EXPERIMENTS ON THE CARBOXYLASE OF PEA ROOTS

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It is known that vitamin B_1 is a growth factor for numerous bacteria and fungi including the yeasts (see the summary in Koser and Saunders (1938)). It has also been demonstrated that vitamin B_1 is essential for the growth of the isolated roots of higher plants (Bonner, 1937; Robbins and Bartley, 1937). Because of this general vitamin B_1 requirement of living organisms, it would seem *a priori* probable that the vitamin plays a rôle in some basic cellular process. That this is indeed the case was shown conclusively by the work of Peters and coworkers (see Peters and O'Brien (1938)) and of Lohmann and Schuster (1937). The latter workers found that the prosthetic group of yeast carboxylase is vitamin B_1 pyrophosphate. In the case of yeast, vitamin B_1 is, then, a constituent of a respiratory enzyme and vitamin B_1 pyrophosphate is hence commonly referred to as "cocarboxylase," a terminology used throughout this paper. Although considerable information is available concerning the rôle of vitamin B_1 as a growth factor for roots, there is little known about the carboxylase of such roots. The present work was undertaken with the hope of elucidating possible relationships between vitamin B_1 and the carboxylase of pea roots.

The reaction system under consideration is defined by four experiments which show (1) that the respiration of whole pea roots is affected by the supply of vitamin B_1 in the medium in which the roots are grown (Table I), (2) that pea roots contain an enzyme (carboxylase) capable of decarboxylating pyruvic acid, (3) that by proper treatment of the enzyme it can be made to lose the greater part of its activity and that the activity is restored by

addition of vitamin B_1 pyrophosphate (Tables II and III), (4) that pea roots contain vitamin B_1 pyrophosphate, as determined by the cocarboxylase activity of filtrates of boiled pea root carboxylase with washed yeast. A once precipitated pea root preparation was found to contain approximately 2.1 γ of extractable cocarboxylase per gm. of dry weight. These findings indicate that pea roots contain a carboxylase similar in some respects to that found in yeast.

Technical—Preliminary investigation showed that it is essential to extract pea roots at low temperatures if denaturation of the carboxylase is to be avoided. The roots used were from etiolated plants grown for 7 days in coarse sand at 25°. At the end of this time the plants were removed from the sand and cleaned with running water. The roots were then cut off with a pair of scissors

TABLE I

Effect of Vitamin B_1 on Oxygen Consumption of Isolated Pea Roots Grown for 4 Weeks on Medium Containing All Growth Substances Except Vitamin B_1 As Noted

Roots supplied with	O_2 consumption per 100 mg. dry weight in 1 hr.
	c.mm.
No vitamin B_1	170
Vitamin B_1 (0.1 mg. per liter)	450

and dried superficially. They were next transferred to a large porcelain mortar which was placed in an alcohol-dry ice mixture. After the mass had frozen solidly, it was ground, transferred to centrifuge tubes, and centrifuged for 10 to 20 minutes. The temperature of the material rose to 3–4° during this process. The supernatant liquid was next transferred to a cold flask and the residue extracted with 0.1 M phosphate buffer of pH 6.2. This extract was combined with the preceding supernatant. To each 100 cc. of the combined extract were added 35 gm. of ammonium sulfate. This addition precipitated proteins, among them the carboxylase, which could then be centrifuged off. The supernatant liquid possessed only slight carboxylase activity and was discarded. The precipitated proteins were redissolved in water and again precipitated by ammonium sulfate. Significant losses

of carboxylase activity occurred if the operations were carried out above 5°.

The determinations of carboxylase activity were carried out with Warburg manometers. 2 cc. of the enzyme preparation suspended in phosphate buffer at pH 6.2 and containing 60 to 90 mg. of protein were placed in each Warburg vessel, together with 0.5 cc. of coenzyme solution, containing approximately 100 γ of vitamin B_1 pyrophosphate (Merck). 1 cc. of buffered 0.05 M solution of sodium pyruvate, containing 0.1 per cent $MgCl_2$, was placed in the side arm of the vessel and tipped in after attainment of temperature equilibrium. In some experiments, the coenzyme solution was placed in the side arm, and the pyruvate added with the enzyme. Suitable controls for the CO_2 evolution and for the small O_2 consumption of the preparation were run in every experiment. The CO_2 evolution in the absence of pyruvate was in all cases zero or negligible. In a typical experiment, the CO_2 evolved in 1 hour in the absence of pyruvate was 0.0 c.mm., and in the presence of pyruvate 106 c.mm. Quantitative determinations of vitamin B_1 pyrophosphate were made according to the method of Lohmann and Schuster (1937) with yeast washed with alkaline buffer to remove the coenzyme. It may be noted here that neither the pea root preparation nor the yeast used in these experiments showed increased activity when 100 γ of vitamin B_1 were added with the coenzyme (compare Ochoa and Peters (1938)). Total vitamin B_1 (phosphorylated and unphosphorylated) was determined in a few experiments by the use of the *Phycomyces* assay (Schopfer and Jung, 1937; Bonner and Erickson, 1938). It was found that under the conditions of the assay vitamin B_1 pyrophosphate possessed an activity per mole equal to that of the vitamin itself.

Removal of Prosthetic Group—Several different procedures were tried in attempts to free the pea root carboxylase preparation from a possible prosthetic group. If a once precipitated preparation was boiled and the denatured proteins filtered off, vitamin B_1 pyrophosphate (as determined by its activity in the yeast test) was found in the filtrate. This indicates that the preparation may contain a vitamin B_1 pyrophosphate-protein combination. Washing of the enzyme preparation with alkaline buffer (by suspending the preparation in 0.1 M Na_2HPO_4 and reprecipitating

with ammonium sulfate) had no effect on the pea root enzyme, even though a similar procedure removed coenzyme from yeast carboxylase (Lohmann and Schuster, 1937). Repeated precipitation with ammonium sulfate (eight or more times) or dialysis through a collodion membrane resulted in denaturation of a large part of the enzyme, while the activity of the undenatured portion was not enhanced by addition of vitamin B_1 pyrophosphate. It was not found possible to destroy the activity of the enzyme preparation by treatment with sulfite, although such treatment is known to destroy vitamin B_1 itself (Williams *et al.*, 1935).

The simplest procedure found for reversible inactivation of the enzyme was the action of the enzyme on pyruvate itself. The enzyme preparation, suspended in phosphate buffer of pH 6.2, was added to a 0.05 M pyruvate solution (containing 0.1 per cent $MgCl_2$) in the ratio of 2 volumes of enzyme to 1 volume of pyruvate solution. This mixture was allowed to stand at room temperature for 2 hours and then placed at 0° overnight. Preparations made according to the above procedure, which will be referred to hereafter as "partially inactivated," lost as much as 70 per cent of their original carboxylase activity. Upon addition of crystalline vitamin B_1 pyrophosphate the original activity was completely restored, indicating that no significant denaturation occurred. The presence of pyruvate was essential for the inactivation.

Activating Effect of Pyrophosphate –It was found that vitamin B_1 pyrophosphate can be replaced by pyrophosphate alone in the reactivation of the pea root enzyme. The action of pyrophosphate is not augmented by addition of the vitamin. This is shown in Table II. Similar experiments with washed yeast showed that pyrophosphate is without cocarboxylase effect on our yeast carboxylase.

That pyrophosphate is actually removed in the pyruvate inactivation of the enzyme is indicated by one experiment in which the supernatant fluid (concentrated to a small volume) from the pyruvate treatment for removal of the coenzyme was found to contain no cocarboxylase (was incapable of activating washed yeast), but this concentrated supernatant was still capable of reactivating treated pea root enzyme to some extent, a property possessed by pure pyrophosphate (Table II).

Nature of Pyrophosphate Effect—On the assumption that pea root carboxylase contains vitamin B_1 pyrophosphate as its functional group, it would seem likely from the above that treatment of the enzyme removed only the pyrophosphate, leaving the vitamin B_1 residue bound to the protein carrier in such a manner as to permit a resynthesis upon addition of pyrophosphate. The following experiment bears upon this hypothesis: 160 cc. of raw juice were precipitated once with ammonium sulfate and taken up in 50 cc. of phosphate buffer. This was divided into two portions. One portion, referred to as Fraction I, was immediately assayed for (*a*) carboxylase activity, (*b*) total vitamin B_1 (phosphorylated and unphosphorylated) by the *Phycomyces* method,

TABLE II

Reactivation of Partially Inactivated Pea Root Enzyme

All the flasks contained pyruvate in addition to the indicated reagents.

	CO_2 in 1 hr., c.mm.		
	Experiment 5	Experiment 6	Experiment 16
Nothing added	18.9	29.7	52.4
85 γ vitamin B_1 pyrophosphate	52.4	60.3	
200 γ Na pyrophosphate·$10H_2O$	40.4		
2000 γ "	57.6	64.0	100
2000 γ " + 100 γ vitamin B_1	54.8	62.7	
0.5 cc. supernatant from inactivated pea root carboxylase			69.4

(*c*) vitamin B_1 pyrophosphate extracted by boiling for 3 minutes on a water bath, (*d*) total vitamin B_1 remaining in the denatured proteins following this extraction. The remaining portion was reprecipitated once and partially inactivated with pyruvate, as described previously. This fraction, known as Fraction II, was again precipitated and assayed in the same ways as Fraction I. The supernatants resulting from the reprecipitations of Fraction II were mixed together to give Fraction III. Fraction III was analyzed for total vitamin B_1 and for vitamin B_1 pyrophosphate as a check on the preceding assays. The results of this experiment are presented in Table III. The carboxylase activity measurements show that the pyruvate treatment caused inactivation of

the preparation to the extent of 51 per cent. This was completely reversed on addition of pyrophosphate, showing that no appreciable denaturation of the enzyme occurred. That reactivation with pyrophosphate is possible in spite of the removal of the greater part of the vitamin B_1 (Table III, 5th line) may indicate that this part of the vitamin B_1 is free; *i.e.*, not combined as cocarboxylase. This view is supported by the failure to find vitamin B_1 pyrophosphate in the supernatants (Fraction III). The portion of the vitamin B_1 which is not removed by the pyruvate treatment, but which remains in the proteins, is so strongly held

TABLE III

Fate of Vitamin B_1 and Vitamin B_1 Pyrophosphate during Treatment of Pea Root Carboxylase with Pyruvic Acid*

The results are expressed per 100 mg. of the dry weight of the solids.

	Fraction I	Fraction II	Fraction III
Dry weight, *mg.*	990	605	385
CO_2 per hr.			
No additions, *c.mm.*	122	60	
104 γ vitamin B_1 pyrophosphate, *c.mm.*	140	116	
2000 γ Na pyrophosphate, *c.mm.*	151	151	
Total vitamin B_1, γ	1.02	0.545	1.45
Extractable vitamin B_1 pyrophosphate, γ	0.30	0.00	0.00
Total residual vitamin B_1, γ	0.42	0.49	

* The vitamin B_1 determinations by the *Phycomyces* method represent the total of the phosphorylated and unphosphorylated vitamin present in the sample.

that it is not extracted even after denaturation by boiling. This evidence, then, supports the hypothesis that pyruvate inactivation removes only pyrophosphate from the enzyme and that resynthesis occurs upon addition of the latter.

The fact that inactivated pea root enzyme preparations can be reactivated by pyrophosphate suggests that it might be possible to detect a significant cocarboxylase synthesis under these conditions. Experiments to test this possibility were carried out, but a synthesis could not be demonstrated with certainty. One negative result of particular interest follows. A pea root preparation was made and treated to remove pyrophosphate. It was

then pipetted into a series of Warburg vessels in the side arms of which had been placed previously water, cocarboxylase, and pyrophosphate solutions, respectively. The measurements were started and the contents of the side arms tipped in. When the next reading indicated an increased rate of CO_2 evolution (and, hence, presumably cocarboxylase synthesis) in the vessel containing pyrophosphate, the vessels were removed and immersed in boiling water. After cooling, 1 cc. of washed yeast was put into the side arm of each vessel and the vessels were replaced on the manometers. After equilibration the yeast was tipped in and the

TABLE IV

Attempt to Demonstrate Synthesis of Vitamin B_1 Pyrophosphate by Pea Root Preparation

Vessels 2, 3, and 4 contained, during the first run, partially inactivated pea root carboxylase, pyruvic acid, and the additions noted below. The contents of each vessel were then boiled, cooled, and 1 cc. of yeast added for the second run. Vessel 6 contained only vitamin B_1 pyrophosphate and pyruvate during the first run.

Vessel No.	Additions	CO_2 in 30 min., c.mm.	
		1st run	2nd run
2	Nothing	64.6	7.3
3	Vitamin B_1 pyrophosphate (10.4 γ)	74.0	23.6
4	Na pyrophosphate (2000 γ)	113.5	7.3
6	Vitamin B_1 pyrophosphate (10.4 γ)	0.0	171.3

cocarboxylase in each vessel was assayed. The results are shown in Table IV.

The failure to demonstrate a synthesis of cocarboxylase in the presence of pyrophosphate under the conditions of this experiment may be attributable to the fact noted earlier, that vitamin B_1 is held very firmly in the protein portion of the pea root carboxylase. Vitamin (and probably cocarboxylase) remains in the protein even after the latter has been denatured by boiling; as shown in the experiment of Table III, 90 per cent of the vitamin present in the partially inactivated preparation was retained by the denatured protein. It is possible that in the experiment of Table IV cocarboxylase was synthesized, but was not liberated by boiling.

The pea root preparation is capable of rapid destruction of

cocarboxylase. This is shown in Table IV by comparing the CO_2 production during the second run in Vessel 3 (pea root enzyme present) with that in Vessel 6 (pea root enzyme absent). This effect may be due to removal of cocarboxylase from the solution by the enzyme or to hydrolysis of the cocarboxylase into vitamin and pyrophosphate, followed by resynthesis from the liberated pyrophosphate and the vitamin bound in the protein.

DISCUSSION

It is shown in the experiments reported above that pea roots contain a carboxylase capable of decarboxylating pyruvic acid. The enzyme preparation contains appreciable amounts of vitamin B_1 and vitamin B_1 pyrophosphate. Treatments designed to dissociate vitamin B_1 pyrophosphate from the preparation reversibly were, however, unsuccessful, as described above. In these experiments it was found that by allowing the enzyme to act on pyruvic acid it could be partially inactivated, and that reactivation could be accomplished by the addition of either vitamin B_1 pyrophosphate or sodium pyrophosphate. It seems highly probable, from analogy with yeast carboxylase, that pea root carboxylase contains vitamin B_1 pyrophosphate as the prosthetic group. On this assumption, the evidence suggests that pyruvate inactivation is due to the splitting of pyrophosphate from the cocarboxylase molecule, and that resynthesis occurs upon addition of large amounts of pyrophosphate to the preparation.

A possible interpretation of the pyruvate inactivation is that a phosphorylation is linked with decarboxylation in the pea root enzyme (compare Lipmann (1939)). Attention should be called to the work of Peters and Sinclair (1933) which showed that addition of sodium pyrophosphate promotes oxidative decarboxylation by brain tissues.

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SUMMARY

1. A protein preparation from pea roots contains a carboxylase capable of decarboxylating pyruvic acid in the presence of Mg^{++}.

Heat denaturation of the preparation liberates cocarboxylase (vitamin B_1 pyrophosphate).

2. The pea root enzyme is partially inactivated by allowing the preparation to act on pyruvate. Vitamin B_1, but not cocarboxylase, is found in the supernatant following precipitation of the pyruvate-inactivated enzyme. Heat denaturation fails to liberate cocarboxylase from the pyruvate-inactivated enzyme.

3. Reactivation of partially inactivated enzyme is accomplished by the addition of cocarboxylase, pyrophosphate, or the concentrated supernatant from the pyruvate inactivation.

4. The reactivation by pyrophosphate suggests that the essential feature of pyruvate inactivation is the removal of inorganic pyrophosphate from the enzyme.

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