LXII. THE RELATION OF VITAMIN B₁ TO COCARBOXYLASE

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LOHMANN [1937] and Lohmann & Schuster [1937] have shown that cocarboxylase is the pyrophosphate of vitamin B₁. They reported that the respiration of brain slices from polyneuritic pigeons could be restored to normal by *in vitro* additions of the phosphorylated vitamin. Peters [1937] has reported that cocarboxylase was only about 10% as effective as the free vitamin in similar *in vitro* experiments.

A problem of fundamental importance is the determination of the nature of vitamin B₁ as it occurs in living tissues, i.e. does the compound occur as the free vitamin, as the pyrophosphate, or is there a dynamic equilibrium between the two compounds?

During the course of the development of a method for the determination of the cocarboxylase content of tissues we have arrived at the conclusion that the two compounds may exist in dynamic equilibrium in living organisms, and have collected some data which relate not only to the mechanism of the phosphorylation of vitamin B₁ but which in a broader sense must have considerable bearing on the whole question of phosphate transfer and carbohydrate metabolism in living organisms.

As far as we are aware there is at present no direct evidence in the literature that cocarboxylase exists as such in animal tissues. Perhaps the best indication is the indirect evidence of Tauber [1937] who has reported a synthesis of cocarboxylase from vitamin B₁ by an enzyme prepared from duodenal mucosa. Auhagen [1933] and Simola [1932] have both reported on the cocarboxylase content of animal tissue extract, but, for reasons explained below, it now appears possible that their results could have been obtained in the complete absence of cocarboxylase from the original tissue. Their method involved the addition of a boiled tissue extract to a washed dried yeast preparation (atizymase) and the measurement of the CO₂ evolved in the presence of magnesium, glucose, cozymase, acetaldehyde, hexosediphosphate and a phosphate buffer. When we attempted to determine the cocarboxylase content of boiled tissue extracts by means of the simplified system described by Lohmann (atizymase, magnesium and pyruvate), we found that the addition of crystalline vitamin B₁ to the system resulted in a synthesis of cocarboxylase if a boiled tissue extract were also present. This method therefore did not distinguish between the free vitamin and the vitamin pyrophosphate but merely measured the sum of the two in the boiled extract. Thus it does not seem valid to conclude from the results of Auhagen and of Simola that cocarboxylase does in fact exist as such in animal tissues. The validity of Auhagen's demonstration of cocarboxylase in partially purified

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boiled tissue extracts is dependent upon the amount of vitamin B₁ and phosphorylating agents which they contained. The more recent work of Lipmann [1937] showed that cocarboxylase was necessary for the oxidation of pyruvic acid by acetone-washed *B. delbrückii*. Vitamin B₁ was without activity but boiled tissue extracts catalysed the reaction. Before it can be concluded that these results prove that cocarboxylase was present in the tissue extracts, it would seem desirable to show that the *B. delbrückii* preparation supplemented by the extracts is unable to carry out the synthesis which we have demonstrated to occur with the yeast preparation.

The present paper deals with the factors concerned with the phosphorylation of vitamin B₁ in the presence of atiozymase and boiled tissue extracts, together with a method for differentiating between the phosphorylated and the unphosphorylated vitamin in tissue extracts.

EXPERIMENTAL

Methods and apparatus. Cocarboxylase activity was measured in terms of the rate of CO₂ evolved, using an atiozymase preparation with sodium pyruvate as substrate and magnesium as activator [Lohmann, 1937]. CO₂ output was measured by means of the Barcroft differential respirometer [Dixon, 1934]. In these experiments considerable acetaldehyde is formed which would be expected to have some effect on the manometer readings. We have checked this point by placing semicarbazide at pH 7.0 in the inner cup. The manometric readings were affected but slightly. Since the O₂ uptake was also demonstrated to be practically negligible, uncorrected readings were taken for CO₂ production. The experiments were carried out with air in the gas space. The flasks were shaken at 100–120 oscillations per minute at a temperature of 30°. The measurements were continued for from 1 to 2 hr. and the taps were opened momentarily when necessary.

Tissue extracts were conveniently prepared by homogenizing the tissue in an all-glass device which has been described in detail by Potter & Elvehjem [1936]. The use of this device greatly expedites the grinding of the tissue and ensures complete cell disruption.

Enzyme. The atiozymase employed in these experiments was prepared from brewer's yeast by the method of Auhagen [1933]. The yeast was spread in shallow pans and was dried for 3 hr. at room temperature with the aid of a fan. This preparation is stable for months when stored in a desiccator. 20 g. dried yeast were washed twice for half an hour with 700 ml. water and centrifuged. The yeast was then taken up in 700 ml. *M*/10 Na₂HPO₄ buffer, pH 7.9, and was further washed three times for 20 min. A short final washing with water followed, in order to remove the alkaline phosphate. The washed yeast (about 10 g.) was then spread on a glass plate and dried rapidly before a fan. This atiozymase preparation was not completely stable, and it was found advisable to prepare it weekly. The preparation was homogenized in *M*/10 Na and K phosphate buffer at pH 6.2 for use as atiozymase.

Atiozymase prepared in this manner is not completely free from cocarboxylase, but shows a great increase in activity when boiled yeast or tissue juice is added. It was also found that different atiozymase preparations, made in the same manner, show different activities. It is therefore necessary to include a manometer containing only the atiozymase, magnesium and pyruvate as a control.

Boiled tissue extracts. Boiled tissue extracts were prepared as follows. A chick was killed by decapitation; the liver was removed, blotted free from blood

on a filter paper, dissected free from connective tissue and placed in a weighed homogenizer tube containing 2 ml. water. The tissue was weighed, homogenized and diluted to a concentration of 333 mg. per ml. with more water. It was then plunged into a boiling water bath for 7 min. The interval between the death of the animal and the introduction of the homogenized tissue into the boiling water was 6–9 min.

Chemical preparations. Pyruvic acid was prepared by double distillation of commercial pyruvic acid at 2–4 mm. Hg and 38–41°. Hexosediphosphate was prepared by the method of Robison & Morgan [1930]. The insoluble barium salt was treated with the calculated amount of saturated sodium sulphate for 2 hr., and the barium sulphate was centrifuged down. The solution of the sodium hexosediphosphate was evaporated to dryness *in vacuo* and the salt was stored in a desiccator. Magnesium was added as the chloride, fluoride and iodoacetate in the form of the sodium salts. The buffer was *M*/10 Na and K phosphate, pH 6.2. Winthrop's synthetic vitamin B₁ was employed throughout.

Procedure. 0.4 ml. atiozymase preparation, equivalent to 100 mg. of dry preparation, was made up to 2.0 ml. with the other solutions. 0.5 ml. boiled tissue extract, corresponding to 167 mg. fresh tissue, 0.1 mg. Mg as the chloride, 10 mg. hexosediphosphate in buffer, 10 mg. Na pyruvate in buffer and 5 γ vitamin B₁ were employed, unless otherwise stated.

(1) *The synthesis of cocarboxylase from vitamin B₁*

When boiled tissue extract was added to atiozymase plus pyruvate there was a marked increase in the CO₂ output. However, it was found that when vitamin B₁ was added to the system a still further increase in CO₂ evolution occurred, indicating that the added vitamin B₁ was synthesized to cocarboxylase. The results are shown in Table I. This experiment was repeated a number of times

Table I. *The synthesis of cocarboxylase from vitamin B₁ in the presence of boiled tissue extract and atiozymase*

	Exp. A		Exp. B				
			Normal liver			Avitaminous liver	
Atiozymase (250 mg./ml.) (ml.)	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Mg (1 mg./ml.) (ml.)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Na pyruvate (30 mg./ml.) (ml.)	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Boiled liver extract (333 mg./ml.) (ml.)	—	—	—	0.5	0.5	0.5	0.5
Vitamin B ₁ (50 γ /ml.) (ml.)	—	0.1	—	—	0.1	—	0.1
Phosphate buffer (ml.)	1.2	1.1	1.2	0.7	0.6	0.7	0.6
CO ₂ μ l./hr.	87.0	86.8	117.5	319	430	258	368

with liver and brain extracts from both normal and polyneuritic birds, and the synthesis with the avitaminous tissue extracts was usually greater. Other tissues have not as yet been used. The results in Table I show clearly that atiozymase alone is unable to convert vitamin B₁ into cocarboxylase, but that in the presence of boiled tissue extract the synthesis is effected. It seems likely that if the synthesis of cocarboxylase from vitamin B₁ occurs with atiozymase and boiled tissue extracts in the presence of pyruvate, it will certainly occur with atiozymase and boiled tissue extracts in the presence of glucose, hexosediphosphate, cozymase and acetaldehyde. From this it is apparent that any vitamin B₁

present in the tissue extract will be converted into cocarboxylase in the presence of the atiozymase, and that the method used by Auhagen and by Simola does not differentiate between the free vitamin and cocarboxylase in boiled tissue extracts.

It was clear that one or more thermostable compounds present in the boiled tissue extract were necessary for the phosphorylation of the free vitamin in the presence of atiozymase. Our attention was next turned to the identification of these constituents. The following compounds were considered to be the likely constituents of the boiled tissue extract: hexosediphosphate, cozymase, adenylic acid and adenylypyrophosphate.

(2) *The effect of hexosediphosphate*

In attempting to compare the cocarboxylase contents of normal and avitaminous boiled liver extracts, using simply atiozymase, magnesium, pyruvate and extract, we found that the livers from polyneuritic birds sometimes gave higher values than those from normal birds. Simola had employed hexosediphosphate in all his experiments and had found significant differences between normal and avitaminous tissues. Furthermore, it had been demonstrated by Nagai [1932] that there was a large increase in the hexosediphosphate content of all tissues, except brain, from avitaminous birds. We therefore added hexosediphosphate to the system and found a large increase in CO₂ output (Table II) which was greater with normal than with avitaminous tissues, in confirmation of the results of Simola. However, it must be remembered that these results indicate the sum of free vitamin and cocarboxylase.

Table II. *The stimulating effect of hexosediphosphate upon the synthesis of cocarboxylase in the presence of boiled tissue extract with and without added vitamin B₁*

	Normal liver				Avitaminous liver		
Vitamin B ₁ (50 γ/ml.) (ml.)	—	—	—	0.1	—	—	0.1
Hexosediphosphate (50 mg./ml.) (ml.)	—	—	0.2	0.2	—	0.2	0.2
Boiled liver extract (333 mg./ml.) (ml.)	—	0.5	0.5	0.5	0.5	0.5	0.5
Buffer (ml.)	1.2	0.7	0.5	0.4	0.7	0.5	0.4
CO ₂ μl./2 hr.	144	316	1100	1522	255	500	1353

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

The stimulating effect of hexosediphosphate upon the CO₂ output could be explained by postulating that hexosediphosphate acted as a phosphate donor in the conversion of vitamin B₁ into cocarboxylase. This "phosphate-donating" action might be direct, or indirect. If indirect, several possibilities would have to be considered. The phosphate might be transferred to cozymase [Lennerstrand, 1936] or to adenylic acid, or the hexosediphosphate might break down to phosphopyruvate which could serve as the phosphate donor; the hexosediphosphate might yield phosphoglyceric acid to give energy for the esterification of inorganic phosphate. Further experiments were carried out to check these possibilities.

In order to check the possibility that the transfer was direct, we determined whether or not hexosediphosphate was the only significant thermostable component which the boiled extract added. This was done by comparing the synthesizing powers of the system atiozymase, magnesium, pyruvate, vitamin B₁ and hexosediphosphate, with and without added boiled tissue extract. A small

though definite synthesis was observed without the tissue extract, but the extent of this synthesis was greatly increased by the addition of the extract (Table III). Von Euler & Vestin [1937] had reported synthesis with hexosediphosphate or adenosinetriphosphate and vitamin B₁ added to an unwashed yeast

Table III. *The stimulating effect of boiled liver extract upon the synthesis of cocarboxylase from vitamin B₁ in the presence of hexosediphosphate*

	Exp. A				Exp. B			
Vitamin B ₁ (50 γ/ml.) (ml.)	—	0.1	—	0.1	—	0.1	—	0.1
Hexosediphosphate (50 mg./ml.) (ml.)	—	—	0.2	0.2	—	—	0.2	0.2
Boiled liver extract (333 mg./ml.) (ml.)	—	—	—	—	0.5	0.5	0.5	0.5
Buffer (ml.)	1.2	1.1	1.0	0.9	0.7	0.6	0.5	0.4
CO ₂ μl./hr.	126	149	150	186	232	296	817	1243

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

preparation. Consequently, we repeated our experiments using boiled yeast juice and obtained the same results as were obtained with the tissue extracts. It was readily apparent, therefore, that hexosediphosphate alone was not sufficient to produce the synthesis and that the effect of boiled tissue extracts was due to factors other than hexosediphosphate. It may be concluded that the phosphorylation of the vitamin does not occur as a result of a direct transfer of phosphorus from hexosediphosphate.

(3) *The effect of fluoride*

Another explanation of the hexosediphosphate effect might lie in the ability of phosphopyruvic acid to act as a phosphate donator [Needham & Lehmann, 1937]. Hexosediphosphate might be expected to break down via triosephosphate and phosphoglyceric acid to phosphopyruvic acid. If the latter were the source of phosphate, the hexosediphosphate effect should be abolished by the action of fluoride.

Sodium fluoride in various concentrations was added to the system in an attempt to inhibit the synthesis. The complete system of atiozymase, magnesium, pyruvate, hexosediphosphate, vitamin B₁ and boiled tissue extract was employed. The results are shown in Table IV and Fig. 1.

Table IV. *The effect of graded molarities of sodium fluoride upon the synthesis of cocarboxylase in the presence of hexosediphosphate, vitamin B₁ and boiled liver extract*

	Exp. A						Exp. B	
Vitamin B ₁ (50 γ/ml.) (ml.)	—	0.1	0.1	0.1	0.1	0.1	—	—
Hexosediphosphate (50 mg./ml.) (ml.)	—	0.2	0.2	0.2	0.2	0.2	—	—
Boiled liver extract (333 mg./ml.) (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	—	—
Na fluoride (0.4 M) (ml.)	—	—	0.2	0.1	—	—	—	0.2
Na fluoride (0.1 M) (ml.)	—	—	—	—	0.2	0.1	—	—
Buffer (ml.)	0.7	0.4	0.2	0.3	0.2	0.3	1.2	1.0
CO ₂ μl./2 hr.	356	2012	803	1047	968	1055	207	212

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

The experiments with added fluoride clearly indicate that the synthesis of cocarboxylase is only partially checked by the inhibition of the reaction by

Fig. 1. The effect of various concentrations of iodoacetic acid and sodium fluoride on the phosphorylation of vitamin B₁. For experimental conditions see Tables IV and V.

Fig. 2. The effects of sodium fluoride and iodoacetic acid on the synthesis and action of cocarboxylase. — Control. Curves I and Ia. ---- 0.04 M NaF from the beginning. Curves II and IIa. - · - · - 0.005 M CH₂ICOOH from the beginning. Curves III and IIIa. 10 mg. pyruvate added to I, II and III at 1 hr. 10 mg. pyruvate added to Ia, IIa, and IIIa at 2 hr. CH₂ICOOH to make 0.005 M added to I and Ia 30 min. after pyruvate addition, as shown by arrows. All flasks contained atiozymase, hexosediphosphate, boiled liver extract, magnesium, vitamin B₁ and phosphate buffer from the beginning.

which phosphopyruvic acid is formed. There appears to be a lag in the evolution of CO_2 when fluoride is used, and it is possible that there are two mechanisms for phosphorylating the vitamin, and that while fluoride blocks the reaction involving phosphopyruvate, it has no effect on the other mechanism.

In a further attempt to demonstrate a breakdown of hexosediphosphate to pyruvic acid an experiment was carried out omitting the addition of pyruvate. The results are shown in Fig. 2 (before pyruvate additions). The amount of CO_2 evolved before the addition of pyruvate was very small, indicating that little pyruvate was formed. However, it is possible that phosphopyruvic acid was formed in considerable amounts and was dephosphorylated only to the small extent necessary for the synthesis of cocarboxylase.

(4) *The effect of iodoacetic acid*

Since it appeared that hexosediphosphate might be effecting the synthesis by a mechanism other than that involving phosphopyruvate, we decided to test the effect of iodoacetate. Needham & Pillai [1937] have furnished evidence for the coupling of oxido-reductions with the esterification of inorganic phosphate and have shown that adenylypyrophosphate can be formed from adenylic acid and inorganic phosphate when energy is obtained from the conversion of triose-phosphate into phosphoglyceric acid. It was believed that the reactions were coupled by means of cozymase. The reaction was prevented by iodoacetate which acted by inhibiting the energy-yielding oxido-reductions.

When iodoacetate was added to the system which contained all the components necessary for the synthesis of cocarboxylase, it was found that the synthetic reaction was virtually abolished. These results are shown in Table V

Table V. *The effect of graded molarities of iodoacetic acid upon the synthesis of cocarboxylase by atiozymase in the presence of hexosediphosphate, vitamin B₁ and boiled liver extract*

	Exp. A						Exp. B	
Vitamin B ₁ (50 γ /ml.) (ml.)	0.1	—	0.1	0.1	0.1	0.1	—	—
Hexosediphosphate (50 mg./ml.) (ml.)	0.2	—	0.2	0.2	0.2	0.2	—	—
Boiled liver extract (333 mg./ml.) (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	—	—
Na iodoacetate (0.05 <i>M</i>) (ml.)	—	—	0.2	0.1	—	—	—	0.2
Na iodoacetate (0.017 <i>M</i>) (ml.)	—	—	—	—	0.2	0.1	—	—
Buffer (ml.)	0.4	0.7	0.2	0.3	0.2	0.3	1.2	1.0
CO_2 $\mu\text{l.}/2$ hr.	1828	425	465	528	590	1017	207	200

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

and Fig. 1. Both fluoride and iodoacetate were without effect on cocarboxylase action *per se* as was shown by the complete absence of inhibition of the atiozymase preparation. The effects of fluoride and iodoacetate upon the synthesis of cocarboxylase and upon its action were further demonstrated in the following experiment.

Atiozymase, boiled tissue extract, hexosediphosphate, magnesium and vitamin B₁ in phosphate buffer were shaken in Barcroft vessels in the absence of pyruvate, with and without inhibitors; CO_2 production was measured throughout the experiment. At the end of 1 hr. the manometers were disconnected long enough to introduce pyruvate into all the flasks and iodoacetate in a Keilin dangler into the control flasks. After equilibration, the cocks were closed and the

CO₂ evolution was again measured for 20 min. The Keilin dangle was then shaken down in the control flasks and the measurement of CO₂ evolution was continued. A parallel run was made in which the pyruvate was added at 2 hr. The results are shown in Fig. 2. In the absence of pyruvate a small amount of CO₂ was evolved at an increasing rate from the control flask, indicating that slight amounts of pyruvate were being formed. The presence of either fluoride or iodoacetate inhibited this CO₂ output. When pyruvate was added, there was a rapid evolution of CO₂ from the control flask and from the flask which contained fluoride, indicating that cocarboxylase had been synthesized in these two flasks. In the flask containing iodoacetate there was relatively little CO₂ evolved, indicating that the synthesis was inhibited by the iodoacetate. The slight evolution of CO₂ which did occur can be attributed to the small amounts of cocarboxylase originally present in the atiozymase. The flask which contained fluoride throughout demonstrated that fluoride inhibited neither the synthesis nor the action of cocarboxylase. The flask which contained iodoacetate throughout showed that the compound inhibited the synthesis of cocarboxylase, and the fact that the delayed addition of iodoacetate to the control flask caused no diminution in the rate of CO₂ output clearly demonstrated that the poison had no effect on the action of preformed cocarboxylase.

The inhibitory action of iodoacetate makes it appear likely that an oxidation-reduction is involved in the synthesis of cocarboxylase. The reactions described by Needham & Pillai [1937] were all dismutations and did not require oxygen. Our experiments have all been carried out in the presence of air and in addition effective shaking has always been secured. The O₂ uptake is however very small, and is certainly too small to have much effect on the volume of CO₂

Table VI. *The O₂ uptake and CO₂ production during the enzymic synthesis of cocarboxylase*

Vitamin B ₁ (50 γ/ml.) (ml.)	—	—	0.1	0.1	0.1	0.1
Hexosediphosphate (50 mg./ml.) (ml.)	—	—	0.2	0.2	0.2	0.2
Boiled liver extract (333 mg./ml.) (ml.)	0.5	0.5	0.5	0.5	0.5	0.5
Na iodoacetate (0.05 M) (ml.)	—	—	—	—	0.2	0.2
Buffer (ml.)	0.7	0.7	0.4	0.4	0.2	0.2
CO ₂ μl./2 hr.	153	—	782	—	200	—
O ₂ μl./2 hr.	—	8.1	—	-24.5	—	2.7

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

evolved (Table VI). If the synthesis involves a dismutation between triosephosphate and pyruvate to form phosphoglycerate and lactate, the amount of lactate formed would be very small in comparison with the amount of pyruvate present. The excess pyruvate would be sufficient to inhibit the oxidation of the lactate [Green & Brosteaux, 1936].

Lipmann [1937] has shown that cocarboxylase is required for the oxidative decarboxylation of pyruvic acid by acetone-treated *B. delbrückii*. In this reaction pyruvic acid is dehydrogenated and oxygen is taken up. He has found that flavin phosphate speeds up the reaction. It seems likely that this compound is involved in a hydrogen transport mechanism and there is little reason to believe that it is involved in the simple decarboxylation of pyruvic acid by atiozymase together with cocarboxylase.

The experiments detailed above suggest that the difficulties involved in Simola's measurements might be overcome by the proper use of iodoacetate so as to inhibit the conversion of vitamin B₁ into cocarboxylase. Table VII indicates the type of experiment by which this may be done. In order to determine

Table VII. *The relative vitamin B₁ and cocarboxylase contents of boiled normal liver extract*

Vitamin B ₁ (10 γ /ml.) (ml.)	—	—	—	—	0.2
Hexosediphosphate (50 mg./ml.) (ml.)	—	—	—	0.2	0.2
Boiled tissue extract (333 mg./ml.) (ml.)	—	—	0.5	0.5	0.5
Na iodoacetate (0.05 <i>M</i>) (ml.)	—	0.2	0.2	—	—
Buffer (ml.)	1.2	1.0	0.5	0.5	0.3
CO ₂ μ l./2 hr.	189	189	265	1885	2154

Standard quantities of atiozymase, Mg and pyruvate were present in every flask. See Table I.

whether the boiled extract contains vitamin B₁ or cocarboxylase it is supplemented with hexosediphosphate to give the combined effect of the vitamin and the vitamin pyrophosphate and is poisoned with iodoacetate to measure the vitamin pyrophosphate alone. The results in Table VII indicate that by far the greater amount of the vitamin is in the unphosphorylated form in the boiled extract from normal chick liver. It remains to be shown how closely this approximates to the situation *in vivo*. In a later paper we expect to present data on this point together with the results of an investigation on the relative amounts of the two compounds in the various tissues of normal and avitaminous animals.

DISCUSSION

Although many experiments need to be done to check all the questions raised by the data in the section above, we feel that certain possibilities are definitely indicated regarding the mechanism of the phosphorylation of vitamin B₁.

There seem to be reasons for believing that the vitamin may be phosphorylated either by adenylypyrophosphate or by phosphorylated cozymase. There does not at present appear to be sufficient evidence in the literature to differentiate clearly between the phosphorylating abilities of these two compounds, and we will not attempt to distinguish between them here. It seems fairly definite that phosphorylated cozymase can form adenylypyrophosphate. In this discussion we shall consider the evidence relating to the phosphorylation of cozymase, while at the same time we recognize the possibility that adenylypyrophosphate may be the compound which ultimately completes the synthesis.

Before proceeding further it should be stated that we have no evidence to disprove the possibility that the vitamin can be phosphorylated directly by the various mechanisms which can phosphorylate adenylic acid or cozymase. The recent paper by Needham & Pillai [1937] indicates that the phosphorylation of cozymase by inorganic phosphate can be coupled with certain dismutations. The fact that the thiazole ring in vitamin B₁ contains a bond capable of oxidation and reduction [Lipmann, 1936] may be a further indication of an analogy between the phosphorylations of the vitamin and of cozymase.

We may now proceed to a consideration of the mechanisms which may be involved in the preliminary phosphorylation of cozymase or adenylic acid.

Ohlmeyer [1935] reported that in the presence of Lebedev yeast extract, hexosediphosphate could phosphorylate adenylic acid to yield hexosemono-phosphate and adenylypyrophosphate. The reaction could be inhibited by iodoacetate. This reaction must be considered to provide a possible explanation for our results until experiments with phosphoglyceraldehyde can be done. However, Needham & Pillai were unable to find any support for the occurrence of this reaction with muscle extract.

Needham & Pillai showed that the dismutation of triosephosphate could yield energy for the phosphorylation of adenylic acid by inorganic phosphate via cozymase. This reaction was inhibited by iodoacetate and appears to us to be a likely explanation for one of the mechanisms concerned in the synthesis of cocarboxylase. The dependence of cocarboxylase upon inorganic phosphate [Lipmann, 1937] also seems to support this idea.

The possibility remains that phosphopyruvic acid can be the source of phosphate, although it has been definitely ruled out as the only mechanism, since the vitamin can be phosphorylated in the presence of fluoride, which is known effectively to prevent the conversion of phosphoglyceric into phosphopyruvic acid. It would be desirable to attempt the synthesis of cocarboxylase using phosphopyruvic acid with and without any accessory phosphate carrier.

It is interesting to note that Birch & Mapson [1936] attributed the bradycardia associated with polyneuritis to an increased amount of adenylic acid in the heart, while Nagai [1932] found a decrease in adenylypyrophosphate in polyneuritis. These observations suggest the possibility that cocarboxylase might even be acting as a phosphate donor to adenylic acid rather than that the free vitamin accepts phosphate from adenylypyrophosphate.

In the experiments described in this paper, the synthesis of cocarboxylase was effected by yeast enzyme. The close analogy which is known to exist between yeast and animal phosphorylations would make it seem likely that the vitamin is phosphorylated in a similar manner in yeast and in animal tissues. That it is phosphorylated in the former is indicated by our work and in the latter (in duodenal mucosa) by the work of Tauber [1937]. It remains to be seen whether or not the synthesis can be effected in the presence of the enzymes of muscle extract. If this proves to be the case the present schemes for glycolysis will have to be revised to take into account one more compound which exists in a phosphorylated and in an unphosphorylated state.

SUMMARY

1. The synthesis of cocarboxylase from vitamin B₁ has been shown to take place in the presence of washed dried yeast, hexosediphosphate and boiled tissue extract.

2. The synthesis of cocarboxylase is almost completely inhibited by 0.005 *M* iodoacetic acid, but is only slightly inhibited by 0.04 *M* sodium fluoride. Neither of the inhibitors affects the action of preformed cocarboxylase.

3. By the proper combination of inhibitors, activators and enzyme, it is possible to differentiate between cocarboxylase and vitamin B₁ in boiled tissue extracts. The presence of both compounds has been demonstrated. It is believed that the two compounds may exist in dynamic equilibrium *in vivo*.

4. The possible mechanisms for the synthesis of cocarboxylase from vitamin B₁ are discussed in the light of the data presented.

REFERENCES

- Auhagen (1933). *Biochem. Z.* **258**, 330.
Birch & Mapson (1936). *Nature, Lond.*, **138**, 27.
Dixon (1934). *Manometric Methods*. (Cambridge University Press.)
Green & Brosteaux (1936). *Biochem. J.* **30**, 1489.
Lennerstrand (1936). *Biochem. Z.* **287**, 172.
Lipmann (1936). *Nature, Lond.*, **138**, 1097.
—— (1937). *Enzymologia*, **4**, 65.
Lohmann (1937). *Angew. Chem.* **50**, 221.
Lohmann & Schuster (1937). *Naturwissenschaften*, **25**, 26.
Nagai (1932). *J. Biochem., Tokyo*, **16**, 359.
Needham & Lehmann (1937). *Biochem. J.* **31**, 329.
—— & Pillai (1937). *Biochem. J.* **31**, 1837.
Ohlmeyer (1935). *Biochem. Z.* **283**, 114.
Peters (1937). *Chem. Ind. Rev.* **56**, 934.
Potter & Elvehjem (1936). *J. biol. Chem.* **114**, 495.
Robison & Morgan (1930). *Biochem. J.* **24**, 119.
Simola (1932). *Biochem. Z.* **254**, 229.
Tauber (1937). *Science*, **86**, 180.
von Euler & Vestin (1937). *Naturwissenschaften*, **25**, 416.