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The effect of acetylsalicylic acid, phenylbutazone and indomethacin on the binding of 11-hydroxysteroids to plasma proteins in patients with rheumatoid arthritis

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Plasma concentrations of total and unbound 11-hydroxysteroids in patients with rheumatoid arthritis have been determined using a fluorimetric method before and after administration of acetylsalicylic acid, phenylbutazone and indomethacin. Unbound 11-hydroxysteroids were measured in plasma ultrafiltrates obtained using the Toribara apparatus. Enhancement and quenching of fluorescence by acetylsalicylic acid, phenylbutazone, and indomethacin, and heparin used as an anticoagulant in the blood samples, have been shown to be absent. The results show that acetylsalicylic acid, phenylbutazone and indomethacin given in recommended therapeutic doses for periods of one week have no significant effect on plasma protein-binding of 11-hydroxysteroids.

ACETYLSALICYLIC acid, phenylbutazone and indomethacin are well established as effective analgesic and anti-inflammatory drugs in the management of patients with rheumatoid arthritis, although the mechanism of their action is little understood. It has been suggested (Brodie, 1965) that in the rat they liberate corticosterone from its binding sites on the α-globulin carrier-protein, transcortin, and that their action is due to the then unbound corticosteroid, which is considered to be the physiologically active fraction of the total plasma corticosteroid (Booth, Dixon & others, 1961). Acetylsalicylic acid decreases the protein-binding of thyroxine (Christensen, 1959), and phenylbutazone is bound to plasma proteins to the extent of 98% at therapeutic plasma levels (Brodie & Hogben, 1957). We have investigated the effects of acetylsalicylic acid, phenylbutazone and indomethacin on the plasma levels of total and unbound 11-hydroxysteroids in patients with rheumatoid arthritis.

Experimental

METHODS

The investigation was made on twelve patients with rheumatoid arthritis. Five of these had been receiving high doses (4·5 g per day) of acetylsalicylic acid for several weeks before the experiments began, and continued to receive the same doses during the period in which the investigations were made. Blood was withdrawn at 09.00–09.30 hr on each of several days in the course of a week or 10 days, and an ultrafiltrate prepared from each sample. Total 11-hydroxysteroids in each sample were determined in duplicate by the standard procedure described below; unbound 11-hydroxysteroids were determined similarly in each sample of ultrafiltrate, and the protein-binding then calculated.

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The remaining seven patients had previously received a variety of drug treatments for rheumatoid arthritis. Each of these seven patients received a placebo consisting of calcium lactate (600 mg) in tablet form daily for one week, after which blood was withdrawn at 09.00–09.30 hr on the eighth day and examined as in the first series of patients. Throughout the following week the same seven patients were treated with acetylsalicylic acid [Solprin: 3 tablets (each containing 324 mg acetylsalicylic acid) five times a day] and protein-binding of 11-hydroxysteroids was determined in 09.00–09.30 hr blood samples taken on the fifteenth day. In four of of the seven patients aspirin was replaced in the third week by phenylbutazone (Butazolidin, 3 × 100 mg tablets daily) and in the fourth week by indomethacin (Indocid capsules, 2 × 25 mg daily). Protein-binding of 11-hydroxysteroids was determined at the end of each of the third and fourth weeks in 09.00–09.30 hr blood samples taken on the 22nd and 29th days respectively. Protein-binding was also determined on samples taken at 1800 hr from two of the four patients at the end of each regime.

The concentration of salicylates in plasma samples on which 11-hydroxysteroid samples were made was determined by the method of Trinder (1954) before and after treatment with acetylsalicylic acid.

COLLECTION OF BLOOD AND ULTRAFILTRATION OF PLASMA

Blood (60 ml) was withdrawn by venepuncture and immediately distributed in heparinized centrifuge tubes (10 ml). Erythrocytes were separated from the plasma by centrifuging for 10 min.

Protein-free ultrafiltrates of the plasma samples were obtained using the apparatus of Toribara (1953). Plasma was placed in Visking tubing (8/32 inch) in the special centrifuge tubes, and centrifuged at 2000 rev/min for 2 hr at 37°; about 5 ml of ultrafiltrate was obtained from each 20 ml of plasma. Pooled ultrafiltrates (one drop) were tested with salicylsulphonic acid 20% w/v in water (1 ml); only those ultrafiltrates, shown to be protein-free by this method, were used.

DETERMINATION OF 11-HYDROXYSTEROIDS

Plasma concentrations of total* and unbound 11-hydroxycorticosteroids were determined by the spectrofluorometric method of Mattingly (1962), observing his recommendations for the purification of dichloromethane, preparation of reagents and cleaning of glassware. The method is not specific for 11-hydroxycorticosteroids (hydrocortisone), but 90% of the plasma 11-hydroxysteroids is known to be hydrocortisone.

Up to four plasma samples, ultrafiltrates or other test solutions were assayed simultaneously. A reagent blank (de-ionized water, 2 ml) and a standard hydrocortisone solution (0·5 μg/ml; 2 ml) were carried through the procedure with each batch.

* For the purpose of this investigation total hydrocortisone means the total of unbound and protein-bound 11-hydroxysteroids. The term "protein-binding" will be used to mean the ratio of unbound hydrocortisone to the total of unbound and protein-bound hydrocortisone.

Hydrocortisone standards. Hydrocortisone (B.D.H.) (50 mg) was dissolved in absolute ethanol (50 ml) (absolute alcohol, James Burroughs Ltd). A stock solution (5 mg; 100 ml) was prepared by diluting this solution (5 ml to 100 ml) with de-ionized water. Further dilution of this solution as necessary gave a working standard of 50 μg/100 ml.

Apparatus. An Aminco-Bowman Spectrophotofluorimeter was used with 1 cm quartz cells, and slit program No. 3. The fluorimeter was coupled to an XY recorder, and excitation and emission spectra of all standard and test solutions were recorded. For excitation spectra the emission monochromator was set at 522 mμ and for emission spectra the excitation monochromator was set at 466 mμ.

TESTS FOR FLUORESCENCE OR QUENCHING FROM EXTRANEOUS SUBSTANCES

Heparin. De-ionized water (5 ml) was shaken in a heparinized centrifuge tube and an aliquot (2 ml) of the solution submitted to the standard procedure. Water alone was simultaneously submitted to the procedure. A standard hydrocortisone solution was similarly shaken in a heparinized tube and a sample (2 ml) of the resultant solution, and, also at the same time, the solution of hydrocortisone alone, was subjected to the standard procedure. Heparin neither gave rise to fluorescence when examined alone, nor did it quench the fluorescence of hydrocortisone (Fig. 1B).

Drugs. Acetylsalicylic acid (10 mg) and phenylbutazone (10 mg) were each added separately to the fluorescence reagent (5 ml), and the fluorescence measured under the standard conditions. Since the contents of an Indocid capsule fluoresced strongly in the reagent, a saturated solution of indomethacin (18 μg/ml) (2 ml) was likewise put through the standard procedure. The first two compounds gave rise to no measurable fluorescence, the third had the same fluorescence as the de-ionized water blank.

Solutions of acetylsalicylic acid (0·4 mg/ml; 1 ml), phenylbutazone (0·1 mg/ml; 1 ml) and indomethacin (18 μg/ml; 1 ml) were added to standard hydrocortisone solution (1 ml) and the mixture assayed by the standard procedure. The relative fluorescence intensity was the same as that produced by dilution of the hydrocortisone with deionized water.

Results and discussion

Wavelengths of the peaks in the excitation and emission spectra of both plasma and ultrafiltrate samples corresponded with those produced by the standard solution of hydrocortisone in each set of determinations (Fig. 1).

Plasma itself has been reported to produce non-specific fluorescence (Braunsberg & James, 1962; Daly & Spencer-Peet, 1964). Absolute values of hydrocortisone, however, were not required, since the object of the present investigation was to study the effect of the clinical treatment on the proportion of unbound to total 11-hydroxysteroids. Careful examination of excitation and emission curves for several of the treated plasma and ultrafiltrate samples revealed no abnormality as the curves were similar to those produced by authentic samples of hydrocortisone. It is thus reasonable to assume that hydrocortisone, as the major 11-hydroxysteroid, was being measured.

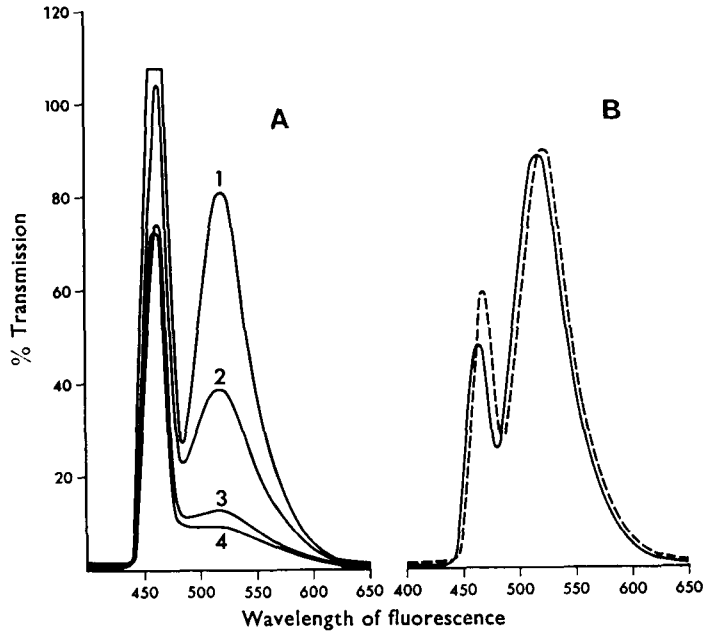

Fig. 1. Fluorescence spectra obtained with Aminco-Bowman Spectrofluorimeter of: A. 1, Standard cortisol, 2, Plasma total 11-hydroxysteroids, 3, Plasma unbound 11-hydroxysteroids. 4, Blank. B. ——— Standard cortisol. ------ Heparin in the presence of standard cortisol. The curves in B are strictly superimposable.

Plasma levels of 11-hydroxysteroids and protein-binding in patients with rheumatoid arthritis treated in the first clinical study with acetylsalicylic acid are given in Table 1. This shows that the protein-binding of corticosteroids in the plasma of rheumatoid arthritic patients treated with acetylsalicylic acid does not differ significantly from previously reported normal values. Using an isotopic technique, Plager, Schmidt & Staubitz (1964) have observed that the ratio of unbound hydrocortisone to the total hydrocortisone in normal adult plasma is 12.64 $\pm$ 3·20% (mean $\pm$ s.d.). We found that in 23 duplicate determinations upon the plasma of five rheumatoid arthritic patients, treated with aspirin, the protein-binding value was 11·20 $\pm$ 2·73% (mean $\pm$ s.d.).

TABLE 1. PLASMA LEVELS OF 11-HYDROXYSTEROIDS IN PATIENTS WITH RHEUMATOID ARTHRITIS TREATED WITH ACETYLSALICYLIC ACID

Patient No.	No. of duplicate determinations	Mean concentration of plasma 11-hydroxysteroids		Mean protein binding (%)
		Unbound µg %	Total µg %	
1	5	2·02	16·4	11·9
2	4	1·99	18·3	11·0
3	4	1·64	14·8	11·3
4	5	1·34	13·3	10·1
5	5	1·23	10·6	11·6

Mean ($\pm$ s.d.) protein-binding (%) for all 23 determinations = 11·20 $\pm$ 2·73.

TABLE 2. PLASMA LEVELS OF TOTAL AND UNBOUND 11-HYDROSTEROIDS IN PATIENTS WITH RHEUMATOID ARTHRITIS TREATED IN EACH OF FOUR SUCCESSIVE WEEKS WITH A CALCIUM LACTATE PLACEBO, ACETYLSALICYLIC ACID, PHENYLBUTAZONE AND INDOMETHACIN RESPECTIVELY

Patient No.	Plasma 11-hydroxy-steroids*		Protein-binding (%)	Plasma 11-hydroxy-steroids*		Protein-binding (%)
	Unbound µg %	Total µg %		Unbound µg %	Total µg %	
	1st week placebo			2nd week acetylsalicylic acid		
6	3·15	21·1	14·9	2·21	15·2	14·5
7	1·40	12·0	11·7	1·16	12·0	9·7
8	1·33	8·3	16·0	1·38	9·2	15·0
9	1·37	11·7	11·7	0·93	9·5	9·8
10	1·82	19·5	9·3	2·07	18·0	11·5
11	1·21	15·1	8·0	1·78	15·1	11·8
12	2·47	19·0	13·0	0·96	10·8	8·9
	3rd week phenylbutazone			4th week indomethacin		
6	2·02	15·3	13·2	2·26	19·1	11·8
7	1·23	12·0	10·2	1·88	17·3	10·9
8	1·88	16·4	11·5	1·67	10·9	15·3
9	1·05	12·0	8·7	1·84	11·7	15·7

* Each result is the average of two determinations.
Mean (± s.d.) protein-binding (%): placebo 12·1 ± 2·85; acetylsalicylic acid 11·6 ± 2·38; phenylbutazone 10·9 ± 2·27; indomethacin 13·4 ± 2·43

Unbound and total hydrocortisone plasma levels (09.00 hr) and protein-binding in the seven patients treated successively with placebo, aspirin, phenylbutazone and indomethacin are presented in Table 2.

The results for these patients treated with a placebo and then with acetylsalicylic acid show no significant difference in protein-binding before and after treatment. The mean salicylate level in the plasma of patients before treatment with acetylsalicyclic acid was 1 mg % and that during therapy, 20 mg %, indicating that therapeutic levels had been achieved. The number of patients studied after treatment with phenylbutazone and indomethacin was too small for full statistical analysis, but the results suggest that no significant difference in protein-binding occurs as a result of treatment with these drugs.

The patients studied experienced definite relief of joint symptoms during treatment. The results therefore suggest that the anti-rheumatic action of these drugs is not associated with a significant degree of displacement of 11-hydroxysteroids from plasma proteins. This conclusion is in accord with the views of Theobold & Domenjoz (1956), of Winter, Risley & Nuss (1963) and of Winter, Risley & Silber (1967), based on experiments with acetylsalicylic acid, phenylbutazone and indomethacin in adrenalectomized rats. It has also been shown (Jansen & Schou, 1967) that neither phenylbutazone, sodium salicylate nor indomethacin influence binding of hydrocortisone to plasma proteins in the guinea-pig.

The level of total (bound and unbound) plasma unconjugated 11-hydroxysteroids is reported to be subject to diurnal variation (Bliss, Sandberg, Nelson & Eik-Nes, 1953). Diurnal variation was observed in the levels of both unbound and total plasma 11-hydroxysteroids of two

TABLE 3. PLASMA LEVELS OF TOTAL AND UNBOUND 11-HYDROXYSTEROIDS AT 09.00 AND 18.00 HR IN TWO PATIENTS WITH RHEUMATOID ARTHRITIS TREATED IN EACH OF FOUR SUCCESSIVE WEEKS WITH A CALCIUM LACTATE PLACEBO, ACETYLSALICYLIC ACID, PHENYLBUTAZONE AND INDOMETHACIN RESPECTIVELY

Treatment	09.00 hr			18.00 hr		
	Plasma 11-hydroxy-steroids*		Protein-binding (%)	Plasma 11-hydroxy-steroids*		Protein-binding (%)
	Unbound μg %	Total μg %		Unbound μg %	Total μg %	
Patent No. 6						
1st week placebo	3·15	21·1	14·9	1·55	14·0	11·1
2nd week acetylsalicylic acid	2·21	15·2	14·5	2·09	11·0	19·0
3rd week phenylbutazone	2·02	15·3	13·2	0·87	9·8	8·9
4th week indomethacin	2·26	19·1	11·8	1·82	18·3	10·0
Patient No. 7						
1st week placebo	1·40	12·0	11·7	0·40	6·0	6·7
2nd week acetylsalicylic acid	1·16	12·0	9·7	0·81	10·2	7·9
3rd week phenylbutazone	1·23	12·0	10·2	0·62	6·9	9·0
4th week indomethacin	1·88	17·3	10·9	0·75	10·7	7·0

* Each result is the average of two determinations.

patients with rheumatoid arthritis during treatment with the drugs (Table 3), but without apparent variation in the protein-binding.

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