CXXXV. PYRUVATE OXIDATION IN BRAIN

VI. THE ACTIVE FORM OF VITAMIN B_1 AND THE ROLE OF C_4 DICARBOXYLIC ACIDS

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THE object of this paper is to present a final proof that the form of vitamin B_1 active in pyruvic acid oxidation is the pyrophosphate and to show that C_4 dicarboxylic acids are an essential part of this system. In so doing not only is the hypothesis of the German workers [Lohmann & Schuster, 1937] proved, but also the importance of recent Hungarian work [Szent-Györgyi *et al.* 1936] fully substantiated. We may also consider that we know now the main facts about the biochemistry of vitamin B_1.

The discovery by Lohmann & Schuster [1937] that cocarboxylase is the pyrophosphate of vitamin B_1, strongly suggested that the phosphoester was the form in which the vitamin is active in the oxidation of pyruvate in animal tissues. It was pointed out in a previous paper [Ochoa & Peters, 1938, 1] that there were two main lines of evidence to support this view: (1) The presence of cocarboxylase in the tissues and the ability of tissues to phosphorylate vitamin B_1; (2) the alleged activity of cocarboxylase in "catatorulin" tests with slices of avitaminous pigeon's brain. Further indirect support was given by the experiments of Lipmann [1937] who showed that the oxidative decarboxylation of pyruvic acid according to reaction (1) or its dismutation [Krebs & Johnson, 1937] according to reaction (2), by alkaline-washed preparations of lactic acid bacteria (*Bacterium Delbrückii*), was catalysed by the pyrophosphate but not by free vitamin B_1. More recently Barron & Lyman [1939] have confirmed this for various strains of gonococcus and staphylococcus.

$$CH_3.CO.COOH + \tfrac{1}{2}O_2 = CH_3.COOH + CO_2 \quad \text{.....(1)}$$

$$2CH_3.CO.COOH + H_2O = CH_3.CH(OH).COOH + CH_3.COOH + CO_2 \text{.....(2)}$$

As regards the presence of cocarboxylase in animal tissues, Ochoa & Peters [1938, 1] showed that in rat's and pigeon's tissues there is cocarboxylase but little free vitamin B_1; brain contains not more than 10% at most of the total vitamin as free vitamin (or monophosphate) and the same is the case with liver.[1] Similar results were obtained by Westenbrink & Goudsmit [1938] using a different method. The amount of cocarboxylase was much reduced in the B_1-avitaminous conditions. Injections of vitamin B_1 led to marked synthesis of cocarboxylase in the liver of avitaminous animals [Ochoa & Peters, 1938, 1; confirmed by Westenbrink & Goudsmit [1938], who also showed this in the

[1] Recent observations show that the vitamin in pigeon's breast muscle not present as cocarboxylase is very likely monophosphoric ester. The whole of the thiochrome-yielding substances in boiled extracts is precipitated by Pb and no thiochrome can be extracted by *iso*butyl alcohol [Ochoa, unpublished results].

kidney]. The vitamin is also readily phosphorylated to cocarboxylase by the liver *in vitro*; with brain and muscle the synthesis was much less [Ochoa & Peters, 1938, 2].

Direct proof of the activity of vitamin B_1 pyrophosphate in animal tissues was however not available, since Peters [1937] failed to confirm Lohmann & Schuster's [1937] contention that cocarboxylase produced equal "catatorulin" effects to vitamin B_1 using avitaminous pigeon's brain brei. That the pyrophosphate is really the active form we have now proved by the use of two different types of finely ground brain preparations I and II. Whereas the brei previously used showed a much larger effect with free vitamin B_1, preparation I is activated by cocarboxylase but not by free vitamin B_1, and preparation II, while being well activated by cocarboxylase, only responds to relatively large amounts of vitamin B_1. With preparation II the activity of vitamin B_1 is due to its synthesis to cocarboxylase, as is also the case with the brei. With both preparations I and II the oxidation of pyruvate is found to be catalysed by the system of C_4 dicarboxylic acids.

Experimental methods

Brain slices or brain brei were used as in previous experiments upon the catatorulin test [Peters, 1938]. Finely ground brain preparations (dispersions) were prepared as follows.

(I) The fresh brain was thoroughly ground up in an ice-cold mortar and successively extracted by grinding with small volumes of ice-cold (1) Ringer-phosphate *p*H 7·3, (2) phosphate buffer (*M*/10) *p*H 7·3, and (3) and (4) distilled water. After each extract the whole was centrifuged; the extracts were combined and finally mixed with the solid residue.

(II) The brain, ground as in I, was extracted with ice-cold Ringer-phosphate and the mixture pressed through muslin.

In either case a fine suspension is obtained which can be accurately pipetted into the manometer bottles, the dispersion being the final addition. One pigeon brain (cerebrum + optic lobes) can make as much as 21 ml. of which 2·0 can be taken per bottle.

Cocarboxylase was determined by the method of Ochoa & Peters [1938, 1] in boiled samples. In some cases, when the amounts of tissue used were small or the dilution high, the contents of the bottles were transferred quantitatively into centrifuge tubes and spun sharply; the residue was then mixed with the appropriate amount of water and boiled in the usual way. All the cocarboxylase is present in the solid residue.

Reduction of oxaloacetic to malic acid was determined by the method of Massart [1939].

Both Barcroft and Warburg manometers have been used, with air or O_2 as gas. Temp. 38°.

Preparations

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

Cocarboxylase. One pure specimen from Prof. K. Lohmann (Berlin). This was occasionally used in the experiments but mainly employed to standardize the other preparation, a synthetic specimen prepared by the method of Weijland & Tauber [1938] by Messrs Merck and Co., U.S.A. The purity of this specimen as tested in this laboratory proved to be 65%. All figures given in tables, etc. are calculated for pure cocarboxylase.

Vitamin B_1 monophosphate. A synthetic specimen prepared in this laboratory by Mr L. A. Stocken (vitamin B_1-free; 2% cocarboxylase).

Crystalline Na pyruvate as prepared in this laboratory was used throughout. The fumarate was Kahlbaum's Na salt and the Na oxaloacetate a pure specimen from the Biochemical Laboratory of Szeged, Hungary.

Presentation of results.

The ideal method of presenting results in tables probably does not exist. In the catatorulin tests with slices and brei, the previous use of rates expressed as μl. O_2/g. × hr. (wet wt.) has been followed here; in the experiments with dispersions it has been thought more informative to express the actual O_2 uptake in μl. for the sample taken, as it is easier so to relate it to the amount of cocarboxylase placed in the bottle.

1. Experiments with brain slices and brei

Doubt was thrown upon the view of Lohmann & Schuster [1937] that vitamin B_1 pyrophosphate was the active form of the vitamin in tissues owing to the failure to confirm the equal activity in the catatorulin tests. As the experiments by the German workers were done with brain slices, we here record in detail in Table 1 (A) three out of eight experiments of our own using small amounts of vitamin B_1 and approximately equivalent amounts of cocarboxylase. In no case is there a catatorulin effect of cocarboxylase approaching that of the free vitamin.

Table I. *Comparison of catatorulin effects of vitamin B_1 and cocarboxylase using slices of pigeon's cerebrum*

Figures represent rate of O_2 uptake in μl./g. tissue × hr. for each period (0·018 *M* pyruvate).

					Average catatorulin effect	
Successive periods (min.) ...	15	15	30	30	First 30 min.	Last 60 min.
(A) Small equivalent amounts of cocarboxylase (0·5 μg.) and vitamin B_1 (0·35 μg.)						
1. No addition	2680	2630	2202	1798	—	—
Cocarboxylase	2592	2500	2245	1802	−109	+ 23
Vitamin B_1	2808	2910	2810	2550	+204	+680
2. No addition	2322	2010	1742	1037	—	—
Cocarboxylase	2156	2060	1888	1265	− 80	+188
Vitamin B_1	2403	2620	2578	2177	+345	+988
3. No addition	1500	1400	1076	956	—	—
Cocarboxylase	1440	1355	1252	1155	− 52	+188
Vitamin B_1	1823	1690	1752	1773	+306	+746
In all cases cocarboxylase or vitamin B_1 are triplicates; no addition, duplicate.						
(B) Large equivalent amounts of cocarboxylase (7·5 μg.) and vitamin B_1 (5 μg.); the latter is much in excess of maximum vitamin B_1 needed.						
4. No addition	2740	2650	2420	1970	—	—
Cocarboxylase	3730	3800	3633	3140	+1070	+1192
Vitamin B_1	3670	3740	3567	3170	+1010	+1173
Figures are the mean of duplicates, in all cases carefully shuffled.						

That the apparently positive results of Lohmann & Schuster can be imitated by using excessive amounts of cocarboxylase is shown by Exp. 4 of Table I (B). Here a large catatorulin effect is produced which is identical with that of vitamin B_1.

Two further brei experiments were done at very alkaline reaction (*p*H 8·2). Table II shows that even here cocarboxylase is not so active as vitamin B_1.

Table II. *Comparison of catatorulin effects of vitamin B_1 and cocarboxylase with pigeon's brain brei at alkaline reaction* (p*H 8·2*)

Cocarboxylase 5 μg.; vitamin B_1 2 μg. Figures represent average rate of respiration (μl./g. tissue × hr.) for the period 30–90 min. (0·018 *M* pyruvate).

	O_2 uptake	Catatorulin increase
No addition	759	—
Cocarboxylase	885	+126
Vitamin B_1	1226	+467
Cocarboxylase + vitamin	1237	+478

Hence it is definite that with brei, under all conditions, or with slices, cocarboxylase is less active in the catatorulin test than free vitamin B_1. When this fact was first clear with brei, it was suggested as one explanation [Peters, 1937] that a permeability factor might be responsible. Since then Dr H. M. Carleton, in as yet unpublished work, has shown that nerve cells are largely destroyed in preparing the brei, though the nuclei are mainly intact. This made the permeability explanation less acceptable; nevertheless the work now to be considered shows that it is the right one. Using the dispersions of brain tissue, evidence has been obtained that the failure of brei to react with the combined form of vitamin B_1 is due to failure to reach the active centre.

The effects of C_4 dicarboxylic acids on the O_2 uptake of brain brei (pigeon) in the presence of pyruvate are generally small. Table III shows the result of such an experiment with fumarate and also the effect of malonate [cf. Greville, 1936; Weil-Malherbe, 1937] which produces here only slight inhibition. Whereas the net O_2 uptake of fumarate alone is 42 μl. the net O_2 uptake of pyruvate is increased in presence of fumarate by 310 μl. These effects are much more marked in the brain dispersions.

Table III. *Effects of fumarate and malonate on the oxidation of pyruvate in brei from normal pigeon's brain*

Concentration of pyruvate 0·025 *M*.

	μl. O_2 uptake per g. tissue in 1 hr. with		
	No addition	Fumarate [0·005 *M*]	Malonate [0·024 *M*]
Tissue (about 200 mg.)	960	1002	726
Tissue + pyruvate	1760	2110	1360
Net O_2 uptake of pyruvate	800	1110	634

Increase of O_2 uptake of pyruvate in presence of fumarate 310 μl. (+39%). Inhibition by malonate of net O_2 uptake of pyruvate 166 μl. (−21%).

2. Experiments with brain dispersions

A. *Pyruvate oxidation and the role of C_4 dicarboxylic acids*

The O_2 uptake of the brain dispersions, in the presence of pyruvate, is markedly stimulated by addition of fumarate. That this effect is catalytic is shown by the fact that the extra O_2 uptake in the presence of both pyruvate and fumarate is much higher than the sum of the extra uptakes with pyruvate and fumarate separately. The experiment of Table IV shows this very clearly. With

Table IV. *Effect of fumarate on the oxidation of pyruvate in pigeon's brain dispersion (type II)*

Each sample contained 2 ml. enzyme (about 220 mg. brain). Concentration of pyruvate 0·025 *M*.

	μl. O_2 uptake in 1 hr. with	
	No addition	Fumarate [0·005 *M*]
Enzyme alone	154	177
Enzyme + pyruvate	274	384
Net O_2 uptake of pyruvate	120	207

Increase of O_2 uptake of pyruvate in presence of fumarate 87 μl. (+73%).

these preparations a high respiration is obtained with pyruvate + fumarate which however falls off rather rapidly after 30–40 min. at 38°, probably owing to instability of some of the enzymic components of the pyruvate oxidation system.

The inhibition by malonate of the pyruvate oxidation is much more marked in the brain dispersions than in the brei and reaches values about 70% (Table V); this is as high as found by Szent-Györgyi *et al.* [1935] for the inhibition of respiration in muscle dispersions. Curiously enough the residual respiration of the brain dispersion is not so strongly inhibited (cf. Table V).

Table V. *Malonate inhibition of pyruvate oxidation in pigeon's brain dispersion (type II)*

Each sample contained 2 ml. enzyme (about 220 mg. brain). Concentration of pyruvate 0·025 *M*.

	μl. O_2 uptake						
Time min.	Enzyme (1)	Enzyme + malonate [0·024 *M*] (2)	Enzyme + pyruvate (3)	Enzyme + malonate + pyruvate (4)	Net O_2 uptake of pyruvate (3–1)	Net O_2 uptake of pyruvate with malonate (4–2)	% inhibition by malonate of pyruvate oxidation
10	48	28	104	48	56	20	64
30	85	60	230	93	145	33	78
50	114	91	310	148	196	57	71

The better results obtained with the brain dispersions, as compared with brei, are probably due to the higher degree of division of the tissue in the former which would allow the C_4 acids (which are rather firmly bound by the enzymes [cf. Banga, 1937]) to diffuse more readily into the surrounding fluid, their concentration thus easily falling below the optimum required for their catalytic activity; also the malonate should reach the succinodehydrogenase more easily.

The negative results of Schoen & Gerard [1937], who used "solutions" of rabbit's brain in water (brain sols), are difficult to explain. With lactate as substrate they found neither a catalytic effect of succinate nor an inhibition by malonate. Although their lactate oxidation might stop short at pyruvate, possibly owing to low pyruvate dehydrogenase and to the low salt content, yet one would expect the lactic dehydrogenase, being a coenzyme I system, to be activated by the C_4 dicarboxylic acids. Recent experiments with dialysed brain dispersions [Banga *et al.* 1939] show still more clearly the indispensability of these acids for the pyruvate oxidation in brain. Further, the reduction of added oxaloacetate to malate which rapidly takes place in muscle preparations [Szent-Györgyi, 1937; Massart, 1939] is also observed in brain brei as well as in our brain dispersions as is shown in Table VI. The experiments of Table VI were done by

incubating 500 mg. pigeon's brain brei suspended in 1·5 ml. Ringer-phosphate (*p*H 7·3) or 1·5 ml. dispersion II (500 mg. brain) with 0·013 *M* oxaloacetate for 5 min. at 38°. The total volume was 4 ml. The samples were deproteinized with 0·5 ml. 10% trichloroacetic acid, oxidized with 0·1 *N* $KMnO_4$, and the neutralized solution was treated with fumarase, the fumarate formed from malate being titrated with 0·01 *N* $KMnO_4$ [Massart, 1939].

Table VI. *Reduction of oxaloacetate in brain*

No.	Enzyme	Fumaric acid found after fumarase mg.	Calculated malic acid (as fumaric) after fumarase mg.
1	Brei	0·218	0·87
2	Dispersion II	0·216	0·86
3	Dispersion II	0·200	0·80
		Average 0·21	0·84

The total fumarate was therefore 1·05 mg. as fumaric acid or 1·22 mg. as malic acid. Massart [1939] found in muscle under identical conditions 1·80 mg. malic acid formed from oxaloacetate, thus brain forms about 70% of the amount that muscle forms.

B. *Activity of cocarboxylase*

Cocarboxylase in very small amounts strongly stimulates the oxidation of pyruvate in the salt dispersions of avitaminous pigeon brain. Free vitamin B_1 is either quite inactive or very little active in dispersions of type I but shows some activity in those of type II. Typical experiments with dispersion I are given in Table VII and Fig. 1; in Table VII one out of four similar experiments with a dispersion (type I) prepared from brain of "rice-fed" pigeons (showing no symptoms) is also quoted (Exp. 7). Experiments with type II dispersions are shown in Tables VIII, IX and XI and Figs. 2 and 3. It may be seen that with this type of dispersion free vitamin B_1 shows small but definite effects.

Table VII. *Effects of vitamin B_1 and cocarboxylase on the O_2 uptake of dispersions (type I) from avitaminous pigeon brain in the presence of pyruvate (0·018*M*) and fumarate (0·004*M*)*

1 ml. enzyme (140–190 mg. brain) to 2·5 ml. with additions. Air; 38°.

		μl. O_2 uptake in 30 min. with				
No.	Condition of birds	No addition	Vitamin B_1 (μg.)	Cocarboxylase (μg.)	Vitamin B_1 + cocarboxylase	Increase μl. O_2 30 min. with cocarboxylase
1	Avitaminous	149	(2) 135	(3·2) 194	186	+45
2	,,	120	(4) 122	(1·0) 198	209	+78
3	,,	100	—	(0·16) 118	—	—
		—	—	(0·53) 133	—	—
		—	—	(1·6) 133	—	+33
4	,,	107	(20) 119	(1·6) 157	—	+50
5	,,	68	(4) 71	(1·6) 117	117	+49
		—	(20) 70	—	—	—
6	,,	87	—	(0·15) 150	—	—
		—	—	(0·2) 156	—	—
		—	—	(0·3) 153	—	—
		—	—	(0·5) 163	—	+76
7	Rice-fed (no symptoms)	143	(10) 144	(1·6) 150	153	+ 7

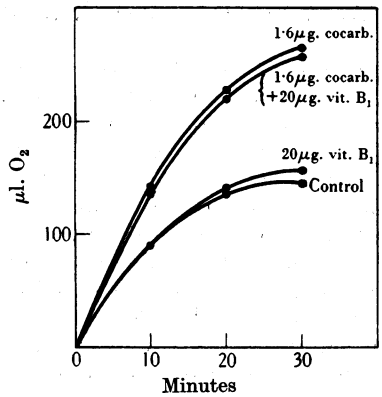

Fig. 1. Comparison of the effects of vitamin B_1 and cocarboxylase on the oxidation of pyruvate (0·018 *M*) in dispersions (type I) from avitaminous pigeon brain, in the presence of fumarate (0·004 *M*). (Samples as in Table VII.)

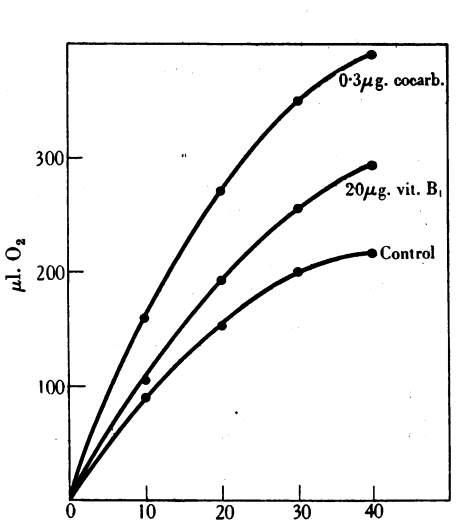

Fig. 2.

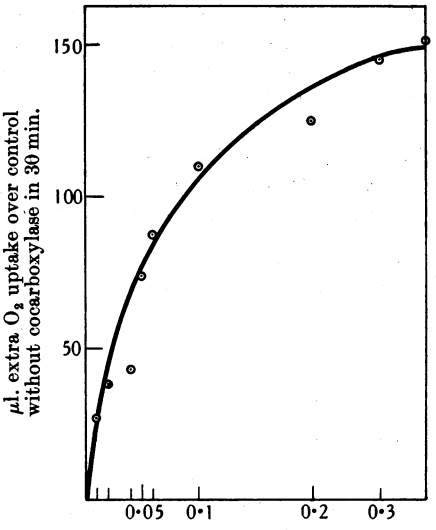

Fig. 3.

Fig. 2. Comparison of the effects of vitamin B_1 and cocarboxylase on the oxidation of pyruvate (0·027 *M*) in dispersions (type II) from avitaminous pigeon brain, in the presence of fumarate (0·005 *M*). (Samples as in Table VIII.)

Fig. 3. Relation between extra O_2 uptake and cocarboxylase concentration in dispersions (type II) from avitaminous pigeon brain. 1·5 ml. enzyme to 2 ml. with additions. 0·027 *M* pyruvate, 0·005 *M* fumarate. O_2, 38°. 320 mg. brain.

Table VIII. *Effects of vitamin B_1 and cocarboxylase on the O_2 uptake of dispersions (type II) from avitaminous pigeon brain in the presence of pyruvate (0·027* M*) and fumarate (0·005* M)

1·5 ml. enzyme (about 320 mg. brain) to 2 ml. with additions. Gas, O_2; 38°.

	μl. O_2 uptake (average of duplicates)			Extra O_2 uptake caused by	
Time min.	No addition	10 μg. Vitamin B_1	1·3 μg. cocarboxylase	Vitamin B_1	Cocarboxylase
10	119	126	174	7	52
20	190	207	288	17	114
30	258	291	401	33	189
40	314	354	476	40	192
50	362	396	522	34	138

Even the smallest amount of cocarboxylase used in the experiments of Table VII (cf. specially Exp. 6), 0·15 μg. for 140 mg. tissue (equivalent to 1·07 μg. for 1 g. tissue), produced practically maximum effects; hence the average maximum catatorulin effect may be calculated for the first six experiments of Table VII with avitaminous brain. The value is 55 μl. O_2 in 30 min. or 580 μl. O_2/g. tissue × hr., which is about the same value as is reached with maximum amounts of vitamin B_1 in brei for the period 1–2 hr. In the dispersions the initial values for the first 10 min. are usually much higher; thus in Exp. 6 the effect reached the phenomenal increase of 1500 μl. O_2/g. tissue × hr. This is a larger increase than has ever been recorded in a catatorulin test with brei and yet it was produced, in this case, by 0·15 μg. cocarboxylase equivalent to approx. 0·1 μg. vitamin B_1; the latter would not produce a maximum rise of more than 100 μl. O_2/g. × hr. with the same amount of brei.

In dispersions of type I vitamin B_1 is inactive (some activity however occurred in Exp. 4, Table VII, with 20 μg.); as there is some cocarboxylase present in the avitaminous brain this shows that there is no stimulation by vitamin B_1 of the action of cocarboxylase similar to that found by Ochoa [1938] for yeast. Moreover, addition of vitamin B_1 together with cocarboxylase (Table VII) does not increase the effect of the latter alone.

Table IX. *Comparison of the effects of vitamin B_1 monophosphate and cocarboxylase on the O_2 uptake of dispersions (type II) from avitaminous pigeon brain, in the presence of pyruvate (0·027 M) and fumarate (0·005 M)*

1·5 ml. enzyme (about 320 mg. brain) to 2 ml. with additions. Gas, air; 38°. (Figures in brackets to the right give the extra O_2 taken up in the presence of catalyst.)

	μl. O_2 uptake in 30 min. (average of duplicates)			
	Exp. 1		Exp. 2	
No addition	473		364	
Vitamin B_1	(0·5 μg.)	474 (–)	(1·0 μg.)	390 (+26)
Vitamin B_1 monophosphate	(0·3 μg.)	469 (–)	(0·6 μg.)	388 (+24)*
Cocarboxylase	(0·01 μg.)	484 (+11)	(0·025 μg.)	398 (+34)
			(0·3 μg.)	463 (+99)

* 0·6 μg. vitamin B_1 monophosphate contained 0·012 mg. cocarboxylase.

As with brei, there is no action upon dispersions from the brain of the "rice-fed" bird (Exp. 7, Table VII) not yet showing symptoms. Hence it seems clear that the 1·2 μg./g. cocarboxylase, found in the brain of the "rice-fed" pigeon, are sufficient to supply the full needs of the enzyme system and that the extra 2 μg./g. in normal brain [Ochoa & Peters, 1938, 1] constitutes a reserve. Since a decrease of 0·8 μg./g. is associated with the appearance of symptoms (0·4 μg./g. cocarboxylase in avitaminous brain) if cocarboxylase is the active form it would follow that for 140 mg. avitaminous tissue a maximum effect should be produced in the bottle by 0·11 μg. This is reasonably close to the amount giving maximum action in Exp. 6 (Table VII).

The effects of increasing concentrations of cocarboxylase below the maximum are shown in Fig. 3 for a dispersion of type II. Amounts as low as 0·01 μg. have a definite effect. 0·3 μg. (with 320 mg. tissue) gives practically maximum activation, no increase occurring by using 1·3 μg. 0·3 μg. cocarboxylase in 320 mg. brain would correspond to about 0·9 μg./g. tissue; this amount is again remarkably close to the 0·8 μg./g. required to raise the cocarboxylase content of the avitaminous to that of the "rice-fed" brain.

Vitamin B_1 monophosphate is not more active than is vitamin B_1 itself in dispersions from avitaminous brain. Table IX shows the results of two experiments with dispersions of type II. The synthetic specimen of vitamin B_1 monophosphate used was free from vitamin B_1, but contained 2% cocarboxylase as tested with yeast.

C. *Synthesis of cocarboxylase by brain tissue* in vitro

The effect of vitamin B_1 in dispersions of type II, as well as in brei, is due to its enzymic phosphorylation to cocarboxylase. Although this only occurs to a very small extent, it is fully sufficient to account for the observed catatorulin effects. At the end of the experiments given in Table VIII and Fig. 2 cocarboxylase was determined (in quadruplicate) in the control samples as well as in those which had received vitamin B_1. The results are shown in Table X. In

Table X. *Synthesis of cocarboxylase from vitamin B_1 in dispersions (type II) from avitaminous pigeon brain (experiments of Table VIII and Fig. 2)*

Cocarboxylase determined at the end of experiments in samples with no addition and vitamin B_1 addition.

		μg. cocarboxylase per sample (320 mg. brain)		
Exp.	Duration min.	In control sample	In vitamin B_1 sample	Synthesis
Table VIII	50	0·26	0·30	0·04
Fig. 2	40	0·16	0·21	0·05

320 mg. brain tissue (being the approximate equivalent of each sample) about 0·05 μg. cocarboxylase is synthesized. In a fresh experiment therefore the activities of 10 μg. vitamin B_1 and 0·05 μg. cocarboxylase were compared. Table XI shows that they produced almost the same O_2 uptake. But whereas

Table XI. *Comparison of the effects of vitamin B_1 and cocarboxylase, in dispersions (type II) from avitaminous pigeon brain, on the O_2 uptake in the presence of pyruvate (0·027 M) and fumarate (0·005 M)*

(A) 1·5 ml. enzyme (about 320 mg. brain) to 2 ml. with additions. Gas, O_2; 38°.

	μl. O_2 uptake (average of duplicates)				Extra O_2 uptake caused by		
Time min.	No addition	10 μg. vitamin B_1	0·05 μg. cocarboxylase	0·1 μg. cocarboxylase	10 μg. vitamin B_1	0·05 μg. cocarboxylase	0·1 μg. cocarboxylase
10	148	158	180	200	10	32	52
20	256	297	310	344	41	54	88
30	327	385	400	452	58	73	125
40	385	487	465	525	140	80	140

(B) Rates of extra O_2 uptake (in μl./hr.) caused by

Time period min.	10 μg. vitamin B_1	0·05 μg. cocarboxylase	0·1 μg. cocarboxylase
0–10	60	190	310
10–20	186	130	216
20–30	100	114	222

the effect due to a given amount of cocarboxylase is practically maximum at the end of the first 10 min. and then decreases, the effect of the free vitamin is not maximum at the end of that time; it increases for the next 10–20 min. This, while adding further support to the view that the effect of the vitamin is due to

its progressive phosphorylation to cocarboxylase, makes a strict comparison of the vitamin and cocarboxylase effects difficult. Still, the agreement between the catatorulin effects of both in Table XI is reasonably good especially at the end of 20–30 min. Unpublished experiments of one of us (S. O.) show that at 38° the amount of cocarboxylase synthesized *in vitro* by various tissues from vitamin B_1 does not increase much after 30 min.

It remains to be shown that the brei used in the ordinary catatorulin tests will synthesize sufficient cocarboxylase to produce the observed action. A previous estimate of this synthesis by one of us [Peters, 1937] was in error owing to lack of knowledge of the stimulant action of vitamin B_1 itself in yeast. The experiments of Table XII show that with brei too there is small but sufficient

Table XII. *Synthesis of cocarboxylase from vitamin B_1 under the conditions of a typical catatorulin test with brei from avitaminous pigeon brain*

Each bottle contained an average of 140 mg. tissue in 3 ml. Ringer-phosphate *p*H 7·3. Gas, O_2; 38°. Pyruvate 0·018 *M*.

No.	Time min.	μl. O_2 uptake/g. tissue (average of duplicates) No addition	2 μg. vitamin B_1	Extra due to vitamin
1	10	377	499	122
	20	682	850	168
	30	946	1270	324
2	10	326	363	37
	20	655	786	131
	30	878	1172	294
		μg. cocarboxylase/g. tissue (determined at end)		
1	—	0·63	0·82	0·19
2	—	0·60	0·70	0·10

Average cocarboxylase synthesis about 0·15 μg./g. brain or 0·02 μg. in an actual sample (140 mg. tissue).

synthesis. The average catatorulin effect at the end of 30 min., in the two experiments of Table XI, was 618 μl. O_2/g. × hr. and the cocarboxylase synthesized 0·15 μg./g. Reference to the curve of Fig. 3 will show that 0·15 μg. cocarboxylase produces in dispersions of type II in 30 min. an approximate extra O_2 uptake of 125 μl. for 320 mg. tissue. This corresponds to 780 μl./g. tissue × hr. a figure very close indeed to the value obtained with brei in Table XII. Hence the agreement is complete that oxidation of pyruvate here is catalysed by the cocarboxylase synthesized from the added vitamin B_1.

Discussion

C_4 dicarboxylic acids. The effect of fumarate is a striking confirmation of views advanced in Szeged of the fundamental importance of the system of C_4 dicarboxylic acids. Szent-Györgyi and his colleagues have suggested that the catalytic activity of these acids involves the two systems succinic-fumaric and malic-oxaloacetic acids. According to them H from the substrates (donators) is transferred to oxaloacetate which is thus reduced to malate; from malate H is transferred to fumarate which is reduced to succinate and this, activated on the succino-dehydrogenase, gives up its H to the cytochrome system. This explains why all these acids are effective in catalysis. It would also give a biological reason for the presence in brain tissue [cf. especially Weil-Malherbe, 1937] of a system capable of forming succinic acid from pyruvic acid, because

this would ensure the formation (from pyruvic acid) of the C_4 acids necessary for catalysis. Such an explanation would make it unnecessary to believe that the formation of C_4 acids from C_3 acids means that the pyruvate is oxidized through the stages of C_4 acids, a view which from several angles has been shown to be untenable [cf. Long *et al.* 1939].

Reaction in which cocarboxylase is involved. This paper adds final evidence to the view that cocarboxylase is the active form of vitamin B_1 concerned in the oxidation of pyruvate by animal tissues. The analogy with yeast and all the evidence derived from work with bacteria [Lipmann, 1937; Hills, 1938; Barron & Lyman, 1939] suggest that cocarboxylase is involved in a stage of (oxidative) decarboxylation of pyruvic acid. Recent work from this laboratory [Long & Peters, 1939] on the metabolism of α-keto-acids (and especially α-ketobutyric acid) in brain supports this view, since the extra O_2 uptake caused by the α-keto-acid in avitaminous brain brei is stimulated by vitamin B_1 and oxidative decarboxylation is the only metabolic change that this α-keto-acid undergoes in brain.

Catalytic activity of cocarboxylase. The catalytic activity of cocarboxylase with pyruvate in our brain dispersions is of a very high order. Assuming 0·01 μg. to be the smallest effective amount under our experimental conditions, this corresponds to a minimum effective concentration of 5×10^{-9} *M*. With $1{\cdot}5 \times 10^{-7}$ *M* maximum effects are obtained. It can be calculated that, under optimum conditions, 1 mol. cocarboxylase catalyses the uptake of 1500 mol. O_2 per min. This is the same order of activity as found by Warburg & Christian [1938] for the coenzyme of the *d*-amino-acid oxidase. Also the activity of cocarboxylase in yeast, in the presence of an excess of vitamin B_1 is of the same order [cf. Ochoa & Peters, 1938, 1], but only about 1/10 of that in the absence of vitamin.

The brain enzyme is saturated with about 1 μg. cocarboxylase per 1 g. fresh brain. In yeast it can be calculated from data from various sources [i.e. Lohmann & Schuster, 1937; Ochoa & Peters, 1938, 1] that 1 g. fresh yeast is saturated with approximately 90 μg. cocarboxylase in the absence of free vitamin B_1, or with 9 μg. in the presence of the latter. In any case the concentration of pyruvate dehydrogenase in brain seems to be much lower than that of carboxylase in yeast.

Vitamin B_1 metabolism. Now that the nature of the vitamin B_1 component of the pyruvate oxidation in animal tissues appears to be settled, it will be possible to consider with greater confidence the metabolism of the vitamin in a wider sense. The difficulty of penetration of the tissue brei by the phosphorylated form which is here shown, supports the idea that vitamin B_1 permeates the tissue walls as such, and is then phosphorylated within the cell. This is consistent with recent findings of Sinclair [1939] on the absence of cocarboxylase (and presence of vitamin B_1) in cerebrospinal fluid, as well as with the estimations of cocarboxylase in blood showing its presence in cells and its absence from plasma [Goodhart & Sinclair, 1939]. The physiological significance of the high phosphorylating powers for vitamin B_1 shown by liver and kidney is probably that these organs act as traps and store the vitamin, supplying it in the free form to the tissues according to their needs; both these organs show a high phosphatase activity towards cocarboxylase [Ochoa, unpublished].

It is difficult to understand why no catatorulin effects have ever been obtained with the avitaminous liver. Obviously the cocarboxylase in liver must also be playing the usual part in the oxidation of pyruvate. It is possible that it might also be concerned there with fat synthesis. McHenry & Gavin

[1938] have shown that administration of vitamin B_1 leads to storage of fat from carbohydrate; this effect is probably secondary to the oxidative decarboxylation of pyruvic acid.

Although it is shown in this paper that vitamin B_1 pyrophosphate is the active form concerned with oxidation of pyruvate, and free vitamin B_1 (or its monophosphate) occurs only in small amounts in tissues, it appears that vitamin B_1 in combination with protein is present in some biological fluids (plasma [Goodhart & Sinclair, 1939]; milk [Houston & Kon, 1939]). Whether this might be a transport form of the vitamin, or whether it has a different biological significance, it is impossible at present to decide.

Summary

1. The system of C_4 dicarboxylic acids of Szent-Györgyi *et al.* is catalytically concerned in the oxidation of pyruvate in brain.

2. With brain slices and brei (avitaminous pigeon) cocarboxylase is less active in the catatorulin test than free vitamin B_1.

3. With two types of finely ground preparations of avitaminous brain, cocarboxylase is much more active than free vitamin B_1, one of the preparations reacts to cocarboxylase and not at all to vitamin B_1. Vitamin B_1 monophosphate is not more active here than is vitamin B_1 itself.

4. Brain preparations (including brei) which respond to vitamin B_1 can be shown to synthesize cocarboxylase in amounts which account for the changes in O_2 uptake found.

5. A maximum response is produced with a concentration of $1{\cdot}5 \times 10^{-7}$ *M* cocarboxylase; each mol. cocarboxylase catalyses optimally the uptake of 1500 mol. O_2 per min.

6. The hypothesis of Lohmann & Schuster that the active form of vitamin B_1 in animal tissues is the pyrophosphate is considered to be now proved.

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