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STUDIES ON K ANTIGEN OF *VIBRIO PARAHAEMOLYTICUS* I. ISOLATION AND PURIFICATION OF K ANTIGEN FROM *VIBRIO PARAHAEMOLYTICUS* A55 AND SOME OF ITS BIOLOGICAL PROPERTIES

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SUMMARY Simple evaporation followed by fractionation by a combination of chromatography on DEAE cellulose column and zone electrophoresis yielded a purified K antigen preparation from an unheated culture filtrate of *Vibrio parahaemolyticus* A55, grown in a completely dialysable medium. This antigen showed a high degree of serological activity and specificity but formed two precipitation lines against K antiserum on an Ouchterlony's agar plate. It had low antigenicity in rabbits even though it blocked K antibody strongly. Contamination of the preparation with O antigen was demonstrated in immunized rabbits, despite its apparent absence in Ouchterlony's test. This antigen was non toxic for mice but was effective in the protection of mice against challenge with the living homologous strain. This protection was type-specific due to K antigen. Chemical analyses showed that the antigen is acidic but it does not contain uronic, sialic or phosphoric acid residues. In this antigen considerable amounts of hexosamine and acetyl groups were found but hexoses and pentoses were not detectable.

INTRODUCTION

In 1950 FUJINO *et al.* (1951, 1953) isolated a new species of microorganism during an outbreak of food poisoning due to " Shirasu " or half dried young sardine, in Osaka prefecture, Japan, and it was considered that mixed infection with this organism and *Proteus morganii* might have caused the severe food-poisoning. Since then evidence has been accumulated showing that such organisms are responsible for food-poisonings with acute gastro-enteritis occurring in Japan particularly in the summer season. TAKIKAWA (1958) first noted that these organisms have unique characteristics and differ from hitherto known enteric pathogens in that they grow most profusely on media containing 3 per cent sodium chloride. SAKAZAKI *et al.* (1963) and FUJINO *et al.* (1965) classified them in the genus *Vibrio* and named them *Vibrio paraheamolyticus*. In serological studies, it was noted that a living culture of *Vibrio parahaemolyticus* is not agglutinated by homologous O antiserum. If the culture is

heated, preferably at 100°C for 1–2 hours, O-inagglutinability disappeares and strong agglutination is obtained. This is interpreted as due to the presence of K antigen which covers the cell surface interfering with the reaction of O antigen with its antibody.

Because of its type specificity this antigen has been a useful tool for type differetiation within the species by agglutination. However, very little is known about its chemical and immunological properties.

Since it was thought that precise immunochemical information would be of great importance in the better undersanding of the true significance of this K antigen in the serology and immunology of infection by this particular organism, the present study was undertaken to isolate and purify the K antigen.

This paper describes a procedure for obtaining it in quite high purity. Some of its biological properties are also reported.

MATERIALS AND METHODS

1. *Bacterial strains*

The strains used were *Vibrio parahaemolyticus* A55, its mutants and *Vibrio parahaemolyticus* A28. The original strains were kindly sent to us by Dr. I. TAKIKAWA.

1) *V. parahaemolyticus A55 (O : 5 ; K : 15)*

This organism is refered to as A55K$^+$ because it contains K antigen and is not agglutinated in the living state by the homologous O aitiserum. It grows well on nutrient agar containing 3 per cent sodium chloride and developes turbid and opaque colonies.

2) *V. parahaemolyticus A55K$^-$ 105*

This K$^-$ mutant was isolated from an ultraviolet-irradiated suspension of a A55K$^+$ culture. This strain is agglutinated readily by O antiserum prepared from a boiled culture of the parent K$^+$ strain. In the reciprocal agglutinin absorption test it was found to have the same O antigen as the parent K$^+$ strain but to lack the K antigen of the parent. Colonies on an agar plate are clear and translucent.

The K$^+$ strain and its mutant K$^-$ strain are both actively motile.

3) *V. parahaemolyticus A55H$^-$ 107*

This non-motile mutant was isolated from the original K$^+$ strain by ultraviolet irradiation. It retained the same O and K antigens as the original A55K$^+$ strain as shown by the agglutinin absorption test.

4) *V. parahaemolyticus A28 (O : 5 ; K : 14)*

This strain has the O antigen in common with the A55 strain but differs in the K antigen.

2. *Antisera*

1) OKH antisera

Antisera were prepared by injecting rabbits with formalin-treated culture of A55K$^+$ grown on nutrient broth containing 3 per cent sodium chloride. Injections were at 7 day intervals in increasing doses from 0.5 ml to 2.0 ml. These antisera contained both anti-O agglutinin and anti-K agglutinin by which the homologous living culture was agglutinated. Their agglutinin titers against homologous living cells usually reached 1 : 3000 or more. Production of H agglutinin was poor and its titer was usually 1 : 200 or less.

2) O antisera

O antisera were prepared from a cell suspension of A55K$^+$ which had been heated at 100°C for 2 hours and then washed thoroughly with saline. A similar injection schedule was used as for OKH antisera. The agglutinin titer of O antisera was usually 1 : 1000 or more.

3) K antisera

K antisera were obtained from OKH antisera by absorbing O antibody with A55K$^-$ cell suspensions or A28 cell suspensions. In both cases absorbed K sera retained almost the original agglutinative titers against homologous living cells.

4) H antisera

H antisera were obtained from OKH antisera by absorbing them with A55H$^-$ cell suspensions.

3. *Gel diffusion precipitation*

Serologic analyses were carried out by double diffusion in agar using the method of OUCHTERLONY (1949). A solution of 0.7 per cent Difco agar in physiological saline was solidified with several wells of 0.8 cm diameter 0.7 cm apart from each other. In the diffusion tests, 0.1 ml of undiluted serum and 0.1 ml of test antigen were placed in respective wells. The concentration of antigen used differd in different experiments. The plates were kept in a closed damp jar and examined daily from the 2nd to the 7th days.

4. *Culture media*

A completely dialysable medium was used to facilitate the separation of cell constituents from nutrient, as described by GOEBEL and BARRY (1958). The medium used consisted of the dialysate from a solution with the following composition per liter: Difco technical casamino acid 15 g, Difco yeast extract 10 g, maltose 10 g and sodium chloride 30 g. The medium was sterilized by passage through a Seitz filter.

5. *Culture filtrates*

Stock cultures of the organisms used were maintained on sterilized sea-water at room temperature and transfered every 3 days to nutrient broth containing 3 per cent sodium chloride before inoculation into experimental media. The culture of A55K$^+$ in nutrient broth was first transferred to 100 ml of the dialysable medium in a flask. Then 10 liters of the dialysate medium in a jarfermenter was inoculated with culture from the flask in the logarithmic phase of growth. The jarfermenter was incubated at 37°C with aeration and stirring. The pH of the growing culture was maintained at 7.2 by means of an electric pH control device. After 4 hours' incubation, the culture had almost reached the end of the logarithmic phase of growth and had a viable count of about 8×10^8 organisms per ml. It was then centrifuged at 5,000 rpm and filtered through a Seitz filter.

6. *Crude antigen of A55K$^+$*

10 liters of the culture filtrate from the Seitz filter was rapidly concentrated to about 1 liter in vacuo at below 40°C and then dialysed against distilled water at 4°C for 24 hours. The dialysate was again concentrated to about 100 ml in vacuo, redialysed and lyophilized. The freeze-dried material, containing only non-dialysable products of bacterial origin, was a yellowish, amorphous powder. This material, refered to as crude antigen, was used as the starting material for purification of K antigen. The average yield of crude antigen from 10 liters of culture was 1,215 mg.

7. *DEAE cellulose column chromatography*

DEAE cellulose (SERVA 0.74 meq/g) columns were equilibrated with 1/40 M tris buffer, pH 8.0. Samples were dissolved in the same buffer and eluted from the column with this buffer followed by gradient elution with NaCl in the same buffer.

The NaCl gradient was achieved with 1.0 M NaCl in 1/40 M tris buffer in the upper reservoir and 1000 ml of 1/40 M tris buffer in the mixing vessel. The eluate was collected with an automatic fraction collector at a flow rate of 2 ml per minutes in a cold room.

8. *Zone electrophoresis*

Zone electrophoresis using Pevikon C-870 (copolymer of polyvinyl chloride and polyvinyl acetate: Stockholms Superfosfat Fabriks A-B) as the supporting medium was employed for fractionation of the sample. Experiments were performed at pH 6.8 in M/15 phosphate buffer, or at pH 8.3 in 0.23 M sodium borate- 0.035 M sodium monobasic phosphate buffer. A current of 2 mA/cm^2 was applied for 16 hours at 4°C. After electrophoresis the Pevikon block was cut into 1 cm segments and each segment was eluted with 10 ml of distilled water.

9. *Chemical assays*

Protein content was determined by Folin-Lowry's method (1953) using crystalline bovine serum albumin as standard. Nitrogen was measured by a micro-Kjeldahl method (YOKOI and AKASHI, 1955) and phosphorus by that of ALLEN (1940). Total carbohydrate was measured by the phenol-sulfuric acid method of DUBOIS (1961) using glucose as standard. The MOLISCH reaction and anthrone reaction were employed for qualitative determination of carbohydrate. Hexosamine was determined by the procedure of ELSON-MORGAN (1933) after 2 hours hydrolysis in 6 N HCl at 100°C. Reducing sugars were measured by the method of NELSON and SOMOGYI (1944) after 2 hours hydrolysis in 6 N HCl at 100°C. Uronic acid was measured by the carbazol-sulfuric acid method (1947) and by the naphthoresorcinol method (1948). Sialic acid determination was made by the thiobarbituric acid method (1959), the resorcinol method (1958) and by the direct Ehrlich method (1952). *o*-Acetyl groups were estimated according to the method of HESTRIN (1949). Nucleic acid was determined from the absorption at 260 mμ in a Shimazu Beckmann-type spectrophotometer.

10. *Enzyme treatment*

The proteolytic enzymes used were trypsin (N.B.C.) and pronase (Kaken). 10 mg of enzyme were added to 10 mg of sample in 10 ml of water. The mixture was adjusted to pH 8.0 with trypsin and to pH 7.1 with pronase. After incubation at

37°C for 20 hours, the mixture was dialysed and concentrated to the original volume and boiled for one hour.

RESULTS

1. *Analysis of antigen in the crude antigen preparation*

The crude antigen is a yellowish water soluble amorphous product which yields a slightly colored, viscous solution when dissolved in water. A 1 per cent solution of the antigen gave positve Molish and biuret reactions. The material contained 6.2 per cent nitrogen, 0.2 per cent phosphorus, 4.1 per cent carbohydrate and 9.1 per cent protein. On hydrolysis it yielded 3.2 per cent hexosamine.

The crude antigen was first analysed serologically by applying the Ouchterlony gel diffusion technique. In the plate, a solution of 10 mg crude antigen/ml was placed in the center well surrounded by anti-OKH serum, anti-O serum and absorbed sera specific for K and H. As shown in Fig. 1, several precipitation lines were formed against the anti-OKH serum.

These lines can be classified into three groups. The first line, nearest to the antigen well, is diffuse and faint. This line fused with the line formed between the antigen and the anti-O serum, and was not observed between absorbed K and H anti-sera. So this line must be due to a reaction between the somatic O antigen and its antibody. In the second group there are two lines, which were the most intense and sharp and fused with the lines formed against K antiserum. They were not seen with O and H antisera. Therefore, the second lines may represent the precipitation of K antigen and its corresponding antibody. The third and faintest line, which does not show clearly in the figure, appeared closest to the antiserum well. As this line fused with the line arising against H antiserum, it was considered to be due to H antigen. Data obtained in this experiment indicate that the crude extract contains at least three distinct antigenic components and that each component could be followed by agar diffusion techniques using the corresponding antiserum.

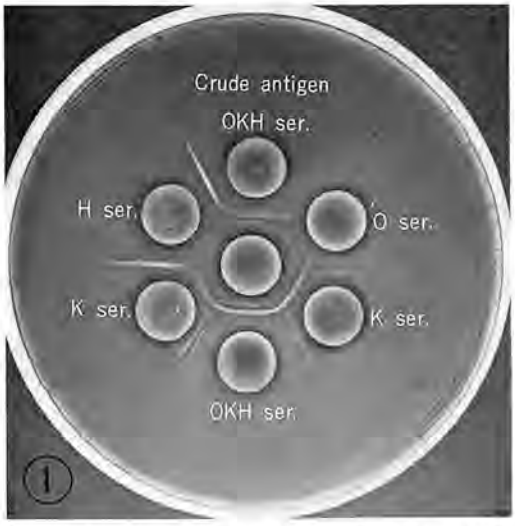

FIGURE 1 Agar diffusion precipitin pattern of the crude antigen.
Center well : Crude antigen
OKH ser. : Anti-OKH serum
O ser. : Anti-O serum
K ser. : Anti-K serum
H ser. : Anti-H serum

Next attempts were made to estimate the relative contents of antigenic components in crude antigen. A series of decreasing concentrations of crude antigen from 10 mg/ml was titrated on the diffusion agar plate against each of the test sera used in the above experiments. The test revealed that the minimal concentrations of crude antigen developing precipitin lines on the plate were approximately 1 mg/ml against K sera and 10 mg/ml against H and O sera.

The effects of various physical and chemical treatments on the precipitability of each of the antigenic components in the crude antigen were studied. First the effect of heat was investigated. A solution of 10 mg/ml of antigen was boiled for 2 hours. On the Ouchterlony agar plate the treated antigen showed no precipitin line against H antiserum,

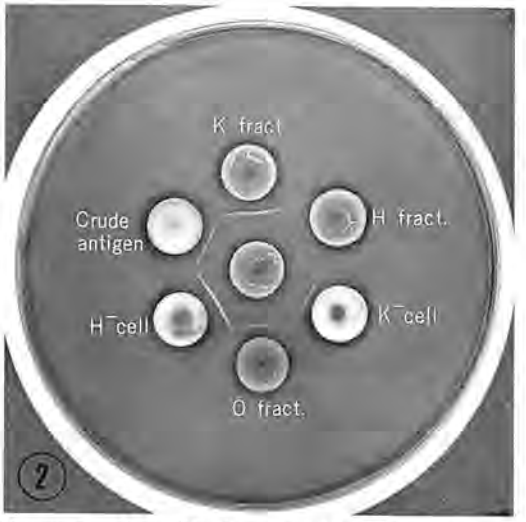

FIGURE 2 Agar diffusion precipitin pattern of fractions from the crude antigen on a DEAE cellulose column.
Center well: Anti-OKH serum
K fract.: Fraction reactive to anti-K serum
O fract.: Fraction reactive to anti-O serum
H fract.: Fraction reactive to anti-H serum
K^- cell: Broth culture of A55K^-105
H^- cell: Broth culture of A55H^-107

indicating the heat-lability of H antigen. Against the O antiserum, the precipitin line now appeared as two lines. The one which was closer to the antigen well fused with the original O-line of the untreated antigen served as a control. The other line was a new one which was somewhat fainter than the former. It seems that boiling unmasked a serologically active component of O antigen which otherwise would have remained inactive.

The K precipitin lines of the boiled antigen did not differ from the control lines in intensity and completely fused with them. This finding seems in contrast to the fact that O-inagglutinability caused by the presence of K antigen was completely removed by boiling the culture for one hour. However this discrepancy was resolved. The packed cells of K^+ culture, after thorough washing and resuspending in saline, were boiled for one hour and centrifuged. The resultant supernatant showed intensive precipitin lines against K serum corresponding to the K lines. So it was reasonably concluded that heating of K^+ bacteria results in release of the K antigenic substance.

The effect of HCl treatment was next investigated. A solution of 20 mg/ml of crude antigen was mixed with an equal volume of N HCl and allowed to stand at 37°C for 18 hours. After removal of the added acid by dialysis, samples were concentrated to the original volume and their immune diffusion tested. In this case, the K antigen underwent some serological change. The line close to the antigen well could no longer be seen and the line near the serum well became somewhat weaker, though it could still be seen even after 10 fold dilution. The O and H antigens completely lost their serological reactivity. From these results, the K antigen appears to differ from the O antigen and H antigen in its resistance to heat and HCl. These characteristics were useful in distinguishing the K antigen from other antigens in subsequent experiments.

2. *DEAE cellulose column chromatography*

The possibility of separating the K antigenic component from the crude antigen by DEAE cellulose column chromatography was studied. Twenty mg of crude antigen were dissolved in 5 ml of 1/40 M tris buffer at pH 8.0. The solution was adsorbed on a column of 3.5 g DEAE cellulose (0.8×10 cm) which had been equilibrated with the same buffer and then eluted by gradient elution with sodium chloride. The serological activity against K, O and H antisera and the quantity of carbohydrate, protein and hexosamine in the eluate fractions were measured. Ouchterlony's test revealed that H antigen was first eluted with 0.05–0.1 M NaCl. O antigen was eluted with the somewhat higher molarity of 0.1–0.3 and K antigen at 0.3–0.4 M. Fig. 2 illustrates the precipitin lines formed with each of the eluates against OKH antiserum on an Ouchter-

lony plate. OKH antiserum was placed in the center well surrounded by each of the eluates together with the curde antigen and cultures of A55K⁻105 and A55H⁻107, which served as standards. As seen in this figure, the K fraction (eluates at 0.3–0.4 M NaCl) formed intense precipitation lines which fused with the K precipitin lines formed by the crude antigen and A55H⁻107 broth culture. The O precipitin line formed by the O fraction (eluates at 0.1–0.3 M NaCl) was not observed with the K fraction. Chemical analyses showed a distinct peak of hexosamine corresponding with the zone of K antigenic activity. A good correlation between precipitation of K antigen and hexosamine content was confirmed in a series of experiments. Therefore, hexosamine positive material may be associated with some of the serological activity of K antigen. Next, large scale experiments were performed and hexosamine positive eluates were collected. One hundred mg of crude antigen dissolved in 20 ml of the above buffer was applied on a 15 g DEAE cellulose column (3×25 cm) and eluted as in the preceding experiments. The same chromatographic pattern was obtained. The procedure was repeated several times and the hexosamine positive fractions were pooled. The combined fraction was dialysed and freeze-dried.

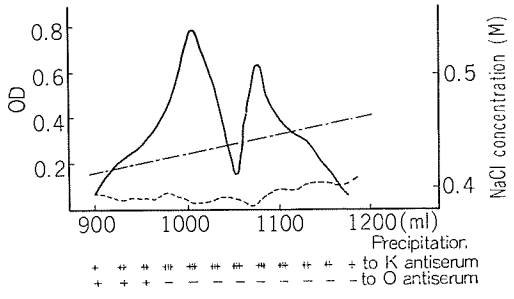

FIGURE 3 Pattern on rechromatography of antigen on DEAE cellulose column.
———— Hexosamine measured by the Elson-Morgan method at 530 mμ
--------- Carbohydrate measured by the phenol-sulfuric acid method at 490 mμ
—·—·— NaCl gradient

To purify this material it was rechromatographed on DEAE cellulose in the same way. The recovery was about 2.6 per cent of the crude antigen applied. As illustrated in Fig. 3, the material which was considerably concentrated in terms of hexosamine content showed two peaks of hexosamine which were almost superposed on each other. On Ouchterlony's plate the eluates from these peaks both formed two precipitin lines against K antiserum which fused with the two lines of the crude antigen. A concentrated solution (10 mg/ml) of this material formed weak precipitation lines against O serum in the gel diffusion agar test.

3. *Zone electrophoresis*

Since it was not possible to eliminate minor contaminants of O antigen from the K antigen preparation by DEAE cellulose chromatography, attempts were made to achieve this by zone electrophoresis. A 1 per cent solution of the partially purified K antigen obtained by DEAE cellulose column chromatography was subjected to zone electrophoresis, at pH 6.8 in M/15 phosphate buffer and at pH 8.3 in 0.23 M borate buffer. In both instances the eluates of the Pevikon segments were analysed for hexosamine, carbohydrate and for serological activity. Representative electrophoretic patterns are illustrated in Figs. 4 and 5. It will be seen from the diagrams that a single sharp peak of hexosamine was obtained with both buffers. The peak appeared about 12 cm from the origin toward the anode at pH 8.3 and about 7 cm from the origin at pH 6.8. On Ouchterlony's plates K lines of precipitation were found exclusively in the peak fraction of hexosamine in both electrophoreses, in conformance to the findings reported in the preceding section. The migration of the serologically active substance toward the anode suggests that it is acidic.

In the subsequent experiment electrophoresis was carried out at pH 8.3, using the same procedure and eluates from the hexosamine peak were collected. The combined eluates

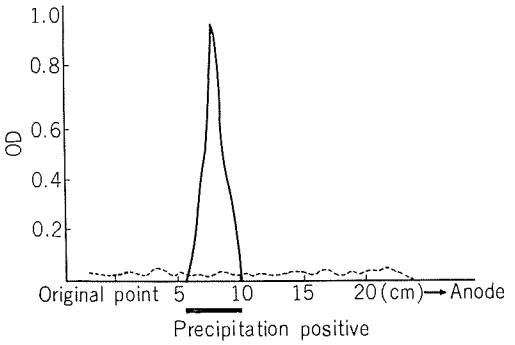

FIGURE 4 Zone electrophoresis pattern of partially purified antigen at pH 6.8.
———— Hexosamine measured by the Elson-Morgan method at 530 mμ
--------- Carbohydrate measured by the phenol-sulfuric acid method at 490 mμ

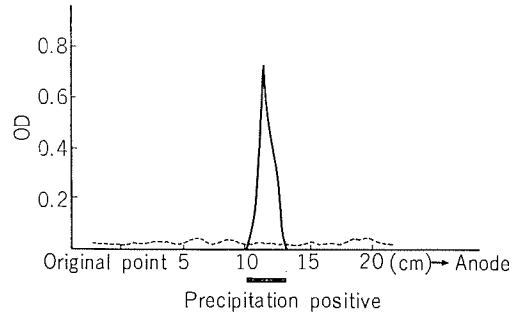

FIGURE 5 Zone electrophoresis pattern of partially purified antigen at pH 8.3.
———— Hexosamine measured by the Elson-Morgan method at 530 mμ
--------- Carbohydrate measured by the phenol-sulfuric acid method at 490 mμ

were dialysed thoroughly against water and then freeze-dried. In this way a total of about 210 mg of the substance was recovered representing about 30 per cent of the partially purified antigen applied. This material was a white powder which was readily soluble in water. A solution of this material was again tested for contamination with O antigen. No precipitin lines were detected against O antiserum even at a concentration of 10 mg/ml on Ouchterlony's agar plates. The material had strong antigenic activity against K antiserum, developing precipitin lines at a concentration of 5 μg/ml, and it is refered to hereafter as the purified antigen. It should be mentioned that the purified antigen showed two precipitin lines against K antiserum.

The purified antigen was tested for its capacity to block agglutination by K antibody. A series of decreasing concentrations of anti-K serum in saline were preincubated for 30 minutes at 37°C with an equal volume of solution containing 10 mg/ml of purified K antigen, and a living culture of homologous K^+ strain was then added. No agglutination was observed after overnight incubation at 37°C. On the other hand O antiserum preincubated with the purified antigen completely retained its agglutination titer against boiled homologous culture. The purified antigen appears to be entirely responsible for the agglutinability of living cells against K antisera.

4. *Chemical properties of the purified antigen*

Data concerning the chemical nature of the purified K antigen are presented in Tables 1 and 2. This material is non-crystalline, water soluble and readily precipitable with ethanol. It contains 3–5 per cent nitrogen and no phosphorus. No nucleic acid components can be detected from the ultraviolet absorption spectra. It gives only weak anthrone and Folin-Lowry reactions and negative biuret and Molisch reactions. The Nelson-Somogyi test was negative on nonhydrolysed material. The total content of carbohydrate except amino sugar was 1.1–2.0 per cent by the phenolsulfuric acid method and that of protein was 0.4–1.7 per cent by the Folin-Lowry method. On hydrolysis it yielded 15–20 per cent reducing sugar. The hexosamine content of hydrolysed material was 17–22 per cent. The *o*-Acetyl content was estimated as 14.5–15.7 per cent by the method of HESTRIN. Despite its acidic nature indicated by its electrophoretic mobility, tests for uronic acid and sialic acid were negative.

The purified antigen is remarkably heat stable. In solution it can be boiled for 2

TABLE 1 *Chemical data on the purified K antigen and the crude antigen*

Antigen	Nitrogen (%)	Protein (%)	Carbohydrate (%)	Hexosamine* (%)	Reducing sugar* (%)	*o*-Acetyl (%)	Minimal amount of antigen giving precipitation on Ouchterlony plate
Purified	3–5	0.4–1.7	1.1–2.0	17–22	15–20	14.5–15.7	5 μg/ml
Crude	6.2	9.1	4.1	3.2	Not tested	Not tested	1000 μg/ml

* Hydrolysed in 6 N HCl at 100°C, 2 hr.
Analyses wer performed on specimens dried in vacuo at 60°C to a constant weight. Samples of 2 mg dry weight were used for nitrogen determination, 5 mg dry weight for protein and carbohydrate. 3 mg dry weight for hexosamine and reducing sugar and 10 mg dry weight for *o*-Acetyl groups.

TABLE 2 *Qualitative characterization test on the purified K antigen (solution: 10 mg/ml)*

Molisch test	Negative
Anthrone test	Negative or weakly positive
Biuret test	Negative
Folin-Lowry test	Weak
Uronic acid test	
Naphthoresorcinol	Negative
Carbazole	Negative
Elson-Morgan test	Strongly positive
Sialic acid test*	
Direct Ehrlich	Negative
Resorcinol	Negative
Thiobarbituric acid	Negative
o-Acetyl Group test	Positive
Phosphorus test	Negative
Ultraviolet absorption at 260 mμ	No absorption maxima

* Either unhydrolysed or hydrolysed (0.1 N H_2SO_4 for 1 hr. at 80°C)

hours without any loss in serological activity. After deproteinization by treating with an equal volume of aqueous 90 per cent phenol at 68°C for 30 minutes. (WESTPHAL, 1954) as well as after proteolytic digestion with pronase and trypsin it retained its serological activity. On agar diffusion plates, antigen treated in this way gave two dictinct lines of precipitation against K antiserum which fused with the lines given by crude antigen. These findings provide conclusive evidence that the very small amount of protein which was found in the purified antigen is not responsible for the serological activity.

5. *Antigenic activity of the purified antigen in vivo*

1) *Antigenicity for rabbits*

The antigenicity of the purified antigen was determined by inoculation of groups of rabbits. Four rabbits were given a series of six injections of a total of 35 mg of the purified antigen during a period of 40 days. None of the animals produced any demonstrable K antibody as measured by either the agglutination test with a homologous living cell suspension or by the agar diffusion test with purified antigen.

In a further experiment the purified antigen was injected with Freund's complete adjuvant into the back muscles of rabbits. A total of 40 mg of the purified antigen was given in four injections. In this case the antigen also failed to evoke K antibodies in rabbits. It should be noted that when the sera obtained were tested against crude antigen, a faint precipitin line was observed which fused with the line developed between O antiserum and crude antigen. This shows that O antigen was in fact present in minute amounts in the purified antigen although not detectable by other methods.

2) *Toxicity for mice*

Toxicity was estimated by injecting groups of ten dd white mice (body weight 18–22 g) intraperitoneally with graded doses of 10

TABLE 3 *Survival of mice immunized with purified K antigen and challenged with V. parahaemolyticus A55K+*

Challenge dose (suspended in 3% NaCl)	Immunizing dose μg/mouse							ED_{50} μg	Control
	200	100	50	10	5	1	0.1		
5×10^7 cells	6/10*	8/10	8/10	10/10	7/10	3/10	1/10	3.2	0/10
3×10^7									0/10
1×10^7									2/10
5×10^6									3/10
1×10^6									10/10

* Survivors/total mice tested.

–2,000 μg of the purified antigen. All the mice survived.

3) *Immunogenicity for mice*

The purified antigen was assayed for its potency as an immunizing agent by the mouse protection tests. Groups of dd white mice, weighing 18–22 g, of both sexes, were each injected intraperitoneally with a single dose of various amounts of the purified antigen. Eight days later they were each challenged with 5×10^7 cells of homologous strain suspended in 3 per cent NaCl solution through the intraperitoneal route. Following the challenge, mice were observed for a period of 72 hours. The results are presented in Table 3. It will be seen that the purified K antigen is effective in immunization of mice against challenge with the homologous strain. The percentage survival indicates that the 50 per cent protection dose (ED_{50}) is about 3.2 μg/mouse.

It should be recalled, however, that the purified antigen used in these experiments was not entirely pure but was contaminated with a small amount of O antigen as evidenced by the *in vivo* tests already mentioned. To exclude the possibility that the protective effect of the purified antigen was due to this contamination, an additional experiment was carried out. Mice immunized with the antigen were challenged with the heterologous strain A28, which possessed only O antigen in common. The immunized mice showed no protection against this heterologous strain. This finding also indicates that the protection afforded by K antigen may be type specific.

Since purified antigen did not stimulate antibody production in rabbits, the presence of antibodies in immunized mice was tested.

Three groups of five mice were injected intraperitoneally with single doses of 5, 10 and 20 μg of the purified antigen respectively. Eight days later, the mice were bled and the blood samples from animals in each group of mice were pooled. Production of K antibody in mice was confirmed on Ouchterlony's plates.

Inspection of data revealed that 5 μg of antigen was more effective in protection than either 100 or 200 μg. This may be interpreted to be the result of immuno-paralysis, as described by FELTON (1949).

DISCUSSION

The K antigen obtained showed a high degree of serological activity and specificity, developing precipitin lines at a concentration of 5 μg/ml against K antiserum on an Ouchterlony's agar plate. It appeared to be electrophoretically homogeneous.

In the course of experiments the hexosamine content was found to be closely proportional to the serological activity through all the steps of fractionation. It was considered, therefore, that hexosamine positive material might be responsible for the antigenic activity. However, chemical analysis showed that the hexosamine content measured by the method of Elson and Morgan accounts for only 17–22

per cent of the purified K antigen, suggesting the presence of other unidentified components in the purified antigen. It should be pointed out, however, that in this study we employed glucosamine but not galactosamine as a standard for measuring the hexosamine content and this may give too low a value for the hexosamine content (ROSEMAN and DAFFNER, 1956). It may also be possible that low values for the hexosamine content are caused by the hydrolysis conditions employed. Further studies are needed to determine these points.

From the fact that the purified K antigen described above is of acidic nature and contains acetyl groups but appears not to contain uronic or sialic acid residues, the K antigen of this organism seems to be principally a polymer of acetylated acidic aminosugar units, provided that hexosamine is proved to be a major constituent of this antigen.

Although the purified K antigen was electrophoretically homogeneous, it showed some serological heterogeneity. Despite the apparent absence of residual contamination with O antigen in the technique of Ouchterlony, *in vivo* tests by immunization of rabbits provided evidence that O antigen was in fact present in minute amounts in the product. The very small amount of O antigen which accompanied the K antigen could not be eliminated by the fractionation procedures used.

Furthermore, the antigen gave two distinct precipitation lines against K antiserum on the Ouchterlony's plate. When the antigen was properly diluted, the line closer to the serum well disappeared while the other line could still be seen. When the antigen was treated with N/2 HCl, the line closer to the serum well remained and the other line disappeared. This indicates that the K antigen obtained consisted of two major serologically active components, differing in nature and concentration. In this connection it should be pointed out that the antigen formed two major peaks of hexosamine on DEAE cellulose column chromatography which coincided with the serologically active zones for K antiserum.

BAKER *et al.* (1959) and WHITESIDE *et al.* (1960) presented evidence that the two lines arising in the reaction of Vi antigen and antibody may be due to the presence of deacetylated Vi antigen. Whether a similar explanation holds for the present K antigen remains to be investigated. With the heterogeneity of the material on the DEAE cellulose column in mind, attempts are now being made to separate the two antigenic components on resins.

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