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# Reevaluation of the products of tryptamine catalyzed by rabbit liver *N*-methyltransferases

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A non-specific *N*-methyltransferase [1] from rabbit liver has been described which, by reason of the wide range of xenobiotics that are acceptable substrates, suggests a possible role for this enzyme in the detoxication of exogenous amines. This catalytic activity had been referred to previously as indolethylamine *N*-methyltransferase and arylamine *N*-methyltransferase [1–6].

Interestingly, one set of data offered for the methylation of one of its substrates, tryptamine [1], implied that methylation occurred at the ring nitrogen rather than at the primary amino group of this compound. The evidence was based mainly on the observation that methylation took place without a concomitant decrease in the free amino group of tryptamine [1] when amines were measured by fluorescence after reaction with fluorescamine [7]. The unusual nature of these findings, and the present availability of two separate enzymes, amine *N*-methyltransferases A and B, of distinct but overlapping specificity [1, 8], prompted our reinvestigation of the methylation of tryptamine by both crude and homogenous rabbit liver *N*-methyltransferases. By means of a new, high-performance liquid-radiochromatographic procedure we have been able to resolve side chain and ring *N*-methylated derivatives of tryptamine. Reexamination was also expected to and did reveal the presence of enzymes responsible for the known formation of α-*N*-methyltryptamine.

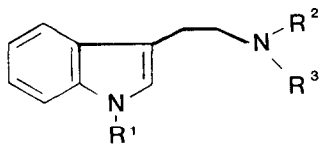
## *Materials and methods*

*Chemicals.* Tryptamine hydrochloride (see Scheme 1) and α-*N*-methyltryptamine (NMT) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). α,α-*N*,*N*-Dimethyltryptamine (DMT) and *S*-adenosyl-L-methionine (AdoMet) were purchased from the Sigma Chemical Co. (St. Louis, MO). $N^1$-Methyltryptamine (RMT) was prepared from α-*N*-methyltryptophan (Aldrich Chemical Co.) using the method of Leete [9]. *S*-Adenosyl-L-[*methyl*-$^3$H]-methionine was obtained from ICN Radiochemicals (Irvine, CA; 50 Ci/mole) or from New England Nuclear (Boston, MA; 75 Ci/mmole).

*Preparation of tissue homogenates.* Male New Zealand rabbits (2.0 to 2.3 kg) were killed by ear vein injection of a mixture of sodium pentobarbitone solution (4 ml; 50 mg/ml) and heparin sodium solution (0.25 ml; 10,000 units/ml). Liver and lungs were removed; lungs were inflated with room air and immediately perfused with 200 ml of saline. Perfused lungs and liver were each homogenized in three parts of 1.15% (w/v) KCl solution, and the resulting homogenate was centrifuged at 25,000 *g* for 45 min. The supernatant liquid was dialyzed against three changes, 3 liters each, of 1 mM sodium phosphate at pH 7.9 over a total of 36 hr. The final preparation was stored at −20°.

Enzyme activity in the dialyzed lung and liver preparation was determined by using AdoMet and L-tryptamine as substrates. A mixture of the dialyzed supernatant fluid (63 μl, 7 mg/ml protein), *S*-adenosyl-L-[*methyl*-$^3$H]-methionine (66 μM, 50 μCi/ml in 1 mM HCl), and 0.2 M sodium phosphate at pH 7.9 (33 μl) were incubated at 37° for 2 min. Reaction was initiated by addition of tryptamine (13 μl of a 1 mg/ml solution in 0.2 M sodium phosphate at pH 6.9) to the mixture. Incubations were carried out at 37° for 60 min and terminated by addition of an equal volume of 0.125 M potassium borate at pH 10. A control incubation was performed in the absence of tryptamine, as well as one in which enzyme heated to 100° for 5 min was substituted for an active preparation. The reaction mixtures were extracted with ethyl acetate (2 × 250 μl), the organic phase was separated and washed with 0.13 M potassium borate at pH 10, and the solvent was evaporated at room temperature under a stream of nitrogen.

Amine *N*-methyltransferases A and B were prepared by




Tryptamine — a) $R^1 = R^2 = R^3 = H$
(Ring) $N^1$-Methyltryptamine (RMT): — b) $R^1 = CH_3$, $R^2 = R^3 = H$
α-*N*-Methyltryptamine (NMT): — c) $R^1 = R^3 = H$, $R^2 = CH_3$
α,α-*N*,*N*-Dimethyltryptamine (DMT): — d) $R^1 = H$, $R^2 = R^3 = CH_3$

Scheme 1. Structure of methylated tryptamines.

a method [8] that represents a variation of a procedure previously described [1]. Partially purified liver transferases (Table 1) refer to enzymes at the stage of hydroxylapatite column chromatography [8]. The incubation mixture for assay, in a total volume of 400 μl, contained 50 mM Tris–HCl at pH 7.8, 34 μM AdoMet with 0.1 μCi [*methyl*-$^3$H]-AdoMet, and either 1 mM tryptamine or 1 mM α,α-*N,N*-dimethyltryptamine. Incubations were carried out at 37° for 30 min and were terminated by addition of 0.5 M potassium borate at pH 10. Control incubations were performed in the absence of substrate, and others in the absence of enzyme. The reaction mixture was extracted with ethyl acetate (2 ml), and the ethyl acetate layer was separated and evaporated under a stream of nitrogen.

The residues resulting from the evaporation of the ethyl acetate extracts from both homogenate and purified enzymes were taken up in HPLC buffer (100 μl) containing standard markers of NMT, DMT and RMT (1 mg/ml of each), and an aliquot (30 μl) of this resulting solution was subjected to radiochromatography.

*High-performance liquid radiochromatography.* High-performance liquid-chromatographic analyses were carried out on an Altex programmable HPLC system that included two model 111A pumps, a model 420 solvent programmer and a model 15 UV detector operating at 254 nm. Separations were carried out on a Partisil-10 ODS-2, 10 μm particle, reversed-phase column (Whatman, 25 cm × 4.6 mm i.d.) to which was attached a pellicular guard column (Whatman, 17 × 0.4 cm). Samples were introduced with a Rheodyne loop injector. Essential chromatographic operating parameters are shown in the legend to Fig. 1. Radioactivity in column effluents was determined using a model HS Flo-1 radioactive flow-through detector (Radiomatic, Tampa, FL) equipped with a Radiomatic model ES Stream Splitter. Flo-Scint III (Radiomatic, Tampa) was used as scintillation medium in a mixing ratio of 4 parts to 1 by volume of a 50% split of the effluent stream. The output from the ultraviolet and radioactive detectors were recorded simultaneously on an Omniscribe model 5000 dual channel recorder (Houston Instruments, Austin, TX).

Recovery of radioisotope from analytical columns was determined by injecting an identical volume of radioisotopic material directly into the radioactive flow-through unit, thereby bypassing the analytical column. In general, recoveries were in the range of 97–98% of the radioactivity applied.

*Results*

Although several methods for the analysis of indoleamine metabolites by HPLC have been proposed [10–12], these systems proved unsatisfactory for us in the resolution of the three possible methylated tryptamines: RMT, NMT and DMT. RMT and DMT exhibited similar retention characteristics with a variety of mobile phases on Partisil-10 SCX cation-exchange chromatography, and a Partisil-10 ODS-1 column could not completely resolve RMT and

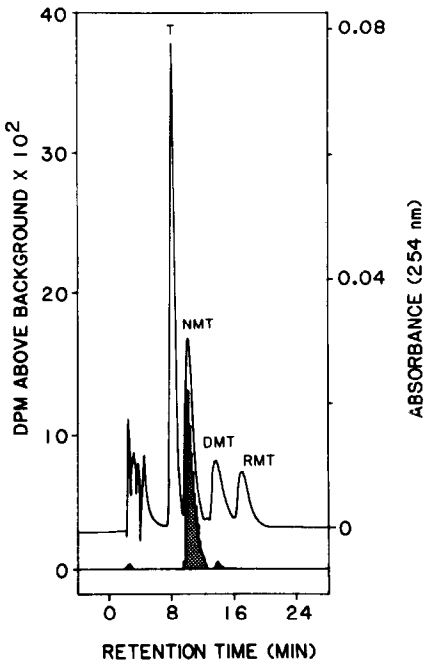



Fig. 1. HPLC analysis of ethyl acetate extractable products after incubation of rabbit lung homogenate with tryptamine and [$^3$H]AdoMet. Column: Partisil-10 SCX (25 × 0.46 cm) with 10 μm particle packing. Eluent: 0.1 M ammonium phosphate–acetonitrile (82.5:17.5), adjusted to pH 2.5 with phosphoric acid. Flow rate: 1.5 ml/min. Shaded areas denote radioactivity in eluate after incubation with enzyme.

Table 1. *N*-Methylated products from the incubation of tryptamine with rabbit liver and lung homogenate and purified rabbit liver amine *N*-methyltransferases

| Preparation | Percent of total extractable $^3$H | | | |
|---|---|---|---|---|
| | RMT | NMT | DMT | Other |
| Dialyzed lung homogenate | 0 | 93 ± 3* | 0.7 ± 0.3* | |
| Dialyzed liver homogenate | 0 | 46 ± 5* | 0.2 ± 0.7* | 40 ± 5*† |
| Partially purified transferases (borate treated‡) | 0 | 99.6 | 0.3 | |
| Partially purified transferases (heated‡) | 0 | 78 | 4 | 10§ |
| Control (no enzyme; borate treated‡) | 0 | 0 | 0 | |
| Control (no enzyme; heated‡) | 0 | 0 | 0 | |
| Amine *N*-methyltransferase A | 0 | 100 | 0 | |
| Amine *N*-methyltransferase B | 0 | 100 | 0 | |

* Mean ± S.E.M., N = 3.

† Percent of tritium eluting in the void volume of the column effluent.

‡ Borate treatment or heating (2 min in a boiling water bath) were the methods used to terminate the reaction.

§ Unidentified radioactive band eluting slightly earlier than the RMT standard.

DMT peaks even after extensive modification of the mobile phase. A satisfactory separation of NMT, DMT and RMT could be obtained by utilizing a Partisil-10 ODS-2 column and a mobile phase consisting of 0.1 M ammonium phosphate–acetonitrile (82.5:17.5), adjusted to pH 2.5 with phosphoric acid. Under isocratic conditions, base-line separation of all three derivatives was achieved (Fig. 1).

The results of parallel incubations utilizing crude rabbit lung and liver homogenates with tryptamine and [$^3$H]AdoMet as substrates are shown in Fig. 1 and Table 1. In both cases, the formation of low levels of NMT was observed as has been reported previously [5], but measurable amounts of DMT were also detected (*ca.* 0.5%) with homogenates of lung. In none of the above incubations was any RMT formation observed.

The purified liver *N*-methyltransferase B, described as catalyzing the transfer of a methyl group from AdoMet to the indolic nitrogen of tryptamine [1], is shown in Table 1 to yield data entirely similar to those obtained with crude preparations; only NMT was observed as the enzymic product. In addition, no ethyl acetate extractable methylated product was observed when DMT was used as substrate. With a related enzyme, homogenous amine *N*-methyltransferase A from rabbit liver [8], the results were the same (Table 1). Clearly, the enzyme-catalyzed methylation of the ring nitrogen has not been demonstrated, and the results of Lyon and Jakoby [1] require explanation.

The data supporting $N^1$-methyltryptamine as the reaction product was based on the measurement of a fluorescent product by the interaction of primary amine, i.e. tryptamine, with fluorescamine. If tryptamine were methylated at the primary amino nitrogen to form $\alpha$-*N*-methyltryptamine, a decrease in concentration of primary amine, and hence in fluorescence, should be observed. If tryptamine were methylated at the ring nitrogen, the concentration of primary amine would remain constant, as would the extent of fluorescence. Experimentally, fluorescence remained constant throughout the enzyme-catalyzed reaction, leading to the conclusion that the secondary amine, i.e. the ring nitrogen, was being methylated [1].

Although $\alpha$-*N*-methyltryptamine is neither intrinsically fluorescent, nor will it react with fluorescamine to form a fluorescent compound, low concentrations of $\alpha$-*N*-methyltryptamine added to tryptamine result in an *increase* in fluorescence of the mixture. This is shown in Fig. 2 wherein the conditions of the enzyme-catalyzed reaction are reproduced. The increased fluorescence upon addition of $\alpha$-*N*-methyltryptamine would directly compensate for the decrease in free primary amine and would thereby lead to the incorrect conclusion that the indole nitrogen of tryptamine was methylated.

The method chosen for terminating the reaction prior to analysis deserves a word of caution. With purified enzyme and tryptamine as acceptor substrate, termination by heating, i.e. placement in a boiling water bath for 2 min, resulted in the formation of a chemical degradation product that had retention characteristics similar to RMT in our analytical system. This product was not observed in extracts from controls that were free of enzyme. Small amounts of the degradation product were also detected after boiling when DMT was the substrate, but were not observed with either tryptamine or DMT when potassium borate at pH 10 was used to halt the reaction.

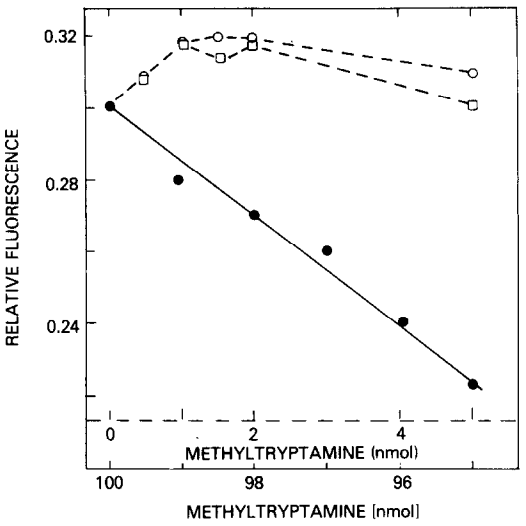



Fig. 2. Change in fluorescence of 100 mM tryptamine solutions upon reaction with fluorescamine in the presence (dashed lines) of increasing concentrations of $\alpha$-*N*-methyltryptamine (○) or $N^1$-methyltryptamine (□); and fluorescence after reaction with fluorescamine of decreasing concentrations of tryptamine (solid line).

## *Discussion*

The enzymatic transfer of a methyl group from AdoMet to the ring nitrogen of tryptamine is difficult to understand mechanistically since the indolic nitrogen atom is known to have very weak basicity (p$K_a$ of −6.5 compared to a p$K_a$ of 8.0 for the side chain nitrogen [13]), by reason of the lone pair of electrons being incorporated into the 10 electron $\pi$-orbital of the hetero-aromatic system. It appears unlikely, therefore, that the latter nitrogen is sufficiently nucleophilic to capture the methyl group of AdoMet by the usual direct $SN_2$ displacement reaction [14].

These factors prompted the present reexamination of the findings of Lyon and Jakoby [1], in establishing transfer of a methyl group from AdoMet to the indolic nitrogen of tryptamine by the rabbit liver enzyme. The results of our studies clearly demonstrate that such methyl group transfer does not occur.

Reevaluation of the interpretation that led to the suggestion of methylation at the ring nitrogen disclosed that, although the concentration of primary amine does decrease as measured fluorometrically, an increase in fluorescence due to enzyme-catalyzed formation of $\alpha$-*N*-methyltryptamine compensates, and leads to an erroneous conclusion. From radiochromatographic studies, the only methylated products of tryptamine observed in incubations with both crude lung and liver homogenates, and with two homogenous non-specific amine *N*-methyltransferases, were $\alpha$-*N*-methyltryptamine and, to a lesser extent, $\alpha$,$\alpha$-*N*,*N*-dimethyltryptamine.

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# The effect of organophosphorous insecticide Wofatox 50 EC on the adenylate cyclase activity of chicken embryo muscle

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The organophosphorous insecticides like parathion or W 50 (methylparathion)* are widely used for plant protection. However, above a certain level, these drugs also poison the warm blooded animals posing thereby potential danger to most wild animals and man [1, 2]. One of the main toxicological effects is the damage of muscles [3]. We also found the cervical muscles [4], including the musculus complexus major (unpublished), from chicken atrophized after treatment of eggs with W 50.

Although the organophosphates are well known inhibitors of various esterases, including most importantly the acetylcholinesterase, in many cases their toxicological effects (particularly if they are present at relatively low concentrations) do not appear to correlate with the inhibition of the cholinergic system [5–7].

The organophosphorous insecticides accumulate in membranes especially in muscle membranes [8]. Due to such asymetric distribution the membrane bound enzymes are the most likely targets for their effects. In the present work we tested the effect of W 50 on the activity of adenylate cyclase (AC), an important plasma membrane bound enzyme, in the chicken embryo musculus complexus major which is a main target of organophosphates.

($\alpha$-$^{32}$P)ATP (40–80 TBq/mmol) was obtained from Amersham International. ATP, cyclic AMP, GTP, GPP(NH)P, crystalline glucagon, creatine phosphate and creatine kinase were purchased from Sigma (St. Louis, MO). Neutral aluminium (90% active; 70–230 mesh) was obtained from Merck, Darmstadt, F.R.G. Wofatox 50 EC (active substance: 50% methylparathion, VEB Chemiechombinat, Bitterfeld, G.D.R.). Chicken eggs of the Shaver Starcross 288 strain were used.

Eggs were incubated in a Ragus automatic incubator and opened two days before hatching. The musculus complexus major was excised from the embryos and homogenized in 10 vol. of ice-cold 20 mM Tris/HCl buffer pH 7.6. For homogenization a motor-driven Waring blender at about 7000 rpm was used. The homogenates were filtered through two layers of cheesecloth and used immediately at 0.2 mg protein per assay for the measurement of AC activity. The latter was determined by measuring the conversion of ($\alpha$-$^{32}$P)ATP (1 mM) to $^{32}$P-cyclic AMP as described previously [9]. Formation of cAMP was linear for at least 15 min. Rat liver plasma membranes were prepared by the method of Neville [10]. Protein was determined according to the method of Lowry *et al.* [11].

AC activity in the muscle homogenate was only slightly stimulated by a relatively high concentration (10 $\mu$M) of GTP (Table 1). The most likely reason for this is that $N_s$, the regulatory protein through which GTP stimulates the enzyme, has GTP-ase activity [12, 13]; the resultant GDP, which binds strongly to $N_s$, blocks the stimulatory cycle [14, 15]. The small effect of GTP in this muscle is comparable to that observed when catalytic subunit preparations and pure $N_s$ were reconstituted in phospholipid vesicles [16]. The non-hydrolysable analog of GTP, GPP(NH)P, has stronger stimulatory effect both in our case (Table 1) and in the reconstituted system [16]. The $\beta$ receptor-specific agonist, isoproterenol, is known to increase the affinity of $N_s$ for GTP thereby inducing and sustaining new cycles of stimulation [14–16]. Isoproterenol, according to this mechanism of action, is not supposed to have any effect in the absence of GTP which agrees with our observation (Table 1). In our system, however, isoproterenol also failed to stimulate the AC activity in the presence of GTP indicating that the $\beta$ receptors were not coupled to $N_s$. Instead we observed the inhibition of GTP effect by isoproterenol (Table 1). No such inhibition of GTP effect occurred in the presence of 10 $\mu$M propranolol, a $\beta$ receptor antagonist (not shown).

It is known that the $\beta$ receptor interacts with $N_i$ [16, 17], the inhibitory guanine nucleotide binding protein, through which GTP inhibits AC activity [18]. As a result isoproterenol occasionally inhibits AC activity (for example in fat cells [19]) presumably due to the dominance of $N_i$ structure. A similar mechanism could also occur in the chicken embryo muscle.

Low concentrations (1–10 $\mu$M) of W 50 did not significantly affect the cyclase activity in the absence of effectors or in the presence of isoproterenol alone (Table 1).

* Abbreviations used: AC, adenylate cyclase; W 50, Wofatox 50 EC; $N_s$, the stimulatory guanine nucleotide binding protein; $N_i$, the inhibitory guanine nucleotide binding protein.

Enzymes: Adenylate cyclase or ATP:pyrophosphate lyase (cyclizing) (EC 4.6.1.1); creatinc kinase or ATP:creatine phosphotransferase (EC 2.7.3.2.).