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N-METHYLATION OF NORMORPHINE BY RAT TISSUES *IN VITRO*

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Abstract—A N-methyl transferase that catalyzes the transfer of methyl groups from S-adenosylmethionine to normorphine has been purified from rat liver and brain. The enzymic activity purified from liver is five times more active than that from brain. However, preparations from hypothalamus and adjacent structures of brain are more active than those from liver. Evidence on the identity of this enzymic activity with nicotinamide methylferase is not conclusive at the present time.

THE N-methylation of normorphine by preparations from rat liver and brain has been described with methionine of S-adenosylmethionine as the methyl donors.[1] Further studies on this reaction measured either by labeled methyl transfer, or by a colorimetric determination of the product, are reported here.

METHODS

Separation of morphine and normorphine from incubation mixtures

The general procedure for the isolation of morphine and normorphine involves organic solvent extraction from an alkaline aqueous medium. In our studies with ^{14}C-(methyl) compounds, it was essential to eliminate highly radioactive compounds with high water solubility from the organic phase. The extraction procedure of Axelrod, in which ethylene dichloride containing *iso*amyl alcohol is the organic solvent,[2] was effective in removing the alkaloids from an aqueous incubation medium which had been brought to pH 9·5 by the addition of sodium hydroxide. When the organic layer was extracted with 0·1 N HCl and the whole procedure repeated, the slight loss of recovery of the compounds (5 to 10 per cent) was compensated by the elimination of contaminating radioactive compounds, particularly the labeled methyl donor itself. This procedure was used in the experiments involving ^{14}C-(methyl) transfer.

The procedure of Milthers[3] was particularly effective in producing a sample for thin-layer chromatography relatively free of lipids and salts. The organic extract, made by a mixture of chloroform and *iso*propanol (3:1), was passed through a column of sodium sulfate, then equilibrated with 0·1 N HCl and the extraction repeated. This procedure was used to prepare a sample for thin-layer chromatography and subsequent colorimetric measurements.

Chromatography

For paper chromatography the solvent systems were (1) tertiary amyl alcohol:n-butyl ether:H_2O (50:7:43); (2) n-butanol:acetic acid:H_2O (60:15:25); (3) ethanol: acetic acid:H_2O (65:1:34).

In early experiments unbuffered Whatman No. 1 paper was used. However, optimal separation of labeled compounds in the first solvent system was obtained when the paper was buffered at pH 6·7 with 0·5 M phosphate buffer, and when a drop of 1 N NH_4OH was added to each spot before chromatography.

Morphine and normorphine were located by their ultraviolet absorption and the iodoplatinate color reaction. Ninhydrin, naphthoresorcinol, and phosphomolybdate reagents were used to locate amino acids, ribose, and S-adenosylmethionine respectively.

Radioactivity of the isolated alkaloids was measured after separation by paper chromatography in an automatic chromatogram 4π scanner (Vanguard Instrument Co.) and compared to standard ^{14}C-methionine spots on paper measured at the same instrument settings. Under our conditions (slit width, 1 cm; chart speed, 12 in./hr; time, 100 sec) a peak of 1 count per min (cpm) is equivalent to $1{\cdot}28 \pm 0{\cdot}09 \times 10^{-5}$ μc of ^{14}C-labeled compound.

For thin-layer chromatography on silica gel the solvent system was (4) ethanol: dioxane:benzene:NH_4OH (5:40-50:5).[4]

The area on the gel at the R_f of morphine was scraped into a centrifuge tube in methanol containing a drop of ammonia, and the silica gel was removed by centrifugation. For the quantitative determination of morphine, the alkaloid was converted to its hydrochloride salt, and the methanol was removed by evaporation.

Morphine and normorphine

Normorphine hydrochloride was obtained through the courtesy of Dr. N. B. Eddy of the National Institutes of Health. Normorphine hemimethanolate was obtained through the courtesy of Dr. Karl Pfister, of Merck Sharp & Dohme, and was converted to the hydrochloride. Both preparations were tested for morphine content by chromatography on paper. When 20 μmoles of normorphine was chromatographed on paper, morphine could not be detected. Morphine was therefore not present at a level of 0·01 μmole, the lower limit of its detection on paper, so that any contamination must be less than 0·05 per cent. In some experiments "purified" normorphine, isolated from a thin-layer chromatogram, was used as substrate.

Morphine was measured quantitatively, after isolation by thin-layer chromatography, by the color reaction with methyl orange after nitrobenzoylation.[5] This method had a sensitivity of 0·002 μmole morphine/ml. The recovery of morphine from tissue incubations was between 80 and 90 per cent. After isolation by chromatography, normorphine was reacted with the Folin–Ciocalteau reagent under the conditions for tyrosine.[6] Normorphine recovery from either a zero-time or 60-min incubation was between 30 and 50 per cent with most of the compound lost during the extraction procedures.

Preparation of ^{14}C-(methyl) S-adenosylmethionine

A methionine-activating enzyme was prepared according to the isolation procedures described by Cantoni for this enzyme and, with ^{14}C-(methyl) methionine as a

methyl donor, was used to catalyze the formation of labeled S-adenosylmethionine.[7] Several preparations with specific activities ranging from 0·2 to 1 μc/μmole were employed in the various experiments reported here.

Enzyme preparations

A partially purified methyltransferase activity was found in the supernatant fraction prepared by centrifuging a 25% homogenate of rat liver in 0·25 M acetate buffer, pH 5·0, at 13,000 $\times$ *g* for 15 min. The supernatant fraction was adjusted to pH 5 by adding cold 1 M acetic acid, and the preparation was heated in a water bath at 52° for 5 min. The preparation was cooled, then centrifuged at the same speed for 10 min, and the denatured material removed. This preparation corresponds to step 1 in the purification of nicotinamide methylferase.[8]

Brain methyltransferase activity was in a fraction prepared by extracting whole rat brain with acetate buffer and denaturing the extract by heat in the same way as the liver preparation.

Purified methyltransferase activity from brain or liver was isolated by following the procedure of Cantoni for the isolation of nictinamide methylferase through step 4. (Briefly, the steps of the Cantoni procedure for the purification of nicotinamide methylferase are: (1) The supernatant fraction after centrifugation at 8,200 $\times$ *g* for 15 min of a liver homogenate prepared in pH 5 acetate buffer is heat-denatured at 50° for 5 min. (2) The fraction precipitated between 0·4 and 0·6 saturation with ammonium sulfate is dissolved in buffer and dialyzed free from sulfate ion. (3) The material is then adsorbed on calcium phosphate gel at pH 5 and eluted at pH 6·8. (4) The ammonium sulfate fractionation is repeated.) In addition, rat brains were dissected into gross anatomical areas: cortex, cerebellum, hypothalamus, medulla, brain stem, and midbrain, and combined areas from ten rat brains were purified through step 2. Other brains were divided into hypothalamus (a block of tissue containing hypothalamus and adjacent tissue) and remaining brain, and purified through step 4.

Liver homogenates were fractionated by centrifugation into particulate fractions. A 0·25 M sucrose homogenate of rat liver was centrifuged at 10,000 $\times$ *g* for 20 min. The supernatant fraction was centrifuged at 100,000 $\times$ *g* for 60 min to sediment the microsomal fraction. In addition, by the same method used for the acetate extract, aliquots of both the soluble fraction and the entire microsomal supernate were heat-denatured in acid solution.

Enzyme activity

Nicotinamide methylferase activity was measured by determining the product, N^1-methyl nicotinamide, by the Huff reaction.[9] Protein was measured by the method of Lowry *et al.*[6] Enzyme activity was expressed as micromoles of product formed per hour per milligram protein.

Normorphine methyltransferase activity was measured in two ways. The product, morphine, was estimated colorimetrically or by ^{14}C-methyl transfer. Enzyme activity was expressed as micromoles product formed per hour per milligram protein.

Incubation mixtures

In the light of experiments on the effect of substrate concentration and pH on the reaction, the final incubation medium used in most experiments consisted of: 10 μmoles normorphine, 125 μmoles Tris–maleate buffer (pH 5·5), 10 μmoles $MgCl_2$, 4 μmoles reduced glutathione, 5 μmoles S-adenosylmethionine (either unlabeled for colorimetric assays or at a specific activity of 0·2 to 1 μc/μmole), and the methyltransferase preparation in a volume of 5 ml.

Duplicate tubes were incubated in a shaking incubator in air at 37° for 30 or 60 min, after which the reaction was stopped with the addition of 0·5 ml concentrated hydrochloric acid. The protein was removed by centrifugation, and the acid-soluble fraction was treated as described in the section on extraction methods. Suitable control tubes and morphine recovery tubes were added to each assay.

RESULTS AND DISCUSSION

Distribution of ^{14}C from labeled S-adenosylmethionine

The incubation of labeled S-adenosylmethionine without normorphine was found to form additional radioactive compounds which were carried through the extraction procedure and which interfered with the identification and measurement of morphine. Aqueous solutions of S-adenosylmethionine were partially hydrolyzed and the products chromatographed in solvent systems 1, 2 and 3. S-Adenosylmethionine was heated in a boiling water bath for 5 min at pH 5, which removes the homocysteine moiety from the molecule,[10] and at 98° for 90 min in 0·01 N HCl which causes more extensive hydrolysis. The whole aqueous sample of the hydrolysates as well as the untreated S-adenosylmethionine was chromatographed in the three solvent systems and examined for ultraviolet absorption, and ninhydrin-, phosphomolybdate-, and iodoplatinate-reacting spots, and was compared with compounds described by Schlenk and dePalma[10] and Duerre[11] in order to identify the compounds that were formed in the absence of tissue. It was apparent (Table 1, section 1) that untreated S-adenosylmethionine had been partially hydrolyzed, losing the homocysteine moiety during the chromatographic procedures. Methylthioadenosine was the resulting product containing radiocarbon. Under more vigorous hydrolysis both S-adenosylmethionine and methylthioadenosine were split into smaller components including ribose, adenine, and methionine. Two unidentified spots were seen: compound X was radioactive, ninhydrin-positive, iodoplatinate-negative, and not visible under ultraviolet examination. Compound Y was radioactive, ninhydrin-positive, and adsorbed in ultraviolet light.

All these compounds were found in the aqueous phase when S-adenosylmethionine alone was present originally. A larger amount of labeled S-adenosylmethionine was included in a reaction mixture with a heat-denatured, inactive tissue preparation in order to identify the compounds that were not formed enzymically. An organic extract, using a double Milthers extraction procedure, was prepared and chromatographed in the three solvent systems (Table 1, section 2, lines 4 to 7). The only radioactive spots were those resulting from the hydrolysis of S-adenosylmethionine. In an inucbation mixture containing an inactive enzyme preparation, stopped at zero time (line 4) only S-adenosylmethionine and methylthioadenosine were radioactive. After 60 min incubation of the same mixture (line 5) little of the label was found in the organic phase. In a zero-time incubation with an active purified enzyme preparation (line 6) there were five radioactive spots as there were when an active enzyme preparation was incubated

for 60 min and the reaction stopped with acid before any S-adenosylmethionine was added (line 7). Thus in incubations which did not include an active methyltransferase activity, the five radioactive compounds which have been localized in three solvent systems have been shown to be derived from reactions not catalyzed by transferase.

TABLE 1. DISTRIBUTION OF ^{14}C FROM LABELED S-ADENOSYLMETHIONINE

Treatment of sample	Recovery of label as					
	S-AM 3*	MTA 5	Methionine 4	X 6	Y 7	Morphine 2
	(counts per minute)					
1. Whole aqueous fraction (1730 cpm, S-AM added)						
1 S-AM, untreated	1000	600	0	0	0	0
2 S-AM, heated at pH 5	160	1000	0	290	100	0
3 S-AM, heated at pH 1	160	0	280	450	300	0
2. Organic extract (10,400 cpm, S-AM added)						
4 Inactive enzyme, zero time	10	93	0	0	0	0
5 Inactive enzyme, 60 min	6	0	0	0	0	0
6 Active enzyme, zero time	6	41	17	6	6	0
7 Active enzyme, 60 min, then S-AM added	5	47	17	14	9	0
8 Active enzyme, 60 min	0	102	29	34	6	52

* S-AM = S-adenosylmethionine; MTA = methylthioadenosine; X and Y are unidentified compounds described in the text. Counts per minute = radioactivity measured in a strip counter, the average of the values in three solvent systems. The compounds are numbered as in the legend to Fig. 2.

Methyl transfer

In the literature are described several methyltransferase preparations that catalyze the transfer of methyl groups to the nitrogen atom of histamine,[12] ethanolamine,[13] and nicotinamide.[8] When these enzyme activities were prepared from rat liver and assayed with normorphine as substrate, only the nicotinamide methyltransferase preparation catalyzed the transfer of radioactivity from the methyl group of S-adenosylmethionine to morphine.

When a liver preparation purified through step 1[8] was incubated at pH 5 with normorphine, the counts in the autoscan of the chromatogram averaged 10 cpm, equivalent to a specific enzyme activity of $6{\cdot}1 \times 10^{-5}$ μmoles of labeled methyl group transferred/hr per mg protein. This tissue preparation was used to establish optimal substrate concentration (Table 2). From 2 mM to 10 mM final concentration of normorphine there seemed to be a plateau of enzyme activity.

Liver transferase purified through step 4 was used in constructing a pH curve of activity (Table 3). A broad maximum ranging from pH 5·5 to 7·0 was found. An approximate 20-fold increase in transferase activity was obtained in purifying from steps 1 to 4.

Preparations from whole rat brain were also active in catalyzing the transfer of labeled methyl group from S-adenosylmethionine to normorphine.

To answer the question of whether nicotinamide N-methylpherase was the enzyme catalyzing the present reaction, both brain and liver enzymes were purified through

steps 1 to 4 of the Cantoni procedure and assayed at three of the steps for relative methylating activity versus normorphine and nicotinamide by quantitative colorimetric and fluorimetric procedures respectively (Table 4). In liver, normoprhine methylating activity was purified 10-fold whereas nicotinamide methylating activity was increased 18-fold. In brain, the low level of morphine formed in incubations with enzymes purified to steps 1 and 2 made the colorimetric determination unreliable in these assays.

TABLE 2. THE EFFECT OF SUBSTRATE CONCENTRATION ON N-METHYLATION BY LIVER

Substrate concentration (mM)	Radioactivity (cpm)
0	0
0·1	2
0·2	5
1	6
2	10
4	8
10	8

The enzyme was purified to stage 1. The radioactivity is the average of two assays at each substrate concentration chromatographed on buffered paper (pH 6·7) in solvent system 1. The R_f of morphine in this system is 0·52. Ten counts per minute is equivalent to $6{\cdot}13 \times 10^{-5}$ μmoles/hr per mg protein.

TABLE 3. N-METHYLATION BY LIVER AS A FUNCTION OF pH

	pH	Radioactivity at R_f 0·70–0·74 (Morphine = 0·73) (cpm)
Acetate buffer	5·0	0
	5·5	17
Tris-maleate buffer	5·5	22*
	6·0	20
	6·5	18
	7·0	17
	7·5	11
	8·0	2

* Twenty-two cpm is equivalent to $28{\cdot}15 \times 10^{-4}$ μmoles of ^{14}C transfer/hr per mg protein.

TABLE 4. RELATIVE METHYLATION OF NORMORPHINE AND NICOTINAMIDE BY BRAIN AND LIVER

Enzyme purification	Brain		Liver	
	Normorphine	Nicotinamide	Normorphine	Nicotinamide
Step 1	+	29	54	166
2	+	94	143	368
4	161	550	544	3005

Units: μmoles $\times 10^4$ N¹methylnicotinamide formed/hr per mg protein; μmoles $\times 10^4$ morphine formed/hr per mg protein. Plus sign = positive, but too low to be measured accurately.

However, the nicotinamide methylating activity was increased 19-fold from steps 1 to 4, and at step 4 the ratio of normorphine to nicotinamide methylated by brain (0·29) was similar to that by liver (0·18).

This study of the relative methylation of nicotinamide and normorphine by the same preparations from either brain or liver was not conclusive since the purification of the two activities proceeded at similar but not identical ratios. In favor of the identity of the two activities are the nature of the methyl donor, pH optima, and purification procedures. Against it is the lack of competitive inhibition of normorphine methylation by nicotinamide (unpublished experiments and Table 6). The absence of normorphine methylation by preparations catalyzing methylation of other compounds suggests the presence of several methyltransferases in both liver and brain. An amine N-methylpherase that can catalyze the transfer of methyl groups to normorphine has been isolated and purified from rabbit lung.[14] At 12-fold purification this enzyme has activity of 56×10^{-3} μmoles of serotonin methylated/mg protein per hr—about as active as our liver enzyme purified to step 4. However, the pH optimum (7·8) and tissue localization (lung, spleen, kidney, and adrenal gland) distinguish this activity from the one described here.

A comparison of the enzyme activity measured quantitatively (Table 4) with that found by measuring ^{14}C-methyl transfer (Table 3) shows that considerably less enzyme activity was found by the radioactive labeling technique. In transmethylation it is possible that added S-adenosylmethionine is not the immediate precursor of the N-methyl group of morphine, so that dilution with unlabeled endogenous methyl groups explains the low ratio of labeled to total methyl groups transferred in these experiments. In spite of this lower value for enzyme activity, the radioactive assay was used because it was of greater sensitivity and involved less expenditure of time.

The nature of the labeled compounds found in an organic solvent extract after an incubation with labeled S-adenosylmethionine has already been described. When a liver methyltransferase preparation purified to step 4 was used as the enzyme source, and the resulting organic phase chromatographed in the three solvent systems described earlier, an additional labeled spot was found at the Rf of morphine in all three systems (Table 1, line 8, and Fig. 1). In the figure are tracings of automatic scanning of chromatogram strips in each system. Line 1 in each system is the tracing of a sample of S-adenosylmethionine, line 2 an assay, and line 3 a control incubation without normorphine. The peak numbered 2 is quite distinct in each assay and absent from the control sample.

In the fractionation of sucrose homogenates of rat liver by centrifugation, it became apparent that the enzyme was found in the supernatant fraction after sedimentation of microsomes (Table 5, line C). When the supernatant fraction was heat-denatured in acid solution in the same way that the acetate extract had been, the activity increased (line D). A two-dimensional chromatogram, first in solvent system 1, then in system 2, was used to isolate labeled compounds in this assay. For the assay of preparation D and a control assay without substrate (Fig. 2), six areas of radioactivity coincided in spots numbered 3, 4, 5, 6, 7, and 8 (this last area is the outer edge of 5). In the assay an additional spot at the R_f of morphine in each direction was found. Thus, the radioactivity of morphine can be measured on a chromatographic strip after separation from other labeled compounds.

Whole rat brain purified through step 4 had a normorphine transmethylferase activity about one-third that from liver. Preparations dissected from anatomical areas varied in this activity. The area containing hypothalamus promoted the methylation to an extent more than twice as great as the next highest activity. For quantitative studies, the brain was divided into hypothalamus (a block of tissue containing hypothalamus and adjacent structures) and rest of brain, and each sample was purified

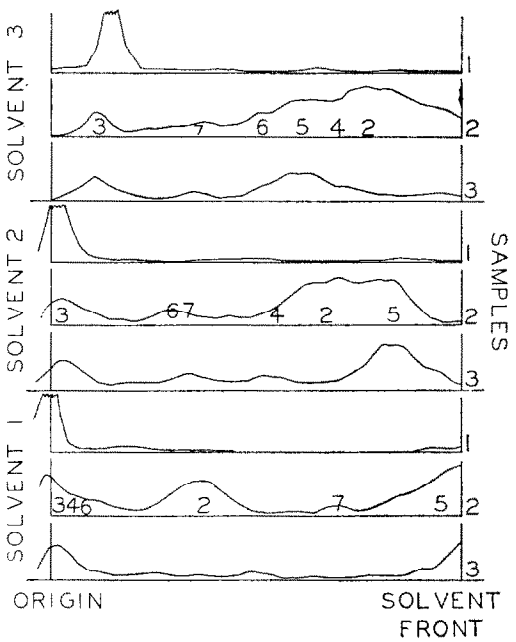

FIG. 1. Autoscanner tracing of chromatographic strips in three solvent systems (described in the text). A full-scale deflection is equal to 1000 cpm for sample 1 and 100 cpm for samples 2 and 3. Sample 1 is an aqueous solution of S-adenosylmetnionine (1730 cpm) placed directly on paper without artificial drying. Samples 2 and 3 are the final organic extracts of an assay and control incubation respectively. The compounds are numbered as described in the legend to Fig. 2.

TABLE 5. ACTIVITY OF LIVER FRACTIONS

	Preparation	Radioactivity* (cpm)
A.	Microsomal-supernatant fraction	16
B.	Fraction A, heat-denatured at pH 5	0
C.	Soluble fraction	16
D.	Fraction C, heat-denatured at pH 5	27

* The radioactivity is the average of two assays chromatographed in two directions and scanned in the second direction, solvent system 2. Twenty-seven counts per minute is equivalent to $6{\cdot}9 \times 10^{-5}$ μmoles/hr per mg protein.

through step 4 and, after an incubation with "purified" normorphine, the amount of morphine was measured by thin-layer chromatography and the colorimetric method. The activity of the preparation from hypothalamus was 0·175 μmole/mg per hr, more than ten times that from rest of brain. The purified hypothalamic preparation was also

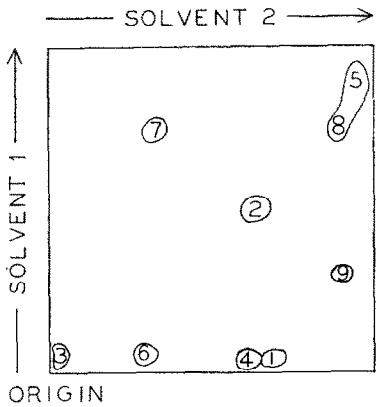

FIG. 2. A diagram of a two-dimensional chromatogram from an assay of preparation D of Table 5 with a control incubation without substrate. The chromatograms were cut into five equal-width strips in the direction of solvent 2, so that the radioactivity of compounds 3, 6, and 4 was measured on the first strip, no compounds on strip 2, compound 2 on the third, compounds 7 and 8 on the fourth, and compound 5 on the last strip. The radioactivity so recorded for the control and assay are given together with the identification, as far as possible, and reactivity of compounds found in the organic phase. The ninhydrin reaction was negative in this experiment for each compound as was the iodoplatinate reaction for compounds 2 and 3. However, when these compounds are present in higher concentration, the reactivity is as described. An ultraviolet-absorbing spot, numbered 9, appeared regularly in both control and assay chromatograms.

Number, compound	Iodoplatinate	Ninhydrin	UV abs.	Radioactivity Control	Radioactivity Assay
1. Normorphine	+	−	+	0	0
2. Morphine	+	−	+	0	27
3. S-adenosylmethionine	+	+	+	3	2
4. Methionine	−	+	−	8	2
5. Methylthioadenosine	+	−	+	100	11
6. X	−	+	−	10	80
7. Y	−	+	+	33	34
8. Extension of 5	+	−	+	100	31
9. Z	−	−	+	0	0

incubated with "purified" normorphine in an assay with labeled S-adenosylmethionine (Table 6). As in liver, the enzyme activity measured by this technique was much lower than the quantitative measurement. Nicotinamide in equimolar concentration was only slightly inhibitory in this experiment.

Whether methyl transfer is actually involved in the pharmacological activity of morphine and normorphine has been discussed extensively, but no unequivocal evidence on the point has been introduced.[15] Milthers has described the isolation of normorphine from brain when morphine was injected *in vivo* in eviscerated animals.[16]

TABLE 6. N-METHYLATION BY PURIFIED HYPOTHALAMIC PREPARATION*

Sample	Normorphine (μmoles/assay)	Radioactivity (cpm)
Blank	0	0
Assay	5	50
Assay + 5 μmoles nicotinamide	5	41

The enzyme was purified to stage 4. The radioactivity is the average of two assays for each figure chromatographed in two dimensions with scanning in solvent system 2, the second direction. Morphine has a Rf of 0·62 in this system. Fifty counts per minute is equivalent to $53{\cdot}2 \times 10^{-4}$ μmoles/hr per mg protein.

Morphine has been found in brain after the intracerebral injection of normorphine as described in our earlier experiments.[1] Thus, N-methyl transfer can be presumed to be possible in brain even though no requirement for transfer for pharmacological activity has been demonstrated.

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