Deacylation of *N*-Formylanilines and *N*-Acetylanilines by Rat Liver Formamidase

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The deacylation of *N*-formylanilines and *N*-acetylanilines to aniline derivatives was examined in rat liver preparations. When N^4-formylsulfanilamide or N^4-acetylsulfanilamide was incubated with rat liver cytosol, the deacylated metabolite, sulfanilamide, was formed. These deacylating activities were inhibited by paraoxone and diisopropyl fluorophosphate, inhibitors of formamidase. N^4-Formylsulfanilamide and N^4-acetylsulfanilamide were deacylated by formamidase purified from rat liver cytosol. *N*-Acetyl- or *N*-formylanilines bearing an electron-withdrawing group at the *para* position were deacylated to anilines by formamidase. In contrast, anilines bearing an electron-donating group at the *para* position were formylated to *N*-formylanilines in the presence of *N*-formyl-L-kynurenine. Formamidase catalyzed both *N*-formylation of aniline derivatives, and deacylation of *N*-formylanilines and *N*-acetylanilines.

Key words —— formamidase, deformylation, deacetylation, formylamino compound, acetylamino compound

INTRODUCTION

Some arylamines are acylated to *N*-arylacetamide and *N*-arylformamide, which are themselves toxic.[1] In contrast, these *N*-arylacylamides are deacylated to the corresponding arylamines, and this process also contributes the toxicity of *N*-arylacylamides.[2–5] 2-Acetylaminofluorene and 2-formylaminofluorene, typical carcinogenic aromatic acylamines, were reported to be deacylated in animal bodies.[6–8] The deacetylation of drugs such as acetanilide and phenacetin was also reported.[9,10] In the deacetylation, some enzymes were purified from livers of animals.[11,12] These deacetylases were shown to be identical with carboxylesterase. In contrast, we previously detected formylamino and acetylamino derivatives as *in vivo* metabolites of polycyclic arylamines.[13] Furthermore, we showed the interconversion between 2-acetylaminofluorene and 2-formylaminofluorene in rats and fish, and suggested that deacetylation of 2-acetylaminofluorene and deformylation of 2-formylaminofluorene contribute for this conversion.[8,14,15] The formylation of arylamines is catalyzed by cytosolic formamidase in the presence of *N*-formyl-L-kynurenine, and some arylformamides formed are concomitantly deformylated by liver preparations.[13] However, deformylation of *N*-arylformamide was not extensively studied.

In the current study, we examined the deacylation of *N*-formylanilines and *N*-acetylanilines in rat liver preparations, and showed that formamidase plays an important role in these deacylation. Furthermore, influence of electronic property of substituents of anilines on deacylation and formylation was also demonstrated.

MATERIALS AND METHODS

Chemicals —— Acetanilide, *p*-aminophenol, toluidine, acetyltoluidine, *p*-aminoacetophenone, *p*-acetylaminoacetophenone, sulfanilamide (SA), N^4-acetylsulfanilamide (ASA), methyl *p*-aminobenzoate, *p*-aminobenzoic acid, *p*-acetylaminobenzoic acid, acetaminophen, *p*-anisidine, *p*-acetylanisidine and methyl *p*-acetylaminobenzoate, which were > 98% chemically pure, were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Kynurenine was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*-Formyl-L-kynurenine (99%) was prepared from L-kynurenine by the method of Dalgliesh.[16] N^4-Formylsulfanilamide (FSA; 99%) and other *N*-arylformamides (> 98%) were prepared from arylamines by formylation according to the method of Tatsumi *et al*.[13]

***In Vivo* Study of SA Metabolism** —— SA was suspended in 5% gum arabic and given orally to male Wistar (Slc:Wistar/ST) rats (170–190 g, 5 weeks old; Japan SLC Inc., Shizuoka, Japan) at a single dose of 100 mg/kg. After administration, the urine and fe-

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ces were collected separately for 48 hr. The urine (1 ml) was extracted twice with ethyl acetate (5 ml). The extract was evaporated to dryness *in vacuo* and the residue was subjected to HPLC. The feces were dried over P_2O_5 *in vacuo* and pulverized in a mortar. The pulverized feces (1 g) was extracted twice with methanol (20 ml) and the combined extract was evaporated to dryness *in vacuo* and the residure was subjected to HPLC.

Liver Preparations —— Male Wistar rats (180–230 g, 6–7 weeks old) were exsanguinated and the livers were immediately perfused with 1.15% KCl, then homogenized in 4 volumes of the KCl solution with a Potter-Elvehjem homogenizer. Microsomes and cytosol were obtained from the homogenate by successive centrifugation at $9000 \times g$ for 20 min and $105000 \times g$ for 60 min. The microsomal fraction was washed by resuspension in the KCl solution and resedimentation for 60 min at $105000 \times g$ for 60 min. These procedures were carried out at 4°C. Protein contents in liver preparations were determined by the method of Lowry *et al.*[17]

DEAE-Cellulose Column Chromatography of Rat Liver Cytosol —— The rat liver cytosol (40 ml, 500 mg protein) was dialyzed against 100 volumes of 0.01 M K,Na-phosphate buffer (pH 7.4) for 12 hr. The dialyzed solution was adsorbed on a column (1.5×12 cm) of DE-52 which was equilibrated with 0.01 M K,Na-phosphate buffer (pH 7.4). The column was washed with 50 ml of the buffer and eluted with a 100 ml linear gradient of 0–0.2 M sodium chloride in the buffer. These procedures were carried out at 4°C. The fractions collected were assayed for deacylase activity towards FSA and ASA, and for formamidase activity.

Purification of Formamidase from Rat Liver —— Formamidase was purified from rat liver cytosol by the method of Shinohara and Ishiguro.[18] Briefly, formamidase was purified from rat liver cytosol by ammonium sulfate fractionation, heat treatment (55°C for 15 min), and DEAE-cellulose and hydroxyapatite column chromatographies. The specific activity was 0.06 unit/mg protein. Formamidase activity was measured in terms of the increase in absorbance at 366 nm due to hydrolysis of *N*-formyl-L-kynurenine according to the method of Arndt *et al.*[19]

Assay of Deacylase Activity —— The incubation mixture consisted of 0.1 μmol of FSA or ASA and a liver preparation equivalent to 0.25 g of liver (5–12 mg protein) in a final volume of 2 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). The incubation was performed at 37°C for 30 min in air. In control experiments, boiled liver preparations were used. After incubation, 30 μg of *p*-aminobenzamide was added to the mixture as an internal standard, and then the mixture was extracted with 5 ml of ethyl acetate. The extract was evaporated to dryness *in vacuo* and the residue was subjected to HPLC. SA formed was determined from its peak area. The time course of the cytosolic reductase activity toward FSA and ASA was linear at least for the initial 30 min. *N*-Acetylaminoanilines and *N*-formylaminoanilines were also incubated with liver cytosol or formamidase, and deacylated metabolites formed were determined by HPLC as in the case of ASA or FSA.

Assay of *N*-Formylase Activity towards Aniline Derivatives —— The incubation mixture consisted of 0.1 μmol of an aniline derivative, 0.5 μmol of *N*-formyl-L-kynurenine and liver cytosol equivalent to 0.25 g of liver (8–12 mg protein) in a final volume of 2 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). The incubation was performed at 37°C for 30 min. After incubation, an internal standard was added, and then the mixture was extracted with 5 ml of ethyl acetate. The extract was evaporated to dryness *in vacuo* and the residue was subjected to HPLC. *N*-Formylanilines formed were determined from the peak area.

HPLC —— HPLC was performed in a Hitachi L-6000 chromatograph (Tokyo, Japan) fitted with a column of 150×4.6 mm Inertsil ODS-2. For the determination of aniline derivatives, *p*-aminobenzamide, benzamidoxime or benzamide was used as an internal standard, and the detector was set at 235 or 254 nm. The assay conditions were as follows: SA and the acylated compounds at a flow rate of 0.5 ml/min in the mobile phase of acetonitrile-0.1 M KH_2PO_4 (3 : 7); *p*-aminophenol and the acylated compounds at a flow rate of 0.3 ml/min in the mobile phase of acetonitrile-0.1 M KH_2PO_4 (1 : 9); *p*-toluidine and the acylated compounds at a flow rate of 0.5 ml/min in the mobile phase of acetonitrile-0.1 M KH_2PO_4 (2 : 8); *p*-formylaminoacetophenone and the acylated compounds at a flow rate of 0.4 ml/min in the mobile phase of acetonitrile-H_2O (2 : 8); methyl *p*-aminobenzoate and the acylated compounds at a flow rate of 0.8 ml/min in the mobile phase of acetonitrile-H_2O (2 : 8); aniline and the acylated compounds at a flow rate of 0.6 ml/min in the mobile phase acetonitrile-H_2O (1 : 9); *p*-anisidine and the acylated compounds at a flow rate of 0.8 ml/min in the mobile phase of acetonitrile-0.1 M KH_2PO_4 (1 : 9); *p*-aminobenzoic acid and the

Table 1. Retention Time (Rt) of Aniline Derivatives

Aniline derivative	Rt (min)	Aniline derivative	Rt (min)
p-aminophenol	6.5	methyl *p*-aminobenzoate	34.5
p-acetylaminophenol	14.9	methyl *p*-acetylaminobenzoate	37.6
p-formylaminophenol	16.5	methyl *p*-formylaminobenzoate	43.6
sulfanilamide	9.6	aniline	12.9
N^4-acetylsulfanilamide	35.4	*p*-acetylaniline	19.2
N^4-formylsulfanilamide	20.1	*p*-formularizing	18.1
p-toluidine	18.7	*p*-anisidine	20.9
p-acetoluidine	27.5	*p*-acetylanisidine	33.8
p-formylaminotoluidine	27.8	*p*-formylanisidine	35.9
p-aminoacetophenone	23.6	*p*-aminobenzoic acid	12.2
p-acetylaminoacetophenone	32.2	*p*-acetylaminobenzoic acid	30.1
p-formylaminoacetophenone	22.8	*p*-formylaminobenzoic acid	16.6

acylated compounds at a flow rate of 0.8 ml/min in the mobile phase of methanol-0.1 M KH_2PO_4 (5 : 95). Retention times of these chemicals are summarized in Table 1.

RESULTS

Metabolism of FSA and ASA by Rat Liver Preparations

The *in vitro* metabolism of FSA and ASA by rat liver preparations was examined. When FSA or ASA was incubated with rat liver cytosol without cofactors, one metabolite, having a retention time corresponding to that of SA, was detected in HPLC chromatograms of the extracts of these incubation mixtures. When FSA or ASA was incubated with boiled liver cytosol, the metabolite was not detected. The metabolite isolated from the incubation mixture of FSA or ASA by HPLC was identified as SA by comparison of its mass and UV spectra with those of authentic SA (data not shown).

Liver cytosol exhibited a deacylase activity toward FSA, but little activity toward ASA. In contrast, liver microsomes exhibited little deacylase activity toward FSA (Fig. 1A). The cytosolic deacylase activities toward FSA and ASA were inhibited by the addition of paraoxone (diethyl 4-nitrophenylphosphate), diisopropyl fluorophosphate (DFP) and potassium cyanide, inhibitors of formamidase.[19,20] However, the activity was not inhibited by bis(4-nitrophenyl)phosphate (BNPP) and phenylmethylsulfonyl fluoride (PMSF), inhibitors of carboxylesterase[21] (Fig. 1B). These results suggest that the cytosolic activities towards FAS and ASA were exhibited by formamidase.

DEAE-Cellulose Column Chromatography of Rat Liver Cytosol

Next, we examined the components in liver cytosol that mediate the deacylation of FSA and ASA. The liver cytosol of rats was subjected to DEAE-cellulose column chromatography, and the fractions were assayed for deacylase activity towards FSA and ASA, and for formamidase activity as described in Materials and Methods. The fractions that exhibited deformylase activity toward FSA also showed formamidase activity, which was assayed in terms of the hydrolysis of *N*-formyl-L-kynurenine, as described in Materials and Methods. The active fractions also showed weak deacetylase activity toward ASA (data not shown). These facts suggest that formamidase in rat liver cytosol was involved in the deacylation of FSA and ASA.

Deacylation of *N*-Arylformamides and *N*-Arylacetamides by Formamidase

Formamidase purified from rat liver cytosol exhibited deformylase activity toward FSA, and also deacetylase activity toward ASA (Tables 2 and 3).

Furthermore, deformylating activity of formamidase in rat liver cytosol toward some *N*-formylanilines and *N*-formylating activity of formamidase towards some anilines were examined. When various *N*-formylanilines were incubated with rat liver cytosol or formamidase, *N*-formylanilines substituted at the *para* position with an electron-withdrawing group, *i.e.*, carbonyl, sulfonamide, car-

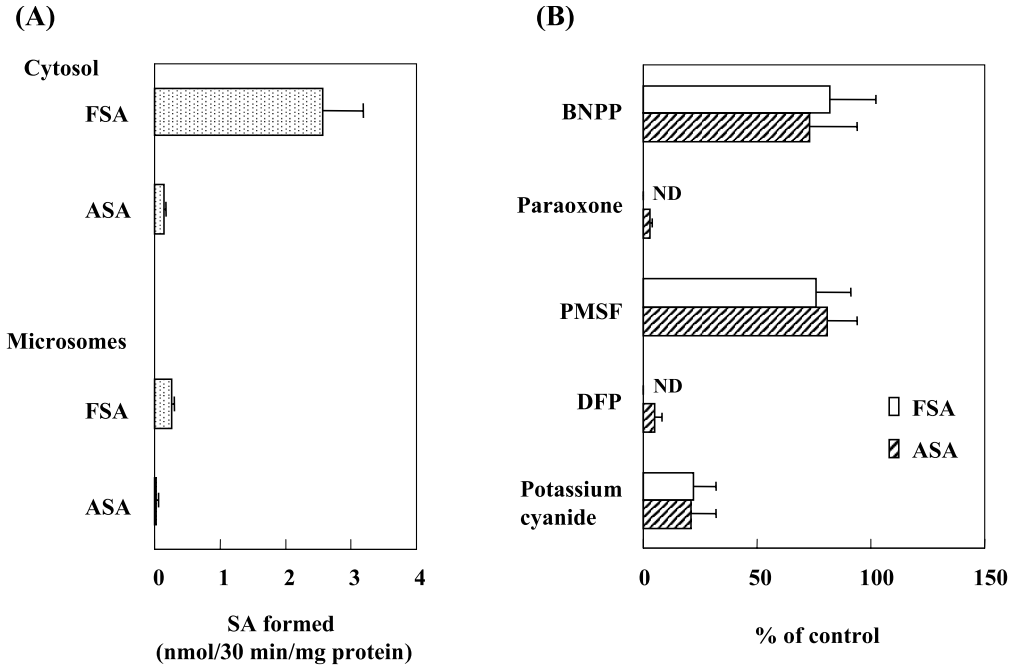
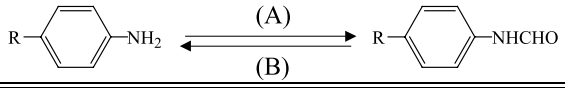


Fig. 1. Deacylation of FSA and ASA by Rat Liver Preparations

Each bar represents the mean ± S.D. of four rats. FSA or ASA was incubated with rat liver preparations as described in Materials and Methods. The amounts of SA formed were determined by HPLC. Paraoxone. bis(4-nitrophenyl)phosphate (BNPP), phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP) and potassium cyanide were added at the concentration of 1×10^{-4} M.

Table 2. Formylation and Deformylation of Arylamines or Acylarylamines by Rat Liver Cytosol and Formamidase



R	Formamidase activity (A)		Deformylase activity (B)	
	Liver cytosol	Formamidase	Liver cytosol	Formamidase
	(μmol/30 min/mg protein)		(nmol/30 min/mg protein)	
H	—	—	1.17 ± 0.20	126 ± 18
OH	0.05 ± 0.02	24.3 ± 3.2	ND	ND
CH_3	0.25 ± 0.06	139.8 ± 22.9	ND	ND
OCH_3	0.16 ± 0.04	67.6 ± 10.4	ND	ND
SO_2NH_2	ND	ND	2.62 ± 0.31	209 ± 33
$COCH_3$	ND	ND	2.72 ± 0.40	192 ± 12
$COOCH_3$	ND	ND	3.72 ± 0.56	159 ± 19
COOH	ND	ND	1.67 ± 0.24	142 ± 17

Each value represents the mean ± S.D. of three experiments. ND : not detected. — : not determined. In the case of (A), an aniline derivative was incubated with enzyme source in the presence of *N*-formyl-L-kynurenine for 30 min. In the case of (B), a formylaniline derivative was incubated with enzyme source for 30 min. Formylanilines and anilines formed were determined by HPLC as described in MATERIALS AND METHODS.

boxylic acid and methyl ester, were efficiently deformylated to anilines, but *N*-formylanilines substituted with an electron-donating group were not deformylated. In contrast, when various anilines were incubated with liver cytosol or formamidase in the presence of *N*-formyl-L-kynurenine, anilines which were substituted at the *para* position with an electron-donating group, hydroxyl, methoxyl or methyl, were formylated to the corresponding *N*-formylaniline derivatives with high efficiency, but anilines substituted with an electron-withdrawing group were not formylated (Table 2). These facts suggested that deformylation and formylation by formamidase are markedly influenced by the electronic properties of the *para* substituent. Furthermore, deacetylation of *N*-acetylanilines by

formamidase was examined using various *N*-acetylated aniline derivatives. Deacetylation of *N*-acetylanilines by formamidase was also influenced by the substituent at the *para* position. *N*-Acetylanilines substituted at the *para* position with an electron-withdrawing group were readily deacetylated to anilines, as was the case for *N*-formylanilines (Table 3).

Based on these results, we suggest that formamidase plays a major role in the deformylation of *N*-formylanilines, and the deacetylation of *N*-acetylanilines, in addition to the formylation of aniline derivatives.

In Vivo Metabolism of SA in Rats

When SA was orally dosed to male rats at the dose of 100 mg/kg, two metabolites corresponding ASA and FAS were detected in the urine and feces. The majority of the metabolites was excreted in the urine. ASA was detected in the urine as a major metabolite of SA. However, FSA was excreted in only little amounts (Table 4). This *in vivo* study supports that formamidase play an important role in the deformylation in the metabolism of SA, as shown in above *in vitro* study.

Table 3. Deacetylation of Acylarylamines by Rat Liver Cytosol and Formamidase

R	Deacetylase activity (nmol/30 min/mg protein)	
	Rat liver cytosol	Formamidase
OH	ND	ND
CH_3	ND	ND
OCH_3	ND	ND
SO_2NH_2	0.74 ± 0.09	128 ± 14
$COCH_3$	0.52 ± 0.10	60 ± 8
$COOCH_3$	0.67 ± 0.13	68 ± 10

Each value represents the mean ± S.D. of three experiments. ND : not detected. An acetylaminoaniline derivative was incubated with enzyme source for 30 min. Anilines formed were determined by HPLC as described in MATERIALS AND METHODS.

Table 4. Urinary and Fecal Excretion of ASA, FSA and SA by Rats Given Orally SA

	ASA	FSA	SA
		(% of dose)	
Urine	23.62 ± 5.47	0.03 ± 0.01	7.56 ± 1.72
Feces	1.06 ± 0.29	0.02 ± 0.01	0.07 ± 0.03
Total	24.68 ± 5.19	0.05 ± 0.01	7.63 ± 1.70

Each value represents the mean ± S.D. of six animals. ASA: N^4-acetylsulfanilamide, FSA: N^4-formylsulfanilamide, SA: sulfanilamide.

DISCUSSION

Amide bond cleavage of arylacylamides is important in the study of the metabolism and toxicology of nitrogenous foreign compounds. *N*-Arylacetamides are deacetylated by enzymes which were named arylacylamide amidohydrolase or arylamidase in the early stage of the study concerning deacylation. These amidase are capable of hydrolyzing *N*-arylacetamides, such as acetanilide, ASA, *p*-acetamidobenzoic acid and 2-acetylaminofluorene, to their unconjugated forms. Some deacetylases involved in the deacetylation of acetanilide or 2-acetylaminofluorene were purified from liver microsomes of animals.[11,12] The microsomal enzymes involved in the deacetylation of *N*-arylacetamides were classified as carboxylesterases.[21] The serine moiety in the active site of these enzymes plays an important role in the reaction, and they are called serine hydrolases; their molecular mass is about 60 kDa. Metabolic activation of acylarylamides such as 2-acetylaminofluorene and phenacetin by deacetylation has also been reported.[3,10,22,23] In this study, we demonstrated that deformylation and deacetylation of FSA and ASA are catalyzed by formamidase. However, formylation of SA was not catalyzed by this enzyme. Furthermore, ASA was detected as a major metabolite of SA after the oral dose of SA, but FSA was not detected as *in vivo* metabolite. Our present findings suggest that formamidase play a predominant role in the deformylation of FSA *in vivo*.

Formamidase, a cytosolic enzyme, is known to participate in tryptophan metabolism, catalyzing the deformylation of *N*-formyl-L-kynurenine to kynurenine with simultaneous liberation of formic acid.[19] The importance of formamidase in the metabolism of xenobiotics, including arylamines and *N*-arylacylamides, has not been recognized, and the toxicological and pharmacological implications are of significant concern. We previously reported that various types of arylamines such as 2-aminofluorene, 4-aminobiphenyl, 1-aminonaphthalene and 1-aminopyrene were acylated to the *N*-formylamino derivatives in rabbits by formamidase.[13] In the previous study on the metabolism of 2-formylaminofluorene,

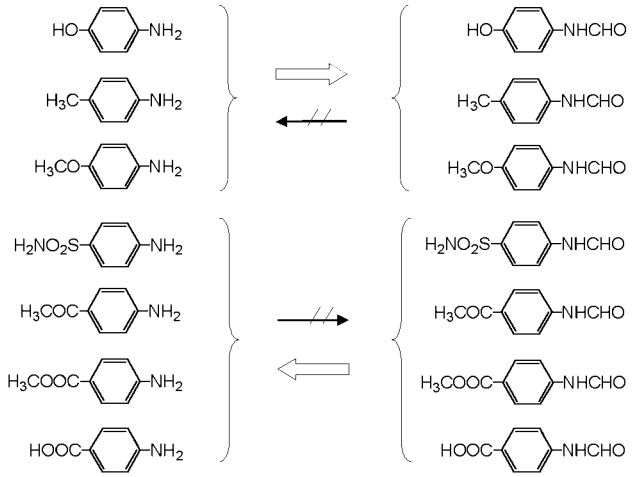


Fig. 2. *N*-Formylation of Anilines and Deformylation of *N*-Formylanilines by Formamidase

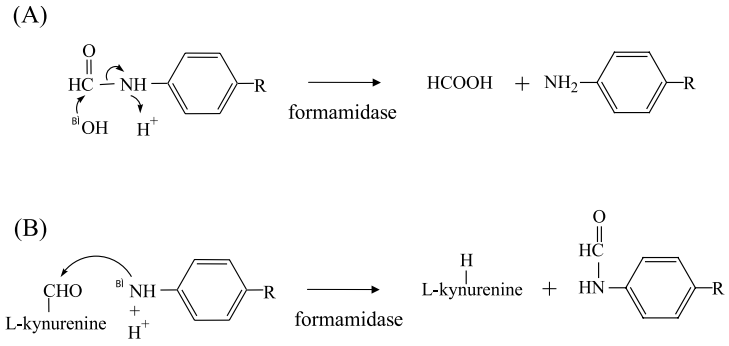


Fig. 3. Postulated Mechanism for the Electronic Effects of the Substituents of *N*-Formylanilines on Deformylation (A), and of Those of Anilines on *N*-Formylation (B)

it was shown that this acylamine was hydrolyzed in the animal body.[8] Recently, we reported that 2-formylaminofluorene was deformylated to 2-aminofluorene by rat liver formamidase.[24] In this study, we have demonstrated that formamidase also catalyzes the deacylation of *N*-formylanilines and *N*-acetylanilines to aniline derivatives, in addition to the *N*-formylation of anilines to *N*-formylanilines. Santti and Hopsu-Havu[25] reported that formamidase catalyzed the hydrolysis of formyl-1-aminonaphthalene. Hydrolysis of *N*-formyl xenobiotics has also been observed, including the acaricides formetanate and chlorphenamide.[26,27] The deformylation of these compounds was catalyzed by a soluble enzyme, and it was speculated that formamidase was involved in the metabolism, because the deformylase activities were inhibited by DFP, an inhibitor of formamidase. Shinohara and Ishiguro[18] reported that formamidase was inhibited by heavy metal ions (Ag, Hg, Cu), sodium fluoride and sodium arsenite. In contrast, Santti and Hopsu-Havu[28] reported that formamidase in guinea pig liver was inhibited by these heavy metals, but not by sodium fluoride. We also observed an inhibitory effect of potassium cyanide. An SH group may be involved in the deacylation, as well as hydrolysis of *N*-formyl-L-kynurenine, in addition to the active serine center.

In this paper, we demonstrated that formamidase catalyzes the deformylation of various *para*-substituted *N*-formylanilines to varying degrees. *N*-Formylanilines bearing an electron-withdrawing substituent at the *para* position of the molecule were effectively deformylated, but those bearing an electron-donating group were not. Formamidase also catalyzed the *N*-formylation of anilines with an electron-donating substituent at the para position (Fig. 2). These electronic effects are reasonable for *N*-acylation and deacylation. An electron-deficient site is easily attacked by the hydroxyl anion (Fig. 3A). In

contrast, an electron-rich site is easily attacked by the formyl moiety of *N*-formyl-L-kynurenine (Fig. 3B). Shinohara and Ishiguro[18] also reported that formylaniline was deformylated by formamidase, but the extent of the reaction was only 1% of that *N*-formyl-L-kynurenine. Arndt *et al.*[19] reported that acetanilide was not hydrolyzed by formamidase. In contrast, Nimmo-Smith[29] examined the deacetylation of aromatic acylamides by mitochondrial deacylase in chicken kidney using various acetanilides, and found that the deacetylation of acetanilides was unaffected by the substituents. The discrepancy with our results may be due to different mechanisms of deacylation. Heymann[6] reported that increasing deacetylation rates were observed for the hydrolysis of substituted acylanilides with increasing lipophilicity of the acyl function. Lipophilicity of the substituents may also influence the deacylation of *N*-arylacylamides.

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