Characteristics of an Endogenous Glucocorticoid Receptor Stabilizing Factor*

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A heat-stable preparation from rat liver cytosol increases the glucocorticoid binding capacity of rat thymocyte cytosol and stabilizes thymocyte binding capacity that has been activated by dithiothreitol. The liver preparation contains at least two heat-stable activities. The activity that increases glucocorticoid binding capacity may represent a rather short lived reducing activity that can be separated from a small M, factor (or factors) that, like molybdate, stabilizes reduced receptors. In unheated cytosol, the majority of the heat stable, receptor-stabilizing activity is present in association with macromolecules but this is released to a small molecular weight form on heating.

The properties of this receptor-stabilizing factor have been examined in a rat liver cytosol assay where the lability of the receptor has been increased by washing the cytosol free of low M, substances by filtration on an Acrodisc UM10 filter. The binding capacity of the washed cytosol preparation is stabilized by addition of boiled cytosol prepared from several rat tissues. The heat-stable factor activity is acid labile and is not affected by incubation with a variety of hydrolytic enzymes, including proteases, nucleases, glycosidases, phospholipase A, lipase, or alkaline phosphatase. The endogenous heat-stable factor(s) is not a nucleotide, a cyclic nucleotide, pyridoxal phosphate, or inorganic phosphate.

Steroid-bound receptors in cytosol washed free of low molecular weight substances are more rapidly transformed to the DNA-binding state on incubation at 15°C than those in normal cytosol. This rapid transformation is inhibited by addition of the heat-stable factor preparation. The two heat-stable activities, inhibition of receptor inactivation and inhibition of receptor transformation, coelute from Sephadex G-10 and from Dowex 1 columns. Both activities have been purified more than 200-fold with respect to Lowry-reactive material.

The heat-stable factor appears to be widely distributed as the activity has been found in primitive eukaryotes, like the lobster and yeast, as well as in avians, amphibians, and mammals. This heat-stable factor(s) may play a role in maintaining the glucocorticoid receptor in the untransformed, steroid-binding form that is found in the cytoplasm.

When cytosols from a variety of tissues are incubated at 25°C, there is a rapid loss of ability to bind glucocorticoids in a specific manner (1, 2). We have shown that inactivated receptors in cytosols prepared from mouse L cells or rat thymic lymphocytes can be reactivated to the steroid binding form by a process that utilizes ATP and a heat-stable factor or factors (3-5). The activation process in rat thymocyte cytosol also requires the presence of a reducing agent, like dithiothreitol, for maximum activation to occur (5). The requirement for a sulfhydryl-protecting agent in cytosols prepared from rat thymocytes (6), lung, and spleen (7) stands in marked contrast to many other well studied systems (e.g. Refs. 4, 7, and 8) where sulfhydryl-protecting agents have no effect on glucocorticoid binding activity.

We have shown that heated cytosol from mouse L cells increases the glucocorticoid binding capacity of rat thymocyte cytosol at 0 to 4°C (3), and Granberg and Ballard (7) have shown that boiled rat liver cytosol increases the specific binding capacity of rat lung cytosol. Although some of this activating effect of heated cytosol preparations can be explained by the presence of sulfhydryl-reducing activity, neither the observations of Granberg and Ballard (7) nor those of our own laboratory (5) are completely explained on that basis alone. We have shown, for example, that a heated rat liver preparation both increases the specific binding capacity of thymocyte cytosol and stabilizes receptors that have been activated with dithiothreitol (3). In this paper we will show that the liver preparation contains at least two heat-stable activities, one of which, like sulfhydryl-reducing agents, activates thymocyte glucocorticoid binding capacity and a second component which, like molybdate, stabilizes the unbound receptor to inactivation and inhibits transformation of the bound receptor to the DNA-binding state.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Stabilization and Activation of Receptors in Thymocyte Cytosol—Addition of dithiothreitol to thymocyte cytosol

1 It should be noted that the term "activation" will be used throughout this paper to describe the process whereby the receptor is converted from a nonbinding form to a form that binds steroids. We use the term "transformation" to describe the process whereby the steroid-bound receptor is converted to a form that binds nuclei, DNA cellulose, etc.

2 Portions of this paper (including "Experimental Procedures," Tables I-VII, and Figs. 3, 8, 10, and 11) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from The Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, MD 20814. Request Document No. 80M-2749, cite authors, and include a check or money order for $6.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
increases glucocorticoid binding capacity and, as shown in Fig. 1, the binding capacity is stabilized by heated liver cytosol or by molybdate. If the binding capacity is partially inactivated by incubating for 30 min at 25 °C and the factor preparation or molybdate is added, stabilization still occurs (Fig. 1). There is no effect, however, if additional dithiothreitol is added. The heated liver preparation has no binding capacity of its own but it can activate thymocyte binding capacity in the absence of dithiothreitol as shown in Fig. 2. Although the activation provided by dithiothreitol is stabilized by molybdate, we have repeatedly observed that the activation provided by the factor preparation is not stabilized by molybdate. Unlike the factor preparation, molybdate alone does not activate or stabilize the binding capacity.

After fractionation of the boiled liver cytosol by Amicon filtration, the stabilizing activity is recovered in the fraction containing components with $M_i$ lying roughly between 1,000 and 10,000 (Fig. 3). As shown in Table I, if the fraction with $M_i < 1,000$ is added to filtered cytosol at twice the concentration used in the experiment of Fig. 3, some stabilization is observed, suggesting that the $M_i$ of the stabilizing factor may be near 1,000 by this method. It is clear from Table I that concentration of components greater than $M_i = 10,000$ or mixing of the nonstabilizing fractions does not inhibit inactivation.

The heated factor preparation and molybdate both have an effect on the receptor in the absence of added reducing activity. This can be seen in the experiment of Fig. 4A where it is clear that in the presence of molybdate the receptor is maintained in a state such that addition of dithiothreitol at any time permits complete reactivation of the binding capacity. The effect of factor in the absence of dithiothreitol is like that of molybdate in that subsequent addition of dithiothreitol will activate the binding capacity well beyond the values observed in the presence of the reducing agent alone (Fig. 4B). It is not clear why the stabilization provided by the heat-stable factor declines after about 1 h. The decline in stabilization is not due

![Fig. 1. Effect of dithiothreitol, molybdate, and a heat-stable preparation from liver on the glucocorticoid binding capacity of rat thymocyte cytosol. Replicate aliquots of thymocyte cytosol were incubated at 25 °C with 2 mM dithiothreitol (DTT), 10 mM sodium molybdate, or an equal volume of heat-treated liver cytosol (f) as noted. At various times, aliquots were removed and assayed for glucocorticoid binding capacity at 0 °C. Incubation conditions: ○, thymocyte cytosol incubated with buffer alone; ●, dithiothreitol added at 0 time; ▼, dithiothreitol added at 0 time and 2 mM dithiothreitol added again at 30 min; △, molybdate added to dithiothreitol-containing cytosol at 30 min; □, molybdate and end dithiothreitol added at 0 time; ▲, f and dithiothreitol added at 0 time.](http://www.jbc.org/)

![Fig. 2. Differences between the effects of molybdate and the heat-stable factor preparation on thymocyte cytosol binding capacity. Thymocyte cytosol was incubated at 25 °C as follows: ○, thymocyte cytosol incubated with buffer alone; ●, plus 2 mM dithiothreitol (DTT); ▼, plus 10 mM sodium molybdate; △, plus dithiothreitol and molybdate; □, plus heat-treated liver cytosol (f); ▲, plus f and dithiothreitol; ●, plus f and molybdate.](http://www.jbc.org/)
to inactivation of the factor as that can be incubated alone or with thymocyte cytosol for many hours at 25 °C without loss of activity (Table II).

It is clear that the stabilizing activity of the heated liver cytosol is different from the activating activity. As shown in Table II, the activating activity (increase in binding activity in the absence of dithiothreitol) is lost when the factor is incubated alone or with thymocyte cytosol for many hours at 25 °C without loss of receptor sulfhydryl groups and it is likely that the activating activity we are observing represents a reducing action that is rather short lived in the system compared to that of dithiothreitol. This would explain why activation provided by the factor preparation is not maintained by molbydate.

In unheated cytosol, most of the stabilizing activity is found in association with components of $M_i > 30,000$. As shown in Table III, if the large $M_i$ components are filtered through an Amicon PM30 filter, little activity passes through the filter. About half of the retained activity is released if the large $M_i$ components are washed once by dilution and refiltration. The results of this experiment suggest that the stabilizing factor is bound to macromolecules in cytosol and that heating denatures these molecules, releasing the small $M_i$ heat-stable factor.

The thymocyte cytosol assay we have used here has two major drawbacks in that the binding capacity is very sensitive to the presence of reducing agents and the cytosol itself contains nucleotides and other small $M_i$ phosphorylated compounds that are known to inhibit inactivation of the binding capacity (1, 5, 8). Accordingly, we have set up an assay system using rat liver cytosol where the binding capacity is not affected by dithiothreitol and where the receptor preparation has been washed in order to eliminate small $M_i$ components.

**Inhibition of Receptor Inactivation and Transformation in Filtered Liver Cytosol**—Specific binding capacity of rat liver cytosol that has been washed by filtration through an Amicon UM10 filter is inactivated more rapidly than that of unfiltered cytosol at both 0 and 20 °C (Fig. 5). When cytosol is washed more than once, the zero time binding value is lowered whereas a single washing results in little or no loss of initial binding capacity. If the filtrate from unfiltered cytosol (data not shown) or the heat-stable factor preparation (Fig. 6A) is added to an equal volume of filtered liver cytosol, the rate of loss is inhibited in a concentration-dependent manner (Fig. 6B). Triamcinolone acetonide-bound receptors in filtered cytosol are transformed to the DNA-binding state more rapidly than those in normal cytosol (Fig. 7A) and the rate of transformation is inhibited by the heat-stable factor preparation (Fig. 7B). If steroid-bound receptors are transformed by heating and the factor is then added, subsequent binding to

![Fig. 5. Inactivation of filtered and unfiltered liver cytosol at 0 and 20 °C.](image-url)

![Fig. 6. Effect of dithiothreitol and heat-stable factor on the rate of inactivation of binding in filtered rat liver cytosol and concentration dependence of factor stabilization.](image-url)
Filtered boiled cytosol was chromatographed on a Sephadex G-10 column as described under "Experimental Procedures." The eluates were monitored at 280 nm (••••); 2-ml fractions were collected, combined, lyophilized, and redissolved in 2 ml of distilled water. A, each fraction was incubated with an equal volume of filtered cytosol for 45 min at 20 °C and the specific binding capacity (○) was assayed. Aliquots of each fraction were also incubated at 15 °C with an equal volume of filtered cytosol pre-bound with [3H]triamcinolone acetonide, and binding to DNA-cellulose (□) was assayed after 1 h, as described under "Experimental Procedures." Each fraction was assayed for (B) conductivity (X--X) and inorganic phosphate (■) and for (C) Lowry (▲) and fluorescamine (Δ--Δ) reactive material.

DNA is not inhibited. Sodium molybdate (10 mM) also blocks both receptor inactivation and transformation in filtered liver cytosol.

As shown in Table IV, incubation of heated cytosol with a variety of hydrolases does not substantially affect the stabilizing activity. Incubation at 37 °C for 120 h with 500 μg/ml of pronase or for 45 min with calf intestine alkaline phosphatase does not affect the factor activity. It is also unaffected by N-ethylmaleimide.

We have previously reported that the binding capacity of rat liver cytosol is partially stabilized by several nucleotides (8). ATP inhibits inactivation of binding capacity in filtered cytosol with a maximum effect observed at 1 to 3 mM (Fig. 8A). The nonmetabolizable ATP analog AMP-PCP produces a modest stabilizing effect and, as shown in Table V, it inhibits part of the stabilization provided by the heat-stable factor preparation, as well as that provided by ATP by decreasing the stabilizing activity of each to the level observed with AMP-PCP alone.

The stabilizing factor is not ATP. The boiled preparation contains only 2 μM ATP, a concentration that is at least two orders of magnitude too low to account for any stabilization. Extraction of heated liver cytosol with activated charcoal does not affect its stabilizing activity while greater than 99% of added [3H]ATP (10 mM) is removed. Other nucleotides and cyclic nucleotides should also be eliminated by the charcoal extraction procedure. Although cAMP does stabilize somewhat, it does so only at concentrations that are at least two orders of magnitude higher than those that exist endogenously. Cyclic GMP has no stabilizing effect. The sum of these observations makes it very unlikely that the factor is a nucleotide.

Sodium phosphate produces a little stabilization with a maximum effect at about 10 mM being only about a third of that provided by the heat-stable factor preparation (Fig. 8B). The concentration of inorganic phosphate in the heated factor preparation is 7 to 8 mM. Inorganic phosphate does not inhibit

1 The abbreviations used are: AMP-PCP, adenylyl (β,γ-methylene)-diphosphonate; Heps, 4-(2-hydroxyethyl)-1-piperazinemethanesulfonic acid.
transformation in filtered cytosol at any concentration and, as with other salts, at 50 mM sodium phosphate and above, transformation is stimulated. Thus, it is clear that neither the receptor-stabilizing nor the transformation-inhibiting activity can be due to inorganic phosphate. Litwack and co-workers (16, 17) have suggested that pyridoxal 5'-phosphate may act as an endogenous receptor "modulator." The factor cannot be pyridoxal 5'-phosphate as this compound (0.01-10 mM) does not inhibit receptor inactivation in filtered cytosol.

Molecular Sieve and Ion-Exchange Chromatography of the Heat-Stable Factor—When boiled cytosol that has been filtered through an Amicon UM10 filter is chromatographed on Sephadex G-10 in 0.1 M Hepes buffer, both transformation-inhibiting activity and receptor-stabilizing activity are recovered in a major peak that elutes after the excluded material (Fig. 9A). A small but consistent peak of elution is stabilized with the major salt peak (determined by conductivity, Fig. 9B). The factor is separated from about 70% of the salt but elutes with both inorganic phosphate and the major peak of Lowry-reactive material (Fig. 9, B and C). The major peak of stabilizing activity is recovered in the same region from longer Sephadex G-10 columns eluted with 200 mM triethylammonium bicarbonate buffer, suggesting that the elution pattern does not reflect ionic interactions with the gel matrix and that the M, is considerably less than 700 by this method.

Boiling the Sephadex G-10 peak material for 5 min in 6 N HCl eliminates both receptor-stabilizing and transformation-inhibiting activities.

Using the receptor-stabilizing assay, we have determined that the factor in boiled cytosol is adsorbed to Dowex AG 1-X8 (HCOO-) but not to Dowex 50W-H+ using 0.1 M Hepes, pH 7.35, for the column wash. In the experiment of Fig. 10, the fractions containing the major peak of receptor-stabilizing activity from Sephadex G-10 were combined and chromato- graphed on Dowex 1. Both the receptor-stabilizing and transformation-inhibiting activities elute in a sharp peak at about 0.25 M triethylammonium bicarbonate. This peak contains all of the inorganic phosphate and a small amount of fluorescamine-reactive material. Although the activity is recovered in a single major peak from both Sephadex G-10 and Dowex 1, it is not yet known if the factor is a single entity or whether the activity represents the action of multiple small M, negatively charged compounds present in cytoplasm.

In the experiment of Table VI, both inhibition of receptor inactivation and transformation were assayed with multiple concentrations of the heat-stable factor preparation at each step through Dowex 1. Inhibition was plotted as shown in Fig. 11 and units of inhibitory activity were calculated from the 50% inhibitory concentration as described under "Experimental Procedures." As can be seen from Table VI, the factor activity is separated from a considerable amount of the Lowry- and fluorescamine-reactive material through the Sephadex G-10 step. Both the rate of receptor inactivation and the rate of transformation are increased by salt, and desalting with Sephadex yields some increase in activity in both assays, commensurate with elimination of the salt effect. Although the Dowex step is useful for defining the charge behavior of the factor, it is not of much value in purification as the apparent yield is low.

As the major peak of receptor-stabilizing activity and the transformation-inhibiting activity co-elute on Sephadex G-10 and Dowex 1 chromatography, it is possible that the same heat-stable component is responsible for both activities. We would speculate that the receptor-stabilizing factor that we have reported in this and previous papers (3, 5) may be identical with the small M, heat-stable transformation inhibitor reported by others (18-21). The heat-stable factor should not be considered to have a function that is unique to glucocorticoid receptor systems. Sato et al. (22) have recently reported that transformation of both androgen and estrogen receptors is also inhibited by a small M, (diabizable) factor (or factors) present in cytosol. As the rates of receptor inactivation and transformation are both increased when the inhibitor is removed, it seems plausible to suggest that the factor plays some role in the regulation of these processes.

Although the work we have presented in this paper has focused on heat-stable factor in rat liver cytosol, we have found the same activity in boiled supernatants prepared from rat brain, heart, kidney, lung, muscle, spleen, and thymus. As shown in Table VII, receptor-stabilizing activity is present in heated liver cytosol from chicken and frog as well as mammals and it exists in primitive systems, like lobster and yeast, which are lower on the evolutionary scale than the point at which glucocorticoid receptors emerged. The boiled preparations from chicken liver, lobster, and yeast have also been tested for inhibition of transformation and found to be active in that assay as well. We have not found factor activity in calf serum or in boiled sonicates prepared from Escherichia coli and Salmonella typhimurium.

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Glucocorticoid Receptor Stabilizing Factor

EXPERIMENTAL PROCEDURES

Cell culture and Polarization - Fetal lymphocytes and liver were obtained from two pregnant, 25- to 30-week-old, female Javanese baboons as previously described (11). The baboon lymphocytes were transferred to RPMI-1640, 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mmol/l L-glutamine, and 10% fetal calf serum at 37 °C in humidified 95% air and 5% CO2. The liver cells were maintained in RPMI-1640, 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mmol/l L-glutamine, and 10% fetal calf serum at 37 °C in humidified 95% air and 5% CO2. The liver cells were harvested and seeded into 10-cm dishes in RPMI-1640, 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mmol/l L-glutamine, and 10% fetal calf serum at 37 °C in humidified 95% air and 5% CO2. The liver cells were maintained in RPMI-1640, 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mmol/l L-glutamine, and 10% fetal calf serum at 37 °C in humidified 95% air and 5% CO2.

In the experiment of Fig. 1, a series of washed unfractionated of seeded liver cultures were prepared according to the following procedure. Twelve 6-cm dishes of each cell type were seeded with 1 × 10⁶ cells/ml on seeding day 0. The cultures were grown in RPMI-1640, 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mmol/l L-glutamine, and 10% fetal calf serum. The cultures were maintained in RPMI-1640, 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mmol/l L-glutamine, and 10% fetal calf serum at 37 °C in humidified 95% air and 5% CO2. The liver cells were maintained in RPMI-1640, 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mmol/l L-glutamine, and 10% fetal calf serum at 37 °C in humidified 95% air and 5% CO2.

Determination of monocyte/macrophage colony forming units - Monocytes/macrophages were determined by counting the monocyte/macrophage colony forming units (CFUs) in 1 ml of RPMI-1640, 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mmol/l L-glutamine, and 10% fetal calf serum at 37 °C in humidified 95% air and 5% CO2. Monocytes/macrophages were determined by counting the monocyte/macrophage colony forming units (CFUs) in 1 ml of RPMI-1640, 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mmol/l L-glutamine, and 10% fetal calf serum at 37 °C in humidified 95% air and 5% CO2.

For the determination of monocyte/macrophage colony forming units (CFUs), the cultures were prepared by counting the monocyte/macrophage colony forming units (CFUs) in 1 ml of RPMI-1640, 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mmol/l L-glutamine, and 10% fetal calf serum at 37 °C in humidified 95% air and 5% CO2.

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Table II

| Glucocorticoid Receptor Stabilizing Factor |
|-------------------------------------------|
| Additions to thrombin optimal | Specific binding capacity |
| | (cpm/µg antigen) |
| 90% | 0 |
| 10% | 0 |
| 0.1% | 0 |
| 0.01% | 0 |

Table III

| Recovery of heat-stable factor activity from heated optimal anti-coagulant optimal anti-coagulant optimals |
|-----------------------------------------------|
| Additions to thrombin optimal | Specific binding capacity |
| | (cpm/µg antigen) |
| 90% | 0 |
| 10% | 0 |
| 0.1% | 0 |
| 0.01% | 0 |

Table IV

| Effect of various eluents on the stabilizing activity of the heated liver preparation |
|-----------------------------------------------|
| Additions to thrombin optimal | Specific binding capacity |
| | (cpm/µg antigen) |
| 90% | 0 |
| 10% | 0 |
| 0.1% | 0 |
| 0.01% | 0 |

Table V

| Effect of ATP and AMP-PCP on the stabilizing activity of the heated liver preparation |
|-----------------------------------------------|
| Additions to thrombin optimal | Specific binding capacity |
| | (cpm/µg antigen) |
| 90% | 0 |
| 10% | 0 |
| 0.1% | 0 |
| 0.01% | 0 |

Figure 1

*Effect of ATP and AMP-PCP on the rate of inhibition of binding capacity of heated optimal anti-coagulant optimal anti-coagulant optimals.* A. Heated optimal anti-coagulant optimal anti-coagulant optimals was isolated for 1 hour at 37°C with various concentrations of ATP (A) or with an equal volume of heated liver optimals (B). The specific binding capacity was measured at 37°C as described under "Methods." B. Heated optimal anti-coagulant optimal anti-coagulant optimals was isolated for 1 hour at 37°C with various concentrations of ATP (A) or with an equal volume of heated liver optimals (B). The specific binding capacity was measured at 37°C as described under "Methods."
Glucocorticoid Receptor Stabilizing Factor

**Table VI**

| Step | Inhibition of Inactivation | Inhibition of Transformation | Total Protein (mg) | Total Fluorescent Receptor (ng) | Total Radioactive Quinones (μg) | Total Quinones (μg) |
|------|-----------------------------|----------------------------|-------------------|---------------------------------|--------------------------------|-------------------|
| Raw chicken | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Boiled supernatant | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Ammonium Sulfate | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Sephadex G-100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Dowex l | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

Assuming that the factor present in the crude extract is entirely recovered in the boiled supernatant.

**Table VII**

| Species | Stabilizing Activity | Specific Binding Capacity |
|---------|----------------------|--------------------------|
| Human | 55 | H.D. |
| Conventional | 95 | H.D. |
| Human monkey | 65 | H.D. |
| Rat liver | 95 | 456 |
| Antia | 100 | 96 |
| Amphibian | --- | --- |
| Fish | 100 | 100 |
| Arthropod | 93 | 0 |
| Plant | 93 | 0 |
| Ummuml | 100 | 0 |

**Figure 1**

Inhibition of receptor inactivation and transformation by partially purified factor preparations. Boiled chicken was lyophilized and reconstituted to half the original volume with distilled water. This solution, which was concentrated two-fold with respect to the original factor preparation, was assayed either undiluted or after dilution with distilled water. Following boiled chicken (7.6 ml) was chromatographed on a Sephadex G-100 column (2.2 ml). The void volume fraction was adsorited to a column of Sephadex G-100, lyophilized, redissolved in 3.7 ml distilled water, and various dilutions were assayed. For the Dowex I preparation, the Sephadex G-100 stabilizing fractions from 20 ml of filtered, boiled chicken were combined, lyophilized, redissolved in 3.7 ml of 10 ml Hepes buffer, and adjusted to pH 7.5 with 1 N Tris base. Assaying that these animals were not pretreated prior to removal of the organ.

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Characteristics of an endogenous glucocorticoid receptor stabilizing factor.
K L Leach, J F Grippo, P R Housley, M K Dahmer, M E Salive and W B Pratt

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