eragrostoid lines of phylogeny, formed C_4 dicarboxylic acids (malate, aspartate, and oxaloacetate) as initial photosynthetic products, but did not evolve carbon dioxide by photorespiration (*5, 11*). The amount of ^{14}C incorporated into aspartic and malic acids ranged from 85 to 92 percent of the total ^{14}C fixed. Consistent with this, leaves of *Panicum miliaceum* contained much PEP carboxylase activity (28.15 μmole of CO_2 fixed per milligram of extractable chlorophyll per minute). This enzyme is used for the synthesis of C_4 compounds (*10*). The parenchyma bundle sheath was extensively developed and contained large starch-laden chloroplasts. The mesophyll cells accumulated little starch.

The dichantheloid species, *Panicum commutatum, P. lindheimeri,* and *P. pacificum,* like the previously studied temperate grasses, synthesized phosphorylated compounds typical of the Calvin cycle, as major products of carbon dioxide fixation (*11*). Phosphorylated compounds accounted for 86 to 95 percent of the total ^{14}C fixed. The leaves also had an active photorespiratory pathway manifested by the evolution of carbon dioxide during photosynthesis (*5*). As expected (*10*), leaf extracts of *Panicum pacificum* had low PEP carboxylase activity (3.16 μmole of CO_2 fixed per milligram of extractable chlorophyll per minute). This value was virtually identical to that found for wheat leaves. Unlike many temperate grasses, however, leaves of the subgenus Dichanthelium contained extensively developed parenchyma bundle sheaths. Despite this elaboration, chloroplasts were absent from the tissue. Leaves of the rosette, vernal, and autumnal growth phases were similar in structure and photosynthetic physiology.

The absence of detectable photorespiration in plants of tropical origin could result from a deficiency of the pathway found in temperate species (*12*), or from a mechanism that prevents carbon dioxide loss. The great affinity of PEP carboxylase for carbon dioxide is well known (*13*). The high activity of this enzyme in tropical plants might preclude the release of carbon dioxide to the outside of the leaf. The specialized chloroplasts within the parenchyma bundle sheath could also be a determinant. Whatever the correct explanation may be, the important point is that tropical grass species do not lose carbon during photosynthesis and temperate species do. The discovery of different physiological and cytological phenotypes within the same genus may now permit genetic analysis of photorespiration and assessment of its importance to the carbon budget of the plant.

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Induction of Brain D(—)-β-Hydroxybutyrate Dehydrogenase Activity by Fasting

Abstract. *D(−)-β-hydroxybutyrate dehydrogenase activity is very low in normal adult rat brain; but during fasting it increases severalfold in parallel with the ketosis. The increase may represent part of a mechanism by which the brain adapts to changing patterns of substrate supply during starvation.*

Owen *et al.* (*1*) have observed that, in obese human subjects after a 5 to 6 week fast, the brain uptake of β-hydroxybutyrate and acetoacetate assumes significant proportions. If completely oxidized to carbon dioxide and water, these metabolites would have accounted for 60 percent of the total cerebral oxygen consumption—52 percent by β-hydroxybutyrate alone—whereas glucose utilization would have required at most only 29 percent of the oxygen uptake. This is in marked contrast to the situation in normal adult brain in which ketone bodies are only negligibly utilized and glucose uptake accounts for almost all the oxygen consumed (*2*). There is, in fact, considerable evidence that no substrate other than glucose can be oxidized sufficiently rapidly by mature brain to maintain its normal energy metabolism and function (*2*). Immature brain may be different. Drahota *et al.* (*3*) have observed that acetoacetate can support nearly the same rate of oxygen consumption as glucose in cerebral cortical slices from 5-day-old rats. Recent studies in our laboratory (*4*) have revealed high levels of activity of the mitochondrial enzyme, D(−)-β-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30, D-3-hydroxybutyrate: NAD^+ oxidoreductase) (BDH) in brains of unweaned rats, but the enzyme activity declines to very low levels when the brain matures. The question then arises how, in view of the apparent limited ability of the normal mature brain to oxidize substrates other than glucose and of its low D(−)-β-hydroxybutyrate dehydrogenase activity, β-hydroxybutyrate utilization could reach such high levels in the brains of the fasting adult patients studied by Owen *et al.* (*1*). Our results demonstrate that fasting leads to an increase in D(−)-β-hydroxybutyrate dehydrogenase activity in adult brain.

We used normal adult male Sprague-Dawley rats approximately 8 months of age and having a mean weight ± standard error of 580 ± 24 g. The animals were housed in individual wire cages; they were allowed free access to Purina Laboratory Chow for 2 weeks before the study began and free access to water at all times. Daily intraperitoneal injections of their minimal daily requirements of thiamine, riboflavin, niacinamide, pyridoxine, and pantothenic acid (*5*) were instituted 1 week before the beginning of fasting. The study was begun by the initiation of fasting; all food (but not water) and feces were removed from the cages at 9:00 a.m. of the selected day. At various times after

Table 1. Effects of fasting on total body weight, blood D(−)-β-hydroxybutyrate concentration, brain weight, and protein content of crude mitochondrial enzyme preparation. Data presented are the means ± standard errors.

Duration of fast (hr)	Animals (No.)	Decrease in body weight (%)	Blood D(-)-β-hydroxybutyrate (μmole/ml)	Brain weight (g)	Protein content of brain fraction* (mg/5 ml)
0	6	—	0.24 ± 0.01	$2.01 \pm 0.00_5$	31.1 ± 3.1
48	3	12	0.87 ± .09	$1.92 \pm .00_3$	30.1 ± 3.3
72	3	19	1.39 ± .25	$1.98 \pm .00_5$	35.3 ± 3.8
120	3	22	1.04 ± .17	$1.93 \pm .00_6$	34.1 ± 4.1

* Five milliliters of the crude brain fraction containing the enzyme activity were derived from 1.0 g of wet weight of brain.

the beginning of fasting, the control and fasting animals were decapitated; their brains were removed, chilled to 0°C in an ice-cold solution of 0.25*M* sucrose and $10^{-4}M$ ethylenediaminetetraacetate (EDTA) (*p*H 7.4), and homogenized by hand for 30 seconds in a Tenbroeck homogenizer in the same solution (5 ml per gram of fresh brain). The preparation of the homogenates was carried out in a cold room maintained at 2° to 4°C. The homogenates were first centrifuged at 700*g* for 10 minutes in a refrigerated centrifuge to remove the intact cells and cell debris; and the supernatant was centrifuged again at 25,000*g* for 20 minutes. The resulting pellet, which contained most of the mitochondria and more than 90 percent of the total BDH activity of the homogenate, was then suspended in 5 ml of the 0.25*M* sucrose and $10^{-4}M$ EDTA solution per gram of original brain weight and sonified (Branson model No. 75 sonifier at maximum plate current) for 5 minutes at −25° to −30° in an ethanol-dry ice bath to maintain the temperature of the suspension at 0° to 10°C.

The BDH activity was assayed in the sonified preparations by measurement of either acetoacetate production by the method of Walker (*6*) or D(−)-β-hydroxybutyrate-dependent nicotinamide-adenine dinucleotide (NAD^+) reduction. Since both assay methods yielded essentially similar results, only those obtained with the method based on NAD^+ reduction are presented. Production of NADH was followed continuously by measurement of absorbance at 340 nm in a Gilford model No. 2000 spectrophotometer. Corrections were made for substrate-free and enzyme-free controls (Table 2). The assay system was at optimum *p*H, and the initial rates were proportional to enzyme concentration over a tenfold range.

Concentration of D(−)-β-hydroxybutyrate in blood was determined by Young and Renold's (*7*) fluorimetric modification of the method of Williamson *et al.* (*8*) in samples obtained at the time of decapitation. Protein was measured by the method of Lowry *et al.* (*9*).

Fasting caused the expected progressive decrease in total body weight and a marked ketosis, the blood β-hydroxybutyrate concentration increasing more than fivefold by 72 hours of starvation (Table 1). Total brain weight tended to decline slightly during the fast, but the total protein content of the sonified, crude fraction which contained the BDH activity did not change significantly (Table 1).

The BDH level in brain increased during the fast, reaching a peak of five times its normal activity at 72 hours (Table 2). The degree and time course of its change, therefore, closely paralleled that of the concentration of D(−)-β-hydroxybutyrate in blood. The changes in enzyme activity are equally apparent whether expressed as the amount per unit weight of total brain or as the specific activity of the total protein recovered in the subcellular fraction known to contain the D(−)-β-hydroxybutyrate dehydrogenase (Table 2).

Sekuzu *et al.* (*10*) have demonstrated that beef heart BDH has an absolute requirement for lecithin, and Gotterer (*11*) has observed a similar phenomenon with the rat liver enzyme. The rat brain enzyme also probably has a lecithin requirement in that all the sonified preparations of rat brain enzyme studied in our laboratory show an increase above "native" activity after the addition of lecithin. The increased enzyme activity which accompanies fasting is not, however, merely the result of increased lecithin cofactor in the tissues; the effect of fasting is, if anything, even more pronounced when the enzyme is assayed in the presence of saturating amounts of added lecithin (Table 2). In fact, the lecithin sensitivity of the BDH activity of brain increases during fasting (Table 2), suggesting that the lecithin cofactor may become more limiting during fasting.

Our results suggest an interesting adaptation of the brain to the effects of starvation. D(−)-β-Hydroxybutyrate dehydrogenase activity is very high in developing brain but becomes very low after maturation (*4*). The normal adult brain is, in fact, almost completely dependent on glucose as the oxidizable substrate for its energy generation (*2*). During starvation the body's carbohydrates stores become depleted, and the supply of glucose then becomes limited only to that made available through gluconeogenesis. On the other hand, a ketosis develops, with marked increases in the concentration in the blood of D(−)-β-hydroxybutyrate and acetoacetate derived from the breakdown of stored fat. The brain apparently adapts to this altered pattern of substrate supply with an increase in its D(−)-β-hydroxybutyrate dehydrogenase activity. In rats the enzyme level may reach 90 percent of the maximum present in the brain during the postnatal developmental period. The brain can then utilize D(−)-β-hydroxybutyrate in place of glucose as an oxidizable substrate. Owen *et al.* (*1*) have estimated, on the basis of cerebral arteriovenous differences, that β-hydroxybutyrate oxidation accounted for a least 50 per-

Table 2. Effects of fasting on D(−)-β-hydroxybutyrate dehydrogenase activity in crude mitochondrial fractions from adult rat brain. The contents of the reaction mixtures included 180 μmole of NAD^+, 33 μmole of mercaptoethanol, 25 μmole of nicotinamide, 126 μmole of antimycin, 1.8 mmole of sodium DL-β-hydroxybutyrate, 1.5 mmole of tris chloride buffer (*p*H 8.6), and 0.05 ml of sonified enzyme preparation in a final volume of 3.0 ml. Lecithin prepared from adult rat brain (*12*) was included where indicated; the amount was equivalent to 0.375 μmole of lecithin phosphate per flask. Incubation temperature was 37.5°C. The values presented are the means ± standard errors of the number of animals indicated in Table 1.

Duration of fast (hr)	Enzyme activity (μmole NADH/100 min)			
	Per gram of total brain		Per milligram of protein in enzyme preparation	
	Native	Lecithin added	Native	Lecithin added
0	24 ± 5	51 ± 7	0.81 ± 0.22	1.8 ± 0.3
48	139 ± 3	334 ± 1	4.70 ± .47	11.3 ± 1.3
72	192 ± 6	452 ± 58	5.56 ± .81	13.2 ± 2.9
120	73 ± 23	298 ± 87	2.09 ± .45	6.9 ± 2.2

cent of the cerebral oxygen consumption in the starving adult human patients studied by them. A similar adaptation may occur in regard to acetoacetate, but it is probably of lesser quantitative significance in that the concentration of acetoacetate in blood is only a small fraction of that of β-hydroxybutyrate during fasting (*1*).

The mechanism of the increased brain D(−)-β-dehydrogenase activity during fasting is still unknown.

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Giant Hepatic Mitochondria: Production in Mice Fed with Cuprizone

Abstract. *Giant mitochondria in hepatocytes that have the average size of nuclei can be consistently produced in the liver of weanling mice by feeding them cuprizone (bis-cyclohexanone oxaldihydrazone). The simplicity of the procedure and the consistency of the results make the feeding of cuprizone a new and useful experimental tool for the study of mitochondrial metabolism.*

Cuprizone (bis-cyclohexanone oxaldihydrazone) is a chelating agent used for quantitative determination of copper (*1*). It is also known as a strong inhibitor of amine oxidase of bovine hepatic mitochondria (*2*). When administered orally, this compound produced severe status spongiosus and enlarged glial nuclei resembling Alzheimer's glia in the cerebellum and the brainstem of mice (*3*). Alzheimer's glia are usually associated with hepatic damage. During the course of our ultrastructural study on cuprizone-induced encephalopathy, we examined livers of mice that had been fed cuprizone and found that the compound consistently produced extremely enlarged mitochondria in hepatocytes.

Weanling Swiss-Webster male mice were fed with a mixture of 0.5 g of cuprizone (*4*) in 100 g of Rockland Mouse/Rat Diet (*5*) from 3 weeks of age. Water was freely available. Compared to control animals that were fed with the same diet, except without cuprizone, experimental animals became inactive in 1 to 2 weeks, showing marked retardation of their growth and weakness of posterior limbs. Most of them died within 3 weeks when cuprizone feeding continued. All of them exhibited brain edema. Details of the neuropathological aspects of cuprizone intoxication will be reported separately (*6*). For electron microscopic examination of the liver, the tissue was fixed by perfusion with 5 percent glutaraldehyde, tnen post-fixed with osmium tetroxide or directly fixed with osmium tetroxide, and embedded in Epon; the sections were stained with uranyl acetate and lead citrate.

All hepatocytes contained extremely enlarged mitochondria that varied in size from 2 to 10 μm in diameter (Fig. 1). Some of the larger mitochondria measured up to 15 μm in diameter. The mitochondria had abundant, slightly osmiophilic granular matrix. Cristae were often arranged irregularly at the periphery, and they appeared to remain normal in size, but the intercristal space was enlarged, giving the relative appearance of short cristae. The outer mitochondrial membranes were in most instances intact. All mitochondria in all hepatic cells in all experimental animals were invariably affected. These giant mitochondria could be seen in preparations stained with hematoxylin-eosin by light microscope as well-defined pale areas in the cytoplasm, but they could easily be overlooked unless the observer was aware of the electron microscopic finding.

Although enlarged hepatic mitochondria have been reported in several other conditions (*7*), the magnitude of the change seen in cuprizone-fed mice appears to be unique. I believe that cuprizone should serve as a useful tool to study mitochondrial metabolism and its relation to their morphology. Since cuprizone binds copper strongly and deprives the tissue of copper required for its normal function, it is reasonable to suspect that mitochondrial copper metabolism may in some way be in-

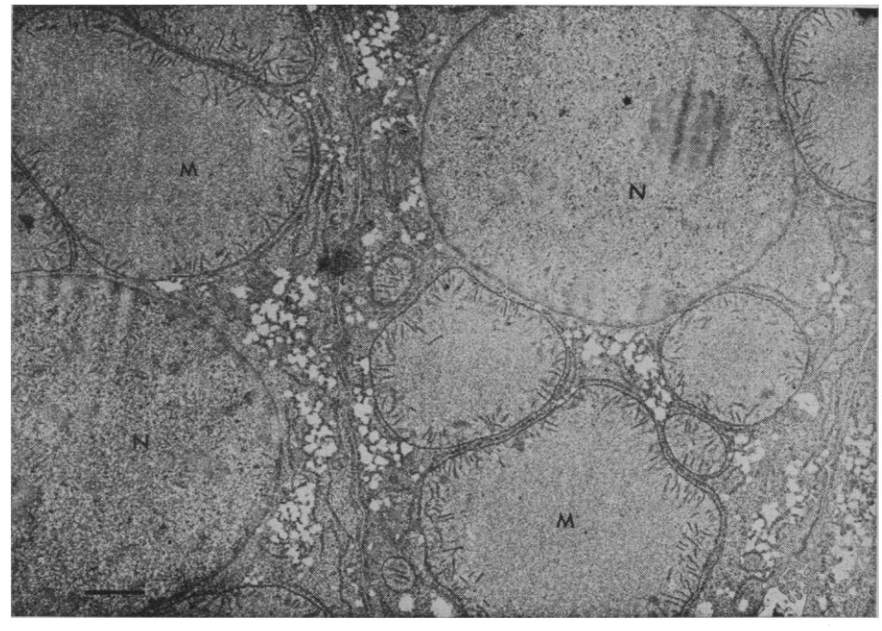

Fig. 1. Electron micrograph of the giant mitochondria in the liver of mice fed with cuprizone. The tissue was fixed by perfusion with 5 percent glutaraldehyde and then post-fixed with osmium tetroxide. *N*, nucleus; *M*, mitochondria. Scale is 1 μm.