CCLXXV. PYRUVATE OXIDASE IN BRAIN

IV. CO-CARBOXYLASE

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THE discovery by Lohmann & Schuster [1937] that co-carboxylase is a pyrophosphoric ester of vitamin B₁ led him to suggest the hypothesis that pyruvic acid accumulation in our experiments is explained by the lack of co-carboxylase. There are really two points to be considered separately; first, whether vitamin B₁ is active in the brain pyruvate oxidase system as the "diphosphate", and secondly, whether this latter, if present, is acting as a co-carboxylase to a carboxylase system such as is found in yeast. If the first point is true, then vitamin B₁ must be phosphorylated during our *in vitro* experiments. Recent work had actually led us, previous to the publication of their discovery, to the view that vitamin B₁ was some kind of prosthetic group of pyruvate oxidase [see especially Westenbrink & Polak, 1937].

In the attempt to throw light upon this important problem, (1) the catatorulin effect of co-carboxylase and the phosphorylation by brain tissue of vitamin B₁ have been examined and also (2) the effect of the brain enzyme system upon α -ketobutyric acid.

(1) *Catatorulin effect of co-carboxylase*

In support of the "co-carboxylase" hypothesis, they stated that this has as good an effect as vitamin B₁ in the catatorulin test. This particular contention has not been here confirmed. In work with crude co-carboxylase, which was prepared by Kinnersley & Peters [1937] by the action of dry yeast upon vitamin B₁,¹ catatorulin effects were obtained; but some later preparations seem to be less effective. Owing to the kindness of Prof. Lohmann, to whom my thanks are due, I have been able to test a specimen of pure co-carboxylase supplied by him against a synthetic specimen of vitamin B₁ chloride hydrochloride. In another paper, reasons will be given for using as standards amounts of the latter not greater than 0.5 μ g.;² some brains do not show much increase in oxygen uptake for the range of concentration 0.5–1.0 μ g.; the latter seems to be maximum when working with pyruvate as substrate.

Contrary to the experience with crude co-carboxylase, the pure specimen showed very poor catatorulin effects, not greater than 10% of those produced by the vitamin itself. The diagram (Fig. 1) shows in detail three of the experiments out of seven made; for the remaining four the relevant details are shown in catatorulin curves;³ all gave decisively a much lower result⁴ for co-carboxylase. It is difficult to be certain that some of the positive effects found were not due to conversion of some of the diphospho-vitamin B₁ into the base by phosphatase action; but I think that the effect is genuinely due to the phosphate

¹ In presence of phosphate-pyrophosphate.

² Written now μ g. instead of γ .

³ The extra O₂ uptake for 1–2 hr. plotted against added vitamin or co-carboxylase.

⁴ 1.0 μ g. vitamin B₁ chloride HCl is equivalent in base to 1.42 μ g. vitamin B₁ pyrophosphoric ester (co-carboxylase here), $\text{C}_{12}\text{H}_{21}\text{O}_8\text{N}_4\text{P}_2\text{SCl}$.

Fig. 1. *Catatorulin tests.* Falling curves: rate of oxygen uptake/time. Rising curves: extra O_2 uptake ($\mu\text{A/g/hr.}$) due to addition of vitamin B_1 for period 1-2 hr. Respiration level for co-carboxylase marked with arrow (CoC.). 1294, average of quadruplicate estimation 0.5 μg . vitamin B_1 and 10 μg . co-carboxylase. 1299, 1.0 μg . co-carboxylase single determination. The remaining points are average of duplicates. In 1298 and 1299 duplicates were exceptionally close. Values from the charts are as follows: 1288, 1.0 μg . CoC $\equiv$ 0.05 μg . V. 1291, 5 μg . CoC $\equiv$ 0.15 μg . V. 1292, 10 μg . CoC $\equiv$ 0.65 μg . V. 1293, 10 μg . CoC $\equiv$ 0.2 μg . V. 1294, 10 μg . CoC $\equiv$ 0.2 μg . V. 1299, 10 μg . CoC $\equiv$ 0.35 μg . V. For equivalence 10 μg . CoC. should be 7.0 μg . vitamin B_1 CHCl_3 values found are 0.3, 0.65, 0.2, 0.2, 0.6, 0.35, approx. 0.4 μg ., or under 6%.

for the following reasons: it is characteristic of vitamin B₁ itself that a sub-maximum effect is converted into maximum by increase of the vitamin B₁; this is not always so with co-carboxylase (cf. Exps. 1293, 1328, Fig. 1). Under our conditions therefore it is definite that the latter is less effective. So far I have not been able to obtain duplicates with slices which were sufficiently close to give quantitative satisfaction. It seems however possible to reconcile in part the apparent discrepancy between these results and those of Lohmann. If the brain slices used were reacting to 0.5 μ g. or less maximally, then the tests were made at too high a level for quantitative work, i.e. vitamin chloride 2 μ g. approx.

Though it weakens the evidence, the failure to confirm the equal efficiency of co-carboxylase in the cataturulin test is not decisively against Lohmann's hypothesis, because it can be argued that conditions of permeability do not

Table I. *Formation of co-carboxylase by avitaminous brain tissue*

Exp. 1268. Each Barcroft bottle contained 0.25–0.35 g. brain brei from avitaminous pigeon in 2.0 ml. Ringer-phosphate, pH 6.2, containing pyruvate 5 mg. At this pH it has been found previously that the cataturulin effect occurs. No filter-paper with KOH was used; after filling with oxygen, the whole was shaken for 72 min. at 38° as usual, with and without vitamin (10 μ g.). The whole was then heated for 2–3 min. in a boiling water-bath and cooled. Washed (Lohmann) yeast was added and MgCl₂ (0.1 mg.) making the total volume 2.4 ml. The bottles were filled with N₂/CO₂ and the evolution of CO₂ followed at 28°. Controls were set up of the same tissue containing a crude co-carboxylase, pyruvate and yeast alone. CO₂ evolution was as follows in 40 min.

	μ l. CO ₂	
Control	$\begin{cases} 78 \\ 78 \end{cases}$	
Tissue. No vitamin	$\begin{cases} 98.5 \\ 107.0 \end{cases}$	Weight of brain used 348 mg. 270 mg.
Vitamin	$\begin{cases} 176.0 \\ 174.0 \end{cases}$	254 mg. 264 mg.
Crude co-carboxylase	$\begin{cases} 445 \\ 440 \end{cases}$	

Increase for no vitamin +25 μ l.; vitamin +97 μ l.

Exp. 1385. The preliminary period of shaking in O₂ was here reduced to 27 min. (12 + 15 min.) at 38°; vitamin 4 μ g. Flasks were heated for 3 min. exactly on the water-bath to inactivate the brain enzyme system and cooled before addition of washed yeast. Other details as before.

CO₂ evolution in 50 min. at 28°

	μ l.	Tissue wt. mg.
No vitamin	9.3 0.7	275 296
Vitamin	86 92	276 260

Controls showed that the washed yeast was active. Addition of crude co-carboxylase gave uptake of 573 μ l. in same period.

Exp. 1386. The preliminary period of shaking in O₂ was 30 min. at 28°; vitamin 7 μ g. Remaining details as before.

CO₂ evolution in 40 min.

	μ l.	Tissue wt. mg.
No vitamin	6.3	291
Vitamin	70.0	292

Controls showed that the washed yeast was active.

permit attachment of the "diphosphate" to the active centre; and it is difficult to advance a satisfactory counter plea, except that much of the tissue reaches a fine state of subdivision in the tests. As previously stated [Peters, 1937; Kinnorsley & Peters, 1937], it can be shown that under our conditions phosphorylation of vitamin B₁ takes place.

Three experiments are recorded in Table I; all show more co-carboxylase effect in bottles previously shaken with vitamin. The object of experiment 1385 was to make certain that phosphorylation could be sufficiently rapid to account for the early appearance of the vitamin effect. It is clear that this is so; in 1386, even 30 min. at 28° suffices for a marked degree of synthesis. Hence the view that vitamin B₁ is first converted into the diphospho-compound is possible and eminently plausible, if no other compound of vitamin B₁ can act as co-carboxylase. Nevertheless the question will not be rigidly proved until a preparation is obtained, which will show the catatorulin effect with co-carboxylase and not with vitamin B₁. It could still be argued that the phosphorylation is only one of many others, due to phosphorylating capacity of the brain extract, as has been shown by Euler *et al.* [1936]. Meanwhile, we may consider provisionally that vitamin B₁ acts in the phosphorylated form in our brain preparations and turn to the next point, whether the remainder of the system resembles yeast carboxylase.

(2) α -Ketobutyric acid ($\text{CH}_3\cdot\text{CH}_2\cdot\text{CO}\cdot\text{COOH}$)

We have stated recently [McGowan & Peters, 1937] that some α -keto-acids give no catatorulin effects. Since then, I have tested the monocarboxylic acid most nearly allied to pyruvic acid, namely α -ketobutyric acid. This was especially important because of the observation of Neuberg & Kerb [1912] that this compound reacted well with yeast carboxylase. Hence the question whether α -ketobutyric acid reacted with avitaminous brain might become a crucial point in determining the specificity of the pyruvate oxidase system in question.

In confirmation of Neuberg & Kerb, I have found that α -ketobutyric acid reacts excellently with yeast carboxylase, and have been able to add the observation that co-carboxylase (diphospho-vitamin B₁ of Lohmann) is also essential for this decarboxylation (Table II). But the behaviour with brain was different (Table III); there was no catatorulin effect; a marked inhibition occurred which is being investigated further. It has been a constant feature of several preparations and has been given by the purest. It is therefore not likely to be due to impurity. In one experiment (1362), the catatorulin effect with pyruvate was less affected by the α -keto-acid than the general level of respiration. It

Table II. *Co-carboxylase and α -ketobutyric acid. Yeast*

Exp. 1367. Dried yeast, washed according to Lohmann. Neutralized pyruvic acid (or α -ketobutyric acid) + MgCl_2 (0.05 mg.) placed in Keilin tubes suspended in a Barcroft bottle, filled with N_2 and containing yeast (approx. 100 mg.) + 1.5 ml. Ringer-phosphate, pH 6.2, $\pm$ co-carboxylase 3.0 μg . Temp. 28°. Keilin tubes displaced after 5 min. equilibration and readings started at 10 min. Conc. α -ketobutyric acid 0.021 *M*; pyruvic acid 0.028 *M*. Average of duplicates.

CO ₂ evolution in 2 hr.	
	$\mu\text{l.}$
No addition	- 5
α -Ketobutyric acid alone	11.4
Same + vitamin B ₁	8.8
Same + co-carboxylase	218.0
Pyruvate alone	2.0
Same + co-carboxylase	156.0

The co-carboxylase used was a pure specimen very kindly given by Prof. Lohmann.

Table III. *α -Ketobutyric acid and brain respiration*

		O ₂ uptake μ l./g./hr.					
		Acid alone (2 expts. quoted out of 7)					
Exp. no.	Additions	Respiratory period			Vitamin differences		
		$\frac{1}{2}$ -1 hr.	1-1 $\frac{1}{2}$ hr.	1 $\frac{1}{2}$ -2 hr.	μ l.		
1374	Nil*	1185	834	652	- 16	+ 44	6
	4 μ g. V.*	1169	870	658			
	Na α -ketobutyrate 0.21 M	608	397	304	83	79	72
	Same + V.	691	476	376			
1371	Nil	1150	863	640	160	79	112
	V.	1310	1042	752			
	α -Ketobutyrate	840	575	395	153	8.5	45
	Same + V.	687	490	350			
	α -Ketobutyrate alone	630	476	305	—	—	—
Acid added to pyruvate (2 expts. quoted out of 4)							
1362	Pyr.	1245	933	755	695	706	693
	Pyr. V. (4 μ g.)	1940	1639	1448			
	Pyr. α -ketobutyrate	882	642	511	618	709	686
	Pyr. V.	1560	1351	1197			
1373	Pyr.*	1262	928	671	768	789	785
	Pyr. V.*	2030	1717	1456			
	Pyr. α -ketobutyrate	985	698	506	515	516	544
	Same + V.	1500	1214	1050			

* Triplicate.

might be thought that a brain carboxylase was converting the acid into prop-aldehyde, like yeast, but that this aldehyde was not further metabolized by the brain tissue; and that accordingly the inhibition was due to the accumulating aldehyde, a class of substances which I have found to be undoubtedly toxic to these systems. This view is excluded by the values found; the inhibition does not increase with time. If anything it is reduced. Further evidence against conversion into aldehyde was found by measuring aerobic CO₂ evolution, with and without vitamin (Table IV). Though some uncertainty in the exact amount of CO₂ formed arises from the failure to stop absorption of gas by addition of HCl to brain tissue, it is clear that no CO₂ at all commensurate with conversion in the bottles into as much as 0.1-0.2 mg. aldehyde takes place, as a result of the presence of α -ketobutyrate; nor could evidence be obtained that extra α -ketobutyrate disappeared with vitamin action (Table IV). Hence, unlike its behaviour to yeast, the compound seems to be unattacked by the brain enzyme system.

So far there is a consensus of opinion that no acetaldehyde can be detected in brain pyruvate metabolism, and that there is no anaerobic decarboxylation [see e.g. Elliott *et al.* 1937; Lipmann, 1937, 1, 2, 3]; our own experience for pigeon brain is in thorough agreement with this. Taking the evidence together therefore the answer to our second point appears to be that even if co-carboxylase is part of the system, the latter is so highly specialized in brain that it hardly wears the same face as the carboxylase in yeast. Lipmann's observation¹ that co-carboxylase in bacteria produces not acetaldehyde, but acetic acid and CO₂ from pyruvic acid is clearly very relevant.

EXPERIMENTAL

α -Ketobutyric acid was prepared after Neuberg & Kerb [1912] by the method of Mebus [1905]. The acid distilled at 74-75° under 18-20 mm. pressure (more

¹ His complete paper [1937, 3] reached me after completion of the above.

Table IV

Exp. 1375. Aerobic CO₂ evolution with α -ketobutyric acid. 10 amounts of 90–100 mg. tissue (washed) in Barcroft bottles containing Ringer-phosphate, pH 7.3, and O₂ were used to obtain the following values in duplicate: (1) O₂ uptake with and without vitamin (4 μ g.); (2) initial CO₂ content of tissues of substrate-Ringer; (3) CO₂ content after 2 hr. respiration with and without vitamin. The initial CO₂ content of substrate-Ringer solution only (4) was separately estimated. The values (2) and (3) were obtained by displacement of Keilin tubes containing 0.3 ml. *N* HCl at 12 and 120 min. respectively, the CO₂ content in (3) being estimated after calculating the O₂ uptake from (1) for the amount of tissue present. Uncertainty is introduced in the CO₂ value by the fact that acid does not stop exchange of gas with this system [cf. Needham, 1932]; in every case after adding the acid to bottles containing brain tissue a rapid displacement of gas in the sense of CO₂ evolution occurred, followed by a steady decrease which indicated an uptake of oxygen; it was not due to leak because it does not occur with (4) at all. The backward creep amounts to some 10–11 mm. for tissue respired 12 min. and some 4–7 mm. for tissue respired 2 hr. By extrapolation of the values for CO₂ to a maximum at zero addition of acid, the following values of O₂ and CO₂ were reached, reduced to 100 mg. tissue (wet weight), for 2 hr. period, after subtracting (1), average of duplicate values.

	μ l. CO ₂	O ₂	R.Q.
No addition	71.9	85.2	0.84
Vitamin (4 μ g.)	106.0	103.0	1.03

These figures are obtained by subtracting the full residual CO₂ and do not allow for the O₂ uptake, due to non-enzymic processes, which has probably occurred during the experiment. Such a figure is covered for O₂ uptake. If approximately half the non-enzymic O₂ has been reckoned with O₂ uptake, then less initial CO₂ should be subtracted and the values for CO₂ might be increased by 20 μ l. The R.Q. would then be correspondingly raised. The amounts are, of course, extremely small, much less than the usual residual values, and the errors correspondingly large. But the vitamin increase in CO₂ is balanced by a similar increase in oxygen. It is therefore mainly residual. The formation of 0.2 mg. propaldehyde, an amount of aldehyde which might be regarded as inhibitory from other experiments, would give an evolution of CO₂ of some 77 μ l., an amount far in excess of the values shown, taking into account the expected increase for the residual. If pyruvic acid had been present, the amount of CO₂ similarly evolved in 2 hr. by the tissue containing vitamin might have been expected to be at least 350 μ l. Hence, though this experiment can only indicate the order of values involved, there is no ground for thinking that CO₂ evolution takes place with the α -ketobutyric acid aerobically without O₂ uptake.

Exp. 1374. Disappearance of α -ketobutyric acid in 2.0 hr. The acid was measured into the flask with a 0.5 ml. Ostwald pipette; 0.5 ml. approx. 5 mg. Na α -ketobutyrate. At completion of this experiment, for which the values are given in Fig. 1, 1 ml. of 25% trichloroacetic acid was added to the bottles. After standing they were filtered, using one filter-paper for the three of (1) residual, (2) residual + vitamin, respectively, (3) one for α -ketobutyric and (4) α -ketobutyric + vitamin, and (5) one for 1.0 ml. of control. The latter contained two separately measured amounts of 5 mg. to which 2.5 ml. RPO₄ were added, and later 1 ml. of 25% trichloroacetic acid. Each bottle was washed with 1 ml. + 1 ml. trichloroacetic acid; the amounts for each of 1–5 were made to 25 ml., 10 ml. of 1 and 2 and 2 ml. of the rest being taken for each B.B.S. value.

Bisulphite-binding substance (B.B.S.) (determined by usual methods [Clift & Cook, 1932]).

	No. of bottles	ml. N/100 iodine	Tissue wt. mg.	Disappeared ml.	Disappeared per 100 mg. α -ketobutyrate + vitamin
Residual	3	0.275	284.4	—	—
Residual + vitamin	3	0.250	300.5	0.013	—
α -Ketobutyrate	2	14.06	208.0	0.45	—
α -Ketobutyrate + vitamin	2	14.00	196.4	0.51	0.05
Control α -ketobutyrate	2	15.00	—	—	—

The amount 0.05 ml. represents 0.026 mg. α -ketobutyric acid and is within the error of the titration. 2 ml. samples of (3), (4) and (5) were also converted into 2:4-dinitrophenylhydrazones and taken through Peters & Thompson's [1934] modification of Clift & Cook's [1932] method. Comparing the colours produced by the control (5) with those of (3) and (4), the results gave control 1.0, (3) 0.88 and (4) 0.85. The slight diminution for (4) is not significant and is of the same order as that for the B.B.S. estimation, indicating that the substance estimated is keto-acid rather than aldehyde.

than one preparation was used); the melting-points were 29–29.6° instead of 32° as stated in the literature. The acid could not be freed from the last traces of ester by distillation. In order to make certain that these traces were not producing the observed inhibition, the sodium salt was prepared by neutralizing cautiously to pH 4.5 with 20% NaOH, and crystallizing from acetone-water mixtures, containing traces of ether. The recrystallized salt, shining plates, melted with decomposition at 195° (uncorrected) and gave the following values for bisulphite-binding power: theoretical 8.9 mg., found 8.84 mg.; Na determined as Na₂SO₄¹; 4.74 mg. used. Calc. 57.2%, found 55.5%. It showed the same biochemical effects as solutions prepared from the freshly distilled acid.

Other experimental details have followed the usual plan. Unless stated bottles have contained 3.0 ml. Ringer-phosphate, pH 7.3. Vitamin B₁ has been 4 µg. per bottle and pyruvic acid (neutralized) approximately 0.02 M.

SUMMARY

1. The catatorulin effect (increased oxygen uptake upon addition of vitamin B₁) of Lohmann's pure co-carboxylase (diphospho-vitamin B₁ or pyrophosphoric ester of vitamin B₁) with avitaminous pigeon's brain tissue is much less than that of vitamin B₁ itself.

2. Vitamin B₁ is phosphorylated rapidly under the conditions of a catatorulin test.

3. α-Ketobutyric acid requires co-carboxylase for its decarboxylation by yeast, but it does not act as a substrate for the catatorulin effect.

4. The hypothesis that vitamin B₁ acts as the diphospho-compound in brain is possible, though not yet rigidly proved; but the other components of the system are dissimilar from those of yeast.

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¹ I am much indebted to Mr Kinnersley for this estimation.

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Note added 6 December 1937. Analysis of Na ketobutyrate (Schoeller) found C, 38.35; H, 4.08%. Calc. C, 38.71; H, 4.03%. In a paper just published (*Bioch. Z.* (1937) **294**, 188) Lohmann & Schuster record evidence of the phosphorylation of vitamin B₁ by normal (?) brain tissue at alkaline reactions; they consider that the brain system is different from yeast carboxylase and therefore reach similar conclusions to mine.