Lack of Dissociation between Insulin and Antibody from Guinea Pig* (33026)

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During recent studies involving the separation of antibody-bound and free insulin-^{131}I by enzyme proteolysis (1), exchange between labeled and stable insulin in antiserum of guinea pig origin was questioned. Although the behavior of insulin in antiserum from insulin-treated individuals has received considerable attention (2), and the displacement of insulin-^{131}I by native hormone was clearly demonstrated (3), comparable studies with antibody from guinea pigs have apparently not been done. Therefore a series of experiments were designed in which the exchange between labeled and stable insulin could be evaluated by comparing patterns of insulin-^{131}I radioactivity after gel electrophoresis of antiserum.

Materials and Methods. Samples of undiluted antiserum obtained from guinea pigs immunized against crystalline pork insulin were divided equally among a series of tubes which were then separated into two groups. One group contained tubes into which stable pork insulin (1.0 and 16.0 units/ml) was added, whereas pork insulin-^{131}I (2.0 μC/ml, specific activity 30 mC/mg) was added to the tubes in the other. Both sets of tubes were placed in a water bath at 37°C for 45 min, after which pork insulin-^{131}I was mixed in with the stable insulin and crystalline insulin was added in turn to the tubes containing only radioinsulin. All tubes were placed in the water bath once again for 45 min and then set aside at room temperature for an additional 75 min. At the end of that time and for 6 consecutive days thereafter, samples of serum were removed and subjected to electrophoresis on agar gel. Between times the tubes were stored at 4°C.

A 1.5-gm sample of oxoid "Ionagar" number 2 agar was dissolved in 100 ml of barbital buffer (pH 8.6, ionic strength 0.05, diluted 1:1 with distilled water) by heating in a boiling water bath for approximately 20 min. Two to 3 ml of the hot gel were spread evenly over the surface of a glass microscope slide (7.5 × 2.5 cm) and a vertical slit was made 3.0 cm from one end by pressing the heated metal tip of a spatula blade into the cooled gel.

Twenty μl of serum were delivered into the slit with a micropipette and after all serum samples had been applied, the slides were placed on a horizontal support in a closed leucite cell. A sheet of heavy blotting paper, previously moistened with buffer solution, covered the ends of the gel and extended into baffled compartments containing barbital buffer (pH 8.6, ionic strength, 0.05) and platinum wire electrodes. An electric potential of 200 V (approximately 25 mA/10 slides) when applied for 2 hours at room temperature was usually sufficient to produce the desired migration. At the conclusion of the electrophoresis the slides were placed on the moderately heated surface of an electric hot plate to coagulate the serum proteins and to expedite drying of the gel. After autoradiographs were made on X-ray film, the zones of radioactivity along the slide were quantified by means of an integrated strip scanner, and the protein bands stained with amide black (Fig. 1).

Results. The characteristic pattern obtained when insulin-^{131}I was added to guinea pig antiserum was distinguished by a zone of radioactivity surrounding the origin (Fig. 2). Although subsequent enrichment with stable insulin caused a slight shift in the major peak of insulin-^{131}I, and the appearance of a minor band of radioactivity in the region of albumin, the overall pattern was completely different from that seen when

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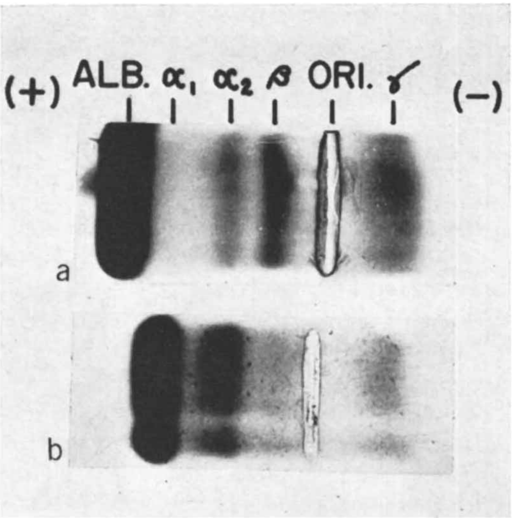

FIG. 1a. Human serum; 1b, Guinea pig serum. Serum protein stained with amide black after agar gel electrophoresis. Antiserum obtained from an insulin-resistant diabetic patient and from guinea pig immunized against pork insulin.

stable insulin was added before the labeled hormone. In the latter instance, insulin-^{131}I was found to migrate in zones that corresponded to albumin and prealbumin; areas that have been shown previously to be associated with free insulin-^{131}I in agar gel (4).

Figure 3 shows that extending the period of reaction to 96 hours (or longer) resulted in patterns of radioactivity that were essentially the same as those obtained with the 2-hour serum sample. This emphasizes the relatively irreversible nature of the bond between antibody and the labeled or stable insulin. The results were reproducible not only with the identical lot of antiserum, but antisera from different guinea pigs immunized against pork insulin also yielded similar patterns.

Antiserum, however, obtained from insulin-resistant diabetic patients reacted completely differently from that of the guinea pig antibody. Exchange between labeled and stable insulin was almost immediately apparent in undiluted serum from resistant patients. Whether the labeled or the stable insulin was added first made little difference in the outcome of the patterns of distribution of radioactivity for the patterns were virtually identical (Fig. 4). Most of the exchange between

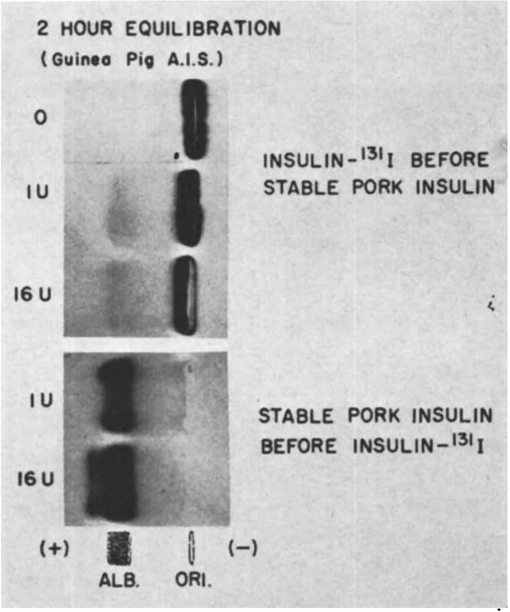

FIG. 2. Autoradiographs made from dried agar gel slide. Upper set of autoradiographs obtained from guinea pig antiserum to which radioinsulin had been added before the stable hormone. Lower set of autoradiographs show electrophoretic distribution of radioactivity when stable insulin was added prior to the insulin-^{131}I.

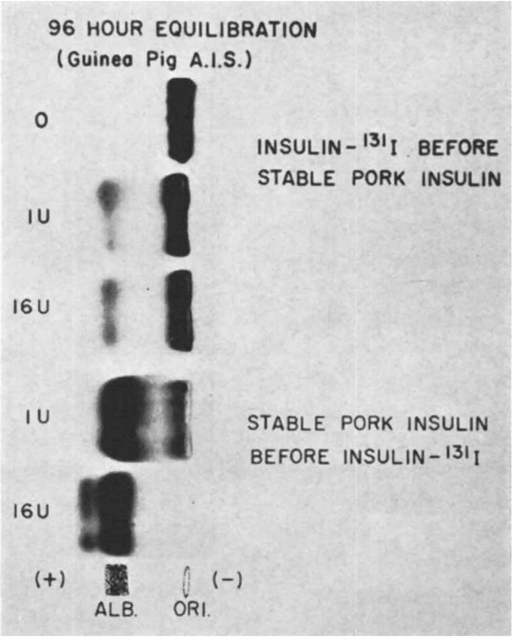

FIG. 3. Autoradiographs obtained from electrophoresis of guinea pigs antiserum after 96 hours of reaction with labeled and stable insulin.

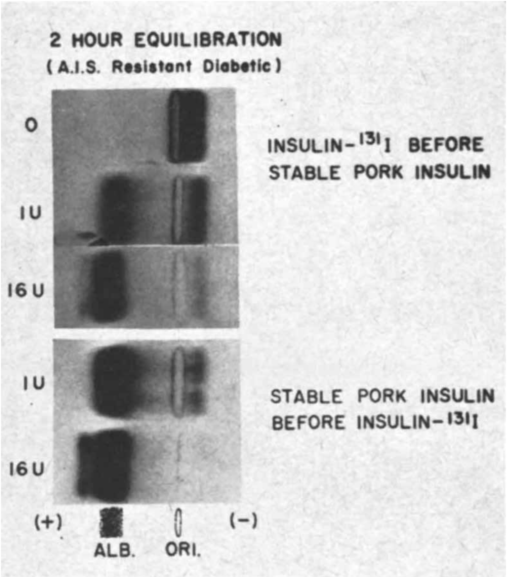

FIG. 4. Autoradiographs made from agar gel electrophoresis of serum from an insulin-resistant diabetic patient. Two hours of reaction with labeled and stable insulin.

insulin-^{131}I and crystalline insulin took place rapidly; the sample of serum taken from the 96-hour period of equilibration (Fig. 5) resulted in a pattern that was virtually the same as that obtained with the 2-hour sample.

The various peaks of radioactivity seen in the radioautographs under the conditions outlined for both guinea pig and human antiserum correspond closely to the percentage values of radioactivity which were obtained by calculations from the integrated strip scanner.

Discussion. In view of previous results demonstrating the relatively unimpeded dissociation–association phenomena of insulin in human serum from insulin-treated diabetic patients (2), it was somewhat unexpected to find an almost total absence of exchange between insulin-^{131}I and stable insulin in antiserum from immunized guinea pigs. This was made all the more striking by the rapid exchange that took place between the same lots of insulin in antiserum from insulin-resistant diabetic patients.

The results obtained with guinea pig antiserum suggest that dilution rather than exchange may be the more important consideration in the mechanism that underlies the radioimmunoassay of insulin. The fact that antiserum in most immunoassay systems is diluted hundreds or thousands of times probably minimizes the importance of the sequence in which various constituents are added. However, our unpublished results suggest that the results of our immunoassay for insulin (1) can be influenced significantly and the sensitivity of the system enhanced if the labeled insulin is added several hours (6 or more) after the addition of the stable hormone. If, on the other hand, insulin-^{131}I and stable insulin are added to the diluted antiserum within a short time of one another the results are essentially the same as those obtained by the addition of the antiserum directly to a mixture of labeled and stable hormone.

The lack of exchange between insulin-^{131}I and crystalline insulin in antiserum from guinea pigs has implications not only from the standpoint of immunoassay procedures in general but also in terms of kinetic systems that have been described for antigen–antibody reactions in other species (2). Whether this also applies to other hor-

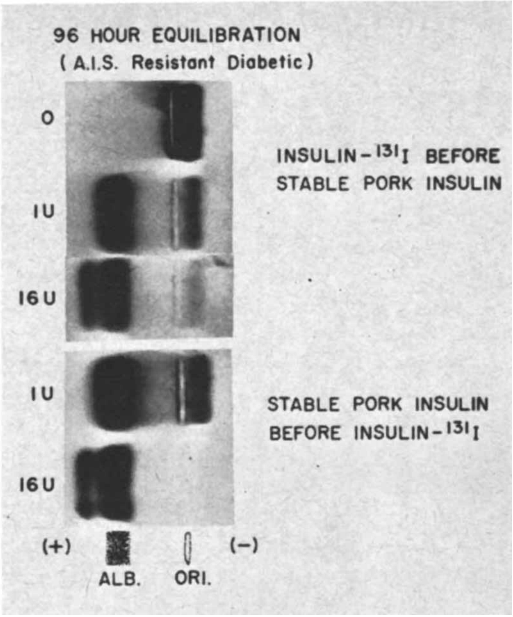

FIG. 5. Autoradiographs from electrophoresis of serum after 96 hours of reaction.

mones and their specific antibodies should make an interesting subject for further investigation.

Summary. Studies assessed the extent to which insulin-^{131}I and unlabeled insulin exchanged in antiserum from immunized guinea pigs. Comparisons of patterns of radioactivity obtained after agar gel electrophoresis of antiserum containing labeled and stable insulin, added in reversed sequences, revealed almost no exchange between insulin-^{131}I and crystalline insulin.

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Passive Hemolytic Antibody Response of Primed, Partially Resistant Mice to Heat-Killed *Salmonella typhimurium* Vaccine* (33027)

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There is considerable literature concerning the response to *Salmonella typhimurium* of mice in terms of increased resistance and antibody formation following administration of various types of immunizing agents. In some cases, attempts have been made to correlate these two parameters. Kenny and Herzberg (1) were able to demonstrate that the rise in bactericidal antibody corresponded to development of resistance against challenge with viable organisms. They also indicated that appreciable levels of resistance and antibody formation occurred whether the immunizing agent was a killed vaccine, or a live avirulent one. Other workers have felt that inactivated vaccines are of relatively little value in enhancing resistance as compared to live organisms (2,5).

The present study suggests that the efficacy of a heat-killed vaccine depends to a considerable extent on the spacing of priming dose and challenge, and that circulating antibodies formed against the "O" antigen (lipopolysaccharide) do not correlate particularly well with the resistance induced.

Materials and Methods. Bacterial cultures. *S. typhimurium*, strain SR-11[1] was routinely maintained by subculture on Trypticase soy agar[2] (TSA). The subcultures were held at 5°C. New stock cultures were revived from lyophilized suspensions at approximately 6-month intervals.

Viable bacterial suspensions for challenging mice were prepared by growing the organisms on TSA for 16–18 hours at 37°C, and then suspending the growth in 0.85% sodium chloride solution. The optical densities of the suspensions were adjusted to a reading of 440 using the blue (no. 47) filter on the Klett photoelectric colorimeter. Tenfold serial dilutions were prepared and were counted by the method of Miles *et al.* (6) using calibrated 18-gauge dropping needles. The counts of such suspensions remain constant within the limits of error of the counting method for at least 3 hours. Counts of the Klett-adjusted suspensions varied between 5.22 to 5.89 × 10^9 viable cells/ml.

Determination of the 50% lethal dose (LD$_{50}$). Graded doses of the suspension were inoculated in 0.2-ml quantities intraperitoneally into mice. Deaths among the animals

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[1] Kindly furnished by Dr. R. E. Chamberlain, Smith Kline and French Laboratories, Philadelphia, Pennsylvania.

[2] Difco Laboratories, Detroit, Michigan.