INCORPORATION OF MANNOSE INTO MOUSE BRAIN LIPID

M. Zatz and S.H. Barondes

Departments of Pharmacology, Psychiatry and Molecular Biology

Albert Einstein College of Medicine,Bronx,New York 10461

Received June 20, 1969

SUMMARY:

Microsomal fractions from mouse brain incorporate mannose-C^{14} from GDP-mannose-C^{14} into lipid material. The extent of incorporation is limited primarily by the endogenous acceptor. The incubation mixture includes phosphoenol pyruvate and Mn^{++}. Protease has no effect on the product. The mannolipid is extremely sensitive to acid but is resistant to base.

Mammalian brain is rich in glycoproteins. Although these lipids have been extensively investigated, none has been described which contains mannose. Several mannolipids have, however, been described in bacterial systems[1,2]. This communication describes the enzymatic incorporation of mannose from guanosine diphosphate mannose-C^{14} (GDP-mannose-C^{14}) into lipid material by a microsomal preparation from mouse brain.

MATERIALS AND METHODS:

GDP-mannose-C^{14} (151 mC/mM) was purchased from the New England Nuclear Corp. Paper chromatography with isobutyric acid: 1N NH_4OH, 5:3 showed only one peak, corresponding to GDP-mannose standard and containing over 98% of the radioactivity spotted. D-mannose-1-C^{14} (48.8 mc/mM) was purchased from International Chemical & Nuclear Corp.

Male Swiss albino mice (25-30 gm., 3 months old, from The Charles River Breeding Co.) were decapitated and their cerebral hemispheres were removed and homogenized in 0.32M sucrose. Microsomes were prepared[3] and resuspended

---

Abbreviations used: C, chloroform, M, methanol, HAc, acetic acid, PEP, phosphoenolpyruvic acid, TCA, trichloroacetic acid, PTA, phosphotungstic acid.

in 10 ml 0.32M sucrose. Reaction mixtures routinely contained 0.5 ml of this preparation (approximately 350 μg protein).

Mannolipid was prepared by incubation of the reaction mixture described in Table 1. The acid insoluble products were precipitated and washed at least twice with 6% TCA-0.5% PTA and the pellet extracted twice with 2 ml chloroform-methanol 2:1, v/v. The organic extracts were washed with 0.2 volumes of 0.9% NaCl and then with 0.2 volumes of the theoretical upper phase as described by Folch, Lees, and Sloane-Stanley[4]. The distribution of radioactivity was followed by liquid scintillation counting.

TABLE 1 - Incubation of Mouse Brain Microsomes with GDP-Mannose-C^{14}.

Reaction Mixture	cpm Incorporated
Complete system	1120
$-Mn^{++}$	232
$-Mn^{++}$, $+ Mg^{++}$	672
- PEP	78
- PEP, + pyruvate	308
- PEP, + ATP	46
- PEP, + $PO_4^=$	721
- GDP-mannose-C^{14}, + mannose-C^{14}	90

The complete reaction mixture contained , in the final volume of 1 ml, 5μM $MnCl_2$; 15μM Tris HCl pH 7.4; 0.5μM PEP; 20mμC GDP-mannose-C^{14} (approximately 20,000 cpm) 0.16 mM surcose; and 0.5 ml of microsomes. In other reactions $MnCl_2$ was replaced by 5μM $MgCl_2$; PEP by 5μM pyruvate, 2μM ATP, or 5μM phosphate buffer pH 7.4. In still other reactions GDP-mannose-C^{14} was replaced by 200 mμC mannose-1-C^{14} (48.8 mC/mM). Reactions were incubated for 60 minutes at 37°. Reactions were stopped by bringing the reaction mixture to 5 ml 6% TCA-0.5% PTA. After precipitation at 0° the mixture was run through a Millipore HA filter and washed 4 times with 6% TCA-0.5% PTA. The filter was placed in a counting vial, scintillation fluid was added, and the sample counted in a liquid scintillation counter.

Protease treatment consisted of adding 1 ml (200 μg/ml) of repurified protease from Streptomyces griseus (Sigma-Type IV) in 0.2 phosphate buffer, pH 7.3 to the reaction mixture after 30 minutes incubation and then counting the incubation for 2 hours[8] at 37°. At the end of the incubation 1 mg of albumin was added to experimental and control tubes, and analysis proceeded as described above.

Thin layer chromatography was performed on 20x20 cm glass plates coated with 0.25 mm silica gel (Brinkmann). Solvent systems used were (A)C:M:H_2O, 65:25:4; (B)[2] C:M: 7N NH_4 OH, 12:60:10; (C) C:M: 15N NH_4OH, 75:25:4; (D)[2] C:M HAc:H_2O, 60:40:3:5; (E)[5,6] C:M:HAc:H_2O, 60:30:8:4; and (F)[5] C:M:HAc: H_2O, 40:25:3:4. Systems E and F were run on Silica gel H, the other systems on Silica gel G.

Descending paper chromatography was performed using solvent systems (G) butanol:pyridine: 0.1N HCl, 5:3:2; (H)[7] isobutyric acid: 15N NH_4OH: H_2O, 57:4:39; (I)[2] ethyl acetate:pyridine: H_2O, 12:5:4 , and (J) phenol:H_2O, 5:2.

Mild base hydrolysis was performed by adding 0.8 ml of 0.125N NaOH in methanol to the sample in 0.2 ml chloroform-methanol and incubating for 20 minutes at 37°. Three ml water were added, the pH was above 11; and the solution was neutralized with Dowex 50-H^+. After removal from the resin, 2 ml of EtOH and then 4 ml of $CHCl_3$ were added, the mixture shaken, and the phases separated by brief centrifugation.

RESULTS AND DISCUSSION:

Requirements for the incorporation of mannose into acid precipitable

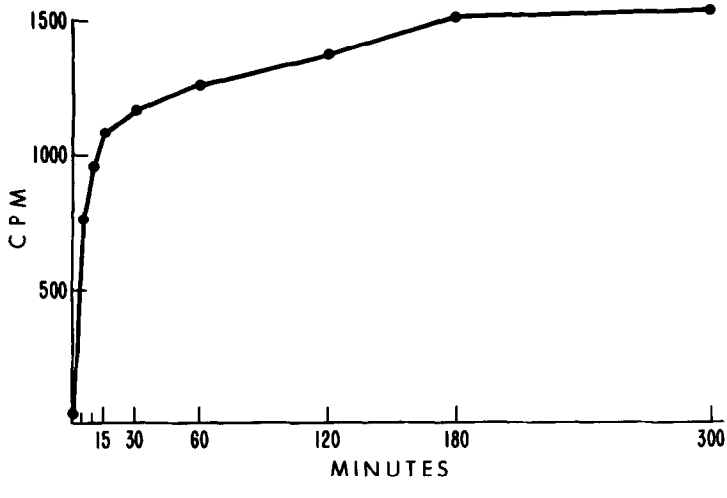

FIGURE 1 - Time Course of Mannose Incorporation into a Lipid Soluble Fraction. Reaction mixtures were those of the complete system described in Table 1; except that they contained 5μM Tris buffer. Reactions were incubated at 37° for various times, stopped, and assayed as described in Table 1.

material by mouse brain microsomes are shown in Table 1. Mn^{++} is required for maximal activity and cannot be replaced by Mg^{++}. The role of PEP is unexplained. Since inorganic phosphate was more effective than pyruvate in substituting for PEP, it may be acting as a phosphate donor. Incorporation is inhibited by ADP, ADP + pyruvate kinase, or ATP. ATP, GTP, UTP, and CTP each inhibit incorporation in the presence of PEP, and none compensates for its absence. GDP-mannose cannot be replaced as the mannosyl donor by mannose, even at much higher concentrations.

The time course of incorporation is shown in Figure 1. It has reached 50% of maximum in less than 5 minutes and 75% of maximum in 15 minutes. The extent of incorporation was linear with microsome concentration up to about 1 mg of microsomal protein (Figure 2). Figure 3 illustrates the effect of GDP-mannose-C^{14} concentration on the extent of incorporation after 30 and 60 minutes incubation. If additional GDP-mannose-C^{14} is added to the reaction mixture after 60 minutes incubation, there is no increase in incorporation. Incubation without GDP-mannose for 60 minutes did not destroy enzyme activity. These data indicate that the incorporation of mannose by this system is limited primarily by the quantity or accessibility of endogenous acceptor. This acceptor is calculated to be present in quantity sufficient to accept approximately 30 μμM mannose per mg of microsomal protein.

Almost all of the radioactivity incorporated into acid precipitable material is lipid soluble. Over 90% of the radioactivity is extracted into the organic solvent after treatment with a small volume of chloroform-methanol 2:1 or ethanol:ether 1:1. This is true after incubation for 5 minutes, 30 minutes, or 60 minutes. Tris buffer pH 7.4 fails to extract the radioactivity from the acid pellet. Polar lipids are known to coprecipitate with proteins in TCA and require solvents such as methanol or ethanol to release them from their association with proteins. Neither chloroform nor petroleum ether readily solubilized the

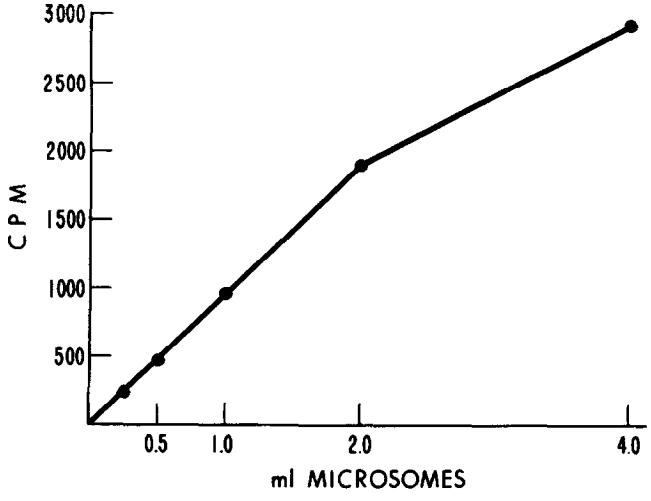

FIGURE 2 - Effect of Microsome Concentration on the Extent of Incorporation. Reaction mixtures contained 5μM $MnCl_2$, 5μM PEP, 20mμC GDP-mannose-C^{14} and varying quantities of microsome suspension. Microsomes from 2 brains were suspended in 10 ml 0.32M sucrose and an aliquot was diluted with an equal volume of 0.32 surcose. All reactions were brought to a final volume of 2.24 ml with 0.32M sucrose. Incubation was for 60 minutes at 37° and incorporation was assayed as described in Table 1. The abscissa refers to volume equivalents of the more dilute microsomal suspension.

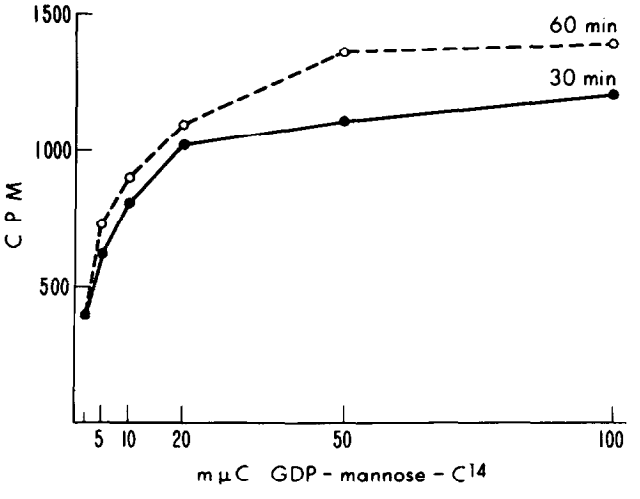

FIGURE 3 - Substrate Curve. Reaction mixtures were as described for Figure 1, except that the quantity of GDP-mannose-C^{14} was varied. Volumes were brought to 1 ml with water. Reactions were stopped after 30 minutes (● -- ●) or 60 minutes (0 -- 0) and assayed as described in Table 1.

incorporated radioactivity from the acid pellet.

Non-lipid material, including proteinacious material, may accompany lipids into organic solvents. Treatment of the reaction mixture after 30 minutes incubation with protease did not reduce the extent of incorporation of radioactivity into acid precipitable material. Furthermore, protease treatment did not reduce the amount or proportion of radioactivity extractable from the acid pellet into chloroform-methanol.

The mannolipid synthesized by mouse brain microsomes is quite labile to acid. All of the radioactivity was released from the mannolipid-C^{14} by hydrolysis in 1N HCl (60 minutes at 100°). Chromatography in three solvent systems (G,H,I) revealed only mannose. Mild acid hydrolysis (0.02NHCl, for 2 minutes at 100°) released about 85% of the radioactivity as mannose (System I). There were traces of material which migrated as mannose phosphate. Unhydrolysed material ran as a single spot beyond fucose in system J but in the acidic system G, 50% of the radioactivity was released and ran with mannose. This acid lability is also consistent with a phosphodiester bond.

The mannolipid synthesized by mouse brain differs from the mannophosphoinositides of Mycobacteria [1,5,6] and from the mannosyl diglycerides of Micrococcus lysodeikcticus[2] in its mobility in the thin layer chromatographic systems used for the analysis of those lipids. It further differs in its resistance to saponification by mild base. The small proportion of material that partitioned into the upper phase after treatment with 0.1N NaOH gave the same single peak in system J as did the unhydrolysed material in the lower phase.

Caccam, Jackson and Eylar[9] have synthesized a mannolipid using particulate fractions from liver, oviduct, and tumor cells. They consider it to be similar to the mannosyl-1-phosphoryl-polyisoprenol precursor of mannan biosynthesis described by Scher, Lennarz, and Sweeley[7], and suggest that it may be a precursor for soluble mannose containing glycoproteins. We have not observed significant glycoprotein synthesis in our system.

However, the rapid rate of synthesis and the fact that mannose-containing lipids have not been observed in mammalian systems may indicate that this mannolipid is an intermediate in the synthesis of some other product. Whether the mannolipid synthesized by mouse brain microsomes is the same as that synthesized by the preparations from Micrococcus lysodeikticus[7] or from liver[9] is currently not known.

Acknowledgment: - This work was supported by grants MH 12773, NIH 5T5 GM 1674, and Career Development Award K3-MH 18232 from the U.S.P.H.S.

REFERENCES:

1. Lee, Y.C.,and Ballou, C.E., Biochem. 4, 1395 (1965).
2. Lennarz, W.J.,and Talamo, B., J. Biol. Chem., 241, 2707 (1966).
3. Barondes,S.H., J. Neurochem., 15, 343 (1968).
4. Folch, J., Lees, M. and Sloane-Stanley,G.H., J. Biol. Chem., 226, 497 (1957).
5. Brennan, P., and Ballou, C.E., J. Biol.Chem., 242 3046 (1967).
6. Brennan,P., and Ballou, C.E., Biochem. Biophys. Res. Communs., 30, 69 (1968).
7. Scher, M., Lennarz,W.J., and Sweeley, C.C., Proc. Natl. Acad. Sci.U.S. 59, 1313 (1968).
8. Barondes,S.H., J. Neurochem., 15, 699 (1968).
9. Eylar, E.H., personal communication (1969)