CCXXVIII. STUDIES IN AVIAN CARBOHYDRATE METABOLISM.

IV. FACTORS INFLUENCING THE MAINTENANCE OF RESPIRATION IN SURVIVING BRAIN TISSUE OF THE NORMAL PIGEON.

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It is characteristic of the pigeon's brain that the rate of respiration falls very rapidly during survival in Ringer-phosphate solution. The addition of glucose [Gavrilescu and Peters, 1931] and more so of lactate [Gavrilescu *et al.*, 1932] increases the uptake over periods of an hour; even in this case the maintenance of respiration claimed by a series of workers for mammalian brain is not seen [*cf.* Loebel, 1925; Meyerhof and Lohman, 1926; Warburg *et al.*, 1924; Holmes, 1930; Quastel and Wheatley², 1932]. During the course of study of the catatorulin action of vitamin B₁ concentrates [Passmore *et al.*, 1933], we were obliged to attend to this matter. The problem was to determine the nature of the factors responsible for the gradual fall in respiration in phosphate buffer solutions. Though CO₂-bicarbonate gives different results with some tissues, the advantages of phosphate for control of p_H and rapid technique are great, and the differences from CO₂-bicarbonate may be less fundamental than they appear. The finding that there is a direct parallelism between the behaviour to vitamin B₁ in Ringer-phosphate of the minced brain and the functional state of the living brain shows that the behaviour under our biochemical conditions is not too artificial to be of value [Meiklejohn *et al.*, 1932].

Fig. 1 shows the behaviour of minced pigeon's brain, the hourly rate being plotted against the time. This shows changes better than the accepted method of plotting total respiration and time. It will be seen that none of the substrates induces maintenance. This is not a question of concentration of substrate; Gavrilescu *et al.* [1932] showed that K_m for *dl*-lactate was 0.024 *M*, making the concentration used here (0.033 *M*) practically maximum. The addition of galactose has only slight effect, though it was found by Sherif and Holmes [1930] to prolong respiration in the nerve (only after 4 hours).

This behaviour was interpreted as a gradual inactivation of some essential enzyme system, until the observations upon cyanide (Fig. 1, curve for succinate + KCN) showed that a respiratory system could remain in abeyance and then return to an original level³. Further study has shown that the addition of certain phosphorus compounds has a marked influence upon maintenance of respiration, when lactate is also present.

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² Quastel and Wheatley's [1932] curves fall off quite quickly (*e.g.* pig brain).

³ This interesting recovery from cyanide deserves more study; the % inhibition noted is close to that of Dixon and Elliott [1929]. It probably indicates formation of aldehyde [*cf.* Wieland, 1932].

Lactate essential. The influence of lactate upon prolonging respiration of brain tissue as well as muscle tissue is well known, as is also the fact that the action of glucose is mainly due to the lactate formed from it. Fluoride, for instance, stops glucose respiration but not that of lactate [Holmes, 1930; Krebs, 1931]. Nevertheless lactic acid is still very generally regarded as a product of anaerobiosis, not essential *per se* in cell metabolism. The discovery that the "catatorulin" action of vitamin B₁ was generally maximum only when lactate was present supports strongly the hypothesis that this acid is essential for normal cell respiration.

Fig. 1.

Fig. 1. Effect of various substrates upon respiration of minced cerebrum in Ringer-phosphate p_H 6.9. (Concentrations: lactate, 0.033 M ; succinate, 0.011 M ; galactose, 0.037 M ; mannitol, 0.015 M ; KCN 0.01 M .)

Fig. 2.

Fig. 2. Effect of concentration of candiolin upon respiration of minced brain in lactate, p_H 7.3.

It must be realised that no examination of a tissue has ever shown less than 10 mg./100 g. lactic acid; we may quote Davenport and Davenport [1928]; Fisher by use of iodoacetate in pigeon's tissues [1931]; Kinnersley and Peters in pigeon's brain [1930]; Owles [1930] found the minimum amount present in circulating blood to be 11 mg./100 cc. = 0.0012 M ; this may be concentrated at a surface to give 0.01 M in the tissue; comparison with the K_m curve referred to above, gives 0.024 M as concentration for maximum effect of the *dl*-acid.

If so, we can only reconstruct the normal respiration of the cell by adding to lactate other substrates. This kind of experiment has been done by others to study the "sparing" effect of one substrate upon another [Quastel and Wheatley, 1932], but not with the avowed purpose of using lactate as a basal "Ringer" metabolite.

It can be shown that addition of some substances to a basal Ringer containing lactate gives a much increased respiration, as compared with the respiration in presence of lactate or of the substrate alone. This was found to be the case with

crude Na hexosediphosphate (prepared from candiolin)¹. The increase is more marked in the 2nd hour than in the 1st, and is maximum when 10–20 mg./3.0 cc. (0.03 *M* approx.) are added to the solution (see Fig. 2).

Such an addition effect is of two possible types; it may either (*a*) be due to independent respiration with two substrates; in this case the respiration of lactate *plus* the other substrate will not exceed the sum of the independent respirations with lactate and substrate separately; or (*b*) the increased respiration may occur only when the lactate and substrate are together; it may be a definite interaction. Exp. 1 (Table I) was designed to test this point.

Table I. *Exp. 1.*

O₂ uptake mm.³/g./hr. for successive half-hour periods. 1000 mm.³ = $Q_{O_2}5$.

Hours	...	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	
Ringer-phosphate*		950	800	650	550	
Ringer + candiolin		1145	1015	845	710	About 200 mm. ³ difference in each
Lactate only		1970	1750	1455	1275	
Lactate + Mg		1935	1750	1460	1280	No effect of magnesium
Lactate + pyruvate		2160	1920	1640	1500	
Lactate + candiolin		1995	1870	1660	1505	
Lactate + candiolin + pyruvate		1940	1820	1620	1480	
Lactate + candiolin + vitamin		1945	1840	1655	1505	

Conc. MgCl₂ 0.0016 *M*. Pyruvate 0.0038 *M*. Candiolin 0.33 %. Vitamin 1y/3.0 cc.

* Calculated from a similar experiment.

The experiment is instructive; it shows increases by addition of pyruvate which equal that of candiolin; no increased effect with candiolin *plus* pyruvate, and as usual in the normal no change with vitamin addition². But since candiolin increases respiration with the Ringer-phosphate solution alone, there is no evidence that the candiolin interacts only in presence of lactate; the effect is of type *a*. The feeling that there was some factor at work influenced by lactate led to the trial of hexosediphosphate. Exp. 2 (Table II) shows that hexosediphosphate as purified from commercial candiolin does not even give maintenance effect. (The action of fluoride will be discussed later.)

We believe that the factor especially concerned is pyrophosphate, to which we were led by experiment upon the "catatorulin" effect (see a further paper).

Table II. *Exp. 2.*

(Calculated hourly.) mm.³/g./hr.

	Hours	...	$\frac{1}{2}$	1	$1\frac{1}{2}$	2
Ringer-phosphate			960	820	640	545
R.P. + hexosediphosphate			1030	940	805	635
Lactate			2140	1960	1735	1500
Lactate + hexosediphosphate			1980	1900	1795	1525
Fluoride { R.P. + fluoride			650	530	360	265
(0.008 <i>M</i>) { R.P. + hexosediphosphate + fluoride			670	575	390	280
{ Lactate + fluoride			1480	1270	950	625
{ Lactate + hexosediphosphate + fluoride			1340	1200	920	630

Conc. of hexosediphosphate 0.008 *M*.

¹ The effect is much larger than that described by Quastel and Wheatley [1932] for the combination of succinate and lactate, though comparison is difficult as they worked with tissue previously autolysed for 3 hours.

² Sometimes vitamin addition shows a slight decrease in the normal.

Exp. 3 (partly shown in Fig. 3 and Table III) shows the effect especially well. It is supported by other experiments on the normal, which illustrate it in greater or less degree, and by the work upon the avitaminous brain. Here the action of pyrophosphate is additive only in presence of lactate¹.

Table III.

	mm. ³ /g./hr. 1000 mm. ³ =Q _{O₂} 5.							
	Hours	...	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3
R.P.			1000	650	480	405	305	260
R.P. + pyrophosphate			810	530	470	380	320	350
L. + pyrophosphate + fluoride			1030*	(940)	530	490	390	310
L.			1960	1360*	1130	950*	790*	690*
L. + pyrophosphate			2070	1475	1390	1220	1210	1100
L. + pyrophosphate + Mg			2280	1860	1600	1510*	1430*	1280
L. + pyrophosphate + α -glycerophosphate			2655	2200	1900	(1880)	1710	1530
Same + Mg			2625	2100	2060*	1950*	1550	1400

* Bad duplicates.

L. = Lactate.

Fig. 3.

Fig. 4.

Fig. 3. Effect of pyrophosphate upon respiration of minced brain, p_H 7.3. (Concentrations: lactate, 0.033 M ; pyrophosphate, 0.0093 M ; α -glycerophosphate, 0.024 M .)

Fig. 4. Effect of various substrates upon respiration of minced brain p_H 7.3. (Concentrations: lactate, 0.033 M ; α -glycerophosphate, 0.024 M ; pyrophosphate, 0.019 M ; hexosemonophosphate, 0.013 M ; β -glycerophosphate, 0.023 M .)

¹ Controls with lactate + pyrophosphate and Ringer-phosphate solution with and without boiled brain show no oxygen uptake.

The additive action is an interaction of type *b*. So far this is the only case found and distinguishes the action of pyrophosphate from other active stimulants of respiration. It supports again the hypothesis that lactate is an essential component of the normal respiration system.

Exp. 4 (Fig. 4) shows the action of pyrophosphate; this and Exp. 5 (in Table V, also give the influence of α - and β -glycerophosphate and of Robison's hexosemonophosphate.

Davies and Quastel [1932] showed that bullock brain reduced methylene blue at about the same rates in presence of Na lactate and Na glycerophosphate, more slowly with succinate. Ashford and Holmes [1931] obtained twice the rate of respiration with lactate as compared with Na glycerophosphate.

We have found the β -glycerophosphate to be practically inactive. The effects obtained by others must have been due entirely to the α -glycerophosphate component of their preparations. This has a remarkable action, as can be seen by the abstract Table IV.

Table IV. *Exps. 4a and 4b.*

α -Glycerophosphate. (0.024 M) mm.³/g./hr.

Hours	...	(4a)			(4b)		
		1½	2	2½	1½	2	2½
Lactate only		1155	1070	900	1230	1140	890
α -Glycerophosphate only		900	720	705	920	850	610
L. + α -glycerophosphate		1810	1690	1665	1720	1620	1305
Ringer-phosphate only		—	—	—	475	400	330

Table V. *Exp. 5.*

mm.³/g./hr. 1000 mm.³ = Q_{O_2} 5.0.

Hours	...	½	1	1½	2	2½
R.P.		840	720	475	400	330
R.P. + α -glycerophosphate		1285	1100	920	850	610
R.P. + hexosemonophosphate		1140	900	650	550	440
L.		1955	1580	1230	1140	890
L. + α -glycerophosphate		2160	1980	1720	1620	1305
L. + hexosemonophosphate		2100	1670	1300	1160	900

The effect is largely independent of lactate, *i.e.*, it is of type *a*, and therefore differs from that of pyrophosphate in this respect. Exp. 5 shows that it has marked action apart from the lactate. As will be seen in a further paper, there is confirmation of this difference between the two substrates with the avitaminous brain, where vitamin interacts specifically with pyrophosphate but not with α -glycerophosphate.

Hexosemonophosphate promotes an increased oxygen uptake in the first hour, which soon drops either to the level of Ringer-phosphate or of lactate only, according to the constitution of the medium. It appears that there must be a rapid inactivation of the hexosephosphatase, but it is certain that this substrate (like hexosediphosphate) added to lactate alone induces no maintenance.

The finding that α -glycerophosphate is an independent additive substrate with lactate introduces at once the idea that we are dealing in brain with a system behaving like the new fermentation scheme of Embden *et al.* [1933], in which Meyerhof and McEachern [1933] concur. It is easy to understand that if free pyrophosphate is necessary in these experiments it may quickly diffuse from the cell and so rapidly fall to an ineffective concentration. The sudden

drop in metabolic rate would be then due to lowered concentration of essential substrates (lactate, pyrophosphate) for the energy metabolism, maintained by the cycle of carbohydrate change.

Exp. 6 (Fig. 5) and Exps. 7 and 8 in Table VI were designed to test whether a supply of several of the elements of Embden's fermentation scheme in presence of

Fig. 5. Effect of combination of various substrates upon respiration of minced brain, in lactate, p_H 7.3. (Concentrations: lactate, 0.033 *M*; pyrophosphate, 0.0093 *M*; α -glycerophosphate, 0.024 *M*; hexosemonophosphate, 0.0072 *M*.)

Table VI. *Exp. 7. Combined effect of substrates.*

Hours ...	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3
L.	1430	1100*	970	785	630	555
L. + α -glycerophosphate	2150	1450*	1130*	1070	1050	825
L. + hexosemonophosphate	1510	1220*	930	750	690	530
L. + pyrophosphate + α -glycerophosphate	2130	1900	1815	1410*	1290*	1140*
L. + pyrophosphate + α -glycerophosphate + fluoride	1555	1225	1010	900	750*	710
L. + pyrophosphate + hexosemonophosphate	1710	1430	1300	1160	1040	980
L. + pyrophosphate + α -glycerophosphate + hexosemonophosphate	2200	2000	1805	1700	1370	1400

Conc. α -glycerophosphate 0.024 *M*; hexosemonophosphate 0.013 *M*; pyrophosphate 0.0093 *M*; fluoride 0.008 *M*.

* Mg.

Exp. 8. Cerebrum only.

Hours ...	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$
L.	2500	1970	1605	1350	1080*	990	790
L. + pyrophosphate	2455	2170	1940	1830*	1480*	1850*	1435
L. + α -glycerophosphate	2700	2330	2110	1880	1250*	1780*	1510
L. + pyrophosphate + α -glycerophosphate	2625	2405	2170	1985	1670*	1800	1670
L. + pyrophosphate + α -glycerophosphate + hexosemonophosphate	2665	2400	2170	1970	1900	1775	1600

Conc. as in Ex. 7.

lactate would give sustained respiration in the "brei"; Exp. 6 (Fig. 5) shows that addition of pyrophosphate to lactate remarkably improves maintenance of respiration, and that α -glycerophosphate further increases respiratory rate. The addition of hexosemonophosphate sometimes has no effect (Exps. 6, 8), or a slight one in the first hour only. Probably there is inactivation of the phosphatase present. Though as yet not proved, it is hard to believe that these substances are not those originally present in the tissue which cause so high an initial level of respiration. There is still more to be learnt, as Exp. 8 shows; the respiration in cerebrum only is still not properly maintained; but for these three experiments we have as average at the 2nd hour lactate alone 1142 mm.³/g./hr., + pyrophosphate and α -glycerophosphate 1791; and at 3rd hour lactate alone 828, with additions 1525, the remarkable average differences being 57 % and 84 % respectively.

Influence of fluoride. The presence of active fermentation following a scheme such as that of Embden and his colleagues in the respiring brain tissue can also be inferred from the effect of fluoride. This may be expected to influence any stage involving interaction with hexosephosphates. So, in Exp. 2, it is seen that some of the residual respiration is fluoride-sensitive and therefore due to hexosephosphate; this becomes some 5 % of the whole after 2 hours with added hexosephosphate. In presence of lactate, about one-third of the respiration is inhibited by fluoride; this is much more than even for Ringer-phosphate + hexosediphosphate. Hence, the improvement upon adding lactate is in part due to an increase in fluoride-sensitive respiration, the hexosephosphate (or triosephosphate) system.

In Exp. 7, fluoride reduced lactate + pyrophosphate + α -glycerophosphate to the level practically of lactate alone; all of this "extra respiration" is fluoride-sensitive. In Exp. 3, lactate + pyrophosphate is reduced to the level of Ringer-phosphate alone, again evidence of the interaction of the fluoride-sensitive system. The effect of α -glycerophosphate is to some extent independent of this: it is not fluoride-sensitive¹. According to Meyerhof and McEachern [1933] fluoride stops either the breakdown of triosephosphate to α -glycerophosphate and phosphoglycerate, or the breakdown of phosphoglycerate. So oxygen rather than pyruvic acid appears to be acting as the hydrogen acceptor.

The above experiments have been done at p_H 7.3. Change of p_H to 6.6 causes 30 % or more fall in the respiratory rate, see Table VII.

Table VII. *Effect of p_H on respiration rate (mm.³/g./hr.), 0-1 hour.*

Exp.	p_H 7.3	6.6	Decrease (%)
9	1730	1390	- 20
10	1990	1491 (4)	- 25
11	1990	1080	- 46

(4) = average of 4.

Magnesium. In all the experiments except Exps. 8 and 3, Mg was added in some form, to avoid any possible complications induced by lowered concentration in the action of the adenylyl triphosphate coferment. In Exp. 3, tests with and without Mg do not show any consistent change. Since Lohmann [1931] found that the "lactic acid" coferment was not that of respiration, this is not surprising. In Exp. 1 MgCl₂ was added and in the others $\frac{1}{8}$ - $\frac{1}{15}$ vol. of Ringer-lactate, saturated with magnesium phosphate.

¹ This has been confirmed by experiments on the avitaminous brain.

DISCUSSION.

This work at present lacks the direct proof that the compounds added are the actual components of the tissue system; meanwhile, much may be done to help the study of this particular system by adding to Ringer-lactate pyrophosphate and α -glycerophosphate; 2–2½ hours of survival are so given. It seems unlikely that the effects are not general for brain tissue. The relation of pyrophosphate to active respiration may have a connection with the finding of Drury [1933] that pyrophosphate has stimulant effects upon the heart. For other tissues results may prove different. In phosphate buffers alone, Dixon and Elliott [1929] found that 0.033 *M* pyrophosphate inhibited markedly the respiration of muscle and liver tissue; there was a slight activation with 0.01 *M*. Engelhardt [1932] on the other hand inferred the participation of pyrophosphate from experiments with avian blood corpuscles showing the anaerobic splitting and aerobic synthesis of the easily hydrolysable phosphorus. Of the two paths of glycolytic change, one from glucose and one from glycogen, described by Ashford and Holmes [1929], it may be speculated that the precise function of that from glucose is to supply the essential component of the system, lactic acid; the other would then provide Embden's triosephosphates.

The case of pyrophosphate draws attention again to the necessity of bearing in mind possible concentration of diffusible constituents in the cell itself; this has been instanced recently by Jowett and Quastel [1933] for glyoxalase and glutathione. Comparison of tissue metabolism in different volumes may be dangerous near the critical margin for substances not strongly absorbed by the cell. A good quantitative illustration of this was given by Passmore *et al.* [1933] in the case of the critical level of residual tissue vitamin B₁. The optimum amount of pyrophosphate in the medium, 0.2 %, is of the same order as that possible in the cell, if we allow for some concentration at the active surface, from Lohmann's figures about 0.1 %.

EXPERIMENTAL.

Normal pigeons have been used throughout; they have been usually fed for 7–14 days upon the standard mixed laboratory diet. The technique of Passmore *et al.* [1933] has been used for the setting up of samples and obtaining oxygen uptake. 16 bottles can be filled from one mixed brain, making 8 duplicate estimations as a maximum. Values recorded are the mean of duplicates unless otherwise stated, calculated half-hourly.

Salts used. Na *dl*-lactate, made up in tubes from recrystallised zinc lactate and sterilised as before; 0.033 *M*.

Ringer-phosphate as before, but allowed to stand 2 days at p_H 7.3 before filtering.

Candiolin. "Ca hexosephosphate." Decomposed either by adding the required amount of Na oxalate, to the neutral suspension and allowing to stand, or by adding just sufficient HCl to dissolve and a solution of oxalate, and then immediately neutralising. The results did not differ. Tests showed that excess oxalate was not present.

Hexosediphosphate prepared as Ba salt from candiolin by Robison's method. Decomposed by Na sulphate.

Hexosemonophosphates. Specimen kindly supplied by Prof. R. Robison. Decomposed by Na₂SO₄ and standing some hours.

Ca α -glycerophosphate. Synthetic specimen supplied by Messrs Boots [King and Pyman, 1914]. Calcium removed by addition of oxalate; tests showed that excess oxalate was not present. Data supplied by Messrs Boots and Co. Loss at 140° 0.3 %. Residue on ignition 60.4 %. CaO 26.6 %. P₂O₅ 34.0 %.

Na β -glycerophosphate. Pure salt, supplied commercially (Messrs Boots).

Na pyrophosphate. Prepared by igniting Na_2HPO_4 (Sørensen). This gave no phosphate reaction with molybdate unless it had stood.

The concentrations of salts are those giving approximately maximum effects.

Temperature $38^\circ \pm 0.1$. p_{H} 7.3, unless otherwise stated. For experiments at p_{H} 6.6, the Ringer-phosphate prepared at p_{H} 7.3 was treated with HCl.

In making additions to the Ringer-lactate solution, the salts have been made up in 0.2–0.3 cc. of solution and so added to the bottles, making allowance for this by addition of less Ringer-lactate. The changes in concentration of lactate so produced would not influence results, but this has been controlled in the latest experiments by adding the necessary amount for lactate in 2.0 cc. Ringer-phosphate and making up to 3.0 cc. with the various additions. The small variations in tonicity have not been corrected. It was found by one of us with Dr Gavrilescu that very wide variations in strength of a glucose solution had little influence upon oxygen uptake. This is not so surprising when it is remembered that in a recognised surgical method, hypertonic solutions are injected into the blood to produce shrinkage of the brain.

SUMMARY.

1. The decrease in respiration rate in minced avian brain systems is substantially reduced in presence of lactate.

2. It is not reduced by galactose, mannitol, sodium β -glycerophosphate, hexosed- and hexosemono-phosphates. The last two substrates may influence the rate of fall initially.

3. Na pyrophosphate added alone has little influence upon maintenance of respiration in the surviving tissue. Added with lactate, it much increases the respiration rate, values considerably in excess of those with lactate alone being obtained. Lactate and pyrophosphate are essential constituents of the respiration system.

4. α -Glycerophosphate has a remarkable additional influence. Ringer-phosphate to which lactate, pyrophosphate and α -glycerophosphate have been added gives much better maintenance than lactate alone.

5. Experiments with fluoride indicate that survival respiration in lactate-phosphate solution involves in part a hexosephosphate stage.

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