CCCIX. PYRUVIC ACID OXIDATION IN BRAIN.

I. VITAMIN B₁ AND THE PYRUVATE OXIDASE IN PIGEON'S BRAIN.

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THE object of the present paper and others to follow is to present the evidence for the pyruvate oxidase theory of the action of vitamin B₁. In brain tissue treated with lactate, we now believe that oxidation mainly follows the expected sequence

and that the action of the vitamin is exerted at stage B.¹ In so exerting an effect, which can be observed noticeably only in the avitaminous brain (or kidney),² this vitamin is either acting as a coenzyme to pyruvate oxidase, or constitutes the prosthetic group of the enzyme itself. At present evidence does not decide between these possibilities, though favouring the latter.

At first, in this laboratory, we favoured the lactate oxidase theory of the action of vitamin B₁ [Kinnersley & Peters, 1930], based upon the large accumulations of lactic acid, which are a feature of vitamin B₁ deficiency. Later this view was discarded; especially we may mention that Meiklejohn [1933] did not find an increased disappearance of lactic acid associated with increased oxygen uptake in presence of the vitamin. There came subsequently the discovery [Peters & Sinclair, 1933] of a substance giving the colour reaction of pyruvic acid as an abnormal product in avitaminous brain respiring in lactate, the subsequent study and identification of this [Peters & Thompson, 1934], and finally the finding of abnormal amounts of pyruvic acid in avitaminous blood [Thompson & Johnson, 1935; Johnson, 1936]. From the time of this work, there has always been a tempting body of evidence in favour of the importance of pyruvate (as an intermediate) in brain respiration systems, and of the close correlation if not identification of vitamin B₁ with the pyruvate oxidation system. The evidence of abnormal amounts of pyruvate in blood was important as it indicated that the phenomenon shown *in vitro* by teased brain also took place *in vivo*, a complete answer to any criticism of the value of such *in vitro* work. In spite of these indications, there seemed to be certain facts which could not be reconciled with the direct pyruvic oxidase view; this led us to hesitate in its adoption. As the majority of these have now been removed, there remain no outstanding objections to the pyruvic oxidase theory. We can now consider some of the objections in detail; the attempt to answer them has brought to light a few new facts about brain enzyme systems.

¹ This scheme was presented by the author [1936], and with McGowan [1936].

² The catatorulin effect is shown by pigeon's kidney [Thompson, 1934].

The relation of lactate and pyruvate.

It is natural of course to believe that respiration in brain tissue follows the sequence (1). If this is so, and if the vitamin acts at stage B, then we should expect (a) the accumulation of pyruvate from lactate in absence of vitamin B₁ as is now known to occur; (b) less O₂ uptake with pyruvate than lactate in the avitaminous brain, and the reverse with normal brain, because in the latter we might expect that conversion of lactate into pyruvate was not sufficiently rapid to saturate the pyruvate oxidase with pyruvic acid; (c) a more pronounced catatorulin effect (vitamin B₁ extra O₂ uptake) with the pyruvate than with lactate. In early work upon this point, Meiklejohn *et al.* [1932] found that (b) occurred, but that (c) did not apply; there were cases in which brain tissue apparently reacted with vitamin B₁ in presence of lactate and not of pyruvate. This seemed to be irreconcilable with scheme (1). Let us first consider work upon (c).

Vitamin B₁ effect and pyruvate. In the last two years a very large number of catatorulin and other tests have been done with pyruvate. These are summarized separately. In over 50 consecutive experiments, there has been no case in which large vitamin effects have not been shown with this substrate. On the other hand, I have occasionally noticed failures with lactate. This is consistent with scheme (1), and not with the early pyruvate work, and suggests that the early failures were due to some special feature of the experiments. In a footnote, Peters & Thompson [1934] considered that the action of pyruvate at pH 7·3 must be linked with that of pyrophosphate (as was lactate at pH 6·6 [Peters & Sinclair, 1933]). This was because it seemed to be a possible difference between the earlier and later experiments. I have now submitted this point to direct tests, using even the more alkaline pH 7·7 in order to emphasize any differences, and can obtain no support for this opinion. In the experiments of Table I, at pH 7·3, Exp. 965 (one of two similar ones with normal brain) is confirmatory of previous work by Meiklejohn *et al.* [1932] who worked without pyrophosphate. There is a larger uptake for normal brain with pyruvate than with lactate in complete agreement with postulate (b). The significant vitamin effect in pyruvate, Exp. 965, is unusual, but it is interesting that it happens to occur with pyruvate; the slight inhibition with vitamin and lactate observed in 1935 has often been noticed in the normal brain and is not yet explained. At this pH of the avitaminous brain the lower O₂ uptake with pyruvate than with lactate is reproducible (Exp. 967). Even with pyruvate plus vitamin there is a higher total oxygen uptake than with the lactate, contrary to the normal. Nevertheless, the other feature of the earlier experiments is not reproduced: there is a marked catatorulin effect with pyruvate, even larger than that with lactate. At pH 7·7 (Table I (b)) the relations with lactate and pyruvate in the five experiments quoted are substantially the same. There is always a catatorulin effect with pyruvate.

There is if anything a greater restoration with pyruvate than with the lactate.

The relations to pyrophosphate at this pH are perplexing. There is no evidence from Exps. 959 and 956 that it is necessary for respiration with pyruvate. It is in fact inhibitory in these experiments both to lactate and to pyruvate. The inhibition to pyruvate persists right through the period of respiration though with diminishing intensity. That for lactate is over (as in the case of the pH 6·6 respiration) (see below) after about 1½ hours.

For the moment it is not possible to explain all these points, but it is clear that they do not support the explanation of the older experiments of Meiklejohn *et al.*, who reported occasional failure to obtain the vitamin effect with pyruvate.

Table I. *Comparison of O₂ uptakes with pyruvate and lactate as substrates at varying pH values. (Normal and avitaminous pigeons' brains.)*

μl./g./hr. P=pyruvate; L=lactate; V=vitamin; pp=pyrophosphate; Res=residual.

(a) pH 7.3. Cool preparation (14°).

Exp. 965. Normal cerebrum.

Period (min.) ...	15	15	30	30	30	30	Vitamin difference	
							0-60	60-120
P	2740	2460	2110	1745	1410	1210	+ 270	+ 225
PV	3035	2675	2390	2000	1600	1435		
L	2810	2465	1995	1460	1070	855	- 82	- 30
LV	2650	2350	1945	1450	1020	850		
Res	1010	935	870	595	376	300		

Experiment quite exceptional in size of the vitamin effect shown with pyruvate by the normal brain.

Exp. 967. Avitaminous cerebrum.

P	1170	1040	660	545	415	367	+ 458	+ 530
PV	1480	1425	1225	1080	945	837		
L	(1405)	1335	1055	860	640	525	+ 350	+ 375
LV	1630	1650	1485	1235	1010	965		

(b) pH 7.7. All experiments avitaminous. Exps. 957, 961, 959 prepared "cool", remainder "warm".

Exp. 957.

Period (min.) ...	15	15	30	30	30	30	Vitamin difference	
Res	1270	1000	785	540	350	330	+ 124	+ 100
ResV	1250	1090	900	610	480	390		
P	1840	1435	1120	750	551	370	+ 470	+ 600
PV	2090	1945	1680	1315	1140	914		
L	1870	1705	1330	976	742	519	+ 664	+ 650
LV	2535	2305	2050	1625	1395	1100		

Exp. 961.

L, LV, single estimations.

Cerebrum.

P	2120	—	1520	—	1090	745	490	+ 410	+ 710
PV	2355	—	2110	—	1815	1435	1130		
L	2100	—	1775	—	1330	1010	700	+ 505	+ 590
LV	2550	—	2335	—	1950	1575	1225		

Optic lobes, rest.

P	1590	—	1150	—	1070	710	485	+ 390	+ 480
PV	1910	—	1610	—	1485	1250	1060		

Exp. 959.

P	2190	1865	1260	945	750	—	+ 518	+ 595
PV	2570	2280	1900	1620	1265	—		
Ppp	2040	1670	1210	940	620	—	+ 223	+ 365
PppV	2105	1540	1540	1255	1035	—		
L	2640	2680	1830	1380	1040	—	+ 114	+ 347
LV	2625	2410	2200	1740	1375	—		

L, LV, single estimations.

Exp. 958.

Period (min.) ...	15	15	30	30	30	30	Vitamin difference	
P	—	1900	—	1200	900	624	+ 825	+ 838
PV	—	2670	—	2100	1760	1425		
L	—	2210	—	1645	1115	895	+ 588	+ 537
LV	—	2720	—	2310	1725	1360		
Lpp	—	2170	—	1590	1200	986	+ 190	+ 465
LppV	—	2290	—	1850	1725	1390		

Exp. 956.

P, PV, single estimations.

P	2260	1635	1160	775	470	440	+ 756	+ 905
PV	2760	2415	2032	1770	1280	1080		
Ppp	1970	1550	1180	940	560	395	+ 472	+ 490
PppV	(2350)	2110	1655	1320	1160	930		

How are the results of the previous work to be explained? There is no difference in the time of year at which the experiments were carried out. The older work was done with brain minced upon a warm plate at room temperature, which might have been rather low upon occasion. I have therefore repeated four such experiments, and quote two, Table II (a) and (b), Exp. 971. In all cases a good

Table II. *Effect of cool preparation and other changes.*

pH 7.3. Pyruvate as substrate always present.

(a) Increasing amounts of pyruvate (cool preparation).

Exp. 968. Normal cerebrum.

Period (min.)...	15	15	30	30	30	Vitamin difference	
						0-60	60-120
Pyr 0.023 M	3065	—	2505	2230	1780	—	—
Pyr 0.046 M	3095	—	2595	2235	1750	—	—
Pyr 0.069 M	2890	—	2680	2195	1670	Slight inhibition	

(b) Pyruvate: acid neutralized without dilution (WP).

Exp. 971. Avitaminous cerebrum (cool preparation).

P	1650	1050	800	616	508		
PV	2030	1650	1405	1240	1040	+ 550	+ 580
WP	1352	1090	785	610	500	+ 450	+ 410
WPV	1725	1550	1250	1070	850		

(c) Effect of calcium addition. Exp. 1, 0.015 M; Exp. 2, 0.0003 M CaCl₂.

Exp. 1. Avitaminous.

P	1410	1085	815	576	—		
PV	1860	1630	1475	1140	—	+ 580	564 (30 min.)
CaP	1105	870	745	560	—	+ 425	540 (30 min.)
CaPV	(1450)	1275	1220	1100	—		

Exp. 2. Avitaminous.

P	2300	1710	1355	972	765		
PV	2710	2390	2035	1720	1360	+ 610	+ 670
CaP	1990	1600	1250	1010	790	+ 530	+ 605
CaPV	2370	2090	1880	1650	1457		

(d) Comparison of crystalline vitamin (4γ) with old vitamin concentrate (46).

Exp. 970. Avitaminous.

P	—	990	—	698	505	372	
PV	—	1500	—	1210	968	810	+ 525 + 500
P	—	960	—	655	480	373	
P (46)	—	1430	—	1235	1020	835	+ 512 + 450

pyruvate effect was found. In Exp. 970, there was used the crude preparation of vitamin B₁ which had been previously employed by Meiklejohn *et al.* in their work. It had been stored for 3 years without change so far as could be ascertained. There was no evidence of any toxic effect either in this or in other experiments. We can therefore exclude the vitamin and also the conditions of mincing. This leaves us with possible variations in the medium, either in the inorganic constituents or in the pyruvate itself. Previously we had employed a Ringer solution which originally contained calcium. After bringing to pH 7.3 approximately, the precipitated Ca phosphate was filtered off. Later we used Ringer without calcium. We had not thought that there would be much difference in view of the smaller amounts of Ca involved, though Sinclair had found that presence of Ca produced slight inhibition. Further tests showed that the addition of traces of Ca make a difference, see Table II (c), but this is not enough

to remove the catatorulin effect with pyruvate.¹ This leaves us with only one possibility, the pyruvate itself. As was stated at the time, the pyruvic acid was highly purified by distillation and though not crystallized was considered to be pure enough for the purpose. But we did not realize then the profound change which can be made in pyruvic acid by neutralizing direct with *N* alkali before previous dilution with water. Peters & Thompson [1934] found that upon occasion a large change in bisulphite-binding power might rapidly occur. In the early experiments, in order to save volume, 80–100 mg. of purified pyruvic acid were weighed out into a tube and the alkali added direct. This seems to be dangerous, though the conditions under which the change occurs have not been worked out. In the experiment in Table II (b) the vitamin effect is reduced with pyruvic acid neutralized before dilution. With smaller amounts of pyruvate the reduction in pyruvate itself might be quite sufficient to account for the results. The early experiments lasted only for 1 hour: if continued they might even then have shown an effect. I think that they can be safely set aside as due to unrecognized experimental error. The present pyruvate gives only slight inhibition at 0.067 *M* (Table II (a)).

If the view is correct that the vitamin action is upon the pyruvate only, it should be possible to find that, in the early stages of an experiment, there is a larger catatorulin effect with pyruvate than with lactate. At a later stage, when sufficient pyruvate is present, formed from lactate, there should be no difference. A decisive experiment of this kind has proved difficult to make; it will clearly be much governed by details such as the rate of diffusion of either the pyruvate or lactate to the active centres concerned. By keeping for some time at the low temperature diffusion effects will be minimized, but there will be a possibility of sufficient action of the lactate oxidase even in the initial 12 min. required for equilibrium at 38°. Some trials of this point proved rather inconclusive, though there was a balance in favour of slightly increased catatorulin effect in the first half-hour only with pyruvate.

The evidence of the delayed substrate experiment.

As the result of delayed substrate experiments (Quastel and Wheatley technique), Peters *et al.* [1935] advanced the following view, considering respiration as a chain of reactions.

This hypothesis was an attempt to explain the following facts. With tissue suspended in Ringer phosphate only, with and without addition of vitamin, the increase upon addition of substrate was greater for tissue previously incubated with vitamin and greater still for vitamin plus pyrophosphate. These facts have been confirmed recently, but at the same time other findings have led to the substitution for (2) of the alternative interpretation, first proposed by the same authors in a footnote. According to the latter, the facts observed in this type of experiment are due to the protection of the essential factor from irreversible destruction. This interpretation now supports instead of opposing the pyruvic oxidase theory.

¹ The effect of Ca is related probably to the colloidal ion effect, see Ashford & Dixon [1935], Dickens & Greville [1935].

The sequence lactate-pyruvate at acid reactions.

I was led to revise views upon hypothesis (2) by fruitless attempts to confirm it directly (with Passmore), and by experiments with the delayed technique, at acid reaction. Peters & Sinclair [1933] had shown that at *pH* 6.6 (instead of the normal *pH* 7.3) there was no catatorulin effect with lactate in absence of pyrophosphate, whereas with pyruvate an effect was found. They had however been unable to demonstrate formation of pyruvic acid by colour reaction; this had been another point irreconcilable at the time with the pyruvic oxidase theory. In the more recent and similar Exp. 861, Table III, made under more modern

Table III.

Acid Ringer phosphate *pH* 6.4. Lactate and pyrophosphate.

Exp. 861. Warm preparation. Avitaminous.

Period (min.) ...	0-15	15-30	30-60	60-80	Vitamin differences			
L	1480	940	650	430	170	160	140	134
LV	1600	1100	790	564				
Lpp	1260	1010	750	570	80	130	260	330
LppV	1340	1140	1010	900				
P	1310	950	595	365	250	300	415	435
P + V	1560	1250	1010	800				

Exp. 866. Warm preparation. Normal brain.

Period (min.) ...	0-15	15-45	45-75	75-105	105-135
(1) O	1530	1170	708	537	400
(2) L 0.041 <i>M</i>	1580	1440	1030	725	615
(3) L 0.082 <i>M</i>	1160	1270	1115	775	627
(4) L 0.164 <i>M</i>	865	1035	800	740	526
(5) Lpp 0.041 <i>M</i>	1430	1340	1080	1020	918
(6) Lpp 0.082 <i>M</i>	1150	1000	875	815	675
(7) Lpp 0.064 <i>M</i>	755	755	615	514	527

Designed to explore the optimum concentration of lactate at this *pH* and the relation to pyrophosphate.

Exp. 863. Warm preparation. Normal brain.

Period (min.) ...	15-45	45-75	75-105	105-135
Nil	1090	576	422	400
L 0.0035 <i>M</i>	1520	1040	785	615
L 0.015 <i>M</i>	1082	925	700	580
PyT 0.017 <i>M</i>	1800	1570	1330	1142

conditions, we see that with the "warm" minced tissue there was a slight catatorulin effect with lactate without pyrophosphate. With lactate + pyrophosphate, the vitamin effect as also the respiration is slightly reduced at first, but later increased over that of lactate alone. With pyruvate alone a large difference was present. The experiment is therefore in the same sense as the earliest ones, but the loss of vitamin effect in lactate is not so pronounced.

Initial reduction of respiration in lactate at *pH* 7.3 by pyrophosphate has now been well established by Johnson [1936].

Exp. 866 (Table III) is a complete experiment upon normal tissue and shows again the inhibition with pyrophosphate which is present for $1\frac{1}{4}$ hours only. After this the pyrophosphate has exerted a marked improvement upon the "lactate" respiration. Inhibition of the respiration, especially during the initial

periods, is seen with increasing concentration of lactate alone, but this becomes much more marked when pyrophosphate is present; compare for instance L and Lpp (0.164 *M*). In the latter case, with the action of pyrophosphate the increase of O₂ uptake over the residual has been entirely eliminated. The lactate at this *pH* (with the normal brain) does not nearly reach the level of pyruvate respiration, as is shown by Exp. 863. Note again that even at a higher concentration of lactate there is inhibition only for 1½ hours. It is clear that the relations with pyrophosphate are complex.

The above facts are consistent with the view that the substrate also at this *pH* producing the largest respiratory effect is pyruvate. Exp. 866 is explained if pyrophosphate is helping the conversion of lactate into pyruvate.

It remains to be proved that pyruvic acid is formed at *pH* 6.6. Table IV gives the few experiments made upon this point. With normal brain (two experiments) there was a slight formation of pyruvate (B. B. S.) from lactate

Table IV. *Formation of pyruvate at pH 6.4.*

Brain tissue allowed to respire in presence of L/pyrophosphate, V or iodoacetate, 0.1 mg./ml. for 2 hours; after cooling 1 ml. of 25% trichloroacetic acid added, filtered and bottles washed with 1 ml. + 1 ml. 5% trichloroacetic acid. Samples (2 ml.) analysed for bisulphite-binding substances (Clift & Cook) or by 2:4-dinitrophenylhydrazine. (Case as modified by Peters & Thompson.)

Figures are mg. pyruvic acid/g. tissue formed in 2 hours, and are means from duplicate bottles estimated separately, except in 1146 where the two bottles were mixed and so estimated.

Exp.	Condition	L	LV	Lpp	LppV	LIAA	Cool preparation	
							LIAApp	Method
995	Normal	0.9	—	0.53	—	—	1.24	Bisulphite-binding (BBS). Duplicate poor
996	"	0.51	—	0.33	—	0.47	0.81	"
998	Avitaminous	1.66	1.16	2.00	1.10	—	—	"
1146	"	1.42	0.45	1.17	0.35	—	—	Hydrazone
1153	"	0.91	—	0.38	—	1.67	1.21	BBS

only (Exps. 995, 996); this was reduced by pyrophosphate, which increased O₂ uptake in 2 hours, but was again increased by adding iodoacetic acid. In the one experiment tried, there was more pyruvate formed in presence of pyrophosphate. With the avitaminous brain there was less pyruvic acid present with the pyrophosphate in spite of the increased O₂. The few results justify the conclusion that pyruvic acid is formed, though in lower amounts than at *pH* 7.3; but there is no experimental support for the suggestion that pyrophosphate improves the yield of pyruvate; actually it was decreased.

Interpretation of the delayed substrate experiments.

At first sight these experiments are much more difficult to reconcile with the view that the sequence of oxidation is lactate→pyruvate. By working at *pH* 6.6, however, it is possible to dissect the reactions better.

Exp. 863 (Table V) shows the extent of variations in the initial respiration which may be expected to be due to experimental error and that addition of vitamin to the normal has no influence, a now well-established fact; again in the delayed substrate experiment there is a much larger respiration with pyruvate than with lactate at this *pH*.

The effect of pyrophosphate (Exp. 878) in the initial stages appears to provide a much clearer argument for the scheme proposed by Peters *et al.* even than before. In absence of the substrate, respiration with and without pyrophosphate

Table V. *Delayed substrate.*

Normal cerebrum. pH 6.6. Warm preparation.
Additions during course of experiment at arrow ↓.

O=Ringer phosphate alone; pp=pyrophosphate; P=pyruvate; L=lactate. Addition at arrow made to bottles after removal from the bath. Bottles refilled with O₂ and returned to bath. Single estimations.

Exp. 863.

Period (min.)...	30	30	30	↓	20	25	36	30
O	2220	1575	960	LV	765	905	725	602
V	2020	1475	920	L	800	815	645	512
O	2130	1485	925	PV	940	1115	970	850
V	2060	1462	932	P	1030	1095	985	860

Exp. 878.

Period (min.)	30	30	30	↓	15	15	20	15	30	30
O	1685	1180	730	Ppp	674	800	665	515	585	460
pp	1370	940	650	P	1280	1330	1330	1215	1270	1170

Period (min.)	30	30	30	30		30	↓	15	15	15	15	15	30	15
O	1450	1040	754	480		360	Ppp	290	195	70	218	140	140	175
pp	1310	855	674	440		370	P	870	880	912	962	1070	960	960

reaches approximately the same level, but upon addition of the substrate the difference in respiration indicates the extent of the underlying difference in the state of the tissue.

The longer the addition of the substrate is delayed (Exp. 878, Table V) the more marked is the effect, even after an initial period of 1½ hours in which respiration of O and pp have been identical within experimental error for at least an hour. The reaction of the pyruvate oxidase is not a function of the respiratory state of the tissue, but of some reaction here improved by pyrophosphate.

The next experiment (862, Table VI) adds the vitamin effect but introduces a new point. Without substrate, we see a slight effect of vitamin. When substrate is added after 1½ hours, pyruvate is more effective than lactate. Lactate in the initial period without addition of pyrophosphate was inhibitory, in this case even with vitamin. Subsequent addition of pyruvate was not effective in giving a large respiration in the last hours unless pyrophosphate was present.

In Exp. 1161 again lactate is less effective than pyruvate in restoring respiration after some 2 hours. This suggests that some part of the lactate oxidase system has broken down in this time. Further, some part of the system is better maintained in the presence of lactate, pyrophosphate and vitamin, viz. that which interacts with pyruvate.

For stabilization of pyruvate oxidase we do not need to use large amounts of lactate. In Exps. 896 and 897 the chief points which need comment are that 1 mg. of lactate in the initial period added with pyrophosphate gives a low respiration compared with pyruvate after 1½ hours of respiration, but maintains the pyruvate oxidase system intact. This can be seen by the recovery to the value given by pyruvate alone in the initial period upon adding pyruvate as a delayed substrate. For demonstration of this maintenance 0.5 mg. of lactate upon occasion has proved to be effective for a period up to 2 hours (Exp. 899), the action of lactate in this respect requiring pyrophosphate and vitamin. In other

Table VI. *Avitaminous and normal brain. Delayed substrate. Vitamin and effect of small amounts of lactate upon stability of pyruvate.*

pH 6.4. Warm preparation.

Additions at arrows.

Exp. 862. Avitaminous (single estimations).

Period (min.) ...	30	30	30	30	26	15	30	30
O	1400	1200	1095	795	↓ LV	630	407	365
OV	1520	1440	1135	895	L	765	585	506
O	1490	1310	960	685	PV	585	464	460
OV	1600	1520	1195	950	P	945	780	700
L	965	—	660	—	543 PV	755	632	610
LV	1455	—	1155	—	795 P	655	504	467
Lpp	1160	875	805	705	PV	495	412	370
Lpp, V	1320	1170	1060	990	P	1045	985	878

Exp. 1161. Avitaminous.

Period (min.) ...	15	23	29	↓	60
pp	1235	950	780	PV	798
ppV	1680	1244	1075	P	1362
pp	(1275)	(990)	750	LV	880
ppV	(1545)	(1200)	(1120)	L	1182

Exp. 896. Normal cerebrum.

Period (min.) ...	15	45	30	30	30	15	20	20	20
pp	1400	995	640	455	↓ Pyr	980	1060	1030	990
Pyr pp	2730	2460	2150	1950		1700	1550	1460	1350
L 0.0037 M pp	2335	1980	1720	1480	Pyr	1810	1680	1620	1560
L 0.0055 M pp	(2170)	(1950)	(1700)	(1550)	Pyr	1680	1615	1540	1500

Exp. 897. Normal cerebrum.

Period (min.) ...	30	30	30	30	30	15	15	20	20	20
Pyr 0.015 M pp	2375	2180	2050	1855	↓ Pyr	1630	1500	1370	1300	1205
Pyr 0.015 M, L 0.0037 M pp	2275	2050	1900	1760	Pyr	1550	1415	1265	1250	1120
L 0.0037 M pp	1955	1645	1575	1360	Pyr	1655	1520	1475	1290	1225
L 0.011 M pp	1915	1760	1610	1540	Pyr	1610	1580	1460	1300	1300

Pyruvate at arrow added to total concentration of 0.023 M.

Exp. 899. Avitaminous cerebrum.

Period (min.) ...	30	30	30	30	30	15	15	30	30
L 0.0018 M pp	1420	1125	980	840	↓ PyrV	850	790	760	800
LppV	1730	1385	1215	1080	Pyr	1275	1300	1140	1110

Table VII. *Pyruvate and pyrophosphate.*

Exp. 871. Normal brain; pH 6.4; warm preparation.

Period (min.) ...	0-15	15-45	45-75	75-105	105-120
O	1170	1180	840	750	355
pp	1195	910	672	625	390
Pyr 0.016 M	2055	1910	1870	1780	1535
Pyr 0.016 M pp	2200	1990	1860	1753	1625
Pyr 0.032 M	1925	1780	1660	1485	1335
Pyr 0.032 M pp	2010	1890	1645	1510	1290

experiments it has been found that at this *pH* pyrophosphate has very little influence upon the respiration of pyruvate, see Exp. 871, Table VII.

There is inhibition here with excess of pyruvate but the pyrophosphate has very little influence upon O_2 uptake. The essential point at this *pH* then is the linkage of pyrophosphate and lactate and the independence of pyruvate and pyrophosphate. In absence of pyruvate, lactate and pyrophosphate together with vitamin are essential for the maintenance of the stability of the pyruvate oxidase system.

The experiments given in Table V have been selected from a large number of attempts to decide these points, and I regard them as significant. In general I have reached the conclusion that the delayed substrate experiment is so much affected by problems of diffusion that it is not a valuable technique. It will be clear that the demonstration of these discontinuities between the lactate and pyruvate systems requires rather fine adjustment. The pyruvate oxidase is very unstable in absence of either pyruvate or vitamin at 38° , hence the decisive point in the experiment may often be missed. The peculiar details of the survival respiration in these mixed enzyme systems indicate again that the most important component is pyruvate. In absence of either lactate plus pyrophosphate or of pyruvate itself, the pyruvate oxidase system appears unstable; rapidly disappearing at 38° .

Further it is clear that some component of the lactate oxidase system is also unstable at this *pH*; this might be the coenzyme in view of recent work by Green & Brosteaux [1936] but this needs proof. Another important component might be lactoflavin; hence the importance of preparing specimens under dark conditions, as has of late been uniformly our practice. The picture fits scheme (1) modified as (1*a*), but not the old scheme (2).

provided we assume that the pyruvate oxidase is unstable in absence of pyruvate or of vitamin B_1 .

Both lines of evidence now strengthen the pyruvate oxidase theory instead of being opposed to it. There still remain some unreconciled data. (a) Peters & Thompson [1934] could find no consistency in the ratio $\frac{\text{Pyruvate disappearing}}{\text{Extra oxygen uptake}}$ either at *pH* 7.3 or 6.6. Further, the vitamin effect was larger with lactate than with pyruvate; this is not immediately to be reconciled with the action of vitamin B_1 only on the pyruvate either preformed or arising from lactate. (b) Peters *et al.* [1935] found that fluoride inhibited the appearance of pyruvate in presence of lactate, as well as inhibiting the O_2 uptake with pyruvate; but fluoride has not been supposed to stop the action of lactate oxidase. (c) The failure to increase pyruvate at acid *pH* values with pyrophosphate.

It is believed that these difficulties can be met in future work, and that the bulk of the evidence is now sufficient to advance the pyruvate oxidase theory of vitamin B_1 action. In subsequent papers, we intend to deal with the question whether the effect is direct or indirect; our present view is that it is direct and that there is no evidence that it is concerned with anaerobic change in our experiments. Rydin [1935] it will remembered could obtain no evidence at all for the view that vitamin B_1 catalysed any anaerobic reaction with methylene blue.

Another interpretation is now given to the experiments of Quastel & Wheatley [1932], with the autoxidized brain tissue; it must be true as they thought that they were studying the oxidation of pyruvate (or lactate), but the

extent of this oxidation does not depend only upon the actual oxidation level before adding the substrate; it is also a function of the inherent stability of factors in the tissue as influenced by the preliminary period; the effect of traces of substrate in this period cannot be neglected.

In case there may still be those who are uncomfortable about the applicability of these results upon teased brain to those obtained with organized slices of tissue, we may stress the similar behaviour of pyruvate *in vivo* and *in vitro*, the fact that we can show up calcium effects in the teased tissue, and more important still that the phenomena as regards pyruvate can be demonstrated perfectly well with slices of tissue. There is really no ground at all for thinking that the particular reaction of pyruvate involving the vitamin which has been here studied does not take place *in vivo* or in the more highly organized tissue, or that the results are not applicable. This does not mean that in the more organized tissue slice some of the products of oxidation may not be brought into contact with other enzyme systems, and so modified. It is likely that the results are general for brain, as O'Brien and Peters showed formation and disappearance of pyruvate from lactate in rat's brain; and very recently Sherman & Elvehjem [1936] report a study upon chicken's brain which confirms in detail the work of the author and Thompson [1934].

In connexion with work upon the relation of the action of vitamin B₁ and its chemical structure, it is noteworthy that the action of the vitamin can be exerted at an acid reaction as well as at a *pH* at which there will be a profound alteration of the ionization of the quaternary grouping. Hence a direct relation to the action of this grouping is not indicated, though it is hard to believe that the two are not related. Possibly the ionization at the surface of the enzyme is changed as compared with that in solution; the author has shown elsewhere [1931] that this is so for an amine group at an interface; the apparent dissociation constant was found to be altered by 3 *pH* units for palmitic amide at the interface benzene-buffer solution. If this applies to the quaternary group of the vitamin the region of dissociation would be shifted to the acid side, i.e. in the right direction.

Summary of effects with pyrophosphate.

The following facts must be covered by an explanation of the action of pyrophosphate.

At acid pH values. Lactate respiration is initially reduced and later stabilized. Vitamin effects improved. No evidence that pyruvate formation is increased. Pyruvate respiration unaffected. It interacts with lactate.

At pH 7.3. Lactate respiration initially reduced, and later improved. Vitamin effect improved later. Pyruvate partly inhibited. Disappearance of α -glycerophosphate improved [Johnson, 1936].

At pH 7.7. Lactate respiration inhibited up to 1.5 hours: pyruvate respiration inhibited.

Though the oxidation of lactate via pyruvate is almost a postulated textbook step, it is important to have this clearer proof that it is an important channel of oxidation for lactate in brain. This work does not decide whether it is the only method of oxidation; a problem which awaits quantitative investigation.

Peters *et al.* [1935] found that from the 2nd to the 3rd hour with avitaminous tissue in lactate solution (*pH* 7.3), poisoned with iodoacetate, 0.924 mg. pyruvate was formed and some 400 μ l. O₂/g. taken up. The conversion of lactate into pyruvate would need about 115 μ l.; the residual respiration for such a period is usually about 250–300 μ l. Hence, the extra O₂ uptake is of the right order for

conversion of lactate mainly through pyruvate. Much of the maintenance produced by pyrophosphate with lactate must be due to the modifications in the early rate of oxygen uptake.

EXPERIMENTAL.

In the experiments here described, the technique of Kinnersley *et al.* [1935, Appendix] has been followed throughout; only essential points are therefore noted in the Tables. Pyruvic acid has been prepared from crystals, kept frozen in cold store; it was made up to be about 5 *M* in amounts of 10 ml. as required and kept at 0°. Samples were neutralized immediately before use, Ringer phosphate being added before the *N* alkali. Pyrophosphate was also freshly made up, from the dried Na salt. Of late it has been my practice to add the substrate immediately before mashing the tissue, after weighing. After this the tissue can be allowed to stand for some time at room temperature. In several experiments it has been found that the O₂ uptake at 30° and also the vitamin effect are not much influenced, even by a long period of shaking, up to 2 hours in air at 15–20°. Evidently the enzyme systems may be considered to be fixed at this pH, so that the only necessity is to allow time for diffusion to take place.

Unless otherwise stated, oxygen uptakes (Barcroft differential technique) are the average of duplicates. Na lactate 0.035–0.04 *M* has been made from Zn lactate; Na pyruvate concentration has been 0.020 ± 0.004 *M* according to convenience, and Na pyrophosphate (brought to pH used) 0.0075 *M*. In Exp. 1161 the amounts were: Li lactate 0.001 *M*, Na pyrophosphate 0.01 *M*. These variations do not influence the present results. Latterly it has been the practice to neutralize the pyruvic acid with a mixture of KOH and NaOH (*N*) in ionic concentration equivalent to the rest of the Ringer phosphate to avoid possible K complications [Ashford & Dixon, 1935]. The expression "brain" refers to the mixed cerebral hemisphere and optic lobes. Crystalline vitamin (natural) 2.5 ± 0.5 γ has been added when indicated; the exact amount is not recorded, as the concentration is maximum and wide increase produces no further change.

SUMMARY.

1. Evidence against the conception that lactate is directly oxidized to pyruvate in pigeon's brain tissue as a preparatory stage to complete oxidation has been re-examined and rejected, so that the view can now be advanced that the action of vitamin B₁ is related specifically to pyruvate oxidase in its aerobic reaction.

2. The course of reaction can now be represented as

3. The best substrate for demonstrating the catatorulin effect is pyruvate.

4. Lactate and pyruvate oxidase systems can be separated at acid pH values.

5. Vitamin B₁ and pyruvate are necessary to ensure stability of pyruvate oxidase at pH 6.6.

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