repeating the drying procedure. The yield of the pure lactone was 0.9 g. (46%). It titrated entirely as a lactone, with ring opening at pH 7.5–8.

L-Fuconolactone had m.p. 104–106°; $[\alpha]_D^{21} + 74^\circ$ in water (c, 2) (Equivalent weight by titration with NaOH, 171. Calc. for $C_6H_{10}O_5$, 162). Muther & Tollens (1904) give m.p. 106–107°; $[\alpha]_D + 78^\circ$ in water. D-Fuconolactone had m.p. 104–106°; $[\alpha]_D^{21} - 70^\circ$ in water (c, 2) (Equivalent weight by titration with NaOH, 163. Calc. for $C_6H_{10}O_5$, 162). Votoček (1902) gives m.p. 106°; $[\alpha]_D - 76^\circ$ in water.

SUMMARY

1. The α - and β -D- and L-fucosides of *p*-nitrophenol have been prepared as substrates for enzyme studies, by using the Helferich reaction for the α - and the Koenigs–Knorr reaction for the β -compounds. The corresponding aldonolactones have also been made.

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Mammalian Fucosidases

2. α -L-FUCOSIDASE

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The mammalian glycosidases, β -D-glucuronidase, β -N-acetyl-D-glucosaminidase, α -D-mannosidase and β -D-galactosidase, form a group of enzymes with the following features in common. They are present in all mammalian tissues that have been examined (Conchie, Findlay & Levvy, 1959*a, b*), and on structural grounds there is reason to believe that they will attack mucosubstances or their degradation products. The activity in sex organs, and to a lesser extent in other organs, is under endocrine control (Conchie & Findlay, 1959), and cancer tissue is usually higher than most normal tissues in its activity (Conchie & Levvy, 1957*a*; Conchie *et al.* 1959*a*). In the liver cell, all four enzymes are largely confined to the cytoplasmic granules (Conchie & Levvy, 1960; Sellinger, Beaufay, Jacques, Doyen & de Duve, 1960), but for β -glucuronidase at least a large part of the enzyme activity is free in the cytoplasm of cancer cells (Conchie & Levvy, 1959). Each enzyme mentioned is subject to powerful and selective competitive inhibition by the aldonolactone that corresponds to the substrate in structure and configuration (Conchie & Levvy, 1957*b*; Findlay, Levvy & Marsh, 1958). β -Glucuronidase hydrolyses the β -glucuronides that are formed *in vivo*

from alcohols, phenols and certain carboxylic acids (for review, see Levvy & Marsh, 1959). It thus may have an additional function that the other three enzymes lack, since there are no known natural substrates for them that correspond to the β -glucuronides, in the animal kingdom at least.

L-Fucose is a frequent constituent of mucosubstances (Bettelheim-Jevons, 1958), and is of particular interest in connexion with the serological specificity of the blood-group substances (Kabat, 1956; Watkins, 1959), in which it is believed on indirect evidence to have the α -configuration. The α -L-fucose residue is also found in the human-milk oligosaccharides (Kuhn, Baer & Gauhe, 1955, 1958). It therefore seemed feasible that the glycosidases discussed above are accompanied by an α -L-fucosidase, and this possibility has now been examined, by using materials described in the preceding paper (Levy & McAllan, 1961).

EXPERIMENTAL

Enzyme preparation. The fresh tissue was suspended in water with the aid of an all-glass homogenizer (Jencons Ltd.) and made 0.1M with respect to acetic acid–NaOH

Table 1. α -L-Fucosidase in the mouse and the rat

For method of assay see text. Results are expressed as μ g. of *p*-nitrophenol liberated/100 mg. of moist tissue in 1 hr. M, male; F, female; i, infant; C, tissue from castrate animal.

	Mouse				Rat			
	M	F	iM	iF	M	F	iM	iF
Liver	53	57	105	94	217	163	549	570
Liver (C)	—	78	—	—	199	—	—	—
Spleen	51	48	38	44	148	185	392	413
Kidney	242	130	185	201	860	532	820	820
Preputial parts	Nil	30	—	—	1215	960	340	440
Testis	148	—	40	—	1045	—	270	—
Epididymis	384	—	130	—	2780	—	210	—
Epididymis (C)	—	—	—	—	184	—	—	—
Ovary	—	124	—	40	—	642	—	340
Uterus	—	54	—	70	—	149	—	233
Uterus (C)	—	Nil	—	—	—	—	—	—
2146 Tumour	27	20	—	—	—	—	—	—
S37 Tumour	40	—	—	—	—	—	—	—

buffer, pH 5.2. After 1 hr. at 37°, the homogenate was centrifuged at 1500g for 15 min. The cell-free supernatant was fractionated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate obtained between 20 and 80% saturation was dissolved in water (5–10 ml./g. of tissue), and diluted as required. The recovery was 75% with rat epididymis, but only 50% with ox liver. Incomplete extraction of the enzyme accounted for the poor recovery in the latter case, and increasing the period of incubation at 37° from 1 to 4 hr. did not improve the extraction. On storage at 0° or –20°, the concentrated preparation started to lose activity after about 3 weeks, and buffering to pH 5.2 with acetic acid–NaOH made no improvement in this respect.

Enzyme assay. Crude homogenates were used to survey different tissues for enzyme activity. In studying the properties of the enzyme from rat epididymis and ox liver, fractionated preparations were usually employed.

Unless otherwise stated, the incubation mixture contained 1 ml. of 0.2M- Na_2HPO_4 –0.1M-citric acid buffer (McIlvaine, 1921), 2 ml. of 2 mM-*p*-nitrophenyl α -L-fucoside and 0.5 ml. of enzyme solution. The volume was made up to 4 ml. with water or inhibitor solution and the final pH was 6.1. After 1 hr. at 37°, 4 ml. of 0.4M-glycine–NaOH buffer, pH 10.5, was added, the mixture centrifuged and the liberated *p*-nitrophenol measured on the Spekker photoelectric absorptiometer with Ilford no. 601 violet filters (peak transmission 430 m μ). The substrate and the aglycone were quite stable in the alkaline medium.

RESULTS

Distribution of the enzyme

Table 1 shows mean figures for the distribution of α -L-fucosidase in the mouse and the rat, based on three to five results. With very small organs, each result was obtained by pooling the tissues from several animals. Infant animals were 2–14 days old, and operated animals were left at least 4 weeks before use. The tumours were subcutaneous.

In its distribution in the mouse and the rat, α -L-fucosidase resembled the other mammalian

Table 2. α -L-Fucosidase in pigs and cattle

Results are expressed as in Table 1.

	Pigs		Cattle	
	M	F	M	F
Liver	208	321	176	160
Kidney	243	213	381	305
Testis	341	—	90	—
Epididymis	231	—	316	—
Ovary	—	103	—	353
Uterus	—	43	—	99

glycosidases we have studied (Conchie *et al.* 1959*a, b*; Conchie & Levvy, 1957*a*; Conchie & Findlay, 1959), with a particularly high activity in rat epididymis. The epididymal enzyme was low in infant animals and after orchidectomy; a fall in the uterine enzyme was seen after ovariectomy. Castration caused no change in liver α -L-fucosidase activity in either sex.

The figures for cancer tissue were relatively low. This was reflected in the figures for human vaginal fluid from patients with cancer of the uterine cervix. In a series of 15 specimens (supplied by Dr J. G. Lawson), there was no difference in α -L-fucosidase activity between cancer patients and controls. β -Glucuronidase and α -mannosidase are usually elevated in activity in patients with cancer of the cervix (Lawson, 1959, 1960). Like these two enzymes, however, α -L-fucosidase did show high post-menopausal activity in vaginal fluid, with a mean of 377 μ g. of *p*-nitrophenol/g./hr., compared with an average pre-menopausal figure of 47.

Table 2 gives figures for α -L-fucosidase in pigs and cattle. Ox liver was selected for further work because it was freely available in the fresh state. Other preparations examined were extracts from the visceral hump of the limpet, *Patella vulgata* (Conchie & Levvy, 1957*b*), with an activity of about 40 000 μ g. of *p*-nitrophenol/g./hr., and from

Trichomonas foetus (Watkins, 1959), supplied by Miss Winifred Watkins, which displayed no measurable α -L-fucosidase activity.

When the anomeric *p*-nitrophenyl β -L-fucoside was employed as substrate, no hydrolytic activity was detected in the rat epididymis, the limpet or the *T. foetus* extract.

Fig. 1. Percentage hydrolysis of: ○, 1 mM- and ×, 2 mM-*p*-nitrophenyl α -L-fucoside after incubation for 1 hr. with various amounts of enzyme.

Fig. 2. Effect of increasing the period of incubation on the percentage hydrolysis of *p*-nitrophenyl α -L-fucoside by various amounts of enzyme: ○, 1 mM-substrate and 1 arbitrary unit of enzyme preparation; ×, 2 mM-substrate and 1 arbitrary unit of enzyme preparation; ●, 0.6 unit of enzyme.

Properties of the enzyme

Effects of varying the enzyme concentration and the duration of hydrolysis. The concentration of substrate that could be employed in this work was limited by its cost and by its low solubility in water. Fig. 1 shows that, up to about 10% hydrolysis of the substrate, the reaction rate was directly proportional to the amount of enzyme used. From Fig. 2 it can be seen that, below the same limit, the rate was unchanged for periods of incubation of up to 3 hr., after which there was some inactivation. These experiments were done with fractionated preparations from rat epididymis. Experiments with similar preparations from ox liver gave identical results. The 'arbitrary unit' shown in Figs. 1 and 2 corresponded to the liberation of about 30 μ g. of *p*-nitrophenol, or 5% hydrolysis of the substrate under the standard conditions of assay. This was the degree of hydrolysis aimed at elsewhere in this work.

Variation in activity with pH. Fig. 3 shows the pH optima for fractionated rat-epididymis and ox-liver preparations in the standard phosphate-

Fig. 3. Effect of pH on the rate of hydrolysis of 1 mM-*p*-nitrophenyl α -L-fucoside in phosphate-citrate buffer by preparations from: ○, rat epididymis; ×, ox liver. ●, Relative activity of the epididymal preparation in 0.05 M-tris-HCl buffer (final concn.).

citrate buffer. Similar curves were obtained in each case when crude homogenates were used, or when the 1 ml. of phosphate-citrate buffer was replaced by 1 ml. of 0.2M- $\text{Na}_2\text{HPO}_4\text{-HCl}$, 0.2M- $\text{H}_3\text{PO}_4\text{-NaOH}$, 0.1M-citric acid- NaOH or 0.2M-acetic acid- NaOH buffer. The activity at the optimum varied by as much as 15% in different buffers, being highest in the mixture of phosphate and citrate, and lowest in acetate. Although the peak for ox liver was at a slightly lower pH than for rat epididymis, the same buffer, pH 6.1, was used for other work with both tissues. 2-Amino-2-hydroxy-methylpropane-1:3-diol (tris) buffer interfered badly, as shown in Fig. 3.

Variation in stability with pH. Fig. 4 shows the loss in α -L-fucosidase activity of fractionated rat-epididymis and ox-liver preparations at varying pH. Specimens were adjusted to the required pH at 0° with NaOH or HCl , incubated for 1 hr. at 37°, cooled to 0° and brought back to the original pH (5.2). The activities were measured in the usual way at pH 6.1 and compared with that of an untreated control that had been kept at 0°. Within experimental error, the results for the rat and ox preparations were indistinguishable, and the limits of pH-stability were 4.5 and 6. It would appear that the activity at the subsidiary peak in the pH-activity curves (Fig. 3) at pH 3-4 was limited by the instability of the enzyme in this region.

Effects of varying the substrate concentration. These experiments were limited on the one hand by the insolubility of *p*-nitrophenyl α -L-fucoside, and on the other hand by the need to keep below 10%

hydrolysis, while still obtaining measurable activities. Provided the latter condition was fulfilled, linear plots were obtained when K_m , the Michaelis constant, was calculated by the method of Lineweaver & Burk (1934). The mean values obtained were 0.21 mM for rat epididymis and 0.22 mM for ox liver. Fractionated enzyme preparations were used. As illustrated in Fig. 5, the limiting velocity was never attained, and a substrate concentration of 1 mM was selected for routine purposes.

Inhibitor trials. Hydrolysis of 1 mM-*p*-nitrophenyl α -L-fucoside under the usual conditions by rat-epididymis, ox-liver and limpet preparations was unaffected by 1 mM-L-galactono-1 $\rightarrow$ 4-lactone (supplied by Dr W. W. Reid) and by 1 mM-L-fucono-1:4-lactone. There is some evidence for the possibility that in the galactose series the inhibitory power of a lactone may be increased on changing from the 1 $\rightarrow$ 4- to the less stable 1 $\rightarrow$ 5-ring structure (Findlay *et al.* 1958). The ring in L-fucono- and L-galactono-1 $\rightarrow$ 4-lactone was opened by titration with alkali, and the solutions were brought back to pH 3 or 6. Such solutions were still non-inhibitory, even after short periods of heating.

DISCUSSION

By analogy with the action of β -glucuronidase and β -N-acetylglucosaminidase on hyaluronic acid (Linker, Meyer & Weissmann, 1955), one might expect α -L-fucosidase to act on natural polymers

Fig. 4. Effect of incubation at varying pH for 1 hr. at 37° on the α -L-fucosidase activity at pH 6.1 of: rat-epididymis (O) and ox-liver (x) preparations.

Fig. 5. Effect of varying the substrate concentration on the rate of hydrolysis of *p*-nitrophenyl α -L-fucoside in phosphate-citrate buffer, pH 6.1, by rat-epididymis (O) and ox-liver (x) preparations.

only after they have been degraded by a polysaccharase. An interesting situation, however, arises with the blood-group substances, from which L-fucose is liberated by *T. foetus* extracts (Watkins, 1959). These extracts do not hydrolyse methyl α - and β -L-fucopyranoside, nor do they liberate the α -L-fucopyranosyl residue from reducing fucose disaccharides prepared from fucoidin. On the other hand, human-milk oligosaccharides, e.g. α -L-fucosidolactose, in which the α -L-fucopyranosyl group is linked to glucose, galactose or N-acetylglucosamine, were completely broken down by the *T. foetus* extracts. Our own failure to observe any action of such an extract on *p*-nitrophenyl α -L-fucoside is in agreement with the lack of action on the methyl glycoside.

Professor W. T. J. Morgan (personal communication) has found that the mammalian α -L-fucosidase preparation failed to destroy the serological properties of, or liberate fucose from, the blood-group A, B and H specific mucopolysaccharides, whereas it rapidly liberated fucose from oligosaccharides such as α -L-fucosidolactose.

In its widespread distribution in mammalian tissues, α -L-fucosidase resembles the other mammalian glycosidases we have studied (see introduction). Whatever their actions may be, the high activity of these enzymes in epididymis argues for some role in insemination. Those glycosidases previously studied have been shown to be free in seminal plasma (Conchie & Mann, 1957), in which case their activities should be governed by the respective pH optima observed *in vitro*. In other words, a variety of effects may be produced by the mixture, depending upon the ambient pH. The pH optimum of α -L-fucosidase is closer to neutrality than that of any of the other enzymes in question.

It seems possible that the activities of these enzymes in semen may alter in pathological conditions of the male genital tract. Unlike the other members of the group, however, α -L-fucosidase did not have a high activity in cancer tissue, although to judge by the effects of castration the activity was under endocrine control.

The failure to observe inhibition of α -L-fucosidase by L-fuconolactone merely adds to the number of α -glycosidases which, in contrast with the β -glycosidases, are at most only feebly inhibited by the appropriate aldonolactones. In our experience, only α -mannosidase and α -glucosidase approach the β -glycosidases in their susceptibility to inhibition by the lactones (Conchie & Levvy, 1957b). This is not a simple question of sugar configuration since α -glucuronidase is not inhibited by saccharo-1 $\rightarrow$ 4-lactone (Marsh & Levvy, 1958).

SUMMARY

1. The α -L-fucosidase activity of mammalian tissues has been studied, with *p*-nitrophenyl α -L-fucoside as substrate. All species and tissues examined displayed appreciable activity. The highest activity was seen in rat epididymis, but this was exceeded by the figure for the limpet, *Patella vulgata*.

2. The values for epididymis and uterus were drastically decreased by orchidectomy and ovariectomy respectively.

3. Properties of the enzyme from rat epididymis and ox liver were examined. The enzyme preparations were indistinguishable, except in the figures for the pH optima, which were 5.6 for ox liver and 6.1 for rat epididymis.

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