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# Drug-binding properties of rat α-foetoprotein

## Specificities of the phenylbutazone-binding and warfarin-binding sites

Françoise HERVÉ,*‡ Krzysztof M. RAJKOWSKI,* Marie-Thérèse MARTIN,* Philippe DESSEN† and Nicole CITTANOVA*

*Unité Associée 586 du Centre National de la Recherche Scientifique, U.E.R. Biomédicale, 45 Rue des Saints-Pères, F-75006 Paris, France, and †Laboratoire de Biochimie, École Polytechnique, 91128 Palaiseau, France

Rat α-foetoprotein (α-FP) strongly binds the drugs warfarin and phenylbutazone, as does albumin; however, the binding sites for the two drugs seemed to be different. This possibility and the specificity of this/these drug-binding site(s) of rat α-FP were investigated by competitive protein-binding experiments with a variety of drugs, representing different pharmacological groups, and biomolecules that are strongly bound by the foetal protein and that are suspected to play a specific role during foetal development. The binding mechanisms were further investigated by using comparisons between computer-derived theoretical displacement curves and experimental points in order to distinguish different possible binding models. The results indicate: (1) that warfarin and phenylbutazone are bound at two distinct sites on rat α-FP and (2) that a negative modulatory effect is exerted between the two sites; (3) that the degree of specificity of these two drug-binding sites is different, since the warfarin-binding site appears to be specific for the binding of coumarinic and anthranilic drugs whereas that for phenylbutazone is able to bind substances of very varied chemical structure and is more hydrophobic; (4) that the phenylbutazone-binding site is the site that binds oestrogens; (5) that thyroid hormones and, probably, fatty acids and bilirubin are bound at (an)other site(s) but exert negative modulatory effects on phenylbutazone binding. The nature of the different binding areas of rat α-FP is compared with that of those already proposed for albumin. The potential risks of toxicity of such interactions between drugs and/or biomolecules on foetal development are also discussed.

## INTRODUCTION

In the foetal rat the serum protein binding of drugs is essentially undertaken by two transport proteins that present very strong structural analogies (Gorin *et al.*, 1981): serum albumin and α-foetoprotein (α-FP). We have shown (Hervé *et al.*, 1984) that, although rat α-FP does not have either the specific albumin drug-binding site II (for indoles and benzodiazepines) or its site III (for digitoxin and bile acids), the foetal protein strongly binds the drugs warfarin and phenylbutazone, as does albumin. However, only an average of 0.4 strong binding site/molecule was found for these drugs, which may mean that only 40% of the heterogenous α-FP populations bind these drugs at the same number of sites (1) as albumin. In these studies (Hervé *et al.*, 1984) the results suggested that, unlike albumin (Fehske *et al.*, 1982), the high-affinity binding sites for warfarin and phenylbutazone are different on α-FP. Here this possibility and the specificity of this/these site(s) have been investigated by competitive binding experiments with a variety of drugs representing different pharmacological groups, and biomolecules that are known to be strongly bound by rat α-FP and that are suspected to play a specific role during foetal development: oestrogens (Nunez *et al.*, 1979), fatty acids (Aussel, 1985), bilirubin (Versée & Barel, 1979) and thyroid hormones (Hervé *et al.*, 1982).

The interpretation of competitive binding results, which is complicated by the fact that many of these compounds are bound by the protein at two classes of sites, of high affinity and of low affinity, was undertaken by using a computer program capable of calculating the expected degree of displacement between two ligands when the binding parameters for both ligands are known. This allowed experimental ligand displacements due to competition for the same binding site(s) to be distinguished from those due to negative modulatory effects.

The potential risks of toxicity and other undesirable effects on foetal development of such interactions between drugs and/or biomolecules are discussed.

The nature of the different binding areas of α-FP is also discussed on the basis of these studies on the foetal protein's binding-site specificities and is compared with the site classification already proposed for albumin (Kragh-Hansen, 1983, 1985).

## EXPERIMENTAL

### Materials

Rat α-FP was prepared as previously described (Cittanova *et al.*, 1974) and has a purity greater than 97%. This protein contains (an) endogenous ligand(s) that is/are extractable by charcoal at neutral pH (Hervé *et al.*, 1981) and that strongly compete(s) with the binding of phenylbutazone and, to a lesser extent, of warfarin

Abbreviations used: α-FP, α-foetoprotein; Dns, 5-dimethylaminonaphthalene-1-sulphonyl.

‡ To whom correspondence and requests for reprints should be sent.

(Hervé *et al.*, 1984). Consequently the α-FP was treated with activated charcoal as previously described before use.

[U-$^{14}$C]Phenylbutazone (19.9 μCi/mg) and [U-$^{14}$C]azapropazone (17.9 μCi/mg) were generously given by CIBA–GEIGY (Basel, Switzerland) and Ziegfried A.G. (Zofingen, Switzerland) respectively. [$^{14}$C]Warfarin (177 μCi/mg) and [2,4,6,7-$^{3}$H]oestradiol (0.33 Ci/mg) came from The Radiochemical Centre (Amersham, Bucks., U.K.). The radiochemical purity of these compounds was higher than 98% as determined by t.l.c. The following drugs were gifts generously supplied by different laboratories: acenocoumarin, oxyphenylbutazone and phenylbutazone from CIBA–GEIGY, azapropazone from Ziegfried A.G., diazepam and sulfafurazol from Roche Laboratory (Neuilly/Seine, France), ibuprofen and flurbiprofen from Boots–Dacour Laboratories (Courbevoie, France), sodium valproate from Sanofi Recherche (Toulouse, France), iodipamide from Guerbet Laboratory (Aulnay/Bois, France), iopanoic acid from Winthrop Laboratories (Longvic, France) and glibenclamide from Hoechst Laboratories (Paris La Défense, France). Warfarin, sulphinpyrazone, sulfadimethoxime, tolbutamide, flufenamic acid, naproxen, furosemide, cloxacillin, Dns-sarcosine, digitoxin, 4-hydroxycoumarin, acetylsalicylic acid, ethacrynic acid, indomethacin, bilirubin, lauric acid, myristic acid, arachidonic acid, L-thyroxine and L-tri-iodothyronine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dns-$NH_2$ was purchased from Fluka A.G. (Buchs, Switzerland), oestrone and oestradiol-17β were from Roussel U.C.L.A.F. (Courbevoie, France) and 17α-ethinyloestradiol was from Steraloid Ltd. (Croydon, Surrey, U.K.). All these compounds were used without further purification.

### Binding experiments

The protein concentrations of charcoal-extracted rat α-FP solutions were checked spectrophotometrically at 280 nm by using an $A_1^{1\%}{}_{cm}$ value of 7.6 and assuming an $M_r$ of 70000 for the foetal protein.

All solutions were prepared in 0.1 M-sodium phosphate buffer, pH 7.4, containing gelatin (50 mg/l), used to avoid adsorption of ligand on the dialysis membrane without significantly binding ligand itself. The same buffer containing 10% (v/v) ethanol or 10% (v/v) dioxan was used for experiments with digitoxin, diazepam, 4-hydroxycoumarin, acetylsalicylic acid, Dns-$NH_2$, myristic acid, ethacrynic acid, glibenclamide, oestrone, oestradiol and 17α-ethinyloestradiol because of their poor solubility in aqueous media; neither 10% ethanol nor 10% dioxan seems to affect α-FP binding (Desfosses *et al.*, 1983). As previously shown (Hervé *et al.*, 1982), inclusion of 0.3 mM-EDTA in the buffer was necessary for the studies with L-thyroxine and L-tri-iodothyronine. When necessary the ligands were dissolved in a small volume of 0.1 M-NaOH and diluted with buffer to yield solutions in the pH range 7.4–7.6.

Competitive binding experiments were as previously described (Desfosses *et al.*, 1983). Charcoal-extracted rat α-FP was 3.54 μM for experiments with labelled warfarin and 15.1 μM for those with labelled phenylbutazone. To study the capacity of drugs or biomolecules to displace warfarin or phenylbutazone, the displacing agent was added to a constant amount of warfarin (0.403 MBq; 2.5 μM) or phenylbutazone (0.364 MBq; 20 μM) per well at [displacer]/[protein] ratios of 1:1, 5:1 and 10:1. When ethanol or dioxan was present in the buffer the displacer concentrations did not exceed 75.4 μM in the studies with labelled phenylbutazone. In order to avoid interference of these solvents in the displacement results the binding of labelled warfarin and phenylbutazone by α-FP in the presence of ethanol or dioxan was measured and served as a reference.

### Calculations

All results are given as percentage displacements of the respective marker by displacer. In cases where the parameters for the binding of both marker and displacer by rat α-FP were known the experimental results were compared with the theoretically expected percentage displacements, determined from the binding parameters, for the various competitive binding models by using the appropriate forms of the general competitive binding equation (Feldman, 1972):

$$[A_T] = \sum_{j=0}^{p} \frac{n_j k_{A_j}[P][A_F]}{1+k_{A_j}[A_F]} + \sum_{i=0}^{p} \frac{n_i k_{A_i}[P][A_F]}{1+k_{A_i}[A_F]+k_{B_i}[B_F]} + [A_F]$$

$$[B_T] = \sum_{k=0}^{r} \frac{n_k k_{B_k}[P][B_F]}{1+k_{B_k}[B_F]} + \sum_{i=0}^{q} \frac{n_i k_{B_i}[P][B_F]}{1+k_{A_i}[A_F]+k_{B_i}[B_F]} + [B_F]$$

where [P] is the total protein concentration, $[A_T]$, $[A_F]$, $[B_T]$ and $[B_F]$ are the total and free (unbound) concentrations of ligands A and B respectively, $n_j$, $n_k$ and $n_i$ are the numbers of equivalent sites in each class and $k_{A_j}$, $k_{B_k}$, $k_{A_i}$ and $k_{B_i}$ are their apparent intrinsic association constants for A and B respectively.

A doubly iterative computer search procedure (Nelder & Mead, 1965) was used to calculate the bound concentrations of both marker and displacer ligands from their total concentrations and the binding parameters. These binding parameters were only known within a given error range, and the computer program developed was able to calculate the ensuing error range associated with the theoretical displacement curves. This computer search procedure was preferred to that described by Feldman (1972) because the latter involves a Scatchard transformation and such transformations magnify errors (Weber & Anderson, 1965). Also, this search, though slow, always converges and avoids problems of ill-conditioning.

## RESULTS

We have previously suggested (Hervé *et al.*, 1984) that the high-affinity binding of warfarin and phenylbutazone by rat α-FP may occur, unlike that to albumin (Fehske *et al.*, 1982), at different sites. Competitive binding experiments were carried out to investigate this possibility further and to study the specificity of this/these site(s). The displacement of labelled warfarin and phenylbutazone bound by rat α-FP by a variety of drugs and

**Table 1. Displacement of labelled warfarin and phenylbutazone from rat α-FP by other drugs and biological ligands**

For experimental details see the text. Standard deviations were in the range 2–5%.

| | Displacement (%) | | | | | |
|---|---|---|---|---|---|---|
| | Warfarin-binding site | | | Phenylbutazone-binding site | | |
| Displacer [Competitor]/[rat α-FP] . . . | 1:1 | 5:1 | 10:1 | 1:1 | 5:1 | 10:1 |
| Anticoagulant coumarinic drugs | | | | | | |
| Warfarin | 41 | 73 | 85 | 22 | 32 | 38 |
| Acenocoumarin | 50 | 76 | 87 | 31 | 40 | 56 |
| 4-Hydroxycoumarin | 14 | 20 | 32 | 8 | 23 | ND* |
| Pyrazolic anti-inflammatory drugs | | | | | | |
| Phenylbutazone | 5 | 7 | 12 | 23 | 59 | 76 |
| Oxyphenylbutazone | 6 | 14 | 17 | 20 | 52 | 63 |
| Azapropazone | 2 | 15 | 20 | 17 | 45 | 60 |
| Pyrazolic uricosuric drugs | | | | | | |
| Sulphinpyrazone | 8 | 18 | 23 | 18 | 27 | 44 |
| Anthranilic anti-inflammatory drugs | | | | | | |
| Mefenamic acid | 29 | 43 | 58 | 37 | 55 | 69 |
| Flufenamic acid | 34 | 69 | 81 | 28 | 58 | 72 |
| Propionic anti-inflammatory drugs | | | | | | |
| Flurbiprofen | 4 | 9 | 8 | 39 | 61 | 76 |
| Naproxen | 2 | 6 | 11 | 50 | 70 | 85 |
| Ibuprofen | 1 | 3 | 5 | 12 | 22 | 23 |
| Indolic anti-inflammatory drugs | | | | | | |
| Indomethacin | 5 | 18 | 27 | 23 | 50 | N.D.* |
| Hypoglycaemiant sulphonamides | | | | | | |
| Tolbutamide | 10 | 31 | 40 | 21 | 27 | 35 |
| Glibenclamide | 0 | 8 | 13 | 22 | 41 | N.D.* |
| Bacteriostatic sulphonamides | | | | | | |
| Sulfafurazol | 6 | 7 | 8 | 7 | 8 | 8 |
| Sulfadimethoxime | 7 | 7 | 11 | 10 | 20 | 31 |
| Analgesics | | | | | | |
| Acetylsalicylic acid | 18 | 24 | 34 | 5 | 13 | N.D.* |
| Biliary contrast agents | | | | | | |
| Iodipamide | 0 | 6 | 8 | 19 | 19 | 21 |
| Iopanoic acid | 9 | 28 | 48 | 53 | 81 | 87 |
| Diuretics | | | | | | |
| Furosemide | 2 | 9 | 17 | 144† | 149† | 154† |
| Ethacrynic acid | 0 | 6 | 12 | 19 | 41 | N.D.* |
| Penicillins | | | | | | |
| Cloxacillin | 4 | 7 | 11 | 13 | 25 | 30 |
| Antiepileptic drugs | | | | | | |
| Sodium valproate | 2 | 5 | 5 | 0 | 0 | 0 |
| Benzodiazepines | | | | | | |
| Diazepam | 0 | 0 | 0 | 0 | 0 | N.D.* |
| Cardiotonic drugs | | | | | | |
| Digitoxin | 0 | 0 | 0 | 0 | 0 | N.D.* |
| Dyes | | | | | | |
| Dns-$NH_2$ | 9 | 18 | 33 | 3 | 12 | N.D.* |
| Dns-sarcosine | 7 | 11 | 15 | 20 | 37 | 47 |
| Oestrogenic hormones | | | | | | |
| Oestrone | 9 | 10 | 13 | 56 | 93 | N.D.* |
| Oestradiol | 6 | 8 | 10 | 59 | 93 | N.D.* |
| 17α-Ethinyloestradiol | 18 | 20 | 20 | 52 | 96 | N.D.* |
| Fatty acids | | | | | | |
| Lauric acid | 8 | 9 | 13 | 26 | 55 | 65 |
| Myristic acid | 8 | 18 | N.D.* | 65 | 66 | N.D.* |
| Arachidonic acid | 6 | 17 | 30 | 50 | 85 | N.D.* |
| Biliary pigments | | | | | | |
| Bilirubin | 6 | 15 | 13 | 18 | 32 | 34 |
| Thyroid hormones | | | | | | |
| L-Thyroxine | 0 | 10 | 25 | 32 | 58 | N.D.* |
| L-Tri-iodothyronine | 0 | 20 | 22 | 33 | 34 | N.D.* |

* Not determined because of poor ligand solubility.
† Percentage increase in the bound fraction.

**Table 2. Binding parameters used in the theoretical calculations of the expected displacements of one ligand by another from sites on charcoal-pretreated rat α-FP**

$n_1$, $k_1$, $n_2$ and $k_2$ are the numbers of equivalent sites and their apparent intrinsic association constants for the high-affinity and low-affinity classes of binding sites respectively.

| Ligand | $n_1$ | $10^6 \times k_1$ ($M^{-1}$) | $n_2$ | $10^4 \times k_2$ ($M^{-1}$) | Reference |
|---|---|---|---|---|---|
| Phenylbutazone | 0.41±0.03 | 1.19±0.19 | 1.5±0.5 | 2.3±0.2 | Hervé *et al.* (1984) |
| Azapropazone | 0.44±0.07 | 0.86±0.14 | 1.3±0.2 | 1.0±0.4 | Hervé *et al.* (1984) |
| Warfarin | 0.47±0.02 | 1.81±0.10 | 2.5±0.5 | 0.52±0.04 | Hervé *et al.* (1984) |
| Oestradiol-17β | 0.44±0.06 | 49.2±5.2 | 1.7±0.7 | 56.3±29.5 | Hervé *et al.* (1986) |
| L-Thyroxine | 1.65±0.08 | 0.82±0.009 | — | — | Hervé *et al.* (1982) |

biomolecules is shown in Table 1. The ligands can be grouped into five categories on the basis of these results. Four of these categories are: (*a*) ligands that strongly displace phenylbutazone but not warfarin (pyrazolic, propionic and indolic anti-inflammatory drugs, glibenclamide, iopanoic acid, ethacrynic acid, cloxacillin, Dns-sarcosine, oestrogens, fatty acids and thyroid hormones); (*b*) ligands that poorly displace phenylbutazone and/or warfarin, probably from the protein's secondary low-affinity sites (bacteriostatic sulphonamides, acetylsalicyclic acid, iodipamide, Dns-$NH_2$ and bilirubin); (*c*) ligands that displace neither phenylbutazone nor warfarin (sodium valproate, digitoxin and diazepam), used here as negative references; and (*d*) ligands that appear to exert a positive modulatory effect on phenylbutazone binding (furosemide).

These results are consistent with the hypothesis that warfarin and phenylbutazone are not bound at the same high-affinity site on rat α-FP. One may note that, so far, no ligands have been found that strongly displace warfarin but not phenylbutazone. However, it will be seen from the Discussion section below that the fifth category of ligands, those that displace both warfarin and phenylbutazone (coumarinic and anthranilic drugs, tolbutamide), may indeed be these specific markers of the warfarin-binding site.

Although these competitive binding results give some information and possibly permit the prediction of the effects of drug binding in the foetal state, they do not permit the determination of which molecular mechanisms are acting. For some ligands having chemical structures analogous to that of the displaced drugs (e.g. sulphinpyrazone, oxyphenylbutazone and azapropazone with respect to phenylbutazone, and acenocoumarin and 4-hydroxycoumarin with respect to warfarin) it is reasonable to propose that the measured displacements correspond to competition for the high-affinity and low-affinity sites of the respective marker. Also, a positive modulatory effect of furosemide on phenylbutazone's high-affinity site can be proposed.

Interpretation at the molecular level of the displacements found with drugs strongly or weakly displacing phenylbutazone and/or warfarin, while having different structures, is more difficult: competition for high-affinity and/or low-affinity sites, as well as negative modulatory effects on binding, may be proposed. The strong displacement of phenylbutazone by warfarin compared with the weak displacement of warfarin by phenylbutazone, despite their comparable affinities, is a case in point. To interpret such results a more refined theoretical tool is required, and one such tool is available when the individual binding parameters for both marker and displacer have been determined precisely and by a method identical with that used in the competition experiments. The previously determined binding parameters for such ligands (oestradiol, warfarin, phenylbutazone, azapropazone, L-thyroxine) are shown in Table 2. For these ligands our computer program allows the calculation of the expected displacement of one ligand by another from a protein with any number of different classes of binding sites if displacement is due to competition for binding at one or more of these site classes. A series of plausible competitive binding models is proposed (Table 3), and the expected displacements, over the experimentally used ranges of ligand concentrations, have been calculated for each model. These

**Table 3. Competitive binding models used here for the theoretical calculations of the expected displacement of a ligand L by a competitor C from a protein with $n_{L_1}$ high-affinity sites ($S_{L_1}$) and $n_{L_2}$ low-affinity sites ($S_{L_2}$) for the ligand and $n_{C_1}$ high-affinity sites ($S_{C_1}$) and $n_{C_2}$ low-affinity sites ($S_{C_2}$) for the competitor**

| Model | Site distribution | |
|---|---|---|
| I | Sites $S_{L_1} \equiv$ Sites $S_{C_2}$ (competition) | Sites $S_{L_2} \equiv$ Sites $S_{C_2}$ (competition) |
| II | Sites $S_{L_1} \equiv$ Sites $S_{C_1}$ (competition) | Sites $S_{L_2} \not\equiv$ Sites $S_{C_2}$ (no competition) |
| III | Sites $S_{L_1} \not\equiv$ Sites $S_{C_1}$ (no competition) | Sites $S_{L_2} \equiv$ Sites $S_{C_1}$ (competition) |
| IV | Sites $S_{L_1} \equiv$ Sites $S_{C_1}$ (competition) | Sites $S_{L_2} \equiv n_{L_2}$ Sites $S_{C_2}$ (competition)<br>$(n_{C_2}-n_{L_2})$ Sites $S_{C_2}$ (no competition) |
| V | Sites $S_{L_1}$ (no competition) | $n_{C_1}$ Sites $S_{L_2} \equiv$ Sites $S_{C_1}$ (competition)<br>$(n_{L_2}-n_{C_1})$ Sites $S_{L_2}$<br>Sites $S_{C_2}$ (no competition) |
| VI | Sites $S_{L_1}$ (no competition) | Sites $S_{L_2} \equiv$ Sites $S_{C_1}$ + Sites $S_{C_2}$ (competition) |

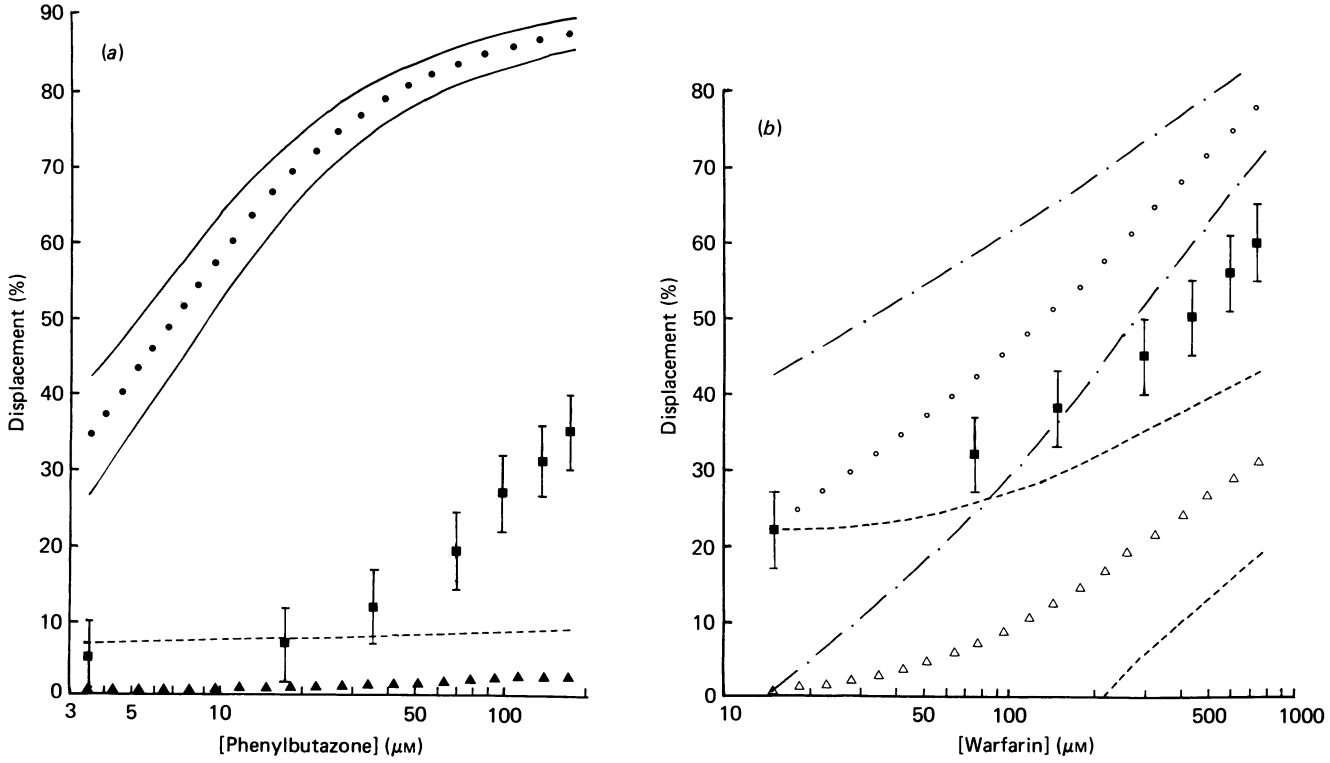


**Fig. 1. Comparison of experimental and theoretical displacements of warfarin bound to rat α-FP by phenylbutazone and of phenylbutazone by warfarin**

(*a*) Displacement of [$^{14}$C]warfarin (2.5 μM) from charcoal-pretreated α-FP (3.54 μM) by increasing concentrations of phenylbutazone (3.54–177 μM); (*b*) displacement of [$^{14}$C]phenylbutazone (20 μM) from charcoal-pretreated α-FP (15.1 μM) by increasing concentrations of warfarin (15.1–755 μM). The medium was 0.1 M-sodium phosphate buffer, pH 7.4, containing gelatin (50 mg/l). Experimental points (■) are the means (with standard deviations) for three triplicate displacement experiments. Theoretical displacement curves (with their standard deviations) are as follows. In (*a*): ●●●, model I with high-affinity sites in common for the two drugs ($n_1 = 0.4$); ▲▲▲, model VI with low-affinity sites for marker that are both the high-affinity and low-affinity sites for the displacer ($n_2 = 0.4 + 1.5$). In (*b*); ○○○, model I with high-affinity and low-affinity sites in common for the two drugs ($n_1 = 0.4$; $n_2 = 1.5$); △△△, model III with only low-affinity sites in common for the two drugs ($n_2 = 1.5$).

theoretical displacements have been compared with the experimental results to see which, if any, model conforms to the experimental results.

### Competition between warfarin and phenylbutazone

If there were competition for binding between warfarin and phenylbutazone according to a given model then the experimental results for displacement of one ligand by another should coincide with both the theoretical curves (displacement of warfarin by phenylbutazone and vice versa) for this model. Fig. 1 shows representative theoretical displacement curves for the competitive binding of warfarin and phenylbutazone, but the curves were calculated for all the models. Comparison of these curves with the experimental results shows that, although the displacements of labelled warfarin by phenylbutazone are closest to the theoretical curves corresponding to models III and IV (Table 3), the displacements of labelled phenylbutazone by warfarin are closer to the curves of models I and II. Azapropazone, which has the same pyrazolic structure and which occupies the same high-affinity and low-affinity sites as phenylbutazone (curves not shown), gave similar results with respect to its displacement of and by warfarin (curves not shown). Thus the experimental results did not coincide with the same theoretical models for the displacements in the two directions. This lack of reciprocity indicates that none of the models represents the actual situation, and the high-affinity sites for the two ligands are not the same and any competition for low-affinity sites is insufficient to explain the measured displacements. It may therefore be proposed that warfarin and phenylbutazone occupy different high-affinity sites on the rat α-FP molecule but that a negative modulatory effect is acting. There are two possibilities for this negative-co-operativity mechanism: either the effect may act between the high-affinity sites for the two drugs or it may be exercised on the warfarin and phenylbutazone high-affinity sites by a low-affinity site that is common for both warfarin and phenylbutazone, with warfarin having a higher affinity for this site than phenylbutazone. Such a negative-co-operativity mechanism should be reciprocal from a thermodynamic point of view (Weber, 1972). Therefore the more important displacement of phenylbutazone by warfarin than vice versa indicates that the effect is acting between the high-affinity sites, since the low-affinity sites have a greater affinity for phenylbutazone than for warfarin, and if they were involved in the co-operativity mechanism one would expect the opposite effect. Nevertheless the calculation of co-

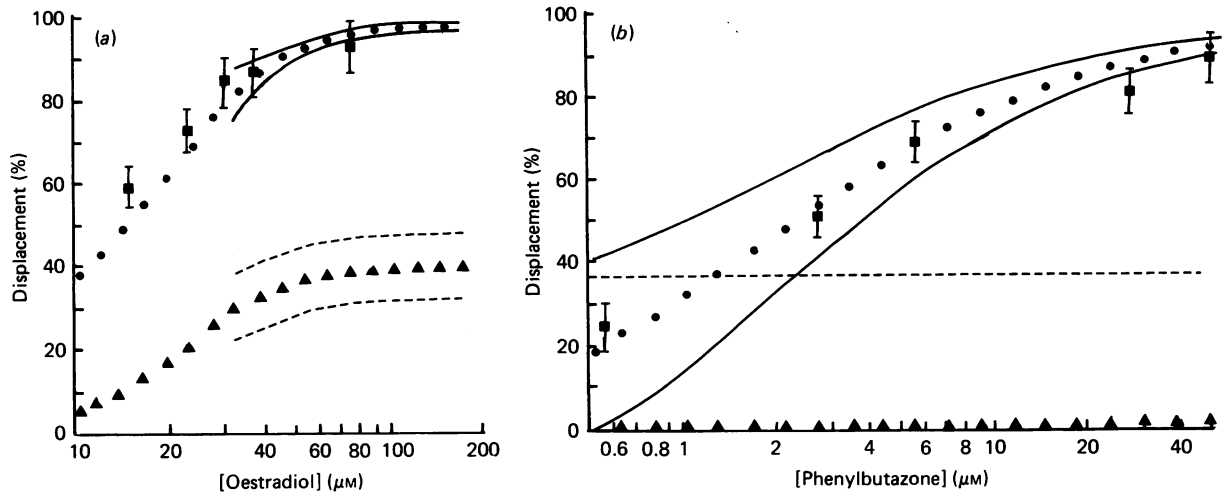


**Fig. 2. Comparison of experimental and theoretical displacements of phenylbutazone bound to rat α-FP by oestradiol and of oestradiol by phenylbutazone**

(*a*) Displacement of [$^{14}$C]phenylbutazone (20 μM) from charcoal-pretreated α-FP (15.1 mM) by increasing concentrations of oestradiol (15.1–75.5 μM); (*b*) displacement of [$^{3}$H]oestradiol (0.01 μM) from charcoal-pretreated α-FP (0.055 μM) by increasing concentrations of phenylbutazone (0.55–55 μM). The medium was 0.1 M-sodium phosphate buffer, pH 7.4, containing 10% (v/v) dioxan. Experimental points (■) are the means (with standard deviations) for three triplicate displacement experiments. Theoretical displacement curves (with their standard deviations) are as follows. In (*a*) and (*b*): ●●●, model I with high-affinity and low-affinity sites in common for the two ligands ($n_1 = 0.425$, $n_2 = 1.5$). In (*a*) and (*b*): ▲▲▲, model III with only low-affinity sites in common for the two ligands ($n_2 = 1.5$).

operativity constants, and thus the affirmation of the existence or absence of reciprocity, requires a knowledge of the free and bound concentrations of both ligands simultaneously, and this can only be found with sufficient precision if ligands are used that are both labelled but with different radioisotopes, and, to date, only $^{14}$C-labelled drugs are commercially available.

### Competition of oestrogens for binding at the phenylbutazone-binding and warfarin-binding sites

Rat α-FP strongly binds oestrogens at two classes of sites, similarly to warfarin and phenylbutazone (Hervé *et al.*, 1986). Here we found that oestradiol strongly displaced phenylbutazone but not warfarin from the foetal protein. By using the binding parameters for oestradiol shown in Table 2 it was possible to calculate the theoretical displacement curves for the various possible models of competition between phenylbutazone and oestradiol, which was chosen as the representative oestrogen because of its greater solubility than oestrone. Comparison of these curves with the experimental diplacements (Fig. 2) shows that oestradiol competes for both the high-affinity and low-affinity sites for phenylbutazone and vice versa. Therefore rat α-FP's binding sites for oestrogens appear to be the same as those for phenylbutazone.

### Competition of thyroid hormones for binding at the warfarin-binding and phenylbutazone-binding sites

Thyroid hormones are fairly strongly bound by rat α-FP and strongly displace phenylbutazone, warfarin only being displaced to a lesser extent. None of the theoretical curves for displacement of labelled phenylbutazone by thyroxine, calculated by using the binding parameters for thyroxine (Table 2), coincided with the experimental results (Fig. 3). The same was found with warfarin (curves not shown). Therefore none of the 1.65 high-affinity sites/molecule binding the thyroid hormones coincides with those binding phenylbutazone and warfarin, indicating that a negative modulatory effect is acting.

### Competition of fatty acids and bilirubin for binding at the phenylbutazone-binding and warfarin-binding sites

It has been shown that fatty acids, particularly the long-chain polyunsaturated fatty acid arachidonic acid (Aussel, 1985) and short-chain saturated fatty acids (Aussel & Masseyeff, 1983), and bilirubin (Aussel, 1985) are strongly bound by rat α-FP. Labelled warfarin was only slightly displaced by these ligands. However, the competitive binding experiments have shown that arachidonic acid, lauric acid and myristic acid were strong displacers of phenylbutazone. Bilirubin displaced phenylbutazone to a much lesser extent. Comparison of the experimental displacements of labelled phenylbutazone by arachidonic acid and bilirubin with theoretical curves, constructed by using the binding parameters given by Aussel (1985) for these biomolecules, indicated that arachidonic acid and bilirubin, though displacers, are not competitors for the phenylbutazone-binding site, and negative modulatory effects may be acting. These results seem to contradict the proposal (Savu *et al.*, 1981; Aussel, 1985) that oestrogens, which we show here to be bound at the same high-affinity site as phenylbutazone, and fatty acids are bound at the same high-affinity sites on rat α-FP. However, this requires confirmation by using binding parameters for arachidonic acid and bilirubin obtained by exactly the same equilibrium micro dialysis method as used for the competitive binding experiments.

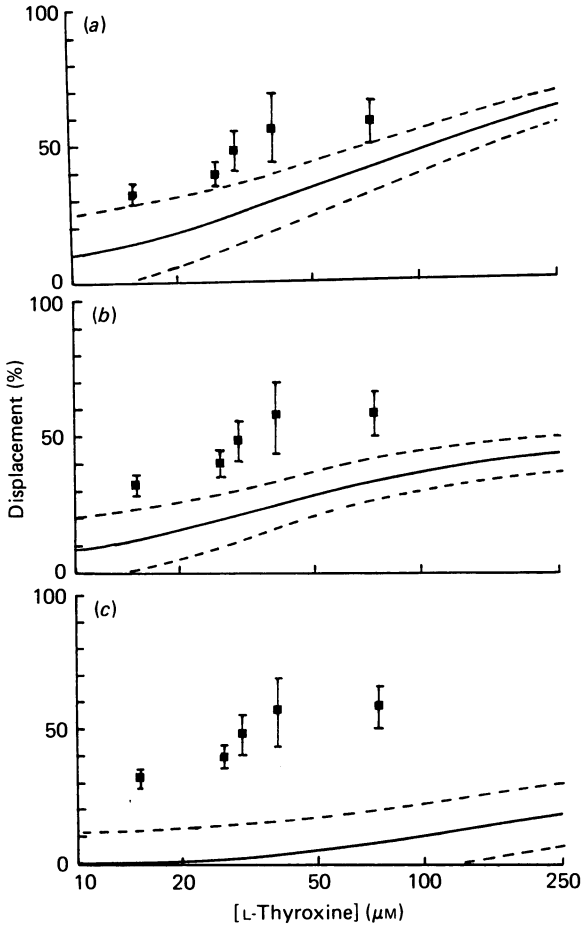


**Fig. 3. Comparison of experimental and theoretical displacements by L-thyroxine of phenylbutazone bound to rat α-FP**

[$^{14}$C]Phenylbutazone (20 μM) was displaced from charcoal-pretreated rat α-FP (15.1 μM) by increasing concentrations of L-thyroxine (!5.1–75.5 μM). The medium was 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.3 mM-EDTA. Experimental points (■) are the means (with standard deviations) for three triplicate displacement experiments. Theoretical displacement curves (with their standard deviations) are as follows: (*a*) the sites for L-thyroxine are the sum of the 0.4 high-affinity and 1.5 low-affinity sites for phenylbutazone; (*b*) 0.4 of the 1.65 sites for L-thyroxine are the high-affinity sites for phenylbutazone, and the remainder are elsewhere on the protein; (*c*) the sites for L-thyroxine are also the low-affinity sites for phenylbutazone.

## DISCUSSION

It has been found that rat α-FP binds warfarin and phenylbutazone at different sites with a negative co-operativity effect either acting between them or via a common secondary low-affinity site. Moreover the degrees of specificity of the warfarin-binding and phenylbutazone-binding sites are very different. Rat α-FP's site for phenylbutazone seems to be composed of a large hydrophobic pocket that binds substances of very varied chemical structure: pyrazolic drugs and oestrogens. In a previous paper (Desfosses *et al.*, 1983) we proposed that the oestrogen-binding site of rat α-FP could be composed of two subsites: a subsite 'A', which binds the aromatic ring of oestrogens, and a subsite 'S', which is more specific for the B–D rings of the oestrogen molecule (C-6, C-16 and C-17). The fact that oestradiol and phenylbutazone compete for the same high-affinity site fits with this hypothesis, since the aromatic rings of phenylbutazone could be bound at subsite 'A' and thus compete for binding with oestradiol. At the same time the much greater affinity of this region for oestradiol than for phenylbutazone would reflect the fact that, whereas the latter is only bound at one subsite in the region (with one binding energy), the former is bound at two (and therefore two binding energies are involved). This hypothesis also fits with the findings that human α-FP binds phenylbutazone (Hirano *et al.*, 1985) and the synthetic oestrogen diethylstilboestrol (Hsia *et al.*, 1986) but does not bind natural oestrogens with high affinity (Nunez *et al.*, 1979), indicating that subsite 'A' is retained in the two proteins, whereas human α-FP has lost subsite 'S'.

Phenylbutazone is also strongly displaced by other drugs (naproxen, iopanoic acid, ethacrynic acid, flurbiprofen, indomethacin), by the dye Dns-sarcosine and by biomolecules such as thyroid hormones, fatty acids and bilirubin. However, thyroid hormones, and probably fatty acids and bilirubin, seem to be bound at (an)other binding region(s) on the rat α-FP molecule but exert negative modulatory effects on the binding of phenylbutazone. The drug furosemide, with a positive modulatory effect on phenylbutazone binding, would also be bound elsewhere on rat α-FP.

Inversely, the warfarin-binding site of rat α-FP seems to be much more specific and less hydrophobic, since it only binds coumarinic drugs and, probably, anthranilic acids, since the latter not only displace warfarin but also displace phenylbutazone in a similar manner to warfarin.

Rat α-FP is often considered as the foetal counterpart to albumin, the two proteins binding biomolecules and drugs similarly. However, the degrees of specificity of their binding sites appear to be different. Unlike rat α-FP, albumin is reported to bind warfarin and phenylbutazone at the same high-affinity site, called site I (Sjöholm *et al.*, 1979; Fehske *et al.*, 1982). However, the existence of a negative co-operativity effect between warfarin-binding and phenylbutazone-binding sites of albumin has been proposed by Madsen and co-workers (Madsen & Tearne, 1980; Madsen & Ellis, 1981). It seems to be probably necessary to use theoretical displacement curves of the type used by Kragh-Hansen (1983, 1985) or the type used here to discriminate between these two possibilities. Nevertheless the degree of specificity of albumin's site I is very different from that found here for rat α-FP: sulfafurazol and sulfadimethoxime and the dye Dns-$NH_2$, which are markers for albumin's site I, bind poorly to rat α-FP's sites for warfarin and phenylbutazone; furosemide, which increases the affinity of rat α-FP for phenylbutazone, decreases warfarin and phenylbutazone binding to albumin. The drugs naproxen, iopanoic acid, ethacrynic acid, flurbiprofen, indomethacin and the dye Dns-sarcosine do not displace either warfarin or phenylbutazone from albumin, since they are markers of albumin's site II for indoles and benzodiazepines, but here they have been found to displace phenylbutazone strongly from rat α-FP. Since albumin's site II is absent from rat α-FP (Hervé *et al.*, 1984), these substances may be bound at the phenylbutazone-binding site. It would be of interest to study the binding of these substances by

human α-FP, since Hirano *et al.* (1985) found that the human foetal protein binds tryptophan and thus retains albumin's site II. Nevertheless their study of tryptophan binding by the quenching of human α-FP's own tryptophan fluorescence appears open to error.

Despite these differences it seems that rat α-FP, like albumin (Kragh-Hansen, 1983, 1985), has a number of definite specific binding regions that can bind exogenous and endogenous ligands and that different binding regions can exert modulatory effects on binding by other regions.

When considering the displacements of endogenous ligands from α-FP by drugs *in vivo* both competitive binding and negative modulatory effects must be considered. Also, the affinities of rat α-FP for phenylbutazone and oestradiol, for example, are such that pharmacological doses of phenylbutazone administered to the mother, alone, would not lead to any appreciable displacement of oestradiol from the protein. Oestradiol is also in competition with endogenous ligands such as oestradiol metabolites and, perhaps, others (Hervé *et al.*, 1981). Thus the effects of phenylbutazone on oestradiol binding would be magnified. This must also be taken in account when considering the endocrinological and pharmacological effects on the foetus of drugs administered to the mother.

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