CXXXIV. THE ESTIMATION OF COCARBOXYLASE (VITAMIN B₁ DIPHOSPHATE ESTER) IN BLOOD

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It is now generally believed that vitamin B₁ must first be converted into its diphosphate ester before it acts as a coenzyme necessary for the degradation of pyruvate [see Banga *et al.* 1939]. Estimations of cocarboxylase in blood in health and disease are obviously of great interest, particularly since nearly 90% of the vitamin in the blood is in the phosphorylated form. Simola [1932] showed that the amount of cocarboxylase in the brain tissue of rats was reduced in avitaminosis; Ochoa & Peters [1938] and Westenbrink & Goudsmit [1938] have published figures for other animal tissues. Apart from a preliminary account of the work in this paper, presented before the Physiological Society in March [Goodhart & Sinclair, 1939], no estimations upon human blood have been published. Both Heyns [1939] and Hennessy & Cerecedo [1939] have also described methods applicable to blood.

Ochoa & Peters [1938] have reported a method for the quantitative determination of vitamin B₁ and cocarboxylase in boiled tissue extracts, based upon the observation of Ochoa [1938] that vitamin B₁ markedly stimulates the decarboxylation of pyruvic acid by alkaline-washed yeast in the presence of cocarboxylase. We have applied their method, slightly modified, to the estimation of cocarboxylase in blood, but we have not found it satisfactory for the estimation of blood vitamin B₁.

Method

Dry baker's yeast was freed from cocarboxylase by rapid washing at room temperature with 0.1 *M* Na₂HPO₄ (1 g. yeast with three lots of 50 ml. phosphate) and once with distilled water. It was then washed twice with 50 ml. acetone and dried *in vacuo*. The activity of this preparation remained constant for at least 3 weeks if kept *in vacuo* when not in use. The production of CO₂ from pyruvic acid, in presence of the yeast preparation (as a source of carboxylase) and excess vitamin B₁, was measured in Warburg manometers. To each 25 ml. bottle were added in order: sufficient phosphate buffer (*pH* 6.2) to bring the total volume to 3.0 ml.; 1.0 ml. blood containing 0.2% neutral potassium oxalate; sufficient HCl to bring the *pH* to 6.2; 0.1 ml. of a solution containing MgCl₂, MnCl₂ and CaCl₂ (0.1 mg. Mg, 3 μ g. Mn, 0.96 mg. Ca); 0.2 ml. vitamin solution (containing 10 μ g. vitamin B₁); 1.0 ml. yeast suspension (100 mg. of the yeast preparation in 0.1 *M* phosphate buffer, *pH* 6.2). The CaCl₂ was omitted from control flasks that did not contain blood. After equilibrating in the bath at 28° in an atmosphere of nitrogen, the reaction was started by tipping in from the side-bulb 0.2 ml. sodium pyruvate (*pH* 6.2, containing 5 mg. pyruvic acid). All determinations were done in duplicate.

Ochoa & Peters [1938] showed that if nearly maximum amounts of vitamin B₁ were added to washed yeast and pyruvic acid, the amount of CO₂ produced

was proportional to the amount of cocarboxylase added. We have found that the height and shape of the curve obtained by adding known amounts of cocarboxylase to the system vary sufficiently with different batches of the yeast preparation to necessitate determining the curve with each new batch (Fig. 1). The evolution of CO_2 was measured 30 min. after tipping in the pyruvate, and the amount of cocarboxylase in the blood was obtained by comparing the difference in evolution of CO_2 between the flasks containing blood and the control flasks with the appropriate standard curve.

For reasons mentioned below, the flasks containing blood were placed for two minutes in a boiling water bath immediately after adding the solution containing MgCl_2 etc. This undoubtedly destroys many substances that would interfere, and it does not destroy the cocarboxylase. The anticoagulant used is important, since both sodium citrate (0.5%) and potassium oxalate (0.2%) inhibit the system. We have used 0.2% potassium oxalate and have added CaCl_2 to precipitate it after the blood has been added to the phosphate buffer. Too much CaCl_2 must not be added, because concentrations of 0.4% decrease the activity of the system. We have checked the method we finally adopted by adding blood rapidly to the phosphate buffer without using any anticoagulant and without adding CaCl_2 . Ascorbic acid (in added amounts up to 0.1%) and nicotinic acid (up to 0.04%) had no adjuvant action; 0.1% nicotinic acid stimulated the system. No effect was observed with urea (up to 0.2%) or with glucose (up to 0.1%). We have tried the effect of addition of various salts; we do not believe that inorganic substances in blood affect our system in presence of the ions mentioned above. Many other substances were tried by Ochoa & Peters [1938]. The presence in blood of vitamin B_1 monophosphate ester or of the pyrimidine or thiazole components of the vitamin would not affect our estimations because we are working in presence of nearly maximum amounts of vitamin B_1 . Adding varying amounts of cocarboxylase to blood produces the expected results [see also Ochoa & Peters, 1938]. A very important factor is the careful control of $p\text{H}$. All the flasks start at $p\text{H}$ 6.2 and the flasks containing blood remain at this $p\text{H}$ throughout the experiment but the control flasks become very slightly more acid ($p\text{H}$ 6.1 to 6.0) after 1 hr. Probably owing to this, rather higher cocarboxylase values are found for blood in the second half hour than in the first, since the activity drops off faster in the control flasks than in the flasks containing blood. For this reason we have used the values obtained at the end of the first half hour.

It will be shown below that blood converts free vitamin B_1 into cocarboxylase. If sterile blood be kept at room temperature for 24 hr. there may be a slight increase in the amount of cocarboxylase; at 0° there is no increase. This is not a likely source of error if the blood is tested as soon as possible after collection or is kept at 0° when necessary, particularly since there is very little free vitamin B_1 in blood.

Fig. 1. Effect of cocarboxylase upon the activity of alkaline-washed yeast.

RESULTS

(1) *Effect of heating blood*

One of us [Sinclair, 1938] found that there were only traces of free vitamin B₁ in unheated blood, using the method of Meiklejohn [1937] for the determination (this method determines both unesterified vitamin and cocarboxylase). Sinclair concluded that practically all the vitamin B₁ in blood was combined, probably with protein. With the method described in this paper we have uniformly obtained appreciable evolution of CO₂ when using unheated blood. Experiments indicate, however, that this is not due to cocarboxylase in the blood. When small amounts (0.13 μ g.) of cocarboxylase are added to samples of human blood that have been previously heated to different temperatures, the percentage recovery varies with the temperature to which the blood is heated, as shown in Fig. 2, curve 1. Curves 2 and 3 in the same figure represent the volume of gas produced by two human bloods over the same temperature range, without added cocarboxylase. If the gas produced by unheated blood represented entirely CO₂ evolved by the action of the cocarboxylase in blood upon pyruvic acid, curves 2 and 3 should approximate in shape to curve 1. Actually they differ considerably. This difference is emphasized by curve 4, which represents the average % yield of cocarboxylase that would obtain in the case of the two bloods used for curves 2 and 3 if it were assumed that the gas produced by unheated blood represented exactly a 100 % yield of cocarboxylase. As there is negligible destruction of the vitamin when boiled for two minutes at pH 6.2, we conclude from these curves that most of the gas evolved by unheated blood is not due to the action of cocarboxylase.

Fig. 2. Effect of heating upon the evolution of CO₂ by blood, and upon the apparent yield of added cocarboxylase.

Table I. *The effect of heat on the cocarboxylase activity of blood*

	μ l. CO ₂ evolved in 30 min.					
	Normal pigeon			Avitaminous pigeon		
	Not heated	70°	100°	Not heated	70°	100°
In bath at once	48.9	77.0	52.3	58.5	37.5	23.2
In bath after 1 hr.	7.0	54.1	55.2	21.3	42.9	31.8

In Table I are given similar results obtained with the bloods of two avitaminous and two normal pigeons. The figures in the lower column were obtained with bloods that had previously been standing for 60 min. in air and at room temperature, in the presence of phosphate buffer (pH 6.2) and Mg, Mn and Ca salts. The volumes of CO₂ evolved by these bloods when not heated were considerably less than the volumes evolved by the corresponding bloods that had not received this extra hour of equilibration. Such differences were not apparent in the case of the corresponding boiled bloods. The boiled avitaminous bloods gave significantly lower values than did the boiled normal bloods, whereas the

results obtained with the unheated bloods did not indicate which were the avitaminous and which the normal birds.

That this activity of unheated blood is no function of vitamin B₁ is also indicated by the fact that it is shown in the absence of yeast, under which conditions the activity is not enhanced by the addition of either cocarboxylase or vitamin B₁. It is depressed to some extent by the addition of alkaline-washed yeast.

From the above facts we cannot entirely exclude the possibility of some cocarboxylase acting in unheated blood but we may safely conclude that this is increased by denaturation of the blood by heating. The increased gas production by bloods that have been heated at 70° for 2 min. compared with boiled bloods is due to the liberation of an adjuvant factor at this temperature, as shown by curve 1, Fig. 2.

In all subsequent experiments we have used oxalated blood that has been boiled for 2 min. in the presence of phosphate buffer (pH 6.2) and Mg, Mn and Ca salts.

(2) *The amount of cocarboxylase in normal human blood*

Estimations of cocarboxylase have been done on 36 different samples of blood taken at least 2 hr. after a meal from 26 presumably normal adult males aged between 20 and 40 years. This group comprised 17 research workers, 4 resident hospital physicians and 5 medical students. These individuals were living on ordinary unrestricted diets and were not receiving any vitamin supplement.

The results are shown in Fig. 3, where each point in column 1 represents a determination upon one of the 36 samples. The average values for the 26 individuals range from 4.5 to 12.0 $\mu\text{g.}$ per 100 ml., the mean is 7.0 and the standard deviation of the individual observations is 2.1. In columns 2, 3 and 4 are given the results of estimations on three different subjects, the samples being collected at intervals of one week to several months. It will be seen that the variation in samples collected from one subject at different times is as great as the variation of the whole group of different subjects. The factors that cause this variation in the amount of cocarboxylase in the blood of an individual are not known and require further study.

Fig. 3. Amount of cocarboxylase in human blood, and its variation in the blood of three subjects.

(3) *The amount of cocarboxylase in the blood of various animals*

The following values (mean in $\mu\text{g.}/100 \text{ ml.} \pm \sigma$) have been obtained for the blood of different animals, including the results mentioned in the last section.

Human (26)	...	...	7.0 ± 2.1
Ox (5)	...	...	5.7 ± 1.5
Pigeon (5)	...	...	20.2 ± 9.8
Avitaminous pigeon (5)	...	...	5.6 ± 0.8

The cocarboxylase in the blood of the pigeons deficient in vitamin B₁, which were all in opisthotonos, is significantly lower than the normal (the difference

between the two means divided by the estimated standard error of this difference is 3.3), and its level is very consistent; this suggests that symptoms of deficiency may arise at a particular level of cocarboxylase in the blood.

(4) *The distribution of vitamin B₁ and cocarboxylase between the plasma and blood cells*

Meiklejohn [1937] stated that about 20% of the total vitamin B₁ in human blood was present in plasma; but as he used as anticoagulant a concentration of potassium oxalate (0.3%) that may be haemolytic, his value may have been too high. However, one of us (H. M. S.), in unpublished experiments in which care was taken to avoid haemolysis, obtained an average value of 14%. We have studied the distribution of both cocarboxylase and vitamin B₁ between plasma and cells. Table II summarizes results with human, ox and pigeon plasma; cocarboxylase is expressed in $\mu\text{g./100 ml. plasma}$. As we have not found the method of Ochoa & Peters [1938] to be sufficiently sensitive for the quantitative determination of vitamin B₁, we have tabulated the results of these estimations merely as $\mu\text{l. CO}_2$ produced in 30 min. by 1 ml. plasma, in presence of 2 $\mu\text{g. cocarboxylase}$ and no added vitamin B₁. The small positive values for cocarboxylase which were obtained in 9 out of the 17 cases tabulated were all associated with partial haemolysis before or during separation of the plasma. Plasma uncontaminated in this manner gave uniformly negative results. If there is no cocarboxylase in plasma there should be none in normal cerebrospinal fluid. In Table II are tabulated the results on 9 samples of c.s.f. Only one positive result was obtained, and this in a fluid that was markedly xanthochromic.

Table II. *The amount of cocarboxylase and vitamin B₁ in plasma and cerebrospinal fluid*

Human plasma		Human c.s.f.		Ox plasma		Pigeon plasma	
Cocarb. $\mu\text{g./100 ml.}$	Vitamin B ₁ $\mu\text{l.CO}_2$	Cocarb. $\mu\text{g./100 ml.}$	Vitamin B ₁ $\mu\text{l.CO}_2$	Cocarb. $\mu\text{g./100 ml.}$	Vitamin B ₁ $\mu\text{l.CO}_2$	Cocarb. $\mu\text{g./100 ml.}$	Vitamin B ₁ $\mu\text{l.CO}_2$
0.5	72	0	—	1.5	—	0	—
—	53	0	—	0	—	3.0	18
0	59	0	—	0	—	—	—
0	37	5.0	—	0	—	—	—
—	—	0	44	—	14	—	—
—	—	0	43	2.3	35	—	—
—	—	0	43	1.5	10	—	—
—	—	0	48	3.5	17	—	—
—	—	0	33	2.0	29	—	—
—	—	—	—	0	—	—	—
—	—	—	—	1.0	—	—	—
—	—	—	—	1.0	21	—	—
—	—	—	—	0	16	—	—

In contradistinction to the results obtained with nearly maximum amounts of vitamin B₁, the gas evolved with a constant amount of cocarboxylase (2 $\mu\text{g.}$) has been consistently more with both plasma and spinal fluid than in the controls, indicating the presence of vitamin B₁ in these fluids.

In Table III the cocarboxylase values ($\mu\text{g./100 ml. blood}$) obtained with ox and pigeon whole bloods are compared with the values given by the erythrocyte and leucocyte layers of the same bloods. These cells were separated from the whole blood by centrifuging at 2500 rev./min. for 1½ hr. Fractionation was not complete, the leucocytes in the "leucocyte layers" of the ox bloods numbering

Table III. *Amount of cocarboxylase in different layers of blood ($\mu\text{g.}/100\text{ ml.}$)*

Source	Ox				Pigeon	
	1	2	3	4	1	2
Whole blood	6.5	5.5	5.0	6.1	19.0	31.7
R.B.C. layer	7.3	14.0	5.0	11.5	67.0	50.6
W.B.C. layer	27.5	23.0	17.0	36.5	14.5	9.5

from 123,000 to 246,000 per $\mu\text{l.}$ with erythrocyte counts of 1,000,000 to 5,760,000. The erythrocytes varied from 11,120,000 to 16,000,000 per $\mu\text{l.}$ in the "erythrocyte layers" of the ox bloods, with leucocyte counts of 1200 to 6700 per $\mu\text{l.}$ Similar variations occurred with the pigeon bloods but, owing to technical difficulties, accurate leucocyte counts are not available for these bloods.

Despite this imperfect separation of cells the results shown in Table III clearly indicate that there is a much larger amount of cocarboxylase in the mammalian leucocyte than in the corresponding erythrocyte. The results on pigeon blood are interesting in that here this difference does not occur. This difference and its explanation are more clearly shown in Fig. 4, where curves 1

Fig. 4. Effect of the cell count upon the cocarboxylase content of blood.

and 2 represent cocarboxylase values obtained with pigeon bloods and curves 3 and 4 the corresponding values of two ox bloods. The smallest erythrocyte count plotted for each blood was obtained with the "leucocyte layer" and represents, therefore, the highest leucocyte count for that blood. In the same way, the highest erythrocyte represents the smallest leucocyte count. The middle values of each curve were obtained with the whole bloods. It can be seen that the cocarboxylase content of pigeon blood tends to vary directly with the erythrocyte count except, perhaps, with very small erythrocyte counts when the presence of large numbers of leucocytes may tend to flatten the curve. It therefore seems likely that, in pigeon blood, the erythrocyte contains as much cocarboxylase as the leucocyte and the increased cocarboxylase content of whole pigeon blood over ox blood is due entirely to this large erythrocyte content.

In the case of ox blood, the rapid fall in cocarboxylase values with decreasing leucocyte counts, despite much greater increases in the number of erythrocytes, indicates the relative importance of these two types of cells in determining the amount of cocarboxylase in mammalian bloods. The tendency of the cocarboxylase value to increase again with further concentration of erythrocytes, despite a slight reduction in leucocytes over the normal value, shows that the mammalian erythrocyte is not entirely devoid of cocarboxylase. In the blood used for curve 4 (Fig. 4) the cocarboxylase content of the "erythrocyte layer" was the same as that of the whole blood. As there were 7,420,000 more erythrocytes and 4700 less leucocytes in the former, it follows that one leucocyte contained as much cocarboxylase, in this instance, as 1600 erythrocytes. This figure is merely illustrative: it is not intended to be taken as more than a very rough approximation of the relative amounts of cocarboxylase in these two types of cells, as neither the method of determining cocarboxylase nor the technique used in doing the cell counts permits exact calculations of this nature.

The amounts of gas evolved by the same samples of blood in the presence of 2 μ g. cocarboxylase are listed in Table IV. In every instance, except one, the figure is largest with the "erythrocyte layers", while the "leucocyte layers"

Table IV. *Amount of vitamin B₁ in different layers of blood*

	μ l. CO ₂ evolved in 30 min.				Pigeon
	Ox				
	1	2	3	4	
Whole blood	53	16	26	—	37
R.B.C. layer	77	29	14	61	76
w.B.C. layer	54	15	27	47	36
Plasma	35	8	17	29	18

give values no greater than those given by the corresponding whole bloods. As the total number of cells in the "erythrocyte layers" is much greater than, and the total number in the "leucocyte layers" is only slightly less than, those in the whole bloods, the results are entirely compatible with the assumption that the blood vitamin B₁ identified in this way readily diffuses and is adsorbed. It should be noted that the values given by pigeon blood, in this instance, are of the same order of magnitude as those given by the ox bloods. The vitamin B₁ thus determined represents only a fraction of the total blood vitamin B₁. We have been able to increase the yield of apparent vitamin B₁ from sheep and ox serum by incubating the serum at pH 3.0 for 7 hr. at 38° in the presence of a concentration of 0.25% of recrystallized pepsin (Table V). This increased yield has been obtained without any change in the amount of cocarboxylase.

Table V. *Effect of pepsin on the amount of vitamin B₁*

	μ l. CO ₂ evolved		
	Not treated	Boiled pepsin	Pepsin
Ox serum	21	34	73
Sheep serum	4	10	35

(5) *Formation of cocarboxylase from vitamin B₁ by blood cells*

The finding that the nucleated blood cells contained large amounts of cocarboxylase, while the non-nucleated mammalian erythrocyte contained very little, and the plasma none at all, suggested to us the possibility that the nucleated blood cells possessed the ability to form cocarboxylase from vitamin B₁. To test this point we incubated samples of 1 ml. oxalated blood for 1 hr. in air at 38° in the presence of 10 µg. vitamin B₁, 0.1 mg. Mg, 3 µg. Mn, 0.96 mg. Ca and 0.5 ml. phosphate buffer pH 6.2. Under these conditions we were not able to demonstrate any synthesis in normal pigeon, ox, or human bloods—a result that is in accord with that of Ochoa, namely, that tissues saturated with cocarboxylase will produce negligible synthesis even under optimum conditions (pH about 8.5 and in an atmosphere of O₂).

The incubation under the same conditions of avitaminous pigeon blood with 10 µg. vitamin B₁ resulted in the synthesis of a considerable amount of cocarboxylase (Table VI). The increase in cocarboxylase that resulted from incubation

Table VI. *Effect of incubation with vitamin B₁ on cocarboxylase content of blood (µg./100 ml.)*

Source	Avitaminous pigeon			Human myeloid leukaemia	Human lymphatic leukaemia
	1	2	3		
Control	5.0	6.0	5.5	124.0	17.5
Incubated with 10 µg. vit. B ₁	25.0	29.0	19.5	174.0	14.5

brought the total amount up to within the limits found for the blood of normal pigeons (see results for whole pigeon blood in Table III).

In Table VI are also listed the results on two human bloods, one from a case of myeloid and the other from a case of lymphatic leukaemia. The erythrocyte count in each instance was approximately the same, 1,500,000 per µl. The total leucocyte count in the myeloid leukaemia was 730,000 per µl. and in the lymphatic leukaemia 360,000 per µl. The much larger amount of cocarboxylase in the former case cannot be accounted for by the difference in cell count alone. It would appear that cells of the myelogenous series contain a great deal more cocarboxylase than do those of the lymphatic series. Also, incubation, in the case of the myeloid leukaemic blood, resulted in a still further increase in cocarboxylase, whereas incubation of the lymphatic leukaemic blood produced no synthesis. One cannot conclude from this that the synthesis of cocarboxylase is a function restricted to those blood cells that originated in the bone marrow, since the state of saturation (before incubation) of the lymphocytes with cocarboxylase in this case of lymphatic leukaemia is not known. More work on this problem is necessary. At present it is safe only to say that those nucleated blood cells that originate within the bone marrow are the most active elements of the blood in synthesizing cocarboxylase from vitamin B₁.

From the above facts, it seems quite probable that the cocarboxylase contained within the mammalian non-nucleated erythrocyte represents the residuum of that acquired by this cell in its nucleated form, while still within the bone marrow.

DISCUSSION

The fact that the cocarboxylase of the blood is contained entirely within the blood cells makes it seem unlikely that the vitamin is carried to the tissues by the blood in the phosphorylated form. It is much more probable that the readily

diffusible vitamin B₁ (or vitamin B₁ monophosphate), which has been demonstrated to be present throughout the blood, is the direct source of both the vitamin B₁ and the cocarboxylase of the fixed tissue cells. The liver and (to a lesser extent) muscle and brain [Ochoa & Peters, 1938] and the kidney [Westenbrink & Goudsmit, 1938] can phosphorylate vitamin B₁. We have shown that the nucleated blood cells that originate in the bone marrow are active in this respect. It seems probable that the phosphorylation is a function possessed by all nucleated animal cells. The liver and kidney appear to be most active in this respect; the former probably phosphorylates vitamin B₁ partly for storage, unesterifying the cocarboxylase when necessary to replenish the blood vitamin B₁. In the kidney phosphorylation may be essential for the reabsorption of the vitamin in the tubules; no cocarboxylase normally appears in the urine.

The inclusion of all the cocarboxylase within the blood cells depreciates to a varying extent the value of total blood vitamin B₁ or of cocarboxylase determinations as a means for determining the presence or absence of deficiency of vitamin B₁ in any individual with a blood dyscrasia. Thus an individual with myeloid leukaemia, with a leucocyte count of 200,000 or more, may be found to have a total blood vitamin B₁ content about six times the normal value, although the plasma vitamin B₁ level may be much less than normal, and the individual may actually suffer from a deficiency of this vitamin. Conversely, an individual with marked anaemia and leucopenia may have a subnormal value for total vitamin B₁ in the blood, with a normal plasma level and no symptoms referable to a deficiency of vitamin B₁.

Admittedly, variations in the cell count, whether red or white, must be large before appreciably affecting the blood level of cocarboxylase. An increased blood value can readily be observed with an increase in the polymorphonuclear leucocyte count of 20,000 or with an increase in the erythrocyte count of 2,000,000 per μ l.

SUMMARY

1. A reliable method for the rapid determination of cocarboxylase in small samples (2 ml.) of oxalated blood has been described.

2. Reasons are given for believing that all the cocarboxylase present in blood is in a combined form.

3. All the blood cocarboxylase is found within the blood cells, the polymorphonuclear leucocytes containing more than the lymphocytes and the non-nucleated erythrocytes containing least.

4. The nucleated blood cells can phosphorylate vitamin B₁; reasons for believing that this is a function common to all nucleated animal cells are stated. Probably the mammalian non-nucleated erythrocytes obtain their cocarboxylase while in the nucleated form within the bone marrow.

5. Evidence is presented for a freely diffusible form of vitamin B₁ in the blood. In addition, there probably exists a form in combination with protein that is not cocarboxylase and is present in serum.

6. The vitamin is probably carried to the tissues as free vitamin B₁ or its monophosphate ester, and not as cocarboxylase.

7. The results of 36 estimations of cocarboxylase in samples of blood from 26 normal male adults are presented. The mean value is 7.0 μ g./100 ml., σ = 2.1.

8. The limitations of estimations of total vitamin B₁ in blood as indices of the presence or absence of deficiency of the vitamin are discussed.

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