of freshly distilled acetic anhydride and allowed to stand in the dark for 2 days. The volatile products were then removed under vacuum, and the last traces of acetic acid were removed by azeotropic distillation with xylene. The remaining oil was distilled under vacuum, b.p. 133–135°/0.5 mm., yield 40%. (See Table II for physical constants, yields, and analyses.)

Partition Coefficients.—The partition coefficients of both the 3-alkylsydnones and the 3,4-dialkylsydnones were obtained between water saturated *n*-amyl alcohol and *n*-amyl alcohol saturated water. Several concentrations of each compound in water saturated *n*-amyl alcohol were equilibrated at 23° with varying quantities of *n*-amyl alcohol saturated water. The concentration in the *n*-amyl alcohol before and after equilibration with the aqueous phase was determined from the ultraviolet absorption at the maximum. An average of at least four determinations for each compound was used. (See Table III).

Determination of CD_{50} Values.—The same procedure previously referred to (5) was used to determine the CD_{50} values. The CD_{50} values for the 3-alkylsydnones were reported in a previous communication (2). (See Table III). The partition coefficient *versus* CD_{50} values are graphically portrayed in Fig. 1.

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Color Reactions of Veratrum Alkaloids with Sulfuric Acid and Sulfuric Acid Reagents

By HORACE D. GRAHAM†

Veratrum alkaloids, when treated with concentrated sulfuric acid, or "sulfuric acid reagents" give red, purple, yellow, or violet with characteristic absorption maxima. This reaction can be used as the basis of a simple, rapid colorimetric method for the determination of isolated samples of this class of alkaloids. The final concentration of sulfuric acid is very critical for maximum color development and, since color intensity is decreased if water is present to the extent of greater than 1 per cent, an anhydrous medium is recommended for maximum development. The typical color is best obtained when ethyl alcohol or methyl alcohol is used as the solvent for the alkaloids. Except for hydrochloric acid, no color is obtained with acids other than sulfuric. After heating for several minutes in hydrochloric acid, some color developed, but the intensity was always much less than that produced after treatment of the same amount of alkaloid with sulfuric acid. Inorganic salts, sodium benzoate, and glucose, above certain limiting concentrations, will interfere with color development.

CONSIDERABLE INTEREST has developed in the veratrum alkaloids because of their potent hypotensive action. This was forcibly emphasized by Kupchan (1), who recently reviewed the hypotensive veratrum ester alkaloids, paying particular attention to the relationship between structure and hypotensive activity. However, despite their growing importance in medicine and pharmacology, simple, rapid methods for the quantitative assay of veratrum alkaloids are still lacking. As far as can be ascertained, present methods of detection and assay involve mainly biological means such as the pigeon emetic response or intricate physical, chemical, or other methods (2–18). Dadlez, *et al.* (19), reported on the color reactions of alkaloids, including veratrine, with sulfuric acid and sulfuric acid in combination with several other reagents such as furfural, *p*-dimethylaminobenzaldehyde, and vanillin. Moraes and Palma (20) employed both concentrated nitric acid and concentrated sulfuric acid in studying the color reactions of several alkaloids including veratrine, following separation of the alkaloids by paper chromatography. Mandelin reagent (concentrated sulfuric acid plus formalin) and Fröehde reagent (concentrated sulfuric acid plus ammonium molybdate) were also used.

Reference has been made (21) to the red, purple, or violet developed by certain veratrum alkaloids on treatment with concentrated sulfuric acid. Since alkaloids of many other classes do not give these colors, a study of the reaction was undertaken to delineate those factors which

Received July 19, 1962, from the George Washington Carver Foundation, Tuskegee Institute, Ala.
Accepted for publication June 27, 1963.
The author extends his thanks to Linnie B. Thomas, Carolyn Sue Kemper, and Patricia A. Smith for technical assistance; thanks are also extended to Dr. John E. Campion, Riker Laboratories, Northridge, Calif., to Dr. Otto Krayer, Department of Pharmacology, Harvard Medical School, Boston, Mass., and to Dr. O. Winstersteiner, Squibb Institute for Medical Research, New Brunswick, N. J., for samples of several of the alkaloids used.
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TABLE I.—COLORS PRODUCED BY VERATRUM ALKALOIDS AND OTHER ALKALOIDS WITH SULFURIC ACID AND SULFURIC ACID REAGENTS

Alkaloid Tested	Reagent No.[a,b] 1	2	3	4	5
Veratrine sulfate	R	P	P	R	R
Veriloid powder	Pk-Y	P	P	R-Br	Pk-O
Total alkaloids of *Veratrum viride*	Pk-Y	Br-Y	Br-Y	R-Br	Pk-O
Jervine	Y	Y	Y-Br	G-Y	Y
Protoveratrines A and B	P-Pk	P	P	R	Pk
Protoveratrine A	Pk-P	P	P	P	Pk
Protoveratrine B	P	P	Pk-P	P	R
Cevadine	R	P	P	R	R
Veratridine	R	P	P	R	R
Veratrine alkaloid	R	P	P	R	R
Alpha veratrine	R	P	P	R	R
Veratramine	Y	Y-Br	G-Y	R-Br	Y-G
Veratric acid	NC	NC	NC	Y	Y
Tomatine	Y	Br	P	Br	G-Br
Tomatidine	Y	G-Br	P	R	G-Y
Solanine	Y	Y-G	Br	O-Br	G-Y
Conessine	Y	Y	Y	Y	G-Y
Colchicine	Y	Y	Y	Y	G-Y
Sitosterol acetate	Y	P-Br	P-Br	O-Br	G-Y
Stigmasterol	O	P-Br	P-Br	O-Br	Br
Cholesterol	O-Y	Br	R-Br	O-Br	O-Br
Ergosterol	O-Br	Br	P-Br	Br	O-Br
Digitonin	Y	Br	Y	O-Br	O-Br
Reagent blank	NC	NC	NC	NC	Y

[a] *1*, Concentrated H_2SO_4; *2*, concentrated H_2SO_4 + $FeCl_3$; *3*, concentrated H_2SO_4 + $FeNH_4(SO_4)_2 \cdot 12H_2O$; *4*, concentrated H_2SO_4 + *p*-dimethylaminobenzaldehyde; *5*, hydroquinone + H_2SO_4. [b] R = Red; Pk = pink; Y = yellow; B = blue; Br = brown; O = orange; G = green; P = purple; NC = no color.

influence the reaction and, specifically, to exploit it for rapid quantitative determination of these alkaloids.

Sulfuric acid alone or in combination with other inorganic or organic reagents has been used frequently for the colorimetric determination of many important alkaloids and steroids (22–24). A preliminary survey showed that, in addition to the colors produced in concentrated sulfuric acid, the veratrum alkaloids gave pronounced colors with some of the reagents reported by Jakovljevic (25) in his method for vinblastine. This communication summarizes the results of experiments on the many factors which may influence the reaction between the veratrum alkaloids and concentrated sulfuric acid. Among those investigated were the final acid concentration necessary for maximum color development, the influence of the presence of water, added salts and other excipients in the reaction medium, color stability, the influence of various solvents for the alkaloids, and the substitution of other acids for sulfuric acid. Color development with various sulfuric acid reagents was also studied.

EXPERIMENTAL

Materials.—The alkaloids are listed in Table I. Sulfuric acid, reagent grade—95–98%, sp .gr. 1.8407 to 1.8437; absolute ethyl alcohol and methyl alcohol; vanillin U.S.P.; ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; ferric chloride, $FeCl_3 \cdot 6H_2O$; *p*-dimethylaminobenzaldehyde, m.p. 74–75°; ferric ammonium sulfate, $FeNH_4(SO_4)_2 \cdot 12\ H_2O$; and inorganic salts, organic salts, and other chemicals (see Table II) were employed. All of U.S.P. or C.P. grade obtained from commercial sources and used without further purification.

The sulfuric acid–ferric chloride reagent was prepared according to the directions given by Zak, *et al.* (23). The ferric ammonium sulfate

TABLE II.—INFLUENCE OF ADDED SALTS, GLUCOSE, AND OTHER COMPOUNDS ON THE COLOR REACTION BETWEEN VERATRINE SULFATE AND SULFURIC ACID

Salt Added	Limiting Concn. (*M*)
(Monovalent)	
Sodium chloride	5×10^{-4}
Sodium biphosphate	5×10^{-4}
Ammonium chloride	1×10^{-4}
Ammonium carbonate	1×10^{-4}
Sodium carbonate	1×10^{-4}
Potassium chloride	2×10^{-4}
Lithium chloride	2×10^{-4}
(Divalent)	
Barium chloride	2×10^{-4}
Magnesium chloride	2×10^{-4}
Magnesium sulfate	2×10^{-4}
Mercuric chloride	2×10^{-4}
(Trivalent)	
Ferric chloride	1×10^{-6}
Aluminum chloride	0.6×10^{-6}
(Organic Salts and Other Compounds)	
Sodium benzoate	3×10^{-4}
Sodium acetate	5×10^{-4}
Hexamethonium chloride	0.5 mg.
Promazine hydrochloride	0.01 mg.
Promethazine	0.01 mg.
Potassium acid phthalate	2×10^{-4}
Glucose	0.01 mg.

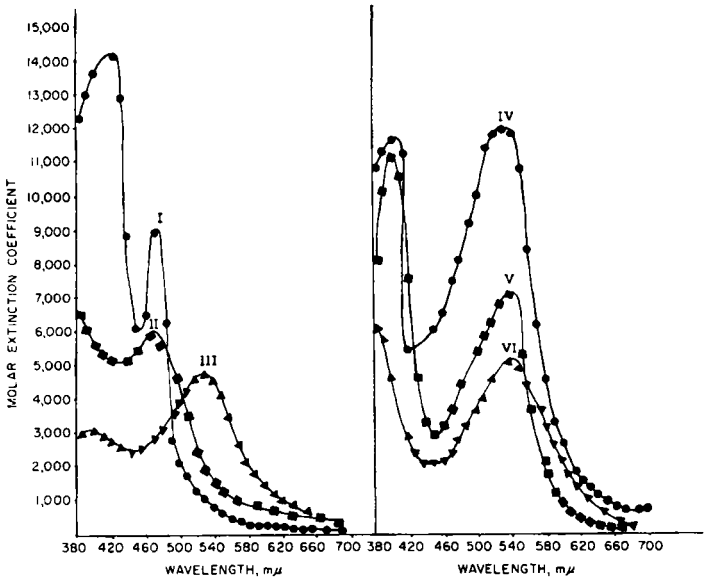

Fig. 1.—Absorption spectra of veratrum alkaloids in concentrated sulfuric acid. Key: I, veratramine; II, jervine; III, protoveratrine A; IV, cevadine; V, veratridine; VI, protoveratrine B.

solution was prepared in a manner similar to the ferric chloride reagent. The vanillin and *p*-dimethylaminobenzaldehyde reagents were prepared as 1% (w/v) solutions of the respective compounds in concentrated sulfuric acid.

Equipment.—Coleman model 14 universal spectrophotometer, 30-ml. Pyrex glass-stoppered test tubes, vacuum oven, and 50-ml. acid buret were utilized.

General Procedure for Screening the Color Reactions of the Various Alkaloids with Sulfuric Acid and Sulfuric Acid Reagents.—For routine screening, the alkaloids were dissolved in absolute methyl alcohol or glacial acetic acid. One milliliter of each stock solution containing 500 mcg. of the particular alkaloid was placed in a glass-stoppered Pyrex test tube. At the end of this period, 9 ml. of concentrated sulfuric acid or sulfuric acid reagent was added and the tubes allowed to stand at room temperature. The color developed was noted immediately after the addition of the sulfuric acid, after 30 and 60 minutes, and again after 24 hours. The results are summarized in Table I.

Determination of Wavelengths of Maximum Absorption of the Colors Developed with the Various Reagents and the Molar Extinction Coefficients or Some of the Veratrum Alkaloids.—One milliliter of a 10^{-3}–10^{-5} *M* solution of the particular alkaloid was placed in a ground-glass-stoppered test tube, and 9 ml. of the sulfuric acid or sulfuric acid reagent was added. The tubes were shaken to mix the ingredients thoroughly and allowed to stand at room temperature for 60 minutes. The wavelength of maximum absorption was then determined by measuring the optical density of the solutions over the wavelength range of 380–700 mμ and plotting the optical density as a function of the wavelength. Molar extinction coefficients were calculated for six of the alkaloids used; the plots

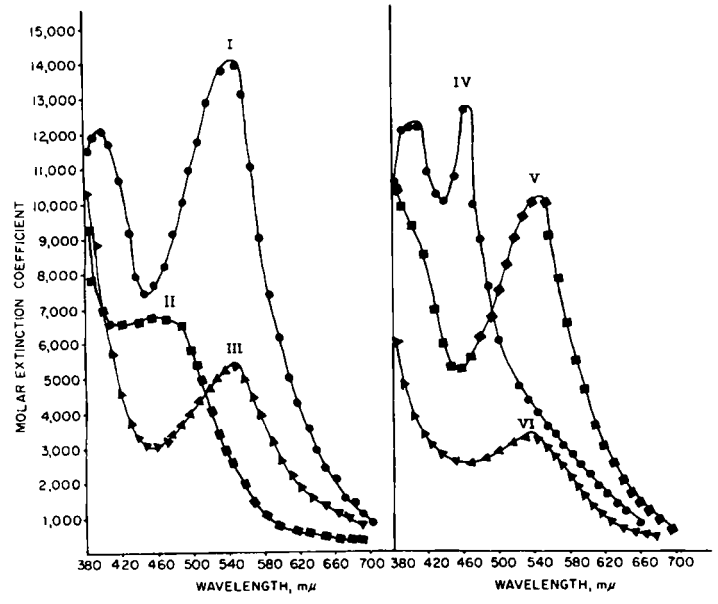

Fig. 2.—Absorption spectra of veratrum alkaloids in concentrated sulfuric acid plus ferric chloride. Key: I, cevadine; II, jervine; III, protoveratrine A; IV, veratramine; V, veratridine; VI, protoveratrine B.

TABLE III.—ABSORPTION CHARACTERISTICS OF VERATRUM ALKALOIDS IN CONCENTRATED SULFURIC ACID AND CONCENTRATED SULFURIC ACID PLUS FERRIC CHLORIDE

Alkaloid	In H_2SO_4		In H_2SO_4-$FeCl_3$	
	λ Max.	Mol. Ext. Coeff.	λ Max.	Mol. Ext. Coeff.
Veratrine sulfate	390	...	380	...
	540	...	550	...
Veriloid powder	410	...	400	...
Total alkaloids of *Veratrum viride*	390	...	380	...
Veratrine	400	...	400	...
	540	...	...	...
Jervine	470	5.95×10^3	420–480	6.7×10^3
Veratramine	410	16.76×10^3	410	13.01×10^3
	470	8.93×10^3	460	12.84×10^3
Protoveratrine A	390	3.20×10^3	530	3.37×10^3
	530–540	4.60×10^3	...	...
Protoveratrine B	540	5.10×10^3	550	5.30×10^3
Cevadine	400	11.70×10^3	400	12.20×10^3
	520–530	11.90×10^3	540	13.98×10^3
Veratridine	400	11.3×10^3	540–550	10.01×10^3
	540	7.00×10^3	...	...

of molar extinction coefficients *versus* wavelength are shown in Figs. 1 and 2.

Data for Figs. 1 and 2 were obtained from measurements against reagent blanks. Data for the other alkaloids listed in Table III were obtained from measurements against distilled water.

Influence of Variables on the Reaction of Veratrum Alkaloids with Sulfuric Acid.—For all experiments on the influence of variables on the reaction, veratrine sulfate only was used. A constant quantity (500 mcg.) of veratrine sulfate was treated with sulfuric acid according to the general procedure, except that the variables were tested over the following ranges: acid normality (3.6 to 32.4*N*), final concentration of water (0.0 to 5.0%), and time for color development (0.0 minutes to 72 hours).

Acids substituted for sulfuric acid were: concentrated hydrochloric, glacial acetic, phosphoric (85%), formic, and lactic acids. The intensity of the color developed was measured after 60 minutes at 540 mμ against a reagent blank.

Influence of Added Salts, Glucose, and Other Compounds on the Color Reaction of Veratrine Sulfate with Sulfuric Acid.—The influence of added salts was determined by adding varying amounts of the particular salt to a tube containing a constant amount (500 mcg.) of veratrine sulfate and developing and measuring the optical density of the resulting color after 60 minutes. This influence is considered of importance since as noted in the "Merck Index" (26) the alkaloids of *Veratrum viride* are usually administered after dilution with saline. Since dilution with 5% glucose may also be done prior to administration (26), the influence of this sugar on color development was also assessed. The severity of interference from other compounds such as promazine hydrochloride, sodium benzoate, etc., was also determined.

The term "limiting concentration" is used here to mean the maximum concentration of added salt or other compound which will cause a deviation in optical density of the color developed of not more than 1% of the color density given by the control system, *i.e.*, by 500 mcg. of veratrine sulfate in 1 ml. of absolute methanol and 9 ml. of concentrated sulfuric acid. The results are summarized in Table II.

Quantitative Response of Alkaloids in Sulfuric Acid.—After the influence of the several variables

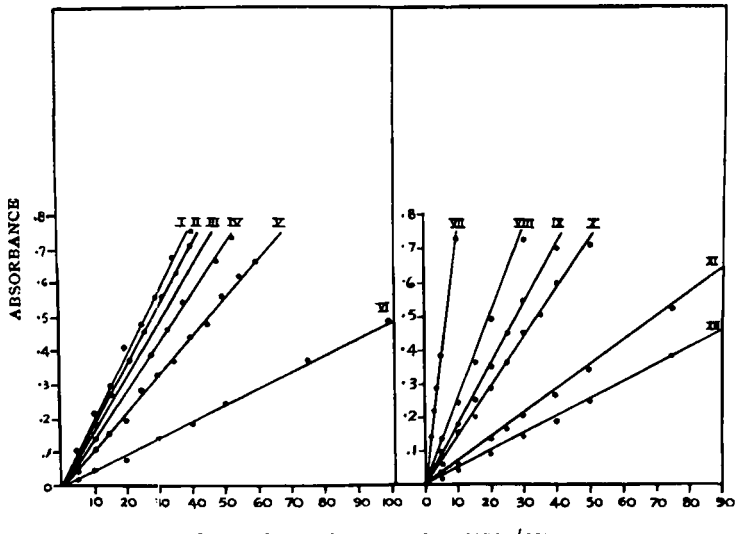

Fig. 3.—Quantitative color response of veratrum alkaloids when treated with concentrated sulfuric acid. Key: I, veratridine[a]; II, veratrine alkaloid[b]; III, jervine[d]; IV, veriloid[c]; V, veratrine sulfate[a]; VI, protoveratrines A and B[a]; VII, veratramine[c]; VIII, α-veratrine[a]; IX, cevadine[b]; X, total alkaloids of *Veratrum viride*[e]; XI, protoveratrine A[b]; XII, protoveratrine B[a]. Letters indicate wavelengths at which measurements of color intensity were made. Key: a, 540 mμ; b, 530 mμ; c, 410 mμ; d, 470 mμ; e, 390 mμ.

was delineated, it was decided to test the quantitative response of the alkaloids in sulfuric acid. For this, 0.0 to 1.0 ml. of a stock solution containing varying amounts of the alkaloids was added to consecutive ground glass-stoppered test tubes. Where necessary, the volume was made up to 1 ml. with absolute methanol. Nine milliliters of concentrated sulfuric acid was added to each test tube and, after standing at room temperature (30°) for 60 minutes, the colors developed were measured at the appropriate wavelengths of maximum absorption. The results are summarized in Fig. 3.

DISCUSSION

In the proposed method, the final concentration of sulfuric acid is very critical for maximum color development. As the acid concentration increased, the density of the color also increased, but over the range tested (3.6 to 32.4 *N*) , no constancy in the density of the color developed was observed. Therefore, it is recommended that the highest final normality of acid (32.4 *N*) be used. Several other acids were substituted for sulfuric acid but, except for concentrated hydrochloric acid, no color development occurred. On heating with concentrated hydrochloric acid, veratrine sulfate gave some color. Water, if present to the extent of greater than 1% of the total volume of the reaction vessel, will diminish color intensity. It can be removed simply by heating with a recovery of 96 to 100.1% of the alkaloid. Maximum color development occurred after 30–60 minutes; the colors are stable for as long as 12 hours.

Salts and glucose interfere with the development of the color. Such interference is minimal at the limiting concentrations or amounts shown in Table II. Substances such as promazine hydrochloride and promethazine interfere because they, too, produce colors in concentrated sulfuric acid. Only slight differences in the sensitivity of the various reagents were noted and, because of its gross simplicity, concentrated sulfuric acid alone is highly suitable. Other related alkaloids—for example, tomatine, tomatidine, solanine, and conessine also give colors with the reagents (Table I), but the colors are different from those produced by the veratrum alkaloids. With most of the veratrum alkaloids, two distinct absorption maxima occurred usually at 390–410 mμ and 530–550 mμ (Table III); with the tomatine, tomatidine, etc., only a single absorption maximum was observed, generally at 400–420 mμ.

Figures 1 and 2 show plots of molar extinction coefficients *versus* wavelength for six defined alkaloids in sulfuric acid and sulfuric acid–ferric chloride. Although slight shifts in wavelengths of maximum absorption of the colors developed with these reagents occurred (Table III), the molar extinction coefficients of any particular alkaloid do not differ greatly from one reagent to the other. However, with either reagent and especially at the higher wavelength of maximum absorption, the molar extinction coefficient of the alkaloids differ greatly, thus allowing for some differentiation. Figure 3 shows that the method as outlined is quantitative and that within the concentrations employed, Beer's law was obeyed.

The sulfuric acid–ferric chloride reagent (reagent *2*) provides some distinction between jervine and veratramine. The former exhibited a broad absorption maximum at 420–480 mμ while the latter absorbed maximally at 410 and 460 mμ. The presence of a benzene ring in veratramine probably could have contributed to the difference in absorption maxima. If these two alkaloids must be separated, veratramine can be precipitated by digitonin, whereas jervine will not be precipitated. In many cases the optical density of the color produced by the veratrum alkaloids was greater at the lower wavelength of maximum absorption, usually 390–410 mμ. However, if the iron reagents are used, these do exhibit some absorption in this region. For this reason, measurements at the higher wavelength of maximum absorption (530–550 mμ) are preferable. Although veratrum alkaloids produced colors with two of the classical steroid reagents—namely, the Lieberman-Burchard and Salkowski reagents, color development was so slow that these reagents are of no advantage. Likewise, sulfuric acid plus vanillin or ammonium molybdate or dichromate are too nonspecific. When veratrine sulfate was used as the representative alkaloid, the typical color was obtained if ethyl alcohol, methyl alcohol, ether, chloroform, or benzene were used as solvents. Best results were obtained when ethyl or methyl alcohol were used. With isopropyl alcohol and amyl alcohols, yellowish pink colors and a muddy appearance of the mixture resulted.

The mechanism of the reaction of the veratrum alkaloids with sulfuric acid and sulfuric acid reagents is not definitely known, but dehydration is certainly involved since water inhibits the reaction. The production of color may also be due partially or wholly to degradation products of the alkaloids (27–30).

The "heat of the reaction" resulting from the addition of the sulfuric acid or sulfuric acid reagents to the alkaloid solution provides enough heat for the immediate development of the red color formed from the reaction of veratrine sulfate. If cold (deep freeze) H_2SO_4 is added to frozen samples of veratrine sulfate and the mixture kept in an ice water bath, a yellow color develops. After 3 hours at room temperature (30°), the typical red color appeared; after 8 hours it reached the intensity of a sample treated by the general procedure. If the tubes to which the cold acid was added were heated, the typical red developed after 10–15 minutes at 40°. At 70°, the intensity of the color developed after 8 minutes of heating was equivalent to that produced from the same amount of alkaloid treated according to the general procedure. This indicates that, although the heat of reaction leads to the immediate production of the typical color, in H_2SO_4 of 32.4 *N*, this typical color will still result even at relatively low temperatures, provided a long enough reaction time is allowed.

SUMMARY

The color produced by veratrum alkaloids in concentrated sulfuric acid or concentrated sulfuric acid containing ferric chloride or ferric ammonium sulfate can serve as a rapid, simple test, for the quantitative determination of these alkaloids

The presence of water in the reaction vessel reduced color intensity which reaches a maximum after 60 minutes. The color developed is stable for at least 6 hours. With most of the alkaloids used, two absorption maxima, usually at 390–410 mμ and 530–550 mμ, were observed. With the sulfuric acid–ferric chloride reagent, jervine and veratramine absorbed at 420–480 mμ and 410 and 460 mμ, respectively. Several of the steroids tested produced colors. However, they exhibited a single absorption maximum which was generally always around 400–420 mμ.

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Stability of the Cobalamin Moiety in Buffered Aqueous Solutions of Hydroxocobalamin

By ARNOLD D. MARCUS and JOSEPHINE L. STANLEY

The degradation of hydroxocobalamin has been studied kinetically in various buffer systems. Degradation was first order with respect to the substrate in all cases. The most stable solution studied was composed of a pH 4.3, 0.05 *M* acetate buffer made isotonic with sodium chloride. The apparent heat of activation has been found in the above system to be 26.95 Kcal./mole. Extrapolations to 25 and 30° show hydroxocobalamin to be sufficiently stable to permit formulation of injectable solutions under practical conditions. Even when the data are constrained to give a poor stability picture at 25 and 30°, the predicted stability remains excellent. At worst, a solution of hydroxocobalamin in pH 4.3 acetate buffer will retain at least 90 per cent of claimed cobalamin at 30° for 170 weeks if a modest 20 per cent overage is included in the solution. The poorer stability in other buffer systems indicates the degradative reaction(s) to be subject to general base catalysis. The influence of some amines on the reaction rate tend to support this inference.

HYDROXOCOBALAMIN is an analog of vitamin B_{12} in which a hydroxyl function has replaced the cyano group in the cobalt coordination complex. Recent clinical studies (1) have provided dramatic evidence that parenterally administered hydroxocobalamin yields considerably more prolonged high blood levels of biologically active cobalamin than does cyanocobalamin. Unfortunately, hydroxocobalamin has acquired over the years the reputation of being unstable in solution (2, 3). We have found that, far from being unstable, hydroxocobalamin in suitably buffered solutions is actually quite stable. Even when viewed pessimistically, hydroxocobalamin is sufficiently stable to allow the preparation of injectable solutions under practical conditions which will retain the claimed cobalamin content for long periods of time.

Preliminary stability tests (4) indicated that an apparently stable solution of hydroxocobalamin could be obtained in pH 4.3, 0.05 *M* acetate buffer made isotonic with sodium chloride. The

Received May 9, 1963, from the Pharmaceutical Research and Development Department, Merck Sharp and Dohme Research Laboratories, West Point, Pa.
Accepted for publication June 13, 1963.
The authors express their thanks to Mr. J. W. Carr and Mr. E. J. Hanus who provided the early formulation data, and to Mrs. H. R. Skeggs, Merck Sharp and Dohme Research Laboratories, for her valuable suggestions concerning the assays. Special thanks is also extended to Dr. T. J. Macek for his considerable help in preparing this manuscript.