Comparison of the *N*-Acetylhexosaminidase Components in the Ram Testis and Epididymis by Isoelectric Focusing

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1. Several enzymic components, with both *N*-acetyl- β -glucosaminidase and *N*-acetyl- β -galactosaminidase activity, have been demonstrated in the ram testis and epididymis by isoelectric focusing. 2. The component (I), which predominates in the testis and caput of the epididymis, is isoelectric at $\text{pH } 6.2 \pm 0.1$, whereas the predominant component (II) in the epididymal isthmus and cauda is isoelectric at $\text{pH } 7.0 \pm 0.1$. 3. The total activity and the relative proportions of the enzymic components vary in the different sections of the epididymis. 4. Although their pH optima are slightly different, the appropriate K_m values for components I and II for the hydrolysis of 4-methylumbelliferyl-*N*-acetyl- β -glucosaminide and *N*-acetyl- β -galactosaminide and the ratios of the maximal velocities towards the two substrates are very similar.

The distribution of *N*-acetyl- β -glucosaminidase (β -2-acetamido-2-deoxy-D-glucoside acetamido-deoxyglucohydrolase, EC 3.2.1.30) in the mammalian male genital tract has been described for several species (Conchie & Mann, 1957; Conchie, Findlay & Levvy, 1959*a,b*; Pugh & Walker, 1961). In all the species examined the adult epididymis has proved to be the richest source of the enzyme, with the activity in the pig epididymis particularly high. The enzyme from this tissue has been partially purified (Findlay & Levvy, 1960) and shown by ion-exchange chromatography to exist in two forms (Leaback & Walker, 1967). Multiple forms of *N*-acetyl- β -glucosaminidase have also been demonstrated in the ram testis by ion-exchange chromatography (Caygill, Roston & Jevons, 1966) and by isoelectric focusing (Winchester, Caffrey & Robinson, 1971). Sandhoff (1968, 1969) and Harzer (1970) have also investigated the *N*-acetyl- β -glucosaminidase activity in several mammalian tissues by isoelectric focusing. The object of this paper is to compare the *N*-acetyl- β -glucosaminidase components in the ram testis and epididymis by isoelectric focusing.

MATERIALS AND METHODS

Tissue. Frozen sheep testes with epididymes intact were obtained from Gland Supplies Ltd., Manchester, U.K., and kept frozen at -15°C until required. After separation of the testis and epididymis a 25% (w/v) homogenate of each organ was prepared separately in

water in an MSE top-drive homogenizer. The homogenates were allowed to stand at 4°C for 3-4 h before centrifugation for 15 min at 4°C and $26000g$ (r_{av} , 10 cm). The supernatant was used in the isoelectric-focusing and starch-gel-electrophoresis experiments. To investigate the distribution of enzymic activity along its length, an epididymis was dissected into six sections, each of which was homogenized in 10 ml of water and analysed separately.

Enzyme assays. The fluorimetric assay procedure of Leaback & Walker (1961) was employed to determine *N*-acetyl- β -glucosaminidase and adapted for the assay of *N*-acetyl- β -galactosaminidase with 4-methylumbelliferyl-*N*-acetyl- β -galactosaminide (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.). pH optima and the kinetic parameters, K_m and V , were determined in 0.05 M-sodium citrate buffers. The thermal stability of an enzymic activity was investigated by preincubating the enzyme sample for 1 h at pH 4.5 at various temperatures before assaying for activity at 37°C in the normal manner. Protein was measured by the Folin method (Lowry, Rosebrough, Farr & Randall, 1951).

Isoelectric focusing. Isoelectric focusing was carried out in an Ampholine type 8100 column (LKB Instruments Ltd., London S.E.20, U.K.) as described by Vesterberg & Svensson (1966). pH gradients were established by using the appropriate ranges of synthetic ampholytes, Ampholines (LKB Instruments Ltd.) in a concentration of approx. 0.83% (w/v). Electrofocusing took place for at least 48 h at 300 V and 5°C . Fractions (2.4 ml) were collected by using a BTL Chromapump and Chromafrac (Baird & Tatlock Ltd., Chadwell Heath, Essex, U.K.) at a rate of 60 ml/h. The column effluent was monitored continuously for E_{280} with a Uvicord II absorptiometer (LKB Instruments Ltd.) and the pH of each fraction

was measured at 5°C with a Pye model 290 pH-meter and a Pye-Ingold spear combined electrode, type 401-M5. The enzyme activity in each fraction was determined by diluting a 0.1 ml sample with 0.9 ml of the appropriate buffer and incubating it with 1.0 ml of the buffered substrate. Although the ampholines did not alter the pH of the incubation mixture at this dilution (20-fold), the resultant sucrose concentrations, up to 3.3% (w/v), did inhibit the enzyme activity. Control experiments showed that at 2% (w/v), the approximate concentration at which the major enzymic components were found, 8% inhibition occurred. To eliminate this inhibition when measuring the kinetic parameters, K_m and V , the enzyme sample was diluted 200-fold in the incubation mixture.

Starch-gel electrophoresis. Starch gels were prepared in 5 mM-sodium phosphate buffer, pH 6.0 or 7.0, by the method of Smithies (1955) by using partially hydrolysed starch (Connaught Medical Laboratories, Toronto, Canada). Electrophoresis was carried out for 4 h at 100 V and 20–25 mA with a buffer concentration of 40 mM in the electrode compartments. Glycosidase activity was detected on the gel surface with the appropriate fluorogenic substrate (Robinson, Price & Dance, 1967).

RESULTS AND DISCUSSION

Isoelectric focusing. The *N*-acetylhexosaminidase activity in the supernatant fractions from homogenates of a testis and its corresponding epididymis was compared by isoelectric focusing. Fig. 1(a) shows that the testis contains several enzymic components with both *N*-acetyl- β -glucosaminidase and *N*-acetyl- β -galactosaminidase activity. The major component has an apparent isoelectric point of pH 6.2 and an *N*-acetyl- β -glucosaminidase/*N*-acetyl- β -galactosaminidase activity ratio of 1.86 at pH 4.5. Minor components have apparent isoelectric points of pH 6.4, 5.7, 5.55 and 3.8. Activity was also observed in the region of pH 6.8–7.4.

To determine whether the main component was homogeneous the material isoelectric between pH 5.7 and 6.7 was re-focused over the range pH 5–8. Before focusing this material was dialysed over 24 h against four changes of 0.05 M-sodium citrate buffer, pH 4.5, to remove the sucrose and some of the ampholines. The non-diffusible material was subsequently concentrated by dialysis against polyethylene glycol 4000 P (Shell Chemicals Ltd.), giving an overall recovery of 62% of the enzyme activity. The concentration of ampholines remaining after this treatment was assumed to be very low as a linear pH gradient was obtained in the re-focusing experiment. A single main component was observed, which was isoelectric at $\text{pH } 6.2 \pm 0.1$ and had a *N*-acetyl- β -glucosaminidase/*N*-acetyl- β -galactosaminidase activity ratio of 1.83 at pH 4.5. A minor second component, which was not completely resolved from the main component, had an apparent isoelectric point of pH 5.9 and a ratio of activities of 2.38 at pH 4.5.

In contrast the major *N*-acetylhexosaminidase component in the epididymis is isoelectric at approx. pH 7.0 (Fig. 1b). It had a *N*-acetyl- β -glucosaminidase/*N*-acetyl- β -galactosaminidase activity ratio of 1.87. A much smaller amount of activity is associated with components isoelectric between pH 6.25 and 6.4, in the region of the isoelectric point of the main testicular component. Other minor components are isoelectric at pH 5.9, 5.7, 5.4 and approx. 3.4. To determine the isoelectric point of the main component accurately and to investigate its homogeneity the material isoelectric between pH 6.9 and 7.6 was re-focused over the range pH 5–8. Ampholines and sucrose were removed as described above, before re-focusing. The main component was found to be isoelectric at $\text{pH } 7.0 \pm 0.1$ with a ratio of the two activities of 3.63 at pH 4.5. A shoulder of activity in the region pH 7.15 and traces of activity in the region pH 6.2–6.4 were also observed.

Thus several *N*-acetylhexosaminidase components can be demonstrated in the ram testis and epididymis. The isoelectric-focusing enzyme pattern for each tissue is characteristic and was obtained consistently on the analysis of several testes and epididymes. A component isoelectric

Fig. 1. Isoelectric focusing of supernatant fractions from homogenates of ram (a) testis (0.5 ml sample), (b) epididymis (0.4 ml sample), over the range pH 5–8. □, *N*-Acetyl- β -glucosaminidase; Δ, *N*-Acetyl- β -galactosaminidase; ○, pH; —, E_{280} . The approximate recoveries of enzymic activity in (a) and (b) were 75% and 80% respectively.

at pH 6.2 (form I) predominates in the testis and a component isoelectric at pH 7.0 (form II) in the epididymis. Several minor components occur in both tissues.

Starch-gel electrophoresis. The difference in isoelectric point between forms I and II has been exploited to effect their separation by starch-gel electrophoresis. After electrophoresis at pH 7.0 most of the epididymal activity remained at the origin but a band of activity migrated towards the anode with a mobility comparable with that of the major testicular component. At pH 6.0 the major epididymal component is seen as the band that has run furthest towards the cathode. Another band of activity with a lower mobility, corresponding to the mobility of the majority of the testicular activity at this pH, is also observed. The existence of minor components in both tissues probably accounts for the diffuse bands of activity also seen on the gel surface.

Distribution of *N*-acetylhexosaminidase activity in the epididymis. Measurement of the *N*-acetylhexosaminidase activity in the distinct anatomical sections of the epididymis revealed that the distribution of activity was not uniform throughout the organ (Table 1). Findlay & Levvy (1960) have also found an uneven distribution in the pig epididymis. Isoelectric focusing and starch-gel electrophoresis have been employed to investigate the distribution along the length of the epididymis of the hexosaminidase components found in the whole organ. Fig. 2 compares the isoelectric-focusing pattern of a piece of epididymal caput (section 1 in Table 1) with the pattern obtained from the most active region of the epididymis (section 3 in Table 1). It can be seen that in the caput section, which contains the efferent ductules, the predominant hexosaminidase components are isoelectric between pH 6.1 and 6.4. This enzyme pattern is more characteristic of the testis than of the whole epididymis. In contrast the

most active region of the epididymis (section 3) contains almost exclusively component II, isoelectric at pH 7.0. Only a trace of activity was found around pH 6.2, the isoelectric point of component I.

This change in the predominant *N*-acetylhexosaminidase component along the length of the epididymis was confirmed by starch-gel electrophoresis of all six sections in Table 1. Only in the caput is form I present in larger quantities than form II. In the isthmus, cauda and the beginning of the vas deferens most of the *N*-acetyl- β -glucosaminidase activity and *N*-acetyl- β -galactosaminidase activity is associated with form II, although some of form I is always present. This distribution of the *N*-acetylhexosaminidase components has been found reproducibly in all organs examined.

To investigate the distribution of components I and II in more detail, another epididymis was dissected into 12 sections. The activity in the supernatant fraction of a homogenate of each section was measured and the nature of the hexosaminidase components determined by starch-gel electrophoresis. The sections at both ends of the isthmus had the highest specific *N*-acetyl- β -glucosaminidase activities. Form II was the predominant component in all these sections.

Comparison of the two major components. The pH-dependence of the *N*-acetyl- β -glucosaminidase and *N*-acetyl- β -galactosaminidase activities of the testis is summarized in Table 2. Although the optimum pH for the hydrolysis of 4-methylumbelliferyl-*N*-acetyl- β -glucosaminide is 5.2, there is a distinct secondary maximum in the activity-pH curve between pH 5.4 and 5.6, within the optimum range of the epididymal activity. This would be consistent with the existence in the tissue of minor components with pH optima different from component I. The presence of these components

Table 1. *Distribution of N-acetylhexosaminidase activity in ram epididymis*

Assays were performed at pH 4.5 and 37°C. One unit of activity was defined as that amount necessary to catalyse the production of 1 μ mol of 4-methylumbelliferone/min.

Section	<i>N</i> -Acetyl- β -glucosaminidase		<i>N</i> -Acetyl- β -galactosaminidase	
	(units/g of wet tissue)	(munits/mg of protein)	(units/g of wet tissue)	(munits/mg of protein)
1 Caput including efferent ductules	0.57	21.4	0.26	9.8
2 Caput	1.15	19.9	0.61	10.6
3 Isthmus	7.2	119.0	4.5	74.5
4 Isthmus	4.28	78.0	2.13	38.8
5 Cauda	1.94	39.2	0.91	18.3
6 Vas deferens	0.25	6.9	0.11	3.0

probably also affects the single apparent pH optimum for the hydrolysis of 4-methylumbelliferyl-*N*-acetylglucosaminide. Woollen, Heyworth & Walker (1961) found that a crude ram testis extract has a single pH optimum (4.3) towards *p*-nitrophenyl-*N*-acetylglucosaminide, but two pH optima (4.45 and 4.7) towards *p*-nitrophenyl-*N*-acetylglucosaminide. The activity maximal at pH 4.7 was not found in a partially purified preparation of the testicular extract (Borooah, Leaback & Walker, 1961), supporting the idea that components with different pH optima account for the shape of the activity-pH curve of the crude extract.

Similarly the *N*-acetyl- β -glucosaminidase activity-pH curve for the whole epididymis reflects the existence in the tissue of enzymic components of different pH optima (Table 2). The activity-pH curve for the caput epididymis in which form I predominates closely resembles that of the testis. In contrast the curve for the most active region of the epididymis closely resembles that of the whole epididymis. The pH-dependence of components partially purified by isoelectric focusing confirms that components I and II have slightly different pH optima. Superimposition of the activity-pH profiles of the individual components, which occur in different proportions in the testis and epididymis, accounts for the pH-dependence of crude extracts of these two tissues.

The kinetic parameters, K_m and V , were determined at the optimum pH for each substrate for preparations of components I and II, which had been partially purified by re-focusing (Table 3). Initial rates of hydrolysis were measured at a series of concentrations of both substrates and the results were analysed by the method of Lineweaver & Burk (1934). V for the hydrolysis of the *N*-acetylglucosaminide is very much greater than that for the *N*-acetylglucosaminide for both components. The ratio of the maximal velocities towards the two substrates and the appropriate K_m values are very similar for both components. Thus there is very little difference between components I and II kinetically.

An attempt was made to differentiate between components I and II by comparing the thermal stability of the *N*-acetyl- β -glucosaminidase activity in crude extracts of the two tissues. The testicular activity was less stable than the epididymal activity. Onset of loss of testicular activity occurred as low as 30°C, whereas loss of epididymal (isthmus) activity was negligible until 45°C. The activity in the caput epididymis has an intermediate stability. As the stability of the activity in a crude extract would depend on that of the major component in the tissue, the two main components can be differentiated on the basis of their thermal

Fig. 2. Comparison of isoelectric-focusing patterns for different sections of the ram epididymis over the range pH 5–8: (a) caput (section 1 in Table 1); (b) isthmus (section 3 in Table 1). Samples of 0.3 ml were analysed. □, *N*-Acetyl- β -glucosaminidase; ○, pH; —, E_{280} . The approximate recoveries of enzymic activity in (a) and (b) were 56% and 53% respectively.

Table 2. pH optima

Substrate	Testis		Epididymis				
	Whole tissue	Component I isoelectric at pH 6.2	Whole tissue	Caput	Isthmus	Component isoelectric at pH 7.0	Component isoelectric at pH 6.2
4-Methylumbelliferyl- <i>N</i> -acetyl- β -glucosaminide	5.2	5.2	5.1–5.6	5.1	5.6	5.1–5.6	5.1
4-Methylumbelliferyl- <i>N</i> -acetyl- β -galactosaminide	4.7–4.8	4.9	4.5	4.5–4.9	4.5	4.65	4.75

Table 3. *Kinetic parameters of partially purified components I and II*

For each component *V* was measured relative to 100 for the *N*-acetylglucosaminide.

	Testicular component I isoelectric at pH 6.2			Epididymal component II isoelectric at pH 7.0		
	pH	<i>V</i>	<i>K_m</i> (mM)	pH	<i>V</i>	<i>K_m</i> (mM)
4-Methylumbelliferyl- <i>N</i> -acetyl- β -glucosaminide	5.2	100	0.6	5.2	100	0.5
4-Methylumbelliferyl- <i>N</i> -acetyl- β -galactosaminide	4.85	22	0.085	4.5	21	0.086

stability. This was confirmed by the results of a limited number of experiments performed on samples of the partially purified components.

Thus *N*-acetylhexosaminidase components I and II differ in their electrophoretic mobility but their kinetic parameters and the pH-dependence of their activity are very similar. The relative proportions of these two main components and the other minor components are different in the testis and in the various sections or zones of the epididymis. The total activity also varies from one zone to another.

The mammalian epididymis has been divided into several zones on the basis of histological studies (Benoit, 1926). In particular Nicander (1958) has divided the ram epididymal duct into six regions by its histology. Zone differentiation has also been shown for many species by the histochemical localization of different enzymes (Maneely, 1955; Allen & Slater, 1957; Fouquet & Guha, 1969). At present the possible relevance of these zones of different metabolic activity in sperm maturation or nutrition is not understood. McGadey, Baillie & Ferguson (1966), studying the distribution of hydroxysteroid dehydrogenases in the hamster epididymis, have demonstrated the possibility of two distinct stereospecific 3 α -hydroxysteroid dehydrogenases and two 16 β -hydroxysteroid dehydrogenases occurring in different zones. This distinction of enzymic activity is based on specificity studies rather than the demonstration of different components. Our data suggest that varying proportions of different enzymic components exist in the zones of the ram epididymis.

Pugh & Walker (1961) have investigated the distribution of *N*-acetyl- β -glucosaminidase in the rat testis and epididymis histochemically. In the testis, the seminiferous tubules show a faint activity and the interstitial cells moderate activity. In the epididymis activity was localized in the tubular epithelium of the caput and cauda, the basal cytoplasm staining particularly heavily in the latter. However, very high activity has also been found in the epididymal seminal plasma of the boar and stallion and much lower activity in epididymal sperm from the stallion (Conchie & Mann, 1957).

In ejaculated ram semen, about 20% of the total seminal activity is associated with the spermatozoa (Conchie & Mann, 1957; Allison & Hartree, 1970). It is noteworthy that those zones of the ram epididymis (sections 3, 4 and 5 in Table 1) with the highest *N*-acetyl- β -glucosaminidase activity and a preponderance of component II are also those in which Nicander (1958) found the lumen to be crowded with spermatozoa. Thus the changes in the total *N*-acetyl- β -glucosaminidase activity and proportions of the enzymic components along the length of the epididymis could be associated with alterations in the composition of the epididymal seminal plasma and/or the characteristic changes that occur in the cellular components, the acrosome and the cytoplasmic droplet, during maturation of the sperm in the epididymis.

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