LXXXVIII. CYCLIC CHANGES IN THE GLYCOGEN CONTENT OF THE LIVER AND THE MUSCLES OF RATS AND MICE.

THEIR BEARING UPON THE SENSITIVITY OF THE ANIMALS TO INSULIN, AND THEIR INFLUENCE ON THE URINARY OUTPUT OF NITROGEN.

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THE physiological changes in the glycogen content of the liver have generally been considered to be the result of changes in the carbohydrate supply of the body. It was formerly regarded as established that glycogen accumulates in the liver after ingestion of food and is eliminated from the liver by fasting, especially if combined with muscular exercise. Accordingly fasting and muscular exercise have been the principal means adopted for removing the glycogen from the liver as well as from the muscular tissue. At a very early date, however, observations were made indicating that the mechanism of this function of the liver was more complex than had been supposed.

Thus it is known that glycogen reappears in the liver and the muscles of rabbits completely deprived of their glycogen by means of strychnine convulsions. If the animals are kept anaesthetised glycogen reappears in the liver within 20 to 28 hours. Pfüger [1903] suggested the possibility of a migration of glycogen from the bony and cartilaginous parts of the body. In his excellent paper on this topic Rolly [1905] indicated the possibility of glycogen formation from body proteins. On the third to fifth day of fasting, rabbits completely deprived of their glycogen by means of strychnine convulsions showed not only the reappearance of glycogen in the liver and the muscles, but also an increase in the nitrogen output through the urine. In Rolly's experiments the excess nitrogen excretion, indicating an increased protein metabolism, occurred at a time when there was no reason to suspect a pre-agonal destruction of proteins.

These early investigations tend to show that the accumulation of glycogen and its disappearance from the liver cannot be explained solely by the storage and mobilisation of the available carbohydrate material. As is contended also by Macleod in his monograph on carbohydrate metabolism, other factors,

hitherto not well-known are involved in this mechanism. Such a view is furthermore supported by recent experimental findings.

In recent times a most valuable contribution to our knowledge of glycogen metabolism has been made by Forsgren [1928]. He has observed cyclic changes in the glycogen content of the liver of rabbits, the maximum being reached at night and the minimum during the daytime. These very striking changes are to a great extent independent of nutritional conditions. Early in the day glycogen may disappear from the liver in spite of an abundant food supply, whilst at night there is an accumulation of glycogen, even if the animals are fasting. There is a corresponding periodicity in the production of bile, the latter reaching its maximum during the day, when the glycogen content of the liver is low. Forsgren obtained these results by histochemical investigations, including staining of the bile-acids and the glycogen in the liver of animals killed at different times of the day. Forsgren's results have been confirmed by chemical analysis of rabbit's liver made by two of the authors of this paper.

In order to check Forsgren's results, we conducted experiments on a rather large scale, analysing the glycogen content of the livers of a considerable number of mice and rats at different times of the day. What particularly aroused our interest in this problem was the observation that mice used in the assay of insulin consistently show a greater resistance to insulin in the evening than at noon. We thought that the diminished sensitivity of the animals to insulin in the evening might be due to an accumulation of glycogen in the liver. This possibility has been suggested by Forsgren himself [1929].

This periodicity, if established, would moreover have an important bearing on our views of carbohydrate metabolism. It is now generally assumed that in a fasting animal the glycogen content of the liver remains constant at a low level or slowly decreases. Thus Cori and Cori [1928], in their studies of the glycogen metabolism, contend that during the second day of fasting (*i.e.* between the 24th and 48th hours) a very minute amount of glycogen only can have been removed from the liver of the rat and converted into sugar. The figures found for the glycogen content of the liver were almost identical on the two different days. This constancy, however, would be quite illusory, had there been in the meantime a considerable increase of the liver glycogen followed by a decrease. The conclusion drawn in regard to the sugar metabolism during that period would consequently be erroneous. The same remark applies to many investigations of recent date on the action of insulin and adrenaline, in which the glycogen content of the body has been studied for periods ranging from 24 to 48 hours, without knowledge of these cyclic changes.

By our experiments on mice and rats we confirmed Forsgren's findings so far as concerns this periodicity in the accumulation of glycogen in the liver. We also found that it is largely independent of nutritional conditions. In spite of 40 hours' fasting a large number of the rats showed an increase in the liver-glycogen during the night. None of the rats, which had fasted more than

24 hours, if killed during the daytime, had any appreciable quantity of glycogen in the liver. The results are thus fully in agreement with the findings of Forsgren in his experiments on rabbits.

In the experiments with insulin the increase in the resistance of the mice to the hormone was found to coincide with the accumulation of glycogen in the liver. In the evening a smaller number of animals showed convulsions than at noon, and at night the dosage of insulin had to be doubled in order to induce severe intoxications in the same percentage of animals.

Having, by our first series of experiments, established the periodicity in the accumulation of glycogen in the liver, we proceeded in the subsequent series to examine also the glycogen content of the muscles. The close connection between the glycogen content of the liver and that of the muscles has been well established, especially by the studies of Cori and Cori [1929]. These authors emphatically assert that glycogen passes from the muscles to the liver and *vice versa*, the lactic acid of the blood and the blood-sugar respectively being the intermediate compounds. On this basis it seemed reasonable to presume that the glycogen content of the muscles would show a periodicity similar to that of the liver. In fact, in our three series of experiments, in which the entire body of the animals (mice) was examined for glycogen in accordance with the method of Lesser, the glycogen content of the muscles was found to be higher during the night than during the day. The difference, however, is not so marked as in the case of the liver. This seems to indicate that the glycogen formed during the night originates from the liver.

In regard to the material which gives rise to this periodical accumulation of glycogen, it may be taken for granted that an important part is played by the carbohydrates. How far the proteins also participate in this synthesis of glycogen is a problem of considerable interest. As stated above, the view that the proteins do contribute to the formation of glycogen has been emphasised by various investigators. The fact that the periodical formation of glycogen is but slowly affected by moderate fasting likewise indicates that the body proteins may participate in the process. If this is actually the case, the urinary output of nitrogen should be affected by the process. For an increased deamination of amino-acids, in connection with the accumulation of glycogen, would naturally result in an increased nitrogen output. As, so far as we know, cyclic changes in the urinary nitrogen output of experimental animals have not previously been demonstrated, we thought it desirable to investigate this question by experiments on rabbits. The results make it evident that in rabbits the nitrogen output does show a periodical variation parallel with that of the liver-glycogen.

EXPERIMENTAL.

The glycogen content of the liver was determined in seven series of experiments on mice and one series of experiments on rats. In each series 120 or 90 animals were taken. Either 10 animals were killed every 2 hours or 15

every 4 hours, beginning at 8 o'clock in the morning. All the animals belonging to one series were kept in a large cage under uniform conditions.

In two of the mouse series the animals were abundantly supplied with food and water. The stomachs of the mice on dissection were always filled with food. In four of the series each animal had fasted 10 hours before being killed. Previously to the latter experiments 10 animals had been placed every 2 hours in smaller cages, supplied with water only. In one of the mouse series the food had been removed 10 hours before the beginning of the experiment, so that those last killed had fasted 34 hours. The rats had fasted 24 hours before the beginning of the experiment.

The animals after weighing were killed by decapitation. The liver was rapidly removed, weighed, cut in pieces and placed in a centrifuge tube together with 1 cc. of 60 % potassium hydroxide. The tube was immersed in a boiling water-bath. The liver of a mouse is sufficiently large for a single analysis. Of the rat's liver about 1 g. was taken.

Where the glycogen content of the whole animal was determined in accordance with the method of Lesser, the mouse, after the removal of the liver, was immediately cut up with a pair of scissors, and placed in a large centrifuge tube, containing 20 cc. of hot potassium hydroxide solution. The alkaline solution, on heating in a water-bath, quickly penetrated the material. The heating was continued for 3 hours; residual bony material was removed before the precipitation with alcohol.

After the hydrolysis 1 cc. of the potassium hydroxide solution was treated with 2 cc. of water and 8 cc. of 95 % alcohol. The precipitate was washed in the centrifuge tube 3 times with 66 % alcohol, 3 times with 95 % and once with absolute alcohol. After washing with ether and drying, it was hydrolysed by heating for 3 hours in a water-bath with 5 % hydrochloric acid. The sugar determination was made according to the method of Bertrand.

By the method used 2-5 mg. glycogen were recovered with a loss not exceeding 3 %. In comparing these results with the figures found on analysis of 25 g. of the liver according to the macro-method, the differences between the figures were found to be less than 5 %.

In the series where insulin was injected into mice, the animals were taken from a stock of several hundreds kept in a large box. In two of the series (Figs. 3 and 4) 50 animals were taken out of the box 10 hours before they were injected with insulin. They were supplied with water only. In one series (Fig. 6) all of them had been deprived of food at 10 p.m. on the preceding day. In each of the experiments recorded in the figures 50 animals were injected with an amount of insulin, known to cause convulsions in 50 % of cases within 2 hours. The amount of insulin necessary was 0.0062-0.0075 units per 20 g. body weight, which was dissolved in 0.5 cc. of saline and injected subcutaneously. The temperature was kept constant at 38°.

The experiments concerning the urinary nitrogen output were performed on female rabbits. The animals were kept in small cages which did not permit

free movements. The cages were provided with bottoms of strong wire netting and were placed in aluminium bowls for the collection of urine. The urine was collected twice daily at 8.30 a.m. and 8.30 p.m. On each occasion the bladder was rinsed with water through a metal catheter. In order that the animals might be supplied with water 60 to 80 cc. of saline was at the same time injected subcutaneously. The urinary excretion was thus followed during a period of 6 days, throughout which the animals were kept fasting. The temperature and the body weight were recorded every other day.

RESULTS AND CONCLUSIONS.

Fig. 1 shows the glycogen content of the liver of animals (mice) abundantly supplied with food. The glycogen content was much higher during the night

Fig. 1. Glycogen content of the liver of mice abundantly supplied with food.

than during the day. The average figures for 10 animals were about 100 % higher in the night than in the day. In Fig. 1 (Table I, A) the figures for the liver-glycogen of the 50 animals killed between 10 a.m. and 8 p.m. were

Table I. *Cyclic variations in glycogen of mice and rats under different conditions.*

(Figures represent mg. glycogen/g. body weight (and average deviation from mean) determined in groups of 10 animals.)

	Time ...	8 a.m.	10 a.m.	12 noon	2 p.m.	4 p.m.	6 p.m.
A. Mouse-liver; animals well-fed		0.93 (± 0.177)	0.56 (± 0.139)	0.62 (± 0.077)	1.10 (± 0.213)	1.02 (± 0.207)	0.86 (± 0.093)
B. Rat-liver; animals fasted 24 hrs. before and during experiment		0.028 (± 0.00225)	0.034 (± 0.00438)	0.033 (± 0.0051)	0.031 (± 0.0062)	0.027 (± 0.0033)	0.115 (± 0.0348)
C. Mouse-liver; animals fasted 10 hrs.		2.22 (± 0.203)	1.67 (± 0.248)	1.20 (± 0.244)	0.54 (± 0.161)	—	0.38 (± 0.049)
D. Mice as under C. Body exclusive of liver		0.57 (± 0.082)	—	—	0.49 (± 0.078)	—	—
	Time ...	8 p.m.	10 p.m.	12 midnight	2 a.m.	4 a.m.	6 a.m.
A. Mouse-liver; animals well-fed		1.28 (± 0.184)	1.94 (± 0.243)	2.06 (± 0.216)	2.51 (± 0.243)	2.04 (± 0.264)	2.08 (± 0.125)
B. Rat-liver; animals fasted 24 hrs. before and during experiment		0.036 (± 0.0047)	0.061 (± 0.0195)	0.076 (± 0.0261)	0.139 (± 0.0272)	0.104 (± 0.0329)	0.083 (± 0.0203)
C. Mouse-liver; animals fasted 10 hrs.		0.34 (± 0.062)	—	1.03 (± 0.158)	—	0.81 (± 0.094)	1.57 (± 0.099)
D. Mice as under C. Body exclusive of liver		0.25 (± 0.0)	—	0.47 (± 0.033)	—	0.62 (± 0.0026)	—

0.83 ± 0.0679 mg./g. body weight and for the 59 killed between 8 p.m. and 8 a.m. 1.97 ± 0.0986 . Thus under normal conditions glycogen accumulates in the liver of the mouse during the night.

Similar changes occur in the liver even if the animals are deprived of food. They could be demonstrated in mice after 10 hours' fasting and in rats which had been kept without food for 40 hours. Fig. 2 shows the results

Fig. 2. Glycogen content of the liver of rats, which had fasted 24 hours before the experiment and were kept fasting.

obtained with rats which had fasted 24 to 48 hours. In the daytime the liver contained about 3 mg. glycogen per 100 g. body weight, whereas the figures during the night were 8–10 mg. The 70 animals killed between 10 a.m. and

Fig. 3. Glycogen content of the liver of mice, which had fasted 10 hours before analysis.

Fig. 4. Glycogen content of the liver of mice, which had fasted 10 hours before analysis.

12 midnight showed an average of 4.8 ± 0.681 , the 50 killed between 12 midnight and 10 a.m. 8.6 ± 1.17 . The figures are found in Table I, B. Figs. 3 and 4

give the glycogen content of the liver of mice, which had fasted 10 hours before killing. The same periodicity is shown in these figures, especially in Fig. 4. Here the average figure for the glycogen content of the liver of the 40 animals killed between 2 p.m. and 12 midnight was 0.42 ± 0.060 mg. per g. bodyweight, whereas the figure for the 70 animals killed between 8 a.m. and 2 p.m. and between 12 midnight and 8 a.m. was 1.32 ± 0.0867 . The figures are given in Table I, C. The glycogen content of the livers of the 60 animals (see Fig. 3) killed between noon and midnight was 0.16 ± 0.078 and of the 57 killed between midnight and noon 0.53 ± 0.057 mg. per g. body weight.

The urinary nitrogen output was followed in three series with 10 rabbits in each. The animals were kept fasting for a period of 6 days, during which time the urine was collected quantitatively and analysed for nitrogen. Throughout all the series it could be established that the urinary nitrogen output was larger during the night than during the daytime. Owing to several circumstances the first two series yielded incomplete results. We therefore report only the third series (see Table II). In the second series the nitrogen output during

Table II. *Urinary nitrogen of rabbits excreted during periods of 12 hours.*

Rabbit	Time	mg. nitrogen.					
		Day of fasting					
		1	2	3	4	5	6
1	8.30 p.m.-8.30 a.m.	779	903	697	721	695	562
	8.30 a.m.-8.30 p.m.	655 + 19	780 + 16	591 + 17	688 + 5	598 + 15	481 + 17
2	8.30 p.m.-8.30 a.m.	680	861	732	592	457	588
	8.30 a.m.-8.30 p.m.	868 - 22	713 + 21	532 + 37	486 + 22	512 - 11	491 + 20
3	8.30 p.m.-8.30 a.m.	778	865	773	628	685	750
	8.30 a.m.-8.30 p.m.	763 + 2	792 + 9	632 + 22	580 + 8	717 - 4	669 + 12
4	8.30 p.m.-8.30 a.m.	835	935	832	758	746	554
	8.30 a.m.-8.30 p.m.	798 + 5	819 + 14	712 + 17	616 + 23	622 + 20	487 + 14
5	8.30 p.m.-8.30 a.m.	1005	1100	1043	632	578	623
	8.30 a.m.-8.30 p.m.	780 + 29	895 + 23	782 + 33	552 + 14	476 + 21	438 + 42
6	8.30 p.m.-8.30 a.m.	875	1010	812	812	654	600
	8.30 a.m.-8.30 p.m.	768 + 14	840 + 20	875	670 + 21	578 + 13	495 + 21
7	8.30 p.m.-8.30 a.m.	698	817	820	674	572	485
	8.30 a.m.-8.30 p.m.	.	691 + 18	669 + 23	590 + 14	509 + 12	466 + 4
8	8.30 p.m.-8.30 a.m.	.	928	882	660	641	634
	8.30 a.m.-8.30 p.m.	673	823 + 13	709 + 24	618 + 7	588 + 9	565 + 12
9	8.30 p.m.-8.30 a.m.	952	853	868	598	529	546
	8.30 a.m.-8.30 p.m.	626 + 52	.	642 + 35	542 + 10	512 + 3	436 + 25
10	8.30 p.m.-8.30 a.m.	840	753	855	.	718	696
	8.30 a.m.-8.30 p.m.	576 + 46	728 + 3	714 + 20	575	540 + 33	640 + 9

the night was, on an average, 16 % larger than in the daytime for the first 3 days and 6.6 % larger during the last 3 days of the fasting. In the third series we succeeded in collecting the urine on each occasion except 6 out of a total of 120. The table shows a constantly increased output of nitrogen during the night, which persists in spite of the continued fasting.

Only on 3 occasions out of 55, on which the morning and the evening figures were recorded, did we observe a lower output of nitrogen in the

morning than in the evening. To judge by the evening figures, the low output on one of these occasions (rabbit No. 2, first day) was certainly due to a defective evacuation of the bladder in the morning, and on another occasion (the same rabbit on the 5th day) it was probably due to the same reason.

In the table the differences between the morning and the evening figures are given as percentages of the evening values. If we take the average for the positive figures, the increased output during the night amounts to 21.2 % for the first 3 days and to 15.8 % for the last 3.

Fig. 5. Glycogen content of the muscular tissue (x x) of mice, which had fasted 10 hours before analysis, as compared with the glycogen content of the liver of the animals (x — x).

In the series recorded in the table the rabbits showed no rise of temperature and a moderate decrease of weight, so that the results were not distorted by any complications.

Fig. 6. Glycogen content of the liver of mice, which had fasted 10 hours before the experiment and were kept fasting.

It may be presumed that this increased nitrogen output during the night is genetically connected with the formation of glycogen. The cyclic accumulation of glycogen in the liver may be attributed partly to a conversion of proteins into glycogen. If we suppose that a rabbit excretes 1.5 g. nitrogen in 24 hours, the increased output during the night would be about 0.1–0.2 g., corresponding to approximately 0.4–1.0 g. glycogen. Thus in a rabbit's liver weighing 60–70 g. up to 1.5 % of glycogen, or possibly somewhat more, could be formed solely from the excess of amino-acids deaminised during the night. It seems reasonable to assume that the body proteins participate in this process, seeing that it is only slightly affected by fasting.

The excess output of nitrogen in these experiments, which is about 10 %

of the total amount of nitrogen eliminated, must represent the minimum amount of proteins thus converted into glycogen. It may, however, be presumed that the proteins metabolised are transformed into glycogen to a much larger extent than can be traced in this way.

SUMMARY.

There are cyclic changes in the glycogen content of the liver of rabbits, rats and mice, which are to a large extent independent of the intake of food. Glycogen accumulates in the liver during the night and disappears again to some extent during the next morning. The same occurs even in fasting animals.

Similar periodical changes occur, though to a minor extent, in the muscles also.

As might have been expected from the observations recorded above, mice show an increased resistance to insulin in the afternoon and particularly during the night.

In rabbits, where these periodical changes are particularly striking, the urinary nitrogen output is increased during the night. In fasting rabbits the excess of nitrogen thus eliminated is about 20 % of the amount excreted during the day. This indicates that an increased deamination of amino-acids occurs during the night and that the formation of glycogen takes place partly at the expense of the body proteins.

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