cells grown in basal medium plus L-threonine or L-lysine. These results clearly indicate that the control exerted by threonine and lysine on aspartyl β-phosphate production in the growing cell occurs through concerted feedback inhibition of enzyme activity and not by a repression mechanism.

In contrast, addition of methionine to the basal medium (glutamate nitrogen source) led to repression of β-aspartokinase formation to the extent that the specific activity was about one-half that of the controls. Repression by methionine has also been noted in *Saccharomyces cerevisiae* (*6*) and may explain, in part, the decrease in growth rate of *R. capsulatus* caused by supplementation with this amino acid only; that is, repression of β-aspartokinase synthesis might be expected to result in deficiency of the common precursors necessary for formation of threonine (and isoleucine) and lysine. The apparent nonparticipation of methionine in feedback control of β-aspartokinase and homoserine dehydrogenase activities implies both that the multifaceted regulatory scheme is such that overproduction of methionine produced biosynthetically cannot occur readily under the range of nutritional conditions usually encountered by *R. capsulatus*, and that a "slow" repression mechanism suffices to ensure modulation of the intracellular methionine pool. Conceivably, under transient conditions of threonine and lysine "excess," continued methionine synthesis may be facilitated by isoenzymes of β-aspartokinase and homoserine dehydrogenase which are inherently insensitive to feedback inhibition, and this remains to be explored.

We conclude that concerted feedback inhibition of β-aspartokinase activity operates as a regulatory device in growing cells of *R. capsulatus* to prevent the overproduction of threonine and lysine. Earlier, we suggested (*2*, *3*) that feedback inhibition of enzyme activity by the concerted action of two or more end products is a phenomenon likely to be of wide occurrence in branched or interconnecting biosynthetic pathways. Concerted feedback inhibition of cell-free β-aspartokinase by L-threonine plus L-lysine has been independently discovered by Paulus and Gray (*7*) during investigation of the *Bacillus polymyxa* enzyme, and the possibility of a similar kind of control of the first enzyme of purine biosynthesis (that is, glutamine phosphoribosylpyrophosphate amidotransferase) is indicated by recent studies with both bacterial (*8*) and pigeon-liver (*9*) enzymes.

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Collagen: Structural Studies Based on the Cleavage of Methionyl Bonds

Abstract. *Eighteen peptides from the α1 fraction and eight from the α2 fraction of rat skin collagen cleaved with cyanogen bromide have been identified by chromatography on carboxymethyl cellulose. The isolated peptides show wide differences in amino acid composition, and their molecular weights vary from several thousand to about 23,000. The data indicate that there are two different chains in the α1 fraction, demonstrating the nonidentity of all three α-chains. The primary structure of each chain appears to be unique throughout its length.*

Physical-chemical studies indicate that the collagen molecule is composed of three chains each with a molecular weight of about 100,000 (*1*). In view of this size, it is not surprising that the separation of peptides in enzymatic digests of unfractionated collagen has not been entirely satisfactory, although useful preliminary information regarding the amino acid sequence in portions of the molecule has been obtained (*2*). The task of structure analysis is further complicated by covalently bonded aggregates in most collagen preparations, and these may contribute additional peptides as a result of interchain cross-links. The chromatographic fractionation of heat-denatured collagen on carboxymethyl cellulose (*3*) has permitted the preparation of single-chain (α) and double-chain (β) components. However, despite the considerable advantage which the use of these components provides, the number of bonds susceptible to the usual proteolytic enzymes is still so large that the satisfactory isolation and analysis of peptides derived from such digests is unlikely (*4*).

The low methionine content of mammalian collagens suggested to us that chemical cleavage at methionyl bonds in collagen chains might provide a relatively small number of protein fragments, amenable to separation and characterization and useful in the study of collagen structure. The ability of cyanogen bromide to cleave methionyl bonds of model peptides in high yields with minimal side reactions (*5*) has been successfully utilized in structural studies of ribonuclease (*6*), sperm whale myoglobulin (*7*), and trypsinogen

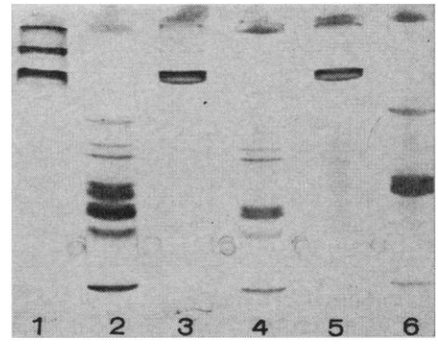

Fig. 1. Acrylamide-gel electrophoresis patterns of acid-extracted rat skin collagen (tubes 1 and 2) and its α1 (tubes 3 and 4) and α2 (tubes 5 and 6) fractions before and after treatment with cyanogen bromide in 0.1*N* HCl. Migration from top to bottom.

Table 1. Amino acid composition and molecular weights of three peptides obtained by methionyl-bond cleavage of the α1 fraction of rat skin collagen*.

Amino acid	Residue (No.)					
	Peptide A		Peptide B		Peptide C	
	Per 1000	Per peptide	Per 1000	Per peptide	Per 1000	Per peptide
4-Hydroxyproline	89	23	99	17	133	5
Aspartic acid	43	11	47	8	(4.7)	
Threonine	25	6	16	3	(1.0)	
Serine	38	10	23	4	55	2
Glutamic acid	66	17	102	18	106	4
Proline	140	36	95	16	183	7
Glycine	349	89	343	59	340	13
Alanine	110	28	135	23	61	2
Valine	17	4	23	4	(3.5)	
Methionine						
Isoleucine	8.4	2	(0.9)		(0.5)	
Leucine	16	4	19	3	26	1
Tyrosine					(0.4)	
Phenylalanine	11	3	18	3	23	1
Hydroxylysine	3.0	1	(0.9)			
Amide nitrogen	44†	11	62†	10	60†	2
Lysine	33	9	32	6	(2.2)	
Histidine	(0.7)		(1.0)		(0.4)	
Arginine	49	13	41	7	28	1
Homoserine	3.4	1	6.0	1	24	1
Molecular weight from amino acid composition						
	23,100		15,500		3,300	
Weight-average molecular weight‡						
	21,500		12,800			

* Numbers in parenthesis indicate fractional residues present as impurities. † Not included in total residues. ‡ Weight-average molecular weights were determined by high-speed sedimentation equilibrium (*12*).

(*8*). The application of nonenzymatic cleavage to collagen appeared particularly promising since secondary structure is lost simply by warming, and the heat-denatured protein remains soluble and is relatively stable under the conditions of cleavage. The absence of cysteine in collagen eliminates a possible source of side reactions (*9*). Rat skin collagen was studied because the amino acid composition of the component chains of the molecule was known from previous work (*3*) and because it is readily prepared in large quantities.

Heat-denatured collagen was treated with cyanogen bromide (100-fold molar excess relative to methionine). The reaction was first performed in different acidic media at 10° to 30°C for periods ranging from 5 to 30 hours. Reaction mixtures were diluted with water and lyophilized. The extent of cleavage of the methionyl bond was evaluated by amino acid analysis (*10*) and acrylamide-gel electrophoresis by a modification of the method described by Nagai *et al.* (*11*). The reaction of the protein in 0.1*N* HCl at 30°C for 15 hours yielded the highest degree of cleavage (from 90 to 97 percent). Some nonspecific degradation of collagen occurred in the absence of cyanogen bromide under these conditions of *p*H and temperature (predominantly of β components as judged by gel electrophoresis and chromatography on carboxymethyl cellulose). This appeared to be less extensive in reaction mixtures, possibly because the initial rapid decrease in chain length brought about by cyanogen bromide–mediated cleavage resulted in proteins less labile than the chains from which they were derived.

The electrophoretic patterns of whole collagen, the α1 and α2 fractions, and the fragments produced by cyanogen bromide are shown in Fig. 1. The sum of fragments from the fractions corresponds to the sum of those produced from whole collagen. The three uppermost bands in tube 2 and the corresponding bands in tubes 4 and 6 represent a small amount of incompletely cleaved collagen chains present to varying degrees in all preparations.

The fragments produced by cleavage of the methionyl bond were initially subjected to molecular-sieve chromatography on columns of Sephadex G-200 (120 by 2.2 cm) maintained at 40°C. Most of the incompletely reacted, methionine-containing protein could be separated from the remainder of the peptides which were retarded owing to their smaller size. The latter peptides (90 to 95 percent of the protein eluted) were chromatographed on columns of carboxymethyl cellulose at 40°C with a sodium acetate buffer (0.02 ionic strength, *p*H 4.8) with a concave gradient of sodium chloride (0 to 0.14*M*). Alternatively, improved resolution of peptides eluted at the start of this gradient was achieved with a sodium acetate buffer (0.01 ionic strength, *p*H 4.8) and a similar concave sodium chloride gradient (from 0 to 0.11*M*). The latter system, however, did not adequately resolve peptides that were eluted at higher ionic strengths.

The prior fractionation of acid-ex-

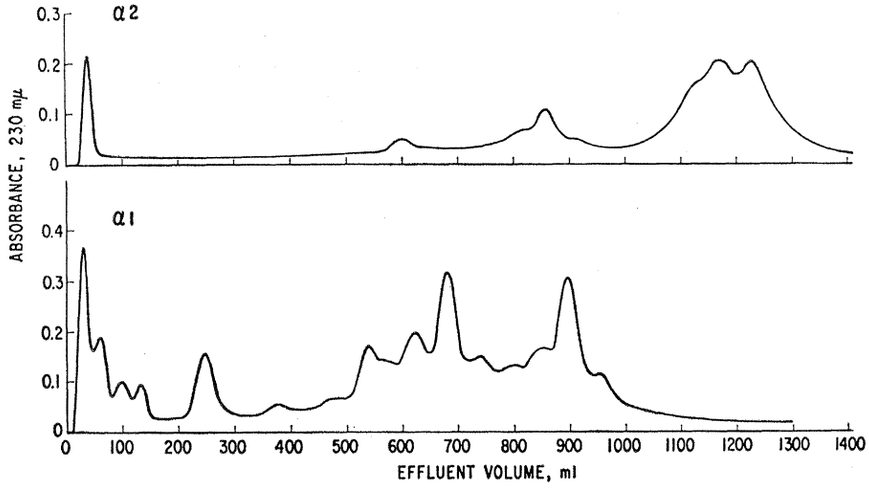

Fig. 2. Elution patterns of peptides produced by treatment with cyanogen bromide from the α1 and α2 fractions of rat skin collagen. Chromatography performed on carboxymethyl cellulose at 40°C with a sodium acetate buffer (0.02 ionic strength) at *p*H 4.8 and a sodium chloride gradient.

tracted collagen on carboxymethyl cellulose permitted examination of the $\alpha 1$ and $\alpha 2$ fractions individually, and resulted in chromatograms of products produced by cyanogen bromide treatment (Fig. 2) which were less complex than those obtained with whole collagen. Sixteen peaks could be distinguished in chromatograms of the reaction products of the $\alpha 1$ fraction. Of these the last peak, which was consistently present, contained one residue of methionine (as the sulfoxide) per fragment. The first four peaks eluted were resolved into at least seven peptides, with the gradient of lower ionic strength yielding a minimum total of 18 peptides in the $\alpha 1$ fraction. Eight peaks were observed in the chromatograms of the reaction products of the $\alpha 2$ fraction. The precise number of peptides in the $\alpha 1$ and $\alpha 2$ fractions after cleavage with cyanogen bromide can only be determined after additional studies, but the final result cannot be very different from these estimates.

Most peptides could be obtained in chromatographically pure form by rechromatography on carboxymethyl cellulose. Although acrylamide-gel electrophoresis of these preparations usually revealed small quantities of other contaminating peptides, these were estimated at 10 percent or less by amino acid analysis. Almost all the fragments derived from $\alpha 1$ have been isolated, and their amino acid composition has been determined. Large variations in composition are present even among the larger fragments. However, glycine is invariably present as one-third of the residues and hydroxyproline, proline, and alanine together consistently account for another third. Most peptides were sufficiently pure to permit the minimum equivalents of a number of amino acids to be calculated for estimation of molecular weight. Since these estimates are subject to rather large errors, particularly for the larger peptides, molecular weights of some of the peptides have been determined by high-speed sedimentation equilibrium (*12*). The amino acid compositions and molecular weights of three typical peptides derived from the $\alpha 1$ fraction are listed in Table 1. Peptides A and C are at the upper and lower size limits, respectively, of the peptides which have been isolated.

The $\alpha 1$ fraction of rat skin collagen contains eight or nine residues of methionine in each chain whose molecular weight is 100,000 (*1*, *3*). The finding of roughly twice this many peptides after methionyl-bond cleavage indicates that this fraction contains two different chains which are not separated by chromatography on carboxymethyl cellulose. The sum of the estimated molecular weights of isolated fragments and the quantitative distribution of amino acids present in small amounts in the $\alpha 1$ fraction are consistent with this conclusion. The fact that all or nearly all of the peptides can be resolved indicates that the two chains are quite different, though of similar net charge. Recently collagen from codfish skin has been shown to contain three different chromatographically separable α-chains (*13*). The similar finding, by a different method, in a mammalian collagen supports the suggestion (*13*) that nonidentity of the three α-chains in collagen is a general property of the protein (*14*). The homogeneity of the $\alpha 2$ fraction from rat skin collagen (6 or 7 methionine residues per chain) is confirmed by the finding of approximately eight fragments resulting from cyanogen bromide treatment.

The α-chains of collagen may be composed of intrachain subunits linked by hydroxylamine-sensitive bonds, these possibly being ester bonds (*15*). The presence of identical repeating intrachain subunits has also been postulated on the basis of a mathematical analysis of electron micrographs of segment-long-spacing aggregates of collagen (*16*). Our data indicate that the amino acid sequence of each of the α-chains is unique throughout its length, and therefore they do not support models of collagen structure based on identical intrachain subunits. If nonidentical subunits are present, this introduces the requirement in the synthesis of collagen for the linear arrangement, in a predetermined sequence, of protein chains linked by nonpeptide bonds.

The location and nature of the cross-links in collagen remains a difficult problem. If interchain cross-links are specific in location, relatively few in number (per chain), and resistant to the conditions employed in methionyl-bond cleavage, limited differences in the peptides derived from single and cross-linked chains might be detectable. No peptides unique to cross-linked chains are visible by acrylamide-gel electrophoresis. Furthermore, the large peptides derived from double chains appear to correspond precisely to the peptides derived from their component single chains. However, differences among the smaller peptides of cross-linked chains as compared with single chains have been observed in their carboxymethyl cellulose elution patterns.

Note added in proof. After our work was submitted for publication, a paper by Nordwig and Dick was published reporting the cleavage of collagen with cyanogen bromide and indicating that prior denaturation of the protein was necessary in order for cleavage to occur.

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