CXV. *IN VITRO* STUDIES ON LACTIC ACID METABOLISM IN TISSUES FROM POLYNEURITIC CHICKS.¹

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THE idea that disorders observed in polyneuritis are due to the accumulation of intermediate substances originating from carbohydrate breakdown which cannot be metabolised in the absence of vitamin B₁ has prevailed since the pioneer work of Eijkman.

The investigations of Peters and his associates have greatly increased our knowledge of the function of the vitamin and indicate that its action is concerned with the metabolism of lactic and pyruvic acids. Kinnersley and Peters [1929] found an increased amount of lactic acid in the brain of polyneuritic pigeons as compared with the normal. Meikeljohn *et al.* [1932] and Passmore *et al.* [1933] have demonstrated that minced cerebrum from polyneuritic pigeons has a low rate of oxygen uptake in the presence of lactate. This lowered rate of respiration was brought back nearly to normal by the addition of small amounts of vitamin concentrate *in vitro*. However, Meikeljohn [1933] found that brain from polyneuritic pigeons readily removes lactic acid and that the increased oxygen uptake with lactate as substrate was not accompanied by an increased lactate removal.

Birch and Mann [1934] have reinvestigated Boyland's [1933] work on the hypothesis that vitamin B₁ may act as a coenzyme for lactic dehydrogenase. Using a more highly purified dehydrogenase preparation they have shown that the coenzyme for lactic dehydrogenase may be divided into two factors designated by them as coenzyme "A" and coenzyme "B". Evidence was presented to indicate that coenzyme "B" contains a factor responsible for the removal of pyruvic acid. Small amounts of pyruvic acid were shown to exercise an inhibitory effect upon the rate of lactic acid dehydrogenation in this isolated enzyme-coenzyme system. Tests for the vitamin B₁ activity of these coenzymes based upon the cure of bradycardia in polyneuritic rats showed that, although "A" was inactive, "B" exercised some temporary curative effect.

From the work quoted above it is generally concluded that in pigeon brain vitamin B₁ is concerned with some phase of lactic or pyruvic acid metabolism. Since all these tissue oxidation investigations have been carried out upon mature pigeons which have been depleted of their vitamin B₁ stores by polished rice feeding, a slow method requiring at least 30 days to produce head retractions,

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we were interested in applying similar methods of study to the polyneuritis produced in chicks by feeding a vitamin B₁-deficient ration developed in this laboratory by Kline *et al.* [1932] and described in detail by Elvehjem [1935]. This ration has the advantage of being so low in vitamin B₁ that polyneuritis is developed in chicks within 2 weeks and yet furnishing ample amounts of the other known factors.

EXPERIMENTAL.

The present investigations have been carried out on young growing White Leghorn chicks weighing 300–500 g. Day-old chicks were placed upon ration 351 [Kline *et al.*, 1934], a complete ration, until they had reached a weight of 250 g. Birds to be used as normals were continued upon this ration, and the experimental chicks were changed to the vitamin B₁-low ration 242 A, which produced acute opisthotonus in 250-g. chicks in 2 weeks. It was found advantageous to grow all chicks to 250 g. weight before placing them upon the vitamin B₁-low ration in order to ensure organs of sufficient size for respiration studies.

Technique employed in respiration studies.

All polyneuritic chicks showing acute opisthotonus and normals were killed by decapitation. After allowing the blood to drain from the neck for 1 min., the cerebrum and other organs to be studied were uncovered and removed to a watch glass where they were gently wiped with filter-paper to remove adhering blood. All tissues were finely minced at room temperature with chromium-plated scissors. Six samples were weighed upon pieces of glass made from coverslips and all introduced at once into the flasks of the Barcroft apparatus,¹ previously filled with buffer, substrate and vitamin (when used) to make a final volume of 3 ml. Rolls of filter-paper were inserted in the absorption tubes which contained 0.5 ml. of 6% NaOH. After the clumps of minced tissue had been broken up with a glass rod, the flasks were attached to the manometers, immersed in the water-bath at 37° and shaken for 10 min. to ensure temperature equilibrium. The stopcocks were closed after the zero reading had been taken, and successive readings were made at 20-min. intervals.

The Ringer phosphate buffer used was made as follows: 20 vols. of $M/2$ Na₂HPO₄ and 80 vols. of Ringer's solution (NaCl 0.9%, KCl 0.025%, CaCl₂ 0.02%, NaHCO₃ 0.015%) were mixed. Enough $N/10$ HCl was added to bring the final solution to p_H 7.3. This solution was diluted to 300 vols. and was then $M/30$ in phosphate concentration.

In many experiments Ringer phosphate *plus* pyrophosphate buffer was used and was made as follows: 20 vols. of $M/2$ Na₂HPO₄, 10 vols. of 0.3 M Na₄P₂O₇, 10H₂O and 70 vols. of the above Ringer's solution were mixed, adjusted to p_H 7.3 with $N/10$ HCl and diluted to 300 vols., making the final solution $M/30$ in phosphate and $M/100$ in pyrophosphate.

The vitamin B₁ concentrate used was Oryzanin Fortior "Sankyo".² An aqueous solution of the concentrate was made to contain 25γ per ml. A 0.1 ml. aliquot containing 2.5γ was added directly to the flasks just before they were attached to the manometers.

Merck's sodium lactate was adjusted to p_H 7.3 and diluted to give 3% lactic acid. 0.2 ml. aliquots were added to the right- and left-hand flasks from which an equal amount of buffer was withheld, making 0.2% lactic acid in the flasks.

¹ The Barcroft apparatus used in these studies has been described by Stare and Elvehjem [1933].

² Obtained from the Takamine Corporation, 132 Front Street, New York City.

EXPERIMENTAL RESULTS.

Oxygen uptake studies.

Table I shows the results from 18 experiments to determine if tissues from polyneuritic chicks show any abnormalities in their utilisation of lactate. Although the oxygen uptake of the avitaminous cerebrum in the absence of

Table I. *Effect of lactate upon the oxygen uptake of normal and polyneuritic cerebrum and heart tissue in Ringer phosphate buffer, p_H 7.3, 37°.*

O ₂ uptake in $\mu\text{l./g./hr.}$									
Exp.		Period (min.)			Exp.		Period (min.)		
		0-40	40-80	80-120			0-40	40-80	80-120
Normal cerebrum.					Avitaminous cerebrum.				
1	No lactate	1210	970	720	7	860	830	600	
	Lactate	1430	1630	1250		1310	1140	930	
2	No lactate	1090	930	810	8	980	880	760	
	Lactate	1380	1230	1060		1400	1510	1030	
3	No lactate	980	730	610	9	650	720	460	
	Lactate	1530	1340	1040		1030	1030	850	
4	No lactate	1040	850	710	10	940	870	670	
	Lactate	1010	1370	1210		1310	1190	1030	
5	No lactate	1140	850	620	11	1150	960	720	
	Lactate	1670	1380	1320		1030	1400	1240	
6	No lactate	1140	750	590	12	940	870	440	
	Lactate	1540	1320	1370		1350	1210	970	
Av.	No lactate	1160	850	680		920	860	610	
Av.	Lactate	1430	1380	1210		1260	1270	1000	
Av.	Increase	270	530	530		340	410	390	
Normal heart.					Avitaminous heart.				
13	No lactate	414	330	283	16	193	198	119	
	Lactate	519	388	330		250	190	129	
14	No lactate	220	175	127	17	276	225	298	
	Lactate	365	300	235		279	181	255	
15	No lactate	377	170	167	18	273	195	168	
	Lactate	453	278	258		243	208	184	
Av.	No lactate	337	225	192		247	206	195	
Av.	Lactate	446	322	274		257	193	189	
Av.	Increase	109	97	82		10	-13	-6	

substrate was consistently lower than that of normal tissue, the increase in oxygen uptake produced by the addition of lactate to avitaminous cerebrum was not significantly less than that produced by similar additions to normal tissue. Avitaminous heart muscle, on the other hand, seemed to show a marked abnormality in its utilisation of lactate in a majority of cases.

In order to determine whether this apparently decreased oxygen uptake was due to a deficiency of the vitamin in the heart muscle, experiments were carried out with additions of vitamin B₁ concentrate to avitaminous heart muscle respiring in lactate substrate. Similar additions of vitamin B₁ were made to avitaminous cerebrum for comparison with results obtained in Peters's laboratory. Typical results are given in Table II. In avitaminous chick cerebrum the addition of 2.5 γ of vitamin B₁ concentrate had no stimulating effect upon the oxygen uptake in lactate-Ringer phosphate *plus* pyrophosphate solution. However, in heart muscle the vitamin significantly increased the oxygen uptake in the

Table II. *The effect of vitamin B₁ concentrate upon the oxygen uptake of avitaminous tissues.*

LRP = lactate-Ringer-phosphate.

PP = pyrophosphate.

RP = Ringer-phosphate.

V = vitamin 2.5γ.

		O ₂ uptake in μ l./g./hr.				
		Period (min.)			Average	% increase with vit. B ₁
Exp.		0-40	40-80	80-120		
Cerebrum:						
19	RP	940	868	441	750	+ 1
	LRP	1347	1203	959	1170	
	LRP + PP	1321	1128	1080	1176	
	LRP + PP + V	1239	1217	1140	1198	
20	LRP	1233	1342	1200	1258	- 14
	LRP + PP	1287	1240	1155	1227	
	LRP + PP + V	965	1065	1108	1046	
21	LRP	1465	1126	1066	1219	+ 2
	LRP + PP	1352	1130	1126	1203	
	LRP + PP + V	1236	1242	1188	1222	
Heart muscle:						
22 a	RP	281	241	159	227	
	LRP	382	272	207	287	
	LRP + V	404	247	225	292	
22 b	RP + PP	385	213	146	248	12
	LRP + PP	538	247	200	328	
	LRP + PP + V	617	212	265	365	
23	LRP	419	209	106	261	71
	LRP + PP	293	188	207	229	
	LRP + PP + V	562	324	289	392	
24 a	RP	225	190	120	178	
	LRP	313	238	263	271	
	LRP + V	345	241	225	270	
24 b	RP + PP	268	155	100	174	12
	LRP + PP	337	188	279	268	
	LRP + PP + V	395	243	264	300	
25	RP	488	202	237	309	
	LRP	547	417	440	468	
	LRP + V	574	355	387	438	
26	RP + PP	312	229	249	263	20
	LRP + PP	349	382	302	344	
	LRP + PP + V	433	377	432	414	

presence of pyrophosphate. In Exps. 22 *b*, 23, 24 *b* and 26 increases of 12, 71, 12 and 20 % respectively were observed in flasks containing added vitamin. In the absence of pyrophosphate (Exps. 22 *a*, 24 *a* and 25) the vitamin appeared to have no significant effect upon the rate of respiration of avitaminous heart.

Lactic acid removal by normal and avitaminous cerebrum and heart tissues.

The ability of avitaminous tissues (especially heart) to utilise lactic acid as substrate for oxygen uptake was so much below normal that it was decided to check this lowered rate of respiration by chemical determination of lactic acid removal.

The technique adopted was similar to that employed by Meikeljohn [1933]. After the minced tissues had respired for 2 hours the respirometers were removed

from the bath, and the contents of the flasks immediately washed into small mortars containing 1 ml. of 20% CCl_3COOH (addition of CCl_3COOH to the flasks before removal of the tissues was avoided since it caused particles of tissue to stick firmly to the walls of the flasks). After thoroughly grinding, the contents of the mortars were washed into 15 ml. centrifuge-tubes and centrifuged. The clear liquid was poured into 25 ml. volumetric flasks and the tissue residue extracted twice more with 5 ml. portions of 4% CCl_3COOH . The extracts were added to the 25 ml. volumetric flask in each case and finally brought to 25 ml. vol. Aliquots were taken for lactic acid determinations according to the method of Friedemann and Graeser [1933]. The values reported represent mg. lactic acid removed in 2 hours and 15 min. since the tissues were respiring in the presence of their substrates for 10 min. before the stopcocks were closed and an additional 5-min. period elapsed between the final reading of the manometers and the emptying of the flask contents into the mortars.

Reference to Table III shows that avitaminous cerebrum was able to remove lactic acid at approximately the same rate as normal. However, the rate of removal in avitaminous heart was less than half that of normal tissue.

Table III. *Aerobic removal of lactic acid by normal and avitaminous tissues.*

Fresh wt. of tissue g.	Lactic acid added mg.	Lactic acid present after 2½ hrs. mg.	Lactic acid removed in 2½ hrs.		Fresh wt. of tissue g.	Lactic acid added mg.	Lactic acid present after 2½ hrs. mg.	Lactic acid removed in 2½ hrs.	
			mg.	mg./g.				mg.	mg./g.
Normal cerebrum.					Avitaminous cerebrum.				
0.130	0	0.41	—	—	0.144	0	0.48	—	—
0.134	5.77	5.43	0.75	5.55	0.142	5.61	5.43	0.66	4.58
0.122	0	0.26	—	—	0.145	0	0.37	—	—
0.124	5.80	5.74	0.32	2.60	0.147	5.61	5.54	0.44	3.00
0.139	0	0.19	—	—	0.205	0	0.38	—	—
0.138	5.39	5.10	0.48	3.48	0.207	5.60	5.41	0.47	2.30
Av. removal			0.52	3.88				0.52	3.30
Normal heart.					Avitaminous heart.				
0.250	0	0.80	—	—	0.256	0	1.15	—	—
0.248	5.77	5.90	0.67	2.70	0.254	5.61	6.37	0.39	1.54
0.210	0	0.74	—	—	0.212	0	0.90	—	—
0.215	5.80	5.97	0.58	2.70	0.209	5.61	6.30	0.21	1.00
0.192	0	0.62	—	—	0.220	0	0.71	—	—
0.188	5.39	5.50	0.51	2.72	0.222	5.60	6.05	0.26	1.17
Av. removal			0.59	2.71				0.29	1.27

A possible fallacy in our calculations requires discussion at this time. It was assumed that the amount of lactic acid which accumulated in the control tissues, to which no additions of lactic acid were made, also accumulated in the flasks containing tissues with added lactic acid. Since this lactic acid accumulates as a result of the action of the glycolytic enzyme system which is dependent upon the oxidation-reduction potential of the suspension [Michaelis and Runnström, 1934], this assumption seems justified. It is believed that the addition of sodium lactate has not inhibited the formation of lactic acid.

Lactic acid dehydrogenase activity of avitaminous and normal tissues.

Lactic acid dehydrogenase activity was measured directly in avitaminous and normal cerebrum and heart tissues by means of the Thunberg [1920] technique. The tissues to be studied were removed from the chick, weighed and

ground with fine quartz sand in 5 cm. mortars. The ground tissues were then washed into tall 50 ml. graduated cylinders with Ringer phosphate solution and diluted with Ringer phosphate to a volume which would make a 5% suspension of the tissue. The contents of the cylinder were thoroughly mixed by shaking, and allowed to settle for 1 min., and 2 ml. aliquots were transferred to Thunberg tubes from the clear tissue suspensions. When substrate was used 2 ml. of 0.5% lactate adjusted to p_H 7.3 were added to the tubes. Ringer phosphate replaced the lactate in experiments where no substrate was used. Exactly 1 ml. of 1:5000 methylene blue was pipetted into the bulb of the Thunberg tube, making the total volume 5 ml. The tubes were evacuated, their contents mixed and placed in a water-bath at 37°. Typical results are given in Table IV.

Table IV. *Decoloration of methylene blue by 5% suspensions of normal and avitaminous tissues in Ringer phosphate and lactate-Ringer phosphate.*

Experiments run at 37°, p_H 7.3 in Thunberg tubes. Values are reported in min. for 80% decoloration of 1 ml. 1:5000 methylene blue.

Normal		Avitaminous		Normal		Avitaminous		
No lactate	Lactate	No lactate	Lactate	No lactate	Lactate	No lactate	Lactate	
Cerebrum.				Cerebellum.				
30	7	25	6	24	6	25	6	
32	8	25	7	29	8	35	7	
29	6	35	7	20	7	30	7	
Av.	31 (10)*	7 (11)	29 (6)	7 (6)	25 (8)	7 (10)	29 (6)	7 (6)
Optic lobes.				Heart.				
30	8	25	8	30	4	90	18	
32	8	30	8	20	6	60	16	
31	10	35	9	40	5	60	24	
				13	9	90	60	
				30	9	35	10	
				60	4	30	12	
				11	4	60	30	
Av.	31 (8)	9 (10)	31 (6)	9 (6)	29 (7)	6 (7)	61 (7)	24 (7)

* Numbers in parentheses indicate the number of experimental animals used.

The ability of chick cerebrum, cerebellum and optic lobes to reduce methylene blue was not decreased in avitaminosis. However, in avitaminous heart muscle, the results indicated that there was a lowered ability to reduce methylene blue. This subnormal reducing rate still persisted in the presence of lactate. It was found that normal heart tissue reduced methylene blue in one-fourth the time required for avitaminous tissue to reduce the same amount of methylene blue.

In order to determine if this lowered reducing activity in avitaminous heart muscle were a primary result of vitamin B₁ deficiency, additions of vitamin B₁ were made to avitaminous tissue in Thunberg tubes. Ringer phosphate-pyrophosphate buffer was used throughout. It may be seen from the results given in Table V that the *in vitro* addition of vitamin B₁ had no effect upon the reaction. The time required for avitaminous heart to reduce methylene blue was not decreased by vitamin B₁. In order to make the conditions more nearly comparable with those in oxygen uptake studies, experiments were carried out using minced instead of ground heart muscle. But with tissue prepared in this way, as with ground tissue, the vitamin did not affect the rate of decoloration of methylene blue.

Table V. *The effect of vitamin B₁ concentrate upon the rate of decoloration of methylene blue in lactate substrate by avitaminous heart tissue.*

Experiments run at 37°, p_H 7.3 in Thunberg tubes with Ringer phosphate-pyrophosphate buffer. Values are reported in min. for 80% decoloration of 1 ml. 1:5000 methylene blue.

Concentration of tissue suspension %	No substrate	Lactate	Lactate + vitamin (2.5γ)
5	40	14	15
5	40	14	15
5	70	16	16
2.5	—	50	50
2.5	—	75	75
2	—	120	120
*	—	75	75
*	250	120	120

* Each tube contained 0.05 g. of scissor-minced heart muscle.

Inhibition by pyruvic acid.

Birch and Mann [1934] showed that small amounts of pyruvic acid inhibited lactic acid dehydrogenation by their purified enzyme-coenzyme system. We have found that pyruvate additions to heart and kidney tissues likewise caused an inhibition of lactic acid dehydrogenase activity, which is especially marked in avitaminous tissue. Typical results are shown in Table VI. In avitaminous tissue

Table VI. *Effect of additions of pyruvic acid upon the lactic dehydrogenase activity of normal and avitaminous tissues.*

Experiments run at 37°, p_H 7.3 in Thunberg tubes. Each tube contained 2 ml. of a 5% suspension of tissue in Ringer phosphate-pyrophosphate buffer, 2 ml. of substrate and 1 ml. of 1:5000 methylene blue.

Substrate	Min. for 80% decoloration of methylene blue	Substrate	Min. for 80% decoloration of methylene blue
Normal heart.		Avitaminous heart.	
Nil	65	Nil	68
Lactate	8	Lactate	30
Lactate + pyruvate (2 mg.)	17	Lactate + pyruvate (2 mg.)	70
Lactate + pyruvate (4 mg.)	35	Lactate + pyruvate (4 mg.)	80
Lactate + pyruvate (8 mg.)	40	Lactate + pyruvate (8 mg.)	100
Normal kidney.		Avitaminous kidney.	
Nil	16	Nil	35
Lactate	6	Lactate	20
Lactate + pyruvate (2 mg.)	9	Lactate + pyruvate (2 mg.)	35
Lactate + pyruvate (4 mg.)	11	Lactate + pyruvate (4 mg.)	45
Lactate + pyruvate (8 mg.)	11	Lactate + pyruvate (8 mg.)	50

2 mg. of pyruvic acid were sufficient completely to inhibit lactic dehydrogenase activity. Normal tissue was able to tolerate higher concentrations of pyruvate without complete inhibition of this reaction.

DISCUSSION.

These results are not entirely in agreement with those of Peters and co-workers, who have found that disturbances in carbohydrate metabolism occur principally in brain tissue in polyneuritic pigeons. Rydin [1935, 1] has shown

that the effect of the vitamin upon oxygen uptake of pigeon brain tissue is less marked in pigeons showing only leg weakness than in birds exhibiting acute opisthotonic symptoms. He [1935, 2] has more recently found that the decreased oxygen uptake in skeletal muscle, liver, kidneys and erythrocytes of pigeons fed with polished rice until opisthotonus developed was due to inanition. It must be remembered that the polyneuritis developed in pigeons by a diet composed exclusively of polished rice is a slowly developing deficiency and may be complicated by the absence of other essential factors of the B-complex besides B_1 .

The nature of the polyneuritis which develops in experimental animals upon a vitamin B_1 -low diet appears to be dependent upon the amount of vitamin previously stored and upon the rate at which these stores are depleted. Birch and Harris [1934] suggest from their studies on bradycardia in polyneuritis that rats given no vitamin B_1 have a very low heart rate and no polyneuritic convulsions, while slight traces of vitamin B_1 act by keeping the heart rate at a high enough level to permit the more slowly developing chronic symptoms to appear. Our results with growing chicks with a very high vitamin requirement indicate that with a more rapidly developing polyneuritis, disturbances in tissue metabolism may appear in heart and possibly other organs before the noticeable effects upon the central nervous system are produced.

Although the oxygen uptake of cerebral tissue from polyneuritic chicks is lower than normal, the oxidation of lactate (as measured by the increase in oxygen uptake produced by the addition of lactate substrate and actual removal of added lactic acid) is not influenced by the avitaminosis nor does the addition *in vitro* of vitamin B_1 have a stimulating effect upon oxygen uptake. No diminution in the rates of reduction of methylene blue by cerebrum, cerebellum and optic lobes in the presence of lactate was observed in polyneuritis. However, with avitaminous heart tissue a lowered utilisation of lactate both as substrate for oxygen uptake and for methylene blue reduction was observed. A lowered rate of lactic acid removal was found in avitaminosis. The subnormal rate of respiration could be improved by *in vitro* addition of vitamin B_1 ; however, measurements of the rate of reduction of methylene blue suggest that the action of the vitamin is not primarily associated with dehydrogenation of lactic acid. It may be, as was suggested by Birch and Mann [1934], that the dehydrogenation of lactic acid is inhibited by the accumulation of its own dehydrogenation products and that an increased rate of oxygen uptake resulting from the addition of vitamin B_1 to avitaminous tissue respiring in lactate may be due to the action of the vitamin in the removal of some intermediate in lactic acid oxidation. Unpublished results from this laboratory indicate that the vitamin is closely associated with pyruvic acid metabolism.

SUMMARY.

1. Lactic acid oxidation by normal and polyneuritic chick tissues has been investigated by means of oxygen uptake studies, determinations of lactic acid removal and estimations of methylene blue reduction time. No significant disturbances in lactate oxidation were observed in brain tissues, but in avitaminous heart a decreased rate of oxygen uptake in lactic acid and a decreased rate of lactic acid removal were found.

2. Lactate oxidation is not affected in avitaminous brain by the addition of vitamin B_1 , but in avitaminous heart similar additions produce increased oxygen uptake.

3. Additions of pyruvate inhibited lactic acid dehydrogenase activity to a greater extent in avitaminous heart and kidney than in normal tissues.

4. The action of vitamin B₁ in lactate metabolism is not primarily concerned with dehydrogenation of lactic acid but may indirectly influence the reaction through its participation in some closely associated reaction.

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