Isolation and Characterization of the Cyanogen Bromide Peptides from the α1 and α2 Chains of Acid-Soluble Bovine Skin Collagen*

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ABSTRACT: The α1 and α2 chains of acid-soluble steer skin collagen were cleaved with CNBr and the resulting fragments were separated by ion-exchange chromatography. The sum of the amino acids of the eight peptides isolated from α1 are in good agreement with the amino acid composition of the α1 chain. The six peptides isolated from the CNBr digested α2 chain also accounted for the amino acid content of α2. In each case, the peptides are similar and clearly homologous to the cyanogen bromide peptides previously isolated from rat skin, human skin, chicken skin, and bone collagens. However, the short, nonhelical, NH_2-terminal end of the α1 chain is variable in that it may lack a NH_2-terminal tetrapeptide (Glu, Leu, Ser, Tyr). The dipeptide α1-CB0, present in chicken and in rat tendon, but absent in human skin collagen, is not present in bovine skin collagen.

The limited cleavage of the individual peptide chains of collagen with cyanogen bromide (CNBr) has become one of the main tools in studying the primary covalent structure of collagen. Data are now available on the compositions and relative ordering, within the intact molecule, of the CNBr peptides from the α1 and α2 chains of rat skin collagen (Butler *et al.*, 1967; Fietzek and Piez, 1969), chick skin and bone collagens (Kang *et al.*, 1969; Miller *et al.*, 1969; Lane and Miller, 1969; Igarashi *et al.*, 1970; Vuust *et al.*, 1970), and human collagen (Click and Bornstein, 1970). Additional information is available regarding some peptides from cod (Laszlo and Olsen, 1969), rabbit (Bornstein and Nesse, 1970), and calf (Rauterberg and Kühn, 1968) collagens.

Most studies have emphasized the compositions of the peptides of isolated α chains and their ordering and comparisons between species, or between different tissues of the same species. Since the degradation of the methionyl residues proceeds most readily and specifically in solution, the majority of investigators have concentrated on the soluble collagens.

Our interest is centered upon the insoluble collagens with regard to two major aspects: the location and nature of the peptide sequences containing cross-linkages, and the locations and nature of moieties such as the sugars and phosphate residues known to be present in insoluble bovine dentine collagen (Schleuter and Veis, 1964), as well as of other possible attachments (Veis and Perry, 1967). Since the bovine collagens are highly cross-linked, these collagens were chosen for study.

In addition to extending the species and tissue comparisons of the soluble collagens to the bovine species, the data presented here on the separation and analysis of the CNBr peptides of the isolated α1 and α2 chains of soluble bovine corium collagens also provide the essential background data for the examination of the insoluble bovine collagens. In view of the marked similarity between the rat and steer collagen peptide data, the nomenclature previously assigned to the rat peptides has been adopted. Components appear in the CNBr digest of insoluble bovine collagen which are not present in the isolated α-chain digests. To facilitate comparison between the present data and that to be presented, a second system of nomenclature has also been indicated which will be applicable to the CNBr digests of the insoluble collagen.

Materials and Methods

Preparation of Steer Skin Collagen. Acid-extracted collagens were prepared from a 2-year-old steer skin according to the procedure of Piez *et al.* (1961). The skin was cut into small pieces. The pieces were extracted at 4° with 10 volumes of 10% NaCl followed by washing with distilled water. This was repeated three times. The suspension was filtered through layers of cheese cloth. The residue was minced in a Wiley mill using Dry Ice chips, extracted overnight with 25% NaCl, and subsequently removed by water washing and filtration through cheese cloth. All extracts were discarded. The residue was extracted with 2 volumes of 3% acetic acid for 24 hr for a total of four times. Each time the suspension was filtered through cheese cloth and the filtrate was retained. The acid extracts were clarified by centrifugation at 27,000*g* for 60 min. Solid NaCl was slowly added to the extract to bring the salt concentration to 10%. The resulting precipitate was collected by centrifugation at 16,000*g* for 30 min. The supernatant was discarded and the sediment was resuspended in 3% acetic acid with stirring overnight. Clarification and reprecipitation were repeated. The final precipitate was redissolved in acetic acid and the solution was desalted by exhaustive dialysis against distilled water. The resulting suspension was lyophilized.

Preparation of α1, α2. The α1 and α2 chains were obtained by chromatography of denatured acid-soluble collagen at 40° on a 25 × 120 mm CM-cellulose column (Whatman CM 32) as described previously (Piez *et al.*, 1963; Bornstein and Piez, 1966). Separation was achieved with a linear gradient of 400 ml of starting buffer (0.06 M sodium acetate, pH 4.8) and 400 ml of limit buffer (starting buffer containing 0.1 M NaCl). The column was eluted at a rate of 150 ml/hr and the effluent was monitored continuously at 230 mμ in a

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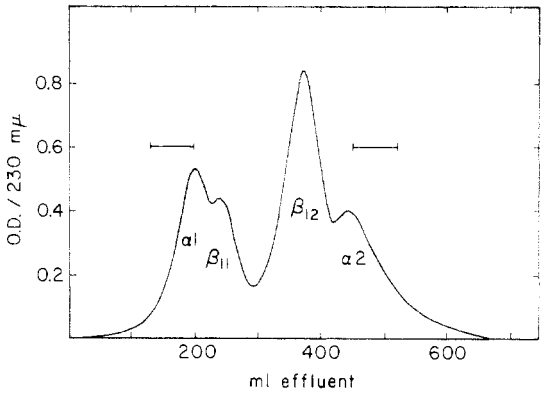

FIGURE 1: Chromatographic pattern of denatured acid-soluble steer skin collagen on CM-cellulose, 40°. Elution was with a linear gradient from 0.06 M sodium acetate (pH 4.8) to 0.06 M sodium acetate–0.10 M NaCl (pH 4.8) over a volume of 800 ml. The fractions of $\alpha 1$ and $\alpha 2$ under the bars were pooled and used for CNBr digestion.

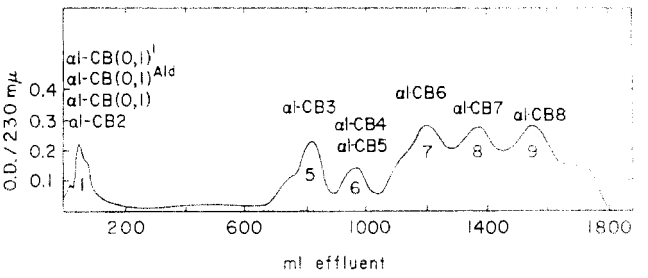

FIGURE 2: Chromatographic pattern of CNBr-cleaved $\alpha 1$ of acid-soluble steer skin collagen on CM-cellulose, 40°. Elution was with a linear gradient from 0.02 M sodium citrate (pH 3.6) to 0.02 M sodium citrate–0.14 M sodium chloride (pH 3.6) over a volume of 2000 ml.

Beckman DBG spectrophotometer. The column effluent was collected in 10-ml fractions (Figure 1). The fractions, indicated by the solid bars in Figure 1, comprising parts of the $\alpha 1$ and $\alpha 2$ peaks were pooled into the two composite fractions, lyophilized, redissolved in 20 ml of 0.15 M acetic acid, desalted at room temperature on a 40 × 400 mm column of Bio-Gel P-2 (Bio-Rad Laboratories), equilibrated with 0.15 M acetic acid, and relyophilized. The homogeneity of each fraction was verified by acrylamide gel electrophoresis.

Cleavage with CNBr. Samples of $\alpha 1$ and $\alpha 2$ chains weighing 100–200 mg were dissolved in 20 ml of 0.1 N HCl. The collagen solution was flushed with nitrogen and 200-fold molar excess (relative to methionine) of CNBr was added. The incubation was carried out at 30° for 4 hr. An alternate procedure, more suitable for the later studies on insoluble collagen, was to dissolve the sample in 70% formic acid. The CNBr reaction was then carried out at room temperature, for 4 hr. In either case, at the conclusion of the reaction period, the digestion mixture was diluted with water and lyophilized.

Chromatography of CNBr Peptides on CM-Cellulose. The CNBr digest was chromatographed on a 25 × 120 mm column of CM-cellulose equilibrated at 40° with 0.02 M sodium citrate, pH 3.6. Samples of CNBr peptides were dissolved in 10–20 ml of the same buffer and applied to the column. The elution was carried out with a linear gradient of NaCl from 0 to 0.14 M over a volume of 2000 ml. Fractions comprising a given peak, following the criterion of disc electrophoresis, were combined, lyophilized, desalted, and relyophilized.

In some cases the peptides were rechromatographed under the same conditions or using CM-cellulose equilibrated with 0.02 M sodium acetate, pH 4.8. In this last case, chromatography was performed using a linear gradient of NaCl from 0 to 0.14 ionic strength over a total volume of 2000 ml.

Chromatography of CNBr Peptides on Phosphocellulose. The first peaks (labeled 1, Figures 2 and 4) from both $\alpha 1$ and $\alpha 2$ CNBr-digest chromatograms were chromatographed on phosphocellulose, essentially according to Kang *et al.* (1969). Identical results were achieved, with regard to the separation of the peak 1 constituents, when the whole $\alpha 1$ or $\alpha 2$ CNBr digest was chromatographed directly on the phosphocellulose without prior CM-cellulose chromatography.

Columns of phosphocellulose were equilibrated with 0.001 M sodium formate buffer, pH 3.6 at 40°. Samples were dissolved in 10 ml of this buffer and pumped onto the column. Chromatography was performed by applying a linear gradient of NaCl from 0 to 0.6 ionic strength over a total volume of 2000 ml. The peptides were then desalted and lyophilized. Resolution of two other small peptides ($\alpha 1$-CB4 and $\alpha 1$-CB5) which did not separate on CM-cellulose was also performed by rechromatography on phosphocellulose with the same conditions.

Disc Electrophoresis. Acrylamide gel electrophoresis was done essentially as described by Reisfeld *et al.* (1962) and Veis and Anesey (1965). In the running gel the concentration of acrylamide was 5% and that of *N,N*-methylenebis(acrylamide) was 2.5%. Migration was toward the cathode, the buffer was sodium acetate, pH 4.8, $\Gamma/2 = 0.05$. The components $\alpha 1$, $\alpha 2$, β_{11}, and β_{12} are clearly resolved in this system.

Amino Acid Analysis. Samples were hydrolyzed in 2 ml of constant boiling HCl at 108° for 22 hr under nitrogen in sealed tubes. The acid was removed under vacuum at about 50° on a Buchler Evapo-Mix. The dried samples were dissolved in water, and a volume containing 0.2–1 mg was used for amino acid analysis on a two-column automatic JEOLCO analyzer. No corrections were made for the possible partial destruction or incomplete release of individual amino acids due to hydrolysis conditions. Lazlo and Olsen (1969) showed such correction factors to be near unity under conditions similar to ours.

Results

$\alpha 1$ Peptides. The CM-cellulose chromatography of the CNBr digest of $\alpha 1$ is illustrated in Figure 2. The peaks are labeled by number, corresponding to the sequence of elution of the peaks from whole *insoluble* bovine skin collagen. They are also identified, above each peak, with the terminology appropriate to the system developed for rat skin, chick skin, human skin, and chick bone collagen. The identity of the peptides was established by amino acid analyses and homology with the above-mentioned results.

The materials in peaks 5, 7, 8, and 9 were subjected individually to rechromatography on CM-cellulose under the same conditions (sodium citrate, pH 3.6) or using sodium acetate buffer at pH 4.8 (see Methods). By this procedure each fraction was divided into one major component and two or three minor components. Polyacrylamide gel electrophoretic data indicated the homogeneity of the isolated

TABLE I: Amino Acid Compositions of CNBr Peptides from the α1 Chain of Acid-Soluble Bovine Skin Collagen.[a]

	α1-CB-(0,1)′	α1-CB-(0,1)	α1-CB2	α1-CB3	α1-CB4	α1-CB5	α1-CB6	α1-CB7	α1-CB8	Peptides	α1[b]
3-Hydroxyproline							0.7			0.7	0.5
4-Hydroxyproline			5.2	14	5.4	3.3	17	23	27	94	97.4
Aspartic acid	1.0	1.2		6.5	2.8	2.7	8.6	12	10	45	45.1
Threonine	0.9	1.0		(0.3)	0.9		3.6	5.0	5.2	16	17.1
Serine	1.8	2.7	1.8	2.8		1.8	7.2	7.4	8.2	32	31.9
Homoserine[c]	1.0	1.0	1.0	1.0	1.0	1.0		1.0	1.0	7	6.3[d]
Glutamic acid	1.0	2.0	4.0	16	2.8	3.3	13	17	19	77	78
Proline	2.0	2.0	6.3	16	5.7	2.6	30	38	33	134	137.7
Glycine	3.1	3.2	12	51	16	12	68	89	89	340	346
Alanine			2.4	22	3.3	3.7	22	36	34	123	126.5
Valine	1.0	1.2		3.8			3.2	5.3	4.6	18	17.8
Isoleucine	0.9	0.9					2.1	2.8	1.9	8	7.6
Leucine		1.1	1.0	3.0	1.9	1.0	4.1	4.3	4.4	20	21.2
Tyrosine	0.7	1.4					(0.2)			2	2.3
Phenylalanine			1.0	3.0		1.0	2.0	3.1	3.1	13	13
Hydroxylysine				(0.3)		1.1	1.3	1.1	1.2	4.7	5.1
Lysine	0.9	1.0		4.8	2.0	1.7	5.4	9.4	9.3	33	32.3
Histidine						0.8	0.8			2	2.4
Arginine			1.0	6.0	3.8	1.2	11	13	15	51	51.7
Total	15	19	35	151	46	38	200	266	265	1020	1040

[a] Values are expressed as residues per peptide. Actual values are listed for amino acids present as less than 10 residues. A space indicates less than 0.2 residue. [b] Values are averages of three determinations and computed for a molecular weight of 95,000 and an average residue weight of 90.6. [c] Includes homoserine lactone. [d] As methionine.

major components. The amino acid compositions of the minor peaks obtained by rechromatography, and their acrylamide gel electrophoretic behavior, generally did not exhibit any striking differences from their respective major components. Similar findings have been reported for the larger CNBr peptides from the α1 chains of other collagens (Butler *et al.*, 1967; Kang *et al.*, 1969).

The last, unlabeled peak of Figure 2 was identified as a large, uncleaved peptide by the presence of methionine. This peptide migrates on gel electrophoresis as a single component in the region of the intact "αs." It had an amino acid composition consistent with either α1-CB(3-7) or α1-CB(8-3).

Peak 1 was resolved by chromatography on phosphocellulose into four peaks, Figure 3. As noted earlier, direct phosphocellulose chromatography on CNBr-cleaved α1 prior to CM-cellulose chromatography yielded only the same four components in the same chromatographic regions. The amino acid compositions of the peaks labeled α1-CB(0,1)^{ald} and α1-CB(0,1) (Table I) differ only in the presence of a single lysyl residue in the latter peptide. The fourth peak of Figure 3, designated α1-CB(0,1)′ has an amino acid composition identical with that of the second peak, α1-CB(0,1), except that in α1-CB(0,1)′, one residue each of glutamic acid, serine, tyrosine, and leucine is missing.

Peak 6 of Figure 1 was resolved into two peptides, α1-CB4 and α1-CB5, by phosphocellulose chromatography under conditions similar to those for the separation of the peak 1 peptides as in Figure 3.

Thus, a total of eight CNBr peptides have been isolated from the α1 chain of bovine skin collagen, consistent with the fact that the α1 chain contains 7 methionyl residues. In addition, both α1-CB(0,1) and a modified α1-CB(0,1)′ have been identified. The compositions of these peptides are listed in Table I, along with the composition of the intact α1 chain. The values for the amino acids present in lowest amount in the CNBr peptides were used to compute the factors for conversion of micromoles into residues per peptide. It was assumed that each CNBr peptide, with the exception of the COOH-terminal peptide, would also contain one residue of homoserine. The homoserine–homoserine

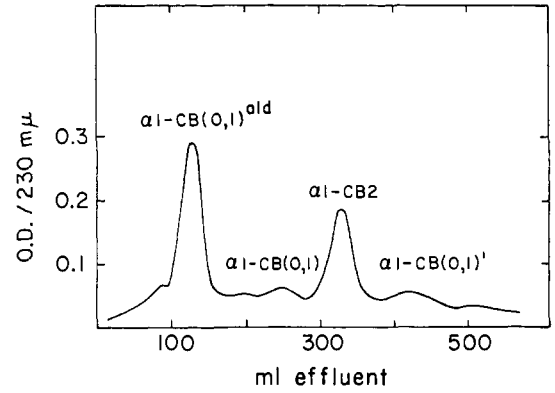

FIGURE 3: Chromatographic pattern of peak 1 from Figure 2 on phosphocellulose at 40°. Elution was carried out with a linear gradient from 0 to 0.6 M sodium chloride in a pH 3.6, 0.001 M sodium formate buffer. The gradient was developed over a total volume of 2000 ml. An identical pattern in the same chromatographic region was achieved using CNBr-cleaved α1.

TABLE II: Amino Acid Composition of CNBr Peptides from the α2 chain of Acid-Soluble Bovine Skin Collagen.[a]

	(α2-CB0)	α2-CB1	α2-CB2	α2-CB3	α2-CB4	α2-CB5	Peptides	α2[b]
4-Hydroxyproline			2.4	25	32	25	84	89
Aspartic acid		1.0	1.8	15	13	16	47	49
Threonine			0.8	5.4	5.8	5.7	18	18
Serine		1.0	1.6	9.6	11	9.6	34	35
Homoserine[c]	0.8	1.0	1.0	1.0	1.0		5	4.2[d]
Glutamic acid		2.0	1.5	24	22	21	70	74
Proline		1.9	2.7	38	34	36	113	120
Glycine	1.2	3.1	10	109	110	104	337	340
Alanine		1.0	3.0	36	35	30	105	105
Valine			0.8	8.2	11	10	30	32
Isoleucine				6.3	4.0	5.3	15	17
Leucine	1.1		0.9	9.0	10	11	32	33
Tyrosine		0.7				1.5	3	3
Phenylalanine		1.0		4.6	4.0	3.6	14	14
Hydroxylysine				2.2	3.7	3.3	9	9
Lysine		0.9		9.0	6.5	5.3	21	24
Histidine				1.6	2.0	4.3	8	8
Arginine			2.6	17	17	17	54	57
Total	3	14	30	321	322	309	999	1031

[a] Values are expressed as residues per peptide. Actual values are listed for amino acids present as less than 10 residues. A space indicates less than 0.2 residue. [b] Values are averages of three determinations and are computed for molecular weight of 95,000 and average residue weight of 92. [c] Includes homoserine lactone. [d] As methionine.

lactone equilibrium was taken into account in all analyses of CNBr peptides.

α-2 Peptides. The CM-cellulose elution pattern for the α2-chain CNBr peptides is indicated in Figure 4. The identity of the peptides was again established by amino acid analyses and by homology to the CNBr peptides from the α2 chains of rat skin, human skin, and chick bone collagens.

The separation of the peptides in peaks 9, 10, and 11 of Figure 4 was not very good upon the initial CM-cellulose chromatography because of their large size and comparable charge and because of the presence of a still higher molecular weight peptide eluting just after peak 11. Gel electrophoresis of this higher molecular weight component showed it to migrate as a single component similar to an α component and it had a composition close to α2-CB5 plus α2-CB3. Similar uncleaved peptides have been isolated from digests of chicken, rat, and human α2 chains (Lane and Miller, 1969; Vuust *et al.*, 1970; Click and Bornstein, 1970).

The peak 9, 10, and 11 peptides were resolved by purification by rechromatography of the central region of each peak on CM-cellulose using the sodium acetate buffer, pH 4.8 system. As in the rechromatography of the larger components of α1 every fraction gave one major, readily isolated component, and two or three easily distinguished minor components. Polyacrylamide gel electrophoretic migration data indicated that each major component isolated was homogeneous. The amino acid compositions of the minor peaks did not show any striking differences from their respective major components. Similar findings have been reported for the larger CNBr peptides from α1 chains (Butler *et al.*, 1967).

The whole CNBr digest of α2, or peak 1 of Figure 4, gave the same chromatogram on phosphocellulose chromatog-

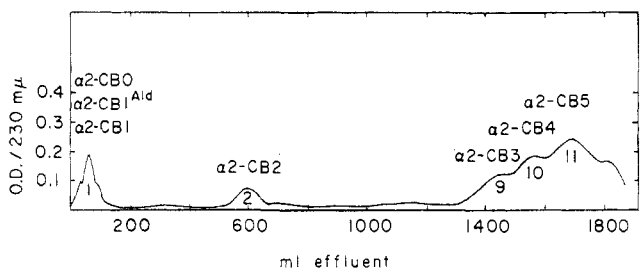

FIGURE 4: The CM-cellulose elution pattern of CNBr-cleaved α2 of acid-soluble steer skin collagen. Conditions were the same as listed in the legend of Figure 2.

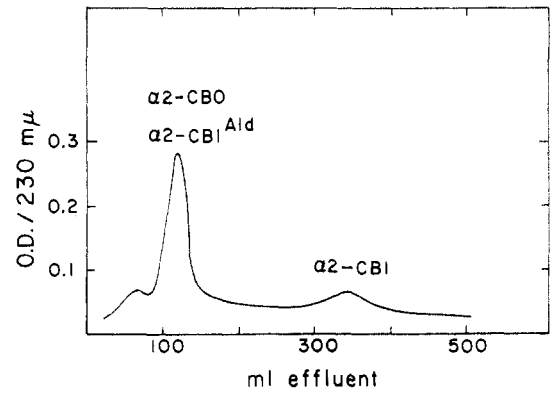

FIGURE 5: Phosphocellulose chromatographic patterns of peak 1 from Figure 4. Conditions were the same as in the legend of Figure 3. An identical pattern in the same chromatographic region was achieved using CNBr-cleaved α2.

raphy, Figure 5. The evidence for the existence of α2-CB0 indicated in Figure 5 is only indirect. However, the first peak of Figure 5 from total CNBr-cleaved α2, analyzed directly without desalting, contained not only α2-CB1^{ald}, but also three additional amino acids, glycine, leucine, and homoserine, which would account for α2-CB0. The doubled content of homoserine in the unseparated peak material before desalting was good evidence of the extra CNBr-cleaved peptide.

Discussion

The CNBr peptides reported in Tables I and II for the α1- and α2-chain peptides account adequately for the entire composition of each chain. The compositions and numbers of residues per peptide are quite analogous to the data reported for rat skin, human skin, and chick collagens.

As in other comparative studies of mammalian and avian collagens, only two methionine substitutions have been noted and the bovine collagen is closer to the rat and human collagens than to chick collagen. Leucine replaces the methionine of α1-CB0 of chick collagen so that only the intact peptide α1-CB(0,1) is present in bovine α1 chains. Similarly, the bovine α1 gives rise to a single α1-CB6 peptide, rather than the α1-CB6A and α1-CB6B of chick skin (Kang *et al.*, 1969).

The NH_2-terminal peptide of α1, α1-CB(0,1), appears in three forms: α1-CB(0,1), α1-CB(0,1)^{ald} differing only in the content of a single lysine residue, and α1-CB(0,1)′ in which α1-CB(0,1) has apparently lost one residue each of glutamic acid, serine, tyrosine, and leucine. These residues probably comprise the NH_2-terminal sequence. This may be an artifact of the preparative procedure used in the isolation of the α1 chain. Bornstein (1969) indicates that the NH_2-terminal sequence of rat skin collagen may have been removed by an *in vivo* physiologic proteolytic mechanism or by an *in vitro* limited degradation of the skin protein during extraction and purification. Bornstein indicates also that rat tendon collagen (which normally does contain the NH_2-terminal tetrapeptide) occasionally in some preparations lacks the tetrapeptide entirely.

Because of the marked homology of all the CNBr peptides of steer skin collagen with the CNBr peptides from rat, chick, and human collagen, the determination of molecular weight by molecular sieve chromatography was omitted.

It can be assumed that the order of the peptides in the chains is the same in all these species (Piez *et al.*, 1969; Vuust *et al.*, 1970; Igarashi *et al.*, 1970): for α1: 0-1-2-4-5-8-3-7-6 and for α2: 1-0-4-2-3-5.

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References

Bornstein, P. (1969), *Biochemistry 8*, 63.
Bornstein, P., and Nesse, R. (1970), *Arch. Biochem. Biophys. 138*, 443.
Bornstein, P., and Piez, K. A. (1966), *Biochemistry 5*, 3460.
Butler, W. T., Piez, K. A., and Bornstein, P. (1967), *Biochemistry 6*, 3371.
Click, E. H., and Bornstein, P. (1970), *Biochemistry 9*, 4699.
Fietzek, P. P., and Piez, K. A. (1969), *Biochemistry 8*, 2129.
Igarashi, S., Kang, A. H., and Gross, J. (1970), *Biochem. Biophys. Res. Commun. 38*, 697.
Kang, A. H., Piez, K. A., and Gross, J. (1969), *Biochemistry 8*, 1506.
Lane, J. M., and Miller, E. J. (1969), *Biochemistry 8*, 2134.
Laszlo, F., and Olsen, B. R. (1969), *Eur. J. Biochem. 11*, 140.
Miller, E. J., Lane, J. M., and Piez, K. A. (1969), *Biochemistry 8*, 30.
Piez, K. A., Eigner, E. A., and Lewis, M. S. (1963), *Biochemistry 2*, 58.
Piez, K. A., Lewis, M. S., Martin, G. R., and Gross, J. (1961), *Biochem. Biophys. Acta 53*, 596.
Piez, K. A., Miller, E. J., and Lane, J. M. (1969), *Biochem. Biophys. Res. Commun. 37*, 801.
Rauterberg, J., and Kühn, K. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 1*, 230.
Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.
Schleuter, R. J., and Veis, A. (1964), *Biochemistry 3*, 1657.
Veis, A., and Anesey, J. (1965), *J. Biol. Chem. 240*, 3899.
Veis, A., and Perry, A. (1967), *Biochemistry 6*, 2409.
Vuust, J., Lane, J. M., Fietzek, P. P., Miller, E. J., and Piez, K. A. (1970), *Biochem. Biophys. Res. Commun. 38*, 703.