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Immunological Studies on the Cellular Constituents of *C. diphtheriae.*

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Since the existence of serological types among *C. diphtheriæ* is well established[1, 2, 3] the isolation of a cellular constituent responsible for type-specificity appears to be possible. Previous reports[4, 5] have shown that the polysaccharides of *C. diphtheriæ* in general are group-specific being shared by members of distinct serological types. From this it may seem obvious that a cellular component other than the polysaccharide is responsible for type-specificity. Because of this a systematic investigation of the various cellular constituents of *C. diphtheriæ* with the object of isolating the type-specific substance was undertaken. On the other hand, it seems also of interest to compare the immunological activities of the various cellular substances.

The organism employed for the present study was the well-known Park 8 strain which belongs to Type D41 of Sia and Huang's serological types.[3] Immune serum was prepared by the intravenous injection of rabbits with well-washed cultures grown on serum-broth, pH 7.6, in accordance with the technic previously used.[4] This will be referred to as the homologous immune serum. Immune sera were also prepared with other serological types of Sia and Huang's such as D25, D30, D40, 6287, 12190, and 2 untyped avirulent strains. This group of immune sera will be designated as heterologous immune sera. Organisms were grown on stomach-digest medium[6] for a week after which they were collected by centrifugation, washed 4 times with distilled water and then with 4 changes of 95% alcohol. The wet bacteria were then suspended in an ether-alcohol mixture (made by mixing equal volumes of each) in the proportion of 20 to 1 respectively contained in a tightly corked flask. The whole mixture was left in the incubator at 37°C for 5 days with frequent agitation

1 Durand, P., and Guerin, J., *Compt. rend. Soc. de biol.*, 1921, **84**, 980.

2 Eagleton, A. P., and Baxter, E. M., *J. Hyg.*, 1923, **22**, 107.

3 Sia, R. H. P., and Huang, C. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 348.

4 Wong, S. C., and T'ung, T., *Ibid.*, 1939, **41**, 160.

5 Wong, S. C., and T'ung, T., *Chinese Med. J.*, Suppl. No. 3, 1939, in press.

6 Young, C. C., *Ibid.*, No. 1, 1936, 143.

during the interim. The supernate was separated from the cells by filtering through ordinary filter paper. The lipoid fraction was obtained by spontaneous evaporation of the solvent and purified by treatment with petroleum ether. The yellow gummy residue obtained was slightly soluble in 95% alcohol but completely in ether and other fat-solvents.

The remaining defatted partially-dried cells were divided into 2 equal portions. One part was extracted with 0.05 N sodium hydroxide while the other was with 1% acetic acid in the proportion of 10 parts of organism to 1 of the extractives. Both extractions were carried out at 37°C with occasional vigorous agitation of the flasks. A few drops of toluol were added as a preservative. The state of cell-autolysis was followed by direct examination of smears made every other day. In the portion treated with alkali, autolysis was found to occur after 3 days of incubation. The fluid, however, was not removed until the 5th or 6th day. The turbid supernate obtained by centrifugation was clarified by filtering through Seitz. The remaining organisms were repeatedly extracted with similar volumes of 0.05 N sodium hydroxide under the above conditions, changes of extractives being made every 5 or 6 days. A total of 3-5 successive changes of extractives sufficed to remove all active substances. To the clear supernate a few crystals of sodium acetate were added as an electrolyte and 1.0 N hydrochloric acid was carefully added until a maximum precipitate occurred. The precipitated protein was collected by centrifugation and washed 3 times with a large volume of sterile distilled water. The precipitate was next suspended in a small volume of distilled water and solution of the precipitate was effected by the slow addition of alkali. Any undissolved material was removed by centrifugation and the solution precipitated once more with acid. The precipitate was again washed thrice with sterile distilled water after which it was suspended in sterile saline. A few drops of alkali were added to dissolve the precipitate and the excess alkali was neutralized with acid. Sterility was obtained by filtering through a sterile Seitz. The total nitrogen was determined by micro-Kjeldahl and the solution was diluted to contain 1% protein. The supernate containing the polysaccharide was precipitated with 3 volumes of 95% alcohol in the presence of sodium acetate. About 5 reprecipitations in 95% alcohol were required to obtain a substance that was easily soluble in 1% solution in saline. The yield for both fractions was comparatively large.

On the other hand, autolysis was found to occur after 7 days in the portion treated with 1% acetic acid but the extractive was not

changed until the 10th day. The supernate was clarified by filtering through a Seitz pad. To the solution was added a few pieces of sodium acetate and one N sodium hydroxide was added slowly until maximum precipitation occurred. The precipitate and the supernate containing the polysaccharide were treated in the same manner as above. The yield for both fractions was comparatively small.

The antigenic activity of the lipoidal, protein, and carbohydrate fractions was determined by the immunization of rabbits, employing 2 animals each. All the animals were first bled and tested for the presence of natural antibodies for the various fractions by the complement-fixation technic. Only those animals whose sera were not anti-complementary and did not contain natural antibodies were used. The immunizing procedure for all the antigens consisted of 3 daily injections followed by 4 days of rest. The process was repeated twice, making a total of 9 injections. Animals were bled 5 days after the last injection. In case of the polysaccharides a total of 9 mg per animal was used while with protein antigens 9 cc of a 1% solution were used. With the lipoid fraction a total of 9 cc of a saturated alcoholic solution diluted 1:4 in saline was employed. The presence of antibodies was determined by agglutination, precipitation, and complement-fixation reactions. In the complement-fixation reaction all immune sera were diluted 1:5 before used. A saturated alcoholic solution of the lipoidal substance was the standard solution upon which various dilutions of the antigen referred in the text were made.

For simplicity of presentation the results are discussed under the following headings:

Lipoid. For the Molisch and protein tests a 1:5 dilution of the lipoid in saline was used. It was found that the lipoid fraction gave a weakly positive Molisch but none of the usual protein reactions. It was non-antigenic and reacted with the homologous immune serum in dilution of 1:160. Approximately similar precipitin-titers were obtained when it was tested with heterologous immune sera. In serological reactions the lipoid fraction is group-specific.

Polysaccharide. It was found that the polysaccharides prepared either by acid or alkali in the absence of high temperature appear to be similar in chemical, serological, and antigenic properties, the total nitrogen for both being 3.1%. Thus the results given for one will be representative of the other. The Molisch reaction was strongly positive when a 1:5000 dilution of the antigen was used and all the usual protein reactions were negative employing a 1% solution. A $1:10^6$ dilution of the polysaccharide reacted with the homologous

serum (ring-test). Approximately the same titer was obtained in similar tests with heterologous immune sera. It was weakly antigenic as shown by the finding that only a complement-fixation titer of 1:50,000 was found when the anti-polysaccharide serum was tested with the polysaccharide. The same immune serum did not give complement-fixation reactions with either the protein or lipoid antigens. No precipitin nor agglutinin could be demonstrated. These results indicate that the polysaccharide is group-specific by serological tests and weakly antigenic in rabbits.

Protein. To the various protein-tests the acid-soluble protein reacted to only the biuret and xanthoproteic although it can be precipitated by ammonium sulfate. Since the fraction is serologically and antigenically inert it need not be discussed further. On the other hand, the alkali-soluble protein reacts with homologous immune serum in dilution of 1:200 (of a 1% solution) but not with heterologous immune sera with a 1% solution. It is weakly antigenic in rabbits giving a complement-fixing titer of 1:500 and agglutinating homologous organism in dilution of 1:10 of the serum. No precipitin could be demonstrated. No cross-reaction was found in complement-fixation reactions when the antiprotein serum (diluted 1:5) was mixed with the polysaccharide or lipoid antigens. This indicates that the antigen is immunologically pure and agrees well with the chemical tests. To show further that the alkaline-soluble protein is type-specific homologous immune serum was absorbed with an organism of the heterologous type. The resulting serum on examination showed that precipitins were completely removed for both polysaccharide and lipoid antigens but not for the alkali-soluble protein.

Since the alkali-soluble protein was found to be type-specific an attempt was made to determine the homogeneity of the protein. Fractionation with various concentrations of ammonium sulfate showed that all the fractions obtained reacted similarly in serological tests, no loss in type-specificity being observed. It is of interest to note that the alkali-soluble protein is heat-labile being destroyed by heating to 56°C for 30 minutes. Protein so treated becomes group-specific, being able to react with homologous as well as heterologous immune sera.

Similar type-specific protein could also be prepared directly from organisms with 0.05 N NaOH at 37°C without previous treatment with ether-alcohol mixture. Such a procedure, however, yielded very little substance even when the time of extraction was extended and the extracting solvent repeatedly changed. Apparently a preliminary

treatment of the organisms with ether-alcohol was necessary in order to render the cells suitable to the extractive action of weak alkali.

Conclusions. A method suitable for the isolation of a type-specific protein in *C. diphtheriæ* is described. Both the lipoid and carbohydrate fractions are group-specific while the alkali-soluble protein is type-specific. The type-specific substance is heat-labile, being converted into a group-specific protein by heating at 56°C for 30 minutes.

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Bactericidal Action of X-Rays in the Presence of Dyes.

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Many dyes undergo color-changes when exposed to X-rays or some forms of visible light.[1] This indicates a process of oxidation-reduction. From this and other considerations it is generally assumed that the bactericidal effect of photosensitization is due to the oxidation of microörganisms.[2] It is conceivable that a non-lethal dose of X-rays in the presence of a dye may exhibit a similar function as that shown by visible light. This view is borne out by the following investigation.

Pneumococcus Type I, *Streptococcus hemolyticus, Staphylococcus aureus, B. subtilis, Mycob. phlei* and *Shigella paradysenteriæ* Flexner, *E. typhi, E. coli* and *Pseudomonas aeruginosa* were chosen as the representative gram-positive and -negative microörganisms. Mercurochrome, eosin, methylene blue, crystal violet, and safranin O were employed. The final concentration of mercurochrome and eosin was expressed in term of percent of the dye while that of methylene blue, crystal violet and safranin O, on account of their poor solubility, was expressed in terms of their saturated aqueous solution. The technic previously reported[3] for making the mixture of dye and organisms was followed. In order to expose a number of dye-bacteria mixtures in a limited space accessible to the X-rays, they were put into small glass cups of 1 cc capacity so that 10 of them could be exposed at the same time.

1 Clark, G. L., and Fitch, K. R., *Radiology*, 1931, **17**, 285.

2 Blum, H. F., *Physiol. Rev.*, 1932, **12**, 23.

3 Tung, T., and Zia, S. H., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 326.