THE INFLUENCE OF DIETARY CARBOHYDRATE ON THE COURSE OF PROTEIN METABOLISM.

by

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A Summary.

A study of the relevant literature reveals that carbohydrate has a beneficial action on the course of "exogenous" and "endogenous" metabolism of protein, for which energy in the form of fat is not a substitute. The objects of the experiments to be described were first, to attempt to determine whether the effects of carbohydrate on the metabolism of dietary and body protein are similar in mechanism and secondly, to gain some insight into the mode of action of this phenomenon.

Experiments were carried out with adult rats, in which the increase in nitrogen output which results when carbohydrate fed with protein is isocalorically substituted by fat, was compared with the change in nitrogen balance which occurs when carbohydrate is transferred from a protein-containing to a protein-free meal. In view of their similar magnitude and transient nature, it was concluded that these two superficially distinct actions of carbohydrate on the exogenous metabolism of protein had probably the same underlying mechanism, which is primarily dependent on the simultaneous feeding of carbohydrate with dietary protein.

The effect of glucose ingestion on the digestibility and rate of absorption of casein was next investigated, as the intestine is a feasible site of the interaction which occurs between carbohydrate and protein fed in the same meal. Faecal nitrogen determinations revealed that glucose did not alter the digestibility of the protein. In addition although glucose did cause a marked delay in the absorption of the casein, the sparing effect of glucose could not be attributed to this effect, as fat ingestion also resulted in a similar delay but without giving rise to nitrogen retention. Experiments were also conducted in which the nitrogen content of the viscera were estimated after the simultaneous feeding of carbohydrate and protein. No significant fraction of the nitrogen which was retained could be accounted for in the viscera and it was suggested that the most probable site of deposition of the retained nitrogen was muscle.

Studies were also made on the effect of glucose and fat administration on the plasma amino acid levels in fasting human subjects and rats. Glucose ingestion caused a 12% reduction in the plasma amino nitrogen which was maximal at 1 hour, whereas fat ingestion resulted in a much more gradual fall, to the extent of 4% of the initial level and of doubtful significance. Similar experiments on rats confirmed the differences in action of glucose and fat. In the fasting human subject, the level of seven essential amino acids

(tryptophan, histidine, leucine, isoleucine, threonine, arginine and valine) fell after glucose ingestion to varying extents. If these depressions were arranged in the form of a ratio with tryptophan as unity and compared with Rose's estimate of the human requirements of these amino acids arranged in a similar way, then a close relationship was observed between the values. It was concluded that the sparing action of glucose was probably due to a stimulation of protein synthesis. Also as the action of glucose on the plasma amino acid level is maximal at one hour and as the level of amino acids in the portal vein will also be maximal shortly after feeding protein, then it was suggested that the action of glucose on the endogenous metabolism of protein was similar to its action on the exogenous metabolism.

The administration of carbohydrate to fasting alloxan diabetic rats, resulted in a very slight decrease in the plasma leucine level compared to that occurring with normal rats. This result was considered to favour the view that insulin is necessary for the sparing action of carbohydrate in the fasting rat.

The effect of glucose and fat on the incorporation of ^{35}S-methionine into tissue proteins was investigated both *in vivo* and *in vitro*. A study was made of the effect of added glucose, succinate, octanoate and acetate on the uptake of ^{35}S-methionine by rat diaphragm and rat liver slices *in vitro*. Glucose increased the incorporation by

9 - 16% and succinate by 59%. Octanoate had little effect on the uptake, but acetate had a definite depressant action. It was concluded that glucose and succinate increased the amount of A.T.P. available for the process of protein synthesis, whereas octanoate and acetate did not.

With the in vivo studies it was found that neither glucose nor fat had any effect on the uptake of ^{35}S-methionine by the intestine and liver. On the other hand it was observed that glucose but not fat greatly increased the incorporation of methionine by skeletal muscle. Two hours after feeding glucose the isotope concentration was slightly lower than that of the control animal, but at the fourth hour, there occurred a marked increase in the uptake which had completely disappeared again by the sixth hour. It was suggested that glucose promotes the synthesis of a peptide or protein which was isolated in some manner from the main mass of muscle protein.

It has been concluded that the action of carbohydrate on the endogenous and exogenous utilisation of protein is due to one underlying mechanism, namely an increase in protein deposition in muscle.

ACKNOWLEDGMENTS.

I am greatly indebted to Professor J. N. Davidson for giving me the opportunity of carrying out this work in his Department and to Dr. H.N. Munro, for his constant help, guidance and encouragement during the course of these studies.

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C O N T E N T S.

" Living bodies are by no means always composed of the same matter at every period of their existence..... .... All parts of the body of man experience a movement which has the double effect of expelling the molecules which can or ought no longer to compose the organs and of replacing them by new molecules..... This process is more or less rapid according to the tissues. The glands, muscles, and skin change with great quickness; the tendons, fibrous membranes, bones and cartilages appear to change but slowly".

Extracts from Magendie's "Textbook of Physiology," 3rd Edition (1829).

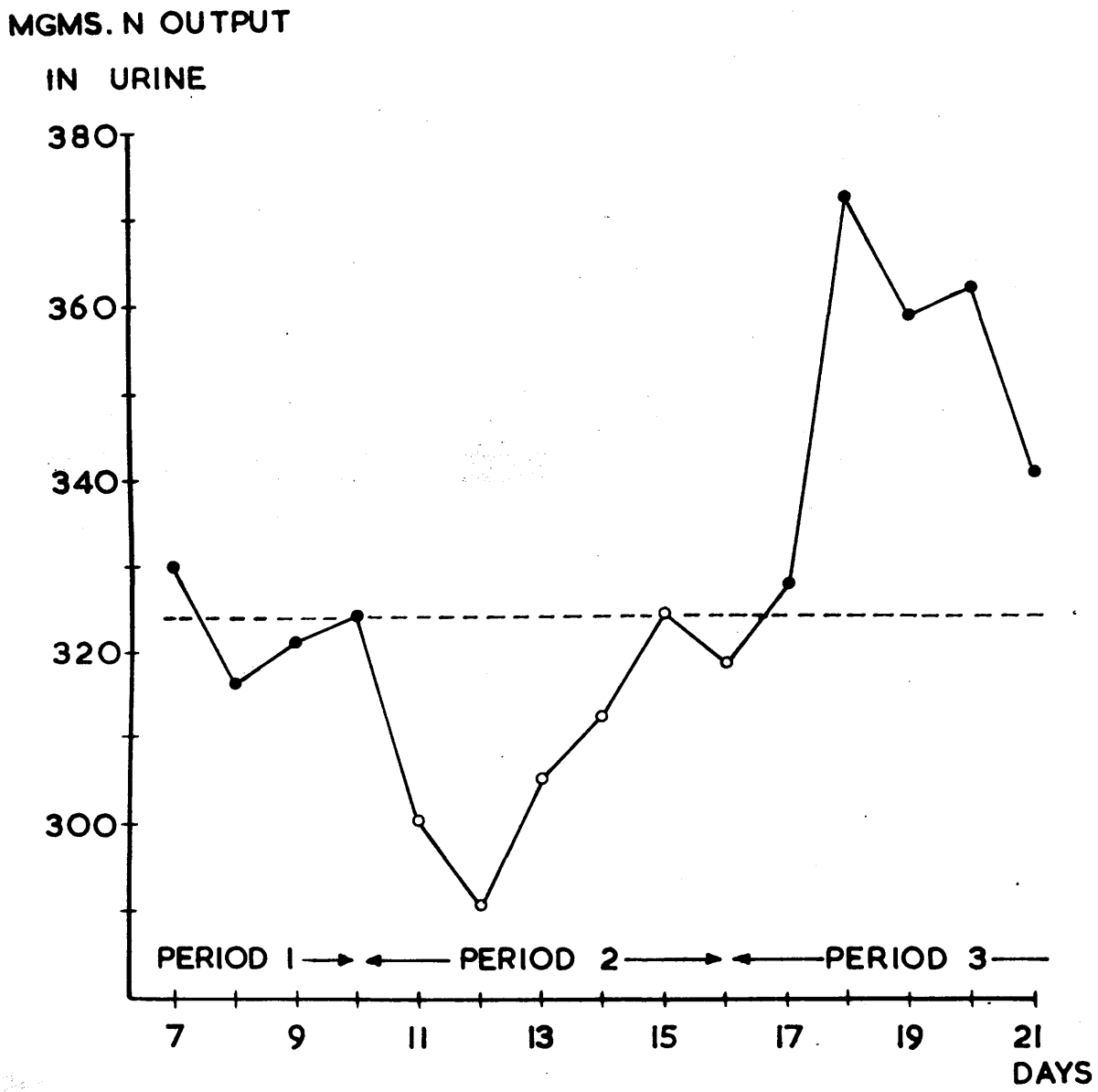
INTRODUCTION

It was Magendie (1786-1855) who early in the nineteenth century first established the essential nature of the nitrogenous constituents of the diet. After feeding dogs with protein free substances such as sugar and olive oil, as their sole source of nourishment, he observed that they developed severe debility and died within a month. Boussingault (1802 - 1887) was so impressed by Magendie's findings that he commenced a line of research which eventually culminated in the creation of the first nitrogen (N) balance study. This technique after some modification by ensuing workers, proved to be of inestimable value in furthering progress in the field of nutrition. Thus Voit and his successors made great use of it in their studies on the influence of carbohydrate and fat on the course of protein metabolism (Sayhun, 1948). The extensive literature on this aspect of protein metabolism, commonly known as the protein sparing action of carbohydrate and fat, has recently been reviewed by Munro (1951). In the first place carbohydrate and fat can spare the utilisation of protein by acting as energy sources, and in this respect their relative merits would appear to be identical, as the N retention which results after superimposing them on a diet already adequate in energy content is of the same order in both cases. On the other hand the literature furnishes abundant evidence of differences in the mode of action of

carbohydrate and fat, and these dissimilarities, which suggest that under certain conditions carbohydrate is superior to fat, will now be briefly referred to.

Substitution of carbohydrate by fat. The well established and long known effect of substituting fat for carbohydrate in the diet will be discussed at length in Section I. Suffice it to say at this juncture that when dietary carbohydrate is replaced by an equivalent amount of fat without altering the total caloric intake, there occurs a marked but temporary loss in N. This suggests that carbohydrate and fat do not affect protein metabolism entirely as energy sources.

Separation of time of ingestion of protein from that of carbohydrate. More recently it has been observed that carbohydrate need not be removed from the diet to produce an adverse effect on N balance; mere separation in the time of eating the carbohydrate and protein being sufficient to cause a marked deterioration. In 1939 Cuthbertson and Munro fed 4 human subjects on adequate diets which could be separated into two portions, one containing all the protein and part of the fat of the diet, the other containing all the carbohydrate and the remainder of the dietary fat. When the subjects were given these two portions at different meals the daily N output was about 2g. greater than when the same amount of food was given in the form of mixed meals. This separation of protein and carbohydrate was only carried out for a few days. Similar experiments with adult rats gave



PERIODS 1 & 3 : PROTEIN & CARBOHYDRATE EATEN SEPARATELY

PERIOD 2 : PROTEIN & CARBOHYDRATE EATEN TOGETHER.

MUNRO (1949)

FIG. 1.

identical results (Cuthbertson, McCutcheon and Munro, 1940). In these experiments with human subjects and rats, attempts to determine the duration of this disturbance in protein metabolism caused by the separation of the dietary protein and carbohydrate, were defeated by loss of appetite. Munro in 1949 overcame this difficulty by offering to rats a very complete synthetic vitamin mixture. The experimental design used was as follows: vitamins, minerals and roughage were given in a morning meal and the protein in an evening meal, and between these two meals carbohydrate or fat was transferred. Fig. 1 shows the result of a typical experiment in which the rats received all the dietary constituents except protein in the morning meal. Carbohydrate was then transferred to the evening meal and this association of protein and carbohydrate resulted in a sharp reduction in the urinary N output. This effect however, was only temporary as the N output returned to its former level within 4 to 5 days. When the dietary carbohydrate and protein were once again separated, there was an immediate loss of N roughly equivalent to the amount previously retained. When fat was similarly transferred from the morning to the evening meal, N output was not affected. An experiment was also carried out in which fat was transferred from the morning meal to a time 1½ to 2½ hours before the protein meal, in order to test the possibility that the fat might be too slowly utilised to spare protein given along with it. The results in this experiment were also

negative. With the exception of the last experiment, these effects were obtained in several independent experiments on groups of rats and the data, when analysed statistically, were shown to be highly significant. Essentially similar results have since been obtained by Geiger, Bancroft and Hagerty (1950). These investigators showed that protein depleted rats regained weight much more rapidly when the dietary protein and carbohydrate were fed together, than when they were offered separately.

Effect of carbohydrate and fat on nitrogen output during starvation. Dogs have been the animals most widely used in studying the effect of carbohydrate or fat administration at the end of a period of hunger. The results of early workers (Voit and others) on this aspect of protein metabolism have tended to be contradictory, as sweeping conclusions were being drawn from one or two brief experiments (Munro, 1951). However, the experiments detailed beneath were technically much more satisfactory and the results are quite conclusive.

Bartmann (1912) fed fat to fasting dogs and concluded that it could spare protein to an extent of 7%; this result, however, is probably due to variation in individual animals and is not statistically significant. Similar experiments were conducted by Richet and Minet (1925), who reported that the average value for the N excretion indicated no sparing effect for fat. On the other hand Wimmer (1912), also working with fasting dogs, obtained evidence for a decided

sparing action with carbohydrate, a finding which was corroborated by Richet and Minet (1925).

A similar difference between the action of glucose and fat has been observed with fasting human subjects. The administration of carbohydrate has been shown to reduce the N output below the level found during fasting (Benedict 1915; Grafe, 1910 and 1914), but no reduction was observed during a short period of fat administration (Thomas, 1910).

The rabbit has also been shown to behave in an analagous manner. The feeding of single doses of sugar to fasting rabbits led to a reduction in N excretion (Heilner, 1906; May, 1894: Voit, 1901), but no effect was obtained with fat (Heilner, 1910; Rubner, 1883).

With regard to the rat the information in the literature is rather meagre and unsatisfactory, no doubt due to the fact that this type of experiment was mainly done before the rat became popular as a laboratory animal. Gregg (1931) has noted that the N outputs of rats fed with butter fat were almost all lower than the nitrogen output of one single rat which he fasted. Kriss, Forbes and Miller (1934) have observed that the N outputs of a group of rats fed fat were lower that the N outputs of a group which were fasting. Unfortunately the authors do not indicate if the two experiments were strictly comparable. Obviously further information is required before it can be definitely concluded that the rat, unlike the man, dog and rabbit, can utilise

fat to spare body protein.

These three classes of experiments described above illustrate very clearly that carbohydrate favourably influences protein metabolism, both of "exogenous" and "endogenous" type (using these terms in a descriptive sense) under circumstances in which fat does not. Thus the preceding evidence shows that removal of carbohydrate from the diet causes a deterioration in N balance, and this also occurs with separation of dietary protein and carbohydrate. It is not clear whether these two phenomena represent different aspects of the same mechanism, namely, a specific effect of carbohydrate exerted on protein eaten in the same meal or whether some differences in mechanism exist. The third group of experiments cited above show that carbohydrate spares the break-down of body protein, whereas fat does not. Again it has to be demonstrated that this action of carbohydrate on "endogenous" protein metabolism is similar in mechanism to its effect on dietary protein.

The experiments which we conducted were designed with two objects in view. First, to try to determine whether these three distinct actions of carbohydrate, which can be distinguished nutritionally are merely three different manifestations of one single phenomenon and secondly to attempt to elucidate the modus operandi of the beneficial action which carbohydrate exerts on protein metabolism.

In the first series of experiments the effect on N

balance of substituting fat for carbohydrate was compared with the change in N balance which occurs when carbohydrate is fed with and apart from dietary protein (Section I). On the basis of our findings it was concluded that most of the effect of substituting fat for carbohydrate could be attributed to failure to eat carbohydrate and protein simultaneously. The next section (Section 2) deals with the site within the body at which carbohydrate exerts this effect on the fate of dietary protein. The digestibility and rate of absorption of protein fed with glucose were thus investigated, as the intestine could feasibly be the site of the interaction which occurs between glucose and protein. In addition, observations were made on the tissue distribution of the N which is retained after simultaneous feeding of carbohydrate and protein. Observations were next made on the action of glucose and fat in the fasting subject, as revealed by changes in the concentrations of the blood amino acids. (Section 3). For comparison with the positive effect on blood amino acid concentration obtained in normal subjects after glucose administration a short series of studies on diabetic rats was carried out. (Section 4). Finally (Section 5) experiments were performed to determine the effect of glucose and fat administration on the incorporation of radioactive methionine into various tissue proteins, either in vivo or in vitro.

From the results of these experiments, we have supplied

fresh evidence which again reaffirms the contrasting effects of glucose and fat on protein metabolism and has thrown new light on the question of the single entity of the various carbohydrate effects. In addition, from the observations made, we have advanced theories on the relationship between the administration of glucose and protein metabolism which may account for the beneficial action of carbohydrate.

SECTION I

THE EFFECT OF CARBOHYDRATE AND FAT ON THE UTILISATION OF DIETARY PROTEIN.

INTRODUCTION

That the addition of carbohydrate and fat to a diet already adequate in protein and energy content leads to N retention has been known for some considerable time. Several authors have compared the efficacy of carbohydrate and fat in this respect and it would appear that from an energetic standpoint these two nutrients can act interchangeably, as broadly speaking they usually cause a similar degree of N retention. (Cuthbertson and Munro, 1937; Forbes Bratzler, Thacker and Marcy, 1939; Forbes and Swift, 1944; Munro and Wikramanayake, 1954). But if their effect on protein metabolism is solely as energy sources then isocaloric substitution of dietary carbohydrate with fat should have no influence on N output. In order to test this hypothesis use has been made of two experimental techniques. In the first type, the subject acts as his own control in that initially he is stabilised for some days on a diet containing normal amounts of carbohydrate, then carbohydrate is withdrawn and replaced by an equivalent amount of energy in the form of fat. The second type of procedure consists of comparing the N outputs in two groups of animals receiving the same amount of protein and energy but differing amounts of carbohydrate and fat. In both types of experiment the exchange of fat for carbohydrate can of course be partial or complete. In Tables 1, 2 and 3 are tabulated the results of complete and

TABLE 1

The effect of substituting fat for Carbohydrate in human subjects.

Species Used	Type of Substitution	Authors	Number Used	DIETS USED: Description	Protein	Fat	Carbohydrate	Energy	Period on diet	N INTAKE AND OUTPUT: Period upon which values are based	Daily N Intake	Mean daily urine N	Mean daily N balance	COMMENTS
					g.	g.	g.	Cal/kg.	days	days	g.	g.	mg.	
MAN	COMPLETE	Kayser(1893, 1894)	1	(Mixed	133	71	338	38	4	last 3	21.2	-	+ 560	On the carbohydrate-free diet the daily N balances were -1.77, -2.48 and -4.98g. respectively.
				(Carb.-free	135	220	0	38	3	all 3	21.6	-	-3080	
				(Mixed	132	71	338	38	3	all 3	21.1	-	+ 930	
		Silwer (1937)	3	(Mixed	155-163	136-137	285	43-49	10-18	last 3	25.2	22.9	-	Food not analysed. Only the first 5 subjects in Silwer's series were on diets adequate in energy and protein content. The two subjects on the higher protein intake returned to the mixed diet and showed a slight N retention as compared with N output during first period.
				(Carb.-low	155-163	260-269	6-7	43-49	11-12	first 11	25.2	25.6	-	
			2	(Mixed	90	126	256	36-49	7	last 3	14.4	12.7	-	
				(Carb.-low	90	237	6	36-49	18	first 11	14.4	16.0	-	
		Tallqvist (1902)	1	(Carb.-rich	102	44	466	35	4	all 4	16.3	-	+ 830	One day of milk diet preceded each period. N balances were -1.58, -1.34, 0.14 and -0.14 during carbohydrate-poorer period.
				(Carb.-poorer	101	140	250	35	4	all 4	16.1	-	- 710	
		Atwater and Benedict(1904)	1	(Carb.-rich	101-105	66-96	800-983	55-68	7-8	last 3-4	16.5	-	+1500	One subject studied during 4 experiments. Complete energy balances determined. Subject did heavy work during experiments.
				(Carb.-poorer	103-106	285-333	318-436	55-68	7-8	last 3-4	16.6	-	-2700	
	PARTIAL	Rosenfeld (1906)	1	(Carb.-rich	-	-	420	-	5	last 3	13.3	-	+2340	Maximum N output on third day of carbohydrate-poor diet but still elevated on 9th (last) day.
				(Carb.-poor	-	-	70	-	9	all 9	13.3	-	-1500	
		Benedict and Milner(1907)	1	(Carb.-poorer	112	346	450	67	3	all 3	18.6	-	+ 580	Complete energy balances determined. Subject did heavy work during experiments. In first experiment, there was a 3-day interval between the two periods; also, more work was done during the carbohydrate-rich period.
				(Carb.-rich	107	87	1002	67	3	all 3	18.0	-	- 220	
			1	(Carb.-rich	101	115	731	59	3	all 3	16.9	-	- 870	
				(Carb.-poorer	103	300	321	61	3	all 3	16.8	-	-2000	
		Umeda (1915)	1	(Carb.-rich	50	30	443	35	7	last 6	8.0	7.81	-	Food apparently not analysed. Uncontrolled diet between periods. Urinary N level stationary within each period.
				(Intermediate	50	165	137	35	7	last 6	8.0	8.17	-	
				(Carb.-poor	50	190	80	35	6	last 5	8.0	8.77	-	
		Boothby, Sandiford, Sandiford & Slosse(1925)	1	(Carb.-rich	54	199	223	43	4	all 4	8.6	7.26	-	Food fully analysed. Daily N output figures not given separately. Basal metabolic rate similar on all three diets.
				(Carb.-poor	54	259	76	42	6	all 6	8.6	7.95	-	
				(Carb.-rich	55	194	215	42	3	all 3	8.8	7.12	-	

See next page for notes.

APPENDIX TO TABLE 1.

1. Where there is a bracket, the same animals were given the two diets successively.
   Where there is no bracket, separate groups were given each diet.

2. A dash indicates that the authors have not provided the necessary information.

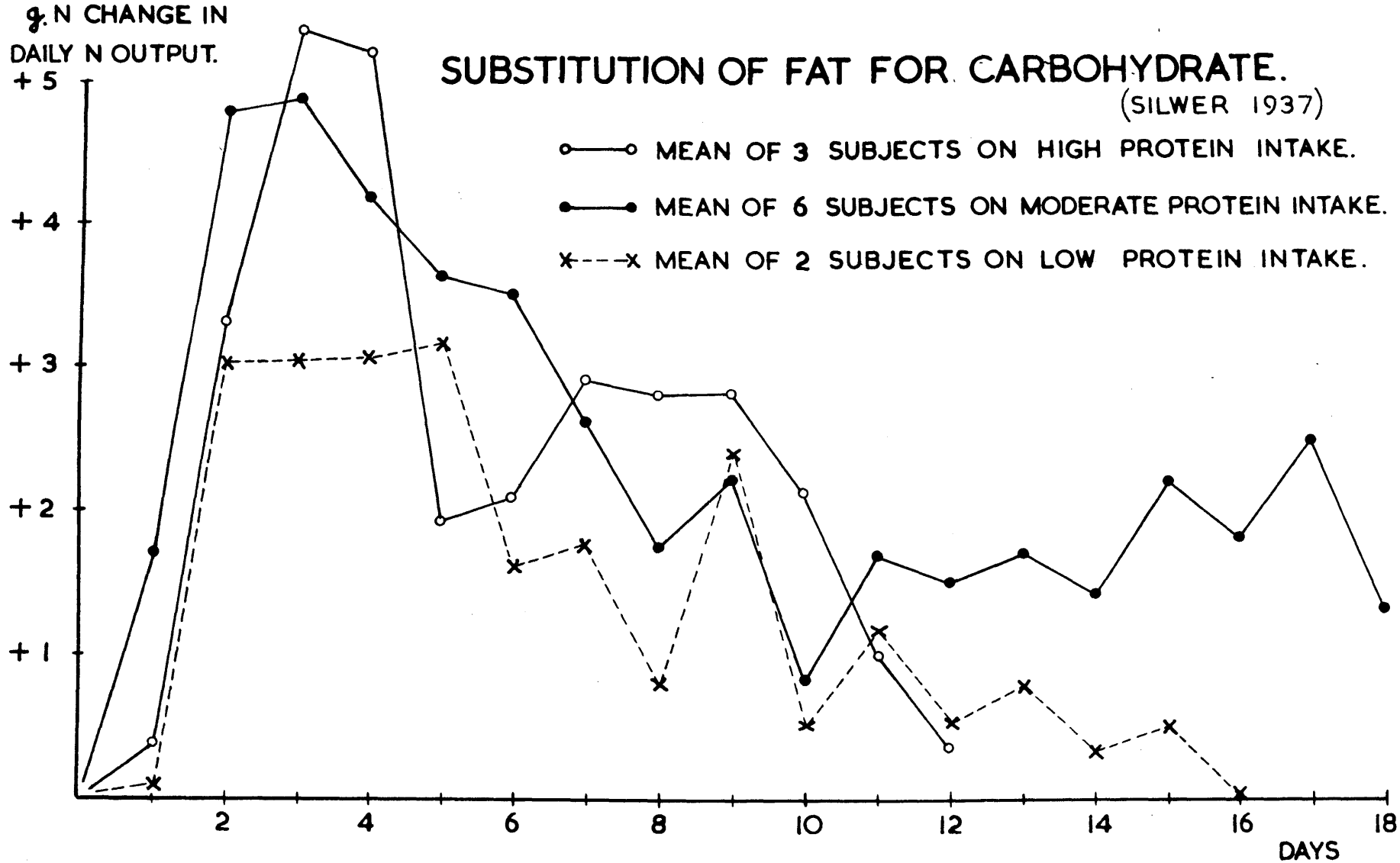

g. N CHANGE IN
DAILY N OUTPUT.
SUBSTITUTION OF FAT FOR CARBOHYDRATE.
(SILWER 1937)
MEAN OF 3 SUBJECTS ON HIGH PROTEIN INTAKE.
MEAN OF 6 SUBJECTS ON MODERATE PROTEIN INTAKE.
MEAN OF 2 SUBJECTS ON LOW PROTEIN INTAKE.
+5
+4
+3
+2
+1
2
4
6
8
10
12
14
16
18
DAYS

FIG. 2.

partial substitution experiments on man, the dog and the rat respectively. These results will now be discussed in some detail.

Experiments on human subjects (Table I). Complete replacement of carbohydrate with fat was accomplished by Kayser (1894) in an experiment on himself. After 4 days on a mixed diet, he isodynamically replaced all the carbohydrate with fat, which resulted in a steadily rising N output for the three days he continued on this regime. On his return to the original mixed diet, the N output fell again to levels slightly lower than those of the first period on the mixed diet. (These results clearly showed for the first time that carbohydrate affects protein metabolism in some specific manner, for which energy in the form of fat is not a substitute). Similar but more prolonged experiments were carried out by Silwer (1937) on several normal men and suitable male patients. The subjects began on diets containing normal amounts of carbohydrate, then after a period of stabilisation, were transferred to diets, which according to food Tables, contained the same amount of protein and energy but were almost free from carbohydrate. This resulted in a sharp rise in N output which was maximal between the second and the fifth day, then diminished and in some cases returned to the levels obtained on the diets containing abundant carbohydrate (Fig.2). Although the subjects were kept on the carbohydrate deficient diet for periods varying from 11 to 18 days, it is difficult

to decide whether protein catabolism was augmented during the whole of this time, since Silwer did not analyse the diets and faeces of his subjects for N content.

Substitution of fat for part of the dietary carbohydrate has been a much commoner type of experiment than the complete substitution referred to above. In 1902, Tallqvist published an experiment carried out on himself in which he changed from a diet containing a fairly large proportion of carbohydrate to one containing much less carbohydrate, but a correspondingly greater amount of fat. During the first diet the mean daily N balance was slightly positive (+ 0.83g.), but when he changed to the diet richer in fat the values were - 1.58, -1.24, + 0.14 and - 0.14g. on the 4 successive days of the regime. His experiment is particularly convincing because, not only did he determine the N and energy value of both diets directly, but he also kept the proportion of protein from animal and vegetable sources the same in each. On the other hand he unfortunately preceded each experimental period with one day of a milk diet, which naturally prejudices the results obtained. However, it appears reasonable to interpret his experiment as showing that the diet lower in carbohydrate content had an unfavourable effect on N balance, which was maximal on the first day, but had not completely disappeared when the experiment closed on the fourth day.

Atwater and Benedict (1904) described four studies made on

one subject who during the experiments did heavy work. Small increments in N output were found to accompany the change from carbohydrate to fat. Rosenfeld in 1906 placed a medical student on an adequate diet for several days and then exchanged fat for part of the carbohydrate during the next nine days. The N output rose, reached a maximum on the third day, but did not return to the level obtaining on the carbohydrate rich diet. Full details of the diets used in this experiment were not given. Benedict and Milner (1907) carried out two experiments during which the subject did heavy work. In their first experiment when a change was made from a carbohydrate poor to a carbohydrate rich diet, instead of the customary N retention resulting, there occurred a loss of N. This may be attributed to the fact that there was a three day interval between the experimental periods and in addition much more work was done during the carbohydrate rich period. The result of their second experiment was in direct contrast to theabove, and demonstrated a N loss when fat was substituted for carbohydrate. In this case, the authors dispensed with the uncontrolled period of three days and also ensured that there was a more equitable distribution of work over the two periods. In 1915 Umeda placed himself for periods of six to seven days on three different diets having the same protein and energy values but different proportions of carbohydrate and fat. In between each period a mixed diet of unknown composition was

consumed. The mean daily N output for each period was found to be least on the diet containing the highest proportion of carbohydrate and greatest on the diet poor in carbohydrate. In considering Umeda's data it must be pointed out that he failed to analyse the food and the faeces, which along with the taking of an uncontrolled diet between the experimental periods constitute three sources of error. In spite of this, his results appeared to agree very well with those of previous workers. Finally, Boothby, Sandiford, Sandiford and Slosse (1925) placed a subject on a carbohydrate rich diet for four days, transferred him to a carbohydrate poor diet for six days, then back to the original diet for a further three days. The N output in the urine was found to increase when fat was substituted for carbohydrate and it returned to normal when the subject was replaced on the original diet.

It must be admitted that few of the substitution experiments performed on man are completely satisfactory. Some authors have relied on tables of food composition to provide an analysis of their diets (Silwer, 1937; Umeda, 1915); this is inadequate for an exact isodynamic substitution of fat for carbohydrate and also for ensuring that the two diets have an identical N content. Thus, Toscani (1948) demonstrated that the differences between N found and N calculated varied in different diets from 0 to - 1.3g.N/day. Mitchell (1949) in an analysis of 12 hospital diets, found that the N content was from +2.6 to -0.2g. N/day

TABLE 2.

The effect of substituting fat for Carbohydrate in Dogs.

Species Used	Type of Substitution	Authors	Number Used	DIETS USED: Description	Protein	Fat	Carbohydrate	Energy	Period on diet	N INTAKE AND OUTPUT: Period upon which values are based	Daily N Intake	Mean daily urine N	Mean daily N balance	COMMENTS
					g.	g.	g.	Cal/kg.	days	days	g.	g.	mg.	
DOG	COMPLETE	Rubner(1883)	1	Carb.-free	64	50	0	39	16	last 2	10.2	9.36	-	Diet adequate to maintain dog in N equilibrium despite low energy intake.
				Fat -free	64	0	64	28	1	1	10.2	8.21	-	
				Carb.-free	64	50	0	39	4	all 4	10.2	10.20	-	
				Fat -free	64	0	80	32	1	1	10.2	7.70	-	
				Carb.-free	64	42	0	35	3	1	10.2	9.20	-	
				Fat -free	64	0	116	40	1	1	10.2	8.07	-	
		Voit and Korkunoff (1895)	1	Mixed	36	22	286	55	3	all 3	5.88	-	+ 860	Each diet preceded by several days fasting. Data from authors' tables 10 and 15 (same dog).
				Carb.-free	36	151	0	55	3	all 3	5.88	-	+ 270	
		Lüthje (1906)		Fat -low	41.6	10	150	100	13	last 6	6.66	-	-100	Protein digest used as N source in diet. No tendency for N loss to diminish during the 5 day period of carbohydrate-free diet.
				Carb.-free	41.0	100	0	124	5	all 5	6.56	-	-840	
				Fat -free	41.4	0	150	90	2	both	6.63	-	+500	
				Carb.-free	41.4	100	0	125	2	both	6.63	-	-590	
		Abderhalden, Messner and Windrath(1909)	1	Mixed	18	45	50	89	7	all 7	2.9	-	+ 700	Protein digest used as N source in diet. Reduction of N balance during second period very gradual, and of doubtful significance.
				Carb.-free	18	75	0	90	25	all 25	2.9	-	+ 380	
PARTIAL		Biernacki (1907)	1	Carb.-rich	45	8	138	84	6	all 6	7.23	-	+1520	An error in Biernacki's N balance figure for first period has been corrected. Second period is divided into three 3-day periods, with N balances of +570, +660 and +1130 mg. N per day respectively.
				Carb.-poor	38	47	56	84	9	all 9	6.07	-	+ 790	
				Carb.-rich	45	8	138	84	6	all 6	7.23	-	+1500	
		Umeda (1915)	1	Carb.-poor	22	73	60	1015 (8)	11	last 10	3.5	2.71	-	Food apparently not analysed. Uncontrolled diet taken in between successive periods, and a single day on each diet allowed before urinary collections begun. No definite trend in N output during any period, the Urinary level remaining stationary throughout.
				Carb.-rich	23	12	206	1051	11	last 10	3.7	2.15	-	
				Carb.-poor	23	86	34	1034	6	last 5	3.7	2.61	-	
			1	Carb.-poor	25	85	34	1032 (8)	6	last 5	4.0	2.92	-	
				Carb.-rich	25	45	125	1034	6	last 5	4.0	2.41	-	
				Carb.-poor	25	74	60	1037	6	last 5	4.0	2.66	-	

See next page for notes.

APPENDIX TO TABLE 2.

1. Where there is a bracket, the same animals were given the two diets successively.
   Where there is no bracket, separate groups were given each diet.

2. A dash indicates that the authors have not provided the necessary information.

8. Actual energy intake (estimated from conversion factors).

different from the N content computed from standard Tables. Secondly, several investigators have failed to determine the N excretion in the faeces (Silwer, 1937; Umeda, 1915; Boothby, Sandiford, Sandiford and Slosse, 1925), on the assumption that the absorption of nitrogen will be the same on carbohydrate rich and carbohydrate free diets. Thirdly, some authors have inserted one or more days of uncontrolled feeding between the various experimental periods, which they wish to compare on the same subject; the readjustment of protein metabolism which must follow this change in diet leads to the introduction of an uncontrolled variable into the experiment. Finally, in many of the experiments no details are given of the actual foods used and it is quite possible that the biological value of the dietary protein may have differed in the carbohydrate rich and the carbohydrate poor periods. Nevertheless, despite these shortcomings, it is abundantly clear that carbohydrate has some action on protein metabolism for which energy in the form of fat is not a substitute.

Experiments on dogs. (Table 2). Several investigators have described experiments with dogs in which carbohydrate has been wholly or partially replaced by fat. In 1883 Rubner brought a dog into N equilibrium on a diet consisting solely of protein and fat. Sugar was then substituted for the fat on three occasions. The N excretion in the urine was found to be lowest on the days when sugar was fed, notwith-

standing the fact that the caloric value of the sugar was less than that of the fat which it replaced. Voit and Korkunoff (1895) determined the minimum amount of meat required to bring dogs into N equilibrium. When meat was the sole constituent of the diet, the dogs needed more than 3½ times the amount of N in the diet than they excreted during a period of hunger. With the addition of fat to the meat the minimum N intake fell to about 1½ times the N excretion of hunger and with the further addition of carbohydrate the minimum figure was about 1.1 to 1.3 times. These results are based on experiments lasting only a few days and do not necessarily mean that the minimum N intake for equilibrium is permanently lower on a carbohydrate containing diet. From these authors' data, it is possible to construct an experiment in which the same dog was starved for several days and then given at one time a protein fat diet and at another time a mixed diet of the same N and energy content for at least three days. The N balance was found to be most favourable on the mixed diet. Luthje (1906) has published data on dogs receiving pancreatic digests as the dietary source of amino acids. One animal was transferred from a diet containing a good deal of carbohydrate to one free from carbohydrate but providing the same amount of N and a slightly higher level of energy. The dog was then transferred back to the carbohydrate containing diet and finally to the carbohydrate free diet. On each occasion, the diet lacking carbohydrate had an unfavour-

able effect on the N balance. Abderhalden, Messner and Windrath (1909), who used digested protein as a N source, have described an experiment on a dog which received digested protein along with carbohydrate and fat. When fat was substituted isodynamically for the carbohydrate, there was no abrupt alteration in N balance, although a slow decline could be seen.

Only two experiments involving partial replacement have been carried out on dogs. Biernacki (1907) carried out partial replacement of carbohydrate by fat in the diet of one dog for nine days and found an adverse effect. Examination of his data shows that the N balance was most severely affected during the first three days of the carbohydrate reduction. In 1915 Umeda carried out two series of experiments on dogs in each of which the animal first received a diet low in carbohydrate, then one rich in carbohydrate, followed finally by the diet poor in carbohydrate. The periods on these diets lasted five to ten days and each diet was preceded by some time on a mixed diet of unknown composition. The carbohydrate rich diet gave the lowest mean output, but in none of the periods was there a definite trend up or down, the urinary level remaining stationary throughout.

From the literature cited above, it can be seen that except for two isolated instances (Benedict and Milner,1907; Abderhalden et al., 1909) there appears to be general

TABLE 3

The effect of substituting fat for Carbohydrate in Rats.

Species Used	Type of substitution	Authors	Number Used	DIETS USED: Description	Protein	Fat	Carbohydrate	Energy	Period on diet	N INTAKE AND OUTPUT: Period upon which values are based	Daily N Intake	Mean daily urine N	Mean daily N balance	COMMENTS
					g.	g.	g.	Cal/kg.	days	days	g.	g.	mg.	
RAT	COMPLETE	Desgrez and Bierry (1921)	?	(Mixed (6)	0.8	1.6	1.9	250	6	-	0.12	-	Positive	Actual N balances not reported.
				(Carb.-free	0.8	2.4	0	250	1	-	0.12	-	Negative	
		Maignon and Jung (1924)	?	Fat-free	-	-	-	-	ca.28	last 5	0.33 (3)	-	-22	Rats were three weeks on diet before collections started, and lost much body weight.
				Carb.-free	-	-	-	-	ca.28	last 5	0.27	-	+15	
		Maignon and Chahine(1931a, 1931b); Maignon & Vimeux(1931) Maignon (1934) Samuels, Gilmore and Reinecke (1948)	10	Fat-free (4)	-	-	-	174	12-20	last 5-9	0.21 (3)	-	± 0	The rats were receiving 20% of energy intake in the form of protein.
			12	Carb.-low	-	-	-	188	12-20	last 5-10	0.20	-	+10	
			12	Fat-free (5)	-	-	-	178	7-19	last 4-5	0.53 (3)	-	-80	50% of the energy intake took the form of protein. N balances differ significantly (t = 4.75; P = 0.01)
			13	Carb.-free	-	-	-	172	7-19	last 4-5	0.51	-	-10	
			?	Fat-low	2.5	0.2	10.1	52	28	last 5	0.40	0.3375		According to standard errors given, difference in N output is on borderline of significance.
				Carb.-free	2.5	4.6	0.1	52	28	last 5	0.40	0.3165		
		Desgrez and Bierry (1920)	?	Carb.-rich (6)	0.8	1.6	1.9	250	3	all 3	0.23	-	+13	
				Carb.-poor	0.8	2.0	1.0	250	1	1	0.23	-	-60	
	PARTIAL	Forbes, Swift, Elliott and James (1946)	12	2% fat (7)	2.20	0.23	7.46	39.64	18	last 8	0.352	-	- 6	Only the authors' data at maintenance intakes are quoted here. Complete energy balances determined. No significant differences between groups.
			12	5% fat	2.20	0.53	6.70	39.54	18	last 8	0.352	-	- 9	
			12	10% fat	2.20	0.99	5.66	39.73	18	last 8	0.352	-	+ 4	
			12	30% fat	2.20	2.33	2.53	39.35	18	last 8	0.352	-	- 8	
		Forbes, Swift, Thacker, Smith, & French(1946)	12	2% fat (7)	2.42	0.22	7.18	39.18	18	last 8	0.387	-	- 4	Same technique as above, with increased intake of several vitamins. No significant difference between groups.
			12	10% fat	2.42	0.98	5.37	38.89	18	last 8	0.387	-	+ 2	
				30% fat	2.42	2.30	2.19	38.14	18	last 8	0.387	-	-15	
		Lathe and Peters (1949)	7	Fat-low (9)	1.7	0.2	10.1	168	5	all 5	0.266	0.168	-	Both groups started on 25% fat diet. After 5 days, group 1 put on low-fat diet by isodynamic replacement of fat by carbohydrate.
			8	25% fat	1.7	2.3	5.4	168	10	last 5	0.266	0.188	-	

See next page for notes.

APPENDIX TO TABLE 3.

1. Where there is a bracket, the same animals were given the two diets successively.
Where there is no bracket, separate groups were given each diet.

2. A dash indicates that the authors have not provided the necessary information.

3. These figures are for absorbed N.

4. Diets consisted of egg albumin 1, sugar 4 and egg albumin 1, fat 2 and sugar $\frac{1}{4}$ parts, respectively; spinach powder, yeast and salts were added to each diet. The N intakes and N balances are expressed per 150g. rat.

5. Diets consisted of egg albumin 1, sugar 1 and egg albumin 1, fat $\frac{1}{2}$ part respectively; spinach powder, yeast and salts were added to each diet. The N intakes and balances are expressed per 150 g. rat.

6. The compositions of the diets are expressed per 100 g. of the rats' body weights.

7. Actual metabolizable energy intake, directly determi ned.

9. The compositions of the diets are based on data given by Lathe and Peters (1949a).

agreement that in the case of man and the dog, the isocaloric substitution of fat for carbohydrate leads to an adverse effect on N balance. But in the case of the rat, which will now be discussed, the evidence in the literature is found to be very contradictory.

Experiments on rats (Table 3). The first complete substitution experiment was carried out by Desgrez and Bierry in 1921 working with adult rats. After receiving a mixed diet for six days they were then transferred to a diet in which all the carbohydrate was replaced by fat. The N balance on the mixed diet was positive but became negative on the carbohydrate free diet. The actual N balances were not reported. Maignon and co-workers (Maignon and Jung, 1924; Maignon and Vimeux, 1931; Maignon and Chahine, 1931 a and b; Maignon, 1934) have reported a series of experiments on rats of about 150g. body weight which lost weight during the course of the experiment. Groups were compared on protein-fat or protein-carbohydrate diets in which protein supplied either 20% or 50% of the calories. At the lower level of protein intake, N balance was similar on the protein-fat and protein-carbohydrate diets. At the higher level of protein intake, the N balance was actually better on the diet rich in fat. These experiments differ from those previously considered in that the rats were some time on the different diets before N balance was determined. If the unfavourable effect of replacing carbohydrate by fat is confined to the first few

days after substitution has occurred, then Maignon's experiments are likely to have missed it. Similar results were also obtained by Samuels, Gilmore and Reinecke (1948). These authors fed groups of rats with two diets of similar protein and energy content, one being almost devoid of carbohydrate and the other very low in fat content. N excretion was found to be greatest in the group receiving the fat low diet. Again it should be pointed out that the rats had been twenty three days on the diet before urine was collected and consequently supply us with no information regarding the immediate effect of carbohydrate deficient diets on N balance.

Several investigators have changed the proportion of carbohydrate and fat in the diet of the rat. Desgrez and Bierry (1920) have described an experiment in which adult rats were brought into positive N balance on a diet containing a fair amount of carbohydrate as well as protein and fat. When fat was substituted isodynamically for half of the carbohydrate, N balance became negative. This change in diet only lasted one day. Forbes, Swift, Elliott and James (1946) and Forbes, Swift, Thacker, Smith and French (1946) have described very carefully controlled experiments in which adult rats received isocaloric diets of different fat contents. In both cases the result is the same, namely, that N balance was not significantly better in the diets richer in carbohydrate. It must again be brought to note that the rats had been 10

days on the diets before urine and faecal collections were commenced and hence any immediate effect of fat substitution would be missed. Lathe and Peters (1949) studied two groups of adult rats which they placed on diets containing 25% of fat. After five days on this diet one group was changed to a similar diet with the fat replaced by carbohydrate, while the other group continued on the fatty diet. Urinary N excretion during the next five days was significantly different for the two groups (after correction of an error in Lathe and Peters calculations), carbohydrate substitution causing a marked reduction; the difference was greatest during the first two days after the change was made.

Examination of the above results show that these rat experiments can be divided into two groups. With one group, in which positive findings were obtained, the excreta were collected immediately following the change in the diet; but with the other, which yielded negative results, a number of days was allowed to elapse before nitrogen balance estimations were initiated.

Objects of present studies. In view of the literature just considered, the question arises as to whether the disturbance in protein metabolism produced by substituting fat for carbohydrate, can be correlated with the N loss which results when carbohydrate is transferred from a protein containing to a protein free meal. For this purpose it was convenient to use rats, but as has been shown above, although there was good

N BALANCE UNIT.

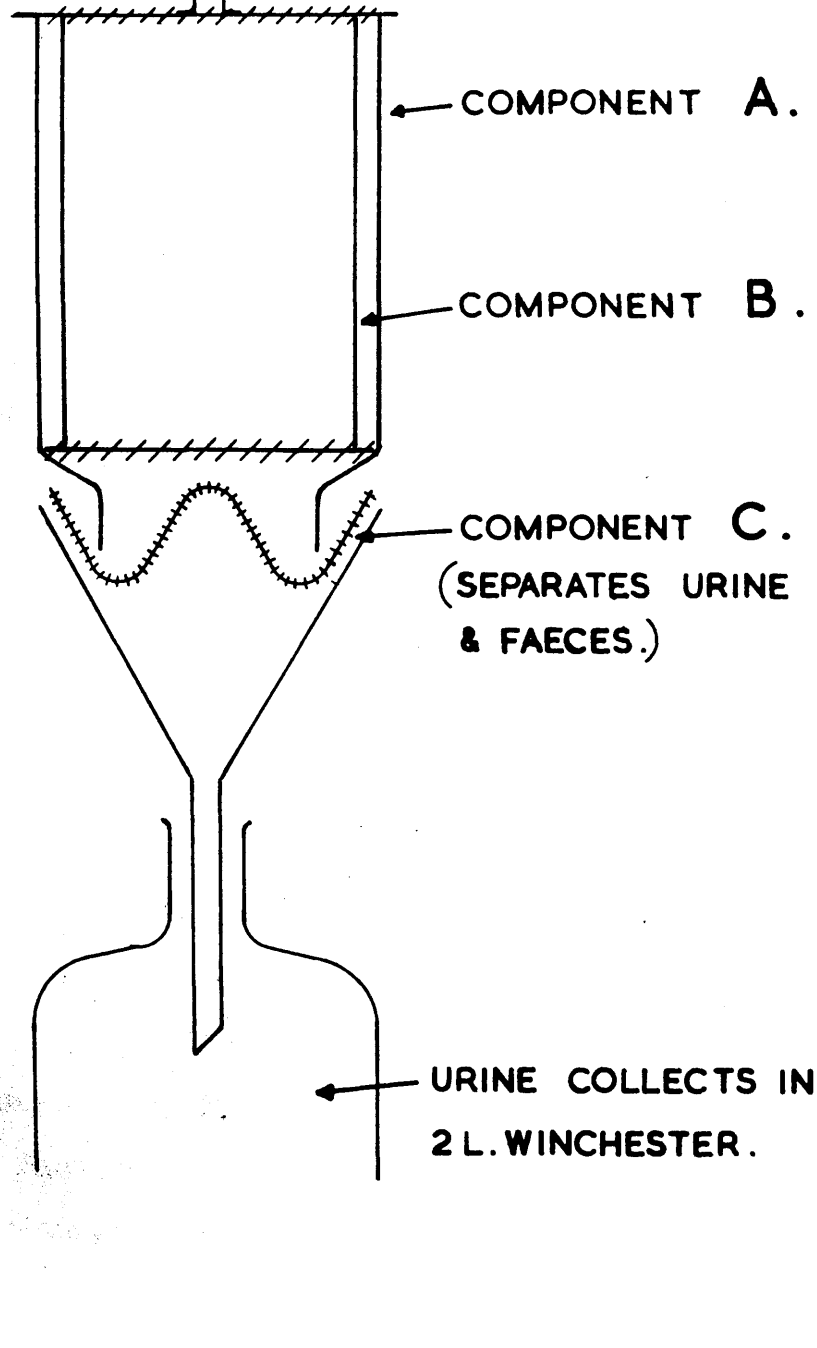


FIG. 3.

evidence in the case of the man and the dog that substitution of fat causes a deterioration in N balance the evidence in the case of the rat was contradictory. It was therefore necessary in the first place to determine the conditions under which substitution of fat for carbohydrate affects the N balance of the rat. When this was successfuly accomplished experiments were then conducted dealing with the effects of partial and complete substitution of fat for carbohydrate in both the protein-containing and protein-free meals. An attempt was then made to correlate these findings with the results obtained with two experiments dealing with the effect of eating the carbohydrates separately from the dietary protein.

EXPERIMENTAL

Animals and diets. Young adult male albino rats were used. They were fasted overnight, weighed and those weighing about 200g. were distributed between 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. They were housed individually under thermostatic conditions in glass containers (Fig.3), and fed the experimental diet twice daily. This consisted of the following purified ingredients: casein, glucose, olive oil and a vitamin-mineral-roughage (V.M.R.) mixture (Munro, 1949). The composition of the vitamin

TABLE 4.

Vitamin Mixture.

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

TABLE 5.

Salt Mixture "446"

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

TABLE 6.

Vitamin-Mineral-Roughage Mixture.

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

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

0.8 ml. of this was mixed with the above mixture.

supplement and of the mineral mixture are in Tables 4 and 5. The proportion in which these were mixed with agar and other constituents to form V.M.R. are found in Table 6. The first meal, containing 2g. of V.M.R. was given at 10 a.m. and the dishes removed at 12 a.m. The second meal given at 5 p.m. provided all the protein of the diet, namely, 2.5g. casein made into a paste with 0.15g. sodium bicarbonate. This meal was eagerly consumed. In addition to these fixed constituents of the diet, carbohydrate and fat were added to one or other meal according to the purpose of the experiment. During each experiment, changes were brought about, either by substituting fat for carbohydrate or by transferring carbohydrate from one meal to the other without altering the total amount of calories given per day. All food was moistened before feeding in order to prevent scattering.

In one experiment stomach tube feeding was used. Two solutions were injected. The first, consisting of 4g. of glucose and 2g. of vitamin-mineral-roughage mixture dissolved in a total volume of 8 mls., was rather thick and tenacious but it was thought undesirable to increase the volume still further, as large volumes are rather liable to result in diarrhoea. The second, the protein solution, consisted of 2.5g. of egg albumen dissolved in a total volume of 10 mls. Casein could not be used because of its very low solubility. The control rats received the two solutions concurrently, whereas with the experimental groups, the interval of time

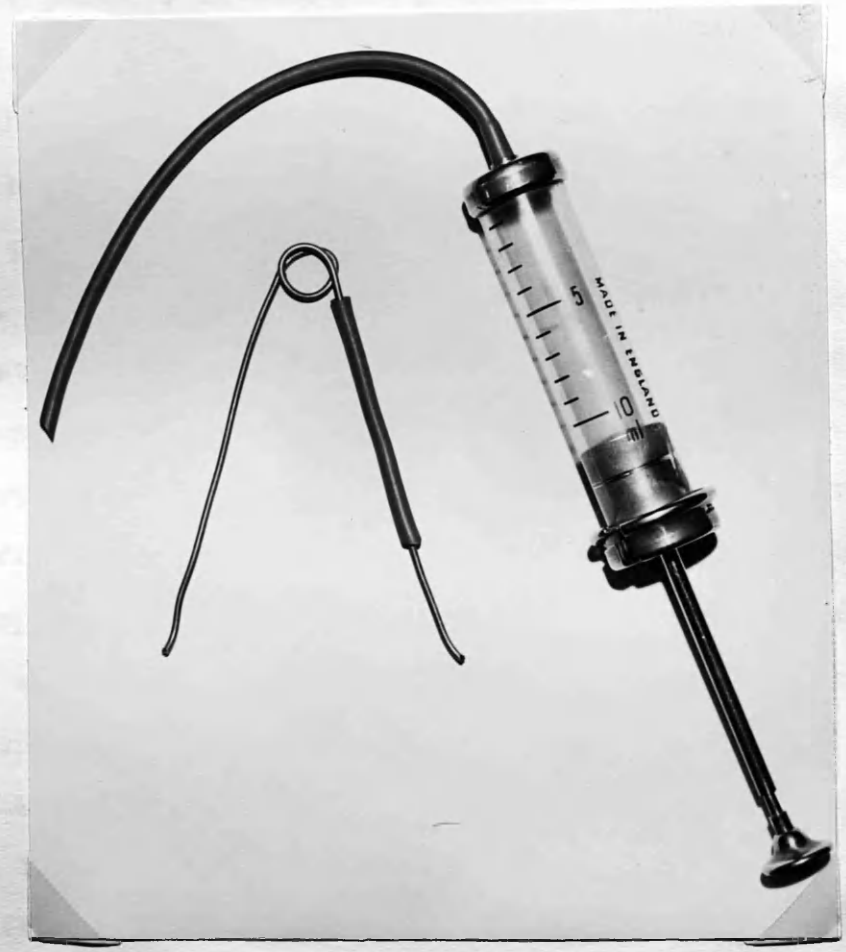


Photo. 1 - Syringe and mouth gag for tube feeding rats.

between feeding the protein and glucose varied very widely.

Initially the method used for stomach feeding was that described by Lathe (1949). The great merit of this method is that it permits intragastric feeding to be performed single handed. This was achieved by dispensing with the usual rubber stomach tube and in its place substituting one made of plastic, which becaused of its increased rigidity, allowed intubation to be performed with one hand whilst the rat was held by the other. This method was extremely convenient, but after some time we abandoned it, since in our hands the hard plastic tube was very liable to perforate the oesophagus. Accordingly, we reverted to the rubber tube and after considerable trial and error we eventually evolved quite a serviceable method. We dispensed with the usual plastic wedged-shaped mouth gag as it was not completely satisfactory and used in its place one constructed from thin copper wire. This was bent into the shape of a safety pin and when inserted into the animal's mouth tended to open out and so held its jaws apart very satisfactorily. Thin rubber tubing was slipped over the wire to prevent possible injury to the rat's mouth (Photo.1).

Collection of excreta. The N balance unit used consisted of three components (Fig.3).

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

B. This component consisting of two pieces of coarse mesh

wire gauze joined together by four metal rods, was designed to fit inside the glass containers. When in place the upper piece of netting overlapped and was in close contact with the glass rim of the bottle, so effectively imprisoning the rat, which sat on the lower piece of netting, the mesh of which was sufficiently coarse to allow the easy egress of urine and faeces. This component was easily lifted out for removing the rat or feeding dish.

C. This comprised a large filter funnel on which was placed a piece of circular fine mesh wire gauze fashioned into the shape of a Mexican hat. This component was placed beneath the neck of the glass cage, its function being the separation of the urine and faeces. The faecal pellets hit the point or sides of the conical portion of the gauze and ran down into the channel which was situated at its periphery. This gutter was placed not directly beneath the rim of the bottle's neck, but a little to the outer side to prevent urine which would normally run down the sides of the jar from constantly moistening the faeces. The filter funnel led directly into a two litre Winchester into which the urine dripped immediately after voiding. At the commencement of each experiment 20 mls. of 11 N H.Cl. were added to each receiver to prevent bacterial decomposition (Addis and Watanabe, 1916).

These individual units were mounted on a metal framework (Dexoin) which was capable of holding 18 glass containers with their accompanying Winchesters. It was mounted on

Photo.2. - Nitrogen balance unit.

TABLE 7

Recovery experiment on nitrogen balance unit.

Experiment No.	Added (mgms N)	Recovered (mgms.N)	Percentage Recovery.
1	831.6	827.4	99.5
2	831.6	826.4	99.4
3	831.6	830.6	99.9
4	831.6	827.4	99.5
5	831.6	829.5	99.7
6	831.6	825.3	99.2
7	831.6	833.7	100.3
8	831.6	828.5	99.6
Mean	-	-	99.6

wheels to allow of ease in transportation (Photo 2).

After collection of the faecal pellets each morning the glass and metal parts of the cages and faecal separators were washed down with 200 mls. of water into the urine bottles. A recovery experiment in which a urea solution was sprayed inside the cages on four successive days, gave an average recovery in 8 experiments of 99.6% with a range of 99.2 to 100.3% for individual cages (Table 7). Since the urine passed during the actual experiments contained about 300 mgms. N per day, this would introduce an error of from -2 to +1 mgms. in the recoveries from individual animals.

Faeces were marked at the beginning and end of each collection period by feeding iron oxide in the morning meal.

Nitrogen estimation. The nitrogen content of the urine, faeces and diets were estimated by the micro-Kjeldhal method as modified by Ma and Zuazaga (1942). The faeces before sampling for analysis were homogenised with water in a Folley blendor, which was very much quicker than the older method of macro-Kjeldhal digestion. All sample volumes were adjusted so that the amounts pipetted into the micro-Kjeldhal flasks contained about 1 mgm. N. This allowed of more accuracy in the subsequent titration with 0.01 N H_2SO_4. The samples were digested with 1½ mls. of concentrated nitrogen free H_2SO_4, with the addition of a bead of mercury as a catalyst. Digestion was continued for half an hour after the sample had cleared (Hiller, Plazin and Van Slyke, 1948), then the flask

was allowed to cool and the sides washed down with 2 mls. of 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 solution was added to precipitate the mercury which would otherwise interfere with the subsequent distillation. The ammonia was received into a conical flask, containing 6 mls. of 2% (w/v) boric acid and 4 drops of the mixed indicator (5 parts of 0.1% bromocresol green in 95% ethanol and 1 part of 0.1% methyl red in 95% ethanol), but collections were started only when the distillate had reached the bottom of the condenser, which eliminated the possibility of any SO_2, liberated by the alkali, collecting in the receiver. The trapped ammonia was now titrated against 0.01 N sulphuric acid.

RESULTS

The effect of complete isocaloric substitution of fat for carbohydrate in a protein containing meal. Two groups of seven rats each were fed initially on the same diet, which provided daily 346 mgms. N and 40 kg.cal./rat. This latter figure is equivalent to 1,311 kg.cal./m^2 body surface area using the formula of Lee and Clark (1929). The morning meal contained 1 ml. of olive oil in addition to 1g. of the vitamin-mineral-roughage mixture; 5g. of glucose together with 2.5g. of casein constituted the evening meal (Table 8).

TABLE 8

The effect of complete isocaloric substitution of fat for carbohydrate in a protein containing meal.

Period (4 day)	Mean Initial Body Wts.(g)		DIET *				Mean Daily Urinary N		Mean Daily Faecal N		Mean Daily N Balance.	
			Exp.		Con.		Exp.	Con.	Exp.	Con.	Exp.	Con.
	Exp.	Con.	a.m.	p.m.	a.m.	p.m.	-	-	-	-	-	-
1	202	205	C5.0g		C5.0g		296	295	23	22	+28	+29
2	-	-	F2.09g		C5.0g		323	290	25	21	- 1	+36
3	-	-	F2.09g		C5.0g		299	293	24	19	+23	+34

* Both the experimental and control groups received 1g V.M.R. and 09g olive oil in the morning meal and 2.5g casein in the evening meal throughout the experiment.

F = fat (olive oil) C = carbohydrate (glucose)

TABLE 8a

Analysis of Variance of N Balance Data.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	Variance Ratio
Total	41	206,244	-	-
Periods	2	17,957	8,979	3.05
Expt. V. Control	1	45,408	45,408	15.44
Interaction	2	37,051	18,526	6.30
Residual	36	105,828	2,940	

$F = 5.25$ at the 1% level ($n_1 = 2$ and $n_2 = 36$)

Therefore the substitution of fat for carbohydrate has a significant effect on the N balance, as shown by the significant interaction ($P < 0.01$).

TABLE 8b

Analysis of Variance of Urine Data.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Total	41	193,437	-	-
Periods	2	16,970	8,485	4.90
Expt. V. Control	1	29,389	29,389	16.99
Interaction	2	31,921	15,961	9.23
Replicates	13	75,369	5,798	3.35
Residual	23	39,788	1,730	-

$F = 5.66$ at the 1% level ($n_1 = 2$ and $n_2 = 23$)

Therefore the substitution of fat for carbohydrate has a significant effect on the urinary excretion of N, as shown by the significant interaction ($P < 0.01$).

TABLE 8c

Analysis of Variance of Faecal Data.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Total	41	10,414	-	
Periods	2	69	35	0.16
Expt. V. Control	1	1,736	1,736	7.9
Interaction	2	698	349	1.59
Residual	36	7,911	220	-

$F = 7.39$ at 1% level ($n_1 = 1$ and $n_2 = 36$)

$F = 3.26$ at 5% level ($n_1 = 2$ and $n_2 = 36$)

Although the experimental group excreted significantly more faecal N than the control group ($P < 0.01$), this was not significantly affected by substitution of fat for carbohydrate. (Interaction $P > 0.05$).

COMPLETE ISOCALORIC SUBSTITUTION OF CARBOHYDRATE FED IN A PROTEIN-CONTAINING MEAL BY FAT.

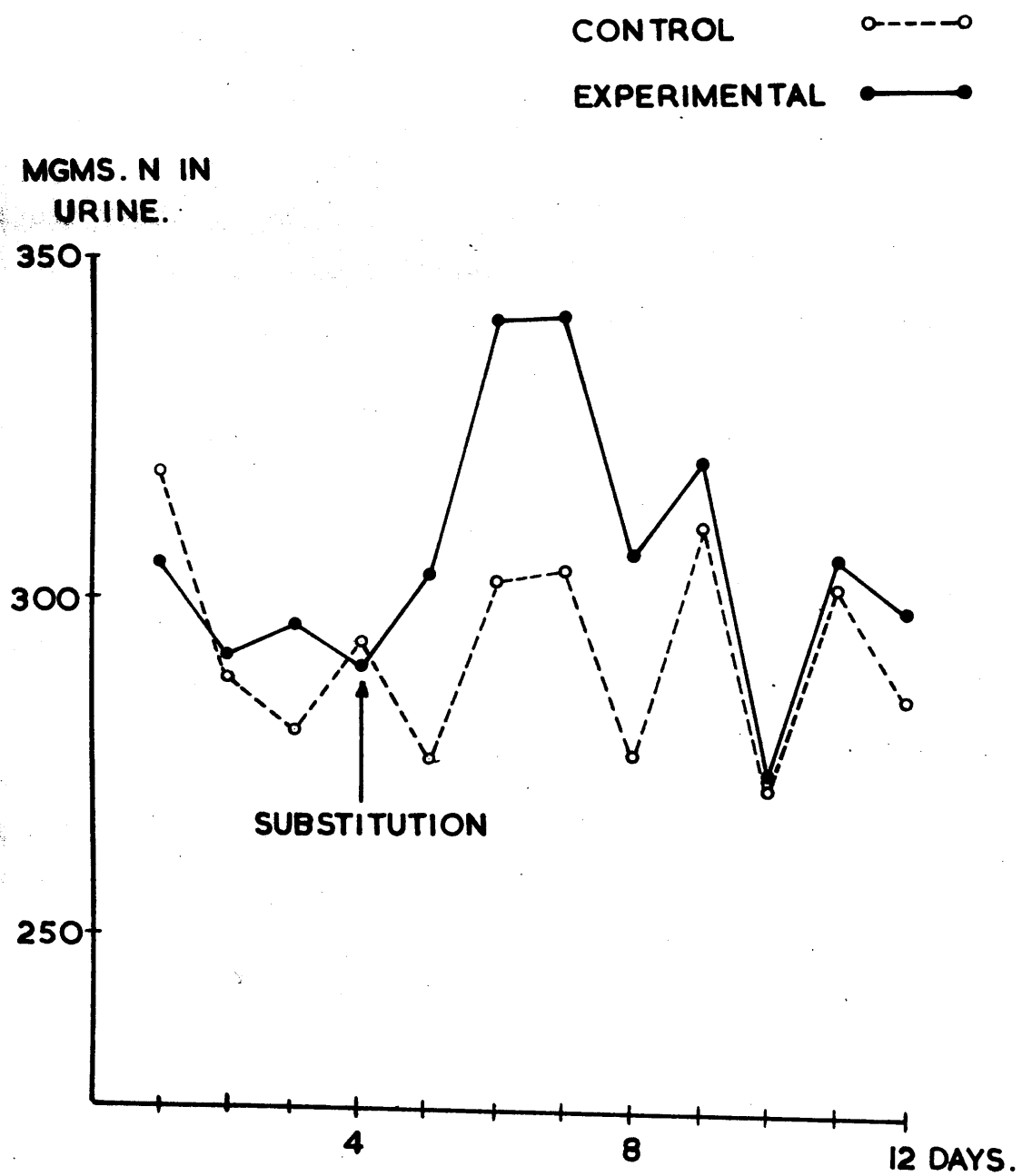


FIG. 4.

In this experiment starch, which normally forms half the bulk of our vitamin-mineral-roughage mixture (Table 6) was omitted, so that the 5g. of glucose fed in the evening meal provided all the carbohydrate in the diet. This diet was given for 7 days before excreta were collected, to allow the N metabolism of the rats to reach a steady state. The N output in the urine was estimated daily, but the faecal N was analysed over the usual four day period, because the ferric oxide marker technique would not permit of daily estimations. During the first four days of collection, both groups continued to eat the same diet and excreted essentially the same amount of N (Fig.4 and Table 8). During the remaining eight days of the experiment the control group continued on this diet, but the experimental group received 2.32 mls. (2.09g.) of olive oil in place of the 5g. of glucose fed in the evening meal. These amounts of carbohydrate and fat are both equivalent to 18.8 kg.cals., if one assumes the physiological fuel value of glucose to be 3.75 kg.cals./g., and of olive oil to be 9.0 kg.cals./g. Although without direct energy balance determinations these conversion factors can only be regarded as approximations, it should be pointed out that a change in energy intake of 1 kg.cal. has been found to result in an alteration in the N balance of the rat of only 3-4 mgms./day (Munro, 1951). An error of this magnitude is small by comparison with the changes produced in the experiments described here. The complete substitution of fat for

carbohydrate caused an immediate rise in urinary N output which persisted for four to five days and then returned to the level of output of the control group (Fig.4). This picture is confirmed by statistical analysis of the N balances (Table 8a) which demonstrates a significant difference between the experimental and control groups only during the first four day period after substitution of fat for carbohydrate. Analysis of the urine data (Table 8b) reveals an essentially similar picture. In consequence of this finding subsequent experiments were limited to the first four days after effecting a change in the diet. There was no significant change in the faecal N output (Table 8c).

The effect of substituting fat for carbohydrate in protein-containing and protein-free meals. In this experiment four groups, each of six rats were given a diet which provided daily 348 mgms. N and 35 kg.cals. per rat (1,174 kg.cals./m^2 body surface area). All groups received 2g. of the vitamin-mineral-roughage mixture together with 1 ml. (0.9g.) of olive oil in the morning meal and 2.5g. of casein in the evening meal. In addition, groups 1 and 2 were given 3g. of glucose in the evening (protein-containing) meal and groups 3 and 4 received 3g. of glucose in the morning (protein-free) meal. After the rats had eaten these diets for seven days, 1.4 mls. (1.26g.) of olive oil were substituted for the glucose fed to groups 2 and 4, the other two groups continuing on the previous diets as controls. Excreta collected over the next

TABLE 9

The effect of substituting fat for carbohydrate in protein containing meals and in meals not containing protein.

Group	Substitution	Mean Initial Body Wt. (g)	DIET *				Mean N Balance over 4 Day Period (mgms)	Difference from Control Group (mgms)
			Preliminary		Experimental			
			a.m.	p.m.	a.m.	p.m.		
1	Control	192	F0.9g	C3.0g	F0.9g	C3.0g	-43	
								-171
2	With Protein	196	F0.9g	C3.0g	F0.9g	F1.26g	-214	
3	Control	200	F0.9g C3.0g		F0.9g C3.0g		- 96	
								- 61
4	Apart from Protein	196	F0.9g C3.0g		F0.9g F1.26g		-157	

* All groups received 2g. V.M.R. in the morning meal and 2.5g. casein in the evening meal throughout the experiment.

F = fat (olive oil) C = carbohydrate (glucose)

TABLE 9a

Analysis of Variance of N Balance Data.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	Variance Ratio
Carbohydrate V. Fat	1	80,736	80,736	32.5
Protein V. No Protein	1	33	33	0.01
Interaction	1	18,260	18,260	7.4
Replicates	5	18,959	3,792	1.5
Residual	14	34,732	2,481	-
Total	22	152,720	-	-

F = 4.6 at the 1% level and 8.86 at the 5% level (n_1 = 1 and n_2 = 14). It is clear then that the substitution of fat for carbohydrate leads to a highly significant alteration in the N balance ($P < 0.01$). Also this effect is significantly greater when carried out in a meal containing protein ($P = 0.02$ for interaction). This is also shown by application of the fiducial limits, Groups 1 and 2 differing by an amount well outside the 1% level, whereas the difference between Groups 3 and 4 is just on the borderline of the 5% level.

four days showed that all four groups were in negative N balance (Table 9). The N balances of both groups 2 and 4 were adversely affected by isocaloric substitution of fat for carbohydrate, but the effect in the case of group 2 (substitution in the protein-containing meal) was much greater than in the case of group 4 (substitution in the protein-free meal). Although the substitution in the caseof group 4 did not lead to a significantly greater N output than the control group (3) it was sufficiently close to P = 0.05 to suggest that larger numbers might have made it significant (Table 9a). It may, however, be concluded from the much greater effect in group 2 that substitution of fat for carbohydrate causes a deterioration in N balance principally by affecting the utilisation of dietary protein at the time of its absorption.

The effect of partial substitution of fat for carbohydrate in a protein-containing meal. The object of this experiment was to determine whether N balance in the rat is affected by partial substitution of fat for carbohydrate in a protein-containing meal, and whether removal of carbohydrate is the sole cause of the change in N output or whether the introduction of fat into the protein-containing meal plays a part.

Four groups, each of seven rats, were started on the same diet, which provided daily 346 mgms. N and 35 kg.cals./rat (1,101 kg.cal./m^2 body surface area). The morning meal consisted of 2g. of the vitamin-mineral-roughage mixture along with 1 ml. (0.9g) of olive oil, while the evening meal

TABLE 10

The effect of complete and partial isocaloric replacement of carbohydrate in a protein-containing meal by fat, the substitutive fat being fed with and apart from the dietary protein.

GROUP.	Mean Initial Body Wt. (g)	* Diet During 4 Day Experimental Period a.m.	p.m.	N Balance over 4 Day Period (mgms)
1 - Control	219		C3.0g	-69
2 - Partial replacement, substitutive fat fed with protein.	219		C1.0g F0.83g.	-152
3 - Partial replacement, substitutive fat fed apart from protein	217	F0.83g	C1.0g	-131
4 Complete replacement, substitutive fat fed with protein.	217		F1.26g	-179

* All groups received 2g V.M.R. and 0.9g olive oil in the morning meal and 2.5g casein in the evening meal throughout the experiment. During the preliminary stabilising period the control diet was fed to all four groups

F = fat (olive oil) C = carbohydrate (glucose)

TABLE 10a.

Analysis of Variance on complete and partial replacement data.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Total	26	114,179	-	-
Treatments	3	45,888	15,296	5.15
Residual	23	68,291	2,969	-

F= 4.76 at the 1% level (n_1=3 & n_2=23) and in consequence the different treatments show a highly significant effect on N balance. The 5% fiducial limits are $\pm$ 30.1. Thus Group 1 differs significantly from the other Groups, which do not differ significantly from one another.

comprised 2.5g. of casein and 3g. of glucose. After a preliminary week on this diet, each group received a different treatment and excreta were collected for the four day period following the change in the diet. Group 1 (controls) continued to receive 3g. of glucose in the evening meal. Group 2 were given only 1g. of glucose and an amount of olive oil 0.92mls. (0.83g.) isocaloric with 2g. of glucose was added to this meal. Group 3 received the same diet as Group 2, except that the olive oil was fed in the morning (protein-free) meal. In Group 4 fat was substituted completely for the carbohydrate in the evening meal. N balance determinations (Tables 10 and 10a) show a significant deterioration in Group 2 as compared with the control Group 1; this indicates that protein utilisation is affected by partial substitution of fat for carbohydrate. The effect of complete substitution (Group 4) was not significantly greater than partial substitution. Comparison of the N balances for Groups 2 and 3 shows that it is immaterial whether the fat replacing the carbohydrate is given with the dietary protein or at another time of the day. It may accordingly be concluded that the effect of substituting fat for carbohydrate is essentially due to removal of carbohydrate from the protein-containing meal and not to an adverse effect of feeding fat with protein.

TABLE 11

The effect of transferring different quantities of glucose from a protein-free to a protein-containing meal.

Group.	Mean Initial Body Wt. (g).	DIET *				Glucose transferred (g).	N Balance over 4 Day Period (mgms)
		Preliminary		Experimental			
		a.m.	p.m.	a.m.	p.m.		
1	200	C3.0g		C3.0g		0	-88
2	196	C3.0g		C2.8g	C0.2g	0.2	-94
3	198	C3.0g		C2.5g	C0.5g	0.5	-35
4	197	C3.0g		C2.0g	C1.0g	1.0	-61
5	194	C3.0g		C1.0g	C2.0g	2.0	-47
6	200	C3.0g			C3.0g	3.0	- 7

* All groups received V.M.R. 2g. and 0.9g. olive oil in the morning meal and 2.5g. casein in the evening meal throughout the experiment.

C = carbohydrate (glucose)

TABLE 11a

Analysis of variance of N balance data.

Source of Variation	Degrees of Freedom.	Sum of Squares	Mean Square	Variance Ratio
Total	56	419,792	-	-
Replicates	9	156,328	17,370	3.48
Treatment	5	53,612	10,522	2.11
Regression	1	36,613	36,613	7.33
Deviations from linearity.	4	16,999	4,250	0.85
Residual	42	209,852	4,996	-

The regression is significant at the 1% level (n_1 = 1 and n_2 = 42 F = 7.27 at 1% level).
There are no significant deviations from linearity (for n_1 = 4 and n_2 = 42 F = 2.59 at the 5% level).

The regression coefficient = 23.8 mg. N/g. carbohydrate. Therefore for 1g. glucose transferred to the protein meal the N balance increases by 23.8 mgms. over the 4 day period.

Nitrogen balance in relation to the amount of carbohydrate fed with protein. Since the most important factor in causing N balance to deteriorate when fat is substituted for carbohydrate appears to be the removal of carbohydrate from the protein-containing meal, it was of interest to determine the relationship between the amount of carbohydrate in the protein-containing meal and N balance. For this purpose, six groups, each of seven rats, were all started on the same diet which supplied 348 mgms. N and 35 kg.cals./rat/day (1,171 kg.cals./m^2 body surface area). The rats were fed in the morning meal with the vitamins, minerals and roughage, together with 3g. of glucose and 1 ml. (0.9g.) of olive oil; the 2.5g. of casein was given alone in the evening meal. After they had been on this diet for a week, different amounts of glucose were transferred from the morning to the evening meal and the excreta collected during the succeeding four days. The results (Table 11) indicate that the presence of small amounts of glucose in the protein meal were insufficient to produce maximal improvement in N balance. Indeed the improvement in N balance was related linearly to the amount of glucose added, analysis of variance showing a significant regression at the 1% level, of N balance against the amount of glucose fed with dietary protein (Table 11a). The slope of this line, 23.8, demonstrated that there were 23.8 mgms. of N retained per g. of glucose transferred over the 4 day period.

N balance in relation to the time of feeding of carbohydrate and protein. Munro's experiments in 1949 demonstrated very clearly that the utilisation of dietary protein was greatly influenced by the simultaneous feeding of carbohydrate, since a separation of 8 hours in the time of ingestion of these two nutrients resulted in a marked increase in N excretion. The purpose of this experiment was to attempt to determine, by a gradual separation of the carbohydrate from the protein-meal, the time interval which must elapse before carbohydrate loses its protein sparing action. It was decided to feed the rats by stomach tube as this was the only means of ensuring the ingestion of two meals separated by a very short time interval, which the experimental design demanded.

Four groups of three rats each were fed initially on the same diet, which provided daily 279 mgms. of N and 31 kg. cals./rat (1,000 kg.cals/m^2 body surface area). The protein solution was fed at 10 a.m. and the glucose and vitamins at 5 p.m.. After a preliminary period of seven days on this diet the rats were divided into four groups. Group 1 received the protein and the carbohydrate together at 12.00; Group 2, the protein at 12.00 and the carbohydrate at 2.00 p.m.: Group 3, the protein at 12.00 and the carbohydrate at 5.00 p.m. and Group 4, the protein at 12.00 and the carbohydrate at 9.00 p.m. Urine and faecal collections were started and continued for four days.

The experiment as a whole was not technically satis-

TABLE 12

The effect on N Balance of separating the time of feeding of carbohydrate from that of protein.

Group.	Mean Initial Body Wt. (g)	Time interval between feeding of carbohydrate & protein (hrs)	N Balance over 4 day period (mgms)
1	203	0	-114
2	219	2	-430
3	235	5	-248
4	224	9	-390

TABLE 12a

Analysis of variance on N Balance data from separation experiment.

Source of Variation	Degrees of freedom	Sum of Squares.	Mean Squares	Variance Ratio
Total	10	425,559		-
Times of feeding carbohydrate.	3	188,176	62,735	1.85
Residual	7	237,383	33,912	-

$F = 4.35$ for 5% level (for $n_1 = 3$ and $n_2 = 7$).
Therefore variation in the time of feeding the carbohydrate with respect to the protein has no significant effect on N Balance.

factory. The rats lost a good deal of weight during the experiment and in addition suffered from some diarrhoea. This was probably due to the volumes injected being too large, but on solubility grounds it was impossible to decrease them to any extent. The results (Table 12) would appear to show that the carbohydrate must be fed within two hours of the protein meal in order to exert its sparing action. This, however, is only supposition as the results were not statistically significant (Table 12a).

DISCUSSION

Our observations show a significant deterioration in N balance when carbohydrate is wholly or partly replaced by fat in the diet of the rat. Data published by other authors fail to agree on this point. Some (Desgrez and Bierry, 1920 and 1921; Lathe and Peters, 1949) also obtained evidence of the superiority of carbohydrate. On the other hand, no beneficial effect was observed by Maignon (1934) in numerous partial and complete substitution experiments, and in an experiment reported by Samuels, Gilmore and Reinecke (1948) N output was not significantly greater on a protein-fat diet than on an isocaloric protein-carbohydrate diet. Similarly in the studies of partial exchange of fat for carbohydrate undertaken by Forbes and his associates (Forbes, Swift, Elliott and James, 1946; Forbes, Swift, Thacker, Smith and

French, 1946), there was no evidence of an increased N output in either series of experiments in which fat was exchanged for carbohydrate.

In considering these experiments as a whole, it is significant that the authors who observed a favourable effect of carbohydrate on N balance had collected the excreta immediately after changing the amount of carbohydrate in the diet, whereas all the negative findings were obtained in experiments in which the rats had been on the high fat intake for a number of days before the study of N output was made. We have observed (Fig.4 and Table 8) that the increase in N output which follows substitution of fat for carbohydrate is quite transitory. It is thus not surprising that the results obtained by other authors are related to the length of time elapsing between the substitution and determination of N balance .

The relationship between the transference of carbohydrate and its substitution by fat. The short duration of the disturbance in protein metabolism caused by substituting fat for dietary carbohydrate is so similar to the transitory effect noted after carbohydrate is transferred from a protein-containing to a protein-free meal (Munro, 1949), that there would appear to be some connection between these two phenomena. That this is so, can be shown by an examination of our data.

First, the amount of fat in the protein containing meal plays no part in the change in N balance when fat is

substituted for carbohydrate, since the effect of feeding the substitutive fat with or apart from protein is similar, (Table 10 - Groups 2 and 3). This finding agrees with the previous observation that the transfer of fat from protein-free to protein-containing meals on the same diet does not alter N balance (Munro, 1949). Thus, when 3g. of glucose, given with a protein meal, are exchanged isocalorically with fat the increase in the N excretion which results, should be due to carbohydrate removal alone and not to the presence of fat. If all the data for complete substitution of fat for carbohydrate in a protein-containing meal (Tables 9 and 10) are averaged, the difference between the control and experimental groups is 140 mgms. N over the four day period. Now if this loss of N is due solely to removal of carbohydrate from the protein meal, then the N retention resulting when 3g. of glucose are transferred from the morning meal to the protein-containing meal should compare with that figure. Using our linear regression data (Table 11a), it can be calculated that 71 mgms. of N should be retained over the four day period. This is only half of the N loss which occurs when carbohydrate, instead of being transferred, is completely replaced by fat. It would thus appear that in the transference experiments the 3g. of glucose which are given seven hours before the protein-containing meal are still sparing protein to an extent of 69 mgms. over the four day period. Reference

to Table 9 (Groups 3 and 4) shows this hypothesis to be correct; when 3g. of glucose given in the protein-free (morning) meal are isocalorically replaced by fat, there is a loss of 61 mgms. N over the four day period. This 61 mgms. of N was being spared by carbohydrate until it was removed and replaced by fat.

Thus these two phenomena (substitution of carbohydrate in a protein-containing meal by fat and transference of carbohydrate from a protein-containing meal) are similar and are primarily due to the amount of carbohydrate fed with the protein. They differ in their magnitude merely because carbohydrate even when fed seven hours before the protein meal, can still cause N retention to a considerable extent. The nature of this sparing effect of carbohydrate when fed apart from dietary protein will be considered later.

SECTION 2

THE DIGESTION, ABSORPTION AND TISSUE DISTRIBUTION OF NITROGEN WHEN CARBOHYDRATE IS FED WITH PROTEIN.

INTRODUCTION

In the preceding section it has been shown that the disturbance in protein metabolism which results when fat is substituted for carbohydrate, is very similar to the effect which occurs when carbohydrate is transferred from a protein-containing to a protein-free meal. It was then suggested that both these effects could be attributed primarily to the amount of carbohydrate fed with the protein. These findings indicate therefore that an interaction occurs between dietary protein and carbohydrate when eaten in the same meal. Two possibilities regarding the site of such an interaction exist. It is conceivable that it may occur (a) in the small intestine during the absorption and digestion of the protein or (b) during their subsequent metabolism. Only the former of these two contingencies will be discussed in this section. In addition, the tissue distribution of the N which is retained due to this interaction effect is at present unknown and so some observations from the literature, which may have a bearing on this problem will also be considered.

A - The intestine as the site of the interaction effect.

In the first place, the various avenues through which glucose might exert its N sparing action on protein, whilst still in the intestinal tract, will be explored and any evidence from the literature supporting or discrediting these

pathways will be cited.

Digestibility of protein. The beneficial action of glucose on protein metabolism could feasibly be due to its causing an increase in the digestibility of the simultaneously fed protein. The final extent of absorption of N would then be augmented and as this is tantamount to an increase in N intake, a transient period of N retention would follow. There is very little relevant material in the literature pertaining to this point, but Mitchell (1934) has stated that faecal N and hence digestibility, is not ordinarily related to the proportions of carbohydrate or fat in the diet.

Rate of release of amino acids during digestion. Even though carbohydrate has no effect on the digestibility of protein, it is becoming increasingly appreciated that mere completeness of digestion is not a sufficient criterion. The essential amino acids must be absorbed simultaneously in order to function successfully in protein synthesis (Cannon, Steffee, Frazier, Rawley and Stepto, 1947; Geiger, 1947) and thus the rate of release of different amino acids during digestion can affect the biological value of a protein (Melnick, Oser and Weiss, 1946; Pader, Melnick and Oser, 1948; Lowry and Thiessen, 1950). There is no reference in the literature to the effect of glucose on this aspect of protein digestion and this question will have to be further

investigated.

Rate of digestion and absorption of protein. Geiger (1951) has made some pertinent observations on the relationship existing between the rate of digestion and absorption of a protein and its efficacy in supporting growth. He observed that diets containing skimmed milk as the only protein source had a stronger growth promoting action on young rats than diets containing beef, pork or veal. Since the amino acid composition of these proteins is practically identical, the possibility was investigated that differences in the rate of digestion and absorption could account for the observed disparities in their effect on growth. It was found that the milk protein disappeared much more slowly from the intestinal tract, than any of the other proteins i.e. the protein with the highest growth promoting action showed a very protracted absorption. Now tissue protein synthesis requires the simultaneous presence of all the amino acids and evidently dietary proteins will be best utilised by the tissues when the rate of supply from the digestive tract corresponds with the tissue requirements. If the rate of supply is too fast then utilisation will suffer, because no excess of essential amino acids can be stored in the tissues for longer than one hour (Cannon et al.,1947); the surplus is then either excreted or irreversibly metabolised (Rose, 1928; Geiger, 1950). Therefore a delay in digestion and absorption of the protein should tend to improve the tissue utilisation. This

concept is also substantiated by the investigations of Leverton and Gram (1949) and Wu and Wu (1950). Their experiments with adult human subjects indicated that protein utilisation was improved when the intake was spread evenly throughout the day, by consuming more frequent, but smaller protein meals. In further support of this hypothesis was the observation by Geiger (1951) that lactalbumin, which supports growth to a higher degree than casein, disappeared more slowly from the digestive tract. The delay which occurs in absorption may be attributed to the fact that some inhibition in the absorption of amino acids may be produced by the presence of other amino acids in the intestinal tract (Pinsky and Geiger, 1952; Orten, Koizumi, France and Johnston, 1951; Kamin and Handler, 1952). Different proteins may therefore be absorbed at different rates because of dissimilarities in their amino acid composition. However, it is clear that a delaying effect of glucose on amino acid digestion and absorption, by improving the tissue utilisation of the liberated amino acids, could possibly account for its N sparing effect. The relevant literature will now be discussed to see if there is any support for this theory.

With regard to the mechanics of digestion, carbohydrate has no consistent effect on the rate of gastric emptying. It has been noted that starchy foods (Cannon, 1911) and small amounts of sucrose (Hunt and Spurrel, 1948) accelerate the rate, whereas larger amounts of sucrose and glucose retard

evacuation (Hunt and Spurrel, 1948; Hurthle, 1930).

Extremely little work has been done on the effect of carbohydrate on the digestion and absorption of proteins. The first comprehensive report was that of London and Sivre in 1909, who working with dogs with fistulae at various points in the small intestine, studied the absorption rate of orally fed starch and raw meat, both alone and in combination. These authors observed that the addition of the carbohydrate resulted in an increased rate of absorption of the protein, as evidenced by the decreased amount of N recovered from the fistulae. Similar experiments with partially digested meat gave identical results. Evidence of a contrasting nature was obtained however by Rothschild and Cera (1938) who used a dog with a Vella fistula of the small intestine to demonstrate that the absorption of a peptone solution was appreciably delayed by the presence of glucose.

The evidence regarding the effect of carbohydrates on the absorption of single amino acids is also rather contradictory. Cori (1926) observed a slight delay in the absorption of glycine when a glucose-glycine mixture was tube fed to rats. He termed this phenomenon "Mutual Inhibition of Absorption" and concluded that sugars and amino acids were absorbed at the same point in the cells of the intestinal mucosa. On the other hand, Wilson (1951) who carried out a similar experiment failed to obtain a significant depression in the absorption rate. The effect of carbohydrate on the absorption of other

single amino acids does not seem to have been explored.

From the literature cited above it can be seen that there is no definite proof for a delaying effect of glucose on protein digestion and absorption and in addition no observations whatsoever have been made on the effect of glucose on the rate of release of amino acids during digestion. The effect of glucose on protein digestibility has also received little attention. It was decided therefore to conduct experiments with the object of demonstrating (a) the effect of glucose on the digestibility of casein, (b) the effect of glucose on the rate of release of amino acids during digestion. The approach to this problem was to see if glucose could spare the utilisation of a simultaneously fed casein hydrolysate. If it does, then obviously the rate of release of the amino acids plays no part in the interaction effect, (c) the effect of glucose and fat on the absorption of a casein hydrolysate.

B - Tissue distribution of N after simultaneous feeding of carbohydrate and protein.

There is good reason for believing that a part of the body protein is much more active metabolically than the rest. The evidence for the existence of this labile protein has been reviewed by Kosterlitz and Campbell (1945); they emphasise that protein of this character is to be regarded as a cytoplasmic component and not as an inert deposit. The proportion of labile to fixed protein varies greatly from tissue to tissue

the viscera in general and the liver in particular having a greater proportion than the carcass (Addis, Poo and Lew, 1936). Isotope experiments (Schoenheimer, 1942) have confirmed this difference in activity between proteins of different tissues.

When protein and carbohydrate are eaten together, N balance only undergoes a temporary improvement (Munro, 1949). Subsequent separation of the protein and carbohydrate leads to an equivalent and rapid N loss. This observation indicates that the continued feeding of carbohydrate with protein is necessary to prevent the retained material from being excreted. A possible explanation of these findings is that they are due to variations in the amount of labile protein.

Experiments with growing rats are also very suggestive in this respect. Cuthbertson, McCutcheon and Munro (1940) have noted that the rate of growth of young rats is not affected by the time of feeding dietary carbohydrate and protein. Now the N accretion, which occurs during growth, is probably in the form of stable body proteins, which according to the above authors, are not influenced by the simultaneous feeding of carbohydrate and protein. This suggests, therefore, that the effect of carbohydrate and protein eaten at the same meal may be on the labile protein fraction of the tissues.

Similar conclusions may be drawn from observations made on fasting dogs. It has been noted that exercise carried out during the early stages of starvation raises the

N output, but that this effect disappears as fasting proceeds (Chambers and Milhorat, 1928; Frentzel, 1897). Chambers and Milhorat (1928) consider that this failure to evoke a response to exercise after prolonged starvation is due to the exhaustion of the labile protein reserves. They have also shown that the administration of glucose prevents this rise in N output which occurs with exercise and it may therefore be suggested that glucose acts by sparing this labile protein.

The observations cited above imply that the action of carbohydrate is on some labile body component, but evidence regarding the site at which these changes occur is lacking. However, the results of experiments by Addis, Poo and Lew (1936) and by Kosterlitz (1947) may be interpreted as indicating a possible location of the labile protein involved. These workers noted that the amount of protein in the liver was considerably influenced by protein intake and that the effect of changing from one dietary level to another was nearly complete within a few days. It may be presumed that the improvement in protein utilisation which occurs when carbohydrate is fed with dietary protein, is equivalent to an increase in protein intake. This effect is limited to a few days and would in this respect suggest the characteristics of protein deposition in the liver, which quickly reaches a new equilibrium level. Thus, there are some grounds for assuming that the simultaneous feeding of carbohydrate and

protein leads to a rapid deposition of labile protein in the liver. This persists until an equilibrium between synthesis and catabolism is reached, the attainment of which is indicated by the return of the N balance to normal levels. If carbohydrate is now removed from the protein containing meal, then conditions become much less favourable for the continuance of protein synthesis and labile protein is broken down and excreted until a new equilibrium is established between synthesis and catabolism.

Working on that premise, we carried out experiments with rats in which carbohydrate was transferred from a protein free meal to a meal containing protein. After four days, during which time the N retention was estimated, the animals were killed. The livers, along with the other viscera were removed and analysed to see if any increase had occurred in their N content; changes in these organs were then compared with the total retention of N.

EXPERIMENTAL

Animals, diets and collection of excreta. Adult male albino rats were used and were fasted overnight prior to use in order to get a reliable body weight determination. They were housed under thermostatic conditions. The diets used were as described in Section 1, except that in some cases an enzymic casein hydrolysate (" Amigen") was used in place of whole

casein. Two feeds were given per day, the morning one containing the vitamin-mineral-roughage mixture (Munro, 1949) and the evening meal the protein (casein or casein hydrolysate). Carbohydrate and fat were incorporated into one or other meal, according to whether it was desired to feed them with or separately from the dietary protein. The collection of urine and faeces and the analysis of urinary, faecal and food N were all carried out as previously described (Section 1).

Special procedures.

(a) Absorption studies. Groups of three rats were fed by stomach tube either

10 mls. of enzymic casein hydrolysate solution (1.25g) or

10 mls. of enzymic casein hydrolysate solution (1.25g) + 1.50 g. glucose or

10 mls. of enzymic casein hydrolysate solution (1.25g) + an isocaloric amount of olive oil, 0.7 mls. (0.63 g.).

After a two hour absorption period, the abdomen was opened under nembutal anaesthesia. The oesophagus and rectum were ligated and the gastro-intestinal tract removed. The gut was slit along its length, placed in a porcelain basin, cut up into small pieces and transferred with washings to a conical flask, where it was shaken vigorously with 100 mls. of distilled water. After settling, the supernatant was filtered through glass wool into a 250 ml. centrifuge bottle. The residue was shaken up twice with 50 ml. portions of distilled water. Sufficient 20% (w/v) trichloracetic acid was now added

to the combined washings in the centrifuge bottle to make the final concentration 6%. It was allowed to stand for 1 hour then centrifuged. The N content of the supernatant was then determined (unabsorbed casein digest). A solution of casein hydrolysate was tested with 20% trichloracetic acid and gave no precipitate. A correction was made for soluble nitrogenous compounds present in the gut of fasting rats. This figure (17.0 mgms. N ± 3.88 S.D.) agrees well with the value of 12.03 mgms. N quoted by Wilson and Lewis (1929) for the N content of the gut of fasting rats.

(b) N distribution experiments. After the completion of the N balance part of some of the experiments, the animals were killed by exsanguination under ether anaesthesia. The liver, kidney, heart and intestine were removed and washed with distilled water. The intestine was slit open and both surfaces quickly washed with distilled water. The tissues were then stored in dilute sulphuric acid. Their N content was estimated by bringing them into solution with N-free sulphuric acid in a macro-Kjeldahl digestion flask. Aliquots were then taken for micro-Kjeldahl estimations, which were carried out as previously described (Section 1).

RESULTS

Effect of glucose on digestibility of casein. The faecal N data from two suitable experiments carried out in Section 1

TABLE 13.

The effect on faecal N output of feeding carbohydrate with and apart from dietary protein.

Expt. No.	No. of Rats	Mean Daily N Intake (mgs.)	Protein and Carbo-hydrate.	Mean Daily Faecal N (mgs.)
1	4	348	Separately (Control)	39
	4	348	Together (Expt.)	37
2	7	348	Separately (Control)	38
	7	348	Together (Expt)	31

The faecal N values for Experiment 1 and 2 were compared with Fisher's "t" test. There was found to be no significant difference between them ($P > 0.05$)

TABLE 14

The effect of feeding carbohydrate or fat with or apart from an enzymic digest of casein.

Exp.	Group	No. in Group	Mean Initial Body Wt. (g)	Period I			Period II			Difference in Mean Daily N Balance.	
				Evening Feed	Daily N Intake (mgms)	Mean Daily N Balance (mgms)	Evening Feed	Daily N Intake (mgms)	Mean Daily N Balance (mgms)	II-I (mgms)	"t"
1.	Carbohydrate transferred	6	342	Digest, fat and carbohydrate.	399	+ 42	Digest and fat	396	+1	-41	4.99
2.	Carbohydrate transferred	4	415	Digest	379	+ 9	Digest and carbohydrate	381	+60	+51	4.04
	Fat transferred	4	418	Digest	378	+18	Digest and fat	380	+18	0	

The effect of carbohydrate transfer from one meal to the other is highly significant ($P < 0.01$)

were utilised here (see Tables 9 and 11). Two experiments were performed, each comprising a control and an experimental group. Both groups were fed initially on the same diet, the morning meal consisting of V.M.R., olive oil and glucose; casein was fed in the evening meal. After a preliminary period of a week on this diet, the glucose in the experimental group was transferred to the protein (evening) meal, while the control animals remained on the original diet. Faeces were then collected for 4 days. It can be observed from Table 13 that no considerable change occurs in the faecal N when carbohydrate is fed with the protein. Statistically there is no significant difference between the respective values ($P > 0.05$). Thus glucose has no effect on the digestibility and hence the final amount of N absorption from casein.

Effect of feeding carbohydrate or fat with or apart from a casein hydrolysate. The object of this experiment was to see if the sparing action of glucose could be demonstrated when a casein hydrolysate was used as an N source instead of whole casein (Table 14). In Period 2 the carbohydrate which had been previously fed with the digest (Experiment 1) was given in the morning meal. This separation resulted in a loss in N which was statistically significant ($P < 0.01$). With Experiment 2 this transfer was carried out in the opposite direction - in Period 2 the carbohydrate was transferred to the hydrolysate containing meal. A marked retention of N occurred which was

TABLE 15.

The effect of glucose and fat on the absorption of casein hydrolysate (rats were tube fed).

Expt. No.	Absorption Rate (mgms./hour)		
	Casein Hydrolysate	Casein Hydrolysate + Glucose	Casein Hydrolysate + Fat
1	56	50	55
2	56	43	66
3	59	43	68
4	56	48	60
Mean	57	46	62

TABLE 15a.

Analysis of Variance of absorption data.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Total	11	696	-	-
Treatments	2	547	2.74	16.5
Residual	9	149	16. 6	-

For $n_1 = 2$ and $n_2 = 9$, $F = 8.02$ at the 1% level.

The 3 treatments are therefore highly significantly different. Application of the 5% fiducial limits reveal that casein hydrolysate + glucose differs significantly from the other 2 treatments, which do not differ amongst themselves.

again statistically significant ($P < 0.01$). In this experiment the effect of transferring fat to the evening meal (hydrolysate containing) was also tried. No change in N balance was observed. The results of these experiments agree with the observations of Munro (1949) and also with our findings in Section 1, when in both cases casein was the protein used. Thus the protein sparing action of carbohydrate is identical when either casein or casein hydrolysate is used as the N source.

The effect of glucose and fat on the rate of absorption of casein hydrolysate. It can be seen from Table 15 that glucose depresses the absorption rate whereas fat does not. This effect is statistically significant (Table 15a). The absence of the delaying effect by fat on the rate of absorption may be related to the fact that, with tube feeding of the digest, the pyloric valve is forced open and hence the effect of fat on gastric evacuation does not occur.

Tissue distribution of N. Two experiments were conducted each comprising a control and an experimental group. Both groups were fed initially on the same diet, the morning meal consisting of V.M.R., olive oil and glucose; casein was fed in the evening meal. After a preliminary week on this diet, the glucose in the experimental group was transferred to the protein (evening) meal, while the control animals remained on the original diet. Urine and faeces were then collected

TABLE 16

The N content of the viscera after feeding carbohydrate with and apart from dietary protein.

	Experiment 1			Experiment 2			Mean difference in 2 expts
	Protein with Carb. (Exptl)	Carb. alone (Control)	Diff.	Protein with Carb. (Exptl)	Carb alone (Control)	Diff.	
No. of rats	8	8	-	4	4	-	-
Mean initial wght.	226	229	-	249	245	-	-
N balance during period (mgms)	+92	+32	+60	-63	-121	+ 58	+ 59
Liver N (mgms)	261	245	+16*	270	283	- 13	+ 2
Intestinal N (mgms)	228	234	- 6	243	249	- 6	- 6
Kidney N (mgms)	51	52	- 1	55	52	+ 3	+ 1
Heart N (mgms)	24	24	0	25	25	0	0
Retained N not accounted for	-	-	51	-	-	58	59

The results in this Table were obtained in collaboration with Mr. D.J. Naismith.

* When adjusted by co-variance analysis for differences in body weight, the livers of the experimental group contained significantly more N than the livers of the control group. ($P < 0.01$).

for four days. At the end of the collection period the animals were killed and the liver, kidney, heart and intestine removed and analysed for their N content as described previously. It can be observed from Table 16 that the amount of N in the liver is significantly increased only in Experiment 1 ($P < 0.01$). Very little weight can be attached to this result as the liver in Experiment 2 showed a loss in N when carbohydrate and protein were fed together. From the mean of the two experiments it can be seen that none of the retained N can be accounted for in the viscera.

DISCUSSION

The effect of glucose on the digestibility of casein. As can be seen from Table 13 the presence of carbohydrate does not interfere with the digestibility and hence the final extent of absorption of casein. However, it is becoming appreciated that mere completeness of digestibility is not a sufficient criterion. As has been previously mentioned differences in the rate of release of amino acids during digestion has been found in some cases to influence the biological values of proteins, but such an effect can play no part in the protein sparing action of carbohydrate, since we have demonstrated the sparing action of carbohydrate when an enzymic digest of casein is used as the source of dietary N (Table 14).

Effect of glucose and fat on the absorption of casein hydrolysate. From Table 15 it can be observed that carbohydrate significantly reduces the rate of absorption of casein hydrolysate (Table 15a). On the contrary fat has no effect, a finding which does not agree with the generally accepted fact that fat delays gastric evacuation. Our results could be attributed to the pyloric sphincter being forced open during the operation of forced feeding. The significance of the reduction in the absorption rate due to carbohydrate can best be appreciated when compared with the effect of carbohydrate and fat on the curve of N excretion after a meal. It has been shown that the addition of either carbohydrate or fat to a protein meal causes a delay in the peak of N excretion in the urine, which is presumably due to retardation of absorption of amino acids from the intestine (Vogt, 1906; Pari, 1908; Falta and Gigon, 1908). But whereas the giving of fat with protein delayed N excretion without reducing the total N output in the urine, the administration of carbohydrate produced a true sparing action besides an alteration in the curve of N excretion. It may be deduced therefore, that simple delay in absorption does not necessarily confer a protein sparing action and the explanation of the interaction of carbohydrate and protein fed together must be sought in some subsequent phase of their metabolism.

That these deductions are correct is shown by the

fact that glucose still exerts its sparing action on N output when given intravenously along with a mixture of amino acids (Elamn, 1953). It is also indicated by the sparing action of carbohydrate on "endogenous" protein metabolism to be dealt with in Section 3.

Tissue distribution of N after feeding carbohydrate and protein together. It can be observed from the data tabulated in Table 16, that when carbohydrate and protein are fed together, the amount of N in the liver is significantly increased only in one of the experiments and in this case it accounts for only approximately 25% of the total N retained. It is felt that little weight can be attached to this result as the liver in the other experiment actually shows a loss in N when carbohydrate and protein are fed together. The simultaneous feeding of carbohydrate and protein did not have any effect on the N content of the other viscera investigated, namely, intestine, kidney and heart and in point of fact the mean of the two experiments indicates that none of the N which is retained can be accounted for in the viscera. It would thus appear that the bulk of the retained N is deposited in the carcass i.e. muscle, skin and skeleton. Of these muscle is by far the most likely site.

These results are not altogether surprising when considered in conjunction with the observations of Addis, Poo and Lew (1936) that after fasting rats for 7 days, the

livers lost 40% of their original protein and the carcasses 8%. This meant, however, that the liver had contributed only 16% of all the protein lost by the body and the carcass 62%. Thus the high percentage of labile protein in the liver should not obscure the fact that the carcass contains a greater quantity by virtue of its larger mass, so that muscle tissue has every chance of incorporating a large percentage of the retained N. It may be noted that an increase in labile protein content of muscle will not be easy to determine, as obviously quite a large change can occur without any evidence being detected on tissue analysis due to the small percentage change involved. For this reason, no attempt was made to estimate changes in carcass N in these experiments.

SECTION 3

THE EFFECT OF GLUCOSE AND FAT ON THE PLASMA AMINO ACID LEVEL.

INTRODUCTION

It was shown in Section 1 by means of N balance studies, that glucose, as distinct from fat, can spare the utilisation of dietary protein and that this action depends at least to a large extent on the feeding of the glucose along with the protein. In Section 2 it was observed that carbohydrate when fed simultaneously with protein produces a delay in its absorption, but no correlation was found to exist between this effect and the sparing action of carbohydrate on protein metabolism. However, the techniques used up to the present have not given any indication of the metabolic pathways through which this effect is mediated. To do so recourse must be made to other experimental methods of exploring protein metabolism. One such approach is the examination of changes in the blood amino acid concentration. It was in 1912 that Van Slyke and Meyer first reported the presence of amino nitrogen in the non-protein fraction of the blood of a dog, and that its concentration increased after feeding meat. In the following year a considerable quantity of free amino acids were isolated from the blood by Abderhalden (1913). The work of these investigators thus established the important point that a correlation existed between the level of free amino acids in the blood and protein metabolism. Accordingly, it was felt that a study of the effect of glucose on the plasma amino acid level

TABLE 17.

Effect of carbohydrate ingestion on blood amino acid N in fasting subjects.

Author.	Carbohydrate Fed	Amount (g)	Time of Maximum Depression (Hrs.	% Decrease in Amino N Level	Comments.
Folin & Berglund (1922)	Glucose	200	2	18.6	
Greene et al.(1923-24)	Glucose Fructose	100	-	-	No data given.Effect stated to be similar in magnitude to that obtained by Folin & Berglund. Minimal Amino N coincided with maximum blood sugar.
Schmitz & Eastland (1935)	Glucose	50	2	12.9	Normal subjects. Blood sugar maximum at 1 hour.
	Glucose	50	3	11.5	Diabetic subjects. Blood sugar maximum at 1 hour
Cossu & Maestri (1936)	Glucose	50	Nil	Nil	Normal subjects
	Glucose	50	1	13.2%	Diabetic subjects.
Harris & Harris (1947)	Glucose	75	$3\frac{1}{2}$	17.4	Normal subjects. Blood sugar maximum at 1 hour.

might assist in the elucidation of the mechanism underlying the protein sparing action of carbohydrate. Several sporadic references to this point are recorded in the literature.

The action of glucose on the plasma amino acids. (Table 17)

In 1922 Folin and Berglund first furnished direct proof of the participation of the amino acids in the sparing effect of carbohydrate. These authors fed 200 gms. of glucose to a fasting youth and noted a reduction in the plasma amino nitrogen. Greene, Sandiford and Ross (1923 - 1924) observed a slight though definite reduction in the plasma amino nitrogen level after the administration of glucose or fructose. The minimal amino nitrogen level was found to coincide with the maximum blood sugar concentration. In 1935 Schmidt and Eastland working with a series of subjects obtained an average reduction of 13% in the plasma amino nitrogen; the haemoglobin level remained unchanged during these experiments proving that haemodilution was not a causative factor. They also showed that control subjects fasted throughout the same period did not exhibit this change. These authors also studied a group of diabetic subjects and made the interesting observation that glucose ingestion resulted in a depression of the blood amino acid nitrogen, which was similar in magnitude to that occurring in normal subjects. However, Cossu and Maestri (1936) failed to find any change in the blood amino acid nitrogen after the admin-

istration of glucose to normal subjects, although, somewhat paradoxically a fall did occur in their diabetic subjects. A fall in the total plasma amino nitrogen was also reported by Harris and Harris in 1947, but in addition, these authors noted that the individual amino acids were not all depressed to the same extent, there being a marked difference in the percentage depression for different amino acids. In contradistinction Schreier and Remsperger (1952) recently claimed that, of all the essential amino acids, isoleucine was the only one whose level was lowered by glucose ingestion. It was also claimed by these investigators that the administration of fat had no effect on the plasma levels of the essential amino acids. There would thus appear to be some discrepancies in the literature. We have therefore made further studies with two objects in view (a) to try to demonstrate the alleged action of carbohydrate on the plasma amino acids of fasting subjects and (b) to determine, in addition whether the administration of fat has a similar effect. The total plasma amino nitrogen was estimated in both instances and in addition the effect of glucose ingestion on the plasma levels of 7 essential amino acids (tryptophan, histidine, leucine, isoleucine, threonine, arginine and valine) was determined by means of microbiological assay.

A similar experiment was then conducted with rats and the results compared with the above.

EXPERIMENTAL

A - Subjects.

Human. Six healthy male adult subjects were used. Each fasted from 6.00 p.m. till 9.00 the following morning and was then given 50 gms. of glucose or 30 gms. of butter. Blood was withdrawn at hourly intervals immediately before the administration of the nutrient and at hourly intervals thereafter for a period of three hours. A control test was also carried out in each case, as there appears to be some doubt as to whether fasting or frequent removal of blood can influence the blood amino nitrogen level (Kerr and Krikoran, 1929; Powers and Reis, 1933; Bischoff and Long, 1929). This was similar in all respects to the above, but with the ommission of glucose or fat. In this way each subject could be compared on the three treatments. Absorption of glucose and fat was followed by means of serial blood sugar determinations (Hagedorn and Jensen, 1923 a & b) and chylomicron counts (Frazer and Stewart, 1939) on the peripheral blood. We are of the opinion that the error of chylomicron counting is considerable and have therefore used an arbitrary score (1,2,3 and 4) to indicate the degree of chylomicronaemia.

Heparin was added to the blood immediately on withdrawal and the plasma separated by centrifugation. Tungstic acid filtrates of the plasma were then prepared according

to the method of Hier and Bergeim (1945) which yields a 1 in 3 dilution. The total plasma amino nitrogen was determined in all four filtrates by the method detailed beneath. The tungstic acid filtrates from the first (zero hour) and third (second hour) samples were assayed microbiologically for essential amino acids as described below. In the case of tryptophan and histidine the 1 in 3 dilution of the plasma often gave assays which were outside the range of our procedure and a 1 in 6 dilution was generally found to be more advantageous. With the remaining five amino acids however, the 1 in 3 tungstic acid filtrates functioned perfectly satisfactorily.

Rats. In addition to these studies in human subjects, a small series of similar experiments was performed on hooded male rats of about 300 gms. body weight. These were fasted overnight and were then fed by a stomach tube 2 gms. of glucose in 4 mls. of water, 1.6 mls. of olive oil, or 4 mls. of water (controls). The rats were then killed at 1, 2 or 4 hours thereafter under pentobarbital sodium anaesthesia, blood being withdrawn from the inferior vena cava for amino N determination. In some cases the plasma levels of tryptophan, threonine, leucine and isoleucine were determined microbiologically.

B – Amino Nitrogen Determinations.

The method used was that described by Hawk (1947). (Folin's original method as modified by Danielson 1933,

Sahyun 1938 - 1939 and Frame, Russel and Wilhelmi 1943). This consists essentially of the formation of a coloured addition complex between the amino acids and sodium - naphthaquinone - 4 - sulphonic acid. This reaction must be carried out at pH 9.2 - 9.4 as much less colour is developed outside this range. This is achieved by adding 0.1 N sodium hydroxide very slowly to the tungstic acid filtrate till it becomes alkaline to phenolphthalein. The pH is then stabilised by the addition of a 1% borax solution (pH 9.2 - 9.4). After the addition of the naphthaquinone colour development is accelerated by heating in a boiling water bath. The excess reagent is then bleached by the addition of sodium thiosulphate in acid formaldehyde solution. Finally the optical density at 490 mu is compared with that of a standard solution treated similarly.

C - Microbiological assay of amino acids.

Organism, medium and procedure. Lactic acid bacilli, being nutritionally exacting towards many amino acids, are admirably suited to their quantitative estimation. Snell and Guirard (1943) have studied in some detail the amino acid requirements of S. Faecalis R and accordingly this organism was used for the assay of the seven essential amino acids mentioned above.

The medium used was that of Henderson and Snell (1948) which contains 18 amino acids in addition to purines,

pyrimidines, vitamins, minerals and glucose. The amino acid to be estimated was of course omitted during the preparation. Before use the pH was adjusted to 6.8 - 7. This is a little higher than the optimal pH for this organism, which is 5.5 to 6.5 but it compensates for the acid production occurring during autoclaving (Orla-Jensen 1933).

The bacteria were carried in a fluid medium, kindly prepared by Dr. I. Lominski of the Western Infirmary, Glasgow. Subculturing was carried out weekly. Cells for inocula were grown on the deficient basal medium supplemented by the deficient amino acid. After incubation for 18 to 30 hours, the culture was centrifuged, the cells washed twice with 0.9% sodium chloride and finally suspended in a volume of sodium chloride 5 to 25 times greater than that of the medium in which they were grown.

Accurate standard solutions of the amino acids under assay are of course a necessity. By trial and error they were prepared at such a concentration that the relationship between bacterial growth and amino acid concentration was as nearly linear as possible over all the dose levels used. The concentrations used were as follows:-

L - tryptophan, 2 µg/ml; L - histidine, 5 µg/ml;
L - threonine, 10 µg/ml; L - arginine, 10 µg/ml;
L - isoleucine, 10 µg/ml; L - valine, 10 µg/ml;
and L - leucine, 10 µg/ml.

The comparison between the standard and the sample can be

made either before or after total growth has occurred. In the former case comparative rates of growth are being measured, in the latter total growth. All the substances which stimulate the rate of growth of S. Faecalis R are not know and therefore cannot be added to the basal medium. However, these substances do not usually interfere with the extent of growth as the organism slowly synthesises them for itself. Assays based on total growth i.e. long incubation, are therefore likely to be more reliable than those based on rates of growth i.e. short incubation time. Consequently, in the present series of assays the tubes were always incubated for 72 hours. Growth was measured by estimating the lactic acid produced by means of standard sodium hydroxide. Generally growth has reached a maximum long before maximal acid production has occurred, but eventually the amount of acid produced is closely related to the total number of cells.

The procedure used was the micromethod of microbiological assay as devised by Henderson, Brickson and Snell (1948), in which the final volume of medium incubated was 0.2 mls. Metal racks were made capable of holding 60 culture tubes of size $\frac{3}{8}$" x 3". The sample and standard were placed in the assay tubes at 5 levels, namely, 0, 0.02, 0.04, 0.06, 0.08 and 0.1 mls., then water added to bring the total volume to 0.1 mls.. All estimations were carried out in duplicate. A metal cover, approximately 3" deep and lined with cotton wool was now placed over the tubes and autoclaving carried

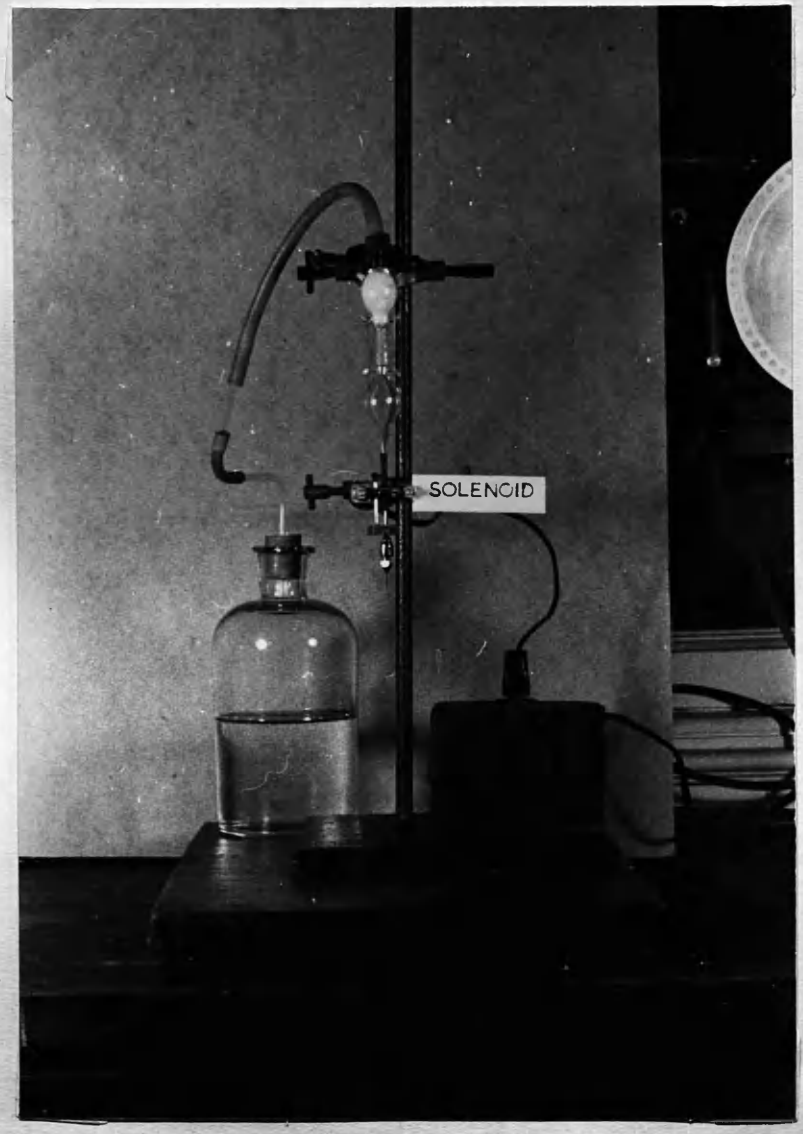


Photo. 3 - The Cannon dispensing unit.

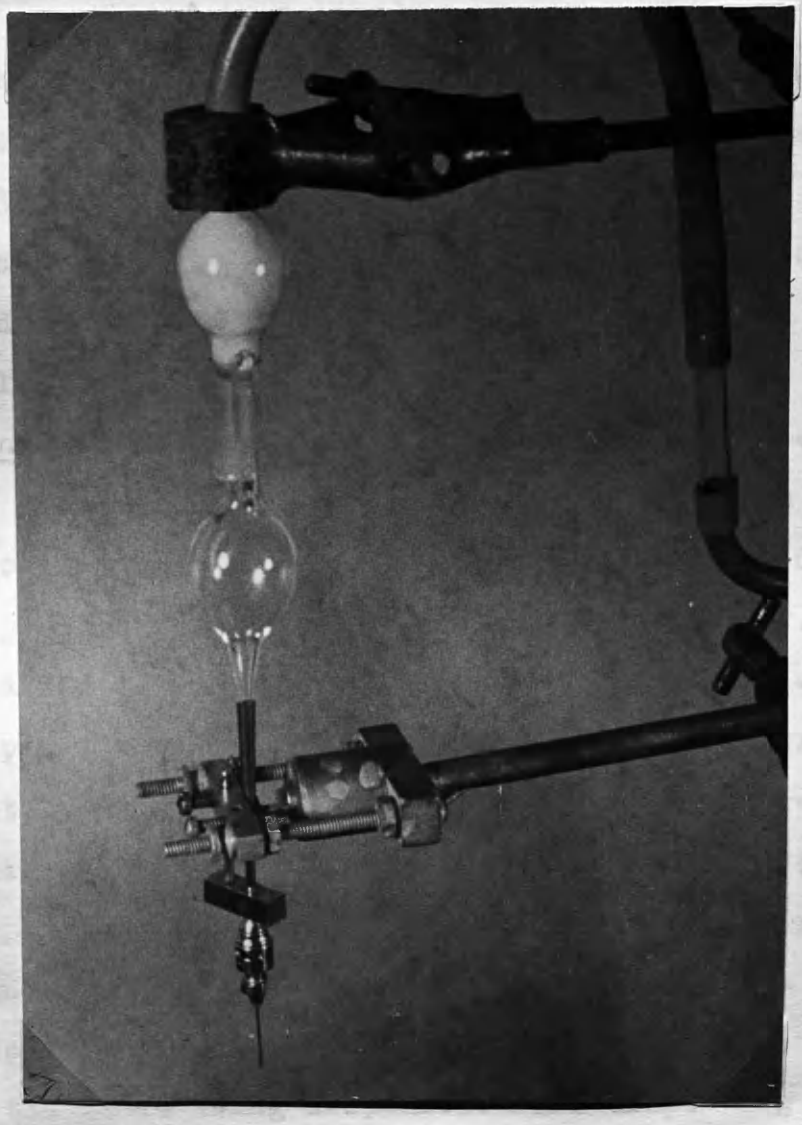


Photo. 4 - Dispensing vessel.

out at 10 lbs. for 10 minutes. Double strength basal medium was sterilised in a similar manner. After cooling it was inoculated with a suspension of the organism and 0.1 mls.of medium pipetted into each tube, which were then incubated at 37°C for 72 hours in a covered water bath. Under such conditions evaporation was negligible. The lactic acid produced by the organisms was then determined electrometrically.

Cannon Dispenser. Microbiological assay is extremely tedious and time consuming if the numerous pipettings and titrations involved have to be performed manually. The Cannon automatic dispenser was used to obviate this difficulty. It consists of a manometer, a solenoid and an electronic unit, and can be adapted for both dispensing and titrating. The dispensing unit (Photo. 3) is composed of a source of pressure (nitrogen cylinder) a dispensing vessel and the Cannon dispenser, the component parts functioning as follows. The manometer is connected between the pressure source and the dispensing vessel and it enables a steady head of pressure to be applied to the fluid being dispensed. The dispensing vessel (Photo.4) consists of (a) a nitrogen inlet tube, the bulb of which is filled with cotton wool and (b) the dispensing vessel proper, of capacity 8 mls.. A ground glass joint connects the two parts, the small glass projections allowing an air tight union to be achieved by means of rubber bands. The dispensing tip is a Luer-type hypodermic needle which has been filed

across just short of the point. It screws into an adaptor which is connected to the vessel by a 2" piece of fine rubber tubing. When not in use for dispensing the adaptor and tip can be removed and the free end of the tubing closed by a glass rod. When the circuit is closed the solenoid valve is drawn back and the contents of the vessel are dispensed under a steady head of pressure. When the circuit is open the solenoid valve is thrust against the rubber tubing compressing it and so interrupting the flow of liquid. The volume dispensed thus depends on the length of time the solenoid valve is open and this is controlled by the electronic unit. This unit contains a large metal disc which is driven by an A.C. electric motor and rotates at a constant rate. Paper discs, from the periphery of which appropriate segments have been removed, can be clamped on to this disc. When the long metal contact arm rests on the paper, the circuit is not completed and the solenoid remains closed, but when it rests on the metal disc, i.e. where a segment has been removed from the paper, the solenoid opens and fluid is dispensed. Thus the volumes dispensed depend on the length of the segments which have been cut from the paper discs. Two main types of paper discs were employed. One had 5 segments of differing lengths removed from it so that volumes of 0.02, 0.04, 0.06, 0.08 and 0.10 mls. could be dispensed in one rotation of the disc. The other had all the segments removed of equal lengths, each segment dispensing 0.10 mls. The

latter one was used for dispensing media. The above description is a very simplified account of the dispensing unit. Actually, no dispensing can occur unless a foot switch is depressed to complete the circuit.

Media, samples, standard and water were all dispensed by the same technique, except that in the case of the media it had to be dispensed in a sterile condition. The appropriate media were pipetted into separate vessels, which were then closed in the conventional manner with cotton wool plugs. The vessels along with the nitrogen inlet tube were placed in a metal boiling tube stand and autoclaved for 10 minutes at 10 lbs.pressure. After the media had cooled they were inoculated with a suspension of the organism, one drop being added for each ml. of medium. The nitrogen inlet tube had now been attached to the manometer and its cotton wool filling enabled aseptic delivery of the inoculated media to be accomplished. Where several different media were being dispensed the nitrogen inlet tube remained in situ, only the vessels being changed. The ground glass joint allowed a rapid replacement of an empty vessel by a fully charged one, the manipulation only taking a few seconds. The surfaces in the ground glass joint are reversed i.e. the nitrogen inlet tube fits outside and not inside the dispensing vessel as is usual. This minimises the risk of media soiling the inlet tube and so eventually contaminating other media as they are dispensed. So far contamination of this nature has not been observed.

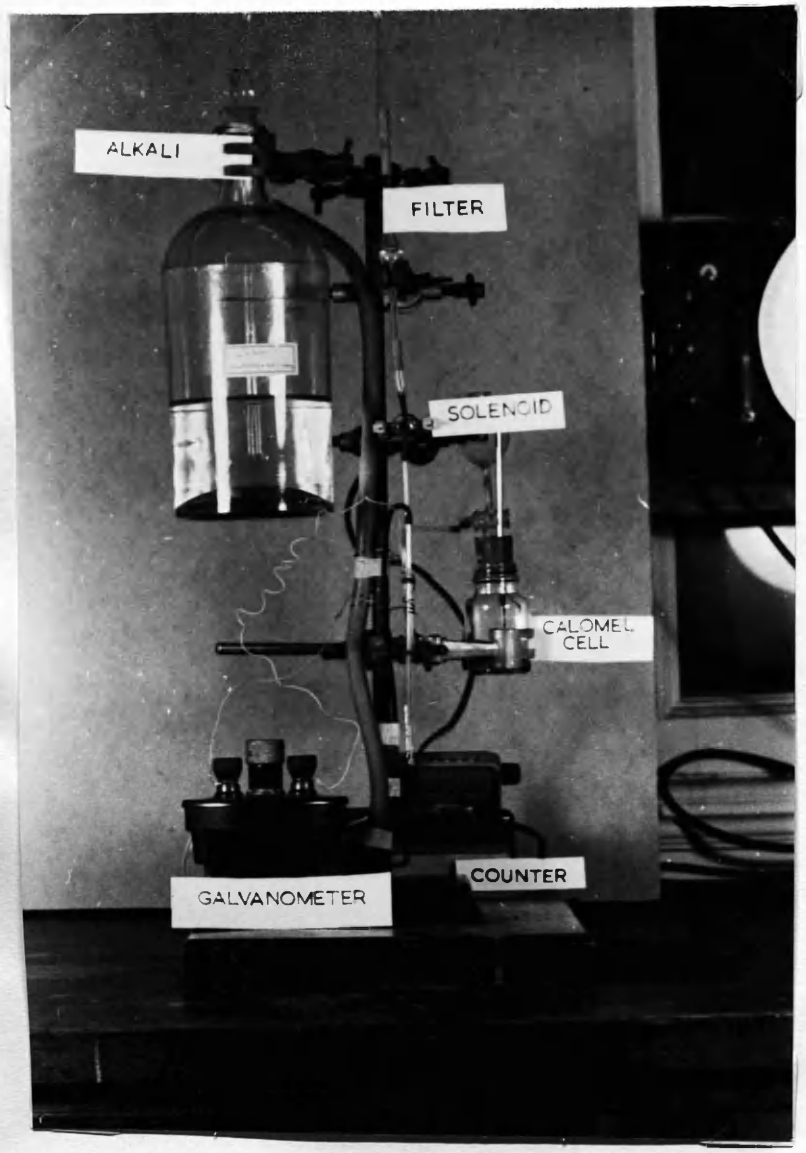


Photo. 5 - Titration unit.

Instead of plugging each of the 60 tubes in one rack individually with cotton wool prior to sterilisation a metal cover lined with cotton wool encloses them all, as has been previously indicated. When the inoculated medium is being dispensed, this cover has to be removed and the tubes are exposed for as long as 30 seconds. However, if reasonable precautions are taken no contamination occurs, for a complete bacteriological examination of the organisms growing in 60 tubes after a 72 hour incubation has failed to reveal any bacteria other than S. Faecalis R.

The Cannon dispenser can also be adapted for titration purposes (Phot.5) in this case a paper disc containing 60 holes around its periphery is clamped on to the metal disc. The circuit is so arranged that if the foot switch is depressed the solenoid remains open permanently and alkali is dispensed under a steady pressure into the assay tube. At the same time an electromagnetic counter is actuated by the holes in the disc and in one rotation of the disc it registers 60 counts. Thus the counter is measuring on a time scale the volume of 0.04 N sodium hydroxide required to neutralise the lactic acid. The end point is determined electrometrically, the essential parts being a quinhydrone electrode connected through a capillary salt bridge to a calomel half cell. The external circuit is closed by a galvanometer of internal resistance 1000 ohms. The potential of such a cell is zero at pH 7.1 and the galvanometer can be adjusted to give no deflection at the desired end

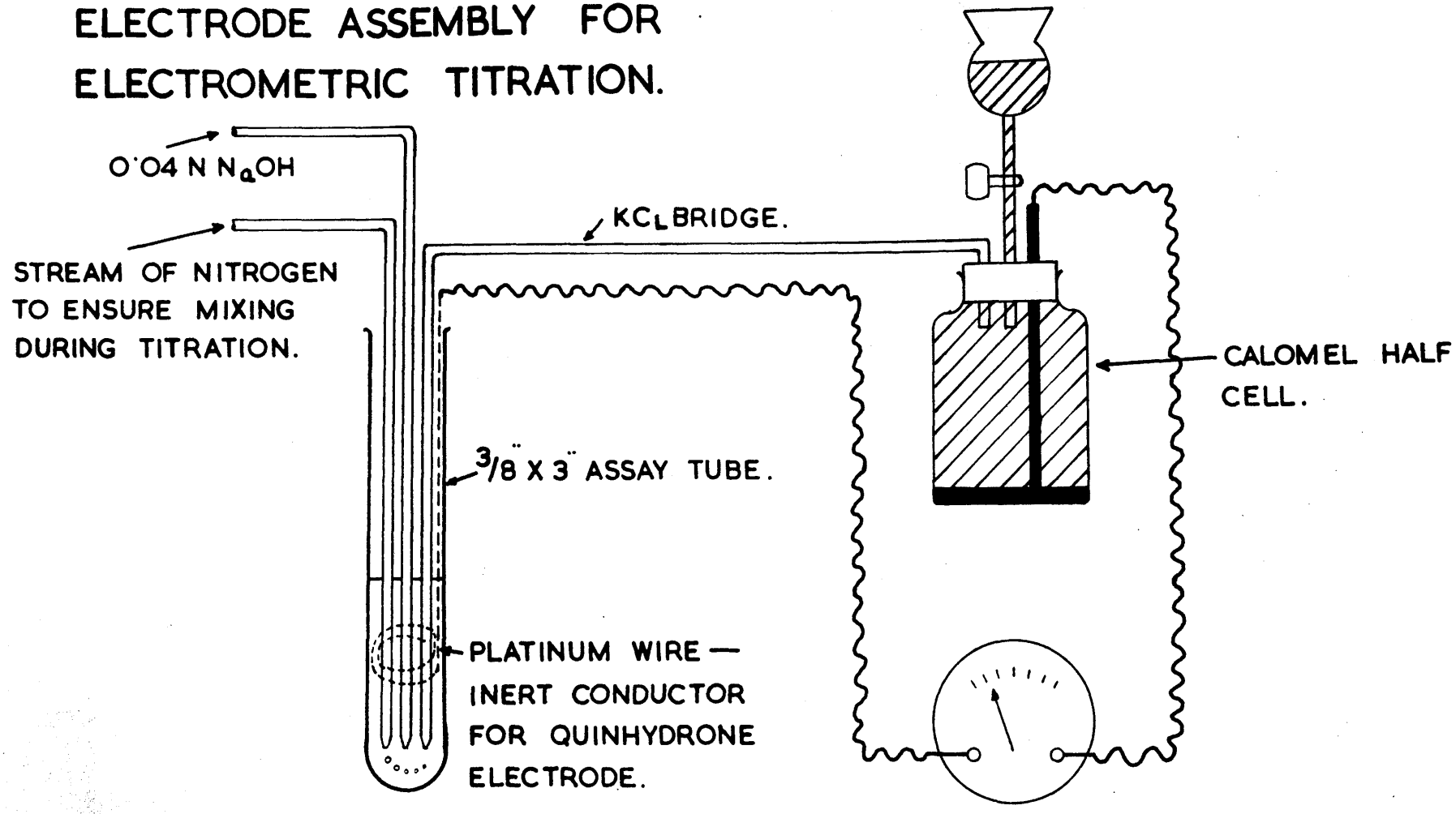

ELECTRODE ASSEMBLY FOR
ELECTROMETRIC TITRATION.
0·04 N NaOH
STREAM OF NITROGEN
TO ENSURE MIXING
DURING TITRATION.
KCL BRIDGE.
CALOMEL HALF
CELL.
3/8" X 3" ASSAY TUBE.
PLATINUM WIRE —
INERT CONDUCTOR
FOR QUINHYDRONE
ELECTRODE.
GALVANOMETER.

FIG. 5.

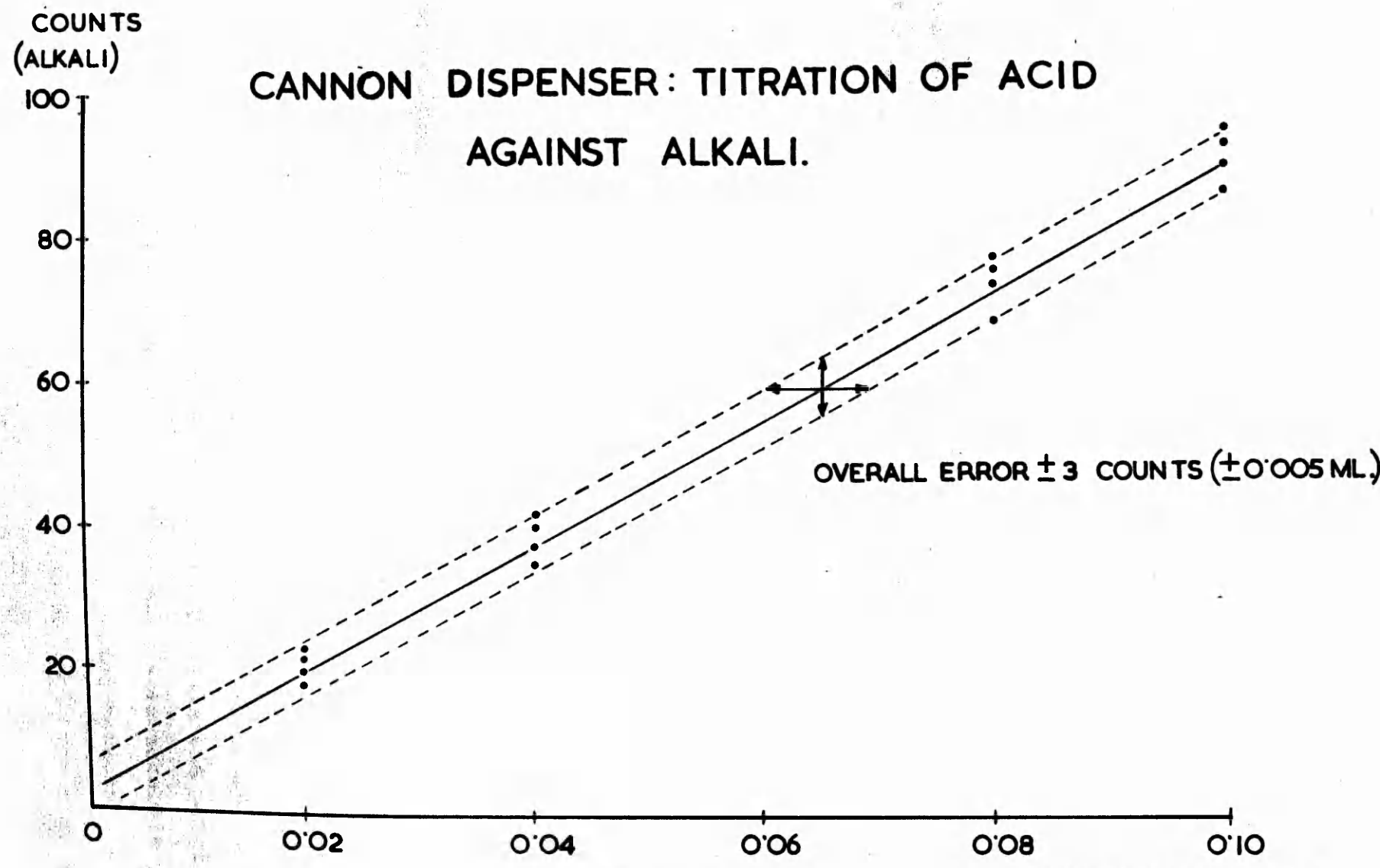

COUNTS
(ALKALI)
CANNON DISPENSER: TITRATION OF ACID
AGAINST ALKALI.
100
80
60
40
20
0
0·02
0·04
0·06
0·08
0·10
OVERALL ERROR ± 3 COUNTS (±0·005 ML.)
MLS. ACID DISPENSED.

FIG. 6.

point. The end point chosen was pH 7.3 and at the pH the addition of 0.005 ml. of 0.04 N sodium hydroxide causes a galvanometer deflection of two scale divisions. To avoid clogging of the very narrow dispensing tip the alkali is filtered through a sintered glass filter as it is being dispensed. Stirring is accomplished by bubbling a fine stream of nitrogen through the solution during titration. The platinum wire which serves as the inert conductor for the quinhydrone electrode is wound round the other three tubes two to three times. Before titration quinhydrone is added to each tube as one drop of a fairly thick suspension in water. The essential parts of the electrode assembly are shown in Fig. 5. As the three tubes have to be introduced into a $\frac{3}{8}$" diameter assay tube, they must be made from fine glass tubing, preferably of 2 mm. external diameter.

Accuracy and reliability of assay. The accuracy of the mechanical aids which have been described above was tested as follows. By means of the dispensing unit, 0.02, 0.04, 0.06, 0.08 and 0.10 mls. of suitably diluted sulphuric acid were added to 0.10 mls. of basal medium. Water was then added to bring the total volume to 0.20 mls. in all cases. Finally a drop of quinhydrone solution was introduced into all the tubes which were then brought to the same end point by means of the titrating unit. If the method is accurate a straight line relationship should be expected between the various amounts of alkali added. As can be seen from Fig. 6

TABLE 18

Constancy of tryptophan assay at ascending levels of the sample.

Mls.of plasma filtrate added.	Tryptophan calculated from standard curve (µg)	Tryptophan present in filtrate (µg/ml).
0.02	0.037	1.85
0.04	0.074	1.85
0.06	0.113	1.88
0.08	0.147	1.84
0.10	0.194	1.94
Mean	-	1.87

The plasma dilution was 1 : 6. Therefore tryptophan in plasma = 11.22 µg/ml

TABLE 19.

Plasma tungstic acid filtrate recovery experiments.

Amino Acid	Added	Found	% Recovery
Arginine	0.0 40.0	25.2 57.6	81
Histidine	0.0 20.0	7.6 29.6	110
	0.0 15.0	22.8 40.8	120
Leucine	0.0 48.0	24.4 72.8	101
	0.0 36.0	18.3 59.4	114
Valine	0.0 20.0	14.6 35.6	105
	0.0 15.0	25.2 37.5	82
Threonine	0.0 20.0	10.6 27.2	83
	0.0 15.0	26.9 44.7	118
Tryptophan	0.0 8.0	7.6 15.2	95
	0.0 6.0	14.4 20.4	100
Range			81 - 120

TABLE 20.

Recovery experiments on plasma.
(Hier and Bergeim,1946).

Amino Acid	Added	Found	% Recovery	Range
Arginine	35.0 18.0 28.6 21.4 18.8	25.8 23.1 37.5 27.0 28.8	101 92 121 105 92	92 - 121
Histidine	8.6 8.6 7.6 8.6 8.6	13.2 11.1 10.5 9.3 11.7	85 100 115 104 91	85 - 115
Lysine	10.0 9.0 14.3 14.3 12.1	15.8 29.0 24.0 31.5 19.5	98 120 89 72 116	72 - 120
Phenylalanine	37.5 9.0 10.7 14.3 10.7	9.0 7.5 11.9 14.3 12.3	84 104 113 93 109	84 - 113

this does exist, the overall error probably not exceeding ± 3 counts (0.005 mls.) in most cases.

According to Snell (1945) the reliability of the assay can be judged by the following criteria. In the first place, there should be agreement with the results obtained by other methods of assay, but so far as the concentrations of amino acids in the blood are concerned, the values obtained by other methods are too insensitive to be of use. Secondly, the values obtained from the various assay levels should agree. The data from a typical tryptophan estimation are tabulated in Table 18. It can be seen that there is very close agreement at all concentrations. Corresponding results were obtained with the other amino acid assays. Finally there should be a satisfactory recovery of a known amount of amino acid added to the plasma. Our results (Table 19) can be seen to compare quite favourably with those of Hier and Bergeim (1946) which are given in Table 20. It seems therefore quite legitimate to draw the conclusion that the assays are reasonably reliable as judged by the two criteria discussed above.

RESULTS

A - Experiments with Human Subjects.

Plasma amino acid nitrogen after glucose or fat administration.

In Table 21 are recorded the mean plasma amino nitrogen values of six subjects during a three hour fasting control

TABLE 21.

Human plasma amino N levels at 1, 2 & 3 hours after the ingestion of 50g. glucose or 30g. fat.

* Group	Amino N (mgms./100 ml.)				Maximum % Depression
	0 hrs.	1 hrs.	2 hrs.	3 hrs.	
Control	6.10	6.12	6.16	5.99	-
Glucose	6.27	5.50	5.54	5.82	12.3%
Fat	6.05	5.85	5.78	5.78	4.4%

* Each group is the mean of 6 subjects.

TABLE 21a

Analysis of variance of amino acid N values for control subjects.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Total	22	225,924		
Replicates	5	223,092		
Time	3	1,037	345.7	2.70
Residual	14	1,795	128.2	

For $n_1 = 3$ and $n_2 = 14$ F = 3.34 at the 5% level.

There is therefore no significant variation in the amino acid level during the control period.

TABLE 21b

Analysis of variance of amino acid N after glucose ingestion.

Source of Variation	Degrees of Freedom.	Sum of Squares	Mean Square	Variance Ratio
Total	23	46,925	-	-
Replicates	5	9,376	1,875	-
Time	3	22,991	7,614	7.9
Residual	15	14,558	971	-

For $n_1 = 3$ and $n_2 = 15$ $F = 5.42$ at the 1% level.

Therefore the effect of glucose on the amino N of the plasma is highly significant ($P < 0.01$).

TABLE 21c

Analysis of variance of amino acid N after fat ingestion.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Total	23	20,137	-	-
Replicates	5	13,301	2,660	-
Time	3	2,817	939	3.5
Residual	15	4,019	268	-

For $n_1 = 3$ and $n_2 = 15$ $F = 3.29$ at the 5% level.

The effect of fat on the plasma amino N is therefore of borderline significance (just above the 5% level).

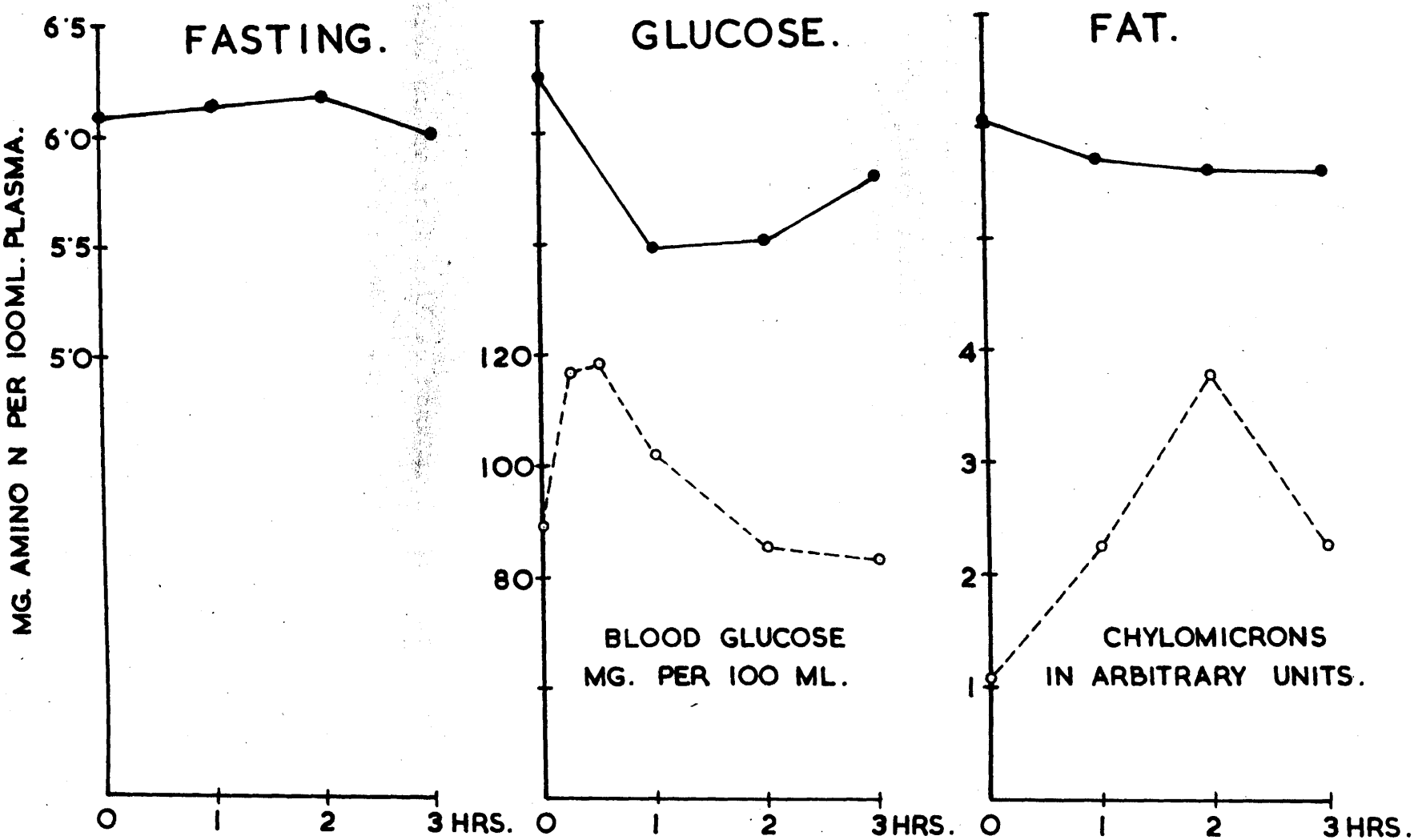

FASTING.
GLUCOSE.
FAT.
MG. AMINO N PER 100ML. PLASMA.
6·5
6·0
5·5
5·0
120
100
80
4
3
2
1
BLOOD GLUCOSE
MG. PER 100 ML.
CHYLOMICRONS
IN ARBITRARY UNITS.
0
1
2
3 HRS.

FIG. 7.

period or following glucose or fat administration. These results are also shown in the form of a graph in Fig.7, which includes the results of the blood sugar estimations and chylomicron counts. The shape of the amino nitrogen curve in the fasting and fat fed subjects is seen to be fairly similar, but following the administration of glucose there is a striking difference, a marked depression occurring in the plasma amino acid level. These three curves will now be considered in more detail.

Over the three hour control period, analysis of variance (Table 21a) failed to reveal any significant variation in the level ($P > 0.05$). This is in agreement with the findings of Schmidt and Eastland (1935) on their fasting control subjects. After the administration of glucose there was a sharp fall in the amino nitrogen level, maximal at the first hour, but still below the fasting level at the end of the third hour. On the other hand, the blood glucose concentration was maximal half an hour after glucose ingestion and in the case of most subjects had returned to the fasting level at the end of one hour. The depression in the amino nitrogen level due to glucose was 12.3% at one hour, which agrees well with the 13% reduction reported by Schmidt and Eastland (1935) (Table 17). Analysis of variance (Table 21b) shows the glucose effect to be highly significant statistically ($P < 0.01$). After fat ingestion the amino nitrogen showed a slight and gradual downward trend, which eventually

TABLE 22

Human plasma amino acid levels in the fasting state and after glucose ingestion*.

Amino acid	Fasting		Glucose		Reduction after glucose	Reduction as percentage of initial level
	0 hrs.	2 hrs.	0 hrs	2 hrs		
	µg/ml.	µg/ml.	µg/ml.	µg/ml.	µg/ml.	
Tryptophan	11.5	10.8	12.0	10.2	1.8	15
Histidine	14.9	16.0	16.7	15.5	1.2	8
Threonine	15.0	16.3	17.0	14.1	2.9	17
Arginine	16.5	17.6	19.6	15.7	3.9	20
Isoleucine	12.4	12.4	13.3	8.5	4.8	36
Valine	27.5	25.1	28.4	21.6	6.8	24
Leucine	21.6	21.8	25.0	17.6	7.4	29

* Mean data obtained on six subjects.

TABLE 22a.

Analysis of variance of percentage change after glucose.

Source of Variation	Degrees of Freedom	Sum of Squares.	Mean Square.	Variance Ratio
Total	41	5,013	-	-
Replicates	5	948	190	5.11
Amino acids	6	2,949	492	13.23
Residual	30	1,116	37.2	-

For $n_1 = 6$ and $n_2 = 30$ $F = 3.47$ at the 1% level.

Therefore the percentage change in the individual amino acids after glucose ingestion is highly significantly different. ($P < 0.01$).

TABLE 22b

Analysis of variance of micromolar change after glucose.

Source of Variation	Degrees of Freedom.	Sum of Squares.	Mean Square	Variance Ratio
Total	41	24,431	-	-
Replicates	5	3,596	719	3.86
Amino acids	6	15,248	2,541	13.65
Residual	30	5,587	186.2	-

For $n_1 = 6$ and $n_2 = 30$ $F = 3.47$ at the 1% level.

Therefore the micromolar change in the individual amino acids following glucose ingestion is highly significantly different. ($P < 0.01$)

represented a fall of 4.4% from the initial level. Statistically (Table 21c) the effect was of border line significance (just above the 5% level); we are therefore disposed to consider it of doubtful physiological importance. The chylomicron count was maximal after two hours, which agrees with the results of Frazer and Stewart (1939). Comparison of the control, glucose and fat experiments at each successive hour with the use of Fisher's "t" test (Snedecor 1946) confirmed this general picture. When the glucose-fed subject was compared with the control there was a highly significant difference at the first and second ($P < 0.01$) but not at the third hour ($P > 0.05$). In contrast the fat-fed subject only differed significantly from the control at the second hour ($P = 0.05 - 0.02$); at the first and third hour $P > 0.05$. Finally when the glucose-fed was compared with the fat-fed subject there was a significant difference at the first and second ($P = 0.05 - 0.02$) but not at the third hour ($P > 0.05$). To summarise, the glucose-fed subject differs from the control and fat-fed subject at the first and second but not at the third hour.

Plasma amino acid levels after glucose ingestion. In Table 22 are tabulated themean plasma levels of 7 essential amino acids at 0 and 2 hours after the completion of a 15 hour fasting period. Inspection of the mean values shows that, over a period of 2 hours, there is no notable fall in the levels, but in some instances there is actually a tendency

TABLE 23

Comparison of amino acid data with that of other authors.*

Amino Acid	Present investigations.	Harris & Harris (1947)	Borden et al. (1950)	Kirsner et al. (1949)	Hier & Bergeim (1946)	Dunn (1945)	Ackermann et al. (1949)	Johnson & Bergeim (1951)
Tryptophan	11.5	11.9	-	-	10.8	11.4	11.8	12.7
Histidine	14.9	13.1	15.2	16.1	14.2	-	14.2	13.8
Threonine	15.0	-	20.7	20.7	20.2	-	-	16.7
Arginine	16.5	25.5	22.1	15.4	23.4	-	-	16.2
Isoleucine	12.4	-	-	-	16.0	-	-	13.4
Valine	27.5	34.3	-	26.8	28.3	-	29.6	27.2
Leucine	21.6	52.5	-	23.8	19.0	-	-	18.6

* All mean values and in fasting state.

TABLE 24

Comparison of the reduction in the plasma amino acid levels with the essential amino acid requirements of man.

Amino Acid.	Reduction in plasma concentration.	Essential amino acid requirements of man.
Tryptophan	1.0	1.0
Histidine	0.7	-
Threonine	1.6	1.9
Arginine	2.2	-
Isoleucine	2.7	2.8
Valine	3.8	3.2
Leucine	4.1	4.3

In each case the value for tryptophan has been taken as 1.0 and the values for other amino acids related to it.

to rise. These fasting levels are compared with the results obtained by other investigators in Table 23.

After the administration of 50 gms. of glucose, the plasma levels of all the amino acids fell (Table 22), the fall varying from 1.2 ug/ml. for histidine to 7.4 ug./ml. for leucine. This drop however, was not a constant percentage of the amount in the circulation, but varied from 8% for histidine to 36% for isoleucine, a variation which was found to be highly significant statistically (Table 22a). A similar variation has been noted by Harris and Harris (1947). Nor did the amino acids disappear from the plasma in equimolar quantities; when the reduction was expressed as micromoles, the depressions in the various amino acids were highly significantly different (Table 22b).

In Table 24 the changes in the amino acid concentrations after glucose ingestion have been arranged in the form of a ratio, the depression occurring with tryptophan having been assigned a value of 1.0. In the next column are Rose's (1949) estimates of the human requirements for these amino acids, also referred to tryptophan as unity. There is obviously a close similarity between the patterns in each series.

B - Experiments with Rats.

Plasma amino acid nitrogen after glucose or fat administration.

In this experiment three groups of 9 rats each were

TABLE 25

The influence of glucose and fat ingestion on the plasma amino N of fasting rats. *

Treatment	Plasma amino N after administration		
	1 hr. (mgms%)	2 hr. (mgms%)	4 hr. (mgms%)
Water	4.5	5.2	5.6
Glucose 2 gms.	4.0	3.9	5.4
Olive oil 1.6 mls.	4.8	5.0	5.1

* Each figure is the mean result obtained with three animals.

TABLE 25a.

Analysis of variance of plasma amino N in rats, following ingestion of glucose, fat & water.

Source of Variation	Degrees of Freedom	Sum of Squares.	Mean Square	Variance Ratio
Total	17	1,396	-	-
Replicates	2	790	-	-
Treatments	2	355	178	10.1
Time	1	34	34	-
Interaction	2	41	22	-
Residual	10	176	17.6	-

For $n_1 = 2$ and $n_2 = 10$ F = 7.56 at the 1% level.

Therefore there is a highly significant difference ($P < 0.01$) between animals receiving glucose and those receiving fat or water at 1 and 2 hours after feeding.

In this analysis the data for the fourth hour was omitted.

TABLE 26.

The plasma levels in the rat of 4 amino acids following the ingestion of carbohydrate, fat and water.

	Amino acid N mgms.%	Tryptophan µg/ml.	Threonine µg/ml.	Leucine µg/ml.	Isoleucine µg/ml.
Control	5.2	20.4	31.2	31.8	19.5
Fat	5.7	22.0	33.0	30.6	20.1
Glucose	4.0	16.8	16.2	13.2	8.1
Depression due to glucose	1.2	3.6	15.0	18.6	11.4

Each assay was performed on a pooled sample each comprising a 1 hour & a 2 hour specimen. The plasma was obtained from 6 of the rats used in the experiment tabulated in Table 25.

starved for 48 hours. Water, glucose or olive oil was then fed by stomach tube as follows:-

Group 1 (controls) received 4 mls. of water,

Group 2 4 mls. of 50% glucose solution, and

Group 3 1.6 mls. of olive oil.

Three rats from each group were then killed at 1, 2 and 4 hours after feeding. The plasma amino nitrogen levels in Table 25 are analysed statistically in Table 25a and demonstrate a highly significant difference ($P < 0.01$) between the animals receiving glucose and those receiving fat at 1 and 2 hours after administration. This difference was obliterated by the fourth hour after feeding. This confirms the results of the human studies described above.

Plasma amino acid levels after glucose or fat administration.

In the above experiment, the plasma from 6 rats was pooled into 3 lots. Each lot comprised plasma specimens from rats killed at 1 and 2 hours after the feeding of glucose, fat and water respectively. A tungstic acid filtrate of the appropriate dilution was then made and the plasma specimens assayed for tryptophan, threonine, leucine and isoleucine. It can be observed from Table 26 that carbohydrate ingestion produces a marked fall in these amino acid levels, whereas fat does not. Moreover, the depression in the different amino acids varies, tryptophan only showing a slight fall whereas the leucine level was markedly lowered. This is substantially the same result as we obtained with our human

TABLE 27.

The plasma levels in the rat of 3 amino acids following the ingestion of fat and water.

Type	Tryptophan µg/ml.	Threonine µg/ml.	Leucine µg/ml.
Rat 1(control)	24.6	25.8	25.2
Rat 2 (fat)	24.6	22.8	29.7
Rat 3 (fat)	26.4	24.6	25.5

Three rats were used. The interval between feeding and killing was 2 hours in all cases.

subjects. With rats, the ratio of the fall in tryptophan to that of leucine is 5:1, whereas with human subjects the comparable ratio is 4:1.

A further small experiment using only three rats was conducted on similar lines. One animal, the control received 4 mls. of water and the other two, 1.6 mls. of fat each. All the animals were killed after two hours. The results (Table 27) show clearly that fat ingestion has no effect on the plasma levels of tryptophan, threonine or leucine.

These latter two experiments substantiate the fact that carbohydrate reduces the plasma amino acid level whereas fat does not.

DISCUSSION.

A - The fall in the plasma amino nitrogen level.

The data presented above show that glucose administered to fasting subjects causes a sharp reduction in plasma amino nitrogen concentration which lasts only a few hours. Administration of fat has no definite effect on the amino nitrogen concentration of the plasma. These findings can be related to the action of carbohydrate on protein utilisation in the fasting and the fed subject.

Fasting subjects. Carbohydrates, when given to fasting human subjects, have been shown to reduce the nitrogen output below the fasting level (Benedict, 1915; Grafe, 1910 and 1914),

but the same effect has not been observed with fat (Thomas, 1910). Thus, in fasting subjects, the action of glucose and fat on the plasma amino acid level is precisely similar to their action on the utilisation of body protein. In both instances carbohydrate has an effect on protein metabolism, which is not shared by fat. It would appear, then, that these two effects are merely two different manifestations of the sparing action of carbohydrate and that the administration of glucose to a fasting subject besides causing a decrease in plasma amino nitrogen also results in a decreased nitrogen excretion.

With regard to the rat the information at hand is rather meagre. Gregg (1931) noted that nitrogen outputs of rats fed with butter were lower than the nitrogen output of a single rat which he fasted. In 1934 Kriss, Forbes and Miller observed that fat ingestion depressed the nitrogen excretion of fasting rats, but the effect was not so marked as that occurring with carbohydrate. Unfortunately with the latter studies the experiments may not be strictly comparable. There is thus not sufficient evidence to conclude with certainty that the rat unlike man can use fat to spare body protein. Our results with rats, which can be interpreted in a similar manner to the human studies, suggest that glucose but not fat can spare the utilisation of body protein in the fasting subject.

This effect of glucose on endogenous metabolism is

proof that the delay in absorption of protein due to simultaneously fed carbohydrate (Section 2), is in no way connected with the sparing effect of glucose.

Fed subjects. It has been observed in the case of man (Cuthbertson and Munro 1939) and also of the rat (Cuthbertson, McCutcheon and Munro 1940; Munro, 1949), that the time at which carbohydrate is eaten in relation to dietary protein influences the course of protein metabolism. Nitrogen balance is better when carbohydrate is taken along with the dietary protein than when the same amount of protein and carbohydrate are eaten separately. When similar changes are made in the time of feeding fat, nitrogen balance is unaffected. There is thus a similarity between the action of glucose on the blood amino acid level of a fasting subject and on the utilisation of dietary protein in a fed subject. In the first place carbohydrate has in both instances an action on protein metabolism which fat does not have. Secondly, the action of glucose on the blood amino acid level of fasting subjects is limited to an hour or so after administration and in fed subjects this would coincide with the main period of amino acid absorption after a meal containing protein and would thus explain why protein utilisation is influenced by the time at which dietary carbohydrate is fed. When the subject is fasting, then, the amino acids spared by carbohydrate are obtained endogenously from the plasma and when the subject is receiving protein in the same meal, utilisation of absorbed amino acids

derived from the dietary protein is enhanced.

This hypothesis has received strong verification by the recent work of Denton and Elvehjem (1954a). These authors studied the absorption of amino acids in dogs, by estimating microbiologically the free amino acid concentrations in the portal vein, after the feeding of different proteins. When casein was fed as the protein of a mixed meal, the amino acid levels in the portal vein rose and the increases were proportional to the amounts supplied by the protein. But when zein was offered as the protein source, a decrease in the amino acid concentrations occurred, which persisted for 1.5 to 4 hours after feeding. Denton and Elvehjem were of the opinion that these changes were due to the absorption of carbohydrate before amino acids were liberated; studies revealed that the sugar content of the blood increased considerably 0.5 hours after feeding the test meal, which besides containing the protein used, also contained sucrose, fat and vitamins. Further experiments with a non-protein mixture containing sucrose were very illuminating (Denton and Elvehjem, 1954b). The amino acid concentrations in the portal blood fell, leucine and isoleucine showing a marked fall, whereas tryptophan was little affected. The latter results are very similar to those obtained by us on the systemic blood. It would thus appear that the glucose effect is distributed throughout both the systemic and the portal circulation. There is also no doubt that it is

TABLE 28

Ratio of depression of essential to non-essential amino acids in plasma after glucose ingestion compared with ratio of essential to non-essential amino acids in muscle.

Plasma Constituent	Initial Level (µg/ml.)	Depression 2 hrs. after glucose. (µg/ml.)	% Depression
Total Amino N	62.7	7.3	13
Amino N of 7 essential amino acids	13.4	3.0	22
Amino N of non-essential amino acids (by difference)	49.3	4.3	9
Ratio of drop in essential to non-essential amino acids.	1 : 1.4		
Ratio of 7 essential to non-essential amino acids in muscle protein.*	1 : 1.4		

* Block and Bolling (1947).

capable of exerting its characteristic action on simultaneously absorbed amino acids.

B - Mode of action of glucose.

The question arises, where do the amino acids go which are removed from the plasma? There is no evidence of increased deamination as Schmidt and Eastland (1935) observed a reduction in blood urea concentration simultaneous with the reduction in amino nitrogen after glucose ingestion. Our study of the relative quantities of different essential amino acids removed after glucose ingestion shows that the pattern is the same as that exhibited by the essential amino requirements of man (Table 24). This suggests that the amino acids are disappearing from the plasma in the proportions required for protein synthesis. It has therefore been concluded that the effect of glucose is either to stimulate protein synthesis or to reduce breakdown - probably the former.

A similar conclusion to the above can be reached by rather a different procedure. In Table 28 the mean initial total amino nitrogen of the glucose fed subjects is expressed in µg/ml., as is the fall occurring two hours after the administration of glucose (calculated from Table 21). The amino acid nitrogen data for the 7 essential amino acids are similarly expressed (calculated from Table 22). By difference the initial level of the "non-essential" amino acids and the reduction due to glucose can be calculated. The ratio of the

fall in the essential amino acids to the fall in the "non-essentials" is then seen to be 3:4.3 = 1:1.4. Muscle tissue constitutes by far the bulk of the protein in the body and if glucose does cause increased protein synthesis we might expect to find some evidence for the laying down of protein in the carcass. The 7 essential amino acids, which we estimated, constitute 35.9% of muscle as against 50.2% contributed by all the other amino acids (Block and Bolling, 1947). In this case the ratio of the essential amino acids to the "non-essentials" is 35.9:50.2 = 1:1.4. The fact that the ratios are identical in both cases suggests that the amino acids are removed from the plasma and synthesised into muscle protein. In addition it also demonstrates that there is extremely good correlation between the amino nitrogen values and the individual amino acid data.

SECTION 4

THE EFFECT OF INSULIN ON THE PLASMA AMINO ACID LEVEL.

INTRODUCTION

Insulin is known to be intimately concerned with carbohydrate metabolism and in addition glucose ingestion is widely recognised as having a markedly stimulant effect on insulin secretion by the pancreas. The point arises then, as to whether this liberated insulin could be a causative factor in the protein sparing effect of carbohydrate. It was shown in Section 3 that one of the manifestations of the sparing effect was the marked fall in the blood amino acid nitrogen level which occurs after glucose administration to a fasting subject. Evidence will now be presented from the literature to demonstrate that the injection of insulin has a precisely similar effect.

In 1928, Luck, Morrison and Wilbur, working with diabetic subjects under treatment with insulin, noted that the blood amino acid nitrogen levels were consistently lower than those of normal subjects. This chance observation led Daniels and Luck (1931) to investigate the effect of insulin injection on the blood amino acid levels of 10 normal fasting medical students. These authors observed that the blood amino acid concentration fell by 25%, the decline roughly paralleling that of the blood sugar. It began to fall promptly after the injection, and remained depressed for more than 4 hours. This finding has been verified by independent investigators (Kerr and Krikoran, 1929; Powers and Reis, 1933). More

recently, Harris and Harris (1947) working with human subjects and Lotspeich (1949) with dogs have demonstrated that after insulin administration the plasma levels of the essential amino acids fall to varying extents, histidine and tryptophan being depressed least and leucine most. These results are very similar to those obtained by us after glucose ingestion. In view of the striking similarity of the action of glucose and insulin on the plasma amino acids, the question may be raised as to whether carbohydrate can still spare protein in the absence of insulin.

The general approach to this problem has been the feeding of glucose to a fasting animal, whose supply of insulin has been removed by a previous pancreatectomy. Under these conditions no sparing action of glucose has been observed on fasting dogs by Barker, Chambers and Dann (1937); Chaikoff and Weber (1927) and Ringer (1912), and on fasting cats by Reid (1936). Similar results have been obtained by Flock, Block, Mann, Grindlay and Bollman (1952), and Bollman, Flock, Grindlay, Mann and Block (1953). These authors subjected dogs to hepatectomy and noted that the accumulation of amino acids which occurs in the plasma can be prevented by the administration of glucose. However, when a pancreatectomy was performed concurrently with the hepatectomy, it was found that glucose had no effect on the rapidly rising plasma amino acid level; for its reduction, insulin injection was necessary. On the other hand, Schmitz and Eastland (1935) and

Cossu and Maestri (1936) have noted a reduction in the plasma amino acid nitrogen following the administration of glucose to fasting human diabetic subjects. Insulin would also not appear to be necessary for the protein sparing action of carbohydrate when fed along with protein, as Bancroft, Geiger and Hagerty (1951) have observed the occurrence of nitrogen retention in animals (rats) so fed, which had previously been rendered diabetic by alloxan or pancreatectomy.

In view of the discrepancies in the literature on this important point, we carried out some experiments on alloxan diabetic rats. As to whether the carbohydrate sparing effect was operative or not was judged by the response to glucose ingestion of the plasma amino acid leucine, which was estimated microbiologically. This amino acid was chosen because previous experiments (Section 3) had shown the concentration of this amino acid in the plasma to be considerably reduced by the administration of glucose.

EXPERIMENTAL, RESULTS AND DISCUSSION

Twelve hooded male rats were starved for 48 hours then rendered diabetic by the subcutaneous injection of a solution of alloxan monohydrate (175 mgms/kilo. body weight) as recommended by Diermeier, Stefand, Tepperman and Bass (1951). They were then returned to normal diet and their urine tested daily for glucose. When the diabetic state was considerably advanced, as judged by the amount of sugar excreted in the

TABLE 29

Effect of glucose on blood amino acids in normal and diabetic rats.

Treatment	Time elapsed. hr.	Normal Rats			Diabetic Rats.		
		Weight g.	Blood sugar mg.%	Leucine in plasma µg./ml	Weight g.	Blood sugar mg.%	Leucine in plasma µg./ml.
Water	1	148	98	32.9	215	322	40.2
Glucose	1	153	145	22.7	222	422	37.8
Difference		-	+47	-10.2(-31%)	-	+100	-2.4(-6%)
Water	2	158	99	40.5	215	548	78.4
Glucose	2	162	133	18.7	207	312	73.4
Difference		-	+34	-21.8(-54%)	-	-236	-5.0(-6%)

Rats fed by stomach tube on 4 ml. water or 2g glucose in 4 ml. water. 3 rats per group.

urine, 1 unit of protamine zinc insulin was injected daily in an effort to stabilise them. This regime was continued for 4 days, then 24 hours before the commencement of the experiment both insulin and food were withdrawn. Six of the rats were fed 4 mls. of water by stomach tube and the remainder received 4 mls. of 50% glucose solution. They were then killed at one or two hours after feeding and blood removed from the inferior vena cava for plasma leucine and blood glucose determinations (these were carried out by the methods previously described - Section 3). The results were compared with those obtained on normal animals similarly treated.

It can be seen from Table 29 that the diabetic rats had a considerably higher blood leucine concentration than the non-diabetic controls. Inspection of the individual results indicated that this was not related to the blood sugar level at the time of death. At one and two hours after the feeding of glucose the control rats showed a fall in plasma leucine of -10.2 and -21.8 µg/ml. respectively (compare Table 26). The comparable figures for the diabetic rats were -2.4 and -5.0 µg/ml.. Thus the fall in the amino acid due to glucose is 4 times as marked with the control as with the diabetic rat, which suggests that insulin is required for the protein sparing action of carbohydrate in the fasting rat. We feel however, that this result should be interpreted with caution, as the diabetic state induced by alloxan appears to vary greatly in severity and consequently it is extremely difficult

to obtain rats suffering from approximately the same degree of diabetes. If an animal happens to be tending towards coma, then because of the accompanying shock the blood volume is markedly reduced and the amino acid assays are of course correspondingly high. We endeavoured to stabilise the diabetic state by the administration of protamine zinc insulin, 1 unit per day, but naturally this had to be withdrawn 24 hours prior to the experiment, as its continued presence would completely falsify the results. We are of the opinion that the only way to solve this problem satisfactorily is to use an extremely large number of diabetic rats, when the variations in the severity of the disease should eventually cancel out. The microbiological assay work required for this purpose would however be prohibitive.

However, if the results are taken as they stand, they fall into line with the observations quoted previously on fasting depancreatised dogs (Barker et al., 1937; Chaikoff and Weber, 1927; Ringer, 1912), on fasting depancreatised cats (Reid, 1936), and hepatectomised and pancreatectomised dogs (Flock et al., 1952; Bollman et al., 1953), suggesting that insulin is necessary for the nitrogen sparing effect of glucose. The findings of Schmitz and Eastland (1935) and Cossu and Maestri (1936) are directly at variance with the above, as glucose when fed to fasting human diabetics produces a fall in the plasma amino nitrogen, comparable in extent to that occurring normally. On the basis of their data it may be suggested

tentatively that human diabetes is not due to a pancreatic deficiency. However, an alternative explanation may lie in the fact that the diabetics chosen had normal blood amino acid nitrogen levels. This suggests that they were either mild cases or else had been under insulin treatment until just prior to the commencement of the experiment, for Luetscher (1942) has shown that in severe untreated diabetics an amino acid nitrogen level of 17 mgms.% (normal 5 - 6 mgms.%) can occur. Excepting human diabetes then, which is an extremely complex subject and may not represent pancreatic deficiency, it may be concluded with a fair degree of certainty that in the fasting animal glucose is unable to spare body protein unless insulin is present.

In the fed animal the results of Bancroft et al. (1951) suggest that a different situation may prevail. These authors fed glucose along with dietary protein and as the liver undoubtedly plays a predominant role in the metabolism of dietary amino acids, it is conceivable that the nitrogen sparing effect of carbohydrate in the liver may differ in its mechanism from that in the peripheral tissues where insulin is essential. If this hypothesis be correct, then it would be thought that when carbohydrate and protein are fed together in the diet, a large proportion of the retained nitrogen would be recovered from the liver, particularly so, as in Bancroft's experiments the degree of nitrogen retention was similar with the diabetic and control rats. But we have shown in Section 2

that under the experimental conditions mentioned, the increase in liver nitrogen was only a very small part of the total amount retained. It would thus appear that the above results of Bancroft et al. (1951) must be interpreted with caution, as they constitute an isolated piece of evidence for the non requirement of insulin in the protein sparing action of carbohydrate.

SECTION 5

THE EFFECT OF GLUCOSE AND FAT ON THE INCORPORATION OF ^{35}S-METHIONINE INTO TISSUE PROTEINS IN VITRO AND IN VIVO.

INTRODUCTION

In an endeavour to gain further information on the action on glucose and fat on protein metabolism, it was decided to adopt the labelled amino acid technique, first utilised by Schoenheimer and his colleagues (1942). Their results, coupled with those of their successors, are of such fundamental significance and are so obviously relevant to the subject under consideration that the general implications of these isotope studies will be discussed in some detail. The subject falls naturally into two groups, in vivo and in vitro studies.

IN VIVO EXPERIMENTS.

Until the late 1930's the prevalent concept of protein metabolism was that propounded by Folin (Folin, 1905; Folin and Denis, 1912). This postulated that protein synthesis only went on to a slight extent in an animal in nitrogen equilibrium. The structural proteins of the body were thought to be subject to a small but continual breakdown and repair, the protein metabolism involved in these changes being termed endogenous. Most of the food protein, since it was not required for the endogenous metabolism, would be oxidised and excreted, this process being called the exogenous metabolism. Now, as the rate of protein synthesis is so slow when compared with ordinary chemical reactions, it was impossible to put this theory to the test until the advent of isotopic amino

acids which could be used as labelling agents. This was accomplished when Schoenheimer and his colleagues synthesised a number of amino acids labelled with ^{15}N (Glycine - Ratner, Rittenberg, Keston and Schoenheimer, 1940; Tyrosine - Schoenheimer, Ratner and Rittenberg, 1939a; Histidine - Tesar and Rittenberg, 1947; Arginine - Block, 1946; Aspartic Acid - Wu and Rittenberg, 1949), and a number containing both ^{15}N and deuterium (Leucine - Schoenheimer, Ratner and Rittenberg,1939b; Ratner, Schoenheimer and Rittenberg, 1940; Lysine - Ratner, Weissmann and Schoenheimer, 1943; Weissmann and Schoenheimer, 1941; Proline - Stetten and Schoenheimer, 1944). If we consider the experiment with leucine to be a representative example, then the following procedure was adopted (Schoenheimer, Ratner and Rittenberg, 1939b). The labelled leucine was fed to an adult rat for three days, then after killing the animal, the various tissues were analysed for their isotopic content. The following relative amounts of isotope were obtained:- serum 100, intestinal wall 89, kidney 82, spleen 65, liver 56, heart 53, testes 46, muscle 18, haemoglobin 17 and skin 11. It was thus demonstrated that the tissue proteins were not inert structural substances,but were continually in a state of flux,amino acids entering and leaving the peptide chain with great rapidity. Moreover,as the tissue isotopic analysis shows, different tissues incorporate the tracer to varying extents. These results, of course, caused the complete downfall of the Folin theory of protein metabolism. As a continuation of the

above experiment Shemin and Rittenberg in 1944 fed ^{15}N-glycine to rats until the tissue proteins were completely saturated with the isotope. The label was then withdrawn from the diet and a steady decline in the isotopic concentration of the tissues was observed, those which had most rapidly incorporated the label showing the most rapid loss.

The advent of the radioactive isotopes ^{14}C and ^{35}S, which possess several advantages over ^{15}N, allowed the pioneering studies described above to be repeated with greater precision and in more detail. The foregoing experiments had of necessity to run over a period of three days or so in order to build up an isotopic concentration which was detectable, but with ^{14}C and ^{35}S, because of the greater sensitivity of their methods of estimation, the incorporation of a labelled amino acid into a protein could be followed hourly after feeding or injection. In addition, since most of the amino acids in the body are continually and rapidly being deaminised and reaminated the ^{15}N exchanges freely with the non isotopic nitrogen from the other amino acids, thus rendering interpretation of the results rather difficult. Using various ^{14}C-labelled amino acids and ^{35}S-labelled methionine and cystine several investigators have substantiated and extended the results obtained with ^{15}N. In 1947 Tarver and Reinhardt injected ^{35}S-methionine into dogs and after two hours demonstrated that the tissue with the highest isotopic concentration was the intestinal mucosa. Five hours after the injection

(in another animal) there was relatively little change in the concentration of ^{35}S in the tissue proteins or in the order of the different tissues with respect to concentration. As the concentration of ^{35}S was highest in the intestinal mucosal proteins after two hours it would be expected that the disappearance of ^{35}S would be fastest in this tissue. That this was so was demonstrated in the rat by Friedberg, Tarver and Greenberg (1948), and Tarver and Morse (1948). At the end of the first day after methionine injection the intestinal mucosa had the highest incorporation of isotope, but by the end of the fourth day, its ^{35}S concentration was actually slightly lower than that of brain and muscle. Similar data on young growing male rats and adult females have been reported by Maass, Larson and Gordon 1949. The same rapid turnover of intestinal mucosal protein as compared with that of any other tissue has also been shown to occur in the rat after administration of ^{14}C-glycine (Greenberg and Winnick 1948). At a quarter and six hours after intravenous injection of glycine there was incorporated in the mucosa 0.99 and 12.20 μ equivalents of glycine per gm. protein respectively; in muscle the respective figures were 0 and 0.5. This marked difference in the rate of incorporation of labelled amino acids by different tissue proteins has also been demonstrated by Neuberger, Peronne and Slack (1951) and Griffin, Bloom, Cunningham, Teresi and Luck (1950), both schools using the rat as experimental animal and ^{14}C-glycine as tracer.

Winnick, Friedberg and Greenberg (1948) injected β - ^{14}C - DL-tyrosine into rats and found that after six hours, intestinal mucosa had the highest incorporation and muscle tissue the lowest. Using the same isotope, Reid and Jones (1948) obtained essentially similar results with mice. The uptake of ^{35}S - DL-cystine by the proteins of intact rats has been examined by two groups of workers (Anderson and Mosker, 1951; Lee, Anderson, Miller and Williams, 1951). Lee and his co-workers found, in confirmation of observations made in vitro by others (Melchior and Tarver, 1947a) that it was possible to remove a significant amount of the activity present in the proteins of the animals by washing the protein with a reducing agent (monothioethylene glycol). Both groups of workers found the specific activities of the tissues to be comparable with those obtained by others using different amino acids. Borsook, Deasy, Haagen-Smit, Keighley and Lowy (1950a) injected ^{14}C-labelled glycine, histidine, leucine and lysine into mice and followed the rate of incorporation into the visceral proteins at intervals from 10 to 240 minutes after injection. Their results indicated that with all four amino acids incorporation was maximal within one hour, thereafter redistribution of the amino acids from the visceral protein resulted in a flattening of the incorporation curve. In the experiments described above, in which different isotopes, different amino acids and different species of animals have been used, one salient point emerges; every

tissue protein is constantly being resynthesised and catabolised and the rate of this process differs from tissue to tissue, intestinal mucosa being the most active and the carcass proteins in general the least active.

The factors governing the rate of protein turnover have not been investigated to any extent. From their urinary data on the rate of excretion of ^{15}N following a dose of labelled glycine to rats, Sprinson and Rittenberg (1949) failed to demonstrate that the protein content of the diet had an influence on the rate of turnover of tissue protein. Their calculated value for the amount of nitrogen going into the synthesis of protein was the same regardless of whether the animals received no protein in the diet or 81% casein. But Solomon and Tarver (1952) demonstrated that the labelling attained with ^{35}S-methionine was higher in the fasted rat than in the fed animal and was greater in an animal on a low protein diet than in an animal on a high protein diet. It would appear that these results can be ascribed to less dilution of the label in the fasting and low protein animals than in the others i.e. the specific activity of the precursor methionine remains higher during the period of incorporation. On the other hand, even though there be lower initial labelling with the high protein diet, the rate of loss of the label is greater i.e. turnover is greater. They suggest that this may be related to the labile protein content of the organ, which will increase when a high protein diet is fed and which is said to have a high

rate of turnover. Norberg and Greenberg (1951) studied the uptake of ^{14}C-glycine into the protein of normal and fasted mice at intervals from 1 to 48 hours after injection. The incorporation was found to be greater with the fasted animals, but the authors ventured no comments on turnover rate. The literature as far as can be ascertained contains no reference to the effect of glucose or fat on the turnover rate of tissue proteins.

IN VITRO EXPERIMENTS

More recently the *in vitro* approach to the problem of protein synthesis using the tissue slice technique has been replacing the *in vivo* studies described above. The great advantage of the tissue slice over the whole animal is in permitting more experimental variables to be controlled, thus providing a greater chance of more rapid progress in uncovering factors which may influence the rate of incorporation of the isotopically labelled compounds. On the other hand there is no guarantee that the protein metabolism of a surviving slice follows the same channels as in the intact animal. Once it had been established that labelled amino acids could be taken up by tissue proteins *in vitro* it was necessary to determine whether the rate of uptake was comparable to that obtaining *in vivo* and if the conditions under which this occurred were physiological. In reviewing the subject, Borsook (1950) showed that the rate of incorporation into intact cells

in vitro (expressed as μ equivalents amino acid incorporated/ gm. protein) was of the same order as occurred with *in vivo* experiments. In addition, he demonstrated that the incorporation rate of a labelled amino acid was a logarithmic function of its initial concentration up to a certain optimal concentration, concentrations above 0.001 M - 0.003 M being inhibitory. In general it may be stated that tissues *in vitro* will incorporate amino acids at concentrations similar to those in the blood and the dependence of the rate of incorporation on concentration is greatest in the physiological range. It would appear therefore that any information obtained from *in vitro* studies may be applicable in some degree to the process *in vivo*.

A considerable amount of work has been done on this subject, both with slices and homogenates, but only the results with tissue slices and diaphragm (itself a natural tissue slice) will be considered here as these are the techniques which we ourselves used. Work in this field was initiated by Melchior and Tarver (1947a). These workers synthesised ^{35}S-cystine and incubated it in Krebs' saline containing 0.2% glucose with liver slices. The isolated protein showed considerable radioactivity but on addition of thioglycolic acid, a reducing agent, the major part of the radioactivity was removed. In view of this finding it was postulated that most of the cystine was joined to the protein by S-S bridges and not by peptide bonds. Cystine would

thus appear to be of little value as a labelling agent in the study of protein synthesis. Shortly afterwards, Melchior and Tarver (1947b) synthesised ^{35}S-methionine and incubated it with liver slices under identical conditions to the above. After isolation of the protein, it was hydrolysed and the cystine isolated by the cuprous salt method of Rossouw and Wilken - Jorden (1935). The methionine was extracted from the supernatant, converted to sulphate and found to be radioactive. Also in 1947 Frantz, Loftfield and Miller demonstrated that ^{14}C-carboxyl-labelled DL-alanine was incorporated into the proteins of rat liver slices. It was noted that oxygen was necessary for this process and that all the radioactivity of the proteins resided in the alanine moiety of the protein. Anfinsen, Beloff, Hastings and Solomon (1947) incubated rat liver slices with radioactive $Na_2{}^{14}CO_3$. The isolated protein was found to be radioactive, the activity being in the glutamic and aspartic acid residues. Again in 1947 Winnick, Friedberg and Greenberg demonstrated the uptake of ^{14}C-glycine by rat intestinal tissue. The incorporation of the isotope was shown to be inhibited by azide, a powerful inhibitor of cytochrome oxidase. Uptake of ^{14}C-alanine and glycine was observed in liver slices by Zamecnik, Frantz, Loftfield and Stephenson in 1948. Simpson and Tarver (1950) now investigated in more detail the uptake of ^{35}S by rat liver slices. The medium was as before i.e. Krebs' saline + 0.2% glucose. Cystine was again

separated from the protein after hydrolysis, in this case by the method of Zittle and O'Dell (1941). The extent of the incorporation was found to depend on the concentration of methionine in the medium. It was also found to vary linearly with time and to be inhibited by respiratory poisons and anaerobic conditions. The label was retained in spite of extensive washing and dialysis procedures. Borsook, Deasy, Haagen-Smit, Keighley and Lowy (1950b) demonstrated that rat diaphragms incubated with Krebs - Henseleit - Ringer solution would incorporate ^{14}C-glycine, leucine and lysine. Anaerobiosis and inhibitors of oxidation and phosphorylation such as arsenite, dinitrophenol and azide were observed to interfere with the uptake of the label.

Evidence for peptide bond formation. A major assumption underlying all the work with labelled amino acids is that the uptakes measured, actually represent the incorporation of the amino acids concerned into the proteins by peptide bond formation. In other words, we must take steps to ensure that the incorporated amino acids are not adsorbed on to the protein, or bound to the protein by disulphide linkages, or by any other linkage except a true peptide bond. The possible involvement of disulphide bonds has been shown by Melchior and Tarver (1947a) in studies in which ^{35}S-cystine was used. Thus when methionine is used as a tracer the protein must be hydrolysed and the cystine precipitated before estimating the radioactivity. However, apart from this it would appear

that most of the amino acid uptake which occurs in slices is due to genuine peptide bond formation for the following reasons:-

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

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

3) Anaerobiosis and inhibitors of oxidation and phosphorylation depress uptake (Winnick et al., 1947; Frantz, Loftfield and Miller, 1947; Frantz, Zamecnik, Reese and Stephenson, 1948; Borsook et al., 1950b).

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

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

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

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

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

When one considers the variety of amino acids used in these studies and the general agreement between the results it would appear quite reasonable to conclude that the major reaction involved is that of peptide bond formation. This conclusion has been substantiated by the work of Anfinsen and Steinberg (1951), who partially hydrolysed egg albumin and isolated a hexapeptide which was shown to contain radioactive aspartic acid.

Effect of various substances on the in vitro incorporation of labelled amino acids.

(a) Addition of amino acids. Borsook, Deasy, Haagen-Smit, Keighley and Lowy (1949 and 1950b) have shown that when labelled glycine, leucine and lysine are incubated with rat diaphragm the isolated protein has a count, which is the sum of the counts of the protein obtained from incubation with the amino acids individually. It may be concluded then that each amino acid is incorporated independently of the others. In addition, these authors have observed that the addition of a mixture of amino acids approximating in composition to that of casein or haemoglobin (with omission of the amino acid corresponding to the labelled amino acid) did not

affect the uptake of labelled glycine, leucine or lysine in the above system. This agrees with the findings of Zamecnik and Stephenson (1950) who noted that the addition of an enzymatic hydrolysate of protein did not increase the incorporation of the label.

It is difficult to reconcile these results with the findings in feeding experiments (Geiger, 1947 and 1948; Schaeffer and Geiger, 1947; Cannon, Steffee, Frazier, Rawley and Stepto, 1947; Yeshoda and Damodarian, 1947) that an indispensable amino acid is ineffective for growth, or for recovery from protein depletion, or for maintenance unless it is fed or injected within a few hours of the other essential amino acids. Similarly, Miller, Bly, Watson and Bale (1950) perfused a rat liver with ^{14}C-DL-lysine and found it essential to have a balanced mixture of amino acids in the perfusion mixture before appreciable incorporation into liver and plasma proteins took place. Again, Hokin (1951) observed that amylase synthesis by pigeon pancreas slices did not occur unless the medium was supplemented with a mixture of all the essential amino acids. This throws some doubt on the significance of the in vitro work, but it should be noted that tissue slices always contain some free amino acids (apparently derived from proteolysis) which may be sufficient in amount to provide for the needs of protein synthesis over the short period of incubation.

(b) Addition of glucose and/or insulin. (Table 30). In 1951

TABLE 30.

Effect of adding glucose, succinate and insulin on the *in vitro* uptake of labelled amino acids by diaphragm, liver and spleen.

Authors.	Tissue Used	State of Animals	Labelled Amino Acids.	Effect of Adding			
				Glucose	Insulin	Glucose + Insulin	Succinate
Kit & Greenberg (1951)	Spleen cells	Not stated	^{14}C-alanine ^{14}C-glycine	+ 10% approx (0.01 M)	-	-	-
Sinex et al. (1952)	Diaph-ragm	Fed	^{14}C-alanine	- 14% (0.0055 M)	+ 57% (0.6 units/ml.)	- 11%	-
Krahl (1953)	Diaph-ragm	Fasting	^{14}C-glycine	+115% (0.0078 M)	No effect (0.1 units/ml.)	+109%	-
	Liver	Fasting	^{14}C-glycine	+126% (0.0078 M)	No effect (0.1 units/ml.)	+ 98%	-
	Diaph-ragm	Fed	^{14}C-glycine	-	+ 32% (0.1 units/ml.)	-	-
	Liver	Fed	^{14}C-glycine	-	No effect (0.1 units/ml.)	-	-
Simpson & Tarver (1950)	Liver	Fasting	^{35}S-methionine	-	-	-	-75% (0.02 M)

continued on next page

TABLE 30 contd..

Effect of adding glucose, succinate and insulin on the in vitro uptake of labelled amino acids by diaphragm, liver and spleen.

Authors.	Tissue Used	State of Animals	Labelled Amino Acids.	Effect of Adding			
				Glucose	Insulin	Glucose + Insulin	Succinate
Melchior & Halikis (1952)	Liver	Fasting	^{35}S-methionine	-	-	-	+40% more than glucose (0.0078 M)
Melchior & Goldkamp (1953)	Liver	Fasting	^{35}S-methionine	-	-	-	+40% more than glucose (0.0078 M)

With glucose and succinate figure in brackets is molarity used.

With insulin figure in brackets is number of units/ml. used. (Soluble insulin used in each case)

it was observed by Kit and Greenberg that the addition of 0.01M glucose increased the uptake of ^{14}C-DL-alanine and glycine by a suspension of normal spleen cells. Sinex, McMullen and Hastings investigated in 1952 the effect of glucose and insulin addition on the incorporation of ^{14}C-DL-alanine by the rat diaphragm. The medium used approximated in composition to Krebs-Ringer and the rats received food up to the time of killing. It was found that insulin (0.6 units/ml.) resulted in an increase of 56.8% in the radioactivity, whereas the addition of both insulin and glucose (5.5 mM/litre) caused a depression of 10.6%. When glucose alone was added the incorporation was reduced by 14% and when pyruvate was added in place of glucose there was a 54% decrease in uptake.

Krahl (1953) compared the uptake of ^{14}C-glycine by the liver and diaphragm of fasted and fed rats and noted that the incorporation was invariably less for the tissue of the starving animals. The addition of insulin (0.1 unit/ml.) did not restore this decrease to normal, but it could be overcome by the addition of glucose (140 mgms.%) to the medium. Insulin + glucose had the same effect as glucose alone. Insulin alone produced a significant increase in uptake only with diaphragms from fed rats in which a rise from 53 to 70 counts occurred, a finding which agrees with the above mentioned results of Sinex et al. (1952). Insulin had no

effect on ^{14}C-glycine uptake by liver slices.

(c) Addition of succinate. (Table 30). When succinate (0.02M) was added to the medium, the amount of ^{35}S-methionine incorporated by rat liver slices fell by 75% (Simpson and Tarver, 1950). This is contrary to the observation of Melchior and Halikis (1952) who were investigating the uptake of ^{35}S-methionine by pituitary tissue. These authors noted that the addition of succinate (0.0078 M) resulted in a 40% increase over the incorporation occurring when glucose was the energy substrate. This latter finding has been corroborated recently by Melchior and Goldkamp (1953), who were working with rat liver slices and ^{35}S-methionine.

In view of the findings by some but not all of the workers mentioned above of a stimulation of amino acid incorporation by glucose, we thought it desirable to see (a) whether the alleged glucose effect occurs and (b) whether fatty acids behave differently from glucose in this respect when added to tissue slices in vitro. When these studies were completed, it was apparent that glucose had no considerable effect on amino acid incorporation and we then turned to studies on incorporation of labelled amino acids by the whole animal. These in fact indicated a considerable stimulation of protein synthesis in muscle shortly after glucose administration but not after giving fat.

EXPERIMENTAL

IN VITRO

Animals. Adult male albino rats weighing about 300 g. were used in these studies. They were starved for 24 – 72 hours before the experiment.

Incubation procedure. The animals were killed by a blow on the head and either the diaphragm or the liver removed as quickly as possible. The diaphragm was washed free from blood in sterile saline and the central tendon removed while suspended in incubation medium. Pieces of liver were sliced in a McIlwain (1953) chopper. The diaphragm or approximately 500 mgms. of liver slices were now transferred to stoppered incubation flasks containing 4 mls. of medium and 2 µc of ^{35}S-methionine. The flasks were gassed with 95% oxygen – 5% carbon dioxide before being incubated at 37°C for 1 hour or longer, during which period they were constantly shaken at 120 cycles per minute. In order to demonstrate that the ^{35}S-methionine was incorporated into the tissue proteins and not merely adsorbed on to the surface of the tissue, a zero time control (Simpson and Tarver, 1950) was carried out in every experiment. This consists of killing the tissue before exposing it to the isotope; 0.1M sulphuric acid was usedto kill the diaphragm and 1.5 mls. of 30% trichloracetic acid (T.C.A.) for the liver. Under these conditions the

radioactivity incorporated was extremely low (just above the background for the counter) showing that the adsorption occurring was negligible.

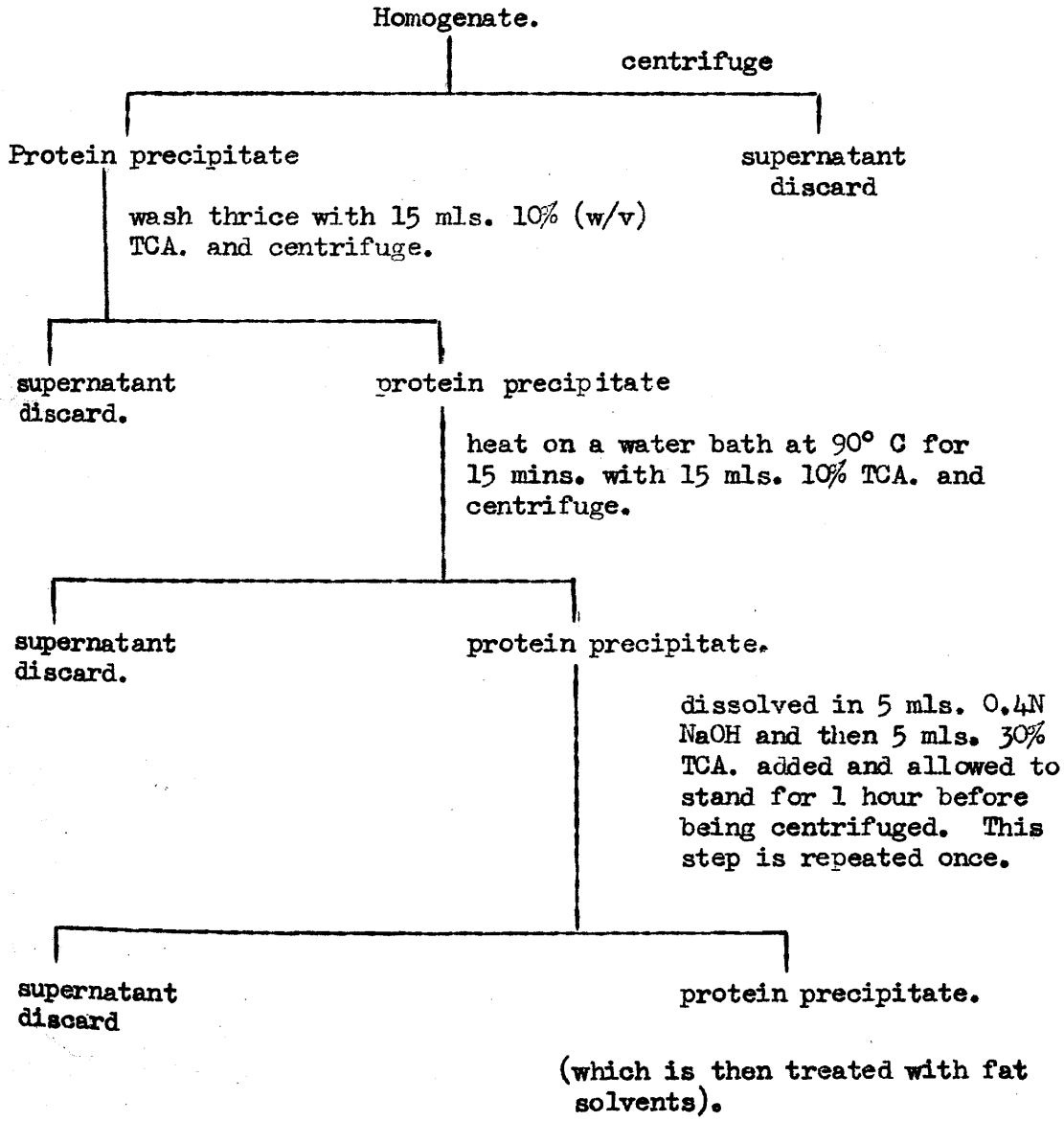
The medium used was that described by Sinex, McMullen and Hastings (1952) and consisted of Na^{+}138, K^{+}5, Ca^{++}10, Mg^{++}5, $HPO_4^{=}$ 2, Cl^{-} 133 and HCO_3^{-} 25, expressed as milli-equivalents/litre. In each experiment the medium was supplemented by the addition of glucose 0.0055M, glucose 0.011M, succinate 0.0078M, octanoate 0.001M or acetate 0.02M either singly or in various combinations. When any of the latter three substances were added, an equal number of milli-equivalents of NaCl were removed from the medium. The medium was Seitz filtered prior to use. All the glassware used was sterilized with heat and every effort was made to exclude bacteria.

Isolation of protein from diaphragm and liver. On the termination of incubation the diaphragm was removed from the flasks and killed by the addition of 0.1M H_2SO_4. After washing with distilled water they were homogenised in a Folley "Nelco" blendor with 15 mls. of 0.4N NaOH for approximately 15 minutes. 20 mls. of distilled water were now added and the proteins precipitated by the addition of 7 mls. of 30% (w/v) T.C.A.. Homogenisation was continued for a further 10 minutes, then the precipitated protein was transferred to a 50 mls. centrifuge tube.

To stop the reaction in the flasks containing liver

Fig. 8

Treatment of homogenates after incubation with ^{35}S-methionine.



slices, 1.5 mls. of 30% (w/v) T.C.A. were added. The slices were then homogenised in a Folley blendor with 20 mls. of 10% (w/v) T.C.A. for 10 minutes. Finally the precipitated protein was transferred to a 50 mls. centrifuge tube.

The subsequent treatment of the protein is described in Fig.8. The salient features are i) repeated washing with 10% (w/v) T.C.A. to remove traces of ^{35}S-methionine adsorbed on to the protein, ii) heating on a water bath for 15 minutes at 90°C with 10% (w/v) T.C.A. to hydrolyse the nucleoproteins (Schneider, 1945), iii) dissolving the protein in 0.4 N NaOH and reprecipitating it with 30% (w/v) T.C.A.. This technique was instituted by Melchior and Halikis (1952) to remove traces of adsorbed methionine from the protein.

The protein was then washed with 15 mls. of fat solvents in the following order:- 95% (v/v) ethanol (twice), ethanol-chloroform (3:1), ethanol-ether (3:1), absolute alcohol and dried overnight with ether.

Method of homogenisation. In our earliest experiments following the recommendation of Sinex, McMullen and Hastings (1952), the diaphragms were homogenised in a Potter-Elvehjem (1936) glass homogeniser. Even after a 24 hour hydrolysis in 6 N HCl of the purified protein, there was seen to be an insoluble sediment in the bottom of the flask. When the diaphragm was homogenised with the glass homogeniser and the precipitated protein fat extracted and dried, then its N

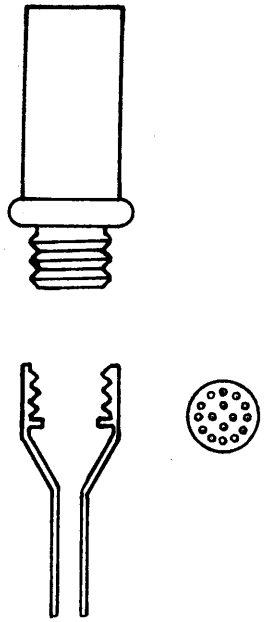
content, determined by the method of Ma and Zuazaga (1942), was found to be 3.51% instead of the expected 16%. If the diaphragm after having been homogenised in 0.4 N NaOH, was centrifuged to remove the insoluble fraction, then the protein precipitated, fat extracted and dried, the N content increased to 9.96%. This figure is still appreciably lower than the theoretical value and it would appear that there is present both a soluble and an insoluble contaminant, the most probable origin of which is the glass of the homogeniser. To test this hypothesis, a sample of casein was weighed out, homogenised in the glass homogeniser, then the protein isolated, fat extracted and dried. A control sample was subjected to similar treatment except that the homogenisation stage was omitted. N estimations showed that the N content of the homogenised and untreated samples were now 8.54% and 13.25% respectively. It was found that when a solution of 0.4 N NaOH was "homogenised" in the glass homogeniser, the solution became cloudy after 30 seconds or so, and when allowed to stand a precipitate eventually settled out. It would appear that the friction of the glass pestle against the glass container rubs away particles of glass, some of which dissolve in the NaOH while some remain insoluble. Since diaphragms require fairly prolonged homogenising (approximately 10 minuted) it is quite apparent that the Potter-Elvehjem homogeniser is not suited for this purpose.

The use of the glass homogeniser was therefore

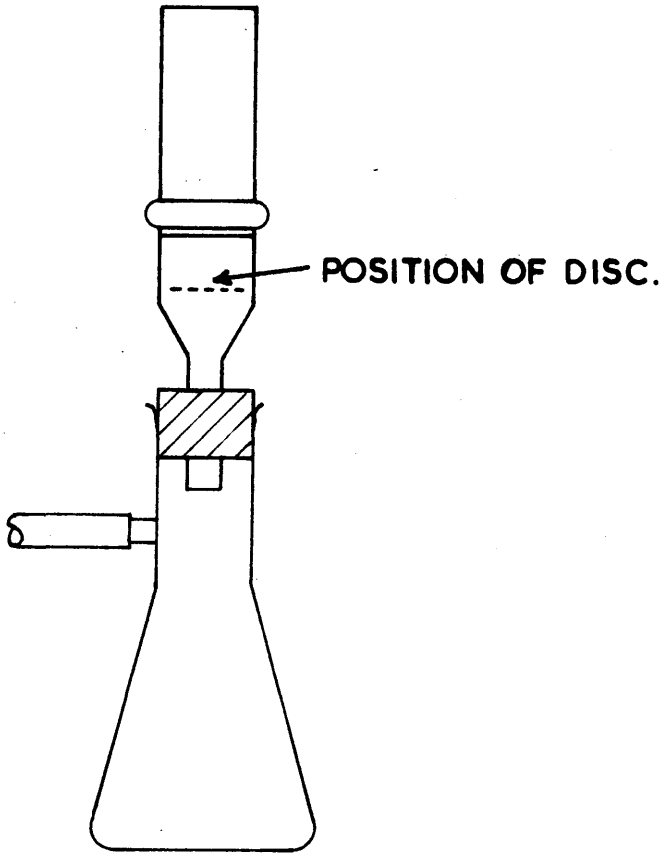
discontinued and the possibility of using a Folley steel bladed blendor investigated. Diaphragms, after homogenisation with this blendor yielded proteins which had an average N content of 13.6%. This indicates that no serious contamination results when a Folley blendor is used and consequently in all further experiments this instrument was used for homogenising tissues.

Isolation and estimation of ^{35}S-methionine from protein. The dried proteins were weighed then hydrolysed with 25 mls. of 6 N HCl for 8 hours in an atmosphere of nitrogen, which prevents the oxidation of cysteine to cysteic acid. The HCl was then removed by distillation in vacuo and the amino acid residue taken up in 40 mls. of distilled water. In order to separate the cysteine from methionine, the method of Zittle and O'Dell (1941) was used, which consists of adjusting the pH of the hydrolysate to 3.0 with solid sodium acetate, heating on a boiling water bath and adding 8 times the theoretical amount of CU_2O. When the pH is adjusted to 4, the cysteine is precipitated as the copper mercaptide and after standing for 40 minutes this precipitate is filtered off. (Cysteic acid is not precipitated by copper, which explains the necessity for hydrolysing in an atmosphere of nitrogen). The filtrate was then evaporated to dryness and 1g. of benzoic acid was added to the residue, which was powdered, formed into a pellet and combusted in an oxygen bomb filled to a pressure of 25 atmospheres of oxygen. (The benzoic acid

MICRO-FILTER.



PERFORATED PLASTIC DISC COVERED WITH CIRCLE OF FILTER PAPER & PLACED IN POSITION.



MICRO-FILTER SCREWED TOGETHER AND PRECIPITATE OF BENZIDINE SULPHATE FILTERED OFF BY SUCTION.

FIG. 9.

acts merely as an inert carrier for the amino acids and enables them to be formed into a pellet). This procedure converts the ^{35}S-methionine into $^{35}SO_4$. The bomb was washed out with distilled water and the washings reduced to small volume (2 mls.); 2 mls. of absolute alcohol and 2 mls. of benzidine hydrochloride solution were then added and the precipitate of benzidine sulphate allowed to settle out overnight in the refrigerator. This procedure has been described by Young, Edson and McCarter (1949). The benzidine sulphate was collected evenly on a filter paper using the micro-filter (Fig.9) and after washing with 95% (v/v) ethanol, was allowed to dry in a dessicator for at least one hour.

The precipitate was then counted with an end window Geiger-Muller counter and afterwards transferred to a conical flask with water. The amount of sulphur was now estimated by the method of Fiske (1921) which consists of titrating the boiling solution with standard NaOH using phenol red as an indicator. The benzidine sulphate is hydrolysed by the boiling water to free benzidine and sulphuric acid which is estimated with the standard alkali.

The radioactivities were then expressed as counts per minute per mgm. of sulphur, due correction being made for the self absorption of ^{35}S Beta particles. (Henriques, Kistiakowsky, Margnetti and Schneider, (1946).

Recovery experiments with the oxygen bomb. Recovery experiments on the oxygen bomb and micro-filter were performed by Mr. James Chisholm and were found to be quite adequate.

The separation of cysteine and cystine from methionine. Mr. Chisholm also investigated the efficacy of the Cu_2O method of separating cysteine from methionine and found it to be quite satisfactory.

The self-absorption of ^{35}S Beta particles. If the activity of a series of samples of varying weight but constant area is measured there is a deviation from a linear relationship between activity and weight of sample, which is due to self-absorption of the Beta particles. A linear relationship between observed activity and sample weight is possible only where the radiations are so penetrating that the upper layers of the sample cannot appreciably absorb the radiations originating in the lower layers. When counting an isotope whose radiations are of such low energy that the ordinary sample thicknesses represent an appreciable fraction of the mean particle range, self-absorption introduces large errors in activity measurements and correction must be made for them.

There are four procedures by which correction can be made or the need for it eliminated (Calvin, 1949). First by counting the sample at "infinite thinness" when the error due to self-absorption can be neglected when compared with other errors in the experiment. This is limited to measuring samples of high specific activities. The second procedure involves reproducing accurately a standard sample thickness.

TABLE 31.

Correction factors for self-absorption of ^{35}S Beta particles.

Thickness - Benzidine SO_4 mg./sq.cm.	Count	Count per mg. Benzidine SO_4/ sq.cm.	Correction Factor	Correction Factor (Henriques)
3.25	1480	455	1.000	1.000
0.46	285	620	1.365	1.370
1.00	600	600	1.318	1.275
5.00	2030	406	0.892	0.830
7.00	2440	349	0.767	0.675
7.40	2500	338	0.743	0.650
10.00	2660	266	0.585	0.530

Divide by correction factor to obtain the corrected count.

This method is technically rather difficult to apply. The third procedure involves counting the samples at "infinite thickness". This method is of very wide applicability, but is wasteful of sample material and cannot be applied if the amount of material available is small. The fourth procedure is to determine the relationship between observed activity and sample thickness and this is the method which we have employed.

A ^{35}S-methionine solution was combusted in the oxygen bomb and the sulphate so formed was converted to benzidine sulphate. Varying amounts of this benzidine sulphate suspension were now filtered, counted and titrated as described earlier. From the data obtained, a graph was constructed by plotting mgms. of benzidine sulphate/cms.^{2} against the counts per minute. Seven arbitrary thicknesses of benzidine sulphate were now selected and the corresponding counts per minute read from the graph (Table 31). The specific activities (the counts per mgm. of benzidine sulphate/cm.^{2}) are seen to differ for every sample, this being due of course to the occurrence of self-absorption. The method of combatting this is to correct every count to a standard thickness, which in this case is 3.25 mgms. of benzidine sulphate/cm.^{2}. (Any standard thickness can be chosen; 3.25 was selected merely because we wished to compare our results with those of Henriques et al. 1946). This is achieved by dividing all the specific activities by 455

(Table 31) and so we arrive at the appropriate correction factors for the various thicknesses of benzidine sulphate. When the specific activity of any sample is divided by its appropriate correction factor, the answer obtained is the specific activity the sample would have had if its thickness had been 3.25 mgms. of benzidine sulphate/cm.^{2}. Our correction factors are seen to agree closely with those of Henriques et al..

IN VIVO

Animals. Adult male albino rats weighing about 300g. were used. They were starved for the 24 hours preceding the experiment.

Procedure. The rats were injected intraperitoneally with a ^{35}S-methionine solution (20 µc in 1 ml. of 0.9% NaCl) then after the lapse of half an hour they were fed either 4 mls. of water (controls) or 4 mls. of 50% glucose solution or 1 ml. of olive oil by stomach tube. At 2, 4 and 6 hours after feeding the animals were killed by exsanguination under ether anaesthesia and the diaphragm, liver, small intestine and a piece of the quadriceps femoris muscle removed.

Isolation of protein from diaphragm, leg muscle, intestinal submucosa and liver. The diaphragm and sample of leg muscle were killed immediately on removal by the addition of 0.1 M H_2SO_4. Thereafter they were both treated as described

under diaphragm in the in vitro section.

The submucosa was scraped from the small intestine with all possible speed and homogenised in a Folley blendor with 30 mls. of 10% (w/v) T.C.A. for 10 minutes. The precipitated protein was then transferred to a 50 ml. centrifuge tube.

The liver was homogenised immediately in a Folley blendor with 40 mls. of 10% (w/v) T.C.A. for 10 minutes. The precipitated protein was then transferred to a 50 ml. centrifuge tube.

The further treatment of these specimens is precisely similar to that described in the in vitro section.

RESULTS

IN VITRO

A - Rat Diaphragm.

Uptake of ^{35}S-methionine by rat diaphragm after the tube feeding of glucose or fat. Twelve adult albino rats which had been fasted for 24 hours were divided into three groups. One group was then fed 4 mls. of water (controls), another 4 mls. of 50% glucose solution and the third group 1 ml. of olive oil by stomach tube. One hour later they were killed by a blow on the head and the diaphragms quickly removed and placed in stoppered conical flasks containing the medium and ^{35}S-methionine. Incubation was carried out at 37°C with constant shaking for one or two hours and the amount of

TABLE 32.

Uptake of ^{35}S-methionine *in vitro* by rat diaphragm after tube feeding of glucose and olive oil.

Time of Incubation.	Counts / min./ mgm. Sulphur.		
	Control	Glucose	Fat
1	1012	777	1181
2	2383	1479	2007

Each value is the mean of 2 experiments. The rats were fed 4 mls. water (control), 4 mls. of 50% glucose or 1 ml. of olive oil by stomach tube and killed one hour later. The diaphragms were then removed and incubated for one or two hours.

TABLE 32a

Analysis of variance of ^{35}S-methionine uptake by diaphragm *in vitro* after the tube feeding of glucose and fat.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square.	Variance Ratio.
Total	11	10,486,457	-	-
Treatments	2	1,431,701	715,850	3.2
Times	1	6,600,833	6,600,833	-
Interaction	2	1,106,513	553,207	2.4
Residual	6	1,347,510	224,585	-

For $n_1 = 2$ and $n_2 = 6$ F = 5.14 at the 5% level.

Therefore different treatments have no significant effect on the uptake of ^{35}S-methionine by rat diaphragm.

^{35}S UPTAKE OF RAT DIAPHRAGM IN VITRO AFTER TUBE FEEDING OF GLUCOSE AND FAT.

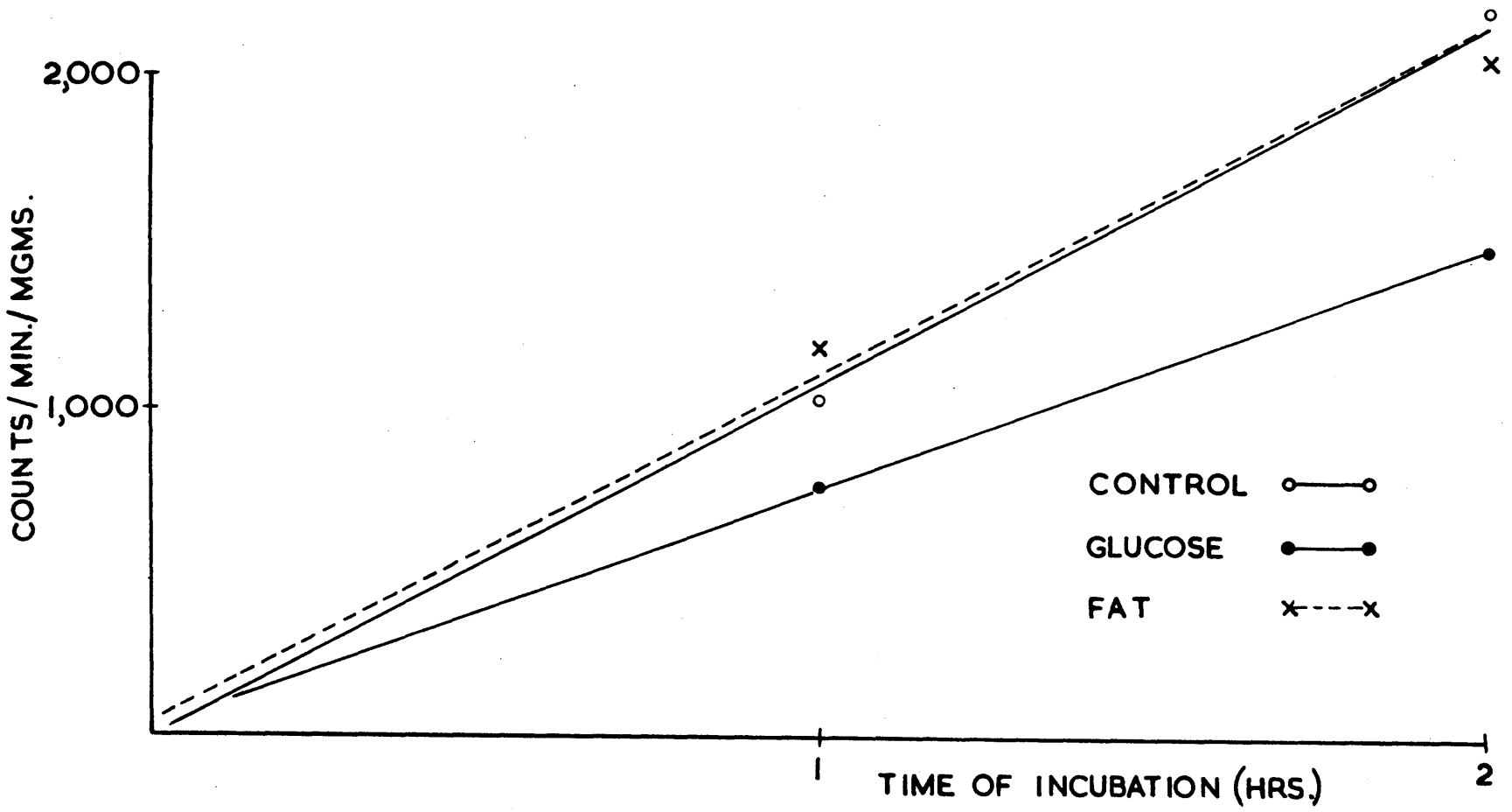


FIG. 10.

TABLE 33.

The effect of glucose, octanoate and acetate on ^{35}S-methionine uptake by diaphragm and liver slices.

Additions to Flask.	Counts / minute / mgm.Sulphur.				% Change	
	Liver Protein	Diaphragm Protein				
		Expt.1	Expt.2	Mean	Liver	Diaph-ragm.
None	468	1317	977	1147	-	-
Glucose 0.0055 M	413	1342	1023	1183	-12%	+ 3%
Glucose 0.0110 M	529	1492	1011	1252	+13%	+ 9%
Octanoate 0.001 M	110	1822	554	1188	-77%	+ 3%
Octanoate 0.001 M + Glucose 0.0055 M	79	1343	532	938	-83%	-18%
Acetate 0.02 M	156	1067	726	897	-67%	-22%
Acetate 0.02 M + Glucose 0.0055 M	78	474	795	635	-83%	-45%

^{35}S-methionine in the muscle protein estimated. It can be observed from Table 32 and Fig. 10 that the incorporation of ^{35}S is quite similar for the fat-fed animal and the control at 1 and 2 hours, but the glucose-fed animal shows a depression at both these times. Statistical Analysis (Table 32a) shows, however, that there is no significant difference between the three treatments. A similar depression in uptake of ^{14}C- DL-alanine has been noted by Sinex et al. (1952) when glucose is added to a medium in which are suspended the diaphragms from fed rats.

Effect of added glucose on the uptake of ^{35}S-methionine by the rat diaphragm. Fourteen adult albino rats were starved for 24 hours, then divided into two groups and killed by a blow on the head. The diaphragms were quickly removed and placed in the various flasks which contained the following additions:- (1) none (control), (2) glucose 0.0055 M, (3) glucose 0.011 M, (4) octanoate 0.001 M, (5) octanoate 0.001 M with glucose 0.0055 M, (6) acetate 0.02 M, and (7) acetate 0.02 M with glucose 0.0055 M. All the incubations were carried out for two hours. From Table 33 it can be seen that the higher concentration of glucose raises the incorporation slightly (9%). This is extremely small when compared with the 115% rise in incorporation obtained by Krahl (1953) working on diaphragms under identical conditions and using ^{14}C-glycine as a labelling agent. Octanoate, on the other hand has a very variable effect, but its mean value is very close to that of

the control. The incorporation due to acetate also fluctuates markedly, but it definitely tends to decrease the uptake. The striking point is that, contrary to expectations, the addition of glucose to octanoate and acetate depresses the count in both cases.

B - Rat Liver Slices.

Effect of added glucose on the uptake of ^{35}S-methionine by rat liver slices.

a) Incorporation after fasting 24 hours. Two adult male albino rats were starved for 24 hours, then killed by a blow on the head. The livers were quickly removed, sliced and the slices pooled. About 500 mgms. of slices were added to flasks containing the same additions as in the last experiment. The results (Table 33) show the same general picture as with the diaphragm. Again there is a slight rise in incorporation with the higher level of glucose (13%). In this case, both octanoate and acetate depress the uptake. Once again, however, a striking feature is the decrease in incorporation which occurs when glucose is added to either octanoate or acetate.

A disturbing feature about these experiments was our failure to reproduce the marked increase in incorporation announced by Krahl (1953). He reported a rise of 126% in the incorporation of ^{14}C-glycine by rat liver slices from starving animals after the addition of glucose to the medium. Krahl obtained these results by starving the animals for an

TABLE 34.

The effect of glucose on the uptake of ^{35}S-methionine by rat liver slices (72 hours starvation)

Time of Incubation.	Counts /minute / mgm.Sulphur.	
	Control	Glucose
1	855	986
2	3360	757
3	3715	1945
4	4838	2525

Glucose was added at a level of 140 mgms.per cent (0.0078 M)

The regression of ^{35}S-methionine uptake on time was calculated for both the control and the glucose fed rats. The values showed no significant difference.

unspecified period. Since Sinex et al. (1952) demonstrated a decrease in ^{14}C-alanine incorporation when glucose was added to diaphragm from fed rats, the discrepancy between ourselves and Krahl might depend on the duration of the fasting period before killing. We therefore decided to starve the rats for three days and then try the effect of added glucose on the amino acid uptake by liver slices.

b) Incorporation after fasting 72 hours. The procedure was identical to the above, except that the only addition was glucose at a concentration of 140 mgms. per cent (0.0078 M) which was that used by Krahl (1953), and the flasks were incubated for 1, 2, 3 and 4 hours. The results (Table 34) were the complete antithesis of what was expected, glucose causing a depression in incorporation over the whole 4 hour period. However, this reduction in uptake was not statistically significant.

Effect of added succinate on the uptake of ^{35}S-methionine by rat liver slices. Succinate was reported by Simpson and Tarver (1950) as depressing the uptake of ^{35}S-methionine by rat liver slices by 75%. Melchior and Halikis (1952) and Melchior and Goldkamp (1953) claimed on the other hand that it resulted in a 40% increase over the incorporation due to glucose. We therefore decided to compare the effect of added succinate with that of glucose and octanoate. The procedure was as above, except that the additions to the flasks were:- (1) none (control), (2) glucose 0.0055 M, (3) succinate

TABLE 35.

The effect of addition of glucose, succinate and octanoate on ^{35}S-methionine uptake by rat liver slices.

Additions to Flask	Counts /minute/mgm.S at		% Change at	
	2 hours	4 hours	2 hrs.	4 hrs.
None (Control)	1823	4027	-	-
Glucose 0.0055 M	2122	4622	+16	+15
Succinate 0.0078 M	2895	5654	+59	+40
Octanoate 0.001 M	2033	5151	+11	+28
Octanoate 0.001 M + Succinate 0.0078 M	1902	4360	+ 4	+ 8

The increase in incorporation when succinate was substituted for glucose was 36% at the second hour and 22% at the fourth hour.

0.0078 M, (4) octanoate 0.001 M, and (5) octanoate 0.001 M with succinate 0.0078 M. Incubation was for 2 & 4 hours. Succinate addition was found to increase the incorporation by 36% over that of glucose in the two hour incubation (Table 35), which compares favourably with the 40% quoted above. Glucose again caused a slight increase in uptake at the 2 & 4 hour periods (16% and 15%). In this experiment addition of octanoate led to a slight increase in incorporation at both 2 & 4 hours, but, just as we had previously found with glucose (Table 33), the addition of succinate to octanoate led to a fall.

If we ignore for the present the first experiment with rat diaphragm, which is in part an in vivo experiment, we may summarise our findings thus:- The addition of glucose leads to a slight increase in incorporation by both diaphragm and liver slices (9% to 16%). The results with octanoate and acetate are variable, but generally speaking octanoate tends to maintain the uptake of the label at the control level, whereas acetate has a definite tendency to reduce the count. When glucose is added to either octanoate or acetate the count is invariably decreased. We feel that this last observation is related to the depression in uptake which occurs when glucose is added to rat liver slices from an animal which has been fasted for a prolonged period (three days), since in such an animal fat metabolism will predominate.

This will be discussed later.

Apart from the results obtained with acetate, the experiments performed above did not show a clear-cut distinction between the action of glucose and that of fat (octanoate) so recourse was made to in vivo studies.

IN VIVO

The in vivo uptake of ^{35}S-methionine by diaphragm, leg muscle, liver and intestinal submucosa. This experiment was repeated 4 times, 9 rats being used in each case. After fasting them for 24 hours, they were divided into three groups. A sterile solution of ^{35}S-methionine (20 µc in 1.0 mls. of 0.9% NaCl) was then injected into each rat intraperitoneally and after a lapse of half an hour groups of three 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 then killed at 2, 4 and 6 hours after feeding.

The diaphragms were dissected out in all 4 experiments and in two of the experiments a piece of the quadriceps femoris muscle was also removed. Both diaphragm and leg muscle were homogenised immediately after extirpation in order to prevent any protein breakdown and consequent loss of radioactive methionine. Liver and intestinal submucosa specimens were removed at the second and the fourth hour in the first two experiments. They were also homogenised with

TABLE 36.

The effect of glucose and fat administration on the incorporation of ^{35}S-methionine by the rat diaphragm in vivo.

Time after feeding.	Counts/Min./mgm.Sulphur.		
	Control	Glucose	Fat
2	698	494	758
4	507	1036	477
6	712	609	625

Each value is the mean of 4 experiments. The rats were starved for 24 hours then ^{35}S-methionine injected intraperitoneally (20 µc/rat) half an hour before feeding 4 mls. of water (control), 4 mls. of 50% glucose or 1 ml. of fat by stomach tube.

TABLE 36a

Analysis of variance of the *in vivo* incorporation of ^{35}S-methionine by rat diaphragm at 2 and 4 hours after feeding glucose or fat.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Total	15	91,832	-	-
Time	1	23,948	23,948	-
Treatments	1	7,268	7,268	
Interaction	1	15,313	15,313	5.40
Replicates	3	19,748	6,583	
Residual	9	25,555	2,839	

The data for the sixth hour were omitted from this analysis as they were incomplete.

For $n_1=1$ & $n_2=9$ F = 5.14 at the 5% level.

The interaction is therefore significant, which indicates that the action of glucose differs significantly from that of fat at the fourth hour.

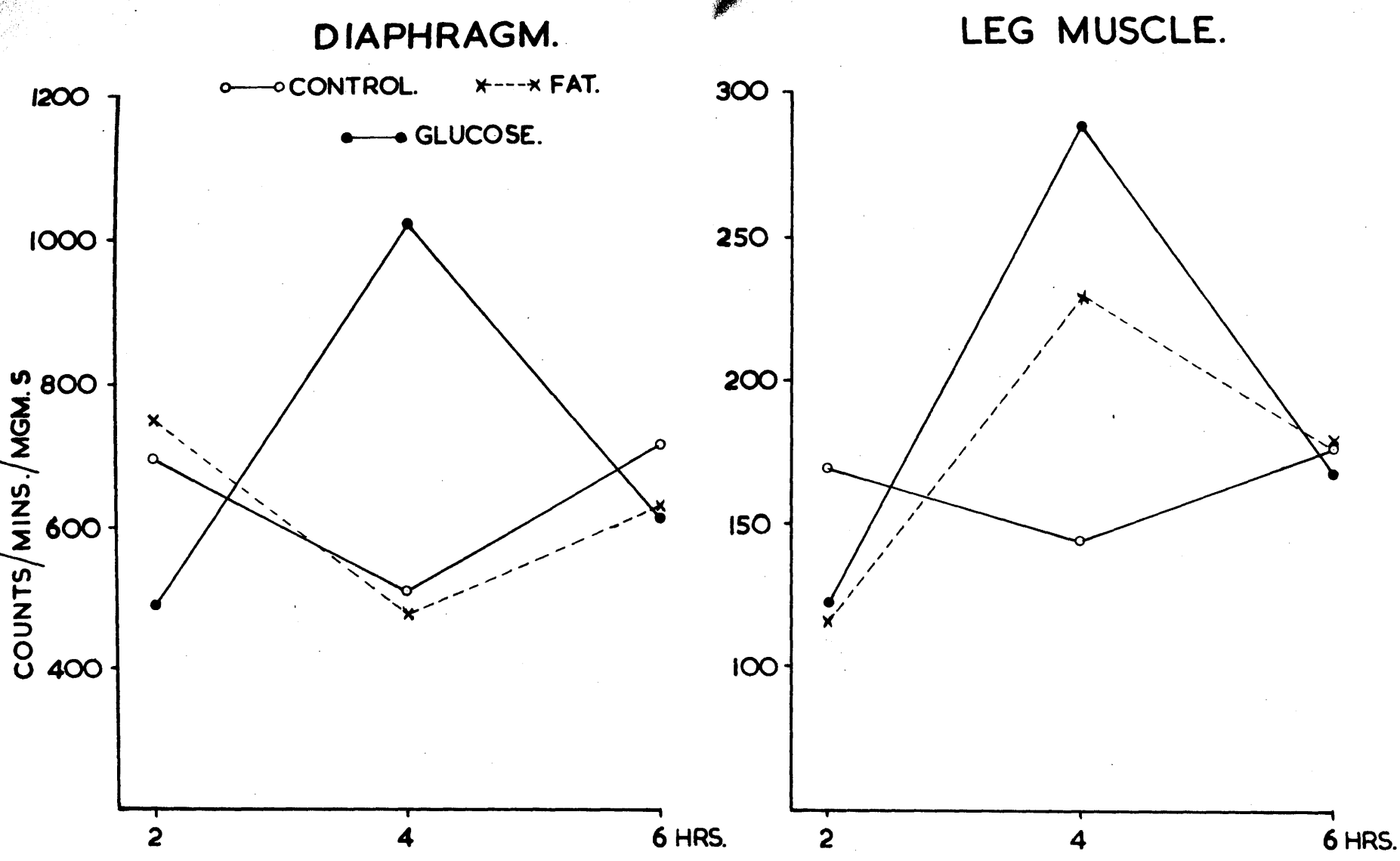

DIAPHRAGM.
CONTROL.
FAT.
GLUCOSE.
1200
1000
800
600
400
COUNTS/MINS./MGM.S
2
4
6 HRS.
LEG MUSCLE.
300
250
200
150
100
2
4
6 HRS.

FIG. 11.

TABLE 37

The effect of glucose and fat administration on the incorporation of ^{35}S-methionine by rat skeletal muscle *in vivo*.

Time after feeding.	Counts/Min./mgm.Sulphur.		
	Control	Glucose	Fat
2	171	121	112
4	144	290	229
6	177	169	175

Each value is the mean of 2 experiments. The rats were starved for 24 hours then ^{35}S-methionine injected intraperitoneally (20 µc/rat) half an hour before feeding 4 mls. of water (control), 4 mls. of 50% glucose or 1 ml. of fat by stomach tube.

all possible speed to prevent loss of methionine.

From Table 36 and Fig.11 it can be seen that the incorporation of ^{35}S-methionine by the control diaphragm remains fairly steady throughout the various time intervals (2, 4 and 6 hours after feeding). The uptake is approximately 4 times that of skeletal muscle (Table 37). This can possibly be attributed to the higher physiological activity of the diaphragm. This effect has been noted previously by Altman, Casarett, Noonan and Salomon (1949), who observed that 24 hours after the intraperitoneal injection of ^{14}C-glycine the incorporation by the proteins of the diaphragm was twice that of the muscle proteins. The ^{35}S uptake by the diaphragm of the glucose-fed animal is very interesting (Fig.11). A slight fall occurs at two hours, but at the fourth hour there is a marked rise to twice the value of the control and by the sixth hour the incorporation has returned again to normal. The fat-fed animal does not differ appreciably from the control. Analysis of variance (Table 36a) demonstrates a significant difference (at the 5% level) at the fourth hour between the glucose and the fat fed animals.

It can be observed from Table 37 and Fig.11 that the incorporation by skeletal muscle follows the same pattern as diaphragm. The control values are relatively constant and the glucose fed animal shows the same peak in uptake at the fourth hour. The feeding of fat also results in an increase at the fourth hour but this is entirely due to a high result

TABLE 38.

Effect of glucose and fat administration on ^{35}S-methionine uptake by liver and intestinal submucosa in vivo.

Tissue.	Counts/min./mgm. Sulphur at					
	2 hrs.			4 hrs.		
	Control.	Glucose.	Fat.	Control.	Glucose.	Fat.
Liver.	4692	5007	5620	5240	5677	4996
Submucosa	6177	7098	7592	11,320	9,185	11,177

Each entry is the mean of 2 experiments.

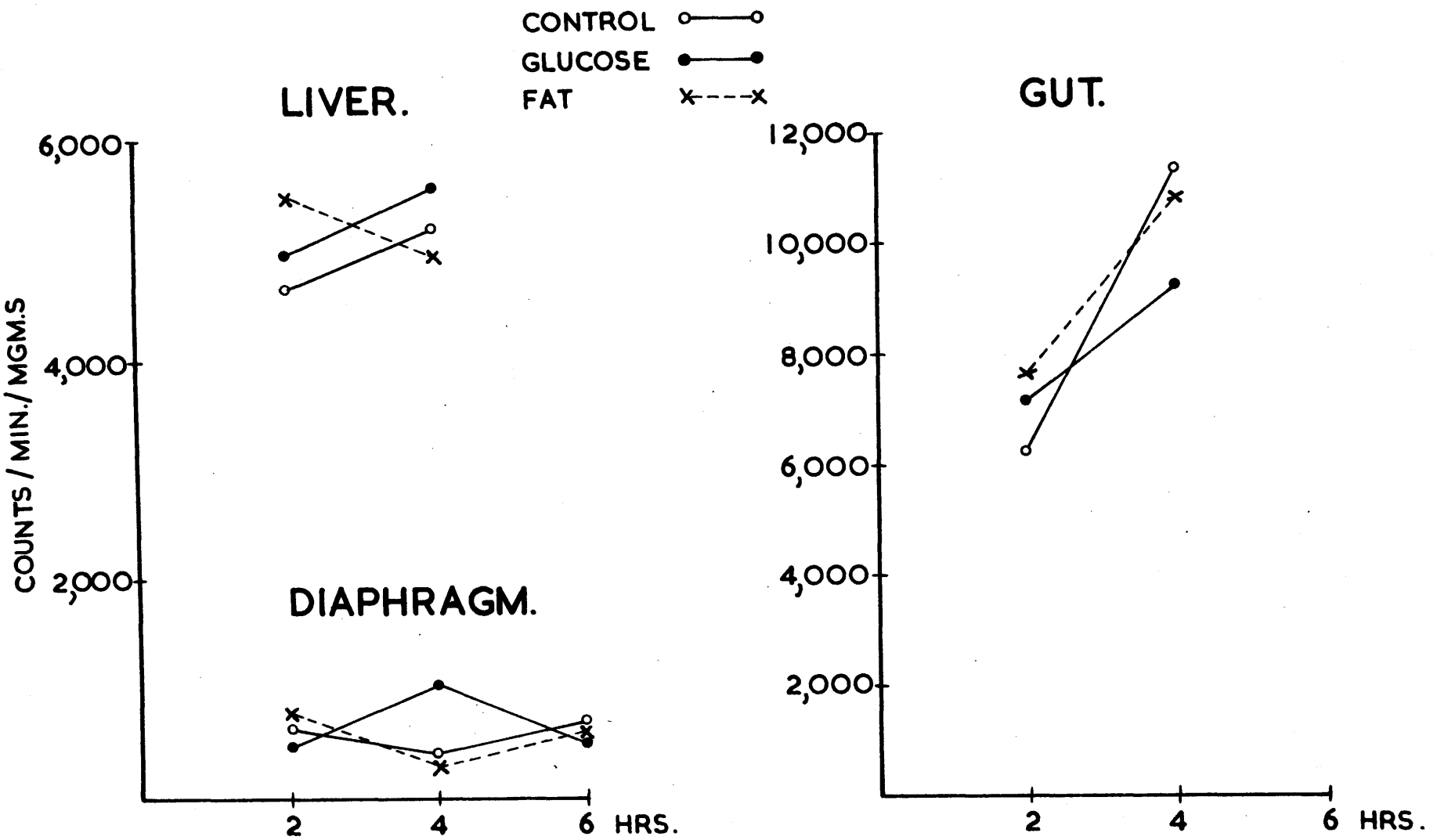

CONTROL
GLUCOSE
FAT
LIVER.
GUT.
DIAPHRAGM.
COUNTS / MIN. / MGM.S
6,000
4,000
2,000
12,000
10,000
8,000
6,000
4,000
2,000
2
4
6 HRS.
2
4
6 HRS.

FIG. 12.

with one of the animals.

The specific activities of the methionine obtained from the proteins of the liver and submucosa are tabulated in Table 38 and the results are compared with those from diaphragm in Fig. 12.

It can be seen that the uptake by these tissues is very much higher than that of muscle (Tables 36 and 37; Fig. 12). In addition the value for the control liver sample increases only slightly from two to four hours, whereas the control value for the submucosa, which is already higher than the liver at two hours, shows a further increase at the 4 hour period. This is in keeping with the very high turnover rate of intestinal submucosa. These observations of differences in rates of amino acid incorporation by various tissues are in agreement with the numerous experiments reported in the literature, and in particular with those of Friedberg, Tarver and Greenberg (1948) with ^{35}S-methionine. A study of the uptake of ^{35}S by liver and submucosa after feeding glucose and fat reveals no obvious departure from the control values. Consequently, as neither glucose nor fat administration appear to have any effect on the turnover rate of the proteins of these tissues, the uptake by liver and submucosa was not investigated in the later studies.

DISCUSSION.

IN VITRO.

The results of the in vitro studies show that the addition of glucose increases the incorporation of ^{35}S-methionine by both liver slices and diaphragm (Table 33) to a slight extent (9 to 16%). It can be deduced from the data of Melchior and Halikis (1952) that these authors also obtained rather small changes with glucose since they observed that the replacement of glucose by succinate stimulated incorporation by 40%, a figure which agrees favourably with the 36% increase we obtained under similar conditions. Kit and Greenberg (1950) have reported a small increase in ^{14}C- DL-alanine uptake similar in extent to ours when glucose (0.01 M) is added to normal spleen cells. A fall of 14% in the incorporation of ^{14}C- DL-alanine into the diaphragm from fed rats when glucose was added, was noted by Sinex et al. (1952). The most probable explanation in this case is the conversion of glucose to pyruvate which is then aminated to give alanine which dilutes the radioactive alanine and so reduces the count. This supposition is supported by their observation that the addition of pyruvate (0.0111 M) inhibited incorporation by 54%. The above results are in marked contrast to the very large increases in the incorporation of ^{14}C-glycine by liver slices (126%) and diaphragm (115%), after the addition of glucose, which has been

reported by Krahl (1953). The discrepancy between his results and ours may be explicable on the grounds that glycine is a non essential amino acid whereas methionine is essential and so added glucose may affect their apparent rates of incorporation differently.

The 37% increase in incorporation over that obtained by glucose, which occurs when succinate is added to liver slices, is in good agreement with the 40% increment quoted by Melchior and Halikis (1952) and Melchior and Goldkamp (1953). Simpson and Tarver (1950) on the other hand reported a 75% reduction in the incorporation of ^{35}S-methionine by rat liver slices when succinate was added to the medium.

One explanation of these increases with glucose and succinate may be obtained by an examination of the current theory of protein synthesis. Peptide synthesis is an endergonic reaction and therefore if the reaction is to proceed it must be coupled with an energy-yielding reaction. The processes of oxidative metabolism are known to constitute the large bulk of the exergonic reactions which occur in tissues. Thus if the uptake of amino acids by tissue proteins represents peptide synthesis, then the abolition of respiration should abolish this incorporation. That this is so has been shown by Winnick et al. (1947); Frantz, Loftfield and Miller (1947); Frantz, Zamecnik, Reese and Stephenson (1948) and Borsook et al. (1950b). In addition, these

authors have observed that arsenite and dinitrophenol also inhibit the uptake of labelled amino acids, which suggests that respiration promotes peptide synthesis by way of phosphorylation. It is now established that phosphorylation does play a part in the synthesis of some types of peptide bonds, adenosine triphosphate (A.T.P.) having been shown to be necessary for the synthesis of (a) glutamine (Frei and Leuthardt, 1949), (b) hippuric acid and p-amino hippuric acid (Cohen and McGilvery, 1947), (c) glutathione (Johnston and Bloch, 1951) and (d) ornithuric acid (McGilvery and Cohen, 1950). However the precise relationship of phosphorylation to peptide synthesis is not known.

The prime requisite for peptide synthesis would therefore be a supply of A.T.P.. When glucose is added to the medium its initial conversion to pyruvate yields two high energy phosphate bonds, then its oxidation via the tricarboxylic acid cycle leads to the generation of a further 30 (Johnston, 1949). The increase in incorporation due to glucose could thus be accounted for. Since the incorporation of ^{35}S-methionine by succinate is 40% greater than that observed with glucose, it presumably generates A.T.P. more rapidly than does glucose. As succinate is an intermediate in the citric acid cycle, it is quite conceivable that its initial rate of oxidation would be greater than that of glucose and so more high energy phosphate bonds would be generated in the short time of incubation, which would

account for the increase in the incorporation of the label.

The results with octanoate and acetate in both liver slices and diaphragm are extremely interesting (Table 33). Octanoate has on the average very little effect on the rate of incorporation, but acetate addition results in a definite decrease. If glucose is now added to either of these two substances a further depression in uptake is observed. A similar result occurs when succinate is added to octanoate (Table 35).

When a depression in incorporation occurs, it may be assumed that there is less A.T.P. available for protein synthesis. Now if acetate is oxidised completely through the citric acid cycle there should be a net gain in the number of high energy phosphate bonds, which should be reflected in a higher rate of incorporation. As this does not occur it may be concluded that either (a) acetate is not completely metabolised during the period of the experiment (2 hours) or (b) acetate is completely oxidised but other endergonic reactions are competing with protein synthesis for the available A.T.P.. In this connection Coniglio, Anderson and Robinson (1952) have observed that acetate metabolism is depressed in the fasting rat, so it is conceivable that the lowered incorporation of ^{35}S in our experiments may be due to a diminution in the rate of acetate oxidation.

It would appear on the other hand that the addition

of octanoate has no effect on the amount of A.T.P. which is available for peptide synthesis, as the rate of uptake of the label remains unchanged.

The addition of glucose to acetate or octanoate results in a depression of the incorporation rate, which implies that there is less A.T.P. available for the proteosynthetic process. Using the same argument as above it may be concluded that either (a) glucose and acetate interfere mutually with one another's metabolism to such an extent that the production of A.T.P. is drastically curtailed or (b) other endergonic reactions compete with protein synthesis for the available A.T.P.. Glucose is assumed to improve the metabolism of fatty acids as it is a source of oxalacetate which is a prime requisite for the entry of acetate into the citric acid cycle. It may well be then that other synthetic reactions are using the available A.T.P.; one such reaction could be the synthesis of higher fatty acids from acetate or octanoate. This hypothesis is in harmony with the observation made by Bloch in 1948 that non-isotopic pyruvate stimulated incorporation of labelled carbon from acetate into the fatty acids of rat liver slices.

When glucose is added to liver slices from an animal which has been starved for 72 hours, a marked depression occurs in the incorporation of ^{35}S-methionine (Table 34). As fat metabolism will predominate in these liver slices due to

prolonged fasting, it can be seen that the situation is analagous to that occurring with glucose and acetate, and it is possible that the A.T.P. generated by the glucose is being used to drive synthetic reactions other than protein synthesis.

It is clear from the results described above that glucose, although it only causes a small increase in incorporation (9 - 16%), has an action which is distinct from that of fat, which either has no effect (octanoate) or results in a depression in the uptake of the label (acetate). Thus it has been demonstrated in yet another manner that glucose has an action on protein metabolism which is not shared by fat.

IN VIVO

The striking feature with the *in vivo* studies is the marked increase (104%) in ^{35}S-methionine incorporation which occurs in the diaphragm 4 hours after the feeding of glucose and which has disappeared completely by the end of the sixth hour (Table 36 and Fig.11). Fat ingestion does not cause any change in the ^{35}S-methionine incorporation. The few experiments so far performed with the skeletal muscle of the leg, show that the response of this tissue to glucose ingestion is precisely similar to that of the diaphragm (Table 37 and Fig.11). In this case, however, fat administ-

ration is seen to result in an increased uptake at the fourth hour, but this is due to a high result with one animal.

There are four possible explanations for this effect of glucose administration on the uptake of ^{35}S-methionine by rat diaphragm and leg muscle:-

(a) Glucose may cause increased absorption from the site of injection (peritoneal cavity), but this explanation can be ruled out because of the absence of a corresponding change in the radioactivity of the proteins of liver and gut mucosa (Table 38 and Fig.12).

(b) The rate of penetration of the labelled methionine into muscle might be enhanced by glucose administration. If this were the case the labelling of muscle protein with the isotope would be higher in the glucose-fed rats at all times throughout the experiment, but inspection of the data (Tables 36 and 37; Fig.11) shows that it is actually lower than the control levels at 2 and at 6 hours and is higher only in the 4-hour specimens.

(c) Glucose could cause an increase in the synthesis of general diaphragmatic protein. If this were so, then the subsequent loss of labelled methionine due to protein catabolism should occur at random. Since the rate of protein metabolism in muscle is very slow, the amount lost during the early hours after administration would represent only a small fraction of the total radioactivity incorporated. For

this reason, a difference between the glucose-fed animals and the control animals, once established, would persist for many hours and not vanish two hours later, as we have found (Tables 36 and 37; Fig.11).

(d) The most feasible explanation is the rapid synthesis of a protein or peptide which is distinct from the main mass of the diaphragmatic protein, as this is the only way in which the marked increase and subsequent decrease in incorporation can be accounted for. When the glucose stimulus wears off, then the peptide or protein breaks down, this process being associated with the rapid fall in radio-activity. If the substance is a peptide, then its molecular weight would require to be sufficiently high to account for its being precipitated with T.C.A.. It is unlikely to be glutathione as this compound would be hydrolysed into its constituent amino acids and its cysteine precipitated as the copper salt in the preliminary stages of isolating the sulphur.

It is to be noted that in the above explanation of our results we have postulated the existence of two isolated and distinct types of protein in rat muscle. This is by no means a revolutionary idea as Shemin and Rittenberg (1944) have concluded from the results of ^{15}N studies, that the turnover rate of rat muscle as a whole is much slower than that of the viscera, but that this is the resultant of a

small fraction that turns over its protein as rapidly as that of the viscera and of a much larger and metabolically inert fraction which turns over extremely slowly. In addition Bidinost (1951), working with ^{14}C-glycine, has noted that the turnover rate of myosin and actin is less rapid than the average for the total muscle proteins.

However, the important point established by these in vivo experiments is that glucose has been shown to have an action on protein metabolism which fat does not have, and that this effect is limited to muscle.

GENERAL DISCUSSION

A - The specific effect of carbohydrate on the course of protein metabolism.

From a nutritional standpoint, carbohydrate has three distinct and beneficial actions on protein metabolism for which energy in the form of fat is not a substitute.

(a) When carbohydrate is fed to a starving animal, the N excretion in the urine is reduced. Under the same circumstances the administration of fat does not affect the N output.

(b) When carbohydrate is transferred from a protein-containing to a protein-free meal, a transient N loss ensues. A similar transfer of fat is without effect on N excretion.

(c) When fat is substituted for carbohydrate in a protein-containing meal, a transient N loss occurs.

The primary object of the studies which we have carried out was to attempt to decide whether these three actions of carbohydrate have one common underlying mechanism i.e. that they are merely three different manifestations of the sparing effect of carbohydrate. We shall first consider the effect of dietary carbohydrate on the utilisation of endogenous and dietary protein i.e. whether (a) and (b) above are identical in mechanism. Secondly, we shall compare the effects on dietary protein utilisation of (i) the time of feeding

carbohydrate and (ii) substituting fat for carbohydrate in a protein-containing meal i.e. (b) and (c) above.

Effect of dietary carbohydrate on the utilisation of endogenous and dietary protein.

(a) In Section 3 we have observed that glucose administered to a fasting subject leads to a reduction in the plasma amino acid N which persists for a few hours (Fig.7). Fat ingestion on the other hand is without any effect on the amino acid level. Now these results may also apply to the action of carbohydrate on dietary protein. The fall in the amino acid level occurs chiefly within two hours of feeding the carbohydrate and thus would coincide with the main period of amino acid absorption after a protein-containing meal. Moreover, the action of glucose is nearly complete by the third hour, as is evidenced by the steadily rising blood amino nitrogen figure. This would account for the necessity of feeding the carbohydrate with the protein in order to obtain an N sparing effect. This is in agreement with the experiment (Section 1, Table 12) in which the time of feeding carbohydrate in relation to protein was varied; this experiment suggested that the carbohydrate must be fed within two hours of the protein in order to spare its utilisation.

(b) Further evidence of a similarity between these two effects is shown by a consideration of the type of protein

which is spared. When protein and carbohydrate are fed at different times, then eaten together, a transient N retention occurs. Subsequent separation leads to an equivalent and rapid N loss. As has been previously noted in Section 2, this indicates that a change is occurring in some labile protein fraction of the tissues. Chambers and Milhorat (1928) have expressed the opinion that glucose when fed to a starving animal spares the utilisation of labile protein. These observations suggest that there is a similarity in the mechanism of the two effects.

(c) In Section 2 attention was drawn to the fact that when carbohydrate and protein were fed together the N retained could not be accounted for in the viscera and the most likely site for this retained N was thought to be muscle. From the depressions which occur in the plasma amino acid levels after glucose administration to a fasting subject, it was deduced that the amino acids were withdrawn into muscle and synthesised into protein(Section 3, Table 28). In addition glucose administration to a fasting rat leads to an increased uptake of ^{35}S-methionine by muscle, but not by the viscera (Section 5, Fig.11 and 12). Therefore with both these actions of carbohydrate the probable site of the retained N is similar, viz. muscle.

(d) Finally, evidence will be presented later that insulin is necessary for the protein sparing action of carbohydrate, both when fed with protein and when fed to the fasting

animal. This again suggests a similarity in mechanism.

We have therefore good reason for believing that the sparing action of carbohydrate on protein fed along with it is based on the same mechanism as that by which it spares body protein.

Relation between time of feeding carbohydrate and its substitution by fat. In Section 1 it was deduced that these two effects of carbohydrate were similar and were primarily dependent on the amount of carbohydrate fed with the protein. If this is so, then the effect on N balance of transferring carbohydrate from the protein-containing to the protein-free meal, should be the same as when a similar amount of carbohydrate fed with protein is isocalorically substituted by fat. As was previously mentioned this does not occur, substitution of carbohydrate by fat leading to a greater N loss than transfer of carbohydrate. It was then suggested that the carbohydrate which was given in the protein-free meal was still sparing protein. This was shown to be correct, as a N loss occurred when it was substituted by fat. It is likely that this carbohydrate which is fed apart from the dietary protein is sparing body protein ("endogenous action"). When it is administered the amino acids will be depressed for 2 - 3 hours and N will be retained as discussed above. Fat, as it has no action on the plasma amino acids, will have no effect on N output. Thus the effect of transferring

carbohydrate from a protein-containing to a protein-free meal is similar to the effect of substituting carbohydrate fed with protein by fat. The two actions differ in their magnitude merely because the glucose when given in the protein-free meal is still sparing protein by its endogenous action; it will be more effective given with the dietary protein because of the more plentiful supply of amino acids which are available.

Summarising these nutritional findings , we may thus conclude that the sparing action of carbohydrate fed to a fasting animal is probably due to the same mechanism as the action of carbohydrate on dietary protein fed along with it; furthermore, this mechanism provides an adequate reason for the N loss which occurs when fat is substituted for carbohydrate in the diet. The question then arises, how does carbohydrate exert this specific action on the course of protein metabolism and in what parts of the body does the effect occur?

B - Mode of action of carbohydrate on protein metabolism.

Site of deposition of retained N. In Section 2, it was shown that no significant fraction of the N retained when protein and carbohydrate are fed in the same meal could be accounted for by changes in the N of the viscera (Section 2, Table 16). This means that the main changes in the N

content of the body must have occured in the carcass i.e. muscle, skin or skeleton. Of these muscle is by far the most likely site. Studies on the plasma amino acids also led to a similar conclusion, as the ratio of the fall in the essential to the non-essential amino acids in the blood after glucose ingestion, has an identical value to the ratio existing between the essential and non-essential amino acids in muscle protein (Section 3, Table 28). The experiments with ^{35}S-methionine furnished conclusive proof on this matter. Glucose administration was shown to have no effect on the ^{35}S uptake by the liver and gut (Section 5, Fig.12), a point which agrees with the observations made in Section 2, but it did cause an increased incorporation by the muscle (Section 5, Fig.11). In addition, the experiments with the plasma amino acids and radioactive methionine imply that the N is retained by a process of increased protein synthesis in muscle.

There is thus every reason for believing that skeletal muscle is involved in the N retention phenomenon. This does not necessarily mean that the action of carbohydrate on protein metabolism actually occurs in the muscle cell. It merely indicates that the end result of the beneficial action of carbohydrate is an increase in the N content of muscle, which is due to increased synthesis of protein in that tissue. This could conceivably be due to an

effect of carbohydrate in another part of the body which ultimately influences the rate of protein synthesis in muscle.

Site of interaction of carbohydrate and protein. It is conceivable that the interaction which occurs between dietary carbohydrate and protein when eaten in the same meal, may occur in the small intestine during the digestion and subsequent absorption of the protein. However, it was demonstrated in Section 2 that carbohydrate had no effect either on the digestibility or the biological value of the protein and its sparing effect could not be attributed to the marked delay which it causes in protein digestion and absorption. In addition, Elman (1953) has shown that glucose still exerts its sparing action on N output when injected intravenously along with an amino acid mixture. We have also shown above that the mechanism of the action of glucose on "endogenous" and "exogenous" metabolism of protein is probably similar in both cases. The intestine can therefore be ruled out as a site of the interaction effect.

The other possible sites of interaction are the remaining viscera and muscle. The liver cannot be the sole site of this action as Flock et al. (1952) were able to demonstrate a reduction in the blood amino acid level when glucose was given to hepatectomised dogs. It has been

observed that the accumulation of amino acids that occurs in the dog (Mirsky, 1938) and the rat (Frame and Russell, 1946; Ingle, Prestrud and Nezamis, 1947) after evisceration, can be controlled by the administration of insulin. We shall shortly show that this effect of insulin on the plasma amino acids is mediated through glucose, so actually these investigators were demonstrating the protein sparing action of carbohydrate in the eviscerated dog and rat. The interaction effect therefore cannot occur solely in the liver, intestine, spleen or pancreas, which leaves either the kidney or the muscle as the only remaining sites. That the kidney is not the site was shown by Barker and Sweet (1937) and Mirsky, Heiman and Swadesh (1937), as these authors demonstrated the sparing effect of carbohydrate in nephrectomised-pancreatectomised dogs.

By a process of exclusion, then, the site of the interaction effect would appear, like the site of deposition, to be muscle.

Mechanism of action of carbohydrate on protein metabolism.

We have demonstrated above that the interaction between carbohydrate and protein occurs in muscle and that the result of this effect is an increase in the N content of the carcass, presumably in the muscle. This could be due to either (a) an increase in protein synthesis or (b) a decrease in protein catabolism following carbohydrate administration.

As regards the possibility of influencing protein catabolism, this idea of control over the amount of protein by changes in the catabolic side has been brought up by Rittenberg and Shemin (1946). However, no substantial data to prove this mechanism have ever been published. Recently, Klingenberg and Rosenkranz (1953) have demonstrated that pyruvic acid is an inhibitor of cathepsins and is therefore a possible mechanism for the protein sparing action of carbohydrate. However, it does not necessarily follow that this in vitro demonstration of enzyme inhibition is the actual in vivo mechanism.

Much more likely is some factor affecting protein synthesis and we shall now consider some of these in detail, namely, the formation of non-essential amino acids, the action of insulin and adenosine triphosphate (A.T.P.).

(a) Synthesis of non-essential amino acids. It has been observed that amino acids can be utilised optimally for protein synthesis only if all the necessary amino acids, the essentials as well as non-essentials, are simultaneously available in all the tissues (Cannon, 1950; Rose, Smith, Womack and Shane, 1949; Cox and Mueller, 1949). In 1939, Foster, Schoenheimer and Rittenberg noted that glucose in the presence of available ammonia, could provide precursors for the synthesis of the non-essential amino acids. This has since been verified by Sprinson and Rittenberg (1949) and Rose et al. (1949). Geiger and Wick (1953) then

suggested that the N sparing effect of carbohydrate could feasibly be explained on the basis of these observations, as the formation of the non-essential amino acids from glucose would tend to favour N retention. They demonstrated that carbohydrate could form non-essential amino acids by feeding ^{14}C-glucose to rats and isolating from the liver labelled arginine, alanine, glutamic acid, aspartic acid, glycine and serine. The crucial point then was whether these amino acids could be formed from carbohydrate in amounts sufficient to affect the utilisation of the essential amino acids. Geiger and Wick (1953) attempted to solve this problem by tube feeding to rats ^{14}C-glucose plus ammonium phosphate and a mixture of the essential amino acids, both together and separately and noting whether there was any difference in the amount of ^{14}C incorporated in the tissue proteins. In order to magnify any changes occurring they tried to establish conditions under which the new formation of protein and therefore the expected uptake of ^{14}C would be localised mainly in one organ. As such a condition they chose the regeneration of liver tissue after partial hepatectomy. The experimental design used was as follows:-

Group A received the ^{14}C-glucose + ammonium phosphate and the essential amino acids simultaneously.

Group B received first the ^{14}C-glucose + ammonium phosphate and 4 hours later the essential amino acids.

Group C received first the essential amino acids and 4 hours later the ^{14}C-glucose + ammonium phosphate.

The animals were killed 9 hours after the first feeding and the ^{14}C content of the liver estimated. The incorporation of ^{14}C in the liver proteins from Groups A and B were found to be equal and higher than that occurring with Group C. Geiger has interpreted these results as favouring his theory that sugar improves the utilisation of the essential amino acids by providing precursors for such non-essentials which are not present in optimal quantity. It must be admitted that at first sight the results with Groups B and C do suggest this as the incorporation is lowered in Group C (glucose fed 4 hours after the essential amino acids). The results of Group A are explained by suggesting that the non-essential amino acids formed from the glucose are stored for 4 hours till the essential amino acids are fed. In support of this contention, experiments with rats are quoted in which it was noted that for an increased rate of growth, it was irrelevant whether the non-essential amino acids were fed simultaneously with or 6 hours before the essentials.

Actually the differences in incorporation are explicable on the grounds that with Groups A and B, the labelled glucose was fed, then 9 hours allowed to elapse before killing the animal, whereas with Group C the corresponding time interval was only 5 hours. As regenerating liver has a high rate of incorporation, obviously contact

with the isotope for a further 4 hours would lead to a large increase in the uptake. In addition these authors also noted that the incorporation of ^{14}C was slightly higher when glucose was fed alone, than when it was given along with the essential amino acids. As the simultaneous presence of the essential amino acids and glucose failed to affect the uptake of ^{14}C, it could hardly be expected that a difference would occur when the times of ingestion of glucose and amino acids were varied. Thus the results of these experiments really demonstrate that the degree of incorporation of ^{14}C into liver protein depends on the length of time elapsing between the feeding of the labelled glucose and the subsequent killing of the animal and not at all on the feeding of the amino acids. If due allowance is made for this factor in Group C, then all the uptakes are found to be approximately equal. The experiments may therefore be dismissed as being essentially negative; they certainly do not favour the view that the N sparing effect of glucose is due to the synthesis of non-essential amino acids.

To summarise, we may say that in cases where the non-essential amino acids are not present in optimal amounts, the formation of such amino acids from glucose could perhaps improve the utilisation of the essential amino acids but it still remains to be proved that glucose is capable of

supplying these amino acids in sufficient quantities for this effect to occur. In any case, this is not the explanation of the N sparing effect of carbohydrate as (a) glucose still exerts its protein sparing effect in well balanced diets, where no deficiency of non-essential amino acids exists and (b) Geiger and Wick (1953) have shown that the non-essential amino acids exert a similar action on the growth rate of rats, when fed either simultaneously with or 6 hours prior to the essential amino acids. Glucose, on the other hand cannot spare dietary protein when fed 6 hours beforehand (Section 1), which proves that the non-essential amino acids formed from it are not exerting a protein sparing action.

(b) Insulin In Section 4 it was concluded, both from the results of our experiments with alloxan diabetic rats and from the evidence cited in the literature, that insulin was necessary for the protein-sparing effect of carbohydrate in the fasting animal. The results of Barker and Sweet (1937) and Mirsky, Heiman and Swadesh (1937), who demonstrated a sparing effect of carbohydrate on nephrectomised-pancreatectomised dogs, do not invalidate this conclusion, as these results can probably be attributed to the extremely high blood sugar levels which occur with this type of experimental animal and it is quite possible that glucose is exerting its action by a mass action effect. On the other hand, Bancroft et al. (1951) concluded that in the fed animal (rat)

the situation was entirely different. They observed that when carbohydrate and protein were fed simultaneously to alloxan diabetic or pancreatectomised rats, the N retention resulting was of the same magnitude as that occurring with the normal rat. As the liver is intimately concerned with the metabolism of dietary amino acids, it is conceivable that the N sparing effect of carbohydrate in the liver may not require insulin, whereas Bollman et al. (1953) have observed that it is necessary in other tissues. If this hypothesis be correct, then it would be thought that when carbohydrate and protein are fed together a large proportion of the retained N would be recovered from the liver. In Section 2, under the experimental conditions mentioned, we were unable to detect the large increase in liver N which this theory demands. Additional evidence bearing on this point was furnished by Krahl (1953) who, working with liver slices from diabetic rats, demonstrated that the uptake of ^{14}C-glycine is restored to normal only by the addition of glucose + insulin. Hence insulin would appear to be necessary for the N sparing effect of glucose in the liver. Bancroft's results must therefore be viewed with suspicion. In view of the evidence which has been presented it would appear that insulin is necessary for the sparing action of carbohydrate both in the fed and the fasting animal. This is in keeping with the conclusions drawn above, where it was

deduced that the action of carbohydrate on "endogenous" and "exogenous" metabolism of protein was similar in mechanism.

The further point arises as to whether insulin acts directly on protein synthesis or is dependent in turn on the utilisation of glucose. Recently, two Italian workers, Pasquinelli and Calzolari (1953) attempted to answer this question. They injected rabbits with increasing doses of insulin and followed the changes in the blood sugar and blood amino acid N. The data was then analysed statistically and because of lack of correlation between the two curves, they concluded that insulin must act directly on the proteo-synthetic process. This appears rather flimsy evidence on which to base a deduction of such moment. Some tissue slice experiments of Krahl (1953) and Sinex et al. (1952) may be interpreted as throwing rather a different light on the matter. These workers incubated rat diaphragm with radio-active glycine and alanine respectively. They found that the addition of insulin to the medium increased the incorporation of the radioactive amino acid only with the diaphragm from a fed animal. This suggests at least, that insulin does not act directly on protein synthesis, but rather that its effect is dependent on its action on some metabolites derived from the nutrients stored in the diaphragm of fed animals. As glycogen is the predominant

foodstuff stored in the diaphragm it would appear eminently possible that the metabolite acted upon is glucose. The theory may then be advanced that insulin is necessary for the protein sparing action of carbohydrate only in so far as it allows better utilisation of glucose.

(c) Generation of A.T.P. Protein synthesis being an endergonic reaction is now generally assumed to be in some way dependent on the utilisation of high energy phosphate bonds (Lipmann, 1949). This hypothesis is in keeping with the results of *in vitro* studies with labelled amino acids, where the inhibition of respiration or phosphorylation leads to a decreased incorporation of the label. An attempt will now be made to correlate this theory with the results of the *in vivo* ^{35}S experiments and with the amino acid studies.

In our experiments on fasting subjects (Section 3), the amino acids in the blood were depressed 1 hour after the administration of glucose and this decrease persisted for another hour before it started to regain its original level (Section 3, Fig.7). With the ^{35}S muscle studies (Section 5, Fig.11), no increase in incorporation was observed until after the second hour, so it may be assumed that the synthesis of a peptide commenced shortly after the administration of glucose, but until some time between the second and the fourth hour its chain length was insufficient for its precipitation by trichloracetic acid. As the plasma amino

N value is very nearly back to the normal fasting level by the third hour, it would appear that the glucose stimulus is wearing off. This is probably related to the rapid fall in ^{35}S activity which occurs in the muscle from the fourth hour onwards. The glucose has generated just sufficient A.T.P. to build up a peptide, then when this supply has been consumed, the energy conditions become unfavourable and the peptide breaks down.

These fasting animals are in negative N and caloric balance. They have no steady supply of foodstuffs to maintain the phosphate bond energy at a level which is capable of maintaining this newly synthesised peptide in position. With fed animals a different situation prevails. It is known (Section 1) that the transfer of glucose from a protein-free to a protein-containing meal results in a retention of N, which persists for as long as the carbohydrate and protein are fed together and is dependent on its magnitude on the amount of carbohydrate fed with the protein. In this case there will be a sharp burst of A.T.P. synthesis when the glucose is given and when the effect wears off (4 hours) the newly synthesised protein does not break down, but can be maintained in its present state, because of the background level of energy from the other nutrients in the diet, until the next dose of glucose, 24 hours later. This process is repeated until after a lapse of 4 days or so, the energy derived from the glucose is unable to synthesise any more

protein. As long as carbohydrate is fed with the dietary protein, the newly synthesised protein remains in status quo, but if the glucose is removed from the protein meal it breaks down as the energy conditions are now unfavourable for its existence.

It may be asked, why does carbohydrate cause this synthesis of protein whereas fat does not? From our in vitro studies (Section 5) it was decided that in the short time of incubation (2 hours) glucose always produced more high energy phosphate bonds which were available for protein synthesis than did acetate or octanoate. This may be due to glucose having a higher rate of oxidation or it may be that with acetate or octanoate other endergonic reactions (in addition to protein synthesis) utilise the A.T.P. which is formed. In any case, the end result appears to be that the energy from glucose produces sharp bursts of A.T.P. activity, whereas with fat the available energy is spread over a much longer period.

Whatever the ultimate outcome of this phenomenon of the protein sparing action of carbohydrate, the mechanism revealed will undoubtedly throw light on the normal mechanism of protein synthesis.

SUMMARY.

Section 1: The effect of carbohydrate and fat on the utilisation of dietary protein.

1. Experiments are described with adult rats, in which the carbohydrate fed in the protein-containing meal was isocalorically substituted with fat. This caused an immediate increase in the urinary N output which persisted for 4 days.

2. When carbohydrate fed in the protein-free meal was isocalorically substituted with fat, the effect on N balance was much less marked.

3. Partial substitution of fat for carbohydrate in a protein-containing meal had a similar effect to complete replacement. The time of feeding the substituting fat was shown to be without effect on the N balance.

4. When increasing amounts of carbohydrate were transferred from a protein-free to a protein-containing meal the N balance was found to progressively improve and a linear relationship was shown to exist between the N retained and the amount of carbohydrate fed with protein.

5. A two hour period of separation in the times of feeding the carbohydrate and protein produced as large an increase in N excretion as a nine hour separation period.

6. It was concluded that the deleterious effect on N balance of substituting fat for carbohydrate in a protein-

containing meal was due entirely to the removal of the carbohydrate from its association with dietary protein, the addition of the fat being without effect. N balance was much less affected when fat was substituted for carbohydrate in the protein-free meal, as the carbohydrate was not exerting its beneficial action on the utilisation of dietary protein.

Section 2: The digestion, absorption and tissue distribution of N when carbohydrate is fed with protein.

1. Experiments are described in which the effect of glucose on the digestibility of simultaneously fed protein was determined by analysis of faecal N. It was demonstrated that glucose did not alter the digestibility of the protein.

2. Experiments were carried out to determine whether the action of glucose on an enzymic digest of casein was similar to its action on whole casein. The results indicated that the sparing effect of glucose on the digest was identical to its action on the whole protein.

3. Fasting rats were fed by stomach tube either an enzymic digest of casein or an enzymic digest of casein + glucose or an enzymic digest of casein + fat. After two hours the animals were killed and the N content of the intestine estimated. Glucose was found to depress the absorption rate significantly whereas fat had no effect.

4. Experiments were also carried out in which the N content of the viscera were estimated after the simultaneous

feeding of carbohydrate with protein. No significant fraction of the N which is retained could be accounted for in the viscera.

5. It was concluded that the sparing effect of glucose could not be attributed to an increase in digestibility of the protein, nor to an alteration in its biological value nor to the marked delay which it causes in absorption. It was also suggested that the site of deposition of the retained N was probably muscle.

Section 3: The effect of glucose and fat on the plasma amino acid level.

1. A study has been made on the effect of glucose and fat administration on the plasma amino acid levels in the fasting human subject and the rat.

2. Glucose ingestion caused a 12% reduction in the plasma amino N which was maximal at 1 hour, whereas fat ingestion resulted in a much more gradual fall to the extent of 4% of the initial value; the effect of fat was of doubtful statistical significance. Similar experiments on rats confirmed the differences in action of glucose and fat.

3. In the fasting human subject, the level of 7 essential amino acids (tryptophan, histidine, leucine, isoleucine, threonine, arginine and valine) fell after glucose ingestion to varying extents. If these depressions were arranged in the form of a ratio, with tryptophan as unity,

and compared with Rose's estimates of the human requirements of these amino acids arranged in a similar way, then there was observed to be a close similarity between the values. With the fasting rat glucose caused a reduction in the plasma levels of 4 essential amino acids (tryptophan, threonine, leucine and isoleucine), whereas fat ingestion had no effect on their concentrations.

4. It was concluded that the sparing effect of glucose was probably due to a stimulation of protein synthesis.

Section 4: The effect of insulin on the plasma amino acid level.

1. Experiments are described in which glucose was administered to fasting, alloxan diabetic rats. This caused a very slight decrease in the plasma leucine level compared to that occurring with the normal rat.

2. It has been concluded that these results favour the view that insulin is necessary for the sparing action of carbohydrate in the fasting rat.

Section 5: The effect of glucose and fat on the incorporation of ^{35}S-methionine into tissue proteins in vitro and in vivo.

In vitro

1. A study was made of the effect of added glucose, succinate, octanoate and acetate, both separately and in various combinations, on the uptake of ^{35}S-methionine by

rat diaphragm and rat liver slices.

2. Glucose was found to increase the incorporation of ^{35}S-methionine by rat diaphragm and rat liver slices by 9 - 16% in a two hour incubation, whereas succinate with rat liver slices increased the uptake by 59%.

3. The effects of octanoate and acetate were observed to be variable, but on the average octanoate had little effect on the uptake, whereas acetate had a definite depressant action. The addition of glucose to either acetate or octanoate invariably resulted in a depression in uptake. Succinate when added to octanoate had a similar effect.

4. The relation of these results to A.T.P. formation and protein synthesis is discussed. It has been concluded that glucose and succinate increased the amount of A.T.P. available for this process whereas octanoate and acetate do not.

In vivo

1. A study was made of the effect of feeding glucose and fat on the incorporation of injected ^{35}S-methionine into the protein of muscle and viscera of fasting rats.

2. It was found that neither glucose nor fat had any effect on the uptake of the label by the intestine and liver.

3. It was also observed that glucose but not fat increased the incorporation of ^{35}S-methionine by skeletal muscle. Two hours after the feeding of glucose the isotopic concentration was slightly lower than that of the control

animal, but at the fourth hour there was a marked increase in the uptake which had completely disappeared again by the sixth hour.

4. It was concluded that glucose was promoting the synthesis of a peptide or protein, which was isolated in some manner from the main mass of muscle protein.

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