Table I. Data for Modified and Unmodified Tropo- and Paramyosin in Various Media[a]

	Medium[b]	$[\eta]^u$	$[\eta]^m$	$10^{13}[s_{20,w}]^u$	$10^{13}[s_{20,w}]^m$	$10^{-3}\ M^u$	$10^{-3}\ M^m$
TM	$KCl_{1.0}K[PO_4]_{0.1}$ (7.3)	34	34	2.59	2.68	74.2[c]	77.2
TM	$GuHCl_{5.0}KCl_{0.6}$-$K[PO_4]_{0.06}$ (6.1)	45[c]	33	2.38		76.2[c]	39.5
PM	$KCl_{1.0}K[PO_4]_{0.1}$ (7.4)	207	206	3.1	2.99	220[d]	200
PM	$GuHCl_{7.5}KCl_{0.2}$-$K[PO_4]_{0.03}$ (7.4)	103	65.6	4.4	2.63		108[e] / 92[f]

[a] Columns whose headings contain superscript m (u) refer to the property obtained when the modified (unmodified) protein is dissolved in the medium given. Intrinsic viscosities are in cc/g; $[s_{20,w}]$ in sec^{-1}. [b] To designate aqueous solvent media, the chemical formula for each constituent (other than water) is given with its molarity as a subscript, followed by parenthetical specification of the pH (ref 3). [c] Reference 3. [d] Reference 2. [e] From the empirical relation: $[\eta] = 0.716r^{0.66}$ as given in C. Tanford, K. Kawahara, and S. Lapanje, *J. Am. Chem. Soc.*, **89**, 729 (1967). [f] From the Scheraga–Mandelkern equation with $\beta' = 2.5 \times 10^6$, as given in H. A. Scheraga and L. Mandelkern, *ibid.*, **75**, 179 (1953).

able helix content, at 25°, up to a guanidine concentration of 7.0 *M*.[7,8] Chemical studies have revealed no disulfide bonds.[6]

To investigate the polypeptide chain make-up of this protein, we prepared modified paramyosin in the same way as tropomyosin, the only difference being that the guanidine concentration of the paramyosin solvent was higher (7.5 *M*). Measurements of the same macromolecular properties were made (Table I, rows labeled PM). Two differences in technique used in the paramyosin study are noteworthy: (1) molecular weights in the benign medium were measured by the Archibald procedure; (2) since it is very difficult to determine the molecular weight in 7.5 *M* guanidine by absolute measurements (because of strong nonideality), we have resorted to use of the intrinsic viscosity–molecular weight relationship for proteins[9] and have confirmed this value by use of the sedimentation–viscosity relationship for random coils.[10]

The results for paramyosin are analogous to those for tropomyosin. Modified and unmodified paramyosins show, for example, markedly different intrinsic viscosities in the denaturing solvent, that of the unmodified protein being larger. Since destruction of cross-linkages in a single-chain random coil would produce the opposite effect,[9] this observation suggests some dissociation of chains in the modified protein. The molecular weights confirm this: the mass of the modified protein molecule in guanidine is one-half that of the native protein. That this chain separation is reversible is apparent from comparison of the data for the modified and unmodified proteins in benign media; they are indistinguishable.

Several conclusions can be drawn from the experimental results: (1) the molecules of both tropomyosin and paramyosin contain two individual polypeptide chains; (2) in both proteins, at least as they are usually prepared, the chains are joined by at least one disulfide linkage (which has *not* been detected chemically in paramyosin[6]); and (3) the disulfide cross-bridging is, in both cases, unnecessary for maintenance of the native conformation—even after all disulfides have been reduced and the resulting sulfhydryls acetylated, the double α-helix is recovered in benign media.

The last of the three conclusions noted above should not be stated without some equivocation. There is a chance that the two chains are unanimously parallel, or unanimously antiparallel, in the unmodified double-helical molecules, but that after modification and redissolution in benign medium a mixture of double helical molecules results, some having parallel chains and some antiparallel. The measurements reported here are responsive only to comparatively gross changes in molecular shape and cannot resolve this ambiguity. However, if a coiled coil owes its stability to knobs-into-holes packing, as has been suggested,[11] it seems quite unlikely that any side-to-side packing other than that characteristic of the native protein would be very stable.

Needless to say, several questions remain; among these is whether the two chains within an individual molecule are identical or different, and, if the latter, whether they can be chemically separated. If so, it would be particularly interesting, for the fundamental theory of stability of protein conformations, to see if a system containing only one of the chains would form an α-helix in a benign medium. Thus far, no instance of a single protein α-helix has been reported.

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(8) M. Noelken, Ph.D. Thesis, Washington University, St. Louis, 1962.
(9) See Table I, footnote *e*.
(10) See Table I, footnote *f*.
(11) F. H. C. Crick, *Acta Cryst.*, **6**, 689 (1953).

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Relief by Modification of Carboxylate Groups of the Calcium Requirement for the Activation of Trypsinogen

Sir:

The activation of bovine trypsinogen involves the removal of a highly anionic hexapeptide from the N-terminal region of the molecule.[1,2] MacDonald and Kunitz[3] had shown earlier that this process is greatly enhanced by divalent cations such as calcium. These ions seem to exert a directing influence by both promoting the formation of active trypsin from trypsinogen and precluding the formation of an "inert protein." Thus in the presence of calcium ions proteolysis is restricted to the single peptide bond between lysine-6 and isoleucine-7, all other lysyl and arginyl bonds being resistant to tryptic cleavage. In the absence of calcium ions proteolysis is retarded and becomes relatively

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(2) P. Desnuelle and C. Fabre, *Biochim. Biophys. Acta*, **18**, 29 (1955).
(3) M. R. MacDonald and M. Kunitz, *J. Gen. Physiol.*, **25**, 53 (1941).

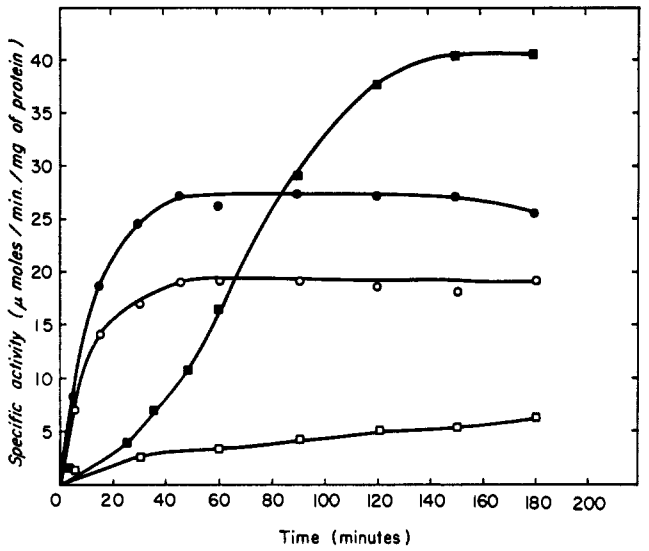

Figure 1. The course of activation in 0.1 *M* Tris-Cl, pH 8.1, at 0° of native (squares) and modified (circles) trypsinogen (3.5 mg/ml) in the presence (filled symbols) and absence (open symbols) of 0.05 *M* $CaCl_2$.

nonspecific.[4] It is not clear how calcium confers specificity on the limited proteolysis.

A possible site of interaction of calcium with trypsinogen is suggested by the amino acid sequence of the activation peptide, which contains a cluster of four β-carboxylates in a tetrapeptide sequence[1] adjacent to the bond being cleaved. The present experiments were designed to examine the effect on the activation process of modification of these free carboxylate groups with amines under the influence of water-soluble carbodiimides. In recent years[5-7] such treatments at acid pH have proven to be specific for other protein carboxylates.

Preliminary experiments showed that trypsin (0.2 m*M*) retained 68% of its activity during a 15-min treatment at pH 4.5, 25°, with a 25-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 1.0 *M* glycine ethyl ester.[8] The reaction was stopped by the addition of 1.0 *M* acetate, pH 3.6, and the reagent was removed by dialysis against 10^{-3} *M* HCl at 0°.

Similar treatment of trypsinogen resulted in a derivative which could be 64% activated relative to the unmodified control (Figure 1). Significantly, even in the absence of $CaCl_2$, the modified protein could be activated to the extent of about 46%, whereas with unmodified trypsinogen in the absence of $CaCl_2$ activation did not exceed 15%. The number of active sites estimated by measuring the ^{32}P incorporation during inactivation with ^{32}P-labeled diisopropyl phosphorofluoridate corresponded to the activity determined by rate assay with benzoyl-L-arginine ethyl ester as substrate.

A series of anionic peptides was isolated from the activation mixture containing glycine ranging from 0.6 to 2.5 g residues per mole in addition to valine, aspartic acid, and lysine (Table I). In contrast, activation of unmodified trypsinogen yielded a single acidic peptide of the expected composition (Val, Asp_4, Lys). The anionic peptides in Table I represent a 60% yield of the N-terminal peptides from the modified trypsinogen. This is greater than the amount predicted from its activability (42%), suggesting that not every tryptic cleavage between lsysine-6 and isoleucine-7 was productive. This observation is in accord with the end group analyses (Table II) which invariably exceeded the expected yield of DNP-isoleucine. However, in the latter case autolysis could account for high values for these N-terminal residues.

Table I. Peptides Derived from the Activation of Modified Trypsinogen[a]

Peptide	g residues of glycine found	Yield of peptide, μmoles	Yield, %
A	0.6	0.106	14
B1	1.7	0.140	18
B2	1.3	0.194	26
C	2.3	0.166	22
D	2.5	0.150	20
Total		0.756	100

[a] The modified protein (13.4 mg/ml) was activated for 1.5 hr at 0° in the absence of $CaCl_2$ in mixtures containing trypsin (0.67 mg/ml) and 0.05 *M* N-ethylmorpholine acetate, pH 8.1. Peptides were separated from the protein on Sephadex G-25 and purified by paper electrophoresis at pH 6.5 and chromatography in 1-butanol–acetic acid–water, 3:1:1. Acid hydrolysis of the anionic peptides yielded valine, aspartic acid, and lysine in the ratio 1:4:1 in all cases, in addition to the indicated amounts of glycine. Peptides B1 and B2 were not completely separated from each other. The stoichiometric yield of activation peptide (assuming 100% activation), after correcting for analytical losses during the isolation procedures, would be 1.31 μmoles.

Table II. Protein N-Terminal Residues in Activation Mixtures[a]

	Moles of DNP-amino acid/mole of protein			
	Trypsinogen	Trypsinogen + Ca^{2+} + trypsin	Trypsinogen + trypsin, no Ca^{2+}	Modified trypsinogen + trypsin, no Ca^{2+}
Val	0.78	0.07	0.77	0.39
Ile + Leu	..	0.67	0.26	0.68
Ser	..	..	0.08	0.32

[a] Trypsinogen or modified trypsinogen was activated with trypsin (3.5 mg/ml) with or without $CaCl_2$ as in Table I for 2 hr. The protein component was precipitated with trichloroacetic acid and end-group analysis was carried out with FDNB (H. Fraenkel-Conrat, J. I. Harris, and A. L. Levy, *Methods Biochem. Anal.*, **2**, 359 (1955)) in 8 *M* urea.

The number of peptides found indicates that the extent of substitution with glycine ethyl ester varied. Further fractionation is required to determine whether the peptide fractions on the electropherogram are molecularly homogeneous. The nonintegral values of glycine may be due to molecular heterogeneity resulting from multiple substitution, partial ester hydrolysis, or side reactions of undetermined character.

The activability of carboxylate-modified trypsinogen in the absence of $CaCl_2$ suggests that the directive influence of calcium on the normal activation may be by an interaction with carboxylate groups of the native zymogen. The present data show that carboxylate

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(8) A similar modification of trypsin had been previously described by Hoare and Koshland.[7]

groups of the activation peptide have been amidated with glycine ethyl ester and suggest that calcium binding and carboxylate modification can be functionally equated in the activation process.

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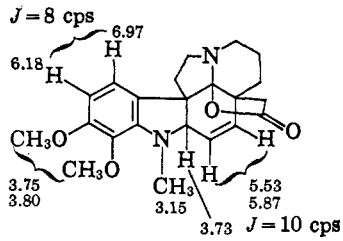
Haplophytine

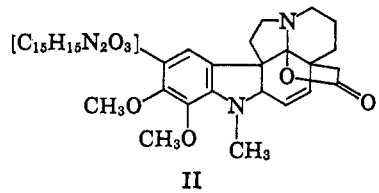
Sir:

Haplophytine[1] was first isolated by Snyder and his co-workers, who carried out an extensive investigation of its chemistry.[2] It is obtained as the principal alkaloid from *Haplophyton cimicidum* (Apocynaceae).[2-4]

High-resolution mass spectrometry[5] required the revision of its formula to $C_{37}H_{40}N_4O_7$ (calcd mol wt, 652.28990; found, *m/e* 652.29148).[6] Acid cleavage gave a crystalline compound (76%), $C_{22}H_{26}N_2O_4$ (calcd mol wt, 382; found: *m/e* 382), mp 201–203°, assigned structure I on the basis of its nmr spectrum (see I for δ values) and the following data. Its infrared spectrum ($CHCl_3$) shows a strong C=O band at 5.73 μ (*cf.* cimicine,[4] cimicidine,[4] and dichotamine[7]),



I



II

but no OH or NH bands. Its ultraviolet spectrum [λ_{max}^{EtOH} 222 mμ (ϵ 28,700), 256 (5900), and 304 (2400)] is in accord with an indoline chromophore. Its mass spectrum shows a strong peak at *m/e* M − 44, characteristic of lactonic alkaloids of the aspidoalbine skeletal type.[7,8] Hydrogenation of the cleavage product gave a zwitterionic tetrahydro derivative, mp 265° dec, formed by saturation of the ethylenic double bond accompanied by hydrogenolysis of the lactone (*cf.* dihydrocimicine and dihydrocimicidine[4]). Esterification with diazomethane gave a glass, $C_{23}H_{32}N_2O_4$, whose mass spectrum showed the molecular ion peak *m/e* 400 (base peak) and a single prominent fragment ion at *m/e* 168, a pattern very characteristic of nonlactonic alkaloids of the aspidospermine type.[9] Reduction of the cleavage product with sodium borohydride gave a zwitterionic product the spectra of whose methyl ester demonstrated that reductive cleavage of the lactone alone had occurred. The mass spectrum of this ester showed a molecular ion at *m/e* 398, confirming the extent of reduction; the absence of significant fragment ions at *m/e* 166 and 179 excluded a formulation in which the ethylenic double bond of I is placed at C-6–7.[9] The position assigned to the double bond is also in better accord with the chemical shift of the C-2 proton and the fine splitting of the olefinic proton signals in the nmr spectrum of I.

The many common features of the spectra of I and those of haplophytine [λ_{max}^{EtOH} 220 mμ (ϵ 48,500), 265 (14,300), and 305 (4500); $\lambda_{max}^{CHCl_3}$ 5.72 and 6.05 μ; δ^{CDCl_3} (ppm) 2.40 (~3 H), 3.00 (3 H), 3.17 (3 H), 3.65 (3 H), 3.72 (1 H), 5.55 (1 H, doublet, J = 10 cps), 5.85 (1 H, doublet, J = 10 cps), 6.27 (1 H, doublet of doublets, J = 7 and 2.5 cps), 6.9–7.2 (3 H, multiplet), 9.04 (1 H, absent after D_2O wash)] showed that the structure of haplophytine is related to that of I by replacement of a hydrogen atom in the latter by a moiety $C_{15}H_{15}N_2O_3$. That the linkage is at C-15 as in II is indicated by the absence of a doublet (J = 8 cps) in the nmr spectrum of haplophytine corresponding to the C-15 proton signal in the spectrum of I. Catalytic hydrogenation of haplophytine gave tetrahydrohaplophytine, $C_{37}H_{44}N_4O_7$, accompanied by spectral changes analogous to those occurring when I was converted to tetrahydro-I; acid cleavage of the methyl ester of O-methyltetrahydrohaplophytine, $C_{39}H_{48}N_4O_7$, gave tetrahydro-I.

The intensities of the ultraviolet maxima of haplophytine indicated that the $C_{15}H_{15}N_2O_3$ moiety also possesses an indoline system. This can be expanded to a 7-hydroxy-1-acylindoline system on the basis of the relationship between the infrared and nmr spectra of haplophytine and the spectra of its O-substituted derivatives, as earlier deduced by Snyder.[2,10] The nmr signal at 6.27 ppm, which can be assigned to an aromatic proton coupled with *ortho* and *meta* protons, requires that the 7-hydroxy-1-acylindoline system be unsubstituted at the C-4, -5, and -6 positions. The signal at 2.40 ppm indicated the presence of an aliphatic N–CH_3 in the $C_{15}H_{15}N_2O_3$ moiety, while the infrared spectrum showed that the third oxygen atom can only be present in an ether linkage.

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(10) The presence of this second aromatic ring is considered to account for the unusual shielding of the protons of one of the methoxyl groups in haplophytine; in I, where this second ring is absent, the chemical shifts of the protons of both methoxyl groups are normal.