CCLXI. STUDIES IN AVIAN CARBOHYDRATE METABOLISM.

FURTHER STUDIES UPON THE ACTION OF CATATORULIN IN BRAIN.

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THIS work originated in an attempt to obtain a stable dry enzyme preparation, which would interact specifically with vitamin B₁ crystals [Kinnersley, O'Brien and Peters, 1933]. Though this object has not been attained, the research has brought to light facts of fundamental interest not only for the vitamin function, but for the respiration of the normal brain itself [Peters and Sinclair, 1933]. It has made clear the necessity of pyrophosphate as such in the respiratory energy system of brain, which could not be so clearly distinguished without the avitaminous animal.

Former researches can be summarised [Gavrilescu *et al.*, 1932; Meiklejohn *et al.*, 1932] by saying that the typical effect of the vitamin is to increase oxygen uptake over a given period *in vitro* specifically with the avitaminous brain²; this is shown in glucose and best in lactate which is known to be formed from glucose. Respiration with succinate behaves normally in the avitaminous brain. This seems to relate the action specifically to lactate. The simple view however that catatorulin³ is the co-enzyme for lactate oxidation is not now tenable for two reasons. Boyland [1933] has found no co-enzyme effect in purified vitamin B₁ concentrates, prepared in this laboratory⁴. More significant still, Meiklejohn [1933] could obtain no evidence of an increased disappearance of lactate corresponding with the extra oxygen uptake. Hence a new hypothesis arose, namely that the effect of catatorulin was related to some other substrate, which may be called substrate X, for the full action of which upon the respiratory system the presence of lactate was indispensable. We soon found that washing the tissue reduced the oxygen uptake and lowered the vitamin effect.

It is well to emphasise that the lesion cannot lie in the cytochrome respiratory enzyme system. Lactate respiration is of course reduced by cyanide, but the catatorulin effect is completely removed. About 70 % of the O₂ uptake is sensitive to *M*/60 and *M*/600 KCN in confirmation of Dixon and Elliott [1929]. Exps. 1, Table I, and 2, Fig. 1, show this, and that *M*/600 KCN abolishes the catatorulin action. Hence this lies definitely in the part of the respiration which is cyanide-sensitive.

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² No other tissue is yet known which shows this effect.

³ Catatorulin is the substance in vitamin B₁ crystals responsible for increased oxygen uptake.

⁴ This is fully confirmed by earlier unpublished experiments by Peters.

Table I. *Cyanide and catatorulin action.*

Exp. 1.	Period	...	1st hour	2nd hour	
	L* only		1130	680	
	L+V		1330	950	
	L+KCN (M/60)		400	220	Av. of triplicates
	L+V+KCN (M/60)		380	200	

* In all Tables L=lactate; V=vitamin B₁ concentrate.

Fig. 1. (Exp. 2.) Effect of KCN and of different p_H upon the catatorulin effect in mixed avitaminous brain. Curves A, E, F and G p_H 7.3; B, C and D p_H 6.8. Curve A, lactate (0.033 M) + vitamin B₁ (1 γ /3 cc.); B, lactate + B₁ (0.2 γ /3 cc.); C, lactate; D, lactate + B₁ (1 γ /3 cc.); E, lactate (calculated from A); F, lactate + KCN (M/600); G, lactate + B₁ (1 γ /3 cc.) + KCN.

Since the "vitamin" effect tended to disappear upon removal of diffusible substances by washing, we turned again to a more careful study of conditions essential for catatorulin action and of the factors concerned in the maintenance of respiration in the minced tissue¹. This has revealed a system of greater complexity than at first realised; but it has also led to knowledge of some substrates which will improve oxygen uptake and so bring the respiration of the minced avian brain more into line with that of sections from mammalian brain as described by others.

The increased respiration, when vitamin B₁ is added *in vitro* to the avitaminous brain in lactate, might possibly be interpreted as a decreased rate of fall in oxygen uptake. Without added lactate, Gavrilescu *et al.* [1932] showed

¹ We prefer the term "respiration," because we feel convinced that it is more correct to consider the minced brain tissue as an organised system than an enzyme mixture.

that the catatorulin effect was present, but reduced in amount; this must be due to the low residual lactate content and shows that lactate is essential for the system.

The effect of known substrates in presence of lactate.

It is clear about the postulated substrate X, with which catatorulin interacts, that lactate is also necessary, and that it is not a substrate competing with lactate. It may arise from a precursor in the tissue; in some experiments for instance there is a distinct tendency for the extra respiration due to vitamin to increase the value $(L + V) - L$. The simplest interpretation of this is that increasing amounts of the unknown substrate are formed during the survival respiration.

Pyruvate. This substrate with the normal pigeon's brain was shown by Meiklejohn *et al.* [1932] to give a larger O_2 uptake than lactate. Hence it increases rather than diminishes respiration when added to lactate with the normal brain; *e.g.* in Exp. 1 [Peters and Sinclair, 1933], pyruvate and candiolin increase the oxygen uptake to the same extent.

The avitaminous brain behaved differently. With pyruvate alone, the oxygen uptake was never as great as with lactate and sometimes not more than the residual. Pyruvate, further, often did not interact with catatorulin. With lactate and avitaminous brain increased respiration always took place (the action has been seen in at least 300 experiments in this laboratory); with pyruvate the effect was variable¹. Further, the pyruvate system in brain recovered more slowly than the lactate, so that this failure is not directly correlated with symptoms. These facts were explained by postulating some secondary factor as necessary for pyruvate respiration.

It is essential to mention here these earlier experiments with pyruvate; they must be taken into account when trying to elucidate the course of the normal oxidative synthesis of lactate. Because vitamin B_1 often fails to improve pyruvate respiration, pyruvate alone cannot be the substrate X for which we are looking.

Three classes of respiration experiments have been tried in this search. (1) Addition of known substances in presence of lactate. (2) Addition of various extracts of brain. (3) Respiratory quotients. Only (1) is considered here². (3) forms the subject of an accompanying communication [Sinclair, 1933, 2]. (2) is not yet complete. All the experiments here recorded were made with the avitaminous pigeon brain.

Phosphorus compounds.

We have shown previously [1933] that together with lactate crude candiolin (hexosediphosphate), α -glycerophosphate and pyrophosphate all have a marked influence upon the respiration of normal brain, the last especially in inducing maintenance of respiration; β -glycerophosphate proved quite inactive.

The avitaminous brain shows general similarity with some interesting differences which are important in trying to trace the course of the biochemical reactions involved in normal respiration.

Crude candiolin and hexosediphosphate (purified). The single experiment 3a, Table II, suffices to show that hexosediphosphate alone may not have much

¹ The previous experiments with pyruvate were done with cruder preparations of vitamin B_1 . It is not known whether this introduces any difference.

² A preliminary account of some of this work was communicated to the International Congress of Cytology on 22. viii. 33.

effect as a substrate. The vitamin effect was here present, but reduced practically to the level of "residual" vitamin effect. Table II, Exp. 3 *b* (Fig. 2), shows the effect of crude candiolin and of purified hexosediphosphate prepared therefrom.

Table II. O_2 uptake in $mm.^3$ of avitaminous brain with various additions.

Avitaminous brain. Purified hexosediphosphate (HP). Vitamin differences.

Exp. 3 *a*.

Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1 $\frac{1}{2}$ -2			
L	1200	1080	720	230	330	400
L + V	1530	1410	1120			
HP	820	760	525	270	260	295
HP + V	1090	1020	820			

Exp. 3 *b*.

Candiolin or hexosediphosphate.

Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	2-2 $\frac{1}{2}$	2 $\frac{1}{2}$ -3
L	1750	1590	1300	1060	885	760
L + V	2050	1855	1550	1370	1240	1155
L + Cand.	1790	1615	1390	1210	1010	875
L + V + Cand.	2045	1940	1770	1600	1470	1370
L + HP	1810	1615	1320	1150	1015	890
L + V + HP	2040	1890	1700	1530	1425	1350

Exp. 4.

Purified hexosediphosphate.

Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	Differences
L	1235	1040	810	715	55 $mm.^3$
L + HP	1185	1065	920	770	
L + V	1305	1190	1060	965	175 $mm.^3$
L + HP + V	1355	1290	1205	1140	

For concentrations, see p. 1924. $Q_{O_2} 5 = 1000 \text{ } mm.^3/g./hr.$

Fig. 2. Effect of candiolin and of hexosediphosphate upon the catatorulin effect in minced avitaminous brain in lactate, p_H 7.3. Curve *A*, candiolin (10 mg./3 cc.) + vitamin B_1 (1 γ /3 cc.); *B*, hexosediphosphate (8 mg./3 cc.) + vitamin B_1 ; *C*, vitamin B_1 ; *D*, hexosediphosphate; *E*, candiolin; *F*, control.

During the first hour no difference is shown; during the second hour there is a difference between preparations with candiolin and with hexosediphosphate; there is a rather greater effect in the uptake where vitamin is present in addition to these substrates. It is beyond the experimental error, but slight. In one experiment, however, out of several the effect genuinely reproduced that of the substrate sought. It is given in detail in Table II, Exp. 4.

In considering any work upon the avitaminous brain, we must keep in mind the fact that animals cannot all be in exactly the same state of avitaminosis; birds have always been selected with the greatest care. If we find animals occasionally showing a maximum effect such as this and at other times none, the interpretation is likely to be that the substrate in question in a normal animal is a remote precursor convertible into the one for which we are looking; but that the enzyme system required is temporarily more or less in abeyance in the avitaminous animal. This point is of importance later. We may note that in Exp. 4, hexosediphosphate increases the respiration mainly in presence of the vitamin.

*Robison's hexosemonophosphate*¹. In two experiments (Table III, Exps. 5, 6) Robison's hexosemonophosphate had no sustaining action, in collaboration with the vitamin. The initial effect seemed reminiscent of the normal; there was a

Table III. *Respiration of avitaminous brain with phosphorus compounds.*
O₂ uptake in mm.³

Robison's hexosemonophosphate (RHP).						
Exp. 5.	Period (hrs.)...	$\frac{1}{2}$ -1	$1\frac{1}{2}$ -2	2-2 $\frac{1}{2}$		
	L	1155	770	695		
	L + V	1280	1030	920		
	L + RHP	1165	770	610		
	L + RHP + V	1270	1000	870		
Exp. 6.	Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	$1\frac{1}{2}$ -2	
	L	1630	1460	1150	920	
	L + V	1730	1585	1360	1165	
	L + RHP	1755	1490	1160	950	
	L + RHP + V	1930	1680	1370	1160	
α - and β -glycerophosphate. (α -gp. and β -gp.)						
Exp. 7.	Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	$1\frac{1}{2}$ -2	2-2 $\frac{1}{2}$ 2 $\frac{1}{2}$ -3
	L	1630	1460	1150	920	800 655
	L + V	1730	1585	1360	1165	980 890
	L + α -gp.	1970	1815	1490	1260	1110 890
	L + α -gp. + V	2090	1975	1800	1620	1450 1310
	L + β -gp.	1430	1325	1145	910	— —
	L + β -gp. + V	1890	1720	1410	1200	— —
Exp. 8.	Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	$1\frac{1}{2}$ -2	2-2 $\frac{1}{2}$ 2 $\frac{1}{2}$ -3
	L	1310	1190	895	725	680 560
	L + V	1530	1425	1245	1150	1045 955
	L + α -gp.	1630	1520	1270	1090	930 800
	L + α -gp. + V	1685	1630	1490	1410	1335 1238
	L + β -gp.	1390	1275	1025	875	755 615
	L + β -gp. + V	1570	1460	1300	1210	1120 1030

¹ We are indebted to Prof. R. Robison for a gift of this material.

slight early stimulation of respiration. The substrate was not studied further, as it showed no promise of interest.

β -Glycerophosphate. No appreciable effect was found in experiments with this substrate which were well controlled by simultaneous experiments with α -glycerophosphate (Table III, Exps. 7, 8).

α -Glycerophosphate. The action of this substrate is in marked contrast to the others considered. It produces a large effect, amounting to some 30–40 %, and even more. But it is to be noticed that the action is not directly related to the vitamin at p_H 7.3. Even at 2–2½ hours, there is no increased catatorulin effect, i.e. $(L + V) - L$ is approximately the same as $(L + \alpha\text{-gp.} + V) - (L + \alpha\text{-gp.})$. It is therefore not peculiarly the substrate sought, but supplies the place of one of the missing entities in the decreased respiration of the vitamin-deficient brain.

Examination of the figures (Table III, Exps. 7, 8) shows that the effect of α -glycerophosphate is to raise the general level of respiration both with and without vitamin. There is slight increase in the differences, indicating small synthesis of X or its precursor, but hardly above the experimental error. Hence with α -glycerophosphate we have a substrate which is additive with lactate, but which shows neither maintenance nor specific interaction with catatorulin.

Na pyrophosphate. This substance is peculiarly important for the vitamin¹. At p_H 7.3, it produces a practically steady respiration for 2–3 hours, and in so doing considerably enhances the vitamin action. At p_H 6.6, where there is normally no vitamin effect, addition of pyrophosphate gives a vitamin effect. Added pyrophosphate therefore behaves as an essential constituent in a respiratory system, of which lactate and vitamin B₁ are necessary components, and therefore functions in part as our substrate.

In more than 10 experiments at p_H 7.3, we have observed the effect of pyrophosphate in a greater or less degree. There is therefore no evidence of the failure in some cases (and not in others) as seen previously with hexosediphosphate. A typical experiment is illustrated in Fig. 3.

Fig. 4 gives the effect of different concentrations (diagram at 2 hours). If we use 6 mg. of the Na salt, the effect is therefore practically maximum.

The conclusion is that lactate and free pyrophosphate in sufficient concentration are essential for the action of vitamin B₁; from this follows naturally the inference that the interaction of these three substances constitutes the basis of the catatorulin effect and is essential for normal tissue respiration. This could only have been learnt by a study of the avitaminous bird.

Mixed substrates. Some experiments upon the effect of mixed substrates have given results rather similar to those in the normal; the additions do much to restore normality in the avitaminous bird.

Table IV shows the very large increases which may be expected by these additions to the avitaminous brain, pyrophosphate, vitamin and α -glycerophosphate together with lactate inducing rises of at least double and in the case of Exp. 11 nearly treble the respiration rate as compared with lactate alone.

Adenosine triphosphate. The pyrophosphate may either function as the free salt, or because it assists in the resynthesis or preservation of the co-enzyme

¹ We were led to this discovery by an attempt to synthesise phospholactic acid by heating lactic acid with metaphosphoric acid. Upon neutralising on ice with 20 % NaOH, crystals separated, a solution of which had marked maintenance activity. The isolated barium salt subsequently proved to be barium pyrophosphate.

Fig. 3. Effect of pyrophosphate upon the respiration of avitaminous brain in lactate p_H 7.3. Curve *A*, control; *B*, pyrophosphate (6 mg.); *C*, vitamin B_1 (1γ); *D* vitamin and pyrophosphate (6 mg.); *E*, vitamin and pyrophosphate (12 mg.).

Fig. 4. Effect of concentration of pyrophosphate upon respiration of avitaminous brain in lactate, p_H 7.3. Curve *A*, with addition of vitamin B_1 (1γ/30 cc.).

Table IV. *Mixed substrates**. *Avitaminous brain. O₂ uptake in mm.³ p_H 7.3.*

	Period (hrs.) ...	0-½	½-1	1-1½	1½-2	2-2½	2½-3
Exp. 9.	L alone	1160	970	710	730	560	500
	L + V	1200	1145	1010	880	860	760
	L + pp. + V	1070	1115	1105	980	1030	880
	Same + α-gp.	1460	1420	1315	1230	1190	1040
Exp. 10.	L + α-gp.	1720	1640	1280	1125	815	—
	L + pp.	1360	1050	700	730	820	—
	L + pp. + V	1640	1360	1300	1260	1290	—
	L + pp. + α-gp.	1930	1495	1290	1140	1000	—
	L + pp. + α-gp. + V	1840	1890	1680	1510	1460	—
Exp. 11.	L	715	600	—	—	—	—
	L + pp.	1010	880	750	715	540	—
	L + V	1030	1020	—	—	—	—
	L + pp. + V	1120	1120	1070	1000	860	—
	L + α-gp.	1225	1100	1040	930	782	—
	L + pp. + α-gp.	1390	1330	1210	1050	870	—
	L + pp. + α-gp. + V	1525	1620	1495	1335	1170	—

pp. = pyrophosphate.

* It seems most probable (but lacks proof), that these are the actual missing substrates in the tissue itself, and that the initial varying rates of respiration in lactate alone are due to varying amounts of these substances in the tissue preformed perhaps during the stimulation of killing and subsequent manipulation. Some preliminary evidence has already been obtained in support of this.

adenyl pyrophosphate [Lohmann, 1928; 1932]. We have obtained no vitamin or vitamin-pyrophosphate effect by addition of the co-enzyme up to concentrations of approximately 2 mg./3.0 cc. = 0.07 %. Some earlier negative experiments with a crude preparation of our own have been confirmed with a specimen very kindly given to us by Dr Boyland¹ (Table V, Exp. 12).

Table V. *Adenosine triphosphate (Adp.) 2 mg./3 cc. Exp. 12.*
O₂ uptake in mm.³

Period (hrs.) ...	0-½	½-1	1-1½	1½-2
L	1210	890	690	550
L + Adp.	1150	800	630	480
L + pp.	1230	910	820	650
L + V	1330	1090	920	710
L + Adp. + V	1510	1090	(925)	715
L + pp. + V	1380	1180	1090	990

Since increase in concentration of adenyly pyrophosphate has no influence in presence or absence of the vitamin, it must be concluded that free pyrophosphate is the essential component of our system, and that this is acting independently of the co-enzyme in this particular respect.

Note. Nagai [1932] has recently studied the pyrophosphate content at death of the muscles of avitaminous pigeons. He found no difference from normal for breast and heart muscle, but a 15 % decrease for avitaminous leg muscle. The latter is barely significant statistically. The figures are in any case of doubtful value, because there is no statement of time elapsing between death and immersion in cooled trichloroacetic acid.

¹ We gratefully record our thanks for a gift of Ba adenosine triphosphate. This was decomposed in acid solution (HCl) and checked by estimation of inorganic and total P and P hydrolysable in 7 minutes.

Hydron concentration and the catatorulin effect.

With normal brain [Peters and Sinclair, 1933] the O_2 uptake at p_H 6.6 is approximately 60–70 % of the value at p_H 7.3. Respiration in lactate solutions is of course lower for avitaminous than for normal brain. The difference between these two hydron concentrations is not nearly so marked for avitaminous brain in absence of added vitamin; but whereas the presence of vitamin increases respiration at p_H 7.3 markedly, this increase with added vitamin is much reduced or even absent at p_H 6.6. Table VI shows typical experiments (a) comparing lactate and lactate + vitamin at p_H 6.6, (b) lactate and lactate + vitamin at p_H 6.6 and 7.3 (see also Fig. 1, Exp. 2).

Table VI. *Effect of p_H on vitamin action. O_2 uptake in mm.³*(a) L + V (1γ at p_H 6.6).

Period (hrs.)...	Exp. 13		Exp. 14	
	0-1	1-2	0-1	1-2
L	810	470	1000	610
L + V	900	550	1135	775

(b) L + V (1γ) at p_H 6.6 and 7.3.

Period (hrs.)...	Exp. 15		Exp. 13	
	0-1	1-2	0-1	1-2
p_H 7.3	1265	1020	1290	1090
p_H 6.6	990	630	900	550

(c) p_H 6.6. V 0.2 γ and 1 γ .

Period (hrs.)...	Exp. 16	
	0-1	1-2
L	955	505 (triplicate averages)
L + 0.2 γ V	1030	620
L + 1 γ V	990	630

It is of considerable interest that the catatorulin effect should be reduced from a maximum at p_H 7.3 (the blood- p_H) to a minimum at p_H 6.6. A further point of importance is that any slight vitamin effects at p_H 6.6 are obtained with small amounts of vitamin (0.2 γ); but increase of vitamin to 1 γ does not give increased respiration, as at p_H 7.3. The equation of Passmore *et al.* [1933] does not hold at p_H 6.6 (see Table VI (c)). This was remarkable, and at first seemed to be explicable only by some abrupt inhibiting effect. The probable explanation has proved more interesting. If pyrophosphate is added, there is a vitamin effect at p_H 6.6, similar to that at p_H 7.3 (see Table VII). Hence, sufficient pyrophosphate must be formed at p_H 7.3 to give the ordinary vitamin effect; this pyrophosphate formation largely fails at p_H 6.6. The reason for this is still obscure; the optimum p_H for pyrophosphatase action was given by Kay [1928] as p_H 7.6, but since then pyrophosphatases with other p_H optima have become known [see Takahashi, 1932].

There is a distinct suggestion that the origin of this pyrophosphate may be normally related to α -glycerophosphate. Though α -glycerophosphate acts independently of the vitamin at p_H 7.3 so far as oxygen uptake is concerned, at p_H 6.6, α -glycerophosphate functions to some extent in place of pyrophosphate (see Table VII, Exps. 17 and 18).

The striking influence of pyrophosphate at p_H 6.6 lends the strongest support to the view that free pyrophosphate is a component of normal cell respiration in brain tissue. The concentration of adenosine triphosphate in 100 mg. tissue

Table VII. *Respiration at p_H 6.6. O_2 uptake in mm.³*

	Pyrophosphate.	α -Glycerophosphate.	Vitamin.
Exp. 17.			
Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$
L	1165	915	710
L + V	(1175)	(985)	(780)
L + α -gp.	1440	1010	740
L + α -gp. + V	1615	1230	970
L + pp.	945	810	730
L + pp. + V	1410	1210	1060
Exp. 18.			
Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$
L	960	800	530
L + pp.	960	770	685
L + V	1150	915	710
L + V + pp.	1200	1115	1050
Exp. 19.			
Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$
L	890	735	640
L + V	1040	835	625
L + pp.	955	840	690
L + pp. + V	1225	1145	1035
L + α -gp.	930	835	770
L + α -gp. + V	1120	1060	955

is low, so that such pyrophosphate can hardly arise from this source, even if the α -glycerophosphate action is otherwise explained. It must be mentioned again in final caution that these findings with substrate additions still require the experimental proof by estimation of concentration in the tissues concerned before we can be quite certain of their application.

The above interesting facts unfortunately do not yet teach us the precise nature of the enzyme system with which the vitamin co-operates. Though pyrophosphate behaves as substrate X, its action must be in some way secondary; *i.e.* the postulated substrate X is multiple. In the attempt to learn whether this was concerned with the accepted hexosephosphate fermentation system, we investigated the action of fluoride.

Fluoride.

The effect of fluoride upon tissue respiration has been much studied [see Meyerhof, 1930]. In mammalian brain, Ashford and Holmes [1929] showed that 0.02 *M* fluoride inhibited lactate formation by 88 %, 0.01 *M* by 81 %, 0.005 *M* by 72 % and 0.002 *M* by 64 %. It is uncertain however that this inhibition is due to stabilisation of hexosephosphates, as even 0.02 *M* did not inhibit the appearance of inorganic phosphate under these conditions (p_H 8.2). At first it was thought that the vitamin effect was relatively little influenced by fluoride. Further experiment showed however that this was a question of concentration.

Table VIII gives the experiments. In each case 0.024 *M* NaF reduced the respiration of L + F + V to that of L + F; this concentration reduced the lactate respiration by 30-35 %, a figure which must be approximately maximum as 0.048 *M* gave no further striking change in L + F. In the second hour period the reduction with 0.024 *M* formed a rather greater percentage of the total respiration, but in actual amount (mm.³/g./hr.) was not much different from the reduction in the first hour. About 300 mm.³/g./hr. are sensitive to fluoride under

Table VIII. *Effect of fluoride (NaF). O₂ uptake in mm.³*

Exp.	Period (hrs.) ...			Calculated differences		Total
		0-1	1-2	1st	2nd	
Exp. 20.	L	940	570	330	280	(610)
	L + F (0.048 <i>M</i>)	610	290			
	L + V	1160	800	380	475	(855)
	L + F (0.024 <i>M</i>)	780	325			
	L + α -gp.	1370	1010	280	320	(600)
	L + F (0.024 <i>M</i>)	1090	690			
Exp. 21.	L	1030	600	190	210	(400)
	L + F (0.008 <i>M</i>)	820	390			
	L + F (0.024 <i>M</i>)	630	290	190	100	(290)
	L + V	1200	780	530	490	(1020)
	L + F (0.024 <i>M</i>)	670	290			
	L + F (0.024 <i>M</i>) + α -gp.	590	310	—	—	
	L + F + α -gp. + V	640	390			
Exp. 22.	L	1160	690	190	200	(390)
	L + F (0.008 <i>M</i>)	970	490			
	L + F (0.024 <i>M</i>)	810	430	160	60	(320)
	L + V	1480	960	420	440	(860)
	L + F (0.008 <i>M</i>) + V	1060	520			
	L + F (0.024 <i>M</i>) + V	855	450	205	70	(278)
	RP	855	460	270	210	(480)
	RP + F	585	250			
Exp. 23.	L	1395	850	160	230	390
	L + F (0.004 <i>M</i>)	1230	600			
	L + F (0.008 <i>M</i>)	1140	450	90	150	240
	L + V	1780	1120	340	390	730
	L + F (0.004 <i>M</i>) + V	1440	730			
	L + F (0.008 <i>M</i>) + V	1150	485	260	245	500
	RP	805	450	140	60	200
	RP + F (0.004 <i>M</i>)	665	350			

RP = Ringer-phosphate.

our conditions. 0.008 *M* NaF inhibited catatorulin action completely in two experiments and only partially in a third. With 0.004 *M* the catatorulin effect was present in Exp. 23, but inhibited some 50 % (reckoned as mm.³/g./hr.). 0.01 *M* therefore appears to be the marginal concentration for complete inhibition of the catatorulin effect; this will only reduce the lactate respiration by 18 %. The catatorulin effect is therefore much more sensitive to fluoride than 70 % of the tissue respiration in the presence of lactate; it appears to follow the concentrations required to inhibit lactate formation more closely, but to be less sensitive than the lactate system.

Some other points deserve notice. From the fluoride action it may be judged that about 70 % of the residual respiration is due to lactate in the first hour, and in the second hour much more. In presence of lactate, the proportion of fluoride-sensitive respiration increases in the second hour. If fluoride inhibits the hexosephosphate-triosephosphate system, this means that more of these compounds are taking part in the respiration in the second hour; they are therefore formed from precursors in the first hour. In presence of vitamin, the

amount of such substances formed must be much greater; in Exp. 22 for instance some 600 mm.³ of the respiration are inhibited by fluoride. This suggests that the vitamin is concerned somehow in the fermentation system of carbohydrate, stimulating a respiration which is fluoride-sensitive¹. The extra respiration is intermediate in sensitivity to fluoride between lactate upon the one hand and the main tissue respiration on the other.

The concentration 0.01 *M* fluoride required to inhibit the vitamin effect is practically the same as that shown by Dickens and Greville [1932] to inhibit glucolysis in the Jensen rat sarcoma.

To recapitulate, the simplest hypothesis that vitamin B₁ is directly concerned with lactate removal was replaced by the hypothesis that it interacted with an unknown substrate X. Now it seems that X is more than one substance.

Further considerations.

The results upon normal and avitaminous brain suggest a close relation to the new fermentation scheme of Embden *et al.* [1933], in which Meyerhof and McEachern [1933] concur. The powerful action of α -glycerophosphate makes this evident.

According to these authors, we have for the anaerobic system:

- (1) Hexosephosphoric acid $\rightarrow$ 2 triosephosphoric acid [dihydroxyacetonephosphoric acid (a) + glyceraldehydophosphoric acid (b)].
- (2) (a) + 2H + (b) + O = α -glycerophosphoric acid + phosphoglyceric acid.
- (3) Phosphoglyceric acid = pyruvic acid + phosphoric acid.
- (4) α -Glycerophosphoric acid + pyruvic acid = triosephosphoric acid + lactic acid
(= 2 lactic acid + phosphoric acid) [Meyerhof and Kiessling, 1933].

In (4) pyruvic acid formed from phosphoglyceric acid in presence of α -glycerophosphate gives lactate (salt at p_H 7.3).

The prominent part played by pyruvic acid (as opposed to methylglyoxal) in these new fermentation schemes makes necessary a further investigation of pyruvate in relation to these brains. The primary object of this paper was confined to investigation of the respiration, but a few other incidental observations have also been made.

Tested by the nitroprusside reaction of Simon and Piaux [1924] as described by Case [1932], no pyruvate reaction is given by the freshly minced avitaminous brain. After shaking with lactate-Ringer at p_H 7.3 for periods up to 2 hours, there is usually found a marked pyruvate reaction.

It is probable that the reaction is actually given by pyruvate, but this cannot be decided until a hydrazone has been separated and identified by melting-point. The reaction is peculiar to avitaminous brain at p_H 7.3. It is not found in experiments carried out at p_H 6.6, or in normal brain tissue. Hence, it is in some way related to the avitaminosis; the obvious connection with Embden's scheme is of great interest.

Upon the assumption that this substance will prove to be pyruvate itself, two problems arise for further work, namely (1) the origin and disappearance of

¹ During the progress of these experiments, Meyerhof's view [1933] that fluoride inhibits phosphoglyceric acid breakdown appeared. It is difficult to reconcile this effect of fluoride with the view that fluoride only influences the breakdown of phosphoglycerate.

the substance responsible for the pyruvate reaction in the respiration experiments, and (2) the nature of the pyruvate lesion.

In regard to (1), two paths are available upon present knowledge, either oxidation of lactic acid or degradation of phosphoglycerate. Origin by oxidation *via* the lactic co-enzyme of Banga, Szent-Györgyi and Vargha [1932] cannot be excluded, unless it proves that the lactic co-enzyme functions well at p_H 6.6; at this p_H no pyruvate reaction appears. The more probable origin appears to us to be *via* Embden's phosphoglycerate; this would account among other things for the ready disappearance of the reaction in presence of α -glycerophosphate; further, since lactic acid would be so formed, it would go far towards explaining the puzzling constancy of lactate in Meiklejohn's experiments [1933]. (2) The problem as to the nature of the pyruvate respiration lesions in these brains has still to be solved. Respiration with avitaminous brain in pyruvate solutions in absence of added vitamin is almost always less than with lactate, in contrast to the normal. The previous results with vitamin additions were variable [Meiklejohn *et al.*, 1932]. A few further experiments show that addition of pyruvate to lactate may be actually inhibitory (see Exp. 24, Fig. 5). At other

Fig. 5. (Exp. 24.) Effect of pyruvate upon catatorulin effect in minced avitaminous brain in presence of lactate (0.033 *M*) and pyrophosphate (7.5 mg./3 cc.). Curve A, vitamin B_1 (1 γ /3 cc.); B, vitamin B_1 + pyruvate (2.8 mg./3 cc.); C, control.

times an experiment such as Exp. 25, Table IX is seen, giving reduced respiration with pyruvate, but identical catatorulin effects with pyruvate and lactate. At present the balance of evidence is towards the view that the pyruvate problem is secondary to that of lactate, so far as the action of the vitamin is concerned. Two paths seem possible for removal of pyruvic acid, synthesis to phosphoglycerate, or reduction to lactate. Exp. 25 is best explained by believing that pyruvate is first reduced to lactate. If then, the presence of vitamin also improves removal of pyruvate by reduction to lactate, the vitamin must be a

Table IX. O_2 uptake in mm.³

Exp. 25.		1	2	V difference
Period (hrs.)	...			
Ringer-phosphate		1030	590	110 mm. ³
Ringer-phosphate + V		1080	700	
L		1600	1010	360 mm. ³
L + V		1810	1370	
Pyruvate		1360	850	330 mm. ³
Pyruvate + V		1580	1180	

Exp. 26.		1	2	3	Difference Total 1-3 hrs.
Period (hrs.)	...				
L		1400	750	615	690
L + V		1560	1040	860	
P		1080	680	380	1020
P + V		1490	960	730	
P + α -gp.		1460	860	690	910
P + α -gp. + V		1810	1160	950	

catalyst in some reducing system. We cannot yet decide which substance reduces pyruvate when oxygen is present. According to Barrenscheen and Beneschevsky [1932] it would be glutathione; whereas according to the scheme of Embden *et al.* [1933] and Meyerhof and McEachern [1933] it would be preformed α -glycerophosphate. If α -glycerophosphate can actually take the place of oxygen, then it might be expected that α -glycerophosphate + pyruvate would give less respiration than pyruvate. In three experiments of which Exp. 26, Table IX, is one, respiration with α -glycerophosphate addition has been increased additively with pyruvate, as with lactate. This independence of α -glycerophosphate respiration is decidedly puzzling, and at the moment difficult to reconcile with the latest fermentation schemes. There is no support at present for the view that the vitamin action is concerned with α -glycerophosphate, or directly with pyruvate, though the fluoride experiments suggest a relation to the fermentation system of carbohydrate¹. We have come to believe that the changes in the tissue induced by lack of vitamin are more profound than at first realised, and that the whole trend of metabolism is altered; this is indeed indicated by Meiklejohn's and also by the R.Q. experiments [Sinclair, 1933, 2]. Since Hopkins and Elliott [1931] showed that pyrophosphate inhibited glutathione oxidation, the interaction with pyrophosphate would be consistent with some such function for the vitamin as mobilising active glutathione in a coupled oxidation system. Knowledge of the exact influence will come only with the patient accumulation of further facts.

The deep-seated changes experienced outwardly by increased lactate in the tissue here studied and variations in normal respiration are especially interesting, because of their close analogy to those in tumour tissue, which has been made closer by work upon the R.Q. Dickens and Šimer [1930] found that this was the great distinction for tumour (a lowering in carbohydrate oxidation in addition to lactic acid formation). Here analogy stops because there is no evidence that polyneuritic pigeons grow tumours in their brains more easily than the normal.

¹ An experiment with ketol [Henze, 1930] gave no indication of change in the respiration either with or without vitamin. We are indebted to Dr Boyland for the specimen of ketol.

The above research has been carried out in Ringer-phosphate solution. The criticism that conditions are too artificial to be of interest is adequately met by the recent finding (H. M. S.) that the catatorulin effect is obtained equally well in bicarbonate- CO_2 solutions.

EXPERIMENTAL.

Birds used for these experiments have been carefully selected to show true opisthotonus symptoms. They have in most cases been already used in vitamin B_1 tests and have therefore been thoroughly depleted of vitamin.

Determinations have been made upon the whole minced brain (up to 16 simultaneous samples) in Ringer-phosphate p_{H} 7.3, containing lactate 0.033 M unless otherwise stated, and in O_2 . The salts used have been described in our previous paper [1933]. The figures given represent the mean of satisfactory duplicate estimations, unless otherwise stated. Periods are calculated for 30 minutes preceding the time stated, *i.e.* $1\frac{1}{2}$ hours means the period $1-1\frac{1}{2}$ hours.

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. Any changes in concentration of lactate so produced would not influence results [*cf.* k_m curves for lactate, Gavrilescu *et al.*, 1932]; but this has been controlled in the latest experiments by adding the necessary amount of 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 Peters and Gavrilescu that very wide variations in strength of a sugar solution had little influence upon oxygen uptake.

The same batch of vitamin crystals has been used throughout. They were made up as described by Kinnersley *et al.* [1933] in acid alcoholic solution, 200 γ /cc. The required amount was taken immediately before an experiment, the alcohol removed, and diluted with Ringer-lactate so that 1 cc. = 2 γ . It was added to the bottles immediately after breaking up the tissue.

Magnesium. As in the previous paper, Mg has been added as 0.05 cc. of saturated Mg phosphate solution in several cases (Exps. 3, 5, 6, 7, 8, 10, 11, 12, 17, 19). It has had no influence.

Calcium concentration. Calcium chloride added to the Ringer-phosphate caused at p_{H} 7.3 inhibitory effects amounting to some 2–10 %, but did not affect the action of the vitamin. In order to work under standard conditions, most of the experiments were done with Ringer-phosphate which was allowed to stand at room temperature for 2 days before filtering from the precipitated calcium phosphate. Latterly, we have worked with a Ca-free Ringer without influence upon the experimental results. For other work upon the influence of calcium see Kisch [1931] and for nerve Gerard [1932].

Concentrations of other substrates. Unless otherwise stated, these have been as follows:

Vitamin B_1 . 1 γ per 3.0 cc. ($\frac{1}{3}$ International vitamin B_1 unit).

Pyruvate 0.019 M . Exps. 26, 27.

Pyrophosphate (Na salt). Anhydrous: 0.0094 M , Exps. 12, 17, 18, 19: 0.0075 M , Exps. 10, 11: 0.0125 M , Exp. 9.

α -*Glycerophosphate* (Ca salt decomposed by oxalate): 0.0143 M , Exps. 7, 8: 0.0238 M , Exps. 9, 11, 17, 19: 0.02 M , Exp. 27: 0.016 M , Exp. 10.

β -*Glycerophosphate* (Na salt): 0.0129 M , Exps. 7, 8.

Hexosemonophosphate (Robison) Ba salt: 0.0076 M , Exps. 5, 6.

Hexosediphosphate Ba salt: 0.0071 *M*, Exps. 3a and b, 4.

Candiolin. 10 mg./3 cc., Exp. 3b.

They were chosen to produce maximum (or approximately maximum) effects.

CONCLUSIONS.

1. The action of vitamin B₁ "catatorulin effect" upon the brain *in vitro* of avitaminous pigeons is maximum at p_H 7.3. It is abolished by cyanide and fluoride in sufficient concentration, being more sensitive to fluoride than most of the respiration.

2. Na pyrophosphate (0.2 %, 0.01 *M*) interacts with vitamin B₁ and lactate to produce large rises in respiration over periods of 2–3 hours; in this way the respiration is largely maintained instead of decreasing rapidly.

3. Hexosediphosphate and Robison's hexosemonophosphate increase the initial rate of respiration in avitaminous as in normal brains, but have no influence in sustaining respiration and show no specific vitamin interaction.

4. α -Glycerophosphate (not β -glycerophosphate) increases the respiration largely; this increase is not specifically related to the vitamin action, but the substance is probably one of the missing tissue substrates in experiments of this type.

5. With lactate alone at p_H 6.6 there is no catatorulin effect unless pyrophosphate is added. To some extent α -glycerophosphate will replace pyrophosphate. It is inferred from this that free pyrophosphate is split off in survival respiration at p_H 7.3.

6. Since vitamin B₁, lactate and pyrophosphate appear to form some coupled oxidation system, the vitamin must influence more than one phase of the energy metabolism of the cell. The facts at present known are the expression of some deep-seated shift in cell equilibria.

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REFERENCES.

- Ashford and Holmes (1929). *Biochem. J.* **23**, 751.
Banga, Szent-Györgyi and Vargha (1932). *J. Physiol. Chem.* **210**, 228.
Barrenscheen and Beneschevsky (1932). *Biochem. Z.* **255**, 453.
Boyland (1933). *Biochem. J.* **27**, 786.
Case (1932). *Biochem. J.* **26**, 753.
Dickens and Šimer (1930). *Biochem. J.* **24**, 1322.
— and Greville (1932). *Biochem. J.* **26**, 1551.
Dixon and Elliott (1929). *Biochem. J.* **23**, 812.
Embden, Deuticke and Kraft (1933). *Klin. Woch.* **12**, 343.
Gavrilescu, Meiklejohn, Passmore and Peters (1932). *Proc. Roy. Soc. Lond.* B **110**, 431.
Gerard (1932). *Physiol. Rev.* **12**, 483.
Henze (1930). *Z. physiol. Chem.* **193**, 88.
Hopkins and Elliott (1931). *Proc. Roy. Soc. Lond.* B **109**, 58.
Kay (1928). *Biochem. J.* **22**, 1448.
Kisch (1931). *Biochem. Z.* **235**, 51.
Kinnersley, O'Brien and Peters (1933). *Biochem. J.* **27**, 232.
Lohmann (1928). *Biochem. Z.* **202**, 466.
— (1932). *Biochem. Z.* **254**, 332.

- Meiklejohn (1933). *Biochem. J.* **27**, 1311.
—— Passmore and Peters (1932). *Biochem. J.* **26**, 1872.
Meyerhof (1930). *Die chemischen Vorgänge im Muskel*. (Springer, Berlin.)
—— (1933). *Nature*, **132**, 373.
—— and Kiessling (1933). *Biochem. Z.* **264**, 40.
—— and McEachern (1933). *Biochem. Z.* **260**, 417.
Nagai (1932). *Biochem. J.* **26**, 353.
Peters and Sinclair (1933). *Biochem. J.* **27**, 1677.
Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 842.
Simon and Piaux (1924). *Bull. Soc. Chim. Biol.* **6**, 477.
Sinclair (1933, 1). *J. Physiol.* **79**, 6 P.
—— (1933, 2). *Biochem. J.* **27**, 1927.
Takahashi (1932). *J. Biochem. Japan*, **16**, 447.