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Keyphrases

Salicylates—hypoglycemic activity
Structure-activity relationship—salicylates
Hypoglycemic acitivity mechanism—salicylates
Fatty acid, nonesterified—salicylate effect
Colorimetric analysis—spectrophotometer

Identification of a Prednisolone Derivative Interacting with Calf Thymus Histones

By KIYOSHI SUNAGA* and S. S. KOIDE

Prednisolone dissolved in 0.2 *M* phosphate buffer, pH 7.4, was analyzed by TLC and found to undergo degradative changes. One of the products on the chromatogram was identified as 21-dehydroprednisolone which interacted with calf thymus histones. Minimal or no binding occurred with prednisolone and the carboxylic derivative. The transformation of prednisolone to 21-dehydroprednisolone was blocked by EDTA and facilitated by cupric ions. However, the binding of histones with prednisolone in phosphate buffer was depressed when cupric ions were added to the media. The results of this study suggest that prednisolone in phosphate buffer is transformed to 21-dehydroprednisolone which interacts with calf thymus histones.

THE BINDING OF glucocorticoids with calf thymus histones was shown to be dependent on a preliminary incubation of the steroids in phosphate buffer (1). In this study, the transformation of prednisolone[1] in phosphate buffer was studied. A derivative was isolated and identified as 21-dehydroprednisolone which interacted with histones. Comparative studies of the binding capacity of histones with 21-dehydroprednisolone and prednisolone in phosphate buffer at various pH values were carried out.

MATERIALS AND METHODS

The material and methods for the determination of the binding of histones with steroids were the same as previously reported. (1–3). 1,2,4-prednisolone-^{3}H with specific activity of 721 mc./mmole was purchased from Schwarz Bio-Research Inc., New York, N. Y. The steroids were purified on silica gel chromatogram (6061, Eastman Kodak Co., Rochester, N. Y.) before use. The 21-dehydro and 21-carboxylic derivatives of prednisolone were prepared according to the method of Lewbart and Mattox (4). The amount of steroid was estimated by measuring absorbance at 240 mμ or by the radioactivities and by the blue tetrazolium (5) and Porter-Silber reactions (6). A liter of phosphate buffer was washed with 100 ml. of 0.001% dithizone in CCl_4, v/v. The aldehyde group was detected by the Sawicki reaction (7).

Chromatogram sheets were developed by the ascending technique. Two solvent systems were used; namely, chloroform–ethanol, 96:4, v/v, and ethyl acetate–*tert*-butanol–5*N* NH_4OH, 50:40:20 by vol. The sheets were developed and dried at room temperature. The unlabeled steroids were detected by UV lamp. The labeled steroids were determined by cutting the sheets in 1-cm. sections and placing each strip in a counting vial. Ten milliliters of phosphor in toluene were added to each vial and the samples were counted as previously reported (1–3). Each steroid component separated on the chromatogram was extracted with 2 ml. of ethanol for 24 hr. at room temperature. The extract was dried under vacuum and the residue was dissolved in distilled water. The eluted components were rechromatographed. About 4% of the material remained at the origin and other metabolites were not detected. The binding of the steroid fraction with arginine-rich histones was performed as previously reported (1–3) with slight

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[1] Trivial names and abbreviations used: Prednisolone, pregna-1,4-diene-11β,17α,21-triol-3,20-dione; 21-dehydroprednisolone, pregna-1,4-dien-21-al-11β,17α-diol-3,20-dione; adrenosterone, androst-4-ene-3,11,17-trione; EDTA, ethylenediamine tetraacetate; dithizone, diphenylthiocarbazone.

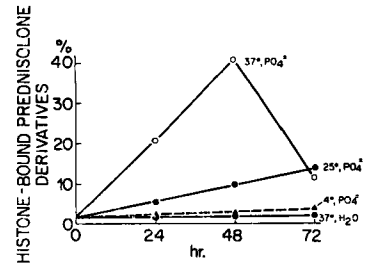

Fig. 1—Binding affinity of arginine-rich histones of calf thymus with prednisolone incubated at different temperatures for 72 hr. Prednisolone was dissolved in 0.2 M potassium phosphate buffer, pH 7.4.

modifications. A mixture was prepared containing 0.2 ml. (3 mg.) of aqueous histone solution, 0.2 ml. of 0.2 *M* potassium phosphate buffer, pH 7.4, and 0.05 ml. of ^{3}H-steroid extracted from the chromatogram. The final concentration of the steroids ranged from 36 to 170 mμM. For comparative study the final concentration was 50 mμM. The mixture was incubated at 37° for 30 min. and filtered on a column (Sephadex G-25) (0.8 × 32 cm.) in the cold (0–4°). The column was eluted with 0.2 *M* potassium phosphate buffer, pH 7.4.

RESULTS

In a preliminary experiment, the monobasic and dibasic potassium phosphates were recrystallized from 0.001 *M* EDTA and several times from deionized water. The transformation of prednisolone to 21-dehydroprednisolone in phosphate buffer prepared with recrystallized phosphates occurred at the same rate as buffer prepared from reagent grade phosphates. Similarly, the transformation occurred in phosphate buffer washed with dithizone.

Figure 1 shows the binding of prednisolone (derivatives) with arginine-rich histones of calf thymus on storage of the steroid dissolved in phosphate buffer or water and incubated at 37° for 72 hr. The maximum binding occurred with the sample stored at 37° for 48 hr. in phosphate buffer. This finding suggested that prednisolone undergoes transformation in phosphate buffer. When EDTA was added

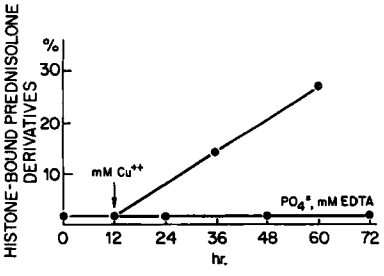

Fig. 2—Binding of arginine-rich histones of calf thymus with prednisolone treated with EDTA and various cations. The following salts were used: $CuSO_4 \cdot 5H_2O$, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, and $CaCl_2$ at a final concentration of 1 mM. The salts were added 12 hr. after the preparation of the prednisolone solution and incubated at 37°. Aliquots of the prednisolone solution were assayed for binding affinity at 12-hr. intervals for 72 hr.

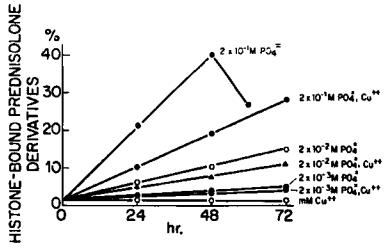

Fig. 3—Binding affinity of arginine-rich histones of calf thymus with prednisolone dissolved in phosphate buffer with and without added cupric ions. Prednisolone was dissolved in potassium phosphate buffer, pH 7.4, and incubated at 37° for 72 hr. The final concentration of cupric ions was 1 mM. The pH of the copper sulfate solution without phosphate buffer was 4.6.

to the solution, the binding capacity was markedly depressed (Fig. 2). The addition of cupric salts reversed the inhibition; whereas Ca^{2+}, Mn^{2+}, and Mg^{2+} ions did not. On the other hand, the addition of cupric salts to prednisolone in various concentrations of phosphate buffer decreased its binding with calf thymus histones (Fig. 3). Other trace metals may be present in analytical grade potassium phosphates which may influence this transformation. No binding was observed when prednisolone was incubated in aqueous cupric sulfate in the absence of phosphate ions (Fig. 3).

Figure 4 shows the TLC of prednisolone dissolved in 0.2 *M* phosphate buffer, pH 7.4, and incubated at 37° for 48 hr. The chromatogram was developed with 4% ethanol in chloroform. Three major and one minor components were detected. These components were identified as the 21-carboxylic derivative (Sp-1), prednisolone (Sp-2), and 21-dehydroprednisolone (Sp-3). The fourth component (Sp-4) which ran with the solvent front was tentatively identified as androsta-1,4-dien-11β-ol-3,17-dione. The Sp-4 spot appeared when 21-dehydroprednisolone was heated at 100° for 10 min. That this component may be androsta-1,4-dien-11β-ol-3,17-

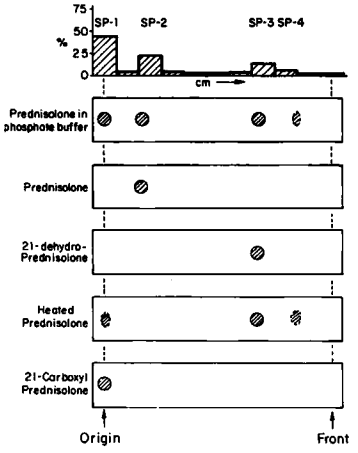

Fig. 4—Thin-layer chromatogram of prednisolone in phosphate buffer. The analysis was performed with prednisolone dissolved in 0.2 M potassium phosphate buffer, pH 7.4, at 37° for 48 hr.

TABLE I—PROPERTIES OF STEROID DERIVATIVES SEPARATED ON THIN-LAYER CHROMATOGRAM

Components	Proportion of 240 mμ: BT: PS	Aldehyde Reaction	R_f
Sp-1	1:0.4:0.4	0	0
Sp-2	1:1:1	0	0.2
Sp-3	1:0.1:0.3	+	0.7

dione is supported by the reports that adrenosterone was formed when cortisone was heated to 90° in ethyl ether peroxides (8) and that the main decomposition product of cortisol-4-^{14}C was androst-4-en-11β-ol-3,17-dione (9). Adrenosterone migrates with the solvent front when developed on silica gel chromatogram with this system. When the chromatogram was developed with a mixture containing ethyl acetate–*tert*-butanol–5 *N* NH_4OH, prednisolone and 21-dehydroprednisolone migrated with the solvent front. With this system, the R_f of Sp-1 was identical with the carboxylic derivative.

Table I shows some of the properties of these three components. When the amounts were determined by measuring the absorbance at 240 mμ and with the blue tetrazolium (BT) and Porter-Silber (PS) reactions, the proportions of the amounts determined by these three methods were markedly different. Only Sp-2 gave equivalent amounts by all three methods and was identified as prednisolone. Only Sp-3 gave a positive aldehyde reaction with the Sawicki reagent identifying it as the 21-dehydro derivative. These results suggest that the α,β-unsaturated 3-keto group remains intact and that the side chain had been altered.

Figure 5 shows the relationship of the binding capacity of histones with prednisolone in phosphate buffer which was incubated at varying lengths of time at 37° to the content of the various steroid derivatives. The transformation of prednisolone in 0.2 *M* potassium phosphate, pH 7.4, and 37° was followed and the contents were analyzed by TLC at 24, 48, and 72 hr. The carboxylic derivative (Sp-1) increased with time; whereas the unchanged prednisolone (Sp-2) decreased. The binding capacity correlated with the content of 21-dehydroprednisolone (Sp-3). These results indicate that the transformation of 21-dehydroprednisolone (Sp-3) to the carboxylic derivative (Sp-1) increased at 72 hr.

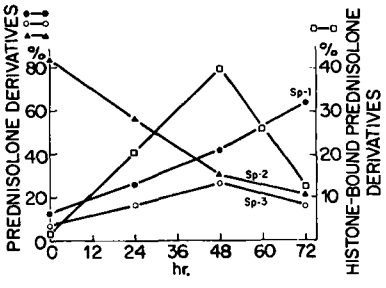

Fig. 5—Content of prednisolone derivatives during storage of prednisolone in phosphate buffer. The binding affinity was determined with the original prednisolone solution dissolved in 0.2 M potassium phosphate buffer, pH 7.4, and with the various derivatives. The final concentration of the steroids was 50 mμM. At time zero, the steroid solution was analyzed immediately after preparation.

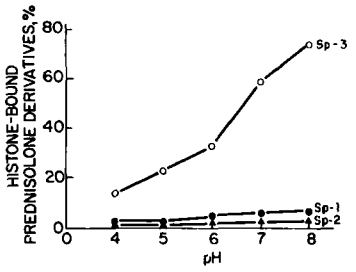

Fig. 6—Binding affinity of prednisolone derivatives with arginine-rich histones of calf thymus at various pH values. The steroids were isolated from the thin-layer chromatogram and dissolved in water. 0.2 M potassium phosphate buffers at the indicated pH values were used as the incubating media. Gel filtration was performed with 0.2 M potassium phosphate buffer, pH 7.4. The final concentration of the steroids was 50 mμM.

Figures 6 and 7 show the binding of these three components isolated from the thin-layer chromatogram with arginine-rich and lysine-rich histones of calf thymus at various pH values. Interaction occurred principally with 21-dehydroprednisolone (Sp-3). Little or no binding was detected with prednisolone (Sp-2) and the carboxylic derivative (Sp-1).

DISCUSSION

Although cupric ions catalyze the degradation of prednisolone to 21-dehydroprednisolone in phosphate buffer, the transformation which proceeds unabated in phosphate buffer washed with dithizone or prepared with recrystallized phosphates remains unexplained. The transformation is in accord with the reports that corticosteroids undergo degradation in aqueous solutions and that the decomposition is catalyzed by cupric ions and inhibited by EDTA (10, 12). It is conceivable that trace amounts of cupric ions are required for the transformation and that an excess may be inhibitory. Cupric ions may influence the binding of prednisolone derivatives with histones. Although the degradation occurs in alkaline media in the absence of cupric ions, the transformation catalyzed

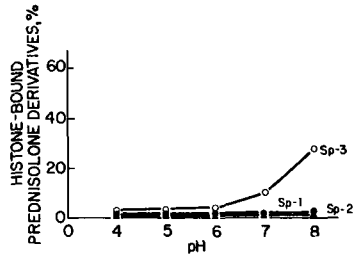

Fig. 7—Binding affinity of prednisolone derivatives with lysine-rich histones of calf thymus at various pH values. The steroids used were isolated from the thin-layer chromatogram and dissolved in water. 0.2 M potassium phosphate buffers at the indicated pH values were used as incubating media. Gel filtration was performed with 0.2 M phosphate buffer, pH 7.4. The final concentration of the steroids was 50 mμM.

by cupric ions in aqueous solutions at neutral pH can be demonstrated only in the presence of phosphate ions.

It has been demonstrated in this study that 21-dehydroprednisolone interacts with histones. This is in accord with the report of Kripalani and Sorby (10) that the degradative products of hydrocortisone interact strongly with human serum albumin. The different binding characteristics of histones with prednisolone and 21-dehydroprednisolone (Fig. 6) suggest that they interact with different loci on the histone molecule. The binding characteristics of these two steroids with transcortin and serum albumin were distinctly different (13). Preliminary studies on the interaction of 21-dehydrocorticosteroids with amino acids in this laboratory suggests that the reaction occurs between the 21-aldehyde and the α-amino group to form Schiff bases.

The possibility that prednisolone (21-dehydrocortisol, is formed under physiological conditions is suggested by the demonstration of 21-hydroxysteroid dehydrogenase in rat and sheep liver and bovine adrenal gland (14–16) although the reaction is strongly in the direction of the alcohol formation. It is noteworthy that the 21-dehydrosteroids are less active or inactive in biological systems in comparison to the parent corticosteroids (17, 18), suggesting that they need to be converted to the alcohol form to be biologically active. Since the biologically active prednisolone did not bind appreciably with histones in comparison with prednisolone, the mechanism of action of prednisolone probably does not depend on its interaction with histones.

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Keyphrases

Prednisolone—degradation products
EDTA, cupric ions effect—prednisolone degradation
21-Dehydroprednisolone binding—calf thymus histones
TLC—separation, identity

Copper-Catalyzed Oxidation of Ascorbic Acid in Gels and Aqueous Solutions of Polysorbate 80

By ROLLAND I. POUST and JOHN L. COLAIZZI

The stability of ascorbic acid in aqueous solution has been studied as a function of polysorbate 80 concentration. The range of polysorbate concentrations studied was sufficiently wide to provide systems ranging from solutions of low viscosity to hydrogels. The study has been conducted at 30, 40, 50, and 60°. First-order rate constants for the oxidative reaction were calculated, and they appear to decrease as a function of polysorbate 80 concentration up to 10 percent, except at 30°, in which case the rate constants decreased up to 30 percent polysorbate 80. The rate generally leveled off at higher surfactant concentrations. It appears that this phenomenon is probably a result of micelle formation, or, more specifically, the increases in micellar aggregation number and micellar concentration which occur up to about 30 percent polysorbate 80. It does not appear that increased viscosity has a significant influence on the rate of ascorbic acid oxidation in these systems.

THE POSSIBILITY OF ENHANCING the stability of drugs by incorporating them into structured vehicles, such as thixotropic gels, has been suggested. Drugs such as vitamins, which are susceptible to decomposition, have been found to be stable for longer periods of time in thixotropic preparations (1). Macek (2) noted that a vehicle of peanut or sesame oil gelled with 2%

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