LXXIII. CONTRIBUTIONS TO THE STUDY OF BRAIN METABOLISM.

I. CARBOHYDRATE METABOLISM. PRELIMINARY PAPER.

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The work about to be described constitutes the preliminary portion of an investigation upon the metabolism of brain cells under conditions both normal and abnormal. The work of Warburg, Posener and Negelein [1924], who showed that brain tissue is capable of converting large amounts of glucose into lactic acid, suggested that a study of the sugar metabolism of the brain might be of considerable interest. It was therefore decided to attempt a comparison of sugar metabolism of the brain in a normal animal, and in an animal suffering from the effects of a convulsant dose of insulin. Rabbits were used for all the experiments.

When this work was already in progress, the papers of Takahashi and Asher [1924] and of Cori [1925] appeared. Takahashi dealt particularly with the total carbohydrate content of the brain, including glycogen, and Cori with the free sugar content. Both were agreed that the lowering of the blood sugar by insulin had very little effect upon the content of reducing substance in the brain.

Takahashi estimated glycogen and "other carbohydrate" (the latter being extracted by a modification of Meyerhof's method) in rabbit brains. The values for reducing substance were obtained by the Folin-Wu technique. His average value for normal animals was: glycogen 38·6, "other carbohydrate" 39·6 mg. %. For insulin-treated animals, prior to convulsions, the average values were: glycogen 46·5, "other carbohydrate" 42·5 mg. %; for "convulsed" animals, he gives three sets of values: glycogen 27, "other carbohydrate" 8; glycogen 6, "other carbohydrate" 41; glycogen 6, "other carbohydrate" 38 (all mg. %).

Cori treated his extracts of rabbit brain with Lloyd's alkaloidal reagent, with the object of removing reducing substances other than glucose, and then estimated the remaining reducing substance by the method of Hagedorn and Jensen. His figures for normal animals (used as controls) range from 43–25 mg. %. He gives only a few values for rabbits, and the variations

between them are large, yet length of time of starvation seems to be the only factor determining the selection of "control" animals, since these do not correspond with the "experimental" in age or weight.

We were able to confirm the fact that no alteration in the amount of reducing substance in the brain need necessarily have taken place at the onset of insulin convulsions. The actual figures we obtained were, however, higher than those given by the two authors mentioned. It is therefore thought advisable to give an account of the methods employed in this work.

The rabbit is killed by a blow on the neck, its brain removed as quickly as possible, and, where an immediate determination is to be carried out, the brain is then placed in a beaker surrounded by freezing mixture, chopped whilst very cold, and weighed out when frozen hard. Whilst still frozen it is placed in 20 % trichloroacetic acid (or alternatively in absolute alcohol). The removal of the brain can usually be accomplished in four or five minutes.

Two methods of extraction were employed, and where possible were both carried out on the same brain.

I. Extraction with trichloroacetic acid (20 or 10 %), a method suggested to us by Mr H. D. Kay. The tissue is ground with sand and ice-cold trichloroacetic acid, allowed to stand for some minutes at 0°, and filtered through muslin. This process is carried out three times. The cloudy solution thus obtained is centrifuged (filtration being very slow or practically impossible), the supernatant fluid poured off, and the precipitate washed three times before being discarded. The beakers, muslin, etc., are also well washed. The solution is next neutralised. It is usually still faintly cloudy, but a final centrifuging after neutralisation leaves it absolutely clear. The reducing substance present in this clear solution is estimated by the method of Hagedorn and Jensen [1923].

II. Extraction with alcohol. The tissue is allowed to soak in 90 % alcohol for some minutes to stop all enzyme action. It is then filtered off through muslin, ground up, placed in 60 % alcohol and left overnight. Next day the tissue is filtered off, ground, soaked for an hour in 60 % alcohol and finally filtered. The process is repeated once more. The cloudy filtrate is centrifuged till it is as clear as possible, the supernatant fluid poured off, and the precipitate washed three times before it is discarded. The solution is next heated gently on a water-bath until all the alcohol has disappeared; it is then full of a flocculent precipitate. Colloidal "iron" is added until the solution is clear; the addition of a little sodium sulphate seems to ensure more successful precipitation by the iron. The solution is filtered while still warm and the bulky iron precipitate is removed from the filter-paper, washed and filtered through the same paper. The washing is carried out three times. The reducing substance in the clear fluid thus obtained is estimated by the method of Hagedorn and Jensen. The estimation is usually done in triplicate, sometimes in quadruplicate.

The clear solutions obtained by these two methods obviously contain no substances that are not soluble in water. It is unlikely that any breakdown

of larger molecules either by enzyme action or by acid hydrolysis could occur during the course of the extraction processes under the conditions described.

The actual values obtained are shown in Table I. The values given by the alcohol extraction method are always higher than those given by the trichloroacetic acid method. The individual variation is obviously considerable. As the two methods show a definite parallelism it seems unlikely that this variation is due to any error of technique.

Glycogen estimations have not as yet been attempted.

Table I.

No.		Wt. brain taken g.	Trichloroacetic acid method. Reducing sub. mg./100 g. brain	Alcoholic method. Reducing sub. Unhydrolysed mg./100 g. brain	Alcoholic method. Reducing sub. Hydrolysed mg./100 g. brain
	Normal				
1	Young	3	100	122	257
2	,,	4	113·8	123·4	216
3	,,	3	109·15	121·3	228
11	,,	2·5	89·1	117	176
13	,,	3	84	117·26	180·6
15	,,	3	76·8	—	189
6	Adult	4	70·6	94·8	152·6
8	,,	4	77·6	—	—
	Insulin				
4	Young	3·5	78·7	93·4	196
5	,,	3	80·03	89·95	186
7	Adult	4	69·5	94·7	146·3
9	,, (C)	4	62·8	84·7	149
10	,,	4	—	90·7	159·4
14	Young	2·5	81·47	104·8	188·2

"C" = convulsed.

The reducing values always rose considerably on hydrolysis, thus indicating the presence of some disaccharide, or polysaccharide, or of some hydrolysable compound yielding a reducing substance.

It will be seen that on an average the rabbits which had received doses of insulin gave somewhat lower values than the normal rabbits, and one or two of the lowest values were given by the former group. It does not seem, however, that this fall in the content of reducing substance always occurs, and it is therefore not likely to be of much importance in accounting for the occurrence of the convulsions.

The extent of the individual variation made comparison of the normal and "insulin" rabbits very difficult. We found, however, that rabbits taken from the same litter gave very constant results, and we adopted the plan of taking both experimental and control animals from the same litter. The figures in Table II show that much clearer results can be obtained in this way; they do not indicate any fall in the amount of reducing substance as a result of treatment with insulin.

To avoid confusion with any possible result of the convulsive muscular movements themselves, the "insulin" rabbits were usually killed in the flaccid condition which immediately precedes the actual convulsions.

Table II.

No.	Litter A	Wt. brain taken g.	Trichloroacetic acid method. Reducing sub. mg./100 g. brain	Alcohol method. Reducing sub. Unhydrolysed mg./100 g. brain	Alcohol method. Reducing sub. Hydrolysed mg./100 g. brain
13	Young normal fed	3	84	117·26	180·6
14	Young insulin starved	2·5	81·47	104·9	188·2
15	Young normal starved	3	76·8	—	189·47
	Litter B				
	Young insulin stood (C)	3	—	145·5	207·3
	Young normal stood	3	—	127·7	187·3
	Young insulin (C)	3	—	137·3	205·6
	Young insulin	3	—	146·88	208·1

"C" = convulsed.

"Stood" indicates that the brain was allowed to stand for 24 hours at room temperature before extraction.

In view of the fact that brain tissue is capable of breaking down large quantities of glucose, and considering also that the convulsions coincide with a low blood sugar, and can be cured by administration of glucose, it seems curious that the reducing substance in the brain tissue should be left untouched when the blood sugar is low.

One possible explanation which suggested itself was that the reducing substance which is extracted from the brain was not glucose.

In order to test this, estimations of the lactic acid content of brain tissue immediately after removal of the brain were carried out, and the figures compared with those given by estimations of the lactic acid content of brain tissue after incubation or standing at room temperature. The method used was that of Meyerhof [1920]. It was necessary to substitute centrifuging for the original filtration through filter-paper. The values given by the distillations are quoted without correction, as comparative rather than absolute values were required.

The values for lactic acid in the brain immediately after death ("resting" lactic acid), expressed as mg. per 100 g. brain tissue, are all very much alike. These values are given in Table III.

Table III.

No.	Rabbit	mg. lactic acid per 100 g. brain	Description	
9 A	Normal	65·29	Resting	Halves of same brain
9 B	,,	62·74	Stood 24 hours room temp. unchopped	
14	,,	98·18	Resting	Animals of same litter
15	,,	84·15	Stood 24 hours room temp. chopped	
18 A	,,	88·73	Resting	Halves of same brain
18 B	,,	85·79	Stood 24 hours room temp. unchopped in N_2	
19 A	,,	73·61	Resting	,, ,,
19 B	,,	86·18	Stood 24 hours room temp. chopped in cyanide Ringer p_H 8·4	
17 A	Insulin	35·29	Resting	,, ,,
17 B	,,	21·48	Stood 24 hours room temp. unchopped in N_2	
20 A	,,	20·90	Resting	,, ,,
20 B	,,	24·64	Stood 24 hours room temp. chopped in cyanide Ringer p_H 8·4	

As far as we could discover, the lactic acid content did not rise on incubation or on standing at room temperature. The brains were either allowed to stand unchopped in the air, or chopped in ordinary Ringer solution containing cyanide ($N/65$ and in some cases $N/650$ KCN).

In one case Ringer solution of p_H 8·4 was used in order to favour the formation of lactic acid as far as possible. It was usually assumed that a whole or half-brain standing unchopped in the air would be lacking in oxygen, in which case any lactic acid formed would not be oxidised. With the intention of testing this point two experiments were carried out in which the half-brains not used for "resting" determinations stood for 24 hours in an atmosphere of nitrogen.

In no case was any detectable rise in the lactic acid content of the brain tissue found as a result of standing or incubation, except possibly in the two half-brains (Table III, 19 B, 20 B) which stood chopped in alkaline Ringer solution. Even here, the rise was so slight as to be almost within the limits of error.

It seems more than probable that, during the life of the animal, glucose diffuses into the brain from the blood, and is present in the brain in estimable amounts, but that in the few minutes elapsing between the death of the rabbit and the freezing of the brain, practically the whole of this is metabolised. Some of this glucose would thus appear as "resting" lactic acid.

When the tissue is allowed to stand unchopped, or to stand chopped, in a Ringer solution of about the p_H of the body, the lactic acid content does not rise above the "resting" level. If it stands chopped in a Ringer solution of p_H 8·4, a trace of additional lactic acid seems to be formed. Probably, therefore, a small amount, but only a very small amount, of glucose remains in the brain after the death of the animal, in which case, a trace, at most, of the reducing substance estimated in the brain after death is glucose.

We have, as yet, had no opportunity of studying the fate of the reducing substances when the brain is kept at p_H 8·4, but the conclusion that there is no further formation of lactic acid on standing is supported by the fact that neither a decrease of the reducing substance in the brain tissue on incubation or on standing, nor any hydrolysis of the hydrolysable fraction can be shown to occur. To confirm this an experiment was performed using two halves of one brain for sugar estimations and two halves of another brain for lactic acid estimations (see Table IV). The two rabbits were of one litter, and the experiments were carried out on the same day. One-half of each brain was used as quickly as possible, in this case about 6 minutes after the death of the rabbit; the other half of each brain was, as usual, covered over to prevent drying, and then left unchopped for 24 hours at room temperature.

If, on the other hand, glucose is supplied to the brain tissue, that is to say if the tissue is chopped and placed in Ringer solution of p_H 7·4 containing glucose but no phosphate, considerable amounts of lactic acid are formed (see Table V).

Table IV.

			mg. per 100 g.
Rabbit 9	9 A	Lactic acid. "Resting" value	65·29
	9 B	Lactic acid. Value after 24 hours room temp. ...	62·74
Rabbit 10	10 A	Reducing substance. "Resting" value; unhydrolysed	107·3
	„	Reducing substance. "Resting" value; hydrolysed ...	165·7
	10 B	Reducing substance. Value after 24 hours room temp.; unhydrolysed	105·6
	„	Reducing substance. Value after 24 hours room temp.; hydrolysed	169·8

Reducing substance calculated as glucose.

Table V.

No.		mg. lactic acid per 100 g. brain
8 B	Stood 24 hours room temp. in Ringer (N/65 KCN)	76·60
8 A	Stood 24 hours room temp. in Ringer (N/65 KCN) + 0·25 % glucose	207·70
21 A	Stood 24 hours room temp. in Ringer (N/65 KCN)	62·70
21 B	Stood 24 hours room temp. in Ringer (N/65 KCN) + 0·25 % glucose	293·30

In every case p_H of Ringer = 7·4.

It seemed that some knowledge of the actual reducing substances present would be very useful at this point, but so far the information obtained has been somewhat scanty, and the matter is still under investigation.

Preliminary experiments indicate the presence of a definite amount of pentose in the aqueous solution obtained after alcoholic extraction (and probably also after trichloroacetic acid extraction) of the tissue. The pentose tests are given very strongly by the solution, whereas Van der Haar's [1918] test, said to be specific for glycuronates, is negative. The method described by Tollens [1910] for the estimation of pentoses, has so far been attempted in one case only. The value obtained in this one case was about 20–30 mg. pentose in 100 g. brain tissue. That such an appreciable amount of pentose is present in our solutions is surprising, since the pentose or pentose-containing substance estimated must be soluble in water, and it is difficult to see how any hydrolysis of substances such as nucleic acids could occur during the extraction process. Work bearing on this question is still continuing.

We have so far no information regarding the nature of the hydrolysable substance. Estimations of the inorganic phosphates before and after hydrolysis have been carried out, and seem to show that the hydrolysable substance is not a phosphate compound, since there is no parallel rise of inorganic phosphates on hydrolysis. Again, the numerical relationship of the reducing values before and after hydrolysis (the method of Hagedorn and Jensen being used throughout) is not such as would be found were the hydrolysable substance a disaccharide such as maltose or lactose. By the Hagedorn and Jensen method maltose and lactose are estimated as about 75 % of their weight of glucose—much higher than they are estimated by copper reduction methods.

Actual figures are as follows:

Amounts of maltose and lactose estimated as 75 mg. before hydrolysis are estimated after hydrolysis as 102 and 91·4 mg. respectively.

Reducing substance from brain (alcohol method). Estimated before hydrolysis as 84·7 mg., and after hydrolysis as 144·9 mg.

Thus the rise in the reducing value after hydrolysis of the substance extracted from brain is far larger than the rise in value of maltose and lactose after hydrolysis.

The hydrolysable substance is also extracted by the trichloroacetic acid method, but the formation of chloroform from the trichloroacetic acid on boiling makes hydrolysis of solutions containing much of this acid inconvenient.

Finally, a comparison was made between the "resting" lactic acid values of normal and "insulin" brains, and also between the values obtained after incubation or standing. It will be seen that the "resting" values of the "insulin" brains are much smaller than those of the normal brains (see Table VI). This supports the view that there is a shortage of glucose in the brain of a rabbit which is on the verge of insulin convulsions.

It is, however, always possible that the low values are due to an increased oxidation of lactic acid as a result of the administration of insulin. It is hoped that this question will be decided by a study of the relationship between the level of glucose in the blood and the amount of lactic acid in the brain after death.

Table VI.

No.		mg. lactic acid per 100 g. brain	Remarks
5	Normal	90·16	Resting
9 A	,,	65·29	,,
14	,,	98·18	,,
18 A	,,	88·73	,,
19 A	,,	73·61	,,
19 B	,,	86·18	Stood 24 hours room temp. $N/650$ KCN Ringer p_H 8·4
1	,,	72·74	Stood 24 hours room temp. chopped; cyanide Ringer
2	,,	85·53	Stood 24 hours room temp. unchopped
4	,,	75·78	Stood 3 hours room temp. unchopped
8 B	,,	76·60	Stood 24 hours room temp. chopped in cyanide Ringer
9 B	,,	62·74	Stood 24 hours room temp. unchopped
15	,,	84·15	Incubated 4 hours unchopped
18 B	,,	85·79	Stood 24 hours in N_2 at room temp. unchopped
6	Insulin	38·70	Resting
7	Insulin (C)	28·16	,,
17 A	Insulin	35·29	,,
16	,,	42·70	,,
20 A	,,	20·90	,,
17 B	,,	21·48	Stood 24 hours room temp. unchopped in N_2
20 B	,,	24·64	Stood 24 hours room temp. chopped in cyanide Ringer

Averages

Normal		Insulin	
Resting	Stood or incubated	Resting	Stood or incubated
83·19	78·66	33·15	23·06

"C" = convulsed

It appears from these preliminary experiments that the reducing substance in brain tissue which is estimated by our methods, and probably, also, by the

methods employed by Takahashi and Cori, is not glucose. The brain is therefore mainly dependent on the blood circulating through it for its supply of glucose; and when the blood sugar level has fallen low, the brain may well be supposed to suffer from glucose starvation. The fact that the reducing substance found in the brain is not necessarily abnormally low at the onset of the convulsions is not significant, since this reducing substance is not glucose.

SUMMARY.

1. The amount of reducing substance which can be extracted from the brains of normal rabbits, and from those of rabbits treated with insulin, has been investigated. Two different methods have been used simultaneously for extraction, and reducing substance has been estimated by the method of Hagedorn and Jensen.

2. It is concluded that there is no marked change in the amount of reducing substance as a result of insulin administration. This confirms the findings of Cori and of Takahashi.

3. The reducing substance of brain is not capable of giving rise to the formation of lactic acid, although, in similar conditions, abundance of lactic acid is formed by the brain from added glucose. Further, the reducing substance consists partly of a pentose, and partly of a substance, the reducing power of which is greatly increased after acid hydrolysis. Probably little or none of it is glucose.

4. Determinations of "resting" lactic acid on the brains of normal and of "insulin" rabbits show a greatly reduced lactic acid formation in the latter case. Neither in "normal" nor in "insulin" brains is there an increase in lactic acid formation over the "resting" value after standing or incubation at body p_H.

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