Whether they differ in any other significant way, and whether the cell culture isolates possess useful characters not seen in previous isolates, is not presently known.

Summary. Five isolates of the trachoma agent have been made by culture in irradiated McCoy cells, without use of the standard method of yolk sac inoculation. These have been passed serially in cell culture at intervals of 3-4, or at 5 days, 9 to 15 times. Increase in level of infection was indicated by increasing counts of inclusions observed regularly at 48 hours after inoculation, and by cytopathic effect in some passages. Passage of cell culture harvests to yolk sacs gave irregular results throughout the series with no indication that successful passage in cell culture was accompanied by adaptation to yolk sac.

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Immunological Properties of Bacterial Cell Wall Mucopeptides.* (29842)

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The mucopeptide component of bacterial cell walls is a common entity among a wide variety of microorganisms and is essential for the rigid structure of the cell wall. There have been several analyses showing qualitative and quantitative variations among microorganisms in the composition of this component(1). To our knowledge there have been no immunochemical studies on mucopeptides, although others have worked with antigens containing this material(2,3). This report presents evidence for the antigenicity of mucopeptides from beta-hemolytic streptococci and describes the mucopeptide-serum reaction. The antigenic relationship between mucopeptides from representative bacterial species, and the structure of the antigenic sites, will be reported later.

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Materials and methods. Cell walls were prepared by the method of Salton and Horne (1). Some preparations were further purified by sucrose gradient centrifugation(4). Treatment included digestion with trypsin and ribonuclease. Mucopeptide was obtained by formamide extraction as described by Krause and McCarty(5). The extraction was repeated 3 times at 170°C for 60 minutes. Preparations were also made from some strains by trichloracetic acid (TCA) extraction(6) or the warm phenol method(7). The following bacteria have been used: group A streptococcus, type 3, strains D-58 and C203/29/4; group A variant streptococcus, strain B455; group C streptococcus, strain H46A; group D streptococcus, strain F-24; *Staphylococcus albus; Bacillus cereus* and *Chromobacterium violaceum.*

Rabbits were immunized by one of 3 meth-

ods: intravenously with a heat-killed bacterial suspension, intradermally with a heat-killed suspension, or subcutaneously with mucopeptide in Freund's adjuvant. An increased antibody concentration was observed in each of the groups. Rabbit globulins were obtained by the method of Nicol and Deutsch (8).

Antibody was determined by the quantitative precipitin technique(9), using 0.2 ml of serum and a total volume of 0.5 ml fluid per tube. Since mucopeptide had negligible absorbance at 280 mμ, the washed precipitate was dissolved in 0.1 N NaOH and the precipitated antibody determined directly after correcting for turbidity at 320 mμ. The mucopeptide preparations were insoluble, but stable suspensions were obtained by sonic vibration for 20 minutes, or by lysozyme digestion. Where appropriate, controls of lysozyme plus serum were included.

Qualitative supernatant tests show excess antibody with lower concentrations of antigen, and the presence of both antibody and antigen in the region of higher antigen concentrations where there is no increase in antibody precipitated. Although this is usually evidence of contaminating antigens, from the results given below it is better ascribed to the fact that the mucopeptide is a very large fragment with several distinct directive groups. Quantitative supernatant tests and cross-absorptions, with homologous and heterologous antigens, are being done to distinguish directive groups common to microbial mucopeptides from those specific for certain microorganisms.

Results and discussion. Although about 20% of normal (pre-immunized) rabbit sera show no reaction with mucopeptide, most pre-immune samples give as much precipitate as immune sera with amounts of antigen up to about 25 to 50 μg/ml of serum. With larger amounts of mucopeptide a plateau of antigen excess is always reached with normal serum (Fig. 1). Studies with fractions from

FIG. 1. Comparison of serum from individual rabbits before and after immunization with group A streptococcal mucopeptides. Two animals selected from 8 immunized in this manner to show range of normal and immune sera. Antigen is group A variant (B455) streptococcal mucopeptide.

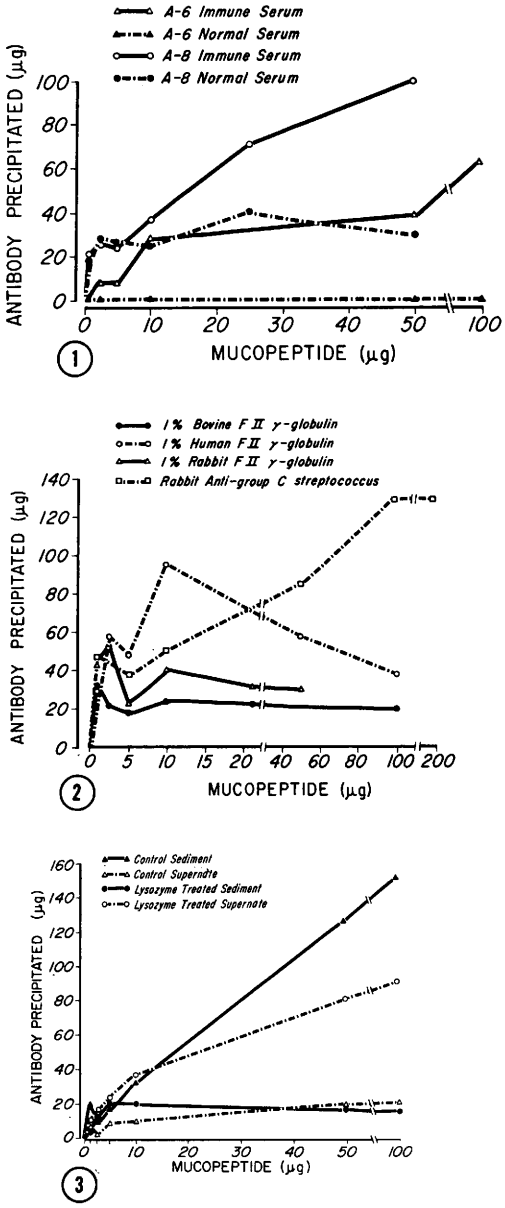

FIG. 2. Quantitative precipitin reaction with mucopeptide from group A variant streptococcus. Gamma-globulin (Cohn Fraction II) from pooled normal rabbit, bovine or human sera. Antiserum from rabbit immunized I.V. with group C streptococci.

FIG. 3. Effect of lysozyme treatment of mucopeptide on quantitative precipitin reaction. Antiserum is from rabbits immunized intradermally with group A streptococci and contains no anti-C polysaccharide. Mucopeptide is from a group A streptococcus. Fifty μg lysozyme per mg mucopeptide, 4 hours at 37°C, centrifuged at 37,000 × g and the sediment resuspended in buffer.

pooled normal rabbit, human,† or bovine‡ serum indicate that the reactivity of normal serum has at least one characteristic of antibody, since only Cohn Fraction II γ-globulins give any reaction with mucopeptide and these give quantitative curves similar to normal serum (Fig. 2). Human serum fractions III, III-O, IV4, V, bovine albumin, and rabbit B_2-globulin give no reaction with mucopeptide.

Immunization of rabbits, on the other hand, results in serum giving an increased precipitate with larger amounts of antigen (Fig. 1, 2). From the evidence below we believe this reaction with immune serum represents at least 2 antigenic groups of the mucopeptide structure and not contaminating antigens. The possibility that the reactive groups are artifacts or formyl groups introduced by the extraction procedure is unlikely since most of the antisera were obtained from animals immunized with intact bacteria. In addition, comparable serological reactivity was obtained with mucopeptide prepared by TCA or warm phenol procedures.

The group-specific C polysaccharide is the most likely antigen on the streptococcal cell wall to persist during the purification procedure and confuse the reaction of antibody with mucopeptide. This possibility was eliminated by the following evidence: (i) antiserum containing anti-C polysaccharide was absorbed exhaustively with isolated C polysaccharide and the curves of absorbed and not absorbed sera plus group A streptococcal mucopeptide were almost superimposable; (ii) treatment of mucopeptide with β-N acetyl glucosaminidase(10) did not affect subsequent reaction with antiserum; (iii) antiserum from animals immunized intradermally with streptococcal vaccine or with mucopeptide in Freund's adjuvant contained no detectable anti-C polysaccharide but gave a quantitative reaction comparable to serum from intravenously immunized rabbits which did have anti-C polysaccharide; (iv) mucopeptide from group A strains or group A-variant strains§ reacted comparably with serum containing antibodies against original C polysaccharide.

Evidence that the antigenic reactants are integral components of the mucopeptide structure rather than protein or other cellular contaminants is: (i) amino acid analysis reveals only components characteristic of cell wall mucopeptide(1) and trace amounts of threonine, valine and leucine; (ii) there is negligible light absorbance by the mucopeptide preparations in the range of 320 to 240 mμ; (iii) treatment with trypsin, papain, pronase, β-N acetyl glucosaminidase or ribonuclease does not affect the serological reaction; (iv) extraction with TCA which removes teichoic acid has no effect; (v) lysozyme treatment of the mucopeptide has a profound effect which varies with the extent of substrate digestion. The mucopeptide antigens of the above studies remain in suspension after centrifugation at 2000 $\times$ g but are sedimentable at 37,000 $\times$ g. After treatment with lysozyme all serological reactivity is soluble, with negligible amounts of antigen sedimentable at 37,000 $\times$ g (Fig. 3). More extensive digestion with lysozyme considerably reduces the capacity to precipitate with antiserum, although inhibition studies show digestion products are present which react with antibody.

Summary. Specific antibodies to cell wall mucopeptide can be obtained which should be useful in elucidating and comparing the structure among microorganisms. Also significant is the observation that nearly all animal sera studied react with most mucopeptide preparations, regardless of source. This represents a "natural" immunological response against an ubiquitous antigenic moiety common to a variety of microbial mucopeptides. In addition to this response, serum from immunized rabbits contains antibodies which react with groupings of smaller available concentration on the intact mucopeptide and hence requires addition of 10-20 times as much antigen for maximal precipitation.

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† Nutritional Biochemical Corp.

‡ Armour Pharmaceutical Co.

§ Variant group A streptococci possess a C polysaccharide antigenically distinct from the original group-specific C polysaccharide(10).

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Diet and Temperature Effects on Excretion of Fat-Mobilizing Substances in Rat Urine.* (29843)

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Chalmers *et al*(1) reported the isolation of a fat-mobilizing substance (FMS) from the urine of fasting humans. Subsequently (2) we reported the extraction of a similar material from the urine of fasting rats (rat FMS). More recently, the separation of rat FMS into 2 chemically and biologically distinct fractions was accomplished in this laboratory and some of the characteristics of these fractions were determined(3,4). One fraction, FMS IA induced hypophagia but not fat mobilization when injected into rats; the second fraction, FMS IB, caused fat mobilization without an effect upon food intake. Both substances exhibited lipolytic activity *in vitro,* the activity of IB exceeding that of IA. Both substances in their present state of purity contain 16-17 amino acids, carbohydrate, phosphorus, hexosamine and a cholesterol-like material but are chemically distinct as shown by thin layer chromatography and electrophoresis(3).

The present communication reports our observations on the effects of previous diet and of environmental temperature on excretion of FMS, FMS IA and FMS IB in the urine of fasting rats.

Methods. Male rats of the Wistar strain were fed one of the 4 experimental diets shown in Table I. FMS, FMS IA and FMS IB were extracted from urine (collected under toluene) and fractionated as previously described(3). Omission of solution in water to separate FMS IA and IB results in extraction of FMS. Lipolytic activity *in vitro* was determined by measuring increase of free fatty acid release when the substance (100 μg) was added to about 250 mg adipose tissue (rat epididymal fat pad) in an incubation medium of Krebs-Ringer bicarbonate buffer (pH 7.3-7.4) with 4% bovine serum albumen and the whole incubated for 3 hours with shaking at 37°C. Free fatty acids in the incubation medium were measured by the procedure of Dole(5).

I. *Effect of previous diet.* Four groups of 8 rats each, weighing 400-450 g, were housed in stainless steel metabolism cages at 23 ± 1°C and each group was fed one of the 4 diets for a period of 6 days. The animals were then fasted for 24 hours during which urine was collected and pooled for each group. FMS IA and FMS IB were separately extracted and assayed for lipolytic activity *in vitro*. The same animals were then refed their experimental diets for a period of 6 days but with a caloric restriction of approximately 50%. Urine was collected during fasting, pooled for each group and FMS was extracted and assayed *in vitro*.

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