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Dissolution and in vitro permeation behaviours of dicumarol nitrofurantoin and sulfamethizole in the presence of protein

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Summary

The dissolution and in vitro transport of dicumarol (BHC), nitrofurantoin (NTF), and sulfamethizole (SMT) in the presence of bovine serum albumin (BSA) and casein was studied. The presence of either protein caused varying effects in both processes for BHC and NTF. Both proteins reduced the dissolution and transport rate of BHC. A protein concentration-dependent increase in dissolution rate of NTF was observed. Although neither protein altered the transport of NTF from solutions, an increase of transport rate was noted when the transport was studied from saturated solutions. No effect was noted with SMT. The effects were attributed to the formation of casein aggregates causing an enhancement of saturation solubility of BHC and NTF. The varying intensity of binding of drugs to BSA was responsible for the influence of BSA on the rate of processes. Studies such as this are valuable in investigating the mechanisms involved in drug–food interactions.

Introduction

It is now well recognized that food, or other substances present in the diet, can increase or decrease drug absorption (Welling, 1977, 1984; George, 1984). This realization has grown over the last 10 years and is based on miscellaneous in vivo studies focusing on the effect of food on the bioavailability of drugs (Welling, 1984). Among these studies, a number has been concerned with the effect of a specific type of food on drug bioavailability (Welling et al., 1978; Ali and Farouk, 1980; Ali, 1981; Ginsburg et al., 1983; Saux et al., 1983; Ogunbona et al., 1985; Macheras and Reppas, 1986a and b).

In parallel, few but not systematic in vitro studies pertinent to the effect of specific food constituents on the dissolution and absorption of drugs have been reported (Nakano, 1971; Lovering and Black, 1974; Donbrow and Touitou, 1978; Rosen and Macheras, 1984; Rosen and Macheras, 1985; Macheras et al., 1986). These studies are valuable for exploring food–drug and/or food–formulation interactions. However, since the mechanisms of the effect of food on drug bioavailability are in the great majority of cases unclear, more in vitro studies are required to elucidate the mechanisms involved. In principle, these studies

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may provide considerable insight into food–drug interactions. In addition, they may prospectively be of more general application in rationalizing the effect of food on bioavailability.

This report describes studies on the effect of two proteins on the dissolution and in vitro permeation behaviours of dicumarol (BHC), nitrofurantoin (NTF) and sulfamethizole (SMT). Since freeze-dried formulations of these drugs in the protein-rich medium milk have been prepared and evaluated in vivo (Macheras and Reppas 1986a, 1986b), it was felt that the in vitro study would give an insight into the mechanisms involved. Bovine serum albumin (BSA) and casein were used as model proteins.

Materials and Methods

Particle size data for the powders of drugs have been reported previously (Macheras and Reppas, 1986a and b). The proteins used were: casein (vitamin-free) from Serva Feinbiochemica, and bovine serum albumin (BSA) from Fluka A.G., fraction V. Sorensen's phosphate buffers (M/15) were used for controlling the values of the pHs of the solutions. Deionized distilled water was used throughout the study and all the reagents used in the assay procedures were reagent grade.

Dissolution

The rotating basket method was used for the dissolution studies. An appropriate amount of drug was placed on a piece of aluminum foil clamped on the bottom of a 250 Mesh screen basket and the whole was immersed in a double-wall beaker containing 600 ml of the buffer (pH 7.5 or pH 7.2) with or without the proteins. The basket was rotated at 60 rpm at 3 cm distance from the bottom of the beaker. Water was circulated from a bath through the double-wall beaker ensuring a constant temperature of $37 \pm 0.5\,^{\circ}C$ throughout the dissolution experiment. Samples of 3 ml were collected at appropriate time intervals. Three ml of dissolution medium maintained at 37 °C were added to the beaker after each sample was removed. Each sample was filtered (Rundfilter MN 816) and protected from light until the assay was performed. In all cases, correction for the dilution was made at the end of each experiment.

The effect of casein on the saturation solubility of all drugs and BSA's on NTF's solubility have been studied previously (Macheras and Reppas 1986a and b). In this study we report solubility data for BHC and SMT in presence of BSA, utilizing the method reported previously.

The relative viscosities of the protein solutions against distilled water were determined using an Ostwald type viscometer at 37 °C and corrected to relative dynamic viscosity by multiplying by the relative density. Density measurements were made using a density meter and cell (Paar, Switzerland).

Transport studies

A plexiglas dialysis cell, similar to that described by Patel and Foss (1965) and consisting of two symmetrical parts clamped together with a dialysis membrane (Serva Feinb., Visking Dialysis tubing) at the plane of symmetry, was employed in the transport-rate studies. Each compartment holds about 95 ml of liquid and the area of the membrane available for permeation was 20.75 cm^2. The cell was immersed in a water bath for temperature control. The donor compartment was stirred with a magnetic bar. The intensity of the agitation was sufficiently low (about 80 rpm) to prevent the formation of a vortex in the compartment. The receiver compartment was agitated and monitored by a flow system consisting of a peristaltic pump (Cole-Parmer Instr. Co., 6-600 T/MN) with polyethylene tubing. The dead volume of the tubings was 1.7 ml and the flow rate 10 ml/min. The permeation cell and solutions were equilibrated with the water bath $(37 \pm 0.1)\,^{\circ}C$ prior to an experiment. To start an experiment, 90 ml of the solution containing the drug or the drug and the protein was transferred to the donor compartment. The receiver compartment contained in all cases 90 ml of buffer with the same pH as in the donor compartment. The solution in the donor compartment contained either a saturated drug's solution in pH 7.2 buffer for all drugs or the drug dissolved in pH 7.5 buffer for BHC (30 μg/ml) and in pH 7.2 buffer for NTF (40 μg/ml) and SMT (90 μg/ml) in presence or absence of protein. All drugs were predissolved in weakly al-

kaline solutions and added in an appropriate volume of buffered protein solution to give the desired drug concentration. Saturated solutions were prepared by adding excess drug to the buffer solutions either with or without protein and equilibrating overnight, or equilibrating overnight and dissolving the appropriate quantity of protein just before the experiment.

In all cases, samples of 1 ml were removed from the receiver compartment at suitable time intervals i.e. 20 min and were protected from light until they were analysed. Immediately after sampling, 1 ml of buffer maintained at 37 °C was added to the receiver compartment and correction for this dilution was made.

Protein binding studies

Since the binding of NTF with BSA as well as the interaction of all drugs with casein have been studied previously (Macheras and Reppas 1986a and b), this study was carried out in a similar manner only for the interactions of BHC and SMT with BSA. Experiments were performed in triplicate and results were analysed using the equation of Scatchard (1949):

$$\frac{r}{D_f} = nK_a - K_a r \qquad (1)$$

where r is the molar ratio of bound ligand to protein, D_f is the concentration of free ligand at equilibrium, K_a is the association constant and n the number of equivalent binding sites with this value of K_a.

The interaction of BHC with BSA was additionally investigated by difference spectroscopy (Perrin and Idsvoog, 1971) using a procedure similar to Rosen (1970). Changes in U.V. absorption at various molar ratios of BHC ($0.149–2.97 \times 10^{-5}$M) and BSA ($1.51 \times 10^{-5}$M) were examined at 270 and 314 nm.

Assay

The concentration of BHC was determined in all studies by UV spectrophotometry at 314 nm. Solutions of BHC which contained either protein were assayed spectrophotometrically for total BHC. Standard curves were prepared by plotting the absorbances at 314 nm. While the presence of casein did not affect the absorbance of BHC, a Beer's plot when BSA (0.1%) was present was linear in the 0–0.8 molar ratio (BHC/BSA) range. By diluting each sample (when BSA was present) to an absorbance value less than 0.300 for BHC, the concentration of BHC was readily found by linear interpolation (Robertson and Madsen, 1974).

Total NTF was determined in all studies by direct spectroscopy at 380 nm by means of a calibration curve and after the appropriate dilutions had been made. Neither proteins interfered with the assay.

The Bratton–Marshal (1939) method was used for SMT. The concentration of drug was followed at 540 nm in all studies.

Results and Discussion

Solubility studies

The solubility of BHC increases almost linearly with BSA concentration (Fig. 1). The presence of BSA did not cause any significant change in the solubility of SMT. The SMT solubility at pH 7.2

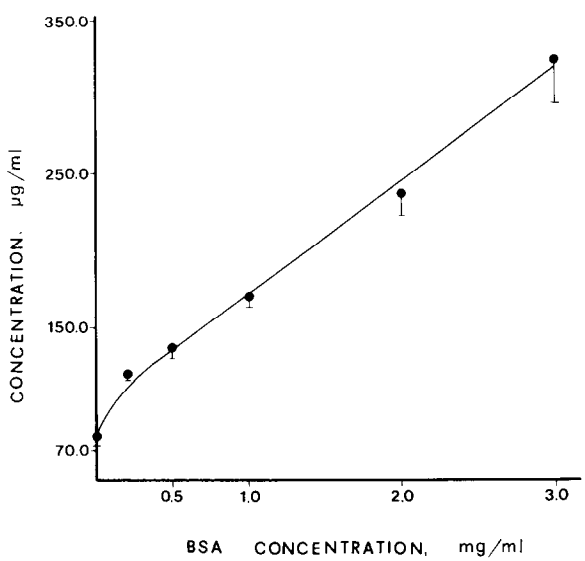

Fig. 1. Effect of BSA on the apparent solubility of BHC at 37 ± 0.1 °C and pH 7.5.

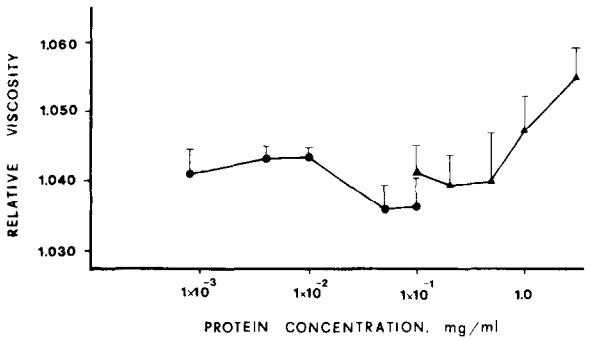

Fig. 2. Relative viscosity vs concentration, for solutions of casein (●) and BSA (▲) in phosphate buffer pH 7.5 (0.066M) at $37 \pm 0.1°$ C. Each experimental point is the mean of 8 measurements. The relative viscosity of buffer solution is 1.035 ± 0.002.

for BSA concentrations of 1–3 mg/ml, was found to be equal to the solubility in absence of protein, 10.36 ± 0.10 (S.D.) mg/ml (Macheras and Reppas, 1986a).

Viscosity

The effect of proteins on the viscosity of the medium is shown in Fig. 2. For casein, it is seen that for concentrations up to 0.01 mg/ml, viscosity remains practically constant while at higher concentrations there is a tendency to lower viscosity values. This is possibly related to the ability of casein to form aggregates under these conditions (Macheras and Reppas 1986a and b). The formation of aggregates would result in a rearrangement of casein molecules and consequently to a reduction of the viscosity (Yamakawa, 1973). BSA appeared to cause a slight increase in viscosity as its concentration increases (Fig. 2). However, the overall changes in the viscosity noted can not be considered adequate to affect the dissolution rate (Wurster and Taylor, 1965).

Dissolution studies

Dissolution data for BHC are shown in Fig. 3. Although not shown, at any one protein concentration 4 replicate runs resulted in a coefficient of variation at each time of $< 31.5\%$. The dissolution data were treated using Weibull's distribution (Langenbucher, 1972) and the results are summarized in Table 1. This table reveals that as protein concentration increases, the values of constants a and b change significantly. These results, in conjunction with the increase of BHC solubility

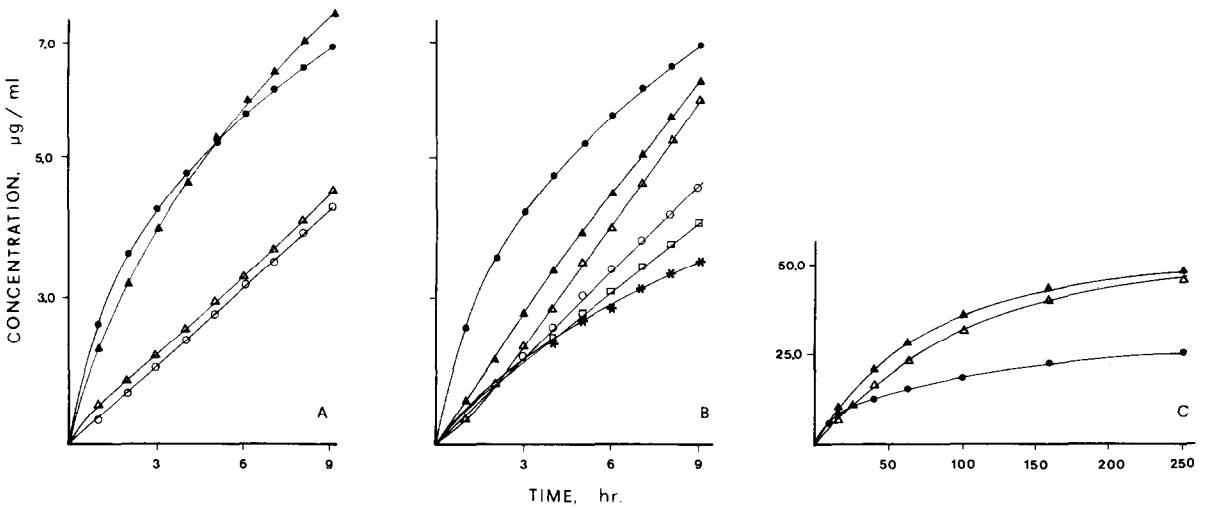

Fig. 3. Dissolution rate data of BHC in presence and absence (●) of proteins at pH 7.5. In cases A and B, the amount of the powder which was brought for dissolution was 30.0 mg. A: effect of casein: (▲), 8×10^{-4} mg/ml; (△), 2×10^{-2} mg/ml; (○), 1×10^{-1} mg/ml. B: effect of BSA: (*), 4×10^{-3} mg/ml; (□), 1×10^{-1} mg/ml; (○), 5×10^{-1} mg/ml; (▲), 1.0 mg/ml; (△), 3.0 mg/ml. C: regenerated dissolution curves based on the corresponding solubility values of BHC in absence and presence of proteins (see Fig. 1 as well as Macheras and Reppas, 1986a): (△), 1×10^{-1} mg/ml casein; (▲), 1.0 mg/ml BSA.

TABLE 1

Results of BHC dissolution after linearization of dissolution rate curves by the Weibull distribution

Protein	Concentration (mg/ml)	T_i (h)	a	b	T_d (h)
–	–	0.43	0.470	18.06	474.7
Casein	8×10^{-4}	0.26	0.650*	24.51*	137.6
	2×10^{-2}	0.00	0.860*	74.85*	150.9
	1×10^{-1}	0.00	1.020*	106.72*	97.4
BSA	4×10^{-3}	0.00	0.690*	67.02*	442.0
	1×10^{-1}	0.00	0.810*	74.93*	206.6
	5×10^{-1}	0.10	0.955*	84.95*	102.2
	1.0	0.05	0.932*	56.35*	75.2
	3.0	0.39	1.109*	82.13	53.2

* Difference is significant at the 0.05 level as determined by the Student's t-test.

in presence of proteins (Fig. 1 and Macheras and Reppas, 1986a), indicate that proteins impede only the initial transport of drug molecules to the bulk solution. To substantiate this argument Fig. 3c has been generated based on the experimentally determined values of constants a and b of Table 1 and assuming that the amount of BHC brought for dissolution was enough to saturate the dissolution medium. Values for the BHC solubility in presence and absence of proteins were obtained either from Fig. 1 or from published data (Macheras and Reppas, 1986a). As seen in Fig. 3c there is a perfect agreement with the corresponding curves of Figs. 3a and b for the first 9 h. Progressively, however, the vast increase of BHC's solubility in presence of proteins causes a dramatic change in the relative position of dissolution curves. A probable explanation for the initial retardation of BHC dissolution in the presence of protein, is the increased interfacial barrier caused by the proteins and/or the much slower diffusivity of drug-protein complexes compared to the diffusivity of free drug molecules (Parrot and Sharma 1967; Prakongpan et al., 1976). The latter explanation, however, can be considered more plausible in view of the extensive binding of BHC to BSA (Figs. 1 and 5) and the experimental fact that casein forms micelles and affects the solubility of BHC (Macheras and Reppas, 1986a).

NTF dissolution data at 37°C and pH 7.2, were expressed in terms of the Hixson–Crowell law (Wurster and Taylor, 1965). Hixson–Crowell cube root plots showed a biphasic pattern. The initial slope gave way to a second lower slope after time intervals ranging from 25 to 32 min in dissolution experiments during 70 min. At any one protein concentration 4 replicate runs resulted in a coefficient of variation at each time point of $<$ 20.7%. Biphasic Hixson–Crowell plots are usually obtained when polydisperse powders are used (Swarbrick and Ma, 1981; Yang and Swarbrick, 1986). Linear regression analysis resulted in equations with correlation coefficients of $0.984 \leqslant r \leqslant 0.999$ ($n = 4$) and $0.990 \leqslant r \leqslant 0.999$ ($n = 5$) for the first and second phase, respectively. Fig. 4 shows the effect of various protein concentrations on the

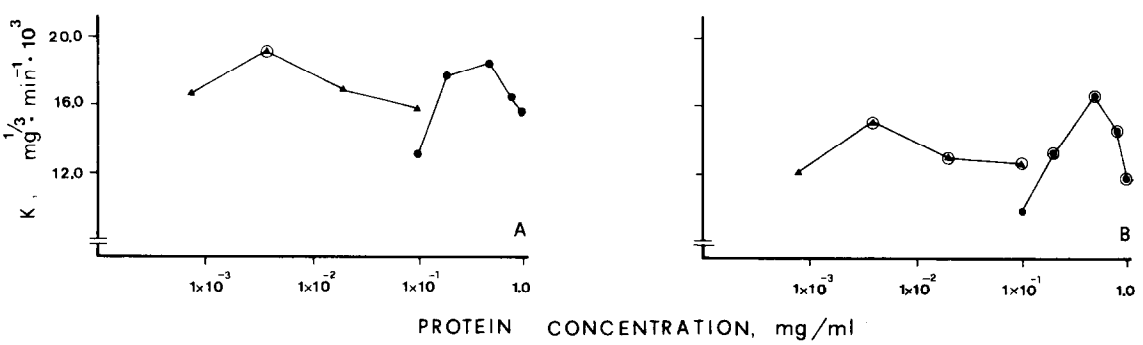

Fig. 4. Apparent dissolution rate constants of NTF K_1 (A) and K_2 (B) as a function of various casein (▲) and BSA (●) concentrations. The corresponding K_1^0 and K_2^0 values, resulting when there was no protein in the dissolution medium, were 0.0142 ± 0.0032 and 0.00885 ± 0.00166 mg$^{1/3}$ · min^{-1}, respectively. Open circles denote that the differences from K_1^0 or K_2^0 are significant at the 0.05 level as determined by Student's t-test. In all cases 50.0 mg of nitrofurantoin powder was used.

apparent dissolution rate constants, K_1 and K_2, of two phases. As it is seen both rate constants are increased with increasing protein concentration. However, maximum values for the rate constants are reached at 0.004 mg/ml casein and 0.8 mg/ml BSA. Further increases in the protein concentration results in decreases of rate constants. Similar results have been obtained by Parrot and Sharma (1967), Short et al. (1972) and Rosen and Macheras (1984), studying the effect of surface active agents and proteins on the dissolution of drugs.

The studies of the effect of proteins in the same range of concentrations of the dissolution of SMT revealed that both proteins did not cause any significant change on the dissolution rate of drug. This is probably due to the unalterable solubility of the drug found in presence of proteins and the high solubility of SMT.

Protein binding

Protein binding results derived from the equilibrium dialysis experiments expressed as percent bound versus total drug concentration are shown in Fig. 5. Graphical analysis of binding data (Klotz and Hunston, 1971) revealed two binding sites for the BSA–BHC interaction ($K_{a,1} = 4.1 \cdot 10^6$ M^{-1}, $n_1 = 3.6$ and $K_{a,2} = 2.3 \cdot 10^4$ M^{-1}, $n_2 = 11.9$) whereas one class of binding sites was found for the BSA-SMT interaction ($K_a = 1.0 \cdot 10^4$ M^{-1}, $n = 4.4$). The estimates of the parameters are in good agreement with those reported in literature (Robertson and Madsen 1974; Rudman et al., 1971; Moriguchi et al., 1968; Hsu et al., 1974).

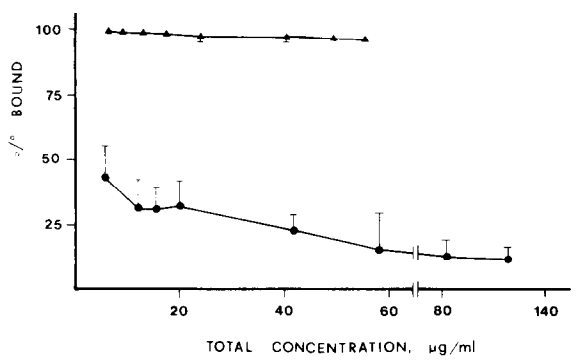

Fig. 5. Percent bound vs total BHC (▲) and SMT (●) concentration.

The differential UV diagrams of the binding of BHC to BSA are shown in Fig. 6. In accordance with the observations of Cho et al. (1971) and Robertson and Madsen (1974), the absorbance of BHC was depressed in the presence of BSA in the region 270–330 nm. To enhance the analytical power of differential UV spectra, 4-cm-length cuvettes were used. It was found that the depression at wavelengths 310–330 nm is evident when the BHC concentration is higher than 4 µg/ml. The same effect has been observed by Perrin and Idsvoog (1971) and Cho et al. (1971). This has been attributed to the different binding of monoionized, diionized, and unionized forms of BHC coexisting at pH 7.4. In other words, the nature of binding sites are basically electrostatic. Since practically all drug is bound with the protein (%bound > 96, Fig. 5), the hypothesis of the expansion of the BSA molecule due to electrostatic character of binding can be considered valid. An important implication of this expansion would apparently be the low diffusivity of drug–protein complex pos-

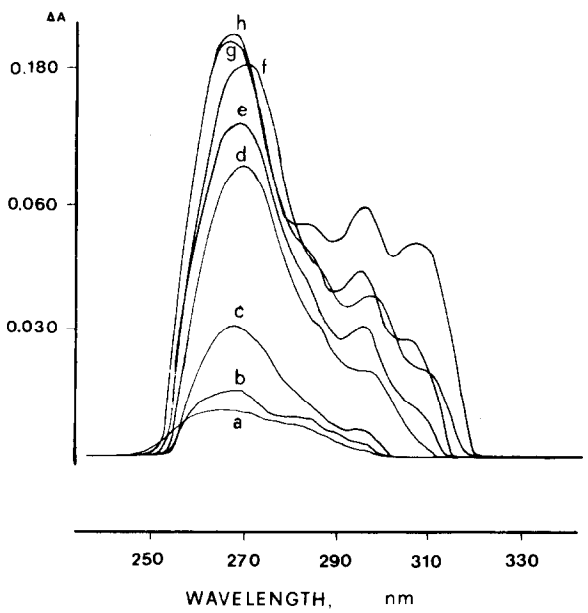

Fig. 6. Differential UV diagrams for the binding of BHC with BSA (1.0 mg/ml). Differential absorbance = absorbance with the drug alone + absorbance with protein alone − absorbance of mixture of drug and protein in 0.066 M phosphate buffer, pH 7.4, in 4-cm cells. a, 0.5; b, 1.0; c, 2.0; d, 4.0; e, 5.0; f, 6.0; g, 8.0; and h, 10.0 µg/ml BHC.

tulated in the dissolution studies with BHC. On the contrary, the binding of NTF and SMT to BSA is too low to affect dissolution in a similar way.

Transport studies

Transport from solutions

Since transport was studied under sink conditions, transport rates were calculated by linear regression analysis of concentration data vs time in the receiver compartment. Correlation coefficients of the regression lines were $0.898 \leqslant r \leqslant 0.999$ (n = 7). At any one protein concentration as well as when no protein was present, two replicate runs resulted in a $C.V. < 12.3\%$.

Transport rates of NTF and SMT were not affected by the presence of proteins and found to be equal to 0.0237 ± 0.0008 and 0.0393 ± 0.0025 $\mu g \cdot ml^{-1} \cdot min^{-1}$ respectively. In contrast the presence of BSA reduced the transport rate of BHC. Nevertheless, this reduction becomes significant only for the higher protein concentration used (Table 2).

A reduction in the rate of transport would be expected as a result of the binding if membrane transport was the rate-limiting step of the whole process. Indeed, this phenomenon was observed only for BHC where the binding is strong (Fig. 5). The percentage of diminution of the transfer rate of BHC in presence of BSA was 83%, a value which correlates well with the percentage bound (98%) of BHC to BSA. Although casein causes a noticeable reduction in the transfer rate of BHC, the influence is not statistically significant. The unaltered transport of NTF and SMT in presence of proteins can be probably attributed to the weak interaction of both drugs with the proteins.

TABLE 2

BHC transport rate in absence and presence of proteins when transport was studied from solutions

Protein	Concentration (mg/ml)	Transport rate ($\mu g \cdot ml^{-1} \cdot min^{-1}$)	Significance
–	–	0.0147 (0.0009)	
BSA	1.0	0.0026 (0.0007)	0.002
Casein	0.1	0.0128 (0.0022)	n.s.

Rate values are means (±S.D.). Significance is determined by Student's t-test; n.s. = not significant at the 0.05 level.

Transport from saturated solutions:

Correlation coefficients of the linear plots of concentration in the receiver compartment vs time varied from 0.986 to 0.999 ($n = 7$). At any one protein concentration as well as when no protein was present, 3 replicate runs resulted in a C.V. < 11.4%.

For SMT, the presence of proteins did not alter the effective permeability coefficient, P. Its value was 0.0016 ± 0.0001 cm $\cdot$ min^{-1} (mean ±S.D. of 9 experiments). Results for BHC and NTF are shown in Fig. 7. As it is seen the qualitative character of the effect of either protein on the permeability coefficient of BHC and NTF is independent from the duration of the interaction between drug and protein prior to the initiation of the experiment. For BHC, it is seen that the presence of BSA causes a dramatic decrease in P. On the contrary, the effective permeability coefficient of BHC in presence of casein was found unaltered (Fig. 7). For NTF, both proteins cause an increase in P values, though at the higher concentrations utilized.

Since only free solute (i.e. non-micellar solute or unbound solute) can cross the membrane, a proposed model for free drug delivery is shown in Fig. 8. The relative importance of the processes in the equilibria has been based on the results of dissolution and transport studies from solutions in presence of proteins and the protein binding studies outlined above or reported previously (Macheras and Reppas, 1986a and b). The results with NTF (Fig. 7), demonstrate that the quantitative character of the effect of BSA on the rate of transfer is almost identical for the two types of experiments. It can be concluded therefore that equilibria in presence of BSA are rapidly established. On the contrary, results with casein are indicative of a slower establishment of equilibrium, Table 3. The table shows the % increase in the effective permeability coefficient in presence of both proteins as compared to the percentage increase in saturation solubility. It can be seen that there is a rough parallel between the percentage increase in the rate of transfer in pres-

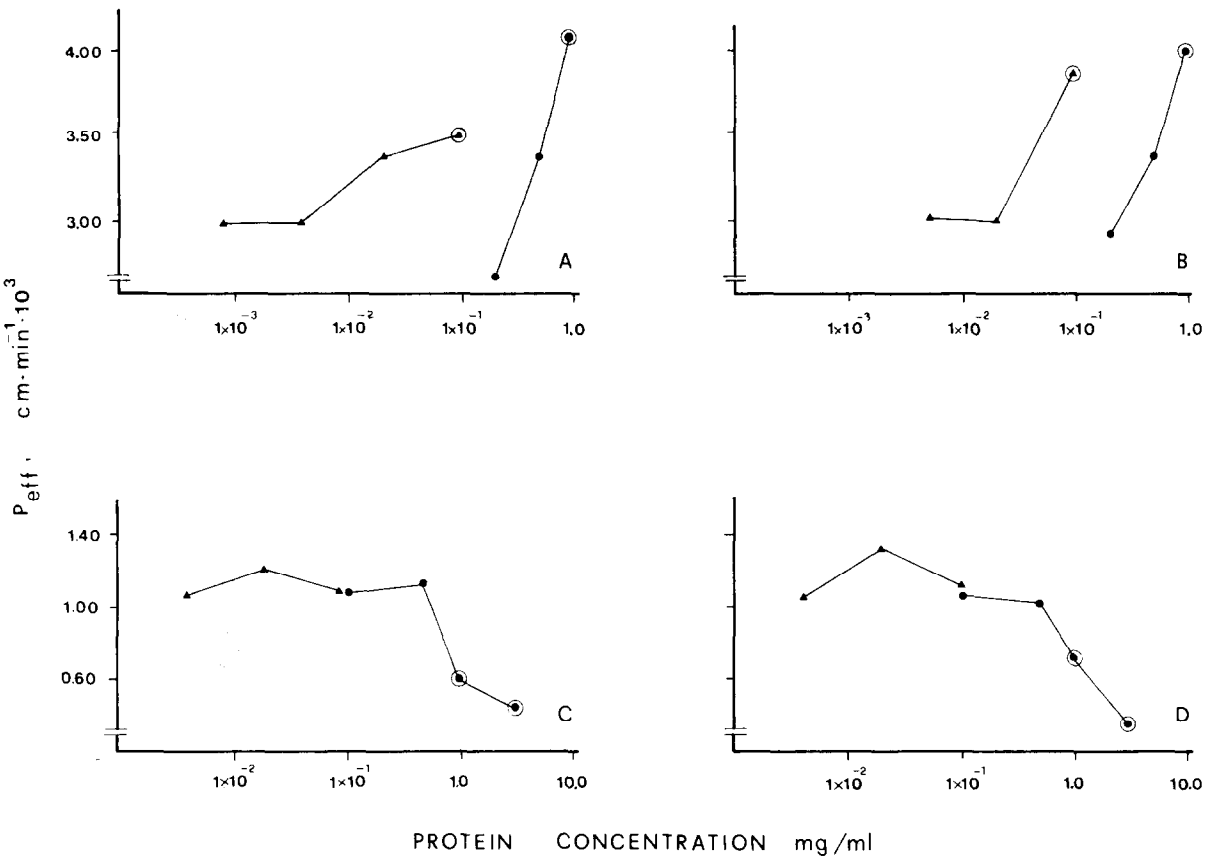

Fig. 7. Effective permeability coefficient of NTF (A and B) and BHC (C and D) as a function of casein (▲) and BSA (●) concentration. A, C: experiments initiated immediately after the addition of protein. B, D: experiments initiated after equilibrating the protein solution overnight. The corresponding P_{BHC} and P_{NTF} values resulted when there was no protein in the donor compartment were 0.001035 ± 0.000073 and 0.00307 ± 0.000204 cm.min^{-1}, respectively. Open circles denote that the differences from P_{BHC} and P_{NTF} values in absence of proteins are significant at the 0.05 level as determined by Student's t-test.

TABLE 3

Percent increase of the solubility and P values of NTF caused by the presence of proteins

Protein	Concentration (mg/ml)	% Increase		
		P_1	P_2	C_s
BSA	1.0	30.6 (12.2)	29.2 (11.7)	17.4 (8.6)
Casein	0.1	14.9 (4.8)	25.1 (6.2)	19.8 (7.5)

Values are means ± S.D. P_1, when the experiment was initiated immediately after the addition of protein; P_2, when the experiment was initiated after equilibrating the protein-drug saturated solution overnight; C_s, saturation solubility (Macheras and Reppas, 1986b).

ence of casein and the increase of saturation solubility caused by casein. Solutions of 1 mg/ml BSA have a more significant effect on the transfer. There is a 1.7-fold higher enhancement of the rate of transfer compared to the one anticipated from the saturation solubility data (Table 3). This can be probably attributed to the ability of BSA-NTF complex to act as an additional reservoir for NTF molecules during the transport process (Fig. 8). The P-values in presence of casein remain practically constant and not dependent upon casein concentration (Fig. 7). This strongly suggests that a mechanism involving solute-containing micelles is operative. It seems likely that the low dissolution rate of BHC in presence of casein during the

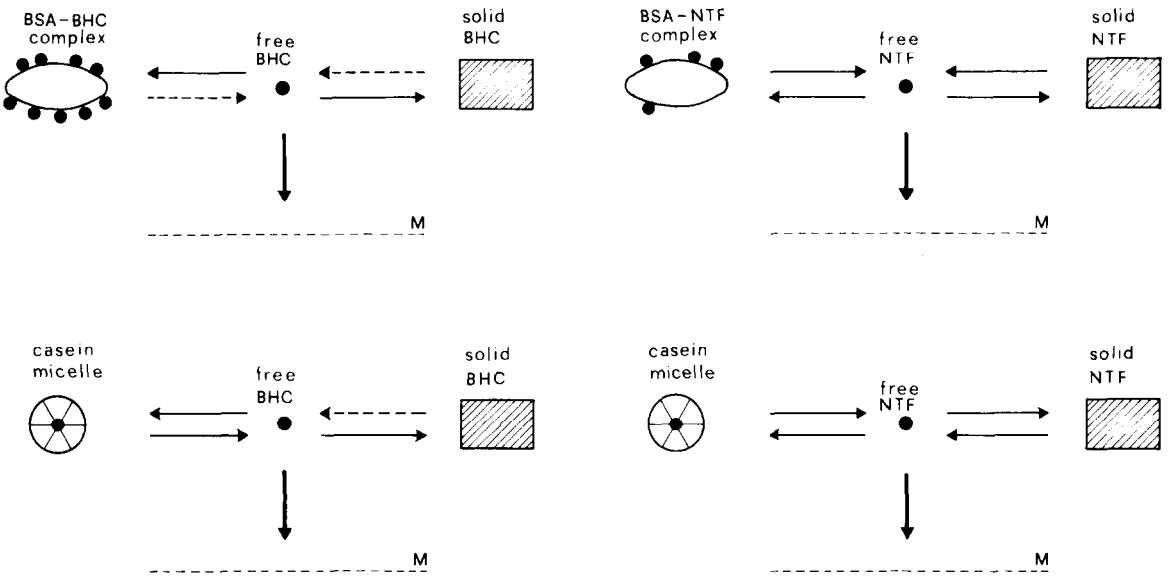

Fig. 8. Proposed model for the transport of solutes from saturated solutions in presence of proteins across the dialysis membrane. Continuous and dashed arrows of the equilibria denote favourable and disfavourable processes respectively. M, membrane.

transport process is balanced by the casein micelles in solution (Fig. 8). The casein micelles contain BHC and therefore provide a replenishment of free BHC molecules as transport proceeds, keeping thus the transfer rate unaltered as compared to that in absence of protein.

The drastic decrease of the rate of transfer of BHC in presence of BSA seems to be concentration-dependent (Fig. 7). This is indicative of a mechanism involving protein binding as the predominant factor for the transport process (Fig. 8). At BSA concentrations of 1 and 3 mg/ml the transfer rate of BHC is reduced by ca. 36% and 61% respectively for both types of experiments (Fig. 7). The low dissolution rate of BHC in the presence of BSA coupled with the depressing effect of BSA on the transfer rate of BHC from solution (Table 2), accounts for the low transfer in the presence of BSA seen in Fig. 7.

The results of the present study give an insight into the mechanisms involved in the dissolution and in vitro transport of BHC, NTF and SMT in presence of BSA and casein. Studies such as this can prospectively be valuable in regard of understanding and elucidating the mechanisms involved in food–drug or food–formulation interactions. The gathering information in this topic would ultimately enable the practising clinician for a pertinent prescription of drugs in relation to mealtimes and type of meal administered.

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