Separation of [^{131}I]Insulin-Antibody Complexes and of Antibodies by Disc Electrophoresis in Polyacrylamide Gels*

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Patterns of soluble antigen-antibody complexes were studied by disc electrophoresis of [^{131}I]-insulin reacted with guinea pig anti-insulin sera, a nonprecipitating system. There was ready separation of free and antibody-bound [^{131}I]insulin and resolution of three or more frictionally distinct antigen-antibody peaks, present in proportions varying with the ratio of reactants. The eluate from the center of each peak showed its original mobility upon re-electrophoresis and could be converted to material of lower mobility by reaction with additional antiserum. Antisera alone were also subjected to disc electrophoresis and antiserum fractions were eluted. Patterns obtained by disc electrophoresis of reaction mixtures of [^{131}I]insulin and these single antiserum fractions showed marked differences among individual antiserum fractions and between each of them and its parent whole antiserum. Results indicated that antibody macroheterogeneity and at least one other mechanism, most likely mass action, were responsible for the [^{131}I]insulin-antibody patterns found with individual or pooled whole antisera. Participation of a ternary component was excluded (in the case of complement and conglutinin) or shown to be very unlikely.

Multiple peaks, presumably consisting of soluble antigen-antibody complexes and varying in proportions with the ratio of reactants, were demonstrated by ultracentrifugation or free electrophoresis of solutions of an antigen-antibody precipitate in excess antigen (Heidelberger and Pedersen, 1937; Pappenheimer *et al.*, 1940; Oncley *et al.*, 1952; Singer and Campbell, 1952, 1953, 1955a,b,c; Singer *et al.*, 1955; Becker, 1953) or excess antibody in the case of a "flocculating" system (Pappenheimer *et al.*, 1940). These phenomena were assumed to be simply manifestations of mass action effects involving one multivalent antigen and one multivalent antibody, despite the likelihood that both were heterogeneous.

Now application of disc electrophoresis in polyacrylamide gels (Ornstein and Davis, 1961) to a nonprecipitating system, [^{131}I]insulin plus guinea pig anti-insulin serum, has permitted improved study of soluble antigen-antibody complexes. Heterogeneity of the [^{131}I]insulin appears negligible. Absence of precipitation precludes errors due to occlusion of inert protein in a specific precipitate (Makinodan *et al.*, 1960; Williams and Donermeyer, 1962) and, together with the sensitivity of detection of labeled antigen of high specific activity, permits study over the complete range of antigen-antibody ratios. Disc electrophoresis affords high resolving power, rapidity, sensitivity, inherent data on molecular frictional properties, and convenient sampling and elution of fractions for further analysis. As a result, multiple [^{131}I]insulin-antibody peaks have been resolved well and their patterns have been related systematically to reactant ratios. With the help of disc-electrophoresis fractionation of antiserum alone, at least two mechanisms have been implicated in the patterns of [^{131}I]insulin-antibody peaks. Application of disc electrophoresis to separating free from antibody-bound antigen has also been illustrated.

Methods and Materials

All procedures, except preparation of protein-free reagent solutions, extrusion and cutting of gel columns after electrophoresis, and counting of radioactivity, were carried out at 0–5° with precooled solutions.

Disc Electrophoresis.—This method of electrophoresis, in cylindrical vertical polyacrylamide gel columns in parallel between buffer-containing electrode dishes, entails discontinuities in voltage gradient, "pore size," and *p*H and solute composition (Ornstein and Davis, 1961). The procedure of Ornstein and Davis (1961) was followed in essence, using standard-*p*H 5 or 7.5% polyacrylamide hard gel, 0.5 cm in diameter by about 5.5 cm, below 0.20 ml of the standard 2.5% spacer gel but no sample gel; sucrose-containing liquid samples were placed on top of spacer gels (Reisfeld *et al.*, 1962). Electrophoresis progressed downward toward the anode at a constant current of 2.5 ma per column. "Stacking" (i.e., formation of a sharp starting protein zone) during traverse of the spacer gel occurred at *p*H 8.3, while electrophoretic separation took place at *p*H 9.5 in the hard gel. Each experiment included six to eight samples over a range of ratios of the given antiserum to [^{131}I]insulin at 2- to 4-fold intervals. "Diluent," [^{131}I]insulin, antiserum, and/or control serum (see subsequent sections for specifications) were added successively to sample test tubes to make a total volume of 40–100 μl, constant in each experiment. Thorough mixing followed each addition. No more than 5 μl of antiserum was used, to avoid protein overloading. After standing overnight, samples received one-fourth to one-third their volume of saturated aqueous sucrose. The radioactivity was then counted and the samples were placed on the spacer gels. Buffer was layered carefully on each sample and electrophoresis was started promptly. Runs continued until the albumin band (in 5% gel) or the leading albumin component (in 7.5% gel) had descended about 4 cm[1] or else two, three, or four times this long. Immediately after electrophoresis the gel tubes were placed in a freezer for 15–30 minutes. Then gel columns were removed under (room-temperature) water, as soon as they began thawing, by means of a 0.56-mm-diameter hard stainless steel wire (Malin) with rounded ends, which was worked gently, with rotation, between gel and glass tube full length, rotated around until free, and pulled out slowly (with further wire rotation if necessary) so as to pull the gel column along and de-

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[1] 40–43 minutes in 7.5% gel, 31–34 minutes in 5% gel.

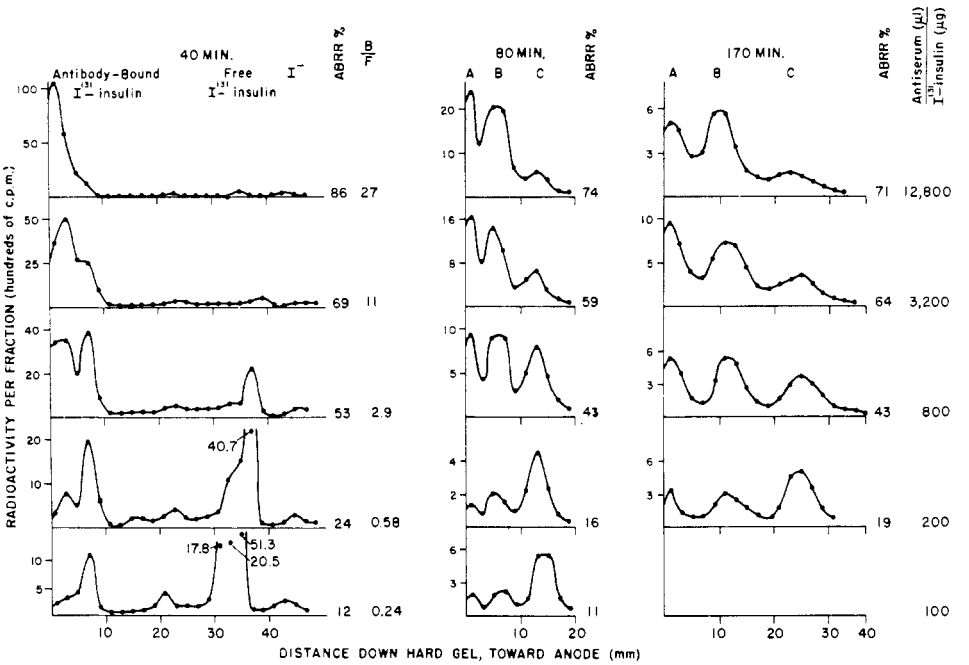

FIG. 1.—Disc electrophoresis patterns of [^{131}I]insulin plus antiserum, at three different times of migration. Berson antiserum. 7.5% gel.

$$\frac{B}{F} = \frac{\text{antibody-bound } [^{131}\text{I}]\text{insulin (cpm)}}{\text{free } [^{131}\text{I}]\text{insulin (cpm)}}$$

Note: Except for Figure 1, left series, and Figure 4-V, only the antibody-bound region of radioactivity is illustrated. These peaks are identified by capital letters. ABRR % = per cent antibody-bound radioactivity recovery.

posit it on the slotted-trough base of the gel cutter.[2] After being lifted from the water on the cutter base and stripped of spacer gel with a small spatula, the hard-gel column (now completely thawed) was cut into 2-mm-thick fractions with the multiwire cutting frame.[2] Each fraction was transferred, with a hardwood applicator stick, into 1 ml of water in a test tube. If fractions were to be eluted, column removal was effected under water at 2–5°, the cutter base was kept on ice during cutting and transfer of fractions, and the latter were put into 300–500 µl of cold "diluent" for elution in the refrigerator 1–3 days, with occasional stirring during the daytime.

By experiments with control guinea pig serum, the total serum concentration (up to 5 µl serum per sample) was shown to be without influence.

Counting of Radioactivity.—Gamma radiation was counted in a transistorized (Picker) well-type scintillation counter, background 180–200 counts per minute (cpm). Unaccounted losses in the sample transfer pipets were minimized by rinsing each one in 4 M urea in 0.15 M NaCl containing 0.25% human serum albumin and adding the rinse to the empty sample tube before counting again.

Representation of Data.—Except in Figure 1, left series, and Figure 4-V, only part of the column, that with antibody-bound radioactivity, is shown. Spacer (soft) gel, although always assayed for radioactivity, is not graphed. For mobility comparisons, the value for a peak is the average of all columns showing the peak clearly in that experiment. Each vertical series of curves represents a single experiment, and each figure includes only experiments on the same lot of [^{131}I]-insulin.

Diluent.—Cold 0.25% human serum albumin in 0.15 M NaCl, freshly prepared from refrigerated stock (Cutter) 25% human serum albumin, was used to dilute antisera or [^{131}I]insulin.

Antisera.—Anti-insulin sera from single guinea pigs were provided by Drs. S. A. Berson and J. R. Leonards, and the latter also supplied a high-titre, pooled guinea pig antiserum. Berson antiserum, elicited by subcutaneous injections of Lilly protamine-zinc beef insulin (Yalow and Berson, 1960), was kept frozen in their laboratory but sent by mail at ambient (spring) temperatures, while the Leonards antisera, elicited by subcutaneous injection of Lilly glucagon-free pork zinc insulin in lanolin plus paraffin (Robinson and Wright, 1961), were kept frozen, even in transit. Sera and their dilutions were kept frozen in this laboratory except when they were thawed at 2–5° for use.

^{131}I-Labeled Insulin.—^{131}I-labeled pork insulin, 150–300 µc/µg, in solution (*ca.* 0.8–1.2 µg/ml) in 1% albumin, was received frozen from Abbott Laboratories. Original and diluted solutions were kept frozen in this laboratory except when they were thawed at 2–5° for use. Results with different lots were closely comparable. No significant changes in disc electrophoretic behavior were evident during the 3–4 weeks a given preparation was used. The content of ^{131}I iodide and radiation-damaged [^{131}I]insulin in each preparation was checked by the paper chromatoelectrophoresis method of Berson *et al.* (1956; Yalow and Berson, 1960) initially and at 2 and 4 weeks. Of the total radioactivity, damaged [^{131}I]insulin remained less than 5%, while ^{131}I iodide, originally less than 5%, increased slowly to 10% or less at 4 weeks. Virtually complete radiochemical

[2] This miniature "egg slicer" consists of two parts: the base, an aluminum block hollowed by a hemicylindrical, column-fitting trough, closed at one end and bearing 0.15–0.25-mm-thick transverse slots with centers 2 mm apart; and a matching aluminum cutting frame bearing taut, parallel 0.10–0.13-mm-diameter hard stainless steel wires (Malin) with centers 2 mm apart.

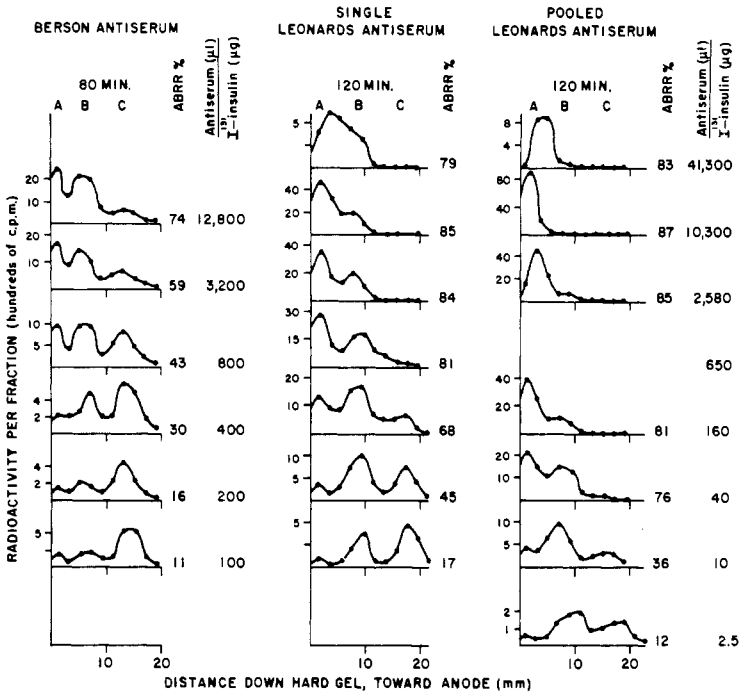

FIG. 2.—Patterns obtained with [^{131}I]insulin plus three different antisera. 7.5% gel. See *Note*, Fig. 1.

purity obtained in the region of interest in the disc-electrophoresis system studied: [^{131}I]insulin alone (with albumin) or with control guinea pig serum or very low proportions of antisera appeared as a single, sharp peak at about the same mobility as the albumin band in 5% gel (1.2–1.3 mm/min) or the faster albumin component in 7.5% gel (0.9–1.0 mm/min); this peak was progressively obliterated by increasing amounts of antiserum (Fig. 1, left). Ahead of the free [^{131}I]insulin was a rather broad, low peak with 4–10% or less of the total sample radioactivity, unchanged by antiserum. Presumably this peak represented ^{131}I iodide, since it was found to have this mobility (slightly faster than [^{131}I]insulin). A few per cent of the total sample radioactivity also remained in the soft (spacer) gel and in a low peak between the free [^{131}I]insulin and the antibody-bound regions; both of these areas were also unaffected by antiserum and might represent damaged [^{131}I]insulin components of different sizes (Berson and Yalow, 1957; Yalow and Berson, 1960). Radioactivity left in the soft gel was high in the case of a bovine [^{131}I]insulin preparation showing a high content of damaged [^{131}I]insulin by paper chromatoelectrophoresis.

RESULTS

Free vs. Antibody-bound [^{131}I]*Insulin.*—Free [^{131}I]-insulin was readily separated from the [^{131}I]insulin-antibody complexes by even rather brief electrophoresis (Fig. 1). With increasing proportions of antiserum to [^{131}I]insulin, the free [^{131}I]insulin peak was reduced progressively and counts increased correspondingly in the antibody-bound area. No precipitate was seen in the soft gel or atop or in the hard gel. With migration of $\leq$45 minutes, so that free [^{131}I]insulin was still on the column (Fig. 1, left), total recovery of radioactivity in different columns of a given experiment varied from 74 to 94% with 7.5% gel and from 66 to 96% with 5% gel. These recoveries were correlated with the proportion of the [^{131}I]insulin bound to antibody.

[^{131}I]*Insulin-Antibody Patterns Obtained with Whole Antisera.*—The maximum per cent antibody-bound radioactivity recovery was 79–89% in various experiments in which free [^{131}I]insulin was run off the columns. Figure 1 illustrates the resolution of components, obtained with Berson antiserum, with increasing distance of migration in 7.5% gel. Figure 2 compares the patterns from the three different antisera in 7.5% gel. Figure 3 compares 7.5% with 5% gel in the farthest migrations studied, with Berson and pooled Leonards antisera; Table I summarizes the mobility data obtained therein.

Patterns observed were the same with or without the presence of 0.010 M, *p*H 7.4 EDTA in the samples throughout the reaction of [^{131}I]insulin with antibody.

Up to the range of antibody excess, the location of highest antibody-bound radioactivity progressed from faster to slower position as the ratio of antiserum to [^{131}I]insulin increased. Further resolution showed this progression passing by way of at least three distinct, reproducible peaks, with the slow peak (A) predominant at high ratios of antiserum to antigen, the intermediate peak (B) predominant at somewhat lower ratios, and the fastest peak (C) predominant at even lower ratios. Corresponding peaks could be identified with different antisera (Figs. 2 and 3), distances of migration (Figs. 1, 2, and 3), or polyacrylamide-gel concentrations (Fig. 3).

In 7.5% gel the A peak did not move beyond the first fraction ($\leq$2 mm). However, in 5% gel, migration for 66–68 minutes permitted resolution into A and A′ (ultraslow) peaks (Fig. 3). Otherwise, peaks were more sharply defined and better separated in 7.5% gel (Fig. 3). The reduction of mobility by greater concentration of gel was more marked for slower than faster peaks (Table I), an effect referable to molecular frictional resistance rather than to charge density (Raymond and Aurell, 1962). Elution data (see next section) demonstrated corresponding differences in average diffusion rate for components of central fractions of A, B, and C peaks in 5% and 7.5% gel.

In the achievable range of increasing antibody excess, although the per cent antibody-bound radioactivity recovery remained maximal, there was a

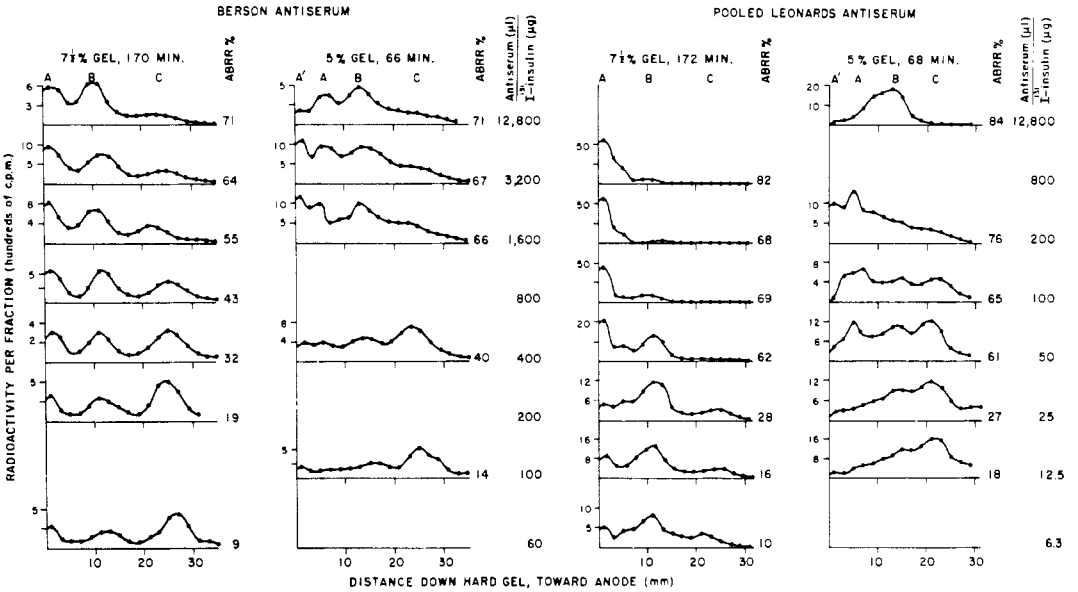

Fig. 3.—Patterns obtained on longer runs in 7.5% gel and 5% gel, with [^{131}I] insulin plus two different antisera. See *Note*, Fig. 1 .

TABLE I
MOBILITIES OF CORRESPONDING [^{131}I]INSULIN-ANTIBODY PEAKS IN 5% AND 7.5% GEL[a]

	Berson Antiserum			Pooled Leonards Antiserum		
	Mobility (100 × mm/min)		Mobility in 5%	Mobility (100 × mm/min)		Mobility in 5%
Peak	5% gel	7.5% gel	Mobility in 7.5%	5% gel	7.5% gel	Mobility in 7.5%
A	9.7	1.2	8.2	8.4	1.2	7.2
B	21.4	6.7	3.2	20.6	6.9	3.0
C	37.1	14.4	2.6	32.4	14.3	2.3

[a] Disc electrophoresis 66–68 minutes in 5% gel or 170–172 minutes in 7.5% gel at 2.5 ma/column. Every mobility figure is the average of several individual data, each of which represents the mid-point of a 2-mm fraction.

TABLE II
ELUTION[a] OF CENTRAL FRACTIONS[b] OF [^{131}I]INSULIN-ANTIBODY PEAKS

Antiserum	Gel (%)	Electrophoresis Time (min)	Elution Time (hr)		Recovery (%) A	B	C
Berson	5	65	22		40	70 (2 fract)	82 (2 fract)
Single Leonards	7.5	120	66		26	52	75
					20	39	
					20	40	
				Mean	22	41	
						55	
						Mean 45	
Pooled Leonards	7.5	120	66		14	48	
					15	49	
					25	58	
					18	52	
				Mean	18	Mean 52	
Pooled Leonards	7.5	170	22		8	38	
						28 (2 fract)	
						Mean 33	

[a] At 2–5° in 400 µl (A) or 300 µl of "diluent." See Methods. [b] Single fraction per peak unless noted to be two fractions pooled (2 fract).

limited and variable degree of reversal of the trend toward increasing dominance of the A peak (Fig. 2, middle and right; Fig. 3, left).

The three antisera showed general similarity of patterns and the same mobility for corresponding peaks (Figs. 2 and 3; Table I) but several definite differences. The pooled, high-titre Leonards antiserum achieved a pattern consisting of only peak A at sufficient ratios of antiserum to [^{131}I]insulin, while the single antisera of Leonards and Berson still showed B present. This was more pronounced with Berson antiserum, which also showed some C when A was maximal. Although

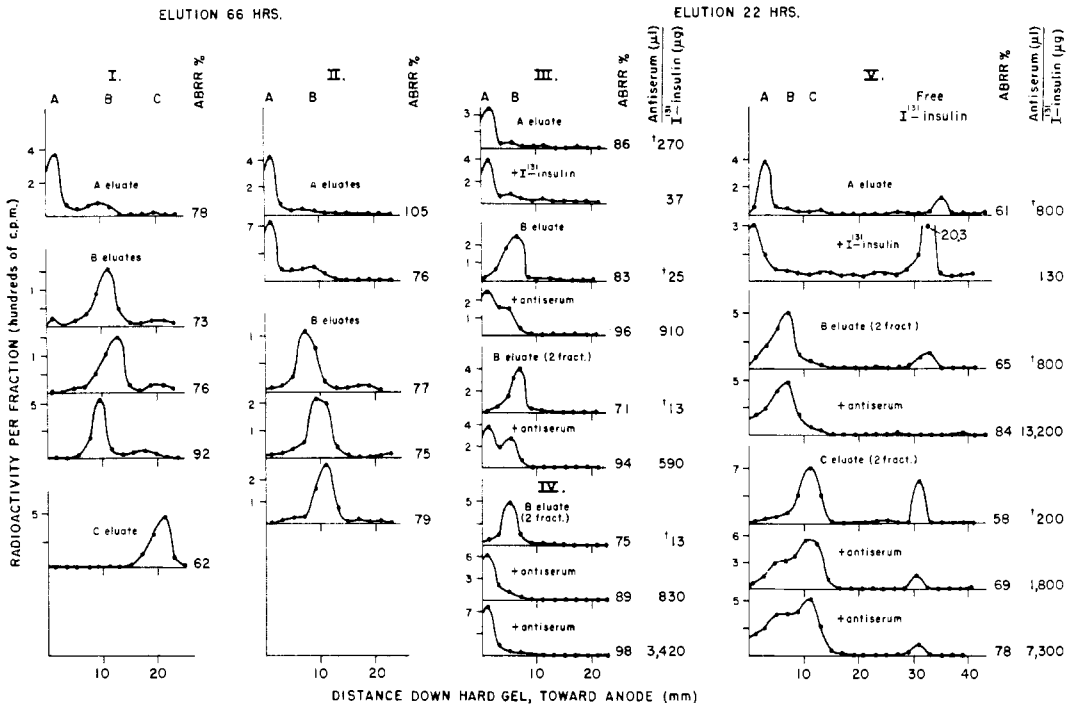

FIG. 4.—Patterns found on re-electrophoresis of eluates from the central fraction (or two fractions if noted) of [^{131}I]insulin-antibody complex peak A, B, or C, alone or with added [^{131}I]insulin or antiserum. See *Note*, Fig. 1.

Antiserum	First Electrophoresis		Second Electrophoresis	
	Minutes	Gel (%)	Minutes	Gel (%)
I. Single Leonards	120	7.5	120	7.5
II. Pooled Leonards	120	7.5	120	7.5
III. Pooled Leonards	170	7.5	86	7.5
IV. Pooled Leonards	65	5	85	7.5
V. Berson	66	5	25	5

† Calculated on the assumption that the given peak had the same ratio of antiserum (µl)/[^{131}I]-insulin (µg) as the whole sample from which it was derived by the first electrophoresis.

these differences correlated with differences in total antibody titre (pooled Leonards > single Leonards > Berson), this factor could not have been the sole cause, since the development of A shown by either single Leonards or Berson antiserum revealed a maximum before the highest ratio of antiserum to [^{131}I]insulin was reached. Conversely, even at low per cent antibody-bound radioactivity recovery the C peak did not predominate with the pooled Leonards antiserum but did with the two single antisera. The three antisera also showed distinctly different points of transition between peaks or of maximum percentage of a given peak (referred to per cent antibody-bound radioactivity recovery to avoid bias due to titre). Finally, when per cent antibody-bound radioactivity recovery was plotted versus logarithm of the antiserum-[^{131}I]insulin ratio, the maximum slope (found in the region between 25 and 50% antibody-bound radioactivity recovery) differed for the three antisera: pooled Leonards > single Leonards > Berson.

Elution and Re-electrophoresis of [^{131}I]*Insulin-Antibody Peaks Obtained with Whole Antisera.*—The central one or two (combined) fractions from each of the three main [^{131}I]insulin-antibody peaks, obtained by disc electrophoresis 65–66 minutes in 5% gel or 120 or 170 minutes in 7.5% gel, were eluted with 400 µl (A) or 300 µl (B or C) of "diluent" at 2–5° for 22 or 66 hours. Recovery from the gel was in the order C > B > A (Table II). Sixty-six-hour eluates were promptly subjected to electrophoresis again. Twenty-two-hour eluates, after separation from their gel fractions, stood 19 or 43 hours further alone or with added amounts of the original antiserum (in the case of B or C eluates) or [^{131}I]insulin (in the case of A eluates) before electrophoresis.

Re-electrophoresis of these eluates showed mostly or entirely one peak with the mobility found in the original electrophoresis (Fig. 4).[3] In one case (not illustrated) a single fraction behind the C peak was eluted, and re-electrophoresis showed substantial amounts of both C and B peaks, distinctly separated without evidence of an intermediate component. If the B or C eluates had been reacted with antiserum, they were converted partly or wholly to slower peaks, depending on the amount of antiserum added (Fig. 4-III, IV, V). By comparison of per cent antibody-bound radioactivity recovery in eluates run alone and after reaction with excess antiserum, it was calculated that 16–23% dissociation of free [^{131}I]insulin from A, B, or C eluates occurred in the 22-hour elution plus 19- or 43-hour further standing.

[^{131}I]*Insulin-Antibody Patterns Obtained with Single Antiserum Fractions Eluted from Previous Disc Electro-*

[3] Because of low total counts in the samples for re-electrophoresis, running times were chosen to afford separation of the three major peaks but minimize excessive spreading. Hence the ability to resolve a minor component in the resulting patterns was somewhat limited.

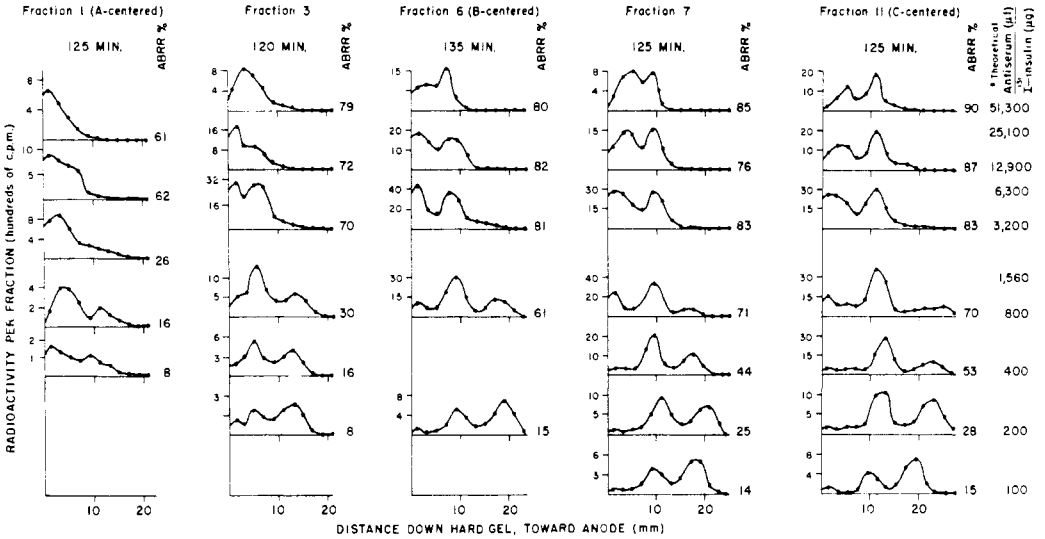

FIG. 5.—Disc electrophoresis patterns of [131I]insulin plus single antiserum fractions eluted 70 hours from previous disc electrophoresis of pooled Leonards antiserum 120 minutes. Both electrophoreses in 7.5% gel. See *Note*, Fig. 1. *Note:* Locations of eluted antiserum fractions are referred to peaks in "guide" columns (samples: [131I]insulin plus the antiserum) run in parallel with antiserum columns. * Theoretical antiserum (µl)/[131I]insulin (µg) was calculated by assuming that each antiserum fraction contained all the original 5 µl of parent antiserum under electrophoresis and elution was 100%.

phoresis.—Samples (5 µl) of pooled or single Leonards antiserum were subjected to disc electrophoresis for 120 minutes in 7.5% gel, in parallel with "guide" samples consisting of various ratios of the same antiserum to [131I]insulin (allowed to stand overnight). The "guide" columns' fractions were counted to indicate the location of peaks A, B, and C for reference. Fractions from the plain antiserum columns were eluted 70 hours in 500 µl of "diluent" at 2–5° and stored frozen. Subsequently, single eluted antiserum fractions from locations corresponding to the centers of A, B, and C or intermediate locations were reacted overnight with various proportions of [131I]insulin. Disc electrophoresis of these samples was then carried out 120–135 minutes in 7.5% gel. Total antibody recoveries, as shown by ability to bind [131I]insulin (Figs. 5, 6), were in this order: fractions from pooled Leonards antiserum > corresponding fractions from single Leonards antiserum, and C-located > B-located > A-located fractions for a given antiserum. Recovery decreased progressively at locations faster than C. Data on recoveries from fractions midway between A, B, and C locations were not sufficient to prove or refute antibody contents lower than those of the above-mentioned fractions.

As shown in Figures 5 and 6, single eluted antiserum fractions differed from their parent whole antiserum (Fig. 2) and among themselves in ways not attributable to titre alone. A-Located fractions (fraction 1) gave rise to [131I]insulin-antibody patterns which resembled those seen with the upper range of parent antiserum (ratio to [131I]insulin) and showed only slight B and negligible C development as per cent antibody-bound radioactivity recovery decreased. The first, dominant peak was somewhat broader (or diphasic) and faster than A in patterns from the parent antiserum. C-Located fractions (Fig. 5, fraction 11; Fig. 6, fraction 9 or 10) had much higher titre, yet their [131I]insulin-antibody patterns resembled those seen with the middle and lower range of parent antiserum; they evolved from predominance of B to predominance of C, while A and B diminished, as per cent antibody-bound radioactivity recovery decreased. The C peak was better developed than it was in patterns from the parent antiserum, especially in the case of pooled Leonards antiserum (Fig. 5). C-Located antiserum fractions also showed a degree of "reversal" phenomena at highest ratios to [131I]insulin, even though the A peak was limited in size. B-Located fractions gave rise to [131I]insulin-antibody patterns intermediate in character, more like those from C-located fractions in the case of pooled Leonards antiserum (Fig. 5).

The location of peak B in [131I]insulin-antibody patterns obtained with various single antiserum fractions usually showed a modest correlation with the location from which the fraction was eluted (Fig. 5).

DISCUSSION

Separation of Free and Antibody-bound Antigen.—Free and antibody-bound [131I]insulin, present in reaction mixtures of pork [131I]insulin and guinea pig anti-pork or anti-beef insulin sera, were separated easily by disc electrophoresis. This rapid method of separation should prove generally applicable to soluble systems, even when the free antigen and its antibody complexes have similar isoelectric points. Since antigen-antibody complexes are always larger than the free antigen, the sieving effect of the polyacrylamide gel (Raymond and Weintraub, 1959) should lead to separation of free from bound antigen unless the antigen is remarkably large, highly asymmetric, or very basic.

Fractionation of [131I]Insulin-Antibody Complexes and Mechanisms of Their Patterns.—Disc electrophoresis for a sufficient length of time separated antibody-bound [131I]insulin into at least three peaks (A, B, and C), representing individual or coherent groups of complexes. Comparison of electrophoresis in 5% with that in 7.5% gel showed the peaks to differ in molecular friction, presumably reflecting molecular size and shape. Repeat disc electrophoresis showed the reproducible mobility of these peaks and did not reveal intermediate peaks, at least between B and C. Since even a 7 S γ-globulin has a molecular size and frictional effect many times that of [131I]insulin (Ornstein and Davis, 1961), it is likely that the mobility of an [131I]insulin-antibody complex in disc electrophoresis reflects chiefly size, shape, and number of antibody molecules contained. The existence of these distinct antigen-antibody peaks

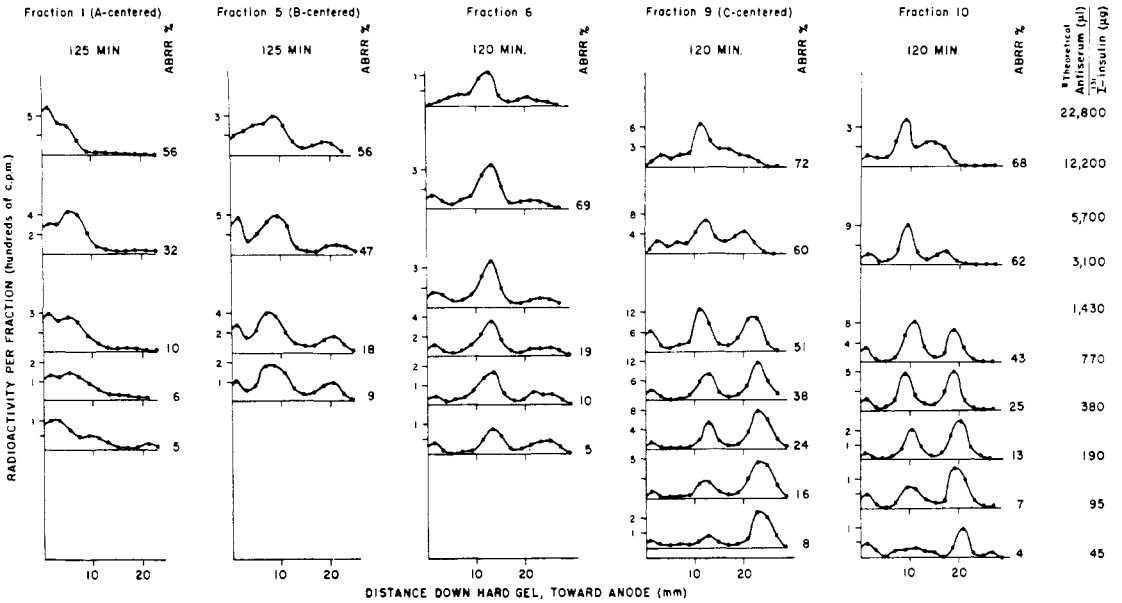

FIG. 6.—Disc electrophoresis patterns of [^{131}I]insulin plus single antiserum fractions eluted 70 hours from previous disc electrophoresis of single Leonards antiserum 120 minutes. Both electrophoreses in 7.5% gel. * See *Notes*, Figs. 1 and 5.

and the variation of their proportions with the ratio of antiserum to antigen reacted might be attributed to either mass action or antibody heterogeneity in the antiserum, if the larger (or more asymmetrical) antibodies had tighter binding but lower total capacity for the antigen (Talmage, 1957; Jerne, 1951; Kabat and Mayer, 1961; Boyd, 1962; Mannik and Kunkel, 1963). These mechanisms were elucidated as follows:

Some differences among their [^{131}I]insulin-antibody patterns suggested that the three antisera contained different "spectra" of antibodies. Disc electrophoresis of reaction mixtures of [^{131}I]insulin and single antiserum fractions, which had been eluted from previous disc electrophoresis of antiserum alone, proved gross antibody heterogeneity (macroheterogeneity) in each antiserum studied, since there were marked qualitative differences among [^{131}I]insulin-antibody patterns obtained with different fractions from a given antiserum. Antibody macroheterogeneity was also shown indispensable to the sequence of [^{131}I]insulin-antibody patterns seen with various ratios of whole antiserum to [^{131}I]-insulin, since single antiserum fractions could give rise to only a limited part of the [^{131}I]insulin-antibody patterns obtained with their parent antiserum. These findings applied to either individual or pooled antisera.

Conversely, mass action effects (multiple compounds of one multivalent antibody with one multivalent antigen) were suggested by the fact that the more mobile antiserum fractions gave rise to [^{131}I]insulin-antibody patterns showing only B and A peaks at high per cent antibody-bound radioactivity recovery but predominately the C peak at lower per cent antibody-bound radioactivity recovery. These effects might instead be attributed to specific microheterogeneity of antibodies, i.e., different antibody molecules specific for different antigenic determinants of insulin and thus able to combine singly or simultaneously with the same [^{131}I]insulin molecule. Such differences in combining site have been shown among whole antisera from different species or different individuals of the same species (Arquilla and Finn, 1963) but have been neither demonstrated nor refuted among multiple antibodies within an individual antiserum. In contrast, Yagi *et al.* (1962) have shown, in individual guinea pig anti-insulin sera, two distinct antibodies readily separated by chromatography on DEAE-cellulose or by electrophoresis on granular starch (and hence presumably differing markedly in charge) but indistinguishable in molecular size or binding properties.

It is unlikely that the observed antibody-bound [^{131}I]insulin patterns were dependent upon the presence of a third component, bound to the antigen-antibody complexes and affecting size, shape, and hence molecular friction of the resolved radioactive components. Participation of either complement or conglutinin in the observed [^{131}I]insulin-antibody complex patterns was excluded by the fact that patterns were the same in the absence or presence of 0.01 M EDTA during reaction (Kabat and Mayer, 1961; Sage *et al.*, 1963). Conglutinin also is not found in significant quantities in guinea pig serum. Macroglobulins would not be expected to enter the 7.5% hard gel (Ornstein and Davis, 1961), and apparently they did not bind to the [^{131}I]insulin-antibody complexes, since only a few per cent of the total sample radioactivity was excluded from the hard gel, regardless of the ratio of antiserum to [^{131}I]insulin. Actually rheumatoid factor (Vaughan, 1956; Vaughan and Butler, 1962), which would not be expected in guinea pig sera, is the only macroglobulin proved to bind actively to antigen-antibody complexes. In contrast, coprecipitation of inert macroglobulin with antigen-antibody precipitates has been reported by several workers (Makinodan *et al.*, 1960; Williams and Donermeyer, 1962) but is irrelevant to the nonprecipitating system used in the present work. Although their possible existence cannot be denied categorically, other components have not been proved to participate actively in ternary reactions with antigen and antibody and thus cannot be given any definitive consideration.

Fractionation of Antibodies.—As mentioned above, disc electrophoresis of either individual or pooled guinea pig anti-insulin sera must have separated two or more antibody entities—individuals or, more likely, coherent groups. These results agree with the consensus of physicochemical evidence that there are a few major groups of antibodies with lesser differences (microheterogeneity) in size, charge, shape, etc. within each group (Mannik and Kunkel, 1963; Mandy *et al.*, 1963).

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Immunochemical Studies on the Tobacco Mosaic Virus Protein. I. The Immunological Relationship of the Tryptic Peptides of Tobacco Mosaic Virus Protein to the Whole Protein*

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The immunochemical activity of the tryptic peptides of tobacco mosaic virus protein was studied in a system composed of the viral protein and antiprotein, using complement fixation and complement-fixation inhibition. A low-molecular-weight fraction ($G_{50}S_2$) of a tryptic digest of the protein was obtained by fractionation on G-50 Sephadex. Peptide mapping revealed that this fraction contained all the characteristic tryptic peptides of tobacco mosaic virus protein except the N-terminal peptide (peptide 1). (The nomenclature of the tryptic peptides of tobacco mosaic virus protein is according to that proposed by A. Tsugita, D. T. Gish, J. Young, H. Fraenkel-Conrat, C. A. Knight, and W. M. Stanley [1960, *Proc. Natl. Acad. Sci. U. S. 46*, 1463].) Peptide 1 was isolated by repeated isoelectric precipitation at *p*H 4.7. Neither the $G_{50}S_2$ nor peptide 1 fixed complement with anti-tobacco mosaic virus protein serum. However, the $G_{50}S_2$ completely inhibited the fixation of complement by tobacco mosaic virus protein and anti-tobacco mosaic virus protein, whereas peptide 1 was inactive. The peptides in the $G_{50}S_2$ fraction were isolated by Dowex 1 × 2 ion-exchange chromatography followed by paper chromatography. The immunochemical activity of $G_{50}S_2$ in the tobacco mosaic virus protein and anti-tobacco mosaic virus protein system could be attributed to tryptic peptide 8 having the amino acid sequence: Ileu-Ileu-Glu-Val-Glu-AspNH_2-GluNH_2-Ala-AspNH_2-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg.

Immunological activity related to that of the whole protein has been obtained from fragments of several protein antigens (Cebra, 1961; Porter, 1959; Goodman, 1963; LaPresle and Durieux, 1957; Press and Porter, 1962). A report from our laboratory demonstrated inhibition of systemic anaphylaxis in egg albumin–sensitive guinea pigs by fragments of egg albumin (Benjamini *et al.*, 1962); however, proteins of known amino acid sequence greatly facilitate the study of antigenic determinants, as exemplified by the study of

* A preliminary report on this study has been presented (Young *et al.*, 1964).