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COMPARISON OF TISSUE FLUID AND PLASMA CONCENTRATIONS OF A PROTEIN-BOUND DRUG SULPHORMETHOXINE BY *IN VIVO* DIALYSIS IN RATS

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The pharmacology of drugs which are strongly bound to serum proteins must obviously be importantly modified by this consideration. Equally clearly, however, protein-binding does not preclude drugs from participation in a considerable range of physiological and pharmacological activities. Nevertheless, protein-bound drug molecules are excluded from direct participation in biological processes (Brodie, 1965) and the pharmacological effect of a protein-bound drug is therefore quantitatively a function of the concentration of the free unbound fraction. The inhibitory effect of protein binding on activity has been illustrated particularly clearly in the case of antibacterial agents—for example, sulphonamides (Davis, 1942, 1943; Anton, 1960; Newbould & Kilpatrick, 1960) and the penicillins (Tompsett, Schultz & McDermott, 1947; Gourevitch, Hunt & Lein, 1960; Rolinson & Sutherland, 1965).

It is usual to assume that the fraction of drug free in the tissues is the same as the free fraction in the plasma, this being the only medium ordinarily accessible for analysis.

The situation *in vivo* is, however, complicated by a number of factors. Binding may occur to tissue proteins—a " relatively unexplored area " (Brodie, 1967)—and a series of equilibria will have to be established between the tissues of various organs and the tissue fluid. This may be a relatively complex situation as in the kidney (Rolinson, 1966). Interactions between drugs and normal plasma constituents bound to plasma proteins in a similar manner, bile pigments, lipids, etc., may also intervene to complicate the situation.

Evidence to support the identity of tissue fluid concentration with the free plasma level in the case of protein-bound drugs has been produced by Verwey & Williams (1962a, b) in dogs; these authors measured the concentrations of protein-bound penicillins in lymph and related these to plasma concentrations. Scholtan & Schmid (1962) provided experimental evidence in mice which indicated that the levels of unbound penicillin and propicillin in serum were very similar to those in tissue fluid, even though the levels of total drug were quite different. Kunin (1965) showed that distribution in the tissues of rabbits of penicillins labelled with carbon-14 was inversely related to their known binding by rabbit sera. The literature on the subject has been reviewed by Rolinson (1966).

In the following experiments direct evidence has been obtained by *in vivo* dialysis to confirm the correspondence between tissue fluid levels of a sulphonamide (sulphormethoxine, " Fanasil," Roche), and the unbound level of drug in the serum at any given total serum concentration.

Sulphormethoxine (Portwich & Büttner, 1964) has been used fairly extensively both for the treatment of bacterial infections—for example, pyelonephritis (Grüneberg & Brumfitt, 1967), chronic bronchitis (Pines, 1967), leprosy (Barclay, Wilkinson & Falciani, 1963)—and for the treatment of malaria (Laing, 1964). It was chosen for the present experiments because its very long half-life in the body permits attainment of a reasonably stable equilibrium in tissue fluids, including the implanted sac, when administered to rats orally in the diet.

METHODS

The technique used involved the implantation of dialysis tubing sacs into the peritoneal cavities of rats. Male white Wistar rats of approximately 350 g weight were used and the sulphormethoxine was given in the food at concentrations of 0.5, 1, 2, 4, and 8 g/kg of mash. The sulphormethoxine was added to the mash by dissolving it in acetone and spraying it over the mash in a mechanical mixer. The rats were given the diets for a period of 1 week before implantation of the sacs. Preliminary experiments indicated that steady drug levels were in fact obtained at 4 days. The rats continued the diet till the sacs were removed. With higher concentrations of drug, addition of a little glucose to the mash was necessary to induce the rats to consume the usual amount.

The sacs were made from sterilized Visking cellulose dialysis tubing (size 8/32) tied at each end and containing 2 ml. of 6% dextran (Intradex, Glaxo). Similar sacs have been used by Wrong, Metcalf-Gibson & Morrison (1965), for *in vivo* analysis of electrolyte content of faeces. Preliminary experiments showed that the dextran had no binding capacity for sulphormethoxine. The sacs were implanted in the peritoneal cavity under ether anaesthesia with sterile precautions through small abdominal incisions which were then carefully closed by suturing. The sacs were left in place for 24 hr, earlier experiments having indicated that the sac concentrations were no greater at 48 than at 24 hr. The animals were then anaesthetized again with ether and the sacs removed. Although the sacs were invested with omentum, irritation of the peritoneum seemed to be relatively slight and there was no excess of free fluid. The rats were then exsanguinated by withdrawing blood from the aorta. The serum and sac concentrations of sulphonamide were estimated by the Bratton & Marshall (1939) technique, conjugated as well as non-conjugated drug being estimated in these *in vivo* experiments. No measurable conjugated drug was, however, detected at any dosage level.

In vitro equilibration was performed using fresh normal rat serum to which sulphormethoxine was added to give a range of concentrations covering those obtained in the *in vivo* experiments. Solution of sulphormethoxine in serum at higher levels was achieved by agitation at 37° C overnight. Under sterile conditions, 2 ml. samples were placed in stoppered 5 ml. tubes together with sacs containing 2 ml. of 6% dextran. The tubes were then agitated at 37° C for 16–24 hr to conform to the *in vivo* experiments. In fact equilibrium was found to be reached at approximately 6 hr. The estimations were performed in an identical manner on sac fluid and on serum after dialysis. There is approximate equivalence between the relative volume of water in serum and in sac-fluid (Intradex), so no correction has been made for this factor in expressing the " free " concentration of drug. All estimations were performed in duplicate. Total serum proteins were measured by the biuret method and protein electrophoresis was performed on polacrylamide gel.

The pH of the sac fluid on removal from the rats was invariably 7.4. After *in vitro* equilibration with fresh rat serum it was also invariably 7.4.

RESULTS

The results of the experiments in rats are shown in Figs. 1–4. Figure 1 shows the relationship between drug levels in sacs and corresponding sera for the whole series (thirty-five *in vivo*, thirty-one *in vitro*), represented arithmetically. Figure 2 shows the same values with the sac concentration plotted on a logarithmic scale, enabling the relationships within the therapeutic range for sulphonamides to be seen more clearly. Inspection demonstrates that there is no significant difference between results obtained *in vivo* and those *in vitro*. The regression line in Fig. 2 is that for the combined series and is the best fitting quadratic for the three series $[(y=\bar{y}+by.\ (x-\bar{x})+by.\ (x^2-\bar{x}^2)]$.

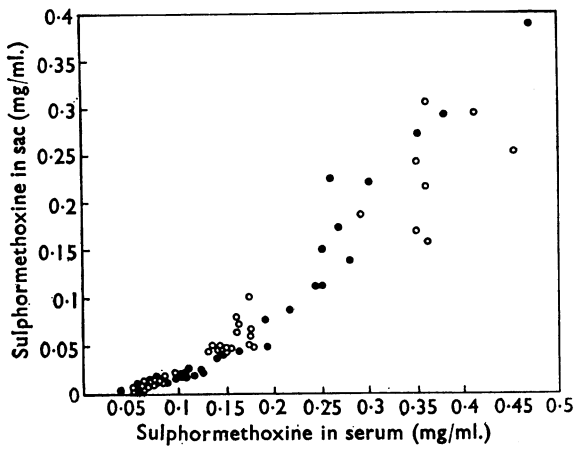

Fig. 1. Comparison of *in vivo* dialysis (○) with *in vitro* dialysis (●). Sac concentrations of sulphormethoxine (mg/ml.) ordinate, serum concentrations abscissa. Arithmetic scale for both.

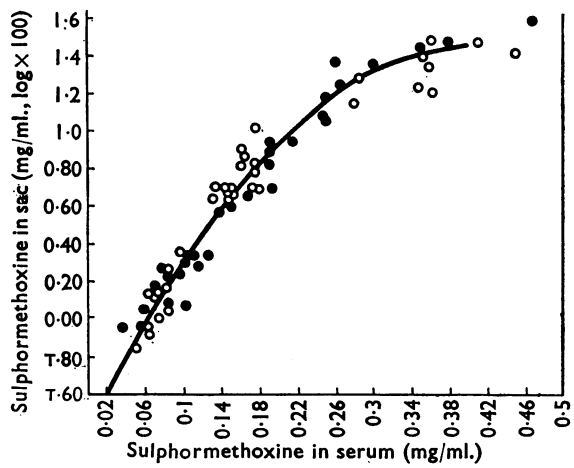

Fig. 2. As for Fig. 1 but with sac concentration (ordinate) multiplied by 100 and plotted logarithmically.

The equations for the three separate curves are as follows:

In vitro $y=\text{antilog}\ (0.6596+\ 9.409\ (x-0.1774)-10.32\ (x^2-0.0421))$

In vivo $y=\text{antilog}\ (0.6761+10.838\ (x-0.1780)-14.65\ (x^2-0.0441))$

Combined series $y=\text{antilog}\ (0.6682+10.03\ \ (x-0.1777)-12.41\ (x^2-0.0432))$

Average serum concentration for various dietary levels of sulphormethoxine in the *in vivo* group are shown in Table 1, together with the total serum protein and albumin levels. It can be seen that there were no significant alterations in total serum proteins with addition of sulphormethoxine to the diet, but that at the higher dose levels the serum albumin tended to be somewhat lower. Control values were for serum proteins 6.6 g/100 ml. and for serum albumin 2.5 g/100 ml.

TABLE 1

DIETARY SULPHORMETHOXINE (g/kg mash) CORRESPONDING SERUM SULPHORMETHOXINE (mg/ml.) TOTAL SERUM PROTEIN AND SERUM ALBUMIN (g/100 ml.)

Number of rats in each group in parenthesis in first column.

Dietary sulphormethoxine (g/kg mash)	Average serum sulphormethoxine (mg/ml.)	Average total protein (g/100 ml.)	Average serum albumin (g/100 ml.)
1 (6)	0·089	6·4	2·6
2 (6)	0·136	6·3	2·1
3 (2)	0·161	6·6	1·9
4 (8)	0·168	6·2	2·0
6 (3)	0·207		
8 (6)	0·358	6·4	1·7

Figures 3 and 4 show the relationship between bound and total drug and bound and free drug for the combined series with the regression lines for the combined series. The level for bound drug reaches a maximum of approximately 0.115 mg/ml. at a serum level of 0.20 mg/ml. or free drug 0.085 mg/ml. Beyond this point in each graph it actually begins to descend, possibly because of the lowered serum albumin in the *in vivo* experiments, but the scatter of values above this point is too great for much significance to be attached to it. For an assumed molecular weight of rat serum albumin of 68,000, the molecular concentration of the albumin in the normal rat serum used for the *in vitro* experiments (2.5 g/100 ml.) was 3.7×10^{-4}. The molar concentration of sulphormethoxine (molecular weight 312) at 0.115 mg/ml. also equals 3.7×10^{-4}. Thus there seems to be a limitation in carrying capacity on the part of rat serum for sulphormethoxine to one molar equivalent of its albumin content (Thorp, 1964) at physiological pH.

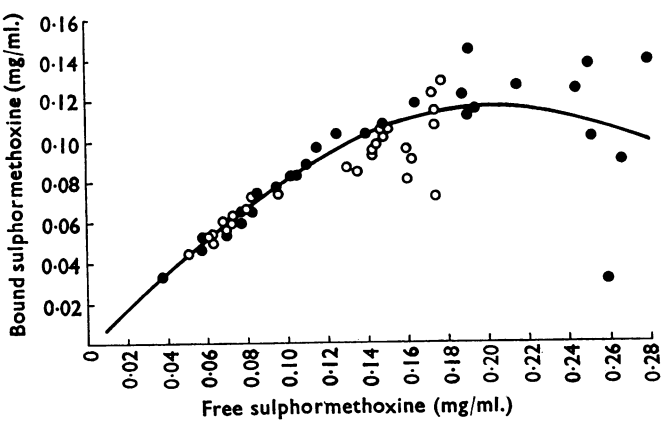

Fig. 3. Bound sulphormethoxine (ordinate) plotted against total serum sulphormethoxine (abscissa) (mg/ml.). (○), Values for *in vivo* dialysis; (●), *in vitro* dialysis. The regression line is for the combined series.

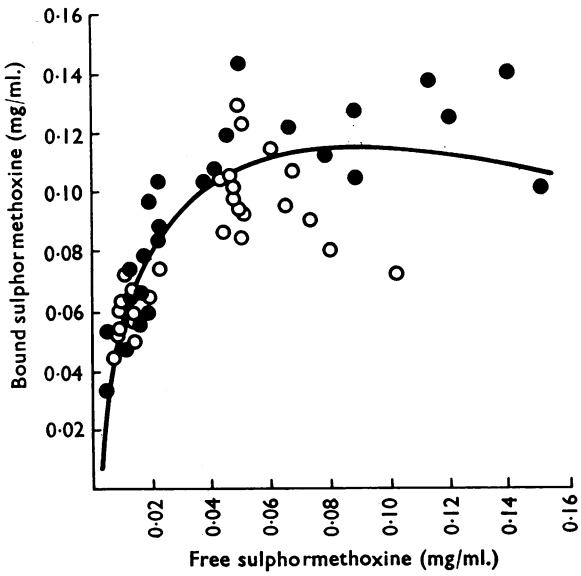

Fig. 4. Bound sulphormethoxine (ordinate) plotted against free sulphormethoxine (abscissa) (mg/ml.). (○ *In vivo* dialysis; (●), *in vitro* dialysis. The regression line is for the combined series.

DISCUSSION

The present experiments indicate that the drug concentration in the peritoneal fluid of the rat corresponds well with the free fraction of the drug in plasma. Results of *in vivo* dialysis in rats receiving sulphormethoxine in the diet gave virtually the same relationships over a wide range between sac fluid and serum as were obtained after *in vitro* dialysis of normal serum to which sulphormethoxine had been added. The relationships found for a single acidic drug such as sulphormethoxine need not necessarily hold in the case of other types of drug or combinations of protein-bound drugs (Gillette, 1967), and further work is in progress to assess the relationships in the case of combinations of drugs similarly bound.

Equilibrium dialysis was used for the *in vivo* experiments, and hence for comparative purposes was also used for the *in vitro* experiments. In the circumstances it was quite satisfactory for it was possible to construct a curve relating serum concentration to sac concentration after dialysis and to use this curve to compare the expected concentration of free drug for any given serum concentration with that found in the sac *in vivo* at the same serum concentration. It must be appreciated that in using equilibrium dialysis the levels of drug in the serum will drop with diffusion of free drug across the dialysis sac so that the relationship observed in the case of any individual sample thus analysed will not apply to the original serum level but to something lower than this by a factor depending on the volume of fluid in the two compartments. Figures for serum concentration shown in the present *in vitro* dialysis experiments are for drug levels after attainment of equilibrium. The disadvantages of equilibrium dialysis for measurement of protein-binding in individual samples of serum are overcome by the use of ultra-filtration techniques (Rieder, 1963; Keen, 1965; Bennett & Kirby, 1965), but these could not, of course, be used in the present experiments.

The quantitative relationships illustrated conform with those expected. The plot of bound versus free drug (Fig. 4) seems to conform to the theoretical curve predicted by Goldstein (1949). The precision of the 1:1 relationship in the rat experiments for maximum binding between serum albumin and sulphormethoxine may have been fortuitous, however, particularly in the light of such data as those of Clausen (1966), which suggest that there may be binding to serum protein fractions other than albumin to a significant extent.

SUMMARY

1. An *in vivo* dialysis technique has been used in rats to make a direct comparison of tissue-fluid concentrations of a protein-bound drug (sulphormethoxine) with that in the free fraction of the serum. Good agreement has been found over a considerable range.

2. The maximum binding capacity of rat serum for sulphormethoxine on a molar basis was equivalent to the molar concentration of the albumin content.

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