Effect of Solvent on Tetrazolium Reaction

R. E. GRAHAM*x, E. R. BIEHL ‡, and C. T. KENNER ‡

Abstract □ The rate of color development of a tetrazolium formazan is shown to be inversely proportional to the dielectric constant of the solvent medium and directly proportional to the hydrogen-bonding capability of solvent mixtures having the same dielectric constant. The geometric isomers of the formazans have different absorbance maxima, and the wavelength of maximum absorbance of a mixture of formazans in different solvents depends upon which isomer predominates in that solvent. The *trans-syn*-isomer (blue form) of blue tetrazolium has a maximum absorbance at 625 nm in dimethylformamide while the *trans-anti*-isomer (red form) absorbs at 517 nm in methanol. The absorbance maxima of the corresponding isomers of the formazans of triphenyltetrazolium occur at 535 and 485 nm, respectively. Water and/or methanol (to a lesser extent) are important in the stabilization of the *trans-anti*-isomer, since the small size of these two substances allows them to form strong intermolecular hydrogen bonds with one or both nitrogen atoms of the azo linkage, thereby preventing the formation of the intramolecular hydrogen bonding exhibited by the *trans-syn*-isomer. The formazan produced by the reaction of corticosteroids with tetrazolium in strongly basic media can lose a reduction unit and be reoxidized to the tetrazolium. This reaction is solvent dependent and occurs at a much faster rate in chloroform than in alcohol USP.

Keyphrases □ Tetrazolium reaction—effect of varying solvents and dielectric constants of solvents on rate of color development and wavelength of maximum absorption of formazan □ Solvent systems—effect on rate of color development and wavelength of maximum absorption of formazan, tetrazolium reaction □ Dielectric constants—solvents in tetrazolium reaction, effect on rate of color development and wavelength of maximum absorption □ Color development—tetrazolium reaction, effect of varying solvents and dielectric constants of solvents □ Absorption, spectrophotometric—maximum wavelength of tetrazolium formazan, effect of varying solvents and dielectric constants of solvents

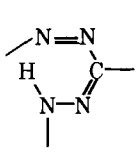
The blue tetrazolium reaction is widely used for the analysis of corticosteroids. USP XIX (1) and NF XIV (2) use a slightly modified procedure of Mader and Buck (3) for corticosteroid analysis. Blue tetrazolium (I), 3,3′- (3,3′- dimethoxy-4,4′-biphenylylene)bis(2,5 -diphenyl-2*H*-tetrazolium chloride), oxidizes the α-keto moiety of the C-17 side chain of corticosteroids in strongly alkaline solution and is reduced quantitatively to a highly colored formazan whose concentration is measured spectrophotometrically.

The analytical procedure is subject to several variables such as temperature (4–6), solvent (1–3, 7–12), concentration of base (13), water (6, 13), and I (6), as well as the steric configuration of the corticosteroid molecule (14). The effect of these variables is minimized by analyzing the blank, standard, and samples concurrently.

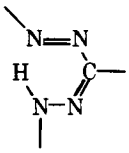
Mechanistic studies (14) showed that the reaction of I with corticosteroids involves a bimolecular reaction in which a reduction unit is transferred from the α-keto moiety of the corticosteroid to I to produce a formazan. The nature of the reduction unit is not known.

The influence of solvent on the production of formazan in this reaction has not been studied extensively. The analysis of corticosteroids by I is generally conducted in alcohol USP (1, 2), although absolute alcohol (7–10) and mixtures of chloroform and alcohol USP (11) have been used. The rate and extent of formazan formation are dependent upon the apparent pH of the solvent medium and are inhibited by increasing concentrations of water (13).

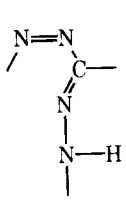
The structure of formazans was reviewed previously (15). The interconversion of the formazan derived from triphenyltetrazolium (II) between different colored forms in the presence of visible light also was reported (16). Formazans contain a basic structure which allows the existence of four possible configurations due to geometric isomerization about the two double bonds.



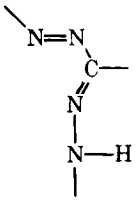
III: *cis-syn*



IV: *trans-syn*



V: *cis-anti*



VI: *trans-anti*

A complete study of the influence of various solvents on the rate of formation and distribution of the various geometric forms of the formazan and their effect on the quantitation of corticosteroids has not been reported. The distribution of the formazan should be highly dependent upon the polarity of the solvent, since only III and IV can exist as intramolecular hydrogen-bonded structures. Consequently, any analytical method using the reduction of a tetrazolium should be solvent dependent.

This paper reports a study of the variation of absorbance with change of solvent in the II and I reaction with selected corticosteroids. Solvent effect on the rate of color development and the wavelength of maximum absorption of the respective formazans produced is discussed.

EXPERIMENTAL

Apparatus—The following were used: UV–visible recording spectrophotometers[1] with 1-cm stoppered quartz cells, glass chromatographic columns for partition chromatography (1.2 and 2.2 × 25 cm constricted at one end to 0.4 × 5 cm), IR spectrophotometers[2], an electrobalance[3], and TLC equipment[4].

Materials—Chloroform (distilled in glass), alcohol USP, and analytical reagent grade absolute ethanol, dimethylformamide, 1-pro-

[1] Cary models 15 and 17.
[2] Perkin-Elmer models 337 and 567.
[3] Cahn model G-2.
[4] Eastman No. 6060 silica gel with fluorescent indicator.

Table I—Solvent Mixtures and Dielectric Constant Range in Reaction of I with Hydrocortisone

Solvent Mixture	Range, %	Dielectric Constant Range
Methanol and 1-propanol	8.3–49.6 90.9–41.3	21.5–26.5
Methanol and methylene chloride	8.3–49.6 90.9–41.3	9.1–23.2
Ethanol and methylene chloride	16.1–98.8 82.6–0.0	9.1–24.8
Ethanol and water	99.6–82.3 0.4–17.7	24.5–33.5

panol, absolute methanol, and methylene chloride were used.

Sodium borohydride[5] [0.8-cm (10/32-in.) pellets], blue tetrazolium[6], 10% aqueous tetramethylammonium hydroxide[7] (VII), triphenyltetrazolium[7], and 70–230-mesh column chromatographic grade silica gel 60[8] were also used.

Reagents—A 1% solution of tetramethylammonium hydroxide was prepared by diluting 5.00 ml of the 10% aqueous solution to 50.0 ml with alcohol USP. Blue tetrazolium, 5 mg/ml, was prepared by dissolving 50.0 mg of I in 10.0 ml of alcohol USP. Corticosteroid standard solutions were prepared to contain 0.010 mg/ml in alcohol USP unless otherwise indicated. The source of each standard is listed in the tables.

General Procedure—The procedure followed, unless otherwise specified, is the official procedure given in USP XIX (1); a 20.00-ml volume of standard corticosteroid in alcohol USP is treated with 2.00 ml of I reagent (5 mg/ml) followed by 2.00 ml of 1% VII. The absorbance is measured against a reagent blank 90 min after the addition of VII.

Separation and Isolation of Formazan and Tetrazolium—An alcoholic solution of the reacted mixture containing the formazan and tetrazolium, diluted with four volumes of water in a separator, was extracted repeatedly with chloroform until colorless. The combined extracts were filtered through chloroform-washed cotton and taken to dryness to recover the formazan. The remaining alcoholic aqueous phase was taken to dryness on the steam bath after being scanned in the UV region.

The resulting residue was washed with cyclohexane to remove the blue formazan of I or the total formazan of II produced during the evaporation. The residue was dissolved in chloroform and filtered into a 100-ml beaker, and the solvent was evaporated under air on the steam bath to yield a crystalline residue.

Rate Studies—A 20.00-ml aliquot of a standard corticosteroid in the appropriate solvent (chloroform or methylene chloride) and a 20.00-ml blank of the same solvent were treated according to the *General Procedure*. Both solutions were transferred to cells as rapidly as possible and placed in the spectrophotometer, and absorbance readings were made each minute at 525 nm until the reaction reached essential completion or 90 min elapsed. This procedure was repeated for selected corticosteroids in various concentrations in other solvents. Rate constants were calculated as previously described (14).

Effect of Dielectric Constant on Rate—The rate of formation of the formazan by the reaction of I with aliquots containing 0.200 mg of hydrocortisone was studied in the solvent mixtures listed in Table I.

The capacitance of several pure liquids was measured according to the procedure of LeFevre (17). A calibration curve was prepared which related the dielectric constants (18) of these liquids to their capacitance. The capacitances of various mixtures of solvents were then measured, and the dielectric constants were obtained from the calibration curve. Fixed amounts of other solvents in these mixtures, such as water and ethanol, are not included in Table I but were used in the determination of the dielectric constants of the solutions. The dielectric constant values for ethanol and water mixtures were obtained from Ref. 19.

Formazan Isolation—*Formazan from I*—

1. One pellet of sodium borohydride was added to a solution containing 55 mg of I and 5 ml of 1% VII in 100 ml of absolute ethanol, and the solution was kept in the dark for 1 week. The blue precipitate which formed (85% of the expected amount) was filtered, washed with absolute ethanol, dissolved in chloroform, and evaporated to dryness.

2. Cortisone (0.5 μmole) and I (0.5 μmole) were allowed to react in chloroform. The resulting solution was placed on a silica gel column (1.2 × 23 cm) and eluted with 25 ml of chloroform. The red formazan remained on the column while the blue was eluted with the chloroform. The silica gel packing was extruded from the column and dampened with equal volumes of methanol and water, and the mixture was allowed to stand for 20 min. It was then mixed thoroughly with chloroform, and the solution containing the red formazan was decanted. Evaporation of both final solutions yielded blue and red formazans.

3. To obtain the formazan of triphenyltetrazolium, one pellet of sodium borohydride was added to a solution containing 100 mg of II in 20 ml of absolute ethanol; the solution was kept in the dark for 24 hr. A crystalline formazan was isolated by the method shown under *Separation and Isolation of Formazan and Tetrazolium.*

Reversibility of Formazan to Tetrazolium—

1. To obtain triphenyltetrazolium from its formazan, 4 drops of 10% VII were added to a solution of the formazan of II in alcohol USP (8 mg/15 ml); the solution was kept in the dark for 8 days. Crystalline II was isolated from the solution following the method described under *Separation and Isolation of Formazan and Tetrazolium.*

2. To obtain blue tetrazolium from its formazan, a solution containing 8.8 mg of the red formazan of I, 10.0 ml of alcohol USP, and 1 ml of 1% VII was kept in the dark for 24 days. The color of the solution changed from red to a brownish yellow. A brownish crystalline substance was obtained from the alcoholic aqueous phase by following the method described under *Separation and Isolation of Formazan and Tetrazolium.*

Effect of Hydrogen Bonding on Formazan Color—A solution of II in dimethylformamide plus 1 drop of 10% VII developed an intense red color, which was scanned in the visible region *versus* a dimethylformamide blank. Approximately 0.5 ml of alcohol USP was then added, and the solution was rescanned four more times at 5-min intervals. The experiment was repeated using I instead of II.

RESULTS AND DISCUSSION

Table II lists the values for the absorbance of the formazan per micromole of corticosteroid from the reaction of I with typical corticosteroid and corticosteroid esters in 83% methylene chloride and in 83% chloroform. The average value of 1.081 (*SD* 0.121) absorbance units/μmole obtained in 83% methylene chloride compares favorably with that [1.031 (*SD* 0.056)] obtained previously in alcohol USP (14), which indicates that one reduction unit is also transferred in the methylene chloride solvent.

The higher average value for absorbance per micromole obtained in methylene chloride was probably due to the increased speed of the reaction, which caused increased absorbance due to less base degradation of I by VII (14). However, the standard deviation was about twice as great in methylene chloride as in alcohol USP, which indicates that the I–corticosteroid reaction is less precise in the former solvent. The high value of 1.195 for flurandrenolide may have been due to slow hydrolysis of the acetonide group to produce a compound that can reduce I.

The formazan absorption band was more symmetric and its wavelength of maximum absorption was displaced to a higher wavelength (532 *versus* 525 nm) in spectra obtained in methylene chloride as compared to those in alcohol USP. In most cases, maximum absorption values were reached in less than 50 min, and the absorption then declined very slowly in methylene chloride. Since methylene chloride can be used to extract corticosteroids from certain pharmaceutical formulations, this solvent may be used advantageously for the direct determination of selected corticosteroids by I.

In contrast, the average value of 0.787 (*SD* 0.206) absorbance unit/μmole for the reaction of the corticosteroid alcohols and esters listed in Table II with I in 83% chloroform indicates that the amount of formazan produced per micromole of corticosteroid is less than that expected with the exception of hydrocortisone. Furthermore, the visible spectra of the formazans produced exhibited wavelengths of maximum absorption at 525 nm as in alcohol USP. In addition, the absorbance reached a maximum value rapidly and then decreased to a constant value within 40 min or more after initial scanning. During this time, the wavelength of maximum absorption gradually shifted

[5] Alpha Products.
[6] Dajac Laboratories.
[7] E. Merck Co.
[8] Eastman Organic Chemicals.

Table II—Absorbance per Micromole Values for Selected Corticosteroids with I in Chloroform and Methylene Chloride

Corticosteroid	Source[a]	83% Methylene Chloride[b] Minutes to A_{max}	Maximum Absorbance	Absorbance per Micromole	83% Chloroform[b] Minutes to A_{max}	Maximum Absorbance	Absorbance per Micromole
Betamethasone	1	30	0.571	1.113	19	0.370	0.726
Betamethasone 17-benzoate	2	50	0.386	0.958	55[c]	0.124	0.306
Betamethasone 21-benzoate	2	50	0.381	0.946	60[c]	0.196	0.491
Cortisone	3	30	0.582	1.049	3	0.524	0.945
Cortisone acetate	4	35	0.541	1.079	6	0.457	0.913
Corticosterone	5	45	0.629	1.056	32	0.410	0.705
Dexamethasone	1	46	0.542	1.056	6	0.438	0.859
11-Desoxycorticosterone	5	71	0.854	1.417	35[c]	0.360	0.583
11-Desoxycorticosterone acetate	4	69	0.664	1.231	40[c]	0.185	0.341
11-Desoxycortisone	5	17	0.576	1.008	11	0.514	0.887
Fluprednisolone	1	8	0.542	1.025	5	0.465	0.877
Flurandrenolide	6	90[c]	0.553	1.195	14	0.399	0.965
Hydrocortisone	4	90[c]	0.598	1.089	12	0.560	1.012
Hydrocortisone acetate	4	44	0.544	1.104	11	0.439	0.884
Methylprednisolone	1	13	0.539	0.988	7	0.435	0.806
19-Norhydrocortisone	7	90[c]	0.536	0.919	9	0.501	0.846
Prednisolone	4	90[c]	0.601	1.087	5	0.485	0.879
Prednisolone acetate	4	45	0.639	1.268	8	0.432	0.866
Prednisone	4	90[c]	0.568	1.020	3	0.528	0.944
Prednisone acetate	5	9	0.504	1.009	5	0.453	0.909
Triamcinolone	4	90[c]	1.497	2.938[d]	40[c]	1.237	2.425[d]
Average				1.081			0.787
SD[e]				0.121			0.206

[a] 1 = NF reference standard; 2 = Warner Lambert Co., Morris Plains, N.J.; 3 = K & K Laboratories, Plainview, N.Y.; 4 = USP reference standard; 5 = Schwarz/Mann, Orangeburg, N.Y.; 6 = Eli Lilly & Co., Indianapolis, Ind.; and 7 = Syntex Research, Palo Alto, Calif. [b] Also contains 8.3% absolute methanol, 7.1% alcohol, and 1.2% water. [c] Absorbance increases beyond the time shown are minimal. [d] Not included in average. [e] Standard deviation = $\sqrt{d^2/(n-1)}$.

to 532 nm, and the resulting formazan spectrum was similar to that obtained in methylene chloride. The bathochromic shift observed in 83% chloroform was probably due to the partial conversion of the red formazan to the blue formazan.

Although the reaction of corticosteroids with I was generally rapid (3 min required for prednisone and cortisone analysis) in 83% chloroform, the spectral shifts and low sensitivities observed indicate that this solvent is not suitable for quantitation of corticosteroids.

Table III shows rate constants and relative rate comparisons for reaction of I with selected corticosteroids in alcohol, methylene chloride, and chloroform. The median rate of the reaction in the less polar solvents, methylene chloride and chloroform, was 6.1 and 6.7 times as rapid for 19 different corticosteroids as it was in the more polar solvent, alcohol; the median rate in chloroform was about 1.2 times as fast as it was in methylene chloride. Hydrolysis of the ester is a prerequisite to the reaction of corticosteroids with I (14). Table III shows that the rates of formazan formation from corticosteroid esters and the corresponding corticosteroid alcohols were similar in methylene chloride and chloroform, indicating that ester hydrolysis occurs rapidly in these solvents.

The absorbance per micromole values and rate constants for six steroids and steroid esters in seven different solvent systems are reported in Table IV. With the exception of 83% methanol and 62.5% chloroform, the average absorbance per micromole values (1.030–

Table III—Rates and Relative Rate Comparisons for the Reaction of Selected Corticosteroids with I in Various Solvents

Corticosteroid	K_a[a]	K_m[b]	K_c[c]	K_m/K_a	K_c/K_a	K_c/K_m
Betamethasone 21-benzoate	0.017	0.113	0.202	6.6	11.9	1.8
Betamethasone	0.024	0.147	0.351	6.1	14.6	2.4
Flurandrenolide	0.056	0.340	0.375	6.1	6.7	1.1
19-Norhydrocortisone	0.072	0.614	0.625	8.5	8.7	1.0
Hydrocortisone acetate	0.081	0.588	0.550	7.3	6.8	1.0
Prednisolone acetate	0.086	0.535	0.610	6.2	7.1	1.1
Hydrocortisone	0.090	0.672	0.431	7.5	4.8	0.64
11-Desoxycorticosterone acetate	0.090	0.086	0.206	1.0	2.3	2.4
Prednisolone	0.092	1.658	1.105	18.0	12.0	0.67
Triamcinolone	0.093	0.206	0.741	2.2	8.0	3.6
11-Desoxycortisone	0.094	0.442	0.477	4.7	5.1	1.1
Methylprednisolone	0.096	0.615	0.746	6.4	7.8	1.2
Corticosterone	0.098	0.110	0.207	1.1	2.1	1.9
Dexamethasone	0.098	0.668	0.885	6.8	9.0	1.3
11-Desoxycorticosterone	0.109	0.072	0.208	0.7	1.9	2.9
Cortisone acetate	0.230	0.657	0.864	2.9	3.8	1.3
Cortisone	0.243	1.123	1.170	4.6	4.8	1.0
Prednisone	0.305	1.140	1.280	3.7	4.2	1.1
Prednisone acetate	0.307	0.745	1.100	2.4	3.6	1.5
Median				6.1	6.7	1.2
Steroid alcohols, median				6.1	6.7	1.1
Steroid esters, median				6.4	6.8	1.5

[a] K_a is the rate in alcohol USP; these solutions contain 86.3% ethanol, 8.3% methanol, and 5.4% water. [b] K_m is the rate in methylene chloride; these solutions contain 83.3% methylene chloride, 8.3% methanol, 7.1% ethanol, and 1.2% water. [c] K_c is the rate in chloroform; these solutions contain 83.3% chloroform, 8.3% methanol, 7.1% ethanol, and 1.2% water.

Table IV—Comparison of Rates of Reactivity and Absorbance per Micromole for Six Different Corticosteroids Reacted with Blue Tetrazolium in Seven Different Solvent Systems

Corticosteroid	Minutes to A_{max}	Absorbance per Micromole	Ethanol, %	Water, %	Average Absorbance per Micromole	Other Solvents
Dexamethasone	70	1.060	99.6	0.4		
11-Desoxycorticosterone acetate	80	1.141	99.6	0.4		
Hydrocortisone	90	1.065	99.6	0.4	1.073	None
Hydrocortisone acetate	80	1.079	99.6	0.4		
Prednisolone	30	1.056	99.6	0.4		
Prednisone	8	1.038	99.6	0.4		
Dexamethasone	50	1.020	90.2	9.8		
11-Desoxycorticosterone acetate	77	1.061	90.2	9.8		
Hydrocortisone	70	1.033	90.2	9.8	1.030	None
Hydrocortisone acetate	67	1.033	90.2	9.8		
Prednisolone	60	1.030	90.2	9.8		
Prednisone	16	0.999	90.2	9.8		
Dexamethasone[a]	70	1.050	91.7	None[b]		
11-Desoxycorticosterone acetate[a]	90	1.107	91.7	None[b]		
Hydrocortisone[a]	90	1.072	91.7	None[b]	1.070	8.3% methanol
Hydrocortisone acetate[a]	90	1.098	91.7	None[b]		
Prednisolone[a]	90	1.055	91.7	None[b]		
Prednisone[a]	70	1.035	91.7	None[b]		
Dexamethasone	ND[c]	0.163	15.1	1.6		
11-Desoxycorticosterone acetate	ND[c]	0.381	15.1	1.6		83.3% methanol
Hydrocortisone	ND[c]	0.127	15.1	1.6	0.190	
Hydrocortisone acetate	ND[c]	0.095	15.1	1.6		
Prednisolone	ND[c]	0.130	15.1	1.6		
Prednisone	ND[c]	0.242	15.1	1.6		
Dexamethasone	12	1.020	7.5	0.9		
11-Desoxycorticosterone acetate	60	1.046	7.5	0.9		83.3%
Hydrocortisone	13	1.086	7.5	0.9	1.039	methylene
Hydrocortisone acetate	14	1.050	7.5	0.9		chloride
Prednisolone	10	0.973	7.5	0.9		8.3%
Prednisone	11	1.061	7.5	0.9		methanol
Dexamethasone	60	1.004	15.1	1.6		
11-Desoxycorticosterone acetate	90	1.109	15.1	1.6		
Hydrocortisone	85	1.059	15.1	1.6	1.052	83.3%
Hydrocortisone acetate	80	1.087	15.1	1.6		1-propanol
Prednisolone	85	1.058	15.1	1.6		
Prednisone	5	0.992	15.1	1.6		
Dexamethasone	20	0.938	34.8	2.7		
11-Desoxycorticosterone acetate	55	0.292	34.8	2.7		
Hydrocortisone	15	0.959	34.8	2.7	0.830	62.5%
Hydrocortisone acetate	48	0.878	34.8	2.7		chloroform
Prednisolone	29	0.972	34.8	2.7		
Prednisone	5	0.943	34.8	2.7		

[a] Contains 5.0 mg of I. [b] Molar equivalent of sodium ethoxide substituted for VII. [c] Not determined.

1.073) indicate that these reactions go essentially to completion during the time required for analysis. In addition, the rate was particularly rapid (5–30 min) in 83% 1-propanol. The visible absorption spectra in most solvent systems were similar to those obtained in alcohol USP, with a maximum absorption at 525 nm (517 nm for 83% methanol).

The average absorbance per micromole in 62.5% chloroform (0.830) was similar to that determined for 83% chloroform (0.820). If it is assumed that the previously reported value of 1.031 absorbance units/μmole for alcohol USP (14) is correct, it appears that only 80% of the theoretical amount of formazan was formed in these solvent mixtures. The average absorbance per micromole value for the same six steroids in 83% methanol was 0.190 after 90 min, or about 18% of the value reported for alcohol USP. The average absorbance per micromole value had increased to 0.413 after 20 hr, which indicates that the reaction was greatly retarded in methanol. However, the absorbance per micromole value of a hydrocortisone solution in 83% methanol after 90 min was 0.498 when 10% VII was used instead of 1% VII. This change represents an approximate fourfold increase in the rate of reaction.

The influence of the dielectric constant of the medium on the rate of reaction of I with corticosteroids is shown in Fig. 1. This figure is a typical plot of the log of the rate constant for the reaction of hydrocortisone *versus* the dielectric constant for several solvent pairs. Two important relationships are exemplified. First, there is an inverse relationship between the log of the rate constant and the dielectric constant for a particular solvent pair; that is, reaction rates increase as the dielectric constant decreases. Second, rates are faster for those solvent mixtures having more hydrogen-bonding capability in media of the same dielectric constant. For example, reactions proceed faster in 1-propanol–methanol than in methylene chloride–methanol mixtures of the same dielectric constant.

Figure 1 also shows that the inhibition effect of water on the reaction rate of I previously noted (13) may in part be a dielectric constant effect. The very rapid reaction rate observed for corticosteroids with I in 1-propanol appears to be due to the low dielectric constant and the hydrogen-bonding properties of this solvent.

The two relationships obtained from Fig. 1, together with the dependence of the absorbance upon the apparent pH in strongly basic media (13) and the evidence that the reaction of corticosteroids with I is not a radical reaction (14), support a mechanism that involves initial enolization of the α-keto group of corticosteroids and subsequent enolate formation. The collision of the negative enolate ion and the positive tetrazolium ion which controls the reaction rate follows ion formation. Reactions involving oppositely charged ions are known to be favored in low dielectric media (20).

The influence of the solvent on the wavelength of the absorption

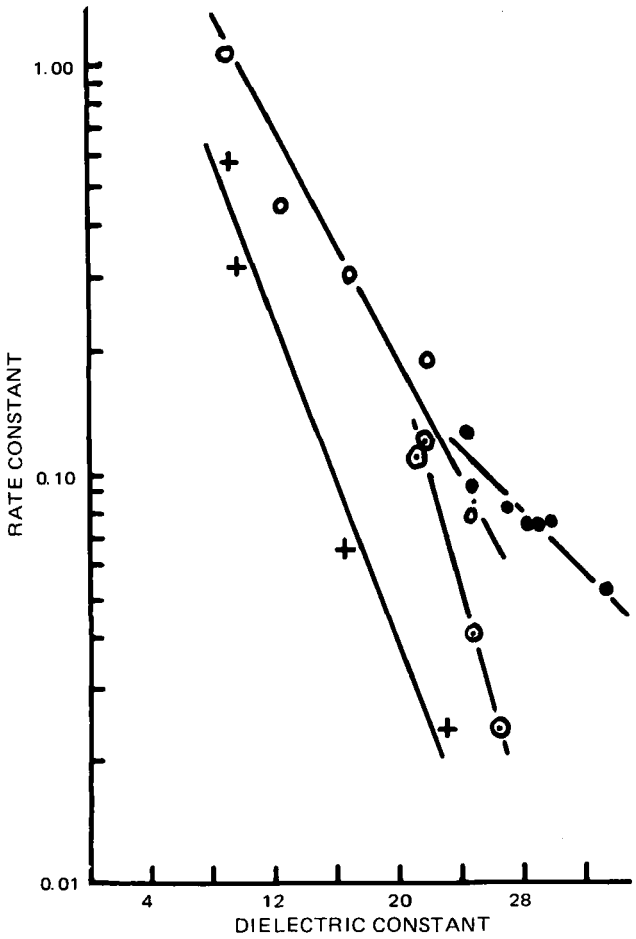


Figure 1—*Variation of rate constant with dielectric constant for the reaction of hydrocortisone with I. Key: ×, mixtures of methylene chloride and methanol; ○, mixtures of methylene chloride and ethanol; ⊙, mixtures of 1-propanol and methanol; and ●, mixtures of ethanol and water.*

maxima of the formazans derived from I and II also was studied. Formazans were prepared in high yield by the reduction of the corresponding tetrazolium with sodium borohydride and were purified by extraction and/or filtration techniques. TLC was used to confirm the absence of the unreacted tetrazolium in the corresponding purified formazan. The reversion of the formazan of II in alcohol USP solution containing 1% VII to II was 94.4% complete in 8 days, and the identity of II so formed was confirmed by UV, IR, and TLC comparison with an authentic sample.

When similarly treated, the formazan of I was also unstable but was converted at a slower rate (36% in 20 days) to a compound that appeared to be a derivative of I. Comparison of this compound with a standard of I showed that the UV curves and R_f values by TLC were similar while the IR spectra were slightly different. These results indicate that under the basic conditions employed in the *General Procedure*, the formazans derived from II and I lose a reduction unit and are oxidized fairly rapidly to II or slowly to a derivative of I.

A similar study of the formazan of II in chloroform (which greatly increases the rate of formazan formation) showed that the rate of reformation of the tetrazolium was much more rapid than in alcohol USP. For this reason, the use of chloroform in the II reaction with corticosteroids would be expected to lead to decreased sensitivity in the measurement of the resultant formazan.

The spectrum of the formazan produced by the corticosteroid reaction with I under the conditions of the *General Procedure* of the official compendia typically exhibit an absorption maximum at 525 nm. TLC analysis of this formazan on silica gel indicates that it is a mixture containing predominantly the red formazan plus a small amount of the blue formazan. The red form, being the more polar of the two, moves with methanol as the mobile solvent while the blue

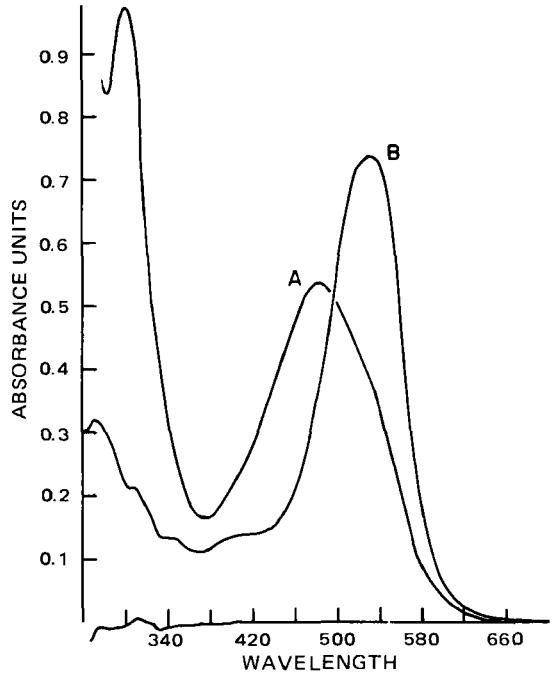


Figure 2—*Spectra of formazans of II. Key: A, spectrum of formazan of II with λ_{max} of 485 nm; and B, spectrum of formazan of II with λ_{max} of 535 nm.*

formazan remains at the point of origin. Conversely, the blue formazan of I moves readily with chloroform as the mobile solvent while the red formazan does not.

Dreiding stereomodels of the possible geometric isomers of the formazans of I indicate that steric interactions are at a minimum in the two *trans*-isomers. Of these, intramolecular hydrogen bonding is possible only in the *trans-syn*-form, which renders this form less polar than the *trans-anti*-form. On this basis, the red and blue formazans of I are assigned the *trans-anti*- and *trans-syn*-configurations, respectively.

The pure red and blue formazans were prepared by the corticosteroid reaction with I in the presence of VII in ethanol and by the action of I plus 1 drop of 10% VII in dimethylformamide. Spectral analysis of the reaction solutions revealed absorption maxima at 517 nm for the red form and 625 nm for the blue form. Furthermore, treatment of I with sodium borohydride in absolute ethanol and treatment of I with 1 drop of 10% VII in 2-propanol yielded the corresponding formazans, which exhibited wavelength maxima of 595 and 580 nm, respectively, indicating that mixtures of the two forms were produced by these reactions. Since the absorbance maximum values are closer to 625 nm than 517 nm, evidently the blue form is formed preferentially under essentially anhydrous conditions.

The addition of either water or methanol to a dimethylformamide solution of the blue formazan of I converts the blue color to purple because some red formazan is produced by reaction of the blue formazan with the added solvent. Thus, water and, to a lesser extent, methanol are important in the stabilization of the red formazan of I. Apparently, the small size of the molecules of water and methanol allows them to form strong intermolecular hydrogen bonds with one or both of the nitrogen atoms of the azo group of the formazan, which prevents the formation of the intramolecular hydrogen bonding exhibited by blue formazan.

Treatment of II dissolved in dimethylformamide with 1 drop of 10% VII produced formazans of intense red color. Immediate spectrophotometric scanning of this solution yielded a nontypical formazan spectrum with an absorption maximum at 535 nm, due to the *trans-syn*-formazan form. Figure 2 shows the effect of the addition of alcohol USP to this solution. Successive scans of the mixture showed that the absorption maximum decreased in absorbance and was shifted to lower wavelengths (from 535 to 485 nm). Also, a new absorption maximum appeared at 300 nm and increased in absorbance with time. Within 20 min, a spectrum was obtained that was similar to that of II formazan obtained in alcohol USP.

REFERENCES

(1) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, p. 622.

(2) "The National Formulary," 14th ed., Mack Publishing Co., Easton, Pa., 1975, p. 976.

(3) W. J. Mader and R. R. Buck, *Anal. Chem.*, **24**, 666(1952).

(4) T. E. Weichselbaum and H. Margraf, *J. Clin. Endocrinol. Metab.*, **15**, 970(1955).

(5) W. Nowaczynski, M. Goldner, and J. Genest, *J. Lab. Clin. Med.*, **45**, 818(1955).

(6) R. O. Rechnagel and M. Litteria, *ibid.*, **48**, 463(1956).

(7) D. E. Guttman, *J. Pharm. Sci.*, **55**, 919(1966).

(8) P. E. Manni and J. E. Sinsheimer, *Anal. Chem.*, **33**, 1900(1961).

(9) J. E. Sinsheimer and E. F. Salim, *ibid.*, **37**, 29(1965).

(10) C. A. Johnson, R. King, and C. Vikers, *Analyst*, **85**, 714(1960).

(11) P. Ascione and C. Fogelin, *J. Pharm. Sci.*, **52**, 709(1963).

(12) J. J. Callahan, F. Litterio, E. Britt, B. D. Rosen, and J. Owens, *ibid.*, **51**, 333(1962).

(13) R. E. Graham and C. T. Kenner, *ibid.*, **62**, 103(1973).

(14) R. E. Graham, E. R. Biehl, C. T. Kenner, G. H. Luttrell, and D. L. Middleton, *ibid.*, **64**, 226(1975).

(15) A. W. Nineham, *Chem. Rev.*, **55**, 355(1955).

(16) R. Kuhn and H. M. Weitz, *Chem. Ber.*, **86**, 1199(1953).

(17) R. J. W. LeFevre, "Dipole Moments," Methusen and Co., Ltd., London, England, 1948, pp. 32, 33.

(18) N. A. Lange, "Handbook of Chemistry," 10th ed., McGraw-Hill, New York, N.Y., 1961, p. 1222.

(19) "International Critical Tables of Numerical Data, Physics, Chemistry and Technology," vol. 6, McGraw-Hill, New York, N.Y., 1929, p. 101.

(20) A. A. Frost and R. G. Pearson, "Kinetics and Mechanisms," Wiley, New York, N.Y., 1961, p. 145.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 25, 1975, from the **Food and Drug Administration, Dallas District, Dallas, TX 75204,* and the *‡Department of Chemistry, Southern Methodist University, Dallas, TX 75275*

Accepted for publication October 10, 1975.

The authors thank Dr. G. H. Luttrell, Jr. (Wyeth Laboratories), for the computer program and for calculation of many rate constants.

[x] To whom inquiries should be directed.

Aggregation of Antihistamines in Aqueous Solution: Effect of Counterions on Self-Association of Pyridine Derivatives

D. ATTWOOD [x] and O. K. UDEALA

Abstract □ The effects of electrolytes on the self-association of the antihistaminic drugs, tripelennamine hydrochloride, thenyldiamine hydrochloride, pyrilamine maleate, pheniramine maleate, chlorpheniramine maleate, and brompheniramine maleate, in aqueous solution were examined by light-scattering methods. The concentration dependence of the light scattering from tripelennamine hydrochloride and thenyldiamine hydrochloride in 0.154 mole of sodium chloride/kg and 0.150 mole of sodium maleate/kg indicated a micellar pattern of aggregation. Higher aggregation numbers and lower CMC's were determined in the presence of the maleate ion. No significant discontinuity in the concentration dependence of the light scattering of the remaining compounds in either of the two electrolytes was evident, and the aggregation of these compounds was treated using a stepwise association model. Values of the association constants and the limiting number of associating species were, in general, increased by the addition of electrolyte in the order water < sodium chloride < sodium maleate. An apparently nonmicellar pattern of aggregation could be induced by chemically changing the counterion from chloride to maleate.

Keyphrases □ Antihistamines—pyridine derivatives, aggregation in aqueous solution, effect of counterions on self-association □ Aggregation—antihistaminic pyridine derivatives, aqueous solution, effect of counterions on self-association □ Counterions—effect on self-association of antihistaminic pyridine derivatives in aqueous solution □ Pyridine derivatives—antihistamines, aggregation in aqueous solution, effect of counterions on self-association □ Electrolytes—effect on self-association of antihistaminic pyridine derivatives in aqueous solution

The self-association in aqueous solution of some antihistamines containing a pyridine nucleus was investigated by light-scattering methods previously (1). No significant discontinuity in the concentration dependence of the light scattering, attributable to a critical micelle concentration (CMC), could be detected. In all cases, however, the scattering intensity exceeded that calculated for the unassociated monomer.

The scattering behavior of three of these compounds, [pyrilamine (mepyramine) maleate, brompheniramine maleate, and chlorpheniramine maleate] could be simulated using a nonmicellar model of association which assumed aggregate growth by stepwise addition of monomers. The intensity of the light scattered by the other compounds studied (pheniramine maleate, tripelennamine hydrochloride, and thenyldiamine chloride) was not of sufficient intensity to establish if association conformed to a micellar or nonmicellar pattern.

Other reports in this series (2–4) concerned antihistamines containing a diphenylmethane nucleus (diphenhydramine hydrochloride, bromodiphenhydramine hydrochloride, chlorcyclizine hydrochloride, and diphenylpyraline hydrochloride). Such compounds have been shown to exhibit typical colloidal behavior in aqueous solution.

This study examined the light scattering of the antihistamines containing a pyridine nucleus in the presence of sodium chloride and sodium maleate. The effect of chemically changing the counterion was investigated for tripelennamine and pyrilamine in an attempt to isolate the cause of their nonmicellar behavior.