EXPERIMENTAL SALMONELLOSIS

III. New Toxic Fraction (L) Obtained from *Salmonella enteritidis* and Its Immunological Properties

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Abstract

Kawakami, Masaya (Gunma University, Maebashi, Japan), Nobutaka Osawa, and Susumu Mitsuhashi. Experimental salmonellosis. III. New toxic fraction (L) obtained from *Salmonella enteritidis* and its immunological properties. J. Bacteriol. **86**:872–879. 1963.—A method is described for the purification of the heat-labile toxins of a fully virulent strain, 116-54, of *Salmonella enteritidis* by ion-exchange chromatography. One component of the heat-labile toxin (L) was homogeneous, as evidenced by the results of the ultracentrifugal analysis and agar gel diffusion test. The mouse LD_{50} was 1.3 μg, and chemical studies indicated that this toxin was a simple protein in nature. It was also evidenced by chemical and immunological tests that this toxin differs from the O antigen (lipopolysaccharide-protein complex).

In a previous paper (Mitsuhashi et al., 1958) it was reported that mice which were superimmunized with live vaccine of *Salmonella enteritidis* resisted 1000 MLD of the same virulent strain injected intravenously. In contrast, a vaccine of killed *S. enteritidis* increased the survival time of mice after challenge, but it was not effective in preventing ultimate death as a result of infection (Ushiba et al., 1953; Mitsuhashi et al., 1958). During the course of the study on immunogenic substances which confer antibacterial protection against the infection of mice with *S. enteritidis*, toxic substances were obtained from the culture filtrate or bacterial cells of *S. enteritidis*.

This paper presents results of studies on the purification and properties of toxic substances.

Materials and Methods

Bacterial strain and culture medium. A virulent strain, 116-54, of *S. enteritidis* was used throughout this study. The strain was lyophilized and stored in a refrigerator at −5 C. About 50 mg of the bacterial cells, which were harvested from an agar medium, were inoculated into a jar containing 10 liters of a semisynthetic culture medium. This medium was composed of $Na_2HPO_4 \cdot 12H_2O$, 80 g; KH_2PO_4, 20 g; $(NH_4)_2SO_4$, 10 g; $MgSO_4 \cdot 7H_2O$, 1 g; sodium citrate, 5 g; glucose, 20 g; Casamino Acids (Difco), 20 g; yeast extract (Difco), 20 g; and distilled water, 10 liters.

The medium was adjusted to pH 7.6. The inoculated medium was incubated at 37 C for 20 hr with aeration. Air was bubbled through at a flow rate of 8.8 liters per min.

Preparation of crude extract. The bacterial cells were harvested and washed with 0.85% saline. Wet packed cells (50 g) were suspended in 200 ml of 0.05 M phosphate buffer (pH 6.8). After heating the bacterial suspension at 56 C for 12 min, it was cooled to 4 C, and the pH was adjusted to 11.0 by the addition of 1 N NaOH. The suspension was maintained at 4 C for 2 hr with gentle agitation by means of a magnetic stirrer, and then was neutralized with 1 N acetic acid. The cells were removed by centrifugation.

To the extract, solid ammonium sulfate was added to 70% saturation, and the precipitated materials were collected by centrifugation. After overnight dialysis of the materials against 0.001 M phosphate buffer (pH 6.0), insoluble materials were removed by centrifugation at 10,000 × *g* for 20 min. The crude extract thus prepared was frozen in a Dry Ice-acetone mixture and stored at −20 C.

Preparation of endotoxin. An endotoxin was prepared from *S. enteritidis* 116-54 by the method of Westphal et al. (1952). The phenol extract of bacterial cells was dialyzed against water, and to it were added 6 volumes of ethanol. The precipitates formed were dissolved in 0.05 M phosphate buffer (pH 6.8) to a concentration of 3%. The

fraction which was precipitated by centrifugation at 76,000 × *g* for 60 min, and was not precipitated at 15,000 × *g* for 10 min, was collected. This fraction was further purified by starch electrophoresis; the material was placed on the midzone of a starch block (1.5 by 7 by 40 cm) steeped with Veronal buffer (pH 8.6; ionic strength, 0.05). After being charged for 16 hr at 200 to 300 v and 16 ma, a fraction, which moved to the cathode and was abundant in methylpentose, was extracted. This fraction was dialyzed and lyophilized. The endotoxin thus obtained was electrophoretically and ultracentrifugally homogenous. Endotoxin prepared by the method of Goebel, Binkley, and Perlman (1945) was also used (i.e., by pyridine extraction, acetone fractionation, and further by starch electrophoresis).

The protein component of the endotoxin, which was separated from 400 mg of endotoxin, was dissolved in 20 ml of water, and an equal volume of 0.2 N acetic acid was added. After hydrolysis in boiling water for 10 min, the resulting precipitates were concentrated by centrifugation at 4 C. The precipitates were washed once with 20 ml of 0.1 N acetic acid and dissolved in 2 ml of 0.1 M phosphate buffer (pH 7.5). The insoluble materials were removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was purified by two successive precipitations with acetic acid as described above. Finally, the precipitates were dissolved in 0.05 M phosphate buffer (pH 7.5) and dialyzed against the same buffer.

Ultracentrifugal analysis. The ultracentrifugal analysis was performed with a model E Spinco ultracentrifuge.

Serological tests. Antibacterial serum was obtained by immunizing rabbits with *S. enteritidis* 116-54; to an overnight broth culture, 0.04 volume of formalin was added and incubated overnight. Each rabbit received an injection of eight increasing doses (0.5 to 3 ml) of this bacterial suspension every 2 days. On the fifth day after the last immunization, the animal was bled. The titers of O and H were 1:800 and 1:12,000, respectively. Another rabbit was immunized with fraction C by administration of a daily dose of 10 to 120 μg for 18 days; the total dose was 1250 μg. A separate rabbit was also immunized daily for 12 days with 20 to 320 μg of the protein component of the endotoxin; the total dose was 3015 μg. The immune sera obtained from these animals were heated at 56 C for 30 min and stored in a refrigerator at −20 C. Agar gel precipitation tests were carried out by the method of Ouchterlony (1953). Different concentrations of antigen and immune sera were placed in the wells appropriately arranged in agar plates. The diameter of the well was 3.8 mm, and the distance between centers of the wells was 15 mm. The precipitation bands were observed after incubation for 1 to 3 weeks at room temperature.

Chemical determinations. Total nitrogen was determined colorimetrically after the wet samples were ashed (Akamatsu, 1952). The amount of phosphorus in the samples was determined by the method of Allen (1940). Reducing sugars were determined by hydrolysis with 1 N H_2SO_4 in sealed ampoules at 100 C for 6 hr, and were evaluated according to the method of Somogyi as modified by Nelson (1944). Pentose and methylpentose were estimated by the method of Dische (Dische and Shettles, 1948; Dische, 1949). Hexosamine and *N*-acetylhexosamine were acetylated with acetylacetone and were determined colorimetrically with Ehrlich's reagent (Elson and Morgan, 1933).

Chromatography. Chromatography was carried out on columns of diethylaminoethyl (DEAE) cellulose, carboxymethyl (CM) cellulose, or Ecteola cellulose (Serva Entwicklungslabor, Heidelberg, Germany). A water suspension of the cellulose powder was allowed to settle for 20 min, and the small particles in the supernatant were removed by decantation. The precipitated celullose was resuspended and packed in a glass tube. About 1 g of the sample was passed through a column packed with 300 ml of wet cellulose. DEAE and Ecteola cellulose were regenerated with 0.1 N NaOH, and CM cellulose was regenerated with 0.1 N HCl. Each of the columns was washed with water and equilibrated against the starting buffer. Samples were also dialyzed against the same buffer. After passing the sample through the column, samples were eluted by stepwise addition of phosphate buffer and buffered NaCl solutions of increasing concentrations. The chromatograph was prepared by plotting the results of the colorimetric protein determination by the modified Folin reaction (Lowry et al., 1951), and of sugar obtained from the anthrone reaction (Morris, 1948).

Toxicity test. Different amounts of each sample were administered intraperitoneally to ddN mice (weighing 15 to 20 g), and deaths were recorded

for 5 days. A group of five mice was used for each amount of test material. The toxicity was expressed by mouse MLD and LD_{50} (Finney, 1952).

RESULTS

Chromatography of crude extract. The crude extract resolved into more than four different fractions (A, B, C, D, and others), depending upon the elution schedule applied on the DEAE-cellulose column (Fig. 1). Toxicity tests were carried out on fractions A, B, C, and D (Table 1). It was observed that the toxicity of fractions A and C was higher than those of the other fractions.

Purification of fraction A. To 1 volume of fraction A, 0.5 volume of saturated ammonium sulfate solution was added. The precipitate formed was removed, and an additional 0.4 volume of saturated ammonium sulfate was added. The precipitate was dialyzed and rechromatographed on another DEAE-cellulose column. A fraction designated as AI, which was eluted by 0.02 M phosphate buffer (pH 7.5), was concentrated by ammonium sulfate precipitation. This fraction had high toxicity, but the toxicity decreased to one-fifth that of the original when heated at 100 C for 10 min (Table 4). Ultracentrifugal studies showed that this fraction contained more than three components involving a substance of high molecular weight. The results of the chemical analysis indicate that the sugar content is proportional to that of the endotoxin (Table 2). This suggests that fraction A contains a polysaccharide which is one of the components of the endotoxin.

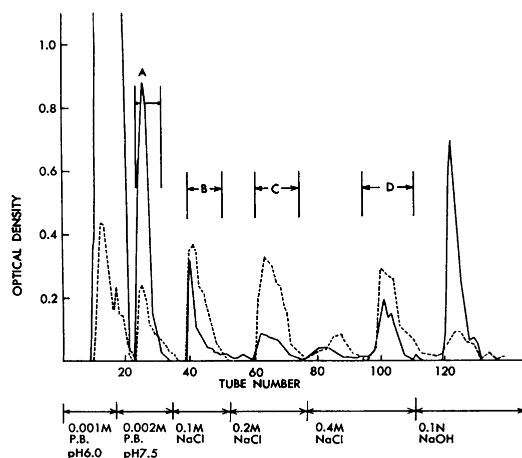

FIG. 1. *Chromatography of a crude extract on DEAE-cellulose column. Crude extract (420 mg) was applied to a column (2.8 by 20 cm), and 7.5 ml of effluent were collected in each tube. Solid line, optical density at 620 mμ in anthrone reaction; dotted line, optical density at 720 mμ in Folin reaction. Total recovery of protein as measured by Folin reaction was 96%. P.B. = phosphate buffer.*

TABLE 1. *Toxicity of fractions A, B, C, and D*

Expt no.	MLD (μg)				
	Crude extract	A	B	C	D
1	220	17.3	129	56.6	280
2	162	22.3	324	62.2	321
3	57.3	19.0	260	64.0	—
4	—	12.4	32.5	58.0	—

TABLE 2. *Chemical composition of each fraction*

Substance	Fraction			Endotoxin
	AI	CI	CII	
	%	%	%	%
Nitrogen	8.3	15.8	14.5	1.5
Phosphorus	1.27	2.25	1.82	0.88
Hexose	22.4	0.68	0	29.8
Pentose	0.96	0	0	—
Methylpentose	18.9	0	0	22.4
Hexosamine	6.36	—	—	1.21
N-acetylhexosamine	3.24	—	—	0.48

Purification of fraction C. Substances of high molecular weight were removed from the crude extract by centrifugation (type 40 P, Hitachi Manufacturing Co. Ltd.) at 76,000 × *g* for 60 min. Then saturated ammonium sulfate was added to the supernatant. Precipitates formed at 33% saturation were discarded, and those resulting from addition to 60% saturation were collected and dialyzed for 6 hr against 0.1 M NaCl-0.01 M phosphate buffer (pH 7.5). The dialyzed portion was applied to a DEAE-cellulose column, which was equilibrated with the same buffer used for dialysis. The addition of 0.15 M NaCl-0.015 M phosphate buffer (pH 7.5) was followed by a 0.2 M NaCl-0.02 M phosphate buffer of the same pH. The second peak, corresponding to fraction C, was obtained and rechromatographed in a similar

manner. From 0.5 g of crude extract, 6.2 to 14.8 mg of the products were obtained. The collected products, corresponding to fraction C, were allowed to flow through a column of CM cellulose

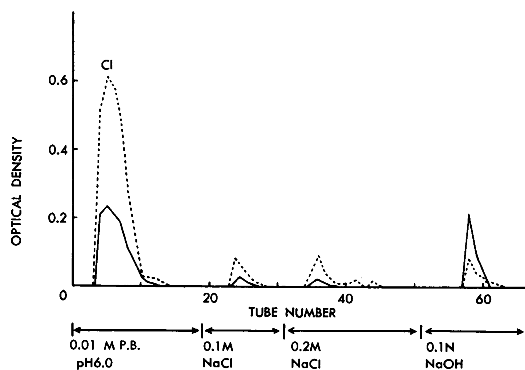

FIG. 2. *Chromatography of fraction C on CM-cellulose column. Fraction C (89 mg) was applied to a column (1.2 by 18 cm), and 7.5 ml of effluent were collected per tube. Solid line, optical density at 620 mμ in anthrone reaction; dotted line, optical density at 720 mμ in Folin reaction. The recovery of protein in the first peak was 75%; in total, 96%. P.B. = phosphate buffer.*

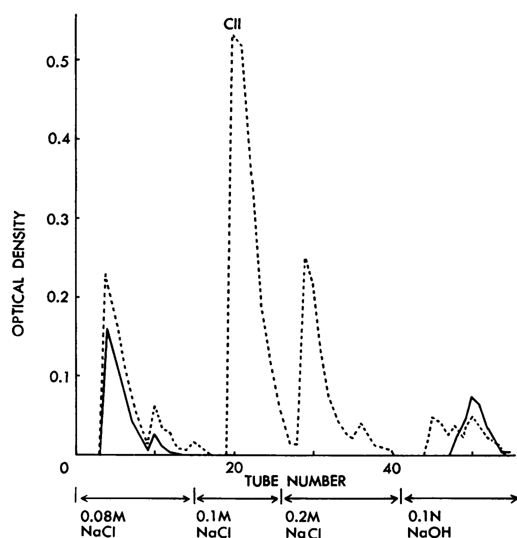

FIG. 3. *Chromatography of fraction CI on Ecteola-cellulose column. Fraction CI (51 mg) was charged on a column (1.2 by 13 cm), and 3.5 ml of effluent were collected per tube. Solid line, optical density at 620 mμ in anthrone reaction; dotted line, optical density at 720 mμ in Folin reaction. Total recovery of protein was 92% as measured by the Folin reaction.*

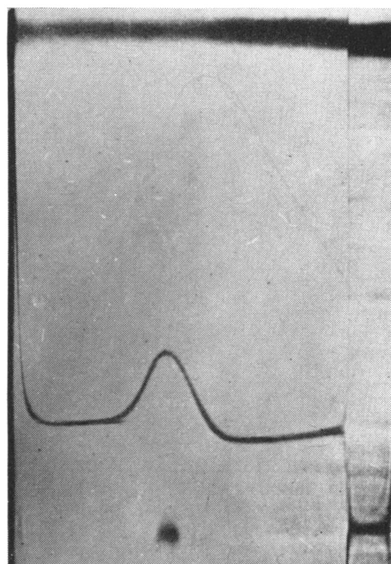

FIG. 4. *Sedimentation pattern of CII. Solution of CII (0.82%) in 0.05 M phosphate buffer (pH 7.5). Rotor speed, 59,000 rev/min; exposure at 75 min.*

buffered with 0.01 M phosphate buffer (pH 6.0) followed by elution with the same buffer (Fig. 2). To the effluent of the first peak, saturated ammonium sulfate was added to 52 to 58% saturation, and a fraction (CI) was obtained. Fractionation of this material was repeated again, and the precipitates were dialyzed against 0.05 M phosphate buffer (pH 7.5). The final purification of this fraction (CI) was performed on a column of Ecteola cellulose. The high peak (CII) which resulted from elution with 0.1 M NaCl-0.01 M phosphate buffer (pH 7.5) contained no sugar detectable by the anthrone reagent (Fig. 3).

Chemical and physical properties of CII. Homogeneity of fraction CII was evidenced by the ultracentrifugal analysis (Fig. 4).

Data shown in Table 2 indicate that CII did not contain any sugar. No peak was found at 380 to 440 mμ, although 480 μg of fraction CII were used for the determination of methylpentose (Fig. 5). A slight rise in the optical density at 570 mμ was shown by the anthrone reaction, but no peak was visible at 620 mμ, which is characteristic for the reaction of hexose (Fig. 6). The CII fraction showed an ultraviolet-absorption spectrum with a maximum at 275 mμ (Fig. 7). No reducing sugar

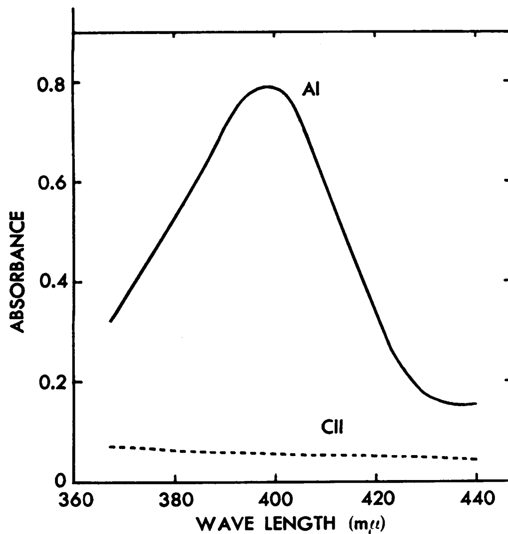

FIG. 5. *Absorption spectra of AI and CII. Fractions AI and CII were dissolved in 0.05 M phosphate buffer (pH 7.5) at concentrations of 602 μg/ml and 600 μg/ml, respectively. Solid line, AI; dotted line, CII.*

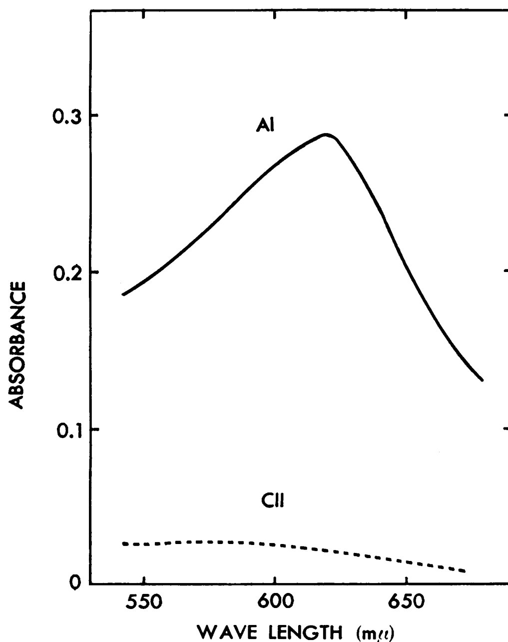

FIG. 6. *Color reactions of fractions AI and CII by Dische's method for methylpentose determination. Solid line, 50 μg of AI; dotted line, 480 μg of CII.*

was detectable in fraction CII when examined by the Somogyi-Nelson method. The nitrogen content of CII was 14.5%.

From these results, it can be concluded that CII may be a protein which is free from nucleic acid and sugar. The lipid content of CII is now under analysis.

Toxicity of CII. The mouse LD_{50} of CII was shown to be 24.4 to 26.2 μg in different samples prepared by the same procedure. The time necessary for death was 20 hr to 4 days. Dullness and occasional diarrhea, but no neuropathic symptoms, were observed.

No decrease in toxicity of the endotoxin after heating at 100 C for 30 min was demonstrable

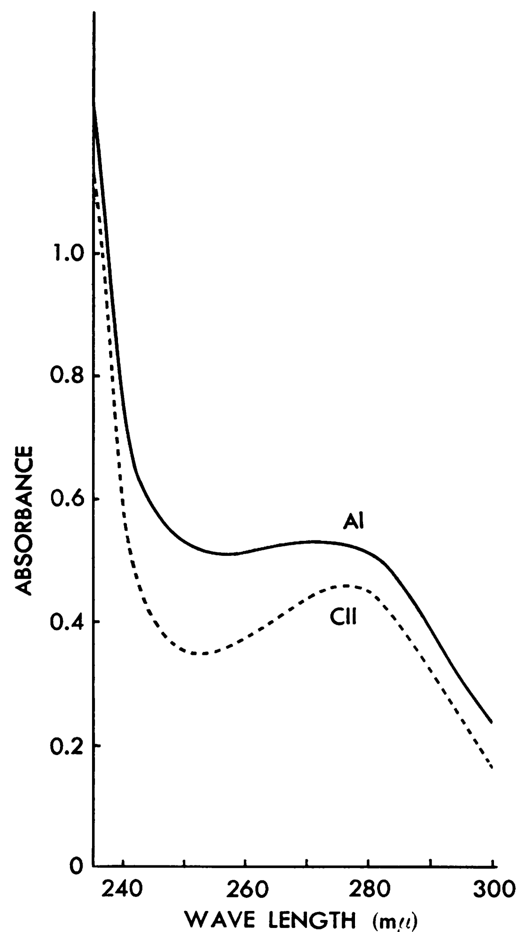

FIG. 7. *Color reactions of fractions AI and CII by anthrone reagent for hexose determination. Solid line, 60 μg of AI; dotted line, 120 μg of CII.*

TABLE 3. *Effect of heating on the toxicity of each fraction*

Heating (at pH 8.0)		LD50 (μg)		
Temp	Time	AI	CII	Endotoxin
C	*min*			
N*		10.3	26.2	185
100	5	7.3	128.5	185
100	10	49.9	134.2	207
100	20	49.9	245.2	162
100	30	124.6	260	222
N		10.3	24.4	185
70	10	18.2	52.3	—
70	30	16.9	107.7	198

* Not heated.

TABLE 4. *Effect of heating at various pH levels on the toxicity of each fraction**

pH	LD50 (μg)		
	AI	CII	Endotoxin
N	13.5	26.1	204
4.8	28.2	124	380
6.0	57.3	178	380
7.2	32.4	152	269
8.0	121	260	195
9.0	—	260	269

* Fractions were heated at 100 C for 20 min at the pH indicated. N = no heating. Buffers used were as follows: pH 4.8 and 6.0, acetate; pH 7.2 and 8.0, phosphate; pH 9.0, carbonate.

(Table 3). However, the toxicity of both AI and CII decreased markedly upon heating.

The decrease in toxicity of AI and CII was most obvious upon heating at an alkaline pH, whereas no change in toxicity of the endotoxin was observed under similar conditions (Table 4).

Serological properties of fraction CII. Serological properties of fraction CII and the endotoxin were compared by the Ouchterlony (1953) technique. A sharp band of precipitate was formed between CII and the antibacterial serum, and it crossed over the precipitation band of the endotoxin (Fig. 8a). A single sharp band was observed between CII and the anti-C serum, but no precipitation was formed between the protein component of the endotoxin and this serum (Fig. 8b). The antiserum of a rabbit immunized with the protein component of the endotoxin formed two broad bands and one weak band when tested against the protein component. In this system, the band of CII fused with the weak band of the endotoxin protein (Fig. 8c). From these results, it is believed that the CII fraction is serologically a single substance which differs from the endotoxin, and that it is not the protein portion of the endotoxin, although both possess the same partial antigen.

Discussion

It has been observed that the water extract of cells from a virulent strain of *S. enteritidis* shows high toxicity, ranging from 31.4 to 220 μg in

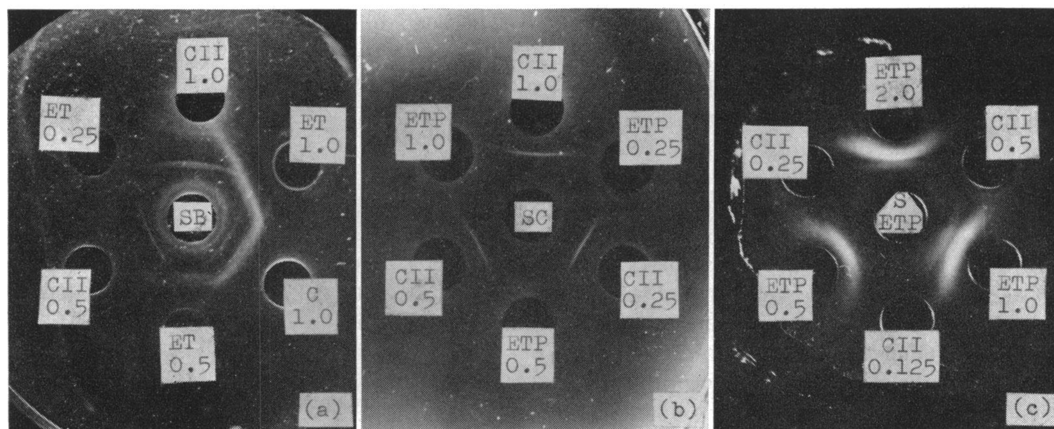

FIG. 8. *Precipitation test of toxins in agar gel. C and CII, toxin fractions of C and CII; ET, endotoxin; ETP, protein component of endotoxin; SB, antibacterial serum; SC, anti-C serum; SETP, anti-ETP serum. Each number indicates the amount of antigen in mg. (a) Photographed on the 18th day, (b) on the 10th day, and (c) on the 24th day.*

mouse MLD, when it is extracted under low temperature at an alkaline pH. In the present study, this extract was resolved into several fractions by means of cellulose chromatography. One of the fractions, designated as AI, gave a mouse LD_{50} of 10.3 to 13.5 μg; its toxicity was 12 times that of the endotoxin obtained from a similar species. The other fraction (C) was purified to a homogenous state by repeated applications of cellulose chromatography. We proposed the term "L" for this purified fraction CII, because CII is labile to heat. The L is protein in nature and free from polysaccharides and nucleic acids. The mouse LD_{50} of the L is 25 μg. The major differences between these toxins (AI and L) and the endotoxin are their high toxicity and sensitivity to heat. Their toxicity was markedly decreased by heating at 100 C for 20 min. The endotoxin was extracted from *S. enteritidis* by the methods of Boivin and Mesrobeanu (1935), Morgan (1937), Goebel et al. (1945), and Westphal et al. (1952). The LD_{50} of each extract was, however, 105 to 224 μg. The toxicity of all these samples was heat-stable (Mitsuhashi et al., 1960).

A toxic component was separated from the endotoxin by Tal and Olitzki (1948) and Tal and Goebel (1950), and was shown to be a conjugated protein or proteinlike component of a lipopolysaccharide-protein complex. On the other hand, some authors (Davies, Morgan, and Record, 1955; Webster et al., 1955; Westphal and Lüderitz, 1954) believed that polysaccharide or lipopolysaccharide is the toxic component of the endotoxin. These components also differ from our toxin in toxicity and heat stability. Serological analysis indicated that the L is not a protein component of the endotoxin, althought it has a partial antigen of the endotoxin protein.

From a culture medium or the cells of *Shigella dysenteriae*, a highly toxic substance, neurotoxin, was separated. Neurotoxin has a mouse LD_{50} of 1.3 μg, and is heat-labile and proteinlike in nature (Boivin and Mesrobeanu, 1937; van Heyningen and Gladstone, 1953). However, such a neurotoxin has not yet been obtained from *S. enteritidis*. Haas (1940) reported on the absence of an exotoxin of the neurotoxin type in *S. enteritidis*. The Vi antigen of some enteric bacteria was also separated by many authors (Webster, Landy, and Freeman, 1952). Recently, a heat-labile toxin was found in a culture medium of *Escherichia coli* (Hohorst, 1953). The toxicity of these toxins, however, is not too high, and no reports on the extraction of such toxins from *S. enteritidis* are available. Thus, the toxic material separated from *S. enteritidis* is a new type of toxin.

It was observed, furthermore, that toxin L has an immune effect by protecting mice against infection due to *S. enteritidis*. The role of toxin L during infection by *S. enteritidis* and the immunity against such infection are the necessary problems to be studied. These will be described elsewhere.

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