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THE BINDING BY BOVINE PLASMA AND PLASMA FRACTIONS OF SALICYLIC ACID AND SOME OF ITS 3-ALKYL ANALOGUES

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Abstract—The extent of binding of salicylic acid and its 3-methyl, 3-*iso*propyl and 3-*tert*.-butyl analogues by bovine plasma and plasma fractions has been determined.

THE extent of binding of salicylic acid and its 3-methyl, 3-*iso*propyl and 3-*tert*.-butyl analogues by bovine plasma has been determined, in parallel with studies of their antidiabetic properties, effect on oxygen consumption and toxicity. Equilibrium dialysis was employed at 37° against iso-osmotic phosphate buffer at pH 7·4. The investigations were continued using the following bovine plasma fractions:

albumin;
γ-globulin;
fibrinogen;
albumin + γ-globulin;
albumin + γ-globulin + fibrinogen.

Each, obtained in powder form, was dissolved in phosphate buffer. In addition, fresh bovine serum was fractionated in half-saturated ammonium sulphate. The precipitated globulins suspended in buffer were also examined, alone, and with added albumin, to determine their binding capacity for 3-methylsalicylic acid. Recently Davison and Smith[1] have described similar work but in acetate buffer at pH 5·4 using albumin at 0·69 g% rather than at a physiological level (3·4 g/100 ml).

METHOD

The same heparinized bovine plasma stored in a deep freeze was used throughout. The total protein was determined by the Biuret reaction and fibrinogen by the method of Foster and Whipple[2]. The proportions of the plasma components were obtained by electrophoresis of a sample of the corresponding serum (Brackenridge[3]). Albumin, γ-globulin and fibrinogen obtained from Armour Pharmaceuticals Ltd., were dissolved in phosphate buffer (2·475 g/100 ml $NaH_2PO_4 \cdot 2H_2O$, 17·5 vols + 1·79 g/100 ml Na_2HPO_4, 82·5 vols.) to give concentrations after dialysis of 3·4 g/100 ml, 0·5 g/100 ml and 0·28 g/100 ml respectively, 22 mg of streptomycin and 6·8 mg of benzylpenicillin were added per 100 ml of plasma or protein solution.

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Quantities of the four drugs were added to each protein solution to provide a range of concentrations of from 10 to 70 mg/100 ml after dialysis. This was performed by placing 5 ml of plasma or protein solution in a Visking bag suspended in 8 ml of buffer in the case of salicylic acid and its 3-methyl analogue, and 3 ml suspended in 5 ml buffer for the higher analogues. Dialysis was allowed to proceed without stirring at 37 ° for 24 hr which had previously been shown sufficient time for equilibration.

The method of Trinder[4] was used for determination of salicylic acid and 3-methylsalicylic acid levels, and an adaptation of the more sensitive ultra-violet spectroscopic method of Stevenson[5] for 3-*iso*propyl and 3-*tert.*-butylsalicylic acids. This involved addition of 0·5 ml N hydrochloric acid to the serum before extraction, and quantitation from standard curves. The size of the samples was chosen so as not to exceed 40 μg of drug. The protein concentrations at equilibrium were obtained by the Biuret reaction.

Fresh bovine serum, diluted to one-half with water was fractionated by addition of an equal volume of saturated ammonium sulphate solution with stirring over 2 hr at room temperature. The precipitate was separated by centrifugation, washed twice with half-saturated ammonium sulphate solution and the cake pressed dry. The solids were then suspended in the minimum volume of phosphate buffer and dialysed five times against 20 vols of the buffer over 5 days at 5°. The dialysed solution was stored in a deep freeze after addition of antibiotics as before. The total protein was determined and the electrophoretic pattern. From this data the solution was diluted with buffer to give the same γ-globulin level as in the whole plasma with which it was to be compared. Solutions of 3-methylsalicylic acid in this mixture of globulins, in albumin + globulins and in albumin + γ-globulin were then dialysed to equilibrium as described.

A measure of the concentration of bound drug at a particular drug level was obtained (Davis[6]) by subtracting from the concentration inside the membrane (bound + free), that in the dialysate (free) corrected for protein volume inside the bag.

RESULTS

The protein concentrations of the solutions are shown in Table 1.

Determinations on the initial mixture and at equilibrium revealed that the levels remained almost constant throughout the dialysis.

TABLE 1. THE PROTEIN COMPOSITION OF THE SOLUTIONS DIALYSED

	Fresh plasma (g %)	Commercial fractions (g %)	Globulin precipitate (g %)
Total protein	8·6		3·46
Fibrinogen	0·5	0·28	
Albumin	3·4	3·4	0·05
Globulin α_1	0·79		—
α_2	0·88		0·49
β	1·04		0·71
γ	2·2	0·5	2·2

β, the fraction (concentration of bound drug)/(concentration bound + free) was calculated for each drug in presence of plasma and plotted against plasma level. There is an increase in plasma binding with increase in size of alkyl substituent, both when the drugs are compared at equimolar concentration and at equal weight per unit

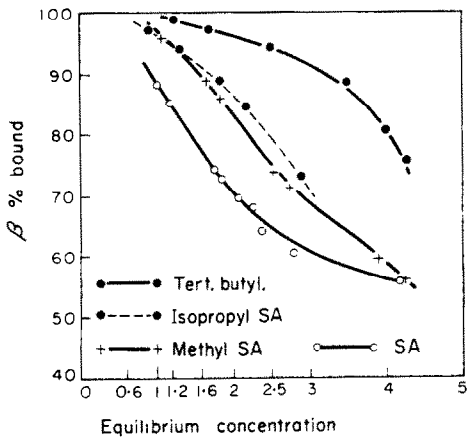

FIG. 1. Binding to whole plasma of salicylic acid, 3-methylsalicylic acid, 3-*iso*propylsalicylic acid, and 3-*teriary*-butylsalicylic acid (equilibrium concentration in moles $\times 10^{-3}$).

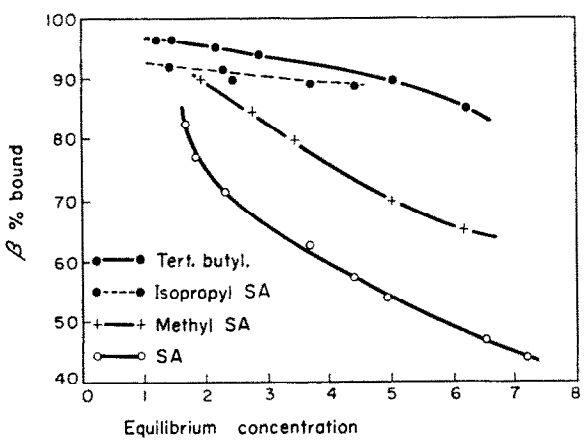

FIG. 2. Binding to albumin (3·4 g/100 ml) of salicylic acid, 3-methylsalicylic acid, 3-*iso*propylsalicylic acid and 3-*tertiary*-butylsalicylic acid (equilibrium concentration in moles $\times 10^{-3}$).

volume. The therapeutically effective levels for salicylic acid and 3-methylsalicylic acid are each approximately 2×10^{-3} M. From Fig. 1, if human and bovine plasma bind to the same extent, the acids would be 70 per cent and 82 per cent bound respectively, corresponding to 6×10^{-4}M and $3{\cdot}6 \times 10^{-4}$M of free drug.

In Fig. 2, the fraction of each drug bound to albumin is plotted against molar concentration for each. Similar results were obtained for salicylic acid and albumin when the antibiotics were omitted and the dialyses performed under sterile conditions. It was therefore assumed that the additives did not interfere in the interaction between the protein and salicylic acid. The curves again lie in the order of increased binding with increase in size of alkyl substituent.

The technique of Scatchard[7] was employed to assess the number of binding sites on albumin available for each compound. Fig. 3 shows the curves obtained for salicylic

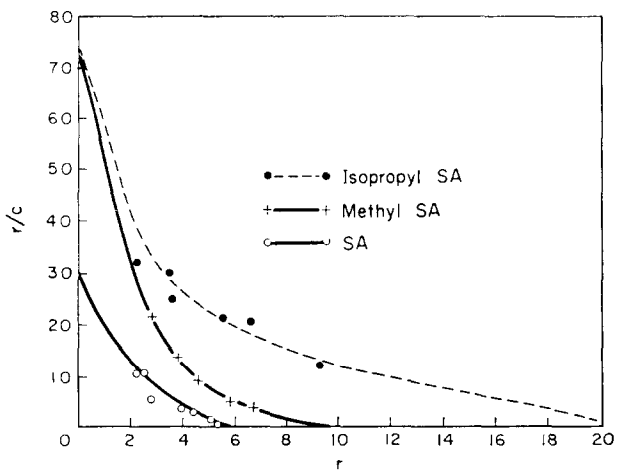

FIG. 3. The ratio (moles bound drug per mole albumin)/moles free drug plotted against moles bound drug per mole albumin at albumin concentration 3·4 g/100 ml, salicylic acid, 3-methylsalicylic acid, 3-*iso*propylsalicylic acid.

acid, 3-methyl and 3-*iso*propylsalicylic acids when the ratio (moles bound drug per mole albumin)/(moles free drug) is plotted against moles bound drug per mole albumin. By this technique the intercept on the abscissa gives the number of occupied sites. However, as the curves are not linear, the conditions which permit the application of the Langmuir isotherm do not strictly obtain; that is, each of the sites on albumin does not have the same affinity for the anion, or else there is repulsion of the later molecules by those already bound. Nevertheless the values of 6, 10 and 20 derived by extrapolating the curves for salicylic acid, 3-methyl and 3-*iso*propyl salicylic acids, respectively, give an approximate picture of the capacity of albumin for each of the compounds. The points calculated for 3-*tert*.-butylsalicylic acid were too far apart for a curve to be drawn with any confidence.

When the graphs of percentage bound against drug level for the systems containing albumin alone and plasma of the same albumin content are compared for each of

salicylic acid and its 3-alkyl analogues, Figs. 4 to 7, it appears that binding by albumin exceeds that by plasma except in the case of salicylic acid at levels above 58 mg/100 ml ($4{\cdot}2 \times 10^{-3}$M). Reynolds and Cluff[8] have found no binding by globulins from solutions of salicylic acid up to 20 mg/100 ml. This has been confirmed for γ-globulin throughout the concentration range for all four compounds investigated, and fibrinogen

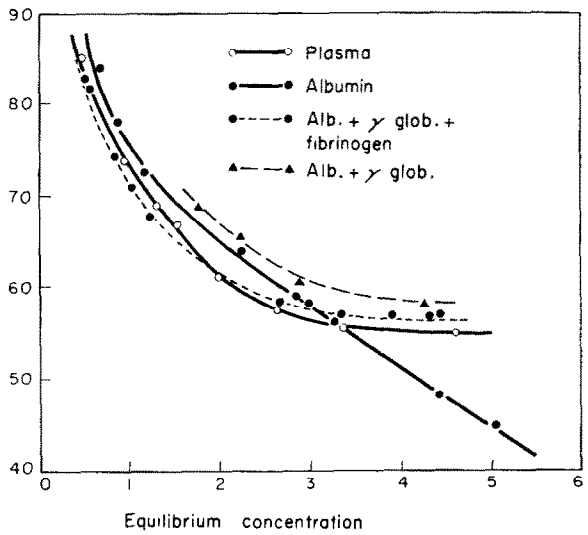

FIG. 4. Binding of salicylic acid to albumin (3·4 g/100 ml), to albumin (3·4 g/100 ml) + γ-globulin (0·5 g/100 ml), to albumin (3·4 g/100 ml) + γ-globulin (0·5 g/100 ml) + fibrinogen (0·28 g/100 ml) and to whole plasma. Equilibrium concentration, moles $\times 10^{-3}$

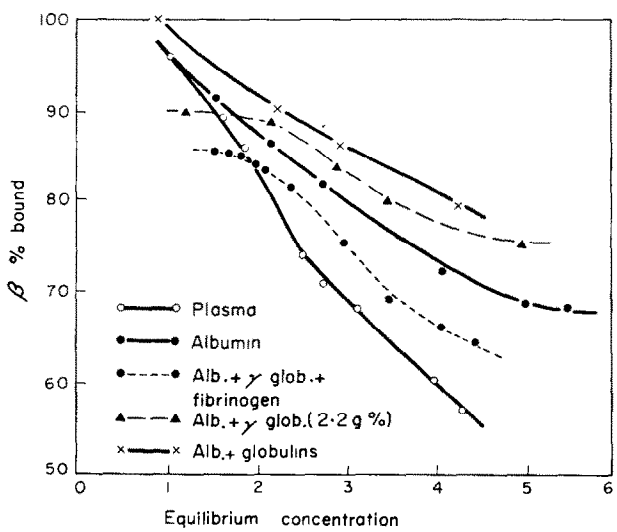

FIG. 5. Binding of 3-methylsalicylic acid to albumin (3·4 g/100 ml), to albumin (3·4 g/100 ml) + γ-globulin (2·2 g/100 ml), to albumin (3·4 g/100 ml) + α_2-globulin (0·49 g/100 ml) + β-globulin (0·71 g/100 ml) + γ-globulin (2·2 g/100 ml), to albumin (3·4 g/100 ml) + γ-globulin (0·5 g/100 ml) + fibrinogen (0·28 g/100 ml) and to whole plasma. Equilibrium concentration, moles $\times 10^{-3}$

has been shown to bind only the tertiary butyl compound and this to a small extent (Fig. 8). However, when mixed with albumin, these two components at 0·5 and 0·28 g/100 ml respectively were found to increase the binding of salicylic acid from that by albumin alone. At drug levels above 50 mg/100 ml they showed a small reduction in the binding capacity for the other three acids.

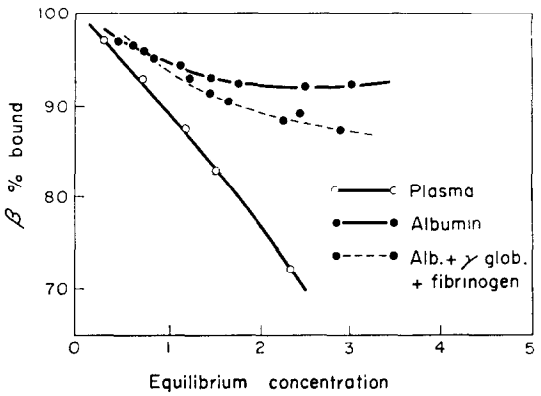

FIG. 6. Binding of 3-*iso*propylsalicylic acid to albumin (3·4 g/100 ml), to albumin (3·4 g/100 ml) + γ-globulin (0·5 g/100 ml) + fibrinogen (0·28 g/100 ml), and to whole plasma. Equilibrium concentration, moles × 10^{-3}

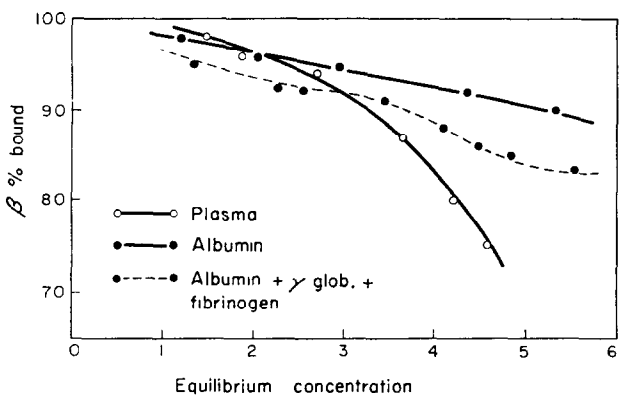

FIG. 7. Binding of 3-*tertiary*-butylsalicylic acid to albumin (3·4 g/100 ml) to albumin (3·4 g/100 ml) + γ-globulin (0·5 g/100 ml) + fibrinogen (0·28 g/100 ml) and to whole plasma. Equilibrium concentration, moles × 10^{-3}

The binding curve for 3-methylsalicylic acid, Fig. 8, shows the capacity of the globulin mixture containing albumin contaminant of less than 0·05 g/100 ml. If the amount bound at a given total concentration is proportional to the albumin content of the solution, then the value calculated for β at 30 mg/100 ml for this quantity of albumin would be 1·3 per cent. Therefore the binding by a mixture of $\alpha^2 + \beta + \gamma$-globulins

appears to be significant. Fig. 6 demonstrates the increase in binding of 3-methylsalicylic acid as the series albumin, albumin + γ-globulin, and albumin + $\alpha^2 + \beta + \gamma$)-globulins, all at the same component concentrations, is traversed.

DISCUSSION

The work of Klotz[9-12] and others leads one to expect that the fraction of the drugs bound by plasma or an albumin solution at a particular drug level rises with increase in size of the alkyl substituent. The results confirm this for the 3-alkyl salicylic acid series and also give an indication of the number of binding sites on albumin available for each of the analogues.

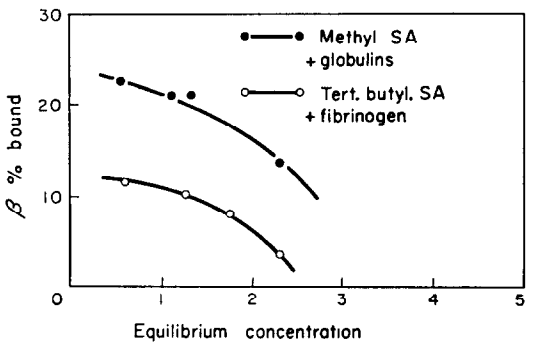

FIG. 8. Binding of 3-methylsalicylic acid to α_2-globulin (0·49 g/100 ml) + β-globulin (0·71 g/100 ml) + γ-globulin (2·2 g/100 ml), and of 3-*tertiary*-butylsalicylic acid to fibrinogen (0·28 g/100 ml). Equilibrium concentration, moles $\times 10^{-3}$

The fact that 3-alkylsalicylic acids unlike salicylic acid, are bound by α- and β-globulins might appear to be contributory to the higher capacity of plasma for these compounds. However, in terms of the amount of 3-methylsalicylic acid bound at 30 mg/100 ml drug level, the increase between albumin and albumin + ($\alpha_2 + \beta + \gamma$)-globulin is only 30(92 — 88·5)/100 or 1·0 mg/100 ml, whereas the mixture of globulins alone bound 4·0 mg/100 ml. Therefore the capacities of the protein components of plasma are not additive, and some masking of binding sites occurs. It thus appears that the considerable difference between the binding capacity of plasma for salicylic acid and for 3-methylsalicylic acid is not simply due to the contribution of α_2 and β-globulins which are only able to bind the substituted acid.

The marked difference observed in the capacity of serum from that of albumin as obtained commercially suggests that the study of the latter is only a qualitative guide to the binding capacity of whole serum for an anionic compound.

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