of Ia to give ring opening analogous to the reaction of IIa in dilute acids observed by Hart and Sandri.[8] The decomposition product is a new ion (λ_{max} 285 mμ, $\epsilon > 3000$), half-formed above 80% H_2SO_4, and tentatively assigned structure Ie. Addition of solutions of Ie to water gives a compound whose b.p. and n.m.r. and infrared spectra are in accord with structure III. The addition of III to H_2SO_4 regenerates Ie.

The identification of Ib is less complete. The ultraviolet spectrum (λ_{max} 293 mμ, $\epsilon > 2600$)[8] is similar to that of Ia and Ie. Ib is half formed above 80% acid as is Ie. Immediate drowning of cold solutions of Ib in ice–10% NaOH gave a 25% yield of polymer and a 20% yield of an olefin which has a b.p. and n.m.r. and infrared spectra in agreement with the alkene (1,1-dicyclopropyl-2-methylpropene) derived from IIb by direct dehydration. In addition to decomposition by polymerization, Ib (or the alkene) is oxidized by 96% H_2SO_4 as evidenced by liberation of SO_2 and rising absorption around 300 mμ.

A number of C_4 and C_8 alkenes and alkanols form a species (possibly several species) in H_2SO_4 characterized by a broad absorption band around 293 mμ.[9] Its absorption and its thermodynamic stability (half-formed in 65% H_2SO_4)[9] are characteristic of alkenyl cations.[2] The slow formation from *t*-butyl alcohol,[10] the same rate of formation from *t*-butyl alcohol and 2-butanol,[9] and our observation that the kinetics of formation are not simply first order identify the 293 mμ species as an alkenyl cation rather than the *t*-butyl cation.[9]

(8) The ϵ values for Ib and Ie are for 96% H_2SO_4 solutions. The ions are not completely formed from their alkenes at these acidities.

(9) J. Rosenbaum and M. C. R. Symons, *Mol. Phys.*, **3**, 205 (1960).

(10) The 293 mμ species is reported to require two hours to half form from a $\sim 10^{-4}$ molar solution of *t*-butyl alcohol in 80 and 98% H_2SO_4 (ref. 9). In our experience with over a hundred carbonium ions including the aliphatic carbonium ions in this communication and ref. 2, the alcohol–carbonium ion equilibria were established within seconds.

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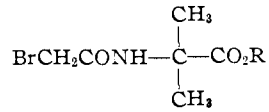
MODIFICATION OF A METHIONINE RESIDUE NEAR THE ACTIVE SITE OF CHYMOTRYPSIN

Sir:

We wish to describe a new method for the selective chemical modification of enzymes, and its application to the pancreatic protease, chymotrypsin. The basic principle is to combine an enzyme with a bifunctional reagent, so designed that it becomes covalently bound to an amino acid side chain at the active site, and then, fixed in position, reacts with another amino acid residue in the vicinity. The reaction of chymotrypsin with nitrophenyl esters,[1] during which the acyl group of the ester becomes attached to the hydroxyl group of the serine residue at the active site,[2] is a good one to demonstrate the utility of the method.

(1) C. E. McDonald and A. K. Balls, *J. Biol. Chem.*, **227**, 727 (1957).

(2) R. A. Oosterbaan and M. E. Van Adrichem, *Biochim. Biophys. Acta*, **27**, 423 (1958).



I, R = *p*-nitrophenyl; II, R = H

When chymotrypsin (1.4 mg./ml.) is treated at pH 5 with a tenfold molar excess (dissolved in 10% by volume of ethanol) of *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate (I), m.p. 149–150° (*Anal.* Calcd. for $C_{12}H_{13}O_5N_2Br$: C, 41.75; H, 3.80; N, 8.12. Found: C, 42.02; H, 3.80; N, 7.88), its activity toward tyrosine ethyl ester[3] slowly decreases: to 40% in 1 hr., to 22% in 3 hr. After overnight dialysis, the activity remains the same, which would be expected by analogy with pivalyl-chymotrypsin.[1] Unlike pivalyl-chymotrypsin, however, chymotrypsin inactivated by I does not regain activity on treatment with a pH 8 buffer containing glycerol.[2] Typical final activities are given in Table I.

TABLE I

INHIBITION OF CHYMOTRYPSIN BY I AT PH 5: EFFECT OF IPA[a] ON THE INHIBITION; PHOSPHORUS CONTENT OF INHIBITED CHYMOTRYPSINS AFTER REACTION WITH DFP

Ratio: I/CT	Ratio: IPA/CT	% Activity	Ratio: P/CT
10.0	0	30	...
10.0	5	35	...
10.0	25	42	...
10.0	100	60	...
0	...	100	1.04
2.5	...	77	...
5.0	...	39	1.05
10.0	...	23	1.04
20.0	...	22	1.03

[a] Abbreviations: IPA = 3-indolepropionic acid; DFP = diisopropylfluorophosphate; CT = chymotrypsin.

At pH 6, the decrease in activity takes place more rapidly to give about the same (30%) end activity, while at pH 7, after a quick drop in activity (to 40% in 1 min.), recovery occurs slowly (3 hr.) to give a product having about 75% activity.[4]

The irreversible inactivation occurs subsequent to a specific reaction at the active site since (a) the rate of the "burst" of nitrophenol during the formation of the acyl enzyme is retarded by the presence of the chymotrypsin inhibitor,[5] 3-indolepropionic acid, which inhibits the inactivation of the enzyme (Table I); (b) bromoacetyl-α-aminoisobutyric acid (II)[6] in a 100-fold molar excess does not cause any inactivation under the usual reaction conditions (4 hrs. at pH 5, overnight dialysis); and (c) methionine (see below) in diisopropylphosphoryl-chymotrypsin is not attacked by I (Table II). A random alkylation reaction is thus excluded.

Reincubation of inhibited chymotrypsin with the inhibitor I failed to reduce its activity further.

(3) G. W. Schwert and Y. Takenaka, *ibid.*, **16**, 570 (1955). The final percentage activity is roughly the same toward N-acetyl-L-tyrosine ethyl ester.

(4) The activities measured during the course of the reaction probably represent the combined activities of reversibly and irreversibly inhibited enzyme, while those measured after long standing (dialysis) are due to irreversibly inhibited chymotrypsin.

(5) H. Neurath, J. A. Gladner and G. De Maria, *J. Biol. Chem.*, **188**, 407 (1951).

(6) E. Abderhalden and E. Haase, *Fermentforschung*, **12**, 313 (1931).

The maximum degree of inactivation so far obtained has been 80 ± 5%, as measured in the standard assay.[3] To determine whether this represents a mixture of 20% of active chymotrypsin with 80% of totally inactive enzyme or an enzyme possessing 20% activity, samples of the inactivated enzyme were allowed to react with diisopropylfluorophosphate. Since it is known that during the complete inactivation of chymotrypsin by diisopropyl fluorophosphate,[7] one mole of diisopropyl phosphate per mole of enzyme[7] is bound to the serine hydroxyl group at the active site,[8] the phosphorus content of inhibited chymotrypsin, allowed to react with diisopropylfluorophosphate until completely inactive, is a measure of the proportion of enzyme molecules capable of reaction at the active site.[9] The phosphorylated proteins, which had less than 0.25% activity, contained[10] one mole of phosphorus per mole of enzyme (Table I).

Amino acid analyses[11] of chymotrypsin and of the irreversibly inactivated chymotrypsin show that one methionine residue in the latter is destroyed, and that the products (after hydrolysis) from it are the same as those obtained by Gundlach, Stein, and Moore (ref. 12, fig. 2c) with ribonuclease inactivated by iodoacetic acid at pH 2.8. In addition, roughly one mole of α-aminoisobutyric acid is found in the hydrolysate (Table II). No other change in amino acid composition has been detected.

TABLE II

METHIONINE, ITS DECOMPOSITION PRODUCTS AND α-AMINOISOBUTYRIC ACID IN HYDROLYSATES OF MODIFIED CHYMOTRYPSIN

Amino acid	Modified CT	CT	DIP-CT[a]
Methionine	1.19[b]	2.08[b]	2.10
S-Carboxymethylhomocysteine	0.5	...	*ca.* 0.01
Homoserine	0.1	...	0.00
Homoserine lactone	0.3	...	0.00
α-Aminoisobutyric acid	*ca.* 1.0[c]	...	trace

[a] The DIP (diisopropylphosphoryl)-CT, which had 0.25% activity, was allowed to react with 25 moles of I for 6 hr. [b] Average of three determinations. [c] This amino acid was difficult to determine with accuracy since it gives a low ninhydrin color yield and falls on one side of the cystine peak in chromatograms.[11]

We conclude that the activated bromine atom of the acyl enzyme alkylates a methionine residue in the vicinity of the active site, either while attached to the serine or shortly after hydrolysis of the acylserine bond. Since the liberated serine hydroxyl group is capable of reaction with diisopropylfluorophosphate, the active site is still capable of functioning, but much less efficient than it was before modification of the methionine residue near it. The inefficiency of the modified enzyme is attributable to a considerable increase (about 11-fold) in the Michaelis constant over that of native chymotrypsin.[13]

Our work corroborates the finding of Ray, *et al.*,[14] that destruction of methionine leads to the inactivation of chymotrypsin. It is unlikely that the sulfide group of the critical methionine behaves as a neighboring nucleophile at the active site of chymotrypsin (though this is entirely possible in the case of phosphoglucomutase[14]), since this activity should be abolished upon alkylation, which gives a ternary sulfonium salt,[12,15] or upon subsequent peptide cleavage.[15,16] The increase in the Michaelis constant indicates that modification of the critical methionine residue results in decreased affinity for substrate.

This general method for the modification of enzymes, as well as the complementary method of Baker, *et al.*,[17] should prove to be a good tool for the "mapping" of enzyme active sites.

Acknowledgments.—We are grateful to the National Institutes of Health for grants A-5299 Bio. and 2E-140 (C1) which helped to support this work.

(7) E. F. Jansen, M.-D. F. Nutting and A. K. Balls, *J. Biol. Chem.*, **179**, 201 (1949); A. K. Balls and E. F. Jansen, *Advances in Enzymol.*, **13**, 321 (1952).

(8) J. A. Cohen, R. A. Oosterbaan, H. S. Jansz and F. Berends, *J. Cell. Comp. Physiol.*, **54**, *Suppl.* 1, 231 (1959), and refs. cited therein.

(9) This method is analogous to the "all or none" assays of (a) W. J. Ray, Jr., J. J. Ruscica and D. E. Koshland, Jr., *J. Am. Chem. Soc.*, **82**, 4739 (1960), and (b) W. J. Ray, Jr. and D. E. Koshland, Jr., Abstracts of Papers, American Chemical Society Meeting, Washington, D. C., March 21–24, 1962, p. 41-C.

(10) "Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists," 6th Ed., A.O.A.C., Washington, D. C., 1945, p. 127.

(11) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(12) (a) H. G. Gundlach, W. H. Stein and S. Moore, *J. Biol. Chem.*, **234**, 1754 (1959); (b) H. G. Gundlach, S. Moore and W. H. Stein, *ibid.*, **234**, 1761 (1959).

(13) Procedure of L. W. Cunningham, Jr., *ibid.*, **207**, 443 (1954).

(14) W. J. Ray, Jr., H. G. Latham, Jr., M. Katsoulis and D. E. Koshland, Jr., *J. Am. Chem. Soc.*, **82**, 4743 (1960).

(15) W. B. Lawson, E. Gross, C. M. Foltz and B. Witkop, *ibid.*, **83**, 1509 (1961); E. Gross and B. Witkop, *ibid.*, **83**, 1510 (1961).

(16) Subsequent peptide cleavage at the carboxyl group of the methionine is likely to be slight under the conditions used (*cf.* ref. 15). This point, as well as the possibility of cleavage upon heating, is under investigation.

(17) B. R. Baker, W. W. Lee, E. Tong and L. O. Ross, *J. Am. Chem. Soc.*, **83**, 3713 (1961); *cf.* also G. Schoellmann and E. Shaw, *Biochem. Biophys. Res. Comm.*, **7**, 36 (1962); *Fed. Proc.*, **21**, 232 (1962).

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A STEREOSPECIFIC TOTAL SYNTHESIS OF 18-SUBSTITUTED STEROIDS. APPLICATION TO THE SYNTHESIS OF *dl*-CONESSINE

Sir:

This communication reports a total synthesis of 18-substituted steroids in which the B-C-D system is constructed following the lines laid in our previous steroid total synthesis,[1] but control of the C_{13} stereochemistry is achieved by the use of an 8-*iso* (*cis* B/C) structure which finally is inverted to the 8-normal (β) configuration. We illustrate the synthesis by building up the steroid alkaloid conessine,[2] but it will be obvious that a variety of 18-substituted 20-ketosteroids would be of considerably simpler access, *e.g.*, *via* IV.

(1) G. Stork, H. J. E. Loewenthal and P. C. Mukharji, *J. Am. Chem. Soc.*, **78**, 501 (1956).

(2) D. H. R. Barton and L. R. Morgan, *J. Chem. Soc.*, 622 (1962), have accomplished a partial synthesis of natural conessine from Δ⁵-pregnene-3β,20β-diol.