Inhibition of Glycosidases by Aldonolactones of Corresponding Configuration

3. INHIBITORS OF β -D-GALACTOSIDASE*

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In spite of the difference in ring-size between inhibitor and pyranosyl substrate, β -glucuronidase is powerfully inhibited by saccharo-1,4-lactone (Levy, 1952). In the somewhat feebler inhibition of β -glucosidase by gluconolactone, the 1,4- and 1,5-lactones are equally effective (Conchie, 1954; Conchie & Levy, 1957). Study of the powerful inhibition of β -N-acetylglucosaminidase by 2-acetamido-2-deoxygluconolactone and 2-acetamido-2-deoxygalactonolactone (Findlay, Levy & Marsh, 1958) revealed changes in the composition of the inhibitor solutions that were attributed to interconversion of the 1,4- and 1,5-lactones. It was concluded that the two forms of 2-acetamido-2-deoxygluconolactone were equally inhibitory, whereas 2-acetamido-2-deoxygalactono-1,5-lactone was considerably more powerful than its 1,4-isomer.

It was decided to investigate the possibility that this difference between the 1,4- and 1,5-lactone was a general phenomenon applying to the galactose series, in contrast with the glucose series, and having its origin in the configuration of the sugar at C-4. Because the hydroxyl groups at C-4 and C-5 are *trans* in galactose, the relative position of the side chain is reversed on changing from a 1,5- to a 1,4-ring (see formulae I-IV). As pointed out by

Hudson (1910, 1911), there is a complete reversal of rotation when galactose or fucose (6-deoxygalactose) is converted into the 1,4-lactone.

Mammalian β -D-galactosidase was used for this work. It is inhibited, although rather feebly, by D-galactono-1,4-lactone (Conchie, Findlay & Levy, 1959); galactono-1,5-lactone has never been isolated. The specificity of β -galactosidase does not appear to be strict for the group at C-6 in the sugar residue (Wallenfels & Malhotra, 1960), and inhibition by D-fucono-1,4-lactone was also examined.

The distribution of β -galactosidase in the mouse and the rat has been studied (Conchie *et al.* 1959), and some properties of the enzyme from rat epididymis have been described (Conchie & Hay, 1959). It became necessary in the course of the present work to investigate properties of the enzyme from other mammalian sources.

EXPERIMENTAL

Enzyme preparations. Ox and pig organs were obtained fresh from the slaughter house. Human tissues were autopsy specimens, supplied by Dr A. J. Carr, Department of Pathology, University of Aberdeen.

As described by Conchie & Hay (1959), the tissue was homogenized in water, incubated for 1 hr. at pH 5.2 in acetate buffer, and precipitated from the clear extract with $(\text{NH}_4)_2\text{SO}_4$ between 20% and 80% saturation. Although this method of preparation was adopted as standard, it was observed that incubation at 37° was unnecessary for the extraction of β -galactosidase in water homogenates of rat epididymis and ox liver, and that the bulk of the enzyme was precipitated between 40% and 60% saturation with $(\text{NH}_4)_2\text{SO}_4$. On centrifugal fractionation of homogenates made in 0.25 M-sucrose solution (Conchie, Hay & Levy, 1961), 80% of the enzyme activity in ox liver was found to be soluble, whereas 80% of the activity in rat liver was bound to the subcellular particles.

Preparations from different sources were kept for several weeks at 0° without appreciable loss in activity.

Enzyme assay. In studying the properties and inhibition of the enzyme, fractionated preparations were usually employed. Crude water homogenates were used in comparing the activity of the enzyme in different mammalian tissues.

The incubation mixture contained 1 ml. of 0.2 M- Na_2HPO_4 -0.1 M-citric acid buffer (McIlvaine, 1921), 0.5 ml. of *o*-nitrophenyl β -D-galactoside solution and 0.5 ml. of enzyme preparation; the volume was made up to 4 ml.

* Part 2: Findlay, Levy & Marsh (1958).

with water or inhibitor solution. For rat epididymis and liver, the pH was 3.0 and the final substrate concentration 2.5 mM, whereas for ox liver and kidney the pH was 7.0 and the substrate concentration 7.5 mM: all other tissues were assayed at pH 4.5 and a final substrate concentration of 2.5 mM. After 1 hr. at 37°, 4 ml. of 0.4M-glycine-NaOH buffer, pH 10.5, was added, the mixture centrifuged and the liberated o-nitrophenol measured on the Spekker photoelectric absorptiometer with Ilford no. 601 violet filters (peak transmission 430 m μ). The usual enzyme and substrate controls were made.

Inhibitors. Galactono-1,4-lactone was obtained from L. Light and Co. Ltd. Different specimens varied in their initial inhibitory power and were recrystallized from ethanol to constant inhibition. The purified material had m.p. 134–137° (corr.) and $[\alpha]_D^{20}$ –76° in water (c, 4); Richtmyer, Hann & Hudson (1939) give m.p. 134° and $[\alpha]_D$ –78°. Sodium galactonate was prepared in solution by treating the 1,4-lactone with NaOH.

The 'most inhibitory solution' was freshly made from sodium galactonate by making the solution pH 2 with HCl and heating at 100° for 7.5 min.; the solution was cooled and made pH 4. It had $[\alpha]_D^{20}$ –20° in water (c, 4), calculated as the lactone. This inhibitor solution could also be prepared by the use of ion-exchange resins. Amberlite IR-120 (H) was stirred into a solution of sodium galactonate until the supernatant had pH 2.9, and the mixture was kept at 100° for 7.5 min.; the cooled filtrate was treated with Amberlite IR-45 (OH) to bring it to pH 4. Attempts to isolate the unknown galactono-1,5-lactone from the most inhibitory solution were unsuccessful.

D-Fucono-1,4-lactone (Levy & McAllan, 1961) that had constant initial inhibitory power was put through similar conversions. Ring-opening with this lactone was slow even at pH 9, whereas galactono-1,4-lactone rapidly reacted with alkali above pH 7.

Inhibitor solutions were prepared at relatively high concentration and diluted 10- to 100-fold immediately before use.

RESULTS

Distribution and properties of the enzyme

Distribution. Mean figures for some pig, ox and human tissues are shown in Table 1. Sex had no effect on the figures for liver and kidney in the ox and pig, and only male human liver was examined. There was not the relatively high activity in epididymis seen in the rat (Conchie *et al.* 1959), but pig and ox liver and pig kidney had high activity compared with the rat. Values for the uterus were variable, as one might expect from the effects of ovarian control, demonstrated with rats and mice (Conchie & Findlay, 1959). The β -galactosidase activity of the ovary itself depends upon the luteal content, and figures for the ox, pig and human are given by Levy, McAllan & Hay (1961).

Variation in activity with pH. Fig. 1 shows pH-activity curves for rat liver, pig ovary and ox kidney. Rat liver resembled rat epididymis (Conchie & Hay, 1959) in having its optimum at pH 3–4. Ox liver and kidney were alike, the activity

rising to a plateau above pH 4, with a peak at pH 7. Ox epididymis, testis, uterus and ovary resembled pig ovary, with an optimum at pH 4–5, and human liver and testis were also in this category. Pig liver and kidney had a similar, but somewhat broader, optimum, from pH 4 to 6. Only the ox showed a real difference between genital and non-genital organs, and the former fell into the same group as the human and pig tissues, whereas rat tissues formed a class of their own.

Altering the substrate concentration from 7.5 to 2.5 mM caused no change in the shape of the pH-activity curves for ox liver and kidney, nor did replacement of the 1 ml. of phosphate-citrate buffer by 1 ml. of 0.2M-Na₂HPO₄-HCl, 0.2M-H₃PO₄-NaOH, 0.1M-citric acid-NaOH or 0.5M-

Table 1. β -D-Galactosidase in some mammalian tissues

For methods of assay, see text. Results are expressed as μ g. of o-nitrophenol liberated/g. of moist tissue in 1 hr.

	Pig	Ox	Human
Liver	27 250	36 900	6 200
Kidney	49 640	2 980	—
Testis	3 660	2 780	4 520
Epididymis	2 350	1 940	1 390
Uterus	1 000	150	—

Fig. 1. Effect of pH on the rate of hydrolysis of o-nitrophenyl β -D-galactoside in phosphate-citrate buffer: x, rat-liver preparation and 2.5 mM-substrate; ●, pig-ovary preparation and 2.5 mM-substrate; ○, ox-kidney preparation and 7.5 mM-substrate.

acetic acid-NaOH buffer. The activity of the enzyme showed little or no change in these different buffers. Tris-HCl buffer (0.05M, final concn.) caused increasing depression of the activity above pH 6.

Attempts to separate the ox-liver enzyme into two fractions with different pH-activity curves were uniformly unsuccessful. Identical curves were obtained at each stage during the extraction of the enzyme, and during its stepwise fractionation with $(\text{NH}_4)_2\text{SO}_4$ between 0% and 80% saturation by increments of 20%. Centrifugal separation of the enzyme in homogenates prepared in 0.25M-sucrose solution into soluble and insoluble fractions led to no changes in the ratio of the activities at pH 4.5 and pH 7. This ratio was also unchanged during progressive inactivation by heat or by exceeding the limits of pH stability (see below). The pH-activity curve for rat epididymis was unchanged by adding an ox-liver preparation that had been inactivated by heat (10 min. at 100°) or acid (1 hr. at 37° and pH 2): the converse experiment was also negative.

Variation in stability with pH. Fig. 2 shows the loss in activity of an ox-liver preparation kept for 1 hr. at 37° and varying pH. Specimens were adjusted to the required pH at 0° with NaOH or HCl, incubated for 1 hr., cooled to 0° and brought back to the original pH (6.3). The activities were compared with that of an untreated control that had been kept at 0°. Assays at pH 4.5 and pH 7 gave identical curves for residual activity. It is evident that the shape of the pH-activity curve (Fig. 1) is largely determined by the pH stability. The limits of stability (pH 5-8) contrast with those for rat epididymis (pH 3-6.5; Conchie & Hay, 1959).

Heat inactivation. Failure to separate the ox-liver enzyme into two components is clearly demonstrated in Fig. 3. The cell-free extract before fractionation with $(\text{NH}_4)_2\text{SO}_4$ was used, in 0.1M-acetic acid-NaOH buffer, pH 5.2. Specimens were treated as shown, chilled and compared in activity with an untreated control assayed at the same pH.

Effect of varying the substrate concentration. Nine experiments with ox liver at pH 7 gave a mean value of 0.6 mM for the Michaelis constant, and the limiting velocity was reached at 15 mM. Inhibition by excess of substrate was not observed. The same values were obtained in a single experiment at pH 3 (0.05M-acetic acid-NaOH buffer). For rat epididymis the corresponding figures are 0.38 mM and 5 mM respectively (Conchie & Hay, 1959).

Hydrolysis for varying periods and with varying amounts of enzyme. Fig. 4 shows that under the recommended assay conditions the rate of hydrolysis by an ox-epididymis preparation was unchanged for nearly 2 hr.; the curve was also linear

Fig. 2. Effect of incubation at varying pH for 1 hr. at 37° on the β -galactosidase activity of ox liver.

Fig. 3. Residual activity of ox-liver β -galactosidase after (a) varying periods at 50° and 60°, and (b) 5 min. at varying temperatures. $\times$, Assays at pH 7; $\circ$, assays at pH 4.5.

for at least this period with rat epididymis (Conchie & Hay, 1959) and ox ovary. In contrast, however, the rate of hydrolysis by ox liver fell continuously with time, no matter how the conditions of assay were varied with respect to pH and substrate concentration. These experiments were with different enzyme preparations that were not standardized against each other. Besides those shown in Fig. 4 experiments were done at pH 5.5 and 6.5. This phenomenon is evidently a question of enzyme instability since the percentage hydrolysis of the substrate was not excessive. It is remarkable, however, that in the absence of substrate (Fig. 2) the enzyme is stable over most of the pH range used here. As to inhibition by the products of hydrolysis, only 10% inhibition was caused by 0.1 mM-*o*-nitrophenol and none by 0.375 mM-D-galactose in the usual 1 hr. assay, which makes this factor of minor importance in considering the results for ox liver in Fig. 4.

In spite of the instability of the ox-liver enzyme, the amount of substrate hydrolysed in 1 hr. was found to be directly proportional to the amount of enzyme added, under the normal conditions of assay. Hydrolysis was as high as 10%, which is three times the maximum usually encountered.

Fig. 4. Effect of increasing the period of incubation on the percentage hydrolysis of *o*-nitrophenyl β -D-galactoside by preparations from: ●, ox epididymis at pH 4.5 and 2.5 mm-substrate; ○, ox liver at pH 7 and 2.5 mm-substrate; ×, ox liver at pH 4.5 and 7.5 mm-substrate; △, ox liver at pH 7 and 7.5 mm-substrate. The different enzyme preparations are not comparable with each other in activity.

Inhibition of rat-epididymis enzyme by galactonolactone

Freshly prepared solutions of galactono-1,4-lactone inhibited β -galactosidase from the following sources to a similar extent: the limpet *Patella vulgata* (Conchie & Levvy, 1957), rat epididymis (Conchie *et al.* 1959), rat liver, ox epididymis. Under comparable conditions, the action on the ox-liver enzyme was relatively feeble (see below).

The enzyme from rat epididymis was employed with the normal conditions of assay in order to study changes in the composition of the inhibitor solution. Fig. 5 shows the relative efficiency of the most inhibitory solution (see Experimental section), sodium galactonate and galactono-1,4-lactone (freshly prepared solution): 50% inhibition was observed at 0.6, 1.4 and 13 mM-inhibitor respectively (all in terms of galactonate ion). It is at once evident that galactono-1,4-lactone is not the most inhibitory entity formed from galactonic acid in the acid assay medium (pH 3). The following experiments describe the conditions for attaining maximum inhibitory power.

Fig. 6 shows the differences in the inhibitory power of solutions of galactono-1,4-lactone made in buffers of varying pH. The experiment at 0° illustrates the immediate formation of a solution equivalent to sodium galactonate in inhibitory power that occurred above pH 7. Heating for varying periods at different temperatures caused no change in such solutions (although they varied slightly in inhibitory power from experiment to experiment; see below). At 38° galactono-1,4-lactone showed itself capable of forming the more

Fig. 5. Inhibition of β -galactosidase from rat epididymis by varying concentrations of galactono-1,4-lactone (×), sodium galactonate (○) and the most inhibitory solution derived from the galactonate ion (●) (see Experimental section).

inhibitory entity directly at acid pH, without intermediate formation of the salt. On heating to 100° at acid pH the lactone solution became more powerful than untreated sodium galactonate. Fig. 6 also shows the same process starting with sodium galactonate solution: formation of the more powerful inhibitor was quicker than in the experiments with galactono-1,4-lactone.

The results in Fig. 6 can be explained by postulating the formation of a more inhibitory lactone,

Fig. 6. Inhibition of rat-epididymis β -galactosidase by solutions pretreated at varying pH: ●, galactono-1,4-lactone after 20 min. at 0° in phosphate-citrate buffer; △, galactono-1,4-lactone after 60 min. at 38° in phosphate-citrate buffer (pH 3–9) or HCl (pH 2); ○, galactono-1,4-lactone after 20 min. at 100° in phosphate-citrate buffer; ×, sodium galactonate after 10 min. at 100° in solutions adjusted to the pH shown with HCl or NaOH. All tests were made at 1 mM concentration.

Fig. 7. Changes in inhibitory power of sodium galactonate solutions adjusted to pH 2 with HCl and kept at: ●, 0° (time in hr.); ○, 38° (time in hr.); ×, 100° (time in min.). All were tested against rat-epididymis β -galactosidase at 0.5 mM concentration.

galactono-1,5-lactone, from sodium galactonate or galactono-1,4-lactone at acid pH before assay. There was a change in optical rotation, as noted in the Experimental section. When the salt is introduced into the acid assay medium, formation of the 1,5-lactone occurs to a limited extent.

Fig. 7 shows the conditions for obtaining the maximum formation of the 1,5-lactone from sodium galactonate at pH 2. Once formed, the 1,5-lactone underwent further transformation at this pH, as shown by the subsequent fall in inhibitory power. Similar experiments were done at pH 3, 4 and 5. The changes became progressively slower and the peak in inhibitory power was lower. Decay of the 1,5-lactone at varying pH can be seen in Fig. 8. Maximum stability was displayed at pH 4. Below this pH there is presumably more rapid conversion into the 1,4-lactone and above it opening of the lactone ring.

Formal proof that the galactonate ion is not itself inhibitory is provided by the velocity curve in Fig. 9. Inhibition by galactonate increased progressively during the incubation with the substrate, showing that the inhibitor was actually produced in the assay medium. The intercept at 1 hr. gives the inhibitory power of sodium galactonate solutions, measured in the usual way. This value tends to vary somewhat from experiment to experiment unless all variables are stringently controlled (cf. Fig. 6). Hydrolysis was linear in the presence of galactono-1,4-lactone or of the most inhibitory solution derived therefrom.

The enzyme from rat liver behaved identically with rat-epididymis preparations with galactono-1,4-lactone and with the most inhibitory solution.

Fig. 8. Stability of the most inhibitory solution (see Experimental section) at varying pH in phosphate-citrate buffer (pH 3–9) or HCl (pH 2): ×, tested at once; ○, tested after 1 hr. at 38°; ●, tested after 24 hr. at 0°. All tests were at 0.5 mM concentration (in terms of galactonate ion) against rat-epididymis β -galactosidase.

Table 2 shows the effects of the two inhibitors on the ox-liver enzyme. At pH 7, both were feeble in their effect. At more acid pH, the ox-liver enzyme was affected only slightly by the 1,4-lactone, but quite strongly by the 1,5-lactone.

Inhibition of the ox-liver enzyme by fuconolactone

Freshly prepared solutions of fucono-1,4-lactone do not inhibit β -galactosidase from rat liver, rat epididymis or ox epididymis. This lactone does inhibit the enzyme from the limpet and from ox liver. In the following experiments with ox-liver β -galactosidase the substrate concentration was reduced to 2.5 mM to facilitate comparison with the work on galactonolactone with rat-epididymis enzyme.

The following points are evident from Table 3. On test at pH 4, fucono-1,4-lactone was not the most inhibitory entity that could be formed in

fuconate solutions. Inhibition by the 1,4-lactone was greater at pH 7 than at pH 4: this was not a property of the enzyme since other experiments showed that dissolving fucono-1,4-lactone in a buffer at pH 7 (cf. Fig. 11) at once yielded a solution that was just as inhibitory on test at pH 4 as at pH 7. The solution obtained on heating fuconate at acid pH was no more inhibitory on testing at pH 7 than was the fresh 1,4-lactone. Fuconate itself was non-inhibitory at pH 7, but slightly inhibitory at pH 4. This was due to ring closure at the acid pH of assay, as illustrated in Fig. 10, which also shows the conversion of fucono-1,4-lactone into the more active inhibitor at pH 4, a process that went as rapidly as with the salt. The more usual type of experiment for this purpose (cf. Fig. 9) was not possible with the ox-liver enzyme, since hydrolysis was never linear with time (cf. Fig. 4).

These results can be explained by assuming formation of the 1,5-lactone at acid pH, and the establishment of an equilibrium between salt, 1,4-lactone and 1,5-lactone at pH 7. Fig. 11 shows the conversion of fuconate and fucono-1,4-lactone into fucono-1,5-lactone at varying pH. The greater

Fig. 9. Hydrolysis of 2.5 mM-*o*-nitrophenyl β -D-galactoside by a rat-epididymis preparation at pH 3 in the absence (●) and presence (○) of 1 mM-galactono-1,4-lactone; in the presence of the most inhibitory solution derived from 0.25 mM-galactonate (x) and of 1 mM-sodium galactonate (Δ) (see Experimental section).

Table 2. *Inhibition of ox-liver β -galactosidase by galactonolactone*

Substrate: 2.5 mM-*o*-nitrophenyl β -D-galactoside. Inhibitor: 5 mM-galactono-1,4-lactone or the most inhibitory solution derived therefrom (see Experimental section).

	Inhibition (%)	
	At pH 4.5	At pH 7.0
Galactono-1,4-lactone	11	16
Most inhibitory solution	65	21

Table 3. *Inhibition of ox-liver β -galactosidase by fucono-1,4-lactone, sodium fuconate and the solution obtained by boiling the latter at pH 2 for 10 min.*

	Inhibition (%)	
	At pH 4.0	At pH 7.0
Fucono-1,4-lactone	31	73
Sodium fuconate	14	Nil
Acid-treated solution	86	69

Fig. 10. Inhibition of ox-liver β -galactosidase by solutions maintained at 38° for varying periods at pH 4.0 in phosphate-citrate buffer: ○, sodium fuconate tested at pH 4.0; x, fucono-1,4-lactone tested at pH 4.0; ●, fucono-1,4-lactone tested at pH 7.0. Assays were with 1 mM-inhibitor.

stability of the lactone ring towards alkali as compared with galactonolactone (see Experimental section) can be seen. Fig. 12 gives the relative efficiency of the most inhibitory solution and freshly prepared fucono-1,4-lactone at pH 4: 50 %

inhibition was observed at 0.09 and 3.6 mM respectively (both in terms of fuconate ion). At pH 7, 0.6 mM-fucono-1,4-lactone caused 50 % inhibition.

The conditions used in Fig. 12 appeared from a few trial experiments to be optimum for obtaining the 1,5-lactone from fuconate at pH 2. The resultant solution did not inhibit rat-epididymis β -galactosidase.

DISCUSSION

If it is assumed that the only new molecular species formed in solutions of galactonic acid, fuconic acid and their 1,4-lactones under our conditions were the 1,5-lactones, it can be concluded that the latter were more powerful inhibitors of β -galactosidase than the respective 1,4-lactones. Although the glycosidase assay offers a more refined method for following changes in the composition of such solutions than do polarimetric measurements, there remain points of detail in the present work for which no explanation can be offered.

It is unlikely that even in our most inhibitory solutions more than a fraction of the anion was present as the 1,5-lactone. Nevertheless, the inhibitory power of these solutions exceeded that of the 1,4-lactone 20-fold with galactonolactone and 40-fold with fuconolactone. It follows that the presence of the 1,5-lactone in traces could alter considerably the apparent inhibitory power of specimens of the aldonic acids or of their 1,4-lactones. The aldonic acids were not themselves inhibitory, and it is not impossible that inhibition by the 1,4-lactones was always due to formation of the 1,5-lactones. Presumably the same argument extends to the inhibition of β -*N*-acetylglucosaminidase by 2-acetamido-2-deoxygalactonolactone (Findlay *et al.* 1958), and of β -glucuronidase by the unknown entity formed in mucic acid solutions under conditions that promote lactonization (Levy, 1952).

It was pointed out by Findlay *et al.* (1958) that the lactone inhibitors for β -*N*-acetylglucosaminidase and β -glucuronidase are much more powerful than those that inhibit the enzymic hydrolysis of unmodified glycosides, such as β -glucosides and β -galactosides, and produce a marked effect at μ M or lower concentration. This was attributed to the presence in the molecule of the acetamido group in the one case and the carboxyl group in the other. Where comparable, the action of fuconolactone on β -galactosidase was more powerful than that of galactonolactone, although the efficiency never approached that of saccharo-1,4-lactone or 2-acetamido-2-deoxyglucosylolactone. The primary alcohol group in the sugar residue is evidently unnecessary for the combination of a β -galactoside

Fig. 11. Inhibition of ox-liver β -galactosidase by solutions maintained at 38° for 1 hr. after adjustment to the pH shown with HCl or NaOH: sodium fuconate tested at pH 4.0 (O) and pH 7.0 (x); fucono-1,4-lactone tested at pH 4.0 (●) and pH 7.0 (Δ). All solutions were tested at 1 mM concentration.

Fig. 12. Inhibition of ox-liver β -galactosidase by varying concentrations of fucono-1,4-lactone tested at pH 4.0 (x) and pH 7.0 (O), and of the solution obtained from sodium fuconate by incubation at pH 2 for 1 hr. at 38°, tested at pH 4.0 (●).

with the ox-liver enzyme, and its replacement by a proton does not cause steric hindrance.

Inasmuch as the inhibition of glycosidases by aldono-lactones closely parallels their substrate specificity (Conchie & Levvy, 1957), the present work supports the statement of Wallenfels & Malhotra (1960) that β -galactosidase, in ox-liver preparations at least, hydrolyses β -D-fucosides. On the other hand, to judge by the differential response of the enzyme in different mammalian tissues to galactonolactone and fuconolactone, the relationship of β -D-fucosidase to β -D-galactosidase activity is a complex one, reflecting the differences observed in the properties of β -galactosidase itself. This problem is at present under investigation.

SUMMARY

1. Mammalian β -galactosidase differs in its pH optimum and other properties according to the tissue of origin. The simplest situation was seen in the rat, where there was apparently the same enzyme in different organs, with a sharp optimum at pH 3–4. The most complex situation was seen in the ox: the enzyme in liver differed from that in the genital organs and had a broad optimum, from pH 4 to 7. Attempts to separate the ox-liver enzyme into two fractions failed.

2. Ox liver differed from the other mammalian tissues examined in that the β -galactosidase activity was relatively resistant to inhibition by galactono-1,4-lactone. This was, however, the only

mammalian tissue studied in which β -galactosidase was inhibited by D-fucono-1,4-lactone.

3. Galactono- and fucono-1,5-lactone are much more powerful enzyme inhibitors than the corresponding 1,4-lactones. This can be explained from the effect of the configuration on the structure of these molecules, as compared with the substrate.

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An Investigation of Suggested Physiological Functions of Carnitine

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Carnitine (β -hydroxy- γ -butyrottrimethylbetaine) was first isolated by Gulewitsch & Krimberg (1905), and simultaneously, under the name novain, by Kutscher (1905). Its significance in metabolism was investigated sporadically during the next 25 years, but interest subsequently waned until Carter, Bhattacharyya, Weidman & Fraenkel (1952) showed that vitamin B₁₂, essential for the development of the meal worm, *Tenebrio molitor*, was identical with carnitine. A further stimulus to the renewed interest in this substance came from its commercial synthesis (Deschamps, Buu-Hoi, Le Bihan & Binon, 1954).

Carnitine is an essential growth factor for at least six organisms, *Tenebrio molitor* (the meal worm), *Tenebrio obscurus*, *Palorus ratzeburgi*, *Gnathocerus cornutus*, *Tribolium confusum* and *Tribolium castaneum*, and is widely distributed in plant and animal tissues (Fraenkel & Friedman, 1957). Although it is present in aqueous extract of animal muscle to the extent of about 3.5% of the dry weight of the protein-free extract (Bender, Wood & Palgrave, 1958) its physiological function has not been ascertained. Various functions have been postulated and some of these have been examined in the present work. It has been reported