sample (as received from a potential supplier) and an instantaneous rerun yield initial *versus* aged data on melting-range shifting after an investment in testing time of <1 hr. Even though the formula ingredients may retard the rate of transition, this test produces a best and worst case melting range to evaluate.

The results of this study in no way reflect on the quality of any base tested or on the ability of any of the manufacturers to supply a range of bases suitable to individual needs.

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Kinetic Study of USP Blue Tetrazolium Assay with Methylprednisolone, Hydrocortisone, and Their Hemisuccinate Esters by High-Pressure Liquid Chromatography

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Abstract □ The reactions of blue tetrazolium (I) with methylprednisolone and hydrocortisone and their hemisuccinate esters at ambient temperature under USP assay conditions in alcohol USP and in absolute alcohol were followed by high-pressure liquid chromatography (HPLC) and spectrophotometry. The disappearance of the ester and the increase and then decrease in the alcohol, as well as the formation of several reaction products with time, were observed by HPLC analysis. The reduction of I was observed spectrophotometrically. A sequential kinetic model was used to describe the overall reaction. The rate constants for the hydrolysis of the hemiester (k_1), the reaction with I (k_2), and the degradation of the parent steroid (k_3) were determined by discrete kinetic experiments using HPLC. The following observations were made: (*a*) k_1 is proportional to $[H_2O]$ and is the rate-limiting step, (*b*) k_2 is about 100 times the value of k_3, and (*c*) k_2 for methylprednisolone is about the same as for hydrocortisone and appears to be independent of the concentration of water. With these rate constants, simulated time–concentration profiles for the reaction of the esters with I favorably compared with experimental data in alcohol USP and absolute alcohol. This study shows that the USP blue tetrazolium assay with these esters has potential for variability and is not stability indicating.

Keyphrases □ Methylprednisolone—and hemisuccinate ester, high-pressure liquid chromatographic and spectrophotometric monitoring of USP assay with blue tetrazolium □ Hydrocortisone—and hemisuccinate ester, high-pressure liquid chromatographic and spectrophotometric monitoring of USP assay with blue tetrazolium □ High-pressure liquid chromatography—kinetic study of USP blue tetrazolium assay with methylprednisolone and hydrocortisone and their hemisuccinate esters

The official USP assay for the corticosteroids, including their esters, is the blue tetrazolium (I) reaction (1). The base-catalyzed reduction of I by the C-17 side chain of corticosteroids is a classical reaction in steroid analysis (2). Meyer and Lindberg (3) established that the α-ketol moiety in corticosteroids is responsible for the reduction of I. However, the reaction mechanism for the C-21 esters of corticosteroids is unknown.

BACKGROUND

Johnson *et al.* (4) reported that color development was unusually slow with hydrocortisone hemisuccinate compared to other steroids. In a study of the reaction of I with cortisone acetate, a C-21 ester, Guttman (5) showed that as the concentration of base catalyst was decreased, the lag time increased and the sigmoid shape of the curve was emphasized. He concluded that hydrolysis of the ester was a prerequisite to a reaction resulting in the generation of formazan. Graham *et al.* (6) also suggested that ester hydrolysis occurs prior to and during formazan development. Their conclusions were derived from graphical kinetic treatment of the spectrophotometric data.

The hydrolysis of the hemiester of corticosteroids in aqueous media has been studied by several investigators. Mauger *et al.* (7) reported that the degradation of its hemisuccinate ester to hydrocortisone was first order. Further degradation to a species devoid of the 17-dihydroxyacetone also was observed. This degradation was followed by separation of the alcohol and ester and subsequent reaction with I. Garrett (8) studied the alkaline hydrolysis of hydrocortisone hemisuccinate by constant pH titration.

High-pressure liquid chromatography (HPLC) has been used recently to study the degradation of hydrocortisone (9). An HPLC method was described (10) that determined simultaneously hydrocortisone (II*a*) and its hemisuccinate ester (III*a*) or methylprednisolone (II*b*) and its hemisuccinate ester (III*b*).

This paper reports a kinetic study of the reaction of I with II and III using this HPLC method. The hydrolysis rates of III to II under compendial blue tetrazolium assay conditions also are reported.

The effect of water in the final reaction mixture of I has been studied. The percentage of water in the official USP method [due to the use of alcohol USP and 10% aqueous tetramethylammonium hydroxide (IV) in the preparation of reagents and samples] is 5.8 (11). Rechnagel and

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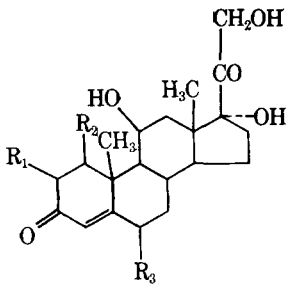
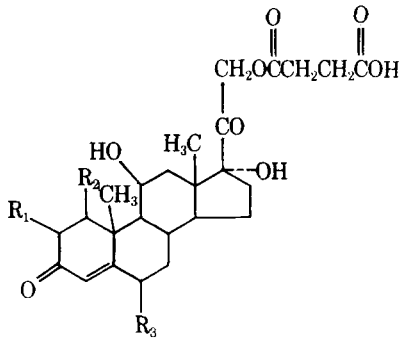


II

IIa: $R_1 = R_2 = R_3 = H$
IIb: $R_1 = R_2$ = missing (unsaturated), $R_3 = CH_3$



III

IIIa: $R_1 = R_2 = R_3 = H$
IIIb: $R_1 = R_2$ = missing (unsaturated), $R_3 = CH_3$

Litteria (12) found that water inhibits the reaction of I and that the extent of inhibition increases as the concentration of water in the final reaction increases. However, Graham and Kenner (11) showed that the effect of water is essentially negligible if it is present at <6%. This paper reports the effect of the water content of the reaction mixture on the rate constants involved in the reaction of I with II and III.

EXPERIMENTAL

Reagents—Acetonitrile[1], acetic acid[2], anhydrous methanol[3], blue tetrazolium[3] (I), and tetramethylammonium hydroxide[3] (IV) were reagent grade. Alcohol and absolute alcohol were USP grade. Compounds IIa, IIIa, and IIIb were USP reference standards. Compound IIb was an NF reference standard.

Apparatus—A high-pressure liquid chromatograph[4] equipped with a 100-μl injector[5], a fixed-wavelength detector[6] (λ = 254 nm) or a variable-wavelength detector[7], a digital integrator[8], a strip-chart recorder[9], and a scanning spectrophotometer[10] were used.

HPLC Conditions—A microparticulate (5-μm) octadecylsilane column[11] was used. The mobile phase was acetonitrile–water–acetic acid (35:65:2) at a flow rate of 1 ml/min.

Sample Preparation—Fifty milligrams of IIIa or IIIb and 39 mg of IIa and IIb were dissolved separately in 100 ml of alcohol (absolute or USP). Then 12 ml of these solutions was diluted to 500 ml with alcohol (absolute or USP) to a final concentration of ~20 μM for each sample.

Reagent Preparation—Compound I, 500 mg, was dissolved in 100 ml of anhydrous methanol. A 10% solution of IV in water was diluted 1:10 with alcohol USP. A 5% acetic acid solution was prepared by diluting acetic acid 1:20 with water.

[1] Glass distilled, Burdick & Jackson.
[2] Mallinckrodt.
[3] J. T. Baker Chemical Co.
[4] Model 3500, Chromatronix, Spectra-Physics, Santa Clara, Calif.
[5] Valco, Houston, Tex.
[6] Model 8200, Spectra-Physics.
[7] Model 970A (λ 242 nm), Tracor, Austin, Tex.
[8] Autolab Minigrator, Spectra-Physics.
[9] Model 7130A, Hewlett-Packard.
[10] Coleman 570, Perkin-Elmer.
[11] Spherisorb 5-μm ODS, Spectra-Physics.

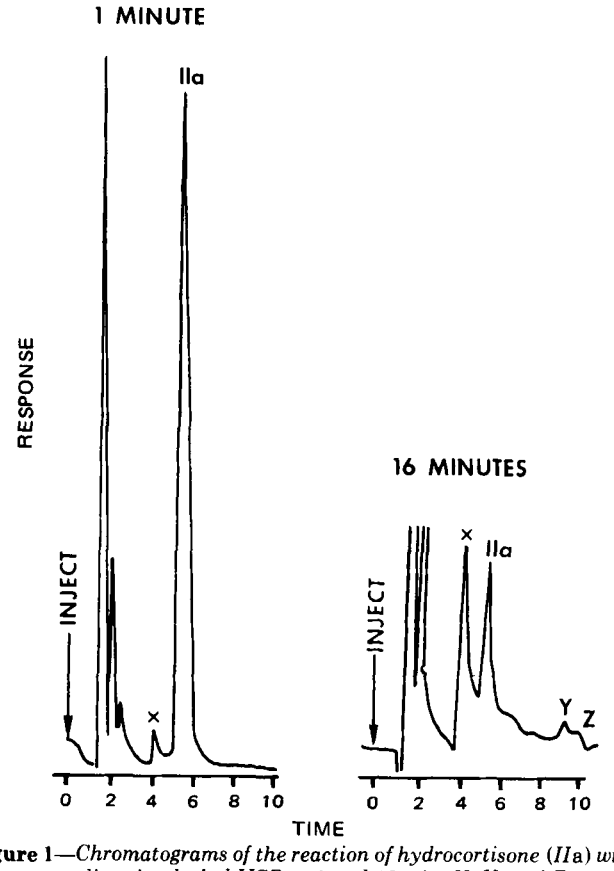


Figure 1—*Chromatograms of the reaction of hydrocortisone (IIa) with blue tetrazolium in alcohol USP at 1 and 16 min; X, Y, and Z are the products of the reaction.*

Kinetic Experiments—*Experiments with I*—Twenty milliliters of the solution of I and 40 ml of diluted IV were added to 200 ml of each sample preparation. The solution was mixed and stored in the dark. At timed intervals, 15 ml of solution was withdrawn and 0.6 ml of acetic acid was added. The absorbance of this solution at 525 nm *versus* a blank preparation was recorded. A portion of the withdrawn solution also was diluted 1:1 with 5% acetic acid and injected onto the HPLC column, and the chromatograms were recorded.

Experiments without I—Twenty milliliters of anhydrous methanol and 40 ml of diluted IV were added to 200 ml of each sample preparation. The solution was mixed. At timed intervals, 15 ml was withdrawn and 0.6 ml of acetic acid was added. This withdrawn solution then was diluted 1:1 with 5% acetic acid and injected onto the HPLC column.

RESULTS AND DISCUSSION

The reactions of I with IIa, IIb, IIIa, and IIIb at ambient temperature under USP assay conditions for III (13) in alcohol USP as well as in absolute alcohol were followed by HPLC and spectrophotometry. The identity and quantitation of each peak were made with respect to standard solutions of IIa, IIb, IIIa, and IIIb.

The percent of water, as determined by the Karl Fischer reaction, was 6.0% in alcohol USP and 0.04% in absolute alcohol. Based on these values, the percent of water in the reaction mixture was calculated to be 6.9% when alcohol USP was used in the sample preparation and 2.3% when absolute alcohol was used.

Chromatograms of these reaction mixtures in alcohol USP at various times are given in Figs. 1–4. Figures 1 and 3 (the reaction of I with IIa and IIb) show a decrease in II with a subsequent increase in peaks X, Y, and Z (possible oxidation products of II) with time. Figures 2 and 4 (the reaction of I with IIIa and IIIb) show a decrease in III with a subsequent increase and then decrease in II and an increase in peaks X and Y with time. These observations suggest that the reaction of the esters of corticosteroids with I is sequential. The intermediate in the reaction sequence is II. Since Gupta (9) showed that IIa is degraded under strongly basic conditions, the decomposition of IIa and IIb under USP assay conditions with alcohol USP and absolute alcohol also was studied.

A kinetic model to describe the overall reaction sequence is given here;

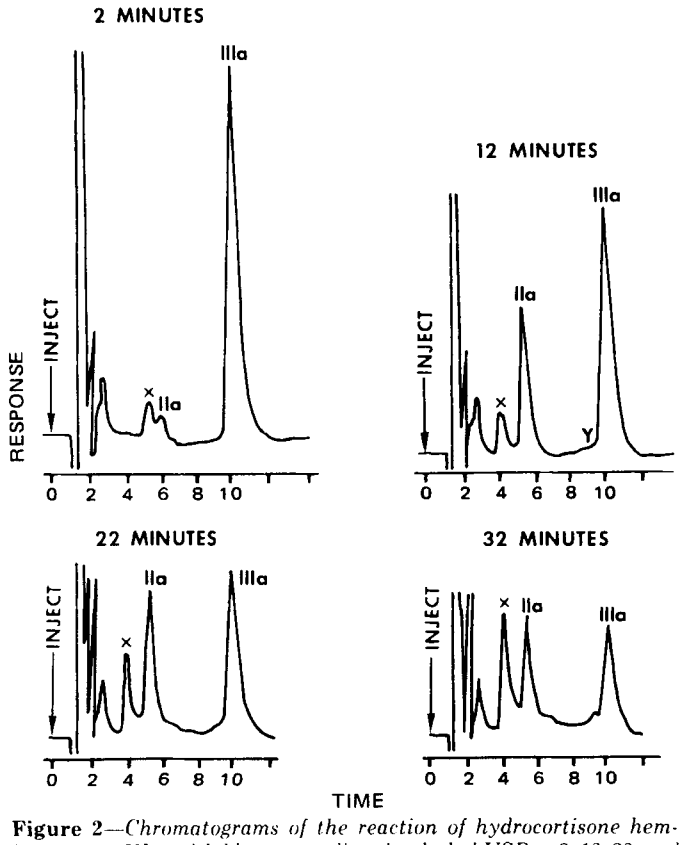


Figure 2—*Chromatograms of the reaction of hydrocortisone hemisuccinate (IIIa) with blue tetrazolium in alcohol USP at 2, 12, 22, and 32 min; X and Z are the reaction products and IIa is hydrocortisone.*

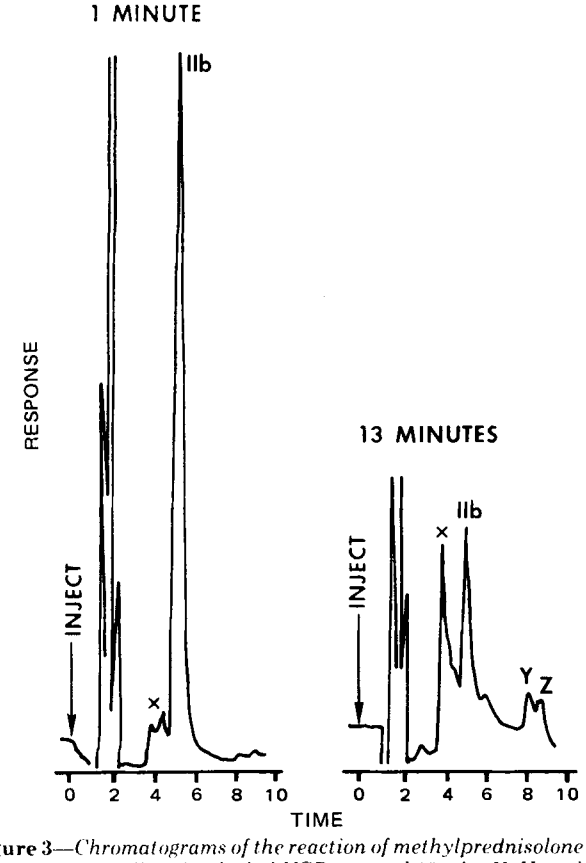


Figure 3—*Chromatograms of the reaction of methylprednisolone (IIb) with blue tetrazolium in alcohol USP at 1 and 13 min; X, Y, and Z are the reaction products.*

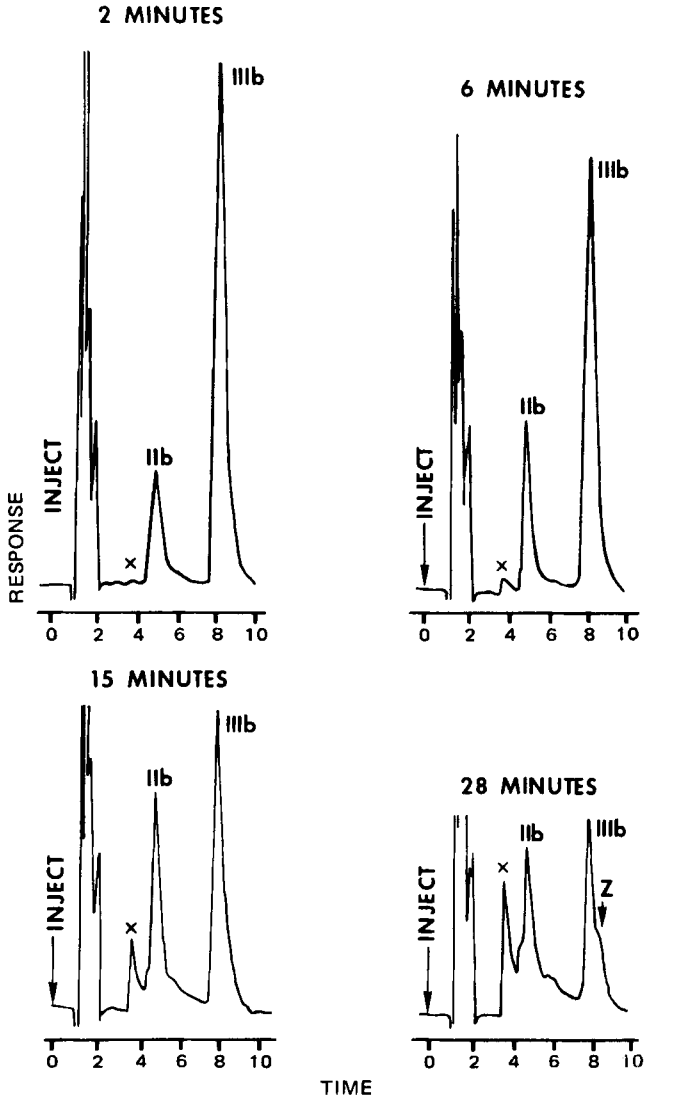


Figure 4—*Chromatograms of the reaction of methylprednisolone hemisuccinate (IIIb) with blue tetrazolium in alcohol USP at 2, 6, 15, and 28 min; X and Z are the reaction products and IIb is methylprednisolone.*

the reformation of I from formazan as postulated by Graham *et al.* (14) is not included. Experimental data of the reaction of II*a* and II*b* with I in 95% alcohol or absolute alcohol suggested that the rate of reformation of I is much slower than the rate of reaction of II with I (Scheme I). Therefore, the loss of formazan (within 100 min of reaction time) would be negligible.

$$\text{III} \xrightarrow[[OH^-]]{k_1} \text{II} \xrightarrow[[I]]{k_2} C + P$$
$$\text{II} \xrightarrow[[OH^-]]{k_3} D$$

Scheme I

P is reduced I; *C* and *D* are decomposition products of II; $[OH^-] = [IV]$, and k_1, k_2, and k_3 are pseudo-first-order rate-constants.

The disappearance of III and the appearance of II and *P* can be described by:

$$\frac{d\text{III}}{dt} = -k_1\text{III} \qquad \text{(Eq. 1)}$$

$$\frac{d\text{II}}{dt} = k_1\text{III} - (k_2 + k_3)\text{II} \qquad \text{(Eq. 2)}$$

$$\frac{dP}{dt} = k_2\text{II} \qquad \text{(Eq. 3)}$$

Table I—Rate Constants of the Blue Tetrazolium–Corticosteroid Reaction

Corticosteroid and Its Ester	Rate Constant, min^{-1}	
	Alcohol USP	Absolute Alcohol
IIa + IIIa		
k_1	0.036	0.014
k_2	0.10	0.10
k_3	0.0015	0.0007
IIb + IIIb		
k_1	0.041	0.018
k_2	0.12	0.13
k_3	0.0021	0.0018

Solutions for these differential equations are:

$$III_t = III_0 e^{-k_1 t} \quad \text{(Eq. 4)}$$

$$II_t = \frac{III_0 k_1}{(k_2 + k_3) - k_1}\left[e^{-k_1 t} - e^{-(k_2+k_3)t}\right] \quad \text{(Eq. 5)}$$

$$P_t = \frac{III_0 k_1 k_2}{k_1 - (k_2 + k_3)}\left[\frac{e^{-k_1 t}}{k_1} - \frac{e^{-(k_2+k_3)t}}{k_2 + k_3}\right] \quad \text{(Eq. 6)}$$

The rate constants k_1, k_2, and k_3 were determined experimentally by following each discrete reaction under USP conditions using either alcohol USP or absolute alcohol as the solvent. The hydrolysis of III, the decomposition of II in IV, and the reaction of II with I were followed independently by HPLC. The results of these kinetic experiments are given in Table I.

These rate constants were used to generate time–concentration profiles for II, III, and *P* to simulate the reaction of III with I under specified solvent conditions. Normalized experimental data obtained by HPLC for the reaction of IIIa and IIIb with I were superimposed on these generated time–concentration profiles (Figs. 5–8). The colorimetric data (*P*) were normalized by dividing each observed absorbance by the absorbance after the reaction was complete (>3 hr). For each ester of II in alcohol USP and absolute alcohol, the predicted concentrations of II, III, and *P* closely agreed with the experimental values. Some estimation of the experimental data (III) occurred since the decomposition products of II (Y and Z) were not totally resolved from III. These observations of the reaction of the hemiester with I corroborate conclusions (5, 6) that hydrolysis of the ester is a prerequisite to the reaction with I.

The following observations can be made about the rate constants for each pseudo-first-order reaction:

1. The rate of decomposition of II in a base (k_3) is ~1% of the rate of reaction with I (k_2) and can be ignored.
2. The rate constants (k_2) under USP assay conditions are about the same for IIa and IIb and are in agreement with the results reported by Graham *et al.* (15) (*i.e.*, k_2 [IIa] = 0.090 and k_2 [IIb] = 0.096).
3. In the ester reaction sequence, ester hydrolysis is the rate-determining step.

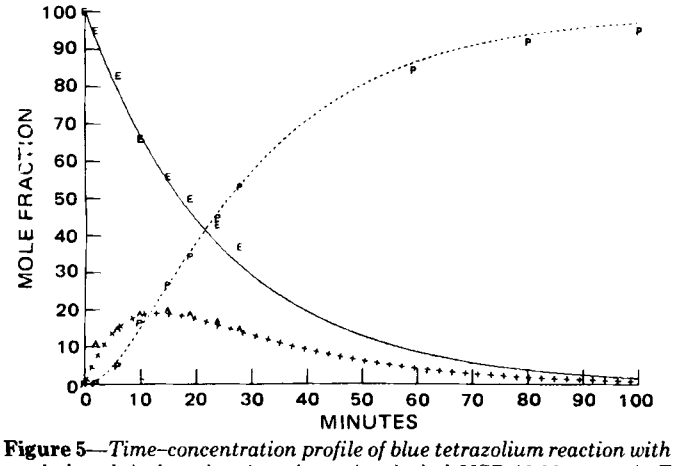


Figure 5—*Time–concentration profile of blue tetrazolium reaction with methylprednisolone hemisuccinate in alcohol USP (6.9% water); E represents the normalized data for methylprednisolone hemisuccinate (IIIb), A represents the normalized data for methylprednisolone (IIb), P represents the normalized data for reduced blue tetrazolium (formazan), and —, +, and . . . are computer-generated curves for E, A, and P, respectively, using rate constants determined independently.*

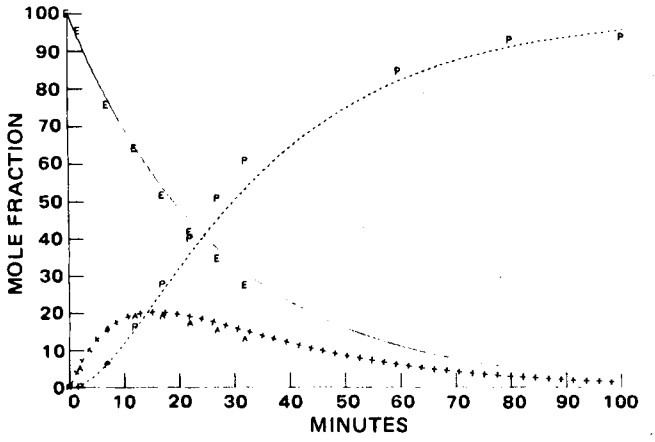


Figure 6—*Time–concentration profile of blue tetrazolium reaction with hydrocortisone hemisuccinate in alcohol USP (6.9% water); E represents the normalized data for hydrocortisone hemisuccinate (IIIa), A represents the normalized data for hydrocortisone (IIa), P represents the normalized data for reduced blue tetrazolium (formazan), and —, +, and . . . are computer-generated curves for E, A, and P, respectively, using rate constants determined independently.*

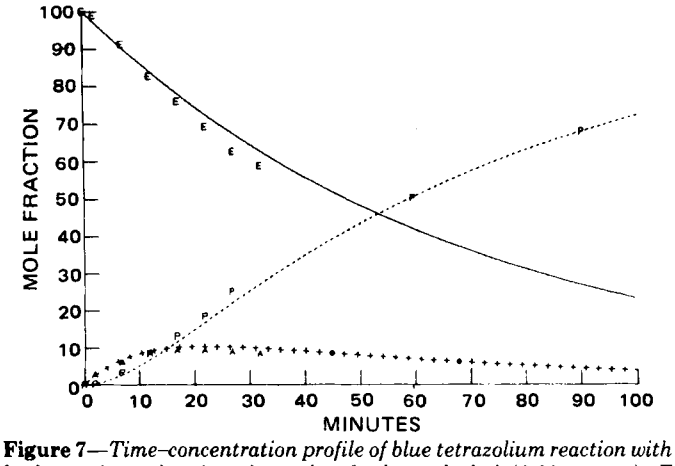


Figure 7—*Time–concentration profile of blue tetrazolium reaction with hydrocortisone hemisuccinate in absolute alcohol (2.3% water); E represents the normalized data for hydrocortisone hemisuccinate (IIIa), A represents the normalized data for hydrocortisone (IIa), P represents the normalized data for reduced blue tetrazolium (formazan), and —, +, and . . . are computer-generated curves for E, A, and P, respectively, using rate constants determined independently.*

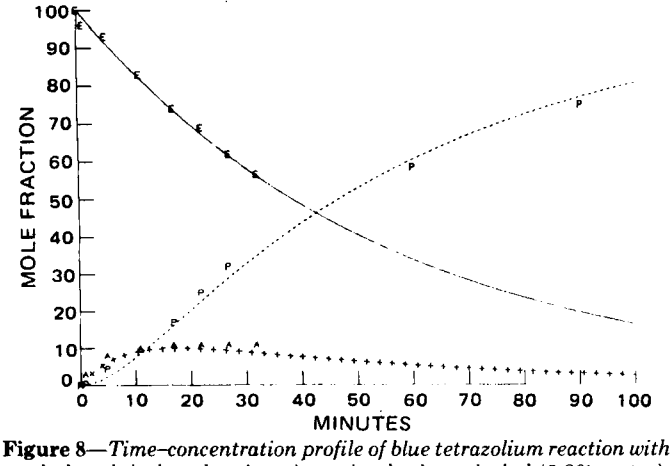


Figure 8—*Time–concentration profile of blue tetrazolium reaction with methylprednisolone hemisuccinate in absolute alcohol (2.3% water); E represents the normalized data for methylprednisolone hemisuccinate (IIIb), A represents the normalized data for methylprednisolone (IIb), P represents the normalized data for reduced blue tetrazolium (formazan), and —, +, and . . . are computer-generated curves for E, A, and P, respectively, using rate constants determined independently.*

4. The water content of the reaction mixture has little effect on the rate of reaction of the corticosteroid (II) with I within the range studied.

5. The hydrolysis rate of the ester (III → II) and subsequently the overall reaction rate appear to be proportional to the water content of the reaction mixture.

These kinetic results imply that the USP blue tetrazolium assay for these corticosteroid hemisuccinate esters has a high variability potential since it is a timed measurement (90 min). These results show that III does not react directly with I and that II must be formed. Therefore, the USP blue tetrazolium assay cannot differentiate between the corticosteroid (II) and its ester (III) and thus is not a stability-indicating assay of III. In fact, if hydrolysis of the ester of these corticosteroids occurred, the results might show an increase in corticosteroid since the reaction rate of the corticosteroid is about three times as fast as the hemiester in alcohol USP.

CONCLUSIONS

1. The reaction of corticosteroid esters (III) with I is sequential. The ester is hydrolyzed first, and the resulting corticosteroid (II) reacts with I. The hydrolysis step is the rate-determining step.

2. The USP blue tetrazolium assay is not stability indicating for the esters of these corticosteroids.

3. As the percent of water in the reaction mixture decreases, the time for complete reaction increases for the USP blue tetrazolium assay of C-21 hemisuccinate esters of corticosteroids.

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New Evaluation of Potential Methylmercury Scavengers

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Received November 5, 1979, from the **Department of Biochemistry and the* ‡*Department of Biophysics, Indiana University School of Medicine*, and the §*Veterans Administration Medical Center, Indianapolis, IN 46202.* Accepted for publication March 24, 1980.

Abstract □ A biological assay was developed to evaluate rapidly the relative efficacy of marketed and experimental mercurial scavengers. Rat liver mitochondrial protein (1.0 mg) was titrated against methylmercuric chloride to the inhibitory level of mitochondrial respiration. Respiration induced by adenosine 5′-diphosphate with succinate (plus rotenone) as the substrate was inhibited consistently by 20.7 ± 3.9 nmoles of methylmercury/mg of protein. Adenosine 5′-diphosphate-stimulated respiration (State 3) was restored with dimercaprol, penicillamine, and cysteine but not with serine. The antagonists glutathione, 3-mercaptopropionic acid, 2-mercaptoethanol, dithiothreitol, thioglucose, mercaptosuccinic acid, and thiosalicylic acid were effective. Glutathione was significantly superior to thiosalicylic acid and mercaptosuccinic acid. Sodium sulfide, thioacetamide, and ethylenediaminetetraacetic acid were completely inactive. Substitution of glutamate (plus malate) for succinate (plus rotenone) as the substrate did not alter the responses significantly. The rat liver mitochondrial assay provides preliminary information about the efficacy and toxicity of water-soluble thiols. Investigations utilizing encapsulated water- and lipid-soluble mercaptans are in progress.

Keyphrases □ Mercurial scavengers—thiol-containing compounds, evaluation of efficacy and toxicity using rat liver mitochondrial assay □ Mercaptans—evaluation of efficacy and toxicity as mercurial scavengers using rat liver mitochondrial assay

Agents currently used to alleviate clinical symptoms and to facilitate the rapid removal of methylmercury include chelators such as dimercaprol, penicillamine, and ethylenediaminetetraacetic acid (1–3). Unfortunately, the effectiveness of these antidotes is limited by their toxicity, chemical instability, and relatively short circulation time. Polythiol resins, thioacetylated steroids, mercaptosteroids, and lipid encapsulation of ethylenediaminetetraacetic acid have been proposed to improve on these mercurial scavengers (4–9).

BACKGROUND

Reports on the analysis of subcellular distribution of labeled mercury indicate significant accumulation of mercury by mitochondria (10). Furthermore, dose and time dependence of the mercurial in relation to the extent of damage incurred by isolated mitochondria has been documented (11–13). In addition, the technique for isolation of viable rat liver mitochondria is well known.

Early reports demonstrating reversal of mercurial inhibition by mercaptans dealt with isolated enzymes (14, 15). Thompson and Whittaker (14) showed significant protection of pigeon brain pyruvate oxidase activity against mercuric chloride inhibition by the addition of dimercaprol and glutathione. Barron and Kalnitsky (15) demonstrated 75% reactivation of mercuric chloride-inhibited succinoxidase by the addition of various dithiols. Sone *et al.* (11) recently studied the effects of methylmercuric chloride on mitochondrial respiration and found nearly complete restoration of succinate oxidation with thiol-containing reagents such as 2-mercaptoethanol, glutathione, cysteine, and dithiothreitol. The concentration required for reversal was not given. Less than 10% recovery of nicotinamide-adenine dinucleotide oxidation was restored by these reagents (11).

In summary, direct effects of mercurials on rat liver mitochondria have been demonstrated (10–13). Furthermore, reactivation of mercurial-inhibited enzymes by thiols has been shown (14, 15). In addition, relief of mercurial intoxication in mitochondria by thiols has been shown but has not been quantified (15). This report describes the use of mito-

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