benzoate. Thus it was found that cyclohexylglucuronide was excreted in the urine of human administered CHS–Na.

Quantitative Investigation of Cyclohexylamine, Cyclohexanol, Cyclohexanone, and Conjugated Cyclohexanol in the Urine

Five volunteers (3 males and 2 females) were given a single dose of 2 g CHS–Na orally. Each volunteer urine was collected for 24 hours after administering CHS–Na. The metabolites of CHS–Na in the urine, cyclohexylamine, cyclohexanol, cyclohexanone, and conjugated cyclohexanol, were determined according to the methods described in experimental.

As shown in Table IV, the results obtained indicated that cyclohexylamine, cyclohexanol, cyclohexanone, and conjugated cyclohexanol were found in the urines of all volunteers receiving CHS–Na, furthermore, that those metabolites excreted in the urine were a small amount.

Table IV. Urinary Excretion of the Metabolites in the 24 hr Urine of Human receiving Orally 2 g CHS-Na

Subject[a]	μg excreted			
	Cyclohexylamine	Cyclohexanol	Cyclohexanone	Conjugated cyclohexanol
K.I. (27—52) ♂	82	150	216	1710
K.S. (25—52) ♂	628	134	212	3990
S.K. (38—51) ♂	10800	45	82	2660
A.S. (21—47) ♀	2370	25	20	3080
Y.O. (21—51) ♀	40	20	10	5140

a) Bracketed quantities are subject's age in years followed by body weight in kilograms.

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Studies on the Glucaric Acid Pathway in the Metabolism of D-Glucuronic Acid in Mammals. V.[1,2] Stimulatory Effect of Diphenylhydantoin and Phenobarbital on the D-Glucaric Acid Synthesis in Man

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Recently an alternative pathway of D-glucuronic acid conversion, in addition to the conversion to L-ascorbic acid or to L-xylulose,[4] which involves the oxidation of D-glucuronolactone (I) to D-glucaric acid (III) by D-glucuronolactone dehydrogenase, has been demon-

1) Part IV: M. Okada, M. Matsui, Y. Watanabe, T. Wanibe, and F. Abe, *Seikagaku*, **39**, 553 (1967). This is the abstract of the paper presented at the 40th Annual Meeting of the Japanese Biochemical Society, Osaka, November, 1967.
2) Part of this work was presented at the 39th Annual Meeting of the Japanese Biochemical Society, Kyoto, November, 1966.
3) Location: *Takada 3-chome, Toshima-ku, Tokyo.*
4) J.J. Burns and A.H. Conney, "Glucuronic Acid," ed. by G.J. Dutton, Academic Press, New York, 1966, p. 365.

strated by Marsh in mammalian systems (Chart 1).[5] On the other hand, a variety of drugs have been reported to stimulate the biosynthesis of L-ascorbic acid,[6] L-xylulose,[7] or III[8] in the rat. The way in which drugs exert this stimulatory effect is not known certainly, but available evidence indicates that many of these drugs act by stimulating the synthesis of the common intermediate D-glucuronic acid or its lactone (I) from UDP-glucose (UDPG) (Fig. 1).

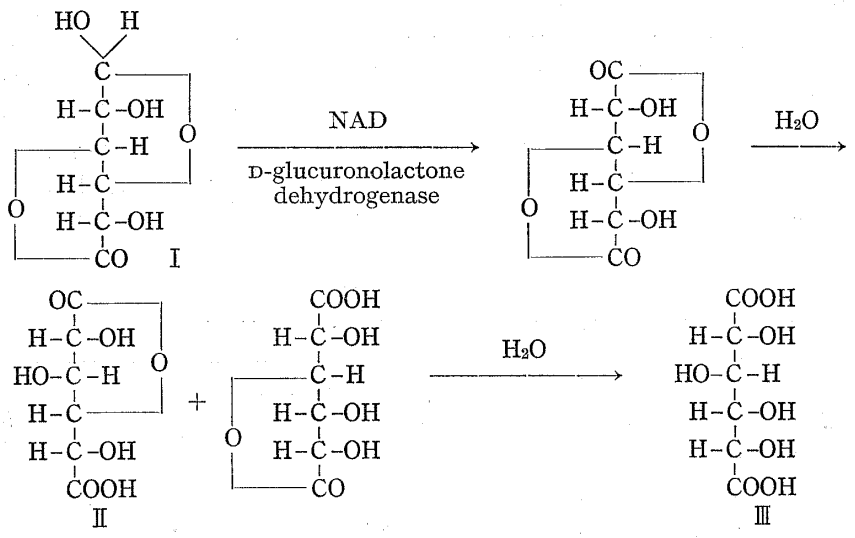

Chart 1

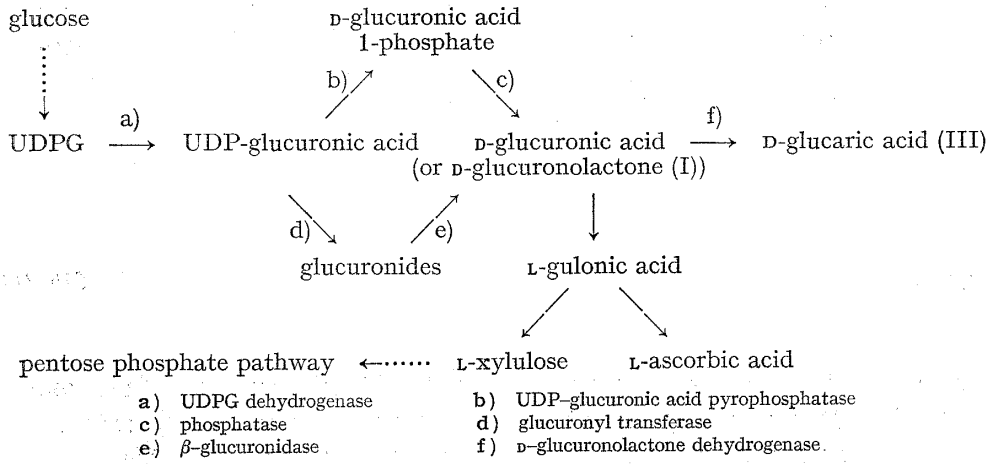

Fig. 1. The Glucuronic Acid Pathway of Glucose Metabolism

The possibility of getting an indication for the effect of drugs on the metabolism of D-glucuronic acid in man, with the aid of L-ascorbic acid as an indicator, naturally fails, since man, monkey, and guinea pig are the only mammals known to be unable to synthesize L-ascorbic acid from D-glucuronic acid. The estimation of L-xylulose, a metabolic intermediate involved in the glucuronic acid–xylulose cycle, does not seem to be a highly suitable indicator, because its levels in the urine or serum of man was found[9] to be very low even after loading

5) *a*) C.A. Marsh, *Biochem. J.*, **86**, 77 (1963); *b*) *Idem, ibid.*, **87**, 82 (1963); *c*) *Idem, ibid.*, **89**, 108 (1963); *d*) *Idem, ibid.*, **99**, 22 (1966).
6) J.J. Burns, A.H. Conney, P.G. Dayton, C. Evans, G.R. Martin, and D. Taller, *J. Pharmacol. Exptl. Therap.*, **129**, 132 (1960); A.H. Conney, G.A. Bray, C. Evans, and J.J. Burns, *Ann. N.Y. Acad. Sci.*, **92**, 115 (1961).
7) M. Enklewitz and M. Lasker, *J. Biol. Chem.*, **110**, 443 (1935).
8) C.A. Marsh and L.M. Reid, *Biochim. Biophys. Acta*, **78**, 723 (1963).
9) Y. Kumahara, D.S. Feingold, I.M. Freedberg, and H.H. Hiatt, *J. Clin. Endocrinol. Metab.*, **21**, 887 (1961).

of the precursor I. Moreover, the sensitive and specific enzymic method[10] for the determination of L-xylulose in biological materials is rather troublesome in the routine of laboratory work. While determination of III in man which has been identified as a normal constituent of human urine[5a] is expected to provide an indication for the matter concerned.[11] This paper deals with determinations of the urine and serum levels of III in epilepsy patients receiving diphenylhydantoin and phenobarbital, both are known as potential stimulator of drug metabolism as well as of carbohydrate metabolism *via* the glucuronic acid pathway in mammals.[12]

Thus determinations of the urinary excretion of III in epilepsy patients under chronic (2—28 years) treatment with the above drugs[13] have been made by the colorimetric method reported earlier.[14] The amounts of III in 24-hour urines of the patients determined by this method are shown in Table I (third column). It is evident that the urine levels of III in these patients are much higher than those of normal human subjects which have been estimated to be about 10—20 mg.[5a,14]

Table I. Urinary Excretion of D-Glucaric Acid (III) before and after Oral Administration of D-Glucuronolactone (I) (1 g) to Epilepsy Patients under Chronic Treatment with Diphenylhydantoin and Phenobarbital

No.	Subjects	D-Glucaric acid in urine (mg/24 hr) Before administration of I	After administration of I 0—24 hr	24—48 hr	Conversion (%)
1	S.S.	154	373	192	21
2	H.I.	54	192	89	14
3	T.K.	60	218	60	13
4	T.T.	249	511	277	24
5	T.T.	275	610	274	28
6	M.U.	148	291	141	12
7	K.W.	47	324	51	23
8	G.S.	32	167	33	11
9	M.W.	52	—	—	—
10	T.O.	31	—	—	—
11	S.K.	180	—	—	—
12	M.S.	146	—	—	—
13	R.M.	36	—	—	—

In order to elucidate whether this stimulation of the synthesis of III resulted from a rise of liver D-glucuronolactone dehydrogenase activity which is involved in the conversion of I into III (Chart 1) or from an increased production of D-glucuronic acid or its lactone (I) available for the enzyme as substrate,[15] loading tests with I (1 g) were performed with some of the above patients. As shown in Table I there was no significant difference between the conversion (%)[16] of I in the patients and that in normal human subjects, which has been estimated to be approximately 16% of the dose.[5a,14] Accordingly, the increased synthesis of III due to the above hypnotics in man could also be explained by an increased formation of D-glucuronic

10) J. Hickman and G. Ashwell, *J. Biol. Chem.*, **234**, 758 (1959).
11) III is considered to be the final metabolite in the glucaric acid pathway of the glucuronic acid metabolism in mammalian systems. D.C. Fish, *Dissertation Abstr.*, **25**, 3211 (1964); *Univ. Microfilms* (Ann Arbor, Mich.), No. 64-12595 (1964); C.A. Marsh, personal communication; S. Takanashi, Y. Watanabe, and M. Okada, unpublished result.
12) A.H. Conney, *Pharmacol. Rev.*, **19**, 317 (1967).
13) When this work had been accomplished, a paper dealing with the similar subject was published. E.M. Aarts, *Biochem. Pharmacol.*, **14**, 359 (1965).
14) M. Ishidate, M. Matsui, and M. Okada, *Anal. Biochem.*, **11**, 176 (1956).
15) Administration of 1 g or 5 g of I to normal human subjects resulted in almost the same per cent conversion of I into urinary III,[14] thus demonstrating that capacity of the liver D-glucuronolactone dehydrogenase in man is high.
16) The conversion (%) was calculated as described previously.[14]

acid or I from UDPG (Fig. 1). In connection with the above result, an incomprehensible observation[17] that a several fold increase in β-glucuronidase concentration was necessary in urine samples from human subjects receiving diphenylhydantoin for maximal hydrolysis of steroid glucuronides as compared to control urine samples, can now be explained unequivocally on the basis of the increased excretion of III in the urines which is converted into D-glucaro-(1→4)-lactone (II), the most potent β-glucuronidase inhibitor,[18] in the incubation conditions employed for the enzymic hydrolysis.

On the other hand, the serum level of III in normal human subjects was so low that accurate determination of it by the colorimetric method was almost impossible.[19] Then a fluorimetric one has been developed recently in this laboratory.[1] By using this method the serum level in normal human adults as well as epilepsy patients chronically under treatment with diphenylhydantoin and phenobarbital was determined. In accordance with expectation it was found to be markedly elevated in these patients compared with normal human subjects, as indicated in Table II. The highest level observed (272 μg/dl) originated from the patient (Subject T.T. in Table I) receiving the drugs for 28 years whose urinary excretion of III was also most increased.

Table II. Serum Level of D-Glucaric Acid (III) in Normal Human Adults (A) and in Epilepsy Patients (B) under Chronic Treatment with Diphenylhydantoin and Phenobarbital

Subject (number)	Serum level of III (μg/dl)	Mean (±S.E.)
A (13)	50, 48, 47, 42, 40, 37, 33, 29, 24, 20, 17, 13, 10	32± 4
B (28)	272, 182, 181, 167, 147, 130, 111, 108, 104, 101, 100, 90, 90, 89, 86, 86, 85, 78, 76, 74, 73, 71, 69, 67, 63, 57, 38, 37	101±11

Additionally, it may be of interest as well as significant that the serum β-glucuronidase level of the epilepsy patients receiving the drugs was mostly elevated,[20] in regard to the postulated pathways for the biosynthesis of D-glucuronic acid in mammals indicated in Fig. 1, though the source of the enzyme and the possible mechanisms by which the enzyme was released are yet obscure.

In conclusion, the urinary excretion as well as the serum level of III might be a useful test for drug–induced alterations in the glucuronic acid pathway in man lacking in the glucuronic acid–L-ascorbic acid cycle.

Materials and Methods

Urine and Serum Samples——Twenty-four hour urines before and after oral administration of D-glucuronolactone (I) (1 g) were collected from epilepsy patients at Yowa Hospital, Tokyo, Shonan Hospital, Yokohama, and Shikiba Hospital, Ichikawa (Chiba prefecture). Sera were collected from epilepsy patients at Yowa Hospital, Tokyo University Hospital, and Shikiba Hospital. Venous blood was taken from the antecubital vein from fasting patients. Blood was allowed to clot, and the serum taken off as soon as possible. The serum was again centrifuged in a conical tube to ensure that all cells were removed.

Determination of Urinary D-Glucaric Acid (III)——This was performed according to "Procedure II" of the method reported previously.[14]

Determination of Serum D-Glucaric Acid (III)——This was carried out by the fluorimetric method[1] using "Procedure II" described previously.[19]

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17) E.E. Werk, Jr., J. MacGee, and L.J. Sholiton, *J. Clin. Invest.*, **43**, 1824 (1964).
18) G.A. Levvy, *Biochem. J.*, **52**, 464 (1952).
19) M. Matsui, T. Kaizu, M. Okada, and M. Ishidate, *Chem. Pharm. Bull.* (Tokyo), **17**, 1871 (1969).
20) T. Kaizu, M. Matsui, F. Abe, and M. Okada, *Seikagaku*, **38**, 648 (1966); C.M. Plum, *Enzym. Biol. Clin.*, **8**, 97 (1967).