A Patient with Nonketotic Hyperglycinemia: Biochemical Findings and Therapeutic Approaches

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Extract

High voltage paper electrophoresis of urine and quantitative amino acid analysis of serum revealed a greatly increased excretion of glycine as well as hyperglycinemia in a patient on the 7th day of life. The cerebrospinal fluid of the infant showed a marked increase in isoleucine concentration as well as elevation of the glycine level. After establishment of the enzymic defect in a liver biopsy with the aid of (1-¹⁴C)glycine and (2-¹⁴C)glycine, several therapeutic approaches have been performed which were based, among other things, on replenishment of the C₁ units pool. Administration of compounds such as leucovorin (*N*⁵-formyl tetrahydrofolate (THF)), methionine, choline, and formate did not result in a normalization of the serum glycine level. During treatment with methionine (350 mg/kg/24 hr) a strong hypermethioninemia (1,240 μmol/liter) and sarcosinemia (5,180 μmol/liter) developed. A supply of pyridoxine, a precursor of pyridoxal phosphate, the latter being a cofactor in the glycine cleavage system, did not influence the serum glycine level. It was concluded that our patient did not show a deficiency of *N*⁵,*N*¹⁰-methylene-THF as a result of a defective glycine cleavage system.

Speculation

In spite of comprehensive investigations which concerns patients who suffer from nonketotic hyperglycinemia, additional studies are required to enhance our knowledge of the biochemical disturbance in these patients. This will provide new therapeutic approaches which are urgently desired, as no efficacious measures are available now.

Introduction

Nonketotic hyperglycinemia is a rare inborn error of metabolism, biochemically characterized by increased concentrations of glycine in blood, urine, and cerebrospinal fluid. Nonketotic hyperglycinemia, which was first described by Gerritsen *et al.* [14], is considered to be a primary defect in the metabolism of glycine. Moreover, elevated concentrations of glycine in plasma have been found in methylmalonic acidemia [16], propionic

acidemia [4], isovaleric acidemia [1], and carbamyl-phosphate synthetase deficiency [12].

In four patients suffering from nonketotic hyperglycinemia, the defect in the glycine cleavage system was demonstrated in liver biopsies [10, 21] and in three patients the disturbed conversion of glycine to serine was established *in vivo* [3, 6].

The purpose of this report is to present a patient with nonketotic hyperglycinemia especially concerning

amino acid analyses, biochemical investigations, and the results of several therapeutic approaches.

Case Report

The patient, a girl, the third child in a family, was born December 22th, 1969. The parents and the sibs, a boy born in 1964 and a girl, born in 1967, are healthy. No miscarriages occurred. Pregnancy was uneventful and a normal delivery occurred in the 38th week of gestation. Birthweight was 2,570 g. Until the end of the 2nd day of life no abnormality was noted. At that time she sucked poorly and began to have signs and symptoms of irritability and somnolence. On the following days the patient became quieter and was admitted to a hospital on the 5th day of life. At that time she reacted poorly to stimuli and was hypotonic. She exhibited frequent myoclonic seizures; however, breathing was still adequate. Oral humanized milk feeding was stopped and glucose and salt solutions were administered intravenously. She continued to exhibit muscular jerks. Anticonvulsive therapy was instituted. From the 7th day of life human milk was given. Hyperglycinemia and hyperglycinuria were found at our laboratory. The patient was admitted to our hospital at January 9th, 1970. On physical examination she was essentially normal except that neurologically she was lethargic with no grasp, Moro, and deep tendon reflexes. There were no signs of increased intracranial pressure. She showed myoclonic seizures frequently. The electrocardiogram (EEG) showed alternately short paroxysm and periods of low voltage activity. At that time, treatment with sodium benzoate was started, with a low protein diet (1 g protein/kg/24 hr). At the age of 5 weeks a synthetic diet free in glycine and serine was given supplemented with 25 ml humanized milk (7 mg glycine). After a short period, in which muscular tonicity varied greatly, she became more hypertonic with exaggerated deep tendon reflexes.

Findings at Age of 1 Year

The patient's weight was 7,500 g, height 66 cm, and head circumference 42 cm. She did not follow with her eyes. She was severely retarded and had no social responses. The muscle tone was still increased. Tendon reflexes were exaggerated. She still exhibited myoclonic seizures. The EEG showed the typical pattern of hypsarrhythmia.

Normal laboratory data were found for blood pH,

pCO₂, blood base excess, serum bicarbonate, sodium, potassium, chloride, urea, hemoglobin, hematocrit, erythrocytes, leucocytes, and platelet counts. Ketoacidosis and ketonuria were never observed. The results of amino acid analyses at different ages are shown in Table I. Ammonia in blood was determined once at the age of about 3 years; a normal value was found (1.3 µg/ml). Oxalic acid excretion at the age of 9 months was less than 10 mg/24 hr.

Methods

Qualitative amino acid analysis of the patient's urine was performed by high voltage paper electrophoresis [22] on Whatman 3MM filter paper, 40 × 20 cm, for 20 min at 100 V/cm in formic acid-acetic acid-water (50/150/850, v/v), pH 2.0. Quantitative amino acid analyses of serum, cerebrospinal fluid, and urine of the patient were performed by elution chromatography with a Beckman/Spinco Unichrom amino acid analyzer [23]. Deproteinization of the samples was carried out with sulfosalicylic acid (5% w/v).

Liver biopsy was taken with a Menghini needle under local anesthesia. A control liver biopsy was obtained surgically. The glycine cleavage system in liver was investigated according to a minor modification of the method described by De Groot *et al.* [10]. The liver biopsy from the patient was preserved at -80° and that from the control subject in liquid nitrogen before use. The biopsy specimens were homogenized in a Potter-Elvehjem homogenizer in 0.05 M potassium phosphate buffer, pH 7.5. A rat liver homogenate was prepared in the same manner. The incubation mixtures contained, in a final volume of 0.6 ml, (1-¹⁴C)glycine (2.1 µmol) and liver homogenate containing 25 mg wet weight for the control liver and rat liver and an unknown amount of the patient's liver. Incubation was performed in 30-ml glass scintillation vials, sealed by rubber stoppers, containing two small glass tubes, one fitting inside the other [13]. The inner tube contained 0.5 ml Hyamine. The scintillation vials contained the components of the reaction mixture. After incubation for 3 hr at 37°, 0.2 ml 2 N HCl was added by injection through the rubber stopper and the vials were kept at 37° for another hour. Thereafter, the stoppers were removed and the inner glass tubes containing Hyamine were removed from the vials and transferred to scintillation vials containing 15 ml scintillation fluid (4 g 2,5-diphenyloxazole (PPO) and 0.1 g dimethyl 1,4-bis[(diphenyloxazolyl)]benzene

(POPOP) in a mixture of 500 ml toluol and 500 ml ethyl-Cellosolve). Counting was performed in a scintillation counter. In this manner the radioactivity of $^{14}\text{CO}_2$ released from (1- ^{14}C)glycine was determined.

The rates of incorporation of ^{14}C from (1- ^{14}C)glycine and (2- ^{14}C)glycine [24] into serine by liver homogenates have been measured in centrifuge tubes containing the same incubation mixture as was used for determination of $^{14}\text{CO}_2$. Two separate experiments have been performed either with (1- ^{14}C)glycine or (2- ^{14}C)glycine. After incubation for 3 hr at 37° the reaction was terminated by addition of 0.6 ml of 0.1 N acetic acid to the incubation mixtures. After placing the tubes for 2 min in a boiling water bath the precipitated proteins were removed by centrifugation for 10 min at $2,000 \times g$. For examination of control and rat liver, 0.2 ml supernatant was subjected to high voltage paper electrophoresis on Whatman no. 3MM filter paper under the same conditions as described before. Thereafter, the strips were dried at 95° and scanned for radioactivity with the aid of a strip counter. The strips were cut into pieces which contained either labeled glycine or serine. The radioactivity of both amino acids was determined with a scintillation counter, using 15 ml liquid scintillation solvent for each vial. For examination of the patient's liver, labeled glycine and serine were separated with the aid of an automatic amino acid analyzer. The supernatant of

the deproteinized incubation mixture was acidified to pH 2 and a 1-ml aliquot of this mixture was applied to the amino acid analyzer. The fractions containing either serine or glycine were collected separately and evacuated to dryness and the residues were dissolved in 1 ml distilled water. These solutions were added to 20 ml liquid scintillation solvent and counted for radioactivity.

Results

Amino Acid Analyses

More than 250 quantitative analyses of amino acids in the serum of this patient have been performed. Significant data are shown in Table I. The most marked abnormality was the strong hyperglycinemia (normal values to 450 $\mu\text{mol/liter}$). The other acidic and neutral amino acids were present in almost normal concentrations. The basic amino acids have been determined once and were found to be normal. Table I also shows the amino acid concentration in the cerebrospinal fluid of this patient. A remarkable increase in the glycine level was found (about sevenfold). In the other amino acids the concentration of isoleucine was markedly raised (about fivefold).

At the age of 11 months the tubular reabsorption of acidic and neutral amino acids was determined in the patient, who was treated at that time with sodium

Table I. Concentrations of amino acids in serum at various ages and in cerebrospinal fluid (CSF) at age of 14 months

	Serum, $\mu\text{mol/liter}$					CSF ¹
	28 Days ²	83 Days ³	13 Months ⁴	25 Months ⁵	Controls, mean $\pm$ SD	
Hydroxyproline	Trace	Trace	Trace	Trace		0
Aspartic acid	Trace	Trace	Trace	21	45 $\pm$ 30	0
Threonine	144	279	264	164	161 $\pm$ 68	50
Serine	168	206	171	154	182 $\pm$ 56	50
Proline	140	226		171	273 $\pm$ 119	0
Glutamic acid			55	66	172 $\pm$ 106	Trace
Citrulline			52	36	31 $\pm$ 12	Trace
Glycine	770	1,540	1,070	835	260 $\pm$ 90	101
Alanine	262	595	940	437	422 $\pm$ 136	51
Valine	131	163	145	142	257 $\pm$ 99	28
Cystine						0
Methionine		27	32	28	38 $\pm$ 18	Trace
Isoleucine	33	152	79	54	86 $\pm$ 36	24
Leucine	82	172	98	77	149 $\pm$ 64	25
Tyrosine	39	89	45	63	89 $\pm$ 40	19
Phenylalanine	57	68	29	46	79 $\pm$ 31	14

¹ Patient received 172 mg sodium benzoate and 36 mg glycine/kg/24 hr.

² Patient received 185 mg sodium benzoate and 14 mg glycine/kg/24 hr.

³ Patient received 280 mg sodium benzoate, 0.95 mg leucovorin, and 2.2 mg glycine/kg/24 hr.

⁴ Patient received 120 mg sodium benzoate and 0.93 mg glycine/kg/24 hr.

⁵ Patient received 130 mg sodium benzoate and 3.9 mg glycine/kg/24 hr.

Liver source	¹⁴ CO ₂ , cpm/mg wet wt/hr	¹⁴ C Serine source, cpm/mg wet wt/hr		Incorporation rates for (2- ¹⁴ C)glycine/(1- ¹⁴ C) glycine
		(1- ¹⁴ C)Glycine	(2- ¹⁴ C)Glycine	
Patient	Not detectable	1,036 ¹	1,164 ¹	1.1
Human control subject	61	137	198	1.5
Rat	170	342	515	1.5

ration of ^{14}C from $(1\text{-}^{14}\text{C})$ glycine and $(2\text{-}^{14}\text{C})$ glycine into serine by liver homogenate. From Table II it appears that no detectable amount of $^{14}\text{CO}_2$ was formed from $(1\text{-}^{14}\text{C})$ glycine, by this patient's liver, whereas a significant $^{14}\text{CO}_2$ production was observed by incubation of human or rat liver with $(1\text{-}^{14}\text{C})$ glycine. The ratio of the rates of incorporation of ^{14}C from $(2\text{-}^{14}\text{C})$ glycine and $(1\text{-}^{14}\text{C})$ glycine into serine was found to be about 1 for the liver of the patient, whereas a value of about 1.5 was found for this ratio for human control and rat liver. These findings are consistent with a defect in the formation of N^5, N^{10} -methylene-THF from glycine (Fig. 1).

Therapeutic Approaches

Several therapeutic approaches were based upon conjugation of glycine and excretion of the conjugates, or restriction of glycine and serine in the diet, or correction of the C₁ units pool which might be diminished as a result of defective cleavage of glycine.

Conjugation of Glycine

This kind of treatment is based upon conjugation of glycine with either benzoate or salicylate and subsequent excretion of the conjugates in urine. Treatment with sodium benzoate was started during the 1st month of life and was continued in a dose varying from 100 to 300 mg/kg/24 hr for about 3 years except for a few days when salicylate was given. We are not able to describe adequately the influence of this treat-

ment on the glycine level in blood, inasmuch as the number of glycine values obtained without any treatment is too small to permit conclusions.

Restriction of Glycine Intake

The patient was fed a diet with a glycine intake which varied from 0.7 to 35.6 mg/kg/24 hr. No significant decrease of the level of glycine in serum was observed with decreasing intake of glycine daily during a period in which the patient received daily 1,500 mg sodium benzoate (Table III).

Supply of C₁ Units

A different kind of treatment was based upon supplying C₁ units [10]. Leucovorin (*N*⁵-formyl-THF), which can be converted into *N*⁵,*N*¹⁰-methylene-THF

Table III. Influence of therapeutic trials on glycine level in serum

Treatment, mg/kg/24 hr		Serum glycine, μmol/liter (mean ± SD)	n	P	Dietary or therapeutic conditions
Glycine (diet)	0.7	687 ± 137	23	>0.05	Sodium benzoate, 1,500 mg/24 hr
	1.3	785 ± 42	6		
	3.9	963 ± 190	7		
	4.9	803 ± 228	7		
	5.8	786 ± 118	12		
	.7	800 ± 14	2		
	395.6	723 ± 135	3		
Leucovorin (i.m.)	0	687 ± 137	23	<0.01	Sodium benzoate, 1,500 mg/24 hr; glycine, 7 mg/24 hr
	0.32	743 ± 188	3		
	0.96	840 ± 149	8		
Methionine (orally)	0	937 ± 168	15	>0.05	Sodium benzoate, 900 mg/24 hr; glycine, 7 mg/24 hr
	45	883 ± 217	7		
	80	827 ± 226	20		
Choline (orally)	0	787 ± 113	13	<0.01	Sodium benzoate, 1,500 mg/24 hr; glycine, 75 mg/24 hr
	71	868 ± 84	5		
	86	750	1		
	107	946 ± 76	5		
Sodium formate (orally)	0	937 ± 168	15	0.025 << 0.05	Sodium benzoate, 875 mg/24 hr; glycine, 7 mg/24 hr
	26	853 ± 117	8		
	42	804 ± 123	5		
Pyridoxine (orally)	0	937 ± 168	15	>0.1	Sodium benzoate, 900 mg/24 hr; glycine, 7 mg/24 hr
	27	822 ± 30	4		
Pyridoxine (orally)	0	827 ± 226	20	>0.05	Sodium benzoate, 900 mg/24 hr; glycine, 7 mg/24 hr; methionine, 480 mg/24 hr
	15	1,051 ± 76	7		
	28	810 ± 144	6		
Folic acid (orally)	0	804 ± 123	5	0.01 << 0.02	Sodium benzoate, 900 mg/24 hr; sodium formate, 180 mg/24 hr; glycine, 7 mg/24 hr
	1.1	1,053 ± 180	9		

¹ Based on regression analysis or Student *t* test.

(Fig. 1) was given intramuscularly in a dosage of 0.32 and 0.96 mg/kg/24 hr (Table III). A small but significant increase of the level of glycine in serum was observed.

Treatment of nonketotic hyperglycinemia patients with methionine as performed by De Groot *et al.* [10] is also based on providing the organism with a sufficient amount of C₁ units. Our patient received methionine combined with sodium benzoate (900 mg/24 hr) and a glycine intake of 7 mg/24 hr. From the results presented in Table III, it can be concluded that treatment with methionine did not result in a significant decrease of the serum glycine level. The dosage of methionine was increased to 350 mg/kg/24 hr but hypermethioninemia (1,240 μ mol/liter) and sarcosinemia (5,180 μ mol/liter) were seen in that period.

Treatment of the patient with choline represents a third method for donation of C₁ units. Choline was administered to the patient during a constant daily sodium benzoate (1,500 mg) and glycine (75 mg) intake. We established a significant increase of the glycine level with increasing choline dosages.

Only one kind of treatment resulted in a small but significant decrease of the serum glycine level, namely, administration of sodium formate (Table III) with a daily intake of sodium benzoate (875 mg) and glycine (7 mg); however, no normalization of the glycine level occurred.

Supply of Cofactors

Because pyridoxal phosphate [19] participates in the glycine cleavage system, pyridoxine was given along with a constant daily sodium benzoate (900 mg) and glycine (7 mg) intake either with or without administration of 480 mg methionine/24 hr (Table III). No dependence on pyridoxine was observed.

Supply of Folic Acid

Treatment of the patient with folic acid, a precursor of N⁵,N¹⁰-methylene-THF, was performed only in conjunction with a constant daily intake of sodium benzoate (900 mg), glycine (7 mg), and sodium formate (180 mg). A significant increase of the serum glycine level was the result.

Discussion

Gerritsen *et al.* [14] supposed originally that nonketotic hyperglycinemia was associated with a defect in the glycine oxidase system; however, later such a defect was ruled out [15]. Thereafter, Ando *et al.* [3] demon-

strated a defect *in vivo* in the formation of ¹⁴CO₂ from (1-¹⁴C)glycine and a complete deficiency in the conversion of (2-¹⁴C)glycine to (3-¹⁴C)serine. Yoshida *et al.* [21] demonstrated a deficiency of the glycine cleavage system in the liver of a patient who suffered from nonketotic hyperglycinemia. The amount of ¹⁴CO₂ formed from (1-¹⁴C)glycine was only 8% of the mean value obtained for control subjects. The amount of (¹⁴C)serine formed after incubation either with (1-¹⁴C)glycine or (2-¹⁴C)glycine was also very small. In their patient a ratio of 1.2 was found between the rates of incorporation of (2-¹⁴C)glycine and (1-¹⁴C)glycine into (¹⁴C)serine, whereas a mean ratio of 1.8 was found for control subjects. Moreover, a nearly equal amount of ¹⁴CO₂ was formed either from (1-¹⁴C)glycine or (2-¹⁴C)glycine. These experiments confirm the *in vivo* findings of Ando *et al.* [3]. From the diminished ratio of the rates of incorporation of (1-¹⁴C)glycine into serine it might be concluded that the formation of N⁵,N¹⁰-methylene-THF from glycine is very important for synthesis of serine from glycine.

Baumgartner *et al.* [6] also investigated a patient with nonketotic hyperglycinemia. After intravenous injection of (2-¹⁴C)glycine and isolation of serine from plasma, they found, as compared with control subjects, a strongly diminished, but detectable conversion to (3-¹⁴C)serine, data in contrast to that of Ando *et al.* [3], who found virtually no conversion. Baumgartner *et al.* [7] postulated the hypothesis of a number of different forms of nonketotic hyperglycinemia. Baumgartner *et al.* [7] investigated the conversion of propionyl-CoA to methylmalonyl-CoA and of methylmalonyl-CoA to succinyl-CoA in fibroblasts and liver homogenate of a patient with nonketotic hyperglycinemia. They found normal conversions and concluded that "nonketotic hyperglycinemia" is a disease distinct from the "ketotic hyperglycinemia" in which one of the previously mentioned conversions does not function. Previously [17] however, a major defect in the conversion of glycine to serine was established after intravenous injection of (2-³H)glycine in a patient who suffered from hyperglycinemia coupled with ketosis and metabolic acidosis.

Recently, Ando *et al.* [2] demonstrated *in vivo* that the conversion of (1-¹⁴C)glycine to CO₂ was defective in three patients with ketotic hyperglycinemia just as in the nonketotic hyperglycinemia patients [3], but, in contrast to the latter, a normal conversion of (2-¹⁴C)glycine to (3-¹⁴C)serine was found after one of the patients received injections of (2-¹⁴C)glycine. From these findings, it might be concluded that a defect in the

conversion of glycine to N^5, N^{10} -methylene-THF results in a defective conversion of glycine to serine in the nonketotic but not in the ketotic hyperglycinemia. A possible explanation for this discrepancy might be the existence of one or more alternate pathways leading to N^5, N^{10} -methylene-THF which could be stimulated in ketotic hyperglycinemia.

De Groot *et al.* [10] studied three patients with nonketotic hyperglycinemia *in vitro*. In liver biopsies they demonstrated a defect in the glycine cleavage system. The authors mentioned that the defective enzyme has a control position in the supply of C_1 units which are necessary for synthetic reactions.

Up to now, several therapeutic measures have been applied to reduce the glycine levels in blood. Rampini *et al.* [18] treated one patient from the age of 6 months with a diet containing only 0.5 g of protein/kg/24 hr. After a period of 3 weeks with unchanged glycine levels, a distinct reduction of the glycine levels was observed with a complete normalization after 2 months. Tada *et al.* [20] treated their patient at the age of 16 months with 3 mg leucovorin every day for a week by intramuscular injections, but they found no change in serum glycine levels. However, significant but temporary decrease in serum glycine concentration was obtained after treatment with 30 mg leucovorin given intravenously by drip infusion. Baumgartner *et al.* [6] could reduce glycine concentration in plasma from 1,600 $\mu\text{mol/liter}$ to 530 $\mu\text{mol/liter}$ by exchange transfusion. Benzoate treatment resulted in a significant but small reduction of the glycine level on a low protein diet (0.5–0.8 g/kg/24 hr). Reduction of the dietary protein content did not result in a significant change of the glycine level in plasma. Bachmann *et al.* [5] also obtained a significant but temporary decrease of the level of glycine in plasma by exchange transfusion. The treatment of their patient with methionine (300 mg/24 hr) or benzoic acid (0.5 g/24 hr) did not prevent early death. Ferdinand *et al.* [11] described a patient with nonketotic hyperglycinemia who survived for 3 years on a protein-deficient diet; however, mental retardation could not be prevented.

One of the most extensive studies concerning treatment of nonketotic hyperglycinemia patients was performed by De Groot *et al.* [10] and by De Groot [9]. They treated patients with a synthetic diet which did not contain glycine and serine. In some, but not all cases, a decrease in glycine level in blood was obtained but mental retardation still developed. Based on the assumption that a shortage of C_1 units existed in the

patients, De Groot [9] treated a patient with methionine and observed a marked decrease of the glycine level in the blood. This decline in glycine concentration could also be explained in terms of a decrease in supply of serine [8].

Treatment of another patient with choline did not influence glycine levels but a reduction to low-normal levels of serine was seen.

As described before, our patient was treated either with compounds which could provide C_1 units, such as leucovorin, methionine, choline, and formate or compounds which could be considered to be precursors of N^5, N^{10} -methylene-THF, *e.g.*, folate (Fig. 1). A slight but significant decrease of the glycine level in serum was obtained only during treatment with sodium formate (Table III). Therefore, it appeared likely that our patient did not show a deficiency of N^5, N^{10} -methylene-THF as a result of a defective glycine cleavage system, inasmuch as no real improvement of the biochemical and clinical conditions was observed during different kinds of treatment.

In contrast to the data of De Groot *et al.* [10], a normal tubular reabsorption of glycine was found during treatment with methionine.

From this study it appears that further investigations will be necessary in order to find a treatment which will restore the biochemical disturbances and prevent physical and mental retardation. Moreover, fundamental biochemical research will help us to clarify the previously mentioned problems of metabolism of glycine in nonketotic and ketotic hyperglycinemia patients.

Summary

A patient suffering from nonketotic hyperglycinemia has been investigated for 3 years. The enzyme defect has been demonstrated by incubation of a homogenate of a liver biopsy with (1- ^{14}C)glycine and (2- ^{14}C)glycine and measurement of the radioactivity of $^{14}\text{CO}_2$ released from (1- ^{14}C)glycine and of the ratio of the rates of incorporation of (2- ^{14}C)glycine and (1- ^{14}C)glycine into serine. The serum of the patient showed a marked increase in glycine level only, whereas a great elevation of both glycine and isoleucine levels was found in the cerebrospinal fluid. Several therapeutic approaches have been attempted which were based upon either conjugation of glycine and excretion of the conjugates (benzoate and salicylate), or restriction of glycine intake, supply of C_1 units (leucovorin, methionine, cho-

line, and formate), supply of a cofactor (pyridoxine), or supply of folate. None of the measures mentioned previously resulted in a real improvement of the clinical or biochemical disturbances.

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