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.

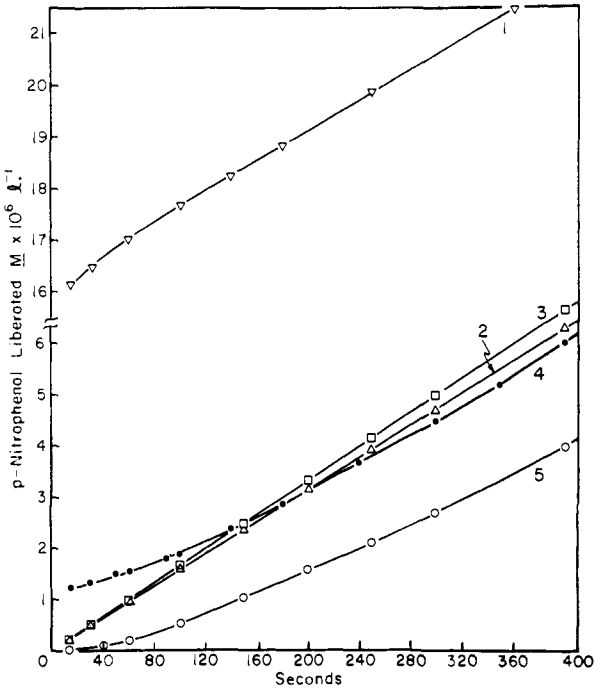


Fig. 1.—Liberation of *p*-nitrophenol in the catalytic hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin and monoacetyl-α-chymotrypsin preparations at 15.6° in tris-(hydroxymethyl)-aminomethane-maleate buffer, *p*H 7.0. Total ionic strength 0.12 *M*; $[E]_0 = 1.6 \times 10^{-6}$ *M*; $[S]_0 = 1.7 \times 10^{-3}$ *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.

(7) This work was aided by grants from the United States Public Health Service and the National Science Foundation.

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

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

RECEIVED DECEMBER 18, 1958

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.

the host, *Escherichia coli*.[2a,b,c] A brief description[3] has been made of some enzymatic events pertaining to the synthesis of DNA in T2 infected *E. coli*. When C^{14} labeled dTTP was incubated with primer DNA and an enzyme system isolated from uninfected cells, radioactive DNA was formed only in the presence of deoxyadenosine triphosphate, deoxyguanosine triphosphate and dCTP[4] as has been demonstrated previously by Bessman, *et al.*[5] We have reported that dHTP will replace dCTP in the system containing the enzyme prepared from uninfected *E. coli* cells. However, with extracts from *E. coli* cells infected with T2 bacteriophage, synthesis of radioactive DNA is observed in the presence of dHTP but *not* when dCTP replaces dHTP. This led to the finding that dCTP is specifically destroyed by an enzyme produced in the infected cell.[3]

We wish to report further details of this system. The dCTP degrading enzyme and the DNA polymerase have been separated by chromatography on a column of hydroxylapatite. The dCTP degrading enzyme fraction, free from interfering phosphatases, catalyzes the reaction

$$\text{dCTP} \longrightarrow \text{dCMP} + \text{inorganic pyrophosphate}$$

Evidence for this reaction is based on the demonstration that one mole of inorganic pyrophosphate is produced for each mole of dCMP formed. This enzyme does not act upon dTTP, deoxycytidine diphosphate, or the ribosyl analog of dCTP, cytidine triphosphate. The appearance of this new enzymatic activity takes place shortly after infection of the cells with T2 bacteriophage, and approximately 50% of the maximum level observed is attained seven minutes after infection of a culture at 37°.

The purified DNA polymerase of infected cells (freed from the dCTP splitting enzyme by the chromatographic procedure and of 30-fold purity compared to the crude extract) utilizes dCTP or dHTP to an equal extent at similar concentrations. At this level of purification it is indistinguishable from the polymerase of uninfected cells by the criterion of substrate specificity.

When dCTP or dHTP was replaced with deoxy-5-glucosylhydroxymethylcytidine triphosphate, there was either little or no synthesis of labeled DNA from C^{14}-dTTP and the other substrates in the presence of either the crude or purified polymerase.[6] It is therefore probable that this glucosyl compound is not an intermediate in the formation of the glucosylated DNA of T2 bacteriophage.

This dCTP degrading enzyme may have a dual function in the promotion of the synthesis of phage DNA. First it degrades dCTP to a form which is not utilized as a substrate for the DNA polymerase and, secondly, it provides dCMP which is known to be the substrate for the synthesis of deoxy-5-hydroxymethylcytidine monophosphate by the reaction of Flaks and Cohen.[7] Furthermore, the production of the dCTP degrading enzyme may be one of the mechanisms responsible for the exclusion of phages other than the T-even variety when *E. coli* cells undergo mixed infection with T2 phage and a phage which contains cytosine in its DNA.[8]

(2) (a) G. R. Wyatt and S. S. Cohen, *Biochem. J.*, **55**, 774 (1953); (b) R. L. Sinsheimer, *Science*, **120**, 551 (1954); (c) E. Volkin, THIS JOURNAL, **76**, 5892 (1954).

(3) J. F. Koerner and M. S. Smith, *Federation Proc.*, in press.

(4) dCTP and deoxyadenosine triphosphate were prepared from the corresponding nucleotides by the method of L. I. Hecht, V. R. Potter, and E. Herbert, *Biochim. et Biophys. Acta*, **15**, 134 (1954). dHTP, deoxy-5-glucosylhydroxymethylcytidine triphosphate, dTTP, and deoxyguanosine triphosphate were prepared by the procedure of V. M. Clark, G. W. Kirby and A. Todd, *J. Chem. Soc.*, 1497 (1957).

(5) M. J. Bessman, I. R. Lehman, E. S. Simms and A. Kornberg, *J. Biol. Chem.*, **233**, 171 (1958).

(6) Experiments carried out in collaboration with Dr. John S. Wiberg.

(7) J. G. Flaks and S. S. Cohen, *Biochim. et Biophys. Acta*, **25**, 667 (1957).

(8) M. Delbrück and S. E. Luria, *Arch. of Biochem.*, **1**, 111 (1942).

(9) Postdoctoral Fellow of the National Cancer Institute, United States Public Health Service, 1956–58.

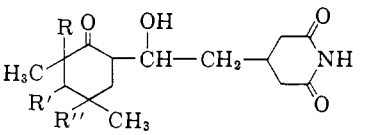
DIVISION OF BIOCHEMISTRY JAMES F. KOERNER[9]
DEPARTMENT OF BIOLOGY MARILYN S. SMITH
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
CAMBRIDGE 39, MASSACHUSETTS JOHN M. BUCHANAN

RECEIVED MARCH 6, 1959

STRUCTURES OF THE STREPTOVITACINS

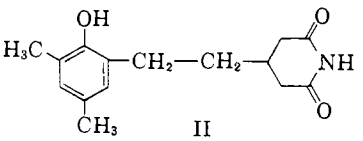
Sir:

The streptovitacins, a group of closely related compounds isolated from a *Streptomyces griseus* fermentation, have been found to have broad-spectrum antitumor activity.[1–6] The chemical evidence described below, coupled with preliminary studies reported elsewhere,[3,7] permit structural assignments (Ia-c) to several components of the complex.



Ia, R = R′ = H; R″ = OH
Ib, R = R″ = H; R′ = OH
Ic, R′ = R″ = H; R = OH

That streptovitacins A and B possess the cycloheximide[8] (I, R = R′ = R″ = H) type structure was shown[7] by (i) spectral comparisons, (ii) the present of an imide system (NMR), (iii) acid catalyzed dehydration to II, identical with material derived



II

from cycloheximide, and (iv) facile alkali-catalyzed retroaldol degradation to six-membered ring ketones. Although certain of the early experiments

(1) J. B. Field, F. Costa and A. Boryczka, "Antibiotics Annual 1958–1959," Medical Encyclopedia, Inc., New York, N. Y., 1959, in press.

(2) W. T. Sokolski, N. J. Eilers and G. M. Savage, *ibid.*, in press.

(3) T. E. Eble, M. E. Bergy, C. M. Large, R. R. Herr and W. G. Jackson, *ibid.*, in press.

(4) J. S. Evans, G. D. Mengel, J. Ceru and R. L. Johnston, *ibid.*, in press.

(5) J. B. Field, A. Mireles, H. R. Pachl, L. Bascoy, L. Cano and W. K. Bullock, *ibid.*, in press.

(6) J. S. Evans, G. D. Mengel and J. Ceru, *Proc. Soc. Exper. Biol. Med.*, in press.

(7) R. R. Herr, "Antibiotics Annual 1958–1959," Medical Encyclopedia, Inc., New York, New York, 1959, in press.

(8) The trade name of The Upjohn Company for cycloheximide is Acti-dione.