Metal Ion-Catalysed Interaction of Peroxidase with Morphine and Protein

In continuation of our work[1] on morphine-protein interaction in central nervous system, evidence has been obtained in the present study that on aerobic incubation of morphine-N-methyl-C^{14} and horseradish peroxidase in presence of certain metal ions, peroxidase can utilize a number of naturally occurring proteins, peptides, and amino acids to generate hydrogen peroxide and subsequently convert morphine presumably by a free radical mechanism into highly reactive intermediates that combine with specific water-soluble proteins and peptide carriers through a chemical bonding different from reversible in vitro binding of morphine with plasma and brain proteins.

Materials and methods. Incubation mixture in 10 ml tubes unless otherwise mentioned, consisted of 5 ml *M*/15 phosphate buffer pH 7.4, 0.2 ml morphine-C^{14} (50 µg/ml) or codeine-C^{14}, or dihydromorphine-H^3, 100 λ $MnCl_2$ (19.8 mg/ml), 100 λ horseradish peroxidase (Sigma, Type II, 135 units/mg; 1 mg/ml), 10 mg human albumin (Sigma, Grade III) or other peptides or amino acids, under oxygen with shaking for 6 h at 37°C, allowing mixture to stand overnight at ambient temperature before extraction. Effect of metabolic inhibitors, nalorphine, 2,4-diamino 5-phenyl thiazole and biogenic amines, etc. were studied in the above 'complete system'. Peptides, amino acids, RNA (Sigma, Type XI, yeast) and DNA (Sigma, Type V, calf thymus, sodium salt) were substituted for albumin and cytochrome C (Sigma, Type VI), mushroom tyrosinase (Sigma, Grade III, 1200 units/mg) and catalase (Sigma C-10, bovine liver, 3000 units/mg) for peroxidase in this system. 5 ml rat plasma or 5 ml rat brain homogenate in phosphate buffer pH 7.4 were substituted for buffer and albumin.

Free morphine was estimated by basifying 2 ml aliquots of incubates and 0.5 ml nonradioactive morphine carrier (500 µg) to pH 9 with dil. NaOH, buffering with 2 ml, 40% K_2HPO_4 solution, extraction by shaking with 15 ml ethylene dichloride containing 30% by volume of n-amyl alcohol, washing the organic phase with 4 ml K_2HPO_4 solution, dissolving the residue from 10 ml organic phase in 0.8 ml n-amyl alcohol and counting with 10 ml toluenephosphor in a liquid scintillation counter. The percentage radioactivity remaining in aqueous phase was calculated from the equation 100 $[(A\text{-}B)/A]$ where A is CPM obtained on extraction of a 2 ml aliquot of incubate of buffer, morphine-C^{14} and requisite amount of water and B that from an actual experiment.

Results and discussion. The pH profile of morphine-peroxidase-albumin-Mn^{++} interaction showed the maxima between 6 and 7. Metal ions had a profound effect on this interaction, Mn^{++}, Fe^{++}, Co^{++}, Fe^{+++} promoted, Cu^{++} and Ca^{++} decreased the yield of water-soluble products while Zn^{++} and Mg^{++} had no effect. Hydrogen peroxide in place of Mn^{++} also gave a considerably high yield and anerobic incubation inhibited the formation of interaction products (Table I). Sodium azide completely inhibited this interaction and inactivation of peroxidase for different time periods in a bath at 100°C led to a reduction in percentage of interaction products (Figure 1). No inhibition was noticed in presence of catalase (10-5000 µg), disodium EDTA, N-ethyl maleimide or urea in incubation mixture. In absence of protein (Table I) the percentage of water-soluble products was very low showing that proteins, peptides and amino acids were involved in a process different from simple adsorption or electrostatic binding. L-tyrosine, L-histidine, L-phenylalanine, αβ-globulin, L-tryptophan, L-cystine, L-hydroxyprolin, reduced and oxidized glutathione all gave high yields of interaction products (74–95%); glycine, L-glutamic acid, L-aspartic acid, L-dopa and L-cysteine showed little or no interaction and other naturally occurring amino acids gave intermediate values. The necessity of a large excess of protein (Figure 2), peptides or amino acids suggested that specific groups of proteins e.g. tryptophane, tyrosine, etc. probably participated in the reaction. The formation of a chemical bond in interaction between morphine and protein was inferred from the fact that acid hydrolysis with 2.4 *N* HCl of dialyzed incubation mixture for 1 h at 15–20 lbs. pressure did not release significant amounts (3–5%) of morphine, an observation that further ruled out the possibility of interaction of unchanged morphine with peroxidase-activated protein. Similar interaction occurred with dihydromorphine-7, 8-H^3 but not with codeine-N-methyl-C^{14} (Table I).

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Table I. Percentage distribution of radioactivity after incubation of morphine-N-methyl-C^{14}, codeine-N-methyl-C^{14} and dihydromorphine-H^3 with artificial systems in absence and presence of peroxidase

Experimental conditions[a]	Radioactivity in aq. phase after morphine extraction (%)
1. Buffer + morphine-C^{14} + water	nil
2. Buffer + morphine-C^{14} + water + human albumin	nil
3. Buffer + morphine-C^{14} + $MnCl_2$	nil
4. Buffer + morphine-C^{14} + $MnCl_2$ + human albumin	7.5
5. Buffer + morphine-C^{14} + Peroxidase + human albumin	18.3
6. Buffer + morphine-C^{14} + Peroxidase + $MnCl_2$	23.5
7. Buffer + morphine-C^{14} + Peroxidase + $MnCl_2$ + human albumin	85.7
8. Buffer + morphine-C^{14} + Peroxidase + H_2O_2 + human albumin	85.1
9. Same as (7) under anaerobic condition	9.0
10. Buffer + codeine-C^{14} + Peroxidase + $MnCl_2$ + human albumin	< 3
11. Buffer + dihydromorphine-H^3 + Peroxidase + $MnCl_2$ + human albumin	80.0

[a] Incubations done using 5 ml *M*/15 phosphate buffer pH 7.4, 0.2 ml morphine-C^{14} or codeine-C^{14} or dihydromorphine-H^3 (50 µg/ml), 10 mg human albumin, 100 λ peroxidase (1 mg/ml), 100 λ $MnCl_2$ (19.8 mg/ml) or 50 λ H_2O_2 (30%) under oxygen with shaking at 37°C for 6 hours, allowing solutions to stand at ambient temperature overnight before extraction.

RNA and DNA (1–10 mg in place of albumin) also acted as carriers to bind reactive intermediates of morphine in the reaction. Cytochrome C, mushroom tyrosinase and purified catalase at same concentration could not replace peroxidase in this interaction. Freshly prepared rat plasma and supernatant from rat brain homogenate prepared in phosphate buffer pH 7.4 gave high yields of interaction products on incubation with morphine-C^{14}, Mn^{++} and peroxidase, but rat brain homogenate itself gave invariably much lower values (11–14%).

5-Hydroxytryptamine even at low molar ratios to morphine (1:1) significantly blocked morphine-peroxidase-albumin interaction; dopamine and to a much lesser extent norepinephrine and epinephrine, were effective only at much higher molar ratios while histamine, acetylcholine and tryptamine showed no effect (Table II).

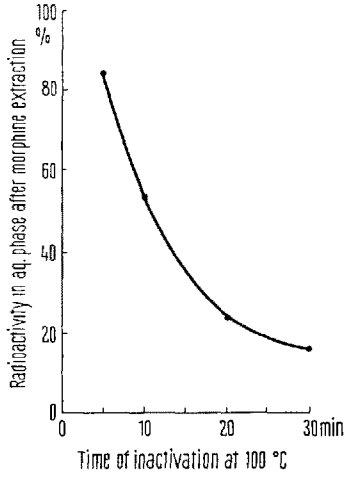

Fig. 1. Rate of inactivation of peroxidase in boiling water as measured by decrease in concentration of water-soluble products formed from morphine-N-methyl-C^{14}.

Table II. Effect of biogen icamines on morphine-peroxidase-albumin interaction

Added substances	Percentage radioactivity in aq. phase after morphine extraction at different molar conc. ratios of amines to morphine					
	0:1	1:1	5:1	10:1	100:1	1000:1
No amines	85.7					
L-norepinephrine		85.3	86.0	74.4	17.9	9.1
L-epinephrine		85.2	87.4	89.2	22.5	9.5
Dopamine		85.5	85.4	40.9	9.5	—
5-hydroxytryptamine		7.0	3.5	3.25	0	—

Biogenic amines were incorporated at different concentrations in incubation medium. Histamine at (600:1), acetyl choline at (700:1) and tryptamine at (100:1) molar concentration ratio to morphine had little or no effect on interaction.

Intracisternally administered Ca^{++} ions markedly suppress morphine analgesia[2] in mice and 2,4-diamino-5-phenyl thiazole hydrobromide (DAPT) administered in conjunction with morphine has been reported[3,4] to counteract narcosis, respiratory depression, intensity of abstinence syndrome and tolerance development but leave the morphine analgesia unimpaired. The blocking effect of DAPT and nalorphine at various concentration ratios to morphine in incubation mixture on morphine-peroxidase interaction is shown in Figure 3. Presence of levorphan or dextrorphan even at 10:1 molar concentration ratio to morphine had no effect on interaction.

Two distinct possibilities[5,6] a) formation of a phenoxy free radical, relocalization of lone electron into aromatic ring and coupling of morphine molecule in 2 position to albumin; b) aromatic hydroxylation of morphine in position 2 to form a catecholyl type of derivative and its subsequent binding to protein or peptide carriers, may be involved in morphine-peroxidase-albumin-Mn^{++} interaction (N-oxide formation ruled out by TLC). Interactions of quinones (or catechols) to proteins[7] are known to take place readily and the consequence of such an interaction in the development of central receptor tolerance to morphine have been mentioned[1]. Further, the simple model system brings out the antagonism of nalorphine, regulatory effects of trace metal ions and certain biogenic monoamines on morphine-peroxidase-albumin interaction and appears interesting in view of occurrence of peroxidase[8] and various trace metals[9–11] in brain and a wide variety of tissues and importance of such metals in maintenance of integrity of myelin sheath of nerve fibers, synaptic transmission, tissue metabolism, enzyme activation and inactivation[12].

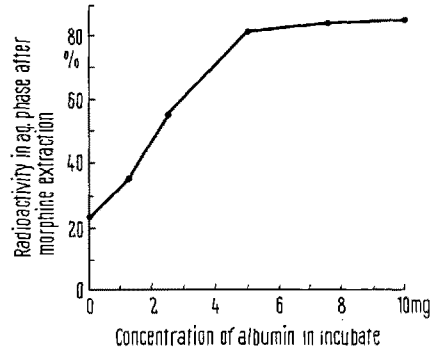

Fig. 2. Effect of different concentrations of human albumin on conversion of morphine-N-methyl-C^{14} to water-soluble products by peroxidase.

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[12] This work was supported by U.S. Public Health Service Grant No. NB-02928. The authors thank Mr. Kevin Kingsbury for skillful technical assistance.

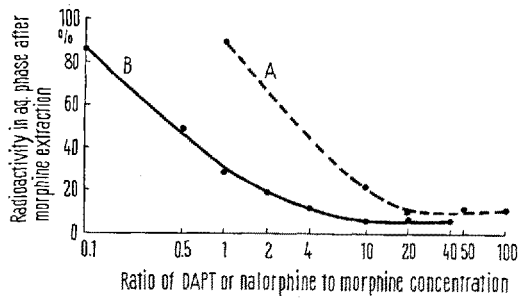


Fig. 3. Effect of different concentrations of 2,4-diamino-5-phenyl thiazole hydrobromide DAPT (A) and nalorphine (B) on conversion of morphine-N-methyl-C^{14} to water-soluble products by peroxidase.

Zusammenfassung. In Modellsystemen von Peroxidase, Proteinen und Metallionen wird Morphium in wasserlösliche (Radikal-)Produkte übergeführt, welche Bindungen mit dem Protein eingehen.

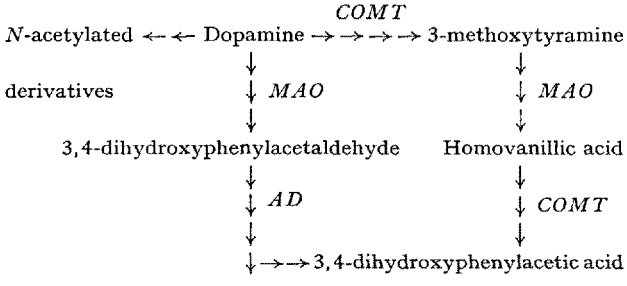
A. L. MISRA[13] and C. L. MITCHELL

Department of Pharmacology, University of Iowa, Iowa City, Iowa 52240 (USA), 21 June 1971.

[13] Present address: New York State NACC Testing and Research Laboratory, 80 Hanson Place, Brooklyn, New York 11217 (USA).

Iproniazid (Marsilid) Reverses Dopamine-Effect and Stimulates the Growth of Mouse Neuroblastoma in vitro

Dopamine markedly inhibits the cell division of neuroblastoma (NB) cell in vitro. On removal of drug 1 h after treatment, the inhibition of cell multiplication persists for 24 h after which dopamine-treated cells grow with same doubling time as that of controls. The precursor of dopamine (L-dopa), metabolite of dopamine (homovanillic acid), norepinephrine or epinephrine produces no such effect[1]. Dopamine at a similar concentration does not affect the cell division of Chinese Hamster-ovary-K1 and Baby-hamster kidney-21 cells in vitro[1]. To elucidate the mechanism of dopamine-effect further, the inhibitor of dopamine metabolism is used in this investigation. The metabolic pathway of dopamine in mammalian cells is shown diagramatically below:



Dopamine is deaminated by monoamine oxidase (MAO) to form aldehyde which is rapidly oxidized by aldehyde dehydrogenase (AD) to produce 3,4-dihydro-xyphenylacetic acid which is converted to homovanillic acid by catechol-*O*-methyl-transferase (COMT). The other metabolic pathway is self explained in the diagramatic presentation. This paper shows that iproniazid (marsilid), an inhibitor of monoamine oxidase, completely reverses dopamine-effect on neuroblastoma cells and by itself stimulates the cell division of neuroblastoma as well as of Baby-hamster kidney cells in vitro. Pyrogyllol, an inhibitor of COMT, markedly reduces the growth of neuroblastoma cells.

Materials and methods. The procedure for culturing and the morphological features of neuroblastoma cell line has been described in the previous publication[1]. When grown in Falcon plastic flask, the average doubling time of neuroblastoma cells is 24 h. This cell line has acetylcholinesterase activity, but no butyrylcholinesterase, indicating its neuronal feature[2]. In addition, tyrosine hydroxylase, a rate limiting enzyme in the biosynthesis of catecholamine is present in the primary tumor[3,4], but is lost in the long-term culture; however the enzyme activity is restored in the dibutyryl cyclic AMP-induced differentiated neuroblastoma cells (PRASAD, WAYMIRE and WEINER, in preparation).

Neuroblastoma cells were treated with marsilid or pyrogyllol 24 h after plating. Marsilid was dissolved in F12 medium without serum immediately before the experiment and added to neuroblastoma cells in vitro at a concentration of 100 µg/ml. Dopamine, 3'4' dihydroxyphenylacetiacid (DPAA) or pyrogyllol was dissolved in the F12 medium without serum containing 1 mg/ml of ascorbic acid immediately before the experiment. Dopamine was added to neuroblastoma cell culture 18 h after the addition of marsilid or 1 h after the addition of pyrogyllol to give a final concentration of dopamine (50 and 100 µg/ml) and of ascorbic acid (20 µg/ml). DPAA was added to neuroblastoma cells (50 and 100 µg/ml) 24 h after plating. After 1 h of incubation, cells were washed twice with F12 without serum and fresh growth medium was added. Control cell population was treated similarly except no drug was added. The addition of ascorbic acid was necessary to prevent the auto-oxidation of dopamine in vitro. Ascorbic acid by itself had no effect on neuroblastoma cells. The cell number 2 days after dopamine-treatment was counted by a Coulter counter. The growth inhibition of neuroblastoma cell population in vitro was calculated as follows:

$$\frac{\text{No. of control cells} - \text{No. of drug-treated cells}}{\text{No. of control cells}} \times 100.$$

If the number of drug-treated cells was greater than the control, the value obtained by the above formula was considered as an index of growth stimulation.

BHK-21 cells in vitro were grown under conditions identical to those of neuroblastoma cells. Marsilid (100 µg/ml) was added to BHK-21 cell culture 24 h after plating (50,000 cells). After 18–20 h of incubation, cells were

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