Biochemical Pharmacology, Vol. 25, pp. 95–97. Pergamon Press, 1976. Printed in Great Britain.

N-acetylation as a route of 2,4-toluenediamine metabolism by hamster liver cytosol

(*Received* 21 *February* 1975; *accepted* 9 *May* 1975)

Many animal species metabolize various drugs and aromatic amines via acetylation [1]. We found that 2,4-toluenediamine, an industrial intermediate which is carcinogenic in rats [2], was selectively *N*-acetylated at the *p*-amino group by various species *in vitro*.* *N*-acetylation of the *o*-amino group was not detected. However, administration of 2,4-toluenediamine to rats resulted in the urinary excretion of various acetylated metabolites [3]. On the basis of these results, it is suggested that the formation of 2,4-diacetylaminotoluene was achieved largely via the intermediate 4-acetylamino-2-aminotoluene. This communication reports the results of studies on the metabolic formation of 2,4-diacetylaminotoluene from 2,4-toluenediamine *in vitro* in the presence of unlabeled acetyl-CoA, acetyl-1-[^{14}C]CoA and hamster liver cytosol [4, 5].

2,4-Toluenediamine was purchased (Aldrich Chemical Co., Milwaukee, Wis.) and purified before use.† 4-Acetylamino-2-aminotoluene, 4-diacetylamino-2-aminotoluene, 2-acetylamino-4-aminotoluene, 2,4-diacetylaminotoluene, 2-amino-4-nitrotoluene and 2-acetylamino-4-nitrotoluene were prepared in this laboratory [6]. Unlabeled acetyl-CoA as a lithium salt (P-L Biochemicals, Inc., Milwaukee, Wis.) and acetyl-1-[^{14}C]CoA, sp. act. 54·7 mCi/m-mole (New England Nuclear, Boston, Mass.) were purchased.

Liver cytosol was prepared from the livers of Syrian golden male hamsters (140–150 g) according to the method of Lower and Bryan [5]. The incubation mixture contained 4·0 μmoles substrate (2,4-toluenediamine, 2-amino-4-nitrotoluene, 4-acetylamino-2-aminotoluene or 2-acetylamino-4-aminotoluene) dissolved in 0·05 ml propylene glycol or 0·1 ml water, 1·0 μmoles acetyl = CoA containing acetyl-[1-^{14}C]CoA (0·25 μCi), 100 μmoles Tris–HCl buffer, pH 7·5 at 37°, and liver cytosol equivalent to 50 mg wet weight of liver.‡ After incubation of the mixture at 37° for 0–30 min, the reaction was stopped by the addition of acetone, and the mixture was extracted with ethyl acetate. The products were separated by thin-layer chromatography and their identities were confirmed by gas chromatography (OV-17, 3% column in Hewlett-Packard type 3) and mass spectrometry (JMS-01SG-2). The products were then quantitatively determined by counting the areas containing the resulting ^{14}C-labeled acetylaminotoluenes.

Since many of the substrates used were only partially soluble in water, propylene glycol was selected as a solvent. However, *N*-acetylation activity from hamster liver cytosol was similar in both water and propylene glycol (Fig. 1) and led to various acetylaminotoluenes. 2,4-Toluenediamine gave several products, mostly 4-acetylamino-2-aminotoluene and traces of 2,4-diacetylaminotoluene and 2-acetylamino-4-aminotoluene. Since only a trace of 2-acetylamino-4-aminotoluene was detected *in vitro* using 2,4-toluenediamine as a substrate§ and only a small amount *in vivo* [3], 2-amino-4-nitrotoluene was selected as a substrate. 2-Acetylamino-4-nitrotoluene was the chief product (Table 1). 4-Acetylamino-2-aminotoluene gave several products, the major one being 2,4-diacetylaminotoluene. The unknown present in largest quantity had an R_f value and retention time like 4-acetylamino-2-aminotoluene but a mass spectrum (parent peak) corresponding to a diacetylamino-aminotoluene. However, it was not 4-diacetylamino-2-aminotoluene (R_f 0·8 and retention time 7·22 min). The mass spectra, the gas chromatographic retention times and the R_f values on thin-layer chromatograms of each reaction product were identical to those of authentic compounds. In addition, it was found that all detectable radioactivity from the products corresponded exactly with the positions on the chromatograms of the authentic acetylaminotoluenes (Table 1).

Hamster liver cytosol *N*-acetylated the *p*-amino group of 2,4-toluenediamine to a much greater extent than the *o*-amino group, yielding the major product 4-acetylamino-2-aminotoluene and a trace of 2,4-diacetylaminotoluene. In addition, 2-acetylamino-4-aminotoluene was also *N*-acetylated appreciably during a long incubation period, but in 5 min hamster liver cytosol had less capacity to *N*-acetylate this compound. Therefore, only 40 per cent of the theoretical amount of 2,4-diacetylaminotoluene was formed. In contrast, hamster liver cytosol has an even lower ability to *N*-acetylate the *o*-amino group of 2,4-toluenediamine, 4-acetylamino-2-aminotoluene and 2-

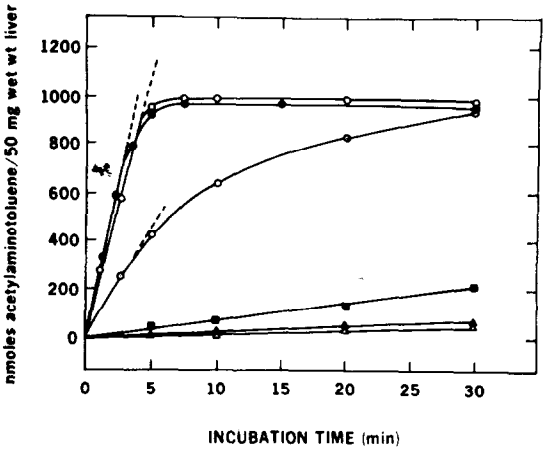

Fig. 1. *N*-acetylation of various substrates as function of incubation times. Key: (○——○) 2,4-toluenediamine (in water) to 4-acetylamino-2-aminotoluene; (●——●) 2,4-toluenediamine (in propylene glycol) to 4-acetylamino-2-aminotoluene; (⊙——⊙) 2-acetylamino-4-aminotoluene (in propylene glycol) to 2,4-diacetylaminotoluene; (■——■) 2-amino-4-nitrotoluene (in propylene glycol) to 2-acetylamino-4-aminotoluene; (▲——▲) 4-acetylamino-2-aminotoluene (in propylene glycol) to 2,4-diacetylaminotoluene; and (△——△) 4-acetylamino-2-aminotoluene (in propylene glycol) to 4-diacetylamino-2-aminotoluene.

* T. Glinsukon, T. Benjamin, P. H. Grantham, E. K. Weisburger and P. P. Roller, manuscript submitted for publication.

† Purification was accomplished by column chromatography on Silica gel G (100–200 mesh) with chloroform–methanol. The compound had a minimum purity of 98·5 per cent and melted at 98–99° [6].

‡ Protein analysis by the method of Lowry *et al.* [7] showed a level of 8·9 mg protein/50 mg wet weight of liver.

§ T. Glinsukon, T. Benjamin, P. H. Grantham, E. K. Weisburger and P. P. Roller, manuscript submitted for publication.

Table 1. Enzymatic *N*-acetylation of 2,4-toluenediamine and its acetyl derivatives by hamster liver cytosol

Substrate	Products	R_f value of product*	Per cent of radiolabeled product	Retention time of product on OV-17, GC column† (min)	Mass spectral data‡ (m e)
2,4-Toluenediamine	2,4-Diacetylaminotoluene	0·34 (A)	2·2	11·95	206 (42), 164 (45), 122 (100), 121 (75)
	2-Acetylamino-4-aminotoluene	0·45 (A)	2·0		
	4-Acetylamino-2-aminotoluene	0·56 (A)	82·8	6·15	164 (82), 122 (100), 121 (80)
	Unknown	0·63 (A)	6·0		
4-Acetylamino-2-aminotoluene	2,4-Diacetylaminotoluene	0·34 (A)	51·8	11·90	206 (40), 164 (41), 122 (100), 121 (70)
	Unknown	0·40 (A)	11·0		
	Unknown	0·49 (A)	2·6		
	Unknown	0·55 (A)	28·5	6·20	206 (parent peak)
	Unknown	0·71 (A)	4·3		
2-Amino-4-nitrotoluene	Unknown	0·33 (B)	4·3		
	Unknown	0·39 (B)	3·2		
	2-Acetylamino-4-nitrotoluene	0·49 (B)	69·0		
	Unknown	0·54 (B)	15·0	7·26	194 (24), 152 (84), 106 (100)
2-Acetylamino-4-aminotoluene	2,4-Diacetylaminotoluene	0·34 (A)	75·0	11·86	206 (46), 164 (52), 122 (100), 121 (79)
	Unknown	0·40 (A)	19·0		
	Unknown	0·49 (A)	1·6		

* Thin-layer chromatography on Silica gel PF_{254} (0·1 mm thickness). Solvent systems: A = chloroform–benzene–ethyl acetate–MeOH (70:15:15:3, v/v) developed three times; B = chloroform–MeOH (97:3, v/v) developed once.

† Gas chromatography: OV-17 (3%) on 80/100 Supelcoport (silicone, 50% phenyl) glass column, injection port temp 322°, flame detector temp 340°, oven temp programmed at 200–220° for 4 min (30°/min); 220° hold for 4 min, then cycle to 270° and hold for 8 min with the gas flow rate as follows: hydrogen, 50 ml/min; air 400 ml/min; and helium 50 ml/min.

‡ Mass spectrometer: source temp 60° and an ionizing potential of 70 eV. Figures in parentheses are relative intensities.

amino-4-nitrotoluene (Fig. 1). In the case of 4-acetylamino-2-aminotoluene, the unknown metabolite was produced to the extent of approximately 50 per cent of the 2,4-diacetylaminotoluene at each incubation period.

The results indicate that 2,4-toluenediamine is mostly *N*-acetylated by hamster liver cytosol to 4-acetylamino-2-aminotoluene and to a much lesser extent to 2-acetylamino-4-aminotoluene. Indirect evidence is furnished by production of 2-acetylamino-4-nitrotoluene from 2-amino-4-nitrotoluene. Both 4-acetylamino-2-aminotoluene and 2-acetylamino-4-aminotoluene then became substrates for *N*-acetyltransferases and were further *N*-acetylated to 2,4-diacetylaminotoluene. Since only a small quantity of 2-acetylamino-4-aminotoluene was formed from 2,4-toluenediamine and in turn was readily *N*-acetylated to 2,4-diacetylaminotoluene, therefore only a trace of 2-acetylamino-4-aminotoluene could be detected *in vitro*. However, it is a urinary metabolite of 2,4-toluenediamine in rats, although to a much lesser extent than 4-acetylamino-2-aminotoluene and 2,4-diacetylaminotoluene.

The major unknown product from using 4-acetylamino-2-aminotoluene as a substrate is not 2,4-diacetylaminotoluene or 4-diacetylamino-2-aminotoluene because it differs in the R_f values and gas chromatographic retention times from the authentic substances. It may be ^{14}C-labeled 4-acetylamino-2-aminotoluene which may have occurred through *N*-deacetylation of the substrate, 4-acetylamino-2-aminotoluene, with subsequent *N*-acetylation with the acetyl-^{14}C moiety from acetyl-[1-^{14}C]CoA. It has been shown that acetylaminoaryl compounds are readily deacetylated by liver and other tissue preparations from several mammalian species [8, 9]. However, since the *N*-deacetyltransferases are located in the microsomal fraction [8], this should not be the case for 4-acetylamino-2-aminotoluene which was incubated with liver cytosol. Another possibility is that a diacetylamino-aminotoluene may be a product, since the mass spectrum had a parent peak of mass 206 which points toward such a substance. Previously, it had been reported that 2-diacetylaminofluorene was formed in small amounts from 2-aminofluorene by rat liver slices [10], supporting our finding. However, the identity of this product requires further investigation.

We conclude that 2,4-toluenediamine is metabolized by hamster liver cytosol to three acetylaminotoluene metabolites, mostly 4-acetylamino-2-aminotoluene, of which a small amount is further metabolized to 2,4-diacetylaminotoluene, and 2-acetylamino-4-aminotoluene which is very readily further metabolized to 2,4-diacetylaminotoluene.

Acknowledgements—We are grateful for the mass spectrometry assistance of Dr. Peter P. Roller and the excellent secretarial assistance of Mrs. Frances M. Williams.

Carcinogen Metabolism and Toxicology Branch, National Cancer Institute, Bethesda, MD 20014, U.S.A.

THIRAYUDH GLINSUKON*
TIMOTHY BENJAMIN
PRESTON H. GRANTHAM
NATHAN L. LEWIS
ELIZABETH K. WEISBURGER

REFERENCES

1. R. T. Williams, *Ann. N.Y. Acad. Sci.* **179**, 141 (1971).
2. N. Ito, Y. Hiasa, Y. Konishi and M. Marugami, *Cancer Res.* **29**, 1137 (1969).
3. P. H. Grantham, T. Glinsukon, L. C. Mohan, T. Benjamin, P. P. Roller, F. E. Mitchell and E. K. Weisburger, *Society of Toxicology Abstrs.*, p. 115 (1975).
4. P. D. Lotlikar and L. Luha, *Biochem. J.* **123**, 287 (1971).
5. G. M. Lower, Jr. and G. T. Bryan, *Biochem. Pharmac.* **22**, 1581 (1973).
6. T. Glinsukon, E. K. Weisburger, T. Benjamin and P. P. Roller, *J. chem Eng. Data* **20**, 207 (1975).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
8. C. C. Irving, *Cancer Res.* **26**, 1390 (1966).
9. P. H. Grantham, E. K. Weisburger and J. H. Weisburger, *Biochim. biophys. Acta* **107**, 414 (1965).
10. J. H. Peters and H. R. Gutmann, *J. biol. Chem.* **216**, 713 (1955).

* Visiting Scientist, Carcinogen Metabolism and Toxicology Branch, National Cancer Institute. Current address: Physiology Department, Faculty of Science, Rama 6th Road, Bangkok, Thailand.

Biochemical Pharmacology, Vol. 25, pp. 97–99. Pergamon Press, 1976. Printed in Great Britain.

Inhibition of a high molecular weight cyclic 3′,5′-nucleotide phosphodiesterase isolated from rat liver

(*Received* 23 *December* 1974; *accepted* 16 *May* 1975)

Butcher and Sutherland [1] first described the relative inhibitory potency of three methylxanthines on a partially purified cyclic 3′,5′-nucleotide phosphodiesterase of beef heart. More recently, a number of substituted xanthines were found to inhibit cyclic AMP phosphodiesterase of epididymal fat pad of rat in a manner corresponding closely to their lipolytic activity [12]. The most active substituted xanthine, 1-methyl-3-isobutylxanthine, was 15-fold more inhibitory than theophylline. A similar order of relative inhibitory potency has also been observed for the highly purified enzyme from beef heart [3].

Several of the cyclic 3′,5′-purine nucleotides and their analogs have also been found to inhibit phosphodiesterase from a number of sources [3, 4]. In most instances, the inhibitory activity of these compounds correlates with their ability to serve as alternate substrates for cyclic 3′,5′-nucleotide phosphodiesterase. In the present study, a soluble cyclic 3′,5′-nucleotide phosphodiesterase with a high degree of specificity for cyclic 3′,5′-purine nucleotide monophosphates was isolated from the soluble fraction of rat liver. Since the molecular weight of the liver enzyme was estimated by gel filtration chromatography to be 380,000 daltons, approximately 3-fold greater than the highly purified phosphodiesterase of beef heart [3, 5], it was of interest to determine the response of this isolated enzyme to substituted xanthines and several cyclic 3′,5′-nucleotide monophosphates and their analogs.

Cyclic 3′,5′-nucleotide phosphodiesterase activity was determined using three assay procedures. Assay procedure I consisted of measuring the release of orthophosphate