EVIDENCE FOR A NEW INTERMEDIATE IN THE CHYMOTRYPSIN CATALYZED HYDROLYSIS OF *p*-NITROPHENYL ACETATE

Sir:

We wish to report that monoacetyl-α-chymotrypsin (AC-A)[1] formed at *p*H 5.0 and isolated by the procedure of Balls and Wood,[2] is a new and previously unrecognized intermediate in the catalytic hydrolysis of NPA. This observation is of considerable significance since it explains two apparently contradictory observations reported previously.[3-5] The kinetic studies of Gutfreund and Sturtevant[3,4] suggest that the acyl enzyme formed at a *p*H greater than 6.5 (AC-I) is deacylated directly. In contrast, Dixon and Neurath[5] concluded from spectroscopic studies that the deacylation reaction involves initially an acyl migration from AC-I to an imidazole nitrogen. The spectroscopic studies,[5] however, were made with AC-A.

The experiments illustrated in Fig. 1 indicate that monoacetyl-α-chymotrypsin exists in at least two stable forms. Curve 1 represents CT,[6] curve 2 represents CT preacetylated for 10 minutes with 100 equivalents of NPA at *p*H 5.0 (AC-II) and curve 3 represents the isolated form of AC-II (AC-III). At *p*H 7.0, zero order steady state liberation of NP was observed within 50 seconds with CT and within 15 seconds with AC-II and AC-III. Curve 4 corresponds to an experiment in which AC-A was mixed with *p*H 7.0 buffer and NPA at zero time. A small burst was observed initially because the enzyme was incompletely acylated. In contrast to the previous experiments (curves 1–3), zero order liberation of NP is not observed until after 350 seconds at *p*H 7.0 when the reaction proceeds essentially with the same rate observed in curves 1–3. The results observed in this experiment are not brought about by the isolation procedure. When CT was allowed to react with NPA at *p*H 5.0 for 4 hours and 15° and then was mixed with buffer at zero time to bring the *p*H to 7.0, curve 5 is obtained which appears identical to the progress curve obtained by completely acylated AC-A.

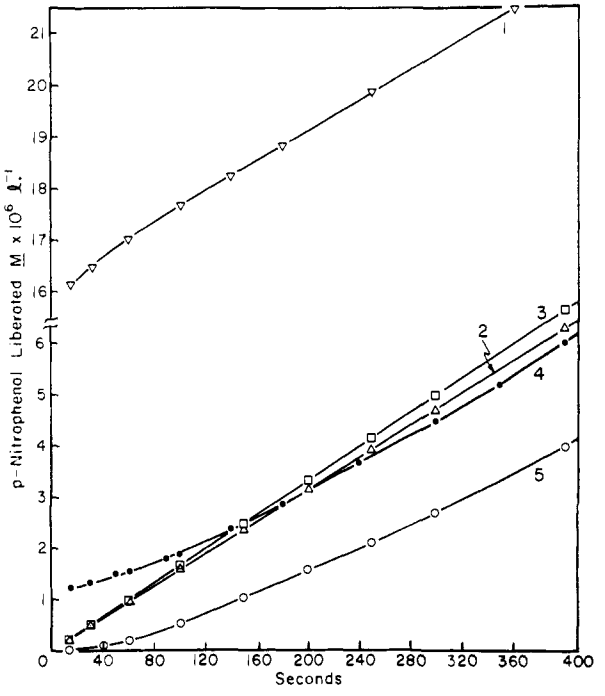

Fig. 1.—Liberation of *p*-nitrophenol in the catalytic hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin and monoacetyl-α-chymotrypsin preparations at 15.6° in tris-(hydroxymethyl)-aminomethane-maleate buffer, *p*H 7.0. Total ionic strength 0.12 *M*; $[E]_0 = 1.6 \times 10^{-6}$ *M*; $[S]_0 = 1.7 \times 10^{-3}$ *M*: Curve 1, chymotrypsin (CT); curve 2, chymotrypsin preacetylated for 10 minutes with 100 equivalents of NPA at *p*H 5.0 and 15.6° and then mixed with buffer at zero time to bring the *p*H to 7.0 (AC-II); curve 3, AC-III; curve 4, incompletely acylated AC-A[2]; curve 5, chymotrypsin preacetylated for 4 hours with 100 equivalents of NPA at *p*H 5.0 and 15.6° and then mixed with buffer at zero time to bring the *p*H to 7.0.

Two further experiments are cited as evidence that at least two stable forms of monoacetyl α-chymotrypsin exist. (1) At *p*H 8.0, a significant change in absorption at 245 mμ accompanies the hydrolysis of AC-A[5] but not of AC-III. (2) At *p*H 5.5 and 23°, 8 *M* urea abolishes the reactivity of the acetyl group of AC-III toward hydroxylamine within two minutes, while AC-A is still fully reactive after two minutes and unreactive after ten minutes of denaturation.

The deviation from zero order kinetics observed in NPA catalysis by AC-A and the spectroscopic changes accompanying its deacylation are interpreted as reflecting the reactions involved in the conversion of AC-A to the form of acyl enzyme predominating at *p*H 7.0 (AC-I). AC-I, AC-II, and AC-III appear kinetically identical. AC-A, however, is a different intermediate, an observation of considerable importance for an interpretation of the mechanism of the α-chymotrypsin catalyzed hydrolysis of NPA.

(1) These abbreviations are used: AC, monoacetyl α-chymotrypsin; CT, α-chymotrypsin; NPA, *p*-nitrophenyl acetate; NP, *p*-nitrophenol.

(2) A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956).

(3) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

(4) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci.*, **42**, 719 (1956).

(5) G. H. Dixon and H. Neurath, THIS JOURNAL, **79**, 4558 (1957).

(6) Crystalline α-chymotrypsin, purchased from Worthington Biochemicals, Freehold, New Jersey, was used.

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(8) Post-doctoral Fellow, National Heart Institute, United States Public Health Service.

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION[7]
CORNELL UNIVERSITY MARIO A. MARINI[8]
ITHACA, NEW YORK GEORGE P. HESS

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A DEOXYCYTIDINE TRIPHOSPHATE SPLITTING ENZYME AND THE SYNTHESIS OF THE DEOXYRIBOSENUCLEIC ACID OF T2 BACTERIOPHAGE[1]

The DNA of T2 bacteriophage contains 5-hydroxymethylcytosine and its glucosyl derivative in place of the cytosine present in the DNA of

(1) This work has been supported by a grant-in-aid from the National Cancer Institute, National Institutes of Health, the United States Public Health Service. These abbreviations are used: deoxycytidine triphosphate, dCTP; deoxycytidine monophosphate, dCMP; deoxy-5-hydroxymethylcytidine triphosphate, dHTP; deoxy-5-hydroxymethylcytidine monophosphate, dHMP; thymidine triphosphate, dTTP; deoxyribosenucleic acid, DNA.