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IMMUNOCHEMICAL STUDIES ON O-ANTIGENS OF *VIBRIO PARAHAEMOLYTICUS*.[1]

2. EFFECT OF ALKALI AND ACID ON SPECIFICITY

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SUMMARY Ten purified O-antigens obtained from *Vibrio parahaemolyticus* pilot strains for O-agglutination were subjected to alkali or acid treatment.

On treatment with 0.2 N sodium hydroxide at 50 C all the O-antigens tested lost their original precipitin lines in agar and produced new lines which still had the respective O-group specificities. Treatment with 0.2 N hydrochloric acid at 50 C gave somewhat complicated results, and the antigens could be divided into four groups according to their behaviors on this treatment. Thus after treatment the first group of antigens had completely lost their ability to precipitate with homologous antisera. The second group gave new precipitin lines instead of the original lines. The third group provided new lines besides the originals. The fourth group was not significantly affected by this treatment.

Cross-reactions were observed among the acid-treated materials, suggesting the presence of a common or very similar antigenic structure.

Formic acid treatment of O3-antigen gave a serologically interesting fragment which inhibited O-cell agglutination by 7S fractions obtained from all the anti O3-sera tested but did not inhibit that by 19S fractions.

INTRODUCTION

In the previous paper it was reported that O-antigens were extracted with phenol-water from boiled cells of 32 strains of *Vibrio parahaemolyticus*, and the specificities of the O-antigens coincided well with those shown by O-agglutination. O-Antigens extracted from 10 pilot strains for O-agglutination were purified and analyzed. These antigens contained

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glucose, galactose, glucosamine, heptose, phosphorus, nitrogen compounds and fatty acid ester. In addition, galactosamine was found in half the 10 samples. Several unidentified materials were also detected by paper chromatography (Torii et al., 1969).

In this work an attempt was made to modify the O-antigens by acid or alkali treatment. In preliminary experiments O3-antigen was subjected to these treatments and antigenic activity was tested by Ouchterlony's agar diffusion technique. When the ability of the antigen to precipitate was lost, its inhibitory activity towards the O3-anti O3 system was examined.

Treatment with 1% acetic acid at 100 C for 60 min or 1 N hydrochloric acid at 100 C for 20 min destroyed the antigenic properties of O3-antigen almost completely. On heating with 0.1 N formic acid at 100 C for 30 min, O3-antigen lost its precipitating activity but inhibited the O3-anti O3 system. When treated with 0.2 N sodium hydroxide at 50 C for 24 hr, the original precipitin line disappeared and a new line appeared. Treatment of the antigen with 0.2 N hydrochloric acid at 50 C for 24 to 48 hr resulted in two lines in agar, one fusing with the original line and the other being new.

The results obtained from the latter two treatments of all the O-antigens of ten groups (O1 to O10) and from formic acid treatment of O3-antigen are reported in this paper.

MATERIALS AND METHODS

1. *O-Antigens*

Ten O-antigens were extracted from boiled cells of the 10 pilot strains (O1 to O10) by the phenol-water method and purified by the method of Westphal et al. (1952 and 1965) as described in the previous report (Torii et al., 1969).

2. *Antisera*

Anti O-sera were obtained by immunizing rabbits with boiled cells according to Burrows' method (Burrows et al., 1946 and cf. Miwatani et al., 1969) as described in the previous report (Torii et al., 1969).

3. *Serological test*

Serological specificity was mainly examined by Ouchterlony's double diffusion technique (Ouchterlony, 1949) with Bacto Special Agar Noble. The agglutionation reaction was performed as previously described (Torii et al., 1969; Torii and Igarashi, 1969). Quantitative inhibition of precipitation was performed in the usual way (Kabat, 1961). Agglutination inhibition was carried out as follows: 0.25 ml of sample in 3% sodium chloride solution was mixed with 0.25 ml of diluted serum fraction and incubated at 50 C for 1 hr. Then the mixture was combined with 0.5 ml of cell suspension, heated at 100 C for 1 hr (O. D. at 550 mμ: 0.3 in a Coleman Junior spectrophotometer with a 16 mm diameter cuvette) and kept at 50 C overnight. The concentration of serum fractions was of the highest dilution at which complete agglutination, with visible aggregates at the bottom of the tubes and a clear supernatant, was obtained without inhibitor.

4. *Fractionation of 7S and 19S antibodies of anti O3-sera*

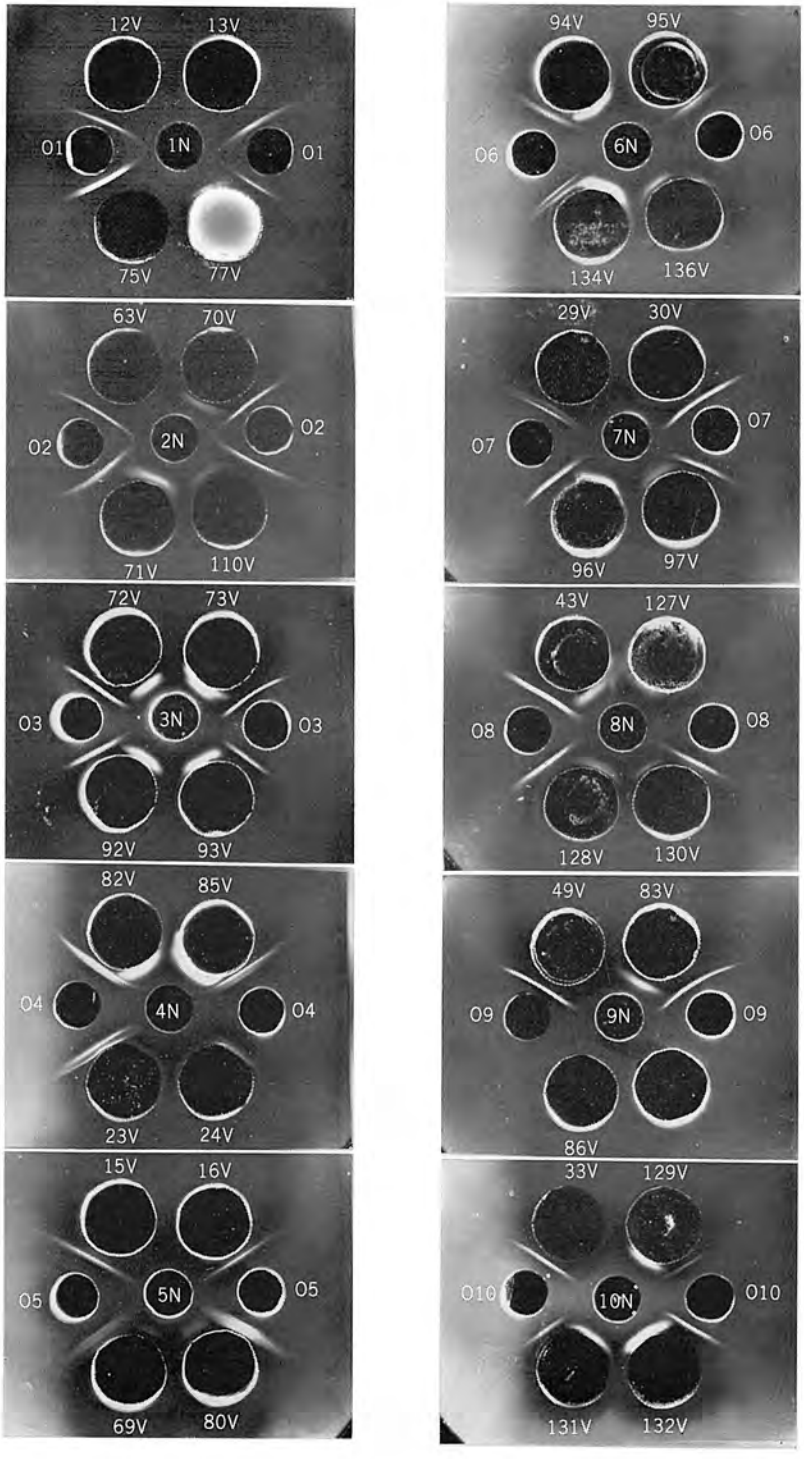
Anti O3-sera (72V, 144V and 157V) were fractionated as follows: sera were treated with DEAE-cellulose equilibriated with 0.01 M phosphate buffer, pH 8.0, according to the method of Stanworth (1960). The 7S fraction in the unadsorbed material was purified by Sephadex G-200 gel filtration (Flodin and Killander, 1962). This fraction was shown by immunoelectrophoresis to contain only γG globulin. Meterials adsorbed on DEAE-cellulose were eluted with 0.3 M phosphate buffer, pH 8.0, and subjected to Sephadex G-200 chromatography according to the method of Flodin and Killander (1962). The 19S fraction was purified by rechro-

FIGURE 1. *Effect of NaOH on antigenicity of O-antigens.*

12V, 13V, 75V, 77V: anti O1-sera, 63V, 70V, 71V, 110V: anti O2-sera, 72V, 37V, 92V, 93V: anti O3*-sera, 82V, 85V, 23V, 24V: anti O4-sera 15V, 16V, 69V, 80V: anti O5-sera, 94V, 95V, 134V, 136V: Anti O6-sera, 29V, 30V, 96V, 97V: anti O7-sera, 43V, 127V, 128V, 130V: anti O8-sera, 49V, 83V, 86V: anti O9-sera, 33V, 129V, 131V, 132V: anti O10-sera*

O1 to O10: O-antigens obtained from ten pilot strains (O1 to O10)

1N to 10N: O-antigens (O1 to O10) treated with 0.2N NaOH at 50 C for 24 hours


12V
13V
O1
1N
O1
75V
77V
94V
95V
O6
6N
O6
134V
136V
63V
70V
O2
2N
O2
71V
110V
29V
30V
O7
7N
O7
96V
97V
72V
73V
O3
3N
O3
92V
93V
43V
127V
O8
8N
O8
128V
130V
82V
85V
O4
4N
O4
23V
24V
49V
83V
O9
9N
O9
86V
15V
16V
O5
5N
O5
69V
80V
33V
129V
O10
10N
O10
131V
132V

matography. Immunoelectrophoresis showed that this fraction contained γM globulin and lipoprotein but not γG globulin. After dialysis against saline both fractions were adjusted to the initial volume of the serum.

RESULTS

1. *Sodium hydroxide-treatment of O-antigens of the ten groups (O1 to O10)*

An aliquot of each O-antigen solution (2 mg/ml of water) was mixed with a half volume of 0.6 N NaOH, incubated at 50 C for 24 hr and neutralized with 0.6 N HCl, and the mixture was tested for precipitation in agar with the homologous antisera. As shown in Fig. 1, each of the NaOH-treated materials gave a rather broad precipitin line closer to the antibody well than the original line.

To test the specificity of the newly produced precipitin line, the materials were examined with both heterologous and homologous antisera. Fig. 2 shows that each of the NaOH-treated materials had the original specificity of the O-groups, a precipitin line only being observed in each homologous system.

O3-Antigen was treated with hydroxylamine according to the method of McIntire et al. (1967) and compared with NaOH-treated O3-antigen. Precipitin lines in agar produced by the two materials fused completely, as seen in Fig. 4A.

2. *Hydrochloric acid-treatment of O-antigens of the ten groups (O1 to O10)*

An aliquot of each O-antigen solution (2 mg/ml of water) was mixed with a half volume of 0.6 N HCl, incubated at 50 C for 48 hr and neutralized with 0.6 N NaOH. Then its precipitation reactions were examined.

After treatment the ten antigens could be roughly divided into four groups on the basis of their precipitation reactions with homologous antisera. O-Antigens of the first groups (O4-, O5- and O8-antigens) had completely lost their ability to precipitate with homologous antisera (Fig. 3D, 3E and 3H). Those of the second group (O2- and O9-antigens) had lost the original precipitin lines but gave new lines (Fig. 3B and 3I). The third group contained O-antigens (O3-, O6- and O10-antigens) which gave new lines besides the original lines (Fig. 3C, 3F and 3J). The antigenicity of the fourth group (O1- and O7-antigens) in agar was not significantly affected by the treatment (Fig. 3A and 3G).

Hydrochloric acid-treated materials were next examined with heterologous antisera. One of the treated antigens cross-reacted with heterologous antisera with which the original antigens did not react (Fig. 4B and 4C). Thus, acid-treated O4-antigen cross-reacted giving a precipitin line with anti O6- and O10-sera and each line fused with those between acid-treated O6-antigen and anti O6-sera and between acidtreated O10 and anti O10-sera, respectively (Fig. 4D and 4E).

Furthermore, O6-antigen, which was subjected to HCl-treatment after periodate oxidation according to the method of Heath, cross-reacted with anti O2- and O4-sera, giving completely fused precipitin lines, as shown in Fig. 4F.

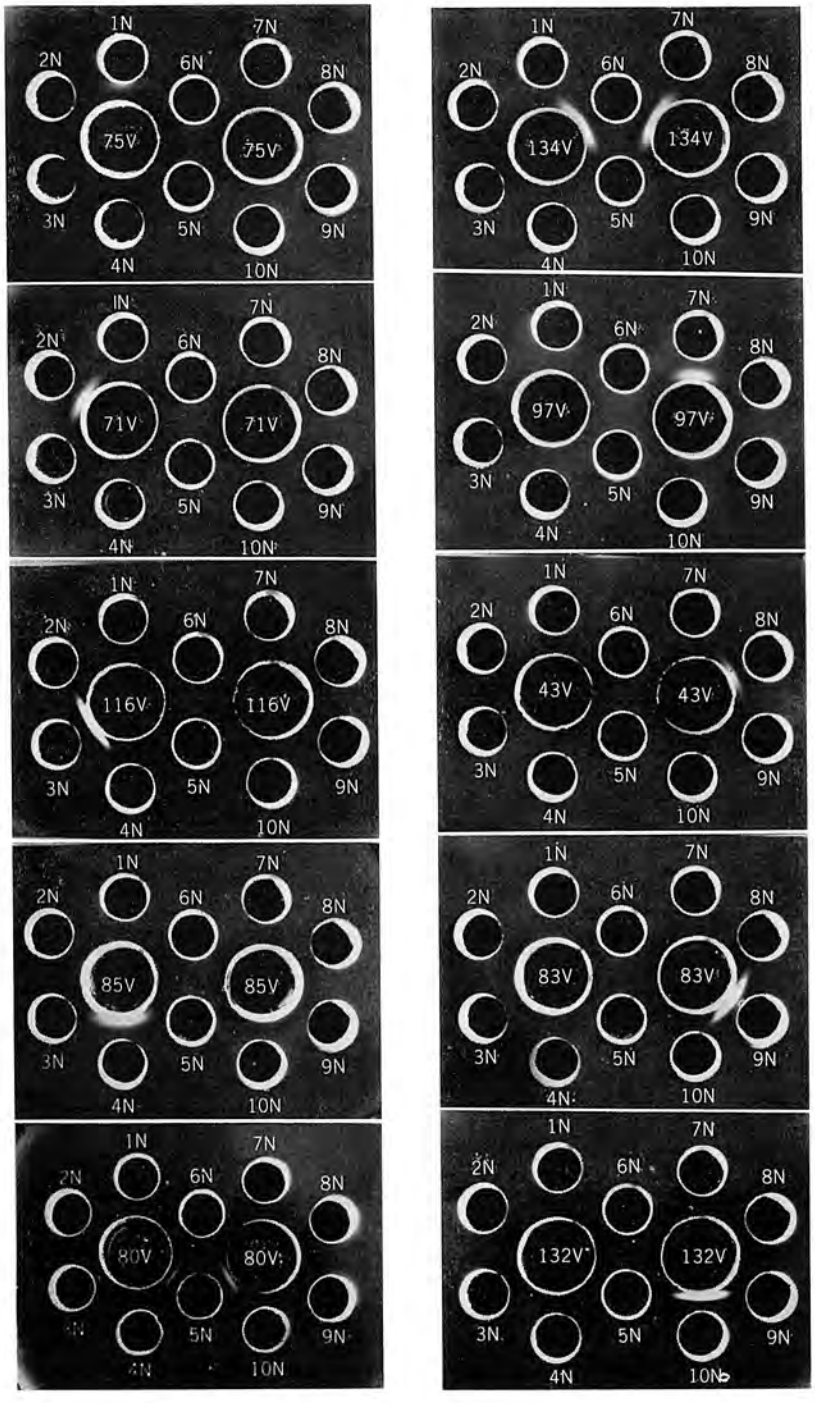
3. *Formic acid-treatment of O3-antigen*

Four hundred mg of O3-antigen were treated with 150 ml of 0.1 N formic acid at 100 C for 30 min. After cooling and several extractions with ether, the resulting highly turbid mixture

FIGURE 2. *O-Specificity of O-antigens treated with NaOH.*

75V: anti O1-serum, 71V: anti O2-serum, 116V: anti O3-serum, 85V: Anti O4-serum, 80V: anti O5-serum, 134V: anti O6-serum, 97V: anti O7-serum, 43V: anti O8-serum, 83V: anti O9-serum, 132V: anti O10-serum,

1N to 10N: O-antigens (O1 to O10) treated with 0.2N NaOH at 50 C for 24 hours

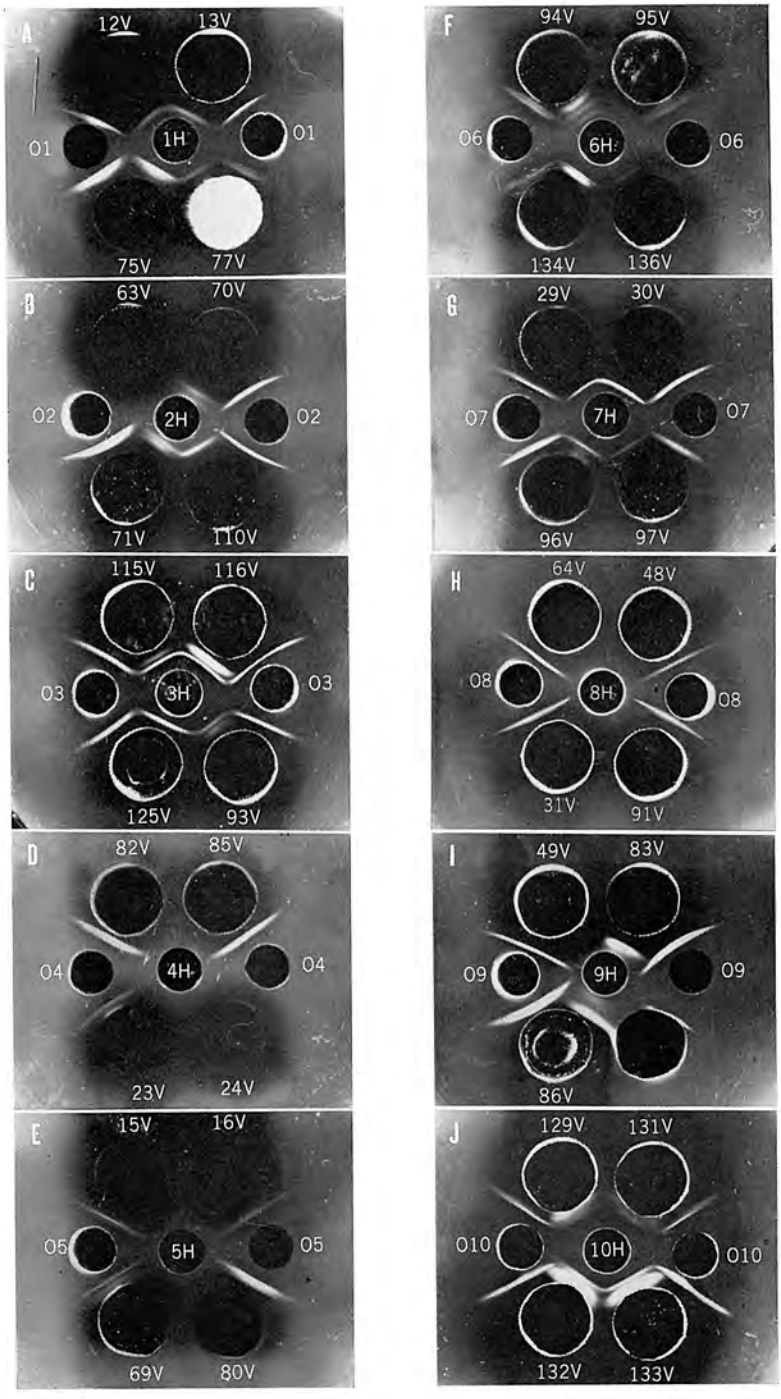

1N 7N 2N 6N 8N 75V 75V 3N 5N 9N 4N 10N
1N 7N 2N 6N 8N 134V 134V 3N 5N 9N 4N 10N
1N 7N 2N 6N 8N 71V 71V 3N 5N 9N 4N 10N
1N 7N 2N 6N 8N 97V 97V 3N 5N 9N 4N 10N
1N 7N 2N 6N 8N 116V 116V 3N 5N 9N 4N 10N
1N 7N 2N 6N 8N 43V 43V 3N 5N 9N 4N 10N
1N 7N 2N 6N 8N 85V 85V 3N 5N 9N 4N 10N
1N 7N 2N 6N 8N 83V 83V 3N 5N 9N 4N 10N
1N 7N 2N 6N 8N 80V 80V 5N 9N 4N 10N
1N 7N 2N 6N 8N 132V 132V 3N 5N 9N 4N 10N

was neutralized and dialyzed against distilled water. The dialysant was concentrated and retreated in the same way. The dialysates were combined, concentrated and chromatographed by a Bio-Gel P-2 column (Fig. 5). Fractions $3D_1$ and $3D_2$ were concentrated and lyophilized (yield: $3D_1$, 24 mg; $3D_2$, 178 mg). The dialysant was centrifuged at 26,000 $\times g$, and the clear supernatant was lyophilized (3R-sup, yield: 116 mg). The precipitate was treated with hydroxylamine by the method of McIntire et al. (1967), and the resulting material was dialyzed and lyophilized (3Rp, yield: 25 mg). None of these fractions precipitated with specific anti O3-sera but some inhibited the specific precipitation (Fig. 6) or agglutination of the O3-anti O3 system.

4. *Agglutination inhibition in the O3-anti O3 system*

Experiments were conducted to see how acid or alkali-treated O3-antigens behaved towards O-cell agglutination. The 7S and 19S fractions of three anti O3-sera were used as agglutinins. The results are shown in Table 1. $3D_1$ only inhibited the agglutination by the 7S fractions of all three anti-sera while $3D_2$ caused no detectable inhibition under the experimental conditions. Inhibition was mainly observed on agglutination by the 19S fraction of serum 144V but not by 7S except with $3D_1$ and 3Rsup. The agglutinations by 7S and by 19S of antisera 72V and 157V were inhibited by 3Rp, $3H_{1,2}$ and $3H_3$.

FIGURE 3. *Effect of HCl on O-antigens.*
12V, 13V, 75V, 77V: anti O1-sera, 63V, 70V, 71V, 110V: anti O2-sera, 115V, 116V, 125V, 93V: anti O3-sera, 82V, 85V, 23V, 24V: anti O4-sera, 15V, 16V, 69V, 80V: anti O5-sera, 94V, 95V, 134V, 136V: anti O6-sera, 29V, 30V, 96V, 97V: anti O7-sera, 64V, 48V, 31V, 91V: anti O8-sera 49V, 83V, 86V: anti O8-sera, 129V, 131V, 132V, 133V: anti O10-sera
O1 to O10: O-antigens obtained from ten pilot strains (O1 to O10)
1H to 10H: O-antigens (O1 to O10) treated with 0.2N HCl at 50 C for 48 hours


A
12V
13V
O1
1H
O1
75V
77V
B
63V
70V
O2
2H
O2
71V
110V
C
115V
116V
O3
3H
O3
125V
93V
D
82V
85V
O4
4H
O4
23V
24V
E
15V
16V
O5
5H
O5
69V
80V
F
94V
95V
O6
6H
O6
134V
136V
G
29V
30V
O7
7H
O7
96V
97V
H
64V
48V
O8
8H
O8
31V
91V
I
49V
83V
O9
9H
O9
86V
J
129V
131V
O10
10H
O10
132V
133V

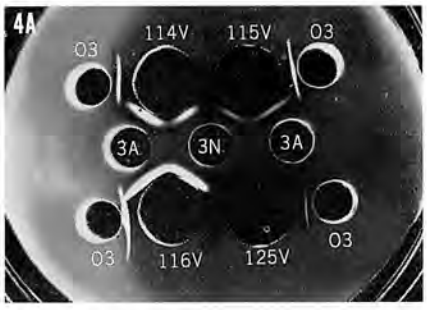
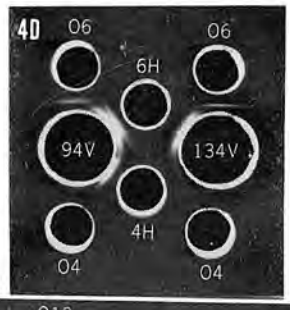


FIGURE 4A. *Fused precipitin lines between 3N and 3A.*
O3: O3-antigen
3N: O3-antigen treated with 0.2N NaOH at 50 C *for 24 hours*
3A : O3-antigen treated with hydroxylamine
114V, 115V, 116V and 125V : anti O3-sera



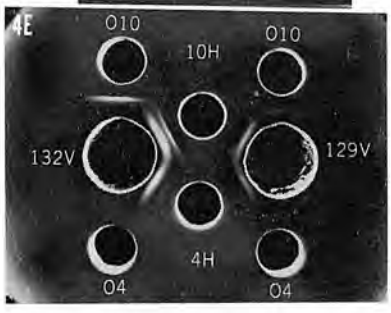


FIGURE 4D and 4E. *Fused precipitin lines between 4H and 6H or 10H.*
O4, O6 and O10 : O4-, O6-, and O10- antigens
4H, 6H, and 10H : O4-, O6-, and O10-antigens treated with 0.2N HCl at 50 C for 48 hours
94V, 134V : anti O6-sera
129V, 132V : anti O10-sera

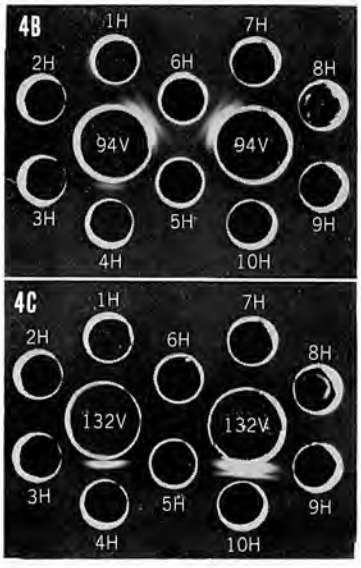


FIGURE 4B. and 4C. *Cross-reaction between 4H and anti O6- or O10-sera.*
1H to 10H : O-antigens (O1 to O10) traeted with 0.2 N HCl at 50 C *for 48 hours*
94V : anti O6-serum
132V : anti O10-serum

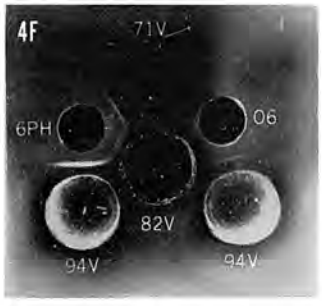


FIGURE 4F. *Fused precipitin lines between 6 PH and anti O2-, O4- or O6-serum.*
O6 : O6-antigen
6PH : O6-antigen treated with periodate and then HCl.
71V : anti O2-serum
32V : anti O4-serum
94V : anti O6-serum

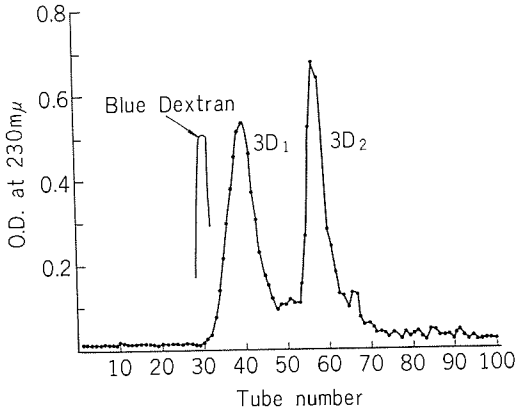


FIGURE 5. *Bio-Gel P-2 gel filtration of the dialysate of formic acid-treated O3-antigen.*

Dialysate was applied to Bio-Gel P-2 (2×80 cm) and eluted with water in a cold room. Fractions of 5 ml were collected every 20 minutes.

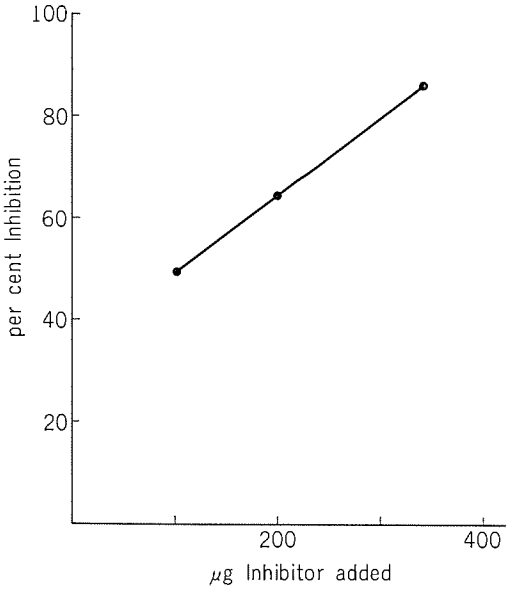


FIGURE 6. *Inhibition by $3D_1$ of O3-anti O3 precipitation.*

100μl of anti O3-serum 157V and 6 μg of O3-antigen were used.

DISCUSSION

In recent years numerous studies on O-specific lipopolysaccharides of *Enterobacteriaceae* have been reported (cf. Landy and Braun, 1964; Nowotny, 1966; Lüderitz et al., 1966; Sharon, 1966). O-Antigenic polysaccharides have been shown to consist of O-specific side chains and a basal core, a common structural entity of polysaccharides from both rough and smooth strains. It has also been shown that when an O-antigen contains more than one O-factor, individual O-factors are not only located on the same molecule but also share part of their determinants (Lüderitz et al., 1966).

It will be of interest to examine whether this is also so in the O-antigen of *Vibrio parahaemolyticus*. On alkali- or acid-treatment of the antigens new precipitin lines appeared suggesting that the determinant groups, at least those involved in the specificities tested, may be located in different parts of the antigen molecule.

The appearance of a new antigenic group after treatment with alkali is probably due to removal of O-acyl groups, because alkali-treated O3-antigen fused completely with O3-antigen subjected to hydroxylamine-treatment which would mainly split O-acyl linkages (Verheyden and Nys, 1962). However, it is not clear whether the O-acyl group itself was responsible for the O-specificities or whether removal of the O-acyl group unmasked antigenic determinants by changing the configuration of the antigen molecule.

The results obtained after hydrochloric acid-treatment were not so simple. But it is of interest that precipitin lines common to several antigens were observed after acid-treatments. This suggests that the antigens may have common, or very similar structures. But it is uncertain at present whether these common or similar structures correspond to the basal core

TABLE 1 *Agglutination inhibition of O3 boiled cells*

Inhibitors \ Antisera		144V		72V		157V	
		7S	19S	7S	19S	7S	19S
	μg						
$3D_1$	125	−	−	+	−	+	−
	250	+	−	++	−	+	−
$3D_2$	250		−	−	−	−	−
	500	−	−	−	−	−	−
$3R_p$	125	−	+	+	+	+	+
	250	−	+	+	+	+	+
$3R_{sup}$	125	−	+		−	+	+
	250	+	+	+	−	+	+
3H	125	−	+				
	250	−	++				
3N	125	−	+				
	250	−	++				
$3H_{1,2}$	125	−	++	+	+	+	+
	250	−	++	+	+	+	+
$3H_3$	125	−	++	−	+	+	+
	250	−	++	+	+	+	+

++ : *No agglutination.*
+ : *Inhibition intermediate between ++ and −.*
− : *Complete agglutination with visible aggregates in the bottom of tubes and clear supernatant.*
3H and 3N: O3-antigen treated with 0.2N HCl at 50 C for 48 hours and with 0.2N NaOH at 50 C for 24 hours, respectively.
$3H_{1,2}$ and $3H_3$ are fractions obtained by chromatography of 3H on Sephadex G-200 (Torii and Igarashi, 1968).

of lipopolysaccharides in *Enterobacteriaceae.*

The results of the agglutination inhibition experiments indicate two important points. One is the difference in specificity between the 7S and 19S fractions of the anti O3-serum, 144V. The 7S and 19S antibodies in this antiserum seemed to be directed to determinant groups contained in $3D_1$ (or 3Rsup) and in others, respectively. This clear difference was not observed in sera 72V and 157V.

The other important point is that the $3D_1$ fragment could only inhibit agglutination by the 7S fractions of all three antisera tested. This suggests that antibody directed to $3D_1$ belonged to the 7S class of antibodies. On O-antigenic stimulation agglutinin produced in the early stage was mainly found in the 19S fraction, and the quite high agglutinin titer of the 19S fraction was maintained on repeated O-stimulation (Pike and Schulze, 1964). So, further investigation will be necessary to ascertain a possibility that only 7S antibody is directed to a particular antigenic determinant on immunological stimulations of an O-antigen.

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