are individually inactive but fully active when combined.

The human complement in one ml of human serum can be deprived of its third component by incubation at 37° C for 1 hour with 1.35 mg of the insoluble carbohydrate of yeast ("zymosan") prepared in this laboratory.[3]

The fourth component is inactivated with 0.2 ml of 0.16 M ammonium hydroxide per ml of fresh serum. The mixture is first shaken and incubated for 1 hour at 37° C; neutralization is then carried out by the addition of 0.032 ml of 1 N HCl. The fourth component can also be inactivated with hydrazine (0.1 ml of 0.16 M hydrazine per ml of fresh serum).

Human complement is inactivated at 52° C for 30 minutes by virtue of the destruction of C'2.

In summary, human complement is composed of four components similar to but not entirely identical with the components of guinea pig complement. It was found that the third component (C'3) is the only component which is effectively mutually substitutive in human and guinea pig complement systems. This is the only component that has features suggestive of enzymic nature. Human C'1 has been purified by ammonium sulfate precipitation and phosphate buffer extraction. It is characterized as a euglobulin with an electrophoretic mobility of $2.9 \times 10^{-5}$ in veronal buffer of pH 7.8 and ionic strength 0.1. This preparation of C'1 comprises 0.8 per cent. of the total serum proteins. A protein fraction comprising 1 per cent. of the total serum proteins and containing nearly all C'2 has been prepared.

Evidence has been obtained to show that the bactericidal action of human serum against *Vibrio comma* is a result of the joint action of complement and antibody; and that this bactericidal effect is destroyed by the inactivation of any one of the four components of complement. The bactericidal action is restored to a specifically inactivated complement by the addition to it of the particular component which it lacks. It has also been found that the bactericidal action of whole human complement against this vibrio can be fortified by the addition of the end-piece ($pH = 5.4 - \mu\ 0.02$ supernate) of human complement.

Fixations of whole human complement and specifically inactivated human complements to viable sensitized *Vibrio comma* have been investigated. From whole human complement nearly all of the C'3 activity is removed during fixation and only small amounts of the activities of the other three components are removed.[4] However, sufficient amounts of the components are fixed so as to cause the destruction of the vibrios. From complement deprived of C'3, the other three components are fixed to the sensitized organisms but bactericidal action is not exerted. However, when C'3 is added to this complex, bactericidal activity occurs.

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## PIGMENT PRODUCTION BY SULFONAMIDE-RESISTANT STAPHYLOCOCCI IN THE PRESENCE OF SULFONAMIDES[1]

IN a previous report[2] the *in vitro* and *in vivo* production of sulfonamide-resistant strains of staphylococci was described. During the past year, investigations have been carried out concerning the mechanism of this resistance. Ten additional resistant strains of staphylococci have been shown to produce a substance which inhibits the antistaphylococcic action of the sulfonamides. It has been found that this inhibitory substance has several of the properties of p-aminobenzoic acid (PAB). These observations confirm the report of Landy and his associates,[3] who studied two sulfonamide-resistant strains of staphylococci supplied by us, and with a microbiological assay concluded that the sulfonamide inhibitor produced by these strains was PAB.

A standard test has been utilized for determining the *in vitro* resistance of staphylococci to the sulfonamides. The organisms were grown for several generations in a water-clear synthetic medium.[4] One tenth cc of a $10^{-3}$ dilution of a 24-hour culture was added to a series of tubes containing 10 cc of the medium with varying concentrations of sodium sulfathiazole. The contents were incubated for 48 hours at 37 degrees C, and bacterial growth ascertained and expressed in terms of turbidity. The growth of non-resistant strains of staphylococci was completely inhibited by less than 1 mg per 100 cc of sodium sulfathiazole; whereas, a concentration of 200 mgs or more was necessary for the sulfonamide-resistant strains.

While the foregoing studies on sulfonamide-resistance were in progress, an interesting and reproducible phenomenon was observed. Control cultures of either non-resistant or sulfonamide-resistant strains of staphylococci in synthetic medium attained their

[1] Aided by grants from the Charles P. DeLaittre Research Fund and from the Committee on Scientific Research of the American Medical Association.

[2] J. J. Vivino and W. W. Spink, *Proc. Soc. Exp. Biol. and Med.*, 50: 336, 1942.

[3] M. Landy, N. W. Larkum, E. J. Oswald and F. Streightoff, SCIENCE, 97: 265, 1943.

[4] G. P. Gladstone, *Brit. Jour. Exp. Path.*, 18: 322, 1937.

[3] *Jour. Biol. Chem.*, 137: 139. 1941.

[4] Human C' has been found by Heidelberger and Mayer (*Jour. Exp. Med.*, 75: 285, 1943) to add roughly as much N to specific precipitates as does guinea-pig C'.

maximum degree of growth within 24 hours of incubation. At the end of this time, and also following a more prolonged period of incubation, the contents of the culture tubes revealed a white, turbid suspension of organisms. In the presence of increasing concentrations of sodium sulfathiazole, the more resistant strains produced a yellow color, which first appeared at the end of 24 hours of incubation. If the cultures remained at incubator or room temperature for another 24 to 48 hours, the color became more intense. The color was so pronounced with some strains that a deep brown-orange pigment was observed. This pigment appeared only in the presence of the higher concentrations of the sulfonamide, and uniformly, shortly after maximum growth had been attained. Strains, whose growth was completely inhibited by 200 mgs per 100 cc of sodium sulfathiazole, showed pigment production occurring in the presence of 40 to 100 mgs concentrations of the drug. There was little or no inhibition of bacterial growth in the tubes showing the presence of the pigment. The pigment appeared also in the presence of sulfanilamide, sodium sulfapyridine and sodium sulfadiazine. It is of interest that strains 7 and 14, which were studied by Landy and his group, did not produce a demonstrable pigment. These strains are not highly sulfonamide-resistant.

The nature of this pigment is being investigated at the present time. The pigment is not of the usual type produced by many strains of staphylococci, since it is not soluble in fat solvents. Evidence at hand would indicate that the pigment is derived from PAB. It would appear that under the experimental conditions described, some strains of staphylococci reproduce readily in the presence of the sulfonamides because of the synthesis of significant amounts of PAB. After maximum growth has been attained, the PAB is changed from a colorless state to a yellow-brown pigment.

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## INTRA-OCULAR VIRUS INFECTIONS

IN investigation of the virus of epizootic fox encephalitis[1,2] we have found that the method of intra-ocular injection is a most useful procedure. This technique appears to be of great potential value in the study of other virus diseases. Observations of infections resulting from intra-ocular injections have been reported by a number of investigators in the virus field, principally in work on the pathogenesis of infections of the nervous system. It is felt, however, that the possibilities of this procedure have not been fully utilized in the approach to the more common problem of demonstrating the presence of viable virus.

Fox encephalitis can be experimentally transmitted to dogs, wolves and foxes. Evidence of infection has, in the past, consisted in death of the animal with characteristic symptoms and the demonstration of inclusion bodies in vascular endothelium in the brain and elsewhere. Studies of fox encephalitis have been hampered by a low or variable mortality of inoculated animals. This ranged from 10 to 80 per cent., depending upon the virulence of the strain of virus used and the natural resistance of the animals injected. Accurate titration of the amount of virus in tissue suspensions and the titration of antisera have been wholly impracticable, since the large number of foxes or dogs required was prohibitive. Employment of the simple technique of intra-ocular injection has changed this picture completely. Intra-ocular inoculation results in 100 per cent. infection of the eyes of foxes; the infection is easily observed grossly and is readily confirmed by demonstration of the inclusion bodies in smear preparations. Previously a 1 to 10 per cent. tissue suspension has been used[3] as an inoculum, but it has now been found that each of the several stock tissue suspensions tested was fully infective by intra-ocular inoculation in a dilution of 1 part in 100,000.

About 0.2 ml. of virus is injected into the anterior chamber after aspiration of a slightly greater volume of aqueous humor. Infection from the fox encephalitis virus is usually apparent grossly by the third day, and in all cases there is a diffuse opacity of the cornea on the fifth day. A significant amount of purulent conjunctival exudate is not seen unless bacterial infection has occurred.

On the fifth day the eye is removed either under anesthesia or after the animal has been killed. Aqueous humor is aspirated with a Pasteur pipette for bacterial culture. Inclusion bodies are demonstrated in the endothelial cells that line the internal surface of the cornea by the technique routinely employed in making smears of the epithelium of the urinary bladder in the diagnosis of canine distemper.[4] Using a modified Shorr's stain,[5] we have frequently found more than half of the cells in a smear to contain large inclusion bodies.

Neutralization tests are carried out by injecting mixtures of serum and virus dilutions into the anterior

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[2] J. C. Barton and R. G. Green, *Am. Jour. Hyg.*, 37: 21–36, 1943.
[3] R. G. Green, N. R. Ziegler, E. T. Dewey and J. E. Shillinger, *Am. Jour. Hyg.*, 14: 353–373, 1931.
[4] R. G. Green and C. A. Evans, *Cornell Vet.*, 32: 190–193, 1942.
[5] W. G. Page and R. G. Green, *Cornell Vet.*, 32: 265–268, 1942.