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STUDIES ON CARCINOGENIC TRYPTOPHAN METABOLITES*—I

ENZYMATIC FORMATION AND HYDROLYSIS OF GLUCURONIDE OF 3-HYDROXYANTHRANILIC ACID

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Abstract—3-Hydroxyanthranilic acid (3-OHAA) is a metabolite of tryptophan which is excreted in the urine as the glucuronide and it has been supposed that 3-OHAA formed by the enzymatic hydrolysis in urine from the glucuronide causes bladder cancer. The enzymatic formation and hydrolysis of this glucuronide were investigated. 3-OHAA and uridine-5′-pyrophosphate-D-glucuronic acid were incubated with the liver microsomes of guinea pigs, mice and rats. The glucuronide of 3-OHAA was formed more slowly than *p*-nitrophenol glucuronide (0·064, 0·026 and 0·025 times in guinea pigs, mice and rats respectively). Marked species difference in the activity of the liver microsomes was found, i.e. guinea pigs > mice > rats. When the glucuronide of 3-OHAA was incubated with β-glucuronidase obtained from human urine, it was hydrolysed more slowly than phenolphthalein glucuronide.

THE TRYPTOPHAN metabolites, 3-hydroxykynurenine, (3-OHKY) and 3-hydroxyanthranilic acid, (3-OHAA) have been described as "endogenous carcinogens", since it was reported that the bladder cancer in mice[1,2] and rats[3] was induced by the implantation of cholesterol pellets containing these compounds and that the leucaemia in mice[4,5] was induced by the subcutaneous injection of these compounds. Since the quantity of these compounds in human urine is increased in patients with bladder cancer,[6] the relation between the tryptophan metabolites in urine and bladder cancer has been investigated by some workers.[7–10] Boyland *et al.*[6,11] presumed that 3-OHKY and 3-OHAA were excreted in urine as the glucuronide and showed carcinogenic action following the enzymatic hydrolysis. This hypothesis is based on the fact that the urinary β-glucuronidase activity, which hydrolyses glucuronides, is increased in patients with bladder cancer.[11] However, the enzymatic formation and hydrolysis of these glucuronides have not been investigated. In this paper, the formation of 3-OHKY glucuronide and 3-OHAA glucuronide (3-OGAA) by the liver enzyme was studied and the formation of 3-OGAA was confirmed. The hydrolysis of 3-OGAA by the β-glucuronidase (EC 3.2.1.31) obtained from human urine was also studied.

The enzyme of glucuronide formation (UDPGA-glucuronyl transferase, EC 2.4.1.17) is present in the microsome of liver and requires uridine-5′-pyrophosphate-D-glucuronic acid (UDPGA) as a glucuronyl donor.[12] This reaction is apparently

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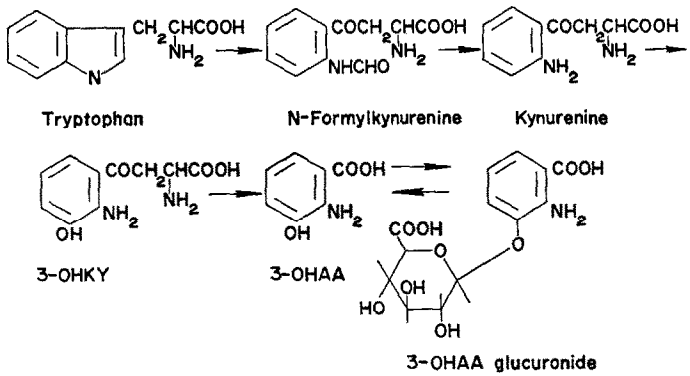

stimulated by the addition of uridine-5'-pyrophosphate-*N*-acetylglucosamine (UDPAG) and ATP because UDPAG and ATP inhibit the breakdown of UDPGA.[13] In this work, UDPGA, UDPAG, ATP and 3-OHAA or 3-OHKY were incubated with the liver microsome of guinea pig and a small quantity of the gum which was identified with 3-OGAA was obtained when 3-OHAA was added. The rates of 3-OGAA formation by the liver microsomes of guinea pigs, mice and rats were estimated by the determination of 3-OGAA formed. At the same time, the rates of the formation of *p*-nitrophenol glucuronide were estimated and compared with those of 3-OGAA formation.

β-Glucuronidase in human urine is precipitated with $(NH_4)_2SO_4$ or organic solvents.[14] In this work, the precipitate by 40 per cent saturation with $(NH_4)_2SO_4$ was used as the enzyme hydrolysing 3-OGAA. The rate of the reaction was estimated by the determination of 3-OHAA liberated. At the same time, the rate of the hydrolysis of phenolphthalein glucuronide was estimated and compared with that of hydrolysis of 3-OGAA.

MATERIALS AND METHODS

Animals. Adult male Hartley guinea pigs (300–350 g), Wistar rats (200–250 g) and DDN mice (15–20 g) were used.

Reagents. Avicel SF cellulose powder and silica gel H from the Merck Co. were used for thin layer chromatography. UDPGA and UDPAG were obtained from the Boehringer & Sohne GmbH Mannheim, ATP from Kowa Co., Nagoya, Japan, bovine β-glucuronidase (570 Fu/mg) from the Worthington Biochemical Corp., New Jersey, DEAE Sephadex (A-25) from the Pharmacia, Upsala, Sweden, 3-OHAA from the Tokyo Kasei Co., Tokyo, Japan, 3-OHKY from the Senju-Seiyaku Co., Osaka, Japan and phenolphthalein glucuronide from the Chugai-Seiyaku, Tokyo, Japan.

Apparatus. Hitachi MPF-2A spectrofluorometer was employed for fluorometry. The sensitivity was adjusted for the meter to show 60 (ex. 350 nm, fl. 450 nm, the sample sensitivity 2) for 10^{-6} M solution of quinine sulfate in 0·1 N H_2SO_4. Hitachi 101 spectrophotometer was employed for photometry.

Preparation of liver microsomes. Animals were killed by decapitation, the livers were removed and 20 per cent homogenates were prepared in 0·154 M KCl. After mitochondria were sedimented at 11,000 *g* for 30 min and discarded, microsomes were

sedimented at 105,000 *g* for 60 min. The microsomes were resuspended in 0·154 M KCl. Protein was determined by the Biuret method.[15]

Detection of 3-OGAA. To 6 ml of 0·125 M Na_2HPO_4–KH_2PO_4 buffer (pH 7·4) containing 16 μmoles of UDPGA, 8 μmoles of UDPAG, 16 μmoles of ATP, 48 μmoles of $MgCl_2$ and 20 μmoles of 3-OHAA or 3-OHKY, 2 ml of the liver microsome suspension from guinea pig (1 ml/g liver, protein 1·2 mg/ml) was added. The mixture was incubated at 37° for 180 min and the reaction was stopped by heat at 100° for 1 min. After centrifugation, the supernatant was concentrated to 0·5 and 0·05 ml of the concentrate was spotted on the silica gel plate (0·25 mm thick). The chromatogram was developed with a mixture of *n*-butanol–*n*-propanol–ethanol–water (3:6:4:2).

Periodate benzidine test for sugars was attempted as follows. After spraying 0·4 per cent aqueous solution of KIO_4 to the plate, 0·1 per cent solution of benzidine in ethanol was sprayed. The appearance of a white spot on the blue ground means the presence of sugars.

For the control experiment, the mixtures from which the substrates or microsome were omitted were treated by the same procedure.

Separation of 3-OGAA. DEAE Sephadex was washed with dil. $(NH_4)_2CO_3$ (the solution of 1 g of $(NH_4)_2CO_3$ in 100 ml of water) and changed to CO_3 type. The remain of the incubation mixture described above was adsorbed on the column of the DEAE Sephadex (1 × 20 cm). After the column was washed with 125 ml of the dil. $(NH_4)_2CO_3$, 3-OGAA was eluted with 80 ml of conc. $(NH_4)_2CO_3$ (the solution of 15 g of $(NH_4)_2CO_3$ in 100 ml of water) and the elute was evaporated to dryness *in vacuo*. The residue was dissolved in a small volume of methanol and applied in a linear fashion to the cellulose powder plate (0·25 mm thick). The chromatogram was developed with a mixture of *n*-butanol–*n*-propanol–ethanol–water (3:6:4:2). From the fluorescent band (R_f 0·35), 3-OGAA was extracted with 10 ml of methanol. After centrifugation, the methanol was evaporated to dryness and a small quantity of the fluorescent gum was obtained.

Treatments of 3-OGAA. The gum was dissolved in 100 ml of methanol and used for the following experiments.

(1) Hydrolysis by bovine β-glucuronidase. One milliliter of the methanol solution of the gum was evaporated to dryness and the residue was dissolved in 1 ml of 0·25 M Na acetate buffer (pH 4·5). To this solution, 0·1 ml of β-glucuronidase solution (1100 Fu/ml) was added. After incubation at 37°, the mixture was cooled in ice and 0·15 ml of 0·5 N H_2SO_4 was added to adjust the pH to 3. 3-OHAA was extracted by shaking with each 2 ml of ether twice. The ether layers were combined and made up to 5 ml with ether, and 3-OHAA was determined fluorometrically (ex. 345 nm, fl. 410 nm).

3-OHAA and 3-OGAA were detected as follows. The ether extract was concentrated and spotted on the cellulose powder plate. The chromatogram was developed with a mixture of *n*-butanol–*n*-propanol–ethanol–water (6:3:2:0·3) and the fluorescent band of 3-OHAA (R_f 0·6) was observed under the 365 nm ultraviolet light. The aqueous layer was evaporated to dryness *in vacuo* and the residue was dissolved in a small volume of methanol. This solution was spotted on the cellulose powder plate and the chromatogram was developed with the mixture of *n*-butanol–*n*-propanol–ethanol–water (3:6:4:2). The band of 3-OGAA was detected.

(2) Naphthoresorcinol test. Glucuronyl group in the gum was determined by the naphthoresorcinol method.[16] One milliliter of the methanol solution of the gum was

evaporated to dryness and the residue was dissolved in 1 ml of water. A mixture of 0·5 ml of this solution, 0·5 ml of 0·35 per cent solution of naphthoresorcinol in 0·1 N H_2SO_4, 0·75 ml of 15 N H_2SO_4 and 0·1 ml of 1 per cent aqueous solution of chloramine T was heated at 100° for 120 min. The sample was cooled in ice for 10 min and shaken with 2 ml of ethylacetate. The absorbance of the ethylacetate layer at 565 nm was estimated.

(3) Alkali treatment. Ten milliliter of the methanol solution of the gum was evaporated to dryness and the residue was dissolved in 0·5 ml of water. A mixture of 0·05 ml of this solution and 0·05 ml of 0·5 N NaOH was kept at a room temperature for 6 hr. Then, 0·3 ml of 0·5 M glycine–H_2SO_4 buffer (pH 3) was added and 0·1 ml of this mixture was applied in a linear fashion to the cellulose powder plate. The chromatogram was developed with the mixture of *n*-butanol–*n*-propanol–ethanol–water (3:6:4:2) and 3-OGAA was extracted with 5 ml of methanol. After centrifugation, 3-OGAA was estimated fluorometrically (ex. 340 nm, fl. 418 nm).

For the control experiment, 0·05 ml of the aqueous solution of the gum, 0·3 ml of 0·5 M glycine–H_2SO_4 buffer (pH 3) and 0·05 ml of 0·5 N NaOH were mixed and 0·1 ml of this mixture was applied to the cellulose powder plate immediately. 3-OGAA was developed and estimated by the same procedure.

(4) Estimation of fluorescence. After the evaporation of 0·2 ml of the initial solution of the gum in methanol, the residue was dissolved in 4 ml of the solvents and the fluorescence spectra were estimated.

Estimation of the rate of 3-OGAA formation. The usual incubation system was as follows. To 0·3 ml of 0·1 M Na_2HPO_4–KH_2PO_4 buffer (pH 7·4) containing 2 μmoles of UDPGA, 0·4 μmole of UDPAG, 0·8 μmole of ATP, 2·4 μmoles of $MgCl_2$ and 0·4 μmole of 3-OHAA, 0·1 ml of the liver microsome suspension from guinea pigs (5 ml/g liver, 0·27–0·32 mg protein/ml), mice (1 ml/g liver, 1·2–1·5 mg protein/ml) or rats (0·5 ml/g liver, 2·0–2·4 mg protein/ml) was added. After incubation at 37° for 30 min, the reaction was stopped by the addition of 2 ml of ethanol. After centrifugation, 0·5 ml of this mixture was applied in a linear fashion to the cellulose powder plate and the chromatogram was developed with the mixture of *n*-butanol–*n*-propanol–ethanol–water (3:6:4:2). 3-OGAA was extracted with 5 ml of methanol and determined fluorometrically (ex. 340 nm, fl. 418 nm).

In order to obtain the standard curve, an aqueous solution of 3-OGAA (the methanol solution was evaporated to dryness and the residue was dissolved in water. The quantity of the glucuronide was derived from the value, 7·85 μg/ml in the methanol solution, which calculated from the result of the hydrolysis by bovine β-glucuronidase) was added to this system in place of 3-OHAA and this mixture was treated by the same procedure. The standard curve was linear within the range of 0·2–5 μg in each tube.

Estimation of the rate of p-nitrophenol glucuronide formation. In the experiment described above, *p*-nitrophenol was added in place of 3-OHAA. The mixture was incubated for 15 min and the reaction was stopped by the addition of 2 ml of ethanol. After centrifugation, 0·5 ml of the supernatant was added into 3 ml of 0·1 N NaOH and the remains of *p*-nitrophenol was determined photometrically at 420 nm.

Precipitation of β-glucuronidase from human urine. One l. of the urine of a normal adult man was centrifuged at 5000 *g* for 20 min and the supernatant was brought to 40 per cent saturation with $(NH_4)_2SO_4$. After keeping in ice for 60 min, the precipitate was collected by centrifugation at 5000 *g* for 20 min and dissolved in 100 ml of water.

The insoluble substance was eliminated by centrifugation and the supernatant was brought to 40 per cent saturation with $(NH_4)_2SO_4$ again. The precipitate was collected and dialysed in 0·05 M Na acetate buffer (pH 4·5) overnight. This enzyme was lyophylized and dissolved in 2 ml of water. In this solution, 15·2 mg/ml of protein was contained (by the Biuret method).

Estimation of hydrolysis of 3-OGAA. After the evaporation of 26·2 ml of the initial solution of the gum in methanol, the residue was dissolved in 0·5 ml of 0·2 M Na acetate buffer (pH 4·5). This solution was treated as $1{\cdot}25 \times 10^{-3}$ M solution of 3-OGAA (from the result of the hydrolysis of 3-OGAA by the bovine β-glucuronidase) and diluted in use.

A mixture of 0·04 ml of the substrate solution and 0·01 ml of the enzyme solution was incubated at 37° for 60 min. Then, the sample was cooled in ice and 1 ml of 0·5 M glycine–H_2SO_4 buffer (pH 3) was added. 3-OHAA was extracted by shaking with each 2 ml of ether twice. The ether layers were combined and made up to 5 ml with ether. 3-OHAA was estimated fluorometrically (ex. 345 nm, fl. 410 nm).

Estimation of hydrolysis of phenolphthalein glucuronide. A mixture of 0·16 ml of phenolphthalein glucuronide solution in 0·2 M Na acetate buffer (pH 4·5) and 0·04 ml of the enzyme solution was incubated at 37° for 60 min. The reaction was stopped by the addition of 10 per cent trichloroacetic acid. Three milliliter of 0·5 M glycine–NaOH buffer (pH 10·5) was added and phenolphthalein was estimated photometrically at 550 nm.

RESULTS

Detection of glucuronides. As shown in Fig. 1, a periodate-benzidine positive spot with purple fluorescence (under 365 nm ultraviolet light) was appeared when 3-OHAA was added as a substrate (the sample A). This result suggests the formation of 3-OGAA. No such a spot was found when 3-OHKY was added (the sample C). As for the sample C, the separation by the paper chromatography was attempted by the method of Dalgliesh.[17] However, the formation of 3-OHKY glucuronide was not proved.

Separation and treatments of 3-OGAA. The gum obtained was treated as follows.

(1) Hydrolysis by the bovine β-glucuronidase. As shown in Fig. 2, 3-OHAA formation reached a constant value after incubation for 30 min. In this condition, 3·65 μg of 3-OHAA was formed from each tube. The quantity of 3-OGAA in each tube (G) was calculated from this result as follows.

$$G(\mu g) = \frac{329{\cdot}24}{153{\cdot}12} \times 3{\cdot}65 = 7{\cdot}85 \text{ (Coefficient is the ratio of the molecular weights).}$$

The spot of 3-OHAA (R_f 0·75) was obtained from the ether extract of the mixture incubated and 3-OGAA could not be found in the aqueous layer. These results show that 3-OGAA was completely hydrolysed in this condition.

This result means that the concentration of 3-OGAA in the initial solution of the gum in methanol is 7·85 μg/ml. This value was employed in the following experiments.

(2) Naphthoresorcinol test. The result of this test shows that the glucuronide equivalent to 4·6 μg of glucuronic acid was contained in each tube (mean of four estimations) and G was calculated as follows.

$$G(\mu g) = \frac{329{\cdot}24}{194{\cdot}14} \times 4{\cdot}6 = 7{\cdot}80.$$

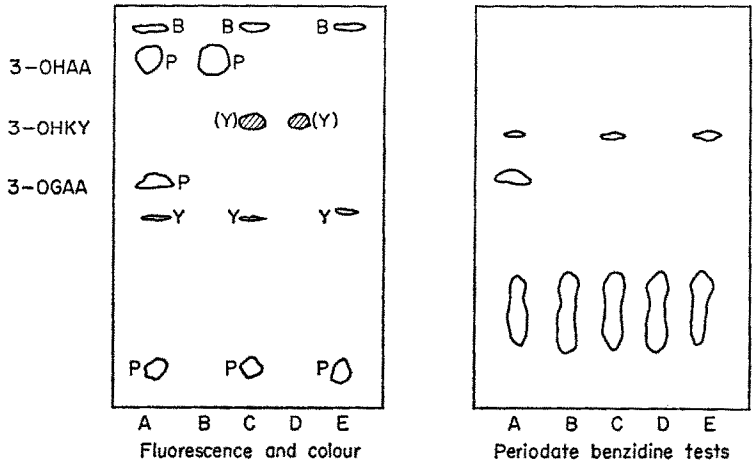

FIG. 1. Detection of 3-OHAA or 3-OHKY glucuronide.
A, 3-OHAA, UDPGA and the microsome were contained;
B, The microsome was omitted from A;
C, 3-OHKY was added in the place of 3-OHAA;
D, The microsome was omitted from C;
E, 3-OHAA was omitted from A.
Fluorescence B: blue, P: purple, Y: yellow.
Color (Y): yellow.

From the above experiments, it is shown that this compound consists of equimolar glucuronic acid and 3-OHAA.

(3) Alkali treatment. The fluorescence intensity was 32·5 (the sample sensitivity 2) in the sample treated with alkali and 33 in the control experiment. This compound was unchanged by the alkali treatment.

Glucuronic acid conjugates to a hydroxyl group or a carboxyl group and the conjugated compounds are called ether type and ester type glucuronide, respectively. The

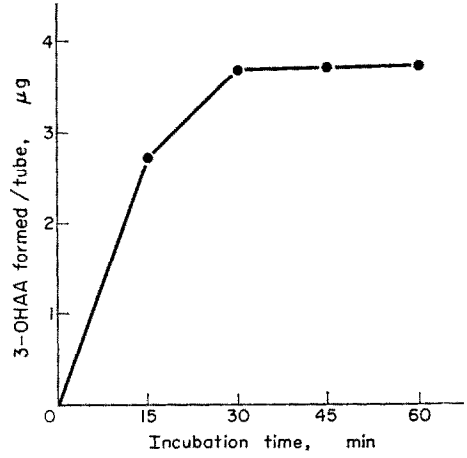

FIG. 2. Hydrolysis of 3-OGAA by bovine β-glucuronidase. Total volume, 1·1 ml, β-glucuronidase: 110 Fu, pH, 4·5.

TABLE 1. FLUORESCENCE OF SOLUTION OF 3-OGAA

Solvents	Max. of excitation spectra (uncorrected) (nm)	Max. of fluorescence spectra (uncorrected) (nm)	Fluorescence intensity
Methanol	340	418	33·2
Ethanol	340	418	37·0
Acetone	328	405	47·6
Distilled water	322	405	23·4
0·1 N HCl	—	—	0
0·1 M Na_2HPO_4	322	405	26·0
0·5 N NaOH	322	405	6·3

The sample sensitivity: 2 (Hitachi MPF-2A).

TABLE 2. ACTIVITY OF FORMATION OF 3-OHAA AND *p*-NITROPHENOL GLUCURONIDE BY LIVER MICROSOMES

Species	Formation of 3-OGAA		Formation of *p*-nitrophenol glucuronide	
	(mole/hr/g liver) $\times 10^{-8}$	(mole/hr/mg protein) $\times 10^{-9}$	(mole/hr/g liver) $\times 10^{-6}$	(mole/hr/mg protein) $\times 10^{-7}$
Guinea pigs	56·8 ± 1·7	39·2 ± 0·57	8·93 ± 0·18	6·16 ± 0·14
Mice	7·78 ± 0·08	6·35 ± 0·36	2·99 ± 0·01	2·45 ± 0·15
Rats	4·29 ± 0·30	3·40 ± 0·06	1·76 ± 0·10	1·35 ± 0·11

Results are expressed as means ± S.E. N = 5. 3-OHAA: 1 mM, UDPGA: mM, UDPAG 1 mM, ATP: 2 mM, $MgCl_2$: 6 mM, pH: 7·4.

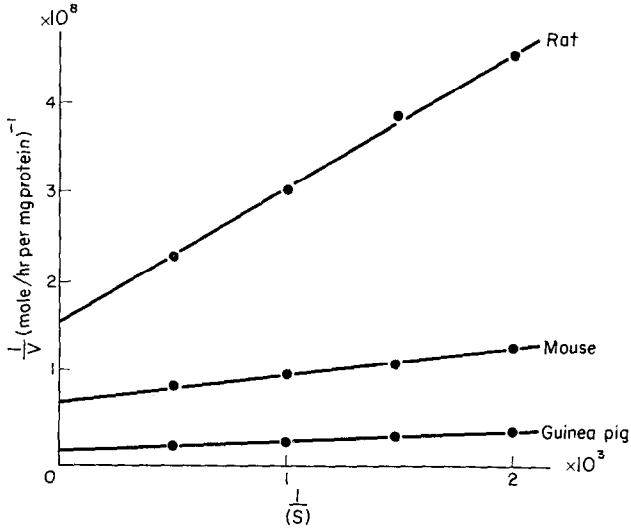

FIG. 3. The Lineweaver–Burk plot of 3-OGAA formation by the liver microsomes. UDPGA: 5 mM, UDPAG: 1 mM, ATP: 2 mM, $MgCl_2$: 6 mM, pH: 7·4. K_m(M) = 2·66 × 10^{-3} (guinea pig), 8·27 × 10^{-4} (mouse), 1·00 × 10^{-3} (rat), V_{max} (mole/hr/mg protein) = 1·57 × 10^{-7} (guinea pig), 1·67 × 10^{-8} (mouse), 7·26 × 10^{-9} (rat).

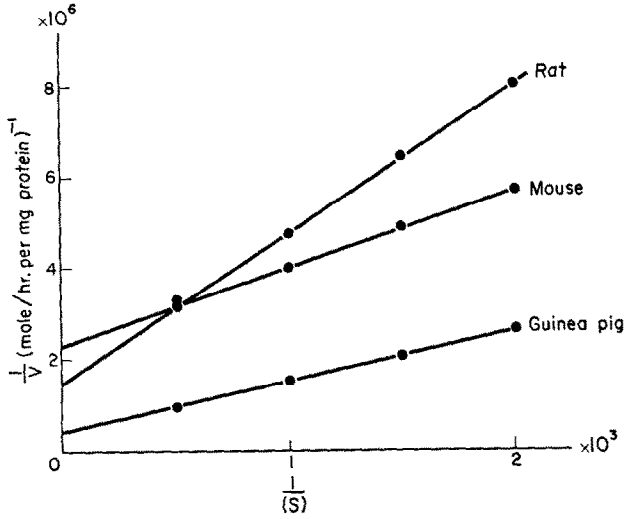

FIG. 4. The Lineweaver–Burk plot of *p*-nitrophenol glucuronide formation by the liver microsomes. UDPGA: 5 mM, UDPAG: 1 mM, ATP: 2 mM, $MgCl_2$: 6 mM, pH: 7·4. K_m(M) = 3·33 × 10^{-3} (guinea pig), 6·77 × 10^{-4} (mouse), 2·17 × 10^{-3} (rat), V_{max} (mole/hr/mg protein) = 2·90 × 10^{-6} (guinea pig), 4·10 × 10^{-7} (mouse), 6·83 × 10^{-7} (rat).

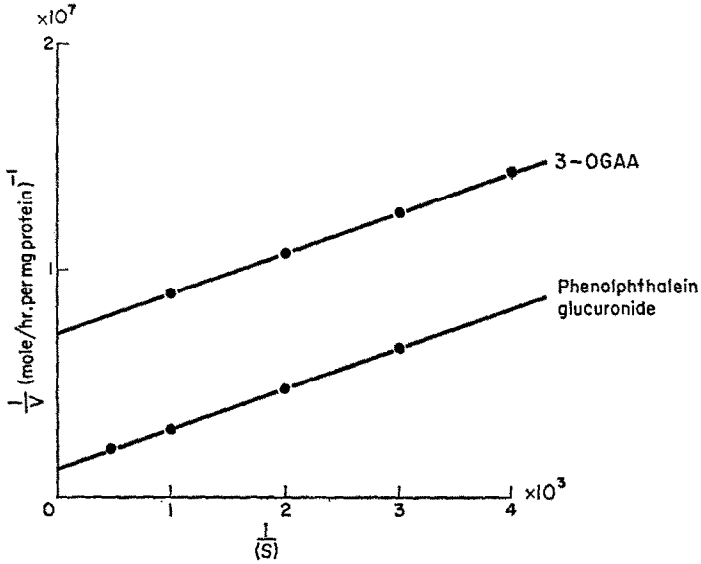

FIG. 5. The Lineweaver–Burk plots of hydrolysis of the glucuronides by the enzyme from huma urine. pH: 4·5, K_m(M) = 2·55 × 10^{-4} (3-OGAA), 1·75 × 10^{-3} (phenolphthalein glucuronide V_{max} = (mole/hr/mg protein) = 1·34 × 10^{-7} (3-OGAA), 7·93 × 10^{-7} (phenolphthalein glucuronide

former is stable in alkali and the latter is easily hydrolysed by alkali treatment. From the result of the alkali treatment, it is shown that the structure of 3-OGAA is the ether type.

(4) Estimation of fluorescence. The fluorescence of 3-OGAA in the various solvents was shown in Table 1. The fluorescence was the strongest in acetone. However, 3-OGAA was determined in methanol in the following experiments because it can be easily extracted with methanol from the plate.

Estimation of the rates of glucuronide formation. The rates of 3-OGAA and *p*-nitrophenol glucuronide formation by the liver microsomes of guinea pigs, mice and rats are shown in Table 2. Marked species difference was found, i.e. guinea pigs > mice > rats. The rates of 3-OGAA formation (per mg protein) were 0·064, 0·026 and 0·025 times of those of *p*-nitrophenol glucuronide formation in guinea pigs, mice and rats respectively. The Lineweaver–Burk plots are shown in Figs. 3 and 4.

Hydrolysis of glucuronides by the enzyme from human urine. The rate of hydrolysis of 3-OGAA (mole/hr/mg protein) was 0·35 times of that of phenolphthalein glucuronide at the concentration of 10^{-3} M and 0·44 times at the concentration of 5×10^{-4} M. The Lineweaver–Burk plots are shown in Fig. 5.

3-OGAA was hydrolyzed by this enzyme more slowly than phenolphthalein glucuronide. However, the difference was comparatively small at the low concentration because the K_m value of the hydrolysis of 3-OGAA was smaller than that of phenolphthalein glucuronide.

DISCUSSION

Glucuronide formation is widely known as a process of detoxication mechanism of foreign compounds in animal body. However, the glucuronide formation of endogenous substances has not been studied in detail except a few compounds, e.g. bilirubin or steroids. Boyland *et al.*[6,11] assumed that the glucuronide of 3-OHAA and 3-OHKY were excreted in urine and cause bladder cancer following the hydrolysis by the urinary β-glucuronidase. The administration of β-glucuronidase inhibitor for the prevention of regeneration of bladder cancer has been discussed on the ground of this hypothesis.[18,19] However, the presence of these glucuronides in animal body has not been confirmed except a trace of 3-OHKY glucuronide in insect[20] or in the urine of pyridoxal deficient rat,[17] and the evidence of enzymatic formation or hydrolysis of them has not been obtained. In this work, it was confirmed that 3-OGAA was formed and hydrolyzed enzymatically, although the rates are comparatively slow. These results indicate that the formation of 3-OGAA in animal body and the hydrolysis of 3-OGAA in urine are possible. The presence of 3-OGAA in urine will be reported later.

REFERENCES

1. M. J. Allen, E. Boyland, C. E. Dukes, E. S. Horning and J. G. Watson, *Br. J. Cancer* **11**, 212 (1957).
2. G. T. Bryan, R. R. Brown and J. M. Price, *Cancer Res.* **24**, 596 (1964).
3. W. Kochen and K. Hochberg, *Z. Krebsforsch.* **73**, 251 (1970).
4. H. Ehrhart, W. Ried, W. Benoit and H. Dörfler, *Z. Krebsforsch.* **71**, 1 (1968).
5. H. Ehrhart, A. Georgi and K. Stanislawski, *Klin. Wschr.* **37**, 1053 (1959).
6. E. Boyland and D. C. Williams, *Biochem. J.* **64**, 578 (1956).
7. R. R. Brown, J. M. Price, E. J. Satter and J. B. Wear, *Acta Un. int. Cancr.* **16**, 299 (1960).
8. J. M. Price and R. R. Brown, *Acta Un. int. Cancr.* **18**, 684 (1962).

9. A. S. KHALAFALLAH and M. A. M. ABUL-FADL, *Br. J. Cancer* **18,** 592 (1964).
10. C. A. BENASSI and L. MUSAJO, *Adv. Clin. Chem.* **7,** 63 (1964).
11. E. BOYLAND, D. M. WALLACE and D. C. WILLIAMS, *Br. J. Cancer* **9,** 62 (1955).
12. G. J. DUTTON, *Glucuronic Acid,* p. 186. Academic Press, New York (1966).
13. B. M. POGELL and L. F. LELOIR, *J. biol Chem.* **236,** 293 (1961).
14. W. PAUL, A. SCHAPIRO and H. GONICK, *Enzym. Biol. Clin.* **8,** 47 (1967).
15. E. LAYNE, *Adv. Enzymology,* **3,** 450 (1957).
16. J. A. L. MEAD, J. N. SMITH and R. T. WILLIAMS, *Biochem. J.* **68,** 61 (1958).
17. C. E. DALGLIESH, *Biochem. J.* **52,** 3 (1952).
18. E. BOYLAND, D. M. WALLACE and D. C. WILLIAMS, *Br. J. Cancer* **11,** 578 (1957).
19. E. BOYLAND, C. H. KINDER, D. MANSON and D. M. WALLACE, *Indust. Urol.* **2,** 439 (1965).
20. K. INAGAMI, *J. Agr. Chem. Soc. Japan* **29,** 918 (1955).