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—Original Article—

IMPAIRED METABOLISM OF METHIONINE IN SEVERE LIVER DISEASES II. CLINICAL AND EXPERIMENTAL STUDIES ON ROLE OF IMPAIRED METHIONINE METABOLISM IN PATHOGENESIS OF HEPATIC ENCEPHALOPATHY

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Summary

Hepatic encephalopathy in patients with severe liver disease was associated with marked elevation of either serum methionine or blood ammonia levels or with simultaneous moderate increases in both parameters. CSF methionine levels also increased in encephalopathic patients with fulminant hepatitis and liver cirrhosis. Increased influx of methionine into the brain over the theoretical values predicted from Pardridge's equation suggested that accelerated transport of serum methionine across the blood-brain barrier was observed in these cases with hepatic encephalopathy.

Hepatic encephalopathy in acute carbon tetrachloride liver injury could be obtained experimentally following intraperitoneal injection of ammonium acetate in rats, which already received intragastric administration of methionine. However, similar encephalopathy could not be observed by the administration of glycine or leucine in place of methionine. These results suggest at least that methionine and ammonia act synergistically on inducing hepatic encephalopathy.

Key Words: *serum and CSF methionine, blood ammonia, hepatic encephalopathy, hepatic failure, severe liver disease.*

Introduction

The previous study[1] on serum methionine concentrations in patients with severe liver diseases indicated that the levels were markedly elevated in fulminant hepatitis and decompensated liver cirrhosis associated with hepatic encephalopathy, ascites or jaundice. The toxic effects of methionine on the central nervous system in severe liver disease have been described[2] and fetor hepaticus by methionine metabolites such as methyl marcaptans and dimethyl disulphide in the breath have been well documented[3]. However, there have been only a few papers[4,5] describing the role of abnormal methionine metabolism in pathogenesis of hepatic encephalopathy.

In this communication, simultaneous analyses of methionine levels in both cerebrospinal

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fluid (CSF) and serum were carried out in patients with severe liver diseases, since the levels in CSF may reflect directly methionine metabolism in the brain. Furthermore, to investigate direct effects of impaired methionine and ammonia metabolism on the pathogenesis of hepatic encephalopathy, an experimental study was also conducted by using an animal model with acute hepatic failure.

Materials and Methods

Simultaneous determinations of methionine levels in serum and CSF were performed on 8 controls, 5 patients with fulminant hepatitis, 8 cirrhotic patients without hepatic encephalopathy and 7 encephalopathic cirrhotics. Influx of neutral amino acids including methionine into the brain has been suggested to be regulated by reciprocal competition across the blood-brain barrier (BBB)[6]. Predicted influx velocity (Vpre) for investigating altered transport of methionine through the BBB was calculated according to the equation of Pardridge[7]. Clinical observations of patients and methods used in this study have been reported in detail[1].

Male Sprague-Dawley rats weighing 250 to 350 g were used throughout the study. Carbon tetrachloride (CCl_4) was given intragastrically to overnight-fasted rats at a volume of 10 ml of 20% solution of CCl_4 in paraffin per kg body weight under light ether anesthesia. The blood for amino acid analysis and other laboratory tests for liver function was obtained through the abdominal aorta. Amino acid analyses in serum and the brain were carried out according to the method already reported[8]. An amino acid dissolved by physiological saline (1.7 M methionine: 23 mmoles/kg body weight, 1.9 M glycine or 3.8 M leucine, each 50 mmoles/kg) was administered intragastrically at a volume of 13.2 ml per kg body weight 24 hours following CCl_4 intoxication, when the maximum extent of acute hepatic injury can be expected. Ammonium acetate (1.0 M, 5 mmoles/kg body weight) was administered intraperitoneally at a volume of 5 ml per kg body weight an hour after intragastric feeding of each amino acid. Synergistic effects of methionine and ammonia on hepatic encephalopathy were examined in acute CCl_4 injury in 3 groups of rats. In the first group of rats, physiological saline was injected intraperitoneally instead of ammonium acetate an hour after intragastrical feeding of methionine. In the second group, ammonium acetate was given an hour after oral methionine administration and in the third group, ammonium acetate was injected an hour after giving saline instead of methionine. All rats were treated with CCl_4 solution 24 hours before starting the above experiment.

Changes in rat behavior after administration of methionine and ammonium acetate were classified into Grade I to IV as follows (see **Fig. 3**): Grade I: Vital activity is generally diminished, but rats can walk and hold up the neck. Grade II: Walking is impossible and the eyes are closed; rats take a sitting position, bending their extremities. Grade III: Animals take a prostrate posture with extension of their arms and legs; they cannot hold up the neck and the eyes are expressionless. Grade IV: Rats are lying on the side and cannot stand, but their response against pain stimuli can still be observed.

Electroencephalogram (EEG) was recorded according to the method previously reported[9]. Serial EEG findings of rats with hepatic encephalopathy were graded from I to IV (see **Fig. 4**). 0: Basic activity is composed of 11-13 c/sec waves with amplitude of approximately 50 μV. I: Ten c/sec waves with 100 μV amplitude are seen temporarily among 11-12 c/sec basic waves. II: Similar 10 c/sec waves with 100 μV are most common. III: Nine-10 c/sec

waves with higher amplitude than 200 μV are mainly observed. IV: Slow waves (7-8 c/sec) with high amplitude similar to Grade III are recognized partially among 9-10 c/sec waves.

The data obtained were statistically analyzed and significant probability of the mean was evaluated by Student t-test.

Results

Serum methionine and blood ammonia levels in patients with liver diseases

Serum methionine concentrations increased significantly in encephalopathic cirrhotic patients without hepatocellular carcinoma (abbreviated simply as hepatoma) but not in the cases with hepatoma (**Table 1**). Blood ammonia levels were significantly higher in the encephalopathic patients with and without hepatoma as compared to respective non-encephalopathic cases with liver cirrhosis. Significant difference in the sum of serum methionine and blood ammonia levels was found between encephalopathic patients and non-encephalopathic cases irrespective of the association of hepatoma. This suggests that simultaneous monitoring of both parameters is im-

Table 1. Serum methionine concentrations and blood ammonia levels and sum of both parameters in cirrhotic patients with and without hepatoma

	Liver cirrhosis without HCC		Liver cirrhosis with HCC	
	Encephalopathy (−)	Encephalopathy (+)	Encephalopathy (−)	Encephalopathy (+)
Serum methionine (μmoles/l)	42 ± 25 (30)	130 ± 170* (18)	27 ± 21 (18)	87 ± 82 (7)
Blood ammonia (μg/dl)	117 ± 46 (21)	169 ± 77* (16)	74 ± 31 (11)	158 ± 46** (6)
Serum methionine + Blood ammonia	155 ± 62 (16)	314 ± 168** (16)	91 ± 32 (11)	247 ± 91** (6)

*: $P<0.05$, **: $P<0.01$. ( ): No. of cases tested.
HCC: Hepatocellular carcinoma.

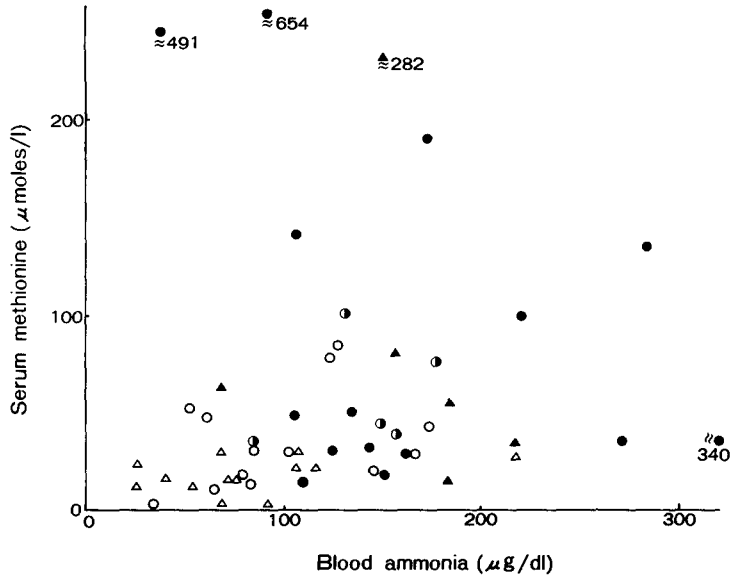

Fig. 1. Two-dimmentional plot of serum methionine concentrations and blood ammonia levels in cirrhotic patients with hepatoma (△) and without hepatoma (○). Solid symbols show the concentrations of these patients with hepatic encephalopathy (▲ or ●). Semisolid simbols represent the levels in patients with neurological symptoms near the times of amino acid measurements. Values from patients with hepatic encephalopathy can be found in the area with marked increases in either of these two parameters or in the area with simultaneous moderate elevations of both methionine and ammonia levels.

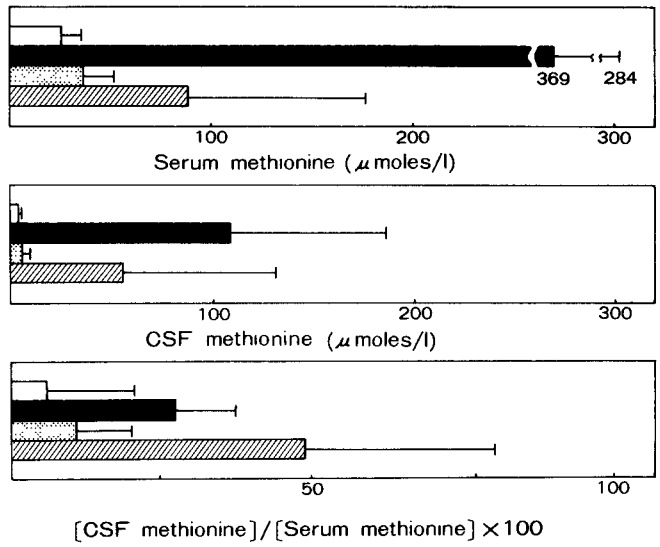

Fig. 2. Methionine levels in serum (upper panel) and in CSF (middle panel) and ratios of CSF methionine concentrations ([CSF methionine]) divided by serum methionine levels ([serum methionine]) (lower panel) in 8 control subjects (□), 5 cases of fulminant hepatitis (■), 8 cirrhotic patients without hepatic encephalopathy (⊞) and 7 encephalopathic cirrhotics (▨).

portant for predicting development of hepatic encephalopathy in cirrhotic patients. Serum methionine and blood ammonia levels in cirrhotic patients were examined in order to investigate synergistic role of these factors in pathogenesis of hepatic encephalopathy (**Fig. 1**). Patients with hepatic encephalopathy were associated with marked increase in either of these parameters, or with moderate elevations of both methionine and ammonia levels, indicating that methionine and ammonia may act cooperatively as one of the factors causing hepatic encephalopathy.

CSF methionine levels in patients with liver diseases

CSF methionine levels in control subjects and cirrhotic patients without hepatic encephalopathy were in the range below 10 μmoles/l (**Fig. 2**). The CSF levels in patients with fulminant hepatitis increased to a greater extent similarly as the blood levels. CSF methionine levels in encephalopathic cirrhotics were also elevated considerably, but the difference between cirrhotic patients with and without hepatic encephalopathy was statistically insignificant ($0.05<P<0.10$). However, ratios of CSF methionine levels/serum concentrations were significantly higher ($P<0.001$) in encephalopathic patients with fulminant hepatitis or liver cirrhosis as compared to non-encephalopathic cases.

Table 2. Predicted influx velocities (Vpre) of methionine into the brain and CSF methionine/Vpre ratios in patients with fulminant hepatitis and in cirrhotics with and without hepatic encephalopathy

	Control subject (8)	Fulminant hepatitis (5)	Liver cirrhosis −HE (8)	Liver cirrhosis +HE (7)
Vpre (nmol·g^{-1}·min^{-1})	1.0 ± 0.4	10.7 ± 7.9*	1.9 ± 1.2	3.4 ± 0.8**
[CSF]/Vpre	1.0 ± 1.1	9.5 ± 2.3**	1.7 ± 1.5	11.2 ± 10.0*

*: $P<0.05$, **: $P<0.01$. ( ): No. of cases examined.
HE: Hepatic encephalopathy.

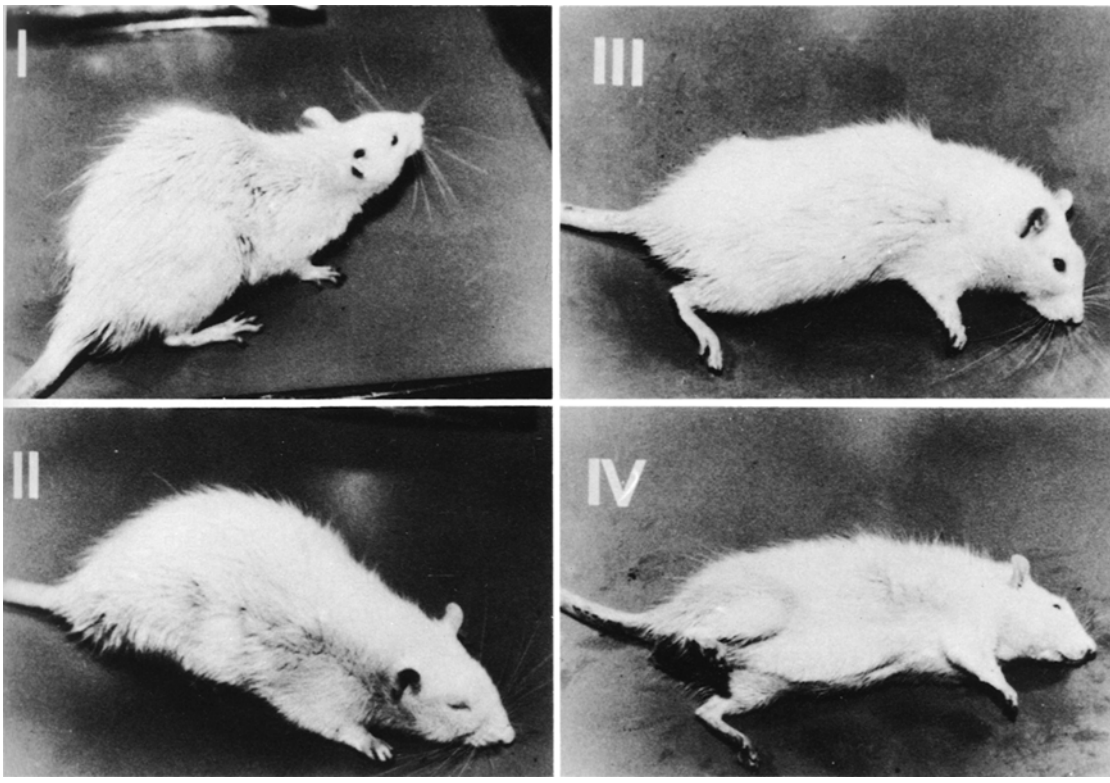

Fig. 3. Classification of hepatic encephalopathy following intraperitoneal infusion of ammonium acetate in methionine-fed rats. Details of 4 Grades were described under Materials and Methods.

Vpre values of methionine as ratios of CSF methionine levels ([CSF]) divided by Vpre values ([CSF]/Vpre) in patients with liver disease were theoretically calculated to analyze alteration of methionine transport across the BBB (**Table 2**). Vpre values of methionine increased significantly in encephalopathic patients with fulminant hepatitis ($P<0.05$) and liver cirrhosis ($P<0.01$). [CSF]/Vpre ratios were also higher in these encephalopathic patients as compared to non-encephalopathic cases, which suggests that in hepatic failure methionine in the circulating blood enters the brain at a greater velocity than that predicted.

Neurological and behavioral changes following administration of methionine and ammonia in rats

Preliminary experiments revealed that changes of behavior could not be observed in CCl_4-injured rats by sole administration of either methionine or ammonium acetate as described under Materials and Methods (**Table 3**). Since some time interval was thought to be necessary for orally administered methionine to be metabolised, ammonium acetate was injected 60 minutes after oral methionine administration. Neurological symptoms could be observed by this protocol in CCl_4-intoxicated rats. However, no abnormal neurological symptoms were developed in CCl_4-untreated rats treated in the same way.

In order to see whether the behavior changes could be induced by oral administration of other amino acids, glycine or leucine was ad-

Table 3. Induction of hepatic encephalopathy by treating with amino acids and ammonium acetate in CCl_4-intoxicated rats

CCl_4 treated	Amino acid administered	Ammonium acetate injected	Hepatic encephalopathy
+	−	+	No
−	Methionine	+	No
+	Methionine	−	No
+	Methionine	+	Yes
+	Glycine	+	No
+	Leucine	+	No

CCl_4, amino acid and ammonium acetate were given intragastrically and intraperitoneally at doses described under Materials and Methods.

Table 4. Grade and duration of hepatic encephalopathy by differently treating with amino acids and ammonium acetate in CCl_4-intoxicated rats

Time of treatment*	EEG	Hepatic encephalopathy	
		Grade	Duration
0	Ⅱ	Ⅱ	10
30	Ⅱ	Ⅱ	35
60	Ⅳ	Ⅳ	60～70
120	Ⅳ	Ⅳ	150

*: Time in minutes from oral administration of methionine to intraperitoneal injection of ammonium acetate. EEG was recorded at the time of hepatic encephalopathy, which was occured within approximately 10 minutes following injection of ammonium acetate. Duration: Periods of duration in minutes of neurological symptomes observed.

Table 5. Effects of methionine and/or ammonia administration on several parameters of liver injury and methionine levels in serum and the brain

	(Ⅰ) Methionine + Saline (3)	(Ⅱ) Methionine + Ammonia (4)	(Ⅲ) Saline + Ammonia (3)
Liver/body weight (%)	4.0 ± 0.5	4.0 ± 0.5	3.7 ± 0.4
G P T (I.U.)	5433 ± 833	7775 ± 2470	4766 ± 404
Insulin (μU/ml)	81 ± 15	193 ± 101	183 ± 97
NH_3 (μg/dl)	259 ± 46	484 ± 36†††	266 ± 9†††
Methionine			
Serum (μmoles/l)	4198 ± 302	5384 ± 740†	158 ± 29‡‡‡
Brain (μmoles/kg)	1175 ± 52	731 ± 200††	73 ± 13‡‡‡

†: Ⅰ vs Ⅱ or Ⅲ, ‡: Ⅱ vs Ⅲ. †: $P<0.05$, ††: $P<0.01$, ‡‡‡: $P<0.001$. ( ): No. of rats tested.

ministered instead of methionine. However, behavior and neurological changes after ammonium acetate injection could not be recognized in these rats treated with glycine or leucine.

Rat behavior and EEG findings were examined after administration of ammonium acetate to CCl_4-treated rats with carying time intervals after oral administration of methionine. CCl_4-treated animals, which were injected with ammonium acetate immediately or 30 minutes after methionine feeding, showed diminished vital activity with eyes closed (hepatic encephalopathy Grade II, **Fig. 3**) approximately 10 minutes after the injection of ammonium acetate. EEG findings also showed Grade II changes at this time (**Fig. 4**). Abnormal neurological symptoms continued for about 10 and 35 minutes in respective rats. On the other hand, rats injected intraperitoneally with ammonium acetate 60 minutes following oral administration of methionine showed Grade III behavior and neurological changes and Grade III in EEG findings 10 minutes after intraperitoneal injection of ammonium acetate. The degree of the symptoms gradually progressed to Grade IV and continued for 60 minutes. In rats which received ammonium acetate 120 minutes following methionine administration, Grade IV behavior and neurological changes with Grade IV EEG findings appeared shortly after injection of ammonium acetate, and severe neurological symptoms persisted for a period of over 150 minutes.

Synergistic effects of methionine and ammonia on hepatic encephalopathy

In order to see synergistic effects of methionine and ammonia on pathogenesis of hepatic encephalopathy, acute CCl_4-injured rats were divided into 3 groups as described under Materials and Methods (**Table 5**). Neurological changes (Grade IV) were observed only in Group II but not in Groups I and III. There

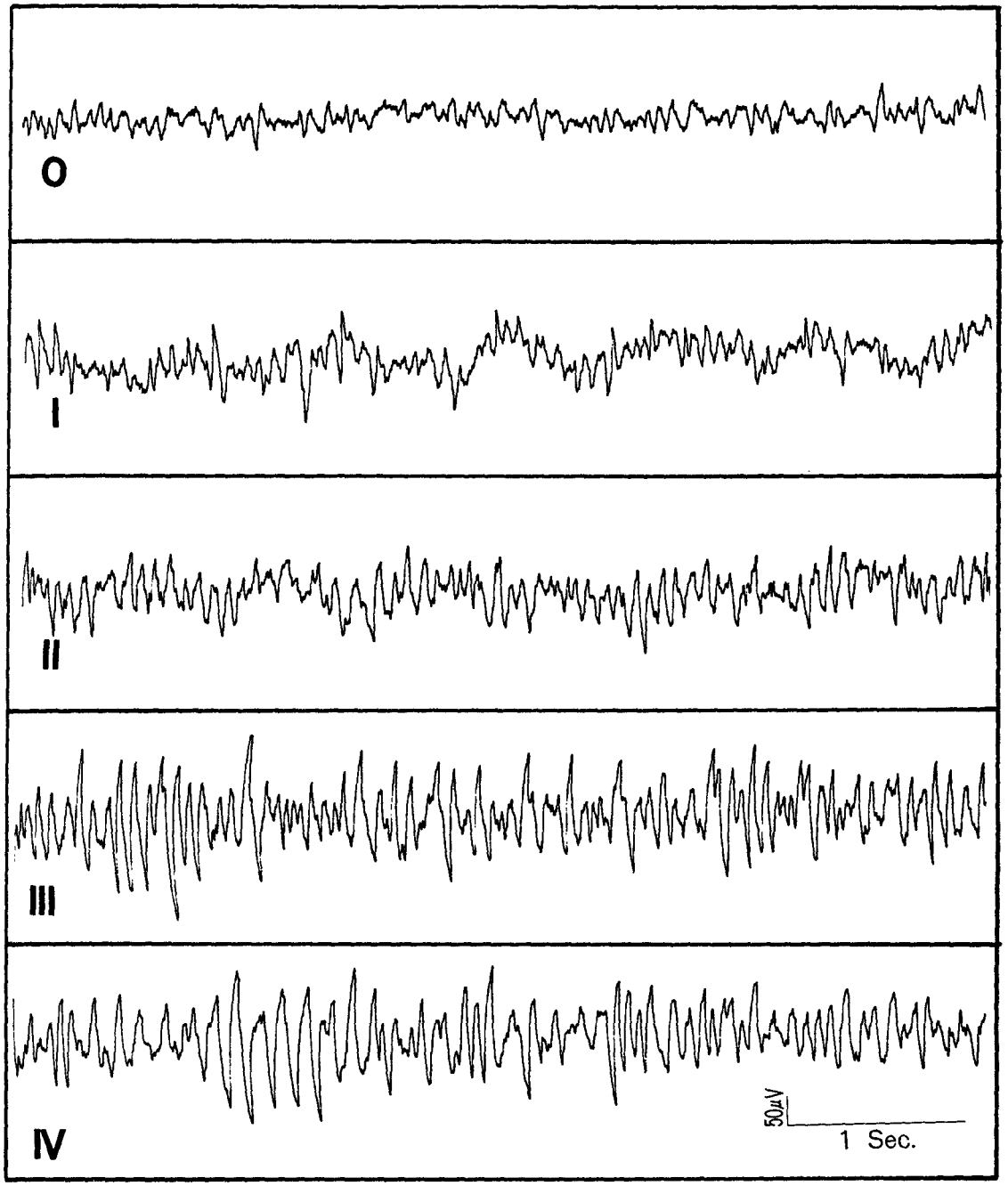

Fig. 4. Classification of EEG findings following intraperitoneal injection of ammonium acetate in methionine-fed rats. Detailed description on EEG findings were shown under Materials and Methods.

were no significant differences of the liver weight/body weight, serum glutamic pyruvate transaminase (GPT) activity and serum insulin levels in these 3 groups, suggesting that liver injury in these 3 groups was similarly occurred. Methionine levels in the serum and in the brain increased to a greater extent in Groups I and II. Blood ammonia levels were markedly elevated in Group II but not in Group III, although both were similarly injected with ammonium acetate.

Discussion

Hepatic encephalopathy is known to be one of the severe complications which are frequently associated with patients with hepatic failure. Various toxic substances such as ammonia[10], fatty acids[11], monoamines[12] and methionine derivatives[13] have been involved in pathogenesis of hepatic encephalopathy. However, it seems to be difficult presently to determine which toxic substance is the causative factor of hepatic encephalopathy in the individual cases; many metabolic abnormalities are probably involved simultaneously in induction of hepatic encephalopathy.

Since development of abnormal neuropsychiatric symptoms was observed in cirrhotic patients during therapeutic administration of methionine[2,13], this amino acid has been suggested as one of the toxic factors which induces hepatic encephalopathy. Synergistic effects of methionine and ammonia or fatty acids on pathogenesis of hepatic encephalopathy have been emphasized from experimental studies by Zieve et al.[4,5]. Detailed clinical and experimental investigations have been needed to reevaluate these observations. Results shown in **Fig. 1** indicated that hepatic encephalopathy could be observed in cirrhotic patients who showed normal concentrations of either blood ammonia or serum methionine. In other words, synergistic effects of both parameters on production of hepatic encephalopathy is suggested also in clinical subjects.

Elevation of CSF methioning levels in encephalopathic patients with fulminant hepatitis and liver cirrhosis are consistent with the previous report[14], in which significant elevations of this amino acid levels in CSF were described in encephalopathic dogs with portacaval shunt. CSF methionine levels did not increase in comatose patients with cerebrovascular diseases (average of 3 cases, 4 μmoles/l). Therefore, analysis of CSF methionine levels rather than the serum levels seems to be a more useful tool for diagnosing hepatic encephalopathy in cirrhotic patients with neurological symptoms. In other changes in CSF aminogram of patients with hepatic encephalopathy, glutamine levels have been reported to be markedly elevated[15,16]. Approximately 2-fold increases in CSF glutamine levels were observed in encephalopathic patients with fulminant hepatitis and liver cirrhosis as compared to non-encephalopathic cirrhotics (data not shown). Elevations of blood ammonia concentrations probably may induce CSF glutamine increases in encephalopathic patients with liver disease.

A validity of the theoretical application of [CSF]/Vpre for analyzing neutral amino acid transport across the BBB has been previously reported[17]. Elevation in [CSF]/Vpre of methionine in encephalopathic patients indicates accelerated transport of this amino acid through the BBB in hepatic failure. Influx of ammonia into the brain is also enhanced in animals with portacaval anastomosis[18]. These may suggest that toxins affecting the central nervous system enter the brain easily and excessively at the time of hepatic failure. Functional[19] or organic change[20] of the BBB may induce abnormal transport of neutral amino acids across the BBB in patients with severe liver disease. However, further studies should be carried out in order to determine the de-

tailed mechanisms of hepatic encephalopathy with respect to the BBB transports of essential substances.

Abnormal behaviors of acute CCl_4-intoxicated rats, which were observed following combined administration of methionine and ammonium acetate, can be defined as methionine and ammonia-induced hepatic encephalopathy for several reasons. No abnormal behaviors could be observed in the absence of hepatic injury. The symptoms were reversible, although its grade was deep. Abnormal EEG findings varied in accordance with degrees of encephalopathy similarly as observed in patients with liver disease[21]. Rats treated with glycine or leucine instead of methionine showed no neurological symptoms, even after injection of ammonium acetate. Thus methionine or its metabolites in cooperation with ammonia is closely participating in production of hepatic encephalopathy.

Preventive effects of antibiotics on methionine and ammonia-induced hepatic encephalopathy[22] have indicated that encephalopathy may develop due to some toxic derivatives of this amino acid, probably produced in the intestine[23]. Methyl mercaptan can be formed from methionine in the liver mitochondria of normal rats[24]. However, it is not known whether mercaptan formation is affected in cirrhotic patients with impaired catabolisms of methionine in the liver[1]. Blood concentrations of methanethiol, one of the mercaptans, increased markedly in severe liver disease, and the levels were elevated progressively according to development of hepatic encephalopathy[25].

Irreversible coma following methanethiol inhalation in a healthy person[26] have strongly suggested that this compound is extremely harmful to the central nervous system. Suppressive effects of methanethiol on the brain mitochondrial respiration[27] and brain (Na^+, K^+)-adenosine 5′-triphosphatase activity[28] are possible mechanisms of methionine and ammonia-induced hepatic encephalopathy. However, apparent neurological changes following oral administration of methionine alone were not observed in this study. Furthermore, CCl_4-treated rats with simultaneous administration of methionine and ammonium acetate showed only slight and short-term encephalopathy. Deep and longer-term encephalopathy in CCl_4-induced rats could only be observed if the ammonium acetate was injected one or two hours after methionine feeding. These evidences indicated that methionine metabolites produced in the intestine possibly act on the central nervous system by enhancing toxic effects of ammonia in the brain or by inhibiting ammonia detoxification, resulting in the longer continuation of encephalopathy. Zieve et al. have recently reported that methanethiol inhibited urea synthesis in the isolated perfused liver[29]. These considerations, therefore, may explain the difference of blood ammonia levels between Groups II and III (**Table 5**), which received the ammonium acetate injection and showed higher glutamine levels in the brain as compared to Group I [Group I, II, III (μmoles/g brain): 6836 ± 1417, 10546 ± 826, 8520 ± 932, respectively].

Effectiveness of branched-chain amino acid (BCAA)-rich solution for arousal from hepatic encephalopathy has been widely recognized[30,31]. Our previous reports[8,17] have described that serum and CSF methionine concentrations, blood ammonia levels and serum and CSF phenylalanine and tyrosine concentrations are diminished in cirrhotic patients following infusion of this type of solution. BCAA are known to be important amino acids which regulate ammonia metabolisms in the skeletal muscle[32]. Further investigations on protective effects of BCAA-rich solution on methionine and ammonia-induced hepatic encephalopathy should be carried out in the future.

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