ENZYMATICALLY ACTIVE COMPONENTS IN TRYPSIN AUTOLYSATES[1]

Sir:

The finding of a low molecular weight protein with enzymatic properties is of considerable interest for a study of the interrelationship of protein structure and function. Autolysis experiments with several enzyme preparations[2,3,4] have suggested the existence of enzymatically active intermediates which are considerably smaller than the original protein. The evidence, however, is only convincing in the case of pepsin.[5] The following experiments were performed to establish whether or not enzymatically active intermediates are formed during the autolysis of trypsin.

Commercial trypsin preparations[6] (I) of low specific activities and highly purified trypsin[7] (II) were used. Enzyme solutions, 5 mg./ml., were autolyzed at 25°, *p*H 9.1, in the 0.05 M sodium borate buffer. After 1.75 hours the reaction was stopped by addition of acetic acid and calcium chloride solutions to give a final concentration of 0.01 N acetic acid, 0.03 M calcium chloride, and 0.03 M borate ion. Diffusion experiments were then carried out with these solutions at 25°. The properties of the 18/32 and 20/32 Visking cellophane membranes and the diffusion technique employed have been studied extensively by Craig.[8,9] The enzymatic activity of the material that had diffused through the membranes was measured by the azo-casein[10] and the more sensitive dye-fibrin method.[11]

It can be seen in Fig. 1 that the proteolytically active component of I, II and autolysate II, diffuses through the 20/32 membrane (permeable[9] to trypsin, mol. wt. 20,000) at the same rate and as a single component. Under identical experimental conditions of temperature, buffer concentration, *p*H, and enzyme concentration (as measured by the azo-casein method), the proteolytically active material of autolysate I diffuses through this membrane as two components. The minor component contains about 2% of the enzymatic activity of autolysate I. Experiments with 18/32 membranes (impermeable to ribonuclease,[9] mol. wt. 13,000) were also consistent with the presence, in autolysate I, of an enzymatically active component which diffuses at a faster rate than trypsin. Five per cent. of the proteolytic activity of the minor component of autolysate I was found to diffuse through this membrane in 12 hours at 0°. Under identical experimental conditions, but during a 24-hour period, these membranes were found to be impermeable to the proteolytically active component of I, II and the autolysate of II.

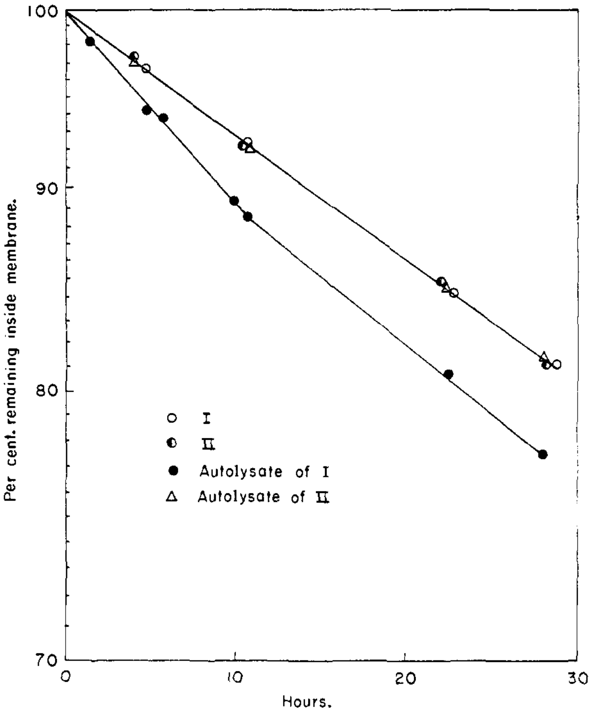

Fig. 1.—Diffusion curves through 20/32 Visking cellophane in 0.01 N acetic acid, 0.03 M calcium chloride, 0.03 M borate ion, at 25°. The difference in the observed initial rates of diffusion of I and the autolysate of I corresponds to the presence of about 2% enzymatically active material.

Diffusion experiments with I indicated that the low specific activities of these preparations were due to the presence of an enzymatically inactive protein which diffuses at the rate of trypsin. During autolysis this protein was found to hydrolyze twice as fast as trypsin. This suggests that this inert protein competes as substrate with the minor enzymatic component leading to accumulation of the latter, and would explain why only one active component is found in autolysate II. In support of these conjectures, it appears that autolysis experiments with equal mixtures of II and heat inactivated II lead to trace amounts of enzymatically active material that diffuses through the 18/32 membranes. These data would also support the hypothesis that the minor enzymatic component is a degradation product of trypsin I and not an impurity in this preparation.

The significance of these findings is that a proteolytic enzyme smaller in size than trypsin is produced through the autolysis of trypsin I, though in small quantities.

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[1] This investigation was supported by a research grant (RG 4842) from the National Institutes of Health, Public Health Service.

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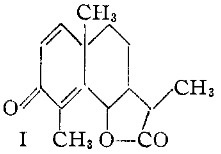
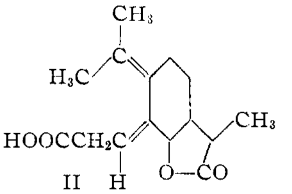
THE STRUCTURE OF PHOTOSANTONIC ACID

Sir:

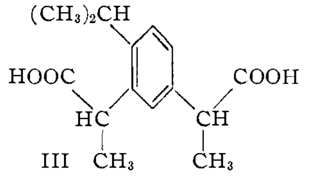
Renewed interest in the irradiation products of santonin (I) and other dienones is evident from the recent appearance of structural proposals for isophotosantonic acid,[1] lumiphotosantonin[2,3] as well as certain steroidal rearrangement or aromatization products.[4,5] *Photosantonic acid* is perhaps the historically best-known member of this group of curious substances; and, largely on the basis of findings summarized below, we wish to suggest for this irradiation-hydration product the expression

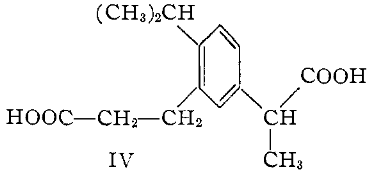

I


II

II, which supersedes the various structures entertained in the past.[6,7,8]

Treatment with hydrogen fluoride of "dehydrophotosantonic acid," an acid-catalyzed isomerization product long regarded as possessing structure III,[7,8] effects cyclodehydration to an *indanone*, m.p. 111–112.5°, $\lambda_{\max}^{\text{methanol}}$ 214 (30,200), 254 (10,600), 302 mμ (3220); carbonyl absorption of the methyl ester: 5.76 and 5.85 μ.[9]


III


IV

(Calcd. for $C_{15}H_{18}O_3$: C, 73.14; H, 7.37. Found: C, 73.26; H, 7.29). This observation serves to exclude structure III and, taken together with previously recorded findings (*e.g.* oxidation to cannabinolactone), allows the formulation IV,[10] an assignment consistent with preliminary nuclear magnetic resonance data.

The nuclear magnetic resonance spectrum of methyl photosantonate is made up of, *inter alia*, signals attributable to sets of non-equivalent protons in: (i) the methyl group and the three methine hydrogens of a substituted lactone ring equivalent to that in santonin (also suggested by various chemical data and by infrared absorption at 5.65 μ); (ii) and one trisubstituted double bond bearing on one of the carbons, a hydrogen and a methylene group joining the double bond and the carboxyl group.[11] Ozonization under carefully defined conditions (in acetic anhydride at −78°) involves formation of acetone in high yield; zinc dust reduction of the ozonide also gives, in lesser amount, acetaldehyde, whereas an oxidative workup yields malonic acid.[12] The ozonization results establish the presence of an isopropylidene grouping and corroborate the partial structure ($HOOC\text{-}CH_2\text{-}CH{=}C<$) initially evidenced by the NMR data. Because of the structural limitations imposed by the convertibility to dehydrophotosantonic acid (IV), the derived partial structures lead directly to the expression II for photosantonic acid.[13]

The chemical features which for so long obscured the constitutional nature of photosantonic acid now become clear: (i) a 1,3-dienoid system which does not exhibit a peak above 210 mμ (apparently due to steric hindrance around the heavily substituted diene system, resulting in out-of-plane distortion and markedly decreased overlap of π-electrons); (ii) a trisubstituted double bond of pronounced inertness (for example, to hot hydrogen peroxide, ref. 10) and therefore easily mistakable for a cyclopropane ring; (iii) in addition to migration of a methyl group from the angular position in santonin to an adjacent position in the side chain of the irradiation product, the net isomerization of the partial structure ($COC(CH_3){=}CH<$) of I to a *normal β,γ-unsaturated carboxylic acid side chain* in II.

Discussion of the mechanism of the change I → II and description of other chemical reactions which photosantonic acid undergoes, will be presented in a subsequent publication.

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(10) The possibility in which the positions of the α- and β-propionic acid substituents are reversed, is untenable since the implied isomeric structure for photosantonic acid cannot accommodate various chemical reactions which the natural product undergoes, *e.g.*, the generation by hydrogen peroxide of an ε-hydroxy-δ-lactone system, during which process the position of the trisubstituted double bond is maintained.

(11) The NMR spectrum was obtained by operating at 30 megacycles in a magnetic field of 7000 gauss, using benzene as a reference. Signals used for the above assignments are found at *ca.* (i) 90–100 (unresolved) and 170 c.p.s.; (ii) 53 (triplet) and 110 c.p.s.

(12) Similar ozonolysis operations on 4-methyl-penten-3-oic acid yield acetone and either acetaldehyde or malonic acid: A. A. Goldberg and R. P. Linstead, *J. Chem. Soc.*, 2354 (1928).

(13) The structural possibility, in which the ethereal oxygen of the γ-lactone ring is attached at the alternate position on the six-membered ring, is highly improbable and moreover is excluded in that this oxygen must be situated allylically to a double bond, as indicated by its catalytic hydrogenolysis (studies in progress) and by a negative tetranitromethane color test on, *e.g.*, the derived hydroxylactone (ref. 10).