Kinetics of Partitioning and Binding of Digoxin and Its Analogues in the Subcompartments of Blood

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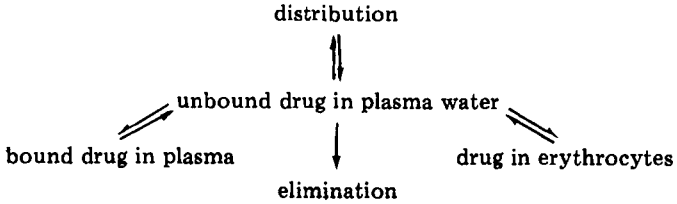
Abstract □ The kinetics of digoxin and analogues (metabolites) in the subcompartments of blood, erythrocytes and plasma, were first order and concentration independent. The rate and extent of red blood cell partitioning of the tested compounds were, according to first-order kinetics, identical in the presence and absence of structurally related compounds and dependent on lipophilicity and temperature; red blood cell penetration was a reversible process. Erythrocyte partitioning of digoxin and its analogues was postulated to be by passive diffusion. There was significant intracellular binding of the compounds, and hemoglobin was the major ligand. The kinetics of red blood cell partitioning of the individual compounds were fitted according to a closed two- or three-compartment model. The latter indicated the existence of two kinetically separable compartments within the red blood cells. Model-independent mean transit times of red blood cell–plasma water partitioning of the compounds depended largely on the lipophilicity and were a thousand times greater for the least lipophilic analogues than for the most lipophilic derivatives. Red blood cell partitioning of digoxin and its metabolites under *in vivo* and *in vitro* conditions, were equivalent. Plasma protein binding of the tested compounds was concentration independent, unaltered in the presence of the analogues, and temperature independent between 24°C and 37°C. Binding to albumin in buffer solution was significantly larger than to albumin in plasma for all the compounds. Binding of digoxin and its analogues to albumin in buffer solution increased with increasing lipophilicity of the compounds.

Keyphrases □ Digoxin—kinetics of red blood cell partitioning, plasma protein binding, analogues □ Red blood cell partitioning—digoxin and analogues, kinetics, plasma protein binding □ Plasma protein binding—digoxin and analogues, kinetics of red blood cell partitioning □ Kinetics—digoxin and analogues in blood, plasma protein binding, red blood cell partitioning

The overall goal of the present study was the further (1) elucidation of the kinetics of digoxin (I) and its analogues (*i.e.*, metabolites) in the subcompartments of blood, plasma and red blood cells (Scheme I). The individual goals were to (*a*) delineate the kinetics of red blood cell partitioning, (*b*) investigate the influence of temperature and drug lipophilicity on cellular uptake, (*c*) assess the gross intracellular distribution of the compounds, and (*d*) make a comparative study of the binding of the compounds to albumin and plasma protein.

Special emphasis was put on the delineation of the kinetics of red blood cell partitioning of the tested compounds. A review of the pharmacokinetic literature on this subject indicated that the significance of the kinetics of drugs in this subcompartment of blood has not been adequately appreciated by pharmacokineticists. The extent of red blood cell uptake has been determined for drugs in some studies; the rate of such uptake has been studied only exceptionally (2). Often tacit assumptions have been made that either red blood cell partitioning of the drug is negligible or equilibration between plasma and cells is instantaneous if drug uptake into the erythrocytes is significant. Concentrations of drugs usually have been assessed in plasma, not whole blood, in pharmacokinetic studies. Obviously, temperature-independent red blood cell partitioning has been presumed for the drugs in most of the studies, since no effort appears to have been made to maintain the temperature of the blood samples after their withdrawal until separation of plasma and erythrocytes. This paper will show conclusively that these *a priori* assumptions are unwarranted and may lead to biased values of pharmacokinetic parameters.



Scheme I—Drug in the blood compartments.

EXPERIMENTAL SECTION

Materials—The following unlabeled and labeled compounds were used: digoxin[1] (I), digitoxin[1] (II), β-methyldigoxin[1] (III), dihydrodigoxin[1] (IV), digoxigenin bis(digitoxoside)[1] (V), digoxigenin digitoxoside[1] (VI), epidigoxigenin[1], digitoxigenin bis(digitoxoside)[1], digitoxigenin digitoxoside[1], digoxin-16′-glucuronide[2] (VII), [12α-^{3}H]digoxin[1], β-methyl[12α-^{3}H]digoxin[1], [12α-^{3}H]digoxigenin bis(digitoxoside)[1], [12α-^{3}H]digoxigenin digitoxoside[1], [21,22-^{3}H]digitoxin[2], dihydro[12α-^{3}H]digoxin[2], and [12α-^{3}H]digoxin-16′-glucuronide[2]. The compounds with the label in the α-position were synthesized according to the method of von Wartburg *et al.* (3) and had the following specific activities: [^{3}H]I, 1460 μCi/mg; [^{3}H]III, 910 μCi/mg; [^{3}H]IV, 12,772 μCi/mg; [^{3}H]V, 552 μCi/mg; [^{3}H]VI, 457 μCi/mg; [^{3}H]VII, 1570 μCi/mg. [^{3}H]II was synthesized according to the method of Haberland and Merten (4) and had a specific activity of 509 μCi/mg. The radiochemical purities of the labeled compounds were investigated using different TLC systems, as described elsewhere (5, 6), and were: [^{3}H]I, >94.5%; [^{3}H]II, 100%; [^{3}H]III, >95.9%; [^{3}H]IV, 100%; [^{3}H]V, >92.5%; [^{3}H]VI, >95.9%; [^{3}H]VII, >98.0%.

n-Octanol was obtained commercially[3]; heparinized (5 USP U/mL) or citrated (3.69 mg/mL sodium citrate and 0.46 mg/mL citric acid) blood and plasma samples were obtained from healthy volunteers who had no prior drug intake[4] for 168 h. Human albumin (fraction V, fatty acid free) (AB_1[5] AB_2[6]) and hemoglobin (HB)[5] (type IV, crystalized twice) were purchased. Three different buffers were used: (*a*) 0.067 M KH_2PO_4, 0.067 M Na_2HPO_4, and 0.113 M NaCl (pH 7.4, 0.300 Osmol, ionic strength: 0.23) (7), (*b*) 0.001 M KH_2PO_4, 0.001 M Na_2HPO_4, 0.157 M NaCl, 0.006 M glucose, 0.004 M KCl, 0.003 M $CaCl_2$, and 0.002 M $MgCl_2$ (pH 7.2, 0.356 Osmol, ionic strength: 0.18), and (*c*) 0.05 M Tris, 0.042 M HCl, and 0.060 M NaCl (pH 7.4, 0.304 Osmol, ionic strength: 0.15). Buffers b and c represented a slight modification of the original buffers employed previously (8, 9).

Determination of Apparent Octanol–Water Partition Coefficients of Digoxin and Analogues—Partition coefficients were determined for [^{3}H]I–[^{3}H]VII at 24°C using twice-distilled water ([^{3}H]I–[^{3}H]VI) or phosphate buffer *a* ([^{3}H]VII). The octanol was allowed to equilibrate with water with gentle shaking for 24 h. Only water-saturated octanol and octanol-saturated water were used in the partitioning experiments. The experiments were performed in 15-mL tubes equipped with glass stoppers. The compounds were added to tubes containing, initially, only the water phase (2.5 mL) spiked with drug concentrations of 40 ng/mL ([^{3}H]I, [^{3}H]III–[^{3}H]VI), 100 ng/mL ([^{3}H]II), and 200 ng/mL ([^{3}H]VII); the octanol phases were added subsequently. The stoppered tubes were then shaken vigorously for 45 min. Preliminary experiments had indicated that this time permitted the compounds to reach equilibrium. After centrifugation, the phases were separated and aliquots (200 μL) were taken from both phases for determination of radioactivity. Two experiments were performed with each of the compounds tested.

[1] Boehringer Mannheim, FRG.
[2] Beiersdorf, Hamburg, FRG.
[3] Fluka AG, Buchs, Switzerland.
[4] Blood Bank, Kantonsspital, Basel, Switzerland.
[5] Miles Laboratories, Elkhart, Ind.
[6] Sigma Chemical Co., St. Louis, Mo.

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Table I—Apparent Erythrocyte–Buffer and Erythrocyte–Plasma Partition Coefficients for Digoxin and Analogues at Different Temperatures[a]

Compounds[b]	37°C	n[c]	24°C	n[c]	14°C	n[c]	4°C	n[c]
[^{3}H]II	2.51 ± 0.061	3	1.95 ± 0.097	5	1.59	1	1.23 ± 0.124	8
	0.090 (0.084, 0.097)	2	0.055 (0.046, 0.064)	2				
[^{3}H]III	1.28 (1.23, 1.33)	2	1.11 ± 0.027	3			0.877 ± 0.095	5
	0.946 (0.917, 0.976)	2	0.882 (0.842, 0.922)	2				
[^{3}H]IV	1.47 (1.44, 1.49)	2	1.34 ± 0.027	4			0.900 ± 0.104	4
	1.09 (1.03, 1.15)	2	1.02 ± 0.013	4				
[^{3}H]I	1.49 ± 0.031	3	1.29 ± 0.056	7			0.884 ± 0.032	7
	1.13 (1.06, 1.19)	2	0.997 ± 0.026	3				
[^{3}H]V	1.31 (1.30, 1.32)	2	1.15 ± 0.061	4			0.875 ± 0.009	4
	1.01 (0.95, 1.07)	2	0.862 ± 0.010	3				
[^{3}H]VI	1.21 ± 0.031	4	1.19 ± 0.032	3	1.02	1	0.932 ± 0.043	3
	0.966 ± 0.028	3	0.838 ± 0.045	3				
[^{3}H]VII	0.762	1						
	0.625	1						

[a] Mean ± *SD*; individual values are reported in parentheses. The upper value is P_{e/c_u}; the lower value is $P_{e/c}$. [b] In order of decreasing lipophilicity. [c] Number of mean values considered; averages of 3–5 individual determinations in blood of 1–7 donors.

Erythrocyte Partitioning of Digoxin and Analogues—The goals of these experiments were to investigate the red blood cell–buffer or –plasma distribution of the compounds prior to and at partition equilibrium, and to determine whether the partitioning was temperature dependent. The experiments used erythrocytes obtained from different donors (n = 2–8) for the partitioning of each individual compound.

Erythrocyte suspensions in buffer *a* or in plasma, with physiological hematocrits of 40% (10), were employed. The procedure used for the determination of drug partitioning into red blood cells was the centrifugal method, which has previously been described in detail (7). Briefly, the suspensions had volumes of 10 mL and were kept in 15-mL glass-stoppered tubes. Immediately after drug addition, the tubes containing the suspensions were shaken vigorously for 15 s and were then rotated for the duration of the experiments. Samples of the suspensions were taken as early as possible following drug addition (≥0.3 min) and at appropriate intervals thereafter. The samples were immediately centrifuged for intervals ranging from 0.5 (early samples) to 5 min (later samples). The time of removal of the separated buffer or plasma phases was taken as the sampling time. The concentrated erythrocyte phases had hematocrits ranging between 50 and 95%. Aliquots (100–500 μL) were taken from the separated erythrocyte, buffer, or plasma phases, and the radioactivity was determined.

Erythrocyte buffer partitioning studies were conducted at 37°C, 24°C, and 4°C with all the labeled compounds; additional experiments at 14°C were performed with [^{3}H]II and [^{3}H]VI. Erythrocyte plasma partitioning was investigated at 37°C and 24°C with all the labeled compounds. The temperatures were maintained throughout the experiments, including the final centrifugation of the red blood cell suspensions. The pH was in the 7.35–7.45 range. Only fresh erythrocyte suspensions were used. For a delineation of the type of kinetics involved, the partitioning of all the compounds with the exception of [^{3}H]VII was investigated systematically at 24°C at drug concentrations of 5, 10, and 100 ng/mL. Additional experiments at 24°C studied the red blood cell partitioning of the (labeled) compounds (10 ng/mL) in the presence of the (unlabeled) analogues (100 ng/mL each). Reversibility of red blood cell partitioning was checked at 24°C by resuspending drug-containing erythrocytes in fresh buffer *a* (11).

The stability of the compounds during their incubation with erythrocytes in buffer or plasma at 37°C was checked for all compounds except [^{3}H]II. Plasma and buffer aliquots taken at different times after incubation were analyzed by specific column chromatography or TLC, as described previously (6). The results showed that the compounds were stable under these experimental conditions. Estimates for the precision of the red blood cell partitioning values were obtained by repetitive determination of the ratios of the erythrocytes to buffer or plasma concentrations in suspensions with red blood cells from a particular donor. The coefficient of variation (CV) values ranged between 1 and 8% for the compounds, except [^{3}H]II. Considerably larger CV values (20–80%) were obtained for this compound with red blood cell plasma suspensions. This was due to the comparatively low erythrocyte concentrations of this drug in the plasma suspensions.

The possible influence of buffer composition on red blood cell partitioning of [^{3}H]I and its analogues was also studied. Red blood cells from different donors were divided up in equal parts and suspended either in buffer *a* or buffer *c*. Red blood cell partitioning from the two buffers at 37°C was then studied with all the compounds except [^{3}H]VII. The results of these experiments showed that red blood cell partitioning from buffer *a* was significantly larger than from buffer *c* for all the compounds tested, although the respective mean red blood cell-to-buffer concentration ratios differed by only 1–15%. These results indicated that red blood cell partitionings of the nonelectrolytic [^{3}H]I–[^{3}H]VI were influenced by buffer composition. Differences in ionic strength, pH, and molecular composition of the two buffers used may have been responsible for the observed discrepancy.

Only buffer *a* was used in the definitive partitioning experiments with [^{3}H]I and its analogues. Buffer *a* was preferred because it had been used successfully in the past (7, 12) in equivalent experiments with other compounds and guaranteed a pH equivalent to that in plasma water (13).

***In Vivo* Erythrocyte Partitioning of Digoxin and Metabolites**—Tritiated digoxin (0.6 and 1.2 mg) was administered intravenously (bolus) and orally to three healthy volunteers aged 21–25 years. Blood was obtained over a period of 144 h after administration of the drug. After blood withdrawal, there was a lapse of 30 min before separation of the plasma and erythrocytes. During this interval the samples were kept at a temperature of 24°C. Plasma of the samples was then divided into two parts. In one part [^{3}H]I and its separable, known, apolar metabolites ([^{3}H]IV–[^{3}H]VI) and unknown polar metabolites were assayed by specific methods (6); in the other part, total radioactivity was measured. Total radioactivity was also determined in the separated erythrocytes of the samples.

Binding of Digoxin and Analogues to Hemolysate, Hemoglobin, Albumin, and Plasma Protein—Details of the equilibrium dialysis method employed in these investigations have been reported previously (1). The experiments were performed at 37°C or 24°C and within a pH range of 7.30–7.50. The hemolysate suspensions and the protein solutions were dialyzed against buffer for 6.5 h. Equilibrium between the free drug concentrations in plasma and buffer was established within this time interval. Aliquots (100–500 μL) were then taken from both chambers of the dialysis apparatus, and the radioactivity was determined.

The binding to hemolysate and albumin was investigated for all the compounds. The binding to hemoglobin was studied with [^{3}H]I and [^{3}H]II. Plasma protein binding was investigated with [^{3}H]II, [^{3}H]III, and [^{3}H]VII. The results obtained with [^{3}H]I and [^{3}H]IV–[^{3}H]VI have been reported previously (1).

Hemolysate Binding—Red blood cells from one donor were separated from

Table II—Linearity of the Red Blood Cell Partitioning Kinetics of Digoxin and Analogues at 24°C

Compound[a]	P_{e/c_u}[b]			
	5 ng/mL	10 ng/mL	10 ng/mL[c]	100 ng/mL
[^{3}H]II	1.82 (1.75, 1.87)	2.03 (2.01, 2.06)	1.93 (1.88, 1.97)	2.06 (2.01, 2.10)
[^{3}H]III	1.10 (1.05, 1.14)	1.13 (1.13, 1.13)	1.19 (1.07, 1.30)	1.13 (1.13, 1.13)
[^{3}H]IV	1.39 (1.38, 1.39)	1.36 (1.33, 1.39)	1.31 (1.30, 1.33)	1.35 (1.32, 1.39)
[^{3}H]I	1.29 (1.22, 1.36)	1.35 (1.33, 1.37)	1.26 (1.24, 1.28)	1.31 (1.24, 1.37)
[^{3}H]V	1.15 (1.08, 1.22)	1.14 (1.13, 1.15)	1.17 (1.10, 1.24)	1.19 (1.16, 1.21)
[^{3}H]VI	1.19 (1.13, 1.25)	1.20 (1.18, 1.22)	1.23 (1.22, 1.24)	1.23 (1.22, 1.23)

[a] In the order of decreasing lipophilicity. [b] n (Number of individual values considered) = 2, obtained from one donor. Individual values are in parentheses. [c] Molar ratio of labeled drug to each of the unlabeled analogues = 0.1.

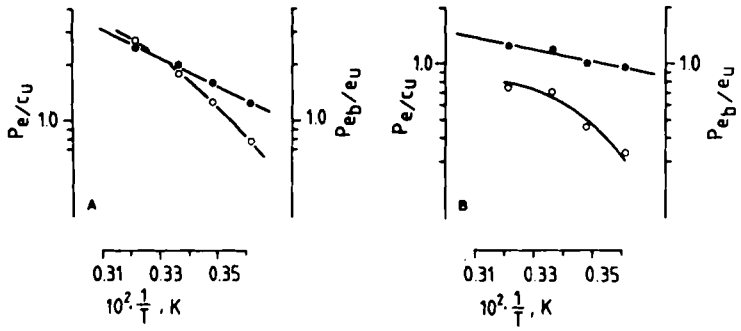
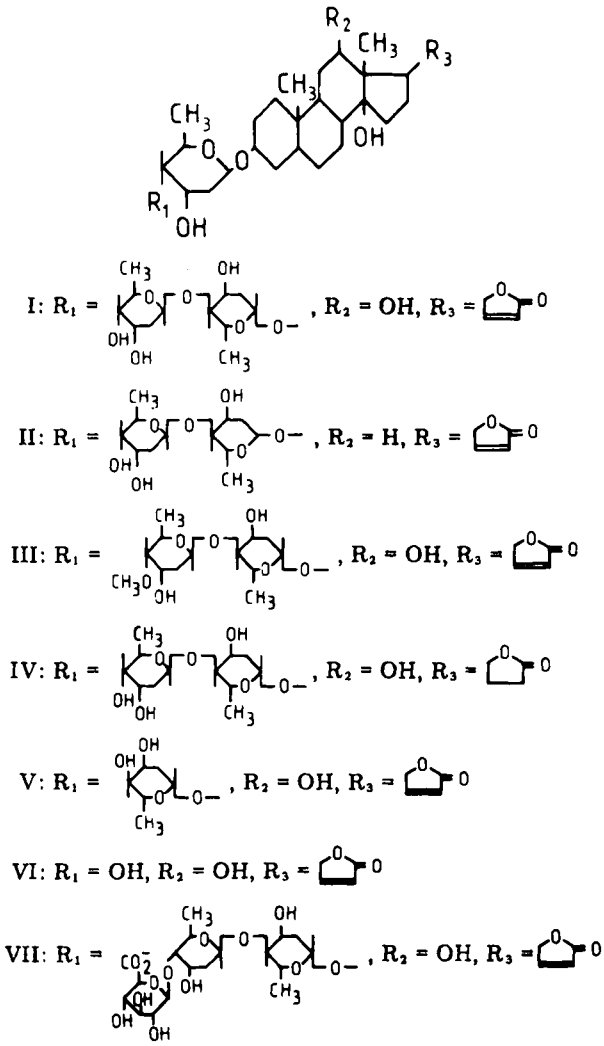


Figure 1—*Semilogarithmic plot of the averaged apparent red blood cell-buffer partition coefficient, P_{e/c_u} (●), and the averaged true red blood cell partition coefficient, P_{e_b/e_u} (○) against the reciprocal of the absolute temperature 1/T for [^{3}H]II (A) and [^{3}H]VI (B). There was an apparent linear dependency of P_{e/c_u} on 1/T in contrast to a nonlinear relationship between P_{e_b/e_u} and 1/T.*

plasma by centrifugation. After removal of the buffy coat, the cells were washed five times and suspended in buffer *a*. Lysis was then induced by exposing the red cells to a temperature of −20°C for 2 h. Membrane-free hemolysates (hemoglobin concentration 140 g/L) were then obtained by ultracentrifugation[7] at 20,000 rpm for 20 min. Binding and/or partitioning to hemolysate was determined for [^{3}H]II (20, 50, and 100 ng/mL) and [^{3}H]I and [^{3}H]III-[^{3}H]VII (20 ng/mL) at 37°C. There was significant net transport of fluid during dialysis, leading to a 10–20% decrease of the initial hemoglobin concentrations in the hemolysates. From 2 to 6 dialysis experiments were run in parallel with the hemolysates for each compound.

Hemoglobin Binding—The binding to hemoglobin was investigated with [^{3}H]I and [^{3}H]II (20 ng/mL) by dialysis against buffer *a* at 37°C. There was significant net transport of fluid during dialysis leading to a 10–20% decrease of the initial hemoglobin concentrations of 140 g/L. The binding to diluted hemoglobin (7 g/L) was also investigated. There was no net transport of fluid in these experiments. Two dialysis experiments were run in parallel.

Albumin Binding—Albumin binding was studied with all the compounds



[7] Sorvall Ultracentrifuge, RC-2B; Ivan Sorvall Inc., USA.

(20 ng/mL) at a physiological concentration of the protein (45 g/L) (14). Buffer *a* was used, and the experiments were conducted at 37°C. Albumin AB_1 and AB_2 were employed in these studies: the binding of [^{3}H]II was with both albumins, the binding of [^{3}H]I and [^{3}H]IV-[^{3}H]VI with albumin AB_1, and the binding of [^{3}H]III and [^{3}H]VII with albumin AB_2. Between 2 and 10 dialysis experiments were run with albumin for each compound. The *CV* values of replicate albumin binding determinations were 1% for [^{3}H]II and 3–7% for the rest of the compounds.

Preliminary experiments had shown that binding of [^{3}H]I (20 ng/mL) to albumin was not influenced by the buffer used. [^{3}H]I was 34.2 ± 2.67% (n = 10) and 35.9% (35.2, 36.6; n = 2) bound to albumin when buffers *a* and *c* were used, respectively. This suggested that the [^{3}H]I-albumin interaction was not influenced by the phosphate ion concentration of buffer *a*.

Plasma Protein Binding—Plasma with physiological albumin content (42–54 g/L) (14) obtained from different donors was used. The binding of [^{3}H]III and [^{3}H]VII was studied at concentrations of 5, 10, and 100 ng/mL (initial concentrations after spiking plasma prior to initiation of dialysis) with the plasma of two donors. [^{3}H]II was investigated at concentrations of 100 and 1000 ng/mL with the plasma of seven donors. In addition, the plasma binding of these three labeled compounds in the presence of unlabeled analogues was studied. The plasma binding of [^{3}H]III and [^{3}H]VII (5 ng/mL) was studied in the presence of all the other analogues (unlabeled I–VII, 100 ng/mL each), and the plasma binding of [^{3}H]II (5 and 100 ng/mL) was determined in the presence of unlabeled digitoxigenin bis(digitoxoside) and digitoxigenin digitoxoside (100 ng/mL each). From 4 to 8 dialysis experiments were run in parallel with the plasma of a particular donor for each compound. The *CV* values for replicate determinations of the plasma protein binding were 1% for [^{3}H]II and 7–15% for the rest of the compounds.

Analytical Procedures—The details of the liquid scintillation method used have been given previously (10). Briefly, 100–500-μL aliquots from plasma buffers *a*, *b*, and *c* and octanol were analyzed in duplicate, either directly[8] or after combustion[9]. The radioactivity in erythrocytes, hemoglobin solutions, and hemolysates was measured only after combustion. The red blood cells were lysed prior to combustion. The hemoglobin concentrations in the hemoglobin and hemolysate binding studies were measured spectrophotometrically after transformation to hemoglobin cyanide (15).

RESULTS

The ± values given in the text and in the Tables refer to the standard deviation, *SD*, of the means, and n is the number of observations considered. Values for *SD* and n are only given in the text for mean values which are not listed in the Tables.

Octanol–Water Partition Coefficients of Digoxin and Analogues—Apparent octanol–water partition coefficients at 24°C (P) of [^{3}H]I and its analogues were calculated from the ratio of the concentration in the organic phase to the concentration in the aqueous phase at equilibrium. The mean P values for the individual compounds were (n = 2): [^{3}H]I, 16.6 (16.5, 16.7); [^{3}H]II, 98.2 (96.8, 99.5); [^{3}H]III, 38.6 (38.3, 38.8); [^{3}H]IV, 17.6 (17.5, 17.6); [^{3}H]V, 15.6 (15.2, 16.0); [^{3}H]VI, 11.6 (11.4, 11.8); [^{3}H]VII, 0.024 (0.023, 0.024).

Erythrocyte Partitioning of Digoxin and Analogues—*Equilibrium Kinetics*—Red blood cell–buffer and red blood cell–plasma partition coefficients, P_{e/c_u} and $P_{e/c}$, respectively, were obtained for the compounds under different experimental conditions and are listed in Tables I, II, and III as mean values for erythrocytes from different donors. Red blood cell partitioning of all the tested compounds was linear and concentration independent (Table II), indicating that first-order kinetics were operative. The values for P_{e/c_u} of the individual compounds were identical in the presence and absence of large concentrations of the respective analogues (Table II). This implied the exis-

[8] Packard Tri-Carb Nos. 3280 and 3255; Packard Instruments, Downers Grove, Ill.

[9] Model 4101; Intertechnique, Plaisir, France.

Table III—Reversibility of the Red Blood Cell Partitioning Kinetics of Digoxin and Analogues at 24°C

Compound	P_{e/c_u}[a] Suspension Experiments	n^b	Resuspension Experiments	n^b
[^{3}H]I	1.39 ± 0.040	8	1.38 ± 0.088	3
[^{3}H]V	1.07 (1.04, 1.10)	2	1.13 (1.11, 1.15)	2
[^{3}H]VI	1.16 ± 0.038	8	1.14 ± 0.061	4

[a] Mean ± *SD*; individual values in parentheses. [b] Number of individual values considered, obtained from one donor.

tence of binding and/or partitioning sites of large capacity within the red blood cells. The equivalency of P_{e/c_u} for the individual compounds in suspension and resuspension experiments (Table III) suggested reversibility of the red blood cell partitioning kinetics. For all the compounds tested, P_{e/c_u} is greater than P_{e/c_l}, indicating significant plasma protein binding of the compounds.

There was no clear-cut relationship between the magnitude of P_{e/c_u} and lipophilicity (P) of the compounds (Table I). Red cell partitioning from buffer or plasma was clearly temperature dependent and decreased with decreasing temperature (Table I). Semilogarithmic plots of P_{e/c_u} against $1/T$, the reciprocal of the absolute temperature, yielded apparent straight lines for all the compounds tested (Fig. 1). The P_{e/c_u} values of [^{3}H]I and its analogues in the experiments conducted at 4°C were >0.70 (Table I), the partition coefficient value predicted for compounds solely distributed within the intracellular water phase of the red blood cells (2, 16). This implied binding and/or partitioning of the compounds in nonaqueous phases within the red blood cells, even at this low temperature.

However, P_{e/c_u}, as defined, did not characterize the distribution of the compounds between a nonaqueous and an aqueous phase as required (17), but defined equilibrium conditions existing in mixed nonaqueous-aqueous (cellular) and aqueous (extracellular) phases. In conceiving the red blood cells as a two-phase (nonaqueous-aqueous) system, a true partition coefficient, P_{e_b/e_u}, was defined as the ratio of the bound/partitioned concentration in the nonaqueous phase to the unbound concentration in the aqueous phase of the cells. Values for P_{e_b/e_u} were calculated (see *Appendix I*). Similarly, the percentage of drug truly bound/partitioned within the nonaqueous phase of the red cells, β_E, was computed (see *Appendix I*) for the different compounds. The values obtained for P_{e_b/e_u} and β_E are listed in Table IV.

When P_{e_b/e_u} was plotted semilogarithmically against $1/T$, curves were obtained with all the compounds (Fig. 1). This was in contrast to the apparent linear relationship obtained previously with semilogarithmic plots of P_{e/c_u} against $1/T$ (Fig. 1).

Nonequilibrium Kinetics—Time-dependent concentration curves were not obtained for the compounds under all the experimental conditions. With the most hydrophilic compound of the series, [^{3}H]VII, erythrocyte partitionings at temperatures of 4°C and 24°C were such slow processes that substantial hemolysis occurred long before the red blood cell partitionings were at equilibrium. With the more lipophilic compounds, however, red blood cell uptake was so fast that partition equilibrium was nearly or completely reached within the interval needed for centrifuging the very first sample taken after admixing the compounds ([^{3}H]I-[^{3}H]V at 37°C, [^{3}H]II and [^{3}H]III at 24°C).

Semilogarithmic plots against time of $(B - B_{eq})/(B_0 - B_{eq})$ and of $(E_{eq} - E)/E_{eq}$, derived respectively from the experimental data obtained in erythrocytes and buffer (see *Appendix II*), gave equivalent results and could be fitted by the same line (Figs. 2 and 3). Red blood cell partitioning of the compounds was characterized by a single distribution phase in most of the experimental settings. However, two distribution phases were clearly distinguishable with [^{3}H]I and [^{3}H]IV-[^{3}H]VI (Fig. 3) at 4°C and with [^{3}H]VII at 37°C. The respective concentration-time data in the erythrocytes, e, in buffer, c_u, and in plasma, c, of all the experiments were fitted using the non-

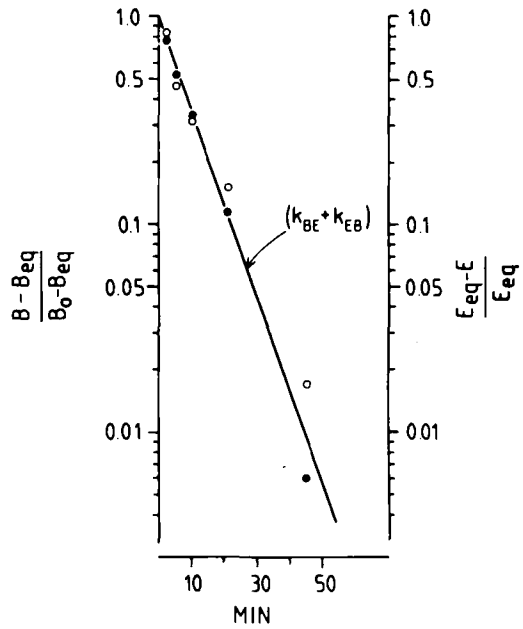


Figure 2—*Semilogarithmic plots of* $(B - B_{eq})/(B_0 - B_{eq})$ *and* $(E_{eq} - E)/E_{eq}$, *calculated from the experimental data for buffer and erythrocytes, respectively, for [^{3}H]III at 4°C. A red blood cell suspension in buffer was spiked with drug, and a single distribution phase was observed. The erythrocyte and buffer data could be fitted to the same line.*

linear least-squares estimation program TOPFIT (18). The program yielded values for the microscopic rate constants (Figs. 4–7) and equilibrium concentrations in erythrocytes, buffer, or plasma. The respective volumes of the red blood cell and buffer/plasma phases, hematocrit (Hc) and "plasmacrit" (1 − Hc), which were known in all the experiments, were fixed and not iterated.

Except for [^{3}H]VII, all data sets from the experiments were fitted individually and the fitting of the concentrations in erythrocytes, buffer, or plasma was simultaneous. The data sets obtained for [^{3}H]VII showed larger variability. The respective results of the two experiments performed with this compound in suspensions of red blood cells in buffer or in plasma were averaged and then fitted. Equal weights were given to all the erythrocyte, buffer, or plasma data. This was justified since the concentration range of all the experimental data was rather small. Attempts were made to fit all the data sets to a closed two-compartment model (Figs. 5–7); however, systematically deviating fits were obtained in those cases where two distribution phases had been seen previously in the graphic analysis of the data (Fig. 3). Adequate fits resulted when these data were fitted to a closed three-compartment model (Fig. 4).

A detailed analysis was performed with the data that had been fitted to the three-compartment model. Attempts were made to interpret, physiologically, the two erythrocyte compartments, E_1 and E_2, which were separable in these experiments (see the Scheme in Fig. 4). Plasma membrane, cellular water, and hemoglobin were expected to contain significant amounts/concentrations of the compounds (9, 19) and, thus, could be the physiological equivalents of the two erythrocyte compartments. However, it must be clearly realized that compartments E_1 and E_2 and the corresponding rate constants obtained in the kinetic analysis were interchangeable; these two compartments could be attributed to the cellular constituents and barriers as follows: (*a*) E_1 = plasma membrane + water, E_2 = hemoglobin; (*b*) E_1 = plasma membrane, E_2 =

Table IV—Compartmental Distribution and True Partitioning of Digoxin and Analogues in Red Blood Cells at 4°C[a]

Compound[b]	$(e_1/c_u)_{eq}$, × 10^2	$(e_2/c_u)_{eq}$, × 10^2	n^c	$(e_1/e)_{eq}$, × 10^2	$(e_2/e)_{eq}$, × 10^2	$(e_1/e_2)_{eq}$	P_{e_b/e_u}	β_E	n^d
[^{3}H]IV	26 (24, 28)	61 (56, 66)	2	30	70	0.43	0.29 ± 0.15	22 ± 8.8	4
[^{3}H]I	22 ± 4.5	62 ± 4.8	5	26	74	0.35	0.26 ± 0.05	21 ± 2.9	7
[^{3}H]V	16 ± 2.0	69 ± 6.1	6	19	81	0.23	0.25 ± 0.01	20 ± 0.8	4
[^{3}H]VI	19 ± 6.7	74 ± 6.5	4	20	80	0.25	0.33 ± 0.06	26 ± 3.6	3
[^{3}H]VII[e]	6.7	69	1	8.8	91	0.10			

[a] $e_{1,eq}$, $e_{2,eq}$, $e_{eq}[(e_1 + e_2)_{eq}]$, and $c_{u,eq}$, respectively the drug concentrations in the erythrocyte compartments (E_1, E_2, E) and plasma water (buffer) (B) at equilibrium, were obtained from fitting suitable data to the closed three-compartment model (see Fig. 4, *Appendix II*). The values for P_{e_b/e_u} and $\beta_E(e_b/e)_{eq}$ were predicted from P_{e/c_u}, depicting the red blood cells as consistingg of a nonaqueous and an aqueous phase of fractional volume 0.7. The values of $(e_1/e)_{eq}$ and $(e_1/e_2)_{eq}$ of the individual compounds should be compared, respectively, to P_{e_b/e_u} and β_E. The values are mean ± *SD*, with individual values in parentheses. [b] In order of decreasing lipophilicity. [c] Number of experiments performed in blood of different donors. [d] Number of mean values considered; averages of 3–5 individual determinations in blood of 3–7 donors.

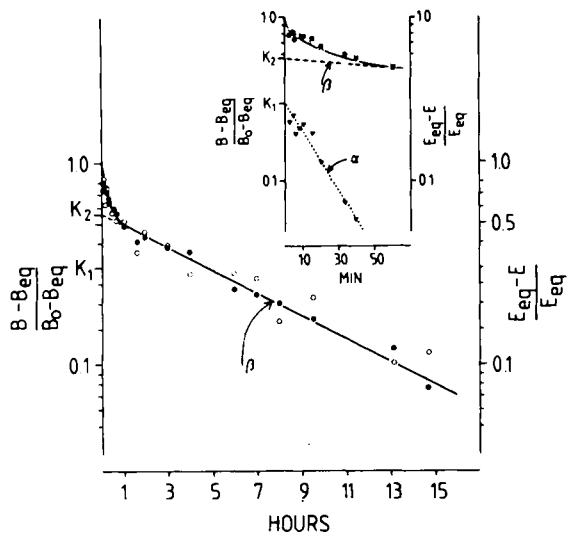


Figure 3 —*Semilogarithmic plot for [^{3}H]VI under same conditions as in Fig. 2. The inset is for the initial data on an expanded time scale. Two distribution phases were separable. The erythrocyte and buffer data could be fitted to the same line.*

water + hemoglobin; (*c*) E_1 = hemoglobin, E_2 = plasma membrane + water; and (*d*) E_1 = water + hemoglobin, E_2 = plasma membrane.

The respective concentrations in the red blood cell compartments, relative to the concentration in buffer [$(e_1/c_u)_{eq}$ and $(e_2/c_u)_{eq}$], were calculated from the microscopic rate constants obtained from the fits (see *Appendix II*). Similarly, the respective compartmental concentrations in the erythrocytes at equilibrium, as a percentage of the total concentration in the red blood cells, $10^2(e_1/e)_{eq}$ and $10^2(e_2/e)_{eq}$, and the ratio of the compartmental concentrations at equilibrium, $(e_1/e_2)_{eq}$, were computed (Table IV). It is interesting to note that, on average, $(e_2/c_u)_{eq}$ was 0.65 ± 0.05 for the tested compounds (Table IV), which is close to 0.7 (2, 16), the value predicted for the ratio of $(e_u/c_u)_{eq}$. In addition, $10^2(e_1/e)_{eq}$ of the individual compounds were similar to the predicted individual values for β_E (Table IV). Furthermore, the $(e_1/e_2)_{eq}$ values for the individual analogues agreed with the individually predicted P_{e_b/e_u} values (Table IV). This suggested that $(e_1)_{eq}$ and $(e_2)_{eq}$ obtained from the kinetic analysis on the assumption of a three-compartment model were equivalent, respectively, to e_b and e_u calculated on consideration of the physiology of erythrocytes and the P_{e/c_u} for the compounds. Hence, the most probable physiological equivalents of compartments E_1 and E_2 were, respectively, hemoglobin and cellular water + plasma membrane (alternative *c* above), on the assumption that the plasma membrane did not contain significant amounts of the drugs.

It became necessary to compare the partitioning kinetics of all the compounds under all the different experimental conditions. Hence, there was a need for a model-independent parameter. Mean transit time, $\bar{t}$, which has been used repeatedly in pharmacokinetic data analysis (20, 21), appeared to be most appropriate for this purpose. The $\bar{t}$ values for the individual experiments were calculated from the ratio of the area under the first moment curve ($AUMC_\infty$) to the area under the plasma concentration time curve (AUC_∞) (21) or from the microscopic rate constants obtained in the fits (see *Appendix II*). The values so obtained for $\bar{t}$ are listed in Table V. An upper limit for $\bar{t}$ was estimated in experiments where red blood cell partition equilibrium of the compounds was reached very quickly. There was wide variation among the $\bar{t}$ values obtained for the compounds in the different experimental settings; $\bar{t}$ ranged between <0.5 and 3800 min. Mean transit time clearly decreased with increasing lipophilicity (*P*) of the compounds (Table V, Fig. 8). For the experiments conducted at 4°C and 24°C, plots of log $(1/\bar{t})$ against log *P* yielded straight lines for the tested compounds (Fig. 8). Mean transit times of [^{3}H]I and its analogues were also highly dependent on absolute temperature. The quantitative evaluation of this dependency, using the data obtained with [^{3}H]VI and plotting log $(1/\bar{t})$ against $1/T$, showed that the relationship was nonlinear (Fig. 9).

The values obtained for $\bar{t}$ with erythrocyte buffer suspensions were significantly larger than with erythrocyte plasma suspensions for all the compounds tested; the differences ranged between 10–25% (Table IV). This implies that plasma protein binding and unbinding were not rate limiting and were much faster processes than red blood cell partitioning and repartitioning. Attempts were made to predict $(\bar{t})$, k_{PE}, and k_{EP} in red blood cell plasma suspension experiments at 24°C for [^{3}H]I and [^{3}H]IV–[^{3}H]VI from $\bar{t}$, k_{BE}, and k_{EB} in red blood cell buffer suspensions, which had been previously obtained in experiments conducted at an equivalent temperature (see *Appendix III*). There was good agreement between predicted and experimentally measured parameters for the tested compounds in the red blood cell plasma suspensions [$(\bar{t})_{pred}$ = 96.9 (±8.50)%·$(\bar{t})_{exp}$, $k_{PE,pred}$ = 103.1 (±10.4)%·$k_{PE,exp}$, and $k_{EP,pred}$ = 104.4 (±10.2)%·$k_{EP,exp}$ (n = 4)].

The similarity of the respectively obtained mean transit time values on partitioning of [^{3}H]VI from buffer into red blood cells ($\bar{t}$ = 5.1 min) and on repartitioning of the compound from erythrocytes into buffer ($\bar{t}$ = 4.9 min) (Fig. 6) suggested complete reversibility of the kinetics. Attempts were made to estimate mean transit times of repartitioning from red blood cells into buffer and plasma at 37°C for the compounds that reached partition equilibrium very quickly ([^{3}H]I–[^{3}H]V: $\bar{t}$ < 1.5 min, Table V). It was assumed that the temperature dependency of $\bar{t}$ for [^{3}H]VI in the range of 4°–37°C was also representative for the other analogues and that the equilibrium of the plasma protein interaction with the compounds was instantaneous. The estimated $\bar{t}$ values for the analogues in the red blood cell buffer suspensions at 37°C were: 0.008 min for [^{3}H]II; 0.036 min for [^{3}H]III; 0.307 min for [^{3}H]IV; 0.536 min for [^{3}H]I; and 1.39 min for [^{3}H]V (*Appendix III*). The corresponding values in the red blood cell plasma suspensions were smaller, on average 80% of the estimates obtained in the red blood cell buffer suspensions, for the slowly plasma-bound compounds ([^{3}H]I and [^{3}H]III–[^{3}H]V). In contrast, the estimated mean transit times in the two suspensions differed widely for the highly plasma-bound [^{3}H]II. The value in suspensions in plasma was clearly smaller [$(\bar{t})$ = 0.001 min] than in suspensions in buffer [$\bar{t}$ = 0.008].

Mean transit times for blood flowing through the sinusoids of the liver and the capillaries of the proximal tubules of the kidney were reported to be 10 and 2.5 s, respectively (22, 23). This suggested that, for the most lipophilic analogues ([^{3}H]II and [^{3}H]III), all of the amounts in the red blood cells could repartition during a single pass through the eliminating organs and, hence, could become available for extraction. For the least lipophilic analogues ([^{3}H]V–[^{3}H]VII), only small fractions of the amounts residing in the red blood cells could repartition and were available for extraction.

***In Vivo* Red Cell Partitioning of Digoxin and Metabolites**—Mean *in vivo*

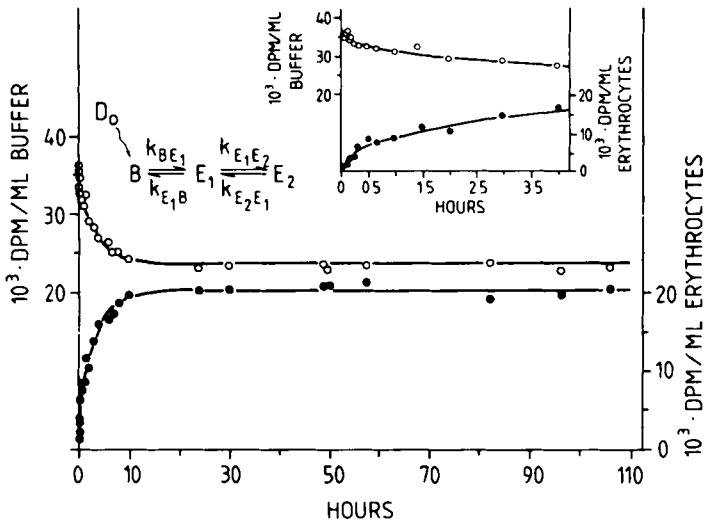


Figure 4—*Typical linear plot of the respective concentrations in erythrocytes (e) (●) and in buffer (c_u) (○) against time for [^{3}H]V at 4°C. A red blood cell suspension in buffer was spiked; the erythrocyte and buffer data were fitted simultaneously to the closed three-compartment model shown. The inset is a plot of the initial data on an expanded time scale.*

Table V—Mean Transit Time of Red Blood Cell Partitioning of Digoxin and Analogues from Buffer or Plasma at Different Temperatures

Compound[a]	$\bar{t}$ in Buffer or ($\bar{t}$) in Plasma, min[b] 4°C	n[c]	24°C	n[c]	37°C	n[c]
[^{3}H]II	1.65 ± 0.32	5	<0.50	2	<0.50	2
[^{3}H]III	7.46 ± 0.98	4	<0.50	2	<0.50	2
[^{3}H]IV	30.3 ± 1.29	3	1.14 (1.03, 1.24)	2	<0.50	2
			0.95 (0.91, 0.98)	2		
[^{3}H]I	63.1 ± 8.64	6	1.99 (1.56, 2.41)	2	<0.50	2
			1.59 (1.44, 1.73)	2		
[^{3}H]V	248 ± 39.1	6	5.17 (5.03, 5.31)	2	<0.50	2
			4.23 (3.85, 4.61)	2		
[^{3}H]VI	407 ± 43.3	4	7.35 (7.21, 7.49)	2		
			6.70 (6.05, 7.34)	2	1.70 (1.63, 1.78)	2
[^{3}H]VII					3807	1
					3098	1

[a] In the order of decreasing lipophilicity. [b] Mean ± *SD*; individual values in parentheses. When multiple values are given, the upper value is in buffer, the lower value in plasma; all single values are in buffer. [c] Number of experiments performed in blood of different donors.

red blood cell-plasma partition coefficients, $\bar{P}_{e/c}^{in\ vivo}$, were determined from the ratio of the total radioactivity in erythrocytes to total radioactivity in plasma in all the blood samples obtained in the pharmacokinetic studies. The $\bar{P}_{e/c}^{in\ vivo}$ values found were dose independent, but showed a clear time dependency: they were only constant for periods of ~2 h after intravenous and oral administration of the drug and increased significantly at later times. This was most probably due to a slow red blood cell partitioning of polar derivatives of [^{3}H]I, which were consistently measurable in the plasma samples of the volunteers. $\bar{P}_{e/c}^{in\ vivo}$ values obtained within the initial 2 h after drug administration were considered for a comparison with *in vitro* red blood cell partitioning values. Estimates for $\bar{P}_{e/c}^{in\ vitro}$ were made from the individual red cell partitioning values of [^{3}H]I and its apolar metabolites ([^{3}H]IV-[^{3}H]VI), previously obtained under *in vitro* conditions at 24°C ($P_{e/c,i}^{in\ vitro}$) and the concentrations of the individual compounds measured in plasma of the volunteers ($c_{eq}^{in\ vivo}$) in accordance with:

$$\bar{P}_{e/c}^{in\ vitro} = \frac{\sum_{i=1}^{4} (P_{e/c,i}^{in\ vitro}) \cdot c_{eq,i}}{\sum_{i=1}^{4} (c_{eq,i})}$$

It was assumed that $P_{e/c} \simeq 0$ for the measured polar derivatives of [^{3}H]I. This was in accordance with the insignificant red blood cell partitioning found for the polar [^{3}H]VII within comparable time intervals at 37°C.

Mean red blood cell partitioning *in vivo* and *in vitro* at 24°C were found to be similar in the intravenous experiments [$\bar{P}_{e/c}^{in\ vivo} = 0.943 \pm 0.154$ ($n = 6$), $\bar{P}_{e/c}^{in\ vitro} = 0.971 \pm 0.012$ ($n = 6$)] and oral experiments [$\bar{P}_{e/c}^{in\ vivo} = 0.975 \pm 0.148$ ($n = 6$), $\bar{P}_{e/c}^{in\ vitro} = 0.955 \pm 0.023$ ($n = 6$)]. This suggested that results on the kinetics of red blood cell partitioning obtained under *in vitro* conditions are valid and allow conclusions regarding *in vivo* conditions.

Binding of Digoxin and Analogues to Hemolysate, Hemoglobin, Albumin, and Plasma Protein—The percentage of drug bound to hemolysate ($\beta_{HB'}$), to hemoglobin (β_{HB}), to albumin (β_{AB}), and to plasma protein (β_{PB}) were obtained with the equilibrium dialysis method. The respective values are given in Table VI (see *Appendix I*). Estimates for β_{PB} of the compounds were also obtained by the red blood cell partitioning method and are included in Table VI (see *Appendix I*).

Binding to Hemolysate—Except for [^{3}H]VII, all the compounds showed significant binding to hemolysate (hemoglobin concentration, 110 g/L) at 37°C (Table VI). The $\beta_{HB'}$ values obtained for [^{3}H]I and its analogues ranged between 47.1% ([^{3}H]II) and −5.1% ([^{3}H]VII). The apparent negative value observed with [^{3}H]VII indicated that this compound was completely unbound and, hence, was excluded from volumes where binding of the other analogues occurred (12, 24). The binding of [^{3}H]II to hemolysate was concentration independent over the range studied (20–100 ng/mL). Estimates of $\beta_{HB'}$ in hemolysates (hemoglobin concentration, 110 g/L) were significantly and consistently smaller than β_E in intact cells (hemoglobin concentration, 330 g/L) (25) (Fig. 10), indicating a diminished binding capacity of the former. This was either due to the much lower hemoglobin concentration in the hemolysates or suggested additional binding/partitioning to constituents other than hemoglobin in the intact cells. There existed a significant positive linear correlation between the values obtained for β_E and $\beta_{HB'}$ [$\beta_E = 27.4\ (\pm 4.71) + 1.11\ (\pm 0.214) \cdot \beta_{HB'}$, $r = 0.919$ (Fig. 10)].

Binding to Hemoglobin—The respective values for the binding of [^{3}H]I and [^{3}H]II to hemoglobin (110 g/L) were $\beta_{HB} = 22.0$ and 71.4% (Table VI). The former value was in agreement with the value previously obtained for the binding of [^{3}H]I to hemolysate ($\beta_{HB'} = 19.7\%$). In contrast, binding of [^{3}H]II was much greater with hemoglobin ($\beta_{HB} = 71.4\%$) than with hemolysate ($\beta_{HB'} = 47.1\%$) (Table VI). It is possible that existing differences in conformation or aggregation of hemoglobin in the two preparations affect the highly bound [^{3}H]II more than the lesser-bound [^{3}H]I. The binding of [^{3}H]II to diluted hemoglobin (7 g/L) was significant [$\beta_{HB} = 19.0$ (15.6 and 22.3; $n = 2$)] and suggested that results on the membrane binding of compounds reported in the literature (19), which had been obtained with red cell ghosts not freed from hemoglobin (14 g/L), must be interpreted with due caution.

Binding to Albumin—The values for the binding of [^{3}H]I and its analogues to albumin are listed in Table VI. The bindings of [^{3}H]II to albumins AB_1 and AB_2 were $\beta_{AB_1} = 97.0\%$ and $\beta_{AB_2} = 97.9\%$. The similarity of these results implied equivalence of the two albumins. Binding of the compounds tested ranged between $\beta_{AB} = 97\%$ ([^{3}H]II) and $\beta_{AB} = 27\%$ ([^{3}H]VII). Albumin binding increased with increasing lipophilicity (P) of the compounds [$\beta_{AB} = 24.3\ (\pm 1.78) + 0.748\ (\pm 0.043) \cdot P$, $r = 0.992$].

Binding to Plasma Protein—The mean values obtained for the plasma protein binding of [^{3}H]II, [^{3}H]III, and [^{3}H]VII together with the previously reported (1) values for [^{3}H]I and [^{3}H]IV-[^{3}H]VI are given in Table VI. All the values were obtained with the equilibrium dialysis method. Plasma protein binding of [^{3}H]II, [^{3}H]III, and [^{3}H]VII was concentration independent over the range studied and was unaltered in the presence of large concentrations of analogues. This was in accordance with the binding characteristics previously found with [^{3}H]I and [^{3}H]IV-[^{3}H]VI (1). Plasma protein bindings of

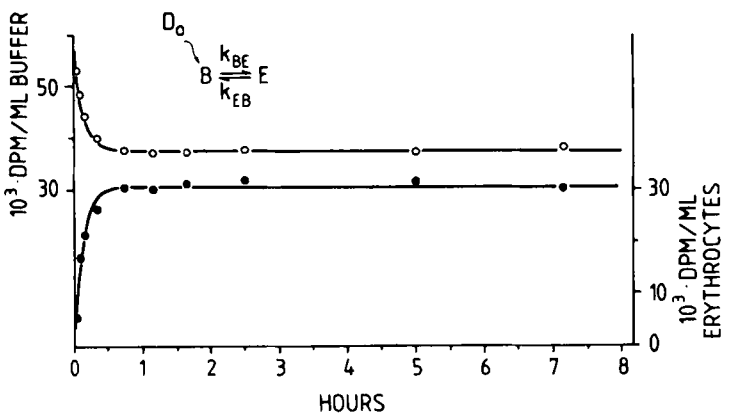


Figure 5—*Linear plot of the respective concentrations in erythrocytes (e) (●) and in buffer (c_u) (○) against time for [^{3}H]III at 24°C. A red blood cell suspension in buffer was spiked; the erythrocyte and buffer data were fitted simultaneously to the closed two-compartment model shown.*

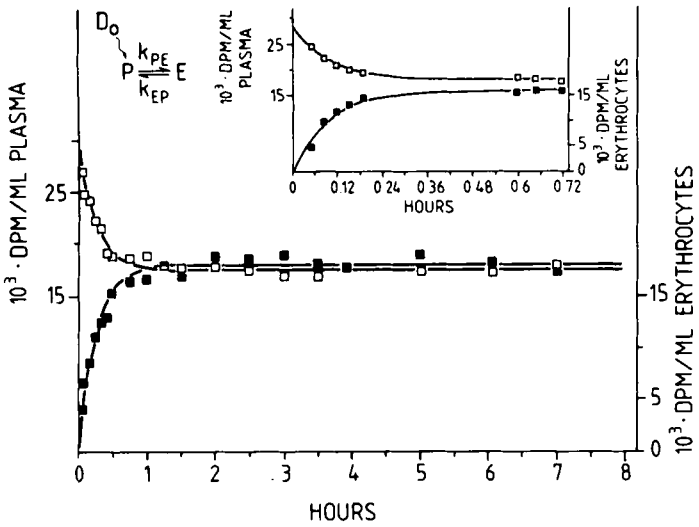


Figure 6—*Linear plot of the respective concentrations in erythrocytes (e) (■) and in plasma (c) (□) against time for [³H]V at 24°C. A red blood cell suspension in plasma was spiked; the data were fitted to the two-compartment model shown. The inset is a plot of the initial data on an expanded time scale.*

[³H]II at experimental temperatures of 24°C [β_{BP} = 96.7 ± 0.38% (n = 11)] and 37°C [β_{PB} = 95.1 ± 1.90% (n = 5)] were similar.

The binding to plasma protein was consistently smaller than to albumin for [³H]I and all the analogues tested (Table VI). A highly significant positive correlation existed between binding to albumin and to plasma protein for the compounds [$\beta_{AB} = -15.9\ (\pm 8.36) + 1.07\ (\pm 0.164)\ \beta_{PB}$, r = 0.894]. There was excellent agreement between the respective plasma protein binding estimates of the compounds obtained from the red blood cell partitioning procedure and the equilibrium dialysis method (Table VI). Plasma protein binding of [³H]I and its analogues using the red blood cell partitioning method was temperature independent within the tested range of 24–37°C (Table VI).

DISCUSSION

Octanol–Water Partition Coefficient of Digoxin and Its Analogues—The partition coefficient values obtained for [³H]I and its analogues in the present study were—except for [³H]II—in agreement with those reported previously by Cohnen *et al.* (26); a significantly higher P value was found for [³H]II in the present study. The reason for this discrepancy was not apparent, since equivalent experimental methods were used in both studies. A significant linear correlation existed between the R_m values by partition chromatography reported by Cohnen *et al.* (26) and the log P values obtained in the present study [$R_{m(30)} = 1.36\ (\pm 0.069) \cdot x - 1.47\ (\pm 0.091) \cdot \log P$, r = 0.995].

Erythrocyte Partitioning of Digoxin and Its Analogues—Red blood cell partitioning kinetics of [³H]I and its analogues were first order over a considerable concentration range, unaltered in the presence of large concentrations of analogues (Table II) and clearly temperature dependent (Table I). This suggested that red blood cell uptake of the compounds was a passive process. Its temperature dependency indicated that partitioning into the plasma membrane, diffusion through unstirred water layers, and/or interaction with hemoglobin could be rate limiting. Membrane diffusion through pores (27) was excluded for [³H]I and its analogues on consideration of the molecular weights of the compounds and the pore radius (4 Å) of the red blood cell plasma membrane (28–32).

The three-compartment model kinetics found for the less lipophilic analogues [³H]V and [³H]VI at 4°C (Figs. 3 and 4) and [³H]VII at 37°C indicated that two rate-limiting steps—one faster, one slower—were operative in these cases. Clearly the processes could be composite. In accordance with the previous physiological interpretation of the erythrocyte compartments, the slower process represented partitioning into the plasma membrane and/or diffusion through intracellular water; the faster process was equivalent to binding to hemoglobin. However, in considering the degree of acceleration of the kinetics of red blood cell partitioning of the compounds found on elevating the temperature, it was concluded that diffusion through water was most probably not a rate-limiting process (31). The three-compartment model collapsed into a two-compartment model with the more lipophilic analogues at the lowest temperature ([³H]II–[³H]III; Figs. 2 and 5) and with all the analogues except [³H]VII at the higher temperatures. This finding suggested that the slower of the two processes was accelerated by an increase in temperature or lipophilicity of the compounds and approached the rate of the faster process, so that apparently two-compartment kinetics with a single rate-limiting process evolved in these cases.

Equilibrium Kinetics—Red blood cell partition coefficients for [³H]-IV–[³H]VII have not been reported thus far in the literature; however, values for [³H]I–[³H]III have been published. Mean P_{e/c_u} and $P_{e/c}$ values of 0.97 (0.93, 1.01; n = 2) and 0.89 ± 0.030 (n = 3), respectively, had been obtained previously by Hinderling *et al.* (5). The latter value was confirmed by the results of the present study [$P_{e/c}$ = 0.88 (0.842, 0.922; n = 2)]; however, the former value was significantly smaller than the corresponding value of the present study [$P_{e/c_u} = 1.11 \pm 0.027$ (n = 3)]. Although it cannot be excluded that the discrepancy was caused by the use of plasma water instead of buffer a in the previous study, there was indications that the value of the former study was biased. Abshagen *et al.* (19), using radiolabeled compounds, reported mean red blood cell partitioning values of six donors for [³H]I and [³H]II at 37°C. The values were obtained on consideration of one drug concentration only ([³H]I: 51 ng/mL; [³H]II: 69 ng/mL). Their respective P_{e/c_u} and $P_{e/c}$ values were 0.90 and 0.90 for [³H]I and 1.37 and 0.09 for [³H]II. The former three values were significantly smaller than those found in the present study ([³H]I: P_{e/c_u} = 1.49, $P_{e/c}$ = 1.13; [³H]II: P_{e/c_u} = 2.51); there was agreement regarding the last value. Attempts to compute estimates for the plasma protein binding of the two compounds using the respective P_{e/c_u} and $P_{e/c}$ values (see *Appendix I*) of Abshagen *et al.* (19) yielded β_{PB} = 0% for [³H]I and β_{PB} = 93.1% for [³H]II. The former value was clearly lower than the values given in the literature for the plasma binding of [³H]I [β_{PB} = 23–29% (equilibrium dialysis, 37°C, normal human serum–plasma) (33–37)] and there was no ready explanation for this bias. The latter value was within the range of the

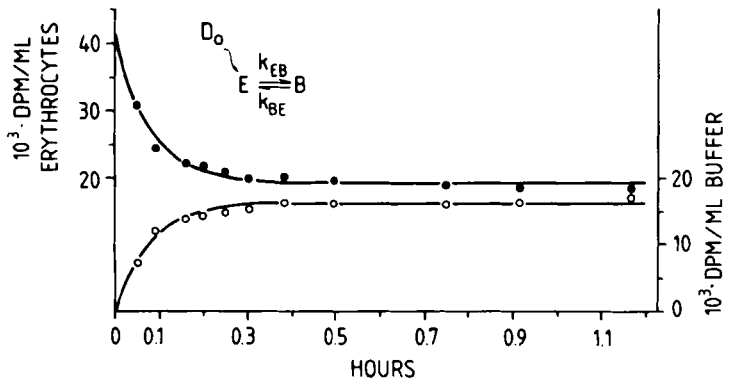


Figure 7—*Linear plot of the respective concentrations in buffer (c_u) (○) and in erythrocyte (e) (●) against time for [³H]VI at 24°C. Red blood cells suspended in buffer and spiked with the compound were separated centrifugally and then resuspended in fresh buffer. The data were fitted according to the two-compartment model shown.*

Table VI—Percent Binding of Digoxin and Analogues to Hemolysate, Albumin, and Plasma Protein at 37°C and 24°C [a]

Compound	β_{HB} 37°C [b]	n [c]	β_{AB} 37°C [b]	n [d]	β_{PB} 37°C [b]	n [e]	37°C [f]	24°C [f]
[^{3}H]II	47.1 (45.1, 49.2)	2	97.2 ± 0.690	12	95.6 ± 1.59	7	96.4	97.2
[^{3}H]III	12.3 (10.3, 14.2)	2	57.3 (55.6, 58.9)	2	25.1 (24.7, 25.4)	2	26.2	20.8
[^{3}H]IV	18.2 (18.0, 18.4)	2	33.9 ± 2.64	6	22.3 ± 2.00	3	25.9	23.8
[^{3}H]I	19.7 (18.8, 20.5)	2	34.2 ± 2.67	10	22.3 ± 1.43	3	24.2	22.8
[^{3}H]V	15.6 (13.4, 17.7)	2	33.0 (32.3, 33.6)	2	22.4 ± 2.55	3	23.0	24.9
[^{3}H]VI	7.45 (6.50, 8.40)	2	35.8 (34.9, 36.7)	2	26.2 ± 1.61	3	20.4	29.5
[^{3}H]VII	−5.05 (−2.10, −8.00)	2	26.8 (24.9, 28.7)	2	13.9 (13.6, 14.2)	2		

[a] Mean ± *SD*; individual values in parentheses. The compounds are listed in order of decreasing lipophilicity. [b] Equilibrium dialysis method. [c] Number of individual determinations considered, obtained in hemolysates of one donor. [d] Number of individual values considered obtained, in plasma of one donor. [e] Number of mean values considered; averages of two determinations in plasma of 2–7 donors. [f] Red blood cell partitioning method.

values reported in the literature for the plasma binding of [^{3}H]II [β_{PB} = 92–97% (equilibrium dialysis, 37°C, normal human serum–plasma) (33–35, 38)].

Nonequilibrium Kinetics—Gardner *et al.* (9) reported mean data on the kinetics of red blood cell partitioning for [^{3}H]I (9–94 ng/mL) at 37°C. An apparent $\bar{t}$ value of 13 min could be calculated from those results. This value was much larger than the corresponding estimate of $\bar{t} < 0.5$ min obtained in the present study. The discrepancy was possibly due to differences in the methods employed. The procedure of Gardner *et al.* used a different buffer and the red blood cell suspensions had much lower hematocrits (5–10%); the incubated red blood cell suspensions were repeatedly washed (at nonspecified temperatures) prior to the final ultracentrifugal separation of the cells and buffer. The surprisingly low values for P_{c/c_u} of 0.65 and 0.40 at concentrations of 9 and 94 ng/mL of the drug, respectively, calculated from their data, indicated that the washing procedure used may have led to a substantial loss of drug from the cells.

Binding of Digoxin and Its Analogues to Hemolysate, Hemoglobin, and Plasma Protein—Hemoglobin (*per se* and in hemolysate preparations) was shown to bind significantly all the compounds tested with the exception of [^{3}H]VII (Table VI). A statistically significant linear correlation existed between binding to hemolysate and binding to intact cells for [^{3}H]I–[^{3}H]VI (Fig. 10). These results suggested that hemoglobin was the major red blood cell constituent that interacted with the compounds. An important binding of the analogues to constituents other than hemoglobin could not be excluded; however, the finding that low concentrations of hemoglobin (7 g/L) bound substantial amounts of [^{3}H]II suggested that reported plasma membrane binding values (9, 19) could be overestimations, since the membrane preparations used in these studies were not free of hemoglobin.

The differences in the binding of [^{3}H]I and its analogues to hemolysate, hemoglobin, and suspensions of intact cells observed in the present study could be explained by the nonequivalence of the three preparations. The cell suspensions had larger hemoglobin concentrations and included additional constituents with binding potential. In addition, conformation and aggregation of hemoglobin in the different preparations were possibly not identical.

The binding of [^{3}H]I and its analogues in albumin preparations was consistently larger than in plasma. Albumin was reported to be the sole protein in plasma to interact with [^{3}H]I and [^{3}H]II (38, 39). The different binding performance observed could be due to a nonidentical conformation or aggregation of the commercially available albumin in buffer *a* and of the "natural" albumin in plasma water. Alternatively, and more likely, this discrepancy was due to a difference in the concentrations of free fatty acids in the albumin preparations and in the plasma used. Free fatty acids at physiological concentrations in plasma were shown to interfere with the binding of drugs to albumin (40). Elevated concentrations of free fatty acids were reported to decrease, significantly, the binding of [^{3}H]I and [^{3}H]II in plasma (41).

Data on the binding of [^{3}H]I and [^{3}H]II to albumin at 37°C, obtained with equilibrium dialysis methods, are reported in the literature. Lukas and de Martino (38) reported β_{AB} = 23% for [^{3}H]I, employing an albumin with a significant free fatty acid content. This value was clearly smaller than β_{AB} = 34.2% for [^{3}H]I found in the present study, which used a fatty acid-free albumin preparation (Table VI). Brock (39) reported a value of β_{AB} = 96.6% for [^{3}H]II with no specification as to the fatty acid content of the albumin used. This value was close to the β_{AB} = 97.2% obtained in the present study (Table VI).

Widely varying plasma protein binding values were reported for [^{3}H]I and [^{3}H]II in the literature (35). The discrepancies were most likely due to differences in the methodologies and experimental conditions used in the studies. For a discussion of the results of the present study, only those data reported in the literature that had been obtained on application of equivalent procedures (equilibrium dialysis, individual plasma of healthy volunteers, temperature 37°C, pH 7.4) were considered. The average percent plasma protein binding found for [^{3}H]II (β_{PB} = 95.6%, Table VI) in the present study was within the

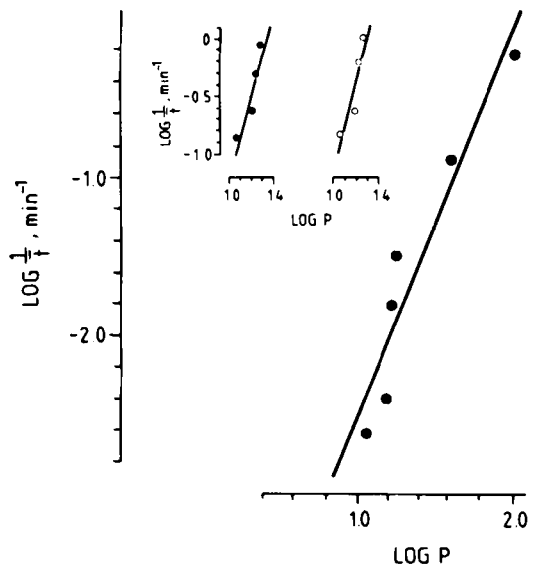


Figure 8—*Logarithmic plot of the reciprocal of the averaged mean transit time, $1/\bar{t}$, against the octanol–water partition coefficient, P, for [^{3}H]I–[^{3}H]VI at an experimental temperature of 4°C. Red blood cell suspensions in buffer were spiked with the compounds. There was an apparent linear dependency of log ($1/\bar{t}$) on log P [log ($1/\bar{t}$) = −5.00 (±0.608) + 2.49 (±0.0428) · log P, r = 0.995]. Corresponding plots are given in the inset for [^{3}H]I and [^{3}H]-IV–[^{3}H]VI in red blood cell–buffer suspensions (●) and in red blood cell–plasma suspensions (○) at an experimental temperature of 24°C.*

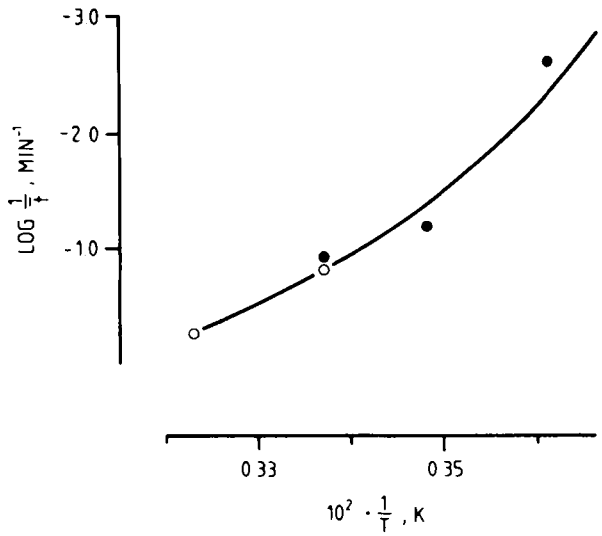


Figure 9—*Linear plot of the logarithm of the reciprocal of the averaged mean transit time, $1/\bar{t}$, against the reciprocal of the absolute temperature, 1/T, for [^{3}H]VI at 4°C, 14°C, 24°C, and 37°C. The relationship between log ($1/\bar{t}$) and 1/T was nonlinear. Key: (●) red blood cell–buffer suspensions; (○) red blood cell–plasma suspensions.*

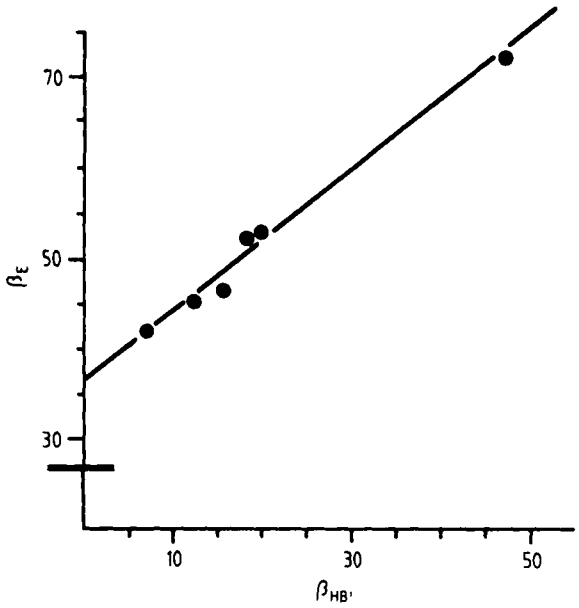


Figure 10—*Linear plot of the percentage bound in intact erythrocytes, β_E, against the percentage bound in hemolysate, $\beta_{HB'}$, for [^{3}H]I–[^{3}H]VI at 37°C. There was an apparent linear relationship between β_E and $\beta_{HB'}$ [β_E: 36.7 (±1.19) + 0.762 (±0.0502) · $\beta_{HB'}$, r = 0.992].*

range of the values given in the literature [92.3–97.3% (33–35, 38)]. The corresponding value for [^{3}H]III, β_{PB} = 25.1%, in the present study was close to the β_{PB} = 25.8% reported by Kramer *et al.* (34), but exceeded the value reported by Dengler *et al.* (42) (β_{PB} = 14%) for this drug. The plasma binding of [^{3}H]VII (β_{PB} = 13.9%) in the present study was similar to the sole value reported in the literature [β_{PB} = 16% (43)] for this drug. The temperature independence of the plasma binding of [^{3}H]I and its analogues for the range of 24–37°C observed in the present study was in agreement with equivalent findings of earlier studies with [^{3}H]II (35, 39).

CONCLUSIONS

The present study showed that red blood cell partitioning under *in vivo* and *in vitro* conditions gave equivalent results. The rate and extent of the red blood cell partitioning of [^{3}H]I and its analogues were according to first-order kinetics, identical in the presence and absence of structurally related compounds, and dependent on lipophilicity and temperature. The kinetics of red blood cell partitioning were reversible. Except for the rather strong temperature dependence found, the results were in accordance with passive diffusion as the underlying process (44, 45). The kinetics of red blood cell partitioning of the compounds from plasma could be predicted from the results obtained in analogous experiments with red blood cell buffer suspensions. The rate constants of red blood cell partitioning and repartitioning were much smaller than the rate constants of plasma protein binding and unbinding for the compounds tested.

Mean transit times of partitioning between red blood cells and plasma water were largely different for the compounds tested and varied at 37°C more than 1000-fold. Similarly, large differences in the kinetics of red blood cell partitioning were reported by Schanker *et al.* (2) for a series of basic compounds which differed in lipophilicity. Measured and estimated values for the mean transit time of the compounds at 37°C indicated that there could be significant repartitioning from red blood cells into plasma during a single passage of blood through the eliminatory organs with the more lipophilic analogues. In contrast, only insignificant red blood cell repartitioning occurred with the less lipophilic analogues.

Plasma membrane partitioning appeared to be the rate-limiting step in the red blood cell penetration of the compounds. All the compounds tested—with the exception of [^{3}H]VII—exerted significant binding/partitioning in a "nonaqueous" phase of the red blood cells. Hemoglobin was the major ligate among the erythrocyte constituents.

Plasma protein bindings of [^{3}H]I and its analogues were concentration independent and unaltered in the presence of structurally related compounds. Binding of [^{3}H]I and the analogues to plasma did not simply reflect the interaction between the individual compounds and albumin, but included the interfering action of fatty acids. Albumin binding of the compounds increased with increasing lipophilicity.

In contrast to plasma protein binding, erythrocyte partitioning of the tested compounds was temperature dependent within the range of 24–37°C. Temperature-dependent repartitioning of the compounds from the red blood cells into plasma on lowering the temperature from 37°C to 24°C was low for the tested compounds, leading to an increase in the plasma concentrations of 2–6%. However, it is entirely feasible that for drugs with more extensive red blood cell partitioning, temperature-dependent red blood cell repartitioning could lead to important artifactual changes in the plasma concentration.

The study demonstrated that the delineation of the kinetics of digoxin and analogues in the subcompartments of blood (erthrocytes and plasma) was mandatory and that *a priori* assumptions regarding the rate and extent of red blood cell partitioning of drugs are unwarranted. In defining clearance in the well-stirred (46) or parallel-tube models (47), it was assumed that drug repartitioning from red blood cells into plasma water was instantaneous, so that the unbound fraction was maintained during the passage of blood through the eliminatory organs. However, the results of this study suggest that this may not generally be true. Hence, in the reported literature, clearance values of drugs estimated without experimentally testing the kinetics of red blood cell partitioning may be biased.

APPENDIX I: Calculations for Partition Coefficients and Percentages Bound

Apparent Red Blood Cell–Buffer (P_{e/c_u}) and Red Blood Cell–Plasma Partition Coefficients ($P_{e/c}$)—Red blood cell–buffer and red blood cell–plasma partition coefficients of drugs have been defined previously (48):

$$P_{e/c_u} = \left(\frac{e}{c_u}\right)_{eq} \quad \text{(Eq. 1A)}$$

$$P_{e/c} = \left(\frac{e}{c}\right)_{eq} \quad \text{(Eq. 2A)}$$

where $c_{u,eq}$, c_{eq} [*i.e.*, $(c_u + c_b)_{eq}$], and e_{eq} [*i.e.*, $(e_u + e_b)_{eq}$] represent, respectively, the unbound concentration of a drug in plasma water (buffer), the total (bound + unbound) concentration in plasma, and the total (bound + unbound) concentration in red blood cells at equilibrium.

True Red Blood Cell Partition Coefficient—The red blood cell partition coefficient, P_{e_b/e_u}, may be defined as the ratio of the unbound to bound red blood cell concentration of a drug at equilibrium:

$$P_{e_b/e_u} = (e_b/e_u)_{eq} \quad \text{(Eq. 3A)}$$

On the assumption that the concentration of a nonelectrolytic drug in the aqueous phase of the red blood cells at equilibrium is equal to the known concentration in the surrounding plasma water (buffer), $c_{u,eq}$, and by setting the fractional volume of the red cell water phase equal to 0.7 (2, 16), e_u can be calculated:

$$e_{u,eq} = 0.7 \cdot c_{u,eq} \quad \text{(Eq. 4A)}$$

In substituting $e_{b,eq}$ with $(e - e_u)_{eq}$ and $e_{u,eq}$ with $0.7 \cdot c_{u,eq}$, an expression for P_{e_b/e_u} is obtained:

$$P_{e_b/e_u} = \frac{P_{e/c_u}}{0.7} - 1 \quad \text{(Eq. 5A)}$$

Percentage of Drug Bound and/or Partitioned in Red Blood Cells—The percentage of a compound bound to and/or partitioned into the nonaqueous phase of red cells, β_E, was defined according to:

$$\beta_E = 10^2 \cdot \left(\frac{e_b}{e}\right)_{eq} = 10^2\left[1 - \left(\frac{e_u}{e}\right)_{eq}\right] \quad \text{(Eq. 6A)}$$

On substitution of Eq. 4A into Eq. 6A an expression for β_E is obtained:

$$\beta_E = 10^2[1 - (0.7/P_{e/c_u})] \quad \text{(Eq. 7A)}$$

Respective Percentages of Drug Bound to Hemolysate, Hemoglobin, Albumin, and Plasma Protein—In the studies which employed the equilibrium dialysis method, the percentage of bound drug, β, was obtained from:

$$\beta = 10^2(c_b/c)_{eq} = 10^2[(c - c_u)/c]_{eq} \quad \text{(Eq. 8A)}$$

In the studies which used the red blood cell partitioning method to estimate plasma protein binding, β_{PB} was calculated from Eq. 9A as reported previously (7):

$$\beta_{PB} = 10^2[1 - (P_{e/c}/P_{e/c_u})] \quad \text{(Eq. 9A)}$$

APPENDIX II: Compartmental Models

Closed Two-Compartment Model (Figs. 4–6)—The integrated equations for this model and the initial conditions, $B = B_0$ and $E = 0$, are in accordance with (49):

$$B = \frac{B_0}{k_{BE} + k_{EB}} [k_{EB} + k_{BE} \cdot e^{-(k_{BE}+k_{EB})t}] \quad \text{(Eq. 10A)}$$

$$E = \frac{k_{BE}B_0}{k_{BE} + k_{EB}} [1 - e^{-(k_{BE}+k_{EB})t}] \quad \text{(Eq. 11A)}$$

where $B_0(D_0)$, B, and E represent the dose and the amounts of drug in the plasma water (buffer) and red blood cells, respectively. Equation 10A can be transformed into:

$$\frac{B(k_{BE} + k_{EB}) - k_{EB} \cdot B_0}{k_{BE} \cdot B_0} = e^{-(k_{BE}+k_{EB})t} \quad \text{(Eq. 12A)}$$

Solving Eqs. 10A and 11A for $t = \infty$ (at equilibrium) yields respectively:

$$k_{BE} + k_{EB} = \frac{k_{EB} \cdot B_0}{B_{eq}} \quad \text{(Eq. 13A)}$$

$$k_{BE} + k_{EB} = \frac{k_{BE} \cdot B_0}{E_{eq}} \quad \text{(Eq. 14A)}$$

where B_{eq} and E_{eq} are, respectively, the amounts in plasma water (buffer) and red blood cells at equilibrium. Substituting Eq. 13A into Eq. 12A and rearranging gives:

$$\frac{B - B_{eq}}{B_0 - B_{eq}} = e^{-(k_{BE}+k_{EB})t} \quad \text{(Eq. 15A)}$$

Equation 15A can be formulated in terms of the amounts of drug in the red blood cells:

$$\frac{E_{eq} - E}{E_{eq}} = e^{-(k_{BE}+k_{EB})t} \quad \text{(Eq. 16A)}$$

Plots of $\ln [(B - B_{eq})/(B_0 - B_{eq})]$ or $\ln [(E_{eq} - E)/E_{eq}]$ against time yield identical negative slopes of $(k_{BE} + k_{EB})$ and intercepts of 1.0 (see Fig. 2).

The mean transit time, $\bar{t}$, for the closed two-compartment model is obtained in accordance with (21):

$$\bar{t} = \frac{AUMC_\infty}{AUC_\infty} = \frac{[(B_0 - B_{eq})/(1 - Hc)]/(k_{BE} + k_{EB})^2}{[(B_0 - B_{eq})/(1 - Hc)]/(k_{BE} + k_{EB})} = \frac{1}{k_{BE} + k_{EB}} \quad \text{(Eq. 17A)}$$

Closed Three-Compartment Model (Fig. 4)—The integrated equations for this model and the initial conditions, $B = B_0$ and $E_1 = E_2 = 0$, are in accordance with (49):

$$B = B_0 \left\{ \frac{k_{E_1B} \cdot k_{E_2E_1}}{\alpha \cdot \beta} + \left[\frac{\alpha^2 - \alpha(k_{E_1B} + k_{E_1E_2} + k_{E_2E_1}) + k_{E_1B} \cdot k_{E_2E_1}}{\alpha(\alpha - \beta)} \right] \cdot e^{-\alpha t} + \left[\frac{\beta^2 - \beta(k_{E_1B} + k_{E_1E_2} + k_{E_2E_1}) + k_{E_1B} \cdot k_{E_2E_1}}{\beta(\beta - \alpha)} \right] \cdot e^{-\beta t} \right\} \quad \text{(Eq. 18A)}$$

$$E = E_1 + E_2 = k_{BE_1}B_0 \left\{ \frac{k_{E_2E_1} + k_{E_1E_2}}{\alpha \cdot \beta} + \left[\frac{k_{E_2E_1} + k_{E_1E_2} - \alpha}{\alpha(\alpha - \beta)} \right] \cdot e^{-\alpha t} + \left[\frac{k_{E_2E_1} + k_{E_1E_2} - \beta}{\beta(\beta - \alpha)} \right] \cdot e^{-\beta t} \right\} \quad \text{(Eq. 19A)}$$

where:

$$\alpha,\beta = \frac{1}{2} [k_{BE_1} + k_{E_1B} + k_{E_1E_2} + k_{E_2E_1} \pm \sqrt{(k_{BE_1} + k_{E_1B} + k_{E_1E_2} + k_{E_2E_1})^2 - 4k_{BE_1} \cdot k_{E_1E_2} + k_{E_1B} \cdot k_{E_2E_1} + k_{BE_1} \cdot k_{E_2E_1}}] \quad \text{(Eq. 20A)}$$

Equation 18A can be transformed and written in a form which is equivalent to Eqs. 15A and 16A of the two-compartment model:

$$\frac{B - B_{eq}}{B_0 \cdot B_{eq}} = \frac{E_{eq} - E}{E_{eq}} = K_1 \cdot e^{-\alpha t} + K_2 \cdot e^{-\beta t} \quad \text{(Eq. 21A)}$$

Similarly plots of $\ln [(B - B_{eq})/(B_0 - B_{eq})]$ or $\ln [(E_{eq} - E)/E_{eq}]$ against time have an intercept of 1.0 and a terminal logarithmic linear phase with a negative slope of β. By, respectively, extrapolating and using peeling-off techniques, the intercepts, K_2, K_1, and the slope α of the faster exponential term can be obtained (see Fig. 3).

The mean transit time for the closed three-compartment model with measurements in compartment B, $\bar{t}_B$, can be computed in accordance with (21):

$$\bar{t}_B = \frac{AUMC_{\infty,B}}{AUC_{\infty,B}} = \frac{[(B_0 - B_{eq})/(1 - Hc)]/(k_1/\alpha^2 + k_2/\beta^2)}{[(B_0 - B_{eq})/(1 - Hc)]/(k_1/\alpha + k_2/\beta)} = \frac{k_1/\alpha + k_2/\beta}{k_1/\alpha^2 + k_2/\beta^2} \quad \text{(Eq. 22A)}$$

Alternatively, $\bar{t}_B$, can be calculated and expressed in terms of the microscopic rate constants in accordance with:

$$\bar{t}_B = \frac{1}{k_{E_1B} + k_{E_1E_2} + k_{E_2E_1}} \cdot \left[\frac{(k_{E_1B} + k_{E_1E_2} + k_{E_2E_1})^2 - k_{E_1B} \cdot k_{E_2E_1} + k_{E_1B} \cdot k_{BE_1}}{k_{BE_1}(k_{E_1E_2} + k_{E_2E_1}) + k_{E_1B} \cdot k_{F_2E_1}} - \frac{k_{E_1B} \cdot k_{E_2E_1}}{(k_{E_1F_2} + k_{E_2E_1})^2 + k_{E_1B} \cdot k_{E_1E_2}} \right] \quad \text{(Eq. 23A)}$$

This alternative way of calculating $\bar{t}$ from the microscopic rate constants of compartmental systems will be presented in a forthcoming paper[10].

Calculation of Equilibrium Concentrations of Drugs in Erythrocytes ($e_{1,eq}$, $e_{2,eq}$, and e_{eq}) and Plasma Water/Buffer ($c_{u,eq}$) Using the Microscopic Rate Constants Obtained from the Fits (Table IV)—*Two-Compartment Model:*

$$e_{eq} = \frac{E_{eq}}{Hc} = \frac{k_{BE}}{k_{EB}} \cdot \frac{B_{eq}}{Hc} \quad \text{(Eq. 24A)}$$

$$c_{u,eq} = \frac{B_{eq}}{1 - Hc} = \frac{k_{EB}}{k_{BE}} \cdot \frac{E_{eq}}{1 - Hc} \quad \text{(Eq. 25A)}$$

$$P_{e/c_u} = \frac{k_{BE}}{k_{EB}} \cdot \frac{1 - Hc}{Hc} \quad \text{(Eq. 26A)}$$

Three-Compartment Model:

$$e_{1,eq} = \frac{E_{1,eq}}{Hc} = \left(\frac{k_{BE_1} \cdot k_{E_2E_1} \cdot B_0}{\alpha \cdot \beta} \right) \cdot \frac{1}{Hc} \quad \text{(Eq. 27A)}$$

$$e_{2,eq} = \frac{E_{2,eq}}{Hc} = \left(\frac{k_{BE_1} \cdot k_{E_1E_2} \cdot B_0}{\alpha \cdot \beta} \right) \cdot \frac{1}{Hc} \quad \text{(Eq. 28A)}$$

$$c_{u,eq} = \frac{B_{eq}}{1 - Hc} = \left(\frac{k_{E_1B} \cdot k_{E_2E_1} \cdot B_0}{\alpha \cdot \beta} \right) \cdot \frac{1}{1 - Hc} \quad \text{(Eq. 29A)}$$

$$(e_1/c_u)_{eq} = \frac{k_{BE_1}}{k_{E_1B}} \cdot \frac{1 - Hc}{Hc} \quad \text{(Eq. 30A)}$$

$$(e_2/c_u)_{eq} = \left(\frac{k_{BE_1} \cdot k_{E_1E_2}}{k_{E_1B} \cdot k_{F_2E_1}} \right) \left(\frac{1 - Hc}{Hc} \right) \quad \text{(Eq. 31A)}$$

$$P_{e/c_u} = (e/c_u)_{eq} = \left(\frac{k_{BE_1} \cdot k_{E_1E_2}}{k_{E_1B} \cdot k_{F_2E_1}} + \frac{k_{BE_1}}{k_{E_1B}} \right) \left(\frac{1 - Hc}{Hc} \right) \quad \text{(Eq. 32A)}$$

APPENDIX III: Prediction of Apparent Mean Transit Time and Rate Constants of a Drug in a Erythrocyte Plasma Suspension from Its True Mean Transit Time and Rate Constants in an Erythrocyte Buffer Suspension Assuming a Two-Compartment Model

The values of the apparent $(\bar{t})$, k_{PE}, and k_{EP} for a drug in a red blood cell plasma suspension are expected to be different from the values of the true $\bar{t}$, k_{BE}, and k_{EB} for the same drug in a red blood cell buffer suspension. Plasma protein binding affects extent and rate of drug partitioning into red blood cells in a suspension in plasma. On the assumption that drug binding to and unbinding from plasma protein is much faster than drug partitioning into and repartitioning from red blood cells, predictions of $(\bar{t})$, k_{PE}, and k_{EP} from $\bar{t}$, k_{BE}, and k_{EB}, respectively, are possible.

The plasma binding-induced alteration of the equilibrium conditions and its impact on the magnitude of the parameters in a red blood cell suspension in plasma relative to a red blood cell suspension in buffer is considered first. Equation 16A can be reformulated:

$$\frac{E_{eq}}{E_{eq} - E} = e^{t/\bar{t}} \quad \text{(Eq. 33A)}$$

for a red blood cell buffer suspension experiment and:

$$\frac{(E_{eq})}{(E_{eq}) - (E)} = e^{t/(\bar{t})} \quad \text{(Eq. 34A)}$$

for a red cell plasma suspension experiment. Solving Eqs. 33A and 34A for $t = \bar{t}$ and $t = (\bar{t})$, respectively, yields:

$$E_{t=\bar{t}}/E_{eq} = (1 - e^{-t/\bar{t}}) = 0.632 \quad \text{(Eq. 35A)}$$

$$(E_t)_{=(\bar{t})}/(E_{eq}) = (1 - e^{-t=(\bar{t})}) = 0.632 \quad \text{(Eq. 36A)}$$

[10] R. Sperb and P. H. Hinderling, unpublished results.

Equations 35A and 36A indicate that after a lapse of $\bar{t}$ after drug administration to buffer or of $(\bar{t})$ after administration of drug to plasma, 63.2% of the amounts, respectively, residing in the erythrocytes at partition equilibrium have reached the cells. (Analogously for a drug "administration" into red blood cells, after a lapse of $\bar{t}$ following drug administration, 63.2% of the amounts, respectively, residing in plasma at partition equilibrium have reached the plasma.)

In the following fractional equilibria $[E_{t=\gamma\bar{t}} = (1 - e^{-\gamma})\cdot E_{eq}, (E_t)_{=\gamma\cdot(\bar{t})} = (1 - e^{-\gamma})\cdot(E_{eq})]$ of a drug and the respective times for their attainment in red blood cell suspensions, $t = \gamma\cdot\bar{t}$ and $(t) = \gamma\cdot(\bar{t})$, where γ is a dimensionless fraction ($0 < \gamma \leq 1$), will be considered. The respective fractional equilibria in a red blood cell suspension in plasma and in buffer are not equivalent for a plasma protein-bound drug. The shift in the equilibrium toward the plasma site in the former suspension affects the magnitude of the parameters. This effect can, however, be assessed theoretically in an erythrocyte buffer suspension. Fractional equilibrium conditions equivalent to those in an erythrocyte plasma suspension are reached at some time, $t = \gamma\cdot\bar{t}'$, after addition of drug to a red blood cell buffer suspension $[E_{t=\gamma\bar{t}'} = (1 - e^{-\gamma})E'_{eq}]$. Intuitively, the corresponding new mean transit time, $\bar{t}'$, should be smaller than $\bar{t}$ (and accordingly k'_{BE}, and k'_{EB} should be larger than k_{BE} and k_{EB}) since the new fractional equilibrium is reached earlier than the true fractional equilibrium in a red blood cell in buffer suspension.

The ratio of $\gamma\cdot\bar{t}$ to $\bar{t}'$, r, can be calculated from:

$$r = \gamma\frac{\bar{t}}{\bar{t}'} = -\ln\left[\frac{E'_{eq} - E_{t=\gamma\cdot\bar{t}}}{E'_{eq}}\right] \qquad \text{(Eq. 37A)}$$

After substituting Eqs. 1A, 2A, 9A, 24A, 25A, and 35A and rearranging, Eq. 37A can be reformulated:

$$r = \gamma\frac{\bar{t}}{\bar{t}'} = -\ln\left\{1 - \left[(1 - e^{-\gamma}) \cdot \frac{[(1 - \beta_{PB}/10^2)\cdot P_{e/c_u}\cdot Hc] + (1 - Hc)}{(1 - \beta_{PB}/10^2)\cdot[P_{e/c_u}\cdot Hc + (1 - Hc)]}\right]\right\} \qquad \text{(Eq. 38A)}$$

and k'_{BE} and k'_{EB} are then obtained from:

$$k'_{BE} = k_{BE}\cdot\frac{r}{\gamma} \qquad \text{(Eq. 39A)}$$

$$k'_{EB} = k_{EB}\cdot\frac{r}{\gamma} \qquad \text{(Eq. 40A)}$$

The plasma binding-induced reduction of the amounts of drug that can partition into the red blood cells is now considered. In a red blood cell plasma suspension only the unbound drug fraction in plasma is available for cell partitioning. The magnitude of the apparent partitioning constant, k_{PE}, referenced to the total (bound + unbound) drug concentration in plasma, will be affected in accordance with:

$$k_{PE} = k'_{BE}\cdot\left(1 - \frac{\beta_{PB}}{10^2}\right) = k_{BE}\cdot\frac{r}{\gamma}\left(1 - \frac{\beta_{PB}}{10^2}\right) \qquad \text{(Eq. 41A)}$$

Hence, if plasma protein binding and red blood cell buffer partitioning of a drug are known, the apparent parameters $(\bar{t})$, k_{PE}, and k_{EP} in an erythrocyte plasma suspension can be predicted from the true parameters ($\bar{t}$, k_{BE}, and k_{EB}) obtained for the drug in an erythrocyte buffer suspension according to:

$$(\bar{t}) = \frac{1}{k_{PE} + k_{EP}} = \frac{1}{\frac{r}{\gamma}[k_{BE}(100 - \beta_{PB}) + k_{EB}]} \qquad \text{(Eq. 42A)}$$

$$k_{PE} = \frac{r}{\gamma}[k_{BE}(100 - \beta_{PB})] \qquad \text{(Eq. 43A)}$$

$$k_{EP} = \frac{r}{\gamma}\cdot k_{EB} \qquad \text{(Eq. 44A)}$$

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Absorption and Disposition of Ethambutol in Rabbits

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Abstract □ The absorption and disposition of ethambutol was examined in six rabbits in a three-way crossover study. Each rabbit received 45-mg/kg doses of ethambutol in three treatments: one intravenous injection, and two oral solutions, ethambutol alone and ethambutol in the presence of aluminum hydroxide (40 mg/kg). Half-lives of ethambutol ranged from 2.26 to 5.20 h when administered alone and 2.18 to 4.00 h when coadministered with the antacid; the difference was not significant ($p > 0.3$). Mean clearance after the oral administrations (189.2 mL/min/kg) was significantly greater than the mean intravenous clearance (43.7 mL/min/kg) ($p < 0.01$), suggesting a first-pass metabolism of ethambutol when administered nonparenterally to rabbits. The volume of distribution ranged from 5.5 to 17.8 L/kg, suggesting an extensive distribution of ethambutol outside the central compartment and, possibly, a localized deposit within the body tissues. Mean bioavailability of ethambutol was ~28% and was not affected by the presence of aluminum hydroxide. The rate of ethambutol absorption, however, was slightly delayed by the antacid.

Keyphrases □ Ethambutol—pharmacokinetics in rabbits, coadministration with aluminum hydroxide □ Aluminum hydroxide—effect on ethambutol absorption in rabbits □ Bioavailability—absorption of ethambutol in rabbits, coadministration with aluminum hydroxide

Ethambutol (I), an antitubercular agent, is prescribed alone or in combination with other drugs for the treatment of tuberculosis. The absorption and excretion of I has been studied in rats and mice (1), dogs (2, 3), and humans (4–6). The median lethal dose (LD_{50}) of racemic I in noninfected adult mice was 12,800 mg/kg when administered orally, 1600 mg/kg when administered subcutaneously, 800–1600 mg/kg when administered intraperitoneally, and 200–400 mg/kg when administered intravenously (1). Since I is well absorbed in mice and the drug metabolites are pharmacologically inactive (3), the remarkable discrepancies in LD_{50} following different administration routes suggest a first-pass metabolism of the drug when taken nonparenterally. A part of this study was thus designed to investigate the effect of first-pass metabolism on the availability of I in rabbits.

Pharmacokinetic studies comparing the intravenous and oral administrations of I to humans have demonstrated rapid and adequate absorption, with bioavailabilities of 70–80% (6, 7). The oral solution and tablets of I were equally well absorbed in humans (6), suggesting a gastric emptying rate- rather than dissolution rate-limited absorption of the drug. Aluminum ion is a known inhibitor of gastric emptying, and its effect on drug absorption has been documented (8). Mattila *et al.* (9) have investigated the effect of aluminum hydroxide on the absorption of I in humans; the results were erratic. Since the results of Matilla *et al.* (9) were inconclusive, this study also seeks to clarify the effect of aluminum hydroxide on the absorption of I using the rabbit model.

EXPERIMENTAL SECTION

Materials—All materials used in this study were the same as those used in a previous investigation (10).

Animal Experiments—Six male New Zealand White rabbits (weight, 2.8–4.1 kg) were studied in a three-way crossover manner. Each rabbit received 45-mg/kg doses of I in three separate treatments: as an intravenous injection, as an oral solution, and as an oral solution in the presence of aluminum hydroxide (40 mg/kg). Gastric emptying of solid food residues was induced by fasting for 38–42 h before drug administration. Water was allowed *ad libitum* during fasting; food and water were withheld over the experimental period. A 2-week washout interval was implemented between the crossover studies.

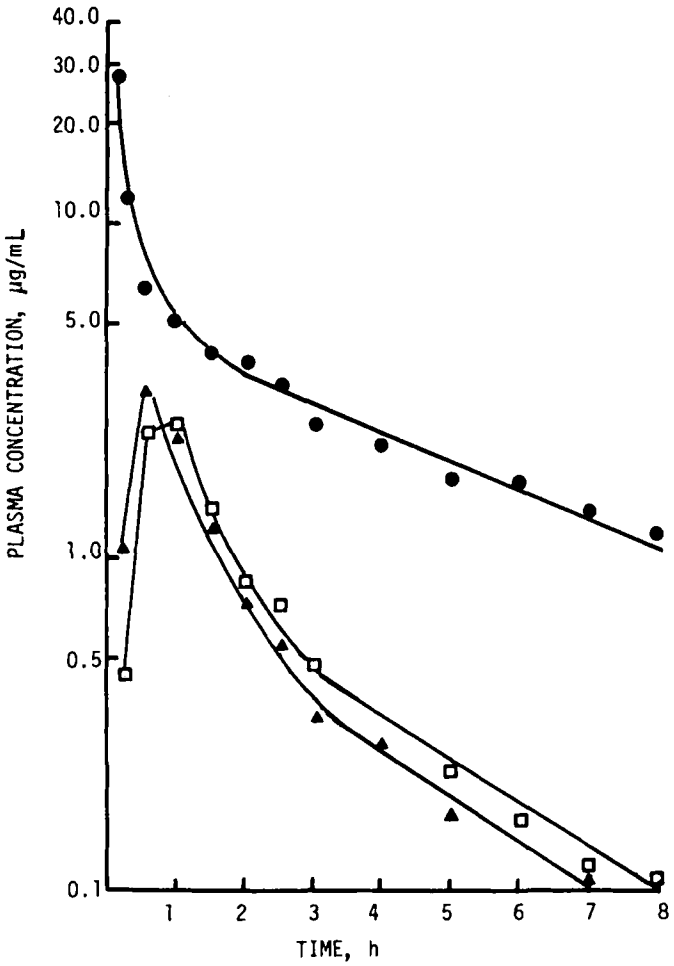

Figure 1—*Semilogarithmic plasma concentration* versus *time plots for rabbit 6 administered ethambutol (45 mg/kg) intravenously (●) and orally (▲, without aluminum hydroxide; □, with the antacid).*

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