Absorbance–pH Relationship in the Steroid–Tetrazolium Reaction

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Abstract □ The absorbance produced by a given weight of corticosteroid in the blue tetrazolium method is shown to be a function of the apparent pH as measured by a glass and saturated calomel electrode. In general, the absorbance decreases as the apparent pH decreases, irrespective of the cause of the apparent pH change. An equation is derived which relates the absorbance to the apparent pH for hydrocortisone solutions, and the relationship is shown to be similar for several representative corticosteroids. It is recommended that the apparent pH be determined on all samples that are run without a prior acidic or basic extraction to determine if low readings are due to interference or to lower than expected quantities.

Keyphrases □ Corticosteroid–blue tetrazolium reaction—absorbance–pH relationship □ Steroid–blue tetrazolium reaction—absorbance–pH relationship □ Blue tetrazolium–steroid reaction—absorbance–pH relationship □ Colorimetry—steroid–blue tetrazolium reaction, absorbance–pH relationship

The blue tetrazolium {3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-bis[2,5-diphenyl-2*H*-tetrazolium chloride]} reaction of Mader and Buck (1) is, with slight modification, the method for corticosteroid analysis in both USP XVII (2) and NF XIII (3). The reaction involves the oxidation–reduction reaction between the C_{17} side chain of the corticosteroid and the blue tetrazolium (I) in alkaline solution to form a colored formazan (4). Since the reaction is not specific and is affected by a large number of interfering compounds (5), extreme care must be exercised in cleanup procedures for pharmaceutical products that include corticosteroids in the formulation. In some cases, interfering substances which are not removed in the cleanup procedures can be detected by the variation of absorbance with time method of Graham *et al.* (6).

The reaction rate and the extent of completion of the formation of the formazan are influenced by numerous factors, including the structural features of the corticosteroid as well as the temperature and solvent used together with the concentrations of base, water, and I. Temperatures ranging from room temperature to 90° with color development times from 2 to 120 min. have been used (1–3, 7–9). Alkalies utilized include alcoholic sodium hydroxide (9, 10), tetramethylammonium hydroxide (II) (1, 11), trimethylbenzylammonium hydroxide[1] (12), and choline (10, 12) in various concentrations.

Rechnagel and Litteria (9) found that water inhibits the reaction and that the extent of inhibition increases as the concentration of water in the final reaction mixture increases. They recommended that reagents be prepared with minimal amounts of water. These investigators also studied the effect of the concentration of I and found that the absorbance due to 9 mcg. of 11-desoxycorticosterone in 3 ml. of reaction mixture increased to a constant value of approximately 120 mcg./ml. and utilized 167 mcg./ml. in their procedure. Concurrent blank, sample, and standards are usually run to offset the effect of these variables as much as possible.

[1] Triton B.

Table I—Effect of Water upon Absorbance and Apparent pH

Sample Number	Water Added, ml.	Water in Reaction Mixture[a,b], % v/v	Net Absorbance[c]	Apparent pH[c]
1	0.00	1.63	0.592	13.75
2	1.00	3.29	0.589	13.79
3	2.00	4.96	0.588	13.75
4	3.00	6.63	0.580	13.72
5	4.00	8.29	0.572	13.70
6	5.00	9.96	0.555	13.67
7	7.00	13.3	0.540	13.58
8	10.00	18.3	0.460	13.40
9	12.00	21.6	0.383	13.31
10	15.00	26.6	0.256	13.23
11	20.00	35.0	0.084	13.03
12	25.00	43.3	0.034	12.83

[a] Each reaction mixture contains 0.200 mg. of hydrocortisone and 0.22 mmole of II. [b] The water in the reaction mixture includes the added water plus the water present in the I and II reagents. [c] Average of duplicates.

This paper reports a study of the relationship between the absorbance and the apparent pH (as measured with a glass and saturated calomel electrode) for the blue tetrazolium reaction with selected corticosteroids. The effect of water, acids, and the concentration of II upon the absorbance and apparent pH of several corticosteroids was investigated.

EXPERIMENTAL[2]

Reagents—The following were used:

1. Tetramethylammonium hydroxide[3], pentahydrate (II).
2. Tetramethylammonium hydroxide[3], 10% aqueous.
3. Tetramethylammonium hydroxide, 10% alcoholic. Dissolve 5.00 g. of II and dilute to 25.0 ml. with absolute ethanol. The resulting solution is 10% anhydrous II and 10% water.
4. Tetramethylammonium hydroxide, 1%. Dilute 5.00 ml. of II, 10% aqueous, to 50.0 ml. with alcohol USP (95% ethanol).
5. Blue tetrazolium, 5 mg./ml. Dissolve 50.0 mg. of blue tetrazolium[4] in 10.0 ml. of alcohol USP.
6. Corticosteroid standards, 0.100 mg./ml. Dissolve 10.0 mg. of the steroid (USP or NF reference standard) and dilute to 100.0 ml.

[2] A Cary model 15 ratio recording spectrophotometer was used. Absorbances were measured in matched 1-cm. quartz cells by scanning from 700 to 490 nm. A Corning model 10 pH meter with a Corning model 476050 combination electrode was standardized with pH 10 standard buffer. Usually 5–10 min. of equilibration was required before the readings became constant.

[3] Eastman Organic Chemicals.

[4] Dajac Laboratories.

Table II—Effect of Salicylamide upon Absorbance and Apparent pH

Solution	Salicylamide in Reaction Mixture[a], mmole	Absorbance[b]	Apparent pH[b]
Reagent blank	0.000	ND[c]	14.40
Standard	0.000	0.584	14.09
1	0.041	0.578	13.70
2	0.082	0.564	13.60
3	0.123	0.515	13.54
4	0.164	0.428	13.50
5	0.180	0.356	13.30
6	0.197	0.232	13.20
7	0.213	0.130	12.86
8	0.230	0.022	12.01
9	0.246	0	11.64
10	0.262	0	11.36
11	0.328	0	10.92
12	0.410	0	10.70
13	0.820	0	10.33

[a] Each reaction mixture contains 0.200 mg. of hydrocortisone and 0.22 mmole of II. [b] Average of duplicates. [c] Not determined.

with alcohol USP or absolute ethanol as required. Lower concentrations are prepared by quantitative dilution of these standards. Many of the 0.01-mg./ml. standards were prepared by dissolving 1.000 mg. of the steroid in 100.0 ml. of alcohol.

All other reagents and solvents were ACS, USP, NF, or analytical reagent grade.

General Procedure - The procedure, unless otherwise specified, is the official procedure given in NF XIII (2) in which a 20.00-ml. volume of standard or sample corticosteroid in alcohol USP is treated with 2.00 ml. of blue tetrazolium reagent followed by 2.00 ml. of II (1%). The absorbance is measured against a reagent blank 90 min. after the addition of II. In this study, the apparent pH of each solution was determined after completion of the absorbance measurements.

Effect of Water Concentration upon Absorbance and Apparent pH—Five-milliliter aliquots of standard hydrocortisone solution (0.10 mg./ml.) in absolute ethanol were transferred to 50.0-ml. volumetric flasks, containing the amounts of water shown in Table I, and diluted to volume with absolute ethanol. Blanks were prepared similarly with the substitution of absolute ethanol for the standard. Duplicate 20.00-ml. aliquots of each standard and a single 20.00-ml. aliquot of each blank were transferred to separate 50-ml. glass-stoppered conical flasks, and the blue tetrazolium reagent and II (1%) were added in the order of standard, standard, and blank for each different amount of water. The time of addition of II was counted as zero time for each set. The flasks were placed in the dark at room temperature (24 ± 1°) for 90 min. and then scanned against absolute ethanol in the same order. The absorbances of the blanks were subtracted from the absorbances of the standards to obtain the net absorbances.

Effect of Salicylamide upon Absorbance and Apparent pH—Solutions of hydrocortisone and salicylamide were prepared in alcohol USP so that 20.00-ml. aliquots contained 0.200 mg. (0.552 μmole) of hydrocortisone and the amounts of salicylamide shown in Table II. These solutions were run in duplicate according to the general procedure by addition of blue tetrazolium reagent (13.7 μmoles) and II (1%, 0.22 mmole) and were scanned after 90 min. against a reagent blank containing no salicylamide.

Effect of Increased Tetramethylammonium Hydroxide Concentration upon Absorbance and Apparent pH—Twenty-milliliter aliquots of a series of hydrocortisone solutions (0.01 mg./ml.) in absolute ethanol were run in duplicate according to the general procedure, except that different concentrations of II in absolute ethanol were used and both the blanks and solutions were scanned against absolute ethanol. The solutions of II were prepared by dilution of II (10% alcoholic) with absolute ethanol. The study was repeated using hydrocortisone solutions in absolute alcohol with conditions adjusted so that the percentage of water in the reaction mixture increased from 4.67 to 6.42% (average 4.96%) and from 5.67 to 6.42% (average 6.00%). (The percentage of water in the reaction mixture in the general procedure is 5.8%.) The study was also repeated using hydrocortisone solutions in alcohol USP and solutions of II prepared by dilution of II (10% aqueous) with alcohol USP.

Effect of Added Acid and Increased Tetramethylammonium Hydroxide Concentration upon Absorbance and Apparent pH—A series of solutions was prepared to contain 0.200 mg. of hydrocortisone and various amounts of salicylic acid per 20.00 ml. These solutions were run in duplicate according to the general procedure, except that increased concentrations of II were used in some determinative steps and all blanks and solutions were scanned against alcohol USP. The study was repeated using stearic acid in place of salicylic acid.

Effect of Various Acids upon Absorbance and Apparent pH—A series of solutions was prepared so that 20.00-ml. aliquots contained 0.200 mg. of hydrocortisone and either 0.100 or 0.200 meq. of one of the following acidic substances: citric acid, tartaric acid, stearic acid, *p*-aminobenzoic acid, salicylic acid, phenobarbital, niacin, or niacinamide. These solutions were run according to the general procedure.

Effect of Added Water and Added Acid upon Absorbance and Apparent pH of Several Corticosteroids—Solutions of several representative corticosteroids were prepared in alcohol USP to contain 0.200 mg. of the steroid in 20.00 ml. For the esters, the weights were adjusted so that the solutions contained the equivalent of 0.200 mg. of the unesterified steroid. These solutions were run according to the

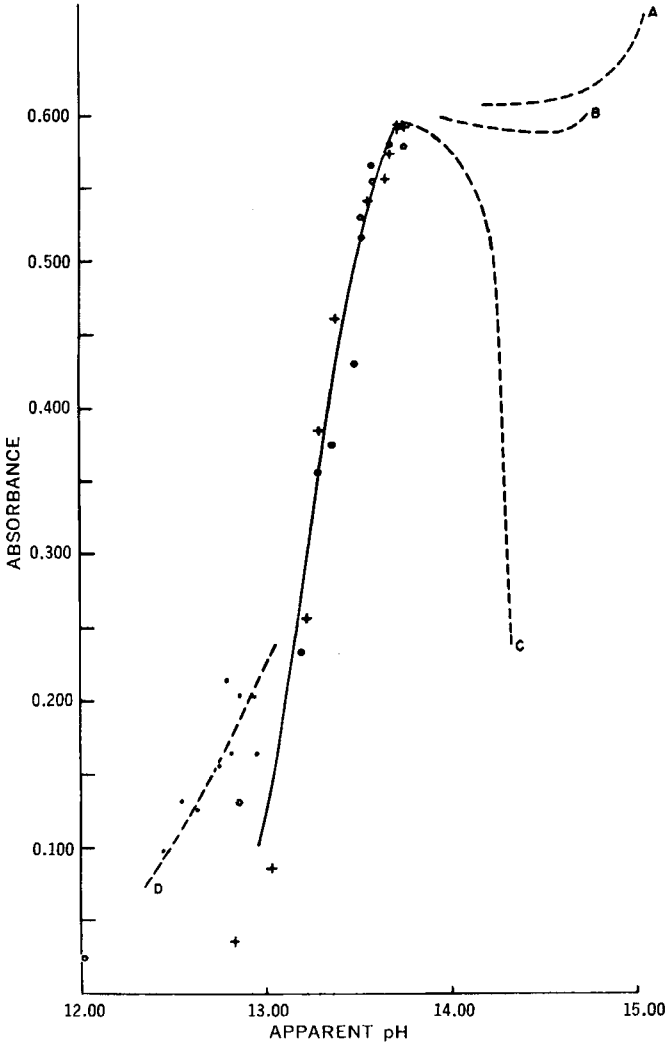

Figure 1—*Variation of absorbance produced by 0.200 mg. of hydrocortisone with apparent pH. Key: ——, derived by calculation using Eq. 1; ○, experimental points from salicylamide study; and +, experimental points from water study. Dashed lines A, B, and C represent the effect of increasing concentrations of II at different water percentage levels: A, low water (0.50–1.24, average 0.84%); B, official amount of water (5.67–6.42, average 6.00%); and C, above official amount of water (5.79–12.92, average 9.09%). Dashed line D represents the effect of adding 0.200 meq. of various acids.*

Table III—Effect of Increased Tetramethylammonium Hydroxide (II) Concentration upon Absorbance and Apparent pH

Percent II Added	Alcoholic II[a]			Alcoholic II[b]		
	Water in Reaction Mixture, %	Net Absorbance[c]	Apparent pH[c]	Water in Reaction Mixture, %	Net Absorbance[c]	Apparent pH[c]
1	0.50	0.605	14.24	4.63	0.599	13.91
2	0.58	0.606	14.50	4.71	0.598	14.24
4	0.75	0.614	14.80	4.88	0.601	14.47
6	0.91	0.628	14.94	5.05	0.604	14.61
8	1.08	0.645	15.02	5.21	0.606	14.70
10	1.24	0.666	15.09	5.38	0.617	14.76
Percent II Added	Alcoholic II[b]			Alcoholic II[d]		
	Water in Reaction Mixture, %	Net Absorbance[c]	Apparent pH[c]	Water in Reaction Mixture, %	Net Absorbance[c]	Apparent pH[c]
1	5.67	0.596	13.99	5.79	0.592	13.76
2	5.75	0.590	14.27	6.58	0.576	13.99
4	5.92	0.588	14.50	8.17	0.548	14.16
6	6.09	0.587	14.64	9.75	0.476	14.24
8	6.25	0.593	14.73	11.33	0.425	14.29
10	6.42	0.598	14.76	12.92	0.243	14.33

[a] Hydrocortisone, 0.200 mg., in absolute ethanol. II prepared by diluting II, 10% alcoholic, with absolute ethanol. [b] Hydrocortisone, 0.200 mg., in diluted ethanol. II prepared as in *Footnote a*. [c] Average of duplicates. [d] Hydrocortisone, 0.200 mg., in alcohol USP. II prepared by diluting II (10% aqueous) with alcohol USP.

general method and also with the general method modified by the use of II (1%) prepared by dilution of II (10% aqueous) with water. Use of water as the diluent in place of alcohol USP raised the percentage of water in the reaction mixture from 5.8 to 12.9%. Solutions containing the same weights of steroids and 0.20 mmole of stearic acid in 20.00 ml. were prepared using alcohol USP. These solutions were run according to the general method.

RESULTS AND DISCUSSION

The results of the study of the effect of water upon the absorbance and apparent pH in the blue tetrazolium determination of hydrocortisone are shown in Table I and Fig. 1. The effect is essentially negligible if the percentage of water is below approximately 6 and the apparent pH is not below 13.75. Above 6% water, both the absorbance and the apparent pH decrease significantly with an increase in the amount of water. In this region, the relatively small changes in apparent pH due to the increase in water are associated with large changes in the absorbance produced by 0.200 mg. of hydrocortisone. The percentage of water in the general or official method [due to the use of alcohol USP and II (10% aqueous) in the preparation of reagents and samples] is 5.8, which is close to, but below, the critical value at which the effect becomes significant.

The results of the study of the effect of salicylamide on the absorbance and apparent pH are shown in Table II and Fig. 1. Both the absorbance due to a given amount of hydrocortisone and the apparent pH decrease with an increase in salicylamide, with as little as 0.041 mmole of salicylamide having a significant effect. There is no development of color in solutions that contain more millimoles of salicylamide than II and in which the apparent pH is less than 12. As in the water study, relatively small changes in the apparent pH below 13.70 are associated with large changes in absorbance.

The values for the net absorbance (A) and apparent pH for these two studies were combined and used to derive Eqs. 1 and 2, which relate absorbance to apparent pH irrespective of the cause of decrease in apparent pH:

$$pH = 6.265A^3 - 5.446A^2 + 2.725A + 12.74 \quad \text{(Eq. 1)}$$

$$A = -1.76679pH^3 + 70.5814pH^2 - 939.004pH + 4160.56 \quad \text{(Eq. 2)}$$

The solid line in Fig. 1 represents values calculated by Eq. 1. The agreement between experimental and calculated points is shown on the figure and by the fact that the coefficient of multiple correlation, R^2, is 0.946 for the combined data from the water study and the salicylamide study.

To determine whether or not the absorbance is affected by the apparent pH only or by both the water and the apparent pH, several studies were made on the effect of an increased concentration of II (Table III). For percentages of water well below the official method amount of 5.8%, both the apparent pH and absorbance increase with an increase in concentration of II. For percentages of water close to the 5.8% of the official method, the apparent pH increases with increase in II but the absorbance is not affected greatly by up to a sixfold increase. Above a sixfold increase in II, the absorbance increases significantly as the apparent pH rises above the 14.60–14.70 range. However, the increase in the percentage of water from the range of 4.63–5.38 (average 4.96) to the range of 5.67–6.42 (average 6.00) as the concentration of II is increased causes an average decrease in absorbance of approximately 0.012 unit per percent water increase for identical concentrations of II. Evidently, an increase in the amount of water above that specified in the official method cannot be offset by increases in the concentration of II alone. This conclusion is further substantiated by the study in which the water was increased from 5.79 to 12.92% as the concentration of II added was increased from 1 to 10% since the absorbance decreased markedly even though the apparent pH increased. The more rapid decrease with increased water concentrations also indicates that the variation in the amount of water has a greater effect upon

Table IV—Effect of Added Acid and Increased Tetramethylammonium Hydroxide (II) Concentration upon Absorbance and Apparent pH

Solution[a]	Acid Added, mmole	II Added, mmole	Net[b] II, mmole	Stearic Acid		Salicylic Acid	
				Net Absorbance[c]	Apparent pH[c]	Net Absorbance[c]	Apparent pH[c]
Blank	0.00	0.22	0.22	0.037	13.70	0.030	13.75
Standard	0.00	0.22	0.22	0.584	13.81	0.584	13.77
Acid blank 1	0.20	0.22	0.02	0.013	ND[d]	0.025	12.90
Sample 1	0.20	0.22	0.02	0.130	12.55	0.203	12.95
Acid blank 2	0.20	0.33	0.13	0.032	13.64	0.044	13.70
Sample 2	0.20	0.33	0.13	0.567	13.65	0.551	13.70
Acid blank 3	0.20	0.44	0.24	0.039	13.85	0.054	13.91
Sample 3	0.20	0.44	0.24	0.579	13.85	0.564	13.93

[a] Each solution contained 0.200 mg. hydrocortisone. [b] Calculated as difference between millimoles of acid and II. [c] Average of duplicates. [d] Not determined.

Table V—Effect of Various Acidic Substances upon Absorbance and Apparent pH

Added Acid[a]	—0.100 meq. Added— Net Absorbance[b]	Apparent pH[b]	—0.200 meq. Added— Net Absorbance[b]	Apparent pH[b]
Standard (no added acid)	0.584	13.77	0.584	13.77
Citric acid	0.567	13.68	0.125	12.63
Tartaric acid	0.573	13.65	0.155	12.75
Salicylic acid	0.554	13.70	0.163	12.96
Niacin	0.573	13.72	0.163	12.82
p-Aminobenzoic acid	0.585	13.72	0.203	12.87
Stearic acid	0.574	13.65	0.097	12.45
Phenobarbital	0.576	13.68	0.214	12.80
Niacinamide	0.637	13.95	0.637	13.95

[a] Each solution contained 0.200 mg. hydrocortisone. [b] Average of duplicates.

absorbance than an increase in the apparent pH or concentration of II. The variations of absorbance with apparent pH for these studies are shown as dashed lines in the upper portion of Fig. 1.

The decrease in absorbance and apparent pH caused by the neutralization of II by acids can be offset, at least in part, by an increase in the concentration of II provided the water concentration does not exceed the amount prescribed by the official method (Table IV). However, the effect of equivalent amounts of various acids is not offset to the same extent by equivalent increases in the concentration of II.

The effect of different acids upon the absorbance and apparent pH is shown further in Table V, which lists the apparent pH and absorbance obtained by the addition of 0.1 and 0.2 meq. of various acids to hydrocortisone solutions. The larger amounts of acid neutralize essentially all of the II present, while the smaller amounts neutralize approximately half. The extent of the effect upon the apparent pH and the absorbance may be essentially negligible for 0.1 meq. of some acids such as *p*-aminobenzoic, while absorbance and apparent pH are actually raised by niacinamide which can act as a strong base in nonaqueous media. The variation of the extent of the effect of various acids is shown more markedly in the solutions containing 0.2 meq. of the acids. In each case, there is a great decrease in absorbance as the apparent pH is lowered to values below 13.00, but the actual absorbances vary from 0.097 to 0.214 and the apparent pH values range from 12.45 to 12.96. The results are shown by the dashed line and experimental points in the lower portion of Fig. 1.

Table VII—Normal Variation of Apparent pH and Absorbance of Hydrocortisone Standards due to Reagents

Date	Apparent pH	Absorbance[a]
10–16–69	13.88	0.581
10–22–69	14.11	0.577
8–14–70	14.15	0.580[b]
9–8–70	14.11	0.578
8–19–71	13.85	0.584
8–19–71	13.87	0.587
8–30–71	13.77	0.586
8–30–71	13.76	0.586
9–2–71	13.80	0.578
10–14–71	13.76	0.587
10–14–71	13.76	0.585
10–22–71	14.06	0.599
10–22–71	14.07	0.598
11–2–71	13.99	0.585[b]
11–2–71	13.99	0.587[b]
11–9–71	13.90	0.598[b]
11–9–71	13.91	0.598[b]

[a] Each standard contained 0.200 mg. of hydrocortisone. [b] The solution of II was prepared from the pentahydrate rather than the 10% aqueous solution.

The results of the study to determine the effect of water and acids upon the absorbance and apparent pH of several representative corticosteroids are summarized in Table VI. For the water study, the average percent change in absorbance per unit change in apparent pH is 47.7, with a range of 40.4–52.9 and with a relative standard deviation of 2.95%. These values indicate that the relative effect of water is essentially the same for all of the corticosteroids studied. The average decrease in absorbance in this study is 0.0477 unit or 0.0067 unit for each unit percent change in water from 5.8 to 12.9%. This value agrees satisfactorily with the average decrease of 0.0060 unit per percent increase in water from 6.6 to 13.3% shown in Table I for hydrocortisone solutions. Desoxycorticosterone

Table VI—Effect of Water and Acid upon Absorbance and Apparent pH of Several Corticosteroids

Corticosteroid[a]	—Official Method— Net Absorbance	Apparent pH	—Water Study[b]— Net Absorbance	Apparent pH	ΔA, %	ΔpH	$\frac{\Delta A}{\Delta pH}$	—Acid Study[c]— Net Absorbance	Apparent pH	ΔA, %	ΔpH	$\frac{\Delta A}{\Delta pH}$
Hydrocortisone	0.600	14.06	0.546	13.89	9.00	0.17	52.9	0.015	11.04	97.5	3.02	32.3
Hydrocortisone	0.599	14.07	0.546	13.89	8.85	0.18	49.2	0.038	11.60	93.7	2.47	37.9
Hydrocortisone acetate	0.598	14.06	0.550	13.89	8.03	0.17	47.2	0.004	11.71	99.3	2.35	42.3
Hydrocortisone acetate	0.596	14.05	0.549	13.89	7.89	0.16	49.3	0.003	11.61	99.5	2.44	40.8
Desoxycorticosterone acetate	0.673	14.05	0.613	13.88	8.92	0.17	52.5	0.005	11.60	99.3	2.45	40.5
Desoxycorticosterone acetate	0.668	14.05	0.614	13.89	8.08	0.16	50.5	0.011	11.65	98.4	2.40	41.0
Prednisone	0.576	14.05	0.533	13.89	7.47	0.16	46.7	0.125	12.04	78.3	2.01	39.0
Prednisone	0.576	14.05	0.530	13.88	7.99	0.17	47.0	0.088	11.75	84.7	2.30	36.8
Dexamethasone	0.535	14.05	0.490	13.89	8.41	0.16	52.6	0.039	11.89	92.7	2.16	42.9
Dexamethasone	0.533	14.05	0.490	13.89	8.07	0.16	50.4	0.021	11.60	96.1	2.45	39.2
Prednisolone	0.589	14.05	0.545	13.88	7.47	0.17	43.9	0.042	12.09	91.9	1.96	46.9
Prednisolone	0.589	14.05	0.544	13.88	7.64	0.17	44.9	0.028	11.81	95.2	2.24	42.5
Prednisolone acetate	0.591	14.06	0.548	13.88	7.28	0.18	40.4	0.013	12.20	97.8	1.86	52.6
Prednisolone acetate	0.591	14.06	0.548	13.88	7.28	0.18	40.4	0.006	12.00	99.0	2.06	48.1
Average					8.03		47.7			94.5		41.6
Standard deviation[d]					0.29		1.41			2.39		2.56
Standard deviation, % of average					3.59		2.95			2.53		6.15

[a] Each solution contained 0.200 mg. of the steroid or the equivalent weight for the esters. [b] General method except that II (1%) was prepared by dilution of II (10% aqueous) with water in place of alcohol USP to give 12.9% water in the reaction mixture. [c] Each solution contains 0.20 mmole of stearic acid in addition to the steroid. [d] Calculated by the difference in duplicate method in which the standard deviation equals the square root of the sum of the squares of the differences between duplicates divided by twice the number of duplicate pairs.

Table VIII—Interference in Samples by Acidic Components

Sample Number	Corticosteroid Present	Blue Tetrazolium Method: Before Extraction — Found, % of Declared	Blue Tetrazolium Method: Before Extraction — Apparent pH	Blue Tetrazolium Method: After Extraction — Found, % of Declared	Blue Tetrazolium Method: After Extraction — Apparent pH: Sample	Blue Tetrazolium Method: After Extraction — Apparent pH: Standard	INH[a] Method, Found, % of Declared
1	Prednisolone[b]	NC[c]	6.74	107.4	14.20	14.14	103.5
2	Prednisolone[b]	NC[c]	6.76	21.2	14.22	14.20	26.9
3	Dexamethasone[d]	NC[c]	5.76	90.0	14.20	14.20	88.5

[a] INH = isonicotinic acid hydrazide. [b] Samples 1 and 2 were declared to contain 0.75 mg. prednisolone, 325 mg. aspirin, 120 mg. salicylamide, and 100 mg. aluminum hydroxide per tablet. [c] NC means no color was developed by the sample. [d] Sample 3 was declared to contain 0.25 mg. dexamethasone, 500 mg. aspirin, and 75 mg. aluminum hydroxide per tablet.

acetate gives unusually high values of absorbance compared to the other compounds since it has a lower molecular weight and a larger molar absorptivity. Omission of the values for desoxycorticosterone acetate and for hydrocortisone from the values of Table VI changes the rate of decrease to 0.0063 absorbance unit, which agrees more closely with the value obtained from Table I.

For the acid study with several corticosteroids, which covers much larger changes in both absorbance and apparent pH, the average percent change in absorbance per unit change in apparent pH is 41.7, with a range of 32.3–52.6 and with a relative standard deviation of 6.15%. These values indicate that the relative effect of 0.200 meq. of stearic acid upon the absorbance and apparent pH is approximately the same for the representative corticosteroids studied. The average decrease in absorbance for all of the steroids is 0.563 unit. This value compares satisfactorily with: (*a*) the average value of 0.424 for the effect of 0.200 meq. of several different acids upon the absorbance of hydrocortisone solutions shown in Table V, (*b*) the average value of 0.418 due to the addition of stearic acid and salicylic acid to hydrocortisone solutions shown in Table IV, and (*c*) the value of 0.454 caused by the addition of 0.213 meq. of salicylamide shown in Table II. Evidently, the almost complete neutralization of II by acids does not affect all of the corticosteroids equally but does cause a great change in both absorbance and apparent pH.

Equations 1 and 2 cannot be used to calculate the absorbance quantitatively from the apparent pH or the apparent pH from the absorbance for a single measurement. This is due to the variation in the response of the various corticosteroids and to the variation in absorbance and apparent pH of standard solutions of the same corticosteroids caused by variations in reagents from batch to batch. The normal variations in the apparent pH and the absorbance of hydrocortisone standards over 24 months are shown in Table VII.

Equations 1 and 2 can be used as a qualitative check upon the absorbance associated with a given apparent pH to determine whether or not a lower than expected absorbance reading is due to decomposition, to less than the stated amounts of the steroid, or to pH variation. It is better, however, to utilize the differences in the absorbance and apparent pH of the standard and the sample or the differences between the actual absorbance of the sample and the expected absorbance of the sample. If the difference between the actual absorbance and the expected absorbance is greater than 0.03 and the apparent pH is below 13.75, the low results may be due to water, acid, or other interference rather than low concentrations of the undecomposed steroid. If, however, the actual and expected absorbances agree and the apparent pH is above 13.75, it is possible that positive interference due to basic components is offsetting lower than expected quantities of the steroid. These checks are possible since the sample size is usually adjusted so that the 20-ml. aliquot used in the color-development step contains approximately 0.200 mg. of the steroid and the absorbance is compared to a standard containing 0.200 mg. of the steroid. As a consequence, apparent pH measurements should be made as a routine procedure in the blue tetrazolium method for corticosteroids.

This study originated due to discrepancies in the analysis of a partially decomposed prednisone capsule preparation before and after basic extraction. The capsule originally contained 2 mg. of prednisone, 225 mg. of salicylamide, 20 mg. of ascorbic acid, and other neutral components. The results by the blue tetrazolium method when run directly by dissolving and adding I and II to a 20-ml. aliquot were zero, but the results were 34.3% of the declared amount when determined after a basic extraction of the capsule contents. Typical examples of samples run since that time in which the apparent pH measurement indicated acidic interference are shown in Table VIII. The results were checked by the isonicotinic acid hydrazide method of Umberger (13), which involves reaction with a different portion of the corticosteroid molecule and which is not affected by acidic components. Due to the low apparent pH, no color was developed during the blue tetrazolium method when the tablet contents were dissolved directly in alcohol USP. After solution of the corticosteroid and acidic components by repeated treatment with acetone followed by evaporation and solution in chloroform, the acidic components were extracted into 0.2 *N* NaOH and the chloroform was evaporated. The residue was dissolved in alcohol USP and gave results by the blue tetrazolium method that agree satisfactorily with the results by the isonicotinic acid hydrazide method.

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