CCXLVI. THE ENZYMIC PHOSPHORYLATION OF VITAMIN B₁

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THE enzymic phosphorylation of vitamin B₁ has been reported by a number of investigators. In most cases these experiments were carried out with yeast cells, dried or fresh, or with sliced or minced animal tissues. Owing to the complexity of the material no clear insight into the mechanism of the phosphorylation has yet been possible. Lohmann & Schuster [1937] described the synthesis of cocarboxylase from vitamin B₁ by minced brain and intestinal mucosa; simultaneous addition of vitamin B₁, adenylyl pyrophosphoric acid, creatine phosphoric acid and phosphopyruvic acid led to an increased synthesis compared with the addition of vitamin B₁ only. Ochoa [1939], on the other hand, failed to obtain an increased synthesis with liver preparations on addition of adenylyl pyrophosphoric acid, phosphoglyceric acid or both together, and adduced this as "evidence against a direct transfer of phosphate from either compound". Lipschitz *et al.* [1938] observed a stimulating influence of hexosediphosphate on the synthesis of cocarboxylase from vitamin B₁ by washed dried yeast in the presence of boiled tissue extracts. Their results are however interpreted by Ochoa & Peters [1938] as merely meaning a removal of acetaldehyde from the reaction mixture by dismutation with triosephosphate.

For the work to be described below a cell-free protein preparation from yeast was used both as promotor and indicator of cocarboxylase synthesis. It was possible to demonstrate that phosphorylation of both vitamin B₁ and vitamin B₁-monophosphoric acid only occurs in the presence of adenylyl pyrophosphoric acid or such reactions as entail its intermediate formation. In its absence no synthesis of cocarboxylase takes place. It is to be concluded that a direct transfer of phosphate from adenylyl pyrophosphoric acid is the mechanism by which vitamin B₁ is phosphorylated.

EXPERIMENTAL

The following abbreviations will henceforth be used: vitamin B₁=B₁; vitamin B₁-monophosphoric acid=B₁-monoP; adenylyl pyrophosphoric acid=ATP.

Preparations: B₁ hydrochloride was a synthetic product from Roche Products, Ltd.

B₁-monoP was prepared from a mixture of B₁-monoP and cocarboxylase obtained by the method which was described by Weijlard & Tauber [1938] for the preparation of cocarboxylase. After acid hydrolysis the product was purified *via* the Au-salt according to Lohmann & Schuster [1937]. M.P. 197° (uncorr.). P=7.5%. The substance was free from B₁ and cocarboxylase.

Cocarboxylase was a synthetic product prepared by a new method shortly to be described. P=13.0%.

ATP was prepared from rabbit muscles by the method of Lohmann [1931]. 10.06 mg. Ba salt contained 0.920 mg. total P, 0.615 mg. pyro-P, 0.02 mg. inorganic P. Purity, 88 %.

Phosphopyruvic acid was synthesized according to Kiessling [1936] and converted into the crystalline Ag-Ba double salt. Ag-Ba salt: P split off by I_2 in alkaline solution: 6.2 %, total P, 6.2 %; inorg. P, 0. Purity, 90 %.

Muscle adenylic acid was a commercial product from Messrs Fränkel and Landau.

Methods: manometric measurements were carried out in the Warburg apparatus at 25° in air. All components were placed in the main part of the vessel with the exception of the substrate which was tipped in from the side bulb after the first reading.

Preparation of enzyme: the method of Warburg & Christian [1938] for the preparation of enzymes free from dissociable prosthetic groups has been adapted for our purpose.

50 g. of air-dried Löwenbräu bottom beer yeast are mixed with 150 ml. water and incubated at 37.5° for 3 hr. After centrifuging, the yeast residue is washed with a volume of water equal to the supernatant extract. The combined extract and washings are cooled to 0° and mixed with 1/2 vol. of cold acetone. The precipitate is discarded. 10 vol. of acetone are now added to the solution. The precipitate is allowed to settle and, after the supernatant liquid has been siphoned off, is centrifuged and dried *in vacuo*. Yield 8.5–9 g. Of this preparation, made according to Axmacher & Bergstermann [1934], a large stock can be prepared and kept in the desiccator for many months without loss of activity.

2 g. of the dry acetone precipitate are dissolved by grinding in a mortar with water and the volume is made up to 60 ml. After cooling to 0° 2 ml. 2 *N* acetic acid are added giving pH about 4.5. The precipitate is centrifuged off and rejected. 30 ml. saturated $(NH_4)_2SO_4$ are added to the solution and the precipitate is again discarded. After a further 30 ml. saturated $(NH_4)_2SO_4$ have been added the active precipitate is centrifuged and redissolved in 20 ml. *M*/20 phosphate buffer pH 6.2.

At this stage the activity of carboxylase is 5 times higher than in the original Lebedew maceration juice (Table I). For a rapid estimation of protein Folin & Ciocalteu's reagent was used, the ratio of the tyrosine content to the dry wt. of the protein having been determined preliminarily at the various stages of purification.

Table I. *Activity of carboxylase preparations*

	μ l. CO_2 /min./mg. protein	Tyrosine content of protein %
Original Lebedew extract	23	9.8
After acetone precipitation	55	12.0
After $(NH_4)_2SO_4$ precipitation	107	12.6

Owing to the increasing fragility of the enzyme it was not found expedient to attempt a higher degree of purification before the enzyme was dissociated by acidification. This process was carried out in the following manner: one-half of the solution of the $(NH_4)_2SO_4$ -precipitate (about 12 ml.) was diluted with 50 ml. water and 40 ml. sat. $(NH_4)_2SO_4$ were added producing but a faint opalescence. The mixture was cooled to -5° and 30 ml. *N*/10 HCl, 1/3 saturated with respect to $(NH_4)_2SO_4$, were added fairly rapidly from a burette while shaking vigorously. After centrifuging the precipitate was once washed with 20 ml. 3/4 saturated $(NH_4)_2SO_4$. This operation was now repeated with the second half of the enzyme.

The yield of active protein was found to be higher when the acidification was thus carried out with two separate portions than when the scale was doubled. The combined precipitates were now triturated with 15 ml. *M*/20 phosphate buffer pH 6.2 and denatured protein was centrifuged off. The protein content of the enzyme solution was in each case rapidly determined colorimetrically with Folin & Ciocalteu's reagent 0.126 mg. tyrosine indicating 1 mg. protein. More phosphate buffer was added until 1 ml. contained 1 mg. protein. The yield of active protein was usually 25–30 mg. It could be increased to 70–80 mg. if after the addition of HCl the precipitation was completed by the addition of a further 30 ml. sat. (NH₄)₂SO₄. But in this case some undissociated protein was precipitated as well, which tended to increase the blank. This step was therefore omitted in most experiments.

Usually the enzyme solution was freshly prepared for each experiment, starting with the dry acetone precipitate. The enzyme is destroyed by dialysis, by drying and even by a few hours' standing at 0°. It can however be preserved by adding an equal volume of sat. (NH₄)₂SO₄ and keeping at 0°. For use the protein precipitate is centrifuged and dissolved in an appropriate volume of phosphate buffer.

The concentration of protein in the experiments to be described was 0.33 mg./ml.

Some properties of the enzyme: pyruvic acid is decarboxylated only after addition of cocarboxylase of which about 6–7 µg./ml. are necessary for saturation (Tables II and III). 0.1 mg. Mg.⁺⁺ and 0.1 mg. Mn⁺⁺/3 ml. were added as a

Table II. *Decarboxylation of pyruvic acid*

Each vessel contained 1 mg. protein, *M*/30 phosphate buffer pH 6.2, *M*/30 pyruvate, 0.1 mg. Mg⁺⁺, 0.1 mg. Mn⁺⁺; total vol. 3 ml.

No addition
30 µg. cocarboxylase

µl. CO₂ in 30 min.
30
1080

Table III. *Decarboxylation of pyruvic acid with and without added B₁*

Reaction mixture as in exp. of Table II.

	µl. CO ₂ in 15 min.	
	Without B ₁	With 10 µg. B ₁
0	21	23
2 µg. cocarboxylase	132	125
6 µg. cocarboxylase	252	265
20 µg. cocarboxylase	349	338

routine, but inorganic phosphate is not necessary for the reaction, since it proceeds just as rapidly in citrate buffer. B₁ and B₁-monoP are both inactive as coenzymes. Contrary to the findings of Ochoa [1938] and Ochoa & Peters [1938] on alkaline-washed yeast no stimulating effect of B₁ was observed even if it was added to quantities of cocarboxylase well below saturation. This at once disposes of the difficulties encountered by these authors in assessing the amount of cocarboxylase with alkaline-washed yeast in presence of B₁.

Other enzymes are present in our protein preparation besides carboxylase. One of these is Negelein's protein A [1936] which catalyses the transfer of phosphate from phosphopyruvic acid to adenylic acid and from ATP to glucose or hexosemonophosphate [Meyerhof *et al.* 1937, 1]. The addition of an active preparation of protein A prepared by Negelein's method to 1 mg. of our protein did not increase the cleavage of phosphopyruvic acid. The protein also contains

the enzyme or enzymes necessary for the transfer of phosphate from ATP to B_1 or B_1 -monoP. The latter may be identical with, or may form a part of "protein A", but this reaction, too, was in no way accelerated by the addition of protein A.

The synthesis of cocarboxylase

Though both B_1 and B_1 -monoP are quite inactive as coenzymes of carboxylase they become, after an induction period of about 15 min., very active if some ATP is added (Fig. 1). ATP alone possesses a slight coenzyme activity, a fact which will be discussed below. Since ATP does not act as a catalyst in this reaction but as a phosphate donor, the amounts required are of the order of 1–3 mg./3 ml. (Fig. 2) whereas the blank reaction of ATP alone reaches its maximum at a much lower concentration. ATP cannot be replaced by adenylic acid (Table IV).

Fig. 1. Synthesis of cocarboxylase from B_1 and B_1 -monoP. Each vessel contained 1 mg. protein, $M/30$ phosphate buffer pH 6.2, $M/30$ pyruvate, 0.1 mg. Mg^{++} , 0.1 mg. Mn^{++} ; total vol. 3 ml. I, with 30 $\mu g.$ cocarboxylase; II, with 10 $\mu g.$ B_1 + 2 mg. ATP; III, with 10 $\mu g.$ B_1 -monoP + 2 mg. ATP; IV, with 2 mg. ATP; V, with 10 $\mu g.$ B_1 .

Table IV. *Effect of adenylic acid on the decarboxylation of pyruvic acid in presence of B_1 and B_1 -monoP*

Each vessel contained 1 mg. protein, $M/30$ phosphate buffer pH 6.2, $M/50$ pyruvate, 0.1 mg. Mg^{++} , 0.1 mg. Mn^{++} ; total vol. 3 ml.

	$\mu l. CO_2$ in 2 hr.	
	Without adenylic acid	With 0.5 mg. adenylic acid
0	58	40
30 $\mu g.$ B_1	48	50.5
60 $\mu g.$ B_1 -monoP	50	57
20 $\mu g.$ cocarboxylase (60 min.)	1068	—
0.5 mg. ATP + 30 $\mu g.$ B_1	440	—

Almost the maximum effect is obtained with a concentration of B₁ as low as 3 μ g./3 ml. It is only slightly higher with 10 μ g./3 ml. Higher concentrations are clearly inhibitory (Fig. 3).

Fig. 2. Activity of ATP. The points of the curves indicate the decarboxylation of pyruvic acid after 80 min. Each vessel contained 1 mg. protein, *M*/30 phosphate buffer pH 6.2, *M*/30 pyruvate, 0.1 mg. Mg⁺⁺, 0.1 mg. Mn⁺⁺; total vol. 3 ml. In addition each vessel contained varying quantities of ATP. I, with 30 μ g. B₁; II, without B₁.

Fig. 3. Activity of B₁. The points of the curve indicate the decarboxylation of pyruvic acid after 80 min. Each vessel contained 1 mg. protein, *M*/30 phosphate buffer pH 6.2, *M*/30 pyruvate, 0.1 mg. Mg⁺⁺, 0.1 mg. Mn⁺⁺, 3 mg. ATP and varying amounts of B₁. Total vol. 3 ml.

It has repeatedly been observed that with B₁-monoP the induction period is longer and the activity slightly lower than with B₁ (Fig. 1). With the latter the activity reaches about 1/3–1/2 of the initial activity of the enzyme saturated with cocarboxylase. This would correspond to the formation of 0.3–1 μ g. cocarboxylase, since with 1 μ g. cocarboxylase the rate of reaction is about half the maximal velocity (cf. Table V). But this is a minimum estimate: the highest activity is only observed after 40 min. when the activity of the enzyme may have declined. There is another fact to be considered: ATP has an inhibitory action on

carboxylase. This inhibition becomes apparent only when an enormous excess of ATP is present compared with cocarboxylase. It increases with increasing concentration of ATP and decreasing concentration of cocarboxylase and it decreases with time (Table V).

Table V. *Inhibition of carboxylase by ATP*

Exp. 1. The enzyme used for this experiment was prepared by suitable dilution from the redissolved $(\text{NH}_4)_2\text{SO}_4$ -precipitate before acidification. Each vessel contained 0.2 mg. protein, $M/30$ phosphate buffer pH 6.2, 0.1 mg. Mg^{++} , 0.1 mg. Mn^{++} , $M/30$ pyruvate; total vol. 3 ml.

	$\mu\text{l. CO}_2$ in 15 min.	Inhibition %
0	248	—
0.1 mg. ATP	249	0
0.3 mg. ATP	235	5
1.0 mg. ATP	200	20

Exp. 2. Enzyme dissociated by acidification as usual. Each vessel contained 1 mg. protein, $M/30$ phosphate buffer pH 6.2, $M/30$ pyruvate, 0.1 mg. Mg^{++} , 0.1 mg. Mn^{++} ; total vol. 3 ml.

	Without ATP $\mu\text{l. CO}_2$		With 2 mg. ATP $\mu\text{l. CO}_2$		Inhibition (%)	
	First 10 min.	Second 10 min.	First 10 min.	Second 10 min.	First 10 min.	Second 10 min.
0	20	19	22	27	0	0
1 $\mu\text{g. cocarboxylase}$	121	129	72.5	98	40	24
3 $\mu\text{g. cocarboxylase}$	182	172	130	148	28.5	14
10 $\mu\text{g. cocarboxylase}$	265	198	193.5	176	27	11
30 $\mu\text{g. cocarboxylase}$	302	200	270	213	10.5	0

This inhibitory action of ATP, which is of great theoretical interest as will be shown later, may well explain the induction period during the synthesis of cocarboxylase from B_1 , but it will tend to become insignificant once full activity is reached.

Though the observed effects can hardly be explained except by a synthesis of cocarboxylase, attempts were made to obtain further evidence of the phosphorylation of B_1 by the thiochrome method. As is well known, only unphosphorylated thiochrome is soluble in *isobutyl* alcohol whereas phosphorylated thiochrome remains in the aqueous phase. After incubation of B_1 with the enzyme in presence of ATP one would expect a decrease of fluorescence in the *isobutanol* phase and the appearance of fluorescence in the aqueous phase. Though the changes observed were in the right direction they were so small that they were almost within the limits of experimental error.

Two reaction mixtures of 15 ml. each were prepared, each containing 5 mg. protein, $M/30$ phosphate buffer pH 6.2, $M/30$ pyruvate, 0.5 mg. Mg^{++} , 0.5 mg. Mn^{++} and 50 $\mu\text{g. B}_1$. To one of them 15 mg. ATP were added. After 80 min. 260 $\mu\text{l. CO}_2$ were formed without, and 4665 $\mu\text{l. CO}_2$ with ATP. A similar experiment was performed, in which the addition of pyruvate was omitted. At the end of the incubation period 1 ml. 10 N H_2SO_4 was added to each solution and they were placed in a boiling water bath for 5 min. After cooling and neutralization 15 mg. ATP were added to the solutions where this addition had previously been omitted. The solutions were filtered and concentrated *in vacuo* to 10 ml. whereby all acetaldehyde was removed. 5 ml. of each solution were now oxidized with 0.2 ml. 1% $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 3 ml. 15% NaOH and extracted with 13 ml. *isobutyl* alcohol. The remaining 5 ml. served as controls after treatment with 13 ml. *isobutyl* alcohol and 3 ml. 15% NaOH . The fluorescence of the aqueous

and the *isobutanol* phases was measured in the light of a mercury lamp with a photoelectric cell connected with a mirror galvanometer. The following results were obtained (corrected for the blanks):

Deflexion in mm.		
	Aqueous phase	<i>iso</i> Butanol phase
In absence of pyruvate:		
Without ATP	0	22
With ATP	4	20
In presence of pyruvate:		
Without ATP	0	24
With ATP	5	21

It was hoped to obtain by this method information about the equilibrium between B₁ and cocarboxylase, but since the results were rather disappointing this line was not pursued further. The only conclusion which can be drawn is that the amount of cocarboxylase formed is very small, probably a few μ g. only.

Synthesis of cocarboxylase with phosphopyruvic acid as phosphate donator

Phosphopyruvic acid is not decarboxylated by the protein preparation described whether cocarboxylase, B₁-monoP or B₁ or any combination of them be present. This is of interest, since it might have been inferred from an analogy with ATP that the pyrophosphate group in cocarboxylase has a function in the mechanism of decarboxylation involving a shift of phosphate. This, however, seems unlikely.

The decomposition of phosphopyruvic acid by our protein requires the presence of (1) a phosphate carrier, adenylic acid or ATP, (2) a phosphate acceptor, e.g. glucose. The progress of the reaction was measured by the decarboxylation of the pyruvic acid formed. In this case cocarboxylase has to be added, too. Even in the presence of the complete system the reaction is slow. Addition of protein A has no effect. If cocarboxylase is replaced by B₁ decarboxylation occurs indicating the synthesis of cocarboxylase. B₁-monoP is active as well, but clearly inferior to B₁. Whereas in the former experiments with ATP as phosphate donator adenylic acid was inactive and considerable amounts of ATP were required, in the experiments with phosphopyruvic acid ATP can be replaced by adenylic acid and both are active if present in catalytic amounts. The reaction is however slower and has a longer induction period when adenylic acid is used instead of ATP (Table VI). Similar observations were made by

Table VI. *Decomposition of phosphopyruvic acid*

Each vessel contained 1 mg. protein, *M*/60 phosphate buffer pH 6.2, *M*/75 phosphopyruvate, *M*/50 glucose, 0.1 mg. Mg⁺⁺, 0.1 mg. Mn⁺⁺; total vol. 3 ml.

	μ l. CO	
	In 1 hr.	In 3 hr.
0.1 mg. ATP	19	27
0.1 mg. ATP + 30 μ g. cocarboxylase	119	342
0.1 mg. ATP + 50 μ g. B ₁ -monoP	31.5	93
0.1 mg. ATP + 50 μ g. B ₁	51	126
0.1 mg. adenylic acid	10	46
0.1 mg. adenylic acid + 30 μ g. cocarboxylase	63	312
0.1 mg. adenylic acid + 50 μ g. B ₁ -monoP	10	84
0.1 mg. adenylic acid + 50 μ g. B ₁	10	108

Meyerhof *et al.* [1937, 1] who explained it by the assumption that the transfer of phosphate occurs between adenosine diphosphate and ATP rather than between adenylic acid and adenosine diphosphate.

It was not possible to replace glucose as phosphate acceptor by stoichiometric amounts of B_1 or B_1 -monoP (Table VII). This is in agreement with the observations recorded above that the synthesis of cocarboxylase stops after amounts in

Table VII. *Decomposition of phosphopyruvic acid with B_1 or B_1 -monoP as phosphate acceptors*

Each vessel contained 1 mg. protein, $M/30$ phosphate buffer pH 6.2, $M/75$ phosphopyruvate, 0.1 mg. adenylic acid, 0.1 mg. Mg^{++} and 0.1 mg. Mn^{++} . Total vol. 3 ml.

	$\mu l.$ CO_2 in 4 hr.
30 $\mu g.$ cocarboxylase	30
30 $\mu g.$ cocarboxylase + $M/75$ glucose	342
15 mg. B_1	28.5
15 mg. B_1 -monoP	38

the region of 1 $\mu g.$ have been formed and that higher concentrations of B_1 even inhibit the synthesis of cocarboxylase. An enzymic synthesis of cocarboxylase on a preparative scale is therefore not feasible. Negative results were also obtained in experiments with dialysed muscle extract arranged on the lines of the experiments described by Meyerhof *et al.* [1937, 2]. Hexosediphosphate was used as phosphate donator. Whereas creatine was highly active as phosphate acceptor both B_1 and B_1 -monoP were inactive.

If alkaline-washed yeast is used as enzyme preparation the results are essentially similar. The breakdown of phosphopyruvic acid is however much faster and no phosphate acceptor is required, no doubt because the ATP formed is broken down by an adenylyl pyrophosphatase contained in the yeast. In presence of adenylic acid, but in absence of a phosphate donator, no synthesis of cocarboxylase from B_1 occurred with alkaline-washed yeast. The results of Table VIII

Table VIII. *Decomposition of phosphopyruvic acid and synthesis of cocarboxylase by alkaline-washed yeast*

Each vessel contained 1 ml. alkaline-washed yeast prepared according to Lohmann & Schuster [1937], $M/30$ phosphate buffer pH 6.2, 0.1 mg. Mg^{++} , 0.1 mg. Mn^{++} and $M/75$ phosphopyruvate. Total vol. 3 ml.

	$\mu l.$ CO_2 in 40 min.
0	6
30 $\mu g.$ cocarboxylase	21
0.2 mg. adenylic acid	65
0.2 mg. adenylic acid + 30 $\mu g.$ cocarboxylase	365
0.2 mg. adenylic acid + 50 $\mu g.$ B_1 -monoP	123
0.2 mg. adenylic acid + 30 $\mu g.$ B_1	169

closely resemble those of Lipschitz *et al.* [1938]. The boiled tissue extract used by these authors was replaced by adenylic acid and the hexosediphosphate by phosphopyruvic acid. The objections of Ochoa & Peters [1938] do not therefore apply to these experiments. Attention may again be directed to the fact that B_1 -monoP is considerably less active than B_1 and that there is a small catalytic effect of adenylic acid alone. The latter effect does not exist in the absence of a phosphate donator.

DISCUSSION

Since the synthesis of cocarboxylase from B₁ requires the intermediation of ATP all the facts known to apply to phosphorylations of this kind also apply to this particular case. Phosphorylation by inorganic phosphate can thus be expected to occur linked up with simultaneous dismutations or oxidations involving a reduction of codehydrogenases. The role of an intact respiration stressed by Ochoa [1939] points in this direction.

The fact that the synthesis of cocarboxylase from B₁-monoP requires a longer induction period than, and does not attain the same intensity as, that from B₁ suggests that the former is not an intermediate in the formation of cocarboxylase from B₁. If this is the correct explanation, one must assume that B₁-monoP is first hydrolysed to B₁ and that this in turn combines with a pyrophosphoric group transferred *in toto* from ATP.

It is probable that under the conditions of our experiments amounts of 1 μ g. cocarboxylase or less are formed. This is in agreement with the maximum rate of decarboxylation reached, with the fact that very small quantities of B₁ suffice for optimal synthesis and with the results of the thiochrome measurements. High concentrations of B₁ inhibit the synthesis, and B₁ is therefore unsuitable as stoichiometric phosphate acceptor for phosphopyruvic acid. Since a higher synthesis of cocarboxylase cannot be forced by an increase of the concentration of B₁ or ATP, the limiting factor is probably the amount of carboxylase protein present. It seems that the synthesis of cocarboxylase stops as soon as the carboxylase present is fully or partly saturated. No preparative use can therefore be made of the reactions described.

An observation which needs some comment is the small catalytic activity of ATP alone. Three possibilities were considered: (1) the preparation of ATP contained B₁, B₁-monoP or cocarboxylase as impurity. Such an explanation was suggested by the fact that the catalytic activity of ATP only appears after an induction period similar to that observed during the synthesis of cocarboxylase from B₁. For that same reason a contamination by cocarboxylase could be dismissed. But also the possibility of a contamination with B₁ or B₁-monoP could be ruled out for the following reasons: the catalytic activity was the same with two different preparations of ATP of slightly different purity. It was also observed with a preparation of adenylic acid under conditions which entailed the intermediate formation of ATP. The thiochrome test made on a solution of 10 mg. ATP was negative. Finally, if this explanation were correct, one would expect the catalytic activity to increase with increasing amounts of ATP until it reached the order attained when B₁ was added. In fact, the maximum-catalytic activity is reached with very small amounts of ATP and is always very much smaller than the effect observed in presence of B₁ or B₁-monoP.

(2) The enzyme preparation may still contain some firmly affixed B₁ or B₁-monoP. This, too, is very improbable. No fluorescence was revealed in a thiochrome test made on a solution of 8.5 mg. protein and carried out in the manner described. Furthermore, the catalytic activity of ATP did not vary substantially with different enzyme preparations.

(3) The only remaining explanation is that the catalytic effect is due to ATP itself. The constitution of ATP resembles that of cocarboxylase in two respects: it possesses a pyrophosphoric group which may enable it to attach itself to the enzyme, and it possesses, at the same position of the pyrimidine ring, a NH₂-group which, according to Langenbeck [1933], is essential to

catalysts of decarboxylation. ATP may thus be assumed to form with the active protein a "pseudo-carboxylase" of much inferior activity.

If ATP has some affinity for the carboxylase protein it would be expected to compete with cocarboxylase. This is indeed the case. The inhibition of carboxylase by ATP, which has been demonstrated, has all the properties of a competitive one. Since it becomes evident only with an excess of ATP over cocarboxylase of 1000 times or more, the affinity of ATP for the carboxylase protein is very small compared with that of cocarboxylase.

It will be recalled that Albers & Schneider [1936] in a short communication claimed a catalytic effect of adenylic acid with alkaline-washed yeast. In my experiments adenylic acid was quite inactive under these conditions unless a phosphate donator such as phosphopyruvic acid was present. The effect observed by Albers & Schneider was probably due to the presence or the formation of ATP too.

SUMMARY

A soluble yeast protein has been prepared from which cocarboxylase was removed by acidification in presence of ammonium sulphate. The addition of cocarboxylase results in a vigorous decarboxylation of pyruvic acid. Vitamin B₁ has no stimulating effect on the reaction even if added to quantities of cocarboxylase well below saturation.

Though both vitamin B₁ and vitamin B₁-monophosphate are quite inactive as coenzymes of carboxylase they become very active in presence of adenylyl pyrophosphate. After about 40 min. the activity reaches 1/3-1/2 of the initial activity of the enzyme saturated with cocarboxylase.

Vitamin B₁-monophosphate was consistently found to be slightly inferior in activity to vitamin B₁. This is interpreted as meaning that the monophosphate is not an intermediary of the synthesis and must first be hydrolysed to the unphosphorylated vitamin.

Adenylyl pyrophosphate alone has a small catalytic action in combination with the carboxylase protein. It is assumed that this is due to the adenylyl pyrophosphate attaching itself to the enzyme and forming a "pseudo-carboxylase". In support of this a competitive inhibition was observed if adenylyl pyrophosphate was added in large excess over cocarboxylase.

Phosphopyruvic acid can act as phosphate donator for the synthesis of cocarboxylase in presence of catalytic amounts of adenylic acid or adenylyl pyrophosphate.

The synthesis of cocarboxylase stops when the carboxylase protein is partly or fully saturated. No preparative use can therefore be made of the reactions described.

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