Plasma Protein Binding of Riboflavin and Riboflavin-5′-phosphate in Man

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Abstract □ Ultrafiltration studies with aqueous solutions of riboflavin (FR) or riboflavin-5′-phosphate (FMN) and each of the major human plasma protein fractions (in concentrations normally found in plasma) show that these flavins interact mainly with albumin. Equilibrium dialysis and ultrafiltration experiments with human plasma containing added FR or FMN, or with serum obtained after parenteral administration of FMN, show that the extent of binding of these flavins is essentially the same as in aqueous solutions containing albumin in a concentration equal to that in the plasma or serum. Both FR and FMN appear to be bound on only a single site on the albumin molecule. The FMN-albumin association constant (3.2×10^4 l./mole at 30°) is considerably larger than the FR-albumin association constant (1.3×10^3 l./mole at 30°). There is indication that the interaction between FR and albumin is nonionic, but that electrostatic forces contribute appreciably to the binding of FMN to albumin.

Keyphrases □ Riboflavin, riboflavin-5′-PO_4—plasma protein binding □ Albumin interaction—riboflavin and -5′-PO_4 □ Ultrafiltration—protein-flavin binding determination □ Fluorometry—analysis □ Refractometry—analysis □ Electrophoresis—analysis

The kinetics of absorption, distribution, and elimination of riboflavin (FR) and riboflavin-5′-phosphate (FMN) in man show interesting and unusual characteristics which have been the subject of continuing investigation in this laboratory (1–3). A knowledge of the plasma protein-binding properties of these compounds is necessary for an accurate assessment of their distribution in, and elimination from, the body. The importance of protein binding in the elimination of riboflavin is exemplified by the occurrence of riboflavinuria as a hereditary defect in a strain of Leghorn chickens due to a deficiency of riboflavin-binding protein in blood serum and in the albumin and yolks of eggs, and the resulting death of their progeny from riboflavin deficiency (4). This demonstrates the potential role of carrier proteins in the maintenance of an adequate riboflavin level in the body.

It appears that there have been only a few semiquantitative studies of the interaction of vitamin B-2 (*i.e.*, FR and FMN) with human plasma proteins. Baker *et al.*, determined the relative amounts of endogenous riboflavin which are precipitated together with various human plasma protein fractions by the Cohn procedure (5). Schubert studied the interaction of FR and FMN with serum proteins by the moving-boundary electrophoresis technique (6). A quantitative determination of the protein binding of FR and FMN was made by Leviton and Pallansch (7), but with bovine serum albumin and with certain milk proteins.

In the study to be described here, the interaction of FR and FMN with the major components of human plasma proteins and particularly with albumin was investigated. In addition, the protein binding of FR and FMN was determined in pooled human plasma to which these compounds had been added, as well as in human serum obtained at various times after parenteral administration of a large dose of FMN.

EXPERIMENTAL

Heparinized pooled whole human plasma[1] from six healthy donors and aqueous solutions of commercial Cohn fractions[2] of human plasma in physiological concentrations (8) were employed in the initial part of this study. The plasma fractions listed in Table I, used directly as obtained from the manufacturer, were dissolved in an isotonic physiological electrolyte solution (8) which was adjusted to pH 7.4 and ionic strength 0.15. Alpha-globulin, fibrinogen, and particularly β-globulin could not be completely dissolved. The first two were mostly in solution but a considerable portion of the latter was in suspension. The protein fractions were stored in a refrigerator and used within 2 days of preparation. The pooled human plasma was stored in a frozen state and was used within 1 month after donation. The plasma was adjusted to pH 7.4 by passing carbon dioxide through it prior to use.

In the detailed study of the interaction of riboflavin and FMN with human serum albumin, crystalline human albumin[2] was dissolved in 0.067 *M* Sorensen's phosphate buffer, ionic strength 0.17, and final pH of 7.4. The flavins were added to the protein solutions about 30 min. prior to ultrafiltration. The desired flavin concentration was obtained by adding 0.5 ml. of aqueous flavin solution to 5.0 ml. of the protein solution. The entire 5.5 ml. was then used for ultrafiltration. To check for possible loss of the flavins due to degradation or binding to the dialysis membrane, controls containing no protein were used routinely.

Ultrafiltration—The extent of protein binding of riboflavin and FMN was determined by centrifugal ultrafiltration through Visking dialysis tubing.[3] Seamless dialysis tubing of 2.5-cm. flat diameter was soaked in distilled water overnight prior to use and cut into 28-cm. segments. One end was knotted, excess water was removed, and the tubing was then inflated and permitted to dry partially for about 10 min. The protein solution was placed into the dialysis bag and the open end was tied. The bag was then suspended in a 50-ml. glass centrifuge tube in a manner such that the two ends extended through the neck of the tube and were fastened by the glass stopper. Centrifugation was carried out at $250 \times g$ for 90 min. at 30 ± 3° in a nontemperature-controlled centrifuge or for 120 min. at 5 ± 1° in a refrigerated centrifuge. After about 10 min., the centrifuge was stopped so that the initial 0.1 ml. of filtrate could be discarded. (This filtrate contained the small amount of water which had been retained by the dialysis tubing.) Subsequently, about 0.9 ml. of filtrate was collected. Filtrates were checked for protein leakage by addition of 3% sulfosalicylic acid. The extent of protein binding was calculated from the concentration of flavin in the ultrafiltrate, and the concentration in the protein solution was calculated by averaging the flavin concentration in that solution prior to ultrafiltration and after ultrafiltration (*i.e.*, when 1 ml. out of the initial 5.5 ml. had been filtered).

Equilibrium Dialysis—Solutions of 50 mcg. % of riboflavin or FMN in 6 ml. of 5% albumin in physiologic electrolyte solution

[1] Kindly supplied by the Children's Hospital of Buffalo Blood Bank and the American Red Cross.
[2] Nutritional Biochemicals Corp., Cleveland, Ohio.
[3] Visking dialysis tubing, Visking Co., Chicago, Ill.

and in whole pooled human plasma, respectively, were prepared. One of the solutions was placed within an invaginated segment of Visking dialysis tubing which was immersed in 6 ml. of the complementary protein solution in a 15-ml. capacity wide-mouthed light-resistant container. The open end of the bag remained outside the bottle and was sealed by closure of the screw cap. The bottles and their contents were rocked gently at 30 ± 1° in a temperature-controlled reciprocating shaker. Determinations of flavin and protein concentrations were carried out on 0.5-ml. aliquots of each solution removed after appropriate intervals up to 10 hr. after the start of dialysis. The rate of equilibration of each flavin in such a system was determined by dialysis of a solution containing 100 mcg. % flavin and 5% albumin against a solution containing only 5% albumin. Possible changes in volume of the plasma or albumin phase were determined by weighing the solutions at the end of the experiment and by determining the relative protein concentration in the aliquots by a biuret assay (9).

In Vivo Binding of Flavins to Serum Proteins—Blood samples were taken periodically from a healthy 25-year-old male subject following parenteral administration of 44 mg. of FMN. Serum was assayed for riboflavin and FMN and the concentration of free flavins was determined by ultrafiltration as described in a preceding paragraph. Plasma protein concentrations and flavin blank values were determined in a serum sample obtained 30 min. prior to FMN administration.

Analytical Methods—Riboflavin and FMN were assayed fluorometrically after appropriate dilution of the samples with 0.067 *M* Sorenson's buffer, pH 7.4, using a fluorometer[4] with primary filter 47-B and secondary filter 2A-12 which are appropriate for the excitation and emission peak wavelengths of 450 and 520 mμ, respectively. Standard curves were prepared by adding 0.5 ml. of solution containing known concentrations of flavins with and without plasma proteins to 3.0 ml. of Sorenson's buffer and obtaining fluorescence readings directly. Blank values were determined from solutions which contained protein or protein ultrafiltrate, but no exogenous flavin.

Riboflavin and FMN in the serum of the subject who received FMN parenterally was assayed by the method of Burch *et al.* (10). Flavin in ultrafiltrates of these samples was assayed directly by fluorometry, using microcells and a high-sensitivity conversion kit.[5]

The pooled plasma and serum samples were assayed for total protein with the Goldberg refractometer; individual protein fractions were determined by cellulose acetate electrophoresis.[6]

RESULTS

The relative binding of riboflavin and FMN to plasma protein fractions at concentrations found in human plasma is shown in Table I. These determinations were made by ultrafiltration. Preliminary experiments had shown that binding of FR and FMN to the dialysis membrane was negligible. It was found that albumin binds riboflavin and FMN more extensively than do the other proteins *at physiologic concentrations*[7] and that FMN is bound to albumin much more extensively than is riboflavin. The binding data for β-globulin can only be considered as approximations since this protein could not be completely dissolved under the experimental conditions. Binding of riboflavin and FMN in whole plasma and in an aqueous solution containing all of the plasma protein fractions was quantitatively very similar to the binding in albumin solution, suggesting that albumin is primarily responsible for the protein binding of these flavins in human plasma. Consistent with this suggestion are the results of equilibrium dialysis studies listed in Table II which show that the concentration ratio of the flavin in human plasma and in 5% albumin solution did not change significantly from unity during 10 hr. of dialysis. The half-time for equilibration of riboflavin in albumin solution (100 mcg. % FR and 5% albumin), when dialyzed against an equal volume of 5% albumin solution, was found to be about 1 hr., while that of FMN was 4 hr. A 10-hr. dialysis period should therefore be sufficient to detect any significant movement of the flavins from one phase to the other. Movement of water between the phases during the dialysis period was less than 10%, based on the final weight of the respective solutions and changes in their relative protein concentrations.

[4] Turner fluorometer, model 111, G. K. Turner Associates, Palo Alto, Calif.

[5] Turner, G. K. Turner Associates, Palo Alto, Calif.

[6] Kindly performed by Mr. Ernest Jablonski, Chemistry Dept., Children's Hospital of Buffalo, using the Beckman Microzone system.

[7] Note that this statement does not refer to the relative extent of flavin binding by these proteins at equal concentrations.

Table I—Binding of Riboflavin (FR) and Riboflavin-5′-Phosphate (FMN) to Human Plasma Proteins and to Plasma Protein Fractions[a]

Protein	Concn., g./100 ml.	Fraction Bound[b] FR	Fraction Bound[b] FMN
Whole plasma	6.5[c]	0.42 (0.02)	0.81 (0.02)
Albumin	4.04	0.41 (0.01)	0.86 (0.01)
Alpha-globulin	0.79	0.13 (0.06)	0.32 (0.06)
Beta-globulin	(0.81)[d]	0.27 (0.02)	0.12 (0.02)
Gamma-globulin	0.74	0.07 (0.05)	0.09 (0.02)
Fibrinogen	0.34	0.04 (0.01)	0.14 (0.04)
Protein fraction composite[e]	6.72	0.45 (0.04)	0.84 (0.02)

[a] Determined by ultrafiltration at 30 ± 3° using 50 mcg. % flavin and protein fractions in physiological electrolyte solution, pH 7.4. [b] Average of three to six determinations; standard deviation in parentheses. [c] Pooled heparinized whole plasma (six donors). Albumin concentration, 3.9 %. [d] Present largely in suspension. [e] A solution containing all of the listed plasma protein fractions in the same concentrations shown in the table.

The fluorescence of riboflavin and FMN was decreased to various degrees in the presence of the plasma proteins so that standard curves had to be prepared for the determination of the two flavins in the presence of each of the proteins. There is an excellent correlation (correlation coefficient, 0.95) between the fluorescence-decreasing effect of the different proteins and the extent of their binding of FR and FMN (Fig. 1).

The interaction of riboflavin and FMN with albumin was studied in greater detail by ultrafiltration at 30° over a 500-fold concentration range, and at 5° in the low concentration range of each flavin. The results of these experiments, shown in Fig. 2, can be described by Eq. 1:

$$\text{fraction bound} = \frac{1}{1 + (D_f)/(nP_t) + 1/(nkP_t)} \qquad \text{(Eq. 1)}$$

which is derived from the general equation describing drug-protein interactions involving one class of binding sites (11):

$$\bar{\nu} = \frac{D_b}{P_t} = \frac{nkD_f}{1 + kD_f} \qquad \text{(Eq. 2)}$$

where $\bar{\nu}$ represents the molar concentration of bound flavin (D_b) divided by the total molar concentration of albumin (P_t), n is the number of binding sites on the protein molecule, k is the association constant for flavin-albumin interaction, and D_f is the molar concentration of unbound flavin.

Table II—Ratio of Concentrations of Riboflavin (FR) or Riboflavin-5′-Phosphate (FMN) in Human Plasma[a] and in 5% Human Albumin Solution Separated by a Dialysis Membrane

Time, hr.	Ratio of Flavin Concentration,[b] Plasma:Albumin Solution FR	FMN
0	1.00	1.00
1	0.99	0.95
2	0.99	0.93
4	0.95	0.89
6	1.01	0.86
8	0.94	0.92
10	0.87	1.01
Mean[c]	0.96	0.93

[a] Pooled from six subjects and containing 4.6 % albumin. [b] Initial flavin concentration, 50 mcg./100 ml. Solutions were agitated mildly in a water bath at 30°. Ratios represent the average of triplicate experiments. [c] Excluding zero-time value.

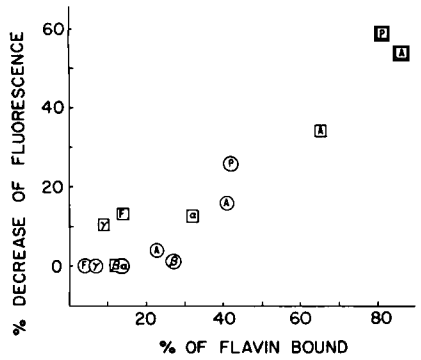

Figure 1—*Relationship between protein binding of riboflavin and FMN as determined by ultrafiltration and the effect of the proteins on the fluorescence of these flavins. Composition of solutions as in Table I; fluorescence readings were made on 7-fold diluted solutions. Key: circles, riboflavin; squares, riboflavin-5'-phosphate; P, plasma; A, albumin; α, alpha globulin; β, beta-globulin; γ, gamma-globulin; F, fibrinogen.*

It can be shown from Eq. 1 that, as the concentration of flavin approaches zero,

$$nk = \frac{F.B.^\circ}{(1 - F.B.^\circ)\, P_t} \qquad \text{(Eq. 3)}$$

where $F.B.^\circ$ is the limiting value of the fraction of flavin bound to the protein. It is also evident from Eq. 1 that the fraction bound will be essentially constant and practically equal to $F.B.^\circ$ at low concentrations of flavin. Values for the association constant, k, were calculated from Eq. 3, based on the assumption that $n = 1$. These values are listed in Table III and the theoretical curves determined from the k values and the known values of P_t [assuming the molecular weight of albumin to be 69,000 (11)] are shown in Fig. 2. These curves agree very well with the experimental data.

Rearrangement of Eq. 2 yields

$$\frac{\bar{\nu}}{D_f} = nk - \bar{\nu}k \qquad \text{(Eq. 4)}$$

which is the basis for the well-known Scatchard plot (12). Such a plot is presented as Fig. 3 for the binding of FMN to albumin. Two regression lines were fitted to the data by the method of least squares, using either the ordinate or abscissa values as the dependent variable. The geometric mean[8] of the two regression lines extrapolates to $\bar{\nu} = 1$ when $\bar{\nu}/D_f$ is zero. Thus there appears to be only a single binding site for FMN on each albumin molecule and the assumption made for calculating the FMN-albumin association constant from the data in Fig. 2, namely that $n = 1$, is justified. The association constant, calculated from the slopes of the regression lines shown in Fig. 3 and from their intercept values on the ordinate, is 3.3×10^4 l./mole, which is in excellent agreement with the value of 3.2×10^4 l./mole obtained from Fig. 2 using Eq. 3.

It was not possible to obtain sufficient data for a Scatchard plot for the binding of riboflavin with albumin due to the low degree of binding and the limited solubility of riboflavin. However, a theoretical line based on $n = 2$ did not fit the riboflavin data in Fig. 2 as well as did the line based on the assumption that $n = 1$.

The association constants for the interaction of riboflavin and FMN with albumin at two temperatures (Table III) were used to calculate the standard free energy of binding, ΔG, and the standard enthalpy change, ΔH (14). These values, which are listed in Table III, are estimations only since binding data could be obtained at only two temperatures.

The protein binding of riboflavin and FMN in pooled whole human plasma at temperatures of 5° and 30° is shown in Fig. 4. These data were obtained by ultrafiltration of plasma to which various amounts of the flavins had been added. The flavin concentrations employed are in the range usually encountered after the administration of moderate doses of vitamin B-2. There was excellent agreement between the experimental data for riboflavin and the theoretically predicted results based on the association constant listed in Table III and the directly determined albumin concentration of 3.9% in the plasma. The agreement between experimental and calculated values was not as close in the case of FMN (Fig. 4).

Table IV shows serum levels of riboflavin and FMN obtained at various times after parenteral administration of a large dose of FMN to a human subject. Listed also are experimental and calculated values for the fraction of *total* flavin bound to the serum proteins. The theoretical calculations were based on the FMN-albumin and FR-albumin association constants determined in the experiments with human albumin solutions, and on the directly determined albumin concentration of the serum. There is excellent agreement between the experimental and the calculated binding values over a 10-fold concentration range of the flavins.

[8] When a single linear relation holds between the true values of two variables and if data for both are subject to error (such that the error in each dimension is proportional to the variation among data in the respective dimensions), then it can be shown (13) that the regression line of best fit has a slope equal to the geometric mean of the two slopes and passes through the intersection of the two least-square estimates obtained by alternatively regressing one variable upon the other as described.

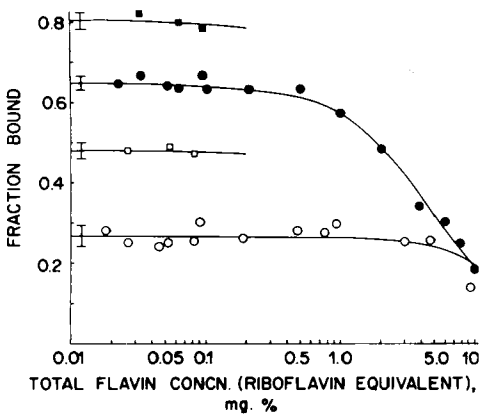

Figure 2—*Binding of riboflavin (○,□) and riboflavin-5'-phosphate (●,■) to human albumin (2 and 0.4% solutions, respectively). Circles, 30°; squares, 5°. The curves were obtained by calculation from Eq. 1, using the association constant value determined from the intercept on the ordinate. Vertical bars represent ±1 standard deviation of the individual data about the horizontal linear portion of each curve. Data points are means of duplicate or triplicate determinations.*

DISCUSSION

The results of the present study suggest that the protein binding of riboflavin and FMN in human plasma is due mainly to the interaction of these flavins with albumin at flavin concentrations likely to be encountered after administration of therapeutic doses of the vitamin. The riboflavin-albumin association constant at 30° found

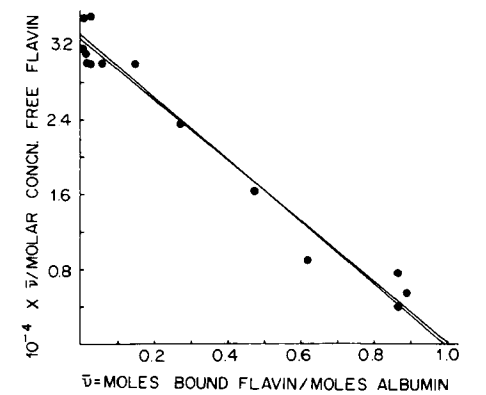

Figure 3—*Scatchard plot of riboflavin-5'-phosphate (FMN) binding to human albumin at 30°. Data were obtained at total FMN concentrations of 0.02 to 10 mg.% in 0.4% albumin solution. The lines are least-square regressions using the ordinate and abscissa values, respectively, as the independent variable.*

Table III—Estimated Effect of Temperature on the Interaction of Riboflavin (FR) and Riboflavin-5′-Phosphate (FMN) with Human Serum Albumin[a]

Flavin	Temp., °C.	Association Constant, l./mole	ΔG, kcal./mole	ΔH, kcal./mole
FR	30 ± 3	1.3×10^3	−4.3	−6.2
FR	5 ± 1	3.2×10^3	−4.5	(−5.4 to −8.5)[b]
FMN	30 ± 3	3.2×10^4	−6.2	−5.4
FMN	5 ± 1	7.1×10^4	−6.2	(−4.7 to −7.4)[b]

[a] Crystalline albumin dissolved in 0.067 *M* phosphate buffer, pH 7.4. Range calculated from the temperature extremes.

in the present study (1.3×10^3 l./mole) is almost equal to that reported by Leviton and Pallansch (1.4×10^3 l./mole) for the binding of riboflavin to bovine serum albumin at pH 6.75 and 20° (7). However, there is a 20-fold difference in the association constant for FMN in the two studies. This may be due to a greater pH sensitivity in the binding of FMN, but it should be noted that the data reported by Leviton and Pallansch are single-point determinations only.

It was found that the interaction of riboflavin and FMN with albumin is appreciably affected by temperature. Thermodynamic considerations, based on the results shown in Table III, suggest that the driving force of the interaction is primarily enthalpic. The enthalpy values are of a magnitude consistent with hydrogen bonding. This, and the temperature dependence of flavin binding, suggest that the interaction of the flavins with albumin is nonionic in character (15, 16). However, addition of the phosphate moiety to riboflavin increases the association constant of the vitamin by more than one order of magnitude under the experimental conditions. This indicates that the binding of FMN to albumin also involves considerable electrostatic interaction. These conclusions are consistent with the known physico-chemical characteristics of the two flavins. Riboflavin is an ampholytic compound with a pKa of 10.2 and a pKb of 1.7 and will thus exist in nonionic form at pH 7.4 (17). FMN, however, possesses the additional phosphate moiety on the ribose portion of the molecule, with pKa's of approximately 4.5 and 8.5 (18). The association of FMN with albumin can therefore be mediated by the nonionic isoalloxazine region as well as by the anionic ribose-phosphate region of the molecule. The more pronounced albumin binding of FMN is consistent also with the mechanism of FMN binding to Old Yellow Enzyme as first proposed by Theorell and Nygaard (19). These investigators postulated the occurrence of hydrogen bonding between the 3-imino group of the

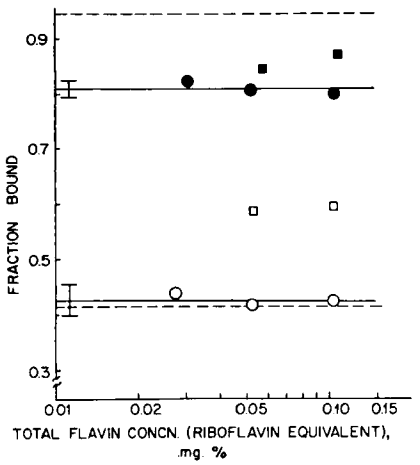

Figure 4—*Binding of riboflavin (FR) and FMN to proteins in whole human plasma at 30° and 5°. Symbols and vertical bars are as defined in Fig. 2. Dashed lines indicate the theoretically calculated binding of FR and FMN at 30° based on the association constant values listed in Table II and the directly determined plasma albumin concentration of 3.9%. Data points are means of 2 to 3 determinations at 5° and 6 to 12 determinations at 30°.*

Table IV—Binding of Riboflavin and FMN to Proteins in Human Serum Obtained After Parenteral Administration of FMN[a]

Flavin Concn. in Serum, mcg./ml. FR	FMN[b]	Total[b]	Fraction of Total Flavin Bound Exptl.	Theoret.[c]
0.859	0.282	1.14	0.58	0.58
0.330	0.085	0.415	0.60	0.56
0.175	0.043	0.218	0.61	0.56
0.091	0.029	0.120	0.63	0.58

[a] 44 mg. FMN, equivalent to 31 mg. FR. [b] Riboflavin equivalent. [c] Calculated by Eq. 1, based on an albumin concentration of 4.6 %.

isoalloxazine ring of FMN and tyrosine hydroxyl groups of the protein, with additional interaction stability being incurred by association of the phosphate anion of FMN with terminal cationic α-amino or lysine ε-amino groups of the protein. This proposal has been reiterated by others (20) including Leviton and Pallansch, who have suggested such a mechanism for the binding of flavins to certain milk proteins (7).

There was excellent agreement between the riboflavin binding data obtained with albumin solutions and the data obtained with human plasma. The reason for the less satisfactory agreement in the FMN binding data is not known. There was excellent agreement also between the experimental and calculated binding values for *total* flavin in serum obtained after parenteral administration of FMN. It was not possible in this experiment to determine the protein binding of riboflavin and FMN individually since the high concentration ratio of riboflavin to FMN did not permit an accurate determination of FMN concentrations in the ultrafiltrate. It is of interest that the fraction of either flavin bound to plasma proteins remains relatively constant over a wide concentration range (Figs. 2 and 4). The rapid conversion of FMN to riboflavin in the body results in a relatively constant value for the fraction of total flavin bound to plasma proteins following administration of FMN. The plasma protein binding of riboflavin and FMN in man is not very extensive and its effect on the distribution and elimination of riboflavin and FMN is likely to be small. The retention of physiological amounts of riboflavin in the body is probably due mainly to the high affinity of the vitamin to flavoprotein enzymes. The association constant for this interaction is at least 1,000 times greater than that for albumin binding (19).

REFERENCES

(1) G. Levy and W. J. Jusko, *J. Pharm. Sci.*, **55**, 285(1966).

(2) W. J. Jusko and G. Levy, *ibid.*, **56**, 58(1967).

(3) *Ibid.*, **56**, 1145(1967).

(4) W. P. Winter, E. G. Buss, C. O. Clagett, and R. V. Boucher, *Comp. Biochem. Physiol.*, **22**, 897(1967).

(5) H. Baker, O. Frank, S. Feingold, and C. M. Leevy, *Nature*, **215**, 84(1967).

(6) R. Schubert, *Zeitsch. Vitaminforsch.*, **19**, 119(1947).

(7) A. Leviton and M. J. Pallansch, *J. Dairy Sci.*, **43**, 1713 (1960).

(8) "Documenta Geigy: Scientific Tables," K. Dien, Ed., 6th ed., Geigy Pharmaceuticals, Ardsley, N. Y., pp. 542–553.

(9) A. G. Gornal, C. J. Bardawill, and M. M. Davis, *J. Biol. Chem.*, **177**, 751(1949).

(10) H. B. Burch, O. A. Bessey, and O. H. Lowry, *ibid.*, **175**, 457 (1948).

(11) A. Goldstein, *Pharmacol. Rev.*, **1**, 102(1949).

(12) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660(1949).

(13) R. Priore, personal communication.

(14) T. S. Lee and L. G. Sillen, "Chemical Equilibrium in Analytical Chemistry," Interscience, New York, N. Y., 1959, pp. 203–230.

(15) I. Klotz, *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 97 (1949).

(16) I. M. Klotz and J. Ayers, *J. Am. Chem. Soc.*, **74**, 6178 (1952).

(17) "The Merck Index," 7th ed., Merck and Co., Inc., Rahway, N. J., 1960, p. 906.

(18) P. E. Sleezer, M. S.Weiss, and G. F. Siemers, *Drug Cosmetic Ind.*, **74**, 196(1954).

(19) H. Theorell and A. P. Nygaard, *Acta Chem. Scand.*, **8**, 1649(1954).
(20) G. R. Penzer and G. K. Radda, *Quart. Rev.*, **21**, 43(1967).

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Ultrasonic Degradation of Aspirin in Mixed Solvent Systems

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Abstract □ The effect of ultrasonic energy on the degradation of aspirin in ethanol–water, diethyl ether–water, and diethylene glycol–water solvent systems at various concentrations and temperatures was studied. It was found that the application of ultrasound to a system undergoing degradation would cause an increase in the rate, but would maintain the same kinetic order as in the control system. The heat of activation seems to be lowered by the mechanical vibrations of the ultrasonic energy. It is postulated that the ultrasonic vibration increases molecular collisions and the movement of the products away from each other, thereby producing a change in the overall rate of reaction. As the concentration ratio was increased in the diethylene glycol system, the subsequent increase in viscosity had a damping effect on the ultrasonic vibration.

Keyphrases □ Aspirin—ultrasonic degradation □ Ultrasonic degradation—aspirin, mixed solvent systems □ Solvent system effect—ultrasonic degradation, aspirin □ UV spectrophotometry—analysis

Ultrasound energy, at frequencies of 20 kc. or above, has been used to affect the rate and yield of a number of chemical reactions. Several hydrolysis reactions can be accelerated by an ultrasonic field including the degradation of procaine (1), ethyl acetate (2), and aspirin (3). Edwards, in a series of papers (4, 5) reported studies of the mechanism of aspirin degradation in aqueous systems in the absence of ultrasound, and found that the overall rate of aspirin hydrolysis followed a pseudo-first-order rate. In the absence of ultrasound, Garrett later studied the effect of alcohol–water and dioxane–water combinations on the hydrolysis of aspirin (6, 7). He reported that as the ethanol concentration was increased, the rate of hydrolysis also increased. He established a mechanism which showed that ethyl acetate was found in the presence of ethanol, causing an increase in the hydrolysis rate. Mario and Gerraughty studied the influence of ultrasound on the degradation of aspirin in an aqueous system (8) and reported that the ultrasonic energy would produce acceleration of the rate and that variation in temperature or pH still produced a pseudo-first-order kinetic rate.

In this study, the effect of ultrasonic energy on the degradation of aspirin dissolved in mixtures of water and ethanol, water and diethylene glycol, and water and ether was investigated. It was decided to study each system at different temperatures so that by using the Arrhenius equation the energies of activation could be calculated for each of the three systems.

EXPERIMENTAL

Equipment—The ultrasonic energy was supplied by a 100-kc. generator,[1] operated at the maximum plate voltage of 1,000 v. The transducer consisted of a mounted barium titanate crystal. Fitted to the inner wall of the ultrasonic bath was a round copper coil connected through an inlet-outlet pump arrangement to a separate constant-temperature water bath,[2] and controlled so that the temperatures of the two baths were both constant and identical, within the limits of ±0.2°, during all individual runs.

Systems and Temperatures Employed—The concentrations of the ethyl alcohol–water solutions were 10, 30, 50, and 70% (v/v), and the three temperatures used were 20, 30, and 40°.

The concentrations of the diethyl ether–water solutions were 1, 3, and 5% (v/v) due to the limited solubility of the ether in water. The temperatures used were 20, 25, and 30°, since higher temperatures were not feasible due to the low boiling point of diethyl ether. Also, condensers were attached to the reaction flasks to prevent volatilization of the ether.

The concentrations of the ethylene glycol–water solutions were 5, 10, 30, and 50% (v/v) and the temperatures used were 20, 30, and 40°.

Procedure—The same procedure was used for the alcohol–water, ether–water, and ethylene glycol–water systems. Two sets of duplicate samples of aspirin buffered to an apparent pH of 3.67 with acetic acid–sodium acetate were used for all degradations. Each sample contained 5.0×10^{-4} moles of aspirin. Of the duplicate samples involved in each degradation, one was subjected to the ultrasound waves, and the other was used as a control by immersing it in the second bath.

After the samples were placed in their respective baths, they were allowed to equilibrate to the selected temperature before a zero time reading was taken. Aliquots were withdrawn from the reaction vessels at accurately measured intervals of time and the absorption values recorded. Since instrumental efficiency could be affected dur-

[1] McKenna model 100 generator, McKenna Laboratories, Santa Monica, Calif.
[2] Catalog No. 3052, Labline Instruments Inc., Chicago, Ill.