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IMMUNOCHEMICAL STUDIES ON O-ANTIGENS OF *VIBRIO PARAHAEMOLYTICUS*.[1,2] 1. PREPARATION, SPECIFICITY AND CHEMICAL NATURE OF THE ANTIGENS

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SUMMARY 1. O-antigens were extracted with the phenol-water method from boiled cells of *Vibrio parahaemolyticus*. The yield of antigens was better than from acetone dried cells.

2. The serological specificities shown by the agar diffusion technique of antigens from thirty two pilot strains coincided well with those of O-agglutination.

3. Chemical studies of purified O-antigens from pilot strains O1 to O10 showed that these antigens were composed of glucose, galactose, glucosamine, heptose, phosphorus, fatty acid ester and nitrogen compounds. In addition galactosamine was found in 5 of 10 samples. The antigens seemed to be lipopolysaccharides.

INTRODUCTION

Vibrio parahaemolyticus is a new species of halophilic pathogenic bacteria found in Japan by Fujino et al. (1953 and 1965) and classified by Sakazaki et al. (1963). These bacteria have heat stable somatic O-antigens and heat labile capsular K-antigens, and were tentatively divided into 10 groups by heated O-cell agglutination and 32 types by live capsulated K-cell agglutination (Sakazaki, 1965). Chemical studies on O-antigens and K-antigens, however, are not yet complete.

It was reported that extraction of O-antigens from acetone dried cells of this microorganism was unsuccessful using any of the usual procedures such as phenol, trichloroacetic acid or pyridine extraction (Miwatani, 1964). Later

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2 A part of this work was presented at the 40th General Assembly of the Japan Bacteriological Society (March 30–31, 1967 at Nagoya).

a few papers appeared indicating that some O-antigens could be obtained with the phenol-water method (Zen-Yoji, 1966; Sakaguchi et al., 1968; Terada, 1968). An effort to obtain O-antigen in good yield was made in this laboratory and it was found that phenol treatment of heat-killed cells resulted in a satisfactory yield of O-antigen. The present studies were undertaken to extract O-antigens from 32 strains and to elucidate their serological specificities and chemical natures.

MATERIALS AND METHODS

1. *Bacterial strains*

Pilot strains from the National Institute of Health, Tokyo, were used including strains O1 to O10 for O-agglutination and strains K1 to K32 for K-agglutination. A tentative classification of the relation between O and K is shown in Table 1.
Strain EB101, first isolated by Fujino et al., was also used.

2. *Bacterial culture*

Bacteria were grown at 37 C with shaking or aeration for 6 to 8 hours in a liquid medium (pH 7.2), containing 1 per cent polypeptone (Daigo), 1 per cent meat extract (Wako special), 0.5 per cent yeast extract (Daigo) and 3 per cent sodium chloride.

3. *Antiseara*

Rabbits, weighing 2.5 to 3.0 kg, were immunized according to Burrows' method (Burrows et al. 1946, and Miwatani et al. 1969) with pilot strains, O1 to O10, heated at 100 C for 2.5 hours. Seven, nine and eleven days after the last injection blood was taken by cardiac puncture. The total volume of blood was usually about 150 ml. Sera were prepared and preserved with 0.25 per cent phenol in a refrigerator.

4. *Serological test*

Serological specificity was examined by Ouchterlony's double diffusion technique (Ouchterlony, 1949) with Bacto Special Agar Noble. Agglutination was carried out as follows: 0.5 ml of cell suspension heated at 100 C for 1 hour (O.D. at 550 mμ: 0.3, in a Coleman Junior spectrophotometer with a 16 mm diameter cuvette) was mixed with 0.5 ml of serial dilutions of antisera and incubated at 50 C overnight.

5. *Chemical analyses*

Nitrogen content was determined with ninhydrin after digestion with sulfuric acid and hydrogen peroxide (Schiffman et al. 1964). Phospborus was estimated according to the method of Dryer et al. (1957). Total sugar was determined by the phenol-sulfuric acid method (Dubois et al. 1951). For hexosamine, samples were hydrolyzed with 3N HCl at 100 C for 6 hours, excess HCl was evaporated off over P_2O_5 and NaOH *in vacuo*, the residue was dissolved in water and hexosamine was assayed after acetylation (Reissig et al. 1955). Heptose was determined according to the method of Osborn (1963). Fatty acid ester was determined as palmitate ester by Snyder and Stephens' method (Snyder and Stephens, 1959).

TABLE 1. *Tentative classification of V. parahaemolyticus by O- and K-agglutination*

O	K	O	K	O	K	O	K	O	K	O	K	O	K	O	K	O	K	O	K
1[a]	1	2[a]	2	3[a]	4	4[a]	8	5[a]	14	6[a]	18	7[a]	19	8[a]	20	9[a]	23	10[a]	24
1	25	2	3	3	5	4	9	5	15					8	21				
1	26	2	27	3	6	4	10	5	16					8	22				
1	32	2	28	3	7	4	11	5	17										
				3	29	4	12												
				3	30	4	13												
				3	31														

This table is according to Sakazaki (1965).
Additional new O-antigens and K-antigens are proposed (Hashimoto et al., 1966; Sakazaki et al., 1968; Shimouchi, 1967 and 1968a, b; Miwatani et al., 1969).
a: Pilot strains for O-agglutination

6. *Paperchromatography*

The multiple ascending technique with pyridine-ethyl acetate-water-acetic acid (5 : 5 : 3 : 1 v/v) and *n*-butanol-acetic acid-water (4 : 1 : 1 v/v) was used. Sugars were detected with silver nitrate-sodium hydroxide (Trevelyan et al., 1950) and hexosamines with ninhydrin.

RESULTS

1. *Extraction of O-antigens*

The bacterial culture was heated at 100 C for 2.5 hours. Then cells were harvested and washed 3 times with 3 per cent sodium chloride solution. Extraction and purification were performed according to the method of Westphal et al. (1952 and 1965) as follows: the cells were suspended in water and stirred with an equal volume of 90 per cent phenol at 65 C for 20 minutes. The mixture was then centrifuged at 4 C and the aqueous layer was dialyzed against deionized water. After several changes of water, the dialysant was concentrated *in vacuo* and lyophilized (crude O-antigen). For purification, 9 volumes of ethanol with a trace of sodium acetate were added to the concentrated crude antigen. The precipitate obtained by centrifugation was washed with 90 per cent ethanol by centrifugation. Then the precipitate was dissolved in water and centrifuged at 105,000 $\times g$ for 4 hours. The precipitate was redissolved in water and recentrifuged. This treatment was usually repeated 4 or 5 times until the absorption peak at 260 mμ disappeared. Generally, 60 to 150 mg of purified material were obtained from 10 l of culture. Analytical ultracentrifugation of the final precipitate usually showed several peaks though in one case there was only one peak. Tiselius electrophoresis also gave several peaks.

2. *Serological behavior of purified O-antigens extracted from pilot strains O1 to O10*

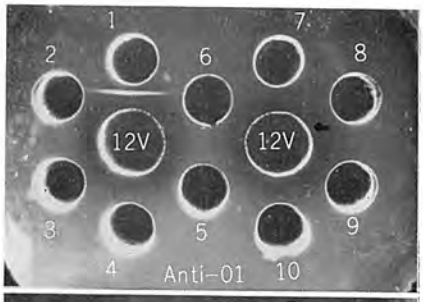
Ten purified O-antigens from strains O1 to O10 were tested against anti-O1 to anti-O10 sera. As shown in Fig. 1, O1-antigen reacted strongly with anti-O1 serum, O2-antigen reacted with anti-O2 serum etc. Thus each O-antigen mainly reacted with the homologous antiserum. So, the specificities of the extracted O-antigens coincided with those of O-agglutination, although various minor cross reactions were observed in different sera.

3. *Comparison of O-antigens extracted from strains bearing the same O but different K antigen*

O-Antigens were extracted from group-O1 (O1 : K1, O1 : K25, O1 : K26, O1 : K32, EB101) group-O2 (O2 : K2, O2 : K3, O2 : K27, O2 : K28), group-O3 (O3 : K4, O3 : K5, O3 : K6, O3 : K7, O3 : K29, O3 : K30, O3: K31), group-

TABLE 2. *Chemical composition of purified O-antigens*

O-Antigen	Nitrogen	Phosphorus	Fatty Acid Ester	Total Sugar	Heptose	Hexosamine
O1	1.0%	4.5%	22.6%	49.6%	2.1%	5.8%
O2	1.9	4.8	22.3	22.0	1.3	5.5
O3	1.8	4.3	17.1	23.8	1.2	5.7
O4	1.8	4.2	16.5	23.8	1.3	5.8
O5	1.4	4.5	15.8	21.4	2.1	5.9
O6	1.5	4.6	17.3	18.5	1.8	8.7
O7	1.2	4.5	15.4	15.7	1.1	6.3
O8	1.3	4.5	20.7	13.7	1.2	7.7
O9	1.2	4.2	22.2	12.7	1.4	5.1
O10	1.6	5.4	19.1	18.5	1.9	7.5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2
6
8
12V
12V
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5
9
4
Anti-01
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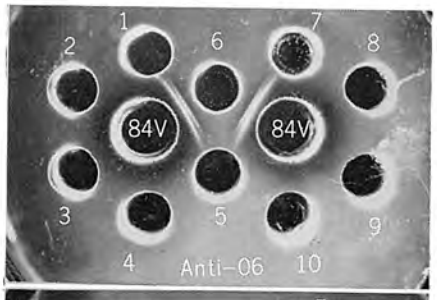
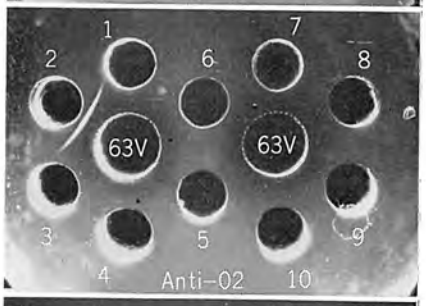
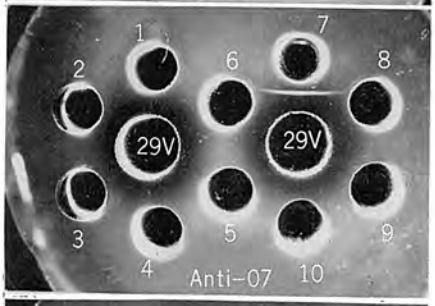

1
7
2
6
8
84V
84V
3
5
9
4
Anti-06
10


1
7
2
6
8
63V
63V
3
5
9
4
Anti-02
10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29V
29V
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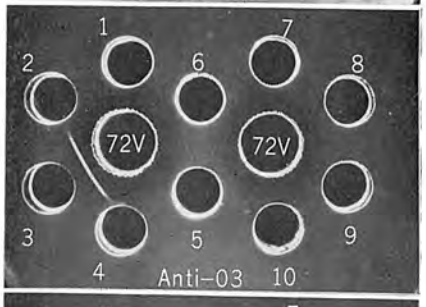
4
Anti-07
10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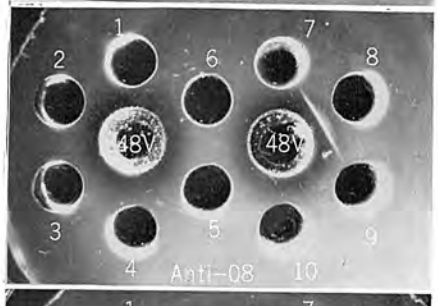
2
6
8
72V
72V
3
5
9
4
Anti-03
10


1
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2
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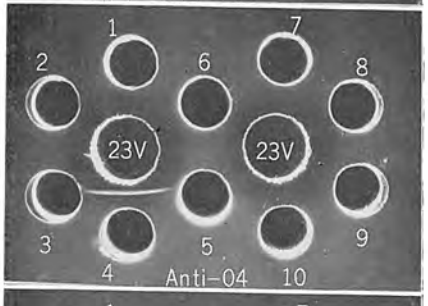
8
48V
48V
3
5
9
4
Anti-08
10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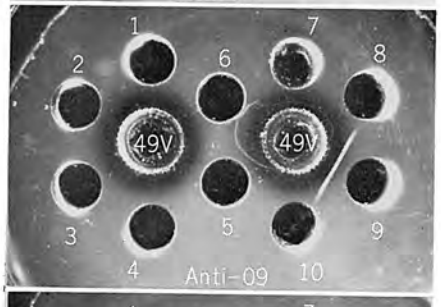
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2
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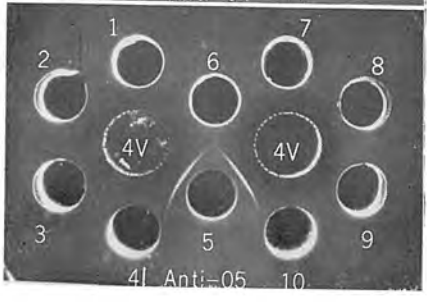
8
49V
49V
3
5
9
4
Anti-09
10


1
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2
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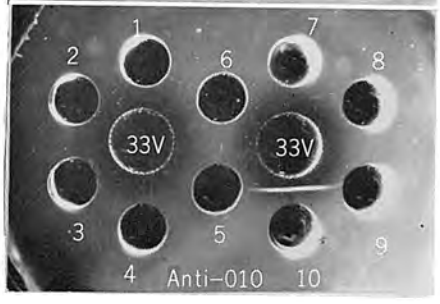
4V
4V
3
5
9
4
Anti-05
10


1
7
2
6
8
33V
33V
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5
9
4
Anti-010
10

FIGURE 1. *Precipitin reactions in agar between purified O-antigens and O-antisera*
1 to 10 are purified O-antigens obtained from pilot strains O1 to O10.
12V, 63V, 72V, 23V, 4V, 84V, 29V, 48V, 49V and 33V are anti-O1, -O2, -O3, -O4, -O5, -O6, -O7, -O8, -O9 and -O10 sera, respectively.

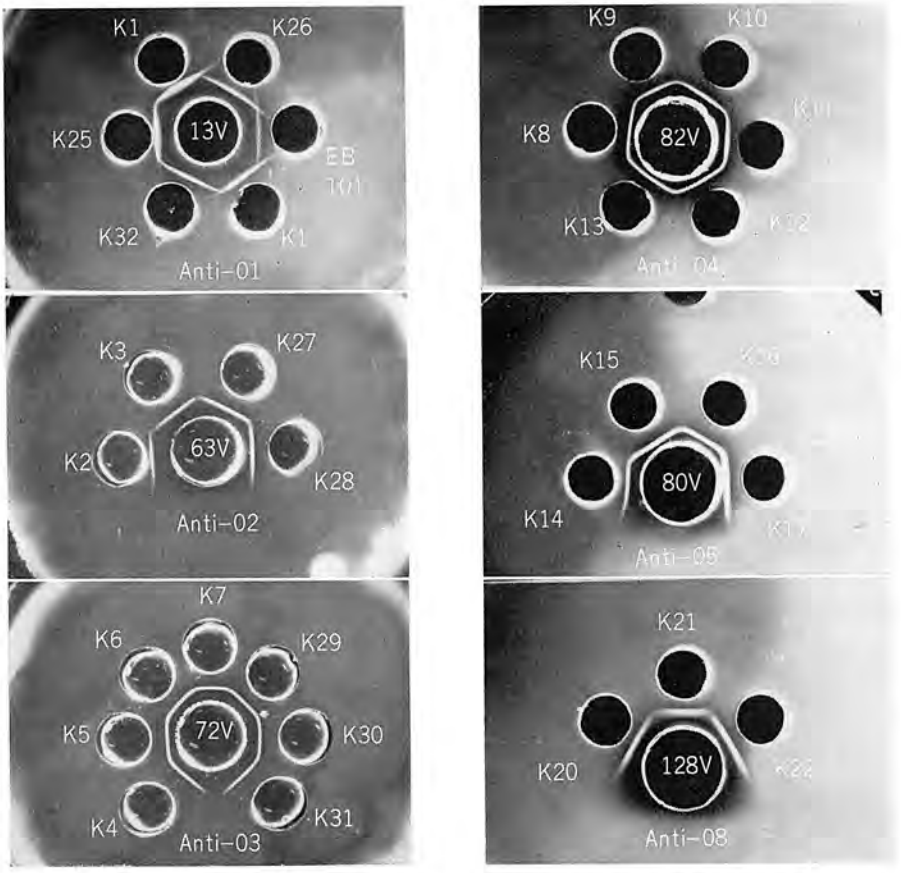

FIGURE 2. *Preciptin reactions in agar of O-antigens from strains bearing same O but different K numbers.*
K1, K25, K26, K32 and EB101 are O-antigens from strains O1: K1, O1: K25, O1: K26, O1: K32 and EB101, respectively.
K2, K3, K27 and K28 are O-antigens from strains O2: K2, O2: K3, O2: K27 and O2: K28, respectively.
K4, K5, K6, K7, K29, K30 and K31 are O-antignes from strains O3: K4, O3: K5, O3: K6, O3: K7, O3: K29, O3: K30 and O3: K31, respectively.
K8, K9, K10, K11, K12 and K13 are O-antigens from strains O4: K8, O4: K9, O4: K10, O4: K11, O4: K12 and O4: K13, respectively.
K14, K15, K16 and K17 are O-antigens from strains O5: K14, O5: K15, O5: K16 and O5: K17, respectively.
K20, K21 and K22 are O-antigens from strains O8: K20, O8: K21 and O8: K22, respectively.
13V, 63V, 72V, 82V, 80V and 128V are anti-O1, -O2, -O3, -O4, -O5 and -O8 sera, respectively.

O4 (O4 : K8, O4 : K9, O4 : K10, O4 : K11, O4 : K12, O4 : K13), group-O5 (O5 : K14, O5 : K15, O5 : K16, O5 :K17) and group-O8 (O8 : K20, O8 : K21, O8 : K22). Crude O-antigens from the same group-O were compared by the agar diffusion technique. The results are shown in Fig. 2. All the antigens of group-O1 reacted with anti-O1 serum, but precipitin lines did not completely fuse, antigens from O1 : K26, O1 : K32 and EB101 lacking some antigenic determinants. In each of the groups O2, O3, O4, O5 and O8, the precipitin lines almost completely fused. These results again indicate that the specificity shown by precipitation of the extracted antigens coincided with the specificity by agglutination.

4. *Effect of salt concentration on agglutination and precipitation in agar*

The agglutination reaction of this microorganism is usually done in 3 per cent sodium chloride medium and the precipitin reaction in 0.9 per cent solution. So, the effect of salt concentration was examined. Agglutination and precipitation of the O3 and anti-O3 system were carried out at both salt concentrations. With O3 heated cells, an equivalent agglutination titre of anti-O3 serum was obtained in both 3 and 0.9 per cent sodium chloride. Agar precipitation of the O3 and anti-O3 system were also the same at both salt concentrations, as shown in Fig. 3.

5. *Chemical analyses of purified O-antigens from pilot strains O1 to O10*

Analytical data obtained using the methods described above are summarized in Table 2. The sugar contents of all but O1-antigen were quite low. The fact that the nitrogen contents were higher than those expected from the hexosamine contents indicates the presence of other nitrogen compounds such as amino acids. The sum of the sugar, hexosamine, heptose, phosphate and fatty acid ester contents were less than 100 per cent of the material. Determination of ketodeoxyoctonate, dideoxysugar and ethanolamine have not yet been carried out.

6. *Detection of major constituent sugars*

Each O-antigen was hydrolyzed with 3 N HCl in a sealed tube at 100 C for 3 hours with occasional shaking. The tubes were opened and the reaction mixtures were concentrated to dryness over P_2O_5 and NaOH. The residues were dissolved in water and applied on paper. Paperchromatography was run as described in the Methods. All the O-antigens contained glucose, galactose and glucosamine as main

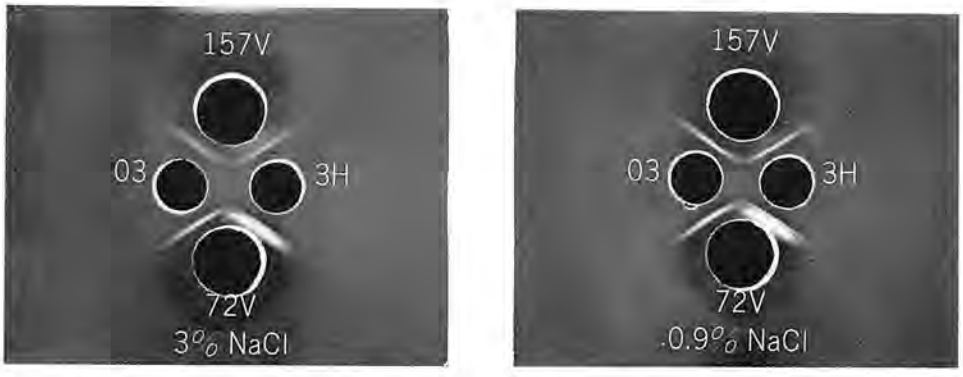

FIGURE 3. *Effect of salt concentration on precipitation in agar in the O3 and anti-O3 System.*
O3 is purified O-antigen of strain O3 : K4.
3H is O3 treated with 0.2 N HCl at 50 C for 48 hours (Torii and Igrashi, 1969).
72V and 157V are anti-O3 sera.

sugar constitutents. O1, O4, O7, O9 and O10 also contained galactosamine. All the hydrolyzates gave faint spots which moved very fast like deoxysugars, and also gave faint slowly moving spots, some of which seemed to be oligosaccharides. Many slightly ninhydrin positive spots were detected besides glucosamine and galactosamine, suggesting the presence of amino acids.

DISCUSSION

The antigens extracted with phenol-water from heated cells of *V. parahaemolyticus* were O-specific antigens of the organism, because the specificity of the precipitin reaction coincided well with that of O-cell agglutination. It is unknown why heating cells increased the yield of antigen. However, heating was a very satisfactory treatment because it increased the yield of O-antigen and facilitated removal of capsular antigens.

Minor cross reactions in precipitation of O-antigens were observed, which in some cases corresponded to the cross reaction in O-agglutination (cf. Miwatani et al., 1969). Further investigation is required to see whether O-antigens are associated with the cross reaction of agglutination.

Chemical analyses showed that these O-antigens were composed of hexoses, hexosamines, heptose, phosphorus, fatty acid ester and nitrogen compounds. The sum of the contents of these materials was less than 100 per cent of the total material. Estimation of total lipid instead of fatty acid ester may give a higher value for the sum, but we have no idea at present about the nature of the rest of the antigen. However, the O-antigen seemed to be lipopolysaccharide like that of *Enterobacteriaceae*.

The sugar constituents detected by paper-chromatography were quite simple. Thus, glucose, galactose and glucosamine, and in some cases also galactosamine, were the major components. If at least 10 specificities of O-antigen can be exhibited by such simple sugar constituents, then the specificities may be largely due to the variety of linkages and sequences of the components. Moreover, minor components which might be overlooked may play a role in the specificities. Chemical studies on antigenic determinants are in progress.

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