Effect of a Nonionic Surface-Active Polymer on Passage of Hydrocortisone Across Rat Intestine *In Vitro*

By WITOLD SASKI

Employing the Crane and Wilson everted sac technique, the passage of hydrocortisone cortisol—in micellar solutions of oxyethylated tertiary octylphenol polymethylene polymer (tyloxapol)—from the mucosal to the serosal side of the rat intestinal membrane was studied. It was determined that the mechanism by which cortisol passes across the membrane is that of diffusion. However, it was found that the relative rate of passage of cortisol dissolved in micellar solutions of tyloxapol is a function of concentration of the solubilizing agent used and is inversely proportional to the concentration of tyloxapol and viscosity of the solution tested.

THE SCIENCE of biopharmaceutics has been well advanced in recent years and excellent reviews on the subject have been published (1–5). However, the part surface-active agents may play in the passage of drugs across biological membranes is far from being understood at the present time. The use of surfactants in the formulation of solubilized systems in pharmacy is rather common, and their effect upon the absorption and biological activity of drugs is of paramount importance.

Originally described by McBain (6), solubilized systems are those in which the solubility of materials otherwise insoluble, or only poorly soluble, in a given medium is increased due to the prior presence of particles of colloidal dimensions, termed micelles. Solubilized systems in pharmacy were reviewed by Swarbrick (7) and Mulley (8). Reports concerning the effects of surface-active agents on drug absorption have been conflicting. As Levy (1) has indicated, enhancement as well as inhibition of the gastrointestinal absorption and the pharmacologic activity of drugs has been observed when amphiphilic compounds were added to a medication. These varied effects may have been due to such factors as the possible interaction with the drug and modification of the physical properties of the dosage form, or possible effects on the absorbing membrane. Recently, the effect of various concentrations of the nonionic surfactant, polysorbate 80,[1] on the absorption of a number of alcohols and barbiturates by goldfish has been studied (9). The absorption rate of the barbiturates was increased significantly in the presence of low concentrations of polysorbate 80, and decreased by higher concentrations of the surfactant. The absorption rate of the alcohols studied was not significantly affected by the surfactant. The retardation of barbiturate absorption at higher polysorbate 80 concentration was interpreted as being indicative of the absence of a dissociating effect of the biologic membrane on the drug–micelle complex. By kinetic analysis, the authors have shown that the modification of barbiturate absorption by polysorbate 80 represents the net effect of enhanced absorption and decreased thermodynamic activity of the drug due to micellar complexation. Kakemi *et al.* (10) reported a decrease of the rectal absorption of sulfonamides by addition of various nonionic surface-active agents, polysorbate 80 included. These workers ascribed the decrease to an entrapment of the drug in micelles. However, contrary to the notion that the only form in which the drug passes the membrane is free drug and not drug in micelle (11), the authors found that the drug in micelle was also absorbed to some extent, and therefore, an overall enhancement of drug absorption was obtained by solubilization. They presented a new model illustrating the proposed mechanism, and developed a formula mathematically expressing the absorption rate of the solubilized drug.

Steroids have been reported to be absorbed by diffusion (12), the more lipoidal the compounds, the greater the absorption rate; the more polar and water-soluble the compound, the less absorption rate. However, there has been no information available as to the effect of amphiphilic, preferably nonionic, compounds on the absorption of steroids. Kavanau (13) suggested that the amphiphilic agents may exert some striking effects by their penetration into the lipid phase of membranes with a consequent alteration of the lipid phase state, the membrane configuration, the rates of transformation of the membranes, and the ability of the membranes to transform between

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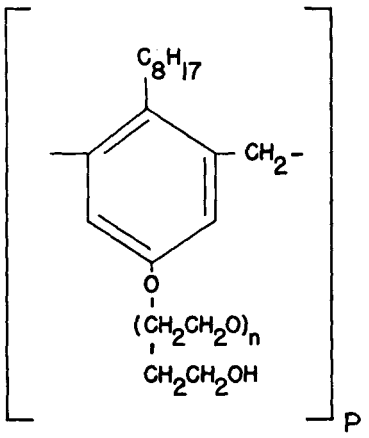
[1] Trademarked as Tween 80, Atlas Chemical Industries, Wilmington, Del.



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different configurations. The penetration of amphiphiles which loosen the liquid-condensed structure of the "bimolecular" disks probably would tend to lead to an increase in the pore size. In some cases, concluded Kavanau, these amphiphiles would tend to promote a localized, or perhaps even an extensive, transformation of membranes to the open configuration and a decreased stability of the lipid–lipoprotein–protein complex. Such compounds, of course, would tend to increase permeability.

The present author set out to investigate the effects a nonionic solubilizer would have on the absorption of poorly soluble corticosteroids. Cortisol (hydrocortisone) was chosen as a representative of the group and, as a solubilizing agent, an oxyethylated tertiary octylphenol polymethylene polymer,[2] better known under its generic name of tyloxapol (14). Tyloxapol was selected as the agent of choice on the basis of its record as practically nonhemolytic (15) and otherwise innocuous to the biologic membranes. It is used as a solubilizer in a sterile ophthalmic solution[3] of cortisol (2 mg./ml.) and in other pharmaceutical preparations. The structure of tyloxapol is shown in I: where $n \simeq 10$ and $p \simeq 4$, and the average molecular weight ($n = 10$, $p = 4$) is 2,812 (16).

The Crane and Wilson (17) everted sac technique seemed well suited for the investigation. Although this method does not give an estimate of the rate of absorption *in vivo*, it can provide useful information about the kinetics of the absorptive process, in addition to showing whether it is active or passive. Several established criteria (18) for adequate characterization of the mode of passage of substances across the biological membrane have been observed in this study. They were, the concentration-effect curve, the time-effect curve, the capacity of the system to transfer both with and against a concentration gradient, experimental conditions such as kind and quantity of added substances, surface tension, viscosity, pH, ionic strength, and verification of the viability or biological integrity of the membrane under the conditions of the study of transfer.

[2] Trademarked originally as Triton A-20, later renamed Triton WR-1339, Winthrop Laboratories, New York, N. Y.
[3] Trademarked as Optef, The Upjohn Co., Kalamazoo, Mich.

EXPERIMENTAL

Apparatus—Eberbach water bath shaker, Beckman DB spectrophotometer with Sargent SR recorder, Central Scientific Co. du Nouy 70545 tensiometer, Federal Pacific Electric Co. Wilhelmy plate tensiometer, Ostwald viscometer, Beckman Zeromatic pH meter, Burrell wrist-action model DD shaker, thermostatically controlled incubator, Crane and Wilson apparatus.

Materials and Reagents—Cortisol (Nutritional Biochemicals Corp., dried at 70° for 24 hr.); tyloxapol (obtained from Ruger Chemical Co., Inc.); triphenyltetrazolium Cl (TTC, Nutritional Biochemicals Corp.), freshly prepared 0.5% solution in aldehyde-free dehydrated ethanol; tetramethylammonium hydroxide 10% solution (Eastman Kodak), freshly diluted 1:10 with aldehyde-free dehydrated ethanol; 1,2-dichloroethane, purified; sodium chloride, reagent grade (Fisher Scientific Co.); benzopurpurin 4B, biological stain grade (Matheson Coleman and Bell).

Preparation of Solutions—Predetermined amounts of cortisol were dissolved in the aqueous solutions of tyloxapol at concentrations of this polymer varying from 0 to 10% w/v. Each solution was made isotonic with the body fluids by the addition of sodium chloride. The solutions were freshly prepared for each experiment using the Eberbach water bath shaker at 37°. The solubility of cortisol in aqueous solutions of tyloxapol has been reported previously (19).

Intestinal Absorption Study—Everted sac technique of Crane and Wilson (17) was used and further perfected in a few important details. Female Sprague-Dawley rats weighing about 250 Gm. were fasted for 24 hr. but had access to drinking water at all times. The animal was killed by a blow on the head and the abdomen cut open by a midline incision, care being taken not to damage the small intestine. The intestine was completely cut across in the region of the upper duodenum and at the ileocecal junction. A 20-ml. syringe cannula was then inserted into the upper end of the intestine and the lumen cleared of its contents by allowing oxygenated saline at room temperature to pass through under moderate pressure. The washed out length of intestine was removed from the animal by carefully stripping off the mesentery at the line of its attachment to the intestine. For the eversion of the intestine, a glass rod of an appropriate length and diameter with a slightly flattened end was used. The intestine was then sleeved onto the glass rod and everted. A tie made with thread at the end of the intestine segment helps the process considerably. Upon completion of eversion a loop was made of the distal end of the sac, and an additional tie using catgut was applied beneath the loop. These precautionary measures practically

exclude the possibility of a leakage. Two segments of approximately 13 cm. length each were obtained from the upper intestinal tract of each animal. Proximal ends of the sacs were attached to the cannula of the Crane and Wilson apparatus.

The sacs were filled with 1–2 ml. of oxygenated saline termed "serosal fluid." Each sac was suspended in 45 ml. of cortisol in saline or cortisol in tyloxapol–saline solution ("mucosal fluid") maintained at 37° and aerated continuously with a mixture of 95% oxygen and 5% carbon dioxide. At indicated times, the total serosal fluid was withdrawn by means of a syringe with a polyethylene cannula attached and the sac rinsed once with about 2 ml. of saline. A fresh 1–2-ml. portion of saline was then placed in the sac. Both serosal and mucosal fluids were assayed each time. Retention within the intestinal wall was low.

The object of everting the sac is twofold. Eversion, with slight distension of the sac, stretches the mucosa and improves its oxygenation. The small volume of fluid inside the sac causes passage of any solute from the mucosal to the serosal side producing a relatively large increase in its concentration in the serosal fluid. This facilitates the detection of transport against the concentration gradient (20).

Analytical Methods—The withdrawn serosal fluid and washing were combined in a separator, boric acid–sodium hydroxide buffer, 0.5 ml. per 5 ml. of solution was added (12), and the fluid extracted twice with 20 volumes of ethylene dichloride, using the Burrell wrist-action shaker for 5 min. each time. The extract was evaporated to dryness in a Petri dish in a stream of hot air. The residue was dissolved in aldehyde-free dehydrated ethanol, transferred to a 10-ml. volumetric flask, and brought up to volume with ethanol. This alcoholic solution was transferred to a 15-ml. capped test tube and 1 ml. of tetramethylammonium hydroxide 10% solution, freshly diluted 1:10 with aldehyde-free dehydrated ethanol, was added, followed by 1 ml. of 0.5% triphenyltetrazolium chloride solution in aldehyde-free dehydrated ethanol. The test tube, after gentle inverting to ensure a thorough mixing, was placed in a dark room (incubator set at 25°) for exactly 25 min. A respective blank solution treated in the identical way served as a control. Color, developed by the reduction of triphenyltetrazolium chloride to form the formazan in a solution buffered with tetramethylammonium hydroxide, was read in a Beckman DB spectrophotometer at 485 mμ. Cortisol concentration was calculated by reference to the respective standard Beer's law curve previously prepared. The effect of air, water, and the concentration of tetramethylammonium hydroxide, have been discussed by Johnson *et al.* (21) and their paper should be consulted to ensure the reproducibility of the tetrazolium assay.

Determination of the Critical Micelle Concentration (CMC) for Tyloxapol—Surface tension method employing a du Nouy tensiometer (22) and a Wilhelmy plate tensiometer (23), and a spectral absorption benzopurpurin 4B method (24) using a Beckman DB spectrophotometer were applied.

Determination of the Viscosity—Relative, absolute, and specific viscosities of the solutions of tyloxapol in physiologic saline at 37° were determined by means of an Ostwald viscometer in a constant-temperature water bath. Densities of the solutions were determined using a pycnometer.

RESULTS AND DISCUSSION

In preliminary experiments, it was found that the integrity of the rat intestine was not impaired by immersion of the everted sac in 1.0% tyloxapol oxygenated saline solution for 30–60 min. in a water bath at 37°. The sacs so treated were subsequently used in several experiments with the mucosal solution containing 0.10 and 0.20 mg./ml. solutions of cortisol in saline (no tyloxapol present). The results obtained were nearly identical with those obtained with the sacs which were not so treated. At rather elevated concentrations of tyloxapol, far above the critical micelle concentration (CMC) of the solubilizing agent, it was found that the relative rate of passage of cortisol is a function of concentration of the surface-active polymer and is inversely proportional to its concentration. This fact served as direct evidence that the membrane did not change so as to allow for the enhancement of passage of cortisol across it.

In an initial series of experiments, the concentration of 200 mcg. of cortisol per ml., that is below the limit of its water solubility, was used while the concentrations of tyloxapol were 0, 0.005, 1.0, 3.0, and 5.0% w/v. The rates of passage of cortisol were observed to be consistently a function of concentration of the surfactant and inversely proportional to its concentration.

The concentration of 0.005% w/v of tyloxapol was included to see whether this amount—below the CMC—would be of any consequence. No significant changes in the rate of passage of cortisol were observed at this concentration of the surfactant. It is conceivable that by means of more accurate methods, such as the use of labeled radioactive cortisol molecules, the effect of the minute amounts of the solubilizing agents could be evaluated more precisely.

The CMC of tyloxapol determined by surface tension measurements was found to be about 0.04 Gm./dl. However, employing the benzopurpurin 4B spectral absorption method, the first sharp inflection indicative of the CMC formation was detected at the concentration of 0.01 Gm./dl.

Detailed data are presented for the series of experiments involving cortisol actually solubilized. Concentration of 500 mcg./ml. was used throughout the series, the only variable being the concentration of tyloxapol, *i.e.*, 1.0, 2.0, 5.0, and 10.0% w/v. The pattern of diffusion of cortisol across the rat intestine is clearly demonstrated by the time-effect curve (Fig. 1) as a function of the concentration of tyloxapol. $C_{serosal}/C_{mucosal}$ on the ordinates denotes the concentration ratio of cortisol passed in a given time from the serosal to mucosal fluid. Number of runs with each particular concentration is indicated, and so are standard deviations for each time interval within each group of the runs, in Table I.

To determine the relative rates of passage, expression of the results in terms of Fick's law was used:

$$\frac{d(C_o - C_i)}{dt} = -P(C_o - C_i) \quad \text{(Eq. 1)}$$

where t is time, and P is permeability coefficient

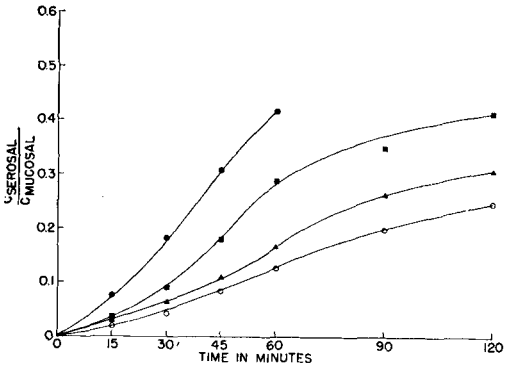

Fig. 1—Passage of solubilized cortisol (0.5 mg./ml.) across the rat intestine in vitro *as a function of the concentration of tyloxapol. Key: Tyloxapol % w/v—●, 1.0; ■, 2.0; ▲, 5.0; ○, 10.0.*

TABLE I—PASSAGE OF SOLUBILIZED CORTISOL (0.5 mg./ml.) ACROSS THE RAT INTESTINE *In Vitro* AS A FUNCTION OF THE CONCENTRATION OF TYLOXAPOL

Tyloxapol w/v, % Concn.	No. of Runs	Time, min.	Mean Ratio of Serosal to Mucosal Cortisol, Concn./ml.	S. D., ±
1.0	(3)	15	0.074	0.021
		30	0.179	0.078
		45	0.313	0.072
		60	0.433	0.078
2.0	(4)	15	0.029	0.010
		30	0.088	0.013
		45	0.178	0.024
		60	0.287	0.067
		90	0.349	0.050
		120	0.417	0.055
5.0	(3)	15	0.026	0.012
		30	0.060	0.009
		45	0.109	0.019
		60	0.165	0.024
		90	0.266	0.024
		120	0.307	0.016
10.0	(5)	15	0.023	0.006
		30	0.043	0.011
		45	0.081	0.024
		60	0.116	0.023
		90	0.184	0.055
		120	0.283	0.099

which is a measure of the relative ease with which the drug crosses the membrane. C_0 represents the drug concentration in the outside (mucosal) fluid, and C_i that in the inside (serosal) fluid. When C_o is made for all practical purposes constant by suspending the everted sac with a very small volume of saline in a relatively large volume of mucosal fluid, the equation may be integrated to give:

$$Pt = -\ln\left(1 - \frac{C_i}{C_o R}\right) \qquad \text{(Eq. 2)}$$

where R is the ratio C_i/C_o at the steady state (25). If R is assumed to be 1, on substituting the absorbed concentration ratios in the equation, and plotting the values of the right-hand side of the equation against time, straight lines are obtained (Fig. 2.). The permeability coefficient P (or the relative rate of penetration of cortisol) as a function of concentration of tyloxapol is given by the slope

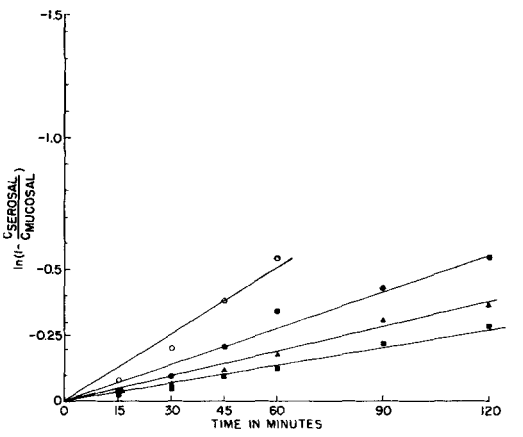

Fig. 2—The relative rates of passage of solubilized cortisol (0.50 mg./ml.) as a function of concentration of tyloxapol. Key: Tyloxapol % w/v—○, 1.0; ●, 2.0; ▲, 5.0; ■, 10.0.

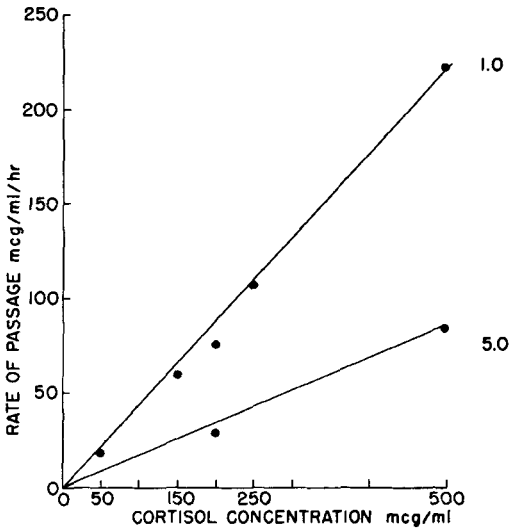

Fig. 3—Effect of concentration of the solubilizing agent on the amount of cortisol passed across the rat intestine in vitro *as a function of the initial concentration solubilized in 1.0 and 5.0 % tyloxapol solution after 1 hr. of incubation.*

of the respective line, $-\ln(1 - C_i/C_oR)/t$. The slopes vary dramatically depending on the concentration of the solubilizer present.

The cortisol concentration effect is illustrated in Fig. 3. It can be seen that the absolute amount of the drug absorbed is directly proportional to its initial concentration, in agreement with the concept of simple diffusion (26). However, it is evident that the amount of the drug absorbed is a function of concentration of the solubilizing agent. The higher concentration of tyloxapol, the lesser amount of cortisol absorbed. It is also evident that the fraction (ratio) of cortisol entering the sac is independent of the concentration of the drug in the outside solution. In other words, the amount passing across the rat intestine is directly proportional to the concentration gradient in accord with the law of simple diffusion. Indeed, it can be seen

from Fig. 3, that the C_i/C_o cortisol ratio in the case of 1.0% tyloxapol is consistently about 0.4 for all five cortisol concentrations, and in the case of 5.0% tyloxapol the ratio is about 0.15.

This series of experiments was carried out in such a way as to minimize variables inherent in using everted sacs from different animals. In one case, the concentrations of cortisol used were 50, 150, 250, and 500 mcg./ml., solubilized in 1.0% solution of tyloxapol, and the determination of cortisol which passed from the mucosal to the serosal side of the sac was consecutively made using the same sac at 1-hr. intervals. Upon repeating the experiment, employing a second segment obtained from the same animal, close results were obtained and averages of the two experiments plotted. A point corresponding to the 200 mcg./ml. concentration plotted on the 1.0% tyloxapol curve was derived from two other experiments involving another rat. Two separate experiments were carried out to obtain the data for the 5.0% tyloxapol curve.

Surface tension of the solutions used throughout the investigation did not seem to play any significant role in the process of cortisol across the rat intestine, differing by a fraction of 1 dyne/cm. in the 1.0–10.0% tyloxapol range (38.7–39.3 dynes/cm. at 21°). The rate of transfer of cortisol from the solutions containing no tyloxapol or just a minute amount of it (below the CMC) was the greatest.

The pH seemed to play no significant part either, although the solutions used were not adjusted to any specific pH. Those containing no tyloxapol were rather close to neutrality (pH 6.5 ± 0.2), while those with tyloxapol had a pH of about 5. The reasons for not adjusting the pH were twofold, cortisol is a nonionizing compound as is tyloxapol, thus, pH-partition theory does not apply. The other reason for not buffering the solutions was a reluctance to introduce ions that might conceivably alter the absorption rates. The ionic strength imparted by sodium chloride used for the sake of tonicity of the solutions was practically uniform throughout the investigation.

The Einstein equation (27) directly relates diffusion to viscosity of the diffusion medium:

$$D = \frac{RT}{N} \cdot \frac{1}{6\pi r\eta} \qquad \text{(Eq. 3)}$$

where D is the diffusion coefficient, N is Avogadro's number, R is the gas constant, T is the absolute temperature, r is the radius of the diffusing molecule, and η is the viscosity.

If it is assumed that except for viscosity all other factors are kept constant, the Eq. 3 may be written:

$$D = K \cdot \frac{1}{\eta} \qquad \text{(Eq. 4)}$$

Absolute, relative, and specific viscosities of 1.0, 2.0, 5.0, and 10.0% w/v of tyloxapol in saline solution at 37° were determined (Table II). When the logarithm of specific viscosity is plotted against tyloxapol concentration, a steep straight line is obtained. It is apparent that the diffusion coefficient of cortisol is inversely proportional to viscosity of the diffusion medium.

According to the views summarized by Levine and Pelican (18), only changes in the biological barrier can influence the rate of absorption of a particular molecular species of a drug transferred by passive diffusion. However, the rate of absorption of a species of a drug molecule which is transferred by facilitated diffusion may be increased or decreased by changes in, or on, the membrane, or by the presence of certain solutes. Consideration of the latter, as the present experiments would indicate, may have important consequences in the therapeutic use of a drug or combination of drugs administered orally. As Stein (28) pointed out, we have no clear idea as to the mechanism of action of facilitated diffusion as yet. At least in part, this is due to our ignorance of the detailed structure of the cell membrane and of the physical chemistry of such a system. In any event, active transport seems to have no place in the system studied since the attempts to detect a movement of solubilized cortisol against the concentration gradient (placing the solutions of the same concentration on both sides of the everted sac) failed.

Physicochemical study of micellar complexation of tyloxapol with cortisol and other steroids, which may throw more light on the mechanism of the passage of these compounds in micellar solutions across the biological membranes, is being carried out in this laboratory.

TABLE II—VISCOSITY OF TYLOXAPOL SOLUTIONS IN SALINE AT 37°

	Absolute, cps.	Relative	Specific
Water	0.6947	...	...
Tyloxapol 1.0% w/v	0.7139	1.0277	0.0277
Tyloxapol 2.0% w/v	0.7754	1.1161	0.1161
Tyloxapol 5.0% w/v	0.8967	1.2910	0.2910
Tyloxapol 10.0% w/v	1.3126	1.8895	0.8895

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Keyphrases

Hydrocortisone diffusion, *in vitro*—rat intestine
Tyloxapol, effect—cortisol diffusion
Everted sac—technique
Colorimetric analysis
Diffusion rate—tyloxapol concentration function

Base-Catalyzed Hydrolysis of Flavins

Effect of Amines and the Particular Role of Position 3

By DONALD B. McCORMICK and WERNER FÖRY

The base-catalyzed hydrolysis of flavins was investigated in 50 percent water–methanol with excess sodium hydroxide or excess amines at different temperatures. Rate constants for the pseudo first-order cleavage of the pyrimidine portion of the isoalloxazine systems were measured and with sodium hydroxide shown to increase from lumiflavin to 3-ethyllumiflavin to 3-carboxyamido(phenylalanyl)lumiflavin. With amines, the size as well as basicity is of prime importance since the general order in efficacy of the nucleophile for hydrolysis of flavins is ethylamine > diethylamine > triethylamine > benzylamine > tributylamine. In general, alkylation of the 3-imino function of the flavin increases its lability because of inability to ionize and undergo resonance stabilization of the anionic tautomers. Moreover, the special case where a 3-carboxyamido function is present allows intramolecular polarization, *via* hydrogen bonding of the amide hydrogen to 4-carbonyl oxygen, which causes a marked increase in hydrolysis rate in sodium hydroxide. From the temperature dependencies of the rates of hydrolysis, the calculated values for energies of activation indicate the decrease expected upon 3-alkylation and further upon intramolecular enhancement with the 3-carboxyamido group. The calculated values for entropies of activation are large and negative in accord with considerable loss of degrees of freedom in the activated complexes formed in the bimolecular mechanism.

FLAVINS CAN undergo hydrolytic decomposition of their isoalloxazine nucleus, particularly in alkaline solution. Kuhn and Rudy (1) isolated urea and 1,2-dihydro-1,6,7-trimethyl-2-keto-3-quinoxaline carboxylic acid from the degradation of lumiflavin in alkaline medium. These results were confirmed by Surrey and Nachod (2) and extended by Svobodova (3) and Wada *et al.* (4) who noted several other unidentified products. Farrer and MacEwan (5) studied the kinetics for hydrolysis of riboflavin over a wide range of pH and showed the reaction to be general acid-base catalyzed. More recently Guttman and coworkers have examined the influence of complexing agents in decreasing the rates for hydrolysis of flavins (6–8) and the intermediates formed following cleavage of the pyrimidine portion of the isoalloxazine system (9–11).

Since most of the earlier studies on the hydrolysis of flavins involved only the use of hydroxyl ion as the attacking species, it seemed desirable to investigate more thoroughly the effects of size and basicity of other nucleophiles such as amines. Also the rate of hydrolysis of a 3-substituted flavin in aqueous sodium hydroxide had been observed to be faster than that of unsubstituted flavin (8), but the primary cause for the difference had not been reported. Furthermore, though such compounds as purines which complex with flavin by planar overlap of their ring systems (12–14) cause a decrease in rate of hydrolysis of flavin (6, 7), certain other types of molecular as-

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