A rapid and sensitive method for the determination of chymotrypsin and trypsin activity

Previous studies[1,2] in this laboratory have indicated the presence of several enzymes in extracts of rat-skin acetone powder with activity toward esters of amino acids and their acylated derivatives fulfilling the specific requirements for chymotrypsin substrates. In order to increase the sensitivity of our assay procedures to the point where rapid rate determinations could be made by direct spectrophotometric observation of the reaction mixture, the possibility of using a chromogenic substrate was investigated. For the detection of chymotrypsin or of chymotrypsin-type activity, a substrate containing the relatively specific amino acid residue, tyrosine, esterified with *p*-nitrophenol (*p*NP) was thought likely to fulfill the necessary criteria since the liberation of *p*NP as the nitrophenolate ion at pH 8 would provide sufficient sensitivity for rate measurements to be made using small amounts of enzyme. Accordingly, the test compound, *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (CT*p*NP), was synthesized by coupling *p*NP with the mixed anhydride of carbobenzoxy-L-tyrosine and ethylchloroformate.

In the enzymic studies, CT*p*NP was dissolved in anhydrous acetone to give a 0.001 *M* solution and the reaction solutions contained 1.00 ml 0.12 *M* $CaCl_2$, 1.00 ml 0.20 *M* tris (hydroxymethyl) aminomethane buffer (adjusted to pH 8.0 with HCl), 0.25 ml methanol, 0.20 ml enzyme (in 0.12 *M* $CaCl_2$), 0.10 ml CT*p*NP solution, and water to 3.00 ml. Spontaneous hydrolysis was reduced to a minimum by the addition of CT*p*NP at zero time, with rapid mixing, to the otherwise complete reaction solution immediately after enzyme addition. Rate measurements were performed in 1 cm quartz cuvettes contained in a thermostated (30°) compartment of a Beckman Model DU spectrophotometer. All rate determinations were corrected for the spontaneous hydrolysis of CT*p*NP and appropriate corrections applied, where necessary, for the pH-dependence of the molar extinction coefficient of *p*NP. Liberation of *p*NP was followed at 400 mμ. Initial reaction velocities were determined from the slopes of optical density *versus* time plots extrapolated to zero time and expressed as the change in optical density/sec or as moles *p*NP liberated/l/sec. Initial substrate concentrations, a_0, were obtained from optical-density measurements, at 400 mμ, of reaction solutions at the time of complete CT*p*NP hydrolysis which, by reference to an appropriate standard curve, could be related to *p*NP concentration. The molar concentration of *p*NP liberated at infinite time was equivalent to the molar concentration of added CT*p*NP. Enzyme concentrations were calculated from optical-density readings at 280 mμ using the factors given by DREYER, WADE, AND NEURATH[3] for chymotrypsin and by GREEN AND NEURATH[4] for trypsin.

The addition of chymotrypsin to reaction solutions containing CT*p*NP resulted in a rapid increase in optical density (Fig. 1, A). Velocity was proportional to chymotrypsin concentration (Fig. 1, B) and the optimum pH was at approximately 8 (Fig. 1, C).

It would appear that, since chymotrypsin treated with diisopropylphosphorofluoridate was inactive toward CT*p*NP, the hydrolysis of this substrate was accomplished in the area of the same active site as concerned with the catalysis of other susceptible substrates[5].

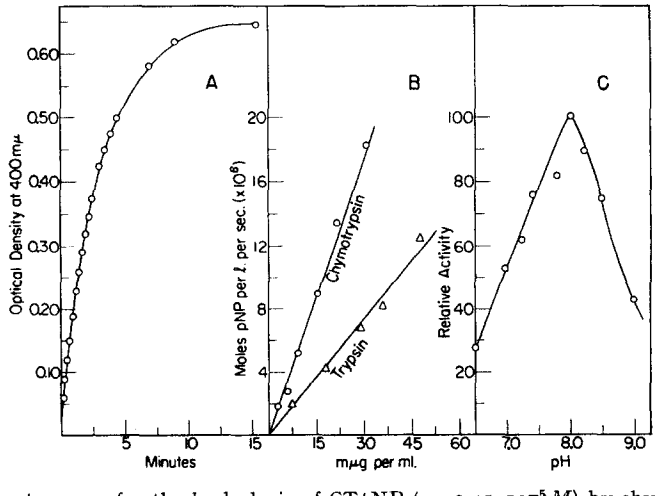

Fig. 1, A. The rate curve for the hydrolysis of CT*p*NP (a_0, $3.45 \cdot 10^{-5}$ *M*) by chymotrypsin (24.5 mμg/ml). B. The relation between velocity of CT*p*NP hydrolysis and enzyme concentration. C. The chymotrypsin-catalyzed hydrolysis of CT*p*NP as a function of pH.

Although cross-reactivity of chymotrypsin and trypsin to certain substrates is not without parallel, it was not anticipated that mμg amounts of trypsin would necessarily produce a rapid hydrolysis of CT*p*NP. However, the rate curve obtained with trypsin was comparable to that observed with chymotrypsin and velocity was proportional to the trypsin concentration (Fig. 1, B). The maximum rate of hydrolysis occurred at about pH 8. As was found for chymotrypsin, tryptic activity toward CT*p*NP was completely inhibited by diiso propylphosphorofluoridate.

Thus, the use of CT*p*NP permits direct and rapid measurements of reaction rates utilizing quantities of chymotrypsin as low as 3 mμg/ml and amounts of trypsin as low as 7 mμg/ml.

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The dissociation of glutamic dehydrogenase by reduced diphosphopyridine nucleotide (DPNH)

In 1952, OLSON AND ANFINSEN[1] showed that crystalline glutamic dehydrogenase exhibited an anomalous sedimentation behavior in the ultracentrifuge. The authors attributed this behavior to either dissociation or unfolding of the enzyme at low concentrations. It has now been shown that dissociation of the enzyme may be obtained by the addition of a substrate for the enzyme, DPNH.

Crystalline glutamic dehydrogenase was prepared from beef liver by the method of STRECKER[2] and recrystallized twice with saturated sodium sulfate at 5°[1]. At pH 7.4 in 0.05 *M* phosphate buffer, the enzyme shows a single peak in the ultracentrifuge. In all experiments, the Spinco Model E analytic ultracentrifuge was used*. The pattern, shown in Fig. 1a, has a hypersharp leading boundary, and is slightly skewed on the trailing edge, similar to that obtained by OLSON AND ANFINSEN. The addition of DPNH to this enzyme solution causes the single peak to split into two distinct peaks as shown in Fig. 1b. In this particular experiment, the molar ratio of DPNH to enzyme was approximately 200:1. Increasing amounts of DPNH decrease the amount

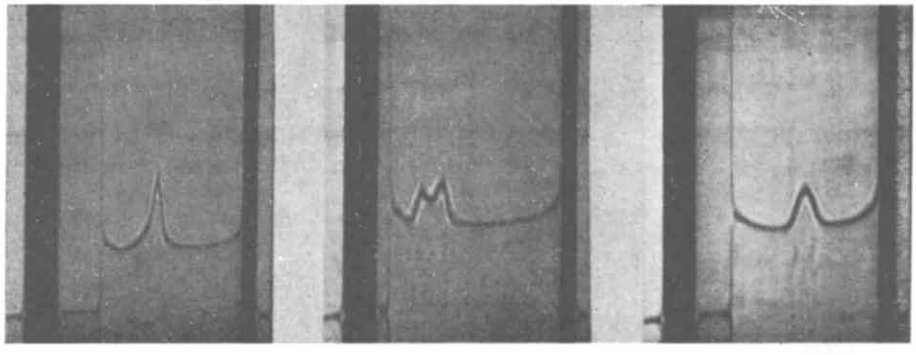

Fig. 1. Sedimentation patterns of glutamic dehydrogenase in 0.05 *M* phosphate buffer, pH 7.4 at 59,780 r.p.m. (a) alone after 660 sec, (b) in the presence of $1.3 \cdot 10^{-3}$ *M* DPNH after 660 sec, (c) in the presence of $5 \cdot 10^{-3}$ *M* *o*-phenanthroline after 1590 sec. The protein concentration was approximately 4.5 mg/ml. Sedimentation is from left to right.

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