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Aspirin binding and the effect of albumin on spontaneous and enzyme-catalysed hydrolysis

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A method of measuring the binding of aspirin to albumin without the interference of hydrolysis was developed. At concentrations of 10 mg litre^{-1}, aspirin is about 85% bound to bovine serum albumin (4 g %), whereas its hydrolysis product, salicylic acid, is 95% bound. Salicylic acid was shown to displace aspirin from albumin binding sites. Both salicylic acid and aspirin bind more strongly to bovine serum albumin than to human serum albumin at protein concentrations of 4 g %. Protein binding protected aspirin against spontaneous hydrolysis although protein-bound aspirin still hydrolysed at a finite rate. In contrast, albumin enhanced the enzyme-catalysed hydrolysis of aspirin. By using a simple model, the rate constants for the individual processes contributing to the overall hydrolysis rate constant in the presence of albumin and esterase are calculated.

It is generally accepted, and substantiated by some studies (Jusko & Gretch 1976; Yacobi & Levy 1977), that the unbound concentration, rather than the total concentration, of a drug which has a small total body clearance, is related to the disposition and pharmacological activity of that drug. Therefore there is a need to study protein binding of drugs and the factors which influence that binding. Salicylic acid is extensively bound to plasma proteins, particularly albumin, and its binding to purified albumin (Muirden et al 1974) and to human serum (Moran & Walker 1968) has been measured. Salicylate is most often administered in the form of acetylsalicylic acid (aspirin) which is less strongly bound than salicylic acid (Hucker et al 1972). As yet there have been no studies reported on the possibility of displacement interactions between salicylic acid and aspirin.

Although the determination of salicylic acid binding is straightforward, the measurement of aspirin binding is complicated by the hydrolysis of aspirin to salicylic acid. The half-life of the spontaneous hydrolysis in Krebs-Ringer pH 7·4 bicarbonate buffer at 37 °C is 17 h (Harthon & Hedström 1971) whereas the half-life of enzyme-catalysed hydrolysis in whole blood is 0·5 h (Harris & Riegelman 1967). The hydrolysis in whole blood is probably catalysed by a group of arylesterases and the kinetics of hydrolysis are first order with respect to substrate at concentrations less than 15 mg litre^{-1} (Harris & Riegelman 1967). Consequently, in the course of an equilibrium dialysis experiment, for example, which may continue in excess of 4 h, considerable hydrolysis will occur and since the hydrolysis product, salicylic acid, is more strongly bound than aspirin, the final equilibrium will continuously alter as salicylic acid competes with aspirin for protein binding sites.

Conversely, the extent of binding should have an effect on hydrolysis rate in that only unbound aspirin is available for hydrolysis.

We have developed a method to measure the extent of aspirin binding to albumin and investigated the effect of albumin binding on both the spontaneous and the enzyme-catalysed hydrolysis of aspirin.

METHODS

Binding measurements

Samples were prepared by dissolving the drug (either salicylic acid or aspirin) in a solution of 4 g % albumin in 0·05 M pH 7·4 phosphate buffer. A trace amount of the appropriate ^{14}C labelled drug was added and the concentration was corrected for this addition. Both drugs were labelled on the carboxyl-carbon and had specific activities of 59 and 31·6 mCi mmol^{-1} for salicylic acid and aspirin respectively (The Radiochemical Centre, Amersham). The radiochemicals were used without further purification. Bovine serum albumin (BSA—Fraction V) was purchased from the Sigma Chemical Company whilst human serum albumin (HSA) was a gift from Kabi AB (Sweden). All other chemicals were of Analar grade. A Packard Tri-Carb scintillation counter was used to measure radioactivity.

The unbound drug and bound drug were separated by ultracentrifugation using a MSE Superspeed 65 thermostated centrifuge (250 000 × g, 37 °C, centrifuging time: 5 h). Aliquots (100 μl) of the drug/

* Correspondence.

protein solution before centrifugation and of the supernatant after centrifugation were counted. The fraction of drug unbound, f_u, was then calculated from the following expression

$$f_u = \frac{\text{Unbound concentration}}{\text{Total concentration}} = \frac{\text{d min}^{-1}\text{ (supernatant)}}{\text{d min}^{-1}\text{ (before centrifugation)}}$$

where d min^{-1} is disintegrations per minute per unit volume.

Hydrolysis experiments

The hydrolysis of aspirin was monitored by following the production of salicylic acid fluorimetrically. All experiments were at 37 °C in a media of 0·05 M pH 7·4 phosphate buffer. The hydrolysis was followed for about 1 h. Timed aliquots from the reaction vessel were acid extracted into ether and back extracted into phosphate buffer (Rowland & Riegelman 1967). Fluorescence of the resulting aqueous phase was measured using a Perkin-Elmer MPF-3L spectrofluorimeter with λ excitation 308 nm and λ emission 420 nm (uncorrected). Aspirin showed negligible fluorescence under the conditions of the assay and negligible hydrolysis (<1%) occurred during the extraction. BSA was used in all hydrolysis experiments as our HSA sample contained a fluorescent impurity which interfered with the assay for salicylic acid.

The fraction of aspirin bound to BSA can be varied either by changing the total aspirin concentration and keeping the BSA concentration fixed or changing the BSA concentration and keeping the aspirin concentration fixed. The latter was adopted as this allowed the unbound fraction of aspirin to be varied over a much wider range. In an initial series of experiments, in the absence of protein, the first-order hydrolysis rate constant was found to be independent of aspirin concentration up to concentrations of 50 mg $litre^{-1}$, consequently the initial concentration of aspirin was set at 50 mg $litre^{-1}$ and the BSA concentration was varied between 0 and 60 g $litre^{-1}$.

The hydrolysis experiments were repeated in the presence of carboxylic-ester hydrolase (Type 1, from hog liver; Sigma Chemical Co.) at a concentration of 4 units ml^{-1}. It was established, in a similar manner to the spontaneous hydrolysis experiment, that the enzyme-catalysed first-order hydrolysis rate constant was independent of aspirin concentration. The esterase did not interfere with the fluorescence assay.

RESULTS

Preliminary binding experiments

To determine binding in an ultracentrifuge experiment, the concentration of drug (in our case the radioactivity) is measured before centrifugation and in the supernatant after centrifugation. Several assumptions are made in evaluating f_u from the results of such experiments. Firstly it is assumed that the drug does not bind to the centrifuge tube. This assumption was checked in the present case by performing centrifugations in which a ^{14}C tracer of drug was used in the absence of both unlabelled drug and protein. With both salicylic acid and aspirin no detectable binding to the tubes was found. Secondly it is assumed that the supernatant truly reflects the unbound concentration of the drug in that the equilibrium is not disturbed by the centrifugation. This hypothesis was tested by the following experiment. Samples of both salicylic acid and aspirin in albumin solution were subjected to ultracentrifugation. Aliquots of the supernatant were taken at various depths down the centrifuge tube and for both drugs the concentration of radioactivity did not change with depth. Hence altered equilibrium in the protein pellet and consequent diffusion back up the tube must be much slower than the time required to complete a run. Thirdly, it is assumed that the radiolabelled drug binds to the protein to the same extent as the unlabelled drug. Although not tested, this assumption should hold to a very good approximation. Finally, in the case of aspirin, it is assumed that hydrolysis does not interfere with the binding determination. Since the aspirin is labelled on the carboxyl carbon, hydrolysis of the supernatant will not alter the total concentration of radioactivity in the supernatant. Also, although in excess of 4 h was required for complete protein precipitation, the bulk the protein (>90%) was spun down within 1 h. Given that the half-life of hydrolysis in buffer is 12 h and even longer, (see later), in the presence of protein, the small amount of hydrolysis that will occur before protein precipitation should be negligible. Therefore the concentration of 'salicylate' in the supernatant should fairly represent the unbound concentration of aspirin in the original sample. This situation contrasts with the long-term equilibrium dialysis experiment in which the hydrolysing aspirin is continuously in contact with the protein and thus the equilibrium is continuously changing.

The effect of salicylic acid on the binding of aspirin to BSA was demonstrated in an experiment using a radiolabelled tracer of aspirin (0·2 mg $litre^{-1}$) in the

presence of varying concentrations of salicylic acid. The results clearly illustrate the ability of salicylic acid to displace aspirin (Fig. 1).

Binding of salicylic acid and aspirin to BSA and HSA

Salicylic acid binds more strongly to BSA than does aspirin, being 95% bound at low concentration whereas aspirin is only 85% bound at low concentration (Fig. 2). The same results plotted in Scatchard form (Scatchard 1949) are shown in Fig. 3. Considerable curvature is evident in both plots which may be interpreted as multiple site binding and/or co-operativity (Schary et al 1978).

The same experiments were repeated using HSA instead of BSA and a comparison of the binding of salicylic acid to these sources of albumin shows the salicylic acid to bind less strongly to HSA; aspirin behaved similarly (Fig. 4).

During the course of our experiments we ran out of the batch of BSA (I) we had been using and had to use a new batch (II) and found considerable inter-batch variation (Fig. 5).

Computations

The binding data were analysed in terms of a classic two-site non-interactive model

$$C_T = C_u + \frac{n_1 k_1 C_u P_T}{1 + k_1 C_u} + \frac{n_2 k_2 C_u P_T}{1 + k_2 C_u} \qquad (1)$$

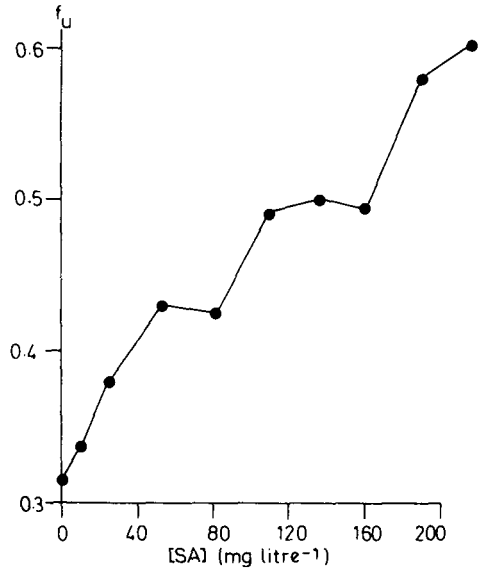

FIG. 1. Displacement of aspirin by salicylic acid. f_u is the fraction of unbound aspirin; [SA] is the salicylic acid concentration; the aspirin concentration was 0·2 mg litre^{-1}; and the bovine serum albumin concentration was 40 g litre^{-1}.

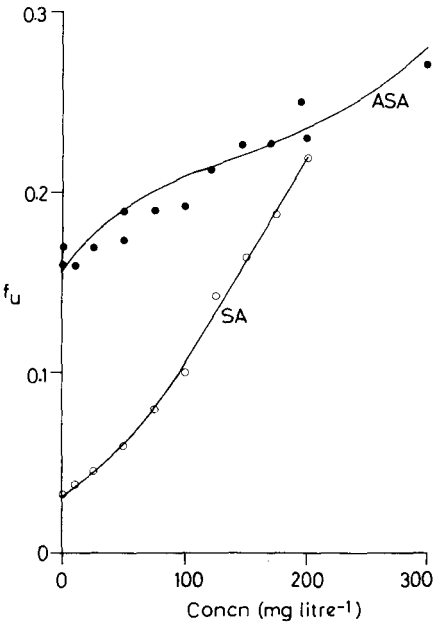

FIG. 2. Binding of salicylic acid (SA) and aspirin (ASA) to bovine serum albumin (40 g litre^{-1}). The lines are computer fits using a two-site non interactive model.

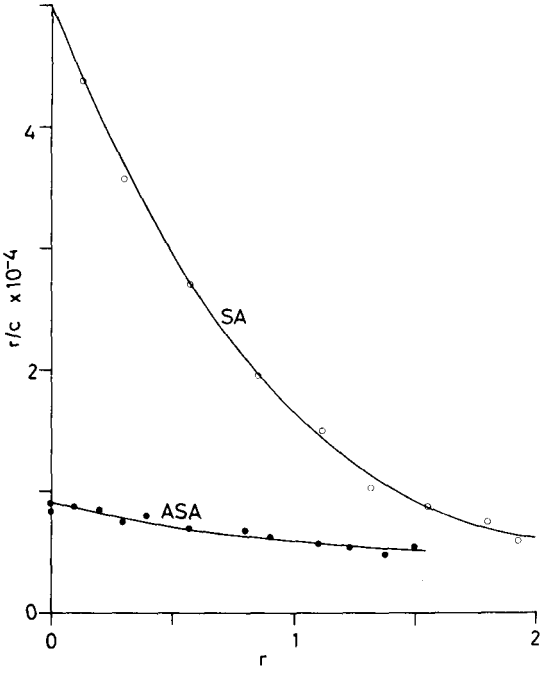

FIG. 3. Scatchard plots of the binding of salicylic acid and aspirin to bovine serum albumin. r is the concentration of bound drug per mole of protein and c is the molar concentration of unbound drug. The data are from Fig. 2.

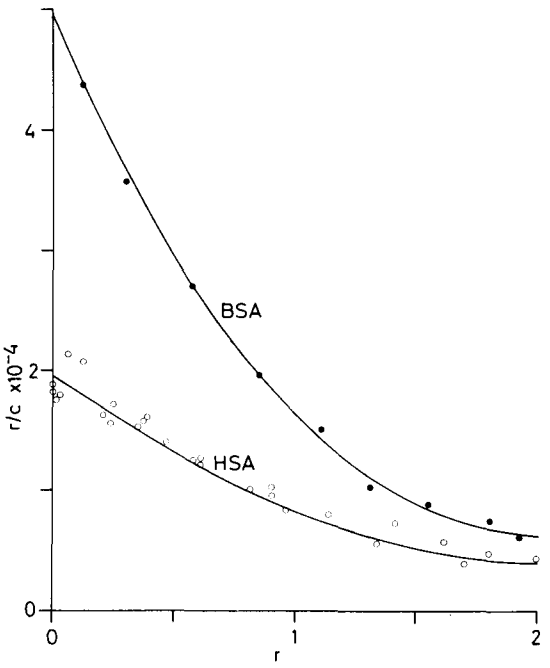

FIG. 4. Comparison of the binding of salicylic acid to bovine serum albumin (BSA) and human serum albumin (HSA). The albumin concentration in both cases was 40 g litre^{-1}.

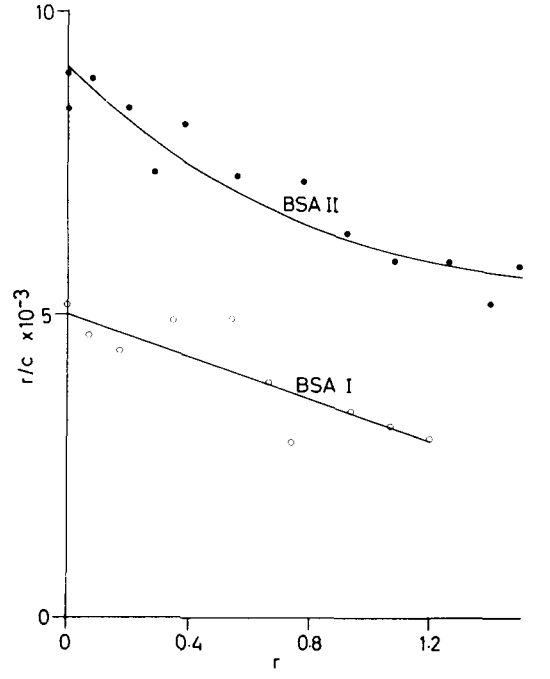

FIG. 5. Comparison of the binding of aspirin to two different batches of bovine serum albumin (BSA I and BSA II). The albumin concentration in both cases was 40 g litre^{-1}.

where C_u and C_T are the unbound and total molar concentrations of drug, P_T is the total molar concentration of protein, n_1 is the number of pre-existing-binding sites which have an association constant k_1 and n_2 is the number of sites with association constant k_2; and also in terms of a three parameter multiple stepwise equilibria model (Spector & Ashbrook 1970).

$$C_T = C_u + \frac{(K_1C_u + 2K_1K_1C_u^2 + 3K_1K_2K_3C_u^3)P_T}{1 + K_1C_u + K_1K_1C_u^2 + K_1K_2K_3C_u^3} \quad (2)$$

where K_1, K_2 and K_3 are the three equilibrium constants. All computations were performed using the NONLIN nonlinear regression program (Metzler et al 1974). The results of these calculations are shown in Table 1 and the fitted isotherms (two-site model) are represented by the solid lines in Figs 2 to 5 (the isotherms calculated from the stepwise equilibria model are virtually superimposable). The two-site parameter estimates for salicylic acid are similar to values in the literature (Jusko & Gretch 1976). In some cases the models are overdetermined in that too many parameters are being fitted to the data. Models with fewer parameters, for example a one- instead of a two-site model, would give an adequate fit. However, since no interpretation is placed on the various parameters in Table 1, these models should be viewed as representations of the data sets.

Hydrolysis experiments

In the first set of experiments, the spontaneous hydrolysis of aspirin in the presence of a range of BSA (Batch II) concentrations was investigated. The extent of aspirin binding in a 50 mg litre^{-1} solution as a function of BSA concentration was determined separately and the binding isotherm obtained, plotted in Scatchard form (Fig. 6), is compared to the binding isotherm obtained previously, in which the aspirin concentration was varied (Fig. 5; BSA Batch II). Clearly the two are not identical indicating that binding does not vary linearly with protein concentration. This effect has been observed before and possible explanations include protein aggregation and/or co-operativity (Bowmer & Lindup 1978). No attempt was made to fit a model to these data and instead the extent of aspirin binding at any particular BSA concentration was obtained by linear interpolation of the experimental binding data.

When the hydrolysis rate constant (k) is plotted against the fraction of aspirin unbound (f_u) (Fig. 7) as expected, the rate of hydrolysis decreased as f_u decreases and there is a significant linear relationship

Table 1. Computer analysis of binding data.[a]

	Two-site model (Equation 1)				Stepwise model (Equation 2)		
	n_1	k_1	n_2	k_2	K_1	K_2	K_3
SA:[b] HSA	1·3	13000	30	80	19500	2800	1900
	(0·4)	(3000)	(400)	(1000)	(800)	(400)	(400)
BSA II	0·99	45000	3·1	1600	50000	4000	2300
	(0·19)	(10000)	(1·1)	(1200)	(3000)	(400)	(400)
ASA : HSA	0·6	5000	3	300	3560	130	1600
	(0·5)	(3000)	(7)	(1000)	(70)	(90)	(1600)
BSA II	1·4	4000	8	300	9200	1500	5700
	(6·5)	(9000)	(80)	(5000)	(500)	(600)	(2800)

[a] Values in parentheses are standard deviations.
[b] SA stands for salicylic acid and ASA for aspirin. BSA II was the second batch of BSA used.

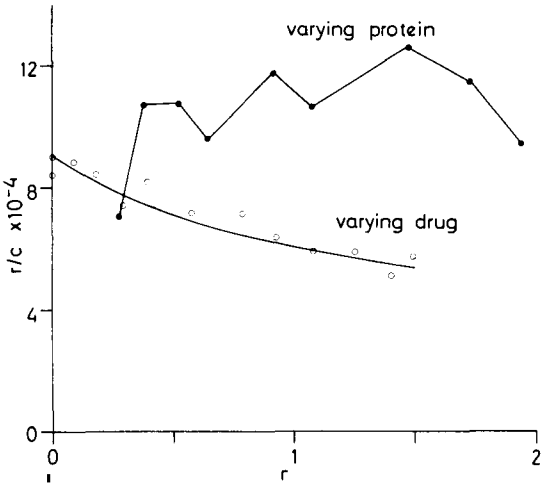

FIG. 6. Scatchard plots for the binding of aspirin to bovine serum albumin obtained by varying the aspirin concentration (BSA concentration: 40 g litre^{-1}) and by varying the protein concentration (aspirin concentration: 50 mg litre^{-1}).

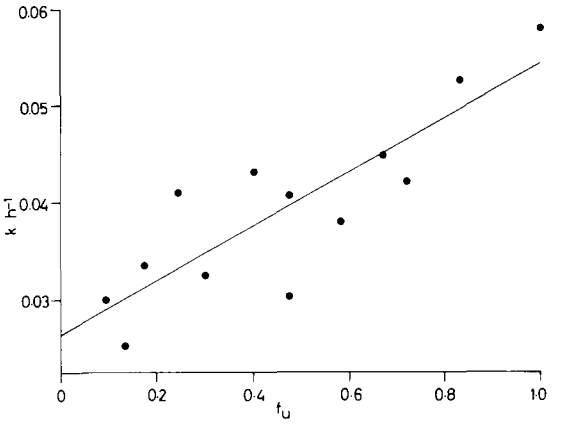

FIG. 7. The dependence of the spontaneous hydrolysis rate constant of aspirin on the fraction of aspirin that is unbound to bovine serum albumin (BSA II).

between k and f_u ($r = 0{\cdot}85$; $P < 0{\cdot}001$). However, suprisingly the line does not go through the origin but has a non-zero intercept. That is when f_u is zero (aspirin is totally bound) there is a finite rate of hydrolysis albeit slower than in the absence of protein. If we designate the hydrolysis rate constant of unbound aspirin by k_u and that for bound aspirin by k_b then the effective rate constant as a function of binding can be determined from the relationship

$$k = f_u \cdot k_u + (1 - f_u) \cdot k_b = k_b + (k_u - k_b) \cdot f_u$$

An analysis of the data in Fig. 7 yields values of the half-lives of hydrolysis of unbound aspirin and bound aspirin of 12 ± 1 h and 27 ± 3 h respectively.

The last experiment was repeated in the presence of esterase. Admittedly, esterase could alter the binding of the aspirin but this possibility is deemed unlikely as the esterase concentration (0·03 g litre^{-1}) is very low compared with the BSA concentration. It would be difficult to measure the binding of aspirin in the presence of esterase as the hydrolysis is so much more rapid. The results of these hydrolysis experiments are shown in Fig. 8, where the hydrolysis rate constant is plotted against f_u. Although there is much scatter in the data, there is nevertheless a significant negative correlation between k and f_u ($r = -0{\cdot}83$; $P < 0{\cdot}01$). The half-life in the presence of esterase and absence of protein (4 h) is much faster than in the absence of the esterase (12 h), as expected. However, BSA seems to have an activating effect on the enzyme in that hydrolysis rate increases as the albumin concentration increases. The result could be explained if either bound aspirin was more suscep-

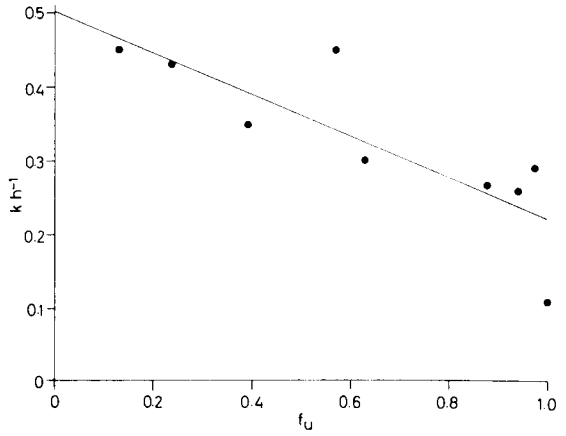

FIG. 8. The dependence of the enzyme-catalysed hydrolysis rate constant of aspirin on the fraction of aspirin that is unbound to bovine serum albumin (BSA II).

tible to enzyme-catalysed hydrolysis than unbound aspirin or if the protein directly activated the enzyme. The present series of experiments cannot distinguish between these alternatives. However, if we accept the former hypothesis, there appear to be four processes contributing to the overall hydrolysis rate constant. They are:

(i) spontaneous hydrolysis of unbound aspirin characterized by a rate constant k_u; (ii) spontaneous hydrolysis of bound aspirin characterized by a rate constant k_b; (iii) the enzyme-catalysed hydrolysis of unbound aspirin characterized by a rate constant k_u^e; and (iv) the enzyme-catalysed hydrolysis of bound aspirin characterized by a rate constant k_b^e.

The overall hydrolysis rate constant is given by the following expression

$$k = f_u \cdot k_u + (1 - f_u) \cdot k_b + f_u \cdot k_u^e + (1 - f_u) \cdot k_b^e$$
$$= k_b + k_b^e + (k_u - k_b + k_u^e - k_b^e) \cdot f_u$$

We already know from the experiment in the absence of esterase that $k_u > k_b$ and so if k is to have a negative dependence on f_u then $k_b + k_b^e > k_u + k_u^e$. Analysing the data in Fig. 8 and using the previously determined values of k_u and k_b, we find that the half-lives of enzyme catalysed hydrolysis of unbound aspirin and bound aspirin are 4 ± 2 h and $1{\cdot}5 \pm 0{\cdot}2$ h respectively.

DISCUSSION

By using ultracentrifugation it was possible to measure the extent of aspirin binding without the interference of significant hydrolysis which could lead to erroneous results in techniques like equilibrium dialysis. At low concentrations, aspirin is about 85% bound to BSA (4 g %) compared with salicylic acid which is 95% bound. It was also shown that salicylic acid is capable of displacing aspirin from albumin binding sites. Since salicylic acid is the major metabolite of aspirin, this result could have some implication on the pharmacokinetics of aspirin after multiple dosing. However, since the half-life of hydrolysis of aspirin in whole blood is about 0·5 h (Harris & Riegelman 1967), binding studies, even using ultracentrifugation, would be difficult. The hydrolyses can be inhibited with potassium fluoride (Rowland & Riegelman 1967) but the effect of fluoride on aspirin binding is unknown.

Several factors that contributed to the variability of aspirin and salicylate binding were investigated. Salicylate and aspirin bind more strongly to BSA than to HSA. There are significant differences in the affinity of salicylate for plasma proteins from different species (Sturman & Smith 1967; Kucera & Bullock 1969). In comparing the binding of a drug between species, or even within a species, differences in protein concentration have to be considered. From the results when the protein concentration was varied, rather than the substrate concentration (Fig. 6), clearly it is not valid to extrapolate the results obtained at one protein concentration to another protein concentration. Hence, differences in binding between species have to be interpreted carefully. We also noticed a marked inter-batch variation in protein. This variation could be due to differing fatty acid contamination: fatty acids have been shown to displace salicylic acid from albumin binding sites (Rudman et al 1971). Dialysis of the albumin before use would reduce this source of contamination. Thus, with all these sources of variability it is not suprising that there is so much variation in the binding parameters appearing in the literature (Jusko & Gretch 1976) and one wonders about the significance of parameters such as n and k, for example, that are quoted, given also that the models from which they are derived are idealized (Schary et al 1978).

In the presence of albumin the spontaneous hydrolysis of aspirin is decreased. However protein-bound aspirin is only partially protected from hydrolysis; the half-life being about twice that for unbound aspirin. The enzyme-catalysed hydrolysis of aspirin is much more rapid than spontaneous hydrolysis but, rather than protecting aspirin, albumin actually activates the enzyme-catalysed hydrolysis. Albumin has an activating effect on β-

glucuronidase (Levvy & Conchie 1966) and the explanation is that the albumin protects the enzyme against heavy metal ions as well as against surface denaturation, whereas bilirubin is completely protected by albumin from oxidation by peroxide in the presence of horse-radish peroxidase and this property is used in determining the extent of bilirubin binding to albumin (Jacobsen & Fedders 1970). From the experiments described here it is not possible, to offer an explanation for the activation of esterase by albumin. The fraction of aspirin bound to albumin can be varied by holding the albumin concentration constant and varying the aspirin concentration. Thus by conducting the hydrolysis experiment in this manner, rather than varying the protein concentration, it should be possible to find out whether the albumin activates the aspirin towards enzymatic attack or activates the enzyme directly. Unfortunately it is difficult to obtain a wide range of f_u values without going to very high aspirin concentration which could lead to saturation of the enzyme-catalysed hydrolysis.

These findings do not invalidate the tenet that the unbound drug is responsible for both disposition and pharmacological effect as these processes require passage across membranes which are not permeable to albumin. In fact, esterase activation by albumin may not have any bearing on the elimination of aspirin from the body as hydrolysis in whole blood only accounts for about 20% of the clearance in man (Rowland & Riegelman 1968). Nevertheless the enzyme catalysed hydrolysis of aspirin cannot be simply related to the fraction of aspirin unbound to albumin.

Acknowledgement

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