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Studies of the Glycine Cleavage Enzyme System in Brain from Infants with Glycine Encephalopathy

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Summary

Glycine content and enzyme activity of the glycine cleavage system were compared in autopsied brain from five infants dying with glycine encephalopathy and four control infants, including two with other types of hyperglycinemia. Glycine content was elevated 2- to 8-fold and glycine cleavage enzyme activity was undetectable in the brains of the glycine encephalopathy patients. Glycine content and enzyme activity were normal in the brains of the control patients, including one with ketotic hyperglycinemia secondary to methylmalonic acidemia. Prolonged dialysis failed to restore glycine cleavage enzyme activity in brain homogenates of glycine encephalopathy patients, and these homogenates failed to inhibit enzyme activity when added to homogenates of control brain. Radioactive bicarbonate was converted to radioactive glycine by control brain, but not by glycine encephalopathy brain. This finding, together with the results of recombination experiments between solubilized human brain enzymes and purified protein components of the bacterial glycine cleavage system of *Arthrobacter globiformis*, indicates that the enzyme defect in glycine encephalopathy involves at least the second or H protein of the 4-protein glycine cleavage enzyme system.

Speculation

The severe neurologic syndrome that characterizes glycine encephalopathy, in contrast to some other forms of hyperglycinemia, is probably caused by absence of glycine cleavage enzyme activity and marked elevation of glycine content in infantile brain. Since symptoms of this inherited disorder only appear shortly after birth, a firmly bound endogenous inhibitor of the glycine cleavage enzyme may accumulate in brain of affected infants, who have been protected during fetal life by clearance of the putative inhibitor across the placenta.

Plasma glycine concentrations are abnormally high in a variety of different genetically determined metabolic disorders of infants and children. These disorders are generally classified as ketotic hyperglycinemias if ketoacidosis is present, and if an unusual organic acid accumulates in blood and tissues and is excreted in the urine. For example, hyperglycinemia often accompanies propionic acidemia (2) and methylmalonic acidemia (12), and it also has been observed in some patients with isovaleric acidemia (1) and β-ketothiolase deficiency (7). When ketoacidosis is absent and no unusual organic acids can be detected in blood or urine, hyperglycinemias are termed nonketotic. However, there are marked clinical differences among patients with so-called nonketotic hyperglycinemia. Some show little evidence of serious illness and have no neurologic symptoms. Others exhibit a severe neurologic disorder which commences within a few days or weeks of birth. It is characterized by lethargy, intractable seizures, spasticity, mental retardation, and usually by early death (16).

We found (20) that three infants with this latter syndrome had greatly elevated glycine concentrations in their cerebrospinal fluid (CSF), in contrast to the normal glycine levels found in the CSF of other hyperglycinemic patients who had no neurologic symptoms. Glycine content was markedly elevated in the brains of two infants who died. Glycine cleavage enzyme activity was not detectable in the brains of these infants, although it was present in their livers at autopsy. We suggested the term glycine encephalopathy to describe this syndrome, and to differentiate it from other nonketotic hyperglycinemias (20).

The glycine cleavage enzyme system is a complex of four different interacting proteins (15). We describe here experiments carried out on autopsied brain from 5 infants with glycine encephalopathy and four control infants that were designed to localize the exact site of the defect in this enzyme system. We have also sought to explain why infants with glycine encephalopathy are apparently normal during intrauterine life, but become severely affected soon after birth.

PATIENTS

Patients *1* and *2* were sisters, aged 10 months and 8 months at death, whom we have described previously (20). Their CSF glycine concentrations during life ranged from 124–203 μmol/liter, and from 82–93 μmol/liter, respectively (34 control infants = 5.2 ± 2.0 μmol/liter).

Patient *3* (21) was born at term after an uneventful pregnancy, and he appeared to be normal during the first 48 hr of life. Thereafter he became listless and fed poorly, and by the age of 72 hr was markedly hypotonic and apneic. Plasma glycine on the 11th day of life was 2027 μmol/liter (normal = 209 ± 46 μmol/liter), and the CSF glycine concentration was 342 μmol/liter. This infant died on the 13th day of life. His only sibling had developed a similar illness on the 3rd day of life and had died on the 8th day, but biochemical studies had not been undertaken.

Patient *4* (21) was normal at birth, but she became ill at the age of 34 hr, and died at 32 days after a course characterized by lethargy, proceeding to coma and seizures. Plasma glycine concentrations ranged from 152–1350 μmol/liter, and CSF glycine concentrations varied between 181 and 300 μmol/liter. A sister of patient *4* had died previously at the age of 8 days with proven hyperglycinemia.

Patient *5* (21) was normal at birth, but he developed neuro-

logic symptoms by the age of 72 hr, and died at 18 days. His plasma glycine concentrations ranged between 116 and 1752 μmol/liter, and his CSF glycine concentrations ranged between 81 and 267 μmol/liter.

Tissues were also obtained at autopsy from four control infants. Two of these infants died without evidence of any metabolic disorder. Control infant *3* had a hyperglycinemia of undetermined type, but showed no neurologic symptoms (20). Control infant *4* died at the age of 2 months with hyperglycinemia secondary to methylmalonic acidemia. The latter diagnosis was confirmed by gas chromatographic demonstration of methylmalonic acid in the infant's urine, and by absence of methylmalonyl mutase apoenzyme activity in a specimen of the infant's liver obtained 2 hr after death (21).

Brain and liver specimens were obtained at autopsy from the five glycine encephalopathy patients and the four control infants (22) within 1–9 hr after death, and were kept frozen at −80° until amino acid analyses and enzyme assays were performed.

MATERIALS AND METHODS

AMINO ACIDS

Frozen brain specimens were homogenized and deproteinized with 0.4 M perchloric acid as previously described (17, 18), and amino acids and related compounds were quantitated on a Technicon amino acid analyzer (21), using the single column lithium citrate buffer elution system of Perry *et al.* (19).

STANDARD GLYCINE CLEAVAGE ENZYME ASSAYS

Activity of the glycine cleavage enzyme system was determined *in vitro* by measuring the rate of formation of $^{14}CO_2$ from [1-^{14}C]glycine, using a modification of the method of Bruin *et al.* (4). Frozen autopsied brain or liver tissue was homogenized in 10 volumes ice-cold 0.32 M sucrose, using 10 strokes in a motor-driven, Teflon pestle tissue grinder. Reaction mixtures (final volume of 2.55 ml) contained 125 μmol Tris-HCl buffer (pH 8.0), 0.5 μmol pyridoxal phosphate, 5 μmol nicotinamide adenine dinucleotide, 5 μmol dithiothreitol, 0.69 μmol tetrahydrofolate, 10 μmol glycine containing 1 μCi [1-^{14}C]glycine (22), and tissue homogenate equivalent to 8–20 mg protein. Protein concentrations were determined by the Lowry method (11).

Incubations were routinely carried out for 30 min at 37°, since preliminary experiments with homogenates of human brain and liver had shown that release of $^{14}CO_2$ was linear with time for at least 30 min. The $^{14}CO_2$ released was trapped in NCS Solubilizer and counted for radioactivity in a Packard Tri-Carb liquid scintillation counter (23). Blank reaction mixtures contained all components except the tissue homogenate, which was replaced by 0.32 M sucrose. All assays were carried out in duplicate or triplicate, and results were corrected for any radioactivity observed in the blanks.

MIXING AND DIALYSIS EXPERIMENTS

Homogenates of brain from control subjects and from glycine encephalopathy patients were mixed in equal proportions in efforts to demonstrate the presence of a possible inhibitor of the glycine cleavage enzyme system in the patients' brains. Mixing was done immediately before enzyme assay in some experiments, whereas in others, the mixed homogenates were stirred at 2° for 24 or 48 hr before being assayed.

Brain tissue homogenized in 10 volumes ice-cold 0.32 M sucrose, containing 0.5 mM dithiothreitol, was dialyzed at 2° for 24 or 46 hr against 100 volumes 0.32 M sucrose containing 0.5 mM dithiothreitol in efforts to remove a putative enzyme inhibitor. The dialysate was changed at 12-hr intervals. In these experiments, undialyzed aliquots of the same brain homogenates, which had stood at 2° during the same intervals, were used to control for deterioration of enzyme activity with time.

EXCHANGE OF CARBOXYL GROUP OF GLYCINE WITH [^{14}C]BICARBONATE

The conversion of radioactive bicarbonate to radioactive glycine by brain homogenates was assayed by a modification of the method of Motokawa and Kikuchi (13). Reaction mixtures (final volume of 2.6 ml) contained 125 μmol Tris-HCl buffer (pH 8.0), 0.5 μmol pyridoxal phosphate, 10 μmol dithiothreitol, 5 μmol glycine, 10 μmol sodium bicarbonate containing 10 μCi of $NaH^{14}CO_3$ (24), and brain homogenate equivalent to 12 mg protein. The mixtures were incubated at 37° for 30 min, and the reactions were stopped and the mixtures deproteinized by addition of perchloric acid to give a final concentration of 0.4 M. After $^{14}CO_2$ had been released from the acidified mixtures, the denatured protein was separated by centrifugation, and the excess perchlorate was removed by addition of potassium hydroxide (17).

Each reaction supernatant was then subjected to preparative chromatography on the amino acid analyzer (19), with collection of the zones of column effluent in which serine and glycine were eluted. These two effluent zones were then dried in a vacuum desiccator, dissolved in a small volume of water and Aquasol, and their radioactivity counted in a scintillation counter.

PREPARATION OF SOLUBILIZED GLYCINE CLEAVAGE ENZYME FROM BRAIN

Frozen brain tissue was ground to a fine powder in liquid nitrogen with use of a mortar and pestle, and was then lyophilized. The dry brain powder was next homogenized with 40 volumes acetone at −15° in a Teflon pestle glass homogenizer, and was centrifuged at 4000 × *g* for 10 min at − 15°. The precipitate was washed 3 times with 3 volumes precooled acetone, and was then dried in a vacuum desiccator at −20°. The dry acetone-extracted brain powder was then stirred for 30 min at 2° with 15 volumes 0.32 M sucrose containing 0.5 mM dithiothreitol. The suspension was centrifuged at 16,000 × *g* for 10 min, and the sucrose extraction was repeated on the pellet. After centrifugation, the two supernatants were combined and were dialyzed for 2 hr at 2° against 100 volumes 0.32 M sucrose containing 0.5 mM dithiothreitol.

PREPARATION OF BACTERIAL GLYCINE CLEAVAGE ENZYME FRACTIONS

The P.T.L-protein and H-protein fractions of a bacterial glycine cleavage complex were prepared from *A. globiformis*, strain 1350 (25), using the methods described by Kochi and Kikuchi (8). These methods employ ammonium sulfate precipitation of proteins from a crude bacterial extract, followed by a separation of the proteins on a DEAE-cellulose column washed with 0.02 M potassium phosphate buffer (pH 7.1) containing increasing concentrations of NaCl. Proteins eluted with buffer containing 0.2 M NaCl were designated P.T.L-protein, and protein eluted with buffer containing 0.5 M NaCl was designated H-protein (8).

BRAIN-BACTERIAL ENZYME RECOMBINATION EXPERIMENTS

Enzyme assays were carried out with combinations of the solubilized glycine cleavage complex from human brain (1.0 ml) and bacterial enzyme protein fractions (0.5 ml). The conditions for these recombination assays were the same as described previously for the standard glycine cleavage assay on brain and liver homogenates, except that incubations were for 30 min at 33.5°, midway between the temperatures optimal for the bacterial glycine cleavage system (30°) and the human system (37°).

ORGANIC ACIDS

Urine and plasma specimens from the glycine encephalopathy patients were acidified to pH 1.0 and extracted into ethyl

acetate. The extracted organic acids were methyl esterified and subjected to gas chromatography in a search for organic acids known to be associated with hyperglycinemias (6).

In a specific search for glyceric acid, other ethyl acetate extracts of acidified urine and of acidified brain (deproteinized in 80% ethanol) were treated with Tri-Sil/BSA (26) to form the trimethylsilyl derivatives of organic acids. An internal standard of 54 μg *o*-toluic acid (27) was added to each of the derivatized samples. These were then analyzed on an LKB-9000 gas chromatograph-mass spectrometer (28) fitted with a glass column (2 m × 6 mm) packed with 6% OV-101 on 100–120 mesh Chromosorb W HP and operated isothermally at 152°. During the chromatography of each derivatized extract, the intensities of ions *m/e* 189 and 193 were monitored. These ions represented, respectively, the $M^{+\cdot}$ − (HCOOTMS + CH_3) fragment of glyceric acid, and the $M^{+\cdot}$ − CH_3 fragment of *o*-toluic acid. The quantities of glyceric acid contained in the original urine and brain specimens before extraction were then estimated from the measured ratios of the intensities of *m/e* 189–193, and the relative increases produced in these ratios when known amounts of glyceric acid were added to samples before extraction.

RESULTS

GLYCINE CONTENT OF BRAIN

The content of glycine was markedly elevated (2- to 8-fold) in all examined regions of autopsied brain from the glycine encephalopathy patients, as compared to the same regions of brain from four control infants (Table 1). It is particularly noteworthy that glycine content was normal in the brain of control infant *4* who died with hyperglycinemia secondary to methylmalonic acidemia. The glycine content of brain rises steadily for 24–48 hr after death, largely because of the hydrolysis of glutathione. Thus the glycine values shown in Table 1 are probably somewhat higher than they were during life. However, the brain of glycine encephalopathy patient *3* was removed and frozen within 1 hr of death, and this patient's glycine elevation in brain must have been almost as striking during life. Except for glycine, the contents of all amino acids and related compounds, serine included, were normal in the brains of the glycine encephalopathy patients.

GLYCINE CLEAVAGE ENZYME ACTIVITY IN TISSUES

Table 2 shows the glycine cleavage enzyme activities found in frontal cortex, cerebellar cortex, and liver of the control infants and the glycine encephalopathy patients. No radioactive glycine was converted to $^{14}CO_2$ in the brains of these patients, although some glycine cleavage enzyme activity was clearly present in their livers at autopsy. Control infant *4*, who died from methylmalonic acidemia, had higher glycine cleavage activity in his brain than did either of the other control infants, or several control adults whose brains we have assayed.

SEARCH FOR POSSIBLE ENZYME INHIBITOR IN BRAIN

Homogenized brain from each of the glycine encephalopathy patients was mixed with homogenate of control infant brain in efforts to demonstrate a possible enzyme inhibitor in the patients' brains. Whether the homogenates were mixed immediately before enzyme assays, or were stirred together at 2° for 24–48 hr before assays, no convincing evidence could be found for inhibition of the control infant's glycine cleavage enzyme system. The amounts of $^{14}CO_2$ formed by such mixtures were usually similar to those formed by the same amounts of control brain homogenate assayed alone.

Table 3 shows the results of a typical dialysis experiment. When homogenate of brain from a control infant was assayed immediately after homogenization, and again after 24 and 46 hr of dialysis, glycine cleavage enzyme activity increased appreciably. That this heightened enzyme activity was not due to contamination by Mycoplasma or other microorganisms was shown by the failure of enzyme activity to increase in homogenates stirred in the same cold room for the same periods. We

Table 2. *Glycine cleavage enzyme activity in brain and liver*[1]

Subjects[2]	Frontal cortex	Cerebellar cortex	Liver
Control *1* (2 days, 2 hr)	110	1030	11760
Control *2* (8 mo, 5 hr)	145	227	3622
Control *3* (hyperglycinemia, ? type) (14 mo, 4 hr)	59	125	165
Control *4* (methylmalonic acidemia) (2 mo, 2 hr)	703	1157	1291
Glycine encephalopathy *1* (10 mo, 3 hr)	0	0	993
Glycine encephalopathy *2* (8 mo, 9 hr)	0	0	1087
Glycine encephalopathy *3* (13 days, 1 hr)	0	0	76
Glycine encephalopathy *4* (1 mo, 2 hr)	0[3]		313
Glycine encephalopathy *5* (18 days, 3 hr)	0[3]		267

[1] Activity expressed as nanomoles of $^{14}CO_2$ formed per hr per g of protein.

[2] Figures in parentheses indicate age at death, and interval from death until tissue was frozen at −80°.

[3] Precise cortical region not identified, probably frontal.

Table 1. *Glycine content of several regions of autopsied brain*[1]

Subjects[2]	Frontal cortex	Occipital cortex	Cerebellar cortex	Caudate nucleus	Putamen globus pallidus	Cervical cord
Control *1* (2 days, 2 hr)	1.65	1.82	1.79	1.69	2.11	1.88
Control *2* (8 mo, 5 hr)	2.09	2.01	2.61	2.44	2.36	2.22
Control *3* (hyperglycinemia, ? type) (14 mo, 4 hr)	2.04	1.97	2.02		2.18	
Control *4* (methylmalonic acidemia) (2 mo, 2 hr)	0.93	1.07	1.45		0.93	2.14
Glycine encephalopathy *1* (10 mo, 3 hr)	4.51	3.55	10.05		7.43	8.90
Glycine encephalopathy *2* (8 mo, 9 hr)	4.84	4.24	8.13		8.40	8.33
Glycine encephalopathy *3* (13 days, 1 hr)	11.29	14.24	17.66	13.07	12.30	14.66
Glycine encephalopathy *4* (1 mo, 2 hr)	4.84[3]					
Glycine encephalopathy *5* (18 days, 3 hr)	2.92[3]					

[1] Glycine content expressed in micromoles per g wet weight.

[2] Figures in parentheses indicate age at death, and interval from death until tissue was frozen at −80°.

[3] Precise cortical region not identified, probably frontal.

think that the increase in glycine cleavage activity in the dialyzed control brain may have been due to removal of some endogenous inhibitor(s) normally present in brain. However, when homogenate of brain from a glycine encephalopathy patient was similarly dialyzed, no enzyme activity whatsoever was detectable after 24 and 46 hr.

ORGANIC ACIDS IN URINE AND BRAIN

We found no unusual organic acids in the urines of glycine encephalopathy patients *1*, *2*, and *3*. Urine specimens were not available from patients *4* and *5*, but patient *4* was reported (21) not to have had propionic, methylmalonic, or isovaleric acid in her urine. Since an abnormally high glycine concentration has been reported in the CSF of a single patient with D-glyceric acidemia (9), the urines of patients *1* and *3*, and the brains of patients *3*, *4*, and *5* were also carefully examined for glyceric acid by combined gas chromatography-mass spectrometry. Table 4 shows the amounts of glyceric acid found in these urine and brain specimens. The concentrations of glyceric acid in the urines of patients *1* and *3* were about 3 orders of magnitude lower than Kølvraa *et al.* (9) found in the urine of their patient with D-glyceric acidemia. Very small amounts of glyceric acid were present in the brains of glycine encephalopathy patients *3*, *4*, and *5*, but a comparable amount was also present in the brain of control infant *4*, who had methylmalonic acidemia.

CONVERSION OF BICARBONATE TO GLYCINE BY BRAIN HOMOGENATES

The glycine cleavage enzyme system in human brain is probably similar to that which has been isolated from rat liver mitochondria by Motokawa and Kikuchi (13–15), and from *A. globiformis* by Kochi and Kikuchi (8). Both of these enzyme systems are composed of four protein components, which the Japanese investigators have named P-, H-, T-, and L-proteins, respectively. Figure 1 illustrates the four reversible reactions in which these enzyme proteins participate. The release of $^{14}CO_2$ from [1-^{14}C]glycine in the *in vitro* assay that we have employed actually measures only the sum of the first two reactions. Since these reactions are reversible, brain homogenates should form radioactive glycine from $NaH^{14}CO_3$, if the first or P-protein, and the second or H-protein, are functioning normally. When a homogenate of control infant brain was incubated with radioactive bicarbonate and unlabeled glycine, radioactive glycine was formed at a rate of 164 nmol/hr/g protein. On the other hand, no radioactive glycine at all was formed when brain homogenate from a glycine encephalopathy patient was similarly incubated. No radioactive serine was formed in either experiment. Failure to produce radioactive glycine from radioactive bicarbonate indicates that the enzyme deficiency in glycine encephalopathy must involve one or both of the first two proteins in the system.

BACTERIAL-BRAIN ENZYME RECOMBINATION

P-protein isolated from *A. globiformis* is capable of participating in glycine cleavage with the other three components isolated from rat liver mitochondria (8). With the procedure we used (8), bacterial P-protein is obtained combined with T- and L-protein, whereas bacterial H-protein is obtained as a separate fraction. Bacterial and mammalian H-protein is heat stable, whereas P-, T-, and L-proteins are rapidly inactivated by heating. Table 5 shows the cleavage of glycine to CO_2 which occurred when bacterial P,T,L and H fractions were combined with the solubilized brain enzyme system obtained from a control subject's brain, and from a glycine encephalopathy patient's brain. The bacterial P,T,L fraction and the bacterial H fraction (heated to destroy any contaminating P-protein) were inactive by themselves, but readily cleaved glycine to CO_2 when combined. Bacterial P,T,L fraction together with heated control brain cleaved glycine to CO_2, thus demonstrating the presence of H-protein activity in control brain. However, no CO_2 was

Table 3. *Glycine cleavage enzyme activity in dialyzed brain homogenates*[1]

	Control infant *2*		Glycine encephalopathy patient *3*	
Time	Stirred	Dialyzed	Stirred	Dialyzed
0 hr	207	207	0	0
24 hr	206	357	0	0
46 hr	174	391	0	0

[1] Homogenates were stirred or dialyzed at 2°. Enzyme activity is expressed in nanomoles of $^{14}CO_2$ formed per hr per g protein.

Table 4. *Glyceric acid content of urine and brain*[1]

Subjects	Urine	Brain
Control *4* (methylmalonic acidemia)	82	64
Glycine encephalopathy *1*	7	
Glycine encephalopathy *3*	21	36
Glycine encephalopathy *4*		113
Glycine encephalopathy *5*		61

[1] Glyceric acid content in urine expressed as micrograms per mg creatinine, and in brain as micrograms per g wet weight. All values are corrected for losses in recovery.

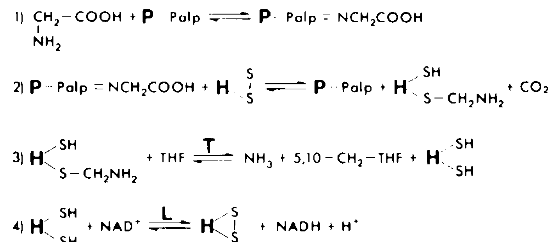

Fig. 1. Diagram of the four reversible reactions in the mammalian glycine cleavage enzyme system, with the four enzyme proteins designated as **P**, **H**, **T**, and **L**. Palp: pyridoxal phosphate; THF: tetrahydrofolate. Modified from Motokawa and Kikuchi (15).

Table 5. *Recombination of solubilized glycine cleavage complex of control and glycine encephalopathy brain with bacterial P,T,L-, or H-protein fractions*[1]

Protein components	$^{14}CO_2$ formed
P,T,L	0
H[2]	0
P,T,L and H[2]	396
Control brain[2]	0
Control brain[2] and P,T,L	93
Control brain[3] and H[2]	0
Glycine encephalopathy brain	0
Glycine encephalopathy brain[2] and P,T,L	0
Glycine encephalopathy brain[3] and H[2]	0

[1] Results are the means of two assays, corrected for any radioactivity observed in the enzyme blanks, and are expressed as counts per min formed for 30-min incubation at 33.5°.

[2] Heated in a boiling water bath for 3 min to destroy all enzyme proteins except H-protein.

[3] Solubilization of the brain enzyme complex results in rapid loss of P-protein activity.

formed from glycine when bacterial P,T,L fraction was combined with glycine encephalopathy brain. This suggested that H-protein was absent or inactive in the solubilized enzyme system from the glycine encephalopathy patient's brain.

The failure to form $^{14}CO_2$ when glycine encephalopathy brain and bacterial H-protein were combined does not necessarily mean that P-protein was abnormal in this brain. $^{14}CO_2$ was also not formed when the solubilized enzyme system from control brain and bacterial H-protein were combined. Unlike the isolated bacterial P-protein, which is relatively stable, partially purified mammalian P-protein has been found to be very unstable. It loses 50% of its initial activity even when stored at −20° for as little as 24 hr (14). We did not attempt to fractionate components of the enzyme system from brain. However, even the solubilization of the enzyme system which was required in order to carry out recombination experiments with the bacterial fractions resulted in rapid loss of P-protein activity. Thus, we were unable to prove whether or not P-protein activity was normal in intact brain of glycine encephalopathy patients.

DISCUSSION

Glycine encephalopathy differs from the ketotic hyperglycinemias, and from other forms of so-called nonketotic hyperglycinemia, in a marked elevation of the glycine content of the brain, which is in turn reflected in an abnormally high concentration of glycine in the CSF. This is illustrated by the high brain glycine levels in our five patients, as well as by the elevated brain glycine values found in a single patient by Bachmann *et al.* (3). On the other hand, brain glycine values at autopsy were normal in a single patient with hyperglycinemia secondary to propionic acidemia (2), and in our hyperglycinemic infants with methylmalonic acidemia and with an undetermined type of hyperglycinemia (Table 1).

It is possible that the high levels of glycine in brain in glycine encephalopathy are directly responsible for the severe neurologic symptoms characteristic of this disorder. Glycine probably functions as an inhibitory neurotransmitter in the spinal cord, and possibly at certain synapses in the brain as well (10), and a great excess of such a transmitter might well disrupt brain activity. A deficiency of N^5,N^{10}-methylenetetrahydrofolate (Fig. 1, reaction 3) seems much less likely to be the neurotoxic factor in glycine encephalopathy, since other pathways lead to the formation of this folate intermediate.

Comparison of *in vitro* glycine cleavage enzyme activity in brain and liver homogenates of glycine encephalopathy patients shows a complete absence of activity in brain, whereas enzyme activity in liver is reduced, but still readily measured (Table 2). This difference between brain and liver is unexplained. It could be accounted for if a necessary activator of the glycine cleavage system failed to penetrate into brain, but was present in reduced amounts in liver, or if a relatively lipid-soluble inhibitor of the enzyme system accumulated in greater amounts in brain than in liver.

Failure of the carboxyl group of glycine to exchange with $NaH^{14}CO_3$ in homogenates of glycine encephalopathy brain *in vitro* demonstrates that an enzyme deficiency must exist within the first two steps of the glycine cleavage system (Fig. 1), although it tells us nothing about the integrity of the third and fourth steps. The recombination experiments using active P-protein derived from *A. globiformis* (in the P,T,L fraction), and H-protein derived from human brain suggest that H-protein is inactive in glycine encephalopathy. H-protein derived from rat liver mitochondria, and presumably the H-protein in human brain, is a small protein with a molecular weight of about 17,000, which is heat stable and contains a functional disulfide group in its molecule (13-15). Its function is to accept the $—CH_2NH_2$ fragment derived from glycine, and to transfer its one carbon unit to tetrahydrofolate (Fig. 1).

Elevated glycine levels in brain appear highly toxic, although elevated glycine concentrations in plasma seem to be harmless. This is borne out by our earlier finding (20) that at least one child with marked hyperglycinemia enjoyed reasonable health and was entirely free from neurologic symptoms, whereas an infant who eventually died from glycine encephalopathy repeatedly had normal glycine concentrations in her fasting plasma. A striking feature of glycine encephalopathy is its onset shortly after birth, often on the second or third day of life (16). Why does an infant with this genetically determined disorder do well during intrauterine life, only to become severely ill in the neonatal period? Infants who subsequently develop glycine encephalopathy apparently exhibit normal intrauterine movements, and do not have microcephaly, decreased birth weight, or inactivity in the first few hours after birth.

This suggests that the absence of glycine cleavage enzyme activity in the brain and the reduced enzyme activity in the liver, apparently caused by failure of the H-protein, are not due to a structural abnormality of the apoenzyme. If this were the case, glycine in plasma might cross the placenta into the maternal circulation during fetal life, but glycine should accumulate in the fetal brain and cause brain damage long before birth. It does not seem reasonable to postulate that glycine could be transported freely out of fetal brain up to the time of birth, and then suddenly fail to cross from brain into the circulation after birth. We believe that the best explanation for the sudden onset of glycine encephalopathy early in infancy is either lack of an enzyme activator which might be supplied by the mother during fetal life, or presence of an enzyme inhibitor which is disposed of across the placenta during fetal life. Another possibility is that production of the hypothetical inhibitor commences only at birth in all infants, but the patients with glycine encephalopathy fail to metabolize it further.

The mixing and dialysis experiments that we carried out on brain homogenates have provided no evidence for the presence in the brain of glycine encephalopathy patients of a loosely bound inhibitor of the glycine cleavage enzyme system. However, they have not excluded the possible presence of an inhibitor which is so firmly bound to protein that activity cannot be restored in homogenates of the patients' brains. Addition of brain homogenate containing such a firmly bound inhibitor to a homogenate of normal brain might also not result in inhibition of the active enzyme system in the latter. Daly *et al.* (5) have recently presented evidence for the existence of one or more nondialyzable endogenous inhibitors of the glycine cleavage enzyme system in rat brain, so that it is reasonable to envisage the occurrence of such compounds in human brain. It is clear, however, that in our patients glyceric acid was not the putative inhibitor.

Future studies of glycine encephalopathy should perhaps focus on a search for such an endogenous enzyme inhibitor. This hypothetical inhibitor might readily be degraded in normal individuals, but not in affected infants. The latter might be protected by the placental circulation during fetal life, only to have the inhibitor accumulate in liver and especially in brain after birth.

CONCLUSION

Glycine content was measured in autopsied brain from five infants dying with glycine encephalopathy and four control infants, including two with other types of hyperglycinemia. Activity of the glycine cleavage enzyme system was determined in autopsied brain and liver from these nine infants. Glycine content was elevated 2- to 8-fold in the brains of the glycine encephalopathy patients. Glycine cleavage enzyme activity was undetectable in their brains, but present at reduced levels in their livers. Dialysis and mixing experiments failed either to demonstrate or to disprove the presence of an endogenous inhibitor of the glycine cleavage enzyme system in the brains of the glycine encephalopathy patients. However, D-glyceric acid was excluded as a possible inhibitor. Failure of brain homogenates from glycine encephalopathy patients to convert radioac-

tive bicarbonate into radioactive glycine *in vitro*, as well as the results of recombination experiments with solubilized human brain enzymes and purified protein components of a bacterial glycine cleavage system, showed that the enzyme defect in glycine encephalopathy must involve at least the H-protein of the 4-protein glycine cleavage enzyme system.

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