fluids. Our data are in accord with the behavior of proteins in plasma and the knowledge that the glomerular membrane is relatively impermeable to protein in spite of a high filtration rate in the kidney.

The data suggest that in diagnosis of poisoning by botulism it is advisable to collect blood for toxicity tests. It should be pointed out, however, that in human cases brought to autopsy toxin has been detected in extracts of liver when blood specimens have been negative(4).

Summary. In rabbits which were given type A botulinum toxin intravenously, and which died rapidly of acute poisoning the toxin was found in lymph and plasma with no tendency to diffuse into erythrocytes. Ratio of toxin in lymph to plasma ranged between 1:6 to 1:12. Sedimentation coefficients of the toxin in these body fluids were similar and had the dimensions of a protein. The particle size was decreased in relation to crystalline toxin, was similar to toxin in lymph from rats given toxin intraduodenally, but was not sufficiently smaller to warrant the assumption of complete dissociation of toxin from the hemagglutinin present in crystalline toxin.

Toxin was absent from fetal blood, but appeared in small quantities in urine and bile. Cerebrospinal fluid was free of toxin except for a small amount at death. In the normal eye none or only a small quantity of toxin appears in the intraocular fluid. By experimental reduction of intraocular pressure toxin in intraocular fluid tends to approach concentrations present in plasma. Inflammation can lead to the occurrence of toxin in intraocular fluid. The findings are compatible with the hypothesis that the toxin *in vivo* has the dimensions of protein and passes tissue barriers by filtration mechanisms normally operative.

1. Heckly, R. J., Hildebrand, G. J., Lamanna, C., *J. Exp. Med.*, 1960, v111, 745.

2. Meyer, E. A., Lamanna, C., *J. Bact.*, 1959, v78, 175.

3. Legroux, R., Levaditi, J. D., *Compt. Rend. Soc. Biol.*, 1947, v141, 996.

4. Schneider, H. J., Fisk, R., *J. Am. Med. Assn.*, 1939, v113, 2229.

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Amino Acid Composition of Mycobacterial Cell Wall.* (26605)

DELLA R. BELKNAP, MERRILL N. CAMIEN AND MAX S. DUNN
Chemical Laboratory, University of California, Los Angeles

Determinations of mycobacterial cell wall composition have been made semi-quantitatively for taxonomic purposes by Cummins and Harris(1), and quantitatively by Kotani, *et al.*(2), who also studied particulate and soluble fractions from the same mycobacterial cells. Glutamic acid, alanine, and α,a'-diaminopimelic acid were not determined quantitatively by the latter authors but were identified as cell wall components. Antigenic properties of mycobacterial cell wall have been examined by Ribi, *et al.*(3). The present report concerns the quantitative determination of amino acids in cell walls from 4 mycobacteria.

Materials and methods. Cells of *Mycobacterium tuberculosis,* strains† $H37R_a$, and

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† All cultures were supplied by Olive View Hospital through the courtesy of Dr. Seymour Froman. Strain numbers other than the familiar $H37R_a$ are those of the Olive View Hospital culture collection catalogue.

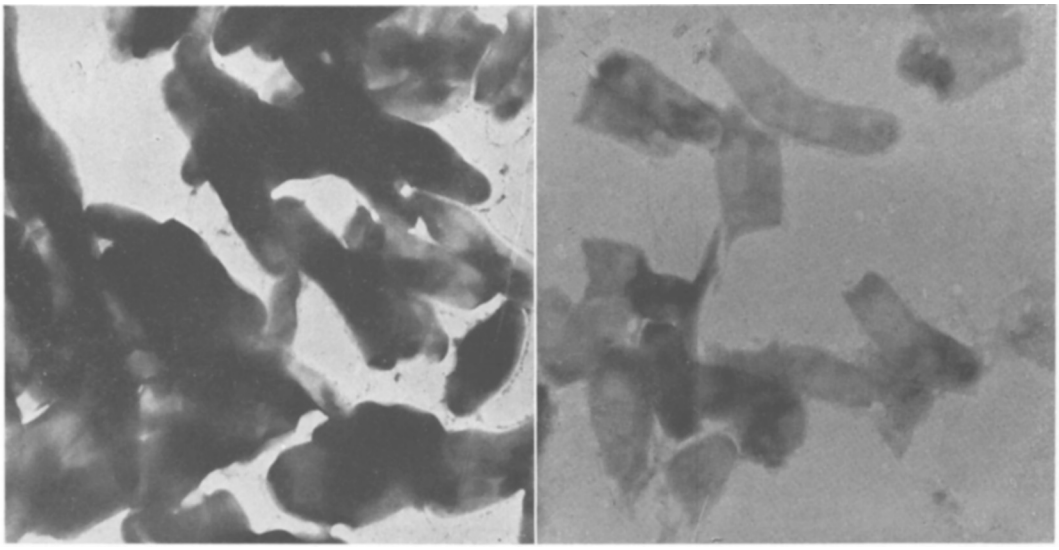

FIG. 1. Electron micrographs of unshadowed and unstained preparations derived from *Mycobacterium sp.*, 'Battey' strain F811. *Left*—partially defatted, air-dried cells which were subsequently subjected to 50 consecutive washings with distilled water (some cell contents are removed by this process). *Right*—cell wall material from the same organism.

F1136, *Mycobacterium ranae,* and the unclassified 'Battey' strain F811, were grown in Roux bottles on Sauton's medium at 37° until maximal pellicle formation had been achieved without onset of degeneration. The cultures of *M. tuberculosis* $H37R_a$ and *M. ranae* were harvested directly, but strains F811 and F1136 (pathogenic) were first sterilized by shaking them with equal volumes of acetone and allowing the mixtures to stand 24 hours. Each lot of cells was harvested by filtration on a Buchner funnel, washed with distilled water, and partially defatted by consecutive 24 hour extractions with acetone and diethyl ether. The air-dried products were stored at room temperature.

Two g portions of cells of each strain were suspended in 30 ml of 0.67 *M* phosphate buffer, pH 7.4, for fragmentation and separation by the method of Kotani, *et al.*(2). Extraneous cellular material was further eliminated by digestion with trypsin and pepsin as described by Cummins and Harris(1), and after final washings the apparently clean cell walls were dried by lyophilization. Absence of cell contents was demonstrated by electron micrography of unshadowed and unstained preparations (Fig. 1). In the case of one batch of cell walls, which will be referred to as "ranae 2," the lyophilized preparation was suspended in phosphate buffer and subjected to an additional separation by the method of Kotani, *et al.*(2).

The products were hydrolysed by heating them in 3 *N* hydrochloric acid in sealed glass tubes for 24 hours at 130°, and the hydrolysates were analysed by the quantitative column chromatographic method of Moore and Stein(4)‡. The chromatographic procedure was checked for precision by running most of the analyses in duplicate and for accuracy by use of recovery samples. The cell wall analyses were verified qualitatively by paper chromatography on Whatman No. 1 paper with the solvent systems, 95% ethanol-concentrated ammonium hydroxide - water 8:1:1, and methanol - 1.25 *N* hydrochloric acid - pyridine 8:2:1.

Results and discussion. Satisfactory pur-

‡ The resin employed was the Analytical Grade of Dowex 50W-x4, minus 400 mesh. Independent ninhydrin color standards prepared with alanine, glutamic acid, diaminopimelic acid and leucine, respectively, were employed in estimating these 4 amino acids in the chromatographically analysed samples. The remaining amino acids were estimated with respect to the leucine ninhydrin color standard, assuming essentially equivalent color yields.

TABLE I. Amino Acid Content of Mycobacterial Cell Wall Preparations.*

Amino acid	Cell wall source†				
	ranae	ranae 2	$H37R_a$	F1136	F811
Glutamic acid	31.8 (5.4, 5.5)	36.5 (5.4)	37.6 (6.8, 6.4)	41.6 (5.4, 5.5)	22.9 (3.2, 3.4)
Alanine	28.9 (3.0, 3.0)	33.5 (3.0)	22.6 (2.4, 2.4)	26.5 (2.1, 2.1)	31.5 (2.8, 2.7)
Diaminopimelic acid	13.6 (3.1, 3.0)	16.1 (3.1)	15.9 (3.6, 3.7)	17.8 (3.1, 3.0)	10.9 (2.1, 2.0)
Aspartic acid	5.8 (.9, .9)	6.7 (.9)	4.4 (.7, .7)	.0 (.0, .0)	11.5 (1.6, 1.4)
Serine	3.3 (.4, .4)	3.8 (.4)	4.8 (.7, .5)	.0 (.0, .0)	5.8 (.7, .5)
Threonine	3.2 (.4, .5)	3.3 (.4)	2.8 (.4, .4)	.0 (.0, .0)	6.0 (.7, .7)
Glycine	6.9 (.6, .6)	.0 (.0)	5.6 (.6, .4)	.0 (.0, .0)	11.5 (.9, .8)
Isoleucine	.0 (.0, .0)	.0 (.0)	6.4 (1.0, 1.0)	5.1 (.6, .6)	.0 (.0, .0)
Leucine	.0 (.0, .0)	.0 (.0)	.0 (.0, .0)	9.0 (1.1, 1.0)	.0 (.0, .0)
Valine	6.6 (.9, .9)	.0 (.0)	.0 (.0, .0)	.0 (.0, .0)	.0 (.0, .0)

* Avg values listed as moles/100 amino acid residues. Original weight % values given in parentheses. Unlisted amino acids enumerated in text could not be detected by either column or paper chromatography, although amounts corresponding to one-tenth the threonine level in $H37R_a$ (column 3) would have been readily measurable if present.

† See text for strain designations and distinction between "ranae" (column 1) and "ranae 2" (column 2).

ity of the cell wall preparations was indicated by their relative freedom from adhering cytoplasmic contents in electron micrographs (representative prints shown in Fig. 1)§ and by absence of chromatographically detectable amounts of arginine, cystine, histidine, lysine, methionine, phenylalanine, proline and tyrosine in their acid hydrolysates (amino acid analyses are given in Table I)‖. Reliability of the amino acid analyses was indicated by good agreement between duplicate determinations (Table I), quantitative recovery of each of the amino acids listed in Table I from known mixtures (typical recoveries ranged from 96 to 104%), and qualitative agreement between analyses by paper chromatography (2 solvent systems) and column chromatography. Essentially complete data from a typical elution analysis are shown graphically in Fig. 2.

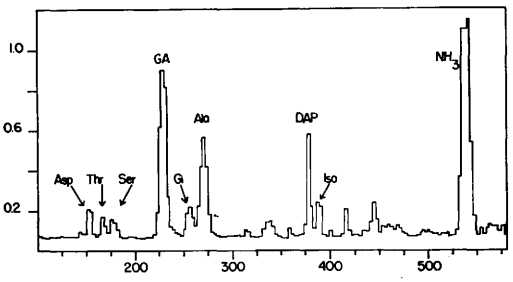

FIG. 2. Elution pattern of amino acids in hydrolysate of cell walls from *Mycobacterium tuberculosis* $H37R_a$. Labeled elution peaks correspond to aspartic acid, threonine, serine, glutamic acid, glycine, alanine, diaminopimelic acid, isoleucine, and ammonia, respectively. Unlabeled irregularities in the base line did not recur in replicate analyses and hence do not appear to have derived from the cell wall hydrolysate.

Glutamic acid, alanine and diaminopimelic acid were major cell wall components in each of the 4 mycobacteria (Table I), occurring in approximate molar ratios of 2:2:1, 2:3:1 and 5:3:2 in *M. ranae* (2 cell wall preparations), the unclassified strain F811, and *M. tuberculosis* (both strains), respectively. Aspartic acid, serine, threonine and glycine (Table I) were either major or minor cell wall components in 3 of the mycobacteria, but none of these 4 amino acids was detected in the preparation from *M. tuberculosis* F1136¶. Isoleucine and leucine were found

§ Most published electron micrographs of cell walls have been produced from metal-shadowed specimens and hence are not readily comparable with the unshadowed materials shown in Fig. 1. Shadowing was purposely avoided in the latter preparations since it seemed probable that the resulting electron-dense coating on the outer surfaces of the cell walls would tend to obscure residual cytoplasmic contents if such were present.

‖ Any of the amino acids listed as absent from the cell walls would have been readily measurable if present at one-tenth the level of threonine in $H37R_a$. (Table I). Even smaller amounts would have been detectable but not accurately measurable.

¶ The hydrolysate of this preparation was regrettably subjected to a decolorization with charcoal. It does not seem likely, however, that this treatment accounts for the apparent complete absence of any amino acid.

in cell walls only from the species *M. tuberculosis* (Table I), isoleucine being present in the material from both strains (leucine only from strain F1136). Valine was found in the cell walls only from *M. ranae*. Ammonia, which was incidentally determined in the chromatographic analyses, was present in all of the cell wall hydrolysates at molar levels approximating that in each case of glutamic acid, suggesting that the latter residue in the cell wall structure is normally amidated.

The analyses given in Table I, together with the semi-quantitative data reported earlier by Cummins and Harris(1), suggest that glutamic acid, alanine and diaminopimelic acid are major cell wall components in most or all mycobacteria, although it is evident (Table I) that the molar ratios of these 3 amino acids vary significantly in the cell walls of different mycobacterial species. Additional amino acids appear to be major cell wall components in certain mycobacteria. This is particularly noteworthy in the cell walls of strain F811 (Table I), which contain aspartic acid and glycine at the same molar level as diaminopimelic acid together with serine and threonine at approximately half this molar level. Similarly it may be seen (Table I) that the cell walls of *M. ranae* contain aspartic acid, glycine and valine, each at half the molar level of diaminopimelic acid and that the material from *M. tuberculosis* F1136 contains leucine at half the molar level of diaminopimelic acid. The remaining amino acids, where present (Table I), are generally at not less than one-fourth the molar level of diaminopimelic acid (the only exception is threonine at approximately one-eighth the diaminopimelic acid level in material from *M. tuberculosis* $H37R_a$) and hence may be considered probably significant.

The differences in composition between cell wall preparations "ranae" and "ranae 2," Table I, appear to be of considerable interest. Both glycine and valine are absent from "ranae 2," but are present in "ranae" at molar levels approximately half that of diaminopimelic acid. Possibly the glycine and valine observed in "ranae" were derived from contaminating material which was eliminated by further processing in "ranae 2," although this seems unlikely because the eliminated contaminating material would have to be constituted almost entirely of glycine and valine in order to account for these results. It is tentatively assumed, therefore, that glycine and valine are normally present in cell walls of *M. ranae* but are rendered sensitive to cleavage from the remaining structure by lyophilization, thus accounting for their absence from "ranae 2," which was subjected to an additional separation procedure after having been lyophilized. An apparently analogous solubilization of bacterial cell wall components following lyophilization has been reported by Brown (5).

It would be desirable, if possible, to find some feature in the cell wall composition of pathogenic strains of mycobacteria distinct from that of non-pathogenic strains, which might, therefore, relate cell wall structure to virulence in these organisms. The present data, although more extensive than previously available with regard to amino acid composition of mycobacterial cell walls, is evidently still too limited to reveal whether or not such a distinction exists.

Summary. The amino acid composition of cell walls from 4 strains of mycobacteria has been determined by quantitative column chromatography. Glutamic acid, alanine and diaminopimelic acid were major components in each of the cell wall preparations. Aspartic acid, serine, threonine, glycine, isoleucine, leucine and valine were present at probably significant levels in cell walls of one or more, but not all of the mycobacteria. Chromatographically detectable amounts of arginine, cystine, histidine, lysine, methionine, phenylalanine, proline and tyrosine were absent from all cell wall preparations.

1. Cummins, C., Harris, W., *J. Gen. Microbiol.*, 1956, v14, 583.

2. Kotani, S., Kitsura, T., Hirano, T., Tanaka, A., *Biken's J.*, 1959, v2, 129.

3. Ribi, E., Larson, C. L., List, R., Wicht, W., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 263.

4. Moore, S., Stein, W. H., *J. Biol. Chem.*, 1954, v211, 893.

5. Brown, A., *Biochim. Biophys. Acta*, 1958, v28, 445.

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