CXCVIII. VITAMIN B_1 AND COCARBOXYLASE IN ANIMAL TISSUES[1]

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THE discovery by Lohmann & Schuster [1937] that cocarboxylase is the pyrophosphoric ester of vitamin B_1 suggested that in animal tissues the vitamin is active like this ester in the oxidation of pyruvic acid. It now seems clear that animal tissues and certain bacteria do not decarboxylate pyruvic acid to acetaldehyde and CO_2 (as yeast does) but instead oxidize it to acetic acid and CO_2, either by dismutation under anaerobic conditions or directly [Krebs & Johnson, 1937; Lipmann, 1937, 1; Weil-Malherbe, 1937]. Lipmann [1937, 2] reports that with acetone preparations from *B. Delbrückii* the simultaneous decarboxylation and oxidation of pyruvic acid require cocarboxylase.

In animal tissues evidence that vitamin B_1 pyrophosphate is concerned in the oxidation of pyruvic acid is derived (1) from catatorulin tests, and (2) from the presence of cocarboxylase in tissues and their alleged capacity to synthesize cocarboxylase from vitamin B_1. The first point, that cocarboxylase can replace vitamin B_1 in catatorulin tests [Lohmann & Schuster, 1937], has not been confirmed in this laboratory using "teased" brain [Peters, 1937]; more recent unpublished experiments with brain slices also gave negative results. This point obviously requires further elucidation.

In regard to the second point, Auhagen [1932] first showed that boiled extracts of animal tissues stimulated the decarboxylation of pyruvic acid by yeast preparations (aetiozymase), indicating the presence of cocarboxylase. Simola [1932] investigated the influence of nutrition upon this phenomenon. Synthesis of cocarboxylase from vitamin B_1 by minced animal tissues or various tissue preparations has been reported from several laboratories [von Euler & Vestin, 1937; Tauber, 1937; Lohmann & Schuster, 1937; Peters, 1937], whereas Stern & Hofer [1937] reported negative results. This work is not yet sufficiently quantitative and further it has not so far been ascertained whether vitamin B_1 is present in tissues in the free form.

We have now developed a method which allows the separate quantitative estimation of cocarboxylase and free vitamin B_1, by means of which the following points have been investigated: (1) the cocarboxylase and vitamin B_1 contents of boiled extracts from normal and avitaminous tissues, (2) the enzymic synthesis of cocarboxylase from vitamin B_1. In the present paper we shall show that there is much more cocarboxylase than vitamin B_1 present in animal tissues, and that it is much reduced when vitamin B_1 is withheld from the diet, and further, that the liver readily synthesizes cocarboxylase from vitamin B_1 *in vivo*. Another paper will deal with the synthesis of cocarboxylase *in vitro*. Various organs (brain, muscle) have only a very limited power of synthesis;[2] intestinal mucosa does not show any activity at all, whereas active preparations can be obtained from the liver.

[1] A preliminary report of this work appeared in the *Trans. Soc. Chem. Ind.* **57**, 470, 1938.

[2] Less in the case of brain than suggested by Peters [1937], who did not know of the stimulating effect of vitamin B_1 upon the action of cocarboxylase (cf. below).

Experimental

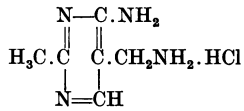
Dry baker's yeast (supplied by Distillers Co. Ltd.) has been used throughout. It was freed from cocarboxylase by rapid washing with $M/10$ Na_2HPO_4 (1 g. yeast 3 times with 50 ml. each time) and once with distilled water at room temperature.[1] The washed yeast was suspended in 10 ml. $M/10$ phosphate buffer of *p*H 6·2. The production of CO_2 from pyruvic acid was measured manometrically using both Barcroft and Warburg manometers. Each bottle contained 1·0 ml. washed yeast suspension (added last), 0·1 ml. $MgCl_2$ (0·1 mg. Mg), 0·4 ml. water or experimental solutions, and 0·2 ml. sodium pyruvate adjusted to *p*H 6·2 (5 mg. pyruvic acid). The reaction was started after 10–12 min. by tipping in the pyruvate solution from the side bulb or dangling tube. The total volume of fluid was 1·7 ml., the temp. was 28° and the gas either air or nitrogen.

Preparations

Cocarboxylase. One pure specimen from Dr K. Lohmann (Berlin); another specimen (70 % purity, vitamin B_1-free) prepared by Mr H. W. Kinnersley from yeast crude cocarboxylase, obtained by enzymic synthesis from synthetic vitamin B_1 by the method of Kinnersley & Peters [1938].

Vitamin B_1 chloride hydrochloride. Synthetic specimens from Messrs Hoffmann la Roche and Messrs Bayer.

Pyrimidines and thiazole vitamin components. From Prof. A. R. Todd (Lister Institute, London).



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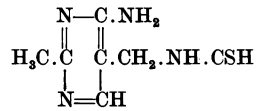

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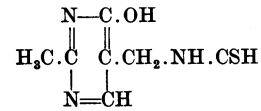
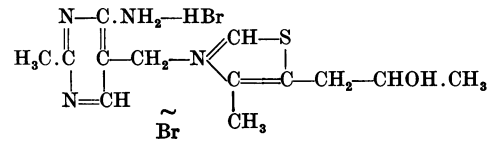


III

Vitamin B_1 analogue from 4-methyl-5-β-hydroxypropylthiazole (referred to in Table II as vit. B_1 analogue IV) from Dr Buchman (Pasadena).

IV 

Cozymase. Prepared from yeast by the method of Meyerhof & Ohlmeyer [1937] by Mr L. A. Stocken in this laboratory; this was 91% pure by comparison with pure cozymase kindly supplied by Dr Ohlmeyer. Other compounds used came from commercial sources.

We are much indebted to those mentioned above for their kind gifts which have made this work possible.

1. *The activation of cocarboxylase action by vitamin B_1*

Whereas vitamin B_1 cannot replace cocarboxylase, it has been found by one of us [Ochoa, 1938] that it markedly stimulates the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of pure cocarboxylase. The effect is apparent with 0·1 γ and increases with increasing concentrations of the vitamin;

[1] The above method of washing was adopted when Lohmann's method failed. At first the latter gave good results.

with 1·0γ cocarboxylase it is nearly maximal for 15γ vitamin B_1 hydrochloride. Fig. 1 illustrates this. The activation is the same in air as in N_2 showing that it is not due to oxidative removal of acetaldehyde. That the vitamin does not induce a disappearance of acetaldehyde by dismutation is shown by the fact

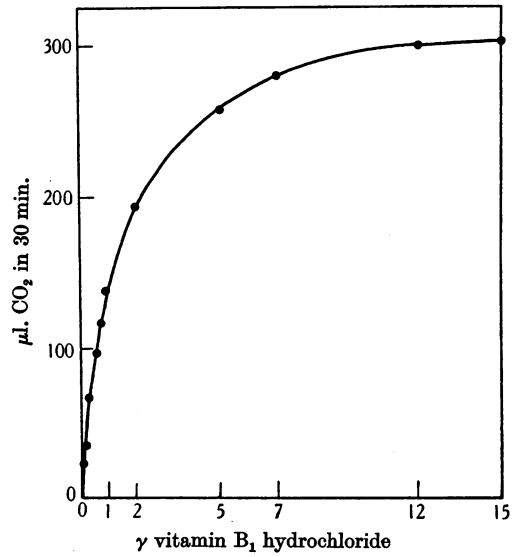


Fig. 1. Activation of cocarboxylase action by vitamin B_1. Ordinate μl. CO_2 evolved in presence of increasing amounts of vitamin and 1γ cocarboxylase, minus CO_2 evolved with 1γ cocarboxylase alone (1γ cocarboxylase = 163 μl. CO_2; control = 26 μl. CO_2 in 30 min.).

that no acid production takes place (measured in bicarbonate solution; gas, pure CO_2). Further, iodoacetic acid, while depressing the decarboxylation to some extent, has practically no effect on the activation by the vitamin, as shown in Table I.

Table I. *Action of iodoacetic acid on the stimulation of cocarboxylase by vitamin B_1*

		μl. CO_2 in excess of control in 30 min.			
Exp. no.	Molarity of IAA	1γ cocarb.	1γ cocarb. + 10γ vit. B_1	Activation by vit. B_1	% inhibition of vitamin activation
1	0	211	505	294	—
	0·0009	187	479	292	0
2	0	162	465	303	—
	0·0050	115	373	258	15

The stimulating effect of the vitamin is due to the pyrimidine part of the molecule and is absent in the absence of the NH_2 group. A number of other compounds investigated were found to be inactive (Table II).

Vitamin B_1 monophosphate has no cocarboxylase activity (as already stated by Lohmann & Schuster), whether alone or together with vitamin B_1, but it was found to stimulate cocarboxylase action. The monophosphate was obtained by acid hydrolysis of cocarboxylase (15 min. in N HCl at 100°); the solution was used for the experiments after bringing the reaction to pH 6·2 with NaOH (Table III).

Table II. *Effect of various compounds on the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of pure cocarboxylase*

Exp. no.	Sample	μl. CO_2 evolved in 30 min.
1	Control	15
	1γ cocarboxylase	160
	1γ cocarb. + 2γ vitamin B_1	344
	1γ cocarb. + 20γ pyrimidine I	385
	1γ cocarb. + 50γ pyrimidine II	318
	1γ cocarb. + 90γ pyrimidine II	385
	1γ cocarb. + 90γ pyrimidine III	161
	1γ cocarb. + 20γ vitamin B_1 analogue IV	432
	1γ cocarb. + 90γ thiazole	150
	1γ cocarb. + 100γ cytidylic acid	168
	1γ cocarb. + 100γ vicine	158
	1γ cocarb. + 100γ adenosine	160
	1γ cocarb. + 100γ nicotinamide	153
2	1γ cocarboxylase	178
	1γ cocarb. + 50γ acetylcholine	174
	1γ cocarb. + 100γ choline	161
	1γ cocarb. + 100γ spermine	154

Table III. *Effect of vitamin B_1 monophosphate on the decarboxylation of pyruvic acid by alkaline washed yeast with and without addition of cocarboxylase. (Quantities expressed for vitamin B_1 monophosphate as γ cocarboxylase; 1γ cocarboxylase = 0·6γ vitamin B_1)*

Sample	μl. CO_2 evolved in 30 min.
Control	9
10γ vitamin B_1	14
10γ vitamin B_1 monophosphate	12
10γ vitamin B_1 + 10γ vitamin B_1 monophosphate	14
1γ cocarboxylase	135
1γ cocarb. + 1γ vitamin B_1 monophosphate	188
1γ cocarb. + 2γ ,,	203
1γ cocarb. + 5γ ,,	257
1γ cocarb. + 10γ ,,	315

Recently Lipschitz *et al.* [1938], observing that addition of vitamin B_1 to alkaline washed yeast containing Mg^{++}, pyruvate and boiled tissue extract, increased the CO_2 output, interpreted this to mean that the vitamin is phosphorylated to cocarboxylase by the yeast enzymes in the presence of the extract. The results quoted above, however, indicate that the increased CO_2 production is due to the stimulation by the vitamin of the effect produced by the cocarboxylase present in the tissue extract. The fact that the pyrimidine components (which cannot be synthesized to cocarboxylase through lack of thiazole) also activate the cocarboxylase action makes it very unlikely that the effect of the vitamin itself is due to synthesis to cocarboxylase. That dry yeast does not phosphorylate vitamin B_1 to cocarboxylase to any extent is also indicated by the experiments of Kinnersley & Peters [1938]. The nature of the vitamin activation is not yet understood and is being further investigated.

Lipschitz *et al.* [1938] also reported that hexosediphosphate markedly increases the decarboxylation of pyruvic acid when boiled tissue extracts are present, an effect which is increased by addition of vitamin B_1; they supposed this action to be due to an influence on the enzymic synthesis of cocarboxylase from vitamin B_1. We find, however, that hexosediphosphate increases the CO_2 output in the complete absence of vitamin if both pure cocarboxylase and cozymase are present. The

effect increases with increasing concentrations of cocarboxylase[1] (Tables IV and V). Since accumulation of acetaldehyde is known to inhibit the enzymic decarboxylation of pyruvic acid the mechanism of action of hexosediphosphate is

Table IV. *Effect of hexosediphosphate on the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of boiled extract from normal pigeon's liver, with and without addition of cocarboxylase. (Hexosediphosphate added, 7·8 mg. as Na salt)*

Sample	μl. CO_2 evolved in 2 hr.
Control	59
0·3 ml. boiled liver extract	235
0·3 ml. boiled liver extract + hexosediphosphate	293
0·3 ml. boiled liver extract + 0·5γ cocarboxylase	601
0·3 ml. boiled liver extract + 0·5γ cocarboxylase + HDP*	840

* HDP = Hexosediphosphate.

Table V. *Effect of hexosediphosphate and cozymase on the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of pure cocarboxylase. (Hexosediphosphate added, 7·8 mg. as Na salt. Cozymase added, 0·2 mg.)*

	μl. CO_2 evolved in 2 hr.				
	γ cocarboxylase added				
Sample	0·5	1·0	2·0	3·0	0·5γ cocarb. + 5γ vit. B_1
Control	48	34	36	73	46
Cocarboxylase	192	361	667	790	427
Cocarb. + HDP*	162	334	657	783	394
Cocarb. + Coz.†	182	354	657	1036	435
Cocarb. + HDP* + Coz.†	200	533	1085	1400	701

* HDP = Hexosediphosphate. † Coz. = Cozymase.

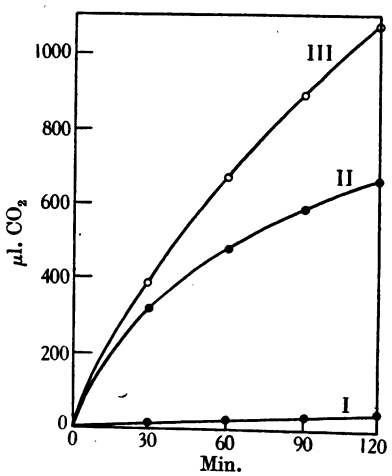


Fig. 2. Effect of hexosediphosphate and cozymase on the decarboxylation of pyruvic acid by alkaline washed yeast in presence of cocarboxylase. Curve I, control. Curve II, 2γ cocarboxylase. Curve III, 2γ cocarboxylase + 7·8 mg. Na hexosediphosphate + 0·2 mg. cozymase.

clear. The yeast enzymes form triosephosphate which dismutates with acetaldehyde in the presence of cozymase to give ethyl alcohol and phosphoglyceric

[1] Essentially the same results have been obtained with brewer's yeast (Löwenbräu, Munich).

acid, so that acetaldehyde is removed and the decarboxylation can proceed at a higher rate. That this effect of hexosediphosphate should be unaffected by fluoride and inhibited by iodoacetic acid [cf. Lipschitz *et al.* 1938] is obvious and requires no further comment. When tissue extracts are added they supply the necessary cocarboxylase and cozymase. Fig. 2 shows that, in presence of hexosediphosphate and cozymase, the production of CO_2 drops less rapidly than it does with cocarboxylase alone, so that the effect of hexosediphosphate markedly increases with time.

2. *Activation by manganese*

Lohmann & Schuster [1937] found that either Mg^{++} or Mn^{++} are necessary for the decarboxylation of pyruvic acid by yeast. Mn^{++} was found by the above authors to be 5–10 times as effective as Mg^{++}, the largest amounts investigated being 10 and 100γ respectively. We find now that whereas 100γ Mg^{++} produce practically a maximum effect, the activation brought about by Mn^{++} continues to increase above 10γ up to approximately 100γ and may be very large (Fig. 3).

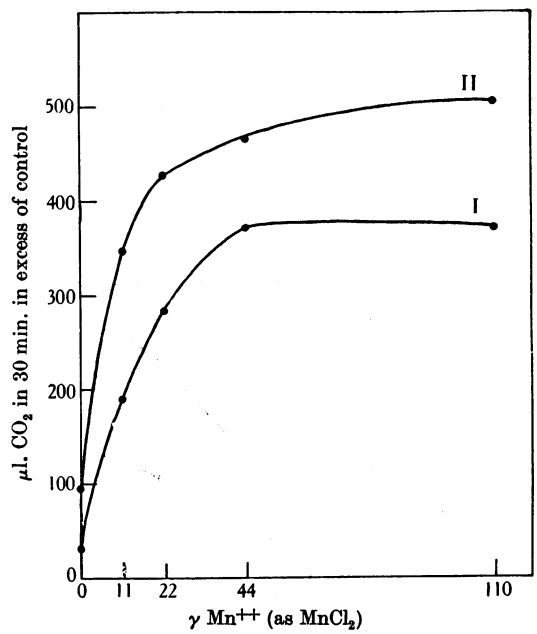


Fig. 3. Activation by Mn^{++} of the decarboxylation of pyruvic acid by alkaline washed yeast in presence of 1γ cocarboxylase, with and without vitamin B_1. Curve I, no vitamin. Curve II, 10γ vitamin B_1 hydrochloride.

This fact would only be of physiological significance if there is uneven distribution in the cell since 1 g. dry bottom yeast, Löwenbräu, Munich, contains only about 5·5γ Mn^{++} according to Ohlmeyer & Ochoa [1937], it is nevertheless interesting in regard to the mechanism of the reaction. Further, small amounts of Mn^{++} increase the activating effect of vitamin B_1. It should be noted that washing of our yeast by the method given above must remove most of the active ions, since if no Mg^{++} or Mn^{++} are added (in presence of cocarboxylase) no significant CO_2

production above the control (without cocarboxylase) takes place. This may account for the fact that if we add the Mg^{++} (100γ) mixed with the pyruvate, as Lohmann & Schuster did, we get very low CO_2 productions.

3. *Method of estimation of cocarboxylase and vitamin B_1*

The activation of the cocarboxylase action by vitamin B_1 makes it possible to determine the two compounds separately if present together. If an amount of vitamin B_1 which produces maximum (or nearly maximum) activation is added to the unknown solution, the cocarboxylase can be determined, since the CO_2 production will not be very much affected by the vitamin which was originally present. The addition of vitamin has also the effect of increasing very markedly the sensitivity of the method. Amounts of 0·01–0·02γ cocarboxylase can thus be estimated. For the cocarboxylase estimation 0·1 ml. vitamin B_1 solution in M/10 phosphate pH 6·2 (10γ vitamin B_1 hydrochloride) and 0·3 ml. unknown solution are added to the bottles, together with the other additions, and the CO_2 production is measured over a period of 30 min. The amount of cocarboxylase is found by reference to a curve obtained with pure cocarboxylase + 10γ vitamin B_1 (Fig. 4, curve II). In our experience, duplicates agree within the usual limits of

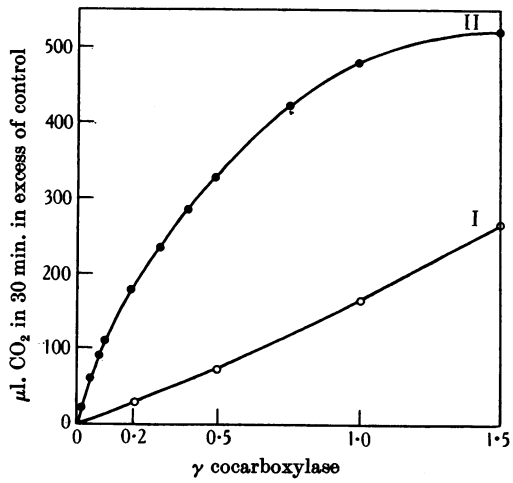


Fig. 4. Decarboxylation of pyruvic acid by alkaline washed yeast with increasing concentrations of cocarboxylase. Curve I, cocarboxylase alone. Curve II, cocarboxylase + 10γ vitamin B_1 hydrochloride.

error of manometric measurement. The assumption must be made that the action of boiled tissue extracts is due to the presence of cocarboxylase. This is at present reasonable as no other compound is yet known which has the same action as vitamin B_1 pyrophosphate.

For the estimation of vitamin B_1 the curve in Fig. 1 is used, working with a constant concentration of cocarboxylase. With an unknown extract, cocarboxylase is first determined, then extra cocarboxylase is added up to a total of 1·0γ in the bottles used. In this way CO_2 in excess of that produced with 1·0γ cocarboxylase alone represents CO_2 due to vitamin B_1 stimulation. The sensitivity of the vitamin estimation is much lower; amounts of vitamin under 0·05γ cannot be detected under our present experimental conditions.

With Mg^{++} (100γ) it is often found that there is little difference between the action of 0·1 and 0·2γ vitamin. In presence of a trace of Mn^{++}, however, the difference becomes distinct as the following experiment shows.

	μl. CO_2 in 30 min. (3γ Mn^{++} as $MnCl_2$. No Mg^{++})
0·5 cocarboxylase only	34·7 (triplicate)
0·5 cocarb. 0·1γ vitamin B_1	44·6 (triplicate)
0·5 cocarb. 0·2γ vitamin B_1	53·1 (duplicate)

This recent finding is being further explored.

Here the assumption that any stimulation by tissue extracts is due to vitamin B_1 cannot be made, as vitamin B_1 monophosphate would produce a similar effect. It is unlikely that free pyrimidines or other vitamin derivatives are present in sufficient concentrations to produce any effect. If, in what follows, we refer to free vitamin B_1 in tissue extracts, the possibility remains that the active substance may be partially or totally the monophosphate.

The main source of error in these methods is that the activity of the washed yeast may vary slightly from day to day or even from experiment to experiment, although washing and other experimental conditions have remained unaltered. It has been our custom therefore to check the cocarboxylase curve frequently and apply a correction if necessary. Some idea of the possible error may be got as follows: for a solution containing 0·25γ cocarboxylase and 1·3γ vitamin B_1 in 0·3 ml. an error of $\pm$0·02γ in estimating the cocarboxylase (an extreme case) might influence the vitamin estimation to the extent of $\pm$0·2γ, the vitamin value thus becoming 1·3$\pm$0·2γ. Our experience with yeast variations is curious. After initial rigid standardization, owing to the steady character of the results we relaxed our precautions of including known amounts of cocarboxylase and vitamin B_1 in each group of estimations; this saved much time. Latterly we have reinstated this precaution because at times unaccountable variations appear.

On the other hand, small variations in the amount of yeast in the bottles in a given experiment are without effect. A significantly lower CO_2 production (as compared with 1 ml.) has been found only with amounts of yeast suspension under 0·5 ml. as shown in Table VI.

Table VI. *Decarboxylation of pyruvic acid by alkaline washed yeast using various amounts of yeast suspension*

Sample	Time min.	μl. CO_2: 1·0 ml. washed yeast	0·5 ml. washed yeast	0·25 ml. washed yeast
0·5γ cocarboxylase	30	72	73	56
0·5γ cocarb. + 10γ vitamin B_1	30	323	293	247
0·5γ cocarboxylase	60	111	115	91
0·5γ cocarb. + 10γ vitamin B_1	60	468	449	383

Recoveries of cocarboxylase and vitamin B_1 added together to various tissues are shown in Table VII. There is a tendency for rather high recoveries with small amounts of cocarboxylase in presence of large amounts of vitamin. Although this is not yet understood, the results show that no significant destruction of either compound takes place during the operations involved in the preparation of the boiled extracts. It must be realized that only 1/9[1] of the amounts added per g. tissue are actually estimated, i.e. in line 5 (Table VII) about 0·14γ cocarboxylase.

It may be added that the method suggested by Lipschitz *et al.* [1938] cannot lead to the desired separate determination of cocarboxylase and vitamin B_1, because it involves for the former the use of iodoacetic acid, which we have shown not to inhibit the vitamin activation, and for vitamin B_1 the use of

hexosediphosphate which can increase CO_2 production in the complete absence of free vitamin.

Table VII. *Recovery of cocarboxylase and vitamin B_1 added together to minced normal pigeon's tissues before preparing boiled extracts therefrom (cocarboxylase and vitamin B_1 are expressed in γ per g. fresh tissue)*

	Cocarboxylase, γ				Vitamin B_1, γ			
Tissue	Added	Found	Recovered	%	Added	Found	Recovered	%
Muscle	0·00	4·14	—	—	0·00	2·43	—	—
	0·75	5·20	1·06	141	18·70	19·50	17·07	91
	3·28	7·20	3·06	93	1·64	4·15	1·72	105
Liver	0·00	4·85	—	—	0·00	0·90	—	—
	1·30	7·02	2·17	167	32·50	32·40	31·50	97
	5·00	9·55	4·70	94	2·51	—	—	—
	0·00	4·38	—	—	0·00	0·99	—	—
	5·00	8·52	4·14	83	5·00	5·68	4·69	94
	2·50	7·30	2·92	117	25·00	20·70	19·71	79
	0·00	4·23	—	—	0·00	—	—	—
	4·00	8·18	3·95	99	8·00	—	—	—

4. *Cocarboxylase and vitamin B_1 contents of animal tissues*

In early experiments Simola [1932] found a decrease in cocarboxylase (about −60 %) in the brain and liver of rats upon synthetic diets as compared with those on normal diets; in the absence of vitamin B the amounts were further reduced; the cozymase content did not vary. Westenbrink [1934] and Leong [1937] have reported estimates of vitamin B_1, by weight and bradycardia tests respectively, in rat tissues; presumably such determinations should include cocarboxylase. Leong's principal values were for rats upon ordinary diet: liver 3·75, heart 3·5, brain 3·5, muscle 1·0γ vitamin B_1 per g. tissue, upon the assumption that 1 international unit = 2·5γ vitamin B_1.

Preparation of boiled extracts. Pieces of pigeon and rat tissues were weighed in weighing bottles, minced or chopped and ground in a mortar with 2 vol. distilled water; the suspension was then heated for 3–5 min. in a boiling water bath with stirring, cooled and centrifuged. The brain, as well as the liver, was sometimes minced on an ice-cold plate, but the results so obtained did not differ from those reached by mincing at room temperature. In the case of one animal the brain was removed under amytal anaesthesia after freezing *in situ* with liquid air [Kerr, 1935]; again there was no significant difference. We think therefore that cocarboxylase remains comparatively stable at room temperature, though we know that slight disappearance occurs on incubation at 38°.

Estimations have been performed upon 0·3 ml. samples of extracts from brain, muscle, liver and heart in animals taken as follows.

Pigeons.

I. Normal.

II. Vitamin B_1-deficient (with symptoms).

III. Rice-fed for 25 days (no symptoms).

IV. Vitamin B_1-deficient (with symptoms) and injected with vitamin B_1 (1 mg. per 100 g. animal) 25 min. before death.

V. Vitamin B_1-deficient (with symptoms) and dosed by mouth with vitamin B_1 (100γ *per diem* per animal) for 3 days before death.

VI. Rice-fed for 26 days (no symptoms) but treated as animals of group V for 3 days before death.

Rats.

I. Normal.

II. Vitamin B_1-deficient (with symptoms).

III. Same as II but vitamin B_1 injected (1 mg. per 100 g. animal) 45 min. before death.

IV. Normal or avitaminous but vitamin B_1 given by mouth (1 mg. per 100 g. animal) 1 to 2 hr. before death.

All animals were killed by decapitation and bled before taking tissue samples.

Birds which have been upon a polished rice diet for 25 days and over and which are receiving daily doses of vitamin B_1, are perfectly well in appearance but they do not regain weight significantly. This is due to lack of other nutritional factors [Carter & O'Brien, 1935; 1936]. They form a good control for the birds with symptoms because of their generally normal behaviour at a much diminished body weight (about 70 % of the normal). 100γ vitamin B_1 *per diem* is a comparatively large dose as 10γ would already produce curative effects.

Results. The average values for pigeons are given in Table VIII and individual values of rats in Tables IX and X. The values for pigeon's brain and liver are shown graphically in Figs. 5 and 6. Individual results for pigeons are given

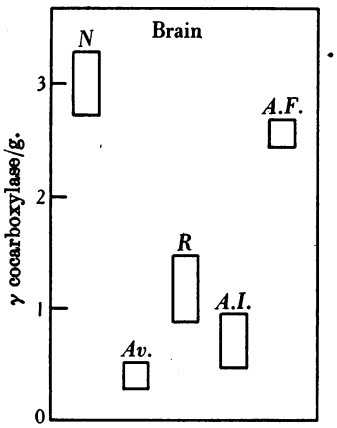


Fig. 5.

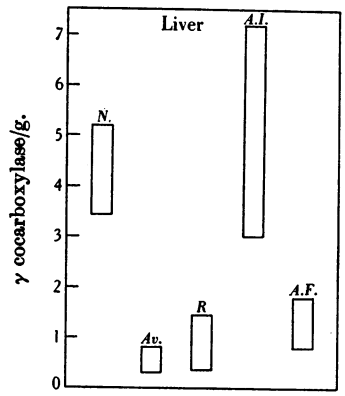


Fig. 6.

Fig. 5. Cocarboxylase in pigeon's brain. *N*, normal. *Av.*, avitaminous (with symptoms). *R*, rice-fed (no symptoms). *A.I.*, avitaminous after injection of vitamin B_1. *A.F.*, avitaminous fed vitamin B_1 for 3 days. Ordinates: mean $\pm 2\epsilon$.

Fig. 6. Cocarboxylase in pigeon's liver. *N*, normal. *Av.*, avitaminous (with symptoms). *R*, rice-fed (no symptoms). *A.I.*, avitaminous after injection of vitamin B_1. *A.F.*, avitaminous fed vitamin B_1 for 3 days.

in the appendix. $\pm 2\epsilon$ (the standard error of the mean) represents $\sigma/\sqrt{n}$ for 6 estimations and over, but $\sigma/\sqrt{(n-1)}$ for fewer estimations than 6. Values are calculated upon the assumption that the fresh tissues contain 70 % H_2O.

A more accurate value would be for brain 80 %, liver 72 %, muscle 74 %, based upon the figures of Roche [1925]: brain, normal 80·7, avitaminous 79·9, inanition 80·2. Liver, normal 71·3, avit. 71·6, inan. 72·6. Muscle, normal 74·8, avit. 73·7, inan. 75·4. A few estimations by us confirm these values. The extreme variations for Roche's tissues were small. For truer values our brain results can be raised by 5 % approx. and the muscle values by 1·5 %.

Though we cannot claim close accuracy for our present method and think it possible that further work may bring to light other sources of error than those referred to above (p. 1503) we consider that our determinations are sufficiently good to demonstrate the points at issue. Even if 50 % of the cocarboxylase estimations (cf. Table VIII) were in error to the extent of +50 % this would mean that the values in Table VIII would be higher by 25 %, but the conclusions below would still stand. As to the vitamin results we can obtain no evidence for the presence of free vitamin B_1 (or its monophosphate) in normal or avitaminous brain, whereas it can be detected after administration. This is therefore a clear difference, but in view of the lower sensitivity of the vitamin method (see p. 1507) it can only be stated definitely that there is less than $0{\cdot}45\gamma$ per g. present.

In the case of the results with brain, and occasionally with liver, showing no vitamin B_1, it has sometimes happened that the tissue extract gave slightly less CO_2 than the control with 1γ cocarboxylase. This has been within the limits of experimental error, so that there is no reason to suspect the presence of inhibitory substances.

In the case of the cocarboxylase values for liver in group IV (Table VIII), it will be seen that they are upon the average over 10 times larger than the corresponding ones of group II. This cannot be due to error introduced by the presence of large amounts of free vitamin, since this (as judged from Table VII) could not raise the results more than 1·7 times.

The outstanding points to note in these results are:

(1) There is much less vitamin B_1 than cocarboxylase in the liver and brain.

(2) The cocarboxylase is reduced in all tissues in both the avitaminous and the rice-fed pigeon, as well as in the avitaminous rat.

Table VIII. *Cocarboxylase and vitamin B_1 in pigeon's tissues (values expressed in γ per g. fresh tissue)*

			Cocarboxylase			Vitamin B_1		
Group	Condition of birds	Tissue	No. of obs.	Mean	Standard error $\pm 2\epsilon$	No. of obs.	Mean	Standard error $\pm 2\epsilon$
I	Normal	Brain	12	3·00	±0·28	7	0·00	±0
		Muscle	9	3·81	±0·42	7	1·60	±0·50
		Liver	9	4·33	±0·88	7	0·32	±0·30
		Heart	8	4·36	±0·57	5	1·54	±0·75
II	Avitaminous (symptoms)	Brain	9	0·40	±0·12	6	0·00	±0
		Muscle	6	1·04	±0·56	5	0·96	±0·71
		Liver	9	0·48	±0·30	7	0·20	±0·20
		Heart	6	0·55	±0·47	5	0·45	±0·27
III	Rice-fed (no symptoms)	Brain	8	1·18	±0·31	—	—	—
		Muscle	2	0·81	—	—	—	—
		Liver	3	0·73	—	—	—	—
		Heart	4	0·91	—	—	—	—
IV	Avitaminous, vitamin B_1 injected 25 min. before death	Brain	4	0·72	±0·24	4	0·55	±0·27
		Muscle	4	2·20	±0·37	4	3·04	±1·90
		Liver	4	**5·10**	±2·10	4	**14·20**	±10·20
		Heart	4	2·05	±0·52	4	4·05	±1·50
V	Avitaminous, dosed 3 days with vitamin B_1	Brain	6	2·55	±0·12	—	—	—
		Muscle	6	1·55	±0·20	—	—	—
		Liver	6	1·28	±0·51	—	—	—
		Heart	6	2·46	±0·49	—	—	—
VI	Rice-fed (no symptoms), dosed 3 days with vitamin B_1	Brain	4	2·82	—	—	—	—
		Heart	4	2·45	—	—	—	—

(3) There is a large increase in both cocarboxylase and vitamin B_1 in the livers of animals shortly after administration of vitamin.

(4) There is a much larger relative increase in the cocarboxylase in brain (and heart) than in the other tissues in pigeons dosed with smaller amounts of vitamin over a period of 3 days. In fact practically normal values are found.

(5) The brain cocarboxylase of pigeons not showing symptoms is higher than that of those which do show symptoms.

Table IX. *Cocarboxylase and vitamin B_1 in rat's tissues. (Individual values. Expressed in γ per g. fresh tissue)*

Group and condition		Brain		Muscle		Liver		Heart	
		Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1
I	Normal	2·93	0·00	0·38	0·90	5·40	0·60	4·15	—
	,,	2·91	0·20	0·32	0·50	4·85	0·60	3·50	0·60
II	Avitaminous	0·68	—	0·10	—	0·53	—	0·52	—
	,,	0·53	—	0·11	—	0·36	—	0·31	—
III	Avitaminous,	0·72	—	0·16	3·80	6·30	24·30	0·74	—
	vitamin B_1 in-	0·54	0·50	0·13	—	2·91	84·60	0·67	—
	jected	0·73	—	0·20	1·10	8·62	7·40	0·77	—

Table X. *Cocarboxylase and vitamin B_1 in the liver of rats after oral administration of vitamin B_1 (Group IV); individual values*

Condition	Animal killed after (hr.)	Cocarboxylase γ per g.	Vitamin B_1 γ per g.
Normal	1	6·50	3·40
Avitaminous	2	6·30	8·10
Avitaminous	2	8·40	8·30

Discussion

We interpret the above facts to mean that cocarboxylase is a very significant form of vitamin B_1 in animal tissues. In some, such as brain and liver, it appears to be predominant; in skeletal and heart muscle there is relatively more vitamin B_1 present, which sometimes approaches the amount of cocarboxylase, hence we cannot say dogmatically that vitamin B_1 itself may not also have some normal function in life. In rat muscle the amount of cocarboxylase is surprisingly low, but there is not much vitamin B_1 either; this agrees with the findings of Westenbrink [1934] and Leong [1937]. As will be noticed by comparing groups II, IV and V of Table VIII, vitamin B_1, if administered, is taken up by the liver and rapidly synthesized to cocarboxylase; with small doses over a period of several days the amount present in the liver again decreases and a preferential increase takes place in the brain and heart, i.e. in the two tissues which are specially affected by vitamin B_1 deficiency. There must therefore be a special affinity for heart and brain tissues reflected in their biological needs. It is evident that even considerable reduction (down to 40 % of the normal) in the cocarboxylase content of the brain can occur before the function of its cells is impaired as shown by the appearance of symptoms. So far as this implies that these symptoms are associated with a low brain cocarboxylase, the evidence reinforces previous views from this laboratory as to the dependence of normal function upon biochemical state. But it is a new point that the liver may co-operate.

What we now want to know is whether in normal functioning there is any local synthesis by brain tissue or whether all the cocarboxylase is transported as such in the blood. The fact that in some birds there is a massive synthesis of

cocarboxylase in the liver before the symptoms clear up is not decisive, because either any local synthesis might be slow, or the cells might take an appreciable time to re-establish sufficient normality.

The finding of Sinclair [1938] that the vitamin B_1 in the blood is in a combined form, though admitting of other interpretations, is consistent with its conveyance as the pyrophosphoric ester; nevertheless there is difficulty in understanding how such an ester could permeate brain and other tissue cells, a difficulty not lessened by the failure to confirm the equal catatorulin activity of cocarboxylase.

We may ask whether the intestine phosphorylates vitamin B_1 *in vivo* in a similar fashion to that suggested for glucose and fat by Verzár & McDougall [1936] or for lactoflavin by the experiments of Rudy [1936]. This needs some direct test, but the experiments of Table X indicate that much vitamin B_1 as such can reach the liver; they are therefore indirect evidence against obligate phosphorylation in the intestine. This conclusion is supported by the failure to obtain *in vitro* phosphorylation of the vitamin with intestinal mucosa, as already referred to in the introduction (unpublished experiments). There seems to be little doubt that the proof that the liver participates in the metabolism of this vitamin is important and suggestive from the clinical standpoint. Its full understanding must wait for more accurate knowledge of the metabolism of pyruvic acid.

Summary

1. Vitamin B_1 (as also does its monophosphoric ester) stimulates the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of cocarboxylase. This action is due to the pyrimidine half of the molecule containing the NH_2 group. It is not due to removal of acetaldehyde by oxidation or dismutation. The nature of this activation is not yet understood and is being further investigated.

2. Hexosediphosphate increases the rate of decarboxylation in presence of cozymase and pure cocarboxylase in the total absence of vitamin B_1. The mechanism of this effect is discussed.

3. Mn^{++} greatly stimulates the carboxylase system if present in sufficient concentrations.

4. The activation of cocarboxylase action by vitamin B_1 has been made use of in a method for the separate estimation of cocarboxylase and free vitamin in the same solution or tissue extract.

5. There is much less vitamin B_1 than cocarboxylase present in boiled extracts from rat and pigeon brain and liver; both occur in those from muscle and heart tissues.

6. The cocarboxylase content of tissues is much reduced in the B_1-avitaminous condition and specifically so in the brain tissue, being soon increased in the latter (and also in the heart) after a short period of administration of the vitamin.

7. Administration of vitamin B_1 to animals leads to an immediate accumulation of both vitamin B_1 and its pyrophosphoric ester in the liver. For the first time this brings the liver into prominence in the metabolism of vitamin B_1.

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APPENDIX

Table XI. *Cocarboxylase and vitamin B_1 in tissues from normal pigeons (γ per g.)*

Brain		Muscle		Liver		Heart	
Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1
3·60	—	4·90	—	2·70	—	4·60	—
3·40	0	3·40	0·90	4·05	0·50	5·22	0·80
2·44	0	—	—	—	—	—	—
2·61	0	—	—	—	—	—	—
2·60	0	2·97	2·25	2·80	0·54	2·90	2·70
2·60	—	4·50	1·60	5·00	—	5·20	1·30
2·96	—	3·70	1·60	3·95	0·00	4·05	—
2·96	—	3·33	0·80	4·40	0·30	5·10	—
3·87	—	2·87	1·80	7·20	0·00	3·38	1·80
3·42	0	4·95	—	4·05	0·00	4·46	1·08
2·76	0	—	—	—	—	—	—
2·70	0	—	—	—	—	—	—
—	—	4·14	2·43	4·85	0·90	—	—

Table XII. *Cocarboxylase and vitamin B_1 in tissues from avitaminous pigeons (γ per g.)*

Brain		Muscle		Liver		Heart	
Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1
0·54	—	2·35	—	1·00	—	1·70	—
0·45	0	0·90	1·20	0·31	0·60	0·36	0·54
0·35	0	1·08	2·10	0·18	0·50	0·45	0·72
0·18	—	0·63	0·40	0·22	0·00	0·18	0·00
0·27	0	0·65	0·50	0·15	0·00	0·21	0·54
0·18	0	0·63	0·60	0·34	0·00	0·40	0·45
0·72	0	—	—	0·23	0·30	—	—
0·40	0	—	—	1·50	0·00	—	—
—	—	—	—	0·40	—	—	—
0·54	—	—	—	—	—	—	—

Table XIII. *Cocarboxylase in tissues from rice-fed pigeons (no symptoms) (γ per g.)*

Brain	Muscle	Liver	Heart
0·99	—	0·45	—
2·20	0·54	0·31	0·99
0·93	1·08	1·44	0·63
0·99	—	—	0·68
0·90	—	—	1·35
1·22	—	—	—
0·95	—	—	—
1·28	—	—	—

Table XIV. *Cocarboxylase and vitamin B_1 in tissues from avitaminous pigeons a short time after injection of vitamin B_1 (γ per g.)*

Brain		Muscle		Liver		Heart	
Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B
0·84	0·40	2·50	5·40	5·75	19·00	2·50	3·60
0·72	0·40	2·07	2·70	2·34	9·50	2·07	4·50
0·42*	0·50	2·40	2·70	5·93	24·00	1·44	5·60
0·90*	0·90	1·80	1·35	6·30	4·50	2·20	2·50
0·81†	0·00	1·71	0·90	2·34	2·70	2·34	2·70
0·54‡	—	0·90	—	1·35	—	0·54	—

* Not recovered at the end of 25 min.
† Bird killed after 1 hr. Not included in average.
‡ Only 25γ vitamin B_1 HCl injected. Killed 25 min. after recovery. Not included in average.

Table XV. *Cocarboxylase in tissues from rice-fed pigeons (19–27 days, with symptoms) dosed by mouth for 3 days with 100γ vitamin B_1 per day*

Date	No. days on rice	Initial wt. g.	Final wt. g.	Wt. after B_1 dosing	γ cocarboxylase per g. Brain	Muscle	Liver	Heart
28 April	27	340	202	203	2·70	1·86	0·81	1·90
	27	402	262	258	2·75	1·26	2·11	1·80
	27	473	316	328	2·43	1·35	0·86	2·70
20 May	19	340	231	232	2·54	1·41	0·92	3·20
	19	371	244	255	2·43	1·35	0·90	3·02
	27	280	194	212	2·43	2·03	2·07	2·12
Average		368	241	248				
Av. % fall		—	65	67				
Av. % increase		—	—	2				

Table XVI. *Rice-fed birds dosed with 100γ vitamin B_1 on successive days*

No. days on rice	Initial wt. g.	Final wt. g.	Wt. after 3 days dosing	γ cocarboxylase per g. Brain	Heart
26	357	252	274	3·40	2·30
26	388	270	299	2·75	1·90
26	395	278	293	2·25	2·90
26	316	210	221	2·90	2·70
Average	364	252	272	2·82	2·45

Av. % fall 69. Av. % rise 6.

Note added 23 August 1938. Similar results to ours for cocarboxylase in tissues have been reached simultaneously by Westenbrink & Goudsmit (*Nature*, 1938, **142**, 151).