A STUDY OF THE CHEMICAL COMPOSITION OF SALMONELLA PULLORUM

Thesis for the Degree of M. S.

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A STUDY OF THE CHEMICAL COMPOSITION OF SALMONELLA PULLORUM

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INTRODUCTION

Although much work has been done by the immunologist to explain the phenomena of specificity and the nature of immunological reactions, he has found them difficult to explain in purely biological terms. However, more definite explanations in terms of modern chemical theories have been presented and are constantly accumulating.

During the past ten years there have been many papers dealing with the isolation of a fraction or a few fractions of microorganisms. However, relatively few workers have attemptedto separate the constituents of the cell as a whole. This is particularly true of work done on the Salmonella group of organisms. The most important work in this group may be summed up by the following papers. In 1928, Furthe and Landsteiner (1) isolated from the typhoid-paratyphoid group specific precipitable substances rich in carbohydrates. Happold (2) prepared a precipitinogen from broth cultures of B. aertrycke, but made no chemical study of it. Again, in 1928, Casper (3) isolated some carbohydrates from B. paratyphosus which he found were not type specific, but which were agglutinated by sera of Schottmulleri and Breslau types of Bacillus paratyphosus B as well as Salmonella typhi murium and typhus serum and in a lesser degree by Gartners' serum. Branham (4) isolated repeatedly a specific carbohydrate from the culture filtrates of B. enteritidus.

Boivan and Mesrobenau (5) have described the extraction of an antigenic polysaccharide from B. aertrycke. Bloor and Miller (6) have given methodsfor the separation of a protein and a polysaccharide from meningococcus.

It has been noticed that in the above papers, the compounds isolated have not been classified any more than as carbohydrates, proteins or lipides. In 1934 Huston, Huddleson and Hershey (7) made a separation of the constituents of the Brucella group of microorganisms in which they attempted to classify further the substances isolated. With modifications, their methods of separation have been used in this work.

In 1929, Bunyea, Hall and Dorset (8) reported a simplified method for the detection of pullorum disease in fowl by a rapid agglutination test, using whole blood. In 1931, Schaffer and MacDonald (9) and Coburn and Stafseth (10) prepared antigens which have given a more satisfactory test. The method of Schaffer and MacDonald is now patented by the Bureau of Animal Industry, U.S.D.A., and recommended for general use. However, it is the contention of Coburn and Stafseth that this patented antigen has not been as satisfactory as the one prepared by Coburn (11).

Thus a twofold object of study is presented: (1) to study the chemical composition of the Coburn and B.I.A. antigens (for they are prepared in quite different manners)

to see if chemical constitution can explain their differences in sensitivity as a diagnostic test, and (2) to arrive at some correlation between the chemical constitution of the bacteria and the various phenomena of specificity exhibited by them.

METHODS OF STUDY

Preparation of Material

History of the Cultures -

The three strains of Salmonella pullorum used in preparing the starting material for this work were obtained from the Department of General Bacteriology, Yale University. They were strains 17, 19 and 20, which had been selected by the Eastern States Laboratory Workers Conference in 1928. All give typical reactions characteristic of their species as to sugar reactions, gas production, etc. All are readily agglutinable.

Preparation of Media -

Medium for preparation of B.I.A. Pullorum Antigen:

Lean beef, trimmed free of fat, is passed through a meat grinder, mixed with twice its weight of distilled water, and allowed to stand at room temperature for 1 hour. The mixture is heated, with stirring, to 50°C., at which point it is maintained, with occasional stirring, for 2 hours. The meat water extract is strained through a cheese-cloth; the last of the extract is removed with a press. The extract

thus obtained is just brought to a boil and immediately filtered through paper. Any solidified fat is removed by filtering through cheese-cloth.

To the infusion are added 2% Bactopeptone, 0.5% sodium chloride and 3% agar. The solution is effected by heating in an autoclave for about 30 minutes, or longer if necessary, in order to dissolve the agar. The reaction is adjusted to pH 7.2 and the medium is then filtered through a good grade of cotton and distributed into flasks. Final sterilization is accomplished in an autoclave at 15 pounds pressure for 30 minutes.

Medium for Preparation of Coburn Antigen:

Skinned and drawn chicken, including the bones was passed through a meat grinder. Ground chicken and tap water in the proportion of one pound of the chicken to 500 ml. of water, were placed in a container and mixed well. The container was covered and placed in flowing steam for 2 hours. After removing from the steamer, the infusion was strained through a wire screen, allowed to cool and chilled thoroughly in the ice-chest. Any solidified fat was removed by filtering through paper. This filtrate was then sterilized in an autoclave at 20 pounds pressure for 30 minutes.

A veal infusion was made in the same manner using one pound of veal to 500 ml. tap water.

The agar was made up with the following ingredients:

Tap water ---------5 parts	1% Neopeptone
Chicken Infusion --3 parts	0.5% Sodium Chloride

Veal infusion ------2 parts 2% Agar

These were placed in a suitable container, covered, and heated in the autoclave for 30 minutes, or longer if necessary. The pH of the solution at this time was brought to 7.2. The medium was distributed into flasks and sterilized by heating in the autoclave at 15 pounds pressure for 30 minutes.

Cultivation of Bacteria:

One quart whiskey flasks were used as culture flasks. Each flask received about 200 ml. of medium. When solidified, and after 24 hour incubation, the agar was seeded with about 2 ml. of a chicken broth suspension of the organism from the inoculating apparatus.

This inoculating apparatus deserves description. The ordinary method of inoculating by transfer from freshly prepared agar slants was too time consuming and resulted in too much loss due to contamination. It is very simple in construction, consisting of the bulbs of two 500 ml. pipettes joined together to a common outlet by a glass Y tube by means of rubber tubing. An inverted funnel is placed over this common outlet to keep away any dust that may settle on the mouth of the bottles being innoculated. Pinch-cocks are so arranged as to allow emptying one pipette at a time, the purpose of the two pipettes serving to make it possible to inoculate each half of a large batch of flasks with two different strains of the organisms.

The apparatus is made ready for inoculating flasks in the following manner. It is first sterilized empty by autoclaving, using cotton plugs and a large paper over the inverted funnel. Then about 200 ml. of chicken broth is placed in each pipette and the apparatus is again sterilized in the autoclave. By means of a long stiff wire, a large loopful of a 24 hour culture of the desired organism is lowered into the top of the pipette and down into the liquid, using all the necessary precautions. This is then aseptically plugged and the apparatus is placed in a 37°C incubator for a period of 6 to 24 hours.

The inoculated whiskey flasks were incubated at 37°C. for 3 days.

The organisms used in preparation of the B.I.A. antigen were harvested using a solution containing 0.85% NaCl and 1% formalin (0.4% actual formaldehyde). About 15 ml. was placed in

the first flask, and the bacteria removed from the surface by gentle rubbing with a long sterile cotton swab. This solution was then transferred to the next flask, continuing in this way until the suspension is so heavy that loss might occur by further transferring. The suspension was then filtered through cheese-cloth to strain out any pieces of agar or other foreign material present, centrifuged in a Sharples super-centrifuge and the bacteria spread in a thin layer on a watch glass and dried in vacuo over Dehydrite. The dried residue was yellow brown in color but appeared almost white when powdered.

The organisms used in the preparation of the Coburn antigen were harvested in the same manner, however using a saline solution containing 0.5% phenol. The suspension of organisms was brought to a boil and allowed to boil slowly for 10 minutes. During the course of the boiling, a scum appeared, which was skimmed off and later dried in vacuo over Dehydrite. This was saved for further study. The cells were recovered in the Sharples centrifuge, spread on a watch glass and dried in vacuo over Dehydrite.

Table 1

Yield of Cultures

Media	Quantity Used	Approximate Yield of Dried Organisms	Ave. gms per 1.
Beef	60 liters	40	0.58
Chicken-veal	90 liters	35	0.44

The cell washings from the two preparations were quite different in appearance. While the washings from the BAI. patented antigen were a pale yellow, those of the Coburn antigen were, after standing a few days, a very dark reddish-brown -- the color probably being due to the phenol. No examinations were made of these washings.

Elementary Composition:

Samples from different lots of the dried cells were used in the determination of a few elements. The moisture content was determined as loss in weight of the material when dried over Dehydrite under reduced pressure at room temperature. Phosphorous determinations were made by the method of Fiske and Subbarow (13).

Table II

Elementary Composition of S. Pullorum
(Cells dried over Dehydrite)

	Coburn		B.A.I.	
Loss in weight over Dehydrite	4.73	4.83	3.31	3.48
*Ash, HNO_3 ignition	4.96	4.98	5.88	5.90
*Nitrogen, micro-Kjeldahl	12.02	12.18	11.84	12.01
*Phosphorous, colorimetric	0.55	0.55	0.79	0.81
*Sulphur, Burgess Parr	0.76	0.72	0.64	0.65

*Values calculated on basis of dry-weight over Dehydrite.

Preparation of Extracts

30 gram samples of the dried organisms were ground in a ball mill for 4 days.

Ether Extract:

The ground cells were then transferred to a beaker with 500 ml. of anhydrous ether and allowed to stand in an ice-chest for 2 days. The ether soluble material was recovered by filtration through an ordinary filter and subsequent evaporation of the ether under reduced pressure. A deep yellow oily solid, having a very sharp odor was obtained, the yield being slightly over 1 per cent.

Water Extraction:

Two water extractions were made. In the first, the cells were transferred directly after partial drying from the filter which had been washed with ether, to a ball mill. Six hundred ml. of water was added, together with 5 ml. of ether and 5 ml. of toluene to prevent the formation of stiff froths which interfere with trituration. The mill was rotated for one hour and the jars were then placed in the ice-chest for six hours.

The water suspended cells were then centrifuged until the supernatant liquid was quite clear. At this stage the liquid had a light opalescent appearance.

About one and a half liters of solution was obtained in this manner. For the second extraction, the cells were resuspended in distilled water by means of a mechanical stirrer. The pH was adjusted to 8.5 with about 10 ml. of N NaOH. The volume was again brought up to about one and a half liters with distilled water and stirring continued for two hours. The suspension was then placed in the ice-chest for 24 hours after which the extract was clarified as before. This time, however, the resulting solution showed a white opalescence.

Lipide Extraction:

The residue from the second water extraction was stirred into 500 ml. of equal parts of absolute alcohol and anhydrous ether. 0.5 milligram of hydroquinone was added to inhibit oxidation. Dehydration was allowed to proceed in the cold. After standing about 24 hours, the mixture was shaken, allowed to settle, and the supernatant liquid replaced by fresh solvent. After allowing the flasks to stand in the ice-chest for two weeks during which time the suspension was daily shaken, a third extraction was made. The residue was collected on a Buchner funnel, washed with alcohol and ether, transferred to chloroform and extracted three weeks in the cold. After final extraction the residue was again filtered off, washed with chloroform, and dried first in the air, and then in vacuo over Dehydrite.

The Foam:

One of the steps in the preparation of the Coburn antigen is the boiling of the suspension of organisms, to which 0.5% phenol has been added, for a period of 10 minutes. During this process there is a foam or scum that collects at the surface, which necessarily must be skimmed off, for it seems that leaving it in the antigen tends to make the antigen progressively more and more sensitive until agglutination will take place with negative serum.

This dried foam, which in the case of each preparation of antigen comprises approximately 5% of the total weight of the cells, was extracted six times with a 50% alcohol-ether mixture over a period of three days. The supernatants were filtered and evaporated, after extracting the phenol with 10% NaOH. The resulting lipides made up 7.9% of the original weight of the dried foam.

The residue gave a strong Biuret, xanthoproteic and Molisch test, indicating abundant protein and carbohydrate present. Sulphur and phosphorous were also present. Microscopic examination, using a Gram stain, showed many whole and fragmented cells.

On the basis that the foam consisted of cells alone, the lipide content should have been approximately 4%; however, about 8% lipide was shown to be present.

Thus one may conclude that upon heating the suspension of cells, some lipides are liberated which tend to concentrate at the surface, and in doing so, bring up some whole and fragmented cells, which comprises the foam.

Separation of Fractions

Acetic Acid Precipitation:

The first and second water extracts when combined made a total volume of 3 liters and appeared as a slightly opalescent light yellow solution. Sufficient 1:1 acetic acid (about 5 ml./liter) was added to bring the pH to 3.5. The resulting precipitate was allowed to settle in the ice chest and two days later was collected in a centrifuge and washed with water. The acetic acid fraction was placed in the cold for further study after adding a few ml. of toluene to prevent decomposition. The protein precipitate was stirred in cold distilled water, and 7 ml. of 1N NaOH was added, bringing the pH of the mixture to 8.0. The material dissolved completely, but the solution remained decidedly opalescent. This was then diluted to 2 liters and enough acetic (1:1) acid added to bring the pH back to 3.5. The protein precipitated immediately. Four hours later the precipitate was again collected and put into solution at pH 8.5. The acetic acid was again added until a pH of 3.5 was reached.

Inasmuch as the protein did not precipitate readily, the solution was placed in the cold overnight. After this third precipitation the solution had cleared up considerably, but still was cloudy at pH 8.0. Reprecipitation liquors after the second precipitation were discarded after finding only a trace of nitrogen present and getting a negative Molisch test. A neutral solution of the reprecipitated protein was forced through a medium Berkefeld filter, the resulting solution being water-clear. This was acidified with acetic acid to a pH of 3.5. After standing overnight the precipitate was centrifuged off, washed with water, rapidly dehydrated with alcohol, followed by acetone and ether, filtered through a small Hirsh filter funnel and desiccated in vacuo over Dehydrite. This fraction was referred to as the nucleoprotein.

Trichloracetic Acid Precipitation:

The acetic acid filtrate from the above precipitate was concentrated in vacuo at 35°C. to about 850 ml. To this was added 42.5 grams of CCl_3COOH to make up a 5% solution. The mixture was allowed to stand overnight in the cold. The precipitate was centrifuged off and washed with 5% CCl_3COOH. This precipitate was suspended in 800 ml. of distilled water and dissolved with 7 ml. of 1N NaOH. Forty grams of CCl_3COOH was added to reprecipitate the fraction.

Alcohol Precipitation:

The trichloracetic acid solution was extracted with ether to remove most of the reagent acids and further neutralized with NH_4OH to pH 7.0. To the concentrate was added 20 volumes of 95% alcohol which precipitated the alcohol insoluble fraction. This was dissolved in 35 ml. of distilled water and reprecipitated with 20 volumes of alcohol. The precipitate was set aside for further examination.

The Alcohol-Soluble Fraction:

The supernatants from the two alcohol precipitates described above were combined and concentrated in vacuo at 35°C. to a volume of 300 ml. To this was added an excess of barium hydroxide and the solution aerated to remove the excess ammonia. The barium was quantitatively precipitated by the addition of sulphuric acid. The filtrate was evaporated at room temperature and dried in vacuo over Dehydrite. A half-portion of it was dialyzed until it was no longer acid to litmus paper. It was also dried in vacuo over Dehydrite.

Precipitation with Four Volumes of Alcohol:

The twice precipitated alcohol insoluble material contained a proteinlike substance, the socalled "S" substance and polysaccharides. The material was suspended

in 25ml. of water, acidified with a few drops of acetic acid, and an equal volume of alcohol was added. The "S" substance and polysaccharides readily dissolved, whereas the protein-like substance was insoluble and was centrifuged out. The latter was subjected to a second and third extraction in the same manner. The combined extracts were concentrated under reduced pressure to about 35 ml. and treated with 20 volumes of alcohol. The precipitate formed was collected in the centrifuge and redissolved in 50 ml. of water with the aid of an equal volume of alcohol. The 50% alcohol solution was clarified by centrifuging. To the supernatant were added 1 ml. of saturated sodium acetate, 0.5 ml. 30% NaOH and 10 volumes of alcohol. Precipitation from alkaline solution with alcohol was complete at this concentration. This conclusion was established by a negative Molisch test. The precipitate obtained from alkaline solution was dissolved in 50 ml. of water without neutralization. An equal volume of alcohol was added, and the solution again clarified. To this 50% alcohol solution were added a few drops of acetic acid, 1 ml. of saturated sodium acetate and 3 volumes of alcohol. The resulting precipitate was washed and dried in the usual manner and designated as the "S" substance.

Polysaccharides:

The remaining solution was made up to 20 volumes with alcohol. The precipitate was dissolved completely in water, remaining perfectly clear upon the addition of four volumes of alcohol. After final reprecipitation with sodium acetate and 20 volumes of alcohol, the substance was dried by alcohol, acetone and ether, and designated as crude polysaccharides.

Water-Insoluble Fraction:

Most of the residue left after extracting the original alcohol precipitate with 50% alcohol was insoluble in water, but formed stable opalescent sols when in a slight alkaline medium. This fraction has been referred to as a protein-like substance although its nitrogen content was considerable below that of a simple protein.

Examination of Fractions

Table III

Quantitative Distribution of Fractions in S.pullorum.

	Coburn (30.00gm)		B.A.I. (27.63gm)	
	gms.	%	gms.	%
Nucleoprotein	0.60	2.0	-	-
Protein Remaining in Extract after Removal of Precipitable Prot.	.14	0.47	0.37	1.34
Alc-Soluble Fraction	0.18	0.60	1.11	4.02
"S" Substance	-	-	0.20	0.73

Table III (Continued)

	Coburn (30.00Gm)		B.A.I. (27.63Gm)	
	Gms.	%	Gms.	%
Crude Polysaccharides	0.20	0.66	0.17	0.62
Ether Extract	0.55	1.81	0.31	1.12
Alc-ether-chloroform Ext.	0.66	2.21	0.65	2.35
Cell Residue	26.70	88.10	23.10	83.60

The yield given with the description of the various fractions represents values obtained in a single analysis. The difficulty in producing an adequate supply of the dry cells precluded further study to confirm the results.

The Nucleoproteins:

This fraction, as isolated from the Coburn antigen was designated "nucleoprotein" inaccordance with common usage, although it is admittedly a mixture of acid-precipitable proteins, including albumins, globulins and perhaps others.

The total yield was approximately 2%. The dried material consisted of a fluffy gray-white powder, insoluble in water, but forming a stable sol upon the addition of NaOH.

The nitrogen content of this preparation was 11.3%. Phosphorous was markedly present as determined by a qualitative test with $(NH_4)_2MoO_4$. The iso-electric point was between 3.5 and 4.0.

The following qualitative tests were very marked: biuret, Rosenheim and Molisch. The following were found positive: xanthoproteic, aniline hydrochloride and purines (isolated as the silver salts). Millions' and sulphur were negative. Hydrolysis with N acid for 4 hours liberated no reducing sugars.

The biological properties were examined using solutions containing weighed amounts of the dried material. On intradermal inoculation in pullorum-sensitive fowl, a 1-1,000 solution produced a specific reaction, whereas no reaction was obtained with non-sensitized birds. Precipitation with antiserum* was visible up to 25,600 dilution of the antigen.

When injected intravenously, this fraction functioned as a complete antigen, giving rise to antibodies which were found to be present by the rapid stained antigen and the tube agglutination methods. The antiserum thus produced agglutinated antigen up to a dilution of 1-800.

No "nucleoprotein" fraction was isolated from the B.A.I. antigen; however, it was presumably present, as a very fine clouding appeared which would not settle. The iso-electric point was sought by taking aliquot portions and adjusting the pH over a range of 2.8 to 5.8, using intervals in pH of 0.2. However, upon standing in the cold, no precipitation occured in any of the tubes.

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* The S.pullorum agglutination titer of antiserum was 1-30,000.

The remaining solution was concentrated to one liter and the work on it continued.

Trichloracetic acid Precipitable Substance:

A substance was precipitated from the Coburn antigen with CCl_3COOH, but upon reprecipitation nothing came down. Several methods were used in an attempt to cause precipitation, but the only one which met with partial success was the addition of saturated ammonium sulphate. However, after isolation, it was proven not to be a protein, having a nitrogen content of less than 0.1%. The biuret, Rosenheim and Molisch tests were negative.

In the case of the B.A.I. antigen, again no precipitation occurred. Attempts to induce precipitation by varying the pH failed.

A Protein-Like Substance Remaining in the Extract after Removal of the Acid-precipitable Proteins:

A grayish-white, very brittle material was obtained from the Coburn antigen. This was extremely insoluble in all neutral solvents and only partially soluble in alkaline solution. Although it was isolated from the alcohol-soluble extract, after drying, it was no longer alcohol-soluble. The yield was extremely small, being 0.47%. Phosphorous was present, sulphur absent, and the nitrogen as determined by a single trial on one preparation was 1.8%. The biuret, Rosenheim and xanthoproteic

tests were weak. The Molisch test was very faint and the aniline hydrochloride test negative. Purines were not detectable.

Biologically this fraction appeared to be inactive. Precipitation with antiserum was observed up to a 1-1,600 dilution, but since the substance was so very insoluble, it might have been due to the settling of the insoluble particles.

However, in the case of the B. A. I. antigen, a much larger yield, 1.34%, was obtained, This was a gray-white powder which was insoluble in water and formed stable sols in dilute alkali.

Phosphorous was present, sulphur absent; the nitrogen by a single analysis was 4.28%. The biuret, Rosenheim and xanthoproteic reactions were positive. The Molisch test was faintly positive but aniline hydrochloride test, negative.

The biological properties differed somewhat from that of the Coburn antigen. Antiserum was precipitated by a 1-25,600 dilution of the antigen. Sensitized and non-sensitized birds gave no skin reaction with this substance when injected intradermally, the injections having been made into the wattles of the hens.

The "S" Substance:

No "S" substance was isolated in the case of the

Coburn antigen. The solution became clouded upon the addition of 4 volumes of alcohol, but no separation of any material occurred either on standing or centrifuging.

In the case of the B.A.I. antigen a yield of 0.73% of the substance was obtained. It appeared as a pure white brittle powder which was fairly soluble in water, but formed a cloudy sol upon the addition of a slight amount of alkali.

The unpurified "S" substance gave no test with I in KI, a slight Molisch test and upon hydrolysis with 2N acid for 6 hours, 15.6% reducing sugars calculated as glucose. The orcinol, phloroglucinol and resorcinol tests were negative. Before hydrolysis, the substance gave no reduction with Benedicts' reagent. The biuret and Millons' tests were negative. The substance contained 4.08% nitrogen.

From this preliminary examination, it was impossible to define the chemical individuality of this "S" substance.

Upon intradermal injection of a 1-1,000 dilution of this substance into the wattles of a hen, a specific reaction was again observed. Precipitation with antiserum occurred in dilutions as high as 1-25,600 of the "S" sub-

stance. If there had been enough of the material it would have been interesting to determine whether repeated purification would have increased the precipitation titer and lowered the carbohydrate content. This observation was made in the work of Huston, Huddleson and Hershey (7). When injected intravenously into non-sensitized birds, this fraction also gave rise to antibodies. The antiserum so produced agglutinated antigen up to a dilution of 1-800.

The Polysaccharides:

This fraction in the Coburn antigen was thought to contain some "S" substance, for upon the addition of 4 volumes of alcohol, cloudiness appeared but could not be made to settle out. However upon the addition of 10 volumes of alcohol, it precipitated readily. Thus, although no "S" substance was isolated from this fraction, it appeared to be present.

The yield of this crude polysaccharide preparation was 0.66%. It was a crumbly powder, readily soluble in water, leaving a slight cloudiness.

The following qualitative tests were made: Molisch and resorcinol tests were positive, but the orcinal and phloroglucinol, negative. I in KI test was negative.

There were no reducing sugars with Benedicts' reagent before hydrolysis. The biuret and Millons' tests were negative. The nitrogen content was found to be 0.6%.

Several portions were subjected to hydrolysis with 2 N acid for 2 and 4 hours and one portion with 1 N acid for 10 hours. Folin-Wu sugar determinations on aliquot portions correspond to glucose values between 23.5 and 30.7% for the various hydrolyses.

This crude polysaccharide had the ability to precipitate antiserum with a 1-6,400 dilution of the antigen. When a 1-1,000 solution of this substance was injected into the wattles of chickens, a negative reaction was observed in the case of both positive and negative birds.

It is believed that the reason this fraction gave a precipitation reaction was because it was not sufficiently purified to remove all of the "S" substance.

The "S" substance in the case of the B.A.I. antigen had been separated, or partially so, from the polysaccharides. This fraction was extremely soluble in water. The yield was 0.63%.

The qualitative tests made gave identical results with those of the Coburn polysaccharides. The per cent nitrogen, however, was appreciably higher, being 1.98%.

After 3 and 4 hour hydrolyses with 2 N acid and one hydrolysis for 10 hours with 1 N acid, Folin-Wu sugar determinations on aliquot portions gave glucose values between 28.8 and 32.2%.

This polysaccharide fraction exhibited identical antigenic properties with those observed in the case of the corresponding Coburn fraction.

The Alcohol-Soluble Fraction:

The mixed substances from the supposed protein-free extract, unprecipitated by 20 volumes of alcohol, formed on desiccation a brown sticky amorphous mass. However, since in the Coburn antigen this fraction comprised about 25% of the total weight of the cells, and therefore probably contained other substances used in the various precipitations, a portion was dialyzed until free from acid, and this upon weighing was found to represent only 0.6% of the total weight of the cells.

This substance gave negative biuret and Millons' tests. The Molisch test was very weak, and the substance did not reduce Benedicts' reagent. Phosphorous was present, but sulphur was absent.

The nitrogen content of this fraction before dialysis was 11.1% and after dialysis it decreased to 3.9%.

This fraction caused no serum precipitation.

The alcohol-soluble fraction isolated from the B.A.I. antigen was dialyzed before it was desiccated. A brown sticky mass, resembling lipide material was obtained, the yield being 4.02%.

The qualitative tests made on this substance gave results identical with those of the Coburn fraction. However, the nitrogen content differed, being 7.4%.

This preparation also did not show any antigenic properties.

The Cell Residue:

The water extracted, defatted and dried cell residues of both antigens was a white powdery material. Both stained with difficulty, but the whole and disintegrated bits of cells could be distinguished. They gave a very strong biuret and Molisch reactions, indicating the abundant presence of protein and carbohydrate.

Precipitation tests could not be carried out because of the extreme insolubility of this residue.

The Lipides:

Upon cold ether extraction of the ground cells, a brown colored waxy solid was obtained, which comprised 1.12% of the cell weight. It was soluble in alcohol, acetone and ether and contained no phosphorus.

The total alcohol-ether-chloroform extract made up the greater bulk of the lipides, being 2.21%. The crude material was a dark brown sticky mass, which was entirely homogenous in appearance. It had a melting point slightly above room temperature.

The nitrogen content of a single sample was 0.62% and the phosphorus was 1.92%. The atomic N:P ratio was approximately 1.4.

A flaky white substance insoluble in ether was obtained from the above lipide. It was also chloroform and alcohol insoluble. Nothing more was determined about the chemical or biological character of this substance, since there was less than 0.1 gram of the material available.

The U.S.P. lipide fraction was much the same as the Coburn lipide. On cold ether extraction of the ground cells, a 1.12% yield of a brown colored waxy solid was obtained. This was soluble in alcohol, ether and acetone and was also phosphorus free.

The alcohol-ether-chloroform extract comprised 2.35% of the weight of the cells. It was a lighter brown substance than that of the Coburn antigen, and also had a melting point slightly above room temperature.

The nitrogen content of this fraction was 0.86% and the phosphorous 1.8%, giving an atomic P:N ratio of about 1.0.

S U M M A R Y

1. A method is given for the separation of several fractions of Salmonella pullorum.

2. Preliminary findings of the chemical and biological study of these fractions are reported. Briefly they are:

(a) A fraction precipitated by acetic acid and designated as "nucleoprotein" had the power to precipitate antiserum at a dilution of 1-25,000. It also elicited a specific skin reaction and showed definite antigenic power.

(b) A fraction insoluble in 20 volumes of alcohol and separated from the other constituents of this fraction by precipitation with acetic acid showed ability to precipitate antiserum up to a dilution of 1-25,600; however, it showed no further biological properties. This fraction cannot be further classified than to designate it as a protein-like substance.

(c) A substance, designated as "S" substance, which was isolated from the alcohol insoluble fraction by precipitation with 4 volumes of alcohol, precipitated antiserum

when being diluted up to 1-25,600. It also gave a specific skin reaction and functioned antigenically.

(d) A fraction insoluble in 20 volumes of alcohol, from which the acetic acid precipitable substance and "S" substance had been separated, termed polysaccharide. Antiserum was precipitated with antigen up to a dilution of 1-6,400. This fraction gave a negative skin reaction.

(e) A fraction soluble in ether, alcohol and chloroform composed the cell lipides, upon which no biological studies were made.

3. In view of the fact that the separation and purification of the constituents of the antigens studied was inadequate, it is not safe to conclude that differences in chemical constitution can explain the difference in their sensitivity.

B I B L I O G R A P H Y

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