REFERENCES

1. Q. R. Bartz, C. C. Elder, R. P. Frohardt, S. A. Fusari, T. A. Haskell, D. W. Johannessen and A. Ryder, *Nature, Lond.* **173**, 72 (1954).
2. S. A. Fusari, T. H. Haskell, R. P. Frohardt and Q. R. Bartz, *J. Am. chem. Soc.* **76**, 2881 (1954).
3. B. Levenberg and J. M. Buchanan, *J. Am. chem. Soc.* **78**, 504 (1956).
4. B. Levenberg, I. Melnick and J. M. Buchanan, *J. biol. Chem.* **225**, 163 (1957).
5. R. R. Ellison, D. A. Karnofsky, S. S. Sternberg, M. L. Murphy and J. H. Burchenal, *Cancer* **7**, 801 (1954).
6. J. F. Holland, E. H. Gehan, C. O. Brindley, M. M. Dederick, A. H. Owens, B. I. Schinder, R. Taylor, E. Frei, O. S. Selawry, W. Regelson and T. C. Hall, *Clin. Pharm. Ther.* **2** (1), 22 (1961).
7. A. C. Bratton and E. K. Marshall, *J. biol. Chem.* **128**, 537 (1939).
8. B. Levenberg and J. M. Buchanan, *J. biol. Chem.* **229**, 1005 (1957).
9. B. Levenberg and J. M. Buchanan, *J. biol. Chem.* **224**, 1019 (1957).
10. M. P. Schulman, J. C. Sonne and J. M. Buchanan, *J. biol. Chem.* **196**, 499 (1952).
11. S. C. Hartman, B. Levenberg and J. M. Buchanan, *J. biol. Chem.* **221**, 1057 (1956).
12. R. P. Herrmann and J. M. Buchanan, *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. VI, p. 71, Academic Press, New York (1963).
13. T. C. French and J. M. Buchanan, *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. VI, p. 78, Academic Press, New York (1963).
14. A. J. Collins and R. J. Ancill, *J. Pharm. Pharmac.* **22**, 150 (1970).
15. A. J. Collins and R. J. Ancill, *Biochem. Pharmac.* **19**, 310 (1970).
16. J. M. Buchanan, *Ciba Foundation Symposium on amino acids and related peptides with antimitotic activity* (Eds. G. W. E. Wolstenholme and C. H. O'Connor) p. 75, J. A. Churchill, London (1958).
17. T. C. French, I. B. Dawid and J. M. Buchanan, *J biol. Chem.* **238**, 2186 (1963).
18. R. J. Ancill, Unpublished results.
19. H. Dahn and H. Gold, *Helv. Chim. Acta* **46**, 983 (1963).
20. W. Bradley and R. Robinson, *J. chem. Soc.* p. 1310 (1928).

Biochemical Pharmacology, Vol. 20, pp. 712–716. Pergamon Press, 1971. Printed in Great Britain

Purification and substrate specificity of indoleamine-*N*-methyl transferase

(*Received* 15 *June* 1970; *accepted* 31 *July* 1970)

An enzyme, present in rabbit lung, which transfers the methyl group from *S*-adenosylmethionine to the amino group of a variety of indoleamines and phenylethylamines, has been described by Axelrod.[1] Recently, a similar indoleamine-*N*-methyl transferase was isolated from brain, although it appears to be more specific in that it does not *N*-methylate phenylethylamines.[2] Although serotonin was reported to be the most active substrate in both cases, the studies presented herein on partially purified indoleamine-*N*-methyl transferase from rabbit lung demonstrate that *N*-methyltryptamine is the substrate with the lowest K_m. These results raise the possibility that a physiological role of the enzyme may be the formation of dimethyltryptamine.

For the purification of indoleamine-*N*-methyl transferase, the 40–60% ammonium sulfate fraction was prepared from albino male rabbit lung according to the procedure of Axelrod.[1] A 12-ml aliquot of the dialyzed enzyme preparation containing about 150 mg protein was placed on a Sephadex G-150 column (3 × 45 cm) equilibrated with 1×10^{-3}M potassium phosphate buffer (pH 7·0) con-

taining 5×10^{-5}M EDTA and 1×10^{-4}M dithiothreitol. The column was eluted with this buffer at a rate of 12 ml/hr and the active fractions (150–200 ml eluate) were pooled and concentrated 10-fold by ultrafiltration. A 1·0–2·0 ml aliquot of the concentrate containing 5–10 mg protein was placed on a column of DEAE-Sephadex A-50 (1·5 × 25 cm) equilibrated with 0·06 M potasssium phosphate buffer, pH 7·0, containing 5×10^{-5}M EDTA and 1×10^{-4}M dithiothreitol. The column was eluted with a gradient of potassium phosphate buffer, pH 7·0 (containing EDTA and dithiothreitol). For the elution, 150 ml of 0·15 M phosphate buffer was introduced into an equal volume of 0·06 M buffer in a linear gradient apparatus; 5-ml fractions were collected at a rate of 6 ml/hr using a drop-counting fraction collector. The fractions containing enzyme activity were pooled, concentrated approximately 10-fold by ultrafiltration and stored at −70°.

Indoleamine-*N*-methyl transferase activity was measured by minor modifications of the method of Axelrod.[1] Substrates were tested over a range from 10^{-6} to 10^{-3}M. A typical reaction mixture contained: 31 μmoles potassium phosphate buffer, pH 7·9; 2·4 mμmoles S-adenosylmethionine-methyl-^{14}C (160,000 counts/min); 34 mμmoles *N*-methyltryptamine; and 0·05 ml enzyme in a final volume of 0·1 ml. Mixtures were incubated 60 min at 37° and the reaction was terminated by addition of 0·2 ml of 0·125 M sodium tetraborate, pH 10. [^{14}C]-methylated products were extracted into 2 ml of water-saturated isoamyl alcohol and the radioactivity of a 1-ml aliquot was determined in a liquid scintillation counter. The indoleamines were identified by co-chromatography with known standards on silica gel thin-layer plates using isopropanol–ammonia–water (160:10:30 and 200:10:20) as solvents.[2,3] Dimethyltryptamine was further characterized by gas–liquid chromatography and mass spectrometry.[4] Boiled enzyme fractions, omission of substrate, and addition of borate at the onset of incubation were procedures used to assess nonenzymatic incorporation of radioactivity into the isoamyl alcohol phase.

Phenethanolamine-*N*-methyl transferase was assayed by the method of Axelrod using normetanephrine as substrate,[5] and imidazole-*N*-methyl transferase was assayed according to the procedure of Brown *et al.*[6] using histamine as substrate. Protein was measured by the method of Oyama and Eagle.[7]

S-adenosylmethionine (specific activity, 42 μc/μmole) was purchased from the New England Nuclear Corp.; serotonin creatinine sulfate was purchased from Mann Research Labs.; *N,N*-dimethyltryptamine and *N*-methylserotonin oxalate were obtained from K & K Laboratories; and tryptamine hydrochloride and *N*-methyltryptamine hydrogen oxalate were obtained from Calbiochem. All chemicals were reagent grade commercial products.

The purification of indoleamine-*N*-methyl transferase from rabbit lung is presented in Table 1. A 50-fold purification has been achieved with approximately a 15 per cent yield. The enzyme preparation is heavily contaminated with imidazole-*N*-methyl transferase through the Sephadex G-150 step. Separation of this contaminant is almost complete after chromatography on DEAE-Sephadex (Fig. 1). The indoleamine-*N*-methyl transferase is eluted between fraction numbers 14 and 23 (phosphate concentration estimated at 0·09 M), while the imidazole-*N*-methyl transferase is eluted earlier.

TABLE 1. PURIFICATION OF INDOLEAMINE-*N*-METHYL TRANSFERASE*

Step	Total protein† (mg)	Total activity	Specific activity
Soluble supernatant fraction	3400	3100	0·92
40–60% Ammonium sulfate fraction	580	1940	3·34
Sephadex G-150 concentrate‡	64	780	12·2
‡DEAE-Sephadex A-50 concentrate	9·5	480	50·4

* Each assay mixture contained: 31 μmoles of phosphate buffer, pH 7·9; 34 μmoles *N*-methyl tryptamine oxalate; 2·4 mμmoles S-adenosylmethionine-methyl-^{14}C; 0·015–0·05 ml enzyme and water to 0·10 ml. Incubation was for 60 min at 37°. The specific activity is defined as mμmoles [^{14}C]-dimethyltryptamine formed/mg protein/hr.

† The starting material was 70 g of rabbit lung.

‡ This preparation was used for most studies.

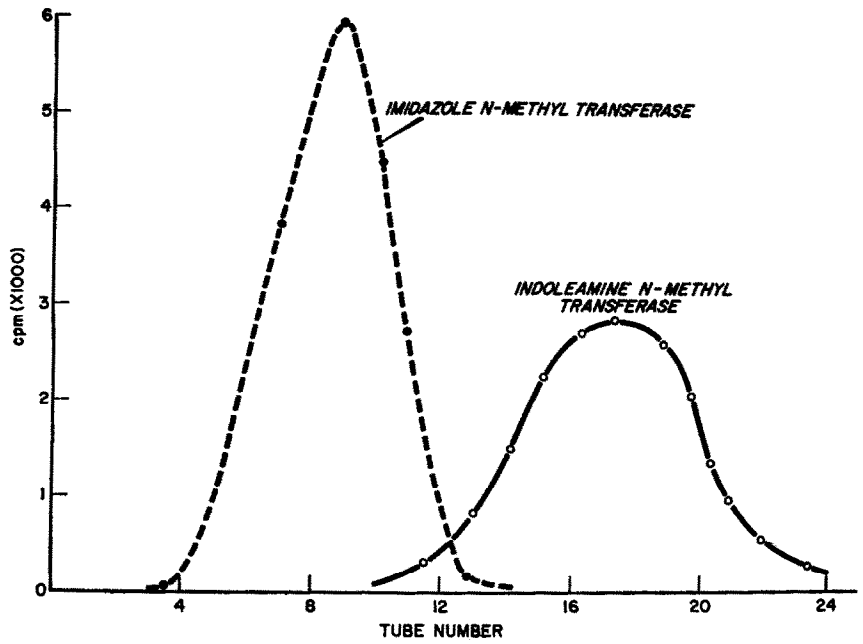

FIG. 1. Separation of imidazole-*N*-methyl transferase from indoleamine-*N*-methyl transferase by chromatography on DEAE-Sephadex A-50. For details of the separation see text.

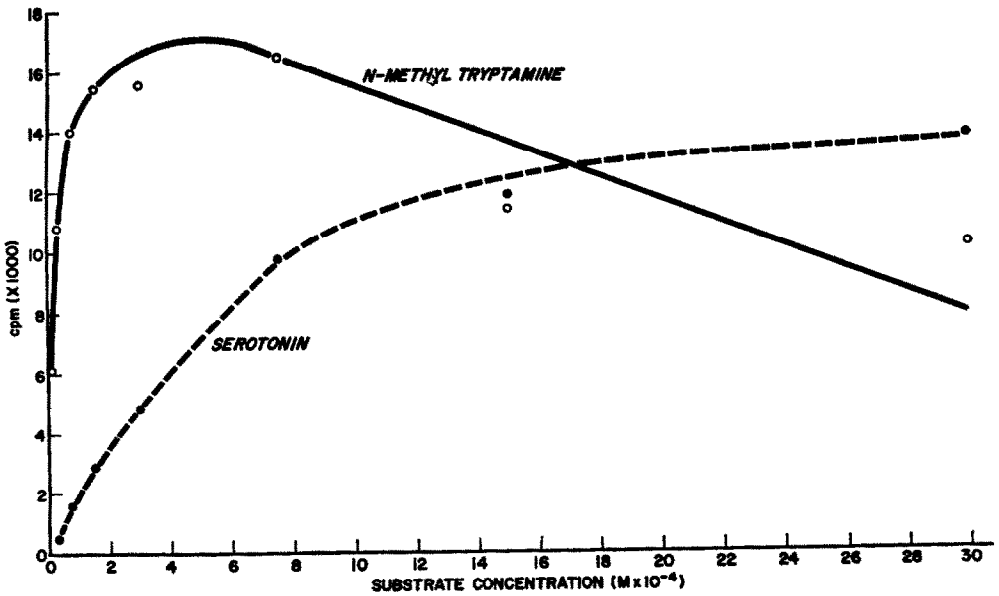

FIG. 2. Methylation of serotonin and *N*-methyltryptamine by indoleamine-*N*-methyl transferase.

Phenethanolamine-*N*-methyl transferase activity, present in trace amounts through the Sephadex G-150 step, could not be detected in the final preparation. The enzyme is stable at −70° for approximately 2 months. The presence of dithiothreitol enhances the stability and addition of bovine serum albumin to 2 mg/ml further stabilizes the enzyme, but was not used routinely.

While serotonin had been reported to be the best substrate for the enzyme,[1,2] the data in Fig. 2 demonstrate that *N*-methyltryptamine exhibits an apparent substrate inhibition, when present at relatively high concentration in the assay ($3{\cdot}3 \times 10^{-3}$M), and has the lowest K_m in the series of four related substrates tested (Table 2). *N*-methyltryptamine has a 20-fold greater affinity for the methylating enzyme than serotonin, while *N*-methylserotonin and tryptamine have respectively a 6-fold and 3-fold greater affinity for the enzyme than serotonin. *N,N*-dimethyltryptamine inhibits enzyme activity by 70–95 per cent when tested at 1×10^{-4}M against substrates at equimolar concentration.

These results confirm the original observations by Axelrod[1] on the occurrence of relatively high concentrations of indoleamine-*N*-methyl transferase in rabbit lung. The enzyme can methylate several indoleamines using S-adenosylmethionine as the methyl donor. Of particular interest is the fact that *N*-methyltryptamine is the best substrate in the series of compounds evaluated. Since the substrate

TABLE 2. SUBSTRATE SPECIFICITY AND *N,N*-DIMETHYLTRYPTAMINE INHIBITION OF INDOLEAMINE-*N*-METHYL TRANSFERASE

Substrate (10^{-4}M)	K_m	% Inhibition produced by 10^{-4}M dimethyltryptamine
N-methyltryptamine	$5{\cdot}0 \times 10^{-5}$	70
N-methylserotonin	$1{\cdot}6 \times 10^{-4}$	72
Tryptamine	$3{\cdot}3 \times 10^{-4}$	93
Serotonin	$1{\cdot}0 \times 10^{-3}$	95

with the lowest K_m is often considered to be the more natural substrate for an enzyme, these studies point to the possibility that at least in rabbit lung the indoleamine-*N*-methyl transferase may have the physiological role of forming dimethyltryptamine. The apparent substrate inhibition produced by *N*-methyltryptamine and the product inhibition produced by dimethyltryptamine imply stringent substrate and product control on enzyme activity *in vitro*.

N,N-dimethyltryptamine has been recognized to be a potent psychotogenic agent in man.[8] The report of its presence in human blood and urine[9,10] suggests that, if formed in substantial quantities, it could cause mental aberrations. The recent communication on the occurrence of *N,N*-dimethyltryptamine and 5-methoxy-*N,N*-dimethyltryptamine in the urine of acute schizophrenics[11] suggests that alkylated indoleamines other than *N,N*-dimethylserotonin (bufotenin) may play a role in psychoses.[12] The presence in mammals of an enzyme which forms hallucinogenic compounds takes on additional significance, since it has recently been found in human brain tissue.[2] Studies on the preparation and properties of the brain enzyme are currently in progress.

Acknowledgements—The authors are grateful to Dr. Harry W. Dougherty for valuable suggestions for purifying the enzyme and to Mr. J. Smith and Dr. W. J. A. VandenHeuvel for the gas–liquid chromatography–mass spectrometry studies for characterizing dimethyltryptamine.

Department of Biochemistry,
Merck Institute for Therapeutic Research,
Rahway, N.J. 07065, U.S.A.

LEWIS R. MANDEL
SIMON ROSENZWEIG
FREDERICK A. KUEHL, JR.

REFERENCES

1. J. AXELROD, *J. Pharmac. exp. Ther.* **138**, 28 (1962).
2. M. MORGAN and A. J. MANDELL, *Science, N.Y.* **165**, 492 (1969).
3. J. AXELROD, *Science, N.Y.* **134**, 343 (1961).
4. W. J. A. VANDENHEUVEL, *J. Chromatog.* **36**, 354 (1968).
5. J. AXELROD, *J. biol. Chem.* **237**, 1657 (1962).

6. D. D. BROWN, R. TOMCHICK and J. AXELROD, *J. biol. Chem.* **234**, 2948 (1959).
7. I. OYAMA and H. EAGLE, *Proc. Soc. exp. Biol. Med.* **91**, 305 (1956).
8. S. SZARA, in *Amines and Schizophrenia* (Eds. H. E. HIMWICH, S. S. KETY and J. R. SMYTHIES), p. 181, Pergamon Press, Oxford (1967).
9. F. FRANZEN and H. GROSS, *Nature, Lond.* **206**, 1052 (1965).
10. H. GROSS and F. FRANZEN, *Z. klin. Chem.* **3**, 99 (1965).
11. B. HELLER, N. NARASIMHACHARI, H. E. HIMWICH and J. SPAIDE, *Physiologist* **12**, 252 (1969).
12. H. D. FABING and S. R. HAWKINS, *Science, N.Y.* **123**, 886 (1956).

Biochemical Pharmacology, Vol. 20, pp. 716–718. Pergamon Press, 1971. Printed in Great Britain

Dihydrofolate reductase activity of leukemia L1210 during development of methotrexate resistance

(*Received* 18 *May* 1970; *accepted* 31 *July* 1970)

DEVELOPMENT of resistance to folate analogs in murine leukemia has most often been found to be associated with an increased dihydrofolate reductase (EC 1.5.1.3) activity of the leukemic cells. The selection process leading to these events has been discussed in detail.[1–4] Consecutive passage through treated mice seemed required to achieve resistance to a given drug regimen,[1,4,5] in contrast to human leukemia, which becomes refractory in the patient during therapy.[6] It became, therefore, of interest to determine whether dihydrofolate reductase levels were in fact already increased during the initial transfer generation of systemic leukemia L1210 under treatment with methotrexate (MTX).

CDF_1 hybrid male mice were inoculated s.c. with spleen suspensions of leukemia L1210, as described previously.[1] MTX (0·75 mg/kg s.c.) was injected daily, and tumor reinoculations were carried out as in the establishment of Friedkin's subline FR-1.[1] Acetone powders were prepared from the solid tumors growing at the site of inoculation and from the spleens infiltrated with leukemic cells, stored at −20°, and extracted with buffer, pH 7·4, for determination of dihydrofolate reductase activities.[1,7] In comparing the present results with Friedkin's data,[1] it should be noted that carrying out the rate determinations at 28°[7] instead of 32° and using a $\Delta\epsilon$ value of 12,300[8] instead of 11,300[1] in the spectrophotometric assay have resulted in somewhat lower specific activities.

Dihydrofolate reductase activities in the local tumors and spleens of leukemic mice treated daily with MTX from day 5 after tumor inoculation were measured 48 hr after the last injection of the drug (Table 1).

The decrease during the first 18 days after inoculation, as compared to the specific activities in untreated animals, was attributable to partial binding of the enzyme to the drug.[9,10] When treatment was continued to day 20 after inoculation or longer, dihydrofolate reductase activity in the local tumors was increased markedly. Resistance, as reflected by increased enzyme activity, thus became evident only after at least 15 days of treatment and shortly before the expected death of the treated animals. The activity in the tumors of animals treated until day 20 was not increased further when the interval between the last injection of MTX and tumor removal was extended to 72 hr (Table 1), and it was identical with that observed in animals of the next untreated transfer generation (Table 2). Therefore, bound MTX produced no significant inhibition of enzyme activity 48 hr after injection once the cells had become partly resistant, possibly because of synthesis of new enzyme during that period or because of the emergence of cells with decreased permeability to MTX.

The increased dihydrofolate reductase activity in the local tumors of mice treated from day 5 to 20 was not observed in the spleens (Table 1). In agreement with previous findings,[11] these spleens became much larger in leukemic mice treated with MTX than in untreated control animals. The much lower enzyme activity per unit of protein in the spleens, as compared to the tumors, on days 22 and