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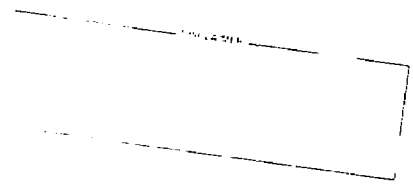

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ABSTRACT

Using a technique in which substrate fatty acids are incorporated into microsomal membranes followed by comparison of their rates of desaturation or elongation with those of exogenous added fatty acids it has been found that the desaturation rate is more rapid for the membrane-bound substrate than for the added fatty acid. Moreover, the product of the membrane-bound substrate is incorporated into membrane phospholipid whereas the product of the exogenous substrate is found in di- and triacyl glycerols and in free fatty acids as well. These and other findings point to a normal sequence of reaction of membrane lipids with membrane-bound substrates involving transfer of fatty acid from phospholipid to the coupled enzyme systems without ready equilibration with the free fatty acid pool.

INTRODUCTION

Most lipid metabolic reactions in the cells of living organisms involve membrane lipids and membrane-bound enzymes (1). However, only a few papers discussing the utilization of lipid substrates by their respective enzymes have attempted to describe the mechanism of the interactions between membranous enzymes and membranous lipid substrates (2).

Research from several laboratories has elucidated the biosynthetic mechanisms that determine the composition of the brain lipids. Reactions such as fatty acid elongation and desaturation occurring in brain membranes have been investigated "in vitro" with added fatty acids or fatty acyl-CoA thioesters with no assurance that this is the actual mechanism of the reaction as it proceeds in the membrane (3).

We have developed an "in vitro" technique for preparing brain microsomes labeled in the membrane phospholipids with ^{14}C-fatty acids. These membranes were then used as substrates for a study of desaturation and elongation of fatty acids as a new approach toward understanding these important reactions as they probably occur in the membranes.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]Linoleic (57 mCi/mmol), linolenic (56.2 mCi/mmol) arachidonic (52.7 mCi/mmol), and [2-^{14}C]8,11,14 eicosatrienoic (55 mCi/mmol) acids were obtained from the Radiochemical Centre, Amersham, England. Radiopurity of the methyl esters was greater than 98% for all fatty acids. Nonradioactive fatty acids and lipid standards were purchased from Supelco, Inc., Bellefonte, PA, or Applied Science, State College, PA. The fatty acid substrates were prepared in micellar solution by dispersal in twice their molar amount of 0.1 N NH_4OH and diluting to the desired volume with 1% triton WR 1339 (Ruger Chemical Co., Irvington, NJ). ATP, CoA, NADH,

NADPH, glutathione, malonyl CoA were products of Sigma Chemical Co., St. Louis, MO. p-Bromophenacyl bromide was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Pentex bovine albumin, fatty acid free fraction V, was a product of Miles Laboratories, Inc. All other reagents used were Baker analyzed reagents. Solvents were ACS grade and redistilled before use.

Preparation of Microsomal Fractions from Rat Brains

Five or six (16-day-old) or ten to twelve (4-5 day-old) male rats (Mission Labs, Rosemead, CA) were killed by decapitation and the brains quickly removed and washed with ice cold 0.32 M sucrose. The whole brains from the 16-day-old rats (for elongation studies) were weighed and homogenized in 9 volumes of 0.32 M sucrose with a Potter-Elvehjem type homogenizer equipped with a teflon pestle. Brain tissues from the 4-day-old rats (for desaturation studies) were homogenized with 0.32 M sucrose containing 0.1 M-phosphate buffer at pH 7.2 and 2 mM reduced glutathione (4 ml medium per g tissue) (4). Preparation of microsomes was as described by M. Gan-Elepano and Mead (5). All the above procedures were done at 4 C. Preparation of membranes and incubations were performed on the same day. Protein was determined by the method of Lowry et al. (6).

Incubations

For the incorporation of [^{14}C]fatty acids into the lipids of microsomal membranes, the incubation mixture contained 4 µmoles ATP, 0.2 µmoles CoA, 4 µmoles $MgCl_2$, 2 µmoles reduced glutathione, 100 µmoles potassium phosphate buffer 0.1 M, pH 7.2, 0.05% triton WR 1339, 7.5 to 17.5 nmoles of [^{14}C]fatty acids (52-57 mCi/mmol), and 2-4 mg of microsomal protein in a final volume of 1 ml. The incubation vials were capped with rubber stoppers and nitrogen was flushed through for at least 10 min, to ensure a nitrogen atmosphere. The vials were shaken at 37 C for 15 min.

For desaturation of exogenous fatty acids, fresh, unlabeled microsomes (2-5 mg protein) were incubated with 4 µmoles ATP, 0.2 µmoles CoA, 4 µmoles $MgCl_2$, 2 µmoles reduced glutathione, 100 µmoles potassium phosphate buffer 0.1 M, pH 7.2, 1 µmole NADH, 0.05% triton WR 1339, 1.0 mg "soluble" protein, and 7.5 to 15 nmoles of [^{14}C]fatty acids (52-57 mCi/mmol), in a final volume of 1 ml. Incubation was carried out for 20 min, at 37 C, under air, with shaking.

For elongation of exogenous fatty acids, fresh, unlabeled microsomes, (3-5 mg protein) were incubated with 8 µmoles ATP, 0.4 µmoles CoA, 8 µmoles $MgCl_2$, 1 µmole NADH, 1 µmole NADPH, 60 nmoles of malonyl CoA, 100 µmoles 0.1 M-phosphate buffer pH 7.2, 0.05% triton WR 1339, and 11 to 23 nmoles of [1-^{14}C]arachidonic acid (52.7 mCi/mmol), in a final volume of 1 ml. Incubation was carried out for 30 min, at 37 C, under nitrogen, with shaking.

For desaturation and elongation studies of endogenous fatty acids, the [^{14}C]labeled microsomal membranes were washed with 1% BSA (bovine serum albumin) by resuspending the labeled microsomes in 1% BSA in 0.32 M sucrose and centrifuging at 50,000 x g for 2 hr, at 4 C.

For desaturation of endogenous fatty acids, the albumin-washed ^{14}C labeled microsomal membranes were resuspended in 0.1 M-phosphate buffer pH 7.2-2 mM reduced glutathione -0.32 M sucrose solution and further incubated with 4 µmoles ATP, 0.2 µmoles CoA, 4 µmoles $MgCl_2$, 1 µmole NADH, 10 µmoles $CaCl_2$, 100 µmoles potassium phosphate buffer, pH 7.2, 1.0 mg of "soluble" protein (50,000 x g x 2 hr supernatant), 2 µmoles reduced glutathione, and 2-4 mg fresh, unlabeled microsomal protein, in a final volume of 1 ml. Two to 4 mg of albumin-washed [^{14}C]microsomal membranes containing approximately 800,000 dpm were used. Incubation was carried out for 30 min, under air, at 37 C, with shaking.

For elongation, the complete system contained 8 µmoles ATP, 0.4 µmoles

CoA, 8 moles $MgCl_2$, 1 mole NADH, 1 μmole NADPH, 2 μmoles reduced glutathione, 10 μmoles $CaCl_2$, 100 μmoles of 0.1 M-phosphate buffer pH 7.2, 60 nmoles of malonyl CoA, 3-5 mg of fresh, unlabeled microsomal protein, and 3-5 mg of albumin-washed [^{14}C]labeled microsomal membranes (approx. 800,000 dpm), in a final volume of 1 ml. Incubation was carried out for 30 min, under nitrogen, at 37 C, with shaking.

A stock solution of p-bromophenacyl bromide in ethanol at a concentration of 4×10^{-2} M was used when needed.

Extraction and Separation of Lipids

The total lipids from the reaction mixtures were extracted with 20 vol of chloroform-methanol (2:1, v/v). The suspension was centrifuged and to the supernatant was added 0.2 vol of water. The upper phase was removed, and the chloroform-rich lower phase was washed 3 times with Folch's theoretical upper phase (7). The lower phase, containing the lipids, was reduced to dryness under a stream of nitrogen, at room temperature, resuspended in C:M (2:1, by vol) and an aliquot was taken for counting.

Neutral lipid classes were separated on precoated 0.25 mm Silica Gel G plates by development with a mixture of petroleum ether-diethyl ether-acetic acid (70:30:1, by vol). For the separation of polar lipids, the plates were developed with a mixture of chloroform-methanol-water (65:25:4, by vol). Areas containing the lipids were identified by comparison with known standards chromatographed at the same time. After a brief exposure to iodine vapors, they were scraped off the plates and transferred to counting vials to which 1.0 ml of water and 10 ml Aquasol (New England Nuclear, Boston, MA) were added. Radioactivity was counted with a Beckman liquid scintillation spectrometer. Correction for quenching was made by the external standard method. Efficiency for [^{14}C] was

about 70%.

The plates were also scanned for radioactivity by a Packard Radiochromatogram TLC scanner (Model 7200). Areas under the peaks were measured by triangulation.

Total phospholipids, free fatty acids, and triacylglycerols were isolated by TLC with the solvent system petroleum ether-diethyl ether-acetic acid (70:30:1, by vol). Migration of monoacylglycerols was facilitated by the procedure described by Rousseau and Gatt (2). Bands were collected and methylated to obtain fatty acid methyl esters of the isolated lipid classes.

Total phospholipids were also subjected to hydrolysis by Crotalus adamanteus venom as suggested by Robertson and Lands (8). After hydrolysis, the mixture of free fatty acids and lyso-phospholipids was separated on TLC plates using the above solvent mixture, petroleum ether (30 to 60 C):diethyl ether:acetic acid (70:30:1, by vol) and the mixture $CHCl_3$:MeOH:H_2O (65:25:4, by vol). The distribution of radioactivity among the lipid fractions was measured as described above.

Gas Liquid Chromatography

The total lipid extracts and and lipid fractions isolated by TLC, were transmethylated by treatment with 5% methanolic HCL solution at 80-90 C in screwcap vials, under nitrogen, for at least 3 hr. The fatty acid methyl esters were extracted with hexane and washed three times with water. The hexane extract of radioactive fatty acids was taken to dryness under a stream of nitrogen.

Gas liquid chromatography was carried out in a Packard Gas Chromatograph Model 7400 equipped with dual flame ionization detectors and a 4 mm x 6 ft coiled glass column. 10% Silar 10 C on 100-120 mesh Gaschrom Q was used for the analyses. The Packard Model 894 Gas Proportional

Counter was used for radioactivity determinations. Identification of the radioactive peaks was accomplished by comparison with known standard fatty acid methyl esters.

$AgNO_3$-Thin Layer Chromatography

The methyl esters of fatty acids were also analyzed by argentation-TLC in ethyl acetate:toluene (70:30, v/v) as described by Cook (4). The Packard Model 7200 radiochromatogram scanner was used for monitoring ^{14}C activity. Areas under the peaks were measured by triangulation.

RESULTS AND DISCUSSION

The distribution of radioactivity among lipid fractions after incubation of rat brain microsomes with ^{14}C fatty acids is shown in Table I. A significant proportion of the fatty acids added to the incubation mixture was incorporated into phospholipids and, to a lesser extent, into diacyl and triacyl glycerols. Within the phospholipids, CPG was several times more highly labeled than EPG. A similar pattern of incorporation was found by Cook (4) with rat brain homogenates using desaturation conditions, although the level of free fatty acids remaining at the end of the incubation period was significantly higher in his experiments. Therefore, microsomes might have a higher specific activity of the acyltransferases involved in the incorporation of acyl-CoA derivatives of fatty acids into phospholipids than did the whole homogenates.

No major differences were found stemming from the nature of the fatty acid substrates used. Incorporation into phospholipids was higher for 18:3 and 20:4, while the incorporation into triacyl glycerols was greater for 20:3 and 20:4.

Since expriments on elongation of arachidonic acid carried out with brain microsomes from 4- or 16-day-old-rats gave similar results, the age of the animals and, therefore, developmental changes in the acyl

transferases are not a factor.

Increased incubation time or substrate concentration (up to 20 nmoles/ml) did not substantially alter the percentages of radioactivity incorporated into lipids nor the pattern of incorporation. Kinetic studies of the incorporation were not carried out since our aim was primarily to obtain labeled microsomal membranes to be used as a source of endogenous fatty acids for desaturation and elongation studies.

The rather high levels of labeled triacyl glycerols found were unexpected, since TG are almost absent in brain lipids. The lipase described in brain microsomes (9-11), which can hydrolyze tri- or diacylglycerols, with a pH optimum at 4.8, would not be active in our "in vitro" experiments carried out at pH 7.2. However, "in vivo", this lipase or other unknown regulatory mechanisms may explain the lack of TG in the whole brain.

The incorporation of fatty acids into phospholipids and neutral lipids was dependent on the presence of added ATP and CoA in the incubation mixture (Table II). The co-factors are necessary for activation of the fatty acid substrates to acyl-CoA derivatives by acyl-CoA synthetases (12). The same effects were shown by adding to the incubation mixture unlabeled fatty acids, which would compete for the needed co-factors and binding sites. TABLE II

The incorporation of fatty acyl-CoA derivatives involves also a second step catalyzed by acyl-CoA – lysophosphoglyceride acyltransferases (13). This "exchange" reaction requires the presence of lysophosphoglycerides in the membrane to accept the fatty acids. Therefore, membrane-bound phopsholipases may also be involved in the overall mechanism of incorporation of fatty acids into the phospholipids. To test this possibility, we added p-bromophenacyl bromide (p-BPB), a phospholipase A_2

inhibitor (14) to the incubation mixture, at a concentration known to inhibit elongation of endogenous fatty acids by rat brain mitochondria and microsomes (5). However, p-BPB was found to have no effect on the incorporation of fatty acids into polar or neutral lipids (Table II). These results may be explained by the reported lack or minimal activity of phospholipase A_2 in brain microsomes (15-18) and suggest that a phospholipase A_1 may be largely involved. However, the incorporation of ^{14}C linolenic acid into the 2-position of the phospholipids of the microsomal membranes, as shown by hydrolysis of the labeled phospholipids with Crotalus adamanteus venom (Table III), rather than in the 1-position, did not support phospholipase A_1 involvement. Other hydrolytic pathways, such as action of a bound phospholipase A_2 inaccessible to pBPE in the microsomes may also be present or the pathway of incorporation involved may be the phosphatidic acid synthesis and hydrolysis to diacylglycerol with consequent formation of PC with CDP choline. TABLE III

The distribution of radioactivity among lipid fractions of the ^{14}C-labeled microsomal membranes, after washing with 1% albumin in 0.32 M sucrose and centrifuging at 50,000 x g for 2 hr is shown in Table IV. Between 78 and 97% of the free fatty acids initially present in the labeled microsomes were removed by this procedure (compare Table I). Larger albumin concentrations were tested but they brought about no further decrease in the amount of free fatty acids left after washing. The small amounts of free fatty acids remaining in the labeled microsomal preparations were taken into account for the calculations of the degree of desaturation of the endogenous fatty acids, in the experiments to be described below. TABLE IV

Assuming that the washing procedure removes only free fatty acids, the relative levels of labeled polar to labeled neutral lipids (DG + TG) before and after washing should remain constant. However, this was not

the case, since the relative levels of labeled TG and DG (NL) were increased after washing (compare Tables I and IV). This means that some polar lipids were removed or lost during the washing procedure along with the free fatty acids. Washing liver microsomes have also been shown to lose a soluble protein factor that is necessary for the full activity of the linoleic acid desaturase (19). This factor contains lipids, mainly CPG. Therefore, it is possible that a similar loss occurs in our washed brain microsomal preparations. This possibility is supported by our findings that the brain washed microsomal preparations also lose most of their desaturation capacity, and by the stimulatory effect of the post-microsomal supernatant on the brain microsome desaturation capacity described by Cook (4). The presence of a lipoprotein factor necessary for desaturation of fatty acids such as the one reported by Leikin, et al. (19) in liver microsomes, has not been investigated in brain but is very likely to be present in this tissue as well.

The distribution of radioactivity among lipid fractions of the albumin-washed labeled microsomal membranes, after incubation of these membranes with fresh brain microsomes is shown in Table V. There were at most only minor changes after 20 min of incubation under desaturating conditions. In the absence of ATP and CoA in the incubation mixture, there was a three-fold increase in the level of free fatty acids at the end of the incubation period, with or without adding pBPB, together with a decrease of radioactivity in polar and neutral lipids. These results may be explained by the hydrolysis of both polar and neutral lipids by a microsomal lipase during the incubation. In the presence of ATP and CoA (complete system) the released fatty acids would be almost completely reincorporated into polar and neutral lipids. The addition of pBPB or unlabeled fatty acids did not affect this reincorporation mechanism, again suggesting that an accessible phospholipase A_2 may not be involved.

TABLE V

"In vitro" desaturation and elongation of exogenous fatty acids by rat brain microsomes is shown in Table VI. TABLE VI

Linoleic (18:2ω6), linolenic (18:3ω3), and 8,11,14-eicosatrienoic (20:3ω6) acids were converted into their corresponding desaturation products, 18:3ω6, 18:4ω3 and 20:4ω6. Arachidonic acid (20:4ω6) was elongated to 22:4ω6. The relative desaturation activities for the 4-day-old rat brain microsomes were 18:3>20:3>18:2. Desaturation of 18:3ω3 was also found by Cook (4) to be greater than for the other fatty acids in whole brain homogenates.

Optimal incubation conditions for desaturation were determined with brain microsomes from 4-day-old rats only for linolenic acid (Fig. 1). FIG. 1 The optimal pH was 6.5. Activities at pH 7.2, 7.5 and 6.0 were 12, 22 and 33% lower, respectively. pH 7.2 was used throughout all incubations since we were concerned not only with desaturation but also with the release, activation, desaturation (or elongation) and reincorporation of fatty acids in the microsomal preparations.

The time response for the enzyme system was linear up to 20 min of incubation, and protein inhibition occurred between 6-8 mg of protein/ml. The highest rate of 18:4ω3 synthesis was reached at approximately 15 nmoles/ml. Optimal conditions were similar to those reported for whole homogenates (4).

ATP and CoA were needed for maximal activity (Table VII). Addition of unlabeled 18:3 (90 nmoles/ml) significantly decreased desaturation of labeled 18:3ω3). Therefore, as was shown for the incorporation of fatty acids into lipids, CoA and ATP were necessary for activation of the added substrates to acyl-CoA derivatives and further desaturation by the desaturases. Activity remaining in the absence of ATP and CoA could be explained by the presence of endogenous cofactors in the microsomal preparations. TABLE VII

As expected, addition of p-bromophenacyl bromide (0.2 μmoles/ml) did not significantly alter desaturation of exogenous linolenic acid.

Desaturation and elongation of endogenous fatty acids by rat brain microsomes is shown in Table VIII. Extent of desaturation of 18:3ω3 to 18:4ω3 was greater than conversion of 20:3ω6 to 20:4ω6 and 18:2ω6 and 18:3ω6. Elongation of arachidonic acid was not detected under the conditions used. TABLE VIII

While ATP and CoA were also needed for maximal desaturation of endogenous fatty acids (Table IX), in the absence of ATP and CoA, with or without added p-BPB, almost 50% of the activity of the complete system was still present. Addition of unlabeled 18:3 also decreased desaturation of fatty acids, but only to about 60%. This level of desaturation activity remaining in the absence of ATP and CoA cannot be explained only by endogenous cofactors left in the microsomal preparations used. Direct desaturation of fatty acids in the phospholipids without their release and conversion to the acyl-CoA derivatives as has been shown in yeast and rat liver microsomes (20), cannot be discarded and is a possibility. Desaturation activity in the complete system could then be the result of direct desaturation plus desaturation of the partially hydrolyzed phospholipids (and TG) after activation of the released fatty acids in the presence of ATP and CoA to their acyl-CoA derivatives.

The addition of a phospholipase A_2 inhibitor, p-BPB, to the complete system, did not modify desaturation of the endogenous fatty acids (Table IX). This fact could be explained either by the lack of an accessible phospholipase A_2 in microsomes (15-18), or the existence of direct desaturation, or both. TABLE IX

The distribution of desaturation substrate and product among lipid fractions after incubation of exogenous and endogenous linolenic acid under desaturating conditions is shown in Table X. If desaturation of TABLE X

endogenous fatty acids takes place only after hydrolysis and equilibration of the released fatty acids into the exogenous fatty acid pool, the same distribution would be expected for the exogenous as for the endogenous fatty acid desaturation products. The mechanisms involved would be hydrolysis, activation, desaturation, and partial reincorporation of the converted fatty acids into phospholipids and neutral lipids. However, this was not the case in our experiments. With the exogenous substrate (18.3ω3), 67% of the product (18:4ω3) was found in the phospholipid fraction, 22% in the free fatty acids and 10% in the neutral lipid fraction (DG + TG).

With the endogenous substrate more than 96% of the 18:4ω3 formed was detected in the phospholipids, and very little, if any, in the free fatty acids and neutral lipids.

These results again suggest the occurrence of a direct desaturation mechanism. However, another alternative system would be possible, involving hydrolysis of the phospholipid fatty acids by a tightly coupled phospholipase and coupling of the released fatty acids to a different desaturation system (and different fatty acid pool) from the one known to operate with the exogenous fatty acids.

This latter possibility is supported by a comparison of the percent desaturation of exogenous and endogenous fatty acids (Table XI). By radio-GLC analysis, 37.7% of the exogenous substrate was converted into 18:4 3 in the complete system, and only 5.2% in the absence of ATP and CoA (Exp. I, column C). Assuming that no hydrolysis has taken place during incubation, (Exp. II, column D) desaturation of endogenous 18:3ω3 without or without cofactors (ATP and CoA) should be minimal. Under these conditions only the small amount (3.9%, Exp. II, column A) of free fatty acids left in the microsomal preparations after washing, would be desaturated

TABLE XI

at the same rate as that shown for the exogenous fatty acid (37.7%). On the contrary, if hydrolysis did take place, more desaturation would be found. The percent desaturation expected under these conditions may be calculated on the asusmption that the levels of free fatty acids remaining at the end of the incubation period, in the absence of ATP and CoA (Exp. II, column B), represent the maximal values of hydrolysis that occurred during incubation, and that these fatty acids were desaturated at the same rate as the exogenous fatty acids. The values obtained in this way Exp. II, column E) do not fully account for the percent desaturation found by radio-GLC of the endogenous fatty acids (Exp. II, column C), with or without co-factors. The differences (Exp. II, column F) may be explained by either of the two mechanisms already discussed: direct desaturation of fatty acids by membrane-bound phospholipid desaturases, or by hydrolysis of the phospholipids followed by activation to their acyl-CoA thioesters, and coupling of these enzyme systems to a different and more efficient mechanism for desaturation. If this latter mechanism is present, it would desaturate the released fatty acids at almost twice the rate as the one desaturating exogenous fatty acids. Similar results were obtained in several experiments with 18:3ω3 or 20:3ω6.

The fact th· under analogous conditions, elongation of endogenous arachidonic acid could not be demonstrated may serve as a control of the techniques and calculations carried out.

Further experiments are needed to support fully the existence of either of these two mechanisms in rat brain microsomes. However, we believe that these studies are, at the very least, significant as a new approach for the elucidation of the mechanisms involved with membrane-bound enzymes acting on membrane-bound lipids as they actually occur in the organism.

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TABLE I

Distribution of Radioactivity among Lipid Fractions After Incubation of Rat Brain Microsomes with [^{14}C] Fatty Acids.[a]

	Substrate			
	18:2	18:3	20:3	20:4
Polar lipids (PL)	43.3	54.4	41.3	56.7
CPG[b]	32.0	47.4	37.4	52.7
EPG[b]	10.9	6.5	3.9	4.0
Others	0.4	0.5	--	--
Neutral lipids (NL)	56.7	45.6	58.7	43.3
DG[b]	6.4	3.4	6.0	--
FFA[b]	35.3	28.2	29.8	21.2
TG[b]	15.0	14.0	22.9	22.1
Ratio PL/NL (TG+DG)	2.0	3..	1.4	2.5

[a]Values are given as percentages of total radioactivity incorporated into lipids.

[b]Abbreviations: CPG - choline phosphoglycerides; EPG - ethanolamine phosphoglycerides; DG - diacylglycerols; FFA - free fatty acids; TG - triacylglycerols.

Incubation conditions are as described in the Experimental Procedures Section.

TABLE II

Effect of Additions to the Incubation Medium on Incorporation of [^{14}C]-Fatty Acids into Lipid Fractions by Rat Brain Microsomes.[a]

Incubation Mixture	PL	FFA	NL
A) Complete system[b]	43.0	46.0	10.9
-ATP and CoA	19.1	80.9	--
+ pBPB[c]	42.8	39.6	17.5
+ unlabeled 18:3[d]	28.1	69.3	2.6
B) Complete system[b]	54.4	28.2	17.4
-ATP and CoA	6.3	93.7	--
C) Complete system[b]	41.3	29.8	28.9
-ATP and CoA	3.8	96.2	--

[a]Values are given as percentages of total radioactivity incorporated into the lipids.

[b]Incubation mixture as in Table I, containing 14.5 nmoles of [1-^{14}C] linolenic acid (A), 10.5 nmoles of [1-^{14}C]linolenic acid (B), and 7.5 nmoles of [1-^{14}C]eicosatrienoic acid (C).

[c]pBPB: p-bromophenacyl bromide (0.2 µmole).

[d]Unlabeled linolenic acid (90 nmoles).

Abbreviations as in Table I.

TABLE III

Distribution of Radioactivity in Phospholipid Classes as Determined by Hydrolysis with Phospholipase A_2 from Crotalus adamanteus Venom after incorporation of [1-^{14}C]Linolenic Acid by Rat Brain Microsomes.[a]

Lipid Fractions	Before Hydrolysis	After Hydrolysis
Lyso-CPG	--	26.6
Lyso-EPG	--	7.4
CPG	85.0	--
EPG	15.0	--
FFA	--	66.0

[a]Values are given as percentages of total radioactivity in lipids, and were obtained by radio-TLC.

TABLE IV

Distribution of Radioactivity Among Lipid Fractions of [^{14}C]-labeled Microsomal Membranes After Washing with 1% Albumin[a]

	Substrate			
Lipid Fractions	18:2	18:3	20:3	20:4
Polar Lipids	66.2	62.5	46.6	57.1
Free Fatty Acids	1.0	3.9	4.1	4.7
Neutral Lipids	32.8	33.6	49.3	38.2
Ratio PL/NL (TG+DG)	2.0	1.8	0.9	1.4

[a]Values are given as percentages of radioactivity incorporated into the lipids after washing of the previously [^{14}C]labeled microsomal membranes with 1% albumin solution. For experimental details see text.

TABLE V

Distribution of Radioactivity among Lipid Fractions of Albumin-Washed [^{14}C-18:3]-labeled Microsomal Membranes, after Incubation with Fresh Rat Brain Microsomes.[a]

Incubation Mixture	PL	FFA	NL
Complete system[b]	61.4	5.7	32.9
Complete, 0 time	62.5	3.9	33.6
-ATP and CoA	57.3	12.6	30.1
+pBPB[c]	62.4	3.9	33.7
-ATP and CoA, +pBPB[c]	58.2	10.6	31.2
+unlabeled 18:3[d]	60.8	5.4	33.8

[a]Values are given as percentages of total radioactivity incorporated into lipids.

[b]Incubation conditions are as described in the Experimental Procedures Section.

[c]pBPB: p-bromophenacyl bromide (0.2 μmoles).

[d]Unlabeled linolenic acid (90 nmoles).

Abbreviations as in Table I.

TABLE VI

Desaturation of [1-^{14}C]-Fatty Acids (A) and Elongation of [1-^{14}C]Arachidonic Acid (B) by Rat Brain Microsomes[a]

	Substrate		Product	
A)	18:2ω6	75.1	18:3ω6	24.9
	18:3ω3	50.8	18:4ω3	49.2
	20:3ω6	66.5	20:4ω6	33.5
B)	20:4ω6	75.2	22:4ω6	24.8

[a]Values were obtained by radio-gas liquid chromatography, and are given as percentages of total radioactivity. Incubation conditions are as described in the Experimental Procedures Section.

TABLE VII

Effect of Additions to the Incubation Medium on Desaturation of [1-^{14}C]Linolenic Acid by Rat Brain Microsomes[a]

Incubation Mixture	Product
Complete system[b]	18.1
- ATP and CoA	6.8
+ pBPB[c]	15.7
+ Unlabeled 18:3[d]	2.0

[a]Values are given as percentage of radioactivity in the desaturation product, as determined by radio-GLC.

[b]Brain microsomes from 4-day-old rats were incubated with 10.5 nmoles of [1-^{14}C]linolenic acid and 2.0 mg of microsomal protein. Incubation conditions were as described in the Experimental Procedures Section.

[c]pBPB: p-bromophenacyl bromide (0.2 μmole).

[d]Unlabeled linolenic acid (90 nmoles).

TABLE VIII

Desaturation of Endogenous [^{14}C]Fatty Acids (A) and Elongation of Endogenous [^{14}C]Arachidonic Acid (B) by Rat Brain Microsomes[a]

	Substrate		Product	
A)	18:2ω6	95.7	18:3ω6	4.3
	18:3ω3	89.5	18:4ω3	10.5
	20:3ω6	93.4	20:4ω6	6.6
B)	20:4ω6	>99.0	22:4ω6	<1.0

[a]Values were obtained by radio-GLC, and are given as percentage of total radioactivity. Incubation conditions are as described in the Experimental Procedures Section.

TABLE IX

Effect of Additions to the Incubation Medium on Desaturation of Endogenous [^{14}C]linolenic Acid by Rat Brain Microsomes[a]

Incubation Mixture	Product	
	GLC	$AgNO_3$ TLC
Complete system[b]	10.5	10.1
Complete, 0 time	0.0	0.0
- ATP and CoA	5.0	4.9
- ATP and CoA, + pBPB[c]	4.9	---
+ pBPB[c]	11.1	10.2
+ Unlabeled 18:3[d]	6.0	6.4
+ 1-^{14}C- 18:3[e]	37.7	36.8

[a] *Values were obtained by radio-GLC and by radio-*$AgNO_3$*-TLC,* and are given as percentage or radioactivity in the desaturation product.

[b] *Incubation conditions are as in the Experimental Procedures* Section.

[c] *pBPB: p-bromophenacyl bromide* (2 μmoles).

[d] *Unlabeled linolenic acid* (90 nmoles).

[e] 10.5 nmoles [^{14}C]18:3.

TABLE X

Desaturation of Exogenous (A) and Endogenous (B) Linolenic Acid. Distribution of Radioactive Substrate and Product in Lipid Fractions[a].

	PL	FFA	NL	TL
A) Exogenous Substrate				
Total [^{14}C]Fatty Acid[b]	54.5	28.2	17.3	100.0
18:3ω3 -Substrate[c]	31.5	20.7	13.8	66.0
18:4ω3 -Product[c]	23.0	7.5	3.5	34.0
18:4ω3 -Product[d]	42.5	26.5	20.4	37.7
B) Endogenous Substrate				
Total [^{14}C]Fatty Acid[b]	61.5	5.7	32.8	100.0
18:3ω3 -Substrate[c]	51.9	5.6	32.5	90.0
18:4ω3 -Product[c]	9.6	0.1	0.3	10.0
18:4ω3 -Product[d]	15.6	<1.0	<1.0	10.6

[a]Incubation conditions are as in the Experimental Procedures Section. Values are given as percentages or radioactivity in lipid fractions.

[b]Values obtained by radio-TLC.

[c]Values calculated from TLC and GLC data.

[d]Values obtained by radio-GLC.

Abbreviations as in Table I. For experimental details see text.

TABLE XI

Comparison between Desaturation of Exogenous and Endogenous Fatty Acids by 4-day-old Rat Brain Microsomes[a]

Substrate	A	B	C	D	E	F
I. Exogenous 18:3 3						
Complete system[b]	100.0	28.2	37.7	--	--	--
- ATP and CoA	100.0	93.7	5.2	--	--	--
II. Endogenous 18:3 3						
Complete system[b]	3.9	5.7	10.6	1.5	5.0	5.6
- ATP and CoA	3.9	13.4	4.2	0.2	0.7	3.5
+ pBPB[c]	3.9	3.8	12.9	1.5	4.3	8.6
- ATP and CoA, + pBPB	3.9	11.3	4.9	0.2	0.6	4.3

[a]Values are given as percentages of total radioactivity.

[b]Inc. conditions are as in the Experimental Procedures section.

[c]pBPB - p-bromophenacyl bromide (2 µmoles).

A. FFA at the start of the incubation period (radio-TLC).

B. FFA at the end of the incubation period (radio-TLC).

C. Desaturation product at the end of incubation (radio-GLC).

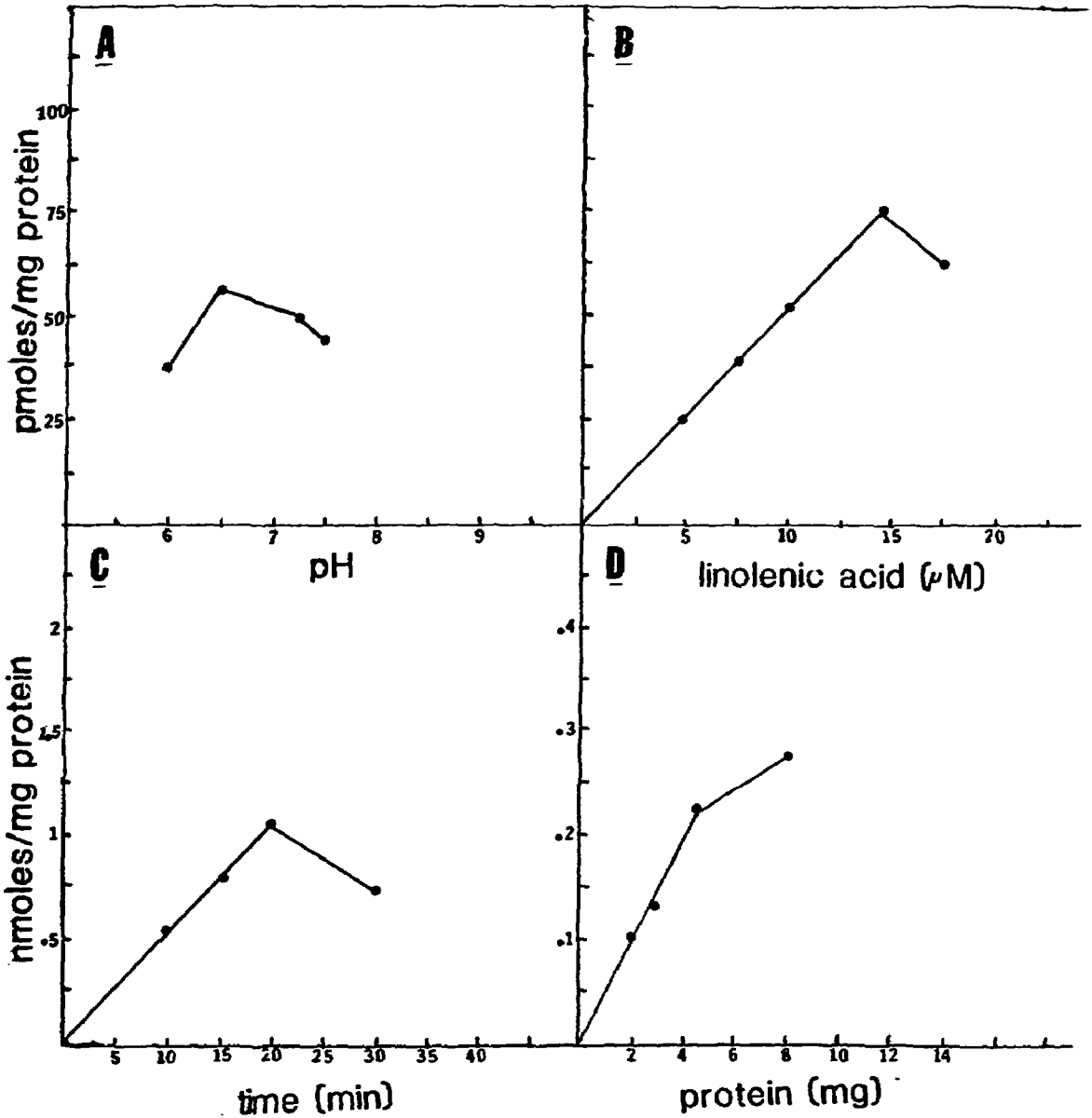
D. Expected desaturation product under null hydrolysis conditions.

E. Expected desaturation product after hydrolysis.

F. Difference between expected values (E) and those found by radio-GLC(C). Expected values were calculated from the desaturation product of exogenous fatty acids.

LEGEND

Fig. 1. Rate of desaturation of 18:3 3 to 18:4 3 by 4-day-old rat brain microsomes. A. Effect of pH. B. Effect of substrate concentration. C. Effect of incubation time. D. Effect of microsomal protein concentration.


A
B
C
D
pmoles/mg protein
100
75
50
25
6
7
8
9
pH
5
10
15
20
linolenic acid (μM)
nmoles/mg protein
2
1.5
1
.5
5
10
15
20
25
30
35
40
time (min)
.4
.3
.2
.1
2
4
6
8
10
12
14
protein (mg)