Precipitating and Complement-Fixing Antibodies to Collagen with Species and Collagen Subunit Specificity* (33010)

E. CARWILE LEROY (Introduced by C. L. Christian)

Department of Medicine, Columbia University College of Physicians and Surgeons, and the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital, New York, New York 10032

Collagen and its denatured derivative gelatin have been considered classic examples of proteins weak or lacking in antigenicity (1, 2). In the last decade, several laboratories have demonstrated the development of complement-fixing antibodies against various extractable portions of the collagen fibril. Watson and Rothbard (3–9) showed that antibodies to acid-soluble collagens possess considerable species specificity, a finding confirmed in more recent studies (10, 11). Schmitt *et al.* found that antigenic determinants of tropocollagen solutions could be removed by proteases which remove peptides essential for several aggregation phenomena unique to the collagen system but which do not affect the helical portion of the molecule (12). These reports suggest that collagen is sufficiently antigenic to permit the development of immunologic techniques to study normal and perhaps abnormal collagens in quantities less than those necessary for precise structural studies. This report describes the preparation of precipitating and complement-fixing antibodies to acid-soluble calfskin collagen; these antibodies precipitate collagen and its derivative gelatin quantitatively from solution. The antibodies show species specificity and also subunit specificity for a portion of the collagen molecule different from the major subunits of collagen. The subunit to which the antibodies react has been implicated in the cross-linking process by previous investigators (13, 14).

Materials and Methods. Collagen preparations. Acid-soluble collagens were prepared from bovine and human skin. Term or near-term fetal calf skin was obtained immediately following the commercial slaughter of pregnant cows. The skin was removed, packed in ice, and transported to the laboratory. All further preparation and storage were carried out at 1–4°C. The skins were cleaned by dissection; minced dermis was ground with dry ice in a hand meat grinder. Citrate-soluble collagen was prepared by the procedure of Gallop, and purified by repeated (3×) precipitation by dialysis against 0.02 *M* Na_2HPO_4 (pH 9.0) and redissolving in 0.1 *M* sodium citrate buffer, pH 4.3 (15, 16). The precipitated collagen was washed free of salt, lyophilized and stored *in vacuo* over P_2O_5 at 4°C. The skin of stillborn infants was removed, cleaned, extracted, and stored in an identical manner.

Analytical techniques. Hydroxyproline was measured by a previously described procedure (17). Disc electrophoresis was by the method of Nagai *et al.* (18). Optical rotation was measured with a Landolt-Lippich half shadow polarimeter with a filtered sodium arc light source. Collagen subunits were prepared by carboxymethyl cellulose chromatography (19, 20). Highly purified Clostridial collagenase (obtained from Worthington Biochemical Corp., Freehold, N. J.) was determined to be free of nonspecific protease activity using the caseinolytic procedure of Kunitz (21, 22).

Collagen antisera. Rabbits were immunized with acid-soluble calfskin collagen in complete Freund's adjuvant as outlined in Table I (see legend to Table I for details of immunization).

Immunological techniques. Double agar diffusion was carried out in Ouchterlony gels (0.7% agar Noble (Difco) in pH 7.6 Veronal buffer containing 0.33 *M* glycine). Immunoelectrophoresis was by the micromethod of Scheidegger (23). Complement

* This work was carried out during the tenure of a NIH Special Fellowship and a PHS Research Career Development Award (1-KO3-AM 28369-01) and was supported by grants from the U.S. Public Health Service and the Hartford Foundation.

TABLE I. Immunization Schedule and Antibody Formation.[a]

Route of injection	Frequency of injection	Period of injection	Antigen dose (mg)	Antibodies: Precipitating	Antibodies: Complement-fixing (1:100 dilutions of sera)
Toe pad	weekly	1 month	0.5	trace	—
Subcutaneous, back	weekly	1 month	1.3	1+	weak (65% with 300 μg of Ag)
Toe pad	weekly	1 month	0.5	4+	strong (80% with 10 μg of Ag)
Intravenous	3×/week	2 weeks	5.0	4+	strong (95% with 5 μg of Ag)

[a] Immunization schedule and antibody formation in four male albino rabbits (2.5–3.0 kg). One-tenth ml of a 0.05% solution of purified acid-soluble calfskin collagen in complete Freund's adjuvant was injected into a toe pad of each paw weekly (×4) both before and after dorsal subcutaneous injection of 0.5 ml of the same collagen adjuvant solutions. After 10 days 1.0 ml of 0.1% collagen in saline was injected into the ear vein three times a week for 2 weeks. The final bleeding was 7 days after the last intravenous injection. Precipitating antibody levels were similar in all 4 rabbits; the quantitative complement-fixing data given in parentheses was obtained with M-13 antiserum. The last two bleedings were comparable in all respects and used interchangeably in the studies presented. Sera were stored at —20°C with 1/10,000 merthiolate as preservative.

fixation was measured with guinea pig complement and sheep erythrocytes using the micromethod of Wasserman and Levine (24).

Results. The acid-soluble calfskin collagen was characterized by disc gel electrophoresis, optical rotation, and hydroxyproline content. The disc gel electrophoresis pattern of acid-soluble calfskin collagen is shown in Fig. 1. The preparation contained predominantly α and β subunits with only a trace of γ or higher aggregates. No faster migrating breakdown products were seen; also, there were no detectable bands when this collagen was subjected to disc gel electrophoresis at pH 9, the system commonly used to detect serum or other acidic proteins. The optical rotation ($\alpha_{589}{}^{21}$) of the collagen preparation was —341° : this value fell to —180° after denaturation at 56°C for 3 hours. These data are similar to those of earlier investigations (25, 26). The hydroxyproline content of the collagen preparations was 10% of the dry weight, and remained constant after threefold reconstitution by salt precipitation.

Precipitating and complement-fixing antibodies were produced after repeated immunization of 4 rabbits with native calfskin collagen (see Table I). Three antisera gave weak reactions against either bovine serum albumin or bovine gamma globulin when reacted against bovine serum in immunoelectrophoresis. These reactions could be removed completely by absorption of the antisera with 5–10 μl of bovine serum per ml of antiserum. A single antiserum, designated M-13 in this study, gave a single line in immunoelectrophoresis with bovine serum and in double diffusion experiments this line showed a reaction of convergence or identity with the single line obtained with native calfskin collagen. Only antiserum M-13 was used for the remainder of the study.

The specificity of antiserum M-13 for calfskin collagen was confirmed by three types of evidence: (a) Antiserum M-13 repeatedly gave a single precipitin line in diffusion and in immunoelectrophoresis when reacted with native calfskin collagen. A direct photograph of the unstained immunoelectrophoresis slide is shown in Fig. 2. These lines were completely abolished by digestion with highly purified bacterial collagenase which was free of nonspecific protease activity. Furthermore, bovine serum albumin and bovine IgG globulin preparations had no precipitin or complement-fixing activity with antiserum M-13. (b) Antiserum M-13 precipitated 95% of collagen hydroxyproline from solution. To study this, native collagen was converted to "parent gelatin" by heating at 56°C for 30 min to prevent the spontaneous precipitation of

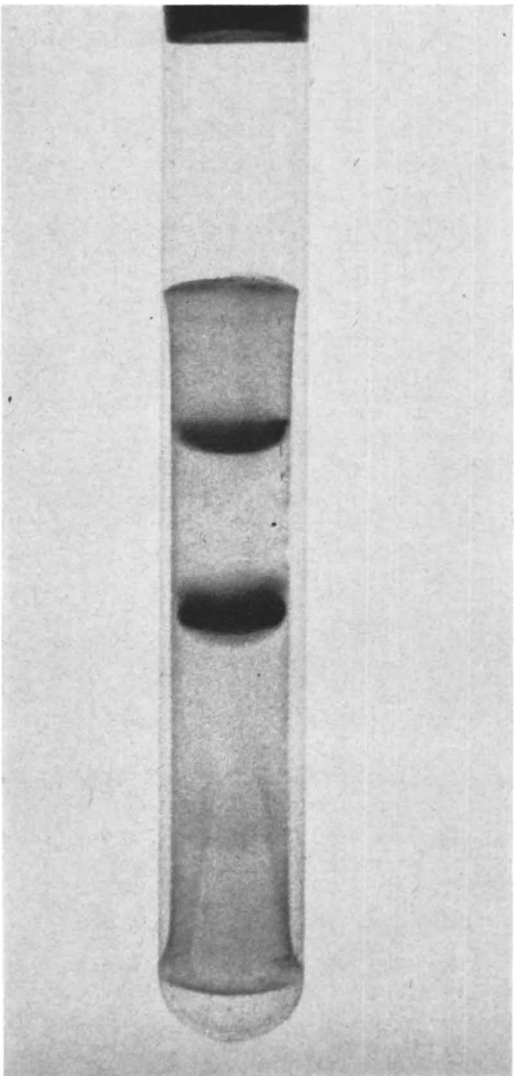

FIG. 1. Disc gel electrpohoresis at pH 4.2 of 200 μg of purified acid-soluble calfskin collagen stained with aniline blue black. The lower, darker band contains both α_1 and α_2 subunits which are partially resolved. The upper, lighter band contains β_{11} and β_{12} subunits. A trace of γ or higher aggregates is at the top of the gel. The spacer gel was removed in washing. The band at the bottom of the gel is tracking dye. (see Ref. 18).

collagen at 4°C. The results are shown in Table II: the experimental details are given in the legend to Table II. Large volumes of antisera were required to precipitate collagen quantitatively from solution; these volumes precluded study of the antibody

TABLE II. Quantitative Precipitation of Acid-Soluble Calfskin Collagen by M-13 Antiserum.[a]

Antigen (μg of hypro)	Antiserum (ml)	Antigen in precipitates (μg of hypro)	%
32	0.15	1.9	6
32	0.3	2.9	9
32	1.2	5.3	17
10.7	0.2	2.2	21
10.7	0.8	5.7	54
10.7	3.2	10.1	95
32	—	0	—
—	1.5	0	—
32	1.2[b]	0	—

[a] Quantitative precipitation of acid-soluble calfskin collagen by M-13 antiserum. Lyophilized acid-soluble calfskin collagen was dissolved in pH 7.6 Veronal buffer by heating at 56°C for 15 min. The concentration of collagen was determined by hydroxyproline measurement (× 7.46). Varying ratios of collagen and antiserum were mixed, heated to 37°C for 30 min and allowed to stand at 4°C for 10 days with the appropriate controls (including nonimmune rabbit serum). The mixtures were centrifuged, washed (×3) with several volumes of cold saline, hydrolyzed in acid, and hydroxyproline was determined by a procedure specific for hydroxyproline in the presence of several thousand parts of other amino acids (17).

[b] Nonimmune rabbit serum.

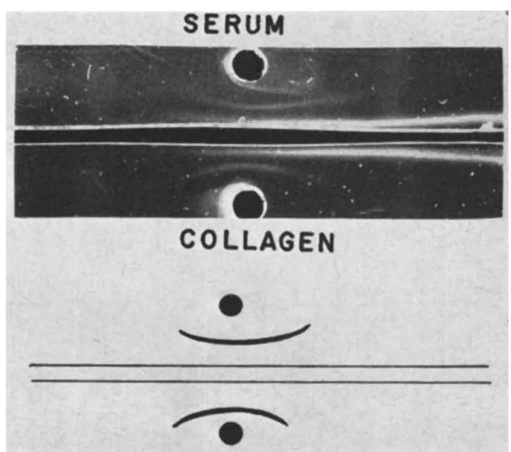

FIG. 2. Microimmunolectrophoresis of bovine serum (upper well) and acid-soluble calfskin collagen (lower well) reacted against M-13 antiserum. The single line with both serum and collagen was absent after treatment with protease-free bacterial collagenase.

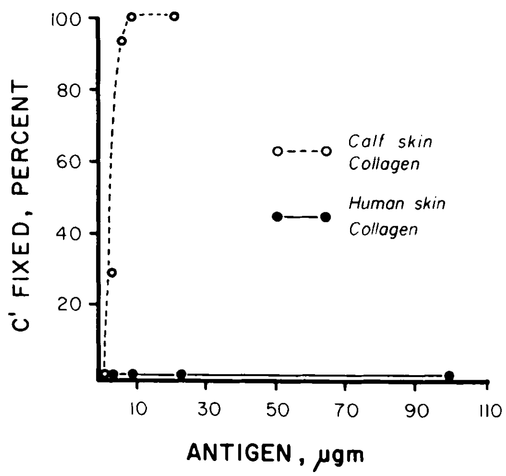

FIG. 3. Complement-fixation of calfskin collagen and human skin collagen in the presence of M-13 antiserum (1:100 dilution). The antigen concentration was determined by hydroxyproline measurement (× 7.46).

excess portion of the curve. (c) The reaction of antiserum M-13 with calfskin collagen was found to be species specific. Collagen was purified from young bovine and young human skin by identical procedures. No precipitin reaction was noted when the antibody to calf collagen was reacted with human collagen in double diffusion agar experiments. Also, quantities of human skin collagen in 10-fold excess of the amount of calf collagen required to fix complement completely fixed no detectable complement, as shown in Fig. 3.

In an attempt to see if the antibodies to collagen would be useful in studying the cross-linking regions of the collagen molecule, calf -collagen was fractionated into subunits by carboxymethyl cellulose chromatography (19). A representative pattern, monitored by the optical density at 230 mμ, is shown in Fig. 4. Three major peaks were consistently obtained. Disc gel electrophoresis of the leading and trailing portions of Peaks 2 and 3 demonstrated α, β, β, and β plus α subunits, respectively. The precipitating activity of the collagen preparations was found only in Peak 1. Figure 5 shows the reaction of convergence obtained when antiserum M-13 was reacted with unfractionated calf collagen and concentrated Peak 1 material. Also, the complement-fixing activity was substantially enriched in the Peak 1 eluates. As shown in Fig. 6, the complement-fixing activity of a concentrate of Peak 1 was 5–10-fold stronger than unfractionated acid-soluble calfskin collagen.

The nature of the Peak 1 material is unknown. It has virtually no hydroxyproline, does not react with the Folin-Ciocalteau protein reagent or absorb light in the region of 280 mμ (suggesting little or no tyrosine or tryptophan), has very little ninhydrin reactivity prior to hydrolysis but substantial amounts of ninhydrin reactive material after hydrolysis and the immunologically active material or materials are not dialyzable and are retained inside collodion tubes in ultrafiltration concentration. The immunological activity is lost at 4°C in acetate buffer (pH 4.8), is lost on lyophilization in this buffer, but is retained for several weeks if the pH is adjusted to 7.6

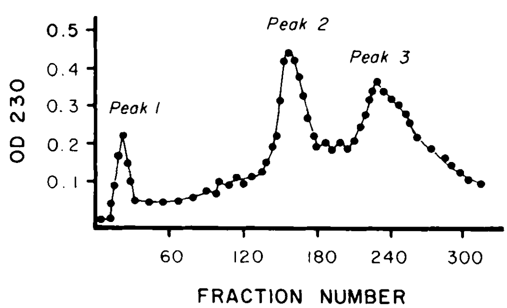

FIG. 4. Carboxymethyl cellulose chromatography of denatured calfskin collagen. A 3.5 × 40-cm jacketed column of Whatman CM 32 microgranular carboxymethyl cellulose was used to fractionate 250 mg of acid-soluble calfskin collagen which was dissolved overnight in 50 ml starting acetate buffer at 4°C and heated at 40°C for 30 min. A continuous ionic gradient was used to elute with 2 conical chambers and a limiting buffer of 0.1 *N* NaCl (19). Total gradient volume was 3000 ml. Ten-ml fractions were collected and read separately in a Zeiss spectrophotometer. The Peak 1 fractions were pooled, the pH was adjusted to 7.6, and the sample was concentrated to 4 ml at 4°C under vacuum in collodion bags. The 15 fractions on either side of peak fractions of Peak 2 and Peak 3 were desalted on G-25 Sephadex with a pyridine acetate buffer and lyophilized. Weighed samples were analyzed by disc gel electrophoresis.

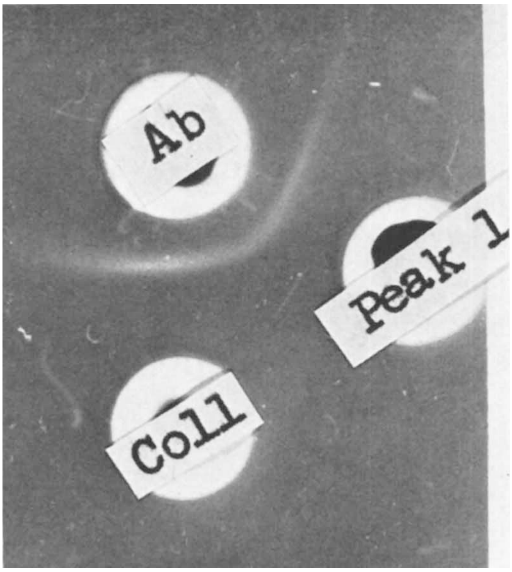

FIG. 5. Ouchterlony gel diffusion comparing the precipitin reactions of denatured calfskin collagen and concentrated Peak 1 material. Ab = undiluted M-13 antiserum, 0.2 ml; Peak 1 = concentrated Peak 1 material (see legend, Fig. 4), 0.2 ml, containing 16 μg of leucine equivalents on quantitative ninhydrin analysis. Coll = acid-soluble calfskin collagen in Veronal buffer heated at 56°C for 15 min, 0.2 ml, containing 440 μg of denatured collagen (hypro $\times$ 7.46). A reaction of convergence was consistently observed; the Peak 1 precipitin line was sharper than the denatured collagen line.

with sodium hydroxide prior to concentration by ultrafiltration and storage is at —20°C. Further studies attempting to elucidate the chemical nature of the immunologically reactive Peak 1 material are in progress.

Initially it seemed possible that the Peak 1 material consistently obtained by many laboratories from the fractionation of calfskin, rat skin, or rat tail tendon collagen was not related to collagen but was simply a more antigenic impurity persisting after extraction and multiple purification steps. To test this possibility, a carboxymethyl cellulose fractionation was carried out on acid-soluble calfskin collagen not subjected to denaturation at 40°C in an unjacketed column at room temperature (23°C). There was a broad peak absorbing at 230 mμ but no immunologically reactive material was present when tested in double diffusion agar after concentration of eluates. This suggests that although the Peak 1 material is not a well recognized and already characterized subunit of collagen, it is an integral part of collagen which is separated from the other subunits by denaturation. These data are confirmatory in all respects with the data of Kessler *et al.* (13, 14) who suggest that this material is related to the nonhelical cross-linking regions of collagen which have been termed telopeptides by Schmitt *et al.* (12). The amino acid data of Kessler show sufficient glutamic acid residues to account for the acidic nature of the material (14).

Discussion. Moderately potent antisera to acid-soluble calfskin collagen have thus been raised in rabbits by extended immunization with native, particulate antigen mixed with complete Freund's adjuvant. These are the first antibodies to native collagen with precipitating ability; this ability will permit more quantitative techniques to study the interaction of collagen and its antibodies. The realization that collagen is antigenic is a relatively recent one. The absence of tryptophan and the presence of tyrosine in only trace quantities were the basis for the early presumption that collagen was nonantigenic. Initial attempts to

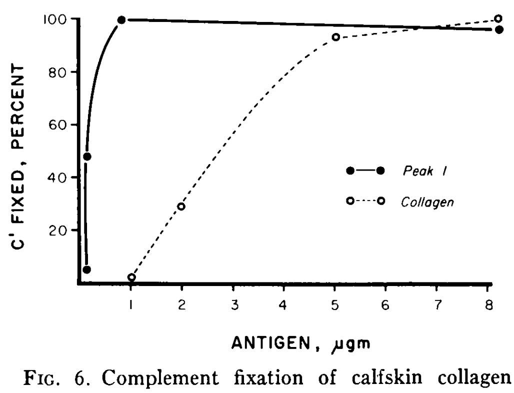

FIG. 6. Complement fixation of calfskin collagen and concentrated Peak 1 material. Antiserum M-13 was used in 1:100 dilution. Antigen concentration of collagen was hydroxyproline content $\times$ 7.46; Peak 1 material was hydrolyzed, ninhydrin reactive material was determined, and results were expressed as leucine equivalents. The Peak 1 material was 5–10-fold more active than collagen.

document this hypothesis used gelatin or other forms of denatured or altered collagen as the immunizing and identifying antigen (1, 2, 27, 28). The introduction of techniques to extract or solubilize collagen without denaturation (29) led to the successful production of antibodies which fixed complement in the presence of collagen in 1930 (30). Extensive studies on the antigenicity of collagen have been carried out more recently by Rothbard and Watson, who have produced complement-fixing antibodies to several types of native collagen. These studies have shown that the collagens of several species differ considerably in their antigenic character and presumably therefore in primary structure. These studies have also shown the presence of a material reacting with collagen antibodies in the renal glomerular basement membrane of the rat and man (9). A crucial feature of the antigenicity of tropocollagen, the major constituent of all extractable or "soluble" collagens, was the demonstration by Schmitt *et al.* that the antigenic determinants of tropocollagen reside largely in regions of the molecule essential to the cross-linking of collagen monomers into the various polymers which can be formed (12). These "telopeptide" regions are not in the helical backbone of collagen and do not contain the typical collagen repeating sequence; they do contain the only precisely demonstrated cross-link yet shown in collagen, the intramolecular linkage which has been carefully delineated by Bornstein and Piez (20).

It is possible but as yet unproved that the antibodies described in this report are reacting to peptides of the telopeptide type described by Schmitt *et al.* Because the immunological techniques used are several hundred times more sensitive than analytical techniques of identification, it has not yet been possible to obtain sufficient immunoreactive material to characterize chemically. It is tempting to speculate that this material may be essential both to *in vitro* aggregation, as suggested by Kessler, and to *in vivo* determination of the function of collagen in the various connective tissues.

Summary. Rabbits immunized with acid-soluble calf skin collagen develop antibodies with both precipitating and complement-fixing activity specific for bovine collagen. The antigenic determinants reside in acidic portions of the collagen molecule which are removed by heat denaturation and may be implicated in the collagen cross-linking process.

I thank Dr. Charles L. Christian for his continued advice and support. Mrs. Esther Gudes and Miss Mabel Wong provided able technical assistance.

1. Starin, W. A., J. Infect. Diseases **23**, 139 (1918).
2. Waksman, B. H., and Mason, H. L., J. Immunol. **63**, 427 (1949).
3. Watson, R. F., Rothbard, S., and Vanamee, P., J. Exptl. Med. **99**, 535 (1954).
4. Rothbard, S. and Watson, R. F., J. Exptl. Med. **103**, 57 (1956).
5. Rothbard, S. and Watson, R. F., J. Exptl. Med. **109**, 633 (1959).
6. Rothbard, S. and Watson, R. F., J. Exptl. Med. **113**, 1041 (1961).
7. Rothbard, S. and Watson, R. F., J. Exptl. Med. **116**, 337 (1962).
8. Rothbard, S. and Watson, R. F., J. Exptl. Med. **122**, 441 (1965).
9. Rothbard, S. and Watson, R. F., J. Exptl. Med. **125**, 595 (1967).
10. Hisa, S. and Suzuki, Y., Acta Pathol. Japon. **9**, 499 (1959).
11. Steffen, C., Timpl, R., and Wolff, I., J. Immunol. **93**, 656 (1964).
12. Schmitt, F. O., Levine, L., Drake, M. P., Rubin, A. L., Pfahl, D., and Davison, P. F., Proc. Natl. Acad. Sci. U. S. **51**, 493 (1964).
13. Kessler, A., Rosen, H., and Levenson, S. M., Nature **184**, 1640 (1959).
14. Kessler, A., Rosen, H., and Levenson, S. M., J. Biol. Chem. **235**, 989 (1960).
15. Gallop, P. M., Arch Biochem. Biophys. **54**, 486 (1955).
16. Gallop, P. M. and Seifter, S., in "Methods in Enzymology", Colowick, S. O. and Kaplan, N. O., eds., vol. 6, p. 635. Academic Press, New York, 1963.
17. LeRoy, E. C., Kaplan, A., Udenfriend, S., and Sjoerdsma, A., J. Biol. Chem. **239**, 3350 (1964).
18. Nagai, Y., Gross, J., and Piez, K. A., Ann. N. Y. Acad. Sci. **121**, 494 (1964).
19. Piez, K. A., Eigner, E. A., and Lewis, M. S., Biochemistry **2**, 58 (1963).
20. Bornstein, P. and Piez, K. A., Biochemistry **5**, 3460 (1966).
21. Kunitz, M., J. Gen. Physiol. **30**, 291 (1947).

22. Nagai, Y., Lapiere, C. M., and Gross, J., Biochemistry **5**, 3123 (1966).
23. Scheidegger, J. J., Intern. Arch. Allergy Appl. Immunol. **7**, 103 (1955).
24. Wasserman, E. and Levine, L., J. Immunol. **87**, 290 (1961).
25. Cohen, C., J. Biophys. Biochem. Cytol. **1**, 203 (1955).
26. Harrington, W. F. and Von Hippel, P. H., The Structure of Collagen and Gelatin, in Adv. in "Protein Chemistry," Anfison, C. B., Anson, M. L., Bailey, K., and Edsall, J. T., Vol. 16 pp. 1–138. Academic Press, New York, 1961.
27. Maurer, P. H., J. Exptl. Med. **100**, 497, 515 (1954).
28. Maurer, P. H., Arch. Biochem. Biophys. **58**, 205 (1955).
29. Nageotte, J., Compt. Rend. Soc. Biol. **96**, 172 (1927).
30. Loiseleur, J. and Urbain, A., Compt. Rend. Soc. Biol. **103**, 776 (1930).

Received Jan. 18, 1968. P.S.E.B.M., 1968, Vol. 128.

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

MORRIS REICHLIN, JOEL J. SCHNURE, AND VERNON K. VANCE
(Introduced by C. Bishop)

Department of Medicine, State University of New York at Buffalo School of Medicine, The Buffalo General Hospital, Buffalo, New York 14203

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

* Supported by USPHS Grants AM-10428, 2-T01-AM-05389, and 5-R01-AM-05581.