## The autolysis of trypsin in the presence of urea

It is known[1] that urea affects the rate of autolysis of trypsin. Knowledge of the autolysis in urea, however, has hitherto been based on the measurement of the absorbancy at 2800 Å of the fraction soluble in dilute trichloroacetic acid. It occurred to us that one could study in some detail the extent of the autolysis of trypsin in urea by reacting the N-terminals directly in the incubation mixture providing that urea would not interfere with the N-terminal analysis. Previous reports of the dinitrophenylation of proteins in the presence of urea have provided limited[2] or no[3] evidence concerning whether or not urea interferes with the reaction between DNFB and the terminal amino groups.

To test the feasibility of this approach, dinitrophenylation of a mixture of several amino acids was carried out in the presence of a large excess of DNFB in water and in 8 *M* urea. The same amounts of DNP-amino acids were recovered in each case in spite of the fact that the urea and the DNFB formed a complex[4]. Similarly, dinitrophenylation of porcine $\gamma$-globulin (Pentex, lot 2007) yielded the same amounts of several N-terminals whether the reaction with DNFB was carried out in water or in various concentrations of urea[4]. Thus, the dinitrophenylation of proteins in the presence of urea proves advantageous. The protein is actually reacted in the conformation brought about by the action of urea, the removal of which (a cumbersome and time-consuming operation[5]) prior to dinitrophenylation would permit the protein to undergo many structural modifications.

Dinitrophenylation of trypsin in aqueous media furnished three products: A, B and C. The main fraction A was the DNP-protein. Fractions B and C were mixtures respectively of acidic and basic DNP-peptides.

Reagent-grade urea, recrystallized from aqueous ethanol, and commercial crystalline salt-free trypsin (Worthington, 2 times cryst., lot TR 685 SF) were used. Protein solutions of 1 mg/ml in glass-distilled water or in unsalted, unbuffered 2, 4, 6, 8 *M* urea were prepared by dilution of dialysed 3 % trypsin in 0.01 *N* HCl. After standing for 6 h at 27° in the dark at pH 7, the reaction mixtures were shaken with DNFB at pH 9 and processed in the conventional way[6] for the recovery of the DNP-protein (Fraction A). Evaporation of the alcohol washings of A yielded Fraction B. The combined acidic yellow supernatants and water washings of A, after exhaustive extraction with ether, were sent through an acidic talcum-celite column to remove inorganic salts and urea; the yellow bands were then eluted with 1 % HCl in acetone. Evaporation of the acetonic solvent yielded Fraction C. Both B and C were chromatographed on paper by means of *tert.* amyl alcohol saturated with 3 % aq. ammonia and were thereby resolved into several DNP-peptides. The study of these DNP-peptides is part of the work in progress.

Products A, B and C were hydrolysed in glass-distilled 5.7 *N* HCl in sealed tubes for 16 h or longer at 108°. The ether and water extracts of the hydrolysates were resolved on paper by the *tert.* amyl alcohol-phthalate system[7], and the DNP-amino acids and DNP-peptides were measured in 1 % $NaHCO_3$ at 3600 Å. The quantities found indicated the extent of autolysis.

Our results are in line with a previous observation[1] concerning the effect of urea upon the rate of autolysis of trypsin. We have found that intermediate concentrations

Abbreviations: DNFB, 2,4-dinitrofluorobenzene; DNP, dinitrophenyl.

of 2 to 4 *M* urea increased (up to 50 % for some N-terminals) whereas concentrations of 6 to 8 *M* urea depressed (to nearly zero for some N-terminals) the extent of the autolysis of trypsin. The ε-DNP-lysine was present in all the Fractions A, B, and C. In Fraction A its amount increased linearly with the concentration of urea above 2 *M*. In Fractions B and C, however, the ε-DNP-lysine had a maximum in 2 *M* urea and the lowest value in 8 *M* urea just like the α-N-terminals; and in B the amount of ε-DNP-lysine was several times as large as in C. These overall results indicate that autolysis was maximum in 2 *M* urea and least in 8 *M* urea. In 2 *M* urea a large number of peptides were split off the protein molecule and ended up in Fractions B and C. In 8 *M* urea the unfolding of the trypsin molecule and the unmasking of the ε-lysine were most extensive.

Of great interest was the observation that the extent of autolysis paralleled the amount of flocculation of the protein in the incubation mixture at pH 7, *i.e.* flocculation was maximum in 2 *M* urea and absent in 8 *M* urea. Whether flocculation was caused by autolysis, or whether flocculated material was more easily autolysed, it is not yet known. The role of urea in these phenomena is being investigated. It was also observed that if the protein was directly suspended in 8 *M* urea small specks of material remained undissolved during the entire incubation period. Fractions B and C were then several times as large (indicating more hydrolysis) as in the case in which the protein was dissolved in 0.01 *N* HCl and dialysed prior to incubation in the 8 *M* urea. It is hoped that experiments now in progress will provide an explanation of these findings.

GIUSEPPE COLACICCO
CHARLES R. DAWSON

*Department of Chemistry, Columbia University, New York, N.Y. (U.S.A.)*

[1] T. VISWANATHA AND I. E. LIENER, *J. Biol. Chem.*, 215 (1955) 777.
[2] F. S. STEVEN AND G. R. TRISTRAM, *Biochem. J.*, 70 (1958) 179.
[3] H. FRAENKEL-CONRAT AND B. SINGER, *Arch. Biochem. Biophys.*, 65 (1956) 296.
[4] G. COLACICCO, Thesis, Columbia University, 1959.
[5] C. W. CATER AND W. E. F. NAISMITH, *Arch. Biochem. Biophys.*, 77 (1958) 98.
[6] F. SANGER, *Biochem. J.*, 39 (1945) 507.
[7] S. BLACKBURN AND A. G. LOWTHER, *Biochem. J.*, 48 (1951) 126.

Received May 19th, 1959

## A mammalian and protozoan electron-transport inhibitor in *Tetrahymena pyriformis*

An electron-transport inhibitor has been found in cell-free preparations of the protozoan *Tetrahymena pyriformis* which affects enzyme systems of *Tetrahymena* and rat liver mitochondria. The inhibitor is "activated" on mild heating or aging, it is resistant to boiling after activation but is not produced in boiled fresh preparations, and it is non-dialyzable.

Cultures were grown in 2.0 % proteose-peptone–0.2 % yeast extract medium. The cells were harvested after 3–6 days as described[1] and passed twice through a

Abbreviation: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide.