STUDIES ON THE BIOSYNTHESIS OF DIPICOLINIC ACID IN SPORES OF BACILLUS CEREUS VAR. MYCOIDES[1]

J. J. PERRY AND J. W. FOSTER

Department of Bacteriology, University of Texas, Austin, Texas

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After it was established that sporogenesis in certain aerobic bacilli could take place in the absence of exogenous nutrients ("endotrophic sporulation") (Hardwick and Foster, 1952; Perry and Foster, 1954; Foster and Perry, 1954), a program was undertaken to fractionate well washed endotrophically produced spores, germinated spores, and vegetative cells of *Bacillus cereus* var. *mycoides*. A rather dense suspension of spores germinated for 50 minutes was boiled for several minutes in 25 per cent ethanol. The hot suspension was clarified by quick centrifugation of the extracted spores. Upon cooling, the ethanol supernatant developed a cloudiness, which slowly settled to the bottom. Microscopic examination of the sediment revealed that it consisted of microcrystalline material. Similarly treated vegetative cells did not yield the crystalline material. The crystalline material was obtained from spores of the one other bacterial species tested, *Bacillus subtilis*. The above preliminary experiments were performed in this laboratory by W. A. Hardwick.

During the course of identification of this material, and before we had ascertained its identity, a paper by Powell (1953) appeared in which isolation from spores of aerobic bacteria and characterization of dipicolinic acid were described. This compound is pyridine-2,6-dicarboxylic acid (PDA). The properties of our compound made it probable that it was the calcium salt of dipicolinic acid. This paper presents evidence for the identity of the substance and describes a method for quantitative microestimation of this compound together with data on the amount synthesized during the time course of a bacterial suspension undergoing endotrophic sporulation. Also presented are experiments testing 2,6-diaminopimelic acid (DAPM) as a possible precursor of pyridine-2,6-dicarboxylic acid.

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EXPERIMENTAL METHODS AND RESULTS

The laboratory stock culture of *Bacillus cereus* var. *mycoides* employed for most of the work was the same strain as that used in our earlier work (Foster and Perry, 1954). General procedures relative to endotrophic sporulation also were substantially those used in previous work. The composition of synthetic glucose-glutamic acid-salts (CGS) medium was per liter distilled water: glucose, 2.0 g; NaH_2PO_4, 1.0 g; glutamic acid, 8.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 5 mg; NaCl, 5 mg; $CuSO_4 \cdot 5H_2O$, 0.1 mg; $MnSO_4 \cdot 5H_2O$, 10 mg; $ZnSO_4 \cdot 5H_2O$, 10 mg; $FeSO_4$, 10 mg.

Isolation and identification of pyridine-2,6-dicarboxylic acid. Maximum recovery of pyridine-2,6-dicarboxylic acid from ungerminated spores required an initial acid hydrolysis. Whether this serves to destroy any impermeability of the spore walls or releases pyridine-2,6-dicarboxylic acid from a bound organic or inorganic combination has not been decided. The water washed spores were suspended in 3 N H_2SO_4 and boiled for 15 minutes. Authentic pyridine-2,6-dicarboxylic acid was completely stable under these conditions. The whole suspension was made slightly alkaline with NaOH and extracted overnight in a continuous liquid-liquid type extractor. This treatment removes interfering neutral and basic ether soluble materials. The suspension was then adjusted to pH 1 with H_2SO_4 and again was continuously extracted with ether. The crude crystalline pyridine-2,6-dicarboxylic acid left after evaporation of the ether was recrystallized twice from hot water and finally dried in an oven at 100 C for 3 hours. The melting point (block) was 238 C with decomposition; the melting point of synthetic (Hess and Wissing, 1915) pyridine-2,6-dicarbox-

ylic acid crystallized in the same way was 238 C with decomposition; mixed melting point was 238 C with decomposition.

Literature values for the melting point of synthetic pyridine-2,6-dicarboxylic acid range from 226 C (Hess and Wissing, 1915) to 235–237 C (Soine and Buchdahl, 1950). Powell (1953) reported 229 C for the bacterial pyridine-2,6-dicarboxylic acid. According to Soine and Buchdahl the lower melting point is characteristic of anhydrous pyridine-2,6-dicarboxylic acid and the higher melting point characteristic of the hydrated (1.5 moles H_2O per mole) pyridine-2,6-dicarboxylic acid form. In any case, there was in these experiments complete identity of bacterial pyridine-2,6-dicarboxylic acid and synthetic pyridine-2,6-dicarboxylic acid tested simultaneously. The absorption spectrum in the Beckman showed maxima at 263, 270, and 278 mμ, the principal peak being at 270 mμ, and was indistinguishable from that of synthetic pyridine-2,6-dicarboxylic acid. The natural material was inseparable from authentic pyridine-2,6-dicarboxylic acid by means of one- and two-dimensional paper chromatography involving the use of several solvent systems. The pyridine-2,6-dicarboxylic acid spots could be located on the chromatogram as dark areas when viewed above a Mineralite ultraviolet lamp. The spore substance is therefore identified as pyridine-2,6-dicarboxylic acid, thereby confirming Powell's (1953) independent discovery.

Quantitative microestimation of pyridine-2,6-dicarboxylic acid. The specimen of cells is boiled in 20 ml 3 N H_2SO_4 for 15 minutes to release bound pyridine-2,6-dicarboxylic acid. This concentration of H_2SO_4 was found to be optimum. An aliquot of about 3 ml of the suspension is transferred to a 125 ml separatory funnel, 15 volumes of ethyl ether added, and the whole shaken to extract the pyridine-2,6-dicarboxylic acid. The partition coefficient between ether-water was found to be 0.46. The ether phase containing the pyridine-2,6-dicarboxylic acid is removed, the ether evaporated, and the residue made up to a small exact volume with water. An aliquot containing 50–200 μg pyridine-2,6-dicarboxylic acid is applied as a spot to a sheet of Whatman no. 1 filter paper, and the chromatogram developed (ascending) with a solvent mixture consisting of ethyl ether 84 ml, formic acid 6 ml, water 4 ml. After development, drying, and location of the pyridine-2,6-dicarboxylic acid with the aid of the Mineralite lamp (Rf = 0.75), the spot is cut out and the pyridine-2,6-dicarboxylic acid eluted with small portions of hot water to a final volume of 5.0 ml. This solution is transferred to a quartz cuvette, and the absorption value determined at the 270 mμ maximum. The content of pyridine-2,6-dicarboxylic acid corresponding to the measured absorption value is read from a standard curve constructed from graded doses of authentic pyridine-2,6-dicarboxylic acid subjected to the entire procedure. As controls for ultraviolet absorbing impurities in the paper, solvents, etc., circular zones of size equivalent to those cut out for pyridine-2,6-dicarboxylic acid elution are cut out from blank portions of the same chromatogram at the Rf value corresponding to pyridine-2,6-dicarboxylic acid, eluted, and used as controls in the Beckman. Figure 1 illustrates the linearity of one such standard curve over the concentration range used.

The step involving paper chromatography of the ether extract was introduced early in this investigation. Pyridine-2,6-dicarboxylic acid appears to be by far the major if not the only ether soluble substance in the spores with an ultraviolet absorption revealed by the Mineralite lamp. For certain types of experiments the Beckman quantitation could probably be performed directly on the acid ether extract of the spores.

Biosynthesis of pyridine-2,6-dicarboxylic acid during endotrophic sporulation. Three hundred ml of a distilled water suspension of washed vegetative cells of *B. cereus* var. *mycoides* in a Fernbach type flask were shaken continuously at 30 C, in a manner which gives the characteristic endotrophic sporulation curve described previously (Hardwick and Foster, 1952; Foster and Perry, 1954; see also figure 2). At zero time the suspension contained per ml 0.39 mg dry weight of vegetative cells. At hourly intervals two 10 ml aliquots were removed, one for dry weight measurement of centrifugable cell material and the other for hydrolysis and quantitative analysis of pyridine-2,6-dicarboxylic acid content by the method described in the preceding section. Before the aliquots were analyzed the percentage of cells which sporulated was determined by microscopic examination (Hardwick and Foster, 1952). Figure 2 shows the typical

sporulation pattern as a function of time and also shows that the rate of biosynthesis of pyridine-2,6-dicarboxylic acid was directly proportional to the rate of spore formation, the two curves being virtually superimposable. Unless one assumes that pyridine-2,6-dicarboxylic acid exists in the vegetative cells in a completely unextractable form, these data show that synthesis of pyridine-2,6-dicarboxylic acid is one of the final acts in the sporulation process and is concomitant with the spore becoming a visible morphological entity. The synthesis of pyridine-2,6-dicarboxylic acid itself evidently does not occur during the period of prespore synthesis described previously (Hardwick and Foster, 1952; Foster and Perry, 1954) although some precursor of pyridine-2,6-dicarboxylic acid could be produced during that period.

At the 12th hour, when sporulation was maximal, the pyridine-2,6-dicarboxylic acid content of the spores in two separate experiments was found as 3.1 and 3.4 per cent of the dry weights of the centrifugable solids. At the 12th hour spores are not completely liberated from sporangia and probably other residuum from the vegetative cells (see figure 7 in Hardwick and Foster, 1952); consequently the above values probably are smaller than the true value for spore content of pyridine-2,6-dicarboxylic acid. In another experiment the incubation was continued for a total of 24 hours, at which time

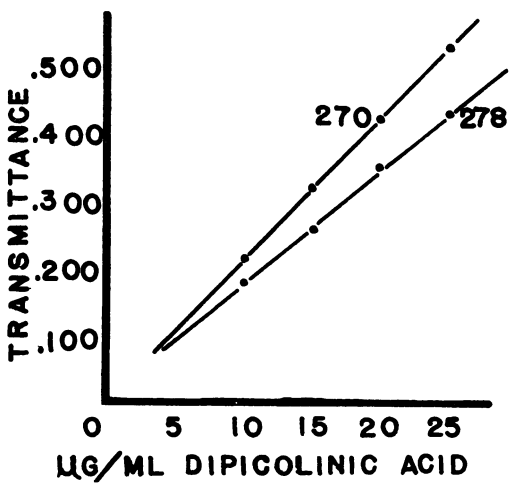


Figure 1. Proportionality between concentration of pyridine-2,6-dicarboxylic acid and absorption at 270 and 278 mμ, respectively, measured in the Beckman spectrophotometer.

iberation of spores from sporangia was more nearly complete. The pyridine-2,6-dicarboxylic acid content of these well washed spores was 4.8 per cent. This compares with the value of approximately 12 per cent computed from the data of Powell (1953) for the pyridine-2,6-dicarboxylic acid content of *Bacillus megaterium* spores. The spores analyzed by Powell were harvested from a complex organic growth medium. This disparity in pyridine-2,6-dicarboxylic acid contents led to examination of the pyridine-2,6-dicarboxylic acid content of spores of our organism produced in two complete growth media: one, the synthetic glutamic-glucose-salts (GGS) medium used routinely in our laboratory for growth of this organism (Hardwick and Foster, 1952) and the same medium supplemented with 0.5 per cent yeast extract. The well washed spores were analyzed for pyridine-2,6-dicarboxylic acid by the procedure described above. Spores produced in the synthetic glutamic-glucose-salts medium contained 5.1 per cent pyridine-2,6-dicarboxylic acid; spores produced in the complex organic medium contained 5.1 per cent pyridine-2,6-dicarboxylic acid. Thus spores produced in the synthetic growth medium contained only slightly more (5.1 versus 4.8 per cent) pyridine-2,6-dicarboxylic acid than spores produced endotrophically. Furthermore, the presence of a complex organic supplement (0.5 per cent yeast extract) did not increase the content of pyridine-2,6-dicarboxylic acid. The larger pyridine-2,6-dicarboxylic acid value obtained by Powell (1953) may have been due to the particular complex growth medium she used, or to the particular strain of bacterium investigated. One additional difference to be noted is that we

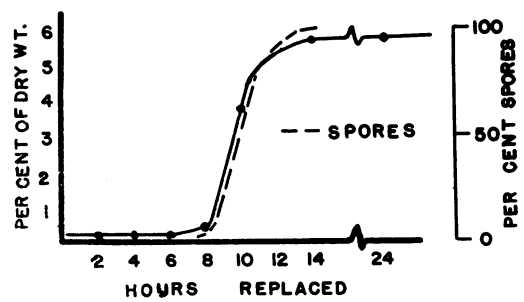


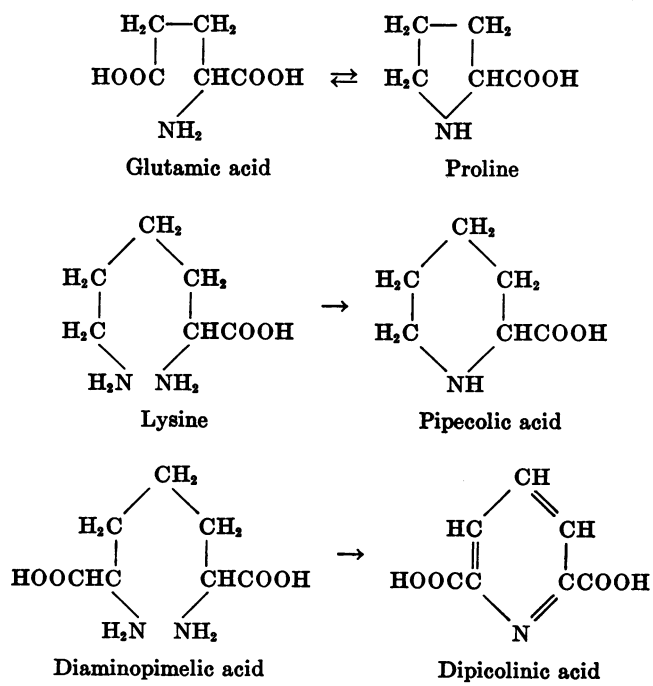
Figure 2. Content of pyridine-2,6-dicarboxylic acid (solid line) in a suspension of *Bacillus cereus* var. *mycoides* undergoing endotrophic sporulation.

analyzed ungerminated spores whereas Powell analyzed the exudate of germinated spores. As a consequence, some pyridine-2,6-dicarboxylic acid may be unextractable by our method, or alternatively some pyridine-2,6-dicarboxylic acid may be synthesized during germination.

2,6-Diaminopimelic acid (DAPM) as a possible precursor of pyridine-2,6-dicarboxylic acid. (*a*) *Theoretical.* Based on the analogous relation of nitrogen containing heterocyclics to the corresponding aliphatic amino acids, it seemed worthwhile to examine 2,6-diaminopimelic acid as a possible precursor for pyridine-2,6-dicarboxylic acid. Illustrations are: the relation of the heterocyclic proline to glutamic acid via pyrroline carboxylic acid and glutamic semialdehyde (Taggart and Krakaur, 1949; Lang and Schmid, 1951; Vogel and Davis, 1952); the relation of proline to δ-aminovaleric acid (Hoogeheide and Kocholaty, 1938); and the relation of pipecolic acid (piperidine-2-carboxylic acid) to lysine (Lowy, 1953). These relations are depicted structurally together with the presumed relation of 2,6-diaminopimelic acid to pyridine-2,6-dicarboxylic acid, as shown in the accompanying diagram. Except for the unsaturation in the heterocyclic ring this ring closure corresponds to that forming pipecolic acid from lysine. Indeed, 2,6-diaminopimelic acid may be thought of as 6-carboxy-lysine.

Apart from comparative biochemistry, serious consideration of 2,6-diaminopimelic acid for the above described role would be aided by a demonstration of the presence of this amino acid in the spore forming bacteria. Work and Dewey (1953) have done this for 3 species of *Bacillus* and 2 of 3 species of *Clostridium*, and Powell and Strange (1953) found 2,6-diaminopimelic acid in hydrolyzates of a nondialyzable peptide excreted by germinating spores of *B. megaterium* and *B. subtilis*. In addition, using two-dimensional paper chromatography, we have demonstrated the existence of 2,6-diaminopimelic acid in acid hydrolyzates of vegetative cells of *B. cereus* var. *mycoides*, the organism used in these studies.

(*b*) *Biosynthesis, isolation, and purification of C^{14}-labeled 2,6-diaminopimelic acid.* The auxotrophic strain of *Escherichia coli* strain 26-26, which is blocked in the synthesis of lysine and which accumulates 2,6-diaminopimelic acid when grown in the presence of lysine (Davis, 1952), was used to synthesize totally labeled C^{14}-2,6-diaminopimelic acid. An extract of radioactive algae was used as a supplementary nutrient in the *E. coli* medium (see below). One hundred milligrams (dry weight) of C^{14}-labeled



Scenedesmus obliques, containing about 40 μc radioactivity, were refluxed with 10 ml of 1 N HCl for 1 hour. This treatment was designed primarily to hydrolyze polysaccharides. After cooling, the liquid was clarified by centrifugation and neutralized with NaOH. This carbohydrate containing hydrolyzate was added to 500 ml of the glucose-salts-lysine medium, recommended by Work and Denman (1953) for growth of the *E. coli* mutant and accumulation of 2,6-diaminopimelic acid. After inoculation and incubation on a shaker for 48 hours at 30 C, the culture medium was freed of cells and suspended matter by centrifugation. The supernatant was adjusted to pH 3, brought to a boil, and filtered through celite for deproteinization. The supernatant was then passed through a "dowex 50" column (25 by 400 mm), and the 2,6-diaminopimelic acid eluted with HCl according to the procedure of Work and Denman (1953). Prior to the radioactive run, a separation of a mixture of glutamic acid and authentic 2,6-diaminopimelic acid was achieved. Elution of the amino acids was followed by ninhydrin reactions on a small aliquot of each fraction (Moore and Stein, 1948). The 2,6-diaminopimelic acid was obtained in the first 300 ml of 2.5 N HCl following 3 liters of 1.5 N HCl. The ninhydrin positive fraction from the radioactive experiment, which corresponded to the position of the 2,6-diaminopimelic acid in the authentic run, was combined and concentrated to dryness *in vacuo* three successive times with addition of water each time, to remove the HCl. This pooled fraction displayed a single ninhydrin positive spot at the same Rf as authentic 2,6-diaminopimelic acid when both were tested side by side on two one-dimensional chromatograms employing different solvents. The radioactivity in the chromatograms was checked by eluting the various portions of duplicate paper strips and counting the dried eluates in planchets. Radioactivity was observable only in that section of the paper strip corresponding to the spots showing ninhydrin positive tests, e.g., 2,6-diaminopimelic acid. Similarly, the radioactivity was found associated with a single ninhydrin positive spot on a two-dimensional chromatogram developed with water saturated phenol and butanol-acetic acid-water (4:1:1 v/v). The aliquot initially applied to the paper contained 98 counts per minute (cpm). Total counts recovered from the two-dimensional chromatogram were 102 cpm. By comparison with a standard curve constructed from the ninhydrin color from graded amounts of authentic 2,6-diaminopimelic acid, the bacterial filtrate yielded 60 mg of labeled 2,6-diaminopimelic acid, with a total radioactivity of 8.6×10^4 cpm (1.4×10^3 cpm/mg).

(*c*) *Conversion of labeled 2,6-diaminopimelic acid to pyridine-2,6-dicarboxylic acid during endotrophic sporulation.* Four flasks each containing 10 ml of a washed suspension of *B. cereus* var. *mycoides* vegetative cells were prepared for a normal endotrophic sporulation and placed on the shaker at 30 C; the suspension was heavier than usual (26 mg dry weight per ml) in order to avoid dilution of the rather low specific activity of the labeled precursor and to obtain sufficient pyridine-2,6-dicarboxylic acid for isolation and counting without addition of carrier. To flask no. 1 was added at zero time 10 mg of the labeled 2,6-diaminopimelic acid described above ($= 1.4 \times 10^4$ cpm). All flasks were then shaken as usual; after 4 hours 10 mg of the labeled 2,6-diaminopimelic acid were added to flask no. 2 and the shaking continued; after 8 hours 10 mg of the labeled compound were added to flask no. 3. Flask no. 4 received no 2,6-diaminopimelic acid. All flasks required a total of 22 hours' shaking to reach maximum sporulation, about 70–80 per cent in this case. The delayed maximum, as well as the low maximum, probably was a consequence of the exceptionally heavy suspension. The sporulation pattern in the flasks receiving the labeled compound was the same as that in the control flask which received none. After centrifuging, the cells from each of the flasks were suspended in 3 N H_2SO_4 and boiled for 20 minutes to liberate pyridine-2,6-dicarboxylic acid from the spores. The supernatants were saved for determination of residual labeled 2,6-diaminopimelic acid (see below). The acid hydrolyzates were adjusted to pH 7.5 and extracted continuously with ether for 12 hours in a liquid-liquid extractor. The residual cell suspension was saved for amino acid analysis (see below). The alkaline ether extract had a negligible radioactive count and was discarded; the water phase was acidified to pH 1 with H_2SO_4 and reextracted with fresh alkali-washed ether for 24 hours. The pyridine-2,6-dicarboxylic acid in the ether extract of

each flask was recovered by evaporation of the solvent and then purified separately by partition chromatography. Used for this purpose were celite columns (12 by 400 mm), packed with 15 grams celite moistened with 12 ml of 0.5 N H_2SO_4. The developing solvent was composed of 97.5 volumes chloroform and 2.5 volumes redistilled *n*-butanol. The developing solvent was collected in 5 ml fractions; each was titrated with 0.0215 N NaOH using phenol red as the indicator. The acid eluted from the columns to which were applied the radioactive ether extracts of the spores displayed an elution curve similar to that of authentic pyridine-2,6-dicarboxylic acid. Radioactivity measurements were made on an aliquot of each fraction; figure 3 shows that the counts were directly proportional to titratable acid in each fraction, indicative of constant specific radioactivity of the acid, and that the acid in each case was apparently radioactively pure. The titration curves indicate the absence of a significant amount of any second acid. The acid containing fractions from each run were pooled, the total radioactivity measured, and the specific radioactivity computed from the titration values. The data are presented in table 1. They show that some labeled pyridine-2,6-dicarboxylic acid was formed from the labeled 2,6-diaminopimelic acid. Using the 4 hour run as an illustration (since more detailed study of this one is reported below) and provisionally assuming a molar conversion of 2,6-diaminopimelic acid to pyridine-2,6-dicarboxylic acid, it may be computed that under the conditions of the experiment only 3.8 per cent of the pyridine-2,6-dicarboxylic acid synthesized during endotrophic sporulation was derived from the exogenously supplied 2,6-diaminopimelic acid ($55/1.4 \times 10^3$ cpm). Identity of the pyridine-2,6-dicarboxylic acid isolated from each of these experiments was confirmed by Rf values in ether-formic acid-water chromatograms, and by the characteristic ultraviolet absorption spectrum in the spectrophotometer. The radioactive preparations isolated from the spores were indistinguishable from authentic pyridine-2,6-dicarboxylic acid by these criteria.

Consumption of the labeled 2,6-diaminopimelic acid during endotrophic sporulation. A quantitative measurement of the color developed with ninhydrin reagent on aliquots of the supernatant liquids from the various flasks at the conclusion of the 22 hour incubation period was then made. The values for the unknown were compared to those of a standard curve constructed from graded levels of authentic 2,6-diaminopimelic acid. Initially each flask received 10.0 mg. Table 1 shows the amounts remaining and consumed in the different time treatments. Although the

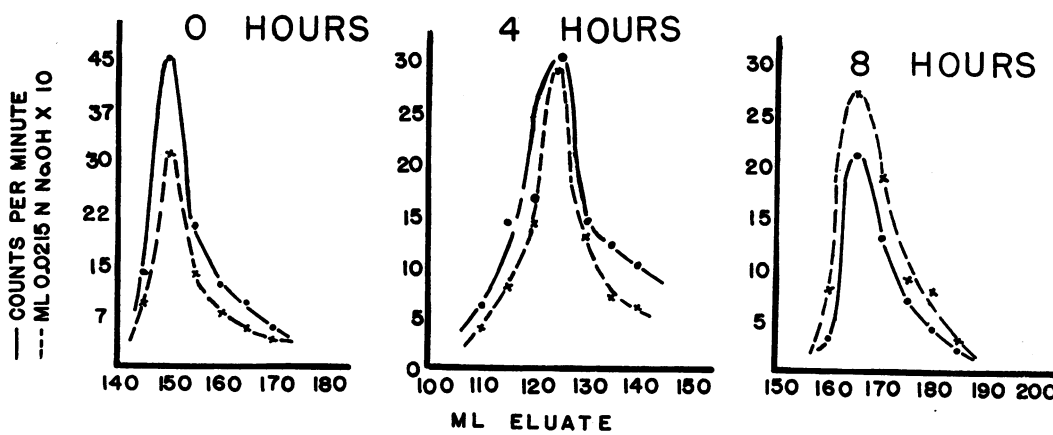


Figure 3. Partition chromatography of radioactive pyridine-2,6-dicarboxylic acid extracted from spores of *Bacillus cereus* var. *mycoides* formed endotrophically in the presence of C^{14}-2,6-diaminopimelic acid. The experiment was performed on 3 aliquots of the cell suspension, each after shaking in parallel for a total of 22 hours. The chart on the left represents analysis of the suspension to which C^{14}-2,6-diaminopimelic acid was added at 0 time; the chart in the center represents that to which C^{14}-2,6-diaminopimelic acid was added after 4 hours shaking, and the chart on the right represents that to which C^{14}-2,6-diaminopimelic acid was added after 8 hours shaking.

TABLE 1

Radioactivity of pyridine-2,6-dicarboxylic acid (PDA) from spores produced in the presence of C^{14}-labeled 2,6-diaminopimelic acid (DAPM)

	PDA from Cells (Spores) Which Had Received Labeled DAPM after Shaking* for		
	0 hours	4 hours	8 hours
Pure PDA recovered, mg	2.1	1.85	1.26
Total radioactivity, cpm above background	113	102	52
Specific radioactivity, cpm/mg	54	55	41
DAPM remaining in supernatant liquid, mg	7.9	8.7	9.6
DAPM consumed, mg	2.1	1.3	0.4

* Total shaking (incubation) time in each case was 22 hours.

recovery of pyridine-2,6-dicarboxylic acid was not designed to be quantitative, using the figures in table 1, one may compute that in the 0, 4, and 8 hour treatments a minimum of 3.8, 5.6, and 9.3 per cent, respectively, of the 2,6-diaminopimelic acid consumed was converted to pyridine-2,6-dicarboxylic acid. It would appear that addition of the 2,6-diaminopimelic acid at a time closer to sporulation allowed a greater portion of it to be converted to pyridine-2,6-dicarboxylic acid. Perhaps this is due to the fact that less opportunity existed for consumption of 2,6-diaminopimelic acid by alternative or competing reactions. Because our interest was solely in the biosynthesis of labeled pyridine-2,6-dicarboxylic acid, no attempt was made to account for the radioactivity of that portion of the 2,6-diaminopimelic acid consumed but which was not converted to pyridine-2,6-dicarboxylic acid.

Radioactivity of various amino acids obtained from spores produced in the presence of C^{14}-labeled 2,6-diaminopimelic acid. Having established that 2,6-diaminopimelic acid can to some extent be converted to pyridine-2,6-dicarboxylic acid during sporulation, it was essential to ascertain the specificity of the intracellular conversion. In other words, is the labeled 2,6-diaminopimelic acid broken down to some common pool from which all of numerous other intracellular spore compounds are indiscriminately synthesized, or is the more direct ring closure pathway to pyridine-2,6-dicarboxylic acid favored? Analysis of the amino acids in the spores was used as a partial guide to the answer to that question. The acidic suspension of cell debris left after ether extraction of the pyridine-2,6-dicarboxylic acid in the 4 hour treatment was made 6 N with HCl and refluxed for 24 hours to hydrolyze proteins. The hydrolyzate was decolorized with a small amount of norite, concentrated *in vacuo*, and applied to a dowex 50 column for separation of individual amino acids. The procedure was similar to that of Stein and Moore (1950). Five ml fractions were collected with an automatic fraction collector. The eluting acid was HCl of normality and volumes depicted

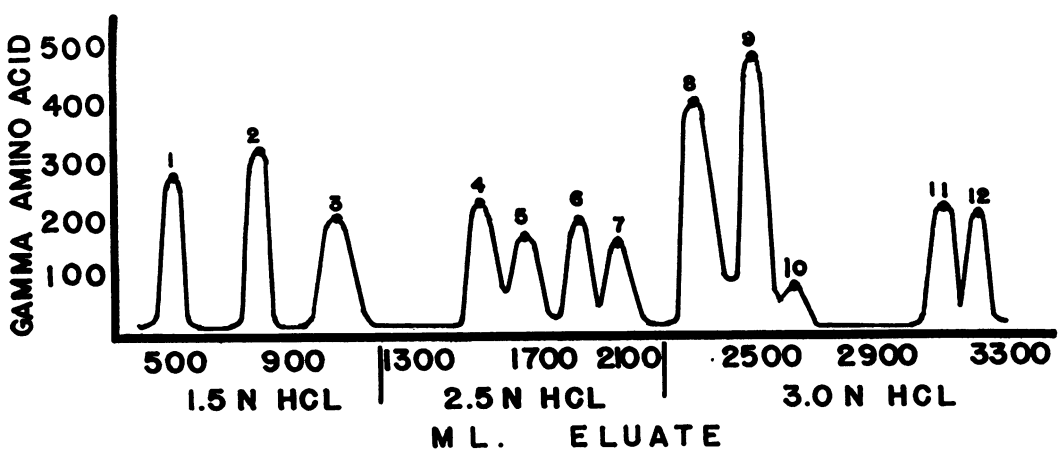


Figure 4. Separation of amino acids in hydrolyzate of spores by means of elution from "dowex 50" column.

TABLE 2

Radioactivity of some amino acids isolated from spores produced in the presence of C^{14}-labeled 2,6-diaminopimelic acid added after the 4th hour

Amino Acid	Duration of Counting	Total Counts Registered	Background Counts Registered During the Interval	Total Counts due to the Amino Acid	Counts per Minute due to the Amino Acid	Amount of Amino Acid in Sample Counted	Specific Radio Activity
	min		*min* × 22			*mg*	*cpm/mg*
Leucine	28	664	616	48	1.7	0.165	10.3
Aspartic acid	30	684	660	24	0.8	0.195	4.1
Glutamic acid	30	636	660	(−24)	0	0.135	0
Glycine	32	666	704	(−38)	0	0.165	0
Arginine	33	759	726	33	1.0	0.120	0.8
Alanine	30	672	660	12	0.4	0.135	3.0
Valine	30	696	660	36	1.2	0.120	10.0
Methionine	30	663	660	3	0.1	0.210	0.5
Lysine	50	1,120	1,200	(−80)	0	0.240	0
Histidine	30	660	660	0	0	0.105	0
Isoleucine	30	660	660	0	0	0.135	0
Unknown	30	684	660	24	0.8	0.150	5.3
Pyridine-2,6-dicarboxylic acid	20	608	440	168	8.4	0.180	46.6

in figure 4. Detection of the amino acids was performed by the ninhydrin test on a neutralized aliquot of each fraction. Twelve distinct "peaks" were obtained up to the point at which elution was discontinued. The fractions in each group were pooled, and the total amino acid content of each peak determined quantitatively by means of the ninhydrin colorimetric method. Although significant radioactivity was detectable in the amino acid fractions, nonamino acid radioactivity also appeared to be eluted from the column since fractions (especially early ones) possessing no ninhydrin reacting material showed significant counts. Consequently, each amino acid was further purified by applying it to paper as a 2 inch band. Depending on the amino acid the amount used was 0.10 to 0.25 mg. The papers were then developed using aqueous phenol as the solvent system. After drying the papers, the individual amino acids were located by cutting a ¼ inch strip from the center of the band and treating it with ninhydrin solution. In all cases, each band showed only a single amino acid upon development. The entire area containing the amino acid was cut out, and the amino acid eluted from the paper with hot water. A small aliquot of each water extract was taken for quantitative determination of the amino acid, and the remainder of the extract was dried in a planchet and its radioactivity measured. The counts per minute for the individual amino acids were relatively small (table 2) but significant because of the absolute number of counts registered. The data of table 2 show that some of the amino acids had a very small count although none approached the specific activity of the pyridine-2,6-dicarboxylic acid produced at the same time. The most active amino acid contained less than one-fourth the radioactivity of pyridine-2,6-dicarboxylic acid. It will be noted that lysine, known to be produced in *E. coli* from 2,6-diaminopimelic acid through the action of a specific decarboxylase (Dewey and Work, 1952), had no significant radioactivity.

DISCUSSION

The experiments with labeled 2,6-diaminopimelic acid fall short of providing a critical test of whether pyridine-2,6-dicarboxylic acid originates via ring closure of the unbroken carbon chain of the amino acid. However, they do show that under the particular conditions as much as 4 per cent of the pyridine-2,6-dicarboxylic synthesized originated from the carbon of the exogenous labeled 2,6-diaminopimelic acid (assuming the latter was uniformly labeled). In this respect they provide a basis for further studies along these lines, now under way in this laboratory.

Now that 2,6-diaminopimelic acid has been

shown to contribute carbon to pyridine-2,6-dicarboxylic acid, it becomes feasible to undertake the experiments involving use of discretely labeled 2,6-diaminopimelic acid, particularly with C^{14} in one or more of the noncarboxyl carbons, and location of the C^{14} in the corresponding carbon atoms of the pyridine-2,6-dicarboxylic acid. Thereby would be provided a means of testing conclusively the "direct" ring closure idea. Until this experiment is done, one could furnish the equally valid hypothesis that the 2,6-diaminopimelic acid carbon chain is broken down to one or more shorter carbon chain compounds in a metabolic pool, this compound(s) being the actual precursor(s) of pyridine-2,6-dicarboxylic acid. Moreover, other amino acids and other compounds could well be a source of the short chain compounds.

That radioactivity was found to have been preferentially incorporated into pyridine-2,6-dicarboxylic acid in relation to some dozen of the amino acids isolated from the spores cannot be used as conclusive evidence for the idea of a specificity of the conversion of 2,6-diaminopimelic acid to pyridine-2,6-dicarboxylic acid. Of all the compounds in spores pyridine-2,6-dicarboxylic acid is very likely a major one which is synthesized *de novo* during sporogenesis; with the methods used there was none detectable in vegetative cells. One could assume that the amino acids of endotrophically produced spores very likely were utilized preformed to some extent at least, as they were made available from breakdown of unlabeled protein in the vegetative cells. This could account for their being relatively unlabeled.

Another experimental approach being employed and having a bearing on this problem is whether other C^{14}-labeled amino acids besides 2,6-diaminopimelic acid, as well as 1, 2, and 3-carbon compounds, can function as precursors of pyridine-2,6-dicarboxylic acid.

In tracer experiments of the type reported in this paper, the relatively small conversion percentage of the exogenous labeled substrate to pyridine-2,6-dicarboxylic acid is not too significant by itself. It is not unexpected that an exogenous precursor is at a competitive disadvantage with the same intracellular (active?) compound in metabolic reactions occurring in the intact cell. If one assumes that utilization of exogenous 2,6-diaminopimelic acid for spore synthesis implies utilization of endogenous 2,6-diaminopimelic acid for the same purpose, one may regard this as partial evidence that the preformed low molecular weight substances in the vegetative cell are reutilized for synthesis of the spore (Hardwick and Foster, 1952). It is hoped that further experiments will give a clearer picture of this process.

SUMMARY

Pyridine-2,6-dicarboxylic acid (PDA) has been isolated from spores of two species of the genus *Bacillus*. The substance has been characterized chemically; this work confirms the discovery made independently by Powell (1953). A method for quantitative estimation of pyridine-2,6-dicarboxylic acid in the range of 20 to 100 μg has been described; it involves liberation of pyridine-2,6-dicarboxylic acid by acid hydrolysis, ether extraction, paper chromatography, and spectrophotometric measurement of pyridine-2,6-dicarboxylic acid in the paper eluate by ultraviolet absorption at 270 mμ. Biosynthesis of pyridine-2,6-dicarboxylic acid during endotrophic sporulation follows the same time pattern as appearance of spores. Endotrophically produced spores contained 4.8 per cent pyridine-2,6-dicarboxylic acid; spores produced in synthetic or complex organic media contained 5.1 per cent pyridine-2,6-dicarboxylic acid. Totally C^{14}-labeled 2,6-diaminopimelic acid (DAPM) was synthesized by Davis' *Escherichia coli* mutant, isolated, and obtained in radioactively pure form. The C^{14}-labeled 2,6-diaminopimelic acid was added to a bacterial suspension undergoing endotrophic sporulation. The pyridine-2,6-dicarboxylic acid isolated from such spores was significantly more radioactive than any of 12 amino acids in the spores. 2,6-Diaminopimelic acid was shown to be present normally in vegetative cells of the sporulating bacilli, and the conclusion was made that 2,6-diaminopimelic acid can be converted to pyridine-2,6-dicarboxylic acid. The data do not discriminate between two possible mechanisms: one is breakdown of the carbon chain of the amino acid to smaller precursors of pyridine-2,6-dicarboxylic acid; the other is ring closure of the carbon chain without prior rupture.

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