

COMMUNICATION TO THE EDITORS

Separation of skin-sensitizing and complement-fixing antibodies from guinea pig anti-bovine insulin sera

It has recently been shown that antibodies to one antigen can be separated in fractions with qualitatively different immunological properties.⁽¹⁾ This is the case even within the group of γ S γ -globulins.

The most clear-cut results have been obtained by Benacerraf and colleagues.⁽²⁻⁴⁾ These authors subjected immune sera to electrophoresis and found two populations of precipitating antibodies of the same specificity in the regions of electrophoretically slow and fast moving γ -globulins. It further appeared that the former population was able to fix complement whereas the latter was inactive in this respect. However, the skin-sensitizing properties were present only in the fraction with the fast moving γ -globulins. In a number of publications chromatography on DEAE-cellulose is described as a means for the fractionation of γ -globulins and consequently, of antibodies.^(5,6) Yagi used this method to fractionate anti-insulin sera into a fraction containing slow-moving γ -globulins and a fraction containing fast-moving antibody globulins together with other proteins. In our investigations this method was adopted because of its simplicity. The fractions from guinea pig anti-bovine insulin serum* thus obtained were characterized by means of immunoelectrophoresis,^(7,8) binding of insulin¹³¹I†,^(9,10) complement fixation, skin anaphylaxis tests and immunofluorescence.

A typical result of an immune serum fractionation is noted in Fig. 1. The two fractions with maximal protein appeared to contain antibodies which bind insulin-¹³¹I. Immunoelectrophoresis of the concentrated fractions of the first and the second peak revealed a difference in electrophoretic mobility of precipitating antibodies present in the two fractions (Fig. 2, left and right slides). In the middle the pattern of unfractionated serum is shown. Note the two curved precipitation lines. Generally the antisera contain two double curved precipitation lines. This suggests the presence in the antisera of four populations of anti-insulin globulins.

Complement fixation was tested by means of quantitative complement fixation analysis.⁽¹¹⁾ It appeared that the immune complex of insulin with antibodies obtained from the second peak eluates (Fig. 1) is unable to fix complement whereas the unfractionated serum, and its first peak are highly active in this respect.

This feature was confirmed in immunofluorescence experiments. Insulin present in the islets of Langerhans binds antibodies to insulin.⁽¹²⁾ In our experiments bovine pancreatic tissue slices containing cells with fixed guinea pig anti-bovine insulin antibodies were incubated (*a*) with fresh human serum and (*b*) with human serum in which thermolabile complement components were destroyed by incubation during 30 min at 56° C. That human complement components were bound to

* Guinea pigs were immunized by intramuscular injections of 10 U. of insulin in complete Freund's adjuvant every other week. Blood (5-6 ml) was taken by cardiac puncture once a fortnight. With some guinea pigs this procedure has been maintained for about a year.

† Highly purified crystalline bovine insulin with 24.9 I.U./mg protein and used for immunization and assay was supplied by Organon, Ltd., Oss, The Netherlands.

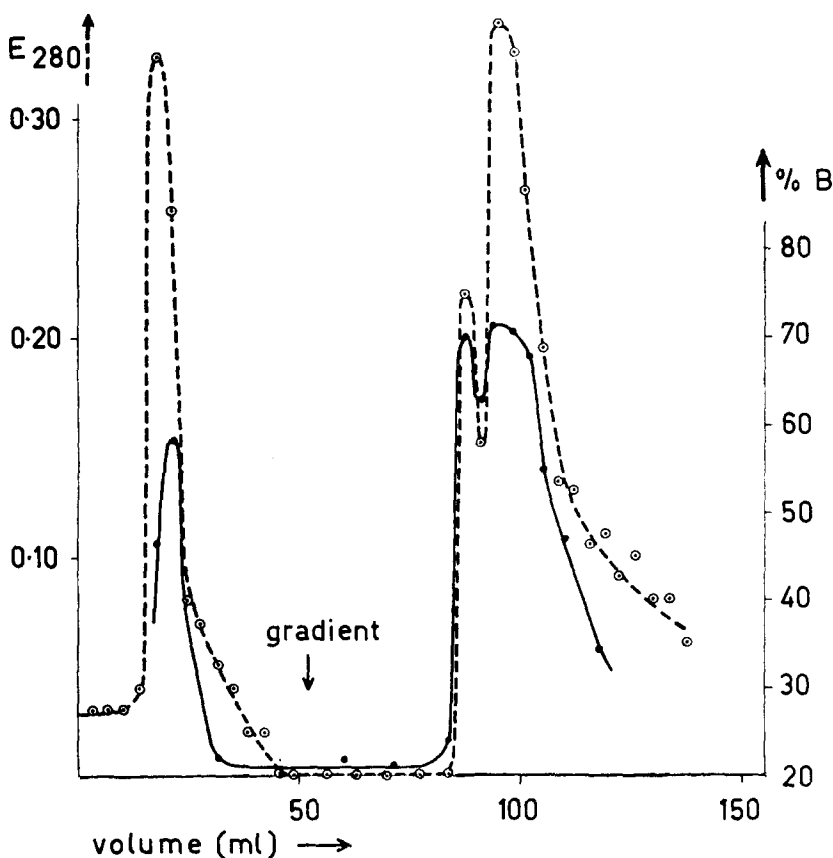


FIG. 1. Fractionation of an anti-insulin serum on a DEAE-cellulose column (20 \times 1 cm). 0.5 ml of antiserum dialysed against the starting buffer (0.035 M Tris and 0.005 M H_3PO_4 , pH 8.4) was first eluted with the same buffer. The gradient was prepared by dropwise addition of mixing buffer (0.50 M Tris and 0.59 M H_3PO_4 , pH 4.0) to 250 ml of starting buffer. The ratio at the end of the elution period was approximately 2 : 1. Fractions of 3.5 ml were collected with a speed of about 1 ml per min. Fractions were incubated with an appropriate amount of insulin- ^{131}I prepared according to the method of Greenwood *et al.*⁽⁹⁾ After the incubation free and bound insulin were separated by chromatography on Whatman 2 MM with veronal buffer 0.1 M, pH 8.6. The results are expressed as:

$$\% \text{ bound} = \frac{\text{radioactivity of the bound fraction}}{\text{radioactivity of the bound and free fractions}} \times 100$$

(Procedure originally described by Yalow and Berson.⁽¹⁵⁾)

Careful examination of the DEAE-cellulose type to be used for fractionation is necessary, since one may meet considerable difficulties with correct protein separation from one DEAE lot to another. DEAE-cellulose used here is a product of Eastman Kodak Co.

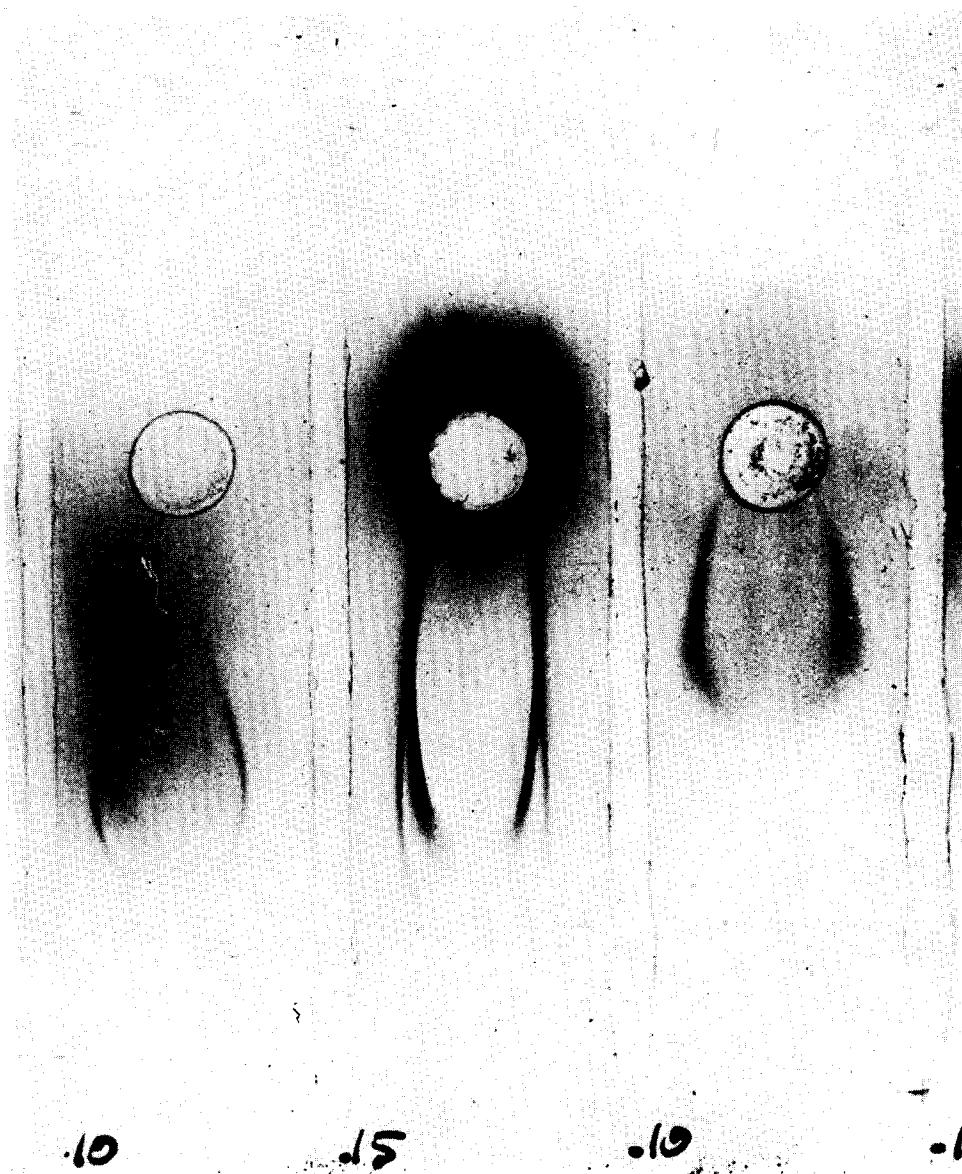


FIG. 2. Immunoelectrophoresis of gp anti-bovine insulin serum (central well) as well as its chromatographic fractions. The four troughs contain 0.10 mg or 0.15 mg/ml of crystalline bovine insulin. Fractions from peak I in left well and peak II in right well (the latter comprising both poorly separated sub-peaks) were first combined and concentrated 10 times by means of polyethylene glycol 4000. Electrophoresis was performed in 1.3 per cent agar in veronal buffer pH 8.6 (50 min at 7-8 V/cm and 8-10 mA per slide).

The semi-circular band around central well with antiserum is probably lipid containing material. Contamination of bovine insulin with other bovine proteins has not been shown in precipitin test with rabbit anti-bovine serum.

the immune complex became apparent by the binding in (a) of fluorescent anti- β_1C and anti- β_1E from rabbit anti-human complement globulins. No fluorescence was observed in (b). The antisera to complement components have been described in earlier work.⁽¹³⁾ Fluorescent immunoglobulins were prepared as described in Ref.⁽¹¹⁾ Fluorescence was observed with cells labelled with the unfractionated antiserum or with slow γ -globulins. With the fast globulins the result was consistently negative.

By passive cutaneous anaphylaxis tests in guinea pigs (antiserum and antigen administered intradermally with an interval of 5 hr and 0.5 ml 2.5% Evans blue intracardially before the second intradermal injection) it was shown that the first eluted globulin populations of the fast moving globulin moiety contained all of the skin-sensitizing antibodies. The complement binding slow moving globulins were completely and consistently inactive in skin tests.

Further fractionation of proteins in second peak eluates by means of disc electrophoresis⁽¹⁴⁾ revealed the presence of 21 identifiable proteins at pH 7.9 and increased pore size of the gel. After electrophoresis 7 out of 8 protein containing gels were cut in 10 or more parts according to the protein pattern in a dyed control gel. (Number 8 of the same run.) Special care was given to γG and γA areas and the proteins therein separated by elution of the gels with a solution containing 0.9% NaCl.

With this technique the fast moving anti-insulin globulins emerging in peak II eluates were separated into three distinguishable groups. These groups of globulins varied with respect to insulin binding capacity, electrophoretic mobility and anaphylactic activity.

The differences in insulin binding capacity of the various anti-insulin antibodies were demonstrated initially by incubation of the whole gel with excess of carrier free insulin ^{131}I , washing and calculation of remaining counts in gel cuts. In a second method anti-insulin antibodies were first eluted and subsequently incubated with carrier free insulin ^{131}I . The differences in insulin binding capacity were then

demonstrated by comparing of $\frac{B}{B+F}$ values per mg of protein in each gel fraction.

Quantitative experiments are underway to determine the dissociation and association constants of each antibody population.

The variation in electrophoretic mobility was determined in immunoelectrophoresis with rabbit anti guinea-pig globulin serum. The slowest-moving first group of antibodies as well as the fast-moving third group contained the strongest insulin ^{131}I binding properties per mg of protein. The second group of antibodies with intermediate mobility had lowest insulin binding capacity and was the only one active in PCA. These antibodies moved with a speed equal to medium speed 7 S γ -globulins. With rabbit anti-gp serum these guinea-pig globulins with anti-insulin activity precipitated in immunoelectrophoresis far below the β -globulins of a normal guinea-pig serum used for reference on the same slide. Whether or not the fast-moving third group of antibodies are γ - or β -globulins is still a matter of speculation.

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