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Unsuitability of Indoxyl Acetate as a Substrate for the Assay of Testicular Hyaluronidase

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The most recently published method for the assay of testicular hyaluronidase preparations was based on the premise that the enzyme also exhibited carboxylesterase activity towards indoxyl acetate. Studies on the relative enzyme activities of various hyaluronidase preparations towards hyaluronate and indoxyl acetate, the relative stabilities towards pH, temperature and mechanical shaking and the behaviour towards a variety of inhibitors, showed that the activities towards the two substrates reflected the presence of at least two different enzyme systems in the preparations. Gel chromatography and polyacrylamide-gel-electrophoresis experiments confirmed these conclusions and the collective findings clearly establish that methods based on the use of indoxyl acetate cannot be employed to measure testicular hyaluronidase activity.

Mammalian hyaluronidases (hyaluronate glycanohydrolase, EC 3.2.1.35) are endo-glycosaminidases acting on the β-(1→4)-*N*-acetylglucosaminide bonds of hyaluronic acid, chondroitin 4-sulphate and chondroitin 6-sulphate. Under certain circumstances the enzymes also exhibit transglycosylation properties. The most widely studied enzyme has been that from testes. However, Bollet, Bonner & Nance (1963) have shown that hyaluronidase activity also occurs in many other mammalian tissues including lung, kidney, spleen and synovial fluid. The activity present in young rat bones (Vaes & Jacques, 1965), rat liver (Aronson & Davidson, 1967; Hutterer, 1966) and canine submandibular gland (Tan & Bowness, 1968) is lysosomal in origin. The properties of enzyme from lysosomal sources are slightly different from those of the testicular enzyme and this affords a means of distinguishing the two.

Assay procedures for hyaluronidase activity depend upon either physical or chemical changes in the macromolecular structure of hyaluronate (see Tolksdorf, 1954). However, all these methods are either time-consuming or lack accuracy and there is a great need for a new rapid and sensitive procedure. These advantages are claimed for a spectrofluorimetric method developed by Guilbault, Kramer & Hackley (1967). The method is based on the claim that testicular hyaluronidase also exhibits esterase activity towards indoxyl acetate. Hydrolysis of this ester under certain conditions gives rise to the strongly fluorescing Indigo White. This method was tested against relatively impure commercial preparations of testicular hyaluronidase and the possibility that contaminating (and possibly unusual) esterases are responsible for the effect obviously needs to be excluded with certainty. It has been noted by Barrett (1969) 'that a finding so unexpected as that of a carboxylesterase activity in an endoglycosidase will demand careful verification'. The criteria for verification adapted by Guilbault and his colleagues (1967) was based on inhibition studies.

The present work demonstrates that indoxyl acetate cannot be used to assay testicular hyaluronidase and establishes unequivocally that the hydrolysis of that compound by hyaluronidase preparations is due to one or more contaminating esterases. A preliminary account of some of the findings has already been made (Rhodes, Olavesen & Dodgson, 1970).

MATERIALS AND METHODS

Enzyme preparations. Commercial preparations of bovine testicular hyaluronidase (activities in the range 300–550 i.u./mg, see Humphrey, 1957) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., Koch–Light

Laboratories Ltd., Colnbrook, Bucks., U.K. and BDH Chemicals Ltd., Poole, Dorset, U.K. Enzyme preparations of much greater purity (activity ranges, 20000–38000i.u.) were prepared in the AB Leo Laboratories. Cholinesterase (Type IV, from horse serum) was obtained from Sigma Chemical Co.

Enzyme substrates and inhibitors. Sodium hyaluronate (Grade III-S) and indoxyl acetate were purchased from Sigma Chemical Co. and Koch–Light Laboratories Ltd., respectively. Di-isopropyl phosphorofluoridate was kindly supplied by Dr H. J. Smith of the Welsh School of Pharmacy, Cardiff, U.K. and paraoxon (diethyl *p*-nitrophenyl phosphate) was a gift from Dr W. N. Aldridge of the Medical Research Council Toxicology Research Unit, Carshalton, Surrey, U.K. Polyphloretin phosphate was prepared in the AB Leo Laboratories.

Measurement of hyaluronidase activity. Three different procedures were employed.

Method 1. This was based on the determination of the *N*-acetylglucosamine reducing end groups liberated from hyaluronate by the enzyme. The method was essentially that of Aronson & Davidson (1967), the liberated hexosamine being determined by the procedure of Reissig, Strominger & Leloir (1955). The enzyme was incubated at 37°C for 15min in the presence of 0.1M-sodium acetate–acetic acid buffer, pH5.4, to which had been added sufficient NaCl to give a final concentration of 0.15M and 1mg of hyaluronate/ml. The total volume of the incubation mixture was 0.5ml. The reaction was terminated by placing the incubation tubes in an ice bath and adding 0.1ml of 0.7M-potassium tetraborate. Suitable control determinations were made in which enzyme and substrate were incubated separately and mixed immediately before adding the potassium tetraborate. On some occasions boiled enzyme–substrate mixtures served as controls. A linear relationship between liberated *N*-acetylglucosamine and hyaluronidase concentration holds over a limited concentration range only. When necessary, enzyme solutions were diluted so that not more than 0.01 hexosamine unit of enzyme activity was present in the incubation mixture. A hexosamine unit of enzyme activity is defined as 1μmol of *N*-acetylglucosamine liberated from sodium hyaluronate/min per mg of protein. One such unit is approximately equivalent to 9600i.u. Method 1, although less sensitive than method 2, can be employed over a wide range of experimental conditions and was the method of choice.

Method 2. In this method reducing material liberated from hyaluronate was determined by the method of Park & Johnson (1949) as adapted for hyaluronidase assay by Rapport, Meyer & Linker (1950). A reducing unit of enzyme activity is defined as 1μmol of reducing material (glucose is the standard) liberated from hyaluronate/min per mg of protein. One such unit is approximately equivalent to 5000i.u. This method was employed in certain experiments (specified in the text) where greater sensitivity was required.

Method 3. This was essentially the spectrofluorimetric method claimed by Guilbault *et al.* (1967) to be applicable to the assay of hyaluronidase. The modifications involved the use of 2.8ml (rather than 2.9ml) of buffer and the Hitachi–Perkin Elmer MPF-2A spectrofluorimeter fitted with a constant-temperature attachment. The wavelengths of excitation and emission of Indigo White, the product of enzyme action, are at 396 and 470nm respectively and the spectrofluorimeter was set at these wavelengths with slit widths of 5 and 10nm and a recorder sensitivity setting of 1.

Indigo White is an unstable compound and it is not possible to prepare samples that are suitable for the standardization of the method. Standardization was achieved by allowing cholinesterase to hydrolyse a fixed amount of indoxyl acetate to completion. In practice 0.03μmol of the ester was treated in this way (incubation volume of 3ml) after preliminary experiments had indicated the amount of cholinesterase required. With the instrument settings described above a recorder deflexion of 100 was obtained. Since it was inconvenient to repeat the standardization procedure for each experiment advantage was taken of the fact that a 1.0μM solution of quinine sulphate gave a recorder deflexion of 40 at its own particular excitation (350nm) and emission (450nm) maxima. Subsequently such a solution was routinely employed to standardize the instrument. The enzyme activity measured by this method is hereafter referred to as esterase activity and 1 esterase unit is equivalent to 1μmol of indoxyl acetate hydrolysed/min per mg of protein. It should be noted that these units cannot be related directly to the units of the other methods because of the different incubation temperature employed. However, when esterase activity was measured at 37°C an approximately twofold increase in activity was observed (cf. Fig. 3). This temperature was not used as a routine because of the relatively high substrate blanks that were obtained.

Determination of protein. This was measured with the Hilger Uvispek spectrophotometer at a wavelength of 280nm (1cm light-path). A number of hyaluronidase preparations (Koch–Light) (1mg/ml) gave an extinction of 0.96 and this was then selected as a standard value to which other readings were referred.

Column chromatography. Gel filtration on Sephadex G-200 at 2°C was carried out according to manufacturer's instructions [Pharmacia (G.B.) Ltd., London W.13, U.K.]. An elution buffer of 0.1M-sodium acetate–acetic acid, pH6.0, to which had been added sufficient NaCl to give a final concentration of 0.15M, was employed in all runs.

Electrophoresis. Polyacrylamide-gel electrophoresis of hyaluronidase preparations (Koch–Light and AB Leo) (38000i.u./mg) was carried out according to the manufacturer's instructions in the Shandon disc-electrophoresis apparatus (Shandon Scientific Co. Ltd., London N.W.10, U.K.) employing a 7.5% gel concentration and pH4.3. Enzyme samples (2mg/ml) were dissolved in the reservoir buffer that also contained 10% (w/v) sucrose and portions (50μl) were layered under the running buffer on to each gel. Electrophoresis was at 4mA per tube for 1.5h. From each group of 4 tubes one gel was stained for protein with a solution of Amido Black (in 7%, v/v, acetic acid) and one was incubated at room temperature in 2.9ml of McIlvaine buffer (0.1M, pH6.4; see Gomori, 1955) to which had been added 0.1ml of an 83mM solution of indoxyl acetate in dioxan and sufficient NaCl to give a final concentration of 0.15M. The Indigo White resulting from esterase action was oxidized slowly to Indigo Blue which precipitated as a band at the site of esterase activity. Both the remaining gels were sliced into 2mm sections which were each triturated with 1ml of 0.1M-sodium acetate–acetic acid

buffer, pH 5.4, to which had been added sufficient NaCl to give a final concentration of 0.15M, and left overnight at 2°C to allow any enzyme present to be extracted. Hyaluronidase and esterase activities of samples of the resulting extracts were then determined.

Glassware. Testicular hyaluronidase is readily adsorbed on to glass surfaces (Rasmussen, 1954) and wherever possible glass containers, columns etc. were avoided and polypropylene, polyacrylic or silicone-treated equipment was employed.

RESULTS

Relative enzyme activities of different hyaluronidase preparations. Enzyme activities towards indoxyl acetate and sodium hyaluronate were determined for various commercial hyaluronidase preparations. All preparations tested contained both enzyme activities but the ratios of the two activities varied considerably and, maximally, by a factor of about 45 (Table 1). These results immediately established that indoxyl acetate could not be employed directly as a substrate for the assay of hyaluronidase preparations. However, they did not entirely exclude the possibility that hyaluronidase was capable of exhibiting carboxylesterase activity and further investigations were therefore made. Two particular preparations (Koch–Light and AB Leo, 38000i.u. preparation) were selected for detailed study.

Effect of pH *on enzyme activities and stabilities.* Under the assay conditions described earlier, maximum hyaluronidase activity for both preparations was obtained at pH 5.4 and maximum esterase activity for both preparations at pH 6.4. The relative stabilities of the enzyme activities towards pH are shown in Fig. 1. In these experiments enzyme solutions of the same concentration (180 μg/ml) were maintained at 0°C for 5min at pH values

Table 1. *Relative activities of various testicular hyaluronidase preparations towards sodium hyaluronate and indoxyl acetate*

Hyaluronidase activity was measured by method 1.

Source of enzyme preparation	Hyaluronidase activity (μmol/min per mg)	Esterase activity (μmol/min per mg)	Ratio of esterase/hyaluronidase activities
Koch–Light Laboratories Ltd.	0.033	0.034	1.0
Sigma Chemical Co.	0.045	0.059	1.3
BDH Chemicals Ltd.	0.056	0.026	0.47
AB Leo Laboratories			
Prep. 1	2.16	1.20	0.56
Prep. 2	2.92	0.95	0.32
Prep. 3	3.88	0.12	0.03

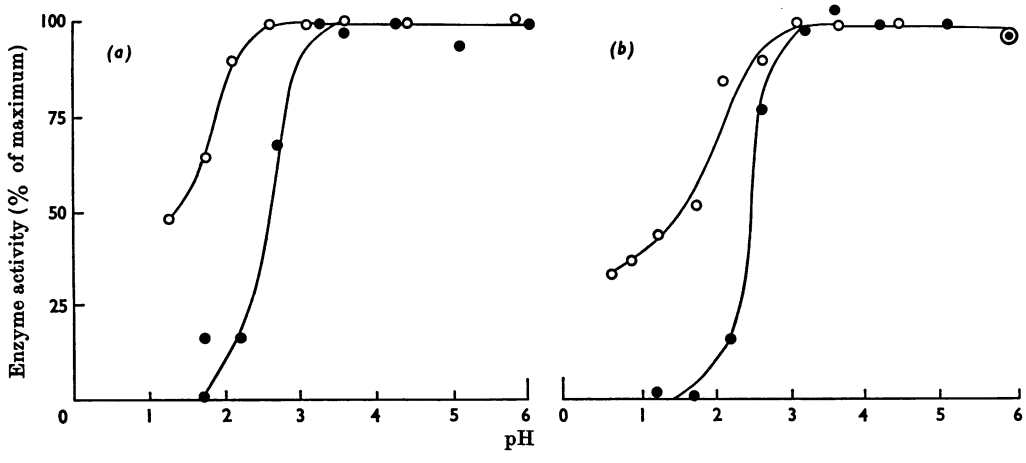

Fig. 1. Effect of pH on the stabilities of hyaluronidase (○) and esterase (●) activities of testicular hyaluronidase preparations. (*a*) AB Leo preparation; (*b*) Koch–Light preparation. Hyaluronidase activity was measured by method 1.

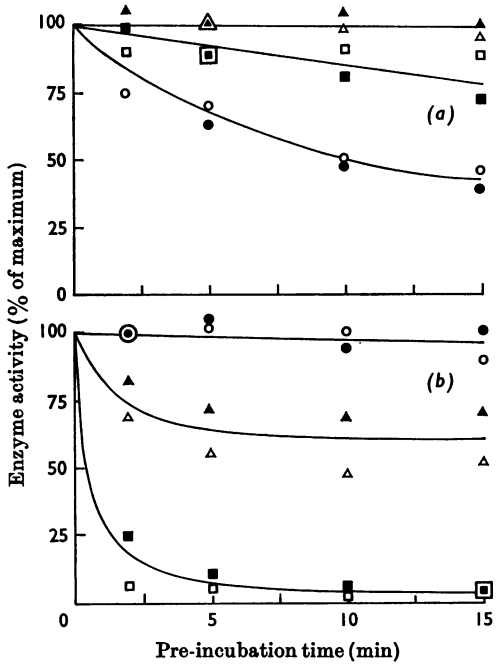

Fig. 2. pH-stability–time curves for hyaluronidase and esterase activities of testicular hyaluronidase preparations. ○, □, △, AB Leo preparations; ●, ■, ▲, Koch–Light preparations. (*a*) Hyaluronidase activities, ○ and ●, pH1.8; □ and ■, pH2.2; △ and ▲, pH2.7. (*b*) Esterase activities, □ and ■, pH2.2; △ and ▲, pH2.7; ○ and ●, pH3.2. Hyaluronidase activity was measured by method 1.

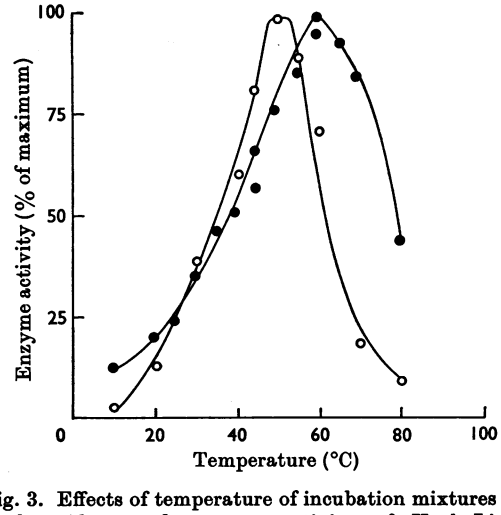

Fig. 3. Effects of temperature of incubation mixtures on hyaluronidase and esterase activity of Koch–Light testicular hyaluronidase. ○, Hyaluronidase activity (measured by method 1); ●, esterase activity.

ranging from 1 to 10. At the end of this period the pH of each solution was rapidly adjusted to approx. pH5.5 and portions were then withdrawn and assayed for hyaluronidase and esterase activities. Marked differences in stability at pH below 3.0 were observed (Fig. 1) and this effect was confirmed in separate experiments in which the rates of inactivation were examined at a few selected pH values (Fig. 2).

Effect of temperature on enzyme activities and stabilities. The effects of incubation temperature on the enzyme activities of the Koch–Light preparation are presented in Fig. 3. Enzyme concentrations of 0.2 and 0.03mg/ml were selected for the assay of hyaluronidase and esterase activities respectively. The former activity was maximal at 50°C and the latter at 60°C. Temperature–inactivation curves for both Koch–Light and AB Leo preparations are presented in Fig. 4. In these experiments enzyme solutions (1mg/ml in 0.1M-sodium acetate–acetic acid buffer, pH6.0, containing NaCl to 0.15M) were incubated at different temperatures for 5min before cooling in an ice bath. Portions were then taken, diluted appropriately and assayed with methods 2 and 3. Again significant differences in stability were noted between the two types of enzyme activity. Further experiments in which the rates of inactivation were studied at certain selected temperatures also revealed significant differences between hyaluronidase and esterase activities (Fig. 5).

Effect of mechanical shaking on enzyme stabilities. An aqueous solution (5mg in 50ml) of the Koch–Light preparation was shaken (Griffin and George wrist-action shaker set at slowest speed) in a 250ml glass flask and samples were withdrawn for assay (Methods 2 and 3) at various times up to 4h. Activity towards hyaluronate decreased virtually to zero in this time whereas esterase activity was unaffected (Fig. 6).

Effect of inhibitors on enzyme activities. Guilbault *et al.* (1967) reported that the hydrolysis of indoxyl acetate by hyaluronidase preparations was strongly inhibited by the heavy metal ions Cu^{2+} and Fe^{3+}, and by CN^-. Both heavy metals were known to act as inhibitors of hyaluronidase (Meyer & Rapport, 1951) and this was taken by Guilbault's group as further evidence for the common identity of the two activities. Table 2 shows that both esterase and hyaluronidase activities of the AB Leo and Koch–Light preparations exhibited very similar behaviour towards the two metal inhibitors. However, CN^- ion inhibited the hydrolysis of indoxyl acetate but was without effect on the hyaluronidase activity at the concentrations tested. Polyphloretin phosphate, a potent inhibitor of several enzymes including hyaluronidase (Diczfalusy *et al.* 1953) was only a relatively poor inhibitor of esterase activity

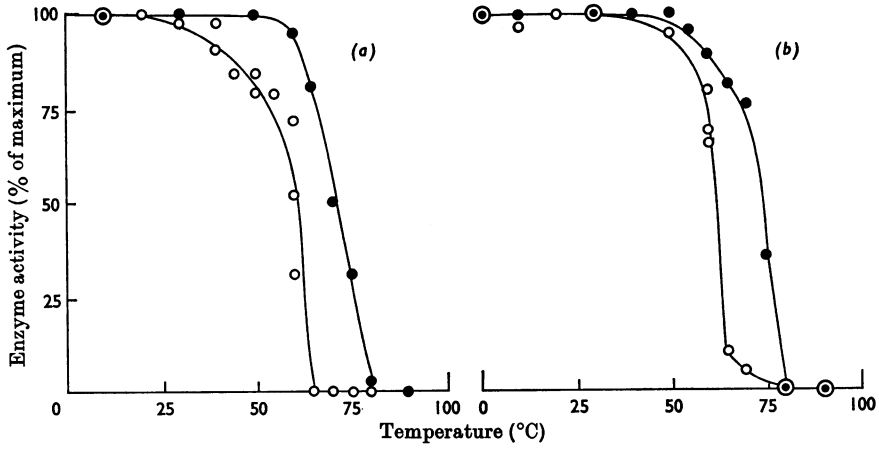

Fig. 4. Effect of pre-incubation temperature on hyaluronidase (○) and esterase (●) activities of testicular hyaluronidase preparations. (*a*) AB Leo preparation; (*b*) Koch–Light preparation. Hyaluronidase activity was measured by method 2.

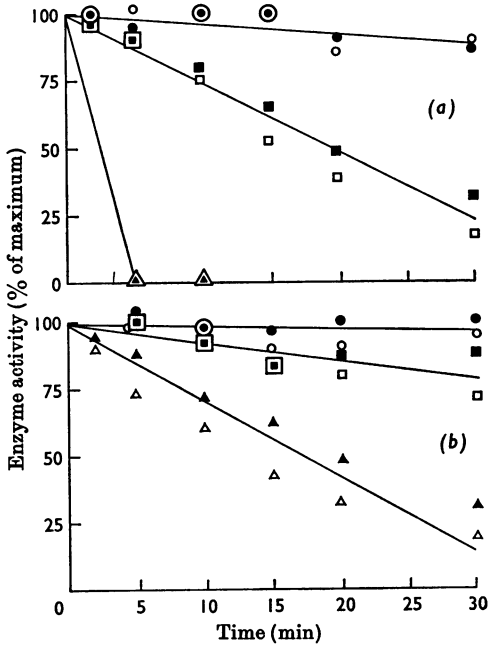

Fig. 5. Temperature stability–time curves for hyaluronidase (*a*) and esterase (*b*) activities of testicular hyaluronidase preparations. ○, □, △, AB Leo preparations; ●, ■, ▲, Koch–Light preparations. ○ and ●, 50°C; □ and ■, 60°C; △ and ▲, 70°C. Hyaluronidase activity was measured by method 2.

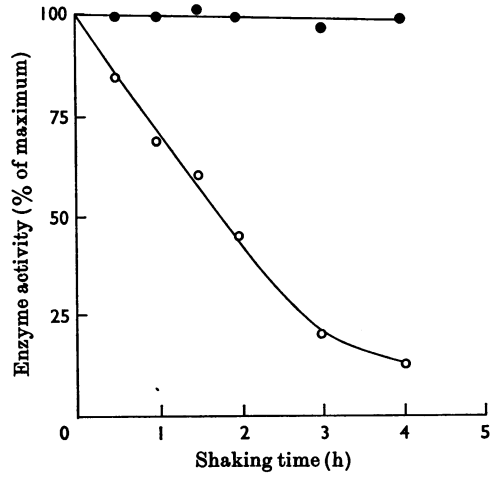

Fig. 6. Effects of mechanical shaking in glass vessels on the hyaluronidase (○) and esterase (●) activities of Koch–Light testicular hyaluronidase. Method 2 was employed to assay hyaluronidase activity.

(Table 2). A noteworthy difference may be noted here between the AB Leo and Koch–Light preparations in that the latter (and much less pure) preparation is markedly less responsive to the inhibitory effects of polyphloretin phosphate. Presumably this is a reflexion of non-specific interaction of inhibitor with inert protein.

Marked differences were also observed in the behaviour of the two enzyme activities towards organophosphorus reagents. In these experiments enzyme preparation (1 mg/ml in the usual acetate–chloride buffer, pH 5.4) was pre-incubated at 25°C for various times with either di-isopropyl phosphorofluoridate (concentration in preincubation mixture

Table 2. *Effects of various inhibitors on hyaluronidase and esterase activities of AB Leo and Koch–Light preparations of testicular hyaluronidase*

Each result is the average of two separate experiments. Hyaluronidase activity was measured by method 1.

Inhibitor	Inhibitor concentration (μg/ml of incubation mixture)	Enzyme activity (%)			
		AB Leo		Koch–Light	
		Hyaluronidase	Esterase	Hyaluronidase	Esterase
None		100	100	100	100
Cu^{2+}	4	52	56	61	58
	8	36	49	46	49
	12	26	34	34	39
Fe^{3+}	4	78	71	74	66
	8	48	42	38	47
CN^-	5	100	85	100	90
	10	100	74	100	78
	20	100	50	100	63
Polyphloretin phosphate	5	70	100	98	100
	10	50	100	95	99
	20	8	97	90	98
	50	0	90	60	97
	100	0	75	30	91
	200	0	45	0	85

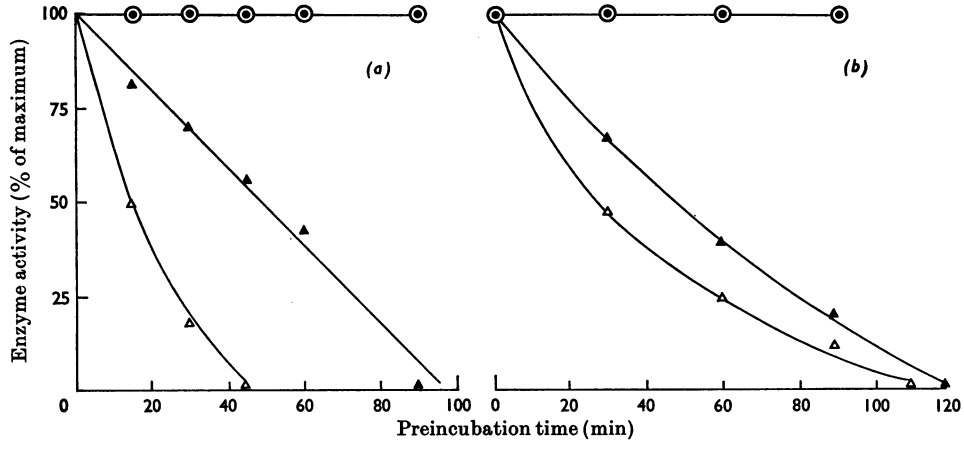

Fig. 7. Effects of preincubation with (*a*) di-isopropyl phosphorofluoridate and (*b*) diethyl *p*-nitrophenyl phosphate (paraoxon) on the hyaluronidase activity and esterase activity of testicular hyaluronidase preparations. ⊙, AB Leo and Koch–Light hyaluronidase activity; △, AB Leo esterase activity; ▲, Koch–Light esterase activity. Hyaluronidase activity was measured by method 1.

of 11 μmol/ml) or paraoxon (concentration of 0.9 μmol/ml). Samples were removed at time-intervals and assayed for enzyme activities. Hyaluronidase activity, in contrast with esterase activity, was completely unaffected by either reagent (Fig. 7).

In substrate-competition experiments it was possible to show that sodium hyaluronate had no effect on esterase activity towards indoxyl acetate even at concentrations as high as 2mg/ml of incubation mixture. This provides further evidence for the separate identity of the two enzyme activities. Unfortunately, it was not possible to do the converse experiment because of the interference of indoxyl acetate with the hyaluronidase assay methods employed.

Gel-filtration experiments. The two enzyme preparations (10mg of each) were subjected to gel filtration on a Sephadex G-200 column (1.8cm × 79

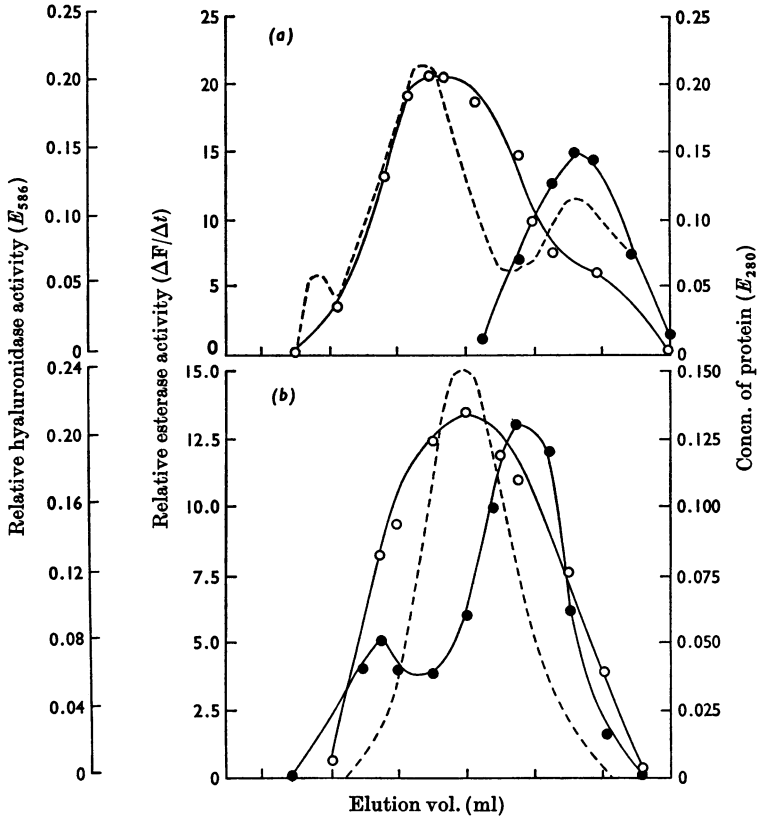

Fig. 8. Gel filtration on Sephadex G-200 of (*a*) Koch–Light and (*b*) AB Leo testicular hyaluronidase preparations. ○, Hyaluronidase activity; ●, esterase activity; ----, protein concentration. Hyaluronidase was measured by method 1 and the results are recorded as spectrophotometric readings obtained per 10μl (AB Leo) or 50μl (Koch–Light) samples of fractions. Enzyme–substrate mixtures were incubated for 1h (AB Leo) and 24h (Koch–Light). Esterase activity is recorded as change in fluorescence/min ($\Delta F/\Delta t$) per 100μl samples of fractions.

cm). The flow rate was 8ml/h and 2ml fractions were collected. The elution diagrams (Fig. 8) provided further evidence of the separate identity of esterase and hyaluronidase activities. The small, fast-running, additional peak of esterase activity in the AB Leo preparation is of note and is referred to again in the next section.

Polyacrylamide-gel electrophoresis. The results for the two enzyme preparations are presented diagrammatically in Fig. 9. A clear distinction between hyaluronidase and esterase activities was obtained in the AB Leo preparation where two bands of esterase activity were actually detected (see the preceding section). The distinct hyaluronidase band contained only a barely detectable trace of esterase activity. With the Koch–Light preparation the distribution of protein bands was somewhat different and one esterase peak only could be detected. Unfortunately, although this particular peak was obtained in the expected place on the electrophoretograms, it proved impossible to detect hyaluronidase activity in any section even after prolonged incubation with substrate. However, a protein band was present in the correct region for hyaluronidase and the failure to detect the enzyme was not unexpected in view of the relatively low activity of the starting material and the general instability of the enzyme. In this connexion the losses of AB Leo hyaluronidase activity during gel electrophoresis were always in excess of 90%. Losses of esterase activity (main peak) were generally about 60%.

DISCUSSION

Several enzyme proteins are now known to exhibit catalytic activity towards substrates that are either unrelated or only distantly related to

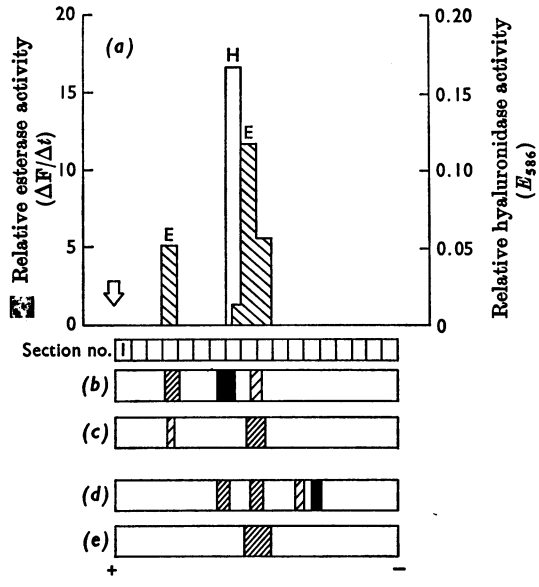

Fig. 9. Polyacrylamide-gel electrophoresis patterns obtained with AB Leo and Koch–Light testicular hyaluronidase preparations. (*a*) Hyaluronidase (H) and esterase (E) activities of the AB Leo preparations; (*b*) protein bands of the AB Leo preparation; (*c*) Indigo Blue (esterase) bands of the AB Leo preparation; (*d*) protein bands of the Koch–Light preparation; (*e*) Indigo Blue (esterase) bands of the Koch–Light preparation. The arrow indicates the origin. Hyaluronidase activity was measured by method 1 and the results are recorded as spectrophotometric readings obtained per 0.4ml sample of gel extract. Esterase activity is recorded as change in fluorescence/min ($\Delta F/\Delta t$) per 0.5ml sample of gel extract.

their true physiological substrates. In some cases this property forms the basis of simple assay methods for enzymes that could otherwise be assayed with difficulty only. In developing such methods there must be clear evidence that a single enzyme is responsible for the activities towards different substrates.

In the case now under consideration the introduction of a simple fluorimetric technique employing indoxyl acetate for the assay of hyaluronidase would have removed much of the tedium and difficulty associated with the established assay procedures. Indeed the method developed by Guilbault *et al.* (1967) has already found its way into one standard volume of fluorimetric methods (Udenfriend, 1969). The present work has clearly established that the hydrolysis of indoxyl acetate by hyaluronidase preparations cannot be attributed to a novel property of the catalytic centre of the enzyme or to the activity of some other region of the enzyme molecule. In fact the collective results show that the hydrolysis can be accounted for by the presence of a contaminating esterase (or esterases). Although separation of hyaluronidase and esterase activities can be achieved on a small scale by gel electrophoresis, conditions have not yet been established for separation on a large scale. Meanwhile, if hyaluronidase preparations that are largely free of esterase activity are required they may readily be obtained by taking advantage of the differences in stability exhibited by the two enzymes at pH below 2.

REFERENCES

Aronson, N. N. & Davidson, E. A. (1967). *J. biol. Chem.* **242**, 437.

Barrett, A. J. (1969). In *Lysosomes in Biology and Pathology*, vol. 2, p. 245. Ed. by Dingle, J. T. & Fell, H. B. Amsterdam and London: North-Holland Publishing Co.

Bollet, A. J., Bonner, W. M., jun. & Nance, J. L. (1963). *J. biol. Chem.* **238**, 3522.

Diczfalusy, E., Ferno, O., Fex, H., Högberg, B., Linderot, T. & Rosenberg, T. (1953). *Acta chem. scand.* **7**, 913.

Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 140. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Guilbault, G. G., Kramer, D. N. & Hackley, E. (1967). *Analyt. Biochem.* **18**, 241.

Humphrey, J. H. (1957). *Bull. World Hlth Org.* **16**, 291.

Hutterer, F. (1966). *Biochim. biophys. Acta*, **115**, 312.

Meyer, K. & Rapport, M. M. (1951). *J. biol. Chem.* **188**, 485.

Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.

Rapport, M. M., Meyer, K. & Linker, A. (1950). *J. biol. Chem.* **186**, 615.

Rasmussen, P. S. (1954). *Acta chem. scand.* **8**, 633.

Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.

Rhodes, C., Olavesen, A. H. & Dodgson, K. S. (1970). *Biochem. J.* **118**, 18P.

Tan. Y. H. & Bowness, J. M. (1968). *Biochem. J.* **110**, 19.

Tolksdorf, S. (1954). In *Methods of Biochemical Analysis*, vol. 1, p. 439. Ed. by Glick, D. New York: Interscience Publishers Inc.

Udenfriend, S. (1969). *Fluorescence Assay in Biology and Medicine*, vol. 2, p. 408. New York: Academic Press Inc.

Vaes, G. & Jacques, P. (1965). *Biochem. J.* **97**, 380.