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Reaction of Sorbic Acid with Nucleophiles: Preliminary Studies

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ABSTRACT

Mercaptoethanol and cysteine were found to react slowly with sorbic acid when heated over several days at 80°C, pH 3·7–7·8. At pH 3·7, both reactions were of overall second order ($k_2 = 0{\cdot}48$ and $0{\cdot}65\,\mathrm{M}^{-1}\,h^{-1}$, respectively) and it is suggested that cysteine behaves as a sulphur-nucleophile. The reaction of cysteine with sorbic acid is shown to proceed with 1:1 stoichiometry. A product isolated from the reaction involving mercaptoethanol gives rise to mass spectroscopic data consistent with nucleophilic attack at position 5 of sorbic acid to give also, the 1:1 adduct. The effect of pH on rate of reaction is small.

*Amines (*n*-butylamine,* t*-butylamine and glycine) were found to be unreactive under the same conditions.*

INTRODUCTION

There is much literature on the uses of sorbic acid in food applications but little is known of its chemical reactivity. The most widely studied reactions are those with nitrite ion (Namiki *et al.*, 1981) and autoxidation (Seow & Cheah, 1985) but the conjugated dienoic acid structure of the additive is susceptible to nucleophilic attack. Examples of known nucleophilic reactions include that with sulphite ion (Hägglund & Ringbom, 1926) and with amines (Kheddis *et al.*, 1981). Studies of the latter reaction involved the heating of sorbic acid with amines under vigorous conditions (200°C, 6 h) when dihydropyridones (I) and (II) were formed as a result of nucleophilic

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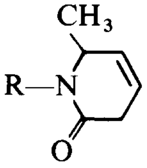
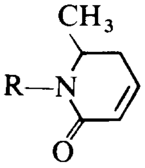


I



II

addition at C_5 followed by cyclisation. Higuchi (1976) suggests that sorbic acid also reacts with cysteine.

The expectation that sorbic acid should react readily with nucleophiles and the widespread occurrence of such compounds (e.g. amines and thiols) in foods makes an investigation of their reactions important and timely because of the increasing use of sorbic acid as a food additive. In this paper we report preliminary studies on the reactions of sorbic acid with amines and thiols.

EXPERIMENTAL

All reagents were obtained from BDH Chemicals Ltd, Poole and, where possible, were of AnalaR grade. Unless otherwise stated, reaction mixtures contained sorbic acid (0·01M) and nucleophile (0·01M) and were buffered in the range pH 3·7–7·8 with acetate or phosphate buffers (1M). The kinetics of the loss of sorbic acid were followed by measuring absorbance at 254 nm and concentrations calculated from calibration data obtained at each pH value used. All reactions were carried out at 80°C in stoppered flasks and aliquots of reaction mixture withdrawn at timed intervals and diluted with water, as required, prior to measurement of absorbance. Since sorbic acid degrades spontaneously or undergoes autoxidation in aqueous solution, control experiments containing no nucleophile were also carried out.

The stoichiometry of the reaction was determined for cysteine as the nucleophile. For analysis of sorbic acid an aliquot of reaction mixture was diluted 10 times with water and the resulting solution (10 μl) subjected to analysis by hplc (Spherisorb 5-ODS reverse phase column with water:acetonitrile, 7:3 solvent containing sodium acetate (0·1 M), adjusted to pH 5·4 with acetic acid) and column effluent was monitored at 254 nm (Varian UV-50). The sorbic acid peak was eluted after 15 min at a flow rate of 0·9 ml min^{-1}. The concentration of cysteine was measured by hplc analysis of its 2,4-dinitrophenyl derivative. An aliquot (50 μl) of reaction mixture was mixed with aqueous solutions of sodium carbonate (200 μl, 10 g litre^{-1}) and dithiothreitol (100 μl, 10 g litre^{-1}) and water (180 μl) added. The mixture was warmed at 37°C for 30 min when an aqueous solution of iodoacetic acid was added (50 μl, 93 g litre^{-1}). After 30 min at room temperature 2,4-dinitrofluorobenzene (10 μl) was added and the mixture heated at 30°C for 80 min with vigorous shaking. Excess reagent was extracted by adding HCl (50 μl, 6M) and toluene (0·5–1·0 ml) and shaking for 4 min. The mixture was allowed to stand overnight at 4°C for separation and the lower aqueous phase (10 μl aliquot) subjected to hplc analysis (Spherisorb 5-ODS reverse phase column maintained at 44°C, eluted with water:acetonitrile, 96:4, containing sodium acetate (0·01M) adjusted to pH 3·65 with acetic acid, as the first solvent for 10 min followed by methanol:water, 7:3). Column effluent was monitored at 375 nm (Varian UV-50) at a flow rate of 1·3 ml min^{-1}.

Preparative scale reactions involved heating a mixture of reactants (0·25 mol of each) in water (1 l) for *c.* 4 weeks at 80°C. The mixture was steam distilled and a product recovered by crystallising the oily residue.

RESULTS

Kinetic measurements

The progress of the reaction of sorbic acid with nucleophiles was followed at the $\lambda_{\max}$ for sorbic acid (254 nm) in the expectation that addition took place with loss of at least one of the conjugated double bonds. In the absence of nucleophile the absorbance of reaction mixtures (initial concentration of sorbic acid = 0·01M) held at 80°C was monitored for 1260 h. At pH > 6 the absorbance decreased by approximately 8% of its initial value over this period of time whilst at pH < 6 the loss became progressively larger with decreasing pH. The change was followed to 63% reduction in absorbance at pH 3·7 and was found to conform well to first order kinetics ($k_1 = 7{\cdot}9 \times 10^{-4}$ and $5{\cdot}6 \times 10^{-4}\,\mathrm{h}^{-1}$ at pH 3·7 and 4·7, respectively).

When reaction mixtures contained equimolar concentrations of sorbic acid and nucleophile (0·01M), no reaction could be observed at 80°C when the nucleophile was *n*-butylamine or glycine in the range pH 3·7–7·8. Indeed, it appeared that even the spontaneous loss of sorbic acid observed at the lower end of the pH range, had been suppressed. On the other hand, a slow loss of sorbic acid was found when *t*-butylamine was used as the nucleophile. In this case, kinetic data obtained at pH 4·5 conformed well to first order kinetics with a rate constant of $5{\cdot}0 \times 10^{-4}\,\mathrm{h}^{-1}$ which is seen to be very similar to that found ($5{\cdot}6 \times 10^{-4}\,\mathrm{h}^{-1}$) for the spontaneous loss of sorbic acid at pH 4·7. The low solubilities in water of simple amines did not allow higher concentrations to be used but the experiments were repeated at initial concentrations of sorbic acid and glycine of 0·1M. It is reasonable to expect that any true nucleophilic attack on sorbic acid will be of first order with respect to each reactant (i.e. a tenfold increase in concentration of each reactant would represent a hundredfold increase in rate of reaction) but no reaction could be detected.

Mercaptoethanol was chosen as a simple thiol and cysteine as a molecule with an amino and a thiol group. The progress of the change in absorbance at 254 nm of reaction mixtures as a function of pH and reaction time for the sorbic acid-mercaptoethanol reaction is shown in Fig. 1 for initial reactant concentrations of 0·01 M. At pH 3·7 the data cover approximately 85% of the reaction and are found to obey second order kinetics ($k_2 = 0{\cdot}48\,\text{M}^{-1}\,\text{h}^{-1}$) as shown by the second order plot (graph of 1/(absorbance) vs time) in Fig. 2. A first order plot was not linear. The data obtained at higher pH values did not conform to first or second order kinetics. It is evident from Fig. 1 that the rates of loss of sorbic acid during the first 113 h are relatively unaffected by pH whilst at longer times the rate of change of absorbance decreases as pH is increased in the range pH 3·7–5·7. At long reaction times it was found that reaction mixtures had undergone browning, particularly at the higher pH values, and this may be responsible for the observed increase in absorbance at 254 nm with pH of such reaction mixtures. A feature of these results is, however, the small pH dependence of the rate in the early stages of reaction.

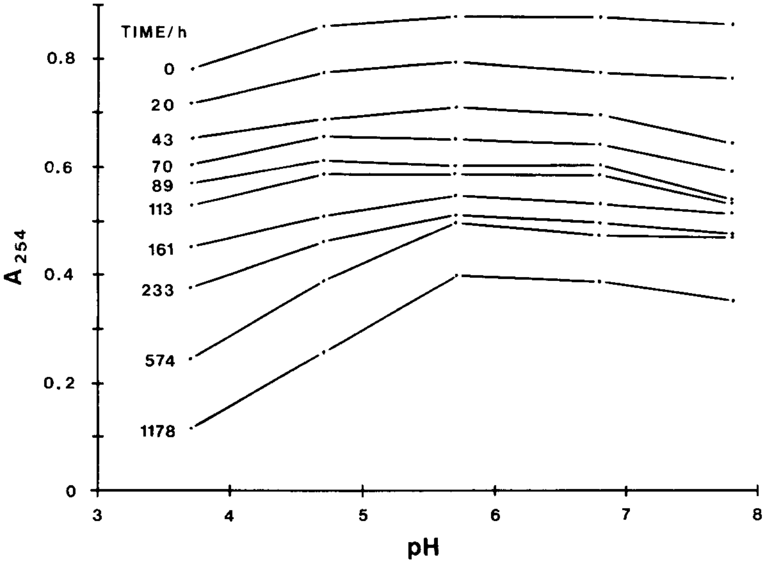


Fig. 1. The effect of pH and time on absorbance at 254 nm (A_{254}) of mixtures of sorbic acid and mercaptoethanol. Initial conditions: [sorbic acid] = [mercaptoethanol] = 0·01 M; 80°C; Buffer: 1 M acetate (pH 3·7–5·7) or 1 M phosphate (pH 6·8–7·8).

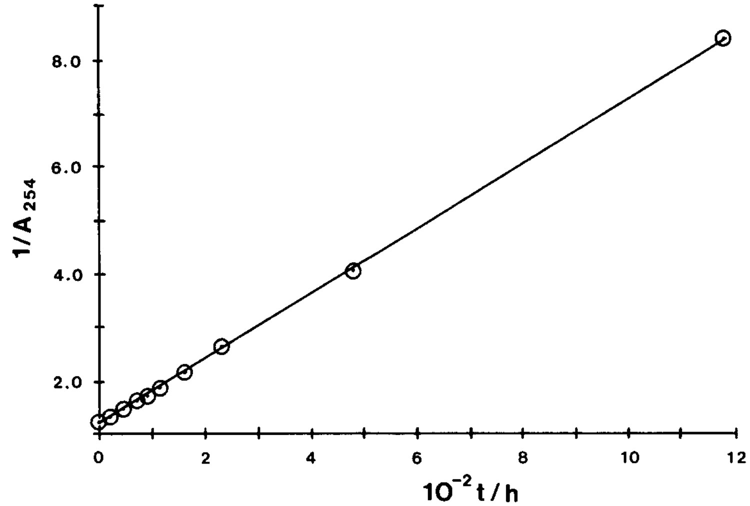
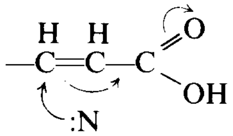


Fig. 2. Second order plot for the reaction of sorbic acid with mercaptoethanol. Data plotted as $1/A_{254}$ vs time where A_{254} is the absorbance due to sorbic acid. Initial conditions: [sorbic acid] = [mercaptoethanol] = 0·01M; pH 3·7, 1M acetate buffer; 80°C.

The addition of nucleophiles to α,β-unsaturated carboxylic acids is directed by the acid group as follows:



and this directing effect is lost when the acid becomes ionised. If the reaction in question is between an acid and a base then it can be shown that the rate of reaction will be a maximum at a pH value given by

$$\mathrm{pH} = (\mathrm{p}K_{a1} + \mathrm{p}K_{a2})/2$$

where $\mathrm{p}K_{a1}$ and $\mathrm{p}K_{a2}$ are, respectively, the $\mathrm{p}K_a$'s of the acid and conjugate acid of the base. The $\mathrm{p}K_a$ of sorbic acid and mercaptoethanol are, respectively, 4·51 (Serjeant & Dempsey, 1979) and 9·7 (Martell & Smith, 1977). Hence, at all pH values studied the thiolate anion, which is expected to be the nucleophile, will be present in trace amounts and its concentration will increase tenfold for every increase in unit of pH. On the other hand, the concentration of sorbic acid will decrease approximately tenfold for every unit of pH above pH 4·7. The net result is that, since the $\mathrm{p}K_a$'s of sorbic acid and mercaptoethanol are far apart, the effects of the ionisation of the acid and thiol on the rate nearly balance each other and the graph of rate as a function of pH is expected to show a very broad maximum.

The progress of the sorbic acid–cysteine reaction as a function of pH is

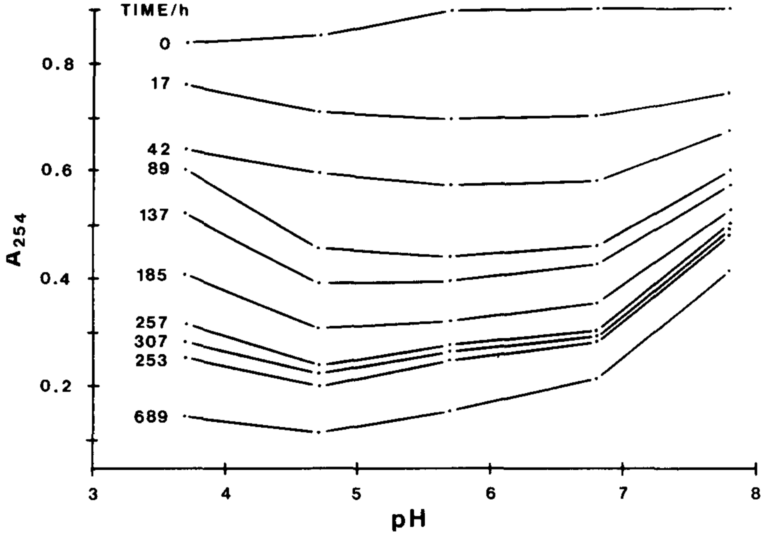


Fig. 3. The effect of pH and time on absorbance at 254 nm (A_{254}) of mixtures of sorbic acid and cysteine. Initial conditions: [sorbic acid] = [cysteine] = 0·01M; 80°C. Buffer: 1M acetate (pH 3·7–5·7) or 1M phosphate (pH 6·8–7·8).

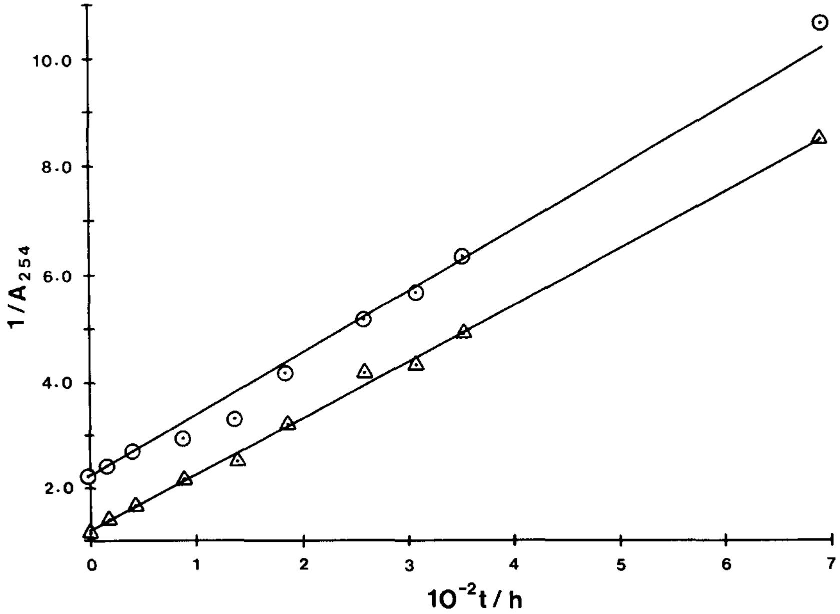


Fig. 4. Second order plots for the reaction of sorbic acid with cysteine at pH 3·7 ⊙ and 4·7 △. Data plotted as $1/A_{254}$ vs time where A_{254} is the absorbance due to sorbic acid. The line for pH 3·7 is displaced by one unit of $1/A_{254}$ for clarity. Initial conditions: [sorbic acid] = [cysteine] = 0·01M; 1M acetate buffer; 80°C. The actual intercept at $t = 0$ is 1·2 (absorbance units)$^{-1}$.

illustrated in Fig. 3 for initial reactant concentrations of 0·01 M at 80°C. In contrast to the reaction involving mercaptoethanol the rate appears to pass through a more clearly defined maximum in the range pH 4·7–6·8 and kinetic data obtained at pH 3·7 and 4·7 follow second order kinetics as shown in Fig. 4. The second order rate constant at pH 3·7 was found to be $0{\cdot}65\mathrm{M}^{-1}\,\mathrm{h}^{-1}$ and is clearly of the same order of magnitude as that for the reaction involving mercaptoethanol. It is suggested, therefore, that cysteine reacts as a sulphur-nucleophile in this reaction.

Stoichiometry

The stoichiometry of the sorbic acid–cysteine reaction was found by measuring the amounts of both reactants lost at various reaction times. Sorbic acid was measured directly by reverse phase hplc whilst cysteine was determined as its 2,4-dinitrophenyl derivative. The analytical procedure for the latter involved the addition of dithiothreitol to cleave any cystine which may have been formed by oxidation of cysteine. All thiol groups were subsequently protected by forming a derivative with iodoacetic acid before a coloured derivative was formed with 2,4-dinitrofluorobenzene. Concentrations of both reactants were calculated directly from peak areas using calibration graphs prepared using known concentrations of sorbic acid and cysteine. A summary of the observed stoichiometries is given in Table 1, where each data point is the mean of three independent determinations. It is evident that the reactants are combining in a ratio close to 1:1 at pH <6·8, but the amount of cysteine bound at pH 7·8 is consistently higher at all reaction times. Control experiments containing no sorbic acid showed no significant loss of cysteine over 161 h at 80°C, pH 3·7 and 4·7 but losses of 10%, 40% and 65% were evident for controls carried out, respectively, at pH 5·7, 6·8 and 7·8. The kinetics of this loss are unknown and the extent to which it takes place in the presence of sorbic acid is unclear but it is possible that the high stoichiometry at the highest pH could be due to losses of cysteine by mechanisms other than reaction with sorbic acid. In contrast, no loss of sorbic acid could be measured in controls containing no cysteine after 168 h.

The analysis procedure ensures that any cystine formed in reaction mixtures is measured as cysteine. Since it is relatively easy for cysteine to undergo autoxidation, it is necessary to examine the reactivity of cystine in this system. In order to compare the reactivity directly with reactions containing initial reactant concentrations of 0·01M, the cystine concentration was made up to be 0·005M. The amounts of sorbic acid and cystine lost after 144 h give stoichiometries (cystine:sorbic acid) of 0·45–0·61:1 at pH 3·7–6·8 and a stoichiometry of 1·32:1 at pH 7·8. The order of magnitude

TABLE 1

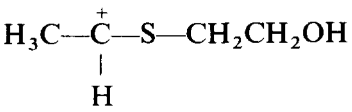
Stoichiometry of the Reaction of Sorbic Acid with Cysteine. Initial Conditions: [sorbic acid] = [cysteine] = 0·01M; 80°C. Buffers: 1M Acetate (pH 3·7–5·7) or 1M Phosphate (pH 6·8–7·8).

Reaction time (h)	*pH*	*% Sorbic acid lost*	*% Cysteine lost*	*Stoichiometry cysteine/sorbic acid*
41	3·7	17·2	20·4	1·19
	4·7	40·5	34·4	0·85
	5·7	44·2	37·5	0·85
	6·8	38·6	35·0	0·91
	7·8	31·2	59·0	1·89
65	3·7	28·4	25·0	0·88
	4·7	48·3	47·4	0·98
	5·7	58·5	52·4	0·90
	6·8	60·0	44·4	0·74
	7·8	55·1	76·4	1·39
113	3·7	52·8	57·8	1·09
	4·7	61·4	63·0	1·03
	5·7	68·5	75·0	1·09
	6·8	65·8	65·0	0·99
	7·8	64·4	87·0	1·35
161	3·7	74·7	70·4	0·94
	4·7	79·9	78·8	0·99
	5·7	79·0	83·0	1·05
	6·8	74·6	87·4	1·17
	7·8	67·2	88·6	1·32

of the amount of reactants lost is similar to that shown in Table 1 at the 65 h reading.

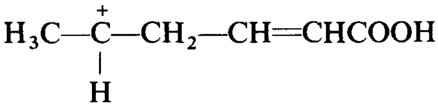
Nature of reaction products

An impure reaction product was isolated from the sorbic acid–mercaptoethanol system. Accurate mass measurements in the mass spectrum of this product confirmed the presence of the monoadduct, found *m/e* 190·0662 (required for the molecular ion $C_8H_{14}O_3S$, *m/e* 190·0664). The likelihood that the mercaptoethanol had added to position 5 is supported by the observation of a fragment at *m/e* 105·0370 (required for C_4H_9OS, *m/e* 105·0374) which is consistent with the following structure:



$$H_3C\text{—}\underset{\textstyle H}{\overset{+}{C}}\text{—}S\text{—}CH_2CH_2OH$$

Also, a fragment at *m/e* 133·0595 (required for $C_6H_9O_2$, *m/e* 133·0603) may be assigned to:



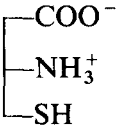
$$H_3C{-}\overset{+}{\underset{H}{C}}{-}CH_2{-}CH{=}CHCOOH$$

as shown or with the double bond in position 3. This ion could arise from the loss of $—S—CH_2CH_2OH$ from the molecular ion. We have no data concerning the yield of the sorbic acid–mercaptoethanol adduct and no product of sufficient purity has yet been isolated from the sorbic acid–cysteine reaction.

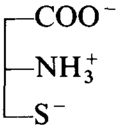
DISCUSSION

The observation that amines are less reactive towards sorbic acid than are thiols is consistent with the much lower nucleophilicity of amines (Davis, 1968). The thiolate ion is likely to be the most powerful nucleophile present naturally in foods. It is established here that the addition reaction involving thiols has a stoichiometry of 1:1 and is the result of a bimolecular attack, probably at position 5 of sorbic acid. A bimolecular mechanism has been observed for the addition of sulphite ion to several α,β-unsaturated carboxylic acids and sorbic acid (Hägglund & Ringbom, 1926).

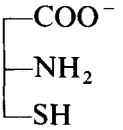
The second order rate constants obtained for the reaction of sorbic acid with mercaptoethanol and cysteine are expressed in terms of the total concentration of sorbic acid + sorbate ion and thiol + thiolate ion. At pH 3·7, a 0·01M solution of 'sorbic acid' and 'mercaptoethanol' contains 0·0087M sorbic acid and 10^{-8}M thiolate ion. The rate constant in terms of the undissociated acid and thiolate ion is, therefore, $0{\cdot}48/(0{\cdot}87 \times 10^{-6}) = 5{\cdot}5 \times 10^{5}\text{M}^{-1}\,\text{h}^{-1}$. The situation is less clearly defined for the reaction involving cysteine. The carboxylate ion of this amino acid may exist in any of the following four forms (III–VI):



III



IV



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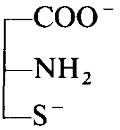
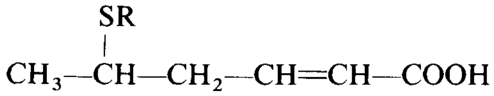


VI

Similarly, there are four combinations of amine/protonated amine and thiol/thiolate for the carboxylic acid form of cysteine. The pK_a values of —SH differ according to whether the proton is lost from structure III or V (8·64 and 10·40, respectively) whilst the ionisation of the NH_3^+ group is similarly affected by the state of the thiol group (pK_a 8·74 and 10·50 for ionisation of III and IV, respectively) (Wrathall *et al.*, 1964). The net result is that, at any pH, similar concentrations of IV and V are present and the conversion of III to IV + V has an overall pK_a of 8·39. The formation of VI has pK_a 10·76. Thus, at pH 3·7, a 0·01 M solution of cysteine contains approximately 10^{-7} M thiolate ion and, on this basis, the rate constant ($7{\cdot}5 \times 10^4 \text{M}^{-1}\text{h}^{-1}$) for the reaction of sorbic acid with the thiolate form of cysteine is approximately seven times smaller than for the reaction with the thiolate form of mercaptoethanol. This treatment of data does not allow for the presence of the protonated carboxyl group of cysteine (pK_a *c.* 2·4, Graham *et al.*, 1972); approximately 5% of the cysteine is in the form of the undissociated acid.

The ability of cystine to react with sorbic acid at a significant rate cannot be explained but it was shown that the apparent stoichiometry of the reaction of cysteine with sorbic acid is not affected by the oxidation of some of the cysteine to cystine.

The position on sorbic acid at which H^+ adds to complete the reaction may be 2 (2,5-addition) or 4 (4,5-addition). Hägglund & Ringbom (1926) suggest the former and it is found that this type of addition normally occurs with dienes which are not conjugated to an electron attracting or withdrawing group, e.g. the addition of bromine to butadiene. This may be explained in terms of resonance. On the other hand, structure VII, which is the result of 4,5-addition, retains a double bond conjugated with the acid group and is presumably of lower energy than that with an isolated double bond in position 3.



$$CH_3{-}\overset{\displaystyle SR}{\underset{}{CH}}{-}CH_2{-}CH{=}CH{-}COOH$$

VII

The product with structure VII is also capable of adding a second thiolate ion in position 3. Whilst no conclusive evidence for such a diadduct has yet been obtained, preliminary work on reaction mixtures containing sorbic acid : mercaptoethanol, molar ratio 1:2, shows the formation of a product with sufficient sulphur (by microanalysis) for it to be ascribed to such a diadduct. However, this is not reflected in the results shown in Table 1 for cysteine (molar ratio sorbic acid : cysteine, 1:1). At long reaction times, when a high percentage of sorbic acid has been converted to the 1:1 adduct, the probability of the nucleophile colliding with a sorbic acid molecule is less than with a molecule of product. This would allow the formation of a diadduct if position 3 was reactive. It is interesting to note that Hägglund & Ringbom (1926) found that sorbic acid reacted with sulphite ion more slowly than did crotonic acid ($CH_3CH{=}CHCOOH$) which confirms that a single double bond conjugated to the carboxyl group is indeed reactive towards nucleophilic attack.

CONCLUSION

The data reported in this paper confirm the expected reaction between sorbic acid and thiols but the conditions required for changes to be observed on a time scale of days (when concentrations of reactants are 0·01M) are vigorous (80°C) compared with those found during the storage of food. If a 'normal' activation energy of the order of 120 kJ mol^{-1} for a reaction involving the making and breaking of covalent bonds is assumed, the rate of loss of reactants will be at least 10^3 times slower at 20°C. Thus, it is expected that any reaction between sorbic acid and thiols in foods will be slow unless the food is heated to a high temperature. Any reaction between sorbic acid and amines is much slower on the same time scale.

The main difficulty in estimating the rates of these reactions is to be able to state reliably the concentrations of reactants. Carboxylic acids are known to partition into non-aqueous phases and may be concentrated in micelles formed by surfactants. The concentration of free sorbic acid is, therefore, not only a function of pH but also the presence of other components. The potentially reactive thiols may be located on peptides or proteins; these may differ considerably in their polarity and hence offer a different environment for the reaction with the hydrocarbon chain of sorbic acid. The contribution of the type of reaction identified in this work to the fate of sorbic acid in food is now being considered.

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