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Effect of Sonic Energy, Ultracentrifugation, and Phenol on *Neisseria gonorrhoeae* Endotoxin.* (24460)

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For certain endotoxin-antigen studies the natural cell-free complex is desired. For other studies the smallest active unit of the original biologic complex is necessary. The present investigations deal with both types of products.

Preparation of soluble nucleoprotein-bound endotoxin by physical methods. Cultivation of *N. gonorrhoeae*† has been previously described(1,2). The gonococcus cells were washed twice with 4 volumes of saline and once with 4 volumes of distilled water. A supply of such cells was kept frozen at –20°C. Washed cells contained 10-12% solids. Three g portions of washed cells were suspended in 47 ml portions of distilled water and subjected to sonic disintegration in Raytheon 10-kc sonic oscillator at 8°C for 60 minutes at maximum intensity. Cell debris, 8.8-9.0%, on dry cell basis, was removed by centrifuging in cold room in Servall angle centrifuge at 3,600 g for 15 minutes. The clear supernatant was placed in cellophane bags and dialyzed for 20 hours with continuous stirring against distilled water. The supernatant, after dialysis, contained 90% of solids originally present in the gonococcus cells (5-6 mg protein/ml). Analysis indicated that this material consisted mainly of nucleo-proteins. Such supernatants were stored at –20°C for several months without loss of toxicity. Precipitates which occasionally formed after thawing contained part of the endotoxin, and were put back in solution by brief sonic treatment. LD_{50} values on the supernatant are listed in Table I. Twenty-five to 50 CFl male mice, weighing 16-18 g, and 5 different endotoxin concentrations were used in the assays(2). Endotoxin solutions were administered intraperitoneally. The animals were observed 5 days. Karber's(3) procedure was employed for calculating LD_{50} values and interpreting their statistical meaning. From sonic treated supernatant a stable endotoxin-containing acetone powder was prepared as follows: a small quantity of sodium chloride was added to the supernatant and

TABLE I. Endotoxin Activities of Fractions Obtained by Sonic Treatment and Ultracentrifuging.

Type of fraction	LD_{50} value, mg
Supernatant at 3,600 g after sonic treatment	1.6
Sediment at 26,500 g in 30 min.	1.1
Idem 60 "	1.3
Supernatant at 26,500 g in 60 min.	1.4
Acetone powder from same supernatant after removal of sediment	1.5
Acetone powder, cells extracted at pH 13	2.3
Protein-free endotoxin	.8

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† The gonococcus strain employed was isolated from male patient by Dr. James D. Thayer of this laboratory.

the endotoxin was precipitated, together with nucleoproteins and a small quantity of other proteins, by addition of 3 volumes of cold acetone. The precipitate was removed by centrifugation and dried over sodium hydroxide *in vacuo*. The yield of dry material was 90% on the basis of dry gonococcus cells. Endotoxin precipitates are only very slightly soluble in water. The dry powder was readily put in solution, however, by suspension in distilled water and brief sonic treatment. An aliquot of the supernatant obtained after sonic treatment of the gonococcus cells, and dialyzed as described above, was centrifuged in the Spinco Model L ultracentrifuge at 26,500 g for 60 minutes at −10°C. Thirty to 35% of the endotoxin sedimented under these conditions. The sediment was put in solution by suspension in distilled water and brief (15 minutes) sonic treatment. When thawed supernatant was used, sedimentation was much heavier (up to 75%). Occasionally endotoxin-containing material precipitated on freezing. This precipitate was not removed before ultracentrifuging. An increase in speed up to 100,000 g did not result in heavier sedimentation. Endotoxin is firmly bound to nucleoproteins and speeds up to 100,000 g did not result in sediments or supernatants of significantly different toxicities (Table I). The endotoxin remaining in the supernatant after centrifugation was removed by addition of small quantity of sodium chloride and 3 volumes of acetone.

Preparation of protein-free endotoxin. Here a phenol-water mixture, first employed by Palmer and Gerlough(4) in preparation of antigenic-substances from other organisms, was used. Fifty g of washed gonococcus cells were suspended in 45 ml of distilled water. The cell suspension was poured into Waring blendor containing a mixture of 110 g phenol and 67 ml distilled water. The contents of the Waring blendor were stirred for 8 min. The temperature of mixture was 25°C at the beginning, and 45° at end of stirring. Then the mixture was cooled to 20°C and centrifuged in cold room in Servall angle centrifuge at 3,600 g. The clear supernatant (aqueous layer) was placed in cellophane bags and dialyzed for 24 hours at 4° against 14 l of distilled water. The latter was stirred continuously and changed occasionally. The lower (phenol) layer containing the proteins was discarded. A small quantity of sodium chloride was added to the dialyzed supernatant. The endotoxin was precipitated by the addition of 1.5 volumes of cold acetone. The precipitate was washed once with cold acetone and once with cold ether, and dried over sodium hydroxide *in vacuo*. The yield of dry material was 350 mg.

Composition. This preparation had an LD_{50} of 0.8 mg (Table I). It contained 52% total saccharide, as measured by Dische's(5) indol method, 53% nucleic acid, and 8.5% nitrogen. It contained 0.2% serum albumin equivalent (probably polypeptide), as found by Folin-Ciocalteu procedure, and 8% lipid. The latter was determined as follows: 200 mg of protein-free material was dissolved in 39 ml of distilled water, 1.1 ml of conc. sulfuric acid added and the mixture boiled with refluxing for 1.5 hours. The cooled hydrolysate was shaken with chloroform and centrifuged. Chloroform phase was removed, filtered and evaporated to yield 16 mg of wax-like lipid. We were unable to remove from the protein-free endotoxin the nucleic acid component with ion-exchangers or by fractionation with acetone or ethanol. Uranium acetate precipitated both the polysaccharide component and the NA moiety. *Absorption curves.* In Fig. 1 is seen the ultraviolet absorption curve of the natural nucleoprotein complex endotoxin, labeled NP, together with the absorption curves of yeast nucleic acid (YNA), solid line, and the protein-free endotoxin (PF). All show a peak at 260 mμ. NP shows an absorption curve typical of plant nucleoproteins. The PF absorption curve indicates removal of protein from the natural product. Only slightly different products were obtained when hydrolysis with phenol water was carried out at lower temperatures, or by applying mechanical mixing instead of extraction in the Waring blendor, or by using phenol-water mixtures of different proportions.

Summary. 1) Sonic disintegration puts about 90% gonococcal endotoxin into a soluble state as contrasted with 50% yields by earlier methods. Most fractions obtained by

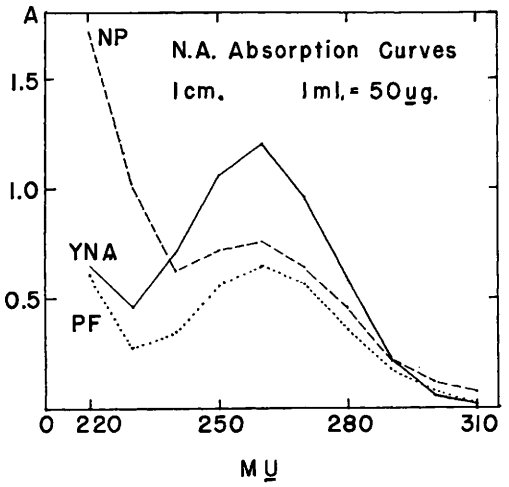

FIG. 1. Absorption spectra. NP = natural nucleoprotein complex; YNA = yeast nucleic acid; PF = protein-free endotoxin.

this physical procedure did not differ statistically among themselves in toxicity, but all of them were invariably more toxic than the product obtained by extraction of gonococcal cells at an alkaline pH. It is believed these methods will be useful in biological studies where cell-free, chemically unchanged fractions are desired. 2) Phenol-water mixtures are suitable for removal of the protein component from nucleoprotein complex of *N. gonorrhoeae* endotoxin. The protein-free endotoxin contains mainly polysaccharide and NA. The dried product is more toxic than those obtained by other methods. It dissolves readily in water resulting in a slightly opalescent solution. This fraction should be useful in studies where portein-free material is desired.

ADDENDUM: While this paper was in press we were able to remove all of the NA from the endotoxin. The method employed, chemical nature and other properties of the product will be described in subsequent publication.

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Effect of 4-Amino Folic Acid Antagonists on Biological Acetylations.* (24461)

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Previous studies have shown that p-aminosalicylic acid (PAS) inhibits acetylation of isoniazid (INH), and produces a marked increase in plasma levels of INH when the 2 drugs are administered in combination(1). The synergistic effect of the INH-PAS combination observed in tuberculosis therapy has been attributed, in part, to inhibition of INH acetylation by PAS(2). It is conceivable that similar events may occur when multiple antimetabolite combinations are employed in cancer chemotherapy. Thus, apart from the anticancer effect contributed by each member of the combination by virtue of its antimetabolite activity, secondary factors may be present to further increase the over-all effect(3). For example: 6-amino-nicotinamide (6-AN), a carcinostatic antimetabolite (4-7) is a competitive inhibitor of arylamine acetylation, and is itself acetylated in rabbits(1,8). Administration of this substance in combination with a second antimetabolite having similar properties with respect to acetylation could give rise to increased plasma and cellular concentrations of either substance, due to inhibition of acetylation. The end result could be

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