THE EFFECT OF NON-IONIC DETERGENTS AND PHOSPHOLIPASE A ON ENZYMES INVOLVED IN ADULT RAT BRAIN STEROL BIOSYNTHESIS FROM (2-^{14}C)-MEVALONIC ACID IN VITRO

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SUMMARY

Incubation of (2-^{14}C)-mevalonic acid with 3000 x g adult rat brain supernate in the presence of Tween 80 or Triton X-100 produced a marked shift in the distribution of radioactivity among squalene, steryl ester and free sterol when compared to controls. Although the conversion of labeled mevalonate to cholesterol was only slightly influenced, the detergents markedly affected the turnover of squalene and methyl sterol precursors. The major neutral isoprenoid material formed had the general properties of geranylgeraniol on TLC and radioactivity-monitored-GLC. Phospholipase A from Vipera russelli, at an optimal tissue to phospholipase ratio, resulted in a 25% increase in mevalonate incorporation into total neutral isoprenoids. An increase in radioactivity of almost 25% was also seen in the free sterol fraction. Phospholipase A from bee venom did not show this type of increase. No labeled geranylgeraniol-type material was found on incubation with either of the phospholipases.

INTRODUCTION

Extensive studies by Gaylor's laboratory (1) in separating, isolating and purifying components of the liver cholesterol enzymatic biosynthetic sequence via the use of ionic, non-ionic detergents and other agents prompted us to attempt the same with brain tissue. As a preliminary step, we felt it necessary to first examine the overall effect of these agents on the brain sterol biosynthetic sequence. The addition of Tween 80 and Triton X-100 to 3000 x g adult rat supernate incubated with (2-^{14}C)-mevalonic acid produced rather surprising results which we wish to report here as a preliminary to more extensive studies in progress. Some results with phospholipase A using similar experimental conditions are also given.

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MATERIALS AND METHODS

Materials - Adult rats of a Wistar-derived strain, were purchased from National Animal Co., St. Louis, Missouri. All cofactors, as well as the Tween 80, Triton X-100 and Phospholipase A, were purchased from Sigma Chemical Co., St. Louis, Missouri. Two types of phospholipase A were examined. One, from Vipera russelli, had an activity of 4 units/mg protein at pH 6.5 and 37°. The second, from bee venom, had an activity of 1550 units/mg protein at pH 8.5 and 37°. The (2-^{14}C)-DL-mevalonic acid (sp. act. 5.82mCi/mmole, as the DBED salt) was purchased from New England Nuclear, Boston, Massachuesetts. Reference synthetic trans-geranylgeraniol was a gift of Dr. Charles A. West. Reference farnesol was obtained from Fritzsche Bros., Inc., New York.

Tissue preparation and incubation - Cell-free 3000 x g supernates were prepared as previously described (2). In several experiments designed to test for microsomal enzyme solubilization, the 3000 x g supernate was pre-incubated for 20 min at 4° in the presence of Tween 80 or Triton X-100. The resultant material was then centrifuged at 15,000 x g for 20 min to remove mitochondria, and the supernate was further fractionated into microsomal and soluble fractions by centrifugation at 112,000 g for 2 hr (3).

Each incubation contained tissue equivalent to 0.5 g wet wt of original whole brain tissue plus (2-^{14}C)-mevalonic acid in the quantities indicated. The following cofactors were also added to each of the Tween 80 and Triton X-100 incubations in 0.5ml of buffer:ATP 4.5 μ moles; NADH, 7.0μ moles; $MgCl_2.6H_2O$; 24.6 μ moles; glucose-6-phosphate; 17.7 μ moles; and reduced glutathione (16.3 μ moles). Half these quantities were utilized for the phospholipase A experiments. The samples were incubated at 37° in a shaking incubator for 20 hr. Incubations were terminated by the addition of benzene. Previous

examination (2) indicated that the metabolism of the labeled precursor was not due to bacterial or other contamination over the lengthy incubation periods. All data presented are the mean of at least two experiments.

Lipid analysis. Neutral lipid fractions were prepared and alumina column chromatography performed as previously described (2,3).

The ^{14}C-free sterol-isoprenoid alcohol fractions were examined on TLC using a system described by Rahman et al (4). Areas of interest on TLC were scraped directly into scintillation vials for radioactive determination as described (2); ^{14}C-Cholesterol content was determined by cholesterol dibromide formation (5).

GLC examination of the ^{14}C-free sterol-isoprenoid alcohol fractions were performed on a Barber-Colman Model 5000 gas-liquid chromatograph equipped with a radioactive monitoring system(6). Separation of sterols was achieved on a 3% OV-17 column at 260°. The material thought to be geranylgeraniol was chromatographed on 3% OV-17 at 155° and 205°, and on 3% SE-30 at 190°.

RESULTS

Effect of detergents. Varying quantities of Tween 80 and Triton X-100 were added to adult rat brain 3000 x g supernates incubated with (2-^{14}C)-mevalonic acid (Table I). It is apparent that detergent concentration was significant, with optimum concentration evident. All concentrations of Tween 80 depressed incorporation into total neutral isoprenoids. In contrast, Triton X-100 had a variable effect, 100 mg per incubation apparently most markedly affecting total incorporation. Tween 80 at concentrations of 30 mg per incubation and higher decreased the amount of squalene and steryl ester formed but increased the total ^{14}C-content of free sterol and the isoprenoid alcohols. The ^{14}C-cholesterol content was not increased by any concentration but a concentration effect of Tween 80 was evident. With Triton X-100 the con-

TABLE I. Incubation of Adult Rat Brain Cell-Free Preparations with (2-^{14}C)-Mevalonic Acid in the presence of Tween 80, Triton X-100 or Phospholipase A.

Addition		Total Neutral	Squalene	Steryl Esters	Free Sterol Fraction	Cholesterol
	mg	dpm	dpm	dpm	dpm	dpm
Tween 80	0	279,000	96,000	123,000	59,000	2580
	30	98,700	1,320	34,600	62,800	280
	60	134,000	1,600	35,800	96,200	560
	100	187,000	2,070	26,600	158,000	1870
	250	166,000	1,900	15,300	149,000	-
	500	156,000	1,950	3,600	150,000	-
Triton X-100						
	0	135,000	19,200	36,400	79,200	-
	0.1	108,000	30,100	41,600	36,900	-
	1.0	10,600	0	3,360	7,200	-
	10	63,200	0	7,780	55,400	-
	100	126,000	0	1,580	124,000	-
	250	54,000	0	440	53,500	-
	500	29,700	0	530	29,200	-
Control For Phospholipases		93,000	5,990	19,400	68,400	814
Bee Venom Phospholipase A	Units					
	31	24,500	0	16,200	8,290	-
	6.2	28,300	0	18,400	9,930	-
	0.62	44,100	431	26,700	16,900	-
	0.062	99,000	5,840	15,200	78,000	527
Vipera russelli Phospholipase A						
	0.040	90,500	102	75,200	15,200	0
	0.020	76,700	563	49,200	27,000	66
	0.0040	115,000	6,400	23,200	85,400	650
	0.00040	87,000	7,270	23,600	56,100	312

Each incubation contained brain 3000 x g supernate equivalent to 0.5g of wet tissue. The Tween 80 experiments contained 5μCi of (2-^{14}C)-mevalonic acid, the Triton X-100 and phospholipases, 1μCi. Total volume of each detergent incubation was 3.4 ml, of each phospholipase incubation 2.0 ml.

centration effect on free sterol and other isoprenoid alcohols was more variable. The effect on ^{14}C-cholesterol content was not determined.

Examination of the free sterol-isoprenoid alcohol fractions from the detergent experiments by TLC indicated the bulk of the radioactivity chromatographed in the 4,4-dimethyl, 4α-methyl sterol regions (R_f, 0.53-0.69). The same was true of control samples. Examination of the ethanol fractions on radioactivity-monitored GLC indicated the usual mixture of ^{14}C-methyl sterols for the controls (6). However, the Tween 80 ethanol fractions gave one radioactive peak, which at 260° on 3% OV-17 appeared at the solvent front. When the column temperature was lowered to 155° no radioactive peak could be eluted in a 20 min period. Trans, trans-farnesol, a C_{15} isoprenoid alcohol, at this temperature had a retention time of 3.2 min. When the 3% OV-17 column temperature was raised to 205° a single radioactive peak was found with a retention time of 3.1 min. Because of its high specific activity, no mass peak was seen for this radioactive peak. Unlabeled reference geranylgeraniol cochromatographed with this radioactive peak. Acetylation of the radioactive material in the presence of cold geranylgeraniol with acetic anhydride-pyridine resulted in mass and radioactive peaks having retention times of 4.3 min on 3% OV-17 at 205°. A 3% SE-30 column, operated at 190°, yielded cochromatographing mass and radioactive peaks also, with the free compound retention time being 2.9 min and the acetate 4.1 min. The same results were found for the Triton X-100 ethanol fraction.

Pre-incubation of 3000 x g supernates was performed with Tween 80 and Triton X-100 (100 mg quantities of either detergent /0.5 g equivalent of wet tissue) for 20 min as described, followed by fractionation into 112,000 x g supernate and microsomes and subsequent incubation of these subcellular fractions in the same manner as the cell-free prepara-

tions in Table I. No significant incorporation of mevalonate into the microsomes was found but incorporation comparable to cell-free preparations into the supernate occurred. The radioactive material present in this supernate was, however, identical to that of the cell-free preparations; namely, the labeled isoprenoid alcohol having this radioactivity monitored GLC properties of geranylgeraniol.

Phospholipase A addition. Varying quantities of phospholipase A derived from two different sources were added to adult brain cell-free preparations in the same manner as the detergents (Table I). High concentrations of both added enzymes reduced overall incorporation of 2-^{14}C-mevalonic acid. Steryl ester fractions tended to be proportionately enriched in comparison to control values, particularly with the Vipera russelli phospholipase A. A concentration of 0.0040 units of this latter enzyme /0.5g equivalent wet wt of tissue was particularly interesting. The level of ^{14}C-total neutral isoprenoid and ^{14}C-free sterol formed was approximately 25% greater than control values. Enzyme concentrations intermediate to those shown have not as yet been examined. TLC examination of the steryl ester fraction verified that it was steryl ester. Unlike the detergent experiments, no labeled isoprenoid alcohol such as geranylgeraniol was found in the free sterol fractions. TLC distribution of label among the major sterol classes indicated little variation from controls. Increased incorporation brought about by 0.0040 units of Vipera russelli phospholipase A did not lead to increased formation of labeled cholesterol.

DISCUSSION

The importance of hydrophobicity to membrane structure is becoming increasingly apparent (7,8). Disrupting this hydrophobicity by means of detergents or phospholipase A has made possible the solubilization and partial purification of liver microsomal enzymes involved in cholesterol biosynthesis (1). Due to the limited extent to which

neural tissue forms sterol in vitro (2) we too have turned to such agents to stimulate, if not solubilize, the particulate enzymes of brain sterol biosynthesis. One particulate brain enzyme, squalene-2, 3-epoxide-lanosterol cyclase, has already been shown to be stimulated by deoxycholate, as is the liver enzyme (9).

The data herein presented suggests a new approach to isoprenoid synthesis by brain. The isolation from the Tween 80 and Triton X-100 experiments of what appears to be labeled geranylgeraniol, or at least a C_{20} isoprenoid alcohol, is difficult to explain. It may be merely an artifact of experimental conditions. Its formation by 112,000 x supernate suggests that whatever the origin of the compound, its formation is promoted by the detergents' modification of a soluble enzyme.

The addition of phospholipase A to whole enzyme systems, appears to have more promise than the detergents. The overall formation of free sterol, but not cholesterol, was stimulated by 25%. Additions of other agents to this system may increase this stimulation.

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REFERENCES

1. Gaylor, J. L. (1972) in Advances in Lipid Research, Vol. 10, (R. Paoletti and D. Kritchevsky, eds.) p. 89-141, Academic Press, New York.

2. Kelley, M. T., Aexel, R. T., Herndon, B. L. and Nicholas, H. J., (1969), J. Lipid Res., 10, 166-174.

3. Ramsey, R. B., Jones, J. P., Naqvi, S.H.M. and Nicholas, H. J., (1971), Lipids, 6, 154-161.

4. Rahman, R., Sharpless, K. B., Spencer, T. A. and Clayton, R. B. (1970), J. Biol. Chem. 245, 2667-2671.

5. Schwenk, E. and Westhessen, N. T. (1952), Arch. Biochem. Biophys., 40, 334-341.

6. Ramsey, R. B., Aexel, R. T. and Nicholas, H. J. (1971), J. Biol. Chem., 246, 6393-6400.

7. Green, D. E. and Perdue, J. F. (1966), Proc. Nat. Acad. Sci., 55, 1295-1302.

8. Glaser, M., Simplins, H., Singer, S. J., Sheetz, M. and Chan, S.I. (1970), Proc. Nat. Acad. Sci., 65, 721-728.

9. Dennick, R. G. and Dean, P.D.G. (1974) J. Neurochem., 23, 261-266.