Purified cytosine deaminase from *Pseudomonas aureofaciens*[8] also has been found to undergo stimulation in the presence of pyrophosphate[9]. As with the *S. marcescens* enzyme, only V_{max} increased for the deaminase reaction. Thus, the *S. typhimurium* deaminase differs from these other deaminases with respect to pyrophosphate activation since its $K_{0.5}$ for cytosine changes.

The best inhibitor of *S. typhimurium* cytosine deaminase activity was determined to be orotidine monophosphate[4]. It can be noted in the table that both the $K_{0.5}$ and V_{max} values are altered as the orotidine monophosphate concentration is increased. The $K_{0.5}$ for cytosine increased from 0.77 mM in the absence of inhibitor to 2.00 mM after 1 mM orotidine monophosphate was added. In contrast, the V_{max} decreased approximately 50% following the addition of 1 mM orotidine monophosphate. These data indicated that orotidine monophosphate is a mixed-type noncompetitive inhibitor of cytosine deaminase. Nucleotides have also been determined to be allosteric inhibitors of the cytosine deaminases isolated from *S. marcescens* and yeast. In *S. marcescens*, cytosine deaminase activity was significantly inhibited by dGMP[7]. This noncompetitive inhibitor of the deaminase affected only the V_{max}. Purified yeast deaminase is inhibited by a variety of purine and pyrimidine nucleotides that have been characterized as mixed-type competitive or noncompetitive inhibitors[10].

The regulatory nature of the *S. typhimurium* cytosine deaminase could be intrinsic to its role in the salvage of pyrimidine bases. Along with a CMP-degrading activity and uracil phosphoribosyltransferase, cytosine deaminase occupies a central role in the recycling of RNA degradation products, such as CMP, in *S. typhimurium*[11]. Depending upon intracellular concentrations of pyrophosphate and orotidine monophosphate, salvage of such nucleotide products might be regulated at the level of enzyme activity. Under pyrimidine nucleotide limiting conditions, the de novo pyrimidine ribonucleotide biosynthetic pathway will be synthesizing UMP at a maximal rate to ensure cellular survival[12]. This pathway will also be producing significant levels of pyrophosphate[13] which may stimulate cytosine deaminase activity. This promotes the salvage of pyrimidine bases since it allows their conversion to the nucleotide level. If pyrimidine nucleotides are present in excess within the bacterial cell, orotidine monophosphate, an intermediate of the de novo pyrimidine ribonucleotide biosynthetic pathway, will most likely be elevated in concentration due to orotidine monophosphate decarboxylase inhibition by UMP[14]. Therefore, cytosine deaminase activity will be inhibited which correlates with the lack of pyrimidine base salvage biosynthesis under such conditions. This type of regulation complements the repression of cytosine deaminase synthesis by pyrimidines in *S. typhimurium* since it ensures an immediate response by the bacterial cell as effector concentrations vary.

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Manipulating the activity of immobilized enzymes with different organo-smectite complexes[1]

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Summary. Enzymes may be immobilized on hydrophobic surfaces of organo-smectite complexes. The immobilized enzyme may be active, partially active, or inactive depending on the nature of the organic surface. These materials may be useful as models for enzymes in natural systems, and in medicine and industry.
Key words. Immobilized enzymes; smectite; organo-smectite complex; hydrophobic bonding; urease; arginase.

Understanding the basic biochemistry of enzymes may be facilitated by studies of immobilized enzymes, for in living cells enzymes exist largely in this state[2]. In nature, enzymes may also exist in the immobilized form by sorption onto natural clay-organic matter complexes[3]. Immobilized enzymes also have many important practical applications in industry and medicine[4,5].

The solid matrices used to immobilize enzymes vary widely in their nature[2,4,5]. The type of enzyme attachment to the matrix material may be through covalent bonding, adsorption, microencapsulation, and matrix entrapment. Adsorption of enzymes on layer silicates, such as smectite[6], may involve a number of interactions, the most energetic of which is ionic bonding[6,7]. This involves the exchange of cations on the mineral surface with positive sites on the enzyme or protein structure.

Our work reported here and in two earlier publications[8,9] has shown that enzymes may be immobilized by a unique process which involves the use of synthetic organo-smectite complexes. These complexes are prepared by saturating the exchange capacity of smectite clay with organic cations such as hexadecyltrimethylammonium$^+$ (HDTMA). This creates a surface of alkyl groups (in the case of HDTMA) which changes the mineral surface from one of a hydrophilic to hydrophobic nature. Enzymes are believed to be immobilized by hydrophobic bonding to the organic surface of the organo-smectite complex as shown schematically in figure 1. Hydrophobic portions of the enzyme interact with the hydrophobic alkyl groups creating an adsorption which is pH independent.

We now describe results which provide the basis for development of 'tailor-made' immobilized enzyme systems based on the use of different organo-smectite complexes. We have found that the enzymes glucose oxidase[8] and urease[9] were strongly bound to HDTMA-smectite and exhibited activities similar (60–

Ammonia released in 1 h at 20 °C from 1 mmole of urea by two ureases (bacterial and jack bean) immobilized on HDTMA-smectite and on [Fe(bipy)$_3$]-smectite after treatment for 15 h at 20 °C and 50 °C. Analyses were made in 0.1 M buffers at the optimum pH of each enzyme, 6.9 for jack bean and 8.5 for bacterial urease

Treatment	Bacterial urease		Jack bean urease	
	HDTMA-clay mmoles NH_3 h^{-1}	[Fe(bipy)$_3$]-clay	HDTMA-clay mmoles NH_3 h^{-1}	[Fe(bipy)$_3$]-clay
20 °C	0.068	0.5	0.08	0.14
50 °C	0	0.04	0	0.04
Activity retained after 50 °C heat treatment	0%	8%	0%	28%

100%) to that of the free enzyme. Horseradish peroxidase and arginase were also strongly adsorbed on HDTMA-smectite, but had no activity whatsoever. This suggests that the mode of binding is such that there is a minimum of interference with the active sites of glucose oxidase and urease, and implies that the binding sites are in no way proximal or related to the active sites. On the other hand for horseradish peroxidase and arginase active sites may indeed be closely related to binding sites.

The above rationale suggests that by providing different kinds of organic surfaces where different bindings might occur, a given enzyme might be active on one kind where the active sites were not affected, and inactive on another kind where interference was great. Such effects are described here utilizing the consecutive two-step reaction:

$$\text{arginine} \xrightarrow{\text{arginase}} \text{ornithine} + \text{urea} \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

When both enzymes were immobilized simultaneously on HDTMA-smectite and arginine used as a substrate, no NH_3 appeared, again confirming the inactivity of arginase on this surface. However, when the two enzymes were simultaneously immobilized on smectite which had its cation exchange sites saturated with $[Fe(2,2'\text{-bipyridyl})_3]^{2+}$ ($Fe(bipy)_3$) cations[10], the consecutive two-step reaction occurred as shown in figure 2. This figure shows that after a lag period, a steady state production of NH_3 occurs, not dissimilar to other results with matrix bound multienzyme systems[11]. When compared with NH_3, an exact stoichiometric level of ornithine was not obtained presumably due to analytical difficulties (e.g. incomplete derivitization) or secondary reactions involving ornithine. Proof that the enzymes were adsorbed was established by assaying the equilibrium solution for enzyme activity, with negative results. The observation of arginase activity on the smectite whose surface was covered with the bipyridyl ligands, suggests yet another type of binding mechanism, perhaps a $\pi - \pi$ interaction between aromatic groups of certain amino acids in the enzyme with the aromatic bipyridyl surface. Whatever the mechanism at least it points to different adsorption processes and different environments since arginase is active on the bipyridyl surface and not on the alkyl surface, while urease was active on both.

Other evidence of differential interactions between organo-smectite complexes and immobilized enzymes is given in the table where effects of heat treatment on enzyme activity are presented. Two ureases, one bacterial, the other from jack bean, are immobilized on either HDTMA-smectite or Fe(bipy)$_3$-smectite. The thermal stability of the enzymes is obviously greater on the bipyridyl-smectite surface. This observation again suggests the microenvironment of the enzymes on the alkyl and the aromatic surfaces is different and results in differential thermal

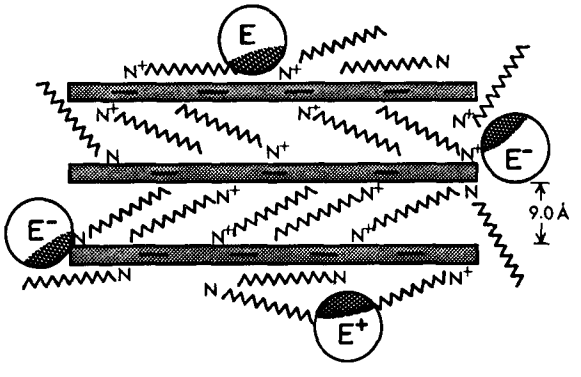

Figure 1. A model for binding of enzymes to hydrophobic HDTMA-smectite. The dark portions of the enzymes (E) are hydrophobic areas.

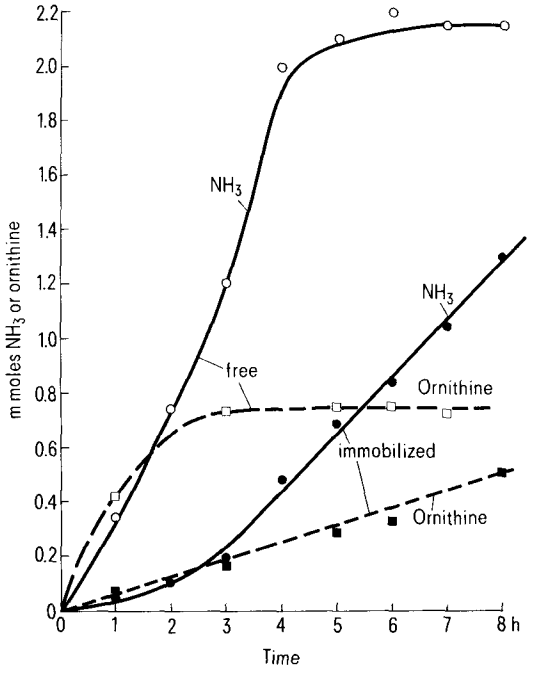

Figure 2. Ammonia from arginine according to the consecutive two-step reaction: arginine $\xrightarrow{\text{arginase}}$ ornithine + urea $\xrightarrow{\text{urease}}$ $2NH_3 + CO_2$ at 20 °C. Arginase and urease (bacterial) were immobilized simultaneously on [Fe(bipy)$_3$]-smectite. Separate urease and arginase solutions containing 1 mg enzyme ml^{-1} were prepared in triethanolamine (TEA) buffer at pH 8.5. 1 ml of each enzyme solution was added to 100 mg [Fe(bipy)$_3$]-smectite contained in 3 ml TEA buffer. The enzyme-clay mixture was shaken gently for 18 h at 20 °C. For comparison, 1 ml of each enzyme solution was also added to 3 ml TEA buffer without [Fe(bipy)$_3$]-smectite (referred to as 'free enzyme') and treated in the same fashion. Enzymatic reactions were initiated by adding 1 ml of either the free or imobilized enzyme to 39 ml of TEA buffer solution containing 1 mmole of arginine. A drop of toluene was added to the assay solution to inhibit microbial growth. Analyses for NH_3 formation were made with an Orion ammonia electrode Model 95-10. Ornithine was derivitized with *o*-phthalaldehyde and mercaptoethanol, and analyzed using high pressure liquid chromatography with UV absorbance detection at 340 nm.

stabilities. Another observation apparent in the table is that the two ureases have greater activity when adsorbed on the Fe(bipy)$_3$-smectite as compared with the HDTMA-smectite, even though equal quantities of enzyme (1% by weight) are immobilized on the two organo-smectite complexes. This suggests easier access to active sites for the substrates.

The use of organo-smectite complexes for immobilization of enzyme systems has several advantages. First, by placing the proper organic cation on the mineral, one can create 'tailor-made' surfaces having a variety of kinds of interactions with proteins and enzymes in particular. Second, since clays possess large surface areas, a large amount of enzyme can be bound on a given quantity of clay mineral[9]. Thirdly, as reported above, different kinds of mineral-organic matrices may have different specificities regarding the activities of various adsorbed enzymes, perhaps giving information about the nature and location of the active sites in the enzyme. Finally, it seems likely that clay-organic complexes could be used as models for a variety of systems such as biological membranes, since surface properties such as hydrophobicity, polarity, and aromaticity can be created as desired. These systems may also be useful in the study of important natural processes such as the stabilization of soil enzymes and soil organic matter, and for industrial applications.

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Effects of acetaldehyde and/or ethanol on neutral amino acid transport systems in microvillous brush border membrane vesicles prepared from human placenta

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Summary. At 20 mM of acetaldehyde, the activities of three transport systems of L-alanine distinguished by the difference in their cation dependence, namely 1) Na^+-specific, 2) Li^+-dependent, and 3) Na^+-independent systems, were significantly reduced in a similar manner. Only the Li^+-dependent system was selectively inhibited at toxic concentrations of acetaldehyde and ethanol.
Key words. L-Alanine; transport system; membrane vesicle; human placenta; acetaldehyde; ethanol.

It has been well documented that the fetal alcohol syndrome (FAS) results from the adverse effects of maternal ethanol consumption on fetal growth and development[1,2]. Principal features of FAS are central-nervous-system dysfunctions, facial characteristics, and growth deficiencies. The pathogenesis of FAS has been currently investigated and reviewed[3-5], considering various factors such as the direct mutagenic effects of ethanol or acetaldehyde, the inhibition of protein synthesis, the alteration of neurotransmitter or hormonal balance, and the inhibition of placental transfer of nutrients. There are several reports that impairment of the placental amino acid transport is one of the causes of the intrauterine growth retardation[6-8].

Although the effects of acetaldehyde or ethanol on amino acid transport have been studied in some organs and cultured cells[9-12], no investigations of direct actions on the transport systems have been reported so far because of possible complications arising from unknown internal compartmentalization and intracellular metabolism. Membrane vesicles provide a simplified system, in which a transport process can be studied under well-defined conditions dissociated from intracellular components[13], and in addition, known magnitudes and polarities of chemical or electrochemical driving forces can be imposed across the membrane[13,14].

In our previous report, it was demonstrated that the Li^+-dependent uptake of L-alanine, presumably by the 'ASC' system, in placental membrane vesicles was selectively inhibited by ethanol[15]. The present communication concerns the direct effects of acetaldehyde and/or ethanol on the neutral amino acid transport systems using 0.1 mM L-alanine and the microvillous brush border membrane vesicles prepared from human placenta.

Materials and methods. L-[^{14}C(U)]-Alanine (174 mCi/mmole) was purchased from New England Nuclear Corp. (Boston, MA); acetaldehyde (99% pure) from Merck (Darmstadt, Germany); ethanol (99.5% pure) from Katayama Chemical Ind. (Osaka, Japan). All other chemicals were of reagent grade. Placentae were obtained at delivery from normal full-term vaginal deliveries. Microvillous brush border membrane vesicles were prepared in mannitol buffer consisting of 0.3 M mannitol and 10 mM Tris-HCl buffer, pH 7.4, by a modification[14] of the method described by Smith et al.[16]. The membrane vesicles were stored at 0 °C until used (within a few days). The experiments on uptake were performed according to the following procedures. Incubations were initiated by adding the membrane vesicles to the incubation medium and carried out for 30 s at 25 °C in a total volume of 100 µl containing 80–100 µg of vesicle protein, 0.1 mM L-[^{14}C(U)]-alanine (10 mCi/mmole), 100 mM NaCl, 100 mM mannitol and 10 mM Tris-HCl buffer, pH 7.4. The addition of acetaldehyde and/or ethanol to the incubation medium was done just before the initiation of uptake in order to avoid evaporation of the substrate(s). In some experiments NaCl was replaced isoosmotically by LiCl or mannitol. Uptake was terminated by the addition of 1 ml ice-cold 10 mM Tris-HCl buffer, pH 7.4, containing 0.8 M NaCl (wash buffer), followed by immediate filtration through a Millipore filter (HAWP; pore size, 0.45 µm). The filter was washed twice 5 ml ice-cold wash buffer. Radioactivity of the dried filter was measured in toluene scintil-