Effects of 2,4-Dinitrophenol on Membrane Lipids of Roots[1]

Received for publication July 7, 1981 and in revised form March 27, 1982

PATRICIA C. JACKSON AND JUDITH B. ST. JOHN
Plant Physiology Institute (P. C. J.) and Agricultural Environmental Quality Institute (J. B. S.), Agricultural Research, United States Department of Agriculture, Beltsville, Maryland 20705

ABSTRACT

Previous work has shown that the undissociated form of 2,4-dinitrophenol (DNP) increases the permeability of barley (*Hordeum vulgare var. trebi*) roots to ions. The present studies were undertaken to determine whether the effects of undissociated DNP were directly on membrane lipids. Relative amounts of the principal fatty acids from the lipids of barley root membranes were assayed as a function of DNP concentration, pH, and time of treatment under conditions similar to the previous studies of DNP effects on permeability. Undissociated DNP increases the proportions of palmitic and oleic acids and decreases linoleic and linolenic acids with no changes in the amounts of total fatty acids. The effects are immediate, as are the effects on permeability. Only the undissociated DNP is effective. Anionic DNP has no effect, although it is the major species taken up by the roots both at pH 5 and pH 7. DNP has no effect on respiration at either pH, indicating that undissociated DNP effects are on the membranes and not a general metabolic effect. The close parallelism between the effects of DNP on the composition of membrane lipids and on permeability suggests that the increase in permeability produced by undissociated DNP is due to a direct effect on the root membranes.

Previous work (14) has shown that undissociated forms of organic acids such as formic, acetic, and propionic increase permeability of barley roots to ions. The permeability increase is associated with changes in the membrane lipids of the roots (13). Undissociated forms of DNP[2] and various phenolic analogs likewise increase permeability of barley roots to ions (10). This was interpreted as direct effects of DNP and its analogs on the root membranes, an interpretation that is shared by others studying effects of various phenols (3, 7, 16). Whether such effects also are associated with changes in the membrane lipids is the premise of the work presented here.

Relative amounts of the principal fatty acids, *i.e.* palmitic (C 16:0), stearic (C 18:0), oleic (C 18:1), linoleic (C 18:2), and linolenic (C 18:3), from the polar lipids of membranes (8) of the roots were determined. The fatty acids were measured as a function of DNP concentration, pH, and time of treatment under conditions similar to those in previous studies on the effects of DNP on permeability of the roots to ions (10). The results show that undissociated DNP causes chemical changes in the root membranes.

MATERIALS AND METHODS

The roots used were from 6-d-old seedlings of barley (*Hordeum vulgare* var. Trebi) which had been grown in aerated 0.2 mM $CaSO_4$ with or without 0.1 mM KCl in the dark at 25°C. The pH was adjusted and maintained at 5.6 by titration with KOH or HCl. The roots were excised and rinsed three or four times with demineralized H_2O just before use in an experiment.

Details of the procedure and conditions for the experiments are the same as in previous studies (12). Briefly, 10 g roots were maintained in aerated salt solutions with or without DNP at 23 to 25°C for 15 min to 6 h. Then the roots were removed, rinsed, and freeze dried. The pH was adjusted and maintained during treatment by periodic titration with an appropriate base or acid.

Lipids from ground freeze-dried roots were extracted according to procedures of Folch *et al.* (6). The polar lipids (membrane lipids) were separated from the nonpolar lipids by rubber membrane dialysis (4) and then were saponified with alcoholic KOH according to the method of Burchfield and Storrs (5). Methyl esters of the resulting fatty acids were prepared for GC (18) with boron trifluoride in methyl alcohol as described by Metcalfe and Schmitz (15). Heptadecanoic acid was added to all samples as an internal standard.

The results are expressed generally as the percent by weight of the total fatty acids (C 16:0, C 18:0, C 18:1, C 18:2, C 18:3) and as ratios of saturated to unsaturated acids. This was done partly because the gas chromatographic data are presented as relative values. The combination of weighing the samples and quantitative introduction of an internal standard so that quantities can be calculated is less precise. However, the total fatty acids are given as μmol acid/g freeze-dried roots. The dry weights are approximately 7% of the fresh weights. Experiments were repeated 1 to 4 times. The data presented are from representative experiments.

RESULTS

Effects of DNP on the fatty acids from polar (membrane) lipids of roots vary with the pH of the solution. DNP at pH 5 increases the proportion of palmitic acid (C 16:0) and decreases the proportions of linolenic acid (C 18:3) and, to a lesser degree, linoleic acid (C 18:2) from the membrane lipids of roots in 10 μM K^+-DNP for 6 h (Table I). Total amounts of the fatty acids do not change so that the effects are entirely distributional. That is, a decrease in C 18:2 and C 18:3 is balanced by an increase in C 16:0. Such changes in the fatty acids are reflected by an increase in the relative saturation and an increase in the ratio of linoleic to linolenic acid. These effects are all significant at the 1% level of confidence. Stearic (C 18:0) and oleic (C 18:1) acids increase slightly but effects on these acids are smaller and less consistent than effects on the three principal fatty acids which comprise more than 90% of the total fatty acids from the membrane lipids.

In contrast to the effects at pH 5, 10 μM DNP at pH 7 has no effect on the fatty acids, distributional or otherwise. Lack of effects at pH 7 is consistent with the much lower concentration of undissociated DNP at pH 7 than at pH 5.

Effects of DNP at pH 5 appear to be produced as soon as the roots are introduced into the solution, as shown by roots treated with 10 μM DNP as a function of time (Fig. 1). Changes in the

[1] This paper is dedicated to the memory of Sterling B. Hendricks.

[2] Abbreviations: DNP, 2,4-dinitrophenol; DNP-H, undissociated DNP, S/U, ratios of saturated to unsaturated fatty acids.

Table I. *Effect of 2,4-Dinitrophenol on Fatty Acids from Membrane Lipids of Roots*

Roots were maintained in 10 μM solutions of 2,4-dinitrophenol for 6 h. S/U is the ratio of the saturated to unsaturated fatty acids. Standard deviations are within ±2% of the values for 16:0, 18:2, 18:3, S/U, and 18:2/18:3; within ±9% of the values for 18:0 and 18:1; and average ±15% for total μmol/g.

Treatment	DNP-H	16:0	18:0	18:1	18:2	18:3	S/U	18:2/18:3	Total
	μM	% total fatty acids							μmol/g
H_2O		26.6	1.7	3.9	49.8	18.1	0.393	2.75	38.8
2,4-DNP									
pH 7	0.009	25.0	2.1	3.9	50.8	18.2	0.372	2.79	34.1
pH 5	0.80	33.0	1.8	4.5	46.1	14.6	0.535	3.17	43.5

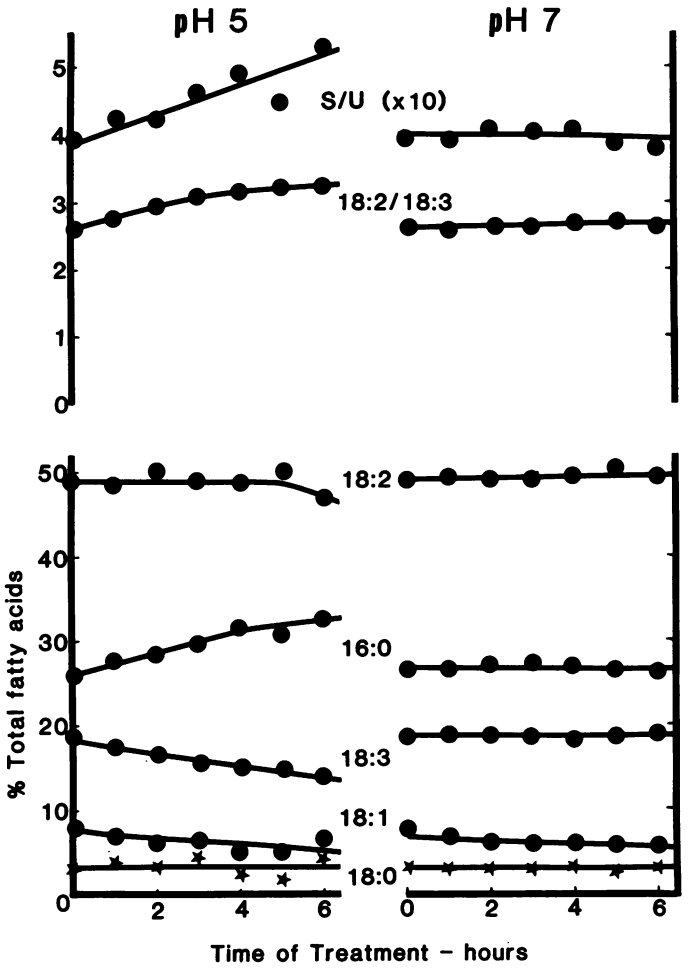

FIG. 1. Effects of 10 μM DNP on proportions of fatty acids from root membrane lipids as a function of time and pH. Percentages of C 18:0 and C 18:1 have been multiplied by 2 on graph. S/U is ratio of saturated to unsaturated fatty acids. Standard deviations are within ±2% of values for 16:0, 18:2, 18:3, S/U, and 18:2/18:3 and within ±9% of values for 18:0 and 18:1.

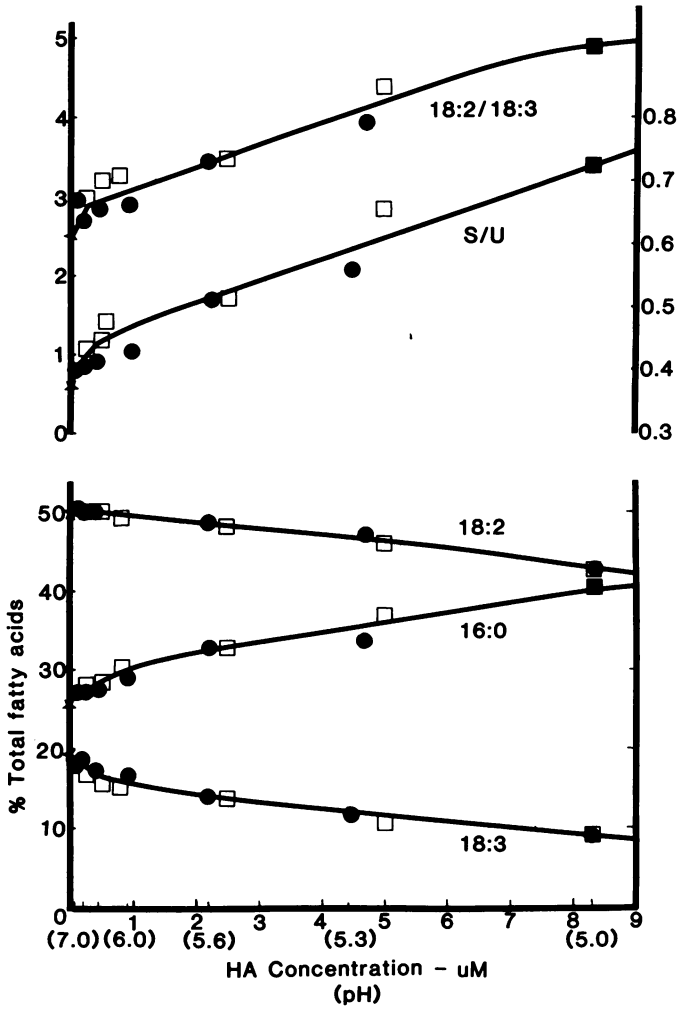

FIG. 2. Effects of various concentrations of DNP-H on proportions of fatty acids from root membranes. (○), Treatment with DNP-H concentrations obtained by varying pH from 7 to 5 at a constant DNP concentration of 0.1 mM. (□), Treatment with DNP-H concentrations obtained by varying DNP concentration from 1 μM to 0.1 mM at a constant pH of 5.0. HA concentration is concentration of undissociated DNP-H. S/U is ratio of saturated to unsaturated fatty acids. Standard deviations are within ±2% of values for 16:0, 18:2, 18:3, S/U, and 18:2/18:3 and within ±9% of values for 18:0 and 18:1.

proportions of C 16:0 and C 18:3 and in the ratios progress at constant rates for 4 h or longer. The curves all pass through zero change at zero time with correlation coefficients of 0.92, 0.95, 0.84, and 0.95 for C 16:0, C 18:3, S/U, and 18:2/18:3, respectively. Only the effect on C 18:2 appears delayed, a feature of its role as an intermediate between C 18:3 and C 16:0. No effects on the fatty acids are evident during the 6 h of treatment when the roots are in 10 μM DNP at pH 7. Individual results at pH 5 differ significantly from comparable results at pH 7, thereby showing consistency only with the concentration of DNP-H. The fatty acids do not respond to presence of anionic DNP, which is the predominant species at either pH.

Dependence of the effects on the presence of DNP-H only was tested by comparison of the effects as a function of various concentrations of DNP-H which were obtained in two ways. The concentration of DNP-salt was varied at a constant pH or, the pH was varied at a constant DNP-salt concentration. The results show that effects of the concentrations of undissociated DNP obtained in these two ways describe the same curve (Fig. 2). The effects

Table II. *Effect of Various Substituted Phenols on Fatty Acids from Membrane Lipids of Roots*

Treatment was for 6 h and the salt concentrations were 0.1 mM. Standard deviations are within ±2% of the values for 16:0, 18:2, 18:3, 18:2/18:3, and S/U; within ±10% of the values for 18:0 and 18:1; and average ±20% for the total μmol/g.

Treatment	Undissociated Acid	16:0	18:0	18:1	18:2	18:3	S/U	18:2/18:3	Total
	μM			% total fatty acids					μmol/g
$CaSO_4$		27.0	1.0	2.9	49.8	19.3	0.390	2.59	45.4
2,5-DNP									
pH 7	1.6	27.6	1.8	3.4	49.9	17.4	0.416	2.86	—
5	62.4	30.3	0.9	3.3	49.3	16.2	0.453	3.04	51.9
2,6-DNP									
pH 7	1.7	27.3	2.2	3.4	48.1	18.9	0.418	2.55	—
5	62.9	29.0	1.0	3.2	50.5	16.3	0.428	3.09	62.9
2,4-Cl, nitrophenol									
pH 7	2.7	29.2	2.9	4.0	47.3	16.8	0.470	2.82	43.0
5	73.8	34.0	1.0	3.5	48.0	13.1	0.548	3.66	36.7

Table III. *Nonreversibility of DNP Effects on Fatty Acids from Membrane Lipids of Roots*

The concentrations of KCl and $CaSO_4$ were 1.0 and 0.1 mM and DNP was 10 μM at pH 5 (0.8 μM undissociated DNP). S/U is the ratio of saturated to unsaturated fatty acids. Standard deviations are within ±2% of the values for 16:0, 18:2, 18:3, 18:2/18:3, and S/U; within ±9% of the values for 18:0 and 18:1; and average ±15% for total μmol/g.

Treatment	16:0	18:0	18:1	18:2	18:3	S/U	18:2/18:3	Total
			% total fatty acids					μmol/g
KCl + $CaSO_4$	26.5	1.2	2.8	51.3	18.1	0.383	2.83	44.1
DNP (3 h)	28.6	1.3	3.3	50.5	16.2	0.427	3.19	44.6
↓								
KCl + $CaSO_4$ (3 h)	29.5	1.6	4.0	50.1	14.7	0.451	3.40	35.7

increase in direct proportion to the DNP-H concentration from 0 to 1.0 μM. The total DNP concentration in this range varied from 0 to 12.5 μM at pH 5 and the pH varied from 7 to 6.0 at 0.1 mM DNP. With further increase in the DNP-H concentration above 1.0 μM, the degree of change lessens as if it ultimately approached a maximum. These results show that the effects of DNP on the fatty acids depend only on the concentration of DNP-H. The effects reflect neither the pH change, *per se*, nor the concentration of anionic DNP.

Three other substituted nitrophenols that also increase permeability of the roots to ions (10) were assayed for the effects on the fatty acids with respect to pH (Table II). Presence of 0.1 mM 2,5-DNP or 2,6-DNP at pH 5 increases the proportion of C 16:0 and decreases the proportion of C 18:3, thereby increasing the degree of saturation and the ratio, 18:2/18:3, but has little or no effect at pH 7 where the concentration of the undissociated species is much lower. On the other hand, the concentration of undissociated 2,4-chloronitrophenol changes less with the pH change and effects are evident at both pH 5 and pH 7. Effects of these phenols are much like those of DNP, except that the changes in the fatty acids are generally not as large as with DNP. Furthermore, the concentration of undissociated phenol required for observable effects is much greater for these phenols than for DNP.

Reversibility of the DNP effects on the fatty acids was tested by maintaining roots in 10 μM DNP at pH 5 for 3 h and then quickly transferring them after a rinse in water to 1 mM KCl + 0.1 mM $CaSO_4$ for 3 h. This experiment is similar to a previous experiment on DNP uptake which showed that less than 3% of the DNP taken up by the roots is lost during transfer and subsequent imbibition in the salt solution without DNP (10). The expected changes in the fatty acid composition occurred during the 3 h in the presence of DNP; however, they continued to progress for 3 h after transfer of the roots to a salt solution without DNP (Table III). Some loss in the total amount of fatty acids occurred during the second 3 h but the loss is barely significant. It would have to be a loss of only C 18:2 and C 18:3 in order to account for the distributional changes.

A time course of the changes in the fatty acids of roots in salt solution without DNP after 3 h of pretreatment in 10 μM DNP at pH 5 was performed with the results shown in Figure 3. Changes in the fatty acid proportions indeed continue after removal from DNP in much the same pattern as fatty acids from roots continually in the presence of DNP (Fig. 1). Total amounts of the fatty acids did not change at all during the first 3 h in the DNP solution, but thereafter, the total amount decreased progressively to 70% of the initial content. Such a loss, although significant at the 5% level of confidence, can account for the distributional changes if only C 18:2 and C 18:3 are lost.

DISCUSSION

Effects of DNP on the root membrane lipids are due solely to the undissociated molecular species of DNP. Anionic DNP has no effect on membrane lipids or ion permeability, although it is the major species taken up by barley roots both at pH 5 and pH 7 (10). Effects of 10 μM DNP are marked at pH 5 where the concentration of DNP-H is relatively high, but effects are absent at pH 7 where the DNP-H concentration is one-eightieth of the concentration at pH 5 (Table I). Involvement of only DNP-H was verified in another way. Various concentrations of DNP-H can be obtained either by varying the pH at a constant concentration of DNP-salt or by varying the DNP-salt concentration at a constant pH. When this is done, the results show that the effects depend only on the concentration of DNP-H, whatever the pH or concentration of DNP-salt (Fig. 2).

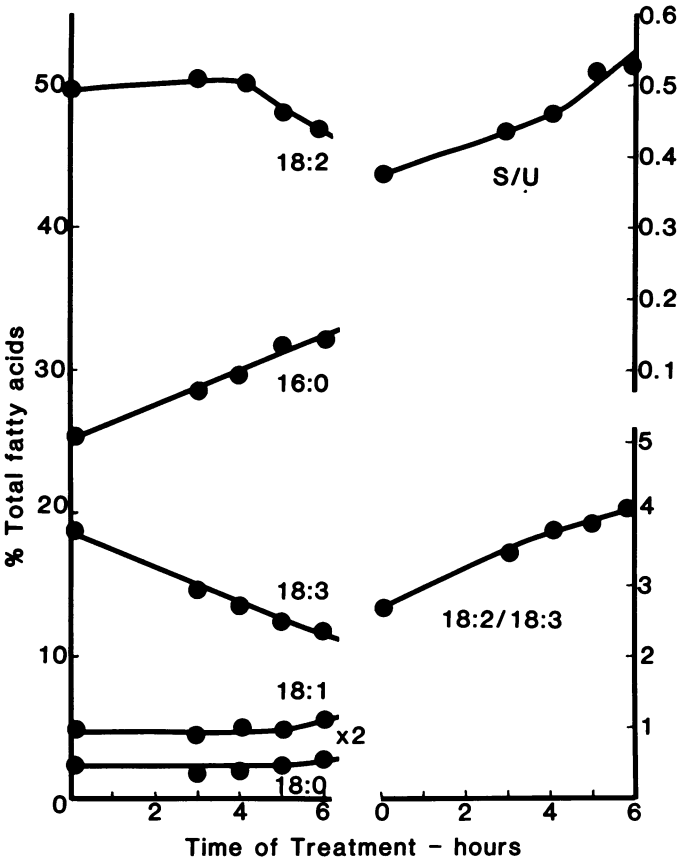

FIG. 3. Effects of treatment of roots for 3 h in 10 μM DNP at pH 5 followed by treatment in 1 mM KCl + 0.1 mM $CaSO_4$ at pH 5.5 on fatty acids from root membrane lipids. S/U is ratio of saturated to unsaturated fatty acids. Standard deviations are within ±2% of values for 16:0, 18:2, 18:3, S/U, and 18:2/18:3 and within ±9% of values for 18:0 and 18:1.

Effects on DNP-H on membrane lipids closely parallel its effects on ion permeability with respect to the effective concentrations of DNP, the rapidity of the action, the relative effects of the other phenols, and lack of any effect of the presence or uptake of anionic DNP, *per se* (10). This might suggest a simple and direct relationship between permeability and membrane lipids except that the roots immediately recover ability to accumulate ions upon removal of DNP from the external solution (10), but the fatty acid distribution does not revert (Table III). This is an important difference between the organic acid effects on ion accumulation that were not immediately reversible (14). The difference may be one of degree of permeability increase but nevertheless it indicates that several factors control ion accumulation. The effects on the root membranes not only involve an increase in ion permeability but also, thereby, may disrupt the gradient for ion accumulation. Loss of buffering capacity from roots in 10 mM acetate at pH 5 for 3 h along with a respiratory decrease (14) indicates much more than inorganic ions were lost from the roots over this time. Presumably, the losses include compounds that provide the gradient for ion accumulation, *i.e.* substrates, ATP, *et al.* Thus, little or no potential for ion accumulation remained, particularly in the epidermal and outermost cortical cells (approximately 25% of the total root cell volume) which are the first to lose or accumulate ions. No loss of buffering capacity or respiratory decrease occurred with roots in 10 μM DNP at pH 5 for 3 h (10) so that much of the potential for ion accumulation is likely to have remained in this case. Furthermore, the hydrogen from the hydroxyl group of undissociated phenols such as DNP forms readily reversible hydrogen bonds with peptides, whereas the hydrogen bonds formed by the hydrogen of the undissociated carboxyl group of organic acids are relatively stronger (19). Thus, effects of DNP are more likely to be reversible than effects of the organic acids even when the effects are the same. These differences between DNP and the organic acids explain the difference in reversibility of the effects on permeability. However, the reversible effects of DNP on ion accumulation without reversion of the fatty acid distribution indicates some separation of the two effects.

The continued change in the fatty acid distribution after removal of DNP from the roots (Fig. 3) can be explained by consideration of the root morphology. Evidently DNP-H does not reach all of the interiors of all of the cells within 3 h and DNP already in the more external portions of the roots continues to progress to the interior. Slow accessibility of DNP to all of the cells is indicated by a loss of only approximately 40% of the K^+ from the roots over the first 3 h, whereas a 75% loss occurred in 6 h and an almost total loss by 24 h (10). As a corollary, the measured effects on the fatty acids are expected to be attenuated and represent only minimal changes because the measurements involve all of membranes of all of the cells that are not readily accessible.

It might be held that DNP-H effects on the membrane lipids reflect the uncoupling action of DNP. Failure of DNP to increase respiration of barley roots (10) might suggest that DNP was not uncoupling. However, the large increases in root respiration produced by substantial uptake of succinate and acetate (14) indicate that the respiration is rate limited by the substrate content. Under such conditions, uncouplers do not stimulate respiration (17, 20). Evidence that DNP uncoupling is not involved with the lipid changes and permeability increase comes from other considerations. Namely, the uncoupling of oxidative phosphorylation of mitochondria by DNP is competitive with Pi (1, 11). This infers that anionic DNP is the effective species. Furthermore, DNP competitively inhibits phosphate uptake by barley roots (9), which is rate limited by oxidative phosphorylation of root mitochondria (11). Thus, DNP anions would be expected to uncouple both at pH 5 and pH 7 in the present experiments, but be without effect on the membrane lipids or permeability. This is not to say that ATP utilization (ATPase) is not involved in ion uptake. It simply does not appear to be rate limiting for these experiments. Rather the effects of DNP are much like those of organic acids (formic, acetic, *et al.*) in that they increase permeability and affect the membrane lipids in the same way (13). That is, the degree of saturation of the fatty acids increases with an increase in C 16:0 balancing the decrease in C 18:3. Only the undissociated molecular species are effective, which contrasts greatly with the uncouplers.

Thus, the changes in the root membrane lipids produced by DNP-H indicate that DNP affects the plasma membranes, among other membranes, of the roots directly and thereby alters the permeability of the roots to ions. This is indicated by the rapid effects of DNP on both the fatty acids from the membrane lipids (Fig. 1) and the ion permeability (10). The time curves of the fatty acid changes show no initial lag. They are linear for at least the first 4 h and extrapolate to zero effect at zero time. The rapid reversibility of the effect of DNP on ion uptake (10) is another indication that its action is a direct effect on the plasma membranes and not a result of internal metabolic changes.

The results show that DNP-H is effective at a lower concentration (1 μM) than undissociated forms of the other phenols tested (60–100 μM), which in turn are effective at lower concentrations than the undissociated organic acids (0.5–1 mM) (14). Among these compounds, DNP-H is the most hydrophilic, and the organic acids are the least hydrophilic. This appears to be a factor in the effects rather than lipid solubility *per se* (10). The common denominator for all of these diverse compounds is that they must carry an acid entity to be at all effective. Compounds such as these are known to bind to proteins, displacing water by hydrogen bonding

of the undissociated hydroxyl or carboxyl group (19). They are all lipid soluble but sufficiently hydrophilic for a protein environment of the membrane where hydrophilic ions permeate. Bakker *et al.* (2) have shown that DNP causes swelling of mitochondria and phospholipid vesicles. An attractive possibility is that hydrogen bonding of DNP-H to membrane protein induces swelling, which both increases ion permeability and inhibits fatty acid desaturases. Previously, we had suggested that the undissociated organic acids change the membrane lipids by inhibiting fatty acid desaturases. This is consistent with the effects of DNP-H as well.

LITERATURE CITED

1. BADDELEY MS, JB HANSON 1967 Uncoupling of energy-linked functions of corn mitochondria by linoleic acid and monomethyldecenylsuccinic acid. Plant Physiol 42: 1702–1710
2. BAKKER EP, EJ VAN DEN HEUVEL, AHCA WIECHMANN, K VAN DAM 1973 A comparison between effectiveness of uncouplers of oxidative phosphorylation in mitochondria and in artificial membrane systems. Biochim Biophys Acta 292: 78
3. BAKKER JL, H LEVITAN 1974 Phenols: effects on membrane permeability of molluscan neurons. Brain Res 67: 555–561
4. BOTTCHER CJ, FP WOODFORD, E BOELSMA-VAN HOUTE, CM GENT 1959 Methods for the analysis of lipids extracted from human arteries and other tissues. Rec Trav Chim 78: 794–814
5. BURCHFIELD HP, EE STORRS 1962 Biochemical Applications of Gas Chromatography. Academic Press, New York, pp 488–568
6. FOLCH J, M LEES, GH STANLEY 1957 A simple method for the isolation and purification of total lipids from animal tissue. J Biol Chem 226: 497–509
7. GLASS ADM 1974 Influence of phenolic acid upon ion uptake. IV. Depolarization of membrane potentials. Plant Physiol 54: 855–858
8. HITCHCOCK C, BW NICHOLS 1971 Plant Lipid Biochemistry. Academic Press, New York
9. HOPKINS HT 1956 Absorption of ionic species of orthophosphate uptake by barley roots: effects of 2,4-dinitrophenol and oxygen tension. Plant Physiol 31: 155–161
10. JACKSON PC 1982 Differences between effects of undissociated and anionic DNP on permeability of barley roots. Plant Physiol In press
11. JACKSON PC, SB HENDRICKS, BM VASTA 1962 Phosphorylation by barley root mitochondria and phosphate absorption by barley roots. Plant Physiol 37: 8–17
12. JACKSON PC, KJ STIEF 1965 Equilibrium and ion exchange characteristics of potassium and sodium accumulation by barley roots. J Gen Physiol 48: 601–616
13. JACKSON PC, JB ST. JOHN 1980 Changes in membrane lipids of roots associated with changes in permeability I Effects of undissociated organic acids. Plant Physiol 66: 801–804
14. JACKSON PC, JM TAYLOR 1970 Effects of organic acids on ion uptake and retention in barley roots. Plant Physiol 46: 538–542
15. METCALFE LD, AA SCHMITZ 1961 The rapid preparation of fatty acid esters for gas chromatography analysis. Anal Chem 33: 363–364
16. RIEMERSMA JC 1968 Effects of sodium azide and 2,4-dinitrophenol on phosphorylation reactions and ion fluxes in saccharomyces cerevisiae. Biochim Biophys Acta 153: 80–87
17. SLATER EC 1963 Uncouplers and inhibitors of oxidative phosphorylation. *In* Metabolic Inhibitors, Vol. II. Academic Press, New York, pp 503–516
18. ST. JOHN JB 1976 Manipulation of galactolipid fatty acid composition with substituted pyridazinones. Plant Physiol 57: 38–40
19. VAN SUMERE CF, J ALBRECHT, A DEDONDER, H DE POOTER, I PÉ 1975 Plant proteins and phenolics in the chemistry and biochemistry of plant proteins, *In* JB Harbone, CF Van Sumere, eds. Proceedings of Phytochemistry Society, University of Ghent, Belgium, September 1973. Academic Press, London, pp 211–247
20. WEINER MW 1979 Uncoupling agents distinguish between effects of metabolic inhibitors and transport inhibitors. Science 204: 187–188