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BIOSYNTHESIS OF D-GLUCARIC ACID IN MAMMALS: A FREE-RADICAL MECHANISM?

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ABSTRACT

In the presence of iron salts and hydrogen peroxide, D-glucuronic acid was converted into D-glucaric acid. The reaction was strongly inhibited by free-radical scavengers and is ascribed to the action of the hydroxyl radical. The formation of D-glucarate was dependent upon pH and occurred in the presence of some iron-complexing agents. The first product of oxidation was a lactone that was a strong inhibitor of β-D-glucuronidase and assumed to be D-glucaro-1,5-lactone. Microsomal preparations in the presence of NADPH also produced D-glucarate from D-glucuronic acid, presumably due to formation of hydrogen peroxide, and the product was an inhibitor of β-D-glucuronidase. Superoxide did not produce D-glucarate from D-glucuronate. The cytochrome P450 system is more likely than "glucuronolactone dehydrogenase" to be responsible for the production of D-glucaric acid *in vivo*.

INTRODUCTION

D-Glucaric acid is a normal constituent in mammalian urine and the excretion by adult human males is 30–100 μmol per day[1]. It is an oxidation product of D-glucuronic acid, since ingestion of D-glucurono-6,3-lactone results in a dramatic increase in the excretion of D-glucarate[2]. Similar changes also occur after treatment with some drugs[3,4], and the measurement of excreted D-glucarate has been proposed[5,6] as a simple non-invasive means of assessment of the general and induced levels of microsomal enzyme activity in the liver.

Although the amount of D-glucaric acid excreted by man is relatively low and this pathway of metabolism of D-glucuronic acid appears to be of minor quantitative significance, compared with the conversions, *via* L-gulonic acid, into L-xylulose and L-ascorbic acid in most mammals other than primates[7], the potential for the biosynthesis of D-glucaric acid is high. The conversion from D-glucurono-6,3-lactone is extensive (20%); in a reported case of hyperglucaricaciduria[8], where the metabolic lesion was suggested to be glucuronate reductase ($NADP^+$-L-gulonic acid dehydrogenase, EC 1.1.1.19), an adult human male was reported to excrete 4–8 g of

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D-glucaric acid daily, whereas the total urinary excretion of D-glucuronic acid (*i.e.*, free + conjugated) was only 2 g per day.

D-Glucaric acid is not a substrate for any known mammalian enzyme, and thus appears to be an end metabolite. As a simple carbohydrate, with a potential conversion into metabolic energy of the same order as that of D-glucose, this poses the question of the reason for its formation. Unlike other excreted end-products (for example, urea and uric acid), D-glucaric acid does not mediate the elimination of potentially toxic substances. It is possible that D-glucaric acid could specifically modify the action of an enzyme, or be produced as a result of participation of D-glucuronic acid or its lactone as a substrate in another unrelated metabolic pathway, or be formed non-enzymically.

Supporting the modifier hypothesis is the finding[9] that, within 1 h of a single oral dose of D-glucurono-6,3-lactone, the measured activity of human serum β-D-glucuronidase decreased by 75%, with only a partial recovery after 3 h. In mice, there was a similar dramatic decrease in the activity of liver β-D-glucuronidase[10]. Whereas D-glucuronic acid is only a feeble inhibitor, and D-glucurono-6,3-lactone and D-glucaric acid are non-inhibitory, D-glucaro-1,4-lactone is a specific competitive inhibitor of β-D-glucuronidase[11], for which it has an affinity much greater than that of any known substrate. If formed *in vivo*, D-glucaro-1,4-lactone could exert an effective feedback control on the hydrolysis of β-D-glucuronides, formation of which is a common mechanism of detoxification, and thereby facilitate their excretion.

A cytosolic liver enzyme, with NAD^+ as co-substrate, has been found[12] to produce D-glucaric acid from D-glucurono-6,3-lactone but not from D-glucuronic acid. However, studies[13] of the enzyme, purified from rat liver, established that this "glucuronolactone dehydrogenase" was an aldehyde dehydrogenase of wide specificity, and it may be assumed to act upon the known *aldehydo* form of D-glucurono-6,3-lactone, for which the K_m value was high.

It is difficult to conceive how D-glucaro-1,4-lactone or other inhibitory lactones could be produced by such a mechanism, and indeed it has been shown that the immediate reaction product is probably D-glucaro-6,3-lactone[14], which is non-inhibitory towards β-D-glucuronidase. Thus, whereas some administered D-glucurono-6,3-lactone would be converted into D-glucaric acid by "glucuronolactone dehydrogenase", it seems unlikely that the reaction is a major source of physiologically produced D-glucaric acid. Furthermore, the excretion of D-glucaric acid was doubled in guinea pigs after prolonged administration of disulfiram[15], a strong inhibitor[14] of "glucuronolactone dehydrogenase".

Another objection to the "glucuronolactone dehydrogenase" hypothesis is that there is no satisfactory evidence for the presence of significant amounts of D-glucurono-6,3-lactone *in vivo* at physiological pH. Specific and non-specific lactonases for this compound are known, but the equilibrium at pH 7 strongly favours D-glucuronic acid[16]. It has been assumed, without supporting evidence, that the lactone, perhaps formed by the reverse action of glucuronolactone reductase

(EC 1.1.1.20), might accumulate in regions of the cell, *e.g.* proximal to membranes, where the local pH may be abnormally low.

Despite these criticisms, this enzymic reaction has been generally accepted as the physiological origin of D-glucaric acid. Other feasible routes are worthy of investigation. The increase in excretion of D-glucaric acid observed after activation of the microsomal enzyme system by xenobiotics suggests a connection between this system and the synthesis of D-glucaric acid *in vivo*, and there is much other supportive evidence. A significant correlation existed between the content of cytochrome P450 in tissue homogenates from liver biopsies and the D-glucaric acid excreted, in cases of normal and cirrhotic livers[17], and of livers after treatment with various drugs, including phenobarbitone[3,18], oral hypoglycaemic agents[4], and antipyrine[17].

Although prolonged treatment of rats with phenobarbitone also resulted in increased urinary excretion of D-glucuronic acid and the products of all the known pathways of metabolism of D-glucuronic acid[19], the excretion of D-glucaric acid returned to normal within 4 weeks of cessation of the treatment, whereas those of D-glucuronic acid, L-gulonic acid, and L-ascorbic acid remained at about twice the normal level. When phase-2 substrates (*i.e.*, those directly amenable to conjugation) were administered to rats, the metabolism of D-glucuronic acid was stimulated, as evidenced[20] by increased glucuronyl transferase activity and excretion of D-glucuronic acid, but without significant changes in the excretion of D-glucaric acid or the level of hepatic cytochrome P450. Changes in the excretion of D-glucaric acid therefore appear not to be solely dependent on the availability of the precursor.

A D-glucarolactone, which was highly inhibitory towards β-D-glucuronidase, has also been detected[21] after platinum-catalysed oxidation of D-glucuronic acid and such a system is known to involve free radicals. Production of the superoxide radical in biochemical systems has long been known, but there is now considerable evidence for formation *in vivo* of the highly reactive hydroxyl radical. The Fenton reaction[22] (hydrogen peroxide and Fe^{2+}) has been implicated in this process. The possible conversion of D-glucuronic acid into D-glucaric acid has therefore been investigated using the Fenton reagents and also a rat-liver microsomal system, since the formation of hydrogen peroxide by hepatic microsomes is well established.

EXPERIMENTAL

General. — Potassium hydrogen D-glucarate was recrystallised and then converted[23] into the dicyclohexylammonium salt, and also used to prepare[24] D-glucaro-1,4-lactone monohydrate (m.p. 89–92°) and D-glucaro-6,3-lactone (m.p. 139–142°). Water, double-distilled from glass, was used throughout to minimise contamination by metal ions.

β-D-Glucuronidase was prepared from rat preputial gland and partially purified[25] by gel chromatography on Sephadex G-100. "Glucuronolactone de-

hydrogenase" was prepared from the soluble fraction of rat-liver homogenates and partially purified[13] by ammonium sulphate fractionation followed by ion-exchange chromatography on a DEAE-Sephadex column. The final product had a specific activity of 1.5 units/mg of protein, where one unit of enzyme activity catalyses the formation of 1 μmol of NADH/h from 2.5mM NAD^+ at 37° and pH 6.5.

The Fenton reaction was investigated by incubation of 5mM D-glucuronic acid with 10mM H_2O_2 and 0.5mM Fe^{2+}. Catalase (final concentration, 150 units/mL) was added and incubation at 30° was continued for a further 5 min in order to destroy excess H_2O_2 before the assay of D-glucarate.

The "glucuronolactone dehydrogenase" product was prepared by incubating 10mM D-glucurono-6,3-lactone, 5mM NAD^+, and partially purified enzyme (2 units/mL) in 0.05M orthophosphate–NaOH buffer (pH 6.5) for 30 min at 37°.

For microsomal preparations, male Wistar rats (130–180 g each) were pretreated (i.p.) for 3 days with phenobarbitone (80 mg/kg) and denied food for 24 h prior to being killed. The microsomal preparations[26] were made from liver homogenates (5 mL/g of tissue) in 0.25M sucrose + 40mM Tris/HCl buffer (pH 7.4), washed once by centrifugation in the same medium, and then resuspended (~10 mg of protein/mL) in 0.15M KCl + 10mM Tris/HCl (pH 7.4).

Production of hydrogen peroxide and D-glucarate by microsomal preparations. — For the production of H_2O_2, the incubation mixture (2 mL) consisted of 10mM D-glucose 6-phosphate, 0.6mM $NADP^+$, 10mM $MgCl_2$, 10mM nicotinamide, mM sodium azide, 2mM ADP, 0.15mM KCl, and D-glucose 6-phosphate dehydrogenase (0.5 U/mL), preincubated for 10 min at 37°, and the microsomal preparation (0.2 mL, containing ~2 mg of protein) was then added. For the investigation of the conversion of D-glucuronic acid into D-glucarate, a further 0.05 mL of D-glucuronic acid solution (final concentration, 5 mM) was added. Before the D-glucarate was assayed, the incubation mixtures were chilled in ice for 5 min, and then deproteinised by centrifugation through a Centriflo membrane cone (Amicon 25000M cut-off).

Assays. — Assays of β-D-glucuronidase were conducted at pH 5.0 using *p*-nitrophenyl β-D-glucopyranosiduronic acid as substrate. For assays of D-glucarate, the incubation mixtures were adjusted to pH 9.0 and stored at 30°/5 min in order to convert D-glucarolactone into the acid; two independent methods were then used: (*a*) conversion of D-glucarate into ketodeoxy-D-glucarate, and then into pyruvate, by a bacterial enzyme preparation, followed by assay using lactate dehydrogenase[1], (*b*) inhibition of β-D-glucuronidase after partial conversion into the 1,4-lactone[27].

Lactones were assayed by conversion into the hydroxamate and colorimetry[28] of the ferric hydroxamate at 540 nm, using D-glucaro-1,4-lactone as the standard. Hydrogen peroxide was measured spectrophotometrically, using potassium thiocyanate and ferrous ammonium sulphate[29], with standards prepared from a stock solution of H_2O_2 determined spectrophotometrically using a molar absorption coefficient of 43.6$M^{-1}.cm^{-1}$ at 240 nm.

Chromatography. — Products of the Fenton reaction and of "glucuronolactone dehydrogenase" action were shaken with Dowex 50-X8 (H^+) resin for 3 min to remove cations. Descending p.c. on Whatman 3M paper was then performed with 2-butanol–ethanol–formic acid–water (4:1:1:5) which had been equilibrated at 20° overnight, and to the upper layer of which 10% of ethanol was then added. Detection was effected with aqueous acetone–silver nitrate. T.l.c. of β-D-glucuronidase inhibitors[30] was performed on silica gel (Gelman ITLC Type SG).

Protein concentrations were measured by the method of Lowry *et al.*[31].

RESULTS

Model experiments using the Fenton reaction. — The conversion of D-glucuronic acid into D-glucaric acid by hydrogen peroxide–ferrous ion was confirmed by two separate methods (Fig. 1). The net rate of formation of D-glucarate decreased rapidly with time and the concentration of D-glucarate became almost constant after 3 h. This effect could be explained in part by a decrease in the concentration of D-glucarate when incubated in the same medium, as was also found with D-glucaro-1,4-lactone. The conversion of D-glucuronic acid into D-glucaric acid, and the decomposition of D-glucaric acid or its 1,4-lactone, were inhibited by free-radical scavengers (Table I). Reduction in the formation of D-glucarate in the presence of D-mannitol at the same concentration (5 mM) as that of D-glucuronic acid was ~50%, indicating that these compounds have similar affinities for HO· radicals, whereas thiourea and methyl sulphoxide were much more susceptible.

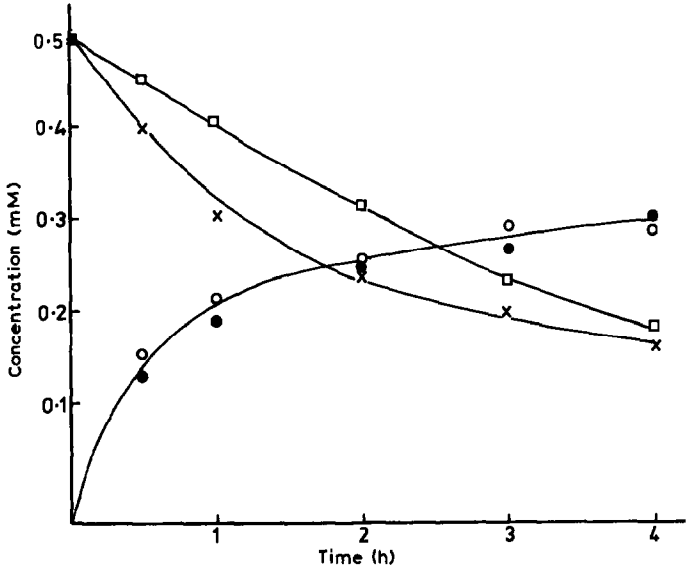

Fig. 1. Formation of D-glucarate [○, method (*a*); ●, method (*b*)] from 5mM D-glucuronic acid, and loss of D-glucarate (□) and D-glucaro-1,4-lactone (×, estimated as D-glucarate) in the Fenton reaction at pH 6.0/30°, with 10mM H_2O_2 and 0.5mM $FeSO_4$.

TABLE I

INHIBITORY EFFECTS OF THE FREE-RADICAL SCAVENGERS D-MANNITOL, THIOUREA, AND METHYL SULPHOXIDE ON THE FORMATION OF D-GLUCARIC ACID FROM D-GLUCURONIC ACID, AND ON THE DECOMPOSITION OF D-GLUCARIC ACID AND D-GLUCARO-1,4-LACTONE, AS A RESULT OF THE FENTON REACTION[a]

Reactant	*Addition*	D-*Glucarate*	
		Final concentration (mM)	*Inhibition (%)*
5mM D-Glucuronate	—	0.31	—
	20mM D-Mannitol	0.093	70
	5mM D-Mannitol	0.16	48
	5mM Thiourea	0.062	80
	mM Thiourea	0.12	61
	mM Me_2SO	0.068	78
	0.4mM Me_2SO	0.14	55
mM D-Glucarate	—	0.71	
	50mM D-Mannitol	0.86	
	10mM Thiourea	0.90	
	4mM Me_2SO	0.89	
mM D-Glucaro-1,4-lactone	—	0.59	
	50mM D-Mannitol	0.72	
	10mM Thiourea	0.75	
	5mM Me_2SO	0.78	

[a]Initial incubations were with 10mM H_2O_2 and 0.5mM $FeSO_4$ in 50mM cacodylate buffer (pH 6.0) for 1 h at 30°, with subsequent measurement of D-glucarate by method (*b*) (see Experimental).

The production of D-glucarate increased markedly as the pH of the medium was lowered (Table II). This result was not due to the conversion of D-glucuronic acid into the lactone since incubation of D-glucurono-6,3-lactone, *e.g.*, at pH 5, in place of D-glucuronic acid then produced no detectable D-glucarate. The formation of D-glucarate from D-glucuronic acid occurred in the presence of either Fe^{2+} or Fe^{3+} and hydrogen peroxide, and was dependent upon the buffer employed. For those buffers (orthophosphate, ACES, Tris/HCl) which also bind Fe ions, a complexing agent (EDTA or ADP) was generally required. However, the presence of the complexing agent diethylenetriaminepenta-acetic acid (DTPA), generally regarded as specific for Fe^{3+}, resulted in negligible production of D-glucarate with Fe^{3+}.

No difference in the production of D-glucarate was observed with the use of either freshly prepared solutions of β-D-glucuronic acid ($[\alpha]_D$ +6°) or solutions which had been stored at 37° for 24 h ($[\alpha]_D$ +38°), when anomeric equilibrium had been attained. Thus, it could be inferred that the rate of conversion of either anomer into D-glucarate was similar.

Lactone formation. — Although D-glucaric acid was identified as an end product of the reaction, there was evidence for the initial formation of a lactone which

TABLE II

EFFECTS OF pH AND DIFFERENT BUFFERS ON THE PRODUCTION OF D-GLUCARATE FROM 5mM D-GLUCURONIC ACID BY 10mM H_2O_2 IN THE PRESENCE OF 0.05mM IRON SALTS AND COMPLEXING AGENTS

Fe Salt	D-*Glucarate production* (μM)[a]				
	pH 5.0	*pH 6.0*	*pH 6.9(a)*	*pH 6.9(b)*	*pH 7.4*
Fe^{2+}	420	179	11.5	15	7
Fe^{2+} + EDTA	53	71	72	45	18
Fe^{2+} + DTPA	53	24	25	17	15
Fe^{2+} + ADP	153	133	6	41	18
Fe^{3+}	440	160	9	18	4
Fe^{3+} + EDTA	7	32	20	47	14
Fe^{3+} + DTPA	5	2.5	0	2	0
Fe^{3+} + ADP	149	142	4	38	17

[a]Buffers employed: pH 5.0, 50mM acetate; pH 6.0, 50mM cacodylate; pH 6.9(*a*), 50mM orthophosphate; pH 6.9(*b*), 20mM ACES; pH 7.4, 20mM Tris/HCl. Assays of glucarate by method (*b*).

strongly inhibited β-D-glucuronidase. The production of D-glucarate, lactone, and β-D-glucuronidase inhibitor determined after destruction of the residual hydrogen peroxide with catalase is shown in Table III. The values for D-glucarate were slightly higher than those for the lactone, particularly at pH 6.0, probably due to partial hydrolysis of the latter. The inhibition of β-D-glucuronidase by the product was such that, if measured as D-glucaro-1,4-lactone, its concentration would have been about thrice that of the determined D-glucarate, and it was thus a much more powerful inhibitor than D-glucaro-1,4-lactone at the equivalent concentration.

The product of oxidation of D-glucuronic acid by the Fenton reaction had a mobility (R_F 0.38) in p.c. which was different from those (0.41 and 0.33, respectively) of D-glucaro-1,4- and -6,3-lactones. The mobility of the product of "glucuronolactone dehydrogenase" was similar to that of the 6,3-lactone. The products were extracted from the appropriate areas of the paper chromatogram with 0.05M acetate buffer and a portion (1 mL) was included in β-D-glucuronidase assays (see Experimental). The extracts of the Fenton reaction product and of D-glucaro-1,4-lactone caused >80% inhibition of the enzyme, whereas those of the 6,3-lactone and of the "glucuronolactone dehydrogenase" product were almost non-inhibitory (<10%). T.l.c. of the β-D-glucuronidase inhibitors[30] also showed that the product of the Fenton reaction (R_F 0.61) was not D-glucaro-1,4-lactone (R_F 0.73) or D-glucuronic acid (R_F 0.43).

Action of superoxide. — No evidence was found for the direct involvement of the superoxide ion in the formation of D-glucarate. When 5mM D-glucuronic acid was incubated with a system generating the superoxide radical, namely, xanthine oxidase (0.05 unit/mL) and 0.3mM xanthine at pH 7.0 and 37° for 30 min, no D-glucarate was produced. However, if superoxide dismutase (60 units/mL) was added, followed after 15 min by mM $FeSO_4$, continued incubation for 30 min yielded 8 μM D-glucarate, presumably due to production of hydrogen peroxide.

TABLE III

COMPARISON OF THE CONCENTRATION OF D-GLUCARATE, LACTONE, AND β-D-GLUCURONIDASE INHIBITOR (EQUIVALENT CONCENTRATION OF D-GLUCARO-1,4-LACTONE) PRODUCED FROM 5mM D-GLUCURONIC ACID BY THE FENTON REACTION AT pH 5.0 AND 6.0[a]

Product	*pH 5.0* (mM)	*pH 6.0* (mM)
D-Glucarate	0.44	0.19
Lactone	0.40	0.145
Inhibitor	1.26	0.50

[a]Incubation conditions as in Table II.

Formation of hydrogen peroxide and D-*glucaric acid by rat-liver microsomal fractions.* — Microsomal fractions of liver homogenates from phenobarbitone-treated rats were incubated at pH 7.1 and 37° in the presence of $NADP^+$, a D-glucose 6-phosphate dehydrogenase system to generate NADPH, and mM azide to minimise the action of any catalase present. The concentrations of the resulting H_2O_2 attained a maximum after 30 min and the rate of net production during the initial 5 min was 4.1 nmol/min/mg of microsomal protein. If $NADP^+$ was excluded and H_2O_2 was added to a final concentration of 100 μM, then, after incubation for 10 min, the concentration of H_2O_2 fell to 23μM (77% loss). Increasing the azide concentration to 5mM had little effect on this loss.

Addition of D-glucuronic acid to the H_2O_2-generating system resulted in the formation of D-glucaric acid (Table IV), and the mean value for 5 microsomal preparations was 0.44 ±0.09 nmol/min/mg of protein. D-Glucarate was not formed

TABLE IV

FORMATION OF D-GLUCARIC ACID FROM 5mM D-GLUCURONIC ACID, ADDED TO A RAT-LIVER MICROSOMAL PREPARATION PREINCUBATED WITH D-GLUCOSE 6-PHOSPHATE, D-GLUCOSE 6-PHOSPHATE DEHYDROGENASE, AND $NADP^+$ (SEE EXPERIMENTAL)[a]

Addition	D-*Glucarate* (μM)	*Relative* β-D-*glucuronidase activity*
None	0	100
D-Glucuronic acid	20	79
D-Glucuronic acid *minus* $NADP^+$	0	98
D-Glucuronic acid + 20mM D-mannitol	3	96
D-Glucuronic acid + superoxide dismutase	23	75
D-Glucuronic acid + 0.5mM Fe^{2+}	38	60
D-Glucuronic acid + 0.5mM Fe^{3+}	35	63
D-Glucurono-6,3-lactone (5mM)	5	98

[a]Apparent β-D-glucuronidase activities were measured directly after incubation with D-glucuronic acid at pH 7.1 for 30 min/37°, using 0.2 mL of the microsomal incubate (final vol., 2 mL); D-glucarate was measured, after deproteinisation, by method (*b*). The values are means of duplicate assays.

when $NADP^+$ was omitted, and only a trace was formed when D-glucuronic acid was replaced by D-glucurono-6,3-lactone, probably due to partial hydrolysis of the lactone, as $NADP^+$ is not a cofactor for any contaminating "glucuronolactone dehydrogenase" activity[13]. As expected, increased formation of D-glucarate resulted on addition of either Fe^{2+} or Fe^{3+} ions to the system containing D-glucuronate. D-Mannitol abolished the formation of D-glucarate, whereas there was little effect when superoxide dismutase was added.

Nature of the product of the microsomal system. — There was evidence for the presence of a strong inhibitor of β-D-glucuronidase in the microsomal system, resulting from the conversion of D-glucuronate (Table IV). Assays of the β-D-glucuronidase activity of the microsomal fractions showed an apparent decrease in the activity of the enzyme when D-glucuronate was added to the H_2O_2-generating system, particularly in the presence of Fe^{2+} or Fe^{3+} ions.

Direct evidence of lactone formation by the microsomal system could not be obtained due to the low concentrations of product, but all the inhibitory effects disappeared if, prior to β-D-glucuronidase assay, a preincubation was performed at pH 8.5 for 15 min at 37°, whereby any lactone present would be hydrolysed.

DISCUSSION

D-Glucaric acid is shown to be formed, *via* a lactone that inhibits β-D-glucuronidase, as a result of the Fenton reaction with D-glucuronic acid. The question remains as to whether intracellular conditions permit a similar transformation.

The production of hydrogen peroxide *in vivo* has been demonstrated by many workers, and, in normal, perfused rat livers, has been calculated to exceed 80 nmol/min/g of liver[32]. Some enzymes, *e.g.*, uric acid oxidase in peroxisomes, form H_2O_2 directly. For other enzymes such as xanthine oxidase, the initial product is mostly the superoxide radical, which may then undergo dismutation, spontaneously or catalysed by superoxide dismutase, to produce H_2O_2 (reaction *1*). No evidence was found that the superoxide radical reacted directly with D-glucuronic acid to produce D-glucaric acid.

$$2\,O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

Most of the H_2O_2 formed in the liver is produced within the microsomal system, however, and involves the action of NADPH and cytochrome P450. It has been suggested[33] that a "short-circuit" of the role of cytochrome P450 in oxygen activation and substrate conversion is responsible, but that the H_2O_2 arises not by dissociation of peroxycytochrome P450 (reaction *2*), but by release of the superoxide radical from oxycytochrome P450 (reaction *3*), followed by dismutation.

$$O_2^-\text{-}Fe^{3+}\text{-}R + 2\,H^+ \rightarrow Fe^{3+}\text{-}R + H_2O_2 \qquad (2)$$

$$O_2\text{-}Fe^{2+}\text{-}R \rightarrow Fe^{3+}\text{-}R + O_2^- \qquad (3)$$

It might then be expected that, if H_2O_2 is the agent responsible, the formation of D-glucarate in the rat-liver microsomal preparation should increase in the presence of added superoxide dismutase. The observed absence of this effect could possibly be explained by the presence of sufficient endogenous dismutase in the crude subcellular fraction.

Hydrogen peroxide, which did not *per se* produce D-glucaric acid from D-glucuronic acid, is a potential source of hydroxyl radicals which readily react with most types of biological molecules. Production of HO· radicals by reaction of H_2O_2 with the superoxide radical (reaction *4*, the Haber–Weiss reaction) was originally suggested[34] as being biologically feasible, but it is now generally agreed that the low rate constant makes this unlikely in view of the steady-state low concentrations of both reactants *in vivo*.

$$H_2O_2 + O_2^- \rightarrow O_2 + HO^- + HO\cdot \qquad (4)$$

However, in the presence of transition metals, and especially iron salts, a catalysed version of this reaction (combination of reactions *5* and *6*) readily produces HO· radicals. Reaction *6* is the Fenton reaction.

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \qquad (5)$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO\cdot \qquad (6)$$

In discussing the possibility that the metal-dependent Haber–Weiss reaction is responsible for known toxic effects of the superoxide radical *in vivo*, Halliwell and Gutteridge[35] concluded that "the small pool of non protein-bound iron between transferrin, cell-cytoplasm, mitochondria, and ferritin could provide iron for the Fenton reaction". In the initial experients in which D-glucuronic acid was converted into a D-glucarolactone by this reaction, inhibition by D-mannitol and other free-radical traps strongly implicated the HO· radical. The presence of the metal was effective in either the ferrous or ferric form, since the latter with H_2O_2 also produces free radicals[22] probably by initial production of superoxide (reaction *7*), which can also participate in reaction *5*.

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^- + 2\,H^+ \qquad (7)$$

The presence of metal-complexing agents (EDTA and ADP) generally stimulated the production of D-glucarate when such metal-binding buffers as phosphate or ACES were used (see Table III). However, inhibition of the reaction was noted in the presence of DPTA, particularly with Fe^{3+}, which is consistent with the finding that DTPA, unlike EDTA, inhibits the iron-catalysed Haber–Weiss reaction[36].

Generation of H_2O_2 by the rat microsomal fraction, in the presence of $NADP^+$, a NADPH-generating system, and mM azide, reached a steady-state con-

dition after 30 min. Increasing the azide concentration to 100mM does not change[33] the net production of H_2O_2, which, presumably, is decomposed by systems other than catalase. Under these conditions, the rate of conversion of D-glucuronate into D-glucarate was low, but was increased by the addition of iron salts as anticipated from the results of the model experiments. The formation of D-glucarate was inhibited when D-mannitol was added to the system, which would support a free-radical mechanism, rather than oxidation of D-glucuronic acid by a possible peroxidic action of cytochrome P450[37]. It was impossible to determine, however, if such radicals were generated by a metal-catalysed Haber–Weiss mechanism, or by a Fenton reaction due solely to the presence of peroxide.

For the microsomal system, indirect evidence for the intermediate formation from D-glucuronic acid of a lactone which inhibited β-D-glucuronidase was the apparent decrease of the activity of this enzyme in the microsomal fraction when $NADP^+$ was added. If this inhibition of β-D-glucuronidase was caused by the formation of D-glucaro-1,4-lactone, the ratios of D-glucarate to D-glucaro-1,4-lactone would be considerably higher than those found in the simple Fenton reaction (see Table III). A D-glucarolactone produced in the microsomal system would, however, be expected to undergo partial hydrolysis to D-glucarate at the pH (7.1) employed.

The product of the Fenton reaction was shown by chromatography to be different from that of "glucuronolactone dehydrogenase" action, which was non-inhibitory to β-D-glucuronidase and is probably D-glucaro-6,3-lactone. It also differed from the known inhibitor D-glucaro-1,4-lactone, compared with which the Fenton product had a considerably higher inhibitory power. This unidentified product is assumed to be D-glucaro-1,5-lactone, which is also the hypothetical product of the oxidation of D-glucuronic acid by the Pt/O_2 system[21], and of uronic acid oxidase found in plant peroxidase preparations[30].

In the Fenton reaction in the presence of D-glucuronic acid, iron remained in the Fe^{2+} state, suggesting that it is involved in the oxidation process, *e.g.*, as in reaction *8*.

$$>CHOH + Fe^{3+} + HO\cdot \rightarrow >C{=}O + Fe^{2+} + H_2O + H^+ \qquad (8)$$

The overall reaction (combination of reactions *6* and *8*) of lactone formation could then be expressed as:

$$\text{D-glucopyranuronic acid} + H_2O_2 \rightarrow \text{D-glucaro-1,5-lactone} + 2\,H_2O,$$

which is analogous to the oxidation of secondary alcohols to ketones by the same agents[38].

This hypothesis for a similar product produced in biological systems and leading to excreted D-glucaric acid could be investigated further using purified hepatic microsomal constituents in order to eliminate, if possible, effects due to endogen-

ous superoxide dismutase and aldose reductase (EC 1.1.1.19) and L-hexonate dehydrogenase. A microsomal ethanol-oxidising system has been reconstituted with partially purified cytochrome P450 and NADPH-cytochrome P450 reductase in phospholipid vesicles[39], and it was concluded that the results were consistent with an iron-catalysed Haber–Weiss mechanism producing hydroxyl radicals which converted ethanol into acetaldehyde. The reaction envisaged in this investigation is analogous, and, if confirmed, would represent the first example of a non-metabolisable compound produced *in vivo* as a result of a free-radical action. It also suggests that the measurement of the excretion of glucaric acid may provide an assessment of a potential mechanism of oxygen toxicity, and that β-D-glucuronidase has a possible function in the provision of free D-glucuronic acid in the cell as an efficient remover of free radicals.

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