ifying potential of these organs when challenged may not be available.

1. Rutenburg, S., Michael, J. G., Proc. Soc. Exp. Biol. & Med., 1964, v117, 301.
2. Skarnes, R. C., Ann. New York Acad. Sci., 1966, v133, 644.
3. Chedid, L. C., Skarnes, R. C., Parant, M., J. Exp. Med., 1963, v117, 561.
4. Ho, M., Kass, E. J., J. Lab. & Clin. Med., 1958, v51, 297.
5. Collins, R. O., Wood, W. B., Jr., J. Exp. Med., 1959, v110, 1005.
6. Trapani, R. J., Waradvekar, V. S., Landy, M., Shear, M. J., J. Infect. Dis., 1962, v110, 135.
7. Keene, W. R., J. Lab. Clin. Med., 1962, v60, 433.
8. Wiznitzer, T., Better, N., Rachlin, W., Atkins, N., Frank, E. D., Fine, J., J. Exp. Med., 1960, v112, 1157.
9. Rutenburg, S., Rutenburg, A., Smith, E., Fine, J., Ann. New York Acad. Sci., 1966, v133, 633.
10. Palmerio, C., Zetterstrom, B., Shammash, J., Euchbaum, E., Frank, E. D., Fine, J., New Eng. J. Med., 1963, v269, 709.

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## The Presence of Diaminopimelic Acid in the Rickettsiae.* (32119)

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Diaminopimelic acid (DAP) is an amino acid peculiar to the mucopeptide of all bacteria with the exception of the Gram positive cocci. It is not found in yeast, fungi, protozoa, viruses, or in mammalian tissues(1,2). With the discovery of muramic acid in rickettsiae (3) it was a reasonable assumption that rickettsial cell walls contained a mucopeptide similar to that in bacteria. Diaminopimelic acid was first described in the cell wall of *Rickettsia mooseri* by Wood and Wisseman (4). The following report describes its presence in *Rickettsia prowazekii, Rickettsia mooseri, Rickettsia quintana* and *Coxiella burneti.*

*Materials and methods. Rickettsial preparations. R. prowazekii, R. mooseri* and *C. burneti* were grown in the yolk sac of embryonated eggs. *R. prowazekii* and *R. mooseri* were purified by a combination of differential centrifugation and treatment with DEAE cellulose by a batch process(5). Additional purification was performed with a sucrose density gradient(6). The cells were inactivated with 0.1% formalin, washed 3 times in distilled water, lyophilized and dried to constant weight over $P_2O_5$. *C. burneti* was obtained as a formalin killed, lyophilized product which had been purified by a somewhat different procedure(7). It was dried to constant weight.

*R. quintana,* Fuller strain, obtained from Dr. Weiss, Naval Medical Research Institute, was grown in an atmosphere at 5% $CO_2$ on Vinson's agar medium(8). After 5 days the cells were harvested, washed 3 times in physiological saline, and inactivated in 0.1% formalin. Finally, they were washed 3 times in distilled water, lyophilized and dried to constant weight.

*General procedure.* Rickettsial samples varied between 20 and 50 mg dry weight. The cells were first extracted in turn with 5 ml volumes of acetone, alcohol, and ether (5 min with shaking). The cells were then hydrolyzed in 6 N HCl (18 hours, 105 C, $N_2$ atmosphere). The hydrolyzed sample was evaporated to near dryness in a stream of $N_2$ (hot plate, 50-60°C). Residual HCl was re-

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moved by lyophilizing from small volumes of water a total of 6 times. The sample was then dissolved in several ml of water and the pH adjusted to 5.5-6.0. A precipitate which formed was removed by centrifugation. The sample was then concentrated to 0.2 ml and subjected to electrophoresis as described below. Following this, the sample was concentrated to 1.0 ml. A portion of this sample, usually 0.3 to 0.5 ml, was diluted in the DAP basal medium and analyzed for DAP content by the microbiological assay described below. Another portion of the 1.0 ml sample was reduced in volume and transferred quantitatively to Whatman #4 paper and applied as a line. Subsequently, DAP was chromatographically isolated using methyl ethyl ketone : butanol : $H_2O$ 2:2:1(9). The solvent was allowed to drip from the serrated bottom edge of the paper (18 hours). The DAP line was located by a reference spot of known DAP, extracted from the paper and concentrated to a small volume, usually 0.2 ml. A quantitative acid ninhydrin DAP assay was performed on a portion of the isolated DAP as described below. Another portion of the isolated DAP was rechromatographed using methanol : $H_2O$ : 10 N HCl : pyridine (80: 17.5 : 2.5 : 10)(10) to determine the isomeric state of the DAP. Known reference samples of meso, DD, and LL DAP were run simultaneously.

*Microbiological assay.* The microbiological assay for diaminopimelic acid was performed with an auxotrophic mutant of *E. coli*(11). The assay procedure followed that of Moulder (12) with the following modifications. The rickettsial hydrolysate was diluted in the basal medium and then sterilized by membrane filtration. A minimum of 3 different volumes of this diluted sample was then combined in pre-sterilized assay tubes with basal medium, which had been heat sterilized. Meso-DAP was employed as the standard. The assay range was 0-5 $\mu$g. The total volume was 1.0 ml to which 0.05 ml of inoculum was added. Growth was measured turbidimetrically in microcuvettes in the Beckman DU spectrophotometer. Assays were considered valid only if there was an absence of any appreciable updrift or downdrift in comparing different levels of sample.

TABLE I. Diaminopimelic Acid Analysis of Several Rickettsial Species.

| Organism | % of whole cell as diaminopimelic acid | |
|---|---|---|
| | Microbiological assay | Ninhydrin assay |
| R. quintana | .24 | .25 |
| " | .22 | .25 |
| R. mooseri | .15 | .14 |
| " | .13 | .12 |
| " | .14 | — |
| R. prowazekii | .17 | .13 |
| " | .23 | .15 |
| C. burneti-Henzerling strain | .35 | .35 |
| " " " | .35 | .35 |
| C. burneti-Ohio strain | .50 | — |
| " " " | .50 | — |

*Ninhydrin assay.* The acid ninhydrin assay for DAP followed the procedure of Work (13), using the modification suggested for analysis in the presence of lysine, ornithine, proline, cystine, or tryptophan. The total volume was reduced to 0.5 ml. Meso DAP was employed as a standard in a range of 0-5 $\mu$g.

*Electrophoresis.* After acid hydrolysis the rickettsial sample was quantitatively transferred to 6 paper strips in the Spinco Model R electrophoresis chamber. Separate strips were run with pure samples of DAP and cystine. The buffer was ammonium acetate, 0.05 M, pH 5.5, the approximate isoelectric point for DAP. A constant voltage of 220 V was applied for 18 hours. After drying the DAP and cystine reference strips were sprayed with ninhydrin. Using the reference strips as guides, the neutral amino acid band containing DAP and the cystine band (isoelectric point pH 5.0) were located on the strips containing the rickettsial sample. The neutral amino acid band was cut from the strips, eluted with water, pooled, and concentrated.

*Results.* Diaminopimelic acid was found in whole cells of *R. prowazekii, R. mooseri, R. quintana,* and *C. burneti* (Table I). The values obtained in repeated assays of a single organism do not vary significantly. There is also good agreement between the values obtained by the two independent techniques, the microbiological assay and the acid ninhydrin assay.

The isomeric state of the DAP in the vari-

ous rickettsiae was determined by an indirect method. The solvent system(10), methanol: $H_2O$ : 10 N MCl : pyridine (80 : 17.5 : 2.5 : 10) permits a partial resolution by paper chromatography of the 3 DAP isomers, meso, LL and DD. The LL isomer shows a somewhat higher mobility than the other two and is easily separated, while the meso and DD isomers have almost identical mobilities. The chromatographically purified DAP obtained from the various rickettsiae showed a mobility identifical to that of meso and DD DAP. It was determined in a separate experiment that the DD isomer did not permit any growth of the assay organism when used over the concentration range of the assay test while equal amounts of growth were obtained when comparing equal amounts of the meso and LL isomers. The acid ninhydrin assay for DAP does not distinguish between isomers.

Since the LL isomer was eliminated by chromatography, the growth obtained in the microbiological assay must be due entirely to the meso form. Considering the close agreement between the DAP data obtained by the microbiological assay and the acid ninhydrin assay, it is believed that the rickettsiae examined contain only the meso variety, although the possibility of trace amounts of the DD isomer cannot be excluded.

*Discussion.* The presence of DAP in all of the rickettsiae tested provides additional evidence for the essentially bacterial nature of these organisms. The quantity of DAP found in the rickettsiae is only slightly lower than that reported for several Gram negative bacteria(14). The fact that DAP is present in the meso form only is not unexpected as this is the situation in the majority of the bacteria. Although no studies were made to localize the DAP to the rickettsial cell wall, previous studies in various bacteria(15) and of *R. mooseri*(4) have shown that DAP was always localized there.

Diaminopimelic acid is now known to be a member of the aspartic acid family(16), with the biosynthetic pathway branching off at the aspartic semialdehyde step. Pyruvate supplies the other 3 carbons of the 7 carbon DAP (16). The subsequent steps in DAP synthesis involve enzymes that are peculiar to bacteria. Since DAP synthesis does not occur in the uninfected host system, these enzymes are likely to be present in rickettsiae. The immediate precursor of meso DAP is LL DAP; therefore a DAP racemase is probably also present in the rickettsiae tested. In bacteria that have no nutritional need for lysine, it is obtained directly by the decarboxylation of DAP. It is not known yet whether rickettsiae have a DAP decarboxylase, although Moulder *et al*(12) have shown its presence in another intracellular parasite, the agent of meningopneumonitis.

Both DAP and muramic acid are found in the mucopeptide moiety of the bacterial cell wall. With the finding of both of these compounds in rickettsiae, the presence of mucopeptide in rickettsial cell walls is a reasonable assumption. It is also probable that the d-isomers of alanine and glutamic acid would be present along with appropriate racemase. Finally, one may assume that the rickettsiae have the complex of enzymes involved in the step-wise synthesis of the mucopeptide.

*Summary.* Diaminopimelic acid is present in whole cells of *R. prowazekii, R. mooseri, R. quintana,* and *C. burneti* in concentrations only slightly lower than those reported for Gram negative bacteria. In each species studied meso DAP is the only isomer present.

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1. Work, E., Biochem. J., 1951, v49, 17.
2. Work, E., Dewey, D. L., J. Gen. Microbiol., 1953, v9, 394.
3. Allison, A. C., Perkins, H. R., Nature, 1960, v188, 796.
4. Wood, W. H., Jr., Wisseman, C. L., Jr., J. Bact., 1967, v93, 1113.
5. Fiset, P., Silberman, R., Arch. de l'Inst. Pasteur de Tunis, 1966, v43, 231.
6. Ribi, E., Hoyer, B. H., J. Immunol., 1960, v85, 314.
7. Ormsbee, R. A., Peacock, M. G., J. Bact., 1964, v88, 1205.
8. Vinson, J. W., Fuller, H. S., Path. Microbiol., 1961, v24, 152.
9. Wolfe, M., Biochim. Biophys. Acta, 1957, v23, 186.

10. Rhuland, L. E., Work, E., Denman, R. F., Hoare, D. S., J. Am. Chem. Soc., 1955, v77, 4844.
11. Gilvarg, C., J. Biol. Chem., 1962, v237, 482.
12. Moulder, J. W., Novosel, D. L., Tribby, I. C., J. Bact., 1963, v85, 701.
13. Work, E., Biochem. J., 1957, v67, 416.
14. Salton, M. R. J., in The Bacterial Cell Wall, Elsevier Publishing Co., Amsterdam, 1964.
15. Cummins, C. S., Harris, H. J., J. Gen. Microbiol., 1956, v14, 583.
16. Gilvarg, C., Fed. Proc., 1960, v19, 948.

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## Antiscorbutic Activity of D-Araboascorbic Acid.* (32120)

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In 1933-36, Dalmer and Moll(1), Zilva(2) and Reichstein and Demole(3) recognized D-araboascorbic acid as a factor with antiscorbutic properties, although considerably less active than L-ascorbic acid.† Recent reports claimed that D-araboascorbic acid was not able to replace the antiscorbutic role of L-ascorbic acid(4,5). In view of the controversy concerning the vitamin activity of D-araboascorbic acid, we investigated this problem on guinea pigs fed a scorbutigenic diet supplemented with various doses of D-araboascorbic acid.

*Methods and results.* Experiments were performed on young adult male animals, 4-5 months-old, weighing 330 ± 40 g. The guinea pigs were initially fed Wayne Guinea Pig diet containing 40 mg L-ascorbic acid per 100 g for 2 weeks. After this adaptation period the animals were given our purified scorbutigenic diet(6,7) for one week (preparatory period) and thus depleted of ascorbic acid. The animals were then divided into 10 groups of seven each. For 38 days, 6 groups were fed the scorbutigenic diet supplemented with daily oral doses of 1, 2, 10, 50, 100 or 200 mg D-araboscorbic acid. However, 3 guinea pigs from the group that received 10 mg D-araboascorbic acid were kept in the experiment for 115 days. Three groups were fed the scorbutigenic diet and daily doses of 1, 2 or 15 mg L-ascorbic acid for 38 or 115 days. Animals of one group were fed the scorbutigenic diet exclusively until they died. In the above studies L-ascorbic acid and D-araboascorbic acid were dissolved in water and administered in volumes of 1 ml by pipet.

During the experimental period we observed the appearance of animals and recorded their weights regularly (Fig. 1 and 2); at autopsy

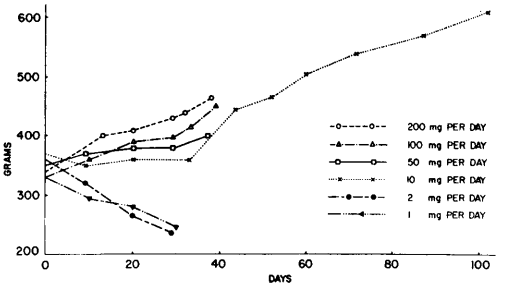


FIG. 1. Average body weight of guinea pigs receiving D-araboascorbic acid.

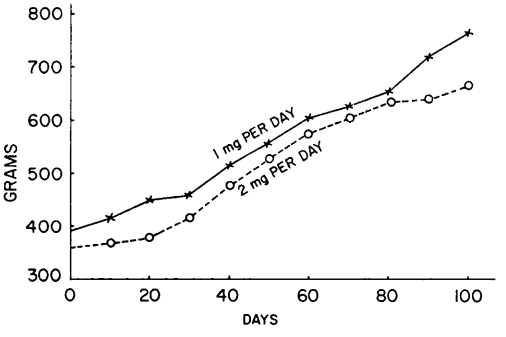


FIG. 2. Average body weight of guinea pigs receiving L-ascorbic acid.

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† L-ascorbic acid or ascorbic acid is also known as L-xyloascorbic acid or vitamin C. D-araboascorbic acid (known also as isoascorbic acid or erythorbic acid) differs from L-ascorbic acid only in the specific configuration of — H and — OH groups around the fifth carbon.