cursors. The results in Table IV might allow for the speculation that the increased availability of tryptophan resulting from reduced protein synthesis could sufficiently alter the binding of cytochrome P-450 for isopentenyl pyrophosphate that alkaloid synthesis might proceed, whereas at lower levels of available tryptophan such would not occur because of the low affinity of the cytochrome P-450 for isopentenyl pyrophosphate. Based on the observation that benzyl thiocyanate, which has been shown to enhance tetracycline formation (16), increased total alkaloid in *C. purpurea* to almost the same degree as phenobarbital (15), it is suggestive that cytochrome P-450 may be involved in the formation of certain other secondary cell metabolites in addition to that which has been discussed in this report.

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DRUG STANDARDS

Detection of Decomposition and Analytical Interferences in Pharmaceutical Preparations Containing Corticosteroids

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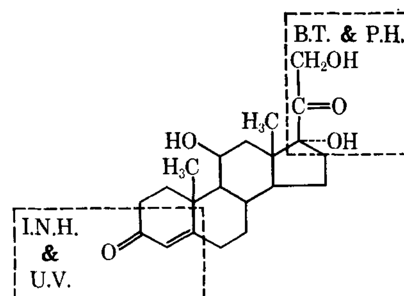
Abstract ☐ Since the blue tetrazolium and phenylhydrazine reagents for corticosteroids react with the intact side chain at C_{17}, and the isonicotinic acid hydrazide method and UV spectrophotometry depend upon conjugation in Ring A at the other end of the molecule, the analytical results by the four methods give information concerning decomposition caused by oxidation of the C_{17} side chain and by deconjugation in Ring A. Methods are proposed which allow the detection and determination of both acidic and neutral decomposition products. Measurement of the variation of absorbance with time can be used to detect unidentified interferences in the blue tetrazolium, phenylhydrazine, and isonicotinic acid procedures. The extent of interference of several substances which interfere in at least one of the color reactions is reported. Several examples of the use of the proposed methods to detect and determine decomposition and/or interference are given.

Keyphrases ☐ Corticosteroids, decomposition determination—methods compared ☐ Decomposition, corticosteroids—C_{17} side-chain oxidation determination ☐ Interference—corticosteroid analysis ☐ Blue tetrazolium, phenylhydrazine, isonicotinic acid hydrazide, UV spectrophotometry methods—analysis

The detection of decomposition and of analytical interferences in pharmaceutical corticosteroid preparations is important in the correct determination of the composition of such preparations. The usual methods of analysis for undecomposed corticoid hormones include the blue tetrazolium reaction (BT) (1–5); the phenylhydrazine–sulfuric acid–alcohol reaction (PH) (6, 7), which is also known as the Porter-Silber reaction; the isonicotinic acid hydrazide reaction (INH) (8), which is also known as the Umberger reaction; and UV spectrophotometry. As is shown in Structure I, the BT and PH reagents react with the C_{17} side chain, while the INH and UV methods depend upon the conjugation of the carbonyl group at C_3 with the double bond between C_4 and C_5 in Ring A of the steroid nucleus. Since



hydrocortisone—portions of molecule measured by the following methods: INH = isonicotinic acid hydrazide, BT = blue tetrazolium, PH = phenylhydrazine H_2SO_4, and UV = ultraviolet

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Table I—Reported Interferences in Corticosteroid Photometric Methods

Interfering Substance	Reference	BT	PH	INH
Acetone	(36)	+	NR[a]	+
Active hydrogen compounds	(37)	+	NR	NR
Allantoin	(38)	+	NR	NR
Alloxantin dihydrate	(39)	+	NR	NR
Amphotericin B	(36)	+	+	+
Bacitracin	(36)	+	+	None
Benzal acetone	(8)	NR	NR	+
Benzyl styryl ketone	(8)	NR	NR	+
Dyclonine hydrochloride	(36)	+	+	+
Erythromycin	(39)	+	+	+
Erythromycin stearate	(36)	+	−	None
Hydroxy ketones	(39)	+	NR	NR
Iodochlorhydroxyquin	(36)	None	+	+
Lanolin	(36)	+	NR	NR
Nystatin	(36)	+	+	+
Phenothiazine	(34)	NR	−	NR
Polyhydroxyphenols	(37)	+	NR	NR
Polyhydroxythiols	(37)	+	NR	NR
Polymixin B sulfate	(36)	None	+	None
Reducing sugars	(1)	+	NR	NR
Sodium novobiocin	(36)	+	+	+
Stearic acid	(36)	−	Table II	Table II
Tetracyclines	(39)	+	+	+
Xylocaine	(36)	NR	−	NR
Zinc bacitracin	(36)	+	−	None

[a] NR = not stated in reference.

the reactions in these four methods occur with different portions of the molecule, they can be used to detect and distinguish between decomposition which occurs at the C_{17} side chain and decomposition caused by deconjugation in Ring A. Quantitative agreement between all four methods indicates that the corticosteroid is not decomposed and that there is no interference in the determinative steps of any of the methods due to components of the preparation. Differences between the values by the four methods indicate decomposition or interference.

The most common type of corticosteroid decomposition encountered in pharmaceutical preparations is caused by the oxidation of the C_{17} side chain by readily reducible organic and inorganic compounds, by metal ions, and by air in alkaline solutions (9–16). Much less common is the destruction or movement of the double bonds in Ring A caused by absorption of UV light (17–20). The degradation products caused by oxidation of the C_{17} side chain are neutral or acidic in character (9, 11, 21–23) and are a complex mixture of organic acids together with neutral compounds such as aldehydes and ketones. These decomposition products can be separated by extraction into chloroform, since the neutral compounds will be extracted along with the undecomposed steroid while the acidic components will remain in the aqueous phase.

The BT reaction is the official method in USP XVII (24) and NF XII (25) for preparations listed in these compendia. It is more specific than the INH or UV method for corticosteroids since the BT reagent reacts with that portion of the molecule usually involved in decomposition. The BT method is subject to many interferences, as shown in Tables I and II. Table I lists reported interferences with each of the three types of reactions used to determine corticosteroids. Interferences in the UV determination are not included, since a large number of ingredients in corticosteroid preparations absorb in the same region and the interference is relatively easy to detect due to changes in the UV spectra. The PH reaction is specific for the 17,21-dihydroxy-20-keto side chain at C_{17} and is less subject to interference than the BT procedure.

Many substances which interfere in the BT, PH, INH, and UV procedures are usually removed during sample cleanup by extraction, by magnesium silicate[1] column chromatography (26), by TLC (17, 27), by paper chromatography (28–30), and by column-partition chromatography (31, 32). Water-soluble acidic decomposition products and interferences are removed by the use of acidic and basic traps in column procedures and by acidic or basic extractions in separator procedures. Neutral decomposition products extract with the undecomposed corticosteroid.

This paper reports several unreported interferences to the determinative methods (Table II) and suggests methods of evaluation of results by the four methods to detect and determine decomposition in corticosteroid preparations and to detect interferences.

EXPERIMENTAL

Reagents and Equipment—The reagents called for in the references for the individual methods were prepared as directed. All reagents were USP or ACS grade. All corticosteroid standard solutions were prepared from USP, NF, or commercial reference standards.

All measurements were made on a Cary spectrophotometer, model 15.

Methods—In all methods used, the samples were carried through any necessary cleanup and preparative steps. The final solution obtained was evaporated to dryness carefully under air on a steam bath. The residue was dissolved and made to volume with alcohol USP, so that the concentration was approximately 0.010 mg./ml. of the corticosteroid. This solution was used for the determinative steps for all four methods. A standard solution of the corticosteroid of the same concentration was also used in the determinative steps of each method. In all cases, the absorbance spectra of the first standard and the first sample were rescanned after all measurements in the series had been completed.

BT Method—The procedure given in USP XVII (24) and NF XII (25) was followed except that 10.0-ml. aliquots and 1.0 ml. each of the BT reagent and the tetramethylammonium hydroxide reagent were used.

PH Method—The procedure of Silber and Porter (7) was followed without modification.

INH Method—The procedure of Umberger (8) was used, except that the INH reagent was modified by using twice the recommended concentration of hydrochloric acid to increase the sensitivity of the reaction (33).

UV Spectrophotometry—The alcohol USP solution was scanned directly in a 1-cm. cell.

Interference Studies—The interference of several ingredients in corticosteroid preparations, which has not been reported previously, was investigated. The interfering substance was added to a standard solution of hydrocortisone, which was then evaporated to dryness and the residue dissolved in alcohol USP prior to color development. In each case, the absorbance was compared to the absorbance of a standard hydrocortisone solution under the same conditions.

Time Study of Absorbance Variation to Detect Interference—The absorbance of the sample solution and of the standard solution was measured periodically during and after the standard color development period.

Detection of Decomposition or Interference—The absorbance of the sample and standard solution was determined by all four

[1] Florisil, Floridin Co., Pittsburgh, PA 15222

Table II—Interferences in Corticosteroid Photometric Methods

Interfering Substance	Level mg. Interference/mg. Hydrocortisone	Absorbance: BT Standard	BT Standard + Interference	BT % Difference	PH Standard	PH Standard + Interference	PH % Difference	INH Standard	INH Standard + Interference	INH % Difference
Sorbitan monostearate	250/1	0.585	0.680	+16.2	0.546	T[a]		0.326	0.357	+9.5
Sorbitan monooleate	250/1	0.585	0.748	+27.9	0.546	T		0.326	0.457	+40.2
Ethyl ether peroxides	22/1	0.551	0.618	+12.2	0.379	0.347	−8.4	0.338	0.331	−2.1
Lanolin	600/1	0.585	0.846	+44.6	0.546	T		0.326	0.746	+128.8
Salicylamide	60/1	0.582	0.570	−2.1	0.546	0.549	+0.5	0.326	0.325	−0.3
Sodium lauryl sulfate	20/1	0.564	0.558	−1.1	0.379	T		0.326	0.324	−0.6
Stearic acid	120/1	0.582	0.568	−2.4	0.546	T		0.326	0.325	−0.3
Sulfur	0.05/1	0.588	0.742	+26.2	0.559	0.559	0	0.332	0.332	0.0
Sulfide	0.133/1	0.585	0.709	+21.2	0.546	0.546	0	0.326	0.328	+0.6
Polysorbate 60	90/1	0.570	0.754	+32.3	0.386	T		0.328	0.333	+1.5

[a] T = solution becomes turbid so that absorbance cannot be determined.

methods. Significant differences between the values obtained indicate either decomposition or interference in one or more methods.

Procedure for Determination of Decomposition at C_{17} Side Chain—Prepare an alcohol USP extract of the sample and determine the amount of corticosteroid by either the INH or UV method. This is the total amount of the undecomposed plus any decomposed corticosteroid present.

Dissolve or suspend the sample in an aqueous solution and extract with chloroform. Retain both solutions.

Determine the acidic decomposition products in the aqueous fraction by evaporation of a known aliquot to dryness, followed by determination by the INH or UV method.

Determine the undecomposed corticosteroid in the chloroform fraction by evaporation of a known aliquot to dryness, followed by determination by the BT or PH method.

Determine the neutral decomposition products plus the undecomposed corticosteroid in the chloroform fraction by evaporation of a known aliquot to dryness, followed by determination by the INH or UV method.

RESULTS AND DISCUSSION

The major portion of the determinations in this investigation has been made on hydrocortisone samples and standards, but the results are equally applicable to other corticosteroids since the decomposition pathways and determinative reactions are similar for all corticosteroids.

Several components of corticosteroid preparations were found to cause interference in the determinative steps of at least one of the four corticosteroid methods. The results are summarized in Table II.

Table III—Analysis of Corticosteroid Preparations

Product	Corticosteroid	Concn.	% of Declared Value by BT	PH	INH
Lotion					
1	Hydrocortisone	0.125%	100.0	99.2	99.7
2	Hydrocortisone	0.125%	24.3[a]	2.5	43.8
3	Hydrocortisone	0.25%	97.6[a]	90.6	100.3
4	Hydrocortisone	0.25%	110.8	108.4	109.6
5	Hydrocortisone	0.5%	95.3[a]	90.7	98.7
6	Hydrocortisone	0.5%	99.2[a]	94.4	96.9
7	Hydrocortisone	1%	107.2	107.5	107.4
Cream					
1	Hydrocortisone	0.125%	48.4[a]	30.2	71.1
2	Hydrocortisone	0.125%	82.2[a]	45.0	60.0
3	Hydrocortisone	0.25%	103.6	105.6	101.8
4	Hydrocortisone	0.5%	111.7[a]	102.7	113.2
Ointment					
1	Hydrocortisone acetate	0.5%	104.2[a]	93.4	96.9
Ophthalmic drops					
1	Hydrocortisone acetate	2.5%	78.4	80.6	79.0
2	Prednisolone	0.2%	89.0	. . .	89.5
Ophthalmic suspension					
1	Dexamethasone	0.1%	106.3	106.8	107.0
2	Prednisolone acetate	0.25%	111.8[a]	99.8	102.1
Tablets					
1	Prednisone	0.75 mg.	90.1		88
2	Prednisone	2.5 mg.	100.0		
3	Prednisone	5 mg.	50.6[a]	45.3	65.1

[a] Interference by variation of absorbance with time.

Table IV—Neutral and Acidic Corticosteroid Decomposition Products Determined in Pharmaceutical Preparations

Pharmaceutical Formulation	Corticosteroid	Corticosteroid Found: % Undecomposed	% Decomposed: Neutral	% Decomposed: Acidic
Capsule	Prednisone (2 mg.)	34	9	57
Tablets (buffered)	Prednisone (5 mg.)	65	15	20
Tablets	Hydrocortisone (4 mg.)	82	5	13
Cream	Hydrocortisone (0.125%)	48	36	16
Cream	Hydrocortisone (0.25%)	53	9	38
Lotion	Hydrocortisone (0.125%)	2	34	64
Lotion	Hydrocortisone (0.125%)	77	10	13
Lotion	Hydrocortisone (0.25%)	84	10	6
Lotion	Hydrocortisone (0.25%)	73	17	10
Lotion	Hydrocortisone (0.25%)	76	12	12

Typical results of determinations by the three methods on samples from different lot or batch numbers of the same preparation are shown in Table III. The difference between the results obtained from samples that have undergone decomposition and the results obtained from undecomposed samples is immediately apparent. The samples of ophthalmic drops are typical of samples in which there is no decomposition but in which the amount of corticosteroid is less than the declared amount. In all cases in which there is a significant discrepancy between the results by the BT and PH methods, a study of the variation of absorbance with time indicated that there was interference in the BT determination. In these cases, the value by the PH method is assumed to be correct.

Decomposition at the C_{17} side chain is indicated whenever the results by BT and PH methods are comparable but are lower than those by the INH or UV method. If no decomposed corticosteroid is found, the difference in values is due to a negative interference in the BT or PH method and/or a positive interference in the INH or UV method. The extent of the decomposition and the amounts of acidic and neutral decomposition products determined for several different corticosteroid preparations are given in Table IV. The amount of neutral decomposition products was determined by subtraction of the values obtained by the BT or PH method from those obtained by the INH or UV method on the chloroform extract of the sample. The value of the total decomposed fraction was calculated by subtraction of the value obtained by the BT or PH method on the chloroform extract from the values obtained by the INH or UV procedure on the alcohol USP extract of the sample.

Decomposition in Ring A is indicated whenever the results by INH and UV methods are comparable and are lower than the values obtained by the BT and PH methods. Decomposition of Ring A is due primarily to absorption of UV light and is not found often in corticosteroid preparations. It is more probable that such a difference in values is due to a positive interference in the BT or PH reaction. Positive interferences in the BT are more probable than negative interferences in the INH or UV procedure, as shown in Tables I and II.

All four methods are subject to interference by components of corticosteroid preparations; detection and elimination of such interferences are important to the correct interpretation of analytical results. Methods of detecting interferences include comparison of the PH and BT results. Differences between these values indicate interference, since the color-development reactions are different even though the reagents react with the same portion of the corticosteroid molecule. The BT reaction is subject to more interferences than the PH reaction, since the BT reagent is less specific for corticosteroids than the PH reagent.

Interference in the BT reaction can be either positive or negative. Positive interferences, such as lanolin, are those that react with the BT reagent to produce the diformazan. Negative interferences are often due to the acidic nature of the interfering substance. The BT reaction is highly pH dependent; substances which lower the pH below the optimum value can cause decrease of color formation or even completely inhibit the color formation. The negative interference of stearic acid and of salicylamide is probably due to pH effect, since increasing amounts of each cause a decrease in the pH of the BT reagent sample solution. As an example, the absorbance of a standard hydrocortisone solution containing 50 mg. of stearic acid was 0.331 compared to an absorbance of 0.608 for the standard alone. The pH of the BT solution containing the stearic acid and standard was 13.15 and that of the BT solution of the standard alone was 13.80.

The PH reaction is also subject to both positive and negative interferences by components of corticosteroid preparations. Interference is caused by some surface-active agents such as polysorbate 60, sorbitan monooleate, and sorbitan monostearate. Interference is also caused by organic acids which are soluble in chloroform but insoluble in the strongly acidic phenylhydrazine–sulfuric acid–alcohol solution used. The insoluble compound raises the base line by light dispersion and causes an increase in the absorbance of the sample which is not always compensated by the blank. Lack of compensation by the blank is probably due to the fact that the particle size of the solid separating from the sample is not always identical to the particle size of the solid separating from the blank. Such differences in size cause differences in the amount of light dispersion and the rate of settling during the actual measurement. The negative interference of phenothiazine (34) in the PH reaction is probably due to a reaction with phenylhydrazine which is not corrected for by the blank used.

Differences between the results by the INH and UV methods also indicate interference in one or both procedures. Positive interference in the INH procedure is caused by substances such as vanillin, which form derivatives with the reagent, or by substances such as oil-soluble vitamins and salicylamide, which absorb in the same region of the spectrum.

Many of the components of corticosteroid preparations interfere with the determination by simple UV spectrophotometry, since they absorb in the same region of the spectrum. Stabilizers such as methylparaben and propylparaben fall into this category. Such interference is usually detected readily by comparison of the absorbance curve of the sample to that of the standard. Interfering substances cause distortions of the spectra which are readily discernible.

Many of the interferences discussed can be detected by time studies of absorbance variation during the color-development period of the samples and standards. This is especially true with the BT procedure, since the absorbance developed by the BT reagent with corticosteroids becomes constant after a certain period of time or continues to increase at a rate dependent upon the individual steroid (35). Remeasurement of the absorbance of

Table V—Detection of Interference in the BT Method by Variation of Absorbance with Time

	Absorbance					Difference
	Sample 1. Hydrocortisone Lotion 0.125%					
Minutes	90			126		
Standard hydrocortisone	0.591			0.592		0.001
Sample	0.491			0.528		0.037
	Sample 2. Hydrocortisone Lotion 0.125%					
Minutes	30	60	90	150	210	Difference (90–150)
Standard	0.551	0.561	0.568	0.565	0.570	0.003
Sample A (2.1 g.)	0.462	0.490	0.515	0.528	0.540	0.013
Sample B (3.0 g.)	0.668	0.720	0.756	0.784	0.798	0.028

both the standards and samples 20 or 30 min. after the specified time for color development will show any major interference present. Minor interferences usually require an extended time study of absorbance variation during and after the color-development period for both standards and samples. Examples of the detection of interference by the measurement of the variation of absorbance with time are shown in Table V. The increase of 0.037 unit in the absorbance of Sample 1 during the 36 min. after the standard had become constant is an obvious indication that some component in addition to hydrocortisone is reacting with the BT reagent. This particular case of interference was detected by a routine rescan of the absorbance of the first standard and sample of a series of determinations after all samples and standards had been measured. The time lapse of 36 min. was required to measure all samples and standards. Sample 2 is an example of interference that is not immediately apparent by a rescan of the first standard and sample after completion of the series of measurements. In this particular case, a small increase in absorbance was noted for the sample when it was rescanned so that the extended time study was made. This study definitely proves that some interfering substance was present.

Many of these interfering substances may be separated from the corticosteroid in samples by use of a new acetonitrile–diatomaceous earth[2] column procedure (40).

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