Fatty acids in esters and cerebrosides of human brain in phenylketonuria

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SUMMARY Fatty acids from cerebrosides and cerebroside sulfates and those bound by ester linkage from white and gray matter of frozen phenylketonuric brains were determined and compared with analyses of nonphenylketonuric brains. A decrease in the major monoenoic acid relative to the major saturated acid (18:1/18:0, or 24:1/24:0, or 24h:1/24h:0) of each fraction analyzed is observed in this disease. The amounts of cholesterol, cerebroside, and cerebroside sulfate in the brain are not altered in phenylketonuria.

The sum of the three polyunsaturated fatty acids 20:4, 22:5, and 22:6 constitute approximately 30% of the ester-bound fatty acids of human cortical gray matter.

KEY WORDS phenylketonuria · fatty acids · oleic acid · cerebrosides · human brain · white matter · gray matter · polyunsaturated fatty acids · hydroxy acids · cholesterol

WHILE THE METABOLIC ERROR associated with phenylketonuria has been well defined biochemically, its relation to the resulting mental deficiency remains obscure. In a previous study, a hypothesis involving the formation of ω-phenyl fatty acids in the brain of phenylketonurics was investigated. Unusual fatty acids were not found (1). In the course of that study, alterations in the fatty acid composition of human brain in phenylketonuria were seen and are the subject of this report. A preliminary report of some of this work has appeared (2).

MATERIALS AND METHODS

Materials

Brain specimens were obtained within 16 hr following death, were wrapped in aluminum foil, and stored at −20° in a sealed polyethylene bag. Brains were analyzed within 1–12 months. In each instance, gray matter and the underlying white matter were obtained by first slicing the tissue into sections 1–2 mm thick. White and gray matter were then separated with a scalpel. Occipital cortex was obtained from patients *C.D.*,[1] O.D., C.L., and L.R., while frontal cortex was obtained from patients *J.D.* and R.B.

J.D. was one of three severely retarded siblings, all of whom were phenylketonuric. He was admitted to a state residential school at the age of 12 years. Blood phenylalanine concentration was 16 mg/100 ml, as determined by the method of Berry (3), and paper chromatographic methods (4) for amino acids showed a marked increase in urinary phenylalanine. Urine tests with 10% ferric chloride and 2,4-dinitrophenylhydrazine were repeatedly strongly positive. Other than developmental and intellectual slowness, no neurological symptoms were apparent and there was no history of convulsions. He was tested psychologically on admission to the residential school and found to have an IQ of 9. At the age of 18, having been previously well, *J.D.* aspirated food during a meal and died suddenly. The autopsy failed to reveal any specific abnormalities. The brain was of normal weight and appearance. Histological studies were normal and no demyelination was apparent.

C.D. was admitted to a state residential school because of severe mental impairment, but was otherwise physically and neurologically normal. There was no history of convulsions. The diagnosis of phenylketonuria was established when he was 45 as a result of a survey of the residential school. The urine ferric chloride test was strongly positive, as was also reaction with 2,4-dinitrophenylhydrazine. Paper chromatographic methods for

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

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[1] Initials of cases of phenylketonuria are italicized.

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the detection of phenolic acids (5) showed large amounts of *o*-hydroxyphenylacetic acid (over 100 μg/mg of creatine) (6) in addition to moderate amounts of *p*-hydroxyphenyl lactic acid, *m*-hydroxyhippuric acid, and *p*-hydroxyphenyl hydracrylic acid (7). Blood phenylalanine concentration was 30.5 mg/100 ml, as established by the method of La Du and Michael (8). He was hospitalized because of repeated pulmonary infections, and died 3 weeks later at the age of 50. Histological examination of the CNS revealed some loss of subcortical myelin predominantly in the post-central areas (occipital, parietal lobes). The frontal lobes were histologically normal.

Age, sex, and diagnosis of the other cases described here are: R.B., male, 33 yr, malignant hypertension; O.D., male, 53 yr, astrocytoma (frontal lobe); C.L., male, 78 yr, myocardial infarction; L.R., female, 86 yr, pneumonia. The latter patient had been hospitalized 4 months prior to death because of senility.

All solvents were redistilled. Chloroform was used within one week. Silica gel for column chromatography was Unisil (Clarkson Chemical Co., Inc., Williamsport, Pa.). For TLC, Silica Gel G was obtained from Brinkmann Instruments, Inc., Great Neck, N.Y.

Extraction of Lipids

A lipid extract was prepared from 0.3 g of brain, filtered on a sintered glass funnel of medium porosity, and washed with 0.1 M KCl (9). The solvent was removed from the sample in a weighed flask with a rotary evaporator. Anhydrous ethanol–benzene 1:1 (5 ml) was added near the end of the evaporation to prevent splashing and to aid in the removal of water. The weight of the lipid was recorded. The washed, air-dried, caked, nonlipid residue of the Folch extract was transferred to glazed paper and weighed. The sum of these two values was used as the total dry weight of the tissue. The weight of material removed from the lipid extract by washing was not determined. The difference between the wet weight and the dry weight was used as a measure of the water content.

Weighed samples of gray matter containing 2–3 g of tissue from patients *C.D.*, R.B., and C.L. were extracted by the same procedure. This larger amount was required for reliable determinations of cerebroside and cerebroside sulfate.

Preparation of Fatty Acid Methyl Esters from Ester-Bound Fatty Acids

The lipids were processed by a method developed in this laboratory.[2] The dried lipid was dissolved in 10 ml of chloroform–methanol 2:1. The methanol used contained 0.21 M NaOH. This solution was stirred magnetically in a closed screw-cap or ground glass tube for 1 hr at room temperature, after which it was washed with 2 ml of 0.36 M acetic acid and evaporated to dryness (10). The dry residue of the lower layer was placed on a 6 mm o.d. column containing Florisil (8% water by weight). One gram of Florisil was used for gray matter and 2 g for white matter. Methyl esters were eluted with petroleum ether–benzene 9:1. Twenty-five milliliters of eluting solvent were used per gram of Florisil. Cholesterol, cerebrosides, and cerebroside sulfates were eluted together from the column with chloroform–methanol 3:1. An internal standard, methyl 2-methylmyristate, was added to the methyl ester fraction for quantification by GLC. The volume of the eluate was reduced in vacuo and the sample was then transferred to screw-cap test tubes, dried, and dissolved in 0.5–1.0 ml of petroleum ether for GLC. The amounts of fatty acids were calculated by triangulation and by comparison of areas of the curves with that of the standard. The sum of the peak areas were corrected for the weight of the methyl group. The esters were stored under nitrogen at 2° no more than 2 days prior to analysis.

Purification and Methanolysis of Cerebrosides and Cerebroside Sulfates

The procedure of Kishimoto et al.[2] was employed. The chloroform–methanol eluate from Florisil was taken to dryness in vacuo and placed on a 6 mm o.d. column containing 1 g of silica gel for lipids of white matter and 0.5 g for lipids of gray matter. Stepwise elution was carried out with the following chloroform–methanol concentrations and volumes per gram of silica gel: 20 ml of chloroform–methanol 98:2 for cholesterol; 40 ml of chloroform–methanol 94:6 for cerebrosides; and 40 ml of of chloroform–methanol 85:15 for cerebroside sulfates. Completeness of separations was checked by TLC.

In the following procedures the amounts of solvent, etc., are for cerebrosides. One-half these amounts were used for cerebroside sulfate fractions. The dried cerebroside (or cerebroside sulfate fraction) was weighed, mixed with 2 ml of 5% dry HCl in methanol, and heated in a tightly sealed tube for 17 hr at 75°. Samples were evaporated to dryness under nitrogen and placed on a 1 g Florisil column. Unsubstituted methyl esters were eluted with 25 ml of petroleum ether–benzene 9:1 and the 2-hydroxy methyl esters were eluted with 25 ml of petroleum ether–diethyl ether 8:2. The unsubstituted esters were dried and 0.3 ml of carbon disulfide was added for GLC. Acetylation of 2-hydroxy esters was performed in small conical screw-cap tubes, taking care to get the whole sample in the bottom of the tube. A mixture of *p*-toluenesulfonic acid in isopropenyl acetate

[2] Y. Kishimoto, W. E. Davies, and N. S. Radin, paper in preparation.

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TABLE 1 LIPIDS FROM PHENYLKETONURIC AND NONPHENYLKETONURIC BRAINS

	Gray Matter			White Matter		
	J.D.	*C.D.*	nonPKU*	*J.D.*	*C.D.*	nonPKU*
			g/100 g dry weight			
Ester-bound fatty acids	14.3	13.1	13.8	13.1	10.7	13.8
Cholesterol	5.75	6.26	6.54	12.5	14.2	13.8
Cerebrosides†	—	2.73	2.92	11.3	10.6	11.0
Cerebroside sulfates†	—	0.65	0.86	3.9	4.1	4.6
Total lipid†	40.7	39.7	40.8	69.8	65.0	69.0
Water (per cent wet wt)	81.8	82.9	82.0	69.0	70.9	71.0

* Mean of four brains, except for gray matter cerebrosides and cerebroside sulfates, which are the mean of two brains. Extremes are within 10% of the mean except cerebrosides (gray), where the deviation is 13% of the mean, and cerebroside sulfates (white), where the extreme values were 3.0 and 7.6.

† Determined gravimetrically. Substances were weighed in test tubes on a semimicro balance, except for gray matter cerebrosides and cerebroside sulfates which were weighed in Teflon cups on a Cahn Electrobalance (La Jolla, California).

(4 mg/ml) was added to the dried sample of hydroxy esters (about 0.1 ml per estimated milligram of esters). The tubes were capped and heated in a water bath at 60° for 30 min, after which a few grains of anhydrous K_2CO_3 were added. The samples were allowed to stand for at least 15 min and the supernatant solution was used directly for GLC.

GLC of Fatty Acid Esters

GLC was performed as previously described (1) using a temperature gradient of 2.3°/min starting at 160° for the unsubstituted esters. For ester-bound fatty acids, the temperature was held at 120° for 3 min after injection and then raised to 160° in 1 min, at which temperature the gradient was started. 2-Acetoxy methyl esters were chromatographed using a gradient of 1.15°/min starting at 200°. National Heart Institute standard mixtures, D and F, were run regularly to check the accuracy of the instrument. Stated and found composition agreed with a relative error of less than 5% for each component. Other compounds used for identification purposes were the methyl esters of 20:4 (arachidonic acid), 22:6ω3, and the acetoxy methyl esters of 18h:0, 22h:0, and 24h:0. Brain acids were identified by comparison of retention times with those of the known standards. Duplicate GLC analysis of methyl esters from ester-bound fatty acids gave absolute values which agreed within 5% for acids comprising 5% or more of the total recovered sample. Duplicates of the fatty acids of cerebrosides and cerebroside sulfates agreed within 10%. GLC analysis was performed in triplicate or quadruplicate for several of the latter samples. The fatty acid composition of the various lipids studied from nonphenylketonuric brains were generally similar, differing less than 10% from the mean calculated for each major component (fatty acids comprising over 10% of the total). These values were therefore tabulated as the means.

Cholesterol was determined by the method of Zurkowski (11).

RESULTS

Total Lipid, Cholesterol, Cerebrosides, and Cerebroside Sulfates

Results are shown in Table 1. Gray matter contained 40–42% lipid by dry weight while white matter contained 65–76% lipid in the 6 brains studied. White matter contained about twice the percentage of cholesterol found in gray matter. No differences were seen between brains of phenylketonuric and nonphenylketonuric patients. There were no striking differences in the amounts of cerebrosides found in the various brains. The cerebroside sulfate in the white matter of O.D. is higher than in the other brains examined.

Ester-Bound Fatty Acids

Seven major peaks, in addition to the peak of the internal standard, were measured. The amounts of fatty acid present in each sample are given in Table 1, and the percentage composition is shown in Table 2. In gray matter of phenylketonuric patients, there is less oleic acid than stearic acid while the converse is seen in the analyses of gray matter from nonphenylketonuric patients. The ratios of 18:1/18:0 are listed in Table 2. For white matter, the percentage of oleic acid is always greater than that of stearic acid, but the 18:1/18:0 ratio is somewhat lower in the brain of phenylketonuric patients.

Cerebroside- and Cerebroside Sulfate-Linked Fatty Acids

The compositions of the major fatty acids of cerebrosides and cerebroside sulfates are shown in Tables 3 and 4. While the differences in the 24:1/24:0 and 24h:1/-24h:0 ratios are in some cases very small, only 1 of 24 brains from nonphenylketonuric patients gives a ratio lower than the highest of 12 corresponding ratios for brains of phenylketonuric patients.

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Studies with Rat Brain

To see whether the ratios of major saturated and monoenoic acids differ at different ages, experiments were performed on ester-bound fatty acids of whole rat brain. Rats of the Sprague-Dawley strain, 21 and 540 days old, were obtained from Camm Research Institute, Inc. (Wayne, N.J.) and were sacrificed 1 day after arrival. Total brain lipids were extracted as described under Methods. Three rat brains were pooled from each group (Table 5). A relative decrease in palmitic acid and an increase in oleic acid is seen in the older rats.

DISCUSSION

Preliminary studies on total fatty acids of the brain in phenylketonuria were performed on formalin-fixed brain samples containing both white and gray matter (2). The inversion of the ratio of oleic to stearic acid observed in those samples led to the present study. The possibility of obtaining, immediately post mortem, unfixed brains from phenylketonuric patients on a normal diet was a rare opportunity. Gray and white areas as well as lipid classes were separated, and fatty acids were isolated quantitatively.

Of interest in the present study were the amounts of cerebrosides and cerebroside sulfates found. The only previous report of a brain lipid alteration in phenylketonuria is that of Crome, Tymms, and Woolf (13), who reported a decrease in cerebrosides (cerebrosides + cerebroside sulfates) as well as cholesterol content. Our results indicate that this decrease is not seen in all cases of phenylketonuria. The two phenylketonuric patients reported here, however, were considerably older than those reported by Crome et al. It is possible that different brain areas are altered to different extents. The total lipid, water, and cholesterol content appear to be unchanged in phenylketonuria.

Differences from reported values for lipids might be caused by our method of obtaining dry weight in which the water wash of the lipid is lost. However, comparison of our results with experiments in which tissue is dried at 110° suggests that little error is introduced (14).

The ratio 18:1/18:0 is lower in the white and gray matter of the two cortical samples from phenylketonuric brain than in corresponding samples of nonphenylketonuric brain (Table 2). The ratio 18:1/18:0 for nonphenylketonuric gray matter, calculated from the study of ester-bound fatty acids of Baker (15) is 1.42, somewhat higher than our value.

The 18:1/18:0 differences found are in the same direction but somewhat smaller than those we found in formalin-fixed brains (2). It is interesting to note that in the fatty acids found in unfixed brain, there are relatively large amounts of 20:4, 22:5, and 22:6 in the ester-bound fatty acids (Table 2). These acids are virtually absent in formalin-fixed brains. The variable amount of long-chain polyenoic acids reported for human brain in the previous literature may be related to the conditions and length of storage. In addition, the use of a temperature program permits improved quantification of acids which are eluted late from the column. Some losses due to oxidation in storage and handling may have occurred despite our precautions. Also, there may be some error in determining the long-chain polyenoic acids introduced by the detector. Both of these errors would give falsely low values. Thus, the amounts of 20:4, 22:5, and 22:6 are minimal values.

Fatty acid analyses of nonsubstituted and hydroxy fatty acids of cerebrosides and cerebroside sulfates (Tables 3 and 4) indicate that the ratio of the principal

TABLE 2 MAJOR ESTER-BOUND FATTY ACIDS OF HUMAN BRAIN

	Gray Matter			White Matter		
	J.D.	*C.D.*	nonPKU*	*J.D.*	*C.D.*	nonPKU*
			%			
16:0	23.7	24.2	23.1	14.5	14.0	14.8
18:0	23.4	22.1	22.0	21.6	22.6	20.6
18:1	20.4	20.1	23.8	43.9	44.0	44.0
20:1	0.6	0.5	1.1	3.8	3.7	4.8
20:4	10.1	10.0	8.8	5.5	4.6	4.6
22:5†	5.7	6.4	4.6	7.2	8.3	7.7
22:6	16.1	16.5	16.7	3.6	2.8	3.5
18:1/18:0	0.87	0.91	1.05	2.03	1.95	2.07
			1.08			2.11
			1.08			2.12
			1.14			2.23

* Mean values from four brains. Individual values of major components are within 10% of the means except 22:6 (gray), in which two samples were 13.5 and 18.9% of the total, and 16:0 (white), in which two samples were 12.7 and 17.1% of the total.

† Assumed to be ω6 (12). A small peak midway between this peak and 22:6 is probably 22:5ω3.

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TABLE 3 NORMAL FATTY ACIDS OF CEREBROSIDES AND CEREBROSIDE SULFATES OF HUMAN BRAIN

	Gray Matter				White Matter					
	Cerebrosides		Cerebroside Sulfates		Cerebrosides			Cerebroside Sulfates		
	C.D.	nonPKU*	*C.D.*	nonPKU*	*J.D.*	*C.D.*	nonPKU*	*J.D.*	*C.D.*	nonPKU*
	%									
16:0	3.9	1.4	5	4	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.
18:0	7.2	8.6	4	4	5.5	5.3	7.8	2.1	1.7	2.9
22:0	5.3	3.7	3	2	2.0	3.1	2.3	1.8	2.7	2.4
23:0	5.2	4.2	4	4	4.3	4.8	4.0	3.4	4.7	3.2
24:0	11.2	11.4	12	11	13.2	16.2	10.6	12.1	15.5	11.1
24:1	38.4	43.4	38	42	47.1	44.4	49.9	46.8	45.1	48.4
25:0	3.4	4.0	6	4	4.6	5.1	3.9	5.6	5.3	4.5
25:1	12.4	14.9	16	16	13.3	12.0	13.8	15.6	13.5	17.0
26:1	13.0	8.4	13	11	10.1	9.1	7.8	12.5	11.4	10.6
24:1/24:0	3.43	3.49	3.2	3.3	3.57	2.74	4.26	3.87	2.91	3.98
		4.18		4.4			4.31			4.14
							4.35			4.16
							6.27			5.21

* Mean values from two brains for gray and from four brains for white. Individual values of major components are within 10% of the mean except for cerebrosides 24:0 (white), in which one sample was 8.3% of the total, and 25:1 (white), in which two samples were 12.2 and 16.3% of the total. In cerebroside sulfates 25:1 (white) one sample was 19.5% of the total.

unsaturated fatty acid to the principal saturated fatty acid present (24:1/24:0 or 24h:1/24h:0) is again somewhat reduced in phenylketonuric brain. While the differences are small, they are consistently in the same direction with only one exception, the ratio of 24h:1/24h:0 in the gray matter cerebrosides of R.B. compared with that ratio found in *C.D.* (Table 4). It is not surprising that the change in C_{18} fatty acid ratios is reflected in C_{24} fatty acids, since it is likely that the latter are formed directly from C_{18} precursors (16–18).

Neither white nor gray matter, nor any lipid class examined, appears to be the primary site of the change in fatty acid composition. Rather, a general modification of fatty acid composition of all brain lipids probably occurs.

The inversion of the ratio 18:1/18:0 in phenylketonuria may reflect an actual change in concentration of one or more phospholipid components. Klenk (19) reported more stearate than oleate in human brain phosphatidyl ethanolamine, while the reverse was true for lecithin and phosphatidyl serine.

O'Brien, Fillerup, and Mead (20) analyzed gray and white matter of human brain by somewhat different procedures. They reported cerebrosides and cerebroside sulfates in somewhat smaller amounts. Our fatty acid analyses show relatively less of the 16:0 and 18:0 than reported by O'Brien et al., and more of the higher homologues. This is particularly true of the hydroxy acids. We find that hydroxy fatty acids below C_{24} account for 21–26% of the total fatty acids of cerebrosides (white or gray) and for 18–21% of fatty acids in cerebroside sulfates. O'Brien et al. found that the shorter acids constitute 30% of the total in gray matter and 43% of white matter cerebroside acids in the brain of a 55 yr old patient, and 51 and 41% in gray and white matter cerebroside sulfates. Studies with a previously analyzed sample, kindly furnished by Dr. O'Brien, lead us to believe that the values reported here more closely resemble the true composition of these lipids.[3]

In previous studies with formalin-fixed brain, we observed a low 18:1/18:0 ratio in a single non-adult nonphenylketonuric brain. In order to investigate the change in 18:1/18:0 with age, we analyzed brains of young and old rats of the same strain (Table 5). This ratio appeared to increase with age. An even more dramatic difference in 18:1/18:0 in rat brain with age can be calculated from the data of Biran and Bartley (21). Possibly the low ratios obtained both with adult brain in phenylketonuria and with young brain reflect a relatively undeveloped brain, a concept consistent with that of arrested development in this disease (22).

Gellhorn, Benjamin, and Wagner (23) observed a relative decrease in the rate of oleic acid synthesis with increasing age in adipose tissue of rats. These authors did not study brain fatty acid synthesis, but it is of note that the effect seen in adipose tissue is the reverse of that expected on the basis of the amounts of these acids present in brains of different ages. Calculation of the 18:1/18:0 ratio from a recent report of fatty acids in adipose tissue of diabetic rats (24) gives 11.1 for normal rats and 6.1 for diabetic rats. The authors suggest a defect in an oxy-

[3] O'Brien has recently published (*Science* **147**: 1099, 1965) new data on the nonhydroxy fatty acids of cerebrosides found in human white and gray matter. He indicates, as we report here, that the shorter fatty acids are minor components.

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TABLE 4 HYDROXY FATTY ACIDS OF CEREBROSIDES AND CEREBROSIDE SULFATES OF HUMAN BRAIN

	Gray Matter				White Matter					
	Cerebrosides		Cerebroside Sulfates		Cerebrosides			Cerebroside Sulfates		
	C.D.	nonPKU*	*C.D.*	nonPKU*	*J.D.*	*C.D.*	nonPKU*	*J.D.*	*C.D.*	nonPKU*
					%					
22h:0	7.7	7.8	7	6	6.4	7.1	7.0	4.6	7.1	5.7
23h:0	15.4	16.8	14	14	15.4	15.8	15.3	12.8	15.5	13.7
24h:0	28.8	29.4	30	28	31.8	33.1	28.9	34.1	36.0	30.0
24h:1	24.0	24.0	21	24	22.9	19.9	25.8	24.2	16.7	24.8
25h:0	7.8	7.1	9	8	7.3	7.0	6.8	8.1	7.3	7.1
25h:1	7.7	6.8	8	8	5.9	4.5	6.5	5.8	5.4	6.9
26h:0	1.2	1.6	2	1	1.2	2.0	1.8	0.9	Tr.	2.3
26h:1	7.3	6.2	10	9	9.2	10.6	7.9	9.6	11.9	10.0
24h:1/24h:0	0.83	0.68 0.96	0.70	0.80 0.93	0.72	0.60	0.78 0.78 0.89 1.18	0.71	0.46	0.76 0.78 0.80 0.98

* Mean values from two brains for gray and from four brains for white. Individual values of major components are within 10% of the mean except for cerebrosides 24h:0 and 24h:1 (white), where samples were 24.8 and 29.3% of the total respectively. In cerebroside sulfates 24h:1 (white), one sample was 27.7% of the total.

genase which could catalyze the synthesis of monoenoic acids from the corresponding saturated acids.

The present findings might result from an impairment of the biosynthesis of oleic acid. Recent reports indicate that oleic acid is synthesized in mammalian liver microsomes (25–27). This synthesis involves the stepwise conversion of stearic acid to oleic acid. Impairment of this system might decrease 18:1/:8:0. Oleic acid has also been reported to be synthesized via an avidin-insensitive pathway in liver mitochondria which does not appear to involve saturated acids directly (28). An enzyme system in yeast that converts stearic to oleic acid has been studied by Bloomfield and Bloch (29), who have suggested that the first step in the mechanism is a hydroxylation, although there is some question about the nature of steps involved (30, 31).

If a mixed function oxidase is indeed involved in the direct conversion of stearic to oleic acid, it is interesting that the present results, which are consistent with a lowered activity of this enzyme, occur in a disease in which another mixed function oxidase, phenylalanine hydroxylase, is missing.

The authors are indebted to Dr. Reinhard Friede of this University for histological studies on brains from phenylketonuric patients. We also wish to acknowledge the help of Dr. E. J. Rennell and his staff at the Coldwater State Hospital. Methyl 2-methylmyristate was kindly furnished by Dr. Edward A. Napier of this University.

This work was supported in part by National Institutes of Health grant NB-3101.

Manuscript received March 19, 1965; accepted May 20, 1965.

TABLE 5 ESTER-BOUND FATTY ACIDS OF WHOLE RAT BRAIN

Age	22 Days	540 Days
16:0	27.2	20.6
18:0	22.5	21.8
18:1	26.0	33.0
20:4	10.4	9.8
22:6	13.8	14.8
18:1/18:0	1.16	1.51

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