preparation used represents a special case and samples taken from other sources and subjected to different chemical treatments might yield a different affinity series. Consequently, strict comparisons of results with those obtained by other workers should not be made.

Up to this point only the possible significance of the affinities for cartilage of 'reversibly bound' cations has been discussed. It is possible that the relative affinities of the 'irreversibly bound' cations might be of importance in the study of the mechanism of calcification, but the experimental data at present available do not permit discussion of this possibility.

SUMMARY

1. Some of the factors which influence exchange reactions between cations and cartilage have been investigated.

2. It has been found that a fraction of the cations initially bound to cartilage does not exchange readily with other cations. Those cations which do exchange readily do so reversibly.

3. The affinities of some cations for cartilage have been measured and recorded in the form of exchange constants, calculated from a Rothmund & Kornfeld (1918) equation. The order of increasing affinity for cartilage is K^+, Na^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Be^{2+} (probably a complex ion) and Cu^{2+} (probably a complex ion).

4. A comparison of this affinity series with those obtained in studies on sulphated mucopolysaccharides and on the mechanism of calcification has been made.

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Purification of β-*N*-Acetylglucosaminidase from the Pig Epididymis

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In previous papers it has been shown that the richest source of β-*N*-acetylglucosaminidase in different mammalian species is the epididymis, and that the activity in this organ is exceptionally high in the pig (Conchie, Findlay & Levvy, 1959*a*, *b*). It is probable that this enzyme, like β-glucuronidase, is concerned in the ultimate degradation of hyaluronic acid and chondroitin (Linker, Meyer & Weissmann, 1955). Pig epididymis, like other pig tissues, displays a remarkably low β-glucuronidase

activity in comparison with the tissues of other mammalian species (Levvy & Marsh, 1959). Since pig epididymis can be obtained in quantity, it seemed a good starting material for the purification of β-*N*-acetylglucosaminidase.

EXPERIMENTAL AND RESULTS

Enzyme assay. The liberation of *p*-nitrophenol and phenol from their *N*-acetyl-β-glucosaminides was followed at 37° by the procedures described by Findlay, Levvy & Marsh (1958). With both substrates, the pH of the incubation mixture was made 4·25, to correspond to the optimum observed in the present work. As before, 0·1 M-NaCl was present in the incubation mixture and exerted the usual 5–10 % activation observed with this enzyme.

Epididymal preparations at all stages of purification showed a progressive fall in net activity with dilution, that was unaffected by NaCl. To overcome this, it was necessary to have albumin, as well as NaCl, present in the incubation mixture. Crystalline bovine-plasma albumin (L. Light and Co. Ltd.) was used, in a final concentration of 0·01 %. At the usual level of enzyme activity for assay, activation by albumin rose progressively from 10 to 100 % or more as the specific activity of the preparation increased. This effect was additive to that of NaCl. The purified enzyme at high dilution was unstable in the absence of albumin, leading to variable activation figures for the latter.

At the final stage of purification 0·02–0·05 μg. of enzyme protein was sufficient for assay with either substrate in the presence of albumin.

Protein determination. Protein was determined at all stages by the method of Lowry, Rosebrough, Farr & Randall (1951), as adapted for the Spekker photoelectric absorptiometer by Levvy, McAllan & Marsh (1958). The colour reaction was done throughout at 25°. Albumin was selected as standard. This choice was confirmed by micro-Kjeldahl determinations of N, which gave figures ranging from 15 to 17 % of the protein in stages 3 and 7*b* of the purification (Table 3). However, the whole tissue and a trichloroacetic acid precipitate from it had an N content of 21 % of the protein, estimated as albumin. It would appear therefore that the epididymis was rich in less chromogenic protein such as protamine (Levvy *et al.* 1958), and that this was removed at stage 3.

β-N-*Acetylglucosaminidase activity of different portions of the pig epididymis*

The epididymides were dissected from the testes immediately after the animal was killed and were stored at −20° until required. There was no loss in enzyme activity during storage. The organ was cut into portions before thawing, as shown in Fig. 1. After thawing at room temperature the tissue was suspended in ice-cold water by treatment in a Waring Blendor for 90 sec. (There was no apparent inactivation of the enzyme during this treatment, as compared with controls suspended with a glass homogenizer.) Table 1 shows the activity of the different portions of the epididymis. One portion of the caput epididymis had a much higher enzyme activity than had the rest of the organ, and this was a perfectly consistent phenomenon. This most active section of the caput was frequently taken for purification of the enzyme, since a high specific activity could be reached at a much earlier stage of purification (Table 3). (Rat epididymis also showed a higher β-*N*-acetylglucosaminidase activity in the caput than in the cauda.)

Table 2 records figures for a whole epididymis compared with values for other pig tissues. Apart from those for the

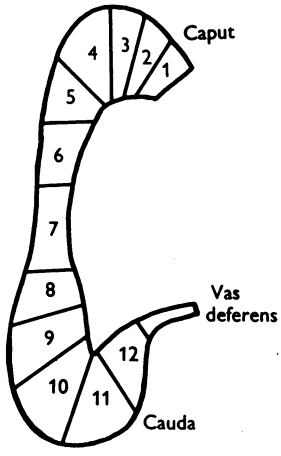

Fig. 1. Division of epididymis for assays (portion numbers shown in Table 1).

Table 1. β-N-*Acetylglucosaminidase activity of pig epididymis divided into portions as shown in Fig.* 1

Results are expressed as μg. of *p*-nitrophenol liberated from its *N*-acetyl-β-glucosaminide in 1 hr. by 1 mg. of moist tissue. Assays were in the presence of albumin.

Portion no.	Moist wt. (g.)	*p*-Nitrophenol (μg./mg. of tissue)
1	5·4	180
2	5·8	768
3	4·7	1980
4	6·1	6680
5	6·3	2620
6	5·5	712
7	4·4	540
8	4·9	448
9	8·4	550
10	18·7	1000
11	13·9	728
12	18·7	224

Table 2. β-N-*Acetylglucosaminidase activity of different pig tissues*

Results are expressed as in Table 1.

	p-Nitrophenol (μg./mg. of tissue)
Liver	44
Spleen	92
Kidney	133
Testis (adult)	48
Testis (infant)	103
Epididymis (adult)	1000–2000
Epididymis (infant)	29

adult epididymis, the figures are not strikingly different from those reported for mouse and rat organs (Conchie *et al.* 1959 *a*).

Purification procedure

General comments. It was borne in mind that the procedure should be equally applicable to the whole epididymis or to selected portions. But for the variable composition of the tissue, the last two stages could have been replaced by a single fractionation. The final procedure is essentially an alternate fractionation with $(NH_4)_2SO_4$ and acetone, and Table 3 shows this applied to the whole organ and to the most active section of the caput. Ethanol was employed in early experiments but proved less reliable than acetone. The specific activity of the starting material was enhanced by its low protein content (determined as albumin), which averaged 4·5 % throughout the tissue.

Precipitates were allowed to settle at 0° for 30 min. before centrifuging, which was done at 0°. The centrifuging of $(NH_4)_2SO_4$ precipitates was done at 10 000 *g* for 10 min.; acetone precipitates were centrifuged for 10 min. at 25 000 *g*. Volume changes were corrected for by adding water after mixing the reagents. Analytical-grade $(NH_4)_2SO_4$ was employed.

General manipulation of the preparation was done at room temperature. At stage 2 the homogenate was centrifuged at room temperature for 15 min. at 1500 *g*. Solutions were dialysed at 0° in Visking sausage casing for 5 days against several changes of 0·05 M-citric acid–NaOH buffer, the pH of which was altered at stage 5. At stage 3 NaCl became unnecessary for complete solution of the enzyme (cf. Pugh, Leaback & Walker, 1957 *a*). There was usually a recovery at stage 7 *b* of 20 % of the original activity, and the purification ranged from 20-fold for the most active section of the caput to 70-fold for the whole epididymis. The specific activities attained were variable at stage 6 but tended towards the same value at stage 7. Generally the final buffered solution was clear and colourless and the concentrated enzyme was stable for several weeks at 0°.

Stage 1 (*homogenate*). The tissue was suspended in about 3 parts of ice-cold water with the Waring Blendor, and the homogenate was made 0·05 M with respect to citric acid–NaOH buffer, pH 4·3, and 0·1 M with respect to NaCl. It was incubated for 4 hr. at 37° and stored overnight at −20°.

Stage 2 (*supernatant*). After the homogenate had been thawed at 37° the insoluble material was centrifuged off and washed on the centrifuge with an equal volume of 0·05 M-buffer, pH 4·3, containing 0·1 M-NaCl. The washing fluid was added to the supernatant, which was cooled to 0°.

Stage 3 (*first ammonium sulphate fractionation*). Cold, saturated $(NH_4)_2SO_4$ solution, pH 4·3, was added to the supernatant to give a final saturation of 20 % and the precipitate was discarded. The preparation was brought to 70 % saturation with $(NH_4)_2SO_4$ and this precipitate was retained. It was dissolved in the minimal volume of 0·05 M-buffer, pH 4·3, and dialysed against the same buffer. Material that separated from solution was discarded.

Stage 4 (*first acetone fractionation*). Cold acetone was added stepwise to give concentrations of 20, 40 and 60 % (v/v). All three precipitates were dissolved in minimal volumes of 0·05 M-buffer, pH 4·3. Any insoluble material was discarded.

Stage 5 (*second ammonium sulphate fractionation*). Fraction 4 *b* was made 40, 65 and 75 % saturated with $(NH_4)_2SO_4$. All three precipitates were dissolved in 0·05 M-citric acid–NaOH buffer, pH 5·5, and dialysed against the same buffer. Insoluble material was discarded.

Stage 6 (*second acetone fractionation*). Fraction 5 *b* was treated with cold acetone in final concentrations of 20, 35

Table 3. *Purification of β-N-acetylglucosaminidase from pig epididymis*

Specific activities are expressed as μg. of *p*-nitrophenol liberated from its *N*-acetyl-β-glucosaminide in 1 hr. by 1 mg. of protein, assayed in the presence of albumin. Percentages of saturated ammonium sulphate solution (SAS) and acetone are by volume.

			Whole epididymis		Most active section of epididymis	
Stage	Description	pH	Specific activity	Recovery (μg. of *p*-nitrophenol/mg. of moist tissue)	Specific activity	Recovery (μg. of *p*-nitrophenol/mg. of moist tissue)
1	Homogenate	4·3	40 800	1580	139 000	6870
2	Supernatant	4·3	61 000	1270	247 000	5630
3	20–70 % SAS	4·3	151 000	1110	895 000	3670
4 *a*	0–20 % acetone	4·3	78 700	40	750 000	85
b	20–40 % acetone	4·3	308 000	782	1 600 000	3250
c	40–60 % acetone	4·3	1 350	18	3 030	2
5 *a*	0–40 % SAS	4·3	201 000	121	749 000	225
b	40–65 % SAS	4·3	670 000	583	2 240 000	2000
c	65–75 % SAS	4·3	271 000	2	2 230 000	305
6 *a*	0–20 % acetone	5·5	500 000	2	1 380 000	2
b	20–35 % acetone	5·5	2 200 000	456	3 260 000*	1860
c	35–50 % acetone	5·5	41 900	18	247 000	45
7 *a*	0–20 % acetone	5·5	1 810 000	3	1 790 000	9
b	20–25 % acetone	5·5	2 890 000	348	3 270 000	1280
c	25–30 % acetone	5·5	2 390 000	51	1 770 000	16

* Lower values were usually observed here.

and 50%. The precipitates were dissolved in 0·05 M-buffer, pH 5·5, in the usual way.

Stage 7 (third acetone fractionation). Fraction 6*b* was fractionated with 20, 25 and 30% acetone, and the precipitates were dissolved in 0·05 M-buffer, pH 5·5, in which they were completely soluble.

Properties of the enzyme

Effects of varying the enzyme concentration and the duration of hydrolysis. These experiments were done at pH 4·25 with 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide in citric acid–NaOH buffer containing NaCl. The rate of hydrolysis by the purified enzyme stayed unchanged for 3 hr. in the presence of albumin, but fell rapidly in its absence after 1 hr. Hydrolysis was proportional to the amount of enzyme added when albumin was present. Without albumin, the net activity rose as more enzyme was added to the incubation mixture (see above).

Effects of pH. Fig. 2 shows that the optima for the hydrolysis of phenyl and *p*-nitrophenyl *N*-acetyl-β-glucosaminides by the purified enzyme in the presence of NaCl and albumin are identical at about pH 4·2. At the optimum, the mean ratio of *p*-nitrophenol (μg.) to phenol (μg.) liberated per mg. of protein was 2·5. Withdrawal of NaCl merely caused a slight fall in activity without any change in the pH optimum. It was impossible to determine the pH optimum in the absence of albumin, owing to the instability of the enzyme. However, a preparation of the most active section of the caput at stage 2 (Table 3), specific activity 194 000 *p*-nitrophenol units/mg. of protein, gave a curve for the hydrolysis of *p*-nitrophenyl *N*-acetyl-β-glucosamide identical with that shown in Fig. 2, whether albumin was present or not. NaCl was present in both cases and the activation produced by albumin was 15%.

The pH optimum for the hydrolysis of *p*-nitrophenyl *N*-acetyl-β-glucosaminide by a rat-epididymis preparation corresponding to stage 3 in Table 3 was again the same as that shown in Fig. 2, in the presence or absence of NaCl. Albumin was unnecessary with this preparation, owing to its relatively low specific activity. The optimum for the hydrolysis of *p*-nitrophenyl *N*-acetyl-β-glucosaminide by a ram-testis preparation is given as pH 4·4 by Heyworth, Borooah & Leaback (1957), and, for the hydrolysis of the phenyl glycoside by a rat-kidney preparation, as pH 4·3 by Pugh, Leaback & Walker (1957*b*).

Fig. 3 suggests that the shape of the pH–activity curve at low pH is determined largely by the instability of the enzyme in this region. The concentrated preparation was diluted several hundredfold with 0·01% albumin, giving a final pH of 5–6.

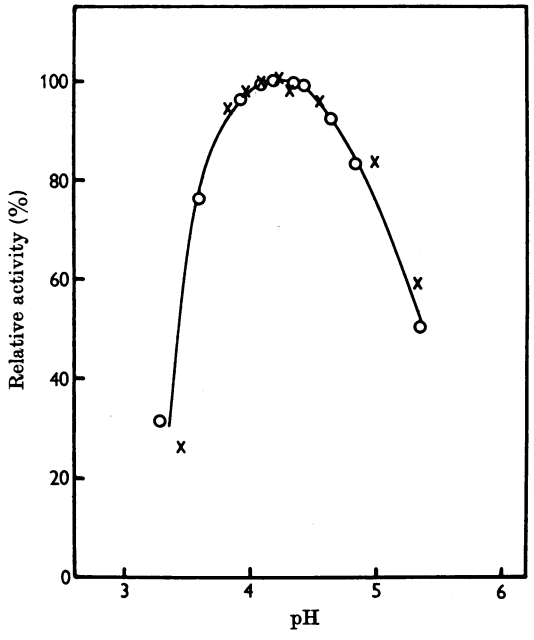

Fig. 2. Hydrolysis of 0·01 M-phenyl *N*-acetyl-β-glucosaminide (○) and 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide (×) at varying pH values in 0·05 M-citric acid–NaOH buffer, in the presence of 0·1 M-NaCl and 0·01% of albumin. Specific activity of enzyme: 3 280 000 (○) and 2 740 000 (×) μg. of *p*-nitrophenol/mg. of protein respectively (cf. Table 3).

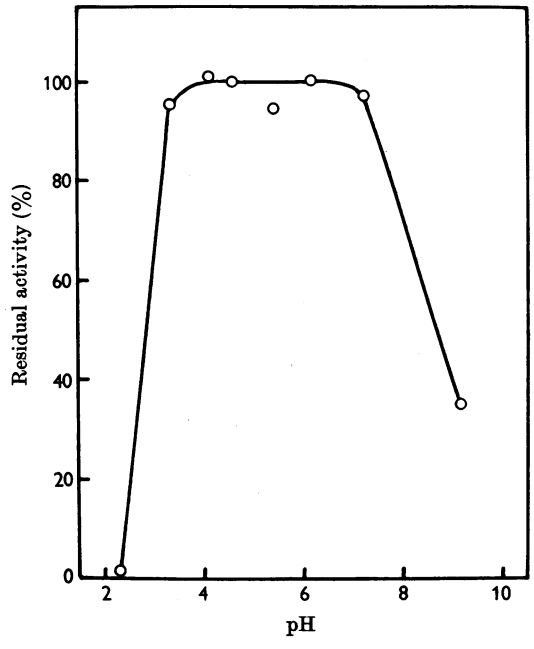

Fig. 3. Stability of the purified enzyme maintained at varying pH for 1 hr. at 37°: specific activity was 3 270 000 μg. of *p*-nitrophenol/mg. of protein (cf. Table 3).

Table 4. *Dissociation constants for the complexes formed by purified β-N-acetylglucosaminidase with substrates (K_m) and competitive inhibitors (K_i)*

Experiments were done in 0·05 M-citric acid–NaOH buffer, pH 4·25. Sodium chloride or albumin or both were added as shown. Enzyme was at stage 7*b* (Table 3), except where otherwise shown.

Substrate aglycone	0·1 M-NaCl	0·01% Albumin	K_m (mM)	K_i *N*-Acetylglucosaminonolactone (μM)	K_i *N*-Acetylgalactosaminonolactone (μM)
p-Nitrophenol	−	−	1·8	.	.
	+	−	2·1	.	.
	−	+	2·1	.	.
	+	+	2·1	0·46	2·0
p-Nitrophenol with enzyme at stage 3	+	+	2·0	0·51	2·1
Phenol	+	+	3·1	0·50	1·4

Specimens were adjusted to the required pH at 0° with NaOH or H_2SO_4, incubated for 1 hr. at 37°, cooled to 0° and brought back to the original pH. After further dilution, they were assayed with *p*-nitrophenyl *N*-acetyl-β-glucosaminide under optimum conditions and compared with an untreated control that had been kept at 0°. A preparation at stage 3 (Table 3) gave a similar curve, as did a rat-epididymis preparation at a corresponding stage of purification.

Dissociation constants. Table 4 gives mean values obtained with a number of different enzyme preparations for K_m for the two substrates, and for K_i *N*-acetyl-2-amino-2-deoxy-D-gluconolactone (*N*-acetylglucosaminonolactone) and *N*-acetylgalactosaminonolactone [for definition of constants, see Dixon & Webb (1958)]. Lactone solutions were prepared immediately before use. The results, calculated by the method of Lineweaver & Burk (1934), are in all respects similar to those obtained with the enzyme from rat epididymis (Findlay *et al.* 1958), except that all constants are five times as great with the pig preparations. As implied by the inhibitory action of *N*-acetylgalactosaminonolactone, our purest enzyme preparation was found to be highly active in hydrolysing *p*-nitrophenyl *N*-acetyl-β-galactosaminide in qualitative experiments.

DISCUSSION

As with purified β-glucuronidase from the female-rat preputial gland (Levvy *et al.* 1958), albumin caused considerable activation of β-*N*-acetylglucosaminidase from pig epididymis, which was not pH-dependent, and abolished the fall in net activity that accompanied dilution of the preparation. With both enzymes, K_m was the same in the presence or the absence of albumin, within experimental error. The simplest explanation of this phenomenon is an increase in the proportion of the enzyme protein in the active form (*e*), resulting in an increase in the rate of hydrolysis ($V = k_3 e$), without change in k_1, k_2 and k_3 in the equations:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{products},$$

where E is the enzyme, S the substrate and $K_m = (k_2 + k_3)/k_1$. The same arguments, of course, apply to the small degree of activation of β-*N*-acetylglucosaminidase caused by sodium chloride.

Apart from its action on acidic mucopolysaccharides containing glucuronic acid, β-glucuronidase hydrolyses the β-glucuronides that are formed *in vivo* from alcohols, phenols and certain carboxylic acids. β-*N*-Acetylglucosaminidase, on the other hand, has no known natural substrates analogous to the β-glucuronides, but its action may be expected to extend to mucosubstances of all classes, since these always contain amino sugars, predominantly, if not entirely, *N*-acetylglucosamine and *N*-acetylgalactosamine.

SUMMARY

1. β-*N*-Acetylglucosaminidase from pig epididymis has been purified to give a final activity of about 3 million *p*-nitrophenol units/mg. of protein on assay in the presence of albumin.

2. Albumin caused extensive activation of the purified enzyme and abolished the fall in specific activity that occurred on dilution of the preparation.

The authors gratefully acknowledge the willing co-operation of Robert Lawson and Sons (Dyce) Ltd. in supplying pig epididymides, the gift of a specimen of *p*-nitrophenyl *N*-acetyl-β-galactosaminide from Dr R. Heyworth and a grant from the Medical Research Council to one of them (J. F.).

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Biochem. J. (1960) **77**, 175

Metabolism of Polycyclic Compounds

16. THE METABOLISM OF 1:2-DIHYDRONAPHTHALENE AND 1:2-EPOXY-1:2:3:4-TETRAHYDRONAPHTHALENE*

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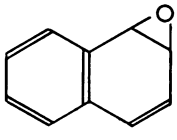
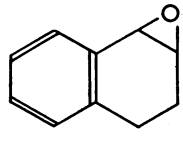
Previous work on the metabolism of naphthalene in animals has led to the suggestion that *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and the mercapturic acid, *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (III) arise in the body through the same intermediate (Boyland & Sims, 1958; Sims, 1959; Booth, Boyland & Sims, 1960). The most probable structure for the intermediate would be the epoxide (I), which has not yet been prepared. The related epoxide, 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (II), is available, however, and the present paper describes the metabolism of this compound and its related hydrocarbon, 1:2-dihydronaphthalene. Previous work (Pohl & Rawicz, 1919; Boyland & Solomon, 1955) indicates that 1:2-dihydronaphthalene is converted by animals into a substance which yields naphthalene on acidification, whereas the present work has shown that both 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene are con-

* Part 15: Booth, Boyland & Sims (1960).



(I)



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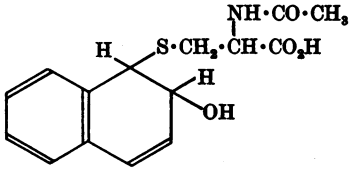

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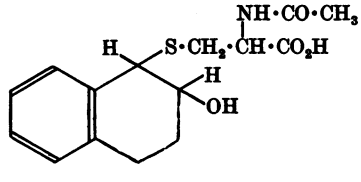

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