Because of the comparatively small dimensions the decrease lasts for an hour or so, and because of its well-defined limits both the decrease and the recovery occur very quickly.

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[1] Escobar, I., N. W. Nerurkar, O. Troncoso, E. Maldonado, A. Lazo, and R. Anda, "Cosmic ray intensity variations during magnetic storms of November, 1960," presented at the symposium on Research in Space held in Buenos Aires, November 28–December 5, 1960.

[2] Carmichael, H. and J. F. Steljes, *Phys. Rev. Letters*, **3**, 392 (1959).

[3] McCracken, K. G. (personal communication).

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## *VITAMIN C AS A COENZYME: THE HYDROLYSIS OF MUSTARD OIL GLUCOSIDES*

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The physiological functions of vitamin C have been obscure and reactions in which it participated as a specific coenzyme unknown. Nagashima and Uchiyama[1] recently discovered that the rate of cleavage of sinigrin by an enzyme preparation from yellow mustard increased by 260 per cent on addition of 0.001 *M* L-ascorbate, whereas an equal concentration of the stereoisomeric D-araboascorbate produced a rise of only 50 per cent. With other, crude plant extracts, they found that L-ascorbate could accelerate attack on the same glucoside by factors as large as 100. We came independently to the domain and sketch the result.

Yellow mustard seed (from *Sinapis alba*) contains at least two enzymes that catalyze the same reaction, hydrolysis of mustard oil glucosides (glucosinolates). One enzyme is, like fungal sinigrinase,[2] indifferent to vitamin C and is the classical myrosin. The other enzyme requires the vitamin as cofactor and will be called an ascorbate-activated glucosinolase. General thioglucosidase activity in mustard[3,4] against 2,4-dinitrophenyl β-D-1-thioglucopyranoside or desulfoglucocapparin (S-β-D-1-glucopyranosylacetothiohydroximic acid) is ascorbate-independent. Although the enzymes have not to our knowledge been completely separated, their relative proportion varies immensely between mustard flour and different extracts. The cleavage of sinigrin or sinalbin by whole mustard is accelerated with 0.002 *M* L-ascorbate by a factor of 80. The response from ordinary myrosin preparations is a rate factor of merely four or so,[1,5] but through a changed procedure an enzyme solution can be obtained with the major properties of the native system and an ascorbate effect of 400. The hydrolysis of glucosinolates can be followed by determination of substrate or any product (isothiocyanate, glucose, sulfate, acid). A convenient method for rate studies is automatic titration with alkali at constant pH.

Defatted yellow mustard flour hydrolyzed sinigrin at zero-order rates on the

magnitude of 0.1 μmole/mg flour/min without coenzyme or 10 fully activated. Extraction of the ascorbate-dependent glucosinolase comprised three steps: treatment of the mustard with a thiol (0.07 *M* 2-mercaptoethanol) and alkaline buffer (carbonate) to neutralize the mixture; washes with a dilute salt (0.006 *M* sodium bicarbonate), exhaustive if desired; removal of the enzyme with water. The solution (1.5 mg of protein/ml) contained 40 per cent of the glucosinolase in the flour, purified fifteenfold. Centrifugation at pH 7 and 30,000 *g*, after dialysis or precipitation from 0.01 *M* phosphate, gave supernatants with specific activity in 0.01 *M* sinigrin—0.002 *M* sodium L-ascorbate—0.001 *M* sodium chloride at pH 7 and 25°C of 300 μmoles of glucoside cleaved/mg protein/min. Without vitamin, the activity was 0.7. The Michaelis constant for sinigrin was roughly 0.0015 *M* in 0.002 *M* ascorbate; it was less without as much cofactor. The enzyme was stable in solution at 5° and partly sedimented during 2 hr at 140,000 *g*. By ultraviolet absorption and accordance of biuret color and chromate reduction equivalent, the material was essentially protein.

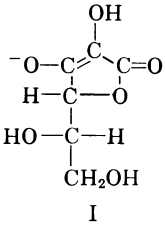
The zero-order rate of sinigrin hydrolysis by glucosinolase was linearly dependent on L-ascorbate concentration from $6 \cdot 10^{-7}$ (doubling the speed compared with blank) to $4 \cdot 10^{-6}$ *M* and followed a Michaelis-Menten equation between $2 \cdot 10^{-4}$ and 0.0015 *M*, with a constant of $2.5 \cdot 10^{-4}$ *M* (25°, pH 7). Over the whole range up to 0.0015 *M*, the velocity agreed to 15 per cent with the Michaelis expression plus the rate without activator, although the full concordance involved the artificial assumption that speed was proportional to enzyme concentration. At more than 0.0015 *M* L-ascorbate, the velocity (then five-sixths of the Michaelis limit) held stationary to 0.003 *M* and thereafter declined, at 0.02 *M* to half maximal. Optimal concentrations increased like the Michaelis constant with temperature or change of cofactor. Spectroscopic and kinetic evidence indicated no disturbing accumulation of any intermediate during the rate studies. The concentrations of cofactor appeared unchanged by the reaction, and the velocities pertained to the enzymatic stage.

Six substances were found for which the relations between cofactor concentration and specific rate of sinigrin hydrolysis with standard enzyme obeyed Michaelis laws with the same upper limiting velocity as for L-ascorbate. The compounds in classical nomenclature, with ratios of the Michaelis constant of L-ascorbate to that of the analogue, were L-rhamnoascorbate (1/6), 6-desoxy-L-ascorbate (1/12), L-glucoascorbate (1/55), D-araboascorbate (1/60), D-erythroascorbate (1/100), and 2-O-methyl-L-ascorbate (1/160), newly obtained as the pure, crystalline acid. Altogether, more than three dozen enediols or derivatives of vitamin C were examined. No substance furnished a higher limiting speed or lower Michaelis coefficient than L-ascorbate, but cofactors with smaller maximal rates appeared possibly to be numerous. The clearest example was 5,6-isopropylidene-L-ascorbate, having a constant of only $5.5 \cdot 10^{-4}$ *M* but an upper velocity 45 per cent of that for L-ascorbate. D-Ascorbate and D-glucoascorbate belonged to a coenzymatically inert group, producing less effect alone than $10^{-4}$ as much L-ascorbate and leaving the activated system undisturbed. Also indifferent were 2-keto-L-gulonic acid, its methyl ester, and the L-hexono-4D-lactones. Dehydroascorbic acid was inactive toward glucosinolase, and ascorbic oxidase could instantly halt the promoted cleavage of sinigrin with as much as $5 \cdot 10^{-4}$ *M* vitamin.

The coenzymatic power of 2-O-methyl-L-ascorbate was notable because it showed that vitamin C was not acting as the conventional reducing agent. With sufficient (0.05 *M*) 2-methyl ether, the rate of sinigrin hydrolysis attained two-thirds of the maximum with L-ascorbate. Addition of the ether to glucosinolase optimally activated with ascorbate caused no increase of velocity and confirmed that the compounds affected the same enzyme and site. The action of 2-O-methyl-L-ascorbate was undeterred by ascorbic oxidase while ascorbate present was destroyed. Likewise, 2-desoxy-L-ascorbate and 3-O-methyl-L-ascorbate were coenzymes, with diminishing efficacy.

The rates of hydrolysis of sinigrin by glucosinolase in $1.5 \cdot 10^{-6}$ or 0.0025 *M* L-ascorbate, otherwise unbuffered, changed less than 20 per cent between pH 6 and 9. The constancy over a pH range between the first and second ionizations of ascorbic acid ($pK_1$ 4.25, $pK_2$ 11.6) demonstrated that the singly charged anion was the effective species. Activities of 2-O-methyl-L-ascorbic acid (pK 3.4) and 2-desoxy-L-ascorbic acid (pK 3.6) were similarly insensitive, and 3-O-methyl-L-ascorbic acid (pK 7.8) showed a tenfold increase of coenzymatic power from pH 6 to 8.

L-Ascorbate appears to act through rapid, reversible formation of a ternary complex with glucoside and enzyme. The reaction starts without induction period when vitamin or glucosinolase are added and promptly stops when the coenzyme is selectively oxidized. Variation of zero-order rate with ascorbate concentration corresponds to a Michaelis-Menten relationship. The existence of six analogues giving the same limiting velocity as L-ascorbate but larger Michaelis constants can most simply be attributed to combination with lower affinities for enzyme plus substrate but at the same site to form the identical catalytic configuration. Inactive compounds like D-ascorbate are not bound. Mutual influence of coenzyme and substrate is shown by dependence of the Michaelis constant of D-arabo-ascorbate on the side chain of the glucosinolate (sinigrin or sinalbin). The increase in the Michaelis constant of sinigrin with L-ascorbate concentration near the coenzymatic coefficient presumably reflects transition from dominant reversible binary combination of sinigrin and enzyme to union of glucoside with the enzyme-ascorbate complex.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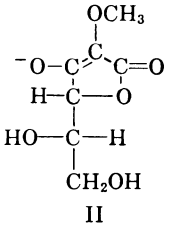
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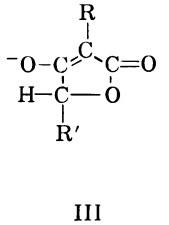
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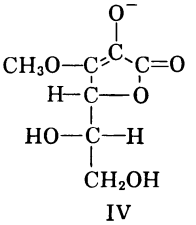

IV

Since the maximal velocity produced by L-ascorbate (I) is approached also with 2-O-methyl-L-ascorbate (II), the 2-hydroxyl group is important for combination with enzyme-substrate but is not a catalytic center. The activity of 5,6-isopropylidene-L-ascorbate, D-erythroascorbate and DL-2-hydroxy-4-methyl-tetronate shows that the side chain hydroxyl groups are not critically reactive. The functional unit is a tetronate ion (III) with 2- and 4-substituents that enhance affinity for the enzyme. The requirement for (III) is not absolute, since other

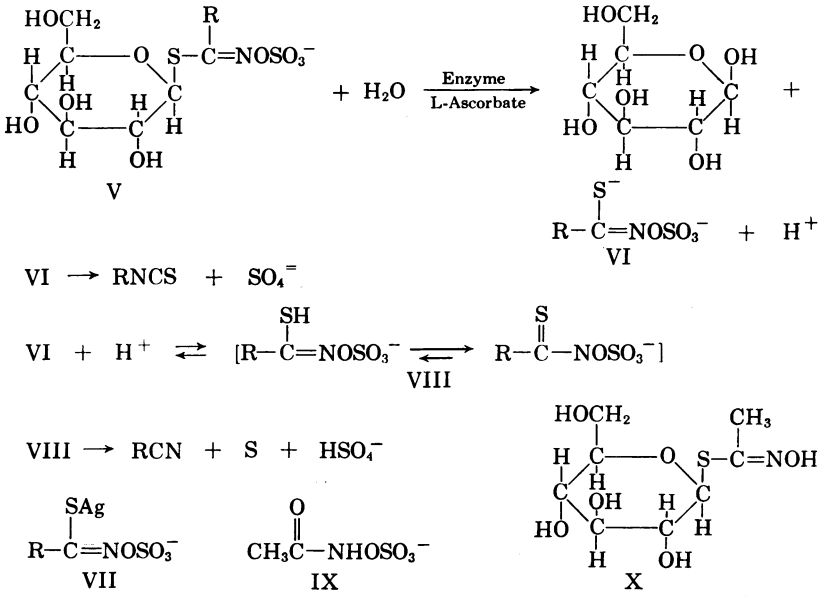
anions resembling (I) have effect, in particular 3-O-methyl-L-ascorbate (IV), which can furnish one tenth of the maximal rate of (I). The general appellation for ions like (III) and (IV) is enolate or base.

Hydrolysis of sinigrin with glucosinolase and L-ascorbate at neutrality gave allyl isothiocyanate, β-D-glucose, sulfate and acid at approximately equivalent rates. The velocity diminished at pH below 6 but to 3.5 it was with sufficient vitamin at least one-tenth of optimal and 50 times faster than without cofactor. With pH 5 or less, allyl cyanide and sulfur[6] appeared among the products, and at pH 3.5–3.6 the routes to element plus nitrile or to mustard oil competed equally. Hydrogen sulfide was also evolved in acid[7] but seemed quantitatively unimportant. The ratio of nitrile or sulfur to isothiocyanate was crudely proportional to hydrogen ion concentration.

During the ascorbate-promoted neutral hydrolysis, temporary absorption appeared that was most obvious at 255 mμ, where starting material and final products had almost equal extinctions.[5] The extra absorbance (0.5-mm light path) ranged up to 0.6 with velocity of glucoside cleavage slightly below 0.003 $M$/min. When the transient was maximal, the rate of cleavage of sinigrin could be measured (at 228 mμ)[5] without interference. With temperature fixed, the ratio of largest absorption or amount of intermediate to concurrent speed was a constant independent of enzyme or ascorbate concentrations or buffer (acetate, phosphate or carbonate, pH 5–9.4). The steady-state condition implied that the absorbing intermediate, formed by the enzymatic process, decomposed in a first-order reaction not involving enzyme, cofactor or other ions. The half-life could be estimated from the rate of appearance at the start, on the assumption that the velocity of formation was constant, or the fall after the enzymatic reaction was quenched by ascorbic oxidase. The probable result at 20°, under allowance that decay appeared slower than rise, within a factor of two was 25–30 sec. The data sufficed, if one mole of sinigrin generated one of intermediate, to calculate its extinction coefficient (log $\epsilon$ 3.9 at 255 mμ; $\lambda_{max}$ 245–250 mμ, log $\epsilon$ 4.0) and concentration.

When a solution containing unstable intermediate was suddenly acidified to pH 2, the enzymatic reaction stopped at once and the substance disappeared within a minute. Sulfur was produced, and from the amount or more accurately from the difference between total sinigrin consumed and isothiocyanate formed, the kinetically inferred concentration of intermediate could be verified.

Since the transient spectral effect diminished beyond 255 mμ and the considerable absorption by L-ascorbate in the 290-mμ region was unchanged during reaction, the cofactor did not appear to furnish part of the intermediate. Furthermore, the intermediate could much exceed ascorbate as well as enzyme. The intermediate therefore is simply a fragment of sinigrin (potassium myronate or allylglucosinolate; V, R = $H_2C{=}CHCH_2$) and is regarded as the sinigrate or vinylacetothiohydroxamate-O-sulfonate ion (VI, R = $H_2C{=}CHCH_2$), the basic form of the aglucone. A model for the spectrum is the absorption maximum of phenylacetothiohydroxamate in methanol at 247 mμ (log $\epsilon$ 3.8).[8] Silver sinigrate (VII, R = $H_2C{=}CHCH_2$) is a well-known[4, 9] product from sinigrin and silver nitrate and is decomposed to isothiocyanate by neutral nucleophiles (thiosulfate, warm chloride) that combine with silver liberating (VI), but to nitrile and sulfur by corresponding acidic reagents (hydrogen sulfide, hydrochloric acid). Enzymatic formation of




(VI) thus explains the observed products. Since the rate of decomposition of the intermediate is unaltered from pH 9 to 5 and appears faster at 2, yet at 3.5 almost half of the material goes through the same reaction as at neutrality, the effective $pK_a$ of vinylacetothiohydroxamic acid-O-sulfonate (VIII, R = $H_2C{=}CHCH_2$) is 3.5 or less. Acetohydroxamic acid-O-sulfonate (IX), which undergoes Lossen rearrangement at pH 9 to isocyanate (eventually N,N'-dimethylurea) with a half-life of 20–25 minutes at 25°, has $pK_a$ 8.0 (cf. acetohydroxamic acid,[10] pK 9.4). If acidifying influences of the vinyl group and particularly of the divalent sulfur atom[11] are superimposed on (IX), it is indeed plausible to assign to (VIII, R = $H_2C{=}CHCH_2$) a $pK_a$ on the order of 3. An outstanding feature of mustard oil glucosides is the great acidity of the aglucones.

Ascorbate-activated glucosinolase is a specific thioglucosidase, and the Lossen rearrangement of (VI) is proved under our conditions to be nonenzymatic. Since desulfoglucocapparin (X) is neither substrate nor inhibitor of glucosinolase, the sulfate group of (V) is important for combination with the enzyme as well as for electron withdrawal. That the enzymatic reaction consists in displacement of a strongly electron-attracting group from the 1-glucosyl residue supports the idea that the coenzyme acts as a base or nucleophile. The ion (VI) liberated is less basic than ascorbate. No evidence exists of a glucosyl-enzyme or glucosyl ascorbate intermediate, but the net retention of anomeric configuration suggests two nucleophilic displacements at the 1-position,[12] first by enzyme-ascorbate, then by water. We conclude that ascorbate behaves toward the thioglucosidase as a reversibly dissociable base, closely connected with the active nucleophilic group.

The ascorbate ion (I) is stable over a wide pH range surrounding neutrality. The negative charge is distributed over the 1- and 3-oxygen atoms, as reaction of the acid with diazomethane witnesses, and 2-carbon atom besides. Amid the basic cloud is the 2-hydroxyl group. We recall the catalytic power, emphasized by Swain and Brown,[13] that can belong to molecules with neighboring acidic and

basic centers. The high reactivity toward acyl halides of a phenolate ion with an *o*-hydroxyl group[14] is independent of any oxidation of the catechol and specially relevant to (I). Besides the sort of coenzymatic reaction now described, involving the acid group of (I) only in the attachment to enzyme, we can anticipate processes in which important acidic catalysis, as well as basic, is exerted on substrate. The coexistence of both functions in the ascorbate system is accompanied by the reducing power, and effective inhibition of enzyme and (I) by the methyl ethers (II and IV) would be an ambiguous criterion of mechanism. The present example is valuable to make clear that the reducing action of ascorbate need not be its major property. The oxidation-reduction equilibrium can serve as means to govern the level of an acid-base catalyst.

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[1] Nagashima, Z., and M. Uchiyama, *Bull. Agr. Chem. Soc. Japan*, **23**, 555, A75, A82 (1959); *Nippon Nôgei-kagaku Kaishi*, **33**, 881, 980 (1959); Nagashima, Z., M. Uchiyama, and S. Nishioka, *ibid.*, **33**, 723 (1959); *Bull. Agr. Chem. Soc. Japan*, **23**, A63 (1959).

[2] Cf. Reese, E. T., R. C. Clapp, and M. Mandels, *Arch. Biochem. Biophys.*, **75**, 228 (1958).

[3] Goodman, I., J. R. Fouts, E. Bresnick, R. Menegas, and G. H. Hitchings, *Science*, **130**, 450 (1959).

[4] Nagashima, Z., and M. Uchiyama, *Nippon Nôgei-kagaku Kaishi*, **33**, 1144 (1959); *Bull. Agr. Chem. Soc. Japan*, **24**, A11 (1960).

[5] Cf. Schwimmer, S., *Acta Chem. Scand.*, **15**, 535 (1961).

[6] Cf. Gmelin, R., and A. I. Virtanen, *Suomen Kemistilehti, B*, **34**, 15 (1961); *Ann. Acad. Sci. Fennicae, Ser. A II*, No. 107 (1961).

[7] Schwimmer, S., *Acta Chem. Scand.*, **14**, 1439 (1960).

[8] Lundeen, A. J., Ph.D. thesis, Rice Institute, 1957.

[9] Will, H., and W. Körner, *Ann. Chem. (Liebigs)*, **125**, 257 (1863); Gadamer, J., *Arch. Pharm.*, **235**, 44 (1897); *ibid.*, **237**, 507 (1899).

[10] Wise, W. M., and W. W. Brandt, *J. Am. Chem. Soc.*, **77**, 1058 (1955).

[11] Hofmann, A. W., *Ber. deut. chem. Ges.*, **10**, 1095 (1877); Leo, H., *ibid.*, **10**, 2133 (1877); Hine, J., and M. Hine, *J. Am. Chem. Soc.*, **74**, 5266 (1952).

[12] Koshland, D. E., Jr., *Biol. Revs. Cambridge Phil. Soc.*, **28**, 416 (1953).

[13] Swain, C. G., and J. F. Brown, Jr., *J. Am. Chem. Soc.*, **74**, 2538 (1952).

[14] Jandorf, B. J., T. Wagner-Jauregg, J. J. O'Neill, and M. A. Stolberg, *J. Am. Chem. Soc.*, **74**, 1521 (1952); Epstein, J., D. H. Rosenblatt, and M. M. Demek, *ibid.*, **78**, 341 (1956); Churchill, J. W., M. Lapkin, F. Martinez, and J. A. Zaslowsky, *ibid.*, **80**, 1944 (1958).