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# Spectral Changes during the Action of Myrosinase on Sinigrin

SIGMUND SCHWIMMER*

*Western Regional Research Laboratory, Albany 10, California**, and Organic Chemical Laboratory, Royal Veterinary and Agricultural College, Copenhagen, Denmark*

When myrosinase acts on sinigrin, the absorbancy at 227.5 mμ (maximum for the substrate) decreases linearly with time. On the basis of the observations a convenient, continuous method for estimation of enzyme activity has been developed. At 255 mμ, an isosbestic point for the spectra of the substrate sinigrin and allyl *iso*thiocyanate, presumably the only UV absorbing product of the enzyme reaction, there is observed an initial rapid rise in absorbance followed by a slower rise and still slower decrease to a final value somewhat above the initial absorbancy of the enzyme reaction mixture. The influence of enzyme and substrate concentration and of pH on the rate of change of absorbance at these two wavelengths was determined. $K_M$ values obtained below neutrality were of the order of 0.1 mM, and the pH optimum was found to be between 6 and 7. At pH 7, the $K_M$ values rose as much as one order of magnitude. The accelerating effect of ascorbic acid on the enzyme action was confirmed by the spectrophotometric method developed herein. The results indicate that the instability of ascorbic acid buffered at pH 6.0 is decreased during the action of the enzyme.

The structure and distribution of the naturally occurring derivatives of S-(glucosyl)-thiohydroximyl sulfates (mustard oil glucosides) have been elucidated in recent years, in large measure as a consequence of the investigations of Kjaer [1] and of Ettlinger and Lundeen [2]. As a result of this work and of more recent investigations of the properties of enzyme preparations responsible for the hydrolysis of these glucosides (myrosinase [2–4]) it has been suggested that the hydrolysis of these substances proceeds *via* simple thioglucosidase action accompanied by a Lossen rearrangement with concomitant sulfate displacement to yield *iso*thiocyanate derivatives. However, results published in the last year or so demonstrate a multiplicity of alternative pathways for the enzymatic breakdown. Thus, organic thiocyanate [5], thiocyanate ion [6], and

* Senior Postdoctoral Fellow, National Science Foundation, 1958–59.

** A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

nitrile [7] have been shown to be products of enzymatic action under appropriate conditions. Gaines and Goering [8], after separating the thioglucosidase and the sulfatase functions of myrosinase, have shown that both are necessary for the formation of *iso*thiocyanate. Furthermore, it has been found that ascorbic acid can influence both the rate of enzyme action and the nature of the products formed [4,7].

The present paper presents results of a spectroscopic investigation of the action of myrosinase on sinigrin, S-(glucosyl)-2-butenothiohydroximyl sulfate. These studies provide a basis for a convenient continuous method for the assay of myrosinase and show the effect of variations of substrate concentration, enzyme concentration, and pH. In addition, they show that a UV absorbing intermediate or side product is formed during the reaction and that added ascorbic acid and the enzymic reaction exert reciprocal effects; *i.e.*, the decomposition of sinigrin is accelerated by ascorbic acid, and ascorbic acid is protected from deterioration (as measured spectroscopically) in the presence of the enzyme reaction mixture.

## MATERIALS AND METHODS

Sinigrin, purchased from Light & Co.* (London), was recrystallized twice from 90 % ethanol to yield sinigrin hydrate (m.p. = 124°–126°). Allyl *iso*thiocyanate was used as a fresh distillate of a commercial sample (Eastman). Ascorbic acid was also purchased from Eastman. The myrosinase preparation used in all these experiments was obtained from Coleman's yellow mustard powder according to the following procedure, carried out at 0°–4°. 100 g of the mustard powder was extracted 10 times with 300 ml portions of acetone. After drying *in vacuo*, the defatted powder was extracted two times with 300 ml of water. The residue was removed by centrifugation and the combined supernates were fractionated with acetone. The portion precipitating between 30 % and 70 % was dialyzed against distilled water for 41 h and the retentate, after removal of small amount of precipitate by centrifugation, was lyophilized to yield 2 g of white enzyme preparation. The spectra reported here were obtained with a Cary Recording Spectrophotometer, which was also used to determine the course and extent of enzyme action (*cf.* Fig. 2). For all the other experiments either a Beckman DU or Zeiss spectrophotometer was employed. Unless otherwise stated 10 mm and 1 mm light paths were used at 255 m$\mu$ and at 227.5 m$\mu$, respectively.

## RESULTS

*Spectra of substrate and product.* (Fig. 1). At the absorption maximum of the substrate sinigrin (227.5 m$\mu$, $\varepsilon = 7\,800$) the molar extinction coefficient $\varepsilon$ of the product, allyl *iso*thiocyanate, is 564 or 7.2 % of that of sinigrin. This suggests that one can conveniently follow the enzyme reaction at 227.5 m$\mu$. The expected decrease in absorbancy at this wave length may be considered attributable to the disappearance of sinigrin. If one assumes that the only UV absorbing material formed is indeed allyl *iso*thiocyanate, then, $S_t = S_o - \Delta OD/\Delta\varepsilon$, and the % hydrolysis is $(\Delta OD/S_o\Delta\varepsilon) \times 100$; where $S_t$ = molar concentration of substrate at time $t$, $S_o$ is the initial substrate

* Mention of specific products does not imply recommendation by the Department of Agriculture over others of a similar nature not mentioned.

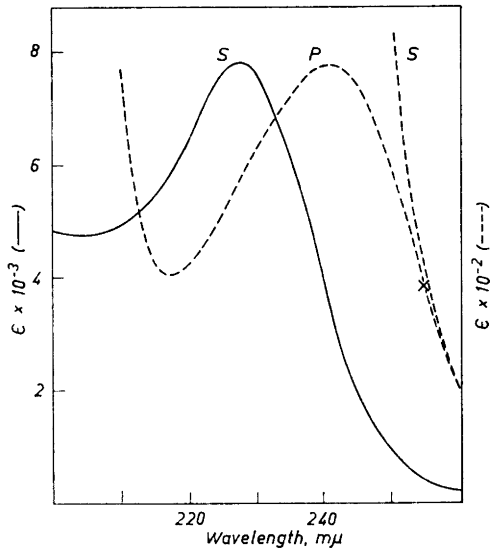


*Fig. 1.* Ultraviolet absorption spectra of aqueous solutions of 0.05 mM sinigrin (S) and 1 mM freshly prepared allyl *iso*thiocyanate (P); $\varepsilon$ = molar extinction coefficient. Solid line (——————) is for $\varepsilon \times 10^{-3}$ and the broken lines (- - -) are for $\varepsilon \times 10^{-2}$. The two spectra are isosbestic at 255 m$\mu$, $\varepsilon$ = 380.

concentration, $\Delta OD$ the observed change in optical density and $\Delta\varepsilon$ the difference in molar extinction coefficients between that of the subtrate and that of the product. The $\varepsilon$ value for allyl *iso*thiocyanate in dilute aqueous solution at its maximum of 240 m$\mu$ amounted to 770, and at this wavelength $\varepsilon$ = 4 000 for sinigrin. It will be noted in Fig. 1 that the two spectra are isosbestic at 255 m$\mu$ ($\varepsilon$ = 380). Therefore, if neither UV absorbing side products nor stable intermediates were formed during the course of the reaction there would be no change in optical density at this wavelength which could be attributed to enzyme action.

Dilute solutions of sinigrin were found to be spectroscopically stable for 3 h and over the pH range (3—9) used in the present studies. Small but significant changes did occur in allyl *iso*thiocyanate solutions. At room temperature, the absorbancy at 240 m$\mu$ of unbuffered solutions (pH 6) decreased 8 % in 2 h. Absorbancy at pH 8.65 (TRIS buffer) increased 10.9 %, 10.6 %, and 5.5 % at 227.5 m$\mu$, 240 m$\mu$, and 255 m$\mu$, respectively. In addition to the 240 m$\mu$ peak, aqueous solutions of undistilled allyl *iso*thiocyanate exhibited a maximum at 298 m$\mu$ as well.

*Enzyme-induced changes in absorbancy.* Upon the addition of myrosinase to sinigrin at pH 6.0, a rapid decrease of absorbancy at 227.5 m$\mu$ was observed proceeding almost linearly during the first 50 % hydrolysis (Fig. 2). The rate of decrease in optical density then slowly decelerated until a minimum optical density was attained, corresponding to 96 % hydrolysis of sinigrin based on

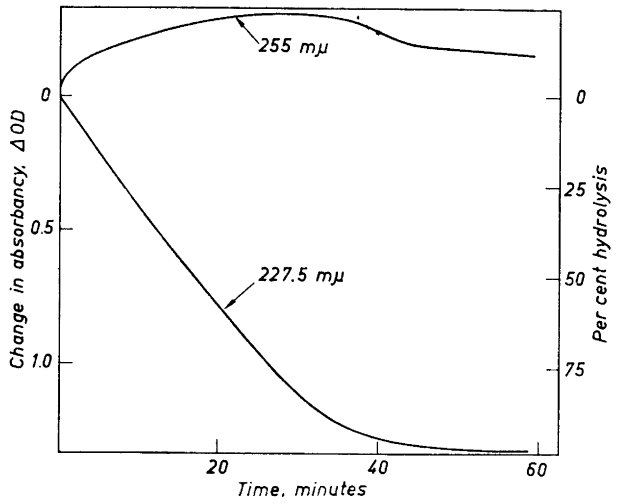


*Fig. 2.* Absorbancy changes with respect to time in a myrosinase-sinigrin reaction mixture. The curves were obtained with a Cary recording spectrophotometer, which began to record 12 sec. after the start of the reaction. The absorbancy values at zero-time used to calculate optical density change were obtained from the sum of the absorbancies of substrate and enzyme measured separately. One ml of enzyme reaction mixture at 25° contained; 1.9 μmoles substrate, 40 μmoles acetate buffer at pH 6.0, and 0.25 mg of myrosinase preparation. Percent hydrolysis of sinigrin is calculated on the assumption that allyl *iso*thiocyanate is the only stable material formed during the reaction absorbing at 227.5 mμ. Light paths at 227.5 mμ and at 255 mμ were 1 mm and 10 mm, respectively.

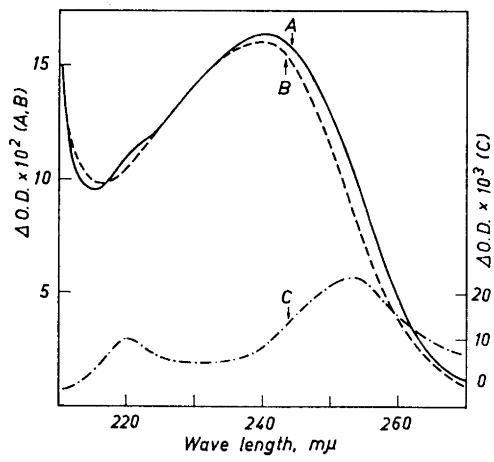


*Fig. 3.* Spectrum of enzyme reaction mixture described in Fig. 2 after 2.5 h. Points obtained after correction for protein (solid line, Curve A). Curve B (- - -) is the calculated spectrum of a mixture of 99 % allyl *iso*thiocyanate and 1 % sinigrin. Curve C (- · - · -) shows the difference spectrum between the two curves. All measurement made with 1 mm light path.

the assumptions outlined above. Instead of finding no change in absorbancy at the isosbestic point 255 mμ as expected, there was observed a very rapid initial rise in optical density followed by a slower increase until a maximum was reached. The optical density of the reaction mixture then decreased to an apparent minimum value somewhat higher than that at zero time.

The spectrum of the sinigrin-myrosinase digest (after correction for enzyme alone) at 2.5 h is shown in Fig. 3, Curve A. The spectrum of a mixture of 99 % allyl *iso*thiocyanate and 1 % sinigrin is shown for comparison, Curve B. The general shapes of the two spectra are similar. It will be noted that beyond 235 mμ, shift of the experimental spectrum in the bathochromic sense is observed. Also to be noted is the appearance of a slight shoulder in the experimental spectrum between 215 and 225 mμ. The difference spectrum, also shown in Fig. 3, indicates two relatively weak absorption bands at 220 and 253 mμ.

*Influence of enzyme and substrate concentrations.* Fig. 4 shows that there is a strict proportionality between rate of enzyme action, as measured by decrease of absorbancy at 227.5 mμ, and enzyme concentration. Applications of simple Michaelis kinetics to the curve describing the effect of substrate concentration on the initial rate yielded a value of $V_{max} = 0.033$ μmoles per ml per min. and a $K_M$ value between 0.12 and 0.14 mM. An estimation of the turnover number as the number of molecules of sinigrin decomposed per min. by one molecule of enzyme (assumed mol.wt. = 100 000) yields a value of 181. This indicates that the enzyme is still in a very low state of purity.

The small but almost instantaneous increase of optical density at 255 mμ shown in Fig. 2 was too rapid to permit estimation of true initial rate. However, it was possible to measure a quasi-initial rate by plotting optical densities

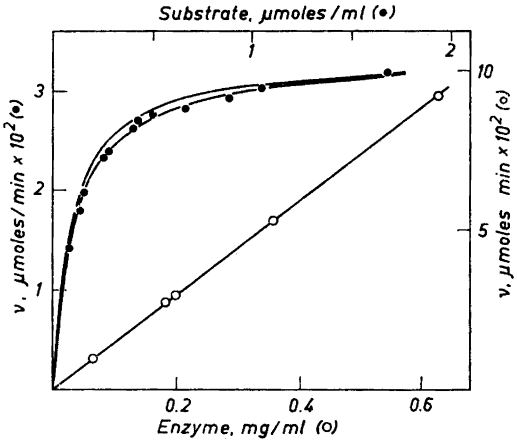


*Fig. 4.* Influence of enzyme and substrate concentration on the initial rate of decrease of absorbancy at 227.5 m*u*. This rate, *v* is expressed as moles of sinigrin disappearing per ml digest per min. The closed circles (●) are the experimentally found rates and the two curves are the theoretical curves for $V = 0.033$ μmoles/ml/min and $K_M = 0.12$ or 0.14 μmole/ml. Enzyme reaction mixture at pH 6.0 (acetate buffer) contained 0.83 μmole/ml of sinigrin for the enzyme concentration curve (O). The amount of enzyme for the plot of substrate *vs.* rate data was 0.185 mg/ml.

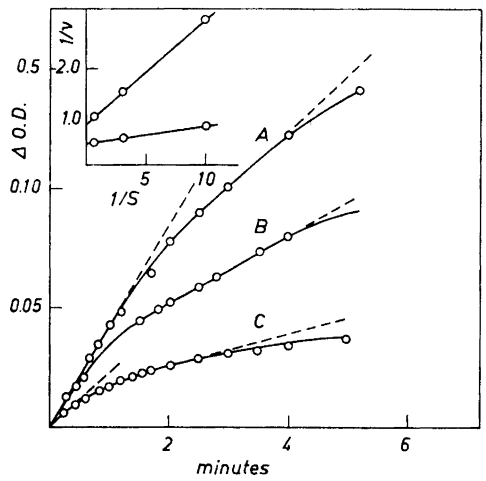


*Fig. 5.* Determination of "fast" and "slow" rates at 255 mμ as affected by substrate concentration. One ml of enzyme reaction mixture at 25° contained 33 μmoles of acetate buffer pH 4.0, 0.15 mg of enzyme preparation and the following μmoles of substrate: A, 1.67; B, 0.33; C, 0.10. The slopes of the broken straight lines (- - -) are estimated for determination of "fast" and "slow" rates. The insert shows reciprocal substrate-velocity plot used to determine $K_M$ and $V$.

against time during the first half-minute of the reaction and extrapolating to zero time (Fast Reaction, Fig. 5). This "fast" reaction was followed by an interval in which the increase in optical density appeared to be linear with respect to time (Slow Reaction). Fig. 5 shows that plots of the reciprocal of these rates against reciprocal substrate concentrations yield straight lines, thus permitting calculation of quasi-kinetic constants.

*Influence of pH.* Table 1 summarizes the influence of pH on the kinetic constants for the decrease of absorbancy at 227.5 mμ and for the "fast" and "slow" increases at 255 mμ. It will be noted that the calculated maximum velocities ($V$ relative) do not exhibit great changes with respect to pH. The value at pH 3 for the rate at 227.5 mμ is probably too low since the decrease in absorbancy due to sinigrin disappearance was counteracted by increase due to the formation of turbidity in the enzyme reaction mixture [7]. Above pH 7.0 the maximum rates for all three reactions decreased relatively rapidly. Below neutrality $K_M$ values were of the order of $10^{-4}$ M and were fairly independent of pH. Perhaps the most remarkable feature observed was the rather dramatic increase in, and appearance of optimum $K_M$ values at pH 6.96, amounting to a 10-fold increase for the "slow" reaction at 255 mμ. One effect of this remarkable increase is to create an apparent optimum pH of around 6.0 at substrate concentration below that required for saturation of the enzyme.

*Influence of ascorbic acid.* Fig. 6 and Table 2 show that the stoichiometric quantities of ascorbic acid used in these experiments appear to stimulate

*Table 1.* Influence of pH on kinetic constants $V$ and $K_M$ [1]

| pH | Wave length, mμ | | | | | |
|---|---|---|---|---|---|---|
| | 227.5 | 255 f | 255 s | 227.5 | 255 f | 255 s |
| | $V$ (relative) | | | $K_M$ (μmole/ml) | | |
| 3.00 | 0.77 | — | — | 0.18 | — | — |
| 4.00 | 1.00 | 1.0 | 1.0 | 0.14 | 0.08 | 0.25 |
| 5.00 | 1.05 | 1.1 | 1.1 | 0.14 | 0.25 | 0.30 |
| 6.00 | 1.21 | 1.5 | — | 0.14 | 0.26 | 0.27 |
| 6.96 | 1.30 | 1.7 | 1.0 | 0.68 | 0.56 | 2.7 |
| 7.75 | — | 1.2 | 0.7 | 0.58 | 0.23 | 1.6 |
| 8.63 | 0.98 | 1.0 | 0.3 | 0.27 | 0.16 | 0.45 |

[1] Reaction mixture contained 0.15 mg/ml of enzyme preparation. Letters "f" and "s" represent "fast" and "slow" reactions as explained in text and Fig. 5. $V$'s set at 1.00 at pH 4. Actual values for 227.5, 255 f and 255 s were, respectively: 0.028 μmole per min. per ml of digest; 0.22 optical density units per 5 min; and 0.11 optical density units per 5 min. The buffer used in final concentration of 0.033 M were as follows: malate for pH 3.0; acetate for pH 4.00, 5.00 and 6.00; TRIS for pH 6.96, 7.75 and 8.63. Other conditions are the same as in Fig. 5.

the action of the enzyme as measured by rate of decrease in optical density at 227.5 mμ. However, the results were complicated by the apparent spectroscopic instability of dilute ascorbic acid solution under the conditions of assay (Fig. 6, Curve A—I). This course of decrease of absorbancy was essentially the same as that of Curve A—I when either enzyme or substrate was added to the ascorbic acid separately (Table 2). The optical density at 227.5 mμ of the standard reaction mixture containing sinigrin and myrosinase (but no ascorbic acid) decreased linearly at a rate of 0.016 optical density units per min. (B—I). In the presence of ascorbic acid the rate of decrease of optical density was accelerated to 0.038 optical density units per min., over double that in the absence of ascorbic acid and the change was linear with respect to time (Curve C—I). It would thus appear that the increased rate did not include

*Table 2.* Absorbancy changes ($\Delta OD$) in the presence of ascorbic acid [1]

| Components [2] | | | $\Delta OD$ | | | | | |
|---|---|---|---|---|---|---|---|---|
| E | S | AA | 227.5 mμ | | | 255 mμ | | |
| | | | 2 min. | 5 min. | 10 min. | 2 min. | 5 min. | 10 min. |
| + | + | + | −0.071 | −0.184 | −0.372 | −0.001 | −0.035 | −0.072 |
| +[3] | + | + | −0.064 | 0.162 | −0.325 | — | — | — |
| + | + | — | −0.030 | −0.079 | −0.157 | +0.003 | +0.006 | +0.004 |
| + | — | + | −0.027 | −0.061 | −0.059 | −0.143 | −0.368 | −0.381 |
| — | + | + | −0.024 | −0.060 | −0.060 | — | — | — |
| — | — | + | −0.026 | −0.058 | −0.060 | — | — | — |

[1] Selected data from curves of Fig. 6.
[2] AA = Ascorbic acid; S = Substrate; E = Enzyme.
[3] E added 20 min after incubation of S + AA.

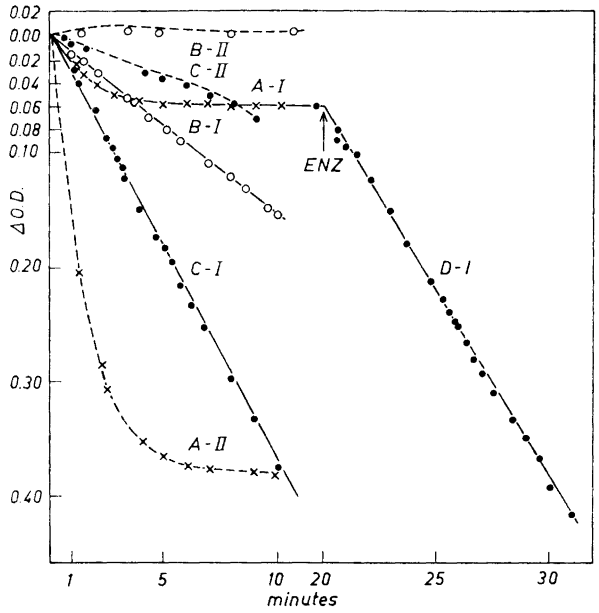


*Fig. 6.* Effect of ascorbic acid on change in absorbancy with time in a myrosinase-sinigrin reaction mixture. Broken lines (II) (- - -) represent date obtained at 255 mμ and smooth lines (I) (————) those obtained at 227.5 mμ (1 mm light path in both cases). Complete system contained per ml; 0.89 μmole of sinigrin, 0.90 μmole of ascorbic acid, 167 μmoles of pH 6.0 acetate buffer and 0.167 mg of enzyme preparation. Curves A (×) complete system less substrate and enzyme; Curves B, (●) complete system less ascorbic acid; Curves C, complete system (O); Curve D, complete system less enzyme at zero time; at 20 min. enzyme added (ENZ).

the decrease due to ascorbic acid. This suggestion is strengthened by the results obtained when enzyme was added to a sinigrin reaction mixture 20 min. after addition of ascorbic acid. The rate of decrease of optical density amounted to 0.032 per min., double that in the absence of ascorbic acid (Curve D—I).

Further substantiation of this conclusion is afforded by the results obtained at 255 mμ (Curves A—II, B—II, C—II, Fig. 6). The slight increase at 255 mμ in the presence of substrate and enzyme alone is shown in Curve B—II (The low values are the result of using a 1 mm light path instead of 10 mm as was used in the previous experiments at this wavelength). At 255 mμ under the conditions of the enzyme reaction, the optical density of ascorbic acid alone dropped precipitously in the first few minutes (Curve A—II). Curve C—II presents the results obtained with the complete system containing sinigrin, myrosinase and ascorbic acid. It will be noted that for the first minute there was practically no decrease in optical density after which the optical density decreased at a rate equal to 4 % of that in absence of enzyme plus substrate. (Comparison of Curve C—II and A—II). Absorbancy changes at 2.5 and 10 min. are shown in Table 2. These data were obtained by interpolation from the curves of Fig. 6.

## DISCUSSION

The almost strict linearity between optical density changes at 227.5 mμ on one hand and both time and enzyme concentration on the other demonstrate the feasibility of a rapid convenient continuous method for assaying the overall activity of myrosinase preparations. In recent publications, one [3] and four [4] hour incubation periods have been used in assaying this enzyme. In the assay procedure proposed herein, easily measurable changes at the initial stages occur within 10 min.

The findings of optical density changes at 255 mμ, the isosbestic point of substrate and product during the action of the enzyme, suggest the formation of side reactions or of relatively stable intermediates. Alternatively, these changes might in fact be due to the spectrum of the enzyme-substrate complex. The extremely rapid initial rise, immeasurably fast with the equipment used in the studies, may be considered as tending to corroborate the latter hypothesis, at least for this very early stage of the reaction. The subsequent rise and fall of absorbancy may then be due to interaction of desulfurylated and/or desugared intermediates with the enzyme. In this connection, Gaines and Goering [8] in discussing the significance of their results on the separation of the sulfatase and glucosidase mixtures of myrosinase, suggest that in the presence of both enzymes (myrosinase), the residual structure from glucosidase action is bound to the sulfatase factor. In any case, the actual concentration of the components giving rise to this absorbancy must be very small in view of the data obtained at 227.5 mμ. If this is so, then the extinction coefficients of the components are very high indeed.

The enzymatic nature of the changes at 255 mμ are further corroborated by the finding that the rates of change obey simple enzyme kinetics and that the effect of pH on these constants are qualitatively the same as those obtained at 227.5 mμ.

In common with other hydrolytic enzymes, the dramatic change in $K_M$ value in the neighborhood of neutrality (Table 1) suggests the involvement in the enzyme reaction of an ionizing group in the enzyme at pH 6.0 to 7.0.

The data of Fig. 6 corroborates previous reports that ascorbic acid accelerates the myrosinase-sinigrin reaction [4]. In addition, the data strongly suggest that in the presence of the enzyme-sinigrin reaction mixture, the break-down of ascorbic acid (as evidenced by decrease in absorbancy) is significantly retarded. The change in the optical density of the buffered solution of dilute ascorbic acid observed here are in agreement with the results of Mohler and Lohr [9].

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