Table VIII—Effects of Nicotinic Acid and Histamine on the Disappearance of Theophylline from the Rat Rectum after 1 hr of Perfusion

Substance	Percent Lost				
	pH 4.5	pH 6.0	pH 6.8	pH 7.4	pH 8.9
Theophylline only	7.8 ± 1.5	5.2 ± 0.3	—	4.7 ± 0.2	4.0 ± 0.2
Nicotinic acid only	49.4 ± 1.3	—	4.6 ± 0.4	8.2 ± 3.2	5.5 ± 0.7
Theophylline in presence of 1% nicotinic acid	9.8 ± 2.8	—	5.9 ± 3.7	5.1 ± 0.9	6.1 ± 5.5
Theophylline in presence of 1% histamine	9.1 ± 4.7	9.1 ± 3.9	—	10.2 ± 3.7	12.2 ± 5.6

bleeding from the rectum, probably as a result of damage to the rectal membrane, and, thus, promoted the disappearance of theophylline from the perfusate.

Citrate and I do not appear to be absorbed from the rectum since the remaining chelating activity in the perfusate, measured according to the method of Ogino and Hayashi (4), was significant. This finding suggests that compounds that are not absorbed well themselves are not suitable adjuvants for rectal drug absorption.

Comparison of Sodium Nicotinate and Histamine with Salicylate—Another possible mechanism involves the effect of vasodilation and inflammation on the disappearance of theophylline from the perfusate. In this regard, the effects of nicotinate and histamine on the disappearance of theophylline were studied.

Neither histamine nor nicotinic acid significantly facilitated the disappearance of theophylline (Table VIII). At pH 4.5, the disappearance of theophylline from the perfusate was not facilitated by nicotinic acid, although nicotinate was lost from the perfusate. The action of salicylate as an adjuvant probably does not depend on vasodilation or an inflammatory action.

Mechanistically, the enhancement of rectal absorption of theophylline by salicylate still is unclear. The nonionic and ionic forms of salicylate apparently have different paths through the membrane. It is possible that salicylate reduces the lipophilicity or increases the permeability of the membrane, perhaps by interacting with some substance in the membrane, *e.g.*, calcium or magnesium ions, which may be present as structural features, and thus concurrently allows theophylline to pass through the rectal membrane.

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Time Course and Disposition of Methazolamide in Human Plasma and Red Blood Cells

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Abstract □ Methazolamide was determined in plasma, whole blood, and urine by a GLC–mass spectrometric method. Temporal patterns of methazolamide concentrations in plasma and red blood cells were obtained following single- and multiple-dose oral administration of the drug. The nonlinearity in the binding of the drug to the red blood cell carbonic anhydrase was evident from a comparison of plasma and red blood cell concentrations. The drug was cleared slowly from the red blood cells. The binding constants to the two isoenzymes of carbonic anhydrase were determined from the plasma and red blood cell concentrations and were in agreement with those determined by previous measurements. The half-life of elimination was 7.5 hr. The urinary recovery of unchanged drug was ~25% of the administered dose.

Keyphrases □ Methazolamide—time course and disposition in human plasma and red blood cells □ GLC–mass spectrometry—analysis, methazolamide, human plasma and red blood cells □ Distribution—methazolamide, time course and disposition in human plasma and red blood cells

Methazolamide (5-acetylimino-4-methyl-Δ^2-1,3,4-thiadiazoline-2-sulfonamide) is a carbonic anhydrase inhibitor used in the treatment of glaucoma. It is the methylated analog of the tautomer of acetazolamide. Both drugs reduce the transport of ions from the secretory cells of the ciliary body into the nascent aqueous humor and decrease aqueous secretion through a local osmotic effect (1). In particular, inhibition of the carbonic anhydrase in the secretory cells of dogs reduces the bicarbonate flux (2) and sodium-ion flux (3) into the posterior chamber of the eye.

The dynamics and distribution of drug following different patterns and routes of administration have been studied much more extensively for acetazolamide than for methazolamide. All work to date on the determination of methazolamide in biological fluids and tissues has employed the indirect enzymatic method developed by Maren and coworkers (4, 5). This method is based on two facts: (*a*) the rate of hydration of carbon dioxide catalyzed by carbonic anhydrase is reduced by an inhibitor present in a biological fluid; and (*b*) the rate of hydration or, equally, the rate of formation of carbonic acid or protons directly affects the time interval required to cause a given change in pH. Although methazolamide is metabolized significantly (only 25% is recovered as unchanged drug in the urine) in contrast to acetazolamide, which is not metabolized, the metabolites appear either to be very weak inhibitors of carbonic anhydrase or to lack inhibitory potential (6).

Plasma and red blood cell concentrations and the uri-

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nary output of methazolamide were determined as a function of time following oral administration of the drug using a specific GLC–mass spectrometric method. The method employed the propionyl analog of methazolamide as an internal standard, methylated derivatives for chromatography, and selective-ion monitoring for detection.

EXPERIMENTAL

Materials—Ethyl acetate, methylene chloride, hexane, benzene, and toluene of high purity were used as received[1]. Methyl iodide[2] was diluted with methylene chloride to prepare a 1 *M* solution, which was used to alkylate methazolamide and the internal standard.

A 0.1 *M* solution of tetrahexylammonium hydrogen sulfate, prepared in this laboratory, was used to catalyze the alkylation reaction.

A saturated aqueous solution of silver sulfate[3] was used to remove tetrahexylammonium iodide, formed during the extractive alkylation, from the organic phase (7).

A 0.5 *M* ammonium acetate buffer (pH 4.5) was prepared by titration of acetic acid with ammonium hydroxide. A 1 *M* aqueous sodium phosphate[3] solution was used for the pH adjustment of the extracted biological fluid prior to extractive alkylation.

Synthesis of 5-Propionylimino-4-methyl-Δ^2-1,3,4-thiadiazoline 2-Sulfonamide—The hydrochloride salt of 5-imino-4-methyl-Δ^2-1,3,4-thiadiazoline-2-sulfonamide (I) was formed by the acid-catalyzed hydrolysis of methazolamide. Methazolamide (1 g) was dissolved in 20 ml of ethanol and 2 ml of concentrated hydrochloric acid, and the resulting solution was heated under reflux for 1.5 hr. After cooling, the solid was filtered off and dried, resulting in 800 mg of a crystalline white material. The NMR spectrum of this material was consistent with I and showed the absence of the acetylimino group present in methazolamide.

Propionic acid (1 ml), propionic anhydride (540 mg), and I (575 mg) were heated at 120° for 2.5 hr. After cooling, 15 ml of water was added to the solution, and the mixture was extracted three times with 20 ml of ethyl acetate. The organic extract was washed with brine (20 ml), dried over anhydrous sodium sulfate, and evaporated. The crude material was dried under vacuum and recrystallized from ethyl acetate–ether to give 143 mg of the desired compound, the propionyl analog of methazolamide. The compound melted at 175–177°.

Apparatus—A mass spectrometer[4] was interfaced with a gas chromatograph[5]. Chromatographic columns were prepared from empty glass columns (183 cm × 2 mm i.d.), silanized, and packed with 3% OV-17 on Chromosorb W (AW, DMCS, 100–120 mesh). The column and injector temperatures were 180 and 190°, respectively. The flow rate of the carrier gas (helium) was 25 ml/min.

The methylated derivatives of methazolamide and the internal standard were detected by selective-ion monitoring of the *m/z* 249 mass fragment, which is the base peak for the methylated derivative of both methazolamide and the internal standard. The ionizing voltage was 70 ev. The retention times for the methylated derivatives of methazolamide and the internal standard were 7 and 11 min, respectively.

Analysis of Methazolamide in Plasma and Whole Blood—A known quantity of the internal standard in methanol was added to a 1-ml aliquot of plasma or a 0.5-ml aliquot of whole blood in a 40-ml conical centrifuge tube. Distilled water (0.5 ml) was added to the whole blood to lyse the red blood cells. Two milliliters of 0.5 *M* ammonium acetate (pH 4.5) was added to the biological fluid. Each sample was extracted twice with 10 ml of ethyl acetate by shaking the tube horizontally for 10 min and then centrifuging it to separate the organic and aqueous phases.

The combined organic extracts were taken to dryness in a second 40-ml centrifuge tube under a nitrogen stream. One milliliter of 0.1 *N* H_2SO_4 was added to the residue, and the aqueous solution was washed twice with 10 ml of hexane. The aqueous solution then was adjusted to pH 10 with ~150 μl of 1 *M* sodium phosphate.

Extractive methylation of methazolamide and the internal standard was effected by adding 50 μl of 0.1 *M* tetrahexylammonium hydrogen sulfate and 2.5 ml of freshly prepared methyl iodide in methylene chloride. The centrifuge tube was sealed tightly with a polytef stopper, and

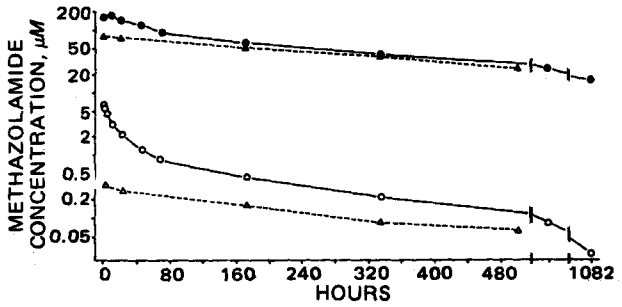

Figure 1—*Temporal patterns of plasma concentrations (open symbols) and red blood cell concentrations (solid symbols) for the subject administered a 50-mg tablet (△ and ▲) and for the subject administered a 150-mg solution of methazolamide (○ and ●).*

the contents were agitated[6] at 40° for 30 min. After centrifugation, the organic layer containing the permethylated derivatives was transferred to a 15-ml centrifuge tube and taken to dryness under a nitrogen stream. The residue was dissolved in 6 ml of benzene, and the benzene solution was washed twice with 2.5 ml of a saturated aqueous solution of silver sulfate. The retained organic layer was taken to dryness under a nitrogen stream. The residue was dissolved in toluene (100 μl), and a few microliters of the solution was injected into the GLC–mass spectrometric system.

The amount of methazolamide in the biological sample was determined from the area ratio of the responses of the permethylated derivatives (methazolamide to the internal standard) and the calibration curve. The concentration of methazolamide in the red blood cells (C_{RBC}) was determined from:

$$C_{RBC} = \frac{C_{WB} - (1 - H)C_p}{H} \qquad \text{(Eq. 1)}$$

where C_{WB} and C_p are the measured concentrations of methazolamide in whole blood and plasma, respectively, and H is the hematocrit.

Analysis of Methazolamide in Urine—Aliquots (1 ml) were analyzed using the method described for plasma and whole blood, except that the residue, when dissolved in 0.1 *N* H_2SO_4, was alkylated directly after adjustment to pH 10 with sodium phosphate and the hexane washes were omitted.

Standard Curves—A standard curve was prepared by spiking 1-ml aliquots of plasma with 0.5, 5, 50, 100, 500, 1000, and 5000 ng of methazolamide and a constant amount (250 ng) of the internal standard. The samples were prepared for GLC–mass spectrometric analysis as already described. The standard curve was obtained by plotting the area ratio of the responses of the *m/z* 249 fragments (methazolamide to the internal standard) *versus* the amount of methazolamide added. The internal standard, when derivatized, exhibited a small peak with the retention time of the methazolamide derivative. The peak area ratio of the minor peak to the major peak was 0.0025, and this ratio was used to correct the peak area ratio for the standards. Linear least-squares analysis of the data yielded a slope of 3.2×10^{-3} and a correlation coefficient of 0.999.

Another standard curve, prepared using 0.5 ml of whole blood, had the same slope as that obtained with the spiked plasma samples.

Aliquots (1 ml) of urine were spiked with 0.1, 0.5, 1.0, 5.0, and 10 μg of methazolamide and a constant amount (1 μg) of the internal standard. The samples were prepared for analysis as already described. The slope of the standard curve using urine was 0.87, with a correlation coefficient of 0.999.

***In Vivo* Studies**—Three male subjects were administered methazolamide. Subjects 1, 2, and 3 weighed 88, 77.3, and 84 kg, respectively. Plasma and red blood cell concentrations and the cumulative urinary excretion of methazolamide were determined in single-dose studies after administration of a 50-mg methazolamide tablet to Subject 1 and after oral administration of 150 mg of methazolamide as a solution to Subject 2 and in a multiple-dose study after administration of 100-mg methazolamide tablets (three times daily) to Subject 3.

Blood from the subjects was drawn into 10-ml evacuated tubes containing heparin. The sample was divided into two parts: one part was centrifuged immediately to obtain plasma, and the other was retained for the analysis of whole blood. Hematocrits were determined. The blood samples were obtained at the times indicated in Figs. 1 and 2. Urine was

[1] Nanograde solvents, Mallinckrodt, St. Louis, Mo.
[2] Eastman Kodak Co., Rochester, N.Y.
[3] Baker Chemical Co., Phillipsburg, N.J.
[4] 1015 SL, Finnigan Corp., Sunnyvale, Calif.
[5] Model 1700, Varian Associates, Palo Alto, Calif.
[6] Evapo-Mix, Buchler Instruments, Fort Lee, N.J.

collected at variable intervals following drug administration. The pH of the urine collections obtained from the multiple-dose study was determined.

RESULTS AND DISCUSSION

Plasma and Red Blood Cell Concentrations (Comparison and Temporal Aspects)—The GLC–mass spectrometric method is sensitive to <1 ng of methazolamide/ml of plasma. The sensitivity is important in the study of drug distribution between plasma and erythrocytes when the concentrations are in the nanogram and microgram per milliliter range, respectively. A typical chromatogram is shown in Fig. 3.

The temporal patterns of the plasma and red blood cell drug concentrations in the subjects administered single doses of methazolamide are presented in Fig. 1. Three hours following administration of a 50-mg tablet to Subject 1, the red blood cell and plasma drug concentrations were 19.1 (80.9 μM) and 0.078 μg/ml, respectively. With the assumption of a red blood cell volume of 2.6 liters (30 ml/kg), 49.7 mg, or nearly the entire dose, was sequestered by the red blood cells. Three hours after oral administration of 150 mg of methazolamide as a solution to Subject 2, the red blood cell and plasma drug concentrations were 36.1 (153 μM) and 1.37 μg/ml, respectively. The red blood cell drug concentration was equivalent to 84 mg or 56% of the dose present in the red blood cells. The plasma drug concentration thus varied by more than 17-fold and the red blood cell drug concentration by less than twofold for a threefold increase in the dose.

Methazolamide binds reversibly to carbonic anhydrase (8). The interaction is describable by the law of mass action. Each enzyme macromolecule has a single binding site for the drug molecule (9). Human erythrocytes have been reported to contain 156 μM carbonic anhydrase, of which 20 μM is the high-activity type C and 136 μM is the low-activity type B (10). Maren *et al.* (6) reported that the total concentration of carbonic anhydrase in the human erythrocyte is variable (range 130–200 μM). Thus, the red blood cell concentration of methazolamide approaches the concentration of carbonic anhydrase in the erythrocytes following administration of the 150-mg dose but not following administration of the 50-mg tablet.

The dramatic increase in the plasma concentration for a less than twofold increase in the red blood cell concentration is a manifestation of the strong, nonlinear interaction of methazolamide with the red blood cell carbonic anhydrase. The free plasma concentration has been reported to be 45% of the total plasma concentration (11). The free drug rapidly penetrates erythrocytes (12). Therefore, the total plasma concentrations reflect the free intracellular concentrations. As the dose is increased and enzyme saturation is approached, the fraction of the dose bound to the carbonic anhydrase decreases due to the nonlinear nature of the binding. Consequently, a larger fraction of the dose is available to distribute in the total body water, and the plasma concentration increases rapidly.

The temporal patterns of the plasma and red blood cell concentrations in Subject 3, administered the multiple-dose regimen of methazolamide, are presented in Fig. 2. The plasma concentration resulting from the first 100-mg dose was insufficient to achieve a high fractional binding of the carbonic anhydrase, as judged by the reported concentrations of red blood cell carbonic anhydrase.

Repeated doses of the drug resulted in steady-state plasma concentrations of ~9.5 μg/ml (40 μM), which was much higher than the maximum plasma concentration observed in the first dosing interval (0.21 μg/ml). The red blood cell concentration at steady state was ~40 μg/ml (169 μM). Under the steady-state condition, the carbonic anhydrase in the red blood cells is near saturation since the binding constants of the isoenzymes (to be described) are much smaller than the free concentration of drug. If it is assumed that the cell water represents 63% of the volume of the red blood cells (10), the concentration of bound drug and, therefore, carbonic anhydrase was ~158 μM [169 μM − (0.63 × 0.45 × 40 μM)] in Subject 3.

The red blood cell concentrations appear to approach steady state significantly faster than the plasma concentrations (Fig. 2). Consideration of a simple system can demonstrate that this difference in the approach of the concentrations to their respective steady-state values is a manifestation of the strong, reversible, nonlinear interaction between the drug and carbonic anhydrase. One can imagine a system in which there is a local equilibrium between bound and free drug:

$$B = \frac{PF}{K + F} \qquad \text{(Eq. 2)}$$

where B is the concentration of bound drug, P is the concentration of binding sites, F is the concentration of free drug, and K is the dissociation

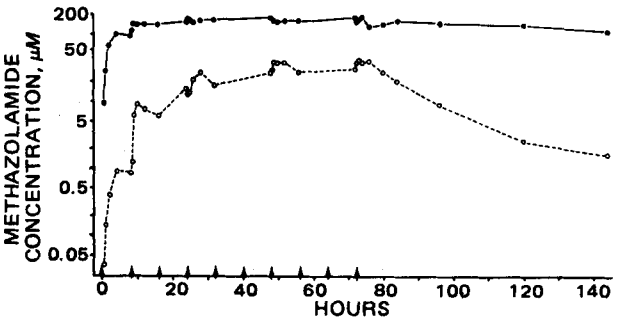

Figure 2—*Temporal patterns of plasma concentrations (O– –) and red blood cell concentrations (●—) for the subject administered a multiple-dose regimen.*

constant of the drug–macromolecule complex. Furthermore, it is assumed that the system is open such that the steady-state concentration of the free drug is determined by its infusion rate (mass flow) divided by the clearance of the free drug. Now at steady state:

$$B_{ss} = \frac{PF_{ss}}{K + F_{ss}} \qquad \text{(Eq. 3)}$$

Dividing Eq. 2 by Eq. 3 yields:

$$R_B = \left(\frac{\alpha + 1}{\alpha + R_F}\right) R_F \qquad \text{(Eq. 4)}$$

where $R_F = F(t)/F_{ss}$, $R_B = B(t)/B_{ss}$, and $\alpha = K/F_{ss}$.

As R_F approaches 1, R_B approaches 1. However, R_B/R_F is always >1, except at steady state, where $R_B/R_F = 1$. In a system with strong binding and an infusion rate sufficient to ensure that $F_{ss} \gg K$, $\alpha \ll 1$. Thus, under these conditions, $R_B \simeq 1$ at times when the free concentration is an order of magnitude greater than K yet substantially less than F_{ss}, whatever the exact temporal dependence of the free concentration on the input rate.

Urinary Recoveries, Renal Clearances, and Total Clearances—The amount of unchanged drug recovered in each dosing interval of the multiple-dose regimen is presented in Table I. The pH of the urine may have influenced the recovery of the drug. The pH of the urine collection and the amount of drug recovered for the sixth and ninth doses were low relative to the values for their respective preceding and following doses. Methazolamide is a weak acid with a pK value of 7.2 (12). Thus, it appears

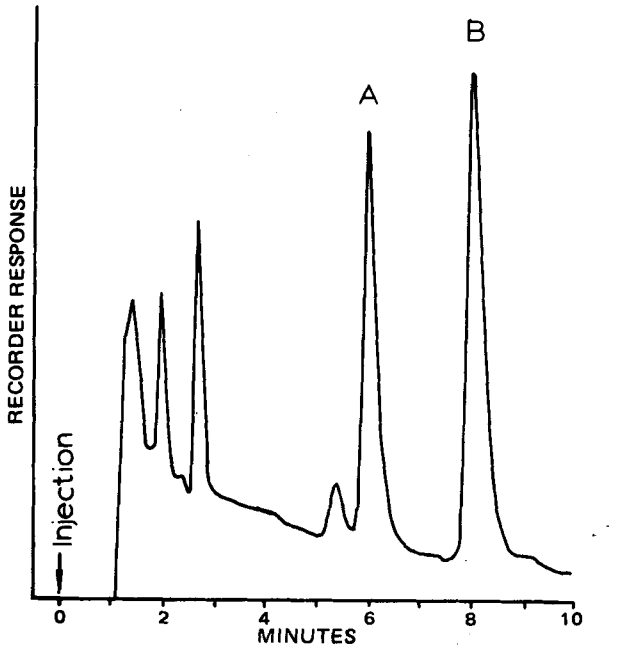

Figure 3—*Chromatogram of plasma sample calculated to contain 7.6 ng of methazolamide. Peaks A and B correspond to the permethylated derivatives of methazolamide and the internal standard, respectively.*

Table I—Renal Clearances following Administration of Methazolamide

Dosage	Time Interval, hr (dose)	Amount Excreted, mg	pH	Volume, ml	Renal Clearance[a], ml/min
100 mg tid	0–8 (1)	0.783	6.71	745	10.9
	8–16 (2)	9.48	7.29	1480	12.3
	16–24 (3)	11.4	7.20	1000	9.2[b]
	24–32 (4)	18.3	7.38	1185	7.6
	32–40 (5)	19.3	7.24	1005	6.7[b]
	40–48 (6)	14.7	6.60	865	
	48–56 (7)	28.6	6.97	880	7.2
	56–64 (8)	22.5	6.62	840	5.0[b]
	64–72 (9)	11.0	5.70	350	
	72–80 (10)	29.1	6.90	750	7.0
	80–144	53.3			8.1
	0–144	218.4			7.1
150 mg	0–72	17.9			8.0
50 mg	0–327	6.5			7.7

[a] Based on total plasma concentration. [b] Plasma concentration not mapped over dosing intervals 3, 5, 6, 8, and 9.

that tubular reabsorption of the drug is an important component in renal clearance. Methazolamide, in contrast to acetazolamide, is not secreted in the proximal tubules (6).

The renal clearances for the dosing intervals also are presented in Table I. The renal clearance was calculated from the amount of unchanged drug excreted during a given time interval divided by the corresponding area under the plasma curve. The area was determined using the trapezoidal approximation. The renal clearances more or less correlated with the urine pH, as they should if tubular reabsorption is important. The average renal clearances for the 50-mg, 150-mg, and 100-mg tid doses were 7.7, 8.0, and 7.1, respectively. These values are similar to the reported renal clearance (9.0 ml/min) expressed relative to the total plasma concentration (6).

Maren *et al.* (13) showed that in dogs chronically administered high doses of acetazolamide, an initial bicarbonate loss ensued, followed by a period in which the dogs became refractory to further bicarbonate loss. The pH and volumetric flow of the urine also appeared to correspond to the rate of bicarbonate excretion. Thus, a similar phenomenon may have been operative during the multiple-dose regimen of methazolamide since there appeared to be an initial increase in both the pH and the volumetric flow.

The total body clearance was estimated by subtracting from the dose the amount of drug remaining in the red blood cells (30 ml/kg) at the time of the last sampling and dividing this value by the corresponding area under the plasma curve. The contribution to the total amount of drug in the body at the last sampling time was small, as reflected in the plasma concentration (Table II). Furthermore, the contribution of the extrarenal carbonic anhydrase pool, which Maren *et al.* (6) claimed was <10% of the total pool but which Lehmann *et al.* (14), suggested was approximately equal to the red blood cell pool was neglected. Furthermore, methazolamide was reported to be well absorbed (6), and absorption was assumed to be complete. The estimated total body clearances are presented in Table II.

It is possible that along with the influence of bicarbonate excretion on the renal clearance of methazolamide, the resulting metabolic acidosis influences its metabolic clearance. Conceivably, changes in the plasma pH relative to changes in the pH of the tissue fluids could cause a redistribution of the drug, as was observed with the weak acid phenobarbital (15), and, hence, alter its clearance. Multiple-dose regimens of 25 mg bid or 50 mg bid reduced the plasma pH from 7.41 to 7.35 (16). Thus, the total clearance is at best an average over the changes of the acid–base balance caused by the drug.

The ratio of the renal clearance to the body clearance should be equal to the fraction of unchanged drug recovered in the urine, provided urine is collected for a sufficient time. The predicted fractional urinary recoveries for the 50-mg, 150-mg, and 100-mg tid doses were 0.25, 0.29, and 0.26, respectively.

In the multiple-dose study, the total amount of drug recovered in contiguous urine collections up to 216 hr was 228 mg or 23% of the administered dose (1000 mg). Excretion rates were determined for 2-hr urine collections centered at 528 and 744 hr. Linear interpolation of excretion rates between the midpoint (204 hr) of the last contiguous urine collection and those determined at 528 and 744 hr gave a total urinary recovery of 248 mg or 25% of the administered dose. This urinary recovery is similar to that reported by Maren *et al.* (6).

Much longer times would be required for urine collections following the single-dose administrations compared to the multiple-dose regimen to obtain fractional urinary recoveries similar to those predicted by the ratio of the renal and total clearances. The amount of methazolamide recovered in contiguous urine collections up to 96 hr after administration of the 150-mg dose was 19.3 mg. Based on the excretion rates determined from short-duration urine collections centered at 170, 319, and 649 hr, 39 mg (26% of the dose) would be expected to be obtained at 649 hr. Times far in excess of 649 hr would be required to obtain a reasonable estimate of the fractional urinary recovery, following administration of the 50-mg dose, solely on the basis of contiguous urine collections. The smaller the dose, the larger is the fraction of the dose that will be bound (reversibly) to the carbonic anhydrase because of the saturable nature of the binding. Thus, the smaller the dose, the longer are the periods required for most of the drug to be cleared because the enzyme effectively buffers the drug, permitting only small free concentrations of the drug to be cleared.

Metabolites—Approximately 75% of the dose was not accounted for as unchanged drug in the urine. Therefore, the urine was analyzed for the presence of metabolites. Deacetylated methazolamide, an intermediate in the synthesis of the internal standard, represented a possible metabolite. The type B isoenzyme of carbonic anhydrase has esterase activity (17). Hence, the isoenzyme might be expected to catalyze the hydrolysis of the acetylimino bond of methazolamide. Deacetylated methazolamide could be extracted with ethyl acetate from urine, converted to a trimethyl derivative, and detected by selective monitoring of the molecular ion (m/z 236). Deacetylated methazolamide was not detected in urine collected during the multiple-dose administration to Subject 3.

Besides deacetylated methazolomide, no other metabolites were detected using mass fragmentography and the described workup.

Methazolamide–Isoenzyme Dissociation Constants—The plasma and red blood cell concentrations obtained following administration of the 150-mg dose of methazolamide were used to estimate the dissociation constants of the isoenzyme–methazolamide complexes. The unbound plasma concentration (45% of the total concentration) was assumed to be in equilibrium with the intracellular free concentration at all times. The mass transport coefficient for the transport of the drug across the red blood cell membrane is ~7 liters/min based on the reported rate constant of 195 hr^{-1} (12). This clearance is much greater than the total body clearance, which is 60 ml/min based on the free plasma concentration. Therefore, a virtual equilibrium of drug between the plasma and intracellular water seems reasonable.

The concentration of intracellularly bound methazolamide was calculated by subtracting the product of the unbound plasma concentration and the fraction of cell water (0.63) from the total red blood cell con-

Table II—Total Body Clearances of Methazolamide

Dosage	Hours	Red Blood Cell Concentration, μg/ml	Plasma Concentration, μg/ml	Total Clearance[a], ml/min
100 mg tid	744	4.53	0.019	27.4
150 mg	1082	4.06	0.0076	27.6
50 mg	504	6.49	0.017	31.3

[a] Based on total plasma concentration.

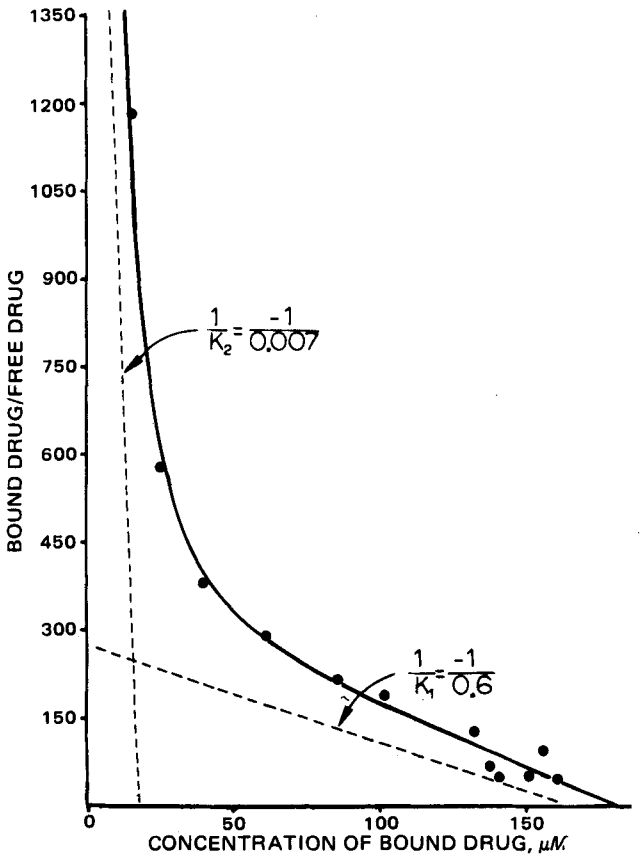

Figure 4—*Binding isotherm of methazolamide to red blood cells.*

centration. A Rosenthal plot (Fig. 4) was constructed by plotting the ratio of the bound drug concentration to the unbound drug concentration in the plasma against the bound drug concentration. The curvature of the plot indicates multiple binding sites for methazolamide. Since it was shown that there is one binding site per carbonic anhydrase molecule (9), the curvature may be a manifestation of the two isoenzymes of carbonic anhydrase present in the human erythrocyte. The binding curve was resolved by a graphical procedure into linear segments that reflect the two isoenzymes (18). The dissociation constants for the type B–methazolamide complex and the type C–methazolamide complex are 0.6 and 0.007 μM, respectively. The estimated concentrations of isoenzyme B and isoenzyme C are 165 and 18 μM, respectively.

The concentrations of methazolamide required to reduce the hydration rates of carbon dioxide by 50% (I_{50}) at 25° were reported to be 0.016 and 0.017 μM for reactions catalyzed by the type C and type B isoenzymes, respectively (6). Since the enzyme concentrations were maintained low relative to the measured I_{50} values (18), these inhibitory concentrations are similar to the dissociation constants of the respective methazolamide–enzyme complexes. The inhibitory activities of methazolamide on the carbonic anhydrase in red blood cells were similar at 4, 22, and 37° (10).

There is reasonably good agreement between the dissociation constant determined from the kinetic measurement and that determined from the binding analysis for the type C isoenzyme (0.016 and 0.007 μM, respectively). However, there is a large difference in these values for the type B isoenzyme (0.017 and 0.6 μM, respectively). Anions have been shown to reduce significantly the catalytic activity of isoenzyme B, whereas they have little effect on the catalytic activity of isoenzyme C (17). The concentration of chloride and bicarbonate ions in the red blood cell reduces the activity of isoenzyme B to 8% of that in the absence of the anions (17). Therefore, the inhibitory constant for isoenzyme B in the presence of chloride and bicarbonate ions is 0.2 μM, which is similar to the value determined from the Rosenthal plot.

The presence of the chloride and bicarbonate anions in the erythrocyte influences the distribution of the drug in the physiological situation. In principle, the same fractional inhibition of a given isoenzyme in various tissues should result at equilibrium. Thus, if 99% inhibition of the type C isoenzyme in the secretory cells of the ciliary process is achieved, a requirement for substantial intraocular pressure lowering (6), the type C isoenzyme in the erythrocyte will be 99% inhibited. However, the percent inhibition of the type B isoenzyme in the erythrocyte will be significantly less due to the presence of the anions, even though the intrinsic binding constants (binding constants in the absence of anions) of the two isoenzymes are similar.

The dissociation constant for acetazolamide and human red blood cell carbonic anhydrase (presumably type B) was determined from *in vitro* equilibration studies; a mean value of 1.6 μM was reported (19). This value is approximately an order of magnitude larger than the I_{50} value of 0.25 μM reported by Maren *et al.* (6). This trend is exactly the same as was observed with methazolamide and, again, probably is the result of competition by the intracellular anions, yielding an apparent dissociation constant that is larger than the true dissociation constant.

Influence of Binding on Decline in Plasma and Red Blood Cell Concentrations—Krüger-Thiemer and coworkers (20, 21) discussed the influence of protein binding on the temporal patterns of plasma and plasma water concentrations of sulfonamide-type drugs. The diminishing steepness in the log plasma concentration–time curves was explicable in terms of the nonlinearity introduced by protein binding. The similar curvature in the temporal patterns of the plasma methazolamide concentrations is probably a manifestation of the binding of the drug to carbonic anhydrase rather than to plasma proteins. The fractional binding to plasma proteins has been reported to be constant over the therapeutic range of plasma concentrations.

The decline in plasma concentrations following the administration of the 10th dose to Subject 3 can be fitted to a model in which the distribution of the drug is rapid relative to the elimination, the elimination is proportional to the free concentration of drug, and the binding of the drug to the two isoenzymes is described by the law of mass action. Similar models were discussed by other investigators (20, 22, 23). The differential equation describing such a model is:

$$\frac{d \ln C_f}{dt} = -\frac{Q_B}{V + \frac{(1-f)}{f} V_p + \sum_{i=1}^{2} \frac{E_i K_i}{(K_i + C_f)^2}} \qquad \text{(Eq. 5)}$$

and the integrated equation is:

$$t = \frac{1}{Q_B}\left\{\left[V + \frac{(1-f)}{f} V_p\right] \ln (C_f/C_f^0) + \sum_{i=1}^{2} (E_i/K_i) \ln \times \left[\frac{C_f(K_i + C_f^0)}{C_f^0(K_i + C_f)}\right] + \sum_{i=1}^{2} E_i \frac{C_f^0 - C_f}{(K_i + C_f)(K_i + C_f^0)}\right\} \qquad \text{(Eq. 6)}$$

where Q_B is the total body clearance expressed relative to the free plasma concentration, V is the volume of distribution of free drug, f is the fraction unbound in the plasma, V_p is the volume of distribution of serum albumin (24), E_i is the molar amount of isoenzyme i, K_i is the dissociation constant of the isoenzyme i–drug complex, C_f^0 is the intial free plasma concentration, and $C_f(t)$ is the free plasma concentration at time t. The times for the observed plasma concentrations were evaluated using the values of the parameters shown at the bottom of Table III, varying V, E_1, and E_2 to obtain the best fit to Eq. 6. The predicted times and the sampling times, expressed relative to the time of the initial concentration, are presented in Table III.

Table III—Predicted and Observed Times [a] in the Decline of Plasma Concentrations following the 10th Dose of the Multiple-Dose Regimen [b]

	Free Plasma Concentration, μM							
	18.2	12.2	9.05	4.10	1.18	0.743	0.0544	0.0362
Calculated from Eq. 6	0	4.45	7.94	18.2	47.3	69.5	480	616
Experimentally determined	0	4	8	20	44	68	452	668

[a] Values are in hours. [b] The parameter values were: Q_B = 3.5 liters/hr, f = 0.45, V = 30 liters, V_p = 6.6 liters, E_1 = 500 μmoles, K_1 = 0.6 μM, E_2 = 150 μmoles, and K_2 = 0.007 μM.

The agreement between the predicted and adjusted sampling times is reasonably good. However, a volume of distribution of free drug of 30 liters rather than the volume of total body water (50 liters) was used to obtain the predicted times. Also, the total molar amount of carbonic anhydrase (650 μmoles) employed was intermediate between the amount suggested by Maren *et al.* (6) (≃400 μmoles) and the amount (≃900 μmoles) suggested by Lehmann *et al.* (14). However, recognizing the limitations of such a simple model, it nevertheless strongly suggests that carbonic anhydrase acts as the storage site for the drug.

The plasma concentrations following the administration of methazolamide in general will not decline as a simple exponential function of time, even if the drug distributes rapidly and the clearance is concentration independent. The interaction of the drug with the carbonic anhydrase masks this behavior. When the free concentrations are such that the concentration-dependent volume terms are made small relative to the concentration-independent volume terms (see denominator of Eq. 5), the plasma concentrations appear to decline in a simple exponential fashion over the initial portion of the time course. Under these conditions, the predicted half-life is 7.5 hr, using the parameters at the bottom of Table III. This predicted half-life is approximately the same as the half-life observed over the first three data points of the decline where the free plasma concentrations are large relative to K_1 (Fig. 2). This half-life was called the intrinsic half-life by Krüger-Thiemer *et al.* (21); in this simple model, it is the half-life for elimination not masked by the drug–macromolecular interaction.

When the free plasma concentration has declined into the range of the dissociation constant of the isoenzyme B complex, the buffering effect of the isoenzyme on the free plasma concentration becomes apparent. The ratio of the bound concentration to the free concentration increases as the plasma concentration decreases with time. The apparent volume of distribution of free drug (total amount in the body divided by the free concentration) increases as the ratio increases (23). The apparent volume of distribution of free drug (V_{df}) for this system is:

$$V_{df} = V + \frac{(1-f)}{f} V_p + \sum_{i}^{2} \frac{E_i}{K_i + C_i} \qquad \text{(Eq. 7)}$$

The reciprocal of the expression on the right side of Eq. 5, which can be considered as the instantaneous time constant, is not simply V_{df}/Q_B. At least for those times when the volume term, which is dependent on the interaction of the drug with isoenzyme 2, is negligible, the denominator in Eq. 5 can be expressed as a relatively simple function of V_{df}, namely:

$$V_f + \frac{K_1}{E_1}(V_{df} - V_f)^2$$

where:

$$V_f = V + \frac{(1-f)V_p}{f} \qquad \text{(Eq. 8)}$$

and:

$$V_{df} = V_f + \frac{E_1}{(K_1 + C_f)} \qquad \text{(Eq. 9)}$$

Thus, as the apparent volume of distribution increases, the instantaneous time constant increases from V_f as a function of the square of the difference between the apparent volume of distribution and V_f. Since the instantaneous time constant increases as a function of an increase in the apparent volume of distribution, which in turn increases with a decrease in the plasma concentration, the slope of the log plasma concentration–time curve decreases strongly with a decrease in the plasma concentration in the transition region.

During the later time periods, the exceedingly slow decline in the plasma concentration is controlled by the strong binding of the drug to the type C isoenzyme. In principle, the plasma concentrations will decline exponentially as a function of time, with a half-life greatly in excess of the intrinsic half-life, when the free plasma concentration becomes small relative to the dissociation constant of the isoenzyme C–drug complex (22, 23). Plasma concentrations more than an order of magnitude less than those observed in the present study are required to observe this phenomenon.

The persistence of methazolamide in the red blood cells 1082 hr after administration of the 150-mg dose is a manifestation of the strong binding between the drug and the high activity type C carbonic anhydrase. At this time, the red blood cell concentration was 4.1 μg/ml; therefore, the red blood cells still contained 6.2% of the dose. The corresponding plasma concentration was 7.6 ng/ml (Table II). Drug is eliminated from the body very slowly, because the intracellular concentration of freely diffusible inhibitor in the erythrocytes and other tissues containing the enzyme is maintained small by the strong interaction of the drug with carbonic anhydrase.

Red blood cell and plasma concentrations were studied as a function of time in dogs administered acetazolamide intravenously (8). Although plasma concentrations were not measured directly after 24 hr because of the lack of assay sensitivity, plasma concentrations were calculated from the urinary excretion rates and the clearance. When the plasma concentration declined to a level such that the intracellular concentration of freely diffusible inhibitor was only a few percent of the total red blood cell concentration, the drug in the red blood cells acted as a source for that in the plasma. The total red blood cell concentration then declined slowly because of the small intracellular concentration of freely diffusible inhibitor.

An exceedingly slow decline ($t_{1/2}$ = 120 days) in human blood of an anticonvulsant drug, 2-amino-4-phenyl-sulphenylbenzenesulfonamide, was reported (25). The drug was shown to bind strongly to both isoenzymes of carbonic anhydrase in the human erythrocyte.

Usefulness of Methazolamide in Comparison to Acetazolamide—Methazolamide and acetazolamide inhibit the high activity type of carbonic anhydrase (type C) present in the ciliary body to the same degree (6). The plasma protein binding of methazolamide is significantly less than that of acetazolamide, 55 compared to 93%, respectively, in the therapeutic range of plasma concentrations (6, 11). Therefore, if the mechanism for the reduction in aqueous secretion and, hence, intraocular pressure were due solely to the inhibition of carbonic anhydrase, equal concentrations of the freely diffusible inhibitors, or a total plasma concentration of methazolamide, only 15% that of acetazolamide would be required for an equivalent response at steady state. Furthermore, since the membrane permeability of methazolamide is at least one order of magnitude greater than that of acetazolamide (12), an even greater disparity may exist in the dosage requirements with the pulsed delivery offered by conventional dosage regimens. Maren *et al.* (6) showed that methazolamide is distributed more uniformly than acetazolamide in rat tissues after oral administration of the drugs.

The systemic acidosis that results from a decrease in bicarbonate reabsorption due to the inhibition of renal carbonic anhydrase contributes to the reduction in intraocular pressure (6, 16). The resulting systemic acidosis is greater for acetazolamide than for methazolamide at equivalent plasma concentrations and was postulated to be due to the active secretion and accumulation of acetazolamide, but not methazolamide, in the proximal tubule (6).

A correlation has been demonstrated between side effects sufficiently severe to cause discontinuation of therapy and the degree of systemic acidosis (plasma carbon dioxide combining power of <21 mEq/liter; control, 27 mEq/liter) following multiple-dose regimens of either acetazolamide or methazolamide (26). The studies of Maren *et al.* (6) and Stone *et al.* (16) showed that the side effects became more severe with an increase in the acidotic state. Maren *et al.* (6) also suggested that the formation of renal stones may be due to the decrease in urinary output of citrate that occurs with an increase in systemic acidosis.

Thus, as pointed out previously (6, 16), low dose regimens of methazolamide, in which the side effects are minimized, rather than the conventional regimens of acetazolamide would be a rational therapy in patients whose intraocular pressures are controlled by the direct action of the inhibitor on the ciliary body carbonic anhydrase.

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^{13}C-NMR Experimental Methods for Determination of Resonance Multiplicities: *Strychnos* Alkaloids

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Abstract □ ^{13}C-NMR spectral assignments of the *Strychnos* alkaloids brucine and strychnine have been reported by numerous investigators. One recent report contained several disparities in the assignments that were attributable to incorrect determinations of spin multiplicities. The source of the inaccuracies in the spin-multiplicity determinations of very complex molecules is discussed, and several additional techniques for the determination of these multiplicities are described that are less subject to interpretational errors than the conventional and routinely employed single-frequency off-resonance decoupling methods. These procedures are applied using brucine as a representative example.

Keyphrases □ ^{13}C-NMR spectroscopy—determination of resonance multiplicities, brucine □ Brucine—determination of resonance multiplicities □ Resonance multiplicities—brucine

Several recent reports dealt with the ^{13}C-NMR spectral assignments of strychnine, brucine, and related *Strychnos* alkaloids (1–9). The assignments in one report (8) for several resonances in both strychnine and brucine are in direct conflict to those reported previously. It is possible that errors occurred in this work that were due to the incorrect determination of spin multiplicities for the resonances in question.

The results obtained from two complementary methods that can provide unambiguous determination of the resonance multiplicities in even the most complex ^{13}C-NMR spectra are presented using brucine as an example. These methods are alternatives to conventional single-frequency off-resonance decoupling techniques and can be executed on modern Fourier transform spectrometers with only minor modification.

RESULTS AND DISCUSSION

A spectral parameter that is readily accessible for the pulsed Fourier transform technique and that provides an alternative method for the determination of spin multiplicities is the spin-lattice relaxation time, T_1 (10–12). Experimental evidence strongly supports the premise that in molecules of moderate size, the ^{1}H–^{13}C dipolar mechanism is predominantly responsible for ^{13}C-NMR relaxation. On this basis, for molecules that tumble at rates in excess of the motional narrowing limit (Larmor resonance frequency), the relaxation rate may be expressed as:

$$1/T_1^{DD} = N\hbar^2\gamma_C^2\gamma_H^2 r_{CH}^{-6}\tau_c \qquad \text{(Eq. 1)}$$

where γ_C and γ_H represent the magnetogyric ratios of the ^{13}C and ^{1}H nuclei, respectively, and $\hbar^2$ is Planck's constant. Thus, relaxation is determined by the terms Nr_{CH}^{-6} and τ_c, where the former term is modulated by the number of protons at specific internuclear distances (typically, relaxation is mediated by the directly attached protons, if any), while the latter term is the reorientational correlation time, typically 10^{-12}–10^{-10} sec for natural products of moderate molecular weight (*i.e.*, mol. wt. 250–1000) (13).

A further requirement is that the molecule tumble in a random fashion (isotropic), which can strictly be true only for spherical molecules or close approximations such as adamantane (14). In practice, however, the relaxation of a relatively large number of molecules has been found to conform to this simple requirement, thereby allowing the use of Eq. 1 rather than the more complex equations necessary to describe ordered (anisotropic) reorientation. This behavior leads to the very useful result that the relaxation time of a given carbon is inversely proportional to the number of directly attached protons. Thus, in relatively rigid cyclic compounds, methylene resonances are expected to undergo relaxation at a rate twice as fast as methine carbons. In contrast, methyl groups are capable of an internal reorientation in addition to the overall isotropic reorientation, which imparts a different reorientational correlation time, τ_i (referred to as τ_μ by some investigators), typically on the order of 10^{-13}–10^{-11} sec, resulting in longer relaxation times. In the extreme case, these relaxation times can be up to three times as long as those observed for methine resonances.

In complex molecules in which large numbers of carbon resonances have substantially similar chemical shifts, thereby preventing the unambiguous interpretation of single-frequency off-resonance decoupling spin multiplicities, relaxation considerations present a viable alternative for the demonstration of this information. This consideration is especially useful where a methylene carbon bears nonequivalent (anisochronous)

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