# DIAMINOPIMELIC ACID DECARBOXYLASE OF THE AGENT OF MENINGOPNEUMONITIS[1]

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ABSTRACT

MOULDER, JAMES W. (University of Chicago, Chicago, Ill.), DOROTHY L. NOVOSEL, AND ILSE C. TRIBBY. Diaminopimelic acid decarboxylase of the agent of meningopneumonitis. J. Bacteriol. **85**:701–706. 1963.—Evidence is presented for the presence in meningopneumonitis particles and extracts of an enzyme decarboxylating $\alpha,\epsilon$-diaminopimelic acid to lysine and for the absence of a corresponding enzyme in the uninfected host. Properties of the enzyme are described and compared with those of bacterial diaminopimelic acid decarboxylases. The significance of these observations with respect to the mode of lysine biosynthesis in the psittacosis group and to its phylogenetic origin is pointed out.

Bader and Morgan (1958) observed that lysine is not required for multiplication of the 6BC strain of psittacosis agent in L cells, although this amino acid is a constituent of the agent (Ross and Gogolak, 1957) and is necessary for growth of the L cells themselves (Eagle, 1955). This suggested that members of the psittacosis group may synthesize their own lysine via $\alpha,\epsilon$-diaminopimelic acid (DAP) as do bacteria (Dewey and Work, 1952; Davis, 1952). This hypothesis was tested by examining concentrated suspensions of the agent of meningopneumonitis, a typical member of the psittacosis group and also known to contain lysine (Jenkin, 1960), for diaminopimelic acid decarboxylase (Dewey, Hoare, and Work, 1954), the enzyme catalyzing the final step in the bacterial synthesis of DAP. The search was facilitated by the absence of this enzyme in the chick embryo host.

[1] A preliminary account of this work was presented at the Annual Meeting of the American Society for Microbiology, Kansas City, Mo., May 6 to 10, 1962.

## MATERIALS AND METHODS

*Organisms.* The Cal 10 strain of meningopneumonitis agent was maintained by passage in chick embryo yolk sac as described by Moulder and Weiss (1951).

A lysine-requiring auxotroph of *Escherichia coli* ATCC 12408 used for microbiological assay of lysine was routinely transferred on slants of Penassay Agar (Difco) to which had been added 30 mg/liter of DL-lysine·HCl.

The *E. coli* auxotroph M-145 (kindly supplied by Charles Gilvarg, Department of Biochemistry, New York University School of Medicine) was used for microbiological assay of DAP. This organism has a multiple amino acid requirement and was therefore maintained on Penassay Agar supplemented with 10 mg/liter of DL-methionine, 50 mg/liter of DL-threonine, 50 mg/liter of DL-lysine·HCl, 50 mg/liter of DL-leucine, and 20 mg/liter of DAP.

*Materials.* Amino acids and pyridoxal phosphate were A grade reagents from the California Corporation for Biochemical Research, Los Angeles. The DAP was a mixture of 40% of the L isomer and 60% of the *meso*. Other reagents were of analytical grade. All solutions were prepared with glass-distilled water.

*Preparation of agent suspensions and extracts.* Concentrated suspensions of meningopneumonitis agent were prepared from infected allantoic fluid as described by Colón and Moulder (1958). Two modifications were introduced. A yolk sac inoculum was substituted for infected allantoic fluid, and 0.33 M ammonium acetate was used as the washing medium instead of phosphate-buffered saline. The final suspension was made in 0.08 M ammonium acetate adjusted to pH 7.0 by addition of NaOH. The infectivity of purified meningopneumonitis agent at 37 C is maintained better in ammonium acetate solutions than in any other simple salt solution tested (J. Litwin, *personal communication*). In addition, the vola-

tility of this salt makes it easy to remove before chromatography and microbiological assay of incubation mixtures.

A typical concentrated suspension of meningopneumonitis agent contained $10^{9.7}$ chick embryo yolk sac $LD_{50}$/ml. Its protein content was 1.12 mg/ml and there was 0.032 mg/ml of total phosphorus. Air-dried and metal-shadowed preparations examined in an electron microscope consisted almost exclusively of agent particles. About two-thirds of the particles were of the large, flat type (Litwin, 1959).

Particle-free extracts were made by disintegrating concentrated suspensions for 30 min in a Raytheon 10-kc sonic oscillator. Titration in chick embryo yolk sac indicated a 90 to 99% loss of infectivity. Electron microscopy revealed many broken particles and much debris. Particle-free supernatants were obtained by centrifugation at 25,000 $\times$ $g$ for 1 hr at 0 C in an angle centrifuge.

Since the enzyme to be studied is present in most bacteria, sterile precautions were observed in preparing particle suspensions and extracts. Bacteriological sterility was established by inoculation of thioglycolate broth and by direct examination of stained smears. All results reported here were obtained with samples sterile at the end of the incubation period at 37 C.

*Analytical methods.* Protein was determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as standard. Bartlett's (1959) procedure was used for total phosphorus.

Samples for determination of total lysine and DAP were hydrolyzed in a sealed tube with 6 N HCl at 100 C for 18 hr.

Samples for microbiological assay and chromatography of free lysine and DAP were prepared by boiling particle suspensions or extracts for 10 min, cooling to 0 C, and centrifuging at 10,000 $\times$ $g$ for 30 min at 0 C in an angle centrifuge. The supernatants were taken to dryness in vacuo to sublime away the ammonium acetate and were resuspended in one-fifth the original volume of water. Samples of 10 to 50 $\mu$liters were withdrawn for paper chromatography, and the remainder passed through small sintered-glass filters and used for microbiological assay.

Paper chromatography of amino acids was carried out by conventional methods. The solvent was phenol-water (80:20, ammonia atmosphere), and amino acid spots were located by reaction with ninhydrin.

For quantitative determination of lysine, Pyrex tubes (13 $\times$ 100 mm) fitted with Morton stainless steel closures were filled with 0.75 ml of the minimal medium of Davis and Mingioli (1950) and 0.15 ml of an unknown sample, autoclaved for 10 min at 15 psi, cooled in running tap water, and inoculated with 0.1 ml of a suspension of *E. coli* ATCC 12408. This inoculum was prepared from a stock slant incubated for 24 hr at 37 C by suspending the growth in 1 ml of minimal medium and diluting 1:500 in the same medium. The inoculated assay samples were kept at 37 C for 24 hr, transferred to 1-ml cuvettes (1-cm light path), and their optical densities were measured at 450 m$\mu$ in a spectrophotometer. A standard curve was constructed for each assay. The growth response was approximately linear in the range from $10^{-5}$ to $10^{-4}$ M L-lysine. DAP gave no growth response in concentrations several times greater than ever encountered in the samples analyzed.

The method of Colón and Moulder (1958) developed for bioautography of folic acid was used for bioautographic identification of lysine on paper chromatograms. The medium and assay organism were the same as in the tube assay of lysine. A spot containing $10^{-4}$ moles of L-lysine gave excellent growth on the assay plate.

DAP was determined with the multiple auxotroph of *E. coli* M-145. The general procedure was the same as in the lysine assay. When the minimal medium of Davis and Mingioli (1950) was supplemented with all the M-145 requirements except DAP ($4 \times 10^{-3}$ M DL-lysine, DL-methionine, DL-threonine, and DL-isoleucine), the growth response was linearly related to DAP concentration in the range from $5 \times 10^{-6}$ to $5 \times 10^{-5}$ M and was not enhanced by the further addition of the other amino acids required by M-145.

## RESULTS

The lysine and DAP content of meningopneumonitis particles was determined by microbiological assay of acid-hydrolyzed samples of three representative preparations. Lysine accounted for 3.9% of the total protein, a level similar to that found in bacteria by Anderson et al. (1958). DAP was not present in significant amounts. Its

concentration could not have been more than 0.3% of the total protein. These findings confirm the observation of Jenkin (1960) that lysine but not DAP was present in paper chromatograms of acid-hydrolyzed meningopneumonitis agent.

When agent suspensions were incubated at 37 C without added substrates or cofactors, paper chromatography showed the accumulation of at least five ninhydrin-positive substances, one of which was lysine (Table 1, Fig. 1). The others have not yet been positively identified. When DAP was added to the incubation mixture, there was a two- to fivefold increase in lysine production, which was further increased by the simultaneous addition of pyridoxal phosphate (Table 1, Fig. 1). There was no lysine accumulation during incubation of fresh agent suspensions at 0 C or of boiled suspensions at 37 C.

Lysine was positively identified by the following criteria: chromatography in phenol-water (80:20, $NH_3$ atmosphere), neutral phenol-water (80:20) and butanol-acetic acid, growth response with the lysine-requiring auxotroph of *E. coli* ATCC 12408, and paper-chromatography-bioautography with the same organism.

These results demonstrated beyond reasonable doubt that suspensions of meningopneumonitis agent contained an enzyme that decarboxylated DAP to lysine. It seemed most unlikely that this activity resided in host-cell contaminants. Lysine is an essential amino acid for the chick, and DAP or enzymes metabolizing it have never been reported in animal tissues. Nevertheless, uninfected chorioallantoic membranes and allantoic fluid were homogenized together, and the homogenate was carried through the same series of differential centrifugations used to prepare the agent suspensions. The uninfected-particle suspensions were diluted so that each milliliter contained the material from 20 to 30 eggs, the same degree of concentration achieved in the final agent suspensions. When incubated at 37 C, these uninfected-host preparations formed no lysine either in the presence or absence of DAP and pyridoxal phosphate.

TABLE 1. *Effect of diaminopimelic acid (DAP) and pyridoxal phosphate on lysine production by meningopneumonitis agent**

| Addition | Lysine produced (mμmoles/mg of protein) |
|---|---|
| None | 25.9 ± 3.2† |
| DAP ($5 \times 10^{-3}$ M) | 58.7 ± 8.2 |
| DAP ($5 \times 10^{-3}$ M) and pyridoxal phosphate ($10^{-4}$ M) | 79.9 ± 14.2 |

* To 0.5 ml of meningopneumonitis agent suspended in 0.08 M ammonium acetate (pH 7) were added DAP and pyridoxal phosphate as indicated above, both in the same diluent. Ammonium acetate was added to a final volume of 1 ml, and the samples were incubated for 18 hr at 37 C.

† Mean ± SE of experiments with seven different preparations of meningopneumonitis agent. As measured by Student's paired *t* test, both DAP and pyridoxal phosphate significantly increased the production of lysine (P = 0.001 and 0.02, respectively).

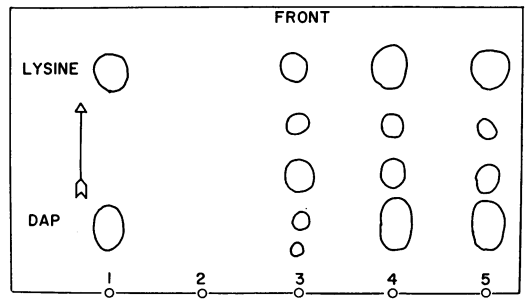


FIG. 1. *Paper chromatography of supernatants from meningopneumonitis suspensions incubated for 18 hr at 37 C; formation of lysine and other ninhydrin-positive compounds.* (1) *Standard DAP and lysine;* (2) *boiled agent suspension, no additions;* (3) *fresh agent suspension, no additions;* (4) *fresh agent suspension plus* $5 \times 10^{-3}$ M *DAP;* (5) *fresh agent suspension plus* $5 \times 10^{-3}$ M *DAP and* $10^{-4}$ M *pyridoxal phosphate. Samples were prepared and chromatographed as described in Materials and Methods.*

Figure 2 shows the rate of DAP decarboxylation at 37 C. The enzyme was active for many hours but at a constantly declining rate. For the first 2 hr of incubation, the $Q_{lysine}$ was 0.1 to 0.2, the same as in the least active bacteria studied by Dewey (1954). At this initial rate, all the lysine of the meningopneumonitis particle would have been produced in 20 hr.

Figure 3 demonstrates that the amount of lysine produced from DAP was proportional to the concentration of meningopneumonitis agent in the incubation mixture.

Lysine synthesis depended on DAP concentra-

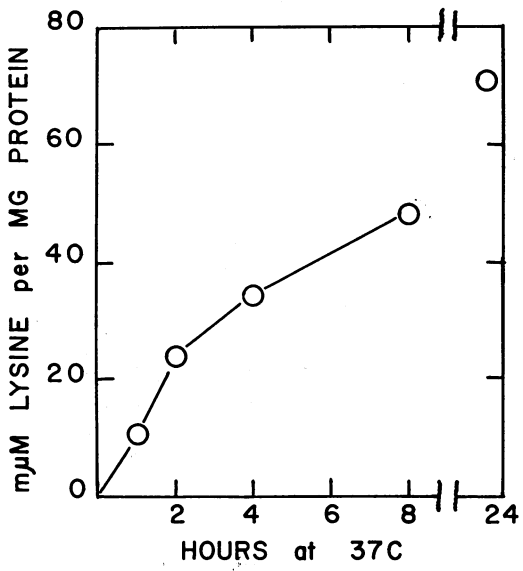


FIG. 2. *Rate of lysine production by suspensions of meningopneumonitis agent incubated at 37 C with 5 × 10⁻³ M DAP and 10⁻⁴ M pyridoxal phosphate.*

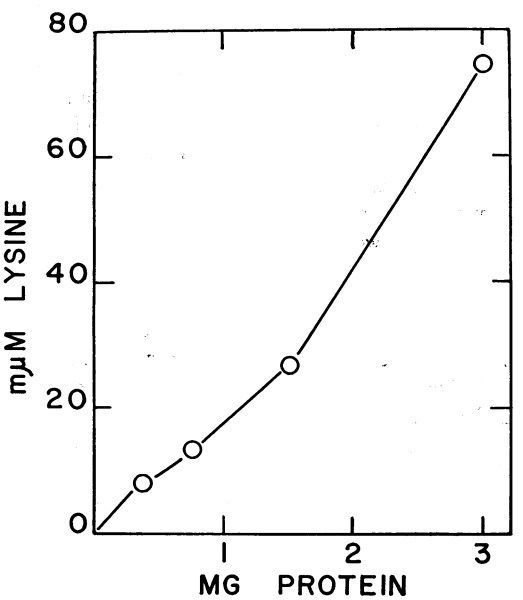


FIG. 3. *Dependency of lysine formation on concentration of meningopneumonitis agent. Suspensions of varying agent concentration were incubated for 6 hr at 37 C with 5 × 10⁻³ M DAP and 10⁻⁴ M pyridoxal phosphate.*

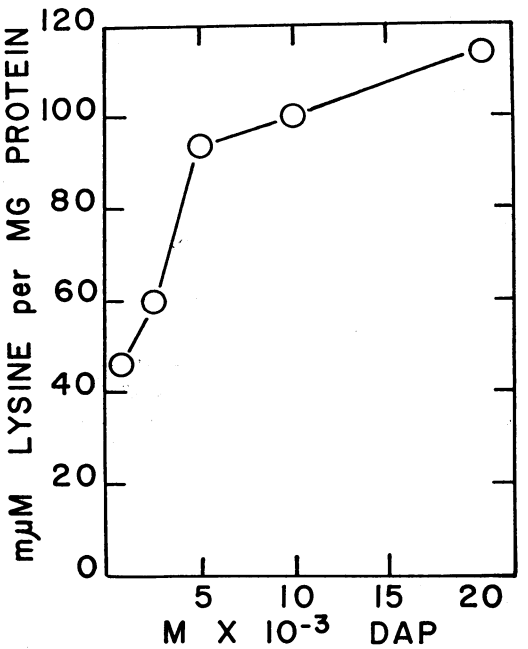


FIG. 4. *Effect of DAP concentration on lysine production by meningopneumonitis agent. Different concentrations of DAP were incubated for 18 hr at 37 C with a constant amount of agent suspension and 10⁻⁴ M pyridoxal phosphate. The values plotted are corrected for an endogenous lysine accumulation of 17 mμmoles of lysine per mg of protein.*

TABLE 2. *Diaminopimelic acid decarboxylase in particle-free extracts of the agent of meningopneumonitis**

| Source | Protein | Lysine formed (mμmoles/mg of protein) | | |
|---|---|---|---|---|
| | | Addition | | |
| | | None | DAP (5 × 10⁻³ M) | DAP (5 × 10⁻³ M) and pyridoxal phosphate (10⁻⁴ M) |
| | *mg/ml* | | | |
| Intact particles | 1.57 | 30 | 58 | 68 |
| Extract | 0.093 | 165 | 496 | 596 |

* Values given are the means of three experiments. Cell-free extracts were prepared as described in Materials and Methods. Each sample consisted of 0.5 ml of extract, DAP, and pyridoxal phosphate as indicated in a volume of 1.0 ml. The diluent was 0.08 M ammonium acetate (pH 7). Samples were incubated for 18 hr at 37 C.

tion as shown in Fig. 4. The Michaelis-Menton constant calculated by the method of Lineweaver and Burk (1934) was 1.7 × 10⁻³ M. The corresponding constant for the DAP decarboxylase of *Aerobacter aerogenes* was estimated to be 2.8 × 10⁻³ M (Dewey et al., 1954).

The meningopneumonitis DAP decarboxylase showed good activity between pH 6 and 8 with an

optimum at about neutrality. At pH 7, 0.08 M ammonium acetate and 0.02 M tris(hydroxymethyl)aminomethane gave the same results.

Active particle-free extracts were readily obtained from sonically disrupted particles (Table 2). Extracts contained one-third to two-thirds the activity of the particles from which they were obtained. Their activity per mg of protein was almost ten times that of intact particles. The addition of DAP to extracts produced a distinctly greater effect on lysine production than it did in particle suspensions, but the stimulatory effect of pyridoxal phosphate remained the same. When the extracts were dialyzed, or aged at 0 C and then dialyzed, there was a slow loss of enzyme activity but no increase in the effect of added pyridoxal phosphate.

## Discussion

The DAP decarboxylases of meningopneumonitis agent and of *E. coli* and *A. aerogenes* (Dewey et al., 1954) have similar pH optima and Michaelis-Menton constants, they all require pyridoxal phosphate for maximal activity, and all are constitutive in nature. The meningopneumonitis agent does not exhibit detectable lysine decarboxylase activity. In this respect, it differs from the bacteria examined by Dewey (1954), all of which contained both DAP and lysine decarboxylases. In possessing DAP decarboxylase but no detectable DAP, the meningopneumonitis agent resembles *Micrococcus lysodeikticus* and *Sarcina lutea* (Dewey et al., 1953).

The principal, and probably only, pathway of lysine biosynthesis in bacteria is via DAP (Dewey and Work, 1952; Davis, 1952; Vogel, 1959*c*; Rhuland and Hamilton, 1961; Finlayson and Simpson, 1961). Therefore, the presence of DAP decarboxylase in the agent of meningopneumonitis and the absence of any exogenous lysine requirement for multiplication of the 6BC strain of psittacosis agent in L cells (Bader and Morgan, 1958) may be explained by assuming that members of the psittacosis group make their own lysine via DAP. Attempts to obtain DAP synthesis in isolated meningopneumonitis particles are now in progress.

In addition to bacteria, some lower fungi (Vogel, 1960), green algae (Vogel, 1959*b*), and higher plants (Vogel, 1959*a*) have also been reported to employ the DAP pathway for lysine biosynthesis. However, the possibility of a phylogenetic link between the psittacosis group and any of these organisms appears remote, and the present results may reasonably be interpreted as adding to the growing weight of evidence for the bacterial ancestry of the psittacosis group (Colón and Moulder, 1958; Colón, 1960, 1962; Jenkin, 1960; Moulder, Novosel, and Officer, 1963).

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