Isolation and Characterization of Diaminopimelic Acid from Culture Filtrate of an *Escherichia coli* Mutant. (20115)

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Work isolated from acid hydrolysates of a number of bacteria, including *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*, a ninhydrin-reactive compound that she tentatively characterized as the optically inactive *meso* isomer of α,ε-diaminopimelic acid (1). Subsequently Davis showed that certain lysine-requiring auxotrophs of *Escherichia coli* accumulate relatively large amounts of a compound that satisfies the lysine requirement of other auxotrophs. In each of four solvents the accumulated compound had an R_F value determined by either ninhydrin or microbiological response corresponding to diaminopimelic acid and was equally stable to acid and alkali. Certain other *Escherichia coli* mutants that require both diaminopimelic acid and lysine were found to accumulate threonine(2). An enzyme that decarboxylates diaminopimelic acid to yield lysine has been observed by Dewey and Work to occur in many bacteria but to be absent from *Escherichia coli* auxotrophs that require lysine(3).

The difficulties involved in obtaining *Corynebacterium diphtheriae* or *Mycobacterium tuberculosis* cells in quantity and in isolating diaminopimelic acid from such sources are formidable. This report confirms the findings of Davis with respect to the accumulation of relatively large amounts of diaminopimelic acid in culture filtrates of a representative *Escherichia coli* auxotroph and extends the work by describing the details of a convenient procedure by which relatively large amounts of the compound may be readily isolated. In the development of an isolation scheme for obtaining diaminopimelic acid it soon became apparent, as indicated by paper chromatography, that diaminopimelic acid is the main ninhydrin-reactive component of the culture filtrate of an *Escherichia coli* mutant (26-26). It was therefore possible to establish the isolation scheme using the ninhydrin reaction as the only assay procedure. In the final procedure the bacterial centrifugate was treated first with Norit on which all colored products, but no diaminopimelic acid, are adsorbed. The clear filtrate was next passed through a strong anion exchange resin. Diaminopimelic acid above its isoelectric point is retained as an anion by the resin. Cations and other unabsorbed components were washed through the column with a large volume of water. The diaminopimelic acid was eluted from the column with hydrochloric acid. This eluate containing diaminopimelic acid, hydrochloric acid, and other acids was stirred with an excess of a strong anion exchange resin in its bicarbonate form. Anion removal from the eluate was forced to completion because of the instability of carbonic acid, one product of the equilibrium. Under the conditions employed in the removal of the anions, the diaminopimelic acid is not taken above its isoelectric point and no absorption on the resin occurs. The resin filtrate thus obtained was freed of traces of anions and cations by passage through weak anion and cation exchange resins. Diaminopimelic acid was crystallized by the addition of ethanol to a concentrated aqueous solution of the amino acid.*

Diaminopimelic acid obtained by the procedures summarized above was found to have by paper chromatography in 10 different solvent systems R_F values indistinguishable from the corresponding values obtained with authentic synthetic diaminopimelic acid. Even on considerably overloading the papers no more than one ninhydrin-reactive component could be observed. Elementary analysis for carbon, hydrogen, and nitrogen indicated the

* After the isolation scheme described in this paper had been developed we received from Dr. E. Work a submitted manuscript describing the isolation of DAP from an *E. coli* mutant. This method, which differs substantially from ours, had appeared in print (E. Work and R. Denman, *Biochim. et Biophys. Acta*, 1953, v10, 183) at the time proof of the present paper became available. The relative merits of the two procedures remain to be explored.

existence of only small amounts of contamination. Diaminopimelic acid obtained as described above was found to have a small amount of dextrorotation which increased with increasing concentration of hydrochloric acid in the solvent. This behavior according to the rule of Lutz and Jirgensons(4) is characteristic of an amino acid of the L or "natural" configuration. A solubility determination of the amino acid as obtained after one crystallization showed that it is about twice as soluble in water as is the purified diaminopimelic acid obtained by Work from bacterial hydrolysates. Repeated recrystallization of the amino acid from water and ethanol gave a product essentially free of optical rotation and approaching the water insolubility described by Work. Pertinent to these findings with respect to optical rotation and solubility is the corresponding data for cystine the only other naturally-occurring amino acid capable of existing in a *meso* form(5). Meso cystine is considerably less soluble in water than either D- or L-cystine. Mixtures of *meso* and D- or L-cystine can be more soluble than either component alone. It is suggested that in the present instance an analogous situation may prevail in that preparations of diaminopimelic acid with optical activity that show only one spot on paper chromatography but are more soluble than anticipated for the *meso* form may be mixtures of more soluble L (+)- and less soluble *meso*-diaminopimelic acid from which the more insoluble *meso* form is obtained on repeated recrystallization. Obviously the final solution would be facilitated by the availability of all forms of diaminopimelic acid or the discovery of a solvent in which the optically active component of once-crystallized diaminopimelic acid preparations is the more insoluble so that recrystallization to constant rotation could be achieved. The isolated diaminopimelic acid was further characterized by a Van Slyke amino nitrogen determination that showed all nitrogen to be present as α-amino nitrogen. In addition the amino acid was reduced by a 2-step procedure to pimelic acid, demonstrating the absence of branching in the carbon chain. As would be expected, diaminopimelic acid obtained from the representative *Escherichia coli* mutant accumulating the compound is active in satisfying the diaminopimelic acid requirement of another *Escherichia coli* mutant with a nutritional requirement for both diaminopimelic acid and lysine. Diaminopimelic acid from *Escherichia coli* is inactive in satisfying the lysine requirement of *Neurospora crassa*-lysineless (33933), *Streptococcus fecalis* or *Leuconostoc mesenteroides*. Diaminopimelic acid is inactive as a source of threonine for the last two species.

Experimental. Assay method. One hundredth ml of suitably diluted solution or fraction to be examined was evaporated to dryness on filter paper and the ninhydrin reaction carried out as described by Consden *et al.*(6). The qualitative test could be made semi-quantitative by preparing a graded dose series of applications of the solution to the paper and comparing the intensities against those from a similar series prepared from a standard solution of synthetic diaminopimelic acid. The synthetic diaminopimelic acid used in several phases of this study was kindly furnished by Dr. Herschel K. Mitchell.

Microbiological production of the amino acid. Mutant strain 26-26 of *Escherichia coli* that accumulates the amino acid under study was kindly made available by Dr. Bernard D. Davis. Microbiological production of the amino acid was carried out in the medium of Davis and Mingioli(7) modified to contain 0.5% glucose instead of 0.2% and to contain 40 γ/ml of added L (+)-lysine. A flask containing 400 ml of the above medium was seeded from a fresh agar slant of the mutant and after incubation at 30°C for 18 hours with continuous shaking was used to seed 12 liters of medium. After growth at 30°C for 18 hours with continuous agitation and aeration the 12 liter culture was used to inoculate 200 liters of the purified medium contained in an industrial fermenter. The microbiological production was carried out at 30°C for 18 hours with stirring at 50 rpm and aeration with sterile air at a ratio of ¼ volume of air per volume of medium per minute. Following growth of the mutant the cells were centrifuged off with a Sharpless centrifuge and the centrifugate concentrated at 40°C to a volume of 60 liters in a Mojonnier concentrator. This solution was stored in the refrigeration

room and aliquots from it worked up for diaminopimelic acid over a period of several months.

Clarification with Norit. One liter of concentrated bacterial filtrate was shaken with 50 g of Norit A for 30 minutes and then was filtered with suction. The filtrate was clear and colorless.

Absorption on Dowex-1 (OH^-). One liter of norit filtrate was poured on to a column containing 600 g of Dowex-1 resin which had been freshly converted to the OH^- form by liberally washing with 4 liters of 2.5 N NaOH and then with distilled water until the washings were free of alkali. The norit filtrate was passed through the column at a rate of about 10 ml per minute. After application of the norit filtrate the column was washed with distilled water at a flow rate of about 10 ml per minute until the washings were free of alkali. This required about 10 liters of water.

Elution from Dowex-1 (OH^-). The Dowex-1 (OH^-) column containing the bound diaminopimelic acid and anions was eluted with 0.2 N HCl at a flow rate of about 10 ml per minute. Fractions of 500 ml each were collected and by the qualitative ninhydrin test those fractions containing the diaminopimelic acid were located. Those fractions containing most of the diaminopimelic acid were combined.

Anion removal with Dowex-1 (HCO^-_3). The Dowex-1 (OH) eluate containing diaminopimelic acid substantially free of cations but containing HCl and acids of other anions contained in the norit-treated bacterial filtrate was freed of these anions by stirring with Dowex-1 (HCO^-_3). The resin was prepared columnwise by washing the commercially available material with sat. $NaHCO_3$ and then distilled water until it was free of alkali. Ordinarily stirring of the hydrochloric acid eluate for 1-2 hours with 200-400 g of Dowex-1 (HCO^-_3) was sufficient to neutralize the anions contained in the solution. When the supernatant solution was alkaline to Congo red paper the resin was filtered off and the filtrate and resin washings were combined and evaporated *in vacuo* to dryness.

Final deionization. The dry residue obtained in the previous step was taken up in 100 ml of H_2O and centrifuged at high speed to remove small amounts of insoluble matter. The centrifugate then was applied to a column containing 100 g of IR-4B freshly prepared in the OH^- form by washing liberally with 2.5 N NaOH and then with distilled water until the washings were free of alkali. The solution to be freed of anions was applied at a flow rate of about 2 ml per minute and was followed by about 300 ml of water until a ninhydrin reaction on the effluent was essentially negative. The combined solution and washings then were applied to a column containing 100 g of IRC-50 freshly prepared in the H^+ form by washing liberally with 1 N HCl and then with distilled water until the washings were free of acid. The solution to be freed of cations was applied at a flow rate of about 2 ml per minute and was followed by about 300 ml of water until a ninhydrin reaction on the effluent was essentially negative.

Crystallization and analysis. The washings from the IR-4B and IRC-50 columns were evaporated *in vacuo* to dryness. The residue was taken up in 30 ml of water and centrifuged at high speed to remove particulate matter. Pure white diaminopimelic acid was then crystallized by the addition of 70 ml of ethanol to the clarified supernatant solution. The cooled suspension was centrifuged and the 70% ethanolic supernatant solution discarded. The product was washed with 7 ml of cold 70% ethanol and then was dried overnight at 105° C. Yield, ca. 260 mg. Analysis:

	C	H	N
Calculated for $C_7H_{14}O_4N_2$	44.20	7.42	14.73
Found (corrected for .62% ash)	43.89	7.48	13.94

A Van Slyke amino nitrogen determination on a separate but equivalent preparation showed 13.63% N. Diaminopimelic acid prepared as described could be recrystallized by solution in water followed by addition of ethanol to a concentration of 70%. One preparation that had been recrystallized five times analyzed as follows:

	C	H	N
Calculated for $C_7H_{14}O_4N_2$	44.20	7.42	14.73
Found (corrected for .44% ash)	44.38	7.95	14.13

Optical activity. The optical activity of once-crystallized diaminopimelic acid with the analysis described above had the rotation summarized as follows:

Solvent	$[\alpha]_{23°}^{D}$ (1.2% solution)
Water	+ 1.2
.05 N HCl	+ 6.3
.2 "	+10.2
2.0 "	+10.5

Other equivalent preparations have shown specific rotations of from +9 to +11.5°. A sample of diaminopimelic acid recrystallized five times from water and ethanol had a rotation of only +1.5° in 2 N HCl.

Water solubility. Thirty mg of once-crystallized diaminopimelic acid was shaken at 23.5°C for 24 hours with 0.4 ml of water in a stoppered tube. The mixture was centrifuged and 0.2 ml of the supernatant was evaporated in a tared weighing dish. A residue of 5.0 mg was obtained, giving a solubility in water of 2.5%. A solubility determination on a sample that has been recrystallized 5 times from water and ethanol gave a solubility, similarly determined except that the temperature was 24.3°, of 1.3%. Work reported that the solubility in water of diaminopimelic acid from a bacterial hydrolysate is 0.92% at 21°C.

Paper chromatography. Three microgram amounts of once-crystallized diaminopimelic acid in 0.01 ml H_2O were spotted near the edge of Whatman No. 1 papers alongside equivalent amounts of synthetic diaminopimelic acid. The paper strips were developed in a number of solvents as indicated below and the position of the isolated and reference diaminopimelic acids was determined by the ninhydrin reaction. In no instance was there any suggestion of more than one ninhydrin-reacting component in the diaminopimelic acid isolated. Separate studies in which 30 μg amounts of diaminopimelic acid were chromatographed with phenol also failed to show the existence of more than one ninhydrin-reactive component even in once-crystallized diaminopimelic acid. RF values are summarized as follows:

Solvent system	RF values: Authentic diaminopimelic acid	RF values: Isolated diaminopimelic acid
Ethanol(7), water(3)	.20	.21
" (1), " (1)	.39	.39
n-Propanol(2), water(1)	.16	.16
n-Butanol (sat. with H_2O)	.01	.01
n-Butanol (sat. with 10% urea)	.01	.01
n-Butanol(4), acetic acid(1), H_2O(5)	.077	.078
Phenol (sat. with H_2O)	.25	.25
n-Butyric acid(9), isovaleric acid(9), H_2O(2)	.061	.062
iso-Amyl alcohol—5% K_2HPO_4	.91	.91
iso-Amyl alcohol—5% KH_2PO_4	.91	.92

Reduction of diaminopimelic acid to pimelic acid. This procedure was carried out stepwise on 107 mg of diaminopimelic acid essentially by the procedure described by Work in which diaminopimelic acid is converted to the corresponding dibromo derivative by reaction with nitrosyl bromide followed by reduction with hydrogen at atmospheric pressure in the presence of platinum black(1). The product obtained after recrystallization three times from benzene and once from water melted at 96.5-98.0°C. An authentic sample of pimelic acid in the same bath melted at 99.0-101.5°C and a mixture of the two melted at 96.5-100.5°C.

Microbiological assays. The activity of diaminopimelic acid as a growth factor for *Escherichia coli* mutant 173-25(2) was determined in the basal medium of Davis and Mingioli(7) according to customary disc-plate assays. Results obtained are summarized as follows:

Conc. of diaminopimelic acid in sol. applied to disc, γ/ml	Zone diameters: Synthetic diaminopimelic acid, mm	Zone diameters: Isolated diaminopimelic acid, mm
100	45	45
50	40	40
25	36	36
12.5	26	27
6.25	18	18
3.125	*	*

* Indistinct.

Determinations of lysine and threonine activity

with *Streptococcus fecalis* R (ATCC 8043) were made according to the procedure of Stokes *et al.*(8) and with *Leuconostoc mesenteroides* (ATCC 8042) according to the procedure of Dunn *et al.*(9). The inability of diaminopimelic acid from *Escherichia coli* to satisfy the lysine requirement of *Neurospora crassa*—lysineless (33933) was determined according to the procedures of Mitchell and Houlahan(10).

Summary. A convenient procedure by which diaminopimelic acid may be readily isolated from the culture filtrate of an *Escherichia coli* mutant is described. The purified diaminopimelic acid resembles that isolated by Work(1) from bacterial hydrolysates with respect to water solubility and absence of significant optical activity. The diaminopimelic acid isolated may be presumed to be the internally compensated *meso* isomer. The possibility that a more water soluble L(+)-diaminopimelic acid may exist in once-crystallized preparations that show only one ninhydrin reactive component was not excluded.

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Effect of Isoniazid on Early Acute Inflammatory Response in Mice. (20116)

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Recent observations on surgical and postmortem lung specimens from patients treated with Isoniazid for tuberculosis revealed extensive healing(1). The granulation tissue in these healing lesions was very vascular and remarkably free from necrosis and inflammation. For this reason it was decided to determine whether Isoniazid had any effect on the host responses apart from its anti-bacterial activity. The local response to inflammation produced by oil of turpentine in mice was investigated.

Experimental procedure. Seventy mice (Swiss Albino) weighing 25-30 g were divided into 2 equivalent groups. One group received 0.25 mg each of Isoniazid (Rimifon) 12 hours prior to subcutaneous injection over the sternal region of 0.025 ml of oil of turpentine. At the time of the oil of turpentine injection, another dose of Isoniazid was administered and thence twice daily until sacrifice. The other group of animals received the oil of turpentine in the same dose and at the same time as the preceding group. Injections of saline were given to this group at the same time that the preceding group received Isoniazid. Fifteen animals in both groups were sacrificed 7 hours and 5 in each group 16, 24, 48 hours and 5 days after oil of turpentine injection. They were autopsied and the area of oil of turpentine injection was excised and prepared for