REACTION OF TRYPSIN WITH ORGANIC PHOSPHATE INHIBITORS

by

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It has been found that the proteolytic activity of trypsin can be inhibited completely by certain *p*-nitrophenyl phosphate esters, and that this process is accompanied by the liberation of *p*-nitrophenol.

Trypsin solutions were prepared in 0.05 *M* borate buffer, pH 7.6, containing 0.025 *M* $CaCl_2$. Crystalline trypsin was used. The proteolytic activity of the solutions was estimated by ANSON's haemoglobin method[1], and the molarity of active trypsin calculated assuming NORTHROP, KUNITZ AND HERRIOTT's[2] value of (0.17 T.U.) $\frac{\text{Hb}}{\text{mg. P.N.}}$ for pure enzyme. The phosphate esters were dissolved in *iso*propanol and diluted 10-fold with the borate-$CaCl_2$ solution immediately before use. Equal volumes of enzyme and inhibitor solution were mixed and incubated at 25°, and the nitrophenol liberated was estimated at intervals by measurement of the absorption at 4000 A using a quartz spectrophotometer. A similar solution without trypsin was incubated in parallel and used as the control in the optical density measurement, so that compensation was automatically made for the nitrophenol liberated by spontaneous hydrolysis of the ester. The calcium ions present stabilized the trypsin[3] and negligible self-inactivation occurred under the experimental conditions used.

Results with two esters are summarised below.

I. *Diethyl* p-*nitrophenyl phosphate*, $(EtO)_2(p\text{-}NO_2.C_6H_4O)P{:}O$ (also known as E600 and paraoxon)

Trypsin concentrations in the incubated solution were varied between $2.7 \cdot 10^{-6}$ and $9.3 \cdot 10^{-6}$ *M* and the ester concentrations between 10^{-2} and 10^{-3} *M*. The ester was in considerable excess and its concentration remained effectively constant during the incubation. Nitrophenol liberation followed pseudo-unimolecular kinetics and reached a constant and maximal value in 4 to 48 hrs depending on concentrations. The ratio of the number of molecules of nitrophenol liberated to number of molecules of enzyme inhibited was found to be 2.16 ± 0.15 (mean of 26 expts.). After incubation, the enzyme was completely inhibited.

II. *O,S-diethyl O*-p-*nitrophenyl thiophosphate*, (EtO) (EtS) $(p\text{-}NO_2.C_6H_4O)P{:}O$

Trypsin concentrations varied between $9.3 \cdot 10^{-6}$ and $1.2 \cdot 10^{-5}$ *M* and the ester concentrations between $5 \cdot 10^{-4}$ and 10^{-3} *M*. This ester was more reactive and complete inhibition and nitrophenol liberation were effected within 1–3 hrs. The molecules nitrophenol: molecules enzyme ratio found was 2.01 ± 0.15 (mean of 21 expts.).

In the above calculations, the most generally accepted value of 36,500 for the M.W. of trypsin has been used. Lower values down to 34,000 have been reported, and a value of 34,900 would give a ratio of exactly 2 from our data.

HARTLEY AND KILBY[4] have shown that when chymotrypsin is inhibited by E600, one molecule of nitrophenol is liberated for each molecule of enzyme inhibited, while JANSEN, NUTTING AND BALLS[5] have shown that chymotrypsin inhibited by di*iso*propyl fluorophosphonate (D.F.P.) contains one equivalent of phosphorus. By analogy, the overall reaction of trypsin with E600 or analogues may be written:

$$\text{Trypsin} + 2\ (EtO)_2PO(NO_2.C_6H_4O) \longrightarrow \text{Trypsin} - [(EtO)_2PO]_2 + 2\ NO_2.C_6H_4.OH \quad . \ . \ . \ . \quad (1)$$

Measurements were made of the rate of liberation of nitrophenol from the system trypsin-E600, using excess E600 and working in the range in which the system followed first-order kinetics. The velocity constants were found to be proportional to the concentration of E600 and not to its square, thus eliminating a termolecular reaction as in the overall equation (1). The observed results might be explained either by postulating a single active centre in the trypsin molecule which reacts initially with one molecule of inhibitor in a slow rate-controlling reaction, followed by a fast reaction with

a second molecule of inhibitor; or by postulating the presence of two active centres which react independently, *i.e.*, trypsin behaves as if its molecular weight was half the accepted value.

Confirmatory and complementary evidence for an equation of the type (1) has been found independently by JANSEN and co-workers[6] who have shown that trypsin is inhibited by D.F.P. and that the amount of phosphorus in the inhibited crystalline trypsin product corresponds to an equivalent weight of 20,000.

A fuller account of this work will be published later.

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BEMERKUNG

ZU DER ARBEIT VON ANNEMARIE WEBER UND HANS H. WEBER,

*Zur Thermodynamik der Kontraktion des Fasermodells**

Der Freundlichkeit von Herrn K. H. MEYER (Genf) verdanken wir die Auskunft, dass die vielfältig und auch von uns als WIEGAND-SNYDER-Gleichung bezeichnete Gleichung schon vorher von GERKE[1] und in verwandter Art von LEBLANC UND KRÖGER[2] abgeleitet wurde.

Ferner hält es Herr K. H. MEYER für möglich, dass die gemeinsame Zitierung von E. WÖHLISCH und K. H. MEYER als Autoren der thermokinetischen Theorie der Elastizität zu Missverständnissen führen könnte. Um solche zu vermeiden, sei ergänzt: Die thermokinetische Theorie als solche stammt von WÖHLISCH[3]. WÖHLISCH aber dachte sich — entsprechend dem damaligen Stand der Faserstoff-Chemie — diese Ketten als Ketten, deren in sich starre Einzelglieder ganze Moleküle, Mizellen oder Kristallite seien. Während WÖHLISCH die Molekularstruktur der Ketten nur andeutend behandelt hatte, baute K. H. MEYER[4] die Theorie gerade nach der Seite der molekularen Struktur aus durch die Einführung der in sich flexiblen Fadenmoleküle. K. H. MEYER gab der Theorie damit die Form, in der sie heute fast allgemein diskutiert wird.

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LITERATUR

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