

GLUTAMIC ACID DEAMINATION IN THE PRESENCE OF MONTMORILLONITE

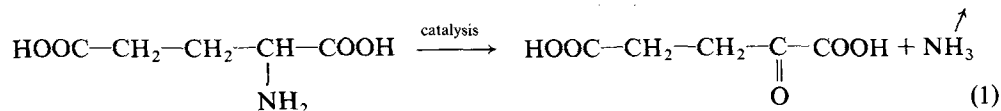
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ABSTRACT: Glutamic acid deamination was realized in the absence and in the presence of montmorillonite saturated with various metallic cations (Na^+ , Mn^{2+} , Cu^{2+}). The catalytic activity depends upon the interlayer exchangeable cation and the pH of the suspension. A maximum was observed near pH = 5–6. In the presence of the enzyme l-glutamate dehydrogenase and the coenzyme nicotinamide adenine dinucleotide, glutamic acid transforms into α -ketoglutaric acid, but in the presence of montmorillonite, it yields α -hydroxyglutaric acid and traces of butyric acid. However, kinetic and reaction yield are significantly reduced compared to the biological system.

The interaction between organic matter and clays has attracted wide attention during the last decades (Adams *et al.*, 1982; Adams, 1987; Ballantine *et al.*, 1981a, b; Cornelis & Laszlo, 1985; Laszlo, 1987). Among recent works are adsorption studies of organic and bio-organic substances on to smectites by Graf & Lagaly (1980), Bzik *et al.* (1983), Garwood *et al.* (1983) and Margulies *et al.* (1988). The swelling behaviour of smectites gives rise to interlayer space incorporation (Micera *et al.*, 1988; Sugahara *et al.*, 1988). The conditions are then requisite for catalytic transformations of the adsorbates (Mortland, 1984; Boyd & Mortland, 1985, 1986; Corma & Perez-Pariente, 1987; Siffert & Naidja, 1987). Nevertheless, the catalytic behaviour of the phyllosilicates in biochemical reactions has still to be elucidated, and the aim of this study is to shed more light on such reactions. The catalytic decomposition of smectite-glutamic acid complexes was examined. In a biological medium, glutamic acid is converted into α -ketoglutaric acid under the action of l-glutamate dehydrogenase enzyme and nicotinamide adenine dinucleotide (coenzyme) according to the reaction:



The objective of the study was to examine if montmorillonite is able to play the role of the enzyme in the preceding reaction. The experiments were achieved with a montmorillonite saturated with different metallic cations (Na^+ , Mn^{2+} , Cu^{2+}).

MATERIALS AND METHODS

Montmorillonite preparation

The clay used was a montmorillonite from Maghnia (Algeria) and its purification and transformation into the homoionic form (Na^+ , Mn^{2+} , Cu^{2+}) have already been described by Siffert & Naidja (1987) and Naidja (1988).

Organic and bio-organic reactants

The reactants used were of high purity grade: l-glutamic acid ($\text{HOOC}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$) and α -ketoglutaric acid ($\text{HOOC}-\text{CH}_2-\text{CH}_2-\text{CO}-\text{COOH}$) were supplied from Merck-Schuchardt (West Germany). α -hydroxyglutaric acid disodium salt ($\text{NaOOC}-\text{CH}_2-\text{CH}_2-\text{CHOH}-\text{COONa}$) was obtained from Sigma Biochemical (USA) and butyric acid ($\text{CH}_3-\text{CH}_2-\text{CH}_2-\text{COOH}$) and nicotinamide adenine dinucleotide (NAD^+) coenzyme ($\text{C}_{21}\text{H}_{27}\text{N}_7\text{O}_{14}\text{P}_2$) from Fluka A.G. (Switzerland). L-glutamate dehydrogenase (l-GDH) enzyme, extracted from bovine liver and lyophilized (100 U.E./mg protein) was supplied by Merck-Schuchardt. Biochemical products (enzyme and coenzyme) were stored at 4°C in order to avoid degradation.

Techniques and methods

For kinetic measurements of ammonia desorption, 0.5 g of montmorillonite were dispersed in polysulfone flasks containing 25 cm^3 of l-glutamic acid aqueous solution (27.2 mmol/dm^3 at $\text{pH} = 3.08$). After addition of clay, the pH was brought to 4.5, montmorillonite acting as a buffer on the system. L-glutamic acid decomposition generates NH_4^+ cations which, to a large extent, remain trapped in the montmorillonite interlayer space. The amount of NH_4^+ cations thus formed was then determined by addition of drops of a concentrated sodium hydroxide solution to the suspension in order to attain pH 12. The ammonia released during the preceding reaction was immediately determined by a specific electrode indicative of NH_3 gas of ORION type (after 1 min). A standardization curve was established for every set of measurements. This technique was previously applied by Mortland (1984).

Adsorption curves of the l-glutamic acid on to M^{n+} -montmorillonite were established by dosing the aminoacid amount (micro Kjeldahl method) remaining in the solution after clay contact (24 h) at $\text{pH} = 3.08$ and 20°C . Three drops of chloroform were added to sterilize the medium.

The reduction kinetic of NAD^+ coenzyme was followed by UV spectroscopy at $\lambda = 340\text{ nm}$ and $\text{pH} = 7.3$ using a LERES-S.30 spectrophotometer. UV spectroscopy could not be used with clay suspension tests as some of the clay mineral remained dispersed even after strong centrifugation.

X-ray diffraction (XRD) patterns of the complexes were recorded using a Philips PW 1009/80 diffractometer with Cu-K α filtered radiation ($\lambda = 0.154\text{ nm}$) at tube settings of 20 mA and 40 kV. The clay-aminoacid samples were sedimented on glass plates and dried at 40°C for 3 days, some being heated to 160°C for 2 h to eliminate hydration water molecules.

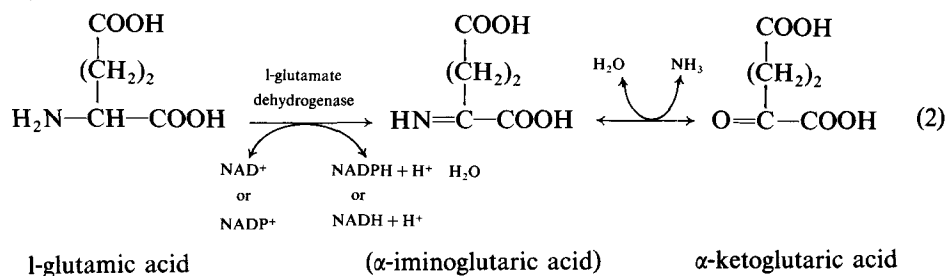
Infrared (IR) spectra of the complexes were recorded on self-supporting thin films obtained by sedimentation of concentrated clay suspensions. The spectrum of pure l-glutamic acid was obtained using a KBr pellet at 1% by weight (Beckman spectrophotometer IR.20, $4000\text{--}300\text{ cm}^{-1}$).

The reaction products were detected by high performance liquid chromatography (HPLC) of the supernatants of the centrifuged clay suspensions. The instrument was a Spectraphysics 3500 apparatus working under the following conditions: (mode: anion exchange; column: Partisil 10 μm SAX; eluent: (a) KH_2PO_4 0.05 M at $\text{pH} = 4.56$, (b) Na_2HPO_4 M/15 and KH_2PO_4 at $\text{pH} = 7.6$; detection: ultra-violet at $\lambda = 208\text{ nm}$.)

RESULTS

Biochemical reaction in the absence of clay

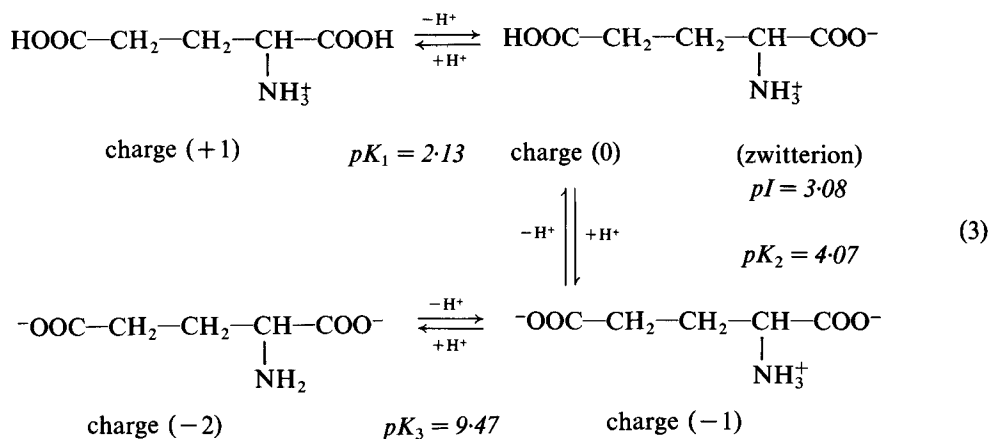
Glutamic acid is an important source of ammonia in biological cycles. Its deamination into α -ketoglutaric acid (2) is catalysed by the specific enzyme, l-glutamate dehydrogenase (l-GDH) (Louisot, 1983). This enzyme is associated with nicotinamide adenine dinucleotide coenzyme (NAD⁺).



In the presence of l-GDH apoenzyme, NAD⁺ coenzyme is reduced into NADH by attracting an H⁺ proton from the glutamic acid molecule. The reduced coenzyme absorbs UV radiation at $\lambda = 340 \text{ nm}$ (Nambiar *et al.*, 1983; De Koke *et al.*, 1986). Fig. 1 shows the optical density *vs.* time of NADH formation, *e.g.* the transformation of l-glutamic acid into α -ketoglutaric acid. The reaction kinetic is high and depends on enzyme concentration, pH, and temperature. The curve obtained serves as a reference for the experiments with montmorillonite.

L-glutamic acid adsorption on to Na⁺-montmorillonite

Protein and aminoacid adsorption on clays, particularly montmorillonite, has already been described by many workers (Morgan & Corke, 1976, 1977; Siffert & Kessaissia, 1978; Graf & Lagaly, 1980; Lagaly & Hermann, 1983; Siffert & Larson, 1983). Aminoacid adsorption depends on clay pretreatment and experimental conditions. The pH of the medium plays an essential role, defining the amphoteric behaviour of aminoacids, *e.g.*, the different species of glutamic acid. For this acid, the following equilibria are observed:



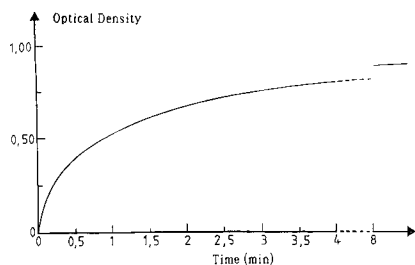


FIG. 1. Glutamic acid transformation into α -ketoglutaric acid in the presence of the enzymatic system ($\text{NAD}^+ \rightarrow \text{NADH}$). Optical density at $\lambda = 349 \text{ nm}$, $\text{pH} = 7.3$ and $T = 20^\circ\text{C}$.

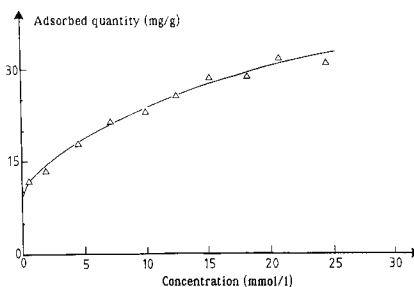


FIG. 2. Adsorption isotherm of glutamic acid on Na^+ -montmorillonite at $\text{pH} = 3.08$ and $T = 20^\circ\text{C}$.

As clay mineral particles are negatively charged, aminoacids would be adsorbed when they are neutral or positively charged. The adsorption curve in Fig. 2 of l-glutamic acid on to Na^+ -montmorillonite was obtained at the isoelectric point ($\text{IP} = 3.08$) of the aminoacid. The adsorbed amount is expressed by the formula of Thomas *et al.* (1983):

$$Q_a = (C_0 - C_e) \cdot V/P \quad (4)$$

where: C_0 is the initial concentration, C_e the equilibrium concentration, V the solution volume and P the adsorbent weight.

The isotherm of the H type, according to Giles *et al.* (1960) shows that the adsorbate displays a high affinity for the montmorillonite surface at low concentration. The isotherm seems to level off at 35 mg/g of clay. This amount corresponds to 0.23 mmol/g and represents one quarter of the total CEC of the mineral (91 mEq/100 g).

XRD analysis

XRD analysis of the montmorillonite-glutamic acid complexes (Table 1) shows that there is a decrease in the interlayer spacing of $\sim 0.190 \text{ nm}$ for Mn^{2+} -montmorillonite. The exceptionally high interlayer spacing displayed by Mn^{2+} -montmorillonite free from glutamic acid at 40°C ($d_{001} = 1.615 \text{ nm}$) and $d_{001} = 1.577 \text{ nm}$ after heating at 160°C stems from the presence of particularly high hydrated Mn^{2+} cationic species (probably $\text{Mn}(\text{OH})_x(\text{H}_2\text{O})_y$). In the presence of l-glutamic acid, the interlayer distance becomes comparable to those of Na- and Cu-saturated minerals ($d_{001} = 1.40 \text{ nm}$). The interlayer spacing does not fall below 1.36 nm after l-glutamic acid adsorption, and the increase in the interlayer space Δd ($\Delta d \neq 1.36 - 1.00 \neq 0.36$) always shows that material has been incorporated into the mineral structure (1.00 nm corresponds to the thickness of an individual 2:1 clay mineral layer). The incorporation shows that diffusion of glutamic acid into the structure and its transformation products outside the interlayer space cannot be neglected in the catalytic activity transformation mechanism. Extraction of some exchangeable cations and their complexation with glutamic acid outside the clay interlayer space can also be expected.

TABLE 1. Basal spacings of M^{n+} -montmorillonite-glutamate 'complexes', after sedimentation and drying at 40°C (3 days) and 160°C (2 h).

Sample	Dried at 40°C for 3 days		Heated at 160°C for 2 h	
	d_{001} (nm)	Δd (nm)	d_{001} (nm)	Δd (nm)
Na ⁺ -montmorillonite	1.32		1.24	
Mn ²⁺ -montmorillonite	1.61		1.57	
Cu ²⁺ -montmorillonite	1.38		1.05	
Na ⁺ -M ^{te} -l-glutamate	1.40	0.07	1.38	0.13
Mn ²⁺ -M ^{te} -l-glutamate	1.42	-0.19*	1.38	-0.18*
Cu ²⁺ -M ^{te} -l-glutamate	1.40	0.02	1.36	0.30
Na ⁺ -M ^{te} -l-glutamate-NAD ⁺	1.40	0.07	1.38	0.13
Mn ²⁺ -M ^{te} -l-glutamate-NAD ⁺	1.42	-0.19*	1.40	-0.17*
Cu ²⁺ -M ^{te} -l-glutamate-NAD ⁺	1.42	0.04	1.40	0.34

* Decrease in spacing.

IR spectroscopy

IR spectra show three new absorption bands for montmorillonite-glutamic acid complexes (Table 2, Fig. 3). The first one is weak at 3060 cm^{-1} and corresponds to protonated NH_3^+ groups of l-glutamate fixed at the clay mineral surface (Little, 1966; Bellamy, 1975; Streitwieser & Heathcock, 1981). The second one at 1720 cm^{-1} absent on the spectrum of pure glutamic acid is probably due to stretching vibrations of unionized acid groups $-\text{COOH}$ (Cross, 1967; Theng, 1974). The third band at 1590 cm^{-1} stems from carboxylate stretching vibrations. This band is large (from 1640 to 1590 cm^{-1}) for Cu^{2+} -montmorillonite-glutamic acid complex and was reported by Bellamy (1975) to appear between 1610 and 1560 cm^{-1} for aminoacids. Another band at 1460–1440 cm^{-1} , though of low intensity, confirms the existence of carboxylate symmetric vibrations. Hence, it seems that the incorporated organic compound may be protonated at the NH_2 groups. Also ionized and unionized carboxyl groups coexist on the organic surface.

Kinetic of ammonia formation

Influence of the exchangeable cation. The kinetic of ammonia release during the transformation of l-glutamic acid incorporated into montmorillonites saturated with different metallic cations (Na^+ , Mn^{2+} , Cu^{2+}) was studied. The same mechanism was examined in the presence of the same cations (introduced in the medium as salts) in an amount equivalent to that existing in the interlayer space, namely 0.91 mEq $M^{n+}/25 \text{ cm}^3$ of aminoacid solution. Experiments were performed with and without the NAD^+ coenzyme. The volume of ammonia gas released was measured as a function of time with a NH_3 specific electrode (Figs. 4, 5 and 6). Cations in their free state (introduced as NaCl , $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ salts) had practically no influence on ammonia gas formation. For Na^+

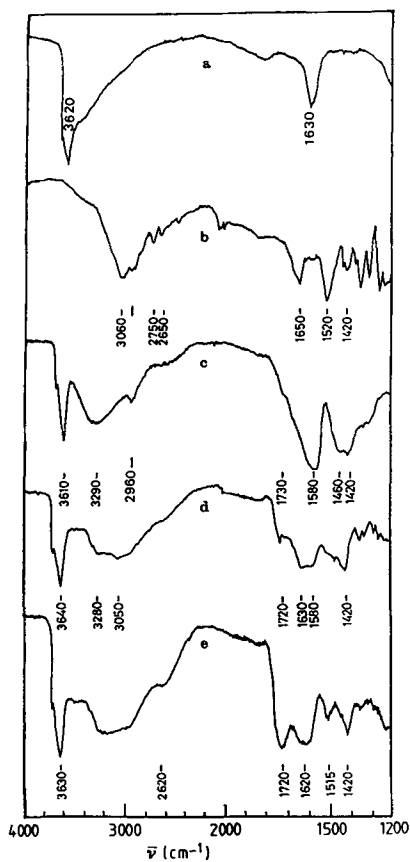


FIG. 3. IR spectra. (a) Pure M^{n+} -montmorillonite; (b) glutamic acid in KBr; (c) glutamate- Na^+ -montmorillonite complex; (d) glutamate- Mn^{2+} -montmorillonite complex; (e) glutamate- Cu^{2+} -montmorillonite complex.

TABLE 2. Details of IR spectra of glutamate- M^{n+} -montmorillonite complexes, $\bar{\nu}$ in cm^{-1} . (a) pure Na^+ -montmorillonite; (b) glutamic acid; (c) glutamate- Na^+ -montmorillonite; (d) glutamate- Mn^{2+} -montmorillonite; (e) glutamate- Cu^{2+} -montmorillonite.

(a)	(b)	(c)	(d)	(e)	Assignment
3620	—	3640	3630	3610	$\nu OH M^{n+}$
—	—	3290	3380–2900	3240–2900	νOH aminoacid
—	3060	—	3050	3040–2900	νNH_3^+ aminoacid
—	2960	2960	2920	2920	νCH
—	2650	2650	2650	2650	$\nu COOH$ aminoacid
—	—	1720	1720	1720	$\nu COOH$
—	1650	—	—	—	$\delta a NH_3^+$
1640	—	—	1630	1640–1600	δH_2O in M^{n+}
—	—	1580	1580	1640–1590	$\nu a COO^-$
—	1520	—	1515	1515	$\delta s NH_3^+$
—	1460	1460	1440	1440	$\nu s COO^-$
—	1420	1420	1420	1415	νCO ; $\delta a NH_3^+$

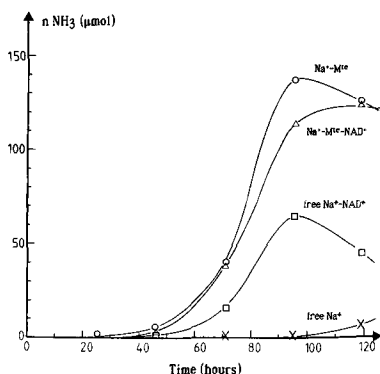


FIG. 4. Kinetic of ammonia formation from glutamic acid at 20°C in the presence of: × Na⁺, □ Na⁺ and NAD⁺, ○ Na⁺-montmorillonite, △ Na⁺-montmorillonite and NAD⁺.

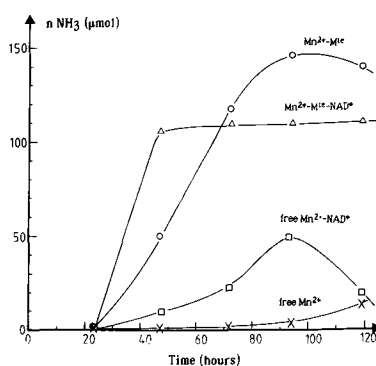


FIG. 5. Kinetic of ammonia formation from glutamic acid at 20°C in the presence of: × Mn²⁺, □ Mn²⁺ and NAD⁺, ○ Mn²⁺-montmorillonite, △ Mn²⁺-montmorillonite and NAD⁺.

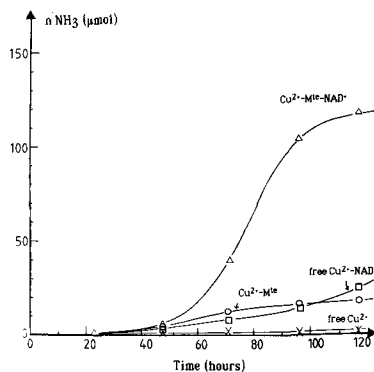
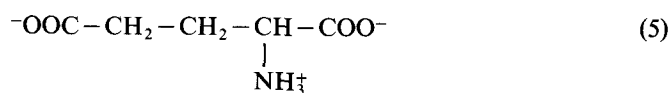


FIG. 6. Kinetic of ammonia formation from glutamic acid at 20°C in the presence of: × Cu²⁺, □ Cu²⁺ and NAD⁺, ○ Cu²⁺-montmorillonite, △ Cu²⁺-montmorillonite and NAD⁺.

and Mn²⁺ cations in the presence of NAD⁺ coenzyme, ammonia gas release was moderate (≈ 60 μmol after 96 h). With Na⁺-montmorillonite and Mn²⁺-montmorillonite, ammonia gas formation was significantly higher but the reaction rate was somewhat low compared to that with enzyme (l-GDH). The ammonia yield became significant after 96 h (140 μmol) and corresponded to 22%. It is likely that the initial sluggishness of the reaction may be due to the time required for the dispersion and swelling of the clay mineral, as well as for the diffusion of the amino acid into the montmorillonite interlayer space, and for ammonia to migrate outside the structure. Surprisingly, ammonia release was very reduced (15 μmol after 96 h) with pure Cu²⁺-montmorillonite and coenzyme NAD⁺ with Cu²⁺ cations (Fig. 6). It seems that the association of Cu²⁺-montmorillonite and NAD⁺ coenzyme may be necessary in order to

obtain significant formation of ammonia. Table 3 lists the amounts of ammonia released after 96 h for the different systems and summarizes the products detected by HPLC in the medium after centrifugation. A very small quantity of α -ketoglutaric acid was formed in the presence of free cations. α -hydroxyglutaric acid and butyric acid were also detected in small quantities in the presence of Na^+ -montmorillonite. But, although the ammonia release was important, no organic product other than glutamic acid was detected for Mn^{2+} - and Cu^{2+} -montmorillonite. The major part of the reaction products probably remained blocked in the interlayer space of the clay mineral.

Influence of pH. Enzymatic activity is generally affected by a variation in the pH of the medium. Optimal pH for enzymatic catalysis is often near neutrality (pH = 6–8). Generally, high or low pH values induce irreversible enzyme denaturation (Berk, 1976). The activity of the montmorillonite *vs.* pH (Fig. 7) was obtained by measuring the ammonia released after 96 h at 20°C. A maximum of activity is observed at pH = 5 for Na^+ -montmorillonite and pH = 5.5 to 6.0 for Mn^{2+} - and Cu^{2+} -montmorillonites. These values are far from the isoelectric point (IP = 3.08) of glutamic acid and are higher than its pK_2 ($pK_2 = 4.07$; equilibrium 3). Between pH = 5 and 6, the predominant glutamic acid species is:



One might suppose, therefore, that this species of glutamic acid is incorporated into the clay mineral space. It would be expected that the carboxylate groups complex the exchangeable cation (Mn^{2+} and Cu^{2+}) and that NH_3^+ groups neutralize the negative sites on the clay interlayer surface.

TABLE 3. Ammonia released after 96 h during glutamic acid deamination and results of HPLC. (ϵ) = small quantity; (m) = medium quantity.

System	Ammonia released after 96 h (μmol)		Products detected by HPLC in the centrifugates
Free M^{n+} -1-glutamic acid- NAD^+	Na^+	60	(ϵ) α -ketoglutaric acid
	Mn^{2+}	45	(ϵ) α -ketoglutaric acid
	Cu^{2+}	15	(ϵ) α -ketoglutaric acid
M^{n+} -montmorillonite-glutamic acid	Na^+	140	(m) α -hydroxyglutaric acid and (ϵ) butyric acid
	Mn^{2+}	145	—
	Cu^{2+}	15	—
M^{n+} -montmorillonite-glutamic acid- NAD^+	Na^+	120	(m) α -hydroxyglutaric acid
	Mn^{2+}	120	—
	Cu^{2+}	110	(m) α -hydroxyglutaric acid and (ϵ) butyric acid

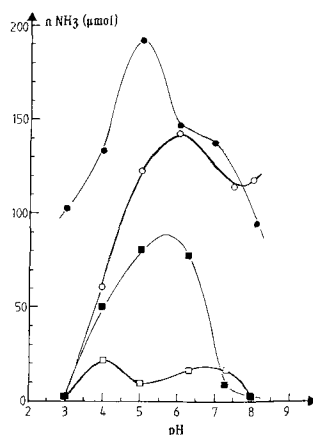


FIG. 7. Influence of pH on ammonia formation after 96 h at $T = 20^{\circ}\text{C}$. ● Na^+ -montmorillonite and NAD^+ , ■ Mn^{2+} -montmorillonite and NAD^+ , ○ Cu^{2+} -montmorillonite and NAD^+ , □ blank.

DISCUSSION

The catalytic activity of montmorillonite is affected by pH, the maximum activity being observed around $\text{pH} = 5$ to 6. To interpret the catalytic behaviour of the clay mineral, it is necessary to take into account the following antagonistic reactions: (i) surface catalysis; (ii) formation of complexes with the exchangeable cations.

Glutamic acid, free cations and coenzyme

In the presence of coenzyme and Na, Mn, or Cu salts, a small amount of α -ketoglutaric acid is formed. The classical biochemical process takes place, but the reaction yield remained low because of the absence of enzyme. In addition, complex formation between the amino acid and free Mn^{2+} and Cu^{2+} cations in solution should occur, which reduces the formation of α -ketoglutaric acid.

Glutamic acid and M^{n+} -montmorillonite

Na⁺-montmorillonite. Provided that no complex formation is possible, α -hydroxyglutaric acid formation (detected by HPLC) implies a catalytic mechanism (Fig. 8). The first step consists of protonation of a carboxylic group. Protons may originate from ionized water molecules in contact with Al cations at the clay mineral surface and constituting acid sites (Weiss, 1981). This protonation is followed by hydrolysis of the $\text{C}-\text{NH}_3^+$ group into $\text{C}-\text{OH}$ and NH_4^+ . This cation may neutralize a surface charge or the remaining carboxylate group. The α -hydroxyglutaric acid formed obviously remains in the interlayer space and only a small part moves out.

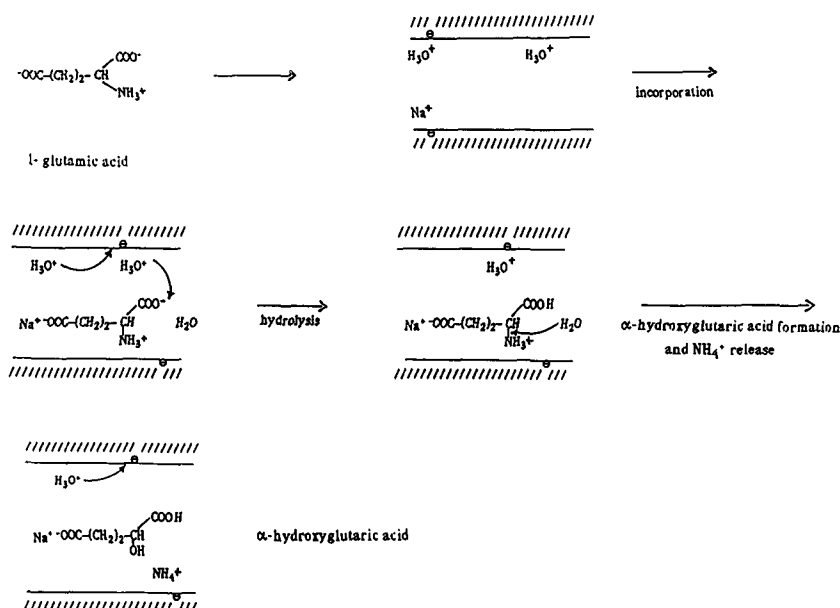


FIG. 8. Glutamic acid transformation into α -hydroxyglutaric acid: mechanism in the presence of Na^+ -montmorillonite.

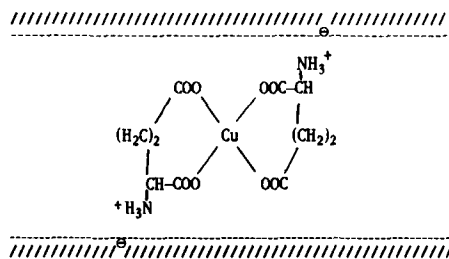
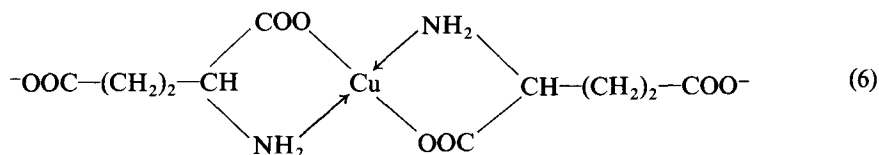


FIG. 9. Interlayer complex.

Mn²⁺- and Cu²⁺-montmorillonites. The deamination reaction is strongly depressed by complexation of Mn and Cu cations with glutamic acid (Das Sarma, 1956; Kirshon & Barsily, 1959; Girdar *et al.*, 1976). There is competition between glutamic acid decomposition and complex formation. Metallic complexes, particularly those existing in living organisms (Mn and Cu), have been extensively studied (Kroll, 1952; Das Sarma & Bailar, 1956; Sabine *et al.*, 1964). Principally two types of complexes exist in which the metal cation is tetracoordinated (6) (Li & Doody, 1953). The complex with $-\text{NH}_2$ ligands probably cannot be incorporated into the structure, as it is negatively charged.



The other complex with four —COO^- ligands (Fig. 9) bears two positively charged —NH_3^+ groups and may be incorporated. In fact, IR spectroscopy results emphasized the presence of —NH_3^+ and —COO^- groups on the incorporated glutamic acid molecule (Laing & Petit, 1975). The Cu glutamic acid complexes are more stable than those of Mn (Girdar *et al.*, 1976) (Table 4). The catalytic decomposition of glutamic acid is delayed as the stability constants of the complexes increase. However, NH_3 measurement with the specific electrode requires pH values ≥ 12 . The addition of NaOH in particular decomposes complexes of Mn, but hardly those of Cu. Thus it is evident that NH_3 formation with Mn^{2+} -montmorillonite would be higher (145 μmoles) than with Cu^{2+} -montmorillonite (15 μmoles).

Glutamic acid, M^{n+} -montmorillonite and coenzyme

Numerous reactions seem to take place simultaneously. The coenzyme seemingly completes the catalytic activity of the clay mineral. This is particularly so considering that the

TABLE 4. Stability constants of M^{n+} -glutamic acid complexes.

Cation	$K_{1:1}$	$K_{1:2}$
Mn^{2+}	$10^{4.07}$	$10^{3.50}$
Cu^{2+}	$10^{8.23}$	$10^{6.07}$

coenzyme can hinder complex formation with Cu and formation of α -hydroxyglutaric and butyric acids. However, we do not have any explanation concerning reactions taking place with Mn^{2+} -montmorillonite.

CONCLUSIONS

Glutamic acid deamination in the presence of M^{n+} -montmorillonite essentially produced α -hydroxyglutaric acid and sometimes traces of butyric acid. The aminoacid transformation depends on the nature of the interlayer exchangeable cation and its ability to form complexes. The catalytic activity of the montmorillonite surface depends on pH and clearly remains lower than that of an enzyme. Nevertheless, the montmorillonite has an advantage over the enzyme as it displays a larger activity pH range (pH = 3–8). Moreover, the activity of montmorillonite is not specific. In the presence of montmorillonite, α -hydroxyglutaric and butyric acids are obtained, whereas l-GDH enzyme only yields α -ketoglutaric acid. The results show that the catalytic contribution to the deamination process is at a maximum with a mineral saturated with Na cations.

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