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EFFECTS OF pH AND ASCORBATE ON BENZYLGLUCOSINOLATE DEGRADATION IN SEED EXTRACTS OF *LEPIDIUM SATIVUM*

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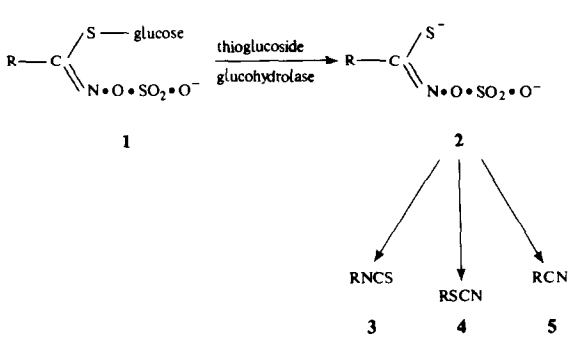
Key Word Index—*Lepidium sativum*; Cruciferae; cress seeds; autolysis; glucosinolate degradation; pH; ascorbate.

Abstract—The effects of pH on the enzymic degradation of benzylglucosinolate in *Lepidium sativum* seed autolysates were investigated both with and without addition of the enzyme co-factor ascorbic acid. Benzyl cyanide, isothiocyanate, thiocyanate and alcohol were identified in autolysates, although only traces of the alcohol were obtained. The nitrile was always the major product (> 80% of total glucosinolate products) even at pH 8 and 9 when the usually accepted, proton-dependent mechanism of nitrile production cannot be operative. Thiocyanate was always the second most abundant product. In the absence of added ascorbate, isothiocyanate production decreased with increasing pH, again contrary to accepted theory. *L. sativum* seeds thus constitute an inherently nitrile-producing system which exhibits 'anomalous' glucosinolate degradation. In the absence of added ascorbate, thiocyanate was the only product which was formed in approximately constant amounts, whatever the pH, so its mechanism of production is not necessarily pH-dependent. The presence of added ascorbate in general promoted enzyme activity and showed a maximum effect at *ca* pH 5, although minimum isothiocyanate formation was observed at that pH. At pH 4 and below, there was less glucosinolate degradation in the presence of added ascorbate than in its absence, and the conclusion is reached that at relatively high acidities the enzyme co-factor behaves as an inhibitor.

INTRODUCTION

Glucosinolates **1** are thioglucosides, mainly found in the Cruciferae. As shown in Scheme 1, they degrade enzymically via an aglucone **2** to yield three main types of products: isothiocyanates **3**, thiocyanates **4** and nitriles **5**. The side-chain R can represent a number of structural possibilities, but all are derived from, or elaborated from, the side-chains of naturally occurring α-amino acids. In addition to those shown in Scheme 1, other products are possible from particular glucosinolates under certain circumstances, but in general they are not relevant to this paper. Of about 80 known naturally occurring glucosinolates, only three appear capable of forming thiocyanates (not thiocyanate ion), whilst all give isothiocyanates and nitriles. These three are allyl-, benzyl- and 4-(methylthio)butyl-glucosinolates.



Scheme 1. Enzymic degradation of glucosinolates.

In recent years much effort has been devoted to studies aimed at elucidating the detailed mechanisms of glucosinolate degradation, but considerable doubt still exists concerning many aspects of the reactions. In fact, some earlier conceptions of glucosinolate degradation have recently been shown to be incorrect or not entirely true, e.g. [1]. One aspect which is particularly confused at present concerns the effects of pH on glucosinolate degradation and the nature and relative amounts of the products formed. The long-accepted theory is that following enzymic hydrolysis (see Scheme 1) the aglucone **2** rearranges either to the isothiocyanate **3** or fragments to the nitrile **5**, depending on the pH of the medium. The nitrile is supposedly formed via a protonation mechanism and so is favoured at lower pH values. Between pH 3 and 5 a mixture is expected, but above pH 5 only the isothiocyanate should be obtained (by a Lossen rearrangement). There is much evidence for this theory, and in some recent model system studies using pure allyl- and 2-phenethyl-glucosinolates together with a mustard thioglucosidase preparation, it was confirmed that nitrile formation was favoured at lower pH levels and that the ratio of nitrile to isothiocyanate was directly related to the hydrogen ion concentration of the reaction medium[2]. However, during some recent studies of glucosinolate

degradation in *Lepidium sativum* seed extracts, nitrile was found to be the major product at pH 7.4[1]. Saarivirta had previously obtained similar results at pH 6.7[3], and in all the *Lepidium* sp. examined by Cole at pH 7–8 the main product was the nitrile[4]. However, it has also been observed that under genuine conditions of autolysis (i.e. at the natural pH of the plant) some Cruciferae give predominantly isothiocyanate whilst some give predominantly nitrile, and in some instances the same glucosinolate provides a different major product depending on the particular crucifer[5]. It was suggested, therefore, that some plants are mainly isothiocyanate-producing, whilst others are mainly nitrile-producing[5], since in these cases pH could not be a significant contributory factor. It should also be mentioned that it was observed some time ago that nitrile could also be produced non-enzymically[6], and recently it has been shown that isothiocyanate as well as nitrile can be formed by a purely thermal mechanism[7].

The previous investigation of glucosinolate degradation in *L. sativum* seed extracts[1] covered a number of experimental variables and only a very narrow pH range was studied (6.69–7.42). In the light of the above comments and the present uncertainties regarding the effect of pH on glucosinolate degradation *in vivo*, it was decided to extend this pH range and to investigate in more detail the products of glucosinolate degradation in *L. sativum* seed extracts, as affected by varying pH.

Ascorbate has long been recognized as a co-factor of thioglucoside glucohydrolase, although again there is some confusion concerning its exact mode of action and of the extent of its influence[8–16]. In some recent investigations of the degradation of some pure glucosinolates by an *L. sativum* thioglucosidase preparation, it was shown that ascorbate was not in fact essential for enzyme activity, but its presence did considerably enhance isothiocyanate formation[17]. Nitrile production, however, was erratic in the presence of ascorbate but this was not the case in its absence when the amount of nitrile formed increased regularly with time. These data suggest that ascorbic acid might not necessarily be involved in promoting enzymic degradation to nitrile although it would seem that it can be involved in isothiocyanate formation. In addition to the survey of *in vivo* glucosinolate degradation at different pH levels, it was therefore decided to carry out these experiments both in the presence and absence of added ascorbate. It was known that the concentration of free endogenous ascorbic acid in *L. sativum* seeds (determined as *ca* 0.03 mg/g by the routine 2,6-dichlorophenolindophenol method) would not be sufficient for promotion of thioglucosidase activity[17], but it was considered that this factor ought, in any case, to be taken into account and investigated further.

RESULTS AND DISCUSSION

It has long been known that *L. sativum* contains relatively large amounts of benzylglucosinolate, and although allyl- and 2-phenethyl-glucosinolates have also recently been determined in the seeds they were found in only trace amounts and only when using a sensitive analytical procedure[1]. In this work, using less sensitive but quantitatively more accurate techniques, only the products of the major glucosinolate were detected, and thus discussion is limited to this one precursor. Benzyl cyanide, isothiocyanate, thiocyanate and alcohol were all positively identified by means of GC and GC/MS in all *L. sativum* seed autolysates, but in no case was any other benzyl compound recognized. Previously, benzaldehyde, benzyl mercaptan and benzyl methyl sulphide were also detected in *L. sativum* seed extracts, but only when using a rather more sensitive analytical procedure[1]. There is, however, no proof that these three benzyl compounds or the alcohol are also derived from benzylglucosinolate, although some evidence of this origin has been presented for benzyl alcohol and benzaldehyde[1]. In this work the amounts of benzyl alcohol which were obtained in comparison with the other products were very small (too small for accurate measurement), so consideration is limited to the three main compounds. Benzyl thiocyanate was obtained in all experiments, in agreement with much previous work which has shown benzylglucosinolate to be one of the few thiocyanate-forming glucosinolates[1, 3, 5, 18–23].

Table 1 gives data for the amounts of benzylglucosinolate degradation products obtained on autolysis of crushed *L. sativum* seeds (1 g) at ambient temperature for 15 min in buffers of various pH but in the absence of any added ascorbic acid. Table 2 gives similar results for autolysis carried out in the presence of added ascorbate, using ascorbate buffers such that the concentration of co-factor was constant (6×10^{-2} M). All experiments were performed in triplicate and results were reproducible to better than $\pm 5\%$.

In the presence of added ascorbate the total amount of glucosinolate products increased with increasing pH to a maximum at *ca* pH 5 and then decreased. In the absence of added ascorbate the total amount decreased approximately linearly with increasing pH, although a distinct maximum was observed at *ca* pH 8. Identical trends were shown in both cases for the nitrile product. Thus, it is immediately obvious that added ascorbate does affect the mechanism of glucosinolate degradation and that it seems to enhance the process at pH values approaching the optimum for the enzyme. However, the total amounts of glucosinolate products formed were about the same, whether or not ascorbate was added, and on average *ca* 10 mg of products were obtained per g of seeds. This corresponds to a benzylglucosinolate content in *L. sativum* seeds of *ca* 35 mg/g (i.e. a rather surprising 3.5%).

It can be seen from Tables 1 and 2 that benzyl cyanide was always the major degradation product whatever the pH of the medium, and it was never less than 80% of the total glucosinolate products. This is in general agreement with previous work[1]. It has already been mentioned that there has been a suggestion that some Cruciferae are inherently predominantly nitrile-producing whilst others are predominantly isothiocyanate-producing[5], and if this is the case clearly *L. sativum* seeds belong to the former group. Benzyl thiocyanate was always the second most abundant product of the three, and this too substantiates previous findings for *L. sativum*

Table 1. Amounts of benzylglucosinolate products formed in autolysates of *Lepidium sativum* seeds (1 g) at different pH without added ascorbate

pH	Benzyl cyanide ($mg \times 10^{-2}$)	(%)	Benzyl thiocyanate ($mg \times 10^{-2}$)	(%)	Benzyl isothiocyanate ($mg \times 10^{-2}$)	(%)	Thiocyanate + isothiocyanate ($mg \times 10^{-2}$)	(%)	Thiocyanate/ isothiocyanate	Total products ($mg \times 10^{-2}$)
3	832	81.3	104	10.2	87	8.5	191	18.7	2.0	1023
4	799	82.2	98	10.1	74	7.7	172	17.8	1.32	971
5	784	84.4	98	10.5	47	5.1	145	15.6	2.09	929
6	733	84.3	99	11.4	37	4.3	136	15.7	2.68	869
7	631	83.1	110	14.5	18	2.4	128	16.9	6.11	759
8	797	85.9	117	12.6	14	1.5	131	14.1	8.36	928
9	656	83.8	115	14.7	12	1.5	127	16.2	9.58	783

Table 2. Amounts of benzylglucosinolate products formed in autolysates of *Lepidium sativum* seeds (1 g) at different pH in the presence of added ascorbate (6×10^{-2} M)

pH	Benzyl cyanide ($mg \times 10^{-2}$)	(%)	Benzyl thiocyanate ($mg \times 10^{-2}$)	(%)	Benzyl isothiocyanate ($mg \times 10^{-2}$)	(%)	Thiocyanate + isothiocyanate ($mg \times 10^{-2}$)	(%)	Thiocyanate/ isothiocyanate	Total products ($mg \times 10^{-2}$)
3.25	722	86.0	60	7.2	57	6.8	117	14.0	1.05	839
4.12	738	83.8	123	14.0	19	2.2	142	16.2	6.47	880
4.98	1107	88.0	145	11.5	6	0.5	151	12.0	24.17	1258
6.02	923	86.0	136	12.7	14	1.3	150	14.0	9.71	1073
6.93	882	88.5	94	9.4	21	2.1	115	11.5	4.48	997
7.40	853	89.2	71	7.5	32	3.3	103	10.8	2.22	956

seeds[1, 3]. However, these *in vivo* results do not agree with certain model system studies on enzymic degradation of pure glucosinolates (including the benzyl derivative), when isothiocyanate production normally exceeded nitrile, except at low pH[2, 17]. Furthermore, considerable quantities of nitrile were produced even at pH values as high as 8 and 9 (Tables 1 and 2) when the protonation mechanism of the basic theory of glucosinolate degradation is clearly impossible. So the generally accepted theory and model system studies are in conflict with these particular *in vivo* results. Thus, there must be some quality about this natural system which directs glucosinolate degradation more to the nitrile and which is not significantly pH dependent (if at all), and this factor was missing from the model system experiments on, in particular, benzylglucosinolate[17], when the simple basic mechanism must still have been operative. It has been suggested that the metal ion content of the natural system might afford an explanation of this type of behaviour[2], and although there are certainly other possibilities there is some supporting evidence for this proposal[12, 24, 25]. Whatever the cause, it is either not present or does not function with all Cruciferae, since on autolysis some genuinely conform with the original theory of glucosinolate degradation and are predominantly isothiocyanate-producing (e.g. *Brassica oleracea*[26]).

The amounts of nitrile produced (in the absence of added ascorbate) decreased with increasing pH, although the overall percentage remained about the same (Table 1). The interruption in the decrease at *ca* pH 8, which is the reason for the previously mentioned similar maximum for total glucosinolate products, can be explained as follows. Whilst citrate–phosphate buffers were employed to obtain acid and neutral pH levels, it was necessary to use borate buffer to obtain pH 8 and 9. According to Gronowitz *et al.* sodium tetraborate is very efficient at increasing the rate of degradation of 2-hydroxybut-3-enylglucosinolate[27], and presumably the same phenomenon occurred with benzylglucosinolate of *L. sativum* and hence produced this artificial maximum. Whilst isothiocyanate decreased considerably with increasing pH, thiocyanate production remained about constant (Table 1). This is demonstrated by the regular increase in the ratio of thiocyanate to isothiocyanate. This decrease in isothiocyanate production with increasing pH is completely the reverse of what would be expected on the basis of the main theory of glucosinolate degradation and also on the basis of appropriate model system studies[2, 17], but since *L. sativum* does not conform to the theory with regard to nitrile production, it is not particularly unexpected that neither does it conform with regard to isothiocyanate production. It may well be that the cause of the 'anomalous' behaviour is the same in both cases.

Since only thiocyanate formation was almost constant over the pH range studied, this means that glucosinolate degradation to thiocyanate, but not to the other products, was independent of pH between 3 and 9. Glucosinolate catabolism to thiocyanate is not understood at present, and this finding might therefore prove of value in further attempts to discover the mechanism of thiocyanate formation.

Table 2 gives the results for the experiments carried out in the presence of ascorbic acid (6×10^{-2} M). In general, glucosinolate degradation was promoted by ascorbate (cf. Table 1) except at pH values of 4 and less, but that point will be discussed later. The extent of activation was relatively small and was, on average, *ca* 30%. Some previous work has shown much greater enhancement of thioglucosidase activity by ascorbate, but the extent of activation can vary over a wide range depending on a number of factors [12, 13, 15, 17, 28–30]. It certainly depends at least on the concentration of co-factor relative to substrate[17], and hence the limited activation observed in this work might well have been due to the concentration of co-factor not being appropriate for optimum activation. However, this relatively low degree of activation does not affect the validity of the findings, since consistent activation was obtained, except at pH 4 and below.

In contrast to the results obtained in the absence of ascorbate (when increase in pH caused uniform decrease in the extent of glucosinolate degradation and the amounts of products formed, except thiocyanate), there was a marked change in behaviour at *ca* pH 5 in its presence (Table 2). At this pH there was a peak of glucosinolate degradation and a maximum in the production of both nitrile and thiocyanate. Only isothiocyanate did not show identical behaviour, but formation of this product exhibited a minimum at the same pH. Saarivirta has suggested that benzyl isothiocyanate is generated from the glucosinolate only if the formation of nitrile and thiocyanate is limited or prevented in some way[3], and this would explain this minimum, although on the data presented here this type of behaviour could apply equally to either of the other two products and not necessarily both. With respect to enzyme activity, this isothiocyanate minimum is not significant, in that the total amount of thiocyanate plus isothiocyanate and their ratios to each other (Table 2) both show the same maximum at *ca* pH 5. This is due to the extent of the thiocyanate maximum considerably exceeding that of the isothiocyanate minimum. These data provide some evidence concerning the origins of these two products. It is usually assumed that both compounds are formed directly in a single step from a common precursor (e.g. **2**, Scheme 1) or that they are mutually interconvertible. However, in either case it would be reasonable to expect constant values for the two sets of data just mentioned, in that variations in isothiocyanate and thiocyanate formation (and concentrations) would compensate for each other. Although production of the precursor itself or the first of the two products in an equilibrium situation could, of course, also vary with pH in the presence of ascorbate and hence explain the overall maximum at pH 5, it seems a little less likely that the two different phenomena would both be observed to show maximum effect at the same pH. Therefore, these results provide some further limited evidence in support of an independent mechanism for the formation of thiocyanate.

The results in Table 2 confirm that ascorbic acid promotes the activity of *L. sativum* thioglucosidase

and show that the optimum pH is *ca* 5. Above this pH, glucosinolate degradation decreases in the same manner as was observed for autolysis without added ascorbate (Table 1). It is only below pH 5 that the two sets of results differ in this respect. Ascorbic acid is stable over a wide pH range around neutrality[9], but it is known to be a better activator of glucosinolate hydrolysis at values near 7 than at lower values, and the ratio of singly ionized ascorbic acid to the non-ionized form is much greater at 7 than at 4[15]. This may well explain the lower activation of enzyme below pH 5 and hence the decreased extent of glucosinolate degradation, but it does not explain how activity continued to rise below pH 5 in the absence of ascorbate (Table 1) and give greater amounts of glucosinolate degradation products than in the presence of ascorbate, all other factors being constant. The obvious conclusion is that even though ascorbate is a proven enzyme co-factor, it is also an inhibitor at low pH values (i.e. below *ca* pH 4.5). Ascorbate-inhibition of thioglucosidase activity has been observed before although under slightly different circumstances[13, 31]. Originally it was considered that ascorbate acted as a base or nucleophile in its co-factor rôle[9], but recent work has shown that it functions more through induced conformational changes in the enzyme–substrate system rather than in dissociation–association of the genuine enzyme[32], and binding of ascorbic acid to an iso-enzyme, activated by the acid, has been determined[33]. This type of mechanism could also account for an inhibitory mode of action at relatively high acidities[13].

Previous studies have shown that the exact pH optimum for ascorbate-activated thioglucosidase seems to vary depending on the source of the enzyme and certain other factors. Most older work found it to be somewhat higher than that determined here (usually between pH 6 and 9), although with mustard thioglucosidase Schwimmer observed much decreased activity above pH 7[8]. However, an optimum of between pH 4 and 5.5 has been reported for the thioglucosidase of *Sinapis alba*[13] and of pH 5.5 for that of *Brassica napus*[34], and more recently, using mustard thioglucosidase and two pure glucosinolates, maximum activity was obtained at pH 5.95 with both glucosinolates[2]. The pH optimum observed in our work is thus in reasonable accord with these more recent findings.

EXPERIMENTAL

L. sativum seeds were obtained from Suttons Seeds Ltd, Reading, U.K. ('curled cress') and were authenticated by basic seed microscopy studies.

Buffers. Citrate–Pi buffers of the required pH (3, 4, 5, 6 and 7) were prepared from aq. solns of 0.1 M citric acid and 0.2 M Na_2HPO_4. Borate buffers (pH 8 and 9) were prepared from aq. solns of 0.2 M boric acid and 0.05 M $Na_2B_4O_7 \cdot 10H_2O$. Ascorbate buffers were prepared by mixing various aq. solns of (−)-ascorbic acid with aq. solns of Na_2HPO_4 varying in concn from 0.05 to 0.5 M, such that in each buffer the final concn of ascorbic acid was always 6×10^{-2} M.

Seed autolysis. L. sativum seeds were ground to a fine powder in a coffee grinder. To 1 g was added 50 ml of buffer and the mixture shaken at room temp. for 15 min. Seed residue was removed by centrifugation and the aq. layer extracted with CH_2Cl_2 (2 × 30 ml). Following centrifugation the organic layer was separated, dried (Na_2SO_4) and reduced in vol. to *ca* 5 ml using a rotary evaporator without heat.

GC analysis. Extracts (1 μl) were examined by FID GC using a 1.5 m × 4 mm i.d. glass column packed with 3% Carbowax 20 M coated on 100–120 BSS mesh acid-washed Diatomite C. Carrier gas was N_2 (42 ml/min) and the column temp. 160°. Absolute amounts of compounds produced were determined by standard injections of pure samples of benzyl cyanide, benzyl thiocyanate and benzyl isothiocyanate. Recovery from sample prepn was determined after submitting standard amounts of pure compounds to the whole procedure.

GC/MS. Glucosinolate degradation products were identified by EI-GC/MS using an instrument fitted with a data processing system. The GC conditions above were used but with He as carrier. MS conditions were: ionization potential, 70 eV; ionization current, 300 μA; source temp., 230°; resolution, 600; scan speed, 3 sec/decade (repetitive throughout run).

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REFERENCES

1. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 1369.
2. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 2547.
3. Saarivirta, M. (1973) *Planta Med.* **24**, 112.
4. Cole, R. A. (1976) *Phytochemistry* **15**, 759.
5. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 1657.
6. MacLeod, A. J. (1970) *Flavour Ind.* **1**, 665.
7. MacLeod, A. J., Panesar, S. S. and Gil, V. (1981) *Phytochemistry* **20**, 977.
8. Schwimmer, S. (1961) *Acta Chem. Scand.* **15**, 534.
9. Ettlinger, M. G., Dateo, G. P., Harrison, B. W., Thompson, T. M. and Thompson, C. P. (1961) *Proc. Natl. Acad. Sci. U.S.A.* **47**, 1875.
10. Tsuruo, I., Yoshida, M. and Hata, I. (1967) *Agric. Biol. Chem.* **31**, 18.
11. Lein, K. A. (1972) *Angew. Bot.* **46**, 137.
12. Tookey, H. L. (1973) *Can. J. Biochem.* **51**, 1305.
13. Bjorkman, R. and Lonnerdal, B. (1973) *Biochim. Biophys. Acta* **327**, 121.
14. Bjorkman, R. (1976) in *The Biology and Chemistry of the Cruciferae* (Vaughan, J. G., MacLeod, A. J. and Jones, B. M. G., eds.), p. 191. Academic Press, New York.
15. West, L. G., Badenhop, A. F. and McLaughlin, J. L. (1977) *J. Agric. Food Chem.* **25**, 1234.
16. Tsuruo, I. and Hata, T. (1967) *Agric. Biol. Chem.* **31**, 27.
17. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 2071.
18. Virtanen, A. I. (1965) *Phytochemistry* **4**, 207.
19. Saarivirta, M. and Virtanen, A. I. (1963) *Acta Chem. Scand.* **17**, S 74.
20. Gmelin, R. and Virtanen, A. I. (1959) *Acta Chem. Scand.* **13**, 1474.
21. Virtanen, A. I. (1962) *Arch. Biochem. Biophys.* **1**, 200.
22. Park, R. J. and Armitt, J. D. (1969) *J. Dairy Res.* **36**, 37.

23. Virtanen, A. I. and Saarivirta, M. (1962) *Suom. Kemistil. B.* **35**, 248.
24. Youngs, C. G. and Perlin, A. S. (1967) *Can. J. Chem.* **45**, 1801.
25. Austin, F. L. and Gent, C. A. (1967) *Chem. Commun.* **2**, 71.
26. Kaoulla, N., MacLeod, A. J. and Gil, V. (1980) *Phytochemistry* **19**, 1053.
27. Gronowitz, S., Svensson, L. and Ohlson, R. (1978) *J. Agric. Food Chem.* **26**, 887.
28. Bjorkman, R. and Janson, J. C. (1972) *Biochim. Biophys. Acta* **276**, 508.
29. Lonnerdal, B. and Janson, J. C. (1973) *Biochim. Biophys. Acta* **315**, 421.
30. Ohtsuru, M. and Hata, T. (1972) *Agric. Biol. Chem.* **34**, 2495.
31. Tsuruo, I. and Hata, T. (1968) *Agric. Biol. Chem.* **32**, 1425.
32. Ohtsuru, M. and Hata, T. (1973) *Agric. Biol. Chem.* **37**, 1971.
33. Ohtsuru, M. and Hata, T. (1975) *Agric. Biol. Chem.* **39**, 1505.
34. Henderson, H. M. and McEwen, T. J. (1972) *Phytochemistry* **11**, 3127.