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# THREE TYPES OF *CORYNEBACTERIUM RENALE* CLASSIFIED BY PRECIPITIN REACTIONS IN GELS

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## INTRODUCTION

There have been few reports dealing with the typing of *Corynebacterium renale* (HIRATO, 1933; OCHI & ZAIZEN, 1940; KUME et al., 1956). They investigated biological and biochemical properties of the organisms and reported the existence of two or three types within this species. Serological investigation of this species was performed by MERCHANT (1935), FEENSTRA et al. (1945, 1948) and LOVELL (1946); none of them gave sufficient information (CRUETHLEY et al., 1961).

Despite the sensitiveness of the microorganism to antibiotics such as penicillin, pyelonephritis due to *C. renale* is ineradicable. The presence of the microorganism in the urine of apparently healthy cattle has been reported (MERCHANT & PACKER, 1961; HIRAMUNE et al., 1967). In addition, the mechanism of the pathogenesis of this classical disease is still obscure. We believe that detailed information of the microorganism is necessary to bring light to these problems.

Efforts were made by the authors to produce soluble antigens from the *C. renale* organisms, to show the morphological aspects of extracted bacteria and solubilized antigen, also to use the antigens for classification of *C. renale* by precipitin reactions in gels. Biochemical and cultural behaviors were compared with serological properties of the strains of *C. renale*. Three serologically different types in *C. renale* deserve special emphasis.

## MATERIALS AND METHODS

**Bacterial strains** Fifty-one strains of *C. renale* were primarily used. Later, an additional 27 strains were also employed, mostly for serological typing. Among the total 78 strains, 62 were kindly provided by the Hokkaido Branch, National Institute of Animal Health, Sapporo. These strains were isolated from cattle with pyelonephritis and from the urine or urinary tract of apparently healthy cattle. The remaining 16 strains, which have been maintained in this department, were isolated from bovine pyelonephritis materials.

**Antigen preparation** Strain No. 9, isolated from a case of pyelonephritis, was used

for the preliminary work of antigen extraction. This strain was cultivated in flasks containing 50 ml nutrient broth. After 2 days incubation at 37°C, the bacterial cells were harvested from each flask by centrifugation. The deposit cells were washed once with sterile saline solution and resuspended in 1 ml of saline in most cases. Antigenic fractions were extracted from the washed deposit by the following agents: Ethanol (final 50%, 4°C overnight), n-butanol (final 50%, shaken for 10 min and allowed to stand overnight at room temperature, aqueous phase used), ethyl ether (excess amount, shaken for 10 min and kept overnight at room temperature, aqueous phase used), phenol (final 50%, 65°C for 20 min, aqueous phase used), sodium deoxycholate (Difco, final 1% in aq. dest., 45°C for 4 hr), hot trichloroacetic acid (TCA) (added 0.25 ml 25% or 5% TCA, 90°C for 30 min), cold TCA (added 0.25 ml 25% or 5% cold TCA, 4°C for 10 min), and hydrochloric acid (HCl) (cell deposit was suspended in 1 ml N/20 HCl, boiled for 15 min, neutralized).

In preparing the antigen for typing, each strain of *C. renale* was cultivated on agar medium in petri dishes. Two agar-plates were used instead of 50 ml nutrient broth. Harvested cells were extracted with 0.5 ml 1% sodium deoxycholate.

**Antisera** Rabbits were used for the preparation of antisera. Each inoculation was performed intravenously with 4 ml of bacterial suspension. Initially they were injected twice, at an interval of 4 days, with a formalin killed suspension of *C. renale*, made as follows: One tube of slant agar culture was incubated at 37°C for 2 days. The cells from the slant agar culture were collected and suspended in 5 ml of 0.02% formol saline. They were then placed in the incubator at 37°C for 2 days, followed by one night in the refrigerator before use. Then, a suspension of live bacteria was injected at constant intervals of one week, increasing, respectively, the amount of cells from one to three tubes of slant agar. Usually, 7~8 injections were required to produce serum satisfactory for precipitin reactions.

Strains Nos. 9 and 45 were chosen as the first strains to make the antisera. Both were chosen due to the comparably good growth and the different character they acquired when suspended in liquid. Strain No. 9 readily dissolved in suspension, however No. 45, tended to aggregate in a mass.

By observation of the preliminary precipitin test, strains Nos. 33, 10, 14, 17, 42 and 48 were also selected to immunize rabbits.

**Precipitin reaction** The microgel diffusion methods as reported by MANSI (1958) and MURTY & HANSON (1961) were used with a slight modification, described in Results. After filling wells with antigens and antisera the gels were kept at room temperature.

**Isolation of bacterial cell walls** Shaking the bacterial cells with glass beads (0.1 mm in diameter) by Homogenon (Ohtake) was the method used to isolate bacterial cell walls. After shaking for 20 min, the specimen was centrifuged in a sucrose density gradient. The purity of the cell walls was examined by electron microscopy.

**Sectioning and electron microscopy** Residual cells after washing and further treatment were fixed overnight at 4°C in 1% osmium tetroxide in phosphate buffer, pH 7.2. Fixed bacteria, sedimented by centrifugation and washed twice with distilled water, were mounted on 'Formvar' grids, shadowed with palladium or hardened in 2% agar, dehydrated, embedded in Epon and sectioned with JUM 5 A type ultramicrotome (Japan Electron Optics Laboratory Co.) using glass knives. Sections were stained with uranyl acetate and lead

acetate, and examined in a JEM 7 electron microscope.

## RESULTS

### 1 Preparation of soluble antigens by extraction with various agents and comparison of their antigenicity

The antigens extracted from the culture of strain No. 9 were tested against the homologous immune rabbit serum. The results are shown in figure 1.

Sodium deoxycholate (SDC) produced an antigenic fraction which gave 4 or more lines. Treatment with HCl produced a fraction which formed 3 lines. Two lines appeared in the reactions with the ethanol and n-butanol extracts. The phenol and ether extracts showed a positive reaction, but the line of precipitation was faint. The TCA-hot and TCA-cold extracts showed no reaction at all for the precipitin test.

Thus, the treatment with SDC and HCl produced antigens giving stronger and sharper lines than treatment with other chemicals. According to the greater number of lines in the SDC extract, to which the 3 lines of the HCl extract were connected and fused, SDC was considered to be the most effective method of antigen extraction.

No reaction was observed with the supernatant of the broth culture.

Preliminary work on the factors influencing the precipitin reaction showed that gels made with distilled water with 3 mm distance between wells, and kept at room temperature were the most favorable conditions.

### 2 Localization of the antigen, morphology of antigen particles and morphology of the residual cells

Cell wall fraction was isolated from strain No. 9, and antigen extraction was performed with the cell wall fraction. As shown in figure 2, SDC extract of the cell wall showed similar antigenicity to that of the whole cell. Therefore, it is said that the antigen extractable by SDC is derived from the cell wall fraction.

Figure 3 shows the electron micrograph of the antigens extracted with SDC and then dialysed in distilled water. Many small particles were found, which were not uniform in diameter.

To prove whether or not these particles were antigenic, a filtration test was carried out using 450, 100 and 10 m $\mu$  filter pads (Millipore). The antigenicity of each filtrate was tested by precipitin reaction in gels. The result is shown in figure 4.

Antigens filtered through 10 m $\mu$  pads did not show any antigenicity. Antigenicity was demonstrated in the filtrate when 450 and 100 m $\mu$  pads were used. This finding indicated that the antigen particles were larger than 10 m $\mu$  in diameter. The diameter of the antigen was thus comparable to that of the particles shown in figure 3.

Figure 5 shows the intact cell of strain No. 9, and figures 6 and 7 show the cell wall, and the cell wall treated with SDC of the homologous strain. Figure 8 shows the cell wall fraction isolated from the residual cells of strain No. 8 treated with SDC. From these photographs, it seems likely that cell walls of *C. renale* have a multi-layered appearance. Perhaps SDC could solubilize the upper layer, which possesses an antigenic substance.

Thin sections of *C. renale* showed that SDC treatment for 1 hr would remove intracyto-

plasmic materials almost entirely (figs. 9 & 11). Antigenic substances could be removed not only by treatment for 1 and 4 hr but also by treatment for 15 min. Removal of the antigenic substances from the cell walls was, however, not clear in the photographs of the sections (figs. 10 & 11).

TABLE 1 *Immunodiffusion of antisera to 8 strains of C. renale with the antigens extracted with deoxycholate from 78 strains*

ANTIGENS OF STRAINS NOS.	ORIGINAL NAME OF STRAIN	ANTISERA AGAINST STRAINS NOS.							
		9	10	33	14	17	45	42	48
1	65-122	2*1	4	2	0	0	1	0	0
2	29	5	4	3	1	0	0	0	1
3	56-1	4	4	3	1	0	0	0	0
4	43-7	5	4	2	0	0	0	0	0
5	43-5	3	3	2	0	0	0	0	0
6	38-2	3	4	2	0	0	0	0	0
7	38-1	4	3	3	0	0	0	0	0
8	34-1	3	5	3	1	0	0	0	0
9	111-1	3	4	4	0	0	0	0	0
10	65-123	1	3	2	0	0	0	0	0
11	29-8	3	3	4	1	0	0	0	0
12	66-1	3	2	4	1	0	0	0	0
15	1369	4	3	4	0	0	0	0	0
16	1169	3	3	2	0	0	0	0	0
21	1371	4	4	3	0	0	0	0	0
24	1364	4	2	3	1	0	0	0	0
25	1211	3	4	3	1	0	0	0	0
27	1363	3	3	3	1	0	0	0	0
28	1103	3	4	4	0	0	0	0	0
29	1157	4	5	3	0	0	0	0	0
30	1217	3	5	3	(1)*2	0	0	0	0
31	1221	4	4	4	0	0	1	0	0
32	1152	3	4	4	(1)	0	0	0	0
33	1155	3	4	3	0	0	0	0	0
34	1162	3	5	4	(1)	0	0	0	0
38	1266	4	4	3	0	0	0	0	0
49	XVB	3	3	3	1	0	0	0	0
50	Kameda	3	4	3	0	0	0	0	0
57	1599	3	3	3	0	0	0	0	0
65	1622	3	3	3	0	0	0	0	0
66	1626	3	4	3	0	0	0	0	0
67	1641	3	3	3	0	0	0	0	0
68	1642	3	4	3	0	0	0	0	0
69	1645	4	4	3	0	0	0	0	0
70	1646	3	4	3	0	0	0	0	0
71	1649	3	3	3	0	0	0	0	0
72	1650	3	3	3	0	0	0	0	0
73	1655	3	3	3	0	0	0	0	0
74	1658	2	4	2	0	0	0	0	0
76	1661	3	4	3	0	0	0	0	0
79	568	3	3	3	0	0	0	0	0
106	D-487	3	3	2	0	0	0	0	0

TABLE 1 (concluded) Immunodiffusion of antisera to 8 strains of *C. renale* with the antigens extracted with deoxycholate from 78 strains

ANTIGENS OF STRAINS NOS.	ORIGINAL NAME OF STRAINS	ANTISERA AGAINST STRAINS NOS.							
		9	10	33	14	17	45	42	48
13	38-3	0	0	0	<b>2</b>	<b>1</b>	<b>3</b>	0	1
14	1361	0	0	0	<b>2</b>	<b>1</b>	<b>3</b>	0	0
17	1309	0	0	0	<b>3</b>	<b>2</b>	<b>3</b>	0	(1)
18	1366	0	0	0	<b>3</b>	<b>2</b>	<b>2</b>	0	1
19	1174	0	0	0	<b>3</b>	<b>1</b>	<b>2</b>	0	0
20	1362	0	0	0	<b>2</b>	<b>2</b>	<b>4</b>	0	(1)
22	1205	0	0	0	<b>2</b>	<b>2</b>	<b>3</b>	0	0
23	1373	0	0	0	<b>1</b>	<b>2</b>	<b>4</b>	0	0
26	1218	(1)	0	0	<b>1</b>	<b>1</b>	<b>3</b>	0	0
35	1365	0	1	0	<b>2</b>	<b>2</b>	<b>2</b>	0	0
36	1367	0	1	0	<b>2</b>	<b>2</b>	<b>2</b>	0	0
37	1368	0	0	0	<b>6</b>	<b>6</b>	<b>2</b>	0	(1)
39	39	0	0	0	<b>3</b>	0	<b>4</b>	0	0
40	40	0	0	0	<b>2</b>	<b>1</b>	<b>2</b>	0	0
41	41	0	0	0	<b>2</b>	<b>1</b>	<b>3</b>	0	0
45	Kiku	0	0	0	<b>1</b>	<b>1</b>	<b>3</b>	0	1
46	Hara	0	(1)	0	<b>2</b>	<b>3</b>	<b>3</b>	0	1
52	52	0	0	0	<b>2</b>	<b>2</b>	<b>1</b>	0	0
58	1601	(1)	0	0	<b>2</b>	<b>2</b>	<b>2</b>	0	0
59	1607	0	0	0	<b>2</b>	<b>3</b>	<b>2</b>	0	0
60	1608	0	(1)	(1)	<b>3</b>	<b>2</b>	<b>2</b>	0	0
61	1609	(1)	0	0	<b>2</b>	<b>2</b>	<b>2</b>	0	0
62	1610	0	0	0	<b>1</b>	<b>1</b>	(1)	0	0
63	1611	0	0	0	<b>1</b>	<b>2</b>	<b>2</b>	0	(1)
64	1614	0	0	0	<b>1</b>	<b>1</b>	<b>3</b>	0	0
75	1695	0	0	0	<b>2</b>	<b>2</b>	<b>3</b>	1	1
42	Fukuya	0	0	0	0	0	0	<b>3</b>	<b>3</b>
43	Miyake	0	0	0	(1)	(1)	1	<b>2</b>	<b>2</b>
44	Nagahama	0	0	0	1	(1)	1	<b>2</b>	<b>2</b>
47	Tojo	0	0	0	1	0	2	<b>3</b>	<b>3</b>
48	Takahashi	0	1	0	1	1	1	<b>4</b>	<b>4</b>
51	51	0	0	0	0	0	0	<b>3</b>	<b>3</b>
77	Hayakita-1	0	0	0	1	1	2	<b>5</b>	<b>4</b>
78	Hayakita-2	0	0	0	1	(1)	2	<b>4</b>	<b>4</b>
103	Iwamisawa	0	0	0	1	1	1	<b>3</b>	<b>3</b>
104	Asahikawa	0	0	0	0	0	0	<b>3</b>	<b>4</b>

\*1 Number of precipitin lines, boldface indicates the reactions including strong and sharp lines.

\*2 A very faint line

### 3 Classification of *C. renale* by gel diffusion precipitin test

A total 8 immune rabbit sera were prepared which were immunized against strains Nos. 9, 10, 33, 14, 17, 45, 42 and 48. Antigens extracted with SDC were prepared from 78 strains and were tested by precipitin reaction in gels with these 8 immune sera. The results are summarized in table 1, in which it is found that the strains of *C. renale* could be divided into three main groups. Types I, II and III were used to designate the serological groups,

whose representatives were thought tentatively to be strains Nos. 9, 45 and 42 respectively.

Reactions between antisera and antigen of strains belonging to the same type gave distinct lines while between those belonging to different types, usually no line was observed.

It should be mentioned that the strains belonging type III showed a reaction to anti-type II serum, this was concluded, by the appearance of one or two lines (tab. 1). Other intertype reactions were also found but all of them were very faint and were undoubtedly secondary in nature.

Figures 12~16 are to show the immunodiffusion, homologous (or intratype) and heterologous (or inter-type) reactions. Homologous or intratype reaction usually gave 2 or more distinct lines. Inter-type reaction shown by the antigens of the strains of type III to the antisera to the strains belonging type II was usually characterized by the appearance of 1~2 faint lines. One example is shown in figure 16 which shows the reaction between the antigens of strain No. 44 (type III) and the antiserum of strain No. 45 (type II).

Of the 78 strains examined, 42 belonged to type I, 26 to type II and 10 to type III. Thus type I strains were predominant in number. In order of decreasing frequency of type, they were type I (54%), type II (33%) and type III (13%).

All the strains of type III were from pyelonephritis material, while type I and type II, only 36% (15/42) and 23% (6/26), respectively, were from material of the disease. Accordingly, many strains of types I and II, but none of type III, were from apparently healthy cattle.

#### 4 Relation of the serological typing to the biochemical and cultural behavior

The following tests were performed with 51 strains. Sugar fermentation (glucose, mannose, fructose, maltose, xylose, glycogen, dextrin and trehalose were used), urea splitting, indol and H<sub>2</sub>S production, V.P., M.R., nitrate reduction, gelatin liquefaction, hemolysis of sheep red cells, litmus milk test, growth on 0.3% oleic acid agar, growth on blood tellurite agar and halo formation (proteolytic activity) on 10% milk agar.

From the results obtained the following distinct relationships were found between the biochemical characters and the serological type.

- 1) Xylose was fermented by the strains of type III, but not by strains of other types.
- 2) Nitrate was reduced only by strains of type II.
- 3) Strong alkali was produced in litmus milk by strains of type III.

In addition, some cultural behavior characteristics showed a tendency to relate to the serological type. They were as follows.

- 4) Growth on oleic acid agar plate The strains showing favorable, unfavorable and no growth respectively, were those belonging to types II, I and III.
- 5) Growth on milk agar plate Many strains of type I formed haloes around colonies on the milk agar. The strains of the other types showed no haloes, except 2 of type II, which formed haloes.
- 6) Growth on blood tellurite agar plate The results of this test manifested a limited relationship to the serological classification, in that the size and the features of colonies had a distinguishable character for each type. Colonies of the strains of type III were small, uniform, grey in color; those of type I were either medium size, large, or small and not uniform; those of type II were large and black in color.

- 7) Viscosity Viscosity of the sediment of broth culture and the viscosity of colonies were characteristic of many strains of type II.

#### DISCUSSION

Typing of 78 strains of *C. renale* by precipitin reactions in gels was performed using the antigen extracted with sodium deoxycholate. The authors came to the conclusion that they were divided into 3 types. In addition, some biochemical and cultural behaviors such as xylose fermentation, nitrate reduction and strong alkali production in litmus milk were found to accord with the serological type. There were other behavior characteristics which exhibited a similar tendency. Further studies on the nutritional requirements and morphology of *C. renale*, which have been reported elsewhere (HIRAI & YANAGAWA, 1967; YANAGAWA et al., 1966), also give additional evidence for the adequacy of the typing.

Of the three preceding reports dealing with the classification of *C. renale*, KUME et al. (1956), pointed out that nitrate reduction was characteristic of one of their types. This finding is in agreement with the authors report in that nitrate is reduced by strains belonging to type II. The type B strains of HIRATO (1933) show a similarity to those of type II of this report in that in broth they formed a slimy sediment. Generally, however, an appropriate comparison with preceding reports is impossible because no typing has been done on the basis of serological properties.

There is a possibility that each type could be further divided into subgroups. This might, especially, be true when a careful analysis is made on the overlapping inter-type reaction.

Among the 78 strains tested, type I was predominant in number. From a pyelonephritis origin stand point, so far as the information is available, the strains are in the following order: type III (100%), type I (36%), and type II (23%). Since the extent of collecting the strains were rather limited, only in Hokkaido, the frequency of the types and the proportion of pyelonephritis origin in each type might be changed when a rich accumulation of strains is made in other areas. From the above data, however, we presume that type III, lowest in frequency, is most intimately associated with the disease, and that type I, highest in frequency, and type II are rather widely distributed among healthy cattle and could cause the disease spontaneously in this order.

While conducting this experiment the authors noticed that some strains possessed very many fimbriae, which have been reported elsewhere (YANAGAWA et al., 1966). Antigenicity of the fimbriae, particularly its role in the typing, will be the subject of future study.

The type specific antigen was easily solubilized by sodium deoxycholate. The

localization of the soluble antigen in the cell wall was confirmed by removing the antigen from the isolated cell wall. However, the localization within the cell wall was not clarified morphologically.

It would be of interest to know how the antigens of each type are chemically different. Studies on the chemical nature of the type specific antigens are in progress.

#### SUMMARY

Serologically antigenic fractions were extracted from the cells of *Corynebacterium renale* by various agents. Treatment with sodium deoxycholate was found most satisfactory because it produced fractions containing antigens which formed several lines including all those found in HCl, ethanol, n-butanol, phenol and ether extracts.

The antigens possessed a particle size of more than 10 m $\mu$  after being solubilized by deoxycholate and dialyzed. Antigenicity was similar between the antigens produced from the whole cell and the isolated cell wall fraction. Ultra-thin sections of the cells treated with deoxycholate were shown.

Typing of 78 strains of *Corynebacterium renale* by precipitin reaction in gels was performed using the antigen extracted with deoxycholate. The authors came to the conclusion that they were divided into 3 types. Some biochemical and cultural behavior characteristics were found helpful in explaining the differences between the types. Type I, predominant in number, and type II included strains originating from both normal urine and the material of pyelonephritis. Type III, least in number, was composed of all the isolates from the affected cattle.

#### ACKNOWLEDGEMENT

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## EXPLANATION OF PLATES

### PLATE I

- Fig. 1 Immunodiffusion of rabbit antiserum to *C. renale* No. 9 (center well) with the following extracts of homologous organisms: deoxycholate (a), HCl (b), n-butanol (c), phenol (d), ethanol (e) and ether (f)
- Fig. 2 Immunodiffusion of rabbit antiserum to *C. renale* No. 9 (center well) with the following antigens of homologous organisms: deoxycholate extract from the whole cell (a), deoxycholate extract from isolated cell wall fraction (b) and residue of extracted cell wall (c)
- Fig. 3 Antigen extracted with deoxycholate and dialyzed  
×30,000
- Fig. 4 Immunodiffusion of rabbit antiserum to *C. renale* No. 9 (center well) with the dialyzed deoxycholate antigens filtered through pads of the following pore size: 450 m $\mu$  (a), 100 m $\mu$  (b) and 10 m $\mu$  (c). No filtration control is (d) and original non-dialyzed antigen is (e).

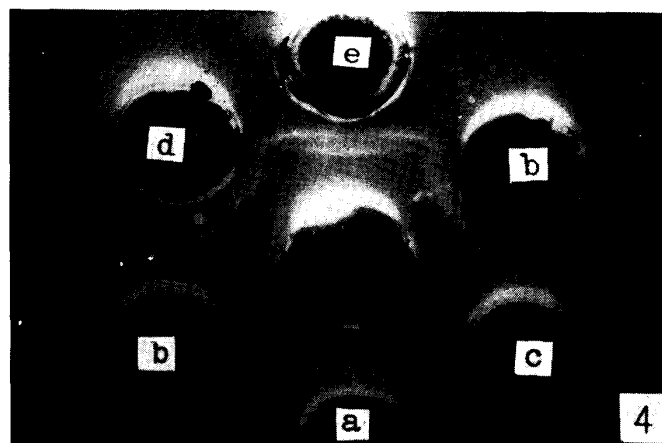
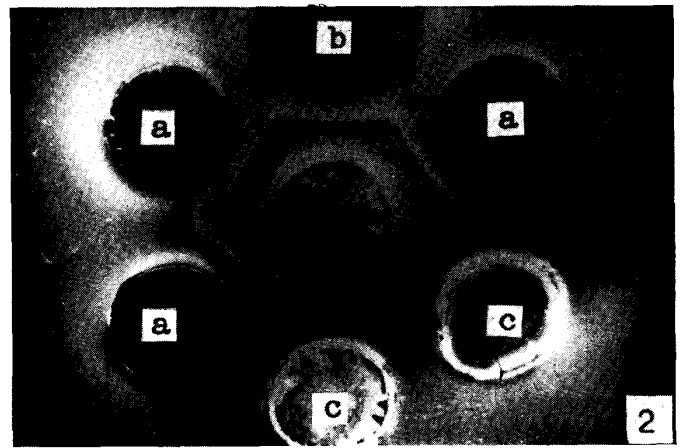
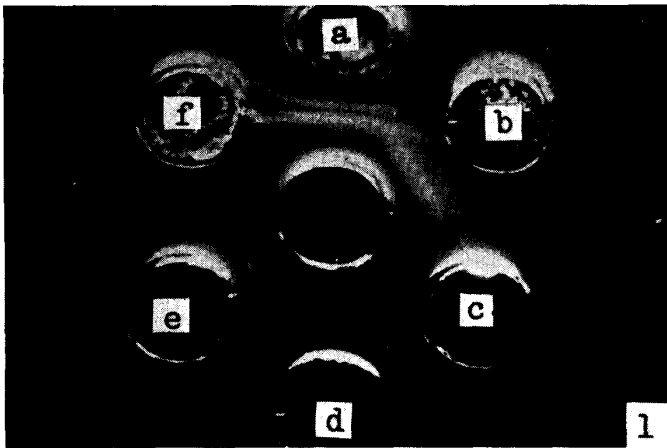


PLATE II

Fig. 5 Whole cell of *C. renale* No. 9  
×30,000

Fig. 6 Cell wall of *C. renale* No. 9  
×24,000

Fig. 7 Cell wall treated with deoxycholate of *C. renale* No. 9  
×25,000

Fig. 8 Cell wall isolated from *C. renale* No. 8 which was treated with  
deoxycholate beforehand  
×30,000

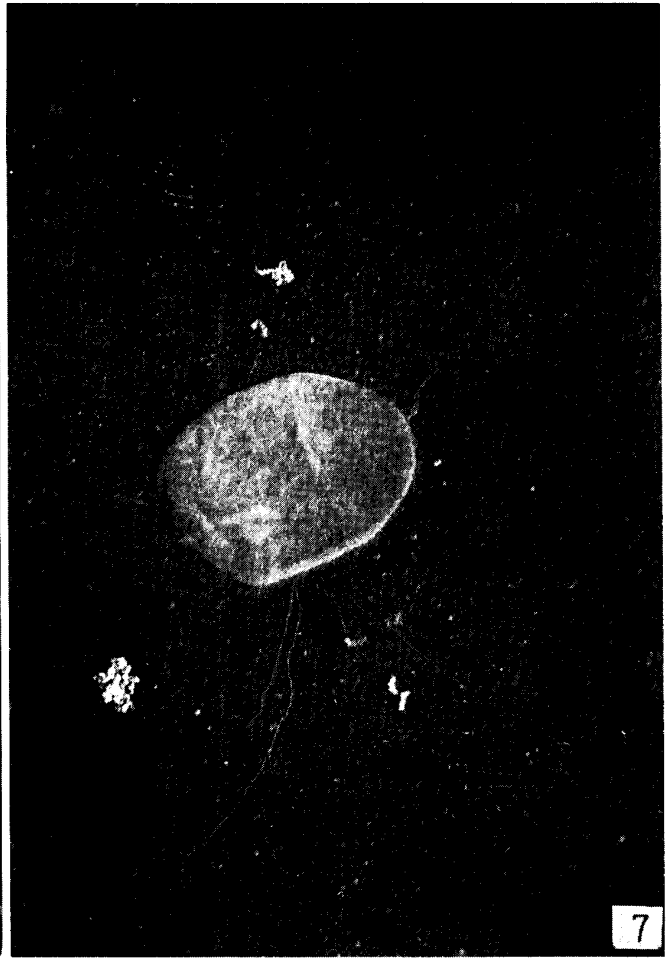


PLATE III

- Fig. 9 Ultrathin section of the cells of *C. renale* No. 45  
×69,000
- Fig. 10 Ultrathin section of the cells of *C. renale* No. 45 treated with deoxy-  
cholate for 15 min  
×72,000
- Fig. 11 Ultrathin section of the cells of *C. renale* No. 45 treated with deoxy-  
cholate for 60 min  
×72,000

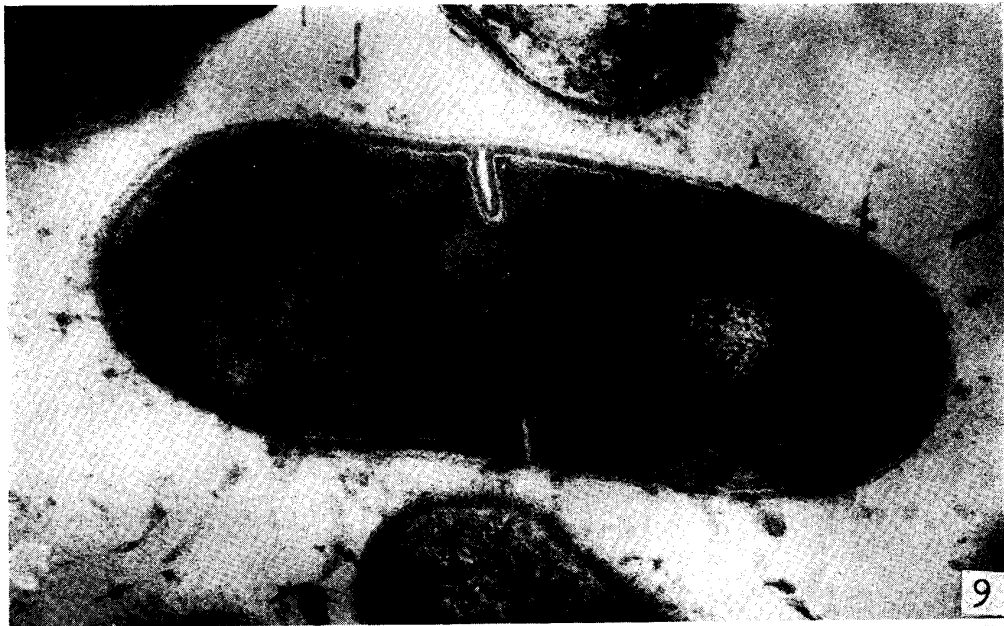


PLATE IV

Immunodiffusion of rabbit antisera to *C. renale* No. 9 (a), No. 33 (b), No. 42 (c) and No. 45 (d), filled in peripheral wells, with antigens of the following strains

No. 9	.....	Fig. 12
No. 33	.....	Fig. 13
No. 45	.....	Fig. 14
No. 42	.....	Fig. 15
No. 44	.....	Fig. 16

filled in each center well

