STUDIES IN PROTEIN SYNTHESIS.

The relationship of energy intake
to protein metabolism.

by

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Thesis submitted for the Degree of
Doctor of Philosophy of the
University of Glasgow.

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ACKNOWLEDGEMENTS.

CONTENTS.

INTRODUCTION.

INTRODUCTION.

The realisation by Magendie (1816) of the differences between the nitrogenous and non-nitrogenous moieties as nutrients undoubtedly opened the way to studies on protein metabolism in the early 19th century. Shortly thereafter, urea was recognised as the principal end product of protein metabolism by Dumas and Cahours (1842) and the first N-balance studies were described by Boussingault in 1839. Techniques were therefore available for studying changes in protein metabolism and experiments by Voit and his associates established the interdependence of carbohydrate, fat and protein metabolism.

These experiments, on which the concepts of protein-sparing action are based, have been extended to explore the nutritional aspects of this relationship under a wide variety of nutritional and physiological conditions thus revealing the profundity of the relationship – the mechanism of which is susceptible to biochemical analyses. The most obvious way in which carbohydrate and fat could mediate in protein metabolism is by their energy-yielding properties, and experiments in which energy in the form of carbohydrate or fat has been added to or subtracted from the diet have been performed on animals and humans under various nutritional regimes varying from undernutrition to surfeit feeding.

The Relationship of Nitrogen Balance to Energy Intake.

The evidence has been summarised below under several headings: (a) experiments on undernourished subjects, (b) experiments on overnourished subjects, (c) evidence of a continuous relationship of N balance and energy intake, and (d) the effect of dietary energy level on protein utilisation.

(a). Experiments involving N balance at inadequate caloric intakes. Short-term experiments on humans, where carbohydrate (Lusk, 1890; Miura, 1892; and Rosemann, 1901) or fat (Neumann, 1899) was removed from a diet adequate in protein and energy content show that the body responds by an immediate increased excretion of N irrespective of the energy source removed. Unfortunately, as these experiments were not performed under controlled conditions, it is not possible to compare the magnitude of the losses after removal of carbohydrate and fat as energy sources. In a study of growing mice, Bosshardt, Paul, O'Doherty and Barnes (1948) found that a reduction in the energy intake had an adverse effect on the gain of body N. This phenomenon was observed irrespective of the nature of the energy source withdrawn, and was similar for equicaloric removal of carbohydrate or fat.

The effects of adding more energy to inadequate diets have also been recorded. In the earliest experiments (Bischoff, 1853; Botkin, 1858; Bischoff and Voit, 1860;

Voit, 1869), dogs received a diet containing insufficient protein but which had been supplemented by carbohydrate or fat. These experiments showed an improvement in N balance on feeding the supplement but are not completely satisfactory because in most cases there was no preliminary adjustment to the dietary regimes. A more satisfactory experiment was performed by Allison, Anderson and Seeley (1946) in which dogs received an **energy** supplement ranging from 25-50 per cent of their normal intake. Both carbohydrate and fat were effective to a similar degree in improving N balance. Improvements in N balance have also been reported in human subjects in negative N balance when carbohydrate (Jansen, 1917) or fat (Zuntz and Loewy, 1918) was added to the diet.

The general conclusions to be drawn from these experiments on undernutrition are that (i) reduction of caloric intake on an adequate diet results in an impaired N balance while, conversely, the addition of energy in the form of carbohydrate or fat reduces the N output when added to a diet which is insufficient to maintain the body in N equilibrium; (ii) carbohydrate and fat are equally effective as energy sources for these purposes.

(b). Protein metabolism during surfeit feeding. A number of experiments on protein metabolism in which carbohydrate or fat has been added to diets already adequate in energy content

as judged by their ability to maintain weight and N equilibrium show essentially the same picture of the relationship between energy intake and protein metabolism. Several studies made on human subjects (Cuthbertson, McGirr and Munro, 1937; Cuthbertson and Munro, 1937; Basu and Basak, 1939; Wikramanayake and Munro, 1954), on the rat (Forbes, Bratzler, Thacker and Marcey, 1939; Forbes and Swift, 1944; Lathe and Peters, 1949; Wikramanayake and Munro, 1954), on the dog (Munk, 1879; Biernacki, 1907; Levene and Kober, 1908; Kochmann and Petzsch, 1911; Larson and Chaikoff, 1937; Allison and Anderson, 1945) and on other species reveal a subsequent improvement in N retention by feeding extra energy.

This phenomenon raises an interesting point, namely if the depression in N output caused by the addition of carbohydrate or fat to adequate diets is due entirely to surplus energy, then equivalent amounts of these substances should affect N balance to the same degree. However, in only a few of the experiments quoted in the previous paragraph, viz., those dealing with adult rats, and some described by Forbes and his co-workers (Forbes, Swift, Elliot and James, 1946; Forbes, Swift, Thacker, Smith and French, 1946) have experimental conditions allowed a permissible comparison between the effect of carbohydrate and fat. These experiments show that there is little to choose between the effect of extra carbohydrate or fat on N balance. On the

other hand, Cuthbertson and Munro (1937) found that carbohydrate added to a diet given to humans had a slightly more beneficial effect on N retention than did surplus fat. Further evidence from experiments on man might elucidate the significance of these few observations.

Another question which must be considered is whether the changes in N excretion is quantitatively related to the amount of energy added to or removed from the diet. It has already been pointed out that both carbohydrate and fat can influence protein metabolism as energy sources, but the quantitative aspects of this interaction have been, to a large extent, overlooked. In only a few experiments on human subjects (Cuthbertson and Munro, 1937; Basu and Basak, 1939) and on young pigs (Terroine and Mahler-Mendler, 1927) has the subject been studied at two levels of surplus carbohydrate. N retention was found in these instances to increase in proportion to the amount of extra energy added. Similar studies in which fat was used as the energy source (Voit, 1869) indicate that the degree of N retention is roughly related to the amount of added fat.

(c). Evidence of a continuous relationship of N balance and energy intake. So far we have only considered the effect of energy intake on comparatively extreme conditions, namely, undernutrition and surfeit feeding. It is pertinent that we should consider whether a continuous relationship exists

g. N. Balance

Fig. 5
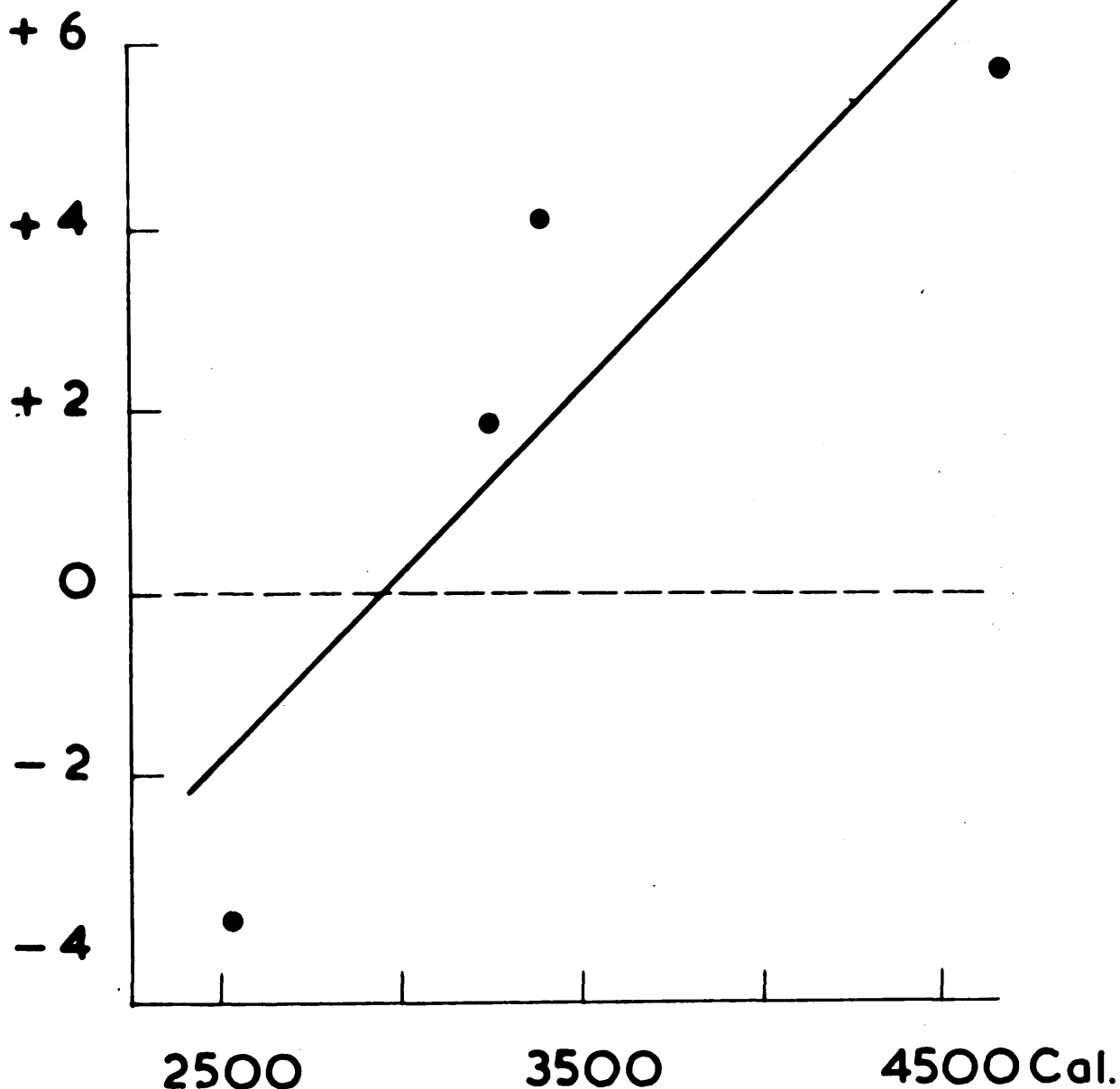
Fig. 1. Variations in N balance produced by adding various amounts of fat to the diet of a human subject. The data are plotted from an experiment by Rubner (1903). The line represents the regression Y= -11.98 + 0.00405 X where X is the energy intake in kg. cal. and Y is the N balance in gm.

g. N. Balance

Fig. 6
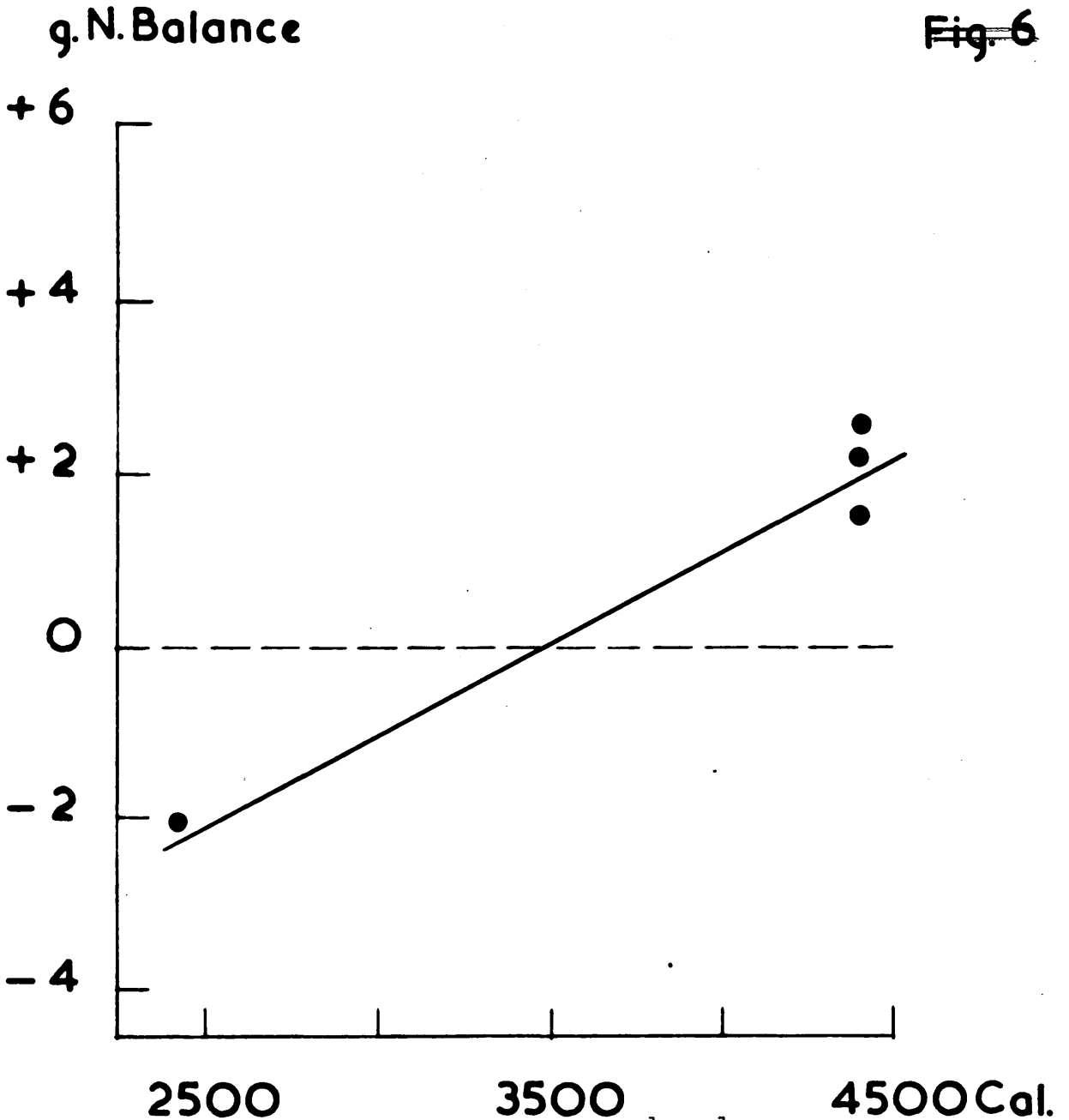
Fig. 2. Variations in N balance/produced by adding carbohydrate
to the diet of a human subject. The data are plotted
from an experiment by Neumann(1919). The line represents
the regression Y= -7.54 + 0.00215 X, where X is the energy
intake in kg. cal. and Y is the N balance in gm.

between energy intake and N balance ranging from a large deficiency to a large excess of energy. In other words, whether the N balance is in a state of dynamic equilibrium with energy intake. In order to test this, Munro and Naismith (1953) studied rats at different levels of energy intake varying from insufficient to surfeit while the protein level was kept constant and adequate. These experiments showed conclusively that a continuous relationship exists between energy intake and N balance on both sides of N equilibrium. These findings are in agreement with data obtained by Rubner (1879, 1903) and Neumann (1919). In these experiments carbohydrate (Neumann) or fat (Rubner) was added to a basal diet which was inadequate to preserve N balance in humans. Analysis of their data reveals that increasing amounts of carbohydrate and fat led to a progressive improvement of N balance from negative through equilibrium to positive (Figs. 1 and 2). Furthermore, the relationship of N balance to energy intake was approximately linear. Rosenthal and Anderson (1951) have described some experiments on dogs in which successive reductions in energy intake caused a decline in N balance from positive at high energy levels to negative at low energy intakes. But quite large changes in energy intake had little effect on N balance when the animals were in or near N equilibrium. It is difficult to understand why the dog should differ from rat and man in this respect.

Studies on the effect of the energy intake of rats

receiving a protein-free diet have also been made (Willman,
Brush, Clark and Swanson, 1947; Swanson and Clark, 1950).
In this instance a somewhat different picture emerges; when
the amount of such a diet is reduced by more than half the
amount eaten voluntarily by the rat, N output rises, but
changes in intake of a less drastic nature seem to be without
effect (Mitchell, 1923; Treichler and Mitchell, 1941; Vars
and Gurd, 1947). Munro and Naismith (1953) have reached
essentially the same conclusions by changing the energy
intake on a protein-free diet from about 900-1700 Cals/m^2
body surface area/day, which was the range of energy intake
used in the experiments in which the protein intake was
adequate. When carbohydrate was used as an energy source,
N balance benefited up to about 1200 Cals/m^2, but not by
further additions. When fat was used as the variable energy
source, no change in N balance was observed. The difference
in action of carbohydrate and fat can be explained in terms
of the special protein-sparing action of carbohydrate, (Munro 1951) which
is not relevant to the present discussion. The conclusion
to be drawn from these experiments of Munro and Naismith (1953)
is that, on a protein-free diet, carbohydrate and fat fail to
produce the beneficial effect observed with a protein-
containing diet. It would therefore appear that, once a
certain energy intake had been reached on a protein-free diet,
further increments of energy have no effect on N balance.
That is to say, lack of an adequate protein supply had

prevented N balance from responding to the changes in energy
intake. Contrary to the findings with rats, various
experiments using dogs and human subjects have been described
which seem to show that addition of energy on a protein-free
diet exerted a protein-sparing action. Johnson, Deuel,
Morehouse and Mehl (1947) found that raising a human subject's
intake from 1200 Cal.- 2000 Cal/day reduced his N output.
Krauss(1926) found that an energy intake of from 170 - 300
per cent above basal requirements produced a degree of N
retention in humans. Experiments with dogs point to the same
conclusion. Murlin (1907) and Wimmer (1912) each studied the
effect of giving increasing doses of carbohydrate to dogs
after a period of starvation. The N output of the dogs fell
as the carbohydrate content of the diet was increased.
Wimmer found that the N output was still falling when dogs
were receiving carbohydrate equivalent to 160 per cent of the
basal requirement. Allison and Anderson (1945) found an
increased N retention when the caloric intake of dogs was
raised from 80 - 100 Cal/Kg.body weight, by adding carbohydrate
to the diet. Osterberg and Wolff (1907)observed a similar
effect over a caloric range of 80 - 160 Cal./Kg. It is
perhaps unfortunate that there are no investigations recorded
where fat, as an energy source, supplemented the protein-free
diet. As carbohydrate displays a protein-sparing action
which is not shared by fat (see review by Munro, 1951), these
experiments in which carbohydrate alone has been used as the

energy source would be more convincing if fat had been
studied under similar experimental conditions.

(d). Effect of dietary energy level on protein utilisation.
From the preceding section it might be assumed that, since N
balance is sensitive to changes in dietary energy intake, then
utilisation of the protein eaten would be less efficient at
low levels of energy intake. This proposition can be tested
by adding the same amount of protein to diets providing low
and high energy levels and comparing the response in N balance.
Allison, Anderson and Seeley (1946) have reported experiments
on dogs in which protein was added at different levels of
energy intake. Their results show a constant response to
changes in protein level at various energy intakes until the
energy intake fell to 25 per cent of requirement when the dogs
failed to respond to the added protein, showing that energy
intake had become the factor limiting the full utilisation
of dietary protein. Above this point, it is remarkable that
energy intake did not affect the response to an increment in
dietary protein level.   Some experiments by Campbell and
Kosterlitz (1948) on the protein content of rat liver, which
we shall consider in more detail later, add strength to this
conclusion.
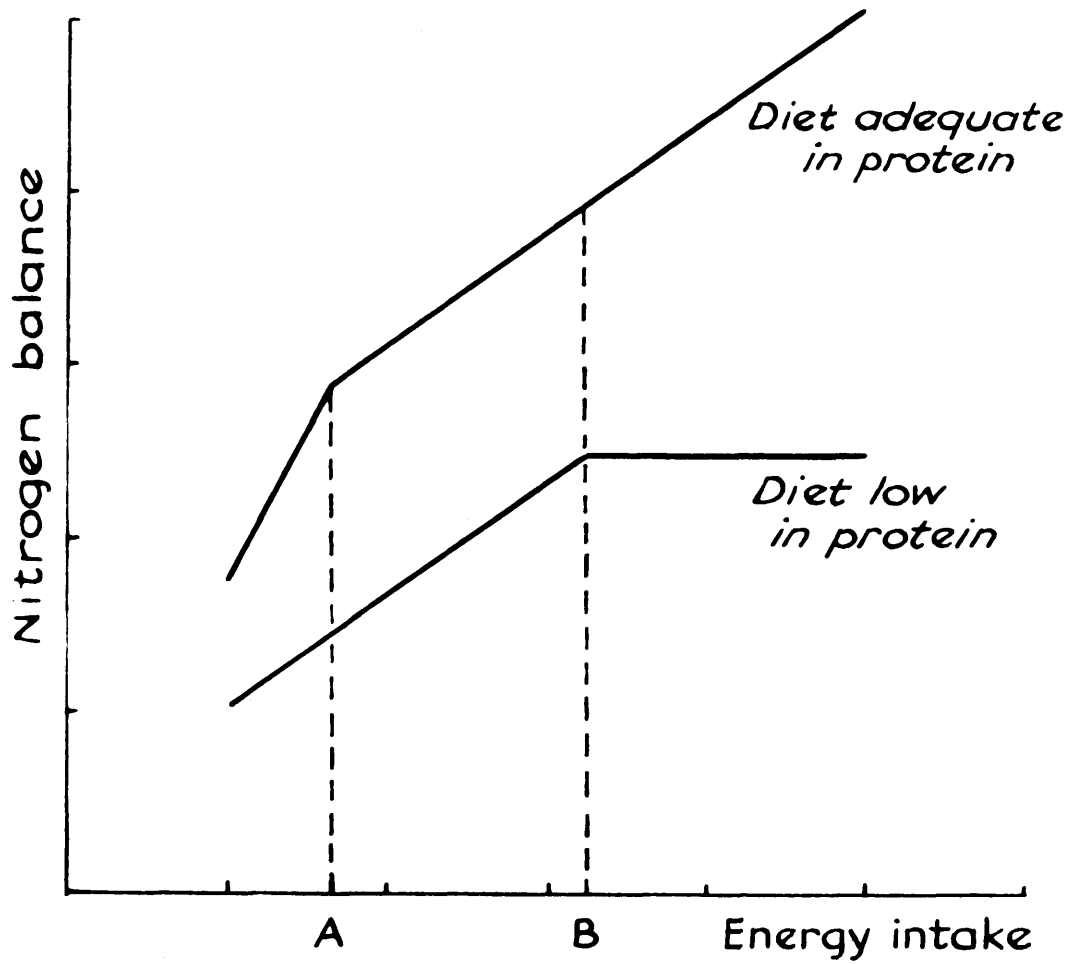
Fig. 3.

The Interrelationships of Energy Intake and Protein Intake in their Effect on N Balance.

The preceding sections suggest the general outlines of the relationship between energy intake and protein metabolism, which reveals both independence and inter-dependence of dietary protein and energy level. "Under normal nutritional circumstances N balance is improved by either an increase in energy intake or in protein intake. However, the beneficial effect of a rise in energy intake can be prevented by an inadequate protein intake and conversely an increased protein intake may not be fully effective because of insufficient energy in the diet" (Munro, 1951). Fig. 3 is an attempt to represent these interrelationships between protein and energy intake diagrammatically, in the form of lines relating N balance to energy intake. The upper line represents the picture obtained with diets adequate in protein content, the lower line depicts the situation with diets deficient in protein. Between energy intakes A and B, the two lines are parallel; this means that, at both levels of protein intake, a given increment in energy intake elicits the same change in N balance. It also follows that a given change in protein intake (i.e., changing from the low-protein diet to the adequate diet) improves N balance to the same extent at all levels of energy between A and B. Outside the parallelogram bounded by A and B, the effects of energy intake and protein intake on N balance are no longer independent of

one another. At caloric intakes less than A there is a
diminished response to raising the protein intake because
of the inadequate energy supply.   For this reason the lines
converge below A.   Conversely, increments in caloric intake
beyond B fail to cause an improvement in N balance on the
diet low in protein, because the inadequate protein supply is
now the limiting factor. The lines relating N balance to
energy intake therefore diverge beyond B.

The picture described in the preceding paragraph was
deduced from a consideration of different types of experiment
on different species and it is therefore gratifying to note
that Calloway and Spector (1954) have recently come to a
similar conclusion from an analysis of recently published N
balance data on human subjects receiving various diets. They
state: "To the general principles set forth - that on a
fixed and adequate caloric intake, protein level is the
determinant - may be added a corollary. That is, at each
fixed inadequate protein intake there is an individual limiting
energy level beyond which increasing calories without protein
or protein without calories is without benefit".

Any explanation of the interaction of energy intake
on protein metabolism must obviously provide a reason why
the effects of dietary energy and protein levels on N balance
are sometimes independent and sometimes interdependent.

The Nature of the Relationship between Energy Intake and Protein Metabolism.

Whilst a complex process like protein metabolism may well be influenced by the available energy in the diet in many ways, it is still worth while to see what factual information there is, and from which, conclusions about its mechanism can be drawn. The current position can be summarised under three headings: (a)Does an increment in energy intake lead to a rise in cellular metabolism? (b) How transitory are the effects of a given level of energy intake on protein metabolism? (c) Does protein metabolism in all tissues respond equally to a change in energy level?

(a). Effect of changes in energy intake on basal metabolism.
The underlying mechanism of the relationship between energy intake and protein metabolism could conceivably be due to changes in cellular metabolism, which would be reflected in the basal metabolic rate. This possibility has been examined by Munro (1950), who studied the basal metabolism of a human subject receiving extra energy on an adequate diet and found no consistent elevation of basal metabolism during the period of surfeit feeding. This finds support in other data recorded in the literature and it is apparent that, to produce N retention in man by overfeeding, no appreciable increase in energy production need occur. In fact, by raising the energy requirement with exercise, N retention can be prevented

(Cuthbertson, McGirr and Munro, 1937). It would appear from these latter data that energy exerts its influence on protein metabolism not by increasing the rate of cellular metabolism but by providing energy-yielding metabolites in excess of requirements.

(b). Effect of varying the time of feeding the additional energy source. Another question germane to this problem is whether the energy supplement must be eaten along with the protein of the diet to show a protein-sparing action or if such an effect exists when the two fractions are eaten separately. Larson and Chaikoff (1937) performed experiments in which dogs received a supplement of carbohydrate for one day only and found a decreased N excretion only when the carbohydrate was eaten within a few hours of the protein containing meal. In a similar experiment Munro and Wikramanayake (1954) confirmed this observation but found that carbohydrate ingestion 12 hours apart from the protein meal eventually led to a N retention. Lathe and Peters (1949) added sucrose to a basal diet for rats some hours after the protein meal and failed to find any difference in N retention between the controls and carbohydrate-fed animals. This is probably due to the fact that the rats had been allowed only 2 days acclimatisation on the basal diet. On the other hand, Munro and Wikramanayake (1954) were not able to observe any differences in N retention in rats in which the energy in the

Table 1.

The effect on N balance when carbohydrate (glucose) is superimposed on a diet adequate for rats, either along with or 12 hr. apart from the dietary protein.

(From Munro and Wikramanayake, 1954).

Treatment during Period 2.	Mean Daily Nitrogen Balances	
	Period 1 mg.	Period 2 mg.
Extra glucose with protein	-0.3	+36.4
Extra glucose apart from protein	-0.8	+31.8
Control group	+3.9	-15.3

During period 1, all the animals received the unsupplemented basal diet; during period 2, extra carbohydrate was given to all except the control group.

form of carbohydrate or fat was fed either together with or 12 hours apart from the dietary protein. These results are shown in Table 1.

In a study of human subjects Munro and Wikramanayake (1954) found a protein-sparing action when extra carbohydrate or fat was fed $5\frac{1}{2}$ hours after the last meal. It would thus appear that the energy supplement need not be given along with protein to induce N retention. Consequently, the effect of energy level cannot be a transitory one, limited to a short period after each meal, but must be prolonged for at least 12 hours, and very probably much longer.

(c). <u>Effect of level of energy intake on the protein content of different tissues.</u> Another point which must be determined is whether the tissues of the body respond to changes in energy intake to an equal extent. This problem has been studied by Munro and Naismith (1953) with rats on an adequate protein intake. It was observed that the total amount of liver protein varied linearly with energy intake. Deposition of N in the liver was compared with the changes in other viscera and in the carcass. Although the carcass was found to represent the largest reservoir, because of the small size of the liver relative to the carcass, the effect on liver composition was considerable, namely a 23 per cent increase in liver N per 1000 Cals. added to the diet compared with a
                                                                                    of the
3 per cent increase in N content of the carcass and/other
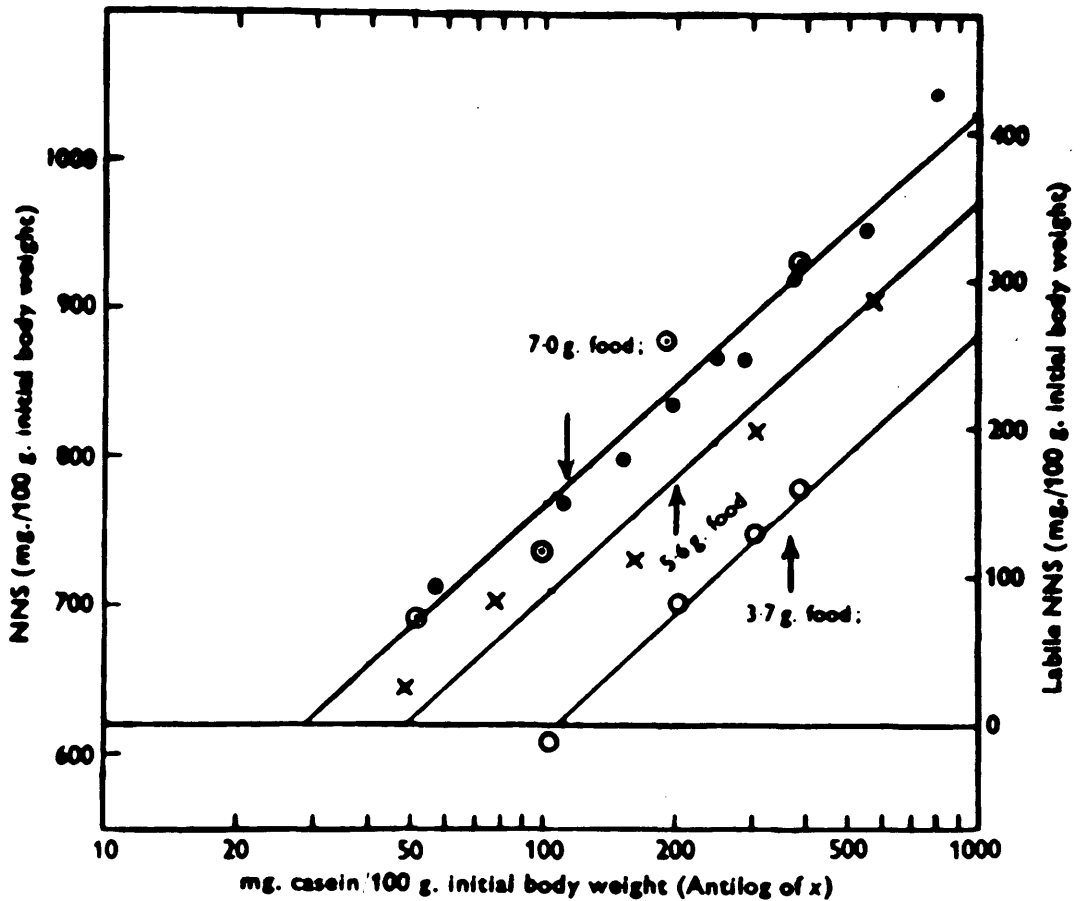
Fig. 4: The effect of feeding different amounts
of casein on the non-glycogen-non-lipid-solids,
i.e. essentially protein. The amount is plotted
against the logarithm of the daily casein N
intakes. Each point is the mean of at least 4
rats (after Campbell and Kosterlitz, 1948).

Table 2.

Regression equations for the lines shown in Fig. 4.
(From Campbell and Kosterlitz, 1948).

Food intake	Regression equation
3.7 gm.	Y = 79.5 + 267.5 X
5.6 gm.	Y =170.5 + 267.5 X
7.0 gm.	Y =233.3 + 267.5 X

viscera. This greater sensitivity of the liver to changes
in energy supply is comparable to its sensitivity to variations
in protein intake as demonstrated by Addis, Poo and Loe (1936),
who studied the effect in rats of feeding a high protein
intake after a period on a protein-free diet. After
five days on the protein diet the liver protein content had
increased by 53 per cent while the carcass had gained only 4
per cent.

The effect of energy intake on liver non-glycogen
non-lipid solids (N.N.S.), i.e. essentially protein, was
studied by Campbell and Kosterlitz (1948). Their findings
are set out in Fig. 4 while Table 2 shows the regression
coefficients of the various lines plotted. Since these
regression lines are parallel to one another, it follows that
equal increments in protein intake result in equal changes in
liver protein content. For example, raising the protein
content from 100-1000 mg. casein/100 g. body weight results
in an increase in N.N.S. of 260 mg. at 3.7 g. food intake;
260 mg. at 5.6 g., and 250 mg. at 7 g. food intake.

These data replicate the changes we have already
noted for the relationship between energy intake and N balance
in the whole animal (Fig. 3), namely an area (A-B) in which
protein and energy increments in the diets produce changes
in the body which are independent of one another. Studies
on the protein metabolism of liver are, therefore, likely to

be representative of the response of the whole animal to energy level.

The Scope of the Present Thesis.

It is obvious from the preceding section that, whatever the nature of the mechanism involved, energy level causes N retention by protein deposition. The large changes in liver protein content produced by varying the energy level make it a suitable organ on which to examine further the mechanism of energy intake on protein metabolism.

Thus the first Section deals with the simple question of whether tissues excised from an animal receiving a high energy intake incorporates amino acids more efficiently than those on a low energy intake. The evidence from these data being positive, this is followed by further _in vitro_ experiments using ^{14}C-glycine which reveal that energy level is more effective in promoting uptake when the previous diet contains protein.

However, this type of approach fails to distinguish between differences in the rate of penetration of the isotope into the cell and differences in the rate of protein synthesis. We therefore turned our attention to whole animal studies with ^{14}C-glycine (Section 3). These investigations supported the idea that energy level in the diet affects protein utilisation during the post-absorptive phase, i.e. reutilisation of amino acids circulating in the

blood between meals, rather than the amino acids which flood the tissues after a meal containing protein.

In view of this conclusion, it became imperative to establish the behaviour of energy donors in the tissues under these various nutritional conditions. This was therefore carried out in Section 4.

As supplementary evidence, we were persuaded that an alternative and non-isotopic approach would be valuable confirmation of the picture. Accordingly, the formation of p-amino hippuric acid, which involves synthesis of a peptide bond, was studied on liver slices excised from animals receiving different energy and different protein levels(Section5)

Finally, the whole picture of utilisation of dietary protein as it is affected by other nutrients is considered in a general discussion.

SECTION I.

THE INFLUENCE OF PREVIOUS ENERGY INTAKE ON THE IN VITRO UPTAKE OF ^{35}S- METHIONINE BY PROTEINS OF LIVER AND MUSCLE.

INTRODUCTION.

The Use of Isotopes in Studying Protein Metabolism.

Until the late 1930's the generally accepted concept of protein metabolism was that expounded by Folin (Folin, 1905; Folin and Denis, 1912). This postulated a distinct dichotomy into endogenous metabolism representing a constant tissue breakdown and repair and a variable exogenous metabolism dependent on the protein intake of the organism. But with the advent of isotopes, studies of protein synthesis, which had been difficult to accomplish, mainly because of the comparative slowness of such a synthesis relative to chemical reactions, could be the more easily performed. The earliest work in this field was done by Schoenheimer (1942) who, after feeding isotopically-labelled amino acids to animals, analysed their tissues for isotope content. The picture he obtained by feeding labelled leucine, expressed relatively, was serum 100, intestinal mucosa 89, kidney 82, spleen 45, liver 56, heart 53, testes 46, muscles 18, haemoglobin 17 and skin 11. It was therefore apparent that the tissue proteins were not inert structural units but were in a continuous dynamic state: amino acids entering and leaving the protein with great rapidity. These findings overthrew Folin's concepts and Schoenheimer's conclusions gained strength from other iso-topic experiments performed in vivo. But such experiments were unlikely to establish the actual modus operandi of such

reactions and so attention was directed to an in vitro approach. This approach, which has been used in this and in a later section, has some advantages to offer over in vivo studies, since it allows of a greater control of experimental variables and various tissues have been investigated in such studies. However, before we can whole-heartedly endorse the findings of such an approach, two questions must be answered: (a) Is the labelled amino acid incorporated into the protein by peptide bonds? and (b) To what extent do tissue slice conditions reproduce those of the intact animal?

(a). Evidence of peptide bond formation. A major assumption underlying all the work with labelled amino acids is that the uptakes measured actually represent the incorporation of the amino acids into the proteins by peptide bond formation. We must preclude any incorporation which is due to adsorption of the labelled amino acid on the protein or which may be bound to the protein by disulphide or any other linkage which is not a true peptide bond. Studies in which ^{35}S-cystine was used have shown that a false estimate of uptake can be given by such S-S linkages (Melchior and Tarver, 1947a). Thus when ^{35}S-methionine is used as a tracer it is imperative that all the cystine must be removed from the protein hydrolysate before estimating the incorporation of the methionine. But if such

a precaution is taken then it appears that the amino acid uptake is due to peptide bond formation for the following reasons:-

1. The uptake decreases as the slices age (Simpson and Tarver, 1950).

2. There is little or no uptake by boiled slices (Winnick, Friedberg and Greenberg, 1947).

3. Anaerobiosis and inhibitors of oxidation and phosphorylation depress uptake (Winnick et al., 1947; Frantz, Loftfield and Miller, 1947; Frantz, Zamecnik, Reese and Stephenson, 1948; Borsook, Deasy, Haagen-Smit, Keighley and Lowy, 1950).

4. Prolonged washing, reprecipitation or protracted dialysis procedures fail to remove the labelled amino acids (Simpson and Tarver, 1950).

5. Proteins labelled with carboxyl-labelled alanine do not lose the label when treated with ninhydrin, which reacts with any free amino acids (Winnick, Peterson and Greenberg, 1949).

6. The uptake is more rapid in foetal (Friedberg, Schulman and Greenberg, 1948) and regenerating tissue (Greenberg, Friedberg, Schulman and Winnick, 1948).

7. Proteins labelled with carboxyl-labelled glycine, histidine, leucine or lysine do not lose the label when subjected to dialysis or performic acid oxidation. Nor is $^{14}CO_2$ released from the protein by ninhydrin treatment.

After hydrolysis, all the radioactivity of the protein can be accounted for in the form of the original labelling agent (Borsook, Deasy, Haagen-Smit, Keighley and Lowy, 1952).

The body of evidence is therefore in favour of the amino acid being incorporated by peptide bonds; there is no evidence against it. This conclusion has been strengthened by the isolation of a hexapeptide containing radioactive aspartic acid from egg albumin after incubating oviduct mince with $^{14}CO_2$ (Anfinsen and Steinberg, 1951).

(b). Is synthesis of protein in tissue slices physiological?
Although tissue slices can incorporate labelled amino acids, is it permissible to assume that incorporation follows the same channels as in the intact animal? If so, the rate of uptake of an isotope _in vitro_ should be comparable to that obtaining _in vivo_ and at the same time the conditions should be physiologically appropriate. In reviewing the subject, Borsook (1950) showed that the rate of uptake into intact cells _in vitro_ (expressed as μ equivalents amino acid incorporated per gm. protein) was of the same order of magnitude as occurred with _in vivo_ experiments. In addition, he demonstrated that such an incorporation was a logarithmic function of the initial concentration of labelled amino acid up to a certain optimal concentration, although concentrations above 0.001M – 0.003M were inhibitory. It is also true to state that tissues _in vitro_ will incorporate amino acids at

concentrations similar to those in the blood and the
dependence of the rate of uptake is greatest in the
physiological range (Borsook, 1950).

An apparent discrepancy between _in vitro_ and _in vivo_
studies arises when we consider the effect of adding amino
acids to the incubation medium. Borsook, Deasy, Haagen-Smit,
Keighley and Lowy (1949 and 1950) have shown that, when
labelled glycine, leucine and lysine are incubated with rat
diaphragm, the addition of a mixture of amino acids
approximating in composition to that of casein or haemoglobin
(with omission of the amino acid being used as a label)
did not affect the uptake of labelled amino acid. This is in
accord with the findings of Zamecnik and Stephenson (1950),
who found no increase in uptake when an enzymatic hydrolysate
of protein was added to the incubation medium. Thus in all
these studies provision of an abundant supply of amino acids
did not apparently promote protein synthesis by the tissue
slices.

A somewhat different picture emerges from the results
of feeding experiments (Geiger, 1947 and 1948; Schaeffer
and Geiger, 1947; Cannon, Steffee, Frazier, Rawley and
Stepto, 1947; Yeshoda and Damodarian, 1947) namely, that an
essential amino acid is ineffective for growth, or for recovery
from protein depletion, or for maintenance unless it is fed
or injected within a few hours of the other indispensable amino

acids. Similarly, Hokin (1951) found that amylase synthesis by pigeon pancreas slices only occurred when the incubation medium was fortified with a mixture of all the essential amino acids. Likewise, Miller, Bly, Watson and Bale (1950) perfused a rat liver with ^{14}C-DL-lysine and found that when a balanced mixture of amino acids was added to the perfusion fluid incorporation into liver and plasma proteins was much increased. In contrast, Jensen and Tarver (1955) failed to observe this stimulation on adding amino acids to the perfusate, and have also pointed out that Miller's data is capable of more than one interpretation. That is to say, the increased incorporation observed by Miller et al. merely represents a change in the specific activity of the ^{14}C-lysine by dilution of the amino acid pool.

It would therefore appear from these findings that protein synthesis in vivo requires a balanced mixture of amino acids and yet the in vitro findings suggest that amino acid supplements have no stimulatory effect on the uptake of labelled amino acids. How then can we reconcile these two approaches? One possible explanation is that there are already sufficient free amino acids in the tissue slices to support protein synthesis (Rutman et al.,1955). Assuming that the synthesised protein contains 2 per cent histidine, 7 per cent leucine and 7 per cent lysine (Block and Bolling, 1951), then 0.005 and 0.018 mg. respectively of these amino acids would be required to support the normal synthetic rate of the fed

animal's liver (12.1 mg./day/gm. of liver.)   If 500 mgms.
of liver are used in the incubation procedure, the amounts
of free histidine, leucine and lysine present are of the
order of 0.04, 0.05 and 0.05 mg. respectively (Solomon,
Johnson, Sheffner and Bergeim, 1951; Wiss, 1949; Schurr,
Thompson, Henderson and Elvehjem, 1950).   Thus, normally,
liver slices contain sufficient amino acids, both free and
derived from the breakdown of protein during incubation,to
maintain a rate of synthesis greater than that observed.
Further addition of amino acids to the medium would therefore
have no effect.   It seems reasonable to assume, therefore,
that in vitro studies do in fact yield the same conclusions
as those found in intact animals.

In Vitro Studies of Protein Metabolism Using ^{35}S.

Early work in this field was performed by Melchior
and Tarver (1947a) who incubated liver slices in Krebs
saline with glucose as an energy source in the presence of
^{35}S-cystine. Considerable radioactivity was found in the
tissue protein but on treatment with a reducing agent, thio-
glycollic acid, the greater part of the radioactivity was
removed. This suggests that the isotopic cystine was
attached to the protein by S-S linkages rather than by peptide
bonds. Cystine, therefore, seems to be of little value as a
labelling agent in studies of protein synthesis. Shortly
thereafter, Melchior and Tarver (1947b) were able to synthesise

^{35}S-methionine and incubated it with liver slices under similar conditions to the above. On hydrolysis of the liver protein and removal of cystine, the methionine sulphur was converted to sulphate, which was found to be radioactive. Further studies by Melchior and Tarver (1950) showed that the uptake of ^{35}S-methionine into rat liver slices was (a) dependent on the concentration of methionine in the medium, (b) varied linearly with time and (c) was inhibited by respiratory poisons and anaerobiosis. More recently, a study of the effect of the previous nutritional state on the incorporation of ^{35}S-methionine into rat liver slices has been made (Rutman, Rutman and Tarver, 1955). The incorporation of the isotope was influenced by the protein content of the diet; as this was raised, so was the incorporation raised. The effect of fasting after feeding a high protein diet, on the other hand, is to reduce the incorporation, while in contrast the effect of fasting after a previous consumption of a low protein or protein-free diet is to stimulate the rate of incorporation.

Scope of the Present Experiments.

In the foregoing summary of the literature, there is no evidence, either from ^{35}S studies or ^{14}C studies, regarding the influence of dietary energy level as such an incorporation rate in tissue slices. A few studies have been made on the effect of fasting. Thus Krahl (1953), using

^{14}C-glycine, obtained a difference in incorporation into
rat diaphragm slices between the fasted and fed rat, the
uptake being invariably less in the former case. Similarly,
as described earlier, Rutman et al.(1955) found such a
distinction between fasted and fed animals in the uptake of
^{35}S-methionine in rat liver slices.

These studies involve complete removal of all nutrients
and it is therefore an assumption to conclude that the caloric
factor is the dominant one responsible for these changes in
the incorporation rate. Indeed, the data of Rutman et al.
indicate differences in the response of rats fasted after
high and low protein intakes.

Our own objective has been to study the uptake of
^{35}S-methionine by pieces of liver and muscle excised from
animals which had been in receipt of different energy levels
in their diets and were fasting some 12-20 hours at the time
of sacrifice. The reason for choosing these conditions was
that earlier studies in this laboratory had demonstrated
better nitrogen balance when the energy intake of an animal
was raised, even if the added source of energy were given 12
hours apart from the dietary protein. Thus the question posed
was whether the tissues, some 12 or more hours after food,
are still exhibiting differences in utilisation of amino acids
in relation to the energy content of the preceding diet.

Table 3.

Vitamin Mixture.

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

Table 4.

Salt Mixture "446".

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

Table 5.

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

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

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

0.8 ml. of this was mixed with above mixture.

TABLE 6.

Details of Dietary Regimes.

A.M.	P.M.
2 g. V.M.R. Protein + carbohydrate + fat mixture yielding 800 Cal/m^2	Olive oil or glucose fed by stomach tube yielding 1000 Cal./m^2 to high energy groups only.

Protein + carbohydrate + fat mixture.

Casein	2.8 g./rat/day
Glucose	1.0 g./rat/day
Starch	1.0 g./rat/day
Fat	0.5 g./rat/day

EXPERIMENTAL.

Rats.   Male white rats (weighing 250-300 gm.) were used in these experiments. They were starved overnight and weighed before being fed the experimental diet for four days. Changes in weight after this period were noted.

Diets.   The rats received 6.5 gm. of a protein-carbohydrate-fat mixture in the morning (10 a.m.) along with 2 gm. vitamin-mineral-roughage (V.M.R.) mixture (see Tables 3, 4, 5 and 6) and where desired the energy intake was increased by feeding varying amounts of olive oil or glucose by stomach tube in the evening at 9 p.m.

Incubation procedures.   The animals were killed by a blow on the head and the diaphragms and livers removed.   The diaphragm was washed in sterile medium and the two lateral segments dissected out while suspended in the medium, then transferred to stoppered incubation flasks. Pieces of the liver were sliced in a McIlwain chopper (McIlwain and Buddle, 1953) and approximately 500 mgm. of liver slices were transferred to stoppered flasks containing 4 ml. of the sterile incubation medium and 2 µc. of ^{35}S-methionine. The composition of the medium was that described by Sinex, McMullen and Hastings (1952) and consisted of Na^+ 140, K^+ 5, Ca^{++} 10, Mg^{++} 5, $HPO_4^=$ 2, Cl^- 133, HCO_3^- 25, expressed as

Fig. 5.

Treatment of Homogenates after Incubation with ^{35}S-Methionine.

Homogenate

centrifuge

Protein precipitate   supernatant
discard

wash thrice with 15 ml. 10% (w/v) TCA

and centrifuge

supernatant
discard   protein

heat on a water bath at 90°C

for 15 mins. with 15 ml. 10%

TCA and centrifuge.

supernatant
discard   protein precipitate

dissolved in 5 ml. 0.4 N

NaOH and then 5 ml. 30%

TCA added and allowed to

stand for 1 hr. before

being centrifuged. This

step is repeated once.

supernatant
discard   protein precipitate

(which is then treated with fat
solvents).

milli-equivalents. The flasks were gassed with 95 per cent O_2 - 5 per cent CO_2 before being incubated at 37°C for one hour or longer. In order to demonstrate that the labelled methionine was incorporated into the tissue protein and not merely absorbed on to its surface, a zero time control was carried out in every experiment. This consists of killing the tissue before exposing it to the isotope; 0.1 M sulphuric acid was used to kill the diaphragm while 1.5 ml. of 30 per cent (w/v) trichloracetic acid (TCA) was used for liver slices (Simpson and Tarver, 1950).

Isolation of protein from diaphragm and liver. At the end of the incubation, the diaphragms were removed and killed by immersing in 0.1 M H_2SO_4, rinsed with distilled water and homogenised in a Folley blendor with 15 ml. 0.4 N NaOH until homogeneous. 20 ml. distilled water was added and 7 ml. 30 per cent TCA. The diaphragm was then homogenised for a further five minutes, and the homogenate transferred to a 50 ml. centrifuge tube.

To stop the reaction in the flasks containing liver slices, 1.5 ml. 30 per cent (w/v) TCA was added. Then the contents of the flasks were homogenised in a Folley blendor with 15 ml. 10 per cent (w/v) TCA for ten minutes. The homogenates were then transferred to centrifuge tubes.

The subsequent treatment of the homogenates is described in Fig. 5. The salient features are (a) repeated

washing with 10 per cent (w/v) TCA to remove traces of
^{35}S-methionine adsorbed on to the protein precipitate;
(b) heating on a water bath for 15 minutes at 90°C with
10 per cent (w/v) TCA in order to hydrolyse nucleoproteins,
(Schneider, 1945); (c) dissolving the protein in NaOH and
precipitating it with 30 per cent (w/v) TCA. Melchior and
Halikis (1952) have advocated this technique for removing
traces of adsorbed methionine from the protein. The protein
was then washed with 15 ml. portions of fat solvents in the
following order:- 95 per cent (v/v) ethanol (twice);
ethanol-chloroform (3:1); ethanol-ether (3:1); absolute
alcohol and dried overnight from ether.

Note on the choice of homogeniser. In our earliest
experiments, following the recommendation of Sinex, McMullen
and Hastings (1952), the tissues were homogenised in a
Potter-Elvehjem (1936) glass homogeniser. It was noticed
that insoluble material remained at the bottom of the tube
even at the end of a 24-hour hydrolysis period of the
purified protein in 6 N HCl. When the diaphragm, homogenised
in 0.4 N NaOH with the Potter-Elvehjem homogeniser as
described by Sinex et al. (1952), was fat-extracted and dried,
the average nitrogen content of the protein by the method of
Ma and Zuazaga (1942) was found to be 3.51 per cent instead
of the expected 16 per cent. If the diaphragm homogenate was
centrifuged to remove the insoluble fraction and the protein

subsequently isolated, fat-extracted and dried, the
percentage nitrogen content of the protein increased to
9.96 per cent.   This is still too low and it would therefore
appear that there is a soluble contaminant as well as an
insoluble one. The most probable origin of these diluents
appeared to be the glass of the homogeniser.   In order to
test this, a sample of casein was weighed out and homogenised
in a Potter-Elvehjem glass homogeniser, then the protein was
isolated, fat-extracted and dried. A control sample was
subjected to similar treatment without homogenising. Nitrogen
estimations showed that the percentage nitrogen content of the
homogenised and the untreated samples were 8.54 and 13.25
respectively.   It was found that when a solution of 0.4 N
NaOH is "homogenised" in the Potter-Elvehjem homogeniser, the
solution becomes cloudy and eventually a precipitate settles
out.   It appears that the friction of the pestle against the
glass container rubs away particles of glass, some of which
dissolve in the NaOH and some remain insoluble.   Since quite
prolonged homogenising is needed for diaphragm samples, the
glass homogeniser is especially pernicious for this purpose.
In view of this, some doubt is cast on data in the literature
obtained through the use of this procedure where whole
protein (so called) is assayed for radioactivity (Borsook et
al., 1950).

The use of the Potter-Elvehjem homogeniser was
therefore discontinued and the possibility of using a Folley

steel-bladed blendor was investigated. Rat diaphragms were therefore homogenised in a blendor, the proteins isolated, fat-extracted and dried. Nitrogen estimations were then carried out on the protein samples and the average nitrogen content was 13.6 per cent. This indicates that no serious contamination results when a Folley blendor is used; therefore in all further experiments a Folley blendor was used for homogenising tissues.

Isolation and estimations of ^{35}S-methionine from protein.

Tarver and his associates have invariably isolated methionine from labelled proteins, because labelled cystine is largely bound by S-S linkage and not as peptide. Their latest procedure (1950) depends on separating the cystine by the copper procedure of Zittle and O'Dell (1941), followed by perchloric acid oxidation of methionine. Young, Edson and McCarter (1949) describe the use of nitric acid in the Carius tubes for oxidation of S-compounds prior to ^{35}S assay.

In the following pages we give an account of the use of the oxygen bomb for the conversion of methionine-S to inorganic sulphate. This is a very efficient conversion, having been used successfully for S-estimations of resistant compounds for many years. Our own experience, detailed below, supports the adequacy of oxidation in the case of methionine.

The dried proteins were weighed out and hydrolysed with 6 N HCl for 8 hours in an atmosphere of nitrogen (to

MICRO-FILTER

Position
of disc

Perforated Plastic Disc
covered with circle of
filter paper and placed
in position

Micro-filter screwed together
and precipitate of benzidine
sulphate filtered off by suction.
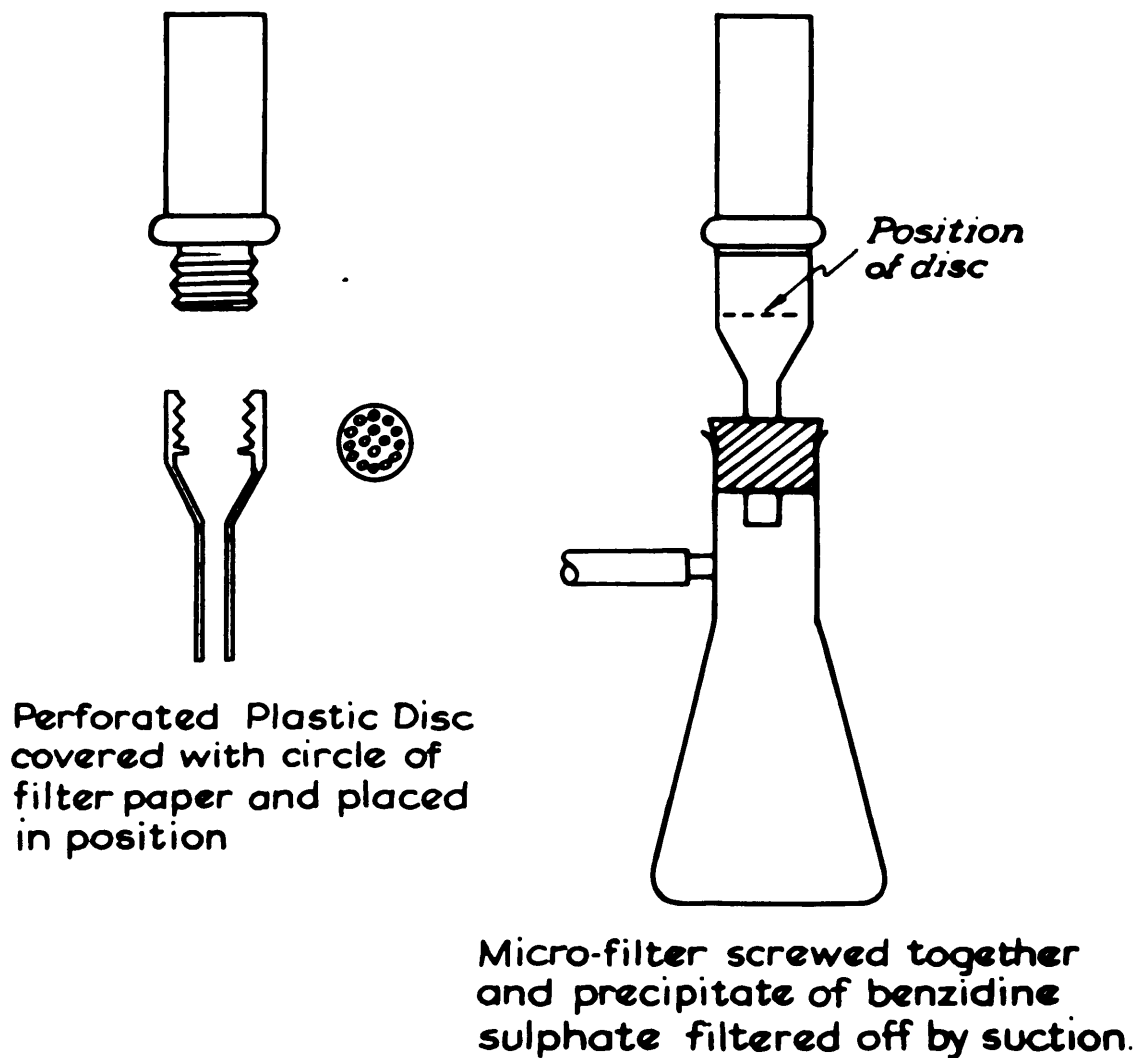
Fig. 6.

prevent the oxidation of cysteine-SH to cysteic acid). The HCl was removed by distillation *in vacuo* and the residue taken up in 40 ml. distilled water. In order to separate cysteine and cystine from methionine in the hydrolysate the method of Zittle and O'Dell (1941) was used, which consists of adjusting the pH of the hydrolysate to 3.0 with solid sodium acetate, heating on a water bath and adding eight times the requisite amount of Cu_2O. When adjusted to pH 4, the copper mercaptide starts to flocculate and after standing for 40 minutes the precipitate of cysteine mercaptide was filtered off. The filtrate was then evaporated to dryness and 1 gm. benzoic acid (analar) was added to the residue which was powdered, formed into a pellet and burnt in an oxygen bomb, 25 atmospheres pressure of oxygen being used for each combustion. This procedure converts the ^{35}S-methionine into $^{35}SO_4$. The bomb was washed out with distilled water and the washings reduced to a small volume (2 ml.); 2 ml. of absolute alcohol, 2 ml. benzidine HCl solution were then added and the precipitate of benzidine sulphate allowed to settle overnight in the refrigerator, as described by Young, Edson and McCarter, (1949). The benzidine sulphate was collected evenly on a filter paper using a micro-filter (Fig. 6) and, after washing with 95 per cent (v/v) ethanol, was allowed to dry in a dessicator for at least an hour.

The precipitate was then counted for radioactivity in an end window Geiger-Müller counter and transferred to a

Recovery Experiments with the Oxygen Bomb and Micro-filter.

(a) Micro-filter.

Sample	Sulphate sulphur added mg.	Sulphate sulphur found mg.	Percentage recovery
1.	2.05	2.11	103
2.	2.05	2.08	102
3.	2.05	2.10	102
4.	2.05	2.09	102
5.	2.05	2.06	100
6.	2.05	2.09	102
Mean	2.05	2.09	102

In this experiment, aliquots of a magnesium sulphate solution containing 2.05 mgm. S per ml. were treated with benzidine-HCl and the precipitate filtered and the amount of S recovered estimated by titration.

TABLE 7b.

Recovery Experiments with the Oxygen Bomb and Micro-filter.

(b) Oxygen bomb + micro-filter.

Addition	Methionine added mg.	Methionine found mg.	Percentage recovery
None 1.	16.3	14.8	91
" 2.	16.3	14.0	86
" 3.	16.3	15.5	95
" 4.	16.3	16.0	98
" 5.	16.3	16.1	99
" 6.	16.3	15.6	96
" 7.	16.3	13.9	85
Mean	16.3	15.0	93
0.5mg.Cu_2O	16.3	12.1	74
1.0mg.Cu_2O	16.3	11.9	73
1.5mg.Cu_2O	16.3	11.7	72
Mean	16.3	11.9	73

In this experiment a known weight of methionine was combusted in the oxygen bomb, in some cases after treatment with Cu_2O. The S formed, being precipitated with benzidine-HCl, filtered and estimated by titration.

volumetric flask with water. The amount of sulphate was now estimated by the method of Fiske (1921), which consists of titrating the boiling solution with standard NaOH using phenol red as indicator. The reaction is:-

$$\left[H_3N-\langle\!\!\langle\rangle\!\!\rangle-\langle\!\!\langle\rangle\!\!\rangle-NH_3 \right]^{++} 2HSO^-_4 \longrightarrow H_2N-\langle\!\!\langle\rangle\!\!\rangle-\langle\!\!\langle\rangle\!\!\rangle-NH_2 + H_2SO_4$$
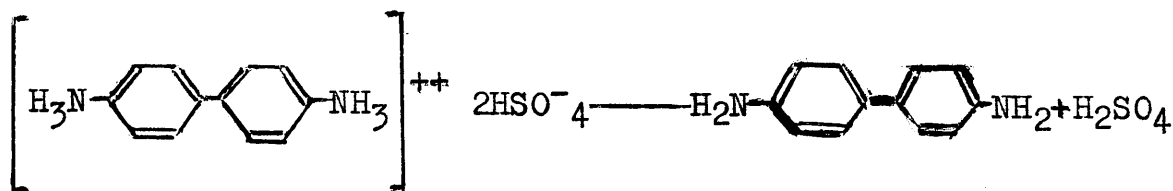
The radioactivities were then expressed as counts per minute per mg. sulphur, due correction being made for self-absorption of the ^{35}S β-particles. (Henriques, Kistiakowsky, Margnetti and Schneider, 1946).

Recovery experiments with the oxygen bomb. The results of recovery experiments using the oxygen bomb and micro-filter are shown in Tables 7a and 7b. In order to test the efficiency of the micro-filter, aliquots of a solution of magnesium sulphate, containing 2.05 mgm. sulphur per ml., were precipitated with the benzidine-HCl reagent as described, filtered using the micro-filter and the amount of sulphur estimated by titration. The results (Table 7a) show that there is no loss of benzidine sulphate when it is filtered with this type of filter. Recovery experiments in which a known amount of methionine was combusted in a bomb, and the resulting benzidine sulphate titrated against standard alkali show that, on the average, 93 per cent of the total methionine present in a sample is recovered (Table 7b). It is interesting that, if the methionine solution is treated with varying amounts of

cuprous oxide under the experimental conditions described
earlier, before being combusted in the oxygen bomb, then the
average recovery of methionine (Table 7b) is reduced to 73
per cent, showing that some methionine is lost with the cysteine
and cystine. Also, when cystine was precipitated from a
synthetic mixture of cystine and $^{35-}$S-DL-methionine, the
cystine precipitate was found to contain 20-30 per cent of the
total radioactivity. Since only the specific activity (counts
per minute per mg. methionine S) was needed in our experiments,
losses of this order do not matter. The amount of radioactivity
(specific activity) in the recovered sulphate is the only
criterion.

Adequacy of the Separation of Cysteine and Cystine from
Methionine. The method of separating the sulphur amino acids
which has been most commonly used is that described by Zittle
and O'Dell (1941) which consists essentially of precipitating
the cysteine as the copper mercaptide by adding the requisite
amount of Cu_2O at pH 3, heating on a water bath and adjusting
the pH to 4 with solid sodium acetate when the cuprous
mercaptide precipitates.

In order to show that all the non-cystine sulphur
in a protein hydrolysate, after treatment with the copper
reagent, is methionine, Simpson and Tarver (1950) added
500 mg. methionine to the copper-treated hydrolysate and
found that the methionine in an aliquot of this solution had
a specific activity of 101. Then 5 mg. amounts of cystine,
homocystine, cystathionine, thiolhistidine and djenkolic acid

Isotope Dilution Experiments showing the Efficacy

of the Copper Precipitation Method for

Separating Cystine from Methionine.

Sample	SO_4-S recovered mg.	Benzidine SO_4 mg./sq. cm.	* Count/min.	Activity cpm./mg.S
Methionine 1	3.21	12.6	6,110	5,092
" 2	3.10	12.2	4,691	4,311
" 3	3.31	13.0	6,368	4,899
Mean	-	-	-	4,767
Methionine 4	2.84	11.2	3,913	4,714
+ cystine 5 (2 mg.S)	2.86	11.3	3,651	4,295
" 6	2.79	11.0	4,111	5,271
Mean	-	-	-	4,760

* Corrected for self-absorption.

In this experiment, 2.01 mg. sodium sulphate sulphur was added to each sample as carrier and therefore, when calculating the activity of each sample, this amount of carrier sulphate must be subtracted from the total sulphate concentration.

TABLE 8b.

Isotope dilution experiment showing the efficacy of the copper precipitation method for separating cystine from methionine.

Sample	SO$_4$-S recovered mg.	Benzidine SO$_4$ mg./cm^2	Count/min.[*]	Activity cpm./mg.S
Methionine 1	3.15	12.24	5,697	1,809
" 2	3.20	12.60	6,572	2,054
" 3	3.05	12.03	6,196	2,031
Mean	-	-	-	1,965
Methionine 4 + cystine 5 (2 mg.S) " 6	2.32	9.19	4,552	1,962
	1.81	7.20	3,705	2,047
	1.51	6.03	2,827	1,872
Mean	-	-	-	1,960

[*] Corrected for self-absorption.

This experiment is similar to that shown in Table 8a but no carrier sulphate was added and hence the activity of the sample is obtained simply by dividing the corrected count by the total amount of sulphate.

were added to the solution before isolating and crystallising the methionine.   After purification by repeated crystallisation, the specific activity of the methionine was found to be 89.5.   This small decrease in the specific activity can be accounted for by traces of the carriers being retained by the methionine.

The method of Zittle and O'Dell (1941) has, however, been criticised by Campbell and Work (personal communcation), who doubt whether all the cysteine and cystine are quantitatively removed by the copper precipitation method. Therefore the efficacy of this method was tested by us by two series of experiments, one using the isotope dilution technique and the other involving a chromatographic analysis of protein hydrolysates prepared from liver and muscle by our experimental techniques.

The isotope dilution experiment consisted of taking a solution of synthetic DL-^{35}S-methionine and dividing it into two parts.   On one part the activity in counts per minute per mg. sulphur was determined.   In the other, a known amount of cystine (2 mg. cystine-S) was added to the methionine solution and the cystine was subsequently precipitated by the copper method of Zittle and O'Dell (1941); the specific activity of the ^{35}S in the methionine recovered from this solution was also determined.   The results are shown in Tables 8a and 8b.   It can be seen that the specific activity of the methionine has not decreased after addition

and removal of cystine, which indicates that the copper
precipitation method separates cystine completely from
methionine since no dilution of the methionine sulphur has
occurred.

The completeness of cystine and methionine separation
by the copper reagent was also tested by chromatographic
analysis.   Liver and diaphragm slices obtained from rats
were incubated with radioactive ^{35}S-methionine for 4 hours
and the proteins subsequently extracted.   These were
hydrolysed. In the first experiment the hydrolysates were
treated with Cu_2O to remove cystine and cysteine and the
filtrate treated with H_2S to remove copper ions. As
inorganic ions interfere with the chromatographic separation
of amino acids (Consden, Gordon and Martin, 1944), the amino
acids were precipitated as the mercury complexes (Campbell
and Work, 1952a) and washed free of inorganic ions with 66
per cent (v/v) ethanol. The mercury complexes were then
decomposed with H_2S and aerated to remove traces of H_2S.
Bromine water was then added to the solution in order to
oxidise any cystine or cysteine present, to cysteic acid.
The filtrate was reduced to a small volume and applied to
Whatman No. 1 "chromatography" paper. A synthetic mixture
of cystine and DL-^{35}S-methionine was treated similarly and
acted as a control when applied to the paper. Suitable
markers were also applied and the paper was eluted with a
phenol:water (5:2 v/v) solvent for 18 hours in the presence
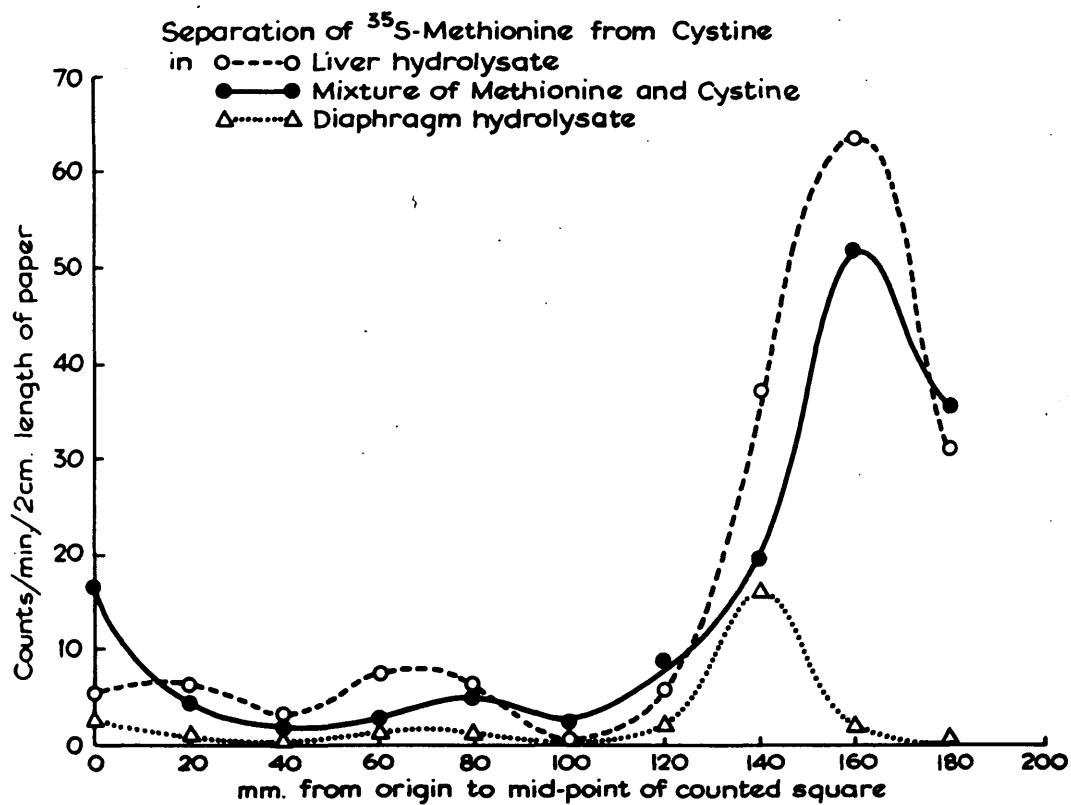
Fig. 7.
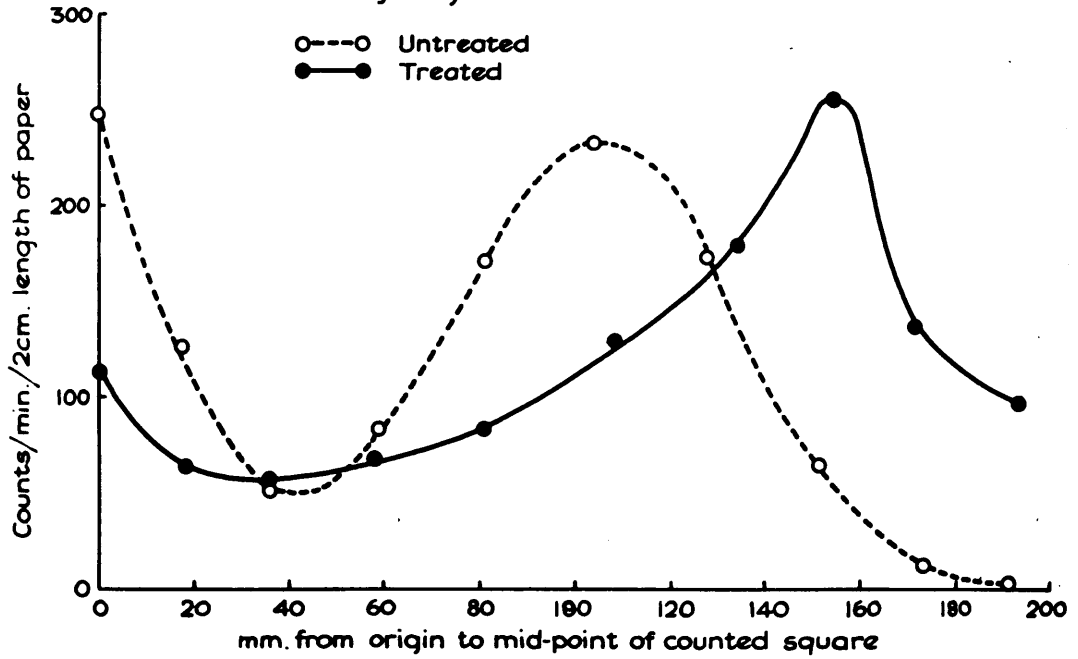
Fig. 8.

of NH_3. The paper was dried and then sprayed with ninhydrin, and the position of the spots noted. The paper was cut into strips and the strips were scanned along their length using an end window counter and the counts per minute graphed against distance from the origin (Fig. 7). The greatest radioactivity corresponds to the methionine spot some distance down the paper. Any cysteic acid present would appear at the origin and there is in fact a small radioactive peak at this point. It is therefore significant that, in the case of the synthetic mixture of cystine and methionine, the only radioactive substance present was methionine and it would appear from the distribution of radioactivity on this strip that a small portion of the radioactivity (methionine) remains at the origin. Thus the slight radioactivity in the cysteic acid position on the hydrolysate strip is not necessarily indicative of cysteic acid unprecipitated.

A further experiment was performed in which radioactive protein hydrolysates were obtained as above and then halved; one half was treated with Cu_2O and the other acted as a control. The samples were then treated as described above and the counts per minute graphed against distance from the origin. The result is shown in the graph (Fig. 8) and indicates clearly that, in the case of liver at least, the activity at the origin is markedly decreased by the copper precipitation method and is probably no more than might be expected from methionine retained at the origin. Unfortunately

the activities of the strip from the diaphragm hydrolysate were too weakly radioactive to be accurately counted. Taking these results with those obtained by the isotope dilution method, it is clear that the copper precipitation method does effectively remove the cysteine and cystine from methionine under our experimental conditions.

Self-absorption of ^{35}S β-particles.　　If the activity of a series of samples of varying weight but constant area is measured there is a deviation from a linear relationship between activity and weight of sample. This is caused by self-absorption.　The linear relationship between observed activity and sample weight is possible only where the radiations are so penetrating that the upper layers of the sample cannot appreciably absorb the radiations originating in the lower strata. The observed activity reaches a maximum when the sample is so thick that radiations originating in the lower layers of the sample are completely absorbed by the intervening layers.　When counting an isotope whose radiations are of such low energy that the ordinary sample thicknesses represent an appreciable fraction of the mean particle range, self-absorption losses introduce large errors in activity measurements and correction must be made for them.

　　　　There are four procedures by which correction can be made or the need for it eliminated (Calvin, 1949). First, by counting the sample at "infinite thinness", where the error due to self-absorption can be neglected when compared

with other errors in the experiment. This is limited to measuring samples with high specific activities. The second procedure involves reproducing accurately a standard sample thickness. This is very difficult to apply. The third procedure involves counting the samples at "infinite thickness". This method, though of wide applicability, is wasteful of sample material and cannot be applied if the amount of material available is small. The fourth procedure is to determine the relationship between observed activity and sample thickness and this is the method we have employed.

The specific activity (counts per minute per mg. S) of each sample is corrected to a standard thickness. In this case, 3.25 mgm. of benzidine sulphate per cm^2 was chosen so that comparison with the factors of Henriques et al. (1946) would be possible. Dr. W.S.T. Thomson investigated the correction factors for self-absorption under our experimental condition and found them to be in agreement with those published by Henriques et al. (1946).

RESULTS.

The experiments described in this Section were carried out to find the effect of previous energy intake on the uptake of ^{35}S-DL-methionine into rat liver and diaphragm slices. In all the experiments, the rats were divided into two groups, one receiving a low energy intake, the other a high intake. The rats were weighed before being fed the

TABLE 9.

The effect of previous calorie intake on the in vitro

incorporation of ^{35}S-DL-methionine into rat liver and

diaphragm slices, 12-14 hours after the last meal.

	Calorie intake Cal./m^2/day	Incorporation of ^{35}S-DL-methionine	
		Liver c.p.m./mg.S	Diaphragm c.p.m./mg.S
Expt.1	973	-	325
	1230[+]	-	391
	1564[+]	-	617
Expt.2	925	154	-
	1270[+]	333	-
	1680[+]	268	-
Expt.3	825	4341	485
	877	6229	334
	1570[*]	6078	-
	1490[*]	4446	517
Expt.4	962	771	513
	1009	1091	486
	1708[*]	2058	469
	1612[*]	2129	664
	1929[+]	1673	711
	1868[+]	1834	241

[*] Fed extra energy as glucose.

[+] Fed extra energy as olive oil.

experimental diet of known caloric value and their surface areas calculated by the method of Lee (1929). The energy intake was adjusted so that the rats in the low energy group received about 800 Cals./m^2/day while those in the high energy group received about 1800 Cals./m^2/day. The details of the morning and evening meals are given in Table 3, and it will be noted that the protein of the diet was all consumed in the morning meal, whereas the variable energy source was in the evening. In the experiments in which the animals were killed 12-14 hours after the last meal, the evening meal was not given till 10 p.m. Where the interval between the last meal and sacrifice was 18-20 hours, the evening meal was administered at 4 p.m. on the preceding day.

In vitro incorporation of ^{35}S-DL-methionine into rat liver and diaphragm slices 12-14 hours after the last meal. The incorporation of ^{35}S-DL-methionine into liver and diaphragm slices for four different experiments is shown in Table 9. It can be seen from this table that the incorporation, expressed as counts per minute per mg. S, is extremely variable, e.g. in Experiment 3, the incorporation into liver proteins at levels of 825 Cal./m^2/day and 1490 Cal./m^2/day are essentially the same; so also are the values obtained at levels of 877 Cal./m^2/day and 1570 Cal./m^2/day. These probably can be explained by the variable response of individual animals. Hence any effect of energy level in the uptake of

TABLE 10.

The data of Table 9 adjusted to the same order of magnitude as the values found in Experiment 3 (see Table 9).

Energy source	Calorie intake Cal./m^2	Uptake of ^{35}S-methionine	
		Liver c.p.m./mg.S	Diaphragm c.p.m./mg.S
Glucose	825	4341	558
	877	6229	384
	962	2688	513
	1009	3804	486
	1490	4446	595
	1570	6078	-
	1708	7176	469
	1612	7424	664
Fat	925	3846	-
	1009	4287	535
	962	3029	564
	1680	6695	-
	1929	6573	782
	1868	7206	265
	973	-	367
	1564	-	697

Method of computation:-
The average incorporation in Expt. 3 (Table 9) is 5274 (liver)
The mean incorporation in the case of the glucose-fed animals
and low energy animals in Expt. 4 (Table 9) is 1512. The
mean ratio in this case is $\frac{5274}{1512}$ = 3.48. To adjust values

in Table 9 to the same order of magniture as in Expt. 3, the
values obtained in Expt. 4 are multiplied by 3.48, e.g.
771 x 3.48 = 2688.

TABLE 11.

Statistical analysis of the adjusted liver data.
(see Table 10).

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	Variance Ratio (F)
Total	13	35,695,544	-	-
Energy levels	1	21,561,134	21,561,134	16.4
Carbohydrate v. fat	1	0	0	-
Interaction	1	1,015,571	1,015,571	-
Residual error	10	13,118,839	1,311,884	-

The value of F at the 5% level of significance is 4.96, and at the 1% level is 10.04. This result indicates that a change in energy intake produces significant changes in the incorporation of labelled ^{35}S-methionine into the rat liver slices.

TABLE 12.

Statistical analysis of the adjusted diaphragm data. (See Table 10).

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio (F)
Total	13	243,612	-	-
Energy levels	1	31,136	31,136	1.5
Carbohydrate v. fat	1	14	14	-
Interaction	1	7	7	-
Residual error	10	212,476	21,248	

The value of F at the 5% level of significance is 4.96, and at the 1% level is 10.04. This analysis reveals no significant increase in incorporation of ^{35}S-DL-methionine into rat diaphragm slices.

^{35}S-DL-methionine could be obscured to some extent by the variability of the individual estimations and by the wide variation experienced from one experiment to another.

The interpretation of the results is therefore difficult but can be made easier by the use of statistical analysis. This can be done in two ways. One (A) is by adjusting the values obtained for the incorporation of ^{35}S-DL-methionine into liver and diaphragm to the same order of magnitude, the other (B) is to prove that the samples analysed come from a population with the same regression coefficient and then finding whether such a regression is significant. Both these methods have been employed.

(A). Table 10 shows the values in Table 9 adjusted to the same order of magnitude. In the case of liver at least, there seems by inspection to be some effect of energy level on the incorporation of labelled methionine. Analyses of variance of the liver and diaphragm data are shown in Tables 11 and 12. Although, in the case of the liver data, a highly significant increase in incorporation is observed as the energy level increases, no significant increase is shown by the diaphragm data. The analyses reveal that carbohydrate and fat are equally effective as energy sources when added to the basal diet.

(B). The method of finding the regression co-efficient of a number of samples is described by Snedecor (1946), who also describes how to find whether all the samples

Table 13.

Regression lines of ^{35}S-methionine incorporation into proteins of liver and diaphragm in relation to the level of energy intake.

Time elapsed since last meal hr.	Liver protein		Muscle protein	
	No. of rats	Regression equation	No. of rats	Regression equation
12-14	13	Y=0.669X+1505	12	Y=0.101X+344
18-20	20	Y=0.731X+ 855	24	Y=0.200X+213
Combined data	33	Y=0.715X+1100	36	Y=0.178X+241

X = energy intake in Cal/sq. m. and
Y = counts /min/ mg. methioine S.

The regression coefficients for liver and muscle 12-14 hours after food are not significant, but at 18-20 hours and for the combined data they are highly significant.

Table 14.

Regression coefficients expressed as a
percentage of the specific activity corresponding
to an energy level of 1200 Cal./m^2.

Time elapsed since last meal hr.	Liver protein Slope	Muscle protein Slope
12-14	29%	21%
18-20	42%	44%
Combined data	36%	33%

are from a population with the same regression coefficients,
and the method of testing the significance of such a regression
"t". The regression equation, i.e.

$$Y = \bar{y} + \frac{Sxy}{Sx^2} (x-\bar{x})$$

where $\bar{y}$ = mean value of all "y" values,

$\bar{x}$ = mean value of all "x" values,

$\frac{Sxy}{Sx^2}$ = regression coefficient

has been calculated for both the 12-14 hour and the 18-20
hour data.   In this case, the "x" and "y" values represent
the caloric intake and incorporation of isotope respectively
and are shown in Table 13.   In the case of the 12-14 hour
data for liver, the value for $\bar{x}$ and $\bar{y}$ are 1,364 and 2,417
respectively. Substituting these values and that of the
regression coefficient (0.6689) in the above equation, we have

$$Y = 2,417 + 0.6689 (x-1,364)$$

which, on expansion, yields

$$Y = 1,505 + 0.669x.$$

The percentage slope, i.e. change per 1000 Cal./m^2 expressed
as a percentage of the value at 1200 Cal./m^2 can therefore be
calculated and in this case was found to be 29 per cent. The
significance of the regression was also tested and in this
instance was not found to be significant. The same procedure
was carried out with the diaphragm data also, and was not
found to be significant.   The percentage slope and "t"
values of the regression lines are summarized in Table 14.

TABLE 15.

The effect of previous calorie intake on the in vitro incorporation of ^{35}S-DL-methionine into rat liver and diaphragm slices, 18-20 hours after last meal.

| | Calorie intake Cal./m^2/day | Incorporation of ^{35}S-DL-methionine into | |
		Liver c.p.m./mg.S	Diaphragm c.p.m./mg.S
Expt. 1	951	–	292
	1257[+]	–	396
	1618[+]	–	509
Expt. 2	768	–	512
	825	3355	880
	1390[*]	–	651
	1655[*]	3569	810
Expt. 3	800	1471	250
	811	1897	595
	830	1866	283
	1561[*]	1659	582
	1624[*]	2344	579
	1837[+]	1465	397
Expt. 4	790	229	214
	830	1111	301
	860	529	288
	870	1309	210
	880	1857	392
	1720[*]	1763	373
	1750[*]	2009	855
	1900[+]	2697	–
	1920[+]	1428	507
	1940[+]	1654	619
	1890[+]	2533	569
	2030[+]	2313	327

[*] Fed extra energy as glucose.

[+] Fed extra energy as olive oil.

Fig. 9.— The Influence of Previous Level of Energy Intake on
^{35}S-Methionine Uptake by the Proteins of Liver Slices and
Diaphragm.
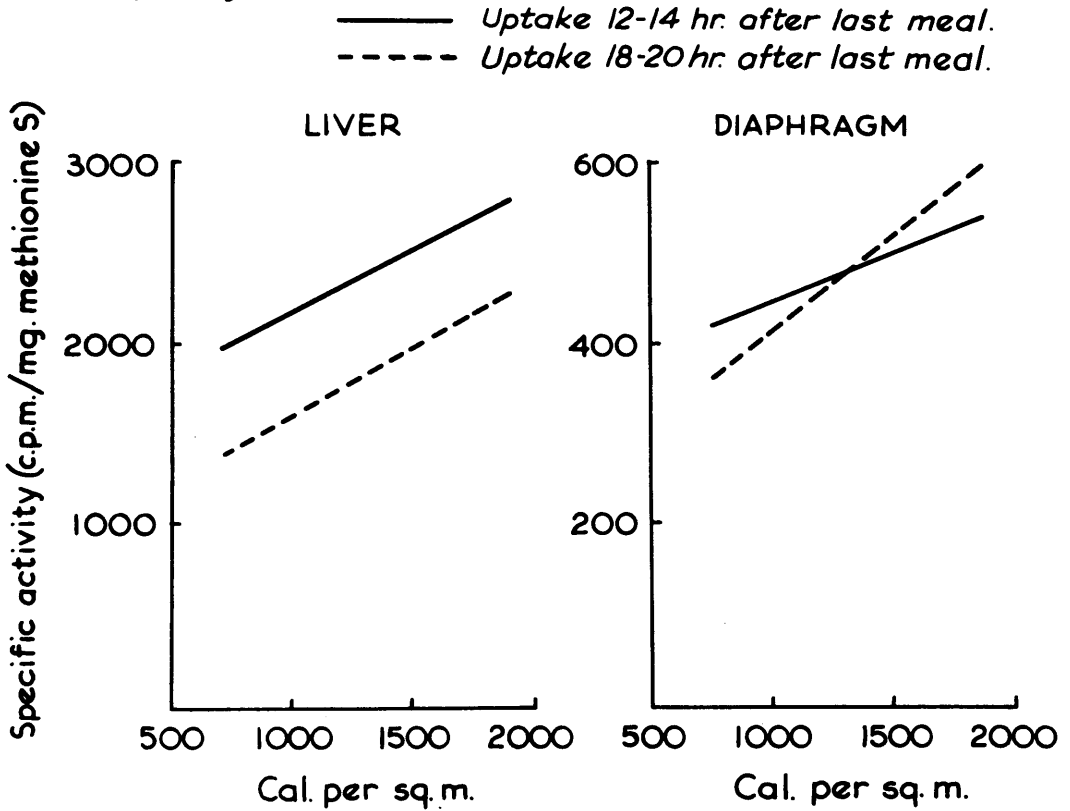
——— Uptake 12-14 hr. after last meal.
- - - - - Uptake 18-20 hr. after last meal.

Incorporation of ^{35}S-methionine into rat liver and diaphragm
slices 18-20 hours after last meal.   The rate of incorporation
of ^{35}S-DL-methionine into rat liver and diaphragm protein
18-20 hours after the last meal is shown in Table 15, which
demonstrates that the incorporation of the label is as
variable as those observed for the 12-14 hour data (Table 9).

In this instance, statistical analysis of the results
was restricted to proving that the samples came from a
population with the same regression coefficient, finding this
parameter and testing its significance.   The regression co-
efficients along with the "$\overline{x}$" and "$\overline{y}$" values are shown in
Table 13, while the regression equations are shown in Table
14.   This clearly shows that for the 18-20 hour data there
is a significant increase in uptake of the labelled amino
acid into rat liver and diaphragm proteins.   These findings
are shown graphically in Fig. 9.   Another point of interest
is that the percentage slope,i.e., the change per 1000 Cal./
m^2 related as a percentage to the value at 1200 Cal./m^2, is
approximately the same in liver and muscle proteins, namely
42 per cent and 44 per cent respectively.

If the 12-14 hour and 18-20 hour data are combined,
the value of "t" is still significant, and the percentage
slope is the same in both liver and muscle.

TABLE 16.

The ratios of incorporation of ^{35}S-DL-methionine into liver to the incorporation into diaphragms after feeding low and high energy diets to rats.

Time after last meal	Low energy		High energy supplied by			
			Glucose		Fat	
		Mean		Mean		Mean
12–14 hrs.	9.0 18.6 2.2 1.5	7.8	8.6 4.4 3.2	5.2	2.4 7.6	5.0
18.20 hrs.	4.7 6.2 3.6 1.8 1.0 5.9 3.2 6.5 3.8	4.1	4.6 2.3 2.8 4.0 4.4	3.6	2.6 2.8 7.0 4.4 3.7	4.1
Mean of combined 12–14 hr. & 18–20 hr. data		5.9		4.4		4.5

The ratio of incorporation of isotope into liver to the incorporation into muscle is shown in Table 16. The ratios have been divided into three groups: those obtained with a low energy intake, a high energy intake when glucose is the energy source, and a high energy intake when fat is the energy donor. These ratios give mean values of 7.8, 5.2, 5.0 for the 12-14 hour data; 4.1, 3.6, 4.1 for the 18-20 hour data, indicating that the ratio of incorporation is approximately the same irrespective of energy level and that the liver protein incorporates five times the amount of isotope incorporated by the muscle protein.

DISCUSSION.

The effect of previous energy intake on the uptake of ^{35}S-methionine into rat liver and diaphragm slices was studied by adding an energy supplement, either carbohydrate or fat, to an already adequate diet. It is clear from these experiments that (a) an increased energy intake does have a stimulatory effect on the uptake of labelled methionine; that (b) such an effect persists for some time after the last meal (18-20 hours or more); and that (c) carbohydrate and fat are equally potent as energy sources. These findings are in accordance with those of Munro and Wikramanayake (1954), who found an improvement in N balance even when the energy moiety of the diet, whether carbohydrate or fat in nature, was fed some 12 hours before or after the protein constituent of the diet.

The only other experiments described in the literature have been of a slightly different pattern to our own. Rutman et al., (1955) studied the uptake of ^{35}S-methionine by liver slices from rats which had received diets ad. libitum with varying amounts of protein and the effect of subsequent fasting. These authors found that incorporation of the labelled amino acid into liver protein increases as the protein content of the diet rises. The effect of fasting was of particular interest to us, in so far as a fast of one day's duration following a protein-free or a low-protein diet had an apparent stimulatory effect on protein synthesis whereas fasting after a protein-rich diet decreased incorporation.

Since in both cases fasting represents a withdrawal of energy from the diet, it would appear that energy deprivation has different effects dependent on the protein content of the preceding diet. However, this type of experiment does not readily lend itself to easy interpretation since no account is taken of the amount of diet consumed, nor can it be concluded that energy is the dominant factor affecting the incorporation when the entire food supply is withdrawn. Although the experiments performed by Rutman et al.(1955) are not strictly comparable to ours, it can be said that they do in fact support our own findings, i.e. they do stress the interdependence of amino acids and energy supply as factors in protein synthesis.

Although the experiments in this Section show that
previous energy intake exerts a prolonged effect on the
incorporation of ^{35}S-methionine into rat tissue slices
in vitro, they fail to distinguish between effects on the
rate of entry of the labelled amino acid into the cell and
its subsequent incorporation into protein. Thus a greater
rate of penetration of the labelled methionine from the
medium into the amino acid pool of the cells could not be
readily distinguished from a direct stimulus to protein
synthesis. This problem is considered in the next Section,
where a more comprehensive series of dietary conditions was
employed and in which an attempt was made to distinguish
between uptake of amino acids into the cell and their
subsequent incorporation into protein.   All that can
legitimately be claimed from the data obtained with ^{35}S-
methionine is that the energy level of the previous diet
continues to affect protein metabolism for at least 18-20
hours after the last meal.

SECTION 2.

THE INFLUENCE OF PREVIOUS ENERGY INTAKE ON THE IN VITRO INCORPORATION OF ^{14}C-GLYCINE INTO LIVER PROTEIN.

INTRODUCTION.

In the previous Section, the effect of adding increments of energy in the form of either carbohydrate or fat to a diet of adequate protein content on the incorporation of ^{35}S-methionine into liver and diaphragm proteins was studied. It was found that a linear relationship existed between energy intake and incorporation of isotope. As the energy intake increased, the incorporation of the labelled amino acid into protein increased.

The objective of the new series of experiments was to answer the criticism of the tissue slice incorporation technique raised in Section 1, namely that we cannot distinguish between differences in rate of protein synthesis and differences in the rate of penetration of the labelled amino acid into the slice. An attempt to distinguish these possibilities depended on incubating slices from the same liver for two different periods of time.

As a further elaboration of the experimental conditions, it was decided to study the in vitro incorporation of a labelled amino acid after various dietary regimes: namely, in conditions (i) where the supply of amino acids was completely endogenous, (ii) where the supply of amino acids was adequate - a protein-containing diet in the post-absorptive state, and (iii) where tissues were being flooded with amino acids - i.e. during the absorptive phase of a protein meal.

TABLE 17.

Composition of Protein-containing Diet.

Potato starch	69 g.
Glucose	69 g.
Margarine	42 g.
Casein	240 g.

4.2 g. given daily.

Composition of Protein-free Diet.

Potato starch	189 g.
Glucose	189 g.
Margarine	42 g.

4.2 g. given daily.

---

1 g. Vitamin Mineral Roughage mixture (for composition see Table 6) fed to each rat daily.

With each of these diets, rats were given low and high energy intakes and the capacity of slices of liver to incorporate ^{14}C-glycine was examined.

EXPERIMENTAL.

Animals.   Male white rats, weighing 190-210 gm., were used in these experiments.   They were starved overnight before being fed the experimental diets and at the end of the dietary period, lasting 7 days, the rats were again weighed.

Diets.   The diets fed had a varying protein as well as energy content.   The composition of these diets and the amounts fed are shown in Table 17.   The animals were randomised into groups, one receiving a protein-free diet while the other received a protein-containing diet.   These groups were sub-divided into a low energy group receiving no additional energy supplement and a high energy group receiving 7 gm.. glucose in the evening.   All food was removed 12 hours before sacrifice so that the animals were in the post-absorptive state.   In order to study animals in an absorptive state some of the rats in the protein-fed group were given 2 gm. casein, solubilised with 0.15 g. $NaHCO_3$, 2 hours before sacrifice.   That these animals were in an absorptive state was verified by the presence of food in the stomach.   By this procedure we were able to compare the effects of energy supplementation on protein-free and protein-containing diets in the post-

absorptive state, as well as its effect on protein-containing diets in the absorptive state.

<u>Incubation procedures.</u>   The animals were killed by exsanguination under ether anaesthesia and the livers removed. Pieces of liver were sliced in the McIlwain chopper (McIlwain <u>et al.</u>, 1953) and 500 mgm. of liver slices were transferred to sterile incubation flasks, which were gassed with 95 per cent O_2 - 5 per cent CO_2 just prior to incubation.   The incubation medium was that described by Sinex <u>et al.</u>(1952), and contained ^{14}C-2-glycine (1 µc. per flask).   The flasks were then incubated for 1 and 2 hours, after which time the reaction was stopped by adding 1.5 ml. 30 per cent (w/v) TCA to each.   A zero time control was prepared by adding the TCA to the flask before incubation.

Sterile precautions were taken throughout, all glassware was sterilised by heating in an oven (100^oC) overnight and the medium was filtered through a Seitz filter.

<u>Treatment of the protein precipitate.</u>   The flask contents after incubation were transferred to centrifuge tubes and centrifuged.   The precipitate was then washed twice with 10 per cent (w/v) TCA, twice heated in a waterbath with 5 per cent (w/v) TCA for 20 minutes at 70^oC. The precipitate was then washed with fat solvents in the following order: ethanol (twice); ethanol:chloroform (3:1); ethanol:ether (3:1) twice; ether (twice). The protein residue was then

The effect of changes in the specific activity of the free amino acid on the uptake of isotope into protein.
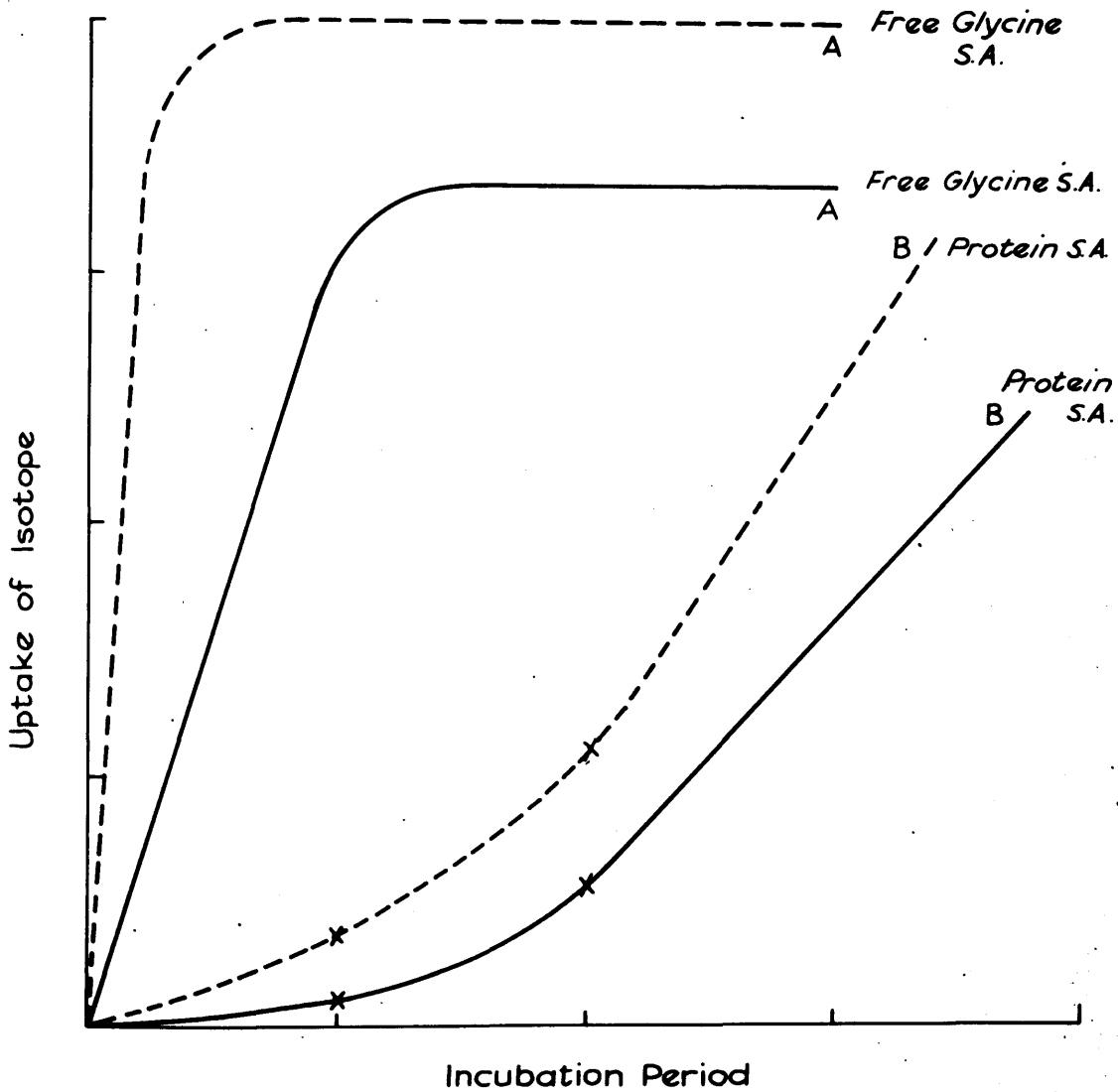
Fig. 10.

dried at 37°C overnight and the protein counted at infinite thickness using an end-window counter.

The radioactivity of the zero time controls in all the experiments was low (ca. 4 counts per minute) showing that this treatment effectively removes all the isotope adsorbed on to the surface of the protein molecule.

RESULTS AND DISCUSSION.

Several factors are involved in a comparison of isotope uptake by tissue slices. Fig. 10 illustrates the changes occurring with time in two parameters, viz. the radioactivity of the free amino acid pool within the slice (line A) and the radioactivity of the protein synthesised from this amino acid pool (line B).

Changes in the latter may be due to (a) real differences in the rate of uptake of amino acids from the pool, i.e. in protein synthesis, or (b) changes in the radioactivity of the free amino acid pool. If the labelling in the pool changes, it will, of course, lead to variations in labelling of the protein formed from the pool and these changes may be erroneously taken for differences in the rate of protein synthesis. The labelling of the free amino acid pool can be altered in two ways, illustrated by the dotted line in Fig. 10. Firstly, there may be more rapid penetration of the labelled amino acid from the medium into the slice, so that a plateau

value is more quickly attained in the pool of free amino acids within the slice (dotted line A compared with solid line A). Secondly, the unlabelled free amino acid concentration in the slice may vary, and thus cause different dilution effects after the plateau has been attained. In Fig. 10 we have assumed a smaller pool for the dotted line A and consequently a higher plateau for the free amino acid specific activity is reached.

The result of these two factors is shown by the uptake into protein (B), which begins to be measurably labelled sooner (because of the more rapid penetration), and exhibits a steeper slope thereafter (because of the lesser dilution of the label at its plateau value). Neither of these effects means that there is a greater rate of protein synthesis under the conditions represented by the dotted line B in Fig. 10.

Thus conclusions from in vitro uptake of labelled amino acids must be cautiously made. In particular, comparison of uptake by pieces of tissue from different animals incubated for a single period of time may exhibit differences in isotope incorporation, but the interpretation of the data is bound to be confused. For example, Rutman et al.(1955) incubated liver slices for a constant period in an investigation of the effects of fasting after various dietary regimes, and then observed differences in the activity of the protein-methionine. Their conclusions regarding the effect of diet on rates of protein synthesis

The effect of differences in the rate
of penetration of isotope into the cell
on the uptake of isotope into protein.
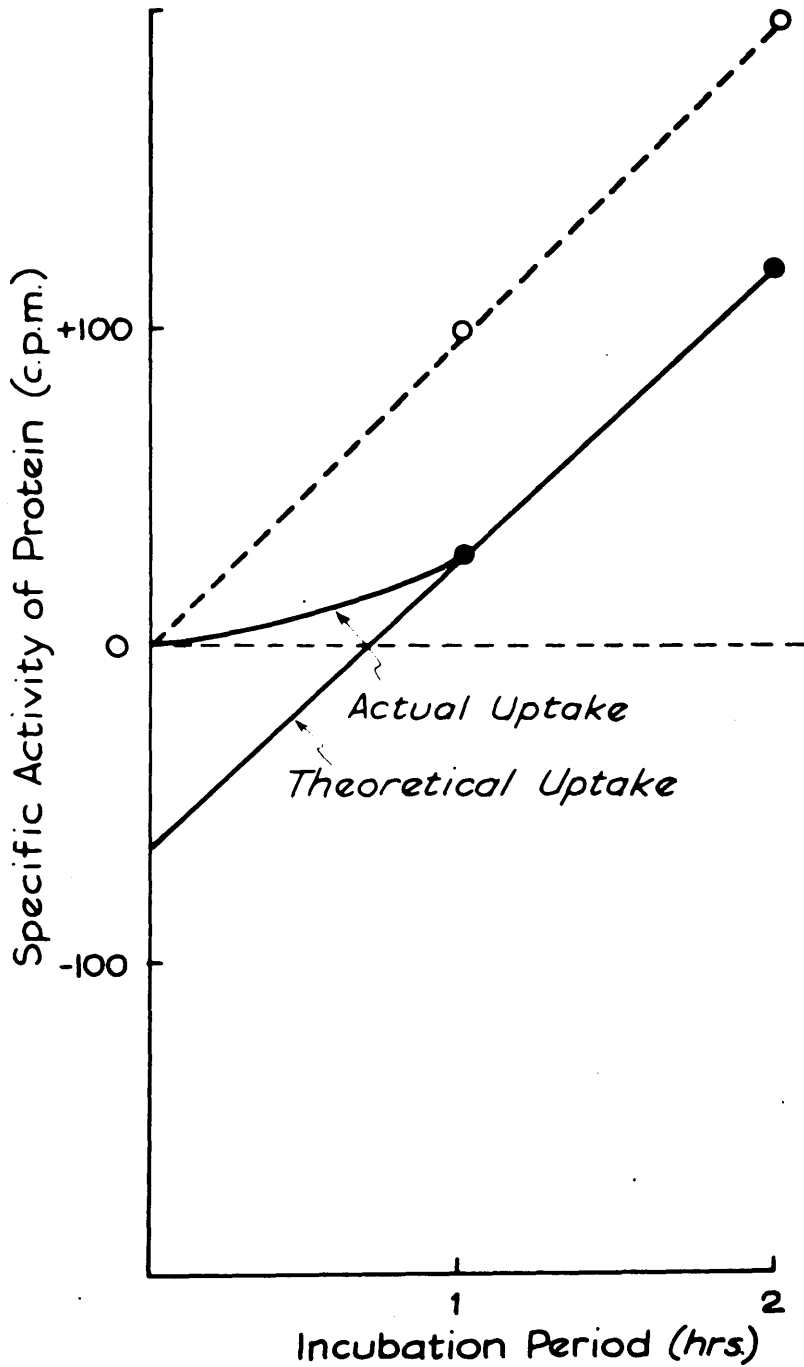
Fig. 11.

TABLE 18.

The effect of previous energy intake on the in vitro ^{14}C-glycine uptake by liver proteins.

Previous Diet	Energy Level					
	Low			High		
	1 hr.	2 hr.	Diff.	1 hr.	2 hr.	Diff.
Protein-free (post-absorptive	183	629	446	113	276	163
	404	698	294	458	1026	568
	134	546	412	890	1592	702
	200	402	202	258	458	200
Protein-containing (post-absorptive)	-	-	-	-	-	-
	311	833	422	656	1377	721
	618	1080	462	1190	1998	808
	558	930	372	410	714	304
Protein-containing (fed casein)	233	538	305	329	695	366
	280	696	416	1497	1787	290
	228	435	207	417	891	474
	312	538	226	272	656	384

The figures are the activities of the proteins (counts per minute at infinite thickness), each line represents a single experiment.

TABLE 19.

Summary of the effect of previous energy
in the _in vitro_ incorporation of ^{14}C-glycine into
liver protein.

Diet	Energy level	Period of Incubation		2 hr-1 hr.
		1 hr.	2 hr.	
Protein-free (post-absorptive	Low	230	569	+339
	High	430	838	+408
	Difference	+200	+269	+69
Protein-containing (post-absorptive)	Low	496	948	+452
	High	752	1396	+644
	Difference	+256	+448	+192
Protein-containing (absorptive)	Low	263	552	+289
	High	629	1007	+378
	Difference	+366	+455	+89

(The figures are the mean activities of the protein
in c.p.m. at infinite thickness.)

TABLE 20.

Statistical analysis of the incorporation data

of Table 18.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio F
Total	43	8,731,184	-	-
Main Effects				
Times (T)	1	1,777,645	1,777,645	42.15**
Energy (E)	1	1,169,689	1,169,689	27.74**
Protein (P)	2	994,897	487,438	11.56**
Interactions				
E x P	2	62,408	31,215	0.74
E x T	1	28,282	28,282	0.67
T x P	2	114,518	57,259	1.36
Experiments	8	3,487,184	435,898	
Error	26	1,096,517	42,174	

For n = 1 and 26. F = 4.22 at 5% level and 7.72 at the 1% level.

n = 2 and 26. F = 3.37 at 5% level and 5.53 at the 1% level.

** Highly significant at the 1% level.

This table shows that there is a highly significant correlation between (i) the time of incubation, (ii) the previous energy, and (iii) protein intake, and the in vitro uptake of ^{14}C-glycine into liver protein. Each of these effects is independent of the others.

are not valid.   Similarly, differences in the _in_ _vitro_
uptake of isotope between liver and tumour slices reported
by Campbell, Greengard and Jones (1957) may well be a
reflection of the differences in the rate of penetration of
isotope into the two types of cell and in the size of the
intracellular amino acid pool.

It is, however, possible to arrange conditions so
that some of the difficulties of interpretation are eliminated.
Considering Fig. 11, also hypothetical, it will be seen that the uptake of isotope
by protein resolves itself into an initial period of delay
followed by a linear rise.   Thus, if we take readings of
protein radioactivity at two time-intervals (e.g. 1 and 2
hours) we can obtain the slope of the line and also its
intercept at zero hours.   This latter figure gives us a
measure of the rate of uptake of isotope from the medium into
the free amino acid pool of the slice.   Thus in Experiment B,
Fig. 11, the radioactivity of the protein is higher at both
1 and 2 hours incubation, but the slope is identical with
that of A;   hence the difference in specific activity is
entirely due to an initial difference in the rate of
penetration.   This is revealed by the difference in intercept
at zero time.

Our own data were obtained by incubating slices of
liver with ^{14}C-glycine for 1 and 2 hours;   all the figures
obtained are recorded in Table 18, and are summarized in
Table 19;   Table 20 represents a statistical analysis of

them. The uptake into liver protein is significantly affected by dietary protein level; this is seen to be due to a higher incorporation in the group subsisting at a normal protein level and killed in the post-absorptive state. Similar animals fed protein just prior to sacrifice show a lower level of uptake, no doubt due to dilution of isotope by a larger intracellular pool of free glycine. Energy level in the preceding diet also had a highly significant effect on isotope uptake. In every instance, whether incubated for 1 or 2 hours or whether fed on protein or none (Table 19), the average uptake by/the high energy group is greater than that for the low energy group. When we consider the difference produced by energy level after 2 hour's incubation, it is seen not to be significantly greater than that at 1 hour of incubation. This means that the entire effect of energy level on the radioactivity of the protein occurs at the start of the incubation and is therefore likely to represent some effect of energy level on the rate of penetration of the labelled amino acid into the slice. Since statistical analysis shows no significant interaction effects (Table 20), this action of previous energy intake takes place irrespective of the protein content of the diet.

To summarize, these experiments have confirmed the conclusions drawn from the data given in the preceding Section, where ^{35}S-methionine was used as the labelled amino acid, namely that previous energy intake affects the uptake

of isotopically labelled amino acids even when 18 hours
have elapsed since the last meal was consumed. Addition
of energy to a protein-containing diet in the post-absorptive
state seems to increase the slope of the line (+192 as
against +92) but this is not significant on the basis of
the present experiments and the number of replications
needed to test it's possibility is prohibitive. Moreover,
because of the absence of any information about the
dilution of the label within the amino acid pool of the cell,
the present data do not offer any hope of identifying real
differences in rate of protein synthesis due to energy
level in the diet. We therefore turned to an <u>in vivo</u>
isotope approach, which is described in the next section.

SECTION 3.

THE INFLUENCE OF PREVIOUS ENERGY
INTAKE ON THE IN VIVO INCORPORATION OF ^{14}C-
GLYCINE INTO RAT LIVER PROTEINS.

INTRODUCTION.

In view of the difficulties inherent in the in vitro study of differences in rates of protein synthesis, we next examined the effect of previous energy intake on the in vivo uptake of ^{14}C-glycine by liver proteins. The system involves determination of the amount of radioactivity in the glycine of the free amino acid pool and in the glycine of the mixed liver proteins. These data, combined with an estimate of the protein content of the liver, allow certain deductions to be made regarding rates of protein synthesis under various nutritional conditions.

Rats were fed on diets containing adequate amounts of protein or devoid of protein, and within each group some were given a low energy diet and others a high energy diet. These animals were injected with ^{14}C-glycine in the post-absorptive state. In addition, animals at each energy level were studied while absorbing the products of a meal of protein. In order to obtain a representative picture, rats were killed at various time-intervals after isotope injection.

EXPERIMENTAL.

Animals and Diets. Male albino rats weighing 185-215 gm. were used in these experiments. They were starved overnight and placed on the various dietary regimes, enumerated in

Section 2, for 7 days. Some of the rats were fed casein one hour prior to injection of the isotope.

<u>Administration of isotope and excision of liver.</u> The rats were injected at 9 a.m. with ^{14}C-2-glycine (10 μc./animal) intramuscularly. They were killed at 3 and 6 hours thereafter by exsanguination under ether anaesthesia. That the animals which had been fed casein were in an absorptive state, was verified by the presence of food in the stomach.

The liver was removed, weighed and homogenised in a Folley blendor with 5 volumes ice-cold 10 per cent (w/v) TCA for 3 minutes. After centrifugation, the filtered supernatant was set aside, while the precipitate was washed twice with 5 ml. ice-cold 10 per cent (w/v) TCA. The combined washings and supernatants were then stored for the determination of free glycine specific activity. The precipitate was then treated with lipid solvents as described in Section 2. The protein residue was then used for determinations of (a) protein N, (b) ribonucleic acid phosphorus (RNAP), (c) deoxyribonucleic acid phosphorus (DNAP), and (d) specific activity of protein glycine.

<u>Analyses of protein N, RNAP, DNAP.</u> A known weight of the dried powder was digested with N NaOH at 37°C for 15 hours and sampled for total N by micro-Kjeldahl estimation. A portion was neutralised with 2.5 N HCl and the DNA and protein precipitated by the addition of ice-cold 30 per cent

TCA to a final concentration of 10 per cent. The
precipitate was centrifuged down at $0^{o}C$ and washed twice
with 1 ml. 5 per cent (w/v) TCA. The supernatant and
washings were pooled and the RNAP estimated by the method
of Allen (1940). The precipitate was dissolved in 1 ml. N
NaOH and the DNAP estimated by the same method. The protein
N was obtained by subtracting the N of RNA and DNA ((RNAP +
DNAP) x 1.69) from the total N of the alkaline digest.

Radioactivity measurement of the carbon compounds. Glycine
was isolated from liver protein and the free amino acid
pool, and the dinitrophenol (DNP) derivative formed using a
procedure based on that of Campbell and Work (1952b). In
this method, the material is reacted with an excess of 1-
fluoro-2:4-dinitrobenzene (FDNB), dissolved in a mixed
organic solvent and the reaction mixture chromatographed on a
buffered celite column. The isolated DNP-glycine is
subsequently purified on celite column developed with ether.
The radioactivity of this pure sample is then determined and
the amount present estimated colorimetrically.

An aliquot of the sample containing about 200 µg.
glycine was dissolved in 1.5 ml. water and solution made
alkaline by the addition of a knife-point of $NaHCO_3$ and
shaken with a 20-fold excess of a 10 per cent solution of
FDNB in methanol for 4 hours. At the end of this period the
reaction mixture was diluted with 5 ml. water, shaken with

20 ml. ether to remove excess FDNB. This ether solution was then shaken with 5 ml. water three times, the washings being added to the original aqueous layer. The latter was then acidified with 3 ml. 2.5 N HCl and extracted 5 - 8 times with 20 ml. portions of ether. The ether extracts were then combined and evaporated to dryness with a current of cold air. Any moisture in the residue was removed by drying over P_2O_5.

The dry residue was dissolved in a mixture of chloroform:n-butanol (93:7) prepared by the method of Krol (1952). This solution was applied to a celite column, 1 cm. internal diameter, 15 cm. long, buffered at pH 5.3, packed in ether, and then washed with chloroform:n-butanol. The column was developed with chloroform:n-butanol, the DNP-glycine band collected and the organic solvents removed by evaporation in a current of air. The residue was dissolved in ether (0.5 ml.) and applied to a celite column prepared as above but using ether saturated with water as the developing solvent. The DNP-glycine band was collected and the ether removed. The dry residue was then dissolved in the minimum of ether and transferred to a planchet on which the sample was counted using an end-window counter. The DNP-glycine was then dissolved from the planchet with chloroform:n-butanol (20 ml.) and then extracted from the latter with 10 ml. 1 per cent $NaHCO_3$. This solution was then read colorimetrically with a "Spekker" absorptionometer,

TABLE 21.

The incorporation of ^{14}C-2-glycine into the free amino acid pool and protein glycine of rat liver after feeding the various diets.

Glycine isolated from	Time of sacrifice after injection (hr.)	Protein-free diet (post-absorptive)		Protein-containing diet (post-absorptive)		Protein-containing diet (fed protein)	
		Low energy	High energy	Low energy	High energy	Low energy	High energy
Free amino acid pool	3	1127	1046	978	1096	588	578
	6	552	738	617	928	265	247
Liver Protein	3	169	221	146	147	99	120
	6	149	254	131	198	83	79
Protein-N concentration	3	91.5	87.7	118.2	106.3	104.0	117.9
	6	100.8	88.4	121.0	122.0	118.7	134.6

The figures for the specific activity of the ^{14}C-glycine are the mean of two replications, and are expressed in counts per minute per 100 µg. of glycine. The protein-N concentrations are also the mean of two replications and are expressed as mg. protein N per mg. DNAP.

using Ilford 601 violet filters. The specific activity of the glycine was expressed as counts per minute per 100 μg. glycine.

Specific activity of free glycine in liver.   The TCA extract of liver was taken and washed free from TCA with ether, as judged by a rise in pH of the aqueous layer to between 4-5. The latter was then evaporated to dryness and the residue dissolved in a known volume of water of which an aliquot was removed, reacted with FDNB and the DNP-glycine isolated.

Specific activity of protein glycine.   About 100 mg. of the lipid extracted powder was refluxed with 20 ml. of 6 N HCl for 14 hours at 130°C. The HCl was subsequently removed by vacuum distillation and the residue dissolved in 10 ml. water. 1.5 ml. of this solution was then treated with FDNB and the DNP-glycine isolated.

RESULTS AND DISCUSSION.

Two independent series of experiments were performed in which the rats on diets containing different amounts of protein and energy were injected with radioactive glycine. The basic information is given in Table 21.   In this are recorded the specific activity of the free glycine and of the liver protein glycine, and also the amounts of protein per liver.   This latter figure is given as mg. protein per mg. deoxyribonucleic acid phosphorus, in this respect

TABLE 22.

The relative specific activity of liver protein glycine from rats receiving various diets.

Time of sacrifice after injection	Protein-free diet (post-absorptive)		Protein-containing diet (post-absorptive)		Protein-containing diet (fed protein)	
	Low energy	High energy	Low energy	High energy	Low energy	High energy
3	15.0	21.2	14.7	12.9	16.6	22.7
6	27.0	35.8	21.1	22.4	31.3	33.3

These figures, which are the mean of two replications, are obtained from the data of Table 21, by dividing the specific activity of the protein glycine by the specific activity of the free glycine and multiplying by 100.

TABLE 23.

The total relative activities of liver protein glycine of rats receiving various diets.

Time of sacrifice after injection	Protein-free diet (post-absorptive)		Protein-containing diet (post-absorptive)		Protein-containing diet (fed protein)	
	Low energy	High energy	Low energy	High energy	Low energy	High energy
3	13.8	18.6	17.4	13.4	17.2	26.0
6	27.2	31.9	26.0	27.6	37.0	44.9
Increments (6–3 hr.)	+13.4	+13.3	+8.6	+14.2	+19.8	+18.9

These figures are obtained by multiplying the relative specific activities given in Table 21 by the amount of protein N given in Table 22, which was expressed per mg. DNAP.

deoxyribonucleic acid being used as a reference standard proportional to the number of liver cells (Davidson and Leslie, 1950).

In confirmation of previous studies (Munro and Naismith, 1953), the amount of protein per liver does not increase when the energy intake of a protein-free diet rises, but, it does increase when the energy level is raised on a protein-containing diet.

As regards uptake of isotope into liver protein, Solomon and Tarver (1952) have already noted that the incorporation of labelled methionine into liver protein is higher in rats fed on a protein-free diet. Our data confirm this picture for ^{14}C-2-glycine (Table 21). However, the explanation for this is apparent when we examine the activities of the free glycine fraction of these livers; the protein-fed animals have a much lower specific activity. The activity of protein-glycine should therefore be viewed against the background of the activity in the precursor pool, i.e., expressed as the relative specific activity as given in Table 22. Finally, the total rate of synthesis is arrived at when we take the amount of protein in the liver into account. The total amount of glycine incorporated per liver can be computed by multiplying the relative specific activity by the amount of protein, as shown in Table 23. The resulting "total relative activity" represents the absolute rate of glycine incorporation per liver, in relation to the precursor

pool of free glycine. The appropriate figure to examine is the rate of increase of the total relative activity between 3 and 6 hours after injection. From a consideration of this figure for the different groups shown in Table 23, it is evident that the actual rate of synthesis of protein is highest in the two groups which had just been fed protein prior to death (+ 19.8 and + 18.9). On comparing the low and high energy groups within the same diet, there is a decidedly higher incorporation at the higher energy level, when the rats had been fasted after a protein-containing diet (+ 8.6 and + 14.2 respectively). On the other hand, the rats on the protein-free diet show no such response to increments in energy level (+ 13.4 at the low level and + 13.3 at the high level).

In summary, we can say that increasing the energy intake on a protein-free diet has no effect on the incorporation of isotope into the liver protein. In contrast, increments in energy level to a diet containing protein results in a greater incorporation of the labelled amino acid in the post-absorptive state, whereas the feeding of protein just prior to death obliterates this difference in incorporation.

These findings imply that <u>the animal's previous energy intake influences amino acid incorporation only when the rat is in a post-absorptive state and does not affect the fate of incoming amino acids at the time of a meal.</u>

SECTION 4

THE INFLUENCE OF PREVIOUS

ENERGY INTAKE ON THE PATTERN OF HIGH-ENERGY

COMPOUNDS IN THE LIVER.

INTRODUCTION.

Although phosphate esters, particularly adenosine polynucleotides, have excited the interest of biochemists for the last thirty years, the estimation of such compounds in pre-war years was extremely crude and relied on the determination of the amount of phosphorus hydrolysed by N acid for 10 minutes – designated the "acid-labile phosphorus".

Acid-labile phosphorus estimation. This fraction is composed of the terminal and sub-terminal phosphate groups of adenosine triphosphate (ATP) and adenosine diphosphate (ADP). When dealing with biological material, which may contain a mixture of ATP and ADP, the estimation of the "acid-labile phosphorus" fraction gives the quantitative amount of energy-rich compounds but gives no information on how these phosphates are partitioned between ATP and ADP. LePage (1945), however, has attempted to estimate ATP and ADP of this fraction separately by hydrolysis curve data and the nitrogen:pentose:labile phosphorus ratio. This method is susceptible to quite large errors due to the possible contamination with other nitrogen, pentose or other labile phosphorus compounds which would affect the ratio.

Nevertheless, the "acid-labile phosphorus" has been examined under a variety of conditions. Thus Rapoport, Leva and Guest (1943) studied the effect of fasting on the "acid-

labile phosphorus" in rat liver; Wagtendonk (1944) and
Wagtendonk and Lamfrom (1945) found that feeding a diet
deficient in "anti-stiffness" factor to guinea pigs decreased
this fraction in muscle, liver and kidneys, while Proger,
Decaneas and Schmidt (1945) studied the effect of anoxia and
cytochrome c injection on the "acid-labile phosphorus" in
rat kidney.

While this method is non-specific, it probably served
its purpose but with the realisation of the importance of
Lipmann's concept of energy-rich bonds (1941) and the
demonstration of appreciable quantities of uridine nucleo-
tides in liver (Dutton and Storey, 1953; Schmitz, Hurlbert
and Potter, 1954; Smith and Mills, 1954), biochemists were
faced with the problems of finding new and more specific
methods for the separation and estimation of the energy-rich
compounds of ATP and ADP.

The phosphate esters are extracted from tissues with
acid, usually trichloracetic acid, and various methods have
been devised for the fractionation of this extract into its
various components. Briefly the methods for the separation
of ATP and ADP from the extract can be divided into (a) those
which depend on the physico-chemical properties of these
compounds (e,g. insoluble salt formation); (b) chromatography;
(c) ionophoresis, and (d) methods which make use of specific
enzymes.

(a). Physico-chemical methods.
Insoluble salt formation. Various attempts to isolate ATP

by precipitation from the acid extract of tissues have been made in the past. The earlier attempts of Fiske and Subbarow (1929), Lohmann (1931, 1932) and Barrensheen and Filz (1932) to isolate ATP from brain led to poor yields or to impure products. Fiske and Subbarow used mercuric acetate as the precipitant, followed by purification by a further precipitation with calcium chloride and ethanol, while Barrensheen et al. separated the ATP as the silver salt. Eggleton and Eggleton (1929), in a study of muscle phosphates, fractionated the muscle extract with solid baryta at pH 9.0.

Stone (1940, 1943) found that this method gave variable results and so he elaborated a scheme involving fractionation with calcium hydroxide and ethanol, which precipitated the nucleotides, and then the ethanol-insoluble fraction was treated with uranyl acetate. This method has some serious disadvantages; in order to precipitate all the "ATP" excess calcium hydroxide has to be added, which hydrolyses ATP as evidenced by the formation of a fraction labelled "decomposed ATP" by Stone (1943). Another serious disadvantage is that calcium hydroxide does not precipitate all the ATP. Stone has assumed the calcium hydroxide precipitation to be adequate because no loss occurred on dissolving and reprecipitating. However, Kerr (1942), in an attempt to determine to what extent the "acid-labile phosphorus" represented the pyrophosphate fraction of ATP, compared Stone's method with a precipitation method involving

mercuric acetate. He found that the amount of "acid-hydrolysable phosphorus" precipitated under Stone's conditions was less than that found on precipitation with mercuric acetate. In view of recent work on uridine compounds, this evidence of more ATP and ADP may be less compelling than it once seemed. Kerr has also found that, if the filtrate from the calcium hydroxide precipitate is made more acid with acetic acid and treated with mercuric acetate, then an additional amount of acid-labile phosphorus is precipitated. This is probably a mixture of ATP, ADP and AMP (N:labile phosphorus ratio of 5.07-1.2). Furthermore, it has been found that calcium hydroxide precipitates only 80 per cent of the ATP from a pure solution under Stones conditions. These then explain Stone's observation that, in the calcium hydroxide filtrate, which he terms the hexose phosphate fraction, 28 to 48 per cent of the phosphorus is hydrolysed in 7 minutes in N HCl at $100^{o}C$. In a similar filtrate after mercuric acetate treatment, it was found that only 8 per cent of the organic phosphorus was hydrolysed in this time.

Kerr(1935, 1940, 1942) advanced a method which has enjoyed some popularity. It involves precipitation of the nucleotides from the tissue extracts by mercuric acetate at pH 5.1. The nucleotide precipitate is suspended in water and hydrogen sulphide is passed into it, thus precipitating the mercury and leaving the nucleotide in solution, which

can then be estimated. A typical application of this method is the work of Venkataraman, Venkataraman, Schulman and Greenberg (1950), who have used this fractionation procedure in an investigation of the effect of thyroxine on ATP levels in liver. After fractionation of the nucleotides, ATP was estimated using myosin.

LePage (1945) published a fractionation method which involves precipitation of the nucleotides with barium acetate. This method has the advantage that small quantities of materials can be analysed, and has been used with success in the investigation of phosphorylated derivatives in muscle, heart, brain, kidney, liver, yeast, bacteria and some plants . The tissue extract is fractionated by adding 0.25 ml. 25 per cent barium acetate per mg. phosphorus at pH 8.2.   In this method, maintenance of a constant pH is vital, variations of $\pm$ 0.5 pH units causes contamination of the nucleotide fraction, normally consisting of ATP and ADP.   These are then estimated by calculating the N:pentose:labile phosphorus ratio. This estimation is based on the assumption that the only nucleotides present are those of adenosine but since uridine compounds are also present in most tissues, and since they too release their labile phosphate in 7 minutes, the nitrogen:pentose:labile phosphorus ratio is not an index of adenosine nucleotides but an estimate of the adenosine + uridine compounds.

In 1948 LePage extended his original method to

facilitate a separation of ATP and ADP. The nucleotides were first precipitated as a mixture with barium acetate, giving a barium-insoluble fraction. This is then dissolved in acid and the nucleotide reprecipitated as the mercury salts, thus removing the inorganic phosphate. The mercury was removed by passing hydrogen sulphide into a suspension of the mercury salts and the resulting filtrate after aeration was again treated with barium hydroxide, only the ATP is precipitated because of the enhanced solubility of ADP in the absence of inorganic phosphate.

Another method is that of Kaplan and Greenberg (1944a), which differentiates the labile and stable phosphate groups of ATP. The nucleotides were first precipitated with barium hydroxide at pH 8.2, then dissolved in N acid. An aliquot was hydrolysed for 7 minutes - "acid-labile phosphorus", thus giving the two labile groups of ATP (and the terminal group of ADP). Another aliquot was similarly hydrolysed and then treated with barium hydroxide, thus giving the non-labile phosphate of ATP (and ADP).

Although these methods are usually adequate as a primary step in analytical procedures, it is not sufficiently selective for radioactive work in which contamination produces relatively more serious errors. Therefore Sacks (1949) designed a method which, although based primarily on the differences in solubilities of the alkaline earth salts, and therefore similar to the methods of LePage (1945) and

Kaplan and Greenberg (1944a), achieves a better separation by using calcium salts as well as barium as precipitants. Sacks (1949) has shown that LePage's procedures do not precipitate adequately the ATP-ADP, by recovering appreciable amounts of ATP and ADP on the addition of calcium trichloracetate to the barium filtrate.

(b). Chromatographic methods.   In view of the wide applicability of both paper and column chromatography to the resolution of the components of biochemical systems, it is not surprising that these techniques have been applied to the separation of nucleotides. Cohn and Carter (1951) have made possible the separation of dibasic acids such as ATP, ADP and phosphoglyceric acid on ion exchange resins by varying the pH or by varying the ionic strength of the eluant. Monophosphate derivatives can be separated by the addition of borate. Volkin, Khym and Cohn (1953), on the other hand, have used ammonium chloride solutions as eluants.

The ion exchange resin used by Cohn and Carter was Dowex-1, on which the nucleotides were absorbed at a pH of 8, while the anion concentration was kept lower than 0.01 N. and eluted thus:-

(i)   adenosine - 0.01 M NH_4Cl in 0.1 M NH_4OH

(ii)   adenine   - 0.01 M NH_4Cl in water

(iii) AMP   - 0.003 M HCl

(iv)   ADP + inorganic orthophosphate - 0.02 M NaCl in 0.01 M HCl

(v)   ATP   - 0.2 M NaCl in 0.01 M HCl.

It can be seen that the ADP fraction is contaminated with orthophosphate and there is a contaminating phosphate compound, in concentrations of 2 to 4 per cent and having labile phosphate groups, in the ATP fraction. Koshland, Budenstein and Kowalsky (1954) have modified this method in so far as they elute ADP with 0.01 M HCl and ATP with 0.1 M HCl.

Deutsch and Nilsson (1953) have used Dowex-2, in the chloride form, as an exchange resin in attempts to separate the adenosine nucleotides from the inosine ones. AMP is eluted with 0.003 M HCl, which is followed closely by inorganic orthophosphate. The inosine monophosphate (IMP) which trails ADP is eluted with 0.02 M NaCl in 0.01 M HCl, the pyrophosphate and inosine diphosphate (IDP) were eluted with 0.05 M NaCl in 0.01 M HCl and the ATP with 0.2 M NaCl in 0.01 M HCl. Obviously these fractions are not homogeneous and a certain amount of cross contamination obtains; between AMP and orthophosphate it is of the order of 2 to 3 per cent; between IMP and ADP 4 per cent, and between IDP and ATP 2.9 per cent. Bergkvist and Deutsch (1954), in an attempt to separate the mono-, di- and triphosphates of adenosine, guanosine, inosine and uridine, used a Dowex-1-formate exchange column. The nucleotides were eluted with formic acid/formate solutions containing increasing amounts of formate. Once more the separations were incomplete; ATP was contaminated with ITP, UMP with orthophosphate. A similar method has been

reported by Schmitz (1954), the main difference being the
eluant, which contains increasing concentrations of formic
acid (0-4 N) followed by 4 N formic acid + 4 N ammonium
formate.

All the methods which have previously been discussed
suffer various shortcomings. The most common one is that
large volumes of eluant are required and therefore either
the nucleotides have to be present initially in relatively
large concentrations or some means of concentrating the
eluates has to be employed. Another disadvantage is the fact
that ion-exchange chromatography takes too long to run to be
of much use as a routine analytical tool where large batches
of samples have to be analysed. Moreover, it can be seen
from the previous discussion that often the fractions are
contaminated to a slight extent by other compounds which
might prove a more or less serious error. Furthermore,
hydrolysis of compounds such as ATP and ADP may occur in the
column. For example, if hexose phosphates, inorganic
phosphate, ATP and ADP are separated by the method of Volkin
et al. (1953) then, in the time taken for the hexose phosphates
and inorganic phosphates to be eluted from the column, the ATP
and ADP still remaining suffer a partial hydrolysis.

Paper chromatography offers some advantages over ion-
exchange chromatography in that smaller samples can be
analysed, the volumes of eluates are much reduced, little if
any decomposition of labile nucleotides occurs and it is much

less cumbersome.   The choice of solvent systems is
determined to a large extent by the separation required.
Thus Carter's method (1950), involving an isoamyl alcohol-
potassium dihydrogen/system separates ATP from AMP, adenosine
                    phosphate
and adenine but fails to separate ATP from ADP. However,
Cohn and Carter (1951) modified this system, replacing the
mono-potassium salt by the disodium one, thus separating ATP
and ADP.   But Guerin (1954) insists that this method is
unsatisfactory since the R_F values of the three adenosine
phosphates lie too close together.

Hanes and Isherwood (1949) were early pioneers in the
chromatography of phosphate esters. Although their method
is satisfactory for some purposes, it fails to separate ADP
completely from inorganic phosphate, and attempts to remove
this contaminant by precipitation as the magnesium ammonium
salt led to considerable and variable losses of the nucleo-
tide. The adenosine phosphates are separated from inorganic
phosphate by their formic acid:isopropyl ether solvent and
the isopropanol:ammonia solvent separates ATP from ADP, but
this has not been obtained by Guerin (1954).

A major consideration in the choice of solvent system
is the low solubility of nucleotides in organic solvents.
Two methods have been devised to overcome this: (1) by the
use of a suitable buffer, and (2) by the use of miscible
solvent system containing sufficient water to keep the nucleo-
tides in solution. Cohn and Carter's method (1951), using

disodium phosphate:isoamyl alcohol is an example of the
buffers used and has been modified by the addition of long
chain amines to the alcohol phase (Turba and Engel, 1951).
Turba, Pelzer and Schuster (1954) have used a citrate buffer
to effect a good separation of the various compounds. The
best miscible solvent systems incorporate isobutyric acid
and various solvent systems have been described.   Turba,
et al. (1954) advocate an isobutyric acid:acetic acid:water
system while Krebs and Hems (1953) use an isobutyric acid:
ammonia:water:versene system.   The latter system is virtually
a modification of the Eggleston and Hems method (1952) which
involves a two solvent-one dimensional technique.   The first
solvent, isopropyl ether:formic acid used in an ascending
direction, separates the hexose phosphates from the
adenosine compounds which remain at the origin. The second
solvent is n-propanol:ammonia:water:versene which separate
the adenosine nucleotides.   But the nucleotide spots are
overlapped by the spots of glucose-1-phosphate, glucose-6-
phosphate, inorganic pyrophosphate and fructose 1:6 di-
phosphate. A serious disadvantage is that pyrophosphate is
confluent with ADP, nevertheless it can be overcome by
separating pyrophosphate in the acid solvent and making due
correction for it when estimating ADP.   But from the figures
published in this paper, it can be seen that fructose-1:6-
diphosphate has the same R_F value as pyrophosphate in the
acid solvent. The application of this method, therefore,

must be limited to analyses of commercial preparations of adenosine nucleotides.

In order to apply this method to tissue extracts, some device must be employed to remove the unwanted phosphorus compounds from the nucleotides. This has been done very simply by Fleckenstein and Janke (1953), who have applied the mixtures to be analysed along a line a short distance from the centre of the chromatography paper. The paper is then folded in the shape of a "V" and placed in the first solvent of Eggleston and Hems (1952). As this solvent ascends it carries with it the contaminating sugar phosphates, while leaving the nucleotides at the origin. After a suitable time, the chromatogram is dried and cut just behind the starting line and the nucleotides can then be resolved in the second solvent. Krebs and Hems (1953) have used this "V" chromatogram method in a modified version of the method of Eggleston and Hems (1952). In this case the second solvent is isobutyric acid:ammonia:water:versene.

In an investigation of the stimulatory effect of ATP on brain adenylic deaminase, Weil-Malherbe and Green (1955) wished to separate the nucleotides at the end of the reaction and found the only suitable means was a combination of two methods. The ethanol:ammonium acetate solvent of Paladini and Leloir (1953) separates ATP from AMP but not AMP and IMP; while the isobutyric acid:ammonia:water: versene solvent of Krebs and Hems (1953) separates IMP and

AMP but not ATP and IMP.   Therefore Weil-Malherbe et al.
ran the chromatogram firstly in the ethanol:ammonium acetate
solvent, the paper was cut between the ATP spot and the
confluent AMP/IMP spots.   The distal portion of the paper
was then run in the second solvent,thus resolving AMP and IMP.

Caldwell (1952) described a two-dimensional method
involving n-propanol:ammonia:water and tert-butanol:picric
acid:water as solvents.   This procedure splits the phosphates
into five fractions, namely, ATP, ADP, orthophosphate, hexose
diphosphate, creatine phosphate + hexose monophosphate + AMP.
If the values of orthophosphate and creatine are determined
by the usual methods, then the individual values for creatine
phosphate, hexose monophosphate and adenylic acid can be
calculated.   This method is rather cumbersome for routine
investigations and Guerin (1954) has found that this method
often fails to separate ATP from ADP.   Caldwell (1955) has
elaborated a system for the separation of phosphorylated
intermediates of glycolysis involving n-propanol:2 M acetate
buffer at pH 4, overcoming some of the disadvantages of his
previous method in that creatine phosphate is resolved from
AMP and fructose 1:6 diphosphate.

In a similar investigation of glycolytic intermediates
in higher plants, Bandurski and Axelrod (1951) used solvent
systems involving methanol:formic acid:water and methanol:
ammonium hydroxide:water, after separating the nucleotides by
LePage's method (1945).   Glycolytic intermediates have been

separated by Omura and Fukushi (1953) using such diverse
solvent systems as iso-propanol:iso-butanol:ammonium
hydroxide; iso-propanol-trichloracetic acid:ammonium
hydroxide:water:butyric acid:ammonium hydroxide:acetone:
monochloracetic acid; tert-butanol:formic acid:water;
n-propanol:ammonium hydroxide:water. Guerin (1954) has
described a method using n-propanol:n-butanol:ammonia:water
by which it is possible to estimate nucleotides in
concentrations of 10^{-9}-10^{-8} M. This method gives a clean
separation of adenosine and inosine phosphates, ortho- and
pyrophosphate and phosphoenol pyruvate, but it has not yet
been applied to tissue extracts. A similar method has been
described by Cerletti and Siliprandi (1955) involving
propanol:water:trichloracetic acid.

Probably the neatest and best chromatographic method
is that described by Bergkvist and Deutsch (1955), which
resolves all the nucleotides with a two-solvent system,
n-propanol:ammonia:water and saturated ammonium sulphate:
water:isopropanol.

To summarize, the most convenient method of separating
phosphates is probably by two-dimensional chromatography,
but requires a reasonable degree of dissimilarity, which can
be provided by differences in pH, in the behaviour of the
two solvent systems. The importance of this can be seen on
comparing the fractionation obtained by Bandurski and Axelrod
(1951) using a combination of basic and acid solvents with

that by Cohen and Oosterbaan (1953) using two acid solvents, which has much less efficient resolving power.

(c). Ionophoretic methods.   Ionophoresis at pH 3.2 was found by Wade and Morgan (1954, 1955) to produce a separation sufficiently different from that produced with an acid solvent system for it to be a useful adjunct to chromatography with the latter system.   In this case an aqueous solution of n-butyric acid and NaOH provides a satisfactory liquid phase for ionophoresis.   In deciding the water content of this system it was necessary to compromise between the low R_F values and compact spots produced by systems with low water content and the high R_F values but diffuse spots produced by systems with a higher water content.   This must also be a consideration in deciding solvent systems for paper chromatography as well as the use of washed papers, thereby removing metallic ion impurities.

Ionophoretic techniques have been described by Turba and Engel (1951), using low voltages (10 v/cm.);   by Turba et al. (1954), using higher voltages (50-100 v/cm.), which gives a sharper separation in a shorter time;   by Boszormenyi-Nagi and Kueber (1955) using citrate buffer (pH 3.9) and by Bock and Alberty (1951) using the conventional Tiselius apparatus.   However, ionophoresis has never achieved such wide application as the other methods described.

(d). Enzyme methods.   While adequate separations of nucleo-

tides from associated metabolites, and even from other
nucleotides can be achieved by the previously described
methods of insoluble salt formation, chromatography and
ionophoresis, the reliable estimation of the individual
nucleotides must depend on enzymic determinations which only
became popular some ten years ago.

The finding of Schmidt and Engel in 1932 that the
purine content of tissues could be estimated by using
specific deaminases and measuring the amount of ammonia
released from the purines was developed in 1934 by Parnas,
Ostern and Mann to estimate ATP by using a phosphatase to
convert ATP to AMP, which was then deaminated. These
observations were forgotten until Kalckar (1944a) described
the determination of AMP by the highly sensitive and specific
adenylic acid deaminase and measuring the drop in extinction
at 265 mμ spectrophotometrically. This was further elaborated
by Kalckar in 1945 to measure adenyl pyrophosphates by
coupling the deaminase with a potato apyrase.

In 1947 Kalckar published a series of papers describing
procedures for estimating hydroxy purines and adenine compounds.
Nucleotides are characterised by intense ultraviolet absorption
and because many different purine derivatives have the same
absorption spectra, e.g., adenine, adenosine, adenylic acid
and adenyl pyrophosphates, direct measurement of the absorption
completely lacks specificity but if changes in the absorption
as a result of specific enzymes are studied then the method,

differential spectrophotometry, is a highly sensitive and specific analytical tool. The method can be appreciated if we consider the application to the estimation of the various adenosine compounds. When AMP is deaminated, IMP is formed and the absorption maximum shifts to 248 mμ; at 265 mμ, the maximum of AMP, the extinction of IMP is only 40 per cent of that of AMP. Therefore, if we follow the deamination of AMP, represented by the drop in extinction at 265 mμ (Δ 265), we have a measure of the AMP concentration, since the drop is directly proportional to the AMP concentration. Similarly, if we wish to estimate ADP, then by using myokinase, which dismutates ADP thus –

$$2 \text{ ADP} \rightleftharpoons \text{ATP} + \text{AMP}$$
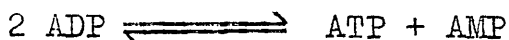
and, adding deaminase, we get a further drop in extinction, which is proportional to half the concentration of ADP. ATP can also be estimated by this method, by adding apyrase which splits ATP to AMP. By adding these enzymes in the order – deaminase, myokinase, apyrase – to a mixture of nucleotides or a tissue extract, we can estimate each separately by the successive drops in extinction at 265 mμ. Some of the ATP estimated will have its origin in the dismutated ADP but this will be equivalent to half the ADP concentration and so due correction can be made.

This elegant method has been criticised by Munch-Peterson (1955) as being too cumbersome to be of use as a routine analytical procedure because of the time taken for

the individual reactions to go to completion. However, the method has been employed either _per se_ or in a modified form. Albaum and Lipshitz (1950) have extended Kalckar's method by measuring the rate of change of extinction rather than the magnitude of the drop and by using hexokinase instead of apyrase to measure the ATP concentration. This method, although quicker, necessitates measuring the rate of change in standard solutions of known purity, and suffers from the disadvantage that some means must be devised for measuring the purity of these standard solutions.

Various other enzymic methods have been described, whose essential feature is that one of the nucleotides, usually ATP, participates in a reaction whose products can be measured. Thus ATP can be measured manometrically in the presence of bicarbonate (Colowick and Kalckar, 1943) or by combining it with glucose in the presence of hexokinase, or the reaction may be coupled thus:-

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} + \text{TPN} \xrightarrow{\text{Zwischenferment}} \text{6-phosphogluconic acid} + \text{TPNH}_2$$
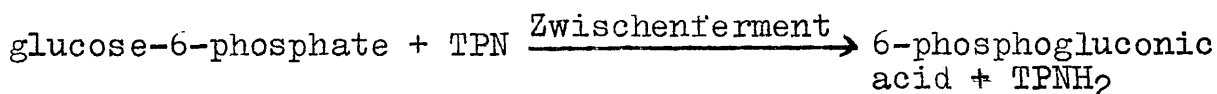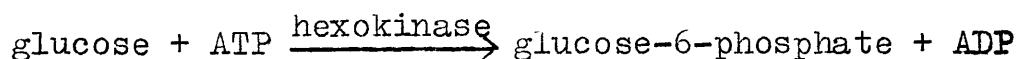
The reduced triphosphopyridine nucleotide ($TPNH_2$) is measured spectrophotometrically (Kornberg, 1950). The hexokinase system is linked with creatine phosphokinase in the method of Chappell and Perry (1954). The reactions involved are -

creatine phosphate + ADP $\xrightarrow{\text{Phosphokinase}}$ creatine + ATP

ATP + glucose $\xrightarrow{\text{hexokinase}}$ glucose-6-phosphate + ADP
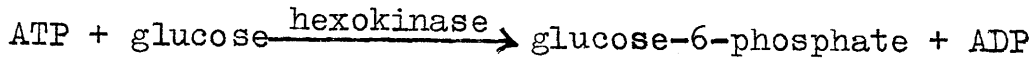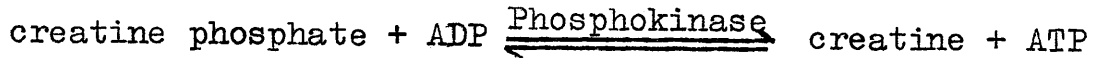
In this case the creatine liberated is determined. The method is sensitive enough to measure 0.5 - 5.0 micrograms of ATP and ADP.

Holloway (1954) has used the conversion of 3:phosphoglyceric acid to the diphospho-compound as a means of estimating ATP. In the reaction

ATP + 3 phosphoglyceric acid $\xrightarrow{\text{phosphoglycerate kinase}}$ ADP + 1:3 diphosphoglyceric acid
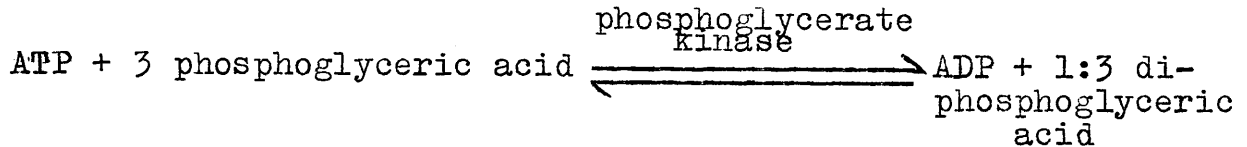
the equilibrium is unfavourable to the formation of 1:3 diphosphoglyceric acid but by trapping this compound with hydroxylamine, the reaction can be driven to completion. The hydroxylamine is combined with ferric chloride and the coloured complex measured colorimetrically. This method has the advantage that only one enzyme is involved and can measure concentrations of ATP from 0.2 - 2.0 μM.

Slater (1953) published a method which depends on the conversion of phosphorylated sugars into dihydroxy acetone phosphate which then reacts, in the presence of glycerol phosphate dehydrogenase, with reduced diphosphopyridine nucleotide (DPNH$_2$), whose disappearance is measured spectrophotometrically at 340 mμ. It consists of three procedures, namely

(A) hexose diphosphate + 2 DPNH$_2$ $\longrightarrow$ 2 glycerol phosphate + 2 DPN

(B) hexose monophosphate + $\sim$ P + 2 DPNH$_2$ $\longrightarrow$ 2 glycerophosphate + 2 DPN

(C) ATP + glucose $\longrightarrow$ glucose-6-phosphate + ADP

By these procedures it is possible to measure hexose mono-phosphate and hexose diphosphate as well as ATP.

A more exotic method, using firefly luminescence, has been described by Strehler and Totter (1952) and can be used to measure ATP, ADP and "any component in a system which can be made to influence the level of ATP". ADP is measured by the simple expedient of adding myokinase to the firefly extract. This method, in a form modified by Born and Bulbring (1955), has been used by Born (1956) to measure the ATP content of blood platelets and by Blaschko, Born, D'Ioro and Eade (1956) in an attempt to correlate ATP levels with pressor amine concentration in adrenal medullary granules.

In the past, it has been a common practice to determine the phosphate liberated from nucleotides by specific enzymes. Rapoport and Nelson (1945), Bailey (1949) and Rowles and Stocken (1950) have used the direct measurement of the phosphate liberated (the terminal phosphate of ATP) by myosin as an index of the ATP concentration. But because of the slowness of hydrolysis (Bailey, 1949; Steyn-Pavre and Gerritsen, 1952), and the uncertainty of removing myokinase activity from the myosin, the method, until recently, had not been widely accepted. By five precipitations Bailey

(1949) succeeded in reducing the myokinase activity to
levels at which the myosin could be used for determining ATP.
Both these difficulties can be overcome by the simple expedient
of adding versene to the reaction mixtures. This chelating
agent accelerated dephosphorylation of ATP by myosin (Friess,
1954; Bowen and Kerwin, 1954a) and completely inhibits
myokinase (Bowen and Kerwin, 1954b; Webster, 1953). Another
factor which contributed to the hesitation in using myosin
was the failure of the myosin preparations to hydrolyse 50
per cent of the acid labile phosphate. The mean values of
43 per cent obtained by Rowles and Stocken (1950) and Steyn-
Pavre et al.(1952) respectively are typical. Ouellet,
Laidler and Morales (1952a) also found discrepancies between
the amount of phosphate split by myosin and that theoretically
possible from spectrophotometric determinations. However, it
was later shown by Ouellet et al. (1952b) that these
discrepancies were due to impurities in the ATP preparation
used. By the myosin method, ADP can also be assayed thus:-

$$2 \text{ ADP} \xrightarrow{\text{myokinase}} \text{ATP} + \text{AMP}$$

$$\text{ATP} \xrightarrow{\text{myosin}} \text{ADP} + \text{P}$$
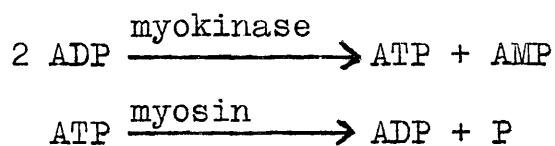
and measuring the inorganic phosphate liberated.

Enzymes have also been used in radioactive tracer
studies to differentiate between the phosphate groups of ATP.
Kalckar (1944b) transferred the terminal phosphate of ATP to
glucose using hexokinase and liberated the second phosphate

with apyrase while the third phosphate was isolated by hydrolysis with barium hydroxide at 100°C. In studies of oxidative phosphorylation by Whittam, Bartley and Weber (1955), the two labile phosphates of ATP were differentiated by apyrase at 0°C, as described by Lee and Eiler (1951) at temperatures below 7°C, apyrase attacks only the last phosphate, while above 7°C it removes both phosphates.

General Aspects of the Problem.

The estimation of adenosine polyphosphates can be divided into two parts - (a) the separation of the polyphosphates from other biological compounds and from each other, and (b) the estimation of the individual nucleotides. The most common practice has been to use both principles in the one assay, e.g., Albaum and Lipshitz (1950) used barium precipitation of the nucleotides from tissue extracts followed by differential spectrophotometry; Trelfall (1957) has used charcoal absorption followed by paper chromatography. The choice of method is undoubtedly wide, depending on the requirements of the investigator, but due consideration must be given to the sensitivity of the method, as all methods are not equally sensitive. In a review by Strehler and Totter (1954), the following sensitivities are given - for the enzymic methods involving hexokinase and deaminase, 20 μg. ~ P, 5-30 μg. for ion exchange methods, 10-50 μg. for the chemical separation methods and 10^{-1} μg. for the firefly luminescence method.

There are some comparisons of the methods in the
literature. Munch-Peterson (1955) has compared the efficacy
of Cohn and Carter's method (1951) with that of Kalckar (1947)
both on a synthetic mixture of ATP and ADP and in tissue
extracts and found good agreement. Rapoport and Nelson
(1945a) compared various enzymic methods, namely, myosin,
snake venom and lobster muscle extract with the value of the
N:pentose ratio in dog and rabbit liver, and found them in
reasonable accord. More recently (1957) Dianzani has
compared fractionation methods (LePage, 1945), chromatographic
methods (Eggleston and Hems, 1952), enzyme methods (Slater,
1953; Holloway, 1954), and found them to be in agreement.
On the other hand, myosin has been frequently used with
results differing from those obtained by other procedures.
Thus Ennor and Stocken (1948) found that the ATP/ADP ratio in
normal liver, as determined with myosin, varied from 90:1
to 2:1 but found a ratio of 1:1 to 1:7 by the N:pentose:
labile phosphorus ratio method. However, this anomoly can be
explained by the findings of Keilley, Kalckar and Bradley
(1956), that the normal myosin preparations contain a
transferase which catalyses the transfer of phosphate from
the uridine phosphates to the adenosine ones.

Nucleotide Content of Various Tissues.

From a consideration of the previous discussion it
is not surprising that some variability exists in the values
for the various adenosine polyphosphates reported in the

TABLE 24a.

TISSUE	NUCLEOTIDE	CONCENTRATION (μM/g.)	METHOD	AUTHOR
Brain	ATP ADP	1.79 0.27	LePage (1945)	LePage (1948)
Brain	ATP ADP	1.73 0.772	LePage's fractionation, acid labile phosphate determined.	Albaum et al.(1946).
Brain	ATP ADP	1.1 0.11	differential spectrophoto-metry (Albaum and Lipshitz, 1950)	Albaum et al.(1953).
Kidney	ATP ADP	1.38 0.48	LePage (1945)	LePage (1948)
Kidney	ATP ADP	0.92 0.27	differential spectrophoto-metry (Albaum and Lipshitz, 1950)	Albaum et al.(1953)
Heart	ATP ADP	2.5 0.42	"	"
Heart	ATP ADP	1.05 0.65	LePage (1945)	LePage (1948)

TABLE 24b.

TISSUE	NUCLEOTIDE	CONCENTRATION ($\mu M/g.$)	METHOD	AUTHOR
Muscle	ATP ADP	5.42 0.59	LePage (1945)	LePage (1948)
Muscle	ATP ADP	6.17 1.05	charcoal absorption and paper chromatography	Trelfall (1957)
Muscle	ATP ADP	3.3 0.092	differential spectrophotometry (Albaum and Lipshitz, 1950).	Albaum et al. (1953)
Muscle	ATP	7.04	column chromatography followed by charcoal absorption	Derache et al. (1956)
Muscle	ATP	1.47	Holloway (1954)	Dianzani (1957)
Liver	ATP	1.17	column chromatography followed by charcoal absorption.	Derache et al. (1956)
Liver	ATP ADP	1.1 0.45	differential spectrophotometry (Albaum and Lipshitz, 1950)	Albaum et al. (1953)
Liver	ATP ADP	0.08 3.3	LePage (1945)	LePage (1948)
Liver	ATP ADP	2.12 1.82 1.81 1.85 .70 .56	LePage (1945) Holloway (1954) Slater (1953) Eggleston and Hems (1952) LePage (1945) Eggleston and Hems (1952)	Dianzani (1957)

literature. In order to facilitate a comparison of these values they are collected in Tables 24a and 24b , where unless otherwise stated, they refer to normal resting rat tissue. In all tissues, with the exception of heart, the values reported using chemical separation methods yield higher values, which can probably be explained by their relative lack of specificity. Another interesting point is the values reported by LePage (1948) for liver; here the ATP content is remarkably low, but at the same time there is a phenomenal increase in the ADP concentration. This may be the result of enzymic breakdown during the fractionation procedure. The value for muscle reported by Derache, Tremoliere, Griffaton and Lowy (1956) is difficult to explain since their value reported for liver is in reasonable agree-ment with the other values reported. The values obtained by using enzymic methods are probably the truer estimate.

The concentration of ATP and ADP in liver have been studied under a variety of dietary and other conditions. Rapoport et al. (1943) found that fasting increased the easily hydrolysable phosphate. However, using enzymic methods, Rapoport et al.(1945b) found that the effects of fasting were a marked decrease in ATP concentration while that of ADP remained virtually unchanged. Flock, Bollman and Mann (1936) were content to measure the "acid-labile phosphorus" under prolonged fasting and on the prolonged feeding of various dietary regimes, notably protein, carbohydrate and fat. These

authors found a decrease in the acid-labile fraction on fasting, in good agreement with Rapoport et al. (1945). Prolonged carbohydrate ingestion greatly increases the acid-labile fraction, while protein and fat intake leave it virtually unchanged. Flock et al. (1936) have also found that the effect of substituting glucose for fat in the diet of rats increases this fraction.

Kaplan and Greenberg (1943) have studied the incorporation of ^{32}P into ATP and also the level of ATP in liver on administration of glucose, insulin and inhibitors such as malonate, fluoride and iodoacetate (1944b, 1944c). Glucose and insulin, when given either together or separately, increase the amount of ATP while malonate and fluoride, but not iodoacetate, decrease the ATP level. But unfortunately, Kaplan et al. have used the "acid-labile phosphorus" as the measure of ATP, which under their conditions contains ADP. Therefore their results can be regarded as little more than an indication of the various effects. Perhaps a more rigorous analytical procedure such as enzymic assay will yield a much more reliable picture of these interesting phenomena.

Scope of the Present Experiments.

The previous Sections have dealt with the incorporation of isotopes into liver and muscle proteins, presumably by the formation of peptide bonds. Since such a reaction is endergonic, peptide bond formation must be coupled with energy-yielding processes. It is therefore pertinent that we

examine the behaviour of energy-yielding metabolites, e.g., ATP, ADP and glycogen, under the same conditions as our incorporation studies.

EXPERIMENTAL.

Animals. Male white rats, weighing 250-300 gm. in the earlier experiments and 190-210 gm. in the later ones, were used in these experiments. They were starved overnight before being fed the experimental diets and at the end of the dietary period, lasting 7 days, the rats were again weighed.

Diets. In our earlier experiments, in which we were concerned with the effect of adding energy to a diet with a constant protein content, the animals received the same diets as described in Section 1. In our later experiments, the diets fed had a varying protein as well as energy content. The composition of these diets and the amounts fed are the same as those described in Section 2.

Estimation of phosphorus. The method chosen was that described by Berenblum and Chain (1938), in which the phosphomolybdic acid is selectively absorbed by iso-butanol. However, it suffers from the disadvantage that the time taken for extraction is appreciable and this might result in hydrolysis of labile organic phosphates. Weil-Malherbe and

Green (1951) have shown that such a hydrolysis is catalysed by molybdic acid. However, Ennor and Stocken (1950) have modified the method of Berenblum and Chain in an attempt to reduce the extraction time to a minimum, and this modification was used in our earlier experiments.

Isolation of nucleotides from liver. The method used in our earlier experiments was that described by Kerr (1942) in which the nucleotides were isolated as the mercury salts, the mercury removed with H_2S and the resultant filtrate estimated.

Method of estimation of the various nucleotides. The choice of method for the estimation of the adenosine nucleotides requires some consideration since all methods are not equally reliable. In this Section, four methods - ionophoresis, paper chromatography, enzymic hydrolysis combined with estimation of phosphorus released and differential spectrophotometry - were examined and the results obtained considered below.

Ionophoresis.

This method has been described by Turba and Turba (1951) and the technique followed was to apply a suitable aliquot, usually 0.5 ml. of the solution under investigation, along a base line 6 cm. from the end of an ionophoretic paper of length 71 cm. The paper was then soaked in citric acid/

Table 25.

Analysis of a "mercury nucleotide fraction" from rat liver by ionophoretic separation.

Expt.	Compound	Total Phosphate (μg.)	Labile Phosphate (μg.)	Labile Phosphate as % of Total.
1	AMP	18.89	3.10	16.4
	ADP	19.93	11.05	55.4
	ATP	26.36	19.10	72.5
2	AMP	18.91	2.9	15.3
	ADP	39.81	8.96	22.5
	ATP	21.16	12.24	57.8

Table 26.

The effect of energy level on the ATP and ADP concentrations in liver.

Energy level	ATP		ADP		AMP		ATP/ADP
	Total P	Labile P	Total P	Labile P	Total P	Labile P	ratio
28	5.70 10.85	6.70 8.6	13.55 28.50	18.0 11.4	6.70 6.80	6.70 3.20	0.29 0.27
Mean	8.27	7.7	21.03	14.7	6.75	4.90	0.28
42	6.5 23.5	8.4 14.0	17.10 29.00	14.4 14.8	6.20 9.60	7.00 2.70	0.27 0.44
Mean	15.0	11.2	23.05	14.6	7.90	4.80	0.36
56	10.5	5.6	20.2	5.4	6.80	2.10	0.34

These compounds were separated by ionophoresis. The figures represent duplicate estimations of the same sample and represent the total amounts (μg/P) per liver. The energy level is given as Cals./m^2/day.

Table 27.

Analysis of a solution of commercial ATP by paper
chromatography.

Compound isolated	Total Phosphate in eluate (μg/ ml.)
ATP	3.62
ADP	3.26
AMP	1.63
Total	8.51

An estimation of the total phosphate in the commercial
solution gave a value of 77.8μg /ml. of which 13.48μg was
free inorganic phosphate. 200 μl. of this solution was
applied to the paper, in other words 0.2x77.8 μg= 15.56μg of
phosphate was applied of which 2.7μg is inorganic, giving a
total of 12.86μg nucleotide.

Table 28.

Analysis of a solution of commercial ATP by paper chromatography.

Compound isolated	Total Phosphate in 2 ml eluate (µg.)		10 min. labile Phosphate in 2 ml. eluate (µg.)		Inorganic phosphate in 2ml. eluate (µg.)
	'a'	'b'	'a'	'b'	
ATP	3.5	1.1	9.4	7.0	2.4
ADP	8.2	1.8	6.48	0.08	6.4
AMP	8.0	5.8	4.12	1.92	2.2

Figures in columns 'a' represent the total amounts found.
Figures in columns 'b' represent the total amount minus the
free inorganic phosphate.

trisodium citrate buffer (pH 3.5) by passing it through a trough of buffer solution, care being taken that the buffer did not reach the applied test solution. The paper was then slung over a glass rod in an ionophoretic cabinet so that the ends dipped into troughs of buffer. Carbon electrodes were placed in the troughs and a voltage (750 v.) applied so that the potential difference along the paper was 11 v./cm. After 7 hours, the papers were removed, dried and examined in U.V. light. Absorbing areas were marked, eluted with water and the phosphorus estimated.

This method was applied to a study of the liver nucleotides which had been precipitated by mercury. The results are shown in Tables 25 and 26. We decided to reject this method mainly for two reasons: firstly, the AMP fraction, which should contain no labile phosphate, contains 16 per cent labile phosphate, and, secondly, the values reported in Table 26 represent duplicate estimations of the same sample done on successive days which vary as much as 100 per cent.

Chromatography.

Because of the foregoing conclusions, it was decided to try chromatography as a means of separating the nucleo-tides. The method employed was that described by Krebs and Hems (1953) involving a one-dimensional two-solvent system. Analyses of a commercial sample of CaATP are shown in Tables 27 and 28 , the former giving a reasonable result, namely,

Table 29.

Analysis of Whatman no. 1 chromatography paper for free phosphate after the paper has been developed in (a) solvent A and (b) after development in solvents A and B.

Distance from base-line (cm.)	Total Phosphate expressed as μg		Average Phosphate concentration μg./sq. cm.	
	'a'	'b'	'a'	'b'
8	1.05 0.85 0.60	0.97 1.16 -	0.13	0.12
12	0.80 0.35 0.39	0.70 1.17 0.70	0.06	0.09
18	1.05 1.25 1.25	2.5 2.1 1.2	0.13	0.20

Figures in column 'a' represent values found after developing paper in solvent 'a' only, while those in column 'b' represent the values found after using both solvents 'a' and 'b'.

of the 12.86 µg. of organo-phosphate applied to the paper,
8.51 µg. or 66 per cent of the total was recovered as
adenosine compounds, while in the latter, it is rather
disturbing that the phosphate hydrolysed by HCl (10-minute
labile phosphate) of ATP is greater than the total phosphate
present and also that the ADP spot contains only 0.08 µg.
labile phosphate, 1.2 per cent of the total phosphate.

It was decided to find out whether any free inorganic
phosphate was eluted from Whatman No. 1 paper under the
conditions in which ATP and ADP were eluted. Accordingly, a
blank sheet of chromatography paper was developed in the
usual way with both solvents and after drying, spots 9 sq. cm.
in area were cut from the paper, eluted with distilled water
and the phosphate content of the eluate determined. The
results are shown in Table 29 . It would appear from this
table that there is a considerable amount of free inorganic
phosphate eluted from Whatman No. 1 paper. There does not
seem to be any particularly concentrated area of free phosphate
but rather a distribution throughout the paper. The
concentration varies slightly across the paper (vide values
given for a distance of 8 cm. from the base line) and also
along the paper where the variation is from 0.2-0.6 µg./sq.6m.
This concentration of free inorganic phosphate, though small
in itself, can constitute a considerable error when small
quantities of organo-phosphates (ATP and ADP) are being
estimated.

Table 30.

The effect of fasting on the adenosine nucleotides of rat liver.

		Our data				Rapoport's data (1945)			
State	Compound	Content in liver (μM/gm)	~P	$\frac{\sim P}{SP}$	ATP/ADP	Content in liver (μM/gm)	~P	$\frac{\sim P}{SP}$	ATP/ADP
Fasting	AMP ADP ATP	4.3 1.9 1.4	5.2	1.2	0.74	1.9 3.5 0.7	4.7	2.5	0.20
Fed	AMP ADP ATP	3.1 3.1 2.0	7.1	2.3	0.65	0.6 3.2 1.5	6.2	10.3	0.47

~P = amount of high energy phosphate is the sum of 2ATP+ADP.

$\frac{\sim P}{SP}$ = ratio of total high energy phosphate to the stable phosphate (AMP).

Rapoport estimated the nucleotides enzymically using myosin, while we employed the differential spectrophotmetric technique of Kalckar (1947).

The possibility of reducing the inorganic phosphate eluted from chromatography paper by washing with N HCl and versene before use was investigated. Whatman No. 1 paper was treated as above and the concentrations of inorganic phosphate eluted were found to be slightly lower than those values reported in Table 29. This procedure as well as being cumbersome is not entirely satisfactory as the paper tends to wrinkle during washing.

Another possibility was to choose a chromatography paper which, according to the manufacturers, had a low phosphate content. Whatman No. 4 paper was chosen, but it was found that the concentrations of inorganic phosphate eluted from this paper were of the same order of magnitude as those listed in Table 29.

We can therefore conclude that the estimation of ATP and ADP after separation by chromatography is influenced by the variable amounts of inorganic phosphate eluted from the paper used in the process of separation. By the choice of a suitable paper and by pre-washing the paper, we can obviate this difficulty to a certain extent but the concentration of phosphate remaining can still cause a considerable error in the quantitative estimation of ATP and ADP.

Chromatographic separation and estimation by P analysis was used to study the effect of fasting on the nucleotide content of rat liver. The results are compared with those reported by Rapoport et al. (1945b) in Table 30.

Table 31.

The effect of previous energy level on the ATP /ADP.

ratio.

Energy level Cal/m^2	Compound isolated	Absorption at 260mµ	Concentration in µm/liver	ATP/ADP
28	ATP ADP	— .336	— 21	0.63
	ATP ADP	.196 .277	12 17	
42	ATP ADP	.125 .145	8 9	1.34
	ATP ADP	.207 .103	13 6.5	
56	ATP ADP	.448 .152	28 9.5	3.99
	ATP ADP	.680 .131	43 8.2	

ATP and ADP were separated by chromatographic methods.
There were two animals in each energy group.

Although the results do agree to some extent, there are a few discrepancies, e.g., the values for AMP are about three times greater than those found by Rapoport.

Another way in which the concentration of nucleotide in the eluates, after chromatography, can be estimated is by measuring the absorption at 260 mμ. This was done in one experiment and the result is shown in Table 31. From these results it would appear that energy added to an adequate protein intake increases the ATP/ADP ratio. However, these chromatographic procedures do not eliminate other nucleotides, notably uridine derivatives, as possible contaminants of the spots. We therefore felt that a much more reliable approach lay in enzymic methods.

Enzymic Methods.

Preparation of Enzymes.

(1)Myosin.   The method used was that described by Bailey (1942). The enzyme was extracted from minced rat muscle with 0.5 M KCl containing 0.03 N NaHCO$_3$ as buffer, the stroma filtered off and the myosin precipitated by diluting the extract with 20 vols. ice-cold glass distilled water and adjusting the pH to 6.8. The precipitate was centrifuged, and purified by repeatedly dissolving in 0.5 M KCl and re-precipitating by dilution. The enzyme obtained after four precipitations was dissolved in a minimum of 0.5 M KCl and stored at 0ºC. Its activity was tested with a standard

FIG. 12.

Scheme of isolation of apyrase from potatoes.

0.01 M KCN extract from potatoes

$(NH_4)_2SO_4$ added to 0.6 saturation

Precipitate | Supernatant (discard)

dissolved and
dialysed for
24 hrs. against
distilled water

Precipitate (discard) | Supernatant

$(NH_4)_2SO_4$ added to 0.6 saturation

Supernatant (discard) | Precipitate

dissolved in H_2O and
dialysed for 24 hrs.

Precipitate (discard) | Supernatant

$(NH_4)_2SO_4$ added to 0.3 saturation

Precipitate | Supernatant

dissolved in water
and dialysed.
(Fraction I)

$(NH_4)_2SO_4$ added to 0.6 saturation.

Precipitate | Supernatant. (discard)

dissolved in water and
dialysed for 24 hrs.
(Fraction II).

solution of ATP, the incubation procedure of Bailey (1949) being followed. With low concentrations of ATP, Bailey (1949) found that the rate of hydrolysis was extremely slow. We therefore ensured that the solution of ATP extracted from the livers was as concentrated as possible and the volumes of enzyme and substrate used for incubation were so adjusted that 30-33 per cent of the available P of the standard solution of ATP was split off by myosin, during an incubation period of 1 hour at 37°C.

(2) Myokinase. The method of preparation was that described by Colowick and Kalckar (1943). Rabbit muscle was ground with sand and extracted with ice-cold distilled water. The extract was heated to 90°C, cooled and pH adjusted to 6.5. After removal of the precipitate, the filtrate was half-saturated with $(NH_4)_2SO_4$, and the enzyme precipitated by full saturation with this salt. It was purified by dialysing against dilute $(NH_4)_2SO_4$, and its activity assayed against a stock solution of CaATP.

(3) Apyrase. This enzyme was prepared from fresh potatoes according to the procedure adopted by Krishnan (1949). The method is shown in Fig. 12. Most of the activity was found to be in Fraction II, which was used in subsequent assays. When this enzyme was used in conjunction with adenylic acid deaminase, it was used in the concentrated form, but if, as in our earlier experiments, this enzyme was used to liberate

Table 32.

The activity of various enzymes against a solution of a pure commercial preparation of ATP.

Enzyme	Total P. (μg/ml)	Inorganic P liberated by enzyme (μg)	% of Total P present	Theoretical
Myosin	64.2	21.1	35	33
Myokinase	64.2	43.4	67	66
Apyrase	64.2	43.7	67.1	66

phosphate from ATP, an experiment was performed to determine the enzyme concentration and the time required to split two-thirds of the total P in a standard solution of ATP. It was found that a 1:25 dilution of the enzyme liberated the requisite amount of P in 15 minutes, but did not hydrolyse any more P during the next 15 minutes. Therefore in subsequent assays this dilution of enzyme was used and the incubation carried out for 15 minutes at $37^{O}C$.

(4) Deaminase. The method of preparation was that described by Kalckar (1947). Rabbit muscle was minced and extracted with ice-cold distilled water. On standing overnight the lactic acid formed from glycogen acidified the mixture to pH 6, and thus precipitated the deaminase. The precipitate was spun off and extracted twice with 1.0 M ammonium acetate at pH 8 with shaking. The course precipitate was spun down and the turbid supernatant subjected to ammoniacal ammonium sulphate fractionation. The fraction, which was precipitated between 0.3 and 0.5 saturation, was centrifuged off, dialysed and stored at $0^{O}C$.

a) Enzymes combined with phosphorus estimations.

The amount of phosphorus released by myosin, myosin + myokinase and apyrase from a pure commercial preparation of ATP was measured and the activity of the various enzymes are shown in Table 32. It can be seen that the amounts liberated, expressed as a percentage of the total P, agree

with the theoretical amounts and this method of estimation
was therefore deemed satisfactory.

b) Enzymes combined with differential spectrophotometry.

The simplest method of estimating the adenosine
nucleotide is by differential spectrophotometry as described
by Kalckar (1947). The liver was quickly removed from the
animal, weighed and a portion (3 g.) homogenised in 2 volumes
ice-cold water for 30 seconds in a Potter-Elvehjem homo-
geniser. The homogenate was washed quantitatively into a
centrifuge tube with 4 ml. water and 2 ml. ice-cold 18 per
cent (v/v) perchloric acid added. The homogenates were then
centrifuged for 10 minutes and the supernatant collected.
The protein precipitate was washed with 1 volume of 3 per
cent (v/v) perchloric acid. To the combined supernatants
was added an equal volume of succinate buffer pH 6.1, the
pH was brought to 7.0 with 2 N KOH. After adjusting the
volume to give a 1:10 dilution of the liver, the extracts
were allowed to stand overnight at $-10^\circ C$. This removes the
perchloric acid as the insoluble potassium salt. The
samples were centrifuged and a 1:3 dilution made for the
enzymic assay. To 2 ml. of the test solution in a cuvette
were added 50 µl. 0.05 M $MgCl_2$ and 20 µl. myokinase
preparation and the absorption at 265 mµ. determined. 10 µl.
deaminase was added and readings were taken at 5-minute
intervals. The deaminase preparations were found to possess

Table 33.

Analyses of pure preparations of AMP, ADP and ATP separately, or as a mixture by means of differential spectrophotometry.

Solution tested	Theoretical concentration (µM/ml test solution)	Concentration found (µM/ml test solution)		
		AMP	ADP	ATP
AMP	75.6	75.6	–	–
ADP	16.0	1.9	12.0	1.8
ATP	18.0	–	–	17.9
Mixture of				
AMP +	.794			
ADP +	.240	.728	.208	.490
ATP	.396			
Total	1.430	.728	.208	.490

The upper set of data represent analyses on the individual nucle
-tides and reveal some impurites in the ADP preparation.   The
lower half of the table summarises an analysis of a mixture of
these three.

some myokinase activity. But this was overcome by adding
versene to a final concentration of 0.008 M, which inhibits
myokinase (Bowen et al., 1953) to a similar aliquot of the
test solution. It was also found that this concentration
of versene is without effect on deaminase activity, since
the values obtained for a stock solution of AMP assayed
with and without versene were 75.6 and 74.4 µM/ml. respectively
The deaminase reacts with AMP to give IMP (inosine mono-
phosphate) with a concomitant drop in extinction. Thus in
the inhibited sample we are measuring the true amount of
AMP and in the uninhibited sample we measure the amount of
AMP + ADP. Subtraction of these two values gives the true
amount of ADP. When the reading has stopped falling the
drop in extinction is noted and 20 µl. apyrase added to the
uninhibited sample. When the reading again becomes steady,
the drop in extinction is noted. The reactions involved are:-

$$AMP + \xrightarrow{\text{deaminase}} IMP + NH_3$$

$$2\ ADP + \xrightarrow{\text{myokinase}} ATP + AMP$$

$$ATP + \xrightarrow{\text{apyrase}} AMP + H_3PO_4$$
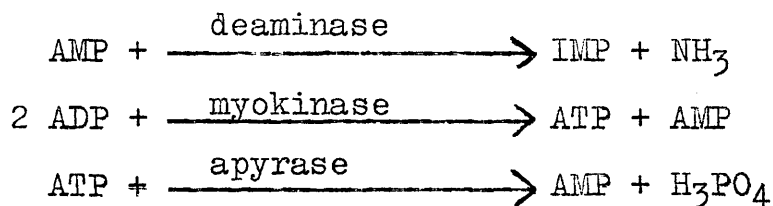
Since the drop in extinction is proportional to the
concentration of the various substrates, their amounts can
be readily ascertained.

The efficacy of this method of estimation was checked
against pure preprations of AMP, ADP and ATP separately and
in a mixture. The results are shown in Table 33. Good

agreement with the theoretical values was found both with regard to amounts of each individual nucleotide and in the total amounts present. This method was therefore used in subsequent analyses.

Estimation of Total Carbohydrate.

The liver extract which was used for the determination of adenosine nucleotides was also used for the determination of total carbohydrate by the Anthrone method (Trevelyan and Harrison, 1952). 1 ml. of this extract was layered on to 5 ml. anthrone reagent (0.2 gm. anthrone in 100 ml. 25 N H_2SO_4) in a boiling tube. The layers were well mixed before immersing the tubes in a boiling water bath for exactly 10 minutes. After cooling the tubes, the solutions were estimated colorimetrically at 620 mμ, and the concentrations read from a standard curve.

RESULTS.

The Effect of Previous Energy Intake on Liver Adenosine Nucleotides during Fasting.

Our first experiments were carried out by adding energy as fat to a diet providing adequate amounts of protein. The protein-containing meal was fed in the morning and the variable energy source in the evening, i.e., about 12 hours before killing. In these experiments, the nucleotides were separated by precipitation as the mercury salts and sub-

<u>Table 34.</u>

The effect of adding energy supplements to an adequate protein diet on the ATP and ADP concentrations of liver.

Energy intake Cal./m^2/day	Nucleotide concentration		ATP / ADP
	ATP (μM/gm wet wt)	ADP (μM/gm wet wt)	
28	1.17	0.48*	2.44
	0.70	0.88*	0.80
	1.05	3.85+	0.27
	0.80	1.21+	0.66
Mean	0.93	1.61	1.04
42	1.06	0.18*	5.89
	1.10	0.19*	5.79
	0.43	0.78+	0.55
	0.57	0.53+	1.09
Mean	0.79	0.42	3.33
56	1.38	0.34*	4.06
	0.55	0.06*	9.17
	0.78	1.24+	0.63
	0.40	0.15+	2.67
Mean	0.78	0.45	4.11

*apryase used; + myokinase used.
Each figure represents a sigle experiment.

Analysis of variance shows a highly significant increase in the ATP/ADP ratio as energy intake rises.

sequently assayed enzymically by myosin, myosin +
myokinase or apyrase. The results are shown in Table 34.
It can be seen from this table that apyrase gives a
consistently lower estimate of ADP than does myosin +
myokinase, and consequently the ATP/ADP ratios are
correspondingly increased. However, it has been established
(Table 34) that this ratio increases as the energy intake
rises, irrespective of the method used to determine ADP.

Since these high energy compounds were examined under
the same dietary conditions as those obtaining in the ^{35}S-
methionine studies, it seems likely that the in vitro
incorporation of the labelled amino acid into tissue proteins
could be related to the enhanced ATP/ADP ratio.

These experiments may be regarded as being preliminary
in nature, and encouraged us to carry out more extensive
studies in which both the energy and the protein content of
the diet were varied simultaneously.

The Effect of Energy Intake with Protein-free and Protein-containing Diets on the Liver Nucleotides 12 Hours after the Last Meal.

In these experiments, the conditions were similar to
those used above, i.e., two meals per day were offered, the
variable energy source being in the evening meal. On this
occasion, the carbohydrate content of this meal was varied to
give the different energy levels. Three main dietary groups

Table 35.

The effect of previous energy level on the adenosine nucleotides in liver 12 hours after feeding the energy meal.

Dietary protein	Energy level	State at sacrifice	Total adenosine nucleotide ($\mu M/gm$)	AMP ($\mu M/gm$)	ADP ($\mu M/gm$)	ATP ($\mu M/gm$)	ATP/ADP	~P	~P/SP
None	Low	Fasting	1.68	1.04	0.37	0.28	0.81	0.93	0.88
	High	Fasting	1.82	0.91	0.39	0.53	1.54	1.54	1.64
Adequate	Low	Fasting	1.78	0.99	0.50	0.29	0.62	1.08	1.11
	High	Fasting	1.56	0.70	0.35	0.51	1.56	1.37	1.98
Adequate	Low	Fed protein	1.76	1.01	0.44	0.31	0.84	1.06	1.09
	High	Fed protein	1.86	0.77	0.73	0.34	0.51	1.41	1.58

The figures are the mean of 4 replications.

Table 36.

The effect of previous energy level on the total adenosine nucleotide concentration in rat liver.

Dietary protein level	Energy level	State at sacrifice	Total adenosine nucleotide	AMP	ADP	ATP	~P
None	Low	Fasting	7.66	4.69	1.65	1.32	4.29
None	High	Fasting	10.20	5.12	2.14	2.94	7.98
Adequate	Low	Fasting	9.59	5.30	2.75	1.54	5.83
Adequate	High	Fasting	12.08	5.41	2.78	3.90	10.58
Adequate	Low	Fed protein	11.87	6.50	2.92	2.45	7.82
Adequate	High	Fed protein	14.42	6.05	5.52	2.85	11.22

The concentrations are expressed as μM per liver.

Each figure represents the mean of 4 experiments.

Table 37.

Summary of the statistical findings of the data of
Table 35.

Parameter studied	Effects		
	Protein	Energy	Interaction
AMP level	not sig.	highly sig.	not sig.
ADP level	sig.	not sig.	sig.
ATP level	not sig.	highly sig.	not sig.
ATP/ADP	not sig.	sig	sig.

were examined: (a) those on a protein-free diet; (b) those
receiving protein in the diet, but in the post-absorptive
state at the time of killing;   and (c) similar animals which
had just received protein 2 hours before sacrifice. Under each
of these three conditions, rats with previous low or high
energy intakes were studied.

In this instance, the adenosine nucleotides were
estimated by the differential spectrophotometric method of
Kalckar (1947). The results are shown in Table 35 , expressed
at μM per gm. wet weight, while Table 36 gives the total
amounts per liver, though these are probably less significant
than concentrations. Table 37 gives a summary of the
statistical evidence derived from Table 35 .

The following points should be noted: (1) The total
adenosine nucleotide content is affected both by previous
energy and protein level, although no obvious interaction
exists (Table 36);   (2) The effect of increasing the energy
intake on the AMP concentration, expressed as μM per gm.
(Table 35)
wet weight, is to significantly reduce the AMP concentration
on all diets (P < 0.01), whereas the protein level of the
(Table 37)
previous diet has no obvious effect;   (3) Analysis of the
ADP data shows that protein intake influences the con-
centration and that this effect varies with the previous
energy level (P < 0.05). This is due to a fall in con-
centration in animals with a high energy intake but in the
post-absorptive state; contrasted with a rise following

protein administration to this group; (4) Statistical analysis of the ATP data shows a significant rise in concentration with increments in energy level ($P < 0.01$) which is independent of dietary protein. Protein level itself has no significant effect; (5) If we consider the ATP/ADP ratio, analysis of the data shows a significant increase with increments in energy intake ($P < 0.05$) coupled with a significant interaction ($P < 0.05$) due to a decrease of the ratio when protein was fed to the high energy group.

The main conclusion to be drawn from these data is that, whereas high levels of energy intake increase the amount of ATP (especially relative to ADP), the feeding of protein obliterates this benefit. One may conclude that, during absorption of amino acids, the availability of ATP must be much the same irrespective of preceding energy intake.

As supplementary evidence, it was decided to study the effect of interposing a meal of constant energy content, between the variable energy source and sacrifice. This can readily be accomplished by feeding the energy portion of the meal in the _morning_ and the protein in the evening, followed by killing next morning. The result of such an experiment should indicate how long the effects of energy in the preceding diet can be detected.

Table 38.

The effect of previous energy level on the adenosine nucleotides in liver

12 hours after feeding the protein meal.

Dietary protein	Energy level	State at sacrifice	Total adenosine nucleotide	AMP (μM/gm)	ADP (μM/gm)	ATP (μM/gm)	ATP/ADP	~P	$\dfrac{\sim P}{SP}$
None	Low	Fasting	1.50	0.79	0.20	0.53	2.65	1.28	1.65
	High	Fasting	1.52	0.66	0.44	0.40	0.91	1.24	2.03
Adequate	Low	Fasting	1.72	0.89	0.32	0.51	1.58	1.34	1.57
	High	Fasting	1.72	0.73	0.51	0.47	0.92	1.46	2.12
Adequate	Low	Fed protein	1.85	1.03	0.34	0.47	1.38	1.28	1.31
	High	Fed protein	1.94	0.99	0.33	0.62	1.89	1.57	1.64

The figures are the mean of 3 replications.

Table 39.

The effect of previous energy level on the total adenosine nucleotide concentration in rat liver.

Dietary protein level	Energy level	State at sacrifice	Total adenosine nucleotide	AMP	ADP	ATP	~P
None	Low	Fasting	8.28	4.27	1.31	2.69	6.69
	High	Fasting	10.41	4.36	3.20	2.96	9.12
Adequate	Low	Fasting	10.57	5.56	1.97	3.00	7.97
	High	Fasting	12.51	5.05	3.87	3.58	11.03
Adequate	Low	Fed protein	12.15	7.04	2.33	2.78	7.89
	High	Fed protein	15.10	7.67	2.40	4.82	12.04

The concentrations are expressed in μM per liver.

Each figure represents the mean of 3 experiments.

The Effect of Previous Energy Intake on the Liver Nucleotides 12 Hours after feeding the last Protein Meal.

The results, expressed in terms of μM nucleotide per gm. wet weight, are shown in Table 38, while the total amounts of nucleotide per liver are shown in Table 39. In the post-absorptive state, the effect of adding energy in the form of carbohydrate to the diets, either deficient or adequate in protein content, is to cause a slight fall in the AMP concentration, accompanied by an increased ADP concentration, whereas that of ATP tends to decrease (Table 38). The ATP/ADP ratio thus falls as previous energy intake rises. The effect of feeding protein (absorptive state) is to obliterate these changes due to energy level, the nucleotide picture becoming equivalent to that on the high protein, low-energy diet.

Consideration of the ~ P/stable P ratio (i.e., 2 ATP + ADP/AMP ratio) is instructive. In the fasting state, increments in energy level raise this ratio, but such an effect is obliterated by feeding casein just prior to killing. It would thus appear that the feeding of protein alters the distribution of these energy-metabolites. This alone is sufficient to account for the fact that amino acids during absorption are treated differently from amino acids circulating in the post-absorptive state.

Table 40.

The effect of previous energy level on the glycogen content

of rat liver.

Concentration expressed as	Protein-free diet (post-absorptive)		Protein-containing diet (post-absorptive)		Protein-containing diet (fed protein)	
	Low energy	High energy	Low energy	High energy	Low energy	High energy
mg. %	0.08	1.54	0.11	0.75	0.10	0.91
Total (mg.) per liver	5.0	105.9	5.0	55.7	5.6	62.3

These figures are the mean of 4 replications.

Table 41.

Statistical analysis of the effect of previous energy intake on the glycogen content of rat liver.

Source of variation	Degrees of freedom	Total squares	Mean square	Variance ratio(F)
Total	23	11,072,776	–	
Protein	2	489,019	244,510	1.52
Energy	1	5,255,640	5,255,640	32.7***
Interaction	1	573,117	573,117	3.56
Replication	3	2,180,100	726,700	
Error	16	2,574,900	160,931	

For n=1 and 16, F= 4.49 at the 5% and 8.53 at the 1% levels.

n=2 and 16, F= 3.63 at the 5% and 6.23 at the 1% levels.

*** highly significant.

The table shows that energy intake has a significant effect on the glycogen content of liver, increments in energy intake increase the glycogen concentration.

The Effect of Previous Energy Level on the Glycogen Content of Liver.

In this instance, the animals were sacrificed 12 hours after the last meal, which was the variable energy source. Therefore we have a comparison of the glycogen content of liver under the same dietary conditions as the nucleotide picture given in Table 35. The glycogen concentrations are shown in Table 40, and a statistical analysis of this data is shown in Table 41. In all groups, the effect of energy level is to increase significantly the glycogen content irrespective of the protein content of the diet. It is important to note that the glycogen content is still high in the absorptive state following the feeding of protein. There is thus no loss of glycogen coincident with the changes in nucleotide metabolism during the absorptive phase.

DISCUSSION.

Although most of the body protein is found in muscle, we have confined our experiments to the liver because of the finding that, when energy is added to a protein-containing diet, the percentage increase in the protein N is greater in liver than in other organs (Munro and Naismith, 1952). It is implied that our findings may explain changes in other organs.

Studies on the energy-rich compounds in the liver,
carried out by previous investigators who studied the
effects of diets (e.g., Kaplan and Greenberg, 1944b,c),
have been restricted to the estimation of ATP content, taking
no account of the ATP/ADP ratio. We must therefore consider
the significance of this ratio in cell metabolism, and the
concept of a "phosphate potential".

The concept of a phosphate potential. In recent years, a
number of investigators have come to recognise that, in
reactions controlled by energy-rich phosphate compounds,
the presence of a phosphate acceptor is as important as the
amount of phosphate-donor, such as ATP, which is present.
The results of several types of experiments appear to support
this concept.

First, certain agents, such as the dinitrophenols,
have been found to stimulate respiration and glycolysis of
tissue slices in vitro (Ehrenfest and Monzoni, 1933; Dodds
and Grenville, 1934; Meyerhof, 1949). These agents
accelerate the breakdown of energy-rich phosphate compounds
and increase the amount of acceptor (e.g., ADP). The effect
of adding acceptor systems has also been studied. Lennerstrand
(1936) observed a stimulation of respiration by AMP, and the
addition of creatine was shown to increase the respiration
of muscle extracts (Belitzer, 1939). By contrast, the
omission of adenylic acid from the medium diminishes the

oxidation of α-ketoglutarate (Green, 1949). Later, ADP
was shown to be more effective as a phosphate acceptor
than AMP (Slater, 1950), while Barkulis and Lehninger (1951)
and Keilly and Keilly (1951) produced more definite evidence
that the actual acceptor is ADP. All this evidence points
to the importance of phosphate acceptor systems, such as
ADP and AMP, in these reactions.

If the reaction

$$ATP \rightleftharpoons ADP + H_3PO_4$$
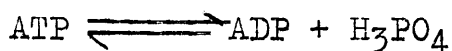
is regarded as analagous to

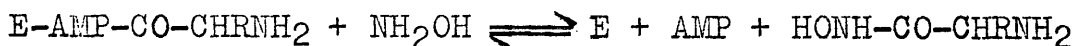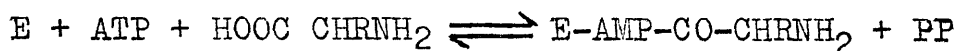$$AH_2 \rightleftharpoons A + H_2$$
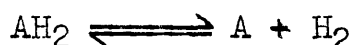
then, just as we have a hydrogen pressure in the tissues,
given by AH_2/A, we may also conceive of a "phosphate
pressure" or a "phosphate potential" (Dixon, 1948),
represented by the ATP/ADP ratio. Since this reaction is
reversible, the ratio will indicate whether there is a
tendency for ATP breakdown or synthesis. Thus, the total
amount of ATP means little if we do not know the amount of
ADP present at the same time. In the experiments listed
above, what governed the rate of reaction was not the amount
of ATP or of ADP, but the proportion of one to the other,
i.e., the ATP/ADP ratio; the rates of reaction are
apparently limited by the rate of transfer or hydrolysis
of energy-rich compounds. This ratio therefore assumes a
central position in P metabolism, and has been suggested as
a possible control mechanism in the economy of the cell

(Lardy and Wellman, 1952). Johnson (1949) showed that the ATP/ADP ratio controls the mechanism of glucose oxidation and concludes that "the primary rate-governing factor is the concentration of inorganic P and ADP, respiration becoming very slow when the value of the expression $(ADP)(H_3PO_4)/(ATP)$ approaches that corresponding to equilibrium". It is conceivable that in a similar manner this ratio controls other mechanisms dependent on ~P, including the equilibrium between amino acids and peptides or proteins synthesised from them.

The effect of the level of energy intake on the nucleotide pattern. The data presented in Table 35 show a rise in the ATP/ADP ratio with energy increments when the last meal contained the variable energy source. This is compatible with Dixon's concept of phosphate potential. However, when a meal of constant composition was interposed between the last energy intake and death, the ATP concentration remains unchanged but the ADP content was increased with increments in energy intake (Table 38). Thus the ATP/ADP ratio goes down. Both of the above conditions reveal some changes in nucleotide pattern with increasing energy intake, largely inexplicable in the latter set of experiments. However, in both instances, the differences at the two energy levels become minimised or even reversed on feeding protein shortly before death. We can thus confidently say that a temporary equalisation of intracellular energy state occurs during the

absorption of amino acids - temporary, because Table 38 indicates an energy -dependent effect on the nucleotide pattern in animals killed 12 hours after a protein-containing meal, i.e. in the post-absorptive state.

The reason for this distortion in nucleotide pattern during amino acid absorption may be related to amino acid activation. The generally-accepted theory is that described by Hoagland, Keller and Zamecnik (1956) and postulates that ATP combines with enzyme, giving an active complex. This complex then reacts with the amino acid, which is activated as the amino-acyl derivative and the ATP broken down to AMP. The reactions may be thus formulated:-

$$E + ATP + HOOC\ CHRNH_2 \rightleftharpoons E\text{-}AMP\text{-}CO\text{-}CHRNH_2 + PP$$

$$E\text{-}AMP\text{-}CO\text{-}CHRNH_2 + NH_2OH \rightleftharpoons E + AMP + HONH\text{-}CO\text{-}CHRNH_2$$

Similarly, Holley (1957) has suggested that the amino-acyl compound could undergo a reaction with another substance (X) with the production of an amino acid -X compound simultaneously with the liberation of AMP and free enzyme.

$$E + AA + ATP \rightleftharpoons E\text{-}AA\text{-}AMP + PP$$

$$E - AA - AMP \rightleftharpoons E + AMP + AAX$$
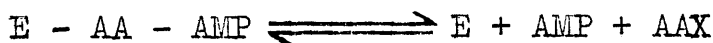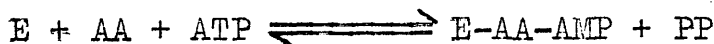
In this instance Holley showed that on the addition of labelled AMP, labelled ATP was formed. Reactions of this type may well be very extensive during the absorptive period after a meal, and consequently cause extensive changes in nucleotide pattern.
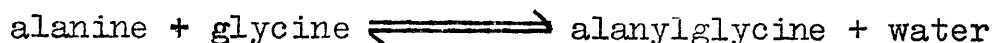
SECTION 5

THE INFLUENCE OF PREVIOUS

ENERGY INTAKE ON THE SYNTHESIS OF p-AMINO

HIPPURIC ACID.

INTRODUCTION.

Although the mechanism of the biosynthesis of peptide bonds is not yet fully understood, the problem is now recognised to be one of finding biological systems capable of providing the energy necessary for these endergonic reactions (Cohen, 1951). Borsook and Dubnoff (1940) showed that the position of equilibrium of a system such as

$$\text{alanine + glycine} \rightleftharpoons \text{alanylglycine + water}$$

was so far in the direction of hydrolysis that no amount of increase in concentration of substrates could lead to a significant amount of peptide bond synthesis. These considerations provided an impetus for the search for systems which would yield the energy to satisfy the requirements for peptide bond synthesis. Model systems have been studied in which one or both of the substrates have been amino-acid analogues or derivatives. Those studied in greatest detail have been (i) acetyl sulphanilamide or other aromatic amines (Lipmann, 1945); (ii) hippuric acid or p-amino hippuric acid (Cohen and McGilvery, 1946, 1947); (iii) glutamine (Frei and Leuthardt, 1949); (iv) glutathione (Johnstone and Block, 1951); (v) ornithuric acid (McGilvery and Cohen, 1950).

The model system studied in the present investigation was the formation of p-amino hippuric acid from p-amino

benzoic acid. This system, which Borsook et al. (1940)
have suggested closely resembles from both chemical and
thermodynamic standpoints, the synthesis of peptides, has
further interest because p-amino benzoic acid has not only
been reported as a constituent of natural peptides (Ratner,
Blanchard, Coburn and Green, 1944) but is generally
distributed in native proteins in a form which can be
hydrolysed only with difficulty (Lewis, 1942; Pennington,
1946).

The requirements for the synthesis of p-amino
hippuric acid from p-amino benzoic acid have been studied
in detail using liver homogenates (Cohen et al., 1947).
While the former authors found the synthetic activity to be
in the insoluble cell fraction, further work by Kielley
and Schneider (1950) using sucrose as a medium for all
fractionation, showed that the activity resided in the
mitochondrial fraction only. However, all these authors
have shown that the system is ATP dependent and furthermore
Chantrenne (1951) showed that the rate of hippuric acid
synthesis was a function of coenzyme A concentration.
Recently, Schachter and Taggart (1953) have shown that
synthesis can be maintained in the presence of benzoyl CoA,
glycine and enzyme. It would thus appear that ATP
participates in the formation of the active CoA compound
rather than in the direct activation of either the carboxyl
or amino group.

The possible nature of the intermediate or intermediates in this reaction is not altogether germane to this investigation. Our main interest lay in the fact that ATP played some active part in the reaction.

As it has already been shown in the previous section that under our nutritional conditions the "phosphate potential", i.e. the ratio of ATP with respect to ADP, rises with increasing increments in energy intake, and we wished to know whether the synthesis of the peptide bond of PAH, is benefited by this rise, and especially whether the giving of protein just before killing affects the rate of PAH synthesis coincident with the observed changes in nucleotide pattern.

EXPERIMENTAL.

Animals and Diets.   Male albino rats, weighing 180-200 gm. were used. The rats were fed the various diets, which have already been described in Section 2, for a period of 7 days. The rats were fasted for 12 hours before sacrifice, except for those studied in an absorptive state where casein was fed 2 hours before killing.

Incubation procedures.   The animals were killed by exsanguination under ether anaesthesia. The liver was removed and sliced in a McIlwain chopper (McIlwain et al., 1953). Samples of the order of 100 mgm. were accurately

weighed on a torsion balance and then transferred to sterile stoppered incubation flasks containing 4 ml. medium. The incubation medium was that described by Sinex et al. (1952), which contained in addition 0.001 M p-amino benzoic acid (PAB) and 0.01 M glycine. The flasks were gassed with 95 per cent O_2 - 5 per cent CO_2 before incubating in a water bath at 37°C for 4 hours. Sterile precautions were taken throughout.

At the end of the incubation period, the contents of the flasks were washed into centrifuge tubes with 16 ml. 0.200 N TCA. An aliquot (10 ml.) of the supernatant was added to 15.5 ml. 0.100 N NaOH and this neutralised extract was stored for p-amino hippuric acid (PAH) estimations.

The method by which PAH is estimated is that described by Cohen and McGilvery (1946). Since the estimation is essentially a diazotisation and since PAB reacts similarly, these two compounds are separated by differential extraction with ether and benzene which under controlled conditions removes 98 per cent PAB and 9 per cent PAH. By determining the total PAB-PAH before extraction and the total PAH after extraction it is therefore possible to calculate the amount of PAH present from the following formula:-

$$\text{PAH concentration} = \frac{\left(\begin{array}{c}\text{Total after}\\\text{extraction}\end{array}\right) - 0.02\left(\begin{array}{c}\text{Total before}\\\text{extraction}\end{array}\right)}{0.89}$$

Estimation of Total PAB-PAH.   A suitable aliquot (1 ml.)
of the neutralised extract was made up to 10 ml. with 0.1 N
HCl, then 1 ml. 0.1 per cent (w/v) sodium nitrite, 1 ml. of
0.5 per cent (w/v) ammonium sulphamate and 1 ml. 0.1 per cent
(w/v) N-naphyl ethylene diamine hydrochloride were added at
three-minute intervals with shaking.   The tubes were allowed
to stand for 10 minutes and the extinctions of the coloured
solutions at 540 mμ. were measured.   The concentration of
PAB-PAH is then obtained from a standard curve.

Extraction and estimation of PAH.   4 ml. of the neutralised
extract were added to 2 ml. 0.1 M citric acid/0.2 M disodium
phosphate buffer (pH 3.95) and shaken in a stoppered tube with
20 ml. ether for exactly 5 minutes.   The ether layer was
removed by suction and the extraction was repeated.   The
aqueous layer was then shaken with 20 ml. benzene for 2
minutes to remove traces of ether.   The aqueous layer was
then sampled for the estimation of PAH.

Estimation of DNA.   The liver samples of the order of
500 mgm. were removed at death and were accurately weighed.
They were then homogenised with 6 ml. water in a Potter
homogeniser (1936).   The homogenate was then made N with
respect to perchloric acid (PCA) and heated for 30 minutes
on a boiling water bath.   This step was repeated on the
precipitate and the combined supernatants were made up to
50 ml.   Aliquots of this solution were taken for DNA

determinations, by the method of Ceriotti (1952, 1955). In this method 2 ml. of the supernatant was added to 1 ml. 0.04 per cent (w/v) indole solution and 1 ml. concentrated HCl (36 per cent w/w) in a centrifuge tube, which was then placed in a boiling water-bath for 10 minutes. After cooling, the solutions were washed thrice with 4 ml. portions of chloroform. It was found that centrifuging the tubes for a few minutes between washings cleared the emulsions. The clear yellow solutions, obtained after the washing procedures, were estimated colorimetrically at 490 mμ. against a standard DNA solution.

RESULTS AND DISCUSSION.

The experimental design in this section is the same as that adopted in the previous section, thus allowing a comparison of the effects of previous energy level when the diets contain varying amounts of protein. Also, a comparison between animals in post-absorptive and absorptive states has been made. In order to compare the synthetic ability of the liver tissues after these dietary regimes, the livers were sliced immediately after sacrifice, and incubated in a medium containing glycine and p-amino-benzoic acid for 4 hours, after which time the PAB and PAH were separated by differential extraction with ether.

TABLE 42.

The effect of previous energy intake on the synthesis of the peptide bond of PAH.

Concentration	Protein-free diet (post-absorptive)		Protein-containing diet (post-absorptive)		Protein-containing diet (fed protein)	
	Low energy	High energy	Low energy	High energy	Low energy	High energy
PAH synthesized per mg. tissue	2.71	2.07	3.09	2.87	3.04	2.64
PAH synthesized per mg. DNA	5.55	3.39	4.52	5.16	6.98	6.24

These figures are the mean of 4 replications.

Expression of Results.

The results have been expressed in two different ways, namely, PAH synthesised (i) per mg. wet tissue weight, (ii) per mg. DNA. When the results are expressed on the basis of "wet weight" they are subject to several imperfections, since liver tissue is not of a constant composition, e.g. water, glycogen and protein contents vary. This variation without doubt occurs under our experimental conditions, and therefore results based on "wet weight" do not afford the only basis of assessment. A more realistic evaluation of the results may be in terms of a fixed standard, e.g. DNA content of the liver slice (Davidson et al., 1950; Gray and DeLuca, 1956). The results so expressed give an estimate of the PAH synthesised per liver cell.

Effect of Energy Intake.

The results obtained are shown in Table 42, expressed in terms of both wet weight and per liver cell. The results would seem to indicate that on the basis of wet weight, previous increases in energy intake have if anything a slight inhibitory effect on PAH synthesis, while the presence of protein in the diet increases the rate of synthesis. The results are by no means clear-cut.

When the results are expressed in terms of DNA content, then the effect of feeding protein appears to be much more pronounced and some rise in synthesis with energy increments is evident among the protein-fed animals in the post-absorptive state. However, at this point we must interpret the results

cautiously. The differences observed between the data
expressed per mg. wet weight and per cell lies in differences
in cell size and protein content, i.e. enzyme content. The
group showing more synthesis in response to a higher energy
intake has in fact more protein per cell (Munro and Naismith,
1953), and for this reason alone could synthesize more PAH
(assuming that enzyme content and protein content of the cell
run in parallel). It is therefore not possible to say
whether higher energy intake has any beneficial effect per se
on rate of synthesis. Probably the fact that this difference
is no longer apparent when expressed per mg. wet weight is
an indication that energy level is not having an action on
PAH synthesis independent of changes in protein (i.e. enzyme)
content.

At first sight it is rather disconcerting that tissues
which show differences in high-energy nucleotide distribution
(Section 4) should fail to show differences in PAH synthesis.
This need not necessarily mean that formation of peptide
bonds of proteins are similarly unaffected. Firstly, the
site of PAH synthesis is in the mitochondria (Keilley and
Schneider, 1950) while Zamecnik and Keller (1954) have shown
that the greatest incorporation of isotope is into the
microsome fraction. The second point of dissimilarity
between the two systems is their different reactions to
N-mustards. McKinney (1949) found that PAH synthesis was
inhibited about 10 per cent in the presence of 5×10^{-4} M

N-mustard, while Goodlad and Munro (1957) found under identical conditions that this concentration of N-mustard inhibits the incorporation of ^{14}C-glycine about 90 per cent. The third point of dissimilarity arises from the work of Baccari and Guerritore (1954). They found the ADP (0.002 M) was as effective as ATP (0.001 M) in promoting PAH synthesis in guinea-pig liver homogenates. Since the reactions were studied anaerobically, and in the presence of glycolysis and myokinase inhibitors, ADP cannot exert its stimulatory effect by being transformed into ATP. Thus it can be said that PAH synthesis is probably independent of the ATP/ADP ratio (i.e., phosphate potential) whereas the incorporation of isotope appears to be dependent on this potential. Supporting evidence comes from the work of Burlington (1957) who could find no correlation between changes in PAH synthesis and changes in the incorporation of labelled amino acids when thyroxine was administered to rats.

The conclusion to be drawn from this is, therefore, that PAH peptide bond synthesis is not necessarily a suitable model to study in the search for the mode of action of dietary energy level on protein metabolism.

<u>GENERAL DISCUSSION.</u>

GENERAL DISCUSSION.

The Influence of Dietary Energy Level on Protein Synthesis.

The evidence of an interrelationship of energy and protein metabolism has been described in the Introduction to this thesis. Briefly, it can be said that energy intake is in a continuous dynamic equilibrium with N balance. That such an interaction is of a considerable duration was shown by the finding of Munro and Wikramanayake (1954) that N retention occurred in rats when the energy supplement of the diet was given 12 hours apart from the protein meal. The prolonged action of dietary energy level on protein metabolism is supported by the results of in vitro studies with ^{35}S-methionine and ^{14}C-glycine, presented in Sections 1 and 2 of this thesis respectively.

In Section 1 it was demonstrated that, when increments in energy were added to a diet already adequate in protein, the incorporation of ^{35}S-methionine into liver and diaphragm protein was significantly increased, even when the tissues were excised 18-20 hours after the last meal. From Section 2 essentially the same conclusions can be drawn. Not only was the incorporation of isotope into liver protein higher when the previous energy intake was high but the incorporation was significantly increased by the presence of protein in the diet when the animal was in the post-absorptive state.

However, the _in vitro_ type of experiment fails to distinguish between differences in the rates of penetration of isotope into the liver cell and actual differences in the rates of protein synthesis. We therefore approached the problem by _in vivo_ studies using ^{14}C-glycine. The results given in Section 3 confirm the picture obtained from the _in vitro_ studies. Since the results have been expressed in terms of "total relative activity" (Table 23), i.e. taking into account variations in the specific activity of the free glycine in the precursor pool and in the amounts of protein N per liver, it is possible to make certain deductions about the overall rates of protein synthesis under our various nutritional circumstances. Inspection of Table 23 reveals that increments in energy intake are only effective in promoting incorporation when the rats had previously been fed protein but were in a post-absorptive state. In contrast, increments in energy intake failed to elicit a response when the rats had previously been maintained on a protein-free diet or when the rats had just been given a protein meal and were therefore in an absorptive state. Thus the feeding of protein obliterates the differences observed when the rats were in the post-absorptive state. These findings imply that the animal's previous energy intake influences amino acid incorporation only when the rats are fasting between meals and does not affect the fate of incoming amino acids at the time of a meal.

This conclusion may at first seem odd, but it is essentially in agreement with the results of nutritional studies. Thus Allison et al. (1946) found that, when a given amount of protein was added to the diet of their dogs there was a constant improvement in N balance at various levels of energy intake down to 50 per cent of the energy requirements. In other words, the capacity of the animal to utilise dietary protein is not influenced by energy intake, although the absolute N balance becomes more favourable as the energy intake rises. Campbell and Kosterlitz (1948) have provided data on the protein content of the livers of rats on various diets which can be interpreted in the same way. Although the total amount of protein in the liver varied with energy intake, in response to a given increase in protein intake, the protein content of the liver rose by the same amount irrespective of the energy content of the diet (vide Fig. 4). Seen in the light of our studies on the uptake of labelled amino acids, these studies on N balance and on liver protein content would appear to mean that energy intake does not affect the response of the protein-synthesising mechanism to incoming amino acids, but rather affects the reutilisation of amino acids circulating between meals.

Before we can postulate how energy intake might exert its effect we must consider the current theories of protein synthesis.

Current Views on Protein Synthesis.

The first question that must be settled is the nature of the starting material for protein synthesis. A considerable body of evidence has accumulated to suggest that proteins are synthesised from amino acids rather than peptides. Thus Christensen (1950) found that an intravenous infusion of amino acids could maintain an animal in positive N balance, whereas the products of partial hydrolysis could not. Similarly, Gerarde, Jones and Winnick (1952) found the greatest incorporation of labelled amino acids occurred when the tissue culture medium contained, instead of the embryo extract consisting of proteins, peptides and amino acids, a mixture of 19 amino acids. The finding that three enzymes from rabbit muscle with different turnover rates had the same specific activity after the injection of labelled amino acids led Velick and his co-workers (Simpson and Velick, 1954; Heimberg and Velick, 1954) to conclude that the three enzymes had been formed from the same amino acid pool and that a given amino acid is replaced at the same rate in all of the positions it occupies. An investigation of the peptides from partially hydrolysed casein by Campbell and Work (1952b) yielded the same conclusion, namely, that casein is synthesised from amino acids and that no peptide take part as such. Again, experiments on antibody formation and on the formation of adaptive enzymes in micro-organisms argue against more complex precursors than free amino acids

in the foundation of such specific proteins (Halvorson and Spiegelman, 1952; Green and Anker, 1954; Gale, 1955; Hogness, Cohn and Monod, 1955).

Thus the available evidence points to amino acids *per se* as the building units for protein biosynthesis and, since peptide bond formation is endergonic, some energy source must be involved. Borsook (1955) has considered the evidence from *in vitro* studies of peptide bond synthesis and concluded that the carboxyl group of the amino acid is activated either by linkage (a) with Coenzyme A, or (b) directly to the enzyme, or (c) in an enzyme-PO_4 complex, at the expense of one or other of the pyrophosphate bonds of ATP. Some supporting evidence appears in the literature. Peterson and Greenberg (1952) have shown that ATP accelerated the *in vitro* incorporation of amino acids into proteins in an enzyme system composed of mitochondria together with a supernatant fraction of rat liver homogenate. Siekevitz (1952) found evidence for a soluble co-factor produced by mitochondria which stimulated the *in vitro* amino acid incorporation. Hoagland, Keller and Zamecnik (1956) suggested that amino acid activation occurred by means of an enzyme-bound amino-acyl-adenylate compound. More recently, Hoagland, Zamecnik and Stephenson (1957) have suggested that, after the initial activation of the amino acid, the active compound is transferred to S-RNA (a species of RNA with a low molecular weight). The activated amino acids now possess sufficient energy to combine by means of peptide bonds.

Two main theories of protein synthesis exist, namely, the "Transpeptidation" theory (Fruton, 1952) and the "Template" theory of Dounce (1952). Several proteolytic enzymes have been shown to catalyse reactions of esters, amides and peptides with amines or amino acids resulting in the formation of new amides or peptides. The energy requirement is met in this case by the hydrolysis of the initial ester, amide or peptide link. Examples of this type of enzymic reaction are numerous. Thus Jones, Hearn, Fried and Fruton (1952) demonstrated polymer formation when glycyl-L-phenylalanineamide was incubated in the presence of cathepsin-C. Transpeptidation reactions involving peptides as distinct from amides have been shown in the case of γ-glutamyl peptides by Hawes, Hird and Isherwood (1952), and by Foder, Miller and Waelsch (1953). However, there are objections to the "Transpeptidation" theory, namely that (a) it necessitates a peptide pool and that (b) if the proteolytic enzymes responsible for such reactions are as demanding in specificity of their substrates with respect to trans-peptidation as they appear to be when acting as hydrolytic enzymes, then a very complex system of enzymes would be required for the synthesis of a single protein.

The "Template" theory of Dounce (1952) envisages the nucleic acid molecule as forming a master template which can either reproduce itself or produce the requisite pattern of amino acids. He suggested that ATP contributes the necessary

energy by means of a phosphotransferase which transfers the terminal phosphate to the phosphate of the nucleic acid by a pyrophosphate linkage. The amino group of amino acids then reacts to form amino-phosphate compounds on the nucleic acid and for each amino group so combined, the phosphate which came originally from the ATP is displaced and appears as inorganic phosphate. Another enzyme links the free carboxyl group of the adjacent amino acid to the phosphate-bound amino group to form a peptide bond. However, this theory has been modified in view of the evidence for carboxyl-activated amino acids. Borsook (1955) has therefore suggested that the amino acids are attached by their carboxyl groups.

Adenine Nucleotide Pattern on Different Diets.

We are now in a position to examine how our own findings accord with these theories. In Section 4 we examined the livers from rats under the nutritional circumstances obtaining in the earlier sections of this thesis, in an attempt to find differences in the distribution of the adenosine nucleotides. The conclusion to be drawn from this work (Table 35) is that, in the post-absorptive state, the ATP/ADP ratio (the "phosphate potential") is higher in animals which were previously receiving more energy-yielding nutrients. During the absorption of a protein meal, however, the distribution of the adenosine nucleotides becomes

temporarily disturbed, thereby obliterating the differences
obtained between energy levels in the post-absorptive state.

If indeed the ATP/ADP ratio controls protein
metabolism as it does glucose oxidation (Johnson, 1949),
these findings would explain why no differences are observed
in the in vivo incorporation of ^{14}C-glycine after feeding
protein (Table 23). In the post-absorptive state, a
higher level of energy intake benefitted the rate of amino
acid incorporation only when the preceding diet contained
protein (Table 23), yet in both instances the ATP/ADP ratio
was more favourable for animals receiving the higher energy
intake (Table 35). This can be explained in terms of the
two major requirements for protein synthesis, namely a supply
of amino acids and an energy source. When the supply of
amino acids circulating in the tissues comes solely from
endogenous sources (protein-free diet), this becomes the
limiting factor in the rate of protein synthesis. On the
other hand, when the diet supplies sufficient amounts of
protein this limitation no longer exists even in the
fasting state and the amount of energy from the diet now
influences the rate of protein synthesis.

As noted in the Introduction to this thesis, the
interrelationship of energy intake and protein metabolism
(shown diagrammatically in Fig . 3) stresses both their
independence and interdependence. The important feature
of this relationship, as shown in the diagram, is that

between certain limits (A and B), when the two lines are parallel, protein and energy intake are independent of each other. Below the point **A**, the energy intake is inadequate so we have a diminished response to increasing protein intakes i.e. the lines converge; while beyond the point B the lines diverge, showing that an increased caloric intake fails to improve N balance because the protein content of the diet is now the **limiting factor**. The work on isotope incorporation reported in this thesis, adds strength to these arguments; and this study of the adenosine nucleotides suggests that the ATP/ADP ratio is the motivating influence between energy intake and protein synthesis.

<u>SUMMARY</u>.

SUMMARY.

Studies have been carried out on rats to determine the nature of the relationship between the level of energy intake and protein metabolism. The experimental studies are described in five sections, summarised as follows:-

Section 1. The influence of previous energy level on the in vitro uptake of ^{35}S-methionine into the proteins of rat liver and muscle.

1.   Rats were maintained on a diet of adequate protein content, to which had been added increasing energy increments in the form of fat, and after fasting overnight were then killed 12 - 14 hours or 18 - 20 hours later.

2.   The uptake of methionine into liver and diaphragm proteins increased linearly with increasing levels of energy intake.

3.   The conclusion to be drawn from this series of experiments is that previous energy intake exerts a prolonged action on protein metabolism in both liver and muscle.

Section 2. The influence of previous energy intake on the in vitro incorporation of ^{14}C-glycine into liver proteins.

1.   A study was made of the influence of the energy content of diets (both protein-free and protein-containing) on the in vitro uptake of ^{14}C-glycine into rat liver proteins. In this instance, a comparison was made between rats which were in a post-absorptive state for 12 hours and those which had just been fed a protein meal.

2.   The uptake of isotope increased as the/content of

energy

the preceding diet rose; the influence of energy level was

greater when the previous diet contained protein.

3.   Since energy level exerts its greatest effect within

the first hour of incubation, it is suggested that the

differences in ^{14}C-glycine incorporation may be due to

differences in the rate of penetration of isotope into the

cell; it is not possible from such experiments to determine

whether protein synthesis per se is affected by energy level.

Section 3.   The influence of previous energy level on the

in vivo incorporation of ^{14}C-glycine into liver protein.

1.   Rats were maintained on the same dietary regimes as those

described in Section 2 and were sacrificed 3 and 6 hours

after the injection of ^{14}C-glycine.

2.   The only circumstance in which the energy level in

the preceding diet affected protein synthesis was in the case

of the group fasting after a normal protein intake. In

contrast, the energy level of the diet did not affect the

uptake of glycine when the rats had been fed a protein-free

diet or when the rats were in the absorptive state after

a protein meal.

3.   The conclusion has been drawn that differences in

energy content of the diet exert their main effect on protein

utilisation in the post-absorptive state and not at the time

when tissues are being flooded with amino acids from the gut.

Section 4. The effect of previous energy intake on the adenosine nucleotide pattern of rat liver.

1.   Rats were maintained on diets either containing protein or free from protein and providing different energy levels.

2.   In the post-absorptive state, the ATP/ADP ratio was found to improve when the energy content of the preceding diet was raised. Such an improvement in the ratio was obliterated when protein was fed 2 hours prior to sacrifice.

3.   A difference in nucleotide pattern was still apparent even when a meal was interposed between the last energy supplement and sacrifice; again, the feeding of protein before death altered the pattern.

4.   The results were interpreted to mean that the enhanced ATP/ADP ratio observed with increasing energy intakes may well regulate the incorporation of amino acids into protein and consequently the change of nucleotide pattern on feeding protein could account for the lack of influence of energy intake during the phase of absorption.

Section 5. The influence of energy intake on PAH synthesis by liver slices in vitro.

1.   Liver slices from rats which had been fed on the same dietary regimes as above were incubated with p-amino benzoic acid and glycine.

2.   When the results are expressed in terms of the
DNA content, the effect of protein and energy intake appears
to increase the synthesis in the protein-fed group in
the post-absorptive state.

3.   When the results are expressed per mg. wet weight,
it would appear that no differences exist between the
various dietary fators. The fact that differences are no
longer apparent has been taken to mean that energy level has
no effect on PAH synthesis independent of changes
in protein (i.e. enzyme) content.

5.   It has been concluded that PAH peptide bond
synthesis is not a suitable model system for the study of
the effects of energy level on protein synthesis.

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