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PROCESS OF FRACTIONATING MIXTURES OF BACTERIAL ORIGIN

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This invention relates to the fractionation of mixtures of bacterial origin essentially comprising complex carbohydrates and proteinaceous materials; more particularly, it relates to the obtainment of complex-carbohydrate antigens from bacterial organisms. As employed herein, the terms "bacterial organisms" and "bacterial origin" respectively embrace, in addition to their literal meaning, cultures and culture-medium origin; and the term "proteinaceous materials" embraces derived proteins such as the proteoses, peptones, and polypeptides, as well as proteins per se.

It has been found that the chief problem in the obtainment of the complex-carbohydrate antigens from bacterial organisms is the removal therefrom of associated proteinaceous materials. In the processes heretofore employed, various chemical and/or physical treatments have been utilized to effect purification of the largely-carbohydrate antigenic complex, but these processes have been unsatisfactory because of their inefficiency and/or complexity.

It is the object of this invention to provide a simple, rapid and efficient process of fractionating mixtures of bacterial origin essentially comprising complex carbohydrates and proteinaceous materials, especially a process for obtaining complex-carbohydrate antigens from bacterial organisms.

In the practice of this invention, fractionation of the mixture is effected by selectively dissolving the proteinaceous component in a solvent essentially comprising a liquefied phenol, e. g., U. S. P. liquefied phenol (88%). By "a phenol" is meant, of course, a member of the class of aryl hydroxides, including, inter alia, phenol, the cresols (ortho, meta, and para), chlorcresols and naphthols. Where the complex carbohydrates possess a relatively high solubility in phenols, the efficiency of the fractionation may be increased by modifying the liquefied phenol with a member of the group consisting of lower aliphatic alcohols (preferably ethanol) and lower fatty acids (preferably acetic acid).

The fractionation may be effected by subjecting the mixture to extraction with the liquefied phenol, and recovering the insoluble complex-carbohydrate component. Where the complex carbohydrates possess a relatively high solubility in phenols, it is preferable first to dissolve the mixture in the liquefied phenol, and fractionally precipitate the complex-carbohydrate component by adding a lower aliphatic alcohol or a lower fatty acid. The complex-carbohydrate component, in either case, may be further purified by washing with a volatile organic solvent for phenols (e. g. alcohol), drying with acetone, extracting with water or neutral saline solution, clarifying the solution, and precipitating the complex carbohydrate from the solution by means of sodium acetate and alcohol (or an alcohol-ether mixture).

The purified antigens obtained by the process of this invention have obvious utility, e. g., in the immunization of humans and in the potency-assay of antiserums.

The following examples are illustrative of the invention:

Example 1

Obtainment of an antigen from the typhoid bacillus (Eberthella typhosa)

5 g. of the acetone-dried typhoid organisms is suspended in 150 cc. U. S. P. liquefied phenol (88%), and the suspension is vigorously shaken for 8 hours and centrifuged. The supernatant is then removed; and the phenol-insoluble residue is shaken with 125 cc. 88% phenol for three hours, the mixture centrifuged, and the supernatant separated from the phenol-insoluble residue.

This residue is washed twice with a 9:1 mixture of alcohol and ether, twice with acetone, and nearly completely dried, yielding 1.95 g. powder, which is extracted with 150 cc. of a neutral saline solution (0.9% NaCl), and then with 100 cc. and 50 cc. portions of water, separating each extract by centrifuging. The saline and water extracts are combined, clarified by filtration through paper pulp and then through asbestos pulp, and mixed with sodium acetate and 2 volumes of the alcohol-ether mixture; and the resulting precipitate is washed with alcohol and acetone, and dried, yielding 400 mg. of an antigenic substance (A).

[For purposes of comparison as to antigenicity, the phenol-soluble fraction is recovered as follows: The phenolic supernatants are combined and mixed with 2 volumes of a 9:1 mixture of alcohol and ether, and the resulting precipitate is separated by centrifuging, washed twice in the centrifuge with the alcohol-ether mixture, and twice with acetone. The precipitate (which after nearly complete drying weighs about 3.1 g.) is then successively extracted with 150, 100, and 50 cc. of water at pH 7.0, the extract being seprated each time by centrifuging. The aqueous extracts are combined, and clarified by filtration through paper pulp and then through asbestos pulp. The resulting aqueous solution is then

mixed with 2 volumes of a 9:1 mixture of alcohol and ether, and with sodium acetate; and the resulting precipitate is washed with alcohol and acetone, and dried, yielding 580 mg. of the phenol-soluble fraction (B).]

In comparative antigenicity tests in mice, about 100 times as much of the phenol-soluble fraction (B) as of the antigenic substance (A) was needed to protect about half of a group of test animals from death, despite an apparently much lower virulence of the infecting dose of organisms used in the tests with (B). Hence, the greater part of the antigen has been concentrated in the phenol-insoluble fraction, and a large amount of undesired, relatively-inactive material has been eliminated therefrom. (The comparative antigenicity was determined by injecting white mice with 2 doses of the substance being tested, in varying doses, at an interval of a week. One week after the second dose, each mouse was given 50,000 live typhoid organisms suspended in mucin as an infecting dose, and the mice were observed for a week thereafter. Control animals were injected only with varying doses of the live culture to test its virulence.)

EXAMPLE 2

Obtainment of a pneumococcus (Diplococcus pneumoniae) type-specific polysaccharide from type 14 pneumococcus

A suitable culture of type 14 pneumococcus is seeded into buffered peptone broth containing 0.5% glucose (dextrose), and the mixture is incubated at 35 to 37° C. for 7 days, during which time most of the cocci autolyze, and the desired polysaccharide passes into the liquid. The culture is then killed by the addition of phenol to a concentration of approximately 1%, followed by incubation at the same temperature overnight. The killed culture is then passed through a Sharples supercentrifuge to remove any cells, cellular debris, and other insoluble matter, and the clear liquid, containing the type-specific polysaccharide, is stored at 2 to 4° C. until further treated.

The solution (44 liters) is concentrated to 5750 cc. under reduced pressure at a temperature not exceeding 30° C., and is then treated with 575 cc. of a solution containing 0.5 g. sodium acetate per cc., and with 57.5 cc. of glacial acetic acid, making a total volume of approximately 6380 cc. To this is added 8930 cc. (approximately 1.4 volumes) of a mixture of 9 volumes of absolute ethyl alcohol with 1 volume of ether; the resulting precipitate, containing the desired polysaccharide and other substances, is removed by centrifugation and suspended in approximately 200 cc. of 95% phenol solution. The mixture is shaken mechanically for about ½ hour, then centrifuged; and the phenolic liquid is poured off and the residue again suspended in about 200 cc. of 95% phenol. The shaking, centrifuging, and removal of phenolic liquid are repeated, and the entire procedure of suspension of the residue in 95% phenol, shaking, centrifuging, and decanting the phenolic liquid is carried out a third time. (The phenolic extracts contain no appreciable amounts of the desired type-specific carbohydrate, but contain much undesired proteinaceous and highly nitrogenous material, and are therefore discarded.)

The phenol-insoluble residue is washed with alcohol and/or acetone to remove remaining phenol, and then repeatedly extracted with water, until no appreciable quantities are removed by further extraction. The combined extracts are freed of water-insoluble material by centrifugation and filtration through paper pulp, and the clarified extract then treated with $\frac{1}{10}$ its volume of sodium acetate solution (0.5 gm. per cc.) and $\frac{1}{100}$ its volume of glacial acetic acid. To this mixture is added 3 times its volume of the 9:1 alcohol-ether mixture. The precipitate so obtained, containing the desired polysaccharide, is again dissolved in water, using a volume much smaller than that of the aqueous extract above, centrifuged to remove water-insoluble material, if any, and treated in the same way with sodium acetate solution and glacial acetic acid. One volume of alcohol-ether mixture is added, and the resulting precipitate, comprising the desired type-specific polysaccharide in substantially pure form (except for inert, inorganic substances) is washed with absolute ethyl alcohol and dried with acetone.

The procedure of this example is also applicable to the obtainment of pneumococcus type-specific polysaccharides from other types of pneumococcus (except types 4, 7, 11, 15, 25, 27, 31 and 32, as to which types the procedure of Example 4 is especially adapted).

EXAMPLE 3

Obtainment of type-specific polysaccharide from the Friedländer bacillus (Klebsiella pneumoniae) type B

A suitable culture of Friedländer bacillus type B is seeded into buffered peptone broth containing 0.5% glucose (dextrose), and the mixture is incubated at 35 to 37° C. for 7 days, during which time most of the bacterial growth autolyzes, allowing the desired polysaccharide to pass into the liquid. The culture is then killed by the addition of phenol to a concentration of approximately 1% and incubation overnight at the same temperature; and the killed culture is passed through a Sharples supercentrifuge to remove any cells, cellular debris, and other insoluble matter, and the clear liquid, containing the desired polysaccharide, is stored at 2 to 4° C. until further treated. (A further quantity of the polysaccharide may be present in the cells and debris centrifuged off.)

The solution (60 liters) is concentrated to 6000 cc. under reduced pressure at a temperature not exceeding 30° C., and then treated with 600 cc. of a solution of sodium acetate containing 0.5 gm. per cc., and 60 cc. of glacial acetic acid. To this mixture is added half its volume (approximately 3325 cc.) of a mixture of 9 volumes of absolute ethyl alcohol and 1 volume of ethyl ether. The resulting precipitate contains most of the desired polysaccharide of the liquid, and is removed by centrifugation. (A much smaller amount of slightly less pure material may be obtained from the supernatant liquid by the addition of more of the alcohol-ether mixture and purification in substantially the same manner as described below.)

The precipitate is extracted with three successive 350-cc. portions of 95% phenol, and the residue, containing the desired polysaccharide, is washed with three portions of absolute ethyl alcohol to remove phenol. The phenolic extract contains no appreciable quantity of the desired polysaccharide.

The alcohol-washed phenol-insoluble material is then extracted thoroughly with water, the desired polysaccharide being dissolved. Any insoluble material is removed by centrifugation, and

the aqueous solution is treated with $\frac{1}{10}$ its volume of sodium acetate solution (0.5 gm. per cc.) and $\frac{1}{100}$ its volume of glacial acetic acid. Upon the addition of an equal volume of absolute ethyl alcohol to the mixture, a precipitate forms; after washing with alcohol and drying with acetone, the precipitate consists of the desired polysaccharide in substantially pure form except for a small amount of inert inorganic material.

EXAMPLE 4

Obtainment of pneumococcus (Diplococcus pneumoniae) type-specific polysaccharide from type 7 pneumococcus

A suitable culture of type 7 pneumococcus is seeded into buffered peptone broth containing 0.5% glucose (dextrose), and the mixture is incubated at 35 to 37° C. for 7 days, during which time most of the cocci autolyze, allowing the desired polysaccharide to pass into the liquid. The culture is then killed by the addition of phenol to a concentration of approximately 1% and incubation at the same temperature overnight. The killed culture is then passed through a Sharples supercentrifuge to remove any cells, cellular debris, and other insoluble matter, and the clear liquid, containing the type-specific polysaccharide, is stored at 2 to 4° C. until further treated.

The solution (36 liters) is concentrated to 6000 cc. under reduced pressure at a temperature not exceeding 30° C. To 3000 cc. of this concentrate is added 300 cc. of a solution containing 0.5 gm. of sodium acetate per cc., and 30 cc. of glacial acetic acid, and to this mixture is added 6600 cc. (approximately 2 volumes) of a mixture of 9 volumes of absolute ethyl alcohol and 1 volume of ethyl ether. The precipitate formed contains the desired polysaccharide, and is collected by centrifugation.

The precipitate is extracted by three successive portions of approximately 150 cc. of U. S. P. liquefied phenol (88%), the extraction mixture being mechanically shaken each time for approximately ½ hour, and the extract being removed by centrifugation and decantation. The final phenol-insoluble residue contains only a small portion of the desired polysaccharide, the bulk of it having dissolved in the phenolic solution. The phenolic extracts are combined and treated with one-half volume of absolute ethyl alcohol containing approximately 1% lithium chloride; the resulting precipitate contains practically all the desired type-specific polysaccharide of the phenolic extract, and the supernatant liquid contains much undesired highly nitrogenous material.

The precipitate is washed three times with the alcohol-ether mixture, in order to remove any phenol present, and is then extracted with three successive portions of about 100 cc. of water. The aqueous extracts are combined and centrifuged to remove insoluble material, if any, and treated with $\frac{1}{10}$ their volume of sodium acetate solution (0.5 g. per cc.) and $\frac{1}{100}$ their volume of glacial acetic acid. To this mixture is added sufficient (about 1.5 volumes) of the alcohol-ether mixture to cause precipitation of practically all the polysaccharide present in the extract; the precipitate, when washed with absolute alcohol and dried with acetone, consists of substantially pure type-specific carbohydrate, except for inert inorganic substances.

The procedure of this example is also applicable to the obtainment of pneumococcus type-specific polysaccharides from pneumococcus types 4, 11, 15, 25, 27, 31, and 32.

The procedure of Example 1 may be varied as follows: The acetone-dried organisms (*Eberthella typhosa*) are repeatedly extracted with 95% phenol, and then with alcohol to remove the phenol. The phenol-insoluble residue is dried with acetone and extracted with neutral physiological saline (0.9% NaCl) or with water; and the extract is clarified, and precipitated with alcohol or alcohol-ether in the presence of sodium acetate. The precipitate is subjected to a second phenol extraction, and the alcohol-washing, water extraction, clarification and precipitation are repeated. The final product (A1), after drying with acetone, is a fluffy white powder, which readily forms a viscous, faintly opalescent 1% solution in water or physiological saline.

The process of this invention may also be applied to the crude antigenic product obtained from the typhoid bacillus by the tryptic digestion procedure. Thus, a tryptic-digest preparation made according to Wakeman (Military Surgeon, 84: 318, 452, 1939) gives a weak biuret reaction; the small amount of material responsible for this reaction may be removed by a single extraction with 88% or 95% phenol. On extraction of the phenol-insoluble residue with water, clarification, precipitation, and drying as outlined in the preceding paragraph, a product is obtained which closely resembles product (A1) in appearance and properties. Both products, obtained in yields of 10–15% of the weight of the organisms, give negative or very faint biuret reactions and very strong Molisch reactions, have nitrogen contents (corrected for ash and moisture) of 3.4–3.6%—less than 10% of which is accounted for by hexosamine, if present—and give strong precipitin reactions with antityphoid rabbit serum.

The invention may be variously otherwise embodied within the scope of the appended claims.

I claim:

1. The process of fractionating mixtures of bacterial origin essentially comprising complex carbohydrates and proteinaceous materials, which comprises selectively dissolving the proteinaceous component in a solvent essentially comprising a liquefied phenol.

2. The process of fractionating mixtures of bacterial origin essentially comprising complex carbohydrates and proteinaceous materials, which comprises selectively dissolving the proteinaceous component in a solvent essentially comprising liquefied phenol.

3. The process of separating an antigenic polysaccharide from associated proteinaceous materials, comprising selectively dissolving the proteinaceous component in a solvent essentially comprising a liquefied phenol.

4. The process of separating an antigenic polysaccharide from associated proteinaceous materials, comprising dissolving the mixture in a liquefied phenol, and fractionally precipitating the polysaccharide component from the solution by adding thereto a member of the group consisting of lower aliphatic alcohols and lower fatty acids.

5. In the process for obtaining an antigen from the typhoid bacillus, the step of extracting the typhoid bacillus with a solvent essentially comprising a liquefied phenol.

6. In the process for obtaining a pneumococcus type-specific polysaccharide from a culture of a pneumococcus other than types 4, 7, 11, 15, 25, 27, 31, and 32, the step of extracting a mixture

derived from the culture and essentially comprising the polysaccharide and associated proteinaceous materials with a solvent essentially comprising a liquefied phenol.

7. In the process for obtaining a pneumococcus type-specific polysaccharide from a culture of a pneumococcus of the group consisting of types 4, 7, 11, 15, 25, 27, 31, and 32, the step of dissolving a mixture derived from the culture and essentially comprising the polysaccharide and associated proteinaceous materials in a liquefied phenol, and fractionally precipitating the desired polysaccharide from the solution by adding thereto a member of the group consisting of lower aliphatic alcohols and lower fatty acids.

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