servations(1,5), and indicates a release of stored radioactivity. Assuming that the stored radioactivity represents radioactive cyanocobalamin(4) and with a normal intake and absorption of vit. B_{12} this finding suggests an exchange between stored cyanocobalamin in the liver and the rest of the body. Such turnover can be enhanced by parenteral injections of large amounts of vit. B_{12}. The loss of the vitamin in the urine can be explained by the assumption that all available binding capacity was exceeded whereby the free cyanocobalamin was excreted by the kidneys.

Summary. Radioactivity measurements were made over the liver, spleen and precordium for 7½ months after oral administration of test doses of radioactive vit. B_{12}. One subject was given a large series of parenteral injections of nonradioactive vit. B_{12}, while another subject served as a control. The disappearance rate of radioactivity was much faster with the repeated parenteral injections. Radioactivity was lost in the urine. These observations showed that massive parenteral doses of vit. B_{12} exchanged with cyanocobalamin stored in the liver.

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Preparation and Some Properties of *Neisseria gonorrhoeae* Endotoxin.* (23323)

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In the present paper a rapid and simple method for the preparation of a stable fraction containing dry, soluble endotoxin from dry gonococcus cells is described. This material offers a fairly toxic product suitable for *in vivo* and *in vitro* studies. It has been observed that washed gonococcus cells dried over sodium hydroxide *in vacuo* dissolve readily at pH 13. Under these conditions presumably all of the cell-bound endotoxin is freed. Other investigators(1,2) have previously used a much less alkaline pH for the slow extraction of cell-bound endotoxin. Under those conditions only a small portion of the endotoxin is obtained. The gonococcus in these studies was grown in an atmosphere of air using a modified liquid medium which was found to be suitable for the large scale cultivation of the organism.

Materials and methods. Culturing methods. Six different strains of gonococcus were used in this study. Five were isolated from patients by Dr. James D. Thayer of this laboratory, one strain was obtained from the American Type Culture Collection (No. 9827). The strains were maintained on dextrose starch agar (Difco) supplemented with 0.1% yeast extract and 1% nutrient agar

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(Difco) (3). Serial transfers were made every 3 days. From the slants transfers were made to test tubes containing 6 ml portions of liquid medium (described in the procedure to follow). From the test tube cultures which were maintained as a permanent stock, transfers were made to 250 ml Erlenmeyer flasks containing 75 ml portions of liquid medium. These "seeding" cultures and the slants were grown in an atmosphere of CO_2 (candle method). All cultures were grown for 3 days at 36°C. *Large scale cultivation of gonococcus.* The medium had the following composition (g/l): soluble starch, 0.5; yeast extract (BBL, Difco), 1; Bacto nutrient broth, 9; Difco proteose peptone No. 3, 4; dextrose 2; Na_2HPO_4 3; NaCl 5. The pH after autoclaving was 7.2. Low form Pyrex flasks (2.5 l) containing 1.0 l of medium were employed. Final inoculation was made with 20 ml of seeding culture/l. In this medium the organism grew as well in an atmosphere of air as in one of CO_2. After 6 days of growth at 36° the cells were collected in the Sharples centrifuge. They were washed twice with 0.85% sodium chloride solution and once with distilled water. After each washing the cells were collected in the Servall angle centrifuge at 7000 g. The washed cells were dried over sodium hydroxide *in vacuo*. The yield of dry cells was 60 to 100 mg per l of medium, depending on the strain employed. The American Type Culture Collection strain grew best, but its endotoxin producing capacity was low. 25 l of culture were processed at one time. After completion of this paper we found that the nutrient broth may be omitted provided 0.8% proteose peptone No. 3 is used. Then good growth is obtained in 3 days. In the following experiments a single strain (isolated from a patient) was employed. *Preparation of endotoxin.* 500 mg of dry gonococcus cells were ground in a mortar and the resulting fine powder was then dispersed and lysed with two 6 ml portions of 0.05 N sodium hydroxide (pH 13). The resulting mixture was placed in a 125 ml glass-stoppered Erlenmeyer flask. The mortar and pestle were rinsed with 60 ml of 0.05 N sodium hydroxide and the washings added to the lysate. The flask was stoppered and the mixture shaken for ½ minute. The mixture was then adjusted to pH 7.4 with 0.5 N acetic acid. If a precipitate of proteins formed it was redissolved by shaking. Cell debris were removed by centrifuging in a cold room in the Servall angle centrifuge at 6600 g. The supernatant was placed in cellophane bags and dialyzed for 20 hours at 4° against 14 l of distilled water. The latter was stirred continuously. A small quantity of sodium chloride was added to the dialyzed supernatant and the endotoxin was precipitated, together with nucleoproteins and small quantities of other proteins, by the 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 40% on the basis of dry gonococcus cells. This preparation, which consisted mainly of nucleoproteins, contained 14.24% nitrogen and 1.08% phosphorus. This product was dissolved in a small volume of 0.05 N sodium hydroxide. The resulting solution was adjusted to pH 7.40 with 0.25 N acetic acid, diluted to the desired concentration with sterile distilled water and administered intraperitoneally. It had a MLD (minimum lethal dose) of 2 mg/20-22 g mouse. The animals were observed over a 5-day period.

Results. Effect of trypsin on endotoxin. From 1 g of dry gonococcus cells a cell-free endotoxin-containing solution was prepared as described in the preceding paragraph, except that the solution was not dialyzed. It was divided into 2 equal portions (21 ml each). To one portion 5 ml crystalline trypsin (Worthington, 2x crystallized) solution containing 50 mg of trypsin protein was added, and the mixture adjusted to pH 8.6. To the second portion 5 ml of distilled water was added as a control. To each sample 0.25 ml of 2% merthiolate solution was added. The samples were kept at 36° for 7 days and the pH was maintained at 8.6 by the addition of 0.05 N sodium hydroxide. At the end of the digestion period the samples were dialyzed for 24 hours against distilled water. The MLD of the trypsin-digested sample was 1 mg, that of the control was 2 mg, on dry weight basis. In another experiment an endotoxin-containing solution was kept at pH 7.0. After

3 days of tryptic hydrolysis, most of the endotoxin was destroyed. There remained only a small quantity of toxic nucleoprotein.

Yield, toxicity and properties of different gonococcus fractions. MLD values are close approximates because only a small number of mice were used for each quantity of endotoxin. The dry gonococcus cells had an MLD of 5 mg. The lysate after dialysis showed a removal of 48% of soluble (mostly nucleoprotein) material from the cells. On this basis the MLD was 0.5-2 mg. The acetone precipitate's MLD was 2 mg and the yield was 40% on dry cell basis. An acetic acid precipitate was obtained by precipitating the proteins from the dialyzed lysate at pH 4.2, dissolving the precipitate in dilute sodium hydroxide and lyophilizing the neutralized solution. The MLD of such products made at different times from the same cell source varied from 6 to 30 mg and the yield was 40 to 46%. The tryptic digest which was conducted at pH 8.6 for 7 days contained after dialysis only 6% solids and had an MLD of 1 mg. The control had a solid content, after dialysis, of 16% and an MLD value of 2 mg. An aliquot of the control before dialysis had a solid content of 60% and an MLD of 7 mg. Thus, part of the loss appears to be due to tryptic hydrolysis and part to the alkaline pH. In another experiment without trypsin at pH 13 all of the endotoxin was destroyed in 7 days at 36°. This endotoxin, however, is quite resistant to heat. There was no loss of toxicity when a solution containing 3 mg of endotoxin per ml at pH 7.0 was kept at 60° for 60 minutes.

Deoxynucleic acid content of different gonococcus fractions. Because of the importance of deoxynucleic acid in endotoxin formation and because the results may aid in the elucidation of the chemical nature of this endotoxin, DNA assays were made on most gonococcus fractions. Dry gonococcus cells contained 5.8% DNA. The acetone precipitate's DNA content varied in different preparations between 5.6-6.0%. The tryptic digest, after dialysis, contained 14.0% DNA. The lysate at pH 8.6 kept for 7 days at 36° without trypsin contained 2.4% DNA before dialysis. An aliquot of this lysate, after dialysis and concentration to the original solid content contained only a trace of DNA. This indicates that the residual portion became small enough to pass through the cellophane membrane. Here the solid content dropped from 60% to 16%. Tryptic hydrolysis resulted in a different breakdown, because after dialysis, both the MLD value and the DNA value were high. Nevertheless much of the endotoxin was destroyed as indicated by the yield on a solid basis.

For DNA analysis the various fractions were hydrolyzed for 15 minutes with boiling 5% trichloroacetic acid in test tubes covered with appropriate glass bulbs to prevent evaporation. Aliquots of the filtrates were analyzed by the diphenylamine method(5), using the Klett-Summerson photoelectric colorimeter with orange filter No. 721. Thymus DNA, very kindly furnished by Dr. J. L. Irvin, was employed for the preparation of a calibration curve.

Discussion. Tryptic hydrolysis (at pH 8.6) followed by dialysis resulted in the removal of much inert material and a 2-fold increase in toxicity. There is, however, considerable loss in endotoxin mostly because of the action of trypsin. Gonococcus endotoxin is quite sensitive to continued exposure to an alkaline pH but quite resistant to heat at pH 7.0. A comparative study of different endotoxin-containing fractions has been made. Our findings indicate that this endotoxin is a protein. There is another kind, a so-called Boivin-type of endotoxin present in certain Gram negative bacteria which has been named "glucolipid." That endotoxin has been found to contain a polysaccharide combined with phospholipid and is prepared by the extraction of bacterial cells with trichloroacetic acid. A small quantity (1.5 mg per g of bacterial protein) of glucolipid is also present in the gonococcus(5).

Summary. 1. An improved method for large scale cultivation of *N. gonorrhoeae* in an atmosphere of air has been described. 2. A rapid method for the preparation of a stable fraction containing dry, soluble gonococcus endotoxin and some of the properties of this product have been presented. This endotoxin appears to be a protein. 3. DNA equivalents

in different gonococcus fractions do not parallel toxicity.

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Cholinesterases in Human and Ruminant Central Nervous Tissue.* (23324)

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In comparison to acetylcholinesterase (AChE), the role of butyrylcholinesterase (BuChE) in central nervous tissue has been relatively unexplored. This seems to be due to (a) lack of knowledge of the physiological substrate for BuChE, (b) presence of this enzyme in the glial tissue rather than in the axoplasm(1) which would rule out any function for this enzyme in nerve impulse propagation, and (c) its apparent absence from ruminant central nervous tissue, making it difficult to assess the fundamental function of the enzyme. Its reported absence from ruminant tissue has been based on the observation of Mendel, Mundell and Rudney(2), that benzoyl choline is a specific substrate for BuChE, and no benzoyl choline hydrolysis is evident when tissues from this species are assayed with this substrate(3). Recently, Hardwick(4) reported isolation of an enzyme from bovine plasma that possessed properties of a BuChE, except for its inability to hydrolyze benzoyl choline. This investigation was undertaken to determine whether a BuChE could be demonstrated to be present in bovine brain tissue. If this could be established it might be possible to view the role of this enzyme in brain tissue in a new perspective.

Methods. Because of observations that BuChE activity is higher in the white matter of central nervous tissue than in the gray(5), this part of the brain was used for assay. The brains were kept frozen at −15°C until ready for use, and then allowed to thaw, at which time the desired samples were dissected out, a 1:5 homogenate in 0.023 M $NaHCO_3$-0.154 M NaCl prepared, and aliquots ranging between 0.5 and 1 ml were used in the determinations. Activity was measured in the rotary Warburg apparatus by a modification of the method of Ammon(6), and rates were calculated from the initial velocity, although in most cases the velocities simulated a zero-order reaction during the test period which was usually 60 minutes. Differentiation of the 2 cholinesterases was made possible by the use of a specific acetylcholinesterase inhibitor, mytelase† (N′, N′,-bis-[2-diethylamino ethyl] oxamide bis-2-chlorobenzyl chloride)(7). Reaction mixtures with the final concentration of reactants in the vessel are given in the tables. In all cases the substrate was tipped in from the side arm after 5 minutes equilibration. All results are the averages of 4 experiments.

Results. The Q values, expressed as μmols of substrate hydrolyzed/g tissue/hr, for human, bovine and sheep central nervous tissue white matter, are shown in Table I. With acetylcholine as a substrate, hydrolysis occurs in the 3 species to varying extents, with human white matter having the highest activity, and bovine white matter the least. To assess the contribution of each of the 2 enzymes, the tissue was then assayed in the presence of 5×10^{-7} M mytelase. It can be

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