antigen, and that this portion is antigenic. This would lead one to anticipate the possibility of securing, under appropriate conditions, a relative cross-immunity to vascular damage with gram-negative organisms generally. The organisms used in the experiments reported here were selected as a test of this hypothesis, and the results observed support our earlier conclusions.

Summary. Mice immunized with endotoxin preparations of *Shigella paradysenteriæ* Flexner, *Salmonella typhimurium* and *Rhodospirillum rubrum* were found to have been protected against the induction of hemorrhage in implanted tumors.

Protection by immunization was found to be about as effective against the induction of tumor hemorrhage when the heterologous organisms were used as when the homologous were used. This finding supports our earlier hypothesis that a common antigenic toxic component is characteristic of gram-negative bacteria generally.

14360

A Rapid Method for Estimation of Penicillin

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In connection with chemical studies on penicillin and, more particularly, with efficient large scale production of the antibiotic substance, there has been an urgent need for a rapid method for the estimation of the substance. It had been hoped that the antibioluminescent activity of other antibiotic agents might be found to be a property of penicillin since use of this property gave a method for rapid titration of potency; such however did not prove to be the case.[1,2] Methods of titration requiring 3 to 6 hours delay are known and employed,[3], but with the rapid rise in activity seen in the more recent methods of penicillin production, this time interval is still too long for efficient production.

A report from England[4] stated that the group at the Wellcome Physiological Research Laboratories had devised a new method of testing for penicillin based on the hemolysis of red blood cells by hemolytic streptococci. This test was complete within 3 to 4 hours, *i.e.*, was not much faster than tests already in use. Rammelkamp[5] had already described an 18-hour test which was based on the hemolysis of red cells by hemolytic streptococci. In connection with studies on *Aspergillus flavus* in which the hemolytic streptococcus was used as the test organism[6] the present authors had occasion to note the very rapid appearance of hemolysis which occurred if conditions were optimal. This suggested that a method of titrating penicillin or other antibiotic substances could be devised with conditions so arranged that a reading could be made in as little as 60 to 90 minutes.

Of the strains of hemolytic streptococci tested, strain C-203 has produced detectable amounts of hemolysin most rapidly. Up to the present time no other group of bacteria has proven more satisfactory than *Streptococcus pyogenes*. Since penicillin acts only by

1 Rake, G., McKee, C. M., and Jones, H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 273.

2 Rake, G., Jones, H., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 136.

3 McKee, C. M., unpublished data; Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1943, **46**, 187; Foster, J. W., and Wilker, B. L., *J. Bact.*, 1943, **46**, 377.

4 Wilson, U., *Nature*, 1943, **152**, 475.

5 Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

6 Jones, H., Rake, G., and Hamre, D. M., *J. Bact.*, 1943, **45**, 461.

preventing growth of the cocci and has no action on hemolysin already produced, the first problem was to avoid the presence of preformed hemolysin which would act in the presence even of high concentration of penicillin. It has not proven possible to grow the organisms in fluid medium which will allow good growth without the production of hemolysin. In any medium in which the organism grew well, either plentiful hemolysin was produced or the organism developed variants which failed to produce hemolysin even in the presence of red blood cells. Moreover, because the hemolysin is apparently closely adsorbed on the surface of the organisms, it was not possible to remove it from them by throwing them down in a centrifuge and washing them repeatedly. The technic finally adopted was one of using cultures with so little preformed hemolysin as to be insignificant in the test. Cultures for inoculation are taken preferably during the logarithmic phase of their growth. The exact technic is given below.

Optimal conditions for the test have been determined. Beef heart infusion broth has proven the best medium; for red blood cells, 1% defibrinated rabbit's blood is better than any other concentration of the same or other blood; for incubation, most satisfactory results have been obtained by use of a water-bath at 37°C.

In performing the test a 2- to 3-hour culture in 1% rabbit's blood broth of C-203 (*i.e.*, one in which hemolysis is just appearing) subsequently stored overnight at 0°C, is used for the inoculation of a lot of 1% rabbit's blood broth in volumes of 0.1 ml of inoculum for each 2.0 ml of blood broth. As soon as this fresh culture shows detectable hemolysis (*i.e.*, within 2 hours), and the organisms are dividing rapidly, it is ready for use in the test. With such a culture, readings of potency can be read in 55 to 90 minutes. If it is desirable to test filtrates or other preparations without this 2-hour delay, the overnight culture may be used directly, but in this case it may be 2 hours before detectable hemolysin will be formed.

In carrying out the test, 2 ml amounts of 1% rabbit's blood beef heart infusion broth are placed in a series of 13 x 100 mm tubes. To each tube is added 0.1 ml of the appropriate dilution of penicillin standard or unknown filtrate. As a control of culture one tube receives 0.1 ml of distilled water. As a control of preformed hemolysin one tube receives 0.7 Florey units in 0.1 ml, *i.e.*, an amount of penicillin which will certainly inhibit growth of the streptococci. To each tube is now added 0.2 ml of culture. Contents of tubes are well mixed before incubation.

With the test as set out above, between .03 and .05 of a Florey unit in a total volume of 2.3 ml will prevent growth and production of hemolysin. In the standard the range used is from 0.08 to 0.01 Florey units (.08, .06, .04, .03, .02, .015, and .01). Filtrates are diluted and tested, according to their expected potency, in the following manner: 1/2, 1/3, 1/4, 1/6, 1/8, etc., up to 1/256.

In reading the test it has been found that the first signs of hemolysis can be most readily detected by whirling the tubes slightly and throwing a plume of red cells into the clear supernatant broth. In the presence of a trace of hemolysin this plume becomes rapidly hemolyzed. All tubes showing such commencing hemolysin are thoroughly mixed and replaced in the water-bath for 2 or 3 minutes by which time uniform hemolysis will have appeared. In most cases all tubes which are to show hemolysis will do so at almost the same time, and an end-point is obtained by taking the last tube in each series showing no hemolysis. Occasionally a tube shows hemolysis delayed by about 30 minutes over the others. Such a tube is given a ± reading and an interpolated potency.

The test can be set up rapidly and without sterile precautions. It can be performed with turbid or heavily contaminated samples. Potencies obtained by its use have been compared on many occasions with those obtained by more accurate 18-hour tests[7] and a remarkably good correlation has been obtained.

Summary. A rapid test for the estimation of penicillin or other antibiotic agents makes use of the hemolytic property of β streptococci. With optimal conditions, as set out above, results can be read within 55 to 90 minutes.

[7] McKee, C. M., Rake, G., and Menzel, A. E. O., to be published.