# COMMUNICATIONS TO THE EDITOR

## THE REACTION OF *p*-NITROPHENYL ACETATE WITH CHYMOTRYPSIN[1]

*Sir:*

The reaction of NPA with chymotrypsin at *p*H 5.0 results in a stable monoacetyl derivative which is an intermediate in the catalytic hydrolysis of the ester at higher *p*H.[2] Phosphorylating agents (*e.g.*, DFP) have a similar effect and give rise to stable monophosphoryl enzyme derivatives, the hydroxyl group of a serine residue being the final point of attachment of the phosphoryl residue.[3,4,5]

The reaction of NPA with chymotrypsin is a two-phase process,[6] a rapid initial "burst" of *p*-nitrophenol being followed by a slow liberation until NPA is exhausted. At low temperature and *p*H, using rapid mixing techniques, the initial acetylation reaction in isolation has been followed in the Cary recording spectrophotometer and its kinetics have been found to correspond to those of a bimolecular reaction. At higher *p*H and temperature, the slow zero order reaction, which represents the turnover of acetylchymotrypsin (de-acetylation being rate-limiting), has been followed. The acetylation reaction, with a maximum at *p*H 8–9, occurs with a velocity approximately one hundred fold that of de-acetylation (maximal at *p*H 8.5–10). The energy of activation for the acetylation reaction at *p*H 6.0 was found to be 13,700 cal. per mole and for the de-acetylation at *p*H 7.5, 15,700 cal. per mole (as compared to 18,400 cal. per mole for the base catalyzed hydrolysis of NPA[6]).

The following series of experiments were undertaken to study the attachment of the acetyl group to the enzyme. A difference spectrum (600–230 mμ) between two portions of the same acetylchymotrypsin solution, one of which had been allowed to de-acetylate at *p*H 8.0, 25°, showed that the two proteins were spectrally identical. Since a histidine side chain has been implicated in the action of proteolytic enzymes[7,8] and since the properties of acetyl-imidazole are known to include a characteristic ultraviolet absorption with a peak at 245 mμ,[7] the changes at 245 mμ were carefully followed during the acetylation reaction. The quantity of enzyme was chosen to provide an easily measurable change at 245 mμ if acetylimidazole were formed, but no such change was observed.

In contrast to acetyl esters such as ethyl acetate, the acetyl group in monoacetyl chymotrypsin is reactive toward hydroxylamine at *p*H 5.5 with the formation of one equivalent of hydroxamic acid.[2] However, when acetyl-α-chymotrypsin was denatured (reversibly) in 8 *M* urea at *p*H 3.0 and then reacted with hydroxylamine at *p*H 5.5, no formation of hydroxamate could be detected. (NPA reacted normally with hydroxylamine in the presence of 8 *M* urea.) The acetyl group remained bound to the protein, however, and when the urea was diluted out (with 0.001 *N* HCl at 0°), regained its normal properties. However, if chymotrypsin is initially denatured in 8 *M* urea, acetylation by NPA does not occur. These observations focus attention upon the modifying influence of the environment in the native protein upon the reactivity of amino-acid side chains. When the "reactive" side chain in the native state is first acetylated and the acetyl enzyme is then denatured, the modifying influence of the protein configuration is again in evidence, the acetyl group being (reversibly) transformed from a state in the native protein in which it is reactive toward hydroxylamine to an unreactive state in the denatured protein. It is clear, therefore, that the properties of this "specially reactive" group in the protein cannot be interpreted in terms of the known properties of the amino-acid side chains in isolation, the reactivity of the group in question being functionally related to the specific structure of the native protein. This unusual reactivity has also been demonstrated by the observation that at *p*H 5, the same group in chymotrypsin is rapidly acylated by acetic, propionic or butyric anhydride. It is proposed that the successive acylation and deacylation of this uniquely reactive group constitutes an important feature of the mechanism of hydrolysis of NPA by chymotrypsin.

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(1) *p*-Nitrophenyl acetate will be abbreviated to NPA, and diisopropyl phosphofluoridate to DFP. This work was performed under Contract No. Nonr-477-04 between the University of Washington and the Office of Naval Research, Department of the Navy, and was supported also by funds made available by the people of the State of Washington, Initiative 171. Our thanks are due to Miss Dorothy Kauffman for technical assistance. Details of this work will be given in a paper now in preparation.

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(4) R. A. Oosterbaan, P. Kunst, and J. A. Cohen, *Biochim. Biophys. Acta*, **16**, 299 (1955).

(5) G. H. Dixon, S. Go and H. Neurath, *ibid.*, **19**, 193 (1956).

(6) B. S. Hartley and P. Kilby, *Biochem. J.*, **50**, 672 (1952).

(7) E. Stadtmann, "Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, Md., 1954, p. 581.

(8) H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441 (1955).

## POLYPEPTIDES. X. CONFIGURATIONAL AND STEREOCHEMICAL EFFECTS IN THE AMINE-INITIATED POLYMERIZATION OF N-CARBOXY-ANHYDRIDES

*Sir:*

The usual formulation of the amine-initiated polymerization of N-carboxy-α-amino-acid anhydrides predicts a narrow molecular weight distribution provided the propagation rate is fast compared to initiation.[1] Recent work on the poly-

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