Insulin Antibodies: Partial Characterization by Gel Filtration.* (31119)

E. Toro-Goyco, M. Martínez-Maldonado and M. Matos
(Introduced by Conrado F. Asenjo)
Department of Biochemistry and Nutrition, University of Puerto Rico School of Medicine, and Radioisotope Service, Veterans Administration Hospital, San Juan, P. R.

Studies during the past decades have shown that antibodies are produced in animals and man upon repeated exposure to foraneous insulin. A variety of insulin antibodies have been described, but their differences have not been clearly established. Extensive studies using various types of electrophoretic techniques have established their gamma globulin nature(1-4). Their characterization from the point of view of size has, however, been much less thoroughly studied.

This research was undertaken with the aim of learning more about the molecular size and shape of insulin binding antibodies. For this purpose advantage was taken of the technique of gel filtration, an application of the molecular sieve principle which has been shown to be very useful in the separation of proteins of various sizes and shapes(5,6).

Materials and methods. Blood was obtained from patients hospitalized at the Veterans Administration and at the University hospitals. Thirty-seven were male and 11 female. Thirteen had never received insulin. Eight healthy hospital employees served as controls.

Two ml serum samples were incubated with one ml I-131 insulin dilutions containing from 0.3 to 0.5 μg I-131 insulin/ml. Commercial I-131 insulin used was obtained from Abbott Laboratories, North Chicago, Ill. Preparations are made by iodination of zinc insulin in the proportion of one atom I-131 per molecule of insulin (Mw 6,000). Insulin preparations had a specific activity of not less than 4 mc/mg. Incubation was performed at 37°C for 2 hours in a water bath shaker. The incubated mixture was then passed through small anionic exchange resin columns (Amberlite IRA-400 in the chloride phase) to remove any inorganic I-131 that might have detached from the insulin molecule. This step was considered convenient to avoid the introduction of inorganic radioiodine in the gel columns. Two ml of the processed mixture were subjected to gel filtration at a temperature of 25°C.

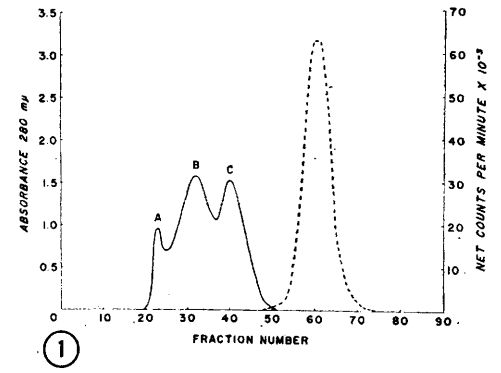
Material used for the gel filtration technique was Sephadex G-200, obtained from Pharmacia Fine Chemicals, New York. The procedures for preparation of columns, collection of samples, determination of protein concentration and radioactivity measurements have been described(7).

Paper electrophoretic studies were conducted on the protein fractions containing bound insulin. These were performed in barbital buffer, pH 8.6, ionic strength 0.075. Prior to electrophoretic analysis the eluted fractions were concentrated using carbowax.† The strips were run in duplicate and stained with bromphenol blue. One of each pair was cut in strips 1 mm in length and these assayed for radioactivity. This procedure allowed the determination of the electrophoretic mobility of the fractions containing the bulk of the bound radioactivity.

In 2 of the cases where the gel filtration pattern showed 2 clearly separable protein bound radioactivity peaks thus suggesting the presence of two γ-globulin-insulin complexes, gel filtration was repeated using 2 ml undiluted serum. The fractions (tubes) containing each radioactivity peak were dialyzed against a hundred-fold excess of 0.1 M NaCl and concentrated by lyophilization. Analytical ultracentrifugation at 20°C and 59,780 rpm was performed after solution of the freeze dried samples in 0.15 M phosphate buffered saline, pH 7.3. Lyophilized samples not dissolving completely were centrifuged at

* Supported in part by Nat. Inst. Health Grant AM-08346-02, USPHS.

† Carbowax is a trade name for polyethylene glycol stearate sold by Fisher Scientific Co., New York City. Fractions were placed on dialyzer tubing and powdered carbowax sprinkled over the bags. It took less than an hour for the fractions to become reduced to half their volume.



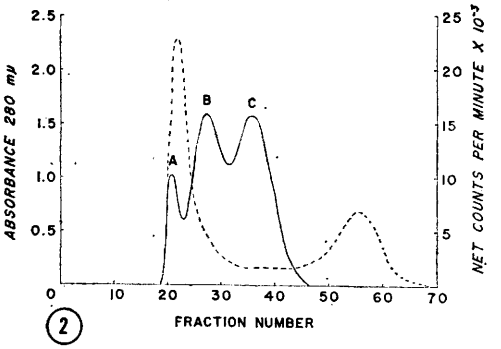


FIG. 1. Sephadex G-200 gel filtration pattern of control serum incubated with I-131 insulin. ——— protein concentration; - - - - - radioactivity. Fractions 3 ml each. Dimensions of column 2.5 × 40.0 cm.

FIG. 2. Sephadex G-200 gel filtration pattern of serum from diabetic patient incubated with I-131 insulin. ———protein concentration; - - - - - radioactivity. Fractions 3 ml each. Dimensions of column 2.5 × 40.0 cm.

2,000 rpm for 10 minutes to remove the insoluble material.

Results. Gel filtration of serum proteins using Sephadex G-200 leads to their fractionation into 3 main groups as shown by peaks A, B and C in Fig. 1. These have been identified as the macroglobulins, 7S globulins and 4S proteins, respectively(5,6). When radioactive insulin was added to normal serum, incubated as described above and subjected to gel filtration, the radioactivity appeared as a single homogeneous peak after the plasma proteins had been eluted from the column (Fig. 1). In none of the sera of 8 normal controls in which this experiment was performed did the results differ significantly from those illustrated in Fig. 1. Neither was it different in diabetic patients not receiving insulin. In those patients currently receiving insulin at least 2 radioactivity peaks appeared (Fig. 2). The fastest eluting radioactivity peak was found to appear together with the serum proteins. The remaining radioactivity appeared after the proteins were eluted as was the case in the controls. These 2 radioactivity peaks were thus described as corresponding to protein-bound and "free" insulin, respectively. In all cases, whether or not binding of I-131 occurred, the recovery of added radioactivity was quantitative, ranging from 90 to 96%.

Under the experimental conditions used, it was shown that only sera from insulin-treated diabetics form complexes with added radio insulin.

The elution behavior of the insulin γ-globulin complexes seemed to differ in various individuals. In some the peak of the protein bound radioactivity coincided with peak A of the fractionation pattern; in some it appeared between protein peaks A and B and in others it coincided with peak B. In all cases, however, paper electrophoresis of the proteins in these peaks indicated that the radioactivity migrated with the mobility of gamma globulins.

An unusual finding was the detection of 2 protein bound radioactivity peaks in several instances (Fig. 3, Table I). This pattern is suggestive of the presence of at least 2 different insulin γ-globulin complexes with different molecular weights or different axial ratios.

Analytical ultracentrifugation of fractions from 2 individuals showing protein bound radioactivity peaks in peaks A and B showed

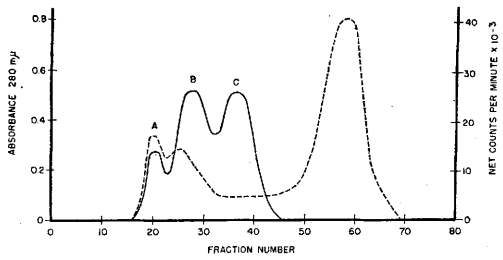


FIG. 3. Sephadex G-200 gel filtration pattern of serum sample from diabetic patient incubated with I-131 insulin. Notice 2 different protein bound radioactivity peaks. ——— protein concentration; - - - - - radioactivity. Fractions 3 ml each. Dimensions of column 2.5 × 40.0 cm.

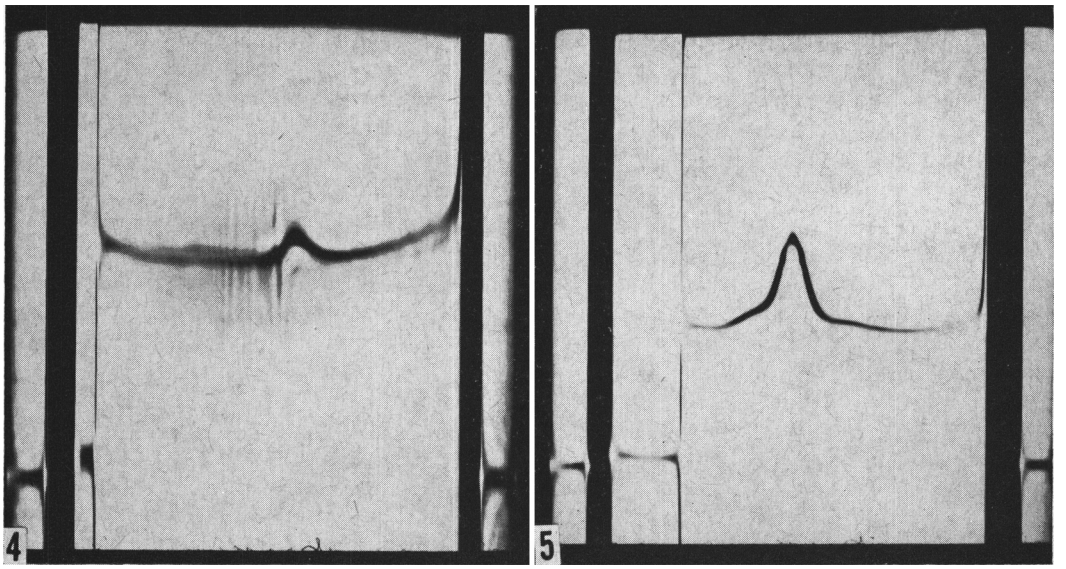


FIG. 4. Ultracentrifuge pattern of soluble lyophilized proteins in a peak A of gel filtration chromatogram. Picture taken 24 min after attaining full speed. Speed 59,780 rpm. Bar angle 70°. Direction of migration from left to right.

FIG. 5. Ultracentrifuge pattern of proteins from peak B of the gel filtration chromatogram shown in Fig. 3. Picture taken 40 min after attaining full speed. Notice slight impurities of 4S proteins. Speed 59,780 rpm. Bar angle 55°. Direction of migration from left to right.

the presence of proteins with different sedimentation rates in each peak thus confirming that the 2 protein bound radioactivity peaks were not artifacts created by the filtration process (Fig. 4 and 5). The fastest eluting peak contained proteins with a sedimentation rate $S_{20,w}$ of 19.6S (Fig. 4) while the second contained proteins with a sedimentation rate ($S_{20,w}$) of 6.7S (Fig. 5).

Discussion. Manipol and Spitzy(8) previously reported the use of the gel filtration technique to separate bound from free insulin and described this technique as superior to older methods. They used Sephadex G-75, which enables the separation of plasma proteins having molecular weight of 50,000 or higher from smaller ones, such as insulin. In this work Sephadex G-200 has been used which, besides allowing the separation of plasma proteins from free insulin, separates the former into 3 groups. This modification allowed the identification of more than one γ-globulin-insulin complex in serum from diabetic patients when their size or shape is appreciably different. As reported by Manipol and Spitzy, recovery of added radioactivity in these studies was also found to be quantitative.

TABLE I. Binding of Radioinsulin by Eluate Fractions in Gel Chromatograms.

Subjects	No.
Normal (no binding)	8
Diabetics:	
Untreated (no binding)	13
Treated:	
Binding in peak A only	5
" in inter AB only	16
" in B only	3
" in both A and B	2
" in inter AB and B	9

Gel filtration does not isolate insulin gamma globulin complexes alone but together with a group of proteins of roughly the same size. It is helpful, however, in supplying a rough estimate of their size. Although no detailed ultracentrifugal work was presented to substantiate their assumptions, Berson and Yalow(9) and Morse and Heremans(10) had already mentioned the 7S nature of insulin antibodies. The former investigators suggested that the sedimentation behavior of a gamma globulin-insulin complex may vary depending on whether the complex has a 1:1 or 1:2 insulin-γ-globulin ratio. The increased

molecular weight and size of an Ag-Ab_2 complex may partially explain the fact that in many of our gel filtration experiments the radioactivity peak was eluted slightly ahead of the bulk of the 7S globulins (Fig. 2). None of their models, however, would explain the appearance of a radioactivity peak in peak A of the gel filtration pattern. Although usually peak A (Fig. 1) contains the β-lipoproteins in addition to the macroglobulins the following evidence indicates that the protein bound radioactivity appearing in said peak was bound to the 19S globulins.

(a) After lyophilization of the proteins in peak A prior to analytical ultracentrifugation, the lipoproteins denature, thus losing their solubility(6). Their removal was accomplished by centrifugation at 2,000 rpm for 10 minutes. The radioactivity bound to the precipitate was negligible, being less than 2% of the total radioactivity in the suspension prior to centrifugation; (b) Since lipoproteins from normal individuals or untreated diabetics did not bind insulin (Fig. 1) binding was not to be expected by lipoproteins in treated diabetics; (c) The radioactivity in the supernatant after centrifugation remained bound to protein as shown by precipitability of radioactivity upon addition of 40% trichloroacetic acid. Upon gel filtration of the soluble lyophilized material through Sephadex G-200, the bulk of the radioactivity appeared with the void volume thus indicating that it was bound to a macroglobulin. These findings verify a previous report by Yagi and collaborators(11), where they reported on the heterogeneity of insulin binding antibodies. Their work, however, involved only ion-exchange work and immunoelectrophoresis, and no attempt was made to identify these antibodies as to size.

Summary. 1. The gel filtration technique was used to separate free from bound insulin in sera and hence to indicate the presence of insulin binding antibodies in the circulation of insulin-treated diabetic individuals. 2. Although most of the sera showed the presence of only one γ-globulin insulin complex, 2 such complexes were observed in 11 of the 35 treated diabetics whose sera was tested for the presence of anti-insulin antibodies. 3. The formation of Ag-Ab and Ag-Ab_2 in some patients(9) is postulated to explain the presence of 2 protein bound radioactivity peaks in the inter AB and B protein peaks, respectively. 4. Analytical ultracentrifugation was used to verify the presence of both 19S and 7S anti-insulin antibodies in 2 cases.

Sincere thanks are due to Messrs. E. Rivera-Collazo for technical assistance, R. Bernabe-Prida for preparation of the illustrations, Dr. L. Haddock for supplying some blood samples and Dr. T. E. Thompson, Dept. of Physiological Chemistry, Johns Hopkins School of Medicine, for making possible the ultracentrifugal analyses.

1. Sehon, A. H., Kaye, M., McGarry, E., Rose, B., J. Lab. & Clin. Med., 1955, v45, 765.

2. Berson, S. A., Yalow, R. S., Bauman, A., Rothchild, M. A., Newerly, K., J. Clin. Invest., 1956, v35, 170.

3. Kalant, N., Gomberg, C., Schucher, R., Lancet, 1958, v2, 614.

4. Welsh, G. W., Henley, E. D., Williams, R. H., Cox, R. W., Am. J. Med., 1956, v21, 324.

5. Flodin, P., Killander, J., Biochim. Biophys. Acta, 1962, v63, 403.

6. Roskes, S. D., Thompson, T. E., Clin. Chim. Acta, 1963, v8, 489.

7. Rivera, Julio V., Toro-Goyco, E., Matos, M., Am. J. Med. Sci., 1965, v249, 371.

8. Manipol, V., Spitzy, H., Int. J. Appl. Radiation and Isotopes, 1962, v13, 647.

9. Berson, S. A., Yalow, R. S., Trans. N. Y. Acad. Sci., 1962, v24, 487.

10. Morse, J. H., Heremans, J. F., J. Lab. Clin. Med., 1962, v59, 891.

11. Yagi, Y., Maier, P., Pressman, D., Arbesman, C. E., Reisman, R. E., Lenzner, A. R., J. Immunol., 1963, v90, 760.

Received February 14, 1966. P.S.E.B.M., 1966, v122.