# STUDIES IN PROTEIN METABOLISM.

## The inter-relationships of energy metabolism and protein metabolism

by

Thomas Walter Wikramanayake, M.B.,B.S.

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

---

October, 1952.

ACKNOWLEDGMENTS.

I wish to express my sincere gratitude to Professor J.N. Davidson for giving me the opportunity of carrying out this work in his Department, and to Dr. H.N. Munro, without whose constant encouragement and patient instruction this work would not have been possible.

I thank Dr. R.M.S. Smellie for introducing me to the techniques of ionophoresis and cell fractionation, and for permission to refer to his unpublished results.

I am also indebted to Dr. F.C. Heagy and Mr. James Chisholm for help given during some of the experiments, to Dr. S.C. Frazer and Messrs. D.J. Naismith and Jack Cameron for being three of the subjects in the human experiment, to Mr. D.R. Cameron and Miss R. Pevie for helping with the photography, and to all who helped with discussions of the various problems.

# C O N T E N T S.

Page

PART V. THE EFFECT OF ENERGY INTAKE ON THE METABOLISM OF RIBONUCLEOPROTEIN AND PHOSPHOLIPID IN DIFFERENT PARTS OF THE LIVER CELL.

"Man ist was er isst"

In the early days, nutritional research was directed towards obtaining information as to which components of man's diet were vital to life and health. The results indicated that proteins are essential to life, that fats and carbohydrates provide energy and that minute traces of accessory substances were needed to prevent certain diseases. In the years that followed, several new vitamins and minerals were added to the list, and it was realised that proteins were not merely sources of nitrogen, and that there were important differences between proteins from different sources. The importance of amino-acids in nutrition was thus established.

During the last two decades, many of the vitamins have been identified chemically and synthesised. Studies in enzymology have linked these catalysts to the vitamins, thus explaining their mode of action. In this way, several of the questions as regards the "what" and the "why" of nutrition have been answered, and more and more investigators turned to the more interesting question of "how". How do various dietary constituents react inside the body to serve their varied functions? How does one nutrient affect the metabolism of others? How do carbohydrate and fat act as sources of energy? And what are the steps in the intermediary metabolism of various dietary components?

The advent of radio-active isotopes saw a rapid advance in our knowledge of intermediary metabolism. By means of this new tool it was demonstrated that there exists a dynamic equilibrium between body constituents. Advances in the study of metabolism showed that carbohydrate, fat and protein had common pathways, and it soon became clear that the metabolism of one influenced that of the other two, and that the problems concerning one constituent cannot be considered independently of problems involving the others. More recent work indicates that carbohydrate and fat influence protein metabolism by acting as sources of energy. In answering the "how" of protein synthesis, one consideration is the provision of energy for such endergonic reactions as the synthesis of peptide bonds, energy which is essential for the building up of a protein molecule from the component amino acids. This energy must be drawn from the pool of energy-yielding metabolites to which carbohydrate and fat are the main contributors.

Our purpose in the series of experiments described below has been to explore the relationship of energy metabolism to protein metabolism, taking the influence of energy intake on nitrogen balance as a starting-point and then analysing metabolic changes which might conceivably explain this relationship. In our investigations, the first step has been to define the conditions under which energy intake

is a factor in protein utilisation; this has been achieved partly by a consideration of the published literature and partly by means of new experiments. In seeking an explanation for this relationship of energy intake to protein utilisation, we have investigated two factors which are currently supposed to play a major role in protein synthesis, namely high-energy phosphate (adenosine triphosphate) and ribonucleic acid. The influence of energy intake on the metabolism of adenosine triphosphate and of ribonucleic acid was examined and related to concurrent changes occurring in protein metabolism. Since phospholipids have some metabolic properties in common with protein and ribonucleic acid (the "labile liver cytoplasm" described by Kosterlitz), we also extended our studies on the influence of energy intake to these. Whilst the results obtained in our investigations cannot be said to have elucidated the whole relationship of energy intake to protein metabolism, they do open up a number of interesting possibilities.

# PART I.

## THE RELATIONSHIP BETWEEN ENERGY INTAKE AND NITROGEN BALANCE.

# INTRODUCTION.

That there is a relationship between the non-protein constituents of the food and the utilisation of protein by the animal organism has been recognised for over a century. A comprehensive review of the literature has appeared recently (Munro, 1951) and shows that, whereas carbohydrate and fat act interchangeably as energy sources affecting protein metabolism, carbohydrate has in addition a special action on protein eaten in the same meal. Only the action of carbohydrate and fat acting purely as sources of energy will be considered here.

Experiments reported in the literature on the effect of energy intake on protein utilisation fall into two main groups: those in which conditions of undernutrition were produced, and those in which the carbohydrate or fat was superimposed on adequate diets.

a. Effect of carbohydrate and fat on protein metabolism during undernutrition: These studies, dealing with the protein-sparing effect during undernutrition, are of two types: one in which energy is withdrawn from an adequate diet by removing carbohydrate or fat, and another in which energy is added to an inadequate diet.

Withdrawal of carbohydrate from an adequate diet results in an immediate increase in the nitrogen (N)

excreted (Lusk, 1890; Miura, 1892; Rosemann, 1901). Only one experimenter (Neumann, 1899) appears to have studied the withdrawal of fat from the diet of a human subject; a similar effect to that obtained by removing carbohydrate was noted. Recently, Bosshardt et al. (1948) found that carbohydrate and fat had similar effects on the gain in body N of growing mice.

Several early experiments on dogs (Bischoff, 1853; Bischoff & Voit, 1860; Voit, 1869) show that addition of carbohydrate or fat to ~~the~~ diets ~~of dogs~~ containing insufficient meat to maintain body weight brings about a reduction in N loss, but these cannot be taken as evidence since the animals were not kept for an adequate length of time on the experimental diet to accustom them to it, and the results obtained might have been affected by the diet prior to the experiment. However, more recent experiments with dogs carried out under strictly controlled conditions indicate that adding fat or carbohydrate to increase their energy intake from 25 to 50% of the normal intake resulted in an improved N balance (Allison et al., 1946). The amount of N retained appears to be the same irrespective of whether carbohydrate or fat was the added nutrient. Similar results have been reported in the case of man (Jansen, 1917; Zuntz & Loewy, 1918) and of the rat (Benditt et al., 1948).

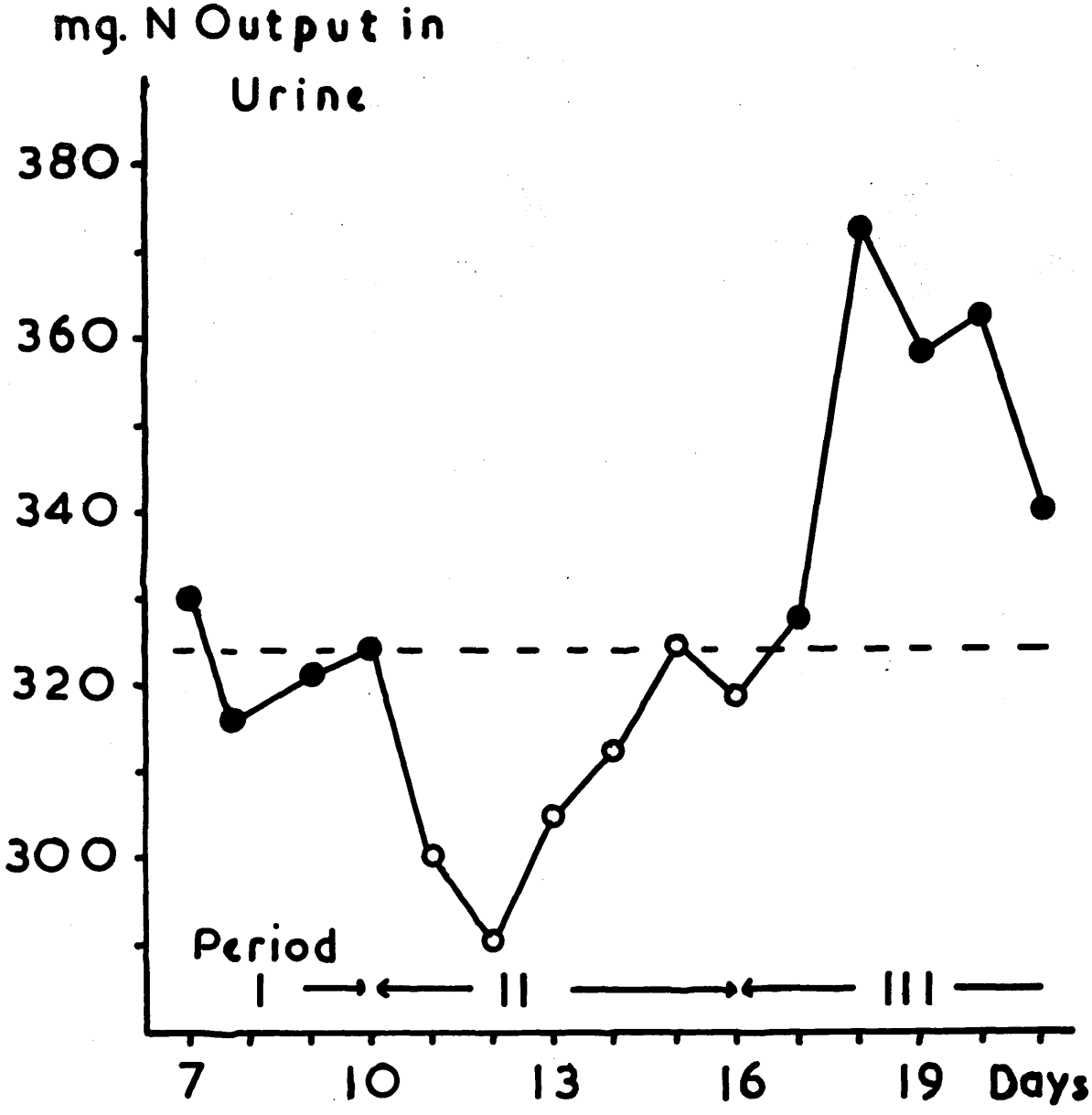
In summary, it is clear that removing carbohydrate or fat from a diet increases N loss, and adding energy in the form of carbohydrate or fat to inadequate diets improves the N balance. There is no adequate evidence to indicate a superiority of carbohydrate over fat in this respect.

b. Effect of addition of carbohydrate and fat to adequate diets: A number of workers have added carbohydrate or fat to diets already adequate in energy content; the results have been summarised by Munro (1951). There is general agreement that N retention occurs when either carbohydrate or fat is added to diets adequate for man, the dog and the rat. Though the data do not show definitely that N retention is related in a linear fashion to the amount of energy superimposed, the observations made by Cuthbertson & Munro (1937) and by Basu & Basak (1939), who studied the same subjects at two levels of superimposed carbohydrate intake, suggest that N retention caused by surfeit energy is roughly proportional to the amount of energy added.

The amount of N retained may vary with time. In man, the first day of surfeit feeding usually has little effect on N excretion (Cuthbertson & Munro, 1937; Cuthbertson, McGirr & Munro, 1937; Basu & Basak, 1939). Different investigators disagree on the length of time during which the improvement in N balance can be maintained. Krug (1893) fed himself

with extra carbohydrate and fat, and obtained as high an N retention on the 15th day as during the first few days of surfeit feeding. In experiments with dogs, Larson & Chaikoff (1937) obtained a considerable retention of N even on the 7th day of surfeit feeding. On the other hand, Voit's (1869) experiments on dogs show that the effect of surfeit fat may be quite short-lived. The effect of surfeit feeding on rats has been shown to be most marked during the first few days of surfeit (Lathe & Peters, 1949a).

c. Effect of the time of feeding carbohydrate and fat: The above studies on undernutrition and overnutrition establish that energy intake affects N balance. However, the question has been raised in recent years of whether the time at which energy-yielding nutrients are given in relation to dietary protein is important. Two types of experiments dealing with this question have to be distinguished. In one, the total daily intake of nutrients remains the same but the time of feeding them is varied. Rats (Cuthbertson, McCutcheon & Munro, 1940) and human subjects (Cuthbertson & Munro, 1939) were fed diets which could be separated into two portions, one containing all the protein and part of the fat of the diet, and the other containing all the carbohydrate and the rest of the fat. When these two portions were fed together, the daily N output was less than when they were given separately.




Periods I & III: protein & carbohydrate eaten separately.

Period II: protein & carbohydrate eaten together.

Munro (1949).

Fig. 1

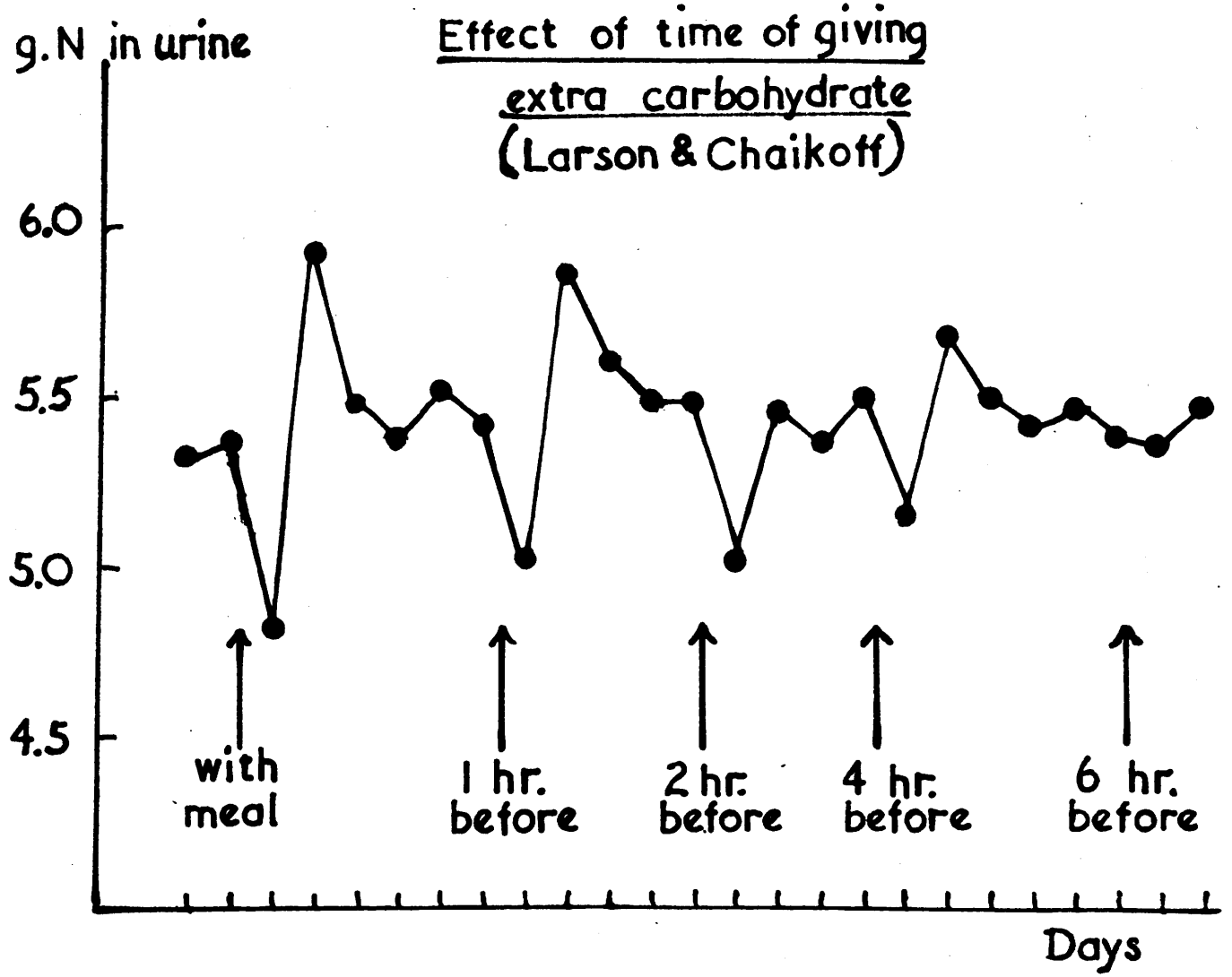

g.N in urine
Effect of time of giving extra carbohydrate (Larson & Chaikoff)
6.0
5.5
5.0
4.5
with meal
1 hr. before
2 hr. before
4 hr. before
6 hr. before
Days

Fig 2

In a later experiment, Munro (1949) established that these changes were due solely to alterations in the time of carbohydrate intake. No change in N balance was observed in experiments involving similar alterations in the time of feeding fat. The N retention obtained when carbohydrate was fed with protein was only transitory, N balance returning to basal values even though the protein and carbohydrate continued to be eaten together. When the two nutrients were again given separately, the N retained earlier was lost in the urine (Fig. I).

The second type of experiment involves a change in energy intake, the extra energy being given at a definite time in relation to the dietary protein. This has led to somewhat conflicting data. Larson & Chaikoff (1937) fed dogs a single daily meal sufficient to maintain them in N equilibrium, and added a single dose of extra carbohydrate given at various time intervals after the meal. Their data have been plotted graphically in Fig.2. It is apparent that there was a lowering of N output only when the surfeit carbohydrate was given within 4 hours of the protein containing meal, no protein-sparing action being observed when single doses of carbohydrate were given outside this period. On the other hand, Cuthbertson & Munro (1939) fed a human subject extra carbohydrate, in one experiment with the

protein-containing meals, and on another occasion 4½ hours after the last meal for the day. The degree of N retention was eventually the same in both experiments. In these experiments, surfeit feeding was continued for several days. In the case of feeding extra carbohydrate apart from the rest of the diet, an effect on N balance was only apparent after the second day of overfeeding. It is possible that Larson & Chiakoff's one-day superimposition experiments would have yielded a different result if the extra sugar had been given on several successive days. Lathe & Peters (1949b), studying the effect of surfeit sucrose given to 4 rats, found that extra sugar given some hours after the protein-containing meal had no protein-sparing action even though it was fed on several successive days. It must be pointed out that their rats were allowed only 2 days to become accustomed to the basal diet - a period far too short for them to reach a steady state on which to base N balance determinations.

Opinion is thus divided as to whether the extra energy must be given with the protein portion of the diet, or whether the protein-sparing effect can be demonstrated even when the surfeit nutrient is given several hours after the protein-containing meals. Accordingly, experiments were carried out on human subjects, rats and dogs, in which additional carbohydrate was given along with or several

TABLE 1.

Vitamin Mixture.

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

TABLE 2.

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, 3 $H_2O$ | 360.0 | g. |
| Copper Sulphate, 5 $H_2O$ | 0.4 | g. |
| Potassium Aluminium Sulphate, 24 $H_2O$ | 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, 6 $H_2O$ | 0.2 | g. |
| Sodium Fluoride | 0.002 | g. |

TABLE 3.

Vitamin-Mineral-Roughage Mixture.

| | |
|---|---|
| Sodium Chloride | 32.5 g. |
| "446" Salt Mixture | 130.0 g. |
| Vitamin Mixture | 250.0 g. |
| Agar Powder | 62.5 g. |
| Vitamised 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 the above mixture.

hours after the dietary protein. In the experiment with rats, an attempt was also made to compare the effect of added carbohydrate with that of added fat.

## E X P E R I M E N T A L.

EXPERIMENT WITH RATS. Adult albino rats were used. They were fasted overnight, weighed, and those weighing about 250 gm. subdivided between the various experimental groups, according to the randomised block technique (Snedecor, 1946), which reduces the effect of slight differences in body weight as a factor in the analyses of the results.

The diet consisted of the following purified ingredients: casein, glucose, margarine, and a vitamin-mineral-roughage (V.M.R.) mixture (Munro, 1949). The composition of the vitamin supplement and of the mineral mixture are given in Tables 1 and 2. The proportions in which these two were mixed with agar (as roughage material) are found in Table 3. The diet provided about 400 mg. of N, 5.3 g. of carbohydrate, 0.8 g. of fat and 40 Cal. (1200 Cal. per sq.m. of body surface area) per rat per day.

They were housed in individual metabolic cages under thermostatic conditions, and fed twice daily, the food being moistened with a little water to prevent spilling. The cages were placed on large funnels which led the urine and

faeces into a receiver, the separation of urine and faeces being carried out by the procedure described by Cuthbertson, McGirr & Robertson (1939). Iron oxide was used as a faecal marker to separate the metabolic periods. After each collection of urine and faeces, which was carried out before giving the morning feed, the funnel was washed with a little dilute acid, which ran into and remained in the receiver, keeping the urine acid during the next 24 hours. The urine and washings collected daily were strongly acidified before storage in Winchester bottles.

EXPERIMENT WITH HUMAN SUBJECTS. The subjects were 4 young adult males. They were allowed to select adequate diets from a limited number of standardised foodstuffs, and this basal diet kept constant throughout the experiment. Three daily meals were taken, between 8.30 and 9 a.m., 12.30 and 1.30 p.m., and 5 and 6 p.m., the weight of each individual item of diet being the same throughout the experimental period. The daily fluid intake and energy expenditure were also kept as constant as possible. From the second day onwards, 24-hour urine collections were made, and the urine analysed for N by the micro-Kjeldhal method of Ma & ZuaZaga (1942).

EXPERIMENT WITH DOGS. The dogs used were two mongrel females, one (Roy) weighing 8 kg. and the other (Jinx) weighing 3.6 kg., belonging to Dr. Mary Lockett and housed

TABLE 4.

Vitamin Mixture for Dogs

based on Allison and Anderson (1945).

The amounts quoted represent a 50-day supply for a dog weighing 10 kg.

Vitamins:

| | |
|---|---|
| Thiamin | 0.25 mg. |
| Riboflavin | 0.25 mg. |
| Niacin | 2.5 mg. |
| Ca pantothenate | 2 mg. |
| Pyridoxine | 0.15 mg. |
| Choline | 150 mg. |
| α-tocopherol | 10 mg. |
| Inositol | 10 mg. |
| *p*-Aminobenzoic acid | 5 mg. |
| Biotin | 0.04 mg. |
| Menaphthone | 0.04 mg. |
| Vitamin A | $5 \times 10^5$ I.U. |
| Vitamin D | $1 \times 10^5$ I.U. |

TABLE 5.

Synthetic Diet for Dogs

based on Cowgill (1923).

| Constituents | Daily Amounts per 10 Kg. body weight |
|---|---|
| Casein | 55 g. |
| Sucrose | 50 g. |
| Lard | 20 g. |
| Margarine | 10 g. |
| Agar | 4 g. |
| Salt mixture (Cowgill No.2) | 3 g. |
| **Cowgill's Salt Mixture No.2:** | |
| Potassium Iodide | 0.75 g. |
| Ferric citrate | 2.7 g. |
| Sodium chloride | 57 g. |
| Magnesium citrate | 49 g. |
| $K H_2PO_4$ | 18.4 g. |
| $CaHPO_4 . 2H_2O$ | 11.7 g. |
| K C L | 10.5 g. |
| Total | 150.0 g. |

in her laboratory. They were perineotomised prior to the experiment for ease of catheterization. The diet was that recommended by Cowgill (1923) and used by Larson & Chaikoff (1937) in their experiment. Vitamin sources were incorporated in amounts similar to those used by Allison & Anderson (1945). The details are given in Tables 4 and 5. The food was weighed and given at about 9.30 a.m. daily, the meal being completed in an hour. To accustom them to the passing of a stomach tube, control stomach tubes were passed during the first 10 days of the experiment, at varying times of the day, and thereafter, only when required. The bladder was emptied by catheter under asceptic conditions every morning before feeding, the urine and washings of the floor of the cage and of catheter being estimated for N daily. The faeces was not estimated, being carefully removed to prevent contamination with cage washings. Roy had to be catheterised twice daily as she could not hold her urine for 24 hours. Throughout the experiment, the animals were under the care of Dr. Lockett, who very kindly attended to the catheterising and feeding herself.

ESTIMATION OF NITROGEN. The method adopted was a modification of the micro-Kjeldhal procedure described by Ma & Zuazaga (1942). The volume of each sample was adjusted to contain about 1 mg. of N. This was digested with 1 ml. conc.

N-free sulphuric acid and a knife-point of a mixed catalyst (3 parts selenium, 2.5 parts potassium sulphate and 2.5 parts copper sulphate). In the case of urine, the digest cleared in 7 or 8 min., and boiling was continued for another 7 or 8 min., the micro-Kjeldhal flask cooled and the sides washed down with 2 ml. distilled water. The distillation was carried out in the apparatus described by Markham (1942), the ammonia liberated by adding 10 ml. 40% (w/v) NaOH being carried by steam and trapped in a conical flask containing 5 ml. 2% (w/v) boric acid and 6 drops of the mixed indicator (5 parts of 0.1% bromocresol green in 95% ethanol to 1 part of 0.1% methyl red in 95% ethanol). Using this method it was found that 99.3% of the N in a standard solution of urea could be recovered with a distillation period of $1\frac{1}{2}$ - 2 min. This period was therefore used in all N determinations. The N starts collecting in the receiver when the first drop of condensed steam creeps down the condenser. The trapped ammonia was titrated against standard 0.01 N sulphuric acid.

The urine was taken for estimation after suitable dilution. The faeces collected during the 4-day periods in the rat experiments were digested in a macro-Kjeldhal flask with about 50 ml. conc. sulphuric acid (analar) and a knife-point of copper sulphate. The digestion was continued for 1 hour after the mixture cleared, the flask cooled, and the

digest and washings of flask made up to a convenient volume. An aliquot of this was again digested with 1 ml. conc. sulphuric acid and selenium catalyst for 15 min. in a micro-Kjeldhal flask, and estimated as before. A similar procedure was used for the food mixtures fed to the rats and dogs, with a drop of mercury as catalyst. The digestion in the macro-Kjeldhal flask was continued for 3 hours after the digest cleared, and digestion continued in a micro-Kjeldhal flask for ½ hour with acid and a small drop of mercury. Selenium has been shown to liberate free $N_2$ when digestion is continued for an hour or more (Patel & Sreenivasan, 1948), but in the short time of digestion necessary for urine and pre-digested faeces, this is not appreciable. Comparison of selenium and mercury as catalyst in estimating food N gave a 5% higher recovery with mercury. As the N in urine and faeces was being compared between experimental periods, selenium was the catalyst used for these estimations, a uniform procedure being followed throughout each experiment.

When mercury was the catalyst employed, the final digest was mixed with 1 ml. saturated solution of sodium thiosulphate in the Markham apparatus, before adding the strong alkali. This precipitates the mercury which would otherwise interfere with the subsequent distillation. When steam is passed through this mixture, the first few gusts of

hot air carry all the sulphur dioxide gas liberated by the alkali, and the collection of the ammonia was started only when the first drop of the distillate reached the bottom of the condenser. This procedure eliminates the possibility of any $SO_2$ collecting in the receiver. A trial was made of using zinc dust to precipitate the mercury, as recommended by Hiller et al. (1948), but this was abandoned in favour of thiosulphate on account of the clogging of the apparatus by beads of zinc.

## R E S U L T S.

a. Experiments with Rats. These were carried out in 2 sets. In the first, 40 animals were distributed into 5 equal groups. The basal diet consisted of 2 g. V.M.R. mixture and 2.5 g. glucose, given at 9 a.m., and a mixture of 3 g. casein, 2 g. glucose and 0.5 g. fat, fed at 5 p.m. This diet was continued for a preliminary period of 7 days to accustom them to the diet, and to the time of feeding. At the end of this period, most of them had learnt to eat their food as soon as it was given, the meals being completed within 1 hour. The collection of excreta was then started. During the first 4-day period, all groups continued on the basal diet. Thereafter, each group was treated differently. One received an extra 3 g. glucose with the evening meal, and

TABLE 6.

The effect on the N balance of rats of adding carbohydrate (glucose) or fat (olive oil) to their diet, along with or apart from dietary protein.

| Group | Treatment during Periods II & III | Mean Initial Body Wt. | Mean Daily Nitrogen Balances | | | | Mean of Periods II & III combined |
|---|---|---|---|---|---|---|---|
| | | | Period I | Period II | Period III | Period IV | |
| | | g. | mg. | mg. | mg. | mg. | mg |
| 1 | Carbohydrate with protein | 242 | -8.4 | +34.3 | +22.9 | -15.6 | +28.6 |
| 2 | Carbohydrate separated from protein | 254 | -0.4 | +28.5 | +18.4 | - 8.8 | +23.4 |
| 3 | Fat with protein | 240 | +19.9 | +24.7 | +21.1 | + 9.1 | +22.9 |
| 4 | Fat separated from protein | 245 | + 5.4 | +27.8 | +25.0 | + 0.2 | +26.4 |
| 5 | No treatment | 247 | - 1.7 | - 8.7 | + 0.5 | - 2.0 | - 4.1 |

One rat in Group 1 died during Period III. This has been allowed for in statistical analysis. There is a significant difference (at the 5% level) between the mean N balances of the 5 groups during Periods II and III, after adjustment by covariance analysis for differences in N balance during Period I. When Group 5 is excluded, the N balances during Periods II and III do not differ significantly.

the second, the same amount of glucose with the morning (protein-free) meal. The 3rd and 4th groups were given 1.34 ml. (1.2 g.) of olive oil with the evening and morning meals, respectively. The 5th group continued on the basal diet with no extra nutrient, and was maintained as the control group. Three rats given extra glucose with the protein-free (morning) meal did not complete the supplemented feed by the end of the period, but the amount left uneaten was not sufficient to reject the data obtained from them. The additional carbohydrate or fat was given daily for 2 successive 4-day periods (periods 2 & 3), and during the last period (period 4), all the groups ate the basal diet only. The feeding dishes were examined regularly, and any portion of the morning meal left unfinished by noon was removed from the cage and given with the diet the following morning. In no instance was the morning meal allowed to be eaten within 5 hours of the protein-containing evening meal. The evening meal was always completed by 6 p.m.

This experiment allows us to compare the effect of isocaloric amounts of surfeit carbohydrate and fat taken along with dietary protein with their effect when taken 5 to 8 hours before the protein containing meal. It also enables us to compare the effect of added carbohydrate with that of added fat. The results are given in Table 6. It will be

seen that during the first 4-day period, when all 5 groups were on the basal diet, the mean N balances of the different groups were essentially the same. The mean value of +19.9 for group 3 is mainly caused by an abnormally high result obtained with one animal, which is probably due to some technical error. In periods 2 & 3, when extra nutrient was given, there was a marked increase in the N balance in all but the control group. Statistical analysis showed that this effect of extra carbohydrate and fat on N balance was significant. The amounts of N retention brought about by surfeit carbohydrate and surfeit fat did not differ significantly; nor did it matter whether the surfeit energy was given with the protein-containing meal or 5 to 8 hours before the protein meal. During the 4th period, when all animals received the basal diet only, there was a decrease in N balance of all the groups, but statistical analysis did not show a greater loss of N by the rats that had received supplements when compared with the control group. We, therefore, see that surfeit carbohydrate and surfeit fat are equally effective in causing N retention, whether given with protein or 5 or more hours before the protein.

In the second experiment, 15 rats were distributed in 3 equal groups. The basal diet was the same as that given in the previous experiments, but the order of feeding

TABLE 7.

The effect on N balance when carbohydrate (glucose) is superimposed on a diet adequate for rats, either along with or 12 hours apart from the dietary protein. During period I, all animals received the unsupplemented basal diet; during period II extra carbohydrate was given except to the control rats.

| Treatment during period II | Initial body weight | Mean daily N Balances | |
|---|---|---|---|
| | | Period I | Period II |
| | g. | mg. | mg. |
| Extra glucose with protein | 239 | -0.3 | +36.4 |
| Extra glucose apart from protein | 249 | -0.8 | +31.8 |
| Control group | 220 | +3.9 | -15.3 |

After adjustment of the values in period II by covariance analysis for differences in N balance during period I, the mean N balances during period II were shown to be highly significantly different ($P < 0.01$). This is entirely due to a difference between the control animals and the other two groups; the groups receiving glucose gave statistically similar results.

was reversed, the protein-containing portion being fed in the morning, and the V.M.R. and glucose at 5 p.m. After a preliminary period of 7 days, urine and faeces were collected for the first 4-day period during which all 3 groups continued to receive the basal diet. During the next 4-day period, one group received an extra 3 g. glucose with the morning (protein-containing) meal, the second was given the same amount of glucose at 9 p.m., while the third group continued on the unsupplemented basal diet. Here we can compare the effect of extra carbohydrate given with protein and given 12 hours after the protein meal. The results, shown in Table 7, confirm that surfeit is equally effective when administered with and apart from the protein, the N balances in Period 2 of the first two groups being statistically similar. Both these groups differ very significantly ($P < 0.01$) from the control group. In this experiment, therefore, glucose caused the same amount of N retention when given 12 hours after the protein as when given along with the protein.

b. Experiment with Dogs: The two dogs, Roy (8 kg. body weight) and Jinx (3.6 kg.) were given 115 g. and 60 g. respectively, of a diet mixed in the proportions given in Tables 4 & 5. From the 2nd day onwards, the urinary N was estimated. During the first 7 days, the N values were very

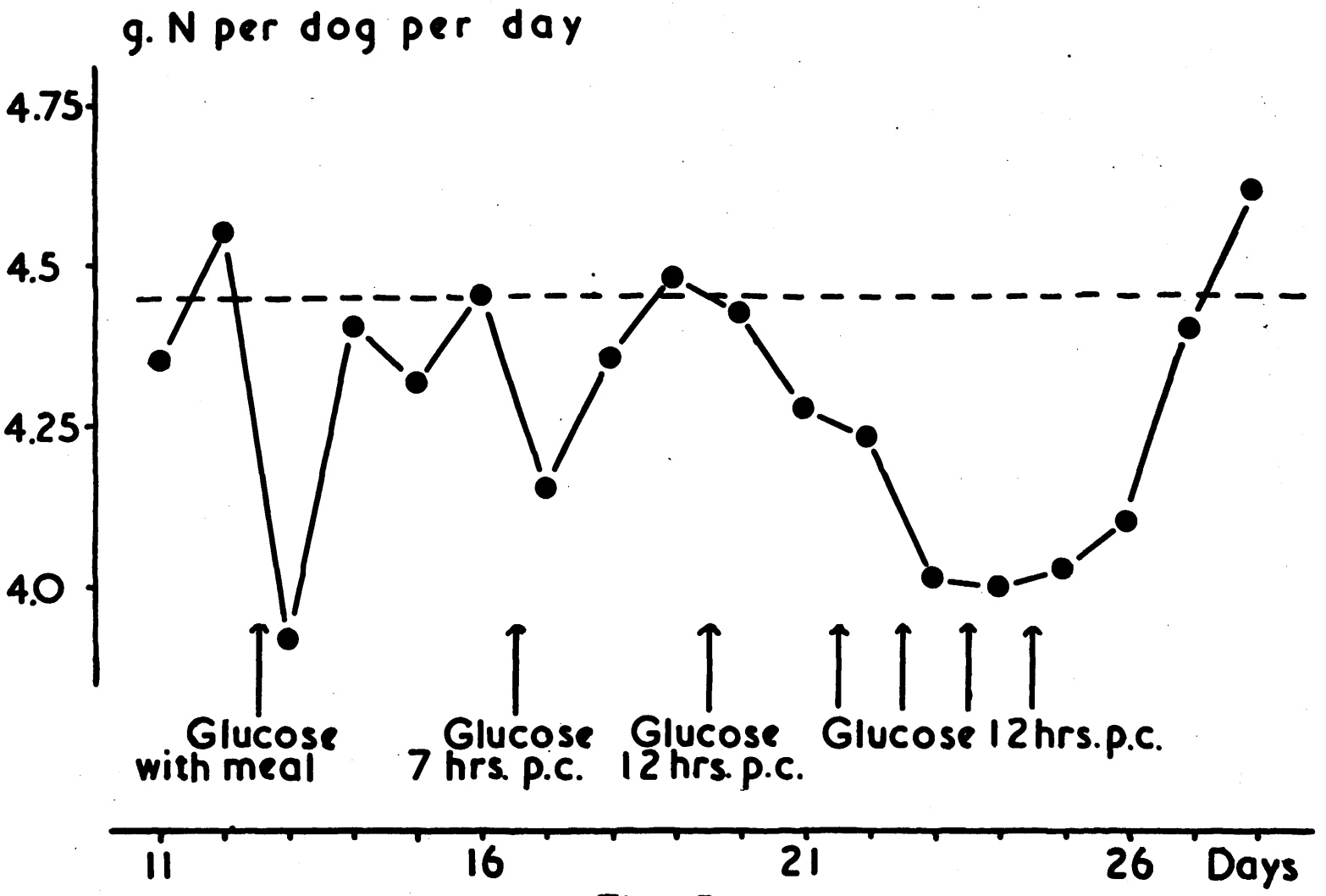



Fig. 3
Mean of two dogs' N output in urine

erratic, due probably to the fact that the animals were getting accustomed to the diet. From the 8th to the 12th day, the N excreted remained at a steady level. On the 13th day, glucose dissolved in a minimum amount of water was given by stomach tube immediately after the single (protein-containing) meal was eaten, Roy getting 50 g. and Jinx 30 g. glucose. This produced a sharp and immediate drop in the urinary N (Fig.3), showing that glucose was highly effective in sparing N. The N excretion regained its former value the next day and remained steady thereafter till the 17th day of the experiment, when the same amounts of glucose were administered as before, but 7 hours after the protein-containing meal. This, again, produced a retention of N, but the amount retained was about half that obtained when glucose was given with protein. Two more days on basal diet alone brought the N excreted to the base line. On the 20th day, the glucose was given 12 hours after the protein meal, but produced no effect on the N excretion on that day. Glucose was again given at the same time the next day and continued for a further 3 days. Fig.3 shows that the continued administration of glucose produced a definite and sustained effect on N balance, the drop in urinary N being of the same order as that obtained when glucose was given with the protein meal. Urinary N remained at this low level as long as the

TABLE 8.

The composition of the basal diets consumed by the subjects in the human experiment. The constituents have been calculated from tables of food composition (McCance & Widdowson, 1946).

| Subject No. | Body Weight | Daily Basal Diet | | | |
|---|---|---|---|---|---|
| | | Protein | Fat | Carbohydrate | Energy |
| | kg. | g. | g. | g. | cal./kg. |
| 1 | 70.8 | 112 | 144 | 454 | 50 |
| 2 | 77.9 | 102 | 115 | 392 | 39 |
| 3 | 67.7 | 102 | 141 | 394 | 48 |
| 4 | 65.7 | 79 | 104 | 398 | 45 |

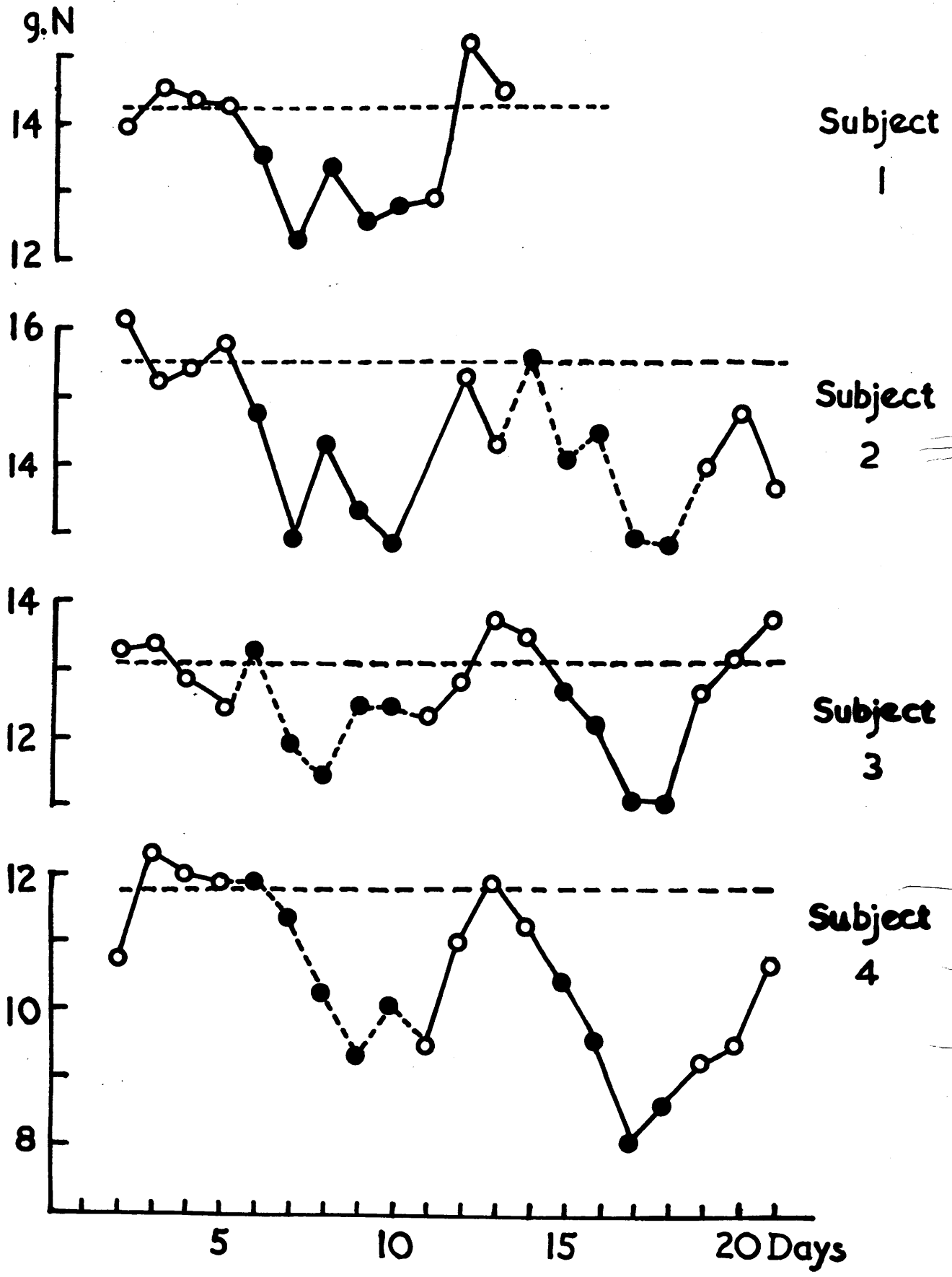

g.N
14
12
Subject 1
16
14
Subject 2
14
12
Subject 3
12
10
8
Subject 4
5
10
15
20 Days

Fig. 4

sugar was given, i.e., till the 25th day, and began to rise to former levels only on the 27th day. Thus, it is clear that surfeit glucose shows an N-sparing effect whether given with the protein or apart from it. The effect on N balance is immediate when carbohydrate is fed with protein, but when given 12 hours after the protein meal, the effect is shown only from the second day onwards.

c. Experiment with human subjects: This experiment was carried out in order to see whether the effect of surfeit energy demonstrated in the case of rats and dogs was the same on humans. The composition of the diets selected by the subjects is given in Table 8. The basal diet was taken for 5 days, till the N in the urine reached a fairly constant level (Fig.4). Assuming the faecal N to be less than 1.5 g. per day, all the subjects were then in positive N balance on the basal diet. For the next 5 days, 200 g. of sucrose was given daily, in addition to the basal diet, subjects 1 & 2 taking the sucrose with the protein-containing meals, at 9 a.m., 1 p.m., and 6 p.m., while subjects 3 &4 received it at 11.30 p.m., 5½ hours after the last meal, in water with the juice of one lemon. Subjects 1 & 2 drank water only at 11.30 p.m. After this 5-day period, the subjects again ate the unsupplemented diet for a few days, until the N output returned to the basal values obtained at the start of the experiment. Then, a second period of surfeit feeding was

TABLE 9.

The effect of superimposing 200 g. of sucrose on an adequate basal diet, at one occasion along with the meals, and at another, apart from the meals.

| Subject | Changes in Output of Urinary Nitrogen | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Extra Carbohydrate with meals | | | | | Extra Carbohydrate apart from Meals | | | | |
| | 1st Day | 2nd Day | 3rd Day | 4th Day | 5th Day | 1st Day | 2nd Day | 3rd Day | 4th Day | 5th Day |
| 1 | +0.73 | +1.99 | +0.87 | +1.67 | +1.44 | - | - | - | - | - |
| 2 | +0.85 | +2.72 | +1.27 | +2.30 | +2.77 | +0.03 | +1.50 | +1.09 | +2.06 | +2.72 |
| 3 | +0.30 | +0.84 | +1.93 | +1.96 | - | -0.24 | +1.06 | +1.54* | +0.53 | +0.56 |
| 4 | +1.34 | +2.16 | +3.64 | +3.09 | - | -0.09 | +0.40 | +1.54* | +2.37 | +1.65 |
| Mean for Subjects 2, 3 & 4 | +0.83 | +1.91 | +2.28 | +2.45 | - | -0.10 | +0.99 | +1.39 | +1.83 | +1.64 |

The change in N output caused by sucrose was measured from the mean N output of the subject during the 1st period on the basal diet. A + sign indicates N retention as a result of adding carbohydrate to basal diet. During the 1st surfeit period, sucrose was taken with the meals by subjects 1 & 2, and apart from meals by subjects 3 & 4. The difference in response to extra carbohydrate fed with meals and apart from meals is statistically significant for the 1st day of surfeit feeding ("t" = 3.00; P = 0.05 - 0.02).

*The 24-hour specimens from these two subjects were accidentally mixed; the figure given is based on the average N content of the combined urines.

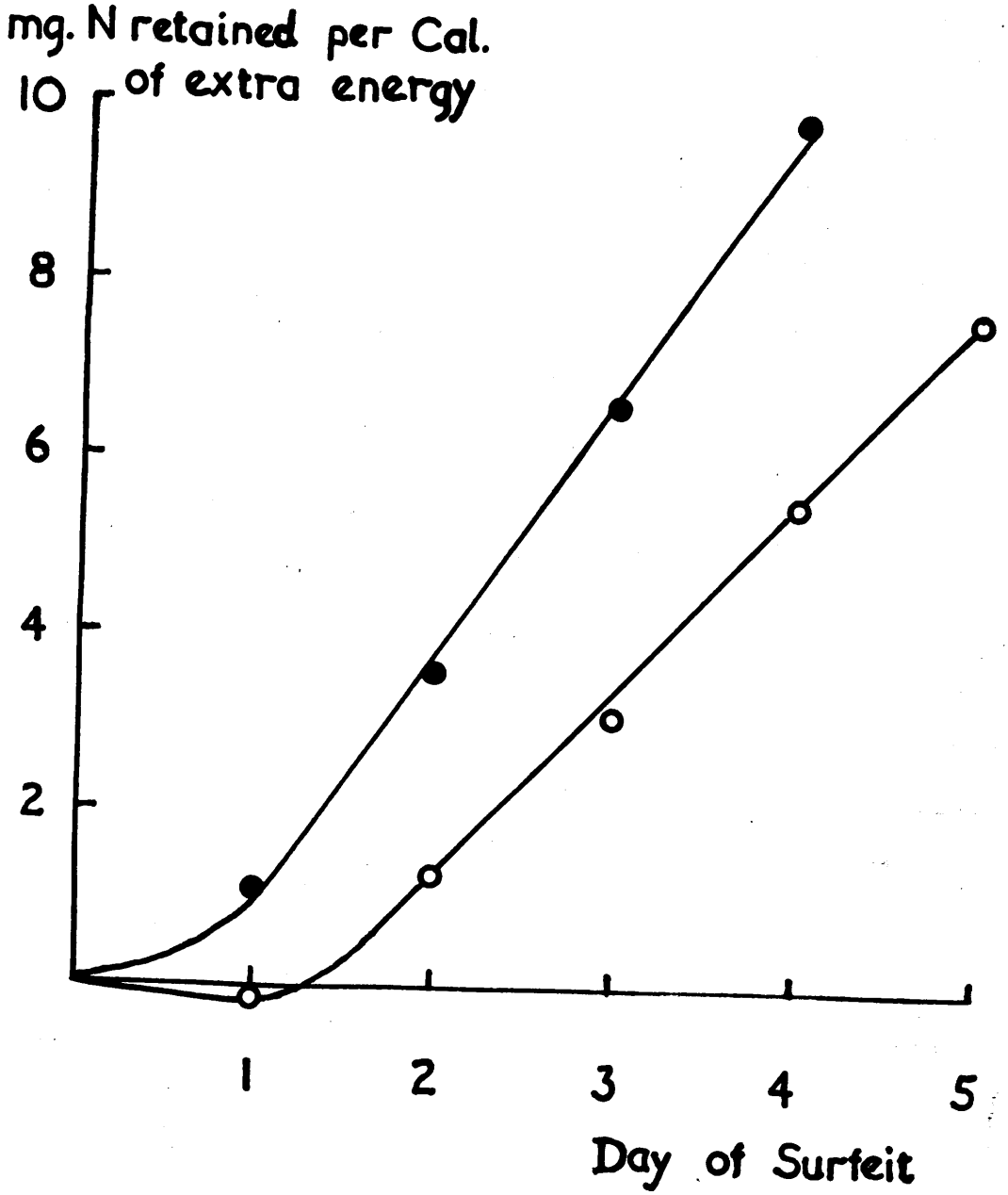

mg. N retained per Cal.
of extra energy
10
8
6
4
2
1
2
3
4
5
Day of Surfeit

Fig. 5

begun, the time of giving the extra sucrose being reversed; i.e., subjects 1 & 2 received the extra carbohydrate at 1130 p.m., in water, and subjects 3 & 4 took it with the other three meals, drinking water with the juice of one lemon at bedtime. Subject 1 developed a febrile illness during this period of surfeit feeding and his experiment had to be abandoned.

This extra carbohydrate caused a fall in N output whether taken along with or 5½ hours after the protein-containing meals. The protein-sparing action of sucrose was evident on the first day of surfeit feeding when it was given along with the other meals, whereas, when the extra sugar was eaten 5½ hours after the last meal, the reduction in N output only occurred from the 2nd day onwards (Fig.4). The changes in urinary N during the two periods are analysed in Table 9. The difference in behaviour on the first day of surfeit feeding was found to be statistically significant. The daily N retentions were transformed into cumulative curves (Fig.5), and the regression lines examined. After the initial delay in starting, the increments in N retained follow a linear slope, which, in the case of carbohydrate fed with protein, averaged 2.9 mg. N per extra Cal. per day, and in the case of carbohydrate given apart from the protein, averaged 2.1 mg. N. To see whether there is a real difference between these slopes the regression equations for

individual subjects were analysed statistically. The slopes were approximately linear for all three subjects, but did not differ during the two surfeit feeding periods for subject 2. In the case of subjects 3 & 4, the rate of increase in N retention was significantly less when the extra carbohydrate was taken apart from the dietary protein. This may be related to the order in which these experiments were carried out. It should also be remembered that only the urinary N was estimated, the faecal N being assumed to be constant. It is possible that the ingestion of a large amount of sugar with the other meals produced an irritation of the bowel and an increase in faecal N. Taking the results as they stand, however, there seems to be a small advantage in giving the surfeit carbohydrate along with the protein-containing meals.

## DISCUSSION.

a. The time factor in protein-sparing by added energy: These experiments prove conclusively that energy added to an already adequate diet spares protein, whether it be given with or separate from the protein-containing meals. Both in the human experiments and in the ones on dogs, it is clear that, when extra energy is given apart from the protein meal,

little or no effect is observed on the N excreted on the first day, the sparing of protein becoming evident only on the second or third day of such addition (Figs. 3 & 4). This agrees with the findings of Cuthbertson & Munro (1939). In the experiment of Larson & Chaikoff (1937) illustrated in Fig.2, surfeit energy given on single occasions 4 hours after the protein meal had no effect on N excretion. Repetition of their experimental conditions shows that this phenomenon is reproducible (Fig.3), but that, with continued administration of extra carbohydrate separately from the dietary protein, there is a beneficial effect on N balance. The reason for the time effect observed by Larson & Chaikoff may be related to the basal diet they used, in which only a small proportion of the total energy is in the form of carbohydrate. It may thus be that their results were due to an interaction effect between the dietary protein and the extra carbohydrate fed at the same meal (Fig.1); this might be expected to come into play if the basal diet were deficient in carbohydrate.

In the experiments with rats, the amount of N retained per Cal. of surfeit nutrient was the same, whatever the time interval between the surfeit food and the protein-containing meal. In the human experiment, however, subject 2 showed no difference in his reaction to the time of taking extra

TABLE 10.

Comparison of the protein-sparing action of extra carbohydrate and extra fat added to adequate diets under comparable conditions.
(Calculated from experiments recorded in the literature).

| Species | Authors | Expt. No.* | Surfeit Fed | | | N Retained** per Cal. extra energy per day |
|---|---|---|---|---|---|---|
| | | | Nature | Extra daily energy | Duration | |
| | | | | Cal. | Days | mg. |
| Growing Rat | Forbes, Bratzler, Thacker & Marcy (1939) | Expt. 1 | Carbohydrate | 11.3 | 7 | 5.3 |
| | | | Fat | 11.5 | 7 | 5.0 |
| | | Expt. 2 | Carbohydrate | 10.8 | 7 | 4.4 |
| | | | Fat | 11.1 | 7 | 4.8 |
| Adult Rat | Forbes, Bratzler, Thacker & Marcy (1939) | Expt. 1 | Carbohydrate | 10.6 | 7 | 3.9 |
| | | | Fat | 10.7 | 7 | 4.5 |
| | | Expt. 2 | Carbohydrate | 10.4 | 7 | 4.3 |
| | | | Fat | 10.4 | 7 | 3.3 |
| | Forbes & Swift (1944) | - | Carbohydrate | 13.4 | 8 | 3.6 |
| | | | Fat | 13.6 | 8 | 7.4 |
| | Present Experiments | Expt. 1 | Carbohydrate | 11.3 | 8 | 2.9*** |
| | | | Fat | 10.7 | 8 | 2.5*** |
| | | | Carbohydrate | 11.3 | 8 | 2.4+ |
| | | | Fat | 10.7 | 8 | 2.8+ |
| Adult Man | Cuthbertson & Munro (1937) | Expt. 2 | Carbohydrate | 780 | 3 | 0.9++ |
| | | | Fat | 700 | 3 | 0.7 |
| | | Expt. 3 | Carbohydrate | 780 | 5 | 1.8++ |
| | | | Fat | 700 | 6 | 1.2 |

Please see next page for notes.

* As referred to in the original publication

** Estimated for the human subjects by comparing the N balance of each subject on the basal diet alone and on the basal diet with supplement; estimated for the rats by comparison with a control group run in parallel and not receiving extra energy.

*** Extra carbohydrate or fat given with protein of diet.

\+ Extra carbohydrate or fat given separately from protein of diet.

++ As measured from the first period on the basal diet.

sugar, whereas subjects 3 & 4 showed a greater retention of urinary N when sucrose was taken with the other meals. Since the faecal N was not estimated in this experiment, the data obtained from the rat experiment are probably the more reliable.

In the experiments on human subjects and on dogs, the amount of N retained daily was maintained constant after the second day of surfeit feeding (compare Figs. 3 & 4 with Fig. 1). These short-term experiments thus agree with the long-term ones reported by Krug (1893) and Larson & Chaikoff (1937), who found that the surfeit energy was effective up to the 15th day in the case of man and the 7th day of adding surfeit to the diet of dogs. In the experiments with rats, however, N retained in Period 3 (Table 6) is slightly less than that in Period 2, in agreement with the finding of Lathe & Peters (1949a) that the effect on rats was most marked during the first few days of surfeit feeding.

b. Comparison of the relative effect of carbohydrate and fat as energy sources: The experiment on rats enables us to compare the effect of extra carbohydrate and of extra fat. Table 10 contains data which are calculations based on results published by other workers. It will be seen that there is a great deal of variation in the values reported. Cuthbertson & Munro (1937) found that surfeit carbohydrate had a

slightly greater beneficial effect on N retention per Cal. than surfeit fat, when only urinary N was considered. When the faecal N is also taken into account, the retention of N per Cal. is seen to be the same with carbohydrate and with fat, for one subject, while it remains slightly better with carbohydrate for the second subject. In studies on the adult rat, Forbes et al. (1939) obtained data which indicate a slightly greater effect due to surfeit fat in one experiment, and a greater effect due to carbohydrate on another occasion. In the case of growing rats, their data reveal no difference between the sparing effect of extra carbohydrate and extra fat. However, Forbes & Swift (1944) found that lard caused a N retention of 7.4 mg. N per extra Cal. per day, whereas the effect of glucose was only a retention of 3.6 mg. N. Later experiments reported from the same laboratory emphasise the difficulty of obtaining consistent evidence that carbohydrate is superior to fat, or vice versa. (Because of their different experimental nature, they are not included in Table 10) Animals were kept for 15 days on a so-called "supermaintenance" (i.e., surfeit) diet and N balance was measured during the last 8 days of that period. The proportion of energy coming from carbohydrate and from fat was varied. In the first series of experiments (Forbes et al., 1946a), N retention was slightly, though not

significantly, better when most of the energy came from fat. In the second series, the amounts of certain vitamins were increased, and the diet containing more carbohydrate now caused a significantly higher N retention than the diet rich in fat (Forbes et al, 1946b).

In our experiments, carbohydrate seems to be more effective than fat when the surfeit is given along with protein, while the position is reversed when the surfeit is separated from the protein. These differences are, however, not statistically significant. Taking into account all the data in Table10, it seems justifiable to conlude that, for equal additions of energy, carbohydrate and fat cause approximately the same degree of N retention when superimposed on a diet already providing adequate amounts of energy.

c. Significance of Carbohydrate and Fat in Protein Utilisation:

In view of our findings on surfeit feeding, it is now possible to construct a picture of the part played by carbohydrate and fat in ~~protein~~ utilisation of dietary protein. First, there is the interaction of dietary protein with carbohydrate given in the same meal (Fig.1). This does not occur with fat. Secondly, there is the relationship between protein metabolism and the energy supplied in the diet. This is independent of the time of feeding the

energy-yielding nutrients, and is shared by both carbohydrate and fat, probably strictly in proportion to the energy they yield. Some time elapses (24 - 48 hours) before an increase in energy intake is fully effective (Figs. 3 & 4). In addition to these two clear-cut phenomena, we probably get a mixed picture, as in the experiments of Larson & Chaikoff. These do not, however, invalidate those other experiments in which the distinction of one effect from the other can be made.

d. Mechanism of Action of surfeit Carbohydrate and Fat:

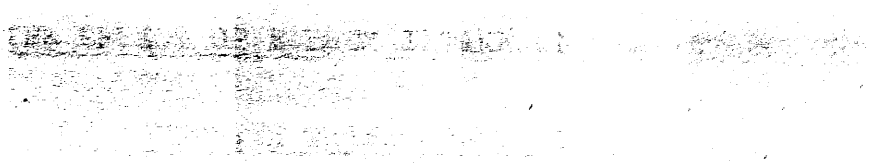
If surfeit carbohydrate and fat cause a retention of N, how is this brought about? Interference with digestion and absorption of proteins is ruled out by the fact that surfeit energy is effective even when the protein of the diet is fed 5 to 12 hours before or after the extra nutrient. It is possible that breakdown products of carbohydrate and fat might help in the production of non-essential amino-acids, but it seems likely that the basal diet has already sufficient carbohydrate for this purpose.

The most plausible explanation is that carbohydrate and fat exercise their effect on N balance by providing extra energy for protein formation. Cuthbertson, McGirr & Munro (1937) showed that when a subject's energy requirements were raised by exercise, surfeit feeding did not improve N

balance. N retention can thus be prevented by using the additional food for supplying energy for exercise. Therefore, the essential factor for N retention is not the intake of extra food, nor the release of surfeit energy, but the presence of energy-yielding metabolites in the tissues, over and above the daily requirements. The N lost when energy is withdrawn from the diet would presumably be due to a subnormal level of these metabolites in the tissues.

It seems possible that giving additional energy results in the laying down of proteins in the body, the energy-yielding metabolites taking part in some phase of amino-acid metabolism or by providing energy for protein synthesis. That peptide-bond synthesis can be stimulated by members of the tricarboxylic acid cycle has been shown by in vitro studies of p-amino hippuric acid formation by rat liver homogenates (Cohen & McGilvery, 1947), and by the formation of ornithuric acid by chicken kidney residues (McGilvery & Cohen, 1950). The rate of synthesis of peptide-bonds in body proteins could similarly be affected by changes in the supply of tricarboxylic acid cycle intermediates. A considerable increase in the synthesis of ATP has been observed shortly after the administration of glucose (Kaplan & Greenberg, 1944), and it seems possible that surfeit carbohydrate and fat may stimulate protein

synthesis in the tissues by providing energy in the form of ATP. This will be developed at greater length in the next section.



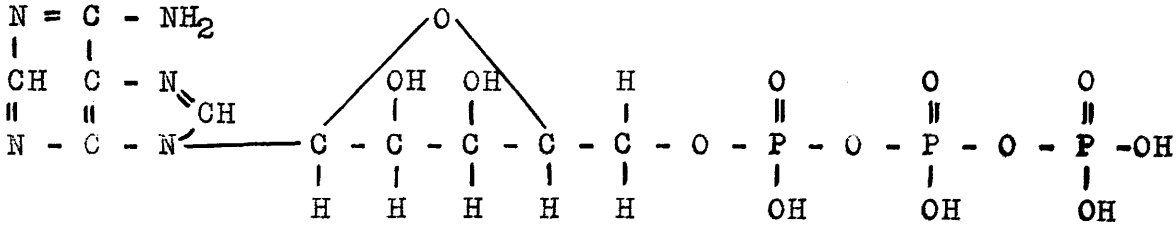
# PART II.

# THE EFFECT OF ENERGY INTAKE ON THE METABOLISM OF ADENOSINE TRIPHOSPHATE IN THE LIVER.

## INTRODUCTION.

In the previous section, it was concluded that the plane of energy intake influences protein metabolism by yielding energy-rich metabolites which help in the formation of body protein, probably through the intermediation of adenosine triphosphate (ATP). The part played by organic phosphates in intermediary metabolism was unknown till Harden (1905) discovered the phosphorylation of hexoses in alcoholic fermentation. Not much importance was attached to this observation until similar hexose phosphates were found in muscle (Embden, 1915), and Harden's dialyzable organic "co-ferment of fermentation" was discovered in muscle and other animal tissues (Meyerhof, 1917). About 10 years later, it was discovered that the formation of the sugar ester was followed by a long series of phosphate compounds by successive metabolic steps (Eggleton & Eggleton, 1927; Fiske & Subbarow, 1929; Warburg & Christian, 1939; Lipmann, 1940). The transfer of phosphate from one compound to anotner took place by transphosphorylation, without passing through the stage of inorganic phosphate, and some of the intermediate phosphate compounds as well as the co-enzymes involved in these reactions were shown to contain phosphate bonds of much higher energy content than "ordinary" esters, releasing up to 10 times as much energy on dissociation as ordinary phosphate ester bonds (Meyerhof & Suranyi, 1927). The

hydrolysis of a high-energy bond yields about 12000 Cal. per g. mol. of phosphate liberated, as compared with the 2000 to 3000 Cal. which are liberated by the hydrolytic fission of energy-poor phosphate esters. The 12,000 Cal. condensed in the energy-rich phosphate bond, " ~ ph", represents a biological energy-unit. Migrating continuously from compound to compound, the quantity of ~ ph in many respects can be regarded as being largely independent of the compound to which it is attached (Lipmann, 1943). The distribution of this unit is handled by a special pooling and transfer system, the adenylic acid system, consisting of adenylic acid or adenosine monophosphate (AMP), adenosine diphosphate (ADP) and ATP. The first phosphate in ATP is an ordinary ester



Adenosine triphosphate

phosphate. The two end phosphates are bound in an energy-rich pyrophosphate linkage (Lohman, 1935); the special importance of ATP arises from the fact that, under the influence of the appropriate enzymes, its terminal phosphate radical, together with the energy of the energy-rich bond, can be transferred intact into other substances, so that the energy is, as it were, forced into the phosphate acceptor.

It is probable that only the terminal ~ph is used directly, and the shuttling of ~ph is facilitated through alternating phosphorylation of ADP and dephosphorylation of ATP (Kalckar, 1941; 1942). Because these reactions were all part of the energy metabolism of the cells and tissues, it became clear that the biological meaning of phosphorylation consists in the generation of high-energy phosphate, whose energy is either transformed into other chemical bonds, or even into other forms of energy. The adenine nucleotides have the ability to take part, as substrate, in a wide variety of enzyme-substrate combinations, so that AMP or ADP can accept phosphate from a large number of energy-rich phosphate compounds such as phosphopyruvate, diphosphoglycerate, and acetyl phosphate (Green & Colowick, 1944) formed at the direct expense of metabolism. The energy-rich phosphate bond of ATP so formed can either be stored as phosphocreatine, or be utilised by hydrolysis of ATP.

Utilisation of Phosphate-Bond Energy: When the metabolically generated ~ph reaches the adenylic acid pool, it becomes available for utilisation. The following examples illustrate how general are the uses of phosphate bond energy:

a. Phosphate bond energy plays an active part in carbohydrate metabolism, taking part in such reactions as the conversion of fructose to glucose (Cori & Shine, 1936) and the formation of glycogen (Cori, 1939; Colowick & Sutherland,

1942).

b. The work of Verzar & Laszt on the intestine, and of Lundsgaard & Kalckar on the kidney indicate a connection between glucose transfer through a cell and its phosphorylation. Energy-rich phosphate bonds have been shown to donate the phosphate group required for the phosphorylation (Lipmann, 1941).

c. In muscle activity, a splitting of ATP initiates the succession of events that results in contraction (Meyerhof, 1944), the energy of the ATP splitting going more or less directly into the mechanical process (Szent-Gyorgi, 1947).

d. Phosphate bond energy can be transformed into light energy. ATP can phosphorylate luciferin, and with the breakdown of luciferin phosphate, light is emitted in an extract of fireflies, the light intensity being proportional to the amount of ATP split (McElroy & Strehler, 1949; McElroy, 1951).

e. ATP is of great importance in the generation of bioelectricity. For the rapid movement of ions during the passage of a nerve impulse, there must be rapid changes in the resistance of the membrane of the nerve. The release and breakdown of acetyl choline have been shown to be essential events in the alterations of the membrane during activity, and ATP provides the energy for acetyl choline formation, the release and removel of acetyl choline

preceding the breakdown of ATP (Nachmansohn, 1951).

f. ATP has been shown to take part in transmethylation. In the biosynthesis of N-methylnicotinamide from l-methionine and nicotinamide, ATP has been shown to activate the methyl donor, l-methionine (Cantoni, 1951a). It has been suggested that this activation of methionine by ATP might be the first step in all transmethylation reactions in which methionine is the methyl donor (Cantoni, 1951b).

g. In the mechanism proposed by Krebs to explain the formation of urea, ammonia and carbon dioxide were added to ornithine to form citrulline (Step I), and a second molecule of ammonia was added to citrulline to give arginine. In the final step, arginase splits off urea, and the ornithine liberated is returned to the cycle (Krebs, 1942). Both Step I and Step II have been shown to require catalytic amounts of ATP (Cohen & Hayano, 1946, 1948; Grisolia, 1951). Step II has been further subdivided into two stages, citrulline and aspartic acid (the specific N donor) condensing to give an intermediate compound, which is then hydrolysed to arginine and malic acid. The condensation is dependent on the high-energy phosphate present, the condensing activity increasing to a maximum with increasing amounts of ATP (Ratner & Pappas, 1949; Ratner & Petrack, 1951; Ratner, 1951).

h. The Synthesis of Peptide Bonds. Though the mechanism of the biosynthesis of peptide bonds is not clearly 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 & Dubnoff (1940) showed that the position of equilibrium of a system such as

alanine + glycine $\rightleftharpoons$ 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 that would release the energy to satisfy the requirements for peptide 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 & McGilvery, 1947); (iii) glutamine (Frei & Leuthardt, 1949); (iv) glutathione (Johnston & Block, 1951); (v) ornithuric acid (McGilvery & Cohen, 1950). In system (iii), no phosphorylated intermediate could be found, but the close relationship between the amount of glutamine formed and the amount of inorganic P liberated indicates that ~P from ATP is utilised. The synthesis of

TABLE 11.

The composition of the rat diets and the times of feeding.

| Group | 10 a.m. P.C.F. | 5 p.m. V.M.R. | 10 p.m. olive oil | Energy Level |
|---|---|---|---|---|
| | g. | g. | ml. | Cal./rat/day |
| 1 | 5.3 | 2 | - | 28 |
| 2 | 5.3 | 2 | 1.7 | 42 |
| 3 | 5.3 | 2 | 3.4 | 56 |

P.C.F. = Protein-Carbohydrate-Fat Mixture

containing casein 2.8 g.

fat 0.5 g.

glucose 1.0 g.

starch 1.0 g.

V.M.R. = Vitamin-Mineral-Roughage mixture

(see Table 3 ).

glutathione comes closest to protein synthesis, and requires ATP, inorganic P, $Mg^{++}$ and $K^{+}$.

As this last section indicates, there are some grounds for supposing that ATP may play a part in protein synthesis, and this is strengthened by Winnick's (1950) experiments. He found that a homogenate of rat liver lost its ability to incorporate $^{14}C$-glycine or alanine when dialysed, but that this was restored by adding ATP along with Mg ions and a mixture of non-labelled l-amino acids. Since our nutritional studies can best be explained by the supposition that the level of some energy donor affects protein synthesis, we carried out experiments to determine whether changes in the ATP concentration in the liver were compatible with its being the intermediary substance.

## EXPERIMENTAL.

Animals, Diet and Management: These were the same as in the rat experiments dealt with in Part I. Table 11 gives the details of the diets given in these experiments.

Estimation of ATP: There are three methods available for estimating ATP in the tissues: by acid hydrolysis, by chromatography and by the use of enzymes.

In the acid hydrolysis method, the solution of ATP is heated with N.HCl for 7 min. (Kaplan & Greenberg, 1944) or

for 10 min. (Bailey, 1949), when the two labile phosphate groups are split off. The value obtained by estimating this P multiplied by 3/2 gives the total ATP P. But the procedure also splits off the terminal P from ADP, and also from other phosphate esters that might be present, and cannot therefore be used when the preparation of ATP is contaminated by these labile P-containing compounds.

In the paper chromatography method (Turba & Turba, 1951), it is possible to separate ATP from ADP, AMP and inorganic phosphate. But it is not known whether any ATP is broken down during the process of separation, and this is quite likely. Any P so liberated might contaminate the ADP eluted from the paper. The method does not appear to be quantitative.

The enzyme method, developed by Kalckar (1947) and Bailey (1949), has the advantage of being specific, provided the enzyme preparation is pure. By a suitable choice of enzymes, it is possible to differentiate between the two labile phosphate groups of ATP. Thus, myosin splits off the end phosphate group:

$$ATP \xrightarrow{\text{myosin}} ADP + H_3PO_4 \qquad (1)$$

and the P set free can be estimated. If the solution is incubated with a mixture of myosin and myokinase, both the labile phosphate groups are set free by a combination of the

following reactions:

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

$$ATP \xrightarrow{\text{myosin}} ADP + H_3PO_4 \qquad (3)$$

At the end of the reaction, AMP and inorganic P remain, and the P estimated less the P split off by myosin alone gives an estimate of the amount of ADP originally present.

Another enzyme, potato apyrase, has been used for the estimation of ADP. It splits off both labile phosphate groups of ATP (Colowick & Kalckar, 1943). Its action is not specific, however, and shows a feeble inorganic pyrophosphatase activity and a still feebler action upon adenylic acid (Bailey, 1949), but, except for very precise analysis, these disadvantages are not serious. Kalckar (1944) successfully applied this enzyme in differentiating the two labile phosphate groups of ATP in which $^{32}P$ had been incorporated. Krishnan (1949) also commented on the non-specific nature of the action of apyrase, and used various dilutions of his enzyme preparation to estimate a known amount of ATP, and obtained a dilution which split off 66% of the P at infinite time. This dilution was then used for assay of unknown ATP solutions.

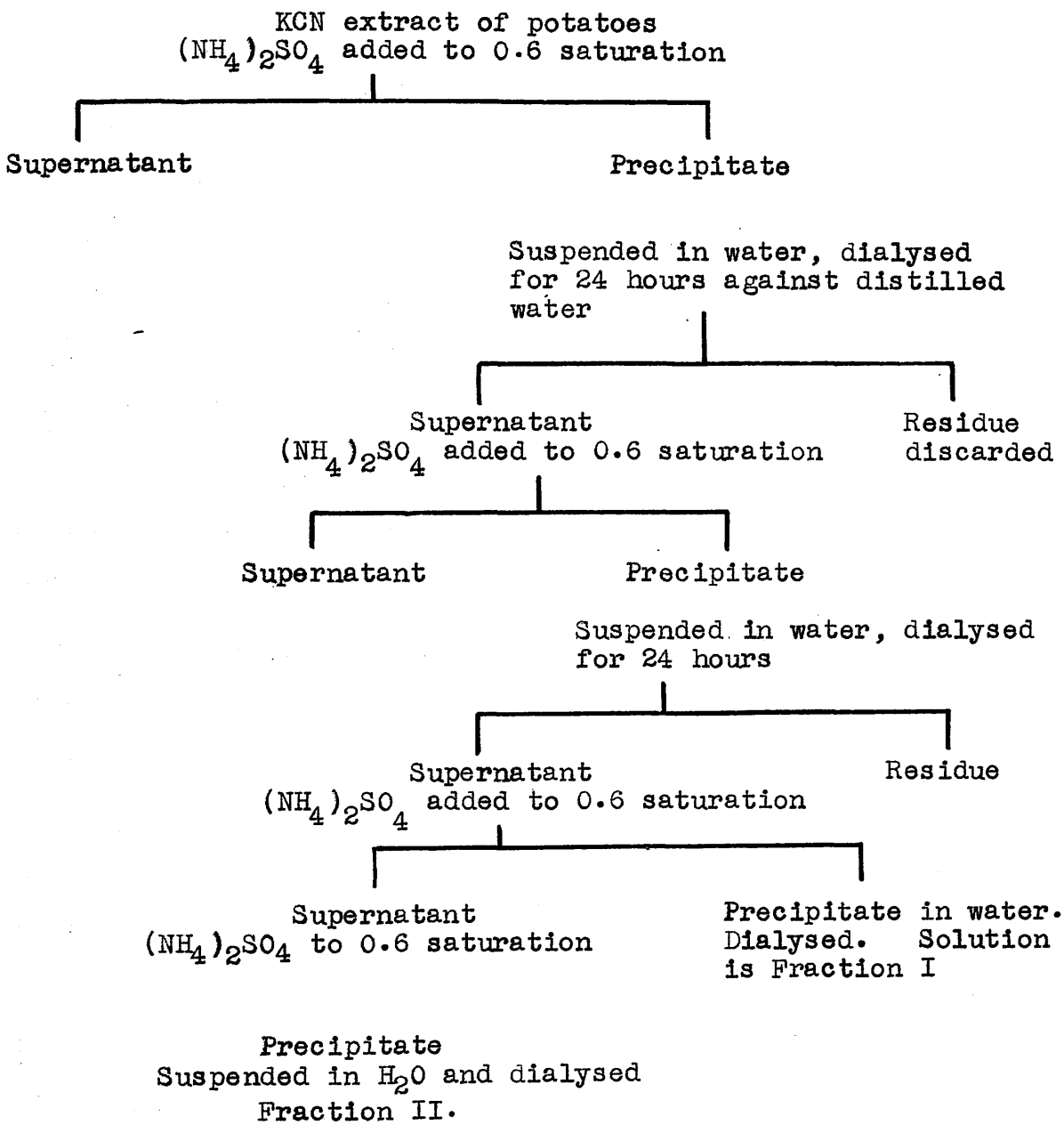
The assay of mixtures of ATP and ADP can also be achieved by the use of hexokinase acting singly and in conjunction with myokinase (Kalckar, 1943). Unfortunately,

the preparation of pure yeast hexokinase is a formidable task (Bailey & Webb, 1948), and less pure preparations may be contaminated with ATP-ase (Keilly & Meyerhof, 1948).

The use of myosin and ATP-ase alone and in conjunction with myokinase thus appears to be the ideal, at any rate, in theory, for the determination of mixtures of ATP and ADP (Bailey, 1949). We, therefore, decided to use these enzymes, and also checked the ADP content by using the potato enzyme.

The Preparation of Enzymes: Myosin: The method used was that of 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 vol. of ice-cold distilled water and adjusting the pH to 6.8. The precipitate was centrifuged, and purified by repeatedly dissolving in 0.5 M KCl and reprecipitating by dilution. The enzyme obtained after 4 precipitations was dissolved in a minimum of 0.5 KCl and stored at 0°C. Its activity was tested with a standard solution of ATP, the incubation procedure of Bailey (1949) being followed. With low concentrations of ATP it was found that long incubation periods were required to hydrolyse all the available P of the terminal group. This was also reported by Bailey (1949), who found that the main disadvantage in the use of myosin was the slow hydrolysis of ATP in the later stages of

## Preparation of Potato Apyrase.



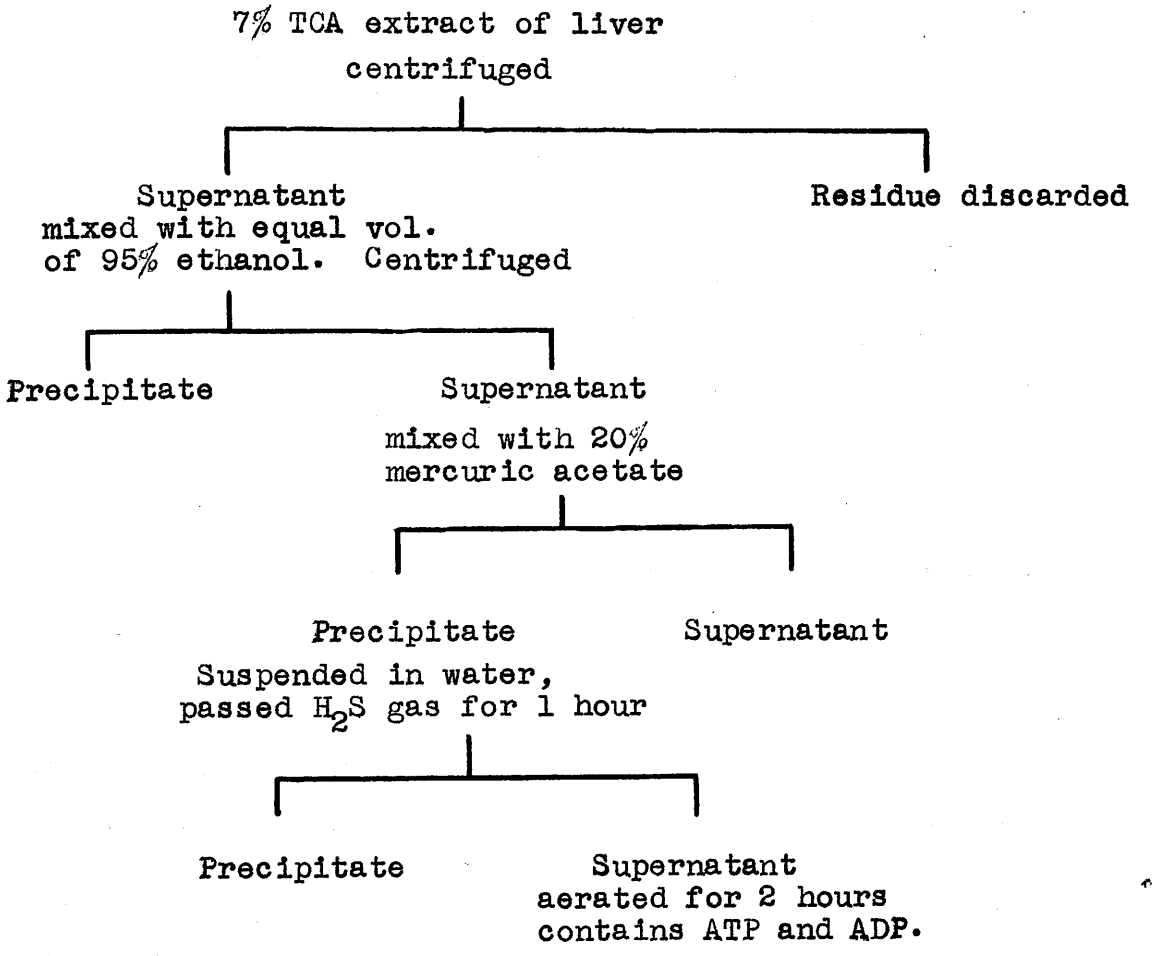

SCHEME 1.

the reaction, when the substrate is almost exhausted and inorganic phosphate has accumulated. We found that, the lower the concentration of ATP, the less P was split by the myosin. Accordingly, care was taken to keep the volume of the solution of ATP extracted from the livers as small as convenient, and the volumes of enzyme and substrate used for incubation were so adjusted that 30 to 33% 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.

Myokinase: The method of preparation was that described by Kalckar (1947). 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, more proteins were separated by half-saturating with $(NH_4)_2SO_4$, and the enzyme precipitated by full saturation with the salt. It was purified by dialysing against dilute $(NH_4)_2SO_4$, and its activity assayed using a solution of CaATP.

Apyrase: This enzyme was prepared from fresh potatoes according to the procedure followed by Krishnan (1949). The method is shown in Scheme I. Most of the activity was found to reside in Fraction II, which was used in subsequent assays. On account of the non-specificity of this enzyme, an experiment was carried out to determine the concentration of enzyme

## Isolation of ATP and ADP from liver.




SCHEME 2.

and the period of incubation required to split 2/3 of the total P in a standard solution of ATP. Various dilutions of fraction II were incubated following the procedure adopted by Krishnan. It was found that a 1 in 25 dilution liberated 2/3 of the P in pure ATP solutions in 15 min., and that the amount hydrolysed did not split off any more P during the next 15 min. Therefore, in subsequent assays, this dilution of enzyme was used, and the incubation carried out for 15 min. at 37°C. In spite of this precaution, it was found that the amount of P split off by this enzyme from ATP isolated from liver was always different from that given by a combination of myosin and myokinase (see Table 15), probably due to the presence of other organic phosphate compounds in the liver ATP preparations which were not present in pure ATP solutions. Ennor (private communication) has also found this.

Isolation of ATP from the Liver: The method was based on that of Ventakareman et al. (1950), and is given in Scheme II. The animals were anaesthetised with nembutal, the livers removed, dropped into 40 ml. ice-cold TCA (7% w/v) in a weighed container for a Nelco blendor, and quickly weighed. They were homogenised for 2 min. at medium speed, centrifuged and the residue washed once with 10 ml. TCA. The supernatant and washings were collected in a measuring cylinder, mixed

with an equal volume of 95% ethanol and allowed to stand for 2 hours. This removed the glycogen from the solution, an essential precaution to be taken before isolating acid-soluble organic phosphates, which are otherwise adsorbed on to the glycogen (Sacks, 1949). The glycogen was removed by centrifugation, the pH adjusted to 5.1 and the nucleotides precipitated by the addition of 20% (w/v) mercuric acetate in 2% acetic acid (0.5 ml. reagent for every 10 ml.). The precipitate was washed with 1/40 dilution of the mercuric acetate solution, suspended in a small volume of distilled water and the excess of mercury removed by passing $H_2S$ gas for 1 hour. It was centrifuged, the $HgS$ precipitate washed, and the supernatant and washings aerated for 2 hours. The pH of this solution, containing ATP and ADP, was adjusted to 7.1 before carrying out estimations. All the steps in the preparation were carried out in the cold.

Estimation of $^{32}P$ incorporation: In the experiment where the incorporation of $^{32}P$ into ATP and ADP was measured, the animals were injected with inorganic phosphate labelled with $^{32}P$ intramuscularly (10 μc. per 100 g. body wt.) 2 hours before they were killed. A very small volume of the TCA supernatant (0.5 to 1 ml.) was taken for estimation of the activity of the inorganic P of the liver before proceeding with the isolation of ATP. The inorganic P liberated by the various enzymes was estimated by the method of Ennor

TABLE 12.

The method of calculating $^{32}P$ incorporation into the different fractions obtained by mercury precipitation from the T.C.A. extract of the liver.

| Fraction | Total P in fraction | Differences | Total activity of fraction | Differences in total activity | ~~Differences in~~ specific activity |
|---|---|---|---|---|---|
| | µg | µg | c.p.m./100 µg P | c.p.m./100 µg. P | |
| Inorganic P | 20.7 | | 2405.34 | | |
| | | 243.6 | | 20335.0 | 8347 |
| Myosin P | 264.3 | | 22740.37 | | |
| | | 1015.7 | | 388944.15 | 38365 |
| Myokinase P | 1279 | | 411684.52 | | |
| | | 1033.7 | | 115912.53 | 11169 |
| Apyrase P | 1298 | | 137652.90 | | |

Differences in columns 3 and 5 are, reading downwards, myosin- inorganic, myokinase - myosin and apyrase - myosin.

$$\text{Column 6} = \frac{\text{Column 5}}{\text{Column 3}} \times 100$$

& Stocken (1950), and the resulting blue-coloured iso-butanol was counted for radioactivity in a liquid counter (Type M6 manufactured by 20th Century Electronics Ltd.). The method of calculating the activity of the different phosphate groups liberated by the various enzymes is shown in Table 12. The specific activity of a fraction is the number of counts recorded per minute per 100 µg. of P in that fraction. The specific activity of the P liberated by, say, myosin is obtained by subtracting the specific activity of the inorganic P present as a contaminant along with the Hg-precipitated nucleotides at the start of the incubation from that of the P found after incubating with myosin. The specific activity of the second phosphate group in ATP and of the first phosphate group of any ADP that is also present in the solution is obtained by subtracting the specific activity of the P liberated by myosin from that of the P set free by myokinase or apyrase.

Estimation of Phosphorus: The method of choice for estimating inorganic P in the presence of organic phosphates is that described by Berenblum & Chain (1938), in which the phosphomolybdic acid is selectively absorbed by isobutanol. It suffers from the disadvantage that the time taken for the extraction is appreciably long, and this might result in hydrolysis of any labile organic phosphates that may be present. This hydrolysis has been shown to be catalysed by

~~phosphe~~molybdic acid (Weil-Malherbe, 1951). The modification of the method, as described by Ennor & Stocken (1950), limits the time taken for the extraction to a minimum. It consists in mixing 5 to 8 ml. of the solution to be estimated with 2 ml. 5% (w/v) NaCl, 0.5 ml. ION $H_2SO_4$, 10 ml. redistilled iso-butanol and 2.5 ml. 5% (w/v) ammonium molybdate. The molybdate is added last, and the separating funnel is shaken vigorously for 15 sec. and the aqueous layer run off quickly. The isobutanol is washed twice with 10 ml. N $H_2SO_4$ and then shaken with a 1 in 200 dilution of a stock stannous chloride solution (10 g. in 25 ml. conc. HCl). The blue coloured isobutanol is transferred quantitatively into a graduated flask and the volume made up to 25 ml. with the washings of the funnel. It is then filtered and the intensity of the colour estimated with a Spekker absorptiometer using Ilford 608 filters.

## RESULTS.

In the single group of experiments carried out, all the animals were fed a protein-containing diet for a preliminary period, and their energy intake was then altered by adding olive oil to the diet, as shown in Table 11. They were killed after 3 days on this diet, and their livers analysed. Great difficulty was experienced in getting all three enzymes to work at the same time. Each assay was

TABLE 13.

The effect of variation in energy intake on the ATP and ADP content of rat liver. The animals received a protein-containing diet, to which increments of olive oil were added.

| Energy Levels | No. of rats | Liver Weight | µg P per liver | | | µg P per g. wet wt. of liver | | | Ratio ATP/ADP |
|---|---|---|---|---|---|---|---|---|---|
| | | | ATP | ADP | ATP+ADP | ATP | ADP | ATP+ADP | |
| Cal/day | | g. | | | | | | | |
| | | 6.9 | 249 | 470 | 710 | 35.6 | 67.1 | 102.7 | 0.53+ |
| 28 | 4 | 7.5 | 163 | 367 | 530 | 21.4 | 48.3 | 69.7 | 0.44+ |
| | | 7.5 | 244 | 1016 | 1260 | 32.4 | 135.4 | 167.8 | 0.24* |
| | | - | 182 | 365 | 547 | - | - | - | 0.50* |
| | Mean | 7.3 | 209.5 | 554.5 | 763.8 | 29.4 | 83.6 | 113.0 | 0.43 |
| | | 7.1 | 234 | 273 | 507 | 32.9 | 38.5 | 71.4 | 0.86+ |
| | | 9.4 | 320 | 376 | 696 | 33.9 | 42.2 | 76.1 | 0.80+ |
| 42 | 4 | 9.5 | 127 | 307 | 534 | 13.4 | 32.2 | 45.6 | 0.41* |
| | | - | 153 | 217 | 370 | - | - | - | 0.71* |
| | Mean | 8.6 | 208.5 | 293.1 | 501.6 | 26.7 | 37.7 | 63.4 | 0.70 |
| | | 7.5 | 320 | 402 | 722 | 42.7 | 53.5 | 96.2 | 0.80+ |
| | | 8.4 | 142 | 160 | 302 | 16.7 | 18.8 | 35.5 | 0.89+ |
| 56 | 4 | 8.5 | 206 | 430 | 635 | 24.4 | 51.1 | 73.5 | 0.47* |
| | | - | 100 | 90 | 190 | - | - | - | 1.11* |
| | Mean | 8.1 | 191.8 | 270.4 | 462.2 | 27.7 | 41.1 | 68.8 | 0.82 |

* Myokinase used. + Apyrase used.

TABLE 14.

Statistical Analysis of the effect of adding fat to a high protein diet, on the ATP/ADP ratio in rat liver.

| Energy Level | 28 Cal. | 42 Cal. | 56 Cal./day |
|---|---|---|---|
| ATP/ADP x 100 | 53<br>44<br>24<br>50 | 86<br>80<br>41<br>71 | 80<br>89<br>47<br>111 |
| Totals | 171 | 278 | 327 |
| Means | 43 | 70 | 82 |

| Source of Variation | Degrees of Freedom | Sums of Squares | Mean Square | Variance Ratio (F) |
|---|---|---|---|---|
| Energy Levels | 2 | 3183 | 1592 | 12.3 |
| Linear Regression | 1 | 3042 | 3042 | 24.2 |
| Deviations from Linearity | 1 | 3141 | 141 | 1.1 |
| Replicates | 3 | 3052 | 1017 | 7.8 |
| Residual Error | 6 | 774 | 129 | - |

For $n_1 = 2$, $n_2 = 6$, $F = 5.14$ at 5% level
and $F = 10.92$ at 1% level.

The statistical analysis indicates that the addition of fat to the diet produces a highly significant increase in the ATP/ADP ratio. There is no deviation from linearity of regression.

checked by estimating a standard solution of ATP with the enzyme preparations used. In the first two experiments, the myokinase preparation was inactive and the apyrase data had to be used instead; in the other two myokinase figures were available and were used as more accurate estimates. The results are shown in Table 13. The ATP and ADP contents have been expressed as "total amount per liver" and "amount per gm. of wet liver." The latter would be influenced by changes in the weight of the liver caused by variations in the amount of glycogen or protein, and would not therefore give a true measure of the changes brought about by increasing the energy intake. Only the "amount per liver" has therefore been taken into consideration. A more accurate mode of expression would be in terms of the DNA content of the liver, thus expressing the ATP as amount per liver cell (Davidson & Leslie, 1950). The ATP content per liver remains constant as the energy intake is increased from 28 to 56 Cal. per day. This agrees with the findings of Albaum et al. (1951) who reported that the ATP content of the liver was not affected by a period of fasting. However, the total ADP in the liver decreases with increasing energy intakes, resulting in an increasing ATP/ADP ratio. Analysis of variance (Table 14) shows that the increase of the ratio is highly significant, and that the change takes place in a

TABLE 15.

Effect of changes in energy intake (fat) on the incorporation of $^{32}P$ into ATP and ADP of rat liver. The rats were killed 2 hours after injection of $^{32}P$.
(1 rat in each group).

| Energy Level | Fraction | Specific Activity | Specific Activity of inorganic P | Relative Specific Activity | Ratio $ATP^{32}/ADP^{32}$ |
|---|---|---|---|---|---|
| cal./rat/day | | c.p.m./ 100 µg P | c.p.m./ 100 µg P | | |
| 28 | ATP terminal P | 8,347 | 19,688 | 42.3 | |
| | ATP-second P | 38,365* | | 194.8 | 0.21* |
| | ATP-second P | 11,169+ | | 56.7 | 0.74+ |
| 42 | ATP-terminal P | 27,120 | 24,716 | 109.7 | |
| | ATP-second P | 17,243* | | 69.7 | 1.5* |
| | ATP-second P | 19,724+ | | 79.8 | 1.5+ |
| 56 | ATP-terminal P | lost | - | - | |
| | ATP-second P | 15,743* | 19,487 | 80.7 | |
| | ATP-second P | 16,703+ | | 85.7 | |

* myokinase used
\+ apyrase used.

The P estimated as ATP - second P includes the terminal P of any ADP already present as such in the solution.

linear fashion, the regression coefficient being 0.01 per Cal.

The radioactive data are shown in Table 15. Only one experiment was performed, and even this is not completely represented due to the accidental loss of one of the specimens. It is interesting to note, however, that the ratio $AT^{32}P/AD^{32}P$ increased as the energy intake was increased, showing that the relative rates of turnover of these two phosphate groups were affected by changes in energy intake in the same manner as the total amounts.

## DISCUSSION.

Our experiments were confined to the liver, even though most of the body protein is found in muscle, for two reasons: the demonstration, by the use of the label $^{15}N$, that the liver is more active in synthesising protein than muscle (Sprinson & Rittenberg, 1949), and that, when energy is added to a protein-containing diet, the percentage increase in the protein N is greater in the liver than in other organs (Munro & Naismith, 1952).

Studies on the energy-rich compounds in the liver carried out by investigators like Kaplan & Greenberg (1944) and Albaum (Albaum et al., 1951) have been restricted to the estimation of the content of ATP, taking no account of the ratio ATP/ADP. Let us first consider the rôle of this

factor in metabolism.

a. The concept of a "Phosphate Potential". During 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 the 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 & Monzoni, 1933; Dodds & Greville, 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 & Lehninger (1951) and Keilly & 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 being analogous 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 ratio ATP/ADP. Since the reaction is reversible, this 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 the 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 thus assumes a central position in P metabolism, and has been suggested as a possible control mechanism in the economy of the cell (Lardy & 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 ex-

pression $(ADP)(H_3PO_4)/ATP$ approaches that corresponding to equilibrium". It is conceivable that in a similar manner this ratio controls other mechanisms dependent on ~~pH~~, including the equilibrium between amino acids and peptides or proteins synthesized from them.

b. Effect of Energy Intake on the Phosphate Potential: Our experiments prove conclusively that increasing the energy intake by adding fat to a well balanced diet results in an increase in the ATP/ADP ratio, in spite of the fact that the total ATP per liver decreased. This is in agreement with the finding that glucose stimulates ATP synthesis (Kaplan & Greenberg, 1944). Under the same circumstances, N balance improves and protein is deposited in the liver (Munro & Naismith, 1952). It seems justifiable to assume that the effect of surfeit energy on protein metabolism is exerted through the increased phosphate potential. Support of such a concept comes from the evidence of the participation of ATP in peptide-bond synthesis (Lipmann, 1949), and from the observation that the uptake of labelled amino-acids into proteins requires the presence of ATP (Winnick, 1950). Siekevitz (1952) showed that the uptake of radioactive alanine into proteins is associated with oxidative phosphorylation. He found that, when liver mitochondria are incubated with $\alpha$-ketoglutarate, or succinate, along with various co-factors

such as AMP, $Mg^{++}$ and phosphate, a soluble co-factor is formed which enables the microsome fraction of the liver to incorporate alanine into its proteins. As this factor is stable when heated at 100°C for 7 min., it is unlikely to be ATP, but Siekevitz thinks it may be a compound derived from ATP.

Our studies on the incorporation of $^{32}P$ into ATP and ADP, though few, corroborate the evidence provided by the quantitative measurements. These results are, however, far from accurate. The specific activity of each compound is arrived at by subtraction of one set of counts from another. Table 12 shows that the %age difference between the counts is small, and so are the differences between the amounts of P liberated by myosin and the P liberated by myokinase present in the sample. Taking into consideration the errors involved in the actual counting, these differences in the activities become negligible. Therefore, though the enzyme method of estimation of ATP and ADP is satisfactory for quantitative work, for radioactive studies a separation of the various compounds by a procedure such as chromatography seems to be the more desirable.

In summary, we see that this universal medium of energy exchange, the adenylic acid system, plays an important part in all reactions involving transphosphorylation and that it is

essential for many synthetic reactions, probably including the formation of peptide-bonds. Our experiments have established that a rise in energy intake increases the phosphate potential. How is this made use of in protein synthesis? A considerable body of evidence suggests that ribonucleic acid is involved in synthesis of proteins, and it has been claimed that this acid or its derivatives are necessary for the transfer of energy with resulting synthesis (Muller, 1947). Spiegelman & Kamen (1946a) think that nucleic acids act as phosphate donors and "funnel energy into the protein synthesising mechanism". Does ribonucleic acid show any change with energy intake? The experiments dealt with in the next section were designed to answer this question.

# PART III.

## THE INFLUENCE OF ENERGY INTAKE ON THE METABOLISM OF RIBONUCLEOPROTEINS.

## INTRODUCTION.

The question has been raised in the previous section whether nucleoproteins play any rôle in mediating the influence of energy intake on protein metabolism. That they may do so is conceivable, since there is an alleged relationship between ribonucleic acid (RNA) and protein synthesis. We shall therefore first consider the evidence relating RNA to protein synthesis, before proceeding to describe our own experiments on the metabolism of RNA under various nutritional circumstances.

a. Evidence relating Ribonucleic Acid to Protein Synthesis.

For the past decade there has been considerable interest in the alleged relationship of RNA to protein synthesis. The suggestion that this might be so was independently propounded in 1941 by Brachet working in Brussels and Caspersson in Stockholm. Both of these investigators used histochemical procedures. Histologists had long been aware that tissues varied in their affinity for basic dyes, and Brachet (1941) identified the principal bosophilic substance in cytoplasm with RNA. This permitted an assessment of the amount of RNA in the cell, and greater precision was obtained when the ribonuclease became available (Brachet, 1941; Davidson & Waymouth, 1946), so that basophilia due to RNA could be distinguished from basophilia of other origin. A different

approach was employed by Caspersson (1939; 1941), who used a technique for the microspectrographic determination of cellular nucleic acids based on the highly selective light absorption of these acids in the central ultraviolet (UV) range. Although this approach has limitations (e.g., a protein having as part of its molecule purines or pyrimidines would have a UV absorption indistinguishable from that of nucleic acid), the results obtained agree in general with Brachet's. The validity of many of the findings has been finally established by Davidson (1947 a & b) using chemical procedures.

The results obtained by these histological and chemical techniques show a striking correlation between the amount of RNA in the cell and the intensity of protein synthesis by the cell. The question of whether RNA really plays an essential part in protein synthesis can be considered under three headings:

i. The correlation of the amount of RNA per cell with the intensity of protein synthesis.

ii. Changes occurring in the amount of RNA with a demand for increased protein synthesis.

iii. Evidence on the metabolism of RNA under various conditions affecting protein synthesis.

i. The amount of RNA per cell and intensity of protein synthesis. There is a striking coincidence between the amount

of RNA contained in a cell and the importance of that cell in protein synthesis. RNA has been found in abundance in (1) secretory tissues like the pancreas, and peptic glands of the stomach (Caspersson, 1947), salivary glands (Caspersson, 1939) and in the pituitary gland during hormone secretion (Harlant, 1943; Abolins, 1952). (2) Cells known to synthesise protein actively, such as liver cells (Davidson & Waymouth, 1946; Davidson, 1947a), developing red and white blood cells (Thorell, 1944; Davidson et al., 1948), nerve cells after stimulation of the corresponding nerve (Hyden, 1943), and the silk glands of the silk worm (Brachet, 1950). (3) Rapidly dividing cells such as growing oocytes (Brachet, 1950), bacteria during the logarithmic phase of growth (Malmgren & Heden, 1947; Boivin, 1947), and the feather-forming cells of birds (Grenson, 1952).

The most widely investigated organ is the liver, on account of its high nucleic acid content. In the fasting animal there is a fall in its protein content accompanied by a decrease in RNA. The same is seen when the animal is placed on a protein-free diet. On re-feeding with protein, both the RNA and the protein in the liver rapidly increase (Davidson & Waymouth, 1944; Campbell & Kosterlitz, 1947; Lagerstedt, 1949). In the fasted or protein-starved animal, the nucleolus is small, but when protein feeding is started, there is a rapid increase in the nucleolar mass and its RNA

content, accompanied by a concentration of RNA near the nuclear membrane (Lagerstedt, 1949).

On the other hand, organs having a high physiological activity but not synthesising large amounts of protein, have only small amounts of RNA, for example, heart and skeletal musde (Davidson, 1950) and oxyntic cells of the stomach (Caspersson, 1947).

ii. Changes in RNA content accompanying a demand for protein synthesis. In tissues such as actively secreting cells, regenerating liver, rapidly growing cells and micro-organisms, there is a great demand for a rapid increase in protein synthesis. This has been found to be accompanied by changes in the RNA concentration in these tissues.

In the immature red blood cell, the amount of RNA and the size of the nucleolus increases till the erythroblast stage, when the growth of the cell ceases, accompanied by a decrease in size of the cell and nucleolar mass. The rate of formation of haemoglobin, which is at first slow, is greatly speeded up when the RNA disappears (Thorell, 1944, 1947).

The exocrine gland cells of the pancreas have conspicuous nucleoli and large quantities of RNA. When the gland is emptied of its contents, by the injection of pilocorpine, the rapid synthesis of new protein is preceded by an increase in size of nucleolus and in the concentration of RNA round the

nuclear membrane (Caspersson, 1947).

In the compensatory hyperplasia following removal of part of the liver, the weight of the organ increases rapidly during the first 24 hours, due to an increase in size of each cell without cell division. The RNA content of the liver increases considerably, reaching a maximum in $1\frac{1}{2}$ to 3 days after the operation. Cell division, which is accompanied by increase in liver protein, is also most active at this period (Novikoff & Potter, 1948; Stowell, 1948; Lagerstedt, 1949). The time relationships show that protein synthesis is preceded by the rapid increase in RNA.

When cultures of chick heart fibroblasts are maintained in a physiological salt solution, the cells stop growing and the RNA content falls. When a growth-promoting medium is added, the RNA content begins to rise immediately. Actual cell division, as evidenced by an increase in the deoxyribonucleic acid (DNA), only occurs 48 hours later (Davidson, Leslie & Waymouth, 1949). Synthesis of RNA begins several hours before the maximum synthesis of protein.

Studies on bacterial growth have shown great changes in the nucleotide content during the early phase of growth, reaching a maximum during the end of the lag phase or beginning of the logarithmic phase. This increase, which corresponds to 5 to 10 times the original content, immediately precedes cell division (Malmgren & Heden, 1947).

Bing, Fagraeus & Thorell (1945) found that the formation of antibody protein proceeds in the same manner, namely, is preceded by an increase of nucleic acids.

In all these instances, therefore, the demand for increased synthesis of protein results in an immediate increase in the RNA content of the tissue, which is followed by the formation of the protein required. This time relationship favours the view that RNA is a factor in the subsequent synthesis of protein.

iii. The metabolism of RNA under various conditions affecting protein synthesis.

The experiments dealt with so far are concerned with changes in the total amount of RNA or protein in the tissues. The metabolic activity of a compound is not, however, measured by the absolute amount present. It is necessary to determine the turnover as well as gross content, and this has been achieved by the use of isotopic tracers. Hahn & Hevesy (1940) were the first to use $^{32}P$ to estimate the turnover of nucleic acids, and since then, several others have used compounds labelled with $^{32}P$, $^{15}N$ and $^{14}C$, to study their biological activity (vide Davidson, 1950). The turnover of RNAP is 5 to 7 times that of DNAP, the figures for hepatoma and regenerating liver being appreciably greater than those for resting liver (Brues, Tracy & Cohn, 1944; Davidson &

Raymond, 1947). The high rates of renewal of RNA in intestine, spleen and liver is in keeping with the importance of these organs in protein metabolism (Davidson, 1950).

Isotopic evidence favouring a relationship between RNA and protein synthesis has been obtained by Spiegelman & Kamen (1946) in yeast. They measured the flow of phosphate from the "nucleoprotein fraction" under various conditions. The yeast was grown in a medium containing $^{32}P$ and then allowed to ferment glucose in a medium containing unlabelled phosphate. The acid-soluble P was lowered in activity, whereas the nucleoprotein P remained constant as long as the cells were maintained amaerobically. Addition of ammonium sulphate to evoke synthesis of new protein produced a marked drop in activity of the nucleoprotein fraction. Reagents preventing the assimilation of N or the formation of new protein inhibited the transfer of phosphate from the nucleoprotein fraction. The validity of these results depended on the composition of this nucleoprotein fraction. On purifying this and estimating the total quantity and turnover of each fraction obtained from it, they found that N assimilation by yeast was accompanied by a drop in the amount of RNA. Other than the "metaphosphate fraction", RNA was the only one thus affected. The turnover rates on the other hand showed a ninefold increase for RNAP. The importance of this was

minimised by the fact that other fractions, including phospholipin, responded in a similar fashion (Spiegelman & Kamen, 1947). They concluded that equally good correlations can be established between protein synthesis and the metabolic activity of the other phosphate-containing fractions.

On the other hand, data which suggest a dissociation between RNA metabolism and protein synthesis have been obtained with bacteria. When cobaltous ions were added to a medium in which Proteus vulgaris were growing, growth ceased, but an increase identical with that seen in growing cells was found in the RNA content of these cells (Levy, Skatch & Schade, 1949). When Proteus vulgaris was grown in a medium containing $^{32}P$ and aliquots were innoculated into media containing no $^{32}P$, it was found that the turnover rate of RNAP was greater in the cells prevented from growing by cobalt, when compared with controls, showing that RNA is actually metabolised faster in the absence of protein synthesis. These experiments at first sight seem fatal to the association between RNA and protein synthesis. However, it has already been pointed out that RNA formation precedes protein synthesis and these experiments may merely show that protein synthesis does not inevitably follow if the conditions for laying down protein are unfavourable.

The action of bacteriophage on E. Coli provides

another example of the lack of relation between RNA metabolism and protein synthesis. Cohen (I95I) found that protein continues to be synthesised in infected bacteria, even though production of RNA has been stopped, as evidenced by no uptake of $^{32}P$. This occurs before the DNA of the phage has begun to form. It is possible that protein synthesis is maintained by pre-existent RNA.

Studies of protein and RNA synthesis after irradiation with X-rays have also been made. Holmes (I95I) injected $^{32}P$ and methionine labelled with 35S simultaneously into rats, some of which were irradiated. Synthesis of DNA was reduced by half, and was the only system affected, RNA showing no change due to X-rays. The results varied widely between individual experiments (due probably to difficulties in removing contaminating $^{32}P$ from the nucleic acid fractions) and showed a complete lack of any relation between the effects of irradiation on the uptake of the two labels by nucleic acids and proteins. To avoid $^{32}P$-containing contaminants, Abrams (I95I) used glycine labelled with $^{I4}C$ in the carboxyl group and studied the turnover of purines. In rabbit bone-marrow, irradiation with X-rays produced a marked drop in the activity of DNA and RNA, though no change was observed in the rate of glycine uptake by proteins. In the rat intestine, when glycine was injected two hours after irradiation, there was an 80% inhibition of DNA synthesis, 49% inhibition of RNA

synthesis and only 3.8% of protein synthesis. When the time interval was increased to 48 hours, the inhibition of DNA was only 33%,of RNA, 26%, and of protein, 17.2%. He concluded that, if RNA does take part in protein synthesis, it may be involved in the synthesis of certain specific proteins, or that only a part of the RNA is involved. It is noteworthy, however, that 48 hours after irradiation, the inhibition of protein synthesis has increased, even though the formation of RNA is now more rapid. This could mean that some time elapses between the synthesis of RNA and that of proteins, the 49% inhibition of RNA noted at 2 hours exerting its effect on protein synthesis only some hours later. This is in keeping with the conclusion, based on purely quantitative studies, that RNA synthesis precedes protein synthesis.

Such a time lag is also shown in the experiments reported by Hammarsten (1951). In liver regenerating after partial hepatectomy, the maximum turnover rates for all the nitrogenous compounds in DNA, nuclear and cytoplasmic RNA, appeared at about 30 hours after operation, i.e., corresponding with the greatest increase in quantity of these compounds. The maximum incorporation of glycine into the proteins, in these experiments, occurred about 30 hours later.

Guberniev and Il'iana (1950) observed that the in vivo stimulation of enzyme secretion in digestive glands resulted in increases in the rate of incorporation of $^{32}P$ into the

nucleoproteins (400% in the parotid, 500% in the liver and 1200% in the pancreas). This could mean that RNA is concerned with enzyme secretion, i.e., active extrusion of enzymes, rather than enzyme synthesis. Hokin (1952), therefore, examined the uptake of $^{32}P$ by pancreas slices *in vitro*, a system in which the synthesis and secretion of enzymes could be studied separately. Addition of an appropriate amino acid mixture to the medium almost doubled the rate of amylase synthesis, but resulted in no appreciable increase in the rate of incorporation of $^{32}P$ into the RNA. On the other hand, a 50 to 100% stimulation of amylase secretion by the addition of carbamylcholine was accompanied by a corresponding increase in the rate of uptake of $^{32}P$ into RNA. He concludes that RNA is not linked with protein synthesis, but functions in the rearrangement and movement of enzymes during the secretory process.

Grenson (1952a) found that RNA in the baseplate of the pigeon's feather was renewed at the same rate as the synthesis of new keratin, but the rate of incorporation of $^{32}P$ into RNA of fowl's oviduct was 5 to 10 times slower than the rate of production of ovalbumin (Grenson, 1952b). One would, however, like to know more details than the author provides about the methods of calculating the rates of RNA and protein synthesis before accepting this evidence.

In summary, it may be said that the case for a relationship between the amount of RNA in a given cell and the intensity of its protein synthesis seems established, and, in cells stimulated to form more protein, there is first an increase in the amount of RNA. On the other hand, a lack of concordance between the uptake of isotopes by RNA and by newly synthesised protein has been demonstrated under certain conditions, namely, for bacteria treated with cobalt salts, for rat tissues following X-irradiation, and in the pancreas during active synthesis of enzymes. Although these observations appear to be fatal to the theory of a rôle for RNA in protein synthesis, it is possible in each instance to envisage an explanation of the data which would allow RNA to retain a place in the protein synthesis mechanism. It therefore seemed desirable to establish whether or not variations in energy intake produce changes in RNA metabolism, and how these may be related to changes in protein synthesis. In devising experiments to test these points, we made use of observations by Munro & Naismith (1952) which show that increments of energy added to protein-containing diets caused an increase in the amount of protein in the liver, whereas the same changes in energy intake produced in a protein-free diet cause, if anything, a reduction in the amount of protein in the liver. Our problem, therefore, was to determine the changes occurring in RNA metabolism under the same

circumstances. The experiments to be described deal with two aspects of RNA metabolism, namely quantitative changes in the amount per liver and changes in the rate of $^{32}P$ uptake by liver RNA.

## EXPERIMENTAL.

### a. Animals.

In all the experiments, young adult male albino rats were used. After an overnight fast, animals weighing between 175 and 200 g. were selected and subdivided between various experimental groups according to the randomised block technique (Snedecor, 1946) which permits one to reduce the effect of slight differences in body weight as a factor in the analysis of results.

### b. Diet and General Management.

Rats were housed under thermostatic conditions in individual metabolism cages. Food was given twice daily in heavy ointment jars and moistened with water to prevent scattering. At 1 a.m., 1 g. of the vitamin-mineral-roughage mixture (see Table 3) was given to all animals. To this glucose was added in an amount required to provide a given level of energy intake. At 5 p.m., rats on the protein-containing diet each received 2.4 g. of casein (Glaxo), 0.69 g. glucose, 0.69 g. potato starch and 0.42 g. fat; for those on a protein-free diet, the casein was replaced by an

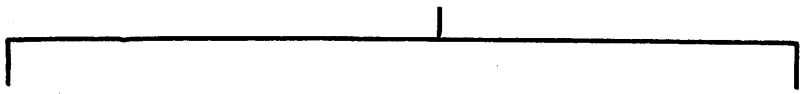
isocaloric amount of carbohydrate (half starch and half glucose).

All rats received an energy intake of approximately 1200 Cal. per sq.m. per day for a preliminary period of seven days, to accustom them to the diet and the times of feeding. By the end of this period, most of the animals had learned to eat their food as soon as it was given, the meal being completed within $\frac{1}{2}$ to 1 hour. At the end of a week the energy intake of different animals was altered by addition or subtraction of glucose fed in the morning meal. Giving the additional energy in the morning made certain that it was the energy effect that was being studied, and not the interaction effect occurring when protein and carbohydrate are fed at the same meal (Munro, 1949). Each rat was maintained for four days at the new level of energy intake, killed by exsanguination under ether anesthesia, and the livers perfused with 0.9% (w/v) saline, and removed for analysis. $^{32}P$ was injected as inorganic phosphate (10 µc per 100 g. body weight) intramuscularly, at either 4, 8 or 24 hours before killing. In most experiments, the rats were killed 24 hours after the injection, i.e., 20-21 hours after the last meal of the 4 day period.

e. Analytical Procedures. i. Fractionation of Liver Constituents. The liver was dried, chilled, weighed and homogenised in 9 vols. of ice cold water in a Nelco blendor at moderate speed for 6 mins., two drops of carpylic alcohol being added

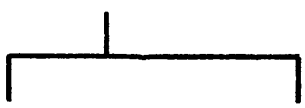
## Isolation of RNA and Inorganic P of Liver.

Liver homogenised in 9 vols. ice-cold water at moderate speed in a Nelco blendor. 4 vols. homogenate mixed with 2 vols. of 30% TCA. Centrifuged



**Supernatant**

To 10 vols. added 1 vol. Mathison's reagent, made alkaline to phenol-phthalein, left overnight at 0°C. Centrifuged and filtered



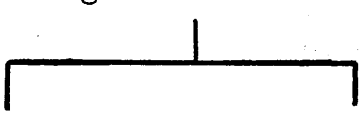
Supernatant $S_1$

Precipitate dissolved in N.HCl $P_1$

**Residue**

Washed twice with 10% TCA.
Washings discarded.
Washed with 10 ml. portion of
- Acetone
- Absolute Ethanol
- Ethanol-Chloroform (3:1)
- Ethanol-Ether (3:1), twice
- Ether.

Washings discarded.
Dry residue incubated with N. KOH at 37°C for 18 hours.
Cooled, added conc. $HClO_4$ to bring pH to 3.
Centrifuged and filtered.



$A_2S$ Fraction

Residue

SCHEME 3.

to prevent frothing. A volume of homogenate equivalent to 3 to 4 gms. liver was pipetted out and sufficient 30% (w/v) trichloracetic acid (TCA) added to make the final concentration 10%. This was then subjected to a modified Schmidt Thannhauser procedure described by Davidson & Smellie (1952). The homogenate was kept in ice for about ½ an hour to ensure complete precipitation of proteins, centrifuged at a low speed for 7 min., and the supernatant filtered. The precipitate was then washed twice with ice-cold 10% (w/v) TCA, centrifuged, and subjected to the procedure shown in Scheme 3. It was mixed well with 20 ml. portions of each of the lipid solvents, centrifuged and the supernatant discarded each time. After the final extraction with ether, the residual ether was allowed to evaporate, and the dry powder containing RNA, DNA and proteins, among other compounds, incubated with N.KOH which hydrolyses the RNA into its nucleotides. These remain in solution when the digest is subsequently acidified, and form the major constituents of the $A_2S$ fraction.

ii. Estimation of Liver Inorganic Phosphate. The radioactivity of the inorganic phosphorus (P) of the liver was obtained according to the method employed by Davidson, Frazer & Hutchison (1951) from the TCA soluble fraction. To a portion of the filtered acid-supernatant, Mathison's (1909) reagent was added (1 ml. to 10 ml. extract) and the mixture

made alkaline to phenolphthalein with $NH_4OH$. After standing overnight at $0^oC$, the precipitate was separated by centrifugation and filtration, washed twice with dilute $NH_4OH$, dissolved in N.HCl and made up to a known volume. The amount of P in this was estimated by the method of Allen (1940), and the radioactivity measured in a liquid counter (Type M6 manufactured by 20th Century Electronics) attached to a conventional probe unit and scaling unit (Type 200 manufactured by Dynatron Radio Ltd.). In a few instances, the supernatant left after removal of the inorganic P from the TCA-soluble fraction was also estimated for amount and activity of its P. This would contain acid-soluble organic P compounds, the $S_1$ fraction of Davidson et al. (1951).

It is possible that some of the compounds in fraction $S_1$, such as adenosine triphosphate (ATP), may break down during the period elapsing between the homogenising of the tissue and the actual separation of the $S_1$ fraction from the magnesium precipitate. Ennor & Rosenberg (1952) estimated the activity of the inorganic P before and after magnesium precipitation and obtained a difference when the livers were removed two hours after the injection of $^{32}P$. These differences were attributed to the magnesium precipitate bringing down some of the organic compounds such as ATP, whose activity would increase the activity of the inorganic P at the two

TABLE 16.

Comparison of the Specific Activity of Inorganic Phosphate isolated soon after Addition of TCA to Liver Homogenates with the Specific Activity after $Mg(NH_4)PO_4$ Precipitation.

| Time between $^{32}P$ injection and killing | No. of Animals | Specific Activity | | Difference |
|---|---|---|---|---|
| | | Immediate | After Precipitation | |
| hrs. | | c.p.m./ 100 µg P | c.p.m./ 100 µg P | % |
| 2 | 8 | 13455 | 13725 | +2.0 |
| 5 | 7 | 5896 | 5841 | -0.9 |
| 8 | 8 | 6666 | 6206 | -6.9 |
| Mean | 23 | 8812 | 8710 | -1.2 |

Statistical Analysis by Fisher's "t" test shows no significant differences between the specific activity immediately after addition of TCA and the specific activity of the $Mg(NH_4)PO_4$ precipitate at any of the time-intervals after injection.

hour interval. In order to test this, in some of our experiments, a small volume of the TCA supernatant (1 to 2 ml.) was removed immediately after the first centrifugation and estimated for $^{31}P$ and $^{32}P$ by a modification of the Berenblum & Chain procedure as described by Ennor & Stocken (1950). The results are given in Table 16; they are expressed as the specific activity of inorganic P; i.e., counts per min. per 100 µg P. They are compared with the specific activities of the same inorganic P after precipitation by Mathison's reagent, estimated by the same method. The studies were made at 2, 5 and 8 hours after ~~the first estimation was made of~~ injection of $^{32}P$. There is no significant difference between the activities of the inorganic P estimated before and after magnesium precipitation, in spite of the fact that the magnesium precipitate was separated from the $S_1$ fraction at least 24 hours after the first estimation was made. This shows that any inorganic P resulting from the breakdown of labile organic compounds does not have a significant effect on the activity of the inorganic P estimated after magnesium precipitation. It should be pointed out that Ennor & Rosenberg added carrier phosphate before carrying out the magnesium precipitation, and the differences observed increased with the amount of carrier added. No carrier phosphate was added in our experiments.

Evidently, in the absence of added carrier phosphate, the magnesium precipitation method is as reliable as the immediate extraction of the inorganic P by the iso-butanol procedure of Berenblum & Chain.

iii. Estimation of RNA $^{31}P$ and RNA $^{32}P$. In 1949, Davidson and co-workers (Davidson et al., 1949a & b) set about devising a method for the estimation of $^{32}P$ in the nucleic acids in the TCA-insoluble, lipid free residue obtained in the Schmidt-Thannhauser (1945) separation of liver constituents. Methods involving the isolation of nucleic acids were not quantitative and required large amounts of tissue. Hydrolysis of the residue with methanolic HCl followed by formic acid hydrolysis to separate the pyrimidines, as proposed by Vischer & Chargaff (1948), is laborious and not quantitative. Digestion of the residue with alkali, as proposed by Schmidt & Thannhauser, on the other hand, was satisfactory for quantitative studies. The alkali split the RNA into its nucleotides which remained in solution when the proteins and DNA were reprecipitated by acid. When examined for radioactive studies, however, a small amount of highly active P was found contaminating the crude RNA fraction ($A_2S$ fraction of Davidson et al. (1951)). This had been reported by several others also (Euler et al., 1948; Jeener, 1949; Friedkin & Lehninger, 1949; Marshak & Calvet, 1949; Jeener & Szafarz, 1950). Washing the original TCA

precipitate several times with TCA did not remove this contaminating P (Davidson et al. 1951), nor did repeated additions of inorganic P to the RNA fraction followed by reprecipitation with magnesium mixture (Jeener, 1949).

Vischer, Magasanik & Chargaff (1949) first used the filter paper chromatography technique of Consden, Gordon & Martin (1944) for the separation of mononucleotides, but they could not separate guanylic ~~from~~ and uridylic acids satisfactorily. Markham & Smith (1951) failed to separate the two pyrimidine nucleotides on paper.

A solution to the problem was found by Smellie (Smellie & Davidson, 1951), who modified the filter paper electrophoresis technique described by Cremer & Tiselius (1950) for the separation of the nucleotides in the alkaline digest in the Schmidt-Thannhauser procedure. By this method he obtained a complete separation of all 4 nucleotides. Moreover, the contaminating inorganic P was removed from the nucleotides. He obtained quantitative recoveries of nucleotides added to the $A_2S$ fraction.

We, therefore, decided to use this technique for the estimation of the incorporation of $^{32}P$ into the RNA. The dry, lipid-free residue was digested for 18 hours at 37°C with N.KOH, cooled, and a few drops of 60% (w/v) $HClO_4$ added to bring the pH to about 3, the digest being kept in ice to

minimise hydrolysis of the nucleotides. The precipitate formed was separated by centrifuging, washed twice with a small volume of N.$HClO_4$, and the combined supernatant and washings made up to a convenient volume. An aliquot of this $A_2S$ fraction, containing 100 to 120 µg P was applied to a spot 6 cm. from one end of a strip of filter paper (Whatman, No.1), 7 cm. broad and 52 cm. long, using an "Agla" micrometer syringe, and the spot dried in a current of cold air. The paper was soaked with buffer solution (0.02 M. citric acid-trisodium citrate, pH 3.5), the end with the $A_2S$ spot being allowed to moisten by capillary attraction, and suspended over a glass rod, the two ends being made to dip into the same buffer solution contained in two glass dishes. The levels of the solution in the dishes were in the same horizontal plane. A steady D.C. supply of 750 volts was applied by means of carbon electrodes, the cathode being in the dish nearest the $A_2S$ spot. At the end of 7 hours the paper was dried in front of infra-red lamps, the nucleotide bands located with the aid of a UV lamp with a special glass filter, as described by Holiday & Johnson (1949), and marked lightly in pencil. The bands were cut out, eluted into graduated tubes with 0.01 N.NaOH (Consden, Gordon & Martin, 1947), and made up to a known volume, 6 ml. of which was taken for counting radioactivity. The solution was then divided into 2 equal

portions and estimated for P by the method of Allen (1940), suitably modified for small quantities of P.

In most cases, this "short run" for 7 hours with a potential gradient of 15 volts per cm. of paper was used for separating the nucleotides, and the 4 nucleotides were cut out, eluted and estimated together. In some experiments, the ionophoretic separation was carried out for 18 hours at a potential gradient of about 11 volts per cm. of paper, when the separation of the individual nucleotides was obtained. As the separation of cytidylic and adenylic acids was still incomplete (due probably to the amount of $A_2S$ put on the paper), these 2 nucleotides were cut out and estimated together.

After this work had been started, Smellie (Davidson & Smellie, 1952) discovered several P-containing compounds running very close to some of the nucleotides. When estimating the individual nucleotides after a long run, therefore, only the central portions of the bands were cut off the paper to minimise contamination of these "concomitants". Smellie also found that these "concomitants" could be got rid of by extracting the TCA-insoluble, lipid-free residue with hot 10% (w/v) NaCl, and precipitating the nucleic acids from this extract with ethanol, before submitting them to alkaline digestion. Further, the use of 0.3 N.KOH instead

TABLE 17.

Comparison between the R.S.A. of the $A_2S$ Fraction and that of the Phosphorus-containing Compounds isolated from Filter Paper after a short Ionophoresis Run.

| Phosphorus containing Fraction | Mg. per Liver* | Relative Specific Activity | |
|---|---|---|---|
| | | 2 hrs. after injection+ | 24 hrs. after injection++ |
| $A_2S$ | 3.97 | 5.9 | 45.6 |
| RNA | 3.42 | 3.1 | 39.2 |
| X | 0.19 | 18.2 | 38.2 |
| $X^1$ | 0.57 | 9.9 | 39.7 |
| RNA + X + $X^1$ | 4.18 | 5.0 | 39.2 |

*Based on analyses of 15 rat livers.

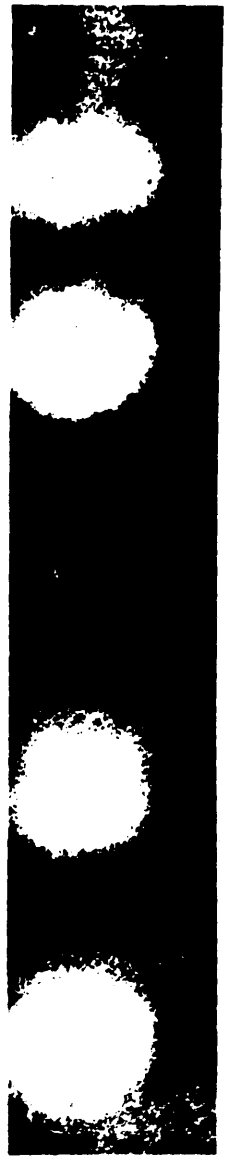
+Based on analyses of 4 rat livers.

++Based on analyses of 11 rat livers.

Fig.6: A- Ultraviolet photograph of the separation of the 4 nucleotides from a rat liver $A_2S$ fraction (Long run).

A'- Ultraviolet photograph of a short ionophoretic run of an $A_2S$ fraction from rat liver. X is the crescent-shaped area preceding uridylic acid and X' the area between X and uridylic acid.

Photographs reproduced by kind permission of Dr. R.M.S.Smellie.


CYTIDYLIC ACID
ADENYLIC ACID
GUANYLIC ACID
URIDYLIC ACID

A


CYTIDYLIC ACID
ADENYLIC ACID
GUANYLIC ACID
URIDYLIC ACID
X'
X

A'

Fig. 6

of N.KOH for the digestion prevented any deamination of cytidylic to uridylic acid (Davidson & Smellie, 1952). This refined technique was, therefore, used in the experiments dealing with the incorporation of $^{32}P$ into the RNA of different cell fractions. It could not, however, be used for any quantitative studies, as quantitative recovery of RNA is not possible after NaCl extraction.

To gauge the recovery of P applied to the paper, the nucleotides as well as the contaminants were cut out, eluted and estimated for total P and activity (Table 17). This was done at a time when Dr. Smellie was developing his technique. X refers to the crescent-shaped, ultraviolet absorbing band running in front of uridylic acid; i.e., between the uridylic acid band and the anode end of the paper (see Fig.6), while X' is that area of paper lying between X and the uridylic acid band after a short run. The amount and activity of the P in a volume of $A_2S$ equal to that applied to the paper was also estimated. The table shows that, when the livers were removed 2 hours after injection of $^{32}P$, the activity of the $A_2S$ fraction was much higher than that of the RNA, which is obviously due to the very high activity of both X and X'. In the 24 hour experiments, the activities of the RNA, X and X' were about the same, but the activity of the $A_2S$ was higher than any of these. The total P recovered from the paper was also higher than that actually

applied to the paper. This discrepancy will be understood when it is remembered that the actual quantities of P in X and X' are very small and their estimation is therefore far from accurate. Even a slight inaccuracy in the estimation that gives a higher value for the amount of P will decrease the specific activity, resulting in a low relative specific activity. These results are in agreement with the findings of Dr. Smellie (Smellie, 1952).

iv. Estimation of Phosphorus. Two methods were used for estimating P, the choice of the method depending on the circumstances of the experiment. In the majority of instances, the method described by Allen (1940) was used. When the inorganic P had to be separated quickly from organic phosphates, the modification by Ennor & Stocken (1950) of the method introduced by Berenblum & Chain (1938) was employed. The method of Allen: An aliquot of solution to be estimated containing 20 to 70 µg. P was digested in a micro-Kjeldhal flask with 1.2 ml. 10 N $H_2SO_4$, till the contents of the flask were colourless, digestion being aided by a few drops of $H_2O_2$ (M.A.R.). The flask was allowed to cool, and 6.35 ml. distilled water added, followed by 2 ml. amidol reagent (1% solution of amidol in 20% sodium metabisulphite) 1 ml. 8.3% ammonium molybdate, and 15 ml. distilled water, in that order. The blue colour that developed was estimated with a "Spekker" absorptiometer, using Ilford 608 filters. Details of the

method are given by Allen (1940).

The method of Ennor & Stocken: Described in the previous section.

v. Experiments with liver slices: The liver was sliced soon after its removal from the animal, the slices weighed on a torsion balance and suspended in Krebs-Ringer-Bicarbonate buffer (Umbriet et al., 1945) contained in 25 ml. conical flasks (3 ml. buffer solution for every 250-300 mg. slice). The solution was brought into equilibrium with an atmosphere of $O_2$ and 5% $CO_2$, the flasks firmly stoppered with rubber stoppers, and constantly agitated for 3 hours in a water bath maintained at 37°C. A very small amount of $^{32}P$-labelled inorganic phosphate or $^{35}S$-labelled methionine was added to the solution before incubation was started. The reaction was stopped by adding TCA, the flasks cooled and the precipitated proteins washed thrice with ice-cold 10% (w/v) TCA. The lipids were extracted and the residue incubated in alkali, according to Scheme 3. When $^{32}P$ incorporation was being studied, the activities of the inorganic P in the TCA soluble fraction and of the RNA were estimated as before. In the experiments with $^{35}S$ labelled methionine, the alkali digest was made up to a convenient volume, and an aliquot taken for N estimation by the micro-Kjeldhal method. An infinitely thin film (Calvin et al., 1949) of the digest on a metal disc

was dried and the radioactivity counted using an end-window counter (Type EHM2 manufactured by G.E.C.). The activity was expressed as counts per min. per mg. N.

Method of expressing radioactive data: Early workers using radioactive tracers took the percentage of the administered dose found in 1 g. of tissue to be a measure of the incorporation of the label by that tissue. It is, however, difficult to estimate the administered dose accurately. Further, the speed at which it is incorporated would depend on the richness of the blood supply to the tissue concerned. It is more accurate and often more important to know the proportion of atoms labelled, e.g., $^{32}P$ per mg. of RNAP. This proportion is referred to as the specific activity, and gives the value determined at the end of the experiment; the specific activity of liver RNAP equals the number of counts per min. per 100 µg. RNAP, at the time the liver was taken for analysis. This depends at the rate at which $^{32}P$ penetrated into the cell, among other factors. The amount of $^{32}P$ in the intra-cellular fluid of, say, the liver will depend on the amount of label in circulation in the extra-cellular fluid, which in turn will depend on the amount injected. Therefore, the Relative Specific Activity (R.S.A.), i.e., the specific activity of the compound divided by the specific activity of another compound in the same cell (such

TABLE 18.

The Effect of Changes in Energy Intake (Carbohydrate) on the Amount of RNAP per Liver.

Protein-Free Diet

| Expt. No. | No. of rats per expt. | Energy Levels | | Amount of RNAP per Liver | | Change in RNAP per liver per 1000 Cal. |
|---|---|---|---|---|---|---|
| | | Low | High | Low energy | High Energy | |
| | | Cals./sq.m. | Cals./sqm. | mg. | mg. | mg. |
| 1 | 12 | 855 | 1618 | 3.27 | 3.81 | +0.65 |
| 2 | 8 | 820 | 1790 | 3.18 | 3.70 | +0.47 |
| 3 | 6 | 663 | 1385 | 3.15 | 2.90 | -0.33 |
| 4 | 8 | 645 | 1371 | 2.73 | 2.87 | +0.21 |
| 5 | 6 | 889 | 1905 | 3.13 | 3.35 | +0.17 |
| 6 | 6 | 858 | 1812 | 3.29 | 3.60 | +0.25 |
| 7 | 6 | 832 | 1848 | 2.76 | 3.26 | +0.19 |
| 8 | 6 | 850 | 1847 | 2.77 | 2.86 | +0.11 |
| Weighted Mean Regressions | 58 | - | - | - | - | +0.26 |

Protein-Containing Diet

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 8 | 725 | 1413 | 2.75 | 3.78 | +1.51 |
| 2 | 8 | 627 | 1543 | 3.84 | 4.60 | +0.92 |
| 3 | 8 | 813 | 1715 | 3.25 | 4.29 | +1.03 |
| Weighted Mean Regressions | 24 | - | - | - | - | +1.09 |

as inorganic P) expressed as a percentage, is more useful and eliminates the difference in the rates of incorporation of the label into the intracellular phosphate in different tissues.

The specific activity and the R.S.A. measure the proportion of molecules replaced in a given time. If the amount of the compound present is large, the total amount synthesised may be quite large, but the proportion replaced in a given time may be small. In order to obtain the total amount of RNAP synthesised per liver, we can multiply the R.S.A. by the total amount present. Our results have, therefore, been expressed in the following ways:

i. the specific activity = counts per min. per 100 µg. P,

ii. the relative specific activity (R.S.A.) = specific activity of a compound divided by the specific activity of inorganic P expressed as a percentage,

and iii. the total amount of radioactivity of a compound per liver = R.S.A. multiplied by the amount of that compound per liver.

## RESULTS.

a. Energy Intake and the Quantity of RNA in the Liver:

Table 18 gives the statistical analysis of the effect of changes in energy intake on the amount of RNA in rat liver, when the animals are kept on protein-containing and protein-

TABLE 19.

The effect of changes in energy intake (carbohydrate) on the uptake of $^{32}P$ by liver RNA 24 hrs. after injection of $^{32}P$ as inorganic orthophosphate (4 rats per group).

| Diet | Mean Initial body wt. | Daily Energy Intake | Specific Activity of RNAP | R.S.A. of RNAP | Specific Activity of RNAP adjusted by covariance analysis | Amount of RNAP per liver | Total radioactivity of liver RNAP, adjusted by covariance analysis | R.S.A. of fraction $S_1$ |
|---|---|---|---|---|---|---|---|---|
| | g. | Cal./ sq.m. | c.p.m. per 100 µg P | | c.p.m. per 100 µg P | mg. | counts per min. | |
| Protein-containing | 185 | 810 | 580 | 37.9 | 683 | 3.25 | 22300 | 111 |
| | 186 | 1715 | 685 | 35.8 | 646 | 4.22 | 27600 | 108 |
| Protein-free | 193 | 820 | 761 | 38.2 | 680 | 3.18 | 21300 | 107 |
| | 189 | 1790 | 839 | 48.4 | 857 | 3.70 | 31600 | 101 |

TABLE 19 (Contd.)

Analysis of Variance on Relative Specific Activity of RNAP.

| Source of Variation | Degrees of Freedom | Sums of Squares | Mean Square | Variance ratio, F |
|---|---|---|---|---|
| Between protein levels | 1 | 16641 | 16641 | 9.48* |
| Between energy levels | 1 | 6481 | 6481 | 3.69 |
| Interaction | 1 | 15129 | 15129 | 8.62* |
| Residual error | 11 | 19301 | 1755 | - |

The value of F for the 5% level is 4.84, and for the 1% level is 9.65. The results indicate that the effect of a change in energy intake is different at the two levels of protein intake.

free diets. The last column gives the regression coefficients representing the change produced over a 4-day period by an increment in energy intake of 1000 Cal. per sq.m. of body surface area. The influence of protein intake on the magnitude of this change is striking, and highly significant. When expressed as a percentage of the amount of RNA in the liver present at an energy intake of 1200 Cal., which is just adequate for weight maintenance, the regression coefficients represent changes of 28.6% on the protein-containing diet, and 8.1% on the diet devoid of protein; i.e., when an animal is given protein, a change in energy intake of 1000 Cal. increases the RNA in the liver by 28.6% of what it contains when the energy intake is 1200 Cal., but only 8.1% in the case of protein-free diets.

b. Energy Intake and $^{32}P$ incorporation into RNA of Liver:

i. Variations in energy intake from addition of Carbohydrate. The influence of increasing energy intake on the incorporation of $^{32}P$ into the RNA of the liver is shown in Table 19. In the case of rats receiving protein in the diet, the relative specific activity (R.S.A.) tends to fall slightly as energy intake rises, whereas, on a protein-free diet, there is an appreciable rise in the R.S.A. Calculating the R.S.A. is one method of correcting for differences noticed in the inorganic P activity of different

TABLE 20.

The effect of changes in energy intake (carbohydrate) on the relative specific activity of liver RNAP. $^{32}P$ injected 24 hours before killing.

Protein-Free Diet

| Expt. No. | No. of Rats per expt. | Energy Levels | | R.S.A. of RNAP | | Change in R.S.A. per 1000 Cal. |
|---|---|---|---|---|---|---|
| | | Low | High | Low Energy | High Energy | |
| | | Cal./sq.m. | Cal./sq.m. | | | |
| 1 | 8 | 813 | 1793 | 38.2 | 48.4 | +9.68 |
| 2 | 14 | 653 | 1373 | 39.1 | 42.7 | +4.57 |
| 3 | 8 | 820 | 1891 | 34.1 | 54.2 | +18.34 |
| 4 | 4 | 823 | 1817 | 14.2 | 18.4 | +4.28 |
| | 34 | | | | | +10.33 |

High-Protein Diet

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 8 | 813 | 1715 | 37.9 | 35.5 | -2.44 |
| 2 | 8 | 670 | 1471 | 34.8 | 37.8 | +3.42 |
| | 16 | | | | | +1.23 |

livers. An alternative method of correcting for these differences is provided by the statistical procedure known as co-variance analysis (Snedecor, 1946) which gives values that would have been obtained had the specific activity of the inorganic P been the same for all livers. This correction is made solely from the evidence of the relationship between the specific activities of the RNAP and of the inorganic P provided by the experiment itself. The figures thus obtained (column 6 of the Table) confirm the picture shown by the study of the R.S.A.

The results discussed above are representative of a series of observations on rats killed 24 hours after injection of $^{32}P$. Over the whole series, 33 rats received the protein-free diet and 18 were given the protein-containing diet, the energy intake ranging from 600 to 1900 Cal. per sq.m. of body surface area. The change in the R.S.A. induced by the alterations in energy intake over this range is expressed by the regression coefficients per 1000 Cal. per sq.m. (Table 20), namely + 10.3 for the protein-free diet and +1.2 for the protein-containing diet. Taking the R.S.A. at 1200 Cal. as the reference standard, these regression coefficients represent a change of +26.5% and +3.4% respectively in the R.S.A. The change in the rate of incorporation is significant for the protein-free diet ($P < 0.01$) but not for the protein-containing diet. The

TABLE 21.

The effect of increasing additions of energy (carbohydrate) to a protein-free diet. The animals were first fed the diet at a level of 1200 Cal./sq.m., for 1 week, and then received different energy intakes for 4 days (3 rats per group). $^{32}P$ injected 24 hrs. before killing.

| Energy Intake | R.S.A. of RNAP |
|---|---|
| Cal./sq.m. | |
| 683 | 22.9 |
| 1000 | 25.7 |
| 1407 | 28.5 |
| 1917 | 32.4 |

Regression coefficient: +7.51 per 1000 Cal.

Analysis of Variance.

| Source of Variation | Degrees of Freedom | Sums of Squares | Mean Square | Variance ratio, F |
|---|---|---|---|---|
| Between replicates | 2 | 68,485 | 34,243 | 21.0 |
| Between energy levels | 3 | 14,593 | 4,864 | 2.99 |
| Linear regression | 1 | 14,569 | 14,569 | 8.94* |
| Deviations from linearity | 2 | 24 | 12 | 0.007 |
| Residual Error | 6 | 9,773 | 1,629 | - |

The F value for the 5% level is 5.99, and for the 1% level 13.74 (for $n_1=1$, $n_2=6$). Thus, the regression is significant. There is no significant deviation from linearity.

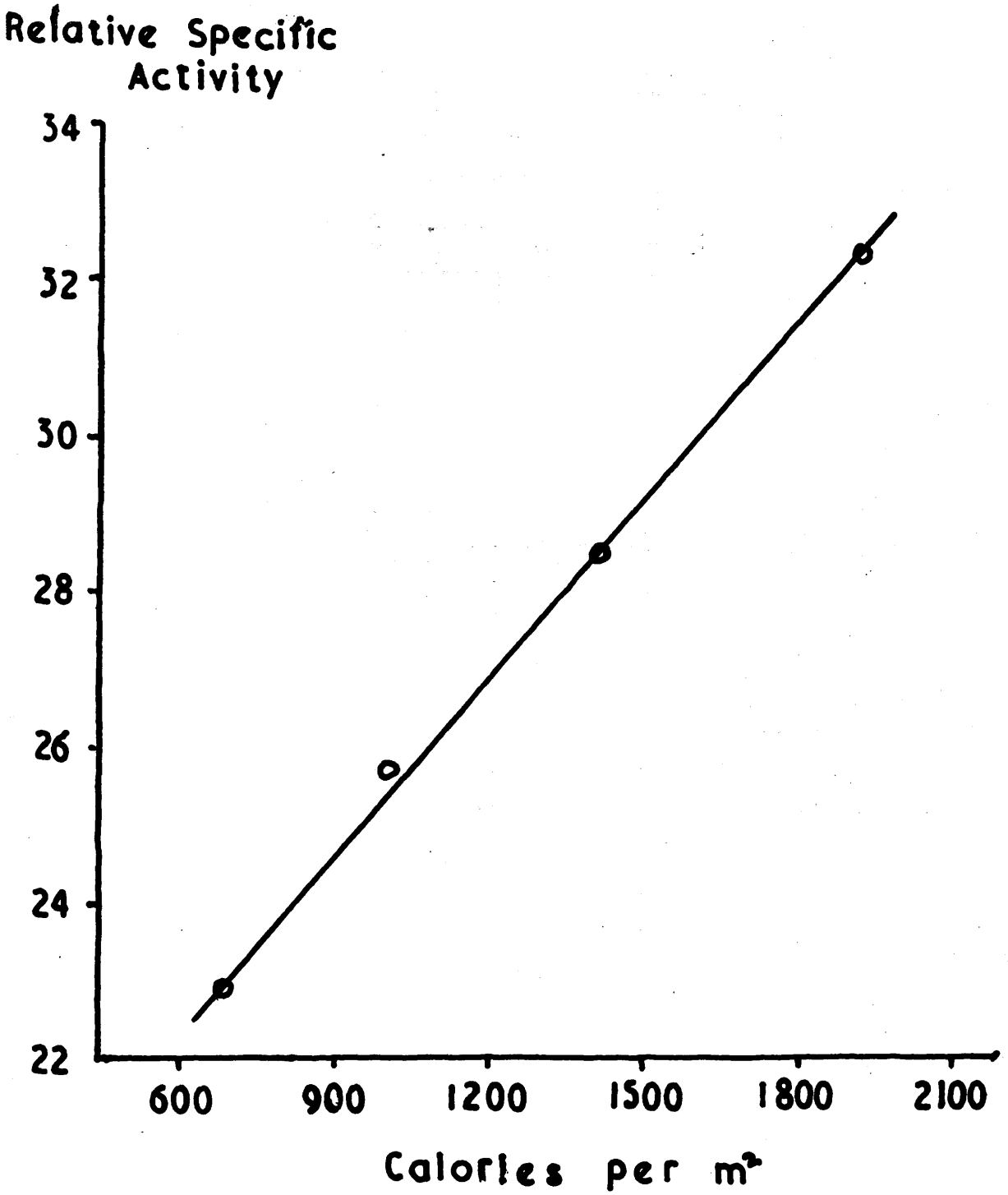

Relative Specific Activity
34
32
30
28
26
24
22
600
900
1200
1500
1800
2100
Calories per m²

Fig. 7

TABLE 22.

The effect of changes in energy intake (carbohydrate) on the uptake of $^{32}P$ by liver ribonucleic acid at 4, 8 and 24 hrs. after injection of labelled P. Each figure is the mean of observations made on 4 rats.

| Time after $^{32}P$ injection | Type of diet | Mean daily energy intake | Mean initial body weight | Specific activity of inorganic P of liver | Relative specific activity of acid-soluble organic P | Relative Specific Activity of Nucleotides | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Whole RNAP* | Adenylic & cytidylic acids | Guanylic acid | Uridylic acid |
| hrs. | | Cal./ sq.m. | g. | c.p.m./ 100 µg P | | | | | |
| 4 | Protein-free | 870 | 179 | 4680 | 84 | 7.5 | 7.6 | 5.2 | 8.7 |
| | Protein-free | 1880 | 179 | 4714 | 85 | 9.8 | 9.4 | 8.2 | 11.3 |
| | Protein-cont'g. | 1840 | 181 | 3638 | - | 4.4 | - | - | - |
| 8 | Protein-free | 950 | 177 | 3063 | 102 | 17.0 | 16.9 | 14.1 | 18.5 |
| | Protein-free | 1850 | 182 | 2419 | 110 | 19.2 | 19.1 | 17.5 | 21.3 |
| | Protein-cont'g. | 1840 | 186 | 2234 | 93 | 13.1 | 12.6 | 10.2 | 16.3 |
| 24 | Protein-free | 840 | 186 | 1769 | 105 | 34.1 | 33.1 | 32.5 | 34.6 |
| | Protein-free | 1890 | 173 | 2237 | 104 | 54.2 | 51.6 | 51.6 | 54.1 |
| | Protein-cont'g. | 1725 | 184 | 1808 | 100 | 32.1 | 32.1 | 28.6 | 34.2 |

*Short run ionophoresis, with nucleotides eluted together.

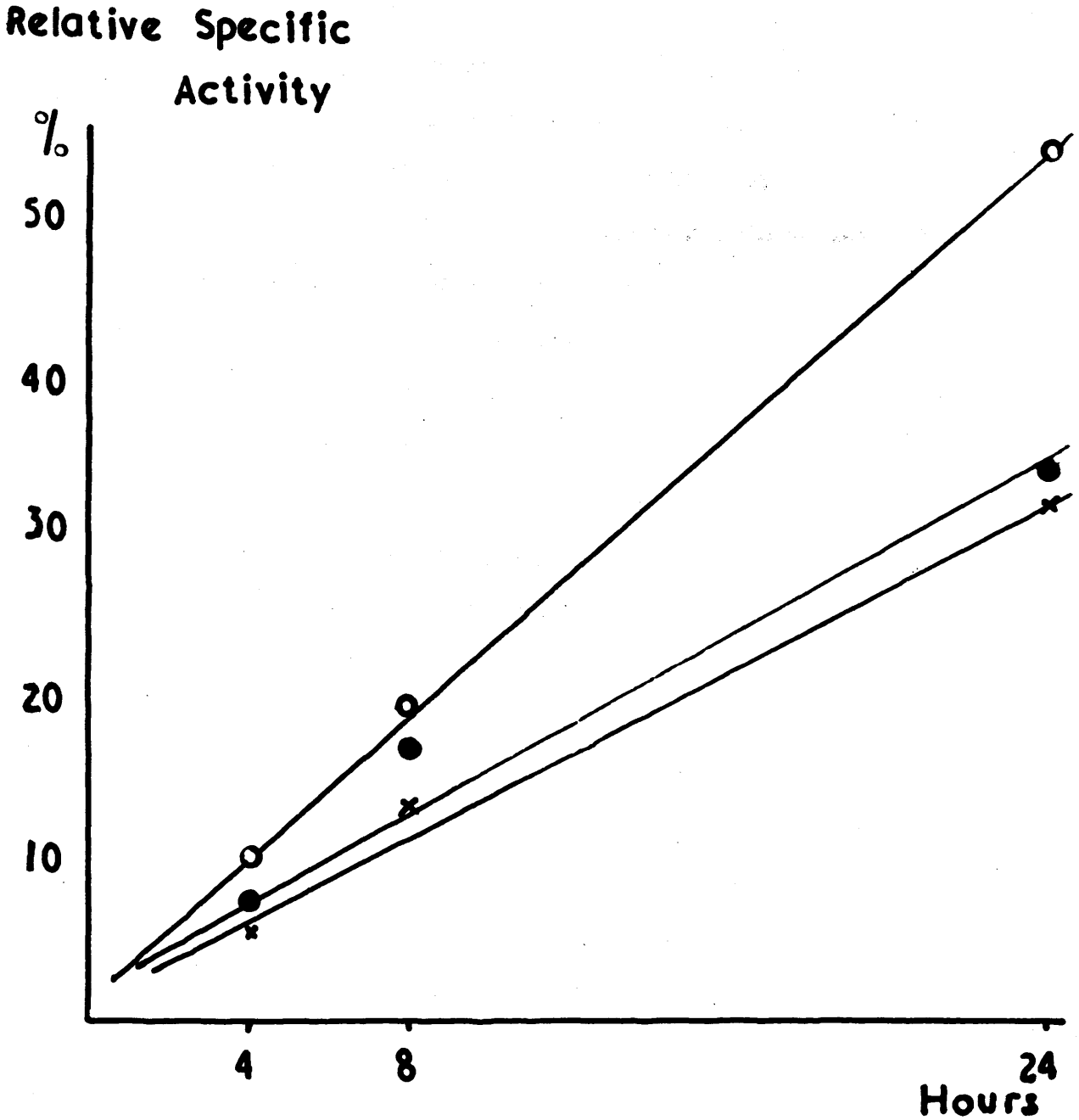



Fig. 8

●——● Low Protein, Low Energy.
o——o Low Protein, High Energy.
×——× High Protein, High Energy.

pooled data from all the experiments is thus in agreement with the single experiment quoted in Table 19.

To confirm the magnitude of the change found in the case of the protein-free diet, a further experiment was carried out in which 4 levels of energy intake were obtained by adding glucose to the protein-free diet. The results shown in Table 21 and Fig.7 remove all doubt as to the linear relationship between energy intake and R.S.A. of the RNAP. Again, using the value at 1200 Cal. per sq.m. as reference standard, and calculating the regression coefficient, it was found that an increase of 1000 Cal. per sq.m. body surface area resulted in a 27.8% increase in the R.S.A. ($P < 0.05$).

The results discussed so far deal with values obtained 24 hours after injection of $^{32}P$. The same picture was obtained with rats killed at shorter time intervals. On account of the amount of work involved, three dietary groups were chosen to provide the necessary contrasts (see Table 19), namely, animals receiving the protein-free diet at high and low levels of energy intake, and those receiving the protein-containing diet at the higher plane of energy intake. They were injected with $^{32}P$ at the usual time on the last day of the 4-day period, and sacrificed at 4 hours, 8 hours and 24 hours after injection. The R.S.A. of animals fed the protein free diet at the higher plane of energy intake were the greatest at all time intervals studied (Table 22 and Fig.8). In this

experiment the specific activities of the individual nucleotides were also studied by carrying out a long ionophoresis run. Table 22 shows, in addition, that under the same conditions of diet and time after isotope injection, the various nucleotides incorporate $^{32}P$ to essentially the same extent. Each nucleotide shows a higher incorporation rate when obtained from animals on the protein-free diet at the higher level of energy intake, than in the other groups. Since contaminants could hardly affect all nucleotide fractions similarly, these figures rule out the possibility that any radioactive contaminant plays a significant part in accounting for the differences observed in P incorporation into RNA on different diets. The specific activity of the acid-soluble organic phosphate fraction ($S_1$), estimated in this experiment, is not significantly altered by the nature of the diet at any of the time-intervals after injection of $^{32}P$ (see also Table 19). This fraction contains compounds such as adenylic acid, ADP, ATP, phospho-glyceric acid, glycerophosphoric acid and glucose-I-phosphate, and from our results we see that, at these time intervals, the changes shown by RNAP in relation to diet are not paralleled by changes in phosphorylation in general. These data from the $S_1$ fraction also provide another important safeguard, namely that the intra-cellular phosphate has a specific activity

TABLE 23.

Comparison of the effect on $^{32}P$ uptake by liver RNA of adding fat to a protein-free diet with the effect of adding carbohydrate (3 rats per group).

| Source of energy variation | Daily energy intake | R.S.A. of RNAP |
|---|---|---|
| | cal./sq.m. | |
| Carbo-hydrate | 1000 | 25.7 |
| | 1920 | 32.4 |
| Fat | 980 | 23.2 |
| | 1900 | 30.5 |

Analysis of Variance:

| Source of Variation | Degrees of freedom | Sums of Squares | Mean Square | Variance ratio (F) |
|---|---|---|---|---|
| Between energy levels | 1 | 14,770 | 14,770 | 7.87* |
| Between carbohydrate & fat | 1 | 1,430 | 1,430 | 0.75 |
| Interaction | 1 | 37 | 37 | 0.02 |
| Replicates | 2 | 51,521 | 25,760 | 13.7 |
| Residual Error | 6 | 11,265 | 1,877 | - |

F = 5.99 at the 5% level and 13.74 at 1% level. Therefore, the influence of energy intake is significant, but the effect of carbohydrate does not differ from that of fat (interaction not significant).

parallel to that of the inorganic phosphate. This is in agreement with the suggestion of Kalckar et al. (1944) that the correction for extra-cellular phosphate in the liver is small on account of the rapid penetration of the phosphate into the liver cells. Venkataraman et al. (1950) hold that any evaluation of the intra-cellular inorganic $^{32}P$ on the basis of the extra-cellular space of the tissue is invalid, but it should be noted that the time intervals at which their studies were made were very small, the animals being killed within 5 min. of the injection of the radioactive label. Further, in all our experiments, the livers were perfused with saline, to reduce contamination of intra-cellular with extra-cellular phosphate.

ii. Variations in energy intake from addition of Fat: The experiments described so far have dealt with changes caused by alterations in energy intake in the form of carbohydrate. The effect of adding fat to a protein-free diet was studied next. One series of rats was maintained on an energy intake of 1200 Cal. per sq.m. by adding fat, and another series by adding carbohydrate to a basal protein-free diet for one week, at the end of which time different energy levels were obtained in each series by varying the amount of fat or carbohydrate fed. The results, given in Table 23, show that fat and carbohydrate are equally effective in

TABLE 24.

The effect of adding carbohydrate or fat to a protein-free diet on the incorporation of $^{32}P$ into RNA of liver. The rats received the diet for 7 days at a level of 1200 Cal. per sq.m. Then energy intake was varied over a 4-day period, so that one (basal) group received 832 Cal. per sq.m. per day, a second group 1879 Cal. per day by adding carbohydrate to the basal diet, and a third group 1818 Cal. per day by adding fat to the basal diet (3 rats per group). $^{32}P$ was injected 24 hrs. before killing.

| Diet | Energy Intake | R.S.A. of RNAP |
|---|---|---|
| | Cal.per sq.m. | |
| Basal | 832 | 19.7 |
| Basal + carbohydrate | 1879 | 23.8 |
| Basal + fat | 1818 | 24.8 |

Analysis of Variance:

| Source of Variation | Degrees of Freedom | Sums of Squares | Mean Square | Variance ratio (F) |
|---|---|---|---|---|
| Between diets | 2 | 4,424 | 2,212 | 3.47 |
| Between experiments | 2 | 69,520 | 34,760 | 54.4 |
| Residual error | 4 | 2,552 | 638 | - |

The F value at 5% level of significance is 6.94. The experiment therefore falls short of a statistical demonstration that carbohydrate and fat both raise the rate of $^{32}P$ incorporation into RNA. However, the magnitude of the change is of the same order as in the preceding experiment.

TABLE 25.

The effect of glucose administration, during a 24 hour period following high and low protein diets, on the amount of RNAP per liver and on the rate of incorporation of $^{32}P$ into RNA (4 rats per group). $^{32}P$ was injected 24 hrs. before killing.

| Diet | Initial body wt. | Daily energy intake on diet | Food during final 24 hrs. | RNAP per liver | Sp.activity of RNAP | Sp.activity of Inorg. P | R.S.A. of RNAP |
|---|---|---|---|---|---|---|---|
| | g. | Cal./sq.m | | mg. | c.p.m./ 100 ug P | c.p.m./ 100 ug P | |
| Protein-containing | 226 | 1680 | Glucose | 5.09 | 611 | 1525 | 39.9 |
| | 230 | 1680 | None | 4.65 | 641 | 1742 | 36.7 |
| Protein-free | 233 | 1705 | Glucose | 4.22 | 986 | 2044 | 48.2 |
| | 230 | 1725 | None | 4.56 | 1182 | 2227 | 53.2 |

Analysis of Variance of Amount of RNAP per Liver:

| Source of Variation | Degrees of Freedom | Sums of Squares | Mean Square | Variance ratio (F) |
|---|---|---|---|---|
| Between protein levels | 1 | 9313 | 9313 | 7.63* |
| Between glucose & no glucose | 1 | 91 | 91 | 0.08+ |
| Interaction | 1 | 6161 | 6161 | 5.05* |
| Residual Error | 12 | 14651 | 1221 | - |

The F value at 5% is 4.75 and at 1% level is 9.33. Thus, a previous intake of protein combined with glucose during the final 24 hrs. (interaction) gave a significantly higher amount of RNA per liver.

TABLE 25 (Contd.).

Analysis of Variance of R.S.A. of RNAP.

| Source of Variation | Degrees of Freedom | Sums of Squares | Mean Square | Variance ratio (F) |
|---|---|---|---|---|
| Between protein levels | 1 | 654 | 654 | 11.28** |
| Between glucose & no glucose | 1 | 6 | 6 | 0.11+ |
| Interaction | 1 | 80 | 80 | 1.37+ |
| Residual Error | 12 | 696 | 58 | - |

F is 9.33 at 1% and 4.75 at 5% level. Thus, the only factor affecting the relative specific activity is the previous protein level, and <u>not</u> the immediate intake of glucose.

stimulating the rate of incorporation of $^{32}P$ into RNA. An additional experiment with fat (Table 24) confirmed this result. In this experiment, however, all the animals were on the same basal diet, and in the 4-day period, the energy intake was altered by adding fat or carbohydrate. The magnitude of the changes produced by the addition of energy were of the same order as in the preceding experiment, though the values fall short of statistical significance.

c. The Immediate Effect of Changes in the Nutritional State:

An attempt was made to study the mechanism underlying the above changes in RNAP synthesis in two further experiments. In the first, rats were fed either a protein-containing or a protein-free diet at a high level of energy intake for a week and then injected with labelled phosphate. After the injection, half of each group were given glucose ad lib. and the rest fasted. All the animals were killed 24 hours after the injection. The data (Table 25) show that the rate of incorporation was significantly influenced by the previous diet and not by the immediate ingestion of glucose. The group on the protein-free diet did not lose their higher incorporation rate when fasted for 24 hours, nor did the high-protein group show an increased incorporation rate when fed glucose ad lib. for a whole day. This picture is amplified by the next experiment, in which all the animals were fed a diet providing an adequate amount

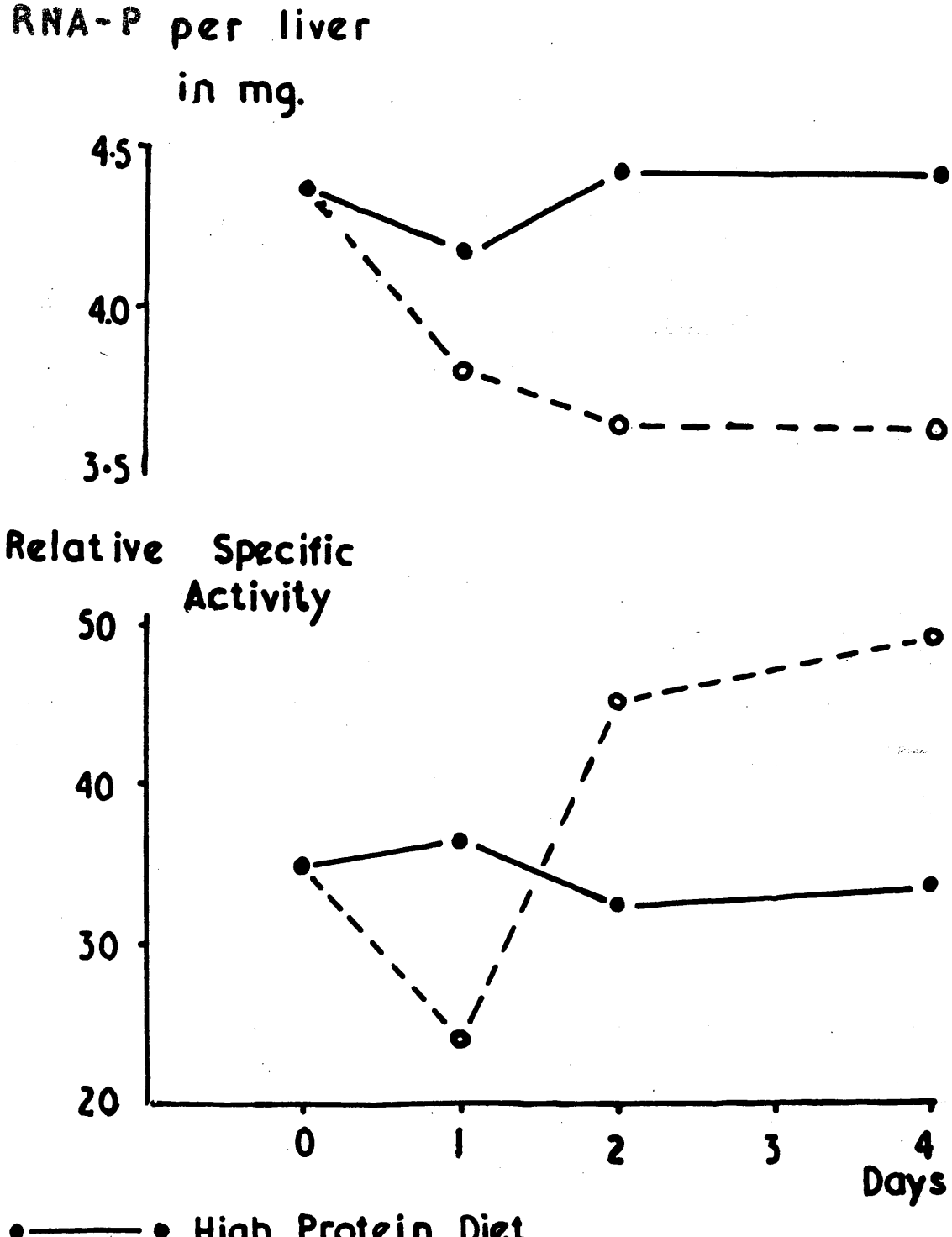

RNA-P per liver
in mg.
4.5
4.0
3.5
Relative Specific
Activity
50
40
30
20
0
1
2
3
4
Days
High Protein Diet
Low Protein Diet.

Fig. 9

of protein and energy (1700 Cal. per sq.m.). At the end of a week half the rats were given a protein-free diet, an iso-caloric amount of carbohydrate being substituted for the protein. Thereafter, groups of three rats on each diet were killed daily, having been injected with labelled phosphate 24 hours previously. The transfer to the protein-free diet produced an immediate reduction in the amount of RNAP in the liver. The rate of incorporation of $^{32}P$, however, registered a fall on the first day, followed by the expected higher level from the second day onwards (Fig. 9). The group that continued to get the protein-containing diet showed a constant amount of RNAP in the liver and no significant alteration in the rate of incorporation of $^{32}P$ (Fig.9). Both these experiments indicate that a change in the nutritional condition is not immediately followed by an alteration in the uptake of P by RNA.

d. The Uptake of $^{32}P$ by RNA in Tissue Slices: Two experiments were carried out with liver slices to find out whether this effect of the addition of carbohydrate on the incorporation of $^{32}P$ into the RNA could be demonstrated in the isolated liver. The animals were prepared as before, being fed a protein-containing or a protein-free diet for a preliminary period, and each group then divided into two, glucose being added to the diets of some to change the level

TABLE 26.

The effect of previous levels of energy intake (carbohydrate) on the uptake of $^{32}P$ by RNA in liver slices. The slices were incubated for 3 hours at 37°C in Krebs-Ringer-bicarbonate buffer containing added $^{32}P$.
(Mean of 2 experiments per observation).

| Diet | Relative Specific Activity of RNAP | |
|---|---|---|
| | Low Energy Level | High Energy Level |
| Protein-containing | 2.27 | 2.38 |
| Protein-free | 2.41 | 4.25 |

of energy intake. At the end of a 4-day period, the animals were killed and slices of liver incubated in Krebs-Ringer-Bicarbonate buffer containing a small amount of $^{32}P$. The results are given in Table 26. In the case of liver slices, as in the case of the whole animal, we find a significant increase in the R.S.A. of the RNAP when the energy intake was raised in the protein-free diet.

e. The Uptake of $^{35}S$ by Protein in Tissue Slices: Two experiments were carried out on liver slices, substituting a small amount of $^{35}S$-labelled methionine for the labelled phosphate in the previous experiments. In the first, two rats were fed a protein-free diet at a high and a low level of energy intake. The activity of the alkali digest of the TCA-insoluble-lipid-free residue was 1866 counts per min. per mg. protein N. in the animal that had been on a high level of energy intake, and 1817 counts per min. in the other animal. In the second experiment, two rats were maintained at the same level of energy intake but fed protein-free and protein-containing diets, and the activities were 1600 and 1491 counts respectively. Taking into account the errors involved in the actual counting, the differences between these activities do not indicate a difference in the rate of incorporation of the label into the liver protein, either with changes in energy intake, or with changes in protein intake. However, these experiments require considerable amplification.

TABLE 27.

Comparison of the changes in amount of RNA per liver and $^{32}P$ uptake by RNA when energy intake from carbohydrate is increased. Regression coefficients indicate the change produced by adding 1000 Cal. per sq.m. body surface area to the diet, and have been expressed as a %age of the amount of RNAP or the R.S.A. of RNAP corresponding to an energy intake of 1200 Cal. Figures in brackets are the number of animals per experiment.

| Diet | Method of RNA isolation | Regression Coefficients | |
|---|---|---|---|
| | | Total RNAP per liver | R.S.A. of RNAP |
| Protein-containing | Ionophoresis | % +28.6 (24) | % +3.4 (16) |
| | Schmidt-Thannhauser * | +29.2 (16)<br>+20.8 (20) | -<br>- |
| Protein-free | Ionophoresis | + 8.1 (58)<br>- | +26.5 (33)<br>+27.8 (12) |
| | Schmidt-Thannhauser * | +10.9 (16)<br>+ 4.4 (24) | -<br>- |

* D.J. Naismith's data.

## DISCUSSION.

a. Energy Intake and RNA Synthesis. The experiments dealing with the amount of RNA in the liver and its incorporation of labelled phosphate indicate that energy intake has a definite influence on the metabolism of RNA. All the data from experiments involving a change in energy intake from carbohydrate are shown in Table 27. The change produced by an increment of 1000 Cal per sq.m. of body surface area has been expressed as a percentage of the amount or of the incorporation rate found at an energy intake of 1200 Cal. per sq.m. This enables one to compare the magnitude of the alterations in the amount and in the incorporation rate caused by various levels of energy. The change in the amount of RNA obtained by the ionophoretic estimation compare favourably with the values obtained by Naismith (Munro, Naismith & Wikramanayake, 1952) using the less accurate Schmidt-Thannhauser procedure; his data are included in Table 27 for comparison. It is clear that an increase in the energy intake by 1000 Cal. per sq.m. produces an increase in the amount of RNAP in the liver of 20 to 30% when the animal is fed a protein-containing diet without significantly altering the rate of incorporation of $^{32}P$, i.e., without a change in the number of P atoms replaced in a given time. When the animal is on a protein-free diet, on the other hand, the amount of RNAP is slightly increased (by 5 to 10%),

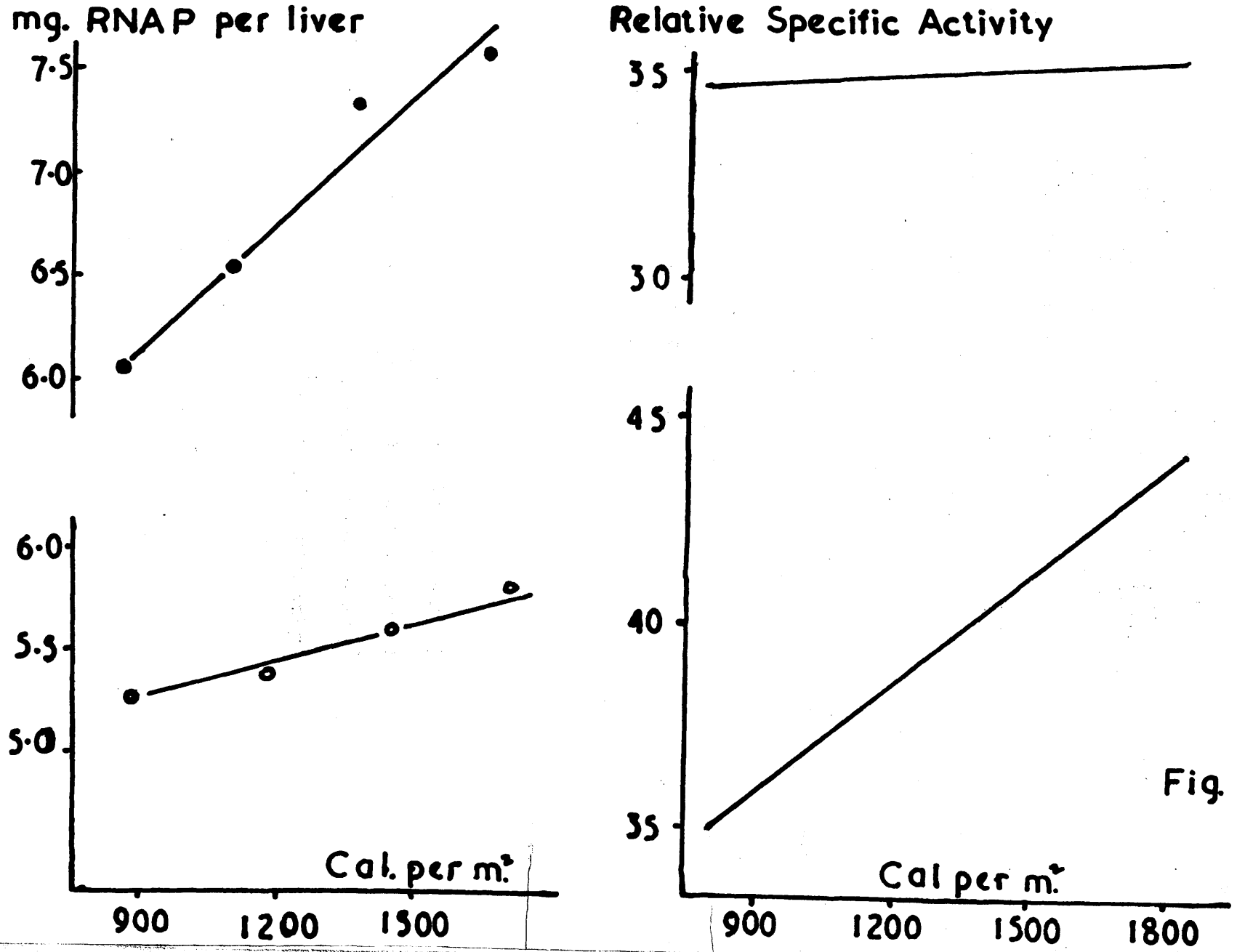

mg. RNAP per liver
7·5
7·0
6·5
6·0
6·0
5·5
5·0
Cal. per m².
900
1200
1500
Relative Specific Activity
35
30
45
40
35
Cal per m².
900
1200
1500
1800

Fig. 10

while the rate of replacement is augmented by about 25%. This indicates that an increase in energy intake causes an increase in the total number of P atoms replaced in a unit of time, the increase being the same whether the animal is on a protein-containing or on a protein-free diet. The increase is produced, in the first case, by an increase in the total amount, and, in the other, by a higher replacement rate (Fig.10). This is also demonstrated in a different and a more direct way in Table 19. Multiplying the specific activity of the RNAP by the total amount in the liver gives a measure of the total amount of $^{32}P$ incorporated per liver, and these values, adjusted by co-variance analysis to allow for differences in the specific activity of the inorganic P in the liver, as given in column 8 of this Table. It is apparent that, whether the diet contains protein or not, a change in energy intake produces an increase in the absolute incorporation of $^{32}P$. Although it may appear from the data that the increment is larger on the protein-free diet, statistical analysis shows that it is not significantly greater than the values obtained on a protein-containing diet. This supports the data given in Table 27 and illustrated in Fig.10, which indicate that the changes in amount and relative specific activity are essentially compensatory, and lead to the same total synthesis of RNA.

We see therefore, that RNA metabolism in the liver is affected by two dietary factors, the amount of RNA being determined mainly by the protein intake, while the rate of incorporation of P is governed by the energy intake. Whether on a protein-free or on a protein-containing diet, there exists a definite relationship between the energy content of the diet and the total incorporation of P into the liver. The incorporation of P may only mean that the P atoms are being replaced without producing a similar change in the rest of the nucleic acid molecule. This question can only be finally settled by the use of labels other than $^{32}P$, such as $^{14}C$. However, Potter, Recknagel & Hurlbert (1951) studied the changes in the specific activity of RNA in the rat liver with time, using $^{14}C$ labelled orotic acid. Their time curves are very similar to those obtained by Davidson & Smellie (1952) using $^{32}P$ as a label. This similarity is an indication that the turnover of RNA as shown by the incorporation of $^{32}P$ is a true measure of the rate of breakdown and synthesis of the compound as a whole. Therefore, it is justifiable to deduce that increasing the energy intake results in a rapid breakdown and re-synthesis of a major portion, if not the whole, of the RNA molecule.

b. RNA and Protein Synthesis: Do these findings throw any light on the relationship of RNA to protein synthesis, especially as regards the influence of energy intake on

protein metabolism?

The amount of protein in the liver is reduced when the animal is transferred from a protein-containing to a protein-free diet (Davidson, 1947; Kosterlitz, 1947) and so is the RNA. On a protein-free diet, the RNA shows a slight increase with increase in energy, but not the protein of the liver (Munro & Naismith, 1952). This lack of change in the amount of protein might, however, mask an increased protein synthesis coupled to an increased rate of breakdown. The experiments with labelled methionine indicate that this is not so. It should be pointed out that these are only preliminary experiments and no attempt was made to separate methionine from cystine and to estimate its activity separately. Yet they agree with the results of Simpson & Tarver (1950) who carried out similar experiments on tissue slices and estimated the activity of methionine in the protein. They found no difference in the uptake of $^{35}S$ between livers from animals fed with protein and those on a protein-free diet. Since, presumably, their animals were fed at adequate levels of energy intake, those on a protein-free diet would have less RNA in their livers but a greater $^{32}P$ incorporation (cf. Fig.19). Yet the protein in the livers studied by them did not show the same change. The data given in Table 26 show that the incorporation of $^{32}P$ into RNA proceeds in tissue

slices as it does in the whole animal.

The possibility that the liver is forming plasma proteins as rapidly on a protein-free diet as on a protein-containing one is negatived by the experiments of Miller et al. (1950) who perfused the isolated intact liver with a solution containing amino-acids, and studied the turnover of labelled lysine in the liver and plasma proteins. They found that the amount of liver and plasma protein synthesised in a given time is dependent on the supply of amino-acids. On a protein-free diet, the supply of amino-acids is low and therefore one would expect a reduced synthesis of both liver proteins and plasma proteins. Thus, all the available evidence suggests that the total protein synthesis of the liver is depressed by the lack of protein in the diet, whereas the RNA synthesis is not.

If the formation of RNA is not depressed by the lack of dietary protein, one would expect the RNA synthesised to accumulate in the liver when the animal is on a protein-free diet, as it does when the animal is fed protein together with increasing amounts of dietary energy. As Table 18 shows, this is not so and this fact suggests that an increase in energy intake creates a demand for RNA, which is removed as fast as it is formed. That this demand is not brought about by immediate changes in energy intake is shown by the results given in Table 25 and in Fig.9. Feeding the animal with

glucose for 24 hours before killing did not produce a change in the incorporation of $^{32}P$ from the values obtained in the case of animals starved during this same period. Again, removal of protein from the diet does not result in an increased incorporation of $^{32}P$ till the level of RNA in the liver has fallen considerably. Campbell & Kosterlitz (1948) carried out a similar experiment as that illustrated in Fig.9. Their results differ from ours only in that the relative specific activity of the RNA begins to rise, in their experiment, from the first day on a protein-free diet, whereas we observed a fall in the R.S.A. on the first followed by an increase on the next day. It should be pointed out that they were taking the $A_2S$ fraction to represent the RNA, no effort being made to remove P-containing contaminants. Our results seem to indicate that the RNA present in the liver cell is sufficient to meet the demands on the first day on a protein-free diet. When this supply has been exhausted, more RNA has to be synthesised to meet these requirements.

In view of the close association between protein, RNA and phospholipid in the liver, which has been termed "labile liver cytoplasm" by Kosterlitz (1947), it seemed of interest to know whether the metabolic changes observed with RNA in relation to energy intake also occurred in the case of

phospholipids. Accordingly, a shorter series of experiments similar to those described above were carried out in which the rate of $^{32}P$ incorporation into phospholipids was studied. These experiments are described in the next section.

# PART IV.

# THE EFFECT OF ENERGY INTAKE ON THE METABOLISM OF LIVER PHOSPHOLIPID.

## INTRODUCTION.

A close relationship exists between the phospholipids and the proteins and RNA of the liver. Fasting, or feeding on protein-free diets, or on diets lacking essential amino-acids, results in a loss of phospholipids, protein and RNA from the liver, while a high-protein diet increases the amount of all three constituents (Kosterlitz & Cramb, 1943; Davidson & Waymouth, 1944; Kosterlitz, 1944). The level of dietary fat influences the total phospholipid content much less than the level of dietary protein, while choline had no effect at all (Kosterlitz, 1951). This similarity in behaviour of phospholipid, protein and RNA led Kosterlitz (1947) to postulate that a fraction which he called the "labile liver cytoplasm" was easily lost from the liver when the protein or energy content of the diet was reduced.

A further suggestive fact was that the decrease in amount of RNA and phospholipid caused by feeding a protein-free diet is accompanied by a rise in the rate of $^{32}P$ incorporation into the phospholipid as well as into the RNA in the liver (Campbell & Kosterlitz, 1948). The liver phospholipids would thus appear to behave metabolically in the same fashion as RNA, the rate of $^{32}P$ incorporation being speeded up to compensate for a reduction in amount. Is energy intake the factor which regulates the rate of

synthesis of the phospholipids as in the case of RNA? In order to determine this, experiments similar to those described for RNA were carried out.

## EXPERIMENTAL.

a. Animals, Diet and Management: These were the same as in the experiments on RNA.

b. Estimation of the Amount and R.S.A. of Liver Phospholipid: The liver was subjected to the fractionation procedure given in Scheme 3. The fat solvents used were, however, slightly different. The TCA precipitate was mixed with 20 ml. portions of absolute ethanol, ethanol-chloroform (3:1), and ethanol-ether (3:1) twice, the mixture being warmed each time for about 10 min. in a water bath maintained at 65 to 70°C. After centrifuging, each solvent was filtered into a measuring cylinder. The precipitate was finally extracted with 20 ml. ether, and the RNA in the residue estimated as before.

The combined lipid extracts were made up to a convenient volume and mixed. An aliquot of this was evaporated in a micro-Kjeldhal tube and estimated for P by the Allen method. The blue solution obtained was counted for radioactivity in a liquid counter. The crude lipid extract (L.E.) was then purified for radioactivity determinations to get rid of any contaminating P-containing compounds, as

recommended by Popjak & Muir (1950). An aliquot was evaporated to dryness at room temperature, and the lipids in the dry residue dissolved in petroleum ether, the extracts being filtered into a measuring cylinder. The P in this fraction (L.P.) was estimated as before for amount and activity. Any organic P-containing compounds, such as phosphoproteins, would be left in the dry residue and not be extracted by the petroleum ether. Any contaminating inorganic P was removed from this L.P. fraction by washing a portion of it with 0.05 N HCl, and then thrice with distilled water (Popjak & Muir, 1950). This washed fraction (L.P.W.) was also estimated for activity. In this method of purification, petroleum ether was used for extracting the dry residue, instead of chloroform (Davidson et al., 1951), on account of the emulsion obtained when chloroform is shaken with water. Petroleum ether, like chloroform, dissolves all the different types of phospholipids in the liver (Zilversmit et al., 1948). Popjak & Muir (1950) evaporated the crude extract in an atmosphere of $N_2$ to prevent oxidation of phospholipids, but this was not found necessary, as we were only estimating the radioactivity of the fractions L.P. and L.P.W., and not absolute quantities.

The specific activities of the phospholipids in these three fractions, L.E., L.P., and L.P.W. were estimated in a

TABLE 29.

The effect of energy intake (carbohydrate) on the amount of phospholipids in the liver.

| Diet | No. of rats per expt. | Low Energy Level | | High Energy Level | | Change per 1000 Cal. | Change per 1000 Cal. as % of amount at 12000 Cal. |
|---|---|---|---|---|---|---|---|
| | | Energy Intake | Phospho-lipin per liver | Energy Intake | Phospho-lipin per liver | | |
| | | Cal./ sq. m. | mg. | Cal./ sq.m. | mg. | mg. | % |
| Protein-containing | 12 | 781 | 6.36 | 1663 | 7.96 | +1.82 | +26 |
| Protein-free | 12 | 810 | 4.68 | 1776 | 4.17 | -0.52 | -12 |

series of animals, and the results are given in Table 28. There is no significant difference between the specific activities of the fractions L.E. and L.P.W. The activity of the L.P. fraction exceeded that of L.P.W. by 3.1%, this difference being statistically significant. But a 3.1% difference in the specific activity is not of practical importance when one considers the errors involved in the method of counting. In all the experiments reported, therefore, the specific activity of the L.E. fraction was taken as that of the phospholipid in the liver. The amount of phospholipid in the liver was calculated from the amount of P in this fraction. The possibility of using this fraction for radioactivity measurements too reduces considerably the amount of work to be done.

## RESULTS.

The experiments were carried out on similar lines as the ones estimating RNA, described earlier. Table 29 gives the change in the amount of liver phospholipid brought about by changes in the energy intake, when the animals were kept on a protein-free or on a protein-containing diet. On a protein-containing diet, increasing the energy intake by 1000 Cal. per sq.m. body surface area produces an increase in the liver phospholipid by 1.82 mg., i.e., an increase of

TABLE 30.

The effect of energy intake (carbohydrate) on the incorporation of $^{32}P$ into the phospholipids of the liver (2 rats per observation).

| Diet | Time after 32P injection | Energy Intake | | Relative Specific Activity | | | Relative Specific Activity x Amount | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Low Level | High Level | Low Energy | High Energy | Change 1000 Cal. | Low Energy | High Energy | Change/ 1000 Cal. |
| | hrs. | Cal./ sq.m. | Cal./ sq.m. | | | | | | |
| Protein containing | 2 | 789 | 1646 | 15.3 | 14.9 | -3% | 0.93 | 1.17 | +27% |
| | 5 | 780 | 1679 | 33.6 | 36.5 | +9% | 2.16 | 2.73 | +26% |
| | 8 | 774. | 1664 | 53.9 | 58.5 | +9% | 2.99 | 4.31 | +41% |
| | Mean | 781 | 1663 | - | - | +5% | - | - | +31% |
| Protein free | 2 | 810 | 1755 | 13.2 | 15.2 | +15% | 0.66 | 0.63 | - 6% |
| | 5 | 815 | 1810 | 38.7 | 49.6 | +25% | 2.03 | 2.15 | + 6% |
| | 8 | 807 | 1765 | 66.9 | 76.2 | +14% | 2.60 | 3.12 | +19% |
| | Mean | 810 | 1776 | - | - | +18% | - | - | + 6% |

26% on the amount present at an intake of 1200 Cal. per sq.m. On the other hand, on a protein-free diet, a similar change in energy intake causes a decrease in the liver phospholipid by an amount equal to 12% of that present at 1200 Cal. This decrease on a protein-free diet differs from the effect observed on the amount of RNAP in the liver, which showed a small though significant increase as the energy intake was raised.

A similar difference between phospholipid and RNA metabolism was observed when the incorporation of $^{32}P$ into these two compounds was studied. The experiments were carried out in the same way as those described in the previous section. After a 4-day period, the animals were injected with labelled phosphate, and killed at 2, 5, and 8 hours after injection. The results are shown in Table 30. At similar energy levels, the incorporation of $^{32}P$ as shown by the relative specific activity (R.S.A.), is somewhat greater when less protein is available in the diet. The change brought about by an increase in energy intake is greater on a protein-free diet than on a protein-containing one. When the R.S.A. is multiplied by the amount of phospholipid in the liver, we obtain a measure of the <u>total</u> number of P atoms replaced in phospholipid per liver in a unit of time. In this way, we find a greater increase (31% per 1000 Cal. per sq.m., taking the value at 1200 Cal. per sq.m. as a

TABLE 31.

The Effect of energy intake (carbohydrate) on the incorporation of $^{32}P$ into the RNA of liver. (2 animals per observation).

| Diet | Time after $^{32}P$ injection | Energy Intake | | Relative Specific Activity | | |
|---|---|---|---|---|---|---|
| | | Low Level | High Level | Low Energy | High Energy | Change/1000 Cal. |
| | hrs. | cal/sq.m. | Cal./sq.m. | | | % |
| Protein-containing | 2 | 789 | 1646 | 2.0 | 1.5 | -33 |
| | 5 | 780 | 1679 | 4.7 | 5.0 | + 7 |
| | 8 | 774 | 1664 | 7.6 | 8.5 | +13 |
| | Mean | 781 | 1663 | - | - | - 4 |
| Protein-free | 2 | 810 | 1755 | 2.8 | 3.7 | +32 |
| | 5 | 815 | 1810 | 6.4 | 8.8 | +33 |
| | 8 | 807 | 1765 | 11.7 | 17.1 | +48 |
| | Mean | 810 | 1776 | - | - | +38% |

standard of reference) due to the energy when the diet contains protein, and only 6% increase when the animal receives no protein. On a protein-containing diet, this change is due to an increase in amount brought about by the augmented energy intake (Table 30) as well as by a slight rise in the R.S.A. of phospholipid. In the case of the protein-free diet, the slight increase in R.S.A. is balanced by a diminution in the total amount as energy intake rises, so that the product of R.S.A. and the amount per liver does not vary significantly as energy intake increases.

The incorporation of $^{32}P$ into the R.N.A. of these same livers was also estimated and the results are shown in Table 31. At all three time-intervals studied, the effect of energy on the rate of incorporation of $^{32}P$ was greater on a protein-free than on a protein-containing diet, the results being in good agreement with those obtained in the previous experiments on RNA. It will be remembered that, unlike phospholipid, the amount of RNA per liver rises slightly as energy increases, even when the diet lacks protein. The rate of RNA synthesis is thus greater on high energy intakes than on low energy intakes even when the diet lacks protein.

## DISCUSSION.

The results indicate some differences in the behaviour of RNA and phospholipid when the energy content of the diet is altered. On a protein-containing diet, the amount of both RNA and phospholipid increases with increase in energy intake. When the diet contains no protein, increase in energy intake results in a significant, though slight, increase in the RNA but not in the phospholipid, which actually decreases slightly. These changes in the phospholipid content follow closely the changes in liver protein: adding energy to the diet increases the liver protein when there is protein in the diet, but produces a slight decrease in liver protein when the diet is protein-free (Munro & Naismith, 1952).

The metabolic activity of RNA and phospholipid is indicated by the rate of $^{32}P$ incorporation. When rats are transferred from a high protein to a protein-free diet, the liver protein, RNA and phospholipid decrease in amount, the fall becoming bigger the longer they are on a deficient diet, but the relative specific activity of the RNA and phospholipid increases (Campbell & Kosterlitz, 1948). This rises to a maximum on the 4th day of the protein-free diet; that of the RNA is maintained at this high level even up to the 7th day, whereas the R.S.A. of the phospholipid has

decreased again by this time. The rise in activity compensates for the fall in amount, so that the total amount of RNA and phospholipid synthesised is kept constant, throughout the whole experiment in the case of the RNA; the speed of synthesis of phospholipid falls off after about 4 days on the deficient diet. It would thus appear, in their own words, that "the total turnover of the phospholipids and of RNA is determined by the metabolic needs of the liver, and is, at least within the limits of these experiments, independent of how much of these substances is present in the liver."

Our experiments indicate to what extent these metabolic needs are coupled with energy intake. In the case of RNA, it has already been established that this is the factor determining total rate of $^{32}P$ incorporation. In the case of the phospholipids, on the other hand, the protein content of the diet seems to have a greater influence. It should be remembered, however, that in our experiments, the rats were on a protein-free diet for 11 days before they were killed. The experiments of Campbell & Kosterlitz suggest that the ability to synthesise phospholipids is limited when the protein-free diet is continued for this length of time. It may be that by the 11th day on such a diet, the liver is not able to respond to an increase in the

energy intake by increasing the synthesis of phospholipids because of some other factor in phospholipid synthesis becoming exhausted after prolonged protein deficiency. Addition of energy to the diet might have produced the same effect on the phospholipid metabolism that it does on the metabolism of RNA, had it been added to the protein-free diet at an earlier stage of protein deficiency. Further experiments are needed to clear up this point. But, taking the results as they stand, it seems justifiable to conclude that RNA is somehow linked with carbohydrate and fat metabolism, while the phospholipid is not so intimately involved. The relationship of these findings to the problem of energy intake and protein synthesis will be considered in the general discussion of all our experiments. Before this, however, data on the influence of diet on the protein, RNA and phospholipid metabolism in different fractions of the liver cell will be presented.

# PART V.

## THE EFFECT OF ENERGY INTAKE ON THE METABOLISM OF RIBONUCLEOPROTEIN AND PHOSPHOLIPID IN DIFFERENT PARTS OF THE LIVER CELL.

## INTRODUCTION.

There is abundant evidence (see below) that the rates of RNA synthesis and of protein synthesis differ in different parts of the liver cell. It therefore seemed desirable, in seeking an interpretation of the RNA data given above, to determine how RNA behaved in relation to energy intake in different parts of the cell. We shall first consider what is already known about the metabolism of RNA, protein and phospholipid in different cell fractions.

a. Metabolism of Protein, RNA and Phospholipids in different parts of the cell: According to the hypothesis put forward by Caspersson and his colleagues, the formation of cellular proteins is regulated by a self-reproducing system which consists essentially of protein combined with nucleic acids. This system functions in all types of cytoplasmic protein formation in the following manner: "A certain part of the chromatin, the so-called nucleolus-associated chromatin, produces substances composed of RNA and proteins, which accumulate and form the main bulk of the nucleolus. From this, proteins rich in basic groups diffuse towards the nuclear membrane, on the outside of which an intensive production of RNA takes place." (Caspersson, 1950). This implies that an increase in the rate of protein synthesis is initiated by a preceding RNA accumulation in the nucleolus

and then at the surface of the nucleus and in the cytoplasm. Evidence supporting this contention has been obtained in experiments with the pancreas (Caspersson, 1947), liver (Lagerstedt, 1949; Barr & Bertram, 1949; Stowell, 1948), nerve cell (Hyden, 1943), immature blood cell (Thorrell, 1947) and mouse embryo (Brachet, 1950). Other authors claim that the nucleolus is the site of all RNA synthesis which then diffuses into the cytoplasm (Pollister & Leuchtenberger, 1949; Marshak & Calvet, 1949; Jeener & Szafarz, 1950); this is consistent with the fact that RNA is replaced at a more rapid rate in the nucleus, the rate of $^{32}P$ incorporation being 10 to 20 times as great in the nucleus as in the cytoplasm (Marshak & Calvet, 1949; Jeener & Szafarz, 1950; Barnum & Huseby, 1950; Davidson, McIndoe & Smellie, 1951). Barnum & Huseby point out however that the replacement rate in nucleus and cytoplasm runs parallel for a considerable part of the experiment, and conclude that the cytoplasmic fraction cannot come from the nucleus, but that both are synthesised at different rates from a pool of acid-soluble P. Further, the nucleotide composition of the nuclear RNA differs from that of the RNA in the cytoplasm (Marshak, 1950; Davidson & McIndoe, 1952). Therefore, it seems likely that the cytoplasm synthesises its own RNA, over and above what might diffuse out of the nucleus.

Hultin (1950) found that the protein N of the nucleus was replaced at a slower rate than that of any other fraction, and therefore cannot be the sole source of cytoplasmic protein. Other evidence leads to the conclusion that the nucleus is not the source of cytoplasmic phosphoprotein (Marshak & Calvet, 1949) or of phospholipid (Ada, 1949; Barnum & Huseby, 1950). Several workers have investigated protein synthesis in the cytoplasmic fractions. Within ½ hour of the injection of a labelled amino-acid, it is found in all cytoplasmic fractions, the greatest incorporation being in the microsome fraction (Borsook & Deasy, 1951; Hultin, 1951), showing that the different fractions have different rates of anabolism, and, presumably, of catabolism. Disintegration of cell structure reduces the incorporation of amino-acid. Some incorporation does occur, however, and the cytoplasmic fractions differ in the rates with which they incorporate labelled amino-acids, indicating functional differences (Borsook et al., 1950). A higher incorporation is shown by the isolated mitochondrial fraction than by the microsome fraction, indicating that mitochondria can synthesise protein in the absence of microsomes. When the two fractions are mixed, the incorporation rate by the mixture of fractions is double that shown by mitochondria, suggesting a synergistic effect (Borsook et al., 1948).

Microsomes are poor in enzymes and it may be that compounds such as ATP are given off by mitochondria and this energy-source is utilised at the sites of synthesis. This has recently been demonstrated by Siekevitz (1952), who incubated mitochondria with α-keto-glutarate or succinate and certain co-factors and obtained a soluble product which, when added to microsomes, enabled them to incorporate alanine. This factor is believed to be a derivative of ATP.

Ada (1949) has studied the uptake of $^{32}P$ by the liver cell fractions and concludes that phospholipids are synthesised separately and metabolised independently of each other in different parts of the cell.

From the above discussion of the metabolism of protein, RNA and phospholipids in the different parts of the cell, it is apparent that the full picture of the influence of energy intake on these cellular constituents can only be obtained by studying their metabolism in different cell fractions.

b. <u>The technique of Cell Fractionation:</u> There are two techniques that could be used in the study of cell fractions -- centrifuging the intact cell, and fractional centrifugation of the homogenised tissue. The former is the more suitable for the study of the cell constituents as they exist <u>in vivo</u> (Holter, 1952), but it is a highly specialised technique and requires the use of an ultra-centrifuge.

The more commonly employed homogenate technique consists in homogenising the tissue in a suitable medium and submitting the homogenate to centrifugation in various gravitational fields. Different media are employed for nuclei and cytoplasmic fractions, on account of the difficulty of freeing the nuclei of contaminating cytoplasmic particles. The technique as a whole is susceptible to various sources of error, which are discussed fully by Holter (1952).

i. Cytoplasmic Fractions: The presence of granules in the cytoplasm was recognised by Schultze as early as in 1861. They were called mitochondria by Benda (1898), and shown by Michaelis (1900) to be the same as the particles stained by Janus green. The first attempt at isolating these by centrifugation was by Warburg (1913). Later, Bensley & Hoerr (1934) obtained large cellular elements by centrifugation of cell extracts, which they identified as mitochondria. Since then, various workers have used differential centrifugation at high speeds for separating the particulate components of the cytoplasm.

Claude (1940) separated particles of diameter 50 to 200 mμ from homogenates in isotonic saline. These granules were chemically similar irrespective of the tissue of origin, and were described as mitochondria. They were smaller than the particles isolated by Bensley & Hoerr and

subsequent work showed him that his granules were not mitochondria, and they were renamed microsomes (Claude, 1943). Details of his method are given in later papers (Claude, 1944, 1946). The mitochondria isolated in saline are both morphologically and histochemically different from those seen in intact cells. Another disadvantage is that the mitochondria and microsomes tend to agglutinate, making their separation difficult.

In 1947, Hogeboom et al. (1947) used hypertonic (0.88 M) sucrose as the suspending medium and isolated rod-like mitochondria which stained with Janus green. These granules resemble in shape, size and staining properties the mitochondria seen in intact cells. Due to the viscosity of the medium, however, a very high centrifugal force is necessary. Further, partial inhibition of the enzyme systems have been reported by Schneider (1948) and Lehninger & Kennedy (1948). With isotonic (0.25 M) sucrose as the medium (Hogeboom et al., 1948), the particles separated in the large granule fraction have the same enzyme systems as those isolated by Claude's technique (Schneider & Hogeboom, 1950), though they are morphologically different from those isolated in hypertonic sucrose. They do not agglutinate, making their separation from microsomes more complete. Comparatively low fields need be employed. Thus, by centrifuging at appropriate fields, the cytoplasmic extract,

free from unbroken cells, nuclei and cell debris, can be fractionated into 3 main fraction portions: (a) Large granule fraction composed of elements of relatively large size, ranging from 0.5 to 2.0 μ in diameter (or length); (b) microsome fraction consisting of submicroscopic particles, approximately 50 to 200 mμ in diameter; (c) supernatant, left after removal of these two fractions. Details of the constituents of these fractions are given by Claude (1950), Hogeboom (1951) and Holter (1952).

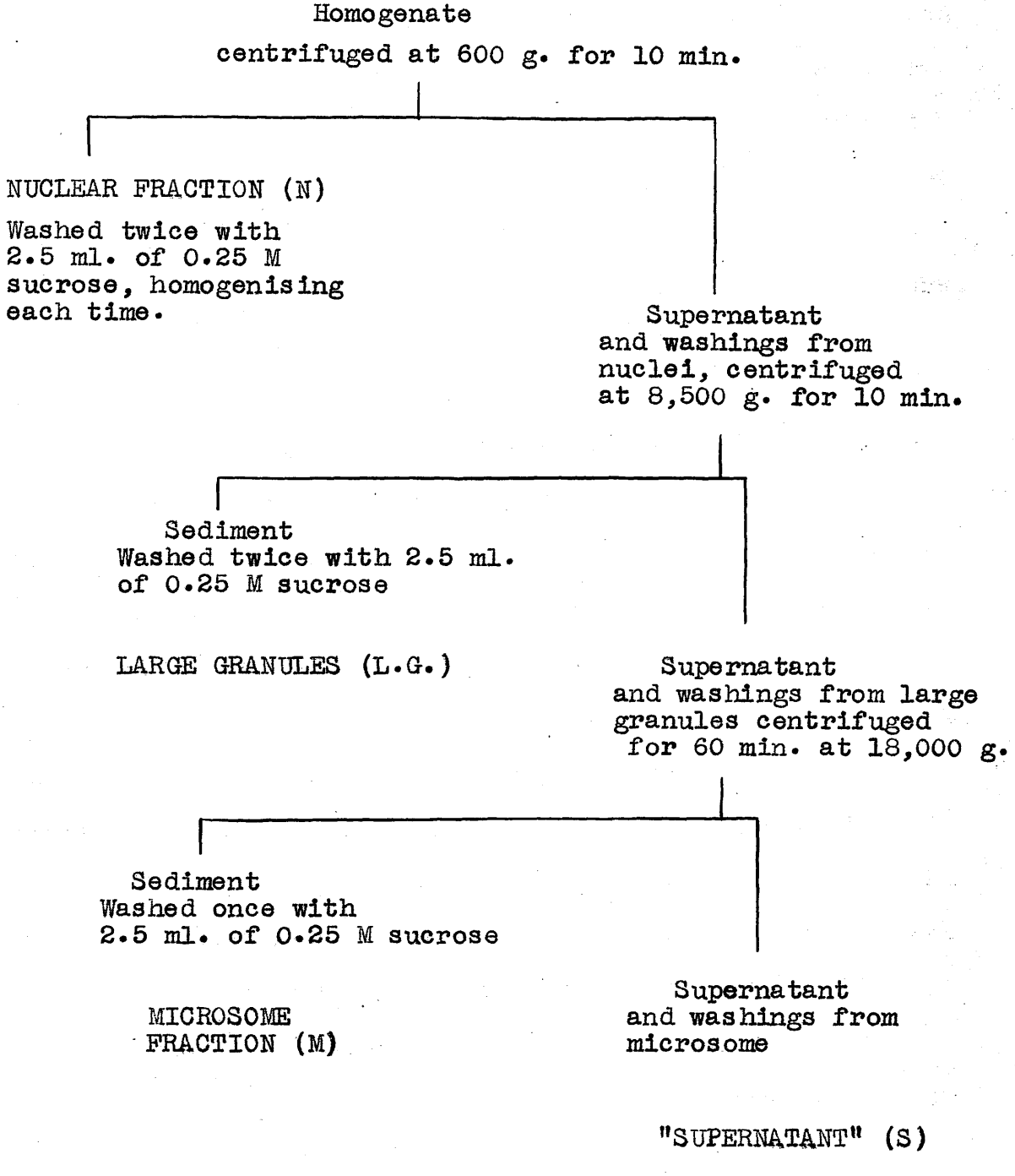
ii. The Nuclear Fraction: Miescher (1896) was probably the first to isolate cell nuclei by a drastic method which included the use of pepsin to digest away the cytoplasm. In more recent times, two methods have been used for the separation of nuclei from tissues of mammalian origin. The first is the method of Behrens (1932), who pulverised the frozen tissue and obtained nuclei by repeated centrifugation from a mixture of benzene and carbon tetrachloride. The second is the method developed by Dounce (1943) who homogenised the tissue in citric acid. Citric acid had been used by previous workers (Stoneberg, 1939; Marshak, 1940; Haven & Levy, 1942) but at a very low pH which would denature proteins and enzymes. Dounce worked at a pH of 6.0 to 6.2, when nearly all enzymes are stable in the cold. Cytoplasmic granules tend to agglutinate between pH 4.0 and

5.9, making complete separation from nuclei impossible. Above a pH of 6.5, nuclei disintegrate. A pH of 3.8 to 4.0 was found to be the best for studies other than enzymatic, as at this pH, there seems to be maximal retention of lipid, nucleic acid and protein (Dounce, 1950). Details of the procedure are found in this last paper.

It is also possible to isolate nuclei from the 0.25 M sucrose medium used for the separation of the cytoplasmic fractions, by utilizing the precipitate obtained by centrifuging off the nuclei and cell debris before proceeding to high speed centrifugation. This is suspended in citric acid and homogenised to remove adherent cell granules. Repeated centrifugations and homogenising in citric acid enable one to get rid of the most of the contaminating granules, but the preparations are less satisfactory than those made directly from citric acid.

In our experiments, we used 0.25 M sucrose as recommended by Hogeboom (1951) to separate all the cell constituents, and also used the citric acid method of Dounce to isolate nuclei from a portion of the same tissue.

The Fractionation of Liver Cell in 0.25 M Sucrose.




Scheme 4.

## EXPERIMENTAL.

a. Animals, Diet and Management: These were the same as those described in Parts III and IV. All animals received a protein-free diet. In the experiments where $^{32}P$ incorporation was studied, $^{32}P$ was injected intramuscularly at the end of the 4-day period, and the animals killed 2, 4 or 18 hours after the injection. The livers of 4 to 6 rats in each dietary group were pooled, minced finely with scissors, portions taken for isolation of nuclei by the citric acid method, and the rest homogenised in 0.25 M sucrose in a Potter-Elvejhem type of homogeniser with a plastic pestle. When studying variations in quantity of protein, RNA and phospholipid in the cell fractions, each liver was treated individually, and the whole of it homogenised to the same extent, and fractionated according to Scheme 4.

b. Fractionation of the Liver Cell: The technique used was that described by Schneider (1948). The homogenate was examined under a microscope to ensure that the majority of cells had disintegrated, and an aliquot was taken for analysis of RNA and phospholipid. This formed the "Whole Cell Fraction". The rest of the homogenate was mixed with a knife-point of sodium fluoride to prevent any enzymatic breakdown of RNA during the interval of time between homogenising the liver and the precipitation of each isolated fraction

with TCA solution. The Nuclear fraction was separated by centrifuging at 600 g. for 10 min. in a refrigerated centrifuge, washed twice with 5 ml. sucrose solution, and the washings added to the supernatant. This supernatant was then fractionated into large granules (L.G.), microsomes (M) and "supernatant" (S), as shown in Scheme 4, after a portion of it (called the "cytoplasmic fraction") had been removed for analysis of RNA and phospholipid. The washed nuclear fraction was taken up in 0.05 M. citric acid and stirred in a Nelco blendor, then centrifuged at 2000 rp.m. for 10 min. The residue was resuspended in the citric acid solution and the process repeated 4 to 5 times, till microscopical examination showed the nuclei to be almost free of contaminating cytoplasmic granules. As a further check on the nuclear RNA, a portion of the minced liver was kept frozen at about -10°C overnight, and a "Nuclear Fraction" prepared from this by homogenising in 2 vols. of 0.05 M citric acid, according to the method of Dounce (1950).

All the fractions so isolated were treated with TCA and analysed for the activity of RNA and phospholipid, as described in Parts III and IV. The RNA was extracted from the dry, lipid-free residue with hot 10% (w/v) NaCl, and precipitated with ethanol, before digestion with 0.3 N KOH. By this procedure, the phosphorus-containing contaminants found in the ionophoresis paper could be got rid of more completely

TABLE 32.

The comparison of $^{32}P$ uptake by RNA and phospholipids 2 hours after injection of labelled P. (Mean activities of nucleotides or phospholipids from pooled livers of 4 rats per group.)

| Fraction | Ribonucleic Acid Relative Specific Activities | | | Phospholipin Relative Specific Activities | | |
|---|---|---|---|---|---|---|
| | 810 Cal. per sq.m. | 1750 Cal. per sq.m. | Difference per 1000 Cal. | 810 Cal. per sq.m. | 1750 Cal. per sq.m. | Difference per 1000 Cal. |
| Whole Cell | 2.04 | 2.41 | +18% | 13.9 | 13.1 | - 6% |
| Nucleus | 10.2 | 13.3 | +29% | 14.0 | 11.1 | -24% |
| Cytoplasm | 1.41 | 1.27 | -11% | 14.9 | 13.3 | -12% |
| Large Granules | 0.57 | 0.57 | ± 0% | 13.0 | 11.4 | -14% |
| Microsomes | 0.57 | 0.65 | +14% | 15.2 | 14.2 | - 7% |
| Supernatant | 2.45 | 2.07 | -18% | 15.5 | 14.5 | - 7% |

TABLE 33.

Comparison of $^{32}P$ uptake by RNA and phospholipid 4 hrs. after injection of labelled P. (Mean activities of nucleotides or phospholipids from pooled livers of 6 rats per group.)

| Fraction | Relative Specific Activity of Ribonucleic Acid | | | Relative Specific Activity of Phospholipin | | |
|---|---|---|---|---|---|---|
| | 830 Cal. per sq.m. | 1800 Cal. per sq.m. | Difference per 1000 Cal. | 830 Cal. per sq.m. | 1800 Cal. per sq.m. | Difference per 1000 Cal. |
| Whole cell | 5.7 | 8.0 | +36% | 35.5 | 35.4 | -0.3% |
| Nucleus | 28.4 | 34.9 | +22% | 28.4 | 26.8 | -6% |
| Cytoplasm | 4.1 | 5.6 | +33% | 36.9 | 36.6 | -1% |
| Large gran. | 2.8 | 4.1 | +40% | 38.8 | 32.0 | -19% |
| Microsomes | 3.1 | 4.2 | +34% | 37.8 | 42.8 | +13% |
| Supernatant | 7.1 | 8.2 | +15% | 48.3 | 52.6 | +9% |

TABLE 34.

Comparison of $^{32}P$ uptake by RNA and phospholipids 18 hours after injection of labelled P. (Mean activities of nucleotides or phospholipids from livers of 6 rats per group.)

| Fraction | Ribonucleic Acid Relative Specific Activities | | | Phospholipin Relative Specific Activities | | |
|---|---|---|---|---|---|---|
| | 780 Cal. per sq.m. | 1440 Cal. per sq.m. | Difference per 1000 Cal. | 780 Cal. per sq.m. | 1440 Cal. per sq.m. | Difference per 1000 Cal. |
| Whole Cell | 28.9 | 33.2 | +20% | 99 | 104 | +8% |
| Nucleus | 45.3 | 56.6 | +33% | 77 | 83 | +11% |
| Cytoplasm | 26.6 | 33.5 | +34% | 104 | 103 | -2% |
| Large Granules | 22.7 | 28.1 | +31% | 99 | 100 | +2% |
| Microsomes | 23.4 | 29.0 | +32% | 106 | 101 | -7% |
| Supernatant | 34.6 | 37.5 | +12% | 102 | 101 | -2% |

(Davidson & Smellie, 1952).

The above description applies to the experiments in which radioactivity was measured. In these, the objective was not quantitative recovery of fractions, but their purification. In experiments where quantitative measurements were made, cell fractionation was the same, but no attempt was made at washing the various fractions, or at removing contaminating granules from the nuclear fraction. The lipid-free TCA precipitate of each fraction was digested in N KOH, made up to a known volume and the total P and N content determined. Simultaneous measurements of ultraviolet absorption in a Beckman spectrophotometer showed that all the absorption could be accounted for by the RNA, and the total P value of the cytoplasmic fractions were taken as a measurement of their RNA content.

## RESULTS.

a. Experiments with labelled phosphate: The incorporation of $^{32}P$ into the RNA and phospholipid of the various fractions were studied at 2, 4 and 18 hours after injection of the label. Since the diet was free from protein, one would expect to find, as in the earlier experiments (Fig.10), an increase in the relative specific activity (R.S.A.) of the whole cell RNA with increasing energy intake. Tables 32,

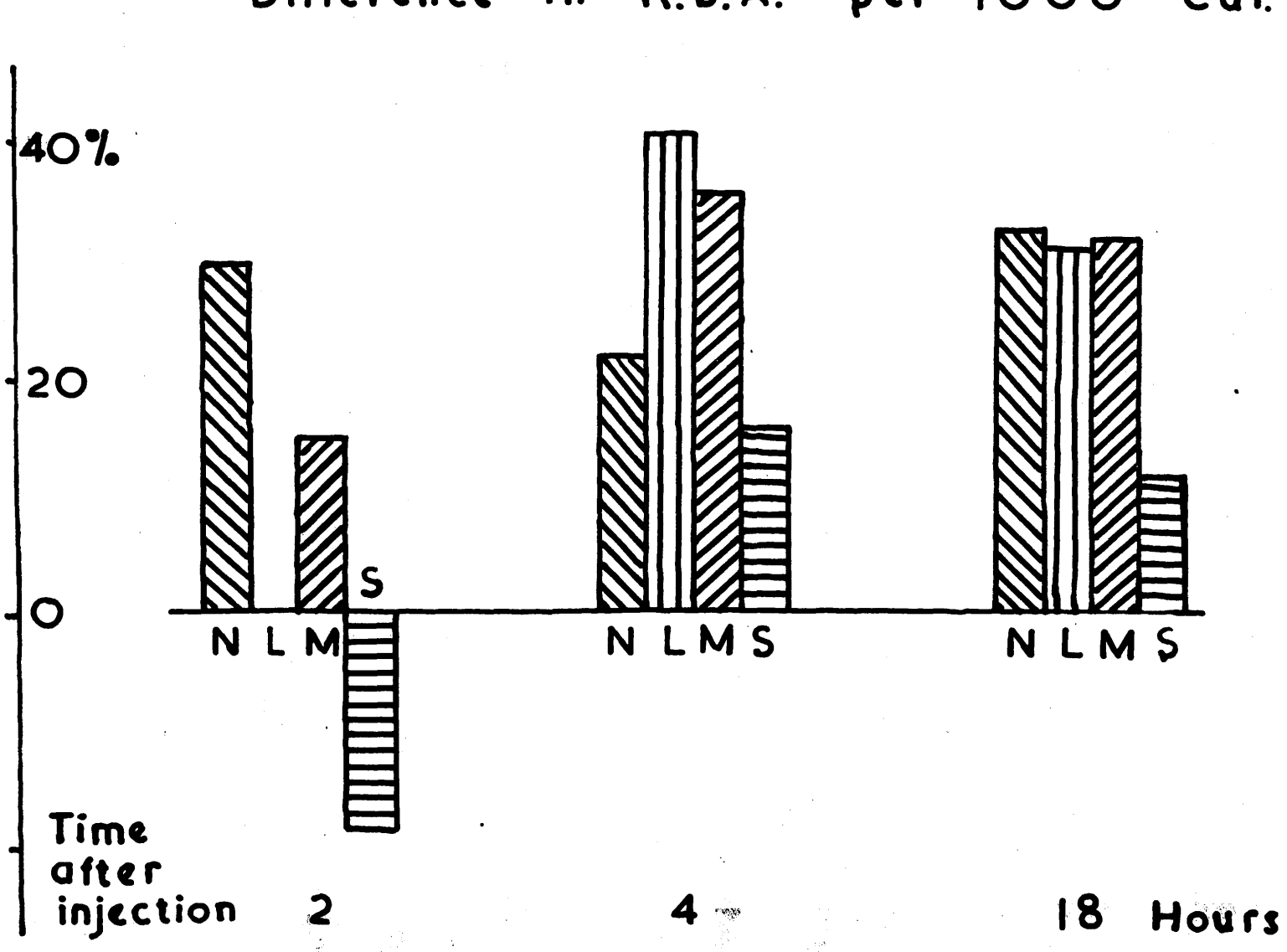

Difference in R.S.A. per 1000 Cal.
40%
20
0
N L M S
N L M S
N L M S
Time after injection
2
4
18 Hours

Fig. 1

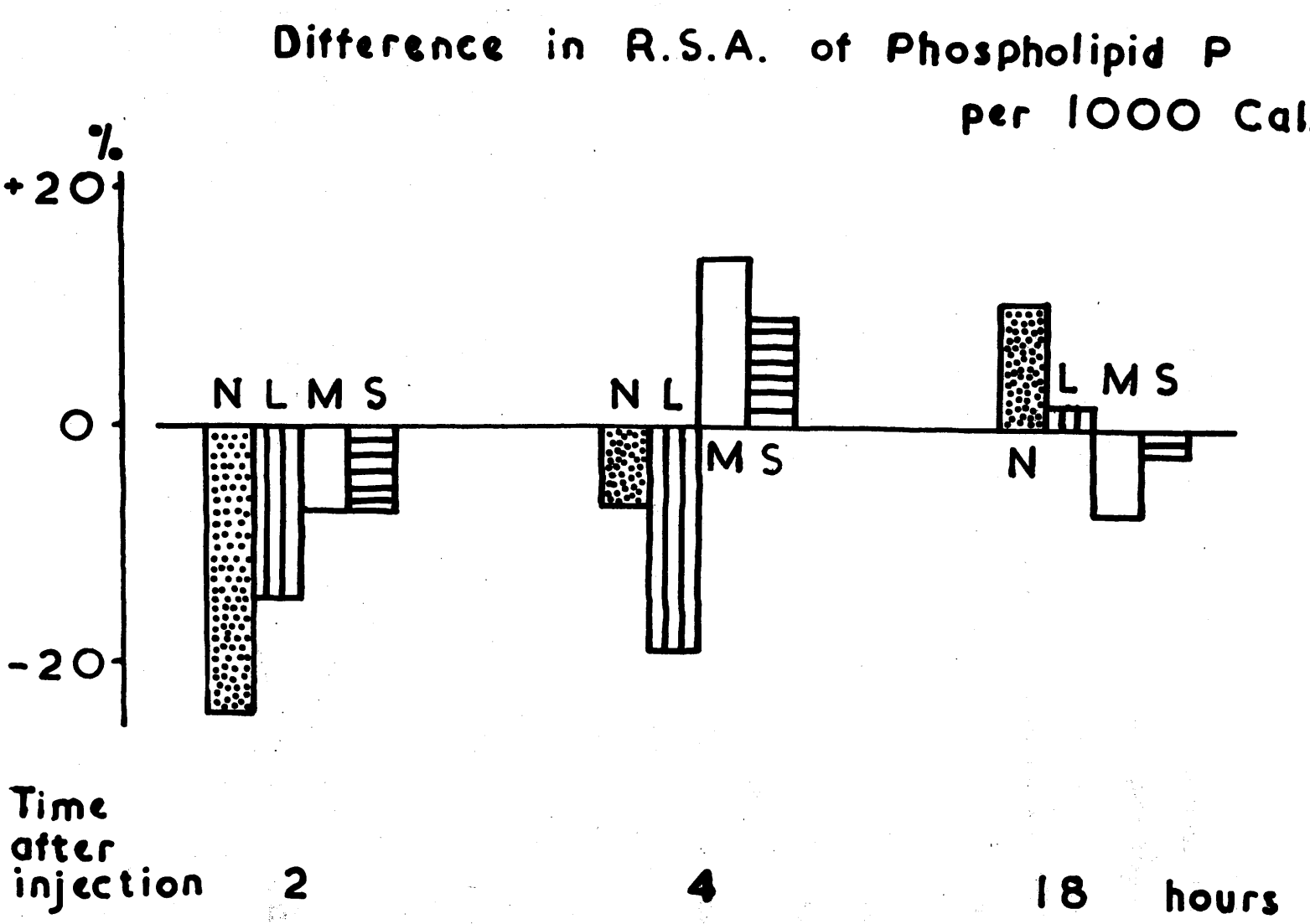
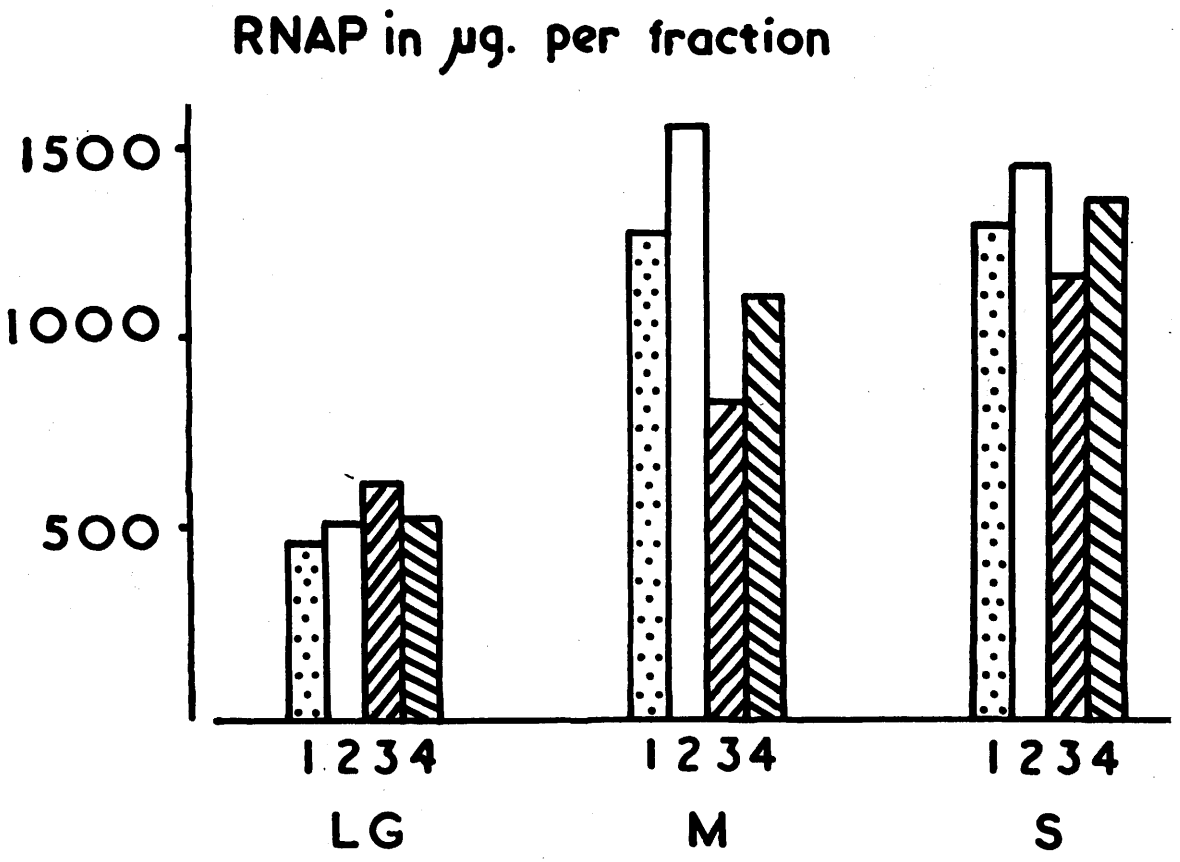

Difference in R.S.A. of Phospholipid P per 1000 Cal.
%
+20
0
-20
N L M S
N L
M S
N
L M S
Time after injection
2
4
18
hours

Fig. 12

33, 34 give the results obtained 2, 4 and 18 hours after injection respectively, and Figs.11 and 12 show the same data in diagrammatic form. The change in the R.S.A. per 1000 Cal. per sq.m. body surface area is expressed as a percentage of the value at an intake of 1200 Cal., and can be compared with the corresponding values obtained for the phospholipids. At 2 hours after injection, the R.S.A. of the nuclear RNA is about 20 times that of the cytoplasmic granule fractions and the R.S.A. of the granular fractions are almost equal. At this time the nuclear RNA is considerably influenced by the level of energy intake while the granular fractions show no important change. The RNA in the supernatant shows a tendency to take up less radioactive P as energy intake rises. At 4 hours after injection, the nuclear RNA continues to show a stimulation produced by the added energy (Table 33), but this is now equalled by changes in the granular fractions. The supernatant RNA is less affected by variations in energy intake than are the other fractions. At 18 hours (Table 34) the RNA in the nuclear, large granule and the microsomal fractions are equally affected by energy intake, while the supernatant RNA is still less susceptible. Tables 32, 33 and 34 show that the phospholipids are not affected in the same characteristic way as the RNA by increasing the intake of energy.



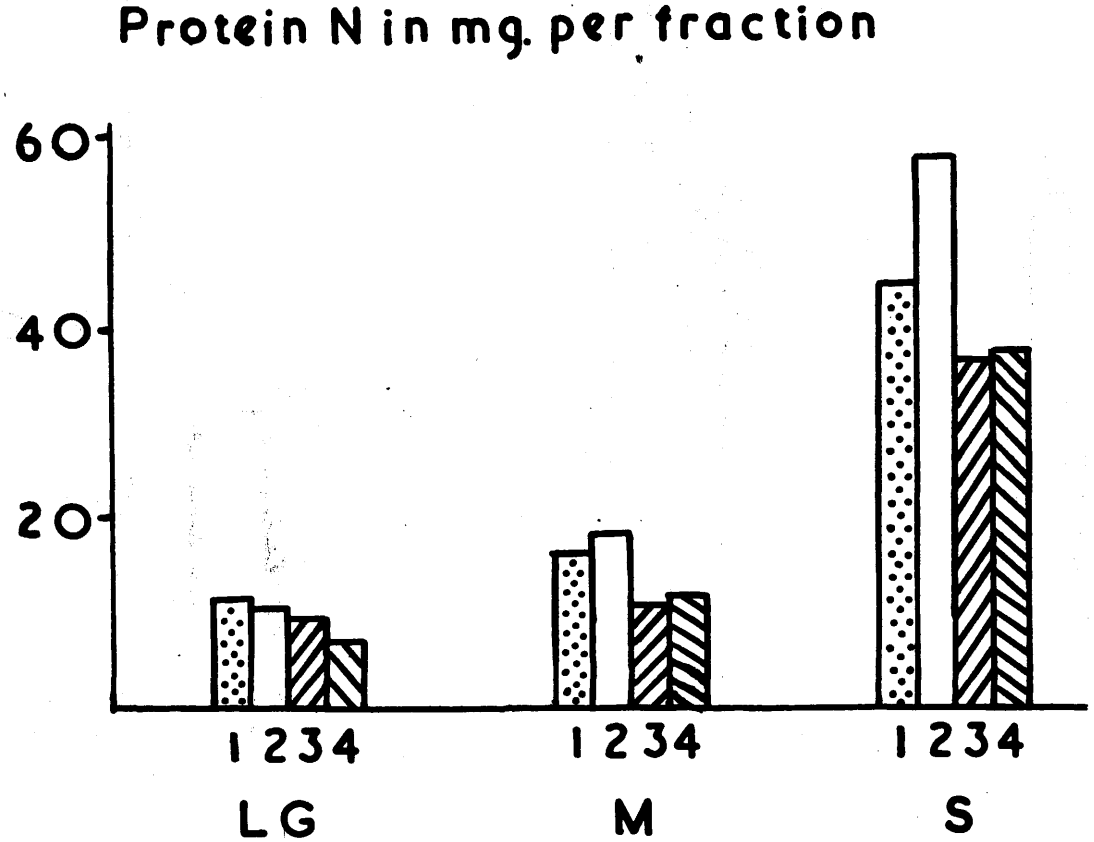

1: High protein, low energy.

2: High protein, high energy.

3: Low protein, low energy.

4 Low protein high energy.

Fig. 13



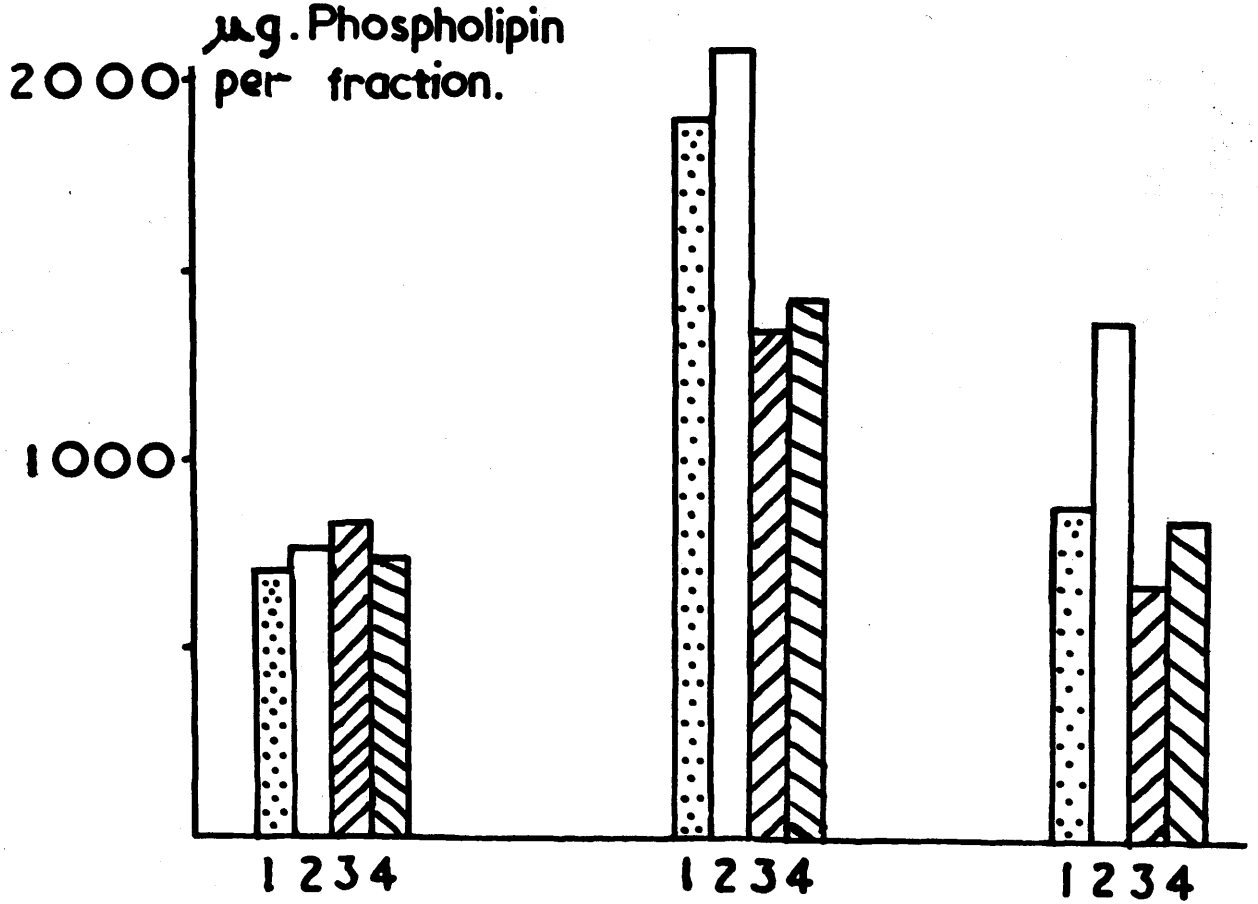

1: High protein, low energy.

2: High protein, high energy.

3: Low protein, low energy.

4: Low protein, high energy.

Fig. 14




1: High Protein, Low Energy. 3: Low Protein, Low Energy.

2: High Protein, High Energy. 4: Low Protein, High Energy.

Fig.15.

b. Quantitative studies: The effect of adding energy to a protein-containing and a protein-free diet on the total amount of protein, RNA and phospholipids in the various fractions was also studied. The nuclear fraction in each case was found to contain about 40% of the total N in the liver, due probably to contamination with large granules and unbroken cells. The results obtained from this fraction were therefore discarded. This greatly limits the value of the data obtained, since variation in the proportion of unbroken cells may obscure changes due to the diet. The results are shown in Figs.13, 14 and 15. Little reliance can be placed on the values obtained from the large granule fraction, because a good proportion of it must have been lost with the nuclear fraction. The protein and phospholipid in the microsomal and supernatant fractions appear to be affected by ~~the intake of~~ both the protein intake and the level of energy. The supernatant RNA is little affected by the protein content of the diet but is influenced by the energy intake, whereas the RNA of the microsome fraction is affected by both protein intake and energy intake.

TABLE 35.

Calculations of Total RNAP (mg./liver) based on Data of Muntwyler et al., (1950).

| Cell Fraction | Low Protein High Energy | High Protein High Energy |
|---|---|---|
| Nucleus | 5.0 | 7.5 |
| Large Granules | 2.9 | 2.9 |
| Microsomes | 18.2 | 27.6 |
| Supernatant | 19.7 | 20.1 |

## DISCUSSION.

Our results agree with the findings of Davidson, McIndoe & Smellie (1951) that the RNA's of the two granular fractions have similar synthetic activities, and that the nuclear fraction is about 20 times as active as the RNA in the granules at 2 hours after $^{32}P$ injection. The change in the R.S.A. with time after injection is also similar to the changes observed by these authors.

The effect on R.S.A. of adding energy to the diet is most marked in the nuclear RNA at 2 hours. The granular fractions show a similar change at 4 and at 18 hours, whereas the supernatant RNA does not seem to be stimulated to the same extent. As in the experiments reported in Part IV, the phospholipids do not show the same type of changes as do the RNA in the various fractions. Our quantitative data, though inadequate, indicate that the amount of RNA in the microsomes is affected by an increase in energy as well as in protein intake, whereas level of protein intake does not affect the amount in the supernatant. Muntwyler et al. (1950) estimated the quantitative changes in the various fractions in protein-fed and protein-starved rats, and values calculated from their data are given in Table 35. Removing protein from the diet produces a loss of RNA from the microsomes and the nuclei, but not from the supernatant. On the

other hand, Singal et al. (1952) found after prolonged protein depletion, that the protein N in all the cytoplasmic fractions was reduced but that the RNA showed no significant change. The R.S.A. of the RNAP was markedly increased in the deficient state, the change being most marked in the granular fractions. Taking all these data together, we see that the RNA in the nuclear and in the microsomal fractions are reduced in amount when the protein content of the diet is reduced, while their R.S.A. of the RNA in these as well as in the large granules is increased. These fractions, therefore, react like the whole liver (Fig.10), the R.S.A. increasing when the amount of RNA per liver is decreased. On the other hand, the amount of RNA in the supernatant is not affected by level of protein intake, and does not therefore need to exhibit a compensatory rise in R.S.A. when energy is added to the protein-free diet. One explanation which would fit this picture is that the other fractions are stimulated to activity by the addition of energy to the diet and that the RNA formed by them is poured into the supernatant, in an attempt to keep the RNA of this fraction constant irrespective of protein intake. The addition of energy to the diet was shown in Part III to increase the demand for RNA in the whole liver, and the experiments described in this section seem to indicate that this requirement is related to

some function in the supernatant. The enzyme systems concerned with glycolysis are localised in this fraction and it is possible that RNA is in some way intimately connected with the glycolysis cycle, and has to be formed in increased amounts when the rate of glycolysis is increased. This will be considered at greater length in the general discussion of all our data which follows.

## GENERAL DISCUSSION.

In our experiments on N balance, it was shown that addition of energy to an adequate diet increased the amount of N retained in the body. Similar experiments carried out by Munro & Naismith (1952) over a wide range of energy intakes indicate that there is a dynamic equilibrium between the energy added and the N retained, the storage of N increasing in a linear fashion with addition of energy. Further, in our experiments we found a lag period between the giving of additional energy and the effect of this energy on N retention (Figs. 3 & 4), indicating that a certain level of the metabolites has to be built up before utilisation of dietary protein begins to be affected. The experiments on ATP show that the same addition of energy increases the "phosphate potential" in the liver, the ATP/ADP ratio increasing linearly with the amount of energy added. It seems probable that the effect of surfeit energy on N balance is in some way brought about by the increase in the phosphate potential, for ATP has been shown to be an important factor in various synthetic reactions, including that of peptide bond formation. This argument can only be finally established by direct experimentation, i.e., by altering the ATP/ADP ratio in protein synthesising systems and studying the effect on amino acid incorporation.

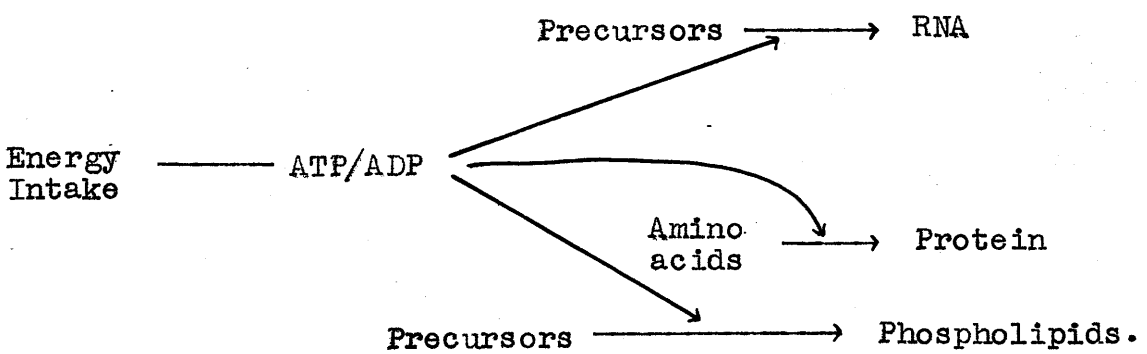
The relationship between energy intake and protein metabolism considered above may be impaired through lack of certain essential factors. On a protein-containing diet, the increase in N balance produced by addition of energy is accompanied by an increase in liver protein, while on a protein-free diet, energy increments do not improve N balance beyond a certain point, and bring about no alteration in the liver protein (Munro & Naismith, 1952). This is presumably related to the amino acid supply; on a protein-free diet less protein formation takes place, the supply of amino acids becoming the limiting factor in protein synthesis. On a protein-containing diet, on the other hand, when the supply of these amino acids is plentiful, energy supply becomes the factor limiting rate of synthesis.

Our experiments on RNA show that, whether the animal is fed protein or not, the total number of P atoms incorporated by the RNA (and, presumably, the rate of synthesis of the whole RNA molecule) is determined by the energy intake and is independent of protein intake. When the diet lacks protein, the relative rate of synthesis is increased to compensate for the fall in the total amount of RNA, while, on a protein-containing diet, the relative rate of synthesis is not increased but the total amount rises instead. This could be due to (a) the fact that the ATP/ADP

ratio acts as a limiting factor in RNA synthesis, or, alternatively, (b) the cellular requirement for RNA being in some way linked to the energy metabolism in general, or to carbohydrate metabolism in particular. The experiments with liver cell fractions can be interpreted along the latter lines (see Part V).

The liver phospholipid, on the other hand, does not appear to be affected in the same way as the RNA, when the energy intake is altered. It is possible that phospholipid synthesis, like other synthetic reactions, is influenced by the ATP/ADP ratio, but under the conditions of our experiments, this could not be demonstrated with the protein-free diet. It is clear that, with prolonged protein starvation, lack of some precursor limits the rate of synthesis (Campbell & Kosterlitz, 1948) and this may explain why increasing the phosphate potential was without effect.

Thus, a single, overall picture of the effect of energy intake on protein, RNA and phospholipid metabolism in the liver might be diagrammatically represented as follows:



The protein level in the diet influences the amounts of RNA and phospholipid in the liver, but the rate of turnover of these compounds appears to depend on the ATP/ADP ratio. Hence, when protein synthesis is limited by lack of essential amino acids, RNA and phospholipid synthesis may proceed as before, but, owing to reduced amounts of protein in the cell, less RNA or phospholipid is held in the cell at any one time. In our experiments, the rate of synthesis of phospholipid was also limited by some factor other than energy supply.

The relationship of the amounts of RNA and phospholipid in the liver to its protein content has been suggested by other authors. Fasting, or feeding a protein-free diet, or a diet lacking in essential amino acids, results in a rapid loss of protein from the liver, as much as 20% during the first 48 hours, and refeeding with protein rapidly increases liver protein (Addis, Poo & Lew, 1936; Kosterlitz & Cramb, 1943; Kosterlitz, 1944, 1947; Lagerstedt, 1949). Along with the loss of protein, the cytoplasmic granules diminish in amount (Berg, 1914; Elman et al., 1943; Kosterlitz, 1947,; Lagerstedt, 1949) and the RNA of the liver is also considerably reduced (Davidson & Waymouth, 1944; Davidson, 1945; Kosterlitz, 1947). The loss of protein, phospholipid and RNA, along with the disappearance of basophilic cytoplasmic granules, has justified the conclusion that some of the phospholipid-ribonucleo-

proteins in the cell cytoplasm constitute a relatively unstable form of protein-containing material, which has been referred to as "labile liver cytoplasm" by Kosterlitz (1947).

Studies of different fractions of the liver cell (Part V) show, however, that removal of protein from the diet does not result in a parallel loss of protein and RNA from all parts of the cell.

A reduction in protein intake results in a loss of protein from all the cytoplasmic fractions, accompanied by a loss of RNA from the microsomal fraction but not the supernatant fraction. The microsomes contain a high proportion of RNA, and the relatively small amount of protein found in them might be nucleoprotein to a very large extent. Therefore, when the amount of protein decreases, one would expect the RNA content also to drop. The supernatant, on the other hand, presumably contains a large amount of protein not associated with nucleic acids, and the loss of this protein might not necessarily lead to an accompanying fall in the RNA. We cannot, therefore, dismiss wholly the concept of a discreet ribonucleoprotein-phospholipid complex with some common metabolic function.

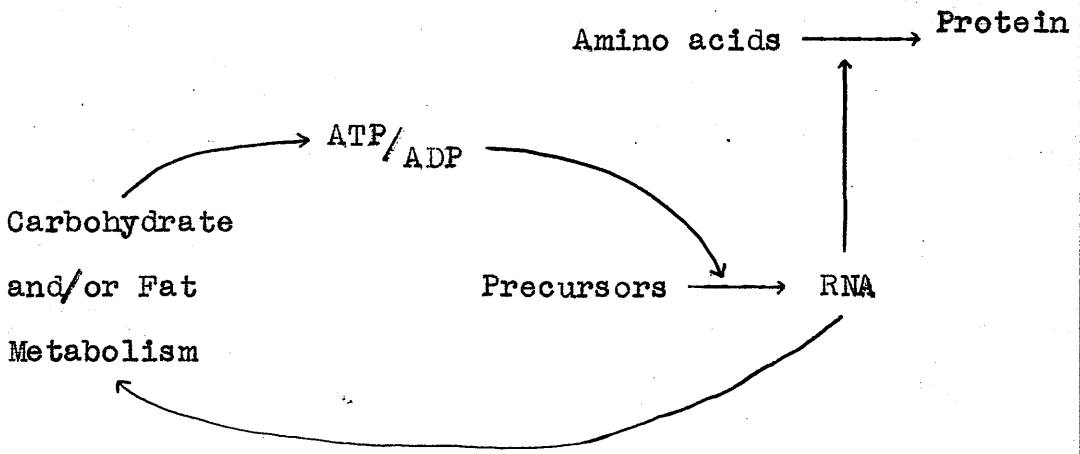
Alternative interpretations of our data must also be entertained. On adding energy to a protein-free diet, we found a marked stimulation of RNA synthesis in the granular

fractions but less so in the supernatant. This may be related to the fact that enzyme systems connected with the tricarboxylic acid cycle are located in the large granules, and that this energy is transmitted to the microsomes (Siekevitz, 1952) which play an important part in protein synthesis (Borsook & Deasy, 1951). But this interpretation is made less probable by the fact that precursors necessary for protein synthesis are lacking when the diet is protein-free, and yet RNA synthesis continues with the same intensity. It is more likely that the rate of synthesis of RNA is stimulated in the nuclear and granular fractions by the addition of energy because more RNA is needed to maintain the RNA content of the supernatant at a constant level. The supernatant contains enzyme systems necessary for glycolysis, and it may be that RNA plays a part in glycolysis. This has also been suggested by Claude (1948, 1950), who pointed out that cells with a high RNA content, such as embryonic and tumour cells, have been shown by Warburg and others to have to a high degree the power of anaerobic glycolysis. Therefore, RNA may be related to the capacity for anaerobic respiration, accounting for the concurrence of large amounts of RNA and active fermentative processes in yeasts and certain bacteria. Claude concludes that RNA might be involved in some phase of the anaerobic mechanism

or act as an intermediate in energy transfer for various synthetic reactions.

Do our experiments definitely exclude the possibility that RNA is a factor in protein synthesis? Examination of the literature suggests that RNA synthesis precedes protein synthesis (see Part III). In our own experiments, we found a slight increase in the liver RNA when energy was added to a protein-free diet, in contrast to protein and phospholipids, which are not affected by a change in energy intake under these circumstances. This tendency towards a build-up of RNA might indicate a preparation for synthesis, which fails for lack of dietary amino acids. Why then does this unused RNA not accumulate in the liver to the same extent as on a protein-containing diet? Energy intake affects the rate of incorporation of $^{32}P$ to the same extent on both diets. This may be due to one of two reasons: it is possible that some unidentified compound subsequent to RNA in the protein synthesis chain of reactions, accumulates. Or, what is more likely, RNA may be chiefly concerned with the metabolism of carbohydrate and fat, or specifically with the glycolytic cycle, and to a lesser extent with protein synthesis. It may be a compound like ATP, capable of performing more than one function or taking part in more than one reaction. This conception of

the role of RNA in cell metabolism may be represented as follows:




In this scheme, the increased phosphate potential promotes the rate of synthesis of RNA, which is in turn connected with the build-up of energy-yielding metabolites from dietary carbohydrate and fat. In the presence of amino-acids, the RNA functions in the reactions leading to the formation of protein.

In view of all the possibilities envisaged to explain our data, it is obvious that much more work will have to be done before one can choose between these alternatives.

## SUMMARY.

Part I: The relationship between energy intake and nitrogen balance.

1. Experiments are described in which additional carbohydrate (glucose) and additional fat (olive oil) were given to adult rats already receiving an adequate diet. This caused a reduction in urinary N output whether the surfeit was given with dietary protein or 5 to 12 hours apart from the protein. N retentions of similar magnitude were obtained whether the surfeit was given in the form of carbohydrate or an isodynamic amount of fat.

2. Similar experiments were carried out on adult dogs, the additional carbohydrate (sucrose) being given with the protein on one single occasion, and 7 and 12 hours apart from the protein on other single occasions. Nitrogen retention was obtained when sucrose was given within 7 hours of the protein meal. Repeated daily doses of sucrose 12 hours after the protein meal eventually produced a retention of N of equal magnitude to the amount stored when sucrose was given with the protein.

3. Experiments were also carried out on 4 human subjects receiving adequate diets. Additional carbohydrate (sucrose) reduced the urinary N output whether it was taken with the diet or 5½ hours after the last meal of the day.

When the sucrose was taken apart from the meals, a lag period of 24 to 48 hours was observed before the urinary N output was reduced.

4. It has been concluded that carbohydrate and fat act interchangeably as energy sources in sparing protein; they do not need to be taken along with dietary protein to exert this sparing action. This N sparing effect is probably produced by increasing the tissue concentration of some energy-yielding metabolite which is necessary for the synthesis of protein.

Part II: The effect of energy intake on the metabolism of adenosine triphosphate in the liver.

1. Experiments are described in which energy in the form of olive oil was given to rats receiving a fixed basal diet. This caused a significant change in the ATP/ADP ratio, which increased linearly with the amount of energy added.

2. The significance of the ATP/ADP ratio is discussed, and it has been concluded that energy intake influences protein synthesis by affecting the "phosphate potential".

Part III: The influence of energy intake on the metabolism of ribonucleoproteins.

1. A study has been made of the effect of variations in energy intake on the amount of ribonucleic acid (RNA) in the rat liver, and on the uptake of labelled phosphorus by RNA.

2. When the diet contained protein, addition of energy in the form of carbohydrate or fat resulted in a considerable increase in the amount of RNA per liver: when the diet lacked protein, an increase in energy intake caused only a slight change in the amount of RNA.

3. The uptake of $^{32}P$ by RNA, as measured by relative specific activity, behaved in the opposite way. Uptake was not affected by variations in energy intake when the diet contained protein, but it was considerably stimulated by addition of energy to the protein-free diet.

4. These results indicate that the absolute rate of incorporation of phosphorus into RNA is dependent on energy intake. At each level of protein intake, addition of energy increases the total number of P atoms incorporated into RNA, in one case by an increase in the amount of RNA per liver without a change in the percentage of P atoms incorporated in a given time, in the other case by an increase in the incorporation rate to compensate for the much smaller change in the amount of RNA per liver.

Part IV: The effect of energy intake on the metabolism of liver phospholipid.

1. Experiments similar to those described in Part III were carried out on rats, to study the effect of variations in energy intake on the amount of phospholipid per liver, and on the uptake of labelled phosphorus by the liver phospholipid.

2. On a protein-containing diet, addition of energy (carbohydrate) produced a marked increase in the amount of phospholipid per liver; when the diet was protein-free, the phospholipid per liver decreased slightly with the addition of energy.

3. The uptake of $^{32}P$, as measured by the relative specific activity, was increased by addition of energy, whether the diet contained protein or not, the change being greater in the latter case.

4. The absolute rate of incorporation of phosphorus was greater on a protein-containing diet, the increase in the relative specific activity on a protein-free diet being balanced by a drop in the total quantity of phospholipid per liver. It has been concluded that the liver phospholipid differs from RNA in that the absolute rate of incorporation of phosphorus is affected by the level of protein intake as well as by the intake of energy.

Part V: The effect of energy intake on the metabolism of ribonucleoprotein and phospholipid in different parts of the liver cell.

1. A study was made of the effect of varying energy intake on the total amount of protein, RNA and phospholipid in different liver cell fractions (nuclear, large granule, microsomal and supernatant), and on the uptake of $^{32}P$ by the RNA and phospholipid in these fractions, when rats were maintained on a protein-free diet.

2. Addition of energy (carbohydrate) was found to increase considerably the uptake of $^{32}P$ by the RNA in the nuclear and granular fractions, the effect on the RNA in the supernatant fraction being less evident. Under the same conditions, there was no appreciable effect on the $^{32}P$ uptake by the phospholipid.

3. This difference in the behaviour of the supernatant fraction with regard to RNA metabolism may be related to the constancy of the amount of RNA in the supernatant at different levels of protein intake, in contrast to the variations in the amount of protein and phospholipids in the supernatant fraction with the level of protein intake.

4. On the basis of our data, it has been suggested

that the nuclear and granular fractions synthesise RNA with the object of maintaining the level of RNA in the supernatant fraction of the cell, and that the RNA of this fraction is in some way involved in energy metabolism.

---

## BIBLIOGRAPHY.

Abolins, L. (1952): Exptl. Cell Research, 3, 1.

Abrams, R. (1951): Arch. Biochem., 30, 90.

Ada, G.L. (1949): Biochem. J., 45, 422.

Addis, T., Poo, L.J. & Lew, W. (1936): J.Biol.Chem., 115, 111, 117; 116, 343.

Albaum, H.G., Hirschfield, A.I., Tonhazy, N.E. & Umbreit, W.W. (1951): Proc. Soc. Expt. Biol., 76, 546.

Allen, R.J.L. (1940): Biochem. J., 34, 858.

Allison, J.B. & Anderson, J.A. (1945): J. Nutrition, 29, 413.

Allison, J.B., Anderson, J.A. & Seeley, R.D. (1946): Ann. N.Y. Acad. Sci., 47, 245.

Bailey, K. (1942): Biochem. J., 36, 121.

Bailey, K. (1949): Biochem. J., 45, 479.

Bailey, K. & Webb, E.C. (1948): Biochem. J., 42, 60.

Barkulis, S.S. & Lehninger, A.L. (1951): J. Biol. Chem., 190, 339.

Barnum, C.P. & Huseby, R.A. (1950): Arch. Biochem., 29, 7.

Barr, M. & Bertram, E. (1949): Nature, Lond., 163, 676.

Basu, K.P. & Basak, M.N. (1939): Indian J. M. Research, 27, 115.

Behrens, (1932): cited by Dounce (1950).

Belitzer, V.A. (1939): Enzymologia, 6, 1.

Benda, (1898): cited by Runnstrom (1952).

Benditt, E.P., Humphreys, E.M., Wissler, R.W., Steffee, C.H., Frazier, L.E. & Cannon, P.R. (1948): J. Lab. & Clin. Med., 33, 257.

Bensley, R.R. & Hoerr, N.L. (1934): Anat. Rec., 60, 449.

Berenblum, I. & Chain, E. (1938): Biochem. J., 32, 295.

Berg, W. (1914): cited by Davidson (1947a).

Bing, J., Fagraeus, A. & Thorrell, B. (1945): Acta. Physiol. Scand., 10, 282.

Bischoff, T.L.W. (1853): cited by Munro (1951).

Bischoff, T.L.W. & Voit, C. (1860): cited by Munro (1951).

Boivin, A. (1947): Cold Spring Harb. Symp. Quant. Biol., 12, 7.

Borsook, H. & Deasy, C.L. (1951): An. Rev. Biochem., 20, 209.

Borsook, H., Deasy, C.L., Haagen-Smit, A.J., Keighley, G. & Lowy, P.H. (1948): Fed. Proc., 7, 147.

Borsook, H., Deasy, C.L., Haagen-Smit, A.J., Keighley, G. & Lowy, P.H. (1950): J. Biol. Chem., 184, 529.

Borsook, H. & Dubnoff, J.W. (1940): J. Biol. Chem., 132, 307.

Bosshardt, D.K., Paul, W.J., O'Doherty, K. & Barnes, R.H. (1948): J. Nutrition, 36, 773.

Brachet, J. (1941): Arch. de Biol., Paris, 53, 207.

Brachet, J. (1950): Ann. N. Y. Acad. Sci., 50, 861.

Brues, A.M., Tracy, M.M. & Cohn, W.E. (1944): J. Biol. Chem., 155, 619.

Calvin, J., Heidelberger, C., Reid, J.C., Tolbert, B.M. & Yanwick, P.F. (1949): Isotopic Carbon, Publish. J. Wiley & Sons, Inc., N.Y.

Campbell, R.M. & Kosterlitz, H.W. (1948): J. Biol. Chem., 175, 989.

Cantoni, G.L. (1951a): J. Biol. Chem., 189, 745.

Cantoni, G.L. (1951b): Phosphorus Metabolism, John Hopkins Press, Baltimore.

Caspersson, T.O. (1939): Nature, Lond., 143, 602.

Caspersson, T.O. (1947): Symp. Soc. Exp. Biol., 1, 127.

Caspersson, T.O. (1950): Cell Growth and Cell Function, Publish. W.W. Norton & Co. Inc., N.Y.

Chantrenne, M. (1952): Symp. sur la Biogenese des Proteines, IInd. International Congress.

Claude, A. (1940): Science, 91, 77.

Claude, A. (1943): Science, 97, 451.

Claude, A. (1944): J. exp. Med., 80, 19.

Claude, A. (1946): J. exp. Med., 84, 51, 61.

Claude, A. (1948): The Harvey Lectures, Series 43, 121.

Claude, A. (1949): Adv. in Protein Chem., 5, 423.

Claude, A. (1950): Ann. N.Y. Acad. Sci., 50, 854.

Cohen, P.P. (1951): Phosphorus Metabolism, John Hopkins Press, Baltimore.

Cohen, P.P. & Hayano, M. (1946): J. Biol. Chem., 166, 239, 251.

Cohen, P.P. & Hayano, M. (1948): J. Biol. Chem., 172, 405.

Cohen, P.P. & McGilvery, R.W. (1947): J. Biol. Chem., 171, 121.

Cohen, S.S. (1951): Fed. Proc., 10, 585. Bact. Rev., 15, 131.

Colowick, S.P. & Kalckar, H.M. (1943): J. Biol. Chem., 148, 117.

Colowick, S.P. & Sutherland, E.W. (1942): J. Biol. Chem., 144, 423.

Consden, L., Gordon, A.H. & Martin, A.J.P. (1944): Biochem. J., 38, 224.

Consden, L., Gordon, A.H. & Martin, A.J.P. (1947): Biochem. J., 41, 590.

Cori, C.F. (1939): Cold Spring Harb. Symp. Quant. Biol., 7, 260.

Cori, C.F. & Shine, W.M. (1936): J. Biol. Chem., 114, xxi.

Cowgill, G.R. (1923): J. Biol. Chem., 56, 725.

Cremer, H. & Tiselius, A. (1950): Biochem. Z., 320, 273.

Cuthbertson, D.P., McCutcheon, A. & Munro, H.N. (1940): Biochem. J., 34, 1002.

Cuthbertson, D.P., McGirr, J.L. & Munro, H.N. (1937): Biochem. J., 31, 2293.

Cuthbertson, D.P., McGirr, J.L. & Robertson, J.S. (1939): Quart.J.Exper.Physiol., 29, 13.

Cuthbertson, D.P. & Munro, H.N. (1937): Biochem. J., 31, 694.

Cuthbertson, D.P. & Munro, H.N. (1939): Biochem. J., 33, 128.

Davidson, J.N. (1945): Biochem. J., 39, lix.

Davidson, J.N. (1947a): Cold Spring Harb. Symp. Quant. Biol., 12, 52.

Davidson, J.N. (1947b): Symp. Soc. Exp. Biol., 1, 77.

Davidson, J.N. (1950): The Biochemistry of the Nucleic Acids. Publish. Methuen & Co. Ltd., London.

Davidson, J.N., Frazer, S.C. & Hutchison, W.C. (1951): Biochem. J., 49, 311.

Davidson, J.N., Gardner, M., Hutchison, W.C., McIndoe, W.M., Raymond, W.H.A. & Shaw, J.F. (1949a): Biochem. J., 44, xx.

Davidson, J.N., Gardner, M., Hutchison, W.C., McIndoe, W.M., Raymond, W.H.A. & Shaw, J.F. (1949b): 1st Int. Congr. Biochem. Abstr., 252.

Davidson, J.N. & Leslie, I. (1950): Nature, Lond., 165, 49. Cancer Res., 10, 587.

Davidson, J.N., Leslie, I. & Waymouth, C. (1949): Biochem. J., 44, 5.

Davidson, J.N., Leslie, I. & White, J.C. (1948): J. Path. Bact., 60, 1.

Davidson, J.N. & McIndoe, W.M. (1952): unpublished results.

Davidson, J.N., McIndoe, W.M. & Smellie, R.M.S. (1951): Biochem. J., 49, iv.

Davidson, J.N. & Raymond, W. (1948): Biochem. J., 42, xiv.

Davidson, J.N. & Smellie, R.M.S. (1952): unpublished results.

Davidson, J.N. & Waymouth, C. (1944): Biochem. J., 38, 379.

Davidson, J.N. & Waymouth, C. (1946): J. Physiol., 105, 191.

Dixon, M. (1948): Multi-enzyme Systems, Publish. Camb. Univ. Press, London.

Dodds, E.C. & Greville, G.D. (1934): Lancet, 112, (1), 398.

Dounce, A.L. (1943): J. Biol. Chem., 147, 685; 151, 221, 235.

Dounce, A.L. (1950): Ann. N.Y. Acad. Sci., 50, 982.

Eggleton, P. & Eggleton, G.P. (1927): Biochem.J., 21, 190.

Ehrenfest, E. & Ronzoni, E. (1933): Proc.Soc.Exptl.Biol.Med., 31, 318.

Elman, R., Smith, M.G. & Sachar, L.A. (1943): Gastroenterology, 1, 24.

Embden (1915): cited by Lipmann (1941).

Ennor, A.H. & Rosenberg, H. (1952): Biochem. J., 50, 524.

Ennor, A.H. & Stocken, L.A. (1950): Austr.J.Expt.Biol. & Med., 28, 647.

Euler, H. von, Hevesy, G. & Solodkowska, S. (1948): Ark.Kemi Min.Geol., 26A, 1.

Fiske, C.H. & Subbarow, Y. (1929): J. Biol. Chem., 81, 629.

Forbes, E.B., Bratzler, J.W., Thacker, E.J. & Marcy, L.F. (1939): J.Nutrition, 18, 57.

Forbes, E.B. & Swift, R.W. (1944): J. Nutrition, 27, 453.

Forbes, E.B., Swift, R.W., Elliott, R.F. & James, W.H. (1946a): N. Nutrition, 31, 203.

Forbes, E.B., Swift, R.W., Elliott, R.F. & James, W.H. (1946b): J. Nutrition, 31, 213.

Frei, J. & Leuthardt, F. (1949): Helv.Chim.Acta, 32, 1137.

Friedkin, M. & Lehninger, A.L. (1949): J. Biol. Chem., 177, 775.

Green, A.A. & Colowick, S.P. (1944): Ann. Rev. Biochem., 13, 155.

Green, D.E. (1949): Respiratory Enzymes; Burgess Publish. Co., Minneapolis.

Grenson, M. (1952a): Biochim. et Biophys. Acta, 8, 481.

Grenson, M. (1952b): cited by Chantrenne (1952).

Grisolia, S. (1951): Phosphorus Metabolism; Publish. John Hopkins Press, Baltimore.

Guberniev, M.A. & Il'ina, L.I. (1950): cited by Hokin (1952).

Hahn, L. & Hevesy, G. (1940): Nature, Lond., 145, 549.

Hammarsten, E. (1951): Isotopes in Biochem., Ciba Foundation Symp.

Harden, A. & Young, W.J. (1905): J. Chem. Soc., 21, 189.

Haven, F.L. & Levy, S.R. (1942): Cancer Res., 2, 797.

Herlant, M. (1943): Arch. de Biol., Paris, 54, 225.

Hiller, A., Plazin, J. & van Slyke, D.D. (1948): J. Biol. Chem., 176, 1401.

Hogeboom, G.H. (1951): Fed. Proc., 10, 640.

Hogeboom, G.H., Schneider, W.C. & Pallade, G.E. (1947): Proc. Soc. Exp. Biol., 65, 320.

Hogeboom, G.H., Schneider, W.C. & Pallade, G.E. (1948): J. Biol. Chem., 172, 619.

Hokin, L.E. (1952): Biochim. et Biophys. Acta, 5, 225.

Holiday, E.R. & Johnson, E.A. (1949): Nature, Lond., 163, 216.

Holmes, B.E. (1951): Isotopes in Biochem.; Ciba Foundation Symp.

Holter, H. (1951): Adv. Enzymol., 13, 1.

Hyden, H. (1943): Acta Physiol. Scand., 6, Suppl. 17.

Jansen, H.W. (1917): cited by Munro (1951).

Jeener, R. (1949): Nature, Lond., 163, 837.

Jeener, R. & Szafarz, D. (1950): Arch. Biochem., 26, 54.

Johnson, M.J. (1949): Respiratory Enzymes; Ed. H.A. Lardy, Burgess Publish. Co.

Johnston, R.B. & Block, K. (1951): J. Biol. Chem., 188, 221.

Kalckar, H.M. (1941): Chem. Rev., 28, 71.

Kalckar, H.M. (1942): J. Biol. Chem., 143, 299.

Kalckar, H.M. (1943): J. Biol. Chem., 148, 127.

Kalckar, H.M. (1944): J. Biol. Chem., 154, 267.

Kalckar, H.M. (1947): J. Biol. Chem., 167, 461.

Kalckar, H.M., Dehlinger, J. & Mehler, A. (1944): J. Biol. Chem., 154, 275.

Kaplan, N.O. & Greenberg, D.M. (1944): J. Biol. Chem., 156, 511.

Keilly, W.W. & Keilly, R.K. (1951): Fed. Proc., 10, 207.

Keilly, W.W. & Meyerhof, O. (1948): J. Biol. Chem., 176, 591.

Kosterlitz, H.W. (1944): Biochem. J., 38, Proc. xiv.

Kosterlitz, H.W. (1947): J. Physiol., 106, 194.

Kosterlitz, H.W. (1951): Liver Disease; Ciba Foundation Symp.

Kosterlitz, H.W. & Cramb, A. (1943): J. Physiol., 102, 18P.

Krebs, H.A. (1942): Biochem.J., 36, 758.

Krishnan, P.S. (1949): Arch. Biochem., 20, 272.

Krug, G. (1893): cited by Munro (1951).

Lagerstedt, S. (1949): Acta Anat. Scand., Suppl. IX.

Lardy, H.A. (1952): The Biology of Phosphorus, Publish. Michigan State College Press.

Lardy, H.A. & Wellman, H. (1952): J. Biol. Chem., 195, 215.

Larson, P.S. & Chaikoff, I.L. (1937): J. Nutrition, 13, 287.

Lathe, G.H. & Peters, R.A. (1949a): Quart. J. Exper. Physiol., 35, 55.

Lathe, G.H. & Peters, R.A. (1949b): Quart. J. Exper. Physiol., 35, 157.

Lehninger, A.L. & Kennedy,E.P.(I948): J. Biol. Chem., I73, 753.

Lennerstrand, A. (1936): cited by Lardy (1952).

Levy, H.B., Skutch, E.T. & Schade, A.L. (1949): Arch. Biochem., 24, 199, 206.

Lipmann, F. (1940): J. Biol. Chem., 134, 463.

Lipmann, F. (1941): Adv. Enzymol., 1, 99.

Lipmann, F. (1943): in W.M. Malsikoff's Dictionary of Biochem., Philosophical Library, N.Y.

Lipmann, F. (1945): J. Biol. Chem., 160, 173.

Lipmann, F. (1949): Fed. Proc., 8, 597.

Lehman, K. (1935): cited by Lipmann (1941).

Lusk, G. (1890): cited by Munro (1951).

Ma, T.S. & Zuazaga, G. (1942): Industr. & Eng. Chem., 14, 280.

Malmgren, B. & Heden, C.G. (1947): Nature, Lond., 159, 577.

Markham, R. (1942): Biochem. J., 36, 790.

Markham, R. & Smith, J.D. (1951): Biochem. J., 49, 401.

Marshak, A. (1940): Science, 92, 460.

Marshak, A. (1950): Amer. J. Physiol., 163, No.3.

Marshak, A. & Calvet, F. (1949): J. Cell. Comp. Physiol., 34, 451.

Mathison, G.C. (1909): Biochem. J., 4, 233.

McElroy, W.D. (1951): Phosphorus Metabolism; John Hopkins Press, Baltimore.

McElroy, W.D. & Strehler, S. (1949): Arch. Biochem., 22, 420.

McGilvery, R.W. & Cohen, P.P. (1950): J. Biol. Chem., 183, 179.

Meyerhof, O. (1917): cited by Lipmann (1941).

Meyerhof, O. (1944): Ann. N.Y. Acad. Sci., 75, 377.

Meyerhof, O. & Suranyi, J. (1927): cited by Lipmann (1941).

Michaelis, L. (1898, 1900): cited by Runnstrom (1952).

Miller, L.L., Bly, C.G., Watson, M.L. & Bale, W.F. (1950): Fed. Proc., 9, 206.

Miura, A. (1892): cited by Munro (1951).

Muller, H.J. (1947): Proc. Roy. Soc., B, 134, 1.

Munro, H.N. (1949): J. Nutrition, 39, 376.

Munro, H.N. (1951): Physiol. Rev., 31, 449.

Munro, H.N. & Naismith, D.J. (1952): in the press.

Munro, H.N., Naismith, D.J. & Wikramanayake, T.W. (1952): in the press.

Muntwyler, E., Seifter, S. & Harkness, D.M. (1950): J. Biol. Chem., 184, 181.

Nachmansohn, D. (1951): Phosphorus Metabolism; John Hopkins Press, Baltimore.

Nachmansohn, D., Cox, R.T., Coates, C.W. & Machado, A.L. (1943): J. Neurophysiol., 6, 383.

Neumann, R.O. (1899): cited by Munro (1951).

Novikoff, A.B. & Potter, V.R. (1948): J. Biol. Chem., 172, 223.

Patel, S.M. & Sreenivasam, A. (1948): Anal. Chem., 20, 103.

Pollister, A.W. & Leuchtenberger, C. (1949): Nature, Lond., 163, 360.

Popjak, G. & Muir, H. (1950): Biochem. J., 46, 103.

Potter, V.R., Recknagel, R.O. & Hurlbert, R.B. (1951): Fed. Proc., 10, 646.

Ratner, S. (1951): Phosphorus Metabolism; John Hopkins Press, Baltimore.

Ratner, S. & Pappas, A. (1949): J. Biol. Chem., 179, 1199.

Ratner, S. & Petrack, B. (1951): J. Biol. Chem., 191, 693.

Rosemann, R. (1901): cited by Munro (1951).

Runnstrom, J. (1952): Trends in Physiol. & Biochem.; ed. E.S.G. Barron, Acad. Press, N.Y.

Sacks, J. (1949): J. Biol. Chem., 181, 655.

Schmidt, G. & Thannhauser, S.J. (1945): J. Biol. Chem., 161, 83.

Schneider, W.C. (1948): J. Biol. Chem., 176, 259.

Schneider, W.C., Claude, A. & Hogeboom, G.H. (1948): J. Biol. Chem., 172, 451.

Schneider, W.C. & Hogeboom, G.H. (1950): J. Biol. Chem., 183, 124.

Siekevitz, P. (1952): J. Biol. Chem., 195, 549.

Simpson, M.V. & Tarver, H. (1950): Arch. Biochem., 25, 384.

Singal, S.A., Hazan, S.J., Snydenstricker, V.P. & Littlejohn, J.M. (1952): Fed. Proc., 11, 289.

Slater, E.C. (1950): Nature, Lond., 166, 982.

Smellie, R.M.S. (1952): personal communication.

Smellie, R.M.S. & Davidson, J.N. (1951): Biochem. J., 49, xv.

Snedecor, G.W. (1937): Statistical Methods; Iowa State College Press.

Spiegelman, S. & Kamen, D. (1946): Science, 104, 581.

Spiegelman, S. & Kamen, D. (1947): Cold Spring Harb. Quant. Biol., 12, 211.

Sprinson, D.B. & Rittenberg, D. (1949): J. Biol. Chem., 180, 707, 715.

Stoneberg, C.A. (1939): J. Biol. Chem., 129, 189.

Stowell, R.E. (1948): Arch. Path., 46, 164.

Szent-Gyorgi, A. (1947): Chemistry of Muscular Contraction. Acad. Press, N.Y.

Thorell, B. (1944): Acta Med. Scand., 117, 334.

Thorell, B. (1947): Cold Spring Harb. Symp. Quant. Biol., 12, 247.

Turba & Turba (1951): Naturwissenschaften, 38, 188.

Umbreit, W.W., Burris, A. & Stauffer, J.F. (1945): Manometric Techniques, p.194. Burgess Publishing Co., Minneapolis.

Venkataraman, P.R., Venkataraman, A., Schulman, M.P. & Greenberg, D.M. (1950): J. Biol. Chem., 185, 175.

Vischer, E. & Chargaff, E. (1948): J. Biol. Chem., 176, 703, 715

Vischer, E., Magasanik, B. & Chargaff, E. (1949): Fed. Proc., 8, 263.

Voit, C. (1869): cited by Munro (1951).

Warburg, O. (1912): cited by Claude (1950).

Warburg, O. & Christian, W. (1939): cited by Lipmann (1941).

Weil-Malherbe, H. & Green, R.H. (1951): Biochem. J., 49, 286.

Winnick, T. (1950): Arch. Biochem., 28, 338.

Zilversmit, D.B., Entenmann, C. & Chaikoff, I.L. (1948): J. Biol. Chem., 176, 193.

Zuntz, N. & Loewy, A. (1918): cited by Munro (1951).