Chemical, Biological, and Structural Properties of Stable *Proteus* L Forms and Their Parent Bacteria

C. WEIBULL,¹ W. D. BICKEL, W. T. HASKINS, K. C. MILNER, AND E. RIBI

Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, Montana

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Proteus L forms were disrupted by osmotic shock, and the sedimentable material present in the homogenate was further fragmented in a Sorvall pressure cell. The pressure cell was also used for disrupting normal *Proteus* cells. The homogenates obtained were fractionated by differential centrifugation. Purified endotoxins were isolated from the major fractions by phenol extraction. Material extracted with phenol from the membrane fraction of the L forms was about as toxic and pyrogenic on a weight basis as the typical enterobacterial endotoxins isolated from cell walls of normal bacteria. The yield of extract from L forms was about one-third of that from an equal weight of normal bacteria. No differences in the gross chemical composition of the phenol extracts from the L forms and the normal cells could be ascertained. A close serological relationship existed between extracts obtained from two L forms and their respective parent bacteria, but no such relationship was found in the case of the third L form studied and its parent bacterium. Diaminopimelic acid was not detected in the membranes of the L forms, but these membranes contained most of the succinic dehydrogenase of the organisms. Only small amounts of this enzyme were present in the wall fraction of normal bacteria. The data obtained suggest that precursors of the *Proteus* endotoxins are formed either in the soluble protoplasm of normal cells and L forms or at sites on the membrane from which they are readily liberated into the protoplasm, whereas the final steps of the synthesis of these toxins take place at the cytoplasmic membrane. In normal cells, much endotoxin is transported to and concentrated in the walls.

The present investigation was planned as a study of the relationship between structure and function in stable *Proteus* L forms and their parent bacteria. Special attention was paid to the endotoxins of these organisms. The toxic properties of a *Proteus* L form were studied by Kotelko (17); *Salmonella* L forms were studied in the same respect by Dasinger and Suter (6) and Diena, Wallace, and Greenberg (8). Nesbitt and Lennarz (22) compared the chemical properties of lipids and lipopolysaccharides extracted from a *Proteus* L form and its parent bacterium. Some preliminary results of our investigation were presented earlier (C. Weibull et al., *Bacteriol. Proc.*, p. 57, 1965).

MATERIALS AND METHODS

Organisms, growth conditions, and harvesting. The L forms used, *P. mirabilis* strains L VI, L 9, and L D52, and their parent bacteria have been described

elsewhere (20). Stock cultures of the L forms were grown in 125-ml Erlenmeyer flasks containing Albimi Brucella Broth (Albimi Laboratories, Inc., New York, N.Y.) with 1,000 units of penicillin G per ml, adjusted to pH 7.5. The cultures were incubated at 37 C for 24 hr and were stored at 4 C. Subcultures were made monthly. Normal bacteria were maintained on Nutrient Agar slants.

Cultures of L forms and normal bacteria for experimental work were obtained by growing the organisms in 4-liter Erlenmeyer flasks containing 3 liters of the Albimi Brucella Broth (no penicillin was added to these flasks). Each flask was inoculated with 200 ml of an overnight culture of the organism to be studied, and the flasks were incubated for 9 to 10 hr on a reciprocating shaker (100 strokes per min). The cultures were harvested with a continuous-flow centrifuge (27,000 × g).

Fractionation of the bacterial material. The main fractionation procedures are outlined in Fig. 1 and 2. All steps were carried out at approximately 4 C. The harvested L forms were suspended in a buffer (Tris) of pH 7.4 containing 0.0078 M NaCl and 0.0025 M tris(hydroxymethyl)-aminomethane (26). A 100-ml amount of buffer was added to 1 g (wet weight) of

¹ Present address: Department of Microbiology, University of Lund, Sweden.

tions (e.g., phenol extracts of cell walls) were treated separately. When tests for homogeneity of variance (35) indicated a similar variation in the content of a certain element, class of compounds, or enzyme (e.g., nitrogen, total carbohydrate, etc.) in two or several sets of fractions, average standard deviations were calculated and, from the deviations, standard errors. Thus, most of the standard errors given in Tables 3 and 4 are calculated with at least 6 degrees of freedom.

Ultracentrifugal analysis. A Spinco analytical ultracentrifuge working at 42,040 rev/min and 20 C was used. Samples were dissolved at a final concentration of 1% in 0.1 M Tris buffer at pH 8.0. The Tris buffer contained 0.01% Merthiolate as a preservative.

Biological assays. Doses producing a defined degree of fever in rabbits (FI₄₀) were estimated by the methods described by Haskins et al. (13). Lethality for 11-day-old chick embryos (LD₅₀) was estimated by intravenous injections of samples according to the method of Finkelstein (10).

Serological tests. Antisera were prepared by prolonged immunization of rabbits with suspensions of L forms or normal cells. The organisms were killed with formaldehyde (final concentration 0.2%) or cold acetone. Gel diffusion tests were performed with agar plates containing 0.5% Ion-agar No. 2 (Oxo Ltd., London, England), 0.85% NaCl, and 0.01% Merthiolate.

Electron microscopy. Unsectioned specimens were prepared by fixing samples, suspended in water, with formaldehyde (final concentration, 4%) for 1 hr and dialyzing the fixed suspension against distilled water for 2 days. The dialyzed suspension was suitably diluted with distilled water and drops of it were allowed to dry on Formvar-coated grids.

For sectioning, specimens were prefixed with formaldehyde as described above, centrifuged, and suspended in OsO₄ fixative (16). Agar-block preparations of the bacterial material were made, treated with uranyl acetate, and embedded in Vestopal W (16). Sections of embedded material were cut on a Porter-Blum model MT-1 microtome and mounted on uncoated 300- or 400-mesh grids. Some sections were stained according to the method of Reynolds (27). All specimens were examined in a Siemens Elmiskop I electron microscope at 80 kv; 30-μ apertures were used.

RESULTS

With the exception of immunological properties and the content of diaminopimelic acid (DAP), no significant differences were revealed among the *Proteus* L forms studied (strains L VI, L 9, and L D52), and only immunological differences were noted among the normal *Proteus* strains.

Most of our studies were performed on fractions obtained by disintegration of normal cells and L forms, and by differential centrifugation of the homogenates. Some of the wall material of normal cells that sedimented during the centrifugations was discarded to avoid contamination

of the final cell wall fractions with unbroken organisms. As indicated by chemical data (see below), additional walls or wall fragments were recovered in the fraction "sediment from protoplasm." Some small fragments probably remained suspended in the fraction designated "protoplasm." Therefore, the values in Table 1 indicating the dry weight of the fraction "cell walls," 6.5 to 8.9% of total cell dry weight, give a low estimate of the percentage of cell wall material in whole, normal *Proteus* cells. However, the correct figure probably is not higher than 10%. Because many L bodies did not lyse during the first treatment with buffer, significant amounts of material were recovered from the washes from the L-form membranes. Preliminary tests indicated that the L forms underwent slightly less complete lysis in distilled water and dilute phosphate buffer than in dilute Tris buffer.

Figure 3 shows electron micrographs of sectioned specimens of the wall fraction of normal *Proteus* cells. As can be seen, triple-layered membranes of the "unit membrane" type (31) represented the main structural element in these specimens. However, some ill-defined granular matter was also present. Figure 4 is an electron micrograph of crude, nonsolubilized membranes

TABLE 1. Amounts of material in fractions obtained by differential centrifugation of disrupted cells from three L forms of *Proteus mirabilis* (strains L VI, L 9, and L D52), and their parent bacteria (strains VI, 9, and D52)^a

Type of cells	Fraction	Yield (% of dry wt of whole organisms)	
		Range	Avg
Normal	Protoplasm	82-85	84
	Sediment from protoplasm	0.5-3.0	2.1
	Cell walls	6.5-8.9	7.7
	Washes, cell walls	3.0-4.8	3.6
L forms	Protoplasm	48-59	52
	Sediment from protoplasm	0.5-2.1	1.5
	Crude, solubilized membranes	20-22	21
	Washes, crude membranes	6.5-15	11
	Granular fraction	3.4-9.2	5.8
	Washes, granular fraction	0.8-1.4	1.1

^a One batch from each strain was studied.

FIG. 3. Sectioned specimen of cell walls of *Proteus mirabilis* VI. The walls were isolated by disrupting cells in a Sorvall Cell Fractionator and differential centrifugation of the homogenate thus obtained. $\times 72,000$ (left), and $230,000$ (right).

of L forms. "Unit membranes" were also predominant in such specimens, but evidently many of the membranes enclosed greater amounts of granular matter than did the broken walls of normal cells.

To obtain *Proteus* endotoxins and substances serologically related to these toxins, fractions of normal *Proteus* cells and *Proteus* L forms were extracted with trichloroacetic acid and phenol. Table 2 shows the amounts of extracted and residual materials obtained. Considerable amounts were extracted with trichloroacetic acid from the protoplasm of both L forms and normal cells (the walls of normal bacteria were not subjected to treatment with trichloroacetic acid). The protoplasm of normal bacteria was precipitated with trichloroacetic acid more completely than the soluble protoplasm of the L forms. The largest amounts of phenol-extractable material were obtained from walls of normal bacteria. However, considerable amounts were also extracted from the protoplasm of normal bacteria and from the crude membranes of the L forms. About 91% of the phenol-extractable material

of the L forms was found in the crude membranes. Conversely, about 94% of the trichloroacetic acid-extractable material of these organisms was located in the fraction "soluble protoplasm."

Our chemical studies were performed to compare the composition of normal *Proteus* bacteria and *Proteus* L forms and to compare the toxic extracts obtained from these organisms with endotoxins extracted from other enterobacteria. Table 3 shows the data obtained from normal *Proteus* cells, and Table 4 gives similar data from *Proteus* L forms. Evidently, the phenol extracts of the wall and protoplasm of normal cells contained heptose, hexosamine, and 3-keto-2-deoxyoctonate in much higher concentrations than the fractions subjected to extraction. In the trichloroacetic acid extract of the protoplasm, also, much hexosamine was found, but no deoxyoctonate or heptose. Dideoxysugars were present in only trace amounts in the normal *Proteus* cells. The small amounts of nucleic acids in the wall fraction probably represented cytoplasmic contaminants. The fraction "sediment from protoplasm" had a composition inter-

FIG. 4. Sectioned specimen of crude membranes of *Proteus mirabilis* L 9. The membranes were isolated by osmotic lysis of the L forms and differential centrifugation of the homogenate thus obtained. The membranes were not solubilized in the Sorvall Cell Fractionator. The section was stained with lead citrate. $\times 48,000$ (inset, 88,000).

mediate between that of the cell wall and protoplasmic fractions, indicating that it consisted largely of wall fragments (this sediment was not washed). As could be expected, the fraction "wash, cell walls" was chemically rather similar to the protoplasmic fraction.

Like the phenol extracts of fractions of normal *Proteus* bacteria, similar extracts obtained from L forms contained considerable amounts of heptose, ketodeoxyoctonate, and hexosamine (Table 4). The trichloroacetic acid extracts of the protoplasmic fractions were, however, strikingly different: extracts from the L forms had a high content of nitrogen, but rather low amounts of hexosamine and fatty acids. The reverse was true for the normal cells. Dideoxysugars and ketodeoxyoctonate were detected in trichloroacetic acid extracts of protoplasm from L forms but not from normal bacteria. The composition of the fraction "wash, crude membranes" sug-

gests that it consisted mainly of soluble protoplasm. Similarly, membrane fragments probably formed the main part of the "sediment from protoplasm."

DAP was not found in the crude, solubilized membranes or the granular fractions of the L forms, but small amounts (about 0.05%) occurred in the protoplasmic fraction of strains L VI and L D52. DAP was found neither in the membrane nor in the protoplasmic fraction of strain L 9.

Results from enzymological and biological assays of normal bacteria and L forms are collected in Tables 5 and 6. In the L forms, the succinic dehydrogenase activity was found in the membrane fraction. This is in agreement with the finding that this activity in bacteria is generally bound to membranous matter (14, 39). In the normal *Proteus* cells, however, the succinic dehydrogenase was mainly located in the

TABLE 2. Yields of materials extracted with trichloroacetic acid or phenol from fractions of normal *Proteus* bacteria and *Proteus* L forms^a

Prepn	Yield, % of dry wt of	
	Starting material for extraction ^b	Whole organisms
<i>Normal bacteria</i>		
Trichloroacetic acid extract of protoplasm	0.6-1.0	0.5-0.8
Residue from trichloroacetic acid extraction of protoplasm	79-92	66-77
Phenol extract (water phase) of the above residue . . .	0.4-3.2	0.3-2.7
Residue from phenol extraction	73-85	61-71
Phenol extract (water phase) of cell walls	29-39	2.2-3.0
Residue from phenol extraction of cell walls	46-56	3.5-4.3
<i>L forms</i>		
Trichloroacetic acid extract of protoplasm	0.9-3.0	0.5-1.6
Residue from trichloroacetic acid extraction of protoplasm	54-67	28-35
Phenol extract (water phase) of the above residue . . .	0.07-0.1	0.04-0.05
Residue from phenol extraction	54-62	28-32
Trichloroacetic acid extract of solubilized membranes. .	0.2-0.4	0.06-0.08
Residue from trichloroacetic acid extraction of membranes	86-97	19-21
Phenol extract (water phase) of the above residue . . .	3.5-11	0.7-2.3
Residue from phenol extraction of membranes	76-87	16-18
Phenol extract of granular fraction	1.3-1.5	0.07-0.08
Residue from phenol extraction of granular fraction . .	60-92	3.4-5.2

^a One batch of each of the strains (*P. mirabilis* VI, 9, D52, L VI, L 9, and L D52) was studied.

^b Starting materials: protoplasm (normal bacteria and L forms), cell walls (normal bacteria), solubilized membranes (L forms), and granular fraction (L forms).

nonsedimentable protoplasmic fraction. Evidently, the cytoplasmic membranes of these cells were solubilized when the cells were processed in the cell fractionator. The succinic dehydrogenase activity present in the wall fraction was probably caused by protoplasmic contamination.

As can be seen from the data in Tables 5 and 6, only the phenol extracts of normal cells and L forms were strongly toxic and pyrogenic. The potencies of these extracts could not be differentiated with certainty from each other. The walls of normal cells were mildly toxic and pyrogenic. The residue from the phenol extraction of the protoplasm of normal cells seemed to be slightly more toxic than the protoplasm itself. The reason for this is not clear, but it is probably not attributable to residual phenol.

To compare more closely the chemical composition of the toxic materials extracted from normal *Proteus* cells and *Proteus* L forms, the pertinent analytical data (Tables 3 and 4) were corrected for the nucleic acid content of these extracts. It was assumed that the nucleic acids (probably mainly RNA) did not contribute to the toxicity and pyrogenicity of the phenol extracts and that they contained 16% nitrogen,

9.5% phosphorus, and 45% pentose. Table 7 gives the corrected data for the extracts from walls and protoplasm of normal cells and from L-form membranes. The extract from the granular fraction of the L forms could not be included in the comparison, since its nucleic acid content had not been determined. Analysis of variance showed that no differences could be ascertained in the gross chemical composition of the extracts studied. This should also be evident from an inspection of the error limits given in Table 7.

To elucidate further the nature of the L form membranes and the localization of the phenol-extractable material in these organisms, an attempt was made to purify these membranes by sedimenting a suspension of crude membranes (not previously lyophilized or solubilized) by centrifuging and resuspending separately the top, the middle, and the bottom portion, each portion constituting about one-third of the original pellet. About two-thirds of the upper part of the top portion was resuspended to form the final "top" fraction, which was transparent. About two-thirds of the lower part of the bottom portion constituted the final, rather opaque "bottom" fraction. The remaining parts of the

TABLE 3. Chemical composition of fractions and extracts of normal *Proteus* bacteria^a

Prepn	Nitrogen	Phosphorus	Total carbo- hydrate	Hexose	Heptose	3-6-Dideoxy- sugars	2-Keto-3-deo- xyoctonate	Hexosamine	Total fatty acids	Nucleic acids
	%	%	%	%	%	%	%	%	%	%
Protoplasm.....	14.3 ± 0.4	2.3 ± 0.2	8.4 ± 0.6	1.4 ± 0.2	1.1 ± 0.1	0.2 ± 0.05	0.3 ± 0.1	1.6 ± 0.4	25.8 ± 1.3	20.4 ± 2.2
Trichloroacetic acid extract of proto- plasm.....	5.9 ± 0.4	0.5 ± 0.2	4.9 ± 0.6	2.6 ± 0.8	0.1	<0.1	0.1	18.3 ± 1.5	34.6 ± 1.3	
Residue from tri- chloroacetic acid extraction of pro- toplasm.....	14.0 ± 0.4	2.0 ± 0.2	7.3 ± 0.6	1.1 ± 0.2	0.9 ± 0.1	0.1	0.3 ± 0.1	1.8 ± 0.4	25.3 ± 0.1	
Phenol extract of the above residue.....	4.4 ± 0.4	2.9 ± 0.6	26.9 ± 1.8	13.8 ± 3.2	4.9 ± 1.2	<0.1	2.5 ± 0.6	18.5 ± 1.7	18.5 ± 1.5	9.0 ± 5.1
Residue from phenol extraction of pro- toplasm.....	14.6 ± 0.4	1.8 ± 0.2	7.0 ± 0.6	1.1 ± 0.2	0.7 ± 0.1	0.1	0.2 ± 0.1	1.4 ± 0.4	26.8 ± 1.3	
Cell walls.....	8.5 ± 0.4	1.2 ± 0.2	10.5 ± 0.6	4.8 ± 0.8	3.0 ± 0.4	<0.1	1.7 ± 0.3	9.5 ± 0.4	35.0 ± 1.3	1.4 ± 0.5
Phenol extract of cell walls.....	3.1 ± 0.4	1.6 ± 0.6	29.2 ± 1.5	14.0 ± 2.8	6.5 ± 0.4	<0.1	4.0 ± 0.5	18.5 ± 1.7	20.2 ± 1.3	<1.0
Residue from phenol extraction of cell walls.....	11.5 ± 0.4	0.9 ± 0.2	1.3 ± 0.6	0.5 ± 0.1	0.8 ± 0.1	<0.1	0.2 ± 0.1	3.4 ± 0.4	38.7 ± 1.3	
First wash, cell walls..	13.3 ± 0.5	2.2 ± 0.2	7.8 ± 0.8	1.9 ± 1.5	1.2 ± 0.1	0.1	0.3 ± 0.1	3.1 ± 0.4	26.8 ± 0.2	
Sediment from pro- toplasm.....	10.8 ± 0.4	1.6 ± 0.2	9.3 ± 0.7	3.3 ± 0.9	2.1 ± 0.4	<0.1	1.0 ± 0.3	5.5 ± 0.4	32.0 ± 1.5	

^a All numbers represent per cent of dry weight of fraction analyzed. The ± signs indicate standard errors. One batch each of *P. mirabilis* strains VI and D52, and two batches of *P. mirabilis* 9, were analyzed.

TABLE 4. Chemical composition of fractions and extracts of *Proteus L. forms*^a

Prepn	Nitrogen %	Phosphorus %	Total carbo- hydrate %	Hexose %	Heptose %	3-6-Dideoxy- sugars %	2-Keto-3-de- oxyoctonate %	Hexosamine %	Total fatty acids %	Nucleic acids %
Protoplasm, soluble...	12.8 ± 0.4	2.4 ± 0.2	5.6 ± 0.6	0.6 ± 0.1	0.5 ± 0.1	0.2 ± 0.05	0.5 ± 0.1	0.4 ± 0.1	17.1 ± 1.3	14.8 ± 2.2
Trichloroacetic acid extract of proto- plasm.....	12.2 ± 0.4	1.8 ± 0.6	12.5 ± 0.6	5.8 ± 0.8	0.1	1.5 ± 0.3	0.5 ± 0.1	1.2 ± 0.3	6.0 ± 1.3	
Residue from tri- chloroacetic acid extraction of pro- toplasm.....	16.2 ± 0.4	2.0 ± 0.2	7.1 ± 0.6	0.4 ± 0.1	0.8 ± 0.1	<0.1	0.2	0.1 ± 0.1	23.3 ± 1.3	
Solubilized crude membranes.....	10.3 ± 0.4	2.1 ± 0.2	5.7 ± 0.6	1.3 ± 0.2	1.2 ± 0.1	<0.1	0.4 ± 0.1	2.1 ± 0.3	38.9 ± 1.3	12.3 ± 2.2
Phenol extract of residue from tri- chloroacetic acid extraction of mem- branes.....	3.9 ± 0.4	1.0 ± 0.2	25.6 ± 1.5	8.7 ± 2.8	6.8 ± 1.2	<0.1	3.4 ± 0.5	17.2 ± 1.5	23.0 ± 1.3	4.3 ± 2.2
Residue from phenol extraction of mem- branes.....	11.0 ± 0.4	2.0 ± 0.2	3.6 ± 0.6	0.4 ± 0.1	0.7 ± 0.1	<0.1	0.1	0.5 ± 0.1	41.5 ± 1.3	
Granular fraction.....	13.9 ± 0.4	0.9 ± 0.2	3.3 ± 0.6	0.6 ± 0.1	0.8 ± 0.1	<0.1	<0.1	0.7 ± 0.3	29.8 ± 1.3	7.3 ± 2.2
Phenol extract of granular fraction....	6.3 ± 1.2		29.6 ± 1.5	7.9 ± 1.1	4.9 ± 0.6	<0.1 ^b	2.9 ^b	9.7 ± 1.7		
Residue from phenol extraction of gran- ular fraction.....	14.5 ± 0.4	1.1 ± 0.2	3.1 ± 0.6	0.5 ± 0.1	0.6 ± 0.1	<0.1	0.1	0.1 ± 0.1	27.4 ± 1.3	
First wash, crude membranes.....	13.4 ± 0.4	2.4 ± 0.2	6.4 ± 0.6	0.9 ± 0.2	0.6 ± 0.3	0.1	0.2 ± 0.1	0.8 ± 0.3	21.8 ± 1.3	17.0 ± 4.4
Sediment from proto- plasm.....	10.6 ± 1.2	1.9 ± 0.2	5.0 ± 0.7	1.2 ± 0.2	1.0 ± 0.1	0.1	0.4 ± 0.1	1.8 ± 0.3	33.2 ± 1.5	

^a All numbers represent per cent of dry weight of fraction analyzed. The ± signs indicate standard errors. One batch each of *P. mirabilis* strains L VI and L D52, and two batches of *P. mirabilis* L 9, were examined.

^b For lack of material, only one determination was made.

TABLE 5. Enzymological, pyrogenic, and toxic properties of fractions and extracts of normal *Proteus* bacteria^a

Prepn	Succinic dehydrogenase activity	Pyrogenicity (FI ₄₀)	Lethality for chick embryo (LD ₅₀)
	%	μg	μg
Protoplasm	84 ± 8	200-540	2.2-7.0
Trichloroacetic acid extract of protoplasm		800->1,000	2.6->40
Residue from trichloroacetic acid extraction of protoplasm		280->1,000	1.8-3.1
Phenol extract of the above residue		0.3-4.6	0.0032-0.0090
Residue from phenol extraction of protoplasm		56->1,000	0.14-1.7
Cell walls	6.0 ± 1.4	23-110	0.032-0.18
Phenol extract of cell walls		0.2-0.5	0.0020-0.0028
Residue from phenol extraction of cell walls		68->1,000	0.72-8.0
First wash, cell walls	4.5 ± 2.0		
Sediment from protoplasm	2.7 ± 1.6		

^a The succinic dehydrogenase activities are expressed as per cent of the activity of the original bacterial homogenate. The ± signs indicate standard errors. One batch each of *P. mirabilis* strains VI and D52, and two batches of *P. mirabilis* 9, were studied.

TABLE 6. Enzymological, pyrogenic, and toxic properties of fractions and extracts of *Proteus* L forms^a

Prepn	Succinic dehydrogenase activity	Pyrogenicity (FI ₄₀)	Lethality for chick embryo (LD ₅₀)
	%	μg	μg
Protoplasm, soluble	3.1 ± 1.4	>1,000	13->40
Trichloroacetic acid extract of protoplasm		96->1,000 ^b	1.1-200 ^b
Residue from trichloroacetic acid extraction of protoplasm		>1,000	3.6-76
Solubilized crude membranes	86 ± 8	250->1,000	0.84-5.4
Phenol extract of residue from trichloroacetic acid extraction of membranes		0.19-3.6	0.0017-0.0076
Residue from phenol extraction		960->1,000	0.49-6.7
Granular fraction	2.0 ± 1.4	>1,000	11-29
Phenol extract of granular fraction		0.65-1.4	0.0052-0.011
Residue from phenol extraction of granular fraction		>1,000	2.1-24
First wash, crude membranes	3.0 ± 1.4	600->1,000	13-15
Sediment from protoplasm	3.8 ± 1.6		

^a The succinic dehydrogenase activities are expressed as per cent of the activity of the original bacterial homogenate. The ± signs indicate standard errors.

^b Three batches had FI₄₀ >1,000 and LD₅₀ >40. One batch each of *P. mirabilis* strains L VI and L D52, and two batches of *P. mirabilis* L 9, were examined.

sediments were combined to form a "middle" fraction. Table 8 shows some chemical properties of the fractionated membranes. It can be seen that the top fraction contained less nitrogen and nucleic acids but more fatty acids, hexosamine, heptose, ketodeoxyoctonate, and succinic dehydrogenase than the bottom fraction. More material could be extracted with phenol from the top fraction, but less granular material was obtained upon solubilization of the membranes in

the cell fractionator. The middle fraction exhibited properties intermediate to those of the top and bottom fractions. Figure 5 shows drop preparations of the latter fractions. In the top fraction, more or less torn membranes constitute the main structural element, but some electron-dense bodies can also be seen. Dense bodies are predominant in the bottom fraction. The chemical and morphological data thus indicate that the phenol-extractable material and the

TABLE 7. Chemical composition of materials extracted with phenol from fractions of normal *Proteus* cells and *Proteus* L forms^a

Source of extract	Nitrogen	Phosphorus	Total carbo- hydrate	Hexose	Heptose	2-Keto- 3-deoxy- octonate	Hexosamine	Total fatty acids
	%	%	%	%	%	%	%	%
Protoplasm of normal <i>Proteus</i> cells.....	3.7 ± 0.4	1.9 ± 0.6	24.7 ± 1.5	14.9 ± 3.2	5.6 ± 1.2	2.9 ± 0.5	19.1 ± 1.7	20.5 ± 1
Walls of normal <i>Proteus</i> cells...	3.1 ± 0.4	1.2 ± 0.6	29.2 ± 1.5	14.0 ± 2.8	6.5 ± 0.4	4.0 ± 0.5	18.5 ± 1.7	20.2 ± 1
Membranes of <i>Proteus</i> L forms...	3.6 ± 0.4	0.7 ± 0.2	25.0 ± 1.5	9.0 ± 2.8	7.0 ± 1.2	3.5 ± 0.5	17.5 ± 1.5	23.8 ± 1

^a The data were corrected for the nucleic acid content of the extracts. The ± signs indicate standard errors.

TABLE 8. Composition of fractionated crude membranes of a *Proteus* L form (strain L VI)^a

Fraction of sedimented membranes	Nitrogen	Heptose	2-Keto- 3-deoxy- octonate	Hexo- samine	Total fatty acids	Ribo- nucleic acid	Deoxy- ribo- nucleic acid	Phenol- extractable material	Granular residue after solubiliza- tion	Succinic dehydro- genase ^b
	%	%	%	%	%	%	%	%	%	
Top.....	9.1	1.7	1.0	3.6	43.1	10.4	0.1	10.0	10.9	0.12
Middle.....	11.8	1.3	0.4	2.3	35.7	19.0	0.2			0.09
Bottom.....	13.8	1.2	0.2	1.1	30.6	26.2	0.4	2.2	23.4	0.06

^a The fractions were obtained by selective resuspension and recentrifugation of membranes previously sedimented by centrifugation.

^b Activity per milligram (dry weight) in arbitrary units.

succinic dehydrogenase were bound to the membranous part of these organisms, which probably consisted of lipoprotein.

In the ultracentrifugal patterns of phenol extracts of walls of normal cells, two major peaks were seen: one with a sedimentation coefficient in the range of 30 to 100S, the other in the range of 8 to 15S (the sedimentation rates were very concentration-dependent). In some extracts, the latter component was split into two peaks, the sedimentation coefficients of which differed by about 10% from one another. A small amount of a third component with a sedimentation coefficient of about 2S was seen in one extract. Similar components were found upon centrifugation of phenol extracts of the protoplasmic fraction of normal cells, but in these extracts the component with a sedimentation coefficient of 2S was regularly seen and represented 5 to 60% of the total macromolecular material. The phenol extracts of the L-form membranes exhibited patterns qualitatively similar to those characteristic for the extracts of the protoplasmic fraction of the normal bacteria. In one extract,

however, the component having a sedimentation coefficient in the range of 30 to 100S was missing. The trichloroacetic acid extracts from protoplasm of normal cells and L forms gave no peaks in the ultracentrifuge when centrifuged at 42,040 rev/min for 2 hr; only a sloping line connected with the meniscus appeared. This indicates that these extracts consisted of polydisperse material of rather low molecular weight.

The serological properties of the phenol and trichloroacetic acid extracts of normal *Proteus* cells and *Proteus* L forms were studied by gel diffusion tests. The phenol extracts of walls of normal cells gave two main precipitation lines with antisera prepared against the homologous bacterial strains (Fig. 6). One of these lines was rather broad in patterns given by strains VI and 9, and could sometimes be resolved into two components. The additional line was fainter. The broad line was closest to the serum well. An additional faint line was seen very close to the antigen well in some experiments.

Phenol extracts of the protoplasm of normal cells gave very much the same patterns as phenol

FIG. 5. Electron micrographs of crude membranes of *Proteus mirabilis* L VI. Drop preparations. The membranes were fractionated by selective resuspension and recentrifugation of membranes previously sedimented by centrifugation. A, top fraction; B, bottom fraction. $\times 59,000$.

extracts of walls, and reactions of identity were observed between the main lines given by the two types of material (Fig. 6). The gel diffusion tests also indicated close serological relationships between the trichloroacetic acid extracts and phenol extracts of normal cells, but the former materials gave essentially only one broad line (sometimes resolvable into two components; Fig. 7, left).

The phenol extracts from two L forms (strains VI and L 9) gave strong precipitation lines with

antisera prepared against their parent bacteria (Fig. 6, left). Extracts from the parent bacteria reacted similarly with antisera prepared against the L forms. Reactions of identity were obtained between the main precipitation lines in patterns obtained by extracts from L forms and normal bacteria when these extracts were tested with antisera prepared either against normal cells or L forms (Fig. 6, left). Phenol extracts from the third L form studied, strain L D52, gave no precipitation lines with antiserum against its

FIG. 6. Gel diffusion tests showing serological relationships between phenol extracts from normal *Proteus* cells and *Proteus* L forms. Left, *P. mirabilis* strains VI and L VI; right, *P. mirabilis* strains D52 and L D52. Wells A, extracts of protoplasm of normal cells; wells B, extracts of walls of normal cells; wells C, extracts of crude membranes of L forms; wells S, undiluted antisera against normal, formalinized cells. Concentration of extracts 1 mg (dry weight) per ml.

FIG. 7. Gel diffusion tests showing serological relationships between trichloroacetic acid and phenol extracts from normal *Proteus* cells and *Proteus* L forms. Left, *P. mirabilis* strain 9; right, *P. mirabilis* strain L 9. Well A, phenol extract of protoplasm of normal cells; well B, trichloroacetic acid extract of protoplasm of normal cells; well C, phenol extract of crude membranes of L forms; well D, trichloroacetic acid extract of protoplasm of L forms; wells S, undiluted antisera against formalinized normal cells (left) or L forms (right). Concentration of extracts 1 mg (dry weight) per ml.

parent organism (Fig. 6, right), nor did extracts from the normal strain D52 react with antiserum prepared against the corresponding L form.

Trichloroacetic acid extracts from the L forms gave rather ill-defined precipitation lines close to the antigen well at a concentration of 1 mg/ml (Fig. 7, right), which probably indicates a lower concentration of antigenic material than in the trichloroacetic acid extracts from normal cells. The patterns obtained, however, showed a serological relationship between trichloroacetic acid and phenol extracts of the L forms.

To correlate some of the serological findings with the appearance of the ultracentrifugal patterns, a phenol extract of walls of normal bacteria was dissolved at a final concentration of 0.1% in 0.1 M Tris buffer and centrifuged for 4 hr at $105,000 \times g$. About 80% of this extract was the very high molecular weight component mentioned above (sedimentation coefficient, 30 to 100S), and the rest consisted of about equal amounts of the two smaller components (2S and 15S). The supernatant fluid and the sediment were studied in gel diffusion experiments. It was found that the fainter of the two lines given by the original extract had almost disappeared in the pattern given by the supernatant fluid. However, the sediment gave a precipitation line showing reaction of identity with the faint line given by the uncentrifuged extract (Fig. 8). This tentatively identifies the component giving rise to the faint line in the gel diffusion

FIG. 8. Gel diffusion tests performed on phenol extracts of walls of *Proteus mirabilis* 9. Well B, original extract; well A, supernatant fluid after centrifugation of extract for 4 hr at $105,000 \times g$; well C, resuspended sediment after centrifugation; well S, antiserum against acetone-killed cells of strain 9. Serum diluted 1:10. Concentration of original extract 1 mg (dry weight) per ml.

FIG. 9. Gel diffusion tests performed on phenol extracts of walls of *Proteus mirabilis* 9 before and after treatment with 0.1 M acetic acid at 100 C for 3 hr. Wells A and C, extract after treatment; well B, extract before treatment; well S, antiserum against acetone-killed cells of strain 9. Serum diluted 1:10. Concentration of extract 1 mg (dry weight) per ml.

studies as the 30 to 100S component in the ultracentrifugal patterns. The heavy lines in the diffusion patterns were probably given by the 2S and 15S components. The faint line disappeared after treatment of the phenol extract with 0.1 M acetic acid at 100 C for 3 hr, indicating a breakdown of the 30 to 100S component (Fig. 9). When the resuspended sediment from the ultracentrifugation experiment was diluted to the same volume as the supernatant fluid, the ratio of toxicity (pyrogenicity) per milliliter of dissolved sediment to toxicity (pyrogenicity) per milliliter of supernatant fluid was about 20. This indicates that the biological activities of the phenol extract studied were mainly associated with the 30 to 100S component.

DISCUSSION

Bacteria can be disrupted by several methods to obtain homogenates suitable for the preparation of purified cell fractions, e.g., cell walls (33). Most values reported for the yield of wall material from gram-negative bacteria disrupted in various ways were considerably higher than those we obtained from *Proteus* (30, 33). However, regardless of possible species differences, many of the wall fractions described elsewhere were probably composed of both walls and cytoplasmic membranes of the organisms studied (14). This was demonstrated with the electron microscope by Boy de la Tour, Bolle, and Kellenberger (3). Salton found most of the succinic dehydrogenase of a *Pseudomonas* sp. in a crude

wall or envelope fraction of organisms disrupted in the Mickle disintegrator (32). This enzyme is generally considered to be located in the membranous part of the bacterial cytoplasm (14, 39). The wall material we studied contained about 5% of the succinic dehydrogenase of the *Proteus* bacteria studied, and about 1% of the nucleic acids. These figures indicated a moderate degree of contamination with cytoplasmic material (cytoplasmic membranes and soluble protoplasm) as was also shown electron microscopically (Fig. 3). It is harder to estimate the purity of the protoplasmic fraction of normal cells. The fraction "sediment from protoplasm" consisted of about 50% each of cell walls and wall fragments, as judged from its chemical composition (Table 3). It thus contained wall material representing about 13% of the material present in the fraction "cell walls" (Table 1). In all probability, considerably less wall material remained suspended in the fraction "protoplasm." This implies that only a small part of the phenol-extractable material obtained from the protoplasmic fraction of normal cells (Table 2) originated from contaminating wall fragments.

The membranous fraction of the L forms, which was obtained by osmotic lysis of the organisms, was undoubtedly heavily contaminated with material from the rest of the protoplasm. This was indicated by their high content of nucleic acids (Table 4) and from their appearance in the electron microscope (Fig. 4). However, about 94% of the trichloroacetic acid-extractable material of the L forms was found in the fraction "soluble protoplasm." This indicates that the osmotic treatment may have released some compounds almost quantitatively into the soluble fraction.

Since the cytoplasmic membrane of the normal cells was solubilized in the cell fractionator, as judged from estimations of succinic dehydrogenase activities (Table 5), the distribution of phenol-extractable material between this membrane and the soluble protoplasm in whole, normal bacteria could not be determined. It is noteworthy, however, that the amounts of material extracted with phenol from the protoplasm of normal cells and the membranes of L forms were about the same when calculated in percentage of the dry weight of whole organisms. Provided that the L-form membranes and the cytoplasmic membranes of normal cells are homologous structures, this suggests that the phenol-extractable material present in the protoplasm of the normal *Proteus* cells was attached to the cytoplasmic membrane. In agreement with the results reported by Nesbitt and Lennarz (22), the total content of phenol-

extractable material in the normal *Proteus* bacteria studied by us was found to be about three times as high as in the corresponding L forms.

In a preliminary communication (C. Weibull et al., Bacteriol Proc., p. 57, 1965), the content of phenol-extractable material in the L-form membranes was given as between 1 and 2%. At that time, the morphology of the fractionated material had not been studied to any considerable extent, and it was believed that the material sedimentable after treatment in the cell fractionator represented the membranous part of the L forms. However, electron microscopy revealed that this material lacked a membranous structure and it was designated "granular fraction." The nature of the granular fraction is not clear. Its low phosphorus and moderate fatty acid content show that it was not composed of volutin or lipid material. Its high nitrogen content rather suggests a proteinaceous nature.

The chemical composition of the *Proteus* cell walls reported here was similar to that of other enterobacterial walls (2, 19, 33). Sectioned *Proteus* walls (Fig. 3) had the appearance of triple-layered "unit membranes" (31). Walls of gram-negative bacteria have often been characterized as "unit membranes," but recent electron microscopic studies have revealed a more complex structure of such walls when they are sectioned in situ (7, 12, 21, 40). The present study thus shows that subcellular structures may have different appearances in the intact cell and after isolation.

No DAP was found in membranes of the *Proteus* L forms, but small amounts were present in the soluble protoplasm of strains L VI and L D52. This is at variance with the findings of Fleck (11), who reported the presence of 0.25% DAP in membranes of a stable L form derived from *P. mirabilis* P 18. These membranes were isolated after the L forms had been disintegrated in a Mickle apparatus. It is difficult to ascertain whether the discrepancy is due to a strain difference or to the mode of disintegration. Our findings also disagree with an earlier finding (38) of considerable amounts of DAP in *P. mirabilis* L 9. However, the organisms studied by Weibull were grown in the absence of penicillin and in a semisynthetic medium. Our present results thus show that many factors may influence the distribution and presence or absence of DAP in L forms.

The chemical composition of the endotoxins isolated from the *P. mirabilis* strains studied by us was rather similar to that of lipopolysaccharides extracted with phenol from another *Proteus* strain and its L form (22) and from *Escherichia coli* (2). Also the trichloroacetic acid extracts

from normal *P. mirabilis* and *E. coli* cells showed some chemical similarity. However, the *Proteus* extracts contained much less carbohydrate. Moreover, they appeared to be polydisperse in the ultracentrifuge and were usually obtained in yields of less than 1% of the extracted protoplasm, as compared with the monodisperse *E. coli* extracts ["native hapten" (2)], which amounted to about 5% of the protoplasm. The trichloroacetic acid extracts of the *Proteus* L forms were chemically very different from the corresponding extracts of *P. mirabilis* and *E. coli* normal cells. They also differed markedly from the trichloroacetic acid extracts obtained by Kotelko, Gromska, and Izdebska (18) from unfractionated *Proteus* L forms. In the latter extracts, heptose and large amounts of hexosamine were found.

The values for the pyrogenicity and toxicity of the *Proteus* endotoxins agree with data obtained from other enterobacteria (2, 29). Dasinger and Suter (6) noted endotoxic activity of penicillin-induced L forms of *Salmonella paratyphi*. These workers injected whole L forms or L-form membranes into mice, and found that the L forms were about one-seventh as toxic as their parent bacteria. Similar results were obtained by Diena, Wallace, and Greenberg (8), who worked with glycine-induced L forms of *S. typhi*. Kotelko (17) reported that antigens of the Boivin type, extracted with trichloroacetic acid from whole *Proteus* L forms, were not toxic for mice. Our data, which indicate that trichloroacetic acid extracts of the soluble protoplasm of *Proteus* L forms are essentially nontoxic, are in agreement with Kotelko's findings.

Our serological studies showed that a close serological relationship exists between the extracts from wall and protoplasm of a particular normal *Proteus* strain. One of the precipitation lines observed in the gel-diffusion tests could be correlated with the toxicity of the phenol extracts. This line was fainter than the lines caused by the material of lower molecular weight present in the phenol extracts, and it was missing in the nontoxic trichloroacetic acid extracts. Our findings are in agreement with the patterns obtained in gel-diffusion tests carried out on extracts from *E. coli* cells (2).

Close serological relationships were also found between two of the L forms and their respective parent bacteria. No such relationships existed between the third L form (strain L D52) studied and its parent organism. This marked change in the serological properties of one *Proteus* strain upon its conversion to an L form was somewhat unexpected. However, Dienes, Weinberger, and Madoff (9) reported that another L form de-

rived from *P. mirabilis* D52 was not agglutinated by antisera against its parent organism and vice versa.

Our data suggest that nontoxic precursors to the *Proteus* endotoxins are formed either in the soluble protoplasm of normal cells and L forms or at sites on the membrane from which they are readily liberated into the protoplasm, probably during growth, since such substances are found in the protoplasm of L forms in the absence of appreciable amounts of endotoxin. The final steps in the synthesis of these toxins take place at the cytoplasmic membrane in both types of organisms. In normal cells, much endotoxin evidently is transported to and concentrated in the walls. This view is in agreement with the results reported by Carey and Baron in studies on *S. typhi* spheroplasts (5). The similarities existing between the normal *Proteus* cells and *Proteus* L forms with respect to the formation, chemistry, and toxicity of their endotoxins indicate that the conversion of normal bacteria into L forms leaves many important functions of the organisms unchanged, as has been pointed out elsewhere (15, 41). However, indications of marked differences in the functions of the *Proteus* L forms and their parent bacteria were obtained during the present study. The trichloroacetic acid extracts from the L forms and the normal cells were chemically very different. The incorporation of DAP into a structural framework was totally inhibited in the L forms, and in one of the L forms the serological properties were strikingly different from those of its parent organism. Thus, as was pointed out by Panos on the basis of his studies on streptococcal L forms (24, 25), it is not justified to regard these forms as simply normal bacteria deprived of their walls.

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ADDENDUM

After completion of our investigation, we encountered the article by Kotelko, Lüderitz, and Westphal entitled "Vergleichende Untersuchungen an Antigenen von *Proteus mirabilis* und einer stabilen L form" (Biochem. Z. 343:227-242, 1965). These workers described the extraction with phenol-water of typical endotoxins from *Proteus* L forms and from normal *Proteus* cells. Trichloroacetic acid extraction of *Proteus* L forms gave nontoxic material, as was also noted by us. In most respects, however, their work and ours are different in content.

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