LXXX. BLOOD PYRUVATE IN VITAMIN B_1 DEFICIENCY.

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IN the course of recent work on the rôle of vitamin B_1 in the metabolism of brain, it was discovered [Peters and Sinclair, 1933] that lactate solutions in which avitaminous pigeon's brain cells had respired for 2 hours gave a positive nitroprusside reaction for pyruvic acid [Simon and Piaux, 1924]. This led to a quantitative investigation of the formation of pyruvic acid during the respiration of normal and avitaminous brain tissue *in vitro* [Peters and Thompson, 1934]. In the course of this work it was discovered that relatively large amounts of pyruvic acid appear during the respiration in lactate of the minced brain tissue from vitamin B_1-deficient pigeons, whereas only a negligible amount (too little to be detected by the nitroprusside reaction) appears during the respiration under similar conditions of the brain tissue from normal pigeons. Further indirect evidence was thereby obtained in support of the recent Embden-Meyerhof scheme, which includes pyruvic acid as a normal intermediary in the metabolism of carbohydrate in the animal organism [see Embden *et al.*, 1933; Meyerhof and Kiessling, 1933; Meyerhof, 1933].

But although it was shown that there was this marked difference in the rates of pyruvate formation during respiration *in vitro*, no significant difference between the contents of pyruvic acid of normal and avitaminous brains could be detected when the estimations were performed immediately after the death of the animal, that is to say, without any period of respiration *in vitro*. A possible explanation is that although avitaminous brain tissue does produce abnormally large amounts of pyruvic acid *in vivo*, this substance is not detectable owing to the fact that it largely diffuses out into the blood stream.

The pyruvic acid in the blood of normal and avitaminous pigeons and rats was therefore investigated. Our results show that there is a marked accumulation of pyruvic acid in the blood of both these animals when in a state of avitaminosis B_1. A preliminary account of this work has already been published [Thompson and Johnson, 1934].

METHOD.

The method employed has been firstly to investigate the bisulphite-binding capacity of trichloroacetic acid centrifugates of the blood of normal and avitaminous animals, which were being used in brain experiments. In order to determine the percentage of pyruvic acid in the total bisulphite-binding compounds present in the blood, parallel estimations were also made by isolating the 2:4-dinitrophenylhydrazone of pyruvic acid and estimating it colorimetrically by a modification of the Neuberg-Case method [see Case, 1932; Peters and Thompson, 1934].

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Determination of the bisulphite-binding capacity of pigeon's blood.

The experiments with pigeons were carried out as follows. The bird was first rested for one hour by being wrapped in a duster and placed in a dosing-box kept in the dark; we have found that by this method more uniform results are obtained than if the bird is taken straight from the cage. At the end of the hour the bird was taken out of the box and killed by guillotining. The blood was allowed to drain into a clean dry beaker, and 3 ml. were then rapidly measured with a clean dry pipette into a weighed 25 ml. centrifuge-tube containing 2 ml. of 25 % trichloroacetic acid and 5 ml. of distilled water. The exact amount of blood taken was obtained by re-weighing the tube. The mixture was then allowed to stand for half an hour, at the end of which time it was centrifuged and re-extracted twice with 5 ml. portions of 5 % trichloroacetic acid. The combined centrifugates were then brought approximately to p_H 2·0 with 40 % sodium hydroxide and made up to 25 ml. in a glass-stoppered volumetric flask.

10 ml. aliquots were taken, treated with sodium bisulphite and titrated with $N/100$ iodine for bisulphite-binding capacity by the method of Clift and Cook [1932]. In order to prevent the frothing which tends to occur on the addition of the sodium bicarbonate to hydrolyse the bisulphite compound, one drop of capryl alcohol was added in some of the experiments immediately before the addition of the bicarbonate; capryl alcohol used in this way has no bisulphite-binding capacity.

All but one of the avitaminous birds used had been dosed with glucose once, according to the usual procedure adopted in this laboratory, and all showed head-retraction at the time of use.

Results.

The values obtained with pigeons are shown in Table I (see also Fig. 1). This table shows that there is a very large and constant increase in the bisulphite-binding capacity over the normal level in the blood of vitamin B_1-deficient pigeons.

Table I. *Bisulphite-binding substances in the blood of normal and avitaminous pigeons.*

mg. pyruvic acid per 100 g. blood (1 ml. $N/100$ I = 0·44 mg. pyruvic acid).

Normal		Avitaminous	
Exp.	B.B.S.	Exp.	B.B.S.
5	4·34	6	10·53
7	4·94	8	17·03
9	3·97	16	12·60
15	4·45	18	11·39
20	3·92	22	10·64
21	3·57	23	13·10
25	4·07	24	10·92
36	2·82	26	8·20
38	5·14	28	6·27
48	3·18	29	11·95
63	3·69	39	9·12
64	3·71	40	6·82
66	3·67	42	12·12
		59	17·70
Average	3·96	Average	11·31

In order to correlate this difference specifically with vitamin B_1-deficiency, estimations were also made on the blood of birds which had developed opisthotonus, but which had been cured by the administration of vitamin B_1 concentrates; in one experiment (Exp. 60) crystalline vitamin B_1 was used. No food

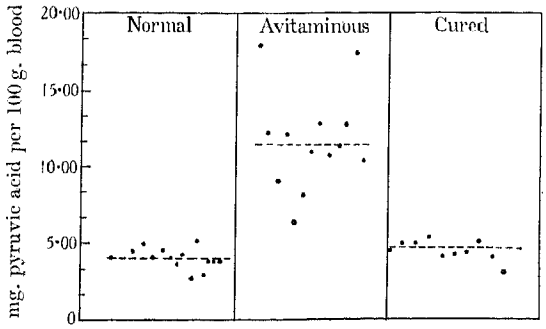

Fig. 1. Bisulphite-binding capacity of pigeon's blood. Broken lines through averages.

was given after dosing, so that their condition of starvation remained strictly analogous to that of the avitaminous birds. The water allowed after dosing was limited to 5 ml. in order to control any possible "washing-out" of the pyruvic acid. The results obtained with the "cured" birds are shown in Table II. Mr H. W. Kinnersley dosed the birds for us. We should like to thank him at this point.

Table II. *Bisulphite-binding substances in the blood of "cured" birds.*

Results expressed as in Table I.

Exp.	B.B.S.	Condition
27	3·77	
30	4·73	
32	5·72	Bird still showed "leg weakness"
33	5·10	
34	4·89	
35	4·77	
37	7·03	"Leg weakness" and partial blindness still present; previously gave anomalous tests
53	6·12	
54	5·55	
60	5·25	Dosed with crystalline vitamin B_1.
Average	**5·29**	

These results show that the bisulphite-binding capacity of the blood returns to a figure only a little above the normal (and not statistically different from the normal) after the nervous symptoms have disappeared on dosing with vitamin B_1. This increase in the bisulphite-binding capacity of avitaminous blood is therefore a specific effect of the avitaminosis and is not merely the result of the accompanying starvation.

The results in Tables I and II have been examined statistically by means of Fisher's "t" test. The difference between the normal and avitaminous levels is certainly significant, whereas no real difference exists between the normal level and that found for the "cured" birds.

Determination of pyruvic acid as 2:4-dinitrophenylhydrazone.

Parallel estimations on pigeon's blood, made by a modification of the Neuberg-Case method [Peters and Thompson, 1934], showed that this increase in the bisulphite-binding capacity of the avitaminous bird's blood is probably due entirely to pyruvic acid. The results are shown in Table III. It was unnecessary to extract the blood of "cured" birds, because even if all the bisulphite-binding capacity of their blood were due to pyruvic acid, which is highly unlikely, the level of pyruvic acid in the avitaminous bird's blood would still be higher than that in the "cured" bird's blood.

Table III. *Amounts of pyruvic acid in the total bisulphite-binding substances in pigeon's blood.*

	mg. pyruvic acid, per 100 g. blood.		
Exp.	B.B.S.	Pyruvic acid	Non-pyruvic acid B.B.S.
	Normal.		
48	3·18	0·82	2·36
63	3·69	0·80	2·89
64	3·71	0·90	2·81
	Avitaminous.		
39	9·12	6·22	2·90
40	6·82	4·38	2·44
26	8·20	6·34	1·86

For these estimations the blood centrifugates, at p_H 2·0, were made up to 25 ml.; 5 ml. aliquots were then taken for bisulphite estimations, the remaining 15 ml. being used for 2:4-dinitrophenylhydrazine extractions. Only six experiments of this type were done because of the unequivocal results.

Identification of pyruvic acid. The blood centrifugates were first tested by the nitroprusside reaction of Simon and Piaux [1924]. Normal pigeon's blood gave a centrifugate showing a faint and evanescent red colour, probably due to sulphhydryl groups. Avitaminous bird's blood showed a slowly developing green colour; in one case the blue of pyruvic acid in fairly large concentrations was seen.

For more certain identification, however, the 2:4-dinitrophenylhydrazone of pyruvic acid was extracted and purified as follows.

Trichloroacetic acid centrifugates were collected from the blood of fifteen avitaminous pigeons. The mixed centrifugates were kept in strongly acid solution in the presence of 2:4-dinitrophenylhydrazine for 24 hours. The mixture of hydrazine and hydrazones was then taken up in ethyl acetate. The ethyl acetate solution was repeatedly shaken with alkaline sodium phosphate solution until no more of the pyruvic acid 2:4-dinitrophenylhydrazone came out. The phosphate solution was then acidified with hydrochloric acid, and the mixture of hydrazones with an accompanying impurity of hydrazine was once more brought into ethyl acetate solution. The whole procedure was repeated until the alkaline phosphate removed the last trace of yellow from the ethyl acetate phase. The ethyl acetate phase was then collected and evaporated to dryness *in vacuo* at 15°. The residue was extracted with light petroleum, and the yellow hydrazone left undissolved was taken up in ethyl acetate and purified by solution in sodium phosphate, acidification with HCl and re-extraction with ethyl acetate. This was repeated five times. The final ethyl acetate solution was washed with distilled water brought to p_H 2·0 with HCl, and the ethyl acetate phase was then drawn off and evaporated *in vacuo* at 15° to a small volume. The hydrazone was precipitated with excess light petroleum and was dried *in vacuo* at about 15° over sulphuric acid.

The final yellow product had the following characteristics:

(1) It gave a red colour with alcoholic potash identical with that given by synthetic pyruvic acid 2:4-dinitrophenylhydrazone.

(2) It melted variously at 210°, 216°, 214°, 220°, 216°, 214° (corrected). The melting-point of pyruvic acid 2:4-dinitrophenylhydrazone is given as 214° [Allen, 1930; Case, 1932]; 216° [Neuberg and Kobel, 1929]; and 219° [Lohmann and Meyerhof, 1934]. We have noticed that different crystals of our purest products melt at different temperatures from 214–220°.

(3) Mixed with synthetic pyruvic acid 2:4-dinitrophenylhydrazone, it melted at 213°, 219°, 216° (corrected). Average 216°.

Enough hydrazone has not yet been collected for a satisfactory analysis, but this is now being carried out. We feel however that there is little doubt that the compound responsible for the rise in the bisulphite-binding capacity of polyneuritic pigeon's blood above normal is pyruvic acid, or some labile compound of pyruvic acid which yields free pyruvic acid in strongly acid or alkaline solution. Phosphopyruvic acid [Lohmann and Meyerhof, 1934] would give the same results as we have found under the conditions of our experiments.

Experiments with rats.

The bisulphite-binding capacity of the blood of normal rats and of rats in acute avitaminosis B_1 was determined in essentially the same way as for pigeons.[1] The rats however were taken straight from the cage, without any preliminary period of resting, and the blood was drained from the neck directly into a weighed 50 ml. centrifuge-tube containing the usual amount of trichloroacetic acid. $N/200$ iodine was used for the titrations instead of $N/100$. The results are shown in Table IV.

Table IV. *Bisulphite-binding substances in the blood of normal and avitaminous rats.*

mg. pyruvic acid per 100 g. blood.

Normal		Avitaminous	
Exp.	B.B.S.	Exp.	B.B.S.
95	3·28	75	11·67
96	3·82	76	7·13
129	4·82	93	11·61
130	4·95	94	9·18
		97	11·21
		120	6·81
		123	8·13
Average	4·22	Average	9·39

These results are in complete agreement with those obtained with pigeon's blood. Hydrazone extractions have not been done in the case of rat's blood, but we have found that in every case the solutions from the avitaminous rats gave the green nitroprusside reaction characteristic of pyruvic acid in low concentration, while the solutions from normal blood gave only the evanescent pink reaction of sulphhydryl compounds.

[1] We thank Mr J. R. P. O'Brien for supplying the rats used.

Discussion.

The results presented in this paper interested us primarily in view of the possible interpretation which they provided for the previous results obtained by Peters and Thompson [1934] with the brain. It is possible that the amounts of brain used by these workers were too small to allow the detection of the increased pyruvic acid in avitaminosis B_1 without a period of respiration *in vitro* to allow for its accumulation, but the raised blood pyruvate suggests that there is also a diffusion of pyruvic acid out into the blood stream.

There now arises the question of whether this raised blood pyruvate is due to metabolic disturbances in the brain alone, or whether it is a reflection of a general lesion throughout the tissues. In view of the work of Fisher [1931] on the lactic acid metabolism of various tissues in avitaminosis B_1, it seems unlikely that the brain alone is responsible for so large an increase in the level of this acid in the blood. In this connection it is also of interest that one of us (R.H.S.T.) found a powerful nitroprusside reaction for pyruvate in lactate solutions in which avitaminous kidney tissue had respired for two hours, whereas the solution in which the normal tissue had respired showed only a very faint colour. Moreover, Embden *et al.* [1933] have shown that pyruvic acid is a normal intermediary in the carbohydrate metabolism of muscle, and Elliott and Schroeder [1934] showed that kidney slices are capable of oxidising pyruvic acid about twice as fast as lactic acid. It seems likely therefore that there is a general lesion throughout the tissues in avitaminosis B_1 resulting in increased blood pyruvate, although we have not attempted to correlate the rise in blood pyruvate with the onset of symptoms.

We feel that the work of Rüter [1923] and of Bornstein and Ascher [1926] excludes any possible interference with our results for avian blood by glycolysis *in vitro*; it was shown by these workers that glycolysis in avian blood occurs very slowly in the absence of inhibitors of cellular respiration. We have ourselves tested this point by allowing the pigeon's blood to drain directly into the trichloroacetic acid and have found that the values obtained did not differ from those obtained when the blood stood for 30–40 seconds before being mixed with trichloroacetic acid. In the experiments with rats the blood has always been drained directly into the protein precipitant.

In view of the possible clinical application of this work as a test for vitamin B_1 deficiency, we are investigating the bisulphite-binding capacity of human blood.

Summary.

1. The bisulphite-binding capacity of the blood of normal and B_1-avitaminous pigeons and rats has been investigated.

2. There are abnormally large amounts of bisulphite-binding substances in the blood of B_1-avitaminous pigeons and rats, an accumulation independent of the accompanying starvation.

3. This accumulation is specifically related to the vitamin B_1 deficiency, and not to the accompanying starvation, because the blood of pigeons cured by vitamin B_1 of the acute symptoms of avitaminosis has a normal level.

4. The increase in bisulphite-binding capacity of the blood of avitaminous pigeons is probably due entirely to pyruvic acid.

We should like to express our gratitude to Prof. R. A. Peters for his encouragement and advice during this research, to Mr R. B. Fisher for the statistical examination of our result and to the Medical Research Council for a grant towards expenses.

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