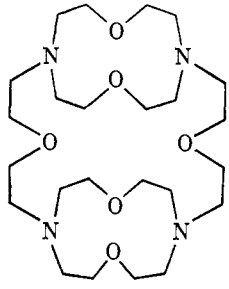
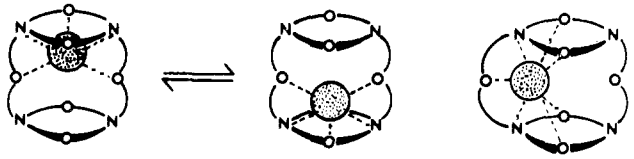
sample. Excess MCl_2 salt does not affect the results described. Finally the trivalent [$La^{3+} \subset$ L] cryptate obtained by addition of $La(NO_3)_3$ gives an eight-line ^{13}C pattern, similar to those observed at +4° with Ca^{2+} (Figure 1) and Sr^{2+}, which remains unchanged at 93°.

Furthermore, in solutions containing about equal amounts of complex and of free ligand, (i) the spectra and coalescence temperatures given by the complexes are unaffected and (ii) the averaged four ^{13}C lines of the [$M^{2+} \subset$ L] complex and the four ^{13}C signals of free L remain sharp and separate even at 99° for M = Sr and Ba; for M = Ca line broadening occurs and coalescence sets in at 99°.

The observations described above may be interpreted as follows. (1) The [$M^{2+} \subset$ L] complexes display an *intramolecular cation exchange* process interconverting two species in which the cation is located unsymmetrically in the molecular cavity. (2) The symmetry-breaking operation converts the D_{2h} symmetry of free L into C_{2v}. The schematic C_{2v} structures **1** and **2** in



L



1 **1′** **2**

which the cation is displaced toward one ring or toward one bridge both agree with the ^{13}C nmr data. However, form **2** looks less favorable on molecular models and the cation location does not correspond to that found in the crystal structure of the [$2Ag^+ \subset$ L] complex[12] which is similar to the location in **1**. Thus, although species **2** may not be rigorously excluded, we favor a structure of type **1** for the [$M^{2+} \subset$ L] cryptates. The cation may complete its coordination shell with one or two anions and/or water molecules. Thus, the spectral changes observed may be attributed to the intramolecular process $\mathbf{1} \rightleftharpoons \mathbf{1}'$. (3) The free energies of activation $\Delta G_c^{\neq}$ for process $\mathbf{1} \rightleftharpoons \mathbf{1}'$ (Table I) decrease with increasing size and decreasing hydration energy of the cations, $Ca^{2+} > Sr^{2+} > Ba^{2+}$. Indeed, the smaller the cation and the higher its surface charge density, the more ligand interaction energy it will loose in the transition state of the $\mathbf{1} \rightleftharpoons \mathbf{1}'$ jump. This is even more so for the La^{3+} complex in which the barrier to intramolecular exchange is higher than 18.5 kcal/mol (Table I). (4) An *intermolecular* cation exchange process is also present, but its rate is much slower and its free energy of activation much higher (>19 kcal/mol) than those of the intramolecular process (see Table I). Cation exchange rates in [2]cryptates are slower the higher the stability of the complex.[13] In the stable [$M^{2+} \subset$ L] cryptates intermolecular cation exchange is indeed slow[14] and the rates follow the sequence $Ca^{2+} > Sr^{2+} \geqslant Ba^{2+}$, *i.e.*, a reverse order with respect to the intramolecular process. This points out the fundamental difference between the two processes: cation jump between binding sites or removal of the cation from its complex. (5) Both intra- and intermolecular cation exchange is fast for the weak complexes of L with alkali cations.

The results described here show that the alkaline earth [3]cryptates of ligand L are "fluxional" type complexes[15] of cations other than transition metal ones. They also indicate that it may be possible to build synthetic molecules which may function as channels for the flow of metal cations.

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(14) [2]Cryptates of Ca^{2+} and Sr^{2+} having log $K_s \sim 7.0$–8.0 show exchange rates and free energies of activation of about 10^{-3}–10^{-4} sec^{-1} and 22 kcal/mol, respectively (at 25° in water).[12b]

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Demonstration of Enzymic Hydrogen Transfer from Substrate to a Flavine[1]

Sir:

Flavoenzymes catalyze the oxidation of $RCH(X)CO_2^-$ (X = OH, NH_3^+) *via* the intermediate formation of reduced flavine. The α-hydrogen removed from substrate during flavine reduction may be either released directly to solvent, transferred to a basic group on the protein,[2] or transferred to flavine itself.[3] We wish to report the first direct evidence for an *enzyme-catalyzed* transfer of substrate hydrogen to a flavine. The direct demonstration of this phenomenon in enzymic reactions, which has until the present not been feasible due to the rapid exchange rates of reduced flavines, has been accomplished by the use of the flavine analog, deazaFMN, in which the N-5 nitrogen is replaced by CH.[4] Bruice and his colleagues have previously used deazaflavines in model reaction studies to demonstrate direct hydrogen transfer between substrate and flavine.[5]

More detailed evidence to be presented in a subsequent communication[6] shows that the flavoprotein *N*-

(1) This work was supported in part by USPHS Grant AM 13443 and Grant I-391 from the Robert A. Welch Foundation.

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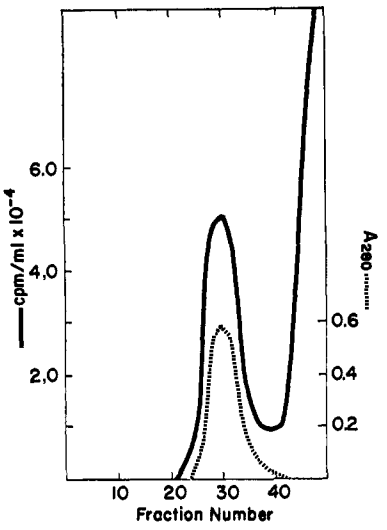
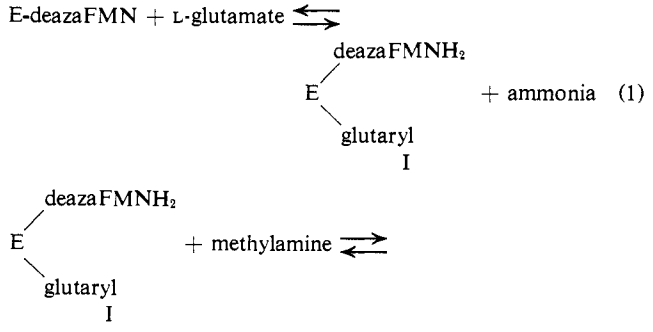


Figure 1. Isolation of deazaFMN glutaryl enzyme intermediate. Enzyme was incubated for 60 min at 10° in 110 m*M* potassium tricine, pH 8.3, 88 m*M* KCl, 1.1 m*M* dithioerythritol, 1 m*M* $MgNa_2$-EDTA, 5 m*M* $MgCl_2$, and 1.1 m*M* [α-^{3}H]glutamate (specific activity = 6.4 × 10^4 cpm/nmol) and then chromatographed at 5° on a Sephadex G-25 column (1 × 57 cm) equilibrated with 10 m*M* sodium pyrophosphate, pH 8.5, 1 m*M* $MgNa_2EDTA$, and 5 m*M* 2-mercaptoethanol. The isolated intermediate contains 4.5 nmol of tritium which corresponds to the incorporation of 0.7 mol of tritium/mole of $deazaFMNH_2$. The $deazaFMNH_2$ formed was determined by the decrease in absorbance at 410 nm ($\epsilon_{deazaFMN} - \epsilon_{deazaFMNH_2} = 9400\ M^{-1}\,cm^{-1}$).

methylglutamate synthetase, containing deazaFMN in place of FMN, catalyzes *N*-methylglutamate formation from glutamate and methylamine *via* the same two-step mechanism previously eludicated for native enzyme,[7] reactions 1 and 2. Reaction 1 and the overall reaction



occur at 1–5% of the rate observed with FMN-reconstituted enzyme. As with native enzyme,[7] the deazaFMN enzyme intermediate (I) can be isolated by Sephadex G-25 chromatography. Incubation of deazaFMN enzyme with [α-^{3}H]glutamate results in the incorporation of tritium into the enzyme, Figure 1. The amount of tritium incorporated is approximately stoichiometric (70–90% yields) with the amount of $deazaFMNH_2$ produced, indicating that deazaFMN is the sole oxidant of the amino acid substrate. No radioactivity is incorporated when the analogous experiment is conducted using apoprotein. In order to identify the radioactive components in the isolated intermediate, denatured enzyme was fractionated to separate glutamate, deazaFMN, and water, as summarized in Figure 2. The total amount of tritium originally present in the

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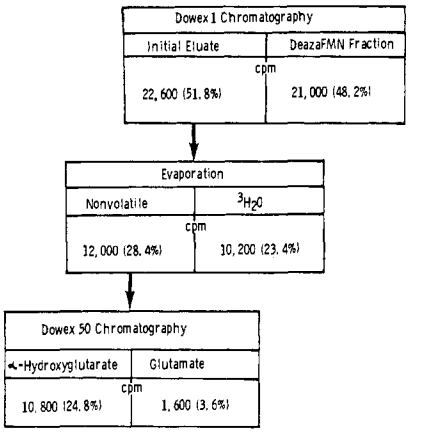

Figure 2. Identification of products released from intermediate by acid denaturation. Intermediate, isolated as described in Figure 1, was denatured with 0.25 *M* formic acid and then chromatographed on a Dowex AG1−X8 (Cl^-, 100–200 mesh) column (0.5 × 3 cm). An initial eluate was collected by washing with 0.5 *M* formic acid and deazaFMN was then eluted with 10.0 *M* formic acid. deazaFMN was identified by paper chromatography [solvent 1 5% $Na_2HPO_4 \cdot 7H_2O$ in water, solvent 2 *tert*-butyl alcohol–6 *N* HCl–water (80:2:20)] and silica gel chromatography [solvent 3 ethyl acetate–formic acid–water (7:2:1)]. deazaFMN and radioactivity were located by fluorescence and fluorography (K. Randerath, *Anal. Biochem.*, **34**, 188 (1970), respectively. The nonvolatile material, remaining after repeated evaporation of the initial eluate from Dowex 1, was chromatographed on a Dowex 50 W–X8 (H^+, 200–400 mesh) column (0.5 × 3 cm). α-Hydroxyglutarate (formation of α-hydroxyglutarate, presumably *via* a side reaction of the catalytically active intermediate, and its identification will be discussed elsewhere[6]) was eluted with water and glutamate was then eluted with 2.0 *M* ammonia. Glutamate, visualized by ninhydrin, was identified by paper (solvent 2) and silica gel (solvent 3) chromatography.

enzyme intermediate as $deazaFMNH_2$ is equal to the amount isolated as deazaFMN plus the amount released as ^3H_2O by oxidation of $deazaFMNH_2$ after denaturation and corresponds to >70% of the radioactivity present in the intermediate. If the intermediate is incubated with methylamine prior to acid denaturation, 85% of the tritium, initially present as $deazaFMNH_2$, is incorporated into *N*-methylglutamate (Table I).

Table I. Reaction of Tritium-Labeled DeazaFMN Enzyme Intermediate (I) with Methylamine

Treatment[a]	Radioactivity incorporated[b] DeazaFMNH₂ (cpm)	Amino acids (cpm)
Unincubated	46,449	
Incubated 3.5 min with 100 mM CH_3NH_2	6,861	39,423[c]
Incubated 3.5 min without CH_3NH_2	40,330	6,184[d]

[a] Intermediate was incubated where indicated at 30° and then denatured with 0.25 *M* formic acid. [b] Values determined by the fractionation procedure of Figure 2. $deazaFMNH_2$ = deazaFMN + ^3H_2O, as described in the text. [c] Identified as *N*-methylglutamate by paper (solvent 2) and silica gel (solvent 3) chromatography. *N*-Methylglutamate was visualized with ninhydrin. [d] The low incorporation of tritium into amino acid, identified as glutamate, is presumably due to trace contamination by ammonia.

These results indicate the following. (1) The isolated reduced deazaFMN enzyme intermediate is catalytically active. (2) $deazaFMNH_2$ functions as the final ac-

ceptor of the α-hydrogen removed from the amino acid substrate. (3) The α substrate hydrogen is most probably attached at C-5 in deazaFMNH$_2$ since the tritium in deazaFMNH$_2$ released from denatured intermediate remains nonexchangeable with solvent and less than 50% is lost during air oxidation. (4) Since deazaFMNH$_2$ has two potential hydrogens at C-5, either of which could be removed during reaction with methylamine, the observed incorporation of 85% of the tritium into *N*-methylglutamate indicates stereospecific enzymic reoxidation of deazaFMNH$_2$. (5) A similar hydrogen transfer appears likely with native enzyme since other aspects of catalysis by FMN and deazaFMN enzymes are similar even though the relative reaction rates are different.

Although the data clearly establish that the α hydrogen of the substrate is transferred to flavine, whether the reaction proceeds *via* hydride transfer or *via* a shielded proton transfer,[1b] cannot be presently determined. The data do show, however, that a proton transfer mechanism as proposed by Brown and Hamilton[8] is inapplicable since this mechanism requires hydrogen transfer to the 5 position of flavine prior to cleavage of the α-carbon–hydrogen bond of the substrate.[9]

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(10) Recipient of Research Career Development Award GM70239 from The National Institute of General Medical Science.

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Structure and Conformation of 4-Peroxycyclophosphamide. A Cytotoxic Oxidation Product of Cyclophosphamide

Sir:

Cyclophosphamide (2-bis(2-chloroethyl)aminotetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide) is used extensively as an antitumor agent.[1,2] The drug has little cytotoxic activity until it is activated in the liver by a mixed function oxidase of liver microsomes.[3,4] Several laboratories have been interested in isolating and synthesizing the active metabolites of cyclophosphamide.[3,5–8] There seems to be some ambiguity as to the identification of a biologically active Fenton oxidation product of the drug. Initially van der Steen, *et al.*,[5] identified this compound as *N*-hydroxycyclophosphamide on the basis of elemental analysis, ir and nmr spectroscopic data, and chemical reactivity of the compound with H_2O_2. However, these investigators, after comparing their ^{13}C nmr data with those of Struck and coworkers concluded that the compound was 4-hydroxycyclophosphamide.[5] In contrast, Struck, *et al.*,[9] presented evidence that this oxidation product is 4-peroxycyclophosphamide. To resolve this ambiguity, we determined the crystal structure of the compound.

Clear crystals, obtained from ethyl ether, were kindly provided by Dr. R. F. Struck.[10] The crystals are monoclinic, space group $P2_1/c$ with four $C_{14}H_{28}Cl_4N_4O_6P_2$ formula units per unit cell. Three-dimensional intensity data, which included 3986 independent reflections, were collected on an automated diffractometer by use of nickel-filtered copper radiation, a scintillation detector, and a θ–2θ scan technique. Three strong, medium-angle reflections (032, 025, 300), chosen as standards, were monitored periodically. Throughout data collection, the continuous and rapid decrease in the intensities of the standard reflections indicated that crystal decomposition was occurring. The three standards decreased at markedly different rates. When any one of the standards lost 33% of its initial intensity, a new crystal was mounted and data collection was continued. Seven different crystals were used to obtain a complete data set. The final crystal was used slightly beyond the 33% decay limit (37% decay for reflection 032, 38% for reflection 025, and 47% for reflection 300). Cell parameters for each crystal were determined by a least-squares analysis of the angular settings for six reflections. Among the seven independent determinations, the maximum variation in cell parameters was about 0.1%. Weighted-average values for the cell parameters are a = 11.206 (2) Å, b = 11.807 (2) Å, c = 19.202 (5) Å, and β = 103.94 (1)°. Intensity values were scaled by a least-squares procedure[11] in which the intensities of the standard reflections were used to calculate scale factors as a function of crystal exposure time.

A suitable trial structure was obtained by direct methods, with the use of the computer program MULTAN.[12] The structure was refined by least squares. Coordinates for hydrogen atoms were calculated by assuming tetrahedral coordination around the carbon atoms, trigonal coordination around the nitrogen atoms, and C–H and N–H bond distances of 1.0 Å. The hydrogen atoms were assigned the isotropic temperature factors of the heavy atoms to which they were bonded and were included in structure factor calculations but not in the least-squares refinement. As the refinement proceeded, new positions for the hydrogen atoms were calculated periodically. Initially refinement was terminated at an R index $(\Sigma||F_o| - |F_c||/\Sigma|F_o|)$ of 0.165 and a goodness-of-fit $([\Sigma w(F_o^2 - F_c^2)^2/(m - s)]^{1/2}$, where w is the weight given each reflection, m is the number of reflections used, and s is the number of parameters refined) of 4.49. At this stage, a difference Fourier map showed a residual peak of 1.9 e/Å^3 at a distance of 2.5 Å from atom C(10′), 3.2 Å from atom

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