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THE FATE OF INTRAVENOUSLY ADMINISTERED HIGHLY PURIFIED BOVINE TESTICULAR HYALURONIDASE (HYALOSIDASE) IN THE RAT

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Abstract—A highly purified commercial preparation of bovine testicular hyaluronidase (GL enzyme, Hyalosidase) was labelled with ^{125}iodine without measurable loss of enzyme activity. The labelled preparation was administered intravenously into rats and the serum half-life of hyaluronidase was determined by measurement of both radioactivity and enzyme activity. The short half-life of the enzyme in plasma could not be accounted for by excretion in the urine and bile. Tissue distribution studies showed that the major site of uptake was the liver (59.7% of the recovered dpm). This rapid uptake by the liver could be reduced significantly by the pre-administration of yeast mannan o- ovalbumin (a mannose-terminated glycoprotein). This suggests that the uptake of hyaluronidase by the liver is mediated by a mannose-specific receptor. Very little radioactivity was found in the heart (0.2% of the recovered dpm).

Mammalian hyaluronidases (hyaluronate 4-glycanohydrolase, EC 3.2.1.35) are endoglycosidases that can hydrolyse the β(1-4) glycosidic bonds in hyaluronic acid, chondroitin 4-sulphate and chrondroitin 6-sulphate. Hyaluronidase has been obtained from a wide variety of sources [1] but the most extensively studied enzyme has been that obtained from bovine testes. Several papers and patents have been published outlining the purification and properties of the enzyme from the latter source [2–4].

Interest has been shown in the use of bovine testicular hyaluronidase for the treatment of myocardial infarction [5] and certain other clinical conditions [6]. Most of the reported work has involved the use of crude preparations of the enzyme although, recently, clinical trials in the U.K. have been conducted using a highly purified preparation of bovine testicular hyaluronidase known commercially as GL enzyme or Hyalosidase [7–9]. Results from the more recent trials have shown that the purified hyaluronidase preparation can reduce significantly the cumulative mortality of patients with myocardial infarction.

Although it has been shown that hyaluronidase has a beneficial effect on patients with myocardial infarction there is very little conclusive evidence that explains how the enzyme functions therapeutically. It has been reported that there is a measurable release of hyaluronic acid degradation products from damaged myocardium after administration of the enzyme [10]. Also, it has been claimed that the enzyme reduces oedema [11] and improves the supply of oxygen and nutrients to damaged tissue [12].

As a first stage in elucidating the therapeutic action of bovine testicular hyaluronidase in the treatment of mycocardial infarction we report, in this paper, on the serum kinetics and tissue distribution of the pure enzyme after intravenous injection into rats.

EXPERIMENTAL

Chemicals. Sephadex G-25, Sephadex G-75, Sepharose 4B and Blue Dextran were purchased from Pharmacia (Milton Keynes, U.K.). ^{125}Iodine (16.2 mCi/μg of iodine; free from carrier and reducing agents) was supplied by Amersham International (Amersham, U.K.). The anaesthetic Sagital was obtained from May & Baker (Dagenham, U.K.). A highly purified (>40,000 I.U./mg) preparation of bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase; EC 3.2.1.35) was kindly donated by Biorex Laboratories, London, U.K. This enzyme preparation is the same product that has been used in clinical trials in the U.K. [7–9] and is also known by the trade names of GL enzyme and Hyalosidase. Potassium hyaluronate for the routine assay of enzyme activity was supplied by Miles Laboratories (Stoke Poges, U.K.). All other biochemicals and chemicals were purchased either from BDH Chemicals (Poole, U.K.) or from the Sigma Chemical Co (Poole, U.K.).

Analytical methods. Protein was estimated semi-quantitatively by measurement of the u.v. absorbance of solutions at 280 nm and quantitatively by the Folin method, using bovine serum albumin as a standard [13]. ^{125}Iodine was quantified by γ-radiation counting using an LKB Minigamma counter (model no. 1275).

Measurement of hyaluronidase activity. The activity of Hyalosidase preparations was measured by the assay of reducing *N*-acetylglucosamine residues liberated from potassium hyaluronate [14]. The assay method was calibrated using the W.H.O. International Standard for hyaluronidase [15] which has a defined activity of 10 International Units per mg (I.U./mg).

Radioactive labelling of enzyme. Hyaluronidase

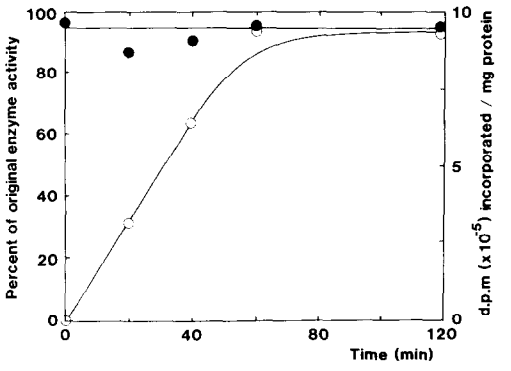

Fig. 1. The effect of iodination on the activity of hyaluronidase. Hyaluronidase was iodinated using a soluble lactoperoxidase preparation. Samples (10 μl) of the reaction mixture were assayed immediately for hyaluronidase activity (●) and for bound ^{125}iodine (○). Iodination using an immobilized lactoperoxidase preparation gave identical results.

was labelled with ^{125}iodine using immobilized lactoperoxidase, prepared by published methodology [16]. A solution of Hyalosidase (300 μl; 5 mg/ml) in 0.05 M-potassium phosphate buffer, pH 7.3, containing 0.15 M-NaCl was placed in a stoppered vial. Immobilized lactoperoxidase (50 μl of packed Sepharose gel) was added followed by 1 mCi of ^{125}iodine. The reaction, which was started by the addition of 4 μl of 8.8 mM-H_2O_2, was allowed to proceed for 2 hr at 20°. Iodination was terminated by adding 25 μl of 0.5 M-L-cysteine HCl, followed by centrifugation at 10,000 *g* for 1 min in order to remove the immobilized lactoperoxidase. The supernatant was applied to a column of Sephadex G-25 (superfine; 0.9 × 30.0 cm) and the radiolabelled enzyme was eluted using 0.154 M-NaCl. Each fraction (0.6 ml) was assayed for hyaluronidase activity and radioactivity. Fractions containing enzyme activity were pooled and applied to a column of Sephadex G-75 (superfine; 0.9 × 30.0 cm) equilibrated with 0.154 M-NaCl. Fractions (0.6 ml vol.) containing hyaluronidase activity were pooled and stored at 4° until required. No significant loss of enzyme activity occurred as a result of the iodination procedure (Fig. 1), and the [^{125}I]-labelled hyaluronidase preparation did not contain unbound iodine.

Polyacrylamide gel electrophoresis. To check the purity of the [^{125}I]-labelled Hyalosidase, samples of the enzyme were subjected to electrophoresis on polyacrylamide gels using non-denaturing conditions at pH 4.3 and 8.5 [17] or under denaturing conditions in the presence of SDS and β-mercaptoethanol [18]. Protein was visualised on intact gels using Kenacid Blue Stain [19] and ^{125}iodine was detected by slicing the gel horizontally into 2 mm thick sections and estimating the radioactivity by γ-counting.

Perfusion of rat hearts. The method used for the perfusion of rat hearts was essentially that of Langendorff as cited by Ross [20], the buffer being that described by Krebs and Henseleit [21]. Rats were killed by asphyxiation with CO_2, hearts were removed immediately and then washed in warm buffer (37°) and cannulated via the aorta. After flushing the heart with buffer to remove any remaining blood, the organ was attached to the perfusion apparatus via the aorta. Each heart was perfused with buffer (40 ml) gassed with 95% O_2 and 5% CO_2 (v/v) containing [^{125}I]-labelled Hyalosidase. The perfusate was recirculated through the heart twice and then replaced by fresh buffer to wash out any unbound [^{125}I]-labelled enzyme. Subsequently the heart was sliced transversely into five sections which were placed into vials to estimate the bound ^{125}iodine by γ-counting.

Estimation of the serum half-life of hyaluronidase in rats. Male rats (approx. 300 g) were anaesthetised with ether and the trachea and right jugular vein were cannulated. A portion of Sagital [0.2 ml of a 50% (v/v) solution in 0.154 M-NaCl] was administered via the jugular vein. Anaesthesia was maintained by further additions of Sagital. The carotid artery was exposed and cannulated towards the heart. A syringe was attached to the cannula so that blood samples could be collected. [^{125}I]-Labelled hyaluronidase was administered (typically 5000 I.U.) via the jugular vein and blood samples (approx. 0.2 ml) were taken from the carotid artery at 1-min intervals for up to 10 min after injection of the enzyme. Each blood sample was added immediately to 50 μl of an anticoagulant solution (2.24 g of D-glucose, 2.20 g of trisodium citrate and 0.80 g of citric acid/100 ml). The packed cell volume of each blood sample was measured using an MSE Haematocrit centrifuge. Each plasma was analysed for enzyme activity and ^{125}iodine. In some of the experiments (see Results for details) 20 mg of yeast mannan or 20 mg of dextran or 40 mg of ovalbumin were injected 2 min before administration of the hyaluronidase.

Tissue distribution of ^{125}I-labelled hyaluronidase. In order to determine the tissue distribution of [^{125}I]-labelled hyaluronidase, the enzyme was administered to the rat via the jugular vein as described above. After exposure to the enzyme for the required period of time the rat was bled via the dorsal aorta. Anticoagulant solution was administered via the jugular vein until the organs were judged to be exsanguinated and the effluent from the aorta was clear of blood. The major organs were removed, weighed and the radioactive content measured. In some cases the gut contents were removed and counted separately. The remaining carcass was digested in 40% (w/v) KOH at 60° for 2 days. The digest was filtered to remove the undigested material. The radioactivity of the filtrate was estimated by γ-counting.

Excretion of ^{125}I-labelled hyaluronidase. In a further series of experiments to those described above the excretion of ^{125}iodine via the bile and urine was estimated. Wholly anaesthetised rats with the ureters and the bile duct cannulated received a 5% (w/v) solution of mannitol (1 ml) via the jugular vein. Urine and bile samples were collected over successive 10-min periods after the injections of ^{125}I-labelled hyaluronidase and the volumes were recorded. A portion of each sample was assayed for total ^{125}iodine and the rest of the material was chromatographed on a column of Sephadex G-75 (superfine; 0.9 × 15.0 cm) to ascertain whether the

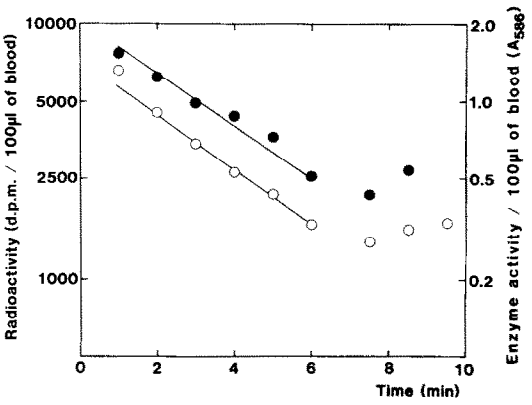

Fig. 2. Estimation of the half-life of hyaluronidase in rat blood. Samples of rat blood (0.2 ml) were taken from the carotid artery and analysed for hyaluronidase activity (●) and radioactivity (○).

^{125}iodine was still associated with protein. Each fraction (0.4 ml) was assayed for radioactivity.

RESULTS AND DISCUSSION

Hyaluronidase was radioactively labelled with ^{125}iodine using an immobilized lactoperoxidase system. Separation of the enzyme from free iodine by sequential chromatography on Sephadex G-25 and Sephadex G-75 showed that there was only one peak of protein and that this was coincident with enzyme activity and ^{125}iodine label. The purity of the labelled enzyme preparation was checked by polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol and at pH 4.3 and 8.5 under non-denaturing conditions. In each case a single radioactive peak was detected coincident with Kenacid Blue staining material.

The immobilized lactoperoxidase method was reproducible and gave specific activities in the range of 13 to 163 μCi/mg protein with no measurable loss of hyaluronidase activity. Routinely, the labelled preparation was stored at 4° and used within several days of preparation. Storage for longer periods resulted in a small but significant dissociation of the iodine. Occasionally, preparations of hyaluronidase that had been stored for extended periods were used in experiments after removal of any unbound ^{125}iodine by rechromatography on Sephadex G-75. These repurified samples gave exactly the same results as fresh preparations of labelled hyaluronidase.

Half-life of [^{125}I]-labelled hyaluronidase in rat blood

The half-life of [^{125}I]-labelled hyaluronidase in plasma was estimated in sequential blood samples taken after i.v. injection of 5000 IU of the enzyme. Measurement of the residual enzyme activity and radioactivity in the citrated blood samples showed that hyaluronidase was removed from the circulation rapidly via two distinct first-order processes (Fig. 2). The faster of the two first order processes, which is responsible for the removal of the bulk of the hyaluronidase from the blood, has a half-life of 2.7 ± 0.7 min (four rats) as determined by radioactive measurement and a half-life of 3.1 ± 0.6 min (four rats) as determined by measurement of enzyme activity. These estimates of the half-life of hyaluronidase in the plasma are comparable to those values obtained by other workers for a variety of animals [22] and for man [22].

The good correlation between the estimates of

Table 1. Tissue distribution of [^{125}I]-labelled hyaluronidase

	Time after intravenous injection		
	2 min	10 min	4 hr
	Percent of recovered dpm/whole tissue		
Liver	59.6	59.7	18.7
Kidneys	4.8	8.0	10.8
Spleen	1.3	2.2	0.2
Pancreas	0.1	0.1	0.3
Lungs	0.2	0.3	0.7
Heart	0.2	0.1	0.1
Brain	0.1	<0.1	—
Testes	0.1	0.1	0.4
Seminal vesicles	0.1	0.1	0.2
Salivary glands	—	0.1	0.1
Stomach	0.2	0.4	3.8
Stomach contents	—	—	3.8
Small intestine	1.0	1.0	1.4
Small intestine contents	—	—	0.9
Large intestine	0.6	0.3	0.8
Large intestine contents	—	—	0.7
Blood	21.2	16.6	4.2
Carcass	10.1	11.0	36.0
Bile	—	—	8.1
Urine	—	—	9.0
Percent recovery of total administered dpm	99.0	90.8	80.0

half-life obtained by measurement of radioactivity and enzyme activity (Fig. 2) shows that [^{125}I]-labelled hyaluronidase is a suitable model for pharmacological studies on the distribution and fate of the enzyme *in vivo*.

It is conceivable that the rapid removal of hyaluronidase from the blood is a result of the breakdown of the enzyme with the subsequent release of the iodine label. The presence of significant quantities of radioactivity in the bile and urine would indicate the presence of breakdown products of [^{125}I]-labelled proteins [23]. To evaluate the extent of breakdown of the enzyme, urine and bile samples were collected for 10-min intervals after i.v. administration of [^{125}I]-labelled hyaluronidase. In the first 10-min period after injection less than 0.5% of the total radioactivity was excreted into urine and bile. During this time more than 80% of the injected hyaluronidase had disappeared from the circulation. Clearly, excretion cannot account for the rapid removal of hyaluronidase from the blood over short time periods. Over 4 hr 9.0% of the administered radioactivity had been excreted in the urine and 8.1% into the bile (Table 1). Analysis of the radioactivity in both fluids by gel filtration on Sephadex G-75 showed that the excreted ^{125}iodine was no longer associated with protein.

Tissue distribution of [^{125}I]*-labelled hyaluronidase*

The tissue distribution of the ^{125}iodine remaining in the rat after i.v. injection of [^{125}I]-labelled hyaluronidase was measured at intervals after the administration of the enzyme. The results (Table 1) show that, over short time periods (2 min) the bulk of the radioactivity (59.6%) accumulated in the liver. However, over a period of 4 hr the radioactivity was gradually redistributed with significant amounts in the kidneys (10.8%) and the gastrointestinal tract (11.4%) with 36.0% remaining in the carcass. Appearance of radioactivity in these tissues would strongly indicate the possible sites of the catabolism of the enzyme, whereas the initial uptake of hyaluronidase by the liver and spleen suggests a carbohydrate-specific receptor mediated mechanism.

It is notable that only a low level of ^{125}iodine was found in heart tissue, an observation that is in agreement with the results obtained by Wolf *et al.* [22]. This result is difficult to reconcile with the known beneficial effects of a single injection of hyaluronidase in the treatment of myocardial infarction [8]. However, preliminary results from our laboratory (Earnshaw, Gacesa, Olavesen and Dodgson, unpublished results) indicate that in damaged myocardium there is a significantly greater accumulation of hyaluronidase. This work will be the subject of a further communication.

Perfusion of the isolated rat heart with [^{125}I]*-labelled hyaluronidase*

The low level of uptake of [^{125}I]-labelled hyaluronidase by the heart, was investigated further in experiments with isolated perfused rat hearts. Hearts were perfused with [^{125}I]-labelled hyaluronidase in Krebs and Henseleit buffer, followed by washing with buffer alone to remove unbound radioactivity. In two separate experiments 3.3 and 1.7% of the administered hyaluronidase was bound to the heart tissue. Further analysis of the myocardium by division into transverse sections revealed a uniform distribution of ^{125}iodine label within the tissue.

The effect of mannose containing compounds on the fate of [^{125}I]*-labelled hyaluronidase*

Recent studies [24] have shown that bovine testicular hyaluronidase is a glycoprotein containing three high mannose oligosaccharide subunits per polypeptide chain. It is well established that certain mannose-terminated glycoproteins are removed rapidly from the circulation by a receptor mediated-process [25]. The mannose-receptors are located in the reticuloendothelial system [26] and the rapid uptake of [^{125}I]-labelled hyaluronidase by the liver and spleen (Table 1) suggests that the fate of the enzyme is governed by the presence of the oligosaccharide subunits. Administration of mannose-containing compounds to rats prior to the injection of hyaluronidase should therefore prolong the serum half-life of the enzyme and reduce accumulation by the liver and spleen.

Administration of yeast mannan prior to the injection of [^{125}I]-labelled hyaluronidase increased the half-life of the enzyme in serum (Table 2). Consequently, reduced accumulation of hyaluronidase in the liver was also observed (Table 3). Ovalbumin, a high-mannose containing glycoprotein, was even more effective than yeast mannan in extending the serum half-life of hyaluronidase. Pretreatment of the rats with dextran produced no significant change in either the serum half-life of hyaluronidase or in the amount of enzyme accumulated by the liver and spleen. The observation that preadministration of mannan or ovalbumin resulted in only partial inhibition of the uptake of hyaluronidase suggests that the capacity of the mannose-receptors is greatly in excess of that required to remove the injected enzyme from the circulation. Although mannan or ovalbumin increased the serum half-life and reduced accumulation of hyaluronidase by the liver and

Table 2. The effect of various compounds on the half-life of hyaluronidase in serum

	Half-life of hyaluronidase in serum (min)	
Competitor	Enzyme activity	Radioactivity
Control	3.1	2.7
Dextran	3.0	2.8
Mannan	5.5	4.2
Ovalbumin	9.2	6.5

Table 3. Tissue distribution of [^{125}I]-labelled hyaluronidase 10 min after intravenous injection

	Percent of recovered dpm/whole tissue			
		Preinjection with		
	Control	Dextran	Mannan	Ovalbumin
Liver	59.7	61.5	18.4	31.4
Blood	16.6	12.5	34.0	27.4
Lungs	0.3	0.6	0.7	0.3
Spleen	2.2	2.8	0.8	2.7
Heart	0.1	0.2	0.4	0.1
Kidneys	8.0	6.8	5.0	14.3
Stomach	0.4	0.4	0.2	0.2
Intestine	1.3	1.7	4.0	2.2
Carcass	11.0	13.1	35.3	21.0
Others	0.4	0.4	1.2	0.4

spleen, there is still only a minute amount of enzyme associated with heart tissue.

The results presented here make it difficult to propose a therapeutic mechanism of action for hyaluronidase in the treatment of myocardial infarction. One possibility is that only a small quantity of enzyme is required to reach the myocardium for protection to occur. Alternatively, the enzyme may be exerting its effects during the short period that it is in the circulation. In either case a lengthening of the serum half-life of hyaluronidase would be expected to increase the efficacy of the enzyme as a drug. However, it is recognised that preinjection with yeast mannan, for example, would not be suitable for this purpose because of the risk of anaphylactic shock. A more promising approach may be to remove the oligosaccharide subunits from hyaluronidase. Certainly with neo-glycoproteins this has been shown to increase dramatically the serum half-life [27].

The possibility that hyaluronidase is exerting therapeutic effects by some process more complex than those described above should not be discounted at this stage. Our own studies indicate (Earnshaw, Gacesa, Olavesen and Dodgson, unpublished results) that damaged myocardium has an enhanced ability to bind hyaluronidase and this is one aspect of the work that is already under intensive study in our laboratories.

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