Journal of Chromatography, 86 (1973) 123–131

CHROM. 6904

SELECTIVE AND SPECIFIC THIN-LAYER CHROMATOGRAPHIC, GAS CHROMATOGRAPHIC AND GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC METHODS FOR THE ASSAY OF INDOLETHYLAMINE N-METHYLTRANSFERASE ACTIVITY

N. NARASIMHACHARI, R.-L. LIN, J. PLAUT and K. LEINER

Thudichum Psychiatric Research Laboratory, Galesburg State Research Hospital, Galesburg, Ill. 61401 (U.S.A.)

(Received May 1st, 1973)

SUMMARY

A simple thin-layer chromatographic (TLC) method for the quantitation of indolethylamine N-methyltransferase activity, using the enzyme preparation from rabbit lung, is described. The products of such N-methylation, N-methylserotonin (NMS) and bufotenin, were identified by gas chromatography–mass spectrometry (GC–MS). It was found that comparable results are obtained with gas chromatography (GC) and TLC. The time, substrate concentration and enzyme concentration curves were obtained by the TLC method.

N-Methyltryptamine (NMT) was found to be a better substrate than NMS for this enzyme, the K_m values being lower. The product of N-methylation, N,N-dimethyltryptamine (DMT), was identified by the GC–MS method and then quantified by GC. GC separations, GC–MS data and the enzyme kinetics data with NMT as the substrate are presented. When tryptamine was used as a substrate, NMT was the major product and a small yield of DMT (10% NMT) was formed.

INTRODUCTION

The occurrence of N-methyltransferase in human brain[1–3], lung[4] and serum[5], and in the brain of different species of animals[1,3] is significant in view of the psychotomimetic properties of the three dimethylated tryptamines, N,N-dimethyltryptamine (DMT), 5-methoxy-N,N-dimethyltryptamine and N,N-dimethylserotonin (bufotenin) and their presence in blood samples of schizophrenic patients[6]. We have recently reported a thin-layer chromatographic (TLC) method for the identification and quantitative determination of all 5-hydroxytryptamine metabolites using a sensitive *o*-phthalaldehyde (OPT) spray[7,8]. This OPT spray method and a modified OPT method provide a sensitivity for identification at the 3-ng level and for quantitation at the 10-ng level. We have used this method in our screening procedure for indolethylamine N-methyltransferase (INMT) in various tissues from different species of animals, and in human serum.

In our efforts to provide a simple and specific procedure for the identification

and quantitation of this enzyme we have investigated the applicability of TLC, gas chromatographic (GC) and gas chromatographic–mass spectrometric (GC–MS) methods and have tested their validity using the preparation from rabbit lung. The methods and the results are described in the chronological order of their development.

EXPERIMENTAL AND RESULTS

Enzyme preparation

N-Methyltransferase from rabbit lung was partially purified according to a modification of the method of Axelrod[9]. 50 g of rabbit lung and 200 ml of 0.1 *M* potassium phosphate buffer, pH 7.4, were blended in a Warburg blender at full speed for 1 min. The homogenate was filtered through cheese cloth to remove the connective tissue and then centrifuged at 60,000 × *g* for 30 min. 24 g of solid ammonium sulfate were added to 100 ml of the soluble supernatant fraction to bring to 40% saturation, and the solution was centrifuged. After the addition of 14 g of solid ammonium sulfate to the supernatant fraction to bring to 60% saturation, the solution was again centrifuged. The precipitate was dissolved in 10 ml of the same buffer and used as the enzyme preparation. One hundred μg of N-methylserotonin (NMS), 100 μg of S-adenosylmethionine and 0.1 ml of the enzyme preparation (1.5 mg protein) in a total volume of 1.0 ml of 0.1 *M* potassium phosphate buffer, pH 7.4, were incubated at 37° for 90 min. At the end of the incubation, the reaction products were extracted by the method described in a previous report[5].

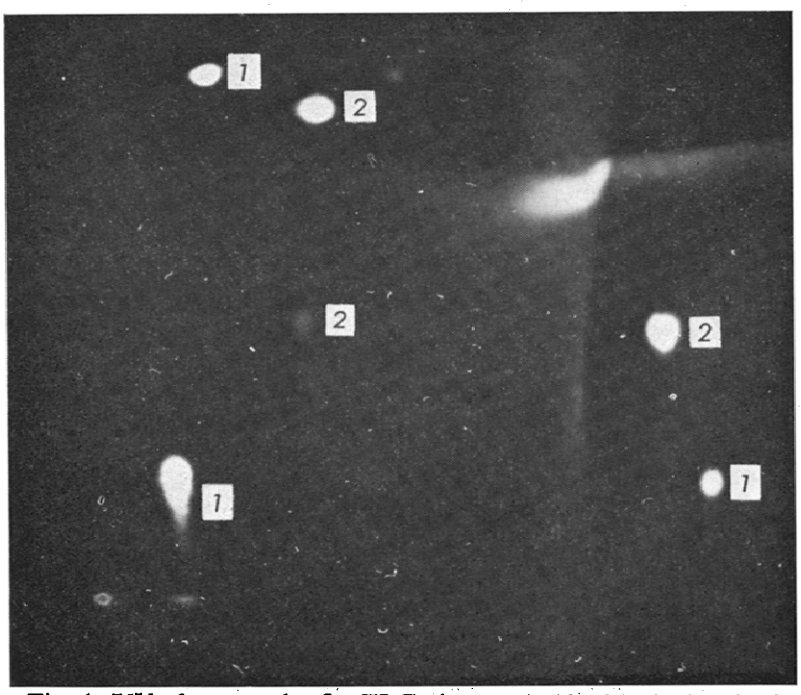

Fig. 1. UV photograph of a TLC plate spotted with the incubation mixture and showing (1) the substrate, NMS, and (2) the product, bufotenin, after OPT spray. The spots on the upper and right hand margins are the reference standards.

Thin-layer chromatography

The dry residue was redissolved in 100 μl of ethyl acetate and a 10-μl aliquot was spotted on a silica gel G TLC plate. After two-dimensional development and OPT spray[7] a distinct fluorescent spot isographic with standard bufotenin was seen (Fig. 1), which on elution with 6 *N* HCl gave the fluorescence maximum at 490 nm (excitation 360 nm).

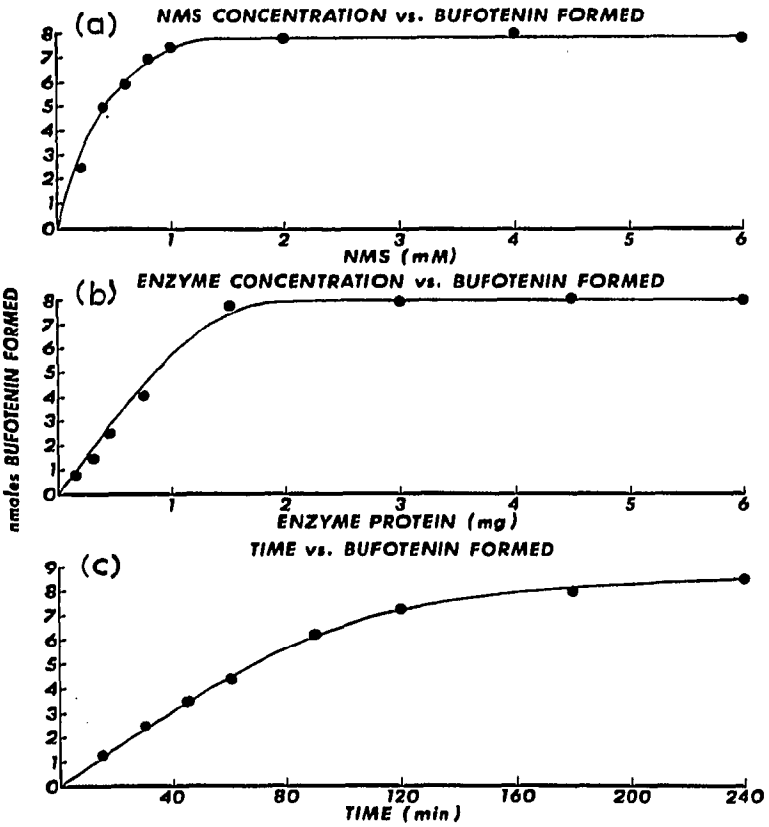

Fig. 2. Graphs showing the substrate (a) and enzyme concentration (b) curves and time curve (c) with NMS as substrate.

A quantitative relationship between the amount of enzyme preparation used and product formation was also established by this method (Fig. 2). An aliquot (20–50 μl) of the incubation mixture directly spotted on a thin-layer plate for two-dimensional TLC gave the same results as the extraction procedure.

Gas chromatography–mass spectrometry

To provide unequivocal proof for such an N-methylation, the pooled extract from four incubations was evaporated to dryness and the residue redissolved in 20 μl of ethyl acetate. A mixture of 2 μl of this solution and 2 μl of the silylating reagent, bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane, was injected into

a GLC column (1% OV-101 on Gas-Chrom Q; isothermal 170°) interfaced with a Varian CH 7 mass spectrometer. Similarly a solution of NMS in ethyl acetate (2 μg/2 μl) was injected along with 2 μl of silyl reagent. In such a column reaction, the OH group is silylated completely and only the peak corresponding to the O-trimethylsilyl (O-TMS) derivative is seen. The O-TMS derivatives of NMS and bufotenin could not be separated on OV-101 or SE-30 columns. However, when the mass spectra of the pure substrate (NMS) and of the incubation mixture were compared, distinct differences were seen. A small peak at *m/e* 276, and a major peak at *m/e* 58 were present in the mass spectrum of the incubation mixture, but not in the spectrum of NMS. These peaks correspond to the molecular ion and the fragment ion of tertiary amines $CH_2=\overset{+}{N}\begin{smallmatrix}CH_3\\CH_3\end{smallmatrix}$ of bufotenin.

In earlier studies with phenethylamines[10] and amphetamines[11] we had observed

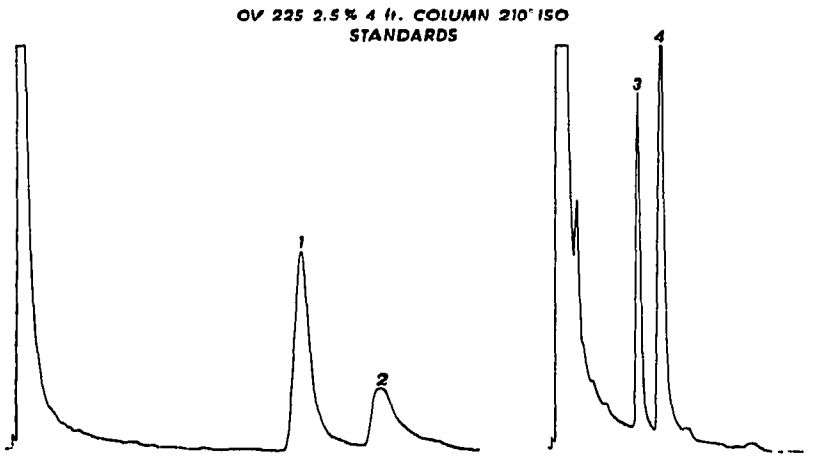

Fig. 3. GC separation of NMS and bufotenin, and of their monoTMS derivatives.

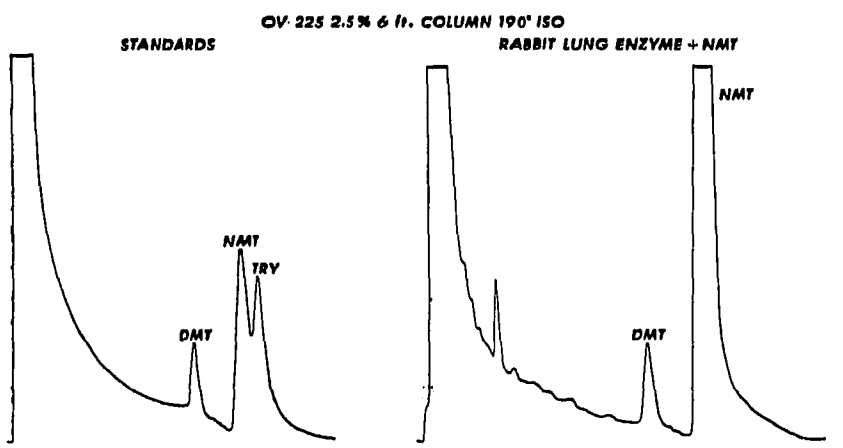

Fig. 4. GC separation of DMT, NMT and tryptamine (TRY).

that OV-225 was an effective column stationary phase for the separation of isomeric compounds. Further the amphetamines had a lower retention time than the corresponding phenethylamines, generally in the ratio of 0.9:1. Therefore we studied the separation of tryptamines, N-monomethylated and N,N-dimethylated compounds using 2.5% OV-225 on Gas-Chrom Q 4-ft. and 6-ft. columns, and 2% OV-225 on a 4-ft. column. A typical separation of NMS and bufotenin and their monoTMS derivatives (210° isothermal) is shown in Fig. 3, and of DMT, N-methyltryptamine (NMT) and tryptamine (190° isothermal) in Fig. 4. The retention data are given in Table I. The identity of bufotenin produced by the reaction of the enzyme prepared from rabbit lung and of NMS was confirmed by GC–MS, and quantitation by GC gave results comparable to those obtained by TLC. The mass spectra of the standard and of the sample of bufotenin were identical. NMS was similarly identified by GC–MS, when serotonin was used as the substrate (Table II).

TABLE I

RETENTION DATA FOR STANDARDS

Compound	*Retention time (min)*
Bufotenin*	11.26
NMS*	12.58
Bufotenin monoTMS*	3.26
NMS monoTMS*	4.04
DMT**	8.20
NMT**	10.40
Tryptamine**	11.34

* Column length, 4 ft.; 2.5% OV-225 on Gas-Chrom Q; 210° isothermal; N_2 flow-rate 40 ml/min.

** Column length, 6 ft.; 2.5% OV-225 on Gas-Chrom Q; 200° isothermal; N_2 flow-rate, 40 ml/min.

TABLE II

RELATIVE ABUNDANCE OF SOME CHARACTERISTIC PEAKS IN THE GC–MS SPECTRA OF BUFOTENIN AND NMS MONOTMS DERIVATIVES OF STANDARDS AND SAMPLES FROM ENZYME INCUBATION

Bufotenin monoTMS			*NMS monoTMS*		
m/e	*Standard*	*Sample*	*m/e*	*Standard*	*Sample*
58	100	100	44	100	100
202	1.4	1.8	203	11.3	10.0
218	5.2	5.8	218	41.0	38.7
261	0.6	1.3	219	75.6	71.7
276	5.6	5.0	247	2.7	1.5
			262	6.0	6.2

N-Methyltryptamine and tryptamine as substrates

In view of the effective separation of DMT, NMT and tryptamine, we investigated the applicability of GC and GC–MS techniques in assaying N-methyltransferase activity with tryptamine and NMT as substrates. On the OV-225 column, the dose response was linear for DMT in a narrow range of concentration, 0.05 to 0.3 μg, with a flame ionization detector (FID). Direct proportionality to peak height can be established when the standards are run at approximately the same level of concentration as the samples.

Procedure. NMT (100 μg), S-adenosylmethionine (100 μg) and enzyme (0.1 ml) were incubated in a final volume of 1 ml of 0.5 *M* phosphate buffer (pH 7.6) for 90 min. The incubation mixture was made alkaline (pH 11.0) by adding 5 drops of 2 *N* NaOH and the mixture was extracted into ethyl acetate (10 ml) by shaking for 15 min on a mechanical shaker. After centrifugation, the ethyl acetate layer was separated, dried over Na_2SO_4 and evaporated to dryness. The residue was redissolved in 100 μl of ethyl acetate and 2 μl of this solution were used for GC analysis. Only a peak with the retention time of NMT (which serves as an internal standard) appeared. After the solution was again evaporated to dryness and redissolved in 20 μl of ethyl acetate, 2 μl were used for GC and for GC–MS. The identity and homogeneity of the DMT peak were established by GC-MS (Fig. 5) and then the GC peak was used for quantitation from a GC dose response curve run daily under the same GC conditions. When an enzyme blank without substrate, and a heated enzyme blank were run, neither showed a GC peak with the retention time of DMT. With this GC method the time curve and the substrate and enzyme concentration curves were obtained for NMT as substrate (Fig. 6). NMT has a lower K_m value and therefore is a more sensitive substrate for this methyltransferase. With NMT as substrate, the enzyme activity is found in liver and serum of rabbit.

When the enzyme preparation from the rabbit lung was incubated with tryp-

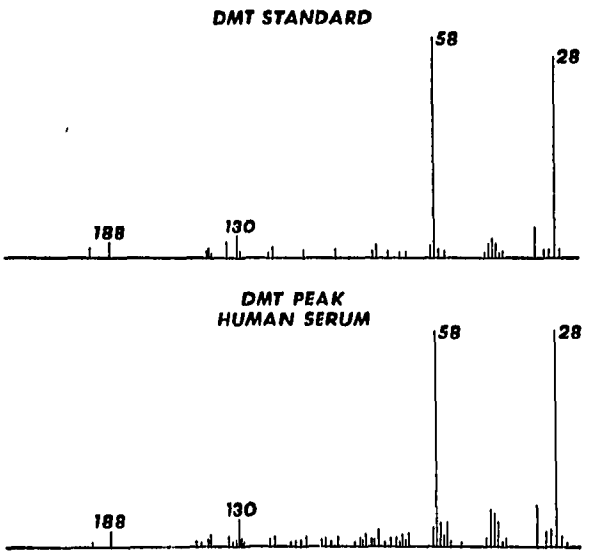

Fig. 5. Mass spectra of standard DMT and DMT peak using the enzyme from human serum.

tamine, NMT was the major product. However, DMT was formed in a small yield, 10% of the value for NMT. Tryptamine was not as good a substrate for this assay, since by GC–MS the sensitivity for the detection of NMT was much lower than that for DMT.

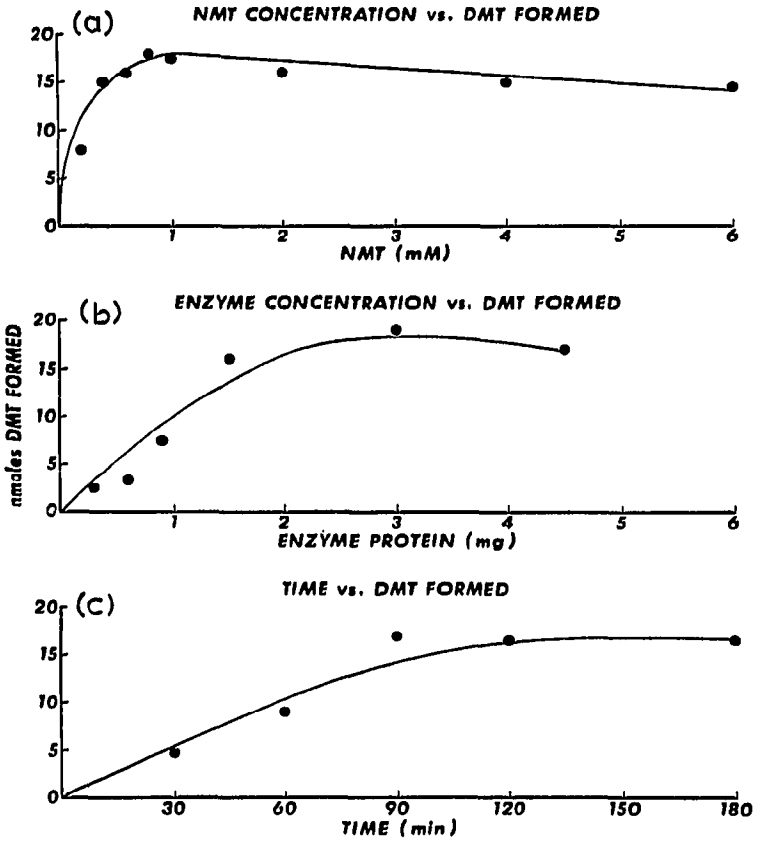

Fig. 6. Graphs showing the substrate (a) and enzyme concentration (b) curves and time curve (c) with NMT as substrate.

DISCUSSION

GC and GC–MS methods have corroborated the applicability of the TLC method for the quantitation of INMT activity. In view of the comparable values and the positive identification of bufotenin by GC–MS, the OPT method should prove valuable in quantitating the enzyme activity with NMS as substrate. However, NMT was found to be a more active substrate than NMS, the K_m value being lower. The use of NMT has an added advantage in that at pH 11 used for extraction, the endogenous phenolic amines are eliminated giving a relatively clear chromatogram. In our experience the FID response on GC for DMT was very satisfactory, levels as low as 0.05 μg giving measurable peak heights. Thus we have not found it necessary to use the silylation and reverse isotope dilution assay tech-

nique[12] for either identification or quantitation. In view of the increased sensitivity provided by NMT as substrate, the unambiguity of the identity of the product and the specificity of the product assay, our technique, unlike the radioactivity count of a total extract, provides both a very selective and sensitive assay method for this enzyme.

We noted a small yield of DMT by GC with rabbit lung enzyme when tryptamine was the substrate, but could not detect bufotenin by TLC when serotonin was used as the substrate. However, when enzyme preparations from toad skin and rat brain were dialyzed and then incubated with serotonin, bufotenin was formed in good yield with the enzyme from toad skin and in low yield with that from rat brain. Bufotenin was formed in higher yields when NMS was used as the substrate for the rat brain enzyme (see ref. 13).

After this method had become available, we examined blood samples from normal and schizophrenic subjects (Fig. 7) and found that, although all of the blood samples tested were positive for this enzyme activity, there were quantitative differences. Samples which were negative for bufotenin with NMS as substrate, were found to be positive for DMT with NMT as substrate.

By using the TLC method with NMS as substrate and the GC with NMT as substrate, we have examined the substrate specificities of the enzyme INMT from different tissues and different species of animals and these results will be reported elsewhere.

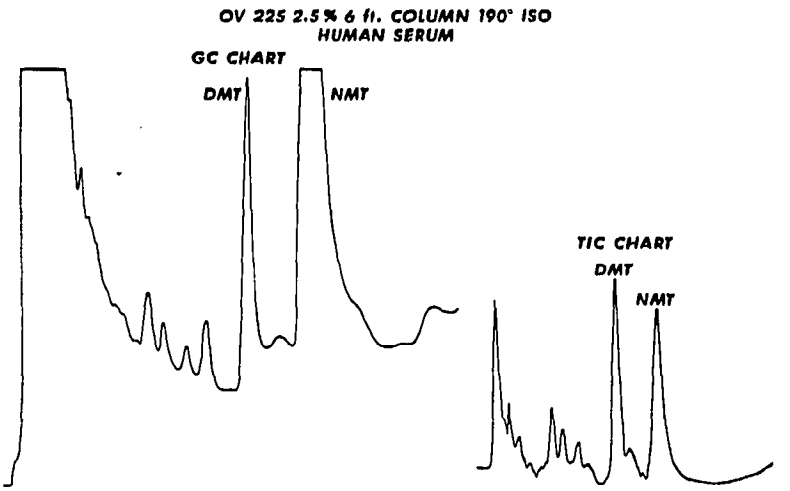

Fig. 7. GC and total ion current (TIC) charts of NMT incubation with human serum.

REFERENCES

1 M. Morgan and A. J. Mandell, *Science*, 165 (1969) 492.
2 A. J. Mandell and M. Morgan, *Nature (London)*, 230 (1971) 85.
3 J. M. Saavedra and J. Axelrod, *Science*, 175 (1972) 1365.
4 L. R. Mandel, H. S. Ahn, W. J. A. VandenHeuvel and R. W. Walker, *Biochem. Pharmacol.*, 21 (1972) 1197.
5 N. Narasimhachari, J. Plaut and H. E. Himwich, *Life Sci.*, 11, Part II (1972) 221.

6 N. Narasimhachari, B. Heller, J. Spaide, L. Haskovec, H. Meltzer, M. Strahilevitz and H. E. Himwich, *Biol. Psychiatr.*, 3 (1971) 21.
7 N. Narasimhachari and J. Plaut, *J. Chromatogr.*, 57 (1971) 433.
8 N. Narasimhachari and R.-L. Lin, *Trans. Amer. Soc. Neurochem.*, 3 (1973) 108C.
9 J. Axelrod, *Science*, 134 (1961) 343.
10 N. Narasimhachari and P. Vouros, *Anal. Biochem.*, 45 (1972) 154.
11 N. Narasimhachari and P. Vouros, *J. Chromatogr.*, 70 (1972) 135.
12 R. W. Walker, H. S. Ahn, L. R. Mandel and W. J. A. VandenHeuvel, *Anal. Biochem.*, 47 (1972) 228.
13 J. M. Saavedra, J. T. Coyle and J. Axelrod, *J. Neurochem.*, 20 (1973) 743.