6. J. E. Brown, J. R. Murray, T. G. Smith, *Science* **158**, 665 (1967).
7. P. Hillman, F. A. Dodge, S. Hochstein, B. W. Knight, B. Minke, *J. Gen. Physiol.* **62**, 87 (1973).
8. In Fig. 3 the recovery of the ERP was measured in isotonic KCl and the photometric measurements were made in artificial seawater. The time course of the absorbance changes remains the same when the cells are in isotonic KCl.
9. The "free retinal" state of rat rhodopsin appears not to produce an ERP response (*10*). This state has yet to be distinguished photometrically from metarhodopsin II (which does produce an ERP response). Spectrophotometric observations do not yet exclude the possibility that both the free retinal state and metarhodopsin II arise from the decay of metarhodopsin I. Hence the free retinal state might form before the development of the LRP.
10. R. A. Cone and W. H. Cobbs, *Nature* **221**, 820 (1969).
11. W. A. Hagins and R. E. McGaughy, *Science* **157**, 813 (1967).
12. See discussion in W. A. Hagins, *Annu. Rev. Biophys. Bioeng.* **1**, 131 (1972).
13. We thank J. Lisman, P. Brown, R. DeVoe, T. H. Goldsmith, and J. Brown for useful criticisms and suggestions on preparing the manuscript. Supported in part by grants from the National Institutes of Health to R.A.C. and by NIH special fellowship 5FO3 GM 42438 to A.F. Part of this work was done at the Marine Biological Laboratory, Woods Hole, while A.F. was a Grass Foundation fellow.
* Permanent address: Marine Biological Laboratory, Woods Hole, Massachusetts 02543.
27 July 1973

Fatty Acyl–Coenzyme A Elongation in Brain of Normal and Quaking Mice

Abstract. *Microsomal enzyme systems from mouse brain that catalyze, respectively, the elongation of palmitoyl-coenzyme A (palmitoyl-CoA), stearoyl-CoA, or arachidyl-CoA appear and reach maximal activity at different times after birth of the animal. A specific C_{20}-CoA elongating system exists in mouse brain in addition to the previously recognized C_{16}-CoA and C_{18}-CoA elongating enzymes. The C_{20}-CoA elongation system is severely reduced in the mutant quaking mouse.*

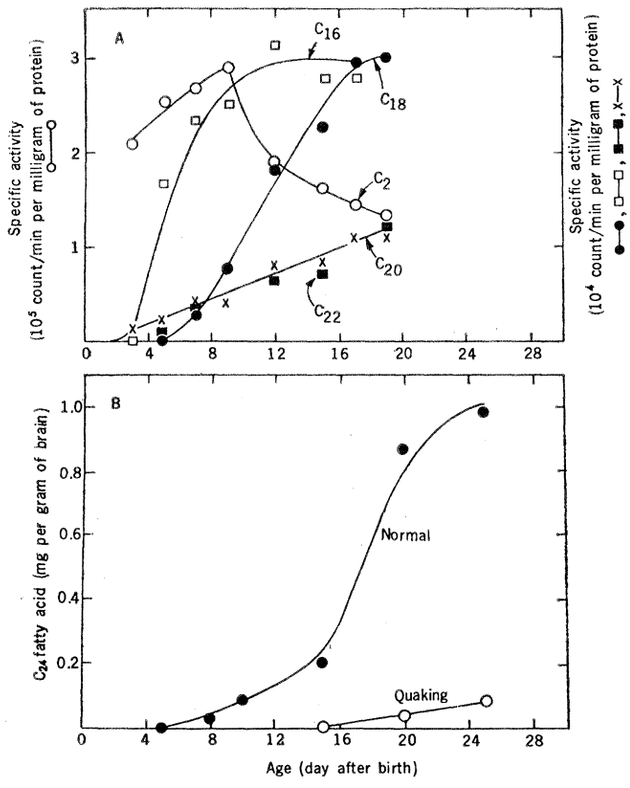
The quaking mouse is a recessive autosomal mutant characterized by defective myelination of the central nervous system (*1*). Baumann and collaborators (*2–4*) have noted that the cerebroside (galactolipid) and sphingomyelin content in the myelin lipids of quaking mice is much less than normal and that the nonhydroxylated long-chain fatty acids typical of these myelin lipids are also drastically reduced in the mutant. Brain lipids of adult quaking mice were found to contain at most one-tenth as much nonhydroxylated C_{24} acids as did normal controls (*3*). In efforts to localize the block in myelin galactolipid and sphingomyelin biosynthesis in the quaking mouse, Baumann and colleagues made two important observations: (i) the activities of the cytoplasmic de novo fatty acid synthetase [which produces palmitate (C_{16})] and of the microsomal enzyme system that catalyzes elongation of palmitoyl-coenzyme A (palmitoyl-CoA) to stearoyl-CoA were only slightly below normal in quaking mice (*5–7*) and (ii) the rate of stearoyl-CoA elongation in mutant brain extracts was also near normal (*6*), but the products of this enzymatic process were not (*6, 7*). Brain microsomes from normal mice incubated with stearoyl-CoA afforded 78.5 percent arachidate (C_{20}), 10.5 percent behenate (C_{22}), and 11 percent lignocerate (C_{24}), while the corresponding enzyme preparation from the quaking mutant produced 99 percent C_{20}, 0.8 percent C_{22}, and 0.2 percent C_{24} (*6, 7*). Baumann and collaborators have also furnished evidence for the existence of two separate microsomal elongating systems in normal mouse brain, one converting palmitoyl-CoA to stearate (C_{18}) and the other converting stearoyl-CoA to longer (C_{20} to C_{24}) acids (*8*).

From the analytical results just mentioned and the fact that the fatty acids having long chains (C_{22} and C_{24}) are greatly diminished in the brain lipids of quaking mice, these investigators concluded that the stearoyl-CoA elongating system is defective in the mutant (*6*). We offer an alternative interpretation of their data. Since stearoyl-CoA elongation, as judged by incorporation of [^{14}C]malonyl-CoA, is not diminished in the mutant (*6*) but stops at the C_{20} stage (*6, 7*), it seemed to us improbable that, in normal mouse brain, one and the same enzyme system elongates C_{18}-CoA not only to but also beyond C_{20}. If it did, the mutation would have resulted in an altered chain-length specificity. It was more likely, in our view, that the conversion of C_{18} to C_{20} is the function of one enzyme system and the extension of C_{20} to C_{22} and C_{24} is the function of another, separate from either of the two elongating activities described by Baumann and co-workers. Diminished levels of the postulated third acyl-CoA elongating system rather than impaired stearoyl-CoA elongation would satisfactorily explain the reduced content of C_{24} acids in mutant mouse brain. The results reported here support our proposal.

Brains from normal mice (Charles River Breeding Laboratory, Wilmington, Massachusetts) and from the quaking mutant (strain C57BL/6J-*qk*, Jackson Laboratory, Bar Harbor, Maine) were washed in cold 0.1*M* potassium phosphate buffer, *p*H 7.0, containing 1 m*M* dithiothreitol, 0.3*M* sucrose, and 0.9 percent NaCl. Three pooled brains were homogenized for 2 minutes with a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Microsomes prepared as described (*8*) were washed, suspended in buffer, and stored at −70°C. The conditions for enzyme assays are described in the figure legends.

In Fig. 1A are plotted activities for de novo fatty acid synthesis and for elongation of C_{16}-CoA, C_{18}-CoA, C_{20}-CoA, and C_{22}-CoA in extracts of brains taken from normal mice at various ages. The soluble fatty acid synthetase (*9*) fluctuates somewhat, rising slightly between 3 and 9 days after birth and declining slowly thereafter. Palmitoyl-CoA elongation activity is first detectable at 3 days and reaches a maximum 12 days after birth. The stearoyl-CoA elongating system appears later; it is not measurable until 5 days after birth and its activity rises to a maximum at 17 days. These differences in time course are additional and confirmatory evidence for the nonidentity of the elongating systems for palmitoyl-CoA and stearoyl-CoA (*8*). Elongation activity for C_{20}-CoA and C_{22}-CoA as a function of age is detectable 3 days after birth and rises steadily but relatively slowly thereafter. This pattern, which is distinct from that for either C_{16}-CoA or C_{18}-CoA elongation, strongly indicates the existence of a separate elongation system for the final steps in the synthesis of C_{24} acids. As shown in Fig. 1B [data taken from Baumann *et al.* (*2*)], C_{24} acids begin to appear in normal mouse brain only after all three elongating activities have become measurable by enzymatic assay.

The steep rise in the content of C_{24} fatty acids in normal mouse brain 15 to 20 days after birth, which coincides with the active phase of myelination (*2*), fails to occur in the quaking mouse, as shown by analysis of mutant total brain lipids (*2*) (Fig. 1B). Proceeding from the assumption that this



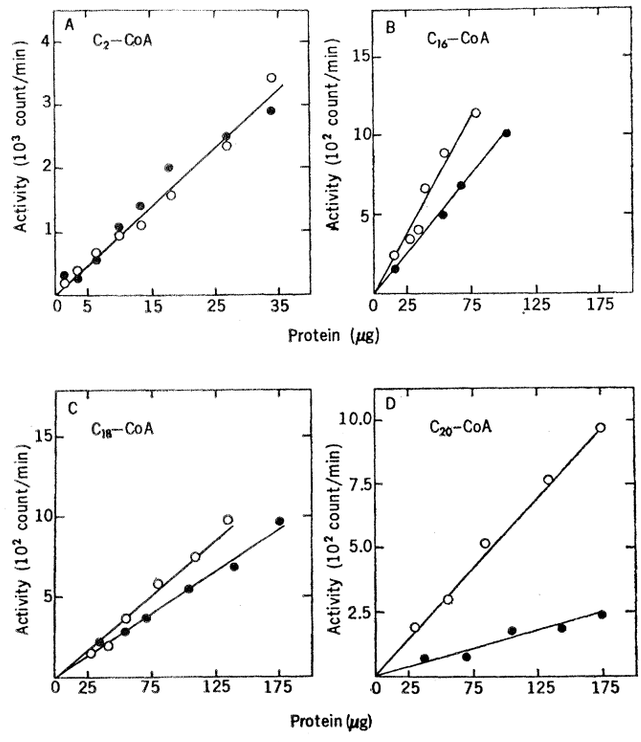


Fig. 1 (left). Fatty acid synthetase and fatty acyl–CoA elongation activities in extracts of normal mouse brain as a function of age. (A) Three brains from each age group were pooled and homogenized, and the cytoplasmic fractions (100,000*g* supernatant) and microsomal fractions were prepared. De novo fatty acid synthesis (○—○) was measured in the supernatant in an assay mixture containing, in a final volume of 0.5 ml, [2-^{14}C]malonyl-CoA (2 μc/μmole), 10 μ*M*; acetyl-CoA, 10 μ*M*; reduced nicotinamide adenine dinucleotide phosphate, 15 μ*M*; reduced nicotinamide adenine dinucleotide, 15 μ*M*; flavin mononucleotide, 0.5 μ*M*; dithiothreitol, 2 m*M*; potassium phosphate buffer, *p*H 7.2, 0.1*M*; and about 30 μg of supernatant protein. Mixtures were incubated at 36°C for 20 minutes. The same assay mixtures were used for measuring acyl-CoA elongating activity except that the [2-^{14}C]-malonyl-CoA (10 μ*M*) had specific activity of 20 μc/μmole and acetyl-CoA was replaced by the optimal concentration of palmitoyl-CoA (2.5 μ*M*) (□—□); stearoyl-CoA (2.5 μ*M*) (●—●); arachidyl-CoA (1.82 μ*M*) (×—×); or behenyl-CoA (1.27 μ*M*) (■—■). For elongation the source of enzyme was about 0.2 mg of microsomal protein. Reaction rates were linear for 40 minutes at 36°C. The microsomal fraction showed a slight activity for incorporating [2-^{14}C]malonyl-CoA into long-chain fatty acids in the absence of added acyl-CoA, presumably due to decarboxylation of the radioactive substrate. Therefore, appropriate corrections were made in calculating the various acyl-CoA elongating activities. Fatty acids were isolated from saponified reaction mixtures in the usual manner. (B) Content of lignoceric acid (C_{24}) in total mouse brain lipids is shown at various intervals after birth for normal (●—●) and quaking (○—○) mice. Data were recalculated from Baumann *et al.* (*2*). Fig. 2 (right). Comparison of fatty acid synthetase and acyl-CoA elongating activities in normal and quaking mouse brains taken from animals 15 days after birth. The enzyme activities, assayed as described for Fig. 1, were for (A) de novo fatty acid synthesis, (B) palmitoyl-CoA elongation, (C) stearoyl-CoA elongation, and (D) arachidyl-CoA elongation; normal mice (○—○); quaking mice (●—●).

abnormality might be due to a deficiency in one or more of the elongating steps, we compared the various enzyme activities in brain extracts of normal and quaking mice 15 days after birth (Fig. 2). The soluble fatty acid synthetase (palmitate production) was as active in the mutant as in the normal mouse; the activities of the microsomal C_{16}-CoA and C_{18}-CoA elongating systems in the brains of quaking mice were 70 and 80 percent of normal, respectively, in confirmation of the results of Bourre *et al.* (*6*). On the other hand, both C_{20}-CoA and C_{22}-CoA (not shown) elongation was more severely impaired in the quaking mouse. These two activities were reduced to 25 percent of normal. The same results (not shown) were obtained when quaking and normal mice were compared 22 days after birth.

While we confirm the findings of Baumann and colleagues regarding the existence of separate mouse brain systems for elongating palmitoyl-CoA and stearoyl-CoA (*8*), we further demonstrate that (i) a third enzyme system is required for elongating fatty acyl–CoA derivatives beyond C_{20} and (ii) this activity is the one that is most noticeably lowered in brain tissue of quaking mice. We therefore suggest that a defect in the elongation of C_{20}-CoA rather than of C_{18}-CoA is responsible for the drastically reduced content of C_{24} acids in brain lipids of quaking mice (*10*).

In comparing the elongation of C_{20}-CoA and C_{22}-CoA we found identical activities for these two substrates both with respect to age dependence (Fig. 1A) and reduced levels in the quaking mouse (Fig. 2). It therefore seems likely that a single enzyme catalyzes the steps from C_{20}-CoA to C_{22} and from C_{22}-CoA to C_{24}.

ISRAEL GOLDBERG*
ISHAIAHU SHECHTER†, KONRAD BLOCH
James Bryant Conant Laboratories,
Harvard University,
Cambridge, Massachusetts 02138

References and Notes

1. R. L. Sidman, M. M. Dickie, S. H. Appel, *Science* **144**, 309 (1964).
2. N. A. Baumann, J. M. Bourre, C. Jacque, S. Pollet, in *Lipids, Malnutrition and the Developing Brain* (A Ciba Foundation Symposium), K. Elliott and J. Knight, Eds. (Associated Science, New York, 1971), pp. 91–105.
3. N. A. Baumann, M. L. Harpin, J. M. Bourre, *Nature* **227**, 961 (1970).

4. C. M. Jacque, M. L. Harpin, N. A. Baumann, *Eur. J. Biochem.* **11**, 218 (1969); N. A. Baumann, C. M. Jacque, S. A. Pollet, M. L. Harpin, *ibid.* **4**, 340 (1968).
5. S. Pollet, J. M. Bourre, N. A. Baumann, *C. R. H. Acad. Sci. Ser. D* **268**, 2146 (1969).
6. J. M. Bourre, S. Pollet, O. Daudu, N. A. Baumann, *ibid.* **273**, 1534 (1971).
7. ——— in Abstracts, 15th International Conference on the Biochemistry of Lipids, The Hague, Netherlands, 1972, p. 32.
8. J. M. Bourre, S. Pollet, G. Dubois, N. A. Baumann, *C. R. H. Acad. Sci. Ser. D* **271**, 1221 (1970); S. Pollet, J. M. Bourre, O. Daudu, N. A. Baumann, *ibid.* **273**, 1426 (1971); S. Pollet, J. M. Bourre, G. Chaix, O. Daudu, N. A. Baumann, *Biochimie*, in press.
9. The soluble fatty acid synthetase has a molecular weight of 600,000, as estimated by chromatography on Sepharose 4-B. Palmitate is the principal product (93 percent), largely in the unesterified form.
10. Brain tissue contains high levels of enzymes that cleave long-chain acyl-CoA derivatives such as palmitoyl-CoA and stearoyl-CoA. These thioesterases occur both in the cytoplasm [S. Kurooka, K. Hosoki, Y. Yoshimura, *J. Biochem.* **71**, 625 (1972)] and in the microsomal fraction [S. Pollet, J. M. Bourre, O. Daudu, N. Baumann, *C. R. H. Acad. Sci. Ser. D* **273**, 1632 (1971)]. We found that microsomal or cytoplasmic mouse brain thioesterases also hydrolyze arachidyl-CoA. Neither their relative substrate specificities (for C_{16}-CoA, C_{18}-CoA, or C_{20}-CoA) nor their total activities differ significantly in normal and quaking mice. Also, the changes in thioesterase activity (with C_{16}-CoA or C_{18}-CoA as substrates) in normal mouse brain as a function of age bear no evident relation to the time course of C_{16}-CoA and C_{18}-CoA elongating activities. It therefore seems unlikely that altered thioesterase levels are responsible for the changed long-chain fatty acid pattern in the brain lipids of the quaking mouse.
11. Supported by grants-in-aid from the Public Health Service, the National Science Foundation, and the Eugene P. Higgins Trust Fund of Harvard University. Address reprint requests to K.B.
* Present address: Laboratory of Applied Microbiology, Hadassah Medical School, Hebrew University, Jerusalem, Israel.
† Present address: Department of Biochemistry, Tel Aviv University, Ramat Aviv, Tel Aviv, Israel.

18 July 1973

Dopaminergic Terminals in the Rat Cortex

Abstract. *The destruction of ascending noradrenergic pathways by bilateral microinjections of 6-hydroxydopamine made laterally to the pedunculus cerebellaris superior completely abolished the in vitro synthesis of [^{3}H]norepinephrine from* L-[^{3}H]*tyrosine in slices and in synaptosomes of the rat cortex. However, normal [^{3}H]dopamine synthesis could still be observed in both cortical preparations from animals with lesions. These results provide the first biochemical support for the existence of dopaminergic terminals independent of noradrenergic terminals in the rat cortex.*

Histochemical and lesion studies have indicated three main ascending dopaminergic systems in the rat brain: the nigrostriatal pathway, which is important in extrapyramidal processes (*1*); the tuberoinfundibular system, which is particularly involved in neuroendocrine mechanisms (*2*); and the mesolimbic system (*3*), whose functions are not yet known. High concentrations of dopamine (DA) have been found in the structures innervated by these systems (*4*). Dopamine has also been observed in other brain regions, but it was considered to be present not as a neurotransmitter but as the precursor of norepinephrine (NE). For instance, the cerebellum and the cortex, both innervated by noradrenergic neurons originating from the locus coeruleus, contain not only NE but also DA (*5*). However, DA, which is almost undetectable in the cerebellum by classical spectrofluorimetric techniques, is present in measurable amounts in the cerebral cortex. In this structure we found that DA concentrations were more than 50 percent of those of NE (*6*). This observation led us to investigate whether cortical DA was localized only in noradrenergic terminals in the rat cortex. In an initial study, we observed that electrolytic or chemical destruction of the noradrenergic ascending pathways resulted in 80 to 100 percent reduction in cortical NE levels, whereas those of DA were only slightly affected (*6*). These results revealed the existence of extranoradrenergic stores of DA. We now report not only the occurrence of extranoradrenergic stores of DA, but also the presence of DA terminals in the rat cerebral cortex.

Groups of eight Charles River male rats (300 to 350 g) were killed by decapitation 5 weeks after bilateral chemical lesions made laterally to the pedunculus cerebellaris superior (LPCS) [plane A_{160}, König and Klippel atlas (*7*)] to specifically destroy the noradrenergic ascending pathways. Chemical lesions were made while animals were under chloral hydrate anesthesia (500 mg per kilogram of body weight) by microinjection of 6-hydroxydopamine (6-OHDA) (*8*) with the use of a Stoelting stereotaxic apparatus. Sham-operated animals were used as controls.

If DA is found in the cortex after destruction of the noradrenergic ascending pathways, DA synthesis from tyrosine should persist whereas that of NE should be markedly reduced. This was first demonstrated in vitro in cortical slices. Immediately after each animal was killed, the cortex was dissected out and 200 mg of cortical tissues was cut in slices (0.4 mm thick) with a McIllwain apparatus. The slices were incubated in 2 ml of physiological medium (*9*) at 37°C for 15 minutes under a constant stream of 95 percent O_2 and 5 percent CO_2 in the presence of 68 μc of L-[3,5-$^3H_2$]tyrosine (40 c/mmole) (Commissariat à l'Énergie Atomique, France). At the end of the incubation period, [^{3}H]DA and [^{3}H]NE in tissues and incubating medium were estimated. Labeled catecholamines were first extracted with ethanol-water (74 : 16) and then separated from ^{3}H-labeled precursors and amine metabolites by successive ion exchange chromatography on Amberlite CG 50 and adsorption on alumina as described (*10*). They were finally separated by ion exchange chromatography on Dowex AG 50 WX4 (*11*). Tyrosine was separated by ion exchange chromatography on Dowex AG 50 (H+ form) and estimated spectrofluorimetrically (*12*).

The combined destruction of the ventral and dorsal noradrenergic ascending pathways induced an almost complete disappearance of [^{3}H]NE formation (2.5 percent of control values) (Table 1). This effect was not associated with a parallel decrease in [^{3}H]DA synthesis. The accumulated [^{3}H]DA in slices and incubation medium still represented 46

Table 1. Synthesis of ^{3}H-labeled catecholamines from L-[^{3}H]tyrosine in cortical slices and in purified synaptosomal preparations of rats with lesions of noradrenergic ascending pathways made by 6-OHDA microinjection. Protein was estimated by the spectrophotometric method of Lowry *et al.* (*21*). Controls were sham-operated rats.

Group	Tyrosine (μg per gram of wet tissue)	[^{3}H]Norepinephrine (nc per gram of wet tissue)	[^{3}H]Dopamine (nc per gram of wet tissue)
	Cortical slices		
Control	15.9 ± 1.6	171.8 ± 20.0	152.2 ± 13.1
6-OHDA	14.5 ± 0.4	4.4 ± 0.7*	70.1 ± 10.0*
	Cortical synaptosomes		
Control	0.210 ± 0.006	5.250 ± 0.200	8.85 ± 0.28
6-OHDA	0.189 ± 0.005	0.340 ± 0.002*	5.78 ± 0.25*

* $P < .001$, Student's t-test.