CLII. CONTRIBUTIONS TO THE STUDY OF BRAIN METABOLISM.

III. CARBOHYDRATE METABOLISM RELATIONSHIP OF GLYCOGEN AND LACTIC ACID.

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In the course of some previous experiments dealing with the carbohydrate metabolism of the brain [Holmes and Holmes, 1925, 1, 2], we observed that the lactic acid content of rabbits' brains showed little or no increase when the tissue was chopped and allowed to stand at room temperature, or was incubated, under anaerobic conditions. This appeared to be the case at p_H 7·6, 8·2, 8·4 and 9·0. In similar circumstances, tissues such as muscle or liver might be expected to show a lactic acid production substantially above the "resting" value, with a corresponding diminution in lactic acid precursor. We further observed that there was no diminution in the "soluble carbohydrate" of brain after the organ had been allowed to stand for 24 hours at room temperature. Since brain tissue readily forms lactic acid from added glucose in these conditions, we deduced that the substances estimated in the extracts did not include glucose. We were subsequently able to show [1926] that the reducing substances which we estimated were largely, if not wholly, creatine and creatinine.

While our experiments were still in progress, Takahashi and Asher [1925] published a paper dealing with the glycogen and "übrigen Kohlenhydrate" content of various tissues, including brain. Since their technique for "soluble carbohydrate" was essentially similar to ours, we have no doubt that the substances (in the brain) which they estimated under that heading were chiefly creatine and creatinine. They found that the glycogen content of the brain, unlike that of other tissues, was not influenced by the treatment to which they subjected their animals, unless the treatment was such as to produce convulsions, in which case the glycogen content diminished. This point is referred to later in this paper. It may here be remarked that, while Takahashi and Asher claim that insulin convulsions markedly reduce the brain glycogen, we found that some of our lowest figures for brain lactic acid were obtained from animals which had convulsed as a result of insulin injection [1925, 2], which is hardly in harmony with their findings.

In view of the criticism which has recently been directed against the application of Pflüger's method for the determination of glycogen to small quantities of tissue [Evans, 1925], we feel it desirable to describe our technique in detail.

As pointed out by Winterstein and Hirschberg [1925], Pflüger's method cannot be applied safely to fresh brain tissue, because the cerebrosides will, partially at least, escape destruction by the potash, and will therefore give rise to galactose in the final hydrolysis and thus vitiate the subsequent estimation of reducing substance. In our experiments the cerebrosides were therefore removed, as suggested by Winterstein, by extraction with alcohol and chloroform. In detail, therefore, our procedure was as follows. The rabbit was killed by a blow on the back of the neck. The brain was removed, freed from membranes and adherent blood clot, and either frozen at once (for "resting" values), or subjected to any desired treatment. The frozen brains were sampled, weighed, and immersed (while still very cold) in 95 % alcohol. To brains that had been in Ringer's solution, three volumes of alcohol were added, and the whole allowed to stand overnight. The tissue, and any precipitate present, was then filtered off through a Jena glass filter (mesh "5–7" or "< 7" was found suitable). If a lactic acid estimation was required, the necessary further extractions were then carried out, the tissue and alcoholic extract being separated by means of the filter; otherwise, the fluid was discarded. The tissue was transferred to a beaker and boiled five times with alcohol and twice with chloroform, being filtered after each extraction through the same filter. The inside of the beaker was carefully scraped and the scrapings transferred to the filter with a little hot chloroform. Finally, the solid was washed twice in the filter with hot alcohol. This treatment removed all substances soluble in hot alcohol or in chloroform.

The powdery mass was next transferred to a 300 cc. Kjeldahl flask. 10 cc. of 60 % potash (Kahlbaum), with which beaker and filter had been rinsed, were added. A condenser was fitted, and the flask heated for 3 hours over a microburner, so adjusted that the potash was a little below its boiling point.

After 3 hours, the contents of the flask were transferred to a 100 cc. beaker: 20 cc. of water, 60 cc. of 95 % alcohol, and a pinch of sodium chloride were added, and the beaker was left overnight. Next day, the precipitate was centrifuged down, washed, taken up in boiling water, filtered, and hydrolysed according to Pflüger's method. The reducing substance was estimated by the method of Hagedorn and Jensen [1923]. Usually 5 cc. of fluid gave convenient readings.

In view of the criticism of Evans [1925] we carried out some experiments to determine whether small quantities of glycogen were precipitated from the comparatively large bulk of fluid (90 cc.) which we used. To a mixture of 10 cc. of 60 % potash, 20 cc. of water, 60 cc. of 95 % alcohol, and a little sodium chloride, known amounts of Kahlbaum's glycogen were added. The subsequent procedure was as described above. Table I shows the results of these experiments.

Table I.

Glycogen added (mg.)	4	3	2	1
„ recovered (mg.)	3·91	3·01	2·15	1·20

As an additional control, a rabbit was killed, and the brain frozen and sampled. Two portions of 4 g. each were weighed out, and glycogen was estimated in each portion, 0·6 mg. of Kahlbaum's glycogen being added to portion A just before the digestion with potash. The figures obtained were as follows:

Portion A	0·882 mg. glycogen
Portion B	0·308 mg. „
Glycogen recovered ...	0·574 mg.
Glycogen added	0·600 mg.

To test out the method, and to endeavour to determine the range of individual variation likely to be met with in normal rabbits, a series of duplicate estimations was carried out on the brains of stock animals. Table II shows "resting" values expressed as mg. of glycogen per 100 g. of fresh brain.

Table II.

Glycogen (mg. %)	A	28·6	41·1	74·8	15·0	14·2	19·6	28·1	43·3
	B	28·9	39·8	70·4	16·9	15·0	15·5	28·7	44·6

It is evident from these figures that, whilst the glycogen content of mammalian brain is very small (a fact noted by previous workers), the variations among normal rabbits, chosen at random from stock, are so large that one cannot regard as valid any conclusions that are based on differences observed between different animals subjected to varying treatment. We were, therefore, compelled to conduct all our experiments on different portions of the same brain.

Table III.

			Resting	Stood or incubated	
No.	Description	p_H	Glycogen mg. %		Remarks
1	Rabbit, normal	9·0	16·22	13·8	Incubated 3 hours at 37°, KCN *M*/500
2	Rabbit, normal	9·0	16·01	12·3	Incubated 3 hours at 37°, KCN *M*/500
3	Rabbit, normal	8·4	28·38	24·38	Stood 24 hours at room temperature, KCN *M*/1000
4	Rabbit, normal	8·2	24·73	22·0	Stood 24 hours at room temperature, KCN *M*/500
5	Rabbit, normal	8·2	13·33	14·92	Incubated 3 hours in nitrogen, no KCN
6	Cat, depancreatised	7·8	43·5	34·7	Stood 24 hours at room temperature, KCN *M*/1000
7	Cat, normal	7·8	26·5	31·1	Stood 24 hours at room temperature, KCN *M*/1000
8	Cat, depancreatised	7·8	31·5	26·0	Stood 24 hours at room temperature, KCN *M*/500
9	Cat, normal	7·8	33·7	25·2	Stood 24 hours at room temperature, KCN *M*/500

In Table III are shown the figures obtained by determining the "resting" value for one-half of a brain, and estimating the other half after it had stood in phosphate Ringer's solution at room temperature, or been incubated under anaerobic conditions.

A few values are included which were obtained in the course of some other work on normal and depancreatised cats.

In Exps. 1 and 2, the "resting" value was obtained from tissue which was frozen and kept in cyanide Ringer's solution at − 2°, while the companion half was kept for the same period in the incubator. This procedure was planned to eliminate any error that might be due to small amounts of glycogen which had dissolved in the Ringer's solution and stuck to the sides of the beaker after precipitation, thus introducing a source of error into the estimation of the incubated half which was absent from that of the "resting" half. Apparently the precaution was unnecessary. In Exp. 5 the tissue was placed, with 10 cc. of Ringer's solution, in a vacuum tube. The tube was evacuated, filled with O_2-free nitrogen, re-evacuated, and again filled with nitrogen. It was thought that by this means all possibility of the conditions being incompletely anaerobic would be avoided.

Table IV shows some "resting" and maximum values obtained for brain lactic acid by Meyerhof's method [1920, 1]. Except in the case of No. 5—which is the same experiment as No. 5 in Table III—the maximum values were all obtained by allowing the tissue to stand, chopped, for 24 hours at room temperature in cyanide-phosphate Ringer's solution. The figures for Exps. 1–4 were, as a matter of fact, obtained before any glycogen estimations were attempted.

Table IV.

Lactic acid in mg. per 100 g. moist brain

No.	Resting	Stood or incubated	p_H	Remarks
1	73·61	86·18	8·4	Stood 24 hours at room temperature in 10 cc. Ringer, KCN $M/1000$
2	116	107·6	9·0	Stood 24 hours at room temperature in 10 cc. Ringer, KCN $M/1000$
3	100·2	106·5	9·0	Stood 24 hours at room temperature in 10 cc. Ringer, KCN $M/1000$
4	124	123	9·0	Stood 24 hours at room temperature in 10 cc. Ringer, KCN $M/1000$
5	123·3	129·5	8·2	Incubated 3 hours at 37° in Ringer in N_2

In most cases, though not in all—and No. 5 is one of the two exceptions—there is a fall in the glycogen on standing. This fall is extremely small in actual amount, though slightly more imposing when calculated as a percentage, owing to the very low "resting" value. Three of the lactic acid values show a slight rise, two a slight fall.

In this respect the metabolism of the brain is evidently quite different from that of muscle or liver. In the case of the lactic acid values the rise is not constant, and, when it occurs, is so small as to approach the limits of experi-

mental error. Though the fall in glycogen seems to be of more regular occurrence, it too is very small. We are, indeed, disposed to regard it with very great reserve, believing that it is possibly due to some hitherto undiscovered flaw in the technique. It may be mentioned here that one of us (B. E. H.) has observed that there is, apparently, but very little fall in the glycogen content of tumour tissue in circumstances similar to those here described.

We carried out a series of experiments to observe the effect on the brain glycogen of incubated chopped brain tissue under aerobic conditions. Meyerhof [1920, 2] has demonstrated the synthesis of glycogen and the simultaneous disappearance of lactic acid in whole amphibian muscle under aerobic conditions. Naturally, it would be unsafe to assume that such a synthesis would occur in our chopped brain preparations, even supposing that it took place in the brain of the living animal, since Meyerhof found it necessary to employ intact muscle, and other workers to employ carefully sliced pieces of tissue.

Winterstein and Hirschberg [1925], however, reported a fall in the glycogen content of frogs' central nervous system when the tissue was kept, unstimulated, in oxygenated sodium chloride solution. Whilst under anaerobic conditions the glycogen content remained unchanged, he observed it to rise when the central nervous system was electrically stimulated in circumstances ensuring an adequate oxygen supply. Our values are arranged in Table V. The brains were halved and the "resting" value was determined on one half, whilst the other was chopped, placed in Ringer's solution in a wide, closed bottle, and shaken in a water-bath at 37° sufficiently vigorously to ensure thorough aeration. The fall in glycogen content observed is not materially greater than that seen under anaerobic conditions.

Table V.

		Glycogen (mg. %)		
			Shaken	
No.	p_H	Resting	3 hours	Description
1	8·0	40·55	33·8	Rabbit
2	7·8	31·7	28·4	Rabbit
3	7·8	43·5	33·1	Cat (depancreatised)
4	7·8	31·5	36·5	Cat (depancreatised)

A few experiments were done to demonstrate the fall in lactic acid, which, under aerobic conditions, occurs readily in a preparation of chopped brain.

Table VI.

	Lactic acid (mg. %)		
No.	Resting	Final	Remarks
1	91·3	15·4	Rabbit, normal. Shaken, as in Table V
2	97·4	36·5	Rabbit, normal. O_2 bubbled through Ringer at 37°
3	104	41	Cat, normal. Shaken, as in Table V

Provided that all cerebrosides are previously extracted from the tissue, the use of Pflüger's method in itself offers some guarantee that the material estimated really is glycogen. In addition, Takahashi [1925], using a different

method, claims to have isolated glycogen from his extracts. We felt, however, that it would be advisable to attempt an identification of our material.

We accumulated, therefore, all the hydrolysed residues left over from the rabbit brain estimations. Finally, about 1300 cc. of fluid were collected, containing some 40 mg. of reducing substance, reckoned as glucose. This was neutralised, concentrated, and treated in the ordinary way with phenylhydrazine. Crystals were obtained having the appearance, under the microscope, of those of glucosazone. Unfortunately they were lost during an attempt at recrystallisation, so we have no data as to melting-point. More material is being accumulated.

Athanasiu [1899] observed glycogen in the central nervous system of the frog, and gave a value of 70 mg. %. Schörndorff [1905] estimated glycogen in dog's brain, finding values of from 190–260 mg. %. From the text it is clear that part of his reducing substance arose from the hydrolysis of cerebrosides. Takahashi [1925] and Takahashi and Asher [1925] report data relating to rat, rabbit, and human brains. They find that there is no fall in brain glycogen after insulin administration (provided convulsions are avoided), peptone feeding, heavy muscular work, thyroid or phloridzin administration, although they find marked diminution of glycogen in other organs in these circumstances. If, however, convulsions have occurred, they believe that the brain glycogen does diminish. We are unable to accept their conclusions for rabbits, because they report only a few experiments, and we believe that the differences which they find might easily be covered by the very wide range of normal variation. Uchida [1925] describes experiments showing a fall in glycogen content of the brains of rats which have been exposed to the action of anaesthetics, as compared with those of normal animals. He uses a method based on a technique used by Rona and Von Eweyk [1924] for isolating pure glycogen from tissues, which, apparently, was not intended to be quantitative. The glycogen values which we have obtained for anaesthetised rabbits fall well within the very wide range of normal values; possibly rats' brains show a less variable glycogen content. Kobori [1926] states that the glycogen content of doves' brains rises markedly after convulsions produced by methylguanidine. He gives some figures which, he claims, support Takahashi's contention that the brain glycogen is diminished by insulin convulsions. Again, so far as rabbits are concerned, the figures fall almost within the range of variation observed by us in normals.

We realised that it is possible that the low values which we, in common with other workers, find for the glycogen content of the brain, may be due, not to a slow or inadequate breakdown mechanism, but to an extremely rapid one. It is conceivable that by no technique hitherto described is it possible to obtain a true "resting" value, since the methods employed for killing animals all involve either the use of an anaesthetic, or a violent, if momentary, stimulus to the central nervous system, which might result in a large glycogen breakdown before the tissue can be fixed. Such an explanation

would not, however, account for the absence of further significant breakdown *in vitro* at alkaline p_H, to explain which it would be necessary to postulate some further mechanism which was operative only in the living animal. We are, moreover, endeavouring to employ other methods of obtaining a true "resting" figure for glycogen, and, although our experiments are incomplete, we have so far failed to find any indication of a higher figure.

In special circumstances, we have found a slight increase above the "resting" value in the lactic acid figure for rabbits' and cats' brains under anaerobic conditions. Gesell [1925] reports a similar rise in the figure for dogs' brains. In our experience, this has never been accompanied by a corresponding fall in glycogen.

Loebel [1925] was unable to demonstrate increased lactic acid production when glycogen was added to brain preparations. This observation, perhaps, lacks very much significance from our point of view, since glycogen added to tissue preparations frequently fails to behave in a manner comparable to that of the glycogen already contained in the tissue, on account, presumably, of its physical properties.

We can find no evidence that glycogen behaves as a lactic acid precursor in the mammalian brain. It has been shown that brain tissue forms lactic acid with great rapidity from glucose [Warburg, Posener and Negelein, 1924; Holmes and Holmes, 1925, 1], and we were also able to show [1925, 2] that, after insulin, the lactic acid content of rabbits' brains runs, roughly, parallel to the blood sugar at the time of death. It seems to us, therefore, that it is quite possible that glycogen plays a comparatively unimportant (or at least quite obscure) part in the carbohydrate metabolism of mammalian brain, and that the organ is dependent for lactic acid precursor directly on the glucose supplied to it by the blood.

Summary.

1. The glycogen content of rabbits' brains is small, and very variable.

2. The lactic acid content of rabbits' brains shows no appreciable rise, nor does the glycogen content show any significant fall, when the chopped tissue is kept at room temperature, or incubated under anaerobic conditions at alkaline p_H.

3. Under aerobic conditions, lactic acid rapidly disappears from chopped brain, but the glycogen suffers no significant change.

4. It is suggested that the brain depends upon the blood sugar, rather than on any substance which it stores itself, for lactic acid precursor.

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