

THE ANTIGENIC PROTEINS OF *SALMONELLA AERTRYCKE*

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It has been established by the work of various investigators—Wells (16, 17), Osborne (15), and others—that purified proteins with few exceptions can be successfully used as antigens. Commonly in the immunization of an animal to a bacterial antigen, however, the whole cell or cell autolysate is used. In the bacterial cell there may be present several different proteins which are antigenic, although little is known in this connection.

The object of this study was to separate various fractions of bacterial protein and determine the antigenic properties of each. The test organism used was *Salmonella aertrycke* which, when used in the form of whole cells, results in the production of a high titred immune serum.

METHOD AND TECHNIQUE

Preparation of the Antigens. The bacteria were grown on two per cent nutrient agar (pH 7.2) in Blake bottles. The agar was allowed to harden with the bottles lying flat. The surface of the agar was flooded with a heavy twenty-four hour broth culture of *Salmonella aertrycke* and was incubated for twenty-four hours at thirty-seven degrees C. The bacteria were then washed down with sterile distilled water, and the suspension was centrifuged in order to concentrate the bacteria, which were placed in a dessicator over phosphorous pentoxide and dried. The weight of the dried bacteria obtained by this procedure was 1.3534 grams.

Methods used in the preparation and isolation of the various fractions were taken chiefly from Mathews' Physiological Chemistry (14) and from a review in Lovett's paper (13) on the quantitative relations of serum albumin.

The fractions which were prepared from the dried bacteria were separated on the basis of solubility. The water soluble substances were separated from the saline soluble, from the alkaline soluble, and from the ether soluble. This was done in the following manner:

A portion of the dried bacteria was suspended in saline and used as Fraction I.

The remaining part of the dried bacteria was ground up in 70 c.c. of distilled water, and the residue was separated by centrifuging. The supernatant fluid, solution A (Chart I) presumably contained both albumin and pseudoglobulin. Some of it was used as Fraction II.

In order to separate the albumin from the pseudoglobulin, the rest of the supernatant fluid was added to an equal volume of ammonium sulphate (100%), which precipitated the pseudoglobulin. This was sepa-

rated by filtration and the filtrate, containing albumin, was dialized free of ammonium sulphate and was diluted up to 70 c.c. Sodium chloride was added to make the salt concentration equal to that of physiological saline. This solution was used as Fraction III.

The pseudoglobulin precipitate was suspended in 70 c.c. of normal saline, dialized free of ammonium sulphate, and used as Fraction IV, sodium chloride being added to replace that lost in dializing.

The residue which would not dissolve in distilled water was ground with 10 c.c. physiological saline, after which the mixture was centrifuged. The saline supernatant fluid, Solution B, presumably contained globulin and probably also other proteins. This was diluted up to 70 c.c. and some of it was used as Fraction V.

In order to precipitate the globulin, the rest of the solution was added to an equal volume of ammonium sulphate (100%). The precipitate was separated by filtration, and the filtrate was discarded. The precipitate was suspended in 70 c.c. physiological saline and dialized free of ammonium sulphate. Sodium chloride was added to replace that lost in dializing, and this suspension was used as Fraction VI.

The residue which did not dissolve in the saline solution (Solution B) was ground in 100 c.c. sodium hydroxide and the mixture was centrifuged. The fluid, which presumably contained conjugated proteins, was diluted up to 70 c.c. and used as Fraction VII. The residue was extracted with ether, which was removed and evaporated. The dry residue from this evaporation was suspended in 70 c.c. physiological saline and was used as Fraction VIII.

Protein and Lipoid Tests. Differential tests were used to determine the protein constituents of the soluble fractions, and a lipid test was made on Fraction VIII. The results of these are shown in Table II.

The Biuret test is not a test for any specific amino acid but is dependent on the peptid linkage. A positive reaction is given by all native proteins and by the larger number of the derived products. It is not so delicate as some of the other tests. Fractions I, II and V gave positive results.

The xantho-proteic reaction is given by tryptophane, tyrosine, and phenyl alanine, the three amino acids having benzene nuclei. Positive reactions were given by Fractions II and VII.

The Adamkiewicz reaction depends on the presence of tryptophane. The results were negative throughout; so probably the positive reaction given by Fraction VII in the xantho-proteic test was due to tyrosine or phenyl alanine, since the Millon test, for tyrosine (benzene nucleus) was also positive, and the tryptophane test was negative. Number VII was the only fraction which gave a positive reaction with the Millon test.

Ehrlich's diazo reaction is a histidine and tyrosine test. A positive reaction for histidine was given by Fraction IV, while all the others except VI were positive for tyrosine.

A test was made for the presence of Cysteine or Cystine by boiling the solutions with sodium hydroxide and adding lead-acetate solution. The results were entirely negative.

Tests were also made for heat coagulation, but there was no perceptible change in any solution.

The ether extract was tested with osmic acid and was negative. It was also negative with all the protein tests, except Ehrlich's diazo reaction, in which it was slightly positive. As shown in tables III and IV, it apparently had no antigenic properties.

Inoculation. Twenty-four rabbits were inoculated intravenously, three rabbits to each fraction. Each rabbit received a total of seven inoculations, which were given at three day intervals. The amount of each was as follows:

Inoculation I	0.1 c.c.
Inoculation II	0.1 c.c.
Inoculation III.....	0.1 c.c.
Inoculation IV.....	0.2 c.c.
Inoculation V.....	0.3 c.c.
Inoculation VI.....	0.4 c.c.
Inoculation VII.....	0.5 c.c.

Since each fraction was diluted up to or suspended in 70 c.c. of fluid, an inoculation of 0.1 c.c. is equal to 1/700 of the total amount of that fraction. The rabbits were bled before the inoculations were started, and the sera were tested for agglutinins and precipitins, with entirely negative results. The dilutions used in determining titres were set up in this way: A series of ten tubes was used for each serum, the tenth tube being a saline control. In the first tube was placed one and eight-tenths c.c. of physiological saline. Each succeeding tube received one c.c. Two-tenths c.c. of serum was placed in tube I and thoroughly mixed. This gave a dilution of one to ten. One c.c. of this was removed and added to the saline in tube II, giving a dilution of one to twenty. This was thoroughly mixed, and one c.c. was removed and added to the saline in tube III, giving a dilution of one to forty, etc.

EXPERIMENTAL DATA

One week and two weeks respectively after the last inoculation the rabbits were bled and the sera tested for agglutinins and precipitins. Each fraction was then tested with each serum for cross agglutinations and precipitations. The results, shown in Tables III and IV, are:

Fraction I:	dried bacteria
	precipitins.....negative
	agglutinins1:2560
	cross agglutination and precipitation...negative.
Fraction II:	albumin and pseudoglobulin mixture
	precipitins.....1:40
	agglutininsnegative
	cross agglutination and precipitation...negative.
Fraction III:	albumin
	precipitins.....1:80
	agglutininsnegative
	cross agglutination and precipitation...negative.

TABLE I—PROTOCOL OF THE FRACTIONATIONS—(NUMBERS INDICATE FRACTIONS)

I. DRIED BACTERIA	
GROUND WITH DISTILLED WATER	
RESIDUE	
GROUND WITH NORMAL SALINE	
RESIDUE	
GROUND WITH NACH	
RESIDUE	
EXTRACTED WITH ETHER	
VIII LIPOID	

II SOLUTION A	
+ (NH ₄) ₂ SO ₄	
SOLUTION	PRECIPITATE
III ALBUMIN	IV PSEUDOGLOBIN

V SOLUTION B	
GROUND WITH NORMAL SALINE	
RESIDUE	
GROUND WITH NACH	
RESIDUE	
EXTRACTED WITH ETHER	
VIII LIPOID	

VI GLOBULIN	
VII CONJUGATED PROTEIN	

SOLUTION	
PRECIPITATE	
DISCARDED	

+ (NH ₄) ₂ SO ₄	
SOLUTION	PRECIPITATE
DISCARDED	VI GLOBULIN
VII CONJUGATED PROTEIN	

SOLUTION	
RESIDUE	

- Fraction IV: pseudo-globulin
precipitins.....negative
agglutininsnegative
cross precipitations and agglutinations...with sera of rabbits eight and nine, immunized to Fraction V...precipitation in a dilution of one to ten.
- Fraction V: globulin and undetermined proteins
precipitins.....1:80
agglutininsnegative
cross agglutination and precipitation...0
- Fraction VI: globulin
precipitins.....negative
agglutininsnegative
cross agglutination and precipitation...with sera of rabbits eight and nine, immunized to Fraction V...precipitation in a dilution of one to ten.
- Fraction VII: conjugated protein
precipitins.....1:10
agglutininsnegative
cross agglutination and precipitation...negative.
- Fraction VIII:
precipitins.....negative
agglutininsnegative
cross precipitation and agglutination...negative.

DISCUSSION AND SUMMARY

In this work attention was focused on the production of antibodies and not on the production of a high titred serum.

The titres produced by antigens other than whole bacteria, although low, were definite.

Cross precipitations, which occurred in only two cases, were in dilutions too low for the use of absorption.

Fraction I, whole bacterial protein, gave positive protein tests with Ehrlich's diazo reaction and the Biuret test, but was negative with all other protein tests applied. This fraction was strongly antigenic. Fraction II, corresponding to a mixture of albumin and pseudoglobulin, gave positive tests with the diazo reaction, the Biuret test, and the xanthoproteic test. This fraction was also antigenic. Fraction III, corresponding to purified albumin, was positive only with the diazo reaction, but was antigenic. Fraction IV, corresponding to pseudoglobulin, was positive for histidine only and was not antigenic. This test was not obtained on any other fraction. Fraction V, corresponding to a mixture of globulin and unknown proteins, was positive with the diazo and Biuret tests and was antigenic, while Fraction VI, separated from V and corresponding to globulin, was negative with all protein tests and was non-antigenic. Fraction V thus contained some other undetermined antigenic protein or proteins. Fraction VI, consisting presumably of conjugated

proteins, gave a positive diazo reaction, positive xantho-proteic and positive Millon. This fraction was antigenic and was the only one which gave a plus Millon reaction. Fraction VIII, presumably lipoid, was negative for lipoids by the osmic acid test, but apparently contained traces of protein, probably as impurities, since a faintly positive diazo reaction was obtained. It was not, however, antigenic.

From a consideration of the above it will be seen that only those fractions which gave tests for the benzene nucleus were antigenic. This is in accord with the accepted idea that the presence of aromatic amino acids is necessary for antigenic activity on the part of a protein.

The protein of *Salmonella aertrycke*, under the conditions here reported, does not appear to contain either globulin, pseudoglobulin, or lipoid.

CONCLUSIONS

1. It has been found possible to separate specific antigenic fractions from the whole bacterial protein of *Salmonella aertrycke*.
2. The whole bacterial protein, under the experimental conditions of this work, was a more powerful antigen than any of the fractions tested.
3. The antigenic fractions obtained corresponded to a mixture of albumin and pseudoglobulin, albumin, a mixture of globulin and undetermined proteins, and conjugated proteins. Since tests for globulin and pseudoglobulin were negative, this indicates that there are present as antigens, albumin, conjugated protein, and some other protein or proteins as yet undetermined.

TABLE II—PROTEIN AND LIPOID TESTS ON VARIOUS FRACTIONS

Fraction	Biuret	Xantho-Proteic	Adam-kiewicz	Millon	Ehrlich's Diazo	Lead Acetate	Heat	Osmic Acid
I	+	0	0	0	+ Tyrosine	0	0	
II	+	+	0	0	+ Tyrosine	0	0	
III	0	0	0	0	+ Tyrosine	0	0	
IV	0	0	0	0	+ Histadine	0	0	
V	+	0	0	0	+ Tyrosine	0	0	
VI	0	0	0	0	0	0	0	
VII	0	+	0	+	+	0	0	
VIII	0	0	0	0	+ (Faint) Tyrosine	0		0

TABLE III—PRECIPITATION AND AGGLUTINATION TITRES

Frac.	One week after last Inoculation			Two weeks after last Inoculation	
	Rabbits	Agglutinins	Precipitins	Agglutinins	Precipitins
I	22 22	1:1280 1:2560 1:320		1:1280 1:2560 1:320	
II	1 2 3	0 0 0	1:40 1:10 1:40	0 0 0	1:40 1:20 1:20
III	10 11 12	0 Dead 0	1:40 1:20	0 0	1:80 1:40
IV	4 5 6	0 0 0	0 0 0	0 0 0	0 0 0
V	7 8 9	0 0 0	1:40 1:40 1:20	Dead 0 0	 1:80 1:80
VI	13 14 15	0 0 0	0 0 0	0 0 0	0 0 0
VII	16 17 18	0 0 0	0 0 0	0 0 0	1:10 0 1:10
VIII	20 21 24	0 0 0	0 0 0	0 0 0	0 0 0

TABLE IV—CROSS AGGLUTINATIONS AND PRECIPITATIONS

H.S.=homologous serum

Fractions	I	II	III	IV	V	VI	VII	VIII
Rabbits 1	0	H. S.	0	0	0	0	0	0
2	0	H. S.	0	0	0	0	0	0
3	0	H. S.	0	0	0	0	0	0
4	0	0	0	H. S.	0	0	0	0
5	0	0	0	H. S.	0	0	0	0
6	0	0	0	H. S.	0	0	0	0
7	0	0	0	0	H. S.	0	0	0
8	0	0	0	1:10	H. S.	1:10	0	0
9	0	0	0	1:10	H. S.	1:10	0	0
10	0	0	H. S.	0	0	0	0	0
11	Dead	0	H. S.	0	0	0	0	0
12	0	0	H. S.	0	0	0	0	0
13	0	0	0	0	0	H. S.	0	0
14	0	0	0	0	0	H. S.	0	0
15	0	0	0	0	0	H. S.	0	0
16	0	0	0	0	0	0	H. S.	0
17	0	0	0	0	0	0	H. S.	0
18	0	0	0	0	0	0	H. S.	0
19	H. S.	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	H. S.
21	0	0	0	0	0	0	0	H. S.
22	H. S.	0	0	0	0	0	0	0
23	H. S.	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	H. S.

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