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Review

Erythrocytes and the Transport of Drugs and Endogenous Compounds

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This review considers the significance and measurement of endogenous compounds and drugs on erythrocytes. Part I examines literature examples where a *direct* measurement of hydrocortisone, phenytoin and valproate was performed on unwashed red cells in vitro and in vivo, showing a consistent contribution of the erythrocyte fraction to the transport of these compounds. *In vitro* partition experiments using systems composed of plasma water, plasma proteins and erythrocytes are discussed. When spiked blood is diluted with blank autologous plasma water, erythrocytes always discharge the compound over-proportionally compared to plasma proteins. *In vivo,* during the distribution phase, the elimination half-life from the erythrocyte is the same as or shorter than that from plasma water, and substantial amounts of drug leaving the circulation originate from erythrocytes. In Part II, the transfer of compounds is considered and evidence for the facilitated exchange of red cell associated substances between the erythrocyte and capillary endothelium presented. Situations where a failure to analyse the erythrocyte compartment leads to the loss of vital information are identified. Part III explores methods for analysing erythrocyte associated substances, most commonly indirect *calculation,* or analysis of *washed* erythrocytes. A *direct* determination is rarely performed, but one such method, allowing concurrent plasma analysis, is discussed. An instrument collects a *fixed and known* quantity of a maximally compressed cell mass, without disturbing the equilibrium between cells and plasma. To isolate compounds associated with the mass of erythrocytes, the red cell sediment can often be extracted quantitatively into a blank protein solution.

KEY WORDS: biological transport; erythrocytes; plasma; protein binding; blood specimen collection; valproic acid; phenytoin; hydrocortisone.

INTRODUCTION

Quantitatively, the three most important transport fractions of blood are plasma water, plasma proteins and cells, mainly erythrocytes. In the laboratory, this three compartmental system, equilibrating in the circulation in the dark and at 37°C, is reduced for practical purposes to the two compartmental system of plasma and analysed at room temperature as if it had only one compartment. The information present in the three separate fractions of whole blood is therefore combined into one value. In the case of a compound which is bound to plasma proteins, this composite value is misleading as a change in the free fraction present in plasma water can be hidden by the large quantity present on proteins. Binding to erythrocytes also occurs, but even less consideration has been given to the role of these cells in the transport of compounds other than oxygen.

There are three reasons for this neglect of the erythrocyte and subsequent lack of knowledge. Firstly, conceptual difficulties exist, as illustrated by the following statement: "The erythrocyte has a cell wall, so it really has its own compartment. Distribution in body fluids does not take effect versus the fraction free in whole blood, but versus the free concentration in plasma water. From the pharmacokinetic point of view, I would say that the red blood cells have the lowest priority" as discussed by a pioneer on erythrocyte studies, M. Ehrnebo (1). As a result, the transport role of erythrocytes is almost invariably investigated using *washed* erythrocytes, as if these cells were indeed separate compartments, and regardless of the fact that washed erythrocytes do not exist in the circulation. Secondly, the transport function of red cells is influenced by physiological factors as the role of erythrocytes will often become more apparent when protein binding is saturated, and thirdly, bioanalytical limitations restrict the investigation of the transport role of erythrocytes.

In the first part of the review, three reports from the literature on the drugs valproate and phenytoin, and the endogenous compound hydrocortisone, are described in detail and the data re-evaluated. All three sudies illustrate that when the above considerations are addressed, erythrocytes do in fact show a consistent and important transport function.

In part II, a review of the mechanisms and significance of substance transport by red cells, and subsequent exchange at the capillary endothelium, is presented.

Part III discusses approaches to the analysis of red cell associated substances, including a method for the analysis of

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drugs or endogenous compounds both directly on red cells and in the plasma of a single blood sample.

PART I

THE TRANSPORT OF SUBSTANCES WITHIN THE MAIN BLOOD FRACTIONS AND THE CONTRIBUTION OF THE ERYTHROCYTE (Three examples from the literature)

Methods of Erythrocyte Investigation

The use of appropriate methods is of vital importance for demonstrating the transport role of erythrocytes. The essence is to measure the compounds present in or on unwashed erythrocytes directly. Three studies in which such an approach was used (2–5) are reviewed in detail. They concern the compounds valproate, an antiepileptic drug and small fatty acid; phenytoin, also an antiepileptic drug; and the steroid hormone hydrocortisone. Both in vitro and in vivo work was described.

The method used to process the erythrocytes in these studies involved harvesting blood into haematocrit capillaries and separating erythrocyte sediments by centrifugation at 10,000 g. The capillaries were then cut with a glass knife, and both the length of the cut fragment and its weight determined. Knowing the weight per unit length of capillary tubing, the mass of the cells was obtained. The cell mass was extracted and its drug content analysed. From these measurements a known concentration in w./v. of cell mass was derived. A correction for the difference in density between red cells and water was not made. During sample pretreatment, care was taken to maintain the same equilibria as present in whole blood or an erythrocyte suspension in plasma water. Hence after sampling, the haematocrit capillaries were centrifuged immediately and the sediment separated from plasma or plasma water. The concentration of drug in the red cell sediment was then adjusted for the residual plasma trapped between the cells. This was determined using ^{14}C-inulin as an extracellular marker and appeared to be 2.0%, in accordance with the literature (6). During sample pretreatment this trapped plasma may be important to maintain the equilibrium that exists in the circulation. The quantity of drug bound to plasma proteins was calculated by subtracting the quantity in plasma water from that in plasma. In vitro, it was confirmed that the total drug recovery from all the fractions was equal to the amount added to blood, plasma or the mixture of red cells and plasma water.

In vitro Partition of Phenytoin, Valproate and Hydrocortisone Between Plasma Water, Proteins and Red Cells

Tables I to III, constructed from the original data of Driessen et al (2–5), show the distribution of these compounds between the three main blood fractions at 20°C; concentrations are expressed in weight per volume of blood, plasma, plasma protein, plasma water or erythrocytes.

(a) Distribution within the Binary System of Plasma Water and Plasma Proteins, i.e. Plasma

Table I shows the plasma concentrations of phenytoin, valproate and hydrocortisone, and the concentrations bound to protein compared to those in plasma water, expressed as the plasma protein/plasma water concentration ratio. As expected, the higher the concentration in spiked plasma, the lower the plasma protein/plasma water ratio of the compound, indicating that the plasma proteins are saturable with all three compounds.

(b) Distribution within an Erythrocyte and Plasma Water Binary System

This is an artificial mixture, in which proteins adhering to erythrocytes are stripped from these cells by washing with autologous plasma water, *before the compounds are added.* Following distribution of the compound between the two fractions, the erythrocytes are not subjected to further washing. The observed red cell/plasma water concentration ratios, over a similar concentration range to that described in Table I, are shown in Table II.

In contrast to the data shown in Table I, the red cell/plasma water ratios are constant, indicating that erythrocytes cannot be saturated by these compounds, not even in the high and toxic concentration ranges used. Compounds which are distributed only in the water phase, and to the same extent both inside and outside the erythrocyte, should have a red cell to plasma water concentration ratio of approximately 0.65; substantially higher ratios indicate accumulation within or on the surface of the red cell.

(c) Distribution within a Ternary System of Plasma Water, Plasma Proteins and Erythrocytes, ie Whole Blood

Table III presents the effect of increasing concentration on the distribution ratios within whole blood.

As the concentration in spiked blood increases, the concentration in plasma water increases relatively to that on plasma protein. Comparing the data in Table III with that in Table I, it must be appreciated that the plasma fraction is now approximately 55% of the total volume, and that the erythrocytes occupy the remaining 45%. Consequently, in addition to competition between the binding sites of the different fractions, the relative volume of these fractions will also influence the observed concentration ratio. This hinders a direct comparison of the observed plasma protein/plasma water ratio in plasma (Table I), with that in blood (Table III). It is striking that as the total concentration in the system increases, the erythrocyte/plasma water ratio remains constant for each drug in the binary system (Table II), and is also constant for phenytoin and hydrocortisone in the ternary system (Table III). *This indicates that for these two compounds, the concentrations on the erythrocytes and in plasma water increase proportionally.* As varying concentrations of phenytoin and hydrocortisone do not alter the red cell/plasma water ratio, the erythrocyte concentration is proportional to the concentration in plasma water.

In Table III, the erythrocyte/plasma water concentration ratios for hydrocortisone and phenytoin appear slightly increased and decreased respectively, in comparison with those in Table II. One must be cautious interpreting this difference, as it seems that the same blood sample was not used in the two experiments, and it is known that other substances, e.g. non-esterified fatty acids, can change the equilibrium between compartments (7–11). Moreover, in the ternary system, the equilibrium between red cells and plasma water was established

Table I. Plasma Protein and Plasma Water Binary System (Plasma) (Temperature 20°C)
Plasma concentration and plasma protein/plasma water concentration ratio (sd)

Valproate		Phenytoin		Hydrocortisone	
Plasma concentration (μg/ml)	Plasma protein/plasma water ratio n = 3	Plasma concentration (μg/ml)	Plasma protein/plasma water ratio n = 3	Plasma concentration (μg/ml)	Plasma protein/plasma water ratio n = 3
2.7	14.0 (0.2)	0.8	14.4 (1.7)	0.18	8.2 (0.4)
27.6	12.5 (0.2)	5.8	14.3 (1.5)	0.68	3.1 (0.2)
48.3	10.8 (0.6)	10.8	13.6 (1.1)	1.18	2.6 (0.1)
69.8	8.3 (0.6)	20.8	13.6 (0.9)	5.18	2.1 (< 0.1)
96.6	5.4 (0.3)	50.8	11.6 (0.7)	10.18	1.9 (0.1)
165.7	3.7 (0.2)	100.8	8.7 (0.6)		
194.9	2.5 (0.1)				
243.0	1.8 (< 0.1)				
1080.2	0.3 (0.1)				

in the presence of plasma proteins, and the cells were not washed beforehand with autologous plasma water.

As the concentration of valproate increases in whole blood, the increasing amount in the water fraction facilitates further accumulation on the erythrocyte, so that the ratio of 0.68 in Table II is approached at the highest valproate concentration in Table III. At low valproate concentrations, this ratio is much smaller in whole blood compared to that of the erythrocyte/plasma water system in Table II. This implies that the plasma protein fraction, which cannot pass the ultrafiltrate membrane employed to obtain plasma water, may contain a competitive inhibitor capable of decreasing the uptake of valproate by erythrocytes, the inhibitor being absent in the plasma water fraction.

Concentration ratios can be calculated from the data of these three studies and compared with other published results. The ratio of red cell to total plasma phenytoin concentration can be obtained from Table III.[4] At a plasma phenytoin concentration of about 8 μg/ml this is approximately 0.3, corresponding with a ratio of 0.28 found by Borondy et al (12) who, using the radioactively labelled drug, also measured the concentration of phenytoin directly on erythrocytes. Kurata and Wilkinson (7), employing indirect techniques and not specifying the plasma phenytoin level, found a ratio of 0.22.

The valproate erythrocyte/plasma water concentration ratio of 0.68 is comparable to a ratio of 0.87 in a red cell/buffer system, as reported by Shirkey et al (13), although in this study the concentration of valproate on erythrocytes was not measured directly, and the observed erythrocyte/plasma partition ratio of 0.20 is far from the ratio of approximately 0.03 that can be calculated from table III, at a blood concentration of 44 μg/ml. The ratios of 0.68 and 0.87 suggest a distribution of valproate merely over water phases inside and outside the erythrocyte, seemingly at odds with the apparent competition for binding sites.

[4] The calculation of the red cell to plasma ratio of phenytoin at a total blood concentration of 5.8 μg/ml, and assuming an haematocrit of 45%, is as follows: 55/100 (13.8 w) + 45/100 (4.0 w) = 5.8 μg/ml, where w is the plasma water concentration. Hence w = 0.618 μg/ml. From this the erythrocyte to plasma ratio is calculated as 0.29.

A substantial association of hydrocortisone with erythrocytes has also been described in other reports in the literature. In similar experiments to those of Driessen et al, Hiramatsu and Nisula obtained an erythrocyte/plasma water coefficient of 2.62 at 36.8°C (14), consistent with the ratio of 2.4, at room temperature in Table III.

(d) In vitro release of substances from the three blood fractions

Driessen et al investigated compound loss from plasma proteins and erythrocytes in spiked whole blood in vitro, following dilution with blank autologous plasma water (2–5). This reproduced the conditions in capillaries with a discontinuous endothelium, simulating the contact of blood with blank intercellular tissue water following the initial exposure of an individual to a compound. Table IV, derived from their data, shows the redistribution of valproate, phenytoin and hydrocortisone within whole blood after dilution with plasma water. Each compound is released relatively easily from the erythrocyte in comparison to plasma proteins, and this finding is supported by other data in the literature for hydrocortisone (14).

(e) Time to equilibrium between blood fractions and effect of temperature

The time to equilibrium of a compound between erythrocytes and plasma has been determined by recombining blank erythrocytes with spiked plasma, measuring the concentration on erythrocytes at several time points, and calculating the rate of migration. In the case of valproate and hydrocortisone, at room temperature and 38°C, equilibrium between blank erythrocytes and spiked plasma is reached within 3 minutes of pooling cells and plasma (4), and it has also been shown that at 36.8°C the dissociation of hydrocortisone from erythrocytes occurs within 2.3 seconds (14). Similarly, the equilibration of pentazocine (15), fentanyl, busulfan and tricyclic antidepressants (16), between erythrocytes and plasma, occurs rapidly within 5 minutes.

Conversely, in the case of phenytoin, the equilibrium between erythrocytes and plasma is apparently not achieved at

Table II. Erythrocyte and Plasma Water Binary System (Hematocrit 0.39 – 0.50; Temperature 20°C)
System concentration and erythrocyte/plasma water concentration ratio (sd)

Valproate		Phenytoin		Hydrocortisone	
System concentration (μg/ml)	red cell/plasma water ratio n = 3	System concentration (μg/ml)	red cell/plasma water ratio n = 3	System concentration (μg/ml)	red cell/plasma water ratio n = 3
2.7	0.68 (0.03)	0.8	4.5 (0.2)	0.18	2.1 (< 0.1)
24.7	0.68 (0.01)	5.8	4.5 (0.1)	0.68	2.0 (0.1)
44	0.60 (0.02)	10.8	4.6 (0.2)	1.18	2.0 (0.1)
66	0.70 (0.01)	20.8	4.5 (0.1)	5.18	2.1 (0.1)
88	0.68 (0.02)	50.8	4.5 (< 0.1)	10.18	2.1 (0.1)
132	0.70 (0.01)	100.8	4.5 (0.2)		
176	0.67 (0.01)				
220	0.69 (0.02)				
978	0.70 (0.01)				

room temperature between 3 and 10 minutes after pooling (3). There are other observations in the literature supporting the slower equilibration of phenytoin. Graves et al reported a consistently lower concentration of phenytoin in capillary serum than in simultaneously sampled venous serum. This difference did not exist for phenobarbital, and the source of this discrepancy was not found (17). In another study, an escalating dosage regimen of phenytoin resulted in significantly lower plasma concentrations compared with the same dose given in a reducing regimen (18). This hysteresis loop may be explained by a slow equilibration of phenytoin between tissue cells and plasma. A prolonged time to equilibrium of 60 minutes, between blood cells and plasma, has also been described for pirarubicin (19).

However, for the majority of drugs in large vessels at 37°C, and in clinical samples drawn from them, it seems likely that red cells are in equilibrium with plasma.

Table III. Plasma Protein, Plasma Water and Erythrocyte Ternary System (whole blood) (Hematocrit 0.39 – 0.5; Temperature 20°C)
Blood concentration with plasma protein/plasma water and erythrocyte/plasma water concentration ratios

Blood Concentration (μg/ml)	Plasma protein/plasma water ratio n = 3	Erythrocyte/plasma water ratio n = 3
	Valproate	
2.7	17.0 (0.6)	0.33 (0.01)
23.1	6.3 (1.1)	0.35 (0.04)
44	7.7 (0.4)	0.24 (0.02)
66	6.0 (0.3)	0.37 (0.02)
88	4.4 (0.3)	0.40 (0.04)
132	2.9 (0.3)	0.51 (0.05)
220	1.5 (0.2)	0.55 (0.03)
978	0.3 (< 0.1)	0.66 (0.04)
	Phenytoin	
0.8	12.7 (0.5)	3.7 (0.3)
5.8	12.8 (0.2)	4.0 (0.3)
10.8	12.0 (0.4)	3.9 (0.3)
20.8	10.8 (0.1)	3.8 (0.3)
50.8	8.6 (0.3)	3.9 (0.3)
100.8	6.4 (0.1)	4.1 (0.2)
	Hydrocortisone	
0.18	7.2 (0.3)	2.3 (0.1)
0.68	3.2 (< 0.1)	2.4 (0.1)
1.18	2.7 (0.1)	2.4 (0.1)
5.18	2.1 (< 0.1)	2.4 (0.1)
10.18	2.1 (0.1)	2.4 (0.1)

In vivo Partition of Phenytoin, Valproate and Hydrocortisone Between Plasma Water, Proteins and Red Cells

Figures 1, 2 and Table V, also compiled from Driessen et al (2,3,5), illustrate the in vivo distribution of phenytoin, valproate and hydrocortisone.

Figure 1 shows the log concentration time curves of valproate in the three blood fractions, after an oral dose of 2.4 g

Table IV. Distribution of Substances Between Blood Compartments in vitro, before and After Expansion of Blood with Blank Autologous Ultrafiltrate

	Before	After	Change
Valproate (VPA)			
volume of whole blood	2.0 ml	2.9 ml	+45%
total amount of VPA	151.0μg	151.0μg	0%
VPA in plasma water	16.8%	29.4%	+75%
VPA on proteins	72.2%	63.4%	−12.7%
VPA on erythrocytes	7.7%	4.7%	−40%
Phenytoin (DPH)			
volume of whole blood	2.0 ml	2.9 ml	+45%
total amount of DPH	21 μg	21 μg	0%
DPH in plasma water	5.4%	8.9%	+65%
DPH on proteins	76.1%	76.6%	+0.7%
DPH on erythrocytes	17.6%	16.5%	−6%
Hydrocortisone (HCOR)			
volume of whole blood	1.5 ml	2.4 ml	+60%
total amount of HCOR	97.5 ng	97.5 ng	0%
HCOR in plasma water	11.1%	18.6%	+68%
HCOR on proteins	68.0%	64.7%	−5%
HCOR on erythrocytes	20.2%	16.3%	−19%

Table V. Rate of Disappearance of Substances from Plasma Proteins, Plasma Water and Red Cells Following a Single Administration in Man Half-life in minutes

Substance	Valproate	Phenytoin	Hydrocortisone
Global time period in which half-life was observed* (min)	30–165	135–1935	30–75
Plasma proteins	300	4074	116
Plasma water	120	2310	24
Erythrocytes	42	1284	26

* The half-lives of valproate in the three blood fractions were obtained graphically. The curves of phenytoin and hydrocortisone were fitted according to a two-component open model.

to a human volunteer, and Figure 2 the same curves expressed as a percentage of the maximum concentration in the fraction.

The data presented in Figure 2 suggest that the rate of change in drug concentration is greater on erythrocytes than on plasma proteins, and the same phenomenon occurs when phenytoin is infused linearly over 30 minutes (3). A more rapid decline of the red cell fraction is observed following a bolus injection of hydrocortisone (5). The rates of decline of valproate, phenytoin and hydrocortisone, in the various blood compartments, are presented in Table V. The elimination half-life from each compartment of whole blood is different; loss from erythrocytes is *faster* than loss from plasma proteins, and it appears that for these three compounds, those molecules associated with erythrocytes, rather than those bound to plasma proteins, are more likely to exchange with plasma water.

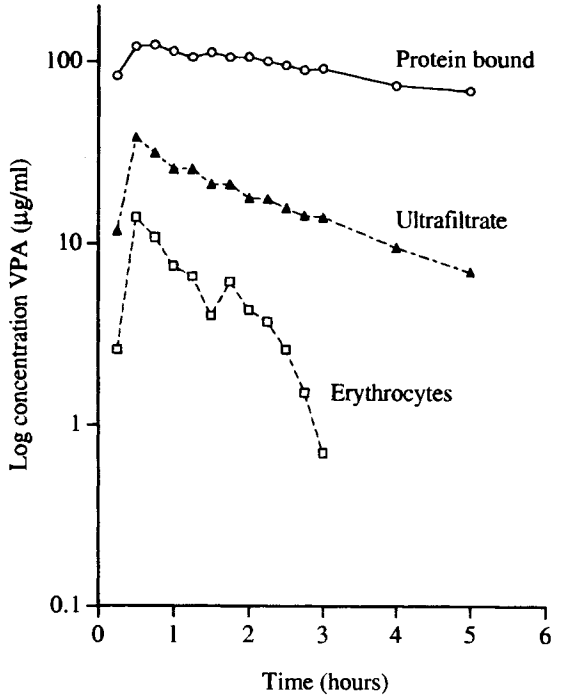

Fig. 1. Concentration time profiles of valproate (VPA) in different blood fractions.

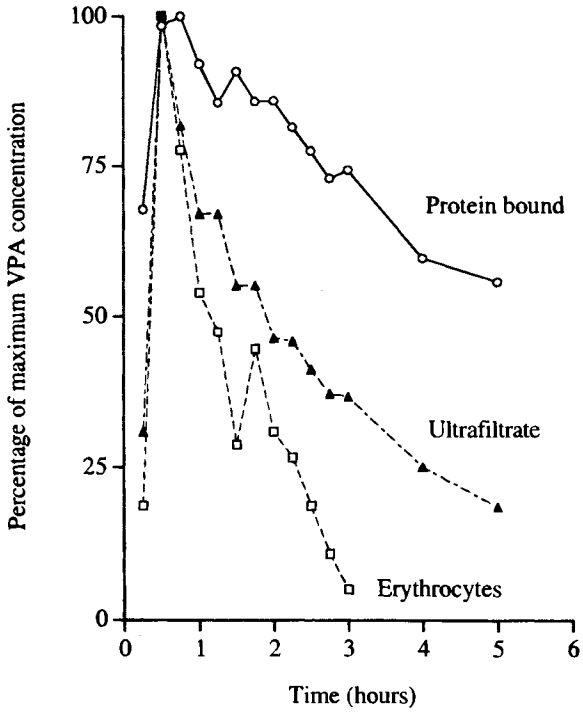

Fig. 2. Percentage change in valproate (VPA) concentration in each fraction.

Differences in the erythrocyte and plasma half-lives of other compounds are reported in the literature (9,20,21). For example, in a pharmacokinetic study of a new sulfonamide, highly bound to erythrocytes, the drug was found to have a *longer* red cell half-life compared to its plasma half-life (20), whereas the half-life of diazepam on erythrocytes is *shorter* than its plasma half-life (9).

Summary

In conclusion, it seems that for certain compounds, erythrocytes form a *secondary* transport system in whole blood which, with plasma water, becomes more significant as the non-protein bound drug concentration increases. Data from dilution experiments indicate that erythrocyte bound valproate, phenytoin or hydrocortisone are more readily available to blank plasma water than the protein bound fraction. In vivo observations, using the same compounds, support the hypothesis that erythrocytes act as a secondary transport system, more reluctant to load and faster to discharge than plasma proteins.

PART II

THE TRANSPORT OF SUBSTANCES IN WHOLE BLOOD AND THEIR EXCHANGE WITH TISSUES

Traditionally, plasma proteins have been viewed as the carriers of drugs and endogenous compounds, in equilibrium with plasma water, whilst the erythrocytes have been regarded as a transport system for blood gases, which bind to proteins within the red cell. However, as described in part I, the erythrocyte can possess a more extensive transport function. Compounds with a strong affinity for intracellular proteins may be sequestrated within the erythrocyte. Such binding does not

necessarily imply that compounds are unavailable to the tissues, but with the exception of oxygen the transport mechanisms have been poorly defined. For most compounds showing an association with erythrocytes, it is not known whether they primarily bind to the outside of the erythrocyte, penetrate the cell membrane, or if both mechanisms are operative. Despite these uncertainties, erythrocytes may be of great importance in transferring such compounds to the tissues. This section discusses the erythrocyte in the context of the classic equilibrium model of plasma proteins, plasma water and tissues.

The Starling Hypothesis

In 1895, Starling introduced the concept of the colloid osmotic pressure in his article: "On the absorption of fluids from the connective tissue spaces" (22). At the same time Hamburger called the absorption of fluid from the tissue spaces "molecular imbibition" (22). The absorbed fluids also contained dissolved electrolytes. The movement of tissue water containing dissolved compounds into a capillary through its endothelium, as a result of the colloid osmotic pressure, was also visualised as occurring in the reverse direction *towards* tissues by virtue of the arterial pressure. Nevertheless, it is not certain if compounds need to be dissolved in plasma water to pass a capillary endothelium into the tissues. An equilibrium between blood and tissues based solely on compounds dissolved in the plasma water phase is a commonly held view in bioscience, (e.g. see the quote of Ehrnebo in the introduction), and is often used in kinetic models (9,23). However, compounds bound to transport proteins in blood may use an additional pathway. Pardridge et al suggest that there are circumstances where the influx of a compound from capillaries into tissues is greater than can be accounted for by the influx from plasma water alone. Their model assumes that an inhibition of protein binding occurs on the capillary endothelium, thereby releasing the compound, and as a result tissue levels correspond more closely to the protein bound fraction than to the free fraction (24,25).

This concept is just as important when considering erythrocytes, as tissues on the other side of a capillary endothelium, or the capillary endothelium itself, may be more exposed to compounds accumulated on these cells (26,27). In the studies reviewed in part I, it was calculated that approximately 40% of the valproate leaving the circulation in the distribution phase originated from erythrocytes; for phenytoin, between one and five hours after administration, this figure was approximately 50%; and for hydrocortisone the value was 28%, between one and twenty minutes after injection. This is in spite of the much lower concentration of drug in the red cell fraction compared to the plasma protein fraction.

Plasma Water as the Central Compartment

Figure 3 shows a dynamic model of whole blood with tissues. It is assumed that the tissues are "empty", and that the redistribution of substances from tissue to blood is negligible.

In this model the plasma water is the central compartment. In a situation of excessive outflow to the tissues, as illustrated in the figure, the concentration in the plasma water compartment depends on the influx from proteins and cells. For a substance bound to plasma protein, the plasma water half-life will be the same as that on proteins when the red cells are empty, whereas it equals that on red cells when loaded erythrocytes discharge and proteins do not deliver substance in any appreciable amounts to plasma water. As binding to proteins is often stronger than to erythrocytes, the half-life measured in plasma water in the case of "empty" tissues will often lie between the longer one of plasma proteins and the shorter one of red cells (see part I, Table V), provided both of these compartments are delivering the substance to plasma water. It seems that the mass of a substance transferred from plasma proteins or red cells to tissues, as depicted in Figure 3, depends firstly, on the relative binding force exerted by these fractions, and secondly, on their storage capacity. A maximum capacity for erythrocytes is sometimes recorded in the literature (28), but often such a maximum cannot be determined (2–4,29,30). Both saturable (hyperbolic) and non-saturable (linear) uptake processes have been described (20,23,31), and the non-linear release from erythrocytes of thiazide diuretics, such as chlorothiazide, on account of binding to intra-erythrocytic carbonic anhydrase, has been well documented (21).

It is likely that the hypothetical transport of substances directly from the red cells to tissues, (dashed arrow in Figure 3), will be difficult to demonstrate using blood samples from large blood vessels.

The Role of the Erythrocyte

As well as generating the colloid osmotic pressure, the higher protein content of plasma, compared to the content of interstitial water, may form a "chemical barrier", to some extent isolating tissues from many compounds in the circulation, and hence from the exterior and each other. Only when the capacity of the plasma proteins is exceeded, are cells—initially blood cells—confronted by higher concentrations. The ability of the erythrocyte to take up and transport molecules is not compromised by its cell wall, provided that such molecules, located inside or outside the wall, can be exchanged with the plasma water fraction or the endothelium during passage through the capillary, a process well illustrated by the transport of oxygen.

Hence the concentration on erythrocytes will often not be significant at low plasma concentrations, and it is only as the plasma concentration increases that erythrocytes carry a physiologically or clinically relevant load. This occurs when plasma protein binding is saturated, especially locally following an intravenous bolus injection, or in blood flowing through the microvilli and transporting substances from the gut lumen to the portal vein. The experimental conditions described in part I, where blood is diluted with autologous plasma water, simulate a capillary with a discontinuous endothelium, found for example in the liver. Erythrocytes, loaded with compounds absorbed from the intestinal microvilli, pass through the portal vein and enter the liver sinusoids, which are lined with fenestrated endothelial cells; when they come into direct contact with the intercellular fluid they release their cargo more readily than the plasma proteins (32) (see Table IV, Part I).

Physical factors, including temperature, affect equilibration within whole blood, but to our knowledge there are only two studies in the literature where partition was observed methodologically at 37°C (19,23). In vitro, the blood to plasma concentration ratio of pirarubicin is 1.8 times higher at 37°C than at 25°C, after 60 minutes incubation, suggesting increased uptake by erythrocytes (19). Furthermore, the binding of phe-

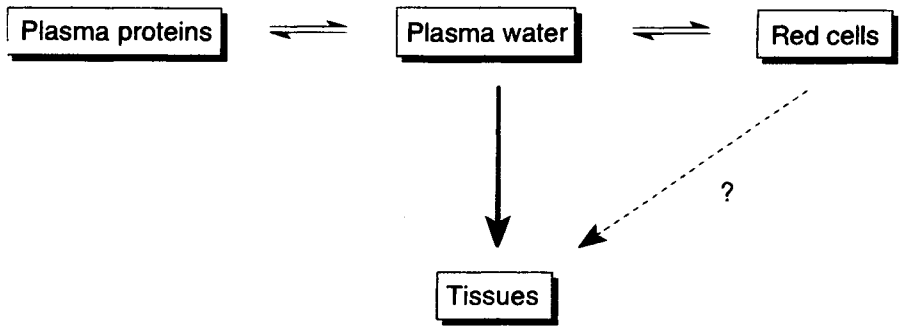

Fig. 3. Erythrocytes and the transport of drugs and endogenous compounds.

nytoin to plasma proteins is less at body temperature than at room temperature. When ultrafiltrate is separated from plasma containing 19.5 μg/ml of phenytoin, the concentration of phenytoin in the ultrafiltrate is 64% higher at 30 to 40°C compared to room temperature (3). Hence in vivo, a systematic shift towards increased concentrations in plasma water, and therefore also in the red cell, is to be expected when compared with data obtained at room temperature. From this point of view, the role of red cells in the transfer of substances is likely to be underestimated.

However, an important question remains:-Can the exchange of whole blood with tissues be described sufficiently in the physico-chemical terms of a system of water, dissolved proteins with a high binding affinity and a low capacity, and suspended cells often with a low binding affinity but a high capacity? Superimposed is a complex rheological system. The flow in capillaries where exchange takes place is intermittent. Capillaries are often smaller than the red cell diameter, and cells are squeezed to fit into the capillary (33). In periods of stasis, the lubricating plasma layer between erythrocytes and the capillary membrane breaks down, and the red cells line up against the capillary membrane. Extra force is needed to start the blood moving again in the capillary (33). In view of these characteristics of the microcirculation, it is possible that a molecule adhering to the relatively fluid red cell membrane, which maintains a dynamic and intimate contact with the endothelial cells, is better placed energetically and/or spacially to cross a continuous capillary endothelium, (for instance the blood/brain barrier), as opposed to being dissolved in plasma water. This may be apparent in the increased rate of change described for erythrocyte bound xenon, compared to the distribution of labelled water, when both are administered in the same injection (34). Thus, during a period of stasis, a transient disequilibrium between red cells and plasma water may occur in the capillary.

It is not at all certain, though, that molecules carried by erythrocytes are presented favourably to the capillary endothelium during the arrest of capillary flow. In some models plasma flow never stops, and pre-capillary sphincters are narrowed so that erythrocytes cannot enter the capillary. As a result, erythrocytes are washed from the capillary system, without experiencing stationary contact with the endothelium (35).

Once capillary flow is resumed, any disequilibrium between cells and plasma will be reversed immediately, and the special transport function of erythrocytes, *if present at all,* will be undetectable in the effluent of larger veins from which blood samples are taken. The time elapsing between blood sampling and the separation of cells from plasma will also mask any specific role the blood components possess. One must further consider that the haematocrit in small capillaries is about half that in large vessels, whereas flow is significantly slower (33). Therefore, although the lower haematocrit reflects the diminished number of erythrocytes in the capillaries, this may be balanced by the increased time available for discharge into the intercellular fluid.

Indications for Analysis of the Red Cell Compartment

In many clinical situations the analysis of plasma alone is adequate. As the relationship between erythrocyte cargo and plasma water concentration is often stable, if desired the quantification of the amount associated with the erythrocyte is a satisfactory alternative to the measurement of concentrations in plasma water. One can also envisage some circumstances in which a more elaborate analysis of the transport system in whole blood is indicated.

a Studies where the erythrocyte itself is the subject of investigation (36). Red cells have been used as drug carriers, particularly of cytotoxic agents (37), and for certain compounds they can act as a bioreactor (19,38–41). In the treatment of malaria, the penetration of antimalarial drugs into the erythrocyte is obviously a critical step (29), and in nutritional studies erythrocyte vitamin content is frequently measured (42). Events in the erythrocyte can often reflect those in other tissues. For example, the red cell folate level is a widely used indicator of the folate status of an individual. More recently, erythrocyte glutathione levels have been proposed as a predictor of tissue intracellular glutathione concentration (43) and as a biological marker for the severity of cystic fibrosis (44), whilst in children receiving maintenance 6-mercaptopurine for acute lymphoblastic leukaemia, the level of erythrocyte 6-thioguanine nucleotides (ETGN) has been found to be important. One study found a correlation between mean ETGN and the degree of myelosuppression (40), and in another report a multivariate analysis showed that ETGN concentration was an independent prognostic variable (45).

b Studies involving the distribution and mutual displacement of body constituents, foodstuffs and xenobiotics through various blood compartments (8,46–47). The mutual interaction of valproate and phenytoin, instrumental in commencing the erythrocyte studies reviewed in part I, is one such example (11). Sometimes the concentration in the erythrocyte compartment is greater than that in the plasma, as in the case of desethylamio-

darone, a metabolite of amiodarone (48), or cyclophosphamide (49). A physiological model of hydrocortisone transport has been described, in which the erythrocyte is central to the delivery of this compound to tissues, on account of the faster rate of dissociation of erythrocyte bound hydrocortisone compared to that bound to cortisol binding globulin (CBG) (14). One would also expect that in certain abnormal conditions (e.g. hyperbilirubinaemia, uremia), when large quantities of pathophysiological products bind to plasma proteins, the secondary transport system will be loaded more heavily (10,13).

Therefore, in the case of certain compounds and in some pathophysiological conditions, it is worthwhile investigating the relationship between the concentration on plasma proteins, the steady state load on erythrocytes and the physiological consequences; some desired or undesired effects may be more closely related to erythrocyte load than to the concentration on (nearly saturated) plasma proteins. For example, in neonates with erythroblastosis foetalis, an unconjugated plasma bilirubin concentration of 300 μmol/l or more leads to the uptake of bilirubin into the central nervous system, which may result in kernicterus. This pivotal plasma concentration may also be accompanied by a manifest loading of erythrocytes.

PART III

THE ANALYSIS OF THE ERYTHROCYTE COMPARTMENT

Methods Used in the Literature

After collecting a blood sample it is easy to obtain plasma by centrifugation, but the separation of plasma water from plasma is more laborious and not routinely performed in clinical laboratories. The degree of plasma protein binding of a substance is calculated by subtracting the amount present in the plasma water, called the free fraction, from that present in total plasma.

The red cell fraction presents the most obstacles when analysing the three main blood fractions, as the residual cell sediment formed following the centrifugation of blood is difficult to analyse quantitatively using normal volumetric procedures. One approach to this problem is to measure the volume of the cell sediment in a calibrated centrifuge tube. However it is then necessary to wash the sediment with saline or buffer several times before analysis, in order to remove the plasma trapped between the cells; this has the disadvantage of disturbing the normal equilibrium between red cells and plasma water, as presented in Figure 3 Part II. The effects of such a washing procedure on the results subsequently obtained are rarely mentioned, and to our knowledge the washing itself is never analysed. A second approach, in which the erythrocytes remain in their natural environment, but where the analysis is indirect, is also common in the literature. The amount associated with red cells is *calculated* by subtracting the amount present in plasma, corrected for the haematocrit, from that present in whole blood (not a medium in which concentrations are routinely measured). As the quantity associated with red cells is often small compared to the plasma concentration, two large numbers are used to determine a small one. This is an inaccurate method, and occasionally even negative values are obtained when the erythrocyte associated amount is calculated. When analysing the erythrocyte compartment these problems are frequently not discussed, and how the recorded erythrocyte concentrations are obtained is not clear. Of 56 reports in the literature describing the analysis of the erythrocyte compartment, 30 employed the washing technique, 11 used subtraction, and in 15 the method was not evident.

In the three studies reviewed in part I, a known weight of erythrocyte sediment was harvested from haematocrit capillaries, and a correction made for the trapped plasma (2% v./v.). Although surmounting the limitations described above, the method is so inconvenient that it is not acceptable for routine use. Only one other example was found in the literature where a similar procedure was followed (Maling et al 1989) (48). As discussed in part II, there is sometimes a need for a direct analysis of the erythrocyte compartment and a more appropriate method, which also allows the simultaneous analysis of plasma, is now available (50) (see below). This has recently been used to investigate the transport of cyclophosphamide and 4-hydroxycyclophosphamide in blood (49), and further work is being performed with other oxazaphosphorines, the taxanes, antimetabolites, mitomycins and suramin.

Direct Analysis of the Erythrocyte Compartment

A direct determination of red cell associated compounds must be performed on *unwashed* erythrocytes, maintained in their natural environment, without disruption of the normal equilibrium existing between red cells and plasma in whole blood. Washing procedures strip the red cells of their outer layer, which may be important in the transport and exchange process. Before a direct analysis, the red cells must be compressed, reducing the amount of plasma between the cells to a fixed volume, ideally as low as possible, approximately 2% (6). The red cells need to be collected in a reproducible manner, and the volume of the collected cell mass must be known.

An instrument (US patent 1993, no. 5256314) meeting these criteria is described in the literature (50), (Figure 4).

A reservoir A is inserted into a container B, as shown in Figure 4. The reservoir is fitted tightly into the container so

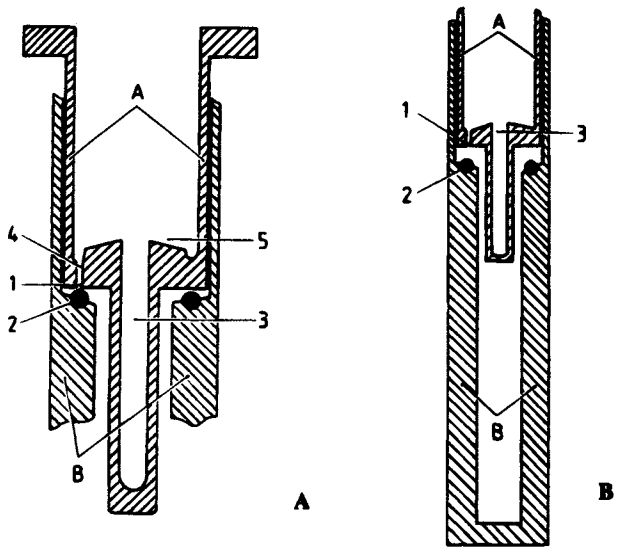

Fig. 4. The erythrocyte compartment.

that the small orifice in the reservoir [1] is sealed by the o-ring in the container [2] (panel **a**). The reservoir is then filled with an aliquot of blood and the unit placed in a swing out rotor. By centrifuging at high speed (≤ 5000 g) red cells are forced into the narrow part of the reservoir [3], into the closed small channel [4] and into the bottom of the broad part of the reservoir [5]. After the first centrifugation some supernatant plasma is removed for independent analysis.

Before the second centrifugation, the reservoir A is detached by turning A in B, so that the o-ring [2] no longer occludes orifice [1] (panel **b**). During the second centrifugation, the flow resistant red cell sludge, together with the remaining supernatant plasma, is forced out of the reservoir A into the bottom of the container B, leaving behind a defined volume of almost pure red cells in the narrow part of A [3].

This mass of cells is transferred to a centrifuge tube by centrifuging the inverted reservoir alone.

The analysis of substances in a matrix of red cells is difficult. Where one is interested mainly in substances which use red cells as a secondary transport system, the red cell mass can often be extracted conveniently into the primary higher affinity transport system i.e. a blank albumin solution, added to the centrifuge tube (Table VI) (5).

CONCLUSION

The studies summarised in Part I show that when a direct determination of valproate, phenytoin and hydrocortisone is performed on unwashed erythrocytes, the transport characteristics of these cells are essentially the same for each compound, i.e. red cells are a transport system with a high capacity and low affinity compared to plasma proteins. Thus erythrocytes are loaded last and unloaded first. Contrary to previous concepts, it appears that the erythrocyte compartment is a readily exchanging blood fraction, with a transfer function at least as important as the plasma water and protein fractions.

This capability of erythrocytes to carry substances other than blood gases has not been fully appreciated or explored. Detailed knowledge of the processes involved is lacking, although the intermittent nature of blood flow in the capillary may be important for the transfer of substances from the erythrocyte to the capillary membrane. The plasma concentration of a substance is usually regarded as the optimum reflection of that in tissue cells, but it is likely that the concentration on erythrocytes is at least equally representative.

Table VI. Percentage Extraction Efficiency of Red Cell Sediments

Substance	Concentration in blood (μg/ml)	Percentage of radioactivity extracted n = 2			
		Human albumin		Bovine albumin	
Valproate	1.8*	94.8	93.3	97.7	95.3
	100	97.3	97.6	98.7	98.8
Phenytoin	0.27*	92.1	92.4	95.9	96.9
	10	92.3	92.7	96.4	96.5
Hydrocortisone	0.018*	83.5	82.8	80.7	77.2
	0.2	94.7	93.9	84.3	83.1

* tracer amounts of radioactive substance.

New improved techniques of erythrocyte analysis now enable the contribution of the red cell compartment to be added to that of the plasma, and consequently the red cell fraction should be ignored no longer.

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REFERENCES

1. M. Ehrnebo. Drug binding to erythrocytes. In J. P. Tillement and E. Lindenlaub (eds.), *Protein binding and drug transport*, Symposia Medica Hoechst 20. F K Schattauer Verlag, Stuttgart-New York, ISBN 3-7945-1123-9, 1986, pp. 49–57. Discussion pp 59–61.
2. O. Driessen, L Treuren, and J. W. A. Meijer. Distribution of drugs over whole blood: I The transport function of whole blood for valproate. *Ther. Drug Monit.* **11**:384–389 (1989).
3. O. Driessen, L. Treuren, J. W. A. Meijer, and J. Hermans. Distribution of drugs over whole blood: II The transport function of whole blood for phenytoin. *Ther. Drug Monit.* **11**:390–400 (1989).
4. O. Driessen, L. Treuren, A. J. Moolenaar, and J. W. A. Meijer. Distribution of drugs over whole blood: III The transport function of whole blood for hydrocortisone. *Ther. Drug Monit.* **11**:401–407 (1989).
5. O. Driessen, L. Treuren, A Moolenaar, J.W.A. Meijer, and P. Verheijen. In vivo distribution of hydrocortisone over whole blood: A novel method for the extraction of erythrocytes. *Meth. Find. Exp. Clin. Pharmacol.* **12**:119–126 (1990).
6. T. C. Pearson and D. L. Guthrie. Trapped plasma in the microhematocrit. *Am. J. Clin. Pathol.* **78**:770–772 (1982).
7. D. Kurata and G. R. Wilkinson. Erythrocyte uptake and plasma binding of diphenylhydantoin. *Clin. Pharmacol. Therap.* **16**:355–362 (1974).
8. W. A. Colburn and M. Gibaldi. Prolonged impairment of the plasma-protein binding of phenytoin in the rat after a single dose of sodium oleate. *Drug. Metab. Dispos.* **6**:452–455 (1978).
9. C. Hariton, G. Jadot, M. Valli, E. Mesdjian, and P. Mandel. Effects of sodium valproate on diazepam: kinetic profiles in plasma, erythrocytes, and different brain areas in the rat. *Epilepsia* **26**:74–80 (1985).
10. H. Mabuchi and H. Nakahashi. A major inhibitor of phenytoin binding to serum protein in uremia. *Nephron* **48**:310–314 (1988).
11. J. W. A. Meijer. Knowledge, attitude and practice in antiepileptic drug monitoring. *A. Neur. Scand.* **83**:Suppl 134,100–104 (1991).
12. P. Borondy, W. A. Dill, T. Chang, R. A. Buchanan, and A. J. Glazko. Effect of protein binding on the distribution of 5,5-diphenylhydantoin between plasma and red cells. *Ann. N. Y. Academ. Sci.* **226**:82–87 (1973).
13. R. J. Shirkey, L. B. Jellett, D. C. Kappatos, T. J. B. Maling, and A. Macdonald. Distribution of sodium valproate in normal whole blood and in blood from patients with renal or hepatic disease. *Eur. J. Clin. Pharmacol.* **28**:447–452 (1985).
14. R. Hiramatsu and B. C. Nisula. Erythrocyte-associated component of blood cortisol. *Ann. N.Y. Acad. Sci.* **538**:159–166 (1988).
15. M. Ehrnebo, S. Agurell, L. O. Boréus, E. Gordon, and U. Lönroth. Pentazocine binding to blood cells and plasma proteins. *Clin. Pharmacol. Ther.* **16**:424–429 (1974).
16. J. I. Javaid, J. M. Davis, and M. Maiorano. Uptake and/or binding of tricyclic antidepressants in human red cells. *Life Sciences* **36**:1761–1769 (1985).
17. N. M. Graves, G. B. Holmes, I. E. Leppik, T. K. Galligher, and D. R. Parker. Quantitative determination of phenytoin and phenobarbital in capillary blood by Ames seralyzer. *Epilepsia* **28**:713–716 (1987).
18. O. Driessen, R. J. Höppener, and E. A. van der Velde. Practical and theoretical aspects of phenytoin administration. *Eur. Neurol.* **19**:353–365 (1980).

19. K. Nagasawa, N. Kitada, C. Tsuji, M. Ogawa, T. Yokoyama, N. Ohnishi, S. Iwakawa, and K. Okumura. Distribution of pirarubicin in human blood. *Chem. Pharm. Bull.* **40**:2866–2869 (1992).
20. T. Ito, T. Yamaguchi, H. Miyazaki, Y. Sekine, M. Shimizu, S. Ishida, K. Yagi, N. Kakegawa, M Seino, and T. Wada. Pharmacokinetic studies of AD-810, a new antiepileptic compound. *Arzneim. Forsch/Drug Res.* **32**:1581–1586 (1982).
21. V. P. Shah, M. A. Walker, J. P. Hunt, D. Schuirmann, V. K. Prasad, and B. E. Cabana. Thiazides XI: partitioning of chlorothiazide in red blood cells after oral administration. *Biopharm. Drug Dis.* **6**:55–62 (1984).
22. E. H. Starling. On the absorption of fluids from the connective tissue spaces. *J. Physiol.* **19**:312–326 (1895).
23. B. Legg, S. K. Gupta, M. Rowland, R. W. G. Johnson, and L. R. Solomon. Cyclosporin: pharmacokinetics and detailed studies of plasma and erythrocyte binding during intravenous and oral administration. *Eur. J. Clin. Pharmacol.* **34**:451–460 (1988).
24. W. M. Pardridge and E. M. Landaw. Testosterone transport in brain: primary role of plasma protein-bound hormone. *Am. J. Physiol.* **249**:E534–E542 (1985).
25. C. M. Mendel, R. R. Cavalieri, and R. A. Weisiger. Letter to the editor: on plasma protein-mediated transport of steroid and thyroid hormones. Reply by W. M. Pardridge. *Am. J. Physiol.* **255**:E221–E227 (1988).
26. E. M. Cornford and K. P. Landon. Blood-brain barrier transport of CI-912: Single passage equilibration of erythrocyte-borne drug. *Ther. Drug Monit.* **7**:247–254 (1985).
27. D. L. Kooyman, G. W. Byrne, S. McClellan, D. Nielsen, M. Tone, H. Waldmann, T. M. Coffman, K. R. McCurry, J. L. Platt, and J. S. Logan. In vivo transfer of GPI-linked complement restriction factors from erythrocytes to the endothelium. *Science* **269**:89–92 (1995).
28. W. H. Reinhart. Binding of cyclosporine by erythrocytes: influence on cell shape and deformability. *Eur. J. Clin. Invest.* **23**:177–81 (1993).
29. F. O. Ajayi, L. A. Salako, and J. O. Kuye. Comparison of the partitioning in vitro of chloroquine and its desethyl metabolites between the erythrocytes and plasma of healthy subjects and those with falciparum malaria. *Afr. J. Med. med. Sci.* **18**:95–100 (1989).
30. P. J. Marroum and S. H. Curry. Red blood cell partitioning, protein binding and lipophilicity of six phenothiazines. *J. Pharm. Pharmacol.* **45**:39–42 (1993).
31. M. Razavi, M. Kraupp, and R. Marz. Allopurinol transport in human erythrocytes. *Biochem. Pharmacol.* **45**:893–897 (1993).
32. C. A. Goresky, G. G. Bach, and B. E. Nadeau. Red cell carriage of label. Its limiting effect on the exchange of materials in the liver. *Circ. Res.* **36**:328–334 (1975).
33. C. G. Caro, T. J. Pedley, R. C. Schroter, W. A. Seed (eds.). The systemic microcirculation. In *The mechanics of the circulation.* Oxford Univ Press, ISBN 0-19-261171-2, 1978, pp. 350–429.
34. C. A. Goresky, A. J. Schwab, and C. P. Rose. Xenon handling in the liver: red cell capacity effect. *Circul. Res.* **63**:767–778 (1988).
35. A. Tajima, H. Nakata, S-Z. Lin, V. Acuff, and J. Fenstermacher. Differences and similarities in albumin and red blood cell flows through cerebral microvessels. *Am. J. Physiol.* **262**:H1515–1524 (1992).
36. W. C. Darbonne, G. C. Rice, M. A. Mohler, T. Apple, C. A. Hébert, A. J. Valente, and J. B. Baker. Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. *J. Clin. Invest.* **88**:1362–1369 (1991).
37. R. Kravtzoff, C. Ropars, M. Laguerre, J. P. Muh, and M. Chassaigne. Erythrocytes as carriers for L-asparaginase. Methodological and mouse in-vivo studies. *J. Pharm. Pharmacol.* **42**:473–476 (1990).
38. R. Dixon, J. Gourzis, D. McDermott, J. Fujitaki, P. Dewland, and H. Gruber. AICA-riboside: safety, tolerance and pharmacokinetics of a novel adenosine regulating agent. *J. Clin. Pharmacol.* **31**:342–7 (1991).
39. D. Ratge, K. P. Kohse, U. Steegmüller, and H. Wisser. Distribution of free and conjugated catecholamines between plasma, platelets and erythrocytes; different effects of intravenous and oral catecholamine administrations. *J. Pharmacol. Exp. Ther.* **257**:232–238 (1991).
40. K. Schmiegelow and I. Bruunshuus. 6-Thioguanine nucleotide accumulation in red blood cells during maintenance chemotherapy for childhood acute lymphoblastic leukaemia, and its relation to leukopenia. *Cancer Chemother. Pharmacol.* **26**:288–292 (1990).
41. E. Rapaport and J. Fontaine. Anticancer activities of adenine nucleotides in mice are mediated through expansion of erythrocyte ATP pools. *Proc. Natl. Acad. Sci. USA* **86**:1662–6 (1989).
42. M. T. Fanelli-Kuczmarski, C. L. Johnson, L. Elias, and M. F. Najjar. Folate status of Mexican American, Cuban, and Puerto Rican women. *Am. J. Clin. Nutr.* **52**:368–372 (1990).
43. A. Hercbergs, F. Brok-Simoni, F. Holtzman, J. Bar-am, J. T. Leith, and H. J. Brenner. Erythrocyte glutathione and tumour response to chemotherapy. *Lancet* **339**:1074–1076 (1992).
44. S. Mangione, D. D. Patel, B. R. Levin, and S. B. Fiel. Erythrocytic glutathione in cystic fibrosis. A possible marker of pulmonary dysfunction. *Chest* **105**:1470–73 (1994).
45. J. S. Lilleyman and L. Lennard. Mercaptopurine metabolism and risk of relapse in childhood lymphoblastic leukaemia. *Lancet* **343**:1188–90 (1994).
46. A. Melander, G. Brante, Ö. Johansson, T. Lindberg, and E. Wåhlin-Boll. Influence of food on the absorption of phenytoin in man. *Eur. J. Clin. Pharmacol.* **15**:269–274 (1979).
47. M. C. Fernández, S. Erill, M. I. Lucena, E. Pita, and N. Pérez-Alférez. Serum protein binding of tolbutamide in patients treated with antiepileptic drugs. *Clin. Pharmacokinet.* **10**:451–455 (1985).
48. T. J. B. Maling, R. W. L. Siebers, C. D. Burgess, C. Taylor, and G. Purdie. Individual variability of amiodarone distribution in plasma and erythrocytes: implications for therapeutic monitoring. *Therap. Drug Monit.* **11**:121–126 (1989).
49. M. S. Highley, P. G. Harper, P. Slee, and E. A. De Bruijn. Preferential location of circulating activated cyclophosphamide within the erythrocyte. *Int. J. Cancer* In press.
50. O. Driessen, M. S. Highley, P. G. Harper, R. A. A. Maes, and E. A. De Bruijn. Description of an instrument for separation of red cells from plasma, and measurement of red cell volume. *Clin. Biochem.* **27**:195–196 (1994).