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SPECIES DIFFERENCES IN THE ALBUMIN BINDING OF 2,4,6-TRINITROBENZALDEHYDE, CHLOROPHENOXYACETIC ACIDS, 2-(4′-HYDROXYBENZENEAZO)BENZOIC ACID AND SOME OTHER ACIDIC DRUGS—THE UNIQUE BEHAVIOR OF RAT PLASMA ALBUMIN

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Abstract—Binding of chlorophenoxyacetic acids and other drugs to albumins from different species was studied by equilibrium dialysis and by two optical probes, 2,4,6-trinitrobenzaldehyde (TNBal) and 2-(4′-hydroxybenzeneazo)benzoic acid (HBABA). Rat plasma albumin behaves abnormally, compared with plasma albumin fraction V preparations from ten other species, in its interaction with HBABA in the presence of certain drugs (thyroxine, indolacetates and chlorophenoxy acids). HBABA may be a useful tool for determining the binding of anionic drugs to various albumins (other than pig and horse albumins). No relationship was discovered between albumin binding and hypocholesterolemic activity *in vivo* in a series of phenoxyacetic acids related to clofibrate (Atromid-S).

CLOFIBRATE[1,2] (Atromid-S, ethyl α-4-chlorophenoxy-α-methylpropionate) is a drug which lowers serum cholesterol in man and other mammals and inhibits hepatic cholesterol synthesis *in vitro*.[3] The two isomeric desmethyl analogues of clofibrate (ethyl D- and L-α-4-chlorophenoxypropionates) differ in hypocholesterolemic activity; only the L-isomer is significantly active in rats.[4] Unpublished experiments in this laboratory have shown that both the D- and L-esters are readily hydrolyzed by rat liver, serum and intestinal preparations, so that the difference in the activity *in vivo* of the two esters cannot be explained merely by a difference in apparent half-life. We therefore examined the albumin binding of the two (optically) isomeric acids and found some distinct differences in their binding to rat albumin, but not to human albumin. These findings seemed rather significant in the light of current theories about the mode of actions of atromid (summarized in ref. 4) invoking displacement of endogenous L-thyroxine, which is surmised to be the active hypolipidemic agent, from its preferential binding site on rat albumin.[5] (In other species, thyroxine is preferentially bound to other plasma proteins.)

As optical probes for studying drug binding to various albumins, we utilized 2,4,6-trinitrobenzaldehyde[6] and 2-(4′-hydroxybenzeneazo)benzoic acid,[7,8] which will be referred to hereafter in this paper as TNBal and HBABA respectively.

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MATERIALS AND METHODS

TNBal (Aldrich Chemical Company, Milwaukee, Wis.) was recrystallized from benzene, m.p. 119–120°. Purified HBABA, m.p. 205–206°, was purchased from Sigma Chemical Company, St. Louis, M. All albumin preparations were obtained from Pentex Inc., Kankakee, Ill.; fraction V preparations were described as >95 per cent pure by electrophoresis at pH 7·0. A sample of rat albumin (fr. V) was also obtained from Mann Laboratories, New York, N.Y. Clofibrate, the derived acid, and the D- and L-α-(4-chlorophenoxy)-propionic acids and their ethyl esters were synthesized from *p*-chlorophenol.[4] Other drugs were the purest grades available (at least 95 per cent pure).

The chlorophenoxyacetic acids (CPA's) were dissolved directly in 0·1 M sodium phosphate, pH 7·4. Thyroxines were dissolved in dimethylsulfoxide (DMSO) to give 10 mM solutions. Other drugs were dissolved in *N,N*-dimethylformamide (DMF) to give 50 mM solutions. These DMSO and DMF solutions were then diluted with phosphate buffer. Drug-free controls contained the same amount of DMSO or DMF (not exceeding 2%, v/v). Solutions were prepared immediately before each experiment and the compounds were shown to be stable under the experimental conditions by spectral analysis.

In man, the serum concentration of α-(4-chlorophenoxy)-α-methylpropionic acid (CPMPA) after administration of 2 g (therapeutic dose) of the corresponding ethyl ester was 200 μg/ml (0·93 mM).[1,9] In rats, the L-desmethyl analog exhibited the same hypocholesterolemic activity as the ethyl ester of CPMPA, but blood level concentrations of the CPA's were not determined. For these albumin binding studies, drug concentrations of 0·5 and 1·0 mM were utilized, since this seemed to be the most appropriate concentration range based on the known concentration of CPMPA in serum after administration of a hypolipidemic dose of the drug. Other compounds were studied in order to compare their binding properties with the CPA's and to probe into the structural requirements for inhibition or stimulation of the HBABA–rat serum protein reaction. Therefore, the same concentrations were employed for all drugs and, except for the CPA's, the concentration range is not related to the therapeutic blood level.

Equilibrium dialysis of CPA's with albumin preparation V powders or human crystalline material was carried out as follows: 4 ml of a mixture containing 6·7 mg protein per ml (approximately 0·1 mM), 1 mM appropriate CPA and 25 mM sodium phosphate, pH 7·4, was dialyzed against 4 ml of 25 mM sodium phosphate for at least 36 hr at 4°. Chlorophenoxyacetic acids and 4-chlorophenol were determined in the appropriate dialysates by measuring the optical density at 280 mμ. α-Phenoxy-α-methylpropionic acid was similarly determined at 270 mμ. As controls, each CPA (1 mM) was dialyzed without albumin and each albumin (approx. 0·1 mM) and phosphate buffer alone were dialyzed without added drugs. Results were calculated as the mean of four experiments with each albumin and each CPA. (The variance between experiments is indicated in Table 1.)

The chromogenic reactions of TNBal with albumins, in the presence and absence of added drugs (1 mM), were studied using conditions described previously, namely 0·1 mM albumin and 0·1 mM TNBal, all in 0·1 M sodium phosphate, pH 7·4, at room temperature.[6]

The chromogenic reactions of HBABA with albumins, in the presence and absence

of added drugs, were studied by adding appropriate solutions of HBABA and drugs to a solution of albumin at room temperature to afford final concentrations of HBABA (0·05 mM), albumin (0·05 mM) and drug (0·50 mM) all in 0·1 M sodium phosphate, pH 7·4. The absorbance was read at 475 mμ.

RESULTS

TNBal reaction. Rat plasma albumin gave rather a poor coloration with TNBal compared with some other readily available albumins. The color formed was very yellow compared with the strong red coloration given by bovine and guinea pig albumins. On mixing the TNBal with rat albumin, the maximum coloration was attained instantly (giving a sharp peak at 410 mμ, a plateau over the range 530–545 mμ and minimum at 475 mμ) and declined steadily thereafter with increasing time. The absorbance was notably inhibited by 1 mM nicotinic acid, a hypocholesterolemic drug in rats and man,[10] which had little effect on the absorbance when TNBal was mixed with bovine albumin (Table 1).

TABLE 1. BINDING OF CHLOROPHENOXYACETIC ACIDS AND OTHER DRUGS TO RAT (R), HUMAN (H), BOVINE (B) AND GUINEA PIG (GP) SERUM ALBUMINS*

Compound	μmoles bound/μmole protein†				% Inhibition of TNBal binding to 0·1 mM albumin‡				% Inhibition of HBABA binding to 0·05 mM albumin§			
	R	H	B	GP	R	H	B	GP	R	H	B	GP
4-Chlorophenoxyacetic acid	4·0	3·2	2·8	2·4	20	20	30	25	−50‖	50	45	40
L-α-(4-Chlorophenoxy)-propionic acid	2·0	1·2	2·6	3·4	10	20	5	40	−55‖	55	25	20
D-α-(4-Chlorophenoxy)-propionic acid	3·6	1·4	3·4	3·0	20	30	25	10	−125‖	50	45	45
α-(4-Chlorophenoxy)-α-methylpropionic acid	3·6	3·8	3·8	3·0	25	30	30	35	−120‖	50	50	60
α-Phenoxy-α-methyl-propionic acid	2·6	1·0	2·8	N.D.	20	30	15	15	−50‖	45	50	35
Nicotinic acid	← N. D. →				10	10	5	50	25	25	50	75
Phenylbutazone	← N. D. →				35	40	40	70	50	80	80	80
Indomethacin	← N. D. →				50	40	75	N. D.	−150‖	30	60	N.D.
p-Chlorophenol	3·2	1·8	2·2	N.D.	15	10	5	15	−5‖	40	35	40

* Drug binding as determined by equilibrium dialysis, by displacement of 2,4,6-trinitrobenzaldehyde (TNBal) and by the effect of the binding of 2-(4′-hydroxybenzeneazo)benzoic acid (HBABA).

† From equilibrium dialysis experiments (see Experimental) using fr. V preparations of plasma proteins or crystalline human serum albumin. N. D., not determined.

‡ With 1·0 mM drug; data accurate to ±0·5 μmole drug bound.

§ With 0·50 mM drug; data rounded to ±3%.

‖ Negative inhibition, i.e. increase in optical density.

HBABA reaction. At neutral pH, HBABA has an absorption maximum at 350 mμ, but when added to solutions of albumin gives a characteristic yellow color with a maximum at 475–480 mμ. This wavelength of maximum absorption does not shift for plasma albumins derived from different species.[8] The absorbance, measured at 475 mμ, of the complex formed on mixing 50 μm HBABA and 50 μm albumin varied considerably with different plasma albumins. Assigning a value of 1·0 to that given by bovine

albumin (fr. V), the relative absorbance with other albumins (fr. V) were: guinea pig, 1·35; mouse, 1·10; rat, 0·80; human, 0·75; dog, 0·70; sheep, 0·60; rabbit, 0·40; pig, 0·30; horse, 0·10. Baxter[8] also showed a difference in relative absorption of the HBABA protein interaction for a series of plasma albumins. A similar relative order of absorbance was observed for those proteins which were studied in common. Bovine α-lactalbumin and hen egg albumin gave no coloration with HBABA. The coloration with bovine and human albumins was reduced by preheating the albumins for 10 min at 60° or by adding 6 M urea.

Many acidic drugs inhibited the albumin–HBABA reaction, indicated by a quenching of the absorbance at 475 mμ (Table 1). Those drugs which strongly inhibited color production were generally those which strongly inhibited the TNBal–albumin reaction and were also known from equilibrium dialysis experiments to bind strongly to albumins. Reduction in absorbance by a drug, therefore, affords some indication that the drug itself binds to albumin and, or adjacent to, the HBABA-binding sites.

Rat albumin preparations again exhibited anomalous behaviour. All drugs so far examined by us invariably decreased the absorbance at 475 mμ which arises from mixing albumins (obtained from Pentex) of other species with HBABA. By contrast, certain acidic drugs actually increased the absorbance at 475 mμ on mixing HBABA with Pentex rat albumin preparations and simultaneously decreased the absorbance at 350 mμ (due to unbound HBABA). The same hyperchromic effect at 475 mμ was observed with the sample of rat albumin obtained from Mann laboratories. Chlorophenoxyacetic acids, D- and L-thyroxines and indoleacetic acids or their derivatives (e.g. indomethacin) demonstrated this property (Table 1 and Fig. 1). Phenylacetic acid and other acidic drugs, e.g. phenylbutazone, nicotinic acid, and fatty acids quenched the color formation, just as they do when added to mixtures of HBABA and albumins from other species. A preparation of mouse albumin resembled rat albumin in that mixtures of HBABA and CPMPA gave a greater absorbance than the HBABA–mouse albumin combination alone. However, the hyperchromic effect of 0·5 mM CPMPA with mouse albumin (0·05 mM) was only one-tenth that of 0·5 mM CPMPA with rat albumin (0·05 mM). At higher concentrations of drug (Fig. 1), indomethacin and to a lesser extent CPMPA begins to inhibit the hyperchromic effect (i.e. there is a tendency for the inhibition to become positive). We are currently studying this phenomenon in greater detail and hope to be able to offer an explanation for the anomalous hyperchromic effect with rat plasma albumin in a future communication.

Clofibrate itself (ethyl ester of CPMPA) added to various albumins was held in solution but did not affect the HBABA–albumin absorbance, indicating that competition with HBABA for its binding sites was by anions. The order of adding CPMPA and HBABA to the albumins did not show a difference in absorbance. The displacement of HBABA by CPMPA can therefore be effected even on preloading the albumins with HBABA.

Differences in binding of D- *and* L-α-(4-*chlorophenoxy*)-*propionic acids.* Table 1 shows that these isomers had different binding affinities to various albumins as indicated by (1) equilibrium dialysis studies and (2) the intensities of the colorations formed on mixing these isomers with either TNBal or HBABA and several different albumins. These isomers also differed in their hyperchromic activity (i.e. increasing absorbance) in promoting the HBABA–rat albumin interaction. The D-desmethyl

isomer, in general, behaved like CPMPA and the L-desmethyl isomer behaved like 4-chlorophenoxyacetic acid, the former two binding more strongly than the latter two. Only in the TNBal reaction with guinea pig albumin did the L-isomer exhibit significantly greater inhibition of the reaction than did the D-isomer. However, the optical isomers were bound approximately equally by human albumin preparation (fr. V and crystalline) in contradistinction to rat, guinea pig and bovine albumins.

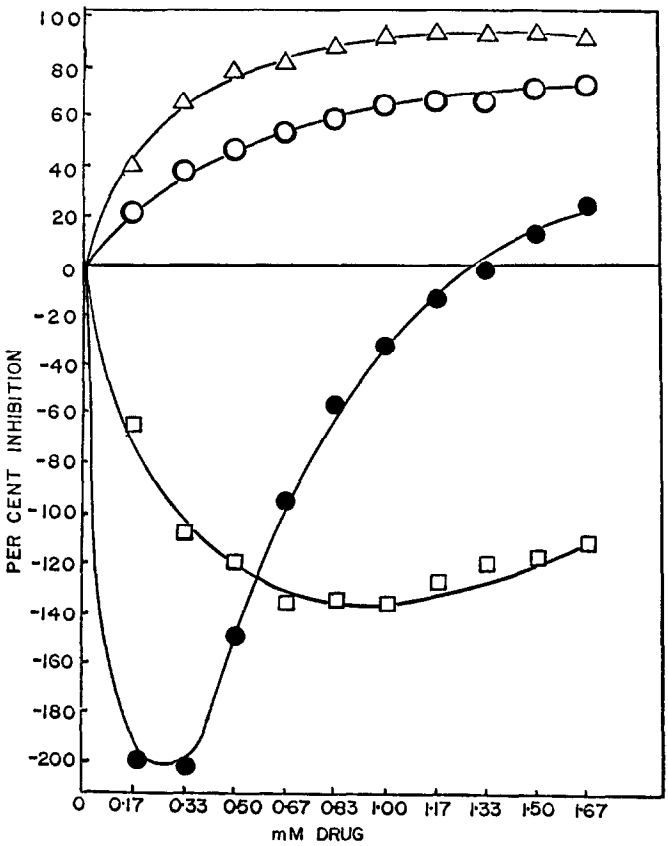

FIG. 1. Effect of drugs on the HBABA (0·05 mM)–serum albumin (0·05 mM) reaction.
○, α-(4-chlorophenoxy)-α-methylpropionic acid with bovine or human albumin; □, α-(4-chlorophenoxy)-α-methylpropionic acid with rat albumin; △, indomethacin with bovine albumin; ●, indomethacin with rat albumin.

D-Thyroxine gives a considerably greater hyperchromic effect than L-thyroxine when added to HBABA–rat albumin mixtures. At a 0·16 mM concentration, D-thyroxine causes a 70 per cent increase in absorbance at 475 mμ, while the hyperchromic effect with L-thyroxine is only 35 per cent.

DISCUSSION

It is fairly common to test drugs *in vivo* in rats and then examine their protein-binding characteristics with bovine fractions before clinically testing their efficacy in man. Our present findings indicate two pitfalls for the biochemical pharmacologist

studying drug action in rats and other small laboratory animals. The rat may be a singularly unrepresentative species as far as drug-binding to its albumin is concerned. This is well known with respect to thyroxine binding. Our experiments have shown that the binding of thyroxine, indoleacetates and phenoxyacetates seems to occur at sites which do not overlap the HBABA-binding site, whereas there is considerable overlapping of these two sites on the albumin molecules from other species. Even when it is clearly recognized that drug binding to rat albumin may be "out of line" with drug binding to bovine albumin, which is extensively used for drug-binding studies, there remains the fact that neither rat nor bovine albumin may be a valid guide to the behavior of human plasma albumin in binding different drug species.

This point is clearly indicated in trying to predict the relative activities of the D- and L-desmethyl CPMPA's and their ethyl esters as hypocholesterolemic agents in man. The L-ester is the active isomer *in vitro* in rats. The D-isomer is not significantly effective,[4] but as these studies have shown, it is the D-acid which binds the more readily to rat albumin. Similarly, the deschloro compound, α-phenoxy-α-methyl-propionic acid, also an effective hypocholesterolemic agent in rats, showed no characteristic binding pattern and was generally bound less than CPMPA. It might be argued therefore that drug binding to albumin represents a site of loss, the other isomer being more readily available to regulate lipid metabolism in the various tissues in the rat. It should be noted that this observed disparity in the binding of the two isomers does not support the hypothesis that clofibrate acts to lower cholesterol levels by displacing thyroxine from its binding site(s) on rat albumin.[5] Osorio *et al.*[11] also found no evidence that clofibrate resembles salicylate and dinitrophenol, two drugs which have been shown to displace thyroxine from its binding sites on plasma proteins in rats. These arguments have little significance when it is realized that human albumin fails to discriminate between the two isomeric acids and does not normally bind L-thyroxine to any great extent. The pharmacologist seeking new drugs for human use should perhaps pay more attention to Alexander Pope's directive (*An Essay on Man*) that "the proper study of mankind, is man" and at least study drug binding to human albumins before predicting the value of a new drug screened in any lower species.

Sturman and Smith[12] have drawn attention to the great variability in plasma protein binding of the salicylate anion over a wide range of animal species. Our more restricted studies indicate that this is probably true also for nicotinic acid, phenylbutazone and indomethacin.

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Addendum—Since we completed this work, a report has appeared from Japan by Moriguchi *et al.*[13] which confirms the usefulness of HBABA for determining the albumin binding of acidic drugs. Drugs studied with bovine serum albumin were substituted benzoic acids[14] and sulfonamides.[15]

Note added in proof—We have recently found that the hyperchromic effect is not observed with rat whole serum; HBABA and CPMPA.

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