In connection with the redox potential requirement, a recent report of an enzyme system of this class stated that both manganese and cobalt ions activated the enzyme, though to a lesser extent than the preferred ferrous ion (Cain, 1962). This is interesting in view of the activity of manganese and cobalt reported here, and may indicate that the redox potential requirement is less severe for this enzyme.

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Biosynthesis of Dipicolinic Acid and of Lysine in *Penicillium citreo-viride**

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The biosynthesis of 2,6-dipicolinic acid by *Penicillium citreo-viride* has been studied using a variety of radioactive substrates. Intermediates from terminal respiration and from the glyoxylate shunt appear in this end product. Radioactive CO_2 was fixed into the ring and into the carboxyl carbons of dipicolinate. The presence of unlabeled propionate enhanced the incorporation of labeled carbonate into dipicolinate and also shifted the ratio of carboxyl to ring activity to favor ring labeling. [1,7-^{14}C]Diaminopimelic acid was transformed by *P. citreo-viride* into dipicolinic acid labeled essentially in its carboxyl groups, but mycelial lysine was devoid of radioactivity. Diaminopimelate could not be detected however, in an acid hydrolysate of the fungal mycelium. Administration of α-[6-^{14}C]aminoadipic acid to *P. citreo-viride* also gave rise to dipicolinate which was predominantly carboxyl labeled. Here, mycelial lysine was revealed by degradation to have been formed directly from α-aminoadipate. D- and L-amino acid oxidases were demonstrated in crude cell-free extracts from *P. citreo-viride;* and it was shown that *meso-* and DL-diaminopimelic acids were oxidized to 2,6-diketopimelate. Chromatographic evidence was found for the presence of diketopimelate in minimal growth medium and in 80% ethanolic mycelial extracts obtained therefrom. The diketo acid was further identified by characterization as its bis-dinitrophenylhydrazone derivative. In replacement experiments which used glucosamine, glutamine, and glutamic acid, respectively, with diketopimelate, increased production of dipicolinic acid resulted. The amino group of L-[^{15}N]-glutamic acid was, however, not incorporated into dipicolinate. It is concluded that dipicolinic acid and lysine are ultimate products which arise from branchpoints in the metabolism of α-ketoadipic acid. This intermediate is postulated either to undergo transamination and reductive amination; or to add a C_2 fragment with subsequent oxidative decarboxylation, to give diketopimelic acid. The nonenzymatic conversion of diketopimelate and ammonia to dipicolinate was confirmed. The last steps of the biosynthesis of dipicolinic acid appear therefore to involve spontaneous reactions from the biologically formed 2,6-diketopimelic acid.

The isolation and characterization of dipicolinic acid (DPA)[1] from aerobic spore-forming bacteria was first reported by Udo (1936) and later by Powell (1953) and by Perry and Foster (1955). From a series of experiments employing radioactive substrates, it was concluded (Perry and Foster, 1955; Martin and Foster, 1958) that the biogenesis of this compound involves the condensation of pyruvate and aspartate (or of alanine and oxalacetate) to yield an unsaturated derivative of α-keto-ε-aminopimelic acid, which then undergoes cyclization and oxidation. It was also shown by these workers that uniformly labeled DAP was incorporated into DPA by *Bacillus megaterium*. Powell and Strange (1959) subsequently pointed out that a facile chemical

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[1] Abbreviations used in this work: DPA, pyridine-2,6-dicarboxylic acid; DKP, 2,6-diketopimelic acid; DAP, 2,6 diaminopimelic acid; α-AAA, α-aminoadipic acid; α-KAA, α-ketoadipic acid; DNPH, 2,4-dinitrophenylhydrazone.

synthesis of DPA could occur spontaneously from ammonium ion and DKP, and thus raised the question of the relevance of this diketo acid to the actual biological process.

The description of a strain of *Penicillium citreo-viride* BIOURGE which produces massive quantities of DPA (Ooyama *et al.*, 1960, 1961, 1962; Ooyama, 1961), stimulated us to study both DPA and lysine biosynthesis in this organism. Evidence has now been gathered to show (a) that DKP is present as a metabolite in *P. citreo-viride*, (b) that the synthesis of DPA proceeds nonenzymatically from DKP; which in turn probably results from enzymatic transformations of α-KAA; (c) that specifically labeled DAP can give rise to DPA via DKP as a result of enzymatic oxidative deamination, but that DAP is not present in this microorganism; and (d) that lysine biosynthesis in the *Penicillia* takes place by way of α-AAA in concordance with Vogel's (1959) phylogenetic scheme.

MATERIALS AND METHODS

Organism.—*P. citreo-viride*, strain 2383, was maintained on Czapek-Dox glucose slants and was grown in Erlenmeyer flasks on liquid medium containing, per liter of tap water, glucose, 100 g; $NaNO_3$, 20 g; K_2HPO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 1 g; and corn steep liquor, 1 ml. After 10 days of stationary culture at 24–27°, 10–12 g/liter of DPA was routinely obtained. Ooyama *et al.* (1961, 1962) have reported comparable yields from this strain and from the related *P. decumbens*.

Syntheses.—DKP (mp 126–128°; yield, 18%) was prepared from the diethylester of oxalacetate and formaldehyde (Cope and Fournier 1957). The bis-DNPH derivative appeared to melt at 185–187°; it then decomposed sharply at 218°.

Anal. Calcd for $C_{19}H_{16}O_{12}N_8$ (548.38): C, 41.63; H, 2.94; N, 20.45. Found: C, 41.41; H, 3.29; N, 20.72.

The synthesis of [1,7-^{14}C] DAP has been described previously (Finlayson and Simpson, 1961). The following changes in this procedure were undertaken to improve the yield and to separate the isomers: A mixture of 4.6 g (20 mmoles) of pentamethylene dibromide, 2.6 g (40 mmoles) of KCN carrier, 15.3 mg of K^{14}CN (2.0 mc), 8 ml of water, and 2 ml methanol was refluxed for 2 days with stirring (Loftfield, 1951). The yield of [1,7-^{14}C]pimelic acid, mp 100–101°, was 2.5 g (80%; 57% radiochemical recovery). This material was diluted with 10.5 g unlabeled pimelic acid and was recrystallized from ether–petroleum ether to give 12.5 g (1.14 mc total activity, 16.7 μc/mmole specific activity). The diacid was treated with 25 g of thionyl chloride and then with bromine (30 g), according to the method of Schotte (1952). Solution in 20 ml hot 98% formic acid followed by standing in the cold for 2 days gave crude crystals of *meso*-α,α'-dibromopimelic acid, 8.0 g, mp 137–145°. These crystals were shaken for 5 minutes with 100 ml ice-cold chloroform and filtered. The residue was recrystallized from 98% formic acid (Schotte, 1952). Purified *meso*-α,α'-dibromopimelic acid, 7.5 g (31%), melted at 152°. The formic acid mother liquors and the chloroform extract were combined and evaporated under reduced pressure, and the residue was crystallized from toluene and then from formic acid. From this was obtained 3.3 g (14%), mp 88–90°, of DL-α,α'-dibromopimelic acid.

The *meso* isomer of dibromopimelic acid was transformed to the diethyl ester via the diacid chloride using thionyl chloride and absolute ethanol. The yield of *meso*-diethyldibromo [1,7-^{14}C]pimelate, bp 141–145°/0.5 mm, was 7.0 g. This intermediate was treated under reflux overnight with 2.0 g of NaN_3 in 40 ml absolute ethanol. After addition of water to the intermediate product and extraction into ether, the extract was dried, evaporated *in vacuo*, and dissolved in 20 ml dry benzene. This solution was refluxed with 9.0 g triphenylphosphine for 5 hours (Lingens, 1960). Following evaporation of benzene, the reaction mixture was hydrolyzed with equal parts of 48% HBr and glacial acetic acid. The hydrolysate was evaporated under reduced pressure, diluted in water, and neutralized with ammonia. *meso*-[1,7-^{14}C]-DAP was precipitated with ethanol, filtered, and reprecipitated twice more from aqueous ethanol to give 2.41 g (yield 9.5% from K^{14}CN) of final product, with a specific activity of 14.97 μc/mmole. This material showed only one ninhydrin-positive spot, corresponding to that for authentic DAP, when examined by paper electrophoresis (pyridine–acetic acid buffer, *p*H 3.5, 40 v/cm, for 1 hour). DL-[1,7-^{14}C]-DAP was prepared in analogous fashion from the racemic dibromodiethyl ester of pimelic acid. The final yield was 1.2 g (5.3% from K^{14}CN) with 16.64 μc/mmole specific activity. Other radiochemicals were commercial products. L-[^{15}N]glutamic acid was synthesized previously (Tanenbaum, 1956).

Lysine Degradation.—The degradation of lysine followed the procedure of Strassman and Weinhouse (1953). One and eight-tenths mmoles (328 mg) of diluted radioactive lysine, obtained from a mycelial mat grown on α-[6-^{14}C]-AAA, was oxidized with acid permanganate to give $BaCO_3$ in 119% yield. After removal of ions by treatment first with $Ba(OH)_2$ and then with CO_2, the neutralized solution was concentrated and was adjusted with HCl to 1.5 N. The acidified solution was chromatographed on a column (3 × 50 cm) of Dowex 50W × 8. Elution was continued with 1.5 N HCl and δ-aminovaleric acid was collected at 1.15–2.0 liters volume. The eluate was evaporated to dryness. The residue was dissolved in a small quantity of water and treated with charcoal, and δ-aminovaleric acid hydrochloride, mp 92–94° (63% yield), was precipitated by addition of acetone. Decarboxylation of 100 mg of δ-aminovaleric acid was effected by the Schmidt degradation to give an 85% yield of $BaCO_3$. The reaction mixture was oxidized with alkaline permanganate. After acidification, the resultant succinic acid was extracted into ether. It was then precipitated from water as the silver salt. On treatment with H_2S, 76 mg succinic acid 76%, mp 180–183°, was obtained. This product was then treated with NaN_3 and concd H_2SO_4 to give $BaCO_3$ (90%) and 40 mg (51%) of ethylenediamine-dihydrochloride.

Dipicolinic Acid Isolation.—Fermentation beers or replacement media were passed through a column of Dowex 1 × 8 (acetate form). The column was washed with 0.5 N acetic acid to remove sugars and other metabolites. The pyridine derivative was then desorbed from the column[2] with 1.0 N HCl, and its appearance was monitored by measurement of the absorbance of effluent fractions at 272 mμ (ϵ_m = 7250). Samples of radioactive DPA thus obtained were decarboxylated by heating in *p,p'*-biphenol with CuO at a bath temperature of 190–240°, under a stream of nitrogen. Pyridine was trapped either in saturated ethanolic picric acid or in a 0.1% solution of perchloric acid in ethyl acetate.

Chromatographic Procedures.—Thin-layer chromatography was carried out either with Silica Gel G or MN-Cellulose (Brinkmann) as the support. After spreading, the plates were dried at 110° for 3 hours and were

[2] This procedure follows that of Finlayson and Simpson (1961), except that it was found necessary to use stronger mineral acid to elute DPA from the column.

kept in a desiccator over $CaCl_2$. Basic amino acids were separated on MN-Cellulose with solvent (A); methanol–water–12 N HCl—pyridine, 80:17.5: 2.5:10 (v/v). Detection was with ninhydrin spray. In these experiments, lysine was detected at R_F 0.45 and histidine appeared at R_F 0.52. In the degradations of radioactive lysine, the solvent system was changed to butanol–acetic acid–water, 4:1:5 (v/v, solvent B). Lysine, δ-aminovaleric acid, and an unknown ninhydrin-positive degradation product were detected at R_F 0.15, 0.42, and 0.32, respectively. Keto-acid-DNPH's were separated on silica gel plates with acetic acid–benzene, 18:120 (v/v, solvent C) or with methanol–acetic acid–ether–benzene, 1:18:60:120, (v/v, solvent D). In the last-named solvent system, analytical samples of bis-DKP-DNPH always separated into two spots having R_F 0.30 and 0.23, respectively (see Fig. 2). Earlier attempts to resolve DNPH derivatives with paper chromatography used butanol–acetic acid–water, 4:1:5, and butanol saturated with 1 N NH_4OH. These solvent systems failed to resolve α-ketoglutaric acid–DNPH from DKP-DNPH.

Lysine Isolation.—Washed, wet mycelial pads were hydrolyzed by refluxing with 6 N HCl for 48 hours. Distillation several times under reduced pressure removed most of the acid. The resultant syrup was placed in a desiccator for several days over NaOH. The water-soluble portion of this residue was passed through a Dowex 50 × 8 column (2 × 47 cm) which had been equilibrated with 0.1 M citrate buffer, *p*H 5.0 (Hirs *et al.*, 1954). The chromatogram was developed with this buffer at an elution rate of 8–10 ml/hr, with collection of 4-ml fractions. This eluate contained the acidic and neutral amino acids. After tyrosine and phenylalanine were removed, the solvent was changed to 0.1 M bicarbonate buffer, *p*H 8.3, in order to obtain histidine. After the *p*H was raised to 9.2 with carbonate, the fraction containing lysine appeared. This fraction was concentrated, and lysine was isolated as the monohydrochloride, in the usual manner.

Keto-Acid Determinations.—Following their separation by thin-layer chromatography, DNPH's of keto acids were measured by the method of Cavallini *et al.* (1949). With bis-DKP-DNPH dissolved in 3 M Na_2CO_3, it was found that Beer's law was obeyed in the range 0.1–0.4 μmole at 470 mμ. All samples were therefore diluted with carbonate solution until such optical range was obtained, and concentrations were read from the standard curve.

Radioactivity Measurements.—All samples were combusted to CO_2 using the Van Slyke-Folch (1940) wet-oxidation method, and were plated in triplicate and counted as $BaCO_3$ at infinite thinness. Suitable corrections for self-absorption, coincidence, and counting efficiency were applied. Radioactivity of samples with high activity was determined in an apparatus with 6.5% efficiency, while radioactivity of those with low activities was measured in a windowless gas-flow counter at 32% efficiency. Using this procedure, radioactivity values were obtained for all samples to within 5% counting error.

Results

Replacement Experiments.—Ten-day-old mycelial pads which had been washed for 3 hours with cold running tap water prior to the addition of supplements were used. With DKP and N_4HCl in potassium phosphate buffer, it was noted (Table I) that more DPA was found in the presence of these substrates than in their absence. Although the endogenous formation of DPA from one mycelial pad to another was highly variable, the net synthesis remained fairly constant in a large number of experiments. In confirmation of the results of Powell and Strange (1959), it was also seen (Table I) that spontaneous chemical formation of DPA from DKP and NH_3 took place. It was further determined in a series of *p*H-dependency studies between 7.2 and 8.0 in phosphate buffer, that the spontaneous formation of DPA from DKP and NH_3 follows a bell-shaped curve, with a maximum at *p*H 7.5. Thus replacement experiments using NH_3 and DKP gave net synthesis of DPA which might have reflected either variables in maintanence of *p*H, or in concentration of metabolites with E_0' values close to those necessary for oxidation of the primary spontaneous condensation products to DPA.

In an attempt to depress the chemical formation of DPA in replacement cultures, organic amino donors such as glutamate, aspartate, glucosamine, glutamine, and lysine were separately incubated with DKP. The best net formations of DPA occurred when DKP and glucosamine, glutamate, or aspartate, in order, were used. In Figure 1 are presented results using glucos-

TABLE I

BIOSYNTHESIS OF DIPICOLINIC ACID FROM DIKETOPIMELATE AND AMMONIA[a]

Series	Supplements to Replacement Media	Dipicolinic Acid (mmoles) Found	Dipicolinic Acid (mmoles) Net "Enzymatic" Synthesis
(A)	10 mmoles DKP + 10 mmoles NH_4Cl	4.13	
	Same (no mycelium)	0.81	
	Mycelium (no supplements)	2.55	0.77
(B)	10 mmoles DKP + 10 mmoles NH_4Cl	2.34	
	Same (no mycelium)	0.86	
	Mycelium (no supplements)	0.78	0.70

[a] *P. citreo-viride* was grown in 500-ml Erlenmeyer flasks, each containing 100 ml medium, under cultural conditions given in the text. Mycelial mats weighing as close as possible to 10.0 g (*ca.* 1.0 g dry wt) were washed in running tap water for 3 hours and were replaced with the appropriate supplement in 100 ml 0.1 M phosphate buffer, *p*H 7.1. Incubation was carried out for 24 hours at 25°. Dipicolinic acid concentrations calculated from OD at 272 mμ.

TABLE II

REPLACEMENT EXPERIMENT WITH L-[^{15}N]GLUTAMATE

Supplements to Replacement Media[a]	Metabolite (mmoles) DPA	Metabolite (mmoles) NH_3[b]	Excess ^{15}N (atom %) DPA[c]	Excess ^{15}N (atom %) NH_3[d]
10 mM DKP + 10 mM L-[^{15}N]glutamate	2.21	0.97	0.006	0.043
10 mM L-[^{15}N]glutamate	0	3.77		0.068
No supplements (endogenous)	0	3.66		

[a] Replacement procedure and cultural conditions were the same as given in Table I except that mycelial mats were washed with tap water for 24 hours. [b] NH_3 determined on aliquot of replacement mixture by standard Kjeldahl distillation procedure. [c] DPA was isolated in this experiment by continuous ether extraction, followed by repeated water recrystallization. [d] We are indebted to I. Sucher and Dr. D. Rittenberg for the mass spectrophotometric ^{15}N analyses.

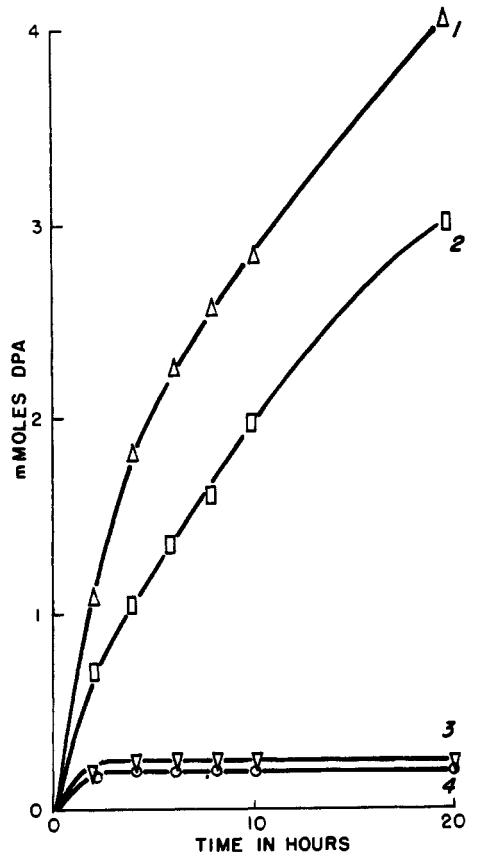

FIG. 1.—Biosynthesis of DPA from DKP glucosamine in replacement cultures. Curve 1, 10 mmoles each DKP and D-glucosamine in 1 × 10^{-3} M phosphate buffer–1 × 10^{-3} M $MgSO_4$–1 × 10^{-3} M fructose 1,6 diphosphate, *p*H 7.25; curve 2, 10 mmoles each DKP and D-glucosamine in tap water; curve 3, endogenous DPA formation; curve 4, no mycelium, reagent blank as constituted for curve 1. Mycelial mats weighing *ca.* 1 g (dry wt) were used.

amine as an enzymatic amino donor to DKP. It can be seen that the endogenous and chemical synthesis of DPA were negligible (curves 3 and 4). To further examine the mechanism of stimulation of DPA formation in these replacements, the extent of incorporation of the amino group of L-[^{15}N]glutamate was investigated. The results of this experiment are given in Table II. Following long-term washing of the mycelium the endogenous formation of DPA was essentially negligible. The presence of labeled glutamate and DKP together resulted in the formation 2.2 mmoles of DPA, and actually repressed the quantity of free NH_3 found. However, neither the DPA nor the NH_3 was labeled with ^{15}N to any extent. Increased synthesis of DPA from glutamate and DKP does not then involve immediate transfer of the amino group of the amino acid into the final product. It was decided therefore to investigate the occurrence and the metabolic pathway to DKP in this microorganism.

Evidence for DKP in P. citreo-viride.—After 9 days' growth on basal medium, the mycelium was removed, and 5 liters of the culture fluid was treated with 300 ml of Brady's solution. The DNPH's of the mixed carbonyl metabolites were collected by filtration and air dried. The washed, dried, harvested mycelium was then extracted with boiling 80% ethanol. After removal of ethanol *in vacuo*, the aqueous residue was also treated with an acid solution of 2,4-dinitrophenylhydrazine. The presence of bis-DKP-DNPH in both the fermentation beer and in the ethanolic mycelial extract was

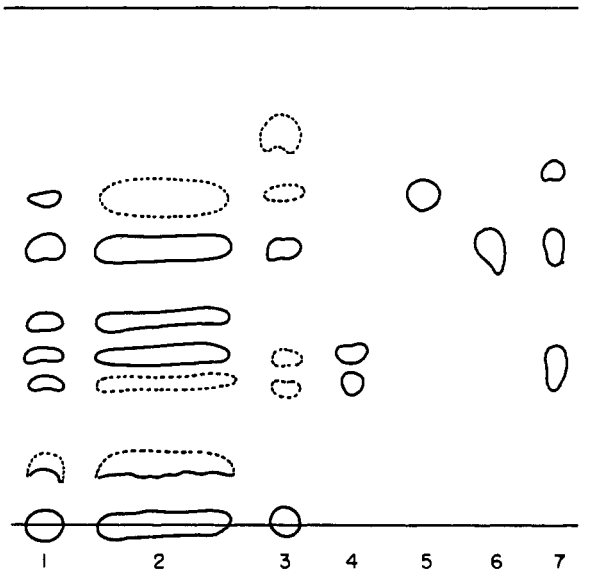

FIG. 2.—Thin-layer chromatography of DNPH derivatives in solvent D (see Experimental). Spot 1, DNPH's from fermentation beer; streak 2, same; spot 3, DNPH's from ethanolic extract of mycelium; spot 4, authentic bis-DKP-DNPH; spot 5, pyruvic acid–DNPH; spot 6, α-ketoglutaric acid–DNPH; spot 7, mixture of pyruvic acid, α-ketoglutaric acid, and DKP-DNPH's.

detected following thin-layer chromatography (solvent D) of these DNPH's on silica gel (Fig. 2). Purification of the bis-DKP-DNPH from the culture fluid of DNPH's was carried out as follows: After solution in ethyl acetate and extraction into 10% Na_2CO_3, the acidic DNPH's were precipitated with 3 N HCl. This mixture of DNPH derivatives was dissolved in acetic acid, insoluble material was removed by centrifugation, and benzene was added until the ratio of benzene to acetic acid was 120:18. Fifty g of silica gel was placed in a chromatographic column (3 × 70 cm). The solution of DNPH's was applied to the column, and development was continued with solvent (C). The bis-DKP-DNPH was eluted at 2.0–2.5 liters volume of column effluent. After removal of solvent at reduced pressure, a portion of the residue was examined by thin-layer chromatography with solvent (D) and was found to be contaminated with small amounts of α-ketoglutaric acid–DNPH and pyruvic acid DNPH. This fraction was again subjected to column chromatography. After stripping off solvents and recrystallization five times from ethanol, 60 mg of DKP–DNPH, mp 218° (decomp), mixed mp with authentic sample 217–218° decomp, was obtained. The infrared spectra (KBr disc) of these samples were identical. In addition, the isolated and authentic bis-DKP-DNPH's were catalytically reduced to DAP (atmospheric presure) with PtO_2 in acetic acid. The catalyst was removed, and the solvents were evaporated. After solution in 1 N HCl, the reaction mixtures were examined by paper chromatography using the solvent system and spray reagent of Rhuland *et al.* (1955). Isolated and synthetic DKP-DNPH's after reduction each gave identical ninhydrin-positive spots at R_F 0.14 (dark yellow-green), and R_F 0.19 (light yellow-green).

Incorporation of ^{14}C Precursors into DPA.—A series of experiments, using growing cultures of *P. citreo-viride* in minimal medium supplemented with radioactive presumptive precursors, is presented in Table III. A standardized procedure for growth of the microorganism and for recovery of DPA was used. Inspection of these data reveals that the best radiochemical

TABLE III
INCORPORATION OF ^{14}C-LABELED PRECURSORS INTO DIPICOLINIC ACID[a]

Expt	Precursor[b]	Total Activity in Final Growth Medium (μc)	Total Amount Isolated (mg)	Radioactivity, DPA	
				$BaCO_3$ (dpm/mg)	Initial Activity Incorporated (%)
1	[1-^{14}C]Acetate	5.0	195	292	0.21
2	[2-^{14}C]Acetate	7.0	206	554	0.43
3	[2-^{14}C]Pyruvate	28.2	187	3508	2.45
4	[3-^{14}C]Pyruvate	28.2	58	2508	0.55
5	[3-^{14}C]Propionate	35.0	237	4323	3.87
6	[1-^{14}C]Glycerol	23.2	255	1423	1.38
7	[2-^{14}C]Glycerol	26.9	206	1879	1.45
8	[4-^{14}C]Succinic acid	14.5	222	2409	2.01
9	[2,3-^{14}C]Succinic acid	33.7	176	5667	3.77
10	[1-^{14}C]Glycine	25.6	188	352	0.25
11	[2-^{14}C]Glycine	19.7	42	1155	0.14
12	[1-^{14}C]Alanine	13.1	227	1105	0.96
13	[3-^{14}C]Aspartic acid	37.3	242	3909	3.57
14	[4-^{14}C]Aspartic acid	23.1	135	2134	1.09
15	[1-^{14}C]Lysine	32.8	70	108	0.03
16	[^{14}C]Carbon dioxide	7.3	27	5385	0.55
17	*meso*-[1,7-^{14}C]Diaminopimelic acid		950	1100	3.9
18	DL-[1,7-^{14}C]diaminopimelic acid		975	985	3.7
19	DL-α-[6-^{14}C]aminoadipic acid	23.1	1060	323	1.28

[a] *P. citreo-viride* was grown for 12 days at 27–29° in 250-ml flasks containing 50 ml basal medium. In expts 17, 18, and 19, the mold was grown in 500-ml flasks containing 100 ml of basal medium. In all experiments except 16 the labeled supplement was added to the appropriate flask prior to sterilization. In expt 16 the mycelial mat was allowed to form on top of basal medium, and at 5 days' growth $^{14}CO_2$ was released into the air space, in a sealed system. [b] All ^{14}C-labeled precursors added at an initial radioactivity of 100 μcuries.

conversion to DPA were from those metabolites which are most closely integrated into the glyoxylate by-pass and to the tricarboxylic acid cycle, e.g., [3-^{14}C]propionate, [2,3-^{14}C]succinate, [3-^{14}C]aspartate, and [2-^{14}C]pyruvate. Those precursors which were tagged in positions capable of undergoing facile carboxylations-decarboxylations (e.g., [4-^{14}C]succinate, [1-^{14}C]acetate, [4-^{14}C]aspartate were incorporated into DPA to a lesser extent than were corresponding species which were marked in more metabolically stable carbons. The poorer isotopic donors included [1-^{14}C]alanine, carbon dioxide, acetate, and glycine. It was found that *meso*- and DL-[1,7-^{14}C]-DAP as well as α-[6-^{14}C]-AAA were not only among the best radioactive precursors of DPA; but that these substrates produced the only noteworthy quantitative increases in the amounts of DPA formed. In contrast, [1-^{14}C]lysine was neither incorporated into DPA nor provided any synergistic action for its production.

The radioactive DPA samples were diluted, and the per cent distribution of isotope into the molecule was determined by assay of the molar radioactivity in the carboxyl groups and in the ring (Table IV). The label from [4-^{14}C]aspartic acid (metabolically equivalent to labeled CO_2, see also Table VI) appeared to be equally distributed in the ring and carboxyl groups of DPA. Relatively less isotope was found in the carboxyl groups of DPA from [3-^{14}C]aspartic acid. Label from [2-^{14}C]pyruvate accounted for a somewhat higher proportion of carboxyl radioactivity than in the comparable experiment which used its 3-tagged species. [2,3-^{14}C]Succinate and [3-^{14}C]propionate supplied small considerable contributions also to the carboxyl groups of DPA; while [4-^{14}C]succinic acid gave rise to a considerable quantity of ring label. It is concluded from these results that these precursors were converted into DPA via reactions of the tricarboxylic acid pathway, and that transformations by way of methylmalonyl-CoA and crotonyl-CoA might account for the randomizations noted.

These hypotheses were substantiated by investigation of the fixation of CO_2 into DPA. In one set of experiments, comparison of HCO_3^- versus CO_2 as an isotopic donor was made. In the second series the effect of the presence of unlabeled propionate on the carbonate incorporation and distribution pattern was examined (Table V). Bicarbonate enhanced DPA production as compared with CO_2 in the gas phase. The presence of unlabeled propionate increased the per cent of initial activity found in DPA. On degradation of these DPA samples (Table VI) it was found, without propionate, that distribution of isotopic label was equal in the ring-carbon and carboxyl-carbon atoms of DPA; while where propionate was present approximately 75% of the radioactivity was found in the pyridine ring.

In Table VII are given the data for degradation of DPA samples obtained from cultures of *P. citreo-viride* which were grown in the presence of presumptive C_6 and C_7 precursors. Whereas DL-α-[6-^{14}C]aminoadipic acid gave rise to DPA which contained 25% of its total radioactivity in the aromatic ring portion, *meso*- and DL-[1,7-^{14}C]-DAP were converted, respectively, to DPA which was labeled exclusively in the carboxyl groups. Apparently these isomers were directly transformed into DPA, without any obligatory intermediary scission or metabolism. It was therefore surmised that amino acid oxidases capable of attacking both α-amino groups in this molecule might be present. This possibility was examined by testing the action of cell-free extracts on DAP and on other amino acids.

D- *and* L-*Amino Acid Oxidases.*—Crude cell-free enzyme extracts were prepared by following the previously published procedures of Horowitz (1944) and Thayer and Horowitz (1951), and of Knight (1948).

TABLE IV

DISTRIBUTION OF ^{14}C IN DIPICOLINIC ACID FROM C_3 AND C_4 PRECURSORS

Precursor and Resultant Products[a]	Expt (Table III)	Radioactivity $BaCO_3$ (dpm/mg)	Radioactivity DPA or Moiety (dpm/mmole $\times 10^{-5}$)	Activity (% and total)	
[3-^{14}C]Aspartic acid	13				
DPA		247	3.4	100	
Carboxyl groups[b]		101	0.4	12	91
Pyridine picrate		126	2.7	79	
[4-^{14}C]Aspartic acid	14				
DPA		412	5.7	100	
Carboxyl groups		587	2.3	40	77
Pyridine picrate		95	2.1	37	
[3-^{14}C]Pyruvate	4				
DPA		234	3.2	100	
Carboxyl groups		103	0.4	13	78
Pyridine picrate		97	2.1	65	
[2-^{14}C]Pyruvate	3				
DPA		353	4.9	100	
Carboxyl groups		310	1.2	24	89
Pyridine picrate		146	3.2	65	
[1-^{14}C]Alanine	12				
DPA		102	1.4	100	
Carboxyl groups		252	1.0	72	100
Pyridine picrate		18	0.4	28	
[3-^{14}C]Propionate	5				
DPA		208	2.9	100	
Carboxyl groups		176	0.7	24	90
Pyridine picrate		135	1.9	66	
[2,3-^{14}C]Succinic acid	9				
DPA		276	4.8	100	
Carboxyl groups		182	0.7	15	98
Pyridine picrate		157	4.0	83	
[4-^{14}C]Succinic acid	8				
DPA		460	6.4	100	
Carboxyl groups		1195	4.7	74	97
Pyridine picrate		85	1.9	23	

[a] Each sample of dipicolinic isolated was diluted 10 times prior to counting and degradation. [b] Total activities reported for carboxyl groups are based upon 2 moles produced per mole of dipicolinate decarboxylated.

TABLE V

INCORPORATION OF CO_2 AND HCO_3^- INTO DIPICOLINIC ACID[a]

Expt	^{14}C Source	Additions	Dipicolinic Acid: Amount Isolated (mg)	Dipicolinic Acid: Specific Activity (dpm/mg $BaCO_3$)	Dipicolinic Acid: Per Cent of Initial Activity
20	CO_2[b]	None	61	313	0.07
21		Propionate[e]	86	478	0.24
22	HCO_3[c]	None	425	17	0.03
23		Propionate[e]	292	460	0.93
24	HCO_3[d]	None	306	1186	1.3
25		Propionate[e]	587	1110	2.4

[a] 100 μc of radioactive precursor added in each experiment. [b] In experiments with $^{14}CO_2$, the gas was generated in a sealed system over mycelial mats on the fifth day of growth, and the system was kept as such until the 12th day. [c] In experiments with $H^{14}CO_3^-$, this metabolite was added in solution as the Na salt. Two-day mycelial mats received isotope; experiments were terminated on the 11th day. [d] Isotope was added on 4th day to mycelial mat; terminated on 11th day. Where propionate was incorporated into the medium, it was added in 1 ml solution as 100 mg of the Na salt simultaneously with the labeled precursor.

TABLE VI

DISTRIBUTION OF RADIOACTIVITY IN DIPICOLINIC ACID FROM [^{14}C]BICARBONATE

Expt	Sample Description	Radioactivity (dpm/mg $BaCO_3$)	Radioactivity (dpm/mM $\times 10^{-6}$)	Per Cent Activity in Fragment and Total	
24 (no propionate)	DPA	1186	1.64		
	DPA, carboxyl groups	2210	0.88	54	108
	DPA, pyridine perchlorate	890	0.88	54	
25 (with propionate)	DPA	1110	1.54		
	DPA, carboxyl groups	1030	0.41	27	105
	DPA, pyridine perchlorate	1220	1.20	78	

TABLE VII

DISTRIBUITION OF RADIOACTIVITY IN DIPICOLINIC ACID FROM C_6 AND C_7 PRECURSORS

Source	Sample	Radioactivity (dpm/mg $BaCO_3$)	Radioactivity (dpm/mmole $\times 10^{-6}$)	Per Cent Activity
meso-[1,7-^{14}C]-DAP	DPA	1100	1.54	100
	DPA carboxyls	4092	1.62	105
	DPA pyridine	0	0	0
DL-[1,7-^{14}C]-DAP	DPA	935	1.38	100
	DPA carboxyls	3845	1.52	110
	DPA pyridine	0	0	0
DL-α-[6-C^{14}]-aminoadipic acid	DPA	323	0.44	100
	DPA carboxyls	681	0.27	62
	DPA pyridine	115	0.11	25

Both methods gave extracts with essentially similar properties. Results with the crude enzymes made by grinding with sand are presented in Table VIII. In experiments with DL-, L-, and D-phenylalanine it was observed that both isomers were oxidatively attacked. In both undialyzed and dialyzed preparations it was shown that oxidation beyond the stage of deamination of the parental compounds occurred. In the experiments with DAP isomers, the oxidation mixtures were divided in half. To one portion an additional 10 μmoles of NH_4Cl was added. This aliquot was allowed to incubate at room temperature overnight, while the first portion was immediately analyzed. In both portions, DPA was estimated spectrophotometrically, while DKP was determined after precipitation of the DNPH's and their separation by thin-layer chromatography, as outlined under Materials and Methods. The results in Table VIII are expressed in terms of μmoles product per original volume in the Warburg flasks. Oxidation of 10 μmoles each of *meso*- and DL-DAP consumed *ca.* 5 μmoles of O_2, and an average of 4.5 μmoles of DKP was produced. Subsequent to incubation with a 2-fold excess of ammonia, the DPA concentration of these extracts rose, while the amount of DKP diminished to barely detectable quantities. These experiments suggested that increased formation of DPA from DAP in growing cultures was probably due to enzymatic oxidative deamination to DKP, followed by a comparable series of nonenzymatic reactions with ammonium ion.

Failure to Detect DAP in P. citreo-viride.—Since the

TABLE VIII
DEMONSTRATION OF D- AND L-AMINO ACID OXIDASES

Extract[a]	Substrate	O_2 Uptake (μmoles)	Subsequent Addition[b]	DPA[c] (μmoles)	DKP[d] (μmoles)
Undialyzed	None	0.1	None	0.3	0.3
			NH_4Cl	0.3	[e]
	DL-DAP	6.3	None	1.7	4.1
			NH_4Cl	2.7	[e]
	DL-Phenylalanine	6.7			
	D-Phenylalanine	7.1			
	L-Phenylalanine	7.0			
Dialyzed	None	0.1	None	0.1	0.2
			NH_4Cl	0.2	[e]
	DL-DAP	6.1	None	1.4	5.3
			NH_4Cl	3.2	[e]
	meso-DAP	5.3	None	2.5	4.5
			NH_4Cl	2.9	[e]

[a] Extracts prepared by sand-grinding 5-day-old mycelia, which had been exhaustively washed with water. Protein concentration was *ca.* 4–7 mg/ml. Warburg flasks contained 1.5 ml cell-free extract and 6.6 mM pyrophosphate buffer (*p*H 8.3) in the main compartments, 0.1 ml 20% NaOH in the center wells, and 10 μmoles of substrate dissolved in 0.2 ml buffer in the side arms. Bath temperature was 30°. [b] After oxygen uptake, the reaction mixture was diluted with 2.0 ml water, and 10 μmoles NH_4Cl was added to an aliquot. After 24-hour incubation at 25°, Brady's solution was added and the DNPH derivatives were collected and separated by thin-layer chromatography. [c] DPA concentration determined spectrophotometrically in aliquot after deproteinization of reaction mixture. [d] DKP concentrations measured by OD determination on alkaline solutions of the bis-DNPH at 470 mμ. The DKP-bis-DNPH was separated from other keto-acid-DNPH's by chromatography on silica (see text). [e] No DKP-bis-DNP could be detected after thin-layer chromatography.

enzymatic potential of this microorganism was demonstrated for oxidation of DAP at both amino groups with concomitant DKP formation, it became of some interest to ascertain whether or not the mycelium contained DAP. Acid hydrolysates and hot 80% ethanolic extracts of the mold mycelium were examined by paper chromatography (Rhuland *et al.*, 1955) with inconclusive results for the presence of DAP. Further examination of the mycelial hydrolysate using an automatic amino acid analyzer (Piez and Morris, 1960) was then carried out. In preliminary trials, authentic DAP emerged from the column at an elution volume of 385 ml to peak (between leucine and isoleucine), when added to a standard mixture. No such peak was found in the mold hydrolysate.

Pathway of Lysine Biosynthesis.—Inasmuch as DAP is not a constituent of *P. citreo-viride*, and in light of the good incorporation of labeled α-AAA (Table VII) into lysine, it appeared that this latter amino acid might be formed from α-AAA, as in the pathway for yeast (Strassman and Weinhouse, 1953). Since none of the members of the *Penicillia* has been examined in this regard, lysine samples were isolated from several pertinent radioactive mycelia. The results are tabulated in Table IX. [3-^{14}C]Propionate is second to best α-AAA as a radioactive precursor of lysine (Table IX), which indicates that there is a biogenetic connection between the formation of α-AAA and the propionate-methylmalonate-succinate series of metabolites. Degradation experiments then showed that α-[6-^{14}C]-AAA was converted to lysine, without redistribution of radioactivity (Table X). It is clear that the pathway to lysine in *P. citreo-viride* is indeed by way of α-AAA, rather than from DAP.

TABLE IX
BIOSYNTHESIS OF LYSINE FROM SEVERAL LABELED COMPOUNDS

Expt (Table III)	Sample Description	Mycelium Dry Wt (g)	Lysine Isolated (mg)	Specific Activity (dpm/mg $BaCO_3$)
13	[3-^{14}C]Aspartic acid	1.5	8.7	1,105
14	[4-^{14}C]Aspartic acid	1.3	5.3	14
5	[3-^{14}C]Propionate	2.0	7.9	10,000
9	[2,3-^{14}C]Succinic acid	1.7	8.2	2,470
3	[2-^{14}C]Pyruvate	1.3	4.9	1,260
4	[3-^{14}C]Pyruvate	0.5	2.5	936
12	[1-^{14}C]Alanine	1.1	3.0	6
17	*meso*-[1,7-^{14}C]Diaminopimelic acid	1.9	9.3	0
18	DL-[1,7-^{14}C]Diaminopimelic acid	2.3	10.5	0
19	α-[6-^{14}C]Aminoadipic acid	3.1	20.1	47,500

TABLE X
DISTRIBUTION OF RADIOACTIVITY IN LYSINE FROM α-[6-^{14}C]NH_2-ADIPIC ACID

Compound	Specific Radioactivity (dpm/mg $BaCO_3$)	(dpm/mmole $\times 10^{-6}$)	Total Activity (%)
Lysine-HCl	2894	3.44	100
Carboxyl group of lysine	95	0.02	0.55
δ-NH_2-valeric acid-HCl	3530	3.50	101.5
Carboxyl group of δ-NH_2-valeric acid	0	0	0
Succinic acid	4230	3.35	97.3
Carboxyl group of succinic acid	8700	3.46	100.6
Ethylenediamine-HCl	0	0	0

DISCUSSION

The discovery (Ooyama, 1961) that the biosynthesis of DPA is not unique to the aerobic (Powell, 1953) and anaerobic (Halvorson, 1961) spore-forming bacteria, and the availability of this DPA-producing strain of *Penicillium* allows us to extend and modify some of the earlier concepts concerning the formation of this pyridine derivative. It was concluded by Martin and Foster (1958) that the biogenesis of DPA in *B. megaterium* involves the entrance of tricarboxylic acid pathway precursors into a sequence of reactions which ultimately provide a nitrogen-containing C_7 precursor.

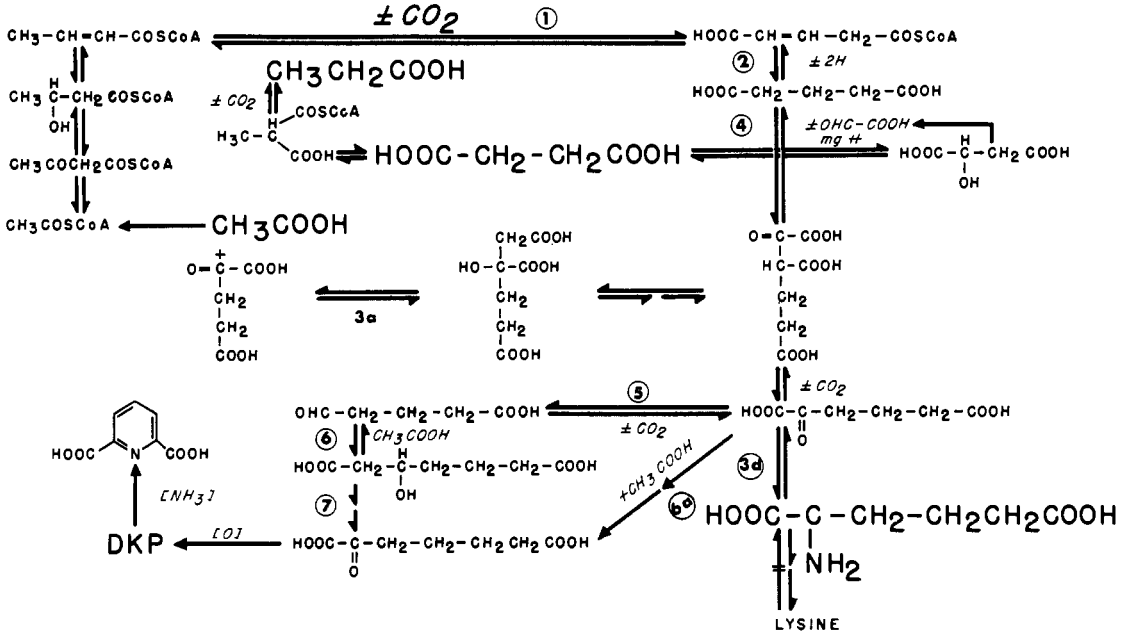

FIG. 3.—Proposed metabolic pathways for DPA and lysine biosynthesis in *P. citreo-viride*.

The involvement of DAP as a possible candidate for this precursor was proposed by Perry and Foster (1955), and was later reinvestigated by Finlayson and Simpson (1961). These workers showed that [1,7-^{14}C]-DAP introduced into growing cultures of *B. megaterium* gave rise to DPA whose specific activity was little higher than that of isolated glutamic acid or of alanine, but that the specific activity of lysine in this experiment was relatively high. Along another line of approach, the possible role of DKP in the formation of DPA was brought out by Powell and Strange (1959), who were able to demonstrate a spontaneous reaction between this diketo acid and ammonia. This chemical synthesis of DPA was found to be enhanced either by cell-free extracts or by quinones. Although DKP could not be detected in the crude *B. megaterium* enzyme extract, its concentration after addition was rapidly diminished by some indeterminate series of chemical or enzymatic changes. Thus its position as a biological metabolite remained equivocal.

In the present work, experiments with *P. citreo-viride* supplemented with [1,7-^{14}C]-DAP demonstrated that this compound was one of the most efficient isotopic precursors of DPA, and that the label appeared exclusively in the carboxyl groups. It was then shown with crude cell-free extracts that there exist oxidative deaminases for *meso-* and for DL-DAP. In contrast to the observations with *Neurospora* (Work, 1955), the oxygen uptake was two, rather than one, atoms per mole of *meso*-DAP, and an almost theoretical quantity of DKP was found. Furthermore, the chromatographic detection and the isolation of DKP–DNPH from *P. citreo-viride* grown in minimal medium provides evidence for the participation of the diketo acid in the normal intermediary metabolism of this microorganism.

The results of these experiments point to the fact that the biogenesis of DPA is a function of DKP formation in *P. citreo-viride*. This conclusion was further reinforced by replacement experiments, which showed that such compounds as glutamine and glucosamine, each with DKP, gave rise to DPA. It is still not yet possible to divorce the spontaneous chemical synthesis of DPA from DKP and ammonia from presumptive enzymatic processes. The conversion of organic amino donors must in some biological fashion furnish or spare ammonia for combination with DKP; whether enzymatic steps are adjunct to the oxidative stages of spontaneous cyclization process remains obscure. The finding that the conversion of DKP into DPA with ammonium chloride is *p*H dependent, with a peak at neutrality, might be further explored for an insight into the mechanism of the chemical transformation.

It is possible to obtain some inkling as to the biological formation of DKP itself from examination of the data for incorporation of isotopic precursors into DPA. The data presented in Tables III to VI support the suggestion of Martin and Foster (1958) that tricarboxylic acid–cycle components are intimately related to DPA biosynthesis. The isotope-distribution pattern into the ring and into the carboxyl groups of DPA from specifically labeled species of aspartic acid, from [1-^{14}C]-alanine and $^{14}CO_2$, reveal possibly parallel pathways for *B. megaterium* and *P. citreo-viride*.

With regard to lysine biosynthesis in *P. citreo-viride*, it was shown first, using radioactive DAP as substrate, that mycelial lysine was devoid of isotopic label; and second, that DAP could not be detected in an acid hydrolysate of the normal mycelium. It is therefore concluded from these results and those for the labeling pattern in lysine derived from the mold grown with α-[6-^{14}C]-AAA that the pathway for lysine biogenesis is from α-AA-A, as is the case for yeast (Strassman and Weinhouse, 1953; Larson *et al.*, 1963), and for *Neurospora* (Mitchell and Houlahan, 1948; Windsor, 1951). This finding is also in accord with Vogel's (1959) hypothesis concerning the relationship between pathway of lysine formation and phylogenetic classification of microorganisms.

Consideration of these data together with the evidence for DKP as a normal metabolite suggests that the following series of metabolic reactions (Fig. 3) may account for lysine and DPA biosynthesis in *P. citreo-viride*. Reactions (1) and (2) have been studied by Tustanoff and Stern (1960, 1963). The intermediary formation of glutaric acid by this pathway provides a mechanism for the incorporation of labeled CO_2 (or of [1-^{14}C]acetate) into the ring carbons of DPA. Because of its symmetry, the involvement of glutarate also offers a basis for randomization of isotope into the final products. Reactions (3a) and those subsequent to α-KAA have previously been proposed by Strassman and

Weinhouse (1953) for the biogenesis of lysine in yeast. Reaction (5) has been demonstrated by Sagisaka and Shimura (1960). It has also been shown by Broquist and Stiffey (1959) that an enzyme system exists in yeast which is capable of transaminating α-ketoadipic acid with glutamic acid to yield α-aminoadipic acid (reaction 3d). The condensation of glyoxylate with glutarate to oxaloglutaric acid (reaction 4, analogous to the isocitratase reaction) is postulated in order to provide an alternative point of entrance for tricarboxylic acid and glyoxylate-cycle intermediates and to provide a mechanism for the distribution of isotope from α-[6-^{14}C]-AAA into both ring and carboxyls of DPA. Reactions (6–7) or (6a) are postulated in order to explain appearance of the isotopic carboxyl of α-KAA into α-ketopimelic acid, and thence into DKP. This series of steps is also compatible with the demonstration (Halvorson, 1961; Gollakota and Halvorson, 1963) that pimelic and α-ketopimelic acids as well as tricarboxylic acid intermediates reversed the inhibitory effect of α-picolinic acid on sporulation and DPA production in *B. cereus* T. As outlined in Figure 3, the biosynthesis of DPA and of lysine would be branch-points of the metabolism of α-ketoadipic acid, which in turn results ultimately from fatty acid intermediates and the products of terminal respiration. This series of reactions might also plausibly explain the fact that although L-[^{15}N]glutamic acid stimulated formation of DPA, none of the stable isotope was transferred into the final product. Transamination of the ^{15}N-labeled glutamate could have provided additional α-ketoglutarate, and in turn more α-ketoadipate and thus DKP. The latter may then have reacted with an unknown specific amino donor. In contrast, Gross *et al.* (1963), in a study of nicotinic acid biosynthesis in *Mycobacterium tuberculosis*, found that aspartic acid can donate three of its carbons together with the amino group to the heterocyclic ring.

The reactions depicted in Figure 3 are consonant with the finding that, in the presence of unlabeled propionate, DPA from radioactive CO_2 achieves a higher ratio of ring-to-carboxyl labeling. An alternative pathway to DPA via Δ′-piperideine-6- (or -2-) carboxylic acid (Aspen and Meister, 1962; Hasse *et al.*, 1962; Larson *et al.*, 1963), can also be postulated. This mechanism is unlikely, in light of the finding that [1-^{14}C]lysine was not incorporated into DPA.

Whether the biosynthesis of DPA from DKP occurs also among the spore-forming bacteria is open to question. The evidence from earlier investigations in no way conflicts with this pathway. It remains to be seen whether DKP can be detected as a true intermediate in the eubacteria by more refined techniques. Another pertinent question is whether cyclization of 1,5-dicarbonyl intermediates is a more general mechanism for the biogenesis of the pyridine ring. The recent experiments of Leete (1963) dealing with coniine biogenesis from four acetate units may bear directly on this point.

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