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HYDROXYL RADICAL SCAVENGING ACTIVITY OF COMPATIBLE SOLUTES

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Key Word Index—Proline, glycinebetaine; sorbitol; mannitol, pinitol, oxidative damage, osmotic stress.

Abstract—Compatible solutes were assessed for their hydroxyl radical scavenging activity by their ability to compete in two different hydroxyl radical generating and detecting systems Hydroxyl radicals were generated by ascorbate–hydrogen peroxide or by xanthine oxidase–hypoxanthine–hydrogen peroxide They were detected by hydroxylation of salicylate or by denaturation of malate dehydrogenase. Of the compatible solutes tested, sorbitol, mannitol, myo-inositol and proline were effective hydroxyl radical scavengers. Glycinebetaine was ineffective. The role of compatible solutes as hydroxyl radical scavengers *in vivo* is discussed.

INTRODUCTION

Compatible solutes are a group of compounds defined by their relatively inocuous effect on metabolism at high concentrations. These solutes accumulate in plant cells in response to a number of stresses and have been mainly studied for their role in the osmotic adjustment of plants subject to salinity and drought They may act as cytoplasmic osmotica which can be accumulated to high concentrations without effect on metabolism [1] The compatible solutes are drawn from several classes of compound and the type accumulated is closely related to the taxonomic position of a species The major types are: (i) proline; (ii) betaines (mainly glycinebetaine) and (iii) sugar alcohols (polyols), mainly mannitol, sorbitol and pinitol [1–4].

Apart from their role as cytoplasmic osmotica, there have been suggestions that these compounds also have other beneficial properties in plants suffering from osmotic stress and water stress *In vitro* studies have shown that they can retard thermal denaturation of enzymes [5, 6], protect proteins from polyethylene glycol-induced precipitation [7], stabilize membranes [8] and protect enzymes against some forms of chemical denaturation [9]. The extent to which compatible solutes have these roles *in vivo*, in addition to their role as osmotica, is not clear.

The observation that mannitol is frequently used by chemists as a scavenger of hydroxyl radicals [10] prompted us to examine the capacity of other compatible solutes to scavenge hydroxyl radicals Hydroxyl radicals are highly reactive and therefore potentially damaging to cells, in particular by initiating lipid peroxidation [11] It has been suggested that increased generation of hydroxyl radicals in damaged tissue elicits phytoalexin synthesis [12]. In plants, superoxide and hydrogen peroxide are generated in illuminated chloroplasts and it is likely that hydroxyl radicals are also formed [13] In normal circumstances, concentrations of these oxygen radicals are likely to remain low because of the activity of protective enzymes including superoxide dismutase, catalase and ascorbate peroxidase [13, 14] Water stress causes an increase in lipid peroxidation and an accumulation of the antioxidant α-tocopherol in grasses [15] and also increases the activity of enzymes of the hydrogen peroxide detoxifying system in chloroplasts [16] This suggests that there is increased generation of oxygen radicals under these conditions. Since compatible solutes also accumulate in droughted plants, their ability to scavenge hydroxyl radicals is of interest

RESULTS

The hydroxyl radical scavenging activity of a range of solutes was assessed by allowing them to compete in two different types of hydroxyl radical generating and detecting systems In the first system they were generated by a mixture of ascorbate, hydrogen peroxide and iron, and detected by their ability to hydroxylate salicylic acid [17] In the second system they were generated by hypoxanthine, xanthine oxidase and hydrogen peroxide [17] and detected by the denaturation of malate dehydrogenase

The ability of compatible solutes to compete with salicylate for hydroxyl radicals generated by the ascorbate system is shown in Fig. 1. A reduction in hydroxylation indicates effective competition for hydroxyl radicals As concentrations of proline, mannitol, sorbitol and myo-inositol (used as an analogue of commercially unobtainable pinitol which is a methylated derivative of myo-inositol) increased, their hydroxyl radical scavenging activity increased. Glycine betaine, in contrast, was ineffective A wider range of solutes, including some non-compatible solutes which also accumulate in plants, were examined at a fixed concentration (Table 1) Sucrose and the polyols were the most effective scavengers, followed by γ-aminobutyric acid, glutamine and proline. Glycine, as well as glycinebetaine, was ineffective Asparagine and glutamate were tested but interfered with the hydroxyl radical generating system

To confirm these results, the solutes were tested with the xanthine oxidase–malate dehydrogenase system. The rate of denaturation of malate dehydrogenase was increased by xanthine oxidase-dependent hydroxyl radical

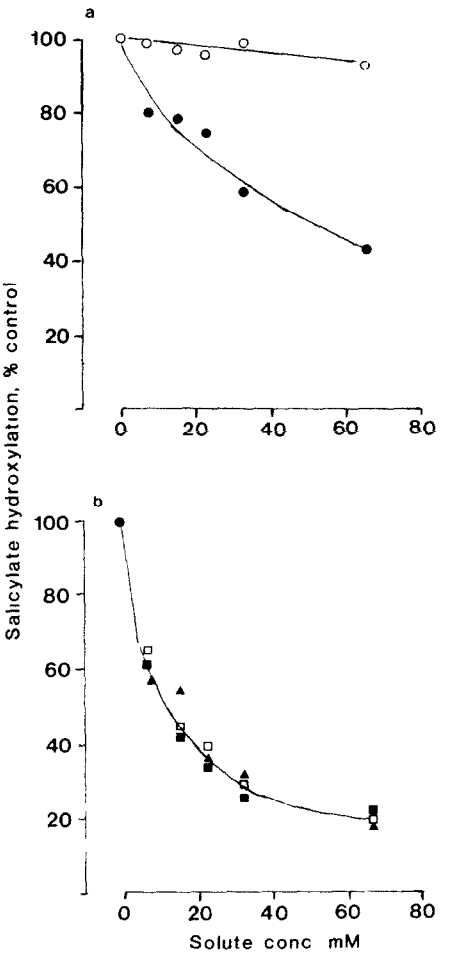

Fig. 1 The effect of various metabolites on salicylate hydroxylation by hydroxyl radicals Hydroxyl radicals were generated by the ascorbate system (a) ● Proline, ○ glycine betaine (b) ▲ Sorbitol, □ mannitol, ■ myo-inositol

generation (Fig 2a) Addition of proline both decreased the rate of denaturation in the absence of hydroxyl radicals and removed the effect of hydroxyl radicals (Fig 2b) The compatible solutes and asparagine were compared using this system (Table 2) Malate dehydrogenase activity after incubation with or without the solutes and with or without xanthine oxidase (hydroxyl radicals) was compared To allow for the stabilizing effect of the compatible solutes on malate dehydrogenase, a scavenging index was calculated (Table 2) Myo-inositol, sorbitol, mannitol and proline protected the enzyme from denaturation by hydroxyl radicals, while glycine betaine and asparagine were ineffective None of the solutes interfered with the xanthine oxidase reaction The relative effectiveness of the compatible solutes as hydroxyl radical scavengers was very similar in both assay systems

DISCUSSION

Among the compatible solutes examined, sorbitol, mannitol, myo-inositol (pinitol analogue) and proline were effective scavengers of hydroxyl radicals Glycinebetaine, however, was ineffective Non-compatible solutes

Table 1 The effect of various metabolites on salicylate hydroxylation by hydroxyl radicals

	$A_{510} \pm$ s d		%
	Control	+ metabolite	control
Sucrose	1 56 ± 0 04	0 04 ± 0 01	25
Glycerol	1 90 ± 0 06	0 55 ± 0 06	28
Myo-inositol	1 71 ± 0 05	0 72 ± 0 02	42
Mannitol	1 52 ± 0 18	0 69 ± 0 04	45
Sorbitol	1 36 ± 0 40	0 61 ± 0 03	55
GABA	1 56 ± 0 04	0 92 ± 0 04	59
Glutamine	1 56 ± 0 04	1 05 ± 0 07	68
Proline	1 16 ± 0 08	0 92 ± 0 05	79
Glycine	1 56 ± 0 04	1 40 ± 0 10	90
Glycinebetaine	1 10 ± 0 02	1 07 ± 0 09	97

Hydroxyl radicals were generated by the ascorbate system and metabolites were added at 16 mM Absorbance values indicate salicylate hydroxylation

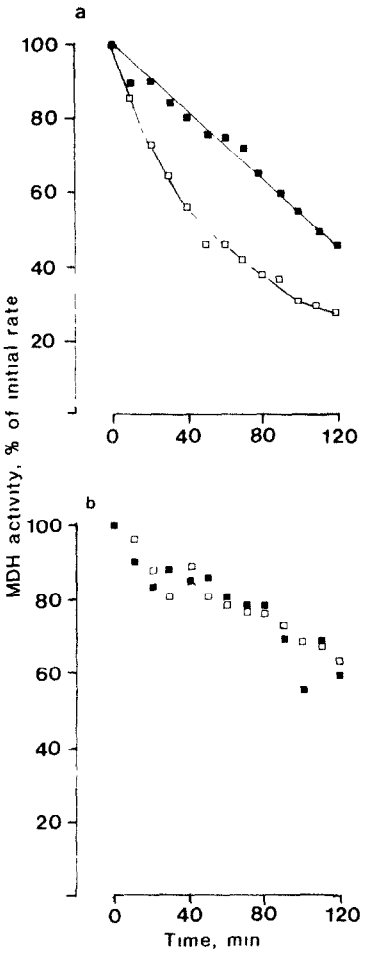

Fig 2 The effect of proline on malate dehydrogenase (MDH) inactivation by hydroxyl radicals Hydroxyl radicals were generated by the xanthine oxidase system (a) Inactivation of MDH by hydroxyl radicals- ■ control, □ hydroxyl radicals generated by xanthine oxidase (b) Effect of proline on MDH inactivation by hydroxyl radicals- ■ proline (33 mM), □ hydroxyl radicals generated by xanthine oxidase + proline (33 mM)

Table 2 The effect of various metabolites on malate dehydrogenase (MDH) inactivation by hydroxyl radicals

	Control		+ xanthine oxidase		
	No metabolite (a)	+ metabolite (b)	No metabolite (c)	+ metabolite (d)	Scavenging index
Myo-inositol	650 ± 18	660 ± 0	261 ± 16	536 ± 4	5 32
Sorbitol	584 ± 34	661 ± 35	266 ± 23	530 ± 9	5 05
Mannitol	1002 ± 34	1061 ± 35	592 ± 47	841 ± 17	4 83
Proline	1002 ± 34	1090 ± 17	592 ± 47	804 ± 29	4 03
Glycinebetaine	648 ± 11	701 ± 34	344 ± 13	361 ± 53	1 06
Asparagine	650 ± 18	425 ± 11	261 ± 16	144 ± 18	0.90

Hydroxyl radicals were generated in the xanthine oxidase system and metabolites were added at 33 mM Values in the table are MDH activity (arbitrary units) ± s d The scavenging index is $\frac{b(a-c)}{a(b-d)}$ A value of 1 indicates no effect of added metabolite and values of > 1 indicate effective competition for hydroxyl radicals

such as sucrose, glutamine and γ-aminobutyric acid were also able to scavenge hydroxyl radicals The products of reactions between compatible solutes and hydroxyl radicals were not considered, but hydroxyproline could be formed from proline [18]

The results show that all the compatible solutes except glycine betaine could, in principle, protect cells against internally generated hydroxyl radicals A similar role has been suggested for flavonoids [19] Further experiments are needed to determine the extent to which compatible solutes could have this role *in vivo* in plants under conditions of drought (see Introduction) or low temperature. Low temperature as well as drought can cause proline accumulation [20] and plants could be subject to oxidative damage under these conditions [21] If it is to be postulated that hydroxyl radical scavenging ability has been one of the selection pressures over evolutionary time for the type of solute accumulated, why is glycinebetaine, a major compatible solute, ineffective? The simplest explanation is that the scavenging ability is incidental and physiologically unimportant. An alternative, and tentative, explanation can be based on the observation that those species which accumulate glycinebetaine will also accumulate proline, for example wheat, barley [22, 23] and a number of halophytes [2], particularly if they are subject to rapid or severe osmotic stress. The same appears to be true of some members of the Chenopodiaceae which are otherwise exclusively betaine accumulators [24]. Proline could perhaps provide extra protection to these plants since a rapid stress to which there is no time for adaptive response could lead to oxygen radical damage These conclusions are necessarily speculative but merit further investigation

EXPERIMENTAL

Two methods were used for generating and detecting hydroxyl radicals The hydroxyl scavenging activity of solutes was determined by adding them to a generating/detecting system and observing their competition with the detector In the first system, hydroxyl radicals were generated from ascorbate and detected by their ability to hydroxylate salicylic acid [17] The reaction mixture contained, in a total vol of 3 cm^3, 150 mM K-Pi buffer, pH 7 4, 0 26 mM ascorbic acid, 0 15 mM Fe EDTA, 0 6 mM H_2O_2, 2 mM Na salicylate and varying concns of test solutes After incubation for 100 min at 25°, hydroxylated salicylate was measured by the method of ref [17] In the second system, also based on the method of ref (17), hydroxyl radicals were generated by xanthine oxidase and hypoxanthine and detected by their ability to denature malate dehydrogenase The reaction mixture contained, in a total vol of 3 cm^3, 150 mM K-Pi buffer, 0 24 mM hypoxanthine, 0 6 mM H_2O_2, 0 15 mM Fe EDTA, 0 1 units malate dehydrogenase (Bovine Heart, Sigma The reaction was started by 0 42 units xanthine oxidase (Sigma) Xanthine oxidase was omitted from control incubations in which no hydroxyl radicals were generated After incubation at 25° for a set time interval (see individual experiments), a 0 1 cm^3 aliquot was taken for assay of malate dehydrogenase activity The MDH assay mixture contained, in a total vol of 3 cm^3, 50 mM K-Pi buffer, pH 7.5, 0 1 mM oxaloacetate and 0 1 mM NADH The rate of NADH oxidation was monitored at 340 nm

Preliminary experiments indicated that the compatible solutes did not interact with superoxide radicals

REFERENCES

1 Wyn Jones, R. G (1984) in *Recent Advances in Phytochemistry*, Vol 18 *Phytochemical Adaptations to Stress* (Timmerman, B N, Steelink, C and Loewus, F A, eds), pp 55–78 North Holland, Amsterdam
2 Stewart, G R, Larher, F, Ahmad, I. and Lee, J A (1979) in *Ecological Processes in Coastal Environments* (Jefferies, R L. and Davy, A J, eds) pp 211–227. Blackwell, Oxford
3. Briers, M and Larher, F (1982) *Plant Cell Envir* **5**, 287
4 Popp, M (1984) *Z Pflanzenphysiol* **113**, 411
5 Paleg, L G, Douglas, T J, Van Daal, A and Keech, D B (1981) *Aust J Plant Physiol* **8**, 107
6 Smirnoff, N and Stewart, G R (1985) *Vegetatio* **62**, 273
7 Paleg, L G, Stewart, G R and Bradbeer, J W (1984) *Plant Physiol* **75**, 974
8 Jolivet, Y, Hamelin, J. and Larher F (1983) *Z Pflanzenphysiol* **109**, 171
9. Yancy, P. H and Somero, G. N (1980) *J. Exp Zool* **212**, 205
10 Czapski, G (1984) in *Methods in Enzymology*, Vol 105 *Oxygen Radicals in Biological Systems* (Packer, L, ed), pp 209–215 Academic Press, New York
11 Halliwell, B and Gutteridge, J M C (1984) *Biochem J* **219**, 1
12 Epperlein, M M, Noronha-Dutra, A A and Strange, R N. (1986) *Physiol Mol Plant Pathol* **28**, 67

13 Halliwell, B (1984) *Chloroplast Metabolism* Clarendon Press, Oxford
14 Asada, A (1984) in *Methods in Enzymology*, Vol 105 *Oxygen Radicals in Biological Systems* (Packer, L, ed), pp 422–428 Academic Press, New York
15 Price, A and Hendry, G A F (1987) in *Free Radicals, Oxidant Stress and Drug Action* (Rice-Evans, C, ed), pp 443–450 Richelieu Press, London
16 Smirnoff, N and Colombe, S V (1988) *J Exp Botany* **39**, 1097–1108
17 Halliwell, B and Gutteridge, J M C (1984) in *Methods in Enzymology*, Vol 105 *Oxygen Radicals in Biological Systems* (Packer, L, ed), pp 47–59 Academic Press, New York
18 Floyd, R A and Z S Nagy (1984) *Biochim Biophys Acta* **790**, 94
19 Husain, S R, Cillard, J and Cillard, P (1987) *Phytochemistry* **26**, 2489
20 Chu, T M, Iuraitis, M, Aspinall D and Paleg, L G (1978) *Physiol Plant* **43**, 254
21 Patterson, B D, Payne, L A, Chen, Y-Z and Graham, D (1974) *Plant Physiol* **76**, 1014
22 Wyn Jones, R G (1981) in *The Physiology and Biochemistry of Drought Resistance in Plants* (Paleg, L G and Aspinall, D, eds), pp 171–204 Academic Press, Sydney
23 Aspinall, D and Paleg, L G (1981) in *The Physiology and Biochemistry of Drought Resistance in Plants* (Paleg, L G and Aspinall, D, eds), pp 205–241 Academic Press, Sydney
24 Coughlan, S J and Wyn Jones, R G (1980) *J Exp Botany* **31**, 883