This work was supported by Research Grant no. AT (30-1) 2184 from the U.S. Atomic Energy Commission and by Grant no. 5K3-GM-242-03 from the U.S. Public Health Service.

REFERENCES

- Borek, E. & Rittenberg, D. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 777.
- Botter, R. & Nief, G. (1959). In *Advances in Mass Spectrometry*, p. 515. Ed. by Waldron, J. D. New York: Pergamon Press.
- Chorney, W., Scully, N. J., Crespi, H. L. & Katz, J. J. (1960). *Biochim. biophys. Acta*, **37**, 280.
- De Giovanni, R. (1960). *Ann. N.Y. Acad. Sci.* **84**, no. 16, 644.
- De Giovanni, R. (1961). *Z. VererbLehre*, **92**, 389.
- De Giovanni, R. & Zamenhof, S. (1959). *Rec. Genet. Soc. Amer.* **28**, 65.
- Graff, J. & Rittenberg, D. (1952). *Analyt. Chem.* **24**, 878.
- Konrad, M. (1960). *Ann. N.Y. Acad. Sci.* **84**, no. 16, 678.
- Marmur, J. & Schildkraut, C. L. (1961). *Nature, Lond.*, **189**, 636.
- Marshak, A. & Vogel, H. J. (1951). *J. biol. Chem.* **189**, 597.
- Moses, V., Holm-Hansen, O. & Calvin, M. (1958). *Biochim. biophys. Acta*, **28**, 62.
- Rittenberg, S. M. & Borek, E. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1772.
- Vischer, E. & Chargaff, E. (1948). *J. biol. Chem.* **176**, 703.
- Weinberger, D. & Porter, J. W. (1954). *Arch. Biochem. Biophys.* **50**, 160.
- Wyatt, C. R. (1951). *Biochem. J.* **48**, 584.
- Zamenhof, S. (1957). In *Methods in Enzymology*, vol. 3, p. 696. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Zamenhof, S., Reiner, B., De Giovanni, R. & Rich, K. (1956). *J. biol. Chem.* **219**, 165.

Biochem. J. (1963) **87**, 82

Metabolism of D-Glucuronolactone in Mammalian Systems

2. CONVERSION OF D-GLUCURONOLACTONE INTO D-GLUCARIC ACID BY TISSUE PREPARATIONS*

By C. A. MARSH

Rowett Research Institute, Bucksburn, Aberdeen

(Received 22 October 1962)

Normal mammalian urine contains D-glucaric acid, which is partly responsible for the inhibitory effect of boiled urine on β -glucuronidase (Marsh, 1962, 1963). Excretion of D-glucaric acid by normal human adults was about 10 mg./day, but it was increased approximately 100-fold within 24 hr. of the oral administration of a 5 g. dose of D-glucurono-(6 $\rightarrow$ 3)-lactone, whereas the administration of D-glucuronic acid had little effect. From their close structural similarity it seemed probable that D-glucuronolactone was a direct precursor of biosynthetic D-glucaric acid, and mammalian tissue preparations were examined with the purpose of identifying the enzyme system that would accomplish such a conversion. The probable source of D-glucuronolactone *in vivo* is free D-glucuronic acid, which appears to be formed in tissues solely from UDP-glucuronic acid, either by direct action of phosphatases (Ginsburg, Weissbach & Maxwell, 1958) or by the hydrolysis of glucuronides formed by transfer of the D-glucuronic acid moiety to unknown acceptors (Hollmann & Touster, 1962). The additional possibility of dietary sources of D-glucuronic acid, e.g. from breakdown of acidic mucopolysaccharides, cannot be excluded.

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

METHODS AND MATERIALS

Substrates. D-Glucurono-(6 $\rightarrow$ 3)-lactone (Corn Products Refining Co., New York, U.S.A.), recrystallized once from water, had m.p. 175–177°. It was converted in solution into sodium D-glucuronate as described by Marsh (1963). NAD and NADH₂ (sodium salt) were 'Sigma Grade' (Sigma Chemical Co., St Louis, Mo., U.S.A.), for which 97.5–100% purity was claimed. Phenolphthalein β -glucuronide was prepared biosynthetically (Levy & Marsh, 1959).

Tissue preparations. Adult human tissues, supplied by Dr A. J. Carr, Pathology Department, Aberdeen University, were apparently non-diseased autopsy specimens obtained within 6 hr. of death and stored at -20° . Fresh pig liver was provided by Robert Lawson and Sons (Dyce) Ltd. The rats used were hooded Lister strain, and the white mice were from the Institute stock (Tyler's original, Mill Hill colony). The C3H mice were originally from the Institute of Physiology, Glasgow University.

For the assay of 'glucurone dehydrogenase' the tissues were treated for 1 min. (average speed 5000 rev/min.) in a chilled glass homogenizer (Jencon's Ltd.) with sufficient ice-cold 0.17M-KCl (to preserve intracellular particles) to give a moist-tissue concentration of 10%. All further manipulations were carried out at 0° . The homogenate was centrifuged at 75 000g for 40 min., and the clear non-

* Part 1: Marsh (1963).

particulate supernatant was the 'crude preparation' used for enzyme assays with D-glucuronolactone. Partial purification of the crude preparation from rat liver, buffered with 0.1M-NaH₂PO₄-NaOH, pH 6.5, was accomplished by slowly adding solid (NH₄)₂SO₄ to achieve 80% saturation (Dixon, 1953); it was important to maintain pH 6.5 by frequently adjusting with 3N-NaOH during this addition. Insoluble protein was sedimented, resuspended in 0.05M-NaH₂PO₄-NaOH buffer, pH 6.5 (0.1 vol.) and dialysed for 3 hr., with stirring, in a closed sac of Visking tubing, against the same buffer. The dialysed solution was treated with (NH₄)₂SO₄ solution, pH 6.5, saturated at 0°, to give a salt saturation of 35%, and, after centrifuging, the supernatant was brought to 60% salt saturation by further addition of saturated (NH₄)₂SO₄. The second precipitate was redissolved in 0.05M-NaH₂PO₄-NaOH buffer, pH 6.5; the final volume was one-half that of the original crude preparation. Crude preparations contained 6.0-6.5 mg. of protein/ml. and the 'partially purified preparation', after (NH₄)₂SO₄ fractionation, 5.0-5.4 mg. of protein/ml., measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine plasma albumin (L. Light and Co. Ltd., Colnbrook, Bucks.) as standard.

β-Glucuronidase assay. The general procedure for the assay of soluble rat-liver β-glucuronidase preparations, with and without the inclusion of inhibitors, was as described by Marsh (1963). Assays were for 1 hr. at pH 5.2 with 0.63 mM-phenolphthalein β-glucuronide as substrate. The incubation mixture (4 ml.) contained 35-40 μg. of liberated phenolphthalein in control assays where material inhibitory to the enzyme was omitted.

Measurement of dihydronicotinamide-adenine dinucleotide. This was determined by its extinction at 340 mμ, with the Hilger Uvispek quartz spectrophotometer. The extinction coefficient of NADH₂ at this wavelength was assumed to be 6.25×10^4 cm.²/mole (Rafter & Colowick, 1957).

Other experimental procedures. The assays of β-galactosidase, α-mannosidase and β-N-acetylglucosaminidase, as well as the determination of total uronic acid and the paper chromatography of sugar acids, were as described by Marsh (1963).

Other materials. Potassium hydrogen D-glucarate (potassium hydrogen saccharate, British Drug Houses Ltd., Poole, Dorset), the source of standard D-glucarate solutions, was recrystallized once from water. D-Glucaro-(6→3)-lactone, m.p. 143-145°, was prepared as described by Smith (1944), and D-glucaro-(1→4)-lactone was obtained from Pfizer Ltd.; this appears to be the ammonium salt. L-Gulonolactone (British Drug Houses Ltd.) and L-ascorbic acid (Roche Products Ltd.) were used without further purification.

Analysis. Determinations of C, H and N were made by Weiler and Strauss, Oxford.

All melting points are corrected.

EXPERIMENTAL AND RESULTS

Conversion of D-glucuronolactone by rat-liver preparations into a potential β-glucuronidase inhibitor

Freshly prepared D-glucuronolactone solutions when incubated with rat-liver preparations were converted into an entity that, after being boiled at acid pH, was a potent inhibitor of β-glucuronidase.

NAD increased the production of this entity by crude enzyme preparations, and was essential when partially purified preparations were used.

General procedure. The incubation mixture consisted of 1 ml. of 0.25M-NaH₂PO₄-NaOH buffer, pH 6.5, 0.5 ml. of 50 mM-D-glucuronolactone, 0.5 ml. of 25 mM-NAD adjusted to pH 6.5, 1 ml. of the enzyme preparation and water to give a total volume of 5 ml., in 10 ml. centrifuge tubes. After incubation for 1 hr. at 37°, the tubes were closed with glass bulbs and heated on a boiling-water bath for 15 min. to destroy enzyme activity. Incubated controls contained all ingredients except the enzyme, which was added immediately after the tubes had been placed in the boiling-water bath. After all tubes had been cooled to 15°, denatured protein was sedimented, the supernatants were decanted into graduated tubes and the volumes were measured. The solutions were then adjusted with 3N-HCl to pH 2.0-2.2, heated at 100° for 40 min. and cooled, and the pH was adjusted carefully with 3N-NaOH to pH 4.0-4.5, to eliminate interference with the pH of the subsequent β-glucuronidase assay. It was essential that the pH did not rise above 5.5 during the addition of alkali, to avoid destruction of the entity inhibitory to β-glucuronidase. The solutions were then diluted to twice the original volume (i.e. before treatment with acid), and a suitable portion was included in the β-glucuronidase assay, to determine the amount of inhibitory material present.

The inhibitory effects of the controls were due in part to D-glucuronic acid, arising from hydrolysis of D-glucuronolactone during the heat treatment at pH 6.5, reconversion into non-inhibitory lactone in the subsequent treatment at pH 2.0-2.2 being only partial. However, when 2 ml. of the control solution was included in the β-glucuronidase assay (i.e. equivalent to a final concentration of 1.25 mM-glucuronolactone), a 35-40% inhibition of the enzyme occurred, whereas 1.25 mM-D-glucuronic acid alone caused only 9% inhibition. D-Glucuronolactone or D-glucuronic acid heated alone in phosphate buffer at pH 6.5 produced solutions with elevated inhibitory powers. A tentative explanation is that glucuronic acid phosphates, which are inhibitors or substrates of β-glucuronidase (Levy & Marsh, 1952), may be formed. The effect was not investigated further. NAD alone did not interfere in the β-glucuronidase assay, and to conserve NAD it could be omitted from controls. Preincubation of the enzyme before its addition to the control did not add to the inhibitory power of the latter.

Comparison of effects with D-glucuronolactone and D-glucuronic acid as substrates and with different tissue fractions. In order to give demonstrable effects when D-glucuronic acid replaced D-glucuronolactone as substrate, it was necessary to increase the usual enzyme-tissue concentration five-fold and the period of incubation to 2 hr. Table 1 compares the production of β-glucuronidase inhibitor from D-glucuronolactone with that from D-glucuronic acid, both under these modified condi-

tions, with a crude iso-osmotic rat-liver homogenate and preparations derived therefrom. The digestion products after treatment were tested against β -glucuronidase. The control digest was not tested for inhibitory power, but it was included in the β -glucuronidase assay which was taken as standard. If the enzyme preparations were boiled before use no more inhibitory material was produced than in controls. The crude homogenate (E_1) and cell-free supernatant (E_2) converted D-glucuronolactone into the inhibitor in the absence of NAD, but effects were small with the particulate fraction (E_3) and partially purified preparation (E_4). (In some experiments the effect with E_4 was negligible.) The addition of NAD to the incubation mixture greatly increased the production of inhibitor in all cases and was essential for the production of more than traces of the inhibitor when partially purified preparations were employed. Inhibitor formation with crude preparations, but without added NAD, was probably due to the presence of endogenous nucleotide. In general, effects with D-glucuronic acid as substrate were similar to, but much smaller than, those with the lactone; these might be due to the presence of traces of unhydrolysed lactone, but a specimen of crystalline sodium D-glucuronate (L. Light and Co. Ltd.) gave similar results. The production of inhibitor, in the presence or absence of NAD, by E_2 was greater than that by E_1 ; this was probably due to the removal of nicotinamide-adenine dinucleotide nucleosidase present in liver microsomal fractions (Kaplan, 1960), since the addition of 40 mM-nicotinamide, an inhibitor of this enzyme, stimulated production of the β -glucuronidase in-

hibitor by fraction E_3 . The effect was absent in incubations containing only fraction E_2 , which lacked the nucleosidase.

Properties of the acid-potentiated β -glucuronidase inhibitor

Reversal of inhibitory power by alkali. Omission of the acid treatment after incubation of D-glucuronolactone with NAD and rat-liver enzyme prevented the appearance of the β -glucuronidase inhibitor. If, after potentiation by treatment with acid, the incubation mixture was adjusted to pH 7.5–8.0 with 3N-sodium hydroxide, boiled for 15 min., re-adjusted to pH 6.0 with hydrochloric acid and added to the β -glucuronidase assay, then inhibition, compared with assays containing the control, was again negligible; it was re-established in full by a second treatment with acid. The entity produced in the incubation was thus only inhibitory after treatment with acid, and its formation was reversed at mildly alkaline pH.

Dialysis. After dialysis of the heat-inactivated incubation mixture against an equal volume of water for 48 hr. at 0°, the diffusate and residue after treatment with acid were equally inhibitory, whereas after dialysis under similar conditions against several changes of large volumes of water the dialysis residue was no longer inhibitory. The inhibitory factor was thus fully diffusible.

Specificity of inhibition. The inhibitor appeared to be specific for β -glucuronidase; its inclusion after treatment with acid in assays of β -galactosidase, α -mannosidase and β -N-acetylglucosaminidase, at concentrations that produced more than 50 %

Table 1. *Conversion of D-glucuronolactone and D-glucuronic acid into acid-potentiated β -glucuronidase inhibitor by rat-liver preparations*

Rat-liver homogenate [E_1 ; 10 % (w/v) in 0.17M-KCl] was centrifuged at 75 000g for 40 min. to give a cell-free fraction (E_2) and a sedimented particulate fraction (E_3) resuspended in the same volume of 0.17M-KCl; $(\text{NH}_4)_2\text{SO}_4$ fractionation of E_2 as described in text gave the partially purified preparation (E_4). The concentration of E_4 was twice that of other preparations in terms of tissue. Incubation mixtures (5 ml.) contained enzyme (2.5 ml.), D-glucuronolactone or D-glucuronic acid (5 mM), NAD (2.5 mM) and nicotinamide (40 mM) as shown below. All incubations were for 2 hr. After treatment at pH 2.0–2.2 as described in text, products were tested as inhibitors (at 25 % of the original concentration in incubation mixtures) of rat-liver β -glucuronidase, with 0.63 mM-phenolphthalein β -glucuronide as substrate. Inhibition by the incubation products was measured by comparison with β -glucuronidase assays containing the products of the appropriate control incubation (see the text); 33.8 and 32.5 μg . of phenolphthalein were liberated in these reference assays with D-glucuronolactone and D-glucuronic acid respectively.

Incubation mixture	Inhibition of β -glucuronidase (%)			
	E_1	E_2	E_3	E_4
Enzyme + D-glucuronolactone	36	48	5	6
Enzyme + D-glucuronolactone + NAD	69	81	30	85
Enzyme + D-glucuronolactone + NAD + nicotinamide	72	75	47	—
Boiled enzyme + D-glucuronolactone + NAD	0	1	1	0
Enzyme + D-glucuronic acid	1	8	0	0
Enzyme + D-glucuronic acid + NAD	8	18	4	21
Enzyme + D-glucuronic acid + NAD + nicotinamide	21	15	10	—
Boiled enzyme + D-glucuronic acid + NAD	2	0	1	0

inhibition in the β -glucuronidase assay, had no effect.

Variation of substrate and inhibitor concentration in the β -glucuronidase assay. Analysis of the β -glucuronidase inhibition by the method of Line-weaver & Burk (1934) showed that the inhibitory factor competed with the substrate. Fig. 1 shows the variation of β -glucuronidase inhibition with various concentrations of the acid-potentiased inhibitor, produced by incubation of D-glucuronolactone and NAD with the crude rat-liver preparation (i.e. the non-particulate fraction). The activity was compared with that in the straightforward β -glucuronidase assay, containing only enzyme, substrate and buffer. To provide quantitative measurement of the amount of inhibitor produced during the incubation, an arbitrary unit was chosen, similar to that employed to measure the acid-potentiased inhibitor in mammalian urine (Marsh, 1963). One unit of inhibitor (U.I.) was defined as that quantity which caused 50% inhibition in the standard assay of β -glucuronidase. Fig. 1 could thus be used to measure the amount of inhibitor (in U.I.) produced in incubation mixtures of D-glucuronolactone, NAD and enzyme preparations. Corrections for β -glucuronidase inhibition given by controls in this and subsequent sections were read from the same curve, and subtracted

from the U.I. produced in the assay. In practice this 'blank' value did not normally exceed 0.25 U.I. (i.e. 20% inhibition of β -glucuronidase).

Identification of the inhibitor

The above properties of the acid-potentiased inhibitor produced enzymically from D-glucuronolactone are identical with those of the entity observed in boiled mammalian urine before and after the ingestion of D-glucuronolactone (Marsh, 1963). D-Glucaric acid was isolated and identified from human urine in quantities that could account for the acid-potentiased inhibitor present. Acid treatment of D-glucaric acid leads to the formation of D-glucaro-(1 $\rightarrow$ 4)-lactone, the specific β -glucuronidase inhibitor.

Comparison of the formation of dihydronicotinamide-adenine dinucleotide with that of the inhibitor. Duplicate series of incubations of the partially purified preparation (see the Methods and Materials section) with D-glucuronolactone (5 mM) and NAD (2.5 mM) were incubated at pH 6.5 for various periods. The amount (in U.I.) of β -glucuronidase inhibitor produced was determined in one series as described above. The second series of tubes was chilled in ice-water for 1 min. at the end of the incubation period. Controls for this series were incubation mixtures containing buffer, enzyme and NAD, but lacking D-glucuronolactone, which was added immediately the incubation mixtures had been cooled. The tubes were centrifuged at 1500g for 1 min. at 0°, portions of the supernatants were withdrawn and the NADH₂ contents were measured immediately. When NADH₂ (1 mM) was incubated for 1 hr., either with the enzyme at pH 6.5 or with D-glucuronolactone, there was no loss compared with a solution of NADH₂ incubated alone at pH 6.5. However, there was a 15% loss of NADH₂ in this last incubation compared with freshly prepared NADH₂. No correction has been made for this in Fig. 2.

Fig. 2 (a) shows the production of inhibitor (in U.I.) and of NADH₂ with time of incubation in a typical experiment. Both curves were linear up to the maximum period of 75 min. (see below). The production of inhibitor is compared with that of NADH₂ in Fig. 2 (b); the slope of this curve gave a value of 24.5 μ m-moles of NADH₂ produced/U.I. Values of 25.5 and 18.2 μ m-moles of NADH₂/U.I. were obtained in other experiments. This gave a mean value of 22.7 μ m-moles of NADH₂/U.I. According to Scheme 1 (see below) equimolar quantities of NADH₂ and D-glucaric acid arise from the oxidation of D-glucuronolactone with NAD. In fact, when boiled at pH 2.0 for 40 min., 24.1 μ m-moles of D-glucaric acid caused a 50% inhibition of β -glucuronidase (Marsh, 1963), i.e. this quantity

Fig. 1. Inhibition of rat-liver β -glucuronidase activity at pH 5.2 by various concentrations of inhibitor produced enzymically from D-glucuronolactone. The substrate was 0.63 mM-phenolphthalein β -glucuronide. The lower abscissa shows the concn. of boiled digest added in the β -glucuronidase assay, and the upper scale indicates the method of calculation of arbitrary inhibitor units (U.I.) present; 1 U.I. causes 50% inhibition.

was equivalent to 1 U.I. If one assumes the equimolar relationship in Scheme 1, therefore, the inhibitory power of D-glucaric acid is almost identical with that of the inhibitor produced enzymically from D-glucuronolactone, which suggests that the inhibitor might be D-glucaric acid.

Isolation of D-glucaric acid from an enzyme digest of D-glucuronolactone and nicotinamide-adenine dinucleotide. A partially purified rat-liver preparation (80 ml., giving 2140 U.I./hr.) was incubated for 10 hr. at 37° with D-glucuronolactone (50 mM) and NAD (5 mM) in a total volume of 200 ml. of 0.05 M-sodium dihydrogen phosphate-sodium hydroxide buffer, pH 6.5. This incubation mixture was boiled and centrifuged, and the sediment was washed with water. Supernatant and washings (350 ml., containing 16 800 U.I., equivalent to 85 mg. of D-glucaric acid) were concentrated under reduced pressure to 50 ml., then dialysed against 2 l. of water for 72 hr. at 0°. The diffusate was concentrated to 50 ml., and an excess of 20% (w/v) normal lead acetate stirred in. The insoluble lead salt was sedimented, resuspended in water (500 ml.) and decomposed with hydrogen sulphide; after removal of lead sulphide the pale-yellow solution was concentrated to 100 ml., charcoal was added, and the mixture was again concentrated to 20 ml. It then

contained 12 600 U.I., equivalent to 64 mg. of D-glucaric acid. Phenylhydrazine hydrochloride (1 g.) and anhydrous sodium acetate (0.2 g.) were added, and the mixture was heated at 100° for 4 min.; a small amount of tarry material was removed by decantation and the heating was continued for 2.5 hr. The crystalline solid (115 mg.) that separated on cooling overnight was recrystallized from aq. 50% (v/v) ethanol, yielding a colourless product (75 mg.), m.p. 210–211° (decomp.). There was no depression in the m.p. on admixture with authentic D-glucaric acid bisphenylhydrazide, m.p. 210° (decomp.) (Found: C, 55.2; H, 5.7; N, 14.4. Calc. for $C_{18}H_{22}O_6N_4$: C, 55.4; H, 5.7; N, 14.4%). If it is assumed that the inhibitor present before treating with phenylhydrazine was D-glucaric acid, the product obtained represented a yield on condensation of 63%.

Hydrolysis of phenylhydrazine derivative. The derivative (3.35 mg.) was shaken at 20° for 3 hr. with ethanol-aq. ammonia [15 ml. of ethanol + 5 ml. of water + 10 ml. of aq. ammonia (sp.gr. 0.88)], and the resulting yellow solution was evaporated to 10 ml. and extracted with ether to remove phenylhydrazine. The aqueous residue, tested as an inhibitor of β -glucuronidase after the standard treatment with acid, contained 334 U.I., equivalent to 8.05 μ moles of D-glucaric acid. If it is assumed that the initial compound was D-glucaric acid bisphenylhydrazide, the yield on hydrolysis represented a 93% recovery of the D-glucaric acid. Similar treatment of authentic D-glucaric acid bisphenylhydrazide gave a 90% recovery of D-glucaric acid.

Examination by paper chromatography. Descending paper chromatography, on Whatman no. 1 (chromatographic grade) paper, of the above hydrolysis products of the inhibitor and authentic D-glucaric acid bisphenylhydrazide gave results similar to those previously obtained with the urine β -glucuronidase inhibitor. With propan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:3:1, by vol.) both products gave spots of R_F 0.21 and 0.32; freshly prepared ammonium D-glucarate gave a single spot at R_F 0.21, whereas both D-glucaro-(1 $\rightarrow$ 4)-lactone and D-glucaro-(6 $\rightarrow$ 3)-lactone gave strong spots at R_F 0.32 and weaker spots at R_F 0.21. Chromatography in butan-1-ol-acetic acid-water (4:1:5, by vol.), after removal of NH_4^+ ion with Amberlite resin IR-120 (H^+ form), gave strong spots at R_F 0.11 with all these compounds. The two crystallized lactones also gave strong spots at R_F 0.22 and the other compounds weak spots at this R_F .

The above evidence confirmed the belief that the potential β -glucuronidase inhibitor produced enzymically from D-glucuronolactone was D-glucaric acid. In subsequent experiments, therefore, measurements made in U.I. were converted to μ g. of

Fig. 2. Formation of NADH₂ and of β -glucuronidase inhibitor on incubation of D-glucuronolactone (5 mM) and NAD (2.5 mM) with rat-liver enzyme. (a) Increase in NADH₂ (○) and inhibitor (●) with time of incubation; (b) plot of NADH₂ and inhibitor formation.

D-glucaric acid, by employing the experimentally determined factor 1 U.I. = 24.1 μ m-moles of D-glucaric acid.

Further studies of the enzyme oxidizing D-glucuronolactone

Incubation with L-gulonolactone, L-ascorbic acid and D-glucaric acid. The partially purified enzyme was incubated with L-gulonolactone (5 mM) or with L-ascorbic acid (5 mM), together with NAD (2.5 mM), but there was no conversion into the β -glucuronidase inhibitor. The presence of 5 mM-L-gulonolactone in a normal assay medium containing D-glucuronolactone did not affect the inhibitor production. The enzyme converting D-glucuronolactone into D-glucaric acid thus appears to be distinct from the NADP-linked L-hexonate dehydrogenase or L-gulonolactone dehydrogenase, which together convert D-glucuronolactone into L-ascorbic acid (see the Discussion section), and L-ascorbic acid was not a precursor of the β -glucuronidase inhibitor. There was no loss of inhibitory power of 5 mM-D-glucarate when incubated with the enzyme and NAD; the D-glucaric acid produced enzymically from D-glucuronolactone does not therefore undergo further conversion during the incubation.

Variation of incubation period. The production of D-glucaric acid from D-glucuronolactone under the standard assay conditions was linear up to 1 hr. (Fig. 2a). Further incubation, however, with either the crude or partially purified preparation, caused a slight progressive decrease in the rate of formation of D-glucaric acid (Fig. 3). This may be due to enzyme inactivation or to exhaustion of substrate. The maximum production of D-glucaric acid in this experiment was 0.4 μ mole, i.e. less than 4 and 2% respectively of the original quantities of NAD and D-glucuronolactone present. However, there may be a further loss of D-glucuronolactone by hydrolysis to D-glucuronic acid (a) non-enzymically at pH 6.5 and (b) by lactonase present in the soluble fraction of liver homogenates (Yamada, 1959a).

Variation of enzyme concentration. The production of D-glucaric acid in the usual 1 hr. assay was proportional to the enzyme concentration, with either the crude rat-liver cell-free supernatant (concentration in assay equivalent to 5–40 mg. of moist tissue/ml.) or the partially purified enzyme (concentration in assay equivalent to 10–80 mg. of moist tissue/ml.).

Presence of the enzyme in other organs and species. Cell-free supernatants of homogenates (prepared in potassium chloride solution) of rat tissues other than liver, and tissues of other species, were examined, by the usual assay procedure, in the presence of NAD. The production of the β -glucuronidase in-

Fig. 3. Formation of D-glucaric acid on incubation, for various periods, of D-glucuronolactone (5 mM) and NAD (2.5 mM) with enzyme from rat-liver homogenate: O, crude preparation; ●, partially purified preparation (see the Methods and Materials section).

hibitor is recorded in Table 2, where the results are calculated (in U.I.) from the rat-liver inhibitor curve (Fig. 1) and then converted to μ g. of D-glucaric acid. The properties of the inhibitors produced from D-glucuronolactone by other tissue preparations appeared to be identical with those of the rat-liver inhibitor, but formal identification of D-glucaric acid was not made. The enzyme occurred in the liver of all species examined, including man; activities of mouse and pig liver were rather higher than those of the rat, whereas the activity in the liver of C3H ('low-level β -glucuronidase') mice was identical with that of 'high-level' strains. Kidney enzyme activity was high for the rat and appreciable for the guinea pig, but very low in mouse and man. Testis enzyme activity was quite high (30–50% of the liver activity) for those species examined, and the enzyme was detected in mouse, but not rat, ovary. Slight enzyme activity was found in the thalamus area of human brain and a trace in rat brain. There was no detectable enzyme activity in rat preparations of spleen, uterus, thyroid, lung, duodenum or serum.

DISCUSSION

The glucaric acid excreted in mammalian urine (Marsh, 1963) is probably the product of oxidation of D-glucuronolactone *in vivo* by the system investi-

Table 2. *Enzymic formation of acid-potentiased β -glucuronidase inhibitor from D-glucuronolactone by tissue preparations from various species*

Tissue homogenates [10% (w/v) in iso-osmotic KCl] were centrifuged before use to remove the particulate fraction. Assay mixtures (5 ml.) containing D-glucuronolactone (5 mM), NAD (2.5 mM) and enzyme (1 ml.) were incubated for 1 hr. at pH 6.5. The inhibitor produced after boiling at pH 2.0–2.2 (see the text) was measured in the assay of rat-liver β -glucuronidase at pH 5.2 with 0.63 mM-phenolphthalein β -glucuronide. Values (U.I.) read from Fig. 1 were converted to D-glucaric acid (1 U.I. = 24.1 μ M-moles of D-glucaric acid). All non-sex tissues were from male animals, unless otherwise stated. Results are expressed as means $\pm$ S.D., with the numbers of animals in parentheses.

Organ	D-Glucaric acid produced (μ moles/g. of tissue)				
	Rat	Mouse	Man	Guinea pig	Pig
Liver	8.4 $\pm$ 2.3 (4)	12.5 $\pm$ 4.2 (4); 12.6 $\pm$ 3.5 (4)*	7.2 $\pm$ 0.5 (4)	8.7 $\pm$ 5.5 (3)	16.7 $\pm$ 4.7 (6)
Kidney	5.0 $\pm$ 0.6 (4)	0.1 (4)	0.2 (1)†	1.3 $\pm$ 0.4 (2)	—
Testis	3.3 $\pm$ 0.4‡	4.7 $\pm$ 0.5‡	4.2 (1)	—	—
Ovary	0	0.9 $\pm$ 0.1‡	—	—	—
Brain	0.1 (2)	0	0§, 0.6 $\pm$ 0.3 (4)	—	—

* C3H strain (females).

† Mean of two experiments, each with the pooled tissues of five animals.

‡ Thalamus.

† Female.

§ Cortex.

Scheme 1

gated in the present paper, and it is proposed to adopt provisionally the trivial name 'D-glucuronolactone dehydrogenase' (systematic name 'D-glucuronolactone-NAD oxidoreductase') for this enzyme. From the experimental evidence that the production of D-glucaric acid and of NADH₂ are in equimolar proportions, and by analogy with NAD(P)-dependent oxidations of other aldoses (see Mehler, 1957), the tentative reaction mechanism is given in Scheme 1. The dehydrogenation of D-glucuronolactone (I) by NAD would be expected to produce D-glucaro-(1→4), (6→3)-dilactone (II) as the primary product. This compound was isolated by Smith (1944) as a glass by prolonged heating of D-glucaro-(1→4)-lactone; in aqueous solution it rapidly underwent mutarotation, and from the resulting equilibrium mixture both D-glucaro-(6→3)-lactone (III) and D-glucaro-(1→4)-lactone (IV) were isolated. At physiological pH and temperature, hydrolysis of these monolactones to D-glucaric acid is known to take place, but the process could conceivably be accelerated or reversed if lactonases of appropriate specificity were present.

Since the present methods for the quantitative measurement of lactones are non-specific, there are considerable experimental difficulties involved in the identification of (III) and (IV) as the first relatively stable products of enzyme action on a compound that is itself a lactone. It should, however, be possible to identify (IV) by its unique inhibitory effect on β -glucuronidase, since the limits of pH stability of this compound have been investigated (Levvy, 1952).

The enzyme that oxidizes D-glucuronolactone does not appear to be identical with D-glucose dehydrogenase or D-glucose 6-phosphate dehydrogenase. Strecker & Korkes (1952) found that mammalian glucose dehydrogenase had optimum activity at pH 9.8 with NAD as coenzyme, whereas preliminary investigation of glucuronolactone dehydrogenase shows a pH optimum of 6.5. Moreover, the presence of 10 mM-D-glucose in the normal assay medium did not affect the amount of β -glucuronidase inhibitor produced; hence it was unlikely that glucose was a competing substrate unless its affinity for the enzyme was much weaker than

that of glucuronolactone. Liver D-glucose 6-phosphate dehydrogenase is specific for NADP as the coenzyme (Dickens & Glock, 1951).

The physiological significance of mammalian D-glucuronolactone dehydrogenase may lie in the production of an endogenous β -glucuronidase inhibitor, for if D-glucaro-(1 $\rightarrow$ 4)-lactone were present it could play an important role in controlling glucuronide hydrolysis *in vivo*. No evidence for this is yet available. Liver D-glucuronolactone-dehydrogenase activity in pig and C3H mouse preparations was comparable with that in other species, and could not thus explain the low concentrations of β -glucuronidase in the tissues of these animals (see Levvy & Marsh, 1959). The β -glucuronidase inhibitor reported in normal mammalian plasma and serum (Fishman *et al.* 1951) could be due to inhibition caused by the formation of D-glucaro-(1 $\rightarrow$ 4)-lactone *in vivo*; alternatively this compound might be formed by conversion of D-glucaric acid in the serum during the enzyme assay, conducted at acid pH (see Marsh, 1962). It would be of particular interest if D-glucuronolactone dehydrogenase occurred generally in testicular and ovarian tissues (see Table 2) and was under hormonal control.

The enzyme may be implicated in mammalian L-ascorbic acid synthesis; enzymes which mediate *in vitro* all the conversions illustrated in Scheme 2 have been identified by various workers (see Touster, 1962). The final stage in the biosynthesis of the vitamin is the oxidation of L-gulonolactone, but whether this compound arises *in vivo* by lac-

tonization of L-gulonic acid or by reduction of D-glucuronolactone is still obscure. If the main precursor of L-gulonolactone is D-glucuronolactone, oxidation of the latter to D-glucaric acid represents a pathway directly competitive with ascorbic acid synthesis. An obvious approach to this problem is the study of D-glucaric acid excretion after the administration of substances known to affect ascorbic acid excretion (Longenecker, Fricke & King, 1940; Burns *et al.* 1960). The isolation of D-glucaric acid from human urine, and the demonstration (Marsh, 1963) of its probable presence in the urine of rats and of other animals able to synthesize the vitamin, suggests that D-glucuronolactone does occur as a natural metabolite. The hydrolysis of D-glucuronolactone is catalysed by 'lactonases I and II' (Yamada, 1959*a*), synonymous with 'aldonolactonase' and 'uronolactonase' (Winkelman & Lehninger, 1958) respectively, and present in the soluble and microsomal fractions respectively of liver homogenates. Lactonization of D-glucuronic acid occurred spontaneously at physiological pH, however, when a trapping agent such as hydroxylamine was present, and this process was not accelerated by the presence of lactonase I (Yamada, 1959*b*), whereas lactonase II accelerated lactonization but only in the presence of a trapping agent (Kawada & Yamada, 1961). D-Glucuronolactone dehydrogenase might act as such a trapping agent, since a small amount of D-glucaric acid was apparently produced in incubations with D-glucuronic acid.

The conversion of glycuronic acids into glycaric acids in plants and micro-organisms has been reported. Soluble preparations from mung beans when incubated with uniformly labelled D-[14 C]-glucuronic acid and D-[14 C]galacturonic acid produced the corresponding aldarcic acids amongst other products (Kessler, Neufeld, Feingold & Hassid, 1961); D-glucuronolactone was not investigated as a substrate, but the addition of NAD had no effect (E. S. Neufeld, personal communication). The oxidation of D-[1- 14 C]galacturonic acid to [1- 14 C]-galactaric acid occurred in the detached ripening strawberry (Loewus & Kelly, 1961). The cell-free system from *Pseudomonas syringae*, which converted D-glucuronate and D-galacturonate into the respective glycaric acids (Kilgore & Starr, 1959), was NAD-dependent.

SUMMARY

1. Rat-liver preparations converted D-glucuronolactone into a factor that, after treatment with acid, was a powerful competitive inhibitor of β -glucuronidase.

2. Partially purified enzyme preparations required the presence of NAD for this conversion.

Scheme 2

3. D-Glucaric acid was isolated from an enzyme digest, and identified as its bisphenylhydrazide.

4. The same enzyme system appeared to be present in liver of other species, including man, and in testis and in rat kidney.

5. The mechanism of the reaction, and its implication in L-ascorbic acid biosynthesis, are discussed.

6. The name 'D-glucuronolactone dehydrogenase' has been given provisionally to this enzyme.

I wish to express my gratitude to Dr G. A. Levvy for his interest and encouragement in this work, and am indebted to Mr A. J. Hay for help in some experiments and to Miss L. Reid for able technical assistance.

REFERENCES

- Burns, J. J., Conney, A. H., Dayton, P. G., Evans, C., Martin, G. R. & Taller, D. (1960). *J. Pharmacol.* **129**, 132.
- Dickens, F. & Glock, G. E. (1951). *Biochem. J.* **50**, 81.
- Dixon, M. (1953). *Biochem. J.* **54**, 457.
- Fishman, W. H. (1952). In *Ciba Found. Colloq.: Endocrinology*, p. 229. Ed. by Wolstenholme, G. E. W. London: J. and A. Churchill Ltd.
- Fishman, W. H., Altman, K. I. & Springer, B. (1948). *Fed. Proc.* **7**, 154.
- Fishman, W. H., Smith, M., Thompson, D. B., Bonner, C. D., Kasdon, S. C. & Homburger, F. (1951). *J. clin. Invest.* **30**, 685.
- Ginsburg, V., Weissbach, A. & Maxwell, E. S. (1958). *Biochim. biophys. Acta.* **28**, 649.
- Hollmann, S. & Touster, O. (1962). *Biochim. biophys. Acta*, **62**, 338.
- Kaplan, N. O. (1960). In *The Enzymes*, 2nd ed. vol. 3, p. 105. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
- Kawada, M. & Yamada, K. (1961). *J. Biochem., Tokyo*, **50**, 419.
- Kessler, G., Neufeld, E. F., Feingold, D. S. & Hassid, W. Z. (1961). *J. biol. Chem.* **236**, 308.
- Kilgore, W. W. & Starr, M. P. (1959). *Nature, Lond.*, **183**, 1412.
- Levy, G. A. (1952). *Biochem. J.* **52**, 464.
- Levy, G. A. & Marsh, C. A. (1952). *Biochem. J.* **52**, 690.
- Levy, G. A. & Marsh, C. A. (1959). *Advanc. Carbohydr. Chem.* **14**, 381.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Loewus, F. A. & Kelly, S. (1961). *Arch. Biochem. Biophys.* **95**, 483.
- Longenecker, H. E., Fricke, H. H. & King, C. G. (1940). *J. biol. Chem.* **135**, 497.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Marsh, C. A. (1961). *Biochem. J.* **79**, 21P.
- Marsh, C. A. (1962). *Nature, Lond.*, **194**, 974.
- Marsh, C. A. (1963). *Biochem. J.* **86**, 77.
- Mehler, A. H. (1957). *Introduction to Enzymology*. pp. 117, 129. New York: Academic Press Inc.
- Rafter, G. W. & Colowick, S. P. (1957). In *Methods in Enzymology*, vol. 3, p. 887. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Smith, F. (1944). *J. chem. Soc.* p. 633.
- Strecker, H. J. & Korkes, S. (1952). *J. biol. Chem.* **196**, 769.
- Touster, O. (1962). *Annu. Rev. Biochem.* **31**, 407.
- Winkelman, J. & Lehninger, A. (1958). *J. biol. Chem.* **233**, 293.
- Yamada, K. (1959a). *J. Biochem., Tokyo*, **46**, 361.
- Yamada, K. (1959b). *J. Biochem., Tokyo*, **46**, 529.

Biochem. J. (1963) **87**, 90

The Use of Reactive Dyestuffs in Enzymology: New Substrates for Cellulolytic Enzymes

By H. N. FERNLEY*

Rowett Research Institute, Bucksburn, Aberdeen

(Received 3 September 1962)

Present methods for following the enzymic hydrolyses of highly polymerized molecules such as cellulose are often not entirely satisfactory, especially where the initial products are qualitatively similar to the starting material. It was thought that by attaching chromophoric molecules to such a

* Present address: Department of Biochemistry, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex.

substrate it might be possible to derive a convenient assay system for this type of enzyme.

Reactive dyestuffs are a class of substances that react chemically under alkaline conditions with hydroxyl groups of cellulose, forming ester or ether linkages. A brief review of their properties is given by Rattee (1961).

This paper is concerned with the coupling of water-soluble reactive dyestuffs to insoluble cello-