The need for high phosphate concentrations was not explained. Growth was optimal at an atropine concentration of 1.25% and was inhibited by higher concentrations of alkaloid.

The details of atropine utilization are being studied and will be reported at a later date, but there is evidence that an atropinesterase is involved. There is also evidence that growth requirements are quite specific for atropine, hyoscyamine, or their hydrolysis products.

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Analysis of Steroids in Mixtures Using the Kinetics of Blue Tetrazolium Reduction

By DAVID E. GUTTMAN

The rate of formation of formazan resulting from the base-catalyzed reduction of blue tetrazolium by certain steroids was studied spectrophotometrically. With cortisone and hydrocortisone, the reaction rate exhibited a first-order dependency on steroid concentration. The rate constant for cortisone-containing systems was significantly larger than that found for hydrocortisone systems. Studies with cortisone acetate showed that hydrolysis of the ester was prerequisite to reaction with the tetrazolium salt. Differences in rates of color development were used to analyze mixtures of cortisone and hydrocortisone and of cortisone and cortisone acetate.

A WIDELY USED colorimetric method for the determination of the purity of corticosteroids and the potency of dosage forms containing such steroids is based on the formation of a colored formazan resulting from the base-catalyzed reduction of blue tetrazolium by the α-ketol side chain of the steroid molecule. The method consists of determining, after a specified time period, the intensity of color in a test preparation and comparing it with that produced under similar conditions in a standard preparation of the steroid under consideration. There have been a number of published studies of the rates of color formation in systems containing reducing steroids and blue tetrazolium and it has been observed that closely related steroids can exhibit significant differences in their rates of reaction with the tetrazolium salt. For example, Chen, Wheeler, and Tewell (1) presented data which suggested that color generation in cortisone-containing systems was much more rapid than in systems containing hydrocortisone. Meyer and Lindberg (6), in their extensive study, showed that the position and configuration of certain keto- and hydroxy-groups in the steroid molecule influenced reducing characteristics. Recknagel and Litteria (7) also demonstrated differences in reaction rate by their determination of the optimum incubation times for maximum color development for various steroids; *i.e.*, 30 min. for cortisone and 11-deoxycorticosterone as contrasted to 50 min. for corticosterone and hydrocortisone. Similarly, Izzo, Keutmann, and Burton (3) reported that reducing steroids with an 11-keto group developed maximum color faster than those with an 11-hydroxyl group. Martin and Salvador (5) found that acetylation of the 21-hydroxyl group decreased reaction rate relative to the parent alcohol, while Johnson, King, and Vickers (4) reported that with hydrocortisone hemisuccinate, and triamcinolone, color development was unusually slow compared to other steroids.

It has not been generally recognized that, under the conditions usually employed in the assay procedure where the steroid is present at a much lower concentration than that of the catalyst and the tetrazolium reagent, the rate of appearance of formazan exhibits a first-order dependency on the steroid concentration. This behavior is of potential analytical utility because of the lack of direct methods for analyzing mixtures of closely related steroids and because certain steroids which might be found or combined in mixtures do exhibit differences in the rate of this reaction. The present study was conducted to test the feasibility of utilizing such differences as the basis for analytical methods for the determination of steroids in mixtures. Toward this end, this laboratory has studied, under closely

Received May 9, 1966, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, 14214.
Accepted for publication June 28, 1966.
The author thanks Mrs. Gail W. Saloman for competent technical assistance in this study.

controlled conditions, the kinetics of the reaction of cortisone, hydrocortisone, and cortisone acetate with blue tetrazolium and has taken advantage of rate differences to analyze mixtures of cortisone and hydrocortisone, and of cortisone and cortisone acetate. The method of proportional equations, suggested by Garmon and Reilley (2), was used for this purpose and was found to yield reasonably accurate estimations of steroid concentration and to offer a rather convenient approach to what would normally be a difficult analytical problem.

PROCEDURE

Stock solutions of the steroids[1] were prepared in absolute alcohol at a concentration of 10 mcg./ml. Binary mixtures of two steroids were prepared by mixing aliquots of two solutions.

Cortisone–Hydrocortisone Systems.—Absorbance-time plots were obtained with a Beckman DB spectrophotometer and a Beckman linear-log recorder equipped with an event marker. The spectrophotometer was set at a wavelength of 525 mμ. A recorder speed of 1 in./min. was used. The cell compartment was maintained at constant temperature by the circulation of water at 24° ± 0.1° with a P.M. Tamson, N.V. constant-temperature circulating bath.

Twenty milliliters of steroid solution was placed in a 50-ml. glass-stoppered conical flask. Twenty milliliters of absolute alcohol was placed in a similar flask to serve as a blank. One milliliter of a 0.5% solution of blue tetrazolium in absolute alcohol was added to each flask. The recorder was started and 1 ml. of a 1% solution of tetramethylammonium hydroxide in absolute alcohol (prepared from a 10% solution in water) was added to each flask simultaneously and with mixing. The time of addition of base was marked on the recorder chart with the event marker. The solutions were placed in cells which were stoppered and placed in the cell compartment.

Cortisone–Cortisone Acetate Systems.—Absorbance-time plots for cortisone acetate-containing systems were obtained in the manner previously described. For the analysis of mixtures of these two steroids, a somewhat different procedure was used. Here, 20-ml. aliquots of steroid solution were pipeted into each of two glass-stoppered test tubes. Corresponding blank tubes were prepared to contain 20 ml. of absolute alcohol. One milliliter of the 0.5% solution of blue tetrazolium was added to each tube. The tubes were placed in a constant-temperature bath at 25° ± 0.1° and allowed to attain temperature equilibrium. One milliliter of the 1% solution of tetramethylammonium hydroxide was added to each tube, and the time of addition was taken as zero time. Exactly 8 min. after the addition of base, one steroid tube and a corresponding blank were quenched by the addition of 1 ml. of glacial acetic acid. Exactly 20 min. after the addition of base, the remaining steroid tube and its corresponding blank were similarly treated. Absorbance values at 525 mμ were determined for each steroid solution using the corresponding blank solution as the reference blank. Standard solutions of pure cortisone and pure cortisone acetate were treated in an identical manner.

Cortisone Acetate Ophthalmic Suspensions.—Two samples of commercially available cortisone acetate ophthalmic suspension were assayed by the kinetic method. The samples were obtained from a local pharmacy. One sample, according to the pharmacist "has been on the shelf for years." The other "was just received from the supplier." These will be designated as "aged suspension" and "fresh suspension," respectively. One milliliter of suspension was placed in a 50-ml. separator and diluted with 5 ml. of distilled water. The steroid was extracted with three 20-ml. portions of chloroform. The chloroform extracts were drained through a cotton plug into a 100-ml. volumetric flask. Chloroform was added to volume. Five-milliliter aliquots of the chloroform solution were placed in each of two 50-ml. glass-stoppered, conical flasks and the chloroform was evaporated using gentle heat. The residue in each flask was dissolved in 20 ml. of absolute alcohol. The analysis was conducted as previously described.

RESULTS AND DISCUSSION

Cortisone–Hydrocortisone Systems.—The time course for formazan appearance, as reflected by increase in absorbance at 525 mμ, is illustrated for each of the steroids in Fig. 1. It is apparent from this figure that formazan production occurred much more rapidly from cortisone-containing systems than from corresponding systems containing hydrocortisone. The semilog plot of Fig. 2 emphasizes this rate difference and illustrates that, under the conditions employed, the rate of appearance of formazan followed first-order kinetics. Here, the logarithm of the function $(A_\infty - A_t)$ is plotted as a function of time, where A_∞ is the asymptotic absorbance and A_t is the absorbance at a particular time. In each case, excellent linearity was observed over at least three half-lives. The pseudo first-order rate constant for cortisone was calculated to be 18×10^{-2} min.$^{-1}$ while that for hydrocortisone was 5.1×10^{-2} min.$^{-1}$. Treatment of the data by the Guggenheim method, which does not rely on an infinity value for absorbance, yielded the same rate constants.

For the analysis of the two steroids in combination, the method of Garmon and Reilley (2), that of proportional equations, was employed. In a reacting mixture containing both steroids, a common product, the formazan, is produced by two simultaneously occurring first-order processes. The absorbance at 525 mμ, which is specific for the product, is thus established at any time by:

$$A = aC(1 - e^{-k_c t}) + aH(1 - e^{-k_h t}) \qquad \text{(Eq. 1)}$$

where

A = absorbance at 525 mμ,
C = initial concentration of cortisone,
a = absorptivity of formazan at 525 mμ,
k_c = first-order rate constant for cortisone,
H = initial concentration of hydrocortisone,
k_h = first-order rate constant for hydrocortisone.

Measurement of two absorbance values at two

[1] The steroids were obtained from commercial sources and were of U.S.P. or N.F. quality.

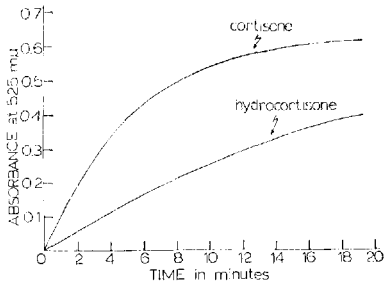

Fig. 1.—A plot illustrating the rate of reaction of cortisone and hydrocortisone with blue tetrazolium.

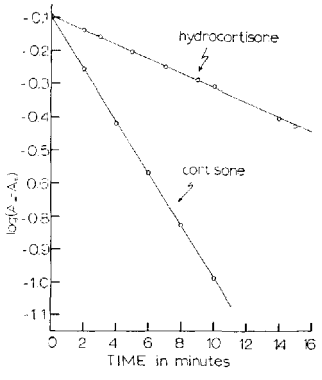

Fig. 2.—A plot illustrating the pseudo first-order appearance of formazan in the reaction of blue tetrazolium with cortisone and hydrocortisone.

times, a shorter time, t, and a longer time, t', allows the formulation of two equations which can be solved simultaneously to yield values for C and H, *i.e.*,

$$A = K_cC + K_hH \qquad \text{(Eq. 2)}$$

$$A' = K'_cC + K'_hH \qquad \text{(Eq. 3)}$$

where

A = absorbance value after the shorter reaction time, t,
A' = absorbance value after the longer reaction time, t',
$K_c = a\,(1 - e^{-k_c t})$; $K'_c = a\,(1 - e^{-k_c t'})$
$K_h = a\,(1 - e^{-k_h t})$; $K'_h = a\,(1 - e^{-k_h t'})$

Although the constants K_c, K'_c, K_h, K'_h can be calculated from a knowledge of rate constant, time, and absorptivity, it is more convenient to determine them directly from absorbance values, at the appropriate reaction times, exhibited by standard preparations of the pure steroids. Thus,

$$K_c = A_c/C_s;\quad K'_c = A'_c/C_s;$$
$$K_h = A_h/H_s;\quad K'_h = A'_h/H_s$$

where

C_s = concentration of the standard solution of cortisone,
H_s = concentration of the standard solution of hydrocortisone,

A_c and A'_c are the absorbances exhibited by the standard preparation of cortisone at times t and t', respectively.

A_h and A'_h are the absorbances exhibited by the standard preparation of hydrocortisone at times t and t', respectively.

Appropriate substitution into Eqs. 1 and 2 and solution of the simultaneous equations for C and H yields:

$$\frac{C}{C_s} = \frac{A - \frac{A_h}{A'_h}A'}{A_c - \frac{A_h}{A'_h}A'_c} \qquad \text{(Eq. 4)}$$

$$\frac{H}{H_s} = \frac{A - \frac{A_c}{A'_c}A'}{A_h - \frac{A_c}{A'_c}A'_h} \qquad \text{(Eq. 5)}$$

For the analysis of mixtures of these two steroids, the longer reaction time was chosen to be 20 min. A shorter reaction time of 5 min. was found to be optimum on the basis of the graphical approach to time selection which was recommended by Garmon and Reilley (2). Absorbance values at these reaction times were read directly from absorbance *versus* time plots which were obtained with mixtures and with standard solutions of the steroids. Results which illustrate the precision and accuracy obtained in the determinations are presented in Table I.

Cortisone–Cortisone Acetate Systems.—Absorbance-time plots obtained with cortisone acetate at a number of different concentrations of base are presented in Fig. 3. A cortisone curve is presented here for comparative purposes. It is apparent from these curves that the rate of reduction of the tetrazolium salt did not exhibit a first-order dependency on the concentration of the steroid ester.

Table I.—Analysis of Cortisone and Hydrocortisone Mixtures

Steroid Concn. Taken, mcg./ml.		Steroid Concn. Found, mcg./ml.	
Cortisone	Hydrocortisone	Cortisone	Hydrocortisone
9.00	1.00	9.30	1.04
		8.72	1.20
		8.55	1.77
		8.91	1.26
7.50	2.50	7.44	2.52
		7.78	2.20
		7.71	2.36
		7.67	2.50
7.00	3.00	7.10	2.93
5.00	5.00	5.12	4.78
		4.93	5.33
		5.41	4.60
		4.86	5.23
4.00	6.00	3.90	6.01
2.50	7.50	2.60	7.48
		2.84	7.40
		2.93	7.25
1.00	9.00	0.68	9.25
		1.33	9.00
		1.18	9.23
		1.07	9.12

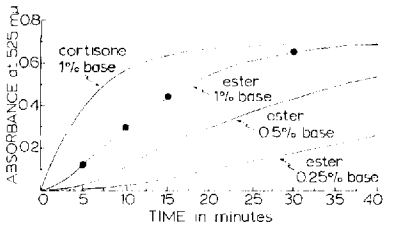

Fig. 3.—A plot illustrating the rate of reaction of cortisone and cortisone acetate with blue tetrazolium.

TABLE II.—ANALYSIS OF CORTISONE AND CORTISONE ACETATE MIXTURES

Steroid Concn. Taken, mcg./ml.		Steroid Concn. Found, mcg./ml.	
Cortisone	Cortisone Acetate	Cortisone	Cortisone Acetate
0.50	9.50	0.41	9.71
1.00	9.00	0.82	9.35
		0.92	9.10
3.00	7.00	3.05	6.85
5.00	5.00	4.61	5.02
		4.75	5.02
7.00	3.00	6.94	2.94
9.00	1.00	8.58	1.07
		8.46	1.07

TABLE III.—ANALYSIS OF CORTISONE ACETATE OPHTHALMIC SUSPENSION, 0.5%

Sample	Label Claim, mg./ml.	Cortisone Acetate Found,[a] mg./ml.	Cortisone Found[a]
"Aged" suspension	5	5.25	0
"Fresh" suspension	5	4.90	0

[a] Average of 3 determinations.

The curves are sigmoidal in shape and a lag-time in the appearance of formazan is observed. It is also seen that as the concentration of catalyst was decreased, the lag-time increased, and the sigmoidal shape of the curve was emphasized. The observed behavior indicates that here production of formazan was the result of a series of consecutive reactions. A logical assumption is that hydrolysis of the ester was prerequisite to a reaction resulting in the generation of formazan, *i.e.*,

$$\text{cortisone acetate} \xrightarrow[OH^-]{k_1} \text{cortisone} + \text{acetate}$$

$$\text{cortisone} + \text{blue tetrazolium} \xrightarrow[OH^-]{k_2} \text{formazan}$$

If the steroid is present in limiting concentration, k_1 and k_2 are pseudo first-order rate constants and the time course for increase in absorbance would be given by:

$$A = aE\left[1 + \frac{1}{k_1 - k_2}(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right] \quad \text{(Eq. 6)}$$

where E = initial concentration of cortisone acetate.

The validity of this model was checked by calculating the value of k_1 by iteration based on a knowledge of k_2, a, E, and absorbance values at particular times. The value, in systems prepared from the 1% solution of tetramethylammonium hydroxide, was determined to be 12×10^{-2} min.$^{-1}$. The solid line of Fig. 3, corresponding to the condition of 1% base, represents observed behavior while the circles represent points predicted from the calculated rate constants.

Although the kinetics of formazan generation in reaction systems containing the cortisone ester is complex, absorbance at constant reaction time is directly proportional to the initial concentration of ester, and the method of proportional equations can, therefore, be used to analyze mixtures of cortisone and cortisone acetate. The derivation and nature of the equations used in this case are essentially identical to those discussed in the case of cortisone and hydrocortisone. Analysis of binary mixtures of cortisone and cortisone acetate were carried out and the results are shown in Table II. Here reaction times of 8 and 20 min. were found to be appropriate.

The method was also applied to aged and fresh samples of commercially available cortisone acetate ophthalmic suspension with the anticipation that the aged preparation might exhibit a detectable concentration of free alcohol. Results are shown in Table III. It was found that the cortisone acetate content in both preparations was consistent with the label claim and that negligible free alcohol was present. The low solubility of the steroid in the aqueous vehicle is probably responsible for the long-term stability of the product.

In applying this differential kinetic method to the analysis of dosage forms or to extracts from biological systems, the same types of interferences that are well recognized in the application of the conventionally used tetrazolium assay of steroids should be anticipated. Thus, for example, the presence in a sample of a nonsteroidal reducing agent, acids, or alkali would be expected to interfere and would necessitate a preliminary treatment by which steroidal components are separated from the interfering substances. In this method, as with other analytical procedures based on reaction kinetics, careful control of temperature and of the concentrations of catalyst and reagent was necessary for reproducible results.

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