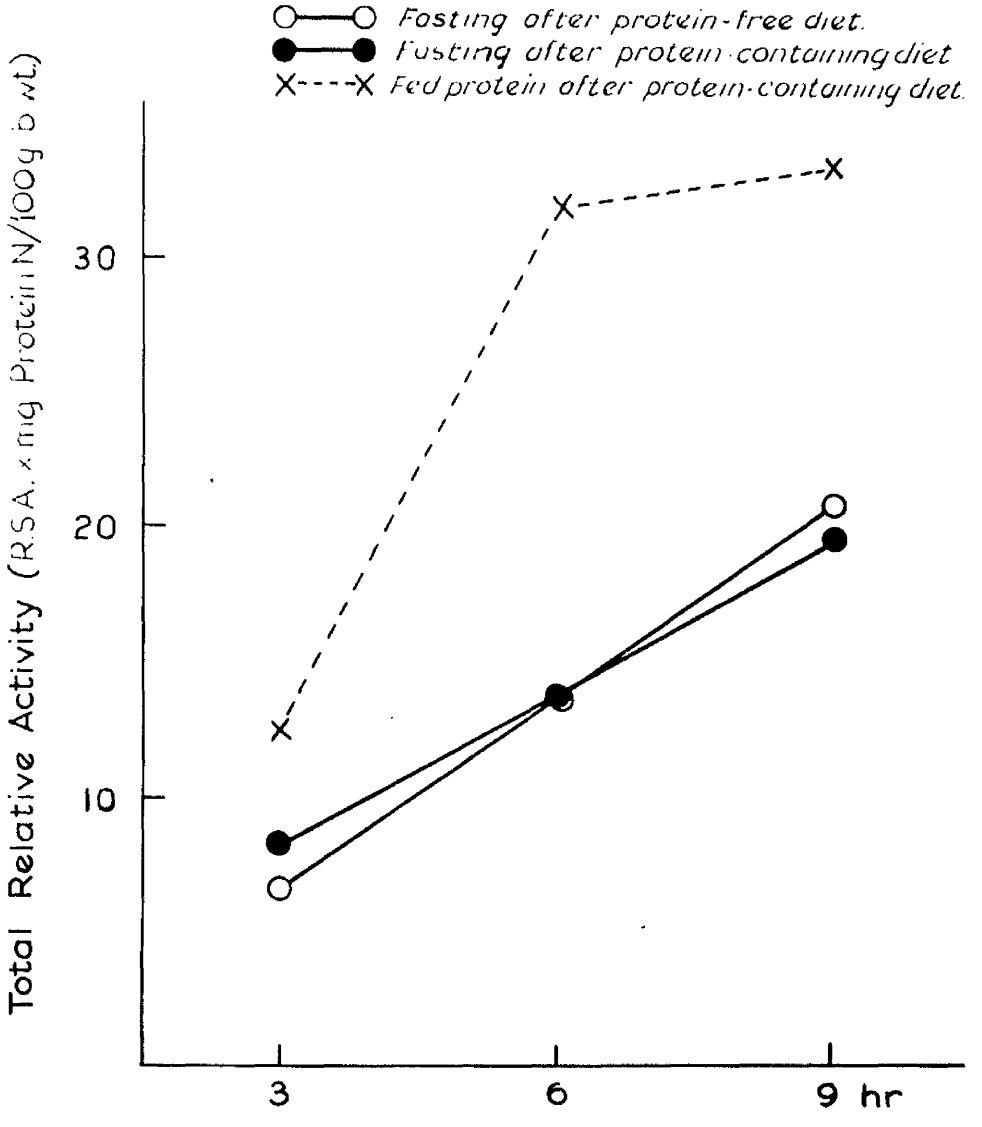
a maximum after 15 minutes and does not fall off appreciably even after 70 minutes.

RESULTS.

The Influence of Dietary Protein on the Metabolism of Liver RNA.

The data on which we can base conclusions about liver protein and RNA metabolism in the present experiments consist of the total amount of protein and RNA per liver, the specific activity of the glycine in the protein, the RNA purine bases and the free amino acid pool of the liver, and the specific activity of ^{32}P in the ribonucleotides and the inorganic P of the liver (Fig.1). From these we can compute the specific activity of ^{14}C-glycine in the liver protein and purine bases as a percentage of the specific activity of glycine in the free amino acid pool of the liver. Similarly, the specific activity of ^{32}P in the ribonucleotides can be related to that in the inorganic P. These relative specific activities allow for differences in the activities of the precursor pools of different animals and permit us to compare the proportion of atoms renewed in the liver protein or RNA in the different nutritional groups. In order to complete the picture it is necessary to allow for the total amount of protein and RNA in which incorporation is taking place: thus an animal with a high relative specific activity and a small amount of RNA may not be synthesising a greater number of new molecules than an animal with a low relative specific activity but with a larger amount of RNA in its liver. As a measure of the absolute

Fig. 5. - Incorporation of ^{14}C-2-Glycine into Liver Protein under various nutritional circumstances.



rate of replacement per liver the relative specific activity (R.S.A.) has therefore been multiplied by the total amount of protein N or HNAP in the liver expressed as mg. per 100 g. initial body weight. This is referred to as "total relative activity" (Campbell, Olley & Blewett, 1949).

At the time of injection with isotopes, the rats were nutritionally in one of three conditions. One group had been fasting overnight after a few days' feeding on a protein-free diet and a second group was fasting after a protein-containing diet. In addition, some animals on the protein-containing diet were fed more casein just prior to injection. Thus we have two groups in the post-absorptive state after diets of different protein content and one group actively absorbing amino acids from dietary protein. Animals from each group were killed at 3, 6 and 9 hours after injection. The whole experiment was repeated three times using rats fed on diets of low energy content and three times with rats on high energy diets.

The Incorporation of ^{14}C-2-glycine into Liver Protein.

The data obtained are given in Fig.5. The results obtained at each energy level provided the same picture and so have been combined: each point therefore represents the average of 6 replications. It is apparent (Fig.5) that the total amount of glycine incorporated into liver protein is of similar

Table 10.

The effect of various nutritional conditions on the amount of RNA in the liver and on the uptake of ^{32}P and ^{14}C-2-glycine into RNA. The results are expressed as specific activities. The data represent the means of 3 replicate experiments.

Energy Level	Protein Intake	Time after isotope injection	Amount of RNA per 100 g. initial body wt.	Specific Activities				
				^{32}P(cpm/100 µg.P)		^{14}C-2-glycine (cpm/100 µg. glycine)		
				Inorganic P	RNA P	Free Glycine	RNA Adenine	RNA Guanine
		(hr.)	(mg.)					
Low	Protein-free (fasting)	3	2.31	4807	204	2568	67	77
		6	2.31	3022	321	1428	132	94
		9	2.30	1999	388	859	111	145
	Protein-containing (fasting)	3	2.87	3695	99	1651	4	8
		6	2.54	2394	125	1173	12	3
		9	2.65	1696	146	772	49	11
	Protein-containing (fed)	3	2.58	4131	201	1265	55	59
		6	3.46	2031	181	648	63	73
		9	2.50	1897	312	479	39	35
High	Protein-free (fasting)	3	2.22	4015	237	2530	39	87
		6	2.29	3379	416	1341	84	193
		9	2.74	2493	518	745	55	83
	Protein-containing (fasting)	3	3.22	3525	109	1580	27	12
		6	3.00	2770	129	1000	16	6
		9	2.94	1864	112	811	42	3
	Protein-containing (fed)	3	3.58	2705	86	1215	-	-
		6	3.34	1804	121	714	63	57
		9	3.36	1273	182	460	49	42

magnitude in the two fasted groups irrespective of the protein content of the preceding diet. This is in agreement with the findings of Geiger (1947) who observed that the utilisation of dietary protein is confined to a short period after its ingestion, since omission of a single amino acid from the diet cannot be compensated for by feeding the missing amino acid a few hours later.

The Incorporation of ^{32}P and ^{14}C-2-glycine into RNA.

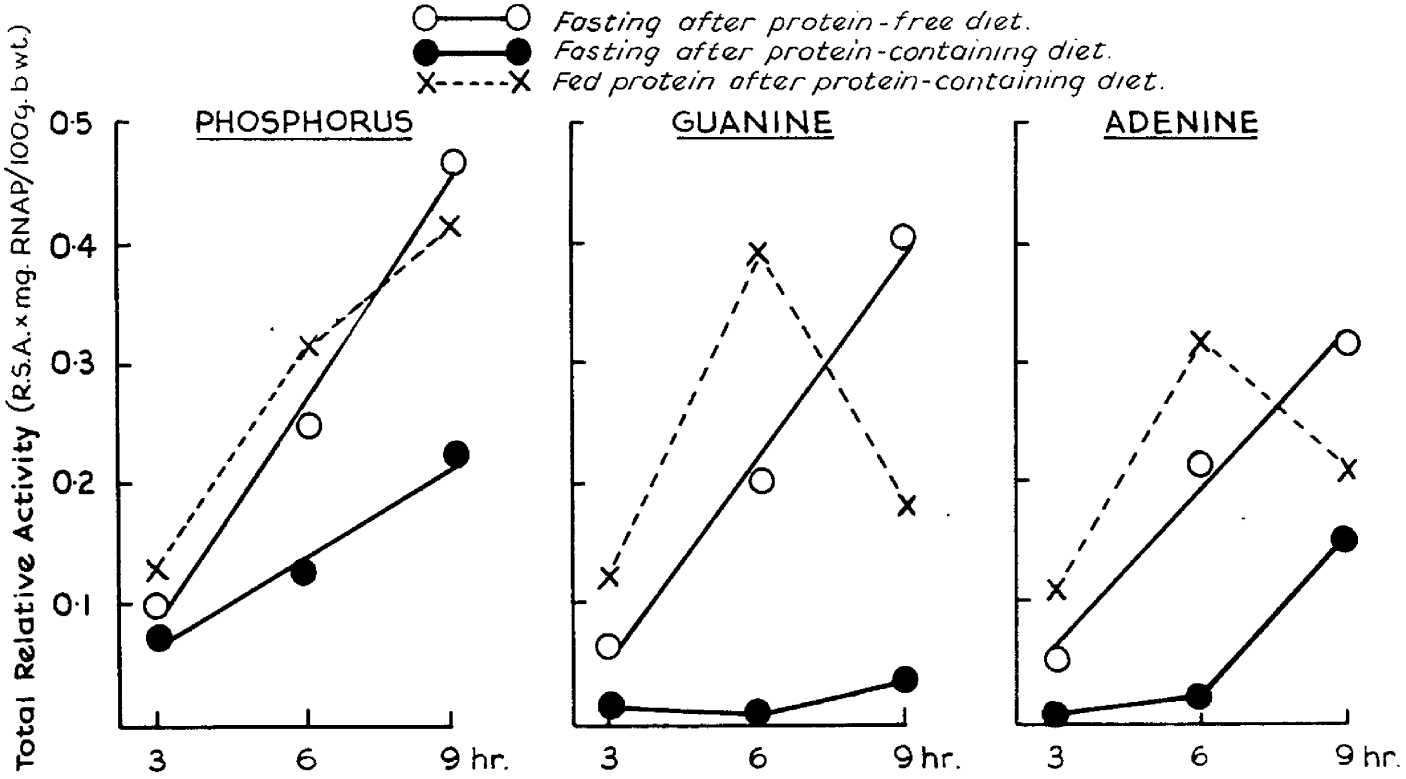
Table 10 gives the basic data obtained from the livers of the various dietary groups for the incorporation of ^{32}P and ^{14}C into RNA and Table 11 gives the total relative activities of RNA for these groups. The data in both tables represent the mean of 3 replicate experiments. Consistent effects of protein intake on RNA metabolism were obtained irrespective of whether the animals had been fed at the low or the high level of energy intake (Table 11). The results obtained at each energy level were therefore combined and presented in Fig.6. It is apparent from Fig.6 that animals fasting after the protein-containing diet showed about half the uptake of ^{32}P into RNA obtained with the rats fasting after the protein-free diet. When protein was fed to the animals at the time of isotope injection, uptake of ^{32}P into RNA rose to about the level found in the group receiving the protein-free diet. Incorporation of ^{14}C-glycine into RNA shows the same picture,

Table 11.

The effect of various nutritional conditions on the uptake of ^{32}P and ^{14}C-glycine into RNA. The results are expressed as total relative activities and the data represent the means of 3 replicate experiments.

Energy Level	Protein Intake	Time after isotope injection	Total Relative Activities (R.S.A. x mg. RNAP/ 100 g. initial body wt.)		
			^{32}P	^{14}C-2-glycine	
			RNA P	RNA Adenine	RNA Guanine
		hr.			
Low	Protein-free (fasting)	3	9.9	5.5	6.5
		6	24.5	21.9	20.6
		9	46.3	31.4	40.7
	Protein-containing (fasting)	3	7.8	0.8	1.7
		6	12.9	2.5	0.5
		9	22.8	16.0	4.3
	Protein-containing (fed)	3	12.6	10.8	12.1
		6	31.7	31.9	39.1
		9	41.2	20.5	18.0
High	Protein-free (fasting)	3	13.1	4.1	8.2
		6	28.4	13.2	31.7
		9	56.6	19.6	30.3
	Protein-containing (fasting)	3	10.5	5.2	1.7
		6	14.6	4.8	1.6
		9	23.7	12.9	1.4
	Protein-containing (fed)	3	10.9	-	-
		6	17.0	29.9	27.4
		9	47.1	35.6	29.8

Fig. 6.- Incorporation of ^{32}P and ^{14}C-2-Glycine into Liver Ribonucleic Acid under various nutritional circumstances.



except that the changes are more striking. The group fasted after the protein-containing diet had an uptake of ^{14}C into adenine which was about one-third of that exhibited by the group on the protein-free diet, and uptake of ^{14}C by guanine was even lower, averaging about one-tenth of the uptake on the protein-free diet. On re-feeding protein, incorporation into guanine rose to about the level obtained with the protein-free diet and incorporation into adenine to a somewhat higher level. It should be emphasised that these dramatic changes in glycine uptake are not artefacts due to the method of calculating total relative activity, but can be seen in the specific activities of the purine bases without reference to the precursor pool of glycine (Table 10).

From these data it is apparent that rats which had been receiving no dietary protein for several days incorporated as much ^{32}P and, in the case of guanine, as much ^{14}C-glycine into liver RNA as did rats which had been on the protein-containing diet and were eating protein at the time of injection with isotopes. This lack of association between dietary protein supply and RNA metabolism agrees with the results of previous experiments (Munro, Naismith & Wikramanayake, 1953) carried out on rats <u>receiving food during the period of labelling</u>. In these earlier studies the rate of ^{32}P uptake by liver RNA was found to be independent of the amount of protein in the diet. In contrast to this, we have found in the present work

Table 12.

Analysis of variance of the effect of protein and energy intake on the total amount of RNA in the liver over the 9 hr. period of isotope injection. Data obtained from Table 10.

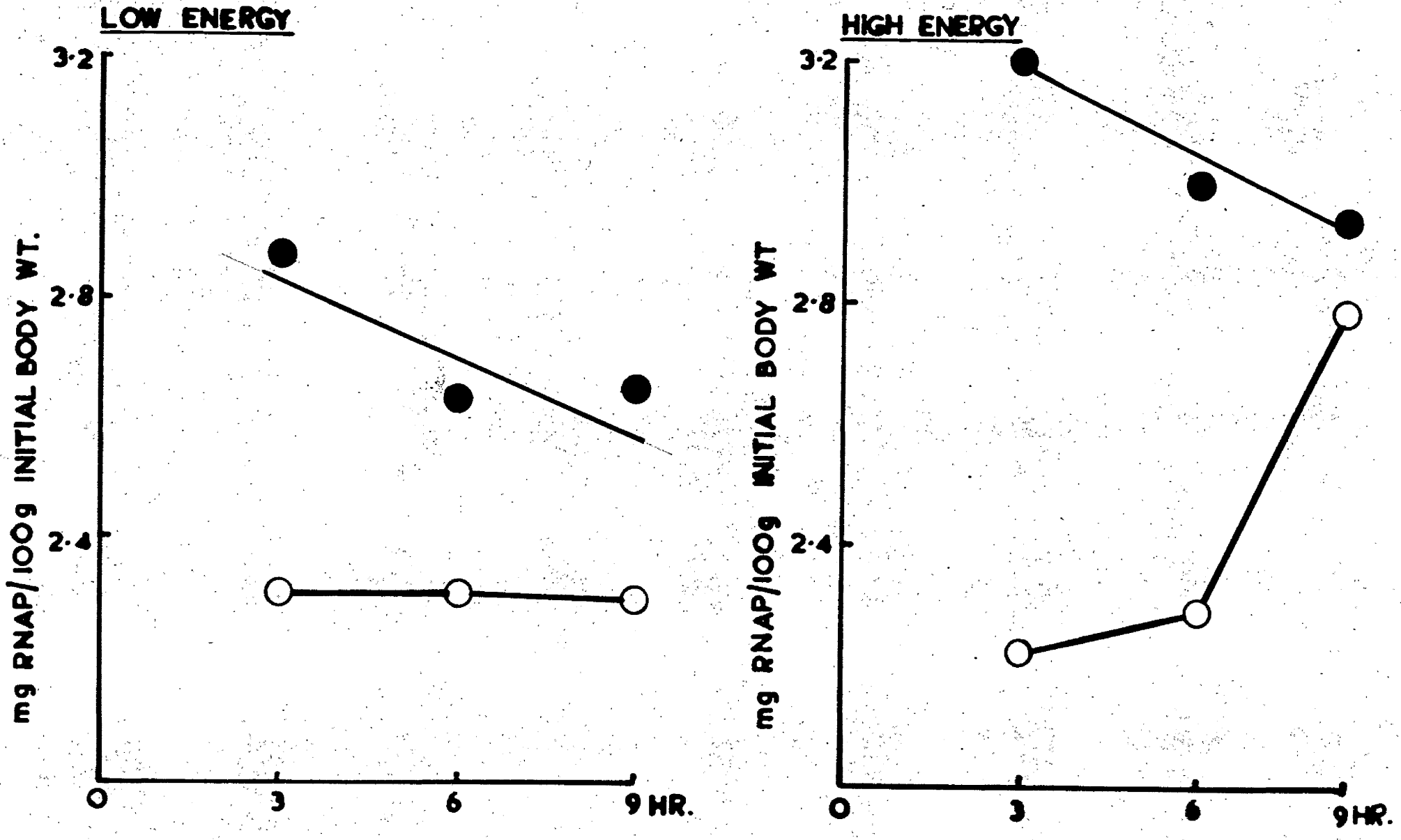
Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio (F)
Total	33	50,581	-	-
Replications	2	6,268	3,134	8.1**
Main Effects:				
Times (T)	2	735	368	< 1.0†
Energy Levels (E)	1	4,247	4,247	11.0**
Protein Levels (P)	1	25,228	25,228	65.3**
Interactions:				
T x E	2	746	373	< 1.0†
T x P	2	3,536	1,768	4.57*
E x P	1	1,332	1,332	3.45†
Residual Error	22	8,489	386	

** Highly significant. For 2 and 22, F at 5% = 3.44
at 1% = 5.72.

* Significant

† Not significant. For 1 and 22, F at 5% = 4.30
at 1% = 7.94

Fig.7. - Total amount of RNAP at 3, 6 and 9 hrs. after isotope injection in the livers of rats either fasting after a protein-free diet (o——o) or fasting after a protein-containing diet (●——●) at both low and high levels of energy intake.



that the protein content of the diet has a profound effect on the incorporation of both ^{32}P and ^{14}C-glycine when the rats are in the post-absorptive state throughout the period of labelling: thus uptake of isotopes by animals fasting after the protein-containing diet was much inferior to uptake by the group fasting after the protein-free diet.

Changes in the amount of RNA in the liver.

These effects of protein intake on the incorporation of isotopes into RNA can be explained by considering changes in the amount of RNA in the liver produced in the preceding experiments by fasting after each type of diet. Fig.7 contains the data obtained and represents the mean of 3 replicate experiments. The results are expressed as mg. RNAP per 100 g. initial body weight and it is apparent that at both levels of energy intake, the rats fed on a protein-containing diet started the fast with more RNA per liver than did the rats fed on the protein-free diet and moreover there was a significant fall during the 9 hr. period elapsing after injection of isotope (see Table 12 for statistical analysis). On the other hand, the rats on the protein-free diet started fasting with less RNA per liver, but the amount was not further reduced by fasting for the 9 hr. period. In fact, at the high level of energy intake in this group, there is a tendency for the total amount of RNA to rise over the 9 hr. period, but this is not

Table 13.

Changes in the amount of RNA in the livers of rats fasted or fed protein during a 24 hr. period following different diets. The data are the means of three replications of the experiment.

Preceding diet		Fed during period of observation	mg. RNAP/100 g. initial body weight		
Energy level	Protein content		0 hr.	24 hr.	Diff.
High	Protein-free	Nil	2.33	2.63	+0.30
	Protein-containing	Nil	3.06	2.52	-0.54
	Protein-containing	Protein	3.06	3.01	-0.05
Low	Protein-free	Nil	2.37	2.10	-0.27
	Protein-containing	Nil	2.62	1.78	-0.84
	Protein-containing	Protein	2.62	2.52	-0.10

Table 14.

Analysis of variance of effect of dietary protein and energy level on the amount of RNA in the liver over a 24 hr. period. The percentage changes in amount of RNA, given in Table 13, are the data used for analysis.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio (F)
Total	17	59,534	-	-
Main effects				
Protein levels	2	17,667	8,834	3.99*
Energy levels	1	4,262	4,262	1.9†
Interaction	2	2,081	1,041	0.47†
Residual Error	12	26,571	2,214	

Statistical analysis shows that only the protein level of the diet has any significant effect on amount of RNA in the liver; it is obvious from the data that the significant effect lies in the group fasted after the protein-containing diet.

significant statistically (Table 12).

This picture was confirmed in a separate series of experiments in which we studied the effect of dietary protein and energy intake on the total amount of RNA in the liver over a 24 hr. period (Table 13). The data are expressed as mg. RNAP per 100 g. initial body weight and each figure is the mean of 3 replicates. Examination of the results shows that only the group fasted after the protein-containing diet lost a significant amount of RNA (see Table 14 for statistics). The effect of feeding protein to animals fasting after a protein-containing diet was to diminish greatly the fall in the amount of RNA which occurred during the fasting (see Table 14 for statistics). The tendency for the RNA of livers of rats fasting after a protein-free diet at a high level of energy intake to increase in amount is again apparent: however, analysis of variance (Table 14) shows that this is once more not significant. The results are in keeping with the findings of Kosterlitz (1947), who studied combined RNA and DNA and found that rats fasted after a stock diet lost nucleic acid from their liver but not when fasted after a protein-deficient diet. Since it is known from the work of Thomson et al. (1953) that DNA does not participate in these changes, RNA must be responsible.

This evidence suggests that during fasting after a protein-containing diet there is an accelerated breakdown of RNA. If this is true, the most likely explanation for the

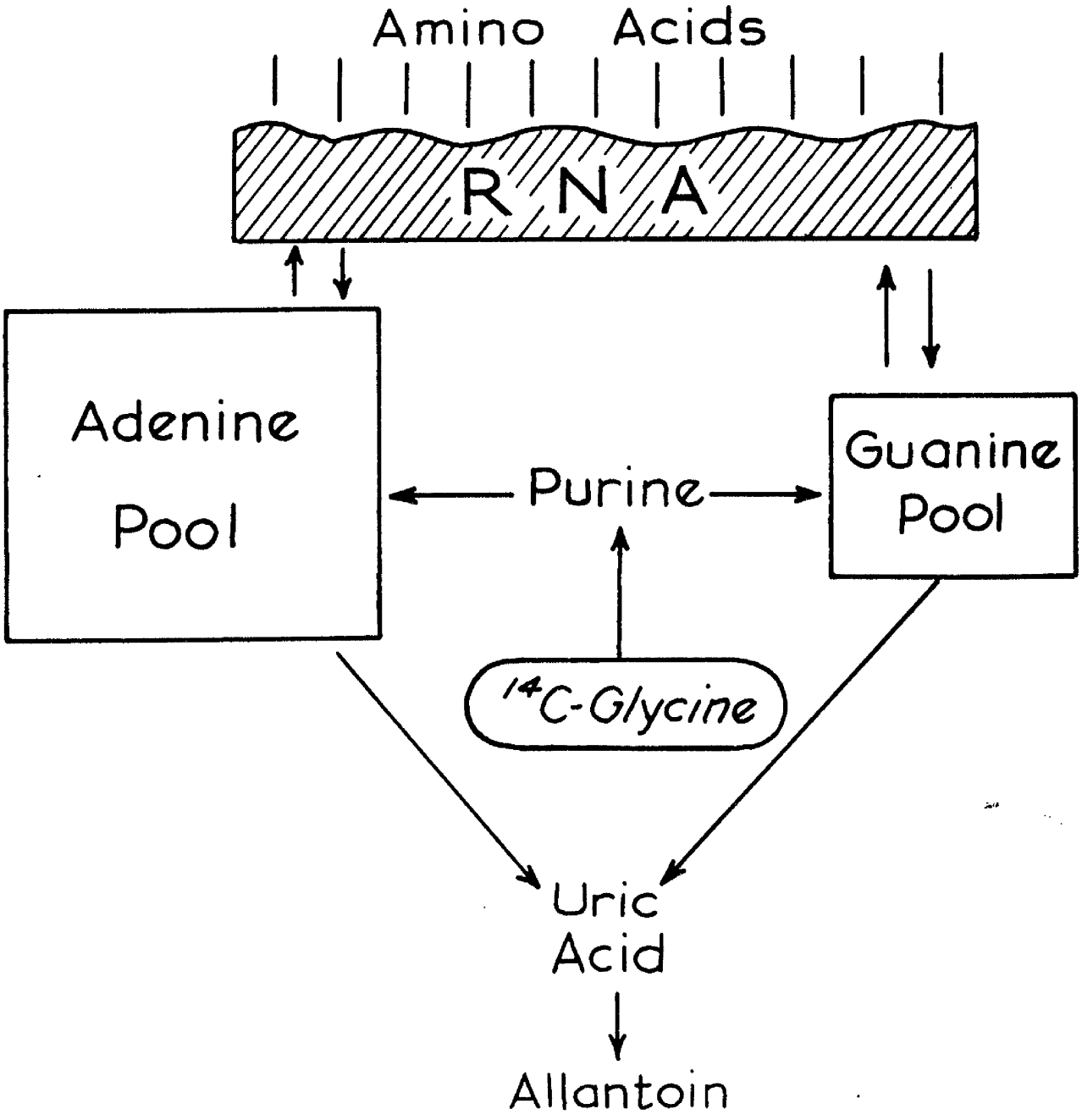


Fig.8. - Relationship between RNA and precursor pools of adenine and guanine.

low uptake of isotopes by RNA in such animals is that the labelled RNA precursors become diluted by the breakdown products entering the same pool as the precursors (Fig.8). When rats from the same dietary group were fed protein just prior to injection with isotopes, uptake of ^{32}P and ^{14}C-glycine rose rapidly to the levels obtained with rats on the protein-free diet, and in such animals liver RNA did not fall significantly (Table 13). This suggests that, as soon as amino acids start to be absorbed from the gut, RNA breakdown is halted with consequent cessation of dilution of the labelled RNA precursors. In other words, protein intake influences the amount of RNA in the liver by regulating its rate of breakdown.

In support of this interpretation of the data, we have examined the effects of diet on labelling in the precursor pools on allantoin excretion and on the uptake of ^{32}P by the RNA of different fractions of the liver cell.

Uptake of ^{14}C-glycine by RNA Precursors.

If the low uptake of isotopes by RNA in animals fasted after a protein-containing diet is in fact due to dilution with breakdown products, one would expect to find evidence of such dilution effects among the precursors of RNA. As a first approximation to the necessary data, we examined glycine uptake by the pooled adenine and guanine compounds of the perchloric-acid soluble fraction of liver. It has been shown by Bennett and Krueckel (1955) that most of the acid-soluble adenine is

Table 15.

The effect of protein intake on the uptake of ^{14}C-2-glycine by the acid-soluble adenine and guanine 3 and 6 hr. after isotope injection.

Previous Diet	Fed after isotope injection	Time after isotope injected	S.A. of Free Glycine	^{14}C-2-glycine from Adenine		^{14}C-2-glycine from Guanine	
				Specific Activity	Relative Specific Activity	Specific Activity	Relative Specific Activity
		hr.	cpm/100 µg. glycine	cpm/100 µg. glycine		cpm/100 µg. glycine	
Protein-free	Nil	3	925	57	6.2	75	8.1
Protein-containing	Nil	3	1780	106	6.0	18	1.0
Protein-contain-	Protein	3	1830	770	42.1	350	19.1
Protein-free	Nil	6	1073	127	12.0	124	11.5
Protein-containing	Nil	6	2980	128	4.3	43	1.5
Protein containing	Protein	6	1560	1082	69.4	508	32.6

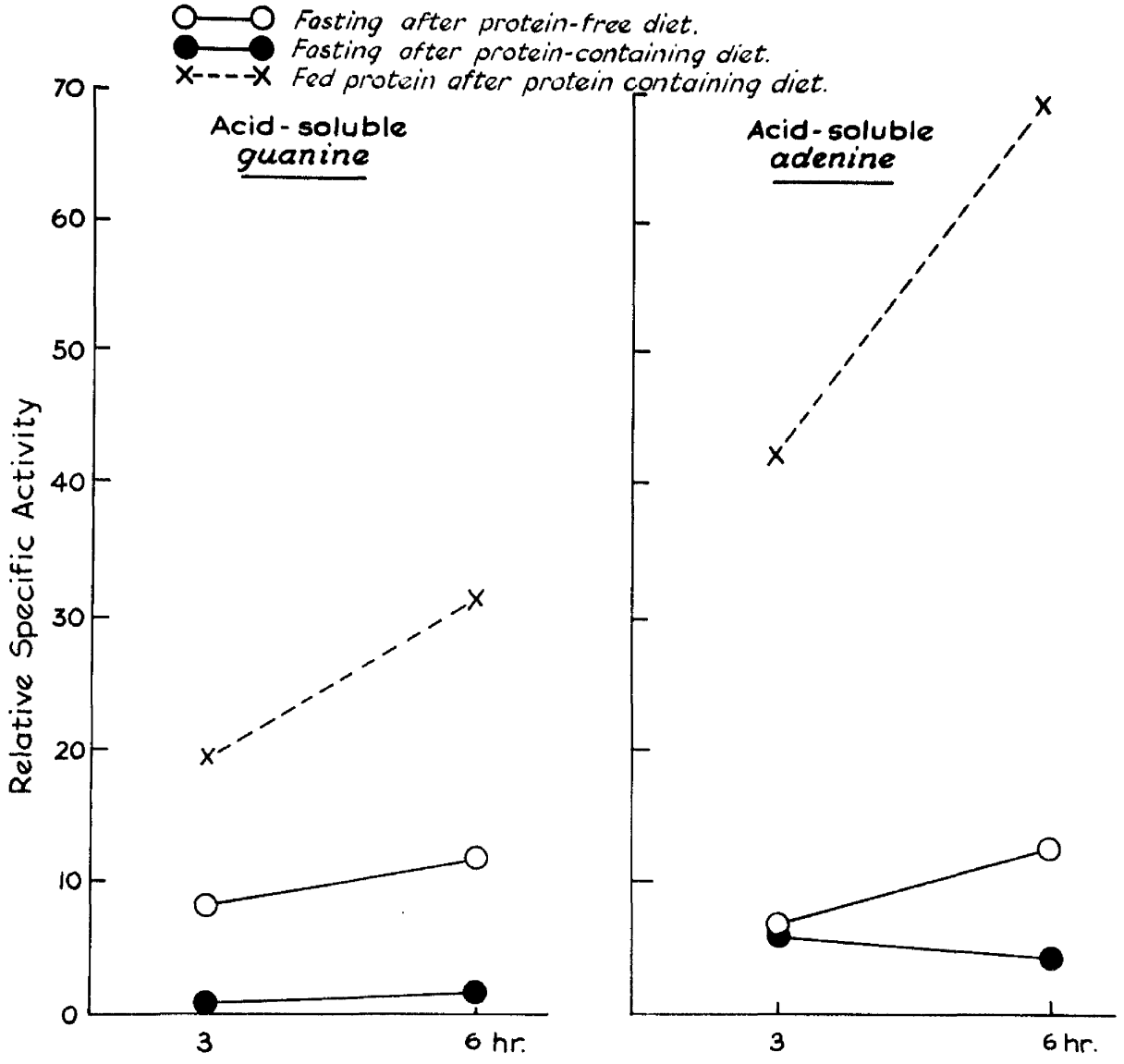


Fig. 9. - Effect of various nutritional conditions on the uptake of ^{14}C-2-glycine by acid-soluble adenine and guanine of rat liver, 3 and 6 hr. after isotope injection.

present in the form of adenosine mono-, di- and tri-phosphates and that guanine takes corresponding forms almost exclusively. After administration of labelled adenine, they observed that the pools of adenosine compounds rapidly reached equilibrium. Similar results were obtained for the guanosine derivatives. Examination of the total adenine and the total guanine of the perchloric acid-soluble fraction of liver will therefore provide a reasonable approximation to the general state of the acid-soluble adenine and guanine precursors of RNA, if we accept adenosine and guanosine phosphates as likely precursors.

Since the action of dietary protein on RNA metabolism was not found to differ appreciably at low and high caloric intakes, the study of changes in labelling in the precursor pools with variations in protein intake was made at an intermediate caloric intake, namely, 1200 cal./sq.m./day. The total amounts of adenine and guanine in the acid-soluble fraction were not measured: consequently the results are expressed, not as total relative activities, but as the specific activity of glycine isolated by hydrolysis from these purines relative to the specific activity of the free glycine.

The data obtained are presented in Table 15 and Fig.9 and it is evident that in the case of rats fasted after the protein-containing diet the uptake of glycine by the adenine and guanine of the acid-soluble pool was much less than in the case of rats fasted after the protein-free diet. On feeding

Table 16.

The effect of different nutritional conditions on the relative uptake of ^{14}C-2-glycine by the purine bases of the acid-soluble fraction and RNA.

Previous Diet	Fed after isotope injection	Uptake of ^{14}C-glycine	
		Acid-soluble guanine/adenine ratio	RNA guanine/adenine ratio
Protein-free	Nil	1.15	1.41
Protein-containing	Nil	0.26	0.48
Protein-containing	Protein	0.47	0.96
Analysis of variance		$P=0.05-0.01$ (6 observations)	$P < 0.01$ (30 observations)

Statistical analysis shows that there is a significant effect of protein level in diet on the G/A ratio for both the acid-soluble fraction and RNA.

protein just prior to injection, incorporation of glycine was markedly stimulated. The changes observed in RNA labelling are thus, in the main at least, reflections of corresponding changes in the precursor pools of adenine and guanine compounds.

Relative Changes in Uptake of Precursors by Adenine and Guanine.

The hypothesis of variations in RNA breakdown receives further support when we consider the relative effects which dilution with RNA breakdown products would have on the acid-soluble adenine and guanine compounds. The pool of adenine compounds is much larger than that of guanine compounds (cf. Fig.8), whereas liver RNA contains slightly more guanine than adenine (Crosbie, Smellie & Davidson, 1953). Breakdown of RNA would therefore have a greater diluent effect on the pool of guanine compounds.

Table 16 contains the data expressed as the relative uptakes of radioactive glycine into guanine and into adenine of the acid-soluble fraction and of RNA. The data for the acid-soluble purines are taken from the experiment recorded in Fig.9; the data for ^{14}C-glycine uptake by RNA purines are from the experiments described in Table 10 and Fig.6. The ratios obtained at different times after isotope injection followed essentially the same pattern and have been averaged. Examination of Table 16 shows that our prediction that breakdown of RNA would have a greater diluent effect on the pool of guanine compounds is fulfilled. Fasting after the protein-containing

Table 17.

The uptake of ^{32}P by liver RNA of rats on various nutritional conditions. The results are given relative to adenylic acid. Each ratio represents the average ratio from 6 replicates and the data were obtained from Fig.6.

Previous diet	Fed after isotope injection	Uptake of ^{32}P by RNA-nucleotides		
		Cytidylic Acid / Adenylic Acid	Guanylic Acid / Adenylic Acid	Uridylic Acid / Adenylic Acid
Protein-free	Nil	1.01	0.95	1.05
Protein-containing	Nil	0.95	0.96	1.27
Protein-containing	Protein	0.99	0.93	1.02

Table 18.

Statistical analysis of the effect of protein intake on the uptake of ^{32}P by the mononucleotides of RNA relative to adenylic P of RNA. (Analysis of data in Table 17).

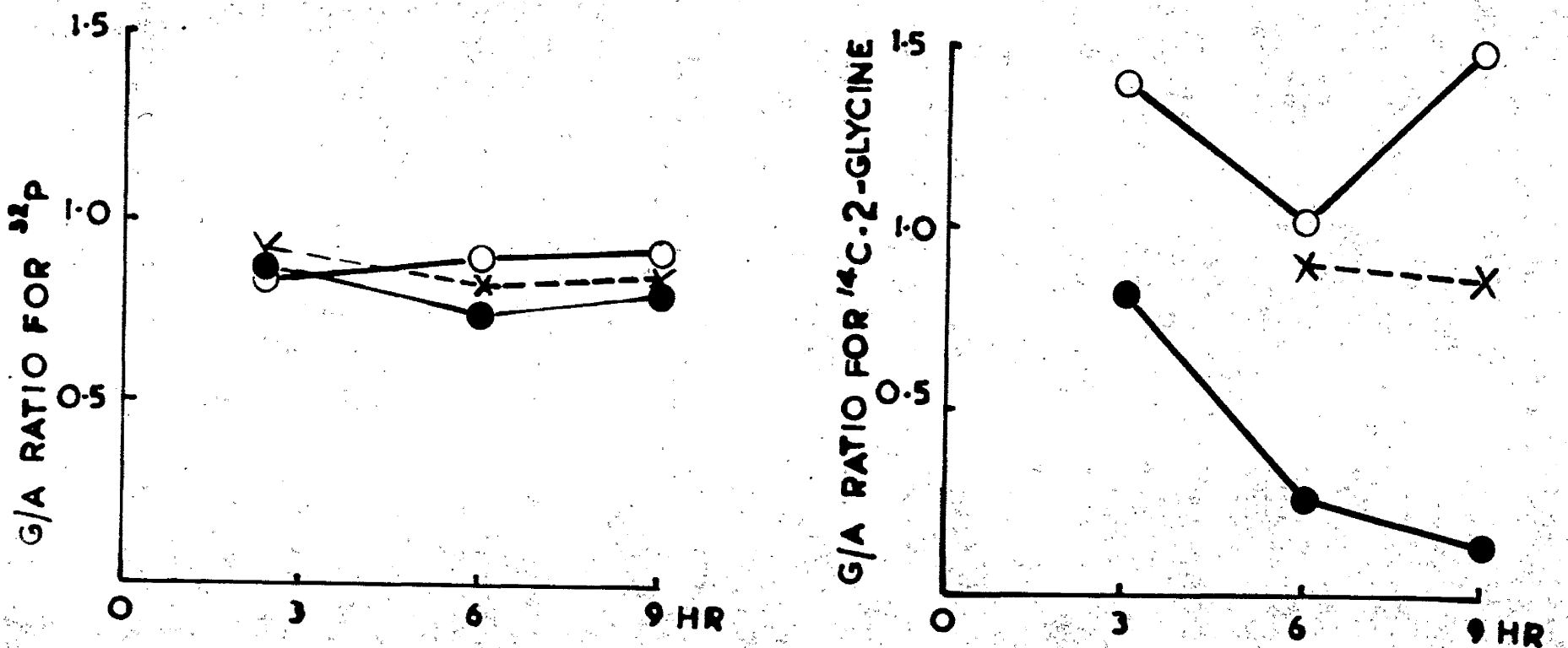
Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio (F)
Total	53	420	-	-
Nucleotides	2	20	10	1.25
Protein	1	4	4	0.50
Interaction	4	21	5	0.61
Residual error	46	375	8	

For 4 and 45 F at 5% = 2.58

at 1% = 3.78.

Statistical analysis shows that protein intake has no significant effect on the uptake of ^{32}P by RNA nucleotides relative to RNA-adenylic acid.

Fig.10. - The $^{G}/_{A}$ ratio for ^{32}P and ^{14}C-2-glycine uptake by RNA 3, 6 and 9 hr. after isotope injection in the livers of rats either fasting after a protein-free diet (O——O) or fasting after a protein-containing diet (●——●) or fed protein after a protein-containing diet (×——×).



diet reduced the uptake of glycine into guanine much more severely than into adenine, as evidenced by a fall in the guanine/adenine ratio ($^{G}/_{A}$). The ratio rose again when protein was fed, i.e., when dilution from RNA breakdown products was held in check. Statistical analysis confirms that the $^{G}/_{A}$ ratio for ^{14}C uptake was significantly affected by diet in the case of the acid-soluble purines and the purines of RNA (Table 16).

By comparison with these data for ^{14}C incorporation, Table 17 shows an absence of effect of diet on the ratios of the specific activities of the ^{32}P of cytidylic, guanylic and uridylic acids of RNA to that of the ^{32}P of the RNA adenylic acid. In each instance the reported values are the average of the data obtained at 3, 6 and 9 hrs. after isotope injection, since a similar picture was obtained at each time interval (see Table 18 for statistical analysis). Figure 10 illustrates this point diagramatically by showing the $^{G}/_{A}$ ratio for both ^{32}P and ^{14}C uptake into RNA at 3, 6 and 9 hrs.

Isotope Uptake by Different Liver Cell Fractions.

Rats fasting after a protein-containing diet exhibit a low uptake of isotopes into liver RNA (Fig.6). If this is due to dilution of RNA precursors by RNA breakdown products, it is likely that precursors of RNA in all parts of the liver cell will be affected. For this reason, we examined incorporation of ^{32}P into the RNA of different cell fractions under

Table 19.

Incorporation of ^{32}P into the ribonucleic acid of different fractions of the livers of rats receiving various diets and killed 6 hr. after the injection of 50 μc of ^{32}P. The data are the mean specific activities of the ribonucleotides in each fraction relative to the specific activity of liver inorganic P expressed as a percentage; each dietary group consisted of 3 rats.

Previous Diet	Fed after isotope injected	Whole homogenate	Nuclei	Mitochondria	Microsomes	Cell Sap
Protein-free	Nil	11.2	33.0	4.7	6.7	9.4
Protein-containing	Nil	5.7	18.3	2.7	3.0	5.9
Protein-containing	Protein	14.2	38.2	8.5	9.4	12.4

Table 20.

Incorporation of ^{32}P into the lipid P of different fractions of the livers of rats receiving various diets and killed 6 hr. after the injection of 50 μc ^{32}P. The data are the specific activities of the lipid P in each fraction relative to the specific activity of liver inorganic P expressed as a percentage; each dietary group consisted of 3 rats.

Previous Diet	Fed after isotope injected	Whole homogenate	Nuclei	Mitochondria	Microsomes	Cell Sap
Protein-free	Nil	61.7	58.6	57.7	63.3	51.9
Protein-containing	Nil	49.0	49.0	43.8	50.6	49.8
Protein-containing	Protein	65.0	62.3	57.4	65.5	49.4

various dietary conditions (Table 19). In the case of rats fasting after the protein-containing diet, the RNA in all cell fractions exhibited a low uptake of the isotope. On feeding protein to such animals, ^{32}P incorporation rose proportionally in the different fractions. This may be contrasted with the effect of changes in protein intake on the total amount of RNA in these fractions. Wikramanayake, Heagy and Munro (1953) found that only the RNA of the microsomal fraction (sedimented at 20,000g) and possibly the nuclear RNA underwent alterations in amount when protein intake was altered. This finding is not compatible with the isotopic data given in Table 19 if we believe that the increased ^{32}P uptake observed after feeding protein is due to an increased rate of RNA synthesis; in that case, increased ^{32}P incorporation ought to be confined to the fraction changing in amount. The quantitative data and the isotopic evidence can, however, be reconciled if we assume that variations in dilution by microsomal RNA breakdown affect the labelling of a precursor pool common to all cell fractions.

A similar study on the effect of protein intake on the uptake of ^{32}P by the lipid P (LP) of the different cell fractions (Table 20) indicates that dietary protein affects the uptake of ^{32}P by LP in a similar manner to the effect on RNA, but this occurs to only a very slight extent in the whole homogenate, nuclei, mitochondria and microsomes and not at all in the cell

Table 21.

Incorporation of ^{32}P into RNA of different fractions of the livers of rats receiving various diets and killed 6 hr. after the injection of 50 μc ^{32}P. The data are expressed as mean R.S.A. of ^{32}P of RNA nucleotides relative to R.S.A. of LP expressed as a percentage.

Diet	Cell Fraction				
	Whole Homogenate	Nuclei	Mitochondria	Microsomes	Cell Sap
Protein-free (fasting)	18.2	56.4	8.2	10.6	18.1
Protein-containing (fasting)	11.6	37.4	6.2	5.9	11.9
Protein-containing (fed)	21.8	61.4	14.8	14.4	25.1

sap. However, a plot of RNA-^{32}P against LP-^{32}P shows that the RNA effect is much more profound (Table 21). This is in agreement with the findings of Wikramanayake et al. (1953) that uptake of ^{32}P by lipid P is not affected by the feeding of protein.

Diet and Allantoin Excretion.

Since allantoin is the principal excretory product of purine metabolism in the rat, it was thought that urinary output of allantoin might reflect changes in the rate of RNA breakdown. Urine was collected from rats over a 24 hr. period of fasting following diets containing protein or free from protein: in addition, one group was fed protein throughout the 24 hr. period. In order to minimise variations in allantoin output resulting from incomplete voiding of urine, the results were expressed in relation to creatinine output, which was assumed to be unaffected by diet. Table 22 shows that the feeding of protein reduced output by comparison with the output of rats fasting after the protein-containing diet. Statistical analysis shows that this effect is significant at the 5% level (Table 23). This finding is compatible with the hypothesis that RNA breakdown is diminished by feeding protein. In contrast to the findings in these two groups, the allantoin excretion of rats fasting after the protein-free diet was found to vary significantly with the energy content of the preceding diet (Table 23). Energy level in the preceding diet did not

Table 22.

The urinary output of allantoin by rats fasted or fed protein during a 24 hr. period following different diets. The data are the means of six replications of the experiment.

Previous diet	Fed during period of collection	mg. Allantoin/mg. creatinine	
		High energy group	Low energy group
Protein-free	Nil	3.97	5.93
Protein-containing	Nil	4.40	4.64
Protein-containing	Protein	4.02	3.99

Table 23.

Statistical analysis of effect of diet on the excretion of allantoin in the urine. Data for analysis obtained from Table 22.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance (F)
Total squares	35	387,718	-	-
Main Effects				
Energy levels	1	47,089	47,089	6.5**
Protein levels	2	53,613	26,807	3.7*
Interaction	2	70,685	35,343	4.9*
Residual Error	30	216,331	7,211	

For 2 and 30 F at 5% = 3.32

at 1% = 5.39

Analysis shows that protein level of the diet has a significant effect and energy level a highly significant effect on allantoin excretion in the urine.

have this effect on rate of loss of RNA from the livers of fasting animals (Tables 13,14) and the effect of previous energy intake on allantoin excretion is presumably due to other causes.

DISCUSSION.

As already indicated, the participation of RNA in protein synthesis was originally suspected because of the large amount of RNA found in cells with a high rate of protein synthesis. Nevertheless, attempts to demonstrate a relationship between rate of protein synthesis and uptake of isotopes by RNA have sometimes yielded negative results, as summarized in the introduction to this thesis.

The present experiments indicate the occurrence of a similar dissociation between protein synthesis and RNA synthesis in the case of liver. Comparison of rats receiving a meal of protein at the time of isotope injection with animals fasting after several days on a protein-free diet revealed a considerable stimulation of ^{14}C-glycine incorporation into the liver proteins of the protein fed group (Fig.5). Nevertheless, the two groups showed an essentially similar uptake of ^{32}P and of ^{14}C-glycine into RNA. This is in agreement with results obtained by Munro et al. (1953) who found that, when rats received either a protein-free or protein-containing diet during the period of labelling the absolute rate of ^{32}P uptake by RNA was independent of the protein content of the diet but dependent on the energy content. However, our present experiments showed that rats fasting after a diet containing protein had a much reduced uptake of isotopes into RNA (Fig.6). Unlike the other two groups, the RNA content of the livers of these animals was

diminishing rapidly during the period of labelling (Fig.7 and Table 13) and it has been concluded that the low isotope uptake of this group was due to dilution of precursors with breakdown products. Subsequent experiments showed that the purine bases of the precursor pool did in fact exhibit a low uptake of glycine under these conditions (Fig.9 and Table 15), that reduction in the labelling of adenine and guanine differed in the way one would anticipate from dilution (Table 16 and Fig.10), that changes in ^{32}P uptake were spread over all subcellular fractions in a manner compatible with dilution of a common precursor pool (Table 19), and that allantoin excretion was greater in fasting than in protein-fed animals (Table 22). Since evidence of dilution was swiftly obliterated on feeding protein, we have concluded that breakdown of RNA is partly or wholly suspended while the dietary amino acids are being absorbed from the gut.

Presumably there is some sort of relationship between the stability of liver RNA and the availability of amino acids for protein synthesis, so that a given level of amino acid supply confers stability on a certain quantity of RNA. Thus in the case of protein-depleted rats, the diminished amount of RNA in their livers is equilibrated with the reduced supply of amino acids in circulation. Similarly, the larger amount of RNA in the livers of rats receiving a protein-containing diet can be maintained in a stable state so long as the animals

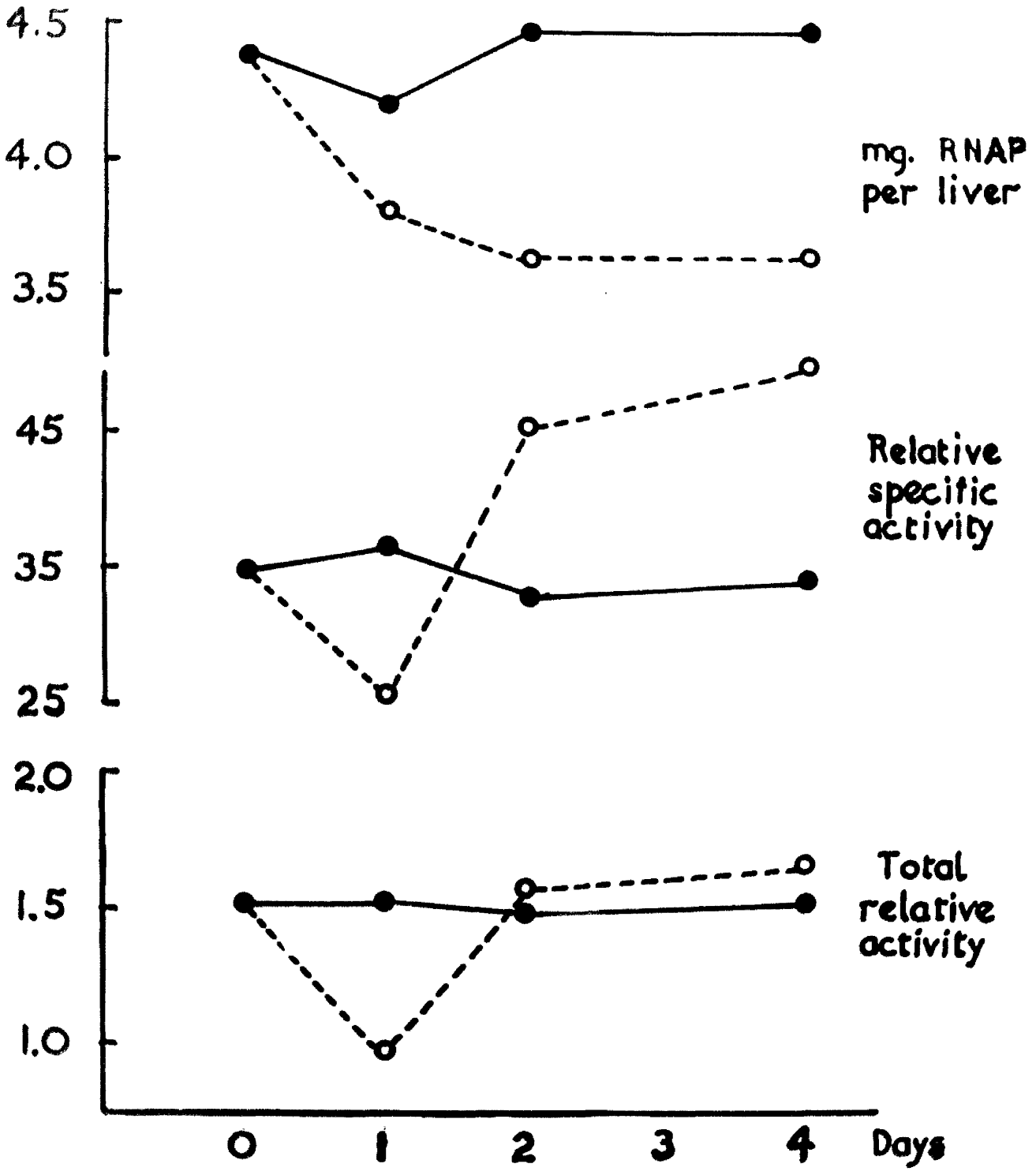


Fig.11. - The effect of a transition from a protein-containing diet (●——●) to a protein-free diet (○——○) on the total amount of RNAP and the uptake of ^{32}P by RNA in rat liver over a period of 4 days (according to Munro et al., 1953).

continue to receive protein. It is during transition from one nutritional state to another (e.g., during a short period of fasting after a diet containing protein) that we observe effects due to imbalance between amino acid supply and the amount of RNA. These effects will continue to be evident until a new equilibrium between the amount of RNA and the supply of amino acids is achieved. The findings of Munro et al. (1953) are a striking illustration of this since they found (Fig.11) that when rats previously receiving adequate amounts of protein were then given a protein-free diet, the uptake of ^{32}P by the RNAP of the liver, expressed as relative specific activity, fell by more than half in the first 24 hr. This was followed by a rise to the expected high uptake on the protein-free diet from the second day onwards. This fall in relative specific activity coincided with the fall in total amount of RNAP in the liver and could be explained by the fact that breakdown of RNA during the first 24 hr. of protein deficiency would flood the liver with breakdown products of RNA and this would have a diluent effect on RNA precursors, with consequent reduction in the labelling of RNA.

The picture of RNA metabolism which emerges from the present study is thus one of a steady synthesis of RNA in proportion to the supply of energy, variations in the amount of RNA being brought about by alterations in the rate of its breakdown in response to changes in the supply of amino acids

available for protein synthesis. This view of the relationship between RNA metabolism and protein synthesis would account for the fact that tissues with a high rate of protein synthesis have a large content of RNA, yet do not exhibit a raised rate of RNA formation (cf. Kihara, Amano, Ikemoto & Sibatani, 1955).

In view of the recent discovery (Grunberg-Manago, Ortiz & Ochoa, 1956) of an enzyme system which can polymerise nucleoside diphosphates to RNA-like compounds it is of interest to consider whether our present data are compatible with RNA precursors of this type. Table 16 shows that the various dietary conditions used in our experiments affected the labelling of adenine and guanine differently, whereas (Table 17) the specific activity of ^{32}P was essentially similar in all four ribonucleotides under these different nutritional conditions. This is precisely what one would expect if adenosine- and guanosine-diphosphates are immediate precursors of RNA. Recent studies by Brumm, Potter and Siekevitz (1956) have shown that the acid-labile atoms of adenosine- and guanosine-diphosphates, as well as those of the other nucleoside-diphosphates, are in facile equilibrium. In consequence, even if RNA breakdown leads to unequal dilution of the pools of adenosine- and guanosine-diphosphates, the terminal P atoms would at once equilibrate and result in similar specific activities of ^{32}P.

However, the rate of conversion of adenine to guanine is much slower (Bennett & Krueckel, 1955), so that unequal dilution of the bases of ADP and GDP during RNA breakdown would persist. It has been shown by Brumm et al. (1956) that the P atoms of adenosine- and guanosine-monophosphates are not so readily exchanged as are the high energy P atoms; consequently, under our experimental conditions, they are less acceptable as likely precursors. Grunberg-Manago et al. (1956) have shown that their enzyme is reversible and can lead to nucleoside diphosphate formation from the RNA polymer. It is thus possible that breakdown products responsible for the dilution of RNA precursors in our experiments are in fact ADP and GDP themselves. If so, we have the interesting situation in which the point of equilibrium between nucleoside diphosphates and RNA is determined by the supply of amino acids available for protein synthesis.

SECTION II.

UPTAKE OF ^{14}C-GLYCINE AND ^{32}P BY LIVER RIBONUCLEIC ACID WHEN PROTEIN IS ADMINISTERED TO PROTEIN-DEPLETED ANIMALS.

INTRODUCTION.

In the previous section we found that protein depletion resulted in a breakdown of liver RNA as evidenced by a fall in RNA content and dilution of the precursors of RNA synthesis. It was concluded that the amount of RNA held in stable form is related to the supply of amino acids. From the work of Munro et al. (1953) we know that this loss of RNA reaches a steady state, since they found that, when there is a transition from a protein-containing diet to a protein-free diet, the fall in amount of liver RNA is followed by a constant lower level (see Fig.11, Section I of this thesis). We must assume therefore that there is a readjustment between the amino acid supply and the amount of RNA held in stable form and when this is achieved a steady state or plateau region will result. We know from the work of Munro et al. (1953) that, once this stable state is obtained, ^{32}P incorporation into RNA is the same as uptake on a protein-containing diet (Fig.11, total relative activity). Since RNA does not accumulate during protein deficiency, this must mean that breakdown (turnover) continues on a protein-deficient diet at a normal rate. Feeding of protein to these depleted animals should therefore result in the same cessation of RNA breakdown noted in Section I. In consequence of alterations in dilution of the precursor pool with breakdown products, this

should therefore produce changes in RNA labelling compatible with the picture presented in Section I. We have accordingly carried out experiments on rats depleted by feeding a protein-free diet and refed protein on the day of injection with ^{32}P and ^{14}C-glycine.

EXPERIMENTAL.

Animals and Diets. Young male albino rats weighing 180-200 g. in the fasting state were used. The rats were housed individually in metabolism cages and maintained under thermostatic conditions. The rats were fed for 7 days on a diet free from protein. The diet was given in two portions, 1.0 g. V.M.R. (see Table 5) and 3.5 g. glucose at 9 a.m. and 4.2 g. protein-free diet (Table 2) at 4 p.m. The energy content of the diet was maintained at 1200 cal./sq.m./day throughout the 7 days. The food was prepared and given to the rats as previously described in Section I.

Isotope Administration. The rats received their last meal at 4 p.m. on the 7th day and on the following morning they were injected with ^{32}P (10 μc. inorganic P/100 g. body wt.) and ^{14}C-2-glycine (10 μc/100 g. body wt.), one injection into each thigh. In addition, half the animals were fed 2.5 g. casein solubilised with 0.15 g. $NaHCO_3$ one hour prior to injection of isotope, the rest being kept in the fasting state. The animals were killed at 3, 6 and 9 hours after isotope injection. The experiments on the labelling of the purine precursor pools were carried out separately and the animals were killed at only two time intervals, 3 and 6 hr. after injection.

Liver Analysis. The livers of the animals were analysed by the methods described in Section I for total amounts of protein N, RNAP and DNAP; specific activity of inorganic P and free-glycine; uptake of ^{14}C-glycine into protein; uptake of ^{32}P and ^{14}C-glycine into RNA; and uptake of ^{14}C-glycine into acid-soluble purines.

Table 24.

The effect of feeding protein to protein-depleted animals on the uptake of ^{32}P and ^{14}C-2-glycine into RNA, 3, 6 and 9 hr. after isotope injection. The data are expressed as relative specific activity (R.S.A.). Each rat received 10 μC/100 g. body wt. of each isotope.

Feeding prior to isotope injection	Time killed after injection	S.A. of inorganic P counts/min./ 100 μg.	S.A. of free glycine counts/min./ 100 μg.	R.S.A. of ^{32}P of RNA-purine nucleotides		R.S.A. of ^{14}C-glycine of RNA purines	
				Adenine	Guanine	Adenine	Guanine
-	3	13,750	2202	2.6	3.4	-	2.8
-	6	15,590	1040	6.3	6.0	3.9	9.0
-	9	12,440	746	11.6	8.0	8.0	11.8
Protein	3	19,340	1528	-	8.1	4.4	13.6
Protein	6	10,880	525	13.2	14.2	26.1	53.6
Protein	9	8,470	555	17.9	25.6	-	47.5

Table 25.

The effect of feeding casein to rats fasting after a protein-free diet on the uptake of ^{14}C-2-glycine by protein and RNA-guanine and of ^{32}P by RNA of the liver at 3, 6 and 9 hrs. after isotope injection (10 μc ^{14}C-2-glycine and 10 μc ^{32}P/ 100 g. body wt.).

Time killed after injection	Total Relative Activities						Ratio of T.R.A. fed to fasted animal		
	^{14}C-protein		^{32}P-RNA		^{14}C-guanine				
	Fed	Fasted	Fed	Fasted	Fed	Fasted	Protein	RNA-^{32}P	^{14}C-guanine
hr.									
3	15.3	9.3	28.1	10.3	47.1	8.5	1.64	2.73	5.52
6	41.2	26.6	53.2	21.0	189.0	32.1	1.55	2.54	5.89
9	43.3	34.6	90.4	39.7	180.3	44.9	1.25	2.28	4.02

RESULTS AND DISCUSSION.

Table 24 contains the basic data obtained from these experiments and Table 25 gives the total incorporation rates expressed as total relative activity (T.R.A.). Owing to losses, the adenine data are too incomplete to be worthy of inclusion in Table 25. Table 25 shows that feeding of protein to animals fasting after a protein-free diet causes increased incorporation of ^{14}C-glycine into protein and an increased incorporation of ^{32}P and ^{14}C-glycine into RNA. If these increased T.R.A.s of RNA reflect an increased rate of synthesis of RNA, it might be anticipated that the ratio of the increase in ^{32}P incorporation (i.e., ratio of T.R.A. of fed animals/T.R.A. of fasted animals) would be the same as that for ^{14}C-glycine. However it will be seen (Table 25) that the T.R.A. of ^{32}P of RNA in fed animals is twice that of the fasted group, whereas the T.R.A. of ^{14}C-glycine of RNA-guanine is five times greater in the fed animals. We feel, therefore, that these findings can be more adequately explained in terms of dietary protein retarding the rate of breakdown of RNA. The different effects on ^{14}C-glycine and ^{32}P uptake would then be explained as due to differing dilution effects on the precursor pools of guanine and of ^{32}P in the manner discussed in the preceding section.

This interpretation is confirmed by an examination of

Table 26.

The effect of feeding casein to rats fasting after a protein-free diet on the uptake of ^{14}C-2-glycine by acid-soluble purines of liver 3 and 6 hr. after isotope injection (10 μc ^{14}C-2-glycine/100 g. body wt.).

Fed after isotope injection	Time killed after isotope injection	S.A. of free glycine	Acid-soluble adenine		Acid-soluble guanine		Ratio of S.A. guanine
			S.A.	R.S.A.	S.A.	R.S.A.	S.A. adenine
			counts/ min./ 100 ug.		counts/ min./ 100 ug.		
Nil	3	925	57	6.2	75	8.1	1.32
Protein		630	463	74.0	338	53.7	0.73
Nil	6	1075	127	12.0	124	11.5	0.98
Protein		903	861	96.0	438	48.4	0.51

^{14}C-glycine incorporation into the acid-soluble purines (Table 26). The feeding of casein causes a dramatic rise in ^{14}C-glycine uptake to which the RNA picture must be secondary. A point of difference from the results noted in Section I lies in the response of the $^{G}/_{A}$ ratio to the feeding of protein. We find (Table 26) that, when protein is fed to animals depleted of protein, there is a fall in the $^{G}/_{A}$ ratio at each time interval, whereas the feeding of protein to rats fasting after a high-protein diet (Table 16 and Fig.10) resulted in a rise in the $^{G}/_{A}$ ratio. This difference is obviously related to the protein content of the preceding diet. It is possible to explain this difference by a consideration of some results obtained by Allison (1956), who showed that feeding of methionine to protein-depleted rats had a conserving effect on body N. This is presumably due to the fact that the amino-acid pool of the livers of rats receiving a protein-free diet becomes quickly deficient in methionine as a result of the high demand for this amino acid for the synthesis of sulphur-containing proteins. In this way, the feeding of a protein-free diet might be regarded as similar to supplying the animal with an amino-acid mixture deficient in one essential amino acid. When casein is fed, the amino-acid mixture circulating to the liver will no longer exhibit the deficiency. This interpretation is compatible

with studies on amino acid mixtures reported in Section III of this thesis. These data show that the addition of the missing amino acid to an incomplete amino acid mixture results in a change in $^{G}/_{A}$ ratio in the same direction as that observed when casein is fed to the protein-depleted animals.

We must conclude, therefore, that feeding of protein to animals depleted of protein produces a decrease in the rate of RNA breakdown similar to that obtained by feeding protein to animals fasting after a protein-containing diet.

SECTION III.

THE EFFECT OF FEEDING COMPLETE AND INCOMPLETE AMINO-ACID MIXTURES ON THE UPTAKE OF LABELLED PRECURSORS INTO THE PURINE AND PYRIMIDINE BASES OF RAT LIVER.

INTRODUCTION.

Our data indicate that feeding of protein to animals fasting after either a protein-free or protein-containing diet causes a reduction in RNA breakdown, irrespective of the protein content of the preceding diet. As a logical sequel to the work in Sections I and II, we decided therefore to study the effect of feeding amino-acid mixtures to rats previously depleted of protein. A comparison was made between a complete amino-acid mixture and one deficient in one essential amino acid, on the supposition that a complete amino-acid mixture would stimulate protein synthesis and a deficient one would not. ^{14}C-2-Glycine and ^{14}C-6-orotic acid were the labelled precursors used in these studies and their uptakes were measured in two independent series of experiments, one for labelled glycine and the other for orotic acid. Thus ^{14}C-glycine was used to measure the uptake of ^{14}C into protein and purines of RNA and the acid-soluble fraction and ^{14}C-orotic acid was used to follow the uptake of ^{14}C into RNA pyrimidines only. Owing to the much larger isotope uptakes obtained after injection of ^{14}C-glycine into protein-depleted rats (see Section II), the animals used in the initial experiments were first fed on a protein-deficient diet.

EXPERIMENTAL.

Animals and Diets. Young male albino rats weighing 180-200 g. in the fasting state were used. The rats were housed individually in metabolism cages and maintained under thermostatic conditions. The rats were fed for 7 days on a protein-free diet as described in Section II. In a few later experiments, rats were also fed during the preparatory period on the protein-containing diet (Section I).

Isotope Administration. The rats received their last meal at 4 p.m. on the 7th day of the diet and on the following morning they were injected into the thigh muscles with either ^{14}C-2-glycine (10 μC/100 g. body wt.) or ^{14}C-6-orotic acid (in the first experiment the dose level was 1 μC/100 g. body wt. and in subsequent experiments this was increased to 2μC/100 g. body wt. However, the final activities in these later experiments were calculated to a standard dose of 1 μC/100 g. final body wt.). A few minutes prior to isotope injection, each rat was fed an amino-acid mixture by stomach tube. The animals were killed at 1½, 3 and 6 hr. after injection of isotope.

Amino-Acid Mixtures. These mixtures were based on the mixture of essential amino acids recommended by Rose, Oesteiling and Womack (1948) for maintenance of growth of rats except that

Table 27.

Composition of complete amino-acid mixture administered to rats.

	Amount per rat	Relative proportions of essential amino acids for rats*
L-Valine	100 mg.	83
L-Leucine	120 mg.	100
L-Isoleucine	80 mg.	67
L-Cystine	20 mg.	83
L-Methionine	80 mg.	
L-Threonine	70 mg.	58
L-Phenylalanine	120 mg.	150
L-Tyrosine	60 mg.	
L-Lysine.HCl	25 mg.	100†
L-Histidine.HCl	95 mg.	58†
L-Arginine.HCl	50 mg.	33†
L-Alanine	20 mg.	-
L-Proline	20 mg.	-
L-Aspartic acid	20 mg.	-
L-Glutamic acid	200 mg.	-
L-Tryptophan	50 mg.	33
Sod. bicarbonate	127 mg.	-

*After Rose et al. (1948)

†As free amino acid.

glycine was omitted to prevent dilution of ^{14}C-glycine. Table 27 shows the contents of this amino-acid mixture and the amounts of each fed to one rat. This table also gives the mixture of Rose et al. for comparison. It might be pointed out that 2.5 g. casein, i.e., the amount of protein fed in previous experiments, is equivalent to 1.4 g. of the ten essential amino acids plus L-tyrosine and L-cystine. By comparison, 1.1 g. of essential amino acids was supplied to each rat in these experiments. The procedure for preparing this mixture for use was to mix the total amino acid requirement for the whole experiment, omitting L-tryptophan, with some distilled water, heat gently until all the amino acids were suspended in solution, cool, add the sodium bicarbonate and make up to a known volume (the rat received its requirement of amino acids in 5 ml. of solution). The solution was halved, one half receiving the L-tryptophan. Thus we have two mixtures, one containing all the essential amino acids, the other being an identical mixture minus tryptophan. The deficient amino-acid mixture was tested for the presence of traces of tryptophan, which might occur as impurities in other amino acids, by paper chromatography and microbiological assay, and yielded negative results.

Liver Analysis.

(a) Experiments with ^{14}C-2-glycine. The livers of the

animals were analysed by the methods described in Section I for total amounts of protein N, specific activity of free glycine, uptake of ^{14}C-glycine into proteins and purines of RNA and acid-soluble fraction.

(b) Experiments with ^{14}C-6-orotic acid. The liver was removed and RNA isolated by the modified procedure of Schmidt and Thannhauser (1945) as described in Section I. It was not necessary to carry out the full purification of RNA free from protein using sodium dodecyl sulphate, as described in Section I, since the ^{14}C of orotic acid is not incorporated into liver protein (Hurlbert & Potter, 1952). Accordingly, the following procedure was adopted after lipid extraction. The lipid-extracted powder was suspended in 30 ml. M-NaCl and 3 ml. 5% S.D.D.S., and heated in a boiling water-bath for 10 minutes with stirring. The supernatant was filtered and the residue again extracted. The nucleic acids were precipitated from the combined extracts by the addition of 2 volumes of ethanol and allowed to stand for several hours at 0°. The precipitate was spun down and washed with 66% ethanol (twice), absolute ethanol and ether. The dry residue was suspended in 0.3 ml. 0.5 N KOH and incubated at 37° for 18 hr. The digest was cooled in ice and the pH adjusted to 1 with a drop of ice-cold 60% ($^{w}/_{v}$) PCA. The precipitated $KClO_4$ was removed by centrifugation at 0° and washed twice

with small amounts of water and supernatant and the washings pooled.

The specific activities of the pyrimidine bases were measured by two independent procedures. The solution of RNA was divided into two; one half was evaporated to dryness and the RNA nucleotides digested to the constituent bases by heating with 0.1 ml. 72% PCA for 1 hr. at 100° with occasional stirring (Wyatt, 1951). The digest was cooled in ice and ice-cold 5 N KOH added to bring the pH to between 5 and 7. The $KClO_4$ and carbon were separated by centrifugation and the adenine, guanine, cytosine and uracil were separated by two-dimensional chromatography on Whatman No.1 using isopropanol/HCl/water (65:16.5:18.5) (Wyatt, 1951) and n-butanol/water (86:14). The chromatograms were developed in the same direction with absolute ethanol, which was found to remove ultra-violet-absorbing contaminants with little movement of the bases.

The other half of the solution was made N with respect to HCl and heated at 100° for 1 hr., resulting in a mixture of purine bases and pyrimidine nucleotides (Markham & Smith, 1949). This mixture was separated by two dimensional chromatography on Whatman No.1 paper, using the same solvent systems described above.

The positions of the pyrimidine bases or nucleotides separated by either method were detected by ultra-violet ab-

sorption and these areas cut out and eluted by shaking in a stoppered test-tube with 4 ml. 0.1 N HCl and incubating at 37° for 18 hr. Tubes were again shaken, paper centrifuged down and supernatant removed and evaporated to dryness. The dry residue was dissolved in 0.3 ml. HCl (cytosine and uracil, 0.1 N; cytidylic and uridylic acid, 0.01 N). 0.1 ml. was plated out on a metal planchette using an 'Agla' micrometer syringe and dried under infra-red lamps. 0.1 ml. was taken for ultra-violet absorption. Blank areas of the same chromatograms, corresponding in size to the area occupied by the base, were also prepared in the same way for correction of ultra-violet absorption. However, these were not evaporated to dryness. The procedure described by Crosbie, Smellie and Davidson (1953) for the determination of the amount of base or nucleotide present by ultra-violet absorption measurements was employed. The final specific activities (expressed as counts/min./μ mole) by the two different methods of isolation showed an agreement to within ±10% in all cases (Table 29). All samples were counted at infinite thinness.

Table 28.

The effect of feeding amino-acid mixtures either containing L-tryptophan or deficient in this amino acid to rats previously depleted of protein on the uptake of ^{14}C-2-glycine by protein and purines of acid-soluble fraction and RNA of the liver. Each rat received 10 μC/100 g. body wt.

Time after ^{14}C-2-glycine injected	Amino-acid mixture fed	S.A. of free glycine	Protein		R.S.A. of acid soluble bases		R.S.A. of RNA purines		Ratio of uptake of ^{14}C-glycine guanine/adenine	
			Amount per 100 g. initial body wt.	Total relative activity	Adenine (glycine)	Guanine (glycine)	Adenine (glycine)	Guanine (glycine)	Acid-soluble bases	RNA bases
hr.		cpm./100 μg.	mg.							
1½	Incomplete	3430	51.6	29.4	8.5	19.7	0.5	1.9	2.32	3.57
	Complete	9460*	56.0	45.0	16.1	(lost)	1.4	4.1	-	2.92
3	Incomplete	2610	50.9	55.0	23.0	53.3	2.7	6.2	1.45	2.32
	Complete	2100	54.5	79.0	29.8	31.3	2.8	4.8	1.05	1.71
6	Incubation	1135	57.5	210.0	74.3	95.1	16.2	31.5	1.28	1.95
	Complete	1055	69.0	218.0	110.0	88.1	26.7	35.8	0.80	1.34

*Large dose of isotope accidentally administered.

X INCOMPLETE

RESULTS AND DISCUSSION.

In this section the two amino-acid mixtures used (Table 27) differ only in that one contains tryptophan and is nutritionally complete, whereas the other is deficient in this amino acid. In the following discussion the former mixture will be referred to as the "complete" amino-acid mixture and the latter as the "incomplete" amino-acid mixture.

Table 28 compares the effect of feeding the complete and incomplete amino-acid mixtures to rats fasting after a protein-free diet, on the uptake of ^{14}C-glycine into proteins and the purines of RNA and the acid-soluble fraction of liver at various time intervals after injection. This comparison shows that the addition of tryptophan to the amino-acid mixture caused a general increased synthesis of protein, as evidenced by a rise in both protein N and total relative activity. This increase in protein synthesis is accompanied by an increased uptake of ^{14}C-glycine into the adenine of the acid-soluble fraction. On the other hand, the addition of tryptophan to the mixture does not appear to increase ^{14}C-glycine uptake into the acid-soluble guanine. As a result, the guanine/adenine (G/A) ratio (Table 28) falls when the amino-acid mixture is completed by addition of tryptophan. This change in G/A ratio is confirmed when we turn to the ^{14}C-glycine uptake by the bases of RNA. Once more, the ratio

Table 29.

The effect of feeding a mixture of essential amino acids either containing tryptophan or deficient in tryptophan on the uptake of ^{14}C-6-orotic acid into the pyrimidines of liver RNA of rats fasting after a protein-deficient diet. The S.As. of both base and nucleotide are given and the data represent the mean of 3 replications of the experiment.

Time after ^{14}C-orotic acid injection	Amino-acid mixture fed	Specific activity (c.p.m. per μ Mde.) of base standardized to a dose of 1 μc/100 g. final body wt.						U/C *
		Cytosine	Cytidylic Acid	Mean	Uracil	Uridylic Acid	Mean	
hr.								
1½	Incomplete	165	164	165	1049	980	1015	6.2
	Complete	202	224	213	1930	997	1014	4.8
3	Incomplete	324	351	338	1078	1070	1074	3.2
	Complete	429	417	423	1361	1265	1313	3.1
6	Incomplete	541	564	553	1386	1449	1418	2.6
	Complete	949	908	929	1670	1764	1717	1.9

*This ratio (U/C) represents the ratio of the mean of specific activities of uracil and uridylic acid to the mean of the specific activities of cytosine and cytidylic acid.

falls with the nutritionally complete mixture.

It will be remembered that, in Section II, a fall in G/A ratio occurred when casein was administered to protein-depleted animals receiving ^{14}C-glycine. This was attributed to the deficiency of methionine in the free amino-acid pool of such animals and the effect of casein was considered to be due to relief of this deficiency. The present data, indicating a fall in G/A ratio with complete amino-acid mixtures, is wholly compatible with this interpretation of the experiments in Section II.

We thought it would be of interest to make a similar study of the pyrimidines of RNA, using ^{14}C-6-orotic acid as a labelled precursor. As previously described in the Experimental of this section, we measured the uptake of ^{14}C-orotic acid into RNA pyrimidines by determining the activity of both pyrimidine nucleotide and base, to ensure that the method of isolation was effective. Table 29 contains the data obtained from these experiments and are the mean of 3 replications of the entire experiment. It can be seen that the two different procedures give results which do not vary by more than $\pm$10%. Since some of the radioactivities in the bases were low, this degree of agreement is reasonable. Although the complete amino-acid mixture produces an increased incorporation of ^{14}C-orotic acid into cytosine (i.e., average of S.A. of base

Table 30.

The effect of feeding amino acid mixtures containing L-tryptophan or deficient in this amino acid to rats on the uptake of ^{14}C-glycine into guanine and adenine and uptake of ^{14}C-orotic acid into uracil and cytosine of liver RNA.

Time after isotope injection	Ratio of $\frac{\text{S.A. adenine}}{\text{S.A. guanine}}$ $\left(A/G\right)$			Ratio of $\frac{\text{S.A. cytosine}}{\text{S.A. uracil}}$ $\left(C/U\right)$		
	Complete Mixture	Incomplete Mixture	Difference %	Complete Mixture	Incomplete Mixture	Difference %
hr.						
1½	0.34	0.28	-18	0.21	0.16	-24
3	0.59	0.43	-27	0.32	0.31	-2
6	0.75	0.52	-31	0.55	0.39	-26
Mean	-	-	-25	-	-	-17

Statistical analysis:

C/U ratio:- The addition of tryptophan to the amino-acid mixture increases the ratio C/U by an average of 17% over the three time intervals. This is significant ($t = 3.00$, $P = 0.02 - 0.01$).

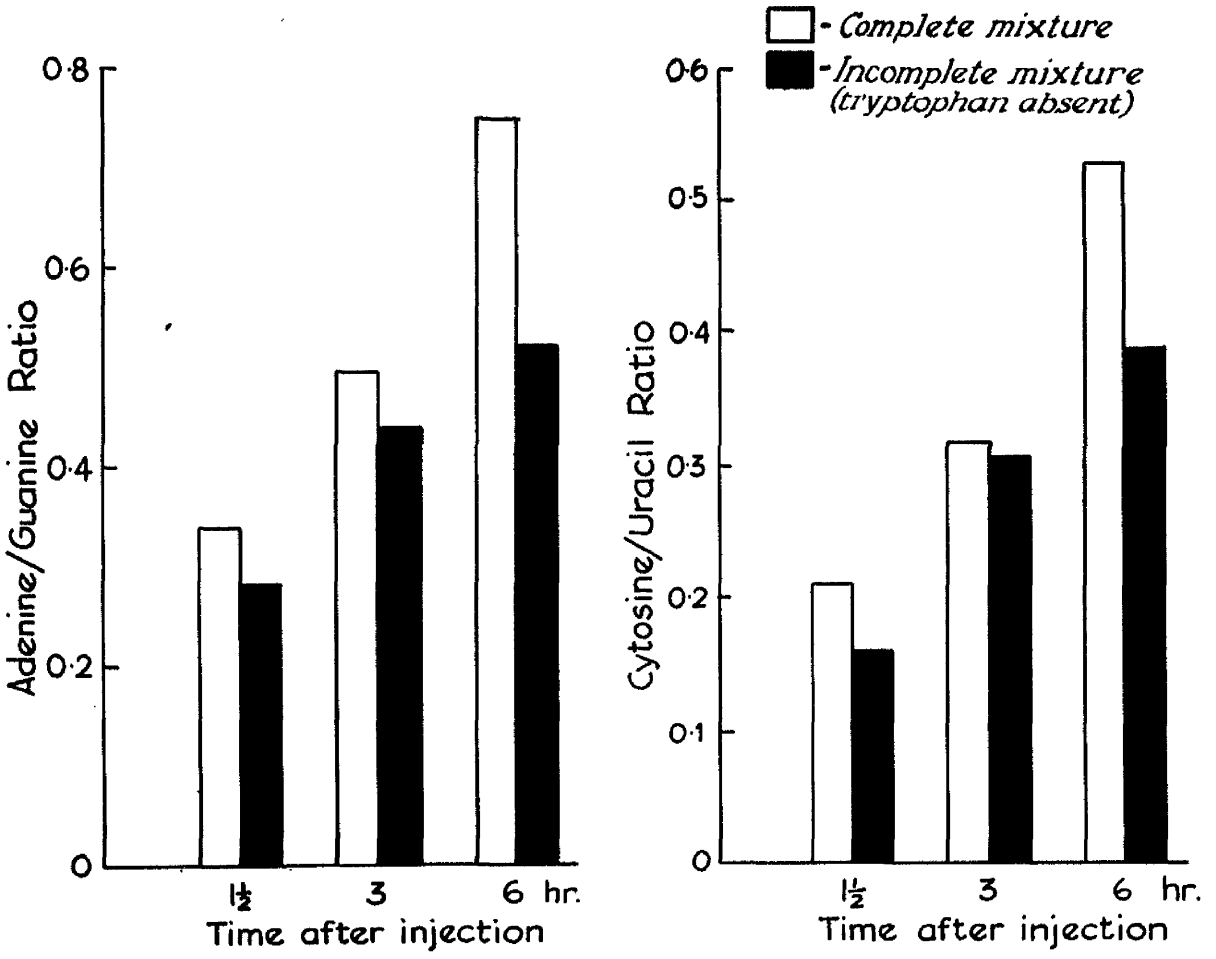
A/G ratio:- The addition of tryptophan to the amino-acid mixture increases the ratio A/G by an average of 25% over the three time intervals. This is significant ($t = 6.37$, $P = 0.05 - 0.02$).

and nucleotide) the activity of uracil (average of S.A. of base and nucleotide) remains relatively unaffected. In saying this, we must assume that the concentration of labelled orotic acid in the liver is essentially similar for animals fed on deficient and on complete amino-acid mixtures. Thus the overall effect of the complete amino-acid mixture is to produce a fall in the ratio of the uptake of ^{14}C into uracil relative to the uptake of ^{14}C into cytosine ($^{U}/_{C}$ ratio). However it must be noted that for some, at present, obscure reason this fall in the $^{U}/_{C}$ ratio is less evident at 3 hr.

We have, therefore, obtained a change in uptake of labelled precursors into adenine relative to guanine, and into cytosine relative to uracil with addition of a single amino acid to the nutriment. In Table 30 are gathered all the relevant data, together with statistical analysis of the significance of the changes. Since the changes appear to be due primarily to alterations in the labelling of adenine and of cytosine rather than of guanine and of uracil, we have chosen to reverse the ratios, giving them in Table 30 and Fig.12 as $^{A}/_{G}$ and as $^{C}/_{U}$, guanine and uracil being regarded as the reference bases. It is apparent that the decrease in the two ratios produced by omitting tryptophan is of similar magnitude for both ratios, although at the 3 hr. interval the fall in the $^{C}/_{U}$ ratio is not so marked as the

Fig.12.

Effect of Complete and Incomplete Amino Acid Mixtures on the Uptake of ^{14}C-Glycine into Guanine and Adenine and of ^{14}C-Orotic Acid into Uracil and Cytosine of Rat Liver Ribonucleic Acid.



change in the $^{A}/_{G}$ ratio. Statistical analysis (Table 30) shows that both ratios are affected significantly by omitting tryptophan from the amino-acid mixture. Fig.12 illustrates graphically the changes produced in the ratios by feeding the two amino-acid mixtures.

Since it appeared that it was the activities of the 6-amino bases (adenine and cytosine) which were increasing when the complete amino-acid mixture was fed, the possibility that these results could be explained in terms of the effect of previous protein depletion on the enzymic systems responsible for the amination step in the formation of those bases must be considered. While the activities of most liver enzymes have been shown to decrease during protein depletion either in proportion to the loss of liver protein or to a greater extent (Rosenthal, Rogers, Vars & Ferguson, 1950; Bargoni, 1950; Meikleham, Wells, Richert & Westerfeld, 1951), the activities of some enzymes remain unaltered, e.g., dipeptidase activity (Bargoni, 1950) and an actual increase in the case of alkaline phosphatase activity has been reported by Rosenthal, Fahl and Vars (1952). Thus it would appear that protein depletion might produce marked alterations in the metabolic patterns of the liver cell. While we have no specific information on the effect of protein depletion on the purine and pyrimidine synthesising systems, Abrams and Bentley (1955a, b) have shown that the biosynthesis of adenylic

Table 31.

The effect of feeding complete and incomplete amino-acid mixtures on the incorporation of ^{14}C-2-glycine into the purines of liver RNA of rats fasting after either a protein-free or a protein-containing diet $1\frac{1}{2}$ hr. after isotope injection. Data are the mean of 4 replications of the experiment.

Diet	Amino-acid Mixture	S.A. of free glycine c.p.m./100 µg glycine	R.S.A. of RNA purines		
			Adenine	Guanine	G/A
Low Protein	Incomplete	3785	1.16	2.25	2.08
	Complete	3448	1.99	3.53	1.81
High Protein	Incomplete	2199	1.15	0.56	0.53
	Complete	2538	1.00	0.55	0.60

Table 32.

A comparison of the effects of feeding mixtures of essential amino acids either containing L-tryptophan or deficient in this amino acid on the uptake of ^{14}C-6-orotic acid into RNA pyrimidines of livers of rats fasting after either a diet free from protein or containing protein - 1½ hr. after isotope injection.

Previous Diet	Amino-acid mixture fed	S.A. of RNA pyrimidines (counts/min./μ Mole) standardized to a dose of 1 μc./100 g. body wt.		S.A. Uracil / S.A. Cytosine
		Cytosine	Uracil	
Low Protein	Incomplete	354	1194	3.38
	Complete	374	1068	2.86
High Protein	Incomplete	236	900	3.82
	Complete	168	670	4.00

and guanylic acids from inosinic acid occurs by two different pathways in bone-marrow preparations. Thus there is a possibility that protein depletion may affect one pathway to a greater extent than the other.

For this reason we decided to examine the effect of feeding the different amino-acid mixtures to rats fasting after a high-protein diet, on the assumption that such a short period of fasting would not produce any marked changes in enzymic activities. The data in Table 31, which are the means of 4 replications, indicate that the complete amino-acid mixture fed to rats fasting after a high-protein diet produces no decrease in the G/A ratio of RNA comparable to that of rats prefed a protein-free diet (cf. Table 28). The effect of feeding complete and incomplete amino-acid mixtures to rats receiving either a protein-free or a protein-containing diet on the U/G ratio is shown in Table 32. While there is again a fall in the ratio when the complete amino-acid mixture is fed to protein-depleted animals, feeding of the complete mixture produces no change in the ratio U/G when the animals are not protein-depleted. Since these figures are derived from only one experiment, we feel that they should not be considered as conclusive evidence that the U/G ratio changes found in the preceding experiments are due to depletion of the enzyme system leading to cytosine formation.

It is thus seen that the changes in $^{G}/_{A}$ ratios and $^{U}/_{C}$ ratios observed with protein-depleted rats have not been replicated with rats previously fed a normal protein intake. We are, however, not convinced that this points inevitably to an enzymic depletion, since animals fasted overnight after a protein-containing diet show such violent fluctuations in $^{G}/_{A}$ ratio when fed protein (cf. Table 16). On these grounds we feel that rigorous proof of the significance of our findings with incomplete amino-acid mixtures depends ultimately on assaying the activities of these enzymes under our various nutritional conditions.

It is thus perfectly possible that the giving of an incomplete amino-acid mixture can lead to abnormalities of protein synthesis accompanied by changes in the ratio of the specific activities of 6-amino to 6-keto bases ($^{A}/_{G}$ and $^{C}/_{U}$). Since we know, in the case of the purines, and must presume in the case of the pyrimidines, that these changes occur primarily in the precursor pools, we are faced with a choice of two possibilities. Either the synthesis of adenine and cytosine is hampered by the omission of one amino acid from the mixture - and one can only envisage this if the enzyme systems leading to these 6-amino bases are especially sensitive to tryptophan deficiency - or else the precursor pools of 6-amino bases and 6-keto bases are affected differently by dilution with RNA breakdown products when the amino acid

mixture is complete or incomplete. That is to say, an RNA rich in adenine and cytosine is sensitive to completeness or incompleteness of the available amino acids in circulation, whereas an RNA fraction rich in guanine and uracil responds to amino acids in bulk.

These results are of interest in view of some recent findings of Elson and Chargaff (1955). These workers found that the bases with 6-amino groups are approximately equal in number to those with 6-keto groups in RNA isolated from many sources. They postulate a structure for RNA involving this relationship and discuss its possible importance as a template for protein synthesis, pointing out that this relationship between the 6-amino and 6-keto bases of RNA is only found in preparations from which protein has not been removed. This suggests that the relationship of these two sets of bases may be associated with the function of RNA in protein synthesis.

SECTION IV.

THE ACTION OF β-2-THIENYLALANINE ON PROTEIN SYNTHESIS AND RNA TURNOVER IN RAT-LIVER SLICES AND CYTOPLASM.

INTRODUCTION.

In the previous section we saw that omission of a single essential amino acid from the mixture in some unknown way affects RNA metabolism. We thought the nature of this effect might become clearer if we attacked the problem from another angle, namely, to block protein synthesis by administering an amino acid analogue.

-2-Thienylalanine (β-2-TA) is known to cause cessation of growth when fed to rats, by preventing the utilisation of its structural analogue the essential amino acid, phenylalanine (Ferger & Du Vigneaud, 1949). We therefore chose this analogue as an inhibitor likely to suit our purpose.

These experiments were carried out primarily on rat-liver slices. The advantage of an in vitro system of this type is that it is dependent on the additions made to the system for its nutritional requirements, unlike the whole animal studies where the picture is complicated by lack of control of the conditions in the organ under study. Thus Simpson, Farber and Tarver (1950) found that injection of ethionine, supposedly a specific antagonist of methionine, caused inhibition of incorporation of radioactive methionine into liver protein but had a negligible effect on the incorporation of methionine into kidney protein. These workers point out that the major limitation in vivo studies is the uncertainty of the

antagonist/metabolite ratio existing in any one tissue over a period of time.

Accordingly we employed liver slices as our system for the study of the action of β-2-TA on protein and RNA synthesis in an attempt to establish whether any relationship could be demonstrated between protein synthesis and RNA metabolism.

The effect of β-2-TA on liver-slice metabolism was of a more general nature than anticipated and the possibility arose that an action of β-2-TA other than on protein synthesis might account for changes in RNA metabolism. Accordingly, we decided to study the action of β-2-TA on a cell-free system, namely, rat-liver cytoplasm, in an attempt to establish whether the action of β-2-TA on incorporation of radioactive amino acid was accompanied by changes in RNA metabolism.

EXPERIMENTAL.

Animals and Diet. Young male albino rats weighing 180-200 g. in the fasting state were used. The rats received no preparatory diet but were taken from stock and fasted overnight before killing.

Preparation of Liver Slices. The rats were killed by stunning and exsanguination and the livers removed, dried and chilled. The slices (0.3 mm. thickness) were prepared using the tissue-chopper described by McIlwain and Buddle (1953) and 400 mg. of slices, weighed on the torsion balance, were suspended in 4 ml. Krebs-Ringer bicarbonate, pH 7.4 (Umbreit, Burris & Stauffer, 1947) containing the various additions described below, in 25 ml. conical flasks.

Preparation of Cytoplasmic Fraction. The liver was removed, chilled, minced with scissors for 20 sec. and homogenised in ice-cold Krebs-Ringer bicarbonate (1 g. liver/2 ml. Krebs-Ringer) for 30 sec. using a Potter-Elvejhem (1936) glass homogeniser. The homogenate was centrifuged at 600 g. for 10 min. at 0° (M.S.E. Major Refrigerated Centrifuge) and the supernatant removed. 3 ml. of this cytoplasmic fraction were added to each 25 ml. conical flask. The final volume in each flask after the necessary additions was 5 ml.

Additions to Flasks. In all experiments ^{14}C-2-glycine (1 µc. per flask) was used to study the uptake of isotopes into the protein of the liver-slices and cytoplasm. In a few experiments ^{35}S-methionine (2 µc. per flask) was also used to establish effects on protein synthesis. ^{32}P (25 µc. per flask) was used to study the turnover of lipid P and the P of RNA nucleotides and ^{14}C-adenine (2 µc. per flask) to study the RNA-purine turnover. Each isotopic study was carried out independently on specimens from the same liver. β-2-Thienyl-alanine was added to some flasks (5 mg. per flask) and in certain cases L-phenylalanine (5 mg. per flask) was added in conjunction with β-2-TA. In the experiments using rat-liver cytoplasm 3-phosphoglyceric acid (50 µ M per flask) and ATP (5 µM per flask) were added as a source of energy in certain cases. Both reagents (supplied by Light & Co.Ltd.) were converted to the potassium salt before addition.

Incubation Procedure. When all the necessary additions had been made, the flasks were gassed with a mixture of 95% O_2/CO_2 and securely stoppered. They were incubated at 37° for 3 hr. with constant shaking. (120 strokes per min.). At the end of the incubation period the reaction was stopped by the addition of 0.5 vol. ice-cold 30% ($^{W}/_{V}$) TCA. Zero time controls were prepared for each isotopic study by the addition of 0.5 vol. ice-cold 30% TCA immediately following all the additions to the flask. As far as was possible, these in-

cubation experiments were carried out under aseptic conditions. The extent of bacterial growth during the incubation period was estimated by plating out a sample of the incubated material on agar and incubating this plate for 24 hr. at 37°. Bacterial contamination was shown to be negligible. After addition of 30% TCA to the slices the precipitate was homogenised in the cold in a Nelco Blendor for 2 min.

Preparation of Protein for Counting. The precipitated protein was washed twice with 10 ml. portions of ice-cold 5% ($^{W}/_{V}$) TCA. The nucleic acids were not removed from these preparations because it was thought that the incorporation of ^{14}C-glycine into the nucleic acids would be negligible in 3 hr. The residue was extracted with 10 ml. portions of the lipid solvents, twice with absolute ethanol, once with ethanol: chloroform (3:1), twice with ethanol:ether (3:1) and once with ether. The residue was dried in air and submitted to performic acid treatment. This is used for the removal of methyl mercaptan (CH_3SH), a breakdown product of ^{35}S-methionine, linked with the sulphydryl groups of protein by a disulphide linkage (Greenberg, 1950). However, the protein labelled with ^{14}C-glycine was also submitted to this treatment, when it was found that the contamination of protein by free ^{14}C-glycine in the zero time specimen could be reduced to negligible counts by this treatment. The protein was dissolved in 98% ($^{W}/_{V}$) formic acid (1 ml./15 mg. protein) and 30 vols. hydrogen per-

oxide (0.2 ml./15 mg. protein), allowed to stand for 30 min. at room temperature. 10 volumes 5% ($^{w}/_{v}$) TCA were added and the precipitate centrifuged, washed twice with 5% TCA and twice with 20 ml. absolute ethanol and once with 20 ml. ether. The precipitate was dried in air and then ground to a fine powder in an agate morter. Samples were counted at infinite thickness and the results expressed as counts per min. at infinite thickness for a standard area of planchette.

^{32}P Determinations. Inorganic P, lipid P and RNAP (as individual nucleotides) were isolated as described in Section I and the specific activities determined.

^{14}C-Adenine Determinations. The RNA nucleotides were isolated by ionophoresis as described in Section I. Adenylic and guanylic acids were eluted from the paper with distilled water, eluates evaporated to dryness and the residue taken up in a small volume of water. An aliquot was plated out on a metal planchette and counted and an aliquot of the same volume used for P estimation by the method of Allen (1940) (Section I). The results were expressed as counts per min. per 100 µg. P. The activities obtained for ^{14}C-adenine incorporation into RNA are somewhat crude, since the presence of citrate in the eluate from the ionophoresis paper contributed an uncorrected thickness factor to the counts. However, the changes produced by β-2-TA are of such magnitude that this effect can be discounted.

Table 33.

The effect of adding β-2-TA to rat-liver slices on the uptake of ^{14}C-2-glycine and ^{35}S-methionine by protein (1 μc. ^{14}C-2-glycine and 2 μc. ^{35}S-methionine added per flask). The protein was treated with performic acid before radio-activity determined. Incubation carried out at 37° for 3 hrs.

Flask	Specific activity of Protein counts/min. at infinite thickness	
	^{14}C-2-glycine	^{35}S-methionine
Control	149	591
β-2-TA (5 mg.)	57	217
Zero time control	3	51
%age inhibition by β-2-TA	63	69

Table 34.

The effect of the addition of β-2-TA and of a mixture of β-2-TA and L-phenylalanine to liver slices on the uptake of ^{14}C-glycine (1 μc. per flask) by protein and the uptake of ^{14}C-adenine (2 μc. per flask) by RNA and the uptake of ^{32}P (25 μc. per flask) by phospholipid and RNA. Incubations carried out at 37° for 3 hr.

Flask	S.A. of protein (after treatment with performic acid) counts per min. at infinite thickness	S.A. of inorganic P counts/min./100 μg P	R.S.A. of RNA		R.S.A. of Lipid P
			^{32}P	^{14}C-adenine	
Zero-time control	4	899,000	0.01	8	0.02
Control	539	587,000	0.37	38	1.80
β-2-TA (5 mg.)	242	550,000	0.15	8	1.20
β-2-TA (5 mg.) + L-phenylalanine (5 mg.)	445	617,000	0.24	21	1.90
%ge inhibition by β-2-TA	55	-	49	100	33

RESULTS AND DISCUSSION.

The uptake of labelled amino acids by liver slices over a 3 hr. period of incubation is illustrated in Table 33. This shows that β-2-TA inhibits the uptake not only of ^{14}C-2-glycine, but also of ^{35}S-methionine in this system. The latter finding indicates clearly that changes in ^{14}C-glycine uptake into protein described in later tables of this section are not attributable to the presence of nucleic acids in the protein preparation, since changes in the uptake of ^{35}S-methionine cannot be accounted for as due to altered nucleic acid metabolism.

Table 34 contains the results obtained with rat-liver slices, and is typical of several such experiments carried out. It is apparent that β-2-TA inhibited the uptake of ^{14}C-glycine into protein and of ^{32}P and ^{14}C-adenine into RNA to a marked extent, and that the presence of phenylalanine in equal concentration to β-2-TA prevented the inhibitory action of the latter to a considerable extent. Lipid P turnover is also inhibited by β-2-TA and the inhibitory effect is again abolished by concurrent addition of phenylalanine to the medium. Obviously inhibitory effects are not confined to protein and nucleic acid but also occur with lipid P. It is thus clear that β-2-TA has a more widespread effect on cellular metabolism than anticipated. Rabinovitz, Olsen and Greenberg (1954) have

shown that even the action of β-2-TA on the uptake of amino acids by the protein of Ehrlich ascites carcinoma cells is a complicated one, since there is evidence that it interferes not only with the actual incorporation of amino acids by the protein but also with their assimilation into the cells. A likely way in which this could occur would be through interference with energy production or utilization. A hint of such an effect in our experiments is that the slices in contact with β-2-TA, unlike the others, retained a pink colour after TCA precipitation, suggesting interference with a respiratory pigment. This suggestion is further strengthened by the findings of Gifford, Robertson and Syverton (1954), who noted that β-2-TA caused inhibition of respiration of Hela-cell cultures over a 72 hr. period of incubation. When these cells were infected with Type I poliomyelitis virus, β-2-TA inhibited the synthesis of this virus only at concentrations which were inhibitory to the respiration of the host cell.

In order to eliminate this irrelevant action of β-2-TA and to study its effect on RNA metabolism in relation to changes in protein synthesis alone, we decided to use a cell-free system, namely, liver cytoplasm, in which 3-phosphoglyceric acid (3-PGA) and ATP are present as energy donors, thus making the system independent of the need for continuing respiration (cf. Zamecnik & Keller, 1954).

Table 35.

A comparison of the effect of β-2-TA on the uptake of isotopes by the protein RNA and phospholipids of rat-liver slices and cytoplasm. The effect of the addition of 3-PGA and ATP to cytoplasm is also shown. Incubations carried out at 37° for 3 hr.

System	Addition to system	S.A. of protein (after treatment with performic acid) counts/min. at infinite thickness	S.A. of Inorganic P counts per min. per 100 µg. P.	R.S.A. of RNA		R.S.A. of Lipid P
				^{32}P	^{14}C-adenine	
Liver Slices	nil (zero-time control)	(2)	(1,476,860)	(0.00)	(0)	(0.01)
	nil	797	914,680	0.28	213	1.94
	β-2-TA (5mg.)	183	1,072,350	0.13	115	1.07
	Inhibition by β-2-TA	77%		53%	46%	45%
Liver Cytoplasm	nil (zero-time control)	(0)	(1,752,000)	(0.000)	(1.64)	(0.04)
	nil	10.3	849,000	0.014	0	0.18
	3-PGA (50 µ mole) & ATP (5 µ mole)	19.0	227,400	0.003	0	0.85
	β-2-TA (5 mg.) 3-PGA (50 µ mole) & ATP (5 µ mole)	15.7	177,750	0.002	0	1.10
	Inhibition by β-2-TA	17%	-	-	-	0%

Table 35 illustrates the effect of β-2-TA on protein, RNA and lipid P metabolism of liver slices and of liver cytoplasm. The results obtained with the liver slices confirm our findings in Table 34, namely, that β-2-TA inhibits the uptake of isotopes into protein, RNA and phospholipid. In the cell-free system (cytoplasm) we were able to demonstrate uptake of ^{14}C-glycine by the protein and this process was stimulated by the addition of 3-PGA and ATP. Furthermore, addition of β-2-TA to the system inhibited the uptake of glycine into protein. The uptake of ^{32}P by lipid P was also augmented on addition of 3-PGA and ATP to the system, but β-2-TA failed to affect this process. This suggests that we are correct in interpreting the action of β-2-TA on the metabolism of phospholipid in the intact cell as a general one involving supply of energy for synthesis. In the intact cell, β-2-TA will reduce ^{32}P uptake by such interference, whereas in the cell-free system 3-PGA and ATP eliminate the necessity for an intact respiratory mechanism. The crucial question is, of course, will the effect of β-2-TA on RNA metabolism observed with liver slices also be abolished in the cell-free system for the same reason? Unfortunately, incorporation of ^{32}P and of ^{14}C into RNA is negligible in our cytoplasmic system, in spite of the presence of 3-PGA and ATP, and numerous attempts to devise an active system have been made by us

with complete lack of success. These negative experiments need not be laboured here.

Thus we do not know whether the inhibition of isotope uptake by RNA in intact slices, when β-2-TA is added to the medium, is connected with changes in protein synthesis or with some other action of this inhibitor. Consequently, until a better cell-free system is evolved for concurrent investigation of protein and RNA turnover, this question cannot be settled.

SECTION V.

STUDIES ON RNA BREAKDOWN IN ISOLATED MICROSOMES.

INTRODUCTION.

In Section I of this thesis we found that fasting after a protein-containing diet was accompanied by a breakdown of RNA in the liver and we obtained evidence to suggest that feeding protein, that is, ensuring an ample supply of dietary amino acids to such animals, caused a cessation of this breakdown. If any attempt is to be made to elucidate the mechanism of this action of dietary protein on RNA stability, it is apparent that a simpler system than the one provided by the whole animal must be devised. The *in vitro* breakdown of RNA was therefore explored. We know from the findings of Wikramanayake et al. (1953) that, when rats are fasted after a protein-containing diet, the reduction in RNA content of the liver occurs mainly in the microsomal fraction. Accordingly we decided that this fraction would be the most sensitive to changes in amino acid supply and we have therefore used a microsomal preparation as our *in vitro* system for studying RNA breakdown.

Our intention in this series of investigations was to examine the effect of previous diet on RNA breakdown in microsomes maintained *in vitro* over a period of a few hours. Breakdown was estimated from the amount of RNA disappearing from the system. As an additional measure of the breakdown of RNA in the system, we used the isotope dilution technique.

We incubated the microsomes in a medium containing ^{14}C-adenine and investigated any dilution effect produced on this isotope by RNA breakdown liberating unlabelled adenine compounds into the medium. However, this work is in the early stages of development and this section contains only a few preliminary experiments involving the use of this *in vitro* system.

EXPERIMENTAL.

Animals and Diet. Young male albino rats weighing 180-200 g. in the fasted state were used in all experiments. The diets administered to the rats were identical with those described in Section I, namely, either protein-containing or protein-free. Throughout the dietary period the animals were fed at a single intermediary energy level, i.e., 1200 cal./sq.m./day for four days. Some of the animals fasting after the protein-containing diet were fed casein 1 hr. prior to isotope injection. Thus the animals were in one of three nutritional states at time of killing, namely, fasting after a protein-free diet, fasting after a protein-containing diet and actively absorbing after a protein-containing diet.

Isolation of microsomal fraction and incubation procedure. Animals in each group were killed by exsanguination under ether anaesthesia, livers perfused with 0.25 M-sucrose (ice-cold), removed, pooled and minced with scissors. This mince was then homogenised in ice-cold 0.25 M-sucrose (1 g./10 ml. 0.25 M-sucrose) for 30 seconds using a Potter-Elvehjem (1936) homogeniser. The homogenate was submitted to differential centrifugation as described in Section I (Fig.3) and the microsomal fraction isolated. The isolated microsomes were homogenised in ice-cold 0.25 M-sucrose (8 ml.) for 45 secs.

and aliquots (3 ml.) placed in 25 ml. conical flasks containing 1.655 μM ^{14}C-8-adenine (2 μc.) in 1 ml. 0.25 M-sucrose solution.

Contents of flasks were gassed with a mixture of 95% O_2 and 5% CO_2. The flasks were stoppered and incubated at 37° for 2 hr. with shaking (120 strokes/min.). At the end of the incubation period the reaction was stopped by the addition of 0.5 volume ice-cold 2.1 N PCA. The precipitated protein and nucleic acids were separated as quickly as possible from the acid supernatant by centrifuging at 3,000 r.p.m. for 5 min. at 0° (M.S.E. Major refrigerated centrifuge) and the residue was washed twice with 1 ml. portions 0.7 N PCA. The supernatant and washings were filtered and combined. Zero time controls were carried out with each dietary group and this was done by precipitating the contents of the flask with 0.5 vol. ice-cold 2.1 N PCA after the addition of the microsomes and ^{14}C-adenine and separating the precipitated proteins and nucleic acids as described above.

Total RNA estimations. In several experiments the total RNA content of the flasks was estimated. The precipitated proteins and nucleic acids after the last washing with 0.7 N PCA were submitted to a lipid extraction by washing with 5 ml. portions of the lipid solvents as described in Section I. The dry residue was digested in N. NaOH (1.0 ml.) for 18 hr. at 37° and the digest made up to a suitable volume (25 ml.)

with water and duplicate aliquots taken for ribose estimation by the orcinol method (Kerr & Seraidarian, 1945). Orcinol reagent is prepared fresh daily by dissolving orcinol in a solution of 0.02% ($^{w}/_{v}$) $FeCl_3.6H_2O$ in concentrated HCl (6 mg. orcinol per ml. of $FeCl_3$ solution). A known volume of digest was added to a clean tube and the volume adjusted to 3 ml. with water. 3 ml. of the orcinol reagent were added and the tubes heated in a boiling water-bath for 30 min. The tubes were removed from the bath and cooled for a few minutes in a water-bath at 20° and the optical densities measured in the S.P. 600 at 665 mμ. Reagent blanks and standard ribose solutions were estimated with each series of tubes.

Isolation of acid-soluble adenine. The filtered supernatant fluid and washings were adjusted to pH 7 with ice-cold 5 N KOH. The precipitated $KClO_4$ was centrifuged down and washed twice with a small volume of water. The supernatant fluid and washings were pooled and evaporated to dryness at 100° on a steam bath. The residue was taken up in 1.0 ml. of 72% PCA and digested for 1 hr. at 100° in a stoppered tube. This converts all adenine-containing compounds to free adenine. The digest was cooled in ice and the pH adjusted to 7 with ice-cold 5 N KOH and the precipitated carbon and $KClO_4$ centrifuged down and the supernatant fluid removed. The residue

was washed twice with a small volume of water. The pooled supernatant fluid and washings were evaporated to a smaller volume *in vacuo*. This material was applied to Whatman No.1 chromatography paper and submitted to two-dimensional chromatography as described in Section III. Adenine was detected on the chromatograms in ultra-violet light, eluted and the S.A. determined as described in Section III. The activity of adenine is expressed as counts per min./μ mole adenine.

In the first two experiments carried out under these conditions, the amount of adenine isolated was very small. Since 1.655 μ moles of adenine were added to the medium as radio-active adenine and this alone should provide sufficient adenine for isolation by this procedure, it was realised that the adenine in the medium was being largely lost in the course of isolation. A possible reason for this loss was by adsorption either onto the microsomes in the course of PCA precipitation or onto the large amounts of carbon formed during PCA digestion from the sucrose present from the homogenising medium. The former possibility was eliminated when counting of the precipitated protein and nucleic acids of the microsomes after the removal of the supernatant fluid and washings indicated that this precipitate gave a negligible count. We have concluded therefore that our main source of loss was for the latter reason, i.e., by adsorption onto the carbon formed during PCA digestion. At the present time we

have devised no method of circumventing this difficulty, and its effect will be apparent when the results are considered.

RESULTS AND DISCUSSION.

Table 36 contains the results obtained when we studied the breakdown of RNA in rat-liver microsomes *in vitro* at intervals over an 8 hr. period of incubation when the rats had been in one of three nutritional conditions, namely, fasting after a protein-free diet, fasting after a protein-containing diet and actively absorbing amino acids from the gut. RNA was measured as ribose by the orcinol reaction. It is suggestive that the RNA breakdown is greatest in the microsomes of those animals which were fasting after a protein-containing diet and that the feeding of protein to such animals was accompanied by a decrease in this breakdown. This finding is in keeping with the results obtained in Section I with whole animal investigations, in which the evidence suggested cessation of breakdown on feeding protein. It is obvious from Table 36 that this method of estimating RNA breakdown *in vitro* is somewhat insensitive for the small changes which occur. For this reason we decided to adopt an isotope dilution technique, i.e., we measured the dilution of ^{14}C-adenine in the medium by breakdown products of RNA. This method ought to be a very sensitive one, given suitable conditions. The principle is to suspend the microsomes in a medium containing ^{14}C-adenine and then to recover the total acid-soluble adenine from the medium. We know from Section IV that labelled adenine is not incorporated into the RNA of cell-free preparations.

Table 36.

The effect of various nutritional conditions on the breakdown of RNA in rat liver microsomes *in vitro* over a 2 hr. and an 8 hr. period of incubation. The livers of 2 animals were pooled in each dietary group. The results are expressed as μg. RNA purine ribose per flask.

Diet	Incubation Time			Difference (%)	
	0 hr.	2 hr.	8 hr.	0-2 hr.	0-8 hr.
Protein-free (fasting)	150.1	152.0	142.6	+1.4	-5
Protein-containing (fasting)	198.5	187.1	175.0	-5.7	-11.8
Protein-containing (fed)	163.5	174.7	161.0	+6.8	-1.5

Therefore, if we recover the total acid-soluble adenine at the beginning and at the end of the period of incubation, any reduction in its specific activity can be attributed to dilution with unlabelled adenine-containing compounds derived from RNA breakdown.

Table 37 contains the data obtained from such an experiment. Owing to technical difficulties we were unable to obtain any data on the changes occurring in the microsomes of those animals actively absorbing amino acids. However, it is apparent (Table 37) that, in those animals which were fasting after a protein-containing diet, there is considerable dilution of acid-soluble adenine of the medium during the 2 hr. period of incubation, whereas there is no change in the S.A. of acid-soluble adenine in microsomes from animals fasting after a protein-free diet. This experiment was not an entirely satisfactory one, due to the fact that the amount of adenine isolated was rather small. We therefore decided to repeat this experiment in conjunction with ribose estimations of total RNA in the system, in order to compare each procedure for changes in RNA breakdown.

Table 38 contains the data from this experiment and shows the changes occurring in the total amount of RNA over a 2 hr. period of incubation. The data have been expressed as u moles of adenine by calculation from the ribose figures, obtained by the orcinol estimation, using values for the

Table 37.

The effect of protein-level in the diet on RNA breakdown in liver microsomes as indicated by changes in the S.A. of acid-soluble adenine after 2 hr. incubation. The livers of two animals were pooled in each dietary group.

Diet	Incubation time (hr.)	S.A. of acid-soluble adenine c.p.m./μ mole adenine	Difference (%) over 2 hr. incubation period
Protein-free (fasting)	0 2	155,769 148,784	-2.6
Protein-containing (fasting)	0 2	189,561 122,038	-35.6

Table 38.

The effect of various nutritional conditions on the RNA of rat liver microsomes, as indicated by changes in the total amount of RNA (expressed as μ moles of adenine) and in S.A. of acid-soluble adenine (expressed as c.p.m. per μ mole adenine) over a 2 hr. period of incubation. Each dietary group contributed the livers of 2 rats.

Diet	Total amount of RNA (expressed as μ moles of adenine)			S.A. of acid-soluble adenine (c.p.m./μ mole adenine)			Adenine in the acid-soluble pool (μ moles)		
	Control	Incubated	Diff.	Control	Incubated	Diff. (%)	Before incubation	After incubation	Diff.
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Adenine added to medium	-	-	-	207,000	-	-	1.655	-	-
Protein-free (fasted)	0.905	0.867	-0.038	173,500	172,500	-1	1.972	1.988	+0.016
Protein-containing (fasted)	1.200	1.073	-0.127	197,000	208,000	+6	1.739	1.649	-0.090
Protein-containing (fed)	1.415	1.378	-0.037	180,200	174,000	-3	1.900	1.968	+0.068

adenine content of rat liver microsome RNA found by Crosbie, Smellie and Davidson (1953). These figures agree with previous results in showing that the greatest breakdown of RNA occurs in the microsomes of animals fasting after a high-protein diet, the change in the RNA content of the microsomes of the other two nutritional groups over the 2 hr. period of incubation being much smaller. Table 38 also shows the changes in the S.A. of acid-soluble adenine over the 2 hr. period of incubation. It is apparent that these figures are not in agreement with the changes in total amount of RNA in so far as the accelerated breakdown of RNA in the group fasting after a protein-containing diet is no longer apparent.

In order to decide whether the isotope dilution technique was adequate to our problem, we made some further calculations on the radioactive data contained in Table 38. Since we know the S.A. of ^{14}C-adenine added to the medium (208,000 c.p.m. per μ mole), activities of adenine isolated from the acid-soluble pool at the beginning of incubation will represent the effect of dilution with ATP and other adenine-containing compounds present in the microsomal preparation. Since we added 1.655 μ mole of adenine to each flask, we can compute the total acid-soluble adenine from this dilution effect (column 7 of Table 38). By similar calculations we can arrive at the total adenine content of the acid-soluble fraction at

the end of incubation (column 8 of Table 38). The differences between columns 7 and 8 can then be directly compared with the u moles of adenine released from RNA, as measured by ribose estimation (column 3). It should be emphasised that a loss from RNA (minus sign in column 3) should be equivalent to a gain to the medium (column 9). Thus the slight losses of RNA-adenine by the first and third dietary groups (-0.038 and -0.037 respectively) coincide with a gain to the medium of 0.016 and 0.068 μ moles of adenine respectively. The second group, which exhibited the largest loss of RNA-adenine (-0.127 μ moles), showed a loss of acid-soluble adenine of a large order of magnitude (-0.090 μ moles). It is impossible to conceive of any route of loss of acid-soluble adenine in this preparation other than through technical errors. In fact the adenine-pool figure for the second group (incubated) is the same as the amount of adenine added, namely, 1.656 μ moles, a rather surprising figure. We must therefore accept the ribose figures as being the more accurate ones, until a method allowing us to use very much less added adenine is devised, in which case we may anticipate greater sensitivity to breakdown products.

The experiments recorded in this section have been of necessity of a preliminary nature. However, they do indicate that when the experimental difficulties have been surmounted, the system may well prove suitable for future investigations.

GENERAL DISCUSSION.

THE RÔLE OF RNA IN PROTEIN SYNTHESIS.

The purpose of this thesis was to investigate the relationship between RNA and protein synthesis. The system adopted for this study, rat liver, was utilised because it had been shown that the protein and RNA of this organ were sensitive to changes in the level of protein in the diet. Our investigations have shown that animals fasting after a protein-containing diet lose RNA from the liver until a new lower level is reached at which it remains constant. On the other hand, animals fasting after a protein-free diet show no change in content of liver RNA. Thus it would appear that the amount of RNA in the livers of animals fasting after a protein-free diet represents the basal state and that addition of protein to the diet causes an increase in RNA content which disappears when the animal is again fasted. This control of the amount of RNA in the liver by dietary protein level appears to reside in changes in the rate of RNA disintegration. Evidence leading to this conclusion has been fully dealt with in Section I; this concept also gains support from the preliminary <u>in vitro</u> studies of Section V. The remaining point of importance established by our work is that nucleic acid metabolism is sensitive to removal of a single essential amino acid from the diet. We have interpreted the data from this part of the work to mean that more than one RNA molecular species is involved in protein

synthesis, one type being responsive to amino acids in bulk, another to the completeness of the amino-acid mixture in the precursor pool.

Before we discuss the significance of these results in relation to the possible rôle of RNA in protein synthesis, it might be convenient at this juncture to consider the current theories of protein synthesis.

There exists a considerable amount of evidence in favour of the view that the starting materials for protein synthesis are free amino acids and not more complicated molecules such as peptides. Thus an intravenous infusion of a suitable amino-acid mixture can maintain animals in positive N-balance whereas an infusion of the products of partial hydrolysis of protein cannot (Christenson, 1950). Among micro-organisms, strains are known which require specific amino acids, but as yet there is no conclusive evidence that a peptide is required as a specific growth factor for any organism. Furthermore, analyses of tissues from many sources have failed to demonstrate the presence of any generally-distributed peptide apart from glutathione.

If we accept the view that amino acids <u>per se</u> are the units for protein biosynthesis, then any theories of protein synthesis must pay due consideration to the following two points:-

1. Since the formation of peptide bonds is an endergonic reaction, some energy source must be involved.

2. Each protein would appear to have a particular and invariable arrangement of amino acids in its molecule, therefore a "sequentialization" step (Zamecnik, Keller, Hoagland, Littlefield & Loftfield, 1956) must be present.

Two reactions involving the formation of peptide bonds have been studied in liver preparations. Lipmann (1945) found the acetylation of sulphonamide in pigeon liver was much reduced under anaerobic conditions but that addition of ATP increased the rate of synthesis. ATP was also found to stimulate the formation of hippuric acid from glycine and benzoic acid in guinea-pig liver (Borsook & Dubnoff, 1947). Similar types of reaction where ATP has been shown to have an activating effect are the synthesis of glutamine from glutamic acid (Speck, 1947; Elliott, 1948) and the synthesis of glutathione from glutamylcysteine and glycine (Bloch, 1949; Johnson & Bloch, 1951).

Recently an amino-acid activating system has been demonstrated in rat liver by Hoagland and his colleagues (Hoagland, Keller & Zamecnik, 1956). The reaction appears to be:-

$$\text{ATP} + \text{amino acid} + \text{enzyme} \rightleftharpoons (\text{aminoacyl AMP})\ \text{enzyme} + \text{pyrophosphate}.$$

ATP could not be replaced by any other nucleotide or phos-

phorylated nucleotide. It would appear also that a separate activating enzyme is necessary for each amino acid rather than one common activating site.

Thus the formation of a peptide requires the presence of some energy-donating system. In this connection we must also consider another theory resulting from investigations carried out by several groups of workers, namely, the "Transpeptidation" theory (Fruton, 1951). Several proteolytic enzymes have been shown to catalyse reactions of esters, amides and peptides with amines or amino acids resulting in the formation of new amides or peptides. The energy requirement in this case is obtained from the hydrolysis of the initial ester, amide or peptide link. Examples of this type of enzymic reaction are numerous. Thus Jones, Hearn, Fried and Fruton (1952) demonstrated polymer formation when glycyl-L-phenylalanineamide was incubated in the presence of cathepsin-C. Transpeptidation reactions involving peptides as distinct from amides have been demonstrated in the case of γ-glutamyl peptides by Hanes, Hird and Isherwood (1952), and by Feder, Miller and Waelsch (1953). Brenner, Müller and Pfister (1950) found that esters of methionine in the presence of chymotrypsin resulted in the formation of the di- and tri-peptide. The factor governing the possibility of this 'transpeptidation' reaction being a mechanism for the building-up of peptide chains would be the presence of a preformed

pool of amides or peptides. Since evidence for such a pool is not forthcoming and since there is such considerable evidence for amino acids as the basic units of protein synthesis, it cannot be said that this mechanism has been established at present. A further objection to the 'transpeptidation' theory arises when we consider the specificity of protein structure. If the proteolytic enzymes which are responsible for transpeptidation are as demanding in specificity of their substrates with respect to transpeptidation as they appear to be when acting as hydrolytic enzymes, one would have to imagine a very complex system of enzymes for the synthesis of only one protein.

An hypothesis has been propounded by Dounce (1952) which not only utilises amino acids as the starting material for protein but also explains the formation of specific proteins and introduces a possible rôle for RNA. According to this author, the nucleic acid forms a template which determines the arrangement of amino acids in peptide chains and can similarly govern the order of nucleotides in nucleic acid chains. He postulates phosphorylation of the nucleic acid to a polyphosphate form containing energy-rich phosphate bonds. Nucleosides or amino acids will react with these phosphate bonds to give a nucleic acid-nucleic acid complex or a nucleic acid-amino acid complex. If these added nucleosides or amino acids then undergo polymerisation, a

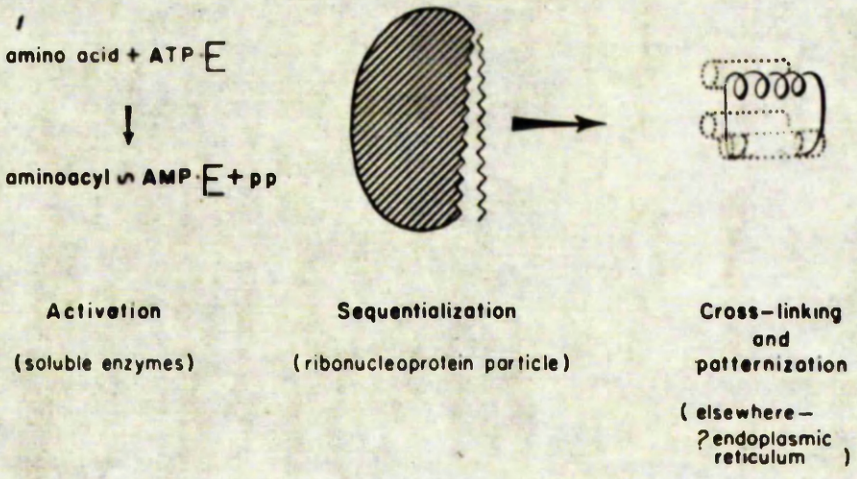


Fig.13. - Steps in protein synthesis in rat liver cytoplasm postulated by Zamecnik et al. (1956).

new nucleic acid molecule or a peptide chain is formed. The nature of the original nucleic acid template would determine the specificity of the nucleic acid or the arrangement of amino acids in the peptide. This theory allows for the template to be a self-reproducing unit. Zamecnik and his co-workers (1956) have extended this theory slightly. In view of their discovery of an amino acid-activating system which involves the formation of an aminoacyl nucleotide, they suggest that these aminoacyl compounds align themselves along the ribonucleoprotein template, their side chain groups governing the position taken up on the ribonucleoprotein surface. They are in favour of the ribonucleoprotein particle of microsomes being this template. Zamecnik's concept of the rôle of RNA in protein synthesis is represented in Fig. 13 taken from a recent article by this investigator.

Details of the structure of the RNA involved in protein synthesis have been considered by Chargaff in a series of studies (Chargaff, 1956). From rat liver microsomes he has isolated a ribonucleoprotein molecule of constant composition through successive reprecipitations. It contained two nucleotides to each amino acid residue. He suggests that this is a genuine compound which plays a fundamental rôle in protein synthesis. Study of the uptake of labelled amino acids by this molecule showed that it had a more intense labelling than the rest of the microsomal protein.

How do our data relating stability of RNA to intensity of protein synthesis contribute to this picture? In the first place, our findings show a dissociation between the rate of synthesis of protein and of RNA in the liver; in other words, the speed of replenishment of the template is independent of its function, just as in the case of an enzyme. On the other hand, our evidence leads us to believe that the stability of the RNA is determined by the intensity of protein synthesis. The number of RNA molecules which can be maintained in a stable form at any one time appears to be a function of the amino-acid supply. Our concept is that the RNA molecules are only stable when the template surface is fully engaged in protein synthesis. This implies that the disengaged RNA molecule is a very unstable structure and requires the presence of the linked amino acids on its active surface to render it more robust. This instability could be due to either inherent chemical instability of RNA or alternatively the naked molecule may be more susceptible to attack by ribonuclease within the cell. In connection with the chemical properties of RNA, it is of interest to note that Elson and Chargaff (1955) have found that native RNA possesses equal numbers of 6-amino and 6-keto bases, whereas RNA which has been deproteinised no longer shows this equivalence of the two groups of bases. This argues in favour of the idea that a part of the ribonucleic acid molecule is dependent for

its chemical stability on the presence of protein.

Evidence from studies on animals fed with incomplete amino-acid mixtures (Section III) suggests that the effect of amino-acid supply on RNA metabolism is not an all-or-none phenomenon, but that a part of the RNA may be sensitive to amino acids in bulk and another part to the completeness of the mixture. RNA may thus play more than one rôle in protein synthesis, but the possible nature of these different functions is obscure. There may, of course, be alternative explanations of the effects observed with the incomplete amino-acid mixture. Further experimental work is needed before any weight can be placed on these suggestions.

SUMMARY

Studies have been carried out on rat liver to determine the nature of the relationship between ribonucleic acid (RNA) and protein synthesis. In particular, an attempt was made to decide why the level of protein in the diet caused changes in the amount of liver RNA. The experimental studies are described in five sections, summarised as follows:-

Section I. The influence of dietary protein on the incorporation of ^{14}C-2-glycine and ^{32}P into the ribonucleic acid of rat liver.

1. Rats were maintained on diets either containing adequate amounts of protein or free from protein, and after fasting overnight, were injected with ^{32}P and with ^{14}C-2-glycine. They were killed at 3, 6 and 9 hr. thereafter. A further group of rats which had received the protein-containing diet was fed protein at the time of injection with the isotopes.

2. The uptake of glycine into liver protein was essentially similar in the two groups in the post-absorptive state, but rose considerably when protein was fed at the time of injection.

3. Labelling of RNA with ^{32}P and with glycine was much reduced in rats fasted overnight after the protein-containing diet. On feeding protein to this group, uptake of both isotopes rose to the level found with the protein-free diet.

4. It is suggested that the low level of labelling in the

groups fasted after the protein-containing diet was due to breakdown of RNA as soon as the supply of amino acids from the gut ceased, the products of breakdown causing dilution of isotopically labelled precursors of RNA in the acid-soluble fraction of liver. The feeding of protein, by restraining RNA breakdown and thus terminating dilution of these precursors, restored the level of labelling in RNA. This hypothesis is supported by a study of the effect of diet (a) on changes in the RNA content of the liver, (b) on ^{14}C-uptake by the purines of the acid-soluble fraction of liver, (c) on the uptake of ^{32}P by the RNA of different liver cell fractions and (d) on allantoin excretion.

5. It is concluded that the rate of synthesis of liver RNA is a function of the available energy, whereas the stability and therefore amount of RNA in the liver is determined by the supply of amino acids for protein synthesis.

Section II. The uptake of ^{14}C-2-glycine and ^{32}P by liver ribonucleic acid when protein is administered to protein-depleted animals.

1. A study was made of the effect of feeding protein to animals fasting after a protein-free diet on the uptake of ^{14}C-2-glycine into protein and acid-soluble purines and the uptake of ^{14}C-2-glycine and ^{32}P into the purines of RNA in rat liver.

2. Feeding of protein to protein-depleted animals resulted in an increase in the incorporation of ^{14}C-2-glycine into protein and into the acid-soluble purines. This increase in the latter was reflected in an increased uptake of ^{14}C-2-glycine and ^{32}P into the purines of RNA.

3. The total uptake of ^{32}P by RNA in the protein-fed animals was twice that of the fasted group, while the uptake of ^{14}C-2-glycine was five times greater in the case of the fed animals. This finding again suggests that supply of amino acids to the liver is accompanied by a decrease in the RNA breakdown, since these changes can best be explained as due to different dilution effects on the respective precursor pools.

Section III. The effect of feeding complete and incomplete amino-acid mixtures on the uptake of labelled precursors into the purine and pyrimidine bases of rat liver.

1. Since the feeding of protein is accompanied by a decrease in RNA breakdown, the effect of feeding amino-acid mixtures was studied. The two amino-acid mixtures fed differed only in that one contained tryptophan and was nutritionally complete, whereas the other was deficient in this amino acid. The main studies were carried out on rats which had previously been fed a protein-free diet.

2. The complete amino-acid mixture produced an increase in protein synthesis accompanied by an increased uptake of ^{14}C-2-glycine into RNA and the acid-soluble purines, together with a fall in the $^{G}/_{A}$ ratio for ^{14}C-2-glycine uptake.

3. The effect of the two amino-acid mixtures on the incorporation of ^{14}C-6-orotic acid into RNA-pyrimidines was similar, in that the $^{U}/_{C}$ ratio for ^{14}C-6-orotic acid uptake fell when the complete amino-acid mixture was fed.

4. It was thought that previous feeding of a protein-free diet might produce a depletion of the liver enzymes, which might account for the changes in the base ratios. A study was therefore made of the effect of the two amino-acid mixtures on the $^{G}/_{A}$ and $^{U}/_{C}$ ratios of RNA when the animals had previously been fed a protein-containing diet. When the two amino-acid mixtures were fed to rats fasting after a protein-containing diet, no fall occurred in the $^{G}/_{A}$ ratio or $^{U}/_{C}$ ratio of RNA with the complete amino-acid mixture.

The implications of these results were discussed.

Section IV. The action of β-2-thienylalanine on protein synthesis and RNA turnover in rat liver slices and cytoplasm.

1. A study was made of the effect of β-2-thienylalanine, a structural analogue of phenylalanine, on the uptake of ^{14}C-2-glycine and ^{35}S-methionine into protein, and ^{32}P and ^{14}C-8-adenine into RNA, and ^{32}P into lipid P of rat-liver

slices and cytoplasm over a 3 hr. period of incubation.

2. When β-2-thienylalanine was incubated with liver slices, the uptake of both ^{14}C-2-glycine and ^{35}S-methionine into protein was inhibited. A corresponding inhibition of the uptake of ^{32}P and ^{14}C-8-adenine into RNA, and ^{32}P into lipid P also took place. It was concluded that β-2-thienylalanine had a widespread effect on cellular metabolism and its action in liver slices was not confined to inhibition of protein synthesis.

3. In order to simplify the system a study was made of the action of this amino-acid analogue on RNA and protein metabolism in a cell-free system, namely, rat liver cytoplasm. 3-PGA and ATP were added to the cytoplasm as energy donors and these substances stimulated isotope uptake by protein and lipid P. The addition of β-2-thienylalanine to this system caused inhibition of the uptake of ^{14}C-2-glycine into protein, but had no effect on lipid P metabolism. This suggests that the action of β-2-thienylalanine on lipid metabolism in liver slices is due to a general effect on respiration. The uptake of isotopes by RNA was negligible in the cell-free system, and therefore it could not be determined whether β-2-thienylalanine inhibited incorporation of precursors into the RNA of slices because of a general metabolic disturbance in consequence of a more intimate relationship of RNA metabolism to protein synthesis.

4. It has been concluded that the inhibitory action of β-2-thienylalanine in liver slices is not limited to an action on protein synthesis, but also involves more general changes, probably affecting energy metabolism. The inability to promote the uptake of isotopes by RNA of liver cytoplasm precludes any possibility of determining whether the changes in RNA metabolism following β-2-thienylalanine treatment of whole cells belongs to this general category.

Section V. Studies on RNA breakdown in isolated microsomes.

1. The effect of previous diet on the breakdown of RNA in liver microsomes incubated in vitro was studied, and a few preliminary experiments have been recorded.

2. Breakdown of RNA was studied by quantitative estimation of RNA in the system (orcinol method) and by the diluent effect of adenine-containing breakdown products of RNA on the activity of ^{14}C-8-adenine added to the suspending medium.

3. Using the orcinol method, the RNA of the microsomes of rats fasting after a protein-containing diet exhibited a higher rate of breakdown during incubation. There was only a small decrease in the RNA content of the microsomes from rats fasted after a protein-free diet and also from the microsomes of rats actively absorbing amino acids at the time of killing. However, the isotope dilution method failed to give satisfactory

results in our hands.

4. These findings with the orcinol method are compatible with the thesis propounded in Section I, namely, that there are changes in the rate of breakdown of RNA in response to variations in the supply of amino acids.

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APPENDIX.

COMPARISON OF TRICHLORACETIC ACID AND PERCHLORIC ACID AS PRECIPITANTS IN NUCLEIC ACID ISOLATION PROCEDURES.

As pointed out in the Experimental of Section I, the use of PCA as a precipitant affected our recoveries of nucleic acids from rat liver. Since the use of this acid was desirable for the isolation of the acid-soluble bases, it was essential that we learn the limitations of its use. Accordingly we have carried out a series of experiments to investigate the stability of the nucleic acids in the presence of PCA, comparing our results with those obtained when TCA was utilised as the protein and nucleic acid precipitant.

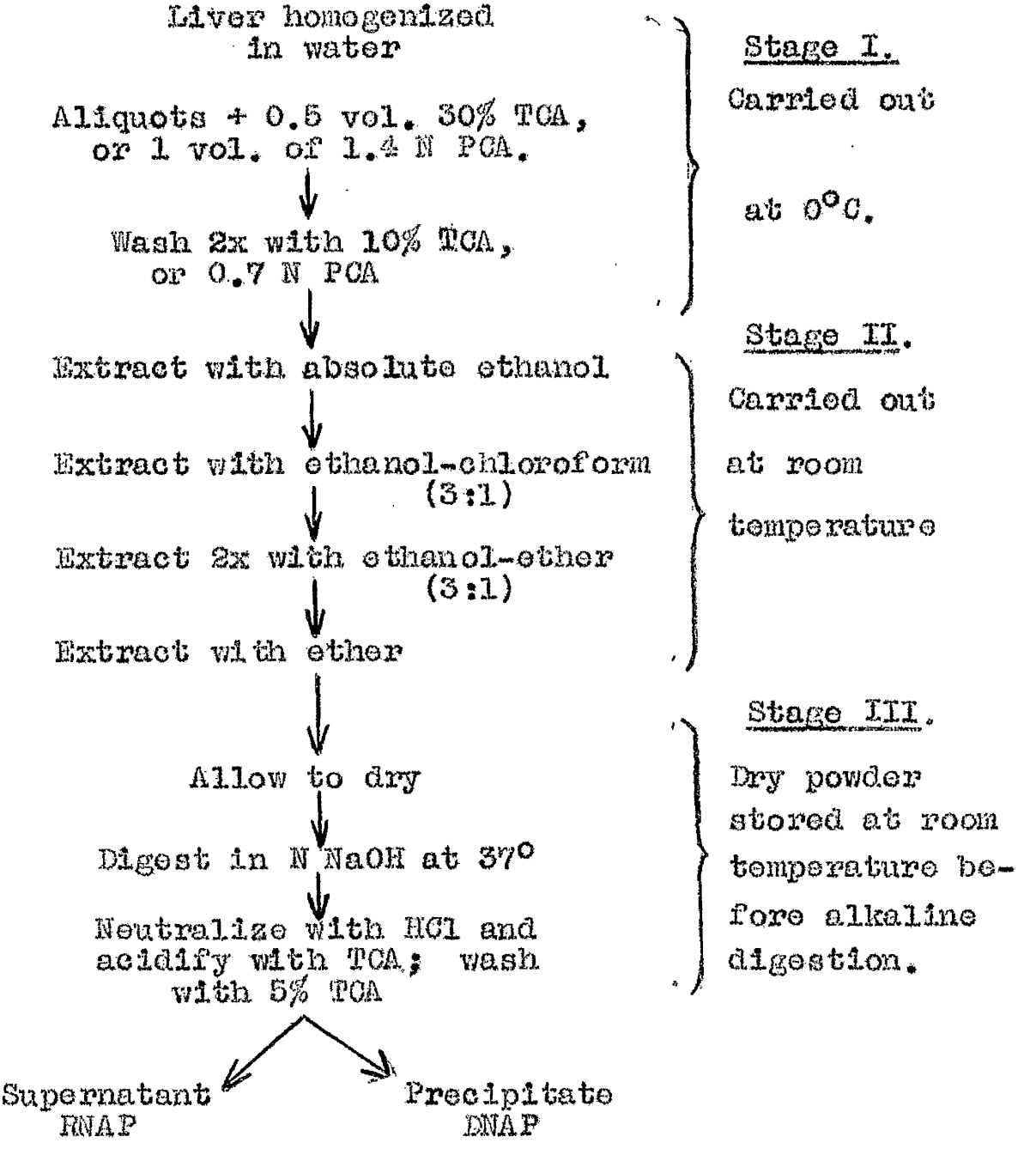
The slightly modified Schmidt-Thannhauser procedure detailed in Table 39 was used for estimating RNAP and DNAP. In studying the action of perchloric acid, the Schmidt-Thannhauser method for nucleic acid isolation may be considered to consist of three stages.

1. Precipitation of proteins, nucleic acids, etc. The effect of prolonging contact with PCA or TCA before centrifuging is shown in Table 40. Up to 2 hr. contact with PCA has no significant effect on the nucleic acid content of the samples, but after 6 hr. contact RNA is seriously reduced (in view of our findings at stage III, it may be mentioned that digestion with alkali was carried out immediately after fat extraction).

Although RNA is not significantly reduced by contact with PCA for 2 hr., Table 41 shows that the acid-soluble purines undergo some dilution from breakdown products. This is

Table 39.

Method used for estimating RNA and DNA.



apparent in the lower specific activity of acid-soluble guanine; adenine is not appreciably affected, presumably because there is a much greater amount of acid-soluble adenine compounds and dilution by breakdown of RNA would be correspondingly less.

11. Extraction of Residue with Fat Solvents. The important point here is that TCA is soluble in the solvents, whereas it appears that PCA is not extracted and remains in the dry powder following ether extraction.

111. Separation of RNA and DNA from an alkaline digest of the residue. The practice has been to set aside the dry powder at room temperature until it is convenient to carry out digestion with alkali. Repeated estimations on powders prepared by TCA precipitation have justified this procedure in the past. However, Table 42 shows that a PCA-precipitated residue, after standing for 10 days, gave a DNAP figure much less than is known to occur in rats of this weight. Standing for a further 6 days reduced the DNAP figure still further, whereas the RNAP rose. If we allow for slight drying out of the powder during the 6-day period, we find that the total nucleic acid phosphorus remained unchanged. This is compatible with gradual decomposition of DNA during storage, so that its phosphorus is no longer precipitated on acidifying the alkaline digest, and thus contributes to the RNAP

Table 40.

Effect of length of contact with 10% TCA or 0.7 N PCA on recoveries of protein, RNA and DNA.

(Data obtained on two pooled rat livers; 10 ml. samples of a homogenate were used and left in contact with TCA or PCA for the stated times).

Precipitant	Time of contact at 0°C	Amounts per liver		
		Protein N	RNA P	DNA P
	hr.	mg.	mg.	mg.
T.C.A.	0.5	177	5.97	2.17
	24	167 (-6%)	5.35 (-10%)	2.03 (-6%)
P.C.A.	0.5	181	5.55	2.17
	2	184 (+1%)	5.31 (-4%)	2.29 (+5%)
	6	176 (-3%)	4.24 (-24%)	2.30 (+6%)
	24	172 (-5%)	1.97 (-65%)	1.93 (-11%)

Table 41.

Effect of 0.7 N perchloric acid as a precipitant for liver homogenates.

Length of contact with perchloric acid	Acid-soluble fraction			Precipitate
	Free Glycine	Adenine Glycine	Guanine Glycine	Total Nucleic Acid Phosphorus
hr.	c.p.m./100 µg.			mg.
0.5	3505	2494	3323	6.86
2	3410	2359	2662	6.63
Difference	-3%	-5%	-20%	-3%

A rat was injected with ^{14}C-2-glycine. The liver homogenate was divided into two fractions, one being worked up as rapidly as possible, the other being left in contact with PCA for 2 hr. The glycine was isolated as the DNP-derivative, following degradation to glycine, of the total acid-soluble adenine and guanine; the free glycine was isolated direct from the acid-soluble fraction.

Table 42.

The effect of storage of the lipid-extracted residue on apparent RNAP and DNAP content of liver. (The livers of two rats were homogenized in PCA and samples taken.).

Sample	Time of storage	Amount per liver		RNAP/DNAP Ratio
		RNAP	DNAP	
		mg.	mg.	
A	0 days*	(4.90)	(1.64)	(3.00)
	10 days+	5.48	1.38	3.97
	16 days+	6.10	1.12	5.45
B	0 days*	(4.90)	(1.64)	(3.00)
	10 days+	5.38	1.25	4.30
	16 days+	5.95	1.00	5.95

*Based on data from rats of identical weight on similar diet, the liver being treated with TCA to precipitate protein.

+The total nucleic acid P (RNAP + DNAP) rises slightly at 16 days compared with 10 days; this is due to drying out of the powder, since expression of the total nucleic acid P per 100 mg. protein in the powder yields identical results at 10 and 16 days. Sample A gave 5.09 and 5.06 mg. total P per 100 mg. protein N, and sample B gave 4.83 and 4.78 mg. at 10 and 16 days respectively.

fraction. An even more serious fall in apparent DNAP occurred in a series of 10 powders stored for about a month before analysis; in this case, the mean DNAP content per liver was 0.62 mg., compared with an expected value of 1.73 mg. for animals of this weight. The RNAP/DNAP ratio was 7.31, instead of 3.0. In view of the fact that RNA is more sensitive than DNA to the action of perchloric acid (e.g., Table 40) it is curious that a considerable amount of alcohol-precipitable RNA can still be extracted with hot 10% NaCl solutions from powders prepared with PCA and allowed to stand for some time.

It is obvious that this continuing action of PCA in the dry, fat-extracted residue is not likely to invalidate estimations of RNA and DNA based on specific reactions, such as that with orcinol.

It is apparent therefore that if valid results are to be obtained when PCA is used to precipitate nucleic acids and protein from liver homogenates, the entire procedure should be carried out as rapidly as possible. Thus at Stage I the PCA must be separated in the first 30 min. after addition. The findings at Stage III would indicate that if RNA and DNA are to be determined as RNAP and DNAP, PCA is not the ideal precipitant.