CLII. STUDIES ON THE METABOLISM OF ANIMALS ON A CARBOHYDRATE-FREE DIET.

I. THE DISTRIBUTION OF GLYCOGEN AND FAT IN THE LIVER OF ANIMALS FED ON A CARBOHYDRATE-FREE DIET.

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THOUGH attempts made some years ago in this laboratory [Hynd and Macfarlane, 1927] to alter the maltase content of the blood of animals did not meet with success, the experiments then carried out showed in a definite manner that animals under different nutritional conditions exhibit striking variations in their response to the internal secretions. This discovery, unknown to us at the time, had already been reported independently by Abderhalden and Wertheimer [1924, 1]. Consequently the results of our experiments were not presented for publication, especially as other workers had confirmed the observations of the above-mentioned authors. In the interval, although several suggestions have been made, no adequate explanation seems to have been advanced to account for all the results obtained. Accordingly it seemed desirable to study the response towards the secretions of the more important ductless glands of animals fed on a carbohydrate-free diet, in the hope that a careful comparison of their behaviour with that of normal animals would help to elucidate the parts played by these secretions.

As the work will require a considerable time to complete, it is intended to communicate the results in sections. The first of these, forming the present paper, deals mainly with changes in the distribution of fat and glycogen in the liver following upon the withdrawal of carbohydrate from the diet, and furnishes data essential for the proper understanding of the subsequent sections.

The results of preliminary experiments on mice pointed to there being no marked macroscopic or microscopic differences in the appearance of the organs of animals fed on a carbohydrate-free diet from those of animals on a normal diet, with the exception of the liver and perhaps the suprarenals. The livers of animals fed on a diet in which the carbohydrate was reduced to a minimum appeared on naked-eye examination to be of a coarser texture, and on histological examination were found to be distinctly fatty, especially in the periphery of lobules. Although Mottram [1909] found that the increase of visible fat in the

liver of rabbits was paralleled closely by a rise in the percentage of fat, it is well recognised that a histological appearance denoting an excess of fat may not be due to a true increase of fat but rather to an alteration in the character of the fat. Accordingly, experiments to determine the actual fat and glycogen content of the liver of animals under the above conditions were carried out.

Experimental animals and diets employed.

The animals used for the majority of the experiments were standard albino rats and mice, but a smaller number of experiments were also conducted on kittens. As it has been reported that the glycogen content of animal tissues suffers a seasonal variation, and that animals such as rats show a seasonal ketonuria, it is worth noting that all our experiments were confined to the winter and early spring months. Our results, therefore, are not affected by such variations, and moreover all the experiments were carefully controlled by the use of normal animals either from the same litter or of like age and weight. Reference to Table II will also show that in the experiments with rats the sex of the animals was taken into consideration.

Twenty-three rats on a carbohydrate-free diet have been examined, as well as 13 controls on a normal diet consisting of approximately equal amounts of bread and milk. The "carbohydrate-free" diet selected for rats consisted of grated cheese, a few drops of milk being added to ensure a sufficiency of vitamins. The rats were kept in separate cages, and were provided with water and as much food as they could eat. Records were kept of the amount of food taken per day, and each animal was weighed once or twice per week.

The diets employed, analysed by the usual methods, are indicated in Table I, the weights given being in g. per 100 g. rat per day.

Table I.

Diet	Analyses of diets.					
	Total food	Carbo-hydrate	Fat	Protein	Salts	Calories
Control	28.3	8.01	0.50	1.64	0.26	44.0
"Carbohydrate-free"	9.0	0.09	3.82	2.73	0.36	48.1

The rats used were 9 to 12 weeks old, the average weight at the commencement of the experiment being 102 g. All the animals remained healthy throughout the experimental period, but the animals on the carbohydrate-free diet developed rough and greasy coats and did not grow so well as the control animals. The average weight of the cheese-fed rats, after 2 weeks' feeding, was 124 g., while the control rats averaged 131 g. After longer periods of feeding, the difference in weight became more marked. Levine and Smith [1927] reported normal growth in young rats on a diet devoid of preformed carbohydrate, and observed no particular ketonuria. In our experiments no acetone bodies were at any time detected in the urine.

Similar experiments with mice have also been carried out. A number of

pregnant mice were obtained and divided into two sets. One set was fed entirely on cheese, and the other on bread and milk. The litters obtained were reared on these respective diets. In one experiment, in which the mice were largely of a black or slate colour, the cheese-fed animals thrived as well as, if not better than, those on the control diet of bread and milk. However, in another experiment with white mice, the cheese-fed animals grew much more slowly than the controls and required a longer period to reach adult size. Of the mice reared in this way, 28 cheese-fed animals and 30 controls have been used for chemical analyses. Experiments have also been carried out with mice fed on non-carbohydrate diets consisting of (a) butter-fat plus a little caseinogen (7 animals used for analyses), and (b) caseinogen plus a little milk (2 animals used for analyses). These diets, however, did not prove so satisfactory as the cheese diet. After 5 to 6 weeks' feeding, a large percentage of the caseinogen-fed mice died, and after a similar period the fat-fed mice were in poor condition. These results may be correlated with the findings of Burr and Burr [1930] and of Wigglesworth [1924] respectively. The former found that a diet devoid of fat and of high protein content was harmful for rats, while the latter found that a very definite ketosis occurred on a diet of fat alone, but on fat and protein the ketosis was much less.

Three sets of experiments, involving the use of 12 kittens, have been completed. Carbohydrate-free diets consisting of (a) fish (4 animals used), and (b) cream (2 animals used) have been employed, while an excess carbohydrate diet consisting of bread and milk served as a control diet (5 animals used).

With the exception of the two kittens, which after a time refused to take cream and therefore lost weight, all the animals remained healthy, but those fed on fish showed the most satisfactory growth. The animals used were from 4 to 6 weeks old at the commencement of the experiment, and were kept on the respective diets for 3 to 4 weeks.

Analytical methods employed.

The methods usually adopted for the estimation of the glycogen and fat content of the liver were modified so as to render it possible to make duplicate determinations of both constituents using only one rat or two mice for each experiment.

Each animal was killed between 9 and 9.30 a.m., and, unless specified otherwise in the tabulated data, food was left in the cage during the previous night. The animal, after being stunned, was decapitated, and the blood, which was collected in a small basin containing a trace of powdered potassium oxalate, used for the determination of blood-sugar by the method of Hagedorn and Jensen. After some experience, the first stage of this process was completed within a minute of the death of the animal. A gastrocnemius muscle was then rapidly dissected and transferred to a tared 25 cc. centrifuge tube containing 1.5 to 2 cc. 60 % potassium hydroxide. The liver was next excised and divided into two portions, which were treated in the same way as the

muscle, one being used for the glycogen and the other for the fat determination. After re-weighing, the three centrifuge tubes were heated for 3 hours in a water-bath kept at 100°, which treatment completely saponifies the fat and does not destroy the glycogen. The larger organs were then carefully dissected, weighed and measured. The interval which elapsed between the killing of the animal and the immersion of the tissues in the strong alkali was, as a rule, less than 1 min., while the transference of the tubes to the bath at 100° was completed within 2 mins. Every precaution was thus taken to minimise *post mortem* glycogenolysis, as emphasised by Simpson and Macleod [1927].

In the case of mice, no estimations of muscle-glycogen were carried out. For the blood-sugar estimation the mixed blood from two animals was employed, and the liver of one was used for the glycogen estimation, that of the other for the fat estimation. Otherwise, the methods of analysis were identical with those described above for rats.

With kittens, a number of samples of blood, muscle and liver were taken for the several estimations, and the average values are reported.

(i) *Estimation of glycogen.* From 1 to 1.5 g. of liver, or muscle, was employed for the glycogen estimation, the modifications of Pflüger's method introduced being similar to those detailed by Irving [1928] except that 80 % alcohol was used for the precipitation, and heating for 3 hours at 100° with 5 % hydrochloric acid for the subsequent hydrolysis. These we found to be the optimum conditions in control experiments carried out on a specimen of pure glycogen prepared from rabbit's liver. After neutralisation and filtration the solution was made up to standard volume (50 cc.) and estimations carried out either by Hagedorn and Jensen's method, or by the modification of that method developed by Hanes [1929]. As Hanes's method requires 3.0–2 mg. glucose for each estimation, while Hagedorn and Jensen's upper limit is 0.3 mg. glucose, the two methods overlap and it is thus possible to change directly from the one to the other, if the amount of sugar present renders this necessary.

(ii) *Estimation of fat.* For the determination of fat, 2.5 to 4 g. of liver were used. In the case of rats this represents the remainder of the organ from the glycogen estimation. The liver-fat, after saponification by 3 hours' heating at 100° with 60 % potassium hydroxide, was converted into fatty acids by acidifying with hydrochloric acid, as detailed by Mottram [1909]. The mixture was then transferred to a separating-funnel and thoroughly extracted with small amounts of carbon tetrachloride. Only on rare occasions did troublesome emulsions form, and as a rule the carbon tetrachloride extract was readily tapped off into a second separating-funnel, where the combined extracts were washed with water to remove traces of mineral acid. The carbon tetrachloride solution of fatty acids was then made up to standard volume (usually 100 cc.) and portions used for analysis.

By titrating 25 cc. of the solution, diluted with about an equal volume of alcohol, with *N*/30 alcoholic potassium hydroxide, using phenolphthalein as

indicator, a measure of the acidity is arrived at. Also by treating 20 cc. of the solution with 5 cc. of Wijs's reagent, and subsequently titrating with *N*/50 sodium thiosulphate, the iodine absorption is obtained. Comparison of the titres obtained in this way for livers of normal animals with those for animals on non-carbohydrate diets would give an accurate measure of the changes brought about by withdrawal of carbohydrate from the diet. However, owing to the fact that it would be difficult to assess the value of such changes, it seemed advisable to express the results as percentage of fatty acids. As the determination of the actual weight of fatty acid present in the small amount of liver tissue employed in our experiments would not be very reliable, we have made the reasonable assumption that the mixed fatty acids would have a mean molecular weight equivalent to that of linoleic acid, $C_{18}H_{32}O_2$, namely 280. On this basis, the average percentage of fatty acids equals 3.7 % and the iodine value lies between 130 and 140 for animals on a normal diet. These values agree well with those quoted by Hartley [1909] for liver-fat.

As the method described above is not that usually adopted for the determination of fat or fatty acid, the process has been checked by trial experiments with oleic acid. The results of these control experiments indicate that:

- (1) The end-point of an iodine value estimation is perfectly definite when *N*/50, or even *N*/100 thiosulphate is used, instead of the usual *N*/10 solution.
- (2) It is not essential to dry the carbon tetrachloride extract before proceeding to the determination of the iodine value.
- (3) Three hours' heating at 100° with 60 % KOH followed by liberation of the acids and their extraction with carbon tetrachloride has no effect on the iodine value.
- (4) By diluting with an equal volume of alcohol, the carbon tetrachloride extract can be utilised directly for the acidity determination.

RESULTS.

Reference has already been made to the growth of the experimental animals. As is usually the case, considerable variations in growth were apparent both in the controls and in the animals on a carbohydrate-free diet. As this is naturally reflected in the weights of the individual organs, the figures given in the Tables are calculated for an animal of 100 g. body weight.

In rats a carbohydrate-free diet causes a definite enlargement of both the liver and the kidneys in male and female. In mice the effect on these organs is similar to that in rats and, as shown by the second experiment, becomes more marked the longer the carbohydrate-free diet is continued. This agrees with the results obtained by Osborne and Mendel [1924]. The average values obtained for the spleen in the rat experiments are remarkably constant. In the mice, on the other hand, wide variations are found. No importance can therefore be attached to alterations in the weight of this organ, which is well

Table II.

Weights of organs.				
(A) Rats.	Male		Female	
	Control	Carbohydrate-free	Control	Carbohydrate-free
Organ	g.	g.	g.	g.
Heart	0.52	0.57	0.69	0.62
Kidneys	0.97	1.22	0.91	1.32
Liver	3.93	4.12	3.72	4.65
Lungs	0.68	0.88	0.76	1.15
Pancreas	0.35	0.39	0.35	0.39
Spleen	0.55	0.49	0.54	0.55
Suprarenals	0.035	0.036	0.37	0.058
Thyroids	0.008	0.014	—	—
No. of animals used	7	11	6	12

(B) Mice.	1st Exp.: 6 weeks' feeding		2nd Exp.: 2½ months' feeding	
	Bread	Cheese	Bread	Cheese
Organ	g.	g.	g.	g.
Heart	0.76	0.72	0.67	0.62
Kidneys	1.50	1.78	1.56	1.92
Liver	6.77	7.06	5.10	6.92
Spleen	1.46	0.66	0.54	0.43
No. of animals used	5	5	15	10

recognised to be liable to marked fluctuations. While no change is apparent in the weight of the suprarenals of male rats, these organs are increased in weight and volume in the female. Owing to the method adopted for killing the animals, it was only possible to examine a few of the thyroids. However, those of the animals on carbohydrate-free diet tend to show an increase in weight, but careful histological examinations, for which we are greatly indebted to Professor Herring, revealed no definite changes from the normal in the case of rats or kittens. The other organs weighed appeared in all three species to be normal to naked-eye examination. Further consideration of the above data will be postponed till the publication of a later section of this work.

Table III.

Average values for rats.

Duration of experimental feeding	Period of fasting	Blood-sugar	Glycogen in muscle	Glycogen in liver	Fatty acids in liver	Iodine value of fatty acids
Weeks	Hours	%	%	%	%	
(A) Carbohydrate diet (bread and milk).						
1	0	0.16	0.77	3.4	3.4	140-141
2	0	0.15	0.55	3.0	3.7	130-140
3	0	0.14	0.46	3.0	4.0	121-123
2	12	0.13	0.70	2.5	3.7	130-140
2	24	0.11	0.51	0.9	2.99	130-140
(B) Non-carbohydrate diet (cheese).						
1	0	0.18	0.49	1.7	5.7	130-134
2	0	0.17	0.63	1.9	7.2	120-128
3	0	0.16	0.58	1.95	5.9	97-120
2	12	0.12	0.50	1.4	3.7	130-140
2	24	0.10	0.29	0.68	2.9	123-130

With regard to the chemical analyses carried out, these showed no difference between male and female animals. Consequently this detail is omitted from Tables III and IV, in which the average values of the results obtained are summarised as briefly as possible.

Table IV.

Duration of experimental feeding Months	Nature of diet	Blood-sugar %	Glycogen in muscle %	Glycogen in liver %	Fatty acids in liver %	Iodine value of fatty acids
Average values for mice.						
1½	Bread	0.148	—	—	—	—
2½	"	0.22	—	4.0	3.7	129-135
2½	Cheese	0.31	—	1.75	9.3	91-120
1½	"	0.198	—	—	—	—
1½	Casein	0.172	—	—	—	—
1½	Fat	0.191	—	—	—	—
Average values for kittens.						
Weeks						
3	Bread	0.158	1.4	6.1	3.0	96
4	"	0.21	—	—	—	—
4	Cream	0.377	—	—	—	—
3	Fish	0.162	0.88	6.9	2.8	96
4	"	0.322	—	—	—	—

DISCUSSION.

(1) *Blood-sugar.* It seems somewhat paradoxical that animals on a carbohydrate-free diet should have a higher blood-sugar than control animals receiving abundant carbohydrate in their diet. Nevertheless, our results point to this being the case. While the blood-sugar of control rats varied from 0.14 to 0.16 %, the values for rats on a carbohydrate-free diet for 1 to 2 weeks tended to be higher, 0.17 to 0.18 %, but normal values were obtained after longer periods of feeding. Thus, though the difference found in the case of rats after a fortnight's feeding was not very marked it was always obtained, and with mice a very decided increase in the blood-sugar value resulted with all the non-carbohydrate diets used, while kittens fed on cream showed a very high blood-sugar. It is also interesting to note, in this connection, that with children Salomonsen [1929] has reported an increase in blood-sugar to follow the addition of fat to the diet. Further, it may be mentioned that Abderhalden and Wertheimer [1925] observed that, in rats on a carbohydrate-free diet, the hyperglycaemia produced by a subcutaneous injection of glucose was much greater than that produced in animals on a rich carbohydrate diet. On the other hand, these same authors [1924, 2] gave graphs showing the initial value for blood-sugar in rats on non-carbohydrate diet at the same level (0.11 %) as that of controls on a carbohydrate diet. Though no mention is made of the fact, the animals had probably fasted for some hours prior to the observations on blood-sugar being made, and support is given to this view as we found that after 12 to 24 hours without food, the blood-sugar of non-carbohydrate rats sinks to practically the same level as that of similarly treated controls.

(2) *Muscle-glycogen.* The estimates of muscle-glycogen in both control rats and those on non-carbohydrate diet were somewhat variable, and not much difference was found between the two sets of animals. Greisheimer and Johnson [1929] have recently reported an almost constant value of 0.32 % on five widely different diets. In cheese-fed rats we found the muscle-glycogen perhaps higher, certainly no lower, than in the controls. After 24 hours without food, there was a fall in the glycogen content of the muscle of cheese-fed rats, while no change was detected in that of the controls.

No estimations were carried out on the glycogen content of the muscles of mice, but with kittens on the two types of diet animals on the non-carbohydrate diet had a considerably lower muscle-glycogen.

(3) *Liver-glycogen.* The glycogen content of the livers of control animals was very constant, 6 % in kittens, 4 % in mice, and 3 % in rats, which values agree well with those quoted in the literature [Barbour *et al.*, 1927]. The livers of animals fed on a non-carbohydrate diet did not become glycogen-free. In fact, with kittens on a diet of fish, the livers contained rather more glycogen than those of the control animals on bread and milk. The livers of cheese-fed rats and mice, on the other hand, contained less glycogen than those of the controls, but still contained a considerable quantity, the amount present, though somewhat variable, being within the limits (0.50–2.74 %) quoted by Magnussön [1929] for rats on a fatty diet. Our results are also consistent with the findings of Burn and Ling [1928] in experiments with rats on a diet consisting of butter-fat and salts. From a very low initial liver-glycogen of less than 0.1 %, these investigators obtained, during the first 4 days on the fat diet, a rise to 1.57 %. The origin of the liver-glycogen in animals receiving a diet devoid of preformed carbohydrate does not concern us for the present, but it may be noticed, as pointed out above, that both the blood-sugar and muscle-glycogen tend to be higher in these animals than in the controls.

After the withdrawal of food, there was a steady fall in the liver-glycogen, but the rate of fall was not so great as that found by other investigators. In the case of the animals on non-carbohydrate diet this might be explained by assuming that the process of gluconeogenesis might of necessity become accentuated when no carbohydrate was being supplied. However, as the decrease obtained with the control animals was also smaller and slower than usual, we consider the main factor concerned to be the conditions under which the experiments were carried out. Thus, all the animals took very little exercise during the experimental period, and were kept at ordinary room temperature (15°–16°), whereas Barbour and his co-workers, for example, used a temperature of about 30°, which undoubtedly would assist in depleting the store of glycogen in the liver.

(4) *Liver-fat.* The fat content of the livers of control rats and mice varied only within small limits, and the fat had a high iodine value, though this fell somewhat on extending the period of feeding on the carbohydrate diet.

Naturally the fat content of the livers of animals on the non-carbohydrate excess-fat diet was higher than that of the controls, with a compensating fall in its iodine value. In kittens, however, practically no difference was found in the fat content of the liver of animals fed on bread and milk and on fish. The iodine values obtained were also identical and lower than those for rodents. In rats the maximum amount of fat was present after 10 to 14 days' feeding, but still higher values were obtained with mice after a prolonged period.

On the withdrawal of food overnight, little change was detected in the liver-fat content of the control rats, an almost constant value of 3.7 % being maintained. Rats on the non-carbohydrate diet, on the other hand, showed striking changes, the initial value of 7.2 % falling after a 12 hours' fast to the same level as that of the controls, namely 3.7 %. Further, the fat which remained had a high iodine value, so that the more saturated fat was the first to disappear from the liver.

Reviewing the results as a whole, Rosenfeld's law regarding the antagonism between the fat and glycogen in the liver is supported in a general way in the case of animals which have not fasted, but not in animals which have fasted. Moreover, it is difficult to increase the fat content of the liver beyond twice the normal amount, and the liver-glycogen never in any circumstances disappears entirely. In fasting animals there is a fall in both liver-glycogen and liver-fat, but while the former proceeds slowly, the latter occurs with extreme rapidity. Whether the disappearing fat is utilised or simply transferred to the fat depôts is a problem which awaits further investigation.

Depisch and Hasenöhr [1929] consider that the administration of fat renders the liver-glycogen more labile. Our results suggest that this change in the behaviour of the liver-glycogen may be brought about not only by fat but by any non-carbohydrate diet. Thus, as under these conditions the liver-glycogen is not increased in amount while the blood-sugar level shows a tendency to be high, the liver must discharge glucose into the blood more readily, for as Soskin [1927] has shown, the liver-glycogen is the sole source of supply of the blood-sugar. This is in agreement with Macleod's [1929] statement that the ease with which the liver maintains the blood-sugar level is dependent not so much on the actual glycogen content of the liver as on the "sensitivity of the glycogenolytic mechanism."

Finally, it is rather remarkable that, after a 12 to 24 hours' fast, not much difference exists between rodents on a non-carbohydrate diet and those on the control diet, not only with regard to the liver-fat content but also with regard to blood-sugar, and both muscle- and liver-glycogen. Nevertheless, as will be shown in subsequent sections of this research, the metabolic condition of the animals is widely different.

SUMMARY.

Animals on carbohydrate-rich and carbohydrate-free diets are compared with regard to their blood-sugar, muscle-glycogen, liver-glycogen and liver-fat contents.

In rats on a carbohydrate-free diet, the blood-sugar tends to be high, there is practically no change in the muscle-glycogen but a large increase in the liver-fat content, and the liver-glycogen is reduced to half that on a carbohydrate diet.

With mice similar results are obtained, but with kittens only slight differences are detected on the two types of diet.

The fasting of rats for a period of 12 to 24 hours causes a very rapid disappearance of the excess of fat from the liver, a slow fall in both muscle- and liver-glycogen, and a fall in blood-sugar to the normal fasting level of 0.11 to 0.12 %.

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