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Changes in Serologic Reactivity of Endotoxin Induced by Fraction IV_1 (Cohn) of Normal Human Serum.* (27882)

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Incubation of bacterial endotoxins in human serum or defibrinated plasma has been shown to result in repression of many of the biological properties of endotoxin(1). In our laboratory, the changes following such incubation have been measured by quantitative analysis of the diminution of ability of the lipopolysaccharide to precipitate its homologous, standardized antibody(2), and by loss of pyrogenicity(3). When defibrinated plasma was fractionated with ethanol according to the method of Cohn *et al.*(4), incubation of endotoxin in 2 of the resulting fractions, IV_1, III_0, was shown to result in the above changes(3). In this communication are presented data showing that the alteration of endotoxin, (such that it is rendered less precipitable by homologous antiserum following incubation in Fraction IV_1) does not involve release of the di-deoxyhexose antigenic determinant sugar, tyvelose(5), *per se,* but that the loss in precipitability is associated with changes in the immunodiffusion pattern of endotoxin.

Materials and methods. Endotoxin. Salmonella typhosa 0-901 was grown in a 30-liter fermentor using the synthetic medium of Erlandson and MacKey(6). The cells were harvested by centrifugation and washed twice with ice cold 0.15 M NaCl. The endotoxin was extracted from the cells using the method of Boivin and Mesrobeanu(7), dried from the frozen state, and characterized as pyrogenic, Shwartzman reactive, lethal for mice, and precipitable with homologous antiserum. *O polysaccharide hapten.* This substance was prepared in this laboratory using the method of Staub and Combes (8). Its properties have been reported elsewhere(2). *Fraction IV_1.* The alpha globulin Fraction IV_1 prepared by Cohn's method 10 from normal human serum was generously supplied by Cutter Laboratories, Berkeley, Calif. *Incubation procedure:* Twenty-five milligrams of Fraction IV_1 were dissolved in 1.7 ml 0.15 M NaCl. Two ml of endotoxin solution at a concentration of 200 μg/ml in saline was added, the pH adjusted to 7.1 with 0.1 N NaOH and the volume of the mixture adjusted to 4 ml with 0.15 M NaCl. The mixture was then incubated in a 37°C water bath for 4 hr, aliquot samples removed immediately and tested for recoverable endotoxin as outlined below. The remaining portions of the incubation mixtures were then stored at −30°C until other tests could be performed. *Test for recoverable endotoxin:* The amount of precipitable endotoxin in a test sample was determined by adding an 0.8-ml aliquot of the incubation mixture to 1 ml of rabbit anti-*S. typhosa* serum (bacterial agglutination titer $>$1:20,000) and 1.2 ml of 0.15 M NaCl. Tubes were incubated for 2 hr at 37°C and 18 hr at 4°C. The nitrogen

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TABLE I. Experimental Design for Determining if 3, 6-dideoxy Sugars Are Released from Endotoxin during Incubation with Serum Fraction IV_1.

Materials	Basic incubation experiment	Control for amount of tyvelose in endotoxin	Control for non-specific release of tyvelose	Control for detection of free tyvelose with Fraction IV_1
Endotoxin	400 μg	400 μg hydrolysed	400 μg	400 μg hydrolysed
Fraction IV_1	25 mg	0	0	25 mg
Menstruum	4 ml saline, pH 7.1	4 ml saline, pH 7.1	4 ml saline, pH 7.1	4 ml saline, pH 7.1
Incubation	4 hr, 37°C	—	4 hr, 37°C	4 hr, 37°C
% endotoxin recovered serologically	20	Not done	100	Not done

2.5 ml of the above solutions were added to 1 ml of phosphotungstic acid reagent. The precipitates (if any) were centrifuged in the cold. 2.5 ml of the supernatant fluid was taken for the periodate hydrolysis step of the thiobarbituric acid reaction. After the color was developed the absorbancy spectra of the samples were determined and are represented in Fig. 1.

precipitated in each tube was determined by the micro-Kjeldahl technic and quantity of endotoxin in the precipitate determined by interpolation on a standard curve(2). *Determination of 3, 6-dideoxyhexose:* Cynkin and Ashwell's modification(9) of Waravdekar and Saslaw's sensitive thiobarbituric acid test(10) was used. Essentially it consisted of hydrolyzing the free sugars with periodic acid to form malondialdehyde, which was subsequently reacted with thiobarbituric acid to form a colored complex. The amount of color developed was determined at 532 mμ with a Zeiss A II spectrophotometer. Due to extraneous colors occurring in samples containing Fraction IV_1 (most probably from sialic acid in Fraction IV_1), it was necessary to precipitate such glycoproteins with 2.5% phosphotungstic acid contained in 0.35 N H_2SO_4. This reagent was added to all samples including controls in a v/v ratio of 1: 2.5. *Immunodiffusion studies:* Glass slides, 3¼" × 4", were covered with a 5-mm layer of 1% Ionagar, and patterns cut with a Feinberg cutter (Code No. 1812, Consolidated Laboratories, Chicago Heights, Ill.). The plates were kept at room temperature in an atmosphere of high humidity and examined daily for the formation of lines of precipitate.

Results. Failure of Fraction IV_1 to release tyvelose from endotoxin: This experiment was designed to detect any release by Fraction IV_1 of the major serologic determinant group, tyvelose, of the endotoxin. Release of this determinant group could result in blocking of the reactive sites on the antibody molecule without precipitation, and thus account for the failure of the parent endotoxin to be precipitated following incubation in Fraction IV_1. The procedure followed is outlined in Table I. Three controls were included. They were designed to determine (1) the quantity of potentially reactive tyvelose contained in the endotoxin, (2) whether or not dideoxy sugars were released from the endotoxin spontaneously without incubation in Fraction IV_1, and (3) if free dideoxyhexose could be detected in the presence of Fraction IV_1. The results following testing of these various incubation mixtures for the absorbancy characteristic of tyvelose are depicted graphically in Fig. 1. It may be seen that(1) hydrolysis of the saline solution of endotoxin by 0.1 N H_2SO_4 at 100°C results in release of tyvelose, inasmuch as the maximum absorbancy at 532 mμ, characteristic of dideoxyhexoses in this reaction, was observed. Thus, it is documented that the endotoxin used in this experiment contained such sugars available for release and detection. That such sugars are not spontaneously released following incubation in the saline menstruum is also shown by the absence of absorbancy when this second control was tested. To control the possibility that tyvelose could be released from endotoxin by Fraction IV_1, but rendered non-reactive by Fraction IV_1, the dideoxyhexose was released from endotoxin by hydrolysis with 0.1 N H_2SO_4 and subsequently incubated with Fraction IV_1. Under these conditions, no interference with

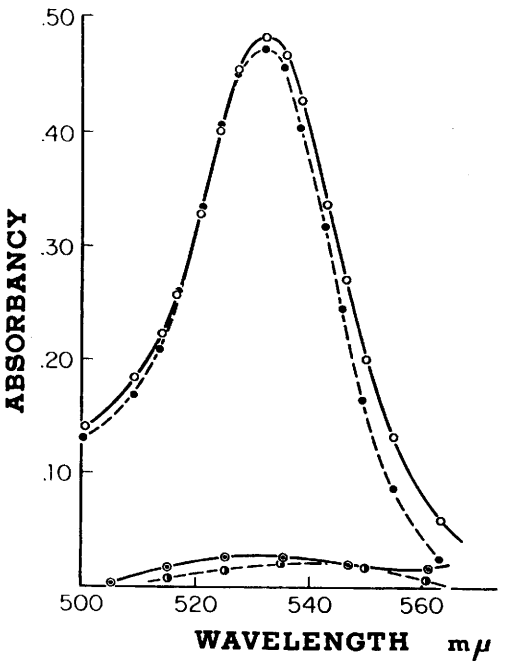

FIG. 1. Test for release of dideoxyhexose from endotoxin following incubation with Fraction IV_1. Samples were treated as indicated in Table I. Legend: ⊙—⊙, endotoxin incubated with Fraction IV_1; ○—○, hydrolysed endotoxin; ◒ -- ◒, endotoxin incubated in saline; ●—●, hydrolysed endotoxin incubated with Fraction IV_1.

the characteristic absorbance was observed (Fig. 1). However, no such absorbancy was exhibited after endotoxin had been incubated in Fraction IV_1. Yet, the ability of this endotoxin preparation to be precipitated by its homologous antibody was reduced by 80% when incubated with 25 mg of this same lot of Fraction IV_1. It was concluded therefore, that the diminution in serologic activity is not associated with the release of the major serologic determinant group of the endotoxin.

Alteration of the immunodiffusion pattern of endotoxin following incubation in Fraction IV_1. Inasmuch as a loss of up to 90% of the precipitating activity of endotoxin occurs following its incubation in Fraction IV_1 of normal human serum(3), the changes in immunodiffusion pattern of the endotoxin were followed by means of the Ouchterlony technic(11). The essential results of many analyses are represented by Fig. 2. It may be seen that endotoxin at a concentration of 100 μg/ml, following incubation for 4 hours in saline, reacts with high titered anti-*S. typhosa* antiserum to give a single line of diffuse precipitation close to the antigen well (Well 1). When this same concentration of endotoxin was incubated with Fraction IV_1, this slowly diffusing line tended to become dispersed and a new, more rapidly migrating line appeared (Well 2). The latter formed a line of identity with the O polysaccharide hapten (Well 3), which had been characterized as hapten in this laboratory previously (2). In addition, endotoxin at a much higher concentration in saline (Well 4) is included in this pattern to show the 3 lines characteristic of this product in high concentrations. One of these lines also forms a line of identity with the hapten, revealing some degradation during isolation of the endotoxin. In Fig. 3 is seen a higher magnification of Wells 1 and 2 as represented in another plate. Typical dispersion of the line produced by intact endotoxin incubated in saline (Well 1) is seen in Well 2. In addition, Well 2 illustrates more clearly the appearance of the line which can be shown routinely to form a line of identity with O polysaccharide hapten.

Centrifugation studies. The new component, (detected as described above with the Ouchterlony technic) appearing following incubation of endotoxin with Fraction IV_1, was identified further as a substance with a much smaller aggregate weight than the parent endotoxin by comparison of the following 4 endotoxin preparations in a high speed centrifuge: 100 μg/ml incubated in saline; 100 μg/ml incubated in Fraction IV_1; 5 mg/ml incubated in saline; and the O polysaccharide hapten. These preparations in a volume of 4 ml, were centrifuged at 10,000 × *g* for 1 hour, and the supernatant fluid decanted and recentrifuged at 20,000 × *g* for 1 hour. The precipitates from the first centrifugation, dissolved in 0.2 ml of 0.15 M NaCl, as well as the supernatant fluid after 20,000 × *g*, were tested for antigenic components by the double diffusion test of Ouchterlony. Fig. 4 is a composite drawing comparing the agar diffusion pattern of each of these tests. It may be seen that the antigen produced following incubation of 100 μg en-

dotoxin in Fraction IV_1, which forms a line of identity with the hapten, is a relatively small molecule, similar to the hapten, whereas the antigen present in the endotoxin preparation at a concentration of 5 mg/ml, which also forms a line of identity with the hapten, is much heavier. This is evidenced by the appearance of the latter in the precipitate after centrifugation at only 10,000 $\times$ g, while the former, as well as the hapten, remained in the supernatant fluid after being subjected to 20,000 $\times$ g. Thus it appears that the component released from endotoxin by incubation with Fraction IV_1 is more closely related in size to the O polysaccharide hapten than to the contaminant found in high concentrations of untreated endotoxin. However, it remains to be shown whether or not these two components are identical.

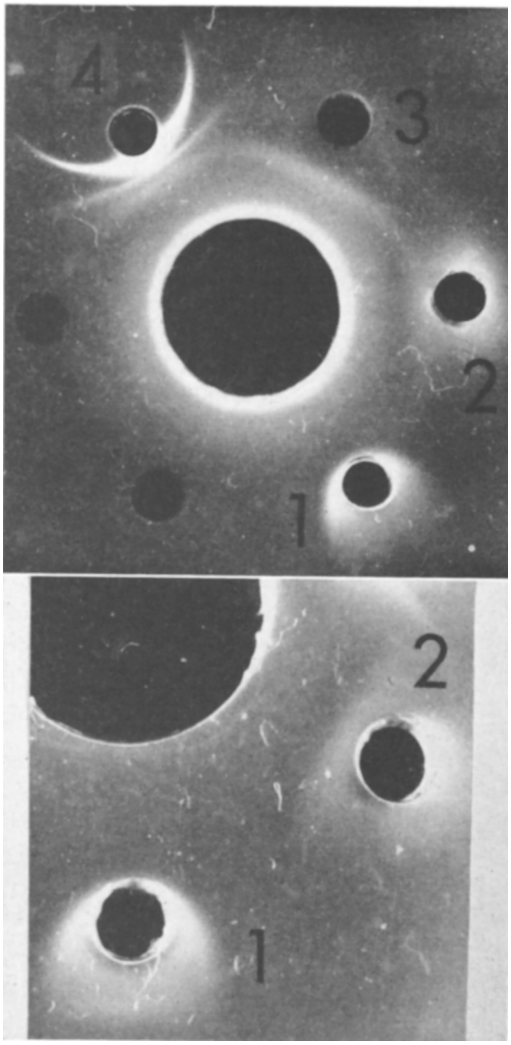

FIG. 2. Immunodiffusion pattern of endotoxin, 100 μg/ml (Well 1); endotoxin, 100 μg/ml, incubated with Fraction IV_1 (Well 2); O polysaccharide hapten, 25 μg/ml (Well 3); and endotoxin, 5 mg/ml (Well 4). The center well contained antiserum against *Salmonella typhosa*. Note line of continuity between endotoxin, 5 mg/ml, the O polysaccharide hapten, and endotoxin which had been incubated with Fraction IV_1. This line is absent when endotoxin, 100 μg/ml, was incubated in saline.

FIG. 3. An enlarged view of the immunodiffusion pattern of endotoxin, 100 μg/ml, incubated in saline (Well 1), and endotoxin, 100 μg/ml, incubated in Fraction IV_1 (Well 2), demonstrating the difference in precipitin patterns.

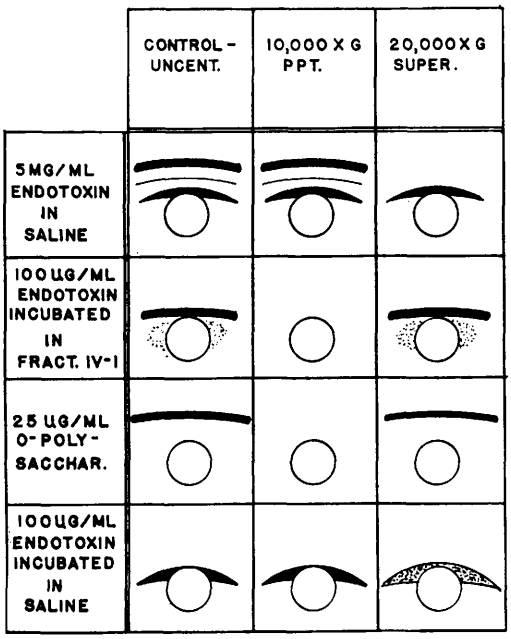

FIG. 4. Diagrammatic representation of immunodiffusion patterns of fractions obtained by high speed centrifugation of endotoxin, endotoxin altered by treatment with Fraction IV_1, and O polysaccharide hapten.

Discussion. The changes in the agar gel diffusion pattern which resulted from incubation of endotoxin with Fraction IV_1 consisted of loss of the major slowly diffusing line of precipitate formed by the endotoxin complex, and the appearance of a more rapidly migrating antigen which formed a line of identity with the O hapten. This is interpreted as evidence for cleavage of the endotoxin complex into smaller subunits by

this serum fraction. Confirmatory evidence on this point was obtained when it was shown that this more rapidly migrating antigen was unable to be sedimented by relatively high centrifugal forces, while the main endotoxin complex and its contaminating components were sedimentable. The formation of lighter components resulting from the incubation of endotoxin with the human plasma altering factor was observed earlier by Stauch and Johnson(2).

This work confirms, and extends with a purified fraction of human serum, the studies of Cluff(12), who recorded similar changes in the agar gel diffusion pattern of Shigella endotoxin when incubated in normal rabbit or human serum. In addition, the results presented herein make a tentative identification of the product released by serum-endotoxin interaction as the O polysaccharide hapten. A similar identification was made independently by using human plasma by Skarnes(13). In addition, Landy, Trapani, and Shear(14) have reported that resin treated plasma destroyed the capacity of endotoxin to stimulate antibody in rabbits, but the ability to precipitate anti-endotoxin antibody was retained. The observed precipitation reaction was characteristic of the haptenic polysaccharide rather than the unaltered endotoxin.

Summary. A substantial loss in the ability of endotoxin to be precipitated by homologous antibody occurs following incubation with Fraction IV_1 derived from normal human serum by cold ethanol fractionation. Evidence is presented that this loss in serologic reactivity is not associated with release of the major determinant sugar, *per se.* Rather, immunodiffusion analysis revealed the dispersion of a major antigen, with formation of a more rapidly migrating component. The latter was shown to form a line of identity with the O polysaccharide hapten.

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Hypoglycemia in Chick Embryos Infected with *S. typhosa via* the Allantoic Cavity.* (27883)

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Since the work of Goodpasture and Anderson which showed that the embryonated chicken egg is susceptible to infection with *Salmonella typhosa,* investigators have utilized this model to study various aspects of

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