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THE HORMONAL CONTROL OF PROTEIN SYNTHESIS.

A study of the action of carbohydrate on protein metabolism.

by

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

April, 1958.

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INTRODUCTION.

The importance of the nitrogenous constituents of the diet has been established since the early nineteenth century. (Magendie 1786 - 1855; Boussingault 1802 - 1887). It was soon realised that the energy-yielding constituents of the diet, carbohydrate and fat, had a considerable influence on the course of nitrogen (N) metabolism, although the underlying mechanism of this dependence was less clearly understood. This influence of carbohydrate and fat on protein metabolism, the protein sparing action of carbohydrate and fat, has recently been reviewed (Munro 1951), where evidence is presented which shows that a difference exists between the ability of carbohydrate and that of fat in sparing protein. This evidence can be considered in 4 parts:

(i) The isocaloric substitution of carbohydrate for fat leads to a transient decrease in urinary N.

(ii) The sparing action of carbohydrate is less effective when the times of carbohydrate and protein ingestion are separated, whereas the time of eating fat is immaterial.

(iii) The administration of carbohydrate to fasting subjects results in a retention of body N, whereas fat is usually not effective in reducing the urinary N output.

(iv) The ingestion of carbohydrate, but not of fat, reduces the level of the plasma amino-acids for some hours.

This evidence of a difference in the action of carbohydrate and fat on protein metabolism will now be considered in more detail.

Substitution of fat for carbohydrate.

The addition of carbohydrate or fat to the diet results in N retention by the body. (Cuthbertson and Munro, 1937; Forbes, Bratzler, Thacker and Marcy, 1939; Forbes and Swift, 1944; Munro and Wikramanayake, 1954). If carbohydrate and fat influence protein metabolism merely by acting as energy sources, then substitution of one for the other, on a caloric basis, should not influence protein metabolism, as reflected by a constant N output. But it has been found that complete (Silwer, 1937) or partial (Umeda, 1915) isocaloric substitution of fat for the carbohydrate in the diet of human subjects results in a loss of body N. Experiments with dogs, in which there was a complete substitution of carbohydrate by fat (Voit and Korkunoff, 1895; Luthje,1906;) or only a partial substitution (Bierhacki, 1907; Umeda, 1915;) also indicate that an enhanced N retention accompanies the period of carbohydrate ingestion. The negative findings of Abderfalden, Messner and Windrath (1909) in a single study form an exception to this.

When/

When carbohydrate was completely replaced by fat in the diets of rats, Maignon and co-workers (Maignon and Jung, 1924; Maignon and Vimeux, 1931; Maignon and Chahine, 1931a and b; Maignon, 1934) found that N balance was actually better on the fat-containing diet. Similar results were obtained by Samuels, Gilmore and Reinecke (1948). In both these groups, the rats were on the diets for some time before urine collections were begun, so that information regarding any immediate effect of carbohydrate on N balance is lacking. This criticism also applies to some investigators (Forbes etal., 1946; Forbes, Swift et al,1946) who partially substituted fat for carbohydrate and found that N balance was not significantly better in the diets richer in carbohydrate. On the other hand, Desgrez and Bierry (1920) and Lathe and Peters (1949) found that N retention was improved during the first few days of a period in which carbohydrate replaced some of the dietary fat. These opposing results were reconciled recently by Munro and Thomson (1955) who showed that the isocaloric substitution of carbohydrate by fat in the diet of the rat results in a transient increase in N output which later resumes its original level. Thus, investigators who collected excreta immediately after the dietary exchange would obtain positive results, whereas those who delayed collection for several days following substitution of fat for carbohydrate would fail/

fail to observe the increased output.

The general picture which has emerged from studies in which fat is substituted isodynamically for carbohydrate, either partially or completely, is that carbohydrate has an effect on protein metabolism for which energy in the form of fat is not a substitute. This effect only occurs immediately after the alteration in carbohydrate ingestion and is only temporary.

Separation of time of ingestion of protein from that of carbohydrate.

It has been observed (Cuthbertson and Munro, 1939) that carbohydrate need not be removed from the diet of human subjects to produce an adverse effect on N balance; mere separation in the time of eating the carbohydrate and protein of the diet being sufficient to cause a deterioration in N balance. The same results were obtained with adult rats (Cuthbertson, McCutcheon and Munro, 1940). Thus the presence of carbohydrate in protein-containing meals causes a retention of N by the body. This has been shown to be a temporary phenomenon (Munro 1949). This author also showed that separating the time of eating protein and fat had no effect on N output. Essentially similar results were obtained by Geiger, Bancroft and Hagerty (1950), who found that protein-depleted rats regained weight much more rapidly when dietary protein and carbohydrate were eaten together, than when they were consumed separately.

Effect of carbohydrate and fat on Nitrogen output during starvation.

Bartman (1912) and Richet and Minet (1925) found that feeding fat to fasting dogs did not significantly reduce the level of urinary N. Wimmer (1912), Richet and Minet (1925) showed that the administration of carbohydrate to fasting dogs was capable to sparing body N. A similar difference in action was apparent when carbohydrate (Benedict 1915; Grafe, 1910 and 1914) and fat (Thomas 1910) were given to fasting human subjects. Heilner (1906), May (1894) Voit (1901) with carbohydrate and Heilner (1906), and Rubner (1883) with fat, drew the same conclusions from their studies on fasting rabbits. The situation with the rat is less clearly defined. Gregg (1931) fed butter fat to fasting rats and noted that the urinary N output of several rats was consistently lower than that of a single control animal. Kriss, Forbes and Miller found that the fasting (control) level of urinary N, 122 mgms. per 100 gms. body weight per 24 hours, was reduced by feeding starch to 30 mgms. per 100 gms. body weight per 24 hours, whereas feeding an isocaloric amount of fat lowered the N output to only 70 mgms. per 100 gms. body weight per 24 hours. Both sets of experimental details are rather vague and a strict comparison of the effect of fat is therefore not possible. As regards carbohydrate administration to fasting/

fasting rats, Rosenthal and Vars (1954) showed that this treatment was accompanied by a significant reduction in urinary N. The meagre data available thus indicate that in the fasting rat, as well as in other species, the ingestion of carbohydrate results in a reduction in N output, but the effect of fat is less certain.

The effect of carbohydrate and fat ingestion on the plasma amino-acids.

When carbohydrate (e.g. glucose) is given to a fasting animal, there is a depression in the plasma amino-acid level, maximal at 1-2 hours after administration. This phenomenon was first observed by Folin and Berglund (1922). Greene Sandiford and Ross (1924) also showed a definite decrease in the plasma amino N level after glucose or fructose ingestion. That this decrease was not caused by haemo dilution was shown by Schmidt and Eastland (1935). Certain authors showed that the level of different amino-acids was not decreased to the same extent after glucose ingestion (Harris and Harris, 1947) and Munro and Thomson (1953) in addition found that fat administration did not have a comparable effect in lowering the blood amino-acid level. The well-known physiological relationship between glucose ingestion and insulin secretion prompted numerous investigations into/

into the role of this hormone in reducing the blood amino-acid level. An exhaustive review of relationship between insulin and the blood amino-acids is beyond the scope of this thesis at this point, but it suffices to say that insulin secretion may be the controlling agent of the blood amino-acid level after glucose ingestion since after pancreatectonny and in alloxan-diabetic animals, no sparing action of glucose has been observed.

It is thus quite feasible that the reduction of the urinary N and the depression of the blood amino-acid level after a meal of carbohydrate are expressions of a single mechanism, inherent in the sparing action of carbohydrate. Fat ingestion, which is not followed by insulin secretion, consequently cannot participate in this effect on protein metabolism, as evidenced by the lack of effect on urinary N output and blood amino-acid level when given to a fasting subject.

Nature of the Problem.

As indicated in the preceding summary of the literature carbohydrate has a specific action on protein metabolism, and we have been led to conclude that this effect probably involves the participation of insulin. It has been our objective to investigate the mode of action of carbohydrate in producing this effect. The simplest system on which to make this study seemed to be glucose administration to

a/

a fasting animal. The following five sections of the thesis therefore represent an analysis of the changes which occur in protein metabolism when a rat receives a dose of carbohydrate.

First, experiments were designed to amplify the meagre data relating to the action of carbohydrate and fat on the N output of fasting rats, in order to establish a specific action of carbohydrate on protein metabolism in this species (Section 1). These were followed by an attempt to discover the site where N is retained after carbohydrate administration Analysis of the liver showed that this organ did not accumulat protein when carbohydrate was given. We therefore turned to the use of labelled amino-acids (Section 2) and examined their uptake by the proteins of muscle and of liver. The results point to the carcass as the main site of action of carbohydrate on protein metabolism. The nature of this action was, however, rather difficult to interpret from the data.

Further experiments were therefore performed using ^{14}C-glycine, and changes in the labelling of the protein were compared with those in the free amino-acid pool (Section 3). These data could most readily be explained by the transitory formation of some peptide or protein in muscle under the influence of glucose.

In/

In Section 4, there is a description of attempts to isolate such a peptide from the acid-soluble fraction of muscle following glucose administration, and in Section 5 some experiments on the incorporation of radioglycine into a muscle protein fraction which is labile towards hot trichloro-acetic acid are discussed.

These sections are followed by a general discussion which attempts to integrate our findings with the more general questions of the action of insulin and other hormones on protein metabolism.

SECTION 1.

THE EFFECT OF CARBOHYDRATE AND FAT ON THE N RETENTION OF THE FASTING RAT.

INTRODUCTION.

It has been recognised for some considerable time that the administration of carbohydrate to fasting animals causes a fall in the urinary nitrogen (N) output.whereas the giving of fat does not have a comparable effect. As indicated in the general introduction to this thesis, although the evidence for this special action of carbohydrate rests on reliable data from various species, it is inadequate and vague in the case of the rat.

The scope of the experiments described in this section is twofold, viz.,

i) to confirm that administration of carbohydrate to fasting rats reduces the N output in the urine in agreement with its action on other species, and in a manner distinct from any changes following fat administration.

ii) to determine which tissue or tissues are responsible for the retention of N produced by feeding carbohydrate. The analysis was confined to the liver and the effect of glucose and fat on its N content determined.

EXPERIMENTAL.

Animals and diets.

Young adult male albino rats were fasted overnight and those weighing about 250 g. were distributed among the various experimental treatments according to the randomised block technique of Snedecor (1946), which reduces the effect of slight differences in body weight as a factor in the analysis of the results. The animals were housed individually under thermostatic conditions in glass containers (fig.1) and fed the experimental diet twice daily. This consisted of the following purified ingredients: casein, glucose, olive oil and a vitamin-mineral-roughage mixture (V.M.R.) (Munro, 1949). The compounding of these constituents in the high and low protein diets are found in table 1a.

The morning feed consisting of 2 g. of V.M.R. and 3.5 g of glucose, was given about 10 a.m. The evening feed consisted of 5.3 g. of a protein-containing or a protein-free mixture (Table 1a)and was given at 5 p.m. Each meal was moistened with water to prevent scattering.

After a period of seven days on the high or low protein diet, urine collections were carried out for 24 hours during which time the animals were fed nothing but water (control) carbohydrate (7 g. glucose a.m. and 5 g. p.m.) or fat (3 mls. olive oil a.m. and 2 mls. p.m.)

TABLE 1a.

Composition of Protein-containing Meal.

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

Composition of Protein-free Meal.

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

Collection of Samples.

The N balance unit used consisted of three components (fig. 1)

A. The glass vessels for housing the rats were large sized chemical reagent bottles, the bottom of which had been removed. They were employed in the inverted position.

B. This component consisted of two pieces of coarse mesh wire gauze joined together with four metal rods and was designed to fit inside the glass containers. When in position, the upper piece of wire gauze overlapped the glass container and effectively imprisoned the rat, which sat on the lower gauze beside its food dish. The mesh of the lower gauze is sufficiently wide to allow easy passage of urine and faeces. The whole component is easily lifted out to remove the rat or to supply it with food.

C. This comprised a large filter funnel on which was placed a circular piece of fine mesh wire gauze in the shape of a Mexican hat. This component was placed beneath the neck of the glass cage and served to separate urine from faeces. The filter funnel led directly to a two litre Winchester which contained 20 mls. 11N.HCl to prevent bacterial decomposition (Addis and Watanabe , 1916) of the urine.

These individual units were mounted on a metal framework of Dexion which was capable of holding 18 such units (photograph 1.)

After/

N BALANCE UNIT.

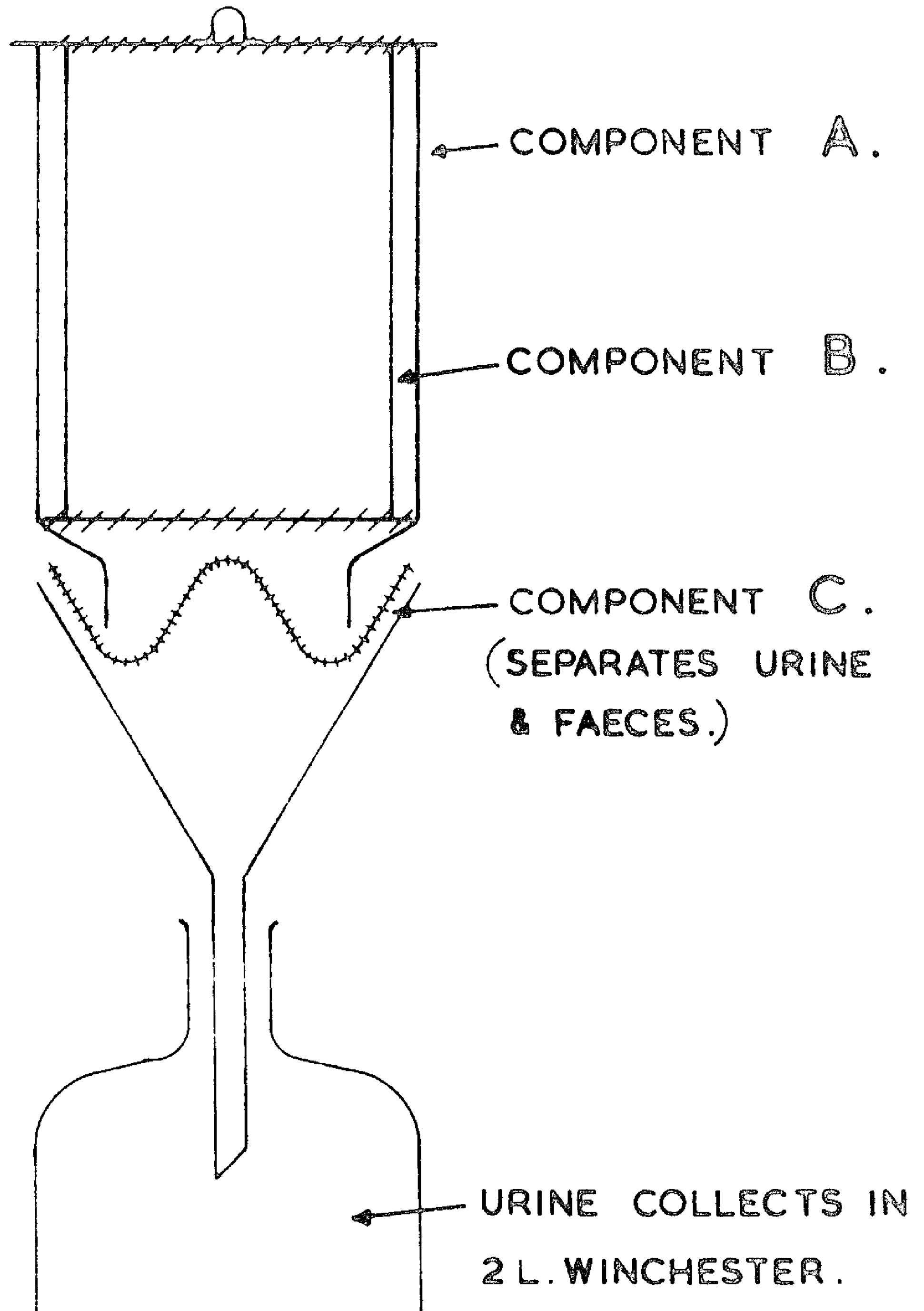
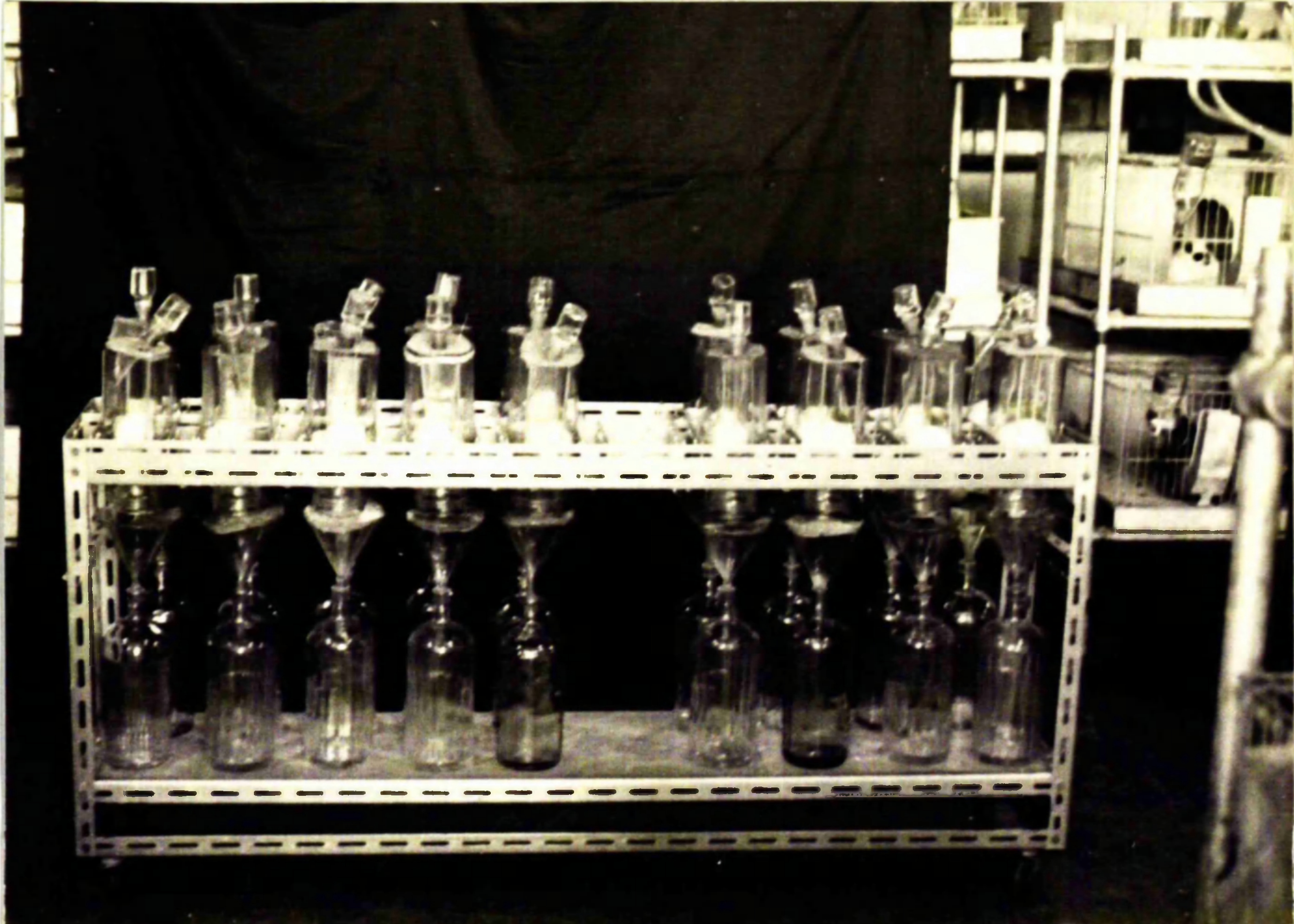


FIG. 1.



PHOTOGRAPH 1.

Nitrogen Balance Unit.

After the 24 hours collection period, the animals were removed from their containers which were subsequently washed down with 200 mls. of distilled water into the urine bottles. The N content of the urine was estimated by the micro-Kjeldhal method as described by Munro and Naismith (1953).

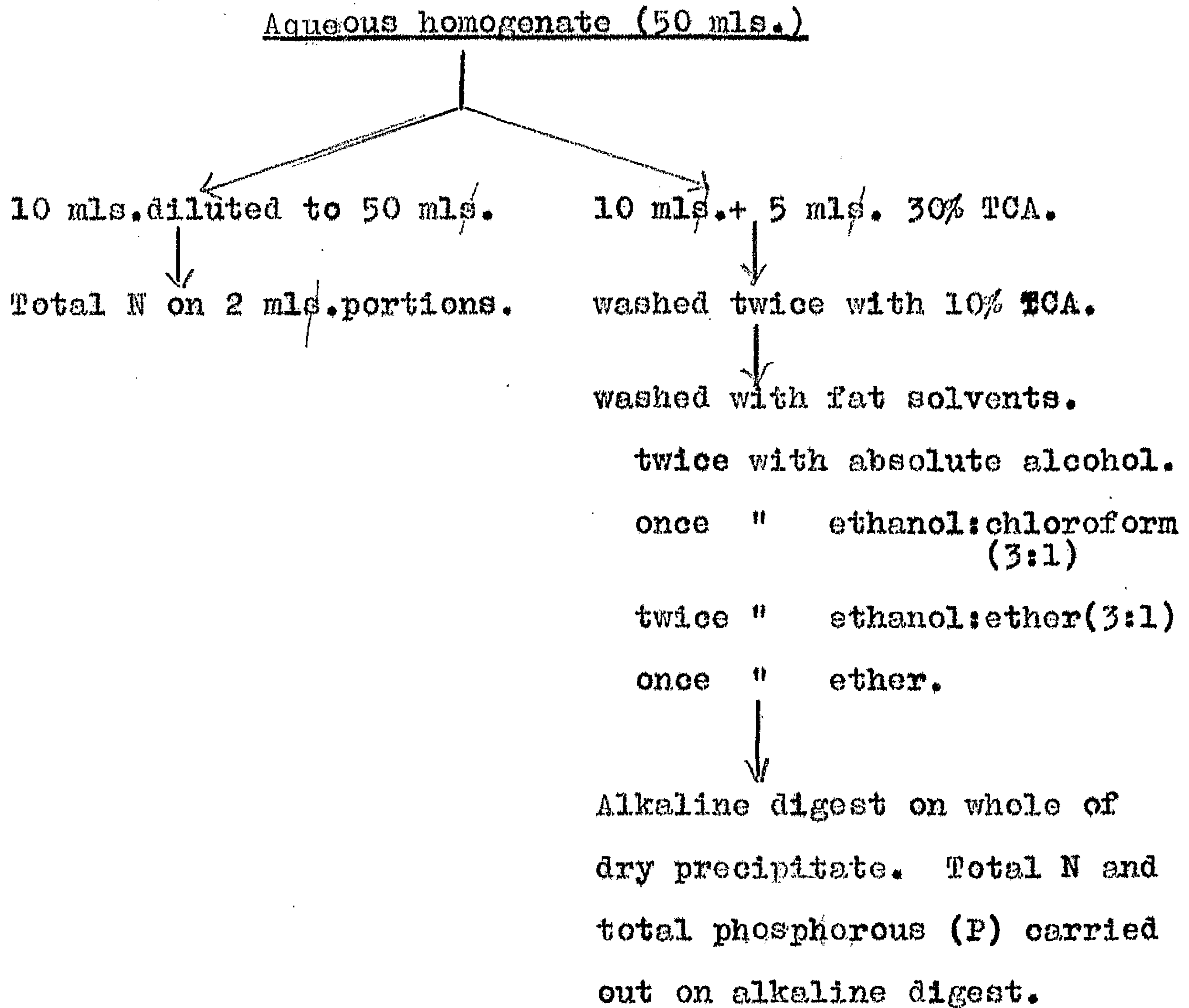
Removal and Analysis of Liver.

The rats were killed by exsanguination under ether anaesthesia and the whole liver excised and washed in distilled water to remove excess blood before homogenising in water. The subsequent treatment of the aqueous suspension is detailed in fig.2.

Total N estimation of urine and liver was carried out by the micro-Kjeldhal method (Munro and Naismith, 1953) on sample volumes which were adjusted to contain about 1 mg.N. This allowed for more accuracy in the subsequent titration with 0.01N.H_2SO_4. The samples were digested in 1.5 mls. of concentrated nitrogen-free H_2SO_4 using a mercury bead as a catalyst. Digestion was continued for half an hour after the sample had cleared (Hiller, Plazin and Van Slyke, 1948) and the flask allowed to cool before the sides were washed down with a little distilled water. Distillation was carried out in the apparatus described by Markham (1942). Before the addition of 10 mls. of 40% (W/V) sodium hydroxide to liberate the ammonia, 1 ml. of saturated sodium thiosulphate was added to precipitate the mercury which would otherwise interfere/

FIG. 2.

Treatment of liver homogenate.



interfere with the distillation. The ammonia was collected in a conical flask containing 6 mls. of 2% (W/V) boric acid and 4 drops of mixed indicator (5 parts of 0.1% bromocresol green in 95% ethanol and 1 part 0.1% methyl red in 95% ethanol but the collection was delayed until the distillate had reached the lower end of the condenser thereby eliminating the possibility of any SO_2, liberated by the alkali, collecting in the receiver. The ammonia content of the sample was estimated by titration with 0.01N H_2SO_4.

Protein N Determination.

The alkaline digest (fig.2) was analysed for total N as described above. A correction for the N contribution by nucleic acids of the digest was obtained by estimating the total P content by the method of Allen (1940), and multiplying this figure by 1.69.

RESULTS.

The effect of carbohydrate and fat administration on urinary N.

The data of six experiments are presented in Table 1. The results indicate that, following both the protein-containing and the protein-free diets, the amount of urinary N is reduced by the administration of carbohydrate and to a lesser extent by the giving of fat. Statistical analysis of the data (Table 2) reveals that the effect of carbohydrate ingestion is very definite at the 5% level following both types of diet whereas the effect of fat administration is of borderline significance after one diet and not significant after the other.

The effect of carbohydrate and fat administration on the N content of the liver.

The mean results for the liver total N (based on six experiments) and liver protein N (on four experiments) are shown in Table 3. Statistical analysis of the data (Tables 4 and 5) shows that, while glucose administration causes a decrease, in both total and protein N which reaches significance, fat administration has an insignificant effect on either the total or protein N. The effect of carbohydrate is perhaps more pronounced following the protein-free diet, though this distinction fails to attain statistical significance.

TABLE 1.

The effect of carbohydrate and fat administration on the urinary N of fasting animals previously fed with protein-containing or with protein-free diets.

Preceding Diet	Experiment. No.	Urinary N (mgms/24 hrs.)		
		Water-fed	Glucose-fed	Fat-fed
Protein containing	1	172	109	143
	2	209	154	196
	3	180	122	159
	4	160	109	142
	5	226	118	80
	6	133	152	160
	Mean	180	127	147
Protein free	1	91	80	97
	2	75	65	54
	3	76	69	90
	4	67	30	45
	5	134	51	50
	6	99	51	105
	Mean	90	58	74

TABLE 2.

Analysis of Variance of Urinary N Data.

Sources of Variance	Degrees of freedom	Sum of Squares	Mean Square	Variance Ratio (F)
Total	35	88,821	-	-
Replicates	5	3,763	753	1.02
Protein levels	1	54,057	54,057	-
Treatment	2	11,035	5,518	7.43
Error	32	23,729	742	-

F = 5.34 at the 1% level ($n_1 = 2$; $n_2 = 32$).

Therefore carbohydrate and fat have a significant effect on the N output ($P < 0.01$).

The minimum significant difference between columns is 32 mg. N/day. Hence the significance of the effect of carbohydrate is high after both diets, while that of fat is border-line in the case of the protein-containing diet and not significant following the protein-free diet.

TABLE 3.

The effect of carbohydrate and fat administration on liver N.

Dietary Treatment	Diet			
	Protein containing		Protein free	
	Total N. (mgms.N/liver)	Protein N.	Total N (mgms.N/liver)	Protein N.
Water	204	141	185	131
Glucose	199	135	175	116
Fat	209	144	192	127

Total N - Each entry is the mean of 6 experiments.

Protein N - Each entry is the mean of 4 experiments.

TABLE 4.

Analysis of Variance of liver total N data.

Source of Variance	Degrees of freedom	Sum of Squares	Mean Square	Variance Ratio (F)
Total	35	31,397	-	-
Replicates	5	22,305	----	----
Protein levels	1	3,580	----	----
Treatment	2	1,143	572	3.53
Error	27	4,359	162	-

F = 3.35 at the 5% level of significance (n_1 = 2 and n_2 = 27).

TABLE 5.

Analysis of Variance of liver protein N data.

Source of Variance	Degrees of freedom	Sum of Squares	Mean Square	Variance Ratio (F)
Total	23	14,199	-	-
Replicates	3	10,873	3624	---
Protein levels	1	1,261	1261	---
Treatment	2	641	322	3.83
Error	17	1424	84	-

F = 3.59 at the 5% level of significance (n_1= 2, and n_2= 17).

DISCUSSION.

Our observations indicate that, when a fasting rat is fed carbohydrate, there is a significant decrease in N output whether or not the previous diet contained protein. Concurrently the liver total N is decreased due to a significant reduction in its protein N content; this effect may be more pronounced when the animal has previously been on a protein-free diet. The administration of an isocaloric amount of fat, on the other hand, while tending to reduce the overall N output by the body, is without effect on the N content of the liver. These results provide more evidence for a difference between carbohydrate and fat in their ability to spare body N (see review by Munro, 1951).

The most interesting aspect of these investigations is the fact that only carbohydrate administration results in a decreased liver N content. Consideration of previous results of Munro and Naismith (1953) lends support to these findings. (See Table 6). They studied the effect of adding extra carbohydrate or fat to protein-free diets and found that an improvement of N balance followed the addition of carbohydrate but was accompanied by a loss of liver protein; this did not accompany addition of fat. The same/

TABLE 6.

Effect of adding carbohydrate or fat, as an energy source, to a protein-free diet. (From Munro & Naismith, 1953).

Variable Source of Energy	Change per 1000 Cal./m^2	
	N Balance	Liver Protein N/rat
Carbohydrate	+ 69	- 11.4
Fat	- 42	- 4.1

same paradox was observed by Rosenthal and Vars (1954), who fasted rats after a period on a protein-deficient diet and observed that the consequent rise in urinary N output was accompanied by an improvement in the N and enzyme content of the liver. In this instance, therefore, the removal of carbohydrate from the diet had a beneficial effect on liver protein content.

The evidence thus indicates that carbohydrate has an effect on protein metabolism which is not shared by fat. Moreover, our experiments and a survey of the literature amplify this by revealing that the N retention occurring under the influence of carbohydrate does not involve deposition of protein N in the liver, but rather its removal.

Further examination of the site of deposition of the retained N by means of total N studies is unlikely to be very rewarding, since only small changes in the N content of tissues such as muscle could account for all the N deposited under the influence of carbohydrate. A more fruitful indication of what tissue or tissues are responsible can be provided by employing radioactive amino-acids and examining their deposition (under the influence of ingested carbohydrate) in different tissues. These studies following in the succeeding sections of this thesis, describe the incorporation of radioactive amino-acids by various tissues, after glucose or fat ingestion.

SECTION 2.

THE UPTAKE OF ^{35}S-METHIONINE BY LIVER AND MUSCLE PROTEIN AFTER CARBOHYDRATE AND FAT ADMINISTRATION.

INTRODUCTION.

The experiments described in this section were begun by Dr. W.S.T.Thomson. As a result of his investigations on the effect of glucose and fat administration on the level of the plasma amino-acids (Thomson and Munro, 1953), he decided to trace the site at which amino-acids disappearing from the plasma after glucose feeding were deposited. By using isotopically labelled methionine, it was found that the uptake of the amino-acid varied from tissue to tissue, being greater in the viscera than in the muscles. Such results have already been found by Friedberg, Tarver and Greenberg (1948) using ^{35}S-methionine. A study of the uptake of ^{35}S by liver and mucosa after feeding glucose or olive oil revealed no obvious departure from the control values. Studies on the incorporation of the radio-active methionine by rat diaphragm and skeletal muscle has never showed a similar response to glucose administration viz. a greater deposition of the isotope in the carbohydrate-fed animals some 4 hours after feeding. The results obtained were very suggestive of an effect but not statistically significant. Fat administration, on the other hand, did not cause any difference in radio methionine uptake from the control animals.

Although/

Although the results of these experiments did not attain statistical significance, the data did suggest that carbohydrate has an action on protein metabolism which is limited to muscle. This seemed well worth following up and it was decided, therefore, to repeat Thomson's experiments in part by feeding to fasting rats, which had previously been injected with radioactive (^{35}S) methionine, either water, glucose or fat and determining the extent of the incorporation of methionine into the proteins of liver and quadriceps muscle after various time lapses.

EXPERIMENTAL.

Animals and Feeding.

Male albino rats, after fasting for 24 hours, were divided into three groups. A sterile solution of ^{35}S-methionine (20 μc in 1.0 ml. of 0.9% saline) was injected into each rat intraperitoneally and after a lapse of half an hour, groups of rats were fed either 4 mls. of water (controls or 4 mls. of 50% glucose solution, or 1 ml. of olive oil by stomach tube. One rat from each group was killed at 2,4, and 6 hours after feeding.

Treatment of Tissues.

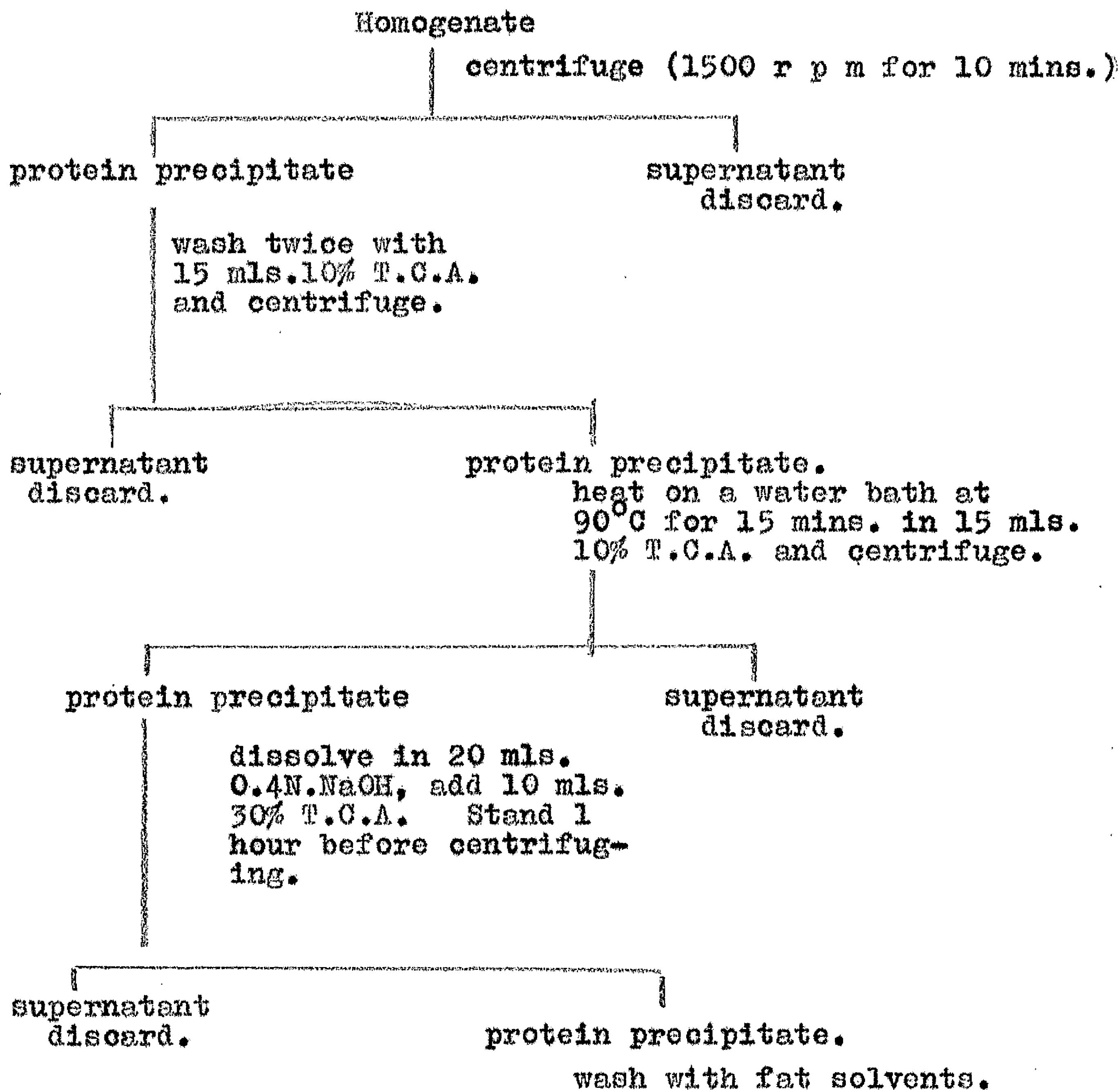
The liver and quadriceps femoris muscles of both hind legs were excised from the rats, which had been killed by a blow on the head. The liver was washed in distilled water, dried and immediately homogenised in 30% T.C.A. The enzymes of the quadriceps muscle were inactivated by a brief wash in 0.1N H_2SO_4 and the muscle well washed in distilled water. The muscle was then roughly minced with scissors and homogenised in 20 mls. of 0.4N.NaOH. The muscle protein was precipitated by the addition of 30% T.C.A.

Isolation of protein from liver and quadriceps muscle.

The subsequent treatment of the muscle and liver homogenates is described in fig.3, the salient features of which/

FIG. 3.

Treatment of Muscle and Liver homogenates.



which are:-

i) washing with 10% TCA to remove adsorbed ^{35}S-methionine.

ii) heating on a water-bath at 90°C for 15 minutes to solubilise nucleic acids (Schneider, 1945).

iii) dissolving (twice) in 0.4N.NaOH and reprecipitating the protein with 30% TCA to remove any adsorbed ^{35}S-methionine (Melchior and Halikis, 1952).

The protein was finally washed with the fat solvents in the following order:-

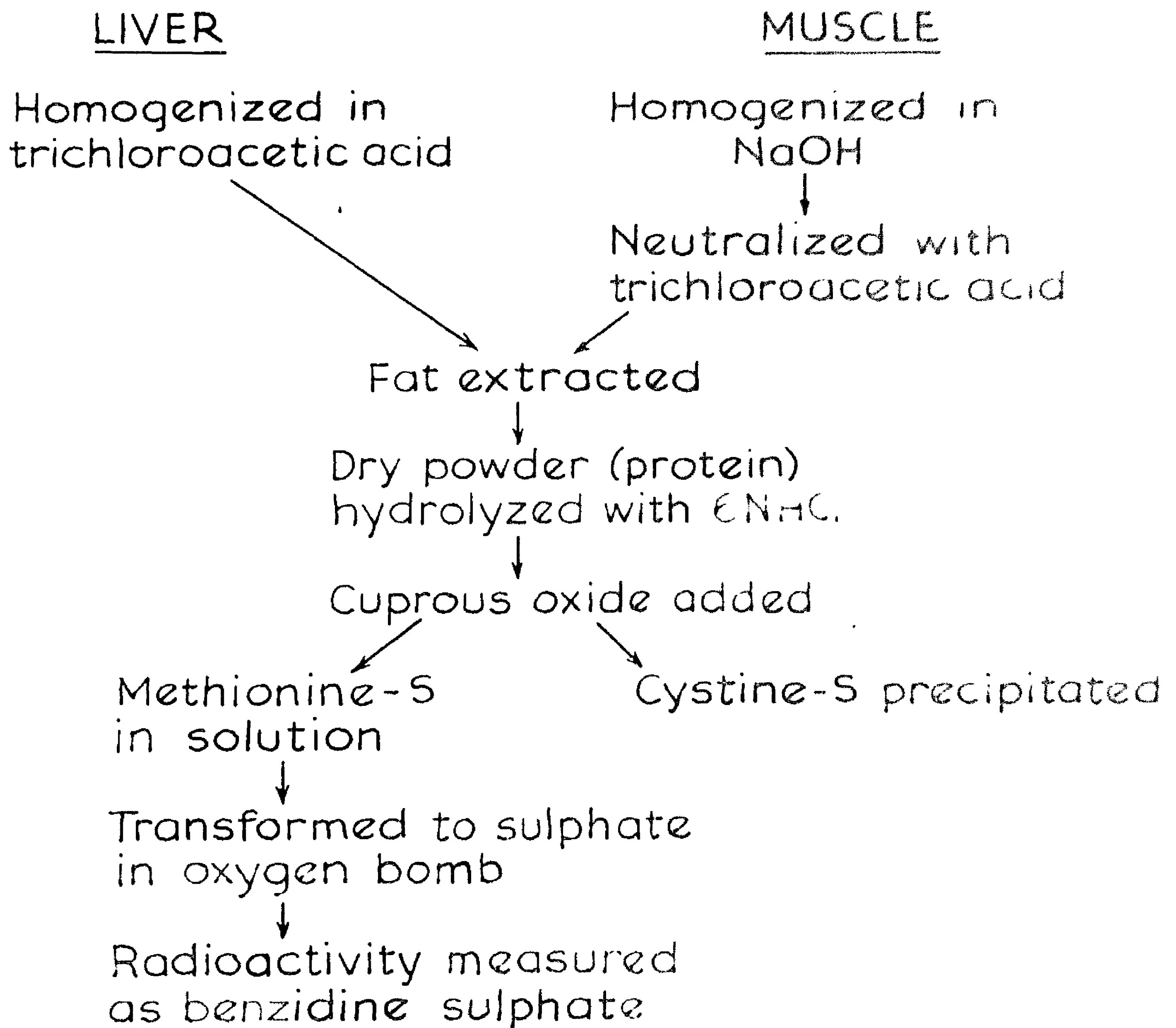
95% (v/v) ethanol (twice),
ethanol: chloroform (3:1),
ethanol: ether (3:1),
absolute alcohol,
ether.

<u>Isolation of ^{35}S-methionine from muscle and liver proteins.</u>
(see fig. <u>3a</u>). The S of the protein is isolated as benzidine sulphate (Young, Edson and McCarter, 1949).

A known weight of the dried protein is refluxed in 6N.HCl for 6 to 8 hours at 130 ± 5°C under an atmosphere of N which prevents the oxidation of cysteine to cysteic acid. The HCl was distilled off under reduced pressure and the dry residue reconstituted with distilled water. The cysteine was separated from the methionine by its precipitation as the/

FIG 3a.

Measurement of Radioactivity of Methionine in Proteins of Liver and Muscle



the copper mercaptide (Zittle and O'Dell, 1941) by adding cystine as a carrier, adjusting the pH to 3 with solid sodium acetate and, after heating in a boiling water bath, adding eight times the theoretical amount of Cu_2O. When the pH was raised to 4, the mercaptide was precipitated and filtered off after standing for 40 minutes. (Cysteic acid is not precipitated by this method). The filtrate was evaporated to dryness and 1 g. of benzoic acid added to the residue. The mixture was compressed into a pellet which was exploded in the oxygen bomb under 25 atmospheres of oxygen. In this manner, any S in the residue is converted to SO_4. The bomb was washed out with distilled water and the washings reduced to a small volume (2 mls); 2 mls. of absolute alcohol and 2 mls. of benzidine hydrochloride solution were added and the precipitate of benzidine sulphate allowed to settle out overnight in the refrigerator.

Determination of the radioactivity of the benzidine sulphate.

The precipitate was collected evenly on a filter paper using a micro filter and after washing with a little 95% (v/v) ethanol was allowed to dry in a desiccator before counting in an end-window Geiger counter.

The quantitative determination of benzidine sulphate. (Fiske, 1921). The precipitate of benzidine sulphate was transferred to a conical flask with water and boiled. The benzidine/

dine sulphate is hydrolysed by this treatment and the sulphuric acid so liberated was estimated by titrating the boiling solution with standard alkali, using phenol red as an indicator.

The specific activities were then expressed as counts per minute per mg. of sulphur, allowing a correction for the self-absorption of ^{35}S Beta particles (Henriques, Kristiakowsky, Margnetti and Schneider, 1946).

The efficiency of the mercaptide precipitation, the recovery from the oxygen bomb and the correction for the self-absorption of ^{35}S Beta particles were all checked by Dr. Thomson and found to be satisfactory.

RESULTS AND DISCUSSION.

I am indebted to Dr. Thomson for permission to quote and incorporate his unpublished observations. The incorporation of ^{35}S-methionine into the proteins of intestinal mucosa, liver, diaphragm and quadriceps muscle are presented diagrammatically in fig.4. The data for intestinal mucosa and diaphragm are entirely his findings. The others, liver (see also Table 7) and quadriceps muscle (Table 8) are compounded from Thomson's and our own data. Inspection of the mucosal and liver results (fig. 4 and Table 7) shows that the incorporation into these proteins is not increased by carbohydrate or fat ingestion.

On the other hand, examination of the muscle data (fig.4 and Table 8) reveals that the dietary treatment has a distinct effect on the incorporation of ^{35}S-methionine. In the case of the diaphragm, the ingestion of fat results in an uptake of ^{35}S-methionine into the protein which parallels incorporation by the water-fed (control) animals. In contrast to this, glucose administration causes the uptake of radio methionine into the diaphragm protein to rise from below the control level at 2 hours to a level which is double that of the control group at 4 hours.

The data for the leg muscle also shows that fat has no/

FIG.4.

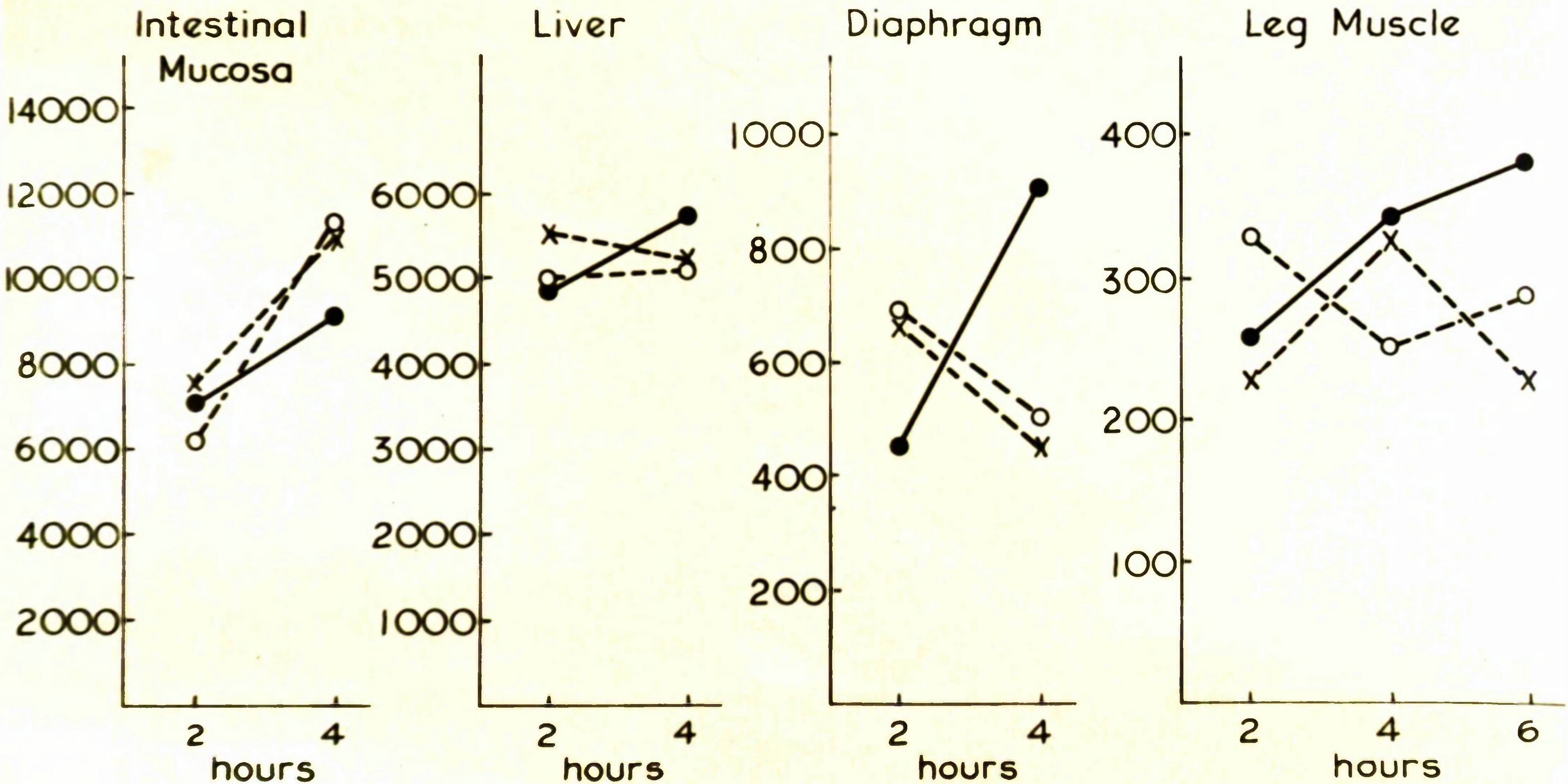


TABLE 7.

The incorporation of ^{35}S-methionine into the protein of rat liver after feeding water, glucose or olive oil.

Time after feeding (hrs.)	Counts/min./mg. sulphur.		
	Water-fed	Glucose-fed	Fat-fed
2	4,979 (4)	4,820 (4)	5,509 (4)
4	5,091 (4)	5,792 (4)	5,182 (4)
6	6,098 (2)	6,080 (2)	3,400 (2)

The figures in brackets indicate the number of experiments.

TABLE 8.

The incorporation of ^{35}S-methionine into the protein of rat quadriceps muscle after water (control), glucose or fat administration.

Time after feeding (hrs.)	Counts/min./mg. sulphur.		
	Water-fed	Glucose-fed	Fat-fed
2	331	262	227
4	254	346	331
6	289	381	229

Each entry is the mean of five experiments.

TABLE 9.

Analysis of variance of the incorporation of ^{35}S-methionine by rat quadriceps muscle after feeding water, glucose or fat.

Source of Variance	Degrees of freedom	Sum of Squares	Mean Square	Variance Ratio
Total	29	125,125	-	-
Times	2	34,122	17,061	4.70
Treatments	1	2,594	2,594	0.70
Replicates	4	15,865	3,966	1.10
Residual	24	87,059	3,627	

F = 3.40 at the 5% level of significance for

$n_1 = 2$ and $n_2 = 24$.

The data were analysed as the percentage change in the protein methionine specific activity from the control after feeding glucose or fat.

no distinct effect, whereas glucose stimulates the uptake of the methionine into the muscle protein at the 4th. and 6th hours.

Thus both types of muscle respond in a similar way to glucose feeding. Statistical analysis of the quadriceps muscle data reveals that the effect of glucose becomes increasingly significant with time (Table 9).

The criticism that glucose ingestion may delay the absorption of amino-acids from the alimentary tract so that the radioactive ^{35}S-methionine only becomes available for incorporation into the proteins of muscle at later times, is unlikely, since no changes in the specific activity of mucosal or liver proteins were noted.

The questions which must now be answered are: Does the feeding of carbohydrate result in an increased synthesis of muscle protein or are the changes in the muscle protein radioactivity due merely to changes in the radioactivity of the methionine pool, free in the muscle cell? This latter would result in changes in labelling of the proteins without any change in rate of protein synthesis. The measurement of activity in the free pool of methionine is difficult to explore for technical reasons, and so the incorporation of ^{14}C-glycine into the protein of the quadriceps muscle was examined. The radioactivity of the precursor pool is much more easily measured, so that the changes in the muscle protein radioactivity can be interpreted with more certainty.

SECTION 3.

THE EFFECT OF GLUCOSE AND FAT ON THE INCORPORATION OF ^{14}C-2-GLYCINE INTO THE PROTEINS OF SKELETAL MUSCLE AND LIVER.

INTRODUCTION.

The experiments described below follow the pattern of those in the preceding section except that ^{14}C-glycine was the labelled amino-acid used. The replacement of ^{35}S-methionine by ^{14}C-glycine allows easy measurement of the radioactive changes in the free amino-acid pool as well as in the proteins. Such knowledge is desirable, as it indicates whether the changes in the labelling of the proteins represents protein synthesis *de novo*, or whether such changes are reflections of the alterations of the radioactivity of the free pool.

EXPERIMENTAL.

Animals.

Male albino rats which had fasted for 24 hours were injected with 20 μc of ^{14}C-2-glycine in 1.0 ml. of 0.9% saline, and divided into three groups. According to this division, the animals received 4 mls. of water (control) or 4 mls. of a 50% glucose solution or 1 ml. of olive oil half an hour after injection. An animal from each group was killed after 2,4, and 6 hours.

Treatment of Tissues.

The animals were killed by a blow on the head and the liver and quadriceps femoris muscle of both rear legs removed. The liver was immediately homogenised in about 20 mls. of 10% TCA. The muscle enzymes were inactivated by a quick wash in 0.1N.H_2SO_4 and the muscle washed in distilled water before mincing roughly with scissors. The mince was then homogenised in about 20 mls. of 0.4N.NaOH and, after frothing had subsided, the protein was precipitated by the addition of 10 mls. of 30% TCA.

Treatment of muscle and liver homogenates.

The original supernatant and two subsequent washings of the protein precipitate were combined and retained for the analysis of the glycine, free in the muscle or liver pool (free glycine estimations). The protein was then purified/

purified as shown in fig.3 of Section 2.

<u>Counting of whole protein samples</u>. See Section 5.

<u>Isolation of Protein Glycine.</u>

A small amount of the dry protein (about 50 mgms.) was refluxed in 6N.HCl for 12 to 14 hours at 130 ± 5°C. The HCl was removed by distillation under reduced pressure, the residue taken up in a little distilled water and transferred to a conical flask, when it was evaporated to dryness in a vacuum desiccator. 5 mls. of distilled water were added and 1.5 mls. used for reaction with 1-fluoro- 2:4 dinitro benzene (FDNB) as described under free glycine.

<u>Estimation of radioactivity of free glycine</u>.

The following procedure is based on that of Campbell and Work (1952).

The T.C.A. washings of the liver or muscle homogenates were washed with ether, until the pH rose to 4. After evaporating to dryness, the residue was taken up in either 5 mls.(liver) or 3 mls.(muscle) of distilled water. 1.5 mls. were made alkaline with $NaHCO_3$ (knife point) and shaken with an excess of diluted FDNB for 4 hours. The subsequent extraction is outlined in fig.5-6. As the ether washings of the first stage remove a little dinitrophenyl (DNP) glycine as well as impurities and unreacted FDNB, the ether layer was washed with distilled water to recover the DNP glycine and the washings retained. The acidification with HCl/

FIG. 5

Procedure for samples reacted with FDNB.

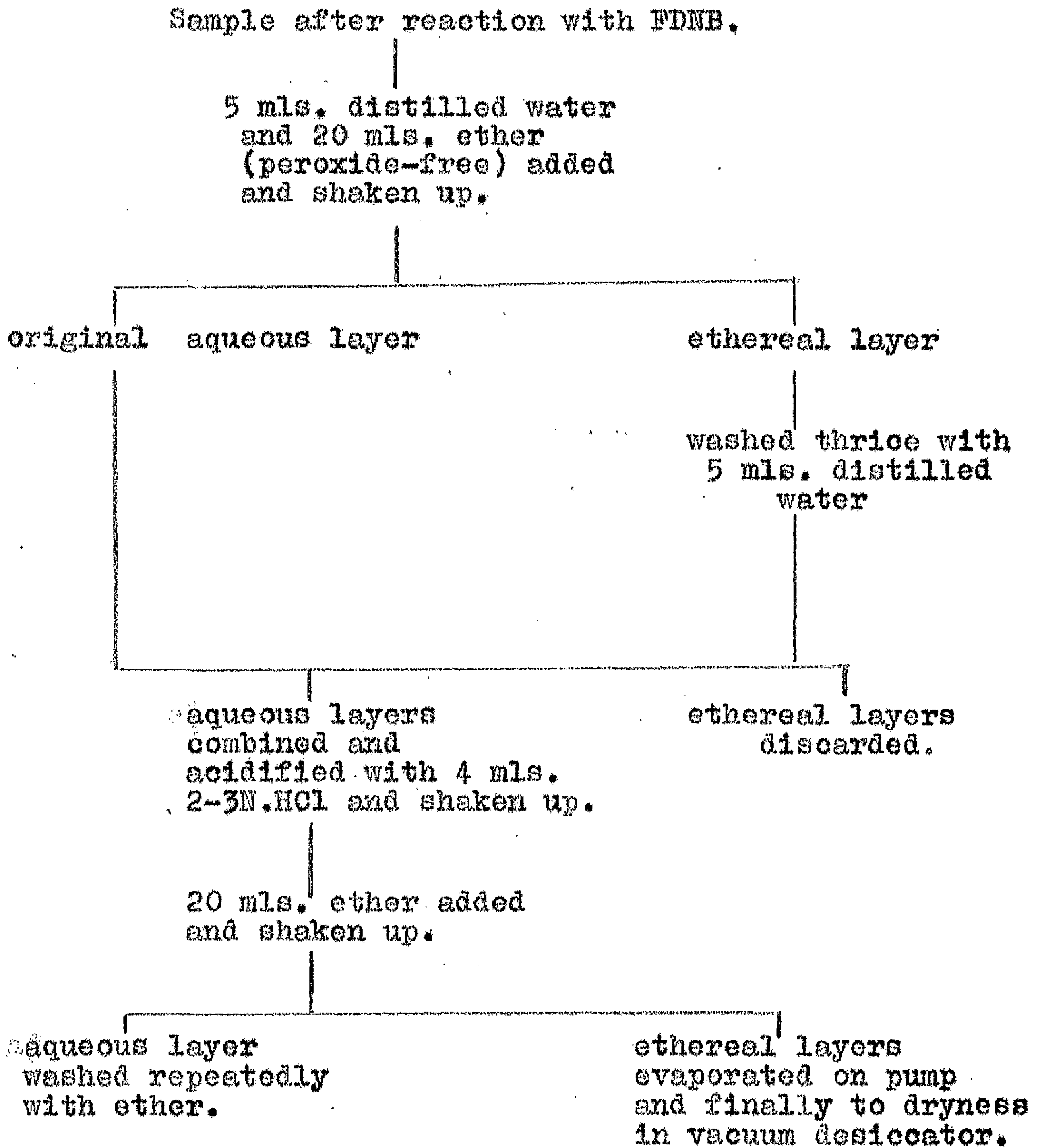
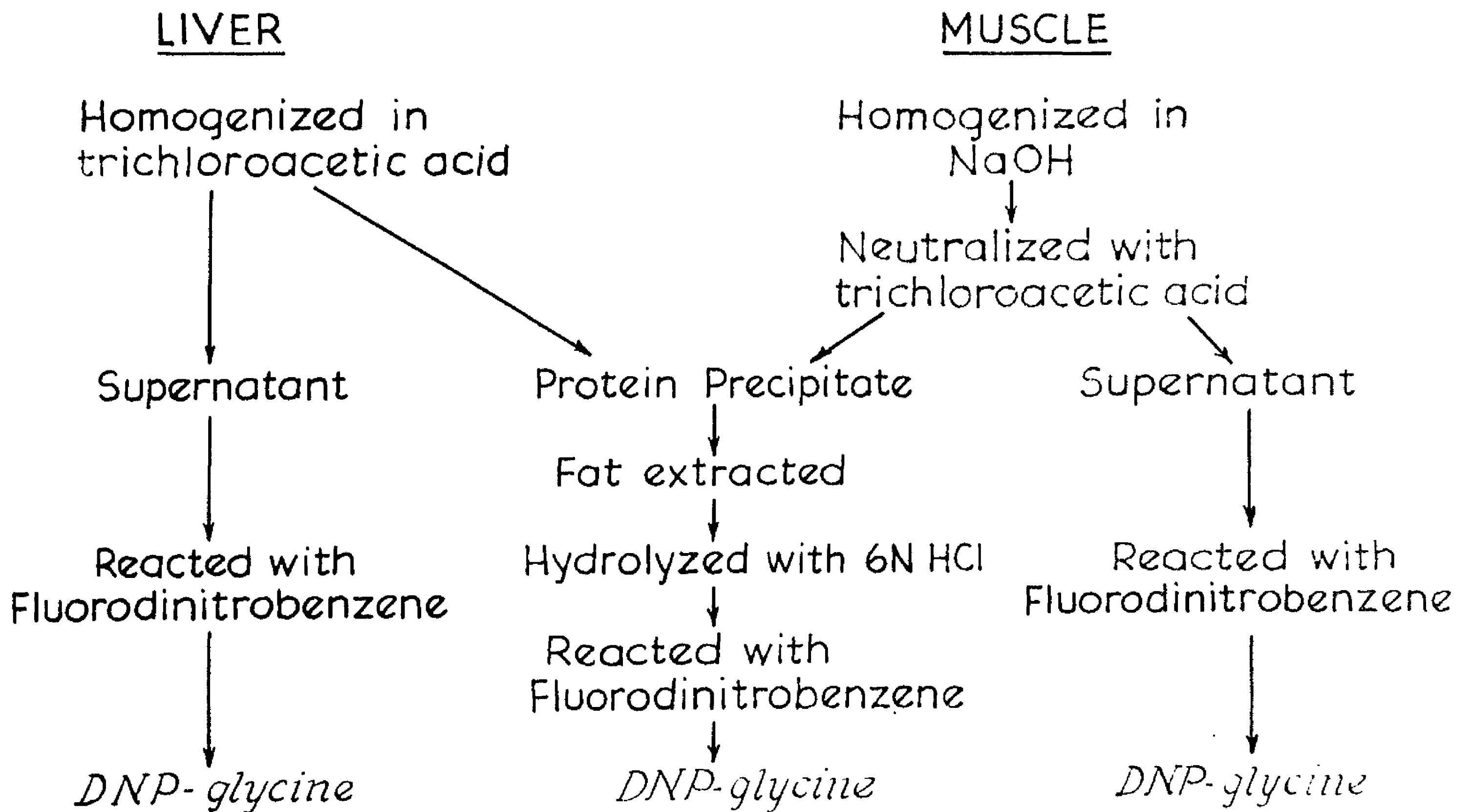


FIG. 6.



HCl favours the extraction of the DNP amino-acids which are separated chromatographically.

Separation of DNP glycine.

The dry sample of DNP amino-acids is dissolved in a small volume of chloroform-butanol (CB) mixture (93:7) as prepared by Krol (1952). This solution is applied to a celite (545) column, of 1 cm. internal diameter, buffered at pH 5.2 with phosphate buffer and packed with washed celite to a height of 16 cms., which had previously been washed with 25 mls. of CB. The DNP glycine separated as a discrete band and was collected. The solvent was evaporated at the water pump. The dry sample was dissolved in dry ether and applied to a second buffered celite column of similar dimensions as the first, in order to purify the DNP glycine further. The developing solvent was ether saturated with water. The DNP glycine band was collected and the ether evaporated as before. The dry sample, in dry ether, was transferred to a counting planchet on which the ether was evaporated by a slight heat. The planchet was counted in a Geiger end-window counter.

Quantitative estimation of DNP glycine.

The DNP glycine was removed from the planchet with a small volume of chloroform-butanol, about 10 mls., which was made up to 20 mls. with washings. 10.0 mls. of 1% $NaHCO_3$ was/

was added and on shaking, the DNP glycine was transferred to the bicarbonate layer. The amount present was estimated spectrophotometrically.

The specific activity of the glycine was expressed as counts per minute per 100 ug. glycine, a correction being made for the self-absorption of ^{14}C Beta particles.

RESULTS.

Some experiments were carried out in which the muscle and liver protein were counted without degradation to glycine. Table 10 gives the specific activities in counts per minute per planchet and fig.7 shows the ratio of activity in muscle protein to that in liver protein. This diagram indicates that, as in the case of methionine (fig.4) the muscle protein of the glucose-fed animals gains radioactivity by comparison with liver protein, the effect being appreciable only some 6 hours after glucose administration. The regression equation for these lines (see legend to fig.7) suggests that glucose depresses uptake into muscle protein 1 hour after administration; this depression is demonstrated by the fact that the protein radioactivity at the intercept (zero time) is 2.89% for the water-fed animal and 1.89% for the glucose-fed animal. This depressing action of glucose is also apparent in the data obtained with ^{35}S-methionine at 2 hours after administration (fig.4) Thus there is no apparent extra deposition of amino-acids in muscle protein at a time when the disappearance of amino-acids from the blood due to the action of glucose is already considerable.

This picture is amplified by studies on glycine isolate from the proteins and from the free amino-acid pools of liver and muscle (Tables 11 and 12). The counts obtained from/

TABLE 10.

The muscle and liver whole protein radioactivity expressed as counts per minute (at infinite thickness) per planchet.

Time After Feeding (hrs.)	Muscle.		Liver	
	Water-fed	Glucose fed	Water-fed	Glucose fed
1	66.1	45.7	2186	1930
2	81.0	93.9	2356	2054
4	99.3	103.2	2217	2267
6	113.1	144.3	1993	1963

The entries at 1 hr. are the mean of 4 experiments whereas at the later times (2,4,6 hrs.) the entries are the mean of 2 experiments.

FIG.7.

Changes in radioactivity of muscle whole protein relative to liver at different times after feeding water (o —— o) or glucose (•——•). The regression equations for the two lines are $Y = 2.89 + 0.40 X$ for the water-fed group and $Y = 1.89 + 0.38 X$ for the glucose-fed group where X = hours after administration and Y = specific activity of the muscle protein expressed as a percentage of the specific activity of the liver protein. Each point is the mean of observations on 2 to 4 animals.

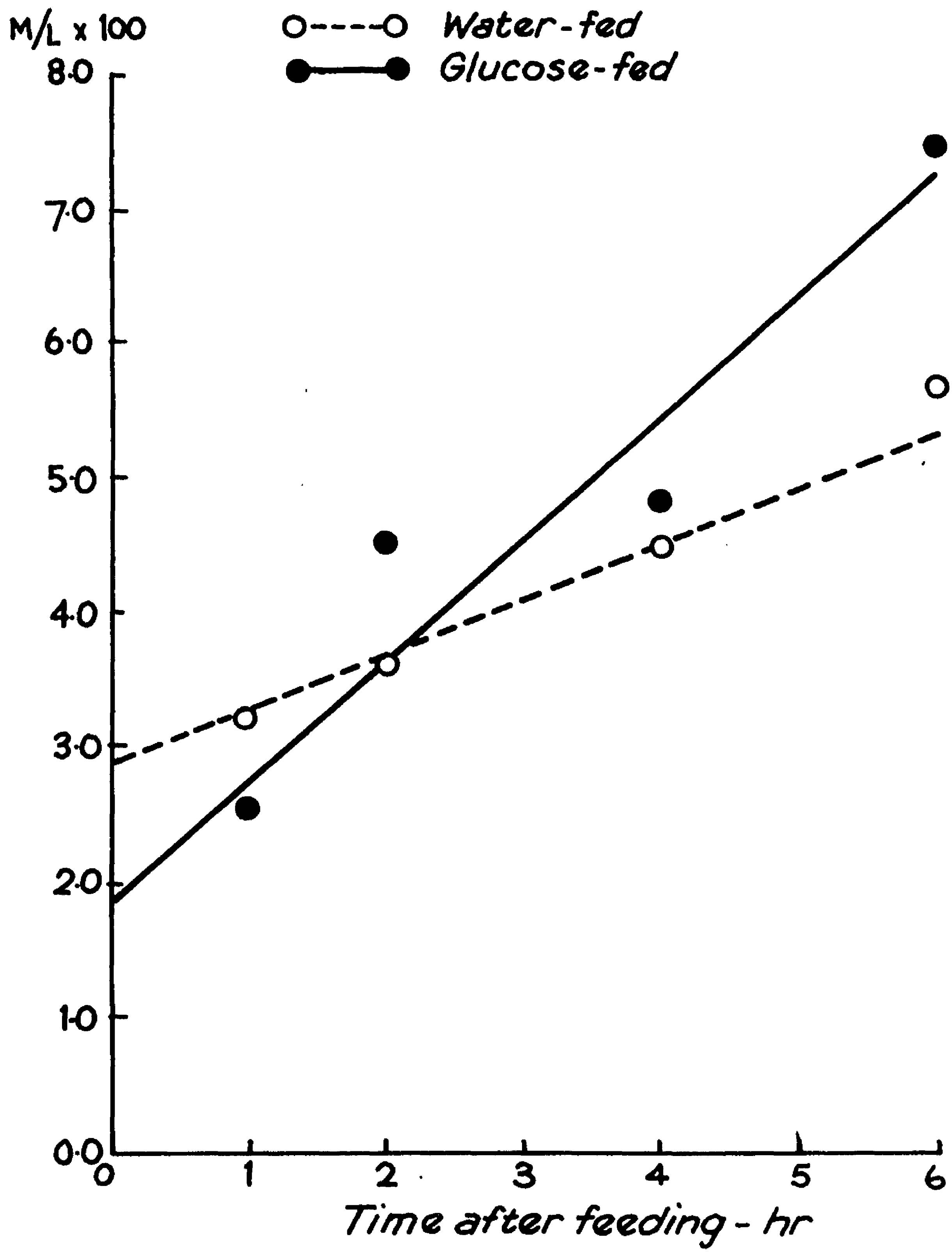

M/L x 100
Water-fed
Glucose-fed
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
1
2
3
4
5
6
Time after feeding - hr

TABLE 11.

The effect of glucose and fat ingestion on the uptake of ^{14}C-glycine by rat liver.

Received	Time after feeding (hrs.)	Specific Activity (Counts/min./100 µg.glycine)		Relative Specific Activity (x 100)
		Protein glycine	Free glycine	
Water	2	284	2076	13.5
	4	239	1224	19.1
	6	280	877	33.6
Glucose	2	218	1493	14.2
	4	240	1011	24.9
	6	288	856	34.1
Fat	2	196	1379	14.5
	4	237	1065	22.0
	6	239	763	34.1

Each entry is the mean of three experiments.

TABLE 12.

The effect of glucose and fat ingestion on the uptake of ^{14}C-glycine by the quadriceps muscle of the rat.

Received	Time after feeding (hrs.)	Specific Activity (Counts/min./100 ug.glycine)		Relative Specific Activity (x 100)
		Protein glycine	Free glycine	
Water	2	15.3	2022	0.91
	4	22.7	1223	1.79
	6	27.6	899	2.98
Glucose	2	11.5	1667	0.67
	4	18.6	1403	1.32
	6	21.6	973	1.68
Fat	2	12.2	1013	1.14
	4	11.1	973	1.05
	6	18.9	885	2.72

from the glycine isolated from muscle protein were very low and we do not propose to lay any weight on them.

The free glycine specific activities are presented graphically in fig.8. The curves in the case of both liver and muscle free glycine follow the usual pattern after a single injection of an isotope, namely, a maximal activity in the tissue fluid shortly after injection, followed by a decline due to removal of the isotope, the decline being initially at least of an exponential character. In the water-fed animals, the decline in specific activity over the 6 hour period is very similar for both liver and muscle pools. After fat administration, the changes in the radioactivity of the muscle and liver pools with time show the same picture. However, in the case of the glucose-fed animals, the changes in the pools of liver and muscle at the various times are quite distinct, for while the level of radioactivity of both liver and muscle pools are initially about the same, the specific activity of the muscle pool is considerably greater at 4 and 6 hours than that of the liver pool. Statistical analysis of the data (Tables 13/13a) reveals that there is a significant difference between the effect of glucose a nd of fat administration on the changes in the radioactivity of the amino-acid pool of muscle. The changes in the liver amino-acid pool after feeding glucose or fat are not significant. In terms of the ratio of the specific

activity/

FIG.8.

SPECIFIC ACTIVITY OF GLYCINE IN THE FREE AMINO ACID POOL

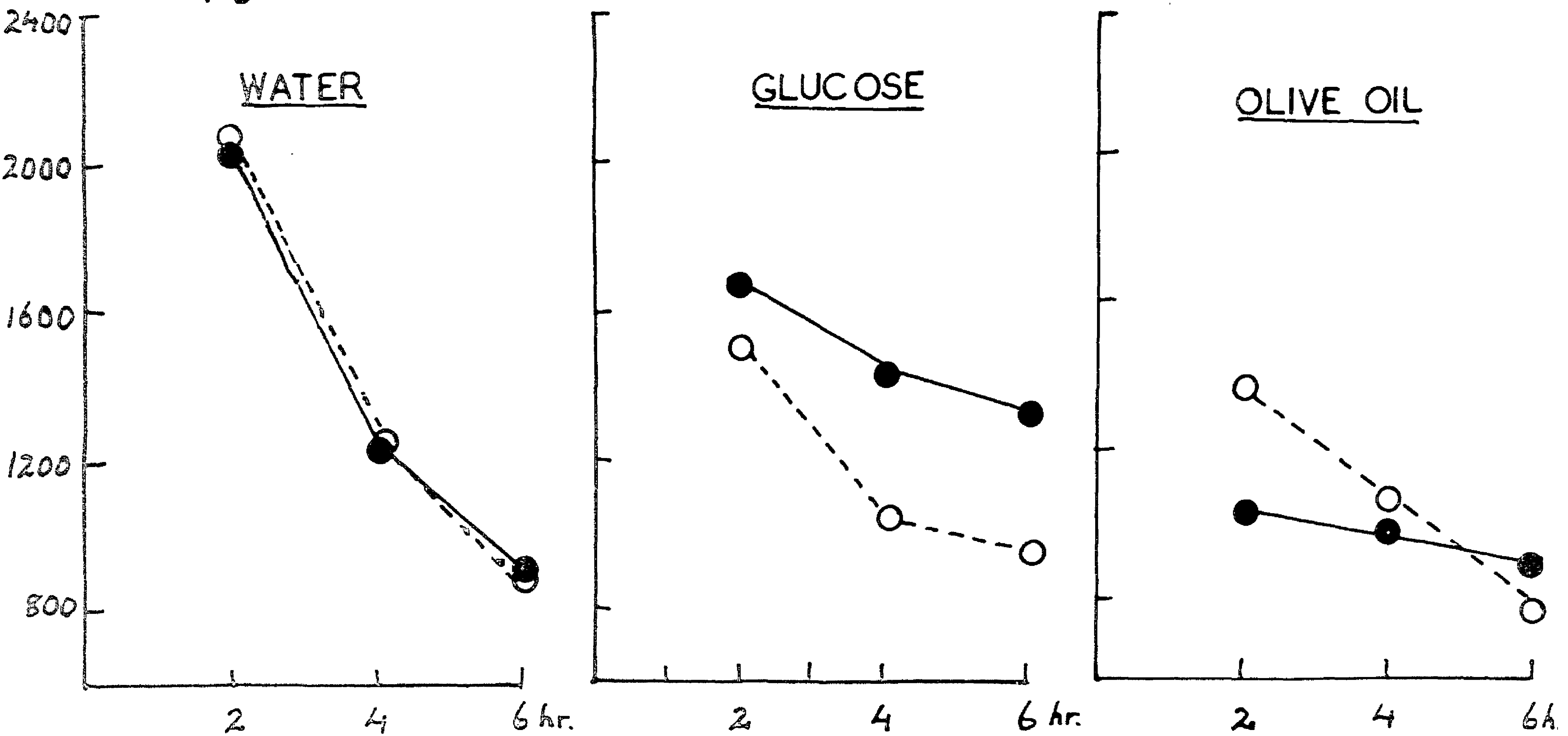


TABLE 13.

Specific activities of free glycine in liver and muscle.

(Specific activity (S.A.) = c.p.m./100 mcg.glycine).

Time after feeding	WATER FED			GLUCOSE FED			OLIVE OIL FED		
	Muscle S.A.	Liver S.A.	Ratio	Muscle S.A.	Liver S.A.	Ratio	Muscle S.A.	Liver S.A.	Ratio
hr.									
2	2022	2076	0.85	1667	1493	1.03	1013	1379	0.74
4	1223	1224	1.05	1403	1011	1.44	973	1065	0.89
6	899	877	1.08	1304	856	1.49	885	763	1.02

Each entry is the mean of 3 experiments.

TABLE 13a.

Analysis of Variance of the changes in radioactivity of the free amino-acid pool of muscle relative to liver given in Table 13, from rats fed either water, glucose, or fat (using 3 substituted figures).

Source of Variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio
Total	26	26,435	-	-
Times	2	5,036	2,518	3.73
Treatment	2	8,609	4,305	5.3
Replicates	2	1,576	788	1.17
Residual	15	12,145	675	-

F = 5.93 at the 1% level of significance for $n_1=2$ and $n_2=18$.

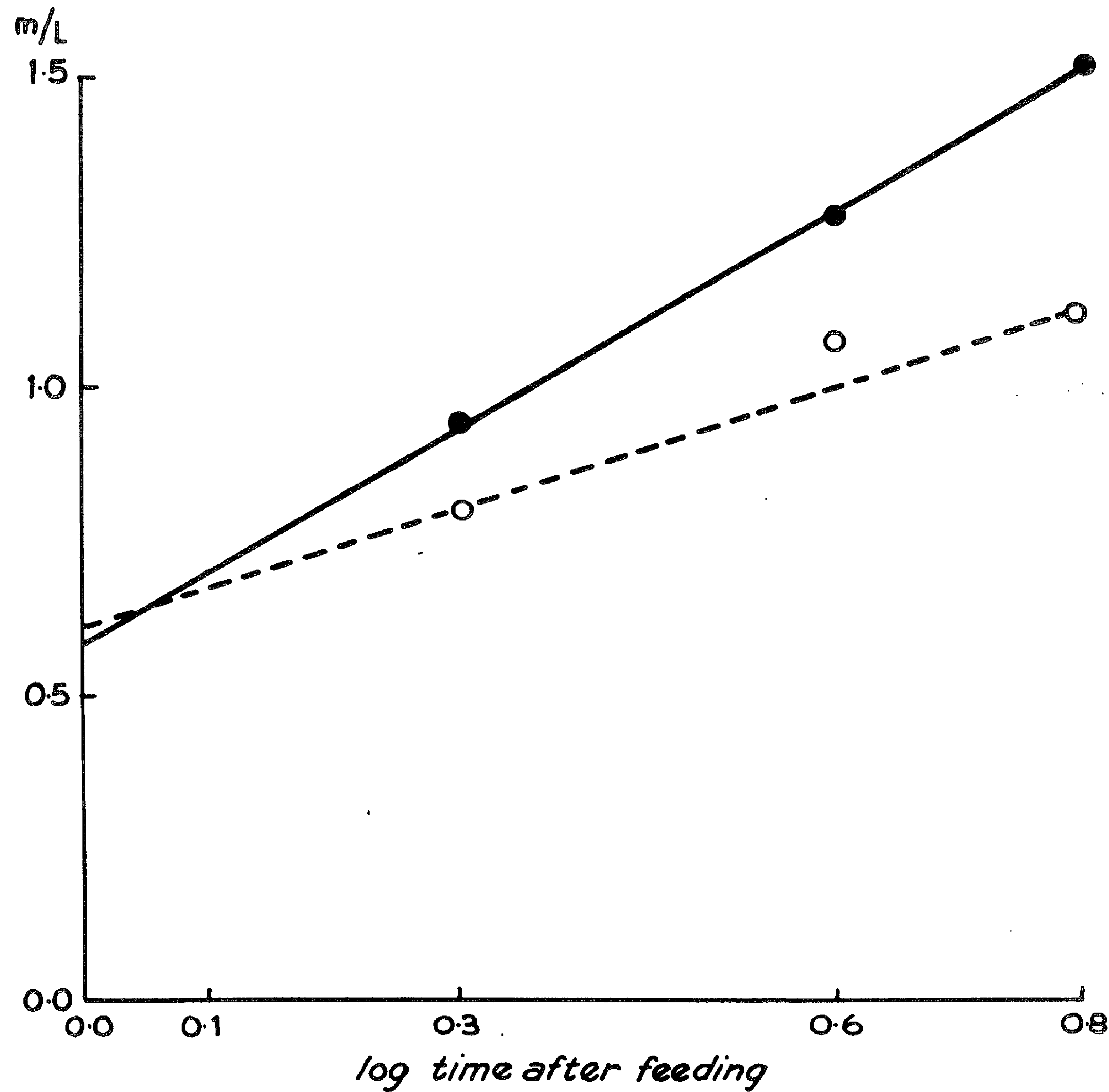
FIG.9.

Changes in radioactivity of muscle free glycine relative to liver free glycine at different times after feeding water (o —— o) or glucose (●——●). The regression equations for the two lines are

Y = 61 + 70 X for the water-fed group and

Y = 58 + 120 X for the glucose-fed group where

X = log.time (hours) after feeding and Y = specific activity of the muscle free glycine expressed as a percentage of specific activity of the liver free glycine. Each point is the mean of 5 observations. Statistical analysis shows that the ratio is significantly greater after feeding glucose ($P < 0.01$).



Water-fed
Glucose-fed
m/L
1·5
1·0
0·5
0·0
0·0
0·1
0·3
0·6
0·8
log time after feeding

activity of muscle free glycine/liver free glycine the action of glucose is to aug ment the ratio about 50% above the level found in the water-fed group at 4 and 6 hours after administration. This picture is confirmed by subsequent estimations on the glycine pools recorded in Section 5. These data will be considered at the appropriate time, but, at the moment, we can express them in fig. 9 as the ratio of muscle to liver specific activities of free glycine. The equations based on these curves, show that the activity at 1 hour (log. 0.0000) is essentially similar in the glucose and water-fed groups.

DISCUSSION.

Previous studies on the changes in the radioactivity of the amino-acid pools of liver and muscle with time have provided incompatible results. Barton (1951) showed that three minutes after the intravenous injection of ^{14}C-glycine into mice, the specific activity of the liver free amino-acid pool was 5 times greater than that of muscle. The activities of the two pools became equal some twenty minutes after injection and the subsequent rates of decline were similar. On the other hand, Henriques et al (1955), found that the labelling in the free pools of liver and muscle from rabbits, which had also been injected intravenously with ^{14}C-glycine, were vastly different, both in respect to the level of radioactivity and their subsequent decline with time. The specific activity of the liver free glycine quickly attained a very high maximum while the muscle free glycine responded much more slowly. This maximum was 2-3 hours after injection and was about 6% of that of the liver. The decline in the liver specific activity was very rapid whereas that of the muscle was much slower, but even after 18 hours the level in the liver was much higher than in the muscle. No record of the changes in the radioactivity of the free pool of rat tissues is available.

It/

It is quite surprising then, that our results, in the case of the water-fed (control) rat, should show that the specific activities of the free pool of liver and muscle are of the same order of magnitude and that the rates of decline are identical in both tissues.

With regard to the action of the administered nutrients on these free glycine pool activities, it is apparent that the only significant changes were those following glucose, which produces a raised free glycine activity in muscle relative to liver, especially at 4-6 hours after administration. Corresponding to this change in the pool, there is greater radioactivity in the protein isolated from the muscles of glucose-fed animals 6 hours after its administration (fig.7). Thus the greater radioactivity of the protein can be attributed to a higher activity in the precursor pool, rather than to a stimulation of protein synthesis following glucose. Presumably the similar changes in the ^{35}S-methionine content of muscle protein 4 and 6 hours after glucose (fig.4) are explained by a parallel change in the free methionine pool activity consequent on glucose administration. In fact, both ^{35}S-methionine (fig.4) and ^{14}C-glycine (fig.7) reveal a depression in muscle protein uptake of amino-acids 1-2 hours after glucose administration. This cannot be attributed to less radioactivity in the free amino-/

amino-acid pool since the glycine data (Tables 11 and 12, and fig.8) show no such depression at 1-2 hours after glucose. Presumably the utilization of A T P for phosphorylation of glucose results in a temporary depletion of available energy for protein synthesis.

From the preceding discussion it is quite clear that there are changes in amino-acid metabolism after glucose administration and that these are limited to muscle. However, these changes in both the protein and amino-acids of muscle are not in evidence until at least 4 hours after the carbohydrate was given. Previous studies under the same experimental conditions (Munro and Thomson, 1953) have shown that the effect of glucose on the blood amino-acid level is much more rapid, the maximal depression occurring 1-2 hours after feeding and returning to the fasting level at 4 hours. Consequently, there must be an intermediate step between the disappearance of the blood amino-acids (1-2 hours) and the changes in radioactivity of the free amino-acid pool (4-6 hours). Indeed, the radioactivity changes coincide with the return of amino-acid levels to normal. On theoretical grounds, it is possible to postulate a mechanism which would account for this. It was already pointed out that the changes in radioactivity in muscle follow the usual pattern for an injected isotope, namely maximal activity in the free pool near the beginning of the experiment, with subsequent/

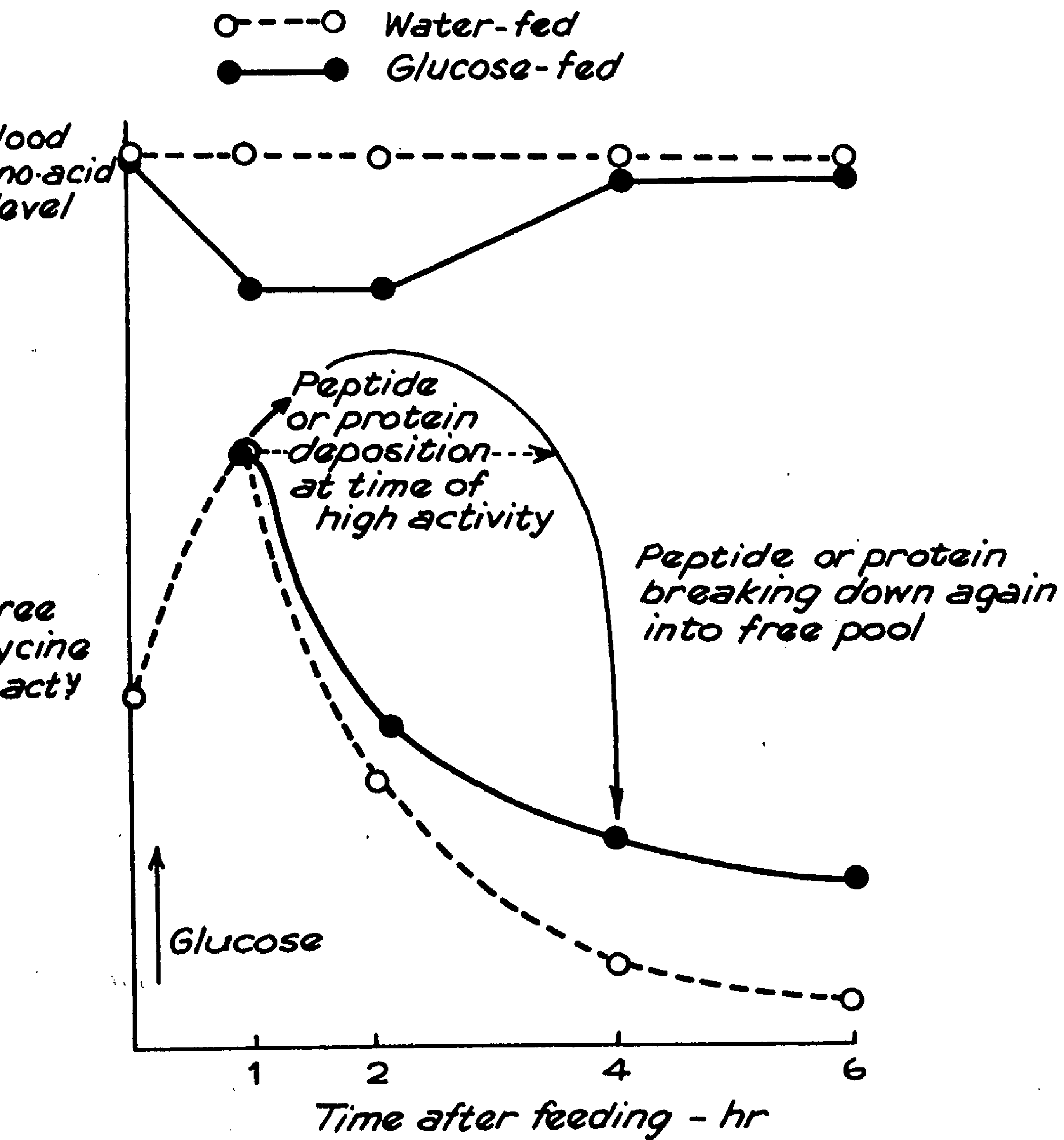


FIG.10. The changes in the blood amino-acid level and free glycine specific activity of muscle after feeding water (o —— o) or glucose (●——●).

subsequent exponential disappearance (fig.8). Thus, if the administration of glucose were to lead to rapid disappearance of amino-acids from the free pool of muscle into some muscle peptide or protein, this material will have a high radioactivity corresponding to the high activity in the free amino-acid pool at the start of the experiment. If, later on, this peptide or protein were to break down again and discharge its amino-acids into the pool, and thence back into the blood stream, it would contribute amino-acids bearing the high radioactivity of the early stages of the experiment and this would keep up the level of radioactivity in the free amino-acid pool of muscle. This conception of the action of glucose is shown diagrammatically in fig.10. This explains why the return of blood amino-acid level to normal coincides with a rise in radioactivity.

In sections 4 and 5 are described experimental studies made in an attempt to identify such peptides or proteins.

SECTION 4.

THE ANALYSIS OF THE ACID-SOLUBLE FRACTION OF MUSCLE AND LIVER AFTER GLUCOSE ADMINISTRATION.

INTRODUCTION.

Nature of the Problem.

Nature of the Problem.

In the preceding section, we put forward a hypothesis to explain the special sparing action of carbohydrate on protein metabolism. This specific mechanism in the fasting animal entails the temporary deposition of amino-acids from the blood stream in skeletal muscle in the form of a labile peptide or protein. Several small peptides do already occur in muscle tissue notably glutathione, anserine and carnosine, but this special peptide or protein must be able to account for all the amino-acids which are removed from the blood stream under the influence of ingested glucose. A good many amino-acids have been studied and all participate to a varying degree in the reduction in the blood level following glucose (Harris and Harris, 1947; Munro and Thomson, 1953); this therefore eliminates the participation of glutathione, anserine and carnosine in this respect.

The extent of amino-N deposition can be calculated from the data provided in section 1, where it was shown (Table 1) that the fasting rat excreted 180 mg. N per 24 hours or 7.5 mg.N per hour, all of which is derived from tissue catabolism. If, for the sake of argument, 50% of these discarded amino-acids are deviated during the 2 hours-period/

period after glucose administration into a labile muscle peptide, this would result in a deposition of 7.5 mg. amino-N in the whole musculature of the body. The skeletal muscle comprises about 40% of the total body weight (Jackson, 1912), or 80 gms. of muscle in a 200 g. rat. Thus, deposition of 7.5 mg. amino-N would represent about 100 µg. N per g. of muscle. The total N content of muscle is about 30 mg. N per g. The deposited amino-N would thus represent about 0.3% of the total N content.

These calculations emphasize the important role which a small change in muscle could play in determining the course of protein metabolism in the body as a whole. From the immediate point of view, they indicate that estimation of total N of muscle after glucose administration would be fruitless as a means of demonstrating amino-N deposition. The only hope is therefore to identify a specific peptide or protein fraction in which such changes are occurring. This section of the work describes examination of the amounts of TCA - and tungstic acid-soluble peptides of muscle following glucose administration.

Examination of non-protein N fractions of muscle for peptides.

An increase of 100 µg. amino-N per g. muscle over the control animal was anticipated as a likely amount to be deposited (see calculation above). Although this amount of peptide material/

material should be readily detectable, the number and size of the peptide molecules which are laid down has considerable bearing on the analysis. The amount of peptide present is calculated as the amino-N difference between hydrolysed and unhydrolysed aliquots of the acid soluble extract. If the amino-N is deposited in the form of a single large peptide molecule, this increased deposition would be readily estimated colorimetrically since it is more than likely that under the experimental conditions only the ends of the molecule will be involved in a coupling reaction. The internal linkages of this large molecule must be ruptured, say by acid hydrolysis, before they are available for colour formation.

On the other hand, should a large number of smaller peptide molecules be produced the difference between the hydrolysed and unhydrolysed aliquots, the peptide amino-N will be much smaller. Also, if a number of smaller peptides do occur, the estimation of the amino-N in the unhydrolysed sample which will be termed "free" amino-N will be falsely high.

Choice of Protein Precipitant.

Before any estimations of amino-N could be made, the choice of the protein precipitatant had to be determined. There are many reagents available for this precipitation, but they do not all act in the same manner so that their extracts may differ to some extent (Hiller and Van Slyke, 1922)/

1922). As we are interested most in the occurrence of peptide material, the reagent which extracts the optimum quantity of this type of compound is the most advantageous for our purpose. The general opinion in the literature is that for peptide extraction trichloro-acetic acid (TCA) is best. (Van Slyke, 1922; Bisset, 1954; Raacke, 1957). Picric acid and tungstic acid give almost as good extractions of peptide material (Van Slyke, 1922; Christensen, 1946). Tungstic acid is by far the best reagent for the extraction of "free" amino-acid. The issue was decided by the fact that TCA had already been used to obtain extracts of liver and muscle in the previous sections.

Method of Amino-N estimation.

The other problem to be settled was the choice of method for the quantitative estimation of amino-N. As it was our intention to include blood amino-N estimations in the initial investigations, the balance was swung in favour of the method first proposed by Folin (1922). This colorimetric procedure was modified by Danielson (1934), Sayhun (1938), Frame Russell and Wilhelmi (1943) and later revised by Russell (1944). The modification employed was essentially that of Danielson (1934)as outlined by Hawk and Oser (1947). It consists of coupling the amino group to a quinone , β- naphthaquinone -4-sulphonate, in alkaline solution and measuring spectrophotometrically the intensity of the reddish brown colour produced, after excess of the quininoid dye has been/

been bleached. The method can be carried out quickly and without difficulty and replicates are in close agreement. As it turned out, the Folin procedure had to be abandoned in favour of the ninhydrin reaction.

General Plan of Experiments.

The search for the form of amino-acid deposition in muscle occurred in three stages (a) The study of free and peptide-bound amino-N in trichloro-acetic acid and tungstic acid extracts.

(b) An attempt, using ion exchange columns, to isolate peptide fractions from the acid-soluble material of muscle.

(c) The abandoning of the Folin reaction in favour of the ninhydrin amino-N reaction and a renewed study with this reaction of the free and peptide amino-N in the acid-soluble fraction of muscle.

A. EXPERIMENTS USING FOLIN'S AMINO NITROGEN PROCEDURE.

Animals and administration of carbohydrate.

Male albino rats were selected and fasted overnight. Animals within a narrow weight range were taken and divided into two groups. According to this division, the rat was then force-fed with either 4 mls. of water, or 4 mls. of a 50% glucose solution. An animal from each group was killed 1,2,4 and 6 hours after feeding, and the whole liver and quadriceps femoris muscles of both rear legs quickly excised.

Estimations of Amino-N in TCA extracts.

The liver was weighed and homogenised in 20 mls. of ice cold 10% TCA. The muscle was briefly minced by hand with scissors, weighed and homogenised in 20 mls. of ice cold 10% TCA. Both homogenates were centrifuged at 1,500 r.p.m. for 10 minutes at $0^{\circ}C$, and the supernatant fluid retained. The protein precipitates were washed twice with 10 mls. of ice cold 10% TCA, the combined supernatant fluid being made up to 50 mls. with distilled water. Samples of the TCA extracts of liver and muscle were neutralised to pH 7 with dilute NaOH and the determination of the free amino-N carried out. Portions of these samples were taken for hydrolysis in 6N.HCl (final conc.) under reflux at $130 \pm 5^{\circ}C$ for 12-14 hours. The HCl was distilled off under reduced pressure and the dry residues, dissolved in distilled water, were estimated for amino-N. Peptide amino-N was calculated as/

as the difference in the amino-N content of hydrolysed and unhydrolysed aliquots.

One of the first difficulties to be encountered was an opalescent liver extract from the glucose-fed animals. This opalescence, due to the deposition of glycogen, increased in proportion to the time which elapsed between feeding and sacrifice. An attempt to remove the glycogen by salivary ptyalin digestion proved unsuccessful. Precipitation of the glycogen by absolute alcohol was more successful but the presence of alcohol was found to reduce the intensity of the colour formed in the amino-N estimation in a non-reproducible way. Subsequently it was found that, when glycogen was added to the liver extract of a water-fed animal in physiological amounts, there was no difference in the optical density of that sample. The presence of glycogen is thus not a serious source of error, presumably because the turbidity in the final coloured solution will be minimised as the liver is assayed for amino-N content at a dilution of 1:50.

A more serious complication, due to the presence of glycogen, is the formation of humin by the condensation of carbohydrate material with amino-acids during acid hydrolysis of the tissue extracts, with a resultant decrease in the amount of amino-N available for estimation. The amount of humin formed is particularly great in the liver extracts of glucose-fed rats. Although the addition of physiological amounts/

amounts of glycogen to the liver extracts of water-fed animals and subsequent hydrolysis showed a slight increase in the amino-N content of the sample, these results are difficult to reconcile with the expected theoretical findings.

<u>Estimation of Amino-N in tungstic acid extracts.</u>

An initial advantage of tungstic acid over TCA is that the tissue extract has a near-neutral pH which simplifies the amino-N estimation. Schurr et al. (1950) have detailed the tissue extraction procedure, the main features of which are as follows:

A weighed portion of liver or muscle in hot water, is heated in a boiling water bath. After cooling, the sample is partially homogenised and a volume of "precipitating reagent" (a freshly prepared mixture of 7 parts of $0.6N.H_2SO_4$ and 5 parts of 10% Na_2WO_4) calculated on the basis of 1.5 mls. per g. liver and 1.2 mls. per g. muscle, is added. Homogenisation is completed and distilled water added to give a 1:10 dilution for liver and a 1:5 dilution for muscle.

Aliquots were taken for total and free amino-N estimations, as before, and the peptide amino-N calculated by difference.

RESULTS.

Amino-N in TCA extracts.

The mean data of four experiments are presented in Table 14. Inspection of the free amino-N from both liver and muscle fails to reveal any gross changes, although glucose administration on the whole seems to reduce the free amino-acid pool. The liver peptide amino-N of the glucose-fed animal can be considered as being unaltered. The muscle peptide amino-N, however, rises to a maximum at 2 hours after feeding glucose and falls finally to below zero level at the sixth hour. The maximum increase of the test over the control at 2 hours is 100 μg. of amino-N per g. of muscle. Such good agreement with the theoretical calculation was very satisfying. However, further study of the possibility of sources of error revealed that TCA had a considerable effect on the colour developed with naphthaquinone. The samples were therefore re-estimated after ether washing to remove TCA and it was found that the presence of TCA raised the apparent content in the free amino-N estimation of the tissue extract (TCA is absent from the hydrolysate, being destroyed during hydrolysis). Interference to this extent throws doubt on all the results, and so the analysis was repeated on tungstic acid extracts.

Amino-N tungstic acid extracts.

The mean data of three experiments are presented in Table 15. The first point worthy of attention is that the extraction/

TABLE 14.

The amino-N estimations on TCA extracts of liver and muscle by Folin's method.

Fraction	Time after feeding (hrs.)	MUSCLE (µg. amino-N/g)		LIVER (µg. amino-N/g)		Difference on feeding glucose	
		Water fed	Glucose fed	Water fed	Glucose fed	Muscle	Liver
Free Amino N	1	596	602	454	485	+ 6	+ 31
	2	559	536	465	439	- 23	- 26
	4	568	546	481	426	- 22	- 55
	6	583	573	505	443	- 10	- 62
Peptide Amino N	1	570	618	452	429	+ 48	- 23
	2	561	661	437	436	+100	- 1
	4	560	560	392	378	± 0	- 14
	6	649	417	454	399	-232	- 55
Total Amino N	1	1166	1219	906	914	+ 53	+ 8
	2	1120	1196	902	875	+ 76	- 27
	4	1127	1104	873	804	- 23	- 69
	6	1232	989	959	842	-243	-117

Each entry is the mean of 4 experiments.

TABLE 15.

The amino-N estimations on tungstic acid extracts of liver and muscle by Folin's method.

Fraction	Time after feeding (hrs.)	MUSCLE (µg.amino-N/g)		LIVER (µg.amino-N/g)		Difference on feeding glucose	
		Water fed	Glucose fed	Water fed	Glucose fed	Muscle	Liver
Free Amino N	1	733	727	689	633	- 6	- 56
	2	667	758	618	637	+ 91	+ 19
	4	750	715	641	606	- 35	- 35
	6	760	672	642	570	- 88	- 72
Peptide Amino N	1	891	977	663	685	+ 86	+ 22
	2	894	877	764	808	- 7	+ 44
	4	807	834	664	676	+ 27	+ 12
	6	873	845	658	597	- 28	- 61
Total Amino N	1	1624	1704	1352	1318	+ 80	- 34
	2	1561	1635	1382	1446	+ 74	+ 64
	4	1556	1551	1305	1281	- 5	- 24
	6	1633	1517	1300	1167	-116	-133

Each entry is the mean of 3 experiments.

extraction of free amino-acids from both liver and muscle is more complete than was achieved withTCA. The total amino-N level is also increased, so that there is a corresponding increase in the peptide amino-N content of liver and muscle. The fact that tungstic acid is more efficient than TCA in extracting amino-acids is in agreement with the findings of Van Slyke (1922) and Raacke (1957).

The results for the various liver fractions bear out those in Table 14. i.e. that a glucose-fed animal does not differ from a control animal in its ability to accumulate amino-acids either in the pool or as aggregates in the liver. The muscle free amino-N picture differs slightly from that previously found (Table 14) in that amino-acids accumulate to a small extent 2 hours after feeding glucose. The time for the maximal occurrence of peptide amino-N has changed from 2 hours to 1 hour after feeding glucose. The results are somewhat erratic and do not reach a significant level. The total amino-N content of muscle does, however, pursue a pattern of greater regularity, being raised at 1 and 2 hours after glucose administration and falling at 4 and 6 hours thereafter. The data for total amino-N is thus very similar in both the TCA extract (Table 14) and the tungstic acid extract (Table 15)

The problem is really a question of finding a 100 µg. of amino-N in a total content of about 1500 µg. Spontaneous variations make this search rather difficult, hence we turned to the use of ion exchange columns to try to concentrate the active fraction.

B. THE USE OF ION EXCHANGE RESINS TO DETECT PEPTIDE ACCUMULATION IN MUSCLE.

Partridge (1952) developed the use of ion exchange columns as molecular sieves for separating the components of biological extracts. Under conditions of low pH (about 1 all the amino-acids and peptides are positively charged, whereas nucleotides are negatively charged and carbohydrate molecules are uncharged. A highly cross-linked cation-exchanging resin will retain amino-acids and small peptides when used under acid conditions while nucleotides and carbohydrate molecules will be allowed to pass through unhindered. Large peptides, however, will also appear in the eluate from the column as their molecular size prevents adsorption. If, in fact, a special peptide is present in the acid-soluble extract of muscle from a glucose-fed animal, it will either be adsorbed on to the resin or allowed to pass through depending on its molecular size. The separation of a peptide molecule from the other constituents of the column eluate could be accomplished by passing the effluent through a second ion exchange resin of the anion type, whereas the resolution of a peptide adsorbed by the column would require a gradient elution. However, the initial gross separation of types and quantitative analyses would indicate whether further investigations would be necessary.

EXPERIMENTAL.

Animals and treatment of tissues.

Water and glucose-fed rats were used as above and tungstic acid extracts of liver and muscle were prepared by the method of Schurr et al. (1950).

Preparation of Cation Exchange Column. (Cohn, 1950).

Dowex-50 x 12 resin, a sulphonated polystyrene resin with 12% cross linking, was employed. After initial water washings and decantations to free resin of fines, the resin was slurried into the column, 1 cm. in diameter with a pad of cotton wool to prevent resinous particles appearing in the eluate, and packed to a height of 4 cms. In this way, disruption of the column by bubbles of air was prevented. The column was washed alternately with acidic and basic solutions of stronger eluting power than any which were to be used in the actual experiment. The column was finally washed with 0.1N.HCl until the pH of the effluent reached 1. Then a tungstic acid extract of muscle (also at pH1) from a water or glucose-fed rat, was applied. The column was finally washed with 0.01N.HCl and the eluate collected. Under these conditions, amino-acids and small peptides should be retained, whereas larger peptides should pass through.

Efficiency of Cation Column.

A column, prepared as described above, was used and a solution containing the amino-acids aspartic acid and serine, and/

and the peptides, glutathione and glycyl-dl-leucine, applied. The eluate was collected and a chromatographic comparison with the test solution before passage through the column showed that it had retained all the constituents of the test solution. Under these experimental conditions therefore, peptides as large as the naturally occurring glutathione, are efficiently retained. The amino-acids, aspartic acid and serine, which are the amino-acids most easily dislodged from such a column, because of their small pK values, are also retained.

Examination of Eluate of Cation Column.

A qualitative ninhydrin test on the eluate from the cation column was positive, indicating that a large peptide or group of peptides is present. The unhydrolysed eluate was then subjected to two dimensional chromatography in butanol-acetic acid - water and phenol - ammonia - water. This revealed a single spot in the glycine-serine area. If this is due to a peptide molecule, it implies that only one peptide is present in the eluate. A quantitative estimation of free amino-N by Folin's naphthaquinone method on the eluate showed that about 200 µg. of amino-N per g. of original muscle were present. Following hydrolysis there was a considerable increase in amino-N which was at first interpreted as evidence of the presence of peptide material but on chromatography of the hydrolysate the original spot at the glycine-serine position persisted and no further spots appeared./

appeared. Thus the increase in amino-N is not due to the liberation of amino-acids from peptide linkage. The probability that the spot is a free amino-acid, which has slipped through the column, is unlikely since the efficiency of the column was already verified and also since the first amino-acid to be eluted would be aspartic acid which occupies an entirely different position on the chromatogram.

Although the eluate had been shown to be free of peptide material, it was decided to undertake a brief investigation into the nature of the compound responsible for the ninhydrin positive area on the chromatogram.

The fact that the chromatographic position of the spot is unaltered after hydrolysis dismissed the possibility of the ninhydrin positive area being phosphoserine since serine and phosphoserine occupy different positions (Dent, 1948) and serine would be liberated from phosphoserine under our conditions of hydrolysis. Unidimensional chromatograms, developed for a considerable time in butanol-acetic acid-water showed decisively that neither an amino-acid-nucleotide complex nor the fructose-amino acid complexes reported by Borsook (1955) were responsible for the ninhydrin-positive area, since there were no coincidental ultraviolet absorbing areas or positive sugar reactions on the chromatograms. Derivatives of creatine, such as creatine phosphate, which could occur in the acid effluent, were dismissed as/

as being responsible for the rise in amino-N after hydrolysis, when it was found that a dilute solution of creatine developed only a slight colour when reacted with Folin's naphthaquinone reagent.

The absence of carnosine phosphate from the cation column eluate was demonstrated by a negative histidine test (Macpherson's Modification - Bolling & Block, 1951) although an authentic specimen of carnosine itself gave a strongly positive reaction to this test. It is extremely unlikely that a peptide consisting of numerous glycine and/or serine molecules is responsible for the increased amino-N after hydrolysis, as such a peptide would not account for the many amino-acids which are known to disappear from the blood on the administration of glucose, (Thomson and Munro, 1953).

Hence the increase in amino-N in the column eluate, which was initially considered to be due to the liberation of amino-acid from peptide linkages, must be caused by interference from other compounds present in the effluent of the cation exchange column.

Interference of Nucleotides with Folin's Amino-N estimation.

As we have already explained, nucleotides occur in the acid effluent of the cation column and are therefore potentially capable of coupling with naphthaquinone. Since carbohydrate derivatives are also present, solutions of inosine monophosphate (IMP) and adenine monophosphate (AMP) in/

in the presence and absence of glucose were examined as regards their ability to couple with the naphthaquinone reagent. The extent of such a reaction was found to be negligible. After acid hydrolysis, however, there was a considerable reaction between the breakdown products of both AMP and IMP and β -naphthaquinone-4-sulphonate. This coupling reaction produced 75%, in the case of AMP, and 50%, in the case of IMP, of the colour formed by 1 μ mole. of amino-N, and was slightly increased (to 85% for AMP) on hydrolysis in the presence of glycogen. The extent of this reaction is much greater than can be accounted for by the degradation of the purine ring to glycine and its coupling with the quinone. Hence the ability of a hydrolysed nucleotide to react with Folin's reagent is the cause of the fallacious amino-N increment in the acid eluate of the cation exchange column. This offers a possible explanation for the greater amount of "peptide" (Folin's reagent) in acid-soluble extracts of muscles obtained from glucose-fed rats, since the extra carbohydrate deposited would lead to an increase in naphthaquinone-reacting products produced from the purine nucleus on hydrolysis.

The absence of peptide material in the acid eluate from the cation column limits the possible occurrence of peptide to the fraction of the muscle extract adsorbed on the/

the column, and studies on this fraction will now be described.

The elution of adsorbed amino-acids and peptides from the cation exchange column.

The removal of the adsorbed compounds from the cation column can be accomplished by washing the resin with any weak solution of a base. In the first instance we used dilute (0.1N) ammonia. Washing was continued with NH_4OH until the pH rose to 9 . The eluate was collected throughout. A portion was hydrolysed and amino-N was determined on the unhydrolysed and hydrolysed specimens. Fig.11 gives the results of a typical run, in which the amino-N put on to the column (both in free and peptide form) is compared with the corresponding data for the material passing through and the material adsorbed and subsequently eluted with NH_4OH. The figures (μg. amino-N/g. wet weight) refer to animals which were fed with water or with glucose 1 hour before killing. In the control animal, the sum of the free amino-N of the eluate and adsorbed fraction is greater than the free amino-N of the untreated sample. This is possibly due to the incomplete removal of NH_3 used in the elution of the adsorbed fraction. In the glucose-fed animal, the sum of the free amino-N of the two fractions is less than that of the untreated sample which may be due to incomplete recovery of the adsorbed material from the column. As regards the total amino-N/

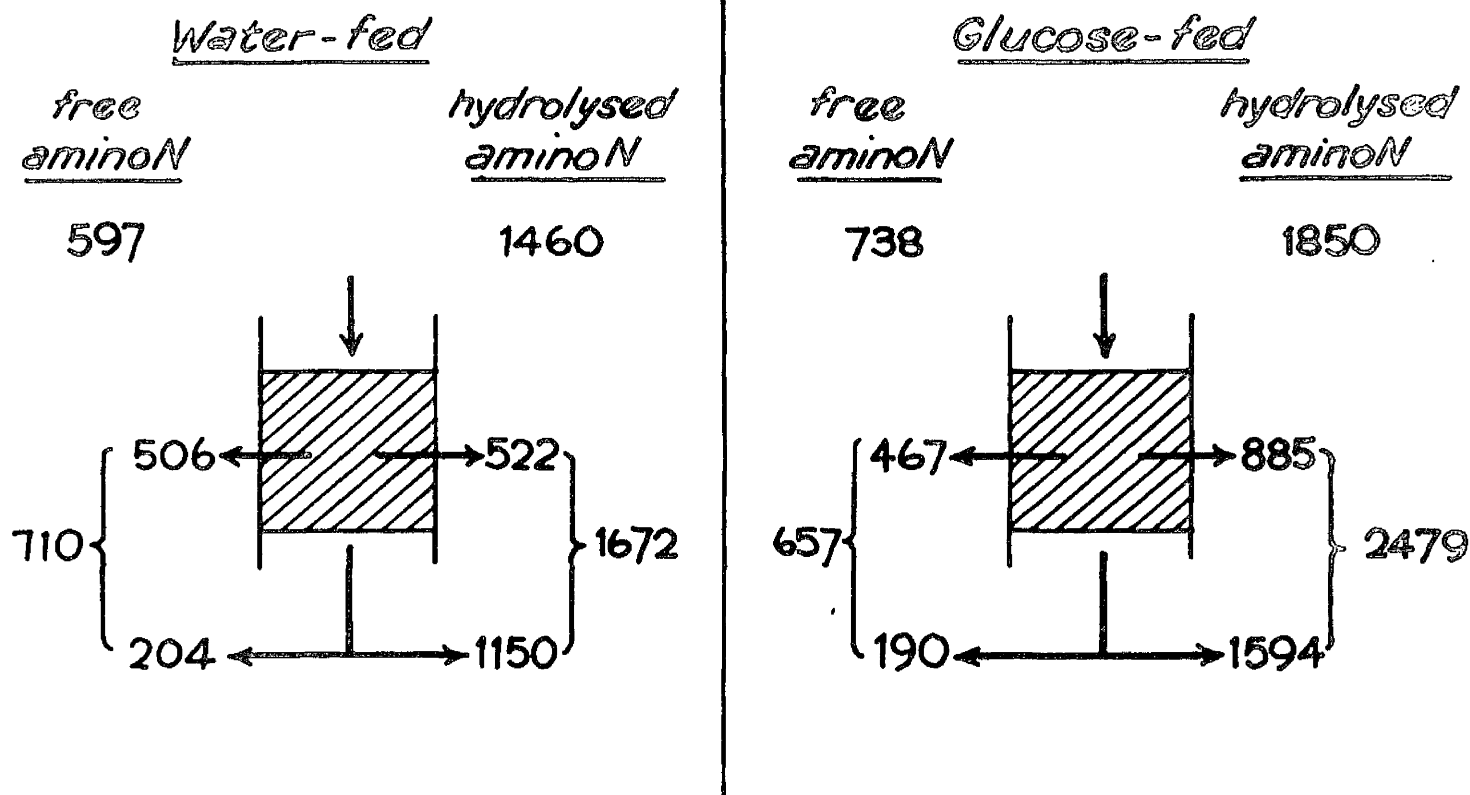


FIG.11.

The cross- hatched area represents the dowex cation exchange resin used to separate the components of the acid-soluble fraction of muscle from water-fed or glucose-fed rats. The free and total amino-N content of the extract were determined by Folin's amino-N method prior to passage through the column. The amino-N content of the eluate and the adsorbed fraction eluted by NH_4OH was determined before and after acid hydrolysis and the quantities obtained in this way compared with the amounts before the extract was passed through the column.

amino-N of both fractions, the sum is greater than the corresponding total amino-N of the samples prior to passage through the column. These results may have been due to certain undesirable complications in technique. Firstly, it was found that a very large volume of the dilute ammonia (0.1N) was required to remove completely all material from the column, and the exact location of the point of complete removal was obscured by the interaction of ammonia with the quantitative ninhydrin test. Secondly, the solvent was found to react with Folin's reagent to a large extent. Repeated estimations on standard NH_4Cl solutions showed that 1 m.mole. gave 80% of the colour of 1 m.mole. of amino-N. The removal of ammonia from the effluents posed a difficult question. The recognised manner of removing ammonia by boiling in alkaline solution gave rather variable amino-N results even when the boiling time was prolonged which suggest that the ammonia was not being completely driven off. The most efficient mode of removal by repeated desiccation over concentrated H_2SO_4 required such a considerable time as to become too tedious for the number of samples to be analysed. Thus a change of solvent was indicated and we turned to the use of dilute caustic soda which should remove the adsorbed fraction in a more concentrated solution and should not interfere with the subsequent amino-N estimations.

The tissue extract was prepared and run through the column as before. The adsorbed fraction was eluted with dilute/

dilute (0.1N) NaOH. The results are presented diagrammatically in fig.12. The analyses of free amino-N were, on the whole, in good agreement with those obtained from the untreated samples. Humin formation was not present during acid hydrolysis of the eluate because glycogen was absent from this material. Nevertheless analysis for total amino-N gave figures which were extremely high. The cause of these very peculiar results is entirely a matter for conjecture as no credible explanation can be advanced.

Thus the information gained from using ion exchange resins as molecular sieves failed to confirm or deny if any peptide amino-N, peculiar to a glucose-fed animal, was being deposited in the acid-soluble fraction of quadriceps muscle. Poor replicability and the discovery of substances interfering with the quantitative estimation, provide enough ground for abandoning Folin's naphthaquinone method and selecting a more specific method of amino-N determination. This is described now.

C. THE USE OF NINHYDRIN FOR AMINO NITROGEN ESTIMATIONS ON MUSCLE EXTRACTS.

One of the more specific reagents for the estimation of amino-acid-N is ninhydrin. It has been employed in two capacities. Firstly, its ability to quantitatively decarboxylate amino-acids has made this method of estimation the most specific available. The analytical problems of this/

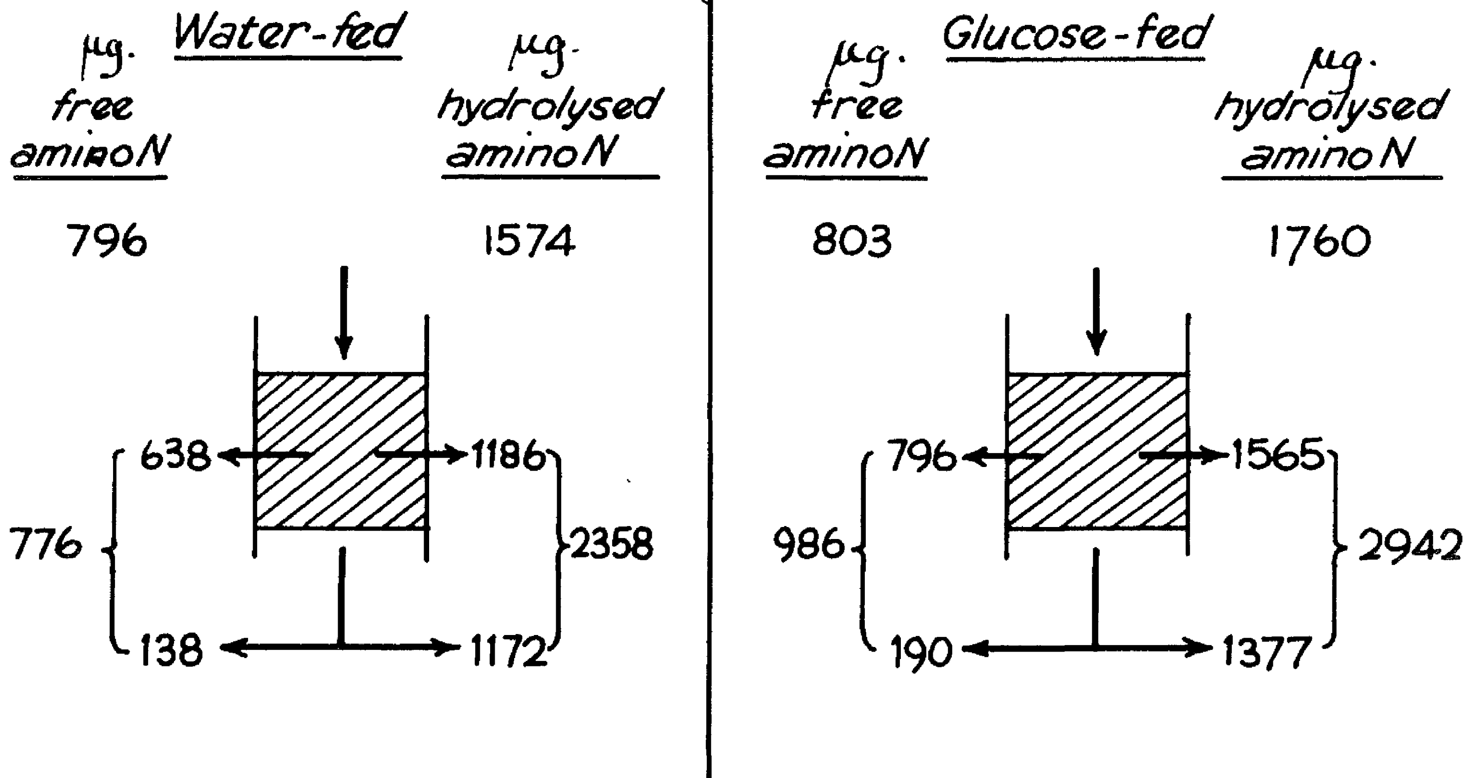


FIG.12. The cross-hatched area represents the dowex cation exchange resin used to separate the components of the acid-soluble fraction of muscle from water-fed or glucose-fed rats. The free and total amino-N content of the extract were determined by Folin's amino-N method prior to passage through the column. The amino-N content of the eluate and the adsorbed fraction eluted by NaOH was determined before and after acid hydrolysis and the quantities obtained in this way compared with the amounts before the extract was passed through the column.

this as a gasometric procedure were solved by Van Slyke et al (1941) and this method was successfully applied to plasma amino-N estimations (Hamilton and Van Slyke, 1943). Barlett and Glynn (1950). Flock et al.(1953), Babson and Winnick (1954) have all used this method successfully on tissue extracts. The liberated CO_2 can be measured in two ways, the titrimetric method being employed in our investigations, since the manometric one is rather more complicated.

In the second place, the ability of ninhydrin to condense with amino-acids to form an intensely coloured product, led to many investigations into the quantitative aspects of the problem. (Moore and Stein, 1948;1954; Troll and Cannon, 1953; Yemm and Cocking, 1955; Yanari, 1956; Meyer, 1957). The modification of Yemm and Cocking (1955) was selected, and although it was primarily designed for the amino-N analysis of column eluates, its application to whole extracts of tissues gave surprisingly consistent results in the majority of cases.

EXPERIMENTAL.

Estimation by the titrimetric procedure.

The choice of the protein precipitating reagent is more limited, as TCA is itself decarboxylated under the experimental conditions. Tungstic acid was given preference over picric acid as it gives the more quantitative extraction of/

of free amino-acids (Van Slyke, 1922).

Animals, feeding, and preparation of extracts were carried out using rats fed either water or glucose as previously described.

Amino-N estimation. Analysis of the free amino-N and total amino-N were performed on extracts containing the equivalent of 1.0 gm. and 0.5 gm. of liver and muscle respectively. The salient features of the analysis are as follows: The pH of the sample is adjusted to 2.5-3.0, and 100 mgms. of citrate buffer (pH = 2.8) added. After boiling to remove atmospheric CO_2, the flask is stoppered and cooled to below 5^oC, when 100 mgms. of recrystallised ninhydrin is added. This flask, and a similar one containing $Ba(OH)_2$, is quickly connected to a special U tube which is immediately evacuated at the water pump. The blank, standard and test solutions are brought to this stage before being boiled together in a rapidly boiling water bath. The distillation of the liberated CO_2 is accomplished by placing the flask containing the $Ba(OH)_2$ in a cold water bath, while continuing to heat the other. The apparatus is cooled before admitting CO_2-free air. The amount of CO_2 liberated and hence the amino-N content of the sample, is estimated by a back titration with standard acid.

Estimation by the colorimetric procedure.

The experiments with this method were carried out in connection with the investigations described in section 5, and,/

and, for the sake of comparison, are included here. Quantitative extracts of liver and muscle were prepared using ice-cold 10% TCA (as similarly described under Folin's amino-N estimations).

Contrary to the findings of Meyer (1957), TCA was found to promote the formation of the amino-acid-ninhydrin complex. Removal of TCA was accomplished by washing the test solutions with ether until the pH rose to 5. This washing was accompanied by a considerable diminution in volume, but after restoring the volume to its original level, the amino-N content of the sample was determined. The interference of TCA (final concentration 1%) was assessed by using hydrolysed muscle extracts <u>in lieu</u> of amino-acid mixtures. Table 16 reports the data and shows decisively that the ether washings effectively remove the TCA without decreasing the amino-N content of the sample. In consequence, the TCA was removed from the extracts before free amino-N analyses were carried out.

The main features of the method of Yemm and Cocking are buffering the solution containing 0.5 - 5.0 μg. amino-N with 0.2M citrate buffer (pH = 5.1) and adding the ninhydrin in a solution of methyl cellosolve which also contains a trace of KCN. After heating in a vigorously boiling water bath for exactly 15 minutes, the capped tubes are cooled and diluted to a suitable volume. The intensity of the blue solution/

TABLE 16.

The effect of various treatments on the amino-N content of hydrolysed muscle extracts (colorimetric ninhydrin procedure).

Treatment	µg.amino-N/g.sample.			
	1	2	3	4
No ether washing No TCA addition	510	601	498	507
No ether washing + 1% TCA(final conc.)	754	708	642	713
Ether washing but No TCA addition	510	587	426	465
Washing after the addition of TCA	506	561	472	469

solution is determined spectrophotometrically. It was found advisable to include two blank estimations and three tubes containing varying amounts of a standard amino-acid solution.

RESULTS.

By the titrimetic method. The mean data of two experiments are presented in Table 17. The most notable feature of the results is that all the estimates of the various liver and muscle fractions are considerably lower than those already reported in Tables 14 and 15. The impression obtained from the muscle data is that glucose generally depresses the amino-N content of the various fractions. There is no evidence of peptide amino-N accumulation after glucose ingestion. A similar conclusion can be drawn for the liver data when it is realised that the high peptide amino-N figures at the 1st. and 2nd. hour are probably due to unreliable estimations.

The level of amino-N, both free and total, is more compatible with the data in the literature than our findings with Folin's reagent (Table 15). The free amino-N in muscle has been reported as 192 µg. per gm. (Friedberg and Greenberg, 1947) with which our data agree. On the other hand Babson and Winnick (1954) estimated the free amino-N of muscle as 330 µg.per gm., while Barlett & Glynn found 340 µg. per gm. The liver

free/

TABLE 17.

Estimation of the amino-N content of the acid soluble extracts of liver and muscle by the titrimetric decarboxylation technique.

Fraction	Time after feeding (hrs.)	MUSCLE (µg.amino-N/g)		LIVER (µg.Amino-N/g)		Difference due to feeding glucose.	
		Water fed	Glucose fed	Water fed	Glucose fed	MUSCLE	LIVER
FREE Amino N	1	202	189	417	415	-13	- 2
	2	197	181	393	324*	-16	-69
	4	191	213	400	413	+22	+13
	6	194	190	402	419	- 4	+17
PEPTIDE Amino N	1	342	304	270	342	-38	+72
	2	239	275	259	317	+36	+58
	4	279	256	253	235	-23	-18
	6	208	199	174	125	- 9	-49
TOTAL Amino N	1	544	493	687	757*	-51	+71
	2	435	456	602	641	+21	+39
	4	470	469	653	648	- 1	- 5
	6	402	389	576	543	-13	-33

Each entry is the mean of two experiments.

* These estimations are technically suspect.

free amino-N results are more consistent. Babson and Winnick (1954) reported the level as 360 μg. per gm. ~~while Ferrari and Harkness ( ) found 413 ug. per gm.~~, and this accords with the data given in Table 17.

Thus ~~although~~ the data are now of the correct order of magnitude, and we may assume that there are no interfering substances present. Under these circumstances, the results fail to reveal the presence of an acid-soluble peptide in muscle after glucose administration and we are prepared to accept this verdict.

By the colorimetric method. These experiments were carried out on TCA extracts. The mean result of two experiments is presented in Table 18. The total amino-N estimates of both liver and muscle at 1 hour are technically unreliable so that the accumulation of peptide amino-N, especially in the case of the liver, is doubtful. Otherwise the results as a whole fail to reveal any increment in muscle peptide amino-N after glucose administration.

The lower capacity of TCA to extract amino-acids is probably compensated by a less specific amino-N estimation and in consequence there is close agreement between the results of this method and those of the decarboxylation technique. Table 19 provides more comparable results of the three methods we have been using (Folin's naphthaquinone method, the titrimetric and colorimetric ninhydrin methods) on/

TABLE 18.

Colorimetric ninhydrin estimations of the amino-N content of the acid soluble fractions of liver and muscle. The muscle and liver were extracted with trichloro-acetic acid.

Fraction	Time after feeding (hrs.)	MUSCLE (µg.amino-N/g)		LIVER (µg.amino-N/g)		Difference due to feeding glucose.	
		Water fed	glucose fed	Water fed	Glucose fed	MUSCLE	LIVER
FREE Amino N	1	261	292	379	402	+31	+23
	2	262	257	419	423	- 5	+ 4
	4	262	261	390	391	- 1	+ 1
	6	263	246	378	406	-17	+28
PEPTIDE Amino N	1	166	195	208	280	+29	+72
	2	146	147	248	294	+ 1	+46
	4	171	169	282	160	- 2	-122
	6	176	160	245	172	-16	-73
TOTAL Amino N	1	427	487	587	682	+60	+95
	2	408	404	667	717	- 4	+50
	4	432	430	672	551	- 2	-121
	6	439	406	623	577	-33	-46

Each entry is the mean of two experiments.

TABLE 19.

A comparison of the free amino-N content of tungstic acid extracts of muscle.

Treatment	Time after feeding (hrs.)	Ninhydrin decarboxy-lation (mean of 2)	Ninhydrin colorimetric (mean of 2)	Folin's naphtha-quinone (single)
Water	1	221	326	798
Glucose		189	238*	798
Water	2	212	296	746
Glucose		212	294	756
Water	4	220	297	744
Glucose		224	291	744

* a single estimation.

on the same tungstic acid extracts of muscle. It can be seen that, of the two colorimetric procedures, the ninhydrin one is by far the more specific, and that the results by this method approach the level obtained by the decarboxylation technique.

DISCUSSION.

The initial studies by Folin's method on TCA and tungstic acid extracts of muscle added weight to the theory that an acid-soluble peptide was deposited in muscle after feeding glucose. Our expectations were destroyed, however, when the findings of the ion exchange columns revealed that substances other than non-protein N compounds were reacting with Folin's reagent and tending to obscure any peptide accumulation. Subsequent amino-N analysis on the acid-soluble fraction of muscle using the more specific reagent, ninhydrin, showed quite decisively that glucose administration did not result in a temporary deposition of a peptide fraction in the acid-soluble portion of muscle. Therefore, since the more reliable estimations had failed to confirm the presence of a special peptide in muscle, we turned to study the possibility of deposition of amino-acids in other muscle fractions. The final section of this thesis describes these studies.

SECTION 5.

THE EXAMINATION OF LIVER AND MUSCLE PROTEIN AFTER GLUCOSE ADMINISTRATION.

INTRODUCTION.

Having failed to obtain evidence of peptide accumulation in the acid-soluble fraction of muscle after glucose administration, we reconsidered other possible methods of amino-acid deposition. The treatment of the homogenate for preparing pure muscle protein samples (see fig.3) was critically evaluated. It appeared to us that two procedures namely heating in TCA and dissolving in NaOH, were possible stages where a labile fraction of the muscle protein might be removed and inadvertently discarded. This period of heating in TCA was introduced as a mild but unspecific way of removing nucleic acids, as their soluble degradation products, from the mass of precipitated protein (Schneider, 1945). A hydrolysis of this nature might easily remove a labile accumulation of amino-acids.

In order to test the hypothesis that a labile fraction was being discarded from the protein of a glucose-fed animal during its "purification", the muscle and liver proteins were treated in one of two ways which either omitted or included the treatment by heating in TCA and dissolving in NaOH. The radioactivities of the proteins prepared by these two treatments were compared with each other. In addition, the loss of amino-N and total N during heating with TCA was compared in the water-fed and glucose-fed rats.

EXPERIMENTAL.

Animals.

Male albino rats, about 200 gms.,were fasted overnight and injected intraperitoneally with 20 μc. of ^{14}C-2-glycine in 1.0 ml. of 0.9% saline. After a lapse of 30 minutes, rats were fed with 4 mls. of water (control) and others with 4 mls. of a 50% glucose solution. In the first series of experiments, animals were killed at only 1 hour after feeding, since this time-interval seemed likely to provide the best evidence of changes following glucose administration, when amino-acids are disappearing from the blood. In subsequent experiments, an animal from each dietary group was killed at 1,2,4 and 6 hours after feeding.

In a later series of experiments, fasting rats were injected intraperitoneally with 10 μc. of ^{14}C-1-leucine and fed as above. All these animals were killed 1 hour after feeding.

Treatment of Tissues.

The animals were killed by exsanguination under ether anaesthesia. The whole liver and quadriceps femoris muscles from both rear legs were excised and from each tissue two portions were taken, from one of which protein was prepared by treating with hot TCA and NaOH, as in previous studies (fig.3) (rigorous treatment) and the other was not subjected to/

to this procedure (mild treatment). As shown diagrammatically in Fig. 13, both portions were initially treated in the same way by homogenising in a Nelco blender with 20 mls. of ice-cold 10% TCA. and the protein precipitate washed twice with 10 mls. of 10% TCA. The supernatant fluids from the ^{14}C-glycine series were retained and analysed for radioactivity in the free amino-acid pool, as described in section 3.

The "mild treatment" protein precipitate was then directly washed with the fat solvents. The "rigorous treatment" protein precipitates were heated in 20.0 mls. of 10% TCA (the supernatant being retained for total and amino-N analysis), dissolved in 20 mls. of 0.4 N.NaOH and reprecipitated with 10 mls. of 30% TCA. This latter step was repeated, and the protein was then washed with the fat solvents. The dry proteins were counted at infinite thickness in a standard planchet in a Geiger end window counter and the effects of the mild and rigorous treatments compared. Analysis on the hot TCA extract was carried out by the micro-Kjeldhal method (see section 1) and by the amino-N method of Yemm and Cocking (see section 4), following hydrolysis for 12-14 hours at 105°C in 6N.HCl.

Uptake of ^{14}C-leucine by subcellular fractions of muscle.

A subcellular fractionation was made in one instance on the carcass muscle of water-fed and glucose-fed rats which had been injected with ^{14}C-leucine. The muscle homogenate (in 0.25 M. sucrose-10 mls. per g.) was centrifuged differentially/

FIG. 13.

The procedure for including or omitting hot TCA and NaOH treatment of muscle and liver homogenates.

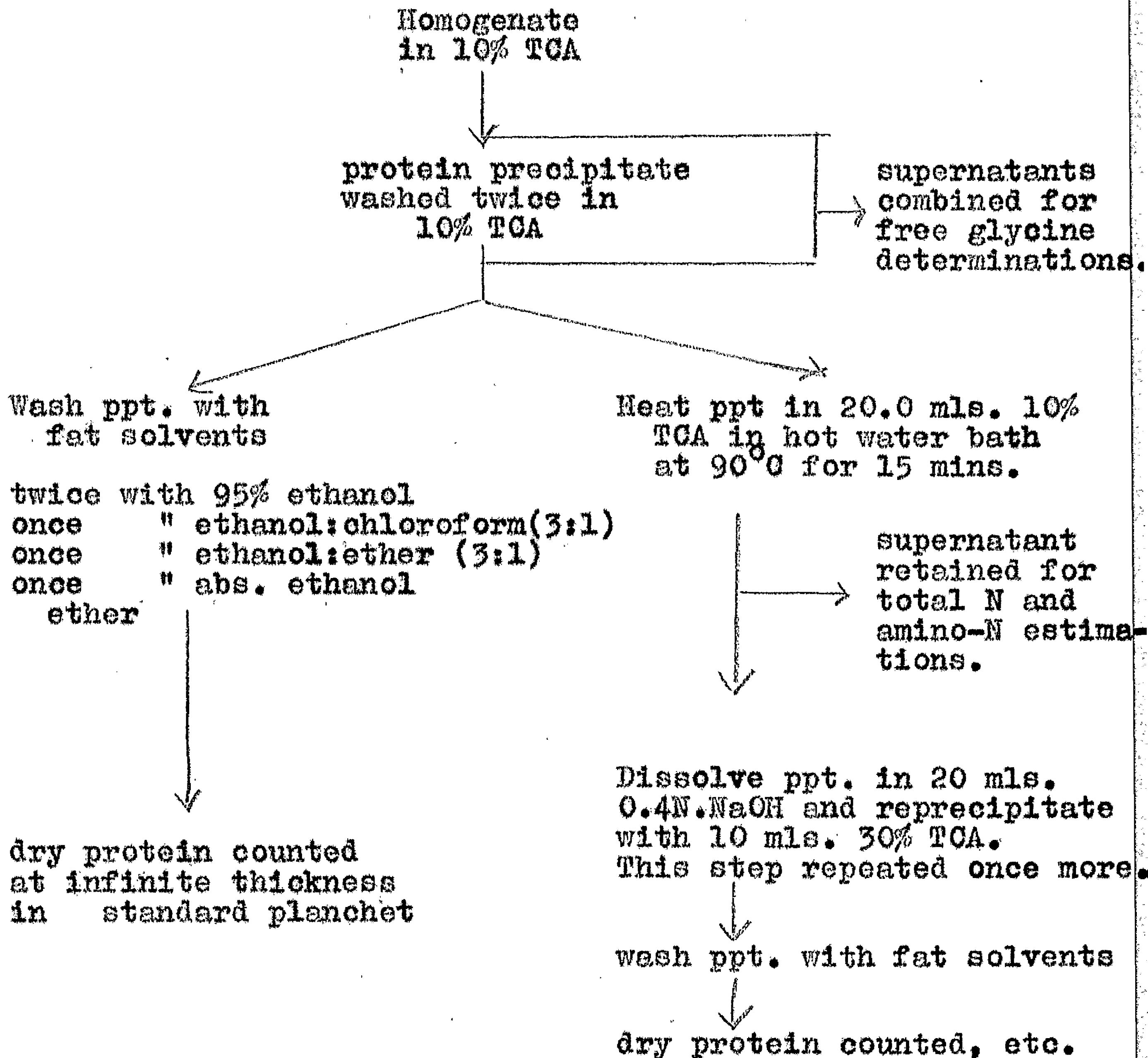
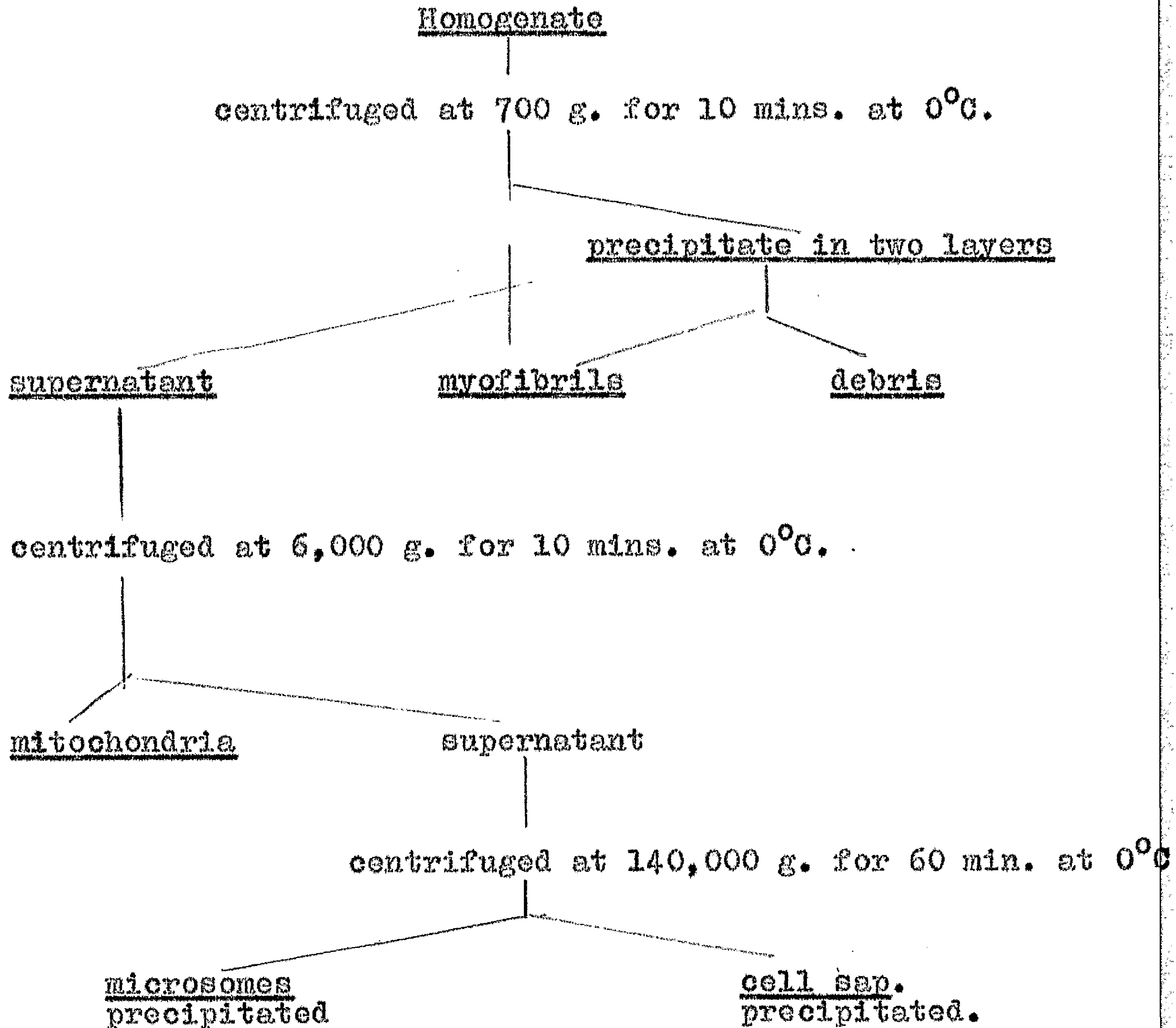


FIG.14.

The differential centrifugation of muscle homogenates in 0.25 M.sucrose.



entially as shown in fig, 14. The proteins of the various fractions were precipitated with 30% TCA and underwent the mild treatment. After measuring their radioactivity, the proteins were given the rigorous treatment with hot TCA and NaOH and recounted in order to determine if the uptake of isotope into labile protein was confined to a particular subcellular fraction.

RESULTS.

The data for the ^{14}C-glycine incorporation into the muscle and liver proteins of animals killed 1 hour after feeding are presented in Tables 20 and 21 respectively. Inspection of the muscle data (Table 20) shows that the radioactivity of the protein from a glucose-fed animal is significantly greater (+35%) when hot TCA and NaOH treatment is omitted while the protein radioactivity from a control animal fed water is not significantly affected by omission of this treatment. The liver protein radioactivity is uninfluenced by the form of treatment, whether the animal has received water or glucose (Table 21). Thus there is some evidence of a labile component in the muscle protein of glucose-fed rats.

In a separate series of experiments, an attempt was made to determine the contribution of hot TCA alone to the total loss of radioactivity from the muscle proteins of a glucose-fed animal. Specimens of protein were prepared from the muscles of water-fed and glucose-fed rats by both the "mild" and "rigorous" procedures and counted. The former was then submitted sequentially to TCA and to NaOH treatment, the protein being dried and counted at each stage. The data are summarized in Table 22. Inspection of the data for the water-fed animals reveals only insignificant changes in the/

TABLE 20.

The effect of TCA and NaOH (rigorous) treatment on the radioactivity of muscle protein from control and glucose-fed animals, killed 1 hour after feeding.

cpm/planchette whole muscle protein					
Water-fed			Glucose-fed		
Rigorous	Mild	Diffce.	Rigorous	Mild	Diffce.
66.0	71.4	+ 8%	62.6	87.5	+24%
67.8	78.2	+15%	88.4	119.9	+36%
76.4	80.5	+ 5%	22.0	39.8	+81%
65.7	78.2	+19%	60.6	74.4	+23%
81.0	68.3	-16%	32.8	41.8	+27%
41.1	55.7	+36%	67.2	80.8	+20%
66.3	72.1	+11%	55.3	73.4	+35%

TABLE 21.

The effect of TCA and NaOH (rigorous) treatment on the radioactivity of liver protein from control and glucose-fed rats, sacrificed 1 hour after feeding.

cpm/planchette whole liver protein					
Water-fed			Glucose-fed		
Rigorous	Mild	Diffce.	Rigorous	Mild	Diffce.
1950	1918	- 2%	819	849	+ 4%
2612	2460	- 6%	3290	2848	-12%
1750	1805	+ 3%	1122	1167	+ 4%
2431	2285	- 6%	2490	2186	-14%
2186	2117	- 3%	1875	1763	- 5%

---

the protein radioactivity with the various treatments. On the other hand, glucose administration results in an increase of 44% in the muscle protein radioactivity when the hot TCA and NaOH treatment is omitted. This increase is only 22% when treatment with hot TCA is included, and is finally reduced to a negligible value when the protein is subsequently dissolved in NaOH. Thus hot TCA and NaOH contribute equally to diminishing the radioactivity of the muscle protein from an animal fed glucose.

A further attempt to remove the excess radioactivity was by the use of salivary ptyalin. This was based on the assumption that the deposition might be connected with glycogen synthesis, either as ^{14}C-glucose formed from glycine or as amino-acid deposited in conjunction with glycogen. Equivalent amounts of the dried protein (prepared without hot TCA and NaOH treatment from a glucose-fed animal) were suspended in an active ptyalin preparation or in distilled water (control) and shaken mechanically for 30 minutes. The proteins were subsequently dried and counted, but no decrease in radioactivity was found in the samples incubated with ptyalin. Thus it is unlikely that carbohydrate or its complexes with amino-acids contribute to the radioactivity of the muscle protein.

<u>Nitrogen analyses of hot TCA extract</u>.

The total N (microKjeldahl) and amino-N (after acid hydrolysis)/

TABLE 22

The effect of TCA and NaOH on the radioactivity of muscle proteins prepared from glucose-fed and water-fed animals.

Treatment of muscle protein	MUSCLE PROTEIN (cpm/planchet)	
	Water-fed	Glucose-fed
hot TCA and NaOH (rigorous treatment)	72.5	55.9
neither hot TCA nor NaOH (mild treatment)	77.1(+6%)	80.4(+44%)
hot TCA alone	71.2(-2%)	67.1(+22%)
hot TCA followed by NaOH	70.5(-3%)	59.8(+ 7%)

Each entry is the mean of observations on 4 animals, killed 1 hour after feeding water or glucose.

hydrolysis) determinations on the hot TCA extract of muscle and liver proteins are presented in Tables 23 and 24 respectively. The total N and amino-N estimations on the hot TCA extract of muscle protein show that more N is extracted by this treatment from animals which had received glucose. The analyses of the hot TCA extract of liver are not so conclusive. The amino-N data shows that after glucose administration less N is removed from the liver protein. The total N results show the converse, which is largely due to a single estimate. Thus the amino-N data appears the more correct.

One difficulty in the N analysis of the hot TCA extract of muscle is that the quantity of amino-N (100 µg. per g. muscle) which is likely to be deposited by the action of carbohydrate on protein metabolism represents a small percentage of the total N present in this extract (about 10% of the amino-N and about 5% of the total N). In the second place, the uncontrolled nature of this mild hydrolysis by hot TCA make consistent results difficult to obtain. It is therefore not easy to achieve statistical significance with this approach. It is, however, interesting that the giving of glucose provides an average increase in amino-N and total N in the muscle extract of the order of magnitude expected.

TABLE 23

The N content of the hot TCA extract of muscle from glucose and water-fed animals, killed 1 hour after feeding. (Figures are μg. total N or total amino-N per g. muscle).

Amino-N			Total N		
Water-fed	Glucose-fed	Diffce.	Water-fed	Glucose-fed	Diffce.
493	652	+159	1128	1315	+187
548	675	+127	1146	1273	+127
990	956	- 34	2040	2045	+ 5
714	964	+250	1680	2040	+360
825	764	- 61	1723	1615	-108
690	709	+ 19	1041	1325	+284
941	905	- 36	1668	1745	+ 77
-	-	-	2110	2120	+ 10
	MEAN	+ 61		MEAN	+118

TABLE 24

The N content of the hot TCA extract of liver from glucose and water-fed animals, killed 1 hour after feeding. (Figures are µg.N per g. liver).

Amino-N			Total N		
Water-fed	Glucose-fed	Diffce.	Water-fed	Glucose-fed	Diffce.
312	296	- 16	2100	2120	+ 20
400	295	-105	2520	2220	-300
262	261	- 1	2000	2040	+ 40
391	409	+ 18	2200	3380	+1180
276	251	- 25	1908	1746	-162
572	441	-131	2630	2520	-110
-	-	-	3040	3175	+135
	MEAN	- 43		MEAN	+115

^{14}C-glycine incorporation into muscle and liver proteins at different times after glucose administration.

In view of the findings at 1 hour after feeding, the whole time course (1-6 hours) was studied. The muscle and liver proteins were counted at infinite thickness in a standard planchet, after both mild and rigorous treatments had been applied. The two treatments were compared and the changes in radioactivity were expressed as a percentage of the radioactivity of the protein which had received the rigorous treatment. The muscle data (Table 25) shows that in the case of the water-fed (control) animals, there is no difference in the radioactivity of the muscle protein after the two treatments. However, the animals which were fed glucose showed that after 1 hour there is a considerable amount of radioactivity which can be removed from the muscle protein by the hot TCA and NaOH treatment, but there is no indication of a labile radioactive fraction at the later times. Table 26 reports the liver data which shows that the protein radioactivity was actually greater after the hot TCA and NaOH treatment without there being any appreciable difference between the proteins from water-fed or glucose-fed rats.

Amino-N analysis on hot TCA extracts of muscle and liver at different times after glucose administration.

The muscle and liver data are compiled in Table 27

The/

TABLE 25.

The changes in the muscle protein radioactivity after treatment with or without hot TCA and NaOH, of animals fed either water or glucose.

Treatment	Time After feeding (hrs.)	MUSCLE PROTEIN (cpm/planchet)		
		Treatment		
		Rigorous	Mild	Diffce.
WATER	1	61.1	62.0	+ 2%
	2	81.0	81.9	+ 1%
	4	99.4	106.1	+ 7%
	6	113.3	121.9	+ 9%
GLUCOSE	1	5.0	61.3	+24%
	2	93.9	96.1	+ 8%
	4	103.2	105.2	+ 2%
	6	144.3	138.7	- 4%

Each entry is the mean of 2 experiments.

TABLE 26.

The changes in the liver protein radioactivity, after treatment with or without hot TCA and NaOH, of animals fed either water or glucose.

Treatment	Time after feeding (hrs.)	LIVER PROTEIN (cpm/planchet)		
		Treatment		
		Rigorous	Mild	Diffce.
WATER	1	2091	2045	- 2%
	2	2356	2038	-12%
	4	2243	1937	-14%
	6	1993	1814	- 9%
GLUCOSE	1	1806	1677	- 4%
	2	2054	1896	- 8%
	4	2267	2173	- 3%
	6	1963	1893	- 2%

Each entry is the mean of 2 experiments.

TABLE 27.

The amino-N content (after acid hydrolysis) of the hot TCA extracts of liver and muscle protein from animals fed either glucose or water.

Time after feeding (hrs.)	LIVER (ug./g wet weight)			MUSCLE (ug./g wet weight)		
	Water fed	Glucose fed	Diffce.	Water fed	Glucose fed	Diffce.
1	413	367	- 46	793	836	+ 43
2	398	353	- 45	835	929	+ 94
4	491	383	-108	860	834	- 26
6	445	380	- 65	919	743	-176

MUSCLE results are the mean of 4 experiments.

LIVER " " " " " 3 "

The results show that after glucose administration a temporary accumulation of amino-N occurs in muscle protein. The maximum deposition occurs 2 hours after feeding, falling to below the control level at the 4th. and 6th.hours; at its maximum it accounts for some 94 μg. amino-N over the control level. This value agrees very well with the calculated figure (100 μg. per g. of muscle) anticipated from the sparing action of carbohydrate. The liver data, on the other hand, show that after feeding glucose there is a consistent decrease in the amino-N content of the hot TCA extract below the control level throughout the course of the experiment. In the case of muscle, the difference in amino-N between the glucose-fed and water-fed animals follows a regression line $Y = 210 - 68X$, when Y = the difference between glucose and water-fed animals in μg. amino-N per g. muscle, and X = time in hours after administration. The slope is significant ($P = 0.02 - 0.01$).

The fact that the time of maximal occurrence of labile amino-N (2 hours) does not coincide with the time of maximum labile radioactivity (1 hour) may be due only to lack of experimental observations, as the radioactive data are from two experiments whereas the amino-N are from four experiments

<u>The incorporation of ^{14}C-leucine into the proteins of muscle and liver.</u>

These experiments were intended to confirm the data obtained with ^{14}C-glycine regarding the accumulation of a labile/

labile fraction attached to the muscle protein after feeding glucose. The mean data of three experiments are presented in Table 28. Inspection of the liver data from animals fed glucose and water reveals that the protein radioactivity is not altered by the heating in TCA and NaOH treatment, a fact in agreement with the previous findings. The muscle protein radioactivity of the control animal is also unaffected by hot TCA and NaOH treatment. After glucose administration the treatment with hot TCA and NaOH fails to reveal the presence of a labile muscle protein fraction. Total N analysis on the hot TCA extracts of muscle proteins from glucose-fed animals also failed to show the presence of extra N.

Distribution of ^{14}C-leucine among subcellular fractions.

The fact that the whole muscle homogenate failed to reveal a labile fraction using ^{14}C-leucine as the amino-acid in question severely decreased the chances of locating a sub-cellular fraction responsible for such a deposition. Nevertheless, we thought it worth while to do such a fractionation. The remaining carcass muscle was excised and homogenised in 0.25 M. sucrose. The technical difficulties involved are great due to the small quantities of protein from certain subcellular fractions (e.g. microsomes). The proteins were first dried and counted without hot TCA and NaOH treatment. The/

TABLE 28.

The incorporation of ^{14}C-leucine into the proteins of muscle and liver of animals killed 1 hour after feeding water or glucose.

Treatment	LIVER PROTEIN cpm/planchet				MUSCLE PROTEIN cpm/planchet			
	treatment				treatment			
	Rigorous		Mild		Rigorous		Mild	
WATER	557 693	625	496 783	640	72.5 46.6	59.6	70.3 46.2	58.3
GLUCOSE	498 593 705	599	470 660 718	616	68.5 39.9 57.2	55.2	61.6 40.4 54.2	52.1

The proteins from this mild treatment were then heated in TCA and dissolved in NaOH, dried and recounted. No decrease in radioactivity peculiar to a single fraction was observed (Table 29) as a result of this treatment. The radioactivities of proteins of the various fractions prepared by both treatments show (Table 29) that the relative uptake of isotope is greatest in the microsomal proteins (cf McLean et al, 1956). However, comparison of counts after mild and rigorous treatments shows no important evidence of a labile protein in any one subcellular fraction.

A feature of the leucine incorporation experiments not evident with the other isotopically labelled amino-acids is the fact that, 1 hour after feeding glucose, the muscle to liver ratio of the whole protein radioactivity is greater than the ratio for the water-fed animal. Table 30 compares the ratios after ^{35}S-methionine, ^{14}C-glycine and ^{14}C-leucine injection. Both the ^{35}S-methionine and ^{14}C-glycine ratios are depressed 2 hours and 1 hour respectively after glucose administration, while that of ^{14}C-leucine at 1 hour is significantly increased. Thus the leucine data does not agree with the glycine data on two separate counts, namely, the absence of a hot TCA labile fraction of muscle protein after glucose administration and the elevated muscle to liver protein ratio 1 hour after feeding.

The data obtained with subcellular fractions submitted

to/

TABLE 29.

The incorporation of ^{14}C-leucine into the proteins of the subcellular fractions of muscle prepared either by including hot TCA and NaOH (rigorous) treatment or by omitting it (mild)

Muscle fraction	cpm whole protein			
	Water-fed		Glucose-fed	
	Rigorous	Mild	Rigorous	Mild
Whole homogenate	72.5	70.3	68.5	61.6
myofibril-nuclear layer	61.1	68.7	56.7	57.5
mitochondria	60.5	68.6	59.4	66.2
microsomes	116.6	110.5	111.7	101.8
cell sap	51.2	62.9	69.3	65.5

Each entry is a single observation.

TABLE 30.

Comparison of the ratio of muscle to liver whole protein radioactivity after injection of different isotopically-labelled amino-acids, of animals fed either water or glucose.

Treatment	Ratio M/L x 100.		
	^{35}S-Methionine uptake	^{14}C-glycine uptake	^{14}C-leucine uptake
Water-fed	21.8	3.2	8.8
Glucose-fed	15.5	2.5	10.3
Difference	-6.3(-25%)	-0.7(-22%)	+1.5 (+17%)

^{35}S-methionine - samples obtained 2 hours after feeding and methionine-S isolated.

^{14}C-glycine and
^{14}C-leucine - samples obtained 1 hour after feeding and whole protein counted.

All proteins treated with hot TCA and NaOH.

TABLE 31.

The radioactivities of the subcellular proteins after heating in TCA and NaOH relative to the activity of the proteins of the whole homogenate.

Muscle fraction	Water-fed	Glucose-fed	Diff.ce
whole homogenate	100	100	-
myofibril-nuclear layer	84	83	-1%
mitochondria	84	87	+3%
microsomes	149	161	+12%
cell sap	71	101	+30%

to the rigorous treatment (Table 29) has been reconsidered in Table 31, to determine which fraction of the muscle cell is responsible for the extra radioactivity from ^{14}C-leucine deposited after glucose administration. It is apparent that the only fraction responding significantly to glucose is the cell sap protein.

DISCUSSION.

The investigations described in this section arose from the failure to locate an acid-soluble peptide in skeletal muscle following glucose administration. Our attention was diverted from the acid-soluble fraction of muscle to the precipitated protein and the procedures to which this protein was subjected during its purification were closely studied. Two steps in this purification procedure, namely treatment with hot TCA and NaOH, were found to have a critical bearing on the final radioactivity of the muscle protein, after the injection of ^{14}C-2-glycine. The liver protein radioactivity from water-fed and glucose-fed animals was not affected when this treatment was omitted. As a result of omitting the hot TCA and NaOH treatment from the purification scheme of muscle protein of a glucose-fed animal, there was a considerable increase in radioactivity over the protein radioactivity when heating in TCA and NaOH was included. No increase in the radioactivity of the muscle protein from a water-fed animal was noted when the hot TCA and NaOH treatment was omitted. Hot TCA and NaOH treatments were found to contribute equally to the loss of protein radioactivity.

The analysis of the hot TCA extract showed that the decrease in muscle protein radioactivity of a glucose-fed animal was accompanied by the loss of amino-N from the muscle protein. Further studies showed that the maximal deposition/

ion of this hot TCA-labile amino-N fraction occurred 2 hours after feeding glucose and accounted for 94 µg. amino-N per g. muscle; after 4 and 6 hours the level of amino-N in the hot TCA extract had fallen below that of the control. Thus there is good evidence for the deposition of amino-acids in skeletal muscle under the influence of ingested glucose as a labile fraction which is attached in some way to the muscle protein. This labile fraction satisfies the requirements of the intermediate step, postulated in Section 3 (see fig.10) to explain the decreased blood amino-acid level 1 to 2 hours after glucose and increased free glycine radioactivity 4 to 6 hours after glucose, since it accumulates soon after glucose administration (1-2 hours) and later breaks down (3-4 hours).

The evidence for the accumulation of a labile fraction in muscle after feeding glucose is convincing but would be more completely so if we had had time to show that the treatment of the precipitate with ribonuclease or deoxycholate failed to alter its radioactivity. Both these treatments specifically remove nucleic acids from the tissue precipitate so that radioactivity due to the incorporation of glycine or its metabolic products into the nucleic acids, can be excluded However, the precipitate was treated with an active preparation of ptyalin which failed to reduce the radioactivity. Thus the contribution of glycogen or its complexes with amino-acids, both of which may contain non-protein radioglycine,/

glycine, either as such or as a carbohydrate derivative, is likely to be negligible.

In order to confirm this labile fraction on muscle protein after feeding glucose, similar experiments were performed using ^{14}C-leucine. Studies on the blood amino-acid level (Thomson and Munro, 1953) revealed that the leucine concentration was most extensively reduced after glucose administration. It was hoped that the radioleucine experiments would therefore be even more striking than the changes found with ^{14}C-glycine after glucose feeding. This was not the case, since no increase in the muscle protein radioactivity was found when hot TCA and NaOH treatment was omitted. It was also found that the muscle to liver ratio of the protein radioactivity was higher than the control at 1 hour after glucose administration, whereas previous experiments with both ^{35}S-methionine (at 2 hours) and ^{14}C-2-glycine (at 1 hour) showed a smaller ratio than the water-fed control (Table 30). In fact, the muscle-to-liver protein ratios were more like these ratios from the glycine experiments some 4 hours after feeding water and glucose. This suggests that perhaps the changes in the radioactivity of the free leucine pool do not follow the pattern of the changes in the free glycine pool, and that 1 hour after feeding, the specific activity of the leucine pool of a glucose-fed animal may be much greater than the specific activity of the leucine/

leucine pool of a water-fed (control) animal. These changes in the precursor pool would be reflected by changes in the radioactivity of the muscle protein so that the ratio to liver protein is increased 1 hour after feeding. A difference in the rates of activation (Hoagland et al, 1956) of the two amino-acids could also account for the observed differences in the rate of incorporation into muscle protein. This gains some slight support from the analysis of radioactivity in subcellular muscle fractions after ^{14}C-leucine administration.

The subcellular fractionation procedure on the skeletal muscle of ^{14}C-leucine-injected animals failed to reveal a subcellular fraction into which the uptake of amino-acids occurred as labile protein under the influence of ingested glucose. This is not surprising since the muscle cell as a whole failed to reveal any labile material with ^{14}C-leucine (Table 29). However, the leucine experiments showed a somewhat greater ^{14}C uptake by muscle protein 1 hour after glucose which was not dislodged by the rigorous treatment. This greater deposition occurs in the protein of the cell sap (Table 31). The occurrence of the pH 5 enzymes, described by Hoagland et al (1956), in the cell sap and the possibility of their increased activity through the agency of "exogenous" glucose, may explain this increased incorporation which is stable to hot TCA and NaOH treatment.

In/

In conclusion, the radioglycine data strongly suggest that a temporary deposition of amino-acids as labile protein takes place in skeletal muscle after glucose ingestion. The ^{14}C-leucine data fail to confirm the presence of a labile fraction, but indicate that an increased deposition of isotope which is stable to hot TCA and NaOH treatment, does occur in skeletal muscle following glucose administration. We tentatively suggest that these effects are different manifestations of the same mechanism, involving the accumulation of a labile protein fraction in muscle which later breaks down liberating radioactive molecules into the free amino-acid pools. These changes in the radioactive pools are reflected by changes in the radioactivity of the muscle protein some 4 to 6 hours after glucose feeding.

GENERAL DISCUSSION.

The investigations described in this thesis arose from the finding that carbohydrate had an effect on protein metabolism which was not shared by fat (see review Munro, 1951). The data concerning the N retention after feeding carbohydrate and fat were rather meagre in the case of the rat and, in section 1, the effect of these energy sources on the level of urinary N of rats on different levels of protein intake was examined. The results obtained from this section reveal that carbohydrate administration significantly reduces the N output in the urine whereas the giving of fat has a minor effect. Thus this difference between the action of carbohydrate and fat on protein metabolism applies in the case of the rat, as in other species.

Role of insulin.

The fact that carbohydrate administration causes insulin secretion from the pancreas suggests that this specific ability of carbohydrate to spare body N may be mediated through the agency of insulin.

One of the manifestations of the sparing action of carbohydrate is its ability to significantly reduce the blood amino-acid concentration of fasting subjects. (Folin & Berglund, 1922; Greene et al., 1924; Schmidt & Eastland, 1935; Harris & Harris, 1947; Munro & Thomson, 1953).

Following/

Following the discovery of insulin, there were reports that it could cause a reduction in the blood amino-acid level (Wolpe, 1924; Wiechman, 1924). This has been amply substantiated in various species (e.g. Luck, Morrison and Wilbur, 1928; Kerr and Krikorian, 1929; Powers and Reis, 1933). More recently it has been shown that the different essential amino-acids of the plasma are reduced to varying extents following insulin administration (Harris and Harris, 1947; Lotspeich, 1949).

The parallelism between the actions of carbohydrate and insulin on the blood amino-acid level raises the question of whether insulin is necessary for this reduction. That this is so is indicated by the fact that following pancreatectomy amino-acids accumulate in the plasma (Barker, Chambers and Dann, 1937; Chaikoff and Weber, 1927; Ringer, 1912; Reid, 1936), and the level is not reduced by feeding carbohydrate. A series of more elegant experiments by Flock et al.(1952) and Bollman et al. (1953), in which dogs were hepatectomised, showed that the consequent accumulation of plasma amino-acids could be prevented by feeding glucose. However, when pancreatectomy was performed concurrently with hepatectomy, glucose had no effect on the plasma amino-acid level; for its reduction, insulin injections were required. This agrees with the observations that the plasma amino-acid levels of alloxan diabetic rats are no longer affected by administering/

administering glucose (Munro, 1956).

Thus the reduction of the blood amino-acid level observed by various authors after carbohydrate ingestion is effected by the secretion of insulin.

Site of deposition.

The evidence, in the case of the fasting rat, so far reveals that the administration of carbohydrate causes a reduction of the urinary N output and at the same time reduces the level of amino-acids in the plasma. The site of deposition of the amino-acids so removed from the plasma was investigated by the analysis of liver following carbohydrate administration to fasting animals (section 1). It was found that the liver actually lost N, due to a significant reduction in its protein content as a result of feeding glucose, so that the amino-acids from the plasma are not deposited in this tissue. Further attempts to localise the site of deposition of these amino-acids involved the use of isotopically labelled amino-acids. Section 2 described the use of radioactive (^{35}S) methionine for this purpose. It was found that, after glucose administration, the uptake of the isotope was increased in muscle tissue, and these findings were extended and confirmed by the use of ^{14}C-2-glycine in section 3

The fact that insulin, secreted in response to the ingestion of carbohydrate, directs amino-acids primarily to the muscle tissues is in agreement with the findings, Hastings et al.(1955) regarding the action of insulin on carbohydrate metabolism./

metabolism. These authors gave insulin to diabetic rats and then excised portions of liver and diaphragm and examined the ability of these tissues to utilize carbohydrate. In the case of the diaphragm, the maximal incorporation of the isotope into glycogen occurred within 10 minutes after insulin administration whereas the liver only showed changes in glycogen labelling some 24 hours later. Thus there is a mechanism in muscle which is sensitive to insulin at a speed commensurate with that at which glucose administration causes changes in the blood amino-acid level of the fasting animal. The fact that insulin can spare body protein by preventing the accumulation of amino-acids in eviscerated animals (Mirsky, 1938; Frame and Russell, 1946; Ingle, Prestud, and Nezamis, 1947) provides another link in the chain of evidence for the action of insulin being primarily on the peripheral tissues. The action of insulin on the plasma amino-acids of such animals is already considerable 15 minutes after injection (Ingle, Torralba and Flores, 1956) a time lapse of the same magnitude as that found with the effect of insulin on carbohydrate metabolism in muscle (Hastings et al, 1956).

The patterns of some of the amino-acids disappearing from the plasma after insulin injection (Harris and Harris, 1947; Lotspeich, 1949) and carbohydrate administration (Munro and Thomson, 1953) are similar and are the same as the/

the proportions of these amino-acids present in muscle protein (Lotspeich, 1949). It would thus appear that in response to glucose ingestion insulin directs amino-acids to the peripheral tissues.

General relationships in protein metabolism following glucose administration.

The preceding evidence shows that glucose administration has several actions on the protein metabolism of fasting animals. These can be linked together as shown in fig. 15. The primary action appears from our evidence to involve deposition of amino-acids in muscle with the participation of insulin. This results in a fall in the blood amino-acid level with a consequent reduction in the amino-acid supply to other tissues. In particular the liver receives less and this results in a reduction in the synthesis of urea (protein sparing action of glucose) and also of protein, as we noted in section 1.

Some evidence of a reduced amino-acid concentration in tissues of animals on protein-free diets is provided by Thompson et al.(1950), who compared the concentrations of various amino-acids, free in different tissues in animals receiving protein-free meals and in fasting animals. Most amino-acid concentrations were lower in the group fed the protein-free diet. More recently, Castro (1955) has shown that/

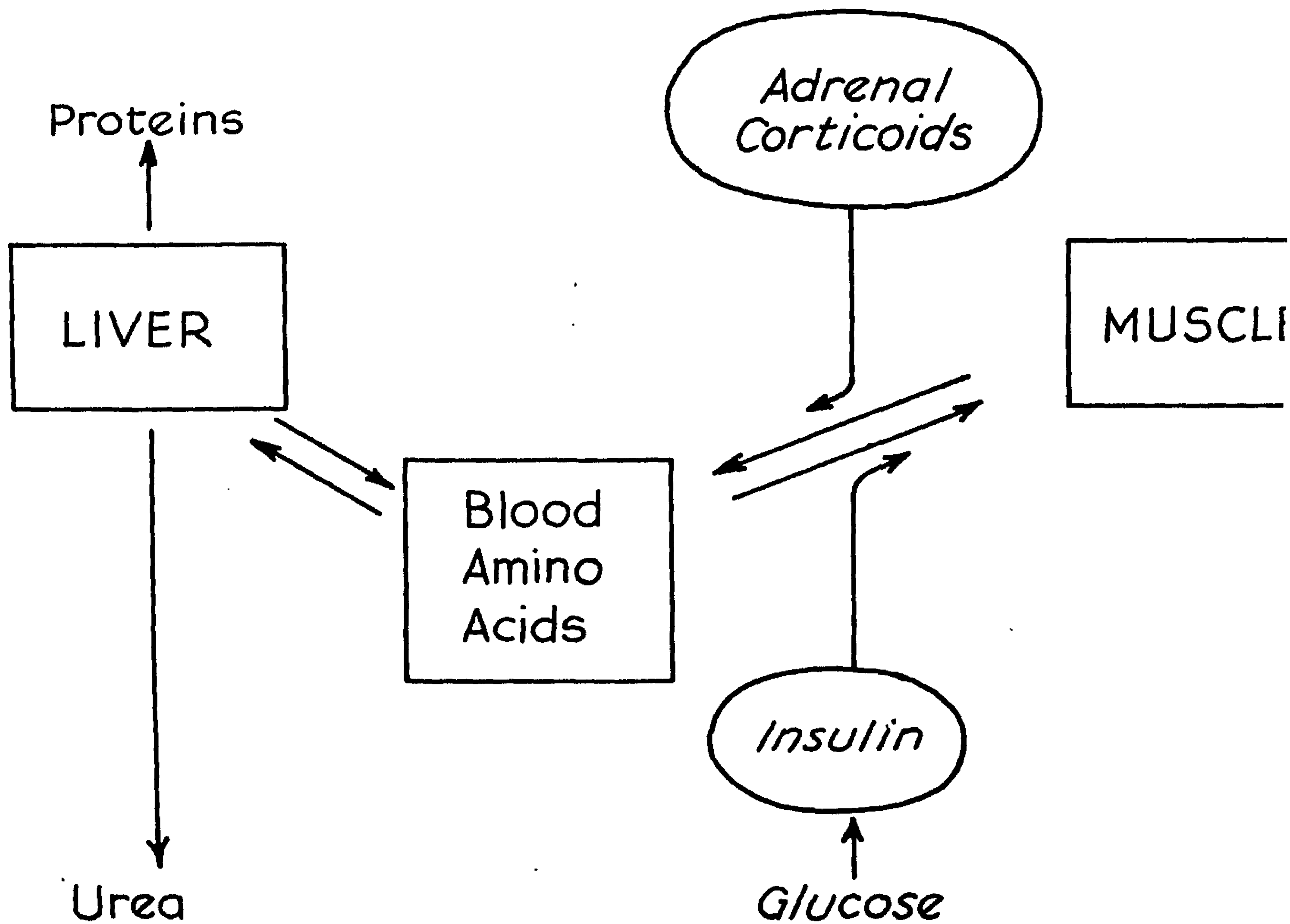


FIG. 15. The mechanism of action of dietary carbohydrate on amino-acid metabolism in fasting animals.

that glucose administration lowers the concentration of the glycogenetic amino-acids in liver and muscle.

The influence of carbohydrate in reducing liver protei content receives support from the literature. Munro and Naismith (1953) found that the addition of energy in the form of carbohydrate to a protein-free diet, while reducing the urinary N output, resulted in a decreased N level in the liver (Table 6). It also explains why Rosenthal and Vars (1954) found that the removal of carbohydrate from the diet increased the total N content of liver as well as the urinary N output. There would indeed appear to be a reciprocal relationship between carcass and liver, in which the gain of protein by the carcass is accompanied by a loss from the liver. As indicated in fig. 15 adrenal corticoids have the opposite action to the glucose-insulin effect, namely a loss of protein from the carcass and a deposition in the liver (Tremolieres, Derache and Lowy, 1955). Indeed, our conception of a balance between carcass and viscera may provide a basis for the hormonal control of protein metabolis

Our experimental observations may also provide an explanation for some of the biochemical characteristics of Kwashiorkor, a serious nutritional disease common in under-developed countries, in which the dietary protein level is low and the carbohydrate content is extremely high. The N content of the liver in such cases is considerably reduced (Burch/

(Burch et al., 1957; Waterlow and Bras, 1957). The condition is usually ascribed to the low intake of poor-quality protein but our data show that the high carbohydrate content of these tropical diets is likely to exaggerate the deficient amino-acid supply to the liver by causing excessive amounts of amino-acids to be deposited in the muscle.

Fig.15 represents the state of affairs when carbohydrate is fed to a fasting animal. When the diet contains adequate amounts of protein as well as carbohydrate, the action of each carbohydrate meal will be complicated by an influx of amino-acids at the same time. Do these dietary amino-acids also undergo temporary storage in muscle? The evidence is in favour of some such mechanism since, as discussed more fuly in the introduction to this thesis, a) substitution of fat for dietary carbohydrate results in a temporary increase in N output and b) separation of the time of carbohydrate and protein ingestion causes a decrease in N retention by the body. These observations lead to the conclusion that dietary carbohydrate influences the utilization of amino-acids ingested in the same meal. The most likely mechanism is an accentuation of the sequence suggested in fig.15 for carbohydrate action on endogenous protein metabolism. This view is strengthened by the finding (Geiger and Pinsky, 1955) that an intact insulin secreting/

secreting mechanism is necessary for the action of dietary carbohydrate on protein fed in the same meal, just as one is required for the action of carbohydrate on the endogenous protein metabolism of the fasting animal. Such a mechanism emphasises the importance of muscle tissue in normal protein metabolism.

Site of action of Insulin.

The evidence in the literature, to support the concept of insulin action on muscle tissue, is meagre and very incomplete. The experimental data available are mostly the results of *in vitro* studies on the incorporation of isotopically labelled amino-acids into the proteins of isolated diaphragm and liver slices. Such studies on these tissues from rats fasted for 48 hours before sacrifice (Krahl,1953) showed that *in vitro* addition of insulin did not increase the uptake of ^{14}C-glycine by either the diaphragm or the liver. Similar experiments performed on the tissues of rats which were fed prior to sacrifice (Krahl,1953) showed that the addition of insulin *in vitro* increased the incorporation into the diaphragm but not into liver protein. Thus, in the case of the normal rat receiving food before sacrifice, the addition of insulin *in vitro* stimulated only the incorporation of amino-acids into the muscle tissue.

Sinex et al.(1952) also showed that the incorporation of ^{14}C-alanine into the diaphragm of rats which received food/

food prior to sacrifice was increased by the addition of insulin to the medium. The uptake of ^{14}C-alanine by the liver was not investigated. Thus although this evidence confirms the stimulatory effect of insulin on the incorporation of amino-acids into the proteins of diaphragm from fed animals, it does not indicate whether this action of insulin is confined to muscle tissue. The same criticism applies to Forker et al.(1951) who showed that the intravenous injection of insulin increased the uptake of ^{35}S-methionine by the skeletal muscle of the eviscerated (pancreatectomised) diabetic dog. A more complete investigation of the *in vivo* action of insulin with fed alloxan diabetic rats is available (Krahl, 1956). These animals were sacrificed up to 18 hours after an injection of insulin and the incorporation of ^{14}C-glycine into diaphragm and liver slices examined. The incorporation into the protein of the diaphragm was greatly stimulated, the maximum uptake occurring when the injection preceded sacrifice by some 10 hours. The corresponding uptake of liver protein was not recorded but liver glutathione showed a relatively slight increment during the same period of time.

Thus in the case of the fed animal, either under normal or diabetic conditions, insulin would appear to affect primarily skeletal muscle tissue. Insufficient evidence makes it impossible to suggest the nature of this action of insulin/

insulin on the fasting animal.

Mechanism of insulin action on protein metabolism.

The alteration in the level of the blood amino-acids in response to glucose or insulin administration has already been discussed and the data favouring insulin as the agent responsible for this reduction has been presented. The evidence which appears to favour the primary action of insulin on muscle tissue has also been considered but the question of whether the increased incorporation of amino-acids into the proteins of muscle is due solely to insulin or whether this action is dependent on consequent changes in carbohydrate metabolism in muscle is more difficult to answer. Evidence in favour of such an interdependence has been presented by Krahl (1956) who studied the incorporation of ^{14}C-glycine into glutathione by liver slices from diabetic rats. The *in vitro* addition of glucose *and* insulin together increased the incorporation, whereas the separate, *in vitro* additions of fructose and pyruvic acid, raised the uptake to an even greater level, inferring that in the case of glucose + insulin addition, the limiting step in the synthesis of glutathione was the entry of glucose into the glycolysis scheme. However, it has been found (Ingle, Flores and Torralba, 1956) that the replacement of the glucose of the blood stream of eviscerated rats by fructose did not diminish the blood amino-acid concentration although fructose is believed to pass into/

into the cell without the aid of insulin and its entry into the glycolysis scheme is not dependent on glucokinase. These findings can be reconciled with the evidence presented by Krahl (that pyruvate and fructose alone stimulate amino-acid uptake) if we assume that only insulin can transfer amino-acids across the cell wall, but once the amino-acids have been introduced into the cell, the rate at which they are utilized is dependent on the energy from glucose oxidation. In addition, Ingle, Torralba and Flores (1955) have found that muscle work transfers glucose into the cells of eviscerated rats, just as insulin does, but only in the case of insulin is there a concomitant reduction in the blood amino-acid level. Thus insulin would appear to have an action on protein metabolism which is independent of its action on carbohydrate metabolism, by transferring amino-acids into the cell, but the subsequent metabolism of the amino-acids is dependent on intracellular glucose utilization. This conception of the mode of action of insulin may explain the differences observed by Krahl (1953) in the uptake of ^{14}C-2-glycine by tissues from fed and fasted rats. He found that in the diaphragm from the fed rat, insulin stimulated amino-acid incorporation, whereas in the diaphragm from a fasted rat, glucose plus insulin was required to raise the uptake to the level of the (fed) diaphragm. (It fails, however, to explain how the addition of glucose alone could raise the level of incorporation to normal). This suggests that the cell must/

must have an adequate intrinsic energy supply before an immediate effect on amino-acid uptake can be observed. Thus the action of insulin on deposition of amino-acids in muscle is probably not dependent initially on the effect of insulin on glucose metabolism, although the energy provided by the latter may be a secondary factor.

The mode of amino-acid deposition in muscle.

A diligent search of the acid-soluble fraction of muscle failed to reveal an accumulation of amino-N after carbohydrate administration (section 4), and in consequence, the treatment of the protein precipitate was closely examined (section 5). We were able to show that carbohydrate administration to animals injected with ^{14}C-glycine, caused a temporary deposition of amino-N associated with the protein of muscle. This extra N could be removed by heating the protein with TCA. In a series of experiments, Hendler (1957) has provided evidence for a hot TCA labile peptide fraction occurring in hen oviduct which he claims is an intermediate in the synthesis of hen oviduct protein. He found that the radioactivity of this fraction was incorporated into hen oviduct protein while the corresponding (radioactive) amino-acid was not incorporated, even when present in high concentration.

The fact that our protein fraction occurring in muscle protein after feeding glucose is labile to hot TCA immediately raises the question of a possible relationship of the labile fraction/

fraction with nucleic acids, especially with the low molecular weight ribonucleic acid (RNA) occurring in the cell sap and reputed to be concerned in the activation of amino-acids for protein synthesis (Hoagland, 1957). This inter-relationship of amino-acids and RNA receives some slight support from the fact that the proteins of the cell sap have a greater radioactivity with ^{14}C-leucine after glucose treatment. It must be admitted that, at present, we have no explanation for the failure to confirm the presence of this labile protein with ^{14}C-leucine.

It is hoped that confirmation of this labile fraction, peculiar to muscle tissue, will shed some light on the hormonal control of protein synthesis at the intracellular level.

SUMMARY.

Section 1: <u>The effect of carbohydrate and fat on the N retention of the fasting rat.</u>

1. Experiments are described on adult rats, which had been stabilised on a protein-containing or a protein-free diet and after a 24 hour fast received either water, glucose or olive oil.

2. Carbohydrate administration significantly reduced the urinary N output in both dietary groups. Fat administration decreased the urinary N output to a less significant degree.

3. The protein N content of the liver was significantly reduced after carbohydrate administration. Fat administration did not significantly reduce the protein N content of the liver.

4. It was concluded that the feeding of carbohydrate resulted in N retention in the body in tissues other than the liver.

Section 2: <u>The uptake of ^{35}S-methionine by liver and muscle protein after carbohydrate and fat administration.</u>

1. A study was made of the effect of feeding glucose or olive oil on the incorporation of injected ^{35}S-methionine into/

into the protein of the skeletal muscles and the livers of fasting rats.

2. It was found that neither glucose nor fat had any effect on the uptake of the labelled amino-acid by the liver. However, it was observed that glucose, but not fat, stimulated the uptake of ^{35}S-methionine by skeletal muscle. Two hours after glucose feeding, the isotopic concentration was lower than that of a fasting animal but the rate of appearance of the label increased more quickly than in the control until the isotopic concentration was significantly greater at the 6th.hour.

3. It was concluded that the amino-acids disappearing from the blood after glucose administration are deposited in muscle.

Section 3: <u>The effect of glucose and fat on the incorporation of ^{14}C-2-glycine into the proteins of skeletal muscle and liver</u>.

1. The specific activity of the free glycine pool of liver and muscle were examined after glucose or fat ingestion.

2. It was found that the specific activity of the free glycine pool of liver and muscle did not change significantly after water or fat administration. It was also found that carbohydrate ingestion did not alter the pattern of changes in the liver free glycine pool, while the muscle free glycine specific/

specific activities were elevated significantly above the control values, especially at the 4th and 6th hour.

3. The ratio of the muscle to liver whole protein radioactivity of the glucose-fed animals was initially below the control level but increased steadily to reach a higher value than the control some 6 hours after feeding.

4. It was concluded that the changes in ^{14}C-glycine and ^{35}S-methionine deposition in muscle protein 4-6 hours after glucose administration were reflections of changes in the labelling of the free amino-acid pool. Since the change in blood amino-acid level is maximal 1-2 hours after glucose feeding, it was concluded that some intermediate compound, such as a labile peptide or protein is deposited in the muscle and its subsequent breakdown causes the observed changes in amino-acid pool radioactivity.

Section 4: <u>The analysis of the acid-soluble fraction of skeletal muscle and liver after feeding glucose.</u>

1. Trichloracetic acid and tungstic acid extracts of muscle and liver were analysed for amino-N by various methods.

2. Folin's β-naphthaquinone method was found to be unreliable by the use of ion-exchange resins.

3. The ninhydrin titrimetric decarboxylation method failed to reveal any changes in the free or peptide amino-N content of the acid-soluble fractions of muscle and liver after glucose administration. These findings were confirmed by/

by the colorimetric ninhydrin method.

4. It was concluded that glucose ingestion did not cause the temporary deposition of an acid-soluble peptide in muscle.

Section 5: <u>The examination of liver and muscle protein after glucose administration</u>.

1. The treatment of the liver and muscle homogenate to obtain pure protein was reconsidered; in particular the stages involving heating in TCA and dissolving in NaOH.

2. It was found that, in animals injected with ^{14}C-2-glycine, a greater amount of radioactivity was present in the muscle protein of a glucose-fed animal, when the hot TCA and NaOH treatment was omitted. The radioactivity of the liver protein was unaltered when the hot TCA and NaOH treatment was omitted, whether or not the animal received glucose. It was therefore concluded that a TCA-labile protein is deposited in muscle following glucose administration.

3. The analysis of the hot TCA extract of muscle protein revealed that, after feeding glucose, more amino-N could be removed from the muscle protein than from the protein of the water fed control. The maximum amount of this extra amino-N occurred 2 hours after feeding glucose and fell to below control level 6 hours after feeding.

4. This hot TCA labile protein fraction of muscle was not/

not demonstrable after ^{14}C-1-leucine injection.

5. It was concluded that labile muscle protein occurred immediately after glucose administration, and that the observed dissimilarities between the uptake of ^{14}C-glycine and ^{14}C-leucine are different expressions of the same mechanism.

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