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# DEGRADATION OF 2-HYDROXYBUT-3-ENYLGLUCOSINOLATE (PROGOITRIN)

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**Key Word Index**—Glucosinolates; 2-hydroxybut-3-enylglucosinolate; progoitrin; thioglucoside glucohydrolase.

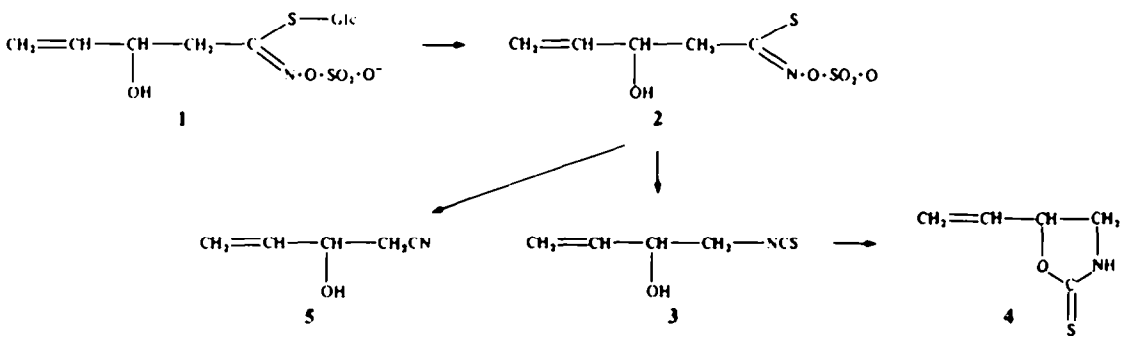
**Abstract**—Using a model system consisting of synthesized 2-hydroxybut-3-enylglucosinolate and a purified thioglucoside glucohydrolase preparation from *Brassica napus*, the effects of ascorbate, of $Fe^{2+}$ and of $Cu^{2+}$ were examined on the extent and course of glucosinolate degradation. Ascorbate was found to promote thioglucosidase activity to a considerable extent over the whole of the wide range of concentrations studied (0.024–400 mM), with a maximum (activation factor *ca* 100) at 1.57 mM ascorbate. $Fe^{2+}$ slightly suppressed reaction, but caused a significant effect by directing degradation to 1-cyano-2-hydroxybut-3-ene rather than to 5-vinyloxazolidine-2-thione, and at low, catalytic, concentrations. $Cu^{2+}$ had similar effect, but was a strong inhibitor of the reaction.

## INTRODUCTION

Glucosinolates degrade enzymically to yield a variety of products, the most common being isothiocyanates and nitriles. 2-Hydroxybut-3-enylglucosinolate, also known by the trivial name progoitrin (**1**, Scheme 1), produces an isothiocyanate which is unstable and which cyclizes spontaneously to give goitrogenic 5-vinyloxazolidine-2-thione (**4**, Scheme 1). Progoitrin is the main glucosinolate of rape (*Brassica napus*), and by this enzymic reaction it is thus responsible for the goitrogenic properties of the plant. Although this important reaction has been the subject of much study, no genuine model system has been reported to date. Therefore, to investigate this reaction properly in a model system, we have synthesized 2-hydroxybut-3-enylglucosinolate [1], and recently we described the isolation of a purified thioglucoside glucohydrolase (E.C. 3.2.3.1) fraction from seeds of *B. napus* cv panter [2]. Ascorbate and certain metal ions are well known to affect the enzymic reaction, and this paper describes our investigations of these factors in our model system.

The activation of thioglucosidase by ascorbate has been known for some time and is well reported, e.g. [3–9], although it is not universal. For example, Vose separated the thioglucosidase of *Sinapis alba* into two isoenzymes, but found that although one was strongly activated by ascorbate the other was unaffected [7]. Similarly, the fungus *Aspergillus sydowi* contains a thioglucosidase that is not activated by ascorbate [10], whilst that of *Enterobacter cloacae* was actually inhibited at all concentrations of ascorbate examined [11], and it may be that there is a major difference here between plant and microbial thioglucosidases. Nevertheless, the majority of the thioglucosidases of plants which have been studied in this respect have been found to be activated by ascorbate, although the extent seems to vary, sometimes being very marked but in other cases being barely significant. Activation factors calculated from data in the literature range from as high as *ca* 50 for the thioglucosidase of *Brassica napus* [12] to as low as *ca* 2 for that of *Crambe abyssinica* [13]. However, it is quite likely that such differences are not due to the plant species, but merely to the different experimental procedures employed.

Previous results have also shown that excess ascorbate completely inhibits thioglucosidases [8]. Thus, there is usually an optimum concentration of ascorbate for maximum enzyme activity, but again there is no great agreement on this level in the literature, and probably for the same reason as that just suggested above. The range of reported optimum ascorbate concentrations extends from *ca* 0.7 mM for the thioglucosidases of *Brassica napus* [12]




Scheme 1. Enzymic degradation of 2-hydroxybut-3-enylglucosinolate.

and of *Sinapis alba* [14] to *ca* 10 mM for those of *Crambe abyssinica* [13], *Brassica oleracea* [15] and *B. juncea* [16]. A convincing mechanism for the interaction of ascorbate with thioglucosidase (and glucosinolate substrate) has been proposed, which also explains inhibition at higher ascorbate concentrations [17] and these ideas have subsequently been refined and extended [18].

The specific objectives of this project were to determine the activation factor of ascorbate with the model system and also the optimum concentration of ascorbate for activity, and to compare these results with the previous studies on biological systems. In addition, the effects of ascorbate on the nature of the products of enzymic degradation and on the course of reaction were also studied.

The effect of metal ions on thioglucosidase activity is unpredictable and, for example, whilst the thiogluosidase of *Aspergillus niger* is stimulated by $Cu^+$, $Cu^{2+}$, $Co^{2+}$ and $Mn^{2+}$, it is inhibited by $Fe^{3+}$, with $Fe^{2+}$ having no effect [19]. Furthermore, some metal ions are activating with thioglucosidases from some species, yet inhibitory with those from others [9, 11, 19, 20]. However, the main interest in the effect of metal ions on enzymic glucosinolate degradation has been more with regard to how they actually affect the course of the reaction and the ratio of products formed, rather than simply their effect on enzyme activity. Although this is clearly a most important aspect of glucosinolate reaction, surprisingly little work has been carried out on this behaviour.

It was as long ago as 1967 that it was shown with *Sinapis alba* and 2-hydroxybut-3-enylglucosinolate that addition of $Fe^{2+}$ to the system caused formation of 1-cyano-2-hydroxybut-3-ene (5, Scheme 1) rather than 5-vinyloxazolidine-2-thione (4, Scheme 1), although the main product in the absence of added $Fe^{2+}$ was the oxazolidinethione [21]. Relatively large concentrations of $Fe^{2+}$ were used in these experiments [21], but subsequently it was shown, using *Crambe abyssinica*, that only catalytic amounts were necessary to cause exactly the same type of behaviour [22]. More recently, catalytic amounts of $Fe^{2+}$ were also shown to promote nitrile formation from benzylglucosinolate in *Lepidium sativum* seeds [23]. Other metal ions have received less study, but their more random effect appears to differ depending on the plant species under consideration [22, 23].

The objective in this project was thus to concentrate on the effects of $Fe^{2+}$ on the model system, and particularly with regard to the nature and proportions of the glucosinolate products obtained.

## RESULTS AND DISCUSSION

### *Effects of added ascorbate*

The effects of a range of ascorbate concentrations on enzymic degradation of synthetic 2-hydroxybut-3-enylglucosinolate are shown in Table 1. Limited hydrolysis experiments (for 1 hour) were carried out to enable changes (increases) in the amounts of degradation products to be feasible and detectable, and Table 1 shows that in no case was all of the substrate consumed. Compared with the blank assay, it can be seen that all levels of added ascorbate over the considerable range that was assessed in this work (0.024–400 mM ascorbate) resulted in much increased thioglucosidase activity, on the basis of increases in total products of enzymic hydrolysis. As anticipated, however, an optimum ascorbate concentration for maximum enzyme activity was shown, and this was on addition of 0.47 μmoles of ascorbate in this experiment, representing a concentration of 1.57 mM. This is well within the range of previously quoted ascorbate optima, that is 0.07–10 mM [9, 12, 13, 15, 16, 24], and quite close to that reported for the thioglucosidase of *Brassica napus*, 0.7 mM [12].

With regard to the extent of thioglucosidase activation by ascorbate, Table 1 shows the very high activation factors obtained in this work, reaching a maximum of *ca* 100. This value is very much higher than any previously reported maximum activation factors, which are generally between *ca* 2 and *ca* 20 [13–16, 24], although levels of 40 and 50 have been reported for some of the purified isoenzymes of *Sinapis alba* [14] and of *Brassica napus* [12]. From Table 1 it can be noted that in this work higher activation factors than these were found extending over a considerable range of ascorbate concentrations, from *ca* 0.1 to *ca* 100 mM. Presumably the reason for the much greater and sustained thioglucosidase activation by ascorbate achieved in this work is the greater purity of the model system (substrate and enzyme) employed in the assays.

Considering now the nature of the products of enzymic degradation of 2-hydroxybut-3-enylglucosinolate in the presence of added ascorbate, it would appear from Table 1 that there is a major effect. In the absence of any added

Table 1. Effects of added ascorbate on the enzymic degradation of 2-hydroxybut-3-enylglucosinolate at pH 5.7

| Added ascorbate (μmol) | 1-Cyano-2-hydroxy-but-3-ene (μmol) | (rel. %) | 5-Vinyloxazolidine-2-thione (μmol) | (rel. %) | Total products (μmol) | Activation factor |
|---|---|---|---|---|---|---|
| 0 (Blank) | | | 0.011 | 100 | 0.011 | |
| 0.0073 | 0.019 | 11 | 0.153 | 89 | 0.172 | 16 |
| 0.029 | 0.041 | 6 | 0.596 | 94 | 0.637 | 58 |
| 0.120 | 0.054 | 5 | 0.970 | 95 | 1.024 | 93 |
| 0.470 | 0.054 | 5 | 1.050 | 95 | 1.104 | 100 |
| 1.87 | 0.054 | 5 | 0.980 | 95 | 1.034 | 94 |
| 7.5 | 0.288 | 32 | 0.602 | 68 | 0.890 | 81 |
| 30.0 | 0.027 | 4 | 0.600 | 96 | 0.627 | 57 |
| 120.0 | 0.003 | 1 | 0.319 | 99 | 0.322 | 29 |

ascorbate (blank) the only product of enzymic hydrolysis was 5-vinyloxazolidine-2-thione, whereas in the presence of added ascorbate, of whatever amount, 1-cyano-2-hydroxybut-3-ene (5, Scheme 1) was also formed. However, the quantities of nitrile produced were much less than those of the oxazolidinethione and, with one exception, at a fairly constant low level. It is likely, therefore, that this formation of nitrile in the presence of ascorbate is due merely to the effect of the activation of the enzyme, rather than any other direct influence.

However, at 7.5 μmoles of added ascorbate an extraordinary peak in nitrile production was obtained. This phenomenon, and the quantitative data, were confirmed in many replicate assays, including using different batches of enzyme and of substrate, so presumably the effect must be genuine. This type of behaviour has not previously been reported and it could, of course, have some implications with regard to the validity of data concerning enzymic glucosinolate degradation. The effect could not be reproduced in enzymic hydrolysis at higher pH, namely 7.0, 7.8, 8.1 and 8.8, when no extra nitrile was obtained in the presence of 7.5 μmoles of added ascorbate in otherwise identical experiments. A possible, if facile, explanation for the effect could be the presence of a pH-sensitive isoenzyme responsible for nitrile production, but clearly the phenomenon requires further, specific study.

*Effects of added* $Fe^{2+}$

The effects of a range of $Fe^{2+}$ concentrations on enzymic degradation of synthetic 2-hydroxybut-3-enylglucosinolate are shown in Table 2. It is clear that the addition of $Fe^{2+}$ has a marked effect on the *course* of the enzymic reaction, since in its absence (the blank) oxazolidinethione was the sole product, whilst whenever $Fe^{2+}$ was present nitrile was formed. This is broadly in agreement with previous work, although in these earlier studies ascorbate was also included, thus introducing two variables [22]. Table 2 shows that only very small relative amounts of $Fe^{2+}$ were necessary to show this effect, and at 0.0625 equivalents of added $Fe^{2+}$, nitrile became the major product of reaction. Eventually, on addition of 0.25 equivalents, the system was completely reversed, and nitrile was the sole product. Unlike ascorbate, $Fe^{2+}$ is certainly not an activator, and indeed it slightly suppresses reaction, the extent increasing with the concentration of added $Fe^{2+}$. However, once nitrile became the sole product, further increases in $Fe^{2+}$ did not then significantly change the amount of nitrile produced, thus implying that there is no further, more severe, inhibition at higher levels of $Fe^{2+}$.

This slight inhibition of reaction by $Fe^{2+}$ can also be seen in Table 3, which gives results comparing the rate of enzymic hydrolysis of the synthetic glucosinolate both in the presence and absence of added $Fe^{2+}$. In these experiments the amount of added $Fe^{2+}$ (0.0625 equivalents) was chosen as a level which afforded roughly equal amounts of the two products (see Table 2), so that any changes in their relative quantities would be more readily detectable. The data show that the rates of hydrolysis were not greatly different whether $Fe^{2+}$ was added or not, but although initially the presence of $Fe^{2+}$ would appear to promote reaction, eventually it does seem to have a slight inhibitory effect. These results do not, however, agree with previous work, in which it was found that added $Fe^{2+}$ increased the hydrolysis rate by a factor of 6 [21]. Although greater amounts of $Fe^{2+}$ were used in these earlier studies (0.25 equivalents) [21], our results (Table 2) would suggest that such a higher concentration would merely slightly increase inhibition. The most likely reason for the difference in these findings is that the previous workers [21] used extracted 2-hydroxybut-3-enylglucosinolate, rather than pure, synthesized material. With regard to the relative rates of formation of the two products under these circumstances, it can be seen from Table 3 that there is little difference. Initially, the rate of formation of the nitrile was faster, but after *ca* 4 hr under these conditions, formation of oxazolidinethione became faster.

With regard to the mode of action of $Fe^{2+}$, a broad comparison of the results in Table 1 with those in Table 2 shows that the two factors ($Fe^{2+}$ and ascorbate) produce quite different effects. Ascorbate is clearly an enzyme activator, whilst $Fe^{2+}$ is not, but instead it actually directs the course of the degradation (it is noticeable, for example, that in the absence of added $Fe^{2+}$—Table 1 nitrile is always the minor product). It is thus unlikely that $Fe^{2+}$ also affects thioglucosidase enzymes, as does ascorbate, but rather it interacts somehow with the enzymically produced aglucone (2, Scheme 1), possibly via catalytic complex formation, and hence directs its decomposition to nitrile rather than isothiocyanate by the Lossen rearrangement. Clearly, there is a number of suitable sites in the aglucone for co-ordination with $Fe^{2+}$. However,

Table 2 Effects of added $Fe^{2+}$ on the enzymic degradation of 2-hydroxybut-3-enylglucosinolate at pH 5.7

| Added $Fe^{2+}$ (μmol) | Equivalents of $Fe^{2+}$ | 1-Cyano-2-hydroxy-but-3-ene (μmol) | 5-Vinyloxazolidine-2-thione (μmol) | Total products (μmol) | Extent inhibition (% of blank) |
|---|---|---|---|---|---|
| 0 (Blank) | 0 | – | 0.540 | 0.540 | 100 |
| 0.0187 | 0.0078 | 0.031 | 0.493 | 0.524 | 97 |
| 0.0375 | 0.0156 | 0.049 | 0.450 | 0.499 | 92 |
| 0.0750 | 0.0312 | 0.153 | 0.290 | 0.443 | 82 |
| 0.150 | 0.0625 | 0.310 | 0.206 | 0.516 | 96 |
| 0.30 | 0.125 | 0.412 | 0.023 | 0.435 | 81 |
| 0.60 | 0.250 | 0.405 | | 0.405 | 75 |
| 1.20 | 0.50 | 0.412 | — | 0.412 | 76 |
| 2.40 | 1.0 | 0.412 | | 0.412 | 76 |

Table 3. Effects of added $Fe^{2+}$ (0.0625 equivalents) on the rate of enzymic degradation of 2-hydroxybut-3-enylglucosinolate at pH 5.7

| Time (hr) | 1-Cyano-2-hydroxy-but-3-ene (μmol) | 5-Vinyloxazolidine-2-thione (μmol) | Total products (μmol) | Control (no added $Fe^{2+}$), 5-vinyloxazolidine-2-thione (μmol) |
|---|---|---|---|---|
| 1.0 | 0.120 | 0.109 | 0.229 | 0.107 |
| 2.0 | 0.200 | 0.103 | 0.303 | 0.226 |
| 3.0 | 0.256 | 0.133 | 0.386 | 0.395 |
| 4.0 | 0.310 | 0.206 | 0.516 | 0.540 |
| 4.5 | 0.379 | 0.260 | 0.639 | 0.691 |
| 6.0 | 0.448 | 0.392 | 0.840 | 1.032 |
| 7.0 | 0.476 | 0.466 | 0.942 | 1.231 |

Table 4. Effects of added $Cu^{2+}$ on the enzymic degradation of 2-hydroxybut-3-enylglucosinolate at pH 5.7

| Added $Cu^{2+}$ (μmol) | Equivalents of $Cu^{2+}$ | 1-Cyano-2-hydroxy-but-3-ene (μmol) | 5-Vinyloxazolidine-2-thione (μmol) | Total products (μmol) | Extent inhibition (% of blank) |
|---|---|---|---|---|---|
| 0 (Blank) | 0 | | 0.540 | 0.540 | 100 |
| 0.0012 | 0.000487 | | 0.493 | 0.493 | 91 |
| 0.0023 | 0.000975 | | 0.473 | 0.473 | 88 |
| 0.0047 | 0.00195 | | 0.493 | 0.493 | 91 |
| 0.0093 | 0.00390 | | 0.430 | 0.430 | 80 |
| 0.0187 | 0.0078 | - | 0.211 | 0.211 | 39 |
| 0.0375 | 0.0156 | 0.005 | 0.070 | 0.075 | 14 |
| 0.0750 | 0.0312 | 0.012 | 0.040 | 0.052 | 10 |
| 0.150 | 0.0625 | 0.016 | .. | 0.016 | 3 |
| 0.30 | 0.125 | 0.020 | | 0.020 | 3 |
| 0.60 | 0.250 | 0.031 | | 0.031 | 6 |
| 1.20 | 0.50 | 0.047 | | 0.047 | 8 |
| 2.40 | 1.0 | 0.047 | .. | 0.047 | 8 |

whatever the mechanism of $Fe^{2+}$ action, its very significant effect at very low concentrations should always be taken into account. It may even be, for example, that at relatively modest natural concentrations of free $Fe^{2+}$ in, say, rape, the supposed formation of toxic oxazolidinethione could, in fact, be completely suppressed.

*Effects of added* $Cu^{2+}$

The effects of a range of $Cu^{2+}$ concentrations on enzymic degradation of synthetic 2-hydroxybut-3-enylglucosinolate are shown in Table 4. It can be seen that whilst $Fe^{2+}$ slightly inhibited reaction, $Cu^{2+}$ drastically inhibits it. Again, this finding is in agreement with previous work [22]. Table 4 shows that even minute concentrations of added $Cu^{2+}$ caused appreciable inhibition, and by comparison with the results in Table 2 it can be seen that equivalent amounts of $Fe^{2+}$ caused much less inhibition. For example, whilst 0.0312 equivalents of $Fe^{2+}$ caused only *ca* 20% inhibition, the same relative concentration of $Cu^{2+}$ caused *ca* 90% inhibition. However, the addition of $Cu^{2+}$ does still affect the ratio of products, and in much the same way as $Fe^{2+}$. Thus, with increasing concentration of added $Cu^{2+}$, the product(s) of enzymic reaction changes from totally oxazolidinethione to mixture to totally nitrile, and these changes occur at much the same relative concentrations for both $Fe^{2+}$ and $Cu^{2+}$. For example, plotting the data in Tables 2 and 4 shows that the point at which equal amounts of the two products are formed is at 0.0480 equivalents of $Fe^{2+}$ and at 0.0515 equivalents of $Cu^{2+}$, which is reasonable agreement. It seems likely, therefore, that $Cu^{2+}$ has the same mode of action as $Fe^{2+}$ in this instance, and probably on the aglucone as just suggested, but that it is in addition a strong enzyme inhibitor whereas $Fe^{2+}$ is only a weak inhibitor. Whether this directional effect on the degradation of glucosinolates shown by these species is a generalized phenomenon shown by other appropriate metal ions needs further study in similar genuine model systems.

## EXPERIMENTAL

*Enzyme, substrate and standards.* Previously reported methods were used to isolate and purify thioglucoside glucohydrolase from seeds of *B. napus* cv panter [2], and to synthesize 2-hydroxybut-3-enylglucosinolate [1], 5-vinyloxazolidine-2-thione [25] and 1-cyano-2-hydroxybut-3-ene [26].

*Enzymic degradation of 2-hydroxybut-3-enylglucosinolate in the presence of ascorbate.* 2-Hydroxybut-3-enylglucosinolate (1.2 μmole) in 275 μl of acetate buffer soln (0.02 M, pH 5.7) containing the appropriate concn of ascorbate as listed in Table 1.

was mixed with 25 μl of *B. napus* thiogluosidase preparation and the mixture incubated at 25° for 1 hr. Products were extracted with $CH_2Cl_2$ (600 μl) and assayed by FID-GC. For 5-vinyloxazolidine-2-thione, a 1.5 m × 4 mm i.d. glass column packed with 5% Apiezon L coated on Celite was used, at a temp. of 130°. For 1-cyano-2-hydroxybut-3-ene, a 1.5 m × 4 mm i.d. glass column packed with 10% neopentyl glycol succinate (NPGS) coated on Celite was used, at a temp. of 150°. In both cases, $N_2$ flow rate was 30 ml/min, injection temp. 150°, detector temp. 250°, and quantification was accomplished by injecting known quantities of the synthetic standards under the same GC conditions.

The same expts were also carried out at pH 7.0, 7.8, 8.1 and 8.8.

*Enzymic degradation of 2-hydroxybut-3-enylglucosinolate in the presence of* $Fe^{2+}$. 2-Hydroxybut-3-enylglucosinolate (2.4 μmol) in 200 μl of acetate buffer soln (0.02 M, pH 5.7) containing the appropriate concn of $Fe^{2+}$ (ferrous ammonium sulphate) (Table 2), was mixed with 100 μl of *B. napus* thioglucosidase preparation and the mixture incubated at 25° for 4 hr. Products were extracted with $CH_2Cl_2$ (600 μl) and assayed as described above.

The same expt was also carried out using a single, standard amount of added $Fe^{2+}$ (0.0625 equivalents), but assays were performed after certain periods of incubation (Table 3).

*Enzymic degradation of 2-hydroxybut-3-enylglucosinolate in the presence of* $Cu^{2+}$. These expts were carried out exactly as described above for those with $Fe^{2+}$, but using instead various amounts of added $Cu^{2+}$ ($CuSO_4$) (Table 4).

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## REFERENCES

1. MacLeod, A. J. and Rossiter, J. T. (1983) *J. Chem. Soc. Perkin Trans.* 1 717.
2. MacLeod, A. J. and Rossiter, J. T. (1986) *Phytochemistry* **25**, 1047.
3. Nagashima, Z. and Uchiyama, M. (1959) *Bull. Agric. Chem. Soc. Japan* **23**, 555.
4. Schwimmer, S. (1961) *Acta Chem. Scand.* **15**, 534.
5. Ettlinger, M. G., Dateo, G. P., Harrison, B. W., Mabry, T. M. and Thompson, C. P. (1967) *Proc. Natn. Acad. Sci. U.S.A.* **47**, 1875.
6. Henderson, H. M. and McEwan, T. J. (1972) *Phytochemistry* **11**, 3127.
7. Vose, J. R. (1972) *Phytochemistry* **11**, 1649.
8. Björkman, R. and Lönnerdal, B. (1973) *Biochim. Biophys. Acta* **327**, 121.
9. Ohtsuru, M. and Kawatani, H. (1979) *Agric. Biol. Chem.* **43**, 2249.
10. Ohtsuru, M., Tsuruo, I. and Hata, T. (1969) *Agric. Biol. Chem.* **33**, 1309.
11. Tani, N., Ohtsuru, M. and Hata, T. (1974) *Agric. Biol. Chem.* **38**, 1623.
12. Lönnerdal, B. and Janson, J. (1973) *Biochim. Biophys. Acta* **315**, 421.
13. Tookey, H. L. (1973) *Can. J. Biochem.* **51**, 1305.
14. Björkman, R. and Janson, J. (1972) *Biochim. Biophys. Acta* **276**, 508.
15. West, L. G., Badenhop, A. F. and McLaughlin, J. L. (1977) *J. Agric. Food Chem.* **25**, 1234.
16. Tsuruo, I., Yoshida, M. and Hata, T. (1967) *Agric. Biol. Chem.* **31**, 18.
17. Tsuruo, I. and Hata, T. (1968) *Agric. Biol. Chem.* **32**, 1425.
18. Ohtsuru, M. and Hata, T. (1979) *Biochim. Biophys. Acta* **567**, 384.
19. Ohtsuru, M. and Hata, T. (1973) *Agric. Biol. Chem.* **37**, 25.
20. Tsuruo, I. and Hata, T. (1968) *Agric. Biol. Chem.* **32**, 479.
21. Austin, F. L., Gent, C. A. and Wolff, I. A. (1968) *Can. J. Chem.* **46**, 1507.
22. Tookey, H. L. and Wolff, I. A. (1970) *Can. J. Biochem.* **48**, 1024.
23. Hasapis, X. and MacLeod, A. J. (1982) *Phytochemistry* **21**, 559.
24. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 2071.
25. Ettlinger, M. G. (1950) *J. Am. Chem. Soc.* **72**, 4792.
26. Bissinger, W. E., Fredenburg, R. H., Kadesch, R. G., Kung, F., Langston, J. H., Stevens, H. C. and Strain, F. (1947) *J. Am. Chem. Soc.* **69**, 2955.