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STUDIES ON CARCINOGENIC TRYPTOPHAN METABOLITES—II

ENZYMATIC FORMATION AND HYDROLYSIS OF SULFURIC ESTER OF 3-HYDROXYANTHRANILIC ACID

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Abstract—Enzymatic formation and hydrolysis of the sulfuric ester of 3-hydroxyanthranilic acid, which was known as a carcinogenic tryptophan metabolite, were investigated. After preincubation of ATP and SO_4^{2-} with the enzymes precipitated with $(NH_4)_2SO_4$ from supernatants of livers, 3-hydroxyanthranilic acid was added and the mixture was incubated. It was confirmed that the formation rate of the sulfuric ester was 0·23, 0·45 and 0·39 times that of *p*-nitrophenylsulfate in guinea pigs, mice and rats respectively.

The enzyme precipitated from human urine was used in the study of the hydrolysis of the sulfuric ester. The sulfuric ester was hydrolyzed at the rate of 0·065 times of hydrolysis of 2-hydroxy-5-nitrophenylsulfate.

These results suggest that this sulfuric ester can be formed in the animal body but that enzymatic hydrolysis of the ester in urine may be small.

IN THE research of carcinogenic tryptophan metabolites, Boyland *et al.*[1,2] presumed that 3-hydroxyanthranilic acid (3-OHAA) and 3-hydroxykynurenine (3-OHKY) were excreted in the urine in the form of glucuronide and that these glucuronides caused bladder cancer after hydrolysis of the conjugate by urinary enzyme. However, formation and hydrolysis of these glucuronides in animal body were not confirmed. In the previous paper,[3] the authors proved the formation of 3-OHAA glucuronide by liver microsomes and the hydrolysis of 3-OHAA glucuronide by enzyme from human urine.

On the other hand, a hydroxyl group can often conjugate with sulfuric acid to form sulfuric ester in detoxication mechanism of animal body. However, the detailed study of the sulfuric esters of the carcinogenic tryptophan metabolites has not been published and the significance of these esters in etiology of cancer has not been discussed enough. In this work, the formation of sulfuric ester of 3-OHAA (3-OSAA) by the enzyme from mammalian liver and the hydrolysis of 3-OSAA by the enzyme from human urine were investigated.

It is known that the enzymatic formation of sulfuric ester proceeds through three steps described below.[4–7]

$$ATP + SO_4^{2-} \xrightarrow{\text{ATPsulfurylase (EC 2.7.7.4)}} \begin{matrix}\text{Adenosine-5'-}\\ \text{phosphosulfate}\\ \text{(APS)}\\ +\\ \text{Pyrophosphate}\end{matrix}$$

$$APS + ATP \xrightarrow{\text{APSkinase (EC 2.7.1.25)}} \text{Adenosine-3'-phosphate-5'-phosphosulfate (PAPS)} + ADP$$

$$PAPS + ROH \xrightarrow{\text{Arylsulfotransferase (EC 2.8.2.1)}} ROSO_3H + \text{Adenosine-3'5'-diphosphate}$$

Sulfuric ester formation was estimated by the method of De Meio.[8] The enzyme mixture of the three steps was precipitated with $(NH_4)_2SO_4$ from the supernatant of liver. ATP and SO_4^{2-} were preincubated with the enzyme mixture to form PAPS, and 3-OHAA was added. After incubation, 3-OSAA was separated by a DEAE Sephadex column and determined fluorometrically. At the same time, formation of *p*-nitrophenylsulfate (NPS) was estimated and compared with 3-OSAA formation.

Three separate enzymes, arylsulfatase A, B and C (EC 3.1.6.1), have been found in mammalian tissues as enzymes hydrolyzing arylsulfuric esters.[9–11] Human urine contains mainly arylsulfatase A and B.[12] The arylsulfatase A and B are known to be precipitated by partial saturation with $(NH_4)_2SO_4$. In this study, the precipitate with $(NH_4)_2SO_4$ from human urine was used as an enzyme hydrolyzing 3-OSAA. The rate of the reaction was estimated by determination of liberated 3-OHAA and compared with that of 2-hydroxy-5-nitrophenylsulfate (nitrocatecholsulfate, NCS).

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. 3-OSAA was prepared from anthranilic acid and $K_2S_2O_8$ by the method of Boyland *et al.*[13] NCS was prepared from 2-hydroxy-4-nitrophenol and chlorsulfonic acid by the method of Roy.[14] Silica gel (Wako, B-0) was obtained from the Wako Junyaku Co., Osaka, Japan, DEAE Sephadex (A-25) from the Pharmacia, Upsala Sweden, ATP from the Kowa Co., Nagoya, Japan and 3-OHAA from the Tokyo Kasei Co., Tokyo, Japan. Avicel SF cellulose powder was used for thin-layer chromatography.

Apparatus. Hitachi MPF-2A spectrofluorometer was employed for fluorometry. Hitachi 101 spectrophotometer was employed for photometry.

Preparation of enzyme system for sulfuric ester formation. The enzyme system was prepared by the method of De Meio.[8] Animals were killed by decapitation, livers were removed and 20 per cent homogenates in 0·154 M KCl were prepared. After sedimentation of mitochondria at 11,000 *g* for 60 min, the microsomes were sedimentated at 105,000 *g* for 60 min and discarded. The supernatants were brought to 25 per cent saturation with solid $(NH_4)_2SO_4$ and the precipitates were separated by centrifugation at 5000 *g* for 20 min after keeping in ice for 60 min. Then, the supernatants were brought to 60 per cent saturation with $(NH_4)_2SO_4$ and the precipitates were collected by the same procedure. The precipitates with 60 per cent saturation were dialyzed in water and used as a enzyme system. Protein was determined by the Biuret method.[15]

Detection of 3-*OSAA*. To 1·4 ml of 0·57 M imidazole-HCl buffer (pH 7·0) containing 15 μmoles of K_2SO_4, 10 μmoles of ATP, 12·5 μmoles of $MgCl_2$ and 10 μmoles of cysteine, 0·2 ml of the enzyme solution (0·5 ml/g liver) was added. After preincubation at 37° for 30 min, 0·4 ml of 2·5 mM 3-OHAA in 0·05 M imidazole–HCl buffer (pH 7·0) was added. After incubation at 37° for 60 min, the reaction was stopped by heat 100° for 90 sec and the mixture was centrifuged. The supernatant was evaporated *in vacuo* to dryness and the residue was dissolved in a small volume of methanol. The methanol solution was spotted on the silica gel plate (0·25 mm thick) and the chromatogram was developed with a mixture of *n*-butanol–*n*-propanol–ethanol–water (6:3:2:1). The authentic 3-OSAA solution in methanol was simultaneously spotted and developed.

Estimation of 3-*OSAA formation*. The sulfuric ester formation was estimated in the condition described by Hilz *et al.*[16] 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 same incubation mixture as described above was prepared and 1·5 ml of the supernatant was adsorbed on the column of DEAE Sephadex (1 × 5 cm) and 3-OHAA was eluted with 60 ml of the dil. $(NH_4)_2CO_3$. Then, 3-OSAA was eluted with 120 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 10 ml of methanol and 3-OSAA was determined fluorometrically (ex. 325 nm, fl. 395 nm).

In order to obtain the standard curve, 3-OSAA 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. Separation from 3-OHAA was complete and 300 μg of 3-OHAA did not hinder the determination of 3-OSAA.

In this condition, the time–activity curve of 3-OSAA formation were linear within the range of 30–90 min.

Identification of 3-*OSAA*. (1) Thin-layer chromatography. The methanol solution which had been used for determination of 3-OSAA in the above experiment was concentrated and spotted on the silica gel plate. The chromatogram was developed with a mixture of *n*-butanol–*n*-propanol–ethanol–water (6:3:2:1).

(2) Acid hydrolysis. Detection of 3-OHAA and SO_4^{2-} ion after acid hydrolysis of the sulfuric ester was attempted. The methanol solution was evaporated to dryness *in vacuo* and the residue was dissolved in 1 ml of 0·5 N HCl. After heating at 70° for 90 min, the pH was adjusted to 3 with 1 M Na acetate and the sample was shaken with 1 ml of ether. The ether layer was concentrated and spotted on the cellulose powder plate (0·25 mm thick). The chromatogram was developed with a mixture of *n*-butanol–*n*-propanol–ethanol–water (6:3:2:0·3). R_f value of authentic 3-OHAA in the system is 0·6.

The water layer was concentrated *in vacuo* to 0·3 ml and a drop of 10% $BaCl_2$ was added. Appearance of white turbidness of $BaSO_4$ means the presence of SO_4^{2-}.

Estimation of NPS formation. NPS formation was determined by adding *p*-nitrophenol in place of 3-OHAA in the same condition of the estimation of 3-OSAA formation. The reaction was stopped by addition of 2 ml of ethanol and the mixture was centrifuged. To 4 ml of 0·05 N KOH, 0·5 ml of the supernatant was added and the remain of *p*-nitrophenol was determined photometrically at 420 nm.

Preparation of arylsulfatase from human urine. As a preliminary experiment, 200 ml of human urine was fractionated as shown in Fig. 1. Each fraction was dialysed in

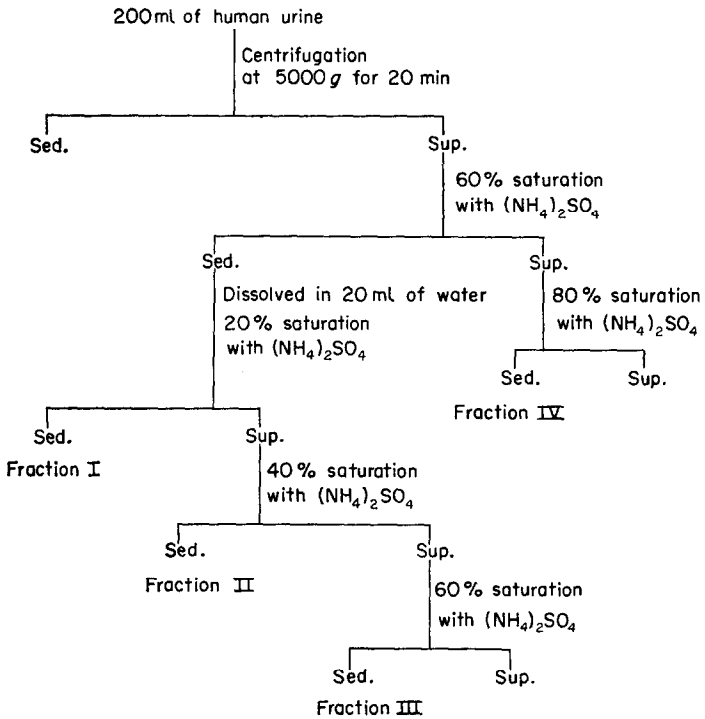

FIG. 1. Precipitation of arylsulfatase from human urine by the parcial saturation with $(NH_4)_2SO_4$. Sup.; supernatants. Sed.; sediments. Sediments were collected by centrifugation at 5000 *g* for 20 min.

water and the activity of arylsulfatase of each fraction was estimated by the method described below. As shown in Table 1, the arylsulfatase activity was the highest in the fraction II.

The arylsulfatase was prepared as follows. One liter of urine of a normal adult man was centrifuged at 5000 *g* for 20 min and the supernatant was brought to 60 per cent saturation with $(NH_4)_2SO_4$. After keeping in ice for 60 min, the precipitate was collected

TABLE 1. ACTIVITY OF ARYLSULFATASE IN EACH FRACTION PRECIPITATED FROM HUMAN URINE

Sample		Hydrolysis of 3-OSAA		Hydrolysis of NCS	
	Protein (mg)	Total activity (μg 3-OHAA/hr)	Specific activity (μg 3-OHAA/hr/mg protein)	Total activity (μgNC*/hr)	Specific activity (μgNC/hr/mg protein)
Urine†	9·77	50·2	5·1	1286	132
Fraction I	2·00	15·0	7·5	448	224
Fraction II	0·86	20·2	23·5	690	802
Fraction III	0·62	0	0	26	42
Fraction IV	0·40	0	0	0	0

* NC: nitrocatechol, 2-hydroxy-4-nitrophenol.
† The urine was concentrated and dialyzed.

by centrifugation at 5000 *g* for 20 min and dissolved in 100 ml of water. This solution was brought to 20 per cent saturation with $(NH_4)_2SO_4$ and the precipitate was separated by the same procedure. The supernatant was brought to 50 per cent saturation with $(NH_4)_2SO_4$. The precipitate was collected and dialyzed in water overnight. The total volume was adjusted to 10 ml with water. This solution contained 6·5 mg/ml pf protein (biuret method[16]).

Estimation of hydrolysis of 3-OSAA. A mixture of 0·05 ml of 3-OSAA solution in water, 0·05 ml of 0·5 M Na acetate buffer (pH 4·5–6) and 0·05 ml of the enzyme solution was incubated at 37°. After incubation, the mixture 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. 3-OHAA was determined fluorometrically (ex. 345 nm, fl. 410 nm).

Estimation of hydrolysis of NCS. The hydrolysis of NCS was estimated by adding NCS in place of 3-OSAA in the same condition of the estimation of hydrolysis of 3-OSAA. After stopping the reaction by addition of 0·2 ml of 2 per cent phosphotungstic acid in 0·1 N HCl, 3 ml of quinol reagent[14] (5 ml of 4 per cent hydroquinone in 0·1 N HCl and 100 ml of 2·5 N NaOH containing 5% $Na_2SO_3 . 7H_2O$ were mixed immediately before use) was added and 2-hydroxy-4-nitrophenol liberated was determined photometrically at 510 nm.

RESULTS

Detection of 3-OSAA. A purple fluorescent spot corresponding to authentic 3-OSAA was detected in the incubation mixtures when the enzymes from all species tested (guinea pigs, mice and rats) were used.

Estimation of 3-OSAA and NPS formation. The results of estimation of 3-OSAA and NPS formation by the enzymes from guinea pigs, mice and rats are shown in Table 2.

TABLE 2. FORMATION OF 3-OSAA AND NPS BY THE ENZYMES PRECIPITATED FROM SUPERNATANTS OF LIVERS

Species	Formation of 3-OSAA		Formation of NPS	
	(mole/hr/g liver) $\times 10^{-7}$	(mole/hr/mg protein) $\times 10^{-8}$	(mole/hr/g liver) $\times 10^{-7}$	(mole/hr/mg protein) $\times 10^{-7}$
Guinea pigs	1·53 ± 0·06	2·64 ± 0·09	7·05 ± 0·54	1·15 ± 0·06
Mice	2·54 ± 0·02	4·36 ± 0·05	6·54 ± 0·18	0·96 ± 0·11
Rats	2·50 ± 0·08	4·44 ± 0·10	6·46 ± 0·37	1·14 ± 0·87

Results are expressed as mean ± S.E.N = 5. SO_4^{2-}: 7·5 mM, ATP: 5 mM, $MgCl_2$: 6·25mM, cysteine: 5 mM, pH: 7·0, preincubation: 30 min. 3-OHAA or *p*-nitrophenol: 0·5 mM.

The ratio of activity per mg protein of 3-OSAA and NPS formation was 0·23:1, 0·45:1 and 0·39:1 in guinea pigs, mice and rats respectively. The activity of 3-OSAA formation of the enzyme from guinea pigs was comparatively low. Marked species difference was not found in the activity of NPS formation.

Identification of 3-OSAA. The methanol solution for determination of 3-OSAA in the above experiment was treated as described in Methods. The same results were

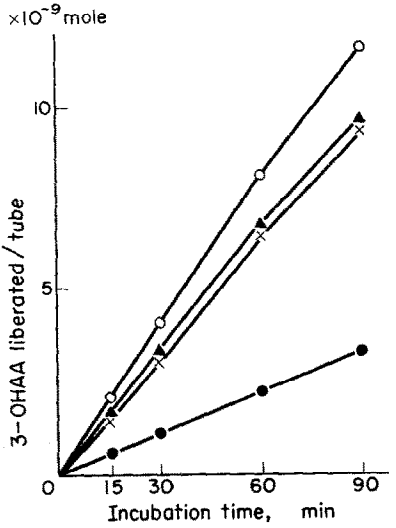

FIG. 2. Time-activity curves of hydrolysis of 3-OSAA at various pH by the enzyme from human urine. Concentration of 3-OSAA: 5 mM. ●——● pH 4·5, ▲——▲ pH 5·0, ○——○ pH 5·5, ×——× pH 6·0.

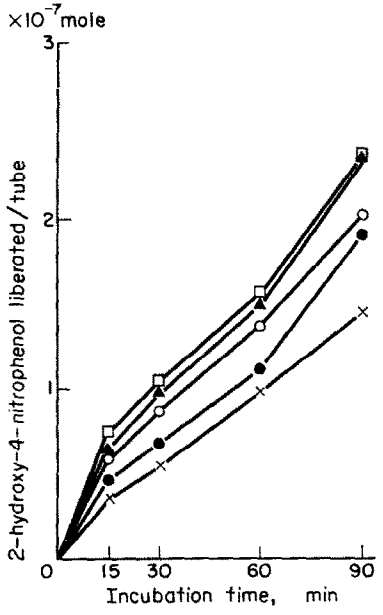

FIG. 3. Time-activity curve of hydrolysis of NCS at various pH by the enzyme from human urine. Concentration of NCS: 5 mM. ●——● pH 4·5, ▲——▲ pH 5·0, □——□ pH 5·2, ○——○ pH 5·5, ×——× pH 6·0.

obtained in the case of three animal species. The fluorescent compound in the methanol solution was identified with 3-OSAA.

(1) Thin layer chromatography. A fluorescent spot which corresponded to 3-OSAA (R_f 0·50) was obtained from the concentrate of the methanol solution for determination of 3-OSAA. No other fluorescent spot was found on the chromatogram.

(2) Acid hydrolysis. A fluorescent spot which corresponded to 3-OHAA was obtained from the ether layer of the hydrolysates. The results of $BaCl_2$ test for SO_4^{2-} in the aqueous layer was also positive.

Enzymatic hydrolysis of 3-OSAA and NCS. (1) Effect of pH and time–activity curve. Time–activity curves of hydrolysis of 3-OSAA and NCS by the enzyme from human urine at various pH were shown in Figs. 2 and 3. The pH optimum was 5·5 in 3-OSAA and 5·2 in NCS. The activity of hydrolysis of 3-OSAA was 0·065 times of that of NCS at pH 5·5 for 60 min. The sulfuric ester linkage of 3-OSAA was shown to be resistant to the enzyme from urine.

It is known that hydrolysis of NCS by arylsulfatase A shows anomalous time–activity curve.[11] Namely, the reaction proceeds the most rapidly in stage I (0–15 min), slowly in stage II (15–60 min) and rapidly again in stage III (after 60 min). The curve of hydrolysis of NCS by the enzyme from human urine showed the similar anomalous form as shown in Fig. 2. However, the curve of hydrolysis of 3-OSAA was quite linear as shown in Fig. 2.

(3) The Lineweaver–Burk plot. The Lineweaver–Burk plots are shown in Figs. 4 and 5. NCS showed substrate inhibition in high concentration as reported by Baum *et al.*[11] However, no substrate inhibition was shown by 3-OSAA.

(4) Effect of PO_4^{3-}, SO_4^{2-} and SO_3^{2-}. Results of estimation of hydrolysis of 3-OSAA and NCS in the mixture containing Na_2HPO_4, Na_2SO_4 and Na_2SO_3 are shown in Figs. 6 and 7. Hydrolysis of 3-OSAA and NCS was inhibited by PO_4^{3-} and

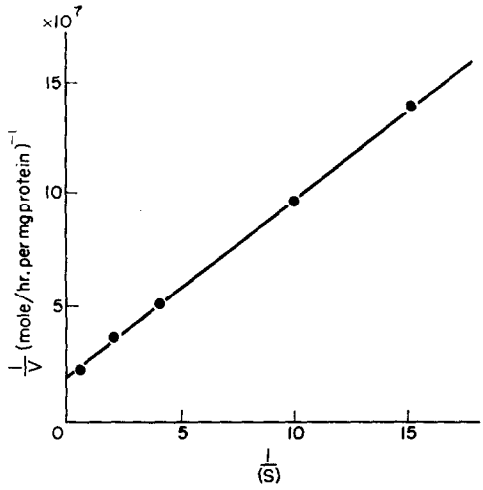

FIG. 4. The Lineweaver–Burk plot of hydrolysis of 3-OSAA by the enzyme from human urine, pH 5·5, apparent $K_m = 4{\cdot}4 \times 10^{-3}$ M. $V_{max} = 5{\cdot}5 \times 10^{-8}$ mole/hr/mg protein.

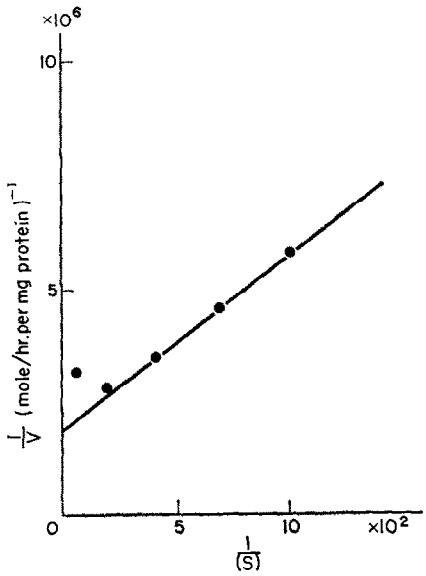

FIG. 5. The Lineweaver–Burk plot of hydrolysis of NCS by the enzyme from human urine. pH 5·2 apparent $K_m = 2{\cdot}0 \times 10^{-3}$ M. $V_{max} = 5{\cdot}24 \times 10^{-7}$ mole/hr/mg protein.

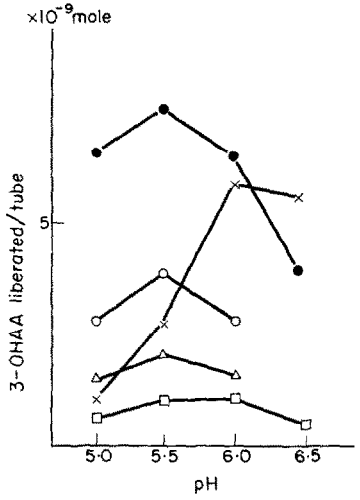

FIG. 6. Effect of negative ions to hydrolysis of 3-OSAA by the enzyme from human urine. ●——● control, ○——○ 0·005 M Na_2HPO_4, △——△ 0·03 M Na_2PO_4, ×——× 0·03 M Na_2SO_4, □——□ 10^{-4} M Na_2SO_3. Concentration of 3-OSAA: 5 mM.

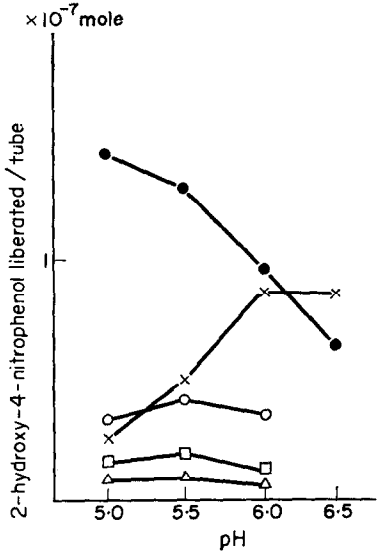

FIG. 7. Effect of negative ions to hydrolysis of NCS by the enzyme from human urine. ●——● control, ○——○ 0·05 M Na_2PO_4, △——△ 0·03 M Na_2HPO_4, ×——× 0·03 M Na_2SO_4, □——□ 10^{-4} M Na_2SO_3. Concentration of NCS: 5 mM.

SO_3^{2-}, and the pH optimum was shifted to alkaline side by SO_4^{2-}. These results are similar to the report by Roy[17] about hydrolysis of NCS by bovine liver arylsulfatase A.

DISCUSSION

Boyland *et al.*[1,2] supposed that liberation of 3-OHAA or 3-OHKY by the enzymatic hydrolysis of the glucuronide conjugate in urine might cause natural bladder cancer. This hypothesis is based on the observation that 3-OHAA, 3-OHKY and β-glucuronidase in urine were high in patients with bladder cancer. On the other hand, sulfuric ester formation is conceivable as a process of conjugation of the carcinogenic metabolites and it has been reported that urinary arylsulfatase activity which hydrolyses the sulfuric ester linkage is also high in patients with bladder cancer. However, the study of sulfuric esters of the carcinogenic metabolites has not been sufficient. Namely, determination of these sulfuric esters in urine was attempted by photometry after separation with charcoal column,[1] but the specificity of this method is unsatisfactory. Enzymatic formation of these sulfuric esters has not been proved. Boyland *et al.*[18] reported that the sulfuric esters of *O*-aminophenol type compounds were resistant to hydrolysis by Takadiastase or in urine, but the rate of the reaction has not been studied in detail.

According to the results in this paper, 3-OSAA formed by the liver enzyme system of De Meio[8] and the ratio of activity to NCS formation (0·23, 0·45 and 0·38 in guinea pigs, mice and rats, respectively) was larger than the ratio of 3-OHAA glucuronide formation to *p*-nitrophenol glucuronide formation by the liver microsomes[3] (0·064, 0·026 and 0·025). On the other hand, 3-OSAA was very resistant to hydrolysis by the enzyme from urine and moreover the hydrolysis was inhibited by phosphate ion at the

concentration similar to those existing in urine. These results suggest that formation of 3-OSAA in animal body is possible but that hydrolysis of 3-OSAA may be negligible in human urine (0·4–1·5 μg/hr/ml of 2-hydroxy-4-nitrophenol was liberated from 16·6 mM NCS)[2] except in urine with extremely high arylsulfatase activity, e.g. infected urine (100–200 μg/hr/ml of 2-hydroxy-4-nitrophenol was liberated from 16·6 mM NCS).[2]

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