Characterization of a Novel Collagen Chain in Human Placenta and Its Relation to AB Collagen†

Helene Sage‡ and Paul Bornstein*

ABSTRACT: A novel collagen chain, termed αC, has been isolated from human placenta by limited pepsin digestion. The collagen containing the αC chain copurifies with placental AB collagen during selective salt precipitation but is virtually absent from fetal birth membranes, which contain relatively larger amounts of AB. Both native AB and αC-containing collagens are resistant to human skin collagenase under conditions that support cleavage of type I by greater than 90%. The αC chain was separated from αB by phosphocellulose chromatography and subsequently from αA by chromatography on CM-cellulose. Its amino acid composition is distinct from αA and αB although all three chains possess compositional features in common; the carbohydrate content of the αC chain was intermediate between those of αA and αB. Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis of peptides produced by CNBr cleavage and by limited digestion with the enzyme mast cell protease indicated different and unique products for the αA, αB, and αC chains. The data support the existence of another collagen chain which is related to the αA and αB chains but which is structurally unique. The proteins containing these chains may in turn comprise a subfamily of collagen isotypes which represents a divergence from and/or specialization of the type IV basement membrane collagens.

Recent studies on vertebrate collagen types have contributed to an understanding of these proteins as members of a family of closely related but structurally distinct gene products. Evidence for this concept has been provided by detailed structural analyses of collagens isolated from a variety of in vitro and in vivo systems [for reviews see Miller (1976) and Kefalides (1975)]. Present knowledge supports the existence of three interstitial collagens (types I, II, and III) and at least two basement membrane types (IV and AB)[1] although further studies are necessary to establish the extent to which these categories are distinct. Only type I collagen has been shown to be a heteropolymer comprised of two different chains; however, the subunit structure of the basement membrane collagens has not been resolved (Bentz et al., 1978; Rhodes & Miller, 1978; Burgeson et al., 1976).

By amino acid composition AB collagen more closely resembles type IV, purified from isolated basement membranes, than it does the interstitial types (Burgeson et al., 1976; Chung et al., 1976; Rhodes & Miller, 1978; Bentz et al., 1978). However, there are consistent structural differences between types IV and AB which argue for the latter protein as a unique isotype in the multigene collagen family. The ubiquitous distribution of AB collagen has been suggested by its presence in diverse locations such as fetal membranes (Burgeson et al., 1976), skin (Chung et al., 1976), placenta and bone (Rhodes & Miller, 1978), synovial membranes (Brown et al., 1978), lung parenchyma (Madri & Furthmayr, 1979), smooth muscle cells in culture (Mayne et al., 1978), dedifferentiated chondrocytes (Benya et al., 1977), embryonic tendons (Jimenez et al., 1978), and possibly neural retinal cells in culture (Linsenmayer & Little, 1978). Its specific localization to morphologically distinct basement membranes has therefore not been established. One interesting role for this collagen has been proposed by Stenn et al. (1979), who suggest that migrating epithelium synthesizes AB collagen and that such activity is necessary for continual movement of these cells.

Questions concerning the possible importance of AB collagen in cell surface associated phenomena, as well as its significance both structurally and functionally as a unique collagen type, have prompted us to continue studies on this collagen. In the course of these studies, a collagen chain was encountered which appeared to be different from αA, αB, and the chains comprising types I–IV collagens. This novel collagen chain behaved similarly chromatographically to a chain called "αC", which was copurified with AB collagen from synovial membranes by Brown et al. (1978), and to minimize confusion we have chosen to retain that nomenclature. This paper describes the location, isolation, chromatographic purification, and preliminary structural characterization of the αC chain and its comparison with the αA and αB chains to which it appears to be related. Additional information concerning the quantitation of AB collagen in several tissues and its susceptibility to vertebrate collagenase is also presented. The data strongly suggest that types AB and C collagens form a distinct subfamily within the group of basement membrane collagens.

Materials and Methods

Isolation of Collagen. AB collagen was isolated from human amnion, chorion, and/or placenta according to the procedure outlined in Figure 1, which represents a composite of methods described by Burgeson et al. (1976) and Rhodes & Miller (1978) for purification of these collagens. The αC chain containing collagen is either absent or present in very low concentrations in amnion and chorion but is extractable from placenta and copurifies with native AB collagen.

Washed fetal membranes were extracted in distilled water containing 0.2 mM PMSF[2] and 0.02 M EDTA at 4 °C for

† From the Departments of Biochemistry and Medicine, University of Washington, Seattle, Washington 98195. *Received April 12, 1979.* This work was supported in part by National Institutes of Health Grants AM 11248 and HL 18745.

‡ H.S. is a recipient of a fellowship from the R. J. Reynolds Industries, Inc.

[1] As the stoichiometry of the αA and αB chains and their possible relationship to each other have not yet been resolved, we refer to the native type as AB collagen. Similarly, native collagen molecules containing the αC chain (regardless of the presence of any other chain type) are referred to as C collagens even though such proteins may also contain αA and/or αB chains.

[2] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; MalNEt, *N*-ethylmaleimide; DTT, dithiothreitol; GlcGalHyl, glucosylgalactosylhydroxylysine; GalHyl, galactosylhydroxylysine.

0006-2960/79/0418-3815$01.00/0

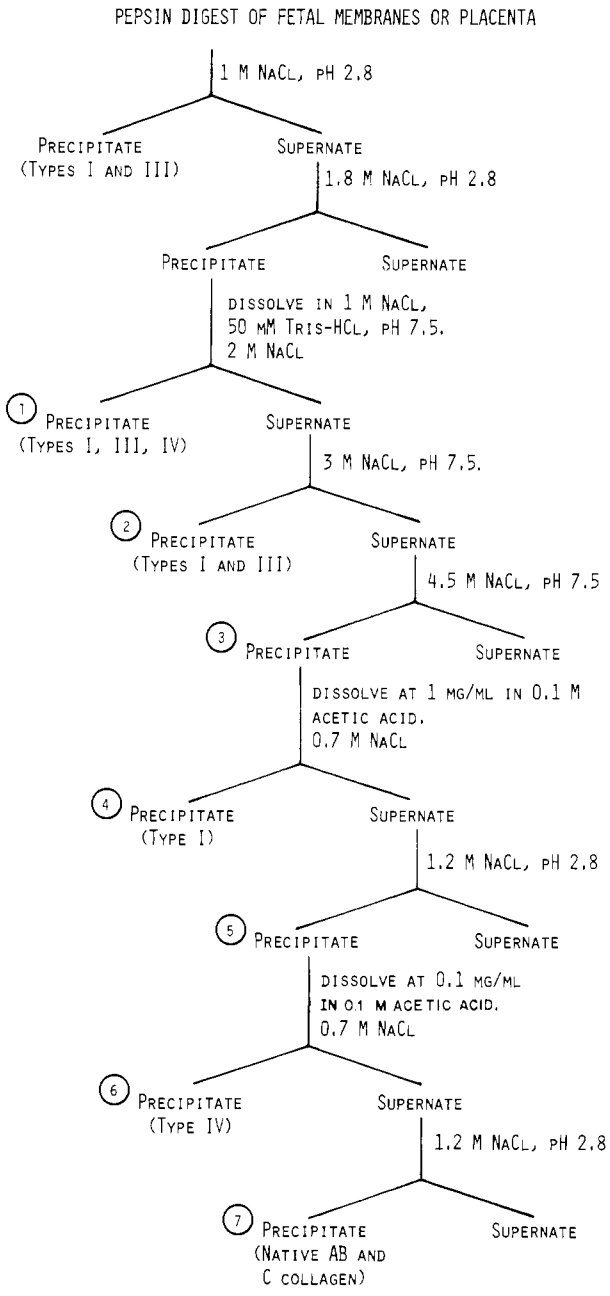


FIGURE 1: Procedure for the isolation of AB and C collagens. Numbers 1–7 identify the successive precipitates examined in Figure 2.

48 h, filtered through cheesecloth, homogenized in a VirTis homogenizer, and lyophilized. All subsequent procedures were also performed at 4 °C. The dried tissue was suspended in 0.5 M acetic acid and digested with pepsin A (Worthington) for 24 h (10 g of tissue–1 L of 0.5 M acetic acid–1 g of pepsin) (Miller, 1972). Alternatively, placentas dissected free of amnion and chorion were washed with distilled water, dispersed in a meat grinder, and sequentially extracted with four changes each of distilled water (24 h), 1 M NaCl, 50 mM Tris-HCl, pH 7.5 (4 days), and 0.5 M acetic acid (3 days), as described by Epstein et al. (1971). Each change was accomplished by filtering the tissue through a colander. The extracted tissue was then squeezed through cheesecloth, weighed, suspended in 0.5 M acetic acid, and digested with pepsin for 24 h (400 g of tissue–2 L of 0.5 M acetic acid–1 g of pepsin) (Rhodes & Miller, 1978). Pepsin digests were clarified by centrifugation for 1 h at 7100*g* prior to salt fractionation. Subsequent procedures are outlined in Figure 1 and are described in detail for the fractionation of type IV collagen from placenta (Sage et al., 1979).

In order to assess purity and recovery, we monitored the protein at each precipitation step by $NaDodSO_4$–polyacrylamide gel electrophoresis, weight of lyophilized precipitates, and amino acid analysis. For studies involving quantitation of AB collagen in different tissues, this collagen was recovered from several other precipitates in which there were small amounts of AB (1 M NaCl, pH 2.8, and 3 M NaCl, pH 7.5) by solution in 0.1 M acetic acid and fractionation with 0.7 and 1.2 M NaCl. Ratios of A/B/C were calculated from densitometric scans of these chains after resolution by $NaDodSO_4$–polyacrylamide gel electrophoresis and from peak areas after resolution by ion-exchange chromatography.

Levels of AB collagen were also measured in membranes from 72- and 135-day-old fetuses according to the procedures described above.

Treatment of Native Collagens with Vertebrate Collagenase. Purified native AB and C collagens were dissolved in 0.1 M acetic acid at a concentration of 1 mg/mL and dialyzed against 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$, 0.2 mM PMSF, and 10 mM MalNEt at 4 °C. The solution was clarified by centrifugation and incubated at 22 °C for 24 h with highly purified human skin collagenase (a gift of Dr. A. Eisen) at an enzyme to substrate weight ratio of 1:100. Another aliquot of enzyme was subsequently added, producing an enzyme to substrate weight ratio of 1:50, and incubation continued for an additional 24 h. Digestion of type I human skin collagen was performed in parallel. The reaction was stopped by addition of an equal volume of $NaDodSO_4$–urea sample buffer containing 50 mM DTT, and the products were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis on 10% or composite 7.5%/12.5% separating gels (Laemmli, 1970).

In addition, the reactivity of this enzyme toward denatured type I and αA and αB chains was examined as described for the native protein by using collagens which had been purified by ion-exchange chromatography in the presence of 6 M urea. The samples were heated at 45 °C for 30 min prior to incubation with the enzyme. A second collagenase, isolated from rabbit articular synovial membranes (a gift of Dr. E. Harris), was also tested on native collagens by using the same conditions as described for human skin collagenase.

Chromatography on Phosphocellulose. Collagen comprised of the αA, αB, and αC chains was chromatographed on phosphocellulose (Whatman P-1, Whatman, Inc.) essentially as described for the αA and αB chains by Rhodes & Miller (1978). A sample (6–10 mg) was dissolved at a concentration of 0.5 mg/mL in 0.1 M acetic acid at 4 °C and dialyzed against the column buffer (30 mM dibasic sodium phosphate, pH 6.3) at room temperature. Prior to column loading, the sample was heated at 42 °C for 30 min and clarified by centrifugation. Gradient elution was performed as described by Rhodes & Miller (1978). Appropriate fractions were pooled, dialyzed against 0.1 M acetic acid, and lyophilized. Type I collagen from lathyritic rat skin was used as a standard.

Chromatography on CM-cellulose. αA and αC collagen chains, which coelute on phosphocellulose, were dissolved in 0.1 M acetic acid and dialyzed against CM-cellulose buffer (6 M urea and 40 mM sodium acetate, pH 4.8) (for lyophilized samples) or dialyzed directly from phosphocellulose buffer. Samples were heated at 42 °C for 30 min and clarified by centrifugation before application to a column (1.0 × 10 cm) of CM-cellulose (CM-52, microgranular) maintained at 42 °C. Bound protein was eluted with a linear gradient from 0–80

mM NaCl in 200 mL at a flow rate of 30 mL/h. Samples of the αB chain were chromatographed under identical conditions. Fractions were pooled, dialyzed against 0.1 M acetic acid, and lyophilized. Type I lathyritic rat skin collagen was used as a standard.

Amino Acid Analysis. Lyophilized protein (100–500 μg) was dissolved in 0.5–1 mL of constant boiling HCl, flushed with N_2, and hydrolyzed in sealed glass ampules in vacuo at 108 °C for 24 h. Samples were analyzed on a Beckman 121 amino acid analyzer which had been modified for single-column microanalysis. Cysteine was determined as cysteic acid (Moore, 1963). Alkaline hydrolysis for determination of hydroxylysine-linked carbohydrate was performed according to Odell et al. (1974). Tryptophan was not determined.

Peptide Mapping Using CNBr Cleavage and Mast Cell Protease Digestion. CNBr digestion of lyophilized αA, αB, αC, and type I collagen, followed by analysis on $NaDodSO_4$–polyacrylamide gel electrophoresis, was performed according to Crouch & Bornstein (1978). In addition, some samples (100–200 μg) were incubated in 250 μL of 0.2 M acetic acid containing 5% β-mercaptoethanol (v/v) at 37 °C for 24 h to reduce methionine sulfoxide (Hudson & Spiro, 1972). These samples were diluted 1:5 with deaerated water, lyophilized, and subsequently digested with CNBr as described above.

Native AB and C collagens were dissolved in 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5, and digested with mast cell protease (a gift of Dr. R. Woodbury) under conditions identical with those described above for vertebrate collagenase. Denatured αA, αB, and αC chains in the same buffer were also incubated with the enzyme at an enzyme to substrate weight ratio of 1:100 at 37 °C for 5–60 min. The reaction was terminated by adding an equal volume of $NaDodSO_4$–urea sample buffer containing 50 mM DTT, and the cleavage products were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis using a 10% separating gel.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Proteins were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis according to the methods of Laemmli (1970) and Studier (1973) as described by Crouch & Bornstein (1978). Protein was stained with 0.25% Coomassie Brilliant Blue R (Sigma) in 20% trichloroacetic acid (w/v), 45% methanol (v/v), and 9% acetic acid (v/v), and the gels were destained in 5% methanol and 7.5% acetic acid (v/v). Protein bands were scanned at 595 nm in their linear range by using a scanning densitometer (Quick Scan, Helena Laboratories).

Results

Isolation and Recovery of AB and C Collagens from Different Tissues. $NaDodSO_4$–polyacrylamide gel electrophoresis illustrating a typical purification of AB and C collagens from pepsin-treated human placenta is presented in Figure 2. At pH 7.5, a concentration of 2 M NaCl resulted in the precipitation of principally types I and III collagens (lane 1). However, two additional bands corresponding to apparent molecular weights of 140 000 and 70 000 were also visible; these proteins have recently been characterized as products of type IV collagen (Sage et al., 1979). Fractionation at 3 M NaCl resulted in additional precipitation of types I and III and some coprecipitation of AB and C (lane 2), but the latter collagens were significantly enriched for in the 4.5 M NaCl precipitate (lane 3). When the latter precipitate was fractionated at a concentration of 0.1 mg/mL at acidic pH, type I was removed at a NaCl concentration of 0.7 M (lane 4), leaving relatively pure AB and C collagens which were precipitated at 1.2 M NaCl (lane 5). However, a second

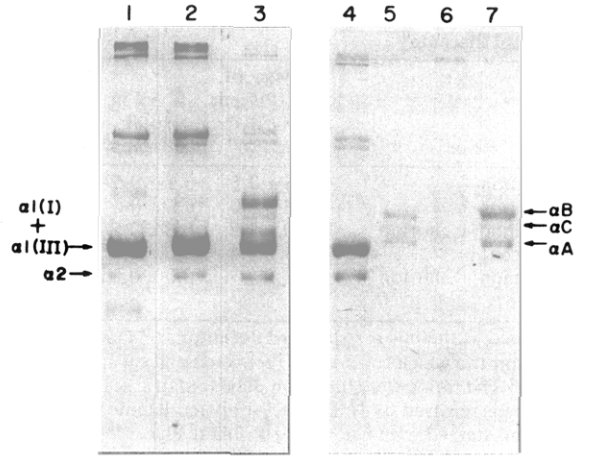


FIGURE 2: Purification of native AB and C collagens from human placenta. Pepsin-treated collagen was selectively salt-precipitated at neutral and acidic pH as described in Figure 1. Protein was resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on composite slab gels (6%/10%) in the presence of 50 mM DTT and stained with Coomassie Brilliant Blue. Numbered lanes refer to precipitates identified in Figure 1. Each lane contained 20 μg of protein. (1) NaCl (2 M) precipitate, pH 7.5; (2) NaCl (3 M) precipitate, pH 7.5; (3) NaCl (4.5 M) precipitate, pH 7.5; (4) NaCl (0.7 M) precipitate, pH 2.8; (5) NaCl (1.2 M) precipitate, pH 2.8; (6) NaCl (0.7 M) precipitate, pH 2.8; (7) NaCl (1.2 M) precipitate, pH 2.8. Positions of migration of several collagen chains isolated from this tissue are indicated by arrows.

exposure of this material, dissolved at 0.1 mg/mL, to 0.7 M NaCl resulted in the removal of the remaining type IV collagen (lane 6), allowing isolation of a preparation containing AB and C collagens which was pure by the criterion of $NaDodSO_4$–polyacrylamide gel electrophoresis (lane 7). The αB, αA, and αC chains were recovered from placenta by using the procedure described in Figure 1 in a ratio of 1:0.9:0.4. The relative mobilities of these chains on $NaDodSO_4$–polyacrylamide gel electrophoresis were not altered by the absence of reducing agent.

Purification of AB collagen from fetal membranes also proceeded as described in Figure 2. We were unable to detect any type IV or C in amnion, but very low levels of these collagens were present in chorion (<0.01% of total collagen). In this regard it is interesting that we have observed, in 4.5 M NaCl precipitates of pepsin-treated human fibroblast and amniotic fluid cell layers, two nonreducible bands on $NaDodSO_4$–polyacrylamide gel electrophoresis with the characteristic mobility of the αA and αB chains (data not shown). In bovine endothelial cell layers which were analyzed in the same manner, a band corresponding to the αC chain was seen, in addition to those comigrating with αA and αB chains (H. Sage, E. Crouch, and P. Bornstein, unpublished experiments).

Recoveries of AB collagen from different tissues are listed in Table I. The results indicate that the yield of total collagen was greater from amnion (28%) than from both chorion and placenta (17 and 20%, respectively). However, the percentage of AB collagen in total collagen was the same for both fetal membranes but was significantly diminished in placenta (6.5 vs. 2%, respectively). Each value reported in Table I was calculated from triplicate determinations on several preparations of each tissue. The tissue combinations (rows 3 and 5) were included as a measure of the reproducibility of the methods and to assess the contribution of each tissue to the mixture in terms of recovered amounts of AB collagen.

The ratios of αB to αA in amnion and chorion were found to be 1.21 and 1.28, respectively, while this value for the

Table I: Recoveries of αA and αB Chains from Amnion, Chorion, and Placenta[a]

tissue	recovery of total collagens from dried tissue (%)[b]	% AB in total collagens[b]	αB/αA[c]
(1) amnion	28.3 ± 2.5	6.5 ± 0.5	1.21
(2) chorion	17.1 ± 2.0	6.5 ± 0.6	1.28
(3) amnion + chorion	19.0 ± 1.9	6.8 ± 0.5	1.28
(4) placenta	19.5 ± 4.4	2.0 ± 0.7[d]	1.14
(5) amnion + chorion + placenta	nd[e]	3.0 ± 0.6[d]	1.15

[a] Expressed as the mean ± standard deviation. [b] Quantitation was based on the weight of protein recovered and on amino acid analysis. [c] The ratios were based on densitometric scans of αA and αB chains resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. [d] Includes C collagen. [e] Not detected.

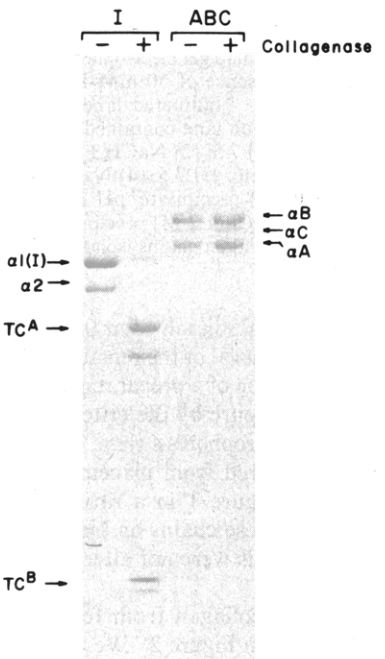


FIGURE 3: Comparative susceptibility of native type I and AB and C collagens to human skin collagenase. Samples were incubated in buffer or with enzyme for 48 h and were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis on a composite (7.5%/12.5%) slab gel in the presence of 50 mM DTT. Protein was stained with Coomassie Brilliant Blue. Each lane contained 20 μg of protein. Type I collagen chains and their cleavage products are indicated.

placental material was 1.14 (Table I). When AB collagen was analyzed from 72- and 135-day-old fetal membranes, the αB to αA ratio in amnion was the same as that which had been calculated for the term amnion, but the chorionic αB to αA ratio was significantly increased (1.5–1.6) over the value seen in the term tissue (data not shown). Recoveries of total collagen were similar to those reported from term tissue in Table I, but the percent of AB in this collagen was 1–2%.

Digestion with Vertebrate Collagenase. The susceptibility of native AB and C collagens to vertebrate collagenase is shown in Figure 3. Under conditions that support 90% cleavage of type I human skin collagen, the AB and C collagens were found to be resistant to human skin collagenase. Identical results were obtained with AB collagen when rabbit synovial collagenase was used and when denatured chains were substituted for native collagen (data not shown). A similar lack of susceptibility of type IV collagen to vertebrate collagenase has also been reported (Sage et al., 1979; Woolley et al., 1978; Crouch & Bornstein, 1979).

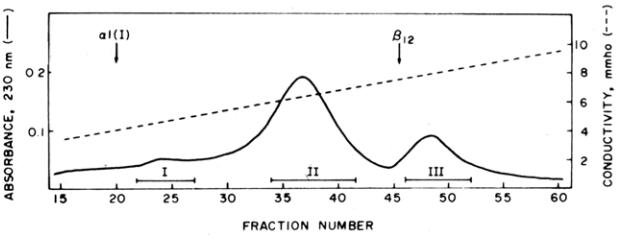


FIGURE 4: Phosphocellulose chromatography of AB and C collagens. The column was equilibrated in 30 mM dibasic sodium phosphate, pH 6.3, and maintained at 42 °C. Protein was eluted with a 0–200 mM NaCl linear gradient over 400 mL at a flow rate of 100 mL/h. Conductivities were read at room temperature. Recovery from this column was 60%. Positions of migration of rat α1(I) and β_{12} chains are indicated. Peak fractions were pooled as indicated (I–III).

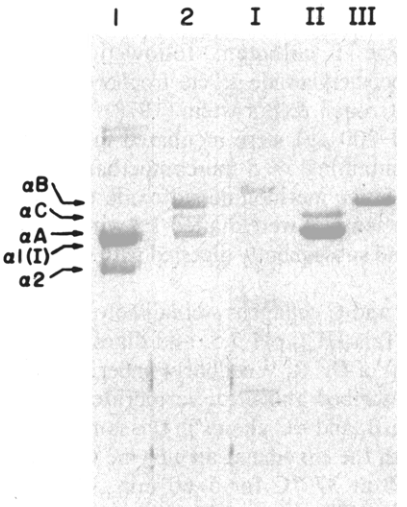


FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of collagens resolved by phosphocellulose chromatography. Samples I–III as indicated in Figure 4 were analyzed on a composite (6%/10%) slab gel in the presence of 50 mM DTT, and protein was visualized by staining with Coomassie Brilliant Blue. (1) Human type I collagen; (2) AB and C collagen sample applied to the phosphocellulose column; (I–III) column fractions pooled as indicated in Figure 4.

Characterization of the αC Chain and Comparison to αA and αB Chains. (*a*) *Phosphocellulose Chromatography.* Figure 4 shows an elution profile which includes three peaks. When analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, peak I contained some residual type IV collagen, as evidenced by two bands which migrated with apparent molecular weights of 140 000 and 70 000 (Sage et al., 1979) (Figure 5). Peak II contained two bands corresponding to the αA and αC chains, while peak III contained the αB chain which appeared homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 5). Complete separation of the αB chain by phosphocellulose chromatography was previously described by Rhodes & Miller (1978). In spite of the disparity in size between peak II and peak III in Figure 4, quantitation of the chains obtained in this system by weight recovery and amino acid analysis has indicated that the ratio of αB to αA is 1:0.9, the same value obtained for the native material before phosphocellulose chromatography.

(*b*) *CM-cellulose Chromatography.* Chromatography of the protein in peak II from the phosphocellulose column on CM-cellulose resulted in the appearance of two peaks (Figure 6A), which were identified by $NaDodSO_4$–polyacrylamide gel electrophoresis as the αA chain and αC chain, respectively (Figure 7). The αB chain was eluted as a single peak from CM-cellulose at only a slightly higher ionic strength than that observed for αC-chain elution (Figure 6B).

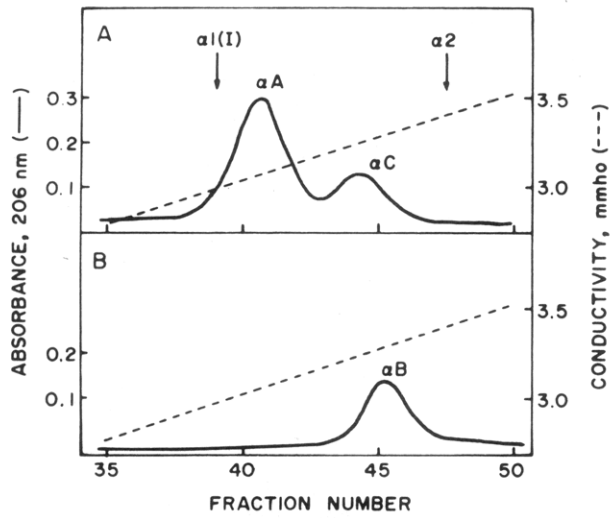


FIGURE 6: CM-cellulose chromatography of αA, αB, and αC collagen chains previously fractionated by phosphocellulose chromatography. The column was equilibrated in 40 mM sodium acetate and 6 M urea, pH 4.8, and maintained at 42 °C. Protein was eluted with a linear gradient of 0–80 mM NaCl in 200 mL at a flow rate of 30 mL/h. Conductivities were read at room temperature. Recoveries were 75–85%. (A) αA plus αC collagen chains (pooled fraction II from the phosphocellulose column; see Figures 4 and 5). (B) αB collagen chain (pooled fraction III from the phosphocellulose column; see Figures 4 and 5). Positions of elution of type I collagen chains isolated from lathyritic rat skin are indicated.

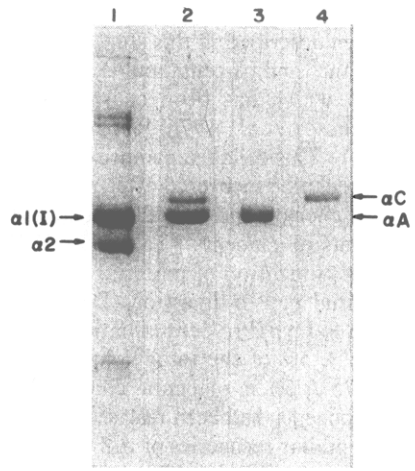


FIGURE 7: $NaDodSO_4$–polacrylamide gel electrophoresis of αA and αC collagen chains after resolution by CM-cellulose chromatography. Protein from each peak obtained after CM-cellulose chromatography was analyzed on a composite (6%/10%) slab gel in the presence of 50 mM DTT and stained with Coomassie Brilliant Blue. (1) Human type I collagen; (2) sample prior to CM-cellulose chromatography (see Figure 5, lane II); (3) αA (see Figure 6A); (4) αC (see Figure 6A).

(*c*) *Amino Acid Analysis.* Amino acid compositions of the αA, αB, and αC chains which had been isolated from placenta and purified by phosphocellulose and CM-cellulose chromatography are shown in Table II. The analyses for the αA and αB chains agree very closely with those reported by Rhodes & Miller (1978) for the same proteins from placenta. However, we were able to detect approximately 1 residue of Cys per 1000 residues in the αB chain by performic acid oxidation (Table II). Our compositions generally support the compositional features reported by Brown et al. (1978) for αA and αB chains isolated from synovial membranes, although we have not observed the difference in glycine level between αA and αB nor the lower arginine content reported by these investigators. The amino acid compositions of the placental

Table II: Amino Acid Compositions of αA, αB, and αC Collagen Chains[a]

	residues/1000		
	αA	αB	αC
Hyl	23	46	43
Lys	13	15	15
His	9.9	6.3	14
Arg	52	42	42
3-Hyp	1.1	0.9	0.9
4-Hyp	107	99	91
Asx	50	50	42
Thr	26	22	19
Ser	34	23	34
Glx	88	100	98
Pro	105	125	99
Gly	325	325	332
Ala	57	41	49
Val	31	21	29
Ile	18	20	20
Leu	39	44	56
Cys[b]	nd[c]	1.2	1.3
Met[d]	10	6.8	8.1
Tyr	2.1	2.5	2.4
Phe	11	12	9.2
GlcGalHyl	9.5	18	17
GalHyl	4.7	12	6.5

[a] Each analysis represents the mean of three to five determinations on several different preparations, in which the coefficient of variation for each amino acid was from 0.2 to 2.4%. Analyses were not corrected for incomplete hydrolysis or for hydrolytic losses. [b] Determined as cysteic acid. [c] Not detected. [d] Determined as methionine plus methionine sulfoxide and sulfone.

αA and αB chains are also very similar to those of the same chains isolated from fetal membranes by Burgeson et al. (1976) and Bentz et al. (1978). Comparison of analyses from placental and fetal membrane αA and αB chains prepared in our laboratory indicated only minor compositional differences, principally in the levels of prolyl and lysyl hydroxylation.

The amino acid composition of the αC chain is similar to those of the αA and αB chains, although it exhibits from 10–15% fewer Pro plus Hyp residues and a higher Leu content (Table II). A small amount of Cys is also present. All the compositions reported in Table II are distinct from that of the α1(IV) chain isolated from lens capsule basement membrane (Dehm & Kefalides, 1978). The hydroxylysine-linked carbohydrate content of αC is intermediate between that of αA and that of αB and reflects the generally elevated levels previously observed for the type IV collagens (Kefalides, 1975). The differences in carbohydrate composition may in part influence the mobilities seen for the αA, αB, and αC chains on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2).

(*d*) *Digestion with CNBr.* The results of an incubation of the αA, αB, and αC chains with CNBr, followed by analysis of the cleavage products by $NaDodSO_4$–polyacrylamide gel electrophoresis, are shown in Figure 8. The patterns obtained from αA and αB chains are quite similar to those reported for the same proteins isolated from fetal membranes by Burgeson et al. (1976). The peptide pattern obtained from the αC chain, however, was quite distinct. Although some lower molecular weight bands were visible, the major cleavage product migrated more slowly than the peptide α2-CB3-5 from the rat skin α2 chains. Numerous digestions under a variety of conditions, including 24-h incubation, prior denaturation of the αC chain by heating at 60 °C for 20 min, and digestion both with and without previous treatment to reduce methionine sulfoxides,

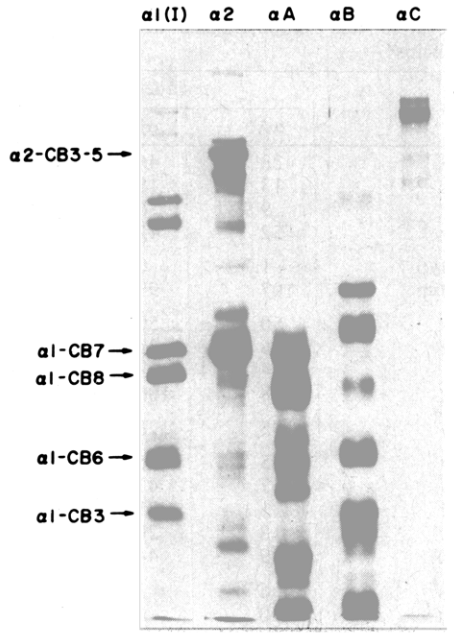


FIGURE 8: $NaDodSO_4$–polyacrylamide gel electrophoresis of cyanogen bromide digests of αA, αB, and αC collagen chains. Collagen was digested with CNBr as described under Materials and Methods, and the cleavage products were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on a 12.5% separating gel in the presence of 50 mM DTT. Protein was stained with Coomassie Brilliant Blue. The major cyanogen bromide peptides from α1(I) and α2 collagen chains are identified. The principal band in the αC lane migrates more rapidly than the αC chain.

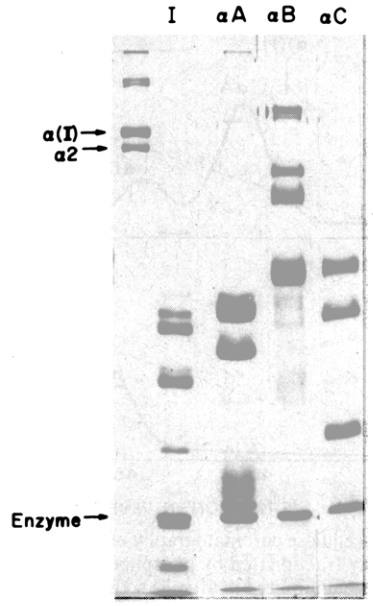


FIGURE 9: $NaDodSO_4$–polyacrylamide gel electrophoresis of mast cell protease digests of type I and of αA, αB, and αC collagen chains. Collagen was digested with mast cell protease for 15 min at 37 °C as described under Materials and Methods. The cleavage products were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on a 10% separating gel in the presence of 50 mM DTT and stained with Coomassie Brilliant Blue. Protein (20 μg) was applied to each lane. α(I) and α2 chains in a type I collagen incubated control are indicated.

all produced the same cleavage pattern. Amino acid analysis of the CNBr digest of the αC chain accounted for 82% of the methionine content as homoserine; the recovery of methionine-derived compounds was 93%. Thus, extensive cleavage at methionyl residues had occurred.

(*e*) *Digestion with Mast Cell Protease.* Mast cell protease isolated from rat skeletal muscle was found to be unreactive toward native AB and C collagens (Sage et al., 1979). However, digestion of the denatured chains at 37 °C produced very reproducible and limited cleavages, resulting in characteristic peptide "maps" which are distinct for each chain (Figure 9). The major cleavage products derived from each chain clearly differ from each other on the basis of molecular size. Time course digestions showed that these profiles remained essentially unchanged for up to 2 h, indicating that the differences observed among the chains as illustrated in Figure 9 were not due merely to unique reaction rates exhibited by the enzyme toward the substrate (data not shown).

Discussion

Preliminary structural studies have been presented which partially characterize the αC chain. This collagen or collagen chain has been compared to AB collagen during the course of this work as it was felt, on the basis of solubility and amino acid composition, that the αC chain more closely resembled αA and αB than it did any other known chain types. It is possible that αC chains exist in association with αA and/or αB chains in the same molecule, since AB and C collagens were copurified by salt fractionation from placenta. However, the absence of αC from fetal membranes indicates that it is not invariably associated with αA and/or αB chains. An analogy to the arrangement observed with type I collagen and type I trimer [$\alpha 1(I)_3$] may exist; modulation of molecular composition could be a function of development or tissue specificity (Crouch & Bornstein, 1978).

The AB collagen described in this study was isolated from both fetal membranes and placenta and is, by several criteria, the same protein which has been characterized by other laboratories (Burgeson et al., 1976; Rhodes & Miller, 1978; Bentz et al., 1978). This collagen comprises only 6.5% of the total collagen that can be recovered after pepsin treatment of both amnion and chorion and only 2% of the total collagen from pepsin digests of placenta (Table I). Reduction, alkylation, and repepsinization of material that was not solubilized by the initial pepsin digestion (Dehm & Kefalides, 1978), in addition to Hyp/Pro determinations on this residue, indicated that 85 ± 5% of the total tissue collagen in fetal membranes and 75 ± 5% in placenta, containing essentially all of the AB components, had been released by the first pepsin treatment. Very similar recoveries of AB collagen have been reported by Rhodes & Miller (1978), using combined fetal membranes and placenta, and by Jimenez et al. (1978), using chick embryo tendons.

It is of greater interest, however, that the data in Table I support the contention that the αA and αB chains need not exist in a ratio of 1:2. These data are based on several criteria, including scanning densitometry of protein resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis, weight recoveries of purified chains, and phosphocellulose chromatography. Rhodes & Miller (1978) have also reported variable ratios for the chains isolated from different tissues and argue that differences in stoichiometry, as well as data obtained from thermal denaturation studies, are most consistent with the presence of αA and αB chains in separate molecules. Ratios differing from 1:2 for αA and αB have also been reported by others (Jimenez et al., 1978; Brown et al., 1978). In contrast, Burgeson et al. (1976) and Bentz et al. (1978), working with fetal membrane collagen, have reported an invariant 2:1 ratio for αB/αA chains and have suggested the existence of the heteropolymer B_2A. It is possible that tissue-specific differences and variability in the conditions of pepsin digestion are at least partially responsible for the present disagreement

in the literature. Also, molecular species such as $\alpha B(\alpha A)_2$ and $(\alpha B)_3$ may exist, in addition to $(\alpha B)_2\alpha A$, and the proportion of these species could differ from tissue to tissue and with age, etc. Finally, if αC can substitute for αB or αA within a molecule, even greater variability may exist, compounding the difficulties in establishing a consistent stoichiometry.

αC is present as a minor component in preparations of native AB collagen ($\alpha A/\alpha C$ is approximately 2:1). Since it displays identical solubility properties with those of AB, it has not been possible to purify it in native form. Phosphocellulose chromatography does not resolve αA from αC, but complete resolution of these chains from the αB chain can be achieved (Figures 4 and 5), as described by Rhodes & Miller (1978). Since these authors did not separate αA and αC, however, the proportion of αA determined in their studies may be somewhat overestimated.

Our preliminary observations suggest that αC is associated with basement membranes, particularly those of vascular origin, as it has been detected in endothelial cell layers, and its occurrence in amnion, chorion, and placenta appears to be related inversely to the degree of vascularity of these tissues. A localization of AB collagen to pulmonary basement membranes was recently demonstrated by immunofluorescence (Madri & Furthmayr, 1979). However, the results of Brown et al. (1978) are not in accord with these findings. These authors claim that αC (in addition to αA and αB) is present in tissues which do not have basement membranes and that equal amounts of this chain can be recovered from both skin and placenta and from inflamed and normal synovial membranes, observations which infer an origin of αC independent from vascular tissue. Further experiments, including characterization of collagens synthesized by endothelial cells, are in progress in an attempt to resolve this apparent discrepancy.

Incubations of the αC chain with CNBr under a variety of experimental conditions invariably produced the same cleavage pattern on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 8). Although all of the starting material was cleaved, only one major high molecular weight band was seen, in addition to some material which migrated at the dye front. Amino acid analysis of CNBr digests of αC has indicated that approximately 80% of the methionine in this chain is recovered as homoserine. The CNBr digestion pattern as analyzed in Figure 8 does not account for all the methionines reported in the αC chain (Table II). It is possible that these amino acids are clustered in the sequence and that cleavage with CNBr yields one very large peptide and a number of smaller ones which are not visible in Figure 8. In this regard we have noticed, particularly with respect to the TC^B peptides and the N- and C-terminal extensions of type I procollagen, that these smaller peptides tend to be eluted from the gels during destaining, making their visualization and quantitation very difficult. There is precedence for a very large collagen-derived CB peptide, in that the $\alpha 2$ chain from calf skin collagen is lacking a methionine between $\alpha 2$-CB3 and $\alpha 2$-CB5 and produces upon CNBr digestion a single peptide, $\alpha 2$-CB3,5, containing 653 amino acids (Fietzek & Kühn, 1974).

The use of mast cell protease provided an additional criterion for establishing structural differences among the αA, αB, and αC chains. The enzyme which was isolated from rat skeletal muscle (Woodbury et al., 1978) has a chymotrypsin-like activity and produces specific and limited cleavages in denatured collagen chains. The cleavage maps generated for types I, AB, and IV collagens have demonstrated that the method is useful for detecting structural differences among these collagens (Sage et al., 1979). The cleavage patterns observed for αA, αB, and αC are different from one another and from that of type I (Figure 9). Each chain gives rise to a limited number of peptides which appear largely distinct when resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis.

It is intriguing to speculate on the origins of this subfamily of collagens which contains the αA, αB, and αC chains. The exact relationship of these chains to one another will only emerge from studies on precursor molecules which have not been truncated by pepsin treatment. Additional confusion could result from such isolation procedures if AB and/or C collagens contain pepsin-sensitive sites within the triple helix, as has been shown for several type IV collagens (Sage et al., 1979; Schwartz & Veis, 1978; Timpl et al., 1978).

The data presented in this study are consistent with the existence of a novel collagen chain, αC, which appears structurally distinct from types I–IV and AB collagen chains. The overall similarity in the amino acid compositions among αA, αB, and αC indicate that these chains are closely related and belong to a distinct subfamily of proteins which are in turn related to the type IV collagens found in basement membranes.

Acknowledgments

Special appreciation is due Theresa Canfield for excellent technical assistance. We thank Ricardo Marroquin for performing the amino acid analyses, Dr. Richard Woodbury for providing the mast cell protease, Dr. Elijah Adams for the 3-hydroxyproline standard, Dr. Billy Hudson for the glucosylgalactosylhydroxylysine standard, Drs. Arthur Eisen and Edward Harris for the vertebrate collagenases, and Dr. Robert Trelstad for initially sending us a placental AB collagen standard. Thanks are also extended to Drs. Ed Crouch and Gary Balian for helpful discussions and to Donna Stewart for typing the manuscript.

References

Bentz, H., Bächinger, H. P., Glanville, R., & Kühn, K. (1978) *Eur. J. Biochem. 92*, 563–567.

Benya, P. D., Padilla, S. R., & Nimni, M. E. (1977) *Biochemistry 16*, 865–872.

Brown, R. A., Shuttleworth, C. A., & Weiss, J. B. (1978) *Biochem. Biophys. Res. Commun. 80*, 866–872.

Burgeson, R. E., El Adli, F. A., Kaitila, I. I., & Hollister, D. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2579–2583.

Chung, E., Rhodes, R. K., & Miller, E. J. (1976) *Biochem. Biophys. Res. Commun. 71*, 1167–1174.

Crouch, E., & Bornstein, P. (1978) *Biochemistry 17*, 5499–5509.

Crouch, E., & Bornstein, P. (1979) *J. Biol. Chem. 254*, 4197–4204.

Dehm, P., & Kefalides, N. (1978) *J. Biol. Chem. 253*, 6680–6686.

Epstein, E. H., Jr., Scott, R. D., Miller, E. J., & Piez, K. A. (1971) *J. Biol. Chem. 246*, 1718–1724.

Fietzek, P. P., & Kuhn, K. (1974) *Hoppe-Seyler's Z. Physiol. Chem. 355*, 647–650.

Hudson, B. G., & Spiro, R. G. (1972) *J. Biol. Chem. 247*, 4229–4238.

Jimenez, S. A., Yankowski, R., & Bashey, R. I. (1978) *Biochem. Biophys. Res. Commun. 81*, 1298–1306.

Kefalides, N. (1975) *J. Invest. Dermatol. 65*, 85–92.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Linsenmayer, T. F., & Little, C. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3235–3239.

Madri, J. A., & Furthmayr, H. (1979) *Am. J. Pathol. 94*, 323–332.

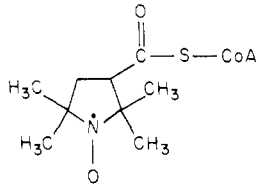
Mayne, R., Vail, M. S., & Miller, E. J. (1978) *Biochemistry 17*, 446–451.
Miller, E. J. (1972) *Biochemistry 11*, 4903–4909.
Miller, E. J. (1976) *Mol. Cell. Biochem. 13*, 165–192.
Moore, S. (1963) *J. Biol. Chem. 238*, 235–237.
Odell, V., Wegener, L., Peczon, B., & Hudson, B. G. (1974) *J. Chromatogr. 88*, 245–252.
Rhodes, R. K., & Miller, E. J. (1978) *Biochemistry 17*, 3442–3448.
Sage, H., Woodbury, R., & Bornstein, P. (1979) *J. Biol. Chem.* (in press).
Schwartz, D., & Veis, A. (1978) *FEBS Lett. 85*, 326–332.
Stenn, K. S., Madri, J. A., & Roll, F. J. (1979) *Nature (London) 277*, 229–232.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237–248.
Timpl, R., Martin, G. R., Brückner, P., Wick, G., & Wiedemann, H. (1978) *Eur. J. Biochem. 84*, 43–52.
Woodbury, R. G., Everitt, M., Sanada, Y., Katunuma, N., Lagunoff, D., & Neurath, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5311–5313.
Woolley, D. E., Glanville, R. W., Roberts, D. R., & Evanson, J. M. (1978) *Biochem. J. 169*, 265–276.

Interaction of a Paramagnetic Analogue of Oxaloacetate with Citrate Synthase†

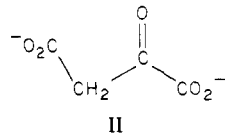
Stuart W. Weidman* and George R. Drysdale

ABSTRACT: Electron paramagnetic resonance studies have indicated that nitrosodisulfonate binds to pig heart citrate synthase. Titration of the enzyme with nitrosodisulfonate revealed several binding sites for the probe per subunit with one site ($K_D \approx 0.1$ mM) having a greater affinity than the others. The substrate, oxaloacetate, competed very effectively for one of the nitrosodisulfonate binding sites ($K_D < 10^{-2}$ mM) at the same time eliminating the weaker probe binding sites. Citrate and (*R*)- and (*S*)-malates also displaced the probe. Failure to resolve low- and high-field shoulders in the high gain–high modulation electron paramagnetic resonance spectra of the enzyme–nitrosodisulfonate system indicated that the bound probe was "weakly immobilized". However, the electron paramagnetic resonance spectrum of the bound probe changed to one typical of a "strongly immobilized" nitroxide upon the addition of a saturating concentration of the substrate acetyl coenzyme A (acetyl-CoA) to the enzyme–nitrosodisulfonate system, indicating the formation of a ternary acetyl-CoA–enzyme–probe complex. Titration of the acetyl-CoA saturated enzyme with the probe indicated one binding site per subunit ($K_D = 0.38$ mM). Thus, nitrosodisulfonate may be considered as a paramagnetic analogue of oxaloacetate in its interaction with citrate synthase. These results are compared with our previous studies with this enzyme, employing a spin-labeled acyl coenzyme A (acyl-CoA) derivative [Weidman, S. W., Drysdale, G. R., & Mildvan, A. S. (1973) *Biochemistry 12*, 1874–1883].

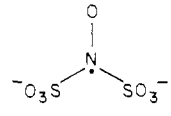
Spin-labeled coenzymes or substrates of enzymes have been utilized as active-site probes by several groups [for a recent review, see Morrisett (1976)]. We have previously examined the interaction of a spin-labeled analogue of acetyl-CoA (R·CoA,[1] I) with the enzyme citrate synthase (Weidman et al., 1973). These studies with R·CoA indicated that citrate synthase bound many di- and trinegatively charged organic ions which could be considered diamagnetic analogues of the substrates oxaloacetate (molecule II) or citrate. These observations aroused our interest in obtaining a paramagnetic analogue of oxaloacetate. Organic nitroxide analogues were not favored because of their large, steric bulk. Consideration of size, structure, and charge led to the choice of nitrosodisulfonate dianion (NDS, molecule III) as a possible analogue of oxaloacetate. Previous use of this substance in the field of biochemistry appears to have been limited to a reagent for modification of tyrosine and tryptophan residues in proteins (Cloughley et al., 1974). To our knowledge this paper is the first report of the application of NDS as an active-site enzyme probe.



R·CoA (I)



II



III

Experimental Procedure

Materials. Acyl-CoA esters were obtained from P–L Biochemicals. Using citrate synthase and oxaloacetate to convert acetyl-CoA or propionyl-CoA to citrate and methyl citrate, respectively, and analyzing the trimethyl esters by techniques previously described (Weidman & Drysdale, 1979),

† From the Departments of Biological Chemistry and Preventive Medicine, Washington University School of Medicine, St. Louis, Missouri 63110. *Received January 30, 1979.* Supported in part by National Science Foundation Grant PCM 74-21871 and NIH Contract N01-HV-2-2916-L (Lipid Research Clinics Program).

* Present address: Division of Gastroenterology, Department of Medicine, University of Tennessee Center for the Health Sciences, Memphis, TN 38163.

[1] Abbreviations used: R·CoA, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy-CoA thioester; NDS, nitrosodisulfonate dianion.

0006-2960/79/0418-3822$01.00/0