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The effect of anti-inflammatory drugs on the protein-binding of 11-hydroxysteroids in human plasma *in vitro*

J. B. STENLAKE*, W. D. WILLIAMS*, A. G. DAVIDSON*, W. DOWNIE† AND K. WHALEY†

** Drug Metabolism Unit, Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, University of Strathclyde, Glasgow and † The Centre for Rheumatic Diseases, Royal Infirmary, Glasgow, Scotland*

The effect of acetylsalicylic acid, ibufenac, indomethacin, oxyphenbutazone and phenylbutazone on the protein-binding of 11-hydroxysteroids *in vitro* at concentrations in excess of normal has been examined in human blood bank plasma, in plasma from women in the third trimester of pregnancy, and in plasma from patients after injections of tetracosactide. Phenylbutazone, ibufenac and indomethacin do not significantly affect the protein-binding of 11-hydroxysteroids. Oxyphenbutazone causes a decrease in the proportion of unbound 11-hydroxysteroids and acetylsalicylic acid produces an increase of unbound 11-hydroxysteroids.

We have already shown that acetylsalicylic acid, phenylbutazone, and indomethacin in therapeutic doses do not significantly affect the binding of 11-hydroxysteroids to plasma proteins in humans with rheumatoid arthritis (Stenlake, Davidson & others, 1968). The total concentration of 11-hydroxysteroids in all these patients was well within the normal physiological range (6·5—26·3 μg/100 ml, Mattingly, 1962), and in consequence the levels of unbound 11-hydroxysteroids were such that in some determinations they came near the limiting concentration detectable by the spectrophotofluorimetric method used. It seemed advisable, therefore, to examine the effect of acetylsalicylic acid and other anti-inflammatory drugs on the protein-binding of 11-hydroxysteroids at higher total steroid concentrations, including concentrations in excess of the normal corticosteroid binding capacity (CBC) of plasma transcortin which was found by De Moor, Heirwegh & others (1962) to be 26 $\pm$ 3·8 μg/100 ml of plasma.

Endogenous levels of 11-hydroxysteroids in human plasma exceed the normal CBC only in abnormal physiological states, such as in Cushing's syndrome and in women in the third trimester of pregnancy, when they frequently rise to two or three times normal values. They are similarly raised after the administration of corticotrophin, and tetracosactide (Synacthen), a synthetic 1–24 polypeptide having adrenocortical stimulating properties identical to those of corticotrophin. Since, however, only a few such patients could be made available to extend our studies of the effect of aspirin and other anti-inflammatory drugs on the plasma protein-binding of 11-hydroxysteroids, we have also used *in vitro* systems in which the 11-hydroxysteroid concentrations have been artificially raised, with the object of extrapolating our results back to normal physiological levels.

EXPERIMENTAL

Materials

In addition to those already described (Stenlake & others, 1968) the following drugs were used; indomethacin (Merck Sharp & Dohme Ltd.); oxyphenbutazone (Geigy); ibufenac (Boots Pure Drug Co. Ltd.); albumin, human lyophilized (Kabi); Depot Synacthen injection (Ciba).

Collection of blood and ultrafiltration of plasma

Plasma (65 ml) was obtained from human blood as previously described (Stenlake & others, 1968). In some experiments plasma from the blood-bank was used.

Ultrafiltration of the plasma or albumin solutions was carried out at 37° by the method of Toribara (1953) as previously reported (Stenlake & others, 1968).

Measurement of 11-*hydroxysteroids*

Standard procedure. The concentration of 11-hydroxysteroids in plasma samples and ultrafiltrates was determined by the spectrofluorimetric method of Mattingly (1962), except that the number of test solutions in each batch was increased to six.

Protein-binding

1. *Effect of anti-inflammatory drugs on the binding of* 11-*hydroxysteroids to the proteins in plasma from patients with rheumatoid arthritis.* Plasma (65 ml) was obtained from each of six rheumatoid arthritic patients treated with a placebo, calcium lactate (600 mg) in tablet form for four days, to ensure that the clinical effect of residual anti-inflammatory drugs from previous therapy was minimal. The level of 11-hydroxysteroids in each plasma sample was increased by 50 μg/100 ml by adding the plasma to the dry residue of standard hydrocortisone solution (0·65 ml; 50 μg/ml in 5% aqueous ethanol). Equilibration of the 11-hydroxysteroids between the protein-bound and the unbound forms was achieved by incubation of the plasma for 60 min at 37°. Aliquots (10 ml) were added to each of six stoppered tubes, one without drug, and the others containing respectively acetylsalicylic acid (10 mg), ibufenac (1 mg), indomethacin (0·2 mg), oxyphenbutazone (2 mg) and phenylbutazone (2 mg). After being shaken overnight, the tubes were centrifuged briefly to disperse the froth formed and each aliquot was then ultrafiltered. The concentration of 11-hydroxysteroids in the plasma samples, ultrafiltrates and controls was determined in duplicate. The results are in Table 1.

2. *Effect of acetylsalicylic acid on the binding of* 11-*hydroxysteroids to proteins of normal, rheumatoid arthritic and blood-bank plasma.* Plasma samples (65 ml), obtained from (a) seven healthy volunteers, (b) a further seven rheumatoid arthritic patients who had received no anti-inflammatory drug in the seven days before the withdrawal of blood, and (c) the blood-bank (four different samples) were similarly equilibrated first with hydrocortisone (50 μg/100 ml), and then with acetylsalicylic acid (100 mg/100 ml) and ultrafiltered. Concentrations of 11-hydroxysteroids in the plasma samples, ultrafiltrates and controls were determined in duplicate. The results are in Table 2.

3. *Effect of different concentrations of acetylsalicylic acid on the binding of* 11-*hydroxysteroids to proteins in blood-bank plasma.* The concentration of 11-hydroxysteroids in blood-bank plasma was increased by 50 μg/100 ml, as described in experiment 1. Aliquots were equilibrated with acetylsalicylic acid to give final concentrations of 20, 40, 60, 80 and 100 mg/100 ml respectively and ultrafiltered. Concentrations of 11-hydroxysteroids in the plasma, ultrafiltrates and controls were determined in duplicate. The results are in Fig. 1.

4. *Effect of acetylsalicylic acid on the protein-binding of* 11-*hydroxysteroids in blood-bank plasma at different total concentrations of* 11-*hydroxysteroids.* The concentration of 11-hydroxysteroids in six samples of the same blood-bank plasma was increased by 0, 5, 10, 20, 30 and 50 μg/100 ml respectively, as described in experiment 1. Each sample was divided into two equal portions, one of which was equilibrated with

acetylsalicylic acid (100 mg/100 ml) and ultrafiltered. Concentrations of 11-hydroxysteroids present in the ultrafiltrates and controls were determined in triplicate. The results are in Table 3.

5. *Effect of incubation time with acetylsalicylic acid on the displacement of* 11-*hydroxysteroids from their protein-binding sites in blood-bank plasma.* The concentration of 11-hydroxysteroids in a sample of blood-bank plasma was increased by 50 μg/100 ml, as described in experiment 1, and acetylsalicylic acid (100 mg/100 ml) dissolved in each of seven fractions. Ultrafiltration of the fractions was begun 0·5, 1, 2, 5, 10, 20 and 50 h respectively after the addition of the acetylsalicylic acid, and concentrations of 11-hydroxysteroids in the control and in each ultrafiltrate were determined in duplicate.

6. *Effect of acetylsalicylic acid on the protein-binding of* 11-*hydroxysteroids in plasma in which the endogenous level of* 11-*hydroxysteroids is high.* Plasma (60 ml) was obtained from two rheumatoid arthritic patients 4 h after an intramuscular injection of tetracosactide (Depot Synacthen) and from two normal, pregnant women, both within one week of delivery. All these patients had received no anti-inflammatory drugs in the seven days before the withdrawal of blood. Acetylsalicylic acid (100 mg/100 ml) was dissolved in samples and each plasma aliquot was ultrafiltered. Concentrations of 11-hydroxysteroids in the ultrafiltrates and controls were measured in duplicate. The results are in Table 5.

7. *Effect of acetylsalicylic acid on the binding of* 11-*hydroxysteroids to human serum albumin.* A solution of serum albumin (4% in 0·1M phosphate buffer, pH 7·4) was prepared and added to the dry residue of standard hydrocortisone solution (50 μg/ml) to give a concentration of 25 μg/ml. After equilibration (60 min at 37°) acetylsalicylic acid (100 mg/100 ml) was added to an aliquot, the solution further equilibrated and ultrafiltered. Concentrations of 11-hydroxysteroids in treated and control ultrafiltrates were measured in duplicate.

8. *Effect of anti-inflammatory drugs on the spectrophotofluorimetric determination of* 11-*hydroxysteroids.* The following drugs were examined as described at the concentrations stated for their effect on the measurement of 11-hydroxysteroid fluorescence.

(a) *Ibufenac* (10 mg/100 ml), dissolved in a standard hydrocortisone solution (20 μg/100 ml). The apparent concentration of 11-hydroxysteroids was compared with the concentration of the same standard solution without added drug.

(b) *Acetylsalicylic acid.* The 18 plasma samples examined in experiment 2 were assayed for 11-hydroxysteroids before and after the addition of acetylsalicylic acid (100 mg/100 ml).

(c) *Oxyphenbutazone* (20 mg/100 ml), dissolved in a standard solution of hydrocortisone (20 μg/100 ml), was examined as under (a).

Blood-bank plasma with the 11-hydroxysteroids artificially increased by 50 μg/100 ml was assayed for 11-hydroxysteroids before and after the addition of oxyphenbutazone (20 mg/100 ml).

Oxyphenbutazone (1·5 mg/100 ml*) was also added to an ultrafiltrate sample from blood-bank plasma in which the 11-hydroxysteroids had been artificially increased by 50 μg/100 ml, and the solution examined as under (a).

(d) *Phenylbutazone* (20 mg/100 ml), dissolved in standard hydrocortisone solution (20 μg/100 ml), was examined as under (a).

* The concentration of oxyphenbutazone in an ultrafiltrate of plasma containing 20 mg/100 ml was found by the method of Burns, Rose & others (1955) to be 1·5 mg/100 ml.

9. *Effect of the anti-inflammatory drugs on plasma pH.* The concentration of 11-hydroxysteroids in blood-bank plasma and rheumatoid arthritic plasma was increased by 50 μg/100 ml as described in experiment 1. Acetylsalicylic acid (100 mg/100 ml), ibufenac (10 mg/100 ml), indomethacin (2 mg/100 ml), oxyphenbutazone (20 mg/100 ml) and phenylbutazone (20 mg/100 ml) were added to separate samples (10 ml) of each plasma. The pH values of the plasma samples and controls were measured.

10. *Effect of pH on the binding of 11-hydroxysteroids to plasma proteins.* Hydrochloric acid (N/2 about 0·2 ml) was added to a further portion of each plasma used in experiment 9 to give the same pH as that produced by the acetylsalicylic acid. The samples containing acetylsalicylic acid and each control were diluted with demineralized water to the same volume as the corresponding samples with hydrochloric acid, and ultra-filtered. Concentrations of 11-hydroxysteroids in each were measured in duplicate.

RESULTS AND DISCUSSION

The use of *in vitro* systems permitted experiments with plasma concentrations of steroids and anti-inflammatory drugs well in excess of those normally found in human plasma after administration of therapeutic doses of the drugs. Drugs were used at plasma concentrations four times those normally found after therapeutic doses (Smith, Gleason & others, 1946; Yu, Burns & others, 1958; Rechenberg & Herrmann, 1961; Holt & Hawkins, 1965; Adams, S. S. & Cliff, E. E., personal communication) and 11-hydroxysteroid levels were raised in most experiments by the addition of hydrocortisone (50 μg/100 ml) giving plasma total 11-hydroxysteroid levels of 55–70 μg/100 ml. For these reasons, it was necessary to check for quenching of fluorescence at the drug concentrations used.

Ibufenac (10 mg/100 ml) and phenylbutazone (20 mg/100 ml) did not interfere with the fluorimetric determination of 11-hydroxysteroids. Both acetylsalicylic acid and oxyphenbutazone, however, caused slight quenching of fluorescence of 11-hydroxysteroids *in vitro* and in plasma. Thus the mean quenching of 11-hydroxysteroid fluorescence in eighteen different plasma samples containing on average 60·6 μg/100 ml of steroid (experiment 2) was 1·82 $\pm$ s.d. 0·63 μg/100 ml after the addition of acetylsalicylic acid (100 mg/100 ml). Since acetylsalicylic acid is appreciably bound to plasma proteins, its concentrations in ultrafiltrates are much less than those in plasma. Concentrations of 11-hydroxysteroids in plasma ultrafiltrates containing acetylsalicylic acid are, therefore, reported uncorrected for this slight quenching effect.

Table 1. *Effect of acetylsalicylic acid, ibufenac, indomethacin, oxyphenbutazone and phenylbutazone on the protein-binding of 11-hydroxysteroids in plasma from rheumatoid arthritic patients*

Patient	Total plasma 11-hydroxysteroids µg/100 ml	Unbound 11-hydroxysteroids µg/100 ml: Control	Acetylsalicylic acid	Ibufenac	Indomethacin	Oxyphenbutazone	Phenylbutazone
1	62·0	17·6	23·9	18·8	17·3	12·9	16·9
2	60·4	14·5	20·0	15·3	14·5	12·2	14·5
3	65·1	16·9	22·4	16·1	16·9	13·7	16·9
4	65·8	19·2	23·9	18·8	20·8	14·5	18·8
5	58·7	15·7	21·6	15·3	15·7	12·2	15·3
6	61·8	14·5	17·3	13·7	14·5	11·0	13·7
Mean ± s.d. ..	62·3 ± 2·72	16·4 ± 1·86	21·5 ± 2·54	16·3 ± 2·06	16·6 ± 2·36	12·8 ± 1·24	16·0 ± 1·87
% Unbound mean ± s.d.		26·3 ± 2·29	34·5 ± 3·71	26·2 ± 2·89	26·6 ± 2·94	20·4 ± 1·44	25·7 ± 2·30
Student's *t* ..			3·95	0·089	0·163	3·96	0·361
P			<0·01	>0·9	>0·8	<0·01	>0·7

Quenching of fluorescence by oxyphenbutazone similarly reduced actual 11-hydroxysteroid concentrations of 20 μg/100 ml in standard hydrocortisone solution, and 65·6 μg/100 ml in plasma to apparent concentrations of 18 and 63·8 μg/100 ml respectively. However, plasma containing oxyphenbutazone (20 mg/100 ml) gave ultrafiltrates containing only 1·5 mg/100 ml, and addition of oxyphenbutazone (1·5 mg/100 ml) to an aliquot of an ultrafiltrate from plasma in which the 11-hydroxysteroids had been increased by 50 μg/100 ml, caused no quenching of fluorescence.

The effect of acetylsalicylic acid, ibufenac, indomethacin, oxyphenbutazone and phenylbutazone on the binding of 11-hydroxysteroids to proteins in the plasma of rheumatoid arthritic patients is recorded in Table 1. Only acetylsalicylic acid produced an increase in the proportion of unbound 11-hydroxysteroids. This displacement of 11-hydroxysteroids was complete after 30 min in contact with the acetylsalicylic acid, and remained constant over a period of 20 h. However, it was convenient to leave the acetylsalicylic acid in contact with the plasma overnight (16 h) and this practice was adopted throughout.

Displacement of 11-hydroxysteroids from their protein-binding sites by acetylsalicylic acid occurs to an equal extent in plasma obtained from normal volunteers and from patients with rheumatoid arthritis (Table 2). The proportion of unbound 11-hydroxysteroids in blood-bank plasma (four samples) was higher than in normal and rheumatoid arthritic human plasma (Table 2), possibly due to protein denaturation on storage. However, the displacement of 11-hydroxysteroids by acetylsalicylic acid in

Table 2. *Comparison of the effect of acetylsalicylic acid on the binding of* 11*-hydroxysteroids to proteins in normal, rheumatoid arthritic and blood-bank plasma*

	No.	Total plasma 11-hydroxysteroids* μg/100 ml	Unbound 11-hydroxysteroids* μg/100 ml: Control	Unbound 11-hydroxysteroids* μg/100 ml: After acetylsalicylic acid	Percentage displacement
Normal plasma	1	59·2	22·3	25·6	5·5
	2	65·2	17·7	22·1	6·8
	3	62·3	19·1	22·3	5·1
	4	53·7	16·3	20·2	7·2
	5	57·6	13·5	15·8	4·0
	6	60·4	14·6	20·6	9·9
	7	56·0	16·2	19·6	6·1
	Mean	59·2	17·1	20·9	6·37
Rheumatoid arthritic plasma	1	59·0	10·8	14·4	6·1
	2	59·8	14·1	18·1	6·7
	3	64·7	17·4	22·4	7·7
	4	63·0	17·9	21·7	6·0
	5	67·2	17·0	21·4	6·5
	6	60·7	20·4	23·1	4·5
	7	59·6	15·7	17·9	3·7
	Mean	62·0	16·2	19·9	5·89
Blood bank plasma	1	61·8	22·4	26·4	6·5
	2	57·4	21·7	24·7	5·2
	3	59·8	16·2	19·7	5·8
	4	64·0	23·0	26·8	6·0
	Mean	60·8	20·8	24·4	5·88

* Each result is the mean of two determinations.

Mean of 18 duplicate total plasma 11-hydroxysteroid concentration = 60·6 μg/100 ml.

Mean of 18 duplicate determinations of 11-hydroxysteroids in the same plasmas after addition of acetylsalicylic acid (1 mg/ml) (not recorded) = 58·8 μg/100 ml.

these samples was similar to that produced in normal and rheumatoid arthritic plasma (Table 2). Blood-bank plasma was used, therefore, in some later experiments to avoid the withdrawal of the large quantities of blood which would otherwise have been required.

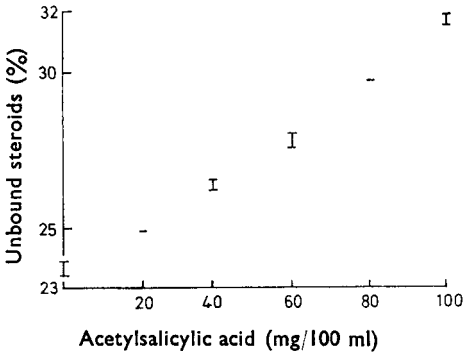

FIG. 1. Changes in the percentage of unbound 11-hydroxysteroids (shown as the range between duplicate determinations) in aliquots of blood-bank plasma at total 11-hydroxysteroid concentration of 63.2 μg/100 ml and varying concentrations of acetylsalicylic acid.

Fig. 1 shows that the displacement of 11-hydroxysteroids from the plasma protein-binding sites in blood-bank plasma is proportional to the concentration of acetylsalicylic acid. However, the curve indicates that the percentage of plasma total 11-hydroxysteroids which remains unbound at concentrations of acetylsalicylic acid normally found in the plasma of rheumatoid arthritic patients (20 mg/100 ml) is only some 1% greater than in untreated plasma. The clinical significance of this is, therefore, doubtful. Competition for binding sites, also is not great as it requires acetylsalicylic acid (1 mg/ml) to displace 37 ng/ml of 11-hydroxysteroids (Table 2). Further, since it is known that acetylsalicylic acid binds to human plasma albumin (Reynolds & Cluff, 1960), it seems likely that in binding, this drug causes displacement of 11-hydroxysteroids from albumin binding sites.

Since the drugs examined are all acidic and the pH of plasma is known to affect the binding ability of transcortin (the principal corticosteroid-binding globulin), with the optimum between pH 7 and 8 (Daughaday & Mariz, 1961), it was necessary also to

Table 3. *The effect of acetylsalicylic acid* (1 *mg/ml*) *on the displacement of* 11-*hydroxysteroids from plasma proteins at different total concentrations of* 11-*hydroxysteroids and calculated displacements from* (1) *all binding sites and* (2) *albumin-binding sites only*

Added 11-hydroxy-steroids μg/100 ml	Total plasma 11-hydroxy-steroids† μg/100 ml (A)	Total plasma 11-hydroxy-steroids not bound to transcortin*† μg/100 ml (B)	Unbound 11-hydroxysteroids† Control (C)	Unbound 11-hydroxysteroids† After acetyl-salicylic acid (D)	Acetylsalicylic acid: Displacement from all binding sites (D–C) μg/100 ml (E)	Acetylsalicylic acid: % Displacement from all binding sites (E/A × 100)	Acetylsalicylic acid: % Displacement from albumin-binding sites (E/B × 100)
0	14·6	0‡	2·71	2·62	−0·09	−0·6	—
5	19·4	0‡	3·16	3·27	0·11	0·6	—
10	24·0	2‡	3·48	3·68	0·20	0·8	10
20	34·3	12·3	8·43	9·09	0·66	1·9	5·4
30	44·1	22·1	12·50	14·30	1·80	4·1	8·3
50	64·1	42·1	23·30	27·40	4·10	6·4	9·7

* Based on an assumed CBC of transcortin = 22·0 μg/100 ml.

† Each result is the mean of three determinations.

‡ Neglecting the unbound concentration.

consider the effect of the drugs on the pH of plasma, and hence on the binding of 11-hydroxysteroids. The pH values of blood-bank and rheumatoid arthritic plasmas, with raised levels of 11-hydroxysteroids, and containing acetylsalicylic acid, ibufenac, indomethacin, oxyphenbutazone and phenylbutazone are recorded in Table 4.

Blood-bank plasma has a lower pH (6·9) than the physiological pH (7·4) because of added sodium acid citrate. Also, fresh rheumatoid arthritic plasma, containing heparin anticoagulant, loses carbon dioxide on exposure to air; its pH is, therefore, slightly higher than physiological pH. Only acetylsalicylic acid had any appreciable effect on the pH of either plasma. Adjustment of plasma pH with hydrochloric acid (in place of acetylsalicylic acid) to the same pH as that obtained with acetylsalicylic acid did not alter the concentration of unbound 11-hydroxysteroids, indicating that their displacement from their protein-binding sites by the drug is not because of the accompanying fall in pH.

Table 4. *Effect of acetylsalicylic acid, indomethacin, ibufenac, oxyphenbutazone and phenylbutazone on the pH of plasma from a patient with rheumatoid arthritis and from blood-bank plasma*

Source of plasma	Plasma aliquot number	Added drug	pH
Rheumatoid arthritic patient	1	None (Control)	7·8
	2	Acetylsalicylic acid (1 mg/ml)	7·3
	3	Ibufenac (0·1 mg/ml)	7·7
	4	Indomethacin (0·02 mg/ml)	7·8
	5	Oxyphenbutazone (0·2 mg/ml)	7·8
	6	Phenylbutazone (0·2 mg/ml)	7·8
Blood bank	1	None (Control)	6·9
	2	Acetylsalicylic acid (1 mg/ml)	6·5
	3	Ibufenac (0·1 mg/ml)	6·8
	4	Indomethacin (0·02 mg/ml)	6·9
	5	Oxyphenbutazone (0·2 mg/ml)	6·9
	6	Phenylbutazone (0·2 mg:ml)	6·9

Displacement of 11-hydroxysteroids by acetylsalicylic acid (100 mg/100 ml) in blood-bank plasma (Table 3) was appreciable only at the higher 11-hydroxysteroid concentrations, apparently when the CBC of transcortin was exceeded; thereafter it increased with increase in total steroid concentration. The displacement from albumin-binding has been calculated (Table 3), assuming the CBC attributable to transcortin in blood-bank plasma to be 22 μg/100 ml (a low value, chosen since the percentage of unbound 11-hydroxysteroids in blood-bank plasma is apparently higher than in normal plasma) (Table 2). Evidence that displacements at these higher 11-hydroxysteroid concentrations only involve albumin-binding sites was obtained from parallel experiments with 4% human serum albumin containing hydrocortisone (25 μg/100 ml) with and without added acetylsalicylic acid (100 mg/100 ml). Ultrafiltrates showed an increase in unbound hydrocortisone levels to 76·4% from 65·2% in the control ultrafiltrate, a displacement of 11·2%, which compares favourably with the calculated displacements in Table 2.

That the displacement is mainly from albumin-binding sites is further confirmed in Table 5 which shows the effect of acetylsalicylic acid on the binding of 11-hydroxysteroids on the plasma from two rheumatoid arthritic patients, in whom the 11-hydroxysteroid levels have been artificially raised by injections of depot tetracosactide

(Synacthen). Seal & Doe (1962) have concluded that transcortin-binding sites are quickly saturated after the administration of ACTH, and that the balance of the 11-hydroxysteroids are either unbound or albumin bound. The results in Table 5 show that aspirin produces displacements of bound 11-hydroxysteroids to similar extents to those observed in the experiments with blood-bank plasma and with human serum albumin solutions.

Table 5. *Effect of acetylsalicylic acid on the protein-binding of* 11*-hydroxysteroids in the plasma from two rheumatoid arthritic patients,* 4 *h after an intramuscular injection of depot synacthen and from two normal women in late pregnancy*

Source of plasma	No.	Total plasma 11-hydroxysteroids* µg/100 ml (A)	Unbound 11-hydroxysteroids* µg/100 ml		Acetysalicylic acid	
			Control	After acetylsalicylic acid	Displacement µg/100 ml (B)	% Displacement $\frac{B \times 100}{A}$
Rheumatoid arthritic patients, 4 h after an intramuscular injection of Depot Synacthen	1	81·4	25·5	30·6	5·1	6·3
	2	40·0	7·1	8·5	1·4	3·5
Normal women in late pregnancy	1	40·2	2·77	2·93	0·16	0·4
	2	47·4	2·09	2·37	0·28	0·6

* Each result is the mean of two determinations.

Endogenous levels of 11-hydroxysteroids and apparently transcortin (or a similar protein) are increased in the plasma of women in the third trimester of pregnancy. Consequently the CBC for 11-hydroxysteroids is also raised. As expected, therefore, displacements (Table 5) of 11-hydroxysteroids by aspirin in the plasma of two women in late pregnancy were small (0·6 and 0·4%) compared with those observed in rheumatoid arthritic patients after injection of tetracosactide (6·3 and 3·5%), although the total levels of 11-hydroxysteroids were comparable.

Phenylbutazone (Burns Rose & others, 1953), indomethacin (Hucker, Zaccher & others, 1966), and ibufenac (Adams, S. S. & Cliff, E. E., personal communication) also bind strongly to plasma proteins. They are similar to many other non-steroidal, acidic anti-inflammatory drugs in that they inhibit heat coagulation of serum albumin and of whole serum as a result of this interaction (Mizushima, 1966; Mizushima & Kobayashi, 1968). In contrast to acetylsalicylic acid, however, the interaction of ibufenac, indomethacin and phenylbutazone with plasma proteins does not interfere with the protein-binding of 11-hydroxysteroids (Table 1). These results are in complete agreement with those obtained for indomethacin by Dr. R. H. Silber (personal communication) employing equilibrium dialysis at 5°.

Contrary to expectation, oxyphenbutazone caused a significant increase in the 11-hydroxysteroids bound to plasma proteins (Table 1). Many examples of one drug displacing another drug or hormone from its protein-binding sites are known (Brodie, 1965), but this, to our knowledge, is the first report of a drug decreasing the unbound concentration of a hormone. The phenomenon is under further investigation.

We conclude that the results of the present work confirm those of the *in vivo* experiments (Stenlake & others, 1968), and lead to the opinion that non-steroidal anti-inflammatory drugs do not exert their effect by causing an increase in the proportion of unbound 11-hydroxysteroids in human plasma.

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