ment varied significantly in different strains but bore no relationship to occurrence of degenerative joint disease, or to ultimate epiphyseal development of knee or head of femur previously found to occur late in second year of life.

Grateul acknowledgement for statistical computations is made to Joan Gurian.

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Heterogeneity of Insulin-Antibody Complexes in Rabbits and Guinea Pigs. (25570)

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Presence of insulin-binding antibodies in human subjects treated with commercial mixtures of beef, pork insulin(1) and in guinea pigs and rabbits immunized with beef or pork insulin(2) has been established with use of insulin-I^{131} and paper chromatoelectrophoresis of insulin antiserum mixtures. Kinetic studies of rates of formation and dissociation of insulin-antibody complexes and analysis of equilibrium mixtures have indicated the heterogeneity of complexes formed in human antiserums(3-6). Heterogeneity in this case appears to be due to presence of 2 (or more) distinct orders of antibody combining-sites rather than to differences in stoichiometric proportions of insulin and antibody in the complexes(5,6). Since human antiserums contain antibodies to both beef and pork insulins, heterogeneity of insulin-antibody complexes might be due to antibodies directed against different antigenic determinants in beef and pork insulin or to separate antibodies directed against multiple distinct antigenic sites in each insulin derived from a single species(6). In our study guinea pigs and rabbits have been immunized with *either* beef *or* pork insulin. Insulin-binding antibodies in antiserums of these animals react with insulin to form heterogeneous antigen-antibody complexes.

Methods. Analytic and technical methods follow procedures described by Berson and Yalow(1,5,6). In "equilibrium state" studies, mixtures of antiserum and beef or pork insulin (containing trace amounts of same species' insulin-I^{131}) were incubated at 37° until equilibrium was reached (usually about 4 hours) and then analyzed by paper chromatoelectrophoresis for insulin bound to antibody(B) and unbound ("free") insulin (F). Considerations based on the law of mass action indicate that when antiserum concentration is kept constant while insulin concentration is varied, the ratio B/F is a linear function of concentration of bound insulin (B) only *if* insulin is univalent *and* all antibody combining-sites are of a single order(5,6). A curvilinear relationship is expected if insulin is multivalent and/or if there is more than a single order of antibody combining-sites. In 5 guinea pig and 3 rabbit antiserums studied, plots of B/F *vs.* B were curvilinear (Fig. 1), a finding which is consistent with either of the last 2 alternatives.

Results. In "transient state" studies, only dissociation experiments are analyzed. Following attainment of equilibrium between

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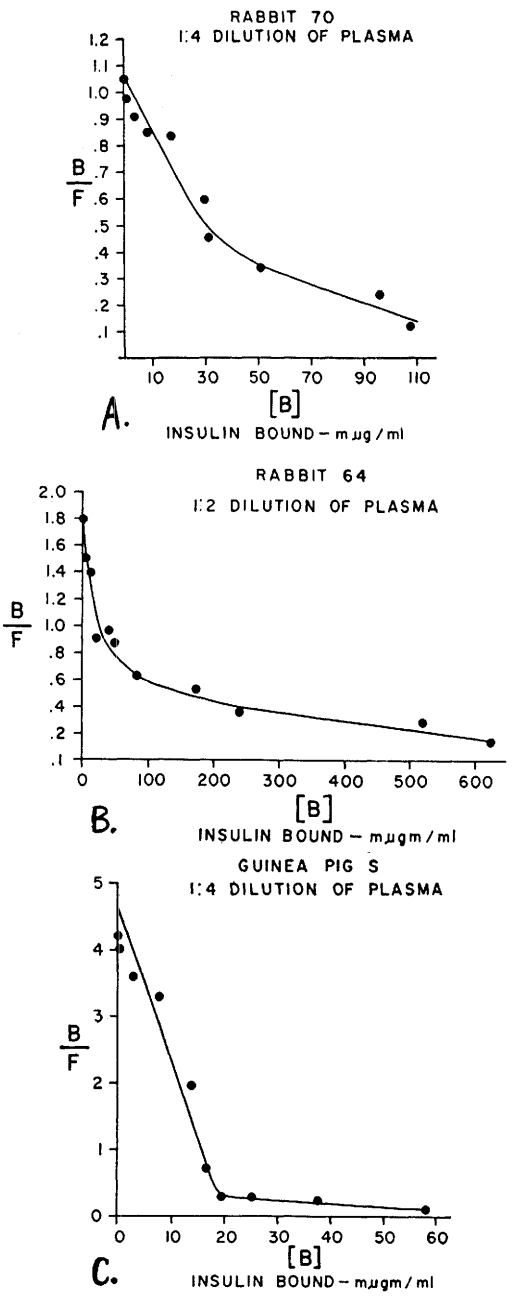

FIG. 1A. Ratio of bound to free insulin as a function of quantity of insulin bound for anti-beef insulin plasma of a rabbit incubated with crystalline beef insulin.

FIG. 1B. Ratio of bound to free insulin as a function of quantity of insulin bound for anti-pork insulin plasma of a rabbit incubated with crystalline pork insulin.

FIG. 1C. Ratio of bound to free insulin as a function of quantity of insulin bound for anti-beef insulin plasma of a guinea pig incubated with crystalline beef insulin.

bound and free insulin, high concentrations of unlabeled insulin were added to the mixtures. Further binding of insulin-I^{131} is now competitively inhibited and rate of "dissociation" of insulin antibody complexes is determined from decrease in concentration of bound insulin-I^{131} with time. The complexes exhibited heterogeneity in rates of dissociation in all experiments. In the 2 rabbit antiserums analyzed, the dissociation curves could be resolved into 2 components with half times averaging 9 and 8 minutes and 2¼ and 7 hours respectively (Fig. 2a). However the 5 guinea pig antiserums appeared to have a greater degree of heterogeneity, and resolution of the experimental curves required more than 2 exponential components (Fig. 2b).

Discussion. That antigen-antibody com-

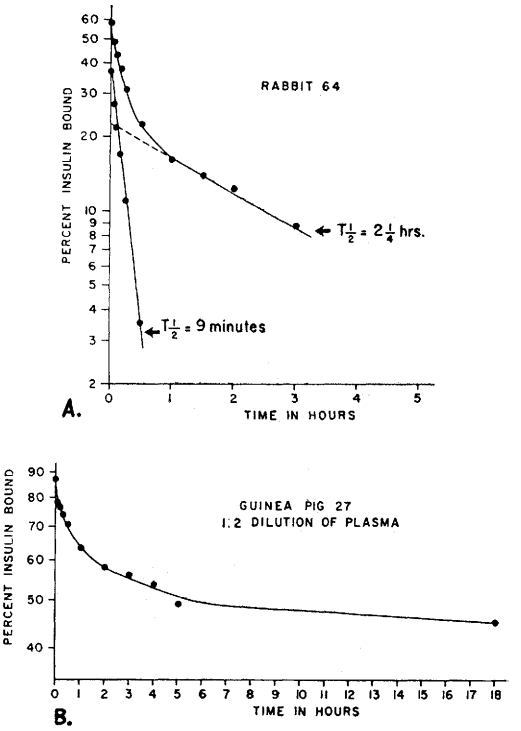

FIG. 2A. A typical rate curve of dissociation for rabbit insulin-antibody complexes. This plasma was from a rabbit immunized with pork insulin and was incubated with crystalline pork insulin-I^{131}.

FIG. 2B. A typical rate curve of dissociation for guinea pig insulin-antibody complexes. This plasma was from a guinea pig immunized with beef insulin and was incubated with beef insulin-I^{131}.

plexes are heterogeneous† is generally not unexpected inasmuch as numerous sites of antigenic determinancy on most antigen molecules are responsible for multivalency of antigen in its reaction with antibody. With insulin as an antigen, however, univalency might be anticipated from studies of Harris, Sanger and Naughton(7). These authors showed that insulins from 5 different mammalian species (cattle, sheep, pig, horse, whale) differ in amino acid sequence only in the 8-10 positions of A chain. Immunologic differences in reaction of these insulins with human anti-beef, pork insulin serums suggest that this region includes a site antigenic for man(8,11). If guinea pig and rabbit insulin likewise differ from other mammalian insulins only in this respect it might be expected that antigenicity would reside only in the relatively small site in question and that the insulin monomer (M. W. 6000) could therefore bind only a single antibody molecule under any circumstance. Moreover, even if beef or pork insulin exhibits more than a single antigenic site to guinea pigs and rabbits, the small size of the insulin molecule might still preclude the binding of 2 (or more) antibody molecules simply because of steric hindrance(11). The possibility that beef or pork insulin possesses other sites of possible antigenicity in guinea pigs and rabbits cannot be excluded until the structures of guinea pig and rabbit insulin are established. (Lacy and Davies(12) obtained evidence, with fluoroscein labeled insulin antibodies, that guinea pig and rabbit insulins are immunologically different from beef insulin and several other mammalian insulins.)

From data presented here it can only be concluded that *either* insulin is multivalent *or* that the antibodies are heterogeneous or, lastly, that both conditions exist. In human antiserums the weight of evidence(6) appears to favor univalency (though not necessarily monoantigenicity). A more complete description of the nature of insulin-antibody complexes in rabbits and guinea pigs must await more extensive studies with these antiserums.

Summary and conclusions. Heterogeneity of insulin-antibody complexes in antiserums of rabbits and guinea pigs immunized with *either* beef insulin *or* pork insulin has been demonstrated. Heterogeneity of complexes in antiserums of human subjects immunized with commercial mixtures of beef *and* pork insulins need not be attributed to presence of distinct antibodies directed against beef and pork insulins.

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† Although heterogeneity of complexes is observed even with univalent haptens(9,10), the heterogeneity is related to Gaussian distribution of binding energies and is rather less marked than in insulin-antibody complexes which, in man(5,6) and rabbits exhibit 2 main groups with grossly different dissociation rates.

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