SOC. EXP. BIOL. AND MED., 1955, v88, 651.
2. Loebl, W. Y., Ullman, T. D., Yaron, A., Sela, M., Berger, A., Katchalski, E., *ibid.*, 1961, v108, 661.
3. Blout, E. R., Farber, S., Fasman, G. D., Klein, E., Narrod, M., *Polyamino Acids, Polypeptides, and Proteins,* Univ. of Wisc. Press, 1963, p379.
4. Evelyn, K. A., Malloy, H. T., *J. Biol. Chem.,* 1938, v126, 655.
5. Mundy, R. L., Heiffer, M. H., Leifheit, H. C., *Radiat. Res.,* 1961, v14, 421.
6. Colowick, S. P., Kaplan, N. O., *Methods in Enzymology,* Academic Press, Inc., 1955, vII, 785.
7. Crosby, W. H., Stefanini, M., *J. Lab. Clin. Med.,* 1952, v40, 374.

Received September 3, 1963. P.S.E.B.M., 1964, v115.

Reaction of *Escherichia coli* Endotoxin with Lithium Aluminum Hydride. (28851)

K. FUKUSHI,* R. L. ANACKER, W. T. HASKINS, M. LANDY, K. C. MILNER AND E. RIBI

U. S. Department of HEW, Public Health Service, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Mont., and Laboratory of Immunology, Bethesda, Md.

Noll and Braude(1) reported that treatment with lithium aluminum hydride ($LiAlH_4$) modified a small portion (about 10%) of Boivin-type endotoxin from *Escherichia coli* to yield a nonpyrogenic and nontoxic but antigenic material (RE fraction). They concluded from infrared absorption data that both the modified portion and the pyrogenic and toxic remainder were free of ester-bound fatty acids. The disappearance of ester absorption bands in the RE fraction was correlated with reduction of pyrogenicity and toxicity. This communication reports the results of studies with the same culture and procedures employed by these workers. We sought to confirm their findings and to obtain additional chemical data on the nature of the changes in bacterial endotoxin produced by treatment with $LiAlH_4$.

Materials and methods. 1) *Culture.* In each experiment, E. coli of serotype 0113, originally isolated from the blood of a patient, was used(1). Organisms from a frozen stock culture (in trypticase-soy broth) were cultivated for 24 hours at 37°C with shaking in synthetic M-9 medium(2) containing 0.5% glucose or without shaking in modified Gladstone medium(3). Three additional transfers were made before the experimental batches were inoculated. These were grown either in 100 ml volumes of M-9 medium in 500 ml Erlenmeyer flasks or in 4000 ml volumes of Gladstone medium in 6000 ml Erlenmeyer flasks. After 18 hours' incubation, the cells grown in M-9 medium were chilled, harvested by centrifugation and washed twice with 0.15 M sodium chloride. The cells harvested after 18 hours from the Gladstone medium were washed with 95% ethanol and then with acetone and were dried in vacuum (1).

2) *Extraction of endotoxin with trichloracetic acid (TCA).* For Experiment I, the wet sediment of saline washed cells grown in M-9 medium was extracted at 0°C for 3 hours with TCA in a final concentration of 0.25 N. The remaining steps were performed at 4-6°C. The extract was clarified by centrifugation and dialyzed. Endotoxin was precipitated by slowly adding ethanol to a final concentration of 68% by volume, and the suspension was allowed to stand overnight. Precipitation was facilitated by adding sodium acetate to a concentration of 0.1 M. The precipitate was collected by centrifugation, dissolved in water, dialyzed, and lyophilized. For Experiment II, dried cells from Gladstone medium were extracted with TCA and refined according to the method used by Noll and Braude(1), except that

* On leave from Tohoku Univ., Sendai, Japan.
Supported by Visiting Research Scientist Program of Nat. Acad. Sciences.

0.25 N TCA, instead of 0.625 N TCA, was employed.

3) *Treatment of endotoxin with lithium aluminum hydride.* Treatment of endotoxin, with $LiAlH_4$ was performed as described by Noll and Braude(1). Endotoxin, which had been dried in vacuum over phosphorus pentoxide, and an equal weight of the $LiAlH_4$ were suspended in dry ether, and the mixture was refluxed for 5 hours. The reaction mixture was then cooled and decomposed by addition of 1 mole hydrochloric acid per mole of $LiAlH_4$. The ether layer was removed, and the aqueous layer containing the voluminous aluminum hydroxide precipitate was collected by centrifugation (1550 $\times$ *g* for 30 minutes) or by filtration. The clear supernatant fluid (or filtrate) was dialyzed against distilled water and lyophilized. The copious precipitate from the reaction mixture was treated, according to the method of Haskins *et al*(4), with an equimolar amount of citric acid, neutralized with sodium hydroxide and centrifuged. The supernatant fluid was dialyzed and lyophilized to isolate the aluminum citrate-endotoxin complex.

4) *Chemical and biological analyses.* Chemical methods are those employed previously (5,6). Median effective doses in various bioassays are expressed as follows: lethality for Rocky Mountain Laboratory male mice as LD_{50}(7) (It should be noted that, coincident with changes in housing and management of the colony, our mice have become more refractory to the lethal effect of endotoxin); tumor damage (production of hemorrhagic necrosis in sarcoma 37 implants in mice) as TD_{50}(7); nonspecific protection against intraperitoneal challenge with *Salmonella typhosa,* strain Ty-2, as ED_{50}(7); pyrogenicity for mature rabbits as FI_{40}, the quantity of substance required to produce a fever index of 40 cm^2 when 1°C and 1 hour are each plotted as 1 inch(8); primary inflammatory effect in rabbit skin as SLD_{50}(9); ability to prepare rabbit skin for the dermal Shwartzman reaction as SPD_{50}(9). Antibody production was measured by agglutination of heat-killed bacterial suspension and by agglutination of chicken erythrocytes sensitized with sodium hydroxide-treated antigens(10). Various products were analyzed by the agar gel diffusion method of Ouchterlony(11). Infrared spectra were recorded with a Perkin-Elmer model 21, double beam spectrophotometer. The samples were prepared by evaporating aqueous solutions on the surfaces of silver chloride windows.

Results. Exp. I (Ec-4). In the first experiment, attempts were made to prepare the nontoxic antigen described as the RE fraction(1). This procedure was followed except that a wet sediment of freshly harvested saline washed cells, which had been grown in M-9 medium was substituted for dried cells grown in Gladstone medium. Treatment of the TCA-extracted endotoxin with $LiAlH_4$ and decomposition of the reaction mixture with hydrochloric acid led to quantitative co-precipitation of organic matter with aluminum hydroxide. The clear supernatant fluid after dialyzing and lyophilizing, expected to contain the RE fraction, yielded merely a trace amount of substance which consisted predominantly of aluminum hydroxide (aluminum oxide recovered after burning on platinum foil). The copious aluminum hydroxide precipitate from the reaction mixture was extracted with citric acid to yield an aluminum citrate-endotoxin complex in 89% yield, the biological activity of which was only slightly less than that of the original product. In contrast to the original endotoxin, this complex dispersed rapidly in water to yield a clear solution. The pertinent chemical and biological data on this preparation and parent endotoxin are compared in Table I. The product contained 2.5% aluminum and 23.2% citrate. Accordingly, hexose, total carbohydrate and hexosamine were reduced in expected ratio; but nitrogen and phosphorus were reduced $\frac{1}{3}$. The content of fatty acid appeared to be reduced more than $\frac{1}{2}$ (10.2% *vs* 23.7%). As reported earlier (4), however, fatty acid analysis of aluminum citrate-endotoxin complexes by the hydroxamic acid method is unreliable, because of interference by citrate. To obtain more meaningful data, the citrate-containing complex was hydrolyzed in 1 N hydrochloric acid for 45 minutes. The water-insoluble portion of the hydrolysate, removed by centrifu-

TABLE I. Analysis of Fraction Resulting from Treatment of *Escherichia coli* 0113 Endotoxin with Lithium Aluminum Hydride.

	Exp I		Exp II		
	TCA extract	Aluminum citrate-endotoxin complex extracted from residue	TCA extract	Filtrate after decomposing $LiAlH_4$ reaction mixture with HCl	Aluminum citrate-endotoxin complex extracted from residue
Chemical	%				
Recovery	100	89	100	10	83
Nitrogen	3.19	2.17	3.72	.19	2.72
Phosphorus	1.35	.92	1.47	.0	.97
Hexose (Anthrone)	23.6	20.0	24.9	—	11.8
Carbohydrate (Tryptophan)	34.9	26.2	43.0	100	28.1
Hexosamine	16.0	13.6	11.2	.95	12.6
FAE + FAA	23.7	10.2*	25.8	.0	7.65†
Aluminum	.0	2.5	.0	—	4.7
Citrate	.0	23.2	.0	.0	19.1
Biological	μg				
Lethality for mice (LD_{50})	810	1230	1000	>2000	1100
Tumor damage in mice (TD_{50})	.31	.34	.46	—	.88
Pyrogenicity in rabbits (FI_{40})	.57	2.0	1.6	> 200	1.0
Protection of mice against *S. typhosa* (ED_{50})	70	85	8.5	—	8.5
Dermal inflammation in rabbits (SLD_{50})	.63	.55	1.4	> 200	1.3
Shwartzman reaction (SPD_{50})	10	50	11	> 200	7.5

* Analysis of lipid from chloroform-ethanol soluble extract of acid hydrolysate (1 N HCl at 100°C for 45 min) 17.6% yield, containing 18.9% FAE + FAA.

† Hydrolysis with 1 N HCl at 100°C for 45 min yielded 8% chloroform-soluble and 5.2% chloroform-insoluble material which contained 36.3% FAE + FAA.

gation, amounted to 17.6% ("crude lipid A"). The major portion of this sediment was soluble in chloroform; the remainder was soluble in ethanol. It contained 18.9% fatty acid ester (FAE) + fatty acid amide (FAA) which would mean that the complex, prior to hydrolysis, contained at least 3.34% FAE + FAA, and that the content of the FAE + FAA had been considerably reduced although only a minute amount of material was recovered from the ether phase. It has been observed that hydrolysis with 1 N hydrochloric acid destroys ester linkages of the original lipid in endotoxin of *E. coli*(12). These free fatty acids present in the hydrolysate are not determined by the hyroxamic acid method. Therefore, we do not have reliable data for the extent of reduction of FAE and FAA of the Boivin-type antigen of *E. coli* as a result of treatment with $LiAlH_4$. An attempt to circumvent these difficulties has been made in Exp. II by determination of FAE + FAA in the endotoxin after citrate was removed by ion exchange resins.

The infrared spectrum of toxic and pyrogenic *E. coli* aluminum citrate-endotoxin complex showed that the 1,735 cm^{-1} ester absorption band present in the original endotoxin had disappeared and that the intensity of the ester band near 1,250 cm^{-1} had decreased considerably (Fig. 1). The magnitude of the latter was similar to that shown in the spectra of "reduced endotoxin" (Fraction RE IV, Fig. 4 in reference 1) where pyrogenicity was reported to be reduced 100-fold. These results indicate that, as in the case of *Salmonella enteritidis*(4), there is no correlation between fatty acid ester content, as estimated from infrared spectra, and toxic and pyrogenic properties of the endotoxin.

Exp. II (Ec-8). In this experiment, performed with an extract of ethanol- and acetone-washed and dried cells from Gladstone medium, filtration of the reaction mixture after treatment with $LiAlH_4$ and hydrochloric acid resulted in a clear filtrate which, upon dialyzing and lyophilizing, produced a white, fluffy powder in 14% yield. When this powder was redissolved in water, a portion remained insoluble and was separated by cen-

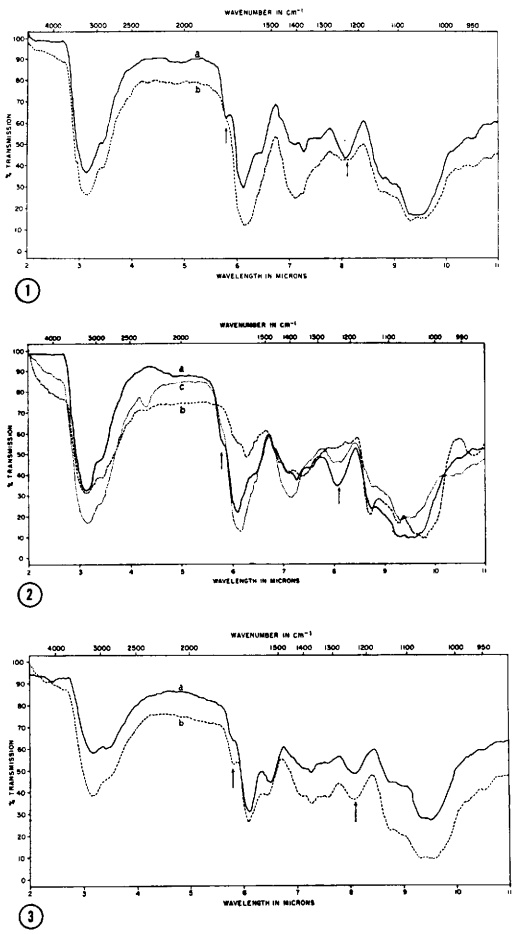

FIG. 1. Comparison of infrared spectra of endotoxin and its corresponding aluminum citrate complex. a) Original endotoxin. b) Aluminum citrate-endotoxin complex.

FIG. 2. Infrared spectra of endotoxin, water soluble material (inactive polysaccharide) after reaction with $LiAlH_4$ and HCl treatment, and aluminum citrate-endotoxin complex extracted from the aluminum hydroxide sediment. a) Original endotoxin. b) Inactive polysaccharide. c) Aluminum citrate-endotoxin complex.

FIG. 3. Comparison of infrared spectra of Freeman-type haptene and endotoxin. a) Original endotoxin. b) Haptene.

trifugation. This insoluble fraction contained 8.6% aluminum, 21.5% carbohydrate and 0.12% nitrogen. It accounted for about ⅓ of the product and was biologically inactive. The soluble portion, which amounted to 10% of the starting material, was nonpyrogenic ($FI_{40} > 200$ μg) and nontoxic ($LD_{50} > 2$ mg, Shwartzman $SPD_{50} > 200$ μg). In contrast to Noll and Braude's RE fraction, it did not stimulate antibody production in rabbits, did not form a precipitation line in agar gel diffusion tests, and did not produce tolerance to endotoxin. Chemically it was an essentially pure nonphosphorylated polysaccharide containing 0.19% nitrogen, 0.95% hexosamine and no fatty acids. None of 6 rabbits given 200 μg of the fraction in a single dose, showed any febrile response. Sera from preinoculation bleedings on these and other rabbits employed agglutinated the parent culture of *E. coli* 0113 to titers ranging up to 1:80 but failed to produce hemagglutination of erythrocytes sensitized with the original endotoxin. Six rabbits each given 200 μg of this polysaccharide, and 6 others given a series of 6 daily injections of smaller doses (2 and 20 μg) failed to develop detectable antibody. In contrast, 2 rabbits given single intravenous injections of 0.5 μg of the complete endotoxin developed agglutinin and hemagglutinin titers of 1:640 and 1:500, respectively. Neither single injections of 200 μg doses nor multiple injections of smaller doses produced tolerance to the endotoxin, as measured by febrile response: the characteristics of the fever curves and the areas enclosed by them (fever indices) in treated and control groups did not differ significantly.

Extraction of the aluminum hydroxide precipitate with citric acid gave a rapidly soluble aluminum citrate-endotoxin complex in 83% yield. The pertinent chemical and biological data for these 2 fractions are compared with those of the starting material in Table I. It is evident that properties of the aluminum citrate-endotoxin complex differ only slightly from the starting material and that much of the lipoid material is retained in the complex. Hydrolysis with hydrochloric acid, as described in Exp. I, yielded a precipitate (crude "lipid A" amounting to 13.2% of the endotoxin complex; 8% of this was soluble in chloroform. The insoluble residue (5.2%) contained 36.3% FAE + FAA. Determination of the FAE + FAA in the aluminum citrate-endotoxin complex was accomplished by passing its aqueous solution through a strong acid and base ion exchange column in the salt form to remove the aluminum cit-

rate. The effluent was dialyzed, lyophilized, and analyzed by the hydroxamic acid method. The FAE + FAA value of the endotoxin after removal of aluminum citrate was 9.45% (7.65% when calculated on the basis of the aluminum citrate-endotoxin complex). Thus, there is an apparent reduction of FAE + FAA from 25.8% in the starting material to 9.45%. Whether this reduction is due to hydrogenolysis alone is questionable and cannot be resolved without isolation and identification of the products of hydrogenolysis.

Although the starting endotoxin used for Exp. II contained a high percentage of esterified fatty acids, the infrared spectrum (Fig. 2a) showed little absorption at 1,735 cm^{-1}. According to Iwahara *et al*(13) this absorption band probably is masked by nucleic acid known to be present in this preparation. The intensity of the ester band near 1,250 cm^{-1}, however, was similar to that of the untreated endotoxin shown in Fig. 2 and 4 in reference 1. Despite the fact that the endotoxin recovered from the aluminum hydroxide precipitate retained the host-reactive properties of the untreated endotoxin, the infrared spectrum (Fig. 2c), again, as in Exp. I, resembled that previously described(1) for the RE IV fraction. The diagram of our inactive polysaccharide (Fig. 2b) differed from that of the other fractions, particularly in giving no absorption band near 1,250 cm^{-1}. In this respect, this diagram corresponded with the least pyrogenic fraction obtained by Noll and Braude (Fig. 2a in reference 1); however, these authors reported this material to be antigenic. It also differed from the absorption pattern of a Freeman-type haptene from *E. coli* (Fig. 3b) whose spectrum closely resembled that of both the parent aqueous ether endotoxin (Fig. 3a) and the Boivin endotoxins (Fig. 1a, 2a). To prepare this haptene, endotoxin(5) was boiled with 0.1% acetic acid for 3 hours, the hydrolysate was neutralized and centrifuged at 60,000 × *g* for 4 hours, and the supernatant thus obtained was recentrifuged, dialyzed and lyophilized. The reconstituted material had a sedimentation constant of 1.1 S, which was similar to that of haptene prepared from *S. enteritidis*(13). This material was nonpyrogenic ($FI_{40} > 250$ μg) and contained 25% FAE + FAA. The parent endotoxin contained 37.6% FAE + FAA. The failure of the latter to absorb strongly at 1,735 cm^{-1} cannot be attributed to masking by nucleic acid, since this was an aqueous ether extract which contained no nucleic acid. By the same token, absorption near 1,250 cm^{-1} must be ascribed to something other than nucleic acid (13), presumably to ester groups.

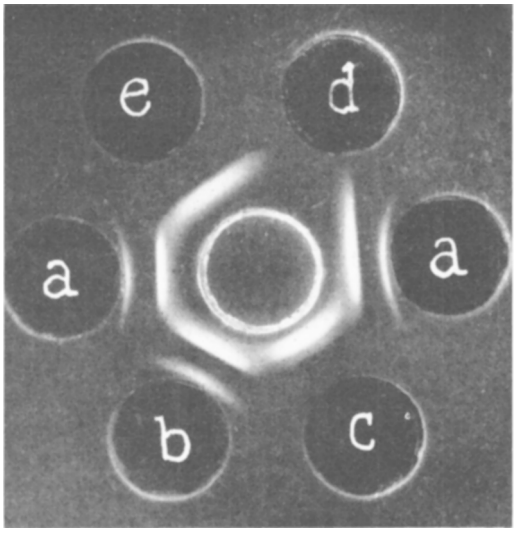

FIG. 4. Gel diffusion patterns of endotoxins from *E. coli* and derivatives. Rabbit antiserum to whole cells in central well. a) Original Boivin endotoxin (1000 μg/ml). b) Aluminum citrate-endotoxin complex prepared from Boivin endotoxin (1000 μg/ml). c) Haptene from aqueous ether extracted endotoxin 62.5 μg/ml). d) Inactive polysaccharide isolated from reaction mixture of Boivin endotoxin treated with $LiAlH_4$ (1000 μg/ml). e) Haptene from aluminum citrate-endotoxin complex (62.5 μg/ml).

The double diffusion procedure of Ouchterlony(11) disclosed 2 components each in the $LiAlH_4$-treated and the untreated endotoxin but none in the inactive fraction when tested at a maximal concentration of 0.1% (Fig. 4). Comparison with haptenes prepared from an aluminum citrate-endotoxin complex (1.3 S) and from an aqueous ether extract (1.1 S) showed that the component of endotoxin which diffused more rapidly (closer to the serum well) was immunochemically identical with the haptenes. Based on these results, together with those reported previously (14), the component which diffused slowly through the gel (indicating a high molecular

weight) is practically indistinguishable from the endotoxin. The existence of substantial amounts of haptene in *E. coli* Boivin extract was not unexpected in view of the earlier results.

Discussion. The present study was originally designed to obtain chemical data for the RE fraction which Noll and Braude(1) had reported as antigenic but nontoxic and nonpyrogenic. For undetermined reasons we have not succeeded in preparing this antigen, although a number of attempts have been made in addition to those reported here. Every effort was made to duplicate their experimental conditions; this included the use of the same strain and culture methods, extraction of Boivin-type endotoxin, and their procedure for cleavage of fatty acid ester bonds with $LiAlH_4$. At the expected stage and in appropriate yield (10%), we obtained an essentially pure nonphosphorylated polysaccharide, which was neither antigenic, pyrogenic, nor toxic.

The endotoxin could be recovered after treatment with $LiAlH_4$ by extracting the aluminum hydroxide precipitate with citric acid. This product was identified as an aluminum citrate-endotoxin complex of undiminished potency analogous to those reported for endotoxin complexes from *S. enteritidis*(4) prepared in the same manner. In contrast to the original endotoxin, the *E. coli* complex dispersed rapidly in water to yield a clear solution.

The origin of the inactive polysaccharide material reported here has not been determined. It may be significant that this fraction was isolated only from acetone-treated dried cells. Our experience has been that treatment of Gram-negative bacteria with acetone or other organic solvents alters the permeability of the cell wall since such cells, when resuspended in aqueous media, release substantial quantities of protoplasmic constituents. Acetone treated bacteria are used extensively as a convenient source for the isolation of bacterial components. Cells preserved in this fashion are both convenient and suitable for a variety of purposes, for example, preparation of cytoplasmic enzymes of microorganisms(15) including *E. coli*(16). However, when these cells are extracted for endotoxin, considerable quantities of protoplasmic material invariably emerge together with the endotoxin. It is conceivable, therefore, that our inactive fraction may be of protoplasmic origin. As reported elsewhere(17), we have subjected to extraction with TCA the protoplasm released from *E. coli* by mechanical disruption and separated from cell walls by centrifugation. Polysaccharide fractions were obtained which had gross chemical composition similar to that of Boivin-type endotoxins; their biological properties were essentially those of acid haptenes.

Since available chemical data are incomplete, comparison of our fractions with the RE fraction can be made only on the basis of infrared absorption studies. In infrared spectra of endotoxin, the absorption bands near 1,735 cm^{-1} and 1,250 cm^{-1} have been attributed to ester carbonyl(1). The reduction of the area under these absorption peaks as a result of treatment with $LiAlH_4$ was used by Noll and Braude as an index for estimating removal of fatty acid ester, which in turn was correlated with reduction of pyrogenicity and toxicity. Material of unaltered potency and appreciable content of lipid, which we recovered after treatment with $LiAlH_4$, gave infrared spectra in which the 1,735 cm^{-1} band had disappeared and the intensity of absorption near 1,250 cm^{-1} was decreased. These spectral changes were approximately the same as those previously reported for the RE IV fraction(1) which was assumed to retain only traces of ester carbonyl and whose pyrogenicity was 1/100 that of the original endotoxin. These authors also described a completely nonpyrogenic material (RE fraction I) whose spectrum showed minimal absorption near 1,250 cm^{-1} (Fig. 2a in reference 1). This diagram most closely resembled that given by a haptenic TCA extract of protoplasm from *E. coli* 0111:B4 which contained 8.3% FAE + FAA.

Our findings on the reaction of $LiAlH_4$ with *E. coli* endotoxin are in general agreement with results of similar treatment of *S. enteritidis* endotoxin(4). Interpretation of such data is limited by the irregularities which have been observed in the course of

infrared absorption analyses. Thus, in addition to observations already mentioned, treatment of *S. enteritidis* endotoxin with $LiAlH_4$ in some instances led to disappearance of the 1,735 cm^{-1} band but to no reduction of the 1,250 cm^{-1} band, and chemical analysis showed no reduction of the lipid content. Furthermore, Freeman-type haptenes and haptenic materials extracted from *E. coli* protoplasm with TCA(17) showed absorption bands of varying intensity near 1,735 and 1,250 cm^{-1}. While the spectra of *E. coli* haptenes reproduced in this paper showed strong absorption at these wave lengths, such absorption was virtually absent in some of the Freeman haptenes we had prepared from *S. enteritidis*. The same observation was also made by Iwahara and co-workers(13) in the spectrum of a Freeman-type haptene prepared from *Sh. flexneri* endotoxin. It is of interest that their parent endotoxin which was a purified phenol extract showed essentially no absorption near 1,250 cm^{-1}. Our findings also are in harmony with earlier work(18) on the infrared spectra of lipopolysaccharide and haptene from *S. typhosa*. Observations such as these emphasize some of the limiting features of the infrared absorption technique as applied to materials as complex as bacterial endotoxins; at present, the method is of questionable value in correlating composition and structure with biological activity.

Summary. Treatment of Boivin-type endotoxin from *E. coli* with $LiAlH_4$ according to the method of Noll and Braude yielded a small amount (about 10%) of an essentially pure nonphosphorylated polysaccharide which was nontoxic and nonpyrogenic. In contrast to their RE fraction this product did not stimulate the production of antibodies in rabbits, precipitate antibody nor produce tolerance to endotoxin. The bulk of the product resulting from treatment with $LiAlH_4$ consisted of biologically unaltered endotoxin. It was isolated as a rapidly soluble, lipid-containing aluminum citrate-endotoxin complex, the infrared spectrum of which was nearly identical to that of an RE fraction with 100-fold reduced pyrogenicity. No correlation was found between fatty acid ester content, as estimated from infrared spectra, and toxic and pyrogenic properties of the endotoxin.

We are grateful to Philip W. Stashak for performing pyrogen and tumor damage potency assays and to William D. Bickel for microchemical analyses.

1. Noll, H., Braude, A. I., *J. Clin. Invest.*, 1961, v40, 1935.

2. Anderson, E. H., *Proc. Nat. Acad. Sci.*, 1946, v32, 120.

3. Braude, A. I., Carey, F. J., Sutherland, D., Zalesky, M., *J. Clin. Invest.*, 1955, v34, 850.

4. Haskins, W. T., Ribi., E., Landy, M., Anacker, R. L., Milner, K. C., PROC. SOC. EXP. BIOL. AND MED., 1963, v112, 113.

5. Ribi, E., Haskins, W. T., Landy, M., Milner, K. C., *J. Exp. Med.*, 1961, v114, 647.

6. Haskins, W. T., *Anal. Chem.*, 1961, v33, 1445.

7. Haskins, W. T., Landy, M., Milner, K. C., Ribi, E., *J. Exp. Med.*, 1961, v114, 665.

8. Keene, W. R., Silberman, H. R., Landy, M., *J. Clin. Invest.*, 1961, v40, 295.

9. Larson, C. L., Ribi, E., Milner, K. C., Lieberman, J. E., *J. Exp. Med.*, 1960, v111, 1.

10. Davies, D. A. L., Crumpton, M. J., Macpherson, I. A., Hutchison, A. M., *Immunol.*, 1958, v1, 157.

11. Ouchterlony, Ö., *Arkiv. kemi. Mineral. Geol.*, 1948, v26B, 1.

12. Wander, A., Process for obtaining pure lipoid through cleavage of crude lipoid obtained from lipopolysaccharides of Gram-negative bacteria, German Patent No. 1,073, 150, Jan. 14, 1960.

13. Iwahara, S., Koshinuma, K., Ishizeki, R., Oba, T., *Japan J. Microbiol.*, 1959, v3, 331.

14. Ribi, E., Haskins, W. T., Milner, K. C., Anacker, R. L., Ritter, D. B., Goode, G., Trapani, R.-J., Landy, M., *J. Bacteriol.*, 1962, v84, 803.

15. Cclowick, S. P., Kaplan, N. O., in *Methods in Enzymology,* Academic Press Inc., New York, 1955, v1.

16. Chuang, Y.-K., *Bull. of Inst. of Chemistry,* Academia Sinica, 1962, v6, 19.

17. Milner, K. C., Anacker, R. L., Fukushi, K., Haskins, W. T., Landy, M., Malmgren, B., Ribi, E., *Bact. Rev.*, 1963, in press.

18. Webster, M. E., Sagin, J. F., Landy, M., Johnson, A. G., *J Immunol.*, 1955, v74, 455.

Received September 3, 1963. P.S.E.B.M., 1964, v115.