Kinetics of Nitrous Acid Induced Reserpine Fluorescence

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The kinetics of the reaction of reserpine with nitrous acid has been studied. The results show a two-step process with nitrous acid reacting with protonated reserpine to form an intermediate complex which under the influence of hydrogen ions forms a colored fluorescent product, identified as 3-dehydroreserpine. A rate expression was derived and reaction rates computed.

MANY CHEMICAL reactions used for the production of chromogenic or fluorogenic products in organic analysis are never studied beyond the point of practical utilization. Yet, further knowledge of their chemistry is desirable to extend their use.

The reaction of reserpine with nitrous acid to produce a fluorogenic-colored product has a long history of effective analytical service. It was first reported on by Szalkowski and Mader (1), who utilized its chromogenic characteristics for the determination of reserpine in pharmaceutical preparations. The procedure was later modified by Banes *et al.* (2–4) and subsequently approved as the official method (5) for pharmaceutical preparations. Since fluorometric techniques are extremely sensitive, a procedure which considered this property of the reaction was adopted by the Association of Official Agricultural Chemists for the microdetermination of reserpine in feeds (6–8). Although there is a wealth of published data on its analytical applications, there seem to have been no earlier attempts to ascertain the mechanism and kinetics of the reaction. In the present investigation, the fluorescent method has been used to study the kinetics involved in treating reserpine with nitrous acid.

EXPERIMENTAL

All fluorescent measurements were made on an Aminco-Bowman spectrophotofluorometer. As previously reported (6), the excitation maximum for the nitrous acid induced reserpine fluorescence is 390 mμ and the fluorescent maximum is 510 mμ.

In studying the effect of nitrous acid on the reaction rate, the initial concentrations of reserpine and of hydrochloric acid in the reaction flask were kept constant at 3.16×10^{-7} moles $L.^{-1}$ and 0.24 mole $L.^{-1}$, respectively. However, the concentration of the nitrous acid was varied from 1.74×10^{-3} to 5.80×10^{-5} moles $L.^{-1}$. All reactions were run and all measurements were taken at room temperature. The procedure was as follows.

Pipet 15 ml. of a 60% chloroform–40% methanol solution containing 4.8 mcg. of reserpine into a 25-ml. volumetric flask. Add several milliliters of methanol. Mix well. Add 0.5 ml. of hydrochloric acid and the desired number of milliliters of sodium nitrite solution (0.1% in 50% methanol–50% water). Dilute to mark with methanol and shake well. Measure the fluorescent development as a function of time.

In studying the effect of hydrochloric acid on the reaction rate the initial concentration of reserpine and nitrous acid in the reaction flask were kept constant at 3.16×10^{-7} moles $L.^{-1}$ and 2.9×10^{-4} moles $L.^{-1}$, respectively. However, the concentration of the hydrochloric acid was varied from 4.8×10^{-2} to 4.8×10^{-1} moles $L.^{-1}$. The procedure as described above was followed with the exception that the milliliters of hydrochloric acid added were varied while the milliliters of sodium nitrite solution added were kept constant.

RESULTS AND DISCUSSION

Postulated Reaction.—It is suggested that the reaction which takes place when one induces fluorescence in reserpine by use of nitrous acid is a two-step reaction which can be expressed as follows:

$$\text{protonated reserpine} + \text{HONO} \overset{K_1}{\rightleftharpoons} [\text{protonated reserpine} : \text{HONO}] \xrightarrow[k_2]{H^+} \text{3-dehydroreserpine (fluorescent product)}$$

That only the protonated reserpine species is involved in the reaction is quite obvious when one considers that the pKa of reserpine is 6.6 (9) and the reaction flask pH never exceeds 1.5.

3-Dehydroreserpine is considered to be the fluorescent product and the following evidence has been gathered to support this contention. (A) The wavelengths for excitation and fluorescence, *viz.*, 390 mμ and 510 mμ, are common to the nitrous acid induced reserpine fluorogen and the natural fluorogen of 3-dehydroreserpine. (B) The fluorescent yields at the above conditions are almost exactly the same for 3-dehydroreserpine and the reaction fluorophor. (C) On different paper chromatographic systems, *viz.*, (*a*) mobile phase, 10% pyridine–90% chloroform, immobile phase, 1%

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benzoic acid in formamide; (*b*) mobile phase, chloroform saturated with formamide, immobile phase, 1% benzoic acid in formamide, the R_f value for the product of the reaction mixture was the same as that for 3-dehydroreserpine.

Nitrous Acid Effects.—Figure 1 is a logarithmic plot of the concentration of unreacted reserpine, expressed as the final fluorescence (F_∞) minus the fluorescence at any time, t, (F_t) *versus* time. This plot demonstrates that at all nitrous acid concentrations a linear relationship is obtained. Thus, this reaction is an apparent first-order reaction with respect to reserpine concentration, *i.e.*,

$$-\frac{d(\mathrm{R})}{dt} = k_{\mathrm{obs.}}[\mathrm{R}_t] \qquad \text{(Eq. 1)}$$

It should be noted that one can use an expression such as $F_\infty - F_t$, because the final fluorescent intensity has been determined to be a certain value which is independent of nitrous acid concentration over a wide concentration range. Figure 2 demonstrates the relationship between the rate of fluorescent formation and nitrous acid concentration. It is seen that with increasing amounts of nitrous acid there is a corresponding increase in the rate of conversion of reserpine to the fluorophor. It is also seen that all the lines asymptotically approach the same final value.

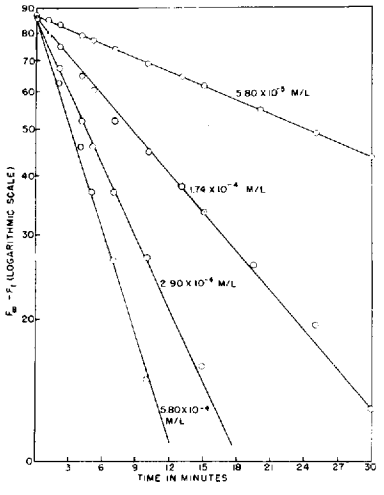

Fig. 1.—First-order plot for loss of reserpine in induced fluorescence reaction at various nitrous acid concentrations.

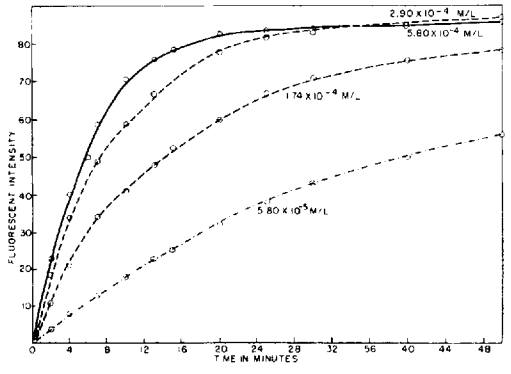

Fig. 2.—Relationship between rate of fluorophor formation and nitrous acid concentration.

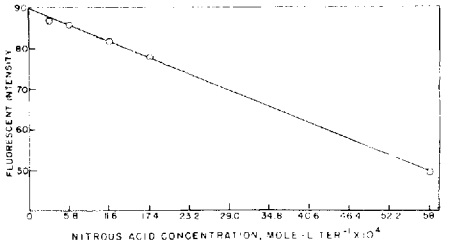

Fig. 3.—Suppression of fluorescent yield as a function of nitrous acid concentration.

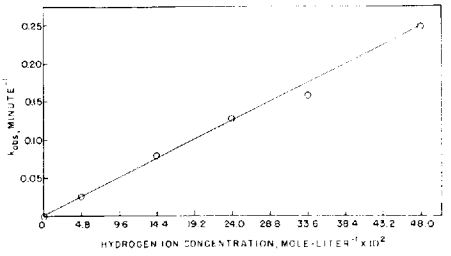

Fig. 4.—Relationship between observed rate constant and hydrogen ion concentration.

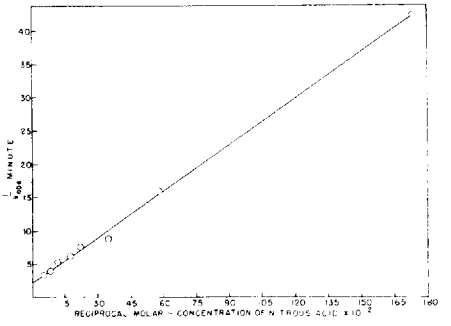

Fig. 5.—Relationship between the reciprocal observed rate constant and the reciprocal nitrous acid concentration.

However, if one extends this study to nitrous acid concentrations greater than 2.90×10^{-4} moles $L.^{-1}$ it is observed that there is a suppression of the fluorescence. The suppression of maximum fluorescence by the higher concentrations of nitrous acid is evident in Fig. 3. This straight line plot indicates that under the experimental conditions used in this study the limiting fluorescent yield is approximately 90 in the absence of the quenching effect of the nitrous acid. This type of quenching has been referred to by Bowman (10) as an inner filter effect. Apparently the excess nitrous acid absorbs some of the activating energy, or some of the fluorescent

energy, or both, thus reducing the amount of fluorescent energy reaching the measuring unit.

Rate Expression.—It was observed rather early in this work that both hydrogen ions and nitrous acid had an effect on the rate of fluorescent formation. Thus, it is obvious that the observed rate constant, $k_{obs.}$, consists of a number of component parts which can be resolved experimentally. If it is assumed that the first step in the reaction, that is the formation of the intermediate complex, is quite rapid, the rate-determining step is the second step, and the over-all rate of reaction is equal to k_2 multiplied ty the product of the concentration of the intermediate complex and the hydrogen-ion concentration, *i.e.*,

$$-\frac{d(R)}{dt} = k_2[R:HONO]\,[H^+] \quad \text{(Eq. 2)}$$

If $[R_t]$ equals the initial concentration of protonated reserpine present in the reaction mixture and [R:HONO] the concentration of the intermediate complex, then $[R_t] - [R:HONO]$ is equal to the concentration of free protonated reserpine remaining. Thus

$$K_1 = \frac{[R:HONO]}{([R_t] - [R:HONO])\,[HONO]} \quad \text{(Eq. 3)}$$

or

$$[R:HONO] = \frac{K_1\,[R_t]\,[HONO]}{1 + K_1\,[HONO]} \quad \text{(Eq. 4)}$$

By substituting Eq. 4 into Eq. 2 one obtains

$$-\frac{d(R)}{dt} = \frac{k_2\,[H^+]\,K_1[R_t]\,[HONO]}{1 + K_1\,[HONO]} \quad \text{(Eq. 5)}$$

If the right hand side of Eq. 5 is substituted into Eq. 1 the following expression for $k_{obs.}$ is obtained

$$k_{obs.} = k_2[H^+]\,\frac{K_1[HONO]}{1 + K_1[HONO]} \quad \text{(Eq. 6)}$$

If the assumptions made thus far have been correct, then one should obtain by plotting the observed rate constant *versus* the hydrogen-ion concentration a straight line which passes through the origin and whose slope is equal to k_2K_1 [HONO]/1 + K_1 [HONO]. Such a linear relationship is obtained as shown in Fig. 4. It is possible to mathematically manipulate Eq. 6 to give the following expression

$$\frac{1}{k_{obs.}} = \frac{1}{k_2K_1[H^+]}\left(\frac{1}{[HONO]}\right) + \frac{1}{k_2[H^+]} \quad \text{(Eq. 7)}$$

The above expression states that, if the original contentions are correct, a plot of the reciprocal nitrous acid concentration *versus* the reciprocal observed rate constant should yield a straight line whose intercept is equal to $1/k_2$ $[H^+]$ and whose slope is equal to $1/k_2K_1[H^+]$. Figure 5 is such a plot, and it is indeed linear. Using the above plot one is able to determine that the equilibrium constant K_1 is equal to 690 L. mole^{-1} and k_2 is equal to 2.2 L. mole^{-1} min.$^{-1}$ at a hydrogen-ion concentration of 0.24 mole L.$^{-1}$ and 25°.

The data presented above support the proposed general mechanism. However, the authors are at present unable to delineate in any great detail the nature of the nitrous acid–reserpine addition product. It apparently forms with great ease and is always in equilibrium with the unreacted alkaloid. Establishment of its chemical structure would be most interesting.

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