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Note

The gel-filtration pattern of eel serum. Separation of haemolysin(s) from haemagglutinins

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In 1927 agglutination of human O-group erythrocytes by absorbed bovine serum was observed[1]; the fact that the same phenomenon occurs with eel serum has been known for decades[2,3]. Agglutinins of human O-group erythrocytes have also been found elsewhere, *e.g.*, in herring serum[4] and in several plants[5–8]. The substance on the O-group erythrocytes responsible for this agglutination was originally thought to be a product of the O-gene and was referred to as the "O-substance". Subsequent evidence, however, refuted this idea and the substance was given the symbol H (ref. 9). The structure of saccharides having the serological specificity of the substance H has been the subject of several papers[10–13]. The early reports on eel blood[14] and proteins of its serum[15] have recently been followed by communications[8,11,16] dealing with the isolation and physico-chemical characterization of the eel immunoglobulin responsible for the agglutination.

Eel serum is used diagnostically to differentiate human-blood group A_1 from A_2 and A_1B from A_2B. The serum must first be heated to suppress its haemolytic action on human erythrocytes, then treated with a mixture of erythrocytes A_1 and B, after which absorption only the anti-H antibody should remain. In spite of the heating, however, the surviving haemolytic activity is sometimes so strong as to make the serum diagnostically worthless. The work described here constituted an attempt at removal of the haemolysin(s) by gel filtration. Another approach to the problem, *viz.*, removal of human haemoglobin from eel serum after the absorption procedure, has also been tried.

EXPERIMENTAL

Eel serum

Blood from 100 to 150 eels (*Anguilla anguilla*) was pooled and centrifuged, about 2.5 ml of serum per eel being obtained. The fresh pooled serum was heated at 56° for 20 min to inactivate the haemolysin(s), then sodium azide (1 g per litre of serum) was added, and the mixture was stored at 4°. Two samples of different pools (referred to as serum I and II) were about 10 months old when available to us for the experiments. The agglutinin titre of serum I for O-group erythrocytes was 128 (the

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reciprocal of the ratio of dilution with a 0.9 % sodium chloride solution at which agglutination still occurred); the titre of serum II was 64. In the procedure normally used for removal of agglutinins anti-A_1 and anti-B by absorption with a mixture of human erythrocytes of these groups (1 volume of erythrocytes plus 3 volumes of eel serum being gently shaken for 20 min, then centrifuged), serum I was haemolytically active, the degree of haemolysis being 35 %; with serum II, no haemolysis was observed.

Gel filtration

The sera (8 ml samples) were filtered in the conventional way on a column (80 cm × 2.8 cm I.D.) of Sephadex G-200 in 0.05 *M* Tris buffer of pH 8 that was 0.5 *M* in sodium chloride. In the gel filtration of serum II, the elution buffer contained 0.5 g of sodium azide per litre.

Immunoelectrophoresis

The contents of the test-tubes representing selected points of the elution peaks were analysed immunoelectrophoretically[17] on glass plates (8 cm × 8 cm) covered with 11 ml of 1.2 % Oxoid agar No. 2 in 0.035 *M* barbitone buffer of pH 8.6; electrophoresis was carried out at a potential gradient of 4 V/cm for 1.5 h, without cooling. Immunodiffusion against a polyvalent anti-eel serum (obtained by short-term immunization of rabbits using Freund's incomplete adjuvant) was allowed to proceed for 24 h at room temperature.

Removal of human haemoglobin from eel serum

Eel serum containing human haemoglobin was dialysed against 0.1 *M* phosphate buffer of pH 6.4, then allowed to pass through a column packed with CM-Sephadex C-50 (nominal haemoglobin capacity 9 g/g of dry exchanger) and equilibrated with the same buffer. The buffer was also used as eluent.

RESULTS AND DISCUSSION

Unlike the normal, three-peak elution pattern, observed with animal sera (fractions 19 S, 7 S and albumin), the elution curve of eel serum had only two peaks. Fig. 1 refers to serum I. The first peak was found to contain agglutinins for human erythrocytes O, A_1 and B, but no haemolysin in the portion not overlapped by the second peak. The numbers on the curve show the agglutinin titres for O-group erythrocytes (the maximum titre was an order of magnitude lower than that of the starting serum). The medium of the elution buffer had no effect on the agglutinin titre. The second fraction contained the haemolytic component(s), but no agglutinin. The haemolytic activity was assessed by the absorption procedure normally used to remove the agglutinins anti-A_1 and anti-B from eel serum (see Experimental). The maximum haemolytic activity coincided with the top of the peak and was similar (estimated visually) to that of the serum. The dashed lines in Fig. 1 delineate a haemolytic region; the thresholds correspond to a just perceptible haemolysis after an extended time of 4 h. It was unnecessary to dialyse the samples against a 0.9 % sodium chloride solution before the assay, since the elution buffer produced no visible haemolysis, even after 10 h.

Prolonged elution of the Sephadex column gave rise to no further peak. A few

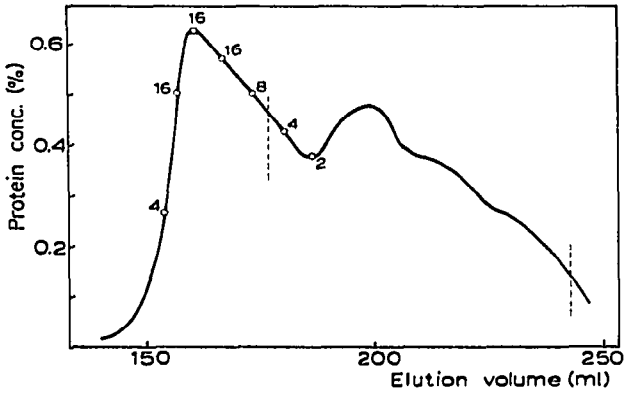

Fig. 1. Gel filtration of eel serum on a column of Sephadex G-200. The numbers on the curve show the agglutinin titres for human O-group erythrocytes; the second peak represents the haemolysin fraction; and the dashed lines delineate the region of haemolytic activity.

days later, however, the Sephadex began to decompose and, within about 10 days, had practically all gone into solution. The Fehling test for glucose in the solution was not positive until after relatively drastic hydrolysis with hydrochloric acid (the concentration of the acid had to be about 10 times greater than that needed to hydrolyse sucrose in boiling solution). Gel filtration of another portion of serum I gave analogous results, as did gel filtration of serum II, including the decomposition of the Sephadex. In the experiments with serum II, this decomposition was rather surprising, as the elution buffer contained 0.05 % of sodium azide. In an attempt to decide whether the decomposition was due to a component of the serum, samples of the Sephadex were left standing in test-tubes with the whole serum, with the agglutinin fraction (top of the peak), and with the haemolysin fraction (top of the peak); lysis was observed in all three instances. With the haemolysin fraction, it appeared to proceed a little faster than with the agglutinin fraction, but the difference (if any) was not significant. This suggests that azide-resistant bacteria may have been responsible for the lysis.

The only difference possibly worthy of mention between the results obtained with serum I and serum II was in the height of the second peak, which was less with serum II. This observation might in some way be related to the fact that this serum showed no haemolytic activity, in contrast to the considerable haemolytic activity of serum I. The effect of heating eel serum to 56° on the second peak could not be investigated, as we had no sample of native (non-inactivated) serum.

Immunoelectrophoresis of the whole eel serum (serum I) is shown in Fig. 2a, and that of the haemagglutinin fraction (top of the peak) in Fig. 2b. The haemolysin fraction gave only one immunoelectrophoretic arc (indicated by the arrow in Fig. 2a). Fig. 2b reveals the presence of two constituents; the one nearer to the anode appeared first in the gel filtration, but was closely followed by the other. We tried to decide which of them was the agglutinin by absorption of eel serum with an excess of human erythrocytes O, A_1 and B, but this treatment had no effect on the immunoelectrophoretic pattern of the serum. Hence it seems that only a small part of the molecules forming one or the other immunoelectrophoretic arc are the haemagglutinins. In

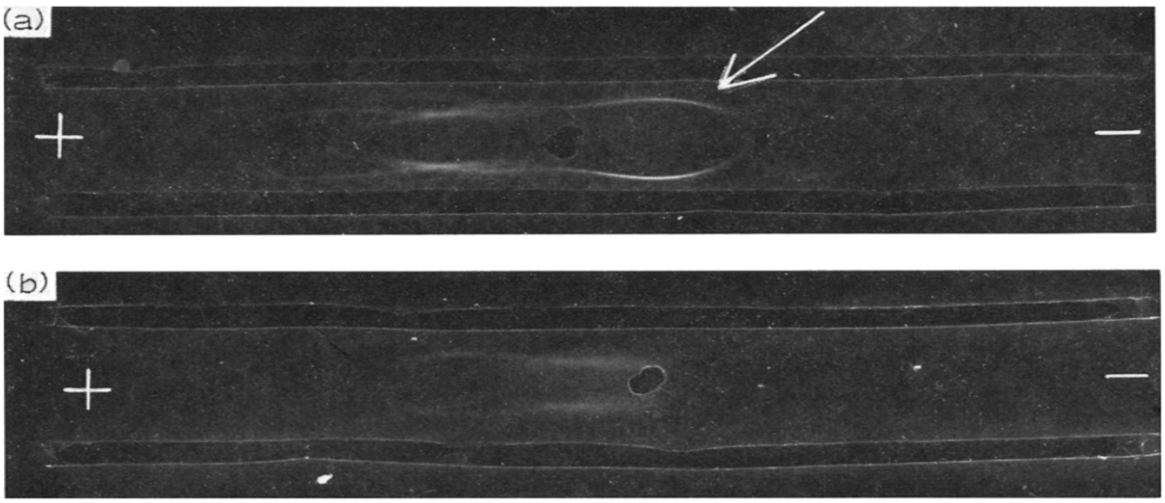

Fig. 2. Immunoelectrophoresis of eel proteins: (a) whole serum (the arrow shows the constituent found in the haemolysin fraction); (b) the agglutinin fraction. Rabbit polyvalent antiserum was used.

either instance, the position of the arc containing the haemagglutinins accords with Springer and Desai's observation[11] that the eel immunoglobulin migrated like an α_2-globulin at pH 8.6. The same immunoelectrophoretic results were obtained with serum II and its fractions.

It can be concluded that gel filtration of eel serum produces a satisfactory separation of the agglutinins from the haemolysin(s), but the agglutinin titre is much decreased. If, consequently, a haemolytically active pool of eel sera is to be used as a source of anti-H antibodies, gel filtration is not a rewarding solution of the problem. For this reason, we have also tried a method based on removal of haemoglobin from the serum after its absorption with human erythrocytes A_1 and B. The haemoglobin was removed on a column of CM-Sephadex C-50, as described in Experimental. After this procedure the serum was no longer haemolytically active and the agglutinin titre for O-group erythrocytes was not decreased.

We do not know why individual pools of eel sera, representing mixtures from at least 100 eels, differ considerably in haemolytic activity after being heated to 56°. It may be that some rare specimens have more heat-resistant haemolysin(s), so that they affect the whole pool, but pollution of the eel blood with mucus (in bleeding the fish) cannot be ruled out as a possible cause. This question, however, is not within the scope of the present paper.

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