INTERRELATIONSHIPS BETWEEN CARBOHYDRATE, FAT AND PROTEIN METABOLISM.

A study of the influence of energy intake on the course of protein metabolism.

By Donald Johnstone Naismith, B.Sc.

This thesis is primarily concerned with the relationship of energy metabolism to protein metabolism. The field of enquiry was subsequently extended to the metabolism of liver phospholipids and ribonucleic acid, since both of these display certain metabolic features in common with liver protein.

The influence of energy intake on protein metabolis in the rat was explored in a study of nitrogen-balance and the protein content of the liver. Rats were fed diets, eithe adequate in protein content or free from protein, in combination with various levels of energy intake provided by carbohydrate or fat. At the adequate level of protein intake nitrogen-balance and the protein content of the liver were affected in a strictly linear fashion by variations in energy supply, whereas on the protein-free diet energy intake appeared to have no considerable effect on these. A study was made of the bodily distribution of the changes in nitrogen balance produced by varying the energy content of th diet containing protein.

As an extension of these experiments, measurements

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were made, under the same nutritional conditions, of the rate of incorporation of an isotopically labelled amino acid (glycine-2-^{14}C) into the mixed proteins of the liver. In the post-absorptive state, the rate of glycine incorporation was essentially the same, at any given plane of energy intake, for animals previously fed the protein-containing and protein-free diets. At each level of protein intake, however, the influence of the energy content of the preceding diet was apparent, the effect being considerably more marked when the preceding diet contained protein. This difference in the response of protein metabolism to energy intake at the two protein levels accounts for the fact that energy intake affects the amount of protein in the liver on one diet, but not on the other. On feeding protein, the pattern of protein metabolism was completely changed. The influx of amino acids into the liver promoted a rapid increase in the rate of incorporation of labelled glycine, independent of both the protein and energy content of the previous diet. These observations suggest that synthesis of liver protein proceeds in intermittent bursts, following the ingestion of protein, while the effect of energy intake on the rate of protein synthesis is a prolonged one, exerted between meals on the so-called "endogenous" metabolism of protein.

The picture obtained in studying phospholipid metabolism in the liver showed some resemblance to that of

liver protein. Only when the diet provided an adequate supply of protein did the synthesis of phospholipid in the liver increase in response to increments in energy intake. It is suggested that the feeding of a protein-free diet so reduces the concentration of some precursor or essential component in phospholipid formation that it becomes the limiting factor in the rate of synthesis. Choline was eliminated as the missing factor.

No such factor restricts the response of ribonucleic acid metabolism to changes in the energy level of the protein-free diet; the rate of synthesis of ribonucleic acid, as measured by combined quantitative and isotopic studies (^{32}P and glycine-2-^{14}C), appears to be determined by the energy content of the diet, rather than by its protein content, a fact of particular interest in view of the alleged relationship between ribonucleic acid and protein synthesis. The stimulating effect of an increased energy intake on ribonucleic acid synthesis has been shown to result from the improvement in energy balance rather than from direct involvement of ribonucleic acid in energy metabolism. Vitamin B_{12} was eliminated as a dietary factor affecting the synthesis of ribonucleic acid under the conditions of these experiments.

As an hypothesis consistent with these observations on the metabolism of protein, phospholipids and ribonucleic acid in the liver, the availability of energy may be picture

as the factor governing their rates of synthesis. Provided no other component of the synthetic mechanism limits the response to a change in available energy, an improvement in energy balance is associated with an augmented rate of synthesis. It would, however, seem that the increased protein synthesis following the ingestion of protein is independent of energy intake.

The findings of these experiments are discussed in the light of current views on the biosynthesis of proteins.

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GENERAL INTRODUCTION.

INTRODUCTION.

Shortly after it was discovered by Dumas and Cahours (1842) that urea was the principal end product of protein metabolism, and an experimental procedure was devised to measure the response of N excretion to various dietary circumstances - the first N-balance studies (Boussingault, 1839), it was established that factors other than the amount of protein ingested had a profound effect on the course of protein metabolism. The early experiments of Voit and his associates showed that under certain circumstances carbohydrate and fat could also influence N-balance, and in this way established a relationship between protein metabolism and energy intake.

Although subsequent experimentation has been concerned almost exclusively with nutritional aspects of this relationship, these studies have been carried out on a variety of species in diverse nutritional and physiological states, thus revealing the extent of the relationship, and providing a starting point from which a biochemical attack on the mechanism of the interaction of fat and carbohydrate in protein metabolism could be made.

The most obvious way in which carbohydrate and fat

affect protein metabolism is through their energy-yielding properties, and experiments in which energy was either added to or subtracted from the diet have been performed on animals and human subjects in nutritional states ranging from undernutrition to surfeit feeding (see review by Munro, 1951).

In a number of short term experiments carried out on human subjects, carbohydrate or fat was removed from diets providing adequate amounts of protein and energy (Rusk, 1890; Rosemann, 1901; Neumann, 1899). Restriction of energy consumption in either form resulted in impaired N-balance. Similarly in experiments on growing mice, Bosshardt, Paul, O'Doherty and Barnes (1948) observed that a reduction in calorie intake produced by alterations in either dietary carbohydrate or fat caused a decrease in the rate of growth. Thus withdrawal of energy in the form of carbohydrate or fat from adequate diets results in an immediate loss of N from the body. Conversely, addition of energy in either form to inadequate diets leads to a reduction in N excretion. Thus in some of the earliest experiments on protein metabolism conducted by Voit and Bischoff, the effect of energy supplements on a negative N-balance was studied in the dog. The protein-sparing action of fat and carbohydrate was demonstrated when either

was used to supplement an amount of meat insufficient to maintain body weight and N equilibrium. Again, Allison, Anderson and Seeley (1946), also using dogs, were able to demonstrate a favourable response of N-balance, when energy intake was raised from 25% to 50% of the animal's normal consumption, and furthermore, equivalent amounts of carbohydrate and fat had similar effects on N-balance. In the few instances in which man was the experimental subject, essentially the same picture was obtained. Addition of carbohydrate or fat to submaintenance diets (Jansen, 1893; Zuntz and Loewy, 1918), resulted in a considerable improvement in N-balance. With the exception of the studies of Bosshardt and his colleagues on growing mice, in none of those experiments dealing with undernutrition was there sufficient evidence on which to base a rigorously quantitative comparison of the protein-sparing effect of fat and carbohydrate.

Studies on animals receiving diets already adequate in energy content, as judged by their ability to maintain weight and N equilibrium, provide a similar picture of the relationship between N-balance and energy intake. Several studies have been made on man (Cuthbertson, McGirr & Munro, 1937; Cuthbertson & Munro, 1937; Basu & Basak, 1939;

Wikramanayake & Munro, 1954) on the dog (Munk, 1879; Biernacki, 1907; Levene & Kober, 1908; Kochmann & Potzsch, 1911; Larson & Chaikoff, 1937; Allison & Anderson, 1945), on the rat (Lathe & Peters, 1949; Forbes, Gratzler, Thacker & Marcy, 1939; Forbes & Swift, 1944; Wikramanayake & Munro, 1954) and on other species, in which a diet rich in protein and calories was supplemented with carbohydrate or fat. In most cases, a substantial retention of N was noted.

The occurrence of these variations in N output with changes in energy intake raises a number of questions. In the first place, if the depression in N output caused by adding carbohydrate or fat to adequate diets is due entirely to the surplus energy, then equivalent increments of these two nutrients would be expected to affect N-balance to the same degree. In only a few of the experiments mentioned above, namely those in which adult rats were used, and in some further experiments on rats by Forbes and his colleagues (Forbes, Swift, Elliot & James, 1946; Forbes, Swift, Thacker, Smith & French, 1946), were experimental conditions such that comparison could be made between the protein-sparing action of fat and carbohydrate. These show that there is little to choose between the effect of surfeit carbohydrate and surfeit fat on protein metabolism

On the other hand, Cuthbertson & Munro (1937), working with two human subjects, studied the relative effects of surplus fat and carbohydrate on the excretion of N and S, and found that carbohydrate provided a slightly greater retention of N and S than did fat. In the absence however of more extensive evidence from experiments on man, the significance of these few observations must remain unsettled.

The second question arising from a consideration of these experiments is whether the alteration in N excretion is directly related to the amount of energy added to or subtracted from the diet. Although from the literature cited above it is evident that both fat and carbohydrate can influence protein metabolism as energy sources, the quantitative aspects of the relationship between the amounts and proportions in which fat and carbohydrate were incorporated in the diet, and the effects produced on protein metabolism have been to a large extent neglected. In only a few experiments on human subjects (Cuthbertson & Munro, 1937; Basu & Basak, 1939) and on young pigs (Terroine & Mahler-Mondler, 1927) in which the same subject was studied at two levels of surfeit carbohydrate, was N retention found to increase in proportion to the amount of extra energy added. Similar studies in which fat was used

as variable energy supplement (Voit, 1869) also suggest that the degree of N retention produced is related to the amount of the energy supplement.

The aim of the experiments presented in this thesis has been to elucidate, with the aid of a wide variety of experimental techniques, certain fundamental aspects of protein metabolism, with special reference to protein synthesis. The central problem has been to investigate the relationship between energy metabolism and protein synthesis, and subsequently, these studies have been extended to the metabolism of substances believed to be closely associated with or directly concerned in the processes leading to protein formation.

In order to clarify the nutritional relationship between energy consumption and protein metabolism, it was proposed, in the first place, to explore the range of energy intake over which N-balance is affected. In addition it seemed of interest to determine to what extent the N content of different organs and tissues would respond to the beneficial effects of fat and carbohydrate. In Part I of this thesis, such experiments are described in which studies of N-balance and the protein content of the liver and other viscera were made on rats receiving diets either rich in protein, or deficient in protein, in combination

with various levels of energy intake. In addition, comparison was made between the efficiency of carbohydrate and fat in depressing N output, and in promoting N retention in the tissues under identical conditions.

Kosterlitz (1944) in an attempt to determine the chemical nature of the changes in the N content of the liver, has correlated, under a variety of nutritional conditions, the amount of protein in the liver with other constituents of the cytoplasm, namely phospholipids and nucleic acids. He showed that in the fasting animal, or in animals fed diets qualitatively or quantitatively deficient in protein, the loss of protein from the liver was accompanied by a corresponding loss of cytoplasmic ribonucleic acid (RNA) and phospholipid. Furthermore, feeding a high protein diet caused an increase in liver protein, RNA and phospholipid. As an interpretation of these findings, Kosterlitz suggested that variations in the quantity of N in the liver in response to altered dietary conditions, were due to losses and gains of whole cytoplasm (protein + ribonucleic acid + phospholipid) rather than of an inert storage protein.

The comprehensive nature of the nutritional conditions employed in the study of protein metabolism (i.e. variations in both protein and energy intake) seemed

ideally suited to a similar study of RNA and phospholipid metabolism. Part II of this thesis therefore contains descriptions of a variety of experiments dealing with the effect of nutritional conditions on ribonucleic acid metabolism and Part III with the influence of energy and protein intake on phospholipid metabolism.

The experiments described in Parts II, Sections 1-3 are concerned with two independent aspects of RNA metabolism. Firstly (Part II, Section 1) changes in the amount of RNA in the liver cell resulting from variations in the energy content of diets either rich in or free from protein were correlated with changes in liver protein under the same conditions, in an attempt to throw some light on the nature of the alleged relationship between RNA and protein synthesis (Brachet, 1950; Caspersson, 1950). In agreement with the considerable body of evidence in the literature offered in support of a role for RNA in protein synthesis, changes in the amount of RNA in the liver were found to parallel closely the variations in protein content.

A second aspect of RNA metabolism, incorporated in Part II, Section 2, was a study of the uptake of radioactive phosphorus into the RNA molecule. As a result of the introduction of isotopically labelled molecules into

biochemical research (Schoenheimer, 1942), it has become obvious that a complete picture of the metabolic activity of any constituent of a living cell cannot be obtained on the basis of quantitative data alone. Those experiments with isotopic phosphorus were performed therefore, under the various dietary conditions described above, with a view to providing a complete picture of the relationship between RNA metabolism and energy intake. In these investigations, it was established that the absolute rate of synthesis of RNA was determined not by the protein content of the diet, but by energy intake; this is in marked contrast to the amount of RNA which is directly related to the protein content of the diet. The problem was then to determine whether the stimulating influence of energy on RNA synthesis in the liver might be due to an involvement of RNA in energy metabolism, or whether it might be explained as an effect of increased energy-yielding nutrients in the tissues. In a series of experiments in which comparison was made between RNA metabolism in animals fed a high energy diet and in animals receiving the same diet but under conditions of increased energy expenditure, it has been possible to make a decision on this point.

Before the effect on liver RNA could be attributed without reservation to the protein content of the diet,

an additional nutritional factor had to be taken into account. A diet devoid of protein is also devoid of natural sources of Vitamin B12, the "animal protein factor". In the past few years, this vitamin has come into prominence as a factor involved in the biosynthesis of nucleic acids, and consequently the possibility was considered that the content of this vitamin in protein-rich diets, rather than increased protein consumption per se, might account for a rise in the amount of RNA in the liver. To test this point, the animals distributed among the various energy and protein experimental groups, were further subdivided so that comparison could be made between those receiving B12 and those receiving an unsupplemented ration. These experiments are described in Part II, Section 3 of this thesis.

Kosterlitz(1944) has demonstrated certain similarities between the metabolism of phospholipid, RNA and protein in the livers of rats transferred from one level of protein intake to another. It was thought that some insight might be gained concerning the interrelationships between protein, phospholipid and RNA metabolism by a study of changes in the amount of phospholipid in the liver produced by alterations in the protein and energy content of the diet. These experiments, discussed in conjunction with

studies on the incorporation of isotopically labelled phosphorus into the phospholipid molecule under the same nutritional circumstances, constitute Part III of this thesis.

Finally, to complete the investigation of the relationship between RNA metabolism and protein metabolism, it was considered desirable to measure the rate of incorporation of an amino acid into the proteins of the liver under the same conditions of energy and protein intake as outlined above. Radioactive glycine appeared to be the amino acid of choice, since it is not only taken up by tissue proteins, but is also incorporated in the purine rings of adenine and guanine, used in the biosynthesis of the nucleic acids. In these experiments, described in Part IV, it was therefore possible to study simultaneously, the rates of RNA and protein synthesis in the same animal.

PART I.

THE INFLUENCE OF ENERGY INTAKE ON NITROGEN BALANCE, AND THE PROTEIN CONTENT OF THE LIVER.

INTRODUCTION.

Although it is now well established that protein metabolism is influenced by energy intake, the underlying biochemical mechanism remains obscure. Examination of the literature (see introductory section of this thesis) suggests that N-balance is affected both by restriction of energy intake below normal requirements, and by supplementing adequate diets with energy-yielding nutrients. It has not however been established whether a large deficiency or a large surplus of energy is required to effect these changes in N-balance; in other words, whether energy intake must be raised or lowered beyond a critical level before N output is altered, or whether N-balance is continuously in equilibrium with energy intake, responding to any increase or decrease in the caloric content of the diet.

An indication that the latter hypothesis might be the correct one is given in the few scattered reports in the literature dealing with this problem. Rubner(1879 ; 1903) and Neumann(1919) both presented evidence that the degree of improvement in N-balance produced by adding carbohydrate or fat to the diets of human subjects, was

directly related to the magnitude of the energy supplement.

If protein is omitted from the diet, a somewhat different picture of the relationship between N output and energy supply is obtained. Thus a reduction in the consumption of such diets seems to have little effect on the N-balance of rats (Mitchell, 1923; Teichler & Mitchell, 1941; Vars & Gurd, 1947), at least until intake represents less than half the amount eaten voluntarily (Swanson, 1951).

It therefore seemed desirable to determine, by means of N-balance measurements and liver protein estimations, firstly the relationship of protein metabolism to a wide range of energy intakes of diets adequate in protein content, and secondly, the effect of the protein content of the diet on this relationship. To this end, experiments were carried out on a single species (the rat) with protein-rich, protein-deficient and protein-free diets, in all of which energy intake was varied from a low level to a surfeit level by changing the amount of carbohydrate or fat fed.

Finally, by estimating the total N content of the liver and other viscera, it was possible to provide some information about the partition within the body of

changes in N-balance brought about by variations in the energy content of protein-containing diets.

EXPERIMENTAL.

Animals. For all experiments, male albino rats about five months old were obtained from a single commercial source. The stock was fasted overnight and animals were selected for each experiment with weights as close to 250 g. as possible. They were then distributed between the various experimental groups according to the randomized block technique (Snedecor, 1946). This statistical procedure consists in selecting enough animals of closely similar weight to provide one rat at each energy level. The process is then repeated with another batch of rats. By a statistical calculation it is possible to eliminate the weight differences between batches as a factor in the results. The mean weights of the animals at the start of the six experiments were 251, 255, 251, 253 and 258 g. respectively, and the maximum range of weights within a single experiment was 12 g. from the average.

Diets. In the six series of experiments to be described,

Table 1.

Composition of the basal Diets for Experiments 1 - 6.

Time of Feeding	Constituent	High Protein Diet A	Protein-free Diet B	Low Protein Diet C
Morning	V.M.R.**(g.)	2.0	2.0	2.0
Evening	Crude casein (g.)	2.8	0.0	0.2
	Fat* (g.)	0.5	0.5	0.5
	Glucose (g.)	1.0	2.4	2.25
	Starch (g.)	1.0	2.4	2.25
N intake per rat per day (mg.) including V.M.R.		368.9	3.3	26.6
Physiological fuel value (Calories)		28.3	29.3	29.3

* The fat used was "Spry", a hydrogenated vegetable oil.

** The Vitamin-mineral roughage mixture (V.M.R.) contributed 7 calories to the basal diets in the form of starch and olive oil (see Table 4).

Table 2.

The Vitamin Supplement

	To prepare 1 kg. (g.)	Daily dose. (µg.)
Calcium pantothenate	0.4	368
Choline hydrochloride.	20.0	18.1mg.
Pyridoxin hydrochloride.	0.05	45.0
Riboflavin.	0.05	45.0
Aneurin hydrochloride.	0.05	45.0
Nicotinic acid.	0.2	180.0
Biotin.	0.01	9.0
p-Aminobenzoic acid.	1.0	900.0
Folic acid.	trace.	trace.
Inositol.	2.0	1.8mg.
Menaphthone.	trace.	trace.

Starch to 1 kg.

This vitamin supply compares favourably with that recommended by Kosterlitz (1947).

Table 3.

Salt Mixture "446".

	(g.)
Sodium chloride.	243.198
Potassium citrate.	533.000
Dipotassium phosphate.	174.000
Dicalcium phosphate.	800.000
Calcium carbonate.	368.000
Magnesium carbonate.	92.000
Ferric citrate.	36.000
Cupric sulphate (pentahydrate).	0.400
Manganese sulphate.	2.800
Potassium aluminium sulphate (Alum).	0.200
Cobalt chloride (hexahydrate).	0.200
Potassium iodide.	0.100
Zinc carbonate.	0.100
Sodium fluoride.	0.002
	2250.0 g.

Table 4.

The Vitamin-mineral-roughage Mixture.

	(g.)
Sodium chloride.	65
Salt mixture "446".	260
Vitamin supplement.	500
Cod liver oil.	125
Wheat germ oil.	50
Agar.	125
	1105

the influence of energy intake on N-balance and on the protein content of the liver was investigated. Some of these experiments represent the sum of two smaller experiments carried out at different times. Since the results obtained on repetition of the individual experiments were similar, this procedure is justified.

Variations in energy intake were effected by having a fixed basal ration (Table 1) providing about 29kg. cal. per rat (800-900 kg. cal./sq.m. of body surface area) per day, to which carbohydrate or fat was added to provide the desired plane of energy intake. Body surface areas were computed from the formula $S = 12.54 \times W^{0.60}$, where S is the surface area and W is the body weight (Lee, 1929). Other essential nutrients in the basal diets were provided by a vitamin-mineral-roughage mixture (V.M.R.). The compositions of the vitamin supplement and salt mixture are given in Tables 2 and 3, and the proportions in which the vitamin and salt preparations were mixed with agar (as roughage material) and wheat germ and cod liver oils as sources of vitamins E, A and D, are recorded in Table 4.

The animals were fed twice daily, at 10 a.m. and 10 p.m. (Table 1). The morning meal consisted of the V.M.R. mixture and any additional energy supplement as required by the experiment; the evening meal contained

all the dietary protein. The food was weighed with great care into non-spilling heavy ointment jars, and moistened with water to prevent scattering. The cages were inspected every morning, and on the rare occasion when food was scattered, this was returned to the dishes. Although it was the aim of the experiment to effect a complete separation of the energy supplement from the protein-containing meal, in order to prevent the interaction effect which occurs when protein and carbohydrate are fed at the same meal (Munro, 1949), a small amount of the morning feed (usually when olive oil was used as source of additional energy) had occasionally to be re-fed with the protein meal in order to maintain a constant calorie intake within each group. Tap water was given ad libitum.

In the first two experiments, the basal diet provided abundant protein; in the third and fourth experiments a protein-free diet was fed, while in a supplementary experiment carried out to confirm and amplify the results of Expt. 4, a small amount of protein was incorporated in the diet. In Expt. 6., in which only the liver was studied, both protein-containing and protein-free diets were used.

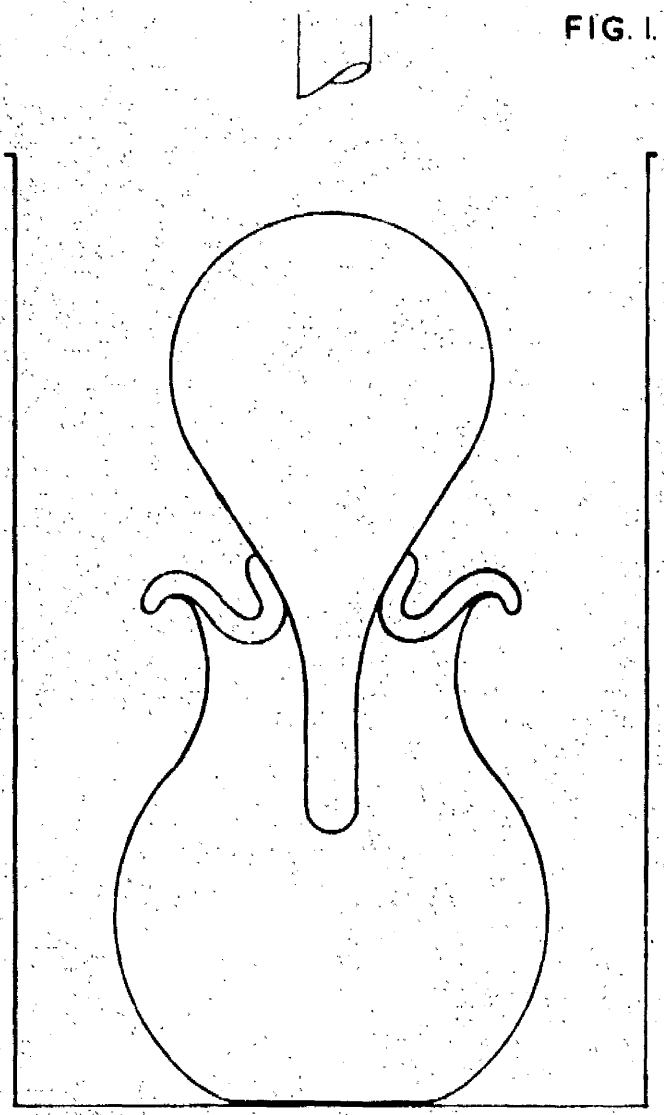
In each experiment, during a seven day preliminary period, the animals received the appropriate basal

ration (Table 1) with the addition of either glucose (3.5 g.) or olive oil (1.5 ml.) depending on which of these constituents was to be varied subsequently as a means of adjusting the energy level of the diet. The rations thus supplied about 40 kg. cal. per day (1200 kg. cal./sq.m./day) and in this way the animals were accustomed to the diet. At this level of energy intake, many of the rats on the protein-containing diet experienced a slight loss of weight. However comparison of the results obtained with these animals with the results from rats which showed a weight gain during the same period did not suggest any influence of these changes in body weight on the subsequent response of protein metabolism to variations in the dietary energy level. As was expected, the animals receiving a protein-free ration experienced a considerable loss of weight during the preliminary period. At the end of this period the rats were distributed at random among the various energy groups. Fat and carbohydrate was then added to or subtracted from the supplemented basal diet in order to provide these different levels of energy intake.

In the first experiment, the animals were arranged in four groups receiving basal Diet A (Table 1) alone, or with glucose supplements of 2.5, 5.0 and 7.5 g.

respectively. In the second experiment fat was used as the variable energy source, in the protein-rich diet, and three groups of animals were fed basal Diet A, or the basal diet with 1.7 or 3.4 ml. of olive oil. In the third experiment, four groups of rats received the protein-free basal Diet B, and the basal diet with 2.5, 5.0 and 7.5 g. of glucose; in the fourth experiment, basal Diet B was fed alone or with supplements of 1.1, 2.2 or 3.3 ml. of olive oil respectively. In the fifth experiment, in which a small amount of protein was incorporated in the diet, the four groups of animals received basal Diet C, and olive oil supplements as for Expt. 4, viz. 1.1, 2.2 and 3.3 ml. In Expt. 6, which was essentially a repetition of Expts. 1 and 3, glucose was added to basal Diets A and B. Thus in each of the six experiments, energy intake varied from about 800-900 kg. cal./sq.m. of body surface area (basal rations alone) to a maximum level of about 1700 kg. cal./sq.m. per day and in this way, different energy levels were provided under otherwise identical conditions. Because of the preliminary week of training to the diet, the animals could readily be persuaded to consume diets providing up to 1700 kg. cal./sq.m. per day. Each animal was maintained for four days at the new level of energy intake, during which urine and faeces were

FIG. I.



HOPKINS SEPARATOR

The urine drips from the funnel on to the pear-shaped glass bulb and passes round into the inner beaker. The faeces fall off the pear into the outer beaker.

collected.

General management and collection of Urine and Faeces.

The rats were housed in a thermostatically controlled environment (maximum temperature range 23-25°C) in single zinc metabolic cages, the bases of which consisted of $\frac{1}{8}$" mesh.

Urine and faeces were collected separately each morning before feeding. At the beginning of the 4-day collection period, the cages were placed on large filter funnels. The mesh of the wire grid forming the base of the cages was such that urine and faeces could drop into the funnel to be collected separately. The separation of urine and faeces was effected by the Hopkins method (Fig. 1.). When the collection was completed, the funnels supporting the cages were washed down with dilute sulphuric acid, and concentrated acid was always added to the collecting vessels to prevent any loss of N as ammonia from the bacterial decomposition of the urine.

Ferric oxide was used as a faecal marker. On the first and last days of the 4-day experimental period, a small amount of ferric oxide was mixed with the morning meal. The beginning and end of the collection period

was then indicated by the passing of coloured faeces. The acidified urine was stored in Winchester bottles, the faeces in beakers, and at the end of the collection period, analyses of urine and faeces were carried out for total N.

Tissue Analyses. The rats were sacrificed between 11.15 and 11.45 a.m., after the last day of collection of excreta, by exsanguination under ether anaesthesia. The livers were rapidly removed, washed in cold water and dried between filter papers, weighed and finely chopped with scissors on a chilled watch-glass. A sample was then taken for determination of protein N and nucleic acids. In Expt. 2., the viscera, comprising the kidneys, heart, spleen, pancreas, testes and intestine (from the lower end of the oesophagus to the beginning of the rectum) were also removed and washed in distilled water. The intestine was slit along its length, and both surfaces quickly washed with water. The tissues were then digested with sulphuric acid for total N estimation.

Nitrogen determinations. N was estimated by a modification of the micro-Kjeldahl method described by Ma and Zuazaga (1942). The volume of each sample was so adjusted

Table 5.

A Comparison of the Methods of N Determination.

1 ml. blood plasma (1 in 5 dilution) digested in each case with 1.5 ml. conc. H_2SO_4

Tube No.	Catalyst	Time of heating after clearing	Removal of Mercury	Titration N/100 H_2SO_4	
		min.			
1	Cu/Se	30	-	8.76	8.76
2	Cu/Se	30	-	8.76	
3	Hg.	30	0.0ml. thiosulphate	8.26	
4	Hg.	30	0.5ml. thiosulphate	8.76	
5	Hg.	30	1.0ml. thiosulphate	8.81	8.79
6	Hg.	30	2.0ml. thiosulphate	8.79	
7	Hg.	15	1.0ml. thiosulphate	8.76	
8	Hg.	15	1.0ml. thiosulphate	8.82	
9	$HgSO_4/K_2SO_4$	30	Zn dust.	8.94	8.98
10	$HgSO_4/K_2SO_4$	30	Zn dust.	9.02	
Blank 1	Cu/Se	30	-	0.14	
2	Hg.	30	1.0ml. thiosulphate	0.14	
3	$HgSO_4/K_2SO_4$	30	Zn dust.	0.28	

Summary of results.		Titration - blank.	As % of (c)
(a)	Cu-Se catalyst (1 & 2) :	8.76 - 0.14 = 8.62	99.1
(b)	Hg. (4, 5, 6, 7 & 8) :	8.79 - 0.14 = 8.65	99.4
(c)	$HgSO_4/K_2SO_4$ (9 & 10) :	8.98 - 0.28 = 8.70	100.0

that the aliquot used for analysis (1-5 ml.) would contain notmore than 1.0 mg. N. Digestion was carried out with 1.5 ml. N-free sulphuric acid in the presence of metallic mercury as catalyst. In spite of its undoubted value as a catalyst in promoting a rapid conversion of organic to inorganic N, the use of selenium appears to result in a definite loss of N if digestion is prolonged for several hours (Patel & Sreenivasan, 1948). On the other hand there is no objection to the use of a selenium catalyst in the micro-Kjeldahl procedure, since the period of digestion is so short. Miller, Plazin and Van Slyke (1948) have however suggested that mercury is preferable to selenium as a catalyst for tissue protein estimations, and so, to clarify the picture, a series of experiments was carried out in which comparison was made of the various methods of N determination, using diluted blood plasma as test substance (Table 5). Although maximum recovery of N was attained with a mercury catalyst, using zinc dust for the decomposition of the mercury-ammonium complex formed during digestion, the use of 1 ml. of a saturated solution of sodium thiosulphate was preferred, since considerable difficulties were encountered in washing from the distillation apparatus the zinc amalgam formed by the zinc dust.

For the distillation of ammonia, the Markham apparatus was used (Markham, 1942). The ammonia liberated in the distillation with 10 ml. of 40% NaOH was trapped in 6 ml. of 2% boric acid, containing the mixed indicator (about 0.05 ml. per estimation) and subsequently titrated with 0.01 N. sulphuric acid. Employing this method it was found that 99.3% of the N of a standard urea solution could be recovered with a distillation period of 1½-2 minutes. This period was used in all N determinations.

Food, faeces, urine and tissue samples were taken for N-determination. The urinary N was determined directly by the micro-Kjeldahl process; the food, faeces and tissues were first brought into solution by digestion with concentrated sulphuric acid in large Kjeldahl flasks, using metallic mercury as catalyst, and samples subsequently taken for the micro-Kjeldahl estimation.

Liver protein-N and nucleic acid estimations.

Estimation of liver protein N and nucleic acid phosphorus were carried out by a slight modification of the Schmidt-Thannhauser (1945) procedure. Each liver was weighed on a chilled watch-glass and coarsely minced with scissors. About 0.5 g. of this was accurately weighed and transferred to a centrifuge tube containing 5 ml. ice-cold 10%(w/v) A.R.

trichloroacetic acid (T.C.A.). The liver sample was thoroughly ground with a glass rod and centrifuged. The precipitated material was washed thrice more with 5 ml. portions of 10% T.C.A. to remove acid-soluble inorganic and organic phosphates. For the extraction of the phospholipids, the residue was extracted in succession with 5 ml. of 80% ethanol and absolute alcohol at room temperature. This was followed by three extractions with 5 ml. portions of a 3:1 ethanol-chloroform mixture, the extractions with this solvent being carried out for periods of 30 minutes at 75°C. The lipid extraction was completed with 5 ml. of redistilled ether. The extracts were combined and the phosphorus content determined by the method of Allen(1940) -(lipid P). The extracted residue containing protein and nucleic acids was allowed to dry, and then incubated with 5 ml. N. NaOH at 37°C for 18 hours. The volume of the alkaline digest was made up to 10 ml. with distilled water, and portions taken for N determination and nucleic acid separation.

Liver protein-N determination. 1 ml. of the alkaline digest was used for liver protein-N determination by the micro-Kjeldahl procedure described above.

Nucleic acid estimations. To 2.5 ml. of the alkaline

digest, neutralized with 2.5 N. sulphuric acid, sufficient 30% ice-cold T.C.A. (1.5 ml.) was added to give a final concentration of 10% T.C.A. The precipitate consisting of deoxyribonucleic acid (DNA) and some protein was centrifuged, and washed twice with 1 ml. portions of 5% ice-cold T.C.A. The combined supernatants containing ribonucleic acid (RNA)were digested with 1.2 ml. 10N sulphuric acid, using hydrogen peroxide as catalyst. The DNA precipitate was dissolved in 1 ml. N. NaOH before digestion. The contribution of phosphorus made to the DNA fraction by tissue phosphoprotein is too small to warrant a further separation, and so may be disregarded in these estimations. The phosphorus content of the digests was estimated by the method of Allen. In calculating the tissue protein N, allowance was made for contribution made by the nucleic acids to the N contained in the alkalin digest. (RNA phosphorus + DNA phosphorus × 1.69).

Statistical Analysis. The relationship between energy intake and N balance or liver protein-N under the different dietary treatments was obtained by calculating the regression coefficients, using the analysis of variance technique to determine the significance of the results (Snedecor, 1946). The regression coefficients represent the change produced over the 4-day experimental period by an increment in energy intake of 1000 kg. cal. /sq.m. of

Table 6.

An example of the statistical methods (analysis of variance) used to analyze the data from Expts.1-6. (The data used are for liver weight in Expt.1; see Table 12 for the original data of which this is the analysis).

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio (F)
Replications	3	159	53.0	2.55
Energy levels	3	553	184.3	8.86
made (Linear regression	1	531	531	25.6
up of((Deviation from linearity	2	22	11	0.53
Residual error	9	187	20.8	-
TOTAL	15	899	-	-

For degrees of freedom of 3 and 9, the variance ratio (F) corresponding to 5% probability and 1% probability are 3.86 and 6.99 respectively. Thus the replications of the experiment are not significantly different. For 1 and 9 degrees of freedom, F is 10.56 at the 1% level of probability; thus the linear regression is highly significant. For 2 and 9 degrees of freedom, F is 4.26 at the 5% level, thus deviations from linearity of regression are not significant.

Table 7.

The effect of varying energy intake by changes in dietary carbohydrate, using a diet rich in protein. Exp. 1.

(4 rats per group)

GROUP	Mean Initial Body Weight	DAILY ENERGY INTAKE		Mean Body Weight Change	N INTAKE AND OUTPUT DURING THE 4-DAY PERIOD.			
		Per rat	Per sq.m. surface area		Intake	Urine	Faeces	Balance
	(g.)	(Cal.)	(Cal./sq.m.)	(g.)	(mg.)	(mg.)	(mg.)	(mg.)
I	238.2	28.2	854	-13.1	1424.8	1509.6	141.0	-225.8
II	248.5	37.5	1103	-6.0	1424.8	1418.6	148.6	-142.4
III	247.9	47.0	1372	+1.4	1424.8	1270.6	167.7	-13.2
IV	239.3	56.3	1664	+8.4	1424.8	1161.1	164.3	+99.4

Statistical Analysis

Difference between energy levels	22.37***	24.99***
Regression coefficient	+26.6***	+410***
Deviations from linear regression	0.06*	0.15*

*** Highly significant (P<0.01)
** Significant (P = 0.05-0.01)
* Not significant (P>0.05)

Table 8.

The effect of varying energy intake by changes in dietary fat, using a diet rich in protein. Exp. 2.

(5 rats per group)

GROUP	Mean Initial Body Weight	DAILY ENERGY INTAKE		Mean Body Weight Change	N INTAKE AND OUTPUT DURING THE 4-DAY PERIOD.			
		Per rat	Per sq.m. surface area		Intake	Urine	Faeces	Balance
	(g.)	(Cal.)	(Cal./sq.m.)	(g.)	(mg.)	(mg.)	(mg.)	(mg.)
I	248.8	28.2	832	-9.8	1494.6	1475.9	123.7	-105.0
II	248.1	42.1	1232	-0.7	1494.6	1425.1	119.0	-49.5
III	252.6	56.1	1610	+5.2	1494.6	1296.6	144.7	+80.3

Statistical Analysis

Difference between energy levels	22.62***	42.25***
Regression coefficient	+19.3***	+237***
Deviations from linear regression	4.06*	4.91*

*** Highly significant ($P<0.01$)
** Significant ($P = 0.05-0.01$)
* Not significant ($P>0.05$)

Table 9.

The effect of varying energy intake by changes in dietary carbohydrate, using a protein-free diet. Exp. 3.

(4 rats per group)

GROUP	Mean Initial Body Weight	DAILY ENERGY INTAKE		Mean Body Weight Change	N INTAKE AND OUTPUT DURING THE 4-DAY PERIOD.			
		Per rat	Per sq.m. surface area		Intake	Urine	Faeces	Balance
	(g.)	(Cal.)	(Cal./sq.m.)	(g.)	(mg.)	(mg.)	(mg.)	(mg.)
I	239.6	29.3	898	-18.5	13.1	303.6	117.2	-407.7
II	238.3	38.6	1179	-16.6	13.1	249.3	132.2	-368.4
III	238.2	48.1	1454	-9.5	13.1	240.1	126.6	-353.6
IV	238.0	56.7	1710	-6.5	13.1	226.8	140.5	-354.2

Statistical Analysis

Difference between energy levels	-7.09 ***		-4.76 **
Regression coefficient	+15.7***		+69.1** ‡
Deviations from linear regression	0.48**		1.78 *

*** Highly significant ($P<0.01$)
** Significant ($P=0.05-0.01$)
* Not significant ($P>0.05$)

‡ Although the linear regression coefficient is statistically significant, the regression coefficient based on log. energy intake is more highly significant ($P<0.01$)

Table 10.

The effect of varying energy intake by changes in dietary fat, using a protein-free diet. Exp. 4.

(4 rats per group)

GROUP	Mean Initial Body Weight	DAILY ENERGY INTAKE		Mean Body Weight Change	N INTAKE AND OUTPUT DURING THE 4-DAY PERIOD.			
		Per Rat	Per sq.m. surface area		Intake	Urine	Faeces	Balance
	(g.)	(Cal.)	(Cal./sq.m.)	(g.)	(mg.)	(mg.)	(mg.)	(mg.)
I	226.5	29.3	918	-13.1	13.1	185.9	129.9	-302.7
II	227.0	38.3	1193	-9.0	13.1	197.2	130.4	-314.4
III	228.5	47.3	1464	-7.1	13.1	214.1	135.0	-336.0
IV	227.8	56.3	1727	+0.5	13.1	191.4	152.1	-333.3

Statistical Analysis

Difference between energy levels	9.46***		1.95**
Regression coefficient	+15.8***		-42.1**
Deviations from linear regression	0.97*		0.41*

*** Highly significant ($P < 0.01$)
** Significant ($P = 0.05$-0.01)
* Not significant ($P > 0.05$)

Table 11.

The effect of varying energy intake by changes in dietary fat, using a diet low in protein. Exp. 5.

(2 rats per group)

GROUP	Mean Initial Body Weight	DAILY ENERGY INTAKE		Mean Body Weight Change	N INTAKE AND OUTPUT DURING THE 4-DAY PERIOD.			
		Per fat	Per sq.m. surface area		Intake	Urine	Faeces	Balance
	(g.)	(Cal.)	(Cal./s.m.)	(g.)	(mg.)	(mg.)	(mg.)	(mg.)
I	247.0	28.3	840	-12.8	105.9	247.6	141.9	-283.5
II	241.0	37.3	1117	-7.5	105.9	178.6	171.7	-243.9
III	246.8	46.3	1366	-6.8	105.9	209.2	141.4	-244.7
IV	238.8	55.3	1656	-2.5	105.9	218.5	146.3	-258.9

Statistical analysis

Difference between energy levels	1.36*
Regression coefficient	+26.8*
Deviations from linear regression	1.52*

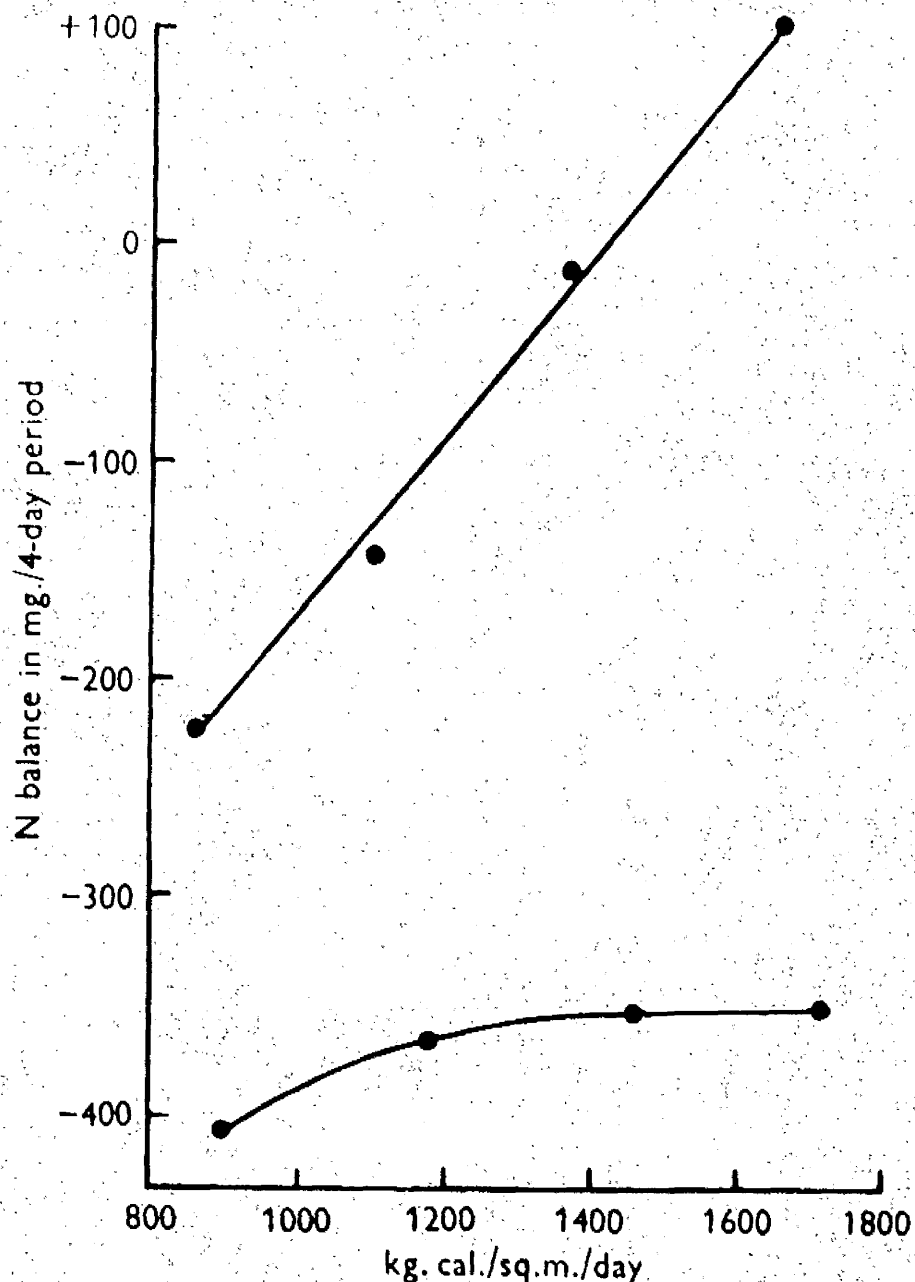
* Not statistically significant ($P > 0.05$)

body surface area. In none of the experiments did the regression lines deviate significantly from linearity. Table 6 gives an example in detail of one such analysis of variance.

RESULTS.

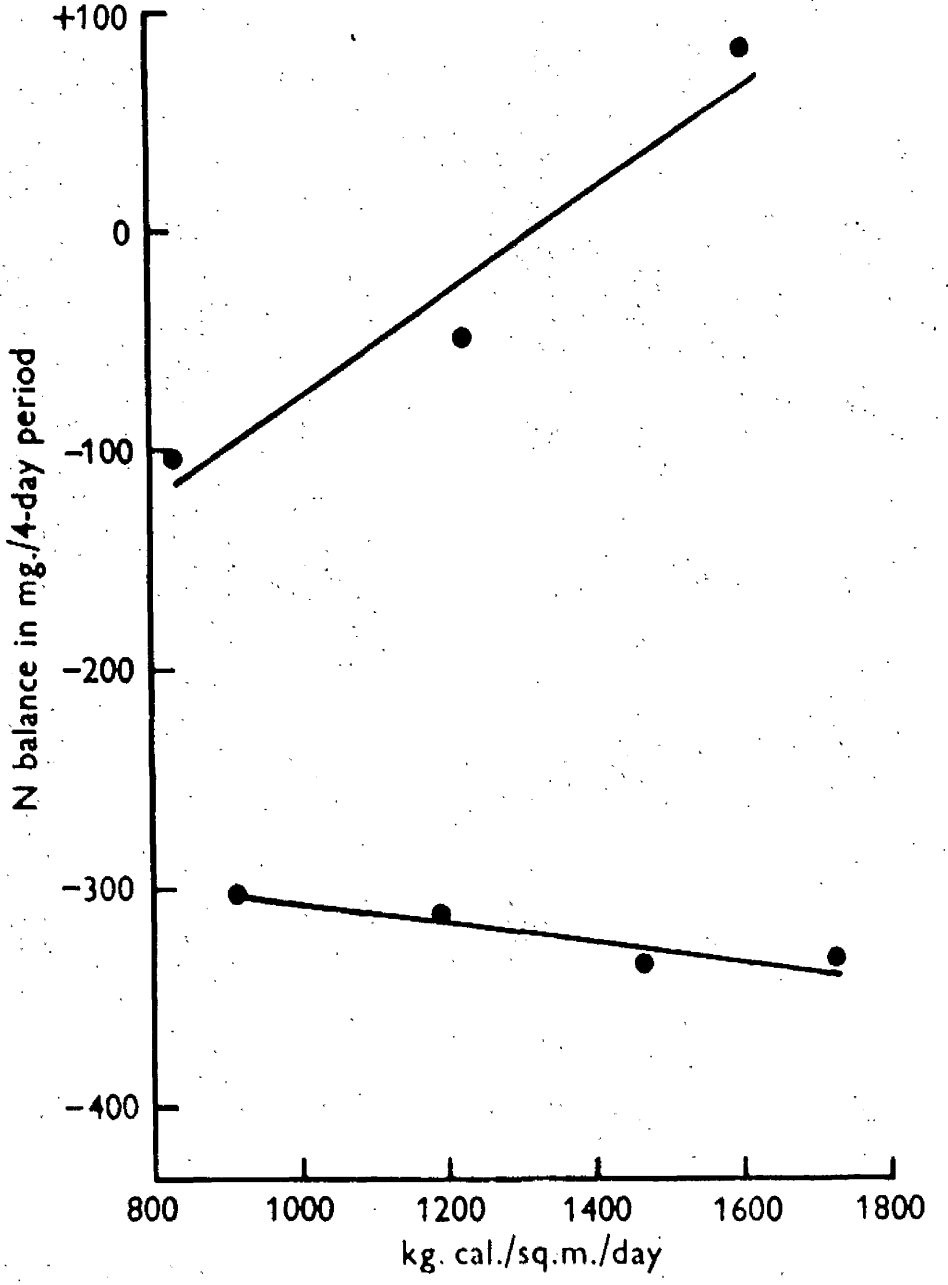
In these six experiments, the relationship of protein utilisation to caloric intake was clearly defined. Within each experiment the different groups of animals were fed the same amount of protein but at different planes of energy intake, the variations being achieved by addition to the basal diet of extra carbohydrate or fat. The daily energy intake and the changes in body weight and N-balance was recorded in Tables 7-11, and show that there was a linear relationship between energy intake and change in body weight. Body weight was influenced by dietary energy level to a greater extent in experiments in which a protein-containing ration was fed than in the experiments in which the diets were low in, or free from protein. This difference, which is statistically significant when carbohydrate but not when fat was used as variable energy source, may be attributed to the deposition of protein in the body, as revealed by the considerable effect of energy intake on N-balance

Fig. 2.



The effect on N balance of adding energy in the form of carbohydrate to the diet. Upper line - protein-containing diet (Exp. 1). Lower line- protein-free diet (Exp. 3).

Fig. 3.



The effect on N balance of adding energy in the form of fat to the diet. Upper line - protein-containing diet (Exp. 2). Lower line - protein-free diet (Exp.4)

when the diet contained protein, but not with the protein-free diet.

The influence of energy intake on N-balance was found to be conditioned by the level of protein in the diet. When carbohydrate was used as variable energy source in a diet providing adequate amounts of protein N-balance was affected in a strictly linear fashion by the increments in energy intake (Table V and Fig. 2). On the other hand, when the animals were maintained on a protein-free ration, it appeared that the same additions of carbohydrate to the diet influenced N-balance up to a certain point only (about 1200 kg. cal./sq.m. per day) and to a very limited extent. In fact under these conditions, the relationship could be described more accurately as a dependence of N-balance on the logarithm of energy intake than on energy intake itself. This suggests a curvilinear relationship as shown in Fig. 2, which indicates that when a diet devoid of protein is fed, the effect on N-balance of additions of carbohydrate is progressively decreased as energy intake is increased.

In the case of additions of olive oil to the diet (Tables 8 and 10; Fig. 3.) at adequate levels of protein intake, increasing amounts of fat brought about a decrease in the negative N-balance, through equilibrium to positive balance; the relationship was again a linear one, and

using equivalent increments of fat, improvement in N-balance was of the same order of magnitude as with carbohydrate. Fat added to the protein-free diet caused no significant change in N-balance. In a further experiment (Expt. 5) in which a small amount of protein was incorporated in the diet, the inability of energy in the form of fat to improve N-balance on a protein-poor diet was again demonstrated (Table 11).

Thus we find that supplementation of a diet, providing adequate amounts of protein, with additional calories, irrespective of the source of the energy supplement, will bring about an improvement in N-balance, whereas on a protein-free diet, the caloric effect is dependent on the nature of the energy source as well as its magnitude, carbohydrate having an effect over a limited range only, and fat being completely without effect. Comparison of the regression coefficients (Tables 7-10) shows that the influence of protein level is highly significant in the case of both carbohydrate and fat.

In Expts. 1-3, improvements in N-balance due to the various dietary treatments were due essentially to a fall in urinary N output, the faecal N excretion actually showing a slight tendency to rise with increasing energy intake (Tables 7-9).

Table 12.

The effect of varying energy intake by changes in dietary carbohydrate, using a diet rich in protein.

(4 rats per group)

GROUP	DAILY ENERGY INTAKE Per sq.m. surface area	LIVER ANALYSIS	
		Mean Weight	Protein N
	(Cal./sq.m.)	(g.)	(mg.)
I	854	6.6	183.5
II	1103	7.4	197.5
III	1372	7.7	206.5
IV	1664	8.2	210.5
Statistical Analysis			
Difference between energy levels		8.86 ***	3.68 **
Regression coefficient		+1.91 ***	+53.0 ***
Deviations from linear regression		0.53 *	0.42 *

*** Highly significant ($P<0.01$)
** Significant ($P = 0.05 - 0.01$)
* Not significant ($P>0.05$)

Table 13.

The effect of varying energy intake by changes in dietary fat, using a diet rich in protein.

(5 rats per group)

GROUP	DAILY ENERGY INTAKE Per sq.m. surface area	LIVER ANALYSIS	
		Mean Weight	Protein N
	(Cal./sq.m.)	(g.)	(mg.)
I	832	7.1	184.6
II	1232	7.9	206.4
III	1610	8.9	220.4

Statistical Analysis

Difference between energy levels	19.03***	21.81***
Regression coefficient	+ 2.21***	+46.1 ***
Deviations from linear regression	0.21*	0.51 *

*** Highly significant ($P<0.01$)
** Significant ($P = 0.05-0.01$)
* Not significant ($P>0.05$)

Table 14.

The effect of varying energy intake by changes in dietary carbohydrate, using a protein-free diet.

(4 rats per group)

GROUP	DAILY ENERGY INTAKE Per sq.m. Surface area	LIVER ANALYSIS	
		Mean Weight	Protein N
	(Cal./sq.m.)	(g.)	(mg.)
I	896	6.0	146.0
II	1179	5.7	142.5
III	1454	6.2	136.7
IV	1710	7.2	139.4

Statistical Analysis

Difference between energy levels	10.54***	2.62*
Regression coefficient	+1.41***	-11.4*
Deviations from linear regression	6.80**	2.11*

*** Highly significant ($P<0.01$)
** Significant ($P=0.05-0.01$)
* Not significant ($P>0.05$)

Table 15.

The effect of varying energy intake by changes in dietary fat, using a protein-free diet.

(4 rats per group)

GROUP	DAILY ENERGY INTAKE Per sq. m. surface area	LIVER ANALYSIS	
		Mean Weight	Protein N
	Cal./sq.m.	(g.)	(mg.)
I	918	6.0	139.7
II	1193	6.3	137.4
III	1464	6.8	148.3
IV	1727	6.8	134.3

Statistical Analysis

Difference between energy levels	6.54**	0.68*
Regression coefficient	+1.00***	-4.13*
Deviations from linear regression	0.80*	0.82*

*** Highly significant (P<0.01)
** Significant (P=0.05-0.01)
* Not significant (P>0.05)

Table 16.

The effect of varying energy intake by changes in dietary fat, using a diet low in protein.

(2 rats per group)

GROUP	DAILY ENERGY INTAKE Per sq.m. surface area	LIVER ANALYSIS	
		Mean Weight	Protein N
	(Cal./sq.m.)	(g.)	(mg.)
I	918	6.0	139.7
II	1193	6.3	137.4
III	1464	6.8	142.3
IV	1727	6.8	134.3

Table 17.

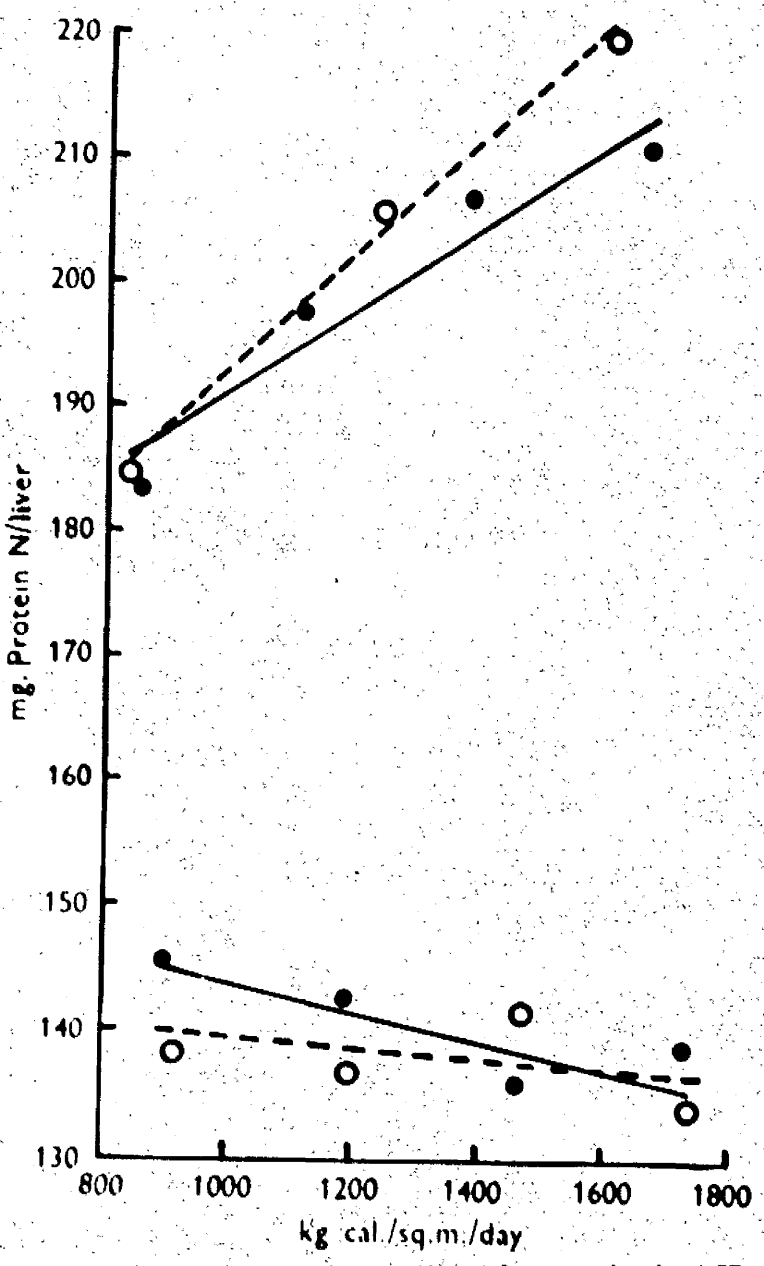
The response of liver protein to changes in energy intake (carbohydrate) at different levels of protein intake.

(10 rats per group)

GROUP	DIET	DAILY ENERGY INTAKE	Liver-protein N per rat	Regression coefficients
		(Cal./sq.m.)	(mg.)	(mg.)
I	Protein-containing	820	191	+28.4
II		1570	213	
III	Protein-free	880	142	-12.3
IV		1650	133	

Statistical Analysis. Analysis of variance demonstrates a significantly different response to changes in energy intake at the two levels of protein intake ($P<0.01$ for interaction). Both regression coefficients differ very significantly from zero and from one another ($P>0.01$)

Fig. 4.



Changes in the total amount of protein N per liver produced by variations in intake of carbohydrate (●——●) or fat (O....O). The upper two curves (Exps. 1 and 2) represent the regression lines obtained when the diet contained protein. The lower two regression lines (Exps. 3 and 4) were obtained when the diet was free from protein.

Liver Analyses. Changes in the total amount of protein in the liver were found to follow closely the variations in N-balance (Tables 12-16). In the experiments in which a high-protein diet was fed, the only ones in which a progressive improvement in N-balance was found, the protein content of the liver was also found to increase linearly with changes in the caloric value of the diet (Fig. 4), whether produced by addition of carbohydrate or fat. On the other hand, on protein-free and low protein diets, where the excretion of N was largely independent of the energy level of the diet, there was a slight, although not statistically significant tendency for the amount of liver protein to fall as energy intake rose (Expts. 3 and 4). These findings were supported by the results of a confirmatory experiment (Expt. 6) in which the effect of additional energy (carbohydrate) on the livers of animals receiving diets either rich in or free from protein were studied simultaneously (Table 17). Basal diets A and B were supplemented with glucose to provide two planes of energy intake, representing about 850 and 1600 kg. cal./sq.m. of body surface area. On the high-protein diet, liver protein increased in amount with the rise in carbohydrate intake, while on the protein-free diet, the amount of protein in the liver fell to a significant extent.

Table 18.

The effect of adding fat to a protein-containing diet on the N content of the liver and other viscera, compared with the changes produced in the N-balance of the whole rat.

(5 rats per group)

GROUP	DAILY ENERGY INTAKE Per sq.m. surface area	N-balance per 4-day period	Total liver N per rat	Total viscera N per rat
	(Cal./sq.m.)	(mg.)	(mg.)	(mg.)
I	830	-105.0	240.9	460.9
II	1230	-49.5	271.9	480.1
III	1610	+80.3	287.7	471.9

Statistical Analysis

Regression coefficients +237 ** +60.3** +14.5*

** Statistically highly significant ($P<0.01$)
* Not statistically significant ($P>0.05$)

Comparison of the regression coefficients at the two protein levels shows that the influence of protein level on the effect of energy was again highly significant.

In Expt. 2 in which energy from fat was added to a protein-containing diet, the partition of the change in N-balance was studied. In addition to analysis of the liver for total N, estimations of the N-content of the combined viscera, comprising the kidneys, heart, spleen, pancreas, testes and intestine, were also performed. From a consideration of the results summarized in Table 18, it is apparent that energy intake had no significant effect on the N content of the viscera. Moreover, comparison of the changes in total liver N with the alteration in N balance suggest that of the total amount of N retained, no more than about a quarter may be accounted for by the liver. The major change in N balance caused by energy increments must have occurred in the carcass.

DISCUSSION.

The purpose of the present investigation was to assess in what measure protein utilization was dependent on the availability of energy in the diet. The main problem to be settled was whether a severe restriction or a large

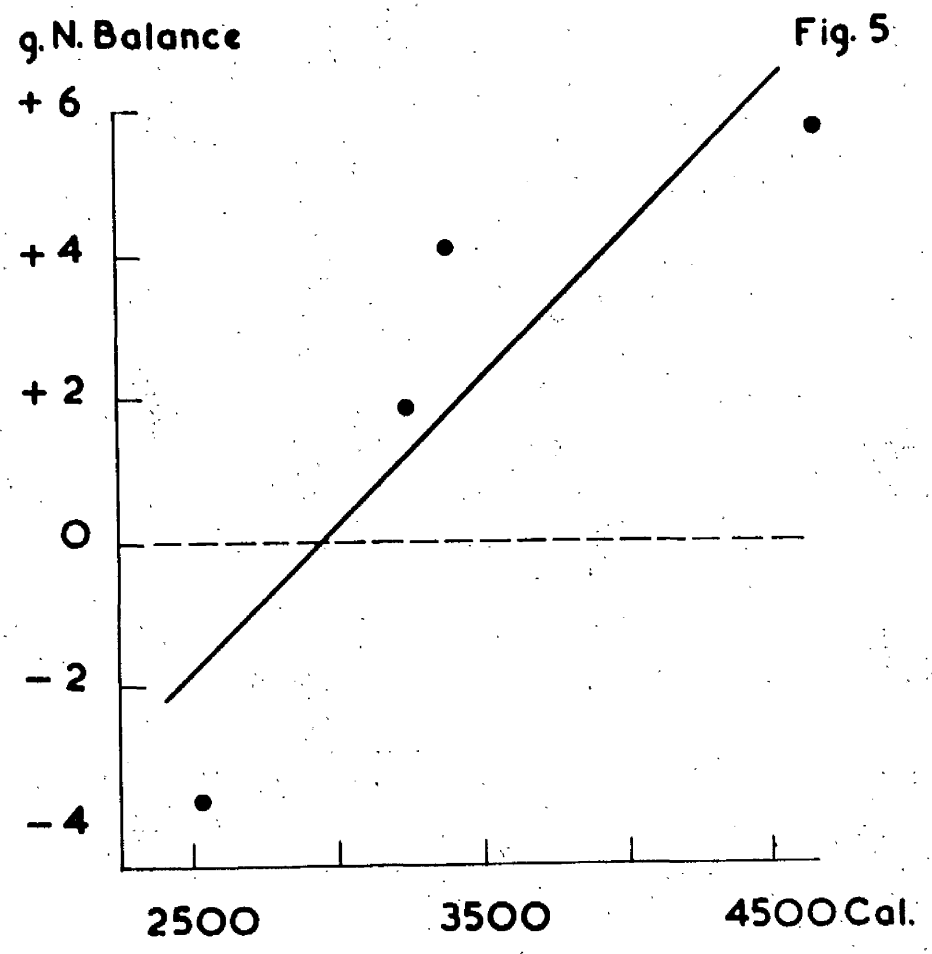
excess of energy was required to produce changes in N-balance, or whether N-balance, and hence protein metabolism in general, was in a state of unstable equilibrium with caloric intake, responding to the slightest variation in energy supply. Furthermore it was proposed to determine whether fat and carbohydrate are equally effective in bringing about these changes in protein metabolism. From the results, a clearly defined picture of the metabolic interaction of fat, carbohydrate and protein was obtained.

Nitrogen balance. When the energy content of an otherwise constant diet, providing adequate amounts of protein, was varied so that it ranged from sub-maintenance levels to an energy surplus promoting an increase in body weight, (Expts. 1 and 2) there was an alteration in N-balance, the magnitude of which was related to the magnitude of the energy change in a strictly linear fashion. (Figs. 1 and 2; Tables 7 and 8). Under the experimental conditions described, fat and carbohydrate, fed in equivalent amounts, were found to affect N-balance to a degree which is not statistically different. This is in complete agreement with the experiments on growing rats of Bonshardt, Paul, O'Doherty and Barnes (1948) who noted that if some quantity of carbohydrate were already present in the diet, further supplements of carbohydrate were no more effective than equivalent amounts of

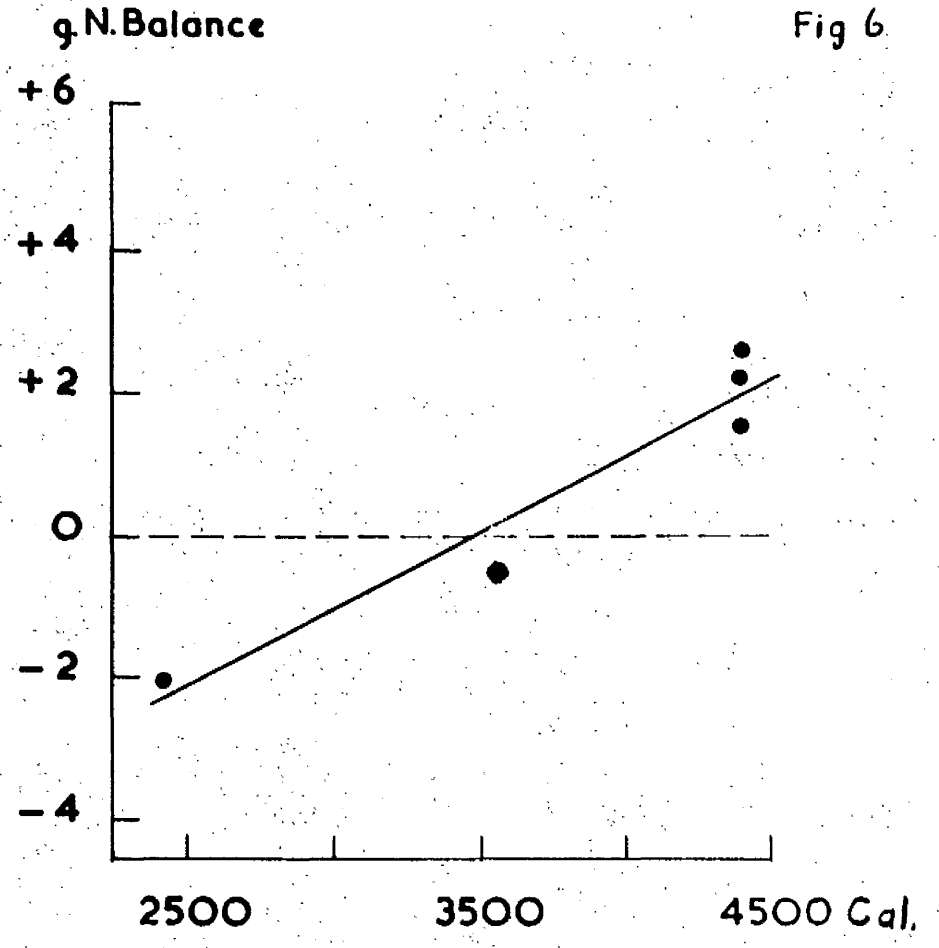
fat in encouraging N retention. Similar negative results with regard to a demonstration of the superiority of carbohydrate are also seen in the data reported by Forbes and his colleagues (Forbes, Swift, Elliott & James, 1946; Forbes, Swift, Thacker, Smith & French, 1946) for rats receiving isocaloric maintenance diets containing various proportions of carbohydrate and fat, and in the experiments of Samuels, Gilmore and Reinecke (1948) in which two diets of similar protein and energy content were fed, one being almost devoid of carbohydrate, and the other low in fat.

Under normal conditions of nutrition then, when the diet contains adequate amounts of protein and energy (some of which at least is supplied by carbohydrate), the dependence of protein utilisation on energy intake can readily be demonstrated.

These studies using protein rich diets are for the most part in agreement with scattered observations in the literature. Only a few experiments have been designed to investigate the extent to which the utilisation of amino acids for protein synthesis, derived either from dietary protein or tissue catabolism, was influenced by caloric intake, but these, viewed in conjunction with the results of the present investigations, suffice to permit a general picture of the quantitative aspects of relationship



Variations in N balance produced by adding fat to the diet of a human subject. The data are plotted from an experiment reported by Rubner (1903). The line represents the regression Y = −11.98+0.00405X, where X is the energy intake in kg. cal. and Y is the N balance in g.



Variations in N balance produced by adding carbohydrate to the diet of a human subject. The data are plotted from an experiment reported by Neumann (1919). The line represents the regression $Y = -7.54 + 0.00215X$, where X is the energy intake in kg. cal. and Y is the N balance in g.

to be presented. Support comes from experiments reported by Rubner (1903) and Neumann (1919) in which diets containing proteins were fed to human subjects at a level inadequate to maintain N equilibrium. In each case, addition to the basal diets of increasing amounts of fat or carbohydrate caused a progressive improvement in N-balance through N equilibrium to a considerable N retention. Inspection of their data, recalculated in graph form in Figs. 5 and 6, shows that the relationship of N-balance to energy intake was approximately linear. No comparison can be made however of the beneficial effect of calories supplied as fat with those supplied in the form of carbohydrate, since the experiments were not carried out under identical conditions. Cannon and his associates (Benditt, Humphreys, Wissler, Stephee, Frazier & Cannon, 1948) encountered the same phenomenon in the protein-deficient rat, while studying the repletion of tissue proteins. The extent to which a constant level of dietary protein could be utilized for protein synthesis by these animals was used as a measure of the degree of protein sparing achieved by increasing the calorie intake. A linear relationship between protein utilization and calories consumed was found to hold over a wide range of energy intake up to a certain critical energy level. At this point we may assume that

protein intake became the limiting factor in the repletion of tissue proteins, since further deposition was induced by raising the protein intake. The limits imposed on protein synthesis by a deficiency of calories are clearly illustrated in a recent publication of Rosenthal and Allison (1951). These authors described studies with dogs in which a progressive reduction in calorie intake, from a high to a low energy level, produced an elevation in N output from a positive to a negative N-balance. From their data however it appears that when the animals were near N equilibrium, considerable changes in energy consumption had little effect on N-balance. The reason for this difference in behaviour of the dog from that of man and the rat remains obscure.

When protein was omitted from the diet (Expts. 3 and 4) the picture of the metabolic interaction of fat, carbohydrate and protein was completely changed. The variations in the diet brought about by altering the consumption of fat were without effect on N-balance. It appeared that under the conditions of a protein free regimen, the tissue amino acids, in this case derived solely from endogenous sources, were present in amounts insufficient to permit the demonstration of an interaction. In order to test this point, a small amount of casein was incorporated in the diet in a

fifth experiment. Essentially the same result was obtained. At low levels of protein intake, the relationship between N-balance and the energy content of the diet(supplied principally as fat) could no longer be demonstrated. On the other hand, in the case of addition of carbohydrate to a protein free ration, a significant improvement in N-balance was evident at levels of energy intake up to 1200 kg. cal./sq.m. of body surface area. Under these conditions then, carbohydrate appeared to have a specific protein-sparing effect which was not shared by fat. An explanation of this phenomenon may be found in a recent publication of Munro and Thomson(1953). These investigators observed that administration of glucose to fasting human subjects and rats caused a considerable reduction in plasma amino-N concentration. Ingestion of fat on the other hand had only a slight effect on amino N which was of doubtful significance. Furthermore, estimation of individual amino acids in the plasma following the administration of glucose revealed a striking similarity between the proportions in which the amino acids were removed from the plasma and the proportions in which the same amino acids are required for the maintenance of N-balance, according to the data of Rose (1949). It was concluded that glucose was able to stimulate protein synthesis by promoting the retention of amino acids circulating in the

blood.

Reports in the literature on the effect on N-balance of the amount fed of a protein-free diet are generally in agreement with the results of the present studies. Although it has been shown that when the consumption of such a diet is reduced to a level less than 50% of the amount eaten voluntarily by the rat, N-balance is adversely affected (Swanson & Clark, 1950; Willman et al., 1947) alteration in intake of a less drastic nature seems to be without effect on N output (Mitchell, 1923). Later experiments (Treichler & Mitchell, 1941; Vera & Gurd, 1947) have supported this conclusion. In investigations with protein-free diets on species other than the rat, N output appears to be more dependent on the amount of the diet eaten. One of the earlier reports in which an attempt was made to correlate N excretion and energy intake was that of Murlin (1907). To dogs which had been subjected to a two-day fast, he fed graded amounts of carbohydrate. With each successive increment of carbohydrate, a comparable improvement in N-balance occurred. He further observed that when the animals' energy requirements had been satisfied, additional energy supplements were even more efficient in causing a fall in N output. In Wimmer's experiments on dogs (1912) essentially the same picture was obtained. Allison

and Anderson (1945) added carbohydrate to a protein-free diet to raise the energy intake of their dogs from 80-100 Cal./kg. body weight, and observed a depression in N out-put.

Conversely, restriction of intake of a protein-deficient diet beyond a certain minimum results in a progressive decrease in N retention. For example Johnson, Deuel, Morehouse and Mehl (1947) in an attempt to determine the caloric requirement to establish a minimum N excretion in human subjects on protein-deficient diets, presented evidence that this minimum could be achieved on an intake of 2000 cal., but not of 1200 cal. or less. This work was substantiated in the more recent study of Deuel (1948) who noted that a maximum protein-sparing effect of non-protein calories might be obtained with intakes of 1500 cal. per day, but not when the level was reduced to 600 cal. per day.

These various studies on man and the dog show that, in contrast to the rat, N-balance may be improved on protein-poor diets by increasing the energy consumed in the form of carbohydrate up to and even beyond the animal's normal requirements. There are no comparable data available for fat as energy source.

An explanation for the relation between energy intake and protein metabolism revealed in experiments 1 and 2, in which the diet contained adequate amounts of protein, might

at first might seem to lie in the demands for energy made by the animal organism. Thus in a state of undernutrition, N loss might be attributed simply to catabolism of protein for energy-yielding purposes, and with an increase in energy from other dietary sources, a depression in N output to sparing of protein in such reactions. If this were so, one would expect that, as caloric intake is progressively diminished, a greater and greater proportion of the dietary protein would be diverted to yield energy. This however is not so. Studies on dogs have shown that for a given increase in protein consumption, a similar improvement in N balance occurs over a wide range of energy intake. Only with a drastic reduction of caloric intake (to less than 25% of the animal's normal consumption) was it found impossible to attain N-balance at any level of protein intake (Allison & Anderson, 1945; Allison, Anderson & Seeley, 1946). Similar results were obtained by Bosshardt et al.(1948) using rats and mice. It was demonstrated that with increasing restriction of calories, protein utilization remained constant until a critical caloric intake was reached. Beyond this point reduction resulted in a rapid decrease in N retention. The critical level of energy intake was represented by 50% of normal requirement in the experiments of Swanson, Willman & Bush(1946). Below this level, the beneficial effect of a

protein supplement (whole egg) to a low protein diet was completely lost, while a further reduction to 25% of the voluntary consumption increased protein catabolism to a point equivalent to starvation. The hypothesis then that the amount of dietary protein catabolised for the production of energy is inversely proportional to the amount of energy provided from other dietary constituents is untenable in the light of these experiments.

An alternative and more comprehensive explanation is in terms of the factors which affect protein synthesis. The synthesis of protein depends on the simultaneous presence in the tissues of both amino acids and calorific energy, either of which may be a limiting factor. In the absence of amino acids from a dietary source (Expt. 3), energy intake was found to influence N retention up to a certain point only, probably by promoting the reutilization of amino acids circulating in the blood. The limited nature of this source however prevents the demonstration of the metabolic interaction beyond this point. When protein is included in the ration, this limitation no longer obtains, and a correlation between the efficiency of utilisation of dietary protein for tissue protein synthesis and the availability of energy in the tissues is readily demonstrated.

Liver Protein. In the experiment under discussion, a study

was made of the changes in liver protein which accompanied N-balance variations. Liver protein N was found to respond to energy intake in an analogous fashion, a rise or fall in N excretion being reflected in the amount of liver protein. The protein content of the liver varied in amount with increments in the energy content of the diet rich in protein (Expts. 1, 2 and 6) but similar increments in the diets either free from, or containing little protein were without beneficial effect.(Expts. 3, 4, 5 & 6). The results obtained in the case of protein-containing diets are in agreement with the observations of Campbell and Kesterlitz(1948) that a reduction in energy intake on a diet containing substantial amounts of protein reduced the amount of liver protein in proportion to the extent of the caloric restriction. Although in Expt. 2 a slight improvement in N-balance was obtained by addition of carbohydrate to the protein-free diet, liver protein showed a slight tendency to decrease (see Fig.4). This negative response of liver protein to increasing energy intake on a protein-free diet was even more marked in the confirmatory Experiment 6, in which the regression coefficient was statistically significant. A similar finding has recently been noted by Rosenthal and Vars(1954). Some unpublished experiments of Munro and Thomson(1955) may help in the interpretation of these findings. In a study of the

effect of feeding glucose and fat on the incorporation of isotopically labeled methionine(^{35}S) into the proteins of muscle and viscera of fasting rats, it was found that while neither glucose nor fat had any effect on the uptake of the label by the liver, glucose, but not fat, caused a marked increase in the rate of incorporation of the amino acid into skeletal muscle. It was concluded that glucose had promoted a synthesis of muscle protein, presumably at the expense of the plasma amino acids, which would reduce the amount available to the liver.

Distribution of retained N. The bodily distribution of the N retained when energy increments were added to the protein-rich diet have been investigated in Experiment 2. Analyses of the viscera revealed that those tissues, with the exception of the liver, could be excluded as potential stores of the protein N retained (Table 18). It would appear that under the conditions of the present studies, as in the experiments of Addis and his colleagues (Addis, Poo and Lew, 1936a, b and c), the N deposited during a positive N-balance is not explained by a large increase in the protein content of any one tissue, but that most of the spared N is distributed throughout the body. Although changes in the total N of the liver accounted for no more than about a quarter of the alteration in N-balance, the effect of this on liver

PART II.

THE INFLUENCE OF PROTEIN AND ENERGY INTAKE ON RIBONUCLEIC ACID METABOLISM IN THE LIVER.

Section 1

On the Amount of Ribonucleic Acid in the Liver.

composition was considerable, because of its small size. If we take as reference standard the amount of protein in the liver at an energy intake of 1200 kg. cal./sq.m., then a change in energy intake of 1000 kg. cal./sq.m. produced a change of 23% in the total N content of the liver. The N in the carcass of a 250 g. rat amounts to about 6 g., so that the N retained or lost from the body per 1000 kg. cal./sq.m. and not accounted for by an alteration in liver N would represent a change of only 3% in carcass N. The relative magnitudes of the change in the N content of the liver and carcass are comparable to those found by Addis and his associates. These authors analysed the tissues of rats which had been fasted for 7 days in an attempt to assess the contribution made by each tissue to the total loss of protein(Addis et al., 1956a). Although 62% of the loss could be accounted for by the muscles, skin and skeleton, and only 16% by loss of protein from the liver, this represented a change of only 6% in the carcass and muscle proteins, while the liver lost 40% of its initial total protein content. The similarity in the ratio of the change in liver protein content to carcass protein content, to the relationship found for the effect of changes in energy intake lends support to the hypothesis that the influence of protein intake and of energy intake on protein metabolism operates through a common mechanism, namely their effect on protein synthesis.

INTRODUCTION.

In Part I of this thesis, experiments are described in which the influence of energy intake on protein metabolism was studied in the rat. Diets either rich in protein or deficient in protein were fed, in combination with various levels of energy intake (from about 850 to 1700 kg. cal./ sq. m. body surface area). A positive response of N balance and the amount of protein in the liver to increments in energy intake was observed when the diet provided adequate amounts of protein. On the other hand, when protein was omitted from the diet, variations in energy intake had little or no effect on N-balance, high energy intakes causing if anything a slight reduction in the amount of protein in the liver. It was thought that the conditions provided in these experiments might be suitable for studying the alleged relationship between ribonucleic acid (RNA) metabolism and protein synthesis. Before describing these studies however, a brief survey of the evidence, which has led to the view that RNA is in some way concerned in the processes leading to protein formation, will be presented.

One of the earliest and basic observations in this field was that of Brachet (1933) who noted a parallel between nucleic acid content and embryonic development. As interest in this subject spread, and now and specific

techniques for the cytochemical detection of both types of nucleic acid and protein were developed, it was revealed that a high content of nucleic acid was not peculiar to embryonic tissues, but that all cells actively engaged in the production of protein, irrespective of their source, animal, plant or microorganism, are characterized by a cytoplasm rich in nucleic acid. Progress in this field has been largely at the hands of Caspersson and Brachet. During the past decade, these investigators working simultaneously and independently with techniques based on entirely different physicochemical principles, have amassed a considerable body of circumstantial evidence implicating RNA in the protein biosynthetic mechanism.

For the investigation of cellular nucleic acid and protein metabolism, Caspersson developed a microspectrographic method of determination, based on the high selective light absorption of the nucleic acids and certain amino acid residues, in the central ultraviolet region (Caspersson & Schultz, 1939). The absorption at 260 mμ, due to purine and pyrimidine bases was used as a measure of the amount of polynucleotide material, while that at 280 mμ, due to absorption by aromatic amino acids, was used to estimate protein. This method, which has the advantage of being applicable to living tissue, has been so refined that it

permits the estimation of protein and nucleic acid in different portions of a single cell, but does not allow a distinction to be made between the two main types of nucleic acid.

For his attack on the problem, Brachet (1940) has used histochemical methods. Specific staining techniques were supplemented with a study of the effects on microscopic preparations of purified nucleases, hydrolysing one or other of the nucleic acids.

With the application of these quantitative cytochemical procedures to a great variety of tissues, Brachet, Caspersson and their associates have succeeded in demonstrating a striking correspondence between the RNA content of a cell and its activity in synthesizing proteins. These findings have since been repeatedly confirmed in numerous chemical studies (see Davidson, 1947).

Since protein formation is quantitatively the primary metabolic process of growth, embryonic tissue was an obvious choice for the study of the relationship between nucleic acids and protein synthesis. Growth in mammalian tissues is not however restricted to the embryo; in the adult animal growth occurs normally in some tissues, as for example during the production of new egg cells in the ovary, or of blood cells in bone marrow tissue. A similar process

occurs during the regeneration of damaged tissues. Moreover the normal function of some cells is associated with a very intense metabolism of protein; most notable are the cells whose task it is to produce a continuous and abundant secretion of enzymes. The relationship between RNA and protein synthesis in these various tissues has been explored and studies extended to the processes leading to protein formation in microorganisms. Reference to the results of some of these experiments will now be made.

Growth in mammalian tissues. Embryonic growth proceeds with very great speed, especially during the earliest stages of development; for example, between the second and tenth day of incubation, the chick embryo increases more than twenty times in weight (Caspersson, 1950). When growth is most rapid, the cells have enormous nuclei, and show very high nucleotide absorption at 260 mμ in the cytoplasm. Corresponding measurements on adult liver show considerable concentrations of nucleotides, but not more than one tenth of that found in the rapidly growing cells. Brachet(1941) combining a cytochemical technique with the chemical estimation of furfural has also revealed an increasing RNA concentration during embryonic growth. Confirmation is found in the work of Novikoff and Potter (1948a) who by quantitative chemical estimations showed that the content

of nucleic acid, especially of RNA, rises only during the period of embryonic development when protein metabolism is most conspicuous.

Davidson and Waymouth (1943, 1944 and 1945) have compared the RNA and deoxyribonucleic acid (DNA) content of embryonic and adult tissues, and have found the concentration of both nucleic acids to be higher in embryonic tissues than in the corresponding adult tissues. The same investigators (Davidson, Leslie & Waymouth, 1949) have correlated changes in the concentration of both ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) with growth in chick embryo fibroblast explants growing in vitro. In the resting state, when the cultures were maintained in Tyrode solution, the ratio RNAP/DNAP was found to lie between 2.2 and 2.8. In contact with a growth-promoting medium, however, there was an immediate and rapid positive response of RNAP; DNAP on the other hand increased only slowly after an initial delay of at least 48 hours, at which time the RNAP/DNAP ratio had risen to 7.4. After 120 hours, when maximal growth of the culture was reached, the ratio had fallen to 5.2 where it remained steady. In these experiments then, there appears to be a marked correlation between the amount of RNA and the activity of the cell in synthesizing protein. Similarly growing

oocytes contain large amounts of RNA at the moment when protein synthesis commences (Brachet, 1952).

In the adult animal, a high concentration of RNA is readily demonstrated in the cytoplasm of the tissue responsible for the production of new egg cells in the ovary; the egg nurse cells of the adult female Drosophila have been studied by Caspersson (1950), and present a typical picture of cells engaged in intense production of cytoplasmic proteins.

Another example of growth in the adult animal is afforded by the haematopoietic system. Circulating blood cells have a limited span of life, and new ones are continuously being formed in the bone marrow. The formation of new cells takes place from a relatively small and constant number of stem cells by means of division and maturation through morphologically characteristic stages. From a study of absorption spectra at points in the cytoplasm of red blood cells at various stages of development, Thorell (1947) found that it was only prior to and during the principal increase in cell substance that the cell contains a high concentration of RNA in its cytoplasm, that is, during the growth phase, when formation of basic cell protein substances is taking place, the cell has a chemical organization resembling that of growing cells in general -

e.g. embryonic cells. During maturation, the activity of the cell in protein generation decreases, parallel with a continuous diminution in the concentration of cytoplasmic RNA and in the nucleolar mass. Only when the RNA content of the cell has reached a low level are the first signs observed of the processes which lead to the differentiation of the cellular proteins, characterized by the formation of haemoglobin. Data obtained for white blood cell renewal show essentially the same relations.

A third type of growth to be considered is tissue regeneration. Regenerating rat liver is a particularly favourable tissue in which to study the relationship between nucleic acids and growth, since it provides relatively abundant quantities of homogeneous rapidly growing tissue. Following the removal of about two thirds of the liver, enlargement of the remainder takes place, which according to the work of Brues, Drury and Brues(1936) is due to true hyperplastic growth, until it attains the size of the original liver in the span of a few days. Stowell (1948) using photometric histochemical methods demonstrated the presence of larger nucleoli and higher concentrations of RNA in the cytoplasm of regenerating liver cells than in normal liver tissue. Furthermore, the rate of increase of RNA has been found to be greatest during the period of most

rapid growth (Novikoff & Potter, 1948b; Lagerstedt, 1949).

Protein synthesis associated with function. The RNA content of different organs of the adult animal show considerable variations (Davidson, 1947). Certain tissues which are physiologically very active, for example the heart, the kidneys, and voluntary muscles, are poor in RNA, while glandular organs, such as pancreas, salivary glands, and the intestinal mucosa are relatively rich in RNA. It is significant that the function of certain cells of the latter mentioned tissues is combined with a very intense production of protein. In the rabbit pancreas for example, where a cell can produce its own weight of protein in less than 24 hours, the nucleoli and cytoplasmic nucleotides are very conspicuous. The rapid resynthesis of protein in the exocrine cells of the pancreas following discharge by aid of pilocarpine is preceded by nucleolar growth and the appearance of RNA in the cytoplasm. In the pancreas we have an opportunity of comparing cells which are highly specialised for the rapid production of protein (the exocrine cells) with cells producing protein slowly (the islets of Langerhans) in the same preparation. Although the islet tissue produces insulin, the total amount of this protein produced in a given time is very much less than that produced by the enzyme secreting cells. Measurement

in the ultraviolet microscope at the absorption maximum of the nucleotides, reveals a much higher content of cytoplasmic nucleotides in the exocrine parts of the gland (Caspersson, Landström & Aquilonius, 1941). These spectrophotometric measurements have been substantiated by nucleotide estimations (Norberg, 1942).

The same picture is furnished by the gastric mucosa, where the peptic cells, producing pepsin, can be compared with the oxyntic cells producing hydrochloric acid. The former cells have on the average 3 to 4 times as much cytoplasmic RNA as the latter (Caspersson, 1950). In the salivary gland of Drosophila larvae, nucleic acid content has again been correlated with function (Caspersson, 1939). A further example is provided by the nerve cell. After embryonic development, the neuron retains the appearance of a cell prepared for active protein formation, as judged by spectrophotometric measurements (Caspersson, 1947), a feature which is unique among the different tissues of the mammalian organism. As a result of the studies of Hyden (1947a) however, this apparent exception proved the rule. A conspicuous fall in the protein content of the cytoplasm of the neuron was found to be associated with normal functioning of the nerve cell, and hence, during intense function its protein-forming system, in which RNA appears to be involved

must be able to replace used proteins very rapidly.

Protein synthesis in plants and microorganisms. The examples mentioned so far have provided evidence for a relationship between RNA and protein synthesis in the metazoan cell. That the same relationship holds for lower organisms has been revealed in the experiments on plant tissues and on the yeast cell by Caspersson (1950), on bacteria (Malmgren & Heden, 1947; Caldwell, Mackor & Hinshelwood, 1950) and on viruses (Hyden, 1947b).

In the adult plant organism rapid growth occurs in various tissues. Cells from the growing tip of Allium root for example, show a very high nucleotide absorption at 260 mu, while cells from a zone of no growth show an absorption curve following that of proteins in general (Caspersson, 1939).

As soon as yeast cells start growing in a suitable medium, large amounts of RNA appear in the cytoplasm. This increase in RNA occurs only during the growth process; if the yeast carried out an intense metabolic process not accompanied by growth, for example fermentation in the absence of a nitrogen source, there is no increase in RNA. Similarly in bacteria, enormous changes occur in nucleic acid content during the course of growth. In the lag phase,

before the commencement of cell division, nucleotide synthesis begins; when the nucleotide content has reached a maximum value it begins to fall until, when division ceases, it has reached approximately the same threshold value as when division started. This strict correlation between RNA metabolism and bacterial growth is comparable in every way to the protein synthetic mechanism functioning during the growth of metazoan cells.

The last organisms to be considered are the viruses. This group contains organisms of varying degrees of complexity, from the simplest viruses, the plant viruses which are composed of ribonucleoproteins, to large forms representing transitions to the bacteria, and containing either DNA, or both types of nucleic acids. The importance of nucleic acid in virus reproduction has been emphasised in experiments by Markham and Smith (1949) who showed that the protein component of a plant virus was no longer infectious if deprived of the ribonucleic acid with which it was normally associated. One of the first signs of virus infection is a stimulation of the cytoplasmic protein-forming system of the host cell, as evidenced by a large increase in RNA content. The product of this process however is consumed by the viruses themselves. Thus the virus appears to exploit the protein synthetic mechanisms of the host

cell in order to fulfil its own needs for reproduction. In these investigations, interest was confined to the quantitative relationship between the amounts of RNA and protein in the cell. The use of radioactive isotopes however has made possible a study of the metabolism of these compounds, but has contributed little more to substantiate the role of RNA in these processes. Presentation of this aspect of the relationship will be delayed until a later section.

In summary then, this mass of circumstantial evidence, derived from both cytochemical measurements and chemical estimations, strongly suggests that RNA may be part of the equipment of all cells for the formation of protein, and compels one to persist in seeking for the role of RNA in protein biosynthesis.

Scope of present experiments. In an attempt to throw some light on the problem of RNA and protein synthesis, a study was made of the influence of energy intake on the total amount of RNA in the liver, both when the diet contained adequate amounts of protein and when it was free from protein, and the results correlated with the pattern of protein metabolism under the same variety of experimental conditions.

EXPERIMENTAL.

Total nucleic acids in the liver. For this series of investigations, livers were obtained from the animals used to study the relationship between energy intake and protein metabolism, described in Part I of this thesis. Full details of their selection and feeding, and methods of tissue analyses employed, may be found there. Briefly, adult male albino rats weighing about 250 g. were fed diets, either providing adequate amounts of protein or free from protein, at an energy level of 1200 kg. cal./sq. m. of body surface area for a preliminary 7-day adjustment period. Subsequently, during a 4-day experimental period, the caloric value of the diet was varied from about 850 to about 1700 kg. cal./sq. m. by alterations in the consumption of either fat or carbohydrate. At the end of this period, the animals were killed by exsanguination under ether anaesthesia, and the livers removed for analyses.

Nucleic acids and protein were estimated by a modified Schmidt-Thannhauser separation procedure (see Part I); the tissue, from which acid soluble phosphates and phospholipids have been removed by extraction with trichloracetic acid and lipid solvents, is submitted to a mild alkali digestion, in which process RNA is broken down to its con-

stituent acid-soluble nucleotides, while DNA is not appreciably affected. When the alkaline digest is acidified, ribonucleotides remain in solution, while DNA is centrifuged down in the protein precipitate. The nucleic acid content of the tissue is then determined from phosphorus estimations (Allen, 1940) on the supernatant (RNA) and on the precipitate (DNA).

The first five experiments deal with the addition of carbohydrate or fat to protein-rich, protein-poor and protein-free diets. These were carried out as independent experiments, and correspond to Experiments 1 - 5 discussed in Part I. In a sixth confirmatory experiment (corresponding to Experiment 6 in the studies on protein metabolism) comparison was made simultaneously between the effect of additions of carbohydrate to protein-containing and protein-free diets. In addition a further confirmatory experiment was performed in which the effect on the nucleic acid metabolism of the liver of energy increments, supplied as carbohydrate, was compared with the effect of equivalent increments of fat, when the diet was devoid of protein. This last experiment is not described in the studies on protein metabolism, since protein N was not estimated.

The amounts of nucleic acid found are expressed as RNA or DNA per liver. Since the animals were of approximately

Table 19.

The effect of varying energy intake by changes in dietary carbohydrate, using a diet rich in protein.

(4 rats per group) Exp. 1.

GROUP	DAILY ENERGY INTAKE Per sq. m. surface area	LIVER ANALYSES (Amounts per liver)		
		Mean Weight	RNAP	DNAP
	Cal./sq.m.	(g.)	(mg.)	(mg.)
I	854	6.6	6.05	1.75
II	1103	7.4	6.56	1.89
III	1372	7.7	7.32	1.85
IV	1664	8.2	7.57	1.90
Statistical Analysis				
Difference between energy levels		8.86**	13.49***	1.71 *
Regression coefficient		+1.91**	+1.96***	+0.15 *
Deviations from linear regression		0.55*	0.61*	1.09 *

*** Highly significant (P<0.01)
** Significant (P = 0.05-0.01)
* Not significant (P>0.05)

Table 20.

The effect of varying energy intake by changes in dietary fat, using a diet rich in protein.

(5 rats per group) Exp. 2.

GROUP	DAILY ENERGY INTAKE Per sq. m. surface area	LIVER ANALYSES (Amounts per liver)		
		Mean Weight	RNAP	DNAP
	Cal/sq.m.	(g.)	(mg.)	(mg.)
I	832	7.1	6.47	1.92
II	1232	7.9	7.55	2.09
III	1610	8.9	8.03	2.01
Statistical Analysis				
Difference between energy levels		19.03***	20.12***	2.05*
Regression coefficient		+2.21***	+2.01***	+0.10*
Deviations from linear regression		0.21*	1.61*	0.04*

*** Highly significant (P<0.01)
** Significant (P = 0.05-0.01)
* Not significant (P>0.05)

Table 21.

The effect if varying energy intake by changes in dietary carbohydrate, using a protein-free diet.

(4 rats per group) Exp. 3.

GROUP	DAILY ENERGY INTAKE Per sq.m. surface area	LIVER ANALYSES (Amounts per liver)		
		Mean Weight	RNAP	DNAP
	Cal./sq.m.	(g.)	(mg.)	(mg.)
I	896	6.0	5.28	1.88
II	1179	5.7	5.39	2.04
III	1454	6.2	5.62	2.03
IV	1710	7.2	5.80	2.05
Statistical Analysis				
Difference between energy levels		10.54***	4.44**	3.73*
Regression coefficient		+1.41***	+0.60***	+0.17**
Deviations from linear regression		6.80**	0.68*	2.29*

*** Highly significant (P<0.01)
** Significant (P = 0.05-0.01)
* Not significant (P>0.05)

Table 22.

The effect of varying energy intake by changes in dietary fat, using a protein-free diet.

(4 rats per group) Exp. 4.

GROUP	DAILY ENERGY INTAKE Per sq. m. surface area	LIVER ANALYSES (Amounts per liver)		
		Mean Weight	RNAP	DNAP
	Cal./sq.m.	(g.)	(mg.)	(mg.)
I	918	6.0	5.42	1.85
II	1193	6.5	5.12	1.84
III	1464	6.8	5.44	1.94
IV	1727	6.8	5.25	1.82
Statistical Analysis				
Difference between energy levels		6.54**	1.27*	1.15*
Regression coefficient		+1.00***	-0.07*	0.00
Deviations from linear regression		0.80*	1.75*	1.72*

*** Highly significant (P<0.01)
** Significant (P = 0.05-0.01)
* Not significant (P>0.05)

Table 23.

The effect of varying energy intake by changes in dietary fat, using a diet low in protein.

(2 rats per group) Exp. 5.

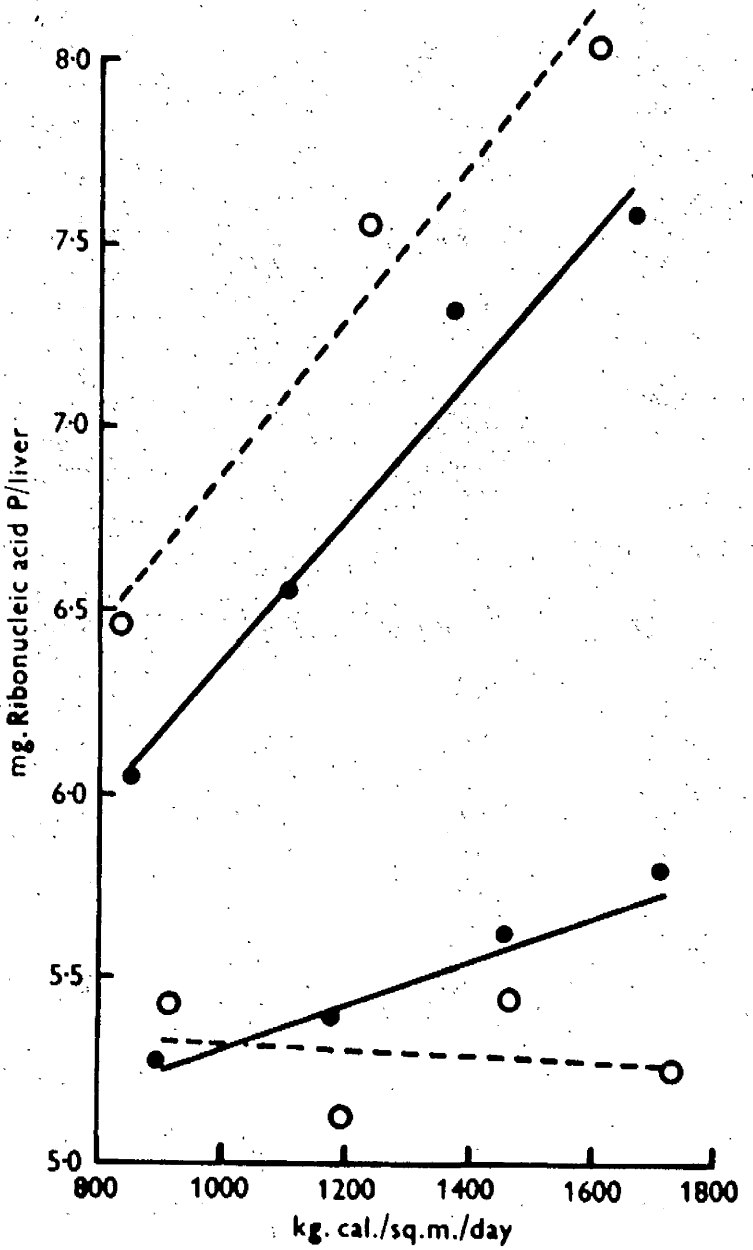
GROUP	DAILY ENERGY INTAKE Per sq. m. surface area	LIVER ANALYSIS (Amounts per liver)		
		Dean Weight	TCHAP	TFAP
	Cal./sq.m.	(G.)	(mg.)	(mg.)
I	940	8.5	5.69	1.92
II	1117	8.9	5.79	2.03
III	1300	9.8	6.03	1.93
IV	1656	7.4	6.18	2.01

Statistical analysis

Difference between energy levels	0.21*	0.16*
Regression coefficient	+0.60*	+0.07*
Deviations from linear regression	0.01*	0.35*

* Not statistically significant ($P > 0.05$)

Fig. 7.



Changes in the total amount of ribonucleic acid P per liver produced by variations in intake of carbohydrate (●——●) or fat (O-----O). The upper two curves (Exps. 1 and 2) represent the regression lines obtained when the diet contained protein. The lower two regression lines (Exps. 3 and 4) were obtained when the diet was free from protein.

the same body weight at the beginning of each experiment, consistent variations in the total amounts of the nucleic acids per liver were attributed to the different nutritional conditions employed. Statistical analyses were carried out as before. The regression coefficients presented represent the change produced by an alteration in energy intake of 1000 kg. cal./sq. m. of body surface area. There were no significant deviations from linearity of regression.

RESULTS

The influence of energy intake on the amount of RNA in the liver was found to be dependent on the protein content of the diet (Tables 19 - 23). When a diet providing adequate amounts of protein was fed, it was observed that the total amount of RNA in the liver increased in a linear fashion with the caloric content of the diet (Fig. 7). This response to energy intake was identical whether carbohydrate or fat was used as the variable energy source in the diet, the regression coefficient being 1.96 mg. RNAP/1000 kg. cal. in the case of additions of carbohydrate and 2.01 mg. in the case of fat; both coefficients are highly significant ($P < 0.01$). When protein was omitted from the diet, increments of energy in the form of carbohydrate were found to cause a small though still significant rise in the amount of RNAP/liver (0.60 mg./1000 kg. cal.; $P < 0.01$), but additions of

Table 24.

The effect of changes in energy intake (carbohydrate) at different levels of protein intake on the ribonucleic acid content of the liver.

(10 rats per group)

GROUP	DIET	DAILY ENERGY INTAKE Per sq. m. surface area	Total RNAP/Liver
		(Cal./sq.m.)	(mg.)
I	Protein-containing	820	6.42
II		1570	7.51
III	Protein-free	880	5.26
IV		1650	5.48

Analysis of variance indicates a highly significant ($P<0.01$) effect of protein intake and of energy intake on the quantities of RNAP and a very significant difference in the influence of energy intake at the two levels of protein intake ($P<0.01$ for interaction).

fat were without effect (-0.07 mg. RNAP/1000 kg. cal.; $P > 0.05$). On the other hand, when a small amount of protein was incorporated in the diet, in a fifth experiment (Table 23), there was a slight tendency for the total liver RNAP to rise with increasing fat consumption. This increase however was not statistically significant. The regression coefficients obtained in the case of the protein-free diets are both significantly less than those obtained with diets containing protein ($P < 0.01$). Comparison of the response of total liver RNAP to the addition to the protein-free diet of carbohydrate, with that produced by addition of fat, as illustrated in Fig. 7, suggests a difference in action of these two nutrients, though in this case the level of statistical significance is not conclusive ($P = 0.05$-0.02).

The results on which these conclusions are based were obtained in five separate groups of experiments performed at different times. It therefore seemed desirable to confirm these findings by a simultaneous study of the effects of the different dietary treatments. In the first of these experiments (Table 24), after the usual preliminary 7-day adjustment period, the rats were maintained on the protein-rich or protein-free diets (Diets A and B, Table 1) at two levels of energy intake only, obtained by feeding the basal ration alone, or with a glucose supplement of 7.0g.;

Table 25.

Comparison of the effect on liver ribonucleic acid of adding carbohydrate or fat to a protein-free diet.

(5 rats per group)

GROUP	Source of energy variation	DAILY ENERGY INTAKE Per sq. m. surface area	Total liver RNAP/rat
		(Cal./sq.m.)	(mg.)
I	Carbohydrate	880	5.34
II	Carbohydrate	1650	5.29
III	Fat	880	5.28
IV	Fat	1660	5.49

Analysis of variance does not reveal any significant difference between the action of carbohydrate and of fat.

addition of carbohydrate to the protein-containing diet was found to exert a significantly greater influence on the RNA content of the liver than its addition to the protein-free diet. In a second experiment (Table 25), the effect of adding carbohydrate (glucose) to a protein-free diet was compared with the effect of adding fat (olive oil). Two levels of caloric intake were again studied, representing a daily consumption of 880 and 1660 kg. cal./sq. m. body surface area. In this experiment it was not possible to demonstrate a significant difference in their influence on the total amount of RNA in the liver.

Thus the essential point emerging from these studies is that the amount of RNA in the liver is much more sensitive to changes in energy intake when the diet contains protein than when it is devoid of protein.

Liver DNAP was neither altered by the level of protein fed nor by variations in energy intake in any of the experiments with the exception of Experiment 3 (Table 21). However, the level of significance in this instance is so low that it can be regarded as of no practical importance. This was not unexpected in view of the many demonstrations of the stability of nuclear DNA under conditions producing profound changes in the composition of the cell cytoplasm (Davidson, 1947; 1952).

DISCUSSION.

The results of those experiments will be discussed in conjunction with the data from the studies with radio-active phosphorus, described in the following section.

PART II.

THE INFLUENCE OF PROTEIN AND ENERGY INTAKE ON RIBONUCLEIC ACID METABOLISM IN THE LIVER

Section 2

On Uptake of Radioactive Phosphorus by Liver Ribonucleic Acid.

INTRODUCTION.

In Section I, the influence of energy intake on the amount of ribonucleic acid (RNA) in the rat liver was described. When the basal (sub-maintenance) diet contained protein, addition of energy in the form of either carbohydrate or fat resulted in a considerable increase in total RNA per liver. On the other hand, when protein was omitted from the diet, an increase in energy intake caused only minimal changes in the amount of RNA. In these studies, however, no indication was given of the effect of protein and energy intake on the metabolism of RNA. As has been mentioned previously, a complete account of the metabolism of any constituent of a living cell can be presented only if a simultaneous study of quantitative changes and turnover rates (as measured with radioactive isotopes) is made. To this end, the influence of energy intake on the rate of incorporation of isotopic phosphorus (^{32}P) into liver RNA has been investigated, both when the diet contained adequate amounts of protein, and when it was free from protein.

When the results of these experiments were considered in conjunction with the quantitative data reported in the previous section, it was established that the metabolism of RNA, in contrast to the amount of RNA in the liver, was

determined not by the level of protein in the diet, but by energy intake. In an attempt to throw some light on this relationship between energy metabolism and RNA metabolism, a further series of experiments was performed in which comparison was made between RNA metabolism in animals fed a protein-free diet at a high level of energy intake, and animals fed the same diet, but in which energy expenditure was increased by reducing the environmental temperature. These experiments are subsequently referred to as the "cold-room studies".

The radioactivity data described in this section, with the exception of the cold-room studies, were the work of Dr. T. W. Wikramanayake, who collaborated with me in this phase of my work. Dr. Wikramanayake has kindly permitted the use of these data abstracted from the work jointly published with him (Munro, Naismith & Wikramanayake, 1953).

EXPERIMENTAL.

Incorporation of ^{32}P into ribonucleic acid. For this investigation a different series of animals were used from the ones described in the study of the influence of energy intake on the total RNAP of the liver. Young male albino rats were again used, but the choice was not restricted to those of 250 g. body weight. Animals weighing from about

180 to 230 g. were selected, but within each experiment, the animals were of closely similar weight.

The general management and feeding arrangements were similar to those employed in the experiments mentioned above. After a preparatory 7-day period, during which the rats were fed either the high protein or low protein diets at an energy level of 1200 kg. cal./sq. m. of body surface area, energy supply was altered so that some animals received a low intake of energy while others received a high intake, all other constituents of the diet being kept constant. Unless otherwise stated, these changes in energy consumption were produced by varying the amount of carbohydrate in the diet. The animals were maintained at the new energy level for 4 days. On the last day, each rat received ^{32}P as inorganic phosphate by intramuscular injection (10 µc./ 100 g. of body weight) and was killed by exsanguination under other anaesthesia after 4, 8or 24 hours, in most cases after 24 hours.

Cold-room studies. In this series of experiments, 3 groups of rats were studied. During the preliminary 7-day period, all received an energy intake of 1200 kg. cal./sq. m. of body surface area, from a protein-free diet. The energy intake of the first group of animals was then restricted

to about 800 kg. cal./sq. m. for a 4-day period. A glucose supplement was added to the diet of the second group during this period, to bring the energy level up to about 1600 kg. cal./sq. m. The third group also received the glucose supplement, but were transferred for the entire 4-day period from an environment thermostatically controlled at about 25°C to a cold-room, the temperature of which ranged from 2 - 4°C. All animals were sacrificed 18 hours after injection of ^{32}P.

Analytical procedures. The liver was quickly perfused with 0.9% saline through the portal vein, dried between filter papers, weighed, and homogenized in 9 vols. of ice-cold water in a Nelco blendor at moderate speed for 6 minutes, with addition of a few drops of capryl alcohol to prevent frothing. Fractionation was then effected by the modified Schmidt-Thannhauser procedure described by Davidson, Frazer & Hutchison (1951). To a volume of homogenate, accurately measured, corresponding to 3 or 4 g. liver, sufficient 30% (w/v) trichloracetic acid (TCA) was added to give a final concentration of 10%. After standing for half an hour in ice, the acidified homogenate was centrifuged at a low speed for 7 minutes, and the precipitate of protein, phospholipids and nucleic acids washed twice with ice-cold 10% (w/v) TCA, the supernatant in each case being filtered and kept for

estimation of the radioactivity of inorganic phosphorus (P). Phospholipids were then removed from the precipitated material by extraction with lipid solvents as previously described (see Experimental section of Part I).

Estimation of liver inorganic phosphate. The radioactivity of the inorganic phosphate of the liver was determined in the TCA soluble fraction by the method of Davidson, Frazer & Hutchison (1951). To the acid solution, Mathison's (1909) reagent was added (1 ml. to 10 ml. extract) and the mixture made alkaline with NH_4OH. After standing overnight in the refrigerator, the precipitate of $Mg.(NH_4)PO_4$ was centrifuged, washed twice with dilute NH_4OH, and filtered off through a Whatman No. 42 paper and dissolved in N HCl. A suitable portion was taken for determination of P by the method of Allen (1940), and radioactivity measured in a liquid counter (Type M6 manufactured by 20th Century Electronics) attached to a conventional probe unit and scaling unit (Type 200; manufactured by Dynatron Radio Ltd.). In view of the criticism of some forms of the $Mg(NH_4)PO_4$ precipitation procedure by Ennor and Rosenberg (1952) it should be noted that no carrier phosphate was used. In a few instances, the activity of the acid-soluble organic phosphorus fraction (S1 fraction of Davidson et al., 1951), the supernatant of the inorganic P precipitation, was determined.

Estimation of ^{32}P incorporation into ribonucleotides. For the purification of RNA and separation of its constituent nucleotides, the method of Davidson and Smellie (1952) was adopted. This procedure permits the isolation of the four nucleotides in a form relatively free from traces of radioactive contaminants. Previous methods of separation had failed to achieve a complete resolution of the nucleotides; for example, employing methods of chromatographic separation on filter paper, Magasanik, Vischer, Doniger, Elson and Chargaff (1950) were unable to separate guanylic acid from uridylic acid, while in the procedures of Carter (1950) and Markham and Smith (1951) cytidylic and uridylic acids were found to occupy the same position on the chromatogram. Smellie's method of fractionation depends on the ionophoretic mobilities of the nucleotides, and is based on the original technique of ionophoresis described by Consden, Gordon and Martin (1946). The procedure was as follows: the dry lipid free residue was incubated with N KOH for 18 hours at 37°C. The digest containing amongst other things undegraded DNA and ribonucleotides, was cooled in ice and brought to pH 1 by addition of 60% (w/v) perchloric acid. The precipitate of protein, DNA and potassium perchlorate was centrifuged and washed twice with a small volume of N perchloric acid, and the supernatant and washings combined for ionophoretic

separation of the ribonucleotides. A suitable aliquot of this fraction, containing from 100 to 120 µg of P, was adjusted to pH 3 with N.KOH, and applied to a spot 6 cm. from one end of a strip of Whatman No. 1 filter paper, 7 cm. broad and 52 cm. long, from an Agla micrometer syringe (Burroughs Wellcome Ltd.); the spot was dried in a current of warm air. The paper was moistened with 0.02 M citric acid/trisodium citrate buffer (pH 3.5) and suspended over a glass rod, the two ends being immersed to a depth of 1 cm. in the buffer contained in two glass dishes. A steady direct current was then applied by means of carbon electrodes, placed in each dish so that the anode was in the dish further away from the nucleotide spot. In most cases, separation was carried out over a 7 hour period with a potential gradient of 15 V/cm. of filter paper. This was sufficient to provide a considerable separation of contaminant P compounds from the ribonucleotides but did not allow elution of the individual nucleotides separately. On completion of the 7-hour run, the paper was dried before infrared lamps, the nucleotide bands located with the aid of an ultraviolet lamp according to the procedure of Holiday and Johnson(1949) and marked lightly in pencil. The bands were cut out, the nucleotides eluted by the method of Consden, Gordon and Martin (1947) into graduated tubes, and made up to a known

volume, 6 ml. of which was taken for measurement of radio activity. The solution was then divided into two equal portions, and P determined by the method of Allen (1940) suitably modified for small quantities of phosphorus. In some cases, the digest was submitted to ionophoresis over a period of 18 hours at a potential gradient of 11 V/cm., in order to achieve separation of individual nucleotides. As the separation of cytidylic and adenylic acids was rarely complete, due probably to the relatively large amounts of material applied to the paper, these two nucleotides were eluted and estimated together.

Expression of Radioactivity Results. The results have been expressed as specific activities (counts/minute/100 µg. P) and also as relative specific activities (specific activity of P compound divided by the specific activity of liver inorganic P multiplied by 100). In addition, the total incorporation per liver has been assessed by computing the "total relative activity", a term suggested by Campbell, Olley and Blewett (1949), for the product of relative specific activity multiplied by the amount of RNA phosphorus.

The data have been analysed by recognized statistical procedures (Snedecor, 1946).

Table 26.

The effect of changes in energy intake (carbohydrate) on the uptake of radioactive phosphorus by liver ribonucleic acid 24 hr. after injection of ^{32}P. The effect of diet on relative specific activity is shown. (4 rats per group)

Diet	Mean initial body weight	Daily energy intake	Specific activity of RNAP	Specific activity of liver inorganic phosphate	Relative specific activity of RNAP	Specific activity of RNAP adjusted by covariance analysis.
	(g.)	(kg.cal./ sq.m.)	(counts/min./ 100 ug.P)	(counts/min./ 100 ug.P		(counts/min./ 100 ug.P)
Protein-containing	185	810	580	1527	37.9	683
	186	1715	685	1896	35.8	646
Protein-free	193	820	761	2004	38.2	680
	189	1790	839	1747	48.4	857

Analysis of variance shows that the effect of changes in energy intake was significantly different ($P < 0.05$) for interaction at the two levels of protein intake for the relative specific activity of RNAP and for the specific activity adjusted by covariance analysis.

RESULTS

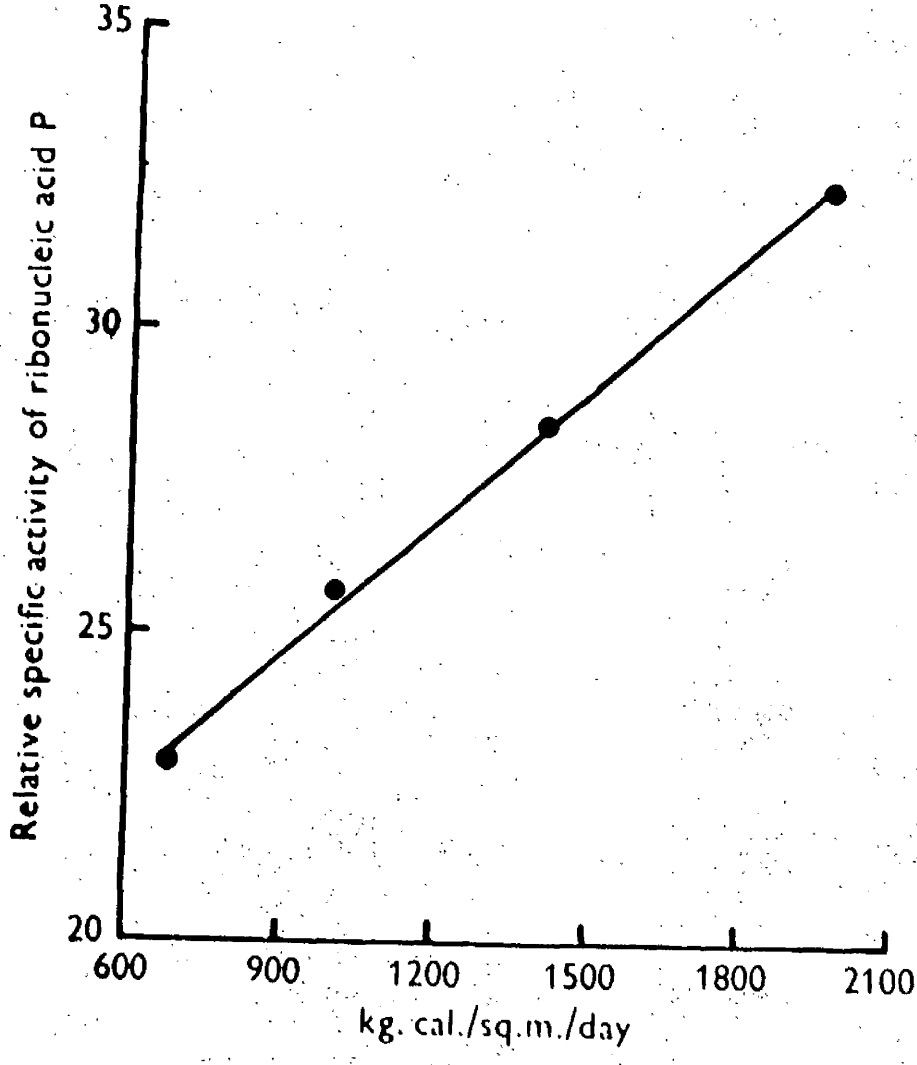
The influence of energy intake on the incorporation of ^{32}P into liver RNA is illustrated in Table 26. The animals were killed 24 hours after injection of the isotope. Since the phosphorus of the polynucleotide molecule is derived ultimately from the pool of inorganic phosphate in the liver cell, the specific activity of the RNAP will be influenced by the specific activity of the inorganic phosphate. In order to eliminate any variations which might be attributed to this factor, the results have been expressed relative to the specific activity of liver inorganic phosphate, i.e. relative specific activity. When the animals received a diet providing an adequate amount of protein, there was a tendency for the relative specific activity of the RNA to fall with rising energy intake. On the other hand, in the case of rats receiving a protein-free diet, there was an appreciable rise in relative specific activity. The statistical method of covariance analysis (Snedecor, 1946) provides an alternative means of correcting the specific activity of RNAP for variations in the activity of inorganic phosphate. This procedure allows one to adjust the observed specific activity of RNAP to give the value which would have been obtained had the specific activity of inorganic phosphate

been the same for all animals. The correction is made solely from evidence of the relationship between the specific activity of RNAP and inorganic phosphate provided by the experiment itself. The results of such an analysis are included in Table 26, and completely confirm the picture obtained from the calculations of relative specific activity.

The experiment just described is representative of a series of observations on rats killed 24 hours after injection of ^{32}P. In the complete series of experiments, 33 animals were fed the protein-free diet, and 18 received the protein-containing diet, energy intake ranging from 650 to 1900 kg. cal./sq. m. of body surface area. The change in relative specific activity induced by alterations in energy intake over this range is expressed by the regression coefficient/1000 kg. cal./sq. m., namely -10.3 for the protein-free diet and +1.2 for the protein-containing diet. If the relative specific activity at 1200 kg. cal. is taken as reference standard, these regression coefficients represent changes of -26.5 and +3.4% per 1000 kg. cal. respectively in relative specific activity. The change in the rate of incorporation is significant for the protein-free diet ($P < 0.01$) but not for the protein-containing diet.

In order to compare the magnitude of the change in uptake of ^{32}P into the RNA molecule with variations in energy

Fig. 8.



The effect of adding carbohydrate to a protein-free diet on the relative specific activity of ribonucleic acid phosphorus. The rats were injected with ^{32}P as orthophosphate 24 hr. before killing.

consumption of a protein-free diet, a further experiment was performed in which carbohydrate was added to the protein-free diet at four levels of energy intake. Inspection of the results, shown in graph form in Fig. 8, leaves no doubt of the linear relationship between energy intake and relative specific activity of RNAP. Using the value at 1200 kg. cal./sq. m. again as reference standard, calculation of the regression coefficient showed that an increase of 1000 kg. cal./sq. m. of surface area resulted in an alteration of 27.8% in relative specific activity.

In the studies so far described, only the results obtained from animals killed 24 hours after ^{32}P was administered have been presented. The same relationships however have been found by Dr. Wikramanayake to hold with animals killed at shorter time intervals (see Munro, Naismith and Wikramanayake, 1953).

It was also established that the various nucleotides, separated by prolonging the period of ionophoresis to 18 hours, incorporate ^{32}P to essentially the same degree at all time intervals after injection. Each nucleotide shows a higher incorporation rate when obtained from the livers of animals fed the protein-free diet at the higher level of energy intake. This would appear to eliminate the possibility that contaminants play a significant part in

Table 27.

Comparison of the effect on ^{32}P uptake by liver ribonucleic acid of adding fat or carbohydrate to a protein free diet.

(3 rats per group)

Source of energy variation	Daily energy intake	Relative specific activity of RNAP
	(kg.cal./sq.m.)	
Carbohydrate	1000	25.7
	1920	32.4
Fat	980	23.2
	1900	30.5

Analysis of variance shows that the influence of energy intake is significant ($P < 0.05$) but that the action of carbohydrate does not differ from that of fat (interaction not significant).

accounting for the differences in phosphorus incorporation into RNA, since they would hardly be expected to affect all nucleotide fractions similarly.

The observations so far described refer to changes in energy intake produced by alterations in dietary carbohydrate. The effect of adding fat to a protein-free diet has also been determined. In these experiments one series of animals was maintained at an energy intake of 1200 kg. cal./sq. m. by adding carbohydrate to a protein-free diet, and another series by adding fat; after a week at this level, different energy intakes were obtained in each series by varying the amount of carbohydrate or fat fed. Table 27 shows that the addition of fat was at least as effective as the addition of carbohydrate in stimulating the rate of incorporation of ^{32}P into the RNA of the liver. It may be concluded therefore that the effect is the consequence of an increase in available energy rather than of a specific function of carbohydrate.

Cold-room Studies: In addition to these experiments carried out in an environment thermostatically controlled at about 25°C (range 23-27°C), a study was made of the effect on RNA metabolism of increasing energy expenditure, by placing some of the animals in a refrigerated room (2 - 4°C).

Table 28.

The effect of lowered environmental temperature on uptake of ^{32}P by liver ribonucleic acid.

(13 rats per group)

GROUP	Environmental temperature	Mean initial body weight	Daily energy intake	Weight change	RNAP/ liver	Relative specific activity of RNAP	Total relative activity of RNAP	Change in total relative activity per 100 kg. cal./ sq. m. added *
		(g.)	(kg.cal./ sq.m.)	(g.)	(mg.)			
Low-energy	25°C	165	820	-16	3.38	25.7	0.84	-
High energy	25°C	165	1600	-5	3.86	29.9	1.11	+41%
High energy	3°C	169	1760	-19	4.01	24.5	0.96	+15%

* The change is expressed as a percentage increase above the low energy value.

Statistical analysis: Analysis of variance shows the following significant differences between the groups.

For RNAP/liver : Variance ratio 4.03; (fiducial limits 0.50)
For relative specific activity: Variance ratio 3.77; (fiducial limits 0.44)
For total relative activity : Variance ratio 5.48 ; (fiducial limits 0.17)

(At the 5% level of significance, the variance ratio is 3.47 and at the 1% level it is 5.78; all values thus lie between these levels of significance, the figure being close to the 1% level in the case of total relative activity).

All the rats were fed a protein-free diet, and three groups were studied (Table 28) (a) at a low energy intake in the warm environment; (b) at a high energy intake in the warm environment, and (c) at a high level of energy intake in the cold environment. As was anticipated, the increase in energy consumption in the warm environment reduced the weight loss, but this advantage was lost if at the same time the environmental temperature was lowered. The amount of RNA per liver increased with the rise in energy intake, but was not further altered when the environment was chilled. On the other hand, uptake of ^{32}P, as indicated by the relative specific activity rose in response to the increased energy intake in the warm environment, but fell if the additional energy of the diet was dissipated under the stress of cold.

DISCUSSION.

It is apparent from the evidence presented that energy intake influences the metabolism of RNA. In order to compare the magnitude of the alteration in amount and in the rate of incorporation of ^{32}P caused by variations in energy supply, the change produced by an increment of 1000 kg. cal./sq. m. of body surface area has been calculated and expressed as a percentage of the amount or of the incorporation rate ob-

Table 29.

Comparison of the changes in total ribonucleic acid/liver and ^{32}P uptake by ribonucleic acid when energy intake from carbohydrate is increased.

(Figures in brackets are the number of animals in each experiment)

DIET	Data recorded in	Method of RNA isolation	Regression coefficients**	
			Total RNAP/liver	Relative specific activity of RNAP
			(%)	(%)
Protein-containing	Fig.7	Schmidt-Thannhauser	+29.2 (16)	-
	Table 24	Schmidt-Thannhauser	+20.8 (20)	-
	-	Ionophoresis	+28.6 (24)	+3.4 (16)
Protein-free	Fig. 7	Schmidt-Thannhauser	+10.9 (16)	-
	Tables 24 and 25*	Schmidt-Thannhauser	+4.4 (24)	-
	-	Ionophoresis	+8.1 (57)	+26.5 (33)
	Fig. 8	Ionophoresis	-	+27.8 (12)

* The animals shown in Table 24 as receiving a protein-free diet include most of those given in Table 25 (carbohydrate added).

** Regression coefficients indicate the change produced by adding 1000 kg.cal./sq. m. body surface area to the diet, and have been expressed as a percentage of the amount of RNAP or the relative specific activity of RNAP corresponding to an energy intake of 1200 kg. cal.

taining at an energy intake of 1200 kg. cal./sq. m. In Table 29 all experiments involving a change in energy intake from carbohydrate are presented in this way. In addition to the results obtained in the study of changes in total RNA per liver using the older Schmidt-Thannhauser procedure, the more accurate estimations of the amount of RNA obtained in the course of the experiments with the radioisotope by ionophoretic separation of the ribonucleotides have been included. Experiments with fat were fewer, but show the same general trends.

The data summarized in Table 29 show that when the diet provides an adequate amount of protein, an increase in energy intake of 1000 kg. cal. causes a rise of some 20-30% in the total amount of RNAP in the liver, but has no significant effect on the rate of incorporation of ^{32}P into the RNA molecule. In the case of the protein-free diet, the total amount of RNAP is only slightly increased (about 5-10%) when consumption of energy is raised by 1000 kg. cal./sq. m., but the rate of replacement of phosphorus atoms is augmented by about 25%. It is thus apparent that at each level of protein intake, the total number of phosphorus atoms incorporated into RNA is increased by raising the intake of energy, in one case by an increase in the total amount of RNA in the liver without a change in the percentage of

Table 30.

The effect of changes in energy intake (carbohydrate) on the uptake of radioactive phosphorus by liver ribonucleic acid 24 hr. after injection of ^{32}P. The table indicates the effect of diet on the total amount of ^{32}P incorporated per liver.

(4 rats per group)

Diet	Daily Energy Intake	Total RNAP/liver	Relative specific activity of RNAP	Total relative activity	Specific activity of RNAP	Total radioactivity of liver RNAP adjusted by covariance analysis
	kg.cal./ sq.m.	(mg.)			(counts/min./ 100 μg.P)	(counts/min.)
Protein-containing	810	3.25	37.9	1.23	580	22,300
	1715	4.22	35.8	1.51	685	27,600
Protein-free	820	3.18	38.2	1.21	761	21,300
	1790	3.70	48.4	1.78	839	31,600

Analysis of variance reveals that the total radioactivity/liver showed a highly significant response to energy intake ($P < 0.01$), but that this effect was not significantly greater for the protein-free diet ($P > 0.05$ for interaction).

phosphorus atoms incorporated in a given time, in the other case by an increase in incorporation rate with a smaller change in total RNAP per liver. It is clear from Table 29 that the magnitude of the change in incorporation rate on the protein-free diet is fully adequate to compensate for the lack of alteration in total amount. Further evidence for this has been obtained by calculations based on the data given in Table 26. If the specific activity of RNAP is multiplied by the total RNAP of the liver, a measure of the total amount of ^{32}P incorporated into the liver is obtained. This procedure has been carried out in Table 30, the figures being adjusted by covariance analysis for differences in the specific activity of the inorganic phosphate of the livers. It will be seen that at each level of protein intake, a rise in energy consumption is accompanied by an increased absolute incorporation of ^{32}P. Although the response to increased energy supply appears to be greater in the case of the animals fed the protein-free ration, statistical analysis shows that the effect is not significantly different at the two levels of protein intake. A similar conclusion can be drawn from calculation of the total relative activity (Table 30). Since this is obtained by multiplying relative specific activity by total amount, total relative activity is a measure of the total amount

incorporated.

These experiments demonstrate clearly that two dietary factors influence the course of RNA metabolism in the liver. In the first place, protein intake plays a dominant role in determining the amount of RNA in the liver. Variations in the total nucleic acid content of adult liver, with the nutritional state of the animal have frequently been observed. (Addis, Poo & Law, 1936c; Kosterlitz & Cramb, 1943; Davidson & Waymouth, 1944; Campbell & Kosterlitz, 1948; 1950; 1952). A fall in nucleic acid content on fasting (in relation to original body weight) was observed in the liver of the rat by Kosterlitz and Cramb (1943). Davidson and Waymouth (1944) also noted a significantly lower concentration of RNAP and a higher DNAP content in liver preparations from starved rats in comparison with livers from fed animals. This investigation was extended by Davidson (1947) who compared the composition of the livers of well fed rats with a comparable group which had been fasted for a period of two days and with a third group of animals refed for two days after a two day fast. The total nucleic acid content of the liver was found to fall when the animals were fasted, and rise again on feeding in parallel with corrosponding changes in liver protein, the variation in nucleic acid being due entirely to fluctuations in RNA: the

DNA content remained unchanged. Subsequent experiments by Kosterlitz (Campbell & Kosterlitz, 1952) have amplified these findings. The RNAP content of the liver cell (represented by the ratio RNAP/DNAP) was observed to be a linear function of its protein content, and hence of the protein content of the diet; furthermore, alterations in dietary fat and choline were without effect on RNAP content. The absence of nutritional effects on nuclear DNA was also noted by Kosterlitz. Estimations of DNA per liver presented in this thesis (Tables 19-23) give further support to the view that the DNA content of the liver of adult animals is not readily influenced by changes in the composition of the diet.

The second dietary factor affecting RNA metabolism is energy intake, which determines the absolute rate of phosphorus incorporation. Campbell and Kosterlitz (1948, 1952) were the first to note an inverse relationship between the percentage turnover of RNAP and the amount of RNAP in the liver. In their experiments they obtained evidence that when part of the RNAP was lost from the livers of rats maintained on protein-deficient diets, the turnover of the remaining molecules was accelerated. In view of the possible contamination of the RNA with other radioactive fractions, (the acid soluble fraction from the Schmidt-Thannhauser

alkaline digest was used), Kosterlitz refrained from attempting to explain the phenomenon.

The observation that energy intake determines the absolute rate of ^{32}P incorporation into RNA raises the question of whether this reflects real differences in the rate of RNA synthesis, or whether the phosphorus is incorporated into the molecule by an exchange reaction, analogous to the exchange of amino acids with corresponding residues in protein molecules. In view of the commonly accepted structure of RNA however (see Davidson, 1953), it seems unlikely that most of the phosphorus atoms can be exchanged with the free inorganic phosphate of the cell without a complete breakdown and resynthesis of the entire molecule. Decisive evidence on this point can only be obtained by a simultaneous study of the incorporation of isotopes into different parts of the polynucleotide structure; such experiments have been carried out and will be presented in a later section of this thesis (Part IV).

To sum up then, it was found that the amount of RNA in the liver was affected by energy intake only under circumstances which have been shown (Part I) to produce an alteration in liver protein; moreover these variations in RNA closely paralleled the changes in liver protein. At first sight these experiments would seem to confirm the

the numerous quantitative studies, which have dealt with this relationship, and lend further support to the concept that RNA is fundamentally involved in the protein biosynthetic mechanism. Viewed in the light of the isotopic experiments, however, the picture is completely changed. The absolute rate of incorporation (a true indication of the metabolism of the RNA molecule) appears to be controlled by energy intake and to be independent of the level of protein in the diet or of the amount of RNA. This suggested at first that the cell might have a requirement for RNA associated with the metabolism of energy. The cold-room studies were designed to investigate this relationship. It was reasoned that if RNA has a function in the metabolism of energy, then an increased energy consumption might be expected, under normal conditions, to lead to an augmented metabolism of RNA; moreover, this postulate would be equally applicable in a case where the beneficial effect of the extra energy intake was abolished by increasing energy expenditure. An analogy is provided by the increased requirement for thiamine exhibited by rats maintained at low temperatures (Hegsted & McPhee, 1950). A further example is provided by the work of Mitchell and his colleagues (Mitchell, Johnson, Hamilton & Haines, 1950) who established that the requirement of the pig for riboflavin was increased

in the cold. In both cases, energy output rises in the cold.

To test this point then, comparison was made between the metabolism of RNA in the livers of rats receiving a protein-free diet at a high level of energy intake, and of a similar group of animals fed the same ration, but maintained at a temperature of 3°C (Table 28). A third group of animals receiving the protein-free diet at a low level of energy intake were studied as controls. In confirmation of previous findings, a marked stimulation of RNA metabolism (as determined by measurement both of relative specific activity and of total relative activity in Table 28) was observed when energy was added to the diet under normal conditions (at an environmental temperature of 25°C) but when energy expenditure was increased by lowering the environmental temperature to 3°C, the metabolism of RNAP in the liver was not significantly raised above that of the control animals receiving no supplement of energy. It would seem then that RNA metabolism is not related to the metabolism of energy, but simply to the availability of energy in the liver, a positive energy balance favouring an increased rate of synthesis. Under the cold-room conditions, the increased energy intake was offset by the additional output;

energy balance thus became less favourable, which is also reflected in the greater weight loss of the cold-room group (Table 28). A similar association between available energy and protein synthesis has been proposed by Munro (1951).

PART II.

THE INFLUENCE OF PROTEIN AND ENERGY INTAKE ON RIBONUCLEIC ACID METABOLISM IN THE LIVER.

Section 3

(a) Relationship to Vitamin B_{12} Intake.

INTRODUCTION.

Crude casein was used in all experiments in which protein was fed. Since casein is known to be contaminated with vitamin B_{12}, a factor which has variously been reported to be involved in the metabolism of energy, protein and nucleic acids, it seemed important to establish to what extent this contamination with vitamin B_{12} might be responsible for the changes in RNA metabolism, attributed in these experiments to the protein of the diet. The function of vitamin B_{12} in intermediary metabolism is by no means clear, as a survey of the literature reveals.

It is now well known that vitamin B_{12} is associated with growth in many species of animals. One of the earliest observations in which an indication of a possible metabolic function of the vitamin was given was that of Cary and his associates, who showed that a nutritional deficiency, now known to be that of vitamin B_{12}, was intensified in rats by raising the protein level of the diet (Cary, Hartman, Drydon & Likely, 1946). This finding, in conjunction with subsequent observations of the effect of the vitamin on growth (Hartman, Drydon & Cary, 1949), was taken as an indication of the involvement of vitamin B_{12} in the metabolism of protein. Hove and Hardin (1951) found that rats which were consuming a 10% protein diet gained more weight per gram of protein

ingested when vitamin B12 was included in the diet than when it was omitted, and so suggested that efficiency of protein utilization was influenced by the vitamin. Further relationships were reported by Zucker and Zucker (1948) who described high blood non-protein N and urea levels in rats, maintained on an all vegetable diet, at the time when the animals showed the most marked signs of deficiency. McGinnis, Hsu and Graham (1948) noted that the blood non-protein N in deficient chicks was greatly elevated, but was lowered to control levels when an alcohol soluble liver fraction was added to the basal diet. Similarly Schultz (1949) found that the acute uraemia occurring in new-born rats from mothers maintained on diets devoid of animal protein, could be prevented by injecting the young with vitamin B12 soon after birth. Charkey, Wilgus, Patton and Gassner (1950) suggested that vitamin B_{12} might have a function in the utilization of circulating amino acids, since it was found that the blood levels of non-protein N and of several amino acids were lower in chicks receiving a supplement containing B12 than in chicks on a basal diet deficient in the vitamin. In a more recent study, plasma protein levels were found to be higher in chicks given B12 than in deficient controls, and it was concluded that the vitamin might function in protein metabolism by stimulating

the formation of plasma proteins and ribonucleoproteins (Hsu, Stern & McGinnis, 1953).

A connection between vitamin B_{12} and nucleic acid metabolism was first noted by Shive. Further evidence was provided in the work of Stern, Taylor and Russel (1949) who investigated the effect of vitamin B_{12} on nucleic acids in the rat, as measured by the concentration of basophilia in liver tissue. It was observed that rats which were deficient in B_{12} showed little or no basophilia, whereas those which received vitamin B_{12} grew well, and presented the normal histological picture in their liver cells. This finding was substantiated in Popper's studies on the effect of B_{12} on hepatic injury due to carbontetrachloride poisoning (Popper, Koch-Weter & Szanto, 1949). Ribonucleic acid disappears from the cells of the liver in the early stages of carbon tetrachloride intoxication. Injection of B_{12} to rats prior to the administration of a toxic dose of carbon tetrachloride was found to inhibit the development of histological changes.

Vitamin B_{12} has also been found to affect the phosphorus metabolism of microorganisms (Roberts, Roberts & Abelson, 1949). When L. leichmannii was grown in a medium containing radioactive phosphorus, vitamin B_{12} was observed to increase the uptake of phosphorus into the DNA fraction

of the cell to a more pronounced extent than into other phosphorus-containing fractions, an observation in accord with the belief that the vitamin is involved in nucleic acid synthesis. This concept was first expressed by Shive as a result of studies on the growth of certain microorganisms. Since the early demonstration of the effect of thymine on growth, much attention has been given to the pyrimidine base and its deoxyriboside thymidine. In the appropriate basal medium, thymidine was shown to permit the organism L. leichmannii to grow ~~have~~ effectively in the absence of vitamin B_{12}, while the parent pyrimidine thymine under the same conditions of growth was inactive (Shive, Havel & Eakin, 1948). By way of explanation, Wright suggested that vitamin B_{12} might function as a coenzyme in carrying out reactions leading to the conversion of thymine into thymidine. (Wright, Skeggs & Huff, 1948). However, with certain bacteria, the ability of thymidine to support growth in the absence of B_{12} is duplicated by deoxyribosides of various purines and pyrimidines (Kitay, McNutt & Snell, 1949). The interchangeability of the deoxyribosides and also the activity of the phosphorylated products the deoxyribotides (Shive, Sibley & Rogers, 1951) suggested that these compounds might simply be providing the groups which they have in common (D-2-deoxyribose) in transdeoxyribosidation reactions (McNutt,

1950). However there is no convincing evidence concerning the precise mechanism of this reaction, or indicating that B_{12} functions as a coenzyme in the biosynthesis of deoxyribosides.

As yet only one fact has been established concerning the role of vitamin B12 in nucleic acid metabolism, namely that it is involved in the metabolism of one-carbon units. The participation of certain sources of a"single-carbon fragment", including formate, formaldehyde and methanol in a number of biochemical reactions has been demonstrated in several species, and in particular in the rat. For example the carbon atoms in the purine ring in the 2 and 8 positions of uric acid excreted by the pigeon have been shown to be capable of being derived from formate carbon (Buchanan & Sonne, 1946). This has been found to apply also to the purines of the rat (Heinrich & Wilson, 195)), and in addition, to the 5-methyl group of thymine (Totter, Volkin & Carter, 1950). From the work of Kratzer (1953) and Stokol and his associates (Stokol, Hsu, Weiss & Smith, 1953) it seems likely that the function of vitamin B_{12} is to control the actual de novo synthesis of one-carbon fragments, and hence the rate of nucleic acid synthesis. The study of the nutrition of certain microorganisms provides further evidence for a function of B12 in purine synthesis. It

has been reported that in a mutant of E. coli which requires purines for growth, vitamin B12 stimulates the conversion of aminoimidazolecarboxamide to purine (Ben-Ishai, Volcani & Bergmann, 1951).

Purposes of the present experiments. In view of these many claims that vitamin B12 is involved not only in the metabolism of protein, but also in the biosynthesis of both types of nucleic acid, it seemed desirable to determine to what extent this factor was responsible for the effects on RNA metabolism attributed to the protein content of the diet in the experiments described in Section 1. In these experiments it was found that addition of energy to the diet of the rat resulted in a considerable increase in the total amount of RNA in the liver, but only when the basal diet contained protein. Since the protein used was crude casein, which is known to contain vitamin B12, the possibility existed that the factor limiting the response of RNA to energy intake was not dietary protein, but vitamin B12, which was completely lacking in the protein-free diet. The present short series of experiments were intended to exclude this possibility. Comparison was made between rats receiving a protein-containing or protein-free diet at high and low levels of energy intake with corresponding control groups receiving a supplement of vitamin B12.

EXPERIMENTAL.

The design of the experiment was identical with those in which the effect of energy intake on the total amounts of the nucleic acids in the liver was investigated. Full details of the selection of animals, composition of the diets, feeding and analytical procedures are given in Part I and Part II (Section 1). Briefly, male albino rats of about 250 g. body weight were given either a diet containing adequate amounts of protein, or a protein-free diet, fed for the first week at a level of 1200 kg. cal./sq. m. of body surface area. Then for a 4-day period the animals were divided, one group receiving an energy intake of about 800 kg. cal. (the unsupplemented basal ration) and a second group receiving an intake of 1600 kg. cal., the extra energy being supplied as carbohydrate. In addition, during this period, the animals were further subdivided; to half of the animals 5 µg of vitamin B12 (Glaxo) were administered daily by intraperitoneal injection. A similar volume of distilled water was injected into the remaining (control) group of animals. At the end of this period, the rats were killed by exsanguination under ether anaesthesia and the livers removed. The livers were then submitted to the Schmidt-Thannhauser separation procedure as described previously to give the amounts of protein, RNA and DNA.

Table 31.

The effect of Vitamin B_{12} on the response of liver ribonucleic acid phosphorus (RNAP) deoxyribonucleic acid phosphorus (DNAP) and protein-N to variations in dietary energy and protein intake. The corresponding data from experiments 1 and 3 of the original series (Section 1 of Part II) are shown for comparison.

Exp.	Diet	Protein-N		RNAP		DNAP	
		Low energy level	High energy level	Low energy level	High energy level	Low energy level	High energy level
		(mg.)	(mg.)	(mg.)	(mg.)	(mg.)	(mg.)
B_{12} given	Protein-rich	189.3	199.7	6.73	7.27	1.93	1.79
	Protein-free	133.4	144.4	5.22	6.24	1.74	2.04
No B_{12} given	Protein-rich	179.1	204.2	6.07	7.29	1.84	1.81
	Protein-free	141.4	132.7	5.33	5.67	1.74	1.79
Exp. 1	Protein-rich	183.5	210.5	6.05	7.57	1.75	1.90
Exp. 3	Protein-free	146.0	139.4	5.28	5.80	1.88	2.05

Statistical analysis (analysis of variance) demonstrates no significant changes in Protein-N or RNAP which can be attributed to vitamin B_{12} administration.

Table 32.

Results from Table 31 expressed as mg. per mg. DNAP.

(4 rats per group)

Exp.	DIET	Protein-N		RNAP	
		Low energy level	High energy level	Low energy level	High energy level
		(mg.)	(mg.)	(mg.)	(mg.)
B_{12} given	Protein-rich	98	112	3.49	4.06
	Protein-free	77	71	3.00	3.07
No B_{12} given	Protein-rich	98	113	3.30	4.03
	Protein-free	81	74	3.06	3.17
Exp. 1	Protein-rich	105	111	3.46	3.98
Exp. 2	Protein-free	78	68	2.81	2.83

The quantities of protein-N RNAP and DNAP are expressed as amounts per liver (Table 31) and the protein-N and RNAP in relation to the amount of DNAP (Table 32).

RESULTS and DISCUSSION.

The results from estimations of protein-N, RNAP and DNAP are summarized in Table 31. In addition, the results from comparable experiments described in Section 1(Exps. 1 and 3) have been included in this table for comparison. Since the amount of DNA in the liver has repeatedly been shown to be insensitive to alterations in the composition of the diet, DNA has been used as a reference standard, and the results of the protein-N and RNAP determinations expressed in Table 32 as mg. per mg. DNAP. In this table, the data from previous experiments has been recalculated on the basis of liver DNAP content and again included for comparison.

Protein-N. Under the circumstances of the present investigation, it is obvious, from inspection of the data included in Table 32 that vitamin B_{12} had no effect whatsoever on the protein metabolism of the liver. Addition to the protein-containing diet of energy promoted a marked deposition of protein in the livers of both control animals

and of those receiving a supplement of vitamin B_{12}, the magnitude of the effect being in each case essentially identical. On the protein-free diet, the vitamin was again shown to be without effect on protein metabolism. In both cases, the tendency of liver protein to fall with rising energy intake on a protein-free diet was confirmed. The individual results from the various groups of rats on the vitamin B_{12} supplemented ration were for all practical purposes identical not only with the results from their counterparts fed the control diet, but with the data re-calculated from previous experiments 1 and 3 reported in Section 1 of Part II. They are completely confirmed when, instead of computing the results per liver (Table 31), the data are expressed per mg. of DNAP as reference standard (Table 32). Similar negative findings have been reported by Chow and Borrows (1950). These authors concluded, from work with rats, that carbohydrate or fat metabolism was more affected by vitamin B_{12} than was protein metabolism, since the vitamin was found to have no influence on N retention. In more recent work (Black & Bratzler, 1952; Moenel & Black, 1952) the effect of vitamin B_{12} on protein and energy metabolism was again investigated in the rat. It was reported that with diets providing high (18%) and low (10%) levels of protein of vegetable origin, there was

no indication that the metabolism of protein was specifically affected.

Ribonucleic acid phosphorus. The response of liver RNAP to an augmented energy intake, on both the protein-rich and protein-free diets, was found to conform to the pattern established in previous experiments (see Section 1 of Part II) in both control animals and in those injected with vitamin B_{12}. In accord with the changes observed in Expts. 1 and 3 included in Table 31, addition of energy to the diet produced a significant increase in RNAP only in animals receiving protein. There was again a marked similarity in the individual results representing the various corresponding groups in the control series, in the series receiving the vitamin supplement, and in the groups included from earlier studies. This is also confirmed when the results are calculated per mg. DNAP (Table 32). Under the present experimental conditions, then, vitamin B_{12} appears to have no measurable effect on the metabolism of RNA in the liver. It has been claimed (Sahasrabudhe & Rao, 1951) that administration of vitamin B_{12} enhanced the liver protein, RNA and DNA in terms of mg. per unit of body weight, but Rose and Schweigert (1952) have shown that while the amounts of RNA and DNA per g. of liver were increased by supplementing the ration of vitamin B_{12} deficient animals, the amounts per cell

were not altered.

It may therefore be concluded that the stimulating effect on the net synthesis of RNA in the liver of energy added to a diet containing animal protein, is conditioned not by the content of vitamin B_{12} in such a diet, but by the protein itself. Furthermore, with the synthetic protein-free diet used, the absence of vitamin B_{12} does not account for the failure of the liver to respond to an increment in energy intake.

PART III.

THE INFLUENCE OF PROTEIN AND ENERGY INTAKE ON PHOSPHOLIPID METABOLISM IN THE LIVER.

INTRODUCTION.

It has been established in a previous section of this thesis, that although the amount of ribonucleic acid in the liver of the rat is largely determined by the protein content of the diet, the rate of synthesis of ribonucleic acid, as measured by the incorporation of isotopically labeled phosphorus (^{32}P) into the molecule, is controlled by energy intake. Certain similarities between the metabolism of ribonucleic acid and phospholipid have been revealed under a variety of nutritional conditions in experiments by Campbell and Kosterlitz (1948; 1952). In view of our findings with ribonucleic acid, it was thought of interest to extend these studies to an investigation of the influence of energy intake on phospholipid metabolism in the liver, in experiments of similar design. Accordingly a study was made of the effect of variations in energy intake on the amount of phospholipid in the livers of rats, and on the rate of incorporation of ^{32}P. These experiments were done in collaboration with Dr. W. C. Hutchison and Dr. T. W. Wikramanayake. I am indebted to Dr. Hutchison for the data reported in Fig.9, and the data reported in Table 34 were jointly obtained by Dr. Wikramanayake and myself.

EXPERIMENTAL.

Energy intake and the quantity of phospholipid in the liver.

The rats employed in these studies were the same animals as were used to investigate the relationship of protein metabolism and ribonucleic acid metabolism to energy intake. The selection and general management of the rats, the composition of the diets and analytical procedures have been described previously in the appropriate sections. Briefly, rats weighing about 250 g. were fed either a protein rich diet or a diet lacking protein, for a preliminary 7-day period at an energy level of 1200 kg. cal./sq. m. of body surface area. Energy in the form of either fat or carbohydrate was then added to or subtracted from the ration to provide two levels of energy intake (approximately 800 and 1600 kg. cal./sq. m.) at each of the protein levels. The animals received the adjusted diets for a further period of 4 days, before they were sacrificed under ether anaesthesia, and livers removed for analysis.

The phospholipids were extracted from the livers by a modification of the method described by Schmidt and Thannhauser (1945) (see Section I). Before submitting the tissue to extraction with lipid solvents, acid-soluble inorganic and organic phosphates were removed by grinding

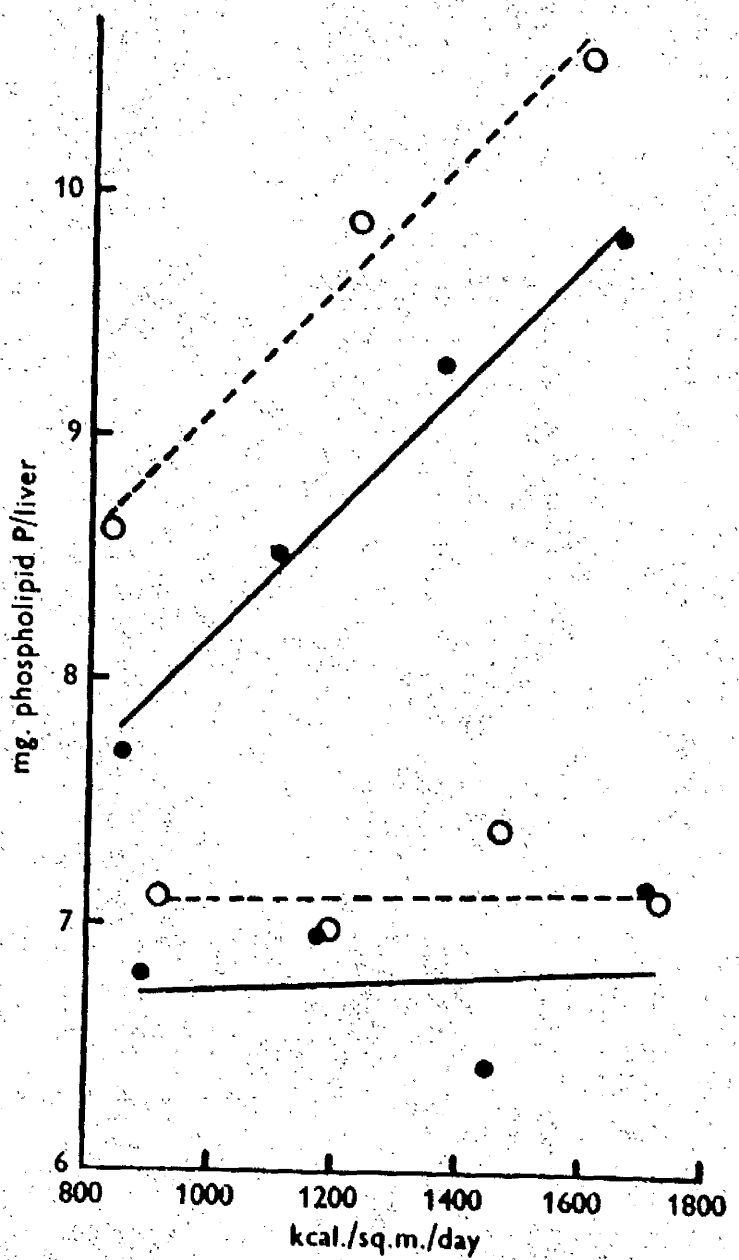
a weighed portion of the liver in ice-cold 10% (w/v) trichloroacetic acid (TCA), centrifuging the sample, and washing the precipitate twice with ice-cold 10% TCA. The residue was then successively extracted with 80% ethanol, absolute ethanol, three times with a 3:1 mixture of ethanol-chloroform (this extraction being carried out at 75° in a water bath for 30 minutes) and finally with redistilled ether. The phosphorus content of the combined extracts was measured by the method of Allen (1940).

Energy intake and the uptake of ^{32}P by the liver phospholipids. Rats having an initial body weight close to 180 g. were used. The nutritional design of the experiment was identical with procedure briefly described above. On the morning following the last day of feeding, ^{32}P (as inorganic phosphate) was administered to each animal by intramuscular injection, (10 uC/100 g. body weight) and the animals killed thereafter by exsanguination under ether anaesthesia at intervals of 2, 5 and 8 hours. The livers were quickly perfused with warm 0.9% (w/v) NaCl via the portal vein, excised, weighed and homogenized in 9 volumes of ice-cold water in a Nelco blendor. Sufficient ice-cold 30% TCA was added to give a final concentration of 10%. After two washings with 10% TCA, the precipitate was

successively extracted with 20 ml. portions of cold absolute alcohol, ethanol-chloroform (3:1) and twice with ethanol-ether (3:1) extraction with the last two solvents being carried out at 65-70°C in a water bath for 10 minutes. Finally the precipitate was extracted with ether. The extracts were combined, the phosphorus content determined by the Allen (1940) method, and the radioactivity of the solution used for the photometric measurement of phosphorus, estimated in a liquid counter (Veall, 1948). This method of phospholipid extraction is essentially that used by Campbell and Kosterlitz (1952) differing only in the use of ethanol-chloroform as one of the solvents. The efficiency of the procedure is indicated by the finding that further treatment of the precipitate with a mixture of chloroform, ethanol and ether (1:3:1) for 10 minutes at 60° removed only traces of P.

A test of the purity of the phospholipid fraction was carried out by Dr. Wikramanayake. The combined lipid extract was first evaporated to dryness at room temperature and the residue then taken up in light petroleum (b.p. 40-60°). This was washed once with 0.05 N HCl, and three times with water (Popjak & Muir, 1950). This purification was found to have altered the specific activity of the lipid phosphorus by no more than 0.5%, and consequently

Fig. 9



Changes in the total amount of phospholipid P per liver produced by variations in intake of carbohydrate(●——●) or fat(O-----O). The upper two curves (Exps.1 and 2) represent the regression lines obtained when the diet contained protein. The lower two regression lines (Exps. 3 and 4) were obtained when the diet was free from protein.

it was abandoned as a routine procedure. It was assumed that the initial washings with TCA had successfully removed any radioactive contaminants which might otherwise have found their way into the lipid extracts. The specific activity of the inorganic phosphate of the liver was obtained from the trichloroacetic acid-soluble fraction by precipitation as $Mg.(NH_4)PO_4$ (Davidson, Frazer & Hutchison, 1951) as previously described (see Part II, Section 2).

The results are expressed as relative specific activities (specific activity of lipid P divided by specific activity of liver inorganic P and multiplied by 100), and as total relative activities (the amount of phospholipid P per liver multiplied by its relative specific activity; Campbell, Olley & Blewett, 1949).

RESULTS.

Energy intake and the quantity of phospholipid in the liver.

The influence of increasing energy intakes on phospholipid is illustrated in Figure 9. When the diet contained an adequate amount of protein, the quantity of phospholipid in the liver varied considerably with changes in the intake of carbohydrate and fat. The regression coefficients were

Table 33.

The influence of variations in energy intake (carbohydrate) on the amount of phospholipid in the liver at different levels of protein intake, and the relationship of these changes to alterations in the amount of protein and ribonucleic acid in the liver.
(7 rats per group)

Diet	Daily energy intake	Phospholipid P/liver	Phospholipid P/100 mg. protein N	Ribonucleic acid P/100 mg. protein N	Ribonucleic acid P/100 mg. phospholipid P.
	(Kg.cal./sq.m.)	(mg.)	(mg.)	(mg.)	(mg.)
Protein-containing	820	8.29	4.29	3.32	77.5
	1570	9.91	4.59	3.51	76.7
Protein-free	880	6.80	4.76	3.64	76.7
	1650	6.45	4.86	4.06	83.7

Statistical analysis*

Protein level	+	+	+	+
Energy level	+	+	+	+
Interaction**	+	-	+	+

* A '+' sign indicates a probability of less than 0.01, and a '-' sign indicates a probability greater than 0.05.

** Significant interaction implies that a change in energy intake had a different effect at the two protein levels

2.51 mg. phospholipid P/1000 kg. cal. in the case of addition of carbohydrate, and 2.57 mg./1000 kg. cal. in the case of fat, both being statistically highly significant ($P<0.01$). In contrast with this finding, neither the addition of carbohydrate nor of fat significantly increased the amount of phospholipid per liver when the rats were fed the protein-free diet (Regression coefficients of +0.01 and +0.02 mg. P/1000 kg. cal. respectively were obtained). Since these results were obtained in 4 separate series of experiments, in which no simultaneous study was made of animals receiving protein at the two levels of intake, a further experiment was performed in which groups of rats on protein-containing and on protein-free diets were studied at the same time. It was confirmed that a beneficial effect of carbohydrate can only be demonstrated when the diet contains protein (Table 33). The pattern of the response of liver phospholipid to changes in energy intake is thus very similar to that of liver protein and ribonucleic acid. Dietary protein appears to have a dominant role in determining the extent to which an increment of energy will affect phospholipid content, but whereas with the addition of carbohydrate to the protein-free diet, the amount of RNA per liver tends to rise slightly, phospholipid, like protein, is unaffected, or even slightly

Table 34.

The effect of variations in energy intake on the incorporation of ^{32}P into the phospholipids of the liver.
(3 rats per group)

Diet	Time after ^{32}P injection	Daily energy intake	Phospholipid P/liver	Relative specific activity		Total relative activity	
	(hr.)	(k.cal./sq.m.)	(mg.)				
Protein-containing	2	789	6.61	14.4)	+10%	0.95)	+35%
		1656	8.23	15.6)		1.28)	
	5	781	7.60	32.0)	+26%	2.41)	+32%
		1671	7.92	40.2)		3.18)	
	8	779	5.88	56.5)	+8%	3.31)	+34%
		1659	7.49	50.4)		4.52)	
	Mean	783	5.70		+15%		+34%
		1662	7.88				
Protein-free	2	802	4.86	15.0)	+13%	0.74)	-6%
		1767	4.07	17.2)		0.70)	
	5	827	4.74	45.0)	+24%	2.10)	+7%
		1831	4.01	56.7)		2.26)	
	8	810	4.06	67.9)	+18%	2.79)	+17%
		1803	4.05	80.7)		3.27)	
	Mean	816	4.55		+18%		+6%
		1800	4.04				

Footnote to Table 34.

* The changes in relative specific activity and in total relative activity brought about by increasing the intake of energy have been given as the change per 1000 k.cal. added to the diet. In each case, the change has been expressed as a percentage of the activity corresponding to 1200 kg. cal./sq.m. body surface area.

Statistical analysis shows that increments in energy intake produced highly significant increases in relative specific activity ($P<0.01$) on both the protein-containing and protein-free diets; the effect was similar on both diets ($P>0.05$). A highly significant change in total relative activity occurred with addition of energy to the protein-containing diet ($P<0.01$) but not in the case of the protein-free diet ($P>0.05$). This difference in the effect of energy intake on the two diets was highly significant ($P<0.01$)

reduced in amount. This is confirmed by computing the relationships between the amounts of protein, RNA and phospholipid P (Table 33).

Energy intake and uptake of ^{32}P. Data showing the amount of ^{32}P incorporated at 2, 5 and 8 hours after injection of labeled phosphorus is given in Table 34. When energy intake was raised, a corresponding increase in relative specific activity was observed, and there was no significant difference in this respect between the protein-containing and protein-free diets. Determination of relative specific activity (the amount of radioactivity/100 µg.P) however represents only one aspect of the metabolic picture of liver phospholipid. In order to obtain an indication of the total number of P-atoms incorporated into liver phospholipid in a given time the relative specific activity was multiplied by the amount of phospholipid in the liver (total relative activity). When protein was provided in the diet, the total relative activity showed an increase of 34% in response to an increment to the diet of 1000 kg. cal., whereas in the case of the protein-free diet, addition of energy produced no significant change. The larger change in the case of the protein-fed animals was the result of a combination of two factors; in addition to its stimulating

effect on relative specific activity, the augmented energy supply promoted a considerable increase in the amount of phospholipid per liver. In the case of the protein-free diet on the other hand, the beneficial effect of energy on relative specific activity was offset by a diminution in the total amount of phospholipid.

DISCUSSION.

When rats were placed on a diet free from protein but adequate in every other respect, phospholipid was lost from the liver, in agreement with the findings of Kosterlitz (1947) and Davidson and his associates (Thomson, Hoagy, Hutchison & Davidson, 1953). Moreover, addition of energy to such a diet in the form of either carbohydrate or fat, tended if anything to reduce the phospholipid level even further, while with a similar increment in energy intake, liver phospholipid rose when a protein-containing diet was fed (Fig. 9; Tables 33 and 34). The level of protein in the diet has also been shown to be a factor governing the response of liver protein and RNA to changes in energy intake (see Part I and Section 1 of Part II). Small differences have been noted however in the responses of these liver constituents to dietary conditions which result in

changes in the ratio of one constituent to another, as the dietary protein and energy levels are varied (Table 33). The ratio RNAP/protein N was observed to decrease with increasing protein content of the diet, in agreement with observations of Campbell & Kosterlitz (1950) but in contrast to the findings of the present investigation, these authors found no change in the ratio phospholipid P/ protein N over a wide range of protein intake.

The influence of energy intake on the metabolism of phospholipid in the liver (assessed by calculation of the total relative activity) has also been shown to be conditioned by the amount of protein in the diet (Table 34). In the case of the protein-containing diet, there was an increase in the total amount of ^{32}P incorporated per liver as energy intake was raised, but energy added to the protein-free diet had no significant action. In this respect, the incorporation of ^{32}P into liver phospholipids displays a different pattern from its uptake by liver RNA, the latter being determined not by the amount of protein in the diet, but by the level of energy intake. Campbell and Kosterlitz (1948; 1952) have also noted a difference in the effect of protein deprivation on the rates of synthesis of phospholipid and RNA by the rat liver. During the first few days on a protein-free diet, it was found that the loss of RNAP and phospholipid P was accompanied by

an increase in the relative specific activity of both liver constituents. As a result, there was the same total amount synthesized per liver on a protein-free diet (as indicated by calculation of total relative activities), as on a protein-containing diet. They concluded that the total turnover of the phospholipids and of RNA was determined by the metabolic needs of the liver and was independent of the amount of each substance present in the liver. In the case of RNA, this rate of synthesis was maintained over a prolonged period of protein deficiency, but after about 4 days, the rate of phospholipid synthesis declined. Campbell and Kosterlitz (1952) ascribe this to the lowered metabolic rate found in such animals, but this explanation is incompatible with the fact that the rate of incorporation into RNA continued to be maintained at the same level throughout the entire period of protein starvation. A more likely explanation is that administration of a protein free ration reduces the concentration of some essential precursor in phospholipid synthesis to such a degree that it becomes the limiting factor in the rate of synthesis. This would explain why the addition of energy to a protein-free diet fails to stimulate phospholipid metabolism. In the present experiments, choline was eliminated as the limiting factor in phospholipid formation, since it was provided in the diet at a level of 40 mg. per rat per day.

PART IV.

THE INFLUENCE OF PROTEIN AND ENERGY INTAKE ON UPTAKE OF GLYCINE-2-^{14}C BY LIVER PROTEIN AND RIBONUCLEIC ACID.

INTRODUCTION.

The experiments described in Part I indicate that the energy level in the diet affects N balance and the amount of protein in the liver favourably only if the diet contains protein. This would imply that the available energy in some way influences utilization of the dietary protein. In order to investigate this further, it was thought desirable to study the rate of incorporation of labelled amino acids into liver proteins at different levels of energy intake, both at the time of feeding protein and between meals. In this way, it was hoped to determine whether the influence of energy intake is confined to a period shortly after protein administration or is spread over the periods in between meals, the so-called "endogenous" metabolism of protein.

It was convenient at the same time to study RNA metabolism. The hypothesis that ribonucleic acid plays a role in the protein synthetic mechanism rests largely on observations of a quantitative relationship between the amounts of protein and ribonucleic acid in the cell. In experiments previously discussed, it has been established that while the amount of ribonucleic acid in the liver varies only under circumstances favouring a parallel change in

liver protein, a finding which lends support to the hypothesis, isotopic studies have revealed that the metabolism of RNA as measured by total relative activity, is governed by energy intake (or more specifically by available energy) and is independent of the amount of protein in the diet. That ribonucleic acid metabolism can proceed independently of the metabolism of protein, would seem to argue against such an hypothesis, unless the function of RNA in protein synthesis does not involve its metabolism. Before a final decision could be made on this point, it was obvious that a simultaneous study of protein and RNA metabolism would have to be made. In the experiments now to be described such a study was carried out.

For determination of the rates of protein and RNA metabolism in the liver, radioactive isotopes of phosphorus (^{32}P) and carbon(^{14}C) were used. Of the many isotopically labeled amino acids available, glycine-2-^{14}C seemed to have many points in its favour. In the first place, it is not only taken up by the mixed proteins of the liver, but is also incorporated into the ring structure of the purines of nucleic acids(Davidson, 1953). Using both isotopes then, it was possible not only to make a simultaneous study of the metabolism of protein and ribonucleic acid under identical conditions, but to determine,

by measuring the incorporation of the isotopes into two different components of the polynucleotide structure, namely the phosphorus "backbone" and the purine"branches" (see Davidson, 1953) whether the RNA molecule responds as a whole to variations in energy intake.

Secondly, methods are already available for the isolation of glycine from protein and from the purines of nucleic acids. Consequently, by devising a method for the separation of glycine from the amino acid pool of the liver cell, it was possible, by measuring its specific activity, to calculate the relative specific activity of glycine obtained from protein and ribonucleic acid. In view of the considerable variations that occur in the free amino acid levels of the tissues under different nutritional conditions (Wu, 1954), the importance of measuring the specific activity of free glycine cannot be overstressed if a proper interpretation of the uptake by protein is to be obtained.

The nutritional design of the experiments was essentially that employed in the study of the influence of energy intake on ribonucleic acid metabolism. Protein-containing and protein-free diets were administered to rats at two levels of energy intake. Some were injected with ^{14}C and ^{32}P in the postabsorptive state, others were allowed to consume protein immediately prior to and following

injection of the isotopes. In this way it was possible to obtain a picture of protein synthesis as it is affected by (a) previous level of protein and energy intake; (b) its immediate reaction to an influx of amino acids from the gut; and (c) the corresponding alteration in RNA metabolism.

EXPERIMENTAL.

Animals and Diets. Male albino rats of 180-200 g. were fed on diets either containing adequate amounts of protein or no protein at 1200 kg. cal./m^2 for a preliminary period of 7 days, followed by a change in energy intake during a subsequent 4 days produced by altering carbohydrate in such a way that they received either circa 800 cal./m^2 or circa 1600 cal./m^2. The rats were housed individually under thermostatically controlled conditions (23-27°) and fed the diet in two portions, the vitamins (Munro, 1949) and variable energy source, in the morning, with the rest of the diet (including any protein present) at 5 p.m. The diet was fully consumed, so that on the morning after the 4-day period, the rats had completed all the previous day's diet before the isotopes were injected - in the case of the protein-containing diet, the meal of the previous evening was consumed with avidity. The importance of

the fasting state will emerge when the data are considered.

Administration of Isotopes and Excision of Liver. The rats were injected intramuscularly under light ether anaesthesia with ^{32}P (10 μC inorganic phosphate/100 g. body weight) and glycine-2-^{14}C (10 μC/100 g. body weight), one injection into the thigh muscles of each leg. The animals were then killed by exsanguination under ether anaesthesia at 3, 6 or 9 hours thereafter. In most experiments the rats were kept fasting during this period, but in certain cases 2.5 g. casein solubilized with 0.15 g. $NaHCO_3$ was fed immediately prior to and following the injections of isotopes.

Following exsanguination, the abdomen was opened and the liver perfused with 0.9% NaCl solution via the portal vein. The liver was excised, washed briefly in water, dried between filter paper, weighed and homogenized in a Waring blendor using about 5 vol.ice-cold 10% (w/v) Analar trichloroacetic acid (TCA) for 3 mins. The homogenate was quantitatively transferred to a chilled centrifuge tube and after spinning in the cold the filtered supernatant fluid was set aside for determinations of the specific activity of inorganic phosphate and free glycine (see below). The precipitate was then washed twice with chilled 10% TCA and extracted as in the procedure of Schmidt and Thannhauser

(1945) with 20 ml. portions of cold absolute ethanol (twice), ethanol:$CHCl_3$(3:1), ethanol-ether(3:1) twice, and ether, and allowed to dry in the air. This precipitate was used for determinations of (a) protein N; (b) ribonucleic acid (RNA) phosphorus; (c) deoxyribonucleic acid (DNA) phosphorus; (d) specific activity of protein glycine; (e) specific activity of ^{32}P in RNAP; (f) specific activity of ^{14}C in RNA. Procedures (b), (c) and (e) were entirely carried out by Miss C.M. Clark, and procedure (f) was jointly carried out with her. I am grateful to her for permitting me to use her results in order to complete the picture I have obtained.

Analysis of total Protein N, RNAP and DNAP. A known weight of the dry powder was digested with N NaOH at 37° for 15 hr. and sampled for total N by micro-Kjeldahl estimation. After neutralizing a portion with N HCl, the DNA and protein were precipitated with ice-cold TCA to a final concentration of 10%. The precipitate was separated centrifugally and washed twice with ice-cold 5% TCA. The supernatant and washings were estimated for RNA-P by the method of Allen (1940) and the precipitated DNA was dissolved in N NaOH and the P content estimated. Protein N was determined by subtracting the N of the RNA and DNA (RNA-P + DNA-P x 1.69) from the total N of the alkaline

digest.

Radioactive phosphorus determinations. The radioactivity of the inorganic phosphate was obtained by precipitation of $Mg(NH_4)PO_4$ as described by Davidson, Frazer and Hutchison (1951).

The specific activity of the ribonucleic acid phosphorus was obtained from the lipid-extracted residue by the ionophoretic method of Davidson and Smellie (1952), using α 11 V./cm. for 18 hours on a 72 cm. strip of Whatman 3 MM paper. This gave satisfactory separation of all four ribonucleotides. After elution, P estimations and determinations of radioactivity were made. The P radioactivity was determined in the Veall (1948) liquid counter.

Radioactive carbon determinations. Glycine was separated for radioactivity determinations from liver protein, RNA and the acid soluble fraction of liver as the dinitrophenyl (DNP) derivative, the procedure being based on that of Campbell and Work (1952). The material was allowed to react with excess 1-fluoro-2,4-dinitrobenzene (FDNB). The reaction mixture was dissolved in a mixed organic solvent and DNP-glycine separated on a buffered Celite column, followed by subsequent purification on other columns. The glycine was then estimated colorimetrically as the yellow DNP derivative and radioactivity related to this.

An amount of the sample equivalent to about 200 µg. of glycine in 1.5 ml. water made alkaline with knife-point $NaHCO_3$ was reacted with a 20-fold excess of a 10% solution of FDNB in methanol for 4 hours with agitation. The reaction mixture was then diluted with 5 ml. of water and shaken with 20 ml. ether (peroxide-free) to remove unreacted FDNB. The ether extract was washed 3 times with 5 ml. portions of distilled water, the washings being added to the original aqueous layer. This solution was then acidified with 3 ml. of 2.5 N HCl and extracted repeatedly with ether until the ether was colourless. The DNP-glycine migrates into the ether layer from acid solution. The ether extracts were combined and the ether removed by evaporation with a current of air. Traces of water were finally removed in a vacuum desiccator. The dry residue was dissolved in $CHCl_3$-butanol mixture (93:7), using $CHCl_3$ butanol as purified by Kroll (1952). The solution was transferred to a Celite column 1 cm internal diameter buffered at pH 6.6 packed in ether to a height of 15 cm., the ether being then displaced by the $CHCl_3$-butanol mixture (as described in detail by Kroll, 1952). The glycine band was collected and the solvent removed by evaporation in a current of air and the dry residue dissolved in a minimal amount of dry ether (about 0.5 ml.) and transferred to a

Table 35.

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. (The values were read off a graph representing a large number of determinations of the thickness factor).

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

Celite column of similar dimensions as the previous one using ether saturated with water as the developing solvent. The DNP-glycine was eluted, ether removed and the dry sample taken up in a minimum of dry ether for transfer to a counting planchette, on which the samples were dried as an even film with slight heat.

The samples were counted using an end-window counter. The DNP-glycine was then dissolved from the planchette with $CHCl_3$-butanol (20 ml.) and the amount of DNP-glycine extracted into 1% $NaHCO_3$(10 ml.) in which it was estimated colorimetrically. The specific activity of the glycine was obtained by relating the counts to the amount of glycine, and expressed as counts/min./100 µg. of glycine. Owing to self-absorption of the β-rays being an important factor in determination of the activity of ^{14}C-labelled compounds, a series of correction factors (Table 35) were obtained by plating out various thicknesses of a single sample of ^{14}C-labelled DNP-glycine, and relating the counts to the amounts on the planchette. Table 35 represents the combined data of 7 such series of determinations; it will be observed that the factors used are somewhat larger than those published for $Ba^{14}CO_3$ (Calvin, 1949). Corrections for dead-time were never

necessary, since direct determination of the dead-time factor of our end-window counters indicated that this source of error would not exceed 3% with our most active samples (4000 c.p.m.). Since ^{32}P was also given to the rats used in our experiments, the DNP-glycine samples prepared from all sources were periodically checked for possible ^{32}P contamination by interposing a thin plate of copper foil between the sample and the end-window counter. This foil excluded all ^{14}C radiations but permitted the passage of two thirds of the ^{32}P radiations. In no case was there the slightest suggestion of the presence of ^{32}P.

Specific activity of free glycine in liver. This was determined on a portion (about one third) of the TCA extract of the liver. TCA was removed by repeated extractions with ether, until the ether extract was pH 4-5. The aqueous solution was then evaporated to dryness in a desiccator. The residue was then dissolved in 6-10 ml. water and 1.5 ml. taken for reaction with FDNB.

Specific activity of protein glycine. About 100 mg. of the lipid-extracted Schmidt-Thannhauser residue was hydrolysed by refluxing with 20 ml. 6N Analar HCl for 14 hours on an oil bath. The HCl was removed by vacuum distillation and the dry residue taken up in about 10 ml. water. 1.5 ml.

Table 36.

The specific activity of DNP-Glycine prepared from liver protein treated with trichloroacetic acid to remove nucleic acids, compared with the specific activity when not so treated.

RAT No.	SPECIFIC ACTIVITY (c.p.m. per 100 μg. glycine)		
	From untreated protein	From TCA-treated protein	Difference
1	281	306	+9%
3	201	204	+2%
4	195	207	+6%
7	385	412	+7%
			+6%

of this solution was reacted with FDNB.

In some cases, the Schmidt-Thannhauser residue was treated with 5% TCA at 100° for 30 min. to remove nucleic acids, as a possible source of glycine by disintegration of the purine nucleus. Comparison of the radioactivity of glycine in samples so treated with the specific activity in untreated specimens (Table 36) indicates a negligible change in specific activity if the nucleic acids are not removed. Consequently the nucleic acids were not routinely removed. This procedure was further justified by subjecting a sample of a mixture of adenine and guanine to the hydrolytic procedure used for liberating amino acids from the proteins. From 12.4 mg. of the purine mixture, only 0.33 mg. of glycine were obtained, representing only 5.1% of the theoretical yield of glycine if decomposition were complete.

Specific activity of purine glycine. For the isolation of purine glycine, it was essential to have a sample of RNA free from protein, since, under the conditions employed for the degradation of RNA, any contaminating protein or peptide material would give rise on hydrolysis to glycine with a considerably greater specific activity, than glycine arising from the purine ring. For the purification of ribonucleic acid a modification of the

method of Bounce and Kay (1953) was used, in which protein is removed by denaturation with sodium dodecylsulphate. The detergent was purified by recrystallization (twice) from hot ethanol.

The dried liver powder from which acid-soluble phosphates and lipids (including phospholipids) had been removed, was suspended in 30 ml. 0.9% NaCl, and 3 ml. of a solution of sodium dodecylsulphate (5% solution in 45% ethanol) were added. The pH of the mixture was adjusted to 7.0 with a few drops of 10% NaOH, and the solution stirred for one hour at room temperature. After the addition of NaCl to make the concentration molar with respect to NaCl, the solution was centrifuged at 18,000 g. for 15 minutes. The nucleic acids, precipitated from the supernatant in the cold by addition of 2 volumes of ethanol, were centrifuged down, washed with ethanol and acetone, and air-dried. This powder was then dissolved in 10 ml. water, 0.9 ml. of detergent solution added, and after stirring for one hour, the above procedure was repeated. The nucleic acid mixture was dissolved in 5 ml. 0.14% NaCl, cooled to 0°C, and the pH adjusted to 4.5 with 0.1 N HCl. The solution was centrifuged at 0°C for 20 minutes at 18,000 g.; the supernatant was made molar with respect to NaCl, adjusted to pH 7.0, and the nucleic

acids precipitated as described above with ethanol, washed twice with 50% ethanol, then once with ethanol, and finally with ether.

The dried precipitate was incubated at 37°C for 18 hours with 0.3 ml. of 0.5 N KOH. The digest, which contains ribonucleotides and undegraded DNA, was then adjusted to pH 1 with 60% perchloric acid in the cold, and the precipitated DNA removed by centrifugation. After adjusting the pH of the supernatant to 3.0, the ribonucleotides were separated by ionophoresis on paper as previously described (see Part II, Section 2). The method of Hanes, Hird and Isherwood (1951) was employed to wash the 3 MM filter papers used for ionophoresis in order to remove peptides. The efficiency of this treatment was tested by paper chromatography, which showed that a complete removal of contaminating amino acids and peptides had been achieved by this washing procedure.

Following ionophoresis, adenylic and guanylic acids, were eluted from the paper, and the respective bases isolated from the ribonucleotides by the method of Tyner, Heidelberger and LePage (1953). This procedure involves hydrolysis of the nucleotides in N HCl and separation of the bases on columns of Dowex 50 resin. Carrier phosphate was added (a 10 times molar excess) to the hydrolysate before

applying it to the resin column.

Glycine was finally obtained from the samples of adenine and guanine by the procedure of Tinker, Cavalieri and Brown (1949). The base was dissolved in concentrated HCl, and heated in a sealed Carius tube for 18 hours at 180°C. This breaks down the purine nucleus, with liberation of glycine. HCl was removed *in vacuo*, and the specific activity of the glycine determined in the usual way.

The efficiency of the purification procedure was shown by analysis of the eluate from the Dowex 50 columns for protein or peptide contaminants. A sample was heated in a sealed tube at 110° for 18 hours in 6 N HCl. Under these conditions, any protein or peptide would be completely hydrolysed. The digest was submitted to two-dimensional paper chromatography. Only one spot corresponding to glycine was revealed by spraying the chromatogram with ninhydrin. This spot was attributed to slight decomposition of the base.

This method of preparation of RNA was used in Experiments 1 and 2. The yields of purine glycine obtained however were very low, and in many cases made the accurate determination of the specific activity of glycine virtually impossible. The low yields of glycine were thought to be due to incomplete extraction of RNA during the first

Table 37.

Comparison of the specific activity of DNP-glycine after separation on a chloroform-butanol column, with the specific activity after subsequent passage through an ether column. (The DNP-glycine specimens were separated from mixtures of amino acids following hydrolysis of liver protein specimens).

RAT No.	SPECIFIC ACTIVITY (c.p.m.per 100 μg.glycine)		
	From chloroform column	From ether column	Difference
1	355	456	+32%
2	346	392	+13%
3	194	242	+25%
4	199	289	+45%
5	339	513	+52%
6	334	438	+32%
8	265	317	+20%

treatment of the liver powder with sodium dodecylsulphate. Accordingly this step was replaced by the procedure of Porter and Knight (1953). This method, also involving the use of sodium dodecylsulphate, was employed by these authors for the removal of RNA from tobacco mosaic virus. The extracted liver powder was suspended in 30 ml. N NaCl and 3 ml. detergent solution. After stirring at 100°C for 10 minutes, the solution was filtered, and the residue again extracted. The filtrates were combined, and the nucleic acids precipitated by the addition of two volumes of ethanol in the cold, and washed with alcohol and acetone. The nucleic acids were then subjected to the same process of purification as before, and the resultant product tested for the presence of protein as described before, with negative results. The yield of glycine was considerably improved by the modified preliminary extraction procedure.

Reliability of determinations of specific activity. The necessity for using a second (other) column as well as the column run with $CHCl_3$-butanol for purification of DNP-glycine is indicated in Table 37, in which the specific activity of the sample was determined after the $CHCl_3$-butanol column as well as after the second (other) column. There is a marked rise in apparent specific activity, indicating considerable loss of inert material. This

Table 38.

Comparison of the specific activity of DNP-Glycine after separation by column chromatography with its specific activity when subsequently run on filter paper using tertiary amyl alcohol as the solvent. (The DNP-Glycine was prepared from various rat liver fractions, as indicated below).

ORIGIN OF GLYCINE	RAD No.	SPECIFIC ACTIVITY (c.p.m. per 100 µg. glycine)		
		From Column	From Chromatogram	Difference
Free amino acids of cell	6	2090	2115	+1%
	15	675	695	+3% } -2%
	17	395	358	-9%
Hydrolysate of liver protein	1	307	315	+3%
	3	236	236	±0% } +9%
	4	207	231	+12%
	5	155	186	+20%
RNA-guanine	12	73	69	-5%

coincided in most instances with the appearance on the other column of a second band of coloured material.

In order to determine whether further purification was necessary, the product from the ether column was run on a paper chromatogram after counting, using an authetic specimen of DNP-glycine as a marker and tertiary amyl alcohol saturated with phthalate buffer (pH 6) as the developing solvent (Blackburn & Lowther, 1951). The DNP-glycine samples in all cases ran as single spots in the same position as the marker sample. After elution the DNP-glycine solution was acidified, extracted with ether, and the extract evaporated. Since a considerable amount of solid material (extracted from the paper) contaminated the DNP-glycine, and made the accurate plating of the sample for counting impossible, a solution in ether was applied to a Celite column, using ether as solvent, as a means of purification. This procedure was found to remove the contaminant completely and to permit the measurement of specific activity of the DNP-glycine in the usual way. Table 58 summarises the results of a number of these tests on the purity of glycine obtained from the free amino acid mixture of the liver cell, and from degradation of purines and proteins. It will be seen that for all practical purposes, there is no appreciable difference between the

Table 39.

Comparison of the specific activities of duplicate samples of DNP-Glycine prepared from different rat liver fractions.

ORIGIN OF GLYCINE	RAT No.	SPECIFIC ACTIVITY(C.p.m. per 100 µg. glycine)		
		First replication	Second replication	Difference
Free amino acids of cell	20	479	487	+2%
	21	675	659	-2%
Hydrolysate of liver protein	11	208	200	-4%
	13	325	369*	+13%

* Only 23 µg. glycine was used; this is about 1/5th of the usual amount assayed for radioactivity.

specific activity of the DNP-glycine subjected to further purification by paper chromatography, and the sample obtained from the second (other) column.

As a general indication of the reproducibility of the specific activity determinations, duplicate estimations on four samples are shown in Table 39. Only in the case of the last sample, in which one estimation was made on a minute sample, did the variation exceed 10%.

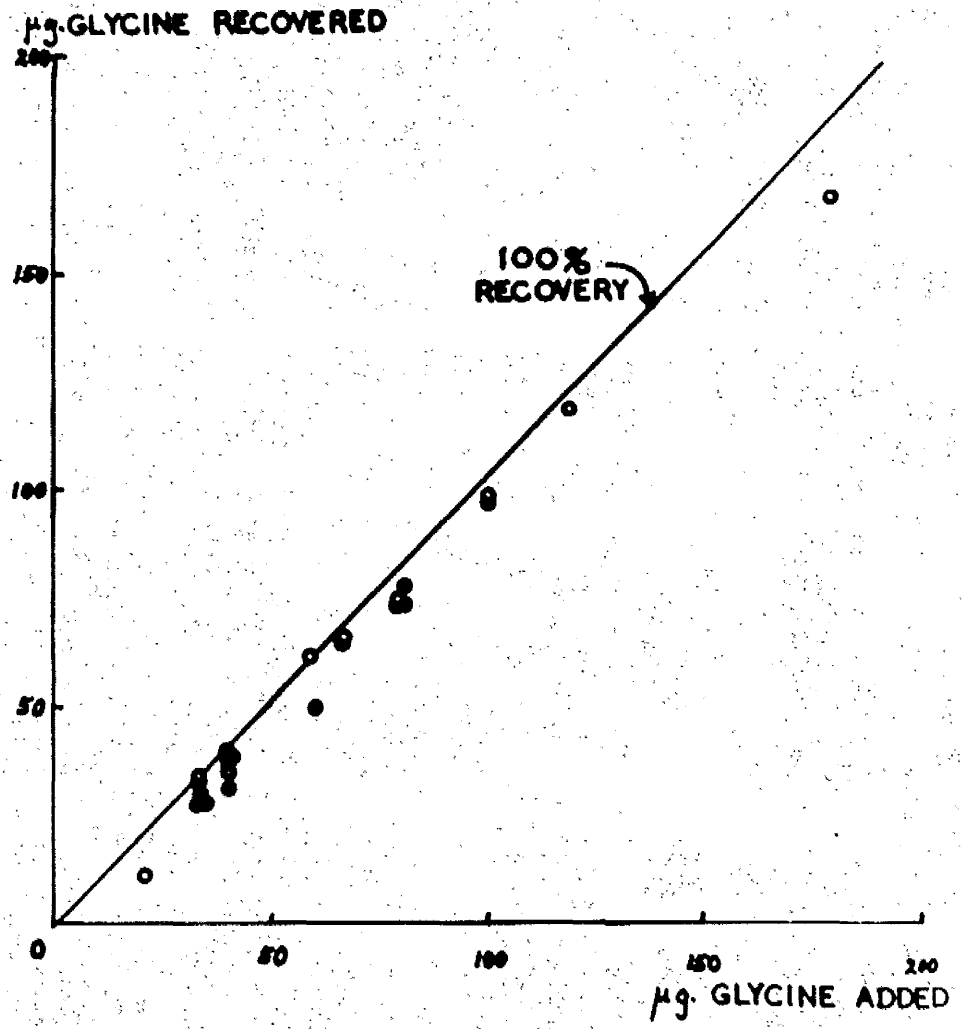
Quantitative Studies of Glycine in Liver Protein. In order to determine whether the various diets used in these experiments altered the glycine content of the liver proteins, quantitative estimations were made by the procedure of Kroll (1952), which utilizes the separation of glycine as the DNP-derivative on Celite columns, by the method already described for the radioactivity determinations. Since Kroll observed a small but constant loss on his Celite columns, it was necessary to provide correction factors for use with our columns; this was especially necessary, since Kroll did not study recoveries of glycine from mixtures of amino acids of the complexity of that obtained on hydrolysis of a protein. Various amounts of glycine were reacted with FDNB and the DNP-glycine separated on Celite columns, the amounts in the effluents being estimated as previously described. In the same way, the recoveries of

Table 40.

The amount of glycine liberated from a sample of liver protein heated in 6 N HCl at 105° for different lengths of time. Each figure is the mean of three estimations of glycine content, which is expressed as g. of glycine per 100 g. of pure protein (containing 16% N).

Time of hydrolysis	Apparent glycine content of protein	Amount released in relation to 24 hr. hydrolysis
hr.	%	%
8	4.63	97
12	4.44	93
16	4.67	98
24	4.79	100

Fig. 10



The recovery of glycine from Celite columns as the dinitrophenyl derivative. o = glycine alone, ● = glycine present in a mixture of amino acids.

different amounts of glycine from a mixture of amino acids were studied. The recoveries under these conditions are shown in Fig. 10. The regression equations obtained are given below, where X is the amount of glycine (in µg.) added, and Y is the amount (in µg.) recovered:

(1) Glycine alone:

$$Y = 1.4 + 0.94X$$

(2) Glycine in amino acid mixture:

$$Y = -4.6 + 0.98X$$

Since the second equation represents the conditions under which glycine is estimated in protein hydrolysates, it was used in all quantitative assays.

In studying the amounts of glycine in liver protein, it was also necessary to establish optimum conditions for hydrolysis. Hydrolysis of a liver protein sample was carried out in 6N HCl at 105°C in an oil-bath for various lengths of time and the amount of glycine liberated was estimated (Table 40). It will be seen that even an 8-hour period is effective in liberating almost as much glycine as a 24-hour hydrolysis period. It was therefore decided to adopt a 16-hour period as adequate.

RESULTS.

Influence of diet on the amount of glycine in liver protein.

Table 41.

Amount of glycine in liver protein obtained from rats on various diets. The glycine content has been expressed in relation to pure liver protein (16.0% N).

DIET	ENERGY LEVEL	Glycine content of protein
Protein-free	Low	4.98
	High	5.06
Protein-containing	Low	5.07
	High	5.33

Before attacking the problem of incorporation of radioactive glycine into the proteins of the liver, it was necessary to determine whether the amount of glycine in the combined proteins of the liver was appreciably affected by the dietary treatments used. If such an alteration did take place, then it would have to be taken into consideration when computing the total amount of radioactive glycine incorporated by the liver ("total relative activity") on the various diets used.

The livers were obtained from rats fed on diets either rich in protein or free from protein and providing either high or low levels of energy during the last 4 days before sacrifice. Protein was isolated in the usual way by precipitation with trichloroacetic acid, followed by removal of nucleic acids with hot 10% trichloroacetic acid and of lipids with organic solvents. The dry powder was examined for nitrogen and for glycine content. The amount of glycine was then related to the protein content of the powder, on the assumption that the mixed liver proteins contain 16.0% nitrogen. Table 41 shows that diet altered the glycine content of the mixture of liver proteins by less than 7% which is probably within experimental error and is from the practical viewpoint negligible. Changes in the protein content of the liver can therefore

Table 42.

The incorporation of glycine-2-^{14}C into the free amino acid glycine and protein glycine of the rat liver. The first four groups of rats were in the post-absorptive state when the isotope was injected; the last two groups were fed protein. Each figure for the specific activity of glycine is the mean of data obtained from two animals (Exps. 2 & 3), with the exception of the fifth group, in which there was only one rat at each time-interval. The results are expressed in counts per minute per 100 ug. of glycine.

Glycine isolated from	Time of sacrifice after injection	Protein-free diet (post-absorptive state)		Protein-containing diet (post-absorptive state)		Protein-containing diet (fed protein)	
		Low energy	High energy	Low energy	High energy	Low energy	High energy
Free amino acids of liver	hr. 3	2362	2188	1825	1490	1265	1215
	6	1523	1332	1173	945	648	714
	9	875	716	733	717	479	460
Liver protein	3	294	355	219	216	195	177
	6	361	371	229	207	212	207
	9	331	349	220	206	193	190

Table 43.

The relative specific activities of liver protein glycine from rats receiving various diets (Computed from the data of Table 42, by dividing the specific activity of protein glycine by the specific activity of free glycine and multiplying by 100).

Time of sacrifice after injection	Protein-free diet (post-absorptive state)		Protein-containing diet (post-absorptive state)		Protein-containing diet (fed protein)	
	Low energy	High energy	Low energy	High energy	Low energy	High energy
hr.						
3	12.6	16.2	12.0	14.5	15.4	14.8
6	24.1	27.9	19.5	21.9	32.8	28.9
9	38.0	47.3	28.4	28.8	40.3	42.7

Table 44.

The total relative activities of liver protein glycine of rats receiving various diets. (Figures obtained by multiplying the relative specific activities given in Table 43 by the amounts of protein per liver. The amount of protein was expressed per mg. of deoxyribonucleic acid phosphorus, used as a reference standard).

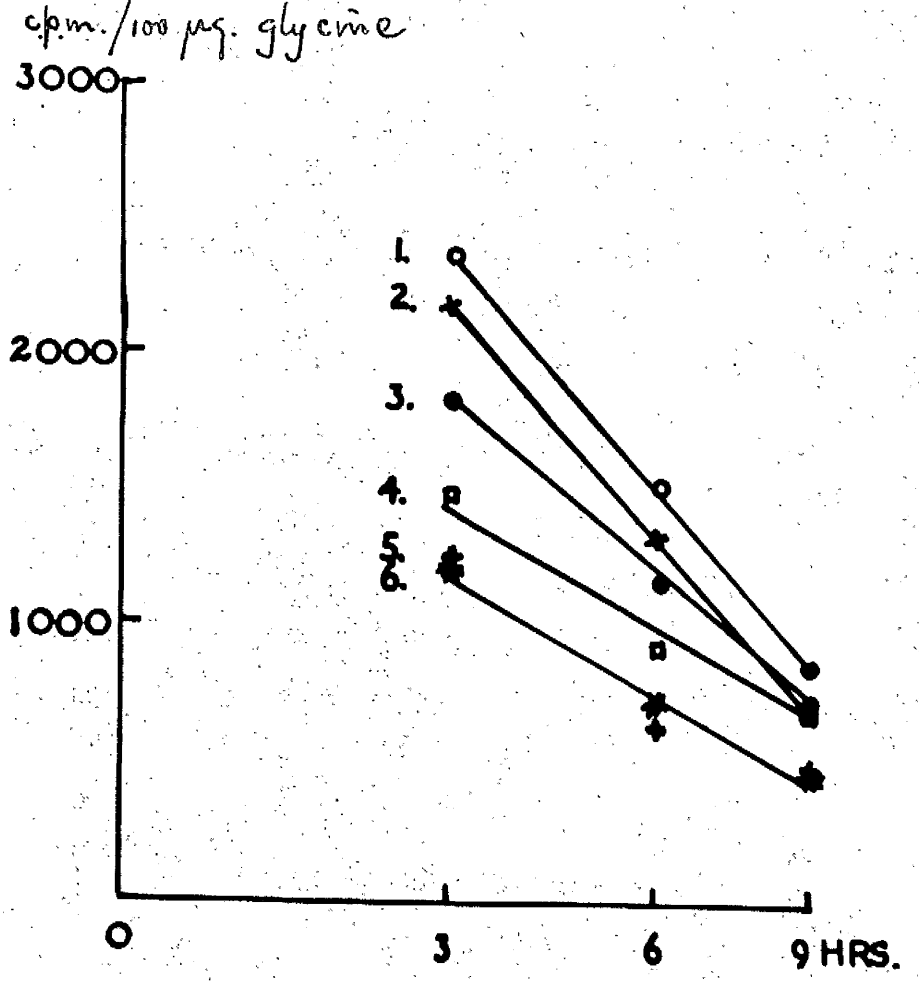
Time of sacrifice after injection	Protein-free diet (post-absorptive state)		Protein-containing diet (post-absorptive state)		Protein-containing diet (fed protein)	
	Low energy	High energy	Low energy	High energy	Low energy	High energy
hr.						
3	83	118	93	159	181	161
6	174	207	189	236	328	291
9	302	345	263	321	466	499

be considered to involve corresponding changes in the amount of protein glycine, and consequently the protein content of the liver can legitimately be used in lieu of protein-glycine determinations when computing total relative activities.

The incorporation of glycine-2-^{14}C into liver protein.

Three independent series of experiments on incorporation were performed, but, owing to a number of technical imperfections, the data from the first of these experiments have been excluded from consideration. In the second and third series, technique was satisfactory and the results are in good agreement. Consequently, the average data from these two experiments is presented in Table 42 to 44. The nutritional groups studied were six in number. Rats were fed either a protein-free diet or a protein-containing diet. During the last 4 days on these diets, the energy content was varied to give either a high or low level of energy intake. On the morning following the last day of this regimen, the animals were injected with ^{14}C and ^{32}P and killed some hours later, without further diet being given since the 5 p.m. feed on the preceding evening. We thus obtain a picture for rats in the post-absorptive state on diets providing two levels of energy at two levels of protein intake. In addition to these four groups protein

Fig. 11.



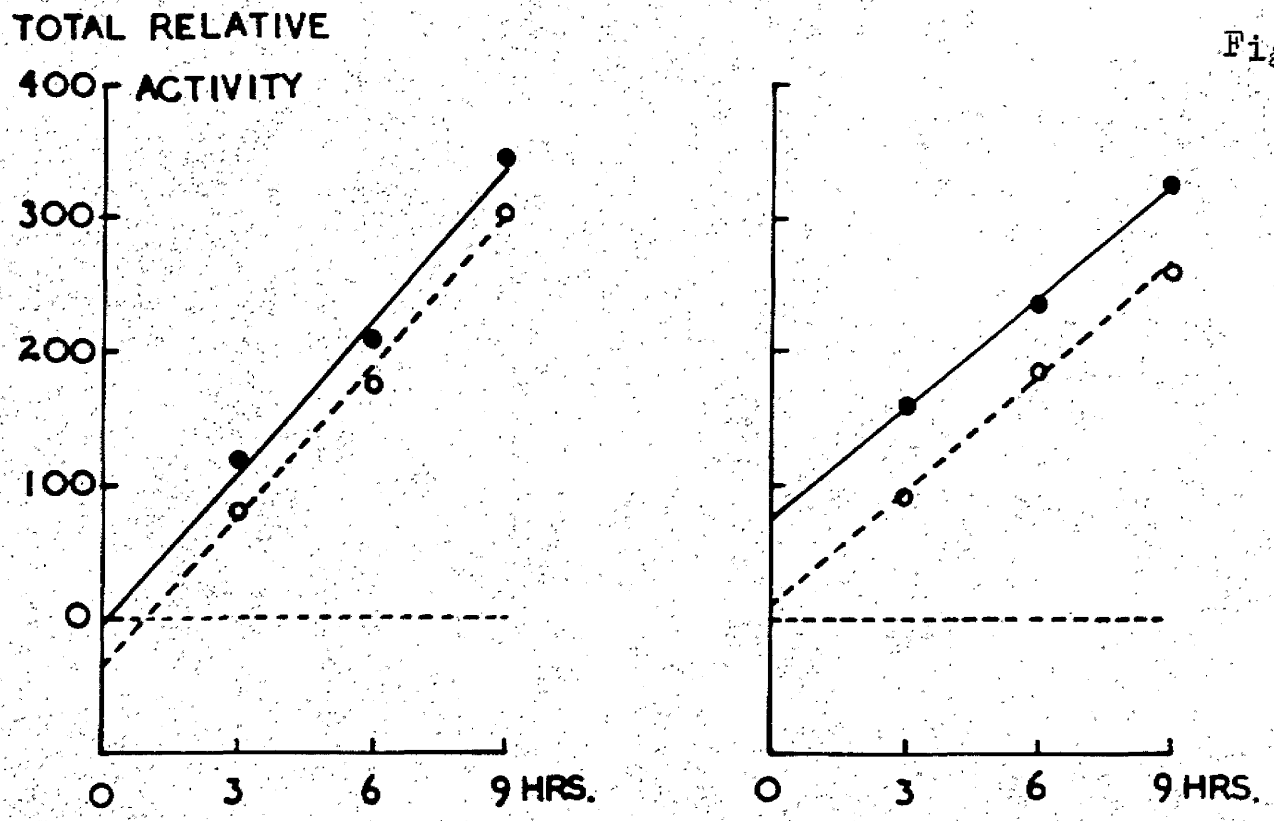
The specific activity of glycine(c.p.m./100 µg.glycine) in the free amino acid fraction of the liver cell. The curves were obtained with animals on the following diets: 1 and 2: protein-free diet (post-absorptive state) at low and high energy intakes respectively. 3 and 4: protein-containing diet (post-absorptive state) at low and high energy intakes respectively. 5 and 6: protein-containing diet (fed protein) at low and high energy intakes respectively.

(casein) was fed at the time of isotope injection to some of the rats on the protein-containing diet at both low and high energy levels, giving a further two groups.

From each animal we obtained a measure of the specific activities of the free (non-protein, non-peptide) glycine of the liver cell and the specific activity of the liver protein glycine (Table 42). The specific activity of the free glycine fraction has also been plotted on semi-log paper (Fig. 11), and it will be seen that it is then linearly related to the logarithm of the time of sacrifice after all types of diet. It is particularly to be noted that an identical line describes the curves obtained in the rats at both low and high energy levels fed protein at the time of isotopic injection.

When we turn to the specific activity of the glycine in protein form, the data (Table 42) show little difference between the figures on any one diet at 3, 6 and 9 hours after injection. This makes it impossible to use the relationship between changes in the specific activity of free glycine, and of protein glycine described by Zilversmit, Entenman and Fishler (1943) in order to compute the rate of protein synthesis. In order to use this method with some degree of confidence a reasonable increment in specific activity of the product (protein) must take place between successive time-intervals after injection

Fig. 12.

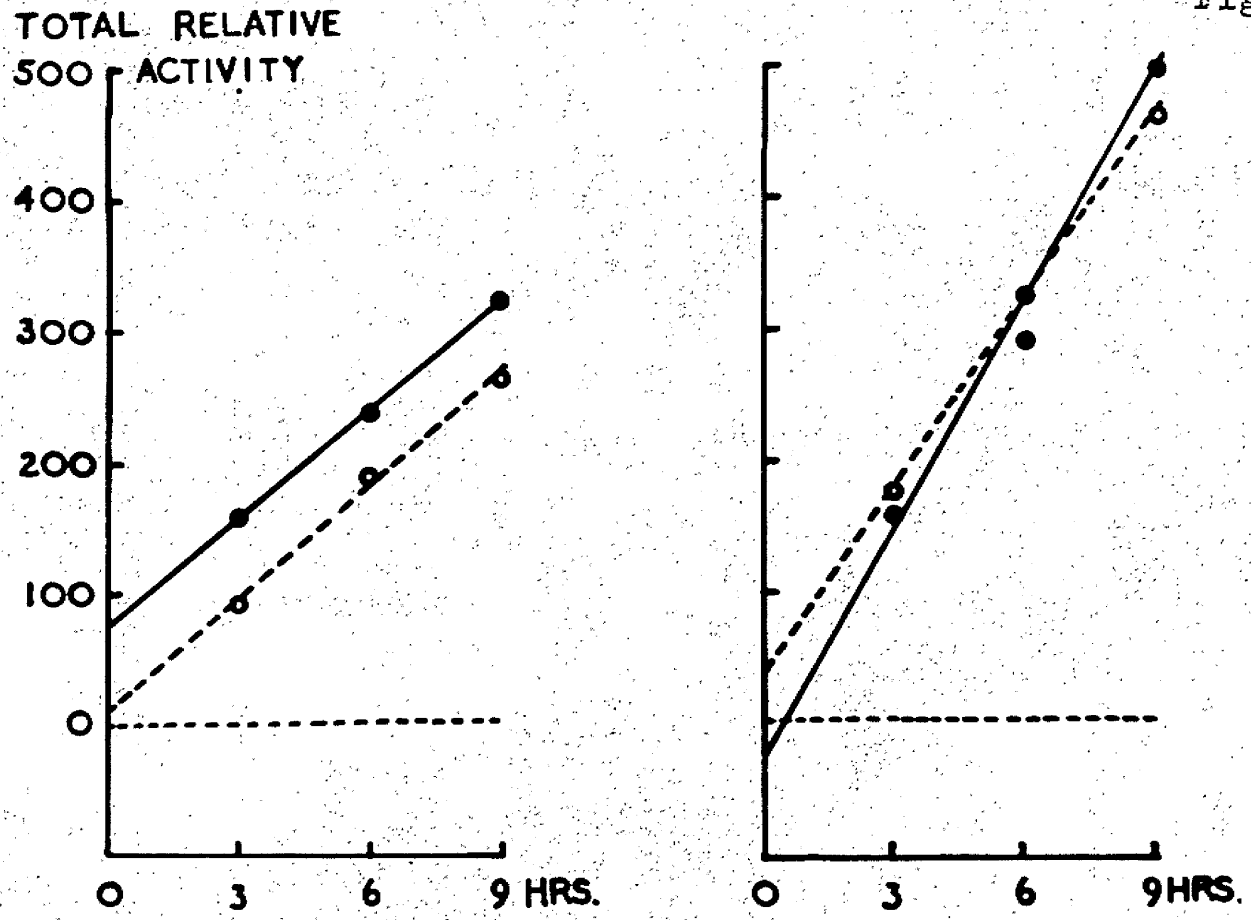


Total incorporation of glycine-2-^{14}C into liver protein (expressed as total relative activities). Left-hand graph obtained from rats on a protein-free diet (post-absorptive state.) Right-hand graph obtained from rats on a protein-containing diet (post-absorptive state). (o-----o = low energy intake; ●——● = high energy intake).

of the isotope.

It will be noted that the specific activity of the protein glycine is actually higher on the protein-free diet, in agreement with the findings of Solomon and Tarver (1952) using ^{35}S-labelled methionine. However, when these specific activities are related to the activities of free glycine isolated from the corresponding livers, i.e. the relative specific activities are calculated, the apparent greater incorporation in the protein-free diet is much less evident (Table 43). The greater uptake of isotope on the protein-free diet is finally accounted for when the total amount of protein synthesized is computed, using "total relative activity" (relative activity x amount). In this case, it was convenient to use the deoxyribonucleic acid content of the liver as a reference standard against which to measure the absolute amount of protein in the liver (Table 44). The lower relative specific activities on the protein-containing diet are balanced by the greater amounts of protein in the liver, so that the total incorporation rate (Table 44 and Fig. 12) is of the same order of magnitude irrespective of the protein content of the preceding diet. At each level of protein intake, there is, however, a distinct effect of the energy content of the preceding diet. When we consider the total relative

Fig. 13.



Total incorporation of glycine-2-^{14}C into liver protein(expressed as total relative activities). Left-hand graph obtained from rats on a protein-containing diet (post-absorptive state). Right-hand graph obtained from rats on a protein-containing diet (fed protein) (o-----o = low energy intake; ●——● = high energy intake).

Table 45.

Regression equations for the total relative activities of liver protein glycine isolated from animals on various diets. The equations have been computed from the data of Table 44 and represent the regression of total relative activity on time of killing, where X = time in hours after injection of isotope and Y = total relative activity of protein glycine.

DIET	ENERGY LEVEL	REGRESSION EQUATION.	
Protein-free (post-absorptive state)	High	Y = 56.8X	-0
	Low	Y = 56.5X	-32
	Difference	+0.3	+32
Protein-containing (post-absorptive state)	High	Y = 27.0	+77
	Low	Y = 28.3	+12
	Difference	-1.3	+65
Protein-containing (fed protein)	High	Y = 56.3	-21
	Low	Y = 47.5	+40
	Difference	+ 8.8	-61

Analysis of variance reveals that the regression lines for the first two diets differ significantly in slope ($P < 0.01$).

Footnote to Table 45 cont'd.

The slope of the regression line on feeding protein is significantly greater than the regression slope for the protein-containing diet in the post-absorptive state ($P < 0.05$). However, the regression coefficients are not significantly affected by energy level within the same diet (i.e., 38.8 and 36.5; 27.0 and 28.3; 56.3 and 47.5 do not differ significantly among themselves). Although this means that the two lines on any one diet do not deviate from parallelism they run at different levels, indicated by the differing constants of the regression equations. In the case of both the protein-free and protein-containing diets (post-absorptive state), the effect of the higher energy level is significantly positive ($P < 0.01$), the change being greater ($r = 0.85$-0.93) in the case of the protein-containing diet. The negative change with energy increment in the case of the protein-fed animals is not significant.

activities for the animals fed protein at the time of injection of the isotopes, it is apparent that the total incorporation is increased by comparison with similar animals not receiving protein (Table 44). However, it is evident (Fig. 13) that, in contrast to the animals studied in the post-absorptive state after the protein-containing diet, previous energy level has no distinct effect on rate of glycine incorporation by these rats.

In comparing these effects of diet on total relative activity, it is helpful to compute the regression lines of total relative activity on time. This relationship is a linear one on the various diets (Figs 12 and 13) and thus is readily susceptible to statistical analysis (Table 45). The two features in the regression equation which have to be considered are the slope of the line and the intercept representing activity at zero time. Table 45 shows that in the post-absorptive state the slope (regression coefficient) is significantly less on the protein containing diet than when the preceding diet is protein free. However, administration of protein to rats previously on a protein diet results in the greatest slope of all. On none of these diets was the slope significantly greater when the preceding diet provided more energy.

As regards the intercept corresponding to zero time, it is

clear that the labelled glycine must take an appreciable time to reach the liver and consequently that total relative activity will still be zero for some time after the injection is given. This means that the projection of the regression line back to zero time (Figs. 12 and 13) should result in a negative value for total relative activity. Table 45 and Figs. 12 and 13 show that this is the case for the animals fed on the protein-free diet and for one group fed the protein-containing diet with extra protein at the time of injecting the isotope. However, rats previously on the protein-containing diet and killed in the post-absorptive state exhibit a positive intercept, i.e. the total relative activity is already appreciable at the time of injecting the isotope. This impossible result, which is nevertheless statistically valid, is due to the rate of incorporation being greater for the 3-hour period than at later times of killing. Presumably the rats previously receiving protein in the diet have still a slightly raised amino acid level in the tissues at the time of injection, but this steadily declines as the animals are killed at successive time intervals. In consequence, those killed at 3 hours after injection have a disproportionately high uptake of glycine into the liver protein, which is not maintained in later samples and this diminishing incorporation rate, when projected back to zero time

leads to a falsely high intercept figure. Whatever the explanation of the differing intercept figures at the different levels of protein intake, there is a slight stimulant effect of previous energy intake on the values obtained for the protein-free diet and a much greater effect when the preceding diet contained protein. However, when protein was fed in addition at the time of injection, there is a non-significant change of opposite sign in the intercept value. This reinforces the conclusion made from inspection of the data, namely that a previous high level of energy intake, notably in conjunction with a diet containing protein, results in a greater initial uptake of glycine into liver protein, whereas the administration of protein at the time of injecting the isotopic glycine obliterates this effect of energy level.

The incorporation of glycine-2-^{14}C and ^{32}P into liver ribonucleic acid. This work was essentially carried out, except for the final assay of radioactive glycine, by Miss C. M. Clark, and it is not intended to do more in this thesis than indicate their bearing on the radioactive phosphorus studies described in Section 2 of Part II of this thesis.

For each animal, glycine has been isolated from

Table 46.

The uptake of glycine-2-^{14}C and ^{32}P by liver ribonucleic acids on various diets. The specific activity of purine glycine was computed relative to that of free glycine, and the specific activity of nucleotide phosphorus relative to that of inorganic phosphate.

DIET	ENERGY LEVEL	Ratio of Relative Specific activities of glycine to phosphorus	
		For Adenylic acid	For Guanylic acid
Protein-free (post-absorptive state)	Low	0.76	0.93
	High	0.39	0.88
Protein-containing (post-absorptive state)	Low	0.60	0.16
	High	0.37	0.13
Protein-containing (fed protein)	Low	0.83	0.92
	High	1.12	1.08
Statistical analysis		P = 0.05-0.01 (30 observations)	P = 0.01 (37 observations)

the purine nucleus of the adenine and guanine of liver ribonucleic acid, and the specific activity of the glycine related to the specific activity of glycine free in the cell (relative specific activity). In addition, the specific activity of the phosphorus of adenylic acid and guanylic acid relative to the specific activity of liver inorganic phosphate was measured. Comparison of the relative specific activity of purine glycine to nucleotide phosphorus is given for adenylic acid in Table 46, which represents the average of the data for 3, 6 and 9 hours. If the relative specific activities are similar, then the ratio is 1.0; if the uptake of glycine into the purine nucleus is less than ^{32}P uptake, the ratio falls below unity. On the diet containing no protein, uptake of glycine and phosphorus are approximately at similar rates for guanylic acid; in the case of adenylic acid the uptake of glycine is less than that of phosphorus. On the protein containing diet in the post-absorptive state, glycine uptake by guanylic acid is greatly reduced in comparison with ^{32}P uptake, but this is not evident with adenylic acid. On feeding protein to the animals on this diet, uptake of glycine by guanylic acid returns to the same level as ^{32}P uptake.

DISCUSSION.

Incorporation of glycine into liver protein. In interpreting the findings with liver protein it is important to consider first of all the exact nutritional state of the animals used in these investigations. Four groups of animals were prepared for the experiment by feeding diets either rich in protein or free from protein at two energy levels. In this way it was proposed to measure the influence of these two dietary factors, namely energy intake (or more precisely the availability of energy) and protein intake, on the course of protein metabolism in the liver, by combining a quantitative determination of the amount of protein in the liver with measurement of the rate of incorporation of an isotopically labelled amino acid into the liver proteins. This combined estimate of protein metabolism is expressed as total relative activity (Table 44). It should be noted however that in all cases, the radioactive labelled glycine was injected at least 14 hours after feeding the last meal and so the animals were in the post-absorptive state. Thus what was measured was the influence of previous dietary treatment rather than the immediate effect of energy and protein on the protein synthetic activity of the liver. From inspection of the data recorded in Table

44, it is obvious that the rate of glycine incorporation in the liver is much the same for animals fed the protein-containing and protein-free diets at each plane of energy intake. On the other hand, at each level of protein intake, the influence of energy intake on protein metabolism is clearly illustrated. More detailed statistical analysis (Table 45) suggests that the influence of previous energy intake is slight in the case of the protein-free diet, and considerable when the preceding diet contained protein. When some of the animals, previously fed a high-protein diet, were allowed to eat casein before they were injected with glycine-2-^{14}C and thereafter until the time of death, the pattern of protein metabolism was completely changed. The total relative activity was elevated to essentially the same value, irrespective of the previous energy content of the diet. Under these conditions, the effect of energy could no longer be demonstrated.

These experiments thus provide evidence of intermittent bursts of increased protein synthesis following the taking of protein (Fig. 13). This is in accord with the evidence provided by Geiger (1950) and others that essential amino acids have to be fed simultaneously in order to effectively supplement one another, which implies that augmented protein synthesis is limited to a short

period after each protein meal.

Energy intake on the other hand appears to have a "between meals" effect in stimulating protein synthesis. Even in the fasting state, the influence of a previous high energy intake can be seen in an augmented rate of incorporation, especially when the preceding diet contains protein. When protein is fed, however, the response of the proteinsynthetic mechanism appears to be independent of previous energy intake. This explains some N balance observations made on dogs by Allison and Anderson(1945). They measured the response of N-balance to an increment in protein-intake at different levels of energy intake (N-balance index). It was found that the N-balance index was unaffected by increasing their energy intake from 80 to 100 kg. cal./kg. body weight or by reducing their energy intake to 50% of the normal requirement. The constancy of the N-balance index means that a given change in protein intake has the same effect on N-balance at these different levels of energy intake. In other words, the capacity of the animal to utilize dietary protein is not influenced by energy intake, although the absolute N-balance becomes more favourable as energy intake rises. A similar observation has been made by Campbell and Kosterlitz (1946)on the response of liver protein to variations

in protein intake; over a wide range of energy intakes, the amount of protein in the liver responded to the same extent to a given increment in protein intake (Fig. 14). The absolute amounts of liver protein (but not the increments) were affected by energy level, so that lower energy intakes did in fact lead to a reduction in liver protein content, an observation which we have been able to confirm in Part I of this thesis.

Incorporation of glycine-2-^{14}C and ^{32}P into liver ribonucleic acid. The results given in Table 46 indicate that glycine uptake and ^{32}P uptake by the adenylic acid and guanylic acid of liver RNA is in approximately the same proportion when the animals are in the post-absorptive state after receiving a protein-free diet (first two dietary groups of Table 46) or when they have just consumed a protein meal (last two groups in Table 46). Since we have already shown (Section 2 of Part II) that total ^{32}P incorporation into liver RNA is independent of the amount of protein laid down in the liver, it follows that the same absence of a relationship holds for the purine bases. The considerable reduction in glycine incorporation relative to ^{32}P incorporation which occurs in the post-absorptive state following a protein-containing diet coincides with the period of disintegration

the period of disintegration of liver RNA and is presumably due to unequal dilution of the bases and ^{32}P in the newly formed RNA by these breakdown products.

GENERAL DISCUSSION.

GENERAL DISCUSSION

The studies presented in this thesis are concerned with certain fundamental aspects of the metabolism of proteins. Interest was primarily focussed on the relationship of energy metabolism to protein metabolism in the liver of the rat, but in subsequent experiments, the study was extended to the metabolism of phospholipids and ribonucleic acid, both of which display certain metabolic features in common with protein. The observations on the metabolism of ribonucleic acid were of particular interest in view of the alleged relationship between ribonucleic acid and protein formation.

The purpose of this section of the work is to discuss the findings of these experiments in the light of current views on the biosynthesis of proteins.

The problems of protein structure and of protein biosynthesis have engaged the attention of biochemists for more than half a century. Shortly after the beginning of this century, Folin (1905) published an interpretation of protein metabolism which seemed to elucidate many of the problems created by the conflicting theories of Voit and Pflüger. From a study of the variations in the composition of urine at different levels of protein intake, Folin was forced to the conclusion that protein metabolism followed

two distinct and independent pathways. The metabolic processes resulting in end-products which tended to be constant in quantity, for example creatinine, he termed tissue or ENDOGENOUS metabolism. Those which gave rise to the variable constituents of urine, for example urea, he termed EXOGENOUS metabolism. It was believed then that in the adult animal only a small amount of ingested protein was needed for the replacement of the daily waste of tissue, reflected by the endogenous metabolism. From the greater part, preliminary removal of nitrogen permitted the oxidation of the carbonaceous residue for the production of energy.

In 1935, however, Borsook and Keighley presented evidence to suggest that the anabolic process was much more extensive than Folin had believed. It was claimed that this "continuing metabolism" of protein, as they termed it, was always in operation, even in the state of N equilibrium, and might normally involve 50 per cent or more of the nitrogen intake. In 1939, Borsook's conclusions were supported by the results of Schoenheimer and his colleagues on the distribution of isotopic nitrogen after feeding labelled amino acids to rats (Schoenheimer, Ratner & Rittenberg, 1939). These experiments revealed that urinary N primarily represents not the dietary N, but tissue N,

and that this catabolic process is associated with a continuous and rapid incorporation of dietary amino acids into tissue proteins.

About the same time, the introduction of new techniques into biochemical research greatly accelerated the accumulation of information on the nature of the protein molecule. Purified proteins were shown with the aid of the high-speed centrifuge to have many of the attributes of homogeneous molecular species. The contribution from chromatographic studies, notably those of Sanger and his colleagues (1948, 1951, 1953) on end-groups and amino acid sequences in the insulin molecule revealed that a purified protein is a single molecular species. Immunological studies support this view; the work of Landsteiner and his associates (1945) suggested that immunological specificity is a reflexion of a specific chemical structure. We may, therefore, suppose that each type of cell possesses enzymic mechanisms capable of the exact control of amino acid composition and sequence of all its proteins.

Although the need for a direct chemical approach to the problem of protein synthesis is obvious, unfortunately such an approach is difficult because of the unavoidable artificialities inherent in the use of excised tissues. For this reason studies on intact animals offer some indication

of the basic features involved. Such an approach, while admittedly peripheral to the core of the problem, has nevertheless been extremely useful in the past, and still offers many advantages in relation to the overall problem.

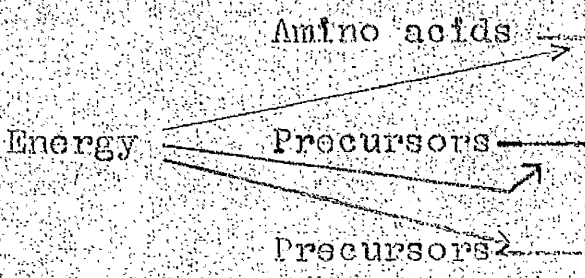
Our original interest in the problem lay in the relationship between energy intake and protein metabolism. Although it is well known that protein metabolism is influenced by energy intake, there had been no systematic study of the relationship between the availability of energy and protein metabolism. We therefore carried out studies of the N-balance and of the protein content of the liver of rats receiving diets either rich in protein or deficient in protein in combination with various levels of energy intake. It was found that the influence of energy intake on N-balance and on the total amount of liver protein was dependent on the protein content of the diet. At an adequate level of protein intake, N balance and liver protein were affected in a strictly linear fashion by variations in energy intake. On the other hand, on a protein-free diet, energy intake appeared to have little effect. These findings can be readily explained in terms of the factors influencing protein synthesis. Protein formation can proceed in the tissues only if there is a supply of both amino acids and energy, either of which

can be a limiting factor in the rate of synthesis. When the diet provides adequate amounts of protein, N balance and the amount of protein in the liver show a linear relationship to energy intake, which in this case is the limiting factor. On the protein-free diet, the amino acid supply is limited to those circulating in the blood, and consequently this factor rather than energy supply limits the extent of protein synthesis. An amplification of this picture has been obtained from a study of the incorporation of glycine-2-^{14}C into liver proteins. The evidence presented in Part IV suggests that, while the influx of amino acids into the liver following the ingestion of protein promotes a rapid (and temporary) increase in the rate of incorporation of amino acids independent of energy intake, the effect of a positive energy balance is a prolonged one, and is exerted between meals. The difference in the "between-meals" effect of energy intake in the case of the protein-containing diet and in the case of the protein-free diet (Table 45) would account for the fact that the dietary level of energy affects the amount of protein in the liver on one diet but not on the other.

Essentially the same picture was obtained in the study of phospholipid metabolism (Part III). Only

when the diet provided an adequate supply of protein was the synthesis of phospholipid in the liver increased in response to increments in energy intake (Table 34). It was suggested that the feeding of a protein-free diet so reduces the concentration of some precursor or essential component in phospholipid formation that it becomes the limiting factor in the rate of synthesis. There appears to be no such factor restricting the response of ribonucleic acid metabolism to changes in the energy level of the protein-free diet (see Part II). The stimulating effect of energy intake has been shown in the cold-room studies to be due to the accompanying increase in energy yielding nutrients in the tissues, i.e. a positive energy balance.

As an attempt to explain these observations on the metabolism of protein, phospholipid and ribonucleic acid metabolism in the liver, we may picture the amount of energy available as the factor governing their rates of synthesis.



Provided no other component of the synthetic mechanism

limits the response to a change in available energy, a positive energy balance is associated with an augmented rate of synthesis. It would, however, seem that synthesis of protein becomes independent of energy intake when the tissues are flooded with amino acids after eating protein.

Energy of Peptide Bond Formation. While it has long been recognized that the formation of peptide bonds involves a supply of energy, there is still no certainty about the mechanism whereby this energy is made available. Because of the difficulties inherent in the study of the synthesis of proteins, which are large molecules of unknown structure, many attempts have been made to study peptide bond synthesis in simple molecules.

The formation of a peptide from two amino acids is accompanied by an increase in free energy, the average value of 3000 cal. per mole being widely quoted. In other words, peptide formation from free amino acids does not proceed spontaneously to any significant extent. Any satisfactory hypothesis of protein synthesis then must explain this coupling of the endergonic reaction to a process providing energy for the system.

Some years ago, Bergmann carried out a brilliant series of experiments in which acetyl, benzoyl and

carbobenzoxy derivatives of a number of amino acids were condensed with aniline or phenylhydrazine, in the presence of intracellular proteolytic enzymes of both plant and animal origin, to form simple peptide bonds. In the same way, two amino acids were made to combine to form a dipeptide by increasing their free energy contents through similar substitutions. For example, chymotrypsin was shown to catalyze the condensation of benzoyl tyrosine with glycine anilide to form benzoyl-tyrosyl-glycine anilide in 65% yield (Bergmann & Fruton, 1944). Brenner a nd Pfister (1951) studied another type of enzyme-catalyzed synthesis. Aromatic and aliphatic esters of amino acids were incubated with chymotrypsin, and the reaction followed by filter paper chromatography. During the hydrolysate large amounts of a mixture of peptides of varying molecular weight were formed. No peptide formation occurred when free amino acids were used as substrate, nor was synthesis observed from the esters without the simultaneous appearance of free amino acids. Although protein biosynthetic mechanism utilizing intracellular proteases is conceivable, as yet no physiological analogues of these substituted amino acids have been found. For example, acetylation and phosphorylation reactions have been known for many years to take place in

animal tissues, but attempts to replace amino acids by their acetylated or phosphorylated derivatives in enzyme-catalyzed reactions have not been successful. Thus Cohen and McGillivray (1947) were unable to synthesize p-aminohippuric acid from p-aminobenzoic acid and N-acetyl glycine in an enzyme system capable of effecting the synthesis with free glycine. Furthermore, N-phosphorylated glycine and benzoylphosphate was found to be no more active than glycine and benzoic acid in the enzymatic synthesis of hippuric acid, although ATP is known to promote the synthesis under anaerobic conditions (Borsook, 1953).

A more profitable line of investigation, also begun by Bergmann and later extended by Fruton, was the study of exchange reactions occurring during the hydrolysis of amide and peptide bonds. Bergmann first noted that during the hydrolysis of hippurylamide by papain in the presence of aniline, considerably more hippuric acid anilide was formed than could be accounted for under the circumstances by direct synthesis from hydrolysis products (Bergmann & Fraenkel-Conrat, 1937). Fruton also demonstrated the replacement of amide nitrogen by isotopic N during the hydrolysis of benzoyl-glycinamide by papain in the presence of isotopically labelled ammonium salts

(Johnston, Mycek & Fruton, 1950a). An extension of the peptide chain at the expense of the amide link was also shown to be catalyzed by proteolytic enzymes. If the hydrolysis of benzoyltyrosinamide by cathepsin or papain in the presence of glycinamide is stopped short of the equilibrium point, benzoyl-tyrosyl glycinamide can be isolated from the reaction medium. This type of reaction Fruton terms "transamidation" (Fruton, Johnston & Fried, 1951). In the same way, "transpeptidation" reactions occur, in which the proteases catalyse the replacement of one of the components of a peptide bond linking two amino acid residues. For example, the replacement of the glycinamide residue of the tripeptide benzoyltyrosylglycinamide by isotopically labelled glycinamide is effected by chymotrypsin, under suitable conditions (Johnston, Mycek & Fruton, 1950b). Peptides may also be lengthened by a transpeptidation reaction. The terminal amino acid residue of carbobenzoxy-glycylphenylalanine can be replaced by the peptide glycylglycine in a papain-catalyzed transpeptidation, leading to elongation of the chain (Fruton, 1952). These experiments have shown that a proteolytic enzyme can act to lengthen a peptide chain, provided that the necessary energy is available in the form of a preformed amide or peptide bond.

Although a scheme for protein synthesis may be

constructed on the transamidation and transpeptidation reactions, an explanation of the synthesis of the amides and peptides which would act as substrates for the transamidases and transpeptidases must still be found. One cannot escape from a synthesis of peptides *de novo* from amino acids, a reaction which must be coupled with an energy-yielding process.

While discussing the many endergonic reactions which occur in living cells and for which energy might be derived from a phosphate bond energy pool, Lipmann (1949) suggested that the energy required for the synthesis of peptides and proteins might also come from this source. In investigating a possible role of high energy phosphate bonds in protein synthesis, the study of model systems - the formation of simple peptides - has again been profitable. The simplest example is afforded by amide synthesis. The free energy of formation of the amide bond of glutamine is of the same order of magnitude as of simple peptides from amino acids. This is a true synthetic reaction, not accompanied, as in the transamidation reactions, by hydrolysis. The formation of glutamine from glutamic acid by cell-free enzyme preparations from a variety of animal tissues was shown by Speck (1949) and by Elliott (1948) to require ATP, and in this coupled reaction, inorganic phosphate was liberated in an amount equivalent to the amide

synthesized.

The simple peptide hippuric acid has long been known to be formed by animals from benzoic acid and glycine. Its *in vitro* synthesis was first attained in a 60-70 per cent yield by Borsook and Dubnoff (1940), using kidney and liver slices as an enzyme source. With this peptide, as with glutamine, the energy requirements for synthesis were again comparable to those for the synthesis of simple peptides from amino acids. Since thermo-dynamic equilibrium point favours only a 1 per cent synthesis, so high a yield could have been attained only by coupling with an energy-yielding reaction. These same authors showed that cyahide, an inhibitor of respiration, abolished the synthesis in their tissue preparations, as did anaerobiosis, and the conclusion that energy for the synthesis was supplied by energy-rich phosphate bonds was confirmed by the demonstration of the stimulation of hippuric acid formation by ATP in guinea-pig liver homogenates (Borsook & Dubnoff, 1947).

The synthesis of the tripeptide glutathione was first studied by Bloch in pigeon liver extracts, and has been separated into two enzyme systems. In the first step, glutamic acid and cysteine are condensed in the presence of ATP to form γ-glutamyl-cysteine. In the second, on the addition of ATP to the enzyme preparation, glycine is added

to the dipeptide to complete the synthesis. One pyrophosphate bond of ATP is used per peptide bond synthesized, and neither of the purified enzymes has been found to display any hydrolytic activity (Johnston & Bloch, 1951). Recently an identical system has been found by Webster (1953) to operate in extracts of bean-seedling hypocotyls. The reactions were followed using isotopically-labelled amino acids, and ATP was found to promote the synthesis even in the presence of respiratory inhibitors. Glutathione represents the one case so far elucidated of any enzyme-catalyzed peptide synthesis from amino acids, in which ATP plays a direct part.

Although these model systems have made an important contribution to our understanding of energetic considerations in peptide bond synthesis, there is an abundance of indirect evidence in favour of a rôle of high energy phosphate in protein synthesis. ATP or generators of high energy phosphate bonds have repeatedly been found to accelerate the incorporation of amino acids into the proteins of various tissue preparations. Furthermore, the most consistent and powerful inhibitors of amino acid incorporation and protein synthesis are inhibitors of respiration, such as dinitrophenol.

While investigating the incorporation of amino

acids into the proteins of bone marrow cells, rat diaphragm and guinea pig liver homogenates, Borsook noted the inhibitory effect of anaerobiosis and numerous inhibitors of respiration and phosphorylation (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950, 1951). The same inhibitors abolished the incorporation of labelled alanine into the proteins of liver slices in the experiments of Frantz, Kamecnik, Reese & Stephenson (1948), the synthesis of serum albumin in liver slices - a demonstration of true protein synthesis - by Peters and Anfinsen (1950) and the formation of the enzymes amylase, lipase and ribonuclease in pigeon pancreas slices by Hokin (Schuchor & Hokin, 1954). Melchior (1951) also found glucose to have a stimulating effect on the incorporation of isotopically labelled methionine into washed E. coli cells, and an inhibition of the labelling by DNP (Melchior, Klioze & Klotz, 1951).

With regard to our own experiments on protein metabolism, it would appear from some experiments carried out by Munro, Chisholm and Wikramanayake (unpublished data) that ATP is probably the form in which energy stimulates the synthesis of protein in the liver. These workers found that the ratio ATP/ADP (the so-called "phosphate potential", Dixon, 1948) increased linearly with increasing increments of energy to the diet, and concluded

that energy intake exerts its effect on protein synthesis through changes in the "phosphate potential".

Use of labelled amino acids in studies of protein synthesis. Mention of the use of isotopically labelled amino acids brings us to a consideration of the general features of this approach to the problem of protein biosynthesis. Such studies with isotopes indicate only end-results, not the intermediate steps of a reaction. When an amino acid is incorporated into a protein molecule, we have no indication as to what happened to the molecule. Schoenheimer (1939) was the first to point this out. In his words, "there are two general reactions which might lead to amino acid replacement, (i) a complete breakdown of the protein into its units, followed by resynthesis, or (ii) only partial replacement of units". In both types of reaction, peptide bonds are broken and resynthesized. To these, a third possibility may be added, a de novo synthesis of peptides from amino acids and subsequent protein synthesis from peptides. The main question arising from the use of isotopes is whether we can consider the incorporation of labelled amino acids to be indicative of protein synthesis. The answer would appear to depend entirely on the conditions of the experiment. In this connection we have first to consider evidence from nutritional studies carried out with

amino acid mixtures in place of dietary protein. Cannon (1948) has reported that, if an adequate diet containing the 10 essential amino acids as principal source of dietary-N is fed to protein-depleted rats, weight recovery is rapid. If however this diet is divided into two parts each containing five amino acids, and these incomplete portions are fed alternately at hourly periods, with an hour's fast between each feed, the animals continue to lose weight. Similarly, Geiger (1950) has shown that, if an animal is fed a diet lacking one essential amino acid only, supplementation with the missing acid will permit growth, maintenance or recovery from depletion only if it is made within a few hours of feeding the main dietary mixture. These experiments suggest therefore that for protein synthesis to take place, all essential amino acids must be available in the tissues practically simultaneously. Few *in vitro* experiments, however, satisfy this condition, yet every tissue tested irrespective of the nutritional state of the animal or composition of the incubation medium in *in vitro* studies, has been found to incorporate every common labelled amino acid presented to it. Thus, Borsook *et al.*, (1950a, 1951) in a series of experiments using intact rat diaphragm, rabbit bone marrow cells and rabbit reticulocytes, have shown that the incorporation rates of

3 amino acids, leucine, lysine and glycine, were affected to essentially the same degree by both accelerating agents and by respiratory inhibitors, a strong indication that incorporation of all amino acids proceeds by a common mechanism. At the same time evidence of an independent incorporation of each amino acid studied was presented, since the replacement of none was affected by the presence of the others. Greenberg and his colleagues, investigating this relationship between the incorporation of a number of amino acids into the proteins of Ehrlich ascites carcinoma *in vitro*, demonstrated the uptake of three amino acids at optimal rates under conditions in which the incorporation of phenyl-alanine was severely inhibited by thienylalanine and fluorophenylalanine (Rabinovitz, Olson & Greenberg, 1954). Gale and Folkes (1953) have shown that although no protein synthesis takes place unless a wide variety of amino acids is provided in the incubation mixture isotopically labelled glutamate is rapidly incorporated into the cells of Staph.aureus in the presence of ATP.

At first sight these experiments suggest that incorporation of any amino acid may proceed without the simultaneous presence of other amino acids, and argue against the all-or-none synthetic mechanism indicated by the

nutritional studies. On the other hand, the tissue content of free amino acids may suffice to invalidate any conclusion based on the assumption that only the added amino acid was present. Furthermore, in none of these experiments was there any evidence that a net synthesis of protein took place; in fact experimental conditions were such that no net synthesis might be expected to occur. Recently, however, certain workers have paid particular attention to this point. Peters and Anfinsen (1950) have combined radioactive measurements with a quantitative immunological procedure to demonstrate not only amino acid incorporation but a net increase in serum albumin synthesized by chicken liver slices.

The strongest evidence in support of the all-or-none hypothesis has come from Yokin's (1951) experiments on enzyme synthesis. He found that pigeon pancreas slices synthesized alpha amylase when incubated in a glucose-saline medium, and that the rate of synthesis may be increased threefold by the addition to the medium of a mixture of 20 amino acids. Of these 20 acids, it was subsequently found that only 10 were needed, these 10 corresponding to the 10 found to be essential for the chick, with the exception of methionine and the inclusion of tyrosine. The fact that methionine may be excluded without

any diminution in the rate of synthesis is consistent with Boissonas's(1950) observation that methionine is the only essential amino acid not present in crystalline amylase. When any one of these 10 amino acids was omitted from the mixture, synthesis fell to the basal level. It was assumed that the non-essential amino acids would either be formed in the pancreas or exist in concentrations sufficiently high to satisfy the requirements of amylase synthesis. Indirect evidence comes from the isotopic data of Greenberg and his colleagues (Greenberg, Friedberg, Schulman & Winnick, 1948). They achieved a twofold stimulation of glycine-^{14}C uptake by adding an unlabelled amino acid mixture to an adult rat-liver homogenate. That tissue stores of amino acids can lead to a completely false picture of amino acid incorporation was demonstrated by Winnick (1950) who obtained a marked stimulation of glycine incorporation into dialysed foetal homogenates when none was obtained with the undialysed preparation.

From a consideration of the results of this wide variety of experiments it seems likely that *in vitro* incorporation of amino acids into proteins may be the result of both net synthesis and an exchange mechanism representing Schoenheimer's "dynamic state", in which no additional protein is formed. The relative contribution of these

factors to the total amino acid turnover will depend entirely on the particular system chosen for study.

With this concept in mind, we may now assess the suitability of the various hypotheses of protein synthesis.

The peptide theory of Protein Synthesis. Linderstrom-Lang (1952) has pointed out that when a peptide is formed by condensation of a carboxylic compound with the amino group of a peptide, the free energy change should be less than that involved in condensation with a free amino acid, e.g. the free energy change is much less when glycylglycine is condensed with benzoic acid than when glycine is used. It follows therefore that the often cited value of 3000 cal./mole of peptide bond synthesized can no longer be accepted. It would be more accurate to state, on the basis of available data that the free energy change in the process of peptide bond synthesis may vary between the limits 400-4000 cal./mole, and individual values will depend on the nature of the components involved in the condensation. Synthesis of protein from peptides might then be a relatively simple affair compared with de novo synthesis from amino acids. Support for this hypothesis comes from a study of plastein formation. If a partial hydrolysate of a protein is merely concentrated, the enzyme responsible for the hydrolysis will apparently catalyze the reverse

reaction to produce a precipitate of a mixture of peptides of high molecular weight. Under the same conditions, no synthesis occurs from free amino acids or small peptides.

If protein synthesis takes place by way of peptides, there should be present in cell extracts a great variety of peptide intermediates. Apart from free amino acids, however, no other possible precursors representing the various amino acids have so far been detected, except in very much lower concentrations. Dialysable amino acid conjugates found in rat liver and muscle represent at most about 2 mg. N./100 g. tissue, after omitting from consideration glutathione, carnosine and anserine (Christensen, Rothwell, Sears & Streicher, 1948). The scarcity of intermediate precursors has been observed also in Saccharomyces cerevisiae by Halvorsen and Spiegelman(1952). They found that none of the free amino acids was depleted from the cells when the utilization of one of them for growth or enzyme formation was prevented by the presence of a synthetic analogue inhibitor. Therefore no appreciable quantity of intermediary precursors appeared to be involved in the synthesis of the enzyme or other proteins, except for possible precursors already so complex as to require the entrance of the amino acid whose utilization was blocked.

Again, if protein formation were from peptides, we would expect the cells would find no difficulty in utilizing synthetic peptides supplied in the nutrient medium. Many experiments suggest that this is not so. For example, an animal can be maintained in positive N balance by intravenous infusion of a suitable mixture of amino acids. When a partial hydrolysate of a protein is used instead, the animal appears to be unable to use much of the peptide, and this is rapidly excreted in the urine. Even a simple peptide like glycylglycine is apparently metabolized only after hydrolysis. A similar difficulty is encountered in studies of the nutritional requirements of microorganisms. Many organisms show loss of ability to synthesize one or other of the 20 amino acids, but organisms have not been found with absolute growth requirements for peptides. In supporting bacterial growth, peptides have usually been found less active than free amino acids, and where a peptide has been found to have growth-promoting properties this has usually been shown to be due to hydrolysis of the peptides. In the few cases where peptides have been found more efficient than amino acids, the advantage has usually appeared on closer examination to lie elsewhere than in the availability of the peptide for direct

anabolic use. Some support for these studies has come from in vitro work on mammalian tissues. Hokin, studying the formation of amylase, ribonuclease and lipase in pigeon pancreas slices, found a partial hydrolysate of fibrin, containing simple peptides and free amino acids, to be equal but not superior to an amino acid mixture in stimulating synthesis (Schacher & Hokin, 1954).

All the evidence we have for the entrance of precursors other than amino acids into protein synthesis has been obtained indirectly. If we suppose a protein is synthesized by step-wise coupling of many peptide units, the incorporation of a labelled amino acid into the protein would be by way of a large number of preformed peptides of varying pool size, each with a different rate of equilibration with the free amino acids, even in the steady state, if a true dynamic equilibrium between free amino acids and peptides is considered. The equilibrium would be further complicated by dilution of the peptide pools from protein catabolism. When these peptides combine to form the protein there would thus be a considerable variation in the activity of the amino acid residue occurring at different points in the protein molecule. If synthesis were by a mechanism using only free amino acids, there would be no substantial difference

in the radioactivity at different points along the chain. Evidence has been found for a considerable difference in the specific activity of an amino acid at different points in a protein molecule in the experiments of Anfinsen and Steinberg (1951). Crystalline radioactive ovalbumin was prepared by *in vitro* incubation with radioactive CO_2 of oviduct mince from hens. By treatment with a proteolytic enzyme from B. subtilis, it was possible to split off less than 2% of the labelled molecule in the form of small peptides for comparison with the remainder. It was found that the specific activity of the aspartic acid of the peptide fraction was considerably higher than the average activity of the aspartate residues in the remaining fraction. The entrance of peptides into the protein synthesising mechanism was offered as an explanation of this asymmetric synthesis. Although in *in vitro* studies a similar result might be obtained in consequence of transpeptidation reactions occurring as an artifact, such an interpretation should be accepted with reserve. The same authors (1951) have observed that radioactive ovalbumin prepared *in vivo* is also not uniformly labelled with respect to both aspartic and glutamic acids; this presents a more serious problem. In contrast, no difference was noted by Hair,

Neuberger and Perrone (1952) in the labelling of haemoglobin. After injection of radioactive valine, the activity of the terminal valine of rat haemoglobin was measured and found no different from the mean value for valine from the remainder of the molecule. Askonas, Campbell and Work (1954) as a result of studies of incorporation of labelled amino acids into the β-lactoglobulin and casein of goat milk consider it unlikely that either protein derives a significant amount of N from blood peptides or from partial hydrolysis and rearrangement of blood proteins. At present there is no conclusive evidence other than that free amino acids are the chief precursors in protein formation. It should be possible by controlled degradation processes for proteins, to determine whether or not a doubly-labelled peptide can serve as direct precursor of the corresponding sequence of two amino acids in a protein.

The transpeptidation theory of protein synthesis. Considerable attention has been given to the possibility that peptides or amides can serve in protein synthesis in another way, by donating amino acids to as yet unidentified acceptors, as summarized in the transpeptidation theory of Fruton. This hypothesis has many points in its favour. Firstly, members of only one well-defined group of enzymes

have so far been shown to catalyze the interaction of amino acid residues with the formation of peptide bonds, namely the proteolytic enzymes. It is known that all cells contain intracellular cathepsins and it may reasonably be asked whether such enzymes could be responsible in part for protein synthesis. From our knowledge of the behaviour of proteases *in vitro* and from a knowledge of the thermodynamics of peptide bond formation, we can say at once that, if the cathepsins do play a part in protein formation, they could only do so when provided with a supply of energy, probably in the form of a pool of pre-formed amide or peptide bonds. The examples of amide and peptide bond formation already mentioned, model systems, make it clear that cells possess a variety of mechanisms for the conversion of phosphate bond energy *into* amide and peptide bonds, and thus, there can be no objection to the theory on thermodynamic grounds. Fruton's(1952) observations on the effect of pH on the exchange reactions make the theory even more attractive. A disparity was found between the pH optimum for hydrolysis and trans-amidation, the latter reaction being favoured by a higher pH. Fruton suggests that at a physiological pH catalysis of replacement may represent a major intracellular function for cathepsins and envisages coupled transamidation and successive additions under different enzymes.

Waelsch (1951) has discovered enzymes in tissue extracts which can catalyze the exchange of substituents of the gamma carboxyl group of glutamic acid(glutamine) and glutathione, and of the beta-carboxyl group of asparagine, and various amino acids, to give gamma or beta peptides. Hanes, Hird and Isherwood(1950) have shown that tissue extracts from many sources catalyze the replacement of amino acids attached to the gamma carboxyl of glutamic acid. They have also discovered an enzyme in cabbage leaves with the ability to catalyse the transfer of glycine from various glycyl peptides to amino acids. The hydrolytic function of the enzyme is actually inhibited by the presence of a suitable acceptor much like the proteolytic enzymes studied by Fruton. The authors suggest a function of these compounds in the biosynthetic mechanism as funnelling agents of biological energy into the synthesis of peptide bonds.

The transpeptidation theory of protein synthesis in general has its limitations. Although the enzymes studied exhibit a specificity, especially for the acceptor molecule, there is little or no specificity for the substituent amino acid or peptide. Again, in all transpeptidation reactions studied, the degree of hydrolysis exceeds transpeptidation. Moreover, metallic ions found

to be essential for amino acid incorporation into proteins have been found either to be without effect or actually inhibitory to transpeptidation. Finally, if protein synthesis were to take place by transpeptidation we are faced with the essentially negative evidence for the existence of peptides as intermediates in protein synthesis.

Template theories of protein synthesis. Any scheme of protein synthesis must take into account immunological and chemical evidence that proteins of each species appear to be unique and structurally distinct from those of other related species. If the specificity of protein structure is inherited within any one species, then it would appear that protein synthesis is under genetic control. As long ago as 1937, Haldane suggested that the genes behaved as master-templates or moulds controlling the specificity of protein structure. This idea was extended by Beadle, who, as a result of his studies on the bread mould Neurospora crassa, suggested a one-to-one relationship between genes and enzymes. Since enzymes are proteins, it was a simple extension of this hypothesis to suggest that genes are master-templates on which proteins are synthesized. Since then, largely as a result of studies on the composition of such self-reproducing

units as chromosomes, viruses and bacterial phage (Caspersson, 1950), the role of organizer in protein synthesis has been transferred from the gene to the nucleic acids. Further evidence was supplied by the demonstration that protein can be synthesized in a system devoid of a nucleus, and that cytoplasmic preparations will incorporate labelled amino acids into their proteins. Brachet and Chantrenne (1951) studied the incorporation of radioactive CO_2 into the proteins of fragments of the alga Acetabularia mediterranea and concluded that the synthesis of protein can proceed for a considerable period in a cell deprived of its nucleus.

Emphasis on nucleic acids as the controlling factors controlling protein specificity has been increased by the demonstration that type-transformation in bacteria can be brought about by cell-extracts from related bacteria, and that the active principles in these extracts are nucleic acids.

Borsook investigating the uptake of a number of amino acids into the particulate fractions of guinea-pig liver, observed that incorporation was more rapid in the microsome fraction, the granules richest in RNA, than in other fractions of the tissue (Borsook et al., 1950b).

Direct proof of the participation of nucleic acids in the

protein synthetic mechanism, however, can only be obtained in a system capable of synthesizing protein and on which an effect of nucleic acid can be demonstrated. Such a system has recently been devised by Gale (Gale & Folkes, 1954). For the study, cells of Staphylococcus aureus, which had been disrupted by supersonic vibrations, were used. This system will incorporate labelled glutamate in the absence of other amino acids, and synthesize protein when a complete amino acid mixture is added to the incubation medium. Removal of nucleic acid, either by NaCl extraction or treatment with ribo- or deoxyribonuclease, decreases both incorporation of glutamate and net synthesis. Furthermore, addition of either RNA or DNA worked up from the insoluble portion of the fragmented cells markedly accelerated the incorporation of glutamate under exchange conditions, and the rate of increase in protein N in the system, in the presence of an amino acid mixture. To support the isotope studies, similar experiments were performed in which an effect of nucleic acids on enzyme production by the fragmented cells was clearly demonstrated.

In similar studies, Beljansky (1954) studied the incorporation of radioactive glycine into the lysed cells of Micrococcus lysodeiktycus. Treatment with ribonuclease

was found to inhibit strongly or completely abolish the incorporation of glycine, but deoxyribonuclease, in contrast to Caleb's observations, resulted in a stimulation of incorporation. Beljansky attributed the augmented incorporation to production of co-factors involved in energy transfer, from DNA breakdown. A role for DNA in protein synthesis in the nucleus analogous to the function of RNA in cytoplasmic protein formation was recently proposed by Allfrey (1954). Calf thymus nuclei isolated in sucrose solution were found to incorporate labelled alanine into nuclear proteins in the presence of an energy source. Treatment with ribonuclease was without effect, but incubation with deoxyribonuclease nearly abolished incorporation. The uptake of amino acids into nuclear protein then appears to require that DNA remains intact. The failure of ribonuclease to block amino acid incorporation into the nuclei is in sharp contrast to its effect on isolated cytoplasmic particles. The uptake of alanine by an *in vitro* system of microsomes and mitochondria were found by Allfrey, Daly and Mirsky (1953) to be impaired by pre-incubation of the microsomes with ribonuclease. Here again we have direct experimental evidence for the dependence of protein synthesis on the presence and integrity of RNA in the cytoplasm.

There have been many attempts to determine the nature of the relationship between nucleic acids and protein synthesis. However the obtaining of definitive evidence of a dependence of protein formation on either nucleic acid content or nucleic acid turnover in a tissue has proved a formidable task and results are in wide disagreement. Our own studies indicate that the metabolism of RNA, both by labelling with ^{32}P and ^{14C} glycine, is independent of the state of protein synthesis in the liver (Table 46).

The question is thus raised, what is the nature of the role of RNA in protein synthesis? The report of Binkley (1952) that cysteinylglycinase is a polynucleotide directly associates protein metabolism with enzyme activity in a nucleic acid. In attempting to account for all the features of protein formation, and a function for the nucleic acids, Dounce (1952) has outlined a reasonably plausible specific chemical mechanism that might account for peptide chain synthesis. The scheme is based on the hypothesis that there are at least as many specific nucleic acids in the cell as there are specific peptide chain arrangements in the proteins of that cell. Furthermore, the specific arrangement of amino acid residues in a given peptide chain is derived from the specific arrangement of

nucleotide residues in a corresponding nucleic acid molecule, and in addition, the nucleic acid molecule is concerned in transferring energy necessary for peptide bond synthesis.

It is first assumed that ATP can combine with RNA or DNA in the presence of a few relatively non-specific enzymes to yield a diphospho-nucleic acid, a reaction analogous to the myokinase reaction between ATP and adenylic acid. The diphosphonucleic acid then reacts with the α-amino groups of amino acids to give amino-phosphate compounds. The enzymes involved at this stage must possess specificity not only for the amino acid, but for a specific purine or pyrimidine base with a suitable nucleotide environment. Finally to form the peptide chain, a relatively non-specific enzyme catalyses a peeling off, transamidation changing phospho-amide links to carboxy-amide links.

The comprehensive nature of this hypothesis is its chief attraction. There is no necessity for assuming any particular relationship between the rate of synthesis of the template and that of the molecule produced by aid of the template; the only requirement is that the template be synthesized rapidly enough to be maintained at a suitable concentration in the cell. Duplication of the

enzymes involved in the synthesis and of the templates themselves is accounted for. In addition an explanation is offered for a role of high energy phosphate bonds in peptide bond synthesis, and most important of all, an acceptable theory of the origin of protein specificity. There is a certain amount of experimental evidence to support the suggestion that nucleic acids have a function in energy transfer, notably the studies of Spiegelman and Kamen (1947) on yeast cells and of Dounce and Kay (1953) on *in vitro* phosphorylation of RNA by ATP. As it is now formulated the hypothesis offers in addition an explanation for the function of DNA, as a template for the synthesis of nuclear protein, corresponding to the role of RNA in the cytoplasm.

At present our knowledge is insufficient to permit us to cling to any one of the various hypotheses. If it can be proved that protein turnover and net protein synthesis are two distinct but closely related processes, as most of the evidence would suggest, then

much of the apparent conflict between the different types of evidence may well disappear. The transpeptidation theory as it stands seems to describe admirably the type of mechanism which would permit an exchange of amino acid residues in tissue proteins with the free amino acids in the cell pool, in the dynamic steady state.

It may even be that there are many mechanisms of protein synthesis in the cell, and consequently the assumption that nature has evolved only one mechanism for protein formation would only serve as an obstruction to further biochemical experimentation.

In one of his novels, Aldous Huxley countered the proposal that "Nature does things in the simplest way" with the suggestion that "Humans understand only the simplest explanations". In exploring the problem of protein synthesis, the biochemist would do well to bear this rebuke in mind.

S U M M A R Y.

SUMMARY.

PART I. The influence of energy intake on nitrogen balance and the protein content of the liver.

1. A study was made of the relationship between protein metabolism and energy intake in the rat, by measuring changes in N-balance and the protein content of the liver at different levels of energy intakes. The diets used were either rich in protein or free from protein, and were fed at various energy levels, ranging from 850 to 1700 kg. cal./sq. m. of body surface area. These planes of energy intakes were obtained by addition of fat or carbohydrate to standard basal diets.

2. The influence of energy intake on N-balance was found to be conditioned by the level of protein in the diet. When the diet provided adequate amounts of protein, additions of energy to a submaintenance ration in the form of either carbohydrate or fat caused a linear improvement in N-balance. When the animals were maintained on aprotein-free diet, addition of carbohydrate to the ration influenced N-balance up to a certain point only (about 1200 kg. cal./sq. m.) and to a very limited degree. On the other hand increments of fat (900-1700 kg. cal./sq. m.) completely failed to influence N-balance.

3. Changes in the total amount of protein in the liver were

found in general to parallel the variations in N-balance. On the high-protein diet, the protein content of the liver was found to increase linearly with changes in the caloric value of the diet, whether produced by addition of carbohydrate or of fat. When the diet contained no protein, addition of energy in either form caused no significant increase in liver protein.

4. An explanation of these findings in terms of the factors affecting protein synthesis is offered. It is suggested that on a protein-containing diet, the factor limiting the synthesis of tissue proteins from amino acids is the availability of energy. When however the diet is free from protein, the supply of amino acids circulating to the tissues comes solely from endogenous sources, and in this case constitutes the limiting factor in protein formation at quite low levels of energy intake. Hence increments in energy intake stimulate protein synthesis with an adequate protein intake but not on a protein-deficient diet.

5. A study was also made of the bodily distribution of changes in N-balance produced by varying the energy content of a diet containing protein. Since alterations in the N-content of liver and other viscera account for only a part of the N retention, it was concluded that the main change must have taken place in the carcass. However, when the changes

in liver and carcass N were considered as percentages of total amount of N in these tissues it was apparent that the change in liver N content was proportionately greater than the change in carcass N.

PART II. The influence of protein and energy intake on ribonucleic acid metabolism in the liver.

Section 1. The influence of energy intake on the amount of ribonucleic acid in the liver.

1. An investigation of the effect of variations in protein and energy intake on the amount of ribonucleic acid (RNA) in the liver was carried out on the rat.

2. The influence of energy intake on the amount of liver RNA was found to be dependent on the protein content of the diet. When the basal (sub-maintenance) diet contained an adequate amount of protein, it was observed that the total amount of RNA in the liver increased in a linear fashion with the caloric content of the diet, whether carbohydrate or fat was added to the basal diet. When the diet lacked protein, increments of energy in either form caused only minimal changes in the amount of RNA.

3. Since the amount of liver RNA was found to change only under circumstances favouring a parallel change in liver protein, these findings provide further evidence for a

quantitative relationship between content of RNA and the amount of protein synthesized by the tissue.

4. In agreement with the many demonstrations of the stability of nuclear DNA, it was found that the DNA content of the liver was altered neither by the level of protein fed, nor by variations in energy intake.

Section 2. The influence of energy intake on the uptake of radioactive phosphorus by liver ribonucleic acid.

1. In order to complete the picture of the metabolism of RNA as opposed to changes in the amount of RNA, a study was made of the effect of energy intake on the rate of incorporation of ^{32}P into liver RNA, both when the diet contained protein and when it was free from protein.

2. The uptake of ^{32}P by liver RNA, as measured by relative specific activity, behaved in the opposite way to changes in the amount of RNA. When the animals received a protein-containing diet, the relative specific activity of the liver RNAP was not significantly altered by variations in energy intake. On the other hand, when the rats received a diet free from protein incorporation was considerably stimulated by addition of energy to the protein-free diet.

3. When considered in conjunction with the quantitative studies on liver RNA, these results indicate that the total

rate of ^{32}P incorporation is dependent on energy intake and not on protein intake. At each level of protein intake, the total number of phosphorus atoms incorporated into RNA is increased by raising energy intake, in one case by an increase in the total RNA per liver without a change in the percentage of phosphorus atoms incorporated in a given time, in the other case by an increase in the percentage incorporation rate accompanied by a smaller change in total RNA per liver.

4. Further experiments were designed to throw some light on this apparent relationship between energy intake and the metabolism of RNA. When energy expenditure was increased by lowering the environmental temperature, the stimulating effect of an energy supplement on the synthesis of RNA was abolished. It was concluded that the metabolism of RNA in the liver of the rat is related not to the metabolism of energy, but to the availability of energy in the tissue, i.e. to the energy balance of the body.

Section 3. Relationship to vitamin B_{12} intake.

1. In the investigations on the influence of energy intake on the amount of RNA in the liver, crude casein, which is known to contain vitamin B_{12}, was used as the protein source in the diet. In view of the many claims that vitamin B_{12} is involved in the metabolism of the nucleic acids, it seemed of

importance to determine whether the effects on RNA metabolism attributed to the protein content of the diet were due to the casein per se or to the contaminating vitamin. Comparison was therefore made between rats receiving diets rich in protein or deficient in protein (as fed in the preceding experiments) at high and low levels of energy intake with similar groups receiving a supplement of vitamin B_{12}.

2. Under these experimental conditions, vitamin B_{12} appeared to have no measurable effect on the amount of RNA in the liver. Similarly there was no indication that the metabolism of protein was affected by the amount of vitamin B_{12} in the diet.

3. It was concluded that the stimulating effect of energy on the net synthesis of RNA was in fact conditioned by protein intake, and not by the vitamin B_{12} content of the diet.

PART III. The influence of protein and energy intake on phospholipid metabolism in the liver.

1. Under a variety of nutritional conditions, other workers have noted certain similarities between the metabolism of ribonucleic acid and phospholipids in the liver of the rat. A study was therefore made of the influence of energy intake on the amount of phospholipid in rat liver, and on the rate of incorporation of ^{32}P.

2. When a diet containing an adequate supply of protein was

administered, the amount of phospholipid in the liver varied directly with energy consumption. In contrast, increments in energy intake failed to increase the amount of phospholipid per liver when a protein-free diet was fed.

3. The relative specific activity of liver phospholipid rose with increasing energy intake, and there was no significant difference in this respect between the two dietary protein levels. The total turnover rate however (as measured by total relative activity) was influenced by energy intake only when the diet contained protein. It is suggested that the failure of energy to stimulate phospholipid synthesis on a protein-free diet is due to a reduction in the concentration of some essential component or precursor in phospholipid synthesis, brought about by the feeding of the protein-free diet, to such a degree that it becomes the factor limiting the rate of synthesis. Choline has been eliminated as the missing factor.

PART IV. The influence of protein and energy intake on uptake of glycine-2-^{14}C by liver protein and ribonucleic acid.

1. In an attempt to extend and amplify the experiments dealing with the quantitative relationship between energy intake and protein metabolism, the rate of incorporation of an isotopically labelled amino acid (Glycine-2-^{14}C) into the mixed proteins of the liver was measured. Since this amino

acid is also incorporated into the purine rings of the nucleic acids, it was possible to study the metabolism of protein and ribonucleic acid simultaneously in the same animal. In addition, further information on the synthesis of the ribonucleic acid molecule was obtained by the use of a second isotope (^{32}P) which permitted the isolation of doubly labelled ribonucleotides

2. These experiments were performed under the same conditions of energy and protein intake as previously described. The isotopes were administered to some animals in the post-absorptiv state, while others were fed protein before the injection of the isotopes, and thereafter until the time of death.

3. The rate of uptake of glycine by liver protein (total relative activity) is slightly greater when the energy content of a protein-free diet is augmented. When energy is added to a protein-containing diet, the effect on glycine uptake is more pronounced. These findings apply to rats in the post-absorptive state. When protein is fed, no influence of previous energy level is demonstrable. It has been concluded that the level of energy provided by the diet influences the rate of protein synthesis in between meals (so-called "endogenous" protein metabolism). On the other hand, the flooding of the tissues with amino acids after a meal leads to an increase in the rate of protein synthesis which is not affected by energy level.

4. The pattern of glycine incorporation into the purine

bases of ribonucleic acid appears to confirm the absence of a relationship between nucleic acid metabolism and protein synthesis.

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