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RECENT STUDIES ON THE BINDING OF CORTISOL IN SERUM*

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SUMMARY
The isolation of CBG by affinity chromatography is described. The amino acid composition of this preparation is in good agreement with CBG isolated by standard techniques. The effect of cortisol on lymphocytes and tyrosine amino transferase activity *in vivo* is shown not to be altered by the presence of CBG. The kinetics of the dissociation of the CBG-cortisol complex is studied in a flowing system at various temperatures and the activation energy for this dissociation calculated to be 21 kcal/mol. Proteins other than CBG which bind to cortisol covalently linked to Sepharose are isolated and identified as IgG, IgM, and IgA. It is not yet clear whether these are naturally-occurring antibodies to cortisol or proteins which bind cortisol at non-antibody combining sites.

INTRODUCTION
THE PRESENCE in human plasma of a macromolecule, corticosteroid-binding globulin (CBG, transcortin), which binds cortisol was established in three different laboratories in the period 1957–1959[1–3]. In the decade following its discovery, the protein was isolated from human[4–6], rat[7], rabbit[8] and cavian[9] plasma. Some of the physicial properties and the amino acid composition of the isolated protein have been described for each of the species from which it has been isolated. The factors affecting the concentration of CBG in plasma, its distribution in vertebrate species, and its affinity for various steroids have been intensively studied. The results of such investigations have been the subject of several recent reviews[10–15], and it will not be the purpose of this communication to review again that which has been summarized both recently and well by others. Rather, we will present a novel method for the isolation of CBG and some experiments conducted with purified human and rat CBG.

In spite of the fact that it has been possible to isolate CBG since 1962[4] there has been no physiological work done with pure preparations of the protein. The reason for this lapse is two-fold. CBG is a trace protein and its isolation in large quantities requires extremely large amounts of starting material. The standard method of isolation, or minor variations on it, is not truly suitable for working with large volumes. In addition, even when working with moderate volumes of plasma, the isolation procedure is quite time-consuming. In the past year we have developed a method for the isolation of CBG using affinity chromatography[16]. The advantage of this method is that it allows one to isolate CBG rapidly and from large volumes of starting material.

ISOLATION AND PROPERTIES OF CBG
Isolation by affinity chromatography[16]

In general, affinity chromatography[17] is a method for protein purification wherein a reagent is synthesized by covalently linking a ligand, with specific

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affinity for a protein, to an insoluble support. This reagent is exposed to the mixture of proteins containing the protein of interest, and the material with little or no affinity for the ligand is washed off the reagent leaving the specific protein bound to it. Suitable conditions for the elution of the bound protein are then determined.

A scheme for covalently coupling cortisol to Sepharose 4B is outlined in Table 1. Note that dioxane is the solvent in which cortisol hemisuccinate is coupled to Sepharose. Although it has been stated that dioxane damages Sepharose [17], we have not found this to be the case, and consider dioxane to be a most convenient solvent for this reaction. To prepare CBG, serum is mixed with cortisol Sepharose in a batchwise fashion, first at 23°C and then at 4°C, allowing sufficient time for the CBG to become bound to the cortisol Sepharose. It is important to work with an excess of cortisol Sepharose since sites on CBG that are occupied by endogenous cortisol will not be available for binding to cortisol linked to the reagent. At equilibrium, an important determinant of the distribution of CBG between cortisol bound to Sepharose and that bound to endogenous cortisol will be the ratio of the concentrations of the two types of cortisol in the reaction mixture. After the CBG has become bound to the cortisol Sepharose, most of the excess plasma is removed by decantation. Almost all of the remaining contaminating proteins are removed at 4°C by washing with buffer in a column on a fraction collector. When the optical density (280 nm) is 0, the eluting conditions are changed in order to obtain the CBG. Four changes are made: cortisol is added to the eluting buffer; the pH of the eluting buffer is raised; the ionic strength of the eluting buffer is increased; and the temperature of the gel is raised from 4 to 24°C. Figure 1 illustrates the manner in which we elute CBG from cortisol Sepharose. Pool I contains CBG and gamma globulins in a ratio of about 1:4. Pool II contains traces of CBG and is mostly gamma globulin. The CBG, in 58 per cent yield, is obtained in pure form after a single chromatography of Pool I on hydroxylapatite [4, 16].

Table 1. Preparation of cortisol sepharose

1. Prepare amino Sepharose according to Cuatrecasas (17) using 200 mmol 3,3′-diaminodipropylamine and 25 g cyanogen bromide/100 ml of packed gel. The yield is approximately 10 μmoles/ml of packed gel.
2. Prepare activated cortisol hemisuccinate by combining 10 mmol of the steroid ester with 10 mmol of dicyclohexylcarbodiimide in 50 ml of dioxane and stirring for 70 min.
3. Filter the reaction mixture from Step 2, add the clear filtrate to 100 ml of amino Sepharose suspended in dioxane, and stir gently for 3 h at room temperature.
4. Wash the gel first with 10 l of dioxane in order to remove the unreacted steroid, and then with about one liter of water in order to remove the dioxane.
5. The final product consists of about 2 μmol of cortisol/ml of gel.

Some properties of human CBG

The CBG which we isolated migrated as a single band on polyacrylamide gel electrophoresis, had 1·01 binding sites/mol, and an association constant at 23°C of $5{\cdot}2 \times 10^7\ M^{-1}$.

The dried protein was subjected to hydrolysis in 6N HCl *in vacuo* at 110°C for 20 h and an amino acid analysis done on a Beckman model 121 amino acid

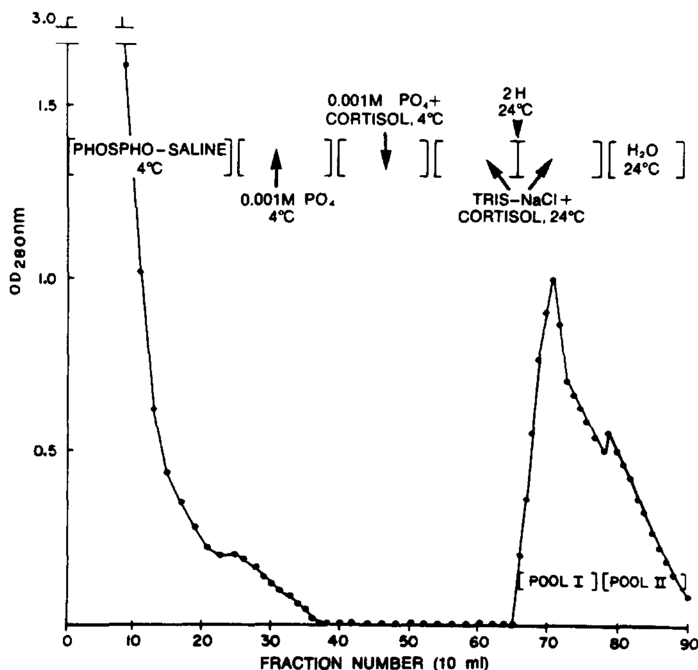

Fig. 1. Elution of CBG from cortisol sepharose. The chromatography of CBG from 2 l. of plasma is shown. Most of the plasma is removed by decantation and washing on a Buchner funnel before the first O.D. is obtained. The conditions in brackets indicate what was added to the column at the stage indicated. Pools I and II were chromatographed further on hydroxylapatite. From Rosner and Bradlow[16].

analyzer. These results along with analyses from two other laboratories are shown in Table 2. As is apparent from the table, our data, with the exception of proline, are in reasonable agreement with the amino acid composition of CBG isolated by standard methods.

Sequence analysis was done on 0·25 μmol of CBG by the automated phenylisothiocyanate degradation method[18] using a Beckman Protein/Peptide Sequencer. The following unique partial NH_2-terminal amino acid sequence was determined:

Arg-Glx-Pro-Asn-Ala-Ala-Tyr-Val-.

Work is in progress to determine the complete amino acid sequence of human CBG.

It has been observed that both rat[7] and cavian[9] CBG polymerize, and specifically denied that the human protein forms polymers[9]. The polymerization of rat CBG can be controlled by the amount of cortisol present whereas the polymerization of the cavian protein seems to be independent of the presence of steroid[9]. We have now observed that human CBG behaves in a manner similar to that obtained from guinea pigs. Figure 2 illustrates this phenomenon. The addition of an 8·5-fold molar excess of cortisol to human CBG followed by incubation for 19 h did not affect the polymeric composition of this preparation of CBG. Electrophoresis of the polymerized CBG on SDS gels[19] showed a single band of molecular weight 56,000. The molecular weight cannot be assumed to be accurate since this system of electrophoresis has not been standardized for

Table 2. Amino acid composition of human CBG

	A	B	C	C/B
Lysine	10	15	15	1·0
Histidine	9	9	10	1·1
Arginine	17	10	10	1·0
Aspartic acid	25	32	32	1·0
Threonine	17	22	19	0·86
Serine	20	22	25	1·1
Glutamic acid	34	38	27	0·71
Proline	24	22	9	0·41
Glycine	21	19	18	0·95
Alanine	23	23	22	0·96
Half-cystine	1	2	X	X
Valine	16	26	20	0·77
Methionine	3	10	10	1·0
Isoleucine	9	16	14	0·88
Leucine	37	40	34	0·85
Tyrosine	6	10	8	0·80
Phenylalanine	14	19	17	0·89
Tryptophan	3	3	X	X

The amino acid composition is reported as moles of amino acid per mole of protein. The data in Column A are from Seal and Doe[4], those in Column B from Muldoon and Westphal[6] and those in Column C from this laboratory.

glycoproteins. The experiment does, however, reveal that the polymers can be deaggregated under the conditions in which these gels are run (1 per cent β mercaptoethanol, 1 per cent dodecyl sulfate) and, further, since subunit structure would be expected to be disrupted under these conditions, it confirms the sequence data which indicate that the protein consists of a single polypeptide chain.

Although the association constants for various steroids with CBG have been documented, only Dixon[20] has attempted to study the rate constants governing this interaction. In his experiments, accurate determination of free steroid as a function of time was rendered difficult by the fact that the bound and free steroid were measured on columns of Sephadex rather than in a flowing system. We have used the flowing system of Colowick and Womack[21] in order to enable us to measure rates, rather than equilibrium constants as suggested by those authors. The apparatus consists of an upper chamber, containing CBG at known concentration, separated by a semi-permeable membrane from a lower chamber through which buffer is pumped at a constant rate. After addition of [^{3}H]-cortisol to the upper chamber the effluent from the lower chamber is sampled for the determination of [^{3}H]-cortisol. The major modification we have made in the design of the apparatus is to alter it so that it can be completely immersed in a constant temperature bath, thus allowing measurements to be made at known precise temperatures. Control experiments with buffer and [^{3}H]-cortisol in the upper chamber are first conducted at several temperatures so that the fraction of the steroid dialyzing, [CPM in effluent]/[CPM in upper chamber], can be related to the concentration of *free* steroid in the upper chamber at any time period. When CBG is present in the upper chamber, then, all other things being equal, the [^{3}H]-cortisol appearing in the effluent from the lower chamber will be a measure of the *free* steroid in the upper chamber. The absolute concentration of bound

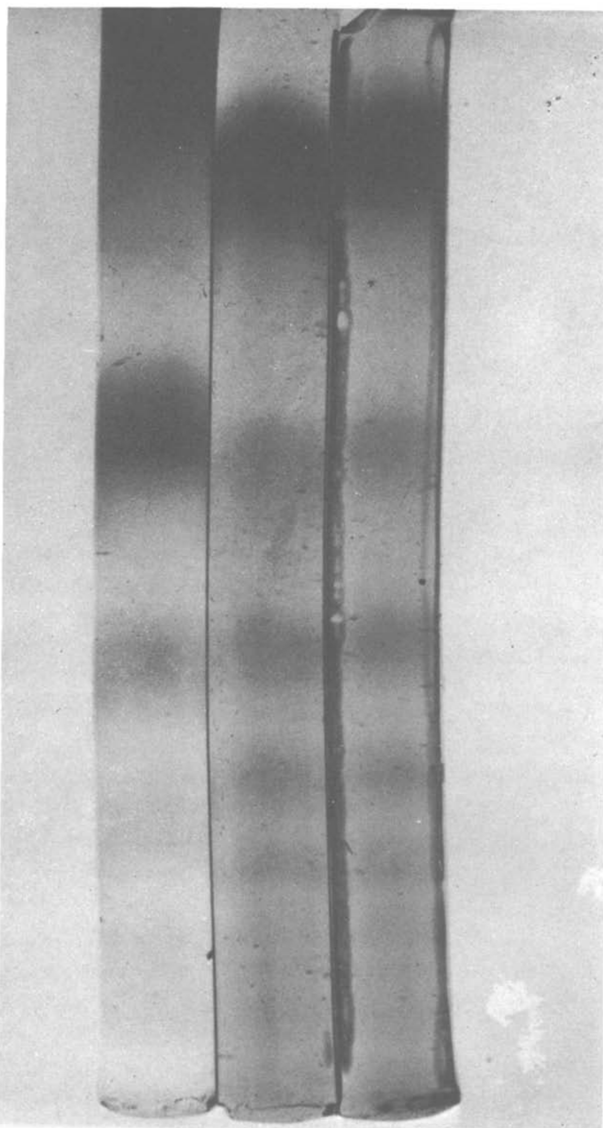

Fig. 2. Polyacrylamide gel electrophoresis. Electrophoresis, on 7 per cent acrylamide at pH 9·5, of a polymeric preparation of human serum albumin (gel on left), human CBG (gel in the middle), and human CBG exposed to an 8·5 molar excess of cortisol (gel on right) for 19 h prior to electrophoresis. The albumin is shown for comparison. The polymeric preparation of CBG was originally shown to be a single band in this same system. It was stored in solution for two days at 4°C and then dried and stored at − 20°C for 24 days.

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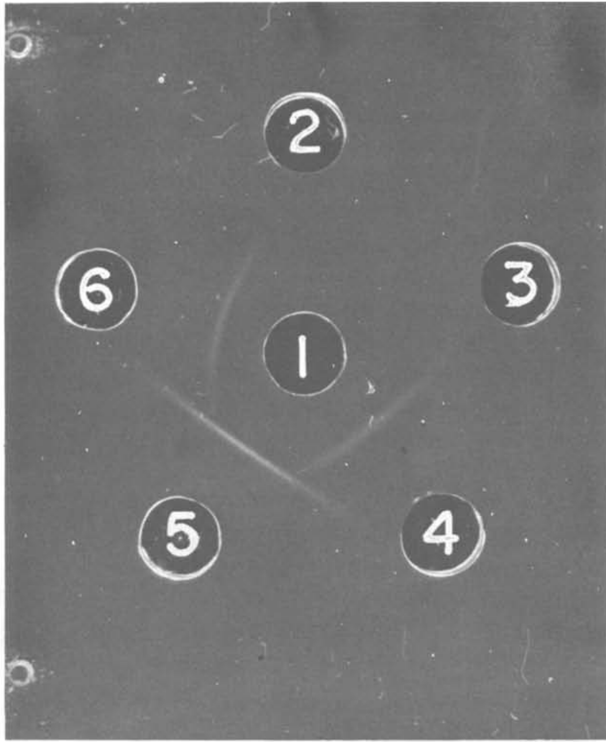

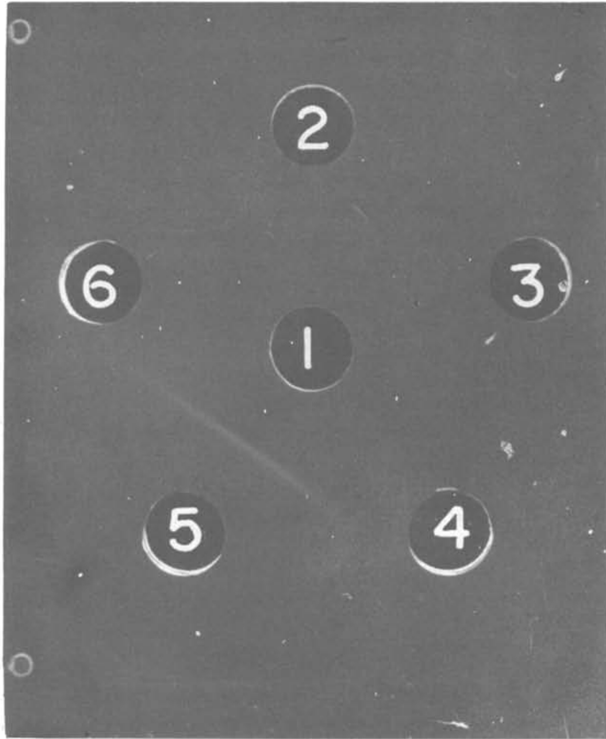

Figs. 4A and 4B. Ouchterlony analysis of gamma globulins. 2–CBG; 3-anti-IgD: 4-anti-IgM; 5–Anti-IgG; 6–Anti-IgA. 1–Peak A from Sephadex G-150 (Fig. 4A): 1–Peak B from Sephadex G-150 (Fig. 4B).

and free steroid in the upper chamber can be calculated from the data obtained in buffer experiments and a knowledge of the total steroid concentration in the upper chamber. In a typical experiment the upper chamber contains $1 \cdot 73 \times 10^{-6}$ M CBG, about 85 per cent of whose sites are occupied by cortisol still present from the isolation procedure. [^{3}H]-Cortisol, $10\times$ less than the concentration of free sites on CBG, is added at time 0 and the system allowed to come to steady state. After the attainment of steady state, radioinert cortisol, in 20-fold excess of the molar concentration of CBG, is added and the rate of change of [^{3}H]-cortisol in the lower chamber determined. The large excess of cortisol assures that we can neglect the reverse reaction in calculating the rate of dissociation of the cortisol-CBG complex. Figure 3 illustrates the results of a typical experiment. Note that

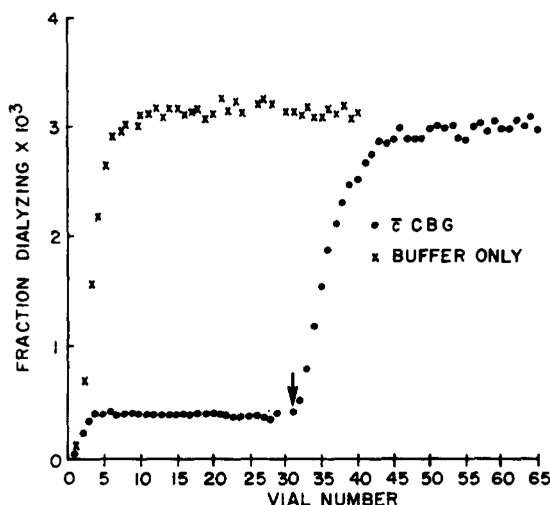

Fig. 3. Rate of dissociation of the CBG-cortisol complex. Two separate experiments are shown plotted on the same set of coordinates. Fraction dialyzing = [CPM in effluent from lower chamber]/[CPM in upper chamber]. One vial was collected every 0·5 min and the flow rate through the lower chamber was 4 ml/min. In both experiments [^{3}H]-cortisol was added to the upper chamber within 2–3 sec of the beginning of the experiment and 5×10^{-5} mol of radioinert cortisol was added (arrow) after the tritium in the effluent from the lower chamber reached steady state.

after the addition of the radioinert cortisol, the fraction of [^{3}H]-cortisol dialyzing in the CBG experiment closely approaches that in the experiment in which protein-free buffer is present in the upper chamber, thus validating the assumption that the reverse reaction, i.e., rebinding of [^{3}H]-cortisol to CBG, is negligible. The rate constant for the dissociation of the cortisol-CBG complex is calculated from:

$$[SP]_t = [SP]_0 e^{-k_2 t}$$

where $[SP]_t$ is the concentration of bound cortisol at any time, $[SP]_0$ is the concentration of cortisol bound to CBG immediately prior to the addition of radioinert cortisol, and k_2 is the rate constant for the dissociation of the complex. The rate of dissociation (k_2) of the complex is too rapid to measure at temperatures above 18°C but can be measured below that temperature. The rate of association of cortisol with CBG (k_1) was too rapid to measure in this system even at 4°C. Measurements at 4°, 10° and 18°C give rise to rate constants (Table 3) allowing

Table 3. Rate constants for the dissociation of the CBG-cortisol complex

	Temp.			
	4°C	10°C	18°C	37°C
k_2(min^{-1})	0·14	0·31	0·87	8·0
t 1/2 (min)	4·95	2·2	0·80	0·087

The rate constants at 4°, 10°, and 18°C were determined experimentally while that at 37°C was obtained by application of the Arrhenius equation.

the construction of an Arrhenius plot which yields an activation energy of 21 kcal/mol. In addition, since the Arrhenius plot is linear, we can extrapolate to the value of k_2 at 37°C. The activation energy for the association of CBG and cortisol can be derived by subtracting the value for the enthalpy change of the reaction, -16 kcal/mole[15], from the activation energy for the dissociation. The value obtained, 5 kcal/mole, is in the region expected for a hydrogen bond. This suggests that the association of CBG and cortisol proceeds through a transition state in which one hydrogen bond has been ruptured.

THE EFFECTS OF CBG *IN VIVO*

Experiments *in vivo*[22, 23] and *in vitro*[24, 25] indicate that CBG acts to prevent cortisol from exerting its effects on cellular processes. However, none of the experiments utilized completely pure preparations of CBG derived from the species in which the experiments were performed. In addition, the *in vitro* experiments do not approximate a system wherein the protein as well as the steroid are in dynamic equilibrium in the extracellular space of a living organism. We therefore purified rat CBG by the use of affinity chromatography and utilized it in the following experiment.

Adrenalectomized, male Sprague–Dawley rats whose average weight was 50 g served as subjects. One group was injected intravenously with cortisol, 14·3 μg/100 g body weight, another with the same dose of cortisol bound to 3·75 mg CBG, and the controls with 0·42 ml buffer/100 g body weight. We determined the concentration of lymphocytes in peripheral blood prior to injection and again at 2 and 5 h. At 2, 3 and 5 h after injection, open biopsies of liver were obtained on each animal for determination of tyrosine amino transferase activity[26]. The results of this experiment are presented in Tables 4 and 5. Both experimental groups show a significantly higher level ($P < 0·01$) of TAT activity than the control group at all time periods, but there are no significant differences between

Table 4. Tyrosine amino transferase activity in liver

	2 h	3 h	5 h
Saline	1·05 ± 0·16	1·05 ± 0·16	1·05 ± 0·16
Cortisol	3·32 ± 0·36	3·46 ± 0·45	4·46 ± 0·65
CBG + Cortisol	3·41 ± 0·33	4·55 ± 0·72	5·69 ± 0·83

The results are expressed as nmol *p*-hydroxyphenylpyruvate formed/min/mg protein ± standard error of the mean.

Table 5. Percentage decrease in lymphocytes in peripheral blood

	2 h	5 h
Saline	5·78 ± 15·4	15·6 ± 11·7
Cortisol	31·5 ± 6·87	64·6 ± 7·00
CBG + Cortisol	31·4 ± 7·31	68·3 ± 6·00

the experimental groups. Similarly, both the cortisol and CBG-injected animals had equal fractional falls in lymphocytes at 2 and 5 h. The difference between both experimental groups and the control group was not significant at two hours but was significant at ($P < 0·01$) at 5 h.

We determined that the average concentration of CBG in the plasma of the rats we used was 100 mg/l. Assuming that the rat plasma volume is 5 per cent of its body weight, then the amount of CBG in the plasma of a 100 g rat is 0·5 mg. We administered 7–8 times this amount of CBG. In addition we determined (equilibrium dialysis at 37°C) that 5 per cent of the cortisol (0·7 μg cortisol/100 g rat) injected was not bound to CBG, an amount insufficient to affect either TAT or lymphocytes if not supplemented by additional steroid from another source. One must therefore conclude that the absence of differences in the two experimental groups is due either to initially bound steroid becoming free or to the fact that cortisol was capable of exerting the two effects studied while still bound to the CBG. In any event, it is clear that the presence of CBG made no difference in the manner in which cortisol behaved in the systems studied, a result at variance with the implications of other published data on the effects of CBG. The question is not one of whose data is correct; it is undoubtedly all correct. The major question is one of experimental design. How does one approach the question of: 'What is the physiological function of CBG?' The design of the present experiment includes the acute introduction of a pharmacologic dose of CBG into cortisol-free animals and might be criticised on that basis. In contradistinction to previous studies, however, the CBG was pure, removing questions of interpretation that might arise if contaminants were present; it was given to the same species from which it was obtained; and it was a dynamic *in vivo* study. It is possible, as has been suggested [27], that the effect of the CBG-cortisol complex is different at different effector sites. We clearly have not examined all possible actions of cortisol. The present work does, however, raise serious doubts about presently held beliefs regarding the function of CBG.

THE BINDING OF CORTISOL TO GAMMA GLOBULIN

We have already seen that when CBG is isolated by affinity chromatography it is contaminated with gamma globulins. If, after the elution of Pools I and II (Fig. 1), the cortisol Sepharose is developed further with 6 M urea, an additional peak of protein is obtained. After removal of the urea by dialysis and gel filtration on Sephadex G-25 the protein was applied to a column of Sephadex G-150 which yielded two reasonably well-separated peaks of protein.

After ascertaining that the peak which eluted earlier (Peak A), i.e. the larger molecules, showed three precipitin bands against anti-whole human serum on Ouchterlony plates, we examined this material against specific types of immunoglobulin antisera. Figure 4A shows that Peak A is a mixture of IgG, IgA and

IgM. In a like manner the protein which was more retarded (Peak B), i.e. the smaller molecules, on Sephadex G-150 showed only one band against anti-whole human serum and this band was identified as IgG (Fig. 4B). Quantitation by means of radial immunodiffusion showed that Peak B consisted of more than 95 per cent IgG with small amounts of IgM and IgA while Peak A consisted of about 50 per cent IgM and roughly equal amounts of IgG and IgA. The association constant for IgG (Peak B) and cortisol (equilibrium dialysis at 23°C) was $0{\cdot}2 \times 10^4\ M^{-1}$ with 1·6 binding sites/mol of IgG. A chromatographically prepared commercial preparation of IgG did not bind cortisol when tested at 1·86 mg/ml with a tracer amount of cortisol.

That an animal can make an antibody to a steroid hormone has already been demonstrated by Beiser *et al.*[28]. The antibody production shown by those investigators, however, was induced by steroids which were covalently coupled to proteins. More recently, Pinto and Rimon[29] have published indirect evidence that antibodies (IgM) to cortisol may circulate in human plasma. We have found that the three common immunoglobulins are all bound to an affinity column using cortisol hemisuccinate as ligand. The quantitative data on IgG indicate an association constant which is not particularly impressive when compared to that of CBG but the fact that such IgG molecules exist at all is surprising. It is of course, possible that the cortisol is not bound to an antibody combining site but rather to another region of the IgG molecule. If this is true, then there must be heterogeneity in regions other than the antibody combining site since IgG prepared by non-specific methods showed little binding. If the IgG is an antibody it does not arise from immunization with cortisol bound to CBG as there was no precipitin line formed (Fig. 4B) when this possibility was examined.

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DISCUSSION

De Moor: Congratulations, Dr. Rosner. Do you think that it is an antibody against cortisol or against CBG?

Rosner: The only evidence we have on that point is the absence of a precipitin line on Ouchterlony plates when purified CBG was examined against the isolated IgG. If one postulates that the species which induces the antibody is cortisol bound to CBG then a precipitin line would be expected. At the present time I would therefore conclude that if the IgG is an antibody, it is directed against cortisol and not against CBG.

De Moor: Do you know anything about temperature dependence of binding of cortisol to your IgG?

Rosner: No, we don't have any studies on that. We've only done it at 23°C.

De Moor: We have the impression that part of cortisol binding is temperature independent, and that this part may be important in respect to some clinical evaluations. But it may be that that part of the binding is only to this IgG.

Rosner: It's possible. I just don't have the data.

Holzbauer: As a physiologist I was particularly pleased to see that Dr. Rosner has done an *in vivo* experiment on rat CBG. There's only one thing which worries me, that is that the rat doesn't produce any cortisol; it only makes corticosterone.

Rosner: That the rat makes corticosterone and not cortisol is certainly correct. They are, however, both reasonably potent glucocorticoid hormones and one would not expect any important differences to ensue in substituting one for the other. In spite of this we would have preferred to use corticosterone but were prevented from doing so by practical limitations. The dose of cortisol which we inject, 14·3 μg/100 g rat, is close to the smallest dose which will give the effects we examine. If we substituted corticosterone we would have had to use 2–5 times as much steroid and hence 2–5 times as much CBG. That much rat CBG would be very difficult to obtain because of the expense of rat plasma.

Munck: Those are pretty experiments, and I'm delighted you have gone into the question of the biological activity of CBG-bound cortisol *in vivo*. The interpretation is difficult, however, and I think you have to use *in vitro* experiments in order to know how much cortisol at any time is free and how much is bound, and therefore how much activity can be ascribed to the bound form. In your experi-

ment, although initially in the presence of CBG the concentration of free cortisol is lower than in the absence of CBG, it is quite possible that after some time—an hour perhaps—the concentration is higher, due to the fact that CBG-bound cortisol is not metabolized and so CBG in effect increases the half-life of cortisol. So even assuming that CBG-bound cortisol is inactive, it is difficult to predict whether the presence of CBG would increase, decrease, or have no effect on the biological activity of a given dose of cortisol, unless one has continuous measurements during the experiments of the levels of free cortisol.

Rosner: It is clearly impossible to have continuous measurements of free cortisol concentration although that would be a highly desireable kind of information. I cannot agree that *in vitro* systems are the proper ones from which to draw physiological conclusions although this is sometimes feasible. The *in vitro* system can tell you what can happen but not necessarily what does happen. I think this is particularly true when investigating the type of system we are considering. An important variable missing in an *in vitro* system is the capillary bed, and that as well as the cell membrane is going to have a major effect on where the cortisol goes. That consideration alone, neglecting the metabolism that goes on *in vivo*, should cause one to pause before extrapolating the *in vitro* data to the *in vivo* situation. The experiment, in addition, was done kinetically (each animal had its liver sampled at each time period) so that we could observe not only any difference in the absolute level of the response but any differences in the time course of the response. There were, as I showed, no differences at any of the time periods we investigated. In regard to your question about CBG prolonging the half life of cortisol in plasma. If CBG does that then the question that follows is: "Can a small but prolonged level of free cortisol in plasma lead to the same magnitude and time course of response as does the more rapidly rising and falling cortisol levels following injection of free cortisol?" I don't know of any data on this point but I would not be terribly surprised if ultimately one saw the same magnitude of response but I would be surprised if the time courses of the responses were the same.

Sitteri: You raised the possibility that cortisol may be active in a bound form. Of course we know that many experiments have now been done in which protein hormones linked to macromolecules, which cannot enter cells, retain their metabolic activity. I wonder whether you, or Dr. Munck, have ever tested cortisol 21 hemisuccinate linked to bovine serum albumin or similar compounds for activity?

Rosner: I think there are several important differences between the work you are referring to and the work I have presented. The work on the peptide hormones used test systems wherein the hormone is covalently attached to large particles so that there is no way for the hormone to get past the cell membrane. This has been used as a strong line of evidence that these hormones mediate their effects through a "second messenger". Cortisol is not covalently bound to CBG and in addition, although it has been looked for, cyclic AMP does not seem to be an important mediator of the action of steroid hormones. I only meant to raise the possibility that both the steroid and CBG could get past the cell membrane and that the cortisol could come off the protein inside the cell as well as in the plasma. We have no experimental data on cortisol covalently bound to any macromolecule. Perhaps Dr. Munck has some data.

Munck: No, we haven't tested protein-linked hormone. But we have tested the

21-hemisuccinate of cortisol, and it is active *in vitro* without being hydrolyzed.
Vermeulen: Dr. Rosner, do you have any idea about concentration of the cortisol-binding antibody compared to the concentration of transcortin?
Rosner: We have very little data on this. I though it was such an interesting observation that I would present it to you, and you've really seen most of what we have.
Slater: I'm intrigued by the difference between the value you have obtained for the half-time of dissociation and those obtained by Dixon (*J. Endocrinol.* **40** (1968) 457). Would you like to explain how your values differ by about a factor of five at 4°C?
Rosner: Although Dixon's approach was extremely clever I think that his data when compared to mine show some internal inconsistencies. He has accurate data at only two temperatures 4° and 22°C and is therefore unable to construct an Arrhenius plot validated by a third point. We have good data at 4°, 10° and 18°C and if one calculates an Arrhenius plot from these data the points all fall on a straight line. Since there is a five-fold disagreement in the 4°C data and fairly good agreement between our numbers at 18°C and his at 22°C we would have quite different slopes for the Arrhenius plots. Our 10°C experiment would not fall on his straight line (calculated from two points). In addition his own data at 37°C would not be in line with his data at 4° and 22°C. Technically he had the disadvantage of having to work with diluted plasma while we used pure CBG. In addition I think the flowing system we use to separate bound from free is probably inherently better than having to stop the reaction and then separate bound from free hormone by gel filtration. These arguments are of course speculative to a certain extent but you did ask me to try and explain the discrepancies.
Crabbé: First I would like to know whether there is independent evidence for ready accessibility of transcortin-bound cortisol to liver cells. By independent evidence, I mean, for example, whether the rate of degradation of cortisol is the same when you inject cortisol as such and when you inject it combined with transcortin. Second, you quite appropriately pointed out that when one works *in vitro*, one dismisses and unduly so, the role, (and probably it is a large one) of the capillary bed. On the other hand, if I'm not mistaken, in the two organs where you've looked for biological evidence for the action of cortisol, the capillary bed is extremely leaky. Is there any evidence from tissues irrigated by more "standard" capillary beds that transcortin-bound cortisol is biologically important?
Rosner: No one has injected cortisol with and without homologous CBG and looked at cortisol half life. The closest anyone has come to that experiment is to study the half life of cortisol in a single patient with a half normal concentration of CBG in plasma. The half life was within the normal range for that laboratory (Lohrenz F. N., Seal U. S. and Doe R. P. *J. Clin. Endocr.* **27**, (1967) 966). This is only one case of course, and there was a reasonable amount of CBG still present so that the appropriate experiment still needs to be done. Your question about the capillary beds is certainly a reasonable one. Cortisol need not cross a capillary to get to a lymphocyte and the liver does have a "leaky" capillary bed. The reason we chose liver was because of the suggestion by Keller *et al.* (Ref. 27 in my manuscript) that cortisol bound to CBG might be effective in this organ. Since this is the only data that goes against the dogma that bound steroid is inactive we thought that we would use our limited supply of rat CBG to investigate their

suggestion. As I mentioned, we have certainly not studied all the target organs for cortisol and results might be different in other test systems. These experiments should be done. In addition we plan to validate these studies by removing endogenous CBG with antibodies and doing the same kinds of experiments. I don't pretend that what we have done is a final answer to the questions raised but hope that this work might cause us to re-examine current beliefs in this area.