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CHARACTERIZATION OF THE TYPE SPECIFIC ANTIGENS OF THREE TYPES OF *CORYNEBACTERIUM RENALE*

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Type specific antigens were obtained by heating at pH 2.8 from three types of *C. renale*. The main antigen, which formed a heavy precipitin line with the homologous antiserum, was isolated by DEAE cellulose and Sephadex G-200 columns. The main antigen of each type contained sugars and protein and was resistant to heat, phenol treatment, and protease digestion. The facts suggest that antigenic determinants are sugars. Arabinose, mannose, and glucose were commonly found in the 3 main antigens.

INTRODUCTION

Three types have been distinguished serologically and biochemically in strains of *Corynebacterium renale*¹⁵). Differences in these three types have also been reported in nutritional requirements⁵), piliation^{16,17}), lysogeny¹⁸), and DNA base compositions⁶).

LOVELL extracted soluble antigens of *C. renale* and found that they contained ribose, arabinose, mannose, and galactose. CUMMINS described the purified cell walls as antigenic in agglutination. However antigenicity of extracts from cell walls was not examined. YANAGAWA et al.¹⁵) extracted soluble antigens of *C. renale* from various strains with sodium deoxycholate and classified the micro-organisms into the three types.

Attempts were made by the authors to clarify the chemical properties of the antigen of the three types of *C. renale*. For this purpose type specific, soluble antigens were isolated and characterized, which were described in this paper.

MATERIALS AND METHODS

Strains *Corynebacterium renale* No. 9 (type I), No. 35 (type II), and No. 42 (type III) were used as the representative of each type.

Antigen extraction These strains were cultivated at 37°C for 48 hr on nutrient agar supplemented with 0.5% glucose. The 20 g amount of wet cell mass harvested was suspended in 40 ml of distilled water. Extraction of the antigen was carried out by disintegrating the suspended cells for 45 min in a water-cooled sonic oscillator (Kubota Co.)

at 10 kc/sec full power. The homogenate was brought to pH 2.8 with 1/10 N HCl and heated at 100°C for 10 min, cooled and adjusted to pH 7.0 with 1/10 N NaOH and centrifuged at $15,000\times g$ for 30 min. The supernatant fluid obtained was dialyzed against 0.02 M phosphate buffer (PB, pH 7.0) overnight at 4°C, which was stored at -20°C as a crude antigen.

Chromatography on diethylaminoethyl (DEAE) cellulose column Each crude antigen was adsorbed on DEAE cellulose column (3 $\times$ 16 cm) equilibrated previously with 0.02 M PB (pH 7.0). The column was washed with approximately 2 times of column-volume of the same buffer and then eluted with an increasing molarity of NaCl stepwisely. Ten ml of each effluent was collected in tubes. The effluents collected in tubes were examined by immunodiffusion as described later. The effluents reacting positively were pooled and dialyzed against 0.02 M PB (pH 7.0) overnight, then again adsorbed on DEAE cellulose column (1.1 $\times$ 26 cm). The column was washed with the same buffer and then eluted with an increasing molarity of NaCl gradiently.

Five ml of each effluent collected in tubes was examined by immunodiffusion and the positive part of the effluents was pooled. The pool was concentrated by dialysis against polyethylen-glycol 8,000. This preparation was chromatographed on a Sephadex column.

Chromatography on Sephadex G-200 column Five ml of the preparation was fractionated with Sephadex G-200 column (2.5 $\times$ 90 cm) with 0.02 M PB (pH 7.0) and the effluent of 5 ml each was collected.

Immunodiffusion The protein content of the effluents in tubes was examined by ultraviolet (UV) absorption at 280 m μ with a spectrophotometer (Shimazu). The antigenicity of the effluents was examined by immunodiffusion using the antiserum of a rabbit immunized with homologous organisms. The immunodiffusion technique and preparation of antiserum was followed according to a previous report¹⁵⁾.

Immuno-electrophoresis A 2.5 ml of 1% noble agar (Difco) dissolved in a barbiturate buffer (pH 8.6, $\mu=0.1$) was poured on a microscope slide. A horizontal trough of 1 $\times$ 64 mm was cut, and a well of 4 mm diameter at a distance of 3 mm from the trough was cut and filled with the main antigen prepared as above (the partially purified main antigen). A power supply (Joko Sangyo) was used to pass an electric current at 0.6 mA/cm for 90 min through the slide connected to the barbiturate buffer (pH 8.6, $\mu=0.05$) with filter papers. The trough was then filled with homologous antiserum and the slide was placed in a moist chamber for 48 hr at room temperature. After washing the slide with saline for 24 hr, the protein band was stained with 0.5% amidoblack. Then the slide was washed for 3 or 5 hr in 3% acetic acid.

Characterization of the partially purified main antigen Protein was estimated by the method of LOWRY et al. with bovine albumin as standard. Total carbohydrate was determined by the phenol sulfuric acid method⁴⁾ with dextrose as standard. Stability to phenol was examined by mixing 2 ml of the partially purified main antigen with an equal volume of phenol saturated with 0.1 M PB (pH 7.0). This was agitated violently for 15 min at room temperature, and then centrifuged at $1,500\times g$ for 15 min. The water phase was collected. The remaining phenol phase was added with 1 ml of 0.01 M PB (pH 7.0). The phenol was removed from both phases by ethyl ether extraction. After the ethyl ether was removed, both phases were added with 0.01 M PB (pH 7.0) so as to obtain the final

2 ml volume, respectively, which was examined by immunodiffusion. Stability of the main antigen to trypsin 1 : 250 (Difco) and prozyme (Kyowa kagaku Co.) was examined as follows. One ml of the partially purified main antigen and 1 ml of 0.25 % trypsin or 0.25 % prozyme, which were dissolved in PB (pH 7.0) containing 10^{-3} M Ca^{++} were mixed and incubated for 4 hr at 37°C. These mixtures were tested by immunodiffusion to determine whether they were still antigenic. Since it was already found, as mentioned above, that the main antigen was resistant to heating at 100°C, stability to autoclaving at 120°C for 20 min was also tested.

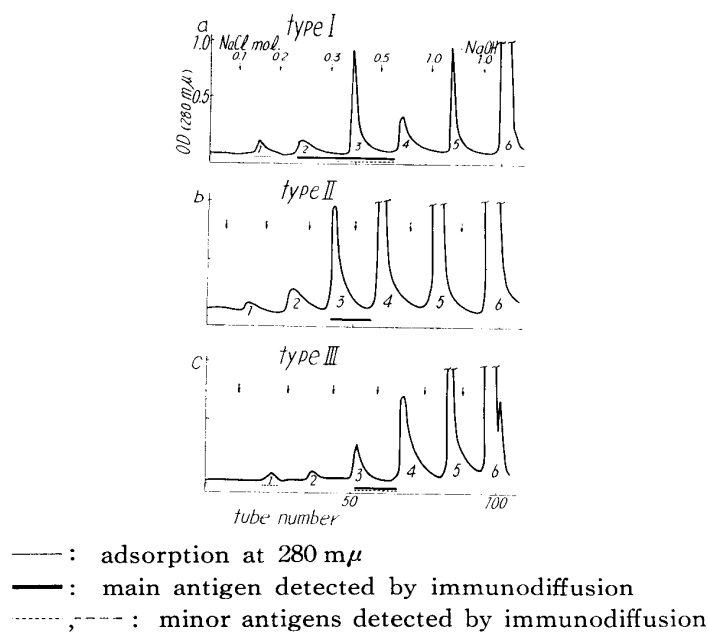
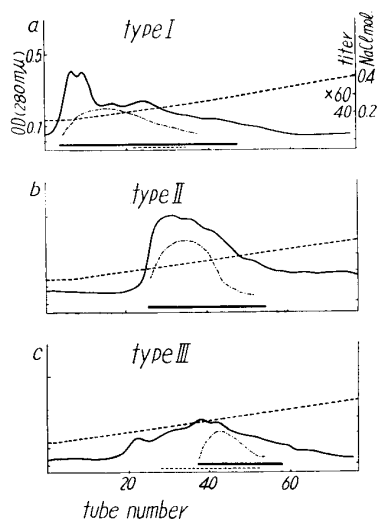
For the identification of sugars, the partially purified main antigens were hydrolyzed with 2 N sulfuric acid at 100°C for 2 hr. After sulfuric acid was removed with $\text{Ba}(\text{OH})_2$, the hydrolyzed main antigens were dried, then dissolved in 0.1 ml of distilled water and applied to paper. Chromatograms were developed on Toyo filter paper No. 51 A in ethylacetate-acetic acid-water (9 : 2 : 2) or in water-saturated phenol. TREVELYAN's silver nitrate reagent was employed for the identification of sugars.

RESULTS

Figures 1~3 show reactions of crude antigens with homologous and heterologous antisera. The main antigens forming the heaviest (types I & III) or a single (type II) precipitin line could easily be distinguished in these figures. The main antigens which were type specific were stable to heat because they were extracted by heating at pH 2.8.

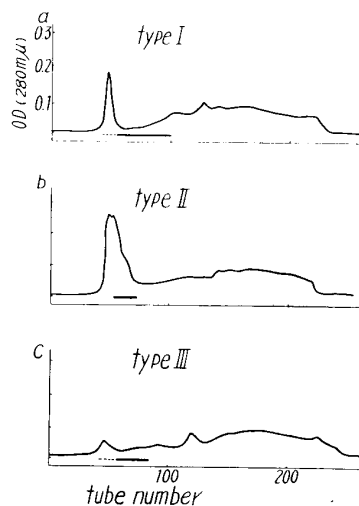
The main antigens at each step of the process of isolation, which is mentioned later, are shown in figures 4~6. A heavy and broad precipitin line among 3 lines was formed by type I (fig. 4), a heavy and sharp line was the only reaction given in type II (fig. 5), and a heavy and sharp line among the 3 lines was formed by type III (fig. 6).

Results of DEAE cellulose chromatography with stepwise elution were shown in figure 7a-c. The figure indicates that the main antigen of type I was found in fractions 2 and 3 while that of type II and III was found in fraction 3. Each of these fractions were rechromatographed on another DEAE cellulose column in a continuous gradient with an increasing molarity of NaCl. The results were graphed in figure 8 a-c. The main antigen of type I was eluted with a wider range of salt concentrations from 0.16 to 0.3M. Serological activity of the main antigen titrated by immunodiffusion did not associate so intimately with the UV absorption peak. The minor antigen of type I was eluted associating with a considerable amount of the main antigen (fig. 8a). The main antigen of type II was eluted with NaCl concentration ranging from 0.22 to 0.3 M. The curve of the main antigen titer and that of UV absorption were almost parallel to each other (fig. 8b). The main antigen of type III was eluted with NaCl concentration of 0.25 to 0.32 M. Serological activity was not correlated so intimately with the UV absorption peak. The minor antigen was detected as associated largely with the main antigen (fig. 8c). Figures 4 and 6 show that the main antigen of types I and III was still accompanied with the minor antigens in spite of rechromatography. In order to isolate the main antigens from minor antigens the fraction containing each main antigen was further fractionated on Sephadex G-200 column. Results of the gelfiltration were shown in figure 9a-c. The main antigens of type I and III were detected, apart from minor antigens, between the first and second UV absorption peaks

FIGURE 7a-c *Chromatography of crude antigen of 3 types on DEAE cellulose*FIGURE 8a-c *Chromatography of main antigens obtained by DEAE chromatography with stepwise elution on DEAE cellulose*

---- : molarity of NaCl
 : titer of main antigen by immunodiffusion

For further information see the footnote for figure 7

FIGURE 9a-c *Chromatography of main antigens obtained by DEAE chromatography with gradient elution on Sephadex G-200*

See the footnote for figure 7

(figs. 9a, 9c, 4 & 6). Type II main antigen was found in the second peak which was close to the first peak (fig. 9b).

Each main antigen thus isolated formed a single precipitin line by immunoelectrophoresis (figs. 10~12).

The minor antigens of types I and III were associated with the first UV absorption peak on Sephadex G-200 chromatograph.

The main antigens were thus partially purified and were characterized.

Heating at 120°C for 10 min did not affect the antigenicity. Phenol treatment of the main antigens resulted in the antigen moving to the phenol phase without demonstrable reduction in antigenicity. Treatments with 0.25 % prozyme and 0.25 % trypsin caused no reduction of the antigenicity, though the precipitin lines slightly diffused toward the serum wells. Whether the antigenicity was affected or not by these treatments was determined by titrating the antigens by immunodiffusion.

Chemical analysis of the main antigens gave the following results. The ratio of total protein to sugar was 26 : 1 (type I), 43 : 1 (type II), and 34 : 1 (type III). Therefore the main antigens were found to consist mostly of protein and small amounts of sugar. The fact that the main antigens were resistant to phenol, proteases, and heat suggest that antigenic determinants are not protein but sugars. Therefore sugar composition of the main antigen of each type was examined. Arabinose, mannose, and glucose were commonly found by paper chromatography. An unidentified sugar was found in type I main antigen, which was supposed to be an oligosaccharide as its R_f value was small. From the size of the spots on the chromatograms the sugar occurring in the highest amount was found to be arabinose.

DISCUSSION

It was suggested from the results that the antigenic determinant of the main antigens of the three types of *C. renale* are sugars. However the antigens contained large amounts of protein.

The elution patterns on DEAE and Sephadex columns were different among the main antigens of the three types; particles of the type II main antigen were considerably uniform, those of type I were most various, and those of type III were intermediate. The different elution patterns of each antigen might be due to the different physical properties of the protein moiety.

LOVELL reported that extracts from phenol-treated cells of *C. renale* with 20% methanol were antigenic in precipitin reaction and the extracts contained ribose, arabinose, mannose, and galactose. Our results showed that the main antigens commonly contained arabinose, mannose, and glucose. The difference between LOVELL's and our results may be primarily due to the fact that we used only the main antigen.

CUMMINS & HARRIS found that among sugars, which they detected in cell walls from *C. renale*, arabinose occurred in the highest amount. This was also

the case with our results. Three sugars, arabinose, mannose, and glucose were commonly detected in each main antigen. An unidentified sugar detected in type I main antigen was the only difference. As in *Salmonella*¹⁰, sequence of the sugars might contribute to the specificity of the three types. This will be a subject of future studies.

Antigens of *C. hofmannii* were studied by BANACH & HAWIRKO. They isolated two antigens, which were heat and acid-stable, and contained 55 and 75 % of protein and 6.7 and 10.2 % of carbohydrate, respectively. The characteristics of the antigens of *C. hofmannii* were similar to those of *C. renale* shown in this study.

From cells of *C. diphtheriae*, WONG & TUNG obtained polysaccharide and protein antigens. The polysaccharide antigens were group specific, while protein antigens were type specific. OEDING & LAUTROP demonstrated that the polysaccharide antigens were heat-stable, while the protein antigens were heat-labile. The type specific antigens from *C. diphtheriae* are heat-labile and proteinous in nature but those from *C. renale* are heat-stable and contain sugars. We have no appropriate explanation for these differences at present.

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EXPLANATION OF PLATES

PLATE I

Figs. 1~3 Immunodiffusion of crude antigen of 3 types to homologous and heterologous antisera I, II, and III indicate the type of *C. renale* described in the text.

A: crude antigen

S: antiserum

These explanations are also applicable to the next figures.

Figs. 4~6 Immunodiffusion of main antigen of 3 types obtained at each step of the isolation process to homologous antiserum.

B: main antigen after 2 times chromatography (stepwise and gradient elution) on DEAE cellulose

C: main antigen further purified by Sephadex G-200 (partially purified main antigen)

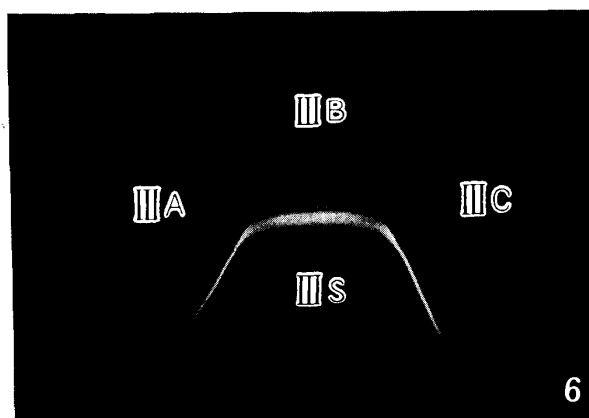
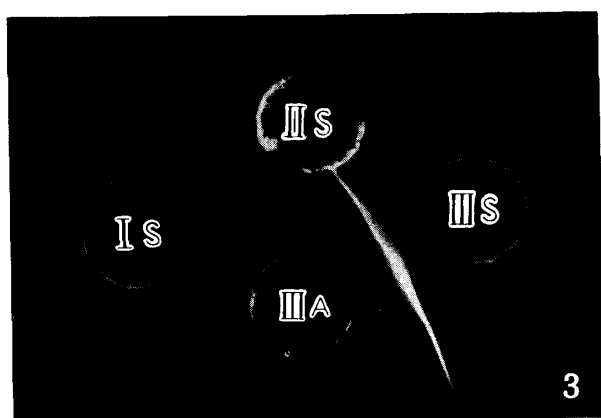
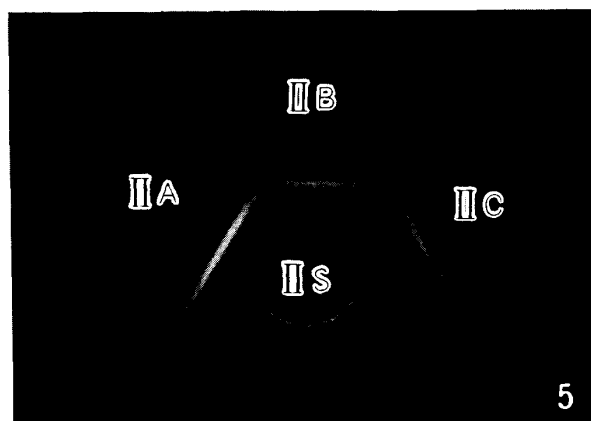
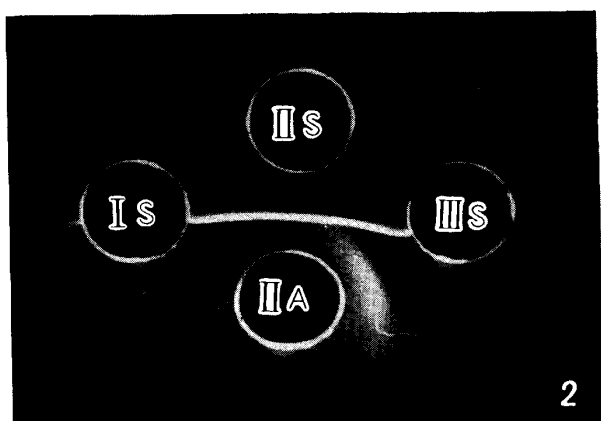
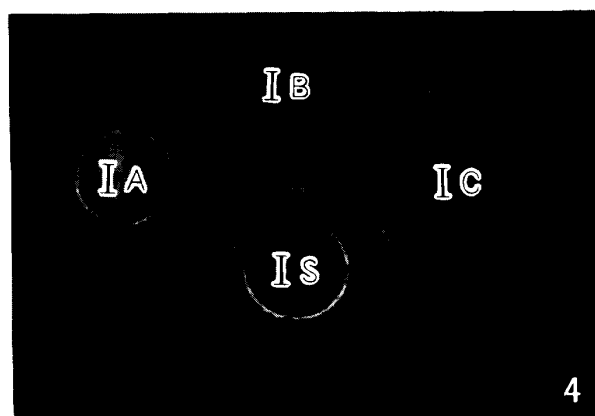
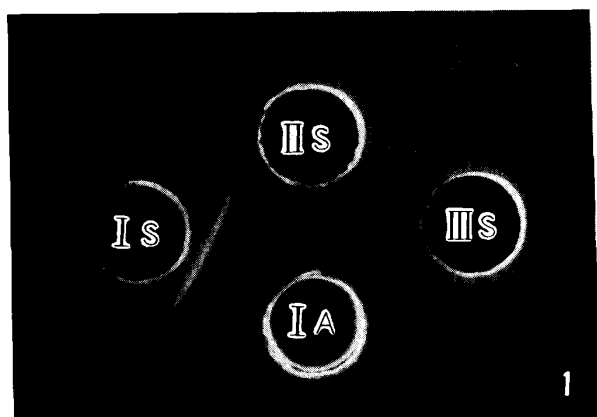


PLATE II Immunoelectrophoresis of partially purified main antigen
to each homologous antiserum

Fig. 10 type I

Fig. 11 type II

Fig. 12 type III

