demiological observations linking clams as possible vectors in hepatitis and suggest that, under special circumstances, shellfish may serve as a reservoir for Au antigen and type B hepatitis virus.

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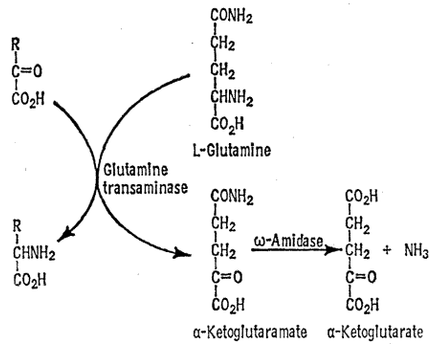
α-Ketoglutaramate: Increased Concentrations in the Cerebrospinal Fluid of Patients in Hepatic Coma

Abstract. *α-Ketoglutaramate, a deaminated metabolite of glutamine not previously identified in biological tissues, was measured in the cerebrospinal fluid of human subjects and found to be increased three- to tenfold in patients with hepatic coma. When perfused into the cerebral lateral ventricles of rats, α-ketoglutaramate (10 mM) depressed the animals' nocturnal locomotor activity, and at higher doses induced circling behavior and myoclonus. The concentration of α-ketoglutaramate in cerebrospinal fluid appears to be a reliable diagnostic indicator of hepatic coma, and its accumulation may contribute to the pathogenesis of this disease.*

Ammonia is believed to play a role in the pathogenesis of hepatic coma, since hyperammonemia is a prominent finding in patients with this condition, and the currently most effective therapeutic measures are directed toward reducing the plasma ammonia concentrations (*1*). Also, a neuropathological picture resembling that of human hepatic encephalopathy can be induced in monkeys after chronic ammonia infusions (*2*). However, a causative relation between the elevated ammonia concentrations in plasma and cerebrospinal fluid (CSF) and the encephalopathy has not been firmly established. A major difficulty in this regard is the observation that patients with liver disease and prominent neurological symptoms can exhibit normal or only moderately elevated concentrations of ammonia in CSF and blood (*3*).

Bessman and Bessman (*4*) suggested that the toxic effect of ammonia in brain results from depletion of α-ketoglutarate, leading to a reduction in tissue adenosine triphosphate (ATP) concentrations. Subsequent studies (*5*) have failed to substantiate this proposal.

Warren and Schenker (*6*) reported that methionine sulfoximine, an inhibitor of glutamine synthetase, protected mice against acute ammonia intoxication, although, paradoxically, ammonia concentrations in brain more than doubled. As this effect is apparently not secondary to energy sparing (*7*), it could conceivably result from the inhibition of the synthesis of a toxic agent formed after the glutamine synthetase step. Glutamine, itself, exhibits no known neurotoxicity (*8*). α-Ketoglutaramate (α-KGM), a metabolite of glutamine formed by transamination with an appropriate α-keto acid (*9*),



has not been explored in this context although glutamine transaminase and ω-amidase are present in brain (*10*). On the hypothesis that α-KGM (or its cyclic isomer 2-hydroxy-5-oxoproline) might be involved in the pathogenesis of hepatic coma, we measured its concentration in the cerebrospinal fluids of human subjects, and assessed its pharmacology by perfusing it into the lateral cerebral ventricles of free-running rats.

Patients diagnosed with (i) hepatic coma, (ii) demonstrated liver disease unaccompanied by neurological symptoms, (iii) respiratory failure and obtundation, and (iv) various neurological diseases but without liver impairment, were studied. CSF was obtained from routine diagnostic lumbar puncture and was immediately frozen at − 80°C. The samples were deproteinized with 3*M* perchloric acid and neu-

Table 1. Concentrations of ammonia, glutamate, glutamine, and α-ketoglutaramate in cerebrospinal fluid of human subjects. The data, expressed as micromoles per liter of CSF, are the mean ± S.D. for the number of patients shown in brackets. The range of individual values is given in parentheses.

Condition	Concentrations (μmole/liter)			
	Ammonia	Glutamate	Glutamine	α-Ketoglutaramate
Nonhepatic controls [9]	67.7 ± 45.6 (21.0–134)	3.6 ± 2.9* (< 1.0–9.0)	422 ± 46.6 (329–477)	3.6 ± 3.2* (< 1.0–8.7)
Respiratory failure with obtundation [2]	28.8, 60.4	6.9, 4.2	1750, 1594	< 1.0, < 1.0
Liver disease without coma [5]	37.9 ± 21.1 (13.5–56.2)	2.4 ± 1.4* (< 1.0–4.8)	576 ± 226 (350–865)	2.6 ± 2.3* (< 1.0–6.2)
Hepatic coma [8]	154.6 ± 61.8† (70.3–233)	7.6 ± 4.2‡ (2.0–14.4)	2056 ± 679§ (1030–2850)	49.0 ± 21.8§ (30.9–95.4)

* Concentrations less than 1 μ*M* were arbitrarily assigned the value of 1.0 μ*M* for statistical purposes. † $P < .01$. ‡ The difference from the nonhepatic control value is significant with $P < .05$. § $P < .001$.

tralized to *p*H 6.6 with $KHCO_3$. Precipitated $KClO_4$ was removed by centrifugation, and the supernatant fluids were stored at −80°C until analyzed. The entire extraction procedure was performed at 0 to 4°C.

Ammonia, glutamate, and glutamine were measured fluorometrically on the neutralized extracts of CSF, by specific enzymatic methods (*11*). For the measurement of α-KGM (*12*), portions (50 μl) of the neutralized extracts, corresponding to approximately 30 μl of CSF, were added to 100 μl of a reaction mixture consisting of 50 m*M* tris-HCl (*p*H 8.5), 0.1 m*M* dithiothreitol and 10 m*M* 2-mercaptoethanol. The reactions were started by the addition of 10 μl of ω-amidase (*13*), and the mixtures were incubated at 37°C for 3 hours. Increasing the enzyme concentration did not reduce the time required for complete hydrolysis, since the rate of deamidation of α-KGM by ω-amidase is limited by a nonenzymatic step, presumably ring opening of the γ-lactam (*9, 13*). The reactions were terminated by the addition of 2 μl of 12*N* HCl, and the mixtures were heated in a boiling water bath for 2 minutes. Extracts of CSF, incubated in the same reaction mixture (ω-amidase omitted) and otherwise treated identically, served as blanks. Reagent blanks which contained 50 μl of neutralized perchloric acid were included with each set of incubations. α-Ketoglutarate was subsequently assayed on 60-μl portions of the boiled mixtures added to 1.0 ml of a reagent which contained 50 m*M* tris-HCl (*p*H 8.5), 0.1 m*M* adenosine diphosphate (ADP), 10 m*M* ammonium acetate, and 0.001 m*M* NADH (reduced nicotinamide adenine dinucleotide). The reactions were started by the addition of liver glutamate dehydrogenase (0.1 mg/ml) and followed fluorometrically. α-Ketoglutarate, derived from α-KGM, was calculated by difference from the blanks (ω-amidase was omitted).

Concentrations of glutamate and glutamine in CSF of nonhepatic control subjects (Table 1) were within the limits reported for normal human CSF (*14*) although ammonia concentrations tended to be higher (*3*). Patients with uncomplicated liver disease had somewhat higher concentrations of glutamine ($P < .1$), while glutamate and ammonia concentrations were within the control range. Patients with hepatic coma had significantly higher concentrations of ammonia and glutamate. Glutamine in patients with hepatic coma was also consistently elevated, in agreement with earlier reports (*15*). However, in two comatose patients in respiratory failure the glutamine concentrations were in the same range as those in patients with hepatic coma. These patients, both of whom were hypoxic and hypercapnic, were classified separately in view of the report (*16*) that glutamine concentrations in CSF are increased in patients in respiratory failure with neurological symptoms. Concentrations of ammonia, glutamate, and α-ketoglutaramate in these two patients were within the limits of nonhepatic controls.

α-Ketoglutaramate in CSF was elevated in all patients with hepatic coma, but the level in nonhepatic coma patients ranged from undetectable (< 1 μ*M*) to a maximum of 8.7 μ*M*. The lowest value in patients with hepatic coma was more than three times higher than the highest value observed in any other subject. α-KGM was undetectable in plasma of any patient.

The effect of α-KGM on the central nervous system was assessed by intraventricular perfusion into rats (*17*). Perfusions were performed in the late afternoon when rats usually sleep 55 to 60 percent of the time. Perfusion with artificial human CSF (*18*) caused a 35 percent decrease in the animals' subsequent nocturnal locomotor activity (Table 2). Fencl *et al.* (*19*) suggested that this effect represents compensation for a sleep deficit incurred during the perfusion process. α-KGM (10 m*M*) caused a further reduction (52 percent) in locomotor activity. When perfused in higher concentrations (100 m*M* in mock CSF), α-KGM induced within 5 minutes abnormal behavior consisting of running in circles and intermittent truncal myoclonus. Animals that received 100 m*M* NaCl (isosmotic with the perfused α-KGM) in mock CSF showed no such behavioral abnormalities.

Since α-KGM can be metabolized in brain with the formation of ammonia, the possibility existed that the depressant effect of α-KGM was mediated by indirectly raising the CSF ammonia concentration. To examine this question, three rats, perfused for 2 hours with 10 m*M* α-KGM, were anesthetized with pentobarbital, and CSF was withdrawn by cisternal puncture. The resulting concentration of CSF ammonia (38.5 ± 8.9 μ*M*, mean ± S.D.) did not differ from noninfused controls (34.7 ± 16.2 μ*M*).

α-Ketoglutaramate concentrations in CSF appear to be a reliable diagnostic indicator of hepatic coma, contents of ammonia and glutamine being less specific in this regard. The cause of the elevated CSF α-KGM in this condition is unclear, but its absence from blood argues in favor of its synthesis in brain. Moreover, the concentration of α-KGM does not appear to be related in simple fashion to concentrations of CSF glutamine, since two patients in whom glutamine was significantly elevated had concentrations of α-KGM within control limits.

Hersh (*13*) proposed that under physiological conditions the enzymes which synthesize (glutamine transaminase) and degrade (ω-amidase) α-KGM are physically linked. According to Meister (*9*), below *p*H 9.0 α-KGM rapidly cyclizes nonenzymatically to its γ-lactam isomer, 2-hydroxy-5-oxoproline. If α-KGM formed by transamination of glutamine normally undergoes enzyme-catalyzed deamidation rather than cyclization in vivo, this could explain why it fails to accumulate even in the face of an elevated glutamine concentration except in hepatic enceph-

Table 2. Effect of intraventricular perfusion of α-ketoglutaramate on the nocturnal locomotor activity of rats. Extradural cannulae (6 mm in length) were permanently implanted into the left parietal bone of 300-g rats (*22*). Animals whose locomotor activity varied less than 20 percent on three successive nights were selected for perfusion. Ventricular perfusion was routinely carried out for 2 hours (3 to 5 p.m.) with the aid of a Harvard infusion pump delivering at the rate of 3.6 μl/min. The location of the perfusion needle was ascertained by continuous pressure monitoring. At the end of the perfusion rats were placed in transparent cages, and locomotor activity was assessed by the number of times the animals interrupted a beam of light focused upon a photocell. The activity was counted for a 12-hour period from 5 p.m. to 5 a.m. Postmortem examination was performed on all test animals and revealed no evidence of hydrocephalus or meningitis. Results, expressed as the ratio of nocturnal locomotor activity after perfusion divided by the mean of the activity on the two nights prior to perfusion, are reported as means ± S.D. for the number of animals shown in brackets.

Treatment	Activity after perfusion/ activity prior to perfusion
Noninfused [16]	0.99 ± 0.14
Artificial human CSF [9]	0.64 ± 0.29*
α-Ketoglutaramate (10 m*M*) in artificial human CSF [7]	0.31 ± 0.14†

* $P < .001$. † $P < .01$; denotes the significance of the difference from rats perfused with artificial human CSF.

alopathy. We suspect that in hepatic coma there is a derangement of cerebral glutamine metabolism involving either disruption of the enzyme linkage, or a decrease in ω-amidase activity.

It has been reported that certain pyrrolidone derivatives including 5-oxoproline, a dehydrated analog of 2-hydroxy-5-oxoproline, selectively antagonize the effects of the excitatory amino acid, L-glutamatic acid, on neural tissue (*20*). If α-KGM (cyclic) behaves similarly, its accumulation might impair neurologic function by competing for glutamic acid receptors in brain. Such a mechanism could account for the reported effectiveness of glutamate administration in the treatment of some patients in hepatic coma (*21*). However, before one seeks potential treatments to counteract α-KGM in hepatic coma, it must be more firmly established that the compound itself depresses the central nervous system.

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12. α-Ketoglutaramate (barium) was prepared according to Meister (*9*). Elemental analysis as calculated from the formula $C_5H_6NO_4Ba_{1/2}$ is C, 28.22 percent; H, 2.84 percent; N, 6.58 percent; Ba, 32.27 percent; the amounts found were C, 27.92 percent; H, 3.02 percent; N, 6.38 percent; Ba, 32.22 percent. One-dimensional thin-layer cellulose chromatography of the free acid in an ethanol, 25 percent ammonium hydroxide, water system (8 : 2 : 1) and an *n*-butanol, acetic acid, water system (10 : 2 : 6) gave a single spot to bromocresol green with R_F values of 0.51 and 0.45, respectively. Solutions of α-KGM were standardized by spectrophotometric measurement of ammonia released on hydrolysis at 98°C in 1*N* HCl, and by deamidation with ω-amidase and determination of α-ketoglutarate or ammonia.
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Transplantation of Allogeneic Bone Marrow in Canine Cyclic Neutropenia

Abstract. *Transplantation of normal bone marrow cells to a gray collie dog with cyclic neutropenia resulted in normal granulocytopoiesis. The finding suggests that cyclic neutropenia occurs because the hematopoietic stem cells are defective. Because of the similarity of human and canine cyclic neutropenia, it also suggests that the human disease may be curable by marrow transplantation.*

Cyclic neutropenia occurs in both man and gray collie dogs. On the basis of marrow morphologic studies and the patterns of fluctuation of blood cell counts, it has been proposed that cycling occurs because of a regulatory defect affecting pluripotential stem cells in both species (*1*, *2*). There are several possible mechanisms for such a defect; the chief possibilities are that the marrow cells are defective or that some host environmental factor causes the marrow cell production to cycle. In order to further investigate the mechanism of cyclic neutropenia, as well as to explore a mode of therapy potentially applicable to the human disease, we have transplanted bone marrow cells from a normal collie to a gray collie. The marrow transplant resulted in normal granulocytopoiesis, ending the cyclic neutropenia.

The donor-recipient pair were a 10-month-old male collie with normal coat color and normal serial blood cell counts and an unrelated 1-year-old female sable-gray collie with well-documented cyclic neutropenia.

Blood cell counts were performed by standard techniques. DL-A typing of cells from the donor-recipient pair was performed by methods described (*3*). One-way mixed leukocyte culture responses were determined by the technique of Rudolph *et al.* (*4*) with minor modifications. The pair of dogs were DL-A identical and mixed leukocyte cultures were mutually nonstimulatory.

One day before transplantation, the recipient received 1000 rads from opposing cobalt-60 sources at 9 rad/min. Under sodium thiamylal and methoxyfluorane anesthesia, 5×10^9 bone marrow cells were aspirated with heparinized syringes from the proximal long bones of the four extremities of the donor; his recovery from this procedure was uneventful.

After filtration of the bone marrow