5. Most of the rabbit antisera (6 out of 7) agglutinated tanned red cells coated with rabbit submaxillary globulin. 6. By hemagglutination inhibition tests, one antiserum (#1565) was found to be inhibited by submaxillary and parotid extracts. The other antisera tested were specific for submaxillary gland antigens. These results agree with those obtained by complement fixation.

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Production of Glucagon Antibodies and their Role in Metabolism and Immunoassay of Glucagon.* (26665)

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Lack of specificity is a significant disadvantage of certain technics for bioassay of glucagon(1-4). Recently developed methods for immunochemical assay of insulin(5,6) appeared adaptable to the specific measurement of glucagon. This report describes the development of antibodies to glucagon in rabbits treated during a 22-month period, the influence of the antibodies on the metabolism of glucagon and their potential use for immunochemical assay of this hormone. Recently, an independent study of similar nature was reported by Unger *et al.*(7).

Methods. Recrystallized porcine glucagon (Eli Lilly & Co., Lot No. 258-234B-93) was used. This preparation contained no detectable amount of insulin when measured by an immunochemical assay described previously(5). Glucagon-I^{131} was prepared by a method used for labeling of insulin-I^{131}(8). Specific activity of the preparations varied from 2.5 to 12.0 mc/mg.

Two of 4 male albino rabbits, weighing 2.5-3.0 kg, were immunized with glucagon suspended in either Bayol-Arlacel or Freund's adjuvants, as described by Hayashida and Li(9). The other 2 animals were immunized with glucagon conjugated to ovalbumin with bis-diazo benzidine(10), similarly suspended in the adjuvants. All 4 animals were given subcutaneous injection of the hormone in doses of 3-10 mg at varying intervals over a 2-year period. Serum samples were obtained 2-3 weeks after each series of injections, frozen and stored.

Titers for glucagon antibodies in serum were determined by a modification of the method previously employed for measuring insulin antibodies(5). Glucagon-I^{131}, 0.02 μg, was incubated for 1 hr at room temperature with 0.2 ml of undiluted serum or of serum serially diluted with 5% human serum albumin. Aliquots, 0.1 ml, were subjected to hydrodynamic flow on paper in veronal buffer, pH 8.6, for 1 hr at room temperature. In this system free glucagon remains at the origin, while antibody-bound glucagon travels as a single band with the serum proteins (11). After the papers had been dried and

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cut into 1 cm segments, distribution of I^{131}-activity was determined by counting the strips in a well-type scintillation counter. Total activity in the bound fraction divided by total activity in the free fraction (B F ratio) was plotted against the dilution of the serum employed. The dilution at which 50% of the chromatographically intact glucagon was effectively bound was taken as the titer value. Chromatographically intact glucagon-I^{131} was taken as the per cent of the glucagon-I^{131} preparation which remained at the origin (hydrodynamic flow) after incubation with pooled normal rabbit serum. For the various glucagon-I^{131} preparations used, this value routinely was 85-90% of the total label.

The influence of immunization on rate of clearance of exogenously administered glucagon was studied in the surviving animal, one of the pair immunized with glucagon conjugated to ovalbumin. Glucagon-I^{131}, 4 μg in 2 ml of saline, was injected into the rabbit's marginal ear vein. Serum was then collected at intervals during a 6-hr period for determination of radioactive content. Four control rabbits of equal age were similarly treated. To allow comparison of results between animals of different weight, the percentage of injected dose in the circulation was calculated as follows:

$$\% \text{ of injected dose} = \frac{\text{Activity/ml serum} \times 0.03‡ \times \text{body wt (g)}}{\text{Total injected activity}} \times 100.$$

Since both glucagon-I^{131} bound to antibody and degraded glucagon-I^{131} move from the origin during hydrodynamic flow at pH 8.6, these two states of the hormone are not differentiated in serum containing circulating glucagon antibodies. Binding of glucagon-I^{131} to antibody, however, is pH dependent (Fig. 1); it does not occur below pH 4 and reaches a maximum at pH 8-8.5. Degradation products of glucagon-I^{131} in both immune and normal sera were therefore quantitated by adjusting serum pH to 2.5 with 1 N HCl and subjecting an aliquot to hydrodynamic flow in 0.3 N acetic acid. Thus degradation could be determined in sera containing relatively large amounts of antibody. No difference in percentage of degradation of glucagon-I^{131} was found in preparations of normal sera subjected to flow at pH 2.5 and at pH 8.6. The preferential salt precipitation technic used previously for immunochemical assay of insulin(5) was applied to glucagon with one modification: precipitation with sodium sulfate at 25°C, which produces a more compact, more easily centrifuged precipitate, was substituted for precipitation by sodium sulfite at 4°C.

‡ Based on serum volume estimated as 3.0% body weight.

Results. Development of glucagon antibodies. None of the sera from untreated animals bound the chromatographically intact glucagon. The development of glucagon antibody titers in rabbits given multiple injections of antigen during the 22-month period is shown in Fig. 2. In all 4 animals, antibody was detectable within 1 month. Two and one-half months later, after the animals had received 3 more injections, the titer of the sera was increased in 3 of the 4 rabbits. At the end of a 5-month rest period all titers had fallen, but they rose significantly again in the surviving rabbits following 2 booster injections. During a second rest period of 9 months, the titers again dropped. At 18 months, the animals still responded to antigen by producing antibody. By 22 months, however, the surviving animals were comparatively unresponsive to the same antigenic stimulus.

Characterization of antisera. None of the sera tested bound insulin-I^{131}, suggesting that the antisera to glucagon did not contain any detectable amount of antibodies to insulin. Although precipitin reactions did not occur when varying amounts of free glucagon were added to the antiserum from each of the rabbits immunized with the glucagon-diazo-ovalbumin, strong precipitin reactions were readily obtained when glucagon-diazo-ovalbumin itself was added. Preincubation of glucagon with large excesses of antisera did not inhibit the hyperglycemic activity of the hormone as determined by injecting the mixture into normal, fasted cats.

Effect of antibodies on clearance of glucagon-I^{131}. Three minutes after injection of

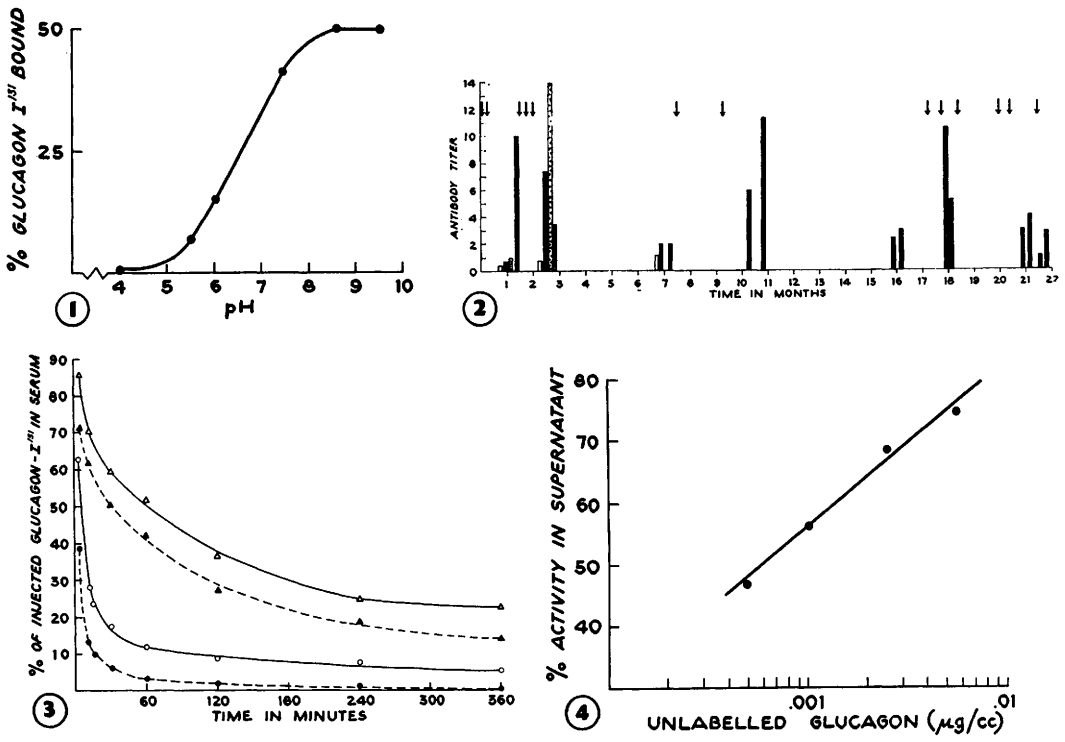

FIG. 1. Effect of pH on binding of glucagon-I^{131} by rabbit antisera. Buffer systems and experimental technic as described by Grodsky *et al.*(8).

FIG. 2. Development of antiglucagon titers in rabbits after intermittent inj. of glucagon preparations suspended in adjuvants. Open bar, striped bar = free glucagon in adjuvants; dotted bar, solid bar = glucagon conjugated to ovalbumin with bis-diazo benzidine and suspended in adjuvants. Arrows indicate inj. of antigen.

FIG. 3. Radioactivity of serum of immunized and normal rabbits after inj. of glucagon-I^{131}. △—△ = immunized rabbit, total serum radioactivity. ▲---▲ = immunized rabbit, serum radioactivity representing chromatographically intact glucagon. Values represent mean of 2 experiments. ○—○ = 4 normal rabbits, total serum radioactivity. ●---● = 4 normal rabbits, serum radioactivity representing chromatographically intact glucagon. Values represent mean of 2 experiments on each of the 4 animals.

FIG. 4. Effect of concentration of unlabeled glucagon on binding of glucagon-I^{131} to antibodies obtained from immunized rabbit.

glucagon-I^{131} into an immunized rabbit, 85% of the label was present in the serum (Fig. 3). As determined by hydrodynamic flow at pH 8.6, 82-95% of the glucagon left the origin as either bound or degraded hormone. Examination of the serum at pH 2.5 disclosed that only 15% of this glucagon was degraded. The glucagon remained comparatively intact and was only slowly cleared from the circulation in the subsequent 6 hr. In the normal rabbits, binding of glucagon could not be demonstrated. Both clearance and degradation of glucagon were rapid; at 1 hr, only 3% of the chromatographically intact hormone remained, compared to 42% in the immunized animal.

Immunoassay for glucagon. When glucagon-I^{131} and sufficient antisera to bind 50% of the label were incubated in glycine-albumin buffer(5) with increasing concentrations of unlabeled glucagon, the unlabeled glucagon displaced the labeled hormone from the antibody. After precipitation of the bound glucagon with Na_2SO_4 through the range 0.0005-0.005 μg of glucagon per ml, the per cent activity displaced into the supernatant was proportional to the log of the glucagon concentration (Fig. 4). Therefore, the preferential salt precipitation technic previously reported for immunoassay of insulin appears adaptable for detecting small amounts of glucagon.

Discussion. Our observations and those of Unger *et al.*(7) leave little doubt that glucagon, when injected into rabbits in suitable adjuvant, produces specific antibodies which bind but do not precipitate glucagon. In our study, measurable antibody appeared in the serum within one month of injection of either glucagon or conjugated glucagon as antigen. Subsequently, the level of antibody increased progressively after repeated antigen injections. After resting periods of 5-9 months, antibody titers declined as expected, but a significant level of circulating antibody was still present in each animal.

The dissociation of glucagon from its antibody *in vitro* with decreasing pH followed a curve almost identical to that previously reported for insulin and its antibody(8) and made possible the development of a technic for measuring both degraded glucagon and immunochemically bound glucagon in aliquots of the same serum. The marked retention of glucagon-I^{131} bound to antibody in the circulation of immunized rabbits was similar to that reported in similar insulin studies(12). The sensitivity of the antibody-bound glucagon to "glucagonase" activity in the liver may be decreased, since degradation products appeared far more slowly in the serum of the immunized rabbit than in sera of the control animals. It is noteworthy that the inhibited clearance of insulin and glucagon in presence of their respective antibodies is the exact reverse of the phenomenon described for most other antigen-antibody systems(13), namely, increased clearance of the complex. Both insulin and glucagon are proteins of low molecular weight and are not precipitated with their specific antisera. Possibly such soluble complexes are removed by the liver through a different mechanism than that required for clearance of precipitating antigen-antibody complexes.

The application of the preferential salt precipitation technic makes possible the use of glucagon-I^{131} of low specific activity for immunochemical assay of extremely small amounts of glucagon.

Summary. The antibody content of sera from rabbits treated intermittently with free glucagon or glucagon conjugated with ovalbumin was measured at intervals during a 2-year period. Circulating nonprecipitating antibody, capable of binding glucagon-I^{131}, was detectable within one month, and was still detectable after 4 to 5 month periods during which no antigen was administered. Glucagon-I^{131} injected into an immunized rabbit was rapidly bound to antibody, remained chromatographically intact and was only slowly cleared from the circulation. Glucagon-I^{131} could be dissociated from its antibody at acid pH. The preferential salt precipitation technic used for immunochemical assay of insulin proved applicable to measurement of minute amounts of glucagon.

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