benzoyl-L-arginine and approaching competitive inhibition with increasing concentrations of this inhibitor.

In the mechanism described here, more than one enzyme-substrate intermediate is involved. It is because part of the substrate molecule (the alcohol portion) is split off during the course of the reaction that, in a later intermediate in the sequence, part of the active center of the enzyme (in this case the anionic site) is free of bound substrate. This intermediate can therefore combine with a substance resembling the portion of the substrate which has been split off. If this substance interacts in a specific way with a region of the catalytic site which takes part in the further reaction of the intermediate, it may act as a non-competitive inhibitor, as has already been shown.

Proposed mechanisms for the non-competitive inhibition of enzymes such as lactic dehydrogenase[13] also invoke a sequence of several enzyme substrate intermediates. Here it is believed that the substrate migrates from one site on the enzyme surface to another during the course of the reaction. It is therefore possible for an inhibitor to become attached to this second site at the same time as the substrate is bound to the first. In this way further reaction is blocked, since the substrate is not free to migrate to the second site.

It may be significant that in both of these mechanisms a reaction sequence involving several steps is an essential part of the explanation for the non-competitive behavior. It is possible that multiple intermediates are a general feature of non-competitive inhibition.

Acknowledgment.—The authors are indebted to the Defence Research Board of Canada for their support of this work under Grant 9510-06.

(13) P. Ottolenghi and O. F. Denstedt, *Can. J. Biochem. Physiol.*, **36**, 1093 (1958); R. M. Krupka and K. J. Laidler, *ibid.*, **38**, 1185 (1960).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF OTTAWA, OTTAWA, ONTARIO, CANADA]

Molecular Mechanisms for Hydrolytic Enzyme Action. IV. The Structure of the Active Center and the Reaction Mechanism

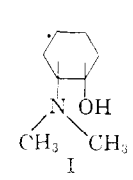
BY RICHARD M. KRUPKA AND KEITH J. LAIDLER

RECEIVED AUGUST 12, 1960

The kinetic results for acetylcholinesterase are briefly summarized, and on the basis of them a suggestion is made (Fig. 1) as to the structure of the active centre of the enzyme. Apart from the anionic site a similar structure is believed to exist in other hydrolytic enzymes. The way in which the substrate is held to the enzyme is deduced. A mechanism for acetylation is given, involving transfer of the acetyl group to the serine hydroxyl group. The acidic and basic sites are believed to play a role in this process, in which there is simultaneous making and breaking of several bonds. A mechanism for deacetylation is also proposed and involves both the acid and basic sites.

The enzyme acetylcholinesterase is a particularly convenient one for the purpose of arriving at a detailed reaction mechanism, owing to its possession of an anionic site. This interacts with cationic centers on substrates and inhibitors, and studies with molecules containing functional groups at known distances from these cationic centres have provided valuable evidence as to the positions of the active sites on the enzyme surface; the anionic centre may be used as an "origin" from which distances may be deduced. This mode of attack has been particularly exploited by Friess[1] and by Wilson.[2a] The deductions from their work combined with those in the preceding three papers[3] lead to a fairly clear-cut picture of the active center of the enzyme and of the way in which the various parts of the active center interact with the groups on substrates and inhibitor molecules. Our conclusions apply particularly to the acetylcholinesterase system, but there is considerable evidence, some of it referred to in Parts I–III of this series, that other enzymes behave in a very similar manner.

Structure of the Active Center.—The results described in the previous papers show that there are two distinct classes of inhibitors for hydrolytic enzymes; those of the first class block deacetylation, while those of the second do not. For the acetylcholinesterase system the inhibitors of the first class are exemplified by *cis*-2-dimethyl-aminocyclohexanol (I), while an example of the second class is prostigmine (II). All of the inhibitors investigated appear to become bound to the free enzyme and to the acyl-enzyme but not to the enzyme–substrate complex. A similar situation seems to exist with inhibitors for other hydrolytic enzymes, as exemplified by the discussion in Part III of the trypsin case. Kinetically the difference between the two



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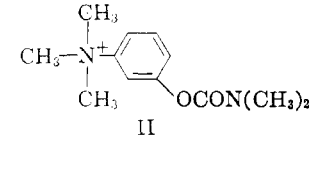

II

(1) (a) S. L. Friess and W. J. McCarville, THIS JOURNAL, **76**, 1303 (1954); (b) S. L. Friess, *ibid.*, **79**, 3269 (1957); (c) S. L. Friess and H. D. Baldridge, *ibid.*, **78**, 199 (1956); (d) D. S. Masterson, S. L. Friess and B. Witkop, *ibid.*, **80**, 5687 (1958); (e) S. L. Friess, E. R. Whitcomb, R. C. Durant and L. J. Weber, *Arch. Biochem. Biophys.*, **85**, 426 (1959).

(2) (a) I. B. Wilson and C. Quan, *ibid.*, **73**, 131 (1958). (b) That such a hydrogen bond occurs is strongly indicated by the results of Wilson and Quan[2a] who found that several compounds containing a hydroxyl group situated about 5 Å. from a tertiary nitrogen atom were very effective inhibitors. One fact that might appear to lead to the opposite conclusion is that the trimethylpropyl ammonium ion is as good an inhibitor as choline[4]; perhaps the end methyl group on the propyl radical interacts with some other site on the enzyme surface.

(3) R. M. Krupka and K. J. Laidler, THIS JOURNAL, **83**, 1445, 1448, 1454 (1961).

(4) I. B. Wilson, *J. Biol. Chem.*, **197**, 215 (1952).

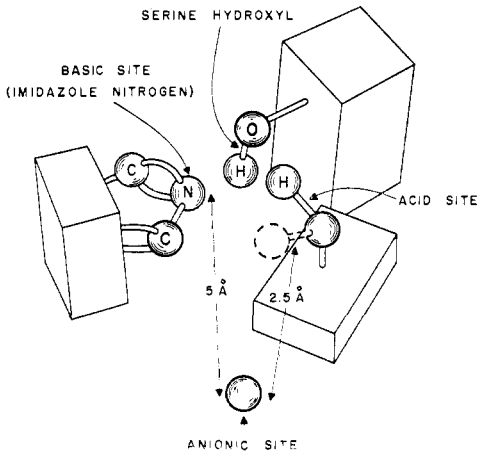

Fig. 1.—Proposed arrangement of functional groups at the active centre of acetylcholinesterase.

types of inhibitors is that those of class I show either non-competitive or mixed inhibition, whereas those of class II show pure competitive inhibition.

The inhibitor *cis*-2-dimethylaminocyclohexanol and its analogs have the common property of possessing, at about 2.5 Å. from the cationic center, a center of high electron density—the oxygen atom in the case of compound I. A reasonable inference is that when attachment to the enzyme occurs a hydrogen bond is formed between this site of high electron density and the hydrogen atom on the acidic site of the enzyme.

Compound II and its analogs (of which choline, carbachol and eserine are examples) contain a functional group at about 5 Å. from the cationic nitrogen atom. Some of them possess a carbonyl group which probably forms a weak electrostatic bond with the basic site on the enzyme, assumed to be an imidazole nitrogen atom. Inhibitors such as choline probably also form a hydrogen bond between the imidazole nitrogen atom and the hydrogen atom of the hydroxyl group.[2b]

The conclusion that the acidic site of acetylcholinesterase is situated about 2.5 Å. from the anionic site, and the basic site some 5 Å. away, is consistent with what would be deduced from the structure of the substrates for this enzyme. When these molecules are in their normal conformations, it is easy to see how there can be simultaneous interaction with the three sites, as will be considered in more detail later.

It remains to consider the relative position of the serine hydroxyl group, to which the acetyl group becomes transferred when the acetyl enzyme is formed from the Michaelis complex. It can be shown using models that in order for it to be possible for inhibitors such as *cis*-2-dimethylaminocyclohexanol to interact with both the anionic site and the acid site the serine hydroxyl must be somewhat further from the anionic site than is the basic site. For the transfer to occur smoothly the serine hydroxyl group must be raised slightly from the enzyme surface.

These considerations lead to the model for the active centre that is shown in Fig. 1. Several of the details of the structure will be discussed further in later sections. Enzymes such as trypsin and chymotrypsin probably have a very similar arrangement except that they lack the anionic site.

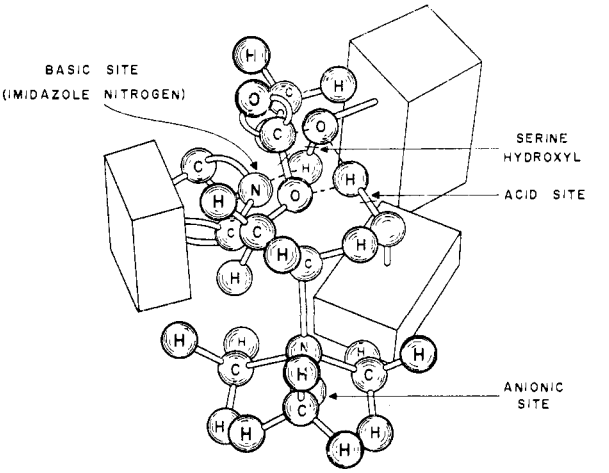

Fig. 2.—A suggested structure for the Michaelis complex between acetyl choline and the enzyme; the dotted lines indicate electrostatic attractions and also bonds that are formed during acetylation. There is also electrostatic attraction between the imidazole nitrogen atom and the carbonyl carbon atom. The three bonds broken are indicated on the diagram.

The Michaelis Complex and the Acetylation Process.—A representation of the Michaelis complex for the acetylcholinesterase–acetylcholine system is shown in Fig. 2. There is seen to be an electrostatic bond between the anionic site and the cationic nitrogen atom. The spatial arrangement in the neighborhood of the acidic and basic groups of the enzyme is such that there are electrostatic attractions between several pairs of atoms; some of these are indicated by dotted lines in the diagram. There is also some attraction between the partially negative imidazole nitrogen atom and the partially positive carbonyl carbon atom. Such interactions have frequently been written as involving the structure N^+—C–O^-, but Prof. Myron L. Bender has pointed out to us that such structures are energetically improbable; furthermore the spectroscopic evidence[5] has given no indication of such structures. In addition it is postulated that there is an electrostatic interaction between the hydrogen atom of the enzyme's acid group and the alcoholic oxygen atom, and the alcoholic oxygen atom of the serine and the carbonyl carbon atom. These various electrostatic interactions probably make the most important contributions to the binding between the enzyme and substrate, but undoubtedly other types of binding are involved at different parts of the molecules; for example, Wilson[6] has shown that the methyl groups of the ammonium ion are important.

It was found for this enzyme system that a proton cannot add on to the imidazole group in the enzyme–substrate complex[7]; a similar result was found by Stewart and Ouellet[8] for the trypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate.

(5) T. Spencer and T. M. Sturtevant, THIS JOURNAL, **81**, 1874 (1959).

(6) I. B. Wilson, *J. Biol. Chem.*, **197**, 215 (1952).

(7) R. M. Krupka and K. J. Laidler, *Trans. Faraday Soc.*, **56**, 1477 (1960).

(8) J. Stewart and L. Ouellet, *Can. J. Chem.*, **37**, 751 (1959).

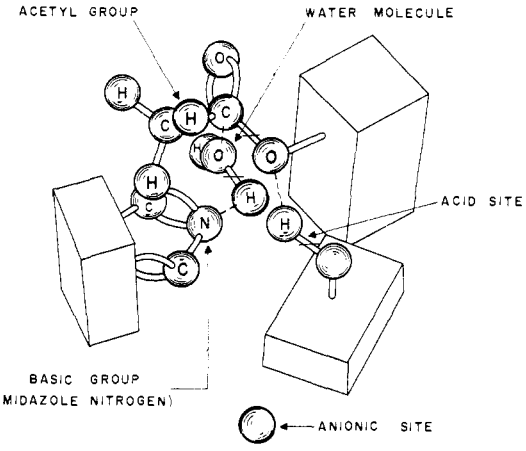

Fig. 3.—Proposed structure for the acetyl enzyme, showing the water molecule that is involved in the deacetylation reaction.

These results may be explained if, for steric reasons, the proton does not have access to the imidazole nitrogen atom.

The nature of the acetylation process is easily envisaged in the light of Fig. 2. New bonds are formed at the dotted lines, and bonds are broken as indicated on the diagram. It is to be noted that there is simultaneous rupture of three bonds and making of three new bonds. Such a process will be highly efficient provided that, as is the case, the atoms are initially held in the appropriate positions.

The Acetyl Enzyme, and the Deacetylation Process.—Figure 3 shows the proposed structure of the acetyl enzyme. Included in the figure is a water molecule arranged in the correct position for deacetylation to occur. The structure of the acetyl-enzyme is such that it is easy to see how inhibitors such as *cis*-2-dimethylaminocyclohexanol can become attached to it as strongly as to the free enzyme. Inhibitors of the second type, however, are too large to become attached other than at the anionic site.

The *p*H results[7] showed that both the acidic and basic groups (in their protonated and unprotonated forms, respectively) are involved in the deacetylation process; that the acidic group is involved is confirmed by the inhibition studies.[3] The role of the imidazole nitrogen atom is presumably to attract a proton from the water molecule, leaving the hydroxide ion available for attack on the carbonyl carbon atom. At the same time the hydrogen atom of the acid site may become transferred to the serine oxygen atom. The various bond breaking and bond making processes are assumed to occur simultaneously, and the process may again be expected to be a very efficient one.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, HARVARD MEDICAL SCHOOL, BOSTON 15, MASS.]

The Synthesis of Azaoxaspirane Steroid Alkaloids[1]

BY FREDERICK C. UHLE

RECEIVED MARCH 15, 1960

The transformations of kryptogenin and of diosgenin to solasodine, of neotigogenin to tomatidine and of sarsasapogenin to 5β-tomatidine, as well as to N-methyl-5β-tomatidine, are described. The conversion of pregnenolone to piperidine derivatives related in skeletal structure to the hydrogenolysis products of the alkaloids is reported.

Structural investigations in several laboratories culminated in provisional formulation of the natural products solasodine[2] and tomatidine[3] as ring F nitrogenous counterparts of the spiroketal sapogenins. The alkaloids merit close study both as members of the steroid family and as representatives of a mode of juncture not encountered elsewhere.

In a first synthetic approach, methods were sought for selective replacement of the 27-hydroxyl of kryptogenin by an amino group. Since no reagent capable of differentiating the primary alcoholic group of the side chain from the unusually reactive 3β-homoallylic secondary function of ring A was known, judicious choice of conditions, followed by fractionation of the expected mixture of products was considered most promising. After persistent experimentation, direct preparation of kryptogenin 27-*p*-toluenesulfonate (II) was achieved in 40% yield through 15 hours treatment with two equivalents of *p*-toluenesulfonyl chloride in anhydrous pyridine at 0°. Use of less, or of more, sulfonyl chloride diminished the yield.

A second, superior preparation of II was developed, however, through selective solvolysis[4] of the 3β,27-di-*p*-toluenesulfonate I, readily available in 90% yield. When a 0.04 molar aqueous acetone solution of I was heated under reflux for 2 hours, the 27-*p*-toluenesulfonate II remained in yields as high as 80%, corresponding to greater than 70% over-all transformation from the sapogenin.

Methanolysis of the 27-*p*-toluenesulfonate in the

(1) Announcements of introductory phases of this work were made in Communications to the Editor of *J. Am. Chem. Soc.*, **73**, 883 (1951); **75**, 2280 (1953); **76**, 4245 (1954); with J. A. Moore, *ibid.*, **76**, 6412 (1954). The expression "azaoxaspirane" has been recommended as a generic term to denote compounds in which appositely placed amino, hydroxyl and keto functions have entered into ketal-like spirane formation: F. C. Uhle and F. Sallmann, *J. Am. Chem. Soc.*, **82**, 1190 (1960).

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(4) *Cf.* the observed[5] and rationalized[6] rapid solvolysis of cholesteryl 3β-*p*-toluenesulfonate.

(5) W. Stoll, *Z. physiol. Chem.*, **207**, 47 (1932).

(6) S. Winstein and R. Adams, *J. Am. Chem. Soc.*, **70**, 838 (1948); R. M. Dodson and B. Riegel, *J. Org. Chem.*, **13**, 424 (1948).