JOURNAL OF BACTERIOLOGY, Feb., 1966
Vol. 91, No. 2
Copyright © 1966 American Society for Microbiology
Printed in U.S.A.

Chemical Degradation of Dipicolinic Acid-C^{14} and Its Application in Biosynthesis by *Penicillium citreo-viride*

MATSUO KANIE,[1] SHIGEO FUJIMOTO, AND J. W. FOSTER

Laboratories of Applied Microbiology, Faculty of Agriculture, Kagoshima University, Kagoshima, Japan, and Department of Microbiology, The University of Texas, Austin, Texas

Received for publication 7 September 1965

ABSTRACT

KANIE, MATUSO (Kagoshima University, Kagoshima, Japan), SHIGEO FUJIMOTO, AND J. W. FOSTER. Chemical degradation of dipicolinic acid-C^{14} and its application to biosynthesis by *Penicillium citreo-viride*. J. Bacteriol. 91:570–577. 1966.—A chemical degradation of dipicolinic acid-C^{14} has been worked out, enabling determination of the specific radioactivity of the carboxyl-carbons (carbons-7 and -8), and of the following carbons of the pyridine ring: carbons-2 and -6 combined, carbons-3 and -5 combined, and carbon-4. The degradation was applied to dipicolinic acid synthesized by washed, submerged mycelium of the mold from glucose and $C^{14}O_2$, and from glucose-*1*-C^{14}, -*2*-C^{14}, and -*6*-C^{14}. The distribution of radioactivity within the labeled dipicolinic acids is consistent with operation of respiratory cycles and with the incorporation of one molecule of CO_2 in the pyridine acid. A C_3 compound is a primary building block. The C_7 chain is believed to result from a C_3 plus C_4 condensation, pyruvic acid and aspartic acid β-semialdehyde being proposed as likely precursors. Other aspects of the biosynthesis of C_7 open-chain compounds and of dipicolinic acid are discussed.

Experiments on the mechanism of biosynthesis of dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA) from C^{14}-labeled precursors (*see* preceding paper for a review of the literature), although yielding much information of value, almost invariably are seriously limited by the lack of information on the distribution of the incorporated C^{14} in the various carbon atoms of the heterocyclic compound. As it stands, only the average specific radioactivities of the two carboxyl-carbons and the five pyridine-carbons have been obtained (5, 7, 16). In consequence, though instructive in some important features, the radioactivity experiments on DPA biosynthesis suffer from the restricted interpretations that are the only ones possible; they entail a disproportionate degree of conjecture. The symmetry of the DPA molecule forestalls the possibility of obtaining the specific radioactivity of each carbon atom separately. However, the procedure described here represents a considerable advance toward the objective of labeling experiments by enabling a determination of the specific radioactivity of the carbon-4 of the pyridine ring, of the carbons-2 and -6 combined, and of the carbons-3 and -5 combined. Carbons-7 and -8 combined, the carboxyl-carbons, are obtained as before (7). Application of the procedure to some typical, labeled precursor experiments is also described in the present paper.

[1] Fulbright Fellow at The University of Texas, 1962–1963.

MATERIALS AND METHODS

Microbiological. The DPA-producing *Penicillium citreo-viride* 2383 was kindly furnished by J. Ooyama, Fermentation Research Institute, Inage, Chiba City, Japan. Growth and washed mycelial suspension procedures were the same as described in the accompanying paper (5).

Chemical. DPA and carbohydrate in the culture medium were respectively determined by the methods of Janssen, Lund, and Anderson (6) and Mokrasch (8). Respiratory CO_2 from closed culture systems was absorbed by 1 N NaOH after addition of HCl to the medium. The carbonate was precipitated as $BaCO_3$, which was filtered and successively washed with water, ethyl alcohol, and diethyl ether. The DPA used for degradation was extracted from the culture filtrates with diethyl ether and was recrystallized from water to constant specific radioactivity (mp, 232 C). Radioactivity measurements were made with a thin-window

Geiger-Müller tube and a Tracerlab "64" scaler. They were corrected for self-absorption when necessary, and also for the dilution resulting from addition of unlabeled carrier compounds at various stages in the degradation.

RESULTS

Specific radioactivities. The overall procedure followed is outlined in Fig. 1. The specific radioactivity of carbons-7 and -8 combined, and of carbons-2 and -6 combined, is obtained in reactions I and II, respectively. The specific radioactivity of carbons-3 and -5 combined is calculated as the difference in the specific activities of the CO_2 liberated in reactions II and III, respectively. The specific radioactivity of carbon-4 is calculated as the difference between the total radioactivity of the succinic acid carbon (Fig. 1) and that portion contributed by carbons-2 and -6, and -3 and -5.

Decarboxylation of DPA (*13*). A 200-g amount of DPA in 1 ml of quinoline was heated with 0.25 g of Cu in a silicone oil bath at 230 to 250 C. The reaction vessel was flushed continuously with a stream of O_2- and CO_2-free nitrogen gas for 30 min. The decomposition products, pyridine and CO_2, were swept out, and the pyridine was precipitated as pyridine picrate by passage of the effluent gas through 40 ml of a saturated solution of picric acid in a 100-ml Erlenmeyer flask. The crystalline pyridine picrate was recovered by filtration on a Büchner funnel and was washed with water (yield, 75%; mp, 162 to 165 C). The gas stream next was passed serially through three test tubes containing, respectively, 20, 10, and 10 ml of 0.5 N NaOH. The absorbed CO_2 was converted to $BaCO_3$ by adding a saturated solution of $BaCl_2$. The precipitate was filtered and washed with water, 95% ethyl alcohol, and diethyl ether (yield, 100%).

Hydrogenation of pyridine (*4*). The pyridine liberated from 1 g of pyridine picrate by addition of 7.5 N NaOH was steam-distilled into 5 ml of 2 N HCl, and the pyridine HCl solution was concentrated in vacuo. The concentrate was made alkaline with concentrated NaOH and was extracted with diethyl ether. The ether extract was dehydrated with anhydrous K_2CO_3, and dry HCl gas was passed through it. The residue obtained by evaporation of the ether was dissolved in 6 ml of absolute ethyl alcohol. Platinum oxide (30 mg) was added, and hydrogenation was effected by H_2 during mixing with a magnetic stirrer for 5 to 7 hr at room temperature. The absorption of H_2 equaled the theoretical. The excess H_2 was removed by suction and the PtO by filtration. Upon evaporation of the alcohol, crystals of piperidine

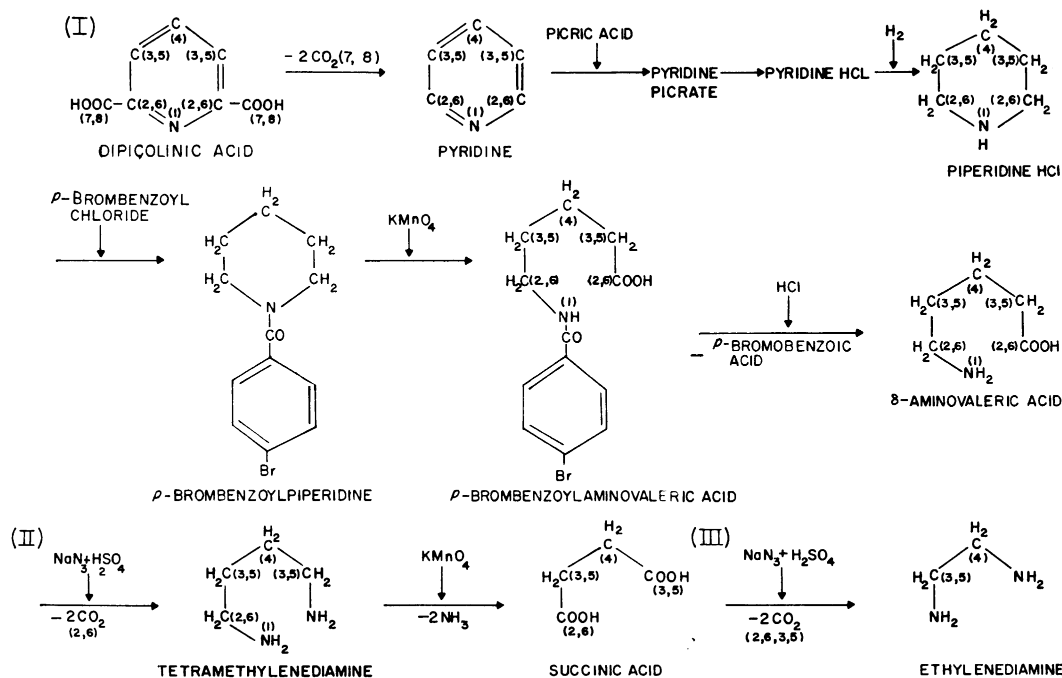

FIG. 1. *Degradation sequence employed to obtain the specific radioactivity of various carbon atoms of dipicolinic acid-C^{14}.*

hydrochloride were recovered by filtration (mp, 242 C).

Synthesis of p-brombenzoylpiperidine (14). To 1 g of piperidine HCl in a three-neck bottle in ice were added 1.13 g of KOH in 2 ml of water, and then 30 ml of diethyl ether. Anhydrous ether (45 ml) containing 2.8 g of *p*-brombenzoylchloride was added dropwise during a 3-hr period with continuous stirring in an ice bath. The ether layer was separated, washed with H_2SO_4 solution and then with water, and was dehydrated with K_2CO_3. After evaporation of the ether, the residue was taken up in 4 ml of chloroform from which crystals of *p*-brombenzoylpiperidine were formed as the chloroform was removed under reduced pressure (yield, 80%; mp, 94 to 95 C).

Conversion to p-brombenzoylaminovaleric acid. To 1.5 g of *p*-brombenzoylpiperidine suspended in 300 ml of water, 100 ml of aqueous 1.25% $KMnO_4$ was added dropwise with continuous stirring while refluxing on a boiling-water bath. After 6 hr of reaction, the color of the $KMnO_4$ disappeared. After cooling, the MnO_2 was filtered off, and the filtrate was made alkaline with dilute NaOH and washed with diethyl ether. Upon acidification with dilute H_2SO_4, crystals of *p*-bromobenzoylaminovaleric acid were obtained. They were recrystallized from 95% ethyl alcohol (yield, 65 to 70%; mp, 179 to 181 C).

Hydrolysis to δ-aminovaleric acid hydrochloride (1). A 1-g amount of *p*-brombenzoylaminovaleric acid and 20 ml of 37.5% HCl were heated in a sealed tube for 36 hr at 100 C. Water (20 ml) was added to the cooled solution, and the *p*-brombenzoic acid was extracted with diethyl ether. The remaining aqueous solution was concentrated in vacuo to a small volume, and then made up to 20 ml so that the resulting solution was 2 N with respect to HCl. This solution was then chromatographed on a column (1.9 by 30 cm) of Amberlite IR-120 resin and eluted with 2 N HCl (70 to 80 ml/hr). The eluate was collected in 20-ml fractions, each of which was tested on paper chromatograms developed with water-saturated phenol. δ-Aminovaleric acid was recognized in tubes 7 to 20. They were pooled and evaporated to dryness under reduced pressure. The residue was taken up in absolute ethyl alcohol, and the δ-aminovaleric acid hydrochloride was crystallized by addition of diethyl ether (yield, 60%; mp, 92 to 93 C).

Decarboxylation of δ-aminovaleric acid (9, 15). An amount of 197 mg of δ-aminovaleric acid hydrochloride was dissolved in 0.6 ml of cold, 38 N H_2SO_4 in a 25-ml flask and warmed in vacuo to remove the HCl. To the cooled solution, 0.6 ml of chloroform was added; then 117 mg of NaN_3 was added, in small portions, with continuous stirring, in an ice bath. The flask was then connected to four tubes in series, the first containing 15 ml of 5% $KMnO_4$ in 1 N H_2SO_4, and the remaining three, 30 ml of 0.5 N NaOH. After all of the NaN_3 had been added, the flask was held at room temperature for 15 hr and then at 55 C for 5 hr. The dissolved CO_2 was recovered as $BaCO_3$ in the usual way (yield, 100%). The reaction mixture in the flask was diluted with water, and then was made alkaline by addition of NaOH and extracted continuously for 72 hr with diethyl ether in a liquid-liquid extractor. The ether was evaporated, and the residue was acidified with HCl and again evaporated to dryness. The resulting crystals of tetramethylenediamine dihydrochloride were dissolved in water and recrystallized by the addition of ethyl alcohol (yield, 64.5%).

Conversion to succinic acid (10). An amount of 130 mg of tetramethylenediamine dihydrochloride was dissolved in 25 ml of water and neutralized with NaOH solution to *p*H 10.0; 15 ml of 1.5 N $KMnO_4$ was added, and the reaction solution was held at 55 C for 4 hr. It was then acidified with 45 ml of 5 N H_2SO_4 and extracted continuously for 48 hr with diethyl ether. The ether was evaporated, and the residue was taken up in acetone. Some insoluble matter was discarded. The residue obtained after evaporation of the acetone was dissolved in 15 ml of water and neutralized with dilute NH_4OH. Silver succinate was precipated by the addition of 3 ml of 10% $AgNO_3$. The precipitate was filtered and suspended in hot water, and H_2S was passed through the suspension. The AgS was filtered off and the filtrate was evaporated in vacuo, whereupon succinic acid crystallized (yield, 87%; mp, 185 C).

Decarboxylation of succinic acid (10). An amount of 60 mg of succinic acid was dissolved in 0.6 ml of ice-cold 38 N H_2SO_4; 0.6 ml of chloroform was added, followed by 100 mg of NaN_3, in small portions, with stirring in an ice bath. The reaction flask was attached to a series of tubes containing NaOH to trap CO_2, as described above. A slow stream of CO_2-free nitrogen was passed through the system, first held at room temperature for 15 hr and then at 55 C for 3 hr. $BaCO_3$ was recovered in the usual way (yield, 92.5%). The reaction mixture was made alkaline to phenol red and distilled in vacuo into picric acid. When almost all of the water had been distilled, 25 ml of xylene was added, and the distillation was continued. This procedure distilled all of the ethylenediamine. The picric acid distillate was concentrated in vacuo to dryness, and the residue was washed with diethyl ether, yielding ethylenediamine dipicrate, which was recrystallized from water (yield, 75%; mp, 232 to 234 C).

Total decomposition of ethylenediamine dipic-

TABLE 1. $C^{14}O_2$ *incorporation in DPA by washed mycelium of Penicillium citreo-viride**

Expt†	CO_2						DPA			CO_2-C incorporated in DPA	
	Initial		Final			Avg‡ count per min per mmole					
	Count/min	Count per min per mmole	Amt (mmoles)	Count/min	Count per min per mmole		Amt (mmoles)	Count/min	Initial $C^{14}O_2$ fixed (%)	Count per min per mole	Amt (mmoles/mole)
1	5,267	1,050	8.85	3,970	449	750	0.68	561	10.7	823	1.10
2	2,668	238	20.1	2,500	124	181	0.71	136	5.1	192	1.06

* All counts per minute are $\times 10^3$.

† Experiment 1: 8 g of mycelium (wet weight) shaken for 6 hr in a 4-liter flask containing glucose, 150 mg; urea, 100 mg; KH_2PO_4, 750 mg; distilled water, 150 ml; CO_2, 3% (v/v) of gas space. Experiment 2: 2 g of mycelium (wet weight) shaken for 12 hr in a 4-liter flask containing glucose, 1.5 g; urea, 170 mg; KH_2PO_4, 250 mg; distilled water, 50 ml; CO_2, 5% (v/v) of gas space.

‡ Calculated from the radioactivities of the initial and the final CO_2.

rate. An amount of 40 mg was decomposed by means of the van Slyke-Folch (17) oxidation mixture, and the CO_2 was recovered as $BaCO_3$ in the usual manner.

$C^{14}O_2$ incorporation in DPA by P. citreo-viride. The results of two experiments (Table 1) in essence confirm those obtained independently with a different strain of the same mold (5), and they likewise resemble the results with bacteria (7). In these two experiments, the mold fixed in DPA 10.7 and 5.1% of the $C^{14}O_2$ initially supplied. In view of the appreciable amount of DPA produced by the fungus, it is apparent that DPA biosynthesis involves a bulk fixation of CO_2, in spite of the net production of CO_2 during the process. In experiments parallel to those reported in Table 1, 3.83 and 8.9 mmoles of CO_2 were produced. The production of metabolic CO_2 under these conditions was found to be essentially linear with time. Hence, the average specific radioactivity of the CO_2 in the system was computed as the mean of the initial and the final values. The last column in Table 1 indicates that about 1 mole of CO_2 was used in the biosynthesis of each mole of DPA.

A very large fraction (86%) of the CO_2-derived label in the DPA was located in the carboxyl and carbon-4 positions (Table 2). The relatively low radioactivity in carbons-2 and -6 and carbons-3 and -5 indicates that they became labeled indirectly and secondarily and do not figure in the primary labeling pattern. The prominent labeling in carbon-4 and in the carboxyl-carbons suggests that these were derived from precursors in relatively close equilibrium with CO_2. In view of the carboxyl labeling, it is likely that carbon-4 also was derived from a carboxyl group in metabolic equilibration with carboxyl-carbons 7 or 8, or both. The most likely intermediate precursor candidate, which is consistent with all prior studies with labeled precursors of DPA (5, 7, 16), is oxaloacetic acid. This acid, or an aspartyl compound derived from it, was believed by Martin and Foster (7) to contribute four of the seven carbons of the DPA molecule. According to Bach and Gilvarg (Federation Proc. **23**:313, 1964), the actual precursor is aspartic acid β-semialdehyde. Martin and Foster's (7) proposal also visualized the carbon-2 (or -6) of DPA as being derived from carbon-2 of the aspartyl group, and carbon-4 of DPA from carbon-4 of the aspartyl group. The latter carbon atom originates from CO_2 via β-carboxylation of pyruvate (12). Equilibration via malic, fumaric, and succinic acids would result in a symmetrical labeling of both carboxyls of oxaloacetate and, also, via loss of the β-carboxyl, in carboxyl-labeled pyruvate. One molecule of pyruvate would condense with the aspartic acid β-semialdehyde to contribute the last three carbons. Figure 2 reconstructs the biosynthesis of DPA from the data in Table 2, based on the pyruvic-aspartyl scheme. The higher labeling in carbon-4 than in carbon-2 (or -6) is not unexpected; it undoubtedly represents fixed CO_2 that was not thoroughly equilibrated with the other

TABLE 2. *Percentage distribution of C^{14} in DPA produced in the presence of $C^{14}O_2$**

DPA	Carboxyl group, carbons-7 and -8	Pyridyl group			
		Total	Carbons-2 and -6	Carbons-3 and -5	Carbon-4
Expt 1, Table 1	51.2	48.8	3.0	11.0	34.8
Expt 2, Table 1	54.2	45.9	—	—	—

* Figures represent percentages of the total DPA radioactivity.

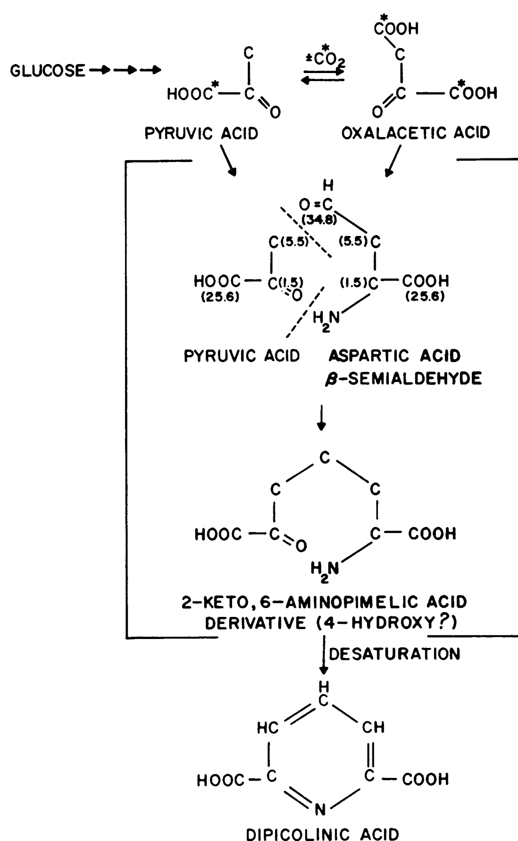

FIG. 2. *Schematic interpretation of the data obtained relative to biosynthesis of DPA from glucose and $C^{14}O_2$. The actual reactants may be activated or may be closely related derivatives of the compounds named. The bracketed structures denote skeletal configurations of proposed intermediates. The figures in parentheses represent percentages of the total radioactivity in the various DPA-carbons computed from the data in Table 2, determined by chemical degradation.*

carboxyl group of the C_4 dicarboxylic acid before being drained off by amination and incorporation in DPA. In fact, this asymmetrical labeling is good evidence for the oxaloacetate mechanism. If the role assigned to pyruvic acid in this scheme is correct, carbons-2 and -6 are equally labeled; so are carbons-3 and -5, and carbons-7 and -8.

C^{14}-labeled glucose and glycerol incorporation in DPA. Efforts were made to determine the contribution of particular carbon atoms in glucose and in glycerol to the biosynthesis of DPA. Experiments were performed like those with $C^{14}O_2$, except that specifically labeled glucose and glycerol were used as the substrates and the atmosphere was enriched with unlabeled CO_2.

Glucose-C^{14}. The weight yields of DPA from the three differently labeled glucose substrates were comparable (20.0 to 23.3%), but the incorporation of their C^{14} differed markedly (Table 3). Of the counts in the glucose consumed, 14.5% of the 1-C^{14}, 22.7% of the 2-C^{14}, and 28.3% of the 6-C^{14} were found in the DPA. This means that carbon-1 was preferentially lost as CO_2 and then carbon-2 and carbon-6, in that order. This is borne out by the percentages of radioactivity in the consumed glucose that were found in the metabolic CO_2.

Carbon-1 could not have entered DPA exclusively via oxidation to CO_2 and reassimilation, since the distribution of label in carboxyl-C versus pyridine-C (Table 4) differed markedly from that obtained in the $C^{14}O_2$ experiments (Table 2). Operation of the hexose monophosphate pathway (HMP) would account for the efficient release of carbon-1 as CO_2. That portion of the carbon-1 that became incorporated in DPA would, consequently, have followed another pathway, assumed to be the Embden-Meyerhof-Parnas pathway (EMP).

The portions of glucose metabolized via the HMP and EMP are computed to be 40.5 and 59.5%, respectively, by

$$\frac{C^{14}O_2 \text{ from glucose-}1\text{-}C^{14}}{\text{total } C^{14} \text{ used}} - \frac{C^{14}O_2 \text{ from glucose-}6\text{-}C^{14}}{\text{total } C^{14} \text{ used}} \times 100$$

TABLE 3. *Glucose-C^{14} incorporation in DPA by washed mycelium of Penicillium citreo-viride**

Labeled substrate	Substrate consumed			CO_2 produced		DPA				
	Count/min	Amt (mmoles)	Count per min per mmole	Count/min	Per cent of substrate counts	Amt (mmoles)	Count/min	Count per min per mmole	Per cent of substrate counts	Relative specific radioactivity†
Glucose-*1*-C^{14}....	1,786	2.13	839	1,040	58.2	0.54	273	505	14.5	0.60
Glucose-*2*-C^{14}....	445	2.19	203	141	31.7	0.58	101	174	22.7	0.81
Glucose-*6*-C^{14}....	1,650	2.10	785	293	17.7	0.63	468	742	28.3	0.95

* All counts per minute are × 10^3. Conditions similar to those described in footnote to Table 1.
† Specific activity of DPA/specific activity of glucose.

TABLE 4. *Percentage distribution of C^{14} in DPA* produced from differently labeled glucose substrates*

Substrate	DPA				
	Carboxyl groups, carbons-7 and -8	Pyridyl groups			
		Total	Carbons-2 and -6	Carbons-3 and -5	Carbon-4
Glucose-*1*-C^{14}........	17.6	82.4	27.8	42.3	12.3
Glucose-*2*-C^{14}.......	27.6	72.4	40.5	26.5	5.4
Glucose-*6*-C^{14}.......	13.4	86.6	30.4	46.2	10.0

* Described in Table 3.

The HMP pathway requires that proportionately more of glucose carbons 2 to 6 be incorporated in products than would be the case with the EMP pathway. Thus, with glucose-*1*-C^{14} as a substrate, the metabolic products should have a lower relative specific radioactivity inasmuch as a larger fraction of the carbon incorporated will have been derived from unlabeled carbons. The last column in Table 3 is in accord with this premise, the relative specific activity of DPA derived from glucose-*1*-C^{14} and -*6*-C^{14} being 0.60 and 0.95, respectively. As expected, the value for DPA derived from glucose-*2*-C^{14} is intermediate.

The 0.95 relative specific activity value for DPA derived from glucose-*6*-C^{14}, being nearly 1.0, indicates that carbons from both halves of the hexose chain were converted to a common precursor in equilibrium, most probably, with pyruvic acid-*3*-C^{14}, carbon 3 of which would thus have approximately the same average specific radioactivity as carbons 1 plus 6 of the initial glucose. Pyruvate probably was formed directly glycolytically and indirectly via a combined HMP-pentose cycle pathway.

The distribution of C^{14} within the DPA molecules produced from the differently labeled glucose substrates (Table 4) provides good evidence of metabolic cycling, with the consequent tendency of the label to randomize in the precursors of DPA. The markedly lower percentage of label from glucose-*2*-C^{14} than from the other labeled glucose substrates found in carbon-4 of the DPA is consistent with the proposed pyruvate-aspartyl condensation mechanism. Other features of the labeling patterns of the DPA are likewise what one would expect from the operation of that mechanism. For example, in each of the glucose experiments, the lower percentage of the DPA-C^{14} found in carbon-4 than in the other pyridyl-carbons is indicative of a nonglucose source of carbon-4, i.e., the unlabeled, diluent CO_2 of the atmosphere. The actual C^{14} fixed via CO_2 was the respiratory $C^{14}O_2$ produced during the experiment (Table 3) and was greatly diluted by the carrier CO_2. Similarly, the metabolic pathways proposed above predict that carbon-6, of all the glucose carbons, would be the least likely to enter the carboxyl groups of DPA, and that was the case (Table 4). Further, carbons-1 and -6 of the glucose ended up mainly in DPA carbons-3 and -5, as was expected. Finally, carbon-2 of the glucose, as expected from glycolytic theory, found its way mainly into DPA carbons-2 and -6. Carbon-2 of glucose would, by operation of a tricarboxylic or a glyoxylic acid cycle, or both, also be expected to make a prominent appearance in the carboxyl groups of DPA via the dicarboxylic acids; this was the case to a considerably greater extent than with carbons-1 or -6 of glucose (Table 4).

Glycerol-C^{14}. The data (Table 5) confirm Hodson and Foster's (5) findings that glycerol is an excellent sole carbon source for DPA biosynthesis in the mold. This is also suggested from other experiments in which labeled glycerol was present as a tracer, along with glucose as the main carbon source in bacteria (7) and in *P. citreo-viride* (16). The specific radioactivities of the DPA produced from glycerol and the distribution of C^{14} between carboxyl-carbons and pyridyl-carbons are substantially what would be expected from Martin and Foster's (7) pyruvate-aspartyl biosynthesis.

TABLE 5. *Glycerol-C^{14} incorporation in DPA by washed mycelium of Penicillium citreo-viride**

Expt	Substrate consumed		DPA			
	Count/min	Count per min per mmole	Count/min	Relative specific radioactivity	C^{14} in carboxyl-carbons (%)	C^{14} in pyridyl-carbons (%)
1. Glycerol-*1*-C^{14}........	131	12	13.1	1.09	41.9	58.1
2. Glycerol-*1*-C^{14}........	—	—	—	—	27.8	72.2
3. Glycerol-*2*-C^{14}........	208	19	24.8	1.31	24.0	76.0
4. Glycerol-*2*-C^{14}........	—	—	—	—	27.0	73.0

* All counts per minute are $\times 10^3$. Conditions similar to those described in footnote to Table 1.

Glycerol may be assumed to be readily converted to pyruvic acid, and the intracellular events involved in DPA synthesis would thereafter be the same as when glucose is the substrate. The significantly higher specific activity of the DPA derived from glycerol-*2-C*^{14} than from *-1-C*^{14} would be the predicted result from loss of the terminal carbon atom via decarboxylation in a tricarboxylic acid cycle; the findings here are in excellent agreement with the corresponding experiments of Hodson and Foster (5). In essence, they mean that glycerol was in part oxidized to a 2-carbon compound, probably the acetyl group, which then participated in the biosynthesis of the oxaloacetic acid-aspartyl precursor of DPA. It will be noted (Table 5) that the carboxyl versus pyridyl distribution of C^{14} differed significantly in the two different glycerol-*1-C*^{14} experiments. This might be the consequence of the relative degree to which CO_2 fixation or metabolic cycling, or both, took place in the two. The ratio of pyridine to carboxyl radioactivity theoretically would be higher with glycerol-*2-C*^{14} than with glycerol-*1-C*^{14}, this difference tending to disappear with increased metabolic cycling. As experiment 2 in Table 5 resembled experiments 3 and 4 in the distribution of radioactivity between the carboxyl- and the pyridine-carbons, it may be inferred that more metabolic cycling took place in this culture than in that of experiment 1. In any event the carboxyl-pyridyl distribution from both 1-C^{14} and 2-C^{14} labeled glycerol is entirely consistent with the labeling expected in DPA formed by the Foster and Martin (7) mechanism.

Discussion

The chemical degradation of DPA, despite its laboriousness and its inability to discriminate between the individual components of three pairs of carbons of the DPA molecule, represents a decided advance in the pursuit of the mechanism of biosynthesis of this heterocyclic compound. The specific radioactivity of carbon-4 of DPA can be obtained, and that happens to be a crucial carbon in the biosynthesis, thus compensating appreciably for the disadvantageous symmetry of the molecule. The utility of the degradation in the interpretation of radioactive precursor experiments was immediately apparent, though only four degradations were done in this work. Notwithstanding its value in the experiments described here, its intrinsic virtues will be realized maximally when it is applied to radioactive DPA produced when metabolic cycling is absent or at a minimum. The experiences described here, though highly instructive, make it clear that ideally the degradation should be applied to DPA produced in short-time, high specific activity experiments of duration no longer than required for incorporation of counts just sufficient for the degradation procedure. The method will be particularly useful with DPA synthesized by cell-free enzymes, since metabolic cycles likely will be precluded.

The data from these experiments, while helping to clarify the small precursor origins of the carbon chain in DPA, do not contribute to the identification of the C_7 straight-chain compound which undergoes cyclyzation. All of the bacterial and mold data are consistent with the keto, aminopimelic acid (KAP) hypothesis (2, 7). However, the same can be said for 2,6-diketopimelic acid (DKP) (11). According to the former hypothesis, a KAP compound is a key intermediate from which are derived 2,6-diaminopimelic acid in *Escherichia coli* (3), DPA, or DKP (5). According to the latter hypothesis, DKP is formed via a postulated novel respiratory cycle analogous to the tricarboxylic acid cycles (16). The DKP spontaneously undergoes ring closure with ammonia, yielding, in bacterial extracts (11), DPA. In support of this hypothesis is the isolation of DKP from a DPA-producing culture of *P. citreoviride*. However, evidence is still lacking that in normal mold cultures DKP is converted nonenzymatically to DPA, that it is the only mechanism for this conversion, and that DKP is not a side product of an alternative precursor, namely, KAP (*see* accompanying paper). On the assumption that the mechanisms of DPA biosynthesis in bacterial spores and in molds are the same, a role for DKP in the bacterial system also awaits demonstration, particularly in view of the cell-free enzymatic synthesis of DPA from pyruvic acid and aspartic acid β-semialdehyde by extracts of sporulating *Bacillus megaterium* (Bach and Gilvarg, Federation Proc. **23**:313, 1964). The conversion of added substrate DKP to DPA in replacement cultures of *P. citreo-viride* (16) may have taken place via KAP in conjunction with an intracellular amino donor.

The evidence regarding KAP versus DKP is ambiguous, and the matter awaits decisive settlement by suitable cell-free mold extracts capable of synthesis of DPA from particular precursors. As regards the precursor origin of the C_7 chain of the DPA molecule, the data in this paper are entirely compatible with the C_3 plus C_4 condensation proposed by Martin and Foster (7). The data neither fit the mechanism proposed by Tanenbaum and Kaneko (16) nor can their theory be made to fit our data.

Whereas the KAP hypothesis has alluded to KAP specifically, if the pyruvic-aspartic semialdehyde condensation is indeed the mode of the C_7 chain formation, the actual intermediate would

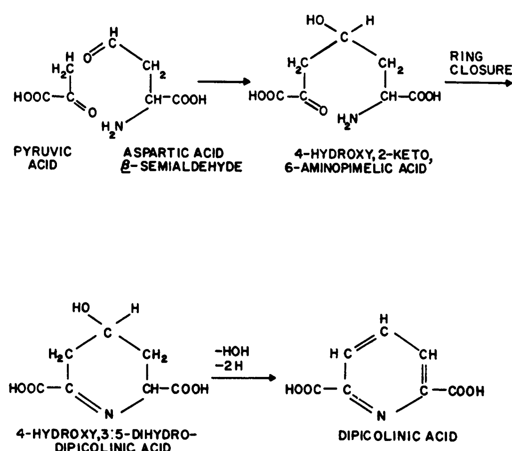

FIG. 3. *Hypothetical pathway of dipicolinic acid biosynthesis.*

most likely be the 4-hydroxyl derivative in a reaction sequence visualized in Fig. 3.

Formation of the two double bonds required by the final stage depicted in the sequence in Fig. 3 is evidently carried out by both bacterial and mold DPA-synthesizing organisms (11, 16).

ACKNOWLEDGMENTS

This investigation was supported by a Public Health Service research grant from the National Institutes of Health, and by grants from the National Science Foundation and the Office of Naval Research.

LITERATURE CITED

1. FISCHER, E., AND M. BERGMANN. 1913. Methylderivate der δ-Aminovaleriansäure und des dl-Ornithins. Ann. Chem. **398**:96–124.
2. FOSTER, J. W. 1956. Morphogenesis in bacteria: some aspects of spore formation. Quart. Rev. Biol. **31**:102–118.
3. GILVARG, C. 1961. *N*-succinyl-α-amino-ε-ketopimelic acid. J. Biol. Chem. **236**:1429–1431.
4. HAMILTON, T. S., AND R. ADAMS. 1928. Reduction of pyridine hydrochloride and pyridinium salts by means of hydrogen and platiumoxide platinum black. J. Am. Chem. Soc. **50**:2260–2263.
5. HODSON, P. H., AND J. W. FOSTER. 1966. Dipicolinic acid synthesis in *Penicillium citreo-viride*. J. Bacteriol. **91**:562–569.
6. JANSSEN, F. W., A. J. LUND, AND L. E. ANDERSON. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science **127**:26–27.
7. MARTIN, H. H., AND J. W. FOSTER. 1958. Biosynthesis of dipicolinic acid in spores of *Bacillus megaterium*. J. Bacteriol. **76**:167–178.
8. MOKRASCH, L. C. 1954. Analysis of hexose phosphate and sugar mixtures with the anthrone reagent. J. Biol. Chem. **208**:55–59.
9. PHARES, E. F. 1951. Degradation of labeled propionic and acetic acids. Arch. Biochem. Biophys. **33**:173–178.
10. PHARES, E. F., AND M. V. LONG. 1955. The complete degradation of carbon-14 labeled succinic acid and succinic anhydride by the Schmidt reaction. J. Am. Chem. Soc. **77**:2556–2557.
11. POWELL, J. F., AND R. E. STRANGE. 1959. Synthesis of dipicolinic acid from α,ε-diketopimelic acid. Nature **184**:878–880.
12. ROBERTS, R. B., P. H. ABELSON, D. B. COWIE, E. T. BOLTON, AND R. J. BRITTON. 1955. Studies of biosynthesis in *Escherichia coli*. Carnegie Inst. Wash. Publ. 607, p. 218–222.
13. RULE, H. G., N. CAMPBELL, A. G. MCGREGOR, AND A. A. WOODHAM. 1950. 4-Benzoyl-*meso*-benzanthrone. J. Chem. Soc., p. 1816–1819.
14. SCHOTTEN, C. 1888. Die Umwandlung des Piperidins in δ-Amino-valeriansäure und in Oxypiperidin. Ber. Deut. Chem. Ges. **21**:2235–2254.
15. STRASSMAN, M., AND S. WEINHOUSE. 1953. Biosynthetic pathways. III. The biosynthesis of lysine by *Torulopsis utilis*. J. Am. Chem. Soc. **75**:1680–1684.
16. TANENBAUM, S. W., AND K. KANEKO. 1964. Biosynthesis of dipicolinic acid and of lysine in *Penicillium citreo-viride*. Biochemistry **3**:1314–1322.
17. VAN SLYKE, D. D., J. FOLCH, AND J. PLAZIN. 1940. Manometric carbon determination. J. Biol. Chem. **136**:509–541.