to be no explanation for the increase or disposition of phospholipid molecules during infection. The matter is further complicated by reason of extensive hyperplasia concomitant with virus replication. In this regard Levin(7) cited previous experiments indicating that phospholipid metabolism is stimulated during certain conditions associated with hyperplasia.

The decreasing specific activity noted in noninfected epithelium and connective tissue might be due either to lack of uptake or to an increased turnover rate. On the other hand, the elevated specific activity (gradually decreasing with time) of infected tissues could be due to an increased synthesis of phospholipid compounds or a normal rate of synthesis with deposition in areas of compounds where there is little turnover.

Summary. Normal control and fowlpox-infected chick scalp were separated by trypsin into connective tissue and epithelium. By appropriate technics the infected epithelial tissue was separated into surface epithelium and follicles. Comparison was made between normal epithelium and connective tissue and between normal and infected tissues with respect to certain lipids and incorporation of P^{32} into phospholipid. Distinct differences in phospholipid and total, free, and esterified cholesterol were found between normal epithelium and connective tissue. The above components were increased 2- to 3-fold at some interval of time in both infected epithelium and connective tissue. The specific activities of phospholipid P from all tissues were approximate at 3 days but that of normal epithelium and connective tissue fell approximately 50% at 5 and 7 days. In contrast the incorporation of P^{32} in infected tissues was 1.5 to 1.7 times that of normal controls.

1. Todd, W. M., Randall, C. C., Coniglio, J. G., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 65.

2. Zilversmit, D. B., Entenman, C., Fishler, M. C., Chaikoff, I. L., *J. Gen. Physiol.,* 1943, v26, 333.

3. Kamen, M. D., *Isotopic Tracers in Biology. An Introduction to Tracer Methodology,* 3rd ed., Academic Press, Inc., 1957, p91.

4. Miroff, G., Cornatzer, W. E., Fischer, R. G., *J. Biol. Chem.,* 1957, v228, 255.

5. Moldave, K., *ibid.,* 1954, v210, 343.

6. Green, M., *Virology,* 1959, v9, 343.

7. Levin, E., Johnson, R. M., Albert, S., *Arch. Biochem. and Biophys.,* 1958, v73, 247.

Received October 20, 1960. P.S.E.B.M., 1960, v105.

Measurement of Cortical Binding Capacity in Human Plasma.*† (26198)

JAMES C. WARREN‡ AND HILTON A. SALHANICK
(Introduced by Thomas F. Dougherty)
Departments of Obstetrics and Gynecology and Biochemistry, University of Nebraska College of Medicine, Omaha

The high levels of cortisol in the plasma of pregnant and estrogen-treated patients have been attributed to an α-globulin named transcortin by Slaunwhite and Sandberg(1) and referred to as corticosteroid binding globulin by Daughaday *et al.*(2). These authors have disagreed on whether binding capacity is elevated during pregnancy(1,2,3). These findings have made pertinent the development of procedures to determine reliably and accurately the binding capacity of plasma.

The protein responsible for binding has not been isolated. The binding process has been studied by ultrafiltration(4), single bag equilibrium dialysis at 4°C(1), "two bag" equilibrium dialysis at 4°C(2), and electrophoresis(5,6). Furthermore, Slaunwhite and Sandberg have indicated that the binding is not readily reversible(1). This has broad implication on the physiologic significance of binding in pregnancy and other conditions.

* Aided by a grant from the Am. Cancer Soc.

† The authors are indebted to the Endocrine Study Section, N.I.H., for cortisol-4-C^{14} and American Red Cross for human serum albumin.

‡ Trainee, Nat. Cancer Inst.

Their conclusions, however, were based upon determinations at 4°C, and time required for equilibrium to be achieved at 4°C has not been determined satisfactorily nor is the effect of temperature completely clear.

This report describes a procedure for determination of binding capacity in terms of standard plasma, relates the equilibrium achieved at the various temperatures in an appropriate time interval, and determines the reversibility of binding, thereby clarifying its physiologic significance.

Materials and methods. Rationale of procedure: Without extensive purification of the cortisol binding globulin, knowledge of a number of adsorptive sites, or removal of endogenous hormone already "bound" to the protein, an absolute determination of binding protein concentration cannot be made. Nevertheless, if an unknown quantity of binding protein is compared by dialysis with a standard preparation, a comparative determination can be made.

Accuracy in comparison requires equilibrium with similar specific activities and levels of free cortisol in all compartments. Under these conditions amounts of bound radioactivity represent concentrations of binding protein in accordance with mass action. The procedure reported here utilizes a "2-bag" dialysis technic under conditions permitting complete equilibration of dialyzable components.

Cortisol-4-C^{14} (4.16 μc/mg) was purified by paper chromatography and stored in ethanol at 4°C. Pools of plasma obtained from healthy individuals were diluted 1:5 or as indicated. Seamless viscose cellulose dialysis tubing 5/8 inch in inflated diameter was used in all experiments. Sterile salt-poor human serum albumin was employed as a 1.0% solution in .89% saline which was the diluent in all cases. Penicillin was added to a final concentration of 3,000 units/ml to prevent bacterial action. This antibiotic has been demonstrated not to affect binding(1). When cortisol was added, final ethanol concentration never exceeded 0.25%.

For dialysis, 10 ml of diluted or undiluted plasma was used in each compartment. After dialysis, the contents of each dialysis bag and a 10 ml aliquot of the serum albumin external compartment were extracted 3 times with 1.5 volumes of redistilled methylene chloride. The extracts were evaporated under a fine stream of air and an aliquot of an ethanol solution was plated on tared 3 cm aluminum planchets. Radioactivity was measured at infinite thinness in a Nuclear-Chicago windowless gas flow counter to a probable error of 3%. Recoveries ranged from 81 to 93%.

TABLE I. Effect of Temperature on Equilibration: 72 Hr of Dialysis at Indicated Temperature after 90 Min. of Equilibration of Radioactive Steroid in Original Compartment. Volume changes between compartments were less than 5%.

			Counts/min.	
Temp.	Exp.	Plasma dilution	Original compartment	Added compartment
4°	A	1:2	273	70
	B	1:2	264	44
	C	1:5	230	101
37°	A	1:5	164	171
	B	1:5	161	156

Results. I. Effect of temperature on rate of dialysis (Table I): To permit "complete" adsorption to occur, .05 μg of radioactive cortisol were added to 10 ml of diluted plasma, and kept at 4°C for 90 minutes. A dialysis bag containing an identical amount of plasma was then placed in the vessel and dialysis carried out for 72 hours. Both compartments were extracted and radioactivity content determined.

At 4°C, regardless of plasma dilution, very little cortisol left the original compartment, indicating either that the steroid was firmly bound or that dialysis had not yet reached equilibrium. At 37°C equal amounts of radioactivity were found in each compartment. Clearly, equilibrium was achieved at this temperature and the steroid binding is reversible. II. Rate of dissociation of bound cortisol at 37°: Two plasma samples were obtained from a healthy male before and after 4 days therapy with dexamethasone (.75 mg every 6 hours) given to lower endogenous plasma 17-hydroxycorticoid levels. To 33 ml of the first (normal) plasma was added .5 μg of cortisol-4-C^{14} and this mixture was dialysed against 50 ml of 5% albu-

TABLE II. Diffusion of Cortisol-4-C^{14} from Equilibrated Normal Plasma to Treated Plasma at 37°.

Time (hr)	CPM/10 ml Normal plasma	Treated plasma	Ratio N:T
0	744*	0	—
2	606	66	9.2
4	506	118	4.3
8	465	169	2.7

* 146 cpm unbound. (With similar experiment using 5% serum albumin in each compartment, equilibrium was 95% complete in 2 hr.)

min at 37° for 24 hours. Aliquots of plasma and albumin were then extracted. The remainder of the plasma was divided into three 10.0 ml portions. These were individually dialysed against 10.0 ml portions of the second (treated) plasma for periods of 2, 4 and 8 hours. At these times dialyses were extracted and amount of radioactivity in each compartment measured. Similar experiments done with cortisol in 5% albumin revealed almost complete equilibrium in 2 hours and ruled out the membrane as a limiting factor. The data indicate that dissociation of cortisol from undiluted plasma even at 37° is a slow process with mass action markedly favoring binding. III. Effect of cortisol concentration on binding (Table III): Non-radioactive cortisol was added to 10.0 ml aliquots of diluted pooled plasma. Amounts added simulated increases of 12.5 and 25 μg/100 ml plasma. Altered plasmas were equilibrated in test tubes at room temperature for 8 hours to assure complete binding, then transferred to dialysis bags. These 2 altered plasmas and 2 control 10.0 ml samples in dialysis bags were assembled in a flask containing 80.0 ml of albumin solution and dialysed at 37°C for 18 hours. Next, 0.3 μg of cortisol-4-C^{14} were added to the albumin compartment and dialysis continued at the same temperature for 30 hours. The objective was to determine if the nonradioactive cortisol would irreversibly saturate some of the binding sites or diffuse equally among all the plasma compartments. The experiment was performed in duplicate.

TABLE III. Effect of Simulated Variable Cortisol Levels on Equilibrium of Dialysis at 37°: 8 Hr of Equilibration, 18 Hr Preliminary Dialysis with an Additional 30 Hr after Addition of 0.3 μg of Cortisol-4-C^{14}. Total volumes of plasma compartments extracted were all 10.0 ± .1 ml. HSA compartment equal 80 ml.

Compartment	Counts/min. Exp. 1	Exp. 2
10 ml aliquot HSA	110	120
Plasma (diluted 1:5)	211	314
"	300	328
" + .25 μg F	301	323
" + .5 " "	318	lost

Equal binding of radioactive cortisol occurred in all compartments. As expected, the albumin medium contained much less hormone. The implication from this isotopic dilution experiment is that binding is reversible at 37°C and that in spite of the load of simulated endogenous cortisol, relative total binding capacities remained constant. IV. Effect of cortisol concentration and protein concentration on binding (Table IV): To confirm the previous observations and to demonstrate the applicability of the procedure, a third series of experiments was devised. First, 1:5 and 2:5 dilutions of plasma were compared; presumably they would represent approximately 2-fold differences in concentrations of binding protein. To one of the 3 compartments of each system, nonradioactive carrier was added in amounts ranging from 0.5 to 4 μg and representing biologic plasma levels of 12.5 to 50.0 μg/100 ml.

The compartments were equilibrated independently at 37°C for 8 hours, then assembled as described in Table IV and dialyzed for 18 hours at 37°C to establish equilibrium. Finally, 0.1 μg of radioactive cortisol was added to the albumin compartment and dialysis continued for another 30 hours.

It is apparent that the non-radioactive cortisol diffused to all compartments, and that even at 37°C significant binding occurs. In almost all cases the 2:1 binding ratio is present, the 2 low ratios probably reflecting experimental error. Correcting for volume changes, calculated distribution ratio is 1.8 and observed ratio is 1.9 ± 0.3 (mean ± standard deviation). Thus, binding does occur at physiologic temperatures, and equilibrium is obtained regardless of endogenous

TABLE IV. Effect of Variable Cortisol Levels and Binding Protein Content in the System: HSA Compartment 20 ml (10 ml Extracted), Cortisol-4-C^{14} Added to HSA Compartment. Volumes of plasma 1:5 extracted = 10.0 ± .1 ml. Volumes of plasma 2:5 extracted = 11.0 ± .2 ml in all cases. Calculated concentration ratio = $\frac{2.0}{1.1}$ = 1.8. Observed ratio = 1.9 ± .3.*

Plasma pool	Exp.	Compartment	mg non-radioactive steroid added	CPM (total)	CPM (bound)	Ratio
I	1	HSA	—	79	—	
		P 2:5	—	348	269	1.9
		P 1:5	.5	218	139	
	2	HSA	—	79	—	
		P 2:5	.5	353	274	2.2
		P 1:5	—	204	125	
	3	HSA	2.0	92	—	
		P 2:5	—	328	227	2.0
		P 1:5	—	212	111	
	4	HSA	4.0	111	—	
		P 2:5	—	278	156	2.1
		P 1:5	—	175	74	
II	1	HSA	—	92	—	
		P 2:5	—	298	197	1.8
		P 1:5	.5	203	112	
	2	HSA	—	97	—	
		P 2:5	.5	252	150	1.5
		P 1:5	—	198	101	
	3	HSA	—	106	—	
		P 2:5	—	265	149	2.0
		P 1:5	1.0	181	74	
	4	HSA	—	100	—	
		P 2:5	1.0	266	156	1.4
		P 1:5	—	210	110	

* Mean ± stand. dev.

plasma levels of cortisol because the protein-steroid complex is dissociable.

Discussion. Two dialysis methods for study of the protein binding system have been published. Slaunwhite and Sandberg (1) employed a single bag of diluted plasma dialyzed at 4°C for 72 hours, performing the experiment in duplicate with one microgram of non-radioactive cortisol in the second system. These conditions do not permit complete equilibrium with similar specific activities in both compartments. The technic tends to measure binding capacity of the unoccupied binding sites. Nevertheless, the dilution of plasma permits some dissociation of the steroid-protein complex, and differences in total binding capacity may be detected.

Daughaday, *et al.*(3) employed a 2 bag dialysis on undiluted plasma at 4°C. If equilibrium is achieved, this system may be employed for determining relative binding capacity of various specimens. But at 4° equilibrium with undiluted plasma is not obtained and observed binding represents for the most part free rather than total binding sites. Endogenous cortisol levels of samples may not be equivalent whereas free sites may be equal. In this situation, a plasma sample may have a total binding capacity greater than the standard but it might not be demonstrable if only the free sites are determined.

An ideal method would determine quantitatively the total number of sites and amount of either bound or free sites. Since the ratio of free and bound sites is not easy to determine, the next preferable procedure would determine number of total binding sites relative to a standard.

The procedure reported here utilizes diluted plasma, dialysis at 37°C for 48 hours

and 2 or more bags to permit comparison in terms of a standard plasma. It has been demonstrated that under these conditions dialysis of the free cortisol through the membrane is rapid. Dissociation of the steroid-protein complex, and therefore equilibrium, is achieved. Specific activity in each compartment is equivalent. Then, by direct measuring the radioactivity in each compartment, an accurate determination of relative total binding capacity may be made.

The procedure has practical significance. Sandberg and Slaunwhite(3) reported that diluted pregnancy plasma has a higher binding capacity than normal, but Daughaday's data from undiluted plasma, dialyzed against a control plasma, revealed that number of available binding sites was approximately the same(2). The difference is readily resolved because the former authors were at least partially measuring total capacity while the latter was determining free binding sites almost exclusively. Thus, the higher concentrations of cortisol which occur in pregnancy plasma were occupying some of the available sites in the latter incubations, and the amount of free sites (and possibly free cortisol) was equivalent.

The physiologic significance of the steroid-protein complex remains undetermined. Presumably, the complex may be physiologically inactive; in this case, it might act as a buffer against excessive concentrations of steroid. On the other hand, the slow rate of dissociation *in vitro* would mitigate against its being a reservoir of readily available steroid. It is possible, however, that the organism has a more efficient method of dissociating the complex or that the complex may be effective in certain systems as an entity. Finally, if one considers synthesis of this protein as an artifact of pregnancy (and it has been demonstrated to be elicited by estrogen in both males and females), it may have no "role" whatsoever and the higher levels of corticoids found in the plasma in pregnancy may reflect nothing other than homeostatic response to lack of available unbound steroid.

Summary. Simultaneous dialysis of 2 diluted plasma samples in the same system at 37°C is an acceptable method for measurement of the relative amounts of corticosteroid binding protein present. Dissociation of the steroid-protein complex in undiluted samples is slow even at 37°. The kinetics of binding do not permit attainment of equilibrium at 4°, even when the plasma is diluted. The differences noted by previous investigators in comparison of pregnant and non-pregnant samples are explainable by this incomplete equilibrium.

1. Slaunwhite, W. R., Jr., Sandberg, A. A., *J. Clin. Invest.*, 1959, v38, 384.

2. Daughaday, W. H., Kozak, I., Biederman, O., *ibid.*, 1959, v39, 998.

3. Sandberg, A. A., Slaunwhite, W. R., Jr., *ibid.*, 1959, v38, 1290.

4. Upton, G. V., Bondy, P. K., *Arch. Biochem.*, 1958, v78, 197.

5. Daughaday, W. H., *J. Clin. Invest.*, 1956, v35, 1434.

6. ———, *ibid.*, 1958, v37, 519.

Received July 21, 1960. P.S.E.B.M., 1960, v105.

Factors Affecting Extractability of Cholesterol from Lyophilized Sera by Cold Chloroform.* (26199)

J. C. FORBES, A. L. FORBES AND O. M. PETTERSON

Department of Biochemistry, Medical College of Virginia, and Veterans Administration Hospital, Richmond

The significance of the fraction of serum cholesterol which is extracted when lyophilized serum is shaken with cold *anhydrous* chloroform for 3 hours has been studied in

* We are indebted to Drs. P. D. Camp, A. Wasserman, and B. B. Kirkland for most of the blood samples used in the study. Supported in part by a research grant from Richmond Area Heart Assn., Inc.