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

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presence of chloride ion seemed important in achieving a stable solution. When the buffer concentration was increased to 0.1 *M* acetate, the cobalamin was less stable. In a buffer composed of 0.1 *M* dibasic sodium phosphate and 0.05 *M* citric acid at pH 4.3, the hydroxocobalamin appeared to be just as stable as in the isotonic 0.05 *M* acetate buffer.

These observations indicated the need for a more careful evaluation of the entire stability picture. It was necessary to determine, if possible, the precise stability of hydroxocobalamin in each system in order to attempt to predict the stability under "ordinary" conditions. It is the purpose of this communication to report such evaluations and the prediction of stability as determined by kinetic studies under accelerated conditions. This report confirms the preliminary observations that hydroxocobalamin is a stable species in the proper solution environment. The response of hydroxocobalamin to various buffers, however, indicates that the degradative reaction(s) may involve general base catalysis.

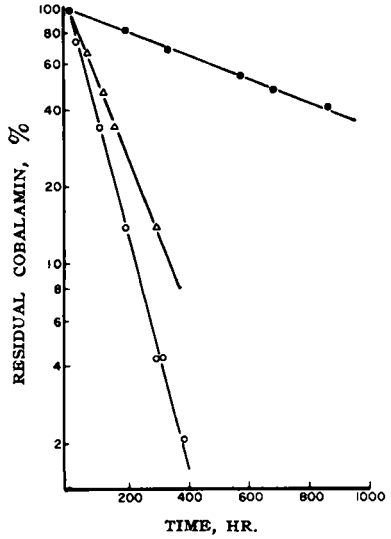


Fig. 1.—Plots showing degradation of hydroxocobalamin to be first order with respect to the substrate under a variety of experimental conditions. Key: ●, pH 4.3, 0.05 *M*, acetate at 70°; ○, pH 4.3, 0.05 *M*, acetate at 90°; △, pH 4.3, citrate-phosphate at 80°.

TABLE I.—RATE CONSTANTS FOR THE DEGRADATION OF HYDROXOCOBALAMIN IN VARIOUS BUFFERS[a]

| Buffer Compn. | Temp., °C. | $k$, hr.$^{-1}$ × $10^{-2}$ |
|---|---|---|
| pH 4.3, 0.05 *M* acetate[b] | 90 | 10.5 |
| pH 4.3, 0.05 *M* acetate[b] | 80 | 3.35 |
| pH 4.3, 0.05 *M* acetate[b] | 70 | 1.07 |
| pH 4.3, 0.05 *M* acetate | 90 | 10.5 |
| pH 4.3, 0.05 *M* acetate | 80 | 3.36 |
| pH 5.5, 0.05 *M* acetate | 90 | 20.0 |
| pH 5.5, 0.05 *M* acetate | 80 | 7.2 |
| pH 4.3, citrate-phosphate | 90 | 25.0 |
| pH 4.3, citrate-phosphate | 80 | 6.90 |
| pH 5.5, citrate-phosphate | 90 | 22.2 |
| pH 5.5, citrate-phosphate, no NaCl | 90 | 22.1 |
| pH 5.5, citrate-phosphate | 80 | 7.85 |

[a] All buffers contained 8.2 mg./ml. sodium chloride unless otherwise noted. [b] Solution contained 200 mcg./ml. hydroxocobalamin initially. These data were used for the Arrhenius-type plot of Fig. 2. All other solutions contained 1000 mcg./ml. initially.

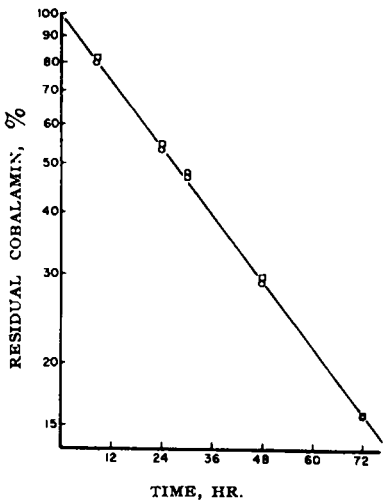


Fig. 2.—A plot showing the degradation of hydroxocobalamin in a pH 5.5 citrate-phosphate buffer (90°) to be unaffected by inclusion of sodium chloride. Key: ○, no chloride; □, 0.14 *M* in NaCl.

In the buffered aqueous solutions of hydroxocobalamin used in this study, the designation *hydroxo*cobalamin is actually inaccurate. Numerous cobalamins or cobalaminium species could actually be present. However, the original term will be retained to simplify the text and discussion. Furthermore, the inherent biological activity resides in the cobalamin moiety; the associated anions, coordinated functions, or presence of a formal charge can influence only stability and compatibility, not the qualitative activity. That the buffer constituents could affect the ability of hydroxocobalamin to provide prolonged high blood levels was recognized, but this would be revealed by clinical studies.

## EXPERIMENTAL

**Reagents.**—All chemicals were Merck, reagent grade, except for the hydroxocobalamin. This was crystalline material assaying at least 95% hydroxocobalamin on an anhydrous basis. All buffers were prepared to give the desired pH at the actual temperature of the run.

**Procedure.**—The solutions were flushed with nitrogen and carefully filled into 5-ml. ampuls to avoid streaking the ampul stems. This prevented charring during the flame-sealing and avoided introduction of extraneous materials which might influence the rate of degradation. The ampuls were placed in constant temperature baths regulated to within ±0.05°. After thermal equilibrium was

attained, three samples were removed, chilled, and stored at 4°. These were designated our "zero" hour samples. Other samples were removed at suitable intervals and quenched in the same manner. All samples were assayed for residual hydroxocobalamin by the microbiological procedure of Skeggs, *et al.* (5, 6).

## RESULTS AND DISCUSSION

As shown in Fig. 1, the degradation of hydroxocobalamin is first order with respect to the substrate under a variety of experimental conditions. The various rate constants are given in Table I. The apparent activation energy for the degradation of hydroxocobalamin in pH 4.3, 0.05 *M* acetate buffer was calculated from the slope of the line in Fig. 2. This value was a rather high 26.95 Kcal./mole. Although the apparent heat of activation could have been used to calculate the rate constants for lower temperatures, the values given in the following discussion were actually obtained by graphical extra-

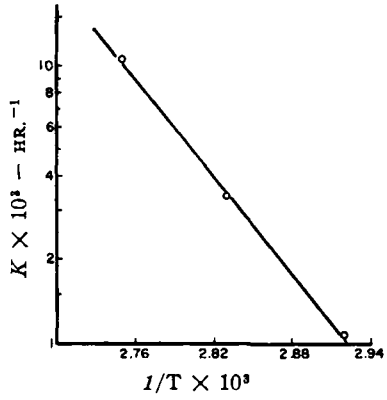


Fig. 3.—An Arrhenius-type plot illustrating the effect of temperature on the degradation of hydroxocobalamin in pH 4.3, 0.05 *M*, acetate buffer. The apparent activation energy calculated from the slope of the line is 26.95 Kcal./mole.

polation. When the calculated and extrapolated values were compared, the differences were insignificant.

Examination of the extrapolated rate constants shows that at 30° the rate is $6.2 \times 10^{-6}$ hours$^{-1}$. As a result, even at this relatively elevated temperature, solutions of hydroxocobalamin in pH 4.3, 0.05 *M* acetate buffers are sufficiently stable to retain at least 90% of the claimed cobalamin content for 125 weeks. The same solution at 25° could be expected to retain no less than 90% of the claimed hydroxocobalamin content for at least 220 weeks. These data make it clear that under suitable conditions hydroxocobalamin is quite stable in aqueous solutions.

Despite the optimistic nature of the preceding treatment, the possibilities for error inherent in microbiological assays and long-range extrapolations were recognized. It seemed judicious, therefore, to try to make predictions from extrapolations tending to give the least favorable 30 and 25° stability patterns. When this was done, the rate constants were $k_{30} = 1.0 \times 10^{-5}$ hours$^{-1}$ and $k_{25} = 4.6 \times 10^{-6}$ hours$^{-1}$. Calculations based on these "new" values indicated a loss of 10% of the initial cobalamin content after 125 weeks at 25° and 60 weeks at 30°. Despite the still good stability at 25°, a temperature of 30° is not unusually high for the storage of pharmaceuticals. It seemed important, therefore, to attempt to insure good stability under such storage conditions.

Calculations based on $k_{30} = 1.0 \times 10^{-5}$ hours$^{-1}$ showed that the use of a 20% average of hydroxocobalamin would yield a solution retaining at least 90% of "label claim" for no less than 170 weeks at 30°. Under such conditions, therefore, and employing extrapolations prejudiced to show greatest degradation, it was apparent that hydroxocobalamin is not "unstable" in solution.

When the degradation of hydroxocobalamin in other buffers was studied, some unexpected results were obtained. In an isotonic pH 5.5, 0.05 *M* acetate buffer, for example, the rate of degradation was approximately twice the rate at pH 4.3. Since in this pH range the hydroxocobalamin-aquocobalamin equilibrium favors the "aquo" form overwhelmingly, pH alone did not appear to be the major degradative influence. This was shown, in part, by rate studies in a buffer prepared to be 0.1 *M* in dibasic sodium phosphate and 0.05 *M* in citric acid. The rate in this buffer was also twice that in the isohydric acetate buffer. Since all three systems contained the same concentration of chloride ion, the differences in rate were inexplicable on the basis of the relative coordinating ability of chloride and other anions. Indeed, as shown in Fig. 3, the actual need for chloride ion is problematical. However, this single run at 90° may not accurately reflect the conditions and reactions which hold at conventional temperatures.

Since it appears somewhat doubtful that chloride ion functions to exert any "protective" effect and acetate is also an unlikely candidate for this type of action, an explanation of the "buffer effect" was sought elsewhere. Although no precise evidence was available, it seemed likely that this response stemmed from nucleophilic attack by the buffer anions. In this sense, acetate is not a "protective" agent but actually a less objectionable species. This would certainly tie in well with the rates at pH 5.5 in acetate systems. The effect of the citrate-phosphate system could be explained in like manner. Another facet of the present study which lends support to this possibility is the observed degradative influences of aniline and pyridine. In a rather qualitative two-point study we have found that both of these nucleophiles, present at 0.1 *M*, in pH 4.3, 0.05 *M* acetate buffer, caused an approximately twofold increase in the rate of degradation of hydroxocobalamin. The precise effects of these and similar agents is being studied further.

The apparent general base catalyzed degradation of hydroxocobalamin is being studied currently in greater detail. The effect of pH, *per se*, also will be evaluated. However, it should not be at all surprising that this type of degradation could occur in the cobalamin moiety. There are seven amide functions and two ester linkages in the molecule. Within the past 10 years, the number of instances of general acid-base catalyzed degradation of amides and esters has been well documented (7–9).

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# Interaction of Some Pharmaceuticals with Macromolecules I

## Effect of Temperature on the Binding of Parabens and Phenols by Polysorbate 80 and Polyethylene Glycol 4000

By N. K. PATEL and N. E. FOSS

**A quantitative evaluation of the effect of temperature on the interaction of methyl, propyl, and butylparaben, phenol and *p*-chlorophenol with polysorbate 80 was obtained with an equilibrium dialysis technique utilizing a plexiglas dialysis cell and a semipermeable membrane. Interactions of parabens with polyethylene glycol 4000 have been studied by the solubility method. The binding affinity of polysorbate 80 for the parabens employed decreased with increase in temperature, whereas that of polyethylene glycol 4000 showed an increase. There was no evidence of temperature dependency of the binding of phenol and *p*-chlorophenol with polysorbate 80. Parachlorophenol showed a greater tendency to interact with polysorbate 80 than with phenol. The significance of these results relative to the possible mechanism of these interactions is considered.**

IN RECENT YEARS considerable attention has been centered on the interaction of preservatives with nonionic macromolecules (1–5) and the importance of considering such interactions when determining proper preservative concentrations (2). These interactions are particularly noticeable with phenolic preservatives in the presence of surfactants which are polyoxyethylene derivatives of fatty acid esters. Several review articles have been published describing the possible mechanism of the interaction of phenolic compounds with surfactants (6–9).

Higuchi and Lach (10) and Guttman and Higuchi (11) hypothesized that the complex formation between phenols and polyethylene glycols could be due to hydrogen bond formation between the hydrogen of the phenolic hydroxyl group and basic oxygen atom of polyethylene glycol. It would thus be expected that proton donors like phenol and *p*-chlorophenol might form molecular complexes with surfactants containing polyoxyethylene groups.

Guttman and Higuchi (11) have indicated that addition of thermal energy to the phenol-PEG interaction might result in a decrease in association between phenol and PEG. Thus, if the principal binding force between a phenolic preservative and a nonionic surfactant is due to hydrogen bond formation, the increase in temperature might decrease the intermolecular association in such a system. Furthermore, nonionic surfactants usually contain a relatively large number of hydrophobic groups in the molecule and contribution of hydrophobic interactions (10) to the magnitude of binding of a phenolic preservative by the macromolecule might be of considerable importance.

In the present investigation an equilibrium dialysis technique was utilized to obtain information regarding the extent of binding of methyl, propyl, and butylparaben, phenol and *p*-chlorophenol with polysorbate 80[1] at various temperatures, for the purpose of relating these interactions to the reported possible mechanism of binding. The solubility method was employed to study the interaction of methyl and propylparaben with PEG 4000 at 20°. The selection of the phenolic compound was based on the relative proton donating power of the compound and on the length of the hydrocarbon chain of the *p*-hydroxybenzoic acid esters.

### EXPERIMENTAL

**Reagents.**—Recrystallized methyl *p*-hydroxybenzoate,[2] m.p. 127–128°; recrystallized propyl

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[1] Polyoxyethylene (20) sorbitan monooleate. Marketed as Tween 80 by the Atlas Powder, Co., Wilmington, Del.
[2] Methyl Parasept, purified; supplied through the courtesy of Heyden Newport Chemical Corp., New York, N. Y.