to-solution shift in the visible spectra) exhibit a broad band in this region at a wave length slightly longer than 3 μ (*cf.* Table II). The remaining dyes, containing bulky substituents in the 4,4'- or 7,7'-positions show a sharp band at a wave length somewhat shorter than 3 μ. This suggests that in those indigo dyes where the —NH— groups partake in intermolecular hydrogen-bonding, the —NH— stretching frequency occurs at slightly longer wave lengths than that observed in the dyes in which the hydrogen-bonding is mainly intramolecular. A similar difference in the —NH— stretching frequencies has also been observed by Blout in a study of the N,N'-dialkylamides of maleic and fumaric acids where, because of their *cis* configuration, only the former are capable of forming intramolecular hydrogen bonds.[16]

In view of the importance of indigo and some of its derivatives as dyestuffs, it is of interest to note that cellophane and gelatin films dyed with indigo also exhibited absorption maxima at 662 mμ, indicative of the presence of the associated form. Since cellophane and gelatin are structurally similar to cotton and wool, respectively, this strongly suggests that it is the polymeric form that is present in fabrics dyed with indigo. This further deepens the mystery surrounding the nature of the forces that hold dyes of this type to fibers, since it is difficult to envisage a mechanism by which indigo molecules, involved as they are in both intra- and intermolecular hydrogen-bonding, are bound to fibers.

Acknowledgment.—The authors wish to record their appreciation to Dr. E. R. Blout who suggested the study of the —NH— stretching frequencies for evidence of intermolecular hydrogen-bonding and to Mrs. D. A. Rogers for carrying out the study of carbonyl frequencies of the two thioindigo dyes included in this paper.

(16) E. R. Blout, private communication.

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, ROHM AND HAAS COMPANY]

The Kinetics of the Decolorization of Anthocyanins by Fungal "Anthocyanase"[1]

BY H. T. HUANG[2]

RECEIVED OCTOBER 3, 1955

Measurements of the decolorization of chrysanthemin at *p*H 3.95 and 30° by a fungal anthocyanase were analyzed kinetically. It was shown that the absorbancy of a decolorizing solution could be adequately described in terms of a_0, the initial absorbancy, t, the incubation time, k', an apparent first-order rate constant for the enzymatic hydrolysis of the glucoside, and k_1 and k_2, two apparent first-order rate constants involved in the reversible transformation of the liberated aglucone into a colorless derivative. k' is a function of enzyme and substrate concentrations. Values of k', k_1 and k_2 were estimated by independent methods and found to agree satisfactorily with those calculated from decolorization data.

Several crude enzyme preparations derived from *Aspergilli* have been found to exert a decolorizing effect on anthocyanins in aqueous solution, within a *p*H range of 3.0 to 4.5.[3] The over-all process was shown to involve firstly, an enzymatic hydrolysis of the anthocyanin to anthocyanidin and sugar, and secondly, a spontaneous transformation of the aglucone pigment into colorless forms. Information was inadequate to characterize clearly the enzyme responsible for the hydrolytic reaction. It was tentatively referred to as "anthocyanase." Detailed kinetic data on the decolorization of chrysanthemin by anthocyanase CN 558 are now available. It will be shown that the kinetics of decolorization are consistent with the above reaction scheme.

Experimental

Chrysanthemin chloride was a crystalline monohydrate isolated from blackberry as described previously.[3] It was shown to be indistinguishable from a synthetic sample of cyanidin-3-β-monoglucoside.[4] Anthocyanase CN 558 was the same preparation as that used in the preceding work.[3] Cyanidin chloride was prepared by hydrolysis of the glucoside in 20% hydrochloric acid.[3]

Decolorization experiments were carried out at 30° in 0.045 *M* sodium lactate buffer at *p*H 3.95. One ml. of enzyme solution was mixed with 5 ml. of substrate solution in buffer in a 12.5 × 125 mm. optically calibrated test-tube. The tube was stoppered and the absorbancy of the reacting solution at 510 mμ was read in a Coleman Junior Spectrophotometer against a lactate-enzyme blank. It was then incubated in a constant temperature bath maintained at 30 ± 0.05°. At selected intervals the tube was removed and a reading of the absorbancy at 510 mμ was again taken. Independent measurements had indicated that within the range employed, concentration of chrysanthemin was directly proportional to absorbancy at 510 mμ. When incubation under N_2 was desired, the tube was flushed well with N_2 during and after the addition of enzyme, and then tightly stoppered.

Reducing sugar was determined colorimetrically by a modification of Somogyi's micro method,[5] using the chromogenic reagent of Nelson.[6] The reducing capacity of chrysanthemin, cyanidin and glucose was found to be equivalent on a molar basis. In direct measurements of the extent of enzymatic hydrolysis, 1-ml. aliquot was mixed well with 1 ml. of Somogyi's reagent, and the mixture kept at 0° until a series of samples was ready for heating and subsequent treatment. The *p*H of the mixture was high enough (>9.7) to arrest all enzyme activity and the over-all procedure produced no chemical hydrolysis of the unchanged glucoside. The extent of hydrolysis was, therefore, proportional to the increase in reducing capacity found in the reacting solution.

Direct observations on the spontaneous decolorization of cyanidin were made by mixing concentrated solutions of cyanidin chloride at *p*H <1 with buffer at *p*H 3.95 at 30° and following the absorbancy of the solution at 510 mμ at regular intervals.

Results and Discussion

Results of most experiments, particularly when the level of enzyme employed was high, suggest that

(1) Presented in part before the Division of Biological Chemistry at the 126th Meeting of the American Chemical Society in New York, N. Y., September 12–17, 1954.

(2) Biochemical Research Laboratories, Chas. Pfizer and Co., Inc., Brooklyn, N. Y.

(3) H. T. Huang, *J. Agric. Food Chem.*, **3**, 141 (1955).

(4) H. T. Huang, *Nature*, **177**, 39 (1956).

(5) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).

(6) N. Nelson, *ibid.*, **153**, 375 (1944).

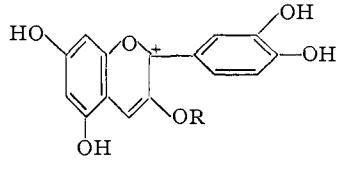
loss of color from 0–10 min. was significantly less than that from 10–20 min. Apparently, an induction period has to elapse before the rate of decolorization, *i.e.*, da/dt, where a denotes absorbancy at time t, reaches a maximum value. After da/dt has fallen to zero, a residual absorbancy a_∞ still remains in the reacting solution. Data from experiments in which the initial substrate concentration, *i.e.*, the initial absorbancy a_0, was varied, indicate that a_∞ is directly proportional to a_0.

Rearrangement of the raw data in the form of plots of log $(a_0 - a_\infty)/(a - a_\infty)$ *versus* time, was found to give rise to curves such as those illustrated in Fig. 1. The interesting feature of these plots is that after an induction period of 10 min., the points for each experiment fall readily on a straight line, from which an apparent first-order rate constant k' can be evaluated. When k' is plotted against enzyme concentration, a straight line is obtained. Thus, k' is directly proportional to enzyme concentration, and must be a function of the kinetics of the enzymatic hydrolysis of the glucoside pigment.

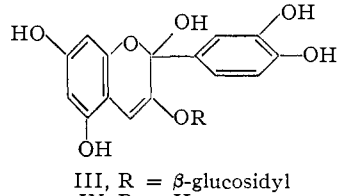
These observations suggest that the over-all reaction may be written as

$$A \xrightarrow{k'} B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \qquad (1)$$

where k', k_1 and k_2 are apparent first-order rate constants. A should represent an equilibrium mixture of the chrysanthemin cation (I)[7] and the pseudobase carbinol (III).[7-9] Similarly, B is regarded as an equilibrium mixture of the corresponding cyanidin forms II and IV. C is a colorless product from B, *i.e.*, from either II or IV or both. Assuming



I, R = β-glucosidyl
II, R = H



III, R = β-glucosidyl
IV, R = H

that k', k_1 and k_2 are small compared with the rate constants involved in the interconversions between I and III and that between II and IV, and that

(7) The chrysanthemin cation should be regarded as a hybrid of a series of resonating structures. In considering its chemical properties the most important structures are (I) and a corresponding expression in which the positive charge is localized on C-4: R. L. Shriner in "The Roger Adams Symposium," John Wiley and Sons, Inc., New York, N. Y., 1955, pp. 103–124, and S. Wawzonek in "Heterocyclic Compounds," Vol. II, Edited by R. C. Elderfield, John Wiley and Sons, Inc., New York, N. Y., 1951, pp. 304–306.

(8) A C-4 carbinol is presumably also formed to a minor extent.

(9) E. Sondheimer, THIS JOURNAL, **75**, 1507 (1953).

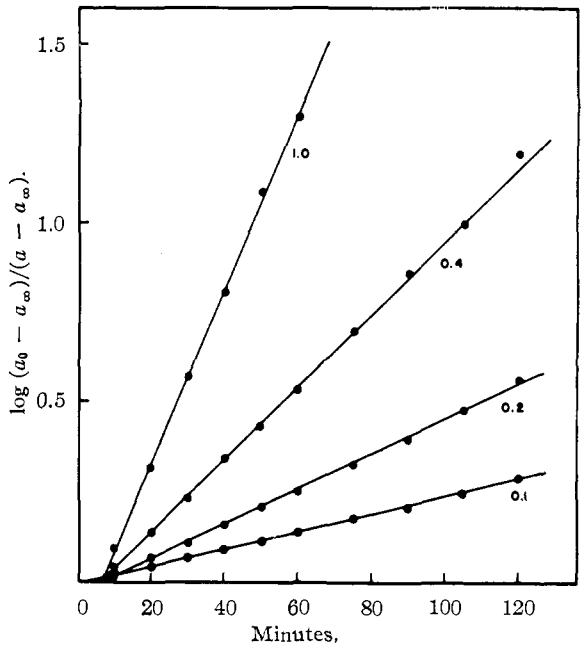


Fig. 1.—Plot of log $(a_0 - a_\infty)/(a - a_\infty)$ *versus* time. Each tube contained 1.03 μmole chrysanthemin and mg. CN 558 as indicated on curve.

all reactants possess unit activity coefficients, it follows from equation 1 that

$$d[A]/dt = -k'[A] \qquad (2)$$

$$d[B]/dt = k'[A] - k_1[B] + k_2[C] \qquad (3)$$

Integration of equation 2 gives

$$[A] = [A_0]e^{-k't} \qquad (4)$$

where $[A_0]$ corresponds to $[A]$ when $t = 0$. Substituting the expression for $[A]$ in equation 3 and rearranging we have

$$d[B]/dt = (k' - k_2)[A_0]e^{-k't} + k_2[A_0] - (k_1 + k_2)[B] \qquad (5)$$

Upon integration, equation 6 is obtained,

$$[B] = \frac{k' - k_2}{k_1 + k_2 - k'}[A_0][e^{-k't} - e^{-(k_1+k_2)t}] + \frac{k_2}{k_1 + k_2}[A_0][1 - e^{-(k_1+k_2)t}] \qquad (6)$$

When $t \to \infty$, that is, when decolorization has ceased, $[B]$ approaches $[B_\infty]$ and equation 6 is reduced to

$$[B_\infty] = k_2[A_0]/(k_1 + k_2) \qquad (7)$$

At any given time, the absorbancy of the solution is due to $[A] + [B]$. We have from equations 4 and 6

$$[A] + [B] = \frac{[A_0]}{k_1 + k_2 - k'}[k_1e^{-k't} - (k' - k_2)e^{-(k_1+k_2)t}] + [B_\infty][1 - e^{-(k_1+k_2)t}] \qquad (8)$$

On the assumption that the molar absorption coefficient of A, and that of B are approximately equal, we may substitute a for $[A] + [B]$, a_0 for $[A_0]$ and a_∞ for $[B_\infty]$ in equation 8 to give

$$a = \frac{a_0}{k_1 + k_2 - k'}[k_1e^{-k't} - (k' - k_2)e^{-(k_1+k_2)t}] + a_\infty[1 - e^{-(k_1+k_2)t}] \qquad (9)$$

Equation 9 is a general expression which relates the absorbancy a of the system at any time to k', k_1 and k_2. In applying equation 9 to our data, two special cases are pertinent. In case I, $(k_1 + k_2)$ is $>> k'$, and equation 9 is reduced to

$$a - a_\infty = k_1 a_0 e^{-k't}/(k_1 + k_2 - k') \qquad (10)$$

From (10) it follows that

$$(a_0 - a_\infty)/(a - a_\infty) = e^{k't} \qquad (11)$$

Thus a plot of log $(a_0 - a_\infty)/(a - a_\infty)$ *versus* t should afford a straight line passing through the origin. In case II, $(k_1 + k_2)$ is $>k'$. When t is small it is necessary to use equation 9 to describe the course of decolorization. As t increases, however, terms involving $e^{-(k_1 + k_2)t}$ would decrease in importance when compared to terms involving $e^{-k't}$, and equation 11 would again become applicable.

It is evident that case II holds for all the experiments described in Fig. 1. Lack of congruity between the initial stages of decolorization and equation 11 is indicated by the fact that when each line is extrapolated to log $(a_0 - a_\infty)/(a - a_\infty) = 0$, t assumes a positive value t'. As enzyme concentration, and hence k', is reduced, t' decreases and eventually approaches zero when case I is reached. Though attainable in practice, reactions under case I conditions are too slow to be studied conveniently.

Two other cases, *viz.*, when k' >> or $>(k_1 + k_2)$, may, of course, also produce data amenable to analysis *via* simplified forms of equation 9. Plots similar to those of Fig. 1 should then permit the evaluation of k_1 and k_2. Preliminary experiments to approach such conditions, however, indicate that the observed rate of decolorization in these cases would be too fast to be measured with any degree of accuracy with the techniques employed in this work. Further experiments were, therefore, confined to conditions of case II.

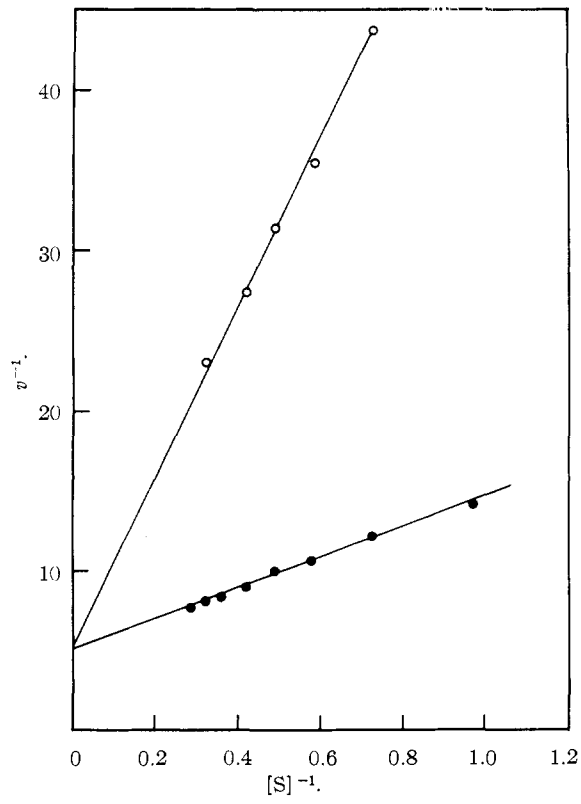


Fig. 2.—Competitive inhibition of anthocyanase-catalyzed hydrolysis of chrysanthemin by glucose: v in units of 10^{-4} M per min., [S] in units of 10^{-4} M: ●, no glucose; O, 5.0×10^{-3} M glucose.

It is possible, however, to calculate k_1 and k_2 indirectly from available case II data. Equation 7 may be written in the form

$$a_\infty = k_2 a_0/(k_1 + k_2) \qquad (12)$$

Substitution of eight sets of values of a_0 and a_∞ in equation 12 gave a mean value of $(k_1 + k_2)/k_2 = 11.0 \pm 0.2$, so that $k_2/k_1 = 0.10 \pm 0.002$. For each experiment a_0, a_∞ and k' are now known. Using corresponding values of a and t, when t is large in equation 10, k_1 and k_2 can be readily calculated. Mean values from 14 separate experiments based on $t = 40$ min. are presented in Table I.

TABLE I

COMPARISON OF KINETIC CONSTANTS DERIVED FROM DECOLORIZATION DATA WITH THOSE DETERMINED DIRECTLY

Constant,[a] min.$^{-1}$	k_1	k_2	k'[b]
Decolorization data	0.18[c]	0.016[c]	0.055[d]
Direct measurement	.23[e]	.020[e]	.054[d]

[a] At *p*H 3.95 and 30°. [b] Initial chrysanthemin concentration = 1.03 μmole/tube and enzyme concentration = 1 mg./tube. [c] Mean of values from experiments summarized in Fig. 2 and Fig. 4; $k_1 \pm 0.04$, $k_2 \pm 0.004$. [d] Mean of 4 determinations, ±0.002. [e] Mean of 3 experiments, accuracy not better than ±50% of value.

Attempts were also made to estimate k', k_1 and k_2 independently. The extent of hydrolysis of chrysanthemin was studied directly by measuring the increase in reducing capacity, as determined by the Somogyi micro method for reducing sugars,[5,6] at regular intervals during a regular decolorization run. Under the conditions employed, close adherence to first-order kinetics was observed, and a k' value was accordingly evaluated. The rate of color fading of cyanidin solutions at *p*H 3.95 was too rapid to be followed accurately. Direct observations were, nevertheless, made and approximate values of k_1 and k_2 calculated. Estimates of k', k_1 and k_2 so obtained are compared to values derived from decolorization data in Table I. Agreement between the two series of values is satisfactory.

The validity of k' as a function of the enzyme activity was further checked by ascertaining the effect of substrate concentration on rate of enzymatic hydrolysis. The initial velocity v for each substrate concentration [S] was calculated from the observed value of k'. The data are summarized as a plot of v^{-1} *versus* $[S]^{-1}$ in Fig. 2.[10] It is evident that v^{-1} is directly proportional to $[S]^{-1}$. This plot yields a value for K_m, the Michaelis constant, of 0.2×10^{-3} M, which is of the same order of magnitude as the substrate concentrations employed. Since the kinetics of an enzyme-catalyzed reaction tend to approach those of a first-order reaction only when K_m is large relative to [S],[11]

(10) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(11) E. Elkins-Kaufman and H. Neurath, *J. Biol. Chem.*, **176**, 893 (1948).

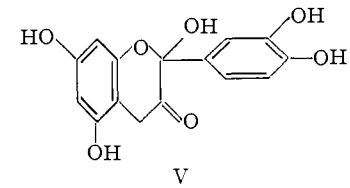
this value of K_m suggests that the reaction was probably competitively inhibited by a split product,[12] *e.g.*, glucose. Glucose was indeed found to be a competitive inhibitor of the enzyme as illustrated by the data presented in the upper plot of Fig. 2. A K_i, *i.e.*, inhibitor–enzyme dissociation constant, of 1.25×10^{-3} M was evaluated.

These results indicate that the over-all decolorization process is adequately described by equation 1. This formulation is also consistent with the chemical properties of the decolorized solution.

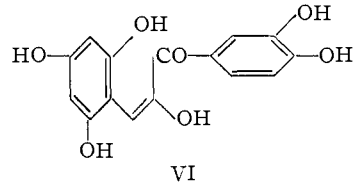
In aqueous solution at *p*H 3.95, chrysanthemin exists as an equilibrium mixture of I and III.[9] Form I is responsible for the characteristic color of the solution and form III can be recognized by its instantaneous conversion to I when the *p*H of the medium is lowered to <1. By analogy, in the enzyme decolorized solution, the presence of II is shown by the residual color. The existence of IV is demonstrated by the fact that upon acidification, an instantaneous, small but always reproducible, increase in absorbancy is noticeable. The slow intensification of color which follows[3] indicates transformation of the colorless form C, which is the predominate molecular species present, into IV and II. Structure V, *i.e.*, the ketonic tautomer of IV, is suggested as most likely for C for two reasons. In the first place, the heterocyclic ring in IV is no longer resonance stabilized, so that isomerization of the enolic group into the more stable keto form V would be expected to occur.[13] Secondly, open chain tautomers such as the chalcone derivative VI may be ruled out, since careful spectrophotometric examination of enzyme decolorized solutions failed to detect any absorption maximum other than that at

(12) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

(13) G. W. Wheland, "Advanced Organic Chemistry," 2nd ed., John Wiley and Sons, Inc., New York, N. Y., 1949, pp. 589–596.



V



VI

510 mμ and C must be regarded as a colorless product. Either chalcone (VI) or the isomeric diketo form would be expected to exhibit a pronounced yellow color.[14] Destruction of C in air, as reflected in losses in the amount of B, *i.e.*, intensity of color, which could be regenerated by acidification of the decolorized solution, has already been noted.[3] This loss was entirely eliminated when decolorization reactions were carried out in an atmosphere of nitrogen. Autoxidation of C is, however, too slow to have a measurable effect on the kinetics of decolorization observed under the conditions reported in this communication.

Acknowledgment.—The author wishes to express his thanks to Mr. Gerard Weiser for technical assistance and to Dr. C. V. Smythe for his interest and encouragement.

(14) An acyclic 1,2-diketone, *e.g.*, biacetyl, is markedly yellow. Chalcone itself is lightly yellow, but a hydroxylated chalcone, *e.g.*, butein is more deeply colored. Butein derivatives, in fact, occur as pigments in nature: T. A. Geissman, THIS JOURNAL, **63**, 2689 (1941), and M. Shimokoriyama and S. Hattori, *ibid.*, **75**, 1900 (1953).

PHILADELPHIA, PA.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLLEGE OF ARTS AND SCIENCES, UNIVERSITY OF LOUISVILLE]

2-Pyrones. XVIII. 5-Aroyl-2-pyrones and 5-Aroyl-2-pyridones

BY RICHARD H. WILEY AND S. C. SLAYMAKER

RECEIVED NOVEMBER 25, 1955

A series of 5-aroyl-2-pyrones I have been prepared by Friedel-Crafts condensations with coumalyl chloride in 20–93% yield and converted to pyridones (II, III, IV) by reaction with ammonia, amines and hydrazines. *para*-Substitution in the aryl nucleus has been established by degradative and spectral data. Pyridone formation is facilitated by the 5-aroyl substitution. The N-aminopyridone IV is converted to the pyridone by nitrous acid. Unique structural features were observed with the analogous N-anilino derivative IV and the hydroxylamine derivative VI. Peroxide-acetic acid oxidation of 5-benzoyl-2-pyridone gives the benzoyloxy derivative V. Infrared and ultraviolet absorption data for all of these compounds are analyzed.

The description[1] of the first known ketone in the 2-pyrone series has initiated a further study of this class of compounds. A variety of such ketones are now available and we wish to report the results of our studies on the preparation, structure, absorption characteristics and reactions of these ketones and the 2-pyridones derived from them.

5-Aroyl-2-pyrones I are readily available from the aluminum chloride catalyzed reaction of coumalyl chloride with those benzenoid compounds which are liquid and can be used in excess as solvents for the reaction. Using such techniques yields of 73–93% of 5-aroyl-2-pyrones have been obtained from benzene, toluene, bromobenzene and chlorobenzene. Substitution in the *para* position is established by oxidation of the tolyl derivative to *p*-toluic acid. With other benzenoid compounds, apparently because they are less stable to aluminum chloride, poor yields are obtained. Thus, ethylbenzene gives but 20% of product and *m*-xylene and anisole give no ketone at all by this process. Furthermore, the reaction is limited to coumalyl chlo-

(1) R. H. Wiley and L. H. Knabeschuh, THIS JOURNAL, **77**, 1615 (1955).