THE EFFECT OF INSULIN ON ACETONURIA.

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The experiments to be described were begun two years ago in order to obtain further information on the part played by the secretion of the posterior lobe of the pituitary body in fat metabolism. Coope and Chamberlain(1) had already shown that injections of pituitary extract caused a rise in the percentage of fat in the liver of rabbits, and the suggestion presented itself of investigating the relation of pituitary extract to the formation of acetone bodies. Observations were first made on the acetonuria occurring in the dog and in the cat when these animals were fed on a fat diet. The finding of Geelmuyden(2) that the acetonuria so produced is very small in amount was confirmed, and attention was turned to the more regular excretion which Wigglesworth(3) first recorded in the rat.

In a preliminary note(4) we have already stated that injections of pituitary extract greatly diminish or inhibit the ketonuria occurring in the rat on the second and third days of a fat diet, in the months of May and June; further, that injections of adrenaline have the same action. Since this was published we have seen the papers of Raab(5), of Hirschhorn and Pollak(6), and of Anderson and Anderson(7).

Raab described a rise in the acetone bodies present in the blood of the dog following the injection of adrenaline. The changes he records, though definite, are not of such a magnitude as to suggest that the phenomenon is of great physiological importance. The work of Hirschhorn and Pollak on the other hand is much more impressive. They describe an increased output of acetone bodies following the injection of adrenaline (*a*) in normal human subjects on a carbohydrate free diet, (*b*) in pyrexial subjects on a low diet, (*c*) in patients suffering from vomiting of pregnancy, (*d*) in certain asthmatic patients, and (*e*) in diabetics. In all cases the rise in the acetonuria observed was striking. Finally Anderson and Anderson(7) have shown that in rats which have been kept for several days on a fat diet, or which are phlorizinised and fed on a carbohydrate-free diet, injections of adrenaline cause an

immediate rise in the output of acetone bodies. Their results are as clear and convincing as those of Hirschhorn and Pollak. The position therefore is that three independent investigations have shown that adrenaline can cause a rise in acetonuria in conditions of carbohydrate deprivation, while we have stated on the other hand that adrenaline inhibits acetonuria. For the present we do not propose to describe our experiments on pituitary extract and adrenaline in detail until we have had a further opportunity to investigate this discrepancy.

In the present paper experiments are described which demonstrate the seasonal variation in the acetonuria, the changes in the liver glycogen, and the action of insulin.

Methods.

The No. 3 diet of Wigglesworth(3) has been used, consisting of butter, filtered when hot to remove traces of casein, and containing 3 p.c. of a salt mixture. The rats were put in a Hopkins metabolism cage in pairs of the same sex, and 24 hour samples of urine were collected. For estimating acetone bodies, instead of the distillation method used by Wigglesworth, we have used the gravimetric method of van Slyke(8).

The rats were selected between the weights of 80 and 150 grm., and for the most part were males of about 120 grm. They were all animals from a Wistar strain bred in the Glaxo Laboratories.

Seasonal variation in the ketonuria.

From March to July 1926 a degree of ketonuria was obtained of the same order as that described by Wigglesworth, whose experiments were carried out at the same time of the year. In the autumn and winter of 1926, however, the ketonuria was much less, and was too small to serve as a basis for the observations we wished to make. We were at a loss to account for the change until the paper of Cori and Cori(9) appeared in April 1927. These workers described a seasonal variation in the ketonuria of rats kept without food for 48 hours. They found that in the summer months there was about two to three times as great an excretion of acetone bodies as during winter. Our observations confirmed this suggestion, though the magnitude of the seasonal difference in our experiments considerably exceeded that recorded by Cori and Cori. When fresh observations were made in the spring of 1927, in which a greater ketonuria was recorded than in winter, there no longer remained any doubt of the existence of this unexpected phenomenon.

In Table I is recorded the average result of all the experiments done in each month of the year.

TABLE I.

Month	No. of observations	Mg. acetone per diem (two rats) 1st	2nd	3rd	4th	5th	6th	7th
Jan.	5	4	12	12	(17)	(6)	—	—
Feb.	2	3	5	7	—	—	—	—
March	1	4	4	7	4	2	2	—
April	2	3	17	32	43	18	6	—
May	5	3	36	67	(68)	—	—	—
June	5	8	41	51	38	(14)	(5)	—
July	10	2	26	36	22	14	(13)	—
Aug.	—	—	—	—	—	—	—	—
Sept.	8*	7	27	22	15	11	14	10
	4†	1	4	5	4	4	2	2
Oct.	3	2	4	5	6	—	—	—
Nov.	6	5	13	7	(15)	(8)	(7)	(6)
Dec.	3	2	6	5	—	—	—	—

*=Males. †=Females.

Brackets indicate that figure is average of smaller number of observations than stated in second column.

If the experiments be divided into two groups, in one of which is included those carried out from October to March, and in the other those carried out from April to July, there are 20 experiments in the first and 22 in the second group. The average third day excretion in the first group corresponded to 8 mg. acetone, while in the second it corresponded to 42 mg. acetone. The standard deviations of these two figures are 1·16 and 6·25 respectively, so that the difference between the two averages is statistically significant. (The ratio of the difference between the averages to the square root of the sum of the squares of the standard deviations is 5·5.)

Cori and Cori(9) investigated the effect of temperature changes on the ketonuria of starved rats and showed that the higher ketonuria of the summer months could not be produced in winter by putting rats for three weeks in a room at 28° C. In our experiments there has been no record of the temperature of the room in which the rats were kept, but there is no obvious wide variation. The September results are of interest because they suggest that female rats have already adopted winter behaviour in advance of the males; but very few observations on female rats have been made.

Effect of successive periods of fat diet.

In order to obtain evidence of the effect of any agent on the ketonuria, it was necessary to observe the behaviour of the same animals on two

occasions, separated by two or three weeks. It has been found that as a rule the ketonuria during a second period of fat diet is appreciably less than that during the first period. The experiment given in Table II is an example of this.

TABLE II.

Two rats (♂), 120 grm. each.

Date when fat diet begun	Mg. acetone per diem			
	1st	2nd	3rd	4th
May 24th	17	70	43	68
June 20th	2	18	6	3

The action of insulin.

According to the time of the year at which the experiment is performed, there are three different responses to injections of insulin.

In Fig. 1 appears the record of an experiment carried out in September in which the pair of rats used were given a preliminary period

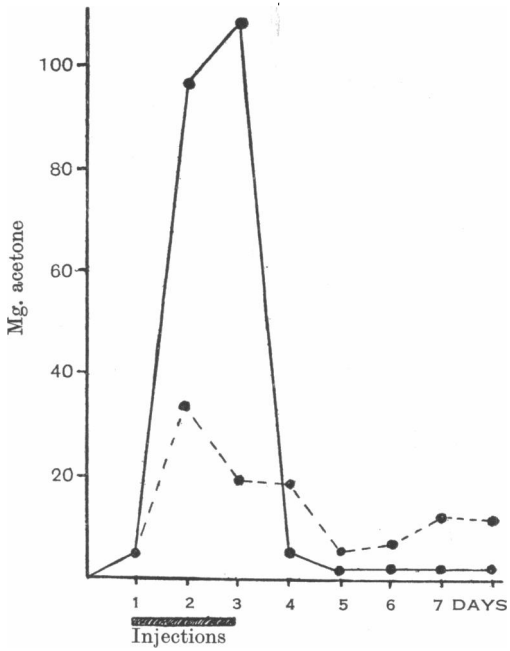

Fig. 1. Broken line shows excretion of acetone bodies by two rats in September. Continuous line shows excretion in same rats when injections of insulin were given. See text.

of fat diet (broken line) to determine the excretion of acetone bodies in the absence of any injection. After a rest of three weeks on a normal

diet, a second period of fat diet was begun, on the second and third days of which injections of insulin were made. The doses employed, per rat, were three doses of 0·4 units at 11 a.m., 1 p.m. and 5 p.m. and one dose of 0·3 units at 3 p.m. on the first day of injections, and three doses of 0·4 units at 1 p.m., 3 p.m. and 5 p.m. following an initial dose of 0·6 units at 11 a.m. on the second day of injections. The figure shows the striking rise in acetone body excretion which the insulin caused. It also shows the very rapid disappearance of the ketonuria when the injections were stopped, the excretion during the five days being similar to that obtained on a carbohydrate diet, and appreciably less than that observed in the preliminary period on the same days. The two rats whose behaviour is described were investigated together with three other pairs. In two of the other pairs the same augmentation of ketonuria was observed, while in the remaining pair there was no augmentation. The smallest dose of insulin following which this effect has been observed was a dose of 0·8 units per day, given in four injections of 0·2 units each to rats weighing 180 and 190 grm. This dosage produced the augmentation without the accompaniment of hypoglycæmic symptoms.

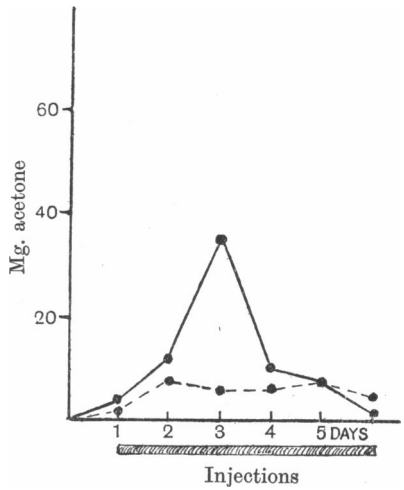

Fig. 2. Showing augmentation of ketonuria by insulin on second day of injections only. No augmentation on 1st, 3rd, 4th and 5th days of injections. Experiment carried out in December. See text.

A second response to the injection of insulin is shown in Fig. 2, which illustrates an experiment carried out in December. In this experiment, as in the other, the rats were given a fat diet for a preliminary period in order to determine the normal ketonuria. This is shown as before as a broken line, and was less in magnitude than the normal September ketonuria. After an interval of 21 days a second period began, and injections of insulin were given from the second to the sixth days of the fat diet. The augmentation of the ketonuria was observed on the third day only. The dosage employed was 0·7 units per rat per day (the rats weighed 160 and 170 grm.) given in three doses of 0·2 units, and one dose of 0·1 unit. A similar experiment on a second pair of rats carried out at the same time gave a precisely similar result. Experiments carried

out in late October gave results resembling the September result in that the augmentation was maintained for two days or, when the dose of insulin was as large as 1·2 units a day, even for three days; but resembling the December results in that the lower doses of insulin produced no augmentation on the first day of injection, while on the fourth day of injection, the ketonuria was no greater than in the preliminary experiment.

The third type of response to the injection of insulin is shown in Fig. 3, which records an experiment carried out in June. There was no

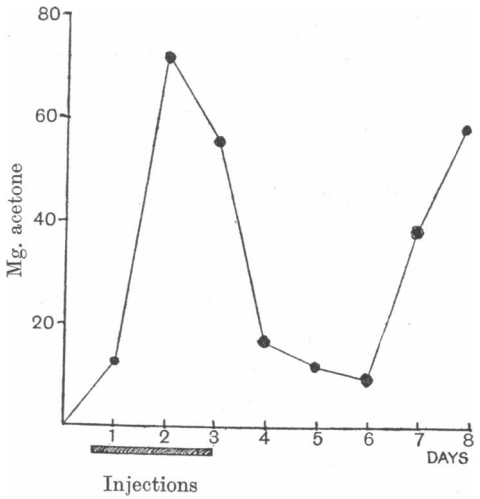

Fig. 3. Showing the second ketonuria occurring in summer about the 6th–8th days of fat diet when injections of insulin are given on the 2nd and 3rd days.

preliminary period of fat feeding, and it is impossible to say whether the large ketonuria observed on the two days of injections was larger than it would have been without the injections. In June and July the average ketonuria on the first day of insulin injections (being the second day of fat diet) was in five experiments 57 mg. (reckoned as acetone). The average ketonuria in the absence of injections was on this day 35 mg., taking this average from seven experiments done during the same weeks as the insulin experiments. This difference is scarcely significant. It may well be that insulin does not augment the already large summer ketonuria.

On the other hand, while the ketonuria was always high on the first day of injections, on the second day of injections in four out of five experiments it fell, at a time when in the absence of insulin it would have

been rising. This fall in one experiment was such as to mean almost a disappearance of ketonuria.

The main interest of the effect of insulin in June and July lay, however, not in its immediate, but in its late effect. When the injections were stopped (see Fig. 3) the ketonuria declined on the fourth, fifth and sixth days, and then again rose on the seventh and eighth days. This curious second rise in the ketonuria occurred in each of the five experiments in which the ketonuria was examined so long after injection.

The relation of ketonuria to hypoglycæmia.

In several experiments in which the augmentor effect of insulin on ketonuria was observed, it was seen that the doses of insulin used produced no obvious symptoms. The most useful information about the level of the blood sugar was obtained from some experiments carried out at the beginning of November. A group of ten rats of similar weight were given a fat diet for six days. The excretion of acetone bodies was determined in two pairs, and the remaining six rats were killed at different stages of the experiment to determine the blood sugar. Injections of insulin, each 0·2 units, were given to each rat four times a day. The blood sugar was determined one hour after the last injection. The rat killed at the end of the first day of injections had a blood sugar of 0·073 p.c. On this day the insulin produced no augmentation of ketonuria. At the end of the second day two rats were killed, the blood sugars being 0·035 and 0·055 p.c. One rat killed on the third day had a blood sugar of 0·050 p.c. On the second and third days of insulin, an augmented ketonuria was observed. On the fourth and fifth days the blood sugar percentages were both 0·060, and on these days there was no appreciable ketonuria, the excretion being less than in the control period. While it appeared that the greatest ketonuria was associated with the lowest blood sugar value, there was no reflection in the blood sugar figures of the striking change from the high ketonuria of the third to the negligible excretion of the fourth day.

The liver glycogen.

The unexpected effect of insulin in increasing ketonuria called to mind its unexpected effect in diminishing liver glycogen first demonstrated by Dudley and Marrian(10). It seemed probable that there was some connection between the two phenomena. To investigate this it was first necessary to determine the changes which took place in the

liver glycogen when rats were fed on a fat diet without insulin injections. These proved to be of considerable interest.

The first observations were made in October. Fifteen male rats, each weighing about 120 grm., were given a fat diet. Three were killed at 24 hour intervals for the determination of liver glycogen. The pieces of liver were transferred to tubes containing hot potash, and the tubes immersed in a boiling water bath within one minute of crushing the skull of the rat. We are indebted to Prof. Lovatt Evans for advice on the method of estimating the glycogen. The results are given in Table III.

TABLE III.

Each figure is the percentage of liver glycogen present in one rat, killed after it had been given a fat diet for the time shown.

	24 hours	48 hours	72 hours	96 hours	120 hours
	0·035	0·37	0·5	0·62	1·48
	0·11	0·41	0·75	1·80	1·51
	0·12	0·555	1·95	2·28	1·68
Average	0·09	0·44	1·06	1·57	1·56

The last line of Table III gives the average of the glycogen percentages, which is seen to rise steadily during the first four days to the

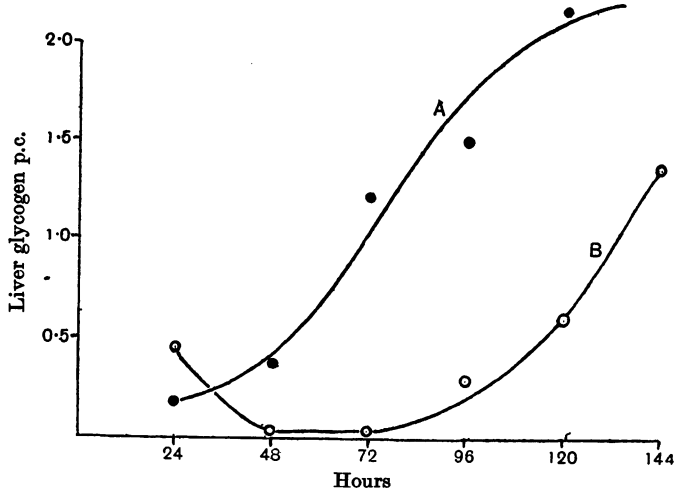

Fig. 4. Curve *A* shows rise of liver glycogen percentage in rats given a fat diet in November. Each point is the average value of three rats. Curve *B* is the record of a similar experiment in which 0·8 units insulin were injected into each rat daily from the second day of the fat diet to the end of the experiment. The curve shows the initial inhibition of the rise by the insulin. The inhibiting action begins to fail on the third day of injections.

appreciable amount of 1·5 p.c., from an initial figure of less than 0·1 p.c. The results of a similar experiment carried out at the beginning of November are shown in Curve *A* of Fig. 4, in which the figure for the rats killed at 120 hours was 2·2 p.c. Two other experiments in November gave similar results. The increase in the glycogen is reminiscent of, though more considerable than, the rise in liver glycogen observed by Barbour, Chaikoff, Macleod and Orr(11) in rats kept without food; they found a percentage of 0·16 at the end of 24 hours, which rose to 0·32 at the end of 48 hours.

A further point of interest lay in the values for the liver glycogen determined at the end of 24 hours of the fat diet. These became higher towards the end of November than they were at the beginning of October. In Table IV are given the results for the individual rats at different times.

TABLE IV.

Percentage of liver glycogen in rats killed after 24 hours' fat diet.

	11 Oct.	1 Nov.	21 Nov.	30 Nov.	12 Dec.	2 Jan.
	0·035	0·03	0·08	0·5	0·12	0·24
	0·11	0·12	0·51	0·5	0·22	0·46
	0·12	0·425	0·57	0·86	0·86	0·63
Average	0·09	0·225	0·39	0·62	0·4	0·44

Taken together with the fact of the gradual decline of the ketonuria from summer to winter, the figures suggest that the liver glycogen after 24 hours of a fat diet is lower in October than in December. The few figures given in Table IV of course do not establish this point.

Origin of the new liver glycogen.

We have as yet been unable to make an exhaustive study of the origin of this liver glycogen, but some observations may be briefly described. From simultaneous determinations of the muscle glycogen, it did not appear that a simple transference of glycogen from the muscles was sufficient to account for the increase in the liver. Table V shows the figures obtained for muscle glycogen in two experiments. Each figure is the average of the glycogen percentages present in the right gastrocnemius muscles of three rats.

TABLE V.

Muscle glycogen during fat diet.

	24 hours	48 hours	72 hours	96 hours	120 hours
Exp. 1	0·25	0·25	0·14	0·16	0·2
Exp. 2	0·23	0·20	0·15	0·18	—

In each of the experiments the final is 0·05 p.c. lower than the initial figure. For a rat of 100 grm., having 50 grm. muscle, this fall represents a disappearance of 25 mg. glycogen. If this were transported to the liver, of weight 5 grm., it would cause a rise of 0·5 p.c. The rise observed in the first of these experiments was 1·87 p.c. (from 0·4 to 2·27) and in the second 1·1 p.c. Hence on the basis of these figures it does not appear that the rise in the liver is due to a fall in the muscle stores.

Similarly the rise does not appear to be due to a formation of glycogen from protein, for observations of the excretion of total nitrogen showed no rise. In Table VI are given the figures for the mg. nitrogen excreted by each of four pairs of rats given a diet of fat and carbohydrate for two days (in order to eliminate the effect of omitting protein) and then given a diet of fat only for four days. The determinations were made by means of Pregl's micro-Kjeldahl apparatus.

TABLE VI.

Diet 1st and 2nd days—75 p.c. rice starch, 25 p.c. filtered butter with salt mixture. Diet 3rd, 4th, 5th and 6th days—filtered butter with salt mixture. Figures are mg. total nitrogen.

Rats	1st day	2nd day	3rd day	4th day	5th day	6th day
1st pair	230	157	191	177	144	138
2nd ,,	324	162	182	113	206	83
3rd ,,	174	195	132	153	179	86
4th ,,	235	224	176	163	120	112
Average	241	184	170	151	162	105

The figures do not show that the protein metabolism of the rats was regularly stimulated by taking them, in January, from a diet of fat and carbohydrate to one of fat alone. There was, on the whole, a gradual fall in the total nitrogen excreted.

We think it probable therefore that the rise in liver glycogen is a new formation from fat, but our evidence does not prove this.

The action of insulin on liver glycogen.

We were now in a position to investigate the action of insulin on the rise of liver glycogen described. Our observations were made in December and January at a time when the effect of insulin on ketonuria was of the kind shown in Fig. 2, in which the augmentation was only witnessed on the second day, or the second and third, of the insulin injections. The injections were given exactly as in the observations on ketonuria, at 10 a.m., noon, 2 p.m. and 4 p.m. daily, and each rat received 0·2 units at each injection. The rats for glycogen determinations were killed one

hour after the last injection. The results of one experiment are given in Table VII, together with the figures for the muscle glycogen.

TABLE VII.

Each figure is the mean percentage of glycogen in two rats. No determinations were made after 1st day of insulin injections, which was 2nd day of fat diet. Days are days of fat diet.

	3rd day	4th day	5th day	6th day
Liver	0·31	0·19	0·62	1·13
Muscle	0·30	0·41	0·34	0·31

The table shows that just as insulin injections cause an augmentation of ketonuria, which ceases after one or two days, so they cause an inhibition of the rise in liver glycogen, which similarly ceases after one or two days.

On the third and fourth days of fat diet, the liver glycogen figures, under the influence of insulin injections, were 0·3 and 0·19 p.c. respectively, whereas in the absence of these injections the figures were about 1·0 and 1·5 p.c. On the other hand, by the fifth and sixth days, the liver glycogen rose in spite of the continuance of the injections. The relation of the rise to that occurring without insulin injections is clearly seen in Fig. 4, in which the values from another insulin experiment are plotted in Curve *B*. At the end of 24 hours' fat diet without insulin the glycogen was 0·45 p.c. Injections on the next two days reduced the glycogen to a negligible quantity, but thereafter failed to prevent a rise to 1·4 p.c. on the fifth day.

In the experiment in Table VII the figures for muscle glycogen are higher than those which were obtained without insulin injections.

DISCUSSION.

The experiments described in the first part of the paper, demonstrating that, in rats eating a diet of fat, insulin increases the ketonuria, appeared at first to add one further complexity to the already sufficiently puzzling phenomena of metabolism. The experiments in the second part, which demonstrate that this effect occurs only in the condition in which insulin is able to reduce the liver glycogen to a low value, show however that there is no new difficulty. The effect on ketonuria might have been foretold from the previously known facts (*a*) that insulin reduces liver glycogen, and (*b*) that the amount of ketonuria is inversely proportional to the amount of liver glycogen. The problem of reconciling the anti-ketogenic action of insulin in the diabetic patient with the ketogenic action in the normal animal on a fat diet, is therefore essentially the

same as that of reconciling the glycogen-forming power of insulin in the diabetic with the reduction of liver glycogen seen in the normal animal.

The observations on the rise of glycogen in the liver of the fat-fed rat are of interest, as they throw further light on the cause of the diminution of liver glycogen by insulin. Burn and Marks(12) have shown that during the recovery from an insulin hypoglycæmia there is certainly a discharge of the existing glycogen stores, and that, when these are exhausted by thyroid feeding, no recovery is possible. But it has always seemed probable that the fall in glycogen was only in part due to this discharge. Many observers (Laufberger(13), Lesser(14), Cramer(15), Best, Dale, Hoet and Marks(16)) have expressed the view that one of the functions of insulin is to inhibit the new formation of glycogen. The evidence presented here is a demonstration that insulin has this property. In rats eating a fat diet there is a rise of liver glycogen, which is a new formation of glycogen, and which is inhibited by injections of insulin for two or three days; the duration of the inhibition depends on the dose employed.

The evidence presented on the origin of the glycogen is incomplete in quantity, but is a further instance of the appearance of carbohydrate in circumstances in which a transformation from fat is the only simple explanation. The figures of nitrogen excretion show that the removal of carbohydrate from the diet does not lead to stimulation of nitrogen excretion in albino rats during the winter, and there is no considerable change in muscle glycogen. In any event it is now accepted by most observers that muscle glycogen cannot act as a source of liver glycogen.

The observations which have been made on ketonuria in the rat by Wigglesworth(3), Levine and Smith(17), Anderson and Anderson(7), together with those here described, make it clear that in this animal Shaffer's views of the ketogenic and anti-ketogenic action of different food materials(18) have no application. Levine and Smith have calculated for one experiment that an observed excretion of 20 mg. acetone bodies should have been, on Shaffer's view, an excretion of 1592 mg.

Finally the seasonal variation in the excretion of acetone bodies by the rat affords matter for speculation. The rats used in our experiments were originally Norwegian rats. It may be supposed that, in their evolution, they have developed a mechanism to enable them to withstand successfully long periods of winter starvation, in which their energy is maintained by combustion of their fat stores. When food becomes plentiful in summer, the mechanism is no longer needed and is in abeyance.

SUMMARY.

1. There is a great seasonal variation in the excretion of acetone bodies by rats fed with a diet of fat; the excretion is high in summer and low in winter.

2. Injections of insulin augment the small ketonuria observed in winter. The augmentation may not be seen until the second day of insulin injections, and it usually disappears by the fourth day of injections.

If insulin injections are given in summer, and then withheld, a second augmentation of ketonuria supervenes after a few days.

3. Experiments in winter months have revealed a steady rise in liver glycogen in rats on a fat diet, beginning at 0·1–0·4 p.c. on the first day, and rising to 1·5–2·0 p.c. on the fourth and fifth day. This rise is not accompanied by a compensating fall in muscle glycogen, nor by a rise in the excretion of total nitrogen in the urine.

4. Injections of insulin delay this rise in liver glycogen for one or two days. The delay in the rise of liver glycogen corresponds in time to the period in which insulin augments ketonuria.

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