inhibited by treatment with actinomycin D in contrast to the partial inhibition found by Notides and Gorski (1966). Surprisingly, actinomycin D evokes the synthesis of an "actinomycin-specific" uterine protein with electrophoretic mobility similar to that of the estrogen-specific proteins.

The synthesis of the estrogen-specific proteins is also detected after *in vitro* treatment of isolated uteri with 17β-estradiol added to the incubation medium. Under these conditions the "secondary estrogen response" is not detected by prolonged treatment with hormone.

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Interactions between Corticosteroids and Histones*

C. Monder† and M. C. Walker

ABSTRACT: This project was designed to test the validity of previous work which concluded that cortisol reacts with histones. In this paper, it is reported that the interaction of cortisol-1,2-*t* and histone was due to the presence of 21-dehydrocortisol-1,2-*t* in the steroid preparations. This conclusion was based on the following observations: Cortisol-1,2-*t* and 21-dehydrocortisol, though reacting with all histone fractions, bound most extensively to arginine-rich histone. Blocking of arginyl residues of histones by glyoxalation greatly diminished binding of 21-dehydrocortisol-1,2-*t* and eliminated binding of cortisol-1,2-*t*. It was concluded that unblocked guanidino residues were essential for the reaction of steroid with histone. Blocking of lysyl residues alone by maleylation had only a small effect on the reaction of either steroid with histone. In model systems using solvent partition analysis, cortisol did not react with any free amino acid, while 21-dehydrocortisol reacted with all free amino acids except proline and hydroxyproline. Net reaction of 21-dehydrocortisol with arginine and histidine was four to five times greater than with most monocarboxylic–monoamino acids, and two times greater than with lysine. Arginine and 21-dehydrocortisol combined in equimolar ratio and irreversibly. Lysine and other amino acids were bound reversibly and less extensively. Preferential reaction of 21-dehydrocortisol with arginyl residues of histone was demonstrated by direct amino acid analysis of the stable steroid–histone complex after acid hydrolysis. This finding was consistent with the behavior of the steroids with modified histones and with free amino acids. 21-Dehydrocortisol-1,2-*t* was a consistent contaminant in all cortisol-1,2-*t* preparations.

It was concluded that binding of corticosteroids to histones occurred only after oxidation of the cortisol ketol side chain and that the reaction involved covalent interactions with the terminal residues of the basic amino acids, arginine and, to a lesser extent, lysine.

Considerable effort has been expended in recent years in attempts to explain how the levels and specific properties of proteins in cells of multicellular organisms are controlled. In 1950, Stedman and Stedman proposed that histones, found abundantly in the somatic nuclei of eukaryotes, could exert such control at the level of gene expression, possibly (as others have suggested (Huang and Bonner, 1962)) by reacting with DNA and thereby diminishing transcriptional activity. It is not yet clear if histones influence RNA synthesis in nuclei by performing such a "repressor-like" role, or whether their main function is to impart rigidity and stability to the intranuclear structures (Mirsky *et al.*, 1968; Chalkley and Jensen, 1968). That histones do inhibit the action of DNA-dependent RNA polymerase in model systems is indisputable. The mechanism *in vitro* probably involves neutralization of the negatively charged groups of DNA by the lysyl and arginyl residues of basic histones. These electrostatic interactions can be diminished if the charge on the histone is decreased by enzymic phosphoryla-

* From the Research Institute of the Hospital for Joint Diseases, Mount Sinai School of Medicine, New York, New York 10035. *Received January 20, 1970.* This work was supported by U. S. Public Health Service Grant AM-09006.

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tion (Ord and Stocken, 1966), acetylation (Allfrey *et al.*, 1964), or methylation (Ord and Stocken, 1966; Tidwell *et al.*, 1968) of the basic residues, or by reacting the histone with acidic proteins (Wang, 1968; Langan, 1967). The DNA molecule, now no longer blocked, becomes more accessible as a template, and the rate of transcription consequently increases.

A number of investigators have been intrigued by the possibility that hormones also might react with histones, and thus loosen their bond with DNA. Such a mechanism could, in principle, explain the increase in RNA and protein synthesis resulting from the action of hormones (Butler, 1966).

The reports by Sluyser (1966a) and Sunaga and Koide (1967b) that corticosteroids reacted with histones *in vitro* was in accord with this possibility. The reaction favored the arginine-rich histones, a selectivity consistent with the idea that steroids may bind to specific intranuclear sites. Although the physiological significance of these observations remains to be evaluated, they do provide a tentative basis for explaining how corticosteroids may influence protein synthesis. Consequently, the nature of the interactions which result in the apparently stable, selective binding of corticosteroids to histones seemed worthy of investigation. The results of studies directed to this goal are presented here.

Materials and Methods

Solvent partition analysis was used to determine the ability of an amino acid to increase solubility of a steroid in the aqueous phase of a two-phase system. In the procedure 4 ml of 0.2 M sodium carbonate buffer (pH 9.2) and redistilled ethyl acetate, each previously equilibrated with the other, and containing the appropriate steroid and amino acid, were mixed in glass-stoppered centrifuge tubes and rocked gently for 120 min at 26 ± 1°. This time interval was adequate to complete any reactions between the components and to establish equilibrium between the phases. The absorbance of the lower (aqueous) phase at 242 mμ was used to estimate steroid concentration, assuming a molar extinction coefficient of 1.5×10^4. None of the amino acids used were soluble in ethyl acetate in the presence or absence of steroids as determined by analyses of the total ethyl acetate layers on the amino acid analyzer. The pH values of the aqueous layers were checked at the end of each determination to be sure they had not changed, since a shift of as little as 0.2 pH unit had a significant effect on the results.

Radioactivity of tritium and ^{14}C-labeled steroids were measured in a Packard 3380 scintillation spectrometer. All reported values are corrected to 100% efficiency. Correction for quenching was made by the external ratios method. Radioactivity was usually counted in 12 ml of a scintillation mixture consisting of 3 g of 2,5-bis(2-*t*-butylbenzoxazolyl)-thiophene, 60 g of naphthalene, 450 ml of toluene, and 300 ml of methyl Cellosolve. Aqueous systems such as column effluents were counted in 10 ml of Aquafluor (New England Nuclear Corp.). The level of water solution added, 0.5 ml, increased quenching from 13 to 20%.

Radioactive steroids were bought from New England Nuclear Corp. and Amersham-Searle Corp. Specific activity and purity were determined as described previously (Monder and Walker, 1970).

Syntheses of 17α-hydroxy-21-dehydrocorticosteroids were performed as described by Monder and Martinson (1969). For 17-deoxy- and 17α-hydroxycorticosteroids the times required for the cupric acetate dependent oxidation were 30 and 120 min, respectively. Synthesis of 21-dehydrocortisol-1,2-*t* from cortisol-1,2-*t* was accomplished by the procedure of Monder and Walker (1970).

Cortisol[1] was acetylated by reacting the steroid for 1 hr with pyridine–acetic anhydride (1:3, v/v). 11β-Hydroxy-4-androstene-3,17-dione (11β-hydroxyandrostenedione) was synthesized as described by Brooks and Norymberski (1953).

Chromatography of radioactive steroids was performed on thin-layer plates coated with silica gel (GF_{250}, E. Merck, AG, Darmstadt) previously purified by continuous extraction in methylene chloride followed by methanol. Maximum resolution was achieved during chromatography by permitting the solvent to rise 15 cm from the origin. To measure the distribution of radioactivity along the developed plate, 0.5-cm segments of gel were scraped off, transferred to scintillation vials, suspended in 2,5-bis(2-*t*-butylbenzoxazolyl)thiophene scintillation mixture, and counted. Best separation of cortisol, 21-dehydrocortisol, 11β-hydroxyandrostenedione, and other degradation products derived from 21-dehydrocortisol was achieved in chloroform–ethanol (25:1, v/v). Cortisol, 21-dehydrocortisol, and cortisol 21-acetate were best separated in ethyl acetate–benzene (9:1, v/v). These last three steroids were not resolved by an extensive series of other systems including various combinations of chlorinated hydrocarbons, acetone, ethanol, methanol, ethyl acetate, and diethyl ether. Some mixtures separated two of the three so that, while unsuitable for purposes of resolution, they were useful for confirming the identities of the steroids.

Reduction of 21-dehydrocortisol to cortisol on a microscale was performed with sheep liver 21-hydroxysteroid dehydrogenase (Monder, 1968b). After the enzymic reaction was completed, the steroid was extracted with ethyl acetate and purified by chromatography on silica gel.

Histones were isolated from calf thymus and separated into lysine-rich (F1) and arginine-rich (F3) fractions by the method of Johns (1964). Corresponding fractions were also purchased from the Worthington Biochemical Corp. To ensure the identity of the fractions, each was subjected to a complete amino acid analysis. All corresponded closely with reported values (Johns, 1964).

Amino acid content of proteins was determined in a Beckman Model 120C analyzer according to Spackman *et al.* (1958).

Amino groups of lysyl residues of histones were blocked by reacting them with maleic anhydride as described by Butler *et al.* (1969). To eliminate binding of maleyl groups to hydroxyl or tyrosyl residues, the modified histones were treated with hydroxylamine as recommended by Freedman *et al.* (1968a). Small molecules were separated from the modified protein by passage of the mixture through a Sephadex

[1] Trivial names used are: prednisolone, 11β,17α-21-trihydroxy-1,4-pregnadiene-3,20-dione; epiandrosterone, 3β-hydroxy-5α-androstan-17-one; 11β-hydroxyandrostenedione, 11β-hydroxy-4-androstene-3,17-dione; 21-dehydrocortisol, 11β,17α-dihydroxy-3,20-dioxo-4-pregnen-21-al. Abbreviation used is: Tricine, *N*-tris(hydroxymethyl)methylglycine.

TABLE I: Quantitative Evaluation of the Purity of Cortisol-1,2-*t*.

Source	Lot No.	Cortisol (%)	21-Dehydro-cortisol (%)	11β-Hydroxy-androstene-dione (%)	Unidentified (%)
New England Nuclear Corp.	321–114	94.0	4.9	0.7	0.4
New England Nuclear Corp.	184–123	98.0	0.9	0.4	0.7
Amersham-Searle Corp.	TRA-133, batch 12	96.5	2.3	1.0	0.2

G-25 column. The number of maleyl groups per mole of histone was calculated using

$$\frac{[(OD_{280\ m\mu})(3300)] - [(OD_{250\ m\mu})(4500)]}{[(OD_{250\ m\mu})(308)] - [(OD_{280\ m\mu})(3360)]}$$

where $OD_{280\ m\mu}$ and $OD_{250\ m\mu}$ are the extinction values for the maleylated proteins, and (308) and (3360) are the relative extinction coefficients of the maleyl imino group at 280 and 250 mμ, respectively. The extinction coefficients for histone at these wavelengths were calculated from the "100 mole" molecular weight *i.e.*, the gram-equivalent weight of the histone mixture assuming that the average molecule contained 100 amino acid residues. The values were 3300 and 4500 at 250 and 280 mμ, respectively, for an equivalent weight of 11,000 at pH 8.0. Within the limits of error (±1.2 residues), 100% of the lysyl residues were bound.

Maleylated histones were modified further to block the arginyl residues by reaction with glyoxal as described Freedman *et al.* (1968b) and Nakaya *et al.* (1967). The number of bound side chains was estimated from the number of arginyl residues remaining in acid hydrolysates of the modified proteins as determined on the amino acid analyzer. Removal of the amino-bound maleyl groups from the maleylated-glyoxalated histone was performed with citrate at pH 3.5 (Butler *et al.*, 1969). The glyoxalated product was insoluble in aqueous solutions from pH 2 to 11.

Binding of steroid to histone was determined by adding the radioactive steroid (2.2 μmoles) in 0.10 ml of ethanol to 1.0 mg of histone dissolved in 0.9 ml of 0.2 M sodium phosphate buffer (pH 7.5) at 30°. After 10 min, the mixture was passed through a column of coarse Sephadex G-25 (45 × 1.0 cm) and radioactivity of effluent fractions of 0.5 ml was measured. To check for the presence of artifacts, steroid was also passed through the column in the absence of histone.

Results

In order to evaluate whether cortisol reacts with histones as has been claimed (Sluyser, 1966a,b, 1969; Sunaga and Koide, 1967b), it is of critical importance to establish if the steroid is homogeneous, or if other components are present that can account for the observed effects. This point is emphasized by the fact that maximum reported binding of cortisol to histone ranged from 8.3×10^{-4} % (Sluyser, 1966a) to about 1% (Sunaga and Koide, 1967a) of the added steroid, an amount that could easily be accounted for by contamination. Several commercial samples of cortisol-1,2-*t* were subjected to thin-layer chromatography to determine their purity. Table I shows that cortisol-1,2-*t* as received from the manufacturer contains variable amounts of 21-dehydrocortisol and 11β-hydroxyandrostenedione. Proof of identity of the various contaminating steroids found has been presented (Monder and Walker, 1970; Westphal *et al.*, 1967).

Cortisol-1,2-*t* was separated from contaminants by thin-layer chromatography on silica gel, and the cortisol region eluted with methylene chloride–methanol (9:1, v/v) (Idler *et al.*, 1966). The cortisol was free of detectable contamination at this stage. Solvent was evaporated off and the steroid was dissolved in a small amount of absolute ethanol. The steroid was incubated with histone in preparation for gel filtration. By the time the mixture was placed in the column, it had accumulated enough 21-dehydrocortisol-1,2-*t* to account for 1.5% of the total steroid. Unpurified cortisol-1,2-*t*, containing 3.6% of 21-dehydrocortisol, and cortisol-1,2-*t* with added 21-dehydrocortisol-1,2-*t* were incubated with unfractionated histone in the same way. The total amount of radioactivity emerging with histone increased in proportion to the level of 21-dehydrocortisol in the steroid, as shown in Table II. It was never possible during the incubation of cortisol under these conditions to avoid the formation of some 21-dehydrocortisol, so that it could not be conclusively established if cortisol itself combined with histone. To test if the ring system common to these 21-carbon steroids

TABLE II: Relationship between 21-Dehydrocortisol-1,2-*t* Content of Cortisol-1,2-*t* and the Binding of Steroid to Histone.[a]

Steroid-1,2-*t* Incubated (moles × 10^9)	21-Dehydro-cortisol-1,2-*t* (% of total steroid-1,2-*t*)	^3H Bound to Histone (moles × 10^{12})
Cortisol (69)	1.5	15
Cortisol (69)	3.6	39
Cortisol + 21-dehydro-cortisol (420)	67	166
11β-Hydroxy-4-androstene-3,17-dione (330)	0.0	0.31

[a] Steroids were incubated with 800 μg of unfractionated histone for 10 min at 30° in 0.8 ml of 0.01 M sodium phosphate buffer (pH 8.0) then passed through a Sephadex G-25 column. Content of 21-dehydrocortisol was determined immediately prior to gel filtration.

TABLE III: Effect of Amino Acids on Solubility of 21-Dehydrocortisol in Aqueous Medium.[a]

Amino Acid	Increase in Steroid in Aq Phase (μmole)	Amino Acid	Increase in Steroid in Aq Phase (μmole)
Arginine	0.297 ± 0.010	Glutamine	0.061 ± 0.005
Histidine	0.284 ± 0.012	Alanine	0.064 ± 0.003
Lysine	0.133 ± 0.007	Aspartic acid	0.069 ± 0.002
Serine	0.132 ± 0.012	Isoleucine	0.096 ± 0.008
Threonine	0.173 ± 0.015	Glutamic acid	0.075 ± 0.004
Proline	0.000	Methionine	0.076 ± 0.006
Hydroxyproline	0.000	Glycine	0.041 ± 0.003
		Valine	0.099 ± 0.005

[a] Four milliliters of ethyl acetate containing 1.000 μmole of 21-dehydrocortisol was equilibrated with 4 ml of 0.2 M sodium carbonate (pH 9.2) containing 137 μmoles of amino acid. Total steroid in aqueous phase was calculated by assuming that the total extinction at 242 mμ was due solely to the steroid, and using an extinction coefficient of 1.52×10^4. The amount of steroid transferred to the aqueous phase in the absence of amino acid, 0.112 ± 0.002 μmole, has been subtracted from the values in the table. Values shown include average deviation from the mean for three separate equilibrations.

TABLE IV: Spectrophotometric Evaluation of the Reaction of 21-Dehydrocortisol with Arginine and Lysine.[a]

Steroid	Steroid Transferred to Aq Phase with: Arginine (μmole)	Lysine (μmole)
21-Dehydro-11-deoxycorticosterone	0.012	0.002
21-Dehydrocorticosterone	0.033	0.008
21-Dehydroprednisolone	0.132	0.051
21-Dehydrocortisone	0.172	0.040
21-Dehydrocortisol	0.190	0.073

[a] Experimental conditions were similar to those described in Table III.

was bound to histone, 11β-hydroxyandrostenedione-1,2-*t* was incubated under the same conditions. As Table II shows only 0.31×10^{-12} mole emerged with histone, out of a total of 3.3×10^{-7} mole of steroid added. This corresponded to a total of 18 cpm, about 7.4×10^{-5} % of that put on the column. It is concluded that, though some 11β-hydroxyandrostene-3,17-dione may have combined with histone, the amount was so small as to be more likely due to nonspecific adsorption. Since the fused-ring system was not, by itself, responsible for the reaction of steroid with histone, it was concluded that the dihydroxyacetone side chain must be involved in some way. The fact that the degree of binding was roughly proportional to the 21-dehydrocortisol content of the preparations suggested that this contaminant may be responsible for the binding, and led us to examine how 21-dehydrocortisol combines with histones. Preliminary to these studies, the reaction of 21-dehydrocortisol with free amino acids was studied.

Reaction of 21-Dehydrocortisol with Amino Acids. Using a two-phase partition system of ethyl acetate and bicarbonate buffer, the specificity of the reaction of 21-dehydrocortisol with L-amino acids was surveyed. These results are summarized in Table III.

The amino acids could be divided into four groups with respect to their ability to react with 21-dehydrocortisol. The most reactive were arginine and histidine. A second group, about half as effective, contained lysine, serine, and threonine. At the opposite extreme were the imino acids proline and hydroxyproline. The remaining acidic and neutral amino acids all were indistinguishable with respect to their ability to combine with 21-dehydrocortisol, and it may be surmized that their common site of reaction with 21-dehydrocortisol was the α-amino group. The more extensive reaction of the basic and hydroxylated amino acids with the steroid undoubtedly involved their side chains. The ε-amino group of lysine probably formed an azomethine linkage, and the hydroxyamino acids may have formed hemiacetals with the steroidal carbonyls. The absence of reaction with hydroxyproline suggests that the amino groups of the hydroxylated amino acids were involved in stabilizing their reaction with steroid. Reaction of 21-dehydrocortisol with arginine is similar to the reaction of other ketoaldehydes with this amino acid (Takahashi, 1968); histidine probably readily forms imidazotetrahydropyridine derivatives with this as it does with other aldehydes (Heyl *et al.*, 1948).

Reaction of 21-Dehydrocorticosteroids with Arginine and Lysine. Table IV shows that, in addition to 21-dehydrocortisol, other 21-dehydrosteroids reacted with basic amino acids. Reaction with arginine was consistently greater than with lysine. The extent of reaction with 17α-hydroxylated steroids was always greater than with the 17-deoxy derivatives.

Effect of pH on the Reaction of Amino Acids with 21-Dehydrocortisol. The reaction of lysine, arginine, and glycine with 21-dehydrocortisol was measured at pH 6.6, 7.4, and 9.5 by the solvent partition method. As expected, the extent of reaction was greatest at pH 9.5 for each amino acid. As the results in Table V demonstrate, arginine reacted most and glycine least at pH 7.4 and 9.5. In order to demonstrate any reaction at all at pH 7.4, amino acid concentrations had to be raised substantially above those at pH 9.5. Even so, there was no detectable effect of glycine on the solubility of steroid in the buffer phase. At pH 6.6, no reaction was detected with any amino acid. The reaction of 21-dehydrocortisol with amino acids probably does not occur to a significant extent below pH 7.

Reversibility of the Reaction between 21-Dehydrocortisol and Amino Acids. The reaction between 21-dehydrocorticosteroids and any amino acid probably involves the formation of an azomethine linkage. This bond is reversible if not complicated by secondary reactions. Under these conditions

TABLE V: Effect of pH on Reaction of Lysine and Arginine with 21-Dehydrocortisol.[a]

Amino Acid	0.274 M Amino Acid–0.2 M Sodium Phosphate (pH 6.6)		0.274 M Amino Acid–0.2 M Sodium Phosphate (pH 7.4)		0.055 M Amino Acid–0.2 M Sodium Carbonate (pH 9.5)	
	Total Steroid Transferred (μmole)	Net Change (μmole)	Total Steroid Transferred (μmole)	Net Change (μmole)	Total Steroid Transferred (μmole)	Net Change (μmole)
L-Arginine	0.105	−0.005	0.154	0.045	0.412	0.300
L-Lysine	0.110	0.000	0.128	0.017	0.242	0.130
Glycine	0.109	−0.001	0.110	0.001	0.152	0.040
None	0.110		1.109		0.112	

[a] Experimental conditions were similar to those described in Table III.

it should be possible to extract 21-dehydrocortisol from an aqueous phase containing amino acid. This proved to be true for lysine, as Figure 1 shows, as well as for monoamino–monocarboxylic acids. In contrast, 21-dehydrocortisol reacted with arginine and histidine to form stable products which resisted extraction from buffer into ethyl acetate.

Reaction between N-Acetylarginine and 21-Dehydrocortisol. Because of the possible relevance of the reaction of 21-dehydrocortisol with arginine to its reaction with histone, the stable product resulting from the condensation of these two components was investigated. The method described by Takahashi (1968) for the reaction of phenylglyoxal with arginine provided the model for these studies. In order to eliminate the possibility of reaction of steroid with the α-amino group, *N*-acetylarginine was used.

To a solution of 400 mg of 21-dehydrocortisol in 3 ml of methanol and 5 ml of 0.2 M Tricine buffer (pH 8) was added a solution of 250 mg of *N*-acetyl-L-arginine dissolved in 5 ml of 0.2 M Tricine buffer (pH 8). The pale yellow solution, obtained after stirring for 18 hr in the dark, was adjusted to pH 3.5, and then passed through a Sephadex G-10 column (90 × 1 cm). The effluent, monitored spectrophotometrically at 300 mμ, showed three peaks (Figure 2). The minor peak emerging earliest was a mixture of substances derived from the steroid and amino acid. No attempt was made to identify them. The third peak was 21-dehydrocortisol, as determined by its chromatographic mobility and by the identity of its infrared spectrum with that of the authentic steroid. The major peak contained a minor component which was easily distinguished under ultraviolet light after chromatography on thin-layer plates of silica gel with 70% aqueous ethanol as the developing solvent. The major fraction was freed of the minor component after passage through a silica gel column (60 × 2 cm) using 70% ethanol as the eluting agent. Elemental analysis of the major component was consistent

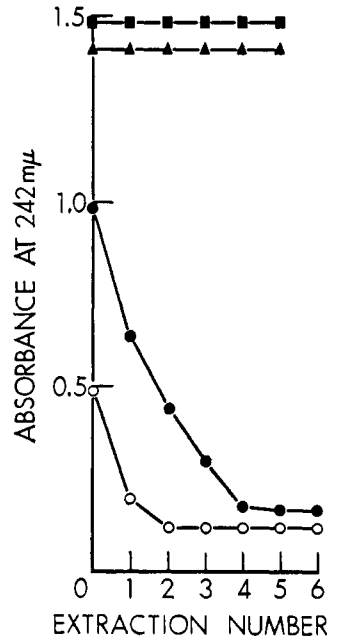

FIGURE 1: Stability of 21-dehydrocortisol–amino acid complexes. The initial equilibration was performed as described in Table III; subsequent extraction of the aqueous phase was done by rocking the tubes for 120 min with equal volumes of ethyl acetate previously saturated with buffer. The amount of steroid in the aqueous phase was measured spectrophotometrically. (○———○) No amino acid, (●———●) lysine, (▲———▲) histidine, and (■———■) arginine.

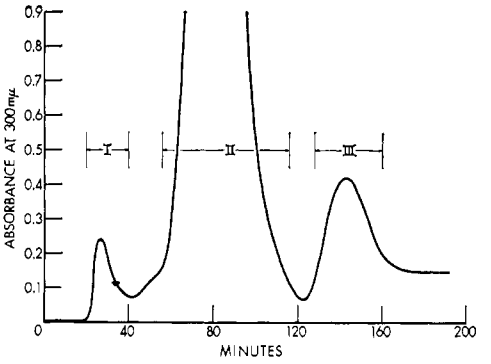

FIGURE 2: Fractionation of the products resulting from the reaction of 21-dehydrocortisol and N^{α}-acetyl-L-arginine. The mixture was passed through a column (1 × 90 cm) of Sephadex G-10 equilibrated with water adjusted with hydrochloric acid to pH 3.5. Fractionation was monitored spectrophotometrically. Flow rate was about 22 ml/hr; temperature, 27°. The fractions under each peak (I, II, or III) were collected together and lyophilized.

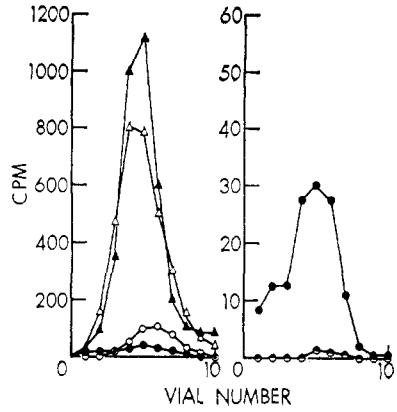

FIGURE 3: Comparison of reaction of 21-dehydrocortisol-1,2-*t* and cortisol-1,2-*t* with histones. Steroid (2.2 μmoles in 0.1 ml of ethanol) was incubated with 1.0 mg of histone dissolved in 0.9 ml of 0.2 M sodium phosphate buffer (pH 7.5) at 30°. After 10 min, the mixture was passed through a column of coarse Sephadex G-25 (45 × 1 cm) and radioactivity of effluent fractions of 0.5 ml was measured. The figures show the region corresponding to the emergence of histone. The secondary peaks of free steroid are not shown. These latter data have been utilized in calculating the values in Table VII. Left panel: (▲——▲) unmodified histone and 21-dehydrocortisol-1,2-*t*, (△——△) maleylated histone and 21-dehydrocortisol-1,2-*t*, (○——○) maleylated-glyoxalated histone and 21-dehydrocortisol-1,2-*t*, (●——●) unmodified histone and cortisol-1,2-*t*. Right panel: (●——●) unmodified histone and cortisol-1,2-*t*. This curve is identical with the one in the left panel, but the vertical axis has been expanded; (◓——◓) maleylated-glyoxylated histone and cortisol-1,2-*t*.

TABLE VI: Amino Acid Composition of Modified Histones.

Amino Acid	Untreated Histone[a]	Maleylated Histone	Maleylated-Glyoxylated Histone	Glyoxylated Histone
Lysine	13.6 ± 1.1	12.9	12.6	13.2
Histidine	1.5 ± 0.2	1.8	1.3	1.1
Arginine	8.9 ± 1.1	8.6	1.7	1.7
Aspartic acid	5.4 ± 0.6	5.3	7.5	6.3
Threonine	5.3 ± 0.2	6.3	5.6	5.5
Serine	5.5 ± 0.2	5.0	5.5	5.8
Glutamic acid	9.8 ± 0.5	10.0	10.0	10.7
Proline	6.1 ± 0.9	6.4	7.2	8.8
Glycine	8.0 ± 0.5	7.9	6.4	8.3
Alanine	14.5 ± 1.0	15.9	15.5	13.5
Half-cystine	0.05	0.1	0.0	0.0
Valine	6.1 ± 0.8	7.3	9.6	6.6
Methionine	0.6 ± 0.2	0.8	0.4	0.3
Isoleucine	4.6 ± 0.6	4.3	4.4	5.0
Leucine	8.2 ± 0.2	8.1	8.0	9.1
Tyrosine	3.2 ± 0.2	3.5	2.9	3.3
Phenylalanine	1.4 ± 0.1	1.6	1.5	0.8

[a] Mean plus and/or minus standard error for three determinations.

with a product containing steroid and arginine in equivalent ratio. *Anal.* Calcd for $N_4H_{46}C_{29}Cl_2O_8$: C, 53.9; H, 7.15; N, 8.65. Found: C, 53.9; H, 7.21; N, 8.69. Spectral maxima were at 243 mμ (ε 14,100) and at 300 mμ (ε 4600). Infrared and nuclear magnetic resonance spectra revealed bands characteristic of amino acid and steroid (J. C. Orr and C. Monder 1969, unpublished observations). The similarity of the extinction coefficient of the complex at 243 mμ to that of free 21-dehydrocortisol provides justification for the use of this value in the solvent partition method to calculate the concentrations of corticosteroids in aqueous media in the presence of amino acids.

Reaction of 21-Dehydrocortisol with Modified Histones. In this section evidence will be presented that 21-dehydrocortisol reacts with histones and that the reaction depends on the presence of basic amino acids, especially arginine. If, as appeared probable from the studies of the reaction of 21-dehydrocortisol with free amino acids, this steroid combined with histones by reacting with the side chains of their basic amino acids, then modified histones with few or no arginyl or lysyl residues should show decreased reaction with steroid. Lysyl and arginyl side chains were blocked by maleylation and glyoxalation, respectively. In addition, a maleylated-glyoxylated derivative was also synthesized. Spectrophotometric analysis showed that 100 ± 10% of the lysyl residues had reacted with maleic anhydride. The maleylated and maleylated-glyoxylated derivatives were all soluble at pH 7.5–8.5, the range in which these studies were performed. The glyoxylated derivative was insoluble in aqueous solution from pH 2 to 11. The amino acid composition of the modified histones are shown in Table VI. Reaction of histone with maleic anhydride was readily reversed in acid. Consequently, the lysine content of the treated histone after acid hydrolysis was the same as that of the untreated histone. The arginine content of the glyoxalated proteins decreased from 8.9 to 1.7 moles per 100 moles of total amino acids. Glyoxalation therefore blocked at least 81% of the arginyl residues.

The ability of 21-dehydrocortisol-1,2-*t* to react with modified and unmodified histones is shown in the gel filtration profiles in Figure 3. Blocking of lysyl amino groups led to a small decrease in the binding compared with unaltered histone. On the other hand, the maleylated-glyoxylated derivative bound very little 21-dehydrocortisol. It was concluded that most of the steroid was bound to arginine. For comparison a profile is shown of the binding of cortisol-1,2-*t* to histone. In striking contrast to its reaction with untreated histone, no detectable binding of cortisol occurred with the maleylated-glyoxylated histone, demonstrating that the basic residues of the protein participated in the reaction with this steroid. The quantitative relationships in these experiments are summarized in Table VII.

Reaction of 21-Dehydrocortisol with Arginine-Rich and Arginine-Poor Histones. Table VIII compares the binding of 21-dehydrocortisol with unfractionated arginine-rich (F3) and arginine-poor (F1) histones. The relationships are in the expected direction, although strict proportionality is not seen. It is very likely that part of the steroid combines with lysyl residues, and that the probability of binding depends on the environment of the reacting groups. However the net reaction of 21-dehydrocortisol with arginine-rich histone is about 2.2 times greater than with arginine-poor histone.

TABLE VII: Reaction of Cortisol and 21-Dehydrocortisol with Modified and Unmodified Histone.

Histone	Steroid-1,2-*t*	Steroid Added		Steroid Bound	
		dpm	μmoles	dpm/mg of Histone	moles × 10^9 per mg of Histone
Untreated	Cortisol	3.64×10^6	3.72	374	0.39
Untreated	21-Dehydrocortisol	3.49×10^6	4.00	11,089	12.9
Maleylated	21-Dehydrocortisol	3.46×10^6	4.00	9,435	10.9
Maleylated and glyoxylated	21-Dehydrocortisol	3.87×10^6	4.00	1,251	1.3
Maleylated and glyoxylated	Cortisol	9.70×10^5	4.44	0	0

Amino Acid Composition of the 21-Dehydrocortisol–Histone Complex. If 21-dehydrocortisol reacted irreversibly with the arginyl residues in histones, the content of arginine in amino acid hydrolysates should decrease. The validity of this assumption depends on the requirement that the 21-dehydrocortisol–arginine condensation product is not split by strong acid to yield arginine. 21-Dehydrocortisol–*N*-acetyl-L-arginine was analyzed in the amino acid analyzer after being subjected to 6 N hydrochloric acid *in vacuo* in a sealed tube for 18 hr at 120°. The profile revealed the complete absence of any ninhydrin-reactive material corresponding to arginine, thus conclusively proving that the reaction product could not be dissociated into the original components. *N*-Acetyl-L-arginine alone, subjected to the same treatment, was recovered as arginine.

In Table IX the amino acid composition of the histone–21-dehydrocortisol complex and histone are compared. Arginine and lysine decreased approximately 80 and 17%, respectively, after reaction of histone with steroid. The small increase in histidine is probably insignificant. In this study, the steroid was in contact with histone for 16 hr prior to analysis. These values therefore probably approach the maximum extent of reaction under the conditions used. These results are consistent with the more indirect evidence suggesting that the steroid reacted preferentially with arginine residues.

TABLE VIII: Reaction of 21-Dehydrocortisol with Arginine-Rich and Arginine-Poor Histone.[a]

Histone	Arginine (mole %)	Lysine (mole %)	Steroid Bound dpm/mg of Histone	Steroid Bound moles × 10^9 per mg of Histone
Unfractionated	10.3	15.6	7333	8.5
Fraction F1	1.6	24.9	4395	5.1
Fraction F3	12.6	10.3	9713	11.2

[a] The experimental conditions were similar to those described in Figure 3 and Table VII.

Discussion

The radioactivity from labeled cortisol incubated with histones is localized preferentially in the arginine-rich fraction of the histone (Sluyser, 1966a; Sunaga and Koide, 1967b; Monder and Walker, 1968). The conditions of incubation or storage of the steroid are those under which oxidation of cortisol to 21-dehydrocortisol readily occurs (Monder, 1968a). It has been suggested (Monder, 1968a), and subsequently confirmed (Sunaga and Koide, 1968), that this conversion could account for the binding of 17α-hydroxycorticosteroids to histones and possibly other proteins (Kripalani and Sorby, 1967). For histones, at least, the data can best be explained by assuming that the steroid reacts specifically with the guanidino residues of the arginyl side chain. The differences between arginine-poor (F1) and arginine-rich (F3) histones with respect to acidic, neutral, and aliphatic hydroxylated residues (8.2 and 14.2, 50.3 and 51.4, 11.9 and 11.6 moles per 100 moles of amino acid, respectively) are not enough to account for the observed differences in steroid binding. Although it has been proposed that cortisol itself reacts with histones (Sluyser, 1969), the argument is not compeling, because the conditions under which the cortisol used in these experiments were stored were precisely those which have been shown to result in extensive oxidation of cortisol to 21-dehydrocortisol (Lewbart and Mattox, 1959). Furthermore, 21-dehydrocortisol-1,2-*t* is present in commercially available cortisol-1,2-*t* (Monder and Walker, 1970).

TABLE IX: Amino Acid Analysis of Histone after Reaction with 21-Dehydrocortisol.

Amino Acid	Untreated Histone[a]	21-Dehydrocortisol + Histone[a]	Net Change[a]
Lysine	12.6	10.5	−2.1
Arginine	8.5	1.9	−6.6
Histidine	1.5	2.1	+0.6
All other amino acids			No significant change

[a] In residues per 100 residues.

In order to establish that the 21-dehydrocortisol contaminant is responsible for the reported binding of cortisol to arginine-rich histones, certain criteria must be fulfilled: (a) it must bind more effectively than cortisol to histone; (b) it must bind preferentially to arginine-rich histone; (c) blocking of the arginyl residues must eliminate or greatly diminish binding; (d) direct demonstration of reaction with arginyl residues must be established. All these criteria have been met. In addition, it must be shown that cortisol itself does not react with histones. Because it is impossible to maintain cortisol completely free of 21-dehydrocortisol under the conditions of our experiments, it has not been possible to demonstrate this directly. Three facts favor the likelihood that cortisol does not react with histone: (a) the degree to which cortisol reacts is directly related to its 21-dehydrocortisol content; (b) maleylated-glyoxalated histone does not combine with cortisol, consistent with the requirement for the availability of arginine and lysine and the behavior of 21-dehydrocortisol; (c) corticosterone, which is not easily oxidized to 21-dehydrocorticosterone (Monder, 1968a) reacts with histones very poorly (Sunaga and Koide, 1967c).

The amount of 21-dehydrocortisol bound to unmodified, unfractionated histone during the 10-min incubation at pH 8 is quite small, equalling about 0.14 μmole of steroid/100 total amino acid residues or 10 arginyl residues. Most, if not all, of the arginine guanidino groups eventually react with 21-dehydrocortisol, since after 18 hr seven out of nine arginyl residues had combined with steroid. The slow rate of reaction may indicate that some arginine residues are more accessible than others.

The fact that the specificity of 21-dehydrocorticosteroids is directed to arginine-rich histones is easily explained on chemical grounds, since glyoxals in general react readily with guanidino residues (Takahaski, 1968; Nakaya *et al.*, 1967). The 21-dehydrosteroids, which are glyoxal derivatives, reacted selectively with the side chain of arginine in a similar way. In model systems using free amino acids, the bond between arginine and 21-dehydrocortisol, unlike that between other amino acids and 21-dehydrocortisol (except histidine) was irreversible. Consequently, the bond between steroid and histone would also be expected to be irreversible, as indeed it was. These results may have relevance to studies on the incorporation of corticosteroids into nuclei. When corticosteroid was either injected into rats or added directly to tissue homogenates, 2–10% of the steroid was incorporated into the nuclear fraction (Dingman and Sporn, 1965; De Venuto *et al.*, 1962). A significant part of the injected steroid was bound to histone in liver (Sluyser, 1966b; De Venuto and Muldoon, 1968; Sekeris and Lang, 1965) and in thymus (Brunkhorst, 1969); in rat liver, the steroid appeared to react preferentially with arginine-rich histone fraction (Sluyser, 1966b).

From the behavior of 21-dehydrocortisol with free amino acids, it can be anticipated that the steroid would react, not only with histones in the cell, but also with other nitrogen-containing substances in physiological fluids, such as urea and amines. Because of this, 21-dehydrocortisol may not be detected as such in biological systems, but would perhaps be found only in a form bound to nitrogen. Voigt and Schroeder (1955, 1956) and others (Eades *et al.*, 1954; Hudson and Lombard, 1955) have reported that nitrogen-containing steroids are present in mammalian tissues and fluids. These steroids, though poorly characterized, may be derived from corticosteroids.

The physiological significance of the preferred reaction of 21-dehydrocortisol with arginine-rich histone *in vitro* cannot be assessed at the present time, since only tentative evidence that corticosteroids may react with histones in such a selective manner *in vivo* is available (Sluyser, 1966b). As a working hypothesis, it may be suggested that the cell is able to oxidize cortisol to 21-dehydrocortisol. Consequent combination of 21-dehydrocortisol with histones should disturb the electrostatic bonds between histone and DNA. Salaganik *et al.* (1969) found, in support of this hypothetical picture, that cortisol injections increased the number of free DNA binding sites in rat liver chromatin without affecting the total amount of histone. Hanoune and Feigelson (1969) also found that cortisol injections did not affect the amount or rate of synthesis of histones in rat liver. By thus "derepressing" DNA at specific sites, the synthesis of specific RNA and subsequently protein could be induced. Unfortunately, repeated attempts to demonstrate increased template activity or increased rate of transcription of RNA with isolated rat liver and thymus nuclei incubated with cortisol or 21-dehydrocortisol have uniformly met with failure in our laboratory, in contrast with the reports of Ohtsuka and Koide (1969) and Dukes *et al.* (1966). Histones can mask DNA in chromatin and prevent it from acting as template for RNA polymerase, but the effect is nonspecific (Paul and Gilmour, 1968). Direct reaction of 21-dehydrocorticosteroids with histones could result in greater specificity, if the structure of the histone directed the steroid to designated binding sites. This could occur if the steroid became bound to selected basic residues on the histone. The data presented here support such a possibility. Nevertheless, if corticosteroids impart specificity at the level of transcription it could equally well be the consequence of their reaction with other macromolecules, such as acidic proteins (Gilmour and Paul, 1969) or protein-bound rRNA (Bekhor *et al.*, 1969; Huang and Huang, 1969) which very probably also have reactive arginyl and lysyl residues. It would require great imagination to fit 21-dehydrocortisol, which contains a functional group as chemically nonselective and highly reactive as the glyoxal side chain, into a scheme which leads to the well-known specific responses of the cell to corticosteroids. Furthermore, physiologically inactive corticosteroids (presumably as the 21-dehydro derivatives) react with histones as well as or better than the metabolically active ones (Sunaga and Koide, 1967c). It is our present belief that the binding of 21-dehydrocorticosteroids with histones that we and others have observed is not biologically meaningful in itself. The possibility that 21-dehydrocortisol represents a physiologically active form of cortisol which functions by combining with specific receptors (not necessarily histone) admittedly retains a certain attractiveness, since it permits one to explain corticosteroid action on the basis of established chemical reactions involving possible site-specific interactions. However, no acceptable evidence is yet available to support such a proposal, and the bulk of experimental work opposes it.

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