Studies on the Mechanism of Activation of Aspartic Acid β-Decarboxylase by α-Keto Acids and Pyridoxal 5′-Phosphate*

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Purified aspartic acid β-decarboxylase from *Clostridium perfringens* is markedly activated by catalytic amounts of pyridoxal 5′-phosphate or α-keto acids. The effects of these activators are not additive, and the added activators may be removed readily by dialysis. Assay of the purified enzyme by microbiological procedures indicates the presence of enzyme-bound pyridoxal 5 -phosphate. Radiation of the purified enzyme with ultraviolet light yields an apoenzyme preparation that no longer responds to the addition of α-keto acid, but which is still activated by pyridoxal 5′-phosphate; microbiological studies show that the radiated enzyme contains no pyridoxal 5′-phosphate. Incubation of the radiated enzyme with pyridoxal 5′-phosphate followed by exhaustive dialysis gives an enzyme preparation which is indistinguishable from the original preparation in that it is activated by both pyridoxal 5′-phosphate and α-keto acids. The experimental data lead to the conclusion that pyridoxal 5′-phosphate functions not only as the prosthetic group of aspartic acid β-decarboxylase but also as a less tightly bound co-factor. The data are consistent with the hypothesis that the enzyme-bound pyridoxal 5′-phosphate is linked to the enzyme in a form which does not react with substrate; reaction of the enzyme with catalytic quantities of an α-keto acid or pyridoxal 5′-phosphate converts the inactive prosthetic group to one capable of forming a Schiff base with aspartate.

The decarboxylation of L-aspartic acid [reaction (1)] by preparations of *Clostridium perfringens*[1] is unique not only because the β-carboxyl group rather than the α-carboxyl group is attacked, but because the enzyme is markedly activated by a variety of α-keto acids as well as by pyridoxal 5′-phosphate (Meister *et al.*, 1951). The present study was undertaken in an attempt to elucidate this activation. The enzyme has now been purified considerably, and the purified enzyme is almost entirely inactive unless either an α-keto acid or pyridoxal 5′-phosphate is added. We have found that the activation of this enzyme by pyridoxal 5′-phosphate differs from that observed with other vitamin B_6 enzymes; thus, the added vitamin B_6 derivative is apparently not tightly bound and may be removed by dialysis. Furthermore, the purified enzyme, inactive in the absence of added α-keto acid or pyridoxal phosphate, contains significant quantities of bound vitamin B_6. Our studies indicate that the bound material is pyridoxal 5′-phosphate and that its presence (as well as that of added α-keto acid or pyridoxal 5′-phosphate) is required for enzymatic activity.

L-aspartic acid ⟶ L-alanine + CO_2 (1)

EXPERIMENTAL

Materials.—L-Aspartic acid was obtained from Mann Research Laboratories. "Uniformly labeled" C^{14}-L-aspartic acid was obtained from Schwarz BioResearch, Inc.; this product was found to contain 45% of its C^{14} in carbon atom 4. Protamine sulfate was purchased from the Nutritional Biochemicals Corporation and carboxymethylcellulose was obtained from the Brown Company. Crystalline pyridoxal 5′-phosphate was obtained from the California Biochemical Research Company; freshly prepared solutions were used.

Methods.—Clostridium perfringens (ATCC 8009) was cultivated as previously described (Meister *et al.*, 1951). The cultures were incubated for 7 to 12 hours at 37° and the cells were then harvested with a Sharples centrifuge. The cells were washed twice by centrifugation with 0.17 M sodium chloride, once with distilled water, and then lyophilized. The yield of dried cells was approximately 1 g per liter of medium.

Microbiological assays for vitamin B_6 were carried out by the methods of Rabinowitz and Snell (1947a,b) and Rabinowitz *et al.* (1948).[2] Ultraviolet radiation of the enzyme preparations was performed as previously described (Meister

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[1] Previously referred to as *C. welchii* SR 12.

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et al., 1951). The concentration of pyridoxal 5′-phosphate was determined spectrophotometrically, the absorbancy values given by Peterson and Sober (1954) being used.

In the studies with C^{14}-aspartic acid, C^{14}-alanine was separated by paper chromatography on Whatman No. 3MM paper; the solvent consisted of 77% ethanol. One-centimeter sections of the paper strips were counted in an automatic gas flow counter; the values were corrected for self-absorption.

Determination of Enzymatic Activity.—Enzymatic activity was determined by the Warburg manometric procedure. The main compartment of the vessel contained L-aspartic acid (15 μmoles), sodium pyruvate (0.5 μmole), pyridoxal 5′-phosphate (0.5 μmole), and sodium acetate buffer (*p*H 5.0, 1100 μmoles) in a final volume of 1.1 ml. The enzyme solution (0.4 ml) was placed in the side-bulb. The rate of carbon dioxide evolution was determined at 38°. Protein was determined by the procedure of Lowry *et al.* (1951). Specific activity is expressed in terms of microliters of carbon dioxide evolved per milligram of protein per hour.

Preparation of the Enzyme.—Several methods of rupturing the cells were investigated, including grinding with small glass beads, grinding with alumina, freezing and thawing, autolysis at 26°, and sonic oscillation. These procedures gave extracts exhibiting specific activity values in the range of 10 to 30 μl of carbon dioxide per milligram of protein per hour. Previous studies (Meister *et al.*, 1951) were carried out with preparations exhibiting specific activities of about 20. The present procedure, described below and in Table I, gives higher initial specific activity values (60–75) and a final preparation with a specific activity approximately 500 times greater than those studied previously.

TABLE I

PURIFICATION OF ASPARTIC ACID β-DECARBOXYLASE[a]

	Volume (ml)	Protein (mg/ml)	Specific Activity[c]	Yield
Step 1	740	17.5	67.0	(100)
Step 2	885	12.0	77.5	97
Step 3	183	14.0	178	53
Step 4	10.9	15.7	1290	26
Fraction 21[b]	12.0	0.265	7330	
Fraction 22[b]	12.0	0.215	9120	

[a] Prepared from 45 g of lyophilized cells; details of the procedures are given in the text. [b] From column chromatography on carboxymethylcellulose. [c] Microliters of CO_2 evolved per mg of protein per hour.

STEP 1.—Forty-five grams of lyophilized cells were suspended in 900 ml of 0.17 M sodium chloride; the suspension was shaken gently at 5° for 6 hours. The mixture was then allowed to stand at 5° for 18 hours, and the resulting gelatinous suspension was centrifuged at 13,000 × *g* at 0° for 20 minutes.

STEP 2.—The clear amber supernatant solution obtained in step 1 was treated with 0.25 volume of a 2% solution of protamine sulfate in 0.17 M sodium chloride. The mixture was stirred at 0° for 15 minutes and then centrifuged at 8000 × *g* at 0° for 15 minutes. The *p*H of the supernatant solution was adjusted to 6.7 by addition of 1.0 N sodium hydroxide.

STEP 3.—The solution obtained in the previous step was cooled in an ethylene glycol–dry ice bath, and 0.25 volume of cold absolute ethanol was added. The temperature of the mixture was allowed to fall to −4°, and it was then centrifuged at 8000 × *g* at −4° for 15 minutes. The supernatant solution was discarded and the precipitate was allowed to drain at −10° for 1 hour. The hard precipitate was broken up with a stirring rod and resuspended in 200 ml of 0.2 M sodium acetate buffer (*p*H 4.6). The suspension was stirred for 15 minutes at 5° and then centrifuged at 8000 × *g* at 0° for 15 minutes. The precipitate was discarded.

STEP 4.—Solid ammonium sulfate (31.3 g per 100 ml) was added to the clear yellow supernatant solution obtained in the preceding step. The solution was stirred continuously in an ice bath, and when the salt had completely dissolved the mixture was centrifuged at 8000 × *g* at 0° for 15 minutes. The precipitate was discarded and additional solid ammonium sulfate (21.4 g per 100 ml) was added to the supernatant solution. The precipitate obtained by centrifugation of this mixture was resuspended in about 2 ml of 0.2 M sodium acetate buffer (*p*H 4.6), and this solution was dialyzed against three changes of 400 ml each of this buffer for 18 hours at 5°. The large precipitate which formed during dialysis was removed by centrifugation.

Column Chromatography on Carboxymethylcellulose.—The dialyzed solution obtained in step 4 was added to the top of a carboxymethylcellulose column (1.7 × 7 cm; prepared from 1.1 g of dry carboxymethylcellulose) (Peterson and Sober, 1961), which had been equilibrated previously with 0.2 M sodium acetate buffer (*p*H 4.6). Gradient elution of the protein was carried out by allowing 300 ml of 0.2 M sodium acetate (*p*H 4.6) to mix with 300 ml of 0.4 M sodium acetate. The flow rate was about 2 ml per minute, and fractions of 10 to 12 ml were collected. The entire operation was carried out in a cold room at 5°. All of the experiments described in this report were carried out with enzyme purified by chromatography on carboxymethylcellulose. The enzyme was stable for at least 1 month when stored at 0°; lyophilization resulted in about a 20% loss of activity. Several attempts to rechromatograph the enzyme led to almost complete loss of activity. The purified enzyme preparation was entirely devoid of glutamic decarboxylase and glutamate-aspartate transaminase activities.

Activation of the Enzyme by Pyridoxal 5′-Phosphate and Pyruvate.—Low concentrations of either pyridoxal 5′-phosphate or pyruvate sufficed

to activate the enzyme. The apparent K_m values for pyridoxal 5'-phosphate and pyruvate calculated by the method of Lineweaver and Burk (1934) were, respectively, 0.9×10^{-5} and 1.2×10^{-5} M. Under the conditions used here for assay of enzymatic activity, pyridoxal 5'-phosphate gave slightly less activation than did pyruvate. When both activators were used together at this concentration, the activity was 10 to 20% greater than when either was used alone; this result is not unexpected since the system is not completely saturated with respect to either activator. The available data therefore indicate that the effects of pyridoxal 5'-phosphate and pyruvate are not additive; representative values are given in Table II. When enzyme preparations were treated with either pyridoxal 5'-phosphate or pyruvate and then dialyzed, the dialyzed enzyme was not appreciably active. However, typical activation was obtained when either α-keto acid or pyridoxal 5'-phosphate was added to the dialyzed enzyme preparation.

TABLE II

EFFECTS OF PYRIDOXAL 5'-PHOSPHATE AND PYRUVATE ON ENZYMATIC ACTIVITY[a]

Additions	Activity
None	8.6
Pyridoxal 5'-phosphate	47.0
Pyruvate	49.7
Pyridoxal 5'-phosphate + pyruvate	56.9

[a] Activity is expressed in terms of microliters of carbon dioxide liberated in 15 minutes. The reaction mixtures consisted initially of L-aspartic acid (15 μmoles), sodium pyruvate (0.5 μmole), pyridoxal 5'-phosphate (0.5 μmole), enzyme (51 μg), and sodium acetate buffer (*p*H 5.0; 1100 μmoles) in a final volume of 1.5 ml; incubated at 38°.

No activation was observed with benzaldehyde, acetaldehyde, salicylaldehyde, or methylglyoxal.

Enzymatic Conversion of C^{14}-Aspartate to C^{14}-Alanine.—Reaction mixtures (0.75 ml) containing L-aspartic acid-C^{14} (10 μmoles; 32,000 cpm), pyridoxal 5'-phosphate (0.5 μmole), sodium pyruvate (0.5 μmole), enzyme (15 μg), and sodium acetate buffer (*p*H 5.0; 450 μmoles) were incubated for 1 hour at 38°, at which time 70% of the aspartate was decarboxylated. After deproteinization, aliquots were chromatographed as described above in the Methods section, and the alanine and pyruvate areas were isolated and counted. Analogous experiments were carried out in which the concentration of sodium pyruvate was increased 100-fold. Within experimental error, the same amount of radioactivity (12,300 cpm) was found in the alanine area in all of these experiments, and no radioactivity was found in the pyruvate area. These results are consistent with earlier experiments in which unlabeled alanine was formed from C^{12}-aspartate in the presence of C^{14}-pyruvate (Meister *et al.*, 1951). The present and previous studies exclude the possibility that the conversion of aspartate to alanine takes place by a mechanism involving transamination of pyruvate with aspartate to yield oxaloacetate and alanine, followed by decarboxylation of oxalacetate to pyruvate. The evidence therefore supports a direct decarboxylation of aspartate to alanine.

Assay of the Enzyme for Vitamin B_6.—The enzyme as purified by column chromatography was assayed for vitamin B_6 with *Saccharomyces carlsbergensis* 4228, which responds to pyridoxine, pyridoxamine, or pyridoxal, *Streptococcus faecalis R*, which responds to either pyridoxamine or pyridoxal (or their phosphorylated forms), and *Lactobacillus casei*, which responds specifically to pyridoxal. As indicated in Figure 1, the values for total vitamin B_6 (response to *S. carlsbergensis*) paralleled those for aspartic acid β-decarboxylase activity. It is of interest that although glutamate-aspartate transaminase was eluted in fractions 12–18 under these conditions, assay of this material did not reveal the presence of vitamin B_6. Enzymatic studies also indicated that the glutamate-aspartate transaminase was completely resolved. Table III gives the values for vitamin B_6 obtained with the several organisms employed. Several comparative assays of this type were carried out on the purified enzyme, and in each case results similar to those given in Table III were obtained. Since *L. casei* responds only to pyridoxal, the data indicate that virtually all of the vitamin B_6 is present in the pyridoxal form. *L. casei* responded poorly to preparations of the enzyme that were not hydrolyzed with dilute hydrochloric acid by the procedure of Rabinowitz *et al.* (1948), but gave a good response when the same preparations were hydrolyzed; it may therefore be concluded that pyridoxal 5'-phosphate rather than pyridoxal is present in the enzyme.

TABLE III

MICROBIOLOGICAL ASSAY OF THE ENZYME FOR VITAMIN B_6[a]

Fraction No.	*S. carlsbergensis*	*S. faecalis R*	*L. casei*
21	54.7	50.3	65.6
22	61.3	56.9	70.0
23	53.6	52.5	62.4

[a] The values are given as mμg of vitamin B_6 (as pyridoxal HCl) per ml of enzyme fraction obtained in the chromatographic separation (Fig. 1).

Resolution of the Enzyme.—Attempts to remove the enzyme-bound vitamin B_6 by dialysis and precipitation of the enzyme under various conditions were not successful. We therefore resorted to the radiation technique (Meister *et al.*, 1951) used previously to destroy the enzyme–vitamin B_6. Although this procedure destroys approximately 50% of the enzymatic activity, the remaining enzyme is almost completely resolved

with respect to pyridoxal 5′-phosphate. A typical result is shown in Table IV. Prior to radiation, the enzyme was activated to about an equal extent by pyridoxal 5′-phosphate and pyruvate. After radiation, the enzyme was activated by pyridoxal 5′-phosphate, but there was virtually no increase in activity on addition of pyruvate. Microbiological assay of the radiated enzyme preparation with *L. casei* and *S. carlsbergensis* failed to reveal vitamin B_6. The radiated enzyme was incubated with an excess of pyridoxal 5′-phosphate and then exhaustively dialyzed. The dialyzed enzyme responded to both pyridoxal 5′-phosphate and pyruvate in typical fashion (Table IV). Thus, virtually the same activity was obtained on addition of either activator. In the course of this work it was found that the apoenzyme was very unstable as compared to the original (unradiated) preparation or the reconstituted enzyme.

TABLE IV
RESOLUTION OF THE ENZYME[a]

Additions	Before Radiation	After Radiation	After Radiation and Treatment with Pyridoxal 5′-Phosphate[b]
None	8.6	5.6	12.4
Pyruvate	49.7	7.8	46.9
Pyridoxal 5′-phosphate	47.0	49.2	44.8

[a] Activity values were obtained under conditions described in Table II; 51 and 102 μg, respectively, of enzyme were used for assay of the enzyme before and after radiation. The enzyme contained 6.8 mμg of vitamin B_6 (as pyridoxal HCl) per 51 μg of protein.
[b] Radiated enzyme (0.58 mg in 2 ml of 0.1 M sodium acetate buffer, *p*H 4.8) was incubated with 0.14 ml of buffer containing 0.7 μmole of pyridoxal 5′-phosphate for 30 minutes at 38°, and then dialyzed at 5° against 5 changes of 500 ml each of 0.2 M sodium acetate buffer (*p*H 4.6) over 3 days. The radiated enzyme contained no vitamin B_6 as determined by microbiological assay.

DISCUSSION

The decarboxylation of aspartic acid exhibits several unusual features. (*a*) In contrast to all other amino acid decarboxylases, the α-carboxyl group is not attacked. (*b*) Although pyridoxal 5′-phosphate activates the enzyme, the added pyridoxal 5′-phosphate is readily removed by dialysis. Nevertheless, the enzyme contains firmly bound vitamin B_6 which is required for enzymatic activity. (*c*) A variety of α-keto acids can replace pyridoxal 5′-phosphate in the activation of the enzyme, and the effects of keto acids and pyridoxal 5′-phosphate are not additive; it appears probable that they act by the same mechanism.

The enzyme-bound vitamin B_6 assays by microbiological methods as pyridoxal 5′-phosphate, and it is destroyed by radiation of the enzyme. The apoenzyme may be reactivated by pyridoxal 5′-phosphate, but not by α-keto acid. However, when the apoenzyme is incubated with pyridoxal 5′-phosphate and then exhaustively dialyzed, the resulting preparation resembles the original untreated enzyme in that it is active provided that either pyridoxal 5′-phosphate or α-keto acid is added. The evidence therefore indicates that activation by α-keto acid requires enzyme-bound pyridoxal 5′-phosphate. Pyridoxal 5′-phosphate reconstitutes the apoenzyme, but it has an additional function, which is also carried out by α-keto acids.

The present findings do not support the suggestion made previously (Meister *et al.*, 1951) that the added α-keto acid yields pyridoxal 5′-phosphate by transamination with pyridoxamine 5′-phosphate present in the enzyme preparation. In the earlier work it was found that added pyridoxamine 5′-phosphate activated the enzyme only in the presence of an α-keto acid; activation under these conditions may be ascribed to pyridoxal 5′-phosphate formed by transamination. The data are consistent with the belief that the enzyme contains pyridoxal 5′-phosphate which is bound in a form that is not accessible to the substrate. By addition of catalytic quantities of α-keto acid or pyridoxal 5′-phosphate the inactive prosthetic group is converted to one that can form a Schiff base with aspartate. It is possible that the aldehyde group of pyridoxal phosphate is linked to a functional group of the protein, which also exhibits relatively high affinity for added α-keto acids and pyridoxal phosphate. Thus, the enzyme-bound pyridoxal phosphate

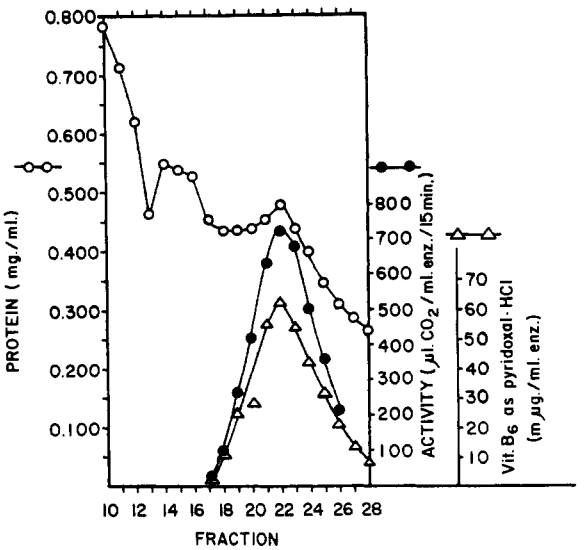

FIG. 1.—Elution of the enzyme from carboxymethylcellulose. Chromatography was carried out as described in the text. Aliquots (1 ml) of the fractions were assayed for vitamin B_6 with *S. carlsbergensis*.

might exist in Schiff base linkage with an amino group of the protein; displacement by added activator would yield a free aldehyde group. Studies on other pyridoxal phosphate enzymes suggest that Schiff base linkages involving the ϵ-amino groups of protein lysine may be at least as reactive with amino acid substrates as the free aldehyde group of pyridoxal phosphate itself. On the other hand, it is conceivable that the reactivity of such Schiff bases may be altered by the tertiary configuration of the protein, and also that a Schiff base involving an amino-terminal α-amino group might be less reactive with substrate than one involving an ϵ-amino group. Alternatively, the inactive form of the enzyme may be similar to the aldimine form postulated for the pyridoxal phosphate of phosphorylase by Fischer *et al.* (1958). The additional linkage on the aldehyde group to the protein might involve a sulfhydryl or imidazolyl group, or possibly another amino group of the protein. Although, in general, aldehydes react more readily with sulfhydryl groups than do α-keto acids, it is evident that such reactivity might be influenced considerably by the orientation of the added activator on the protein. We have attempted to obtain information relating to these possibilities by determining enzymatic activity and vitamin B_6 by microbiological assay after treatment of the enzyme with sodium borohydride in the presence and absence of added α-keto acids and pyridoxal 5'-phosphate under various conditions. Unfortunately, the results of these studies have been difficult to interpret because sodium borohydride caused considerable denaturation of the enzyme in the absence of added activator. Experimental tests of these hypotheses may become more feasible when larger quantities of enzyme are available. Although the enzyme preparation used here is considerably more active than that previously available, it is recognized that it is still relatively impure as compared to certain other vitamin B_6-containing enzymes (*e.g.*, heart muscle glutamate-aspartate transaminase, phosphorylase, cystathionase). Nevertheless, the turnover of aspartic decarboxylase per mole of vitamin B_6 is similar to that of other vitamin B_6 enzymes. For example, it decarboxylates approximately 7000 moles of aspartate per mole of enzyme-bound vitamin B_6 per minute under the conditions employed here; the comparable value calculated from the data of Shukuya and Schwert (1960) for glutamic decarboxylase is 10,000.

In the past, activation of an enzyme by addition of pyridoxal 5'-phosphate has often been accepted as evidence that the added vitamin B_6 compound reconstitutes an apoenzyme. However, this is not true for aspartic β-decarboxylase, for which pyridoxal 5'-phosphate functions not only as a prosthetic group but also as a less tightly bound co-factor. It would be of interest to know whether other vitamin B_6 enzymes are activated by α-keto acids. Such an effect might be difficult to observe with transaminases, since α-keto acids are present in the catalytic systems usually employed. Meadow and Work (1958) have reported that α-keto acids activate the α,ϵ-diaminopimelic decarboxylase of crude bacterial preparations. The increased rate of desulfhydration of cysteine in the presence of α-keto acids observed in certain systems (Delwiche, 1951; Chatagner *et al.*, 1960) may be due to transamination to yield β-mercaptopyruvate, which is in turn enzymatically desulfurated (Meister *et al.*, 1954). On the other hand, this mechanism may not explain activation by α-keto acids in all such systems.

Aspartic β-decarboxylase has been found in a number of other microorganisms (Mardashev *et al*, 1947, 1948a, b, 1949; Cattanéo-Lacombe *et al.*, 1958; Crawford, 1958); a report by Bheemeswar (1955) suggests that it may be present in the silkworm. In contrast, the available data suggest that aspartic α-decarboxylase is less widely distributed. It is of interest that the aspartic β-decarboxylases of *Desulfovibrio desulfuricans* (Cattanéo *et al.*, 1958) and *Nocardia globerula* (Crawford, 1958) are also activated by α-keto acids. The enzyme may be more widely distributed, and may possibly be present in mammalian tissues. The discovery of an abundant source of the enzyme would be important, inasmuch as further studies on the mechanism of its action will doubtless require larger quantities of the enzyme than are now available.

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Investigations on Lignins and Lignification. XXVI.* Studies on the Utilization of Pyruvate in Lignification†

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Uniformly labeled sodium pyruvate-C^{14} was incorporated into a birch sapling by a forced feeding technique. After isolation of the milled-wood lignin, the distribution of radioactivity in the propyl side-chain of its building units was studied by hydrogenation, hydrogenolysis, and vapor phase chromatography. The results indicate that pyruvate is not a direct precursor of the propyl moiety of lignin. Together with specific activities of other wood and leaf constituents, the data reveal certain aspects of the metabolic fate of pyruvate in this plant.

The biogenesis of aromatic compounds is known to occur *via* at least two synthetic routes, *i.e.*, the acetate (Birch, 1960) or shikimate (Davis, 1955) pathways. That the latter reaction sequence is responsible for the formation of phenylpropanoid units is generally acknowledged, and it has been demonstrated (Eberhardt and Schubert, 1956; Acerbo *et al.*, 1958, 1960) that the initial stages of lignification, which also involve the genesis of C_6-C_3 intermediates, follow this same course.

The direct precursor of the C_3 side-chain of the phenylpropanoid type aromatic amino acids, phenylalanine and tyrosine, has recently been reported to be phosphoenolpyruvic acid (Levin and Sprinson, 1960), a compound also proposed as an intermediate in shikimic acid formation (Srinivasan and Sprinson, 1959). While it would be of interest to determine whether phosphoenolpyruvic acid is similarly operative in lignin biogenesis, previously observed difficulties in the permeability of many phosphorylated compounds (Weiss and Mingioli, 1956) prompted the substitution of a closely related compound. The reversibility of the reaction between phosphoenolpyruvic acid and pyruvate (Meyerhof and Oesper, 1949), despite the fact that the equilibrium constant is greatly in favor of formation of the latter, suggested the utilization of pyruvate. In addition, it has been reported (Thomas *et al.*, 1953, 1955) that yeast, when grown on labeled pyruvate as sole carbon source, synthesized tyrosine and phenylalanine with side-chains derived from this α-keto acid as an intact unit. However, the distribution of radioactivity in the aromatic rings of these amino acids indicated that the latter were not formed directly from pyruvate.

Phosphoenolpyruvic acid is also believed to play a key role in the reversal of glycolysis (Krebs, 1954). Hence, the manner in which pyruvate is incorporated into carbohydrates could be analogous to the initial steps of the process under consideration here. Recent studies in this area have shown that the nature of the tissue greatly affects the metabolic route. For example, the fact that C^{14}-labeled pyruvate is converted into rat liver glycogen with randomization of the isotopic carbon suggests that here pyruvate is not a direct precursor (Topper and Hastings, 1949), (Landau *et al.*, 1955). On the other hand, rat diaphragm tissue less efficiently converts radioactive pyruvate directly into glycogen, as exhibited by the localization of activity in the isolated carbohydrate (Hiatt *et al.*, 1958). In addition, extensive studies on various plant tissues (Neal and Beevers, 1960; Brummond and Burris, 1953) have provided evidence for the predominance of an oxidative decarboxylation of this α-keto acid.

Thus, when sodium pyruvate-3-C^{14} was incorporated into a living Norway spruce tree and the lignin was isolated and degraded to vanillin

* For the previous papers of this series see Coscia *et al.*, 1961, and Olcay, 1962.

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