ratio of hyaluronidase/hexosaminidase activities was found to increase to a value approximately the same as that found for a commercial chromatographically purified testicular hyaluronidase (Worthington).

During chromatography on DEAE-cellulose (Fig. 3) the active material from testicular hyaluronidase was more weakly adsorbed than that from serum. Comparing this with the findings on CM-cellulose it appears that the active protein from serum has a lower positive charge under this range of conditions than the active protein from ovine testicular preparations. On the assumption that the same charge distribution is involved in the binding of enzyme to negatively charged hyaluronate molecules this would fit in with the finding that the serum fractions possess optimum activity around pH 3.7, whereas testicular material is more active at pH 5.0.

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A convenient titrant for α-chymotrypsin and trypsin

In order to determine the absolute concentration of active sites in a given enzyme solution, one requires a stoichiometric reaction which can be observed simply and accurately. Ideally, the titrant should react with active enzyme rapidly and irreversibly under conditions as close as possible to those of the kinetic determination for which the knowledge of enzyme concentration is required. Most of the reagents available for the enzymes trypsin and α-chymotrypsin[1,2] suffer from one or more of the following disadvantages: (i) dependence upon enzyme concentration, or pH, or reagent concentration; (ii) operation at pH values outside the normal range of kinetic experiments or in solutions containing organic solvents; (iii) lack of sensitivity (*i.e.*, requiring very concentrated enzyme solutions); or (iv) difficult and lengthy synthesis.

Thus for routine use, the ideal titrant is simple to prepare, and gives accurate and reproducible results under a variety of conditions such that it can be used directly for the assay of enzyme stock solutions. For hydrolytic enzymes, *p*-nitrophenyl esters are attractive candidates for the following reasons: (i) the large extinction coefficient of the *p*-nitrophenate anion makes for a method applicable to stock enzyme solutions of 10^{-5} M or lower, at pH values above 7 so frequently used for kinetic work; (ii) the ratio of the rate constants for acylation and deacylation (the k_2/k_3 ratio) is large[1,3]; and (iii) K_m is relatively small (so that the second kinetic requirement for the titrant, that $[S_0]/K_m$ is large[1], is readily satisfied). The principal disadvantages encountered with *p*-nitrophenyl esters are the high blank rates of hydrolysis, and the low water solubility. To maximise the rate of reaction with the enzyme, *p*-nitrophenyl esters of amino acids specific for the enzyme in question have been used[1,2], but these often suffer from a high 'blank' rate and/or a high turnover rate (k_3) at least above pH 7, resulting in a loss of accuracy in the extrapolation of the steady-state reaction to time zero (and possibly the use of stopped-flow equipment).

We report here the synthesis and use of the *p*-nitrophenyl ester of *p*-(*N*,*N*,*N*-trimethylammonium) cinnamic acid iodide, an analogue of the common chymotrypsin titrant *N*-cinnamoylimidazole[4]. This material is an ester of cinnamic acid, and as such has a much lower rate constant for hydroxide ion-promoted hydrolysis than *p*-nitrophenyl esters of amino acids, while still being a good acylating reagent for the enzymes. Moreover, the *p*-(*N*,*N*,*N*-trimethylammonium) group in the cinnamoyl moiety increases the water solubility to a level which enables the titration to be performed in the absence of organic solvents. The compound is simple to prepare, and is equally useful for trypsin and chymotrypsin. As will be apparent from its structure, it was designed as a complementary reagent to the unsubstituted compound (*p*-nitrophenylcinnamate), and it was hoped that whereas the unsubstituted material is fairly suitable for chymotrypsin, the *p*-(*N*,*N*,*N*-trimethylammonium) compound would be trypsin-specific. In the event, the substituted compound turns out to have a more favourable (*i.e.* larger[1]) k_2/k_3 ratio for each enzyme than does the parent cinnamoyl derivative.

The reagent was synthesised as follows. *p*-(*N*,*N*-Dimethylamino) cinnamic acid[5] (21 g, 0.11 mole) was dissolved in dimethylsulphoxide (60 ml). Methyl iodide (60 ml, 0.5 mole) was added, and the mixture refluxed for 30 min. The volume of the mixture was reduced to about 30 ml by distillation under reduced pressure, and on cooling, crystals of the trimethylammonium derivative separated. These were recrystallised twice from methanol and once from water, and had m.p. 208°. *p*-(*N*,*N*,*N*-trimethylammonium) cinnamic acid iodide (3.3 g, 0.01 mole) and triethylamine (1 g, 0.01 mole) were dissolved in chloroform (40 ml). To this solution was added ethylchloroformate (1.1 g, 0.01 mole), and the mixture was stirred. After 10 min, *p*-nitrophenol (1.4 g, 0.01 mole) was added and the mixture boiled for 2 min. After standing overnight at room temperature, the mixture was filtered, and the filtrate evaporated to dryness. The resulting solid was dissolved in the minimum of hot dimethylformamide, and after cooling, a volume of dry ether about 20% that of the dimethylformamide, was added. After standing for 1 h, the solid (triethylamine hydrochloride) was filtered off. A further volume of dry ether (approximately equal to the volume of dimethylformamide) was added and the mixture kept at 0° overnight. The resulting solid was recrystallised from dimethylformamide–ether to provide pale yellow crystals, m.p. approx. 190°

(decomp.). (Found: C, 47.2; H, 5.1; I, 27.8; N, 6.1. $C_{18}H_{19}IN_2O_4$ requires C, 47.6; H, 4.2; I, 28.0; N, 6.2%.)

Assays of enzyme were normally performed between pH 7 and 8, the appearance of *p*-nitrophenate ion being followed spectrophotometrically. The final pH of the solution must be determined accurately, and the actual *p*-nitrophenol concentration calculated from the extinction coefficient of the *p*-nitrophenate ion at 400 mμ of 18 320 and the pK_a of 7.04 (see ref. 4). Commonly, the sample cuvette contained 3.0 ml of 0.1 M phosphate buffer, 100 μl of 5 mM reagent solution in 1 mM HCl or acetone, and 0.5 ml of enzyme solution (40 μM–1.0 mM in water). The blank rate of hydrolysis of the reagent is negligible under these conditions, but can in any case be allowed for by pipetting the same amount of reagent into the reference cuvette (if a double-beam spectrophotometer is used). The concentration of enzyme in the solution is determined by extrapolating the spectrometer trace back to time zero, and measuring the initial burst. For chymotrypsin, the burst is complete in <3 sec at pH values between 7 and 8 and the deacylation rate is negligible, while for trypsin about 10 min is required for the observation of an adequate portion of the steady-state reaction. The values of enzyme concentration obtained by this method are independent of pH (the method can also be used—with some loss of sensitivity—at low pH, by following the change in the difference spectrum of the ester and the acid *plus* *p*-nitrophenol at 340 mμ (see ref. 1)), and independent of reagent concentration. This shows that both the ratios $[S_0]/K_m$ and k_2/k_3 are high enough for the initial burst of *p*-nitrophenol to be equal to the concentration of enzyme. Under the conditions outlined, α-chymotrypsin (3× crystallised, freeze dried, salt free, from Seravac Laboratories) was found to be 81±3% active on a weight basis (assuming a molecular weight of 25 000), and trypsin (3× crystallised, salt free, from Seravac Laboratories) was found to be 49.5±2 % active on a weight basis (assuming a molecular weight of 24 500). These values agree well with those obtained by other, often more laborious, methods[1].

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