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Binding of Cortisol and Its Degradation Products by Human Serum Albumin

By K. J. KRIPALANI and DONALD L. SORBY

The rate of degradation of the 17-dihydroxyacetone function of cortisol has been measured in a phosphate buffer system, pH 7.4 and ionic strength 0.16, at 15.0°, 25.0°, and 35.0°. The results correspond favorably with data in the literature for degradation of prednisolone under similar conditions. The binding of cortisol and its degradation products to human serum albumin was measured at 25.0° by an equilibrium dialysis procedure. The degradation products were found to be bound more strongly than was cortisol, but did not appear to compete with cortisol for binding sites on the protein molecule. In systems having a constant concentration of total corticosteroid, the apparent binding constant of total steroid increases as the fraction of the material present as degradation products increases. The results of this research are important to the design of experiments which attempt to study the binding of steroids containing the 17-dihydroxyacetone function to serum proteins.

INTERACTIONS with various serum proteins may potentially affect the distribution, metabolism, elimination, and therapeutic effects of a drug. Hence, interactions between drugs and proteins have been subjects for study by many investigators. In his extensive review, Goldstein (1) cites several hundred published studies of drug protein interactions. One particularly active area of interest has been the interactions which occur between various steroid hormones and serum proteins. Such interactions appear to be of major importance to the transport and distribution of these compounds. Cortisol, in particular, has received much attention. Sandberg *et al.* (2), Daughaday (3), Slaunwhite *et al.* (4), Brunkhorst and Hess (5), and Westphal (6), among others, have studied the interaction between cortisol and serum albumin. The literature pertaining to steroid–protein interactions has been reviewed by Daughaday (7).

Two plasma components appear to be involved in binding cortisol (8–10). At physiologic levels, cortisol is bound predominantly by an α globulin commonly termed "transcortin" or "corticosteroid binding globulin" (8, 10). Transcortin is present in relatively low concentrations, however, and rapidly becomes saturated in its binding ability if plasma cortisol concentrations rise much above normal physiologic levels. Serum albumin has a lesser affinity for binding cortisol than does transcortin but plays a major role in binding at elevated serum cortisol levels (11). Effects of cortisol concentration on its binding to plasma proteins have been discussed by Daughaday (7) and by Bush (12). For the most part, research on the steroid–plasma protein interaction to date has employed equilibrium dialysis procedures (7). Equilibration times have varied between 18 and 72 hr. One important criterion which must be met in such a study is that the solute molecules must be sufficiently stable so that chemical degradation does not occur to a significant extent during the equilibration period. Oesterling and Guttman (13) and Guttman and Meister (14) have studied prednisolone degradation. Their data show that the extent of degradation of the 17-dihydroxyacetone function might be significant during the average equilibrium dialysis experiment. While similar information pertaining to cortisol was not available, it was considered that it too should be subject to similar rates of degradation. This research was initiated to determine whether cortisol would undergo significant degradation under conditions of the equilibrium dialysis experiment and whether such degradation would significantly alter the results of

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The authors wish to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

protein binding experiments. Such information was needed for development of suitable experimental procedures for protein binding studies currently under way in this laboratory.

EXPERIMENTAL

Reagents and Materials—Phosphate buffer, pH 7.4, ionic strength 0.16, was prepared by dissolving 16.68 Gm. disodium phosphate, $Na_2HPO_4 \cdot 7H_2O$, and 2.209 Gm. monosodium phosphate, $NaH_2PO_4 \cdot H_2O$, in enough freshly boiled distilled water to make 1 L. Hydrocortisone was obtained from a commercial source and was recrystallized from isopropanol. The melting point was 217–219° (uncorrected). Tetramethylammonium hydroxide reagent was prepared by diluting 1 ml. of a 10% solution in water, obtained from Eastman Chemical Co., to 10 ml. with absolute ethanol. Triphenyltetrazolium chloride reagent was prepared by dissolving 1 Gm. of 2,3,5-triphenyltetrazolium chloride, obtained from Nutritional Biochemicals Corp., in enough absolute ethanol to make 100 ml. Crystalline human serum albumin[1] was purchased from Nutritional Biochemicals Corp. Information furnished by the supplier certified that this material was four times recrystallized and was 100% pure albumin by electrophoresis. The crystalline HSA was stored at −7° and was dried under vacuum over calcium chloride, to a constant weight before use. The molecular weight was assumed to be 69,000. All other solvents and chemicals employed at various stages of the experiment were reagent grade or comparable quality.

Thin-Layer Chromatography of Degradation Products—A 5×10^{-4} *M* solution of cortisol in phosphate buffer was prepared. Ethanol was used to facilitate dissolution of the cortisol and was present in the final sample at a concentration of 2%. The solution was stored at 25.0° for 10 days. An aliquot of the solution was then extracted with four 30-ml. portions of freshly distilled chloroform. The combined extracts were designated "extract I." The aqueous residue remaining after extraction was made strongly acidic with concentrated hydrochloric acid and was again extracted with four 30-ml. portions of chloroform. The combined extracts were designated "extract II." Both extracts I and II were concentrated *in vacuo* to 5 ml. Ten-microliter volumes of the concentrated extracts and of a freshly prepared solution of cortisol of identical concentration extracted in a similar fashion were spotted onto thin-layer chromatographic plates coated with Silica Gel G which had been activated at 110° for 1 hr. After drying, the plates were developed with a mixture consisting of 75 parts benzene, 50 parts acetone, and 0.2 part water. After drying, visualization of the spots was accomplished by first spraying with a solution consisting of equal parts of tetramethylammonium hydroxide reagent and triphenyltetrazolium chloride reagent. The plates were heated for 5 min. at 110°. Steroids reacting with the 2,3,5-triphenyltetrazolium chloride appeared as pink spots on the plate. After the pink zones were marked, the plates were sprayed with 70% w/w sulfuric acid and were heated at 110° for 15 min. All spots were then visible as brown-black areas.

Kinetic Experiments—Kinetic experiments were performed at 15.0°, 25.0°, and 35.0°. Flasks containing cortisol solutions of the appropriate concentration[2] in the phosphate buffer were placed in a thermostat maintained within ±0.05° of the desired temperature. These samples also contained 2% ethanol which was used to facilitate dissolution of the cortisol. At various time intervals, aliquots of the solutions were withdrawn and immediately assayed by a spectrophotometric procedure for their content of total steroid and by a colorimetric procedure for the amount of cortisol still possessing the 17-dihydroxyacetone function.

In the spectrophotometric procedure, the absorbance of the solution was measured at 248 mμ with a Beckman DU spectrophotometer. The apparent concentration of cortisol in the sample was calculated from the absorbance reading, using 16,000 as the molar absorption coefficient of cortisol in the buffer medium.

To determine the amount of steroid still possessing the 17-dihydroxyacetone function, the tetrazolium chloride procedure of Mader and Buck (15) was employed. The appropriate aliquot of the test solution was extracted with four 20-ml. portions of freshly distilled chloroform. The combined chloroform extracts were evaporated under vacuum to dryness. The residue was dissolved in 20 ml. absolute ethanol and 1 ml. each of triphenyltetrazolium chloride reagent and tetramethylammonium hydroxide reagent was added. The assay mixture was allowed to stand in the dark for exactly 30 min. One milliliter of glacial acetic acid was then added to quench further color formation. The absorbance of the mixture was determined with a Bausch & Lomb Spectronic 20 colorimeter at 485 mμ. Ten milliliters of buffer solution, treated in a similar fashion, served as a reagent blank for the procedure. The concentration of cortisol still retaining the 17-dihydroxyacetone function was determined by referring to a standard curve of absorbance *versus* concentration for freshly prepared cortisol solutions assayed in an identical fashion.

Spectrophotometric Estimation of Cortisol Binding—A spectrophotometric procedure was employed for preliminary experiments to determine whether degradation of the 17-dihydroxyacetone function of cortisol significantly affected binding to HSA. The method employed was adapted from one proposed by Klotz and Walker (16) and later used by Westphal (17) in his studies of steroid–protein interactions. Solutions of cortisol in the phosphate buffer, $3.86 \times 10^{-5}M$ and $7.88 \times 10^{-5}M$, were prepared. An aliquot of each solution was mixed with an equal volume of a 4×10^{-5} *M* HSA in phosphate buffer. The absorbance of each solution was measured at 248 mμ with the Beckman DU spectrophotometer. HSA, 4×10^{-5} *M*, diluted with an equal volume of phosphate buffer, served as the reference solution in the spectrophotometer.

The apparent molar extinction coefficient of cortisol ($\epsilon_{app.}$) in the presence of HSA was calculated from:

$$\epsilon_{app.} = \frac{1}{Cd} \log \left(\frac{I_0}{I} \right) \qquad \text{(Eq. 1)}$$

where C is the total molar concentration of cortisol,

[1] In this paper, human serum albumin is henceforth designated as HSA.

[2] See Table I for concentrations of cortisol employed in these experiments.

d is the path length of the cell, and log I_0/I is the absorbance of the mixture at 248 mμ.

The stock solutions of cortisol were then placed in a thermostat at 25.0°. During succeeding days, samples were withdrawn and mixed with freshly prepared HSA solutions. The ultraviolet absorbance was measured and the $\epsilon_{app.}$ was calculated as described.

Equilibrium Dialysis Experiments—The effect of cortisol degradation on the binding to HSA was quantitated by an equilibrium dialysis experiment (18). Eight-inch lengths of Visking cellulose dialysis casings (23/32 in. diameter) were immersed in distilled water and heated on a steam cone for 12 hr. The water was changed three times during this period. The inner lumen of each casing was then flushed with a running stream of distilled water. The casings were soaked an additional 0.5 hr. in distilled water and were then transferred to phosphate buffer solution for 6 hr. at room temperature. The casings were used immediately after this length of time.

Solutions of HSA, 4×10^{-5} M, in phosphate buffer were prepared immediately prior to each experiment. Octyl alcohol, 250 mg./L., was added to HSA solutions to prevent microbial attack. Octyl alcohol between 250 mg./L. and 1000 mg./L. did not interfere with the binding of cortisol by HSA.

Excess buffer solution was carefully expressed from the dialysis casings and a knot was tied in one end to make a small bag. A 10-ml. quantity of the HSA solution was pipetted into the bag and, finally, the open end was knotted. The bag was placed inside a 20 × 200-mm. glass-stoppered test tube containing 20 ml. cortisol solution of the appropriate concentration and age. The tubes were stoppered, dipped in paraffin, and placed on a rocker-type shaker completely immersed in a constant-temperature bath at 25.0°. Six different concentrations of cortisol solution between 5×10^{-5} M and 5×10^{-4} M were used in each experiment and duplicate samples were prepared for each concentration. A time period of 24 hr. was sufficient for attainment of diffusional equilibrium between the solutions inside and outside the dialysis bag. Three samples containing 20 ml. buffer solution in place of cortisol were included in each experiment as controls. There was negligible change in the volumes of solution inside and outside the dialysis sac during the course of the experiment. After 24 hr., the tubes were removed from the bath. The solution external to the dialysis bag was assayed as described previously.

Because cortisol was found to bind to the dialysis casing, parallel experiments were run to obtain corrections for "bag binding." Samples were prepared as described above except 10 ml. phosphate buffer was placed inside the bag instead of HSA solution. At the end of the dialysis experiment, the amount of steroid bound to the bag was calculated as the difference between the amount of compound remaining in solution and the amount originally present before equilibration. Ultraviolet spectrophotometric assay procedures were used to measure the steroid concentration in these experiments. A curve was prepared by plotting the amount of steroid bound by the dialysis bag *versus* the concentration of steroid not bound and served to provide correction values for the test samples containing protein.

To evaluate the effect of degradation of the 17-dihydroxyacetone function of cortisol on the binding of HSA, equilibrium dialysis studies were carried out using cortisol solutions which had been aged for periods of time sufficient to obtain a desired degree of degradation. A 5×10^{-4} M stock solution of cortisol in phosphate buffer was prepared by placing the necessary amount of cortisol in a volumetric flask nearly filled with phosphate buffer. The flask was placed on a shaking device in a constant-temperature room at 25° and agitated vigorously until dissolution of the cortisol was complete. This procedure was usually completed within a 6-hr. period. The solution was then made to volume with phosphate buffer and the flask was placed in a thermostat at 25.0° for the desired period of time. The age of the solution was taken as the time elapsed between first contact of the crystalline cortisol with the buffer solution and the end of the dialysis experiment. Dialysis experiments were performed at 25.0° using cortisol solutions aged for 48, 84, and 288 hr. After diffusional equilibrium was attained, two aliquots of the phase external to the dialysis bag were taken for assay. One portion was assayed by measuring the ultraviolet absorbance at 248 mμ. The second portion was assayed by the triphenyltetrazolium chloride method described above.

Calculations—*Total Steroid Bound*[3]—At equilibrium, the concentration of steroid in the volume outside the dialysis bag may be assumed to be equal to the concentration unbound inside the dialysis casing. The concentration of free steroid in the outside volume is given by the ultraviolet absorbance of this solution after equilibrium is established. The difference between the amount of free steroid in the total volume of the system and the amount of steroid initially placed in the test tube gives the amount of steroid bound. This figure also includes the amount of steroid bound to the dialysis bag. The correction for binding to the bag at any free steroid concentration was obtained from the bag binding correction curve described above.

Undegraded Cortisol Bound—The equilibrium concentration of undegraded cortisol in the volume outside the bag is measured by the triphenyltetrazolium assay method. The total concentration of undegraded cortisol can be obtained from assay of the stock solution at the end of the experiment. The difference between the amount of undegraded cortisol remaining free at equilibrium and the total amount theoretically present at this time gives the amount of cortisol which is bound.[4] A correction for bag binding was obtained by referring to the correction curve described above. It was assumed that the affinity for binding to the dialysis casing was the same for cortisol as for its degradation products.

Degraded Cortisol Bound—The data for the binding of degraded cortisol are calculated in the following manner. The equilibrium concentration of degraded cortisol remaining free in a given sample is obtained by calculating the difference between the equilibrium concentrations of total steroid and undegraded cortisol. In similar fashion, the amount of

[3] The term "steroid" is used here to denote all forms still possessing the steroid ring structure and includes cortisol plus its degradation products.

[4] It was assumed that the rate of degradation of cortisol is unchanged in the presence of HSA. In the actual case, the presence of HSA slows the rate of degradation somewhat (19). However, the difference is slight over the 24-hr. period encompassed by the equilibrium dialysis experiment.

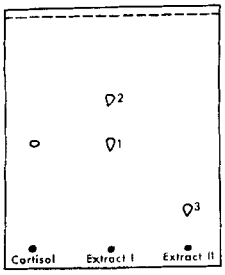

Fig. 1—Thin-layer chromatogram of extracts of a solution of cortisol in phosphate buffer, pH 7.4, μ = 0.16, aged for 240 hr. at 25.0°.

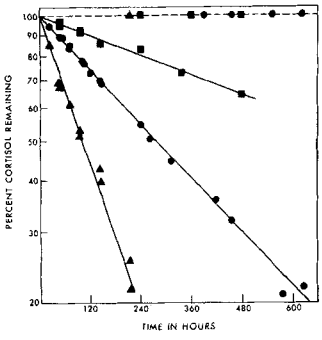

Fig. 2—Disappearance of cortisol from solutions in phosphate buffer, pH 7.4, μ = 0.16, at 15.0° (■), 25.0° (●), and 35.0° (▲), as measured by reaction with triphenyltetrazolium chloride (———) and by ultraviolet absorbance at 248 mμ (– – – –). (See also Table I.)

degradation products bound is calculated from the difference between the concentrations of total steroid bound and the concentration of cortisol bound.

RESULTS AND DISCUSSION

Figure 1 is a diagram of a thin-layer chromatogram of extracts from a cortisol solution aged at 25.0° for 10 days. The freshly prepared cortisol standard produced a spot reacting pink with triphenyltetrazolium chloride. Extract I gave two spots with 70% w/w sulfuric acid marked as 1 and 2 in Fig. 1. Spot 1 reacted with triphenyltetrazolium chloride and has the same R_f as the standard cortisol. It was considered to represent the cortisol remaining undegraded in the test sample. Spot 2 does not react with triphenyltetrazolium chloride and hence must represent a degradation product lacking the 17-dihydroxyacetone function. Extract II gave only one spot, marked 3 in Fig. 1, with sulfuric acid. Spot 3 does not react with triphenyltetrazolium chloride and hence must represent a second degradation product of cortisol also lacking the 17-dihydroxyacetone function. Under aerobic conditions, the degradation of the 17-dihydroxyacetone function of steroids has been reported to involve an oxidative cleavage to yield the corresponding etianic acid (20–22). Chulski and Forist (21) have investigated the effect of solid buffering agents on prednisolone in aqueous solution. Their paper chromatographic examination of the reaction mixture suggested that prednisolone was converted predominantly to a 17β-carboxylic acid and, to a lesser degree, to a 17-keto product. Since the compound represented by spot 3 corresponds to a product possessing acidic characteristics, it is likely that it represents a carboxyl group in place of the 17-dihydroxyacetone function.

It is likely that spot 2 represents a neutral product, probably a 17-keto compound similar to the one reported by Chulski and Forist for prednisolone (21).

TABLE I—RESULTS OF KINETIC STUDIES OF THE DEGRADATION OF THE 17-DIHYDROXYACETONE FUNCTION OF CORTISOL IN PHOSPHATE BUFFER, pH 7.4, IONIC STRENGTH 0.16

Temp., °C.	Apparent First-Order Rate Constant, (hr.$^{-1}$) × 10^3	Half-Life, hr.	Activation Energy, Kcal./mole
15.0	0.896[a]	773	
	0.912	760	
25.0	2.46	282	
	2.69	258	
35.0	6.80	102	17.0[b]
	6.03	115	

[a] The first value at each temperature represents a solution having an initial concentration of 7.9 × 10^{-5} *M*, 5.8 × 10^{-5} *M*, or 7.6 × 10^{-5} *M*, respectively. The second value at each temperature represents a solution having an initial concentration of 4.1 × 10^{-4} *M*, 4.1 × 10^{-4} *M*, or 3.8 × 10^{-4} *M*, respectively. Rate constants were calculated by the method of least squares from experimental data arranged according to the equation, $\ln c/c_0 = -kt$, where c is the concentration of cortisol at any particular time t, c_0 is the concentration of cortisol when t is zero, and k is the apparent first-order rate constant. [b] Calculated from the Arrhenius equation using the method of least squares applied to the rate constant data.

The ultraviolet absorption by cortisol at 248 mμ is characteristic of the Δ^4-3-keto function of the A ring of the steroid nucleus. Under conditions employed in these experiments, the apparent concentration of cortisol determined by the ultraviolet absorption assay remains constant up to a period of 26 days at 25.0° and up to 9 days at 35.0° (see broken line in Fig. 2). This observation indicates that there is no significant degradation of the A ring of cortisol during the periods of time allowed for experiments in this study. The half-life for the photolytic degradation of the A ring of cortisol under ordinary fluorescent laboratory lighting at room temperature has been reported as 160 days (23). The stability of the A ring allows the ultraviolet absorption assay method to be used to show accurately the concentration of total steroid in aged solutions of cortisol. Inspection of Fig. 2 shows that even when considerable amounts of cortisol have degraded, the ultraviolet absorbance remains unchanged.

The results of kinetic studies of the degradation of cortisol in the phosphate buffer are summarized in Table I and Fig. 2. The degradation of the 17-dihydroxyacetone function follows apparent first-order kinetics at all concentrations and temperatures studied. Figure 3 shows an Arrhenius plot of the rate constant data. The activation energy calculated by the method of least squares from the rate constant data is 17.0 Kcal./mole. Data in Table I may be compared with those obtained by Oesterling and Guttman (13) for prednisolone and by Jensen and Lamb (24) for fluprednisolone. The former investigators reported a rate constant of approximately 0.07 hr.$^{-1}$ for degradation of the 17-dihydroxyacetone function of prednisolone in 0.1 *M* phosphate buffer at 70°.[5] Our data predict a value of 0.109 hr.$^{-1}$. The activation energy reported by Jensen and Lamb was 15.2 Kcal./mole. Their rate constant at 70° was reported as 0.0952 day^{-1} (0.004 hr^{-1}). Oesterling and Guttman have pointed out the strong de-

[5] The value for the rate constant was taken from Fig. 4 of the paper by Oesterling and Guttman (13).

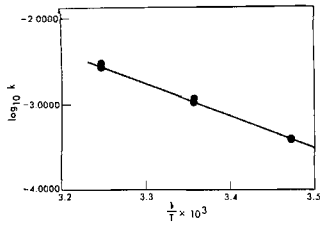

Fig. 3—Arrhenius plot of rate constants for degradation of the 17-dihydroxyacetone function of cortisol in phosphate buffer, pH 7.4, μ = 0.16.

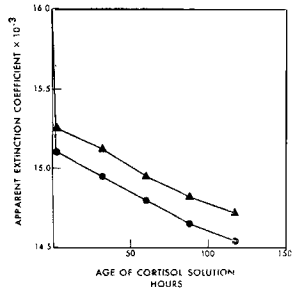

Fig. 4—Effect of aging in phosphate buffer, pH 7.4, μ = 0.16, on the apparent molar extinction coefficient of cortisol at 248 mμ when mixed with freshly prepared solutions of HSA, 2 × 10⁻⁵ M. Initial cortisol concentrations, 1.93 × 10⁻⁵ M (●) and 3.94 × 10⁻⁵ M (▲).

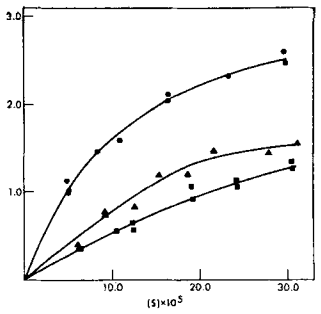

Fig. 5—Binding of total steroid by HSA in systems prepared with solutions of cortisol in phosphate buffer, pH 7.4, μ = 0.16, aged at 25.0° for 48 hr. (■), 84 hr. (▲), and 288 hr. (●).

pendence of the reaction upon the nature of the buffer components, particularly with respect to the content of trace metals in the system. Guttman and Meister (14) have shown the dependence of the reaction upon the hydroxyl-ion concentration. Thus the difference between the rate constants for the different compounds are not unexpected since conditions are not identical in the three different experiments.

Figure 4 shows the results of spectrophotometric studies concerning the effect of sample age on the apparent binding of cortisol by HSA. The large initial decrease in the apparent molar extinction coefficient at 248 mμ can be attributed to the binding interaction between cortisol and HSA (17). As Fig. 4 illustrates, there is a progressive decrease in the apparent molar extinction coefficient of these systems as the cortisol ages. This is indicative of either a greater amount of total steroid being bound or a change in the molar extinction coefficient of the steroid–protein complex. Due to uncertainty about the respective values of the molar extinction coefficients of the various possible steroid–protein complexes, the spectrophotometric method could not be employed to measure the magnitude of the interaction between cortisol or its degradation products and HSA.

Data from equilibrium dialysis studies of protein binding interactions are usually plotted according to:

$$\frac{1}{r} = \frac{1}{nK[S]} + \frac{1}{n} \qquad \text{(Eq. 2)}$$

where r is the average number of molecules of the particular steroid bound per molecule of HSA, n represents the maximum number of binding sites per molecule of HSA, K is the association constant for the interaction, and $[S]$ is the concentration of substrate molecules remaining free in solution when equilibrium between bound and unbound forms is established. This equation is applicable under the conditions that all binding sites have the same intrinsic association constant, K, and that, aside from a statistical factor, the free energy of binding to a particular site is independent of binding at other sites (25). A plot of $1/r$ *versus* $1/[S]$ will yield a straight line with $1/n$ as its intercept on the ordinate axis and $1/nK$ as its slope.

Equation 2 cannot be applied directly to data for aged solutions representing the binding of total steroid by HSA. In these systems, the steroid is present as three different compounds, each of which may bind to the protein with differing affinity. Since data expressing the concentration of each degradation product were not available, an expanded form of Eq. 2 was not employed. Figure 5 shows plots of r *versus* $[S]$ for the binding of total steroid by HSA.

From Fig. 5, it is obvious that the extent of interaction between steroid and HSA increases with the age of the steroid solution. This suggests that the degradation products either bind more strongly than does cortisol or that they are bound to sites not available to cortisol. In the 48-hr. solution, only about 11% of the total steroid is in the degraded form, while approximately 50% is degraded in the 288-hr. material. It is also interesting to note that application of Eq. 2 to the data yields linear plots (Fig. 6). From the slopes of these curves, one obtains values for nK as being 5,700 L./mole, 9,000 L./mole, and 30,000 L./mole at 48 hr., 84 hr., and 288 hr., respectively. Values of n calculated from the intercepts are 5, 5, and 3.6. As stated above, these values are without theoretical meaning; however, they do point out that failure to account for degradation as an experimental variable in these systems may easily go unrecognized. This is particularly true when the assay method employed is not sensitive to the presence of the 17-dihydroxyacetone function.

Figure 7 shows plots of data obtained for the binding of undegraded cortisol in the 48-hr. and 288-hr. samples. It appears that the presence of increased amounts of degradation products in the system at 288 hr. affects the binding of cortisol by HSA. The curves in Fig. 7 represent a visual fit of the data. From these curves, nK values of 4,500 L./mole and 8,900 L./mole are obtained at 48 hr. and 288 hr., respectively. The estimated value of n in each case is 5. Equations for the lines best fitting the experimental data were also calculated by the method of least squares. In this case, nK values were 4,080 L./mole and 10,429 L./mole at 48 hr. and 288 hr., respectively.[6] In this case, however, values for n were 5.3 and 2.1, respectively.

[6] Reciprocals of the 95% confidence intervals of the calculated slopes gave nK values ranging between 5952 and 3106 L./mole at 48 hr. and between 6579 and 26,316 L./mole at 288 hr.

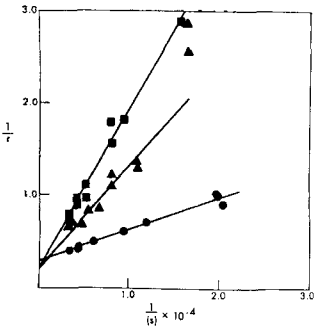

Fig. 6—Plot according to Eq. 2 of the binding of total steroid by HSA in systems prepared with solutions of cortisol in phosphate buffer, pH 7.4, μ = 0.16, aged at 25.0° for 48 hr. (■), 84 hr. (▲), and 288 hr. (●).

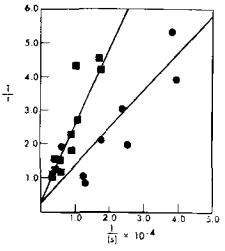

Fig. 7—Plot according to Eq. 2 expressing the binding of undegraded cortisol by HSA in systems prepared with solutions of cortisol in phosphate buffer, pH 7.4, μ = 0.16, aged at 25.0° for 48 hr. (■), and 288 hr. (●).

The values of the intercepts from the least squares calculations indicate that a competition for binding sites may exist between the degradation products and cortisol. This suggests that the affinity between the degradation products and certain sites on the HSA molecule is greater than the affinity of cortisol for the same sites.

This observation is consistent with the observations that the binding of total steroid increases in aged solutions of cortisol. In view of the scatter in the points for the 288-hr. data, there is considerable uncertainty in the value of n obtained from the intercept of the $1/r$ *versus* $1/[S]$ plot. Assigning such significance to the differences in n between 48-hr. and 288-hr. data must be done with caution. Unfortunately, even with repeated experiments, it was impossible to attain the degree of precision in the 288-hr. systems which would allow a more confident assessment of the situation.

Regardless of whether n remains at 5 or indeed is reduced to 2 in systems containing larger amounts of degradation products, it is of interest that the data at 288 hr. suggest that the affinity constant for the interaction between cortisol and HSA must also increase at least by a factor of two or more. This might be explained as being due to an actual difference between the affinity constant of cortisol for some sites as compared to others, especially if one accepts that the decrease in n actually does occur in the 288-hr. system. An alternative explanation would be that binding of the degradation products induces changes in HSA molecules which increase the affinity constant for interaction between cortisol and remaining free sites. Belman (26) has observed changes in the interaction between beryllium and bovine serum albumin when 1% dodecyl sulfate was present. Considerable evidence exists (27–30) that protein molecules are quite flexible and that the secondary and tertiary structure is easily deformed in the presence of certain other molecular species. Karush (27, 30) has suggested that serum albumins possess a number of combining regions, each of which is characterized by a high degree of configurational adaptability. He has also suggested that conformational changes in the protein resulting from binding of a first substrate molecule may induce a capacity for interaction with additional substrate molecules. It appears to be not impossible that an increased binding affinity of cortisol could result from the presence of increasing amounts of its degradation products also being bound by HSA. Brunkhorst and Hess (5) have observed changes in binding capacity caused by increasing concentrations of substrate in their investigations of the cortisol–HSA interaction.

Table II—Values of nK and $\Delta F°$ Reported in the Literature for the Interaction between Cortisol and Human Serum Albumin[a]

Temp., °C.	nK (L./mole) × 10^{-3}	$\Delta F°$ (Kcal./mole)	Ref.
5	5.0	−4.7	(2)
4	6.0	−4.8	(3)
4	2.3	−4.3	(3)
25	5.3[a]	...	(5)
25	4.9[a]	−5.0[b]	(5)
25	4.4[a]	...	(5)
25		−5.5	(31)

[a] The value for nK was dependent upon total concentration of cortisol used in the experiment. [b] Calculated from data given in Table II of *Reference 5*.

It is interesting to compare the results of this experiment with reports of other investigators for the cortisol–HSA interaction (Table II). While nK values in Table II agree favorably with the value obtained in this experiment for the 48-hr. sample, $\Delta F°$ for binding of cortisol in the 48-hr. sample is −4.0 Kcal./mole when n is taken as being 5. The lack of agreement in $\Delta F°$ values between Table II and this experiment is interesting in light of the fact that higher values similar to those in Table II can be obtained if one calculates this parameter from the plots of Fig. 6 for the binding of total steroid from solutions of cortisol aged for varying lengths of time. In such cases, $\Delta F°$ values of −4.1 Kcal./mole, −4.6 Kcal./mole, and −5.4 Kcal./mole are obtained at 48 hr., 84 hr., and 288 hr., respectively. Comparison of these values with those in Table II causes one to question in the experiments cited whether proper control was exerted to insure that cortisol degradation did not take place. Unfortunately, in these reports, no mention was made regarding control of the cortisol samples. Brunkhorst and Hess (5) employed a radiometric assay utilizing cortisol-4-^{14}C. Westphal (17, 31) employed ultraviolet spectrophotometric methods of assay. Neither method would be sensitive to the degradation phenomena reported here.

SUMMARY AND CONCLUSIONS

Data obtained in this research clearly show that the interaction between cortisol and HSA can only be studied by methods which take into account the degradation of the 17-dihydroxyacetone function of cortisol which can occur under usual experimental conditions. These results would also apply qualitatively to other steroids possessing the 17-dihydroxyacetone function.

This research also points out the necessity for considering stability factors in equilibrium dialysis experiments of this type. Competitive binding or other effects imparted by the presence of degradation products can significantly alter experimental results. In addition, since results of dialysis experiments are calculated from concentration differences before and after equilibration, disappearance of solute due to unrecognized chemical instability could be mistaken for binding by the protein and introduce further error into the experiment.

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Mechanistic Implication Between Quenching in Liquid Scintillation System and Photosensitivity with Respect to Energy Transfer

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A basic mechanism for energy transfer in photosensitive biological systems is postulated.

THE USE OF liquid scintillation system for radiation measurement has been widely accepted. Owing to its high counting efficiency, the internal sample method becomes the preferred mode of assaying radionuclides emitting β particles of low kinetic energy, particularly tritium, ^{14}C, ^{35}S, ^{45}Ca, *etc.* The introduction of a sample in the form of an organic compound into the liquid scintillation system frequently causes fluorescence quenching which manifests itself as a decrease in the counting efficiency of the system. The cause of quenching has been attributed to either a diminished light transmission owing to the color of the sample or a molecular interaction between the scintillator and the sample leading to thermal degradation of the excitation energy or a combination of both (1).

The liquid scintillation system is a sensitized fluorescent donor–acceptor system in which the solvent molecule, by virtue of its presence in an overwhelming number in the medium, absorbs the radiation energy and becomes excited; from the excited states, the solvent molecules may undergo de-excitation by fluorescence emission, nonradiative transition, or excitation transfer. In the presence of solute molecules which can accept the excitation energy from the donor solvent molecule, de-excitation can take place by emission of fluorescence of the solute. Because of the inherently greater fluorescence intensity, the spectrum of the solute characterizes that of the entire system. In the presence of a quencher, transfer of excitation energy is adversely affected. According to theory, energy transfer between unlike molecules is brought about by a resonance mechanism over long distances involving dipole–dipole interaction (2, 3) or dipole–quadrupole interaction (3) or by the exchange mechanism (3, 4) in which an overlap of the wave function of the molecules concerned is necessary.

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