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Effect of Probenecid on Renal Clearance of Riboflavin in Man

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Abstract □ The renal clearance of riboflavin was determined in three human subjects at various serum concentrations of the vitamin with and without prior administration of probenecid. Renal clearances of riboflavin exceeded (up to three times) the endogenous creatinine clearances, which indicates that riboflavin excretion involves renal tubular secretion. The clearance of riboflavin was less at low than at high serum concentrations of the vitamin, characteristic of a saturable tubular reabsorption process. Probenecid decreased the renal clearance of riboflavin, and this effect was directly related to the serum concentration of the inhibitor. The serum protein binding of the vitamin was essentially constant (60%) over the concentration range encountered and was unaffected by the presence of probenecid.

Keyphrases □ Riboflavin, renal clearance, man—probenecid effect □ Probenecid, effects—riboflavin, renal clearance, plasma protein binding □ Spectrophotometry—analysis

A pharmacokinetic analysis of literature data by Levy and Jusko (1) yielded renal clearance values for riboflavin in a human subject which were appreciably higher than the normal glomerular filtration rate. Subsequently, it was found that probenecid, an inhibitor of certain specialized renal transport processes (2), decreased the initial rates of urinary excretion of oral and parenteral doses of the vitamin in human subjects (3). These studies suggested that the renal excretion of riboflavin in man involves tubular secretion. The existence of such a mechanism for riboflavin has already been demonstrated in the chicken by Rennick (4) who also noted an inhibitory effect of probenecid on this process.

The purposes of the study to be described were to determine directly the renal clearance of riboflavin in man and to assess quantitatively the effect of probenecid on this process as well as on the plasma protein binding of the vitamin.

EXPERIMENTAL

The studies were carried out in three healthy human subjects: an adult male (Subject J), age 26 years, and two female children (Subjects A and C), ages 8 and 11 years, respectively. The adult subject received a single intravenous dose of riboflavin-5′-phosphate (FMN)[1] equivalent to about 30 mg. of riboflavin (FR) with and without 1 g. probenecid[2] given orally in suspension 1 hr. prior to FMN injection. Urine was collected at appropriate intervals for a total of 48 hr. Blood samples were drawn from the antecubital vein at −0.5, 0.25, 0.75, 1.25, 2.25, 3.25, 4.5, 6.5, 8.5, 13.0, and 25.0 hr. relative to the time of FMN injection. These times were midpoints of urine collection periods.

The two younger subjects received an initial intramuscular dose of FMN[3] equivalent to 16 mg. FR followed by three hourly oral doses of 6 mg. FR as FMN in solution. Urine was collected at hourly intervals and blood was drawn from the antecubital vein at the midpoints of the three urine-collection periods. A 0.5-g. dose of probenecid in tablet form was administered in crossover fashion to the two children 1 hr. prior to the initial dose of FMN. There was an interval of 7 days or more between the control and probenecid studies.

Protein-Binding Determinations—The ultrafiltration technique described in an earlier publication (5) was used to determine the extent of protein binding of the flavins and probenecid in the serum samples.

FMN Stability Study—A series of samples, each containing 1 ml. of FMN in pH 7.4 isotonic Sorensen's buffer and 1 ml. of freshly drawn whole blood from Subject J, were incubated at 37° in the dark. The concentrations of FMN and FR in the samples were determined as a function of time. Control solutions, without blood, were similarly analyzed.

Analytical Method—Riboflavin and FMN were determined fluorometrically by methods previously described (5, 6). Endogenous creatinine levels in urine and serum were determined colorimetrically by the alkaline picrate method (7). Probenecid in serum was assayed by the spectrophotometric method of Dayton *et al.* (8). Initial serum samples were assayed for albumin content as described previously (5).

Data for flavins and probenecid were corrected for blank readings of urine and serum samples obtained prior to administration of the compounds to the test subjects. There was no interference in the assay of any of the compounds due to the presence of the others.

RESULTS

Renal Clearances of Riboflavin and Effect of Probenecid—Riboflavin clearances in Subjects A and C (Table I) were determined

[1] Sodium riboflavin-5′-phosphate, Hoffmann-LaRoche, Nutley, N. J.
[2] Benemid tablets, Merck Sharpe and Dohme, West Point, Pa.
[3] Hyrye injection, S. F. Durst and Co., Inc., Philadelphia, Pa.

Table I—Effect of Probenecid on Riboflavin and Creatinine Clearances in Two Normal Children

Subject	Serum Concentrations[a] Probenecid, mg. %	Total Flavin, mcg./ml.	Clearances, ml./min./1.73 m.² Total Flavin	Creatinine	Clearance Ratio
A (F, 8 yr.)	0.0	0.413	401	142	2.82
	0.0	0.364	337	130	2.59
	0.0	0.298	313	100	3.13
	2.22	0.554	255	98	2.60
	2.34	0.485	136	62	2.19
	3.82	0.422	255	123	2.07
C (F, 11 yr.)	0.0	0.440	291	114	2.55
	0.0	0.318	301	101	2.98
	0.0	0.277	293	99	2.96
	4.13	0.535	153	86	1.78
	5.39	0.369	128	78	1.64
	5.05	0.354	132	114	1.16

[a] Serum samples were obtained at 1.5, 2.5, and 3.5 hr. after initial riboflavin administration and data from each study are listed in that order.

after parenteral administration of the vitamin, with sustaining oral doses given to prevent the usual rapid fall in serum flavin levels. The renal clearances of riboflavin and endogenous creatinine were calculated in the usual way by dividing the urinary excretion rate by the midtime serum concentration. The endogenous creatinine clearance was determined as a measure of glomerular filtration rate (9). For purposes of intersubject comparison, the renal clearance values for the two children were corrected to standard body size of 1.73 m.² by the method of Dubois and Dubois (10).

The riboflavin-creatinine clearance ratios for Subjects A and C ranged from 2.6 to 3.1 in the control periods (Table I). This shows that the vitamin is secreted in the renal tubules. Probenecid caused a decrease in riboflavin clearance and clearance ratios but had no significant effect on the clearance of creatinine. The 0.5-g. dose of probenecid was apparently better absorbed in Subject C than in Subject A as seen by the time course of probenecid serum concentrations in the two children. The relationship between the serum probenecid concentration and riboflavin clearance ratio in the two subjects is shown in Fig. 1.

The experiments in Subject J were designed to determine the renal clearance of riboflavin and the effect of probenecid over a wide range of rapidly changing serum concentrations of the vitamin. The serum concentrations of total riboflavin (FR and FMN) and of FMN alone as a function of time after rapid intravenous injection of FMN with and without prior administration of probenecid are shown in Fig. 2. The corresponding urinary excretion rates as a function of time are shown in Fig. 3. Total riboflavin concentrations in the serum were appreciably higher (Fig. 2) after administration of probenecid while the urinary excretion rates were similar (Fig. 3). Seventy-six percent of the dose was recovered as riboflavin and 14% as FMN in the control experiment. During probenecid administration, 71% was recovered as riboflavin and there was no measurable excretion of FMN.

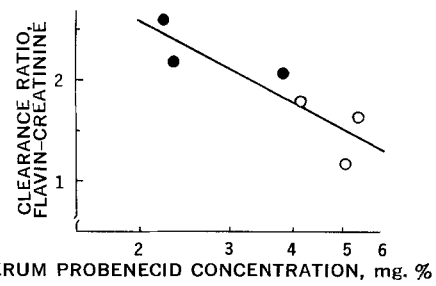

Figure 1—*Effect of probenecid on renal clearances of riboflavin (total flavin) in Subjects A (●) and C (○). The clearance ratio, flavin-creatinine, is plotted as a function of the log serum probenecid concentration, and a least-squares regression line was fitted to the data.*

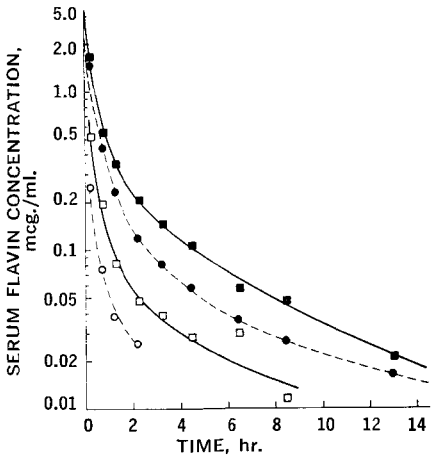

Figure 2—*Serum concentrations of flavins as a function of time after rapid i.v. injection of about 30 mg. FR as FMN to Subject J. Total flavin (solid symbols) and FMN (open symbols) concentrations were determined in a control experiment (circles) and following oral administration of 1 g. probenecid (squares). Total flavin curves were obtained by a computer nonlinear least-squares fit to the data.*

The curves for total riboflavin concentrations in the serum (Fig. 2) and for urinary excretion rates (Fig. 3) were obtained by nonlinear least-squares computer fit to the experimental data. Preliminary graphical analysis by the method of residuals (11) indicated that these data declined in a triexponential manner. Because the serum and excretion values ranged over more than two orders of magnitude, they were converted to their respective logarithmic values to reduce bias. The "NLIN" digital computer program of Marquardt (12) was then used to fit a triexponential curve of the type:

$$F = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + C \cdot e^{-\gamma t} \qquad \text{(Eq. 1)}$$

to the experimental data where F represents either total riboflavin serum concentrations or urinary excretion rates, t is time, and the remaining symbols are constants. The values of the six constants for each of the four sets of data are listed in Table II. The apparent volume of the central compartment, V_c, was calculated from the serum data using the equation:

$$V_c = \text{dose}/(A + B + C) \qquad \text{(Eq. 2)}$$

where the denominator represents the computer derived zero-time serum concentration of the flavins ($C_p°$). This apparent volume

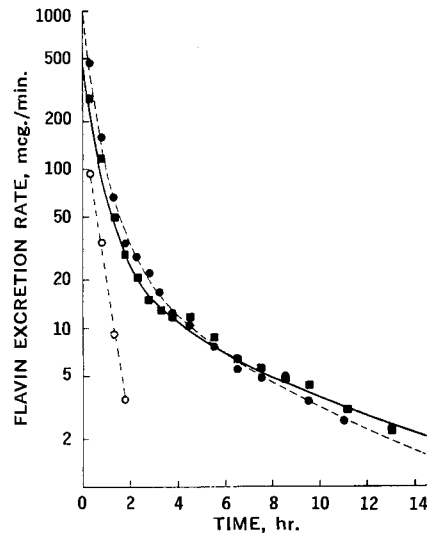

Figure 3—*Urinary excretion rate of flavins as a function of time after rapid i.v. injection of about 30 mg. FR as FMN to Subject J. Lines and symbols are defined as in Fig. 2. No FMN was found in the urine after administration of probenecid.*

Table II—*Pharmacokinetic Coefficients Determined*[a] *from Serum Concentrations and Urinary Excretion Rates of Total Riboflavin after I.V. Administration of Riboflavin-5′-phosphate to Subject J*

Parameter[b]	Serum Levels[c] Control Experiment		Serum Levels[c] During Probenecid	Urinary Excretion Rates[c] Control Experiment		Urinary Excretion Rates[c] During Probenecid
A	1.83	*	3.09	803	*	423
α, hr.$^{-1}$	3.24		3.99	3.02		2.28
B	0.316		0.435	94.7		39.6
β, hr.$^{-1}$	0.717		0.616	0.824		0.956
C	0.072	*	0.140	14.6		18.0
γ, hr.$^{-1}$	0.120	*	0.144	0.148		0.159
Dose, mg.	31.0		30.4			
Apparent volume of central compartment,[d] liter	14.0	*	8.27			

[a] By a least-squares computer fit of Eq. 1. [b] A, B, and C have units of mcg./ml. for serum data and mcg./min. for urinary excretion rates. [c] Asterisk designates those coefficients which do not overlap in the range of their calculated value ± the standard error. [d] Calculated using Eq. 2.

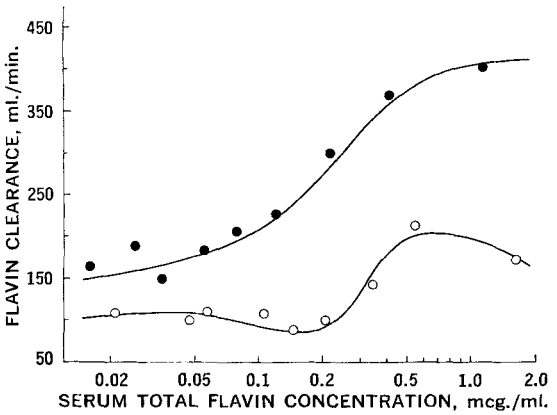

Figure 4—*Renal clearance of total riboflavin plotted as a function of the serum riboflavin concentration in a control experiment (solid symbols) and after oral administration of 1 g. probenecid (open symbols). Curves represent clearances calculated from the least-squares fit of the serum and urinary excretion data. Simultaneously determined endogenous creatinine clearances averaged 124 ± 12 ml./min. and were independent of the serum riboflavin and probenecid concentrations.*

was 14.0 l. in the control experiment and only 8.3 l. in the presence of probenecid.

FMN concentrations in the serum and urine could be determined only during the early times after injection of the vitamin. Available assay methods are inadequate (5) to detect FMN at low concentrations of the vitamin and in the presence of a large excess of FR. It was thus necessary to determine renal clearance in terms of total riboflavin rather than for FMN and FR separately. However, the clearances of FR and FMN determined separately during the early times in the control experiment were quite similar.

The renal clearances of total riboflavin in the presence and absence of probenecid are plotted in Fig. 4 as a function of the total riboflavin concentration in the serum. The points are experimental values and the curves are the results of clearance calculations based on urinary excretion rate and serum concentration values calculated from the parameters of Eq. 1 listed in Table II. The glomerular filtration rate of Subject J averaged 130 ± 10 ml./min. in the presence and 120 ± 11 ml./min. in the absence of probenecid as measured by the endogenous creatinine clearances. As in Subjects A and C, the riboflavin and creatinine clearance values for Subject J indicate that riboflavin excretion involves tubular secretion. Probenecid has a pronounced inhibitory effect on riboflavin excretion as shown by the appreciable decrease in renal clearance when probenecid was given.

The continuous decline in renal clearance of riboflavin during the course of decreasing serum levels of the vitamin is characteristic of a compound which undergoes saturable tubular reabsorption (9). This mechanism occurs with at least three other water-soluble vitamins: thiamine (13), pantothenic acid (14), and ascorbic acid (9). A multiple-compartment pharmacokinetic model embodying saturable renal tubular reabsorption has been developed and applied to riboflavin clearance data from man and dog (15). The relationship which describes the observed (net) renal clearance (Cl_T) of compounds which undergo apparent first-order excretion (clearance) from serum to urine (Cl_f) and saturable tubular reabsorption is

$$Cl_T = Cl_f - \frac{T_m C_u}{C_p(K_m + C_u)} \qquad \text{(Eq. 3)}$$

where C_p and C_u are the serum and urine concentrations of the drug, T_m is the tubular reabsorption transport maximum, and K_m is the Michaelis-Menten constant for the saturable tubular reabsorption process (15). Graphical and computer analysis of the experimental renal clearance data obtained in the absence of probenecid has yielded values of 420 ml./min. for Cl_f, 33.3 mcg./min. for T_m, and 16.3 mcg./ml. for K_m (15). It was not possible to determine these parameters when probenecid was administered because the inhibitory effect of this drug was not constant during the experiment.

Probenecid serum concentrations were determined for Subject J and are shown as a function of time in Fig. 5. These data were used in conjunction with the riboflavin clearance values to construct a drug concentration–response plot which is depicted in Fig. 6. Because of the concentration dependence of riboflavin clearance and the wide concentration range of the data, it was necessary to express the clearances during probenecid administration as a percent of the riboflavin clearance at similar serum concentrations of the vitamin in the control experiment. It should be noted that the pharmacologic effect–serum concentration relationship presented in Fig. 6 (where both riboflavin and probenecid serum concentrations varied markedly) is of a type different from the effect–concentration relationship depicted in Fig. 1 (where the riboflavin concentration was relatively constant). It is of interest, however, that the maximum inhibition of riboflavin clearance[4] in Subject J occurred at a probenecid serum concentration of about 8 mg. % and that the same maximum inhibitory concentration is obtained upon extrapolating the data from Subjects A and C in Fig. 1 to a clearance ratio of unity. Renal clearance studies in dogs (16) also showed that a similar serum concentration of probenecid (7 mg. %) produced maximum inhibition of renal clearance of riboflavin and PAH.

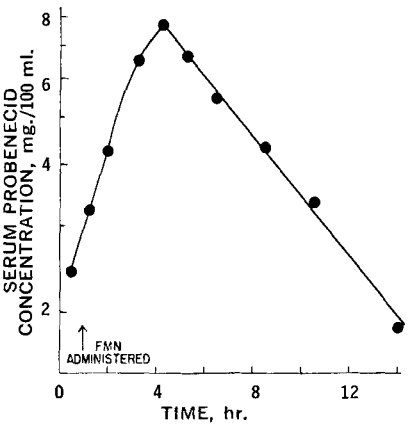

Figure 5—*Concentrations of probenecid in the serum as a function of time after oral administration of 1 g. in aqueous suspension to Subject J. Decline in serum concentration of probenecid represents a half-life of 5 hr.*

[4] This refers to maximum inhibition in terms of actual clearance values (ml./min.) rather than in terms of relative values (percent of control clearance).

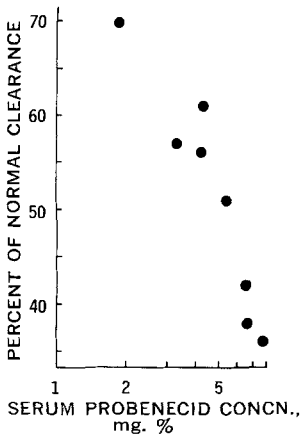

Figure 6—*Effect of probenecid on renal clearance of flavin in Subject J. The percent of normal clearance was calculated (see text) from the data of Fig. 4 and is plotted as a function of the log serum probenecid concentration.*

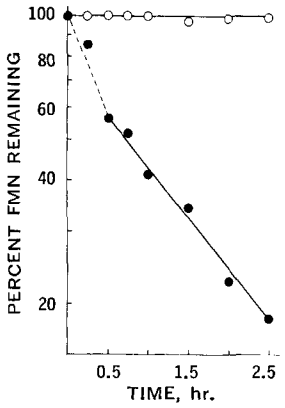

Figure 7—*Rate of dephosphorylation of riboflavin-5'-phosphate during incubation of 72 mcg. % FMN in fresh whole blood (●) and in pH 7.4 Sorensen's buffer (○). Total riboflavin concentration (FR + FMN) remained constant for the duration of the experiment.*

Protein-Binding Results—The serum samples which were obtained in each of the experiments in Subjects A and C were pooled to obtain adequate volume for a determination of the extent of protein binding of the flavins. In Subject A, the flavins were 64 and 57% bound in the absence and presence of probenecid, respectively. The corresponding values for Subject C were 52 and 54%. These data indicate that probenecid had little or no displacing effect on the protein binding of the flavins. The data show also that if the renal clearance values of riboflavin were corrected for protein binding, the net secretion of the flavins would appear to be even more pronounced than indicated by the data in Table I.

In Subject J, individual serum samples were used to determine the extent of protein binding over an appreciable range of serum concentrations of the vitamin. The results of these determinations are summarized in Table III. Over a 10- to 20-fold range in serum riboflavin concentrations, the fraction of total riboflavin bound remained essentially constant and was similar in the control and probenecid experiments. The albumin concentration of the serum was 4.6 g./100 ml., and it was possible to calculate the theoretical degree of binding of total riboflavin using known values (5) for the albumin-FR and albumin-FMN association constants (1.3×10^3 and 3.2×10^4 liter/mole, respectively, at 30°) and the assayed serum concentrations of FR and FMN (Table 3). The basis for such calculations is given in an earlier report on the plasma protein binding of riboflavin and FMN (5). These calculations show that no change in the degree of protein binding of total riboflavin is to be expected over the concentration range of riboflavin encountered in these experiments. Despite the existence of the two forms of the vitamin in the serum, the extent of binding of total riboflavin remained constant due to the relatively constant concentration ratio of FR and FMN in the serum.

The serum protein binding of probenecid was also determined in the serum samples obtained from Subject J. The extent of protein binding of probenecid remained essentially constant during the course of the experiment and the change in serum concentrations of riboflavin did not appear to affect the protein binding of probenecid. Over the serum concentration range of 2 to 8 mg./100 ml., probenecid was 90 ± 4% protein bound, which is in excellent agreement with the data reported by Dayton *et al.* (8).

Table III—Protein Binding of Riboflavin in Human Serum[a] in the Presence and Absence of Probenecid

Serum Flavin Concn., mcg./ml.			Serum Probenecid Concn.,	Fraction of Total Flavin Bound	
FR	FMN[b]	Total[b]	mg. %	Experimental	Theoretical[c]
0.859	0.282	1.141	0.0	0.58	0.58
0.330	0.085	0.415	0.0	0.60	0.56
0.175	0.043	0.218	0.0	0.61	0.56
0.091	0.029	0.120	0.0	0.63	0.58
1.139	0.511	1.650	3.23	0.60	0.59
0.356	0.194	0.551	4.25	0.58	0.62
0.264	0.081	0.346	6.53	0.53	0.55
0.158	0.048	0.206	7.71	0.59	0.55
0.107	0.039	0.146	6.68	0.60	0.57
0.078	0.028	0.106	5.47	0.57	0.57

[a] Albumin content: 4.63 g./100 ml. [b] Riboflavin equivalent. [c] See text.

FMN Dephosphorylation—The data shown in Figs. 2 and 3 indicate that intravenously administered FMN is rapidly converted to riboflavin until a FR–FMN concentration ratio of about 3:1 is attained. This appears to reflect an equilibrium between the rate of dephosphorylation and the rate of phosphorylation of the vitamin. A similar phenomenon has been observed in the rat by Christensen (17). Phosphatase (18) and flavokinase (19) enzymes in the liver may account for these processes, but enzymes associated with erythrocytes also contribute to the very rapid loss of serum FMN and appearance of FR. Incubation of FMN with fresh whole blood from Subject J and with buffer showed that FMN was rapidly converted to FR in whole blood (Fig. 7), but that there was no dephosphorylation of FMN in the control buffer solution. The concentration of total riboflavin in the blood samples remained constant for the duration of the experiment. Previous experiments have shown (5) that incubation of FMN with human plasma produced no change in the relative protein binding of the vitamin which is indirect evidence that FMN is not dephosphorylated in blood plasma.

DISCUSSION

The results of the present study confirm earlier indications (1, 3) that the renal excretion of riboflavin in man involves tubular secretion. Renal clearances of the vitamin were found to exceed markedly the glomerular filtration rate in three human subjects and it was shown that probenecid has a pronounced inhibitory effect on riboflavin excretion. In Subjects A and C, where the riboflavin serum levels were maintained relatively constant, the effect of probenecid could be characterized by a typical dose–response relationship (Fig. 1). In Subject J, the dose–response relationship (Fig. 6) is complicated by the serum concentration dependence of riboflavin clearances resulting from the saturable tubular reabsorption process. Therefore, the clearances during probenecid administration had to be expressed as a percent of the clearance value obtained in the control experiment at the same riboflavin concentration in the serum.

The riboflavin clearances in Fig. 4 have a constant value of about 100 ml./min. during the time when probenecid serum levels declined from 7.7 to 1.8 mg. % and riboflavin serum concentration ranged from 0.2 to 0.02 mcg./ml. This suggests that probenecid inhibits both the tubular secretion and the specialized reabsorption of the vitamin. With inhibition of both active processes, the clearance of flavin would be expected to remain constant at less than the glomerular filtration rate (124 ml./min.) because the vitamin is bound to some extent to serum proteins. In view of the difference in the probenecid serum concentration–effect relationship during increasing and decreasing probenecid serum levels, respectively, it appears that the time course of probenecid concentration in the serum does not adequately reflect the time course of probenecid concentration at its site of action, particularly in the elimination phase of probenecid. The fact that maximum inhibition of riboflavin clearance was observed while probenecid concentration in the

serum declined from 7.7 to 1.8 mg. % suggests that probenecid is retained at its site of action considerably longer than in the serum.

Probenecid had no serum protein-displacing effect on riboflavin and FMN under the experimental conditions. The protein-binding determinations were also done to determine whether the renal clearance of flavin could change as a result of saturation of protein-binding sites at high serum concentrations of riboflavin. This might result in a greater fraction of unbound riboflavin with concomitant increase in renal clearances at high serum riboflavin levels. The actual measurements and theoretically calculated protein-binding data (Table III) showed that the extent of protein binding of total riboflavin is quite constant over the concentration range used in these studies. The protein-binding data do, however, indicate that correction of the renal clearance values for the extent of riboflavin binding would result in even higher clearance values than shown in Table I and Fig. 4 for the three subjects.

The data in Fig. 2 and Table I show that serum concentrations of riboflavin were substantially higher during probenecid administration than during control experiments. Pharmacokinetic analysis of these data indicates that the apparent volume of the central compartment for the vitamin decreased from 14.0 to 8.3 l. when probenecid was administered. This may be a real effect of probenecid on the distribution of riboflavin or it may reflect the limitations of presently available pharmacokinetic techniques in that an inhibition of renal excretion may modify the apparent distribution constants of the pharmacokinetic model. Studies are now in progress to determine the effect, if any, of probenecid on the distribution of riboflavin in anephric subjects (20). It should be possible in this way to determine if probenecid does in fact modify pharmacokinetic parameters other than those reflecting the renal excretion of riboflavin.

In order to examine the mechanism and kinetics of the renal excretion of riboflavin and the nature of the effect of probenecid on these processes in more detail than is feasible in man, additional studies have been carried out in the dog. The results of these studies, which will be reported in other communications (15, 16), demonstrate that riboflavin excretion in the dog also involves both tubular secretion and saturable tubular reabsorption. After attainment of constant serum levels of riboflavin and PAH, a typical log-linear concentration–response relationship was found for the inhibition of renal clearance of these compounds by probenecid. Interestingly, the maximum inhibitory effect of probenecid on both riboflavin and PAH renal clearance in these dogs was reached at about 7 mg. % serum probenecid concentration, similar to the maximum effective concentration found in the subjects of the present study.

Probenecid inhibits the renal excretion of riboflavin in species other than man and dog. Rennick (4) has demonstrated inhibition of tubular secretion of riboflavin by probenecid in the chicken. Markkanen *et al.* (21) have shown that probenecid decreases the basal excretion of riboflavin in man and rabbits whose riboflavin intake was limited to that derived from the normal diet.

There is evidence (16, 22) that the renal transport mechanism for riboflavin may involve a phosphorylation–dephosphorylation process. Probenecid, besides decreasing the renal clearance of various compounds by competitive inhibition, also inhibits directly those enzymes which require a source of high-energy phosphate bond energy (23). This may be the mechanism of the effect of probenecid on the renal clearance of riboflavin, since no FMN was found in the urine when probenecid was administered in the present study. It is of interest that a similar phosphorylation–dephosphorylation mechanism may be responsible for the specialized intestinal absorption of riboflavin (24) which also appears to be inhibited by probenecid (3).

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