A Procedure for Measuring Plasma Binding of Adrenal Corticoids; Effect of Acute and Chronic Stress.* (28444)

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It has become increasingly apparent that knowledge of the state of association of hormones with plasma proteins may be of importance in interpreting physiological mechanisms such as transport of hormones and their metabolites, activity at the cell membranes of peripheral tissues, and feedback control of pituitary tropins(1-5). A simple method of precipitation of plasma proteins with zinc sulfate has been developed which, when used in combination with standard fluorometric procedures, eliminated residual fluorescence normally observed in the plasma of adrenalectomized animals and demonstrated significant quantitative differences in the apparent plasma binding of steroids under different physiological conditions. It is believed that this method or its principle may have application in other areas of study where steroid binding may be an important consideration.

Materials and methods. Female Sprague-Dawley rats and adult female guinea pigs were used as experimental animals. Adrenalectomized animals were prepared by single stage operation of rats and 2-stage operation in the case of guinea pigs, with the left adrenals being removed 7 days after the right. Animals were given 1% saline to drink after adrenalectomy and plasma samples obtained 12-72 hours after operation. Samples for basal plasma corticoid levels were obtained from rats by decapitation, blood being collected in beakers containing 0.1 ml of 2.4% heparin solution. Guinea pigs were sacrificed by cervical dislocation, neck vessels severed with a razor blade and blood collected in heparinized beakers during a 30 second period which began when the animal was removed from its cage. Three to five minutes of ether or 5-20 minutes of pentobarbital anesthesia were employed as acute stressors. Cold exposure was used as a chronically stressful condition, both rats and guinea pigs being house at 4-5° C in individual cages with minimum bedding for 25-30 days. To avoid differences in plasma corticoid levels due to diurnal variation, all samples were collected between 9:00 and 10:00 A. M.

Fluorometric determination of plasma corticoids was carried out on a 0.5-1.0 ml aliquot of plasma according to the basic procedure outlined by Guillemin *et al.*(6). This method is referred to hereafter as the "regular procedure," to distinguish it from modifications involving zinc sulfate precipitation subsequently described. When determining the corticosterone levels of rat plasma, fluorescence was read 30-40 minutes after addition of 30 N sulfuric acid; for cortisol levels of guinea pig plasma, fluorescence was read 25-30 minutes after addition of sulfuric acid. Standard curves for corticosterone and cortisol ranged from 0.01 to 0.15 μg/ml and 0.03 to 0.30 μg/ml respectively.

From numerous precipitation methods investigated, the following is considered the most reliable and readily utilized in conjunction with the regular fluorometric procedure. A suitable aliquot of plasma is diluted to 2.0 ml with water and partitioned with 8 ml practical grade isooctane. The isooctane phase is removed by careful aspiration and discarded; after addition of 7 ml of water, 1 ml of a freshly prepared 10% aqueous solution of zinc sulfate is added and mixed by shaking the tube. After centrifuging, the protein-free supernatant (containing unbound corticoid) is decanted into a 50 ml glass-stoppered centrifuge tube, extracted with 15 ml of chloroform and processed further as in the regular procedure. Appropriate blanks (2 ml water or plasma of adrenalectomized animals) and standards (0.25 μg corticosterone or 0.5 μg cortisol) are processed with each set of samples. In all definitive experiments, duplicate plasma samples of control and experimental animals were processed simul-

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TABLE I. Examination of $ZnSO_4$-Precipitation Method Using Plasma of Adrenalectomized Guinea Pigs to Which Cortisol Is Added.

	Regular procedure			$ZnSO_4$-precipitation		
		Cortisol			Cortisol†	
Sample	Residual fluor*	μg	%	Residual fluor*†	μg	%
Plasma, adrx‡	15.1 ± 2.1 (6)‖	—	—	3.6 ± .5 (6)	—	—
Plasma, adrx + .5 μg cortisol§	—	.44 ± .04 (6)	88	—	.41 ± .03 (5)	82

* Fluorescence equivalent to that of cortisol (μg/100 ml).
† Fluorescence and cortisol of protein-free supernatant.
‡ Plasma of animals adrenalectomized 12-72 hr.
§ Cortisol added 0.5-2 hr before processing of samples.
‖ Mean ± S.D.; No. of samples in parentheses.

TABLE II. Examination of $ZnSO_4$-Precipitation Method Using Plasma of Adrenalectomized, Normal and Acutely Stressed Rats and Guinea Pigs.

		Regular procedure		$ZnSO_4$-precipitation	
Condition	Species	Residual fluor*	Corticoid†	Residual fluor*	Corticoid, μg/100 ml
Adrx	Rat	4.8 ± .7 (7)§	—	0 (7)	—
	GP	14.7 ± 3.1 (8)	—	2.8 ± .4 (7)	—
Normal	Rat	—	11.9 ± 3.0 (5)	—	0 (5)
	GP	—	15.0 ± 2.7 (8)	—	0 (7)
Stressed‡	Rat	—	49.5 ± 4.6 (7)	—	24.2 ± 3.6 (6)
	GP	—	70.2 ± 5.2 (8)	—	51.8 ± 5.1 (8)

* Fluorescence equivalent to that of corticosterone and cortisol (μg/100 ml) respectively.
† Corticoid: rat = corticosterone; guinea pig = cortisol.
‡ Ether anesthesia.
§ Mean ± S.D.; No. of samples in parentheses.

taneously by the regular procedure and by the zinc sulfate precipitation method.

Results. Zinc sulfate precipitation. When processed according to the regular fluorometric procedure, plasma of adrenalectomized rats and guinea pigs exhibited fluorescence of non-steroidal origin (Tables I, II), a phenomenon noted by other investigators. This residual fluorescence is of considerable magnitude in the case of guinea pig plasma, amounting to fluorescence equivalent to 15 μg cortisol/100 ml of plasma. After precipitation of the plasma proteins with zinc sulfate, the residual fluorescence is removed completely in the case of rat plasma and reduced to a cortisol equivalent of 2.8-3.6 μg/100 ml in guinea pig plasma (Tables I, II). The final concentration of zinc sulfate used in the present procedure is approximately 0.084 M. Almost complete removal of the residual fluorescence of plasma of adrenalectomized rats can be accomplished with as little as 0.01 M zinc sulfate; at lower concentrations the fluorescence returns abruptly. Attempts to precipitate plasma proteins with other precipitating agents such as ammonium or sodium sulfate, acetone or ethanol led to erratic fluorometric readings of the supernatant.

In vitro binding of exogenously added cortisol as well as the effect of the additional zinc sulfate precipitation step in the fluorometric procedure on recovery of steroid from plasma was investigated by adding 0.5 μg cortisol to 2 ml of plasma from adrenalectomized guinea pigs. When processed according to the regular fluorometric procedure and corrected for residual fluorescence, 88% of added cortisol was recovered (Table I); after precipitation with zinc sulfate, 82% of added cortisol was recovered in the protein-free supernatant. Incubation of 0.5 μg cortisol with 2 ml of plasma for periods up to 24 hours did not decrease the amount of steroid recovered in the protein-free supernatant. It is of interest to note that exogenously added

TABLE III. Comparison of Unbound and Plasma-Bound ($ZnSO_4$-Precipitated) Corticoids During Acute and Chronic Stress in Rats and Guinea Pigs.

Condition	Species	Corticoid fraction, μg/100 ml‡		
		Total	Unbound	Bound
Normal	Rat	13.2 ± 2.7 (10)§	0	13.2 ± 2.7
	GP	13.6 ± 3.1 (8)	0	13.6 ± 3.1
Acute stress*	Rat	41.1 ± 5.3 (9)	21.0 ± 2.1	20.0 ± 3.0
	GP	66.0 ± 7.3 (8)	52.1 ± 4.2	14.5 ± 2.8
Chronic stress†	Rat	41.0 ± 4.6 (7)	16.6 ± 3.3	24.7 ± 2.8
	GP	58.4 ± 5.0 (9)	14.5 ± 2.8	45.0 ± 2.7

* Ether anesthesia in the case of rats and pentobarbital anesthesia for guinea pigs.
† Cold exposure (4-5°C), 25-30 days.
‡ Corticoids: rat = corticosterone; guinea pig = cortisol.
§ Mean ± S.D.; No. of samples in parentheses.

estrogens likewise do not bind to serum proteins *in vitro*(2) or to proteins of placental origin(7); the liver, however, has been implicated as an important source of proteins for strong steroid-protein complexes(3).

Several supplementary experiments were performed to demonstrate *in vivo* binding of exogenously administered cortisol. One mg of cortisol in saline suspension was administered intraperitoneally to 2 adrenalectomized guinea pigs; 15-30 minutes later the animals were sacrificed and duplicate plasma samples processed by the regular and zinc sulfate precipitation methods. Total cortisol concentrations (regular procedure) were 12 and 18 μg/100 ml; the unbound cortisol concentrations (zinc sulfate precipitation procedure) were 0 and 7 μg/100 ml. The differences between these values, representing significant plasma binding of the cortisol absorbed during the 15-30 minute period after injection, indicate that an *in vivo* mechanism is apparently necessary to effect the protein-steroid association which is demonstrated by zinc sulfate precipitation.

Basal corticoid levels and response to stressors. Table II summarizes corticoid levels observed in plasma of normal and acutely stressed rats and guinea pigs. Basal levels of corticosterone in rats are 11.9 μg/100 ml; after precipitation with zinc sulfate no corticoids are found in the protein-free supernatant. Basal cortisol levels in guinea pigs (15.0 μg/100 ml) similarly appear to be entirely protein-bound since no fluorescence greater than the residual value is present in the protein-free supernatant. After acute stress of ether anesthesia, markedly elevated corticoid levels are observed in both rats and guinea pigs; in both species a major portion of these elevated steroid levels appear to be unbound since they are recovered from the protein-free supernatant after zinc sulfate precipitation (Table II). During acute stress, the increment in plasma-bound steroid over that of the non-stressed level was much greater in the rat than in the guinea pig, the level of bound steroid rising from means of 11.9 to 25.3 μg/100 ml in the rat and from 15.0 to only 18.4 μg/100 ml in the guinea pig. A similar result was obtained in the acutely stressed animals of the experiments shown in Table III.

Zinc sulfate precipitation was used to demonstrate differences in steroid binding under chronically stressful conditions in rats and guinea pigs exposed to cold for 25-30 days. Plasma samples of additional normal and acutely stressed animals were processed simultaneously to provide reliable comparisons. Corticoid values obtained by the regular procedure are listed in Table III as "total"; corticoid values determined in the protein-free supernatant are recorded as "unbound," and the differences are listed as "bound." Chronic cold exposure results in high total corticoid levels in both rat and guinea pig; when analyzed with the zinc sulfate precipitation method, significant differences were noted between acute and chronic stress with respect to the amount of unbound and bound corticoid. The results are most marked in the guinea pig (Table III) where the increment in cortisol concentration ob-

served during acute stress is almost entirely in free form, with only 20% of the total corticoid being removed by precipitation of the plasma proteins. In chronic exposure, although the total level of cortisol is approximately the same as during acute stress, approximately 80% of this appears to be protein-bound.

Discussion. The regular fluorometric procedure used in this study represents basically the method outlined by Sweat(8) and Silber *et al.*(9) with modifications described by Guillemin *et al.*(6). The steroid measured by this method has been referred to as plasma "free" corticoid, a term intended to indicate the unconjugated hormone including that which is unbound and presumably that which is in association with protein. Although the authors are not aware of work which has specifically examined whether all of the protein-bound corticoid is measured by this method, the relatively high values obtained suggest a major portion of the bound hormone can be easily dissociated from protein and extracted with chloroform. With this reservation, the plasma corticoid levels determined in the present work by the regular fluorometric procedure are considered to represent the total unconjugated hormone content. Corticoid levels measured in the protein-free supernatant after zinc sulfate precipitation are considered as unbound, with the difference between them representing an indirect estimate for the protein-bound fraction. No satisfactory way amenable to fluorometric measurement has been found to remove corticoid from the zinc-protein precipitate and measure it directly. Since the level of unbound hormone may have greater physiological importance than the protein-bound form, at least with respect to adrenal corticoids(10,11), the inability to measure the latter directly after zinc sulfate precipitation may not be a serious handicap.

The nature of the protein-steroid complex demonstrated by zinc sulfate precipitation is not known, and is complicated by such phenomena as the reported competition of zinc for corticoid binding sites. In spite of these unknown factors associated with the use of zinc, notable differences could be demonstrated (particularly in acutely stressed and chronically cold-exposed guinea pigs) in the relative amount of cortisol associated with zinc-precipitated proteins and that remaining unbound in the supernatant. During acute stress only 20% of the cortisol was zinc-precipitable whereas 80% was bound in the plasma of cold-exposed animals. Since a complete time study of the relative proportions of bound and unbound hormone during an adrenocortical response to acute stress was not carried out, no inference should be made with regard to potential changes in the number of binding sites or the amount of cortisol-binding protein suggested by the markedly increased fraction of bound cortisol observed during chronic cold exposure. It is, however, of interest to speculate that there may exist adaptive mechanisms which adjust the amount or capacity of steroid-binding protein in association with the classical feedback mechanisms regulating physiological need for hormones.

Summary. A method is described utilizing zinc sulfate precipitation of plasma proteins which, when used in conjunction with standard fluorometric measurements of corticosterone and cortisol, will eliminate residual fluorescence and demonstrate apparent differences in the relative amount of unbound and protein-bound corticoids in plasma of rats and guinea pigs subjected to several different physiological situations. Under basal, non-stressed conditions the corticoids are almost entirely protein-bound. Acute stress of ether or pentobarbital anesthesia and chronic stress of cold exposure increase markedly peripheral plasma corticoid levels; the increment in corticoids during acute stress is predominately unbound, while the increment during chronic cold is associated with protein precipitated by zinc sulfate.

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Serum Protein Change in Response to Positive Tuberculin Skin Test in Humans.* (28445)

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Although tuberculin sensitivity can be transferred passively with considerable regularity by means of leucocytes obtained from sensitive donors, results of attempts at passive transfer through the use of serum or plasma have been rather equivocal. Zinsser and Mueller(1) described passive transfer of tuberculin sensitivity to guinea pigs using serum from tuberculous rabbits. Best results seemed to prevail if the serum was taken from the donor rabbits 24 hours after they had received an intravenous injection of Old Tuberculin. Sandage and Birkeland(2) reported that blood taken from tuberculous rabbits during the response to intravenous tuberculin conferred tuberculin sensitivity to normal recipient rabbits. Blood taken from tuberculous rabbits without tuberculin treatment did not have this capacity. More recently, Cole and Favour(3) described the successful transfer of tuberculin sensitivity in guinea pigs with plasma obtained from sensitive donors. Activity resided in a new plasma fraction which they definded and named fraction IV-10. It is of interest to the present discussion that their donor animals had to be skin-tested with PPD within 48 hours of being bled in order to yield plasma capable of passive transfer.

The various authors mentioned above have postulated that the response to antigen (O.T. or PPD) by tuberculin-sensitive hosts might cause liberation into the circulating plasma of an "antibody-like" substance responsible for passive sensitization. If this is so, then it seemed reasonable to believe that such a substance might be detectable by suitable biochemical techniques. Investigation of the reactivity of a well-defined human population group to 3 types of tuberculin afforded an opportunity to determine whether there is a significant alteration in serum protein components during the response to skin tests in highly sensitive individuals.

Materials and methods. Blood samples were taken from 19 students at the Fort Sill Indian School, Lawton, Okla. The students ranged in age from 16 to 22 years, with an average of 18 years, and consisted of 15 males and 4 females. Immediately after blood samples were taken the entire group was skin tested with intermediate strength (0.0001 mg) of human, avian, and Battey tuberculins (PPD). Forty-eight hours later the skin reactions were measured and a second blood sample was taken. Analysis of the serum proteins was done on filter paper strips using a Spinco electrophoresis apparatus. All electrophoretic separations were done at room temperature at a constant current in pH 8.6 barbital buffer. At the end of migration the strips were oven dried, stained with bromphenol blue, rinsed in acetic acid, and redried. The protein composition and relative

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