

Metabolism of D-Glucuronolactone in Mammalian Systems

IDENTIFICATION OF D-GLUCARIC ACID AS A NORMAL CONSTITUENT OF URINE

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The inhibition of β -glucuronidase by mammalian urine has been noted by a number of workers. This complicates assay of the enzyme in urine, and the use of added β -glucuronidase to liberate excreted phenols and alcohols, including steroids, from the β -D-glucosiduronic acid conjugates. β -Glucuronidase assay is normally conducted with a chromogenic substrate, the enzymic hydrolysis of which may be diminished in the presence of urine which contains competing substrates of different affinities (see Levvy & Marsh, 1954). In addition to natural substrates for β -glucuronidase, however, mammalian urine contains true enzyme inhibitors. Abul-Fadl (1957) found both dialysable and non-dialysable material in human urine which was inhibitory to β -glucuronidase; the dialysable inhibitor was stable to treatment with hot acid. Similar inhibitory fractions were present in rabbit urine (Conzelman & Crout, 1961).

Mammalian β -glucuronidase is inhibited by heavy-metal ions (Levy & Marsh, 1957*a, b*; Fernley, 1962), but not by the common enzymic poisons azide, fluoride, cyanide and iodoacetate. It is, however, a member of the class of glycosidases which are strongly and specifically inhibited by aldolactones of corresponding configuration (Conchie & Levvy, 1957; Findlay, Levvy & Marsh, 1958). Thus the most powerful inhibitors of β -glucuronidase, for which the only known substrates are β -D-glucosiduronic acids and β -D-galactosiduronic acids (Marsh & Levvy, 1958), are D-glucaro-(1 \rightarrow 4)-lactone and the unidentified lactones in boiled mucic (galactaric) acid solution (Levy, 1952). A further examination of the dialysable material in urine indicated the presence of a compound with properties consistent with those of forming a lactone inhibitory to β -glucuronidase, and an attempt was therefore made to isolate and identify it.

A preliminary account of some of this work has been published (Marsh, 1961).

METHODS AND MATERIALS

Urine samples. Human pregnancy urine in bulk and samples of normal human 24 hr. urines (male and female) were obtained from the M.R.C. Obstetric Medicine

Research Unit, Aberdeen Royal Infirmary, to whom the author is indebted. Fresh samples of cat urine were supplied by Dr A. J. Carr, Pathology Department, Aberdeen University; 24 hr. urines of rat and guinea pig, and samples of sheep, pig and calf urines, were collected from animals maintained on normal diets in metabolism cages. The rat and guinea-pig excreta were separated by filtration, and the faeces washed with water, before the combined urine and washings were tested. Urines were stored at 0° if examined within 24 hr. of collection, or otherwise at -20°.

Treatment of urine. The following standard procedure was used in testing the inhibitory powers of mammalian urine. Crude urine (pH 4.5-7.0) was treated (a) at 100° for 40 min. after adjustment with 3N-HCl to pH 2.0-2.2, followed by readjustment with NaOH to pH 4.0-4.5, or (b) at 100° for 15 min. after adjustment with 0.1N-NaOH to pH 7.5-8.0, followed by readjustment with HCl to pH 6.0-6.5. The inhibitory power was maximal after treatment (a) and minimal after treatment (b). Either treatment was sufficient to rid the urine of β -glucuronidase activity, and after the readjustments there was no interference with the pH of the subsequent enzyme assay. Heating at a pH above 2.5 was inadequate for development of maximum inhibitory power of acidified urines, for there was then a considerable rise in pH owing to evolution of CO₂.

β -Glucuronidase assay. Soluble β -glucuronidase preparations were made from the livers of Lister strain rats. The tissue was homogenized in water, and the homogenate (10 ml./g. of tissue), containing 0.1M-acetic acid-NaOH buffer, pH 5.2, was incubated for 1 hr. at 37°, the fraction which sedimented on centrifuging at 1500g for 15 min. was then discarded. The supernatant was diluted for assay to a final volume of 200 ml./g. of moist liver. Biosynthetic phenolphthalein β -D-glucuronide (Levy & Marsh, 1959), concn. 0.63 mM, was the standard substrate for the enzyme assay, which was for 1 hr. at 37° in 0.125M-acetic acid-NaOH buffer, pH 5.2, as described by Levvy & Marsh (1959). The incubation mixture (4 ml.) included 0.5 ml. of enzyme solution, which liberated 35-40 μ g. of phenolphthalein in assays not containing urine or other solutions inhibitory to the enzyme. The assay did not require the presence of an 'activator', as is essential when β -glucuronidase preparations of higher specific activity are used (Bernfeld, Bernfeld, Nisselbaum & Fishman, 1954; Levvy, McAllan & Marsh, 1958). For the determination of their inhibitory power, treated urine samples were added in known concentration to the buffered substrate immediately before the final addition of the enzyme, and the assay was carried out as before. Extra controls were included to compensate for the inherent colour of the urine. All assays were performed in duplicate.

Other glycosidase assays. Assays of α -mannosidase, β -galactosidase and β -N-acetylglucosaminidase, with *p*-

nitrophenyl α -D-mannoside, *o*-nitrophenyl β -D-galactoside and *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide as the respective substrates, were as described by Conchie, Findlay & Levvy (1959), with a partially purified rat-epididymis preparation as the common enzyme source.

Uronic acid determination. Total uronic acid in urine samples was measured by a modification of the carbazole method of Dische (Ashwell, 1957), and colour intensity was measured on the Spekker photoelectric absorptiometer with Ilford no. 605 yellow-green filters (peak transmission 545 m μ). The addition of D-glucuronic acid (20–100 μ g./ml.) gave recoveries of 90–95%. The extinction coefficient given by free D-galacturonic acid in this method was 80% of that given by D-glucuronic acid.

Paper chromatography. The spots given by sugar acids after descending paper chromatography on Whatman no. 1 paper were developed by the aqueous acetone-silver nitrate method of Trevelyan, Procter & Harrison (1950), and fixed with 5% (w/v) sodium thiosulphate (Hulme, 1961).

D-Glucurono-(6 \rightarrow 3)-lactone (Corn Products Refining Co.) was recrystallized from water and then had m.p. 175–177°; it was converted into D-glucuronate in aqueous solution by titration with NaOH to constant pH (8.0).

Potassium hydrogen D-glucarate (potassium hydrogen saccharate; British Drug Houses Ltd.), the source of D-glucarate solutions, and galactaric acid (mucic acid; British Drug Houses Ltd.) were recrystallized once from water. The following were used without further purification: D-galacturonic acid monohydrate and sodium D-gluconate (British Drug Houses Ltd.), and L-ascorbic acid (Roche Products Ltd.).

Determinations of C, H and N were made by Weiler and Strauss, Oxford. All melting points are corrected.

EXPERIMENTAL

Properties of the urine β -glucuronidase inhibitor

Effect of acid treatment. Samples of normal human urine were boiled for 15 min. to inactivate β -glucuronidase, and the supernatant after sedimentation of denatured protein was found to inhibit β -glucuronidase. The inhibitory power of the urine was considerably increased on further boiling after acidification. Thus, in a typical experiment, 25% (v/v) normal male-human urine caused 74% inhibition of rat-liver β -glucuronidase after the urine had undergone standard acid treatment at pH 2.0–2.2 (see the Methods and Materials section), but caused only 43% inhibition after alkali treatment of the urine at pH 7.5–8.0. When preparations were treated thus and added to the enzyme assay in equal final concentration, the inhibitory effects with crude urine were identical with those of both the diffusate and of the residue after dialysis of the urine for 3 days at 0° against an equal volume of water. The residue freed from all dialysable material was non-inhibitory after boiling at acid or alkaline pH. Thus the factors in boiled urine inhibitory to β -glucuronidase were dialysable, and in subsequent experiments only

crude urine samples were employed, unless otherwise stated.

Reversibility of the acid treatment. The high inhibitory power of the urine after acid treatment was decreased by subsequent treatment at pH 7.5–8.0, and then re-established by a second acid treatment. Similar results were obtained by initially treating the urine with alkali and then subjecting it to alternate acid and alkali treatments (Table 1). Acid treatment therefore converted an entity normally present in urine into a more powerful inhibitor of β -glucuronidase, which was again transformed by alkali, presumably to the original compound. Pregnancy urine gave similar results but was more inhibitory after acid treatment.

Effect of extraction with ethyl acetate. Boiled urine, which, when present in 25% (v/v) concentration in the β -glucuronidase assay, caused 69 and 45% inhibition of the enzyme after acid and alkali treatments respectively, was extracted for 2 hr. with ethyl acetate (analytical reagent) in a continuous liquid-liquid micro-extractor (Levy, 1948). The aqueous residue then displayed greater acid potentiation of enzyme inhibition (57% inhibition after acid treatment, 22% inhibition after alkali treatment, at a urine concentration of 25% in the assay). The residue after evaporation of the ethyl acetate extract was, however, equally inhibitory (34% inhibition at a urine concentration of 25%) after acid or alkali treatment.

It would thus seem that there is a dialysable entity present in urine which after alkali treatment has considerably less inhibitory activity towards β -glucuronidase, but which acid treatment converts reversibly into a potent inhibitor. Inhibition after acid or alkali treatments is augmented by the presence of other material, including probably glucosiduronic acids of endogenous origin, soluble in ethyl acetate at pH 2.

Table 1. *Effect of successive acid and alkali treatments on the inhibitory powers of human normal female and pregnancy urine*

The urine was subjected to alternate standard acid and alkali treatments (see the Methods and Materials section) and tested at 25% (v/v) concentration in the β -glucuronidase assay as described in the text.

Procedure	Inhibition after treatment (%)	
	Normal urine	Pregnancy urine
First treatment (acid)	65	82
Second treatment (alkali)	44	46
Third treatment (acid)	66	80
First treatment (alkali)	47	48
Second treatment (acid)	66	84
Third treatment (alkali)	46	49

Table 2. *Inhibition by human pregnancy urine of mammalian glycosidases*

Liberation by a rat-epididymis preparation of *o*-nitrophenol from 2.5 mM-*o*-nitrophenyl β -galactoside, *p*-nitrophenol from 2 mM-*p*-nitrophenyl α -mannoside or 5 mM-*p*-nitrophenyl *N*-acetyl- β -glucosaminide, and by a rat-liver preparation of phenolphthalein from 0.63 mM-phenolphthalein β -glucuronide, were determined as described in the text. Urine was given the standard acid or alkali treatment before addition to the assay mixtures.

Enzyme	Concn. of urine (v/v) in assay mixture (%)	Inhibition by urine (%)	
		After acid treatment	After alkali treatment
β -Galactosidase	25	3	5
α -Mannosidase	12.5	0	0
β - <i>N</i> -Acetylglucosaminidase	6.3	4	3
β -Glucuronidase	25	87	43
β -Glucuronidase	6.3	58	16

Specificity of inhibition. Urine boiled at acid and alkaline pH was tested as an inhibitor of the mammalian glycosidases β -galactosidase, α -mannosidase and β -*N*-acetylglucosaminidase (Table 2). (Human pregnancy urine was employed here since it displayed greater inhibitory activity to β -glucuronidase than normal urine after acid treatment.) There was negligible inhibition of these enzymes at concentrations of urine giving appreciable inhibition of β -glucuronidase. The inhibitory factors present in the urine were thus specific for β -glucuronidase.

Increase of inhibitory power at pH of assay. Development of inhibitory activity of urine also occurred at the pH and temperature of normal β -glucuronidase assay but less rapidly than at pH 2 and 100°.

Fig. 1 shows the effect of preincubating pregnancy urine, previously boiled at pH 7.5, for various periods at pH 4.5, 5.2 and 6.1, when included at 10% (v/v) concentration in the assay of the rat-liver enzyme for 1 hr. Without prior incubation the percentage inhibition by urine was greater at pH 4.5 than at pH 5.2 or 6.1; β -glucuronidase activity at pH 6.1 was less than 50% of the optimum. On incubation before the assay, the inhibition by urine increased appreciably at pH 4.5 or 5.2, but remained constant at pH 6.1, owing to non-conversion into the acid-potentiated inhibitor. The results with normal female urine were similar, but the increase in inhibitory power was less marked since there was a smaller amount of acid-potentiated inhibitor present initially.

Effect of addition of ascorbic acid. Mammalian β -glucuronidase is strongly inhibited by heavy-metal ions, and inhibition by Cu^{2+} ion is greatly potentiated by the presence of L-ascorbate, presumably owing to formation of the inactivating Cu^+ ion (Levy & Marsh, 1957a; Fernley, 1962). In order to eliminate the possibility that the inhibition of acidified urine was partly due to the combination of ascorbic acid and traces of copper (see Levy & Marsh, 1959), the addition of 1 mM-L-

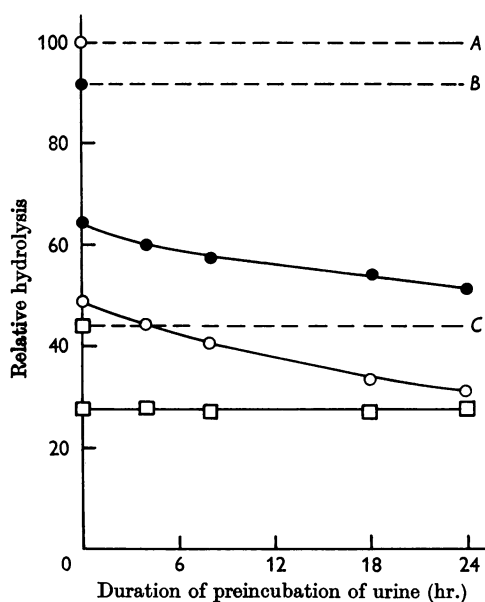


Fig. 1. Hydrolysis of 0.63 mM-phenolphthalein β -D-glucuronide by rat-liver β -glucuronidase in the presence of 10% (v/v) human pregnancy urine previously incubated in buffer at 37° for various periods: O, in 0.25M-acetate, pH 4.5; ●, in 0.25M-acetate, pH 5.2; □, in 0.1M-phosphate, pH 6.1. Enzyme assays were for 1 hr. made in the same buffers. The broken lines, A, B and C, show the levels of enzyme activity at pH 4.5, 5.2 and 6.1 respectively in the absence of urine.

ascorbate to the urine was examined. No effect on the inhibitory powers of the urine after acid or alkali treatment was noted, and the potentiation of the inhibition by acid was fully reversible as before.

Variation of inhibitor and substrate concentration. The inhibition on varying the concentration of the acid- and alkali-treated urine is shown in Fig. 2; to achieve concentrations up to 100% the urine was initially concentrated to 50% of the original volume under reduced pressure at 45°. At high urine con-

centrations, more than 90% inhibition of the enzyme could be achieved, but error due to interference by the inherent urine colour in the assay then became appreciable.

Examination, by the graphical method of Lineweaver & Burk (1934), of the inhibitions at fixed urine and variable substrate concentrations showed that the inhibitory factors in both acid- and alkali-treated urine acted competitively.

Measurement of the acid-potentiated inhibitor in urine

To provide a basis for the comparison of excretion rates of the acid-potentiated inhibitor in urine under different conditions, an arbitrary unit was selected, namely 1 'unit of inhibitor' (U.I.), which was defined as that quantity which produced 50% inhibition of rat-liver β -glucuronidase in the standard assay at pH 5.2 with 0.63 mM-phenolphthalein β -glucuronide as substrate. From the graph (Fig. 2) of inhibition against concentration of acid-treated urine, the U.I. present in a urine specimen could be measured. Correction was made for the augmentation of inhibition due to the alkali-stable factor, this being measured in U.I. from the same curve. The inhibition curves for acid- and alkali-treated urine were approximately

parallel. Thus the inhibitory power (in U.I.) of the alkali-treated urine was subtracted from that of the acid-treated urine at the same concentration, to provide a measure of the acid-potentiated inhibitor present. This method of measurement was justifiable subject to the following assumptions: (a) the inhibitory power of the other substances present in the alkali-treated urine was independent of pH; (b) the entity which displayed inhibitory activity after acid treatment was virtually non-inhibitory at the same concentration after alkali treatment; (c) inhibitions by this entity and the other inhibitory substances were entirely additive.

Substantiation of these assumptions could only be made by complete separation of the acid-potentiated inhibitor from other inhibitory compounds in the urine. However, the removal by ethyl acetate of a fraction equally inhibitory after acid and alkali treatment to leave a residue with more pronounced acid-potentiated inhibition, and the pronounced increase in excretion of acid-potentiated inhibitor after ingestion of D-glucuronolactone (see below), suggested their validity.

Comparison of urines of different mammalian species. An acid-potentiated β -glucuronidase inhibitor, having the same properties as that in human urine, was found in the urines of all other mammalian species tested (Table 3). There was considerable variation in its concentration between individuals of the same species. Estimation of the daily excretion in man, rat and guinea pig was made by the collection of 24 hr. urine samples. The excretion by normal human subjects was fairly constant, being somewhat higher in males than in females.

Influence of ingestion of D-glucuronolactone. Freshly prepared aqueous solutions of D-glucuronolactone given orally elevated greatly the excretion of the acid-potentiated inhibitor within 24 hr. of ingestion (Table 4). With human subjects a single dose of 5 g. of D-glucuronolactone was given, whereas in the rat and guinea pig the dose was repeated over consecutive days. D-Glucuronolactone is a relatively innocuous compound when ingested; Deichmann & Hopfenspirger (1951) state the lethal dose for rats to be 20 g./kg. body wt. after oral administration.

The increase of excretion of the inhibitor in man was 100-fold in the 24 hr. after ingestion of D-glucuronolactone, returning to a normal value after 48 hr. The corresponding increases in the excretion of inhibitor in the guinea pig and rat, given proportionally higher oral doses than the human subject, were 500- and 10-fold respectively during the 24 hr. after the first dose, and were maintained by further doses of D-glucuronolactone. The increased excretion by man of total uronic acid, measured as D-glucuronic acid, within 24 hr.

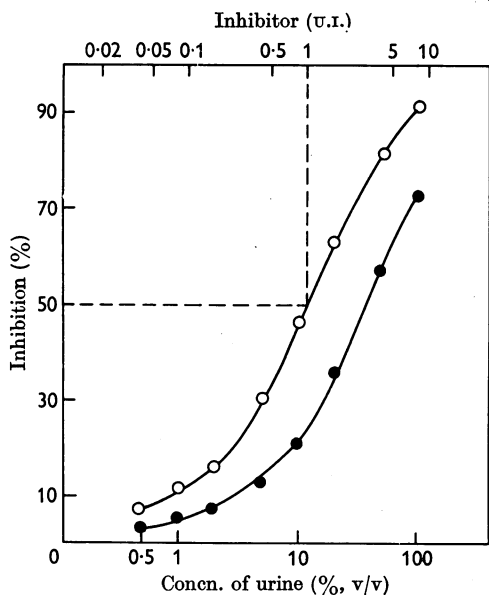


Fig. 2. Inhibition of rat-liver β -glucuronidase activity at pH 5.2 by various concentrations of normal human urine after standard treatment: ○, with acid; ●, with alkali (for details see the text). The assays were carried out in 0.125M-acetate buffer, with 0.63 mM-phenolphthalein β -D-glucuronide as substrate. The upper horizontal scale indicates the method of calculation of arbitrary inhibitor units (U.I.) in the urine; 1 U.I. causes 50% enzyme inhibition.

Table 3. *Excretion of the acid-potentiated β -glucuronidase inhibitor by different species*

Animals were normal adults unless specified, and maintained on normal diets. The inhibitor was assayed as described in the text, and its activity expressed as u.i. (for definition see the text). Results are given per ml. of urine (ranges, with numbers of animals in parentheses) and per 24 hr. urine (means \pm S.D.) where available.

Species	Sex	Excretion of inhibitor	
		u.i./ml. of urine	u.i./24 hr. urine
Man	Male	0.87-3.88 (16)	2197 \pm 425
Man	Female	1.27-3.18 (14)	1698 \pm 337
Rat	Male	6.7 (1)	24
Guinea pig	Male	0.4 (1)	29
Pig	Unspecified	1.9-3.9 (5)	—
Cat	Unspecified	0.2-0.9 (4)	—
Sheep	Unspecified	0.9-2.5 (6)	—
Calf	Unspecified	0.3-1.8 (5)	—

Table 4. *Effect of orally administered D-glucuronolactone on the excretion of acid-potentiated β -glucuronidase inhibitor and of total uronic acid in different species*

A single dose (5 g., 28 m-moles) was given to the male human subject. The rat and guinea pig (both male adults) were given 1 and 2 g. respectively (in aq. 5% (w/v) solution) by mouth and were then allowed unlimited access to water; the doses were repeated after 24 and 48 hr. Assays were carried out as described in the text. For definition of u.i. see the text. Uronic acid was determined as D-glucuronic acid.

Excretion period	Content in 24 hr. urine					
	Inhibitor (u.i.)			Uronic acid (mg.)		
	Man	Rat	Guinea pig	Man	Rat	Guinea pig
0-24 hr. before first dose	1 650	24	29	620	11	23
0-24 hr. after first dose	180 500	266	11 700	1 320	63	178
24-48 hr. after first dose	17 100	480	14 500	810	44	246
48-72 hr. after first dose	2 400	340	10 200	440	21	336

Table 5. *Effect of ingestion of sugar acids on the excretion of acid-potentiated β -glucuronidase inhibitor and of total uronic acid by man*

A single dose (28 m-moles) was given orally to male human subjects; 24 hr. urines were collected immediately before administration, and 0-24 hr. and 24-48 hr. afterwards. Assays were carried out as described in the text. For definition of u.i. see the text. Uronic acid was determined as D-glucuronic acid.

Compound	Content in 24 hr. urine					
	Before dose 0-24 hr.	Inhibitor (u.i.)		Uronic acid (mg.)		
		After dose		Before dose 0-24 hr.	After dose	
		0-24 hr.	24-48 hr.		0-24 hr.	24-48 hr.
D-Glucuronate	1890	2540	1300	384	444	395
D-Galacturonate	1590	1770	1545	414	483	438
D-Gluconate	2340	2360	—	—	—	—

of ingestion of D-glucuronolactone, corresponded to about 18% of the dose, in general agreement with the findings of Fishman *et al.* (1951); the guinea pig and rat excreted 11 and 3% respectively of the total dose of D-glucuronolactone.

The properties of boiled human urine after ingestion of D-glucuronolactone were similar to those of normal urine in that the inhibitory factors were dialysable and that acid potentiation of inhibition was fully reversible. At concentrations of acid-treated urine which gave considerable inhibi-

tion (70-80%) of β -glucuronidase, the inhibitory power of alkali-treated urine was now very low (less than 5% inhibition), suggesting that the acid-potentiated inhibitor was non-inhibitory after alkali treatment. In other words, the residual activities of normal urine are not due to the acid-potentiated factor in its alternative form.

Ingestion of D-glucuronic acid and other sugar acids. The excretion of acid-potentiated inhibitor in human urine was only slightly increased after the ingestion of D-glucuronate, and unchanged after

ingestion of similar quantities of D-galacturonate and D-gluconate (Table 5). Excretion of uronic acid was little affected by ingestion of D-gluconate or D-galacturonate (see the Discussion section).

Isolation and identification of the acid-potentiated β -glucuronidase inhibitor in human urine

The general properties of the acid-potentiated inhibitor in mammalian urine suggested the presence of a sugar acid readily convertible by acid into an inhibitory lactone, and the specific nature of the inhibition for β -glucuronidase led to the belief that the inhibitory entity might be a lactone of D-glucaric acid or galactaric acid, these being the most potent inhibitors of mammalian β -glucuronidase. The great increase in the excretion of the inhibitor after ingestion of D-gluconolactone supported this assumption; oxidation of the C-1 reducing group of D-gluconolactone would lead directly to the formation of D-glucaric acid, whereas inversion at C-4 would also be required to produce galactaric acid. D-Glucaric acid boiled at an acid pH is converted in 30% yield into D-glucaro-(1 \rightarrow 4)-lactone, the specific β -glucuronidase inhibitor (Levy, 1952). When tested, addition of 24.1 μ m-moles (5.06 μ g.) of boiled D-glucaric acid to the assay gave 50% inhibition of rat-liver β -glucuronidase; this quantity was thus equivalent in inhibitory power to 1 unit of the acid-potentiated urine inhibitor. The excretion of 180 000 units of the inhibitor within 24 hr. of a dose of 28 m-moles (5 g.) of D-gluconolactone (Table 4) was thus equivalent to 4.34 m-moles of boiled D-glucaric acid, i.e. 16% of the dose. Since the inhibitory power of boiled galactaric acid is only 2% that of boiled D-glucaric acid (Levy, 1952), conversion into galactaric acid could not account for the high inhibitory power of acid-treated urine after ingestion of D-gluconolactone.

Isolation of D-glucaric acid from pregnancy urine. The acid-potentiated urine inhibitor in human pregnancy urine was concentrated by the procedure given in Scheme 1. This included the partial removal of less soluble inorganic salts etc., removal of endogenous glucuronides by extraction with ethyl acetate after acidification, and precipitation of the inhibitor as a lead salt which was then decomposed with H₂S. The final product consisted of a yellow syrupy aqueous solution containing the inhibitor as an ammonium salt; its inhibitor content (in U.I.) was equivalent to 294 mg. of D-glucarate. This solution (65 ml.) was heated with 10 g. of phenylhydrazine hydrochloride and 2.5 g. of anhydrous sodium acetate for 5 min. at 100°, and the tarry material formed was removed by decantation of the hot mixture. A further 5 g. of phenylhydrazine hydrochloride was added to the aqueous residue, and the mixture heated for a further 150 min. at 100°. When cooled overnight at 0°, it deposited a yellowish crystalline solid (0.31 g.), which after recrystal-

lization from aq. 50% (v/v) ethanol yielded 0.26 g., m.p. 209–211° (decomp.) (Found: C, 55.5; H, 5.4; N, 14.1. Calc. for C₁₈H₂₂N₄O₈: C, 55.4; H, 5.7; N, 14.4%). Authentic D-glucaric acid bisphenylhydrazide, m.p. 210° (decomp.) and galactaric acid bisphenylhydrazide, m.p. 245° (decomp.) were prepared by similar methods; Maquenne (1887) also found the D-glucaric acid derivative to have m.p. 210° (decomp.), and Bülow (1886) gives a value of m.p. 240° (decomp.) for the galactaric acid derivative. There was no depression of m.p. of the product derived from urine when mixed with authentic D-glucaric acid bisphenylhydrazide, and it was appreciably more soluble than the authentic galactaric acid derivative. Assuming the inhibitor before condensation to be all D-glucarate, the recrystallized product represented a yield of 48% on condensation with phenylhydrazine hydrochloride.

Isolation of D-glucaric acid from urine after ingestion of D-gluconolactone. The same product was isolated, by a similar but simpler procedure to that outlined above, from male human urine excreted within 24 hr. of a 5 g. oral dose of D-gluconolactone. The urine (1890 ml. containing 150 000 U.I.) was concentrated under reduced pressure to 800 ml. and boiled for 15 min. The supernatant (containing 130 000 U.I.) after centrifuging was further concentrated to 250 ml., filtered after the addition of charcoal, and excess of aqueous normal lead acetate (20%, w/v) stirred in. The insoluble lead salt was sedimented, washed on the centrifuge with 1% (w/v) lead acetate, suspended in water and decomposed with H₂S. The solution (containing 89 000 U.I.), after removal of lead sulphide, was neutralized with aqueous ammonia, treated with charcoal, filtered, concentrated to 25 ml. and filtered from the small amount of deposited solid. The filtrate (containing 84 000 U.I., equivalent to 425 mg. of D-glucaric acid) was heated with 4 g. of phenylhydrazine hydrochloride and 1 g. of anhydrous sodium acetate for 5 min. at 100°, decanted hot from gummy material and further heated for 150 min. The cream-coloured crystalline solid (0.63 g.) which separated overnight at 0° had m.p. 203–205° (decomp.) and after recrystallization from aq. 50% (v/v) ethanol the m.p. was 210–211° with no depression on admixture with authentic D-glucaric acid bisphenylhydrazide, m.p. 210° (decomp.) (Found: C, 55.2; H, 5.9; N, 14.9. Calc. for C₁₈H₂₂N₄O₈: C, 55.4; H, 5.7; N, 14.4%). The recrystallized material represented a yield of 63% on coupling with phenylhydrazine, if the inhibitor is assumed to be all D-glucaric acid.

Recoveries on addition of D-glucaric acid and D-gluconolactone to urine. To 2 l. of pregnancy urine, containing 4600 U.I., was added 1 g. of potassium hydrogen D-glucarate (equivalent to 168 000 U.I.). Assay of the urine then gave a total of 173 000 U.I.; thus inhibition by the inhibitor in urine and by boiled D-glucarate were fully additive. The urine was then subjected to the same treatment as that given to the urine after ingestion of D-gluconolactone, giving a final solution (containing 114 000 U.I.) which on condensation with phenylhydrazine hydrochloride yielded D-glucaric acid bisphenylhydrazide, m.p. 204–206° (0.65 g., 61% yield).

To a further 2 l. of the same sample of pregnancy urine was added D-gluconolactone (2 g.). The urine when assayed then showed no change in inhibitory power after acid treatment, but inhibition after alkali treatment was

increased slightly owing to conversion into D-glucuronic acid, which, unlike its lactone, is a feeble inhibitor of the enzyme (Levy, 1952). Preparation of the lead salt etc., as above, then yielded only a trace of product after condensation with phenylhydrazine hydrochloride.

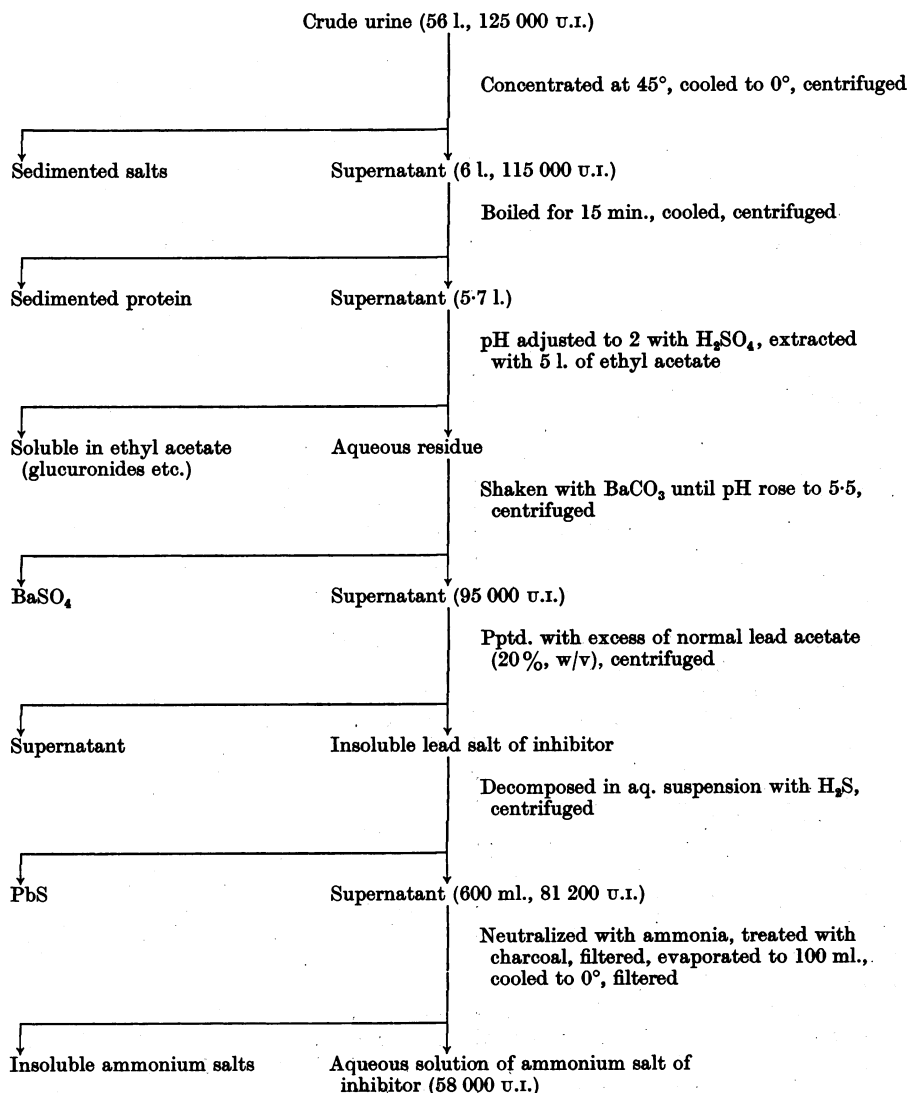
Hydrolysis of phenylhydrazine conjugates from urine and examination of the products by paper chromatography. Finely powdered authentic D-glucuric acid bisphenylhydrazide (500 mg.) was shaken at 20° for 2 hr. with 60 ml. of ethanol-aq. ammonia (sp.gr. 0.88)-water (3:2:1, by vol.); the residual solid after filtration was shaken with a further similar mixture (60 ml.) for 3 hr., when all was dissolved. The combined solutions were concentrated under reduced pressure and extracted with ether to remove traces of phenylhydrazine. A test of a sample for inhibition of β -

glucuronidase after acidifying and boiling indicated a recovery of 90% of D-glucarate from the hydrolysis of the bisphenylhydrazide.

The products (15 mg.) from the condensation with phenylhydrazine hydrochloride of concentrates of pregnancy urine and of urine after ingestion of D-glucuronolactone (see above) were likewise shaken with ethanol-aq. ammonia (sp.gr. 0.88)-water. The resulting solutions, tested as D-glucarate for β -glucuronidase inhibition, and on assuming the starting material to be the bisphenylhydrazide, gave recoveries of 80 and 74% respectively for the hydrolysis.

The three hydrolysis products were examined by paper chromatography and gave the following identical patterns.

(a) In propan-1-ol-aq. ammonia (sp.gr. 0.88)-water



Scheme 1. Partial purification of the acid-potentiated β -glucuronidase inhibitor in pregnancy urine. For definition of U.I. see the text.

(6:3:1, by vol.) (Hanes & Isherwood, 1949). All the products gave a spot at R_f 0.34 with a faint spot at R_f 0.24. Hulme (1961) gives R_f 0.23 for D-glucarate in this solvent. The faster-running spot was probably due to the presence of a lactone, since evaporation of the ammoniacal solution of the hydrolysate gave a final pH of 5.9, and authentic ammonium D-glucarate, prepared from D-glucaro-(6→3)-lactone, gave a much stronger spot at R_f 0.24.

(b) In butan-1-ol-acetic acid-water (4:1:5, by vol.). The hydrolysates, and also authentic ammonium saccharate, were shaken with Amberlite resin IR-120 (H^+ form), to remove NH_4^+ ion, before testing. Each of the four specimens was identical in giving a strong spot at R_f 0.10, presumably the free D-glucaric acid, and much weaker spots at R_f 0.23 and 0.30, presumably the (1→4)- and (6→3)-monolactones.

DISCUSSION

From the experimental evidence it may be concluded that D-glucaric acid is a normal constituent of mammalian urine, and the greatly increased excretion of this compound immediately after ingestion of D-glucuronolactone strongly suggests that D-glucarate is a product of D-glucuronic acid metabolism. Mayer (1901) claimed to have isolated D-glucaric acid as its bisphenylhydrazide from the urine of young dogs after subcutaneous injection of D-gluconate, but this work could not be confirmed by Schott (1911); Stetten & Stetten (1950) were unable to detect radioactive D-glucarate in the urine after intraperitoneal injection of ^{14}C -labelled D-gluconate into rats. If, as seems probable, D-glucarate is entirely responsible for the acid-potentiated component of β -glucuronidase inhibition by urine, the human (non-pregnant) excretion is about 10 mg. of D-glucaric acid per day. The concentration of D-glucarate in urine is thus not inconsiderable, but the lack of a unique chemical reaction for the detection of glycaric acids, coupled with their ready transformation into several lactones, makes it difficult to identify and to isolate them from a complex mixture by conventional methods. The method of enzymic assay, depending on the high specificity of boiled D-glucarate as a β -glucuronidase inhibitor, has now made possible its estimation in concentrations of the order of $1 \mu g./ml.$

Bacterial decomposition of D-glucuronic acid occurs on standing (Keutel, Schweisfurth & Litos, 1961), and Kilgore & Starr (1959) found that cell-free extracts of some phytopathic pseudomonads contained an enzyme capable of oxidizing D-glucuronate to a product believed to be D-glucaric acid. However, there was no change in the acid-potentiated β -glucuronidase inhibition by normal human urine after it had stood for 7 weeks at 0° , or for 2 weeks at 20° (A. J. Hay, personal communication); moreover, after ingestion of D-glucuronolactone the excretion of D-glucarate in man in-

creased 100-fold and that of uronic acid only 10-fold. It is thus unlikely that the D-glucarate in urine is a product of microbial action; this was confirmed by the identification of a mammalian-liver enzyme which in the presence of NAD converted D-glucuronolactone into a product having properties identical with those of the urine inhibitor (Marsh, 1961).

D-Glucuronolactone, but not D-glucuronate, when injected into rats, is partly converted into L-ascorbic acid with retention of the intact carbon chain (Burns & Evans, 1956), and the production of D-glucaric acid from D-glucuronolactone in mammals, including those able to synthesize the vitamin, implies the existence of a pathway which can compete directly with the production of ascorbic acid. This may account for the much greater increase in excretion of D-glucarate in the guinea pig than in the rat after ingestion of D-glucuronolactone (Table 4).

The results of ingestion of D-glucuronolactone contrasted sharply with those of ingestion of D-glucuronate or of D-galacturonate, neither of which elevated appreciably the excretion of either the inhibitor or the uronic acid; obviously there is little conversion of D-glucuronate into D-glucuronolactone in the mammalian stomach, despite the favourable acid conditions before absorption. With D-galacturonate the lack of effect may be due to non-absorption through the gut; Werch & Ivy (1941) found that after introduction of D-galacturonic acid into the ileum or colon of dogs, or into the ileum of a human subject, less than 10% was absorbed after 2 hr. D-Glucuronate may undergo extensive metabolic change during and after a slow absorption process. Packham & Butler (1954) found that negligible [^{14}C]carbon dioxide was respired after intravenous or intraperitoneal injection of uniformly labelled D-glucuronate into rats, and nearly all the radioactivity was excreted in the urine (E. C. Butler, personal communication to Dr G. A. Levvy), whereas, after oral ingestion of the same compound, 58% of the radioactivity was respired within 8 hr., only 5% occurred in the urine and a large proportion was retained in the gut (Packham & Butler, 1952); (see Levvy, 1956, for discussion). Oral administration of D-[6- ^{14}C]-glucuronic acid to humans also resulted in most of the dose being accounted for in the respired carbon dioxide (Baker, Sauberlich, Wolfskill, Wallace & Dean, 1962). Administration by injection or ingestion of ^{14}C -labelled D-glucuronolactone (Douglas & King, 1952, 1953; Packham & Butler, 1954; Baker *et al.* 1962) showed that this compound is metabolized more rapidly than D-glucuronate, and that contrary to the assumption by some workers (see Douglas & King, 1952, 1953; Fishman *et al.* 1951) D-glucuronolactone does not equilibrate

rapidly *in vivo* with the free acid. After intraperitoneal or intravenous injection to rats, approximately half the radioactivity of administered D-glucuronolactone was excreted within 24 hr. in the urine, whereas a minimum of 24–37% of the dose was excreted unchanged (Packham & Butler, 1954). A rather higher value for the proportion of excreted free uronic acid was obtained by Douglas & King (1953), who, however, employed chemical oxidation to D-glucarate to identify D-glucuronic acid; some contamination by D-glucarate in the urine was thus possible.

Ingestion of D-glucuronolactone by man was found by Flaschenträger, Cagianut & Meier (1945) to give a fivefold increase in the excretion of furan-2,5-dicarboxylic acid, and it was suggested that this was another product of glucuronic acid metabolism. However, the method used by these workers to isolate the compound involved heating a dried urine extract with 25% sulphuric acid for 1 hr., followed by steam-distillation. By prolonged heating of potassium hydrogen D-glucarate with strong acid, furan-2,5-dicarboxylic acid may be prepared in good yield (Haworth, Jones & Higgins, 1945; Cope & Keller, 1956); the reaction requires the loss of 3 molecules of water per molecule of D-glucaric acid. It therefore appears probable that much of the furan-2,5-dicarboxylic acid isolated after ingestion of D-glucuronolactone arises by this process.

The presence of D-glucarate in urine may have an important effect on the hydrolysis of urinary β -glucosiduronic acids by added β -glucuronidase preparations; this has been discussed by Marsh (1962). It may also influence the measurement of β -glucuronidase activity in urine, for, though D-glucarate is stable in solution above pH 6.1, more acid conditions cause partial conversion into the highly inhibitory (1 \rightarrow 4)-lactone, even at room temperature (Levy, 1952). The apparent enzyme activity may thus be low, particularly in strongly acid urines, and assay of urinary β -glucuronidase at pH 4.6, the optimum found by Mead, Smith & Williams (1955), will cause further conversion into the inhibitory lactone during the incubation period, as shown experimentally in this paper. This consequence is of particular importance if the usual chromophoric substrates are employed, for these necessitate the use of prolonged incubation periods for the measurement of weak β -glucuronidase activity, e.g. in body fluids. Recourse may thus be necessary to the much more sensitive, if less simple, fluorimetric method with 4-methylumbelliferone β -D-glucosiduronic acid as substrate (Mead *et al.* 1955; Levy, Hay & Marsh, 1957); this method requires short incubation periods and a much lower proportion of enzyme preparation in the assay mixture, thus reducing the inhibitor concentra-

tion. Values published for the normal output of β -glucuronidase in urine are probably therefore low.

SUMMARY

1. Mammalian urine contained a dialysable heat-stable factor which after acid treatment was a specific competitive inhibitor for β -glucuronidase; inhibition became maximal after urine treatment at pH 2 and 100°, but potentiation was significant at the pH and temperature of β -glucuronidase assay. The inhibitor potentiation was reversed by alkali treatment and re-established by further acid treatment.

2. Oral dosage of D-glucuronolactone to man, guinea pig and rat elevated greatly the excretion of the factor, whereas D-glucuronate or D-galacturonate administered to man had no appreciable effect on the excretion of the inhibitor or of uronic acid.

3. D-Glucaric acid was isolated from human pregnancy urine, which contained larger quantities of the factor than normal women's urine, and from human urine after dosage with D-glucuronolactone. It was identified as the bisphenylhydrazide derivative and by chromatographic examination.

4. It was concluded that D-glucarate was responsible for the acid potentiation of the inhibition of β -glucuronidase by normal urine, through partial conversion into the (1 \rightarrow 4)-lactone, and that D-glucaric acid is a product of D-glucuronolactone metabolism in mammals.

5. The significance of this work on the measurement of urinary β -glucuronidase activity, and on the isolation from urine of furan-2,5-dicarboxylic acid, is discussed.

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The Free Iodotyrosines of the Rat Thyroid Gland

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Iodide in the thyroid gland is derived from two sources: (a) the blood, and (b) precursors containing organic iodine, thought to be free iodotyrosines, formed during proteolysis of thyroglobulin. Iodide that enters the gland from the circulation has been called the 'first iodide pool'; iodide derived from breakdown products within the gland, the 'second iodide pool' (Hickey & Brownell, 1954). The former is discharged from the thyroid by perchlorate and thiocyanate; the latter is not.

The iodide of the thyroid has been determined (as $^{131}\text{I}^-$ ion) by a number of workers using different techniques (Wollman & Scow, 1953; Ingbar & Freinkel, 1956; Rosenberg, Athans & Behar, 1960; Wollman, 1962); it appears to represent 0.2–0.3% of the total thyroidal ^{131}I .

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The second iodide pool is larger than the first; its size has been estimated in the rat thyroid (Halmi & Pitt-Rivers, 1962) to be approximately 0.26% of the thyroidal iodine, a finding that has been confirmed by Nagataki & Ingbar (1963); it is nearly 100 times as large as the first pool.

Halmi & Pitt-Rivers (1962) estimated the specific activity of iodide in the second pool at different times up to 24 hr. after single injections of [^{131}I]-iodide and compared the values with the specific activity of thyroglobulin-bound mono- and di-iodotyrosine at corresponding times, determined in a previous study (Pitt-Rivers, 1962). During the first 8 hr., the specific activity of second-pool iodide rose at a faster rate and reached a maximum sooner than that of any organic iodinated compound. It was therefore suggested that monoiodotyrosine and possibly di-iodotyrosine in thyroglobulin might be heterogeneous with respect to turnover; the fraction turning over more rapidly