The Prevalence of Carbon-13

in Respiratory Carbon Dioxide

As an Indicator of the

Type of Endogenous Substrate

The change from lipid to carbohydrate during the

respiratory rise in potato slices

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ABSTRACT Isotope discrimination is a common feature of biosynthesis in nature, with the result that different classes of carbon compounds frequently display different $^{13}C/^{12}C$ ratios. The $^{13}C/^{12}C$ ratio of lipid in potato tuber tissue is considerably lower than that for starch or protein. We have collected respiratory CO_2 from potato discs in successive periods through 24 hr from the time of cutting—an interval in which the respiration rate rises 3–5-fold. The $^{13}C/^{12}C$ ratio of the evolved CO_2 was determined for each period, and compared with the $^{13}C/^{12}C$ ratios of the major tissue metabolites. In the first hours the carbon isotope ratio of the CO_2 matches that of lipid. With time, the ratio approaches that typical of starch or protein. An estimation has been made of the contribution of lipid and carbohydrate to the total respiration at each juncture. In connection with additional observations, it was deduced that the basal, or initial, respiration represents lipid metabolism—possibly the α-oxidation of long chain fatty acids—while the developed repiration represents conventional tricarboxylic acid cycle oxidation of the products of carbohydrate glycolysis. The true isotopic composition of the respiratory CO_2 may be obscured by fractionation attending the refixation of CO_2 during respiration, and by CO_2 arising from dissolved CO_2 and bicarbonate preexisting in the tuber. Means are described for coping with both pitfalls.

INTRODUCTION

Plant tissues are frequently characterized by a reserve of substrate, a condition which is particularly true for a classical object of plant respiration

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study, the storage organ slice. Such tissues do not respond markedly to exogenous substrate. Reserve metabolites are frequently of more than one kind, and it is a matter of some interest to identify the actual endogenous substrate. Respiration in plant tissues may comprise more than one respiratory path. The matter is further complicated by evidence that the relative contribution of coexisting respiratory paths may change with time, as is the case for the sigmoidally increasing respiratory activity of potato discs aerated for 24 hr (1). One piece of evidence pointing to changes in respiratory quality is the concomitant development of malonate sensitivity and cyanide resistance during the aging period (2–4). In addition, aged tissue readily oxidizes glucose and Krebs cycle acids, as measured by $^{14}CO_2$ release from exogenously provided labeled compounds, while fresh tissue totally lacks this ability (5). To date the only intimation of the possible nature of the carbon path comprising the basal respiration has been the evolution of $^{14}CO_2$ by fresh slices from long-chain, carboxyl-labeled fatty acids. The characteristics of the latter degradation point to α-oxidation (6). Since the ability to degrade an exogenous carbon source does not necessarily reveal the nature of the endogenous respiratory substrate, we have sought to identify the endogenous substrate by comparing the $^{13}C/^{12}C$ ratio in the normal respiratory CO_2 with the $^{13}C/^{12}C$ ratio in the various classes of endogenous compounds. The analysis is based on the fact that various classes of tissue metabolites are characterized by their natural relative $^{13}C/^{12}C$ ratios.

Different classes of substrates (e.g. amino acids and lipids) vary from one another in $^{13}C/^{12}C$ ratio as a result of isotopic fractionation during biosynthesis. Extensive fractionation by plant tissue occurs in photosynthetic fixation of carbon dioxide (7), and in dark fixation as well (see below). Such fractionation is indicated by the markedly larger $^{13}C/^{12}C$ ratio of atmospheric CO_2 than of the total plant carbon (7, 8). To a lesser degree, carbon isotope fractionation occurs in the conversion of carbohydrate to lipid (9, 10), and lesser still during the synthesis of cell wall material (10) and amino acids (9). Altogether, isotopic fractionation during biosynthesis results in different $^{13}C/^{12}C$ ratios in the various classes of metabolites. It is these differences which permit us to determine which substrate is utilized by respiring potato discs during any stage of disc aging.

The procedure first requires determination of the $^{13}C/^{12}C$ ratios of the classes of endogenous metabolites which are potential substrates for potato tuber tissue respiration. As respiration of slices proceeds over a 24 hr period, CO_2 is periodically collected, and the $^{13}C/^{12}C$ ratio therein is determined. The metabolite which most nearly resembles the respiratory CO_2 in $^{13}C/^{12}C$ ratio at a given time is taken to represent the predominant respiratory substrate at that time.

Though there have been suggestions regarding the nature of the respira-

tory substrates in aging potato slices from labeling and inhibitor studies (1, 3, 4), direct evidence is lacking. On the basis of a comparison of the $^{13}C/^{12}C$ ratios of respiratory CO_2 through a 24 hr aging period with the ratios of endogenous metabolites, we have been able to deduce that the respiratory substrate is initially lipid and gives way to starch upon aging.

MATERIALS AND METHODS

Tissue discs 1.1 mm thick and 9 mm in diameter were prepared from Idaho Russet Burbank potatoes. Halved tubers were pierced within the vascular ring with a stainless steel cork borer, the core remaining in the tuber. The tuber halves were then sliced with a microtome blade fixed in an appropriate bed (11) to yield discs. At least 100 g of discs washed with 12 liters of CO_2-free water were readied for use in 30 min. Slices were prepared at 2°C.

After slicing, 50–75 g of potato discs were aged in a 1 liter flask containing 200 ml of CO_2-free 10^{-4} M $CaSO_4$. The bathing solution was changed four or five times during the 24 hr aging period. The pH of the solution was maintained at the desired value with an automatic titrator. The electrodes and titrant delivery tube were inserted into the bathing solution through a rubber stopper held in a hole in the side of the incubation flask. A pH of 5.0 was maintained with 0.4 N H_2SO_4, and a pH of 7.2 with 0.4 M Tris.

A rubber stopper in the top of the incubation flask held the air inlet and outlet tubes. The outlet tube led to a liquid nitrogen trap, where CO_2 evolved from the tissue was collected (7). Air entering the flask was scrubbed free of CO_2 by passage through two 3 ft columns, the first containing Drierite, a desiccant, and the second Ascarite, a CO_2 absorbent. The flow of air through the system was 110 ml/min.

Extraction Procedures Though starch is the predominant food reserve in potato tuber, possible respiratory substrates such as lipids and protein were also isolated and analyzed for their $^{13}C/^{12}C$ ratios. Starch was extracted either by centrifugation of starch granules from a slurry of homogenized potato tuber or by formation of an aqueous starch gel. Starch was first gelled by extracting the tissue with hot 80% ethanol. The starch-containing residue was then boiled in water and centrifuged. The supernatant contained aqueous starch gel. The two methods yielded starch with identical $^{13}C/^{12}C$ ratios, indicating there was no isotopic carbon exchange between starch and hot alcohol. Lipid was extracted from air-dried potato tissue with chloroform–methanol (2:1, v/v). Nonlipid material was extracted from the lipid-containing solvent with 0.1 M KCl. Protein was prepared by homogenizing tissue in 2% NaCl. After centrifugation for 15 min at 10,000 g, protein was precipitated by boiling the supernatant for 30 min. The precipitate was dried by lyophilization.

Other types of compounds, e.g. organic acids, present in small amounts in potato, were isolated by ion exchange chromatography as described by Romberger and Norton (4). Compounds adsorbed by the cation and anion exchange resins, respectively, are amino acids and organic acids. The neutral fraction which passes through both resins is composed primarily of sugars. Amberlite or Dowex as exchange resins yielded similar results. All extracted substrates were converted to CO_2 for mass spectrometry (8).

Mass Spectrometer Analysis   The $^{13}C/^{12}C$ ratio in any given sample was compared with a standard. The standard used was the CO_2 from the fossil carbonate skeleton of *Belemnitella americana* (PDB_1). The function defining the values reported here is

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

Thus, a sample with a $\delta^{13}C$ per mil of -10 has a $^{13}C/^{12}C$ ratio less than the standard by 10 per mil, or 1.0 %. For example, the $^{13}C/^{12}C$ ratio of atmospheric CO_2 is smaller than that of the PDB_1 standard by about 7 per mil. Therefore, the $\delta^{13}C$ per mil of this CO_2 is -7. The precision of measuring $\delta^{13}C$ with the mass spectrometer is ±0.1 per mil.

TABLE I
$\delta^{13}C$ PER MIL OF PRIMARY ENDOGENOUS SUBSTRATES
FROM POTATO TUBER TISSUE

Substrate	$\delta^{13}C$ per mil*
Starch	-25.5
Protein	-26.6
Lipid	-34.8

* Average of four separate determinations.

RESULTS AND DISCUSSION

The results which follow show that it is possible to determine experimentally whether the endogenous respiratory substrate in potato tuber discs is either lipid or starch and protein. Comparison of the $^{13}C/^{12}C$ ratio of the evolved CO_2 with that of endogenous tuber metabolites indicates that lipids initially represent the main source of respiratory substrate in fresh tissue discs. Based on our results and previous investigations, it can be deduced that starch becomes the predominant respiratory substrate as aging proceeds. The determination of respiratory substrate during the course of slice aging on the basis of the $^{13}C/^{12}C$ ratio of respiratory CO_2 proved possible only because the $^{13}C/^{12}C$ ratio of lipid is widely different from other endogenous metabolites as a consequence of carbon isotope fractionation during biosynthesis. The effect of fractionation during respiration upon substrate determination will be discussed rigorously in a later section. For the present, it is enough to indicate that carbon isotope fractionation accompanying respiration is relatively inconsequential, and has little effect on the basic question of whether the predominant substrate utilized by aerated potato discs is lipid or starch.

$^{13}C/^{12}C$ Ratio of Metabolites from Potato

The $^{13}C/^{12}C$ ratios of the common macromolecular components of potato tissue are set out in Table I. Lipid was found to have the most negative $\delta^{13}C$

value; i.e. lipid contains less ^{13}C than protein, which in turn contains less ^{13}C than starch. The low $^{13}C/^{12}C$ ratio of the lipid fraction relative to other cellular constituents was previously demonstrated in other higher plants, as well as in various algae (9, 10).

It is interesting to note that when lipids are further fractionated by saponification, the nonsaponifiable fraction (mainly isoprenoids) is on the average

TABLE II

$\delta^{13}C$ PER MIL OF ENDOGENOUS INTERMEDIATE
SUBSTRATES FROM POTATO TUBER TISSUE

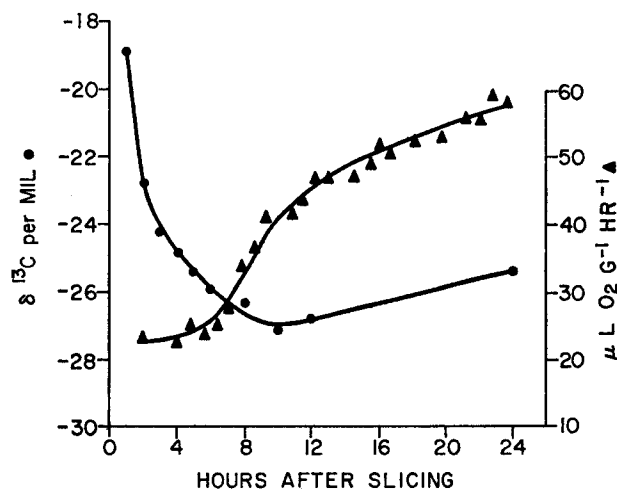| Substrate	$\delta^{13}C$ per mil*
Neutral compounds (sugars)	−23.8
Organic anions (organic acids)	−26.9
Organic cations (amino acids)	−27.1

* Average of three separate determinations.

FIGURE 1. $\delta^{13}C$ per mil of CO_2 collected from potato discs in $CaSO_4$ solution or a moist atmosphere. The respiratory rate was measured with a Beckman O_2 analyzer. ●, $\delta^{13}C$ per mil; ▲, respiratory rate.

2 per mil heavier than the saponifiable fraction (fatty acids). The difference in δ ^{13}C values between the two fractions suggests that ^{12}C-enriched acetate units are utilized more selectively for the synthesis of malonyl coenzyme A than for acetate condensation reactions.

The $^{13}C/^{12}C$ ratios of compounds considered to be metabolic intermediates are listed in Table II. While the δ-values of the major primary components differ from one another (Table I), metabolites derived therefrom closely resemble their macromolecular counterparts. That is, amino acids resemble

protein, and sugars most resemble starch, with respect to their respective $^{13}C/^{12}C$ ratios (Tables I and II).

Change of Predominant Respiratory Substrate during Aging

When potato discs were aged either in $CaSO_4$ solution or in a moist chamber, the $\delta\ ^{13}C$ values of the collected CO_2, which at first were high, decreased rapidly during the first 10 hr and rose slightly during the next 16 hr (Fig.1). While comparison of the $\delta\ ^{13}C$ per mil of the collected CO_2 (Fig. 1) with ap-
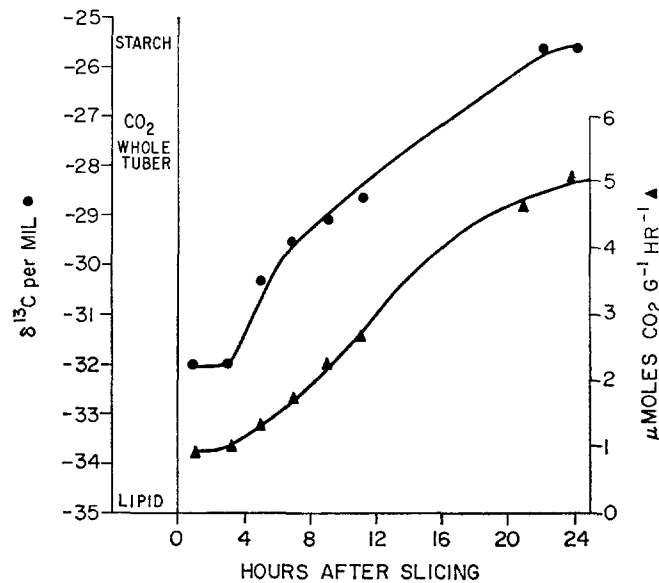

FIGURE 2. $\delta^{13}C$ per mil of CO_2 collected from aging potato discs. The pH of the $CaSO_4$ medium was maintained at 5.0 with H_2SO_4. The CO_2 δ-values for 1 and 3 hr after slicing were determined from Fig. 6. The column at the left indicates the δ-values of the probable primary endogenous substrates and of the respiratory CO_2 from intact tubers. The respiratory rate represents the CO_2 collected from discs for 1 hr at the times indicated.

propriate substrate δ-values (Table I) clearly indicates that starch or protein is the respiratory metabolite in 24 hr old potato discs, determination of the respiratory substrate during the *early* hours of aging (Fig. 1) is obfuscated by two anomalies. The high δ-values of the CO_2 collected during the initial hours of aging do not resemble any of the known δ-values of endogenous substrates (Table I). Furthermore, the *fall* in the δ-values (away from starch) observed in the interval from 4 to 10 hr is inconsistent with other indirect evidence which points to the *development* of starch metabolism (via the tricarboxylic acid cycle) during this period (5).

Two factors distinct from the respiratory process per se have been shown to contribute significantly to the anomalous results of Fig. 1. Both lead to an

enrichment of ^{13}C in the CO_2 collected in the first 0–8 hr. When these factors are experimentally obviated, a totally different trend of the $^{13}C/^{12}C$ ratio with time is noted, as depicted in Fig. 2. One cause of the spuriously heavy CO_2 released in the first hours is the refixation, by carboxylation reactions, of a fraction of the respired CO_2. Incorporation probably favors the lighter carbon isotope, and consequently decreases the relative amount of ^{12}C in the CO_2 subsequently collected. The second factor that leads to an enrichment of ^{13}C in CO_2 evolved by fresh discs is the release of heavy CO_2 from tissue bicarbonate. Tissue bicarbonate was shown to have a higher δ ^{13}C per mil than any other tissue carbon, and the contribution of bicarbonate to respiratory CO_2 would therefore increase the δ-value of the collected CO_2. The bicarbonate in question is in the tuber tissue at the time of slicing, not the product of slice respiration following cutting. Evidence of the release of heavy carbon from dissolved bicarbonate is to be found in the transient relatively high $^{13}C/^{12}C$ values of CO_2 from tomato leaves respiring in the dark following a prolonged period of photosynthesis (7).

Once the effects of CO_2 refixation and of bicarbonate-derived CO_2 are taken into account, it is readily apparent that the change in δ-values of CO_2 collected from tissue during the initial hours of aging is misleading and does not reflect substrate utilization (Fig. 1). When the influence of the extraneous factors noted above is either precluded or corrected for, a totally different trend is noted for CO_2 δ-values with time after slicing (compare Fig. 1 with Fig. 2). Based on the δ ^{13}C values of Fig. 2, the respiratory substrate changes as potato discs age. Assuming for the moment that the corrections which have been made are valid, a comparison of CO_2 δ-values at any given time with values of specific classes of substrates indicates that lipid is the predominant metabolite for fresh tissue respiration, while CO_2 with a δ-value resembling starch or protein characterizes the respiration of aged tissue (Fig. 2 and Table I).

Whether starch or protein is the predominant respiratory substrate of aged discs cannot be discerned from the δ-values alone. However, a choice between the two substrates is possible in the light of previous investigations. The latter have shown that as the respiratory rate of potato discs increases 4–5-fold during 24 hr of aging, there is an inordinately large increase in $^{14}CO_2$ evolution from exogenously provided labeled glucose, concurrent with a similar increase of tricarboxylic acid cycle activity (5, 12). Furthermore, a meticulous balance sheet of potato disc metabolities in relation to time following slicing has shown that extensive protein synthesis during aging occurs primarily at the expense of soluble amino acids and amides (13, 14). The major part of the CO_2 evolved during aging is accounted for, moreover, by a depletion of starch (13, 14). It thus appears that as discs are aerated over 24 hr, starch becomes the predominant respiratory substrate.

Knowing that the choice of respiratory substrates for aging discs is probably either starch or lipid, we may calculate the relative contribution of CO_2 from each substrate consistent with the observed δ-values of the total respired CO_2. Such calculations indicate that 70% of the CO_2 collected from discs during the initial hours of aging is derived from lipid (Fig. 3). As the time of
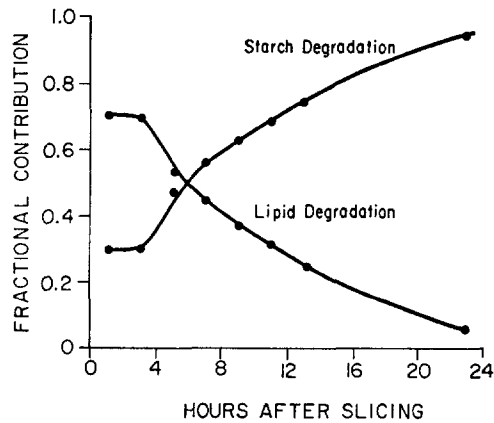

FIGURE 3. The fractional contribution of respiratory CO_2 from starch and lipid degradation in aging potato discs. The values were calculated from results depicted in Fig. 2. (See the text for selection of starch and lipid as substrates.)
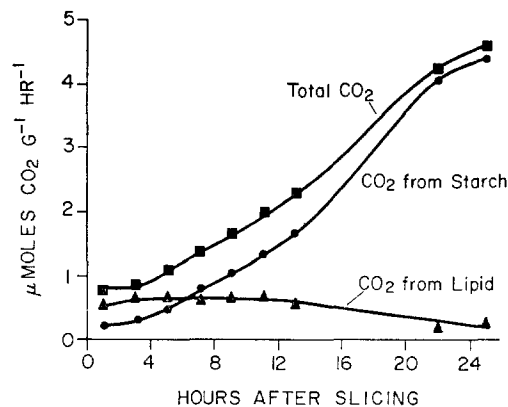

FIGURE 4. The rate of CO_2 evolution from starch and lipid as a function of potato disc age. Calculated from Figs. 2 and 3. (See the text for selection of starch and lipid as substrates.)

incubation increases, the relative contribution from lipid sharply declines while that from starch increases.

It has been recognized for some time that the respiration of aged tissue is qualitatively distinct from the initial, or basal, respiration (1). As aging progresses, the basal respiration rises but little. The respiratory increment is due primarily to the initiation or activation of another oxidative carbon path. From the fractional contributions from lipid and starch to the CO_2 throughout the aging process (Fig. 3), we can calculate the actual amount of CO_2 that should arise from each substrate during the rise in respiration rate (Fig. 4). These calculations suggest that lipid is the primary substrate for

basal respiration, since the rate of CO_2 production from lipid oxidation remains relatively constant for 12 hr. The increase of respiratory CO_2 evolution with time is most likely related to the ever-increasing utilization of starch, and the developed respiration is again distinguished from the basal respiration.

The Basis of Anomalously Heavy CO_2 and Correction Therefor

CO_2 REFIXATION   Since CO_2 fixation is generally accompanied by a significant selective utilization of ^{12}C (7), the refixation of respired CO_2 by potato discs would be expected to raise the $\delta\,^{13}C$ of evolved CO_2 over that arising directly from respiration. It appears that bicarbonate rather than CO_2 per se is involved in dark CO_2 fixation in higher plant tissue. Fixation increases sharply with rising external pH.[1] The extent of ^{13}C enrichment may be expected to be proportional to the amount of refixation, and to the extent of fractionation favoring ^{12}C which occurs during carboxylation.

To assess whether CO_2 refixation in fact increases the $\delta\,^{13}C$ values of respiratory CO_2, potato tissue was incubated at either pH 5.0 or 7.2, and the δ-values of the CO_2 trapped were determined at hourly intervals. It is important to emphasize that δ-measurements were made on the *total* CO_2 produced in the designated period. That is, for experiments at pH 7.2, the bathing solution of a separate sample was acidified at the end of each interval, and the CO_2 so released from bicarbonate was collected and combined with the CO_2 trapped from the air stream during the same interval.

The effect of pH on the $\delta\,^{13}C$ per mil of CO_2 collected from potato discs aged for 24 hr is depicted in Fig. 5. The curves show that the δ-values of the respired CO_2 are at all times more negative at pH 5.0. The difference in δ-value between CO_2 collected at pH 7.2 and CO_2 collected at pH 5.0 is greatest after 3 hr of aging and subsequently diminishes with time. If the isotope fractionation attending dark CO_2 fixation in our experiments is similar to that attending the carboxylation of ribulose diphosphate (7), the higher δ-value of CO_2 collected at pH 7.2 should be due to greater CO_2 refixation, and the amount of CO_2 collected at pH 7.2 (after acidification) should be less than that collected at pH 5.0. Furthermore, one should be able to calculate the amount of CO_2 that must be refixed in order to get the differences in δ-values observed in Fig. 5.

The calculation is simplified by the knowledge that the organic acids synthesized by nonphotosynthetic CO_2 fixation are sequestered in the vacuole and not readily respired (16, 17).[1] If we know the δ-values of the total evolved CO_2, on the one hand, and of the CO_2 emitted with concomitant refixation, on the other, we can readily calculate the relative amounts of emitted and refixed CO_2. The δ-value of the total respiratory CO_2 is taken to be that of

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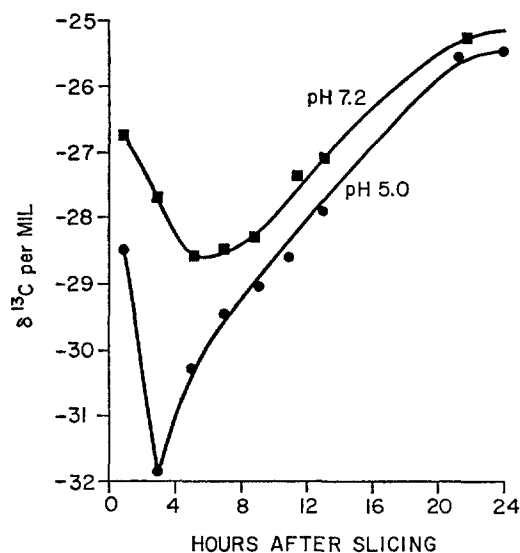[1] Jacoby, B., and G. G. Laties. Unpublished observations (see ref. 15).

FIGURE 5. The effect of pH on changing the $\delta^{13}C$ per mil of CO_2 collected from potato discs. Discs were incubated at either pH 5.0 (●) or 7.2 (■). At the end of each interval, the bathing solution was acidified with H_3PO_4, and the CO_2 so released was combined with the CO_2 trapped during the experimental interval.

the CO_2 collected at pH 5.0, at which pH little net nonphotosynthetic CO_2 fixation by plant tissue takes place (18).[1] By contrast, significant refixation occurs at pH 7.2, and the δ-value of CO_2 collected at pH 7.2 (after acid tipping) is influenced by isotopic fractionation during refixation. If we assume that the carbon isotope fractionation during dark fixation is the same as for the carboxylation of ribulose observed in tomato plants (7), we can calculate the δ-value for the refixed CO_2. The fractionation factor, α, is described as follows (see ref. 7):

$$\alpha = \frac{^{13}C/^{12}C \text{ total } CO_2}{^{13}C/^{12}C \text{ fixed } CO_2} \cong \frac{\delta \text{ total } CO_2 - \delta \text{ fixed } CO_2}{1000} + 1 \qquad (1)$$

The fractionation factor as determined by Park and Epstein (7) is 1.017; that is, the δ-value of the fixed CO_2 is -17 per mil compared with the δ-value of the total CO_2.

The conservation equation for the determination of the relative proportion of refixed CO_2, (x), and emitted CO_2, $(1 - x)$, is as follows.

$$\delta \text{ total } CO_2 \text{ (pH 5.0)} = (x) \, \delta \text{ emitted } CO_2 \text{ (pH 7.2)}$$
$$+ (1 - x) \, \delta \text{ refixed } CO_2 \text{ (pH 7.2)} \qquad (2)$$

In consequence

$$\text{Fraction refixed} = \frac{\delta \, CO_2 \text{ (pH 5.0)} - \delta \, CO_2 \text{ (pH 7.2)}}{1000 \, (\alpha - 1)} \qquad (3)$$

The fraction of CO_2 refixed at pH 7.2 at the time when the difference in δ-values is the greatest (3 hr after slicing, Fig. 5) is 0.24; i.e. 24% of the total respiratory CO_2 is refixed. The fractional decrease of CO_2 (determined with a Warburg apparatus) evolved by tissue at pH 7.2 relative to pH 5.0 was 0.36, which indicates a refixation of 36% of the total CO_2 evolved by respiration. The rate of respiration as measured by oxygen utilization did not differ with pH, indicating that the respiration rate per se is unaffected by the pH of the ambient solution. Thus, since the manometrically observed fractional decrease in emitted CO_2 is at least as great as the calculated decrease, it is reasonable to conclude that the difference between the δ-values of CO_2 collected at pH 7.2 and pH 5.0 is due largely to CO_2 refixation.

These data demonstrate that unless the pH is kept low, CO_2 refixation markedly raises the observed CO_2 δ-values and leads to a spuriously high value which does not reflect the nature of the respiratory substrate (Fig. 1).

RESIDUAL CO₂ Even when care is taken to minimize the effects of CO_2 refixation, the δ-value for CO_2 collected during the first hour remains inordinately high (Fig. 5). As will be seen, the high δ-value for the first hour originates in residual CO_2-bicarbonate, i.e. in CO_2 and bicarbonate in solution in the intact tuber and present in the discs at the time of slicing. The partial pressure of CO_2, and hence the quantity of dissolved CO_2 in potato tubers, may be quite high (19).

Residual CO_2 and HCO_3^- are in time released from discs by diffusive efflux and exchange with respired CO_2. In consequence the initial δ-values of the CO_2 collected from fresh tissue reflect values for metabolic CO_2 adulterated by residual CO_2. Experiments to be discussed will show that the δ-value of the residual CO_2 and HCO_3^- is considerably higher than substrate δ-values, and that removal of the residual fraction lowers the δ-value of initially evolved CO_2 toward that of lipid. Furthermore, since the time required to eliminate residual bicarbonate by exchange with respired CO_2 is decreased by decreasing disc thickness, we were able to determine the δ-value of respired CO_2 without the contribution of the residual fraction.

Residual CO_2 and HCO_3^- were isolated by homogenizing tubers in an air-tight Waring Blendor at $-4°C$. During homogenization, the CO_2 released was swept into a liquid nitrogen trap by passing a stream of CO_2-free air through the blender. The CO_2 collected during the first 10 min of homogenization was considered primarily residual CO_2 per se. Later samples were considered to arise from bicarbonate. The latter samples were collected for 10 min in a stream of CO_2-free air after each of two consecutive 40 min periods in which the homogenate was constantly stirred. Residual bicarbonate differs in δ-value from residual CO_2 by about -7 per mil, a value in accordance with theoretical expectations (8, 9). The values of the δ ^{13}C per mil for CO_2 and HCO_3^- are -26.9 and -19.1, respectively (Table III).

It is apparent that the δ-values of residual CO_2, and particularly of residual HCO_3^-, are sufficiently large that their contribution to respiratory CO_2 leads to CO_2 δ-values greater than those of the substrates (Fig. 1; cf. Fig. 2). Furthermore, the δ-value of -19.1 per mil for residual HCO_3^- is sufficient evidence that neither starch nor protein is the predominant substrate for fresh tissue respiration. Since CO_2 arising from starch or protein will have

TABLE III

$\delta^{13}C$ PER MIL OF CO_2 AND HCO_3^- FROM
POTATO TUBER TISSUE

Compound determined	Time after homogenizing	Tissue content (fresh weight)	$\delta^{13}C$ per mil
	min	*μmoles/g*	
CO_2*	10	0.45	-26.9
HCO_3^-‡	70	1.50	-18.7
	130	1.00	-19.5

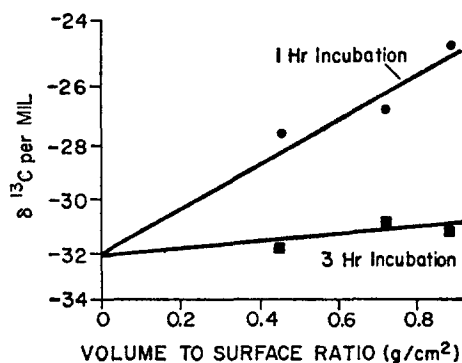* Average of three determinations.
‡ Average of two determinations.

FIGURE 6. $\delta^{13}C$ per mil of evolved CO_2 as a function of disc thickness at constant disc diameter. CO_2 was collected in the intervals from 0.5 to 1.5 hr (●) and from 2.5 to 3.5 hr (■) after slicing. Incubation conditions were the same as in Fig. 2.

a δ-value of about -26 per mil (Table I), any contribution by residual bicarbonate to CO_2 derived from starch or protein will raise the δ-value above -26. Since the observed value for CO_2 from fresh discs is -28.3 per mil (Fig. 5; 1 hr after slicing), it is probable that lipid and not starch or protein is the initial (or basal) substrate.

To estimate quantitatively the contribution of lipid oxidation to total fresh disc respiration, we must know the δ-value for respiratory CO_2 unobscured by refixation or by residual CO_2-bicarbonate. To this end an estimation was made of the δ-value of the respiratory CO_2 of discs of infinite thinness held at pH 5.0. With but a short preincubation period, it is to be expected that respiratory CO_2 from very thin discs should be devoid of con-

taminating residual CO_2, since the rate of purging of preexisting CO_2 from tissue slices will be inversely related to the thickness (20). In Fig. 6 the δ-value for respiratory CO_2 at pH 5.0 has been plotted as a function of relative disc thickness (volume to surface ratio). The influence of disc thickness on the δ-value is readily perceived, as is the effect of preincubation. The unambiguous δCO_2 value for fresh tissue respiration, as determined from the ordinate intercept in Fig. 6, is -32, a value typical of lipid. A precise δ-value for the respiratory CO_2 from fresh slices permits a calculation of the quantitative contribution of lipid metabolism to fresh slice respiration (Figs. 3 and 4).

In summary, when the external pH is kept at 5.0, and correction is made for the contribution of residual CO_2-bicarbonate to the over-all δ-value for respiratory CO_2, the δ-values of respiratory CO_2 accurately indicate whether lipid is the initial endogenous substrate and delineate the shift in substrate with time in aging discs. All deductions are based on the presumption that there is little fractionation during substrate degradation, and, as shown below, such appears to be the case.

Carbon Isotope Fractionation during Substrate Degradation

The difference in carbon δ-values of lipids and carbohydrates reflects fractionation during biosynthesis. The question of concern is whether fractionation during the degradation of a *given* substrate is large enough to obscure any difference between substrates of disparate δ-values. The answer would appear negative, since when substrate is plentiful, i.e. when the decrease in substrate concentration is negligible during the experimental period, the difference in δ-values between the substrate and CO_2 derived therefrom depends solely upon the degree of fractionation in the first step of a unidirectional sequence. Only at metabolic branch points will there be further fractionation in the steady state. Any deviation of the δ-values between CO_2 and substrate during respiration will depend upon the relative prevalence of synthetic side events which do not lead to complete combustion.

While starch metabolism leads to a variety of metabolic intermediates as well as to CO_2, it is noteworthy that all major metabolites other than lipid have a carbon δ-value akin to that of starch (Tables I and II). Thus, when starch is the substrate, fractionation even at metabolic branch points would appear to be of no consequence in degradation. However, were starch converted to lipid and CO_2, the ^{12}C enrichment of lipids would be accompanied by a ^{13}C enrichment of the evolved CO_2 (10). On the other hand, since it could be inferred from a previous investigation with potato discs aged for 3 hr (4) that the rate of lipid synthesis on a carbon basis is less than 1% of the rate of CO_2 production, the enrichment of respiratory CO_2 with $^{13}CO_2$ due to lipid synthesis would be negligible. Based on the δ-values of potato substrates, as much as 10% conversion of starch to lipid would cause but a 1 per mil increase in the δ-value of the respiratory CO_2 over that of starch.

An estimation of the over-all carbon isotope fractionation during respiratory degradation of a given substrate can be obtained from examination of a totally heterotrophic organism. A growing culture of the fungus *Fusarium roseum* was selected as a test organism, since the anticipated prevalence of synthetic reactions concomitant with respiratory metabolism should place an upper limit to the expected deviation of δ between substrate and respiratory CO_2.

Cultures of *F. roseum* were grown aerobically on inorganic salts medium (21) with a sole carbon source of either glucose or palmitic acid. During the logarithmic growth phase, respired CO_2 was collected and analyzed for its δ ^{13}C value. These values were then compared with those of the substrates. As indicated in Table IV, the deviations of the δ-values of the CO_2 from those

TABLE IV

$\delta^{13}C$ PER MIL OF SUBSTRATES AND CO_2 FROM
AEROBICALLY GROWN *F. ROSEUM*

	$\delta^{13}C$ per mil*	
	Substrate	CO_2
Glucose	−10.7	−9.4
Palmitic acid	−22.4	−20.5

* Average of three determinations.

of the corresponding substrates are 1.0 with glucose and 1.9 with palmitic acid. Therefore, the maximum increase of the CO_2 δ-value over that of the substrate should be no greater than 1.9 per mil for potato tuber discs.

If the average maximum deviation between δ-values of substrate and CO_2 derived therefrom were as great as 1.5 per mil for any substrate or mixture of substrates utilized, the change from predominantly lipid to predominantly starch oxidation would not be obscured. For example, when the δ-value of substrate and CO_2 derived therefrom are taken to be identical, the respiratory contribution of starch at 24 hr is estimated to be 95% (Fig. 3). With a 1.5 per mil deviation during degradation, the estimated contribution of starch drops to 86%. The decrease from 95 to 86% is inconsequential in evaluating the change from lipid to starch degradation by potato discs aerated for 24 hr.

CONCLUSION

There is little question that the respiration which develops with time in storage organ slices is different in kind from the basal, or initial, respiration (1, 22). Evidence presented herein has reaffirmed this judgment, and elucidated one of the basic elements comprising the change in respiratory characteristics, i.e. the transition from lipid to carbohydrate as the predominant

respiratory substrate. In contrast to the rise in respiration which develops with time in thin slices, the instantaneous rise on slicing has, tacitly at least, been considered simply an enhancement of tuber respiration. However, the δ-value of the respiratory CO_2 from intact tubers (Fig. 2) is very close to that of the carbon in starch or protein (Table I), and markedly higher than the δ-value of CO_2 evolved by fresh slices, which appears to arise from lipid.

The respiration rate of freshly cut slices may be from 3–5 times that of the intact tuber on a fresh weight basis (1). From measured respiration rates and CO_2 δ-values, we have calculated that the CO_2 produced from carbohydrate (and perhaps protein) is about 0.14 μmole g^{-1} hr^{-1} in the intact tuber and 0.18 μmole g^{-1} hr^{-1} in fresh discs. On the other hand, lipid oxidation increases from less than 0.04 μmole CO_2 g^{-1} hr^{-1} in the tuber to about 0.6 μmole CO_2 g^{-1} hr^{-1} in fresh discs.

The respiratory rise on slicing seems to involve the sudden onset of lipid metabolism. What is the source of lipid? While the lipid content of potato is small [0.13% of the fresh weight (23)], the amount of lipid required to support the calculated rate of lipid oxidation for 25 hr (Fig. 4) would be but 12% of the total lipid present. Jackman and Van Steveninck (24) have shown that the membranous endoplasmic reticulum of beet root virtually disintegrates during the first hours following slicing—later to reappear. Mitochondrial membrane structure is seriously disorganized as well for a limited time (25). Furthermore, the onset of lipase activity has been shown to attend the maceration of seedlings (26). Lipid metabolism *may* reflect the breakdown of cellular membranes, in which case we may have to face the disagreeable task of resurrecting the concept of wound respiration.

Fresh tissue slice respiration is largely cyanide-sensitive and malonate-resistant—characteristics shared by the α-oxidation of long-chain fatty acids (6, 27, 28). The capacity for α-oxidation of fatty acids in potato slices remains fairly constant with aging, as does the malonate-resistant basal respiration. It seems at least possible, therefore, that the α-oxidation of lipids comprises the basal respiration. Cyanide causes a transition from carbohydrate to lipid oxidation in ostensibly cyanide-resistant aged potato slices.[2] In this connection it is interesting that cyanide stimulates the respiration of intact potato tubers (29).

[2] Jacobson, B. S., B. N. Smith, G. G. Laties, and S. Epstein. Unpublished observations.

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