CHANGING RESPIRATORY PATHWAYS IN POTATO TUBER SLICES[1,2]

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It has long been known that freshly cut pieces of tissue from certain tubers and fleshy roots immediately respire considerably more rapidly per unit mass than tissue in the intact organ, and further that sufficiently thin slices of such tissue exhibit an additional respiratory rise during one to several days of incubation. These phenomena have been observed in slices of potato (5, 19, 22, 23, 24, 25), carrot (20, 27), beet (3, 21, 26), Jerusalem artichoke (26), and chicory root (11). Laties (9) made an analysis of some of the factors involved in the secondary respiratory rise.

The slow oxygen uptake of freshly cut potato slices is inhibited by cyanide and carbon monoxide, but the more rapid respiration which develops during aging is quite resistant to these agents (6, 15). It seems likely that a change in electron transport pathways occurs (6). Changes in respiratory carbon pathways also are indicated. The results of early work with chicory root suggest that an increase in tricarboxylic acid cycle (TCAC) activity occurs during aging. The initial respiration is malonate resistant, whereas the respiration induced by aging is malonate sensitive (11). Recent work by Laties (pers communic) has shown that potato slices undergo a similar increase in sensitivity to malonate during aging. The malonate resistance of fresh tissue raises the possibility that the respiration initially is largely mediated by systems other than the TCAC. We have concentrated on this carbon pathway aspect of the problem.

The plan was to develop techniques permitting use of C^{14} labeled sugars as tracers of respiratory pathways in slowly respiring tissues, by extracting and isolating labeled metabolic intermediates arising therefrom; and to employ glucose specifically labeled on the first and sixth carbon atoms, respectively, as a test for respiratory systems, such as the pentose phosphate pathway (PPP), which can discriminate between these two atoms. We concentrated primarily upon fresh tissue because of its greater physiological interest, and because experience with fresh tissue might serve to elucidate other problems concerning dormancy and development.

MATERIALS & METHODS

TISSUE SOURCE & PREPARATION: All potato (*Solanum tuberosum* L.) tubers used were of the Russet variety. Various lots were used during 18 months of preliminary experiments, but in all experiments of our final series we utilized tissue from a single lot of tubers from the crop harvested in the Shafter, Cal., area in early June, 1959. The tubers were stored at 10° ± 1° C until used during the next 3 months.

Tissue for the several treatments of an experiment was prepared as one lot. From the central part of each of 12 tubers, 24 slices 1 mm thick and 8.8 mm in diameter were cut with a cork borer and a table microtome. The slices, cut directly into water at 0° C, were rinsed with four more changes of ice water. Samples were counted onto bibulous paper saturated with ice water and then placed into flasks for treatment. These slices are referred to as fresh. Temperatures near 0° C inhibit development of the metabolic changes characteristic of aging. Thus the physiological freshness of the slices was insured by keeping them at 0° C until the experimental zero time.

Aged slices were prepared by incubating fresh slices in 10^{-4} M calcium sulfate solution on a rotary motion shaking table (70 cycles/min) at room temperature (26°–29° C) in the dark for 24 to 36 hours. The solution (25 ml/g of tissue) was changed hourly for 3 hours, then less frequently for at least ten changes in 24 hours.

Before incubating with radioactive sugar, fresh tissue samples were preincubated at 0° C for an hour either in 10^{-4} M calcium sulfate solution or in the test solutions. The solutions were replaced with fresh aliquots after 30 minutes. Preincubation served as a further washing period to remove contents of broken cells from the cut surfaces and, in the case of malonate treatments, to allow malonate permeation prior to presenting glucose.

The test solutions were of two formulations, for controls (C), and for malonate treatments (M). Both contained 1×10^{-4} M calcium sulfate. The M solution also contained 5×10^{-2} M malonic acid and was adjusted to pH 5.0 with potassium hydroxide. Malonic acid and potassium hydroxide at a hundredth the M concentration were added to C. This was sufficient to hold the pH near 5.0, but had no respiratory effect. Potassium chloride was added to C to

[1] Received May 31, 1960.

[2] This work received financial support from the National Science Foundation, the Rockefeller Foundation, and the W. K. Kellogg Foundation. The work was finished and prepared for publication while the senior author was holding his present position with the U.S.D.A. Forest Service.

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balance the potassium ion concentration of M.

A Warburg apparatus set at 28° C was used for manometric experiments. Each flask contained 3.5 ml of C or M solution, ten tissue slices, and 0.2 ml of 10 % NaOH solution and a fluted paper wick in a sidearm. All treatments were run in triplicate.

INCUBATION WITH RADIOACTIVE GLUCOSE: Glucose randomly labeled with C^{14} and having a specific activity of 45.9 microcuries per micromole ($\mu C/\mu M$) was obtained from Isotopes Specialties Co., Inc., Burbank, Cal. Several lots of D-glucose-1-C^{14} and D-glucose-6-C^{14}, having specific activities ranging from 2.19 to 7.7 $\mu C/\mu M$, were obtained from Nuclear-Chicago Corp., Chicago, Ill. Specific activities of all labeled sugars used in any one series of experiments were adjusted to that of the lowest. Labeled sugars were stored dry and cold in small sublots. Prior to each experiment aliquots were dissolved in water and diluted to the desired specific activity, then transferred to the experimental flasks and evaporated to dryness under nitrogen. The dry sugars were redissolved in the test solutions. This procedure eliminates errors due to spontaneous $C^{14}O_2$ evolution which might otherwise occur.

All tissue incubations with labeled substrates were done at room temperature (26°–29° C) in 125 ml flasks on a rotary shaking table (70 cycles/min). At zero time 40-slice samples (approx 2.2 g fresh wt or 0.4 g dry wt) which had been preincubated in non-radioactive test solutions, were added to the experimental flasks containing the labeled sugars dissolved in 7.5 ml of C or M solution. The flasks then were immediately closed with rubber stoppers equipped with hooks and paper loops (Whatman no. 1, 1 × 8 cm strips) saturated with 10 % sodium hydroxide solution.

At the end of each 10.0 minute period the stoppers were quickly replaced with others bearing alkali-saturated papers. Exposed papers were dropped into tubes containing 10.0 ml of water, which were promptly stoppered. After at least 24 hours, aliquots were assayed for C^{14} by a barium carbonate precipitation technique. The carbonate was plated on discs of Whatman no. 50 paper and the latter were pasted onto aluminum planchets. It was possible to keep the sample thickness uniform and below 0.4 mg/cm², and self absorption was negligible.

At the end of the incubation period the flasks were quickly chilled to 0° C. The radioactive solution then drawn off, and the slices washed for 15 minutes in several changes of 10^{-4} M calcium sulfate at 0° to remove radioactivity from the free space.

EXTRACTION: The chilled discs were dropped into 50 ml of boiling 80 % ethanol and were extracted for 30 minutes. Five more similar extraction periods followed. Extracts were combined and stored at −20° C until fractioned. This procedure extracted about 92 % of the total incorporated radioactivity from the tissue. Residual activity could not be extracted with boiling 20 % ethanol or boiling water.

Alcohol was removed by vacuum evaporation. Aqueous residues were diluted to about 25 ml and clarified by centrifugation if turbid. Fatty substances were removed by extraction with petroleum ether.

FRACTIONATION: Initial separation into basic, acidic, and neutral fractions was achieved by using two superimposed ion exchange resin columns. The columns (12 mm inside diameter) and a sample reservoir were made into a closed system, with ground glass joints. Design was such that solutions did not contaminate the joints, which were sealed with water only.

The upper column contained Amberlite IR–120(H) (a strongly acidic cation exchange resin) 16 to 50 mesh to a depth of 8 cm. The lower column was similarly packed with Amberlite IR–45(OH) (a weakly basic anion exchange resin) 16 to 50 mesh. Analytical grades of these resins, products of Rohm & Haas Co., Philadelphia, were used. Before use the resins were washed in turn with ethanol, concentrated ammonium hydroxide, 2 N sodium hydroxide, and 2 N hydrochloric acid. We found Amberlite IR–45 to have a significant sugar binding capacity. Labeled bound sugar was not removed by any eluting agent employed and could be recovered only very slowly by exchange with non-radioactive sugar. Consequently, isotopic recoveries in early fractionations ranged from 88 to 95 %. In later experiments losses due to sugar binding were greatly reduced by pretreating the resin with a solution containing 1 % each of glucose and fructose for 1 hour and then backwashing with water. After such treatment an average isotopic recovery of 98.75 % was attained in six successive fractionations.

Extracts, diluted to about 50 ml, were passed through the columns at the rate of about 1.5 ml per minute, followed by five bed volumes of water. The neutral fraction emerging from the second column was collected and evaporated to dryness under vacuum. All columns were eluted as soon as possible.

Amino acids and other bases were recovered from the IR–120 resin by elution with 50 ml of 4 N ammonium hydroxide passed through it at the rate of 1 to 2 ml per minute. The eluate was evaporated to dryness under vacuum.

Mixed acids were recovered from the IR–45 resin by elution with 400 ml of an ammonium carbonate-bicarbonate solution passed through at the rate of 2 to 3 ml per minute. This solution was prepared by dissolving 160 g of ammonium bicarbonate in water, adding concentrated ammonium hydroxide to pH 8.5 (about 55 ml), and diluting to 1 liter. The eluate was subjected to vacuum evaporation, whereupon the excess ammonium salts were volatilized. Just before the residue reached dryness a few milliliters of water were added to promote removal of remaining volatile salts. This was repeated if necessary. The final colorless residue consisted of ammonium salts of the mixed acids.

Paper strip electrophoresis was used to effect further separations of both basic and acidic fractions.

Hanging strip cells (28) made by Spinco Division, Beckman Instruments, Inc., were used along with Spinco papers.

The eluate from the IR–120 resin (amino acid fraction) was applied to six electrophoresis strips as a narrow band across the center of each. The electrolyte was 0.1 M ammonium formate adjusted to pH 6.0 with formic acid. These papers were subjected to 250 volts for 3 hours, which resulted in clean separation into discrete bands of acidic, basic, and neutral amino acids.

Paper electrophoresis was also used to separate the mixed acids into low pK (phosphorylated compounds) and high pK (carboxylic and other weak acids) groups. The electrolyte was 0.2 N formic acid (pH 2.2). Each sample (from 2.2 g of tissue) was distributed among eight paper strips as a narrow band 5.5 cm from the negative ends. The eight strip unit was subjected to a constant current of 12 milliamperes for about six hours. The two major bands were separated by 3 to 4 inches. Conversion to the free acid form occurred simultaneously.

Radioactive compounds on the papers were located by recording strip scanning devices. The corresponding active areas from the several papers of each sample were cut out and eluted groupwise between glass strips. Overnight elution with water by this method usually yielded 95 to 100 % recovery of labeled compounds.

The high pK acids were further fractioned by descending paper chromatography. Whatman 3 MM paper previously washed with a mixture of ethyl acetate, acetic acid, and water (10: 5: 2 by volume) was used. The first solvent was the organic phase of *n*-butanol, formic acid (88 %), and water mixed in equal parts by volume and aged for 24 hours. Resolution into seven discrete bands, as revealed by spraying with brom phenol blue, was attained in about seven hours. After strip scanning, the bands were eluted separately, and rechromatographed in other solvents or subjected to electrophoresis as necessary to provide separation and tentative identification. Citric and isocitric acids were separated by the acid amide technique of Ranson (18).

The sugar fractions were banded on 10 cm widths of Whatman no. 3 MM paper previously washed with the solvent. A mixture of ethyl acetate, acetic acid, and water (10: 5: 2 by volume) was used to develop the papers (descending). Radioactive bands were rechromatographed as necessary. Para-anisidine (16) was used as an indicator. The entire area beyond fructose was eluted and the eluate rechromatographed as a narrow band. Thus the small amount of activity in pentoses and other high R_f sugars was easily revealed. The individual sugars in this group were not identified.

The low pK acid fraction, mostly sugar phosphate esters, was not examined in detail.

COUNTING TECHNIQUES: Fractionation procedures were monitored by radioactivity determinations after each step. Samples were taken by evaporating solutions to dryness under vacuum, redissolving in a known volume, and removing duplicate aliquots (1 or 2 % of the total) for counting. Self absorption was not a problem when small samples were used. Samples were plated on glass planchets and counted under a thin window gas-flow detector (D–47, Nuclear-Chicago Corp.). Counting efficiency was maintained at 27 % by frequent use of standards and adjustment of the distance between detector and sample as necessary.

Residual radioactivity in extracted tissue was assayed by oxidizing aliquots by a wet combustion technique (29). The carbon dioxide was trapped in 1 M hydroxide of Hyamine 10-X (*p*-[diisobutyl-cresoxyethoxyethyl]-dimethylbenzylammonium hydroxide) in methanol (prepared by Packard Instruments Co., Inc., La Grange, Ill.). Aliquots of the Hyamine carbonate solution were counted with a Packard Tri-Carb Liquid Scintillation Spectrometer using a scintillator and solvent system based upon that recommended by Hours and Kaufman (8) for tritium. Results obtained at a counting efficiency of 32 % were corrected to 27 % after calibration with standards.

EXPERIMENTAL RESULTS

RESPIRATORY RISE: Potato tuber slices incubated at room temperature for 30 hours exhibit an approximately fourfold increase in respiratory rate. However, similar to the metabolic changes which occur in aging chicory root slices (11), the oxygen uptake of fresh slices is inhibited only about 15 % by 0.05 M

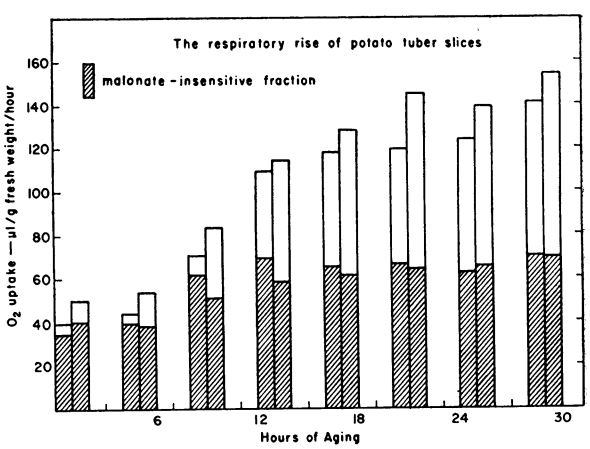

FIG. 1. The respiratory rise of potato tuber slices. After incubation in 10^{-4} M $CaSO_4$ solution for the indicated period the O_2 uptake of samples was measured by conventional manometric techniques at 28° C. Malonate treatment consisted of suspending in test solution containing 5×10^{-2} M potassium malonate (pH 5.0) and 10^{-4} M $CaSO_4$. Controls were suspended in a similar solution containing 5×10^{-2} M potassium chloride but also 5×10^{-4} M potassium malonate at pH 5.0. Slices were put into test solutions 15 minutes before zero time. Each pair of bars represents a separate Warburg run with each treatment triplicated.

malonate at pH 5.0, whereas that of aged slices is inhibited by about 50 %. Thus during the incubation period the malonate resistant fraction of the respiration doubles whereas the malonate sensitive fraction increases 10-to 15-fold (fig 1).

ABSORPTION & UTILIZATION OF RADIOGLUCOSE AS FUNCTION OF AGING: Fresh potato slices, in contrast to aged, have little ability to absorb phosphate (6, 14) and salts in general (10, 13). We found an even greater disparity in the ability of fresh and aged slices to take up and oxidize glucose.

Fresh slices release $C^{14}O_2$ from randomly labeled glucose at a slow rate not initially reduced by treatment with malonate. Slices aged for 36 hours before being presented with labeled glucose release $C^{14}O_2$ at a rate which, during the first 30 minutes, is about 3,000 times greater than that of fresh tissue. In aged tissue, malonate greatly inhibits the evolution of $C^{14}O_2$ from labeled glucose so that it is only 150 to 180 times more rapid than in similar fresh tissue (table I). Hence malonate has no initial inhibitory effect upon $C^{14}O_2$ output in fresh slices but reduces output by aged slices 12-to 15-fold. This occurs even though total respiratory O_2 uptake is reduced by only about 50 % (fig 1). The malonate effect upon $C^{14}O_2$ output in aged slices is the compound result of inhibition of uptake and utilization. In the experiment cited (table I), control and malonate treated slices after 3 hours of incubation in radioglucose solution had taken up 10,800,000 and 2,840,000 cpm, respectively, from 12,000,000 cpm available to each. These results clearly show malonate inhibition of glucose uptake, but this alone is insufficient to account for the observed effect. The remainder is due to inhibition of utilization. Collectively the data suggest that qualitative, rather than merely quantitative, metabolic changes characterize the physiological aging process.

TABLE I

OXIDATION OF EXOGENOUS RADIOGLUCOSE* BY FRESHLY CUT & 36-HR AGED POTATO SLICES**

MIN AFTER ZERO TIME	cpm*** IN RESPIRATORY CO_2		RATIO AGED / FRESH
	FRESH	AGED	
Controls†			
0–10	74	269,000	3640
10–20	121	398,000	3280
20–30	169	455,000	2690
30–40	214	446,000	2080
Malonate treated†			
0–10	94	17,500	186
10–20	174	30,800	177
20–30	199	36,100	181
30–40	265	38,500	145

* Supplied as 20 μC (approx 12,000,000 cpm) of randomly labeled glucose-C^{14}, specific activity 45.9 $\mu C/\mu M$, in 7.5 ml of test solution ambient to 40-disk tissue samples (2.2 g fresh wt). Initial glucose concentration was 5.8×10^{-5} M.

** Fresh slices pre-incubated in non-radioactive test solution for 1 hr at 0° C, aged slices similarly pre-incubated during the 36th hour at room temperature.

*** Counted with a thin window, gas-flow detector at approximately 27 % efficiency.

† Malonate treated disks suspended in test solution containing 5×10^{-2} M potassium malonate (pH 5.0) and 10^{-4} M $CaSO_4$. Controls suspended in similar solution having 5×10^{-2} M potassium chloride but also containinging 5×10^{-4} M potassium malonate to stabilize pH at 5.0.

LABELING PATTERNS IN FRESH TISSUES: By using 10 to 20 μC of high specific activity glucose in 7.5 ml of solution suspending 2.2 g of fresh slices, it was possible to incorporate useful amounts of radioactivity into metabolic intermediates during 1 to 3 hour incubation periods in spite of slow uptake and utilization (tables II, III, & IV). Our results suggest that, if pursued, this approach could yield a great amount of information about the malonate resistant respiration of fresh slices. Such further work will be facilitated by the fact that tissues incubated in the presence of malonate maintain their fresh characteristics for long periods because malonate, like low temperature, prevents the development of the respiratory increment normally induced by aging.

In accord with earlier reports that a rapid sucrose synthesis begins when potato tubers are cut (7, 17), we found that more than half the total extracted activity from fresh slices, following the absorption of uniformly labeled glucose, was in sucrose (table II). Hydrolysis of the sucrose yielded monosaccharides of equal activity. The free fructose was, however, much less active than free glucose (table II) although they were present in similar amounts. In short term experiments with fresh tissue, sucrose behaved as an end product rather than as part of the substrate pool.

Although malonate had only a small inhibitory effect upon sucrose synthesis (table II), it decreased total activity in the acidic and basic fractions and had the indirect effect of increasing the percentage of the total activity appearing in sugars (table IV). Sucrose synthesis in the presence of malonate indicates that the malonate resistant respiration provides energy for some synthetic activity although its ability to support active uptake of glucose and of salts is very limited.

A pronounced accumulation of succinic acid in slices which had been incubated with labeled glucose in the presence of malonate for 3 hours was evident from inspection of chromatograms and from counting data (table II). This indicates that malonate exerts its classical effect upon succinic dehydrogenase in tissue aged for less than 3 hours, although oxygen uptake is little inhibited. It is important to note, however, that in similar experiments of 1 hour duration, malonate caused no obvious increase in amount of succinic acid, and only about a 50 % increase of activity therein. Thus a disproportionate amount of the succinate accumulation occurs during the 2nd and 3rd hours. Hence the metabolic changes induced by aging appear to begin during the first 2 hours. This

idea is supported by the results of our experiments with specifically labeled glucose and by Loughman's work on phosphate uptake by potato slices (14). Further experiments on label distribution after only 1 hour of incubation seem desirable.

EXPERIMENTS WITH SPECIFICALLY LABELED GLUCOSE: Identical 40-slice samples of fresh tissue were incubated for 3 hours in the presence of glucose-1-C^{14}, glucose-6-C^{14}, and randomly labeled glucose (glucose-R-C^{14}), respectively. All the sugars were adjusted to a specific activity of 4.6 $\mu C/\mu M$. A total of 10 μC of radioactivity (approx 6,000,000 cpm) was used in each experimental flask which contained 7.5 ml of test solution having a total initial glucose concentration of 2.9×10^{-4} M. Histograms plotted on the basis of radioactivity in respiratory CO_2 collected during 18 successive 10 minute periods show that the respiratory mechanisms of fresh slices discriminate between the first (C_1) and sixth (C_6) carbon atoms of the glucose molecule both in the controls and malonate treated samples (figs 2 & 3). The ratio of the radioactivity released from glucose-1-C^{14} to that released from glucose-6-C^{14} (C_1/C_6 ratio) was initially high for both control and malonate treated samples and dropped to about three in both after 1 hour. Thereafter, however, the malonate treated slices exhibited an increasing ratio (fig 3). The 3 hour over-all C_1/C_6 ratios of 2.67 and 3.75 for control and malonate treated samples, respectively, reflect the inhibition of C_6 release by malonate, but do not reveal that it is confined mostly to the 3rd hour.

Aged slices also were incubated with glucose-1-C^{14}

TABLE II

EFFECT OF MALONATE UPON DISTRIBUTION OF RADIOACTIVITY FROM RANDOMLY LABELED GLUCOSE* IN FRESH POTATO SLICES**

COMPONENT***	cpm†		MALONATE TREATED AS % OF CONTROL
	CONTROL	MALONATE TREATED	
Respiratory CO_2	22,600	17,300	77
Total ethanol extract	705,000	567,000	80
Lipids	111	367	330
Basic Fraction	96,500	73,700	76
Acidic amino acids	56,100	16,700	29
Neutral amino acids	32,100	56,200	175
Basic amino acids	950	750	79
Acidic fraction	99,400	54,400	55
Phosphorylated compounds	28,000	10,000	36
Weak acids	67,300	41,500	62
Low R_f unknowns	43,300	24,950	58
Citric	4,350	1,280	29
Isocitric	235	757	322
Unknown	102	505	495
Malic	8,900	1,740	20
Glycolic	2,750	1,830	67
Malonic and unknown	205	330	161
Succinic	925	6,300	682
Fumaric	190	125	66
Neutral fraction	399,000	361,000	91
Oligosaccharides	8,050	6,000	75
Sucrose	344,000	306,000	89
Glucose	31,900	26,800	84
Fructose	5,950	5,830	98
High R_f sugars	< 400	< 400	...

* Supplied as described in same footnote table I.

** Slices were preincubated for 1 hour in 10^{-4} M $CaSO_4$ at 0° C then brought to room temperature and incubated for 3 hours in the test solution containing labeled glucose.

*** The respiratory CO_2 was trapped on alkali papers then converted to $BaCO_3$ and counted. Various components were isolated from 80 % ethanol extracts by means of ion exchange, paper electrophoresis, and paper chromatography.

† Duplicate samples on glass planchets were counted with a thin window, gas flow detector. Recoveries were incomplete because only the more active areas of papers were eluted. Counting efficiency was approximately 27 %.

TABLE III

UPTAKE & DISTRIBUTION OF RADIOACTIVITY FROM LABELED GLUCOSE* BY FRESH POTATO SLICES** INCUBATED 3 HOURS WITH & WITHOUT MALONATE

TREATMENT	cpm IN THOUSANDS***			
	RESPIRED CO_2	TOTAL EXTRACT	RESIDUAL IN TISSUE	TOTAL UPTAKE
Control†				
Glucose-1-C^{14}	35.1	462	47.1	544
Glucose-6-C^{14}	13.1	534	33.0	590
Glucose-R-C^{14}	16.7	497	47.0	561
Malonate†				
Glucose-1-C^{14}	30.2	434	28.6	493
Glucose-6-C^{14}	8.1	497	50.1	555
Glucose-R-C^{14}	18.5	405	33.0	457

* Supplied as 10 μC (approx 6,000,000 cpm) of radioactive glucose of the specified labeling pattern in 7.5 ml of test solution ambient to 40 slice tissue samples (2.2 g fresh wt). Initial glucose concentration was 2.9×10^{-4} M.
** Slices were preincubated in non-radioactive test solutions for 1 hour at 0° C, then quickly brought to room temperature and presented with radioactive solutions.
*** See same footnote table I.
† See same footnote table I.

and glucose-6-C^{14}. Because aged slices evolve $C^{14}O_2$ from exogenous glucose several thousand times more rapidly than fresh slices (table I), only 0.4 μC (about 240,000 cpm) instead of 10 μC was used per sample. Specific activities were adjusted to 2.19 μC/μM and the initial total exogenous sugar concentration was 2.44×10^{-5} M in each flask. These experiments were run for only 1 hour.

As in fresh tissue, $C^{14}O_2$ release by aged slices was much more rapid from glucose-1-C^{14} than from glucose-6-C^{14}. That the release of label from aged malonate treated slices was much less than from aged controls (fig 4) was the combined result of malonate inhibition of respiration and of glucose uptake. However, because no discrimination is to be expected during uptake, this inhibition should have no effect upon the C_1/C_6 ratio itself. This ratio was about 4 in the control and about 15 in the malonate treated slices (fig 5).

DISCUSSION

Participation of the PPP in respiratory metabolism may be demonstrated by a technique, introduced by Bloom and Stetten (4), employing glucose-C^{14} preparations specifically labeled in the first (C_1) and sixth (C_6) carbon atoms, respectively. This is possible because, during short times at least, the oxidation and decarboxylation of glucose molecules to pentose phosphate leads to evolution of CO_2 arising specifically from C_1, whereas in the case of molecules degraded to trioses by the Embden-Meyerhof-Parnas pathway (EMPP), and subsequently oxidized, C_1 and C_6 are equivalent and appear in respiratory CO_2 at equal rates. Of course, if the added labeled sugar were completely oxidized during the experimental period, the over-all ratio would be unity regardless of the pathway. Thus it is the ratio and its changes during the early stages of feeding experiments which are significant.

A factor which may lead to elevated C_1/C_6 ratios when both PPP and EMPP-TCAC are present is the incorporation of glycolytic products into compounds which are not part of the metabolic pool. In the presence of PPP such sequestering can raise the C_1/C_6 ratio by retarding release of label from that portion of hexose which has entered the EMPP. In our opinion this effect is of importance only when an attempt is being made to measure relative glucose degradation by the several pathways. We are interested in their relative contributions to respiratory gas exchange. If during a short time period a large fraction of the glycolytic product is being sequestered rather than oxidized via TCAC, the EMPP-TCAC system is in fact making a reduced contribution to respiration (as distinct from glucose degradation) just as indicated by the raised C_1/C_6 ratio for that time period.

We recognize that the C_1/C_6 ratio cannot be used to calculate the fraction of the total respiration proceeding by a particular pathway, and also that a single measurement of the ratio without regard to its rate of change cannot have much validity. Yet the ratio, if it exceeds unity, speaks for the existence of a pathway other than EMPP-TCAC and gives an estimate of the relative contribution of EMPP-TCAC and PPP to the release of CO_2 from C_1 of glucose (when expressed as $\frac{C_1-C_6}{C_1}$). For these reasons we have made ratio determinations at frequent intervals and have based our interpretations mostly upon relative changes with time rather than upon absolute values.

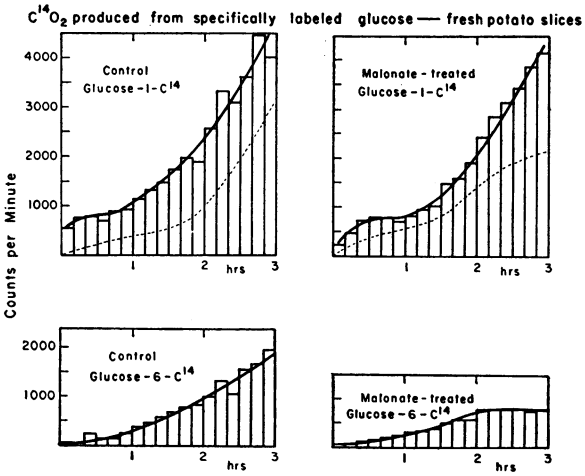

FIG. 2. Relative rates of respiratory $C^{14}O_2$ output from fresh potato slices incubated with specifically and randomly labeled glucose. The dashed curves represent results obtained with the latter. Experimental conditions were as described in the footnotes for table III.

We advance the following tentative interpretations as a basis for further experiments:

In fresh tissue, during the first and part of the 2nd hour, CO_2 release from glucose is mediated largely by the PPP. Although the EMPP may also be functional (1), release of C_6 from glycolytic products is slow. The fact that evolution of $C^{14}O_2$ from glucose-R-C^{14} rises rapidly during the first hour (6.3-fold increase) whereas the increase in label release from glucose-1-C^{14} is less rapid (1.7-fold increase) suggests that glycolytic products contribute increasingly with time. During the first three 10 minute intervals the ratio of label released from glucose-R-C^{14} to that from glucose-1-C^{14} was 1/5.3 (fig 2)—close to the theoretical of 1/6 for oxidation by the PPP exclusively—and in accordance with the suggestion that the PPP predominates initially. The drop in C_1/C_6 ratio from a high of 16.3 to 3.6 during the first hour (fig 3) appears to be the consequence of the onset of oxidation of glycolytic products either via the TCAC or via some other malonate resistant pathway.

In view of the locus of malonate inhibition, malonate would be expected to inhibit the release of radioactivity from glucose-R-C^{14} oxidized via the TCAC more readily than release via the PPP. Because C_1 release is not inhibited appreciably by malonate during 3 hours of incubation, but label release from glucose-R-C^{14} is inhibited considerably after 2 hours (fig 2), it appears that PPP and TCAC are somewhat competitive and that PPP becomes increasingly active as TCAC is inhibited. This may explain why the label distribution after 3 hours of incubation (table II) indicates considerable TCAC activity even though malonate inhibition of O_2 uptake is small. During aging malonate resistant respiration doubles while the total respiration, increasingly malonate sensitive, rises four-fold (fig 1). In aged tissue PPP apparently can no longer compensate for the large TCAC mediated fraction after the latter has been inhibited by malonate, inhibition of O_2 uptake then becomes evident, and the C_1/C_6 ratio rises. This rise in ratio begins to be evident during the 2nd hour (fig 3).

In the fresh controls, the rise in label evolution from glucose-R-C^{14} was as steep as for glucose-1-C^{14} (fig 2). Because equal quantities of label were administered and uptake was similar (table III), then, barring rapid randomization effects for which we have no evidence, the theoretical yield of $C^{14}O_2$ from glucose-R-C^{14} would be about one-sixth that from glucose-1-C^{14} if PPP predominated. Although the ratio was approximately one-sixth during the 1st hour, it rose until it was roughly three-fourths at the end of 3 hours. Again the implication is an increasing contribution by the TCAC.

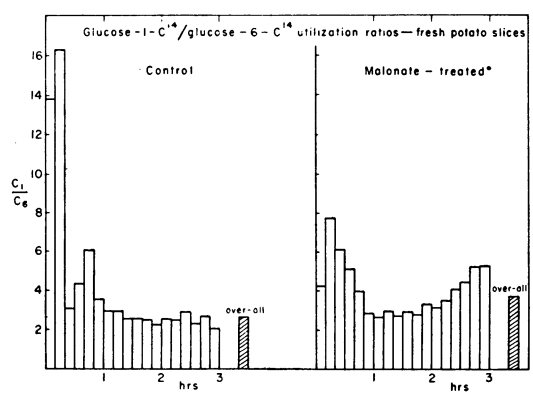

FIG. 3 (*left*). Ratio of $C^{14}O_2$ released from glucose-1-C^{14} versus glucose-6-C^{14} from fresh potato slices. Calculated from the data represented by figure 2.

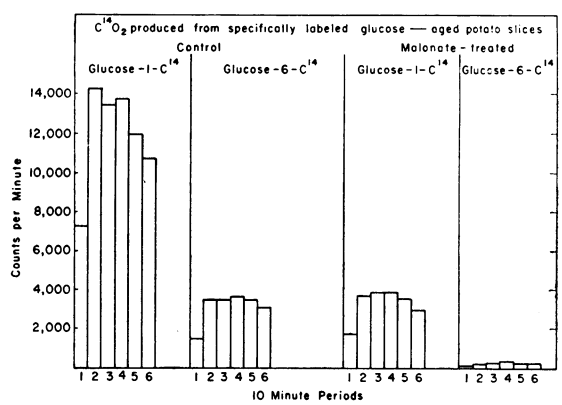

FIG. 4 (*right*). Relative rates of $C^{14}O_2$ output from potato slices aged for 30 hours in 10^{-4} M $CaSO_4$ and then supplied with glucose specifically labeled on C_1 or C_6. The glucose was dissolved in test solutions of the formulation given in the legend to figure 1.

TABLE IV

DISTRIBUTION OF RADIOACTIVITY FROM LABELED GLUCOSE IN EXTRACTS OF FRESH POTATO SLICES INCUBATED 3 HOURS WITH & WITHOUT MALONATE*

TREATMENT	% INITIAL TOTAL EXTRACT			
	BASES	ACIDS	SUGARS	TOTAL RECOVERED
Controls				
Glucose-1-C^{14}	14.3	14.6	70.1	99.0
Glucose-6-C^{14}	17.0	14.9	65.0	96.9
Glucose-R-C^{14}	12.6	13.2	72.0	97.8
Malonate				
Glucose-1-C^{14}	12.8	10.1	75.6	98.5
Glucose-6-C^{14}	16.5	12.3	72.2	101.0
Glucose-R-C^{14}	12.0	9.6	77.6	99.2

* All experimental conditions as described in footnotes to table III.

In the case of glucose-6-C^{14} the inhibitory effect of malonate upon the rise in release of label after 2 hours (fig 2) is striking. Malonate simultaneously depresses release of label from glucose-R-C^{14}, but the latter effect is less evident because randomly labeled hexose releases label from C_1 also.

In fresh controls there was a sharp drop in the C_1/C_6 ratio during the first 2 hours (fig 3). In view of the response to malonate it is likely that this is largely the result of onset of TCAC activity. In the presence of malonate the development of TCAC activity is inhibited and the drop in C_1/C_6 ratio is soon reversed.

Studies of isotope distribution after fresh slices had been incubated with labeled glucose for 3 hours revealed that considerable C^{14} had been sequestered into non-extractable cell material (table IV) or in extractable compounds probably not on direct pathways to CO_2 evolution (table II). Such sequestering is not disadvantageous because in these short term experiments we are not concerned with recovery of large fractions of the added isotope as $C^{14}O_2$. Sequestering of glycolytic products, for reasons mentioned above, is also of minor importance. In any case the ratio $(C_1-C_6)/C_1$ is an expression of the relative contributions of PPP and EMPP-TCAC to the release of label from glucose-1-C^{14}, and thus gives a partial measure of the relative activity of the two pathways in respiration. When the data from figure 2 are expressed on this basis, the percentage contribution of PPP to C_1 release in the controls falls from an initial above 90 to about 70 in 1 hour and then levels off at 60 to 65. In the presence of malonate, the relative contribution of PPP is also initially high and falls to 60 to 65 during the 2nd hour, but then rises to 80 again during the 3rd hour. This is consistent with the idea of onset of TCAC during the 2nd hour. A complicating factor is the presence of a malonate resistant C_6 releasing mechanism throughout the experimental period (fig 2).

There is no evidence of any significant malonate inhibition of label release from glucose-6-C^{14} until the 2nd hour, and the C_1/C_6 ratio then rises (fig 3). The lack of malonate inhibition of label release from glucose-R-C^{14} or glucose-6-C^{14}, lack of succinate accumulation, and the high initial C_1/C_6 ratio all indicate that TCAC activity is negligible during the 1st hour. The observed C_6 release suggests a mechanism other than the TCAC. It could represent release via PPP from hexose made from triose fragments (2), but the possibility of malonate resistant pathways other than the PPP being operative in C_6 release cannot be dismissed. Laties (12) has presented evidence for the existence of two types of malonate resistant respiration in aging chicory root slices. This may be true for potato slices also.

In aged tissue the inhibitory effect of malonate upon C_1 release (fig 4) at first glance suggests that about two-thirds of the total is due to a malonate sensitive system, presumably TCAC. Thus, paradoxically, it seems that C_1 release via the TCAC is about twice as great as total C_6 release in the aged control. The apparent paradox results from inhibition by malonate of glucose uptake per se in aged slices. Such inhibition of uptake, of course, applies equally to C_1 and C_6 and does not alter the C_1/C_6 ratios.

The C_1/C_6 ratio of aged control slices (fig 5) after 1 hour in the presence of labeled glucose is similar to that of fresh tissue after exposure for an equal period (fig 3). But it is not valid to argue that the contribution of the several pathways to respiration is, therefore, the same. Whereas in fresh tissue C_6 release during the 1st hour is not noticeably inhibited by malonate, in aged tissue malonate reduces C_6 release much more than C_1 release and the C_1/C_6 ratio becomes very high (fig 5). These results, together with those of manometric experiments (fig 1) revealing a 10-to 15-fold increase in malonate sensitive respiration during aging, speak for the existence of

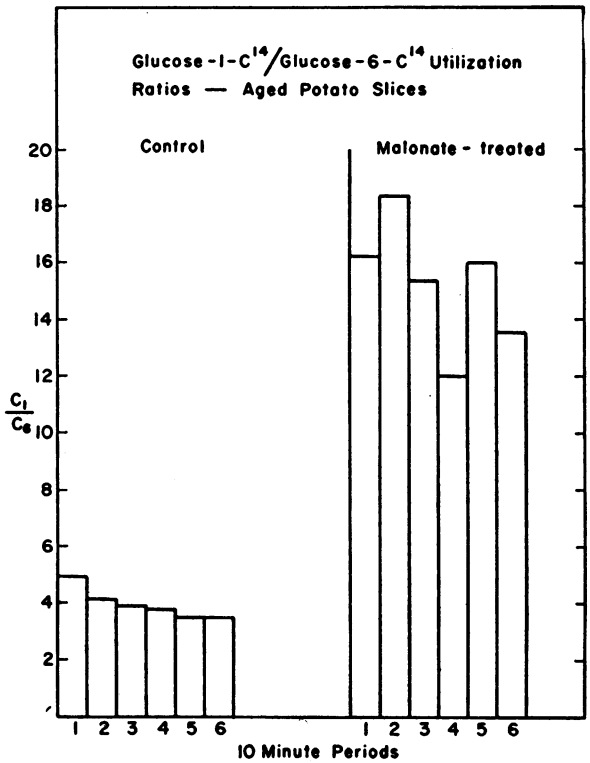

FIG. 5. Ratios of $C^{14}O_2$ released from glucose-1-C^{14} versus glucose-6-C^{14} by 30 hour aged potato slices. Calculated from the data represented by figure 4.

a malonate sensitive, non-discriminating pathway in aged tissue which is initially inoperative in fresh tissue. Thus the respiration of aged slices appears to consist of two major fractions, one, probably EMPP-TCAC, can be inhibited by malonate, the other, probably PPP or related system, is little affected by malonate and accounts for the bulk of the remaining respiratory capacity of aged tissue in the presence of malonate.

The enzymes of the TCAC are not lacking in fresh tissue. Furthermore, it is known that mitochondria from fresh and aged tissue oxidize TCAC acids at similar rates, and also that aging is not accompanied by any measurable increase in cytochrome c oxidase activity (6). Lack of malonate inhibition in fresh and of cyanide inhibition in aged tissue cannot, therefore, be adequately explained by an hypothesis requiring greatly over-dimensioned TCAC enzymes in fresh tissue and a gross excess of cytochrome c oxidase in aged. The fact that slices from newly harvested tubers contain glycolic acid, but lack the acids of the TCAC, whereas the latter appear during aging (Laties, pers communic), is further evidence that the TCAC is inoperative in fresh tissue. The reason for the initial inactivity of the TCAC, even when the acids of the cycle are present, is not obvious. Perhaps ancillary enzymes or cofactors are missing or an inhibitor of an essential step is present.

In contrast to ap Rees and Beevers (1), we believe that the major change which occurs during aging of potato slices is the activation of a malonate sensitive respiratory pathway—presumably TCAC. This development begins early in the aging period so that after only 2 hours the tissue can no longer be considered as strictly fresh. In truly fresh tissue TCAC activity is minimal, and respiratory CO_2 release is mediated primarily via PPP. Both PPP and TCAC activity increase with time, as does sensitivity to malonate. It is especially significant that malonate immediately raises the C_1/C_6 ratio in aged tissue, while in fresh tissue it has little effect until the 3rd hour. It is interesting that when slices are incubated in the presence of malonate, physiological aging does not accompany chronological aging and fresh characteristics can be maintained for long periods. If the major change induced by aging were increased PPP activity unaccompanied by the development of TCAC activity, the observed malonate effects would not be expected.

On the basis of experience gained from this work we believe that this approach can yield valuable information about slowly respiring tissues. Our results indicate that changes in the relative activities of the several respiratory pathways can occur rapidly and that frequent measurements during the early hours of aging are necessary. Total uptake information as a function of time, data on specific activity changes in various metabolic pools, and detailed studies of the distribution of label among metabolic intermediates would be valuable adjuncts; however, we have not been able to continue the work.

SUMMARY

The respiration of freshly cut 1 mm thick potato tuber slices is only slightly inhibited by 0.05 M malonate at pH 5.0. However, during incubation of the slices at room temperature sensitivity to malonate increases. During 30 hours of such physiological aging, total respiration increases four- to fivefold, the malonate resistant fraction doubles, and the malonate sensitive fraction increases 10- to 15-fold over its activity during the 1st hour.

Fresh slices have a very limited ability to absorb and oxidize exogenous glucose, as measured by evolution of $C^{14}O_2$ after feeding labeled glucose. This ability increases more than 3000-fold during 36 hours of aging; however, whereas $C^{14}O_2$ evolution by fresh tissue is not inhibited by malonate, that of aged tissue is reduced by a factor as high as 15.

In spite of limited uptake by fresh slices, techniques were developed which, after use of high specific activity C^{14} labeled glucose as a tracer, permit recovery of labeled metabolic intermediates after 1 to 3 hours of incubation. A method of fractioning extracts is described which by means of ion exchange, paper electrophoresis, and paper chromatography allows isolation of amino acids, carboxylic acids, sugar, and phosphorylated compounds from the same sample.

Glucose-1-C^{14}, glucose-6-C^{14}, and randomly labeled glucose, were fed to tissue slices in the presence and absence of malonate. Respiratory CO_2 was collected and C_1/C_6 ratios were calculated for each 10 minute interval for up to 3 hours. In such experiments fresh slices show initially high but declining C_1/C_6 ratios. Malonate has little effect at first, but reverses the decline during the 2nd hour. By contrast, in aged slices malonate causes an immediate and pronounced increase in the ratio from a control value of about 4 to about 16.

The following tentative interpretations of the data are advanced as a basis for further experimental work: The respiration of fresh slices is largely mediated by the pentose phosphate pathway. Within 2 hours products of glycolysis begin to be oxidized via the malonate sensitive tricarboxylic acid cycle. In aged tissue also the pentose phosphate pathway or similar system is operative, but the tricarboxylic acid cycle mediates a large fraction of the total respiration. The appearance of tricarboxylic acid cycle activity within 3 hours and its continued development to become a major component of respiration is probably the major fundamental change occurring during physiological aging. The rapidity of change during the early hours of incubation must be considered in designing experiments.

Acknowledgments

The authors wish to acknowledge the assistance of Dr. A. E. Flood and Dr. T. O. Perry in some of the experiments, and to thank Dr. G. G. Laties for his suggestions and continued interest in the work.

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