Phytochemistry, 1972, Vol. 11, pp. 3403 to 3411. Pergamon Press. Printed in England.

α-OXIDATION OF ENDOGENOUS FATTY ACIDS IN FRESH POTATO SLICES

G. G. LATIES, CAROL HOELLE and B. S. JACOBSON*

Biology Department and Molecular Biology Institute, University of California, Los Angeles, CA 90024, U.S.A.

(*Received* 16 *May* 1972. *Accepted* 15 *August* 1972)

Key Word Index—*Solanum tuberosum*; Solanaceae; potato slices; fatty acid α-oxidation; imidazole; carbonyl-cyanide *m*-Cl-phenylhydrazone.

Abstract—The respiration of fresh potato slices is inhibited up to 30% by imidazole, an inhibitor of α-oxidation of long chain fatty acids; it is stimulated approximately threefold by the uncoupler, carbonyl-cyanide *m*-Cl-phenylhydrazone (*m*-Cl-CCP). The stimulation by *m*-Cl-CCP is totally inhibited by imidazole. The release of $^{14}CO_2$ from carboxyl-labelled long chain fatty acids is also stimulated by *m*-Cl-CCP in fresh slices, and inhibited by imidazole. The $\delta^{13}C$ value of fresh tissue respiratory CO_2—indicative of the $^{13}C/^{12}C$ ratio of the endogenous respiratory substrate—indicates lipid to be the predominant substrate in fresh slices, as well as in fresh slices treated with *m*-Cl-CCP. Aged slices are considerably less imidazole sensitive in all the above respects. The substrate for aged slice respiration as indicated by $\delta^{13}C$ values is carbohydrate. The basal respiration in fresh potato slices apparently involves considerable lipid oxidation of a mixed nature, while the respiratory increment in the presence of *m*-Cl-CCP represents predominantly α-oxidation of long chain fatty acids.

INTRODUCTION

THE RESPIRATION of thin slices of a variety of bulky plant storage organs immediately on cutting is normally several times that of the intact organ.[1] Subsequently slice respiration rises with time, the rise being prevented by inhibitors of RNA and protein synthesis.[2] The time-related induced respiration has been considered to be qualitatively different from fresh slice respiration in potato, the induced respiration reflecting an enhancement of tricarboxylic acid cycle (TCAC) activity.[1,3] We have determined that the respiration of intact tubers and aged slices involves carbohydrate metabolism, while that of fresh slices comprises predominantly lipid oxidation.[4] Fresh slices fail to oxidize exogenously provided conventional intermediates of glycolysis or of the TCAC.[3] Rather, they selectively metabolize long chain fatty acids[5] in a way which implicates α-oxidation.[6]

Potato tuber is low in total lipid,[7] and in our view lipid substrate, and specifically the long chain fatty acids, originates from the degradation of mitochondrial and cellular

*Present address: Dept. of Biochemistry and Biophysics, University of California, Davis, CA 95616, U.S.A.

[1] G. G. LATIES, in *Control Mechanisms in Respiration and Fermentation* (edited by B. WRIGHT), p. 129, Ronald Press, New York (1963).
[2] M. SAMPSON and G. G. LATIES, *Plant Physiol.* **43**, 1011 (1968).
[3] G. G. LATIES, *Plant Physiol.* **39**, 654 (1964).
[4] B. S. JACOBSON, B. N. SMITH, S. EPSTEIN and G. G. LATIES, *J. Gen. Physiol.* **55**, 1 (1970).
[5] G. G. LATIES and C. HOELLE, *Phytochem.* **6**, 49 (1967).
[6] R. O. MARTIN and P. K. STUMPF, *J. Biol. Chem.* **234**, 2548 (1959).
[7] T. GALLIARD, *Phytochem.* **9**, 1725 (1970).

membranes[8,9] initiated in fresh potato slices by lipase activity induced by cutting.[10] Free fatty acids impair oxidative phosphorylation, substrate level phosphorylation and the transport of adenine nucleotides across mitochondrial membranes,[11] while free fatty acid oxidation products inhibit citrate synthesis, the oxidation of pyruvate and isocitrate,[12] and the activity of phosphofructokinase and of glycolysis in general.[13] The assertion that glycolysis and the TCAC are suppressed in fresh potato slices[3] is explicable in terms of the foregoing.

The respiration of fresh storage organ slices in general,[1] and potato slices in particular,[14] is stimulated by uncouplers of oxidative phosphorylation. This has commonly been ascribed to an enhancement of TCAC activity, since oxidative phosphorylation is linked primarily to electron transport associated with the TCAC. However, Laties[15,16] has argued that the DNP induced respiration of fresh slices is emphatically *not* TCAC related, and the evidence presented below confirms this view. The meaning of uncoupler-stimulated respiration of fresh potato discs has been re-examined utilizing the specificity of imidazole as an inhibitor of α-oxidation of fatty acids,[6] and of the difference in carbon mass isotope composition of the respiratory CO_2 arising from lipid and carbohydrate oxidation respectively.[4] In brief, it has been shown that the raised respiration of uncoupler treated fresh potato slices consists primarily of α-oxidation of long chain fatty acids, while the respiration of untreated fresh slices appears to be a mixture of fatty acid α-oxidation and an undefined respiratory pathway other than the TCAC, which involves both lipid and carbohydrate.

RESULTS

Respiratory Measurements

Experiments by Laties and Hoelle[5] and Jacobson *et al.*[4] on the nature of fresh potato slice respiration were carried out at pH 5·0—on the one hand to facilitate time-course studies of $^{14}CO_2$ evolution from labelled fatty acids,[5] and on the other, to reduce the ambiguity introduced by the presence of tissue bicarbonate in connection with the mass isotope composition of endogenous respiratory CO_2.[4] Imidazole inhibition of fresh slice respiration proved limited or absent at the low pH (Table 1), presumably owing to the impermeativity of protonated imidazole (pK_a *ca.* 6·95). Further, carbonyl cyanide *m*-Cl-phenylhydrazone (*m*-Cl-CCP), an apparently more effective uncoupler of slice respiration than 2,4-dinitrophenol (DNP) (compare Tables 1 and 4), is insoluble at pH 5·0 (pK_a 5·95). Consequently experiments were performed at pH 7·5 or 8·5 depending upon the nature of the experiment and the buffer of choice, pH variation in the indicated range being unimportant with regard to *m*-Cl-CCP stimulation and subsequent imidazole inhibition of respiration. Specifically, stimulation by *m*-Cl-CCP is no more than 15% greater at pH 8·5 than at 7·5, while imidazole inhibition is essentially pH independent in the same range. Maximal respiratory stimulation by *m*-Cl-CCP at pH 5·0 is observed at 10^{-5}M. Imidazole

[8] R. F. M. VAN STEVENINCK and M. E. JACKMAN, *Austral. J. Biol. Sci.* **20,** 749 (1967).
[9] M. E. JACKMAN and R. F. M. VAN STEVENINCK, *Austral. J. Biol. Sci.* **20,** 1063 (1967).
[10] T. GALLIARD, *Biochem. J.* **121,** 379 (1971).
[11] L. WOJTCZAK, K. BOGUCKA, M. G. SARZALA and H. ZALUSKA, *FEBS Symp.* **17,** 79 (1969).
[12] D. B. GARLAND, D. SHEPHERD, D. G. NICHOLLS and J. ONTKO, *Adv. Enzyme Regul.* **6,** 3 (1968).
[13] P. J. RANDLE, R. M. DENTON and P. J. ENGLAND, in *Metabolic Roles of Citrate* (edited by T. W. GOODWIN), p. 87, Academic Press, New York (1968).
[14] D. P. HACKETT, D. W. HAAS, S. K. GRIFFITHS and D. J. NIEDERPRUEM, *Plant Physiol.* **35,** 8 (1960).
[15] G. G. LATIES, *Arch. Biochem. Biophys.* **79,** 364 (1959).
[16] G. G. LATIES, *Arch. Biochem. Biophys.* **79,** 378 (1959).

inhibition at pH 8·5 is considerable at 0·05 M, and for practical purposes maximal at 0·1 M. The total inhibition of uncoupler-evoked respiration by imidazole verifies the effectiveness of the inhibitor and the absence of permeation-related artifacts (Table 1).

TABLE 1. IMIDAZOLE INHIBITION OF CONTROL AND UNCOUPLER-STIMULATED RESPIRATION OF FRESH AND AGED POTATO DISCS

	pH	Fresh		Aged	
		$\mu l\ O_2\ g^{-1}\ hr^{-1}$	% control	$\mu l\ O_2\ g^{-1}\ hr^{-1}$	% control
Experiment 1					
Control	8·5	53	100	145	100
Imidazole, 0·05 M		41	77	136	94
Imidazole, 0·1 M		44	83	128	88
m-Cl-CCP, 10^{-5} M		155	292	240	165
m-Cl-CCP + 0·05 M imidazole		81	152	214	147
m-Cl-CCP + 0·1 M imidazole		48	90	192	132
Experiment 2					
Control	8·5	39	100	130	100
Imidazole, 0·1 M		26	67	101	78
m-Cl-CCP, 10^{-5} M		107	276	194	149
m-Cl-CCP + imidazole		28	72	147	113
Experiment 3					
Control	5·0	33	100	—	—
Imidazole, 0·1 M		37	112	—	—
DNP, 10^{-4} M		58	175	121	93
DNP + imidazole		45	136	142	109

Experiment 1. 10^{-2} M K phosphate, pH 8·5, 10^{-4} M $CaSO_4$.
Experiment 2. Glycylglycine, pH 8·5—0·1 M in absence of imidazole and 0·05 M in presence of imidazole.
Experiment 3. 0·05 M K phthalate, pH 5·0.

The influence of imidazole on the respiration of freshly prepared potato slices and on uncoupler stimulated respiration of fresh slices are compared in Table 1 with the effect of imidazole on aged slices in the presence and absence of uncoupler. The inhibition of fresh slice respiration ranges from 17 to 33% in the experiments reported. Inhibition of uncoupler treated fresh slices is considerably higher, averaging 74%. If attention is focused on the uncoupler evoked increment, the latter is totally inhibited by imidazole in fresh slices. By contrast, aged slices show a much more limited response to uncoupler, and the increment shows but limited sensitivity to imidazole.

Evolution of $^{14}CO_2$ from Carboxyl Labelled Fatty Acid

For experimental reasons the metabolism of long chain fatty acids is best studied by observing the influence of experimental variables on tissue pretreated with fatty acid. In particular, fatty acid absorption is influenced by components of the solution in an unpredictable way, and since fatty acid uptake is rapid, differences in absorption obscure the experimental effects of interest. Further, preincubation precludes a considerable and constant non-metabolic evolution of label from the experimental solution, which varies with the batch of labelled fatty acid and which comprises a volatile component in addition to CO_2. Fatty acids adsorbed on bovine serum albumin can be presented to slices, but

subsequent fatty acid metabolism is low, and the influence of experimental variables is obscured.

Imidazole inhibits the evolution of $^{14}CO_2$ from both laurate and myristate-1-^{14}C acids by fresh slices. The greater effect of imidazole on laurate as compared with myristate oxidation (Table 2) is unexplained. It is noteworthy that inhibition of myristate oxidation in fresh tissue is of the same magnitude as respiratory inhibition (Table 1). In any event label evolution is sharply enhanced by *m*-Cl-CCP, and the bulk of the increment is in turn inhibited by imidazole (Table 2). Aged slices display noticeably higher activity with respect to the oxidation of exogenous fatty acids, but label evolution by aged slices arises in large measure from β-oxidation of the long chain acids followed by the oxidation of acetyl CoA through the TCAC.[5] While imidazole sensitivity is perceptible in aged controls, uncoupler stimulated label evolution is little influenced by imidazole in aged slices, in contrast to the situation in fresh tissue. It will be noticed that imidazole affects label evolution in aged controls (Table 2) to a greater extent than respiration (see Table 1), while influencing respiration and label evolution in fresh tissue to more nearly the same extent. The implication is that whereas aged tissue has the capacity for α-oxidation of fatty acids, it lacks endogenous substrate for the enzyme compared with fresh tissue.

TABLE 2. IMIDAZOLE INHIBITION OF CONTROL AND UNCOUPLER-STIMULATED $^{14}CO_2$ EVOLUTION FROM CARBOXYL-LABELLED LONG CHAIN FATTY ACIDS

	$^{14}CO_2$ evolution			
	Fresh		Aged	
Lauric acid -1-^{14}C	dpm $\times 10^{-3}$	% control	dpm $\times 10^{-3}$	% control
Control	2·1	100	80	100
Imidazole	0·5	24	39	49
m-Cl-CCP	9·8	465	124	155
m-Cl-CCP + imidazole	2·5	119	114	142
External solution: (dpm $\times 10^{-6}$)				
Initial	8·9		11·2	
Final	6·8		6·2	
Myristic acid-1-^{14}C				
Control	5·0	100	31	100
Imidazole	3·9	78	21	68
m-Cl-CCP	18·2	364	62	200
m-Cl-CCP + imidazole	7·3	146	58	187
External solution (dpm $\times 10^{-6}$)				
Initial	11·7		12·0	
Final	4·2		6·2	

Imidazole concentration, 0·1 M; *m*-Cl-CCP, 10^{-5} M. 2·0 g discs pretreated for 30 min in 10 ml 0·1 M Tris, pH 7·5, containing either lauric acid-1-^{14}C (2·9 mCi/mmol) or myristic acid-1-^{14}C (11·25 mCi/mmol) at levels indicated by radioactivity of initial external solutions. Discs subsequently rinsed with water, blotted dry, and placed into 10 ml of solution containing indicated components in addition to 0·1 M Tris, pH 7·5. Discs shaken in 125 ml stoppered Erlenmeyer flasks containing suspended alkali papers (see Experimental). After 30 min, 2·5 ml 5 N H_2SO_4 was added rapidly to the flasks, the flasks restoppered, and $^{14}CO_2$ collected for 60 min. Alkali papers were subsequently treated as described in the Experimental section for determination of radioactivity.

Table 3 compares the effect of malonate on the DNP stimulated oxidation of palmitate 1-^{14}C with the influence of malonate and DNP on glucose oxidation by fresh slices. As with imidazole, malonate inhibits the oxidation of palmitate by fresh slices to a limited extent,

while totally suppressing DNP induced palmitate oxidation. With regard to the relative effect of normal ageing compared with uncoupler treatment on the oxidation of palmitate and glucose respectively, it is to be noted that ageing increases palmitate oxidation approximately 5-fold,[5] while ageing increases glucose oxidation several thousand fold.[1,3] Thus the stimulation of glucose oxidation by DNP as indicated in Table 3 is small, but even so inhibition of the enhanced rate by malonate is only 20%. DNP is expressed in terms of μmol per gram fresh weight because its extensive adsorption makes the conventional designation of concentration ambiguous.

TABLE 3. EFFECT OF DNP AND MALONATE ON THE OXIDATION AND UPTAKE OF LABELED GLUCOSE AND PALMITIC ACID

	% of control			
	Palmitic acid		Glucose	
	$^{14}CO_2$	uptake	$^{14}CO_2$	uptake
Malonate	94	114	85	93
DNP	173	106	298	93
DNP + malonate	98	110	248	98

Fresh slices (3 g) were incubated in 15 ml of solution, pH 5·0, containing either 10 μCi of palmitic acid-1-^{14}C (46 μCi/μmol) or 5 μCi of glucose-U-^{14}C (13·4 μCi/μmol). Malonate was 50 mM and DNP was 0·25 μmol/g fr. wt tissue (see text).

The $\delta^{13}C$ Value of Endogenous Respiratory CO_2 of Control and Uncoupler Treated Fresh Slices

As pointed out previously,[4] the natural ratio of ^{13}C to ^{12}C in lipids of potato tuber tissue is very much lower than the comparable ratio in starch (or protein). The parameter in question is normally expressed as follows:

$$\delta^{13}C \text{ per mil} = \frac{(^{13}C/^{12}C) \text{ sample} - (^{13}C/^{12}C) \text{ standard}}{(^{13}C/^{12}C) \text{ standard}} \times 1000.$$

Since the commonly used standard has a higher $^{13}C/^{12}C$ ratio than any sample of metabolic interest, the $\delta^{13}C$ per ml of respiratory CO_2 is always a negative number. The more negative, the greater the proportion of ^{12}C. In potato the carbon from starch has a δ value of −25·5, and from lipid −34·8. The determination of $\delta^{13}C$, in turn, is reliable to at least the first decimal point. Consequently Table 4 shows that respiratory CO_2 of fresh potato discs is predominantly lipid in origin while that from aged discs is derived primarily from carbohydrate. Furthermore, the large DNP induced respiratory increment reflects lipid oxidation in fresh slices, and the limited DNP elicited increment in aged slices continues to implicate starch as the predominant respiratory substrate.

DISCUSSION

Three lines of evidence support the early assertion[15] that the uncoupler induced respiration of certain fresh plant storage organ slices, in this case potato, represents respiratory metabolism distinct from the TCAC. First, the additional respiratory CO_2 elicited by uncoupler in potato slices has a $\delta^{13}C$ close to that of potato lipid and not of potato starch. The respiratory substrate in aged slices which show vigorous TCAC activity is derived

from starch.[4] Secondly, the uncoupler induced respiratory increment in fresh slices is totally inhibited by imidazole, an inhibitor of long chain fatty acid α-oxidation, while the normal carbohydrate-sustained induced respiration of aged slices shows considerably lower imidazole sensitivity, and the respiration of uncoupler treated aged discs, even less. Finally,

TABLE 4. EFFECT OF DNP ON THE $\delta^{13}C$ PER ml OF RESPIRATORY CO_2 OF FRESH AND AGED POTATO DISCS

	Fresh		Aged	
	μmol CO_2 per g	$\delta^{13}C$ per ml	μmol CO_2 per g	$\delta^{13}C$ per ml
Control				
Total	30	−32·0	176	−26·5
From lipid	21		19	
From carbohydrate	9		157	
DNP				
Total	63	−33·6	204	−26·6
From lipid	55		24	
From carbohydrate	8		180	

DNP concentration: 0·25 μ mol/g fr. wt (see text). Respiratory CO_2 collected in freezing train,[4] and relative prevalence of ^{13}C and ^{12}C determined in a mass ratio spectrophotometer. Each value represents an average of three determinations. The contribution of lipid and carbohydrate respectively to the total respiratory CO_2 is calculated from the over-all $\delta^{13}C$ values.[4] The carbohydrate designation includes any protein derived carbon dioxide.

release of $^{14}CO_2$ from carboxyl labelled myristate-1-^{14}C or laurate-1-^{14}C is stimulated by uncoupler in fresh slices, and the stimulated release is predominantly imidazole sensitive. By contrast, label evolution in aged slices in the presence of uncoupler is inhibited by imidazole to a very small extent (Table 2). While DNP seldom if ever stimulates the respiration of aged potato slices, *m*-Cl-CCP may evoke significant stimulation (Table 1).

The nature of the basal respiration. While it appears that uncoulper stimulated respiration in fresh potato slices is not TCAC-mediated, the meaning of malonate inhibition in the absence of TCAC activity (Table 3) remains unknown. The suggestion made previously[15,16] was that the fumarate-succinate couple may act as a respiratory electron carrier in the old Szent–Gyorgyi sense, in which neither compound is involved as a carbon path intermediate. In this connection nothing is known of the electron transport path related to the oxidation of NADH formed in the second step of fatty acid α-oxidation.[6] There are many microsomal and soluble NADH oxidizing systems in plant tissues[17–19] but there is no indication of their role *in vivo*. Those which reduce cytochrome *c* whether by way of a menadione type intermediate[17] or not[18,19] deliver electrons to a path containing at least one phosphorylative step preceding oxygen involvement. Such a path allows for uncoupler response. Further, there is no evidence one way or the other regarding the existence of fumarate reductase in storage organ tissue, and the propsect of a link between NADH and the mitochondrial electron transport chain by way of a fumarate-succinate couple remains tenable.

Imidazole inhibition of label evolution from carboxyl labelled long chain fatty acids may be more pronounced than respiratory inhibition *per se*. The disparity is greater in

[17] D. P. HACKETT and T. E. RAGLAND, *Plant Physiol.* **37**, 656 (1962).
[18] J. M. RUNGIE and J. T. WISKICH, *Austral. J. Biol. Sci.* (1972) in press.
[19] J. M. RUNGIE and J. T. WISKICH, *Planta, Berl.* (1972) in press.

untreated fresh tissue than in uncoupler treated fresh tissue, in which the uncoupler induced increment is largely inhibited in both cases. The implication is that fresh tissue respiration is due only in part to α-oxidation of fatty acids. This conclusion is supported by the effect of malonate, which like imidazole (Table 1) inhibits fresh tissue respiration to only a small extent, while inhibiting the uncoupler stimulated increment almost totally.[16] By contrast, estimations of the percentage contribution of lipid oxidation to the total respiration of freshly cut potato slices, made on the basis of the $\delta^{13}C$ values of respiratory CO_2, are as high as 70%.[4] There is apparently more extensive degradation and oxidation of lipid in fresh potato slices than can be attributed to α-oxidation. Lipid oxidation in fresh discs, however heterogeneous, constitutes a true wound respiration, for the TCAC-related respiration typical of tubers[20] and aged slices is absent in fresh.[3] The composition of the basal respiration apparently changes with ageing. For as the fractional contribution of lipid oxidation to the total respiration drops below 10% in 24 hr,[4] malonate resistance persists, dropping from 80 to 100% in fresh discs to 30–50% in aged.[1,21] The nature of the malonate resistant-component would appear to be different in fresh and aged tissue slices (see Ref. 16). If the α-oxidase of potato is typical of that in non-green tissue the first step in the oxidation is peroxidative.[6] The source of hydrogen peroxide *in vivo* remains undefined, and may be related to the basal respiration in fresh slices.

Inhibition of the TCAC in fresh slices. Potato tubers have low levels of lipid.[7] The oxidation of long chain fatty acids cannot sustain prolonged respiration without the ultimate pile-up of fatty acids of intermediate chain lengths. There is evidence that β-oxidation is proceeding in fresh slices, but that the oxidation of acetyl CoA is prevented due to an inhibited TCA cycle.[3,5] It is the 12–14 carbon long-chain fatty acids which are the most effective in inhibiting mitochondrial activity,[11] and it is precisely these fatty acids which are most susceptible to α-oxidation.[5,6] The onset of TCAC activity in potato slices after several hours may well be related to the degradation in the cytoplasm of inhibitory free fatty acids, and perhaps of lysolecithin as well.[22] Regarding the cyanide sensitivity of potato slice respiration under various conditions, both control and uncoupler stimulated fresh slice respiration are sensitive, the resistant respiration being 30% of the fresh control rate.[14] Significantly, imidazole inhibition of fresh slice respiration tends to approach 30%. While the fatty acid α-oxidase from peanut cotyledons was shown to be somewhat susceptible to cyanide, requisite cyanide levels for inhibition of the isolated enzyme were relatively high,[6] and the enzyme is considered essentially cyanide insensitive (P. K. Stumpf, personal communication). Fresh potato slices have a poorly developed cyanide resistant electron transport path, while cyanide resistance in aged slices is marked. On treatment of aged slices with cyanide the respiratory substrate changes from carbohydrate to lipid.[23] It would seem that electron transport associated with lipid oxidation can proceed by alternative paths, depending on the age of the tissue.

Finally there is the question of the ubiquity of the anomalous respiration of fresh tissue slices. For example, ap Rees and Royston[24] report conventional glycolytic and TCAC activity in fresh carrot slices. In this connection it may be important that the ultrastructural disintegration and subsequent reconstitution typical of fresh slices of beet[8,9] and Jerusalem

[20] J. Barker, *Proc. Roy. Soc.* **158B,** 143 (1963).
[21] G. G. Laties, *Plant Physiol.* **37,** 679 (1962).
[22] M. J. Earnshaw and B. Truelove, *Plant Physiol.* **45,** 322 (1970).
[23] B. S. Jacobson, G. G. Laties, B. N. Smith, S. Epstein and B. Laties, *Biochim. Biophys. Acta* **216,** 295 (1970).
[24] T. ap Rees and B. J. Royston, *Phytochem.* **10,** 1199 (1971).

artichoke,[25] for example, is absent in swede and carrot.[26] Organelle and endoplasmic reticulum ultrastructure are known to undergo considerable change in ageing potato slices.[27,28] It seems likely that anomalous fresh slice respiration will prove to be related to lipoprotein breakdown in tissue where cutting initiates vigorous lipase activity. The nature of the residual basal respiration in both fresh and aged slices, as well as the reason for the low respiratory level in intact tubers, remain open to question.

EXPERIMENTAL

Materials. Potato (var. Russet Burbank) tubers stored at 7° were halved and pierced within the vascular ring with a 9-mm dia. stainless steel borer, the cores being left in the tuber pieces. Slices 1 mm thick were cut into ice H_2O from the pierced tuber halves with a microtome blade fixed in an appropriate bed. Discs were rinsed with distilled H_2O and either used promptly as fresh discs, or set to age in circularly rotated large Erlenmeyer flasks in 10 vol. 10^{-4}M $CaSO_4$ at 25°, the solution being changed at frequent intervals in the first few hr, and 3–4 × thereafter in a 24-hr period.

Respiratory measurements. Respiration was measured by conventional manometry at 25° with 15 discs (*ca.* 1 g fr. wt) contained in each manometer flask, and 0·2 ml 10% NaOH and a fluted filter paper in the center-well. Buffer was either 10^{-2}M K phosphate, pH 8·5, or 0·1 M gylcylglycine, pH 8·5 as indicated in the table.

Metabolism of labelled fatty acids. Stock solutions of myristate-1-^{14}C and laurate-1-^{14}C (New England Nuclear) were prepared by dissolving 5·1 mg myristic acid (0·25 mCi) and 17·1 mg lauric acid (0·25 mCi) resp. in a final vol. of 50 ml H_2O. The solutions were made alkaline (pH 7–8) with 0·1 N NH_4OH and boiled.[29] Cooled solns were used at once, or stored frozen. Frozen solutions were again boiled following thawing before use. All stocks were optically clear. In experiments, 1·0 ml stock was added to a total of 10 ml experimental solution containing 0·1 M Tris buffer, pH 7·5. Phosphate buffer gave cloudy solutions. 2·0 g fr. wt discs were preincubated in 10 ml labelled solution in 125 ml stoppered Erlenmeyer flasks for 30 min at 25°. 0·1 ml aliquots of the external solution were taken initially and following incubation, and dried at 80° on glass filter paper strips for counting. Dried strips were placed directly in scintillator fluor for estimation of radioactivity. The preincubated discs were rinsed with distilled H_2O and gently surface dried. 2·0 g were placed in 10 ml experimental solution in Tris buffer, pH 7·5, in 125 ml Erlenmeyer flasks. Rubber flask stoppers were fixed with a hook from which a strip of Whatman glass-paper (GF/A) 1 × 8 cm was suspended as a loop. The glass paper was impregnated with 0·2 ml 20% KOH, and hung 5–8 cm above the level of soln. The flasks were incubated in a rotary shaker at 25°. After 30 min, 2·5 ml 5 N H_2SO_4 was quickly delivered to the flask and the stopper immediately reseated. $^{14}CO_2$ was collected for 60 min. Alkaline filter paper strips were subsequently submerged in 4·0 ml CO_2-free H_2O in conical 15 ml glass centrifuge tubes and washed with 1 ml of H_2O delivered in a gentle stream. 5·0 ml 20% Ba acetate was added, and the ppt collected by centrifugation. The Ba ppt was taken up in 50% EtOH in H_2O, re-precipitated by centrifugation, and the precipitate washed twice again in 50% EtOH–H_2O. The final ppt was quantitatively transferred as a H_2O suspension in a small vol. to the sample chamber of one arm of an evacuable Y tube.[30] The other arm contained 1·0 ml KOH, and a side-arm of the sample chamber held 2·0 ml 1 M lactic acid. The Y tube was evacuated, and the lactic acid subsequently tipped into the chamber containing $Ba^{14}CO_3$. $^{14}CO_2$ was collected in 1·0 N KOH which was gently stirred during distillation. 0·2 ml of KOH solution containing ^{14}C-carbonate was dried on glass papers at 80° for 20 min. Papers were subsequently submersed in a PPO–POPOP toluene fluor for counting. Blank values—no more than 200–300 cpm—were determined by carrying out the entire procedure in the absence of tissue.

Carbon mass isotope composition of respiratory CO_2. 50–75 g of freshly prepared rinsed discs were held in a 1 l. incubation flask containing 200 ml CO_2-free 10^{-4}M $CaSO_4$ maintained at pH 5·0 with an automatic titrator. The flask's rubber stopper was fitted with gas inlet and outlet tubes. CO_2-free air was drawn through the flask under slightly reduced pressure (7 mm Hg) at a rate of 110 ml/min. The outlet tube led to a series of traps where H_2O was removed, and CO_2 was collected at liquid N_2 temp. CO_2 was susbequently transferred to special sample containers designed to fit the delivery system of a ratio mass spectrometer.[4] The standard of comparison for the determination of $^{13}C/^{12}C$ ratios in respiratory CO_2 was the fossil carbonate skeleton of *Belemnitella americana* (PDB_1).[4]

[25] L. C. FOWKE and G. SETTERFIELD, in *Physiology and Biochemistry of Plant Growth Substances* (edited by F. WIGHTMAN and G. SETTERFIELD), p. 581, Runge Press, New York (1968).
[26] M. E. VAN STEVENINCK, M.Sc. Dissertation, Univ. Adelaide, Australia (1970).
[27] S. G. LEE and R. M. CHASSON, *Physiol. Plant.* **19,** 199 (1966).
[28] R. BARCKHAUSEN, Ph.D. Dissertation, University of Frankfurt (1971).
[29] P. CASTELFRANCO, P. K. STUMPF and P. CONTOPOULOU, *J. Biol. Chem.* **214,** 567 (1955).
[30] S. ARONOFF, *Techniques of Radiobiochemistry*, Iowa State College Press (1958).

Acknowledgements—This work was generously supported by a grant from the USPHS (to G.G.L.), and B. S. Jacobson is indebted to the U.S. Public Health Service for the award of a pre-doctoral Health Fellowship. Our thanks are due Professor Herman Timm, Dept. Vegetable Crops, University of California, Davis, for the provision of the potato tubers used in this study, and to Dr. P. G. Heytler, E. I. Du Pont Co. Wilmington, Delaware, for the kind gift of *m*-Cl-CCP.