mal *in vitro* development during one schizogonic cycle.

Finally, when the degree of inhibition of DNA synthesis in the absence of the amino acids, which had a significant effect on parasitic growth, is correlated with the molar amounts of the same amino acids in adult human hemoglobin (7), then it can be seen that inhibition of DNA synthesis is most marked in the absence of these amino acids of which hemoglobin contains the least (Table I). The amino acid composition of human rather than monkey hemoglobin is compared because of its availability. The composition of monkey hemoglobin is believed to be similar (8).

Summary. *l*-Isoleucine and *l*-methionine are essential for growth of erythrocytic forms of *P. knowlesi.* An exoerythrocytic source of *l*-cystine, *l*-tyrosine, *l*-arginine, *l*-glutamine, *l*-histidine, and *l*-lysine is required for optimal *in vitro* development of *P. knowlesi* during one schizogonic cycle.

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Relationship of Chemical Structure to Hemodynamic Properties of Endotoxins* (32667)

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Circulatory changes terminating in irreversible shock represent one of the most typical effects of intravenously administered bacterial endotoxins in mammals (1,2). Hemodynamic alterations are characterized primarily by a marked drop in systemic arterial blood pressure and a concomitant increase in portal vein pressure. In the dog, these changes seem to result from pooling of blood in the liver and diminished venous return to the heart (3).

The unusual variety of endotoxic activities and reactions (4) has stimulated attempts to correlate the chemical structure and biological properties of endotoxins isolated from various gram-negative microorganisms (5,6,7). It appears from such studies that the toxic properties of intact endotoxin preparations depend upon certain minimal chemical and physical–chemical requirements (8,9,10).

Recent observations by Johnson and Nowotny (11) that not all biological activities of endotoxins are related to each other are of considerable interest and suggest the possibility of several chemically active centers in the macromolecule. Studies correlating specifically the hemodynamic effects and chemical composition of endotoxins have not been reported. On the assumption that, for the elicitation of endotoxic reactions, active preparations of a certain critical size contain a large polysaccharide moiety and smaller lipid and peptide components, procedures have been developed in this laboratory (12) for the sepa-

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TABLE I. Chemical Composition and Toxicity of Endotoxic Fractions Obtained from Two Strains of *S. marcescens*.

Preparation	Anthrone-positive carbohydrate (%)	Uronic acids (%)	Amino nitrogen (%)	Fatty acids (%)	Lethality for mice (LD_{50}) (μg)
WS-101	23.3	6.9	0.37	20.9	> 850 <1000
WS-102	41.3	6.7	0.83	28.8	> 350 < 450
WS-103	18.3	6.7	0.04	13.7	> 850 <1000
RS-101	26.5	7.9	1.03	26.8	> 750 < 850
RS-102	2.9	3.6	7.9	43.5	—
RS-103	30.9	8.4	0.46	26.9	>1250 <1400
RS-104	29.6	7.5	0.92	28.0	> 750 < 850
RS-105	28.7	7.1	0.79	27.8	>1250 <1400
RS-AP	12.4	21.3	1.4[a]	1.9	>2000 <2500

[a] Glucosamine content.

ration of peptido-lipopolysaccharides from biologically inert components present in crude endotoxin preparations.

This paper describes the fractionation, chemical composition, and hemodynamic properties of endotoxic preparations isolated from a chromogenic and a nonchromogenic strain of *Serratia marcescens*.

Materials and Methods. Two strains of *S. marcescens* were used in this study. The chromogenic strain *S. marcescens* 08 which had been grown in an enriched medium was supplied by Merck Sharp and Dohme. The nonchromogenic *S. marcescens*, strain Bizio, was grown in an inorganic medium (13) in this laboratory. Details of the growth of bacterial cultures and procedure used for the isolation and fractionation of lipopolysaccharide (LPS) have been described elsewhere (12).

The initial products used for further fractionation were partially purified nucleic acid-free peptido-lipopolysaccharides obtained from non-chromogenic (WS-101, Table I) and chromogenic (RS-101, Table I) strains of *S. marcescens* by trichloroacetic acid extraction (14). To remove the peptide moiety, the WS-101 and RS-101 preparations were treated with 90% aqueous phenol (15). This treatment resulted in the isolation of fractions WS-102 and RS-102 (Table I) from the phenol phase, and fractions WS-103 and RS-103 (Table I) from the water phase. Addition of acetone to a 95% saturation of a water solution of RS-103 resulted in the precipitation of subfraction RS-104 (Table I). The subfraction RS-105 (Table I) was obtained as a precipitate by the addition of a saturated solution of NaCl to the filtrate remaining after the removal of RS-104.

Isolation of acidic polysaccharides. Top layers obtained after ultracentrifugation of the TCA-extracted crude endotoxin preparation from chromogenic strain of *S. marcescens* were combined and cetyltrimethylammonium bromide ("Cetavlon") added in portions to produce a 2% solution. The mixture was heated on a water bath at 75°C to ensure

complete dissolution of "Cetavlon," allowed to cool to room temperature and stored overnight at 4°C. The supernatant layer was decanted and the precipitate washed several times with distilled water, then dissolved in 0.15 *M* NaCl and the solution dialyzed against tap and distilled water. Lyophilization of the dialyzate yielded a white powder of acidic polysaccharides (RS-AP, Table I).

Analytical methods. Nucleic acid content was determined by recording the absorption of a 1% aqueous solution of LPS at 260 mμ. The absence of inflection at 260 mμ was interpreted as an indication of a nucleic acid-free preparation. Carbohydrate content was determined by the anthrone method of Koehler (16). Uronic acids were measured according to a modified (17) carbazole method of Dische (18). Determination of amino nitrogen was performed according to the method of Meyer (19) using glutamic acid as standard. Fatty acids were determined by the method of Haskins (20). The results were calculated as the percentage of palmitic acid. Lethality for mice (LD_{50}) and the preparation of antisera were carried out according to the standardized procedures described elsewhere (12). The immunodiffusion experiments were performed in agar gel according to the method of Ouchterlony (21).

Hemodynamic effects in dogs. Thirty-six adult mongrel dogs of both sexes were anesthetized with intravenous sodium pentobarbital, 30 mg/kg. Catheters were placed in the aorta via the femoral artery and the portal vein following laparotomy and cannulation of a splenic vein. Pressures were obtained from Statham pressure transducers and continuously monitored on a Sanborn direct-writing recorder. Mean systemic arterial pressure, portal vein pressure, heart rate, pH, and hematocrit were recorded at specified times after intravenous injections of endotoxic fractions and acidic polysaccharides, as in previously reported studies using crude endotoxin preparations from *Escherichia coli* (22). Endotoxin fractions and acidic polysaccharides were administered in doses causing shock (2 mg/kg) and the animals surviving 4 hours after injection were sacrificed.

Results. Fractionation procedure and chemical composition of endotoxic fractions. The fractionation studies clearly indicate that the crude endotoxin preparations obtained by the trichloroacetic acid extraction of chromogenic and nonchromogenic strains of *S. marcescens* contain a mixture of nucleic acids, acid polysaccharides, peptidolipids and one or more peptido-lipopolysaccharide complexes. Nucleic acids and acidic polysaccharides were readily separated from peptido-lipopolysaccharides by preparative ultracentrifugation. The acidic polysaccharides, isolated from the soluble layer by precipitation with Cetavlon, contained 21.3% uronic acid, 12.4% anthrone-positive carbohydrate, and 1.9% fatty acids (Table I). The content of uronic acid was very similar to that of acidic polysaccharides isolated recently (23) from *E. coli*. A small but significant content of fatty acids (1.9%, Table I) and the identity reaction of one of the two immunoprecipitin lines with those of lipopolysaccharide fractions (Fig. 1, well E) indicates the presence of peptido-lipopolysaccharides in the acidic polysaccharides from *S. marcescens*.

The parent endotoxin preparation WS-101, from the nonchromogenic strain, is a complex consisting of a polysaccharide, peptide, and lipid moieties (Table I). It showed two precipitin lines in gel-diffusion tests (Fig. 1). Treatment of WS-101 with hot aqueous phenol partially removed the peptide moiety and some lipopolysaccharide, yielding a water-soluble, toxic preparation (WS-102, Table I). Immunochemical analysis showed the presence of two distinct lines, one of which gave the identity reaction with WS-101 (Fig. 1). Fraction WS-103, isolated from the water phase, was only slightly soluble in water. Its single precipitin line gave the identity reaction with one of the precipitin lines of WS-101 (Fig. 1). A substantial difference between the WS-103 and WS-101 fractions in amino nitrogen and fatty acid content was without significance in respect to toxicity.

The parent fraction RS-101, from the chromogenic strain, had, in comparison with that from the nonchromogenic strain, a higher content of amino nitrogen and of fatty acids (Table I). However, this quantitative difference in chemical composition was of little

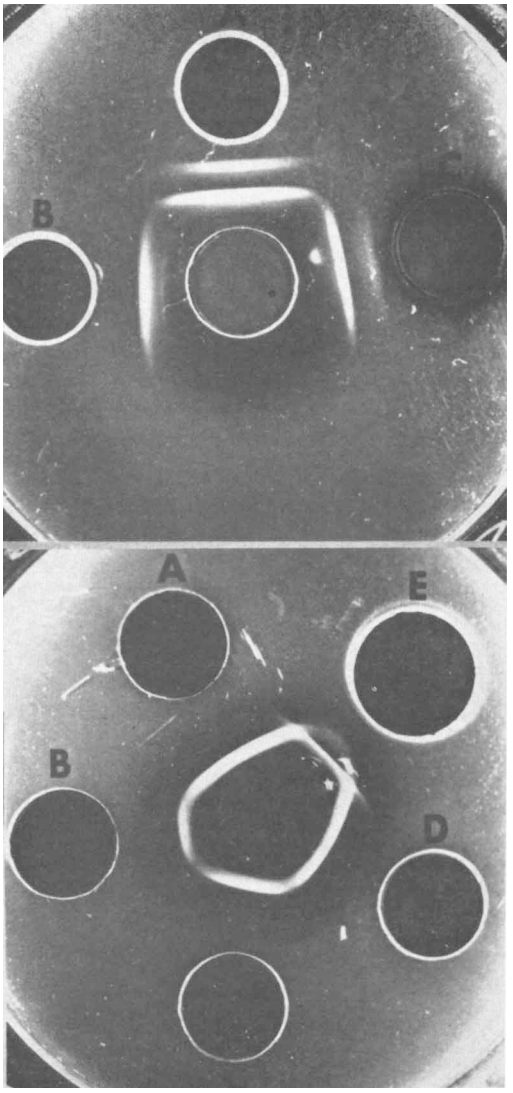

FIG. 1. Gel-diffusion patterns of endotoxin fractions from *S. marcescens*. *Top*: three fractions of nonchromogenic strains: A, WS-101; B, WS-103; C, WS 102. *Bottom*: five fractions of chromogenic strain: A, RS-101; B, RS-103; C, RS-104; D, RS-105; E, RS-AS. Antibodies are placed in the central wells.

significance for the LD_{50} values of these two preparations. The phenol treatment of fraction RS-101 resulted in the isolation from the phenol phase of a complex peptidolipid. In contrast to the fraction WS-102, it contained a high percentage of fatty acids (43.5%) and amino nitrogen (7.9%) and less carbohydrate (2.9%). Unfortunately, due to the insolubility in water, the LD_{50} value and hemodynamic effects of fraction RS-102 could not be determined. The fraction RS-103, isolated from the water phase following phenol treatment, as well as fractions RS-104 and RS-105, obtained by acetone and salt fractionation of RS-103, were all slightly soluble in water. These fractions contained a relatively high percentage of carbohydrate (28–30%), uronic acids (7–8%) and fatty acids (27–28%) and, in comparison with fraction RS-102, a relatively low content of amino nitrogen (0.46–0.92%). The immunochemical analysis indicated the presence of a common antigen in all three fractions (Fig. 1).

Hemodynamic properties of endotoxic fractions. Table II presents the findings following intravenous injections in dogs of the parent (WS-101), phenol (WS-102) and water (WS-103) fractions obtained from the nonchromogenic strains of *S. marcescens*. Significant decreases in mean systemic arterial pressure and elevations in portal vein pressure were observed in the case of each injected substance. Bradycardia occurring in the presence of hypotension, together with a progressive development of acidosis and hemoconcentration, were observed during the 4-hour postinjecting period. Despite the substantially different LD_{50} values of these three fractions, no significant differences were observed among the responses of the reported hemodynamic parameters ($p > .05$).

Table III shows data following injections of the parent fraction (RS-101) and three lipopolysaccharide subfractions (RS-103, RS-104, and RS-105) from the chromogenic strain of *S. marcescens*. Results are similar to those obtained with fractions from the nonchromogenic strain. There were no significant quantitative differences ($p > .05$) among the injected fractions.

Table IV provides data on the effects of intravenously administered acidic polysaccharides (RS-AS), which indicate an earlier and more sustained recovery of systemic arterial pressure following the initial hypotensive phase, in comparison with the responses presented in Tables II and III.

Discussion. The results of this study show that the crude endotoxin preparations ob-

TABLE II. Effect of Fractionation of Endotoxin from *S. marcescens*, Strain Bizio, on Hemodynamic Parameters in Dogs.

Time (min post-injection)	No. of animals	Mean systemic arterial pressure (mm Hg)	Portal venous pressure (mm Hg)	Heart rate (beats/min)	Hematocrit (% RBC)	pH
			Fraction WS-101			
0	6	153 ± 7	10 ± 1	157 ± 11	39 ± 3	7.28 ± .02
30		99 ± 9	13 ± 1	108 ± 8	46 ± 3	7.15 ± .04
120		90 ± 14	11 ± 2	133 ± 19	48 ± 4	7.10 ± .08
240		115 ± 4	12 ± 2	123 ± 9	52 ± 4	7.20 ± .02
			Fraction WS-102			
0	5	139 ± 9	11 ± 2	182 ± 9	44 ± 2	7.27 ± .04
5		88 ± 17	20 ± 1	150 ± 19		
30		106 ± 10	10 ± 1	162 ± 16	46 ± 2	7.16 ± .02
120		77 ± 7	9 ± 2	167 ± 9	50 ± 1	7.19 ± .03
240		104 ± 6	12 ± 1	158 ± 22	49 ± 2	7.25 ± .04
			Fraction WS-103			
0	5	139 ± 9	11 ± 2	182 ± 9	44 ± 2	7.27 ± .04
5		88 ± 17	20 ± 1	150 ± 19		
30		106 ± 10	10 ± 1	162 ± 16	46 ± 2	7.16 ± .02
120		77 ± 7	9 ± 2	167 ± 9	50 ± 1	7.19 ± .03
240		104 ± 6	12 ± 1	158 ± 22	49 ± 2	7.25 ± .04

Note.—Mean values ± SE are given (dose, 2 mg/kg).

tained by the trichloroacetic acid extraction of two strains of *S. marcescens* consist of a mixture of macromolecular compounds which can be separated by fractional ultracentrifugation and precipitation with quarternary ammonium salts into three distinct groups: nucleic acids, acidic polysaccharides, and peptido-lipopolysaccharides. The studies demonstrate that acidic polysaccharides possess relatively low toxicity and elicit negligible hemodynamic responses. They represent, on the basis of these two criteria, relatively inert biological compounds devoid of most of the typical endotoxic properties. On the other hand, it has also been reported that acidic polysaccharides exhibit high pyrogenicity (24) as well as tumor-necrotizing (25) and antigenic properties (23). Since all acidic polysaccharide preparations still contain a certain proportion of lipopolysaccharides, as determined by immunological methods, it is questionable whether some of these biological properties are due to contaminants or to the major products.

Since elimination of nucleic acids and acidic polysaccharides had no effect on toxicity and hemodynamic properties of residual preparations, the complex group of peptido-lipopolysaccharides should be considered as responsible for these endotoxic characteristics. The peptido-lipopolysaccharides isolated from two strains, grown in various media, differed considerably in gross chemical composition but very little in biological properties. Contrary to some recent reports (26,27), the treatment of parent endotoxins with chloroform–methanol resulted in the extraction of negligible amounts of materials, identified almost exclusively as the red pigment or phospholipids in the case of chromogenic and nonchromogenic strains, respectively. However, hot aqueous phenol treatment of the same fractions caused considerable disruption or dissociation of weaker linkages between some components of the peptido-lipopolysaccharide complex. Unexpectedly, a significant difference was found not only in the chemical composition, but also in the solubility properties of the phenol-soluble fractions from the chromogenic and nonchromogenic strains. The toxicity and hemodynamic properties of the phenol fraction

differed very little from those of other fractions. Since one of its two immunoprecipitin lines gave the identity reaction with the fraction isolated from the aqueous phase (WS-103), it is suggested that the phenol phase contained a mixture of an endotoxic lipopolysaccharide and one or more peptides or peptidolipids. In contrast, the corresponding phenol fraction from the chromogenic strain contained primarily amino and fatty acids.

TABLE III. Effect of Fractionation of Endotoxin from *S. marcescens* 08 on Hemodynamic Parameters in Dogs.

Time (min post-injection)	No. of animals	Mean systemic arterial pressure (mm Hg)	Portal venous pressure (mm Hg)	Heart rate (beats/min)	Hematocrit (% RBC)	pH
			Fraction RS-101			
0	5	137 ± 4	8 ± 1	178 ± 12	42 ± 3	7.27 ± .03
5		64 ± 16	15 ± 2	145 ± 13		
30		98 ± 5	9 ± 1	166 ± 12	42 ± 5	7.13 ± .04
120		75 ± 6	8 ± 1	183 ± 12	43 ± 4	7.11 ± .14
240		93 ± 6	8 ± 1	197 ± 13	48 ± 3	7.25 ± .13
			Fraction RS-103			
0	5	141 ± 3	10 ± 1	179 ± 14	44 ± 1	7.31 ± .01
5		86 ± 11	19 ± 2	145 ± 16		
30		100 ± 7	12 ± 1	172 ± 12	51 ± 1	7.19 ± .02
120		78 ± 12	12 ± 1	181 ± 23	51 ± 1	7.16 ± .04
240		93 ± 10	10 ± 2	158 ± 20	52 ± 1	7.22 ± .04
			Fraction RS-104			
0	5	143 ± 14	9 ± 1	160 ± 7	40 ± 3	7.30 ± .03
5		73 ± 16	23 ± 2	122 ± 9		
30		78 ± 17	8 ± 1	143 ± 5	45 ± 3	7.13 ± .03
120		63 ± 13	7 ± 2	156 ± 6	51 ± 2	7.11 ± .05
240		107 ± 28	8 ± 1	170 ± 6	51 ± 4	7.24 ± .03
			Fraction RS-105			
0	5	139 ± 12	10 ± 2	167 ± 13	41 ± 3	7.24 ± .03
5		49 ± 4	23 ± 2	146 ± 13		
30		86 ± 16	10 ± 2	148 ± 15	49 ± 4	7.11 ± .01
120		68 ± 12	11 ± 2	169 ± 15	51 ± 4	7.10 ± .04
240		90 ± 15	7 ± 1	125 ± 50	57 ± 4	7.16 ± .01

Note.—Mean values ± SE are given (dose, 2 mg/kg).

TABLE IV. Effect of Acidic Polysaccharides from *S. marcescens* 08 on Hemodynamic Parameters in Dogs.

Time (min postinjection)	No. of animals	Mean systemic arterial pressure (mm Hg)	Heart rate (beats/min)	Hematocrit (% RBC)	pH
0	5	125 ± 9	146 ± 16	41 ± 2	7.31 ± .02
5		80 ± 15	125 ± 8		
30		120 ± 9	129 ± 9		
120		103 ± 13	144 ± 8		
240		118 ± 11	154 ± 8	56 ± 2	7.33 ± .07

Note—Mean values ± SE are given (dose, 2 mg/kg).

These findings suggest that its water insolubility was probably caused by the high content of a proteolipid of unknown biological properties. Westphal *et al.* (15) have shown that fractions obtained by isoelectric precipitation of phenol phases from various Gram-negative bacteria consist of protein practically free of phosphorus and carbohydrate. Results from the present study have demonstrated that phenol–water treatment of the trichloroacetic acid-extracted endotoxins removes not only the protein moiety, but also other unknown macromolecular components, without significantly altering the toxicity or hemodynamic properties of the compounds remaining in the water phase. These latter fractions from both strains consist mainly of fatty acids and carbohydrates and a very small amount of amino acids which were detected even after phenol treatment. Endotoxic preparations obtained by solvent fractionation of the water phase exhibited qualitatively and quantitatively similar biological properties. Therefore, it is suggested that a macromolecular fatty acid–carbohydrate complex of as yet unidentified structure and size represents the most probable chemical requirement for the elicitation of the hemodynamic properties of bacterial endotoxins.

Summary. The present study was carried out to gain insight into the relationship between chemical structure and hemodynamic properties of endotoxins. Preparations of crude endotoxins, obtained by the trichloroacetic acid extraction of two strains of *S. marcescens,* were fractionated by hot aqueous phenol treatment and sequential acetone precipitation and the carbohydrate, uronic acid, amino-nitrogen, and fatty acid content of each fraction were determined. The effect of fractionation on the endotoxic properties was followed by measuring changes in systemic arterial pressure, portal venous pressure, heart rate, blood pH, and hematocrit produced by intravenous administration of the fractions in dogs. Results indicate that the crude endotoxins of mixture of macromolecular compounds and that sequential removal of nucleic acids, acidic polysaccharides, and peptidolipids had little effect on the hemodynamic properties of remaining lipopolysaccharides. It is suggested that a macromolecular fatty acid–carbohydrate complex of unknown structure and size represents the most probable chemical requirement for the elicitation of the characteristic hemodynamic response to bacterial endotoxins.

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Poliovirus Replication and Cytopathogenicity in Monolayer Hamster Cell Cultures Fused with Beta Propiolactone-Inactivated Sendai Virus* (32668)

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Recently Enders and his associates (1) showed that poliovirus I replicates in primary chick embryo cells and in a line of hamster embryo cells exposed to a high concentration of Sendai virus largely inactivated by ultraviolet (UV) irradiation. It was suggested that poliovirus was incorporated into these naturally resistant cells during the process of fusion brought about by the Sendai virus while the natural barrier to infection presented by the cell surface was temporarily impaired. It was proposed that this technique might prove useful for the propagation of viral agents that hitherto have not been cultivated *in vitro*.

The potential value of the cell-fusion technique for such purposes would obviously be enhanced if it permitted the demonstration of cytopathic effects as well as replication of the agent. In the experiments of Enders and his associates a cytopathic effect could not be recognized with certainty. Although progressive degenerative changes were noted earlier in cells exposed to both polio and Sendai virus, those exposed only to the latter were also ultimately destroyed. Thus the specificity of the change in the poliovirus-exposed cultures remained in doubt. When these experiments were done it was known that the UV-inactivated Sendai virus concentrate contained traces of infectious virus. Later it was found by Dr. George Miller in unpublished experiments in our laboratory that the line of hamster cells employed exhibited cytopathic effects when exposed to unirradiated Sendai virus. Accordingly, the studies reported here were primarily undertaken to determine whether in the absence of all infectious Sendai virus specific cytopathic changes are induced by poliovirus in fused hamster cells. In addition, means were sought to simplify the original procedures which for use as routine were regarded as unnecessarily complicated. As will be described, complete inactivation of Sendai virus without loss of the fusion factor was achieved by addition of beta propiolactone (BPL) and simplification of the technique was effected by adoption of ordinary monolayer cultures instead of cell suspension. In monolayer cultures treated with Sendai virus inactivated in this way cytopathic effects induced by poliovirus were demonstrated.

Materials and Methods. Except as specifically noted hereafter materials and procedures were the same as described by Enders and associates (1). Two lots of poliovirus I (strain Brunhilde) were employed which exhibited titers of $10^{5.8}$ and $10^{6.5}$ ID_{50}/0.1 ml respectively, when assayed in cultures of the AHI line of grivet monkey kidney cells. Sendai virus was concentrated and resuspended ac-

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