Biochemical Pharmacology, Vol. 33, No. 20, pp. 3195–3200, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
Pergamon Press Ltd.

ANTHRANILIC ACID METABOLISM IN THE ISOLATED PERFUSED RAT LIVER: DETECTION AND DETERMINATION OF ANTHRANILIC ACID AND ITS RELATED SUBSTANCES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

JUNKO NAITO,* EMI SASAKI,† YOSHIJI OHTA,‡ RIKIO SHINOHARA§ and ISAO ISHIGURO‡

* Department of Biochemistry, School of Hygiene, Fujita-Gakuen University, 470–11, Toyoake, Aichi, Japan; † Department of Pharmacy, Fujita-Gakuen University Hospital, Aichi, Japan; ‡ Department of Biochemistry, School of Medicine, Fujita-Gakuen University, Aichi, Japan; § Nagoya College of Medical Technology, Japan

(*Received* 28 *September* 1983; *accepted* 10 *May* 1984)

Abstract—In order to elucidate the anthranilic acid metabolism in animal tissue, the metabolism was studied in the isolated perfused liver of rats. A sensitive and rapid method was devised for determination of anthranilic acid and its related substances using high-performance liquid chromatography with electrochemical detection. 5-Hydroxyanthranilic acid and anthranilamide, which have not been detected in animal tissue, were found in the perfusate and in the bile secreted from the perfused liver, respectively. In addition, a non-enzymatic production of anthranilamide from anthraniloyl glucuronide in the presence of ammonium and bicarbonate ions was also observed.

These present results suggest that, apart from undergoing glycine or glucuronide conjugation, anthranilic acid is metabolized to 5-hydroxyanthranilic acid and anthranilamide in the rat liver.

It has generally been accepted that in animals anthranilic acid (An.A),‖ one of the metabolites of L-tryptophan (L-Try), is excreted in urine after conjugation with glycine or glucuronic acid without further metabolism. However, a metabolic pathway from An.A to 5-hydroxyanthranilic acid (5-OHAA) was suggested by Kotake and Kotake in 1942 [1] and the *in vitro* enzymatic conversion of An.A to 5-OHAA in a rabbit liver microsomal fraction with NADPH was demonstrated by Kashiwamata *et al.* [2]. They suggested that the pathway might be physiologically and pathologically important. In addition, Ishiguro and his co-workers found anthranilamide (An.Amide) in the urine of rats treated with An.A [3] and also demonstrated that the hydroxylation of An.Amide to 3- and 5-hydroxyanthranilamide (3- and 5-An.Amide) by hepatic microsomal enzymes in the presence of NADPH and molecular oxygen is stimulated by superoxide dismutase (SOD,EC 1.15.1.1) [4]. Furthermore, some compounds related to An.A are known as putative carcinogens [5] or anti-inflammatory agents [6]. Therefore, in order to elucidate An.A metabolism in animal tissue, we studied the metabolism in the isolated perfused liver of rats.

For this study, a new sensitive method devised for determination of An.A-related compounds including L-Try by high-performance liquid chromatography with electrochemical detection (HPLC) was used.

MATERIALS AND METHODS

Chemicals. The standards of L-Try metabolites and other chemicals were obtained as follows: 3-hydroxyanthranilic acid, kynurenine sulfate, serotonin creatinine sulfate and D-saccharic acid 1,4-lactone from Sigma (St Louis, MO, U.S.A.), 3-hydroxykynurenine and 5-hydroxytryptophan from Calbiochem (Los Angeles, CA, U.S.A.), 5-hydroxyindole acetic acid from Aldorich (Milwaukee, WI, U.S.A.), anthranilamide from Daiichi Chemical Co. (Tokyo, Japan), anthraniloyl glycine from Maruwaka Chem. Ind. Co. (Osaka, Japan), β-glucuronidase from Boehringer (Mannheim, F.R.G.) and ^{15}N-ammonium bicarbonate from Shohkoh Co. (Saitama, Japan). 5-Hydroxyanthranilic acid hydrochloride, of which the purity is 95.5% by atomic analysis, was provided by the Max-Planck-Institute of Biochemistry (Munich, F.R.G.). The chemical properties coincided with those of the 5-hydroxyanthranilic acid reported in the literature [7]. All other materials except for anthraniloyl glucuronide were obtained from Wako Pure Chem. Ind. (Osaka, Japan) and were of reagent grade.

Isolation of anthraniloyl glucuronide from urine. Anthraniloyl glucuronide (AAG) was isolated from the urine of rats collected for 24 hr after injection

* Author to whom correspondence should be addressed.

‖ Abbreviations: L-Try, L-tryptophan; An.A, anthranilic acid; 5-(3-)OHAA, 5-(3-)hydroxyanthranilic acid; AAG, anthraniloyl glucuronide; OAH, *o*-aminohippuric acid (anthraniloyl glycine); An.Amide, anthranilamide; Kyn, kynurenine; 3-OHKyn, 3-hydroxykynurenine; 5-HT, serotonine; 5-OHTry, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindole acetic acid; SOD, superoxide dismutase; HPLC, high-performance liquid chromatography with electrochemical detection.

with An.A (20 mg/100 g body weight) intraperitoneally. The lipophilic substances and An.A in the urine were removed by shaking the urine with the same vol. of ethyl acetate at pH 3.0 three times. One gram of active charcoal was then added to 5 ml of the aqueous solution and the mixture was stirred for an hour at room temperature. The charcoal was obtained by centrifugation at 10,000 *g* for 15 min, washed with the same vol. of deionized distilled water three times and was stirred with 2 vol. of a mixture containing methanol and ethanol (1:1) for 2 hr. After centrifugation, the supernatant was evaporated to dryness under vacuum at 40°. The residue was dissolved in H_2O at pH 5.0. The solution rich in AAG was chromatographed using a silica-gel column and the elution pattern is shown in Fig. 1. Though three peaks containing AAG appeared in the column chromatography, least contamination was found in peak III. Therefore, all fractions of this peak were collected and enriched under vacuum. The concentrated solution was lyophilized over night. The lyophilized sample was dissolved with 5 ml of 50% (v/v) ethanol and was further developed on a silica-gel plate (2 mm thick) with a solvent consisting of *n*-butanol:acetic acid:H_2O (4:1:1, solvent C). AAG at R_f 0.28 was eluted with H_2O (pH 6.0) from the gel and the eluate was mixed with 4 vol. of ethanol, from which insoluble materials were removed by centrifugation. This purification step was repeated, and the finally extracted solution was collected and condensed to a small vol. The concentration of AAG obtained was 73.3% and the yield from the urine was about 8%.

Identification and detection of anthraniloyl glucuronide. A fluorescent substrate at R_f 0.48 on the plate developed with solvent A given in the legend to Fig. 2. was extracted with H_2O at pH 6.0 and the extract was incubated with β-glucuronidase in 0.1 M acetate buffer (pH 4.9) at 37° for 2 hr, resulting in a production of equal amounts of An.A and glucuronic acid. When the reaction mixture contained D-saccharic acid 1,4-lactone, an inhibitor of β-glucuronidase, no production of either An.A or glucuronic acid was observed. From these findings, the fluorescent substance was identified as AAG. In this experiment An.A and glucuronic acid were quantified as follows. The hydrolyzed sample was developed on a silica-gel plate using solvent C. The position of glucuronic acid on the plate was checked by the naphthoresorcinol reaction [8]. Glucuronic acid was eluted with H_2O from the silica-gel corresponding to its position (R_f 0.14) and enriched at least in the concentration of 100 μg/ml and then determined according to the method of Dische and Borenfreund [9]. An.A at R_f 0.94 was extracted in ethyl acetate at pH 3.0 from the plate materials after suspension in H_2O and determined spectrofluorometrically as described below.

Fluorometric determination of anthranilic acid, anthranilamide and anthraniloyl glucuronide. Since AAG was easily hydrolyzed in 0.2 N NaOH by boiling for 10 min, its amount was estimated as equivalent to that of An.A obtained by the alkalic decomposition. Both An.A and An.Amide extracted from the aqueous solution in ethyl acetate at pH 3.0 were analyzed fluorometrically with the wave lengths of 340 nm (excitation) and 395 nm (emission) for An.A and of 359 nm (excitation) and 410 nm (emission) for An.Amide.

Liver perfusion. The liver perfusion was carried out as described by Miller *et al.* [10].

Preparation of sample. The perfusate, 0.2 ml, was mixed with 0.2 ml of 0.01 M EDTA and 0.2 ml of 0.1 M $NaHSO_3$ in a final vol. of 1.8 ml with H_2O, and then 0.2 ml of 2 M $HClO_4$ was added to the mixture. After centrifugation, 1.0 ml of the supernatant was adjusted to pH 6.0 with 2 M KOH and then shaken with 1.0 ml of chloroform to eliminate lipophilic substances. One ml of the aqueous layer separated by centrifugation was mixed with 9.0 ml of 99.5% (v/v) ethanol and evaporated to dryness at 45° under vacuum. The residual solid was then dissolved in 0.2 ml with a solution containing 0.01 mM EDTA and 0.1 mM $NaHSO_3$ (solution I).

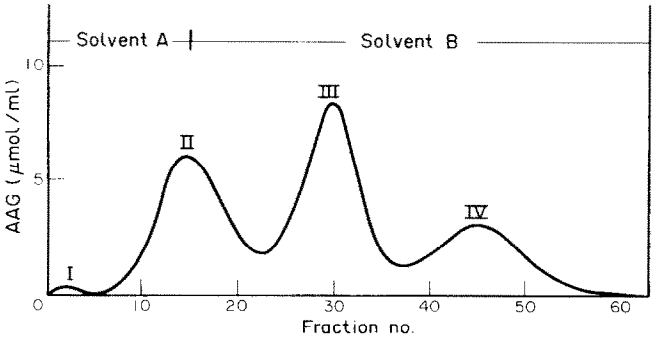

Fig. 1. The separation of anthraniloyl glucuronide from the urine extract by silica-gel column chromatography. Dry powder gel (Wako-gel C 200) was packed as evenly and tightly as possible in a column (2.5 × 20.0 cm) up to the height of 18.0 cm. Before application, 1.0 ml of sample (containing 5 mg AAG) was mixed with 0.5 g of the gel powder and 1.0 ml of acetone in a petri dish and the mixture was dried in an electric oven at 70°. The dried gel was then layered on top of the column. The elution of AAG from the column was carried out with 30 ml of a solvent containing ethyl acetate : isopropanol : ammonia : H_2O (65:25:2:18, solvent A) and followed by 110 ml of 95% (v/v) ethanol (solvent B). Fractions of 2 ml were collected at a flow rate of 1 ml/min and An.A-related compounds in these fractions were monitored by TLC developed with solvent A.

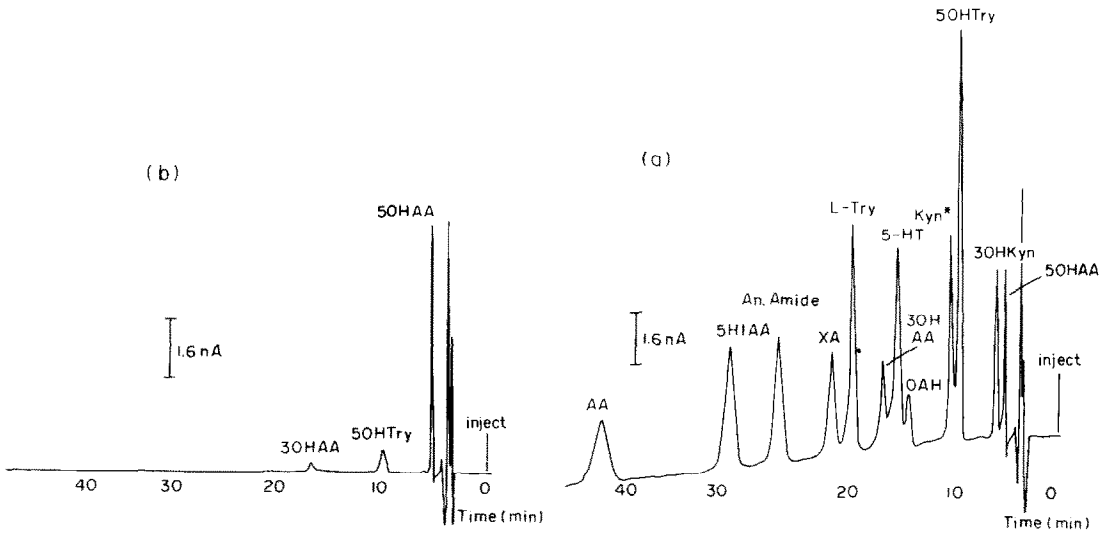

Fig. 2. Separation of standard tryptophan metabolites by reversed phase high-performance liquid chromatography with electrochemical detection. Chromatographic conditions: column: Yanapack ODS-T (4 × 250 mm); eluant: 0.1 M sodium citrate buffer, pH 4.5 containing 0.01 mM EDTA and 5% (v/v) CH_3CN; flow rate: 0.7 ml/min; detection: (a) 0.9 V and (b) 0.3 V vs Ag/AgCl electrode; loads: 5-OHTry, 2pmol; Kyn, 100 pmole; all others, 10 pmole.

In general, the bile was applied to HPLC directly after 20-fold dilution with solution I. The determination and identification of An.Amide were carried out with an enrichment extract obtained by the following procedure: The bile, diluted with 0.1 N NaOH to 10% (2.0 ml), was mixed vigorously with 4 vol. of chloroform in the presence of anhydrous Na_2SO_4 (1.0 g) for 30 min. The organic layer separated by centrifugation was then dried with a rotary evaporator at 45°. The residue was dissolved in 50% (v/v) ethanol (0.2 ml).

High-performance liquid chromatography analysis. Determination of An.A-related compounds was carried out on a Yanaco Model L-3200 liquid chromatograph with a Yanaco electrochemical detector (Yanagimoto Manufacturing Co., Ltd., Kyoto, Japan).

RESULTS

The standards of the main L-tryptophan metabolites including An.A were separated by HPLC at 0.9 V as shown in Fig. 2(a), after injecting 10 μl of each standard solution. The concentration of these solutions was 10^{-6} M, except for 5-OHTry (2×10^{-7} M) and Kyn (10^{-5} M). If analyzed at 0.3 V, the peak of 5-OHAA not only remained, but even increased compared to the peak at 0.9 V, as seen in Fig. 2(b), whereas most of the other compounds could not be oxidized electrochemically at such a low electropotential as 0.3 V. This condition was utilized for the selective determination of 5-OHAA in the sample. Recoveries (means ± S.D., N = 5) of L-Try, 5-OHAA, AAG, OAH and An.A from a pre-perfusate were 73.1 ± 4.7, 66.9 ± 5.3, 75.5 ± 3.6, 74.6 ± 3.9 and 70.8 ± 5.2% respectively.

Time courses of the metabolites appearing during liver perfusion with An.A are summarized in Fig. 3. At 5 hr, the content of OAH in the circulating medium was the highest (44.3 ± 6.1 μmol) of all the compounds and was followed by that of AAG (24.5 ± 3.2 μmol). These two substances, which are the main metabolites of An.A, increased in proportion to perfusion time, after a lag time of 60 min.

On the other hand, the production of 5-OHAA in the perfusate increased linearly with perfusion time until 3 hr, but thereafter the rate gradually decreased (1.63 ± 0.24 μmol at 3 hr). This may be caused by inactivation of the microsomal enzymes, which Kashiwamata *et al.* have described [2], or by autoxidation of 5-OHAA during the experiment. The amount of L-Try increased in the circulating solution until 3 hr after initiation of the perfusion. Since this phenomenon was observed even when livers were perfused in the absence of An.A, it may be due to L-Try being released from the liver in the process of hepatic perfusion.

The excretion patterns in the bile secreted from the liver perfused with An.A are summarized in Fig. 4. Recoveries (means ± S.D., N = 5) of L-Try, 5-OHAA, AAG, OAH and An.A from the bile were over 97%, but that of An.Amide which was extracted and enriched before analysis was only 86.5 ± 4.0%. A remarkable difference of the metabolites in the perfusate from those in the bile was the presence of An.Amide in the bile instead of 5-OHAA in the perfusate.

Besides by HPLC, 5-OHAA in the perfusate and An.Amide in the bile were identified by u.v. spectrophotometry, TLC as well as indophenol reaction for 5-OHAA and the determination of NH_4^+ and An.A for An.Amide which were produced through hydrolyzation of An.Amide by boiling it in an acid solution.

As seen in Fig. 4(b), the highest amount of An.Amide (8.01 ± 0.94 nmol) in the bile was

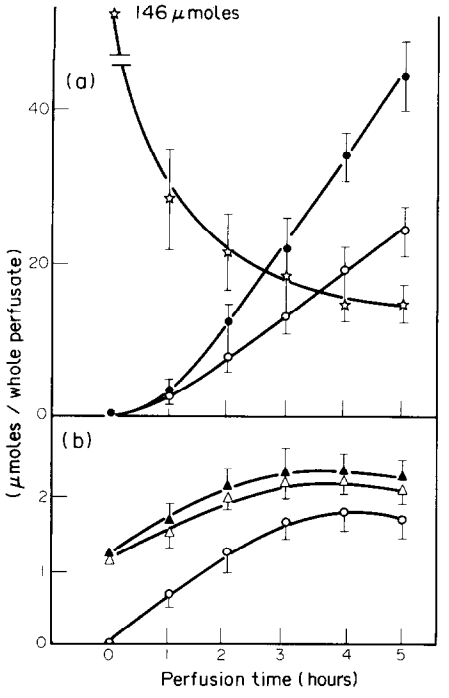

Fig. 3. Accumulative amounts of anthraniloyl glycine, anthraniloyl glucuronide, anthranilic acid, L-tryptophan and 5-hydroxyanthranilic acid in the perfusates gained by liver perfusion with anthranilic acid. Male rats of Wistar strain weighing 280–330 g were used. The livers were perfused in an atmosphere of 95% O_2–5% CO_2 with a semi-synthetic blood which consisted of washed bovine erythrocytes (1 vol.) and Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 2.5% bovine serum albumin and 10 mM glucose (4 vol.). The starting vol. of the solution was 135 ml. After 15 ml of the perfusate had been run through the liver to wash out the remaining blood, the perfusate was discarded. Twenty mg of An.A dissolved in 2.0 ml of physiological saline (pH 7.0) were added to the circulating medium. Flow rate of the perfusate was 2–3 ml/min per g of liver. The hydrostatic pressure in the portal vein was 14 cm H_2O. The pH of the perfusate was adjusted to 7.38–7.42 with 1 M $NaHCO_3$. One ml of the perfusate was removed every hour and prepared as described in the text. Ten μl of each sample were injected into the apparatus. Isoproterenol was added to all samples as chromatographic marker (0.1 nmol). Analytical conditions were identical with those shown in Fig. 2. ●——●, OAH; ○——○, AAG; ☆——☆, An.A; ▲——▲, L-Try; △——△, L-Try in the perfusate without addition of An.A; ○——○, 5-OHAA. Data are represented as means ± S.E. of five experiments.

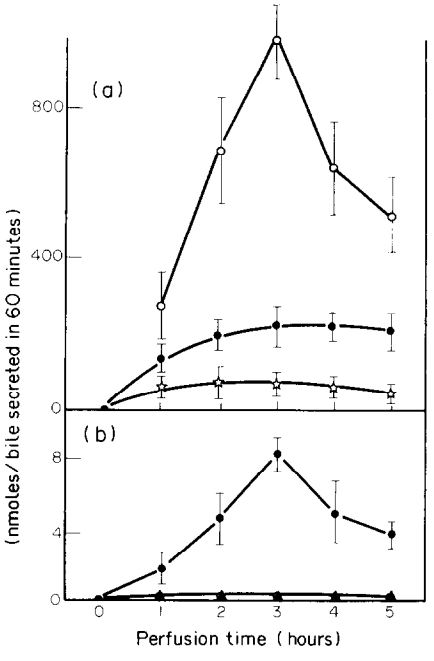

Fig. 4. Contents of anthraniloyl glycine, anthraniloyl glucuronide, anthranilic acid and anthranilamide in the bile for each hour during the liver perfusion with anthranilic acid. The bile obtained for each hour during the liver perfusion was diluted 20-fold with H_2O, except for the case of An.Amide which was enriched as described in the text. The liver perfusion procedure and the HPLC conditions are mentioned in the legend to Fig. 2. (a) ●——●, OAH; ○——○, AAG; ☆——☆, An.A.; (b) ●——●, An.Amide; ▲——▲, L-Try; ○——○, 5-OHAA. Data are represented as means ± S.E. of 5 experiments.

observed between 2 and 3 hr after the beginning of the perfusion and the appearance of AAG was similar to that of An.Amide, suggesting that AAG might be a precursor of the amide.

An attempt to form the amide from AAG using its enriched solution was successful when the solution was incubated with NH_4HCO_3 in the presence of 0.1 M potassium phosphate buffer (pH 7.8) at 37° as shown in Fig. 5. In this An.Amide synthesis An.A or OAH could not substitute AAG. Moreover, in the absence of bicarbonate ions no amide formation was observed, even though ammonium and hydroxyl ions were present.

To verify this reaction, $^{15}NH_4HCO_3$ was used to synthesize the amide and ^{15}N in the amide formed was determined as follows. The amide was extracted with ethyl acetate at pH 3.0, enriched by evaporation under vacuum and separated by TLC with solvent C. Thereafter, a fluorescent substance at R_f 0.86 was

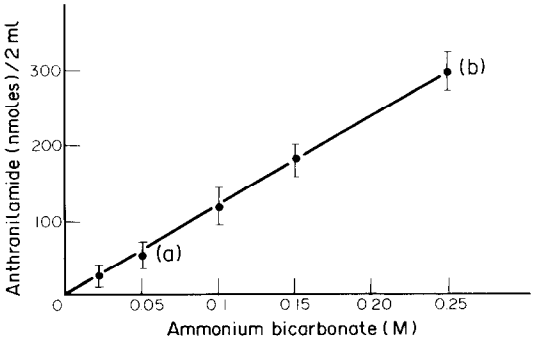

Fig. 5. Non-enzymatic formation of anthranilamide from anthraniloyl glucuronide in its enriched solution as a function of ammonium bicarbonate concentration. The reaction mixture (2.0 ml) consisted of 5 mM AAG, 0.1 M potassium phosphate buffer, pH 7.8 and various concentrations of ammonium bicarbonate as indicated on the abscissa. Incubation was carried out at 37° for 60 min. An.Amide formed was determined fluorometrically as described in the text. pH of the reaction mixture in (a) is 8.02 and in (b) is 8.27. Values represent the means ± S.D. of five experiments.

Table 1. Incorporation of ^{15}N-ammonium ion into anthranilamide

Components of the solution to be distilled	Content (μmol/ml)	Theoretical amount of N released from the component (μg/ml)	Percentage of ^{15}N in total distilled N
Anthranilamide formed from $^{15}NH_4HCO_3$ and the AAG solution	3.575	50	22.3
$(NH_4)_2SO_4$	5.350	150	

^{15}N of the ^{15}N-anthranilamide formed from 0.1 M AAG and 0.25 M $^{15}NH_4HCO_3$ under the conditions given in the legend to Fig. 5 was determined as described in the text. The atomic percentage of ^{15}N in the trapped solution was analyzed by mass spectrometry.

extracted with 70% (v/v) ethanol from the plate-gel and the extract was enriched again. Prior to the analysis of ^{15}N in An.Amide, the concentrated extract was diluted 4 times (with regard to the distillable amount of N) using an ammonium sulfate solution. The diluted solution (200 μg of N/ml) was mixed with saturated NaOH (10 ml) and then distilled utilizing the apparatus of Parnas [11]. By this method, only amidic-N of An.Amide was released as ammonia gas which was collected in 0.1 N HCl (5.0 ml). The atomic percentage of ^{15}N in the distilled solution was analyzed by mass spectrometry at the Shokoh Company Ltd. (Saitama, Japan) (Table 1). The obtained value of ^{15}N was 22.3% which is in good agreement with the theoretically expected value of 25.3%. This indicates that the ammonium of NH_4HCO_3 is being incorporated into the amide formed.

DISCUSSION

The major advantage of the HPLC method for determination of L-Try metabolites, as compared to the techniques known hitherto, lies in their rapid and sensitive separation and determination. The method described in this paper could easily separate eleven metabolites of L-Try and made it possible to determine 5-OHAA and An.Amide which have not been found in animal tissue. Although An.A has been considered as one of the endproducts of L-Try and is excreted in urine after conjugation with glycine or glucuronic acid, the results in this study and other reports published previously [2, 3] suggest that An.A can change *in vivo* to other substances, for example, 5- or 3-OHAA, and An.Amide in mammals (Fig. 6). The failure to find 3-OHAA in the liver perfusate after addition of An.A (Fig. 3) might be attributed to the presence of 3-OHAA oxygenase in the perfused liver.

5-Hydroxyanthranilic acid is well known to be oxidized easily to its quinoimine form. This compound may disturb the metabolism of many physiologically important substances, for instance, unsaturated fatty acid, phospholipids, epinephrine, etc. by its oxido-reductive reaction. In fact, Kotake and his co-workers have reported that 5-OHAA inhibited lipid peroxidation, which is related to membrane damage, and prostaglandin biosynthesis in microsomes and that the hydroxycompound inhibited platelet aggregation induced by arachidonic acid [12].

With respect to the finding of An.Amide in the bile, but not in the liver perfusate, the amide is assumed to be non-enzymatically produced in the bile from AAG based on the following findings: (1) the concentration of AAG in the bile was relatively higher than that in the perfusate (Fig. 3); (2) 0.5–0.6 mM ammonium ion, enough to form An.Amide, was detected in the bile during the liver perfusion with An.A (data not shown); (3) an inevitable anion for the reaction, bicarbonate ion, has been given as a component of the perfusion buffer; (4) even without adding ammonium ion, AAG gave rise to the amide when incubated in the bile (data not shown). In order to assert that AAG is the immediate precursor of An.Amide, however, the examination of the amide

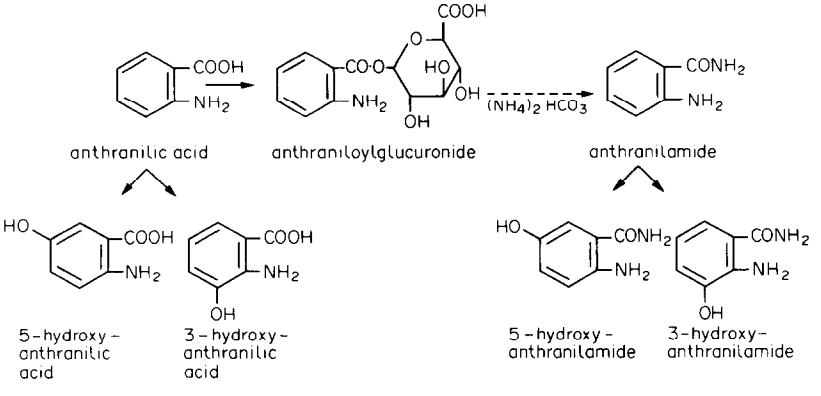

Fig. 6. Proposed metabolic pathway of anthranilic acid in rat liver.

synthesis using AAG with a higher purity than that used in this study is desirable.

Siek and Rieders have reported that an amide of clofibrate is formed when urine from individuals ingesting the compound is treated with NH_4OH or NaOH and ammonium ion [13]. But, in their report the immediate precursor of the amide has not been elucidated.

In our previous report, it was described that An.Amide change to its 3-hydroxy form *in vivo* [14]. We have also clarified that An.Amide is *in vitro* hydroxylated to form 3-hydroxyanthranilamide when the amide is incubated with rat liver microsomal fractions and an NADPH-generating system [15], and that a small amount of the product functions as a catalyzer in the production of H_2O_2 with a consumption of NADPH during the hydroxylation reactions. A prior addition of SOD to the reaction mixture causes a decrease in the NADPH-dependent H_2O_2 formation, while the rate of hydroxylation is markedly increased. These facts suggest that the formation of An.Amide in animal tissue might disturb various reactions which require NADPH, if SOD were non-existent or very little.

REFERENCES

1. Y. Kotake and Y. Kotake, Jr., *Osaka Med. Mitt.* **41**, 1028 (1942).
2. S. Kashiwamata, K. Nakashima and Y. Kotake, *Biochem. biophys. Acta* **113**, 244 (1966).
3. T. Sutamihardja, A. Ishikura, J. Naito and I. Ishiguro, *Chem. Pharm. Bull.* **20**, 2694 (1972).
4. I. Ishiguro, J. Naito, R. Shinohara, A. Ishikura and T. Terai, *Biochim. biophys. Acta* **365**, 148 (1974).
5. G. T. Bryan, *Am. J. clin. Nutr.* **24**, 841 (1971).
6. A. Burkartsmaier and E. Mutschler, *Arch. Pharmac.* **311**, 161 (1978).
7. H. J. Zeitler, *Hoppe-Seyler's Z. Physiol. Chem.* **340**, 73 (1965).
8. C. Neuberg and M. Kobel, *Biochem. Z.* **243**, 435 (1931).
9. Z. Dische and E. Borenfreund, *J. biol. Chem.* **192**, 583 (1951).
10. L. L. Miller, C. G. Bly, M. L. Watson and W. S. Wale, *J. exp. Med.* **94**, 431 (1951).
11. J. K. Parnas, *Z. anal. Chem.* **114**, 261 (1938).
12. K. Goda, T. Ueda and Y. Kotake, in *Biochemical and Medical Aspects of Tryptophan Metabolism* (Eds. O. Hayaishi, Y. Ishimura and R. Kido), Vol. 16, p. 329. Elsevier/North-Holland, Amsterdam (1980).
13. T. J. Siek and E. F. Rieders, *J. anal. Toxicol.* **5**, 194 (1981).
14. I. Ishiguro, J. Naito, A. Ishikura and T. Shiotani, *J. Pharm. Soc.* **94**, 1224 (1974).
15. R. Shinohara, Y. Ohta, J. Naito and I. Ishiguro, in *Biochemical and Medical Aspects of Trytophan Metabolism* (Eds. O. Hayaishi, Y. Ishimura and R. Kido), Vol. 16, p. 325. Elsevier/North-Holland, Amsterdam (1980).