THE DISTRIBUTION OF ANTIGENIC DETERMINANTS IN RAT SKIN COLLAGEN*

By HERBERT LINDSLEY,‡ M.D., MART MANNIK, M.D., AND PAUL BORNSTEIN,§ M.D.

(*From the Division of Rheumatology, Department of Medicine and Department of Biochemistry, University of Washington School of Medicine, Seattle, Washington 98105*)

Immunological studies of collagen have been hampered by the limited solubility and relatively poor immunogenicity of this protein (See review by Kirrane and Glynn [1]). However, antibodies to collagen have been detected by techniques such as immunofluorescence (2, 3), hemagglutination (4–7), complement fixation (8–10), and passive cutaneous anaphylaxis (3, 11). Thus, the immunogenicity of collagen is firmly established.

Despite its large size and complexity, knowledge of the structure of the collagen molecule has progressed sufficiently to permit the determination of the distribution of antigenic determinants in the protein. The collagen monomer is composed of three polypeptide chains, each with a molecular weight of about 95,000. In mammalian collagens two of the chains (termed $\alpha1$) are identical or nearly identical, whereas the third (termed $\alpha2$) is clearly different in amino acid composition and sequence. The helical arrangement of the chains in the native molecule can be disrupted by heat, and the unfolded protein (gelatin) fractionated into its constituent chains by chromatography on carboxymethyl (CM)[1] cellulose (12). Separation of monomeric collagen by this technique yields four fractions, the $\alpha1$ and $\alpha2$ chains and their covalently linked dimers, $\alpha1$-$\alpha1$ (β_{11}) and $\alpha1$-$\alpha2$ (β_{12}). Further analysis of the $\alpha1$ and $\alpha2$ chains has utilized nonenzymatic cleavage at methionyl residues with cyanogen bromide (CNBr). Such studies have resulted in the isolation and characterization of eight fragments which constitute the $\alpha1$ chain of rat skin collagen (13) and six fragments which account for rat skin collagen $\alpha2$ (14). The order of these fragments in the polypeptide chains has been ascertained by a combination of chemical, biosynthetic, and electron microscopic techniques (15, 16).

The present immunological studies were undertaken: (*a*) to determine whether different antigenic determinants exist on the $\alpha1$ and $\alpha2$ chains of rat

[1] *Abbreviation used in this paper:* CM, carboxymethyl.

skin collagen, (b) to localize the antigenic determinants of these chains to specific CNBr-produced fragments, and (c) to examine the extent to which the availability of antigenic determinants is influenced by the conformation of collagen chains. The results described below show that distinct antigenic determinants exist on the α1 and α2 chains. These determinants are located in the amino- and carboxy-terminal sequences of these chains and reflect the predominant interspecies structural differences in collagen. The antigenic determinants of both chains are only partially available in the native collagen molecule.

Materials and Methods

Preparation of Antigens.—Weanling Sprague-Dawley rats were rendered lathyritic by the inclusion of 0.15% β-aminopropionitrile fumarate (Aldrich Chemical Co.,Milwaukee,Wis.) into the diet for a period of 4 wk. Soluble skin collagen was obtained from normal and lathyritic rats by sequential extraction with 1 M NaCl and 0.5 M acetic acid and was purified by repeated precipitation and solution at acidic and neutral pH (17). α1 and α2 chains were isolated from both lathyritic and normal collagens (12) and cleaved with CNBr (17). The resulting fragments were separated by a combination of ion-exchange chromatography on phosphocellulose and CM cellulose and by gel filtration on molecular sieves (13, 14, 17). With the exception of α2-CB4 and α2-CB5, which were incompletely separated, the purity of these fragments exceeded 90%.

To prepare ^{14}C-α2 chains, 0.25 mCi of ^{14}C-glycine was injected into each of three 100 g Sprague-Dawley rats; 48 hr later the rats were sacrificed and skin collagen was extracted with 1 M NaCl and chromatographed as described above. The specific activity of the isolated alpha chains was approximately 5000 cpm/mg.

Rabbit γG-globulin for immunization was prepared from rabbit serum by 40% ammonium sulfate precipitation, diethylaminoethyl (DEAE) cellulose chromatography, and Sephadex G-200 gel filtration.

Preparation of Antisera.—15 New Zealand white rabbits received injections of native rat skin collagen, or preparations of α1, α2, or β_{12} chains. The dose per rabbit per injection consisted of 1–15 mg of protein dissolved in normal saline and mixed with an equal volume of complete Freund's adjuvant. The antigen preparations were injected subcutaneously into four different sites along the back and neck. Booster injections were given every 2–4 wk. The total dose of protein given to each rabbit ranged from 20 to 100 mg during the course of immunization. The rabbits were bled from the ear approximately every 2 wk starting 4–8 wk after the initial injection. One goat was immunized with rabbit γG-globulin. All antisera were rendered 0.01% in sodium azide and stored at 4°C or at −20°C.

Radioiodination of Alpha Chains.—Isolated α1 and α2 chains were trace-labeled by the iodine monochloride method (18), except that iodination was carried out at pH 9.0 in 0.2 M tris(hydroxymethyl)aminomethane (Tris)-HCl and 0.15 M NaCl. Preparations of collagen chains were warmed to 42°C for 20 min just before iodination to assure dissociation of the chains and to permit even distribution of the label. In later experiments this was achieved by iodination in 5 M guanidine hydrochloride (Eastman Organic Chemicals, Rochester, N.Y.) at room temperature. The iodination reagents were adjusted to introduce 1 equivalent of iodine per 10 equivalents of alpha chains. In all experiments carrier-free, reductant-free ^{125}I was used. Free iodine was removed by dialysis against distilled water or by chromatography on Sephadex G-25 equilibrated with 0.1 M acetic acid. Radiolabeled peptides were obtained by CNBr cleavage of iodinated chains and subsequent fractionation of the resulting peptides.

Radioimmunoassay.—An immunoassay was developed in which a rabbit antiserum inter-

acted with radiolabeled rat collagen chains or fragments. The resulting antigen–antibody complexes were precipitated together with nonspecific rabbit γG-globulin by the addition of excess goat antiserum to rabbit γG-globulin. The appropriate ratio of radiolabeled antigen to antibody was determined for each primary system to provide 60–80% precipitation of the antigen in the zone of antigen excess. In subsequent experiments unlabeled chains or fragments were added to detect their ability to inhibit the precipitation of labeled antigen.

In an inhibition assay the following were added sequentially: 100 μl of a buffer composed of 0.1 M Tris HCl (pH 7.5), 0.15 M NaCl, 1% ovalbumin, and 0.01% Na azide; 100 μl of rabbit antiserum; 100 μl of the buffer without azide containing 10–1000 μmole of inhibitor alpha chain or CNBr fragment. After a 2-hr incubation at room temperature, 100 μl of radioactive chain or peptide solution (500–5000 cpm) was added to provide an antigen excess allowing 60–80% precipitation in the absence of an inhibitor. After 1 additional hr of incubation at room temperature, 0.5–2.0 ml of goat antiserum to rabbit γG-globulin was added to provide an excess of goat antibody and the mixture was incubated overnight at 4°C. The precipitate was sedimented by centrifugation at 1000 g for 20 min at room temperature. The supernatant was decanted and the precipitate washed once with 2 ml of 0.15 M NaCl at room temperature. The radioactivity in the precipitate was determined in an automatic gamma counter (Nuclear-Chicago, Des Plaines, Ill.). Controls for each assay included: (a) five separate samples of the labeled material used in the assay to determine the total counts added to each tube, (b) duplicate tubes containing nonimmune rabbit serum in place of an antiserum to collagen or its chains, and (c) duplicate tubes containing antiserum without inhibitor. Control precipitates prepared with nonimmune rabbit serum contained 1–5% of the counts added to each tube.

In experiments with native collagen as an inhibitor the immunoassay was modified as follows to avoid precipitation of the protein. A 0.1 M sodium acetate, pH 5.0, buffer containing 0.15 M NaCl, 1% ovalbumin, and 0.01% Na azide was used. Antisera were dialyzed against this buffer in the absence of ovalbumin. All procedures and incubations were performed at 4°C.

An initial screening of antisera for the presence of antibodies to α1 or α2 chains was conducted as follows. To a tube containing buffer and the antiserum to be tested, but lacking inhibitor, was added 10–50 picomoles of labeled α1 or α2 chain. Total rabbit γG-globulin was precipitated with an excess of goat antiserum and the precipitate counted. Hyperimmune sera, obtained during the 3rd and 4th months of immunization, were pooled to insure adequate volumes of antisera for subsequent experiments.

For liquid scintillation counting the precipitate was washed twice with absolute alcohol, dissolved in 0.2 ml of NCS reagent (Nuclear-Chicago), and transferred into 15 ml of dioxane-based scintillation fluid. Counting was done in an automatic liquid scintillation counter (LS-150, Beckman Instruments Inc., Fullerton, Calif.) to 2% accuracy.

Purification of Antibodies to α2 Chains.—The high protein content of immune precipitates produced quenching during liquid scintillation counting in assays with ^{14}C-α2 chains. Therefore, antibodies to α2 chains were purified by affinity chromatography. α2 chains were coupled to agarose (4B Sepharose, Pharmacia Fine Chemicals Inc., Piscataway, N.J.) by the method of Porath et al. (19). An agarose column containing the covalently bound α2 was extensively washed with 0.01 M HCl in 0.15 M NaCl. Subsequently a potent antiserum to α2 chains was passed over the column and the column washed with 0.2 M sodium borate, 0.15 M NaCl, pH 8.0, until no further protein was detected. Antibodies to α2 were then eluted with 0.01 M HCl in 0.15 M NaCl, pooled, neutralized, and stored at 4°C until further use.

Determination of Protein Concentration.—Noncollagenous protein concentrations were determined by absorbance at 280 nm or with the Folin reagent. Solutions of collagen chains and

fragments were assayed for hydroxyproline content (20) and concentrations determined by their known amino acid compositions.

Demonstration of Chain Specificity.—$\alpha 1$ and $\alpha 2$ chains were immunologically distinct and failed to show any significant cross-reactivity. Evidence for this was obtained from quantitative inhibition of the radioimmunoassay utilizing purified unlabeled $\alpha 1$ or $\alpha 2$ chains as inhibitors of precipitation of radioiodinated $\alpha 1$ or $\alpha 2$ chains. When [125]I-$\alpha 2$ was used as a test antigen with an antiserum to native collagen, addition of 10 picomoles of $\alpha 2$ chains decreased binding to 27% (Fig. 1). These observations were made not only with antisera to whole collagen, but also with antisera to β_{12} and $\alpha 2$ chains. Addition of as much as 100 picomoles of $\alpha 1$ failed to inhibit binding of [125]I-$\alpha 2$ both with anti-rat skin collagen (Fig. 1) and anti-rat β_{12}.
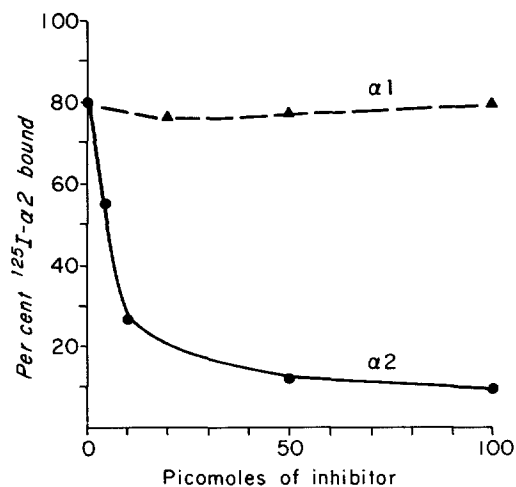

Fig. 1. Inhibition of binding of [125]I-$\alpha 2$ with $\alpha 2$ and $\alpha 1$ chains. The lower curve (●——●) shows decreasing per cent [125]I-$\alpha 2$ bound with increasing quantities of unlabeled $\alpha 2$ chains. The upper curve (▲———▲) demonstrates the absence of inhibition with the addition of increasing quantities of $\alpha 1$ chains. An antiserum to rat skin collagen was used.

Reciprocal experiments were performed with [125]I-$\alpha 1$ as a test antigen using an antiserum to collagen. Addition of 10 picomoles of $\alpha 1$ decreased binding of [125]I-$\alpha 1$ to 34% (Fig. 2). Comparable results were noted with antisera to β_{12}. Antisera to $\alpha 1$ could not be tested directly since rabbits immunized with $\alpha 1$ failed to produce detectable antibodies. Addition of increasing quantities of $\alpha 2$ did produce a slight inhibition of binding of [125]I-$\alpha 1$. Although these results may be due to a shared determinant, a more likely explanation stems from a minor degree of contamination of $\alpha 2$ with β_{12} (which contains $\alpha 1$). The inhibition of

binding of $\alpha1$ by $\alpha2$ was gradual over a wide range of concentrations and was compatible with about 3% contamination. Furthermore, as shown in the reciprocal system (Fig. 1), precipitation of $\alpha2$ was not affected by excess $\alpha1$. A minor degree of contamination (less than 5%) is to be expected particularly in preparations of $\alpha2$ from nonlathyritic collagens which contain a higher proportion of β_{12}.

Localization of Antigenic Determinants on CNBr Peptides of $\alpha1$ Chains.— CNBr cleaves the $\alpha1$ chain of rat skin collagen into eight fragments ranging in molecular weight from 1500 to 24,000 (13; Fig. 3). $\alpha1$-CB1 (mol wt 1500) constitutes the amino-terminal sequence and $\alpha1$-CB6 (mol wt 16,500) represents
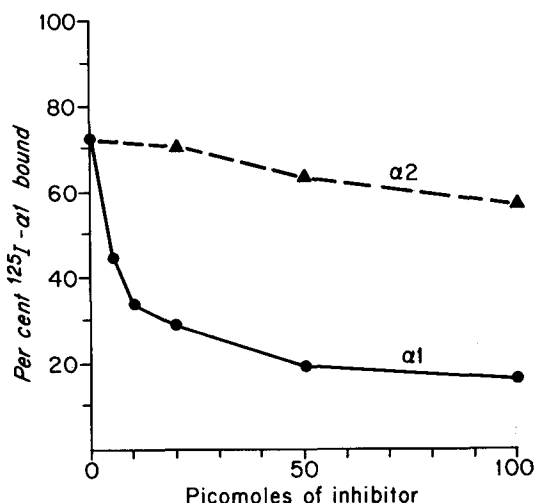

Fig. 2. Inhibition of binding of ^{125}I-$\alpha1$ with $\alpha1$ and $\alpha2$ chains. The lower curve (●——●) shows decreasing per cent ^{125}I-$\alpha1$ bound with addition of increasing quantities of unlabeled $\alpha1$ chains. The upper curve (▲——▲) demonstrates gradual inhibition with addition of increasing quantities of $\alpha2$ chains. An antiserum to rat skin collagen was used.

the carboxy-terminal region of the chain. In order to localize the antigenic determinants of the $\alpha1$ chain to specific CNBr-produced peptides, two approaches were taken. Labeled $\alpha1$ chains were used as test antigens with several antisera, and inhibition was attempted with purified peptides individually or as mixtures. Subsequently, similar studies were performed with labeled peptides as test antigens, and inhibition was attempted with unlabeled peptides and chains. In these experiments antisera against rat skin collagen were used since they were known to contain antibodies to the $\alpha1$ chain from the studies described above.

With ^{125}I-$\alpha1$ chain as a test antigen, the greatest degree of inhibition was seen with $\alpha1$-CB6 (Fig. 4). Lesser and progressive degrees of inhibition were

seen both with α1-CB7 and a mixture of α1-CB4 and α1-CB5. Since these three peptides chromatographed in close proximity to α1-CB6, it was not clear whether they contained separate determinants or whether the observed inhibition was due to contamination with α1-CB6. No inhibition was seen with the four remaining peptides α1-CB1, α1-CB2, α1-CB3, and α1-CB8.

To confirm these inhibition experiments with ^{125}I-α1 chains, radioiodinated α1 chains were cleaved with CNBr and chromatographed on CM cellulose. As expected from the known distribution of tyrosyl residues in the α1 chain of rat skin collagen (13), only α1-CB1 and α1-CB6 were labeled with [125]I. With ^{125}I-α1-CB1 as a test antigen, no antibodies were detected in sera from rabbits immunized with rat skin collagen, β_{12}, or α1 chains. With ^{125}I-α1-CB6 as a test antigen, the greatest degree of inhibition was seen with α1-CB6 (Fig. 5). Lesser and progressive degrees of inhibition were seen with α1-CB4 + α1-CB5 and
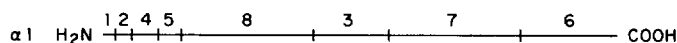

FIG. 3. The order of CNBr-produced fragments in the α1 and α2 chains of rat skin collagen (15, 16). Their numerical designation resulted from an initial sequence of elution from ion-exchange columns. The length of each segment is approximately proportional to the molecular weight of the fragment.

α1-CB7. Again the latter results were thought to represent contamination with α1-CB6 rather than shared determinants. No inhibition was seen with the four remaining peptides of the α1 chain.

Localization of Antigenic Determinants on CNBr Peptides of α2 Chains.— CNBr cleaves the α2 chain into six fragments ranging in molecular weight from 290 (α2-CB0) to 29,500 (α2-CB5) (14; Fig. 3). α2-CB1 (mol wt 1400) represents the amino-terminal sequence and α2-CB5 is formed from the carboxy-terminal region of the chain. In order to localize the antigenic determinants of the α2 chain to specific CNBr-produced peptides, the same approaches were taken as described for the α1 chain. The tripeptide, α2-CB0, was not considered in these experiments.

With ^{125}I-α2 chain as a test antigen, the greatest degree of inhibition was seen with the α2 chain and α2-CB5 (Table I). Essentially no inhibition was noted with the other α2 CNBr-produced peptides in this system. Of note is that α2-CB1 failed to inhibit precipitation of the labeled α2 chain even when
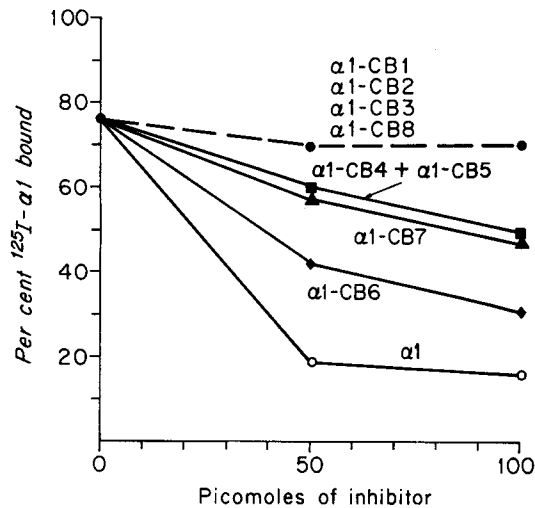
FIG. 4. Inhibition of binding of ^{125}I-α1 with CNBr peptides derived from α1. α1-CB6 (◆——◆) demonstrates inhibition that approaches the inhibition provided by the intact α1 chain (O——O). α1-CB4 + α1-CB5 (■——■) and α1-CB7 (▲——▲) show slight inhibition. α1-CB1, α1-CB2, α1-CB3, and α1-CB8 (●——●) show no inhibition. An antiserum to rat skin collagen was used.

FIG. 5. Inhibition of binding of ^{125}I-α1-CB6 with CNBr peptides derived from α1. α1-CB6 (■——■) and α1 chains (◆——◆) demonstrate strong inhibition. α1-CB7 (●——●), and a mixture of α1-CB4 + α1-CB5 (▲——▲) are far less inhibitory. α1-CB1, α1-CB2, α1-CB3, and α1-CB8 (●——●) show no inhibition. An antiserum to rat skin collagen was used.
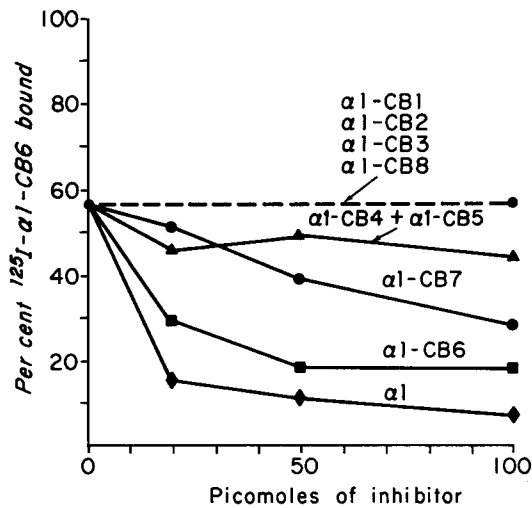
1000 picomoles of the peptide was used as an inhibitor. α2-CB1 was however shown to contain an antigenic determinant by direct assay with the labeled peptide.

To prepare labeled peptides, radioiodinated α2 chains were cleaved with CNBr and chromatographed on CM cellulose. Iodine label was limited to peptides α2-CB1 and α2-CB5 which contain tyrosine (14). With ^{125}I-α2-CB1 as a test antigen, only the α2 chain and α2-CB1 were potent inhibitors, and none

TABLE I

Inhibition of Binding of ^{125}I-α2 and ^{14}C-α2 with α2 CNBr Peptides

Inhibitor*	Per cent ^{125}I-α2 bound	Per cent ^{14}C-α2 bound
None	74	70
α2	11	N.D.‡
α2-CB5	18	⎫
α2-CB4	65	⎬ 22
α2-CB3	62	⎭
α2-CB2	60	N.D.
α2-CB1	70	61

* 100 picomoles were used in assays with ^{125}I-α2 and 5000 picomoles were used in assays with ^{14}C-α2.

‡ N.D., not determined.

TABLE II

Inhibition of Binding of ^{125}I-α2-CB1 and ^{125}I-α2-CB5 with α2 CNBr Peptides

Inhibitor*	Per cent ^{125}I-α2-CB1 bound	Per cent ^{125}I-α2-CB5 bound
None	47	71
α2	2	4
α2-CB1	2	73
α2-CB2	46	71
α2-CB3	⎫	74
α2-CB4	⎬ 41	70
α2-CB5	⎭	6

* 100 picomoles were used in all assays.

of the other peptides was effective at the concentrations tested (Table II). With ^{125}I-α2-CB5 as the test antigen, α2-CB5 was the only peptide that caused inhibition (Table II). Thus antigenic determinants were localized to α2-CB1 and α2-CB5 and were distinguished by these experiments.

The possibility existed that small populations of antibodies directed to other large CNBr peptides were present. To test this possibility, 10% of the usual quantity of ^{125}I-α2 chain was added to mixtures of an antiserum and α2 CNBr peptides (1000 picomoles of each peptide) except either α2-CB3 or α2-CB4.

The mixture of peptides was used to block antibodies to known antigenic determinants and to allow precipitation of labeled $\alpha2$ by antibodies to $\alpha2$-CB3 or $\alpha2$-CB4, if present. In these experiments no such antibodies were detected.

To investigate the possibility that iodination of tyrosyl residues might alter the antigenicity of collagen or depress binding of antibodies, experiments with biosynthetically labeled $\alpha2$ chains were carried out. Antibodies to the $\alpha2$ chain were purified from an antiserum to $\alpha2$ by means of an immunoadsorbent (See Materials and Methods). Since the specific activity of ^{14}C-$\alpha2$ was low, larger quantities of test antigen, antibodies, and inhibitors were necessary in these
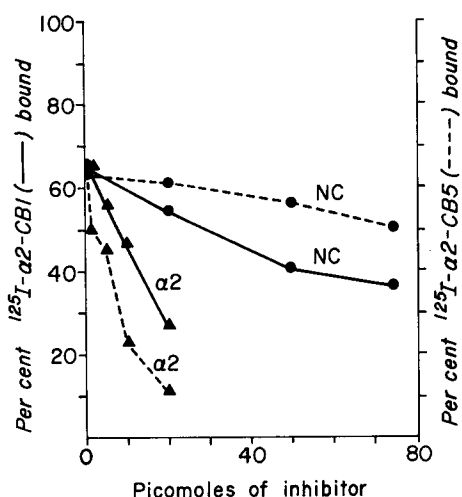

FIG. 6. Inhibition of binding of ^{125}I-$\alpha2$-CB1 and ^{125}I-$\alpha2$-CB5 with native collagen (NC) and $\alpha2$ chains. The $\alpha2$ chain is a better inhibitor of both ^{125}I-$\alpha2$-CB1 (▲——▲) and ^{125}I-$\alpha2$-CB5 (▲———▲) than is native collagen. Native collagen is a more effective inhibitor of ^{125}I-$\alpha2$-CB1 (●——●) than of ^{125}I-$\alpha2$-CB5 (●———●).

assays. The three large $\alpha2$-derived peptides ($\alpha2$-CB3, $\alpha2$-CB4, and $\alpha2$-CB5) were used as a mixture in these experiments. The findings supported the observation with iodinated $\alpha2$ chains in that inhibition occurred with a mixture of $\alpha2$-CB3, $\alpha2$-CB4, and $\alpha2$-CB5 (Table I). No inhibition was noted with $\alpha2$-CB1.

Availability of Antigenic Determinants in Native Collagen.—Experiments were performed with native collagen as an inhibitor to determine whether the triple helical conformation influenced the degree to which antibodies bound to specific antigenic determinants. With both ^{125}I-$\alpha2$-CB1 and ^{125}I-$\alpha2$-CB5 as test antigens, the $\alpha2$ chain was a more effective inhibitor than equimolar quantities of the native protein (Fig. 6). Equimolar amounts of $\alpha2$ chain and the appropriate CNBr-produced peptides resulted in similar inhibition. With ^{125}I-$\alpha1$-CB6 as a test antigen, the $\alpha1$ chain was also more effective as an inhibitor than equi-

molar amounts of native collagen. Thus in the helical collagen molecule the antigenic determinants of both $\alpha1$ and $\alpha2$ are not fully available to react with antibodies. Furthermore, the data in Fig. 6 suggest that native collagen inhibited binding of antibodies to $\alpha2$-CB1 somewhat more effectively than binding to $\alpha2$-CB5, indicating that in the $\alpha2$ chain the amino-terminal antigenic determinant may be more exposed than the carboxy-terminal determinant.

DISCUSSION

Although the immunologic properties of collagen have been studied for many years (see ref. 1 for a review), a precise localization of the antigenic determinants in the molecule required the development of more extensive knowledge of its molecular structure and a sensitive and quantitative means for detecting specific antibodies. In 1964 Schmitt et al. (8) using complement fixation reported that antibodies were directed toward regions of bovine collagen chains which were susceptible to selective attack in the native protein by enzymes such as pepsin and pronase. Presumably these sequences were not in a triple helical conformation. Davison et al. (9) extended these studies and showed that much of the immunologic species-specificity of collagen resided in these protease-susceptible regions and that antigenic determinants were located on both $\alpha1$ and $\alpha2$ chains. Limited cleavage of soluble rat skin collagen with both proteolytic enzymes and CNBr (21) has indicated that such extrahelical peptides include the amino-terminal sequences of the $\alpha1$ and $\alpha2$ chains, a finding supported by structural studies of the protein (17, 22).

Perhaps the most extensive studies of the antigenicity of collagen and its relation to collagen structure are those of Steffen, Timpl, and coworkers (4, 23–26). The immunologic properties of native and heat-dissociated collagens from a number of species were investigated by means of the passive hemagglutination and hemagglutination inhibition techniques. In general, antibody specificities could be divided into those directed toward species-specific determinants and others directed toward non–species-specific or general collagen determinants; the distribution of antibody specificities differed with the nature of the injected antigen (4, 24). The species-specific determinants were subdivided into pepsin-labile and pepsin-resistant determinants. Antibodies specific to the native conformation of collagen were not detected. In accordance with the previous studies of Schmitt, Davison, and coworkers (8, 9), absorption and inhibition studies were consistent with the conclusion that pepsin-labile species-specific determinants were located on extrahelical, protease-sensitive regions of collagen whereas general collagen determinants existed largely in the main body of the collagen helix which in the native protein is degraded only by collagenase (25, 26).

The above studies set the stage for a more precise localization of antigenic determinants on the collagen molecule utilizing inhibition of antigen–antibody

reactions with well characterized collagen chains and fragments. The present studies were undertaken to provide a comprehensive investigation of the best characterized collagen, rat skin collagen, in order to permit correlation between its chemical and immunological properties. During the course of this work reports describing similar approaches with rat (5), guinea pig (6), and calf skin collagen (7) have appeared.

At the inception of this study double diffusion in 1% agar or agarose revealed the presence of weakly precipitating antibodies to $\alpha2$ chains. Absorption of antisera with $\alpha1$ had no effect on this reaction and purified $\alpha1$ chains failed to produce precipitin lines with a variety of antisera (27). However, the limitations of these techniques were apparent. A sensitive radioimmunoassay was therefore developed to provide quantitative information. This assay does not require adsorption of antigen to erythrocytes and is able to detect univalent antigens by subsequent immune precipitation of antigen–antibody complexes.

Our results indicate that antigenic determinants exist on both $\alpha1$ and $\alpha2$ chains of rat skin collagen and that no cross-reactivity exists between antibodies directed toward these determinants. Thus $\alpha1$ chains failed to inhibit binding of ^{125}I-$\alpha2$ with an antiserum to rat skin collagen. Similarly, preparations of $\alpha2$ did not significantly inhibit binding of ^{125}I-$\alpha1$ with an antiserum to rat skin collagen. The minor and progressive inhibition seen in the latter system (See Fig. 2) is compatible with contamination of preparations of $\alpha2$ with $\alpha1$ (as β_{12}) to the extent of 3–5%. However, the studies of Timpl et al. (5) indicate that the $\alpha2$ chain and $\alpha2$-CB5 are capable of inhibiting species-specific antibodies to $\alpha1$ although no inhibition of antibodies to $\alpha2$ with $\alpha1$ was observed. Furthermore, the possibility remains that the non–species-specific antibodies studied by others will cross-react with $\alpha1$ and $\alpha2$ chains.

In our studies the $\alpha2$ chain of rat skin collagen proved to be a better immunogen (in the rabbit) than did the $\alpha1$ chain. Of particular note is that immunizations with isolated $\alpha1$ chains produced no detectable antibodies as tested with the isolated $\alpha1$ chains. However, immunizations with whole collagen produced antibodies to this chain. Since detection of antibodies to $\alpha1$ was readily achieved with the isolated chains, the antibodies produced were not directed specifically to the native conformation of the protein.

Experiments with antisera to rat skin collagen indicate that the main antigenic determinant of the $\alpha1$ chain is located on $\alpha1$-CB6 (Fig. 4). Lesser degrees of inhibition were observed with $\alpha1$-CB7 and with a mixture of $\alpha1$-CB4 and $\alpha1$-CB5. $\alpha1$-CB6 was also identified as the major antigenic determinant in rat and calf skin collagen using hemagglutination inhibition (5, 7).

Studies with $\alpha2$ fragments as inhibitors indicate that the predominant antigenic determinant of the $\alpha2$ chain is located on CNBr fragment $\alpha2$-CB5 (Table I). Antibodies to $\alpha2$-CB1 were also demonstrated but only by direct assay with ^{125}I-$\alpha2$-CB1 (Table II). No inhibition was observed with large

quantities of $\alpha2$-CB1 when antisera were tested with ^{125}I-$\alpha2$ (Table I). The most likely explanation for these findings is the presence, in the antisera tested, of a large proportion of antibodies to $\alpha2$-CB5 in contrast to a relatively small proportion of antibodies to $\alpha2$-CB1. Thus labeled $\alpha2$ chains would be precipitated with antibodies to $\alpha2$-CB5, precluding significant inhibition by $\alpha2$-CB1. Other workers using the hemagglutination technique with calf skin collagen (7) and rat skin collagen (5) as antigens also concluded that the major determinant of the $\alpha2$ chain is located on $\alpha2$-CB5. These findings differ from those of Michaeli et al. (6) who identified the NH_2-terminal region of the $\alpha2$ chain ($\alpha2$-CB1) as the site of the major antigenic determinant in guinea pig collagen.

It is clear that any studies of the antigenicity of collagen must take into consideration the structure of collagen in the immunized species (usually the rabbit) and the degree to which the rabbit will produce antibodies to determinants which exist on its own collagen. Although comparative data are incomplete, the region of the collagen molecule which appears to differ most between species, i.e. the amino-terminal end of the $\alpha2$ chain (28–31), has been identified as the site of an antigenic determinant. Thus, the absence of an antigenic determinant on rat skin $\alpha1$-CB1 and the presence of a determinant on $\alpha2$-CB1 are consistent with the identity in compositions of rat and rabbit $\alpha1$-CB1 and the considerable differences in the compositions of $\alpha2$-CB1 (31).

The finding, in this and other (5, 7) studies, that major antigenic determinants are clustered in the carboxy-terminal regions of the $\alpha1$ and $\alpha2$ chains (represented by $\alpha1$-CB6 and $\alpha2$-CB5 respectively) suggests that considerable interspecies variability may also exist at the carboxy-terminal ends of both α chains. In keeping with this possibility Pontz et al. (7) were able to restrict the location of the antigenic determinants in calf skin $\alpha2$-CB5 and $\alpha1$-CB6 to the last 100 amino acids in the two chains. The presence of tyrosine in $\alpha1$-CB6 of rat (13) and calf (32) collagen and its absence in the carboxyl-terminal sequence of the rabbit $\alpha1$ chain (31) are indicative of interspecies differences in this region of the molecule. Such differences may be immunologically apparent in part because tyrosyl residues are known to enhance the immunogenicity of gelatin, collagen, and other proteins (33, 34).

Although the structure of collagen is highly conserved during evolution, interspecies structural differences exist throughout the collagen helix as well as at the nonhelical ends of the chains (28–31, 35). The former structural differences may account for the general collagen determinants described by Steffen and coworkers (4, 25). Antibodies to such determinants can be expected when the immunized animal is distant on the evolutionary scale from the animal that serves as the source of the antigen.

To some extent the differences in the location of antigenic determinants which have been described by several laboratories may reflect differences in the nature of the immunizing antigen and in the method of detection of the antibody

response. It is likely that the immunogenicity of certain antigenic determinants will vary with conformation of the immunizing protein (i.e. whether native or heat-dissociated) and the ability to detect the presence of antibody populations will similarly be affected by the nature of the test antigen. Thus, our experiments show that antigenic determinants on α chains are less available in the native protein than in the unfolded chains. In addition, early bleedings of immunized animals and test systems, such as hemagglutination and complement fixation, favor detection of γM-globulin antibodies (36). On the other hand, the immunoassay used in this investigation selected for γG-antibodies by the use of hyperimmune antisera to collagen and an antiserum to rabbit γG-globulin.

The consequences of iodination of tyrosyl residues in collagen chains used as test antigens must also be considered. Initial attempts at iodination of collagen led to preparations which were relatively insoluble and resistant to cleavage with CNBr. Aggregation of collagen resulting from iodination of tyrosyl and histidyl residues has been reported (37), although the nature of the bonds involved in this polymerization are unknown. Under the conditions used in the present experiments, chain aggregation was minimized. Radiolabeled chains and peptides coeluted with carrier polypeptides in gel filtration experiments, but consistent differences in elution from carboxymethyl cellulose were observed. Despite the possibility that iodination may alter antigenic sites in proximity to tyrosyl residues and produce subtle chemical changes which are reflected in changes in chromatographic behavior, no evidence was found in these studies for antibody discrimination between iodinated and unlabeled polypeptides.

The experiments reported here, together with parallel studies in other laboratories, indicate that an immunochemical approach will be useful in investigations of the comparative biochemistry of collagen. The ability to produce antibodies to specific regions of the collagen molecule may also assist in the detection of alterations of this protein in human disease.

SUMMARY AND CONCLUSIONS

Immunological studies of rat skin collagen were carried out with a sensitive and quantitative radioimmunoassay. Hyperimmune rabbit antisera to rat skin collagen and isolated α2 chains were used. Iodine-labeled α chains and CNBr-produced peptides served as test antigens, and native collagen, α chains, and CNBr peptides were employed as inhibitors in the assay.

The α1 and α2 chains were immunologically distinct. Although the α1 chain was not immunogenic, antibodies to α1 were detected in antisera to the intact collagen molecule. The major antigenic determinant of the α1 chain was located in α1-CB6 which constitutes the carboxy-terminal region of the chain. The α2 chain contained two non–cross-reacting antigenic determinants, one in the amino-terminal region (α2-CB1) and the other in the carboxy-terminal region

(α2-CB5) of the chain. The native collagen molecule was less effective than isolated α chains in inhibiting binding of labeled peptides to antisera, indicating that antigenic determinants were less accessible in the triple helical molecule.

These immunologic studies are consistent with preliminary comparative biochemical data which indicate that interspecies structural differences in collagen predominate at both the amino- and carboxy-terminal ends of the chains.

BIBLIOGRAPHY

1. Kirrane, J. A., and L. E. Glynn. 1968. Immunology of collagen. *In* International Review of Connective Tissue Research. David Hall, editor. Academic Press, Inc., New York. **4**:1.
2. Rothbard, S., and R. F. Watson. 1961. Antigenicity of rat collagen. Demonstration of antibody to rat collagen in the renal glomeruli of rats by fluorescence microscopy. *J. Exp. Med.* **113**:1041.
3. Rothbard, S., and R. Watson. 1965. Immunologic relations among various animal collagens. *J. Exp. Med.* **122**:441.
4. Steffen, C., R. Timpl, and I. Wolff. 1968. Immunogenicity and specificity of collagen. V. Demonstration of three different antigenic determinants on calf collagen. *Immunology.* **15**:135.
5. Timpl, R., P. P. Fietzek, H. Furthmayr, W. Meigel, and K. Kuhn. 1970. Evidence for two antigenic determinants in the C-terminal region of rat skin collagen. *Fed. Eur. Biochem. Soc., Letters.* **9**:11.
6. Michaeli, D., G. Martin, J. Kettman, E. Benjamini, D. Leung, B. Blatt. 1969. Localization of antigenic determinants in the polypeptide chains of collagen. *Science (Washington).* **166**:1522.
7. Pontz, B., W. Meigel, J. Rauterberg, and K. Kuhn. 1970. Localization of two species - specific antigenic determinants on the peptide chain of calf skin collagen. *Eur. J. Biochem.* **16**:50.
8. Schmitt, F. O., L. Levine, M. Drake, A. Rubin, D. Pfahl, P. F. Davison. 1964. The antigenicity of tropocollagen. *Proc. Nat. Acad. Sci. U.S.A.* **51**:493.
9. Davison, P. F., L. Levine, M. P. Drake, A. Rubin, and S. Bump. 1967. The serologic specificity of tropocollagen telopeptides. *J. Exp. Med.* **126**:331.
10. LeRoy, E. C. 1968. Precipitating and complement-fixing antibodies to collagen with species and collagen subunit specificity. *Proc. Soc. Exp. Biol. Med.* **128**:341.
11. Rothbard, S., and R. Watson. 1956. Antigenicity of rat collagen. Reverse anaphylaxis induced in rats by anti-rat collagen serum. *J. Exp. Med.* **103**:57.
12. Piez, K. A., E. A. Eigner, and M. S. Lewis. 1963. The chromatographic separation and amino acid composition of the subunits of several collagens. *Biochemistry.* **2**:58.
13. Butler, W. T., K. A. Piez, and P. Bornstein. 1967. Isolation and characterization of the cyanogen bromide peptides from the α1 chain of rat skin collagen. *Biochemistry.* **6**:3771.

14. Fietzek, P. P., and K. A. Piez. 1969. Isolation and characterization of the cyanogen bromide peptides from the α2 chain of rat skin collagen. *Biochemistry*. 8:2129.

15. Piez, K. A., E. J. Miller, and J. M. Lane. 1969. The order of the CNBr peptides from the α1 chain of collagen. *Biochem. Biophys. Res. Commun.* 37:801.

16. Vuust, J., J. M. Lane, P. P. Fietzek, E. J. Miller, and K. A. Piez. 1970. The order of the CNBr peptides from the α2 chain of collagen. *Biochem. Biophys. Res. Commun.* 38:703.

17. Bornstein, P., and K. A. Piez. 1966. The nature of the intramolecular cross-links in collagen. The separation and characterization of peptides from the cross-link region of rat skin collagen. *Biochemistry*. 5:3460.

18. Helmkamp, R. W., M. A. Contreras, and W. F. Bale. 1967. I^{131}-labeling of proteins by the iodine monochloride method. *Int. J. Appl. Radiat. Isotop.* 18:737.

19. Porath, J., R. Axén, and S. Ernback. 1967. Chemical coupling of proteins to agarose. *Nature (London)*. 215:1491.

20. Kivirikko, K. I., O. Laitinen, and D. J. Prockop. 1967. Modifications of a specific assay for hydroxyproline in urine. *Anal. Biochem.* 19:249.

21. Bornstein, P., A. Kang, and K. A. Piez. 1966. The limited cleavage of native collagen with chymotrypsin, trypsin and cyanogen bromide. *Biochemistry*. 5:3803.

22. Kang, A. H., P. Bornstein, and K. A. Piez. 1967. The amino acid sequence of peptides from the cross-linking region of rat skin collagen. *Biochemistry*. 6:788.

23. Steffen, C., R. Timpl, and I. Wolff. 1964. Immunogenicity and specificity of collagen. *J. Immunol.* 93:656.

24. Steffen, D., R. Timpl, and I. Wolff. 1967. Immunogenität und Spezifität von Kollagen. III. Erzeugung und Nachweis von Anti-Kollagen-Antikörpern und serologischer Vergleich von nativem und denaturiertem löslichem Kollagen. *Z. Immunitaetsforsch. Allerg. Klin. Immunol.* 134:91.

25. Timpl, R., I. Wolff, H. Furthmayr, and C. Steffen. 1968. Immunogenicity and specificity of collagen. VI. Separation of antibody fractions with restricted specificity from anti-collagen sera using an immunoabsorbent technique. *Immunology*. 15:145.

26. Steffen, C., and R. Timpl. 1970. Immunogenicity and specificity of collagen. X. Investigations on collagen determinants by inhibition experiments with peptides obtained after multiple degradation. *Z. Immunitaetsforsch. Allerg. Klin. Immunol.* 139:455.

27. Lindsley, H. B., M. Mannik, and P. Bornstein. 1969. Immunogenicity of rat and human collagen polypeptide chains. *Arthritis Rheum.* 12:676.

28. Bornstein, P. 1968. Collagen: Relatively invariant (helical) and variable (non-helical) regions. *Science (Washington)*. 161:592.

29. Piez, K. A., H. A. Bladen, J. M. Lane, E. J. Miller, P. Bornstein, W. T. Butler, and A. H. Kang. 1968. Comparative studies on the chemistry of collagen utilizing cyanogen bromide cleavage. *Brookhaven Symp. Biol.* 21:345.

30. Bornstein, P., and A. H. Kang. 1970. Comparative biochemical studies of collagen. *In* Chemistry and Molecular Biology of the Intercellular Matrix. E. A. Balazs, editor. Academic Press, Inc., New York. 1:99.

31. Bornstein, P., and R. Nesse. 1970. The comparative biochemistry of collagen: The structure of rabbit skin collagen and its relevance to immunochemical studies of collagen. *Arch. Biochem. Biophys.* **138**:443.

32. v. d. Mark, K., F. Rexrodt, P. Wendt, and K. Kuhn. 1970. Isolation and characterization of cyanogen bromide-cleaved peptides from a collagenase digest of $\alpha 1$ chain of calf skin collagen. *Eur. J. Biochem.* **16**:143.

33. Arnon, R., and M. Sela. 1960. Studies on the chemical basis of the antigenicity of proteins. II. Antigenic specificity of polytyrosyl gelatins. *Biochem. J.* **75**:103.

34. Kirrane, J. A., and W. Van B. Robertson. 1968. The antigenicity of native and tyrosylated neutral-salt-soluble rat collagen. *Immunology.* **14**:139.

35. Click, E. M., and P. Bornstein. 1970. Isolation and characterization of the cyanogen bromide peptides from the $\alpha 1$ and $\alpha 2$ chains of human skin collagen. *Biochemistry.* **9**:4699.

36. Robbins, J. B., K. Kenny, and E. Suter. 1965. The isolation and biological activities of rabbit γM- and γG-anti-*Salmonella typhimurium* antibodies. *J. Exp. Med.* **122**:385.

37. Bensusan, H. B. 1966. Protein crosslinkages. I. Effect of iodination on the time-dependent solubility changes of reconstituted collagen fibrils. *Arch. Biochem. Biophys.* **115**:77.