LXXXIV. STUDIES ON NERVE METABOLISM.

IV. CARBOHYDRATE METABOLISM OF RESTING MAMMALIAN NERVE.

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In previous papers of this series [Gerard, 1927; Gerard and Meyerhof, 1927], the respiration and lactic acid production of isolated frog nerves, at rest and active, have been considered. An attempt to follow by direct analysis the corresponding changes in carbohydrates was not successful because the methods used were inadequate for the treatment of the small amounts of tissue available. In the present experiments we have had recourse to the much larger nerves obtainable from rabbits. The results are directly comparable to similar studies for mammalian brain at 37° but cannot be accurately compared with those available for amphibian nerve at 15°. On the other hand determinations of oxygen consumption and lactic acid formation for both have helped to bridge the gap.

The present studies are limited to determinations of lactic acid, glycogen and "free carbohydrate" as affected by rest in oxygen and nitrogen.

Methods.

Lactic acid was estimated by two methods; in some experiments the method of Meyerhof [1920] was used, in which the alcoholic extracts of the tissue are deproteinised, and extracted with amyl alcohol; in others, the alcoholic extracts were treated with Schenk's reagent and copper sulphate and calcium hydroxide.

The results obtained by these two methods agree very well. In both cases the final estimation was performed by distillation (without aeration) from the manganous sulphate-sulphuric acid reagent of Friedemann, Cotonio, and Shaffer [1927]. 0·01 *N* permanganate was used, and the "bound" bisulphite liberated with sodium bicarbonate, and titrated with 0·005 *N* iodine. All such estimations were performed in duplicate.

The estimation of "free carbohydrate," *i.e.* carbohydrates other than glycogen, presented considerable difficulty; the amount of tissue was small and the concentration of carbohydrate low, consequently the error due

to non-carbohydrate reducing substances was so large as to invalidate completely any experiments in which it was ignored.

The use of a technique based on the method of Bissinger [1926] offered a way out of the difficulty. The tissue was fixed in iced alcohol, chopped, and ground with sand, and water added to bring the alcohol concentration to 60 %. Next day the alcohol was filtered off through a Jena glass filter (mesh 5–7) and extracted three times with 60 % alcohol. The combined extracts were slowly evaporated on a water-bath; the subsequent procedure depended upon the method chosen for lactic acid estimation. If the amyl alcohol method was contemplated, the extracts were made up to volume (20 cc.) and halved; both halves were evaporated, one being used for lactic acid estimation, the other for carbohydrate: if lactic acid was to be estimated by the Schenk method, no halving of the extract was necessary. The residue was treated with 4 cc. of Schenk's reagent, filtered through asbestos, and the beaker and precipitate were washed with three 1 cc. portions of Schenk's reagent. Hg was removed by H_2S, and H_2S by aeration, washed filter-papers being employed for filtration. (Unwashed filter-paper yields enough reducing substance to introduce an error.)

The carbohydrate was then extracted from the solution as follows.

To the solution (volume about 10 cc.) in a centrifuge tube, was added 1/6 volume of 10 % $CuSO_4$. A few drops of strong sodium hydroxide were sufficient to neutralise the HCl present (shown by commencing precipitation of $Cu(OH)_2$. The tube was placed in ice, and 1/3 volume of 10 % suspension of $Ca(OH)_2$ was added. The tube was left in ice, with occasional shaking, for 30 mins. It was then centrifuged. The supernatant fluid was used, if required, for lactic acid estimation; in that case, both its volume and that of the total contents of the tube were accurately measured. The precipitate was treated with 10 cc. *N* H_2SO_4, then with H_2S, the CuS and $CaSO_4$ were centrifuged, and washed three times with water. After aeration of the fluid and washings, solid Na_2CO_3 was added, until the remaining calcium had been precipitated. The fluid was quickly filtered through a washed filter-paper into a 50 cc. volumetric flask, containing 1 cc. conc. HCl. The precipitate was washed with water, and the contents of the flask made up to volume. 15 cc. portions of the fluid were pipetted out, neutralised, and the reducing substance was estimated by the method of Hagedorn and Jensen [1923].

It is not claimed that this procedure is completely satisfactory. When tested on glucose solutions losses sometimes occur, for which no adequate explanation has been found, and which may amount to 10 % of the amount of glucose added (Table I). No more perfect procedure has, however, been elaborated, and the copper-lime precipitation does afford some assurance of the separation of sugars from the other non-carbohydrate reducing substances in tissue extracts.

Glucose can be recovered by this technique from admixture with creatine and creatinine. Embden and Zimmermann [1924] have shown that hexose-

Table I. *Recovery of glucose (from aqueous solution).*

Glucose added mg.	Glucose recovered mg.	Difference mg.	Error %
1·111	1·048	−0·063	− 5·7
0·666	0·694	+0·017	+ 2·6
0·444	0·420	−0·02	− 4·5
0·192	0·204	+0·012	+ 6·2
0·383	0·350	−0·033	− 8·1
0·575	0·518	−0·057	− 9·9
0·767	0·689	−0·078	−10·2
0·353	0·436	+0·083	+23·0
0·865	0·879	+0·014	+ 1·6
0·240	0·249	+0·009	+ 0·4
0·481	0·428	−0·053	−11·0
0·721	0·719	−0·002	− 0·3

Numerical average error 7 % Algebraic average error −2·3 %

phosphate is carried down on the precipitate, and would therefore be included in our carbohydrate fraction. Apparently creatinine-phosphoric acid is precipitated, even though the free base is not. If creatine-phosphoric acid is present in nerve, it is improbable that it would survive as such until the copper-lime precipitation is carried out, since the "free sugar" fraction always stands for several hours, often overnight, in the acid Schenk reagent. In addition, no creatinine can be detected by the Jaffé or Weyl tests in the final fraction which is used for sugar estimations. (These tests would not show the presence of creatine, but the reducing power of that substance is low.) Finally, the curve obtained by plotting reducing value observed against quantity of fluid taken is linear; in the case of interference by creatinine, the curve falls away as the amount taken for estimation is increased [Holmes and Holmes, 1926]. We therefore feel confident that the values we observed are not distorted by contamination with creatinine.

Glycogen was estimated at first by the modified Pflüger method previously described by one of us [1926]. This was found to be unsatisfactory, and the "copper-lime" precipitation was applied to the glycogen hydrolysates, with satisfactory results, so far as the actual estimation was concerned. Another difficulty, however, arose. We had been accustomed to extract our tissues with 60 % alcohol, to estimate the free sugar and lactic acid in the extract, and to use the extracted tissue for glycogen estimations. Dr P. Eggleton, however, informed us that he had found that glycogen was appreciably soluble in alcohol of less strength than 80 %. This clearly invalidated our glycogen estimations. We take this opportunity of thanking Dr Eggleton for thus generously placing unpublished results at our disposal. By extracting the tissue with absolute alcohol, and estimating glycogen and lactic acid, or with 60 % alcohol, and estimating free carbohydrate and lactic acid, we were able to surmount the difficulty. We were very relieved to find that, although we had been losing at least half of the glycogen in our original experiments, our practice (recommended by Meyerhof) of acidifying the 60 % alcoholic extract had resulted in its subsequent hydrolysis during the rather prolonged process of evaporation, so that it reappeared as "free carbohydrate." Thus,

though our estimations gave an erroneous picture of the distribution of carbohydrate, they presented a reliable one of its total amount—the most important thing from our point of view. Tables II, II*a* and III and III*a* illustrate the point. Needless to say, in our subsequent "free carbohydrate" estimations, we did not acidify the alcoholic extracts.

The technique of a complete experiment was as follows: a rabbit was killed by a blow on the head, the lower part of the body and hind limbs were skinned as quickly as possible and the sciatic nerves dissected out and placed in a dish standing on ice. One to three other animals were treated successively in the same way, the nerves being so arranged that one dish contained the right sciatic nerve of the first, the left of the second, and the right of the third, and so on.

The nerves were well exposed before being handled, and were then held at the distal end with forceps and removed, as far as possible, without being stretched or pulled. One set ("initial") was weighed and at once put into chilled alcohol. The other set was then weighed and placed in a vacuum tube, having a turned-over, hollow stopper containing moist cotton wool (to obviate drying of the nerve). The tube was evacuated and filled with nitrogen[1], evacuation and refilling being repeated three or four times. It was then placed in an incubator at 37° for 1 to 3 hours, or for 1 hour and then left at room temperature over night, or simply left at room temperature. Finally, the tube was cooled, and the nerves were placed in chilled alcohol as already described.

The question arises as to how far these nerves, cooled during dissection, can be regarded as being in a normal condition during the remainder of the experiment. We have tested nerves treated in this manner by their action currents and find that the response remains undiminished for some hours. Also, experiments (to be reported later) have shown that the oxygen consumption of rabbit nerve is not diminished by a preliminary period at 0°; and we believe the findings here reported may therefore be accepted as normal for the resting isolated nerve.

The results of experiments on anaerobic survival of nerve are given in Tables II and II*a*. In Table II, the experiments are those in which the glycogen values are too low, the loss being compensated for by a corresponding increase in the "free carbohydrate." The two values are therefore taken together as "total carbohydrate." In Table II *a* the values for the two carbohydrate reactions are, we believe, correct. It will be seen that the average values for the two tables agree remarkably well. In Table II the lactic acid was estimated by the amyl alcohol method; in Table II *a* by the Schenk technique, except in the case of experiments (1) and (2).

It is plain that lactic acid is produced anaerobically by peripheral nerve at the expense of carbohydrate stored in the tissue. The agreement between the rise in lactic acid and the fall in total carbohydrate is sufficiently good

[1] Purified with red-hot copper filings, and stored over pyrogallol.

Table II. *Rest in nitrogen.*

All values in mg. per 100 g.

	Initial			Final			Differences			
Exp.	Total carbohydrate	Lactic acid	Total	Total carbohydrate	Lactic acid	Total	Lactic acid	Total carbohydrate	Conditions All in nitrogen	Lactic acid method
1	100·6	63·0	163·6	44·1	89·0	133·1	+26·0	−56·5	37°, 1¼ hrs. Room temp. 22 hrs.	[Meyerhof, 1920]
2	83·6	63·7	147·3	68·8	105·4	174·2	+41·7	−14·8	37°, 3 hrs.	,,
3	107·6	101·0	208·6	66·6	134·0	200·6	+33·0	−41·0	37°, 1¾ hrs. Room temp. 20 hrs.	,,
4	85·5	77·5	165·0	57·1	103·8	160·9	+26·3	−28·4	37°, 2½ hrs.	,,
5	109·6	72·3	181·9	71·3	114·2	185·5	+41·9	−38·3	37°, 1½ hrs.	,,
6	104·8	82·4	187·2	50·6	126·6	177·2	+44·2	−54·2	37°, 1¼ hrs.	,,
Av.	98·9	76·6	175·6	59·7	112·1	171·9	+35·5	−39·2		

In Exp. 6, animal was pithed, artificial respiration used, and legs cooled to below 10°, before nerves were dissected.

Friedemann and Kendall [1929] state that they obtain a yield of 95–98 % of lactic acid, using their manganous sulphate reagent, and aeration. In our own experiments we have obtained consistent yields of 91·5 % (without aeration). If the figures in Tables II and II *a* are corrected on this basis, the rise in lactic acid becomes 38·5 mg. per 100 g. and 35·0 mg. per 100 g. respectively, figures very close indeed to the observed fall in total carbohydrate. We have preferred to give the values actually observed, in the tables.

Table II *a*. *Rest in nitrogen.*

All values in mg. per 100 g.

	Initial			Final			Rise or fall				
Exp.	Glycogen	Free sugar	Lactic acid	Glycogen	Free sugar	Lactic acid	Glycogen Fall	Free sugar Fall	Lactic acid Rise	Conditions	Lactic acid method
1	57	—	45	47	—	58	10	—	13	2 hrs. in N_2 at 37°	[Meyerhof, 1920]
2	67	—	87	54	—	116	13	—	30	,,	,,
3	53	—	67	40	—	110	13	—	43	,,	Schenk
4	—	—	68	45	—	101	—	—	33	,,	,,
5	—	37	101	—	17	116	—	20	15	,,	,,
6	—	26	70	—	12	101	—	14	31	,,	,,
7	—	45	76	—	24	119	—	21	43	,,	,,
8	—	61	88	—	23	140	—	38	56	,,	,,
Av.	59	42	75·2	46·5	19	107·5	12·5	23	32·3		
	Total 176·2			Total 173·0							

Average total carbohydrate: Initial 101 Final 65·5

to make it extremely probable that there is no lactic acid precursor concerned other than glycogen and free carbohydrate; of these "free carbohydrate" contributes about twice as much as does glycogen.

In striking contrast to these results are those obtained when the tissue is kept, not in nitrogen, but in oxygen (Tables III and III *a*). In Table III, the glycogen and free carbohydrate are added together and given as "total carbohydrate" since their distribution was not accurately shown. In Table III *a*, the figures give the true relationship between glycogen and "free carbohydrate."

From these two tables and from Table IV, it is clear that the formation of lactic acid, so evident in nitrogen, is entirely suppressed in oxygen, and further, that the oxidative removal of lactic acid does not occur in nerve. To test this point more rigidly, in certain experiments, which appear in Table IV, nerves were kept for varying periods in nitrogen, and were then exposed to oxygen, in the hope that the accumulation of lactic acid might be oxidised away, even if the ordinary "initial" content were not. The results were, however, uniformly negative. The apparent drop in lactic acid in Table III is due to the comparatively small number of experiments, and the random errors of the method. It disappears when a larger group of values is considered, as in Tables III *a* and IV.

Although there is no oxidative removal of lactic acid, there is a very definite fall in carbohydrate, which affects only the "free carbohydrate" fraction (Table III *a*). There is, if anything, a slight rise in glycogen. The contrast between the events which occur in oxygen and in nitrogen are well illustrated by the following figures, which were obtained by dividing the nerves from 6 rabbits each into three portions, which were then distributed to give the best sampling. One set was kept for 1 hour in nitrogen, one for 1 hour in oxygen, and one set was worked up immediately.

Values in mg. per 100 g.

	Initial	Final in N_2	Final in O_2
Total carbohydrate	109·6	71·3	92·6
Lactic acid	72·3	114·2	74·8
Total	181·9	185·5	167·7

We have not been able to gauge successfully the precise time relationships of these events. To construct a curve would necessitate the use of 8 or 10 rabbits, and the time taken in dissection would become unduly long: moreover, it would be necessary, for purposes of sampling, to divide each nerve into four or five pieces, and it is more than likely that the mechanisms concerned would suffer in the process. The tables, however, suggest that the processes taking place in oxygen are more or less complete by the end of the first hour at 37°—the values in Table III are as large as those in Table III *a*, and the same statement applies to those changes which occur in nitrogen.

Table III. *Rest in oxygen.*

Values in mg. per 100 g.

	Initial			Final			Differences				
Exp.	Total carbo-hydrate	Lactic acid	Total	Total carbo-hydrate	Lactic acid	Total	Total carbo-hydrate	Lactic acid	Total	Treatment	Lactic acid method
1	90·5	90·3	180·8	74·1	78·5	152·6	−16·4	−11·8	−28·2	37°, 3½ hrs.	[Meyerhof, 1920]
2	102·0	78·0	180·0	85·5	74·0	159·5	−16·5	− 4·0	−20·5	37°, 1 hr.	„
3	117·5	96·0	213·5	87·6	78·0	165·6	−29·0	−18·0	−47·9	37°, 1 hr.	„
4	109·6	72·3	181·9	92·6	74·8	167·4	−17·0	+ 2·5	−14·5	37°, 1 hr.	„
5*	100·9	84·7	185·6	63·2	90·5	153·7	−37·7	+ 5·8	−31·9	37°, 1 hr.	„
Av.	104·1	84·2	188·3	80·6	79·1	159·7	−23·5	− 5·1	−28·6		

* Nerves from 5 decerebrated cats, 3·5 g. of tissue available for each estimation.

Table III *a*. *Rest in oxygen.*

All values in mg. per 100 g.

	Initial			Final			Differences				
Exp.	Glycogen	Free carbohydrate	Lactic acid	Glycogen	Free carbohydrate	Lactic acid	Glycogen	Free carbohydrate	Lactic acid	Treatment	Lactic acid method
1	—	29	98	—	19	91	—	−10	− 7	2 hrs. at 37° in O_2	Schenk
2	—	45	108	—	25	109	—	−20	+ 1	,,	,,
3	—	72	104	—	24	99	—	−48	− 5	,,	,,
4	—	45	113	—	21	98	—	−24	−15	,,	,,
5	50	—	65	54	—	77	+4	—	+12	,,	,,
6	55	—	57	57	—	62	+2	—	+ 5	,,	,,
7	55	—	63	57	—	65	+2	—	+ 2	,,	,,
Av.	53·3	47·7	86·8	56	22·2	85	+2·6	−25·5	− 1·1		
	Total 187·8			Total 163·2							

Table IV.

Lactic acid (mg. per 100 g. fresh nerve)		
Initial (A)	Final (B)	Treatment
104·2	95·1	Both 1½ hrs. at 37° in N_2 (B) Next 2 hrs. in air at 37°
80·1	102·1	Both, 1 hr. at 37° in N_2 (B) Next 1½ hrs. at 37° in O_2
90·3	78·5	(A) "Resting" (B) 3½ hrs. in O_2 at 37°
36·9	37·7	(A) "Resting" (B) 3 hrs. in O_2 at 37°
87·9	104·4	(A) "Resting" (B) 1 hr. in O_2 at 37°
78·0	74·0	(A) "Resting" (B) 1 hr. in O_2 at 37°
96·0	78·0	(A) "Resting" (B) 1 hr. in O_2 at 37°
72·3	74·8	(A) "Resting" (B) 1 hr. in O_2 at 37°
Average 77·3	78·5	

The question naturally arises as to the precise nature of the "free carbohydrate" estimated. Since the fall in free carbohydrate agrees well with the rise in lactic acid, it seems likely that, in so far as it gives rise to lactic acid, it is really hexose. Clearly, however, the possibility that it is hexose-phosphate must be considered. The reducing power of a molecule of hexose-monophosphate is about 2/3 of that of a molecule of glucose, and that of hexosediphosphate about 1/3[1]. If, therefore, either mono- or diphosphate breaks down to give rise to lactic acid and free phosphoric acid, more lactic acid should appear than can be accounted for by the apparent fall in "free carbohydrate," and there should be a loss of combined phosphate from the free carbohydrate fraction. If, however, as suggested by Meyerhof [1926] two molecules of monophosphate give rise to two molecules of lactic acid and one of diphosphate, then (*a*) one molecule of glucose will appear to have given

[1] Eggleton: personal communication.

rise to an equivalent weight of lactic acid, since the reducing value of 2 mols. of monophosphate = 4/3 mol. of glucose and the reducing value of 1 mol. of diphosphate = 1/3 mol. of glucose (4/3 − 1/3 = 1); and (*b*) there will be no change in combined phosphate.

Table V.

Total P in mg. per 100 g. of fresh nerve.

Exp.	Initial	Final
1	9·6	9·9
2	7·7	10·3
3	7·2	8·4

Table V gives the values obtained in three experiments, where the total phosphate was estimated in the carbohydrate fraction before and after incubation for 2 hours at 37° in nitrogen. In all three experiments the material obtained by the Bissinger precipitation was incinerated with H_2SO_4 and "perhydrol." The phosphorus was estimated in the first two experiments by the Briggs method, in the third by Pregl's micro-method, for the performance of which we have to thank Mr A. Colwell. So far from there being a loss of organic phosphorus, there is actually a rise. In the absence of further data, it is hardly profitable to discuss the possible origin of this increase, but we feel that we are justified in concluding that the lactic acid arises either directly from glucose, or, if from hexosephosphate, then along lines such as those indicated by Meyerhof, and not by any process involving loss of organically combined phosphate.

It is clear that, in any case, the "free sugar" fraction must contain substances other than glucose, since (1) organically combined phosphorus is present, (2) a positive orcinol test (for pentose) can be obtained, and (3) it is capable of yielding lactic acid only to a limited extent (Table II *a*), though in frog nerve [Gerard and Meyerhof, 1927] lactic acid seems to be formed as long as glucose is present. It is of some interest to compare the data presented in this paper with those available from other sources. Calculating from the figures obtained for frog nerve at 15° [Gerard and Meyerhof, 1927], and assuming a temperature coefficient of 2·5, we obtain a value for lactic acid formation by frog nerve at 37° of 35 mg. per hour. Rabbit nerve forms about 36 mg. in two hours, and there is reason to suppose that most of that formation is complete within the first hour.

Gerard and Gruskin (unpublished) find an oxygen consumption for rabbit nerve at 37° of about 280 mm.^{3} per g. nerve per hour, and this has recently been confirmed bv Dr M. Sherif (unpublished). The oxygen equivalent of the sugar disappearing during rest in oxygen, 0·23 mg. per g. per hour (supposing for the moment such sugar to be burned) is 192 mm.^{3} per g. per hour, representing about 68 % of the total oxygen uptake of the nerve at rest in oxygen. Comparing the results here obtained for nerve with those for brain, it is clear that the rate of metabolism in the two tissues is widely different, apart from

the fact that brain oxidises lactic acid freely, while peripheral nerve does not do so at all. Values for oxygen consumption of brain admittedly present great difficulty. Most of those available refer to anaesthetised brain, and are therefore only of very limited value for comparison; the only determinations on unanaesthetised brain of which we are aware are those of Alexander and Cserna [1912, 1913]; they are so enormous that they can hardly be accepted without confirmation. For anaesthetised dog's brain Alexander and Cserna give 144–36 mm.^{3} per g. per min.; Hou [1926], and Hou and Sigiura [1926] 60 mm.^{3}. Schmidt's [1928] figures approximate to 75 mm.^{3} for amytal anaesthesia. The figure for nerve is about 4·6 mm.^{3} per g. per min., and the nerve is not anaesthetised; the contrast is sufficiently striking. Similarly, the rate of lactic acid formation is much greater in brain than in nerve [Holmes and Holmes, 1925; McGinty and Gesell, 1925].

Results on stimulated nerve, obtained by the two authors independently, and using rather different technical procedures, indicate that the carbohydrate changes discussed in this paper bear no part whatever in the extra metabolism of stimulation. In this connection, it is noteworthy that the carbohydrate disappearance in oxygen appears to be complete in two hours; but a nerve, kept in oxygen, will continue to give an electric response to stimulation for at least 4 or 5 hours.

Summary.

1. A modification of the Van Slyke-Bissinger method for separation of carbohydrates from tissue extracts is described.

2. The content of glycogen, "free sugar" and lactic acid of rabbit nerves has been studied in the fresh tissue and after a period of rest in oxygen or nitrogen. We have shown that (*a*) in nitrogen there is rise in lactic acid, and a corresponding fall in carbohydrate; (*b*) in oxygen there is no disappearance of lactic acid, but a definite fall in the carbohydrates.

3. About 60 % of the resting metabolism of rabbit nerve may be maintained by the oxidation of these carbohydrates. The rate of lactic acid formation under asphyxial conditions is comparable to that of frog nerve and much less than that of rabbit brain. No oxidation or resynthesis of lactic acid occurs on admitting oxygen to an asphyxiated nerve.

4. The formation of lactic acid, even if concerned with hexosephosphate, does not involve breakdown of mono- or di-phosphate with liberation of free phosphate.

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