young and old rats, the writer observed an increase with age in the quantity of cortical ash in the individual cells. Scott,[1] using the microincineration technique, observed a concentration of ash at both the nuclear and cell membranes. The following experiments were conducted to determine the nature of the cortical ash with particular reference to calcium.

In using the microincineration technique as a means of localizing the calcium in metazoan cells the question might arise as to whether any calcium at the cell membrane would be intercellular or intracellular. In order to avoid this possibility, the protozoan *Paramecium caudatum* was used as the experimental organism. Paramecium also has the advantage of size; it is large and comparatively easy to work with.

Procedure

The microincineration technique as developed by Policard[2] and Scott is essentially that which was used in the following experiments. The material was fixed in Bouin's Fluid (minus the glacial acetic acid); the absolute alcohol and formalin fixative as recommended by Scott was also used. Both dioxan and alcohol dehydration were employed with no apparent variation in the results. The material was imbedded in paraffin in the usual manner and sectioned at 8 micra. The sections were mounted on clean slides and incinerated in an electric furnace at approximately 600° C., for 30 minutes. The material was then studied by means of the dark-field microscope.

Two specific colorimetric tests for calcium were used; sodium alizarin sulfonate, which produces a red precipitate in the presence of calcium,[3] and the gallo-formic reaction, which produces a blue precipitate specific for calcium.[4] The reagent as used by the writer consisted of a solution of concentrated gallic acid and 40 per cent. formaldehyde (1–1) rendered alkaline (pH 9.0) by the addition of concentrated NH_4OH. The gypsum reaction was also used to a small extent. This consists of dissolving the salt in a microdrop of $0.1N \cdot HCl$ and precipitating the typical $CaSO_4 \cdot 2H_2O$ crystal by adding a microdrop of $0.1H \cdot H_2SO_4$ (Scott).

Results

Random samples of paramecia consistently showed a heavy concentration of salts in the cortex of the cells. The cortical layer of ash was uniform in thickness throughout. A faint pink color was observed, which was imparted to the ash by the presence of iron. Occasional granules of ash were observed in the cell which were probably the inorganic remains of food granules, etc. The thickness of the outer ring of cytoplasmic ash varied considerably. While no actual measurements were done on the thickness of the layer, two distinct extremes were observed, a thin relatively smooth layer of ash on the one hand, and a heavy, granular layer on the other.

Sections treated with sodium alizarin sulfonate showed a distinct change in the color of the cortical layer of ash. The original light pink color gave way to the purplish red, which is characteristic of the alizarin reaction. However, since there does exist a general similarity between the original pink and the final red, the gallo-formic reaction was employed as a check. A bluish-violet color in the cortical ring confirmed the alizarin test for calcium. As previously stated, the gypsum reaction was employed to a small extent. The writer found that the colorimetric methods were sufficiently more positive to warrant their exclusive use. Actually, it was hardly necessary to test for the presence of calcium in the cortical ring of ash. Since almost all the inorganic salts were concentrated in that region of the cell, and since calcium is known to be present in the cell, it would follow that the calcium must be in the cortex. The variation in the thickness of the cortical ring of ash prompted the writer to repeat the experiment using organisms of known ages. (This investigation is being conducted at the present time.)

Summary

By means of the microincineration technique and two specific colorimetric tests for calcium, the writer has demonstrated that most of the cytoplasmic salts are concentrated in the cortex of the cell and that calcium is a constituent of the cortical ash.

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[1] Scott, *Am. Jour. Anat.*, 53: 243, 1933.
[2] Policard, *Protoplasma*, 7: 464, 1929.
[3] Pollack, *Jour. Gen. Physiol.*, 11: 539, 1928.
[4] Cretin, *Bull. Am. Chem. Soc.*, 33: 1551, 1923; *Bull. Hist. Appl.*, 1: 125, 1924.

THE PRESENCE OF A TYPE- AND SPECIES-SPECIFIC CONJUGATED POLYSACCHARIDE IN TYPE I PNEUMOCOCCUS

THE findings of numerous investigators have shown that each pneumococcal type elaborates a type- and a species-specific polysaccharide. They have been isolated individually and characterized chemically. Since heat, acid and alkali have been employed in their isolation, and since autolysates or highly autolyzed organisms have been used as the source of material, it has appeared probable that the polysaccharides thus isolated were fragments or modified parts of a higher complex which might be present in the young and actively growing Gram-positive organisms.

To elucidate this question the isolation of these substances was undertaken from twelve-hour cultures of Gram-positive organisms, by removing the protein from the carbohydrate solution with the chloroform method.[1] The Type I polysaccharide thus isolated produced active immunity in mice and is chemically different from those hitherto described.

The present report is an extension of the above study. To prevent enzymic autolysis strictly Gram-positive, young organisms of Type I were used. They were grown in beef broth containing catalase to remove the hydrogen peroxide produced as a metabolic product. A polysaccharide was obtained which is identical with the one previously isolated.[1] After repeated precipitation from methyl and ethyl alcohol and finally from 60 per cent. ethyl alcohol, the analysis of the polysaccharide gave 6.35 per cent. nitrogen; 2.78 per cent. phosphorus; and 23.0 per cent. reducing sugar.

Serologically, this polysaccharide in dilution of one to one million gives precipitates with monovalent horse sera of Types I, II and III. Type I serum, absorbed with purified species-specific carbohydrate "C" prepared from young Gram-positive rough pneumococci, still reacted to titer with the conjugated polysaccharide derived from Type I, but not at all with a conjugated polysaccharide derived from Type II. Similarly, a Type II serum, absorbed with "C," reacted to titer with Type II but not at all with Type I conjugated polysaccharide. This type I carbohydrate removed antibody against "C" from Type II and Type III sera, and both type and species-specific antibodies from Type I serum.

The presence of phosphorus might be attributed either to inorganic phosphorus, phosphatides or the species-specific "C" carbohydrate as a mixture or in chemical combination with the type-specific polysaccharide.

To remove any phosphatide that might be present in the polysaccharide in one form or another, the following treatments were used:

(a) The dry carbohydrate was refluxed for one hour successively with each of the following solvents: pure acetone, one to one ethyl alcohol and ethyl ether solution, benzene, followed by ethyl ether and alcohol solution, and finally with low-boiling petroleum ether. The carbohydrate was then precipitated from its water solution with two parts of 95 per cent. alcohol.

(b) The carbohydrate was refluxed on a boiling water bath for four hours in N/5 acetic acid, cooled and extracted with ethyl and petroleum ether neutralized and extracted again with the same reagents. The carbohydrate was then precipitated with two parts of 95 per cent. alcohol.

[1] M. G. Sevag, *Bioch. Zt.*, 273: 419, 1934.

The treatments (a) and (b) produced chemically and serologically no change, thus eliminating the possibility of the presence of phosphatide in this polysaccharide. Furthermore, pure "C" in a mixture with the present carbohydrate is readily removed by treatment with two parts of alcohol; therefore the present substance must contain "C" in combination with the type specific carbohydrate.

(c) To remove any inorganic phosphorus which might be present as impurity, the solution of carbohydrate was brought to pH 2.5 with glacial acetic acid and precipitated with two parts of 95 per cent. alcohol. This treatment was repeated five times. Chemically and serologically no change was produced.

(d) The aqueous solution of the polysaccharide prepared as in (C) was treated with five volumes of glacial acetic acid; no precipitate was produced. Upon subsequent treatment with 1.5 volumes of 95 per cent. alcohol and two grams of sodium acetate per 100 cc solution, it flocculated immediately. The aqueous solution of the alcohol and ether-washed precipitate containing 2.0 grams of sodium acetate per 100 cc solution was further fractionated twice with two parts of 95 per cent. alcohol.

(e) 20 cc of the acidified carbohydrate solution was treated with 2 gms of sodium acetate. It was then chilled and treated with 7 cc of chilled 95 per cent. alcohol and centrifuged. The supernatant fluid was further treated with 7 cc of chilled 95 per cent. alcohol. These treatments were repeated several times on the sediments, and the carbohydrates thus obtained were similar to the one obtained above.

These treatments resulted in no change chemically or serologically. These findings would seem to indicate that in actively growing Gram-positive pneumococcus the type- and species-specific carbohydrates exist in a combined complex form. This complex alone or in mixture with swine serum did not produce immunity in rabbits. It is therefore reasonable to assume that this new complex carbohydrate may be in an antigenically active union with a protein component in the organism as the complete antigen. Studies are in progress in this laboratory to isolate this protein-carbohydrate complex in native form.

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THE BITTERLING OVIPOSITOR REACTION TO CORTICOSTERONE

IN 1932 we reported that female bitterlings (*Rhodeus amarus*) show an enlargement of the ovipositor following injection of an estrogenic preparation, while physiological solution of sodium chloride and an anterior pituitary extract yielded no reaction.[1] This

[1] W. Fleischmann and S. Kann, *Pflügers Arch. f. d. ges. Physiol.*, 230: 662, 1932.