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SOME QUANTITATIVE INVESTIGATIONS OF THE BINDING TO AND THE DISPLACEMENT OF BISHYDROXYCOUMARIN FROM HUMAN SERUM ALBUMIN

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Abstract—The binding of dicumarol to human serum albumin fraction V has been investigated at lower drug-to-albumin ratios than in previous investigations by a circular dichroic technique. Two sites having binding constants of $2{\cdot}9 \times 10^6\ M^{-1}$ and $1{\cdot}9 \times 10^5\ M^{-1}$ were capable of inducing optical activity into the dicumarol. A wide range of acidic drugs was found to compete with dicumarol for these binding sites. The binding constant for chlorphenoxy-2-methylpropionate (from clofibrate) at the primary binding site of dicumarol and of warfarin was calculated from the displacement data.

The binding of bishydroxycoumarin (Dicumarol U.S.P.) to human serum albumin (HSA) has been quantitatively investigated by several authors [1–3]. The extremely low solubility in water necessitated the use of pH 10 for the equilibrium dialysis investigations [3], and two equivalent binding sites having association constants of greater than $10^5\ M^{-1}$ were found. Other workers [1, 2] using physiological conditions for their dialysis work found three equivalent binding sites having association constants of greater than $2 \times 10^5\ M^{-1}$. All of these investigations used graphical analysis to obtain the binding parameters; however, a fourth investigation [4], also using dialysis data, used a computer program to find two sets of binding sites, the first of which contained two sites with a binding constant of $2 \times 10^6\ M^{-1}$. All of the investigations presented little data at drug-to-protein concentrations of less than 1·0 because at such concentrations only a very small fraction of the drug is unbound and is at the limit of the dialysis method and the spectrophotometric assay.

Chignell [1], in supporting investigations for the dialysis work, studied the quenching of the single tryptophan residue in HSA and obtained a binding constant of $5{\cdot}2 \times 10^5\ M^{-1}$ for a single site. This investigation suggests that the three sites reported from dialysis data cannot be equal, a statement supported by the dependence of molar absorptivity on the concentration of bound bishydroxycoumarin reported in Cho *et al.* [2]. Investigations in these laboratories with the tryptophan grouping of albumin chemically modified suggest that the primary binding of dicumarol to HSA is to this tryptophan site [5].

Chignell [1] also used circular dichroism (CD) to investigate the extrinsic Cotton effects generated on the binding of dicumarol to HSA. No quantitative interpretation of the CD data was attempted. Chignell also did not report the strong concentration dependence of ellipticity as has been reported for the binding of the drug to bovine serum albumin [6].

In spite of having pK_a values of 4·4 and 8·0 [2], dicumarol is of limited solubility at pH 7·4, and its hydrophobic nature gives it and some of its analogues very large primary binding constants. The almost complete binding of dicumarol to albumin in the clinical situation has necessitated the use of a third compartment in the pharmacokinetic model for the distribution of dicumarol in man [7].

If another strongly bound drug such as 2(chlorphenoxy)-2-methylpropionate (CPMP) is administered concurrently with a coumarin drug, then significant increases in the concentration of the unbound coumarin drug may result, causing an increased pharmacological activity and necessitating an associated dosage adjustment [8]. CPMP is the rapid metabolite *in vivo* of clofibrate and is presumed to be the active lipid-lowering drug [9].

To aid in predicting such competitions, accurate determinations of the first binding constant are necessary as well as investigations of the competition of other drugs with dicumarol for the binding sites.

In view of the large extrinsic Cotton effects generated by the binding of dicumarol to HSA, it was decided to investigate the concentration dependence in a quantitative manner to obtain numbers of binding sites and binding constants as well as the competition from selected drugs for these sites. The concentrations of free and bound drug can be calculated by the method previously used by Rosen [10] and Kostenbauder *et al.* [11].

METHOD

All solutions were prepared in a 0·054 M phosphate buffer, pH 7·4, adjusted to isotonicity with sodium chloride. HSA concentrations of 0·73 to $4{\cdot}35 \times 10^{-5}$ M (using a mol. wt of 69,000) were used, and a concentration range of $7{\cdot}43 \times 10^{-7}$ M to $7{\cdot}43 \times 10^{-5}$ M dicumarol was used. Measurements were made in 1-, 2- and 5-cm cells using a 6003 attachment to a Cary 60 spectropolarimeter with a slit pro-

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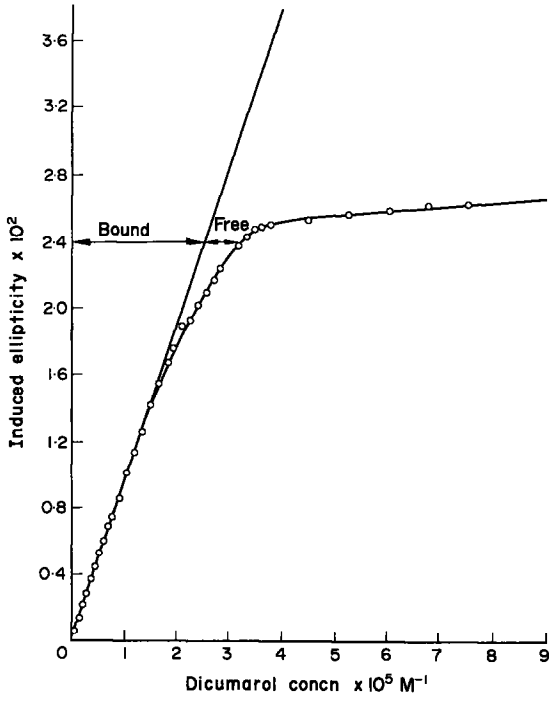

Fig. 1. Estimation of free and bound drug by the method of Rosen [10]. Measurements were made in 20-mm cells and with a constant HSA V concentration of $1{\cdot}45 \times 10^{-5}$ M. Measurements were made at 317 nm.

grammed for a half-band width of 15Å (Cary Instruments, Monrovia, Calif.). The dynode voltage never exceeded 0·35 and the signal-to-noise ratio was never less than 20 to 1 at the wavelength of quantitative measurements. All solutions were scanned from a wavelength at which no optical activity was observed, so that any adjustment for a slight baseline shift could be made.

For the displacement experiments, fixed concentrations of dicumarol and HSA were used, and the concentration of competing drug was varied but the dynode voltage of 0·35 was not exceeded. Concentration and other experimental conditions for these displacements are shown in captions to the figures and tables.

MATERIALS

HSA fraction V (Lot No. K297208) was obtained from Armour, Kankakee, Ill. Bishydroxycoumarin U.S.P. (Dicumarol) was used as supplied by the manufacturer, Abbott Laboratories, North Chicago, Ill., and is at least 98 per cent pure. The drugs for the displacement investigations, sodium warfarin (Endo Lab, Garden City, N.Y.), pyridostigmin bromide and edrophonium chloride (Hoffman–LaRoche, Nutley, N.J.), isopropamide iodide (Smith, Kline & French, Philadelphia, Pa.), decamethonium bromide (Burroughs Wellcome & Co., Greenville, N.C.), tolbutamide (Upjohn, Kalamazoo, Mich.) and chlorpropamide (Pfizer, Groton, Conn.), were used as supplied by the manufacturers. Sodium sulfathiazole, phenobarbital and aspirin were U.S.P. grade. Sodium 2(*p*-chlorophenoxy)-2-methylpropionate was a gift of ICI America, Wilmington, Del., and sodium 2(3-phenoxyphenyl-propionate), a gift of Eli Lilly, Indianapolis, Ind. All other materials were reagent grade and solutions were prepared in de-ionized water.

RESULTS AND DISCUSSION

The induced ellipticity at any wavelength is the observed ellipticity of the drug–albumin mixtures minus the ellipticity of the albumin alone at the same wavelength. The dicumarol itself has no intrinsic optical activity and so the extrinsic Cotton effects can be used to determine the amount of drug bound. Figure 1 shows a plot of induced ellipticity at 317 nm against dicumarol concentration at a fixed albumin concentration of $1{\cdot}45 \times 10^{-5}$ M and cell path length of 20 mm. Drawing the tangent to the curve at zero drug concentration enables the appropriate intensive factor for the bound drug to be determined. This allows the determination of free and bound drug by the method of Rosen [10] and Kostenbauder *et al.* [11].

This method assumes a proportionality between induced ellipticity and amound bound. Such an assumption is only valid when all sites contribute equally to the ellipticity; however, this assumption is not satisfied when more than one class of binding site is observed which contribute unequally to the ellipticity. In such cases, the first binding constant is more accurately determined than the second [10]. These problems are shared with other spectroscopic methods where heterogeneity of binding is involved, and the individual intensive factors cannot readily be determined.

The amount of drug bound/mole of albumin (r) to m classes of independent sites in a non-cooperative process is given by

$$r = \sum_{i=1}^{m} \frac{n_i k_i A}{1 + k_i A} \tag{1}$$

where each class, i, has n_i sites of intrinsic binding constant, k_i, and A is the concentration of unbound drug.

The data are frequently represented in terms of a Scatchard plot [12], and the curvature shown in Fig. 2 for the dicumarol data is indicative of more than one class of binding site. In the simplest case of two classes of binding site, equation (1) modifies to

$$r = \frac{n_1 k_1 A}{1 + k_1 A} + \frac{n_2 k_2 A}{1 + k_2 A} \tag{2}$$

Graphical analysis of data involving multiple binding sites is never unambiguous, and complete separation of the binding parameters is not possible without making numerous assumptions [13].

Figure 2 shows the experimental points on the Scatchard plot, and the extrapolation on the r axis suggests a total of two binding sites. As heterogeneity is seen from the curvature of the plot, this clearly suggests two single binding sites of differing affinity being responsible for the induced CD. The theoretical line in Fig. 2 is drawn using binding parameters calculated by a newly developed computer method [14]. This method gives better estimates of binding constants because the usual assumption that errors in A are negligible compared to the errors in r is not made. The method assumes that the errors in total drug concentration are small compared to the errors in r. The data obtained are summarized in Table 1.

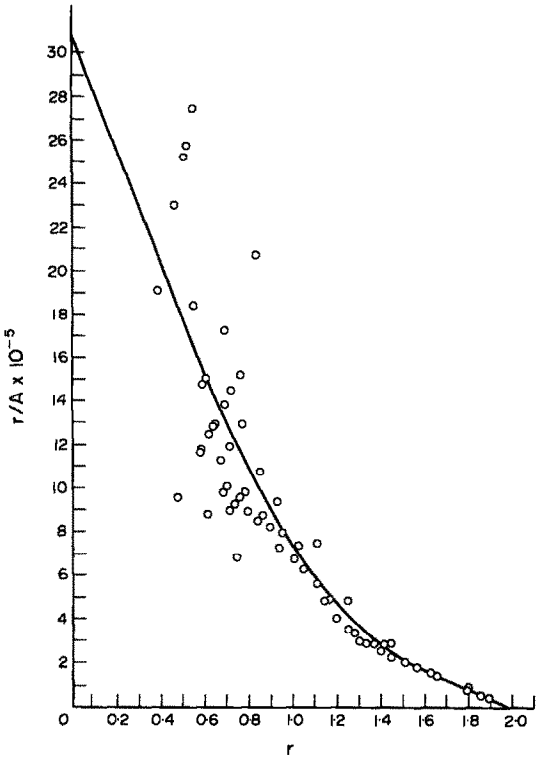

Fig. 2. Scatchard plot of the dicumarol–HSA V interaction. There are 66 data points. The line shows the computer-derived data for two single sites of differing affinity (see Table 1).

For the displacement experiments, three different drug-to-albumin concentrations were used. At the two higher dicumarol concentrations, only drugs showing no measurable induced optical activity on binding to albumin were used as antagonists. Some of these drugs did show some induced activity at the lowest drug-to-albumin ratio used, and appropriate corrections were made. When only one class of site is involved, then the binding constant of the competitor can be calculated by the following equation [15, 16]:

$$k_B = \frac{k_A(A)}{(PA)} \times \frac{[nk_a(A)P_t - k_A(A)(PA) - (PA)]}{B_t k_A(A) - nk_A(A)P_t + k_A(A)(PA) + (PA)} \quad (3)$$

where k_A and k_B are the binding constants for ligands A and B, (PA) is the bound marker drug (dicumarol), (A) the free marker concentration, B_t the total concentration of competitor, P_t the total protein concentration, and n the number of binding sites involved.

Table 1. Binding parameters for dicumarol–albumin complex*

	n_1	$k_1 \times 10^{-6}$	n_2	$k_2 \times 10^{-6}$
1	1·0	2·9 ± 10%	1·0	0·18 ± 5%
2	1·08	2·7	0·97	0·14

* In the first case n_1 and n_2 were fixed at a value of 1, and in the second case were allowed to be variables in the computer program.

The competition data are summarized in Tables 2 and 3.

The ellipticity induced into dicumarol after the binding to albumin is shown at various drug-to-protein ratios in Fig. 3. The lower concentrations show peaks at 296 and 317 nm; at higher concentrations the smaller peak becomes a shoulder and the larger peak is gradually blue shifted. The spectra are in reasonable agreement for the reported u.v. spectra of dicumarol in the presence [1] and absence [1, 2] of HSA. The strong concentration dependence of the shape of the induced CD spectra was also noticed after the binding of dicumarol to BSA [6]. This strong concentration dependence is clearly indicative of heterogeneity in the binding process, more than one site being capable of inducing optical activity into the drug. Unlike the earlier literature, the current investigation measured many data points at drug-to-albumin ratios less than 1 (Fig. 2). The values of $k_1 = 2{\cdot}9 \times 10^6$ and $k_2 = 0{\cdot}18 \times 10^6$ for single sites are very high and among the highest reported for drug–albumin interactions. They are in reasonable agreement with the two equal sites having binding constants of $2{\cdot}0 \times 10^6$ reported by Garten and Wosilait [4] when one considers the severe limitations of their dialysis data at low drug-to-albumin ratios. These values support the observations that in a clinical situation over 99 per cent of the plasma dicumarol is bound to albumin [17] and that erratic therapeutic responses to the drug are found. When drugs are bound so tightly to albumin, they are particularly susceptible to fluctuations in clinical response when concurrently administered with other strongly bound drugs. If a competitor for the primary binding site displaced even 1 per cent of the dicumarol from albumin, then the free, and therapeutically active, concentration would be doubled, assuming a static system, giving the patient an over titration of dicumarol. In practice, other simultaneous biological processes would make the increased free concentration less than this figure.

Unfortunately, the binding constants are close together, and extremely low drug-to-albumin ratios would be necessary to look at the primary binding

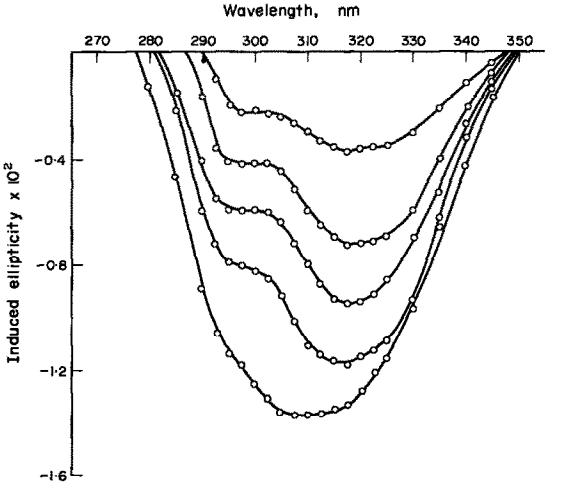

Fig. 3. Induced CD curves for the dicumarol–HSA V interaction. Measurements were made in 10-mm cells using $1{\cdot}45 \times 10^{-5}$ M HSA V. Dicumarol concentrations are: $5{\cdot}96 \times 10^{-5}$ M, $2{\cdot}98 \times 10^{-5}$ M, $2{\cdot}08 \times 10^{-5}$ M, $1{\cdot}49 \times 10^{-5}$ M and $0{\cdot}74 \times 10^{-5}$ M.

Table 2. Displacement data*

Drug	Dicumarol = $2{\cdot}23 \times 10^{-5}$ M†		Dicumarol = $1{\cdot}784 \times 10^{-5}$ M‡	
	Concn (M)	Displacement (%)	Concn (M)	Displacement (%)
Warfarin	$2{\cdot}42 \times 10^{-5}$	5·5	$3{\cdot}89 \times 10^{-5}$	8·3
	$4{\cdot}84 \times 10^{-5}$	9·75	$5{\cdot}83 \times 10^{-5}$	14·3
Tolbutamide	$7{\cdot}42 \times 10^{-4}$	18·7	$3{\cdot}35 \times 10^{-4}$	17·7
			$8{\cdot}38 \times 10^{-4}$	25·7
Chlorpropamide	$7{\cdot}25 \times 10^{-4}$	8·7	$6{\cdot}52 \times 10^{-4}$	14·8
			$1{\cdot}30 \times 10^{-3}$	19·5
Sulfathiazole	$7{\cdot}23 \times 10^{-4}$	2·6	$6{\cdot}30 \times 10^{-4}$	8·6
Sodium trichloroacetate	$2{\cdot}15 \times 10^{-3}$	2·5		
CPMP	$1{\cdot}39 \times 10^{-4}$	3·8	$2{\cdot}22 \times 10^{-4}$	3·6
	$2{\cdot}78 \times 10^{-4}$	8·0	$4{\cdot}45 \times 10^{-4}$	8·5
	$4{\cdot}17 \times 10^{-4}$	11·8	$6{\cdot}67 \times 10^{-4}$	12·7
	$5{\cdot}56 \times 10^{-4}$	13·9	$7{\cdot}78 \times 10^{-4}$	17·4
	$6{\cdot}95 \times 10^{-4}$	17·0		
	$8{\cdot}34 \times 10^{-4}$	21·6		
Phenoxyphenylpropionic acid			$6{\cdot}27 \times 10^{-5}$	6·6
			$9{\cdot}41 \times 10^{-5}$	8·6
Aspirin	$1{\cdot}11 \times 10^{-3}$	None	$1{\cdot}11 \times 10^{-3}$	None
Phenobarbital	$4{\cdot}30 \times 10^{-4}$	None	$4{\cdot}30 \times 10^{-4}$	None
Pyridostigmin bromide	$7{\cdot}67 \times 10^{-4}$	None	$1{\cdot}52 \times 10^{-3}$	None
Edrophonium chloride	$9{\cdot}25 \times 10^{-4}$	None	$1{\cdot}24 \times 10^{-3}$	None
Isopropamide iodide	$4{\cdot}17 \times 10^{-4}$	None	$8{\cdot}36 \times 10^{-4}$	None

* HSA ($1{\cdot}45 \times 10^{-5}$ M) was used in all investigations. The percentage displacement is expressed as the percentage decrease in signal height at 317·5 nm. Measurements were made in 2-cm cells.

† In these initial conditions, approximately 61 per cent of the total dicumarol is bound to the primary site and 30 per cent to the secondary site.

‡ In these initial conditions, approximately 71 per cent of the total dicumarol is bound to the primary site and 24 per cent to the secondary site from equation 2.

alone and at such concentrations the induced ellipticity is very small. Only limited investigations were possible under these conditions; however, at higher drug-to-protein ratios, displacement investigations were possible. The displacements in Table 2 are expressed as a percentage decrease in signal height at 317 nm.

All the drugs shown had negligible extrinsic Cotton effects in the experimental conditions of Table 2. The shape of the curves after competition was consistent with that expected for reduced binding of dicumarol. Basic drugs and the relatively weakly bound acidic drug, phenobarbitone, and aspirin did not displace any dicumarol. Tolbutamide, chlorpropamide, sulfathiazole, sodium salicylate and CPMP displaced some dicumarol, using antagonist concentrations at least 10 times that of the dicumarol. The more tightly bound warfarin and phenoxyphenylpropionic acid give significant displacement of dicumarol at lower concentrations; under different experimental conditions these drugs generate extrinsic Cotton effects on binding to albumin. It is interesting to note that trichloroacetate, a metabolite of chloralhydrate, displaced little of the anticoagulant even at a very high concentration. Although significant quantities of the drug are bound to the second site at the concentrations used in the investigations of Table 1, it is probable that some displacement occurs at both sites. In clinical conditions, it is likely that only the primary site is involved in the binding process, and drugs like CPMP and phenylbutazone [18, 19] have been reported to effectively displace coumarin anticoagulants from albumin. Phenylbutazone has a large primary binding constant [10] of $2{\cdot}37 \times 10^{5}\ M^{-1}$ and unfortunately generates large

Table 3. Displacement data*

Drug	Concn (M)	Displacement (%)	Binding constant
CPMP	$3{\cdot}75 \times 10^{-4}$	8·7	$4{\cdot}57 \times 10^{4}$
	$4{\cdot}38 \times 10^{-4}$	11·0	$5{\cdot}03 \times 10^{4}$
	$1{\cdot}09 \times 10^{-3}$	18·6	$3{\cdot}51 \times 10^{4}$
	$1{\cdot}523 \times 10^{-3}$	20·0	$2{\cdot}69 \times 10^{4}$
Phenoxyphenylpropionic acid†	$4{\cdot}95 \times 10^{-4}$	13·6	
	$8{\cdot}25 \times 10^{-4}$	23·3	
	$1{\cdot}16 \times 10^{-3}$	29·3	

* HSA ($5{\cdot}80 \times 10^{-5}$ M) and dicumarol (3×10^{-6} M) in 5-cm cells were used throughout these investigations. The percentage displacement is expressed as the decreased signal height at 317·5 nm.

† PPPA gave a small induced CD itself under these conditions, making precise quantitation impossible; therefore, no binding constant is shown.

extrinsic Cotton effects on binding to albumin and so cannot readily be investigated by these CD techniques. CPMP does not appear to generate any significant extrinsic Cotton effects down to 310 nm, even when used in the conditions shown in Table 3. A dicumarol-to-albumin ratio of approximately 0·05 was used in these investigations; under these conditions all dicumarol can be assumed to be bound to the primary site. The very high concentration of clofibrate does cause some displacement of dicumarol (Table 3) and the binding constant of $4 \times 10^4\ M^{-1}$ calculated by equation (3) is in good agreement with that of $2{\cdot}5 \times 10^4$ found in the literature for clofibrate and HSA [20]. Phenoxyphenylpropionic acid (PPPA), a new anti-inflammatory drug, displaces the dicumarol; however, a binding constant cannot be determined from the data because PPPA in these concentrations has a small induced optical activity throughout this wavelength region. In other investigations, it has been found to have a binding constant of greater than 10^5 for its primary binding site [5]. Chlorpropamide, sodium salicylate and aspirin all gave small induced curves of their own under the conditions of Table 3, and when mixed with dicumarol the resultant curve appeared to be additive and so no significant displacement was concluded. Warfarin, a monocoumarin, has been reported to undergo a fundamentally different binding than dicumarol [1]; however, in the same report dicumarol was shown to displace warfarin from the albumin. High drug-to-albumin ratios were used, and it is not clear whether or not displacement was from the primary site or not. Although warfarin was earlier reported to show no induced optical activity on binding to HSA, it has been shown [21] to demonstrate the phenomenon at higher albumin concentrations. Figure 4 shows the induced CD curves for warfarin, dicumarol and mixtures of the two after the binding to albumin. The concentrations used are such that only the primary binding sites are involved. Curve 4 is the theoretical curve if the warfarin and dicumarol curves were merely additive. The measured curve is clearly displaced toward the dicumarol curve showing less warfarin to be bound in the mixture, suggesting that the two drugs do share the same binding site. Indirect evidence that the two drugs share the same binding site can be made by comparing the displacement effect of clofibrate on the two drugs. The displacement of warfarin by clofibrate from HSA is shown in Fig. 5. The temperature dependence of the binding of warfarin to a single site on HSA has been investigated by O'Reilly [3], and a value of $1{\cdot}12 \times 10^5$ at 22 can be computed from his data, and this value is supported by the displacement data of Table 2. Using this value in equation (3) allows a binding constant of $3{\cdot}7 \times 10^4$ for the CPMP to be calculated from the data of Fig. 5. This is in excellent agreement with the data from dicumarol

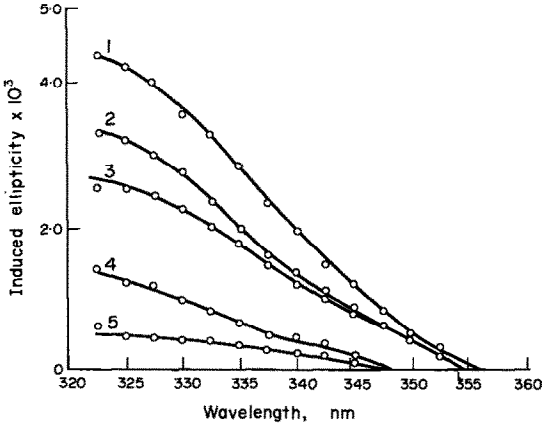

Fig. 5. Induced CD curves for the interaction of warfarin and CPMP with HSA V. Measurements were made in 50-mm cells with a HSA V concentration of $5{\cdot}8 \times 10^{-5}$ M and a warfarin concentration of $2{\cdot}0 \times 10^{-5}$ M. CPMP concentrations are: (curve 1) 0; (curve 2) $1{\cdot}57 \times 10^{-4}$ M; (curve 3) $3{\cdot}13 \times 10^{-4}$ M; (curve 4) $5{\cdot}43 \times 10^{-4}$ M; (curve 5) $1{\cdot}09 \times 10^{-3}$ M.

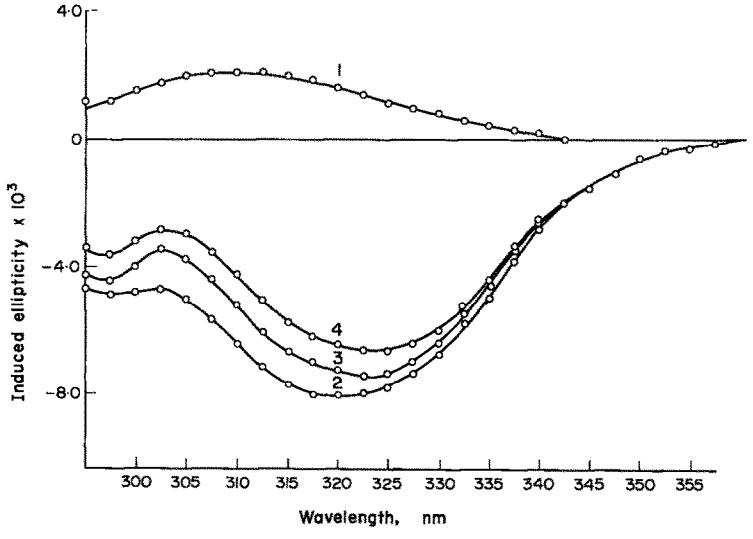

Fig. 4. Induced CD curves for the interaction of warfarin and dicumarol with HSA V. Measurements were made in 50-mm cells with a HSA V concentration of $2{\cdot}9 \times 10^{-5}$ M. (Curve 1) warfarin alone, $1{\cdot}005 \times 10^{-5}$ M; (curve 2) dicumarol alone, $1{\cdot}5 \times 10^{-6}$ M; (curve 3) measured curve for a mixture of 1 and 2; (curve 4) theoretical curve for a mixture of 1 and 2.

displacement. Typical therapeutic doses of dicumarol are 25–200 mg daily, of warfarin 2–10 mg daily, and clofibrate 500 mg 4 times a day. In spite of the significantly lower binding constant than that of the coumarin anticoagulants, the much larger dose of clofibrate makes its metabolite capable of displacing significant amounts of anticoagulant if it is co-administered with the anticoagulants. Whether or not this displacement is responsible for the increased anticoagulant activity of warfarin in the presence of clofibrate will have to await more sophisticated pharmacokinetic investigations than have been conducted up until now. Although it has long been accepted that displacement is the major factor for the potentiation, no previous direct measurements on the primary sites of the anticoagluants have been made, and it has recently been suggested [22] that this effect is due to the more rapid decline of the activity of clotting factors II and X rather than to the displacement phenomenon.

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