Regioselective *N*-Acetylation as a Route of Nitro-*p*-phenylenediamine Metabolism by Rat Liver Cytosol

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Regioselectivity in *N*-acetylation of nitro-*p*-phenylenediamine, a widely used hair dye component, by rat liver cytosolic *N*-acetyltransferases was studied in relation to its substituent effects on enzymatic *N*-acetylation of mono-substituted anilines. Nitro-*p*-phenylenediamine was acetylated specifically at the N^4-position to afford the N^4-monoacetate, a major urinary metabolite in the rat, when incubated with rat liver cytosol fortified with acetyl-coenzyme A. N^1-Acetylation of nitro-*p*-phenylenediamine did not take place even when the N^4-monoacetate was used as a substrate, suggesting a strong steric hindrance effect of the *ortho* nitro group on the enzymatic N^1-acetylation. The steric hindrance effect of the nitro group on the cytosolic *N*-acetylation of the *ortho* amino group was revealed by a comparative study carried out by using aniline, three respective regioisomers of nitroanilines and phenylenediamines as model substrates. The comparative study also indicated the enzymatic *N*-acetylation of the mono-substituted anilines to be strongly influenced by the electronic effect of the substituents.

Regioselective *N*-acetylation in the hepatic cytosol was also investigated with N^1- and N^4-monoacetates of 1,2,4-triaminobenzene. The monoacetates yielded the N^1,N^4-diacetate, another major urinary metabolite of the hair dye component, in the rat, without concomitant formation of the N^2,N^4-diacetate or the N^1,N^2,N^4-triacetate. The triacetate was formed only from the N^1,N^2-diacetate in the enzymatic reactions. A comparative study, carried out by using *N*-mono-acetates of three regioisomeric phenylenediamines, indicated that the *N*-acetyl group had a potent steric hindrance effect on the primary amino group at the *ortho* position.

Thus, the present *in vitro* study strongly suggested that the two major urinary metabolites, nitro-*p*-phenylenediamine N^4-acetate and 1,2,4-triaminobenzene N^1,N^4-diacetate, of the hair dye component could be formed, at least in the rat liver, by the enzymatic *N*-acetylation of the corresponding amines.

Keywords hair dye; nitro-*p*-phenylenediamine; metabolism; rat liver cytosol; *N*-acetylation; regioselectivity; high-pressure liquid chromatography

Introduction

Nitro-*p*-phenylenediamine is a widely used ingredient in permanent and semipermanent hair dye formulations. It has been found mutagenic in certain bacteria[1,2] and *in vitro* mammalian systems[3] and is carcinogenic to female mice.[4,5] We reported in a previous paper that, following the administration of nitro-*p*-phenylenediamine to male rats, two major metabolites, identified as nitro-*p*-phenylenediamine N^4-monoacetate and 1,2,4-triaminobenzene N^1,N^4-diacetate, were excreted into the urine.[6,7] 1,2,4-Triaminobenzene N^1,N^4-diacetate was also isolated and identified as the sole urinary metabolite after the possible metabolic intermediates, the N^4-monoacetates of nitro-*p*-phenylenediamine and 1,2,4-triaminobenzene, were administered to the animals. These results, at least, indicate that in the animals nitro-*p*-phenylenediamine is successively metabolized to nitro-*p*-phenylenediamine N^4-monoacetate, 1,2,4-triaminobenzene N^4-monoacetate, and 1,2,4-triaminobenzene N^1,N^4-diacetate.[7] It thus appears that the metabolism of nitro-*p*-phenylenediamine and 1,2,4-triaminobenzene N^4-monoacetate proceeds by regioselective *N*-acetylation of their amino groups *in vivo*.

The purposes of the present study are 1) to confirm whether the regioselective *N*-acetylation of nitro-*p*-phenylenediamine and its reduction product, 1,2,4-triaminobenzene N^4-acetate, also takes place in rat liver and 2) to obtain evidence for structural factors determining the orientation and the rates of the enzymatic regioselective reactions.

The present study provides evidence that hepatic cytosolic *N*-acetylation of mono-substituted phenylenediamines proceeds highly regioselectively as a result of the steric hindrance effect of the *ortho* substituent. Evidence will be also shown in this paper that the electronic properties of the substituents play an important role in determining the rate of the enzymatic *N*-acetylation.

Experimental

Chemicals Nitro-*p*-phenylenediamine (2-nitro-1,4-diaminobenzene), nitro-*p*-phenylenediamine N^1,N^4-diacetate (N^1,N^4-diacetyl-2-nitro-1,4-diaminobenzene), nitroanilines, nitroaniline *N*-acetates (*N*-acetylnitroanilines), dihydrochlorides of phenylenediamines, *m*-phenylenediamine *N*-monoacetate (*N*-acetyl-1,3-diaminobenzene), *p*-phenylenediamine *N*-monoacetate (*N*-acetyl-1,4-diaminobenzene), *p*-phenylenediamine *N*,*N*′-diacetate (*N*,*N*′-diacetyl-1,4-diaminobenzene), aniline hydrochloride, acetanilide, and N^4-acetylsulfanilamide were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, acetyl-coenzyme A (CoA) as a lithium salt from P-L Biochemicals Inc., Milwaukee, Wisconsin, and bovine serum albumin from Sigma Chemical Co., St. Louis, Missouri. Nitro-*p*-phenylenediamine *N*-monoacetates (*N*-acetyl-2-nitro-1,4-diaminobenzenes), 1,2,4-triaminobenzene *N*-monoacetates (*N*-monoacetyl-1,2,4-triaminobenzenes), 1,2,4-triaminobenzene *N*,*N*′-diacetates (*N*,*N*′-diacetyl-1,2,4-triaminobenzenes), 1,2,4-triaminobenzene N^1,N^2,N^4-triacetate (1,2,4-triacetylaminobenzene),[6,7] *o*-phenylenediamine *N*-monoacetate (*N*-acetyl-1,2-diaminobenzene),[8] *o*-phenylenediamine *N*,*N*′-diacetate (*N*,*N*′-diacetyl-1,2-diaminobenzene)[9] and *m*-phenylenediamine *N*,*N*′-diacetate (*N*,*N*′-diacetyl-1,3-diaminobenzene)[10] were prepared according to published procedures. All other chemicals were of the best available commercial grade.

Preparation of Liver Cytosol Male Sprague-Dawley rats, 6 weeks of age, weighing 200—210 g (Charles River Japan, Inc., Atsugi), were used in all experiments and allowed free access to laboratory food and tap water. The animals were killed by decapitation, and their livers were immediately removed, chilled in ice-cold 1.15% KCl, and homogenized at 0 °C in 3 volumes of 1.15% KCl in a Teflon-pestled glass homogenizer. The homogenates were centrifuged at 105000 × *g* for 60 min with a Hitachi 55P-2 ultracentrifuge. The supernatant was used for the enzyme assay. Protein was determined by the method of Lowry *et al.*[11] using bovine serum albumin as a standard.

Incubation and Extraction Procedures The method of Lower and Bryan[12] was modified for incubations as follows. The incubation mixture contained the following in a final volume of 1.0 ml: 1.0 μmol of an arylamine substrate dissolved in 0.05 ml of propylene glycol, 1.0 μmol of acetyl-CoA,

100 μmol of Tris–HCl buffer, pH 7.5, and 4.3 mg of protein of liver cytosol (0.15 ml). The reaction was initiated by the addition of the substrate and allowed to proceed with gentle shaking for 30 min at 37 °C and then terminated by rapid cooling in an ice bath, followed by the addition of 0.1 ml of 1 N NaOH. The mixture was immediately extracted with two volumes of ethyl acetate or ethyl acetate–*n*-butanol (1 : 1, v/v; saturated with water before use), containing an appropriate internal standard for subsequent high pressure liquid chromatography (HPLC). Ethyl acetate was used only for the extraction of the metabolites of aniline and nitroanilines. The residual aqueous phase was extracted twice with three volumes each of the same solvent without the internal standard. The combined organic phase was evaporated to dryness *in vacuo* below 50 °C. The residue was redissolved in 50% aqueous methanol and subjected to HPLC. Various amounts of acetylated arylamines added to the incubation mixture without the substrate were recovered in a ratio higher than 85% by the aforementioned extraction procedure.

HPLC Separation of the metabolites following incubation was carried out by reverse partition HPLC (a Shimadzu model LC-6A system

Table I. Chromatographic and Spectral Data of Nitro-*p*-phenylenediamine, Its *N*-Acetates, and 1,2,4-Triaminobenzene *N*-Acetates

Compound	HPLC[a] Retention times (min) 1	2	UV $\lambda_{max}^{ethanol}$ nm (ε)	Mass spectral data *m/z* (relative intensity, %)
Nitro-*p*-phenylenediamine	18.2			
N^1-monoacetate	16.2		248 (19100)	195 (M^+, 29), 153 (100), 107 (78), 80 (27), 53 (29)
N^4-monoacetate	25.8		253 (22700)	195 (M^+, 47), 153 (100), 107 (88), 80 (33), 53 (42)
N^1,N^4-diacetate	29.2		253 (23500)	237 (M^+, 24), 195 (58), 153 (100), 107 (43), 53 (15)
Acetanilide[b]	32.8			
1,2,4-Triaminobenzene				
N^1,N^2-diacetate		12.0	223 (18800), 255 (10900), 300 (2300)	207 (M^+, 39), 147 (51), 123 (46), 122 (100), 95 (45)
N^1,N^4-diacetate		21.2	225.5 (29600), 255 (12000), 302 (5100)	207 (M^+, 66), 147 (57), 123 (82), 122 (100), 95 (52)
N^2,N^4-diacetate		14.0	213 (15900), 260 (12000)	207 (M^+, 72), 147 (76), 123 (88), 122 (100), 95 (52)
N^1,N^2,N^4-triacetate		39.6	230 (19300), 260 (16000)	249 (M^+, 61), 165 (62), 147 (63), 123 (64), 122 (100)
N^4-Acetylsulfanilamide[b]		26.0		

a) 1: Mobile phase, acetonitrile–water (15 : 85, v/v), 2: mobile phase, methanol–water (10 : 90. v/v), other conditions as described in the text. *b*) Internal standard for HPLC.

Table II. Chromatographic and Spectral Data of Nitroanilines, Aniline and Their *N*-Acetates

Compound	HPLC[a] Retention times (min) 1	2	UV $\lambda_{max}^{ethanol}$ nm (ε)	Mass spectral data *m/z* (relative intensity, %)
o-Nitroaniline	15.8			
o-Nitroaniline *N*-acetate	11.2		233 (17000)	180 (M^+, 15), 138 (100), 92 (40), 65 (41), 63 (20), 52 (21)
m-Nitroaniline	11.6			
m-Nitroaniline *N*-acetate	14.2		241 (22900)	180 (M^+, 22), 138 (100), 92 (52), 65 (37), 64 (14), 63 (16)
p-Nitroaniline	9.6			
p-Nitroaniline *N*-acetate	15.2		315 (14400), 224 (10860)	180 (M^+, 25), 138 (100), 108 (56), 92 (29), 65 (42), 63 (24)
2,4-Dinitroaniline[b]	19.8			
Aniline		12.0		
Acetanilide		14.6	241 (16000)	135 (M^+, 25), 93 (100), 66 (23), 65 (20)
m-Nitroaniline *N*-acetate[b]		22.0		

a) 1: Mobile phase, methanol–water (1 : 1, v/v), 2: mobile phase, methanol–water (4 : 6, v/v), other conditions as described in the text. *b*) Internal standard for HPLC.

Table III. Chromatographic and Spectral Data of Phenylenediamines and Their *N*-Acetates

Compound	HPLC[a] Retention times (min) 1	2	UV $\lambda_{max}^{ethanol}$ nm (ε)	Mass spectral data *m/z* (relative intensity, %)
o-Phenylenediamine	5.6			
N-monoacetate	11.6		292 (2870), 208 (24100)	150 (M^+, 46), 133 (26), 108 (100), 107 (39), 80 (56), 53 (25)
N,*N*′-diacetate	22.8		214 (20700)	192 (M^+, 20), 133 (35), 132 (36), 108 (100), 107 (31), 80 (32)
p-Phenylenediamine *N*,*N*′-diacetate[b]	31.6			
m-Phenylenediamine		8.0		
N-monoacetate		12.4	296 (2580), 223 (26900)	150 (M^+, 54), 108 (100), 81 (22), 80 (29), 53 (14)
N,*N*′-diacetate		26.4	234 (29300)	192 (M^+, 22), 150 (34), 108 (100), 81 (12), 80 (21)
p-Phenylenediamine *N*,*N*′-diacetate[b]		20.4		
p-Phenylenediamine		6.4		
N-monoacetate		8.8	260 (15000)	150 (M^+, 47), 108 (100), 107 (38), 80 (33), 53 (23)
N,*N*′-diacetate		20.4	265 (23000)	192 (M^+, 32), 150 (22), 108 (100), 107 (31), 80 (23), 53 (16)
m-Phenylenediamine *N*,*N*′-diacetate[b]		26.4		

a) 1: Mobile phase, methanol–water–acetic acid (15 : 85 : 0.1, v/v), 2: mobile phase, methanol–10 mM phosphate buffer, pH 7.5 (2 : 8, v/v), other conditions as described in the text. *b*) Internal standard for HPLC.

chromatograph) on a Shim-pack CLC-ODS (6 × 150 mm, 5 μm in particle size, Shimadzu) column. The flow rate of the developing solvent was 0.6 ml/min, and monitoring of the chromatograms was done at 254 nm with an ultraviolet detector. Chromatographic data for the *N*-acetates are summarized in Tables I—III.

Spectral Measurements Ultraviolet (UV) absorption spectra were recorded in ethanol with a Shimadzu model UV-201 spectrophotometer. Mass spectra were obtained with a Shimadzu model QP-1000 mass spectrometer at an ionization voltage of 70 eV and an ion source temperature of 290 °C by electron impact mode with direct insertion. Spectral data for the *N*-acetates are summarized in Tables I—III.

Results

***N*-Acetylation of Nitro-*p*-phenylenediamine and Its N^4-Acetate by Rat Liver Cytosol** Following the incubation of rat liver cytosol with nitro-*p*-phenylenediamine in the presence of acetyl-CoA and the subsequent extraction of the incubation mixture with ethyl acetate–*n*-butanol, containing acetanilide as an internal standard, only one major peak (t_R 25.8 min) other than that of the substrate was detected as a metabolite by HPLC (Table IV). The peak did not appear in the chromatogram either when boiled (100 °C for 5 min) cytosol was used or when acetyl-CoA was omitted from the incubation mixture. The sole metabolite was eluted from the reverse partition HPLC column and rechromatographed under the same conditions. The metabolite was identical with an authentic specimen of nitro-*p*-phenylenediamine N^4-monoacetate by comparing their UV absorption and mass spectra (Table I). However, neither the regioisomer, nitro-*p*-phenylenediamine N^1-monoacetate, nor the further acetylated product, nitro-*p*-phenylenediamine N^1,N^4-diacetate, was detected in the chromatogram, although they were well separable, if present, under the HPLC conditions used (Table I).

No trace amount of the N^1,N^4-diacetate of nitro-*p*-phenylenediamine was formed even when the N^4-acetate was used as the substrate instead of nitro-*p*-phenylenediamine (Table IV).

***N*-Acetylation of 1,2,4-Triaminobenzene *N*-Mono-, and *N,N'*-Di-acetates** N^4-Acetylation also took place when the N^1- and N^2-monoacetates of 1,2,4-triaminobenzene were incubated under the same conditions as used for nitro-*p*-phenylenediamine. The ethyl acetate–*n*-butanol extracts, containing N^4-acetylsulfanilamide as an internal standard, of the incubation mixtures were shown by HPLC to contain the N^1,N^4-diacetate (t_R 21.2 min) and the N^2,N^4-diacetate (t_R 14.0 min) as the sole metabolites from the N^1- and N^2-monoacetates, respectively (Table IV). Each metabolite was identified with the corresponding authentic specimens by UV absorption and mass spectrometries after being eluted from the HPLC column and then rechromatographed under the same conditions (Table I). Neither the regioisomer, N^1,N^2-diacetate, nor the fully acetylated product, N^1,N^2,N^4-triacetate, was detected as a metabolite in the extract (Tables I and IV), both of which, if present, were detectable by HPLC as peaks well separated from those of the metabolically formed diacetates (Table I).

N^1-Acetylation took place when 1,2,4-triaminobenzene N^4-monoacetate was incubated under the aforementioned conditions. From the incubation mixture, the N^1,N^4-diacetate was isolated and identified as the sole metabolite by the aforementioned chromatographic and spectroscopic methods (Table IV). However, neither the regioisomer, N^2,N^4-diacetate, nor the N^1,N^2,N^4-triacetate could be detected as a metabolite in the incubation mixture by HPLC (Tables I and IV).

The N^1,N^2,N^4-triacetate was formed from the N^1,N^2-diacetate, but not formed from the N^1,N^4- and N^2,N^4-diacetates used as the substrates for the incubation (Table IV).

***N*-Acetylation of Nitroanilines** Rat liver cytosol was incubated with *o*-, *m*-, or *p*-nitroaniline in the presence of acetyl-CoA under the same conditions as used for nitro-*p*-phenylenediamine in order to examine the effect of the nitro group substitution on the acetyl-CoA-dependent acetylation of aniline. Metabolically formed nitroaniline *N*-acetate was isolated from the incubation mixture by extraction with ethyl acetate, containing 2,4-dinitroaniline as an internal standard, and identified with the corresponding authentic specimen by HPLC, as well as by the aforementioned spectroscopic methods, after being eluted from the HPLC column and then rechromatographed under the same conditions (Table II). The HPLC data indicated

TABLE IV. *N*-Acetylation of Nitro-*p*-phenylenediamine, 1,2,4-Triaminobenzene *N*-Mono- and *N,N'*-Di-acetates by Rat Liver Cytosol

Substrate[a]	Possible metabolite	Metabolite formed (nmol/mg protein/30 min)
Nitro-*p*-phenylenediamine	Nitro-*p*-phenylenediamine N^1-monoacetate	0
	N^4-monoacetate	28.7
	N^1,N^4-diacetate	0
Nitro-*p*-phenylenediamine N^4-monoacetate	Nitro-*p*-phenylenediamine N^1,N^4-diacetate	0
1,2,4-Triaminobenzene N^1-monoacetate	1,2,4-Triaminobenzene N^1,N^2-diacetate	0
	N^1,N^4-diacetate	9.4
	N^1,N^2,N^4-triacetate	0
N^2-monoacetate	1,2,4-Triaminobenzene N^1,N^2-diacetate	0
	N^2,N^4-diacetate	11.1
	N^1,N^2,N^4-triacetate	0
N^4-monoacetate	1,2,4-Triaminobenzene N^1,N^4-diacetate	17.3
	N^2,N^4-diacetate	0
	N^1,N^2,N^4-triacetate	0
1,2,4-Triaminobenzene N^1,N^2-diacetate	1,2,4-Triaminobenzene N^1,N^2,N^4-triacetate	27.9
N^1,N^4-diacetate	N^1,N^2,N^4-triacetate	0
N^2,N^4-diacetate	N^1,N^2,N^4-triacetate	0

a) Each substrate (1 mM) was incubated at 37 °C for 30 min with rat liver cytosol (4.3 mg protein/ml) in the presence of acetyl-CoA (1 mM) in 0.1 mM Tris–HCl buffer, pH 7.5, containing propylene glycol (5%, v/v). Propylene glycol which had no effect on the enzymatic reactions was used for dissolution of the substrates.

that introduction of a nitro group into any carbon of aniline led to a marked decrease in its enzymatic *N*-acetylation (Table V). The *N*-acetylation did not take place at all with *o*-nitroaniline, while the other regioisomers underwent the enzymatic *N*-acetylation at significantly different rates whose ratio was about 4:1 for *m*- and *p*-nitroanilines. However, the rate for enzymatic *N*-acetylation of aniline was twice as high as that of *m*-nitroaniline, the best substrate among the three nitro-substituted anilines used (Table V).

N-Acetylation of Phenylenediamines and Their N-Monoacetates To study the effect of amino or acetylamino group substitution on the enzymatic *N*-acetylation of aniline by rat liver cytosol, *o*-, *m*-, and *p*-phenylenediamines and their monoacetates were individually incubated with liver cytosol in the presence of acetyl-CoA under the conditions specified above. The metabolically formed *N*-monoacetates and *N,N'*-diacetates from the phenylenediamines as well as the *N,N'*-diacetates from the *N*-monoacetates were isolated and identified in the same manner as that used for the nitroaniline *N*-acetates (Table III). The HPLC data indicated that introduction of an amino group into any carbon of aniline resulted in a marked increase in the rate of its enzymatic *N*-acetylation, 1.7—1.9 times as high as that of aniline (Table VI). The apparent rate for the enzymatic *N*-monoacetylation of *o*-phenylenediamine exceeded those for *m*- and *p*-phenylenediamines so far as compared with the *N*-monoacetates formed. However, 14% of *m*- and 16% of *p*-phenylenediamine *N*-monoacetates formed metabolically were further acetylated to yield the corresponding *N,N'*-diacetates in the incubation mixture, while the *o*-diamine yielded no detectable amount of the *N,N'*-diacetate. The data, therefore, indicated that enzymatic *N*-monoacetylation took place with the regioisomers in the order of *o*-$\simeq$ *m*-$\geqq$ *p*-phenylenediamines.

Introduction of an acetylamino group into the *ortho* position of aniline markedly decreased its enzymatic *N*-acetylation rate, while the *m*- and *p*-acetylamino groups slightly decreased and increased the enzymatic reaction rate, respectively (Table VI).

o-Phenylenediamine *N*-monoacetate was completely stable and underwent no further metabolism during incubation for 60 min.

TABLE V. *N*-Acetylation of Aniline and Nitroanilines by Rat Liver Cytosol

Substrate[a]	*N*-Acetate formed (nmol/mg protein/30 min)	Relative acetylation
Aniline	38.6	1.00
o-Nitroaniline	0.0	0.00
m-Nitroaniline	19.5	0.51
p-Nitroaniline	4.8	0.12

a) Incubations were carried out under the same conditions as described in Table I.

Discussion

The present investigation of the enzymatic *N*-acetylation of nitro-*p*-phenylenediamine indicated that the diamine was regiospecifically acetylated at the N^4-position in male rat liver cytosol fortified with acetyl-CoA (Chart 1) as had been demonstrated with its metabolism *in vivo* in our previous paper.[7]

From the comparative study carried out by using regioisomers of nitroanilines as model substrates, it was found that the nitro group had a strong blocking effect on the enzymatic N^1-acetylation of nitro-*p*-phenylenediamine, possibly due to its steric and electronic effects. Actually, *o*-nitroaniline was not *N*-acetylated at all, while both *m*- and *p*-nitroanilines were *N*-acetylated (*m*>*p*) to lesser extents than aniline under the same incubation conditions. On the contrary, a primary amino group introduced to the benzene ring of aniline had a markedly accelerating effect on its enzymatic *N*-acetylation. These facts suggest that an electronic effect of the substituent also plays an important role in determining the rate of enzymatic *N*-acetylation.

Rat liver cytosol has been demonstrated by Bray *et al.*[13] to contain deacetylase activity for aromatic amine

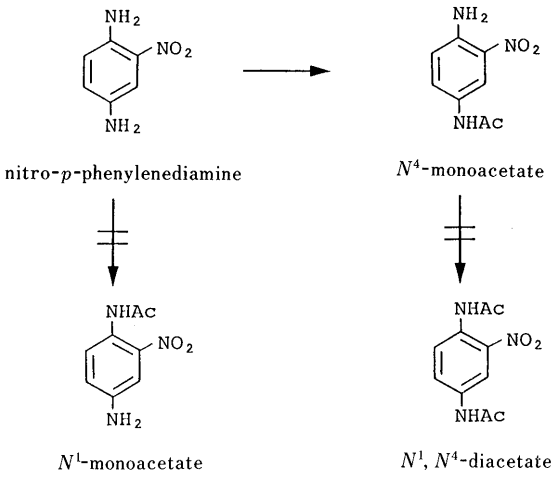

Chart 1. Regioselective *N*-Acetylation of Nitro-*p*-phenylenediamine by Rat Liver Cytosol Fortified with Acetyl-CoA

TABLE VI. *N*-Acetylation of Aniline, Phenylenediamines and Their *N*-Monoacetates by Rat Liver Cytosol

Substrate[a]	*N*-Acetates formed (nmol/mg protein/30 min)		Total acetylation[b]
	N-Monoacetate	*N,N'*-Diacetate	
Aniline	39.4		39.4 (1.00)
o-Phenylenediamine	74.1	0.0	74.1 (1.88)
m-Phenylenediamine	64.1	10.4	74.5 (1.89)
p-Phenylenediamine	56.9	10.9	67.8 (1.72)
o-Phenylenediamine *N*-monoacetate		2.3	2.3 (0.06)
m-Phenylenediamine *N*-monoacetate		32.1	32.1 (0.81)
p-Phenylenediamine *N*-monoacetate		44.0	44.0 (1.12)

a) Incubations were carried out under the same conditions as described in Table I. *b*) Numerals in parentheses represent relative acetylation based on the summed values of *N*-mono- and *N,N'*-di-acetates.

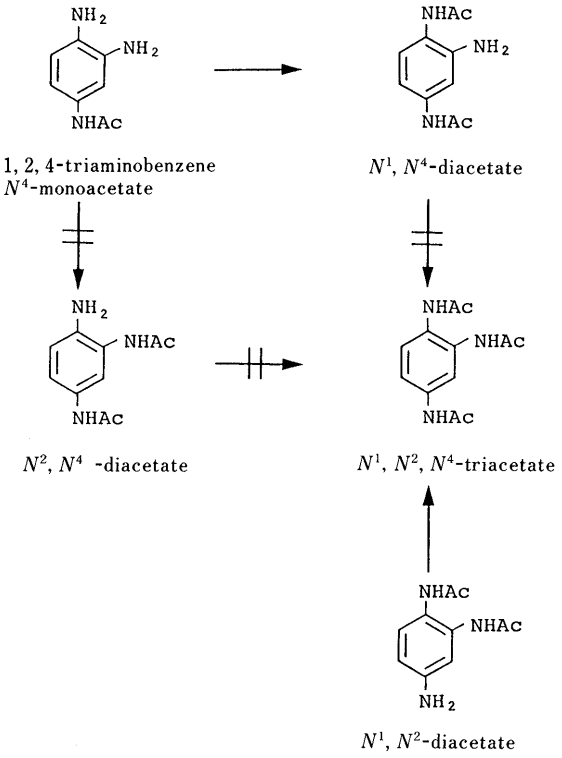

Chart 2. Regioselective *N*-Acetylation of 1,2,4-Triaminobenzene N^4-Acetate by Rat Liver Cytosol Fortified with Acetyl-CoA

N-acetates. They showed *o*-, *m*-, and *p*-nitroaniline *N*-acetates to be deacetylated in the order of $o \gg m > p$ to the corresponding amines by the cytosol. This cytosolic deacetylase activity was strongly inhibited by 0.1 mM paraoxon, *e.g.* *N*-deacetylation of nitro-*p*-phenylenediamine N^1-acetate, which occurred in the absence and even in the presence of acetyl-CoA almost at the same rate, and was inhibited by 92%.[14] In the presence of 0.1 mM paraoxon, no detectable amount of N^1-acetates were formed from nitro-*p*-phenylenediamine, its N^4-acetate and *o*-nitroaniline (data not shown). Therefore, this fact strongly suggests that the nitro group completely inhibits the enzymatic *N*-acetylation of the amino group at the *ortho* position.

1,2,4-Triaminobenzene N^4-monoacetate was regiospecifically acetylated to the N^1,N^4-diacetate without formation of the N^2,N^4-diacetate and the N^1,N^2,N^4-triacetate in the rat liver cytosol fortified with acetyl-CoA (Chart 2) as had been demonstrated *in vivo*.[7] It is evident from the results of the comparative study carried out by using *o*-, *m*-, and *p*-phenylenediamines, their *N*-monoacetates, and 1,2,4-triaminobenzene *N*-mono- and *N,N'*-di-acetates that the primary aromatic amino group substituted at the *ortho* position to the acetylamino group is sterically hindered and hardly acetylated by rat liver cytosolic *N*-acetyltransferases (Tables IV and VI).

The same regiospecificity in further *N*-acetylation has also been demonstrated in our previous *in vivo* study of triacetate formation from the three isomeric triaminobenzene *N,N'*-diacetates in the rat.[7] It is of interest that enzymatic *N*-acetylation of the triaminobenzene N^4-monoacetate occurs specifically at the N^1-position *in vivo* as well as *in vitro*. This would be due to a fixed orientation of the N^4-monoacetate as a substrate on interacting with the substrate-binding site of *N*-acetyltransferase(s) and reacting with the reactive acetyl donor group at its (their) active site, *i.e.* in this strictly oriented situation of the substrate in the enzyme–substrate complex, only the N^1-amino group of the two free vicinal N^1,N^2-amino groups may be interactive with the reactive acetyl group.

Studies to determine the susceptibility of aniline derivatives to *N*-acetylation by *N*-acetyltransferases isolated from pigeon[15] and rabbit[16] livers have also been conducted by Andres *et al.* They found that the rates of the enzymatic *N*-acetylation of 4-substituted aniline derivatives depend on their pK_a values, *i.e.* the substrates with higher pK_a were more susceptible to enzymatic *N*-acetylation. In addition, they demonstrated that mono-substituted anilines with pK_a values higher than 1.0 were *N*-acetylated and also that the aniline derivatives with pK_a lower than 1.0 did not undergo the enzymatic *N*-acetylation although a few exceptions were included. A typical exception in the relationship between the enzymatic *N*-acetylation and pK_a of mono-substituted anilines is found in the 2-substituted anilines, 2-methyl- and 2-ethyl-anilines, both pK_a 4.4, the former of which was a better substrate than aniline and the latter of which did not undergo the enzymatic *N*-acetylation. Moreover, 2-aminobenzoic acid (pK_a 2.2) and 2-aminosulfonic acid (pK_a 2.5) were not *N*-acetylated by their rabbit enzyme. As to these 2-substituted anilines, steric hindrance effects of the substituents would play a more important role in preventing the substrates from the enzymatic *N*-acetylation. Their data and the results of the present study indicate the primary amino group, substituted *ortho* to a nitro or acetylamino group, to resist the enzymatic *N*-acetylation.

Andres *et al.*[15] also maintain that, following enzymatic *N*-acetylation of one of the two primary amino groups of *o*-phenylenediamine, the bicyclic compound 2-methylbenzimidazole is produced from resulting *o*-phenylenediamine *N*-monoacetate (pK_a unknown) through non-enzymatic intramolecular condensation. Therefore, the fact that *o*-phenylenediamine *N*-monoacetate is a very poor substrate for the *N*-acetyltransferases was attributed by them to this facile non-enzymatic conversion of the enzymatically formed *N*-monoacetate to 2-methylbenzimidazole. However, in the present study carried out at the same pH for the incubation medium as used in their study, *o*-phenylenediamine *N*-monoacetate was proved to be stable enough to remain completely unchanged in the incubation mixture even on prolonged incubation. This fact obviously indicates that in *o*-phenylenediamine the first introduced *N*-acetyl group influences a strongly steric hindrance effect on the second enzymatic *N*-acetylation.

Similar steric hindrance effects on the enzymatic *N*-acetylation have been demonstrated with such a smaller size of the substituent as a methyl group with three isomeric toluidines in rabbit liver cytosol[16] and 2,4-toluenediamine *in vivo*[17] and *in vitro*[18] and also as a methoxy group with 2,4-diaminoanisole *in vivo*.[19]

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