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PROTEIN BINDING OF CORTICOIDS STUDIED BY GEL FILTRATION *

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The ability of porous bodies to distinguish between molecules of different sizes has been repeatedly observed. If these porous bodies are devoid of charged groups, solutes placed on top of a column filled with such a material will be partitioned chiefly by molecular sieve effect (1). Molecules with a size above a limiting value will leave the column immediately after the displacement volume. Smaller particles will enter the gel grains and will be retarded and eventually separated into groups of different molecular weight. Such columns, on the other hand, may perform an exhaustive dialysis. The use of dextran gels for these purposes was introduced by Porath and Flodin (2); as suggested by Tiselius the method was named gel filtration.

Nonmetabolized corticoids may be found in human plasma in three different forms: a very small fraction of unbound steroids, a fraction weakly bound to albumin, and a fraction strongly but reversibly bound (3) to a specific protein called transcortin (4). Under basal conditions the latter fraction represents 95 to 98 per cent of total plasma cortisol.[1]

Using gel filtration, the binding of corticoids to plasma proteins could be studied, excluding the albumin binding. The presence of a macromolecular agent strongly binding corticoids was also demonstrated in other biological material. The bound corticoids were quantitated fluorometrically as well as by radioactivity measurements.

MATERIALS AND METHODS

The procedures used for preparation of the dextran gels, packing of the columns, application of the sample, and elution were as described by Flodin (1). Elimination of the "fines" is essential in order to avoid clogging of the columns. Three different gels (Sephadex G-25, Sephadex G-50, and Sephadex DEAE A-50 [2]) were tried with good results; in the experiments to be described only Sephadex G-50 was used. Figure 1 shows the type of results obtained.

In a preliminary study conditions were considered, which could eventually in the course of gel filtration affect decomposition of the protein-steroid complex by dialysis effect, or alter the separation of the protein-bound and unbound corticoids. As far as the former effect (i.e., decomposition) is concerned, temperature and elution rate were found to be important. To assess the influence of temperature, 1-ml plasma samples were "overloaded" with 500 mμg of cortisol and preincubated for 15 minutes at the desired temperature. At 4° C runs were made in a cold room, whereas at 34° C and at higher temperatures columns with a jacket connected to a thermostat were used. From 4° to 34° C there was an almost linear decrease of the amount of steroids in the protein peak. At the latter temperature the mean binding was 28 per cent lower than at 4° C. At a somewhat higher temperature the binding abruptly fell to very low values (Figure 2). This extreme lowering proved to be reversible (4). To show this, gel filtration experiments were done at 46° C and the protein fractions concen-

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[1] The following abbreviations have been employed: cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione); cortisone (17α,21-dihydroxy-4-pregnene-3,11,20-trione); corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione); testosterone (17β-hydroxy-4-androstene-3-one); deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione); progesterone (4-pregnene-3,20-dione); compound S (17α,21-dihydroxy-4-pregnene-3,20-dione); androsterone (3α-hydroxy-androstan-17-one); 17-hydroxyprogesterone (17α-hydroxy-4-pregnene-3,20-dione); tetrahydro-S (3α,17α,21-trihydroxypregnane-20-one); pregnanediol (pregnane-3α,20α-diol); pregnanetriol (pregnane-3α,17α,20α-triol); tetrahydro-DOC (3α,21-dihydroxy-pregnane-20-one); Δ^5-pregnenolone (3β-hydroxy-5-pregnene-20-one); dehydroepiandrosterone (3β-hydroxy-5-androstene-17-one); ethynylestradiol (17α-ethynyl-1,3,5: 10-estriene-3, 17β-diol); stilbestrol (3,4 *bis*(*p*-hydroxyphenyl)-3-hexene); ACTH (adrenocorticotropin); corticoids (cortisol and corticosterone).

[2] Samples of Sephadex powder (medium particle size) were obtained from Aktiebolaged Pharmacia, Uppsala, Sweden.

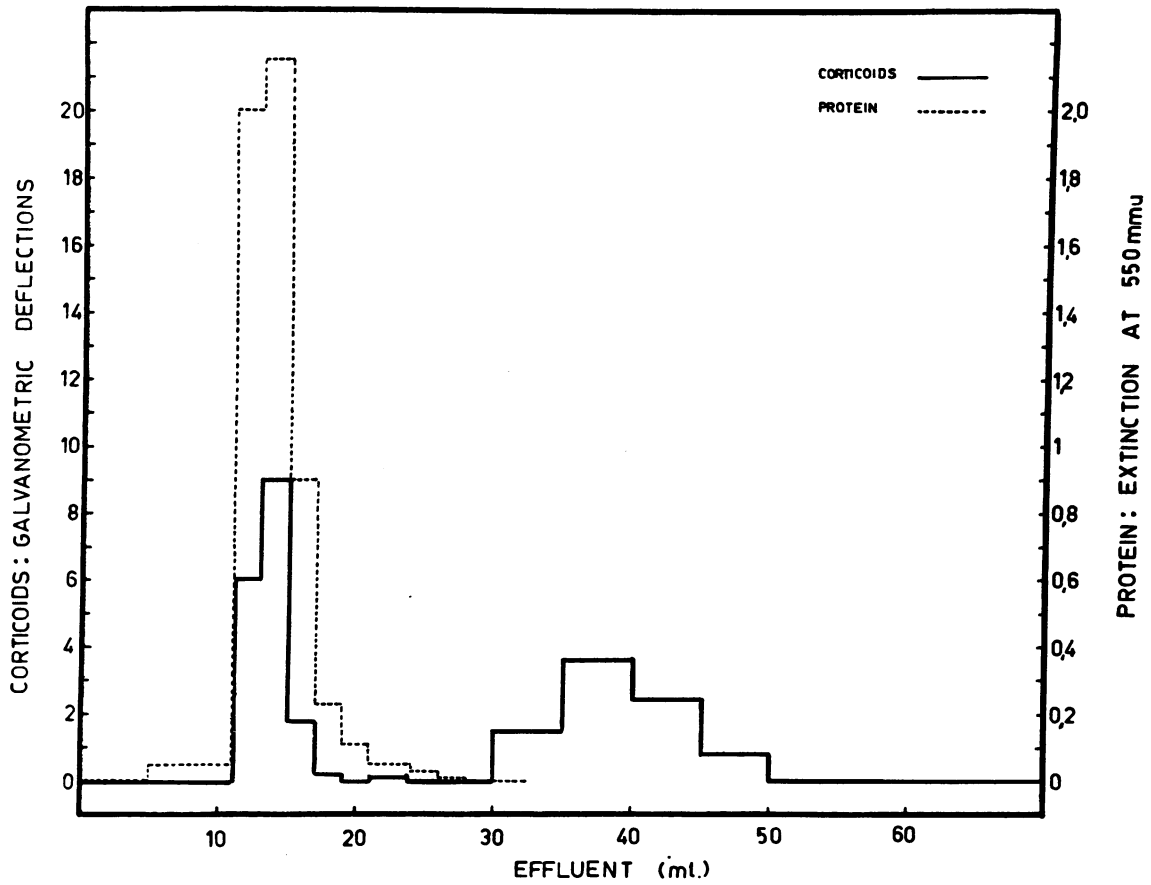

FIG. 1. GEL FILTRATION OF 1 ML HUMAN PLASMA AND 500 MμG OF CORTISOL.

trated by a rapid dialysis *in vacuo,* using an apparatus constructed according to Dr. Mies (Membranfilter Gesellschaft, Göttingen, Germany). These fractions showed the same cortisol-binding capacity per gram protein at room temperature as before. Unless otherwise indicated, all further experiments were made at room temperature (21 to 22° C). The rate of elution, which was regulated by means of an outflow stopcock, also was important. As the fractions were eluted more rapidly a somewhat greater amount of corticoids was found in the protein peak. In seven experiments the amounts of cortisol bound per 100 ml of normal plasma were 22.1, 22.9, 24.4, and 26.1 μg with elution rates of 0.2, 0.5, 1.5, and 2.5 ml per minute, respectively. When four pregnancy plasmas were tested, the results were: 51.8, 53.5, 54.4, and 55.9 μg. For each of these experiments 1 ml of plasma overloaded with 500 mμg of cortisol was used.

The separation of protein-bound from unbound corticoids depends somewhat on the quantity of Sephadex, as related to the volume of the sample, and on the dimensions of the gel bed. Good separations were obtained at a height to diameter ratio of 20:1 and a bed volume to sample volume ratio of 30:1. Under these conditions cortisol concentrations in the sample as high as 1 μg per ml could be used without overlapping of the elution peaks. In the range studied (0.2 to 2.5 ml per minute), the rate of elution did not affect separation of peaks. The effluent pattern was barely changed by the molarity and the nature of the buffer used as eluent. Phosphate buffers of 0.075 and 0.3 M, pH 6.8 and 8, as well as a Tris buffer (0.15 M, pH 7.35), gave almost identical results in 30 comparative tests, each variable being tested against phosphate buffer (0.15 M, pH 7.35). The relative standard deviation in this duplicate study was 7.9 per cent. Addition of glucose (50 mg per ml) in order to increase the viscosity of the sample had practically no influence on the separation.

The following procedure was finally adopted for physiopathological work. One ml of heparinized plasma, with or without added cortisol (500 mμg), was placed on top of the gel bed and eluted with phosphate buffer (0.15 M, pH 7.35) at a flow rate of about 0.5 ml per minute. This flow rate was selected because it could be maintained during at least 50 gel filtration experiments with plasma. The glass column used had an internal diameter of 1.2 cm, and the height of the gel bed was 30 cm. Usually 500 mμg of steroid was used in the overloading experiments; in 20 comparative tests 250 or 750 mμg was used instead,

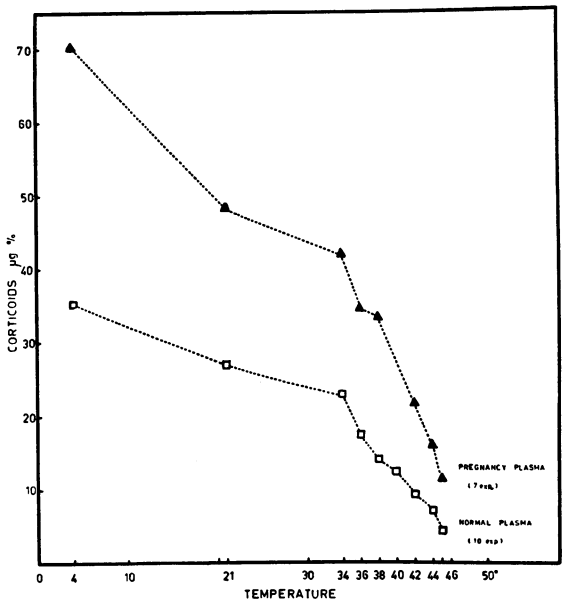

FIG. 2. INFLUENCE OF TEMPERATURE ON THE AMOUNT OF CORTISOL BOUND BY 100 ML OF HUMAN PLASMA AFTER OVERLOADING WITH 500 MµG OF THE HORMONE PER ML.

with very similar results. Serum can also be used; as compared to plasma in eight duplicate overloading experiments the amount of corticoids bound per 100 ml of plasma was 27.8 versus 27.2 µg.

In the eluted fractions, as in the original plasma samples, corticoids were determined with a fluorometric procedure (5, 6) and total protein according to a technique described by Lowry, Rosebrough, Farr and Randall (7), in which a purified β-lactoglobulin was used as a standard. The NaOH wash, normally included in the fluorometric procedure, is not necessary after gel filtration of plasma samples of male patients. In female patients, however, the results were higher when the wash was omitted, and the NaOH step was therefore retained in the routine procedure.

The displacement volume of the columns is about 9 ml and can be estimated visually. During standardization of the method the effluent collected thereafter was examined in 2-ml fractions. The plasma proteins put on top of the gel bed were always found in the first 6 to 8 ml after the void volume; the protein recovery in 20 experiments averaged 95.6 per cent. Together with this protein, a certain amount of corticoids comes off the column. To be quite safe, in the routine technique 10 ml is taken as the protein-bound fraction. As a control procedure the next 2-ml fraction was also examined systematically. In 100 consecutive experiments this tail-fraction was found to contain only traces of fluorescent material [0.33 ±0.071 (SEM [3]) µg per 100 ml of cortisol equivalents], the corresponding mean protein content in 20 determinations being 5 mg per 100 ml. When, occasionally, the latter fraction contains more than 0.8 µg per 100 ml of cortisol equivalents, the column and the gel filtration experiment are discarded. After another 4 ml of buffer eluent was added, the unbound corticoids (small molecules) finally came off the column. Once prepared, a column can be used 50 to 100 times before clogging. After each run it is washed with 40 to 50 ml of eluent and stored well-stoppered at room temperature. Very similar results were obtained in four duplicate determinations with old and freshly prepared columns.

When the test is done without *in vitro* addition of corticoids, the amount of corticoids found in the protein fraction is called the spontaneous protein-binding fraction. The latter parameter can be expressed in micrograms of endogenous corticoids bound per 100 ml of plasma or per gram of protein, and also as percentage of the unconjugated corticoid level. After overloading and equilibration (at least 15 minutes) with cortisol, the amount of corticoids found in the protein fraction is defined as the binding capacity. This parameter is independent of the unconjugated plasma corticoid level and is expressed in micrograms of corticoids bound per 100 ml of plasma or per gram of protein.

Heparinized plasmas were obtained at given times of day, from patients hospitalized in the Department of Internal Medicine. Subjects with endocrinological disorders or who had been treated previously with ACTH or corticoids were eliminated from the control ("normal") series; patients with kidney or liver diseases or with hypertension were also excluded. Infusions of ACTH, catecholamines, or corticoids were performed in certain subjects according to methods described previously (8). Corticoid-poor plasma was obtained by sampling blood at 12 p.m. from "normal" subjects; it was considered adequate when the unconjugated plasma corticoid level was about 5 µg per 100 ml fluorescent cortisol equivalents. Erythrocytes were washed six times with isotonic glucose solution. After hemolysis with an equal volume of water and filtration on Whatman no. 1 paper, the mixture was subjected to gel filtration with or without added cortisol or corticosterone (500 mµg per ml). Pleural, spinal, and ascitic fluids were collected in heparinized tubes. Fresh urine samples were used as collected. Plasma protein fractions were prepared by classical salting-out and electrophoretic techniques (9). Rechromatographed, tritium-labeled steroids were used in certain experiments; the fractions collected were counted in an automatic liquid scintillation spectrometer (Tri-Carb, Packard Instrument Co.).

RESULTS

Reliability of the routine method. The method described meets the criteria put forward by Borth (10). The precision of the cortisol-binding capacity assay can be estimated from the standard deviation between simultaneous and random duplicate determinations. The relative standard de-

[3] Standard error of the mean.

TABLE I

*Capacity of corticoid-poor plasma to bind various steroids**

Steroid added	Amount	Unconjugated plasma corticoids	Routine cortisol-binding capacity	Binding capacity for the steroid examined		As compared to cortisol
				Fluorometrically	Isotopically	
	μg%	μg%	μg%	μg%	μg%	%
Cortisol [3]†	50.0	6.0	20.7	21.3	14.7	100
Corticosterone [17]	50.0	5.5	20.2	21.9		108
Deoxycorticosterone [5]	22.0	6.4	20.6		11.7	80
Testosterone [5]	21.6	5.8	20.5		11.4	78

* Mean values expressed in micrograms of steroid bound per 100 ml of plasma.
† [ ] Number of plasmas tested.

viation of the mean was 3.9 per cent in the former and 8.2 per cent in the latter; at least 30 duplicate determinations were done in each series. Another proof of this precision is given by dilution experiments, in which comparable results were obtained after dilution of plasma (1 : 2, 1 : 4, and 1 : 8) with phosphate buffer (0.15 M, pH 7.35). The relative standard deviation of the differences between undiluted and diluted plasma was 7.6 per cent of the mean. In six experiments 2 ml of plasma (instead of 1 ml) overloaded with cortisol (500 mμg per ml) was placed on top of the gel bed with identical results. The smallest single result which could be distinguished with some assurance from zero was 1.74 μg per 100 ml; this was twice the mean standard deviation of 30 duplicate determinations on plasmas with low cortisol binding (5 to 10 μg per 100 ml.) The mean recovery of fluorescent material placed on the columns was 102.5 ± 2.8 (SEM) per cent for 60 experiments; this gives an idea of the accuracy of the method. As for the efficiency of the procedure, one well trained technician was able to perform 20 cortisol-binding determinations a day. Plasma stored in a deep freezer at − 15° C or in a refrigerator at + 4° C keeps its binding capacity for at least 8 days (2 experiments).

Specificity of the steroid-binding plasma protein fraction. Crude human plasma protein fractions were dissolved in physiological saline so as to match physiological concentrations for the given protein fraction. Cortisol (500 mμg) was added to 1 ml of each of these fractions and the mixture subjected to gel filtration. It was shown on repeated occasions that albumin (3 experiments) and globulins with the electrophoretic mobility of β-globulins (5 experiments) or γ-globulins (2 experiments) did not bind cortisol in the system employed here. Globulin fractions with fast α-mobility (9 experiments) showed a marked binding tendency. Further fractionation of active α-globulin preparations with the aid of ammonium sulfate precipitation showed the cortisol-binding activity to be restricted to a protein soluble in $(NH_4)_2SO_4$ at concentrations up to 2.4 M at neutral pH.

Specificity of the steroid moiety of the complex. Normal plasma obtained at 8 a.m. was partitioned on Sephadex columns without adding any steroid. The protein fractions were pooled, extracted, and evaporated, and the extracts were chromatographed in a Bush C (11) paper system. In three such experiments, done in duplicate, 79.2 per cent of the fluorescent material found in the protein fraction proved to be cortisol and 13.0 per cent corticosterone.

Comparative study of the protein binding of various steroids. In a first set of experiments, corticoid-poor plasma was overloaded with various tritium-labeled steroids (500 mμg per ml plasma) and partitioned on Sephadex columns. The amount of bound steroid was determined by counting the protein-containing fractions. For cortisol, an isotopically measured binding capacity of 14.7 μg per 100 ml was found. If the binding of the endogenous corticoids (i.e., 93.4 per cent of 6.0 or 5.6 μg per 100 ml) is added to this figure, a total capacity of 20.3 μg per 100 ml is obtained. The latter value compares within 2 per cent with the value obtained fluorometrically (Table I). This is another check of the specificity of the procedure.

In a *second set* of experiments, corticoid-poor plasma was overloaded with equal amounts of cortisol and another steroid (competition). After 15 minutes of incubation, gel filtration was per-

TABLE II

*Affinity of corticoid-poor plasma for various steroids in the presence of equal amounts of cortisol**

Steroid examined	Unconj. plasma corticoids	Cortisol-binding capacity after overloading with: Cortisol (500 mμg/ml)	Cortisol (500 mμg/ml) + other steroid (500 mμg/ml)	Cortisol "displaced"
	μg%	μg%		%
Corticosterone	6.1	21.0	11.3 [4]†	54
17-Hydroxyprogesterone	7.7	21.4	10.0 [3]	53
Compound S	7.0	20.2	11.4 [4]	44
Cortisone	6.1	18.9	11.3 [3]	40
Androsterone	7.6	20.8	13.0 [4]	38
Progesterone	6.2	20.3	12.5 [2]	38
Testosterone	7.6	20.8	14.3 [4]	31
Pregnanediol	6.4	21.0	16.0 [3]	24
Deoxycorticosterone	7.0	20.5	15.6 [3]	24
Dehydroepiandrosterone	7.1	19.8	16.1 [3]	19
Tetrahydro-S	7.0	20.5	17.5 [3]	15
Pregnanetriol	5.9	20.5	18.1 [4]	12
Δ^5-Pregnenolone	5.9	20.5	18.0 [4]	12
Tetrahydro-DOC	7.3	20.1	18.8 [2]	6

* Mean values in μg/100 ml plasma.
† [ ] Number of plasmas tested.

formed. The amount of bound cortisol was determined fluorometrically. This could be done, since with the exception of corticosterone, the other steroids tested do not fluoresce under the experimental conditions employed. In some instances the amount of the other steroid bound to transcortin was measured by counting of the protein-bound fraction. The results obtained were compared for each plasma with cortisol overloading alone and are summarized in Table II.

Physiology of the globulin-corticoid binding in human plasma. In 20 gel filtration experiments carried out at room temperature, the spontaneous corticoid binding (i.e., the amount of protein-bound corticoids found in plasma not overloaded with cortisol) amounted to 93.4 per cent of the total unconjugated plasma corticoid level. This parameter, however, was not studied extensively. No significant differences in cortisol-binding capacity were noted between males and females. From 15 to 60 years, no influence of age on the above parameter was found. Children 2 months to 14 years old showed a somewhat higher binding capacity (27.1 μg per 100 ml), whereas much lower values were obtained in umbilical cord blood (10.9 ± 0.45 μg per 100 ml), and somewhat lower values in subjects older than 60. In 115 adults (aged 15 to 60) a mean value of 25.9 ± 3.8 (SD) μg per 100 ml plasma or 3.60 μg per g of plasma protein was found, with a between-person variation of 14.7 per cent (Table III). The within-person variation for normal adults was 8.8 per cent when

blood samples were taken on the same day, and 15 per cent when the venipunctures were repeated in the course of several days. The correlation coefficient between the 12 p.m. unconjugated plasma corticoid level and the cortisol-binding capacity was 0.349 ($p < 0.01$) in 48 subjects. Midnight samples taken from subjects sound asleep were thought to be less prone to be influenced by stress.

In the course of pregnancy a marked increase of the cortisol-binding capacity was noted from the second month on. Four subjects in the second month of pregnancy had a mean cortisol-binding capacity of 39.4 μg per 100 ml; 5 subjects in the third month 42.2; 2 subjects in the fourth month 48.4; 2 subjects in the fifth month 52.4; 6 subjects in the sixth month 44.8; 5 subjects in the seventh month 56.2, and 6 subjects in the eighth month 52.4 μg per 100 ml. Six normal males received either two daily doses of 5 mg stilbestrol (2 subjects) or two doses of 0.5 mg ethynyl-estradiol (4 subjects) for 7 days. The 8 a.m. unconjugated corticoids and the cortisol-binding capacity were determined daily. The transcortin-binding capacity rose more rapidly than the unconjugated corticoid level (Figure 3). The spontaneous binding (not shown in Figure 3) followed the unconjugated corticoid level and remained at a normal 93 per cent of this level.

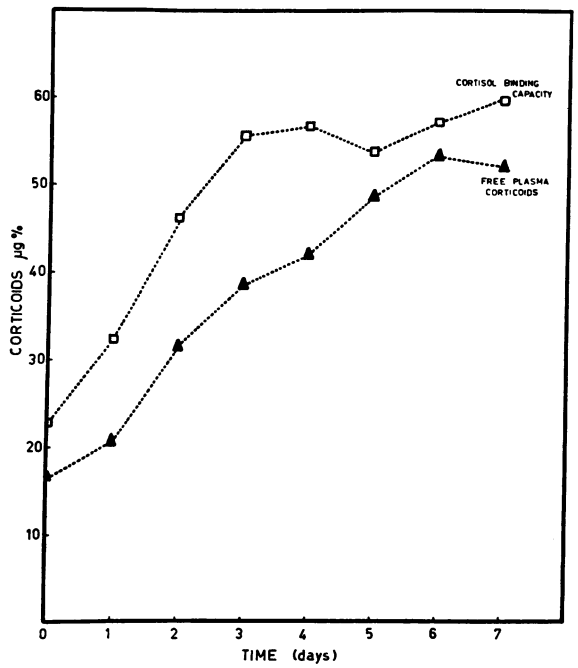

FIG. 3. CORTISOL-BINDING CAPACITY AND UNCONJUGATED (FREE) PLASMA CORTICOIDS IN MALE SUBJECTS RECEIVING DAILY DOSES OF ESTROGEN (6 EXPERIMENTS).

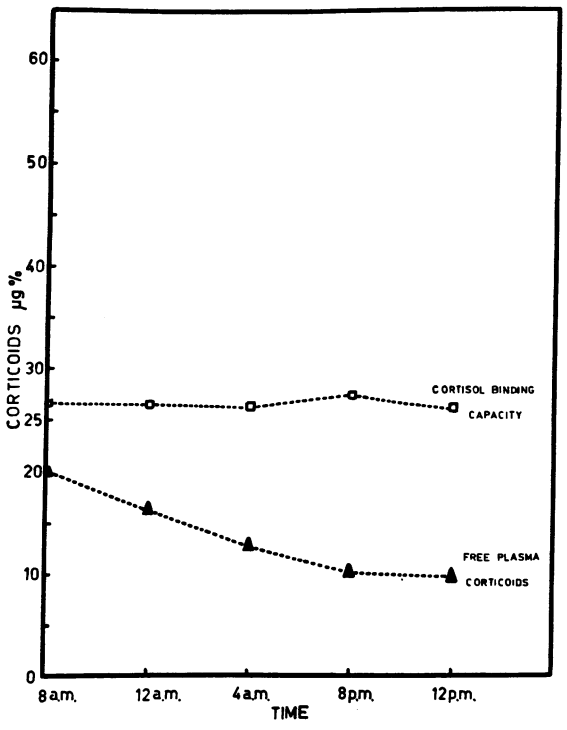

FIG. 4. ABSENCE OF DIURNAL VARIATION IN THE CORTISOL-BINDING CAPACITY OF HUMAN PLASMA (30 EXPERIMENTS).

Plasma cortisol-binding capacity did not show any diurnal variation (Figure 4). Patients whose adrenocortical production had been acutely or chronically suppressed with artificial nonfluorescent steroids (prednisone, prednisolone, triamcinolone or dexamethasone) had a normal cortisol-binding capacity 10 hours after the last peroral administration of the steroid medication. During steroid medication or in the first hours after an intravenous injection of 25 mg prednisolone sodium succinate, an apparent lowering (up to 30 per cent) of the cortisol-binding capacity occurred, possibly caused by competition for the binding sites. The cortisol-binding capacity was not influenced either in males or in females given intravenous infusions of ACTH with or without added catecholamines (Figure 5). Cortisol injected in pharmacological doses in six subjects did not alter the binding capacity in the next 6 hours (Figure 6). Two normal subjects given 2 g of acetylsalicylic acid every 4 hours for a total dose of 6 g, had

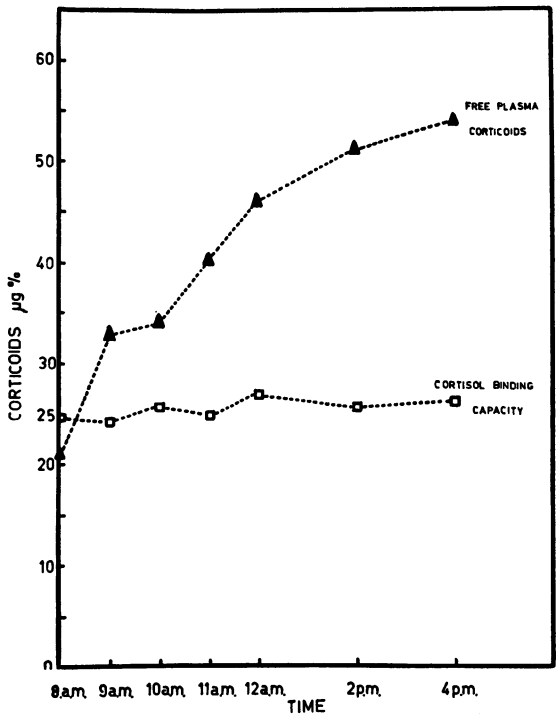

FIG. 5. CORTISOL-BINDING CAPACITY DURING THE INTRAVENOUS INFUSION IN MALES OF ACTH WITH (5 EXPERIMENTS) OR WITHOUT (5 EXPERIMENTS) CATECHOLAMINES. Since almost identical values were obtained in both series, the mean values for the cortisol-binding capacity in the 10 experiments are given. Also shown are the unconjugated plasma corticoid levels of subjects receiving ACTH without catecholamines (5 experiments).

the same binding capacity during and after the test as before.

Cortisol-binding capacity in various pathological conditions. Most of the results obtained are shown in Figure 7. Of interest are the low values found in some uncomplicated cases of obesity with low unconjugated 8 a.m. plasma corticoid levels but normal total plasma proteins. Low levels, on the other hand, were noted in some patients with hypoproteinemia caused by cachexia, hepatic or renal disease, and also in one patient with multiple myeloma. High values were found in some other patients with liver disease and in some patients with pulmonary tuberculosis (mean, 27.1 µg per 100 ml). Normal results were obtained in 26 patients with hypertension (26.8 µg per 100 ml), in 19 patients with diabetes mellitus (25.5 µg per 100 ml), and in 6 patients whose blood was taken immediately and again 2 days after extensive abdominal surgery.

Cortisol-binding capacity of other biological fluids. In three samples of ascitic fluid, binding capacities of 29.3, 13.3, and 12.9 µg of cortisol bound per g of protein were found. In three pleural fluids the values obtained were 18.6, 16.2, and 10.8, and in spinal fluid 43.1, 35.4, 31.3, 26.8, 3.5, and 0.0. In nine fresh urine samples containing up to 4.9 g of protein per L, no cortisol binding could be demonstrated with this system.

Comparative study of the cortisol-binding capacity of plasma of various animals. Heparinized blood was taken from various animals, and the cortisol-binding capacity determined. The values obtained were compared with figures found in the literature for the unconjugated plasma corticoid level of the unstressed animal and also for the biological half-life of cortisol injected in pharmacological doses (Table IV).

Cortisol-binding capacity of red blood cells. In ten experiments with filtered erythrocyte hemolysates, a mean binding capacity of 0.38 µg of cortisol and 0.41 µg of corticosterone per g of protein was found.

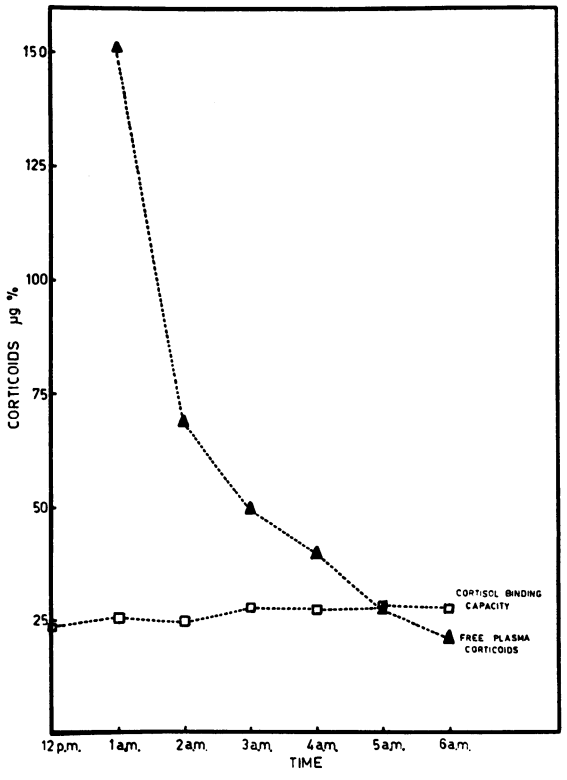

FIG. 6. CORTISOL-BINDING CAPACITY DURING INFUSIONS OF PHARMACOLOGICAL DOSES OF CORTISOL (6 EXPERIMENTS).

TABLE III

Influence of age and sex on the cortisol-binding capacity of human plasma *

Age (yrs):	Cortisol-binding capacity					
	Umbilical cord blood	1–14	15–29	30–44	45–59	60
	μg%		μg%		μg%	
Males		27.2 ± [4]	25.1 ± 1.3 [17]	25.8 ± 0.9 [30]	26.2 ± 0.8 [36]	24.5 ± 0.9 [12]
				25.8 [83]		
	10.9 ± 0.5 [20]					
Females		27.1 [6]	23.4 ± 1.0 [9]	27.6 ± 1.2 [12]	26.8 ± 1.3 [11]	
				26.1 [32]		

* Figures in [ ] indicate number of subjects in each group. Values are means ± SEM.

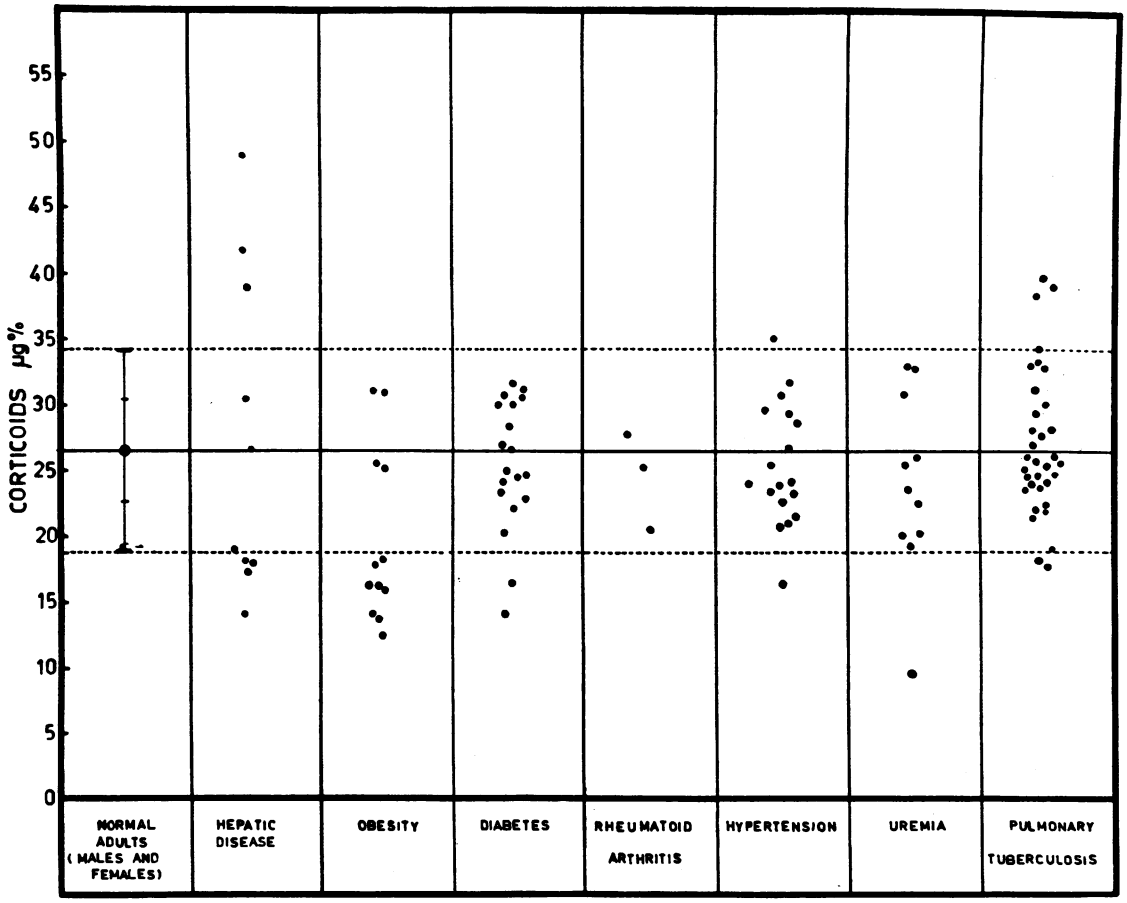

FIG. 7. CORTISOL-BINDING CAPACITY OF HUMAN PLASMA IN VARIOUS PATHOLOGICAL CONDITIONS.

TABLE IV

Correlation of the unstressed, unconjugated plasma corticoid level, the biological half-life of exogeneous cortisol, and the cortisol-binding capacity

Species		Cortisol-binding capacity*	Unconjugated plasma corticoids†	Biological half-life†
		μg %	μg %	*min*
Man	Adults	25.9 [115]	12.1	121 (12, 13)
	Obesity	19.9 [19]	5.9	97 (12, 13)
Guinea pig		29.3 [7]	29.0 (14)	
Rabbit		21.0 [6]		
Rat		15.5 [6]	7.3 (15)	
Hamster		11.4 [3]	1.7 (18)	
Dog		11.0 [6]	1.7	52–61 (16, 17)

* Experimental data reported in this paper; [ ] number of subjects.
† Data from the literature; ( ) reference number.

DISCUSSION

Gel filtration makes it possible to isolate from plasma and other biological fluids a corticoid fraction, which is strongly bound to macromolecular material. In plasma only α-globulin preparations can bind cortisol when overloaded with the hormone and passed through Sephadex columns. These α-globulin preparations can be further purified by ammonium sulfate precipitation. As far as can be inferred from the results obtained hitherto, the latter protein preparations show a physicochemical behavior that is consistent with descriptions of transcortin given in the literature (19). The protein-bound corticoids eluted from the columns proved to be a close estimate of the amount of transcortin-bound corticoids in the plasma sample examined.

The latter affirmation might seem preposterous as one would expect the transcortin-corticoid complex to dissociate continuously by passing through a gel bed devoid of unbound steroid. This exhaustive dialysis effect, however, proved to be unimportant in the case of cortisol and corticosterone, as shown by the clear-cut separation without detectable tailing obtained with these steroids. One would expect this, since it is known that the affinity constant(s) of the transcortin-corticoid binding reaction is very high and that the experimental conditions were set so as to minimize the exhaustive dialysis effect. That some dissociation does indeed occur is shown by the increase of the binding capacity obtained by increasing the elution rate from 0.5 to 2.5 ml per minute and the 93.4 per cent spontaneous binding found in plasma, whereas about 96 per cent binding was to be expected at room temperature. Still better estimation of the bound corticoids could probably be obtained by using somewhat greater elution rates with shorter columns at 4° C. This, however, seemed neither practical nor necessary.

Most current methods for measuring transcortin activity are based on equilibrium dialysis and the use of radioactive steroids. Gel filtration requires no isotopic steroids; being essentially dynamic, only strongly bound corticoids can be separated, but, by the same effect, interference by weakly bound steroids will be largely avoided. In contrast to the relative percentage data obtained by most other methods, the results here can be easily expressed in micrograms per 100 ml of plasma. Increases as well as decreases in binding capacity can be determined, whereas this is difficult with the equilibrium dialysis technique (20).

The parameters defined here must be distinguished from most of those in the literature. Only Upton and Bondy (21) have used a comparable expression to quantitate transcortin activity. Slaunwhite and Sandberg (4, 22) call transcortin binding the percentage of C^{14}-cortisol bound by 10 ml diluted plasma after equilibrium dialysis. Binding capacity is defined as the decrease of C^{14}-cortisol binding caused by addition of 1 μg of non-isotopic cortisol to the approximately 0.3 μg of isotopic hormone used to study transcortin binding. Daughaday (23) defines the combining capacity as the proportion of bound to unbound steroid × protein concentration (in milligrams per milliliter) in the bag after dialysis. In a later paper (20) a double dialysis technique is described and the relative binding activity defined as the amount of bound hormone in the test plasma divided by that in the control sample. In our paper, binding capacity gives a close estimate of the actual amount of steroid which can be bound under "overloaded" conditions per volume of plasma or per weight unit of protein.

According to the literature (20, 24), saturation of transcortin with corticoids must occur at levels

of 20 to 30 μg per 100 ml of plasma. The mean cortisol-binding capacity of 25.9 ± 3.8 μg per 100 ml obtained with gel filtration is in line with this. As suggested by others (22), no significant difference was found between normal males and females or between adults of different age groups (15 to 60 years). The low values noted in umbilical cord blood, and in certain patients with hypoalbuminemia and multiple myeloma, as well as the high ones induced by estrogen impregnation have also been reported already (20, 22, 24). As noted by Sandberg, Slaunwhite and Carter (24), the rise in cortisol-binding capacity induced by estrogen treatment precedes the elevation of unconjugated plasma corticoids. The effect of temperature on transcortin binding described by several authors (e.g. 20) could also be reproduced in gel filtration experiments.

The binding capacity of plasma was not altered by the administration of pharmacological doses of cortisol. This binding capacity also remained unchanged during and after ACTH infusion. Since the unconjugated plasma corticoid level under these circumstances exceeds the transcortin capacity, it is clear that the capacity to bind *added* labeled steroid will be greatly decreased. This explains the results obtained by Sandberg and associates (24). The same results were noted when catecholamines were added to the ACTH infusion. A change in transcortin activity thus cannot explain why the combined administration of ACTH and catecholamines in males produces a much smaller increase of unconjugated plasma corticoids than does the infusion of ACTH alone (8, 25). Neither was transcortin capacity altered after extensive surgery.

Some new information about transcortin has also been gathered. The binding capacity for cortisol remains unchanged during the day, which would mean that the diurnal variation of the unconjugated plasma corticoids is not due to variations in transcortin capacity. The marked decrease in binding capacity observed in some patients with uncomplicated obesity, who also showed a low morning plasma corticoid level, shed new light on the findings of Szenas and Pattee (12). This lowering could be apparent only if transcortin were saturated with abnormal steroids. This mechanism of partial saturation with steroids other than cortisol and corticosterone probably also explains why low cortisol-binding capacities were found during but not after the administration of artificial nonfluorescent steroids.

Some relation seems to exist between the unstressed plasma level of unconjugated corticoids on the one hand, and the cortisol binding capacity and the biological half-life of cortisol injected in pharmacological doses on the other. This was found to be so when blood was taken at 12 p.m. in normal subjects, in patients with obesity, and in various animal species. One might speculate that transcortin binding interferes with liver catabolism (24) and in this way determines the unconjugated plasma corticoid level in the unstressed individual.

In order to study capacity and affinity of plasma to bind steroids other than cortisol and corticosterone, corticoid-poor plasma was taken; thus comparable results could be expected. Even so, the presence of a small amount of endogenous cortisol and corticosterone has to be taken into account. Care was also taken to work under conditions of overloading (at least 200 mμg per ml). Cortisol and corticosterone are bound in comparable amounts. In the near absence of the latter steroids some other steroids can be bound in fair amounts (Table I). The affinity of the binding plasma protein for most of those steroids is definitely smaller than that for cortisol or corticosterone, as can be inferred from the displacement experiments (Table II). Only 17-hydroxyprogesterone and compound S displace about 50 per cent of the cortisol and, thus, most probably have an affinity for transcortin equal to that of cortisol or corticosterone. Tetrahydro compounds, on the other hand, have less affinity for transcortin than their respective parent steroids. Analogous studies have been reported previously (4, 23, 24).

SUMMARY

Gel filtration has been introduced to study protein binding of corticoids (cortisol and corticosterone) and related steroids. A method was devised, which proved to be efficient, accurate, and specific, and which makes it possible to determine, in 1 ml of plasma and without radioactive steroids, the spontaneous binding of corticoids on transcortin and the binding capacity of the latter protein fraction after cortisol "overloading."

The protein moiety of the isolated complex is a fast α_1-globulin with physicochemical properties similar to those attributed to transcortin. Other plasma protein fractions do not bind cortisol in the given circumstances. In native plasmas 92.2 per cent of the steroid moiety of the transcortin complex is either cortisol or corticosterone. In the absence of the latter, other steroids can be bound in almost identical amounts, albeit less strongly.

The cortisol-binding capacity of plasma is the same in male and female adults between 15 and 60 years, the mean value being 25.9 ± 3.8 (SD) μg of cortisol per 100 ml of plasma. There is no diurnal variation, and during or after infusions of cortisol or ACTH no changes in binding capacity, as measured by gel filtration, were found. Low values of binding after cortisol overloading were noted in umbilical cord blood, in patients with hypoproteinemia, in patients treated with high doses of other steroids, and in some cases of uncomplicated obesity; high values were found in some cases of liver disease and pulmonary tuberculosis or after estrogen impregnation.

Transcortin-like binding could also be demonstrated in ascitic, pleural, and spinal fluid, but not in urine. In washed erythrocytes cortisol and corticosterone binding of strong affinity was present, although the capacity per unit weight of protein was much lower than in human plasma.

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REFERENCES

1. Flodin, P. Methodological aspects of gel filtration with special reference to desalting operations. J. Chromatog. 1961, **5**, 103.
2. Porath, J., and Flodin, P. Gel filtration: A method for desalting and group separation. Nature (Lond.) 1959, **183**, 1657.
3. Slaunwhite, W. R. The binding of estrogens, androgens and progesterone by plasma proteins *in vitro in* Hormones in Human Plasma, H. N. Antoniades, Ed. Boston, Little, Brown, 1960, p. 478.
4. Slaunwhite, W. R., and Sandberg, A. A. Transcortin: A corticosteroid-binding protein of plasma. J. clin. Invest. 1959, **38**, 384.
5. De Moor, P., Steeno, O., Raskin, M., and Hendrikx, A. Fluorimetric determination of free plasma 11-hydroxycorticosteroids in man. Acta endocr. (Kbh.) 1960, **33**, 297.
6. De Moor, P., Osinski, P., Deckx, R., and Steeno, O. Further studies of Δ4-3 keto-11 hydroxycorticoid fluorescence. In press.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. biol. Chem. 1951, **193**, 265.
8. De Moor, P., Meulepas, E., Hinnekens, M., Grevendonck, W., and Hendrikx, A. Catecholamines and intermediate corticoid metabolism in normal males and females. Acta endocr. (Kbh.) 1961, **38**, 262.
9. Heremans, J. Les Globulines Sêriques du Système Gamma. Bruxelles, Arscia S.A., 1960.
10. Borth, R. Steroids in human plasma. Vitam. and Horm. 1957, **15**, 259.
11. Bush, I. E. Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. Biochem. J. 1952, **50**, 370.
12. Szenas, P., and Pattee, C. J. Studies of adrenocortical function in obesity. J. clin. Endocr. 1959, **19**, 344.
13. De Moor, P., and Hinnekens, M. The disappearance rate of plasma corticoids in patients with suppressed adrenocortical production. Acta endocr. (Kbh.) 1961, **37**, 1.
14. Silber, R. H., Bush, R. D., and Oslapas, R. Practical procedure for estimation of corticosterone or hydrocortisone. Clin. Chem. 1958, **4**, 278.
15. Yates, F. E., Leeman, S. E., Glenister, D. W., and Dallmann, P. Interaction between plasma corticosterone concentration and adrenocorticotropin-releasing stimuli in the rat: Evidence for the reset of an endocrine feedback control. Endocrinology 1961, **69**, 67.
16. Kuipers, F., Ely, R. S., and Kelley, V. C. Metabolism of steroids: The removal of exogenous 17-hydroxycorticosterone from the peripheral circulation in dogs. Endocrinology 1958, **62**, 64.
17. Samuels, L. T., Brown, H., Eik-Nes, K., Tyler, F. H., and Dominguez, O. V. Extra-adrenal factors affecting the levels of 17-hydroxycorticosteroids in plasma. Ciba Found. Coll. Endocr. 1957, **11**, 208.
18. Schindler, W. J., and Knigge, K. M. Adrenal cortical secretion by the golden hamster. Endocrinology 1959, **65**, 739.
19. Seal, U. S., and Doe, R. P. Purification and properties of "transcortin," the corticosteroid-binding globulin. Fed. Proc. 1961, **20**, 179.
20. Daughaday, W. H., Holloszy, J., and Mariz, I. K. Binding of corticosteroids by plasma proteins. VI. The binding of cortisol and aldosterone by corticosteroid-binding globulin and by the estro-

gen-induced binding system of plasma. J. clin. Endocr. 1961, **21**, 53.
21. Upton, G. V., and Bondy, P. K. The binding of cortisol by plasma protein. Arch. Biochem. **1958**, **78**, 197.
22. Sandberg, A. A., and Slaunwhite, W. R., Jr. Transcortin: A corticosteroid binding protein of plasma. II. Levels in various conditions and the effect of estrogens. J. clin. Invest. 1959, **38**, 1290.
23. Daughaday, W. H. Binding of corticosteroids by plasma proteins. III. The binding of corticosteroids and related hormones by human plasma and plasma protein fractions as measured by equilibrium dialysis. J. clin. Invest. 1958, **37**, 511.
24. Sandberg, A. A., Slaunwhite, W. R., and Carter, A. C. Transcortin: A corticosteroid binding protein of plasma. III. The effects of various steroids. J. clin. Invest. 1960, **39**, 1914.
25. De Moor, P., Hinnekens, M., Steeno, O., Deckx, R., Delaere, K., and Meulepas, E. Corticoid metabolism during the combined administration of ACTH and catecholamines. To be published.