The Adsorption of Diphosphopyridine Nucleotide and Some of Its Model Compounds at a Mercury–Electrolyte Interface*

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ABSTRACT: Diphosphopyridine nucleotide and three model compounds, containing, respectively, methyl, propyl, and benzyl groups in the 1 position of 3-carbamidopyridinium chloride, were examined by current reversal chronopotentiometry for adsorption on mercury from solutions buffered at pH 8. In all cases the data indicated strong adsorption of the dimeric, one-electron, reduction product. No evidence for the adsorption of the methyl reactant was found; however the propyl and benzyl reactants were so strongly adsorbed as to be more easily reduced than their freely diffusing counterpart.

Evidence is presented for the preferential adsorption of the propyl and benzyl dimers. This product film blocks the electron-transfer process in the case of the propyl but not the benzyl compound. Adsorption of the diphosphopyridine nucleotide reactant is not strong enough to produce a potential pause due to its separate reduction. However, using the results of the model compounds it is assumed an "adsorbed reduced followed by a soluble reduced" model applies in the case of diphosphopyridine nucleotide (DPN) as well. From the appropriate relationship the surface excess of diphosphopyridine nucleotide is calculated and conformations on the mercury surface and in solution are discussed.

Recently, a detailed study of the polarographic behavior of diphosphopyridine nucleotide (DPN)[1] was completed (Burnett and Underwood, 1965a). Using the appropriate experimental conditions, these workers showed that the behavior of DPN is polarographically similar to its model compound, 1-methyl-3-carbamidopyridinium chloride (MCPCl) (Burnett and Underwood, 1965a,b). A thorough investigation of the cyclic voltammetric behavior of these compounds has also been completed (A. J. Cunningham and A. L. Underwood, private communication, 1965). The present work was undertaken because it was felt the controlled current studies, *i.e.*, chronopotentiometry, could provide information not available to the controlled potential studies.

Since the present paper will deal exclusively with the first, one-electron reduction, only the pertinent information concerning this reduction will be reviewed here. Additional information, as well as a good review of previous electrochemical work on these compounds, is available in Burnett and Underwood's (1965a,b) original publications. The first polarographic wave observed for DPN corresponds to a one-electron reduction forming a free radical which subsequently undergoes dimerization to form the 4,4′ dimer. This first wave has an $E_{1/2}$ of about -0.93 v *vs.* SCE and is pH independent in the range 4–9. This wave appeared reversible as determined from plots of potential *vs.* log $[(i_d - i)/i]$, and was diffusion controlled as determined by a study of the wave height dependence on the corrected mercury heights. However, when the concentration of the electroactive species was lowered below 10^{-4} M, there appeared a small, concentration-independent prewave at approximately -0.90 v *vs.* SCE. These workers recognized that the usual interpretation of such polarographic prewaves involves adsorption on the mercury surface.

The theory and practice of the chronopotentiometric method for diffusion-controlled mass transport is adequately discussed in Delahay's (1954) monograph. Although a discussion of kinetic complications of mass transport are given, adsorption complications are not.

If an electroactive species is adsorbed on an electrode surface, one might expect the transfer of electrons to be facilitated. Thus, if the electron exchange were reversible, one would expect the adsorbed species to be reduced at more positive potentials than its soluble, *i.e.*, nonadsorbed, counterpart. In systems of interest, significant potential differences are rarely observed. For this reason Laitinen and Chambers (1964) and, independently, Tatwawadi and Bard (1964) have shown, in principle, that it is possible to distinguish three adsorption–reduction orders from the analysis

* From the Department of Chemistry, Emory University, Atlanta, Georgia 30322. *Received April 4, 1966.* This investigation was supported by Public Health Service Research Grant No. GM08282 from the Division of General Medical Sciences, National Institutes of Health. This paper is based in part upon a dissertation submitted by Donald G. Epple in partial fulfillment of the requirements for the Ph.D. degree, Emory University.

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[1] Abbreviations used: DPN, diphosphopyridine nucleotide; MCPCl, 1-methyl-3-carbamidopyridinium chloride; PCPCl, 1-propyl-3-carbamidopyridinium chloride; BCPCl, 1-benzyl-3-carbamidopyridinium chloride; SCE, saturated calomel electrode; HMDE, hanging mercury drop electrode.

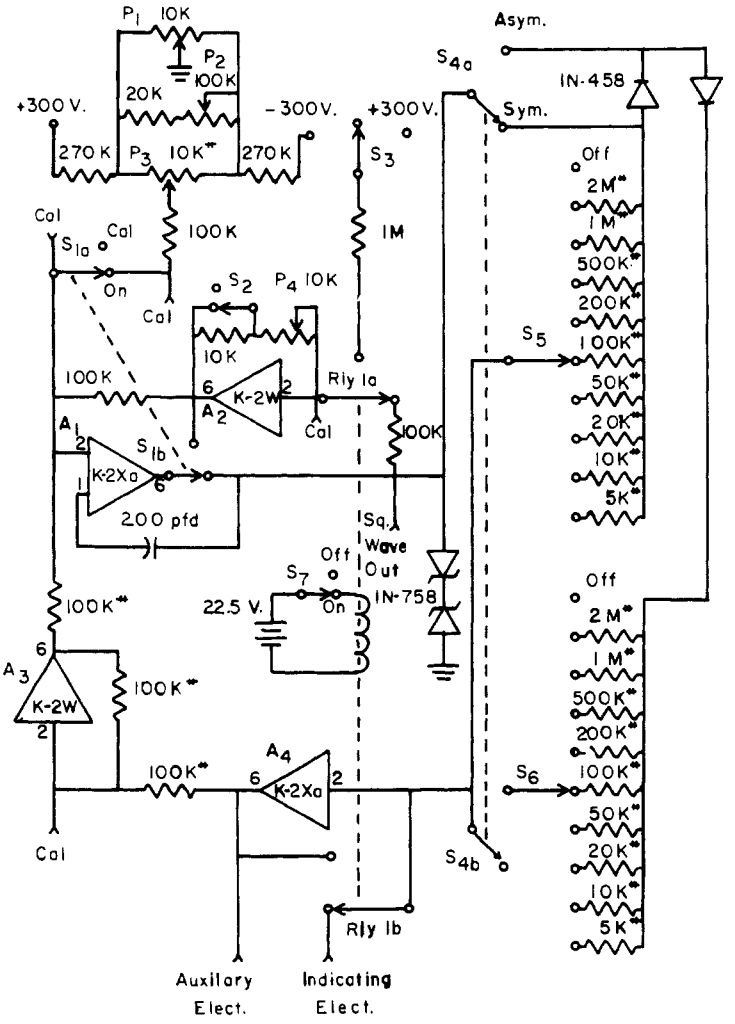

FIGURE 1. Cyclical scan—constant current supply All components ±10% of values except those marked with++. See Experimental Section for discussion of operation and source of components.

of experimental transition times, τ, and current densities, i_0. However, they found, in practice, that their systems gave straight-line fits to all three relationships. If one is to decide whether the adsorbed species reduces before, AR,SR model, simultaneously, AR=SR model, or after, SR,AR model, the soluble species, one needs some independent experimental evidence. Two of the model compounds produced chronopotentiograms with adsorption controlled potential pauses preceding the reduction of the soluble species. On this basis we have analyzed the DPN data with the relationship for the AR,SR model, *viz.*

$$i_0\tau = \frac{n^2F^2\pi^2DC^2}{4}\frac{1}{i_0} + nF\Gamma \qquad (1)$$

where Γ is the surface excess and all other terms have their usual electrochemical significances.

Rapid reversal of the polarity of the electrolysis current allows one to examine the electrode surface for products of the primary electrochemical step. Product removal from the surface by a diffusion mechanism alone results in a ratio of the reverse transition time to the forward transition time, $\tau_{\text{oxidation}}/\tau_{\text{reduction}}$, of 0.333. Testa and Reinmuth (1960) have shown the decrease of this ratio from 0.333 is indicative of a kinetic removal of product. If the product is strongly adsorbed or deposited on the electrode surface, $\tau_{\text{oxidation}}/\tau_{\text{reduction}}$ approaches the limiting value of 1.0 (Herman *et al.*, 1963; Osteryoung, 1963).

Experimental Section

Apparatus. A schematic drawing of the constant current source is given in Figure 1. This circuit is basically a modification of the triangular wave voltage generator of Weir and Enke (1964) to provide current

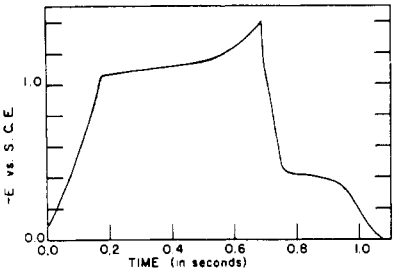

FIGURE 2: Chronopotentiogram of 1-methyl-3-carbamidopyridinium chloride. MCPCl concentration, 0.522 mM, pH 8.0, i_0 = 0.250 ma/cm².

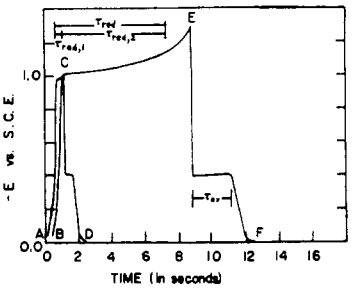

FIGURE 3: Chronopotentiograms of 1-propyl-3-carbamidopyridinium chloride. PCPCl concentration, 1.86 mM, pH 8.0, i_0 = 0.148 ma/cm².

control instead of voltage control and is similar to one described by Herman and Bard (1965). Amplifiers 1 and 2 operate as a potential sensing flip-flop switch, amplifier 4 controls current to preselected levels, and amplifier 3 inverts the signal so that control is maintained. The model designations on the amplifiers are those of G. A. Philbrick Researchers, Inc., Dedham, Mass. The power supply was a Philbrick Model RB-100-B. A previously described potentiostat was used for controlled potential reductions at a large mercury pool (Burnett and Underwood, 1965a).

Potential time curves were measured with a Tektronix Model 564 oscilloscope using a type 2A 63 differential preamplifier in the *Y*-axis input and a type 2B67 time base in the *X*-axis input. A Tektronix C-12 camera equipped with a Polaroid back was used to record potential time curves on type 47-3000 ASA-Polaroid film.

The electrolysis cell was a jacketed beaker of 50-ml capacity with a tungsten contact in a well at the bottom. A mercury pool in this well served as the auxiliary electrode. A rubber stopper was drilled to accommodate a hanging mercury drop electrode (HMDE, the indicating electrode), a dropping mercury electrode, the SCE, a transfer spoon, and a nitrogen inlet. The HMDE was made by imbedding a platinum wire of 0.021-in. diameter in a 7-mm o.d. soft glass tube drawn to a fine point to minimize shielding. Microscopic inspection revealed no cracks about the wire in the finished electrode.

A Leeds and Northrup Model 7401 pH meter was used for pH adjustments. Temperature control to 25.0 ± 0.1° was provided by a Haake Model F thermostat. Framework Molecular Models manufactured and distributed by Prentice-Hall, Inc., Englewood Cliffs, N. J., were used to construct molecular models for theoretical surface coverage calculations.

Chemicals. All buffer solutions were made from reagent grade materials. A pH 8.0 ± 0.1 Tris–hydrochloric acid buffer of ionic strength 0.5 was used in all studies, except where a pH 8.0 ± 0.1 phosphate buffer of μ = 0.27 is noted.

The DPN (coenzyme 1) was obtained from P-L Biochemicals, Inc., Milwaukee, Wis. (formerly Pabst Laboratories), and was used as obtained from this source. The 1-alkyl- (or aryl-) 3-carbamidopyridinium chloride salts were prepared using a slight modification of the procedure used by Burnett and Underwood (1965a). The iodide salts were prepared using the procedure of Karrer *et al.* (1936). The iodide was exchanged for chloride by adding freshly precipitated silver chloride to a saturated solution of the quaternary iodide salt, filtering off the silver iodide precipitate, and precipitating the quaternary chloride salt from the mother liquor with ethanol and ether.

Procedure. Since adsorption equilibrium was desired the fresh mercury drop for each experiment was allowed to equilibrate for a minimum of 10 min in a quiescent solution before a current was applied, except where noted. The surface area of the mercury drop was calculated from the measured mass of individual mercury drops and the spherical geometry. Reported current densities were calculated accordingly. Current densities were selected which permitted transition times to range from about 1 sec to 1 msec. Duplication of results was always verified using the storage mode of the oscilloscope. All transition times were estimated using the $\tau/4$ technique of Delahay and Berzins (Delahay, 1954). All potentials are reported *vs.* the SCE.

Results

Chronopotentiograms for MCPCl, PCPCl, BCPCl, and DPN are shown in Figures 2–5, respectively. The transition times shown in Figures 3 and 4 are atypically long, since these composite curves represent special experiments. Curve ACEF represents the usual reduction process followed by a current reversal at the preset potential on a fresh, equilibrated mercury surface. Curve ACD shows the current reversal being initiated at the transition time of the first reduction process, $\tau_{\text{reduction},1}$. Curve BCEF represents a reduction carried out after a previous reduction had been stopped at point E. Curves similar to BCEF were obtained if large scale potentiostatic reductions of PCPCl and BCPCl were carried out at −1.1 v until one-half the starting material was reduced to dimer.

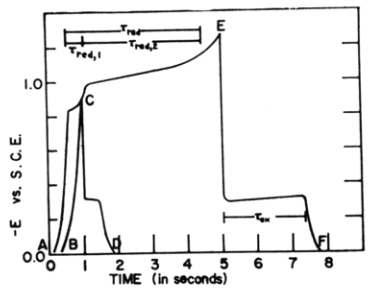

FIGURE 4: Chronopotentiograms of 1-benzyl-3-carbamidopyridinium chloride. BCPCl concentration, 1.12 mM, pH 8.0, $i_0 = 0.125$ ma/cm².

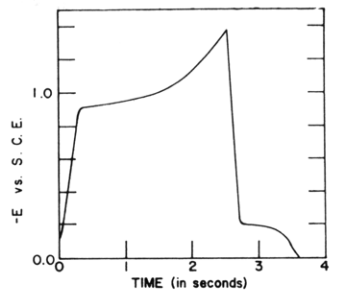

FIGURE 5: Chronopotentiogram of DPN. DPN concentration, 0.999 mM, pH 8.0, $i_0 = 0.125$ ma/cm².

Dimerization of the DPN free radical product is very rapid. In order to demonstrate the presence of the free radical an anodic current density one-tenth of the cathodic current density was used. Since a given number of free radicals will give rise to a $\tau_{oxidation}$ with a given $i_{0,\,anodic}$, using $0.1 \times i_{0,\,anodic}$ will result in a transition time $100 \times \tau_{oxidation}$. This asymmetric mode of operation has in effect made the reoxidation process more sensitive. The results are shown in Figure 6.

Typical chronopotentiometric constants for the total reduction process are plotted in Figure 7 as a function of current density for MCPCl, PCPCl, BCPCl, and DPN. Analysis of $i_0\tau^{1/2}_{reduction}$ *vs.* $1/i_0$ data for DPN in two buffer systems as a function of concentration of DPN yields the surface excess and diffusion coefficient data shown in Table I.

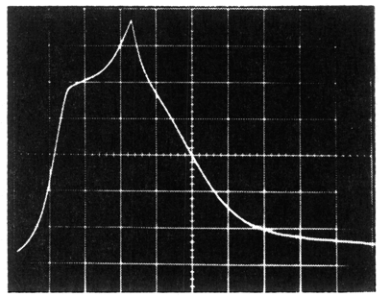

FIGURE 6: Chronopotentiogram of DPN, asymmetric current. DPN concentration, 1.04 mM, pH 8.0, $i_{0,\,cathodic} = 1.25$ ma/cm², $i_{0,anodic} = 0.125$ ma/cm². Vertical axis: 0.20 v/division, midscale = −0.60 v *vs.* SCE. Horizontal axis: 20 msec/division.

TABLE I: Surface Excess of DPN as a Function of Concentration and Buffer.

Buffer	Concn (mM)	Γ (moles/cm² × 10^{10})	*D* (cm²/sec × 10^6)
Tris–HCl (μ = 0.50, pH 8.0)	0.509	0.65	3.3
	0.514	0.49	2.6
	0.754	0.88	2.9
	0.757	0.81	3.6
	0.999	0.73	2.7
	1.013	0.88	3.0
			Av 3.0
Phosphate (μ = 0.27, pH 8.0)	0.506	0.71	5.4
	0.540	0.90	5.5
	0.763	0.86	4.9
	0.956	1.30	4.4
	1.096	1.86	4.6
	1.523	1.78	4.2
			Av 4.8

Discussion

As has been shown before the free radical product dimerizes in the case of all four compounds. The quarter-time potentials of −0.4 and −0.2 v are in good agreement with the reported half-wave potentials for the reoxidation of the 1-methyl-3-carbamidopyridine dimer and the 4,4′ dimer of DPN, respectively (Burnett and Underwood, 1965a,b). Although Figure 6 indicates the free radical has a finite half-life time, we estimate it to be no more than 1 msec.

The ratio $\tau_{oxidation}/\tau_{reduction}$ was determined as a function of current density for all four compounds. In all cases it was found to approach unity at high current densities indicating strong adsorption of the dimeric products. In the case of PCPCl and BCPCl the ratio $\tau_{oxidation}/\tau_{reduction}$ was always unity when the reversal of current was applied at a potential positive enough to prohibit reduction of the soluble species, *i.e.*, point C. This indicates desorption is quite slow. The dimer is also more strongly adsorbed on the surface than is the monomeric oxidized forms of PCPCl and BCPCl. This is proved by the absence of the adsorption potential pause after large-scale reductions convert half the starting material to dimer, and also by the nonequilibrium experiment, in which the dimer product of a previous

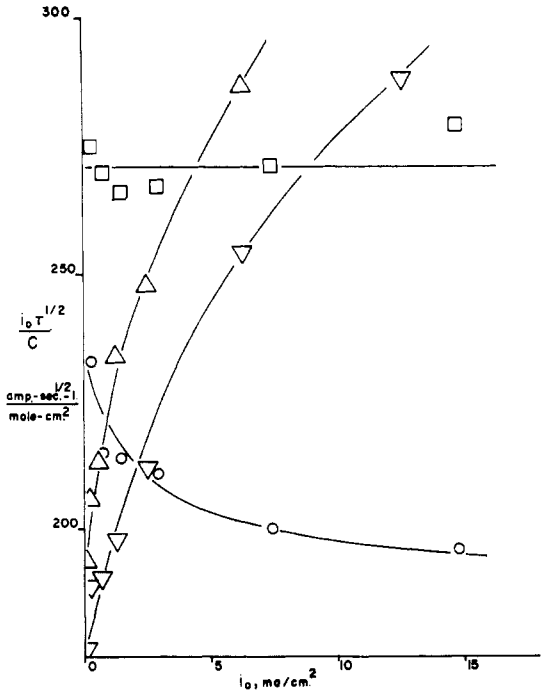

FIGURE 7: Chronopotentiometric constants as a function of current density for: □, 3.00 mM MCPCl; △, 0.913 mM BCPCl; ▽, 1.523 mM DPN; ○, 5.12 mM PCPCl.

electrolysis quenched the adsorption pause of a subsequent reduction, *i.e.*, curve BCEF of Figures 3 and 4.

Although the dimeric product of MCPCl is strongly adsorbed, MCPCl itself is not. The chronopotentiometric constant for six concentration values in the range 0.522–3.00 mM was found to be 271 ± 4 amp $sec^{1/2}$ l./cm^2 mole. The trends in $i_0\tau^{1/2}_{reduction}/C$ as i_0 increases indicates BCPCl and DPN are subject to partial adsorption control whereas PCPCl is kinetically controlled. The values of $i_0\tau_{reduction,1}$ are found to be 52 ± 20 and 42 ± 4 μa sec for the propyl and benzyl compounds, respectively. The constancy of this value indicates an adsorbed species is being reduced in this first potential pause. The over-all kinetic control of the reduction of PCPCl appears at first anomolous. However, Kodoma and Murray (1965) have observed similar kinetic interference to the reduction of copper(II) tartrate complex by adsorbed brucine. In fview o the strong adsorption of the dimeric product, it is possible to picture two models for such an inhibition of electron transfer to the soluble species. First of all the electron may have to "tunnel" through this resistive film of propyl dimer. On the other hand if it was mandatory for the oxidized soluble species to make the closest approach to the mercury surface in order to acquire the electron, then quite possibly the dimer must desorb. We have already noted how slow this desorption process is. The question now arises as to how the benzyl compound avoids this obligatory kinetic step. Apparently the π-electron cloud of the benzyl rings of the adsorbed dimer act as a good conductor of electrons to the soluble oxidized species outside this product film. Thus the electron transfer is not inhibited by the surface film of benzyl dimer.

A comparison of the quarter-time potentials for the reducible species of the four compounds is instructive. For MCPCl $E_{\tau/4} = -1.10$ v. In the case of PCPCl and BCPCl the soluble species are reduced at −1.05 v. However the adsorbed species are reduced at −1.00 and −0.85 v, respectively. Thus although DPN does not exhibit two separate reduction processes, its quarter-time potential of −0.95 v indicates a strong shift in reduction potential due to the reduction of the adsorbed species first, followed by the soluble species.

The DPN data were analyzed according to all three adsorption models. All three gave reasonably consistent values for the diffusion coefficient. However, the AR=SR and SR,AR models, in addition to giving data two to six times smaller than for the AR,SR model, gave surface excess values with decreasing trends as the concentration of DPN increased. This in itself would seem to negate the validity of these models for the order of DPN reduction and only data determined from relation 1 are shown in Table I. The surface excesses and the diffusion coefficients are very much dependent upon the nature of the buffer medium. Using molecular models, surface excesses were calculated for two extreme molecular conformations. The "linear" conformation assumes both the adenine and the carbamidopyridine ring are coplanar with the mercury surface, the ribose and pyrophosphate linkage adjusted for minimum strain. The "folded" conformation places the adenine ring above and coplanar with the carbamidopyridine ring which in turn is coplanar with the surface. Using planimeter and gravimetric techniques to estimate the surface coverage we find the folded configuration to yield a surface excess of 2.5×10^{-10} mole/cm^2 while the linear conformation permits only 0.9×10^{-10} mole/cm^2. It would appear that DPN is linear in the Tris buffer and folded in the phosphate buffer. This prediction assumes the values of Γ are at saturation with monolayer coverage at the highest concentrations reported. Unfortunately studies at higher concentrations are useless because the coulombic contribution from the adsorption process becomes too small compared to the contribution due to diffusion; *cf.* relation 1. However the relative magnitudes of the diffusion coefficients are consistent with our picture of a floppy linear molecule in Tris buffer and a folded more compact molecule in phosphate buffer.

Acknowledgment

The authors wish to thank A. L. Underwood for stimulating discussions.

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Spectral Properties of Schiff Bases of Amino Acid Esters with Pyridoxal and Pyridoxal *N*-Methochloride in Ethanol*

LaVerne Schirch and R. Arden Slotter

ABSTRACT: The reaction of amino acid esters with pyridoxal and pyridoxal *N*-methochloride in ethanol has been studied. The spectra of the imines formed with glycine butyl ester were found to be similar to those formed with amino acids in aqueous solutions. However, a compound was formed between diethyl aminomalonate and pyridoxal *N*-methochloride that did not behave as a typical imine.

Some of the differences which were observed are noted as follows. The compound exhibited an absorption peak at 480 mμ with an ε of at least 40,000. Upon the addition of an excess of a strong base the 480-mμ peak did not shift to a shorter wavelength. The compound disappeared in a few hours at room temperature. Sodium methoxide accelerated both the formation and disappearance of the compound. The possible relation of the data to the mechanism of pyridoxal catalysis was discussed.

The study of the interaction of pyridoxal with amino acids in nonenzymatic systems has been helpful in understanding the role of this coenzyme in enzymatic reactions. However, there is at least one notable difference between the enzymatic and nonenzymatic systems. Several B_6 enzymes have been found to form enzyme–substrate complexes which absorb near 495 mμ (Jenkins, 1961a,b; Schirch and Mason, 1963; Marino and Snell, 1965). Compounds formed between pyridoxal and amino acids in nonenzymatic systems have not been found to absorb at wavelengths in this region of the spectrum. The evidence available from the enzymatic studies indicates that the enzyme–substrate complexes which absorb near 495 mμ are due to a compound in which the α carbon of the amino acid has lost a proton (Schirch and Jenkins, 1964). This structure was postulated by Metzler *et al.* (1954) to be the key intermediate in nonenzymatic transamination. Since the intermediate is a carbanion, one would expect to observe its existence in nonenzymatic systems only in very weakly acidic solvents. Little work, however, has been done with nonenzymatic systems in solvents other than water. Matsuo (1957) reported the spectrum of several Schiff bases of pyridoxal and amino acids in ethanol and showed that transamination occurred at room temperature and in the absence of metal ions. The lack of additional data in nonpolar solvents suggests that this is a fruitful area for further investigation. The purpose of this paper is to study the spectral properties of Schiff bases of amino acid esters with pyridoxal and pyridoxal *N*-methochloride in absolute ethanol. A compound with several unique spectral properties was found to be formed between the amino acid ester diethyl aminomalonate and pyridoxal *N*-methochloride. The possible significance of this compound is discussed.

Experimental Section

Materials. Pyridoxal hydrochloride was purchased from the Sigma Chemical Co. Diethyl aminomalonate was synthesized by the method of Hartung *et al.* (1960) and purchased from the Aldrich Chemical Co. Glycine butyl ester hydrochloride was obtained from Nutritional Biochemicals Corp. and glycine ethyl ester

* From the Department of Chemistry, Bluffton College, Bluffton, Ohio. *Received March 21, 1966.* This investigation was supported by a U. S. Public Health Service grant (GM 11429).