II). The "acid" phosphatase activity of serum, moreover, is less stable on standing than is "alkaline" serum phosphatase and is more slowly inactivated by heating to 60°C.

The "acid" phosphatase of normal serum was found to have a broad range of optimum activity from about pH 4.0 to pH 6.5. This, together with the characteristics summarized in Table II suggest that the enzyme concerned is best classified, for the present, as a phosphomonoesterase A_2. The available data indicate that "acid" serum phosphatase is not identical with erythrocyte phosphatase[1] or with the "acid" phosphatase found in normal prostate tissue[7] and in the skeletal metastases[8] and serum[5] of patients with disseminated prostate carcinoma. As regards phosphatases occurring in the liver and spleen,[9, 10] however, no serious inconsistencies are apparent at this time. These organs may be a source of the "acid" phosphatase content of the serum.

Conclusion. In addition to "alkaline" phosphatase, blood serum contains minute amounts of one or more "acid" phosphatases of the type classified by Folley and Kay as phosphomonoesterase A_2.

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A Micro Precipitin-Technic for Classifying Hemolytic Streptococci, and Improved Methods for Producing Antisera.

REBECCA C. LANCEFIELD. (Introduced by Homer F. Swift.)

From the Hospital of the Rockefeller Institute for Medical Research, New York.

The increasing practice of grouping hemolytic streptococci serologically by the use of the precipitin-reaction has stimulated us to devise a microtechnic whereby the accuracy of the original method is retained with a great saving of material. Potent sera are essential; and for the maintenance of a steady supply, it has been found advantageous to keep on hand rabbits which are repeatedly immunized and bled, with rest periods between the courses of treatment.

It is important to select suitable strains for immunization. The two methods of immunization employed lead to the production of precipitins for both the group-specific carbohydrate (C) and the

[7] Kutscher, W., and Wörner, A., *Z. f. physiol. Chem.*, 1936, **239**, 109.

[8] Gutman, E. B., Sproul, E. E., and Gutman, A. B., *Am. J. Cancer*, 1936, **28**, 485.

[9] Davies, D. R., *Biochem. J.*, 1934, **28**, 529.

[10] Bamann, E., and Riedel, E., *Z. f. physiol. Chem.*, 1934, **229**, 125.

type-specific substances, which are proteins (M) for the types within Group A and probably polysaccharides (S) for the types within most of the other groups. Consequently, those strains that have lost their type-specific substances—either spontaneously or induced by serial subcultures in type-specific serum or by selection of non-type-specific colonial forms (glossy for Group A, rough for other groups)—are the best antigens when the formation of group-specific precipitins is the object. Such strains have been prepared for several of the groups. Group D has proved especially difficult.

When strains devoid of type-specific substance are not available, a strain of the same *group*, but of a different serological *type* from that used in immunization, is employed in preparing the extract for the precipitin-test. This eliminates a possible complicating type-specific antigen-antibody reaction. If the homologous strain, containing type-specific antigen, were used in this test, the extract would contain homologous specific substances for both *group* and *type*, and it would be impossible to distinguish between type- and group-specific precipitins, one or both of which might be present. These precautions are necessary in testing sera which are being prepared, although they are not important in the identification of new strains once suitable group-antisera have been obtained.

Method of Immunization for Preparing Group-Antisera. In Group A, heat-killed cultures have been found at least as satisfactory antigens as formalin-killed bacteria for preparing antisera. Eighteen-hour broth cultures are heated at 56°C for one hour. The first week the rabbits are given 5 daily intravenous injections of 1 cc of whole broth culture followed by a rest period during the second week. In the third week, five 2 cc doses are given, and in the fifth week, five 4 cc doses. The larger doses are given in volumes of 1 to 2 cc prepared by centrifuging the culture and resuspending the bacterial sediment in broth or saline. If further immunization is indicated, it is better to repeat the 4 cc doses of vaccine rather than to increase the amount or to give living culture. Five days after the last injection of the second series, a test bleeding is made. If a sufficiently high precipitin-titer is found, 50 cc of blood are taken from the ear vein into a centrifuge tube lined with a thin layer of sterile vaseline, which, by allowing the clot to separate from the tube spontaneously, favors a large yield of colorless, clear serum. Immunization is continued according to schedule, and 50 cc of blood are obtained every 2 weeks until the antibody-titer begins to fall. The animal is then rested for 2 months or longer. When more serum is needed, injections are begun again, starting with 1 cc doses

as before, with the precaution of giving the first few intraäbdominally or subcutaneously to avoid death of the animal from shock. Animals which have rested from 2 to 12 months usually respond satisfactorily after one series of injections, although 2 or more series may occasionally be required. Furthermore, rabbits that were originally unsatisfactory in their response may on secondary injection produce high-titered antisera. This procedure has proved especially helpful with Groups G and H. It is advisable to keep 2 rabbits on hand for each group and replace them when necessary on account of age or intercurrent infection, such as snuffles. We have kept numerous animals immunized in this way for 3 or 4 years. A typical rabbit producing Group A serum has been under immunization for 3½ years with 6 long rest periods, and has yielded 500 cc of high titered serum, half of which was obtained during the first year.

For preparing antisera to all groups except Group A, formalin-killed cultures have been found much better than heat-killed. The sediment from an 18-hour broth culture is suspended in 0.2% formalin in saline in one-twentieth the volume of the original culture. This is stored in a tightly stoppered container in the icebox. Periods of one to 4 or 5 days are required to kill the streptococci, the time varying according to the serological group of the strain. Immediately before use, a 1:20 dilution of the formalized cell suspension is prepared. Six intravenous injections of 1 cc each are given daily in alternate weeks. Test bleedings are made after the second series, and the main bleedings and rest periods are arranged on the same schedule as that used for Group A animals.

Slight cross-reactions between certain groups have been reported by numerous observers, most often in Group C antisera. These cross-reactions are usually observed only after the tubes have stood in the icebox overnight. When there is a sufficiently high group-antibody content, the cross-reactions are insignificant. When, on the other hand, the homologous group-reaction is weak, these cross-reactions with heterologous groups become relatively more significant and lead to confusion. The cross-reacting capacity of potent group-antiserum may be eliminated by absorbing the serum with heat-killed streptococci from a strain of another serological group, but absorption is not usually found necessary. However, 5 cc of serum can be satisfactorily absorbed with 1 cc of packed bacteria of a heterologous group if the procedure seems advisable.

Preparation of Extracts. Extracts are prepared as previously described.[1] The bacterial sediment from 250 cc of broth culture is sus-

[1] Lancefield, R. C., *J. Exp. Med.*, 1933, **57**, 571.

pended in 10 cc of N/20 HCl in 0.85% NaCl. The suspension is sufficiently acid to turn Congo Red paper blue. The tube is immersed in a boiling-water bath for 10 minutes, cooled under running water, and centrifuged. The supernatant fluid is removed and neutralized and the resulting inactive precipitate is discarded after centrifugation. The final supernatant fluid is used in the precipitin-reaction.

For the micromethod smaller amounts in the same proportion are used. The bacteria centrifuged from 50 cc of a luxuriant growth of streptococci in plain or dextrose broth (or in blood-neopeptone broth for the fastidious organisms in Groups E, F, and G) are extracted in 2 cc volume. More dilute extracts lessen the speed and sensitivity of the reaction especially when the "ring" test in small volumes is used.

Microtechnic. An innovation has been introduced in performing the precipitin-test in a small volume. We have previously hesitated to use micro-methods because of possible misinterpretations due to prozonal phenomena, or to cross-reactions which, in small volumes, might appear to be of the same order of magnitude as the major group-reaction. However, with high-titered sera and sufficiently concentrated extract, we find that a ring-test in a single dilution in a tube of small caliber eliminates the danger of negative reactions due to the prozone by giving mixtures of varying concentrations at the junction of extract and serum. The correctness of the results is further safeguarded by applying the dilution-method to doubtful cases.

A small conical tube is prepared by slightly drawing 7 mm glass tubing in a flame. The drawn tubing is cut in the center to make 2 tubes. The narrow end is sealed into a knob to prevent the tube from slipping through the rack. The 7 mm diameter is retained at the open end to facilitate pipetting, and the lower end has a bore of about 3 mm, holding 0.1 cc in a column 8 to 10 mm high. Good results have been obtained by putting 0.05 cc of the extract into the tube first, and then 0.05 cc of serum. Since the serum is heavier, it sinks below the saline extract and forms a layer with about the right amount of mixing. With the usual technic employed in ring-tests of placing the serum in the tube first, too narrow a ring is formed due to insufficient mixing in a tube of such small caliber. If, due to an air bubble, the fluid fails to lodge at the bottom of the tube, no attempt is made to shake it down. The result can easily be read at any level, and shaking is apt to interfere with the formation of a good plane of junction. Positive reactions are usually obvious at once, and can be safely recorded after ½ hour at room

temperature. In order to save serum, exceptionally potent antisera may be diluted, but not beyond the point where an immediate heavy ring-reaction is obtained with extracts of the homologous group.

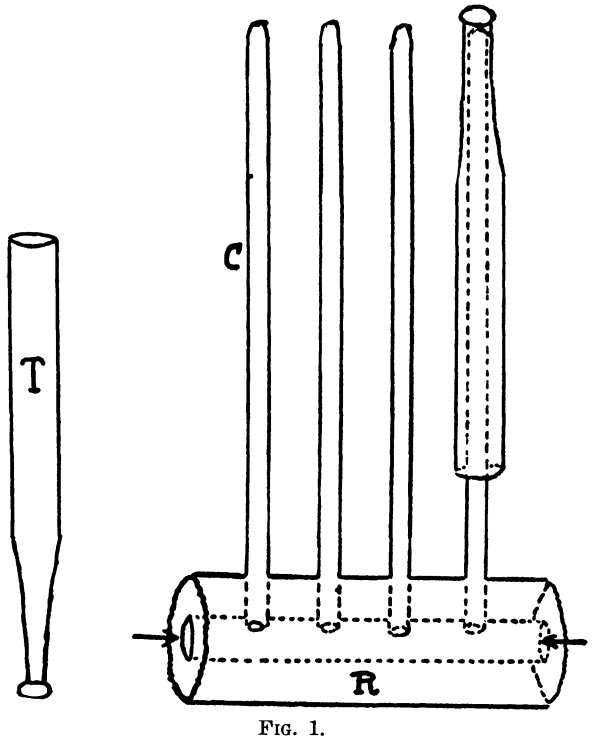

FIG. 1.

Precipitin-tube for microtechnic and apparatus for washing tubes. T. Precipitin-tube; R. Section of rubber pressure-tubing; C. Copper outlet-tube. Arrows indicate water-intake.

In case of doubt, the dilution-method with large volumes is used, or a comparable dilution-method can be employed with the microtechnic in the following manner: 0.05 cc each of undiluted extract and of 1:4 and 1:16 dilutions of extract are pipetted into 3 small tubes, and 0.05 cc of serum is added to each. The small tubes are observed for ring formation, as usual, at the end of a half hour. The tubes are then shaken, incubated at 37°C for 2 hours and placed in the icebox overnight before final readings are made.

The small tubes must be carefully cleaned. A convenient washing apparatus consists of a length of pressure-tubing into which holes have been punched to contain a row of outlets made of ⅛ in. copper tubing about 4 in. long. Running water enters the pressure-tubing at both ends. The small test tubes are first inverted over these outlets to wash out the contents. They are then immersed in bichromate

cleaning solution, washed with running tap water on the apparatus described, and finally rinsed in distilled water and dried for use.

We have recently reëxamined 175 cultures of hemolytic streptococci, which had previously been grouped. The micromethod agreed completely with earlier results and there was no uncertainty in reading the reaction. It seems, therefore, that the method of using a ring-test with small amounts of material is feasible if one observes the safeguards of employing concentrated extracts and potent antisera, absorbed when necessary. In case of doubt the results can always be confirmed by the dilution-method.

The discussion so far has dealt with the classification of hemolytic streptococci into broad serological *groups*. The methods described are, however, also applicable to the differentiation of serological *types* within these groups. Further details of the micromethod for the precipitin-test as applied to the classification into types of members of Group A will be given later.

It should be noted that certain non-hemolytic strains of streptococci may belong to some of the serological groups originally differentiated among the hemolytic streptococci. Stableforth was the first to show that some non-hemolytic streptococci isolated from bovine sources are members of Group B.[2] Sherman has made the same observation with reference to Group D.[3] We also have obtained occasional non-hemolytic members of Groups B, C, D, and G, some of which are serologically identical with known immunological types within the groups represented.[4]

Summary. Methods are described for producing high-titered group-specific antisera for classifying hemolytic streptococci into groups. A description is also given of a reliable micro-precipitin technic which can be used with very small amounts of bacterial extract and antiserum. Attention is called to the occurrence of some non-hemolytic members of Groups B, C, D, and G.

Since this communication was submitted for publication, Fuller has reported a method of extracting streptococci with formamide.[5]

2 Stableforth, A. W., *J. Comp. Path. and Therap.*, 1932, **45**, 185; *J. Path. and Bact.*, 1937, **45**, 279.

3 Sherman, J. M., *J. Bact.*, 1938, **35**, 81.

4 Lancefield, R. C., *J. Exp. Med.*, 1934, **59**, 459.

5 Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 130.