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Induction of Antibodies to Porcine ACTH in Rabbits with Nonsteroidogenic Polymers of BSA and ACTH* (33011)

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The production of antibodies in laboratory animals to adrenocorticotropic hormone has been demonstrated by several techniques including neutralization of the biological activity of ACTH (1–4), hemagglutination inhibition (5) and binding of ^{131}I labeled ACTH (6). Radioimmunoassays for ACTH in plasma have been developed based upon the displacement of ^{131}I from specific antibody by the cold ACTH of plasma (7, 8). Widespread use of these assays has been somewhat inhibited because of the difficulty in regularly obtaining antisera of sufficient titer and appropriate binding properties. Difficulty in obtaining antisera to ACTH can be readily understood. Firstly, ACTH is a small polypeptide of 39 amino acid residues. It is well known that small polypeptides are poor immunogens per se. This may be due to their rapid renal excretion or perhaps other less well understood properties affecting initiation of an immune response.

In addition, and perhaps more significantly, free ACTH is, of course, steroidogenic and the steroids thus elicited result in depression of antibody synthesis (9). This problem has been partially circumvented by the use of mepirapone, a chemical agent which blocks 11-β hydroxylation of the steroid nucleus (7).

We postulated that both of these problems might be overcome by (i) coupling ACTH to a larger molecule such as serum albumin, and (ii) that in analogy to studies with bradykinin coupled to polylysine (10) the complex might be biologically inactive.

Materials and Methods. Coupling of ACTH to bovine serum albumin (BSA). A solution was made containing 20 mg of BSA (Armour fraction V) and 6 mg of porcine ACTH (Wilson lot No. 137106) dissolved in 2 ml of 0.1 *M* phosphate buffer pH 7.0. One ml of a glutaraldehyde solution, 0.021 *M*, was added dropwise to this BSA–ACTH solution with constant stirring. Under these conditions essentially all the ACTH is coupled to BSA. This was proven in 2 ways: (i) Ultracentrifugation of the above complex revealed complete disappearance of slowly sedimenting ACTH. (ii) Gel filtration of the coupled material demonstrated complete disappearance of strongly retarded ACTH from the elution profile of a calibrated Sephadex G 100 column. This Sephadex column completely resolves free BSA from free ACTH. After

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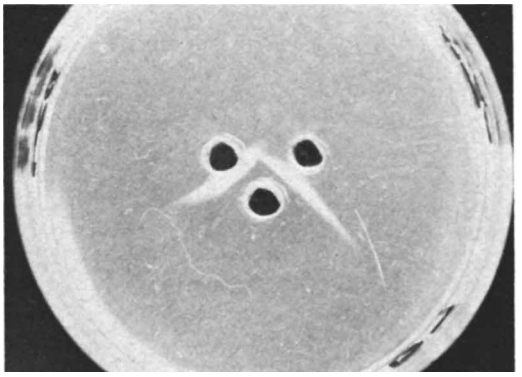

FIG. 1. The center well contains rabbit antiserum No. 139B4. In the upper left is BSA–ACTH and on the right are BSA polymers; both are at a concentration of 1.0 mg/ml.

coupling, both proteins eluted together in the first peak.

Immunization. Eight male New Zealand white rabbits were immunized by the following procedure: 1.2 mg of BSA–ACTH complex in 1.0 ml of saline was emulsified in 1.0 ml of complete Freund's adjuvant. This was injected into the toe pads, intramuscularly, and into multiple intradermal sites. At 10 days, a second injection of 1.2 mg of complex with Freund's adjuvant was given intramuscularly. Thereafter the animals were bled from the central ear artery at 3, 4 and 6 weeks, and biweekly thereafter.

Demonstration of antibodies. Antibodies were shown to be directed against ACTH by agar diffusion and complement fixation (11).

Demonstration of neutralization of the biological activity of ACTH. Bioassay was performed as follows: Female rats of the Holtzman strain weighing 200 ± 25 gm were hypophysectomized. Two hours later, the test substance was injected into a femoral vein and both adrenal glands removed exactly 5 min after the injection. Adrenal steroids were extracted in chloroform: methanol 2:1 (v/v) and corticosterone content was determined by acid fluorescence (12). In such experiments, 0.01 μg of ACTH was incubated with 0.1 ml rabbit antiserum in a volume brought to 0.5 ml with 0.1 *M* phosphate pH 7.0. The incubation was carried out overnight at 4°C. Controls were the same concentration of ACTH and 0.1 ml preimmunization serum from the same rabbit. There were five animals injected in each group.

Results. Figure 1 shows a typical Ouchterlony pattern of serum 139B4 reacting with BSA–ACTH and BSA polymers. Each of the antigen solutions is at a concentration of 1.0 mg of protein per ml. Note the marked spurring of BSA–ACTH over BSA polymers. The BSA polymers were prepared with glutaraldehyde in a manner similar to the BSA–ACTH but with omission of the ACTH. That polymerization of the BSA had occurred was demonstrated by ultracentrifugation. Antisera from all eight rabbits showed such spurring when BSA–ACTH was compared to BSA polymers with each of these antisera. That this spurring represented specific antibodies to ACTH was proven by the following observation: The antisera were each absorbed with 1.0 mg of BSA polymers/ml of antiserum. After this, no antiserum precipitated or fixed complement with either free BSA or BSA polymers. All these sera gave

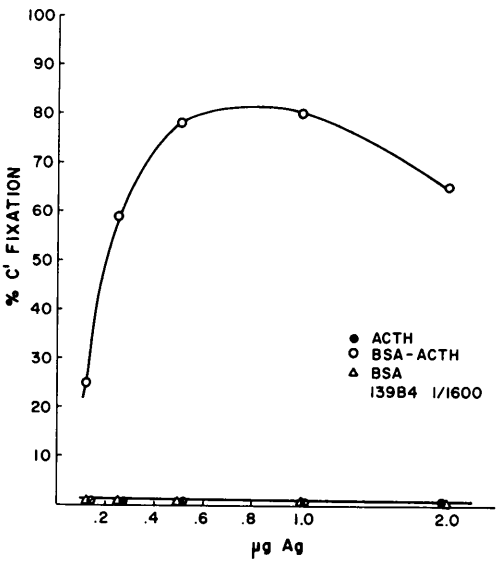

FIG. 2. Complement fixation by increments of BSA–ACTH (○), BSA polymers (△), and free (×) with serum 139B4 at a dilution of 1/1600. This serum had been absorbed with sufficient BSA to remove any reactivity with BSA. The complement fixation buffer has a BSA concentration of 1.0 mg/ml.

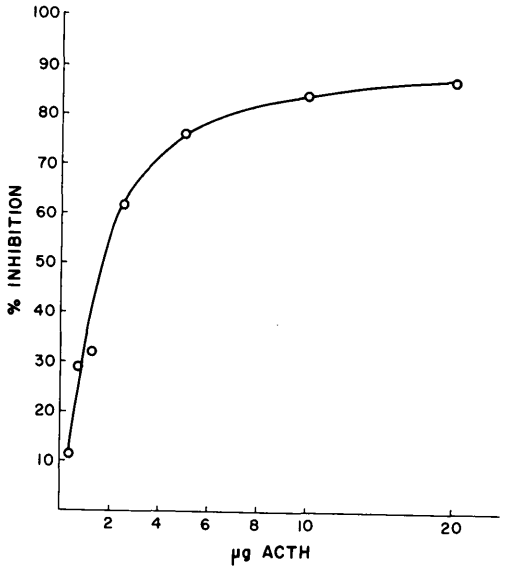

FIG. 3. Inhibiton of complement fixation of 0.5 μg BSA–ACTH with serum 139B4 (absorbed with BSA) by increments of free ACTH. Serum used at dilution of 1/1600.

good complement fixation reactions with BSA–ACTH. Free ACTH neither precipitated nor fixed complement with any of the sera before or after absorption with BSA. However, the entire reaction of BSA–ACTH with these absorbed sera was inhibited by free ACTH. These data are illustrated by typical experiments in Figs. 2 and 3. Figure 2 is a typical complement fixation reaction by the method of Wasserman and Levine (11) at a serum dilution of 1/1600. Figure 3 demonstrates the almost complete inhibition of BSA–ACTH reacting with the BSA absorbed serum 139B4 by free ACTH. Note that 50% inhibition occurs with 1.5 μg of ACTH. All eight antisera showed high complement fixation titers of BSA–ACTH reacting with BSA absorbed serum. In all cases, the reaction was completely blocked by ACTH. All sera from all bleedings have been tested and high complement fixation titers still persist 6 month after immunization.

A second type of experiment which demonstrated that antibodies were directed only toward ACTH after the antiserum was absorbed with BSA was performed: ACTH was coupled to rabbit serum albumin (FV, Pentex Corp., Kankakee, Ill.) in a manner identical to that described above. The equivalent antigenicity of BSA–ACTH and RSA–ACTH when reacting with serum 139B4 (previously absorbed with BSA) is demonstrated in Fig. 4. Thus, the reaction is independent of the carrier and no reactions would be expected or in fact could be demonstrated with RSA, as the sera are in any case prepared in rabbits.

The data regarding the neutralization of the biological activity of ACTH are summarized in Table 1A. All of our sera were titered with BSA–ACTH after absorption with BSA so that the complement-fixing activity which was specifically blockable by ACTH could be compared. The ability of the highest, and lowest titered sera to block the biological activity of ACTH was then tested. There was complete blocking of biological activity of ACTH by both antisera.

Lastly, it was demonstrated that concentrations of BSA–ACTH complex comparable to those used in the immunization were nonsteroidogenic in rats. These data are summarized in Table 1B.

Discussion. Our results are similar to those obtained by McGuire *et al.* (4), with carbodiimide reagent as coupling agent and

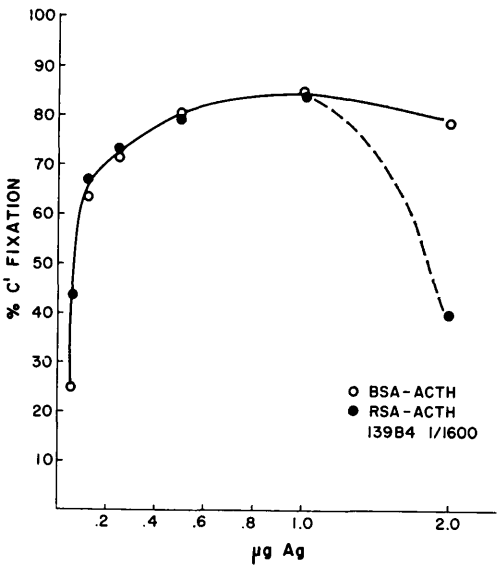

FIG. 4. Complement fixation by increments of BSA–ACTH (○), and RSA–ACTH (rabbit serum albumin ACTH) (●) with serum 139B4 (absorbed with BSA) at a dilution of 1/1600.

TABLE I. A. Comparison of Steroidogenic Effect of ACTH Incubated (20 hours at 4°C) with Control Rabbit Serum (obtained prior to immunization) and Antiserum (obtained after immunization). B. Comparison of Steroidogenic Effect of ACTH and of ACTH–BSA Complex.

Injection	Corticosterone (μg/pair of adrenals)	p
A. ACTH + control serum	4.9 ± 0.36[a]	<0.001
ACTH + antiserum	1.0 ± 0.04[a]	
Control serum	1.1 ± 0.06[a]	
B. ACTH + 0.1 *M* phosphate pH 7.0	7.5 ± 0.42[a]	<0.001
ACTH–BSA + 0.1 *M* phosphate pH 7.0	1.0 ± 0.07[a]	
0.1 *M* phosphate pH 7.0	1.0 ± 0.05[a]	

[a] SE of the mean. Five rats in each group.

rabbit serum albumin as carrier for the ACTH. However, our method seems to offer some distinct advantages. They did not state how many of their rabbits responded to their complex but the complement fixation titer of the one serum they reported is about one tenth to one twentyfifth as potent as our antisera. Our antigenic material is soluble and easily quantitated while their conjugate was insoluble. The carbodiimide reagent reacts with many functional groups in proteins while glutaraldehyde probably reacts only with the epsilon amino group of lysine (13) and possibly also the single α NH_2 group at the N terminus. Moreover, our coupling procedure is as simple as theirs and can probably be adapted to many small polypeptides. Our uniform success in eliciting antibodies and the prolonged response after very small amounts of conjugated hormone are injected would seem to make this approach a desirable one for the elicitation of potent antisera to polypeptide hormones.

Summary. A method is described for the uniform elicitation of antibodies in rabbits to ACTH. Porcine ACTH and bovine serum albumin have been covalently cross linked with glutaraldehyde. This complex is nonsteroidogenic and a potent immunogen for the elicitation of both anti-BSA and anti-ACTH antibodies. A regimen of small doses of the complexes elicited high titers of both types of antibodies in eight out of eight rabbits. These titers are still present 6 months after the last injection of antigen.

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