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THE BINDING OF AROMATIC SULFONAMIDES TO ERYTHROCYTES*

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Abstract—Earlier work *in vivo* showing that sulfanilamide and acetazolamide had an affinity for erythrocytes was confirmed and extended. Eighteen sulfonamides were studied *in vitro*. Twelve unsubstituted drugs (aryl-SO_2NH_2), all carbonic anhydrase inhibitors, had varying degrees of affinity for red cells; dissociation constants varied 10,000-fold. Six substituted drugs (aryl-SO_2NHR), all antibacterial agents and not carbonic anhydrase inhibitors, had no affinity for erythrocytes. Acetazolamide and the other unsubstituted drugs accumulated in canine red cells to 32–92 μmoles/l against concentration gradients. Sulfanilamide and other benzene-sulfonamides could be washed out readily, but acetazolamide and other heterocyclic compounds could not. The K_{diss} of the sulfanilamide complex is 2×10^{-6} M; that of the acetazolamide complex is about 10^{-8} M. Acetazolamide interferes competitively with the accumulation of sulfanilamide in red cells. Formation of the acetazolamide–red cell complex is not dependent on an energy source, pH, or temperature. Red cells of bird, reptile and fish also bind acetazolamide. Binding is to components within the cells, probably not hemoglobin. One component of binding is carbonic anhydrase; for most arylsulfonamides there are also other receptors, in both dog and man. The total $K_{diss.}$ and the K_I for carbonic anhydrase are within one log unit of each other, for a given drug, in the four cases studied. The drug receptors in red cells have a "fit" similar to carbonic anhydrase since they react quite specifically with aryl-SO_2NH_2. In other tissues, such as lens, acetazolamide binds to carbonic anhydrase alone.

Apart from the bound fraction most of the unsubstituted arylsulfonamides appear in erythrocytes, both *in vivo* and *in vitro*, in diffusible forms. The degree of diffusion varies greatly among the drugs. The diffusible or free tissue concentration of drug, and its $K_{diss.}$ or K_I, quantitatively determine the degree of binding or enzyme inhibition.

STUDIES of the pharmacology of acetazolamide in rats, dogs, and man[1, 2] showed it to have an unusual affinity for erythrocytes. Following oral or parenteral administration there is a fairly rapid decline in plasma concentration (half-life in dog and man about 100 min), but when the plasma concentration of drug is no longer detectable (<1 μg/ml), from 15 to 30 μg/ml are found in washed red cells. Thereafter, this value slowly declines; the half-life of acetazolamide in human erythrocytes *in vivo* is about 3 days.[2] A number of other compounds related in various ways to acetazolamide, all of which were carbonic anhydrase inhibitors of potency within about a 100-fold range of acetazolamide, showed qualitatively similar behavior *in vivo*.[3]

These observations appeared to be related to earlier work with sulfanilamide and its derivatives in which it had been found that sulfanilamide and N^4-acetylsulfanil-

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amide showed (both *in vitro* and *in vivo*) a much higher (red cell)/plasma ratio than the N^1-substituted sulfanilamides, such as sulfapyridine, sulfadiazine and sulfathiazole.[4-8]

Thus, it appeared that the binding to red cells in this series of compounds might have the same structural basis as that for carbonic anhydrase inhibition, i.e. $XR—SO_2NH_2$, where R is any homocyclic or heterocyclic ring and X may be any other substituent(s) of the ring (see Maren *et al.*[1] for discussion). But while there is a high concentration of carbonic anhydrase in mammalian (and avian) erythrocytes (about 30 μmoles/l.[9]), there was only circumstantial evidence in favor of the suggestion[10, 11] that it is this enzyme which binds acetazolamide to the red cell.

The following types of experiments have been done in an exploration of red cell binding of sulfonamides. (1) Blood concentrations in dog, following the administration of three representative drugs. (2) Addition of substituted and unsubstituted sulfonamides to dog and human blood, *in vitro*. (3) Effects of time and temperature variation on binding. (4) Competition between acetazolamide and sulfanilamide for binding site. (5) Uptake of acetazolamide by various red cell and lens preparations; effect of metabolic changes. (6) Binding of acetazolamide to non-mammalian red cells. (7) Dissociation constants of drug–red cell complex.

MATERIALS AND METHODS

Drugs were obtained as pure crystals or as powder, in their acid form, from the American Cyanamid Company, with the following exceptions: sulfacetamide was obtained from Schering Corporation; dichlorphenamide and chlorothiazide from Merck Sharp and Dohme; ethoxzolamide from Upjohn; and sulfisoxazole from Hoffman-La Roche. Primary solutions were made by the addition of 1 — 1·6 moles of NaOH per mole of drug. Subsequent dilutions were made in a 0·9 per cent solution of sodium chloride (hereafter referred to as saline).

Analyses for sulfanilamide, N^4-acetylsulfanilamide, sulfadiazine, sulfacetamide, N^1-ethylsulfanilamide, sulfamethoxypyridazine, sulfisoxazole, sulfaethylthiadiazole and chlorothiazide were done on 0·1 — 0·4 ml of fluid by the method of Bratton and Marshall[12]. This procedure measures the arylamino moiety. For chlorothiazide, the trichloracetic acid filtrate (2 ml) was treated with 1 ml of 3 N sodium hydroxide and heated for from 15 to 30 min at 100 °C. Following neutralization, the usual procedure was carried out. Analyses for acetazolamide, methazolamide, ethoxzolamide, CL 13,580, CL, 11,366, CL 5343, CL 13,475, CL 5342, and dichlorophenamide were done by the method of Maren *et al.*[13]; this procedure measures carbonic anhydrase inhibition, presumably an expression of the free arylsulfonamide group. Activity of the compounds as carbonic anhydrase inhibitors was determined in the same way except that equilibrium was achieved between enzyme and inhibitor, and data were reported in terms of K_I.[9] pK values were determined by titration with 0·10 M sodium hydroxide using either a Beckman or a Photovolt pH-meter.

Recovery of drug added to blood was estimated for each experiment and may be calculated from columns (6), (7) and (8) of Table 1a. Hematocrit was from 45 to 50 per cent. It may be noted from these data that recovery is satisfactory except in the case of dichlorophenamide, in which case it was from 60 to 70 per cent.

Male beagles were used for studies *in vivo*. Drugs were injected intravenously. Blood was withdrawn from another vein and immediately centrifuged for 20 min at

850 × *g*, plasma was withdrawn for analysis, the buffy coat discarded, and a sample of unwashed red cells taken for analysis. Following this, the remaining red cells were washed and centrifuged three times with from 4 to 10 vols. of saline. The concentrations of drug reported for "unwashed cells", both *in vivo* and *in vitro*, have been corrected for the plasma (8 per cent) trapped in cells.

Whole blood studies

Freshly drawn, heparinized dog (beagle) blood was used, except when, as noted, other species were investigated. To 10 ml of whole blood in a 25-ml Erlenmeyer flask

TABLE 1b. BINDING OF UNSUBSTITUTED SULFONAMIDES TO HUMAN RED CELLS

Drug	Added (μmoles/l.)	Plasma	R.B.C.	Red cells after W_1 (μg/ml)	W_2	W_3	Drug–receptor complex (μmoles/l.)
Acetazolamide	100	21	19	18	17	17	not saturated
	200	44	40	37	35	35	158
Methazolamide	100	4	40	—	—	34	144
	200	57	52	44	38	38	161
Ethoxzolamide	100	1	26	26	22	22	not saturated
	200	47	47	42	34	36	140

Same procedure as for Table 1a.

was added 0·1 ml of solution of drug, in the appropriate concentration; the drug solution usually contained from 5 to 20 μmoles/ml, in saline; thus, 0·5 — 2·0 μmoles of drug were added to 10 ml of blood. The charged flask was then shaken in horizontal motion on a Burrell Shaker (position 4) for different times and at different temperatures. At the indicated time, about 3 ml of blood were drawn off and immediately centrifuged at 850 × *g* for 20 min; the hematocrit was noted. Plasma was removed for analysis, the buffy coat drawn off and discarded, and a sample of red cells taken for washing and analysis. The red cells were washed and centrifuged three times with from six to eight times their volume of saline, unless noted specially. Data reported are taken from duplicate, and in many cases, triplicate experiments.

Washed cells

Packed cells were washed three times with ten times their volume of saline, then suspended in saline for 16 hr at 4 °C. The cells were removed and suspended, with shaking at 37 °C for 1 hr, in 15 vols. of isotonic saline containing acetazolamide in concentrations of either 11 μg/ml or 44 μg/ml. The mixture was then centrifuged and the cells were washed twice with 50 vols. of isotonic saline and taken for analysis. The final supernatant fluid was free of glucose.

Buffered cells

The preparation was as just described, except that the incubation was carried out in various isotonic phosphate buffers in place of saline.

Bicarbonate

Washed cells were made up, as described, in saline containing 11 μg of acetazolamide/ml. Sodium bicarbonate was added to give 33 m-moles of CO_2/l. and CO_2 was bubbled in to yield a pH of 7·4. The washing and analytical procedure were then carried out as described above for blood.

Iodoacetate

Potassium iodoacetate (10^{-4} M in final solution) was added to 10 ml of a washed cell suspension containing 0·7 ml red cells. After 1 hr, acetazolamide (111 μg) was added and the mixture incubated for 45 min at 37 °C. The cells were separated by centrifugation, washed, and taken for analysis as described above. *Lysed cells* were prepared by treating washed erythrocytes with $\frac{1}{4}$ vol. of anhydrous ether, shaking and centrifuging. The top layer of denatured stromata was removed. The resulting clear solution was dialyzed for 16 hr at 25 °C against saline containing 2 μg of either acetazolamide or sulfanilamide per ml. *Ghosts* were prepared by treating with an equal volume of distilled water, 50 ml of citrated human blood, obtained from the blood bank. The preparation was centrifuged for 20 min at 2520 $\times$ *g*, the supernatant fluid decanted, and the pellet of ghosts remaining washed five times until the rinsings were clear. The viscous pellet (3 ml) was dialyzed with stirring against 100 ml of a solution of acetazolamide in isotonic saline, 0·5 μg/ml, for 15 hr at room temperature.

Equilibrium experiments

(a) The lysed cell preparation following dialysis against sulfanilamide was used. A series of dialysis bags containing such cells saturated with sulfanilamide were suspended at 4° in 5, 10, 20, 50, 100 and 1000 ml of water. Twenty-four hours later the bag contents and solutions were analysed for drug, and the dissociation constant calculated. (b) For drugs with much lower equilibrium constants and thus lower concentration gradient for dialysis of free drug from the bag, it was felt that equilibrium might not be attained. In practice, the system just described for sulfanilamide was applied to acetazolamide, but even when the external solution was 150 l. no drug appeared to be lost from the bag. The red cells were then used as their own dialysis surface; accordingly, 2 ml of red cells containing either acetazolamide, or CL 13,580, or ethoxzolamide in bound form were added to 2 l. of saline, and gently stirred at 4 °C. At intervals, samples of the mixture were withdrawn and both the saline fraction and the centrifuged cells were analyzed.

Carboxyhemoglobin was prepared by passing CO into the lysed cell preparation until it turned bright cherry-red. This preparation in one bag, and the untreated lysed cells in another, were dialyzed against a solution of acetazolamide, 1 μg/ml of saline, for 18 hr at room temperature. *Crystalline hemoglobin* was prepared according to Drabkin[14]. Twenty-five ml of washed human red cells were lysed with $\frac{1}{4}$ vol. of anhydrous ether. The denatured stromata were removed by centrifugation. The clear hemoglobin solution was placed in a dialysis bag and dialyzed against 3 vols. of a saturated solution of ammonium sulfate at 6 °C until approximately 40 per cent by volume was precipitated in the bottom of the bag. The bag was removed and the clear solution above the precipitate was decanted. Crystals of ammonium sulfate were added slowly, while orthorhombic crystallization was viewed through the

microscope. When a crop of crystals had formed, the mixture was re-centrifuged, the supernatant fraction was decanted, and the crystals formed were re-suspended in 3 ml of distilled water. This mixture was dialyzed overnight against 50 vols. of saline at 6 °C. This solution, which was dark red and clear, and was rich in carbonic anhydrase activity (approximately 100 units/ml, as estimated by the changing pH method[13]) was dialyzed against 2 μg of acetazolamide per ml of saline for 18 hr at room temperature.

A hemoglobin-free extract of human red cells was prepared according to Meldrum and Roughton[15], who used the method to concentrate and extract carbonic anhydrase from erythrocytes. It was dialyzed for 10 hr at 25 °C with stirring against a saline solution of acetazolamide containing 0·5 μg/ml.

Lens preparation

Lenses were obtained from either rabbit or dog, thoroughly minced with an equal volume of saline, and placed in a dialysis bag. Fresh dog lens had 309 units carbonic anhydrase per gram, in good agreement with data obtained earlier with rabbit lens.[16] This level of enzyme activity may be compared to 500 — 600 units/ml for dog blood. Lens was dialyzed for from 8 to 20 hr at 25 °C against solutions of various concentrations of acetazolamide. By conducting a control dialysis against saline, it was found that the lens lost about half its carbonic anhydrase activity during the procedure.

RESULTS

1. *Blood concentrations*, in vivo

Figs. 1, 2 and 3 show the distribution of three sulfonamides in plasma and erythrocytes following their injection in the dog. Some of these data are available from the literature, but no studies appear to have been made on washed cells, or on the "diffusible component" in red cells. These data are presented in full, as a way of introducing the problem; each of the three drugs appears to be in a separate category, and representative of others we have studied.

Acetazolamide (*Fig.* 1). Two doses were studied, 5 and 50 mg/kg. Following the low dose (Fig. 1a), 1 hr was required for the maximum concentration of the drug to be attained in red cells; this lag was absent at the high dose (Fig. 1b). It is evident that the component bound to red cells is strongly fixed, as compared to plasma. The amount of drug initially bound is limited, being about 30 μg/ml from very high plasma concentrations (Fig. 1b) and 15 — 20 μg/ml from low plasma concentrations (Fig. 1a). Repeated washing *in vitro* does not decrease these figures. Thus, in a typical experiment, the red cells of the 4-hr sample of Fig. 1(a) were washed repeatedly with from seven to twenty times their volume of saline. Successive values (in μg/ml of red cells) were 16·7 (unwashed), 15·3, 16·6, 15·3, and 14·1. The diffusible component of drug in red cells was evident in the 1-min sample, and its concentration was greatest in the 4-min sample. Thereafter, the concentration of the diffusible component declined more or less parallel to that of drug in plasma. The magnitude of the diffusible component suggests that it may be in equilibrium with unbound drug in plasma, or possibly with the unbound un-ionized fraction, which is about one-third of the plasma concentration.*

* Plasma binding in this species, originally reported as 30 per cent,[1] is from 30 to 60 per cent in our current experiments; the un-ionized fraction is 65 per cent.[17]

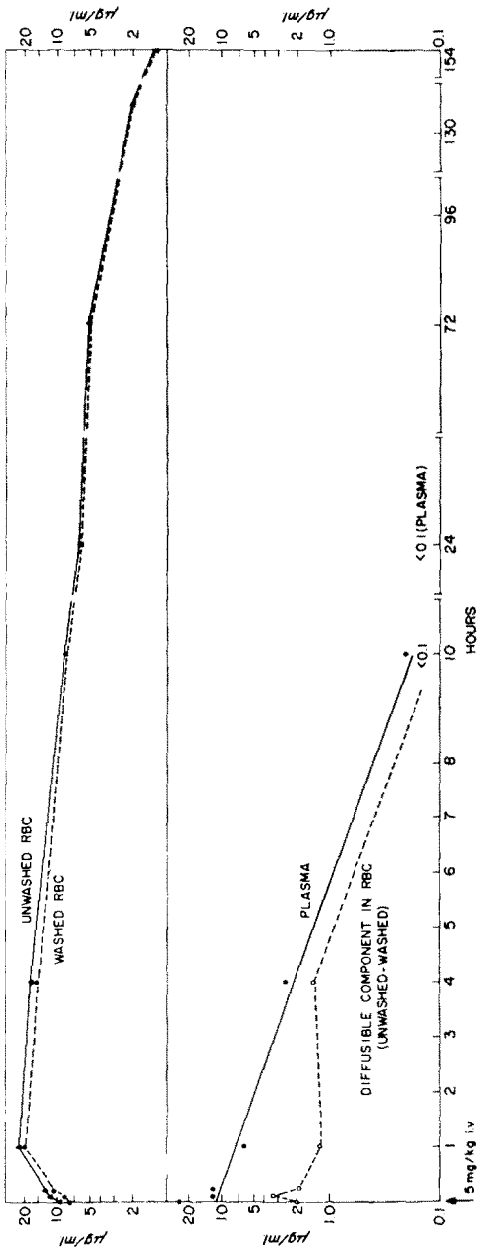


FIG. 1(a). Bound and diffusible components of acetazolamide in red cells: 5 mg/kg intravenously in dog.

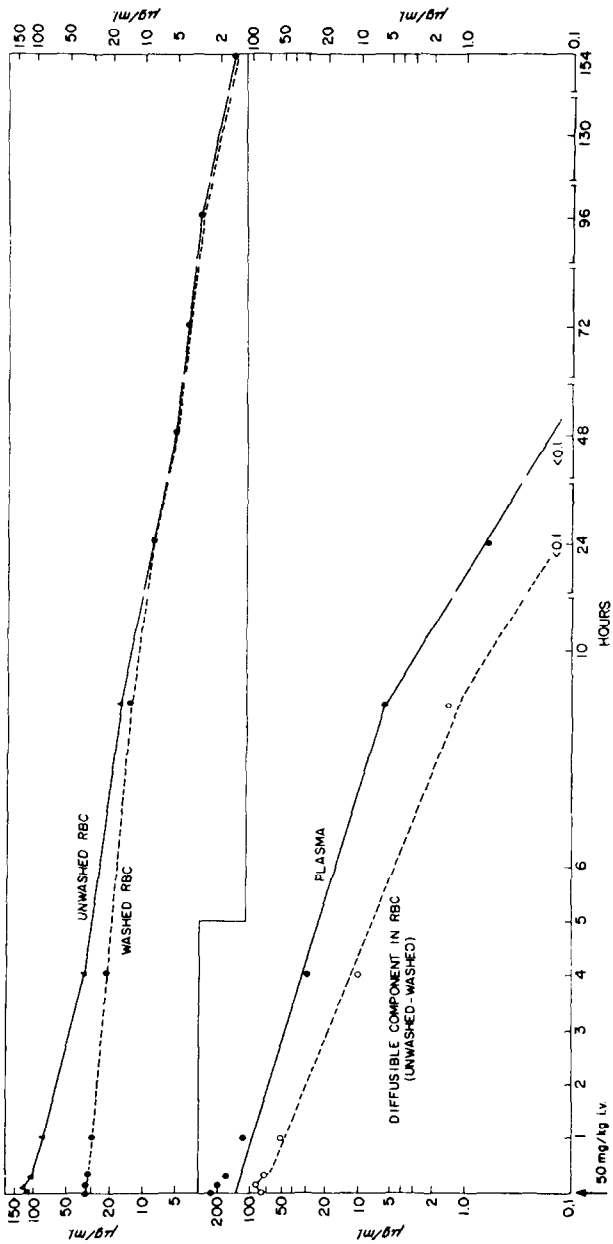


FIG. 1(b). Bound and diffusible components of acetazolamide in red cells: 50 mg/kg intravenously in dog. Three fractions of blood were analyzed at each period: unwashed erythrocytes, washed erythrocytes, and plasma. The "diffusible component" is the difference between unwashed and washed cells.

Fig. 1(c) shows a further characteristic of the red cell binding of acetazolamide, namely, that more than one component seems to be involved. There are two decay slopes, one for the first 12 hr, and one thereafter. The first has a half-life of about 12 hr; the second of about 3 days. The first component is associated with measurable plasma concentrations of drug; the second component still exists when plasma concentrations are less than 0·1 μg/ml. After the first 12 hr, curves for high and low dose

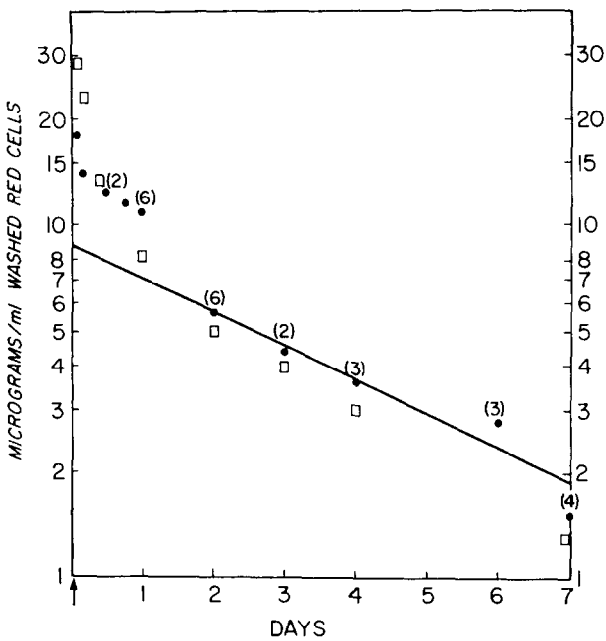


FIG. 1(c). Bound and diffusible components of acetazolamide in red cells: ● Gives average data for three-times washed red cells and number (*n*) of dogs following 5 mg/kg, given intravenously at arrow. Line is least square fit for decay from 2 to 7 days. □ shows expt. of Fig. 1(b), 50 mg/kg, given intravenously at arrow.

are indistinguishable. The same relationship was found when the blood concentrations of acetazolamide were followed in man.[2] Both (or all) of these components are tightly bound, since ordinary washing does not remove them. Analysis of the decay of acetazolamide *in vivo* (see Discussion) is relatively uncomplicated, since the drug is not metabolized in man and only to a minor degree in the dog.[1]

Sulfanilamide (*Fig.* 2). As with acetazolamide, there is evidence that sulfanilamide has an affinity for red cells. After the standard washing technique some drug remains in red cells. This, however, does not persist to the extent observed with acetazolamide. Rather, it declines in parallel fashion with the plasma concentration and is influenced by further washing *in vitro*. In a typical experiment, the red cells of the 4-hr point of Fig. 2(a) (10 mg/kg) were washed repeatedly with from seven to twenty times their volume of saline. Successive values, in micrograms of sulfanilamide per milliliter of packed cells, were 17·2 (unwashed), 8·5, 7, 4·2 and 1.

A particular characteristic of sulfanilamide, both *in vivo* and *in vitro* (Table 3), is that no matter how high the plasma concentration is, the red cell concentration

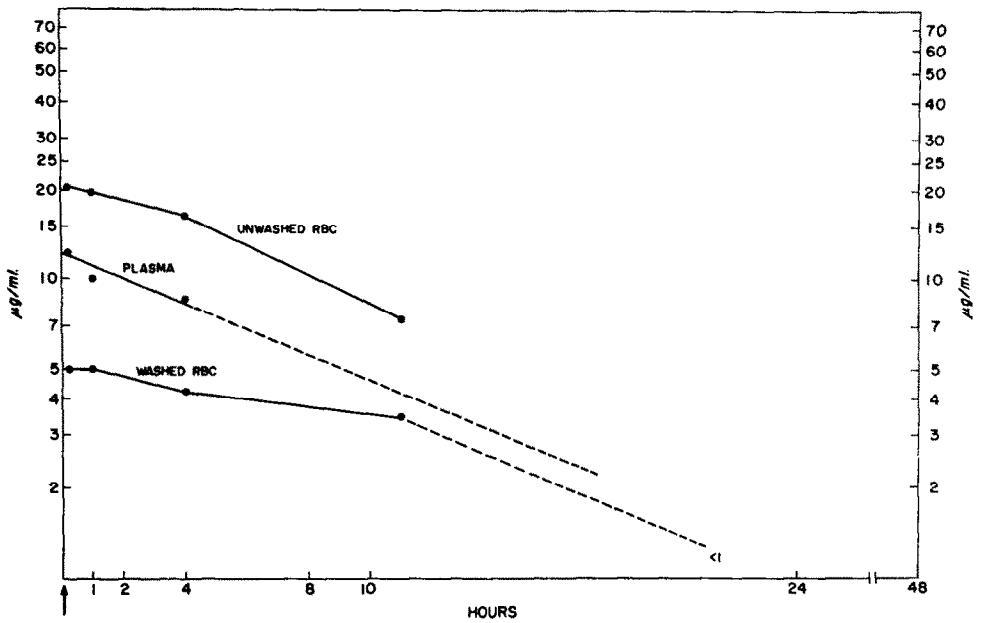


FIG. 2(a)

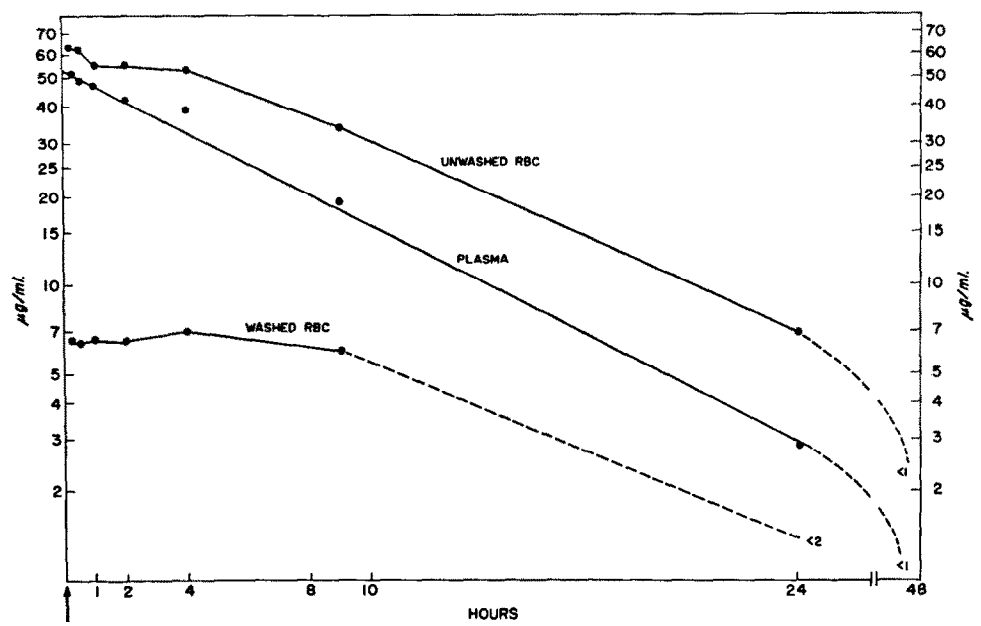


FIG. 2(b)

FIG. 2. Bound and diffusible components of sulfanilamide in red cells: (a) 10 mg/kg intravenously in dog; (b) 50 mg/kg intravenously in dog. See legend for Fig. 1.

exceeds it. The same may be true of N^4-acetylsulfanilamide; the data for these drugs suggest an additional loose reversible binding, perhaps (because of the very large capacity) to hemoglobin.

Sulfadiazine (*Fig.* 3). The data show that some sulfadiazine gains entrance into red cells, but this is entirely removed by washing. Supplementary tests showed that in the samples of Fig. 3, *all* of the drug was removed on the first washing of the three that were done routinely in these experiments.

The data of this section show three patterns of distribution in the blood for sulfonamide drugs. Acetazolamide is firmly bound to red cells, but with a diffusible

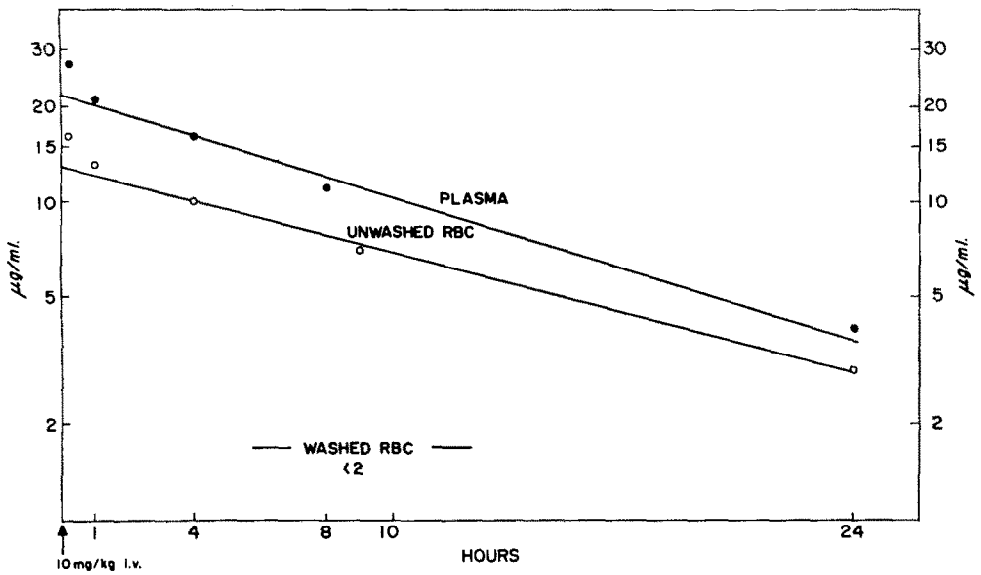


FIG. 3. Lack of binding of sulfadiazine (10 mg/kg given intravenously) to red cells in dog. Procedure as for Fig. 1 and 2.

component also; sulfanilamide accumulates in red cells but is not firmly bound; sulfadiazine has no affinity for red cells. The remainder of this study is an extension of these data to other drugs, and an exploration of the characteristics of the binding phenomenon.

2. *Addition of sulfonamides to blood*, in vitro

Tables 1a, 1b and 2 show the results of addition of eighteen drugs to whole blood. The twelve drugs listed in Table 1a are unsubstituted sulfonamides and have carbonic anhydrase inhibitory activity, as noted in column (5). Drugs of Table 2 (substituted sulfonamides) have no action against the enzyme. All measurements of red cell binding were made after incubation for 1 hr at 37 °C; representative drugs, run at different times, showed no changes in red cell uptake between $\frac{1}{2}$ and 3 hr (Section 3). The variability may be judged by five replicate tests, each for two amounts of acetazolamide added to 10 ml of whole blood. After addition of 0·5 μmoles, plasma concentrations ranged from 4 to 7 μg/ml (av. 6·0), while red cell concentrations ranged from 11 to 14 μg/ml (av. 12). After addition of 2·0 μmoles, the plasma concentrations ranged

from 52 to 60 μg/ml (av. 55), while those of washed red cells were 18 — 19 μg/ml (av. 19). Variations for other drugs were of similar magnitude.

Table 1a shows that all twelve unsubstituted sulfonamides bind to canine red cells; Table 1b shows the same for three of these drugs, using human red cells. Binding is illustrated in two ways. For all but chlorothiazide and dichlorphenamide, drug was shown to move from plasma into red cells against a concentration gradient; and there is partial or complete resistance to washout of drug from the red cells.

There are notable differences among these twelve drugs, although the element of binding to red cells is common to all. There are at least three characteristics of binding, for each drug: (a) The amount of "diffusible component", i.e. the drug which diffuses in is not bound, and is largely or entirely washed out by the first saline treatment. This is measured arbitarily by the difference between concentration of drug in unwashed cells and that following the second wash; where binding is relatively weak, as judged by continuous washout at low concentrations (e.g. sulfanilamide), this is not accurately measurable by this technique. (b) The absolute amount bound, which is indicated by the concentration following the second and third wash. (c) The stability or dissociability of the drug–red cell complex, which is indicated in qualitative terms by the degree of washout in the successive steps. These three modalities appear to be independent of one another.

(a) A sizable diffusible component (difference between columns (8) and (10) of Table 1a) is evident for ten of the twelve drugs when plasma concentrations are relatively high. Under these conditions (1-hr exposure at 37 °C) a certain quantity of drug appears to penetrate the cell by following a concentration gradient; the degree of diffusion is a characteristic of each drug, and possibly dependent on ionization and plasma binding. Two drugs (CL 13,580 and CL 11,366) showed a small or negligible diffusible component within the cell, at high plasma concentrations. These are ionized at body pH, and largely bound to plasma proteins.[18] Chlorothiazide does not enter or bind to cells from a moderate plasma concentration, (11 μg/ml); in this respect, it is unlike all the other compounds. There is diffusion and binding from higher plasma concentrations. With one exception the other nine drugs are less dissociated at pH 7·4; in part, this may explain their greater diffusion. The exception is CL 13,475, which may have special properties, since it is an N^1-substituted sulfanilamide.* The nine diffusible drugs have relatively low protein binding.[18] The diffusible component of acetazolamide (plasma binding from 30 to 60 per cent in the dog) is shown in Fig. 4. It is evident that this drug diffuses along a concentration gradient from plasma to free drug in cell water. The latter fraction washes out readily, leaving the bound component. This situation appears quite analogous to that *in vivo*, when plasma and washed cells are compared (Fig. 1a).

(b) The absolute amount bound to red cells was different among the different drugs. This was not related to potency as a carbonic anhydrase inhibitor, or to any evident structural characteristic. Under these conditions the amount bound was largely, but not entirely, independent of the plasma concentration, a tenfold increment in plasma concentration caused less than a twofold increment in that of red cells. This is precisely the situation observed *in vivo* with acetazolamide (Fig. 1).

* With some exceptions, the N^1-substituted sulfonamides generally diffuse into red cells, albeit to markedly varying degree (Table 2, and Frisk[6] and Fisher *et al.*[7]).

TABLE 1a. BINDING OF UNSUBSTITUTED SULFONAMIDES TO CANINE RED CELLS

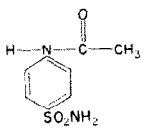
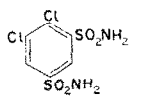
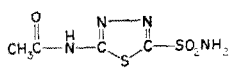
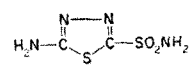
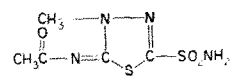
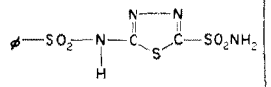
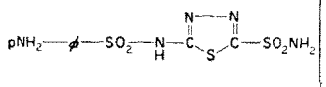
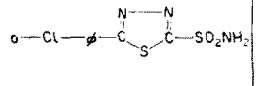
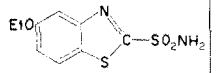
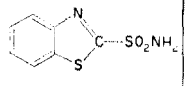
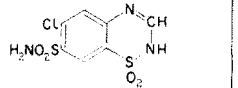
1	2	3	4	5	6	7	8	9	10	11	12
Structure	Name or number	M.W.	pK_a	K_1 ($M \times 10^7$)	Drug added (μmoles/l.)	Concentration of drug (μg/ml) in Plasma	R.B.C.	Red blood cells after Wash 1	Wash 2	Wash 3	Drug–receptor complex (RI) (μmoles/l.)
	Sulfanilamide	172	10·4	28	20 50 200 400 2000	3 8 28 60 304	6 13 39 74 364	3 7 15 28 89	2 4 8 12 19	1 2 4 5 7	—
	N^4 acetylsulfanilamide	214	9·7, 10·4	5	50 200	5 34	16 43	11 22	9 16	8 9	37
	Dichlorphenamide	305	8·3, 9·1	0·25	50 200	9 67	9 31	8 18	6 13	5 11	—
	Acetazolamide	222	7·4, 9·1	0·08	20 50 200	1 6 55	8 14 45	8 12 31	8 12 23	8 12 19	54 85
	CL 5343	180	7·8, 10·4	0·60	50 200	6 36	16 36	14 27	14 21	13 19	72 105
	Methazolamide	236	7·2	0·09	50 200	12 58	14 27	9 13	9 9	9 9	38

TABLE 1a—*continued.*

1	2	3	4	5	6	7	8	9	10	11	12
Structure	Name or number	M.W.	pK_a	K_I (M × 10^7)	Drug added (μmoles/l.)	Concentration of drug (μg/ml) in Plasma	R.B.C.	Red blood cells after Wash 1	Wash 2	Wash 3	Drug–receptor complex (*RI*) (μmoles/l.)
	CL 11,366	330	3·2, 9·0	0·01	50 200	14 116	17 33	18 32	16 31	14 30	43 91
	CL 13,475	345	4·2, 9·0	0·02	50 200	15 77	21 57	21 36	21 35	21 32	61 92
	CL 13,580	276	6·6, 8·8	0·02	50 200	11 80	18 21	17 18	17 18	17 18	63
	Ethoxzolamide	258	8·1	0·01	20 50 200	0·1 13 60	8 10 23	7 9 11	7 9 10	7 9 9	32
	CL 5342	214	7·8	0·01	50 200	7 40	11 23	9 14	8 10	8 9	37
	Chlorothiazide	296	6·7, 9·5	22	20 50 200	11 24 82	<4 8 20	<4 7 13	<4 6 13	<4 6 11	37

* All drugs added in 0·1 ml to 10 ml whole dog blood, and incubated 1 hr at 37 °C.

The final binding figure, designated (*RI*), is the fraction which appears insusceptible to further washing (Table 1a, column (12)). The data suggest that differences between experiments using high and those using low plasma concentrations, with four of the drugs, are largely due to limitation of diffusion or uptake from the low side. Five of the drugs gave the same value, whether approached from the high or the low side. For sulfanilamide, the present technique does not establish a figure for (*RI*), since the bond(s) are so weak that washout appears continuous. For dichlorphenamide, there was evidence that some drug is destroyed during incubation with red cells; the data are approximations only. For chlorothiazide, data are entered only from experiments with high plasma concentration, since there is clearly a major barrier to diffusion from the lower plasma concentrations.

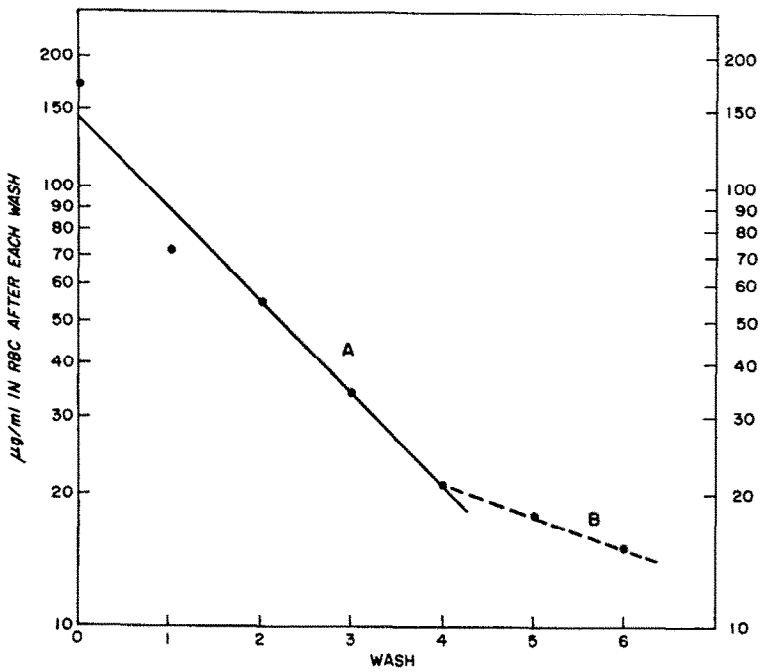


FIG. 4. Partial washout of acetazolamide from erythrocytes; 8 μmoles (1776 μg) of drug were added to 10 ml of whole blood of dog; original plasma concentration, 200 μg/ml. Same procedure as for Table 1a. A shows washout of the diffusible component; B shows the bound component.

Comparing the five drugs in which (*RI*) could be established with the greatest reliability, i.e. the same figure reached from the low or high side, there was evidence that there must be more than one binding site, since (*RI*) for one of these drugs (CL 13,580) was approximately twice that for the others. Data for the four drugs whose (*RI*) values varied somewhat for a single drug depending on the experimental design, also support the idea that the molar binding quantity may differ among drugs, by as much as threefold.

In addition to acetazolamide (Fig. 1), CL 13,580 and ethoxzolamide were studied *in vivo*, in order to compare their red cell binding with data obtained *in vitro*. One hour

following the intravenous injection in the dog, of 5 mg/kg, there were 16 μg of CL 13,580 per ml, and 9 μg of ethoxzolamide per ml, of washed red cells. These data agree closely with those of Table 1a. Further studies of these two drugs *in vivo*, in the manner shown in Figs. 1, 2 and 3 were not profitable in the present context, because these compounds are slowly metabolized in the dog. Thus, despite the extremely low dissociation constant of CL 13,580 and the red cell receptors (see Section 7 below), the concentration of drug in cells at 24 hr was 2·2 μg/ml, while that at 3 days was 0·1 μg/ml.

How carbonic anhydrase may be involved in this binding, and the relation between the external concentration and the amount bound, will be treated in Section 4 of the Discussion.

(c) The data of Table 1a show, by the degree to which the drugs are washed out from the red cells, the difference in "tightness" of binding. Eight of the drugs show no tendency toward washout of the bound fraction. It will be shown later that this would be expected under these conditions if the overall dissociation constant of drug–receptor complex (*RI*) is 10^{-7} M or less.

Table 1b shows experiments similar in design to those of Table 1a for three unsubstituted sulfonamides added to human blood. For each drug considerably more was bound to human than to canine red cells. For acetazolamide, the amount bound (44 μg/ml) agrees closely with that found *in vivo* for this species.[2] The carbonic anhydrase concentration of human and canine blood (or red cells) was found to be identical in this laboratory and by others.[19]

Table 2 shows that the N^1-substituted sulfanilamides are not bound to red cells, in agreement with previous work that has been cited. There is an all-or-none difference between these compounds and those of Table 1a and 1b; *all* of the unsubstituted drugs appear to be bound, whereas none of the substituted ones has this property.

3. *Effects of time and temperature*

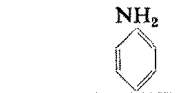
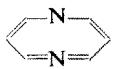
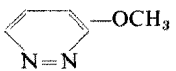
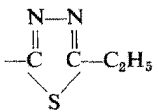
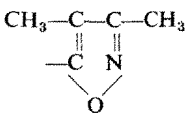
Fig. 5 shows the effect of variations of time and temperature on the binding of both acetazolamide and sulfanilamide. When plasma concentration was high (2·0 μmoles added) essentially full binding of acetazolamide by cells was achieved in 1 hr at 37 °C, 24 °C or 9 °C. With lower plasma concentration, however (0·5 μmoles added), rate of binding appears somewhat dependent on temperature. In this case binding of acetazolamide did not change after 1 hr at 37 °C, but at the lower temperatures the rate was decreased, and saturation was not achieved after 3 hr at 9 °C. With sulfanilamide, neither time nor temperature appears to affect the phenomenon.

The data for acetazolamide suggest that the delay in establishment of binding equilibrium (Fig. 5a) is due to slowness of diffusion through the cell membrane, i.e. early in the experiment drug remains in plasma. There is no evidence that there is penetration of the cell, with a slow reaction between drug and receptor. Were this the case, drug would be washed out from the cells in the periods before 1 hr; this was not observed.

Sulfanilamide, which is generally more diffusible than acetazolamide (less ionized, less plasma binding), shows no comparable delay in getting into the cell, and reaching the binding site (Fig. 5(b)).

It appears from these data that the reaction between drug and red cell receptors may be extremely rapid, and is limited only by the diffusive characteristics of the drug.

TABLE 2. LACK OF BINDING OF N^1 SUBSTITUTED SULFANILAMIDES TO RED CELLS *in vitro*

	Name	M.W.	Plasma binding at 100 μg per ml % bound†	pK_a	μmoles drug added to 10 ml whole blood	Conc. drug found (μg/ml)		
						Plasma	Unwashed* red cells	(Washed twice) red cells
R								
$-C_2H_5$	N^1-ethyl sulfanilamide	200	3	10·7	0·5 2·0	16 41	3 36	<1 1
$-COCH_3$	N^1-acetyl sulfanilamide (sulfacetamide)	214	16	5·4	0·5 2·0	14 49	6 30	<3 2
	Sulfadiazine	272	17	6·5	0·5 2·0	22 71	5 38	<3 <3
	sulfamethoxypyridazine	280	60	6·7	0·5 2·0	28 88	<2 16	<3 <3
	sulfaethyl-thiadiazole	284	86	5·4	0·5 2·0	29 105	<2 <2	<3 <3
	sulfisoxazole	268	68	5·8	0·5 2·0	24 87	1 13	<3 <3

* By difference between total drug added and that found in plasma:

$$\text{drug added} - \frac{(\text{total drug in plasma} + \text{drug trapped in red cell plasma})}{\text{volume of red cells}}$$

It was estimated that 8 per cent of the volume of red cells was plasma.
In some cases, unwashed cells were analyzed directly.

† Kindly carried out by Dr. A. H. Anton, according to method of ref. 25.

4. *Competition between acetazolamide and sulfanilamide for red cell receptors*

Table 3 shows mutual interference of acetazolamide and sulfanilamide for the red cell receptors of the dog. Since no firm binding value for sulfanilamide could be established under these conditions (see also Table 1a), a quantitative estimate of the effect of acetazolamide on the sulfanilamide binding could not be made. However, it is evident that at equimolar concentrations of the two drugs, the binding of sulfanilamide is entirely eliminated by acetazolamide. At a ratio of sulfanilamide to acetazolamide of 40:1, the latter drug clearly interferes with binding of the former. At ratios

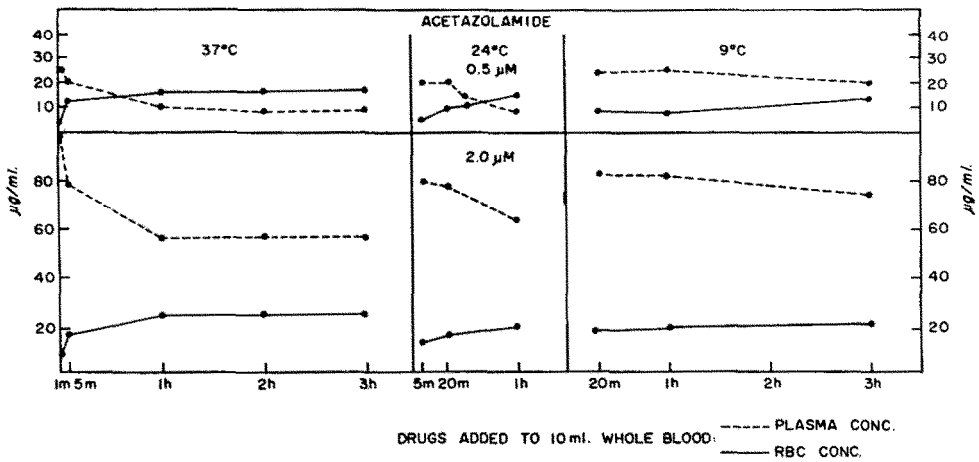


FIG. 5(a)

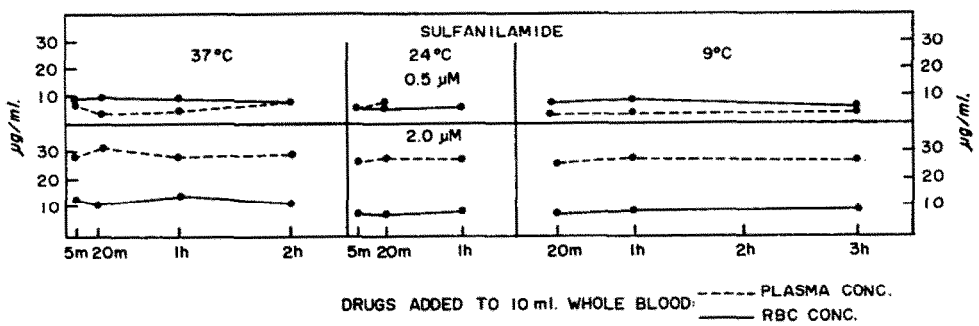


FIG. 5(b)

FIG. 5. Effect of time and temperature on red cell binding by sulfonamides *in vitro*. (a) acetazolamide; (b) sulfanilamide. In these experiments cells were washed twice with five times their volume of a 0·9 per cent solution of sodium chloride.

of 200:1 or greater, the binding of sulfanilamide was uninfluenced by acetazolamide. Using the binding of acetazolamide following the addition of 2 μmoles per 10 ml of blood as a criterion (19 μg/ml of red cells, see Table 1a), the effect of sulfanilamide may be estimated. The last column of Table 3 shows that 100 μmoles of sulfanilamide reduces the binding of acetazolamide by about 50 per cent. From these data, it may be judged that acetazolamide has about 100 times the affinity of sulfanilamide for the red cell receptors.

5. *Uptake of acetazolamide by red cell and lens preparations*

Table 4a shows representative experiments in which red cells were manipulated metabolically, or fractionated, and then tested for their ability to take up acetazolamide. Removal of glucose, alteration of pH or CO_2 equilibria and inhibition of respiration were without effect. Ghosts, which were found to contain no carbonic anhydrase, failed to take up drug; other cell fractions concentrated acetazolamide in

TABLE 3. MUTUAL INHIBITION OF BINDING TO RED CELLS BY ACETAZOLAMIDE AND SULFANILAMIDE

Sulf. (μmoles)	Acet. /10 ml)	Plasma	Red cell concentration after wash* Unwashed	W_1	W_2	W_3	Acetazolamide bound μg/ml R.B.C. (W_3)
				μg sulfanilamide/ml			
0·5	0	8	13	7	4	2	—
0·5	0·1	6	12	4	2	1	3·8
0·5	0·5	8	9	2	<2	<2	10·4
0·5	2·0	9	8	1	<2	<2	18·1
2·0	0	28	39	15	8	4	—
2·0	0·1	28	38	11	5	2	3·1
2·0	0·5	30	36	7	1	<2	10·3
2·0	2·0	31	38	4	<2	<2	16·6
20·0	0	304	364	89	19	7	—
20·0	0·1	300	364	76	15	5	1·7
20·0	0·5	304	364	61	9	2	6·4
20·0	2·0	304	364	68	8	1	11·2
100·0	0	1225	1562	360	71	19	—
100·0	0·1	1188	1475	355	75	13	0·9
100·0	0·5	1225	1475	355	57	18	4·0
100·0	2·0	1213	1475	365	62	10	9·2

* Added within 10 sec after sulfanilamide to whole heparinized dog blood. All mixtures were incubated for 1 hr at 37 °C.

the usual fashion. The "Drabkin hemoglobin" fraction contains carbonic anhydrase; however, the role of hemoglobin as receptor was ruled out by the finding that the hemoglobin-free extract of Meldrum and Roughton[15] also took up drug.

Table 4b compares the reaction of acetazolamide with human red cells to that with lens, and indicates the role of carbonic anhydrase in the red cell binding phenomenon. In one type of experiment, equal volumes of suspensions of fresh minced dog lens and lysed red cells, each in a small dialysis bag, were suspended in a dilute solution of drug. The concentrations of cells and of lens were so adjusted that the enzyme content in the two bags was the same. Preliminary studies had indicated that, at the concentrations of acetazolamide used, diffusion was complete within 8 hr. After 24 hr, the outside solution and the contents of the bags were analyzed. The results showed six times as much uptake into red cells as into lens. Calculations were also made of the molar binding of drug, compared with the molar concentration of enzyme, as derived from kinetic data.[9] The data show that one mole of acetazolamide combines with one equivalent (or mole) of carbonic anhydrase in lens. In the red cell preparation, however, there was about ten times as much drug bound as could be accounted for by

the carbonic anhydrase concentration. The experiments with rabbit lens homogenate confirmed the fact that for this tissue, uptake of drug could be correlated directly with carbonic anhydrase concentration.

6. *Binding to non-mammalian red cells*

Acetazolamide was added to blood of the chicken, alligator (*A. mississipiensis*) and elasmobranch (*S. acanthias*). Results are shown in Table 5. It is evident that red

TABLE 4a. BINDING OF ACETAZOLAMIDE TO HUMAN RED CELL PREPARATIONS

I. *Washed and treated human red cells Incubated 1 hr at 37 °C*	Acetazolamide concentration (μg/ml)	
	Saline medium	Treated R.B.C.
Saline washed, glucose free	12	50
Saline washed, glucose free	64	57
Saline washed, glucose added (100 mg%)	15	49
Saline washed, glucose added (100 mg%)	60	48
Saline washed, 33 m-moles of HCO_3^- per l.: $pCO_2 = 58$	11	38
Saline washed, *K* iodoacetate added (10^{-4} M)	11	42
Phosphate–saline buffers pH 6·1	10	38
6·5	10	40
7·0	10	38
7·5	10	42
8·3	10	40
II. *Erythrocyte preparations in dialysis bag Incubated 25 °C for 18 hr*	Outside conc. in saline	Inside conc.
Lysed cells	2	19
Washed Ghosts	0·5	0·5
CO-treated,lysed cells	1	12
Drabkin hemoglobin	2	19
Meldrum–Roughton	0·5	13

I. Washed cells were suspended in 15 vols. of appropriate medium, containing drug.
II. Inside volume, approximately 2 ml; outside, 100 ml. See Methods sections.

TABLE 4b. BINDING OF ACETAZOLAMIDE TO LENS*

Preparation	Enzyme units/g	(E)† M × 10^{-7}	Drug conc. M × 10^{-7}		
			out	in	bound
Minced dog lens‡	89	15	18	31	13
Ether-lysed red cells, 1:6‡	95	16		192	176
Rabbit lens 1:1 homogenate	100	17	4	18	14
Rabbit lens 1:19 homogenate	10	1·7	1·3	3·4	2·1

* Lens or red cell preparations were put in dialysis bags (5 ml) surrounded by 100 ml of external solution, at 25 °C for 18 hr.
† From Maren *et al.*[9], 1 enzyme unit/g = 0·17 × 10^{-7} M.
‡ Suspended in same outside solution.

cells of all three vertebrate classes bind the drug, although in very different concentrations. The amount of drug bound is not related to the carbonic anhydrase activity of the blood.

7. Dissociation constants of drug–receptor complex

(a) Sulfanilamide. The lysed cell preparation saturated with drug was placed in dialysis bags and suspended in different volumes of water, as described in the Methods Section. In a typical experiment, initially there were 4·5 μg/ml bound to the lysed cells; at equilibrium (24 hr later), there were 2·6 μg/ml inside and 0·5 μg/ml in the

TABLE 5. BINDING OF ACETAZOLAMIDE TO BLOOD OF BIRD, ALLIGATOR AND FISH

	Acetazolamide added to 10 ml blood (μmoles)	Time (min.)	μg/ml		Hematocrit	Carbonic anhydrase units/ml blood*	Notes
			Plasma	washed† R.B.C.			
Chicken	0·5	20	5	27	26	200	Incubated at 37 °C
		110	4	27			
	2·0	20	38	45			
		110	33	44			
Alligator	0·5	20	16	3	27	30	Incubated at 23 °C. *A. mississippiensis* captured in Gainesville, Florida.
		120	13	4			
	2·0	20	45	7			
		120	42	8			
Dogfish	0·5	20	8	11	20	8	Incubated at 23 °C. These experiments were carried out on *S. acanthias* at Mt. Desert Island Biol. Lab. Salisbury Cove, Maine
		100	9	11			
	2·0	20	40	20			
		110	45	23			

* On this basis, dog and human blood (hematocrit = 45) have 600 units/ml.
† Twice with 5–7 vols. 0·9 per cent saline (chicken, alligator) or 1·5 per cent saline–2 per cent urea (dogfish).

outside (5-ml) bath. Thus, if R represents the total receptor sites and RI the bound sites,

$$K_{\text{diss.}} = \frac{R - RI}{RI} \times (I_{\text{free}}).$$

The fraction $\frac{R - RI}{RI}$ is $\frac{4{\cdot}5 - 2{\cdot}1}{2{\cdot}1}$ or 1·14.

I_{free}, measured by the outside concentration, is $2{\cdot}9 \times 10^{-6}$ M, whence $K_{\text{diss.}} = 3{\cdot}3 \times 10^{-6}$ M.

Using the analytical data from the first four dialysis systems (from 5 to 50 ml outside volumes), a reciprocal plot of bound vs. free drug was made according to Klotz *et al.*[20]. A straight line was obtained; from its slope, the dissociation constant was calculated to be $2{\cdot}1 \times 10^{-6}$ M.

(b) Acetazolamide, CL 13,580, and ethoxzolamide. The system using red cells saturated with drug, suspended in saline, proved satisfactory. In a typical experiment. 2 ml of canine red cells containing 16·5 μg of acetazolamide per ml (corrected to 100 per cent hematocrit) were stirred gently in 2 l. of saline at 4 °C for 72 hr. Equilibrium was reached between 4 and 20 hr; thereafter, the concentration of drug in the red cells was constant at 13 μg/ml, and in the saline supernatant fraction, at 5 μg/l. or 10 μg

total.* This latter figure agrees reasonably well with the measured loss of drug from the red cells (16·5 — 13·0) × 2 ml = 7 μg total. Thus, using in the equilibrium expression for I_{free} the average of these two independent measurements, 4·3 μg/l. or 2×10^{-8} M, the dissociation constant for acetazolamide and red cells is

$$K_{diss.} = \frac{16{\cdot}5 - 13}{13} \times 2 \times 10^{-8} = 5 \times 10^{-9}\ \text{M}.$$

Four experiments of this type were done, and yielded results for $K_{diss.}$ ranging between 1 and 10×10^{-9} M.

Similar experiments were done with CL 13,580. In this case there was no measurable decrease in the concentration of drug in red cells; the saline contained at equilibrium 0·7 μg/l. This indicates that each ml of red cells had 0·7 μg less than at saturation; this difference would in fact be undetectable by comparison of red cells initially and at equilibrium. In the experiment cited, the intial saturation concentration was 16 μg/ml and, by difference, at equilibrium was 15·3 μg/ml. The dissociation expression is thus

$$K_{diss.} = \frac{16{\cdot}0 - 15{\cdot}3}{15{\cdot}3} \times 2{\cdot}6 \times 10^{-9}\ \text{M} = 1 \times 10^{-10}\ \text{M}.$$

Since we are measuring an overall dissociation constant, which appears to include several receptors, it was of interest to test further the possibility that there may be decisive differences among the binding constants of the different receptors. For this purpose, human blood was used, since it has a considerably larger binding capacity than canine blood, and conceivably a greater number of different receptors. Ethoxzolamide was selected, since its high activity makes analysis very sensitive, and since its K_I for carbonic anhydrase has been analyzed in detail.[9] At saturation, the red cells (again corrected to 100 per cent hematocrit) contained 48 μg/ml. Two ml of such cells were added to 2 l. of saline at 25 °C and the mixture was gently stirred. In this case, equilibrium was reached within 5 min; for the ensuing 24 hr the saline contained 6 μg/ml. The red cells, analyzed at 1 hr, contained 41 μg/ml. There was close agreement between the amount of drug found in the supernatant fluid (12 μg total) and the difference between drug bound to cells initially and at equilibrium (14 μg total). I_{free} may be taken as the average of these figures, 6·5 μg/l., whence the dissociation constant is

$$K_{diss.} = \frac{48 - 41{\cdot}5}{41{\cdot}5} \times 2{\cdot}3 \times 10^{-8}\ \text{M} = 3{\cdot}6 \times 10^{-9}\ \text{M}.$$

It is evident that most, if not all, of the receptors bind ethoxzolamide in very tight union, since no substantial number released drug under these conditions. The loss of drug to the free state is explicable on the grounds that the average drug–receptor complex has the dissociation constant cited above and that none of the receptors has a

* In this experiment, the saline supernatant fraction was reduced in volume 25-fold in a flash evaporator, in order to bring the concentration of acetazolamide into the detectable range. Appropriate controls were carried out to ensure that drug was recovered quantitatively following this procedure. It was necessary to use only 0·1 ml of concentrated saline in the analytical method to determine this amount of acetazolamide. The concentration procedure was not necessary in the cases of CL 13,580 and ethoxzolamide, which are more active inhibitors and thus detectable in smaller amounts.

markedly higher constant. Carbonic anhydrase, which may account for about one-fourth of these receptors (see Discussion), has approximately the same affinity for ethoxzolamide (Table 1a, column (5)) as the average receptor.

DISCUSSION

1. *Specificity of structures for red cell binding: degree of binding (Results, Sections 1, 2 and 7)*

Since all of twelve unsubstituted arylsulfonamides showed binding to red cells and none of six substituted ones was bound, it is tempting to state that this reaction is a general property of drugs in the class aryl-SO_2NH_2. Identical relations exist with respect to inhibition of carbonic anhydrase; all unsubstituted sulfonamides have activity at least as great as K_I of the order of 10^{-5} M, whereas substituted drugs are devoid of activity.[9]

Among these twelve drugs, variation was found in the molar concentrations bound, and, to a much greater degree, among the dissociation constants. With respect to the molar concentration, there was a threefold range in the same species (dog) between the lowest binding (ethoxzolamide; 32 μmoles/l.) and the highest (CL 13,475: 92 μmoles/l.). There was no obvious chemical or physical correlation among the drugs that bound relatively few sites, or those that bound larger numbers of sites. It was clear, however, that very closely related drugs had similar concentrations bound, i.e. the respective pairs: acetazolamide and CL 5343; ethoxzolamide and CL 5342; CL 11,366 and 13,475. With regard to the dissociation constants of the drug–red cell complex, in the four cases studied these showed an approximate range of 10^4, corresponding roughly to the order of inhibition-strength (as judged by the K_I, Table 1a) against carbonic anhydrase. The relationship between red cell binding and the carbonic anhydrase problem will be discussed in Section 3 below.

2. *Nature of the binding to red cells: diffusion (Results, Sections 3, 4 and 5)*

These data show that the binding of acetazolamide in erythrocytes is not energy-dependent, and it does not appear that "active transport" plays a part. Binding is not to hemoglobin, if acetazolamide is taken as a prototype (Table 4a). The fact that competition for the red cell receptors can be demonstrated between two quite dissimilar drugs of the aryl-SO_2NH_2 class (sulfanilamide and acetazolamide, Table 3) strongly suggests the common nature of the binding among the twelve drugs studied. Results of the competition experiment agree with data on individaul dissociation constants, in placing the relative affinity of acetazolamide to red cells at about 100 times that of sulfanilamide.

We regard the reaction as one of chemical bonding, possibly ionic in nature. On the other hand, diffusion plays an important role in determining the ultimate binding, both *in vivo* and *in vitro*. Certain drugs, such as sulfanilamide and methazolamide, diffuse readily into the red cell, and attain full binding from relatively low concentrations very rapidly. Acetazolamide occupies an intermediate position; full binding requires relatively high plasma concentration, and about 1 hr, both *in vitro* and *in vivo*. The acidic drug, CL 11,366, diffuses poorly. The data suggest that the actual binding may be instantaneous, but that, depending on the chemical type and the particular experimental conditions, the amount of diffusible drug available for binding is the limiting factor. Since this diffusible drug within the red cell (I_{free}) is the fraction

also in equilibrium with carbonic anhydrase (or any other enzyme in cell water), these diffusive characteristics will have a bearing on the activity of such drugs as enzyme inhibitors, *in vivo*. This subject is treated in detail elsewhere.[18]

The diffusive characteristics of the substituted sulfonamides (Table 2) are of interest. Sulfaethylthiadiazole[6] shows very poor diffusion into red cells, parallel to low diffusion into cerebrospinal fluid.[21] Sulfisoxazole[21, 22] and sulfamethoxypyridazine[21–24] show moderate diffusion into red cells and cerebrospinal fluid.* Sulfadiazine, N^1-ethylsulfanilamide and sulfacetamide diffuse well into both red cells and cerebrospinal fluid.[6, 7, 21]

Both plasma binding and ionization limit diffusion. Sulfaethylthiadiazole is 99 per cent ionized and 86 per cent plasma bound. The most diffusible drug of Table 2, N^1-ethylsulfanilamide, is un-ionized at pH 7·4 and little bound. Comparison of two pairs of drugs with similar pK_a values, but different degrees of plasma binding (sulfacetamide and sulfaethylthiadiazole: sulfadiazine and sulfamethoxypyridazine) suggest that diffusion in this series is limited chiefly by plasma binding. The role of ionization in these compounds (pK_a 5·4 to 10·7) appears less critical. It may be that pK_a must be less than about 4 to play a decisive role—compare, for example, CL 11,366 and CL 13,475 in Table 1a. Lipid solubility does not appear to be a major factor: the chloroform partition coefficients of sulfamethoxypyridazine, sulfadiazine and sulfanilamide decrease in that order,[24] while their diffusibility into red cells increases.

3. *Relation of binding to carbonic anhydrase* (*Results, Sections* 5 *and* 6)

Since dog and human erythrocytes contain approximately 30 μmoles (or microequivalents) of carbonic anhydrase per l,[9] it is evident that in many of the present experiments, we are dealing with drug that is bound also to other receptors. There is no indication whether these receptors are on the carbonic anhydrase molecule or on other proteins.

In the data of Table 1a, obtained in dogs, five drugs show a binding concentration of from 32 to 38 μmoles/l. of red cells. It is reasonable to suppose that in these cases the observed binding is all, or nearly all, to the enzyme. It is significant, however, that two of these drugs (methazolamide and ethoxzolamide), when added to human blood (Table 1b), show binding to the extent of 161 and 140 μmoles/l., respectively. Clearly, in these cases most of the binding is not to carbonic anhydrase. Data of Table 1b and the dissociation constant of ethoxzolamide and human red cells (Section 7 above) show that the bond to the non-carbonic anhydrase receptors is generally of the same order of dissociation as that to the enzyme.

Table 1a shows that acetazolamide and four other drugs bind to canine red cells in amounts two to three times greater than that required for saturation of carbonic anhydrase.† Fig. 1(c) shows the same for acetazolamide *in vivo*; two separate decay curves of drug in red cells suggest different binding sites, each with a relatively low

* Sulfamethoxypyridazine achieves considerably higher concentration in cerebrospinal fluid of dog than of man. This appears due to moderate (from 55 to 60 per cent) plasma binding in the dog,[23–25] but high binding (from 80 to 90 per cent) in man.[25]

† The equivalent weight of carbonic anhydrase is based on a study of the inhibition of the catalyzed hydration of carbon dioxide, and agrees with the molecular weight of the enzyme.[9] There may be other binding sites on the enzyme, conceivably related to other reactions. Studies of the kinetics of inhibition of the dehydration of carbonic acid are under way in this laboratory by Dr. Kenneth C. Leibman.

dissociation constant. If the half-lives are taken as an index of the relative affinity for what appear to be two receptors in the red cells, one (data for the first day; half-life 12 hr) would have about six times higher dissociation constant than the other (line drawn for 2- to 7-day data; half-life 3 days). If these latter data are extrapolated back to zero time, the theoretical binding at saturation is 8·8 μg/ml of red cells, or 40 μmoles/l. It is suggested, therefore, that the binding observed in red cells after the second day is to carbonic anhydrase. Support for this idea comes from analysis of data on the pharmacology of acetazolamide in man.[2] In those studies, as in Table 1b, the initial uptake of drug by red cells was high; the 24-hr concentration *in vivo* averaged 32 μg/ml of washed red cells. However, data obtained in the period from 2 days to 2 weeks after drug administration yielded a decay curve for acetazolamide in human red cells which was extrapolated back to zero time to yield a much lower value, 11 μg/ml. Such a curve is similar to that of Fig. 1(c), despite the marked contrast in total binding of drug to red cells in the two species. But since the carbonic anhydrase activity of red cells in dog and man is the same, and in view of the similar drug–red cell decay curves after the first day, it appears that acetazolamide is equally bound to enzyme in the two species. We conclude that in red cells of both species, but to a larger extent in man, there are binding sites for acetazolamide other than carbonic anhydrase, with slightly less affinity for the drug. Such differences in affinity appear below the resolution of the physical methods by which we have measured dissociation or inhibition constants, but are manifest in the sixfold differences between the slopes of the drug decay from red cells on the first day (total binding sites) and that for subsequent days (enzyme site) in the data of Fig. 1(c) and Maren and Robinson[2].

Similarly, the data of Table 5 show that there are receptors other than carbonic anhydrase for acetazolamide in red cells of bird, reptile and fish.

4. *Dissociation constants and "washout". Concentrations of free drug necessary for binding or inhibition* (*Results, Sections* 2 *and* 7)

The washout of drug from red cells (Tables 1a and 1b) reflects the total dissociation constants ($K_{diss.}$) or in the cases where binding is only to enzyme, inhibition constants for carbonic anhydrase (K_I). Other factors which affect the final binding characteristics are diffusion properties of the drug, the concentration of free drug (I_{free}) at equilibrium, and the number of binding sites for the particular drug in the individual species. Examples are given to illustrate these points.

Suppose that the total equivalence of receptors is 100 μmoles/l. and sulfanilamide is used. Both the K_I and the $K_{diss.}$ is 3×10^{-6} M. The loose combination of sulfanilamide with a very high concentration of red cell receptors (see Results, Section 1 and Table 3) may be ignored. If drug is added, as shown in Table 1a, to make a total concentration in blood of 200 μmoles/l., which is assumed to diffuse readily into cell water,* the following will apply:

$$K_{diss.} = \frac{[(R) - (RI)] \times [(I) - (RI)]}{(RI)},$$

* Correction for plasma binding and water concentration of cells are small factors here and are neglected. Differences in the number of receptors over a fivefold range will not seriously influence the equilibria.

where in molar terms (R) is initial receptor sites, (I) is initial drug concentration and (RI) is bound sites. (I_{free}) = ((I) − (RI)). In units of 10^{-6} M, and rounding small fractions:

$$3 = \frac{(100 - 97) \times (200 - 97)}{97}.$$

$I_{free} = 103 \times 10^{-6}$ and the percentage of bound receptors is $RI/R \times 100 = 97$ per cent. Since sulfanilamide is diffusible and reasonably soluble, let us assume that each wash removes 80 per cent of free drug, whence three washes will leave 103 μmoles/l. $\times 0{\cdot}2^3 =$ 0·8 μmoles/l. Using a rearrangement of the above equilibrium expression, we may solve for per cent binding in terms of I_{free}:

$$\frac{RI}{R} \times 100 = \frac{(I_{free})}{(I_{free}) + K_I} \times 100 = \frac{0{\cdot}8}{0{\cdot}8 + 3} \times 100 = 21 \text{ per cent.}$$

In this model, washing would reduce binding very greatly; this was the finding for sulfanilamide (Table 1a). Similar calculations for initial (I) of 2000 μmoles/l. yields an initial binding of 99·8 per cent. In the above model, washing three times reduces this to 84 per cent. The data of Tables 1a and 3 confirm experimentally that only at exceedingly high I_{free} concentrations may binding be maintained with a drug of these K_I and solubility characteristics.

Chlorothiazide (Table 1a) has about the same K_I as sulfanilamide. Presumably, as for other drugs, $K_{diss.}$ is about the same as K_I. The initial equilibrium figures for sulfanilamide will then apply—97 per cent saturation of red cells when 200 μmoles are added per liter of blood. The experimental data show that this is achieved, but also show much less washout than for sulfanilamide. The data (binding from low side) show that this drug is poorly diffusible into cells, and we may suppose that the low washout is a consequence of this. If, in the experiment cited, washing is 50 per cent efficient (instead of 80 per cent, as suggested for sulfanilamide) I_{free} after the third wash will be 103 μmoles/l. $\times 0{\cdot}5^3 = 12{\cdot}5$ μmoles/l., whence

$$\frac{RI}{R} \times 100 = \frac{12{\cdot}5}{12{\cdot}5 + 3} \times 100 = 81 \text{ per cent.}$$

Only a small reduction in saturation is seen, even though the K_I or $K_{diss.}$ is the same as that for sulfanilamide.

Methazolamide has K_I of approximately 10^{-8} M, and, in the dog, the receptor is virtually all carbonic anhydrase. The data show that this compound is readily diffusible. Saturation is achieved and maintained from the low side, i.e. following addition of 50 μmoles/l. The receptor (enzyme) concentration is known with some accuracy, as 30 μmoles/l. Using the same conventions and assumptions as for sulfanilamide, the full initial equilibrium expression is

$$0{\cdot}01 = \frac{(30 - 29{\cdot}98) \times (50 - 29{\cdot}98)}{29{\cdot}98}.$$

$I_{free} = 20 \times 10^{-6}$ M and the percentage of bound receptors is

$$\frac{RI}{R} \times 100 = 99{\cdot}93 \text{ per cent.}$$

If washing is 80 per cent efficient and the usual three washes are carried out, I_{free} will be reduced to 20 μmoles/l. $\times$ $0{\cdot}2^3$ = $0{\cdot}16$ μmoles/l. Percentage binding is then

$$\frac{RI}{R} \times 100 = \frac{0{\cdot}16}{0{\cdot}16 + 0{\cdot}01} \times 100 = 94 \text{ per cent.}$$

Since the analytical error is from 5 to 10 per cent, this cannot be distinguished from the initial saturation value, Thus, washing would be expected to have no noticeable effect for drugs of this K_I (or $K_{diss.}$), even if diffusion and solubility are high. These are the findings of Tables 1a and 1b.

Acetazolamide, as would then be predicted, is not washed out. Lack of complete binding from the low side is attributed to inadequate diffusion.

Similarly, those drugs of low $K_{diss.}$ or K_I which are not metabolized *in vivo* (e.g. acetazolamide and methazolamide) persist in red cells and tissues containing carbonic anhydrase, in equilibrium with exceedingly low concentrations of drug in plasma. Such equilibria, and their relation to enzyme inhibition in the kidney and choroid plexus, will be considered elsewhere.[26]

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