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Basement Membrane Collagens. Cyanogen Bromide Peptides of the D Chain from Porcine Kidney†

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ABSTRACT: An α chain size collagenous component, designated as the D chain, was isolated from the pepsin digest of porcine kidney cortices. Reduction of the D chain with 2-mercaptoethanol and carboxymethylation with iodoacetic acid resulted in the formation of two smaller components of 75 000 and 15 000 molecular weights. The 75 000 and 15 000 molecular weight components were separated by molecular sieve chromatography on a column of agarose A-5M. The 75 000 molecular weight component was cleaved with CNBr in 70% formic acid. The resulting CNBr peptides were isolated by a combination of ion exchange and molecular sieve chromatography and were characterized for amino acid contents and molecular weights. A total of seven CNBr peptides were obtained, which together accounted for the amino acid content of the intact 75 000 molecular weight component.

Collagenous components of basement membrane have been found to be distinct from interstitial types I, II, and III collagens in several respects, including the amino acid composition and the primary structure. To date, the molecular organization and the chain composition of the collagenous component(s) of basement membranes remain controversial. Earlier studies on the material isolated by pepsin solubilization of anterior lens capsule, renal glomerular basement membrane, and descemet's membrane (Kefalides, 1971, 1972; Dehm & Kefalides, 1978) suggested that their structures contained a single type of collagen chains, type IV. The idea that basement membrane collagen may be composed of three identical chains, $[\alpha 1(IV)]_3$, is further supported by biosynthetic studies on procollagen in organ cultures of parietal yolk sac endoderm (Minor et al., 1976) and of lens capsule (Heathcote et al., 1978). However, the above proposed structure is not in agreement with the investigations reported from several laboratories (Hudson & Spiro, 1972; Daniles & Chu, 1975; Sato & Spiro, 1976; Freytag et al., 1976) suggesting the heterogeneity of collagenous component in glomerular basement membrane. Several other investigators (Bailey et al., 1979; Glanville et al., 1979; Kresina & Miller, 1979; Sage et al., 1979) reported on the presence of at least two types of genetically distinct chains, designated C and D chains,[1] in placenta, a tissue rich in basement membrane. Our laboratory also described isolation of the C and D chains from anterior lens capsule and glomerular basement membrane (Dixit, 1978, 1979; Dixit & Kang, 1979). The results of biosynthetic investigations on the basement membrane of murine tumor (Timpl et al., 1978) and on amniotic fluid cell culture (Crouch & Bornstein, 1978) have shown the presence of two electro-

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[1] The nomenclature of the C and D chains presented in this paper corresponds to that used by Kresina & Miller (1979). The recently described C chain from placenta (Sage & Bornstein, 1979) is different from the C chain referred to in this paper.

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phoretically distinct bands indicative of two pro α chains, suggesting the presence of two structurally distinct chains in basement membrane.

Another class of collagen chains (A and B), which are structurally distinct from the C and D chains discussed above, has also been isolated by several investigators from placenta (Burgeson et al., 1976; Chung et al., 1976; Rhodes & Miller, 1978; Bentz et al., 1978) and other basement membrane associated tissues (Duance et al., 1977; Brown et al., 1978; von der Mark & von der Mark, 1979), but their origin from basement membrane remains unproven.

This laboratory has earlier reported on the characterization of the CNBr peptides of the C chain (Dixit & Kang, 1979). The present paper describes the preparation of the 75K (75 000) component from the D chain isolated from porcine kidney cortices, and the isolation and characterization of the CNBr peptides of the 75K component.

Materials and Methods

Preparation of the D Chain. Basement membrane collagen was extracted from porcine kidney cortices by limited pepsin digestion and purified in a manner described previously (Dixit, 1979). The D chain was obtained from the purified basement membrane preparation by gel filtration on agarose A-5M and CM[2]-cellulose chromatography. The details of these procedures as well as the criteria of its purity were documented earlier (Dixit, 1979).

Preparation of the 75K Component by Reduction and Carboxymethylation of the D Chain. The D chain was reduced with 2-mercaptoethanol and carboxymethylated with iodoacetic acid by the procedure described previously (Crestfield et al., 1963; Dixit & Kang, 1979). The reaction mixture was desalted immediately on Bio-Gel P-2 (50–100 mesh, Bio-Rad Laboratories) using 0.1 M acetic acid as eluant and lyophilized. The recovery of the desalted chain was over 95%.

CNBr Cleavage. To maximize the cleavage at methionyl residues by CNBr, the 75K component was treated with 25% 2-mercaptoethanol in 0.2 M NH_4HCO_3, pH 7.0, as described by Aldestein & Kuehl (1970) and as recently used for collagen chains (Kresina & Miller, 1979) for the conversion of methionine sulfoxide to methionine. The protein (4–5 mg/mL) was dissolved in 0.2 M NH_4HCO_3, pH 7.0, containing 25% 2-mercaptoethanol (v/v), bubbled with nitrogen, and incubated at 45 °C for 22 h. At the end of incubation, the protein was recovered by desalting on Bio-Gel P-2 (50–100 mesh, Bio-Rad Laboratories) using 0.1 M acetic acid and lyophilized. The recovery of the protein was over 95%. The lyophilized material was dissolved in 70% formic acid and treated with twice the amount of CNBr under an atomosphere of nitrogen as described previously (Dixit & Kang, 1979). Amino acid analysis of aliquots of the digest documented the conversion of over 90% of the original methionyl residues to homoseryl residues and the absence of methionine sulfoxide residues.

Molecular Sieve Chromatography on Agarose A-5M. The fractionation of 75K and 15K components formed by reduction and carboxymethylation of the D chain was performed on an agarose A-5M column (4 × 120 cm; 200–400 mesh, Bio-Rad Laboratories) using 0.01 M Tris/1 M $CaCl_2$, pH 7.4, as eluant. The sample was dissolved in 8–10 mL of the same buffer by heating at 45 °C for 15 min and applied to the column. The column was eluted at a flow rate of 38 mL/h. The column effluents were continuously monitored at 230 nm in a Gilford spectrophotometer equipped with a flow cell.

CM-cellulose and Phosphocellulose Column Chromatography of CNBr Peptides. The lyophilized CNBr peptides were initially fractionated on a CM-cellulose column (2.5 × 10 cm) equilibrated with 0.02 M sodium citrate, pH 3.8, at 44 °C. Samples of CNBr digest (50–60 mg) were dissolved in 10 mL of starting buffer and applied to the column, and the column was eluted with a linear gradient of NaCl from 0 to 0.12 M over a total volume of 1600 mL at a flow rate of 200 mL/h.

The final purification of individual CNBr peptides obtained after gel filtration on Sephadex G-75S was performed on phosphocellulose columns (1 × 10 cm) using a linear gradient of NaCl from 0 to 0.3 or 0.6 M in 0.001 M sodium acetate, pH 3.6, at a flow rate of 75 mL/h. In all experiments, the column effluents were continuously monitored in a Gilford spectrophotometer. The samples were desalted on Bio-Gel P-2 (100–200 or 200–400 mesh) using 0.1 M acetic acid as the eluant.

Molecular Sieve Chromatography of CNBr Peptides. The various fractions obtained from CM-cellulose chromatography were further fractionated on a Sephadex G-75S column. The column (2 × 120 cm) was equilibrated with 0.04 M sodium acetate, pH 4.8, and eluted at a flow rate of 16 mL/h. The appropriate fractions were pooled, lyophilized, and desalted on Bio-Gel P-2 as described previously. Effluents were continuously monitored as described above.

Molecular Weight Determination. The molecular weights of various CNBr peptides were estimated by molecular sieve chromatography on Sephadex G-75S or G-100 as described by Piez (1968). The medium-size peptides were chromatographed on a Sephadex G75S column precalibrated with a mixture of chick skin α1(I)-CB2, -3, and -7. Larger CNBr peptides were estimated by chromatography on a Sephadex G-100 column (2 × 120 cm) precalibrated with α1(I)-CB2, -3, and -7 and α2-CB3. A drop of tritiated water was used to mark the column volumes.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The purity of the various collagenous fractions (D, 75K and 15K) was determined by $NaDodSO_4$–polyacrylamide gel electrophoresis by the procedure of Laemmli (1970). Slab gels of 7.5% were used for the D chain and 75K component and of 12.5% for the 15K component. Gels were stained for 2 h in 0.1% Coomassie brilliant blue R-250 (Sigma) in 50% methanol and 7% acetic acid and were destained with a solution containing 10% methanol and 7% acetic acid until the background cleared up.

Amino Acid Analysis. The amino acid analyses were performed by the single column method described previously (Kang, 1972). Corrections were made for the incomplete release of valine and for the destruction of labile amino acids, threonine, serine, and tyrosine (Piez et al., 1960). The hydroxylysine glycosides (Glc-Gal-Hyl[2] and Gal-Hyl) were determined as described previously (Askenasi & Kefalides, 1972; Miller, 1972).

Results

Reduction and Carboxymethylation of the D Chain. The purified D chain was reduced and carboxymethylated as described above. The 75K and 15K components formed by reduction of the D chain were isolated by molecular sieve chromatography on an agarose A-5M column. The elution profile of the reduced and carboxymethylated D chain is shown in Figure 1. The 75K component eluted as a single peak when subjected to chromatography on a CM-cellulose column (figure not shown). In later experiments, the 75K component

[2] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; CM, carboxymethyl; Glc-Gal, glucosylgalactosyl; Gal, galactosyl.

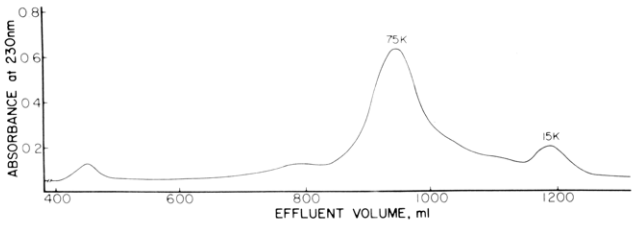

FIGURE 1: Molecular sieve chromatography of 50 mg of the reduced and carboxymethylated D chain on an agarose A-5M column (4 × 120 cm). The column was equilibrated with 0.01 M Tris/1 M $CaCl_2$, pH 7.4, and was eluted with the same buffer at a flow rate of 38 mL/h.

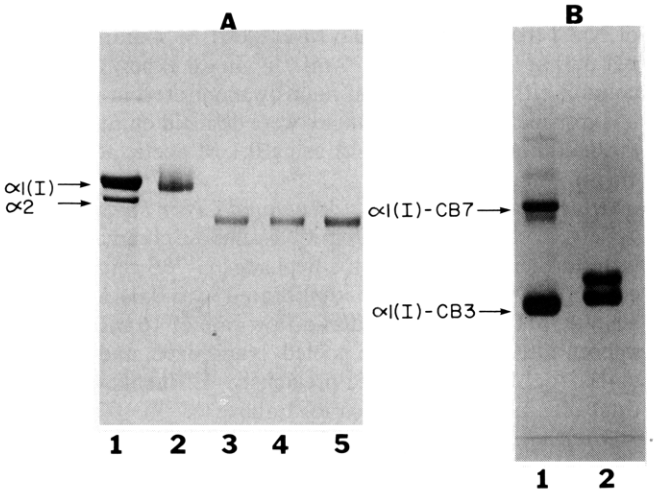

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of the following: (A) (lane 1) type I collagen of chick skin showing α1(I), α2; (lane 2) the D chain without 2-mercaptoethanol; (lane 3) the D chain with 2-mercaptoethanol; (lane 4) the 75K component from Figure 1; (lane 5) the carboxymethylated 75K component without 2-mercaptoethanol. Gels (7.5%) were electrophoresed for 2 h at a current of 50 mA. (B) (lane 1) Standard consisting of α1(I)-CB3 and -CB7 of chick skin collagen; (lane 2) the 15K component from Figure 1. Gels (12.5%) were electrophoresed for 4 h at a current of 25 mA.

obtained from the molecular sieve (A-5M) column was used directly for CNBr cleavage. The purities of the D chain and 75K component were determined by $NaDodSO_4$–polyacrylamide gel electrophoresis, and the results are presented in Figure 2A. The D chain was electrophoresed with and without 2-mercaptoethanol. The amino acid compositions of the D chain and the 75K component are presented in Table I.

The 15K components on $NaDodSO_4$–polyacrylamide gel electrophoresis showed a closely spaced doublet band (Figure 2B). Attempts to resolve the 15K components into individual peptides were unsuccessful, and further characterization was not done. However, the amino acid composition given in Table I has a close resemblance to the D chain and the 75K component described.

Isolation and Characterization of CNBr Peptides of the 75K Component. The initial fractionation of CNBr digests of the 75K component was performed on a CM-cellulose column in sodium citrate, pH 3.8. The elution pattern is illustrated in Figure 3. Four major fractions, 1, 2, 3, and 4–7 (Figure 3) were pooled separately, lyophilized, and desalted. When fractions 1 and 3 were separately subjected to chromatography on Sephadex G-75S, one major peptide peak eluted in each instance (figures not shown). The Sephadex-purified fractions 1 and 3 were then further chromatographed on phosphocellulose to obtain homogeneous CB1 and CB3 (Figure 4). Fraction 2 (Figure 3) was chromatographed on Sephadex

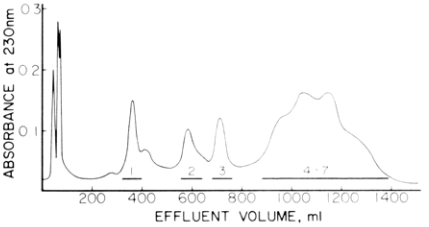

FIGURE 3: CM-cellulose chromatography of a CNBr digest (60–65 mg) of the 75K component on a column (2.5 × 10 cm) equilibrated with 0.02 M sodium citrate, pH 3.8, at 44 °C. The column was eluted with a linear gradient of NaCl from 0 to 0.12 M NaCl over a total volume of 1600 mL at a flow rate of 200 mL/h.

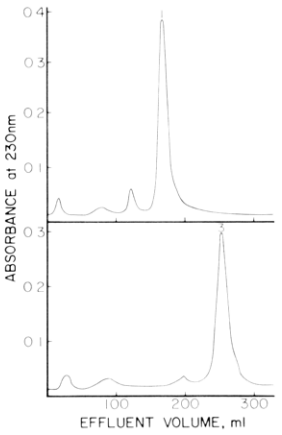

FIGURE 4: Phosphocellulose chromatography of CB1 and CB3 (upper and lower panels, respectively) on a column (1 × 10 cm) equilibrated with 0.001 M sodium acetate, pH 3.6. The column was eluted with a linear gradient of NaCl from 0 to 0.3 M over a total volume of 500 mL at a flow rate of 75 mL/h at 44 °C.

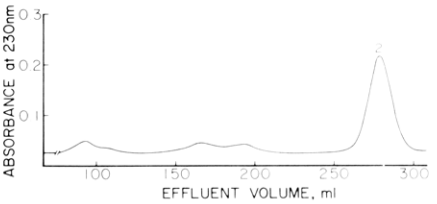

FIGURE 5: Molecular sieve chromatography of fraction 2 (Figure 3) on a Sephadex G-75S column (2 × 120 cm). The column was equilibrated with 0.04 M sodium acetate, pH 4.8, and was eluted at a flow rate of 16 mL/h.

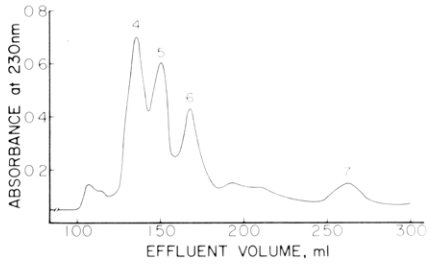

FIGURE 6: Molecular sieve chromatography of fraction 4–7 from Figure 3 on Sephadex G-75S. Conditions were identical with those for Figure 5.

G-75S which yielded a homogeneous peak (Figure 5). The amino acid composition of the peptide CB2 revealed its high degree of purity and was not purified further.

Fraction 4–7 (Figure 3) was further fractionated on Sephadex G-75S. The elution profile is illustrated in Figure 6. The individual peaks 4, 5, 6, and 7 were pooled, desalted, and lyophilized. Further purification of each fraction was performed on a phosphocellulose column in sodium acetate (pH

Table I: Amino Acid Composition of the D Chain and 75K and 15K Components[a]

	D	75K	15K
3Hyp	5.2	5.8	8.7
4Hyp	120	126	130
Asp	56	48	65
Thr	21	19	24
Ser	33	36	30
Glu	56	58	50
Pro	68	64	59
Gly	342	337	335
Ala	48	48	46
Val	34	37	31
$^1/_2$-Cys	2.0	1.8[b]	2.7[b]
Met	10	9	6
Ile	23	21	25
Leu	58	60	52
Tyr	3.8	1.8	0.4
Phe	36	35	33
Hyl	43	40	51
Lys	4.7	6.2	3.7
His	8.0	8.2	11
Arg	36	38	36
Glc-Gal-Hyl	27	28	N.D.[c]
Gal-Hyl	1.7	1.8	N.D.

[a] Residues are expressed as residues per 1000. Values for residues present in numbers greater than 10 were rounded off to the nearest whole number. [b] Half-cystine was determined as carboxymethylcysteine. [c] N.D. denotes values were not determined.

3.6) using a linear gradient of 0 to 0.6 M over a total volume of 500 mL (figures not shown). The peptides CB4 and CB5 were again rechromatographed on Sephadex G-100 for final purification.

The amino acid compositions of CNBr peptides 1 through 7 are presented in Table II. The molecular weights of CB1, 2, 3, 6, and 7 were estimated by their elution position from Sephadex G-75S, whereas those of CB4 and 5 were estimated by their elution position from Sephadex G-100. The observed molecular weights on molecular sieve chromatography and their calculated molecular weights by amino acid contents are presented in Table II. In calculating the molecular weight, if the glycosylation of hydroxylysine residues is considered, the observed and calculated molecular weights are in close agreement with each other within experimental error.

Discussion

The present paper describes the isolation and characterization of the 75K component obtained by reduction of the D chain. The CNBr cleavage of the 75K component at methionyl residues produced a total of seven unique peptides. The seven peptides have been characterized for their amino acid contents and molecular weights. Together, these seven peptides contain 660 amino acid residues. As almost all hydroxylysine residues are glycosylated, the apparent molecular weight of the 75K component as estimated by gel filtration is in good agreement with that calculated from amino acid composition of the isolated CNBr peptides. The peptide CB4 is the COOH-terminal peptide, as it lacked a residue of homeserine. The alignment of the rest of the CNBr peptides is not known at present and is under current investigation.

The 75K component is a major component formed by pepsin cleavage of basement membranes and most likely is derived from a larger size chain which is susceptible to proteolysis. The formation of smaller components than 75K has also been observed by several groups of investigators (Trelstad & Lawley, 1977; Schwartz & Veis, 1978; Kresina & Miller, 1979; Dixit & Kang, 1979). Although no further characterization of 15K component described here was done, the presence of two closely spaced bands (Figure 2B) on Na-DodSO$_4$–polyacrylamide gel electrophoresis is probably indicative of pepsin cleavage at two different sites forming two similar peptides with minor differences in molecular weights. By amino acid composition the 15K component appears to be derived from chain(s) related to the 75K component.

We have earlier reported on the characterization of the CNBr peptides of the C chain (Dixit & Kang, 1979) of bovine

Table II: Amino Acid Composition[a] of CNBr Peptides from the 75K Component of the D Chain

	1	2	3	4	5	6	7	total	75K[b]
3Hyp	1 (0.8)	0	0	1 (1.2)	1 (0.9)	1 (1.3)	0	4	4
4Hyp	10	2 (1.7)	7 (7.0)	25	21	17	3 (3.0)	85	83
Asp	2 (2.2)	0	1 (0.8)	11	8 (8.0)	5 (4.8)	2 (1.8)	29	32
Thr	1 (0.8)	0	0	6 (6.1)	3 (3.0)	1 (0.8)	0	11	12
Ser	1 (0.8)	0	1 (0.8)	8 (7.8)	8 (7.7)	6 (5.6)	2 (1.7)	26	24
Glu	4 (4.2)	0	8 (7.9)	13	7	6 (6.1)	2 (1.9)	42	38
Pro	6 (5.8)	0	8 (5.8)	11	10	10	0	43	42
Gly	22	6 (5.9)	18	67	55	44	11	222	223
Ala	2 (1.8)	0	2 (2.2)	10	8 (8.0)	6 (6.1)	1 (1.0)	29	32
$^1/_2$-Cys	0	0	0	0	0	2 (0.7)[c]	0	2	1.2
Val	2 (1.7)	0	3 (2.7)	7 (6.7)	5 (4.9)	4 (3.8)	1 (0.8)	22	24
Ile	0	1 (0.9)	0	5 (5.0)	4 (4.0)	3 (2.7)	0	13	14
Leu	4 (4.0)	3 (3.2)	1 (1.0)	13	9 (8.8)	8 (7.8)	3 (2.8)	41	40
Tyr	0	1 (0.7)	0	1 (0.8)	0	0	0	2	1.2
Phe	3 (2.8)	0	2	8 (8.0)	6 (6.0)	4 (3.8)	1 (1.0)	24	23
Hyl	1 (1.1)	3 (2.7)	0.2	9 (8.7)	8 (7.7)	6 (5.6)	2 (2.0)	29	26
Lys	0	0	1 (0.8)	0.2	0.2	0.3	0.2	1	0.6
His	1 (1.0)	0	0	1 (1.0)	1 (1.0)	1 (0.8)	1 (1.0)	5	5
Arg	2 (2.0)	0	3 (3.0)	5 (5.0)	5 (5.0)	5 (5.0)	2 (2.0)	23	25
Hse[d]	1 (0.8)	1 (0.9)	1 (0.9)	1 (0.8)	1 (0.8)	1 (0.8)	1 (0.8)	6	6
total	64	17	54	202	162	129	32	660	
mol wt by AA anal.	5958	1734	5194	19 061	15 263	12 186	3140	63 536	
mol wt by gel filtration	6180	1980	5400	23 850	19 980	14 800	2660	74 850	

[a] Residues per peptide to the nearest whole number. Actual values are given where less than 10 residues occur. Lys and Hyl are given as partial residues determined. [b] Residues calculated as residues per 660 residues. [c] Calculated as carboxymethylcysteine. [d] Indicates methionine residues in the case of 75K.

anterior lens capsule. The present paper describes the characterization of the CNBr peptides of the other structurally distinct collagenous component derived from basement membrane, i.e., the D chain, which, for the present study, was prepared for kidney cortices. A similar chain was isolated in earlier studies from basement membrane structures such as anterior lens capsule, glomerular basement membrane (Dixit & Kang, 1979; Dixit, 1979), and placenta (Bailey et al., 1979; Glanville et al., 1979; Kresina & Miller, 1979; Sage et al., 1979). The CNBr peptides of the D chain described in this paper are distinct from the CNBr peptides of the C chain (Dixit & Kang, 1979), further confirming the presence of two distinct collagenous chains in basement membranes.

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Turbidity Measurements in an Analytical Ultracentrifuge. Determinations of Mass per Length for Filamentous Viruses fd, Xf, and Pf3†

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ABSTRACT: An analytical ultracentrifuge has been used to measure light-scattering intensities by the transmittance method. The technique, which is applicable to particles of many sizes and shapes, has the principal advantage that samples can be kept free of dust during the measurements. Also, sample volumes are small, and the scanner and interference optics can be used simultaneously to obtain, for a given sedimenting boundary, turbidity steps at different wavelengths and the concentration step. In the present application the data yield mass per length estimates for three filamentous viruses, 19 100 daltons/nm for fd, 19 600 daltons/nm for Pf3, and 19 100 daltons/nm for Xf.

Measurements of the turbidity, τ, as a function of wavelength provide the same type of information on molecular weights and dimensions of macromolecules in solution as measurements of the angular dependence of the Rayleigh ratio (Heller & Vassy, 1946; Heller et al., 1946; Cashin & Debye, 1949; Doty & Steiner, 1950). Recent papers have dealt with applications to a variety of biological systems [e.g., Camerini-Otero et al. (1974), Gaskin et al. (1974), and Carr & Hermans (1978)] and with methods for interpreting data gathered as a function of wavelength (Camerini-Otero & Day,

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