tide dehydrogenase. III. Catalytic properties of the enzyme from beef heart. J. Biol. Chem. 238: 1529–37.
28. MINAKAMI, S., R. L. RINGLER, AND T. P. SINGER. 1962. Studies on the respiratory chain-linked dihydrodiphosphopyridine nucleotide dehydrogenase. 1. Assay of the enzyme in particulates and in soluble preparations. J. Biol. Chem. 237: 569–76.
29. MITCHELL, P. 1957. A general theory of membrane transport from studies of bacteria. Nature (Lond.) 180: 134–36.
30. PRESSMAN, B. C. 1955. Oxidative phosphorylation with ferricyanide as terminal electron acceptor. Biochim. Biophys. Acta 17: 273–74.
31. SUTCLIFFE, J. F. 1959. Salt uptake in plants. Biol. Rev. Cambridge Phil. Soc. 34: 159–220.
32. VAN ROSSUM, G. D. V. 1961. Effect of growth on potassium uptake and respiration in rat-liver slices. Biochim. Biophys. Acta 54: 403–04.
33. VAN ROSSUM, G. D. V. 1962. The effect of oligomycin on cation transport in slices of rat liver. Biochem. J. 84: 35 P.

The Onset of Tricarboxylic Acid Cycle Activity with Aging in Potato Slices [1]

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Thin slices of potato tuber, as of other plant storage organs, develop a markedly enhanced respiration within a day after cutting (13, 14, 25, 31). Distinct metabolic and physiological changes accompany the rise in respiration. Protein synthesis and starch degradation are initiated (5, 28, 32). Salt-absorbing ability increases sharply (11). A capacity develops for growth in response to auxin (6, 27). The physiological changes are so marked that it has been deemed unreasonable to impute the transformation to the mere quickening of respiratory activity, especially since certain profound changes occur when the respiration rate has risen to but a small extent (14).

Thus the proposal has been put forward that the respiratory increment which arises with time in thin storage organ slices is virtually qualitatively distinct from the basal or initial respiration (11, 13, 14). A change in terminal oxidase characteristics with aging in potato slices has been recognized for some time (25, 31). A subsequent suggestion to the effect that the respiratory carbon path changes as well contends that the tricarboxylic acid cycle (TCAC) is largely inoperative in fresh potato slices and develops with time, so that while the gross respiration rate increases 3 to fourfold during aging, TCAC activity increases to a very much greater extent (13). Apart from the previously mentioned changes in tissue characteristics, the following more pointed observations have led to the above-mentioned hypothesis. A) While a variable modicum of fresh slice respiration may prove malonate-sensitive, fresh tissue respiration is predominantly malonate-resistant. By contrast, the respiratory increment which arises with aging is almost totally malonate sensitive (22). B) Fresh slices release C-1 of 1-C^{14} labeled glucose as CO_2, but fail to release radioactive CO_2 from 6-C^{14} labeled glucose. The ability to convert C-6 of 6-C^{14} glucose to CO_2 arises and develops concomitantly with malonate sensitivity. Malonate inhibits the evolution of radioactive CO_2 from 6-C^{14} glucose. When inhibition of C-6 release by malonate is first manifest, the evolution of $C^{14}O_2$ from C-1 labeled glucose becomes malonate-sensitive for the first time (22). C) Aged slices presented with uniformly labeled glucose-C^{14} accumulate labeled succinate in the presence of malonate. Fresh slices do not (14). D) The evolution of radioactive CO_2 from uniformly labeled glucose increases at least 3,000 times with aging while the respiration rises but threefold (15, 22). E) The absorption of phosphate from dilute solution increases 100-fold with aging, and increases 50-fold within the first 8 hours when respiration has less than doubled. The increase in absorption is attended by incorporation of phosphate into organic forms (4, 16). Current indications associate phophorylation primarily with electron transport attending TCAC oxidations.

In the experiments which follow an attempt has been made to directly assess the extent to which the TCAC is operative in fresh and aged tissue. Components of the TCAC, carbon-labeled in a way necessitating at least one full turn of the cycle for the release of radioactive CO_2, were presented to fresh and aged potato slices. The radioactivity of the respiratory CO_2 was determined in each case, as was the distribution of label among components of the TCAC. The observations indicate that the TCAC may be essentially inoperative in fresh tissue.

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Materials and Methods

Disks 1.0 mm thick and 0.9 cm in diameter were prepared from commercial Russet Burbank potato tubers as previously described, and used either after 20 minutes bathing in running distilled water (fresh disks) or following 24 hours incubation in 10^{-4} M $CaSO_4$ on a rotatory shaker (13, 22).

Experimental Procedure. Three g disks were placed in 10 ml solution in a 125 ml erlenmeyer flask. Twenty microcuries of a given labeled substrate as described in table I was added to each flask, together with K phosphate and $CaSO_4$ at final concentrations of 10^{-2} M and 10^{-4} M respectively. Respiratory CO_2 was absorbed in 0.2 ml 10 % NaOH imbibed on a 1 × 8 cm strip of Whatman No. 3 filter paper bent into a loop and suspended from a hook affixed to a rubber stopper in the mouth of the flask. Alkali papers were changed at 10 minute intervals, the total experimental period being 50 minutes. After each collection period alkali papers were dropped into 9.8 ml 1 % NaOH and allowed to stand in stoppered test tubes overnight. Alkaline solution, 0.2 ml, was plated on copper planchets for estimation of radioactivity with a thin-window gas flow detector (15). Where acetaldehyde was provided as substrate the alkaline solution receiving the filter paper strip was transferred to one chamber of a static wet combustion apparatus (2). The apparatus was evacuated, and 3.0 ml 1.0 M metaphosphoric acid saturated with 2,4-dinitrophenylhydrazine was added to the alkaline solution from the sidearm. CO_2 was distilled with 3 minutes of gentle boiling into 2.0 ml 1 % NaOH in the receiver. In this way contamination of radioactive CO_2 by labeled acetaldehyde was prevented.

Chemical methods. The organic acids were extracted, separated and estimated as described by Romberger and Norton (22). Carrier quantities of succinate and fumarate were added in chromatography to insure precise isolation of the latter for counting. Separate extractions of untreated fresh and aged tissue were carried out, and the acids estimated by titration in the absence of carrier. For some time an acid with an R_F barely in excess of that of glycolate in the solvent systems used was taken to be glycolate. Belatedly the acid was characterized as pyrrolidone-2-carboxylic acid (PCA). Consequently in those cases where radioactive glycolate was provided as substrate the activity ascribed to PCA in all probability pertains to a mixture of PCA and glycolate.

Glutamate and aspartate were isolated from the total amino acid fraction comprising the eluate from the IR 120 cation-exchange column employed in the first stages of fractionation. The eluate was stripped of ammonia and concentrated to 2.0 ml under reduced pressure on a rotary evaporator. The bulk of the concentrated sample was placed on a 30 cm column of Dowex 1 in the acetate form. Elution was affected with 0.5 M acetic acid. Two milliliter eluant fractions were tested for radioactivity, and for amino nitrogen by the ninhydrin method (23). The separation procedure described results in the immediate elution, essentially together, of all amino acids except glutamate and aspartate. The dicarboxylic amino acids, glutamate and aspartate, are subsequently eluted separately in turn (9). With the relatively low levels of glutamic and asparatic acids contained in 3.0 g potato tissue, recovery of the dicarboxylic amino acids from the Dowex 1 column varied from 75 to 80 %.

Fifty microliters of the concentrated total amino acid fraction from 3.0 g tissue was spotted in one corner of 18¼ × 21½ inch Whatman No. 1 papers. Chromatography was carried out for 16 hours in one direction with a mixture of butanol, acetic acid and water (4: 1: 5 by volume), followed by chromatography in the second direction for 16 hours in borate-buffered phenol (113 g 88 % liquid phenol plus 26.3 ml 0.1 M Na borate buffer, pH 8.3). Spots were developed with ninhydrin spray and identified by comparison with standard 2-dimensional chromatograms. Finally the chromatograms and medical X-ray film were appressed in cassets for 2 months. When the X-ray film was developed, areas of radioactivity were matched with ninhydrin-positive areas on the chromatograms, and the relative extent of labeling of the radioactive amino acids was estimated visually.

Table I. *Specifications of C-14 Labeled Substrates*

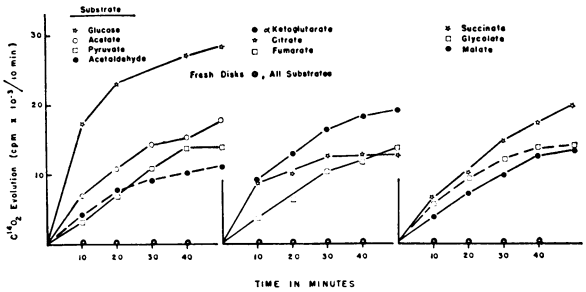
Substrate	Labeled carbon atoms	Specific activity	Final concentration	Source*
		mc/m mol	mM	
Pyruvate	2	4.0	0.50	Cal Biochem
Citrate	1,5	1.43	1.40	"
α-Ketoglutarate	5	2.71	0.74	"
Succinate	2,3	13.7	0.15	"
Fumarate	2,3	6.1	0.33	"
Malate (DL)	3	6.8	0.59	ICN
Acetate	2	11.0	0.18	ISC
Acetaldehyde	1,2	1.03	1.9	Volk
Glycolate	1	2.16	0.93	ISC
Malonate	2	5.0	0.64	ICN
Glucose	U	13.7	0.15	ISC

* Cal Biochem: California Corporation for Biochemical Research, Los Angeles; ICN: International Chemical and Nuclear Corp., City of Industry, Calif.; ISC: Isotopes Specialties Co., Burbank, Calif. Volk: Chicago, Ill.

Results

Figure 1 describes the evolution of $C^{14}O_2$ from a variety of organic acids involved in the operation of the TCAC, as well as from glycolate, acetaldehyde and glucose. The import of the figure is unequivocal: aged disks readily oxidize all substrates to yield $C^{14}O_2$, while fresh disks are totally unable to release radioactive CO_2 from any of the labeled metabolites presented. The distinction is qualitative. Figure 2 gives an indication of the time required for the attainment of diffusion equilibrium in 125 ml erlenmeyer flasks under the experimental conditions. A suspension of washed, glucose-adapted baker's yeast was provided with uniformly labeled glucose at t_0. On the assumption that glucose oxidation begins at once, figure 2 suggests that it takes from 10 to 20 minutes for the attainment of a physical steady-state with respect to $C^{14}O_2$ diffusion. Only after 20 minutes can changes in the measured activity of respiratory CO_2 be taken to reflect the activity of the pertinent substrate pool.

Although fresh slices produce no radioactive CO_2 from any of the substrates provided, there nevertheless is ample evidence that the substrates permeate the cells and in fact undergo metabolic transformation. Taking the radioactivity of the total alcohol extract as a measure of substrate penetration, it is evident from table II that metabolite absorption by fresh disks is appreciable compared with that of aged. The considerable radioactivity in the cationic fraction of fresh tissue extracts (table II), as well as the distribution of radioactivity in organic acids of fresh tissue (tables III, IV) indicate that substrate absorbed by fresh disks is metabolically transformed. Only a small part of the substrate taken up is found unchanged in the tissue. Early deductions regarding the absence of TCAC activity in fresh slices hinged on the malonate-insensitivity of fresh tissue. Since malonate has subsequently proven to be a natural metabolite in certain plant tissues (33) it proved necessary to establish whether malonate resistance perchance reflects malonate metabolism. Neither fresh nor aged potato disks release radioactive CO_2 from labeled malonate. Nevertheless, there is a small amount of malonate conversion, as suggested by tables II and III. In table II, the considerable radioactivity in the acid fraction of disks presented with labeled malonate represents unaltered malonate.



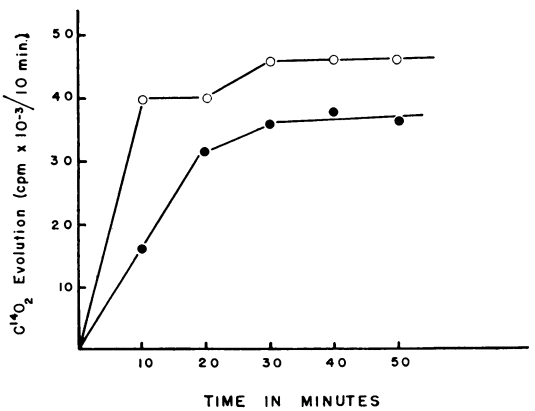


FIG. 1 (*upper*). The evolution of radioactive CO_2 from C^{14}-labeled components of the tricarboxylic acid cycle and from other substrates by fresh and aged potato slices. Experimental conditions as for Table II.

FIG. 2 (*lower*). The apparent time course of radioactive CO_2 evolution from uniformly labeled C^{14}-glucose by suspensions of baker's yeast. Two experiments. 25 mgs net weight washed baker's yeast in 10 ml 0.005 M glucose containing 10 μc glucose-U-C^{14}. Phosphate buffer, 0.01 M, pH 5.0.

The quantity of each acid in fresh and aged disks (table IV) was determined on separate untreated samples and taken to apply to all the treatments within an experiment, on the assumption that the gross tissue acid concentration does not change appreciably in short experimental periods in the presence of small quantities of substrate.

The Interconversion of Organic Acids in Fresh and Aged Disks. The details of $C^{14}O_2$ evolution by potato disks supplied with C^{14}-labeled substrates indicate that in aged tissue the latter are oxidized via the TCAC (14, 15). Since fresh disks readily absorb labeled organic acids, and since the label rapidly appears in a variety of metabolites including other organic acids, the inability of fresh slices to release radioactive CO_2 from any substrate yet provided suggests a block in the TCAC. An incomplete TCAC is further implied by the release of $C^{14}O_2$ from succinate-1-C^{14} by fresh tissue, where a complete turn of the cycle is not necessary for label evolution (table V). In table VI an attempt is made to circumscribe the locus of inhibition. The relative radioactivities of acids isolated from potato slices treated with each of the designated C^{14}-labeled substrates are noted either on the basis of total or specific activity. A value of 100 is assigned to the activity of the acid presented as substrate, or to that of the acid arbitrarily considered next in sequence in the TCAC when the substrate is not among the acids isolated. A comparison of the relative radioactivities is meant to reveal the extent to which label has been transferred from one acid to another.

By comparing the relative activity in citrate in fresh and aged disks it is evident that α-ketoglutarate

Table II. *The Distribution of Radioactivity in the Major Alcohol-Soluble Fractions of Tissue Incubated with C^{14}-Labeled Substrates*

Forty-five disks (3.0 g fr wt) incubated for 50 minutes at 25° in 10 mls solution containing 20 μc labeled substrate, as indicated in table I. $CaSO_4$, 10^{-4} M, and K phosphate, pH 5.0, 10^{-2} M in all cases.

Experiment	Substrate	Tissue*	Total alcohol extract	Eluate cation resin (IR 120)	Eluate anion resin (IR 45)**			Effluent	% Recovery***
					Formate eluate	NH_4HCO_3 eluate	Total		
	Pyruvate	F	83,750	35,500	4,975	4,425	9,400	400	54
		A	150,250	61,200	51,550	4,350	55,900	4,800	80
	Citrate	F	20,500	2,025	11,300	6,000	17,300	800	100
		A	61,250	16,875	24,375	10,000	34,375	400	84
	α-Ketoglu-	F	57,250	34,000	9,075	9,675	18,750	800	95
1	tarate	A	93,500	31,800	40,375	14,225	54,600	400	93
	Succinate	F	316,250	56,500	212,350	11,425	273,775	1,200	89
		A	184,250	45,400	111,100	4,100	115,200	7,200	91
	Fumarate	F	67,500	22,725	30,125	11,075	41,200	400	95
		A	156,500	39,150	76,200	8,500	84,700	6,800	87
	Malate	F	80,000	20,250	40,575	2,300	42,875	400	80
		A	248,000	51,975	171,650	6,025	177,675	7,200	95
	Acetate	F	1,774,000	215,550	187,425	73,175	260,600	2,800	27
		A	270,750	68,625	65,500	5,825	71,325	1,520	52
	Acetalde-	F	723,500	66,600	49,225	15,625	64,850	2,000	18
	hyde	A	293,750	83,025	86,600	7,450	94,050	1,760	61
2	Glycolate	F	84,000	41,850	19,700	2,900	22,600	400	77
		A	114,250	67,950	40,625	1,375	42,000	400	97
	Malonate	F	12,250	1,575	7,700	1,875	9,575	400	94
		A	75,500	3,150	60,825	10,875	71,700	400	96
	Glucose	F	99,250	5,625	9,125	1,175	10,300	64,000	85
3		A	434,500	56,250	76,775	12,050	88,825	187,200	77

* F and A denote fresh and aged disks respectively.

** Formate eluate, primarily organic acids. NH_4HCO_3 eluate, organic phosphates and less readily elutable acidic compounds.

*** Percent recovery indicates the sum of the activity in the individual fractions expressed as the per cent of the total activity in the alcohol extract.

and the dicarboxylic acids bring about citrate labeling in both types of tissue. The disparity is threefold at most and usually less. That the relative activities of citrate are small is of little consequence since the low values apparently reflect a small active metabolic pool (see below). The important feature is that the relative activities in both tissues are of the same order of magnitude. By contrast, when citrate is presented to fresh and aged disks, label transfer to other acids of the TCAC is considerable in aged disks, and minimal in fresh. The block in the TCAC in fresh tissue may thus tentatively be placed between citrate and α-ketoglutarate.

In general the relative activities of all the TCAC acids are considerably higher in aged tissue regardless of which labeled organic acid is provided as substrate. The disparity among acids, and the occurrence of relative activities in excess of 100, are lessened by making the comparison on the basis of specific activities.

Glycolate is metabolized as readily as the other 2-C and potential 2-C (pyruvate) substrates, and serves to label other organic acids. However, since the R_F for PCA is very close to that of glycolate in the solvents used, and since PCA was mistaken for glycolate for some time, the activity assigned to PCA in experiments involving glycolate metabolism in all probability pertains to a mixture of glycolate and PCA. Hence the relative activities have an ambiguous meaning in connection with glycolate utilization.

Comparison of Glutamic Acid and Pyrrolidonecarboxylic Acid Labeling. The data of table VII suggest that glutamate and PCA are to a considerable extent labeled by different paths. The specific activity of glutamate far exceeds that of PCA, while the ratio of label incorporation by aged compared with fresh tissue is considerably greater for PCA. PCA appears to be involved in the TCAC (see Discussion) and its labeling intensity is more nearly akin to that of the TCAC acids than is that of glutamate. Nevertheless the way in which glutamate labeling changes with tissue age suggests that perhaps part of the total cell glutamate may be involved in TCAC activity.

PCA in large measure, if not entirely, arises from

Table III. *The Distribution of Radioactivity in the Organic Acids of Potato Slices Incubated with C^{14}-Labeled Substrates*

Experiment as in table II. Acids were from 45 disks (3.0 g fr wt). Acid quantities are listed in table IV.

Experiment	Substrate	Tissue	Citrate		Succinate		Fumarate		Malate		PCA	
			cpm	cpm/ μmole	cpm	cpm/ μmole	cpm	cpm/ μmole	cpm	cpm/ μmole	cpm	cpm/ μmole
1	Pyruvate	F	802	120	449	118	...	...	342	28	226	11
		A	1,318	61	1,252	394	646	...	16,501	975	10,333	620
	Citrate	F	6,667	1,000	55	15	55	...	120	10	56	3
		A	7,514	344	285	92	103	...	4,064	240	3,735	224
	α-Ketoglutarate	F	95	14	1,508	396	...	...	1,407	114	551	26
		A	392	18	2,377	765	78	...	14,827	875	6,385	384
	Succinate	F	2,312	346	21,318	5,600	...	...	9,926	805	1,271	59
		A	1,318	61	7,299	2,350	1,515	...	33,017	1,950	7,274	438
	Fumarate	F	498	75	276	73	9,466	...	6,721	545	164	8
		A	1,117	51	1,083	349	8,518	...	31,985	1,890	5,374	323
	Malate	F	876	131	718	189	609	...	25,585	2,080	99	5
		A	2,083	96	1,456	469	646	...	85,318	5,050	6,811	410
2	Acetate	F	46,530	2,500	2,597	864	536	214	7,047	616	3,342	242
		A	3,863	358	1,391	660	420	200	21,990	1,880	13,255	690
	Acetaldehyde	F	14,825	795	2,494	830	325	130	2,737	240	870	63
		A	4,114	382	1,861	885	790	376	29,015	2,480	15,530	809
	Glycolate	F	1,508	81	235	78	31	12	569	50	5,029	364
		A	520	48	88	42	113	54	1,738	149	19,925	1,037
	Malonate	F	426	23	377	126	22	9	63	6	606	44
		A	386	36	954	453	101	48	684	58	13,255	690
3	Glucose	F	431	...	120	...	118	...	724	...	152	...
		A	3,985	...	787	...	295	...	8,812	...	4,556	...

Table IV. *Organic Acids in Fresh and Aged Potato Slices*

Experiments as in table III. Acid quantities used for specific activities are listed in table III.

Acid	Experiment 1		Experiment 2	
	Fresh	Aged	Fresh	Aged
	μmoles/3.0 g fr wt			
Citrate	6.7	21.8	18.6	10.8
Succinate	3.8	3.1	3.0	2.1
Fumarate	...	...	2.5	2.1
Malate	12.3	16.9	11.4	11.7
PCA	21.4	16.6	13.8	19.2

Table V. *The Oxidation of Succinate-1-C^{14} by Fresh and Aged Potato Slices*

Conditions as for figure 1, table II. 3.0 g tissue were used.

Time	Radioactivity in respiratory CO_2	
	Fresh	Aged
minutes	*cpm/20 min*	
20	750	62,000
40	1,550	78,500
60	2,550	70,000
80	2,500	79,000
100	3,100	69,000
120	3,600	65,500

glutamine during hot extraction [table VIII, cf (21)]. It might be expected that glutamine in turn derives from glutamate, but a direct interrelation is brought into question by the labeling disparity. It may well be that part of the total cell glutamate is interconvertible with glutamine (see 28), but table VII implies compartmentation. Compartmentation is further suggested by the distinctly different way in which α-ketoglutarate labels glutamate and PCA, respectively, in fresh tissue. The likelihood that glutamate and PCA arise in different ways (see below) as well as being separated spatially helps to explain the difference in labeling between the two.

Discussion

The contention has been made previously on the basis of indirect considerations that the TCAC is largely inoperative in fresh potato slices. Evidence now indicates that in complete contrast to aged slices, fresh potato disks fail to produce radioactive CO_2 from labeled organic acids of the TCAC or from closely related compounds. The presumption that the TCAC is inoperative or relatively inactive in fresh tissue is considerably strengthened. A small TCAC component of fresh tissue respiration may be observed at times, however, varying with the storage history and condition of the tuber. It has been pointed out by apRees and Beevers that a marked increase in the pentose phosphate respiration path

occurs with time in aging potato disks as judged by the excess of C-1 release from C-1 glucose as compared with C-6 evolution from C-6 glucose (1). With respect to the total respiratory rise, however, the point of importance is that whereas the pentose phosphate type of respiration simply increases in magnitude, perhaps several fold, the TCAC is virtually absent in fresh tissue and very active in aged. Since phosphorylative metabolism and its concomitants appear to depend upon the TCAC, it is the de-

Table VI. *The Relative Radioactivity of Organic Acids from Potato Slices Incubated with Labeled Substrates*

Ratios derived from data of table III. Among the extracted acids, the activity of the acid presented as substrate was taken as 100. When the metabolite presented as substrate was not subsequently isolated, a value of 100 was assigned to the nearest component deemed to follow in the classical TCA cycle. Where gylcolate was administered the PCA isolated may in large measure be glycolate (see Methods).

Experiment	Substrate	Tissue	Relative total cpm					Relative specific activities				
			Citrate	Succinate	Fumarate	Malate	PCA	Citrate	Succinate	Fumarate	Malate	PCA
1	Pyruvate	F	100	56	...	43	28	100	97	...	23	9
		A	100	95	49	1250	785	100	650	...	1610	102
	Citrate	F	100	0.8	0.8	1.8	0.8	100	1	...	1	0.3
		A	100	3.8	1.4	54	50	100	27	...	70	65
	α-Ketoglutarate	F	6	100	...	93	37	4	100	...	29	7
		A	17	100	3	630	270	2	100	...	114	50
	Succinate	F	11	100	...	47	6	6	100	...	14	1
		A	18	100	21	450	99	3	100	...	83	19
	Fumarate	F	5	3	100	71	2	...	...	...	...	...
		A	13	13	100	376	63	...	...	...	...	...
	Malate	F	3	3	2	100	0.4	6	9	...	100	0.2
		A	2	2	0.7	100	8	2	9	...	100	8
2	Acetate	F	100	6	1	15	7	100	35	9	25	10
		A	100	36	11	570	342	100	184	56	525	192
	Acetaldehyde	F	100	17	2	19	6	100	104	16	30	8
		A	100	45	19	700	374	100	232	99	650	210
	Glycolate	F	30	5	0.6	11	100	22	21	3	14	100
		A	3	0.4	0.6	9	100	5	4	5	14	100

Table VII. *Comparison of Glutamic Acid and Pyrrolidone Carboxylic Acid Labeling in Potato Slices Incubated with C^{14}-Labeled Substrates*

PCA concentration considered the same for each sample of fresh and aged tissue respectively within an experiment.

Experiment	Substrate	Tissue	Aspartate			Glutamate			PCA		
			cpm	μ moles	cpm/μmole	cpm	μ moles	cpm/μmole	cpm	μ moles	cpm/μmole
1	Pyruvate	F	647	6.5	99	7,221	6.4	1,130	226	21.4	11
		A	11,304	4.2	2,680	16,633	5.1	3,270	10,333	16.6	620
	Citrate	F	186	8.3	23	877	6.5	134	56		2.6
		A	758	3.6	21	7,049	4.8	1,460	3,735		224
	α-Ketoglutarate	F	4,299	7.6	562	24,480	3.8	6,450	551		26
		A	1,931	2.6	743	6,489	3.2	2,020	6,385		384
	Succinate	F	14,329	6.0	2,380	12,908	5.2	2,480	1,271		59
		A	3,337	...	...	15,162	...	...	7,274		438
	Fumarate	F	16,500	4.9	16,500	2,425	3.8	640	164		8
		A	7,810	3.9	7,810	11,487	3.5	3,280	5,374		323
	Malate	F	13,404	6.8	1,980	3,337	5.9	570	99		4.6
		A	4,713	3.5	1,340	14,058	4.2	3,340	6,811		410
2	Acetate	F	13,870	6.3	2,200	74,833	8.0	9,350	3,342	13.8	242
			6,265	2.7	2,320	19,983	4.4	4,530	12,255	19.2	690
	Acetaldehyde	F	5,083	7.2	705	33,543	8.4	4,000	870		63
		A	7,668	4.5	1,700	18,882	2.9	6,500	15,530		809
	Glycolate	F	549	6.6	83	4,083	0.9	4,400	5,029		364
		A	ca 0	1.5	0	440	2.4	183	19,925		1,037
	Malonate	F	117	7.2	16	883	8.3	106	606		44
		A	204	2.7	75	538	4.7	114	13,255		690

Table VIII. *Pyrrolidonecarboxylic Acid Formation by Hot Extraction of Potato Slices*

Hot extract prepared as in Methods. Cold extract: Tissue frozen with dry ice, powdered and lyophilized. Dry powder was extracted with ice cold 70 % ethanol-0.01 N HCl by shaking in the cold for 6 hours (12 g fr wt equivalent extracted with 200 ml ethanol-HCl). Where digestion is indicated, extracts were brought to pH 6.5 and heated for 2 hours at 100°. Under the latter conditions glutamine is converted to pyrrolidonecarboxylic acid (21). Extracts and hydrolyzed extracts were passed through columns, and acids and PCA isolated and estimated as described in Methods.

	Hot extract		Cold extract	
	Control	Digested	Control	Digested
	μmoles/3 g fr wt		μmoles/3 g fr wt	
Citrate	29.3	25.0	26.6	21.0
Malate	18.5	21.2	19.0	18.0
PCA	40.5	51.4	13.0	37.3

velopment of the latter which best explains the change in physiological competence of aging potato slices. Critical metabolic changes are initiated in fresh slices immediately upon cutting and are well along in several hours (5, 13, 14). The suggestion that the TCAC simply increases in magnitude with time much as does the pentose phosphate respiration appears to stem from the fact that apRees and Beevers compared 24-hr slices with 3-hr slices, rather than with freshly prepared ones [(1), cf (22)].

Dinitrophenol (DNP) elicits an immediate substantial respiratory rise in fresh slices of potato and other plant storage organs (7, 12). It may at first sight be supposed that the respiration of fresh tissue is phosphorylative, albeit limited by the rate of phosphate transfer. However, the DNP-induced increment has been shown to be totally malonate-sensitive, in contrast to the basal respiration, and the evidence suggests that in fresh slices DNP evokes an additional respiratory path rather than stimulates the existing one (12). Furthermore, the DNP-elicited respiratory rise in fresh slices, though malonate-sensitive, has been shown not to comprise TCAC activity, indicating that the latter is suppressed in fresh tissue by more than a restraint on phosphorylation (12). The foregoing presumption was verified in an experiment akin to that presented in figure 1, with the difference that DNP was provided fresh potato slices together with glucose-U-C^{14}. Fresh slices produced no $C^{14}O_2$ even in the presence of DNP. Glucose absorption was shown to be unaffected by DNP (15).

Mitochondria from both fresh and aged potato slices readily oxidize TCAC acids (7, 8). The evidence suggests that the TCAC is in fact blocked rather than absent in fresh tissue. The latter supposition, as well as the assignment of the block to a step between citrate and α-ketoglutarate oxidations, is made for the following reasons: A) While the dicarboxylic acids label citrate both in fresh and aged slices to a relatively similar degree, citrate labels the dicarboxylic acids appreciably only in aged tissue. B) The relative radioactivity of the acids of the TCAC is greater in aged than in fresh disks following the presentation of an appropriate labeled substrate. C) Where citrate is heavily labeled directly by acetate or acetaldehyde, label accumulates in citrate in fresh tissue where citrate is not metabolized, and far exceeds the citrate label in aged disks (table III). D) Whereas fresh tissue produces no $C^{14}O_2$ from succinate 2,3-C^{14} which demands a full turn of the cycle for label evolution, $C^{14}O_2$ is evolved to a limited but definite extent by fresh slices from succinate-1-C^{14} (oxalacetate decarboxylation).

Following the observation that certain aldehydes repress the changes which occur with aging in storage organ slices (13, 14) and that glyoxylate and oxalacetate together inhibit the TCAC (24), further investigation led to the isolation of a condensation product of glyoxylate and OAA, namely γ hydroxy-α ketoglutarate, which effectively inhibits citrate oxidation (19). Subsequently the enzymic formation of hydroxyketoglutarate from glyoxylate and pyruvate by potato extracts was shown, which together with the demonstrated oxidation of hydroxyketoglutarate to malate (20) provides a means for 4-C-dicarboxylic acid formation from pyruvate and glyoxylate, for glyoxylate oxidation in a pyruvate-catalyzed metabolic cycle, and for the removal as well as the formation of hydroxyketoglutarate, a factor which lends interest to hydroxyketoglutarate as a metabolic regulator. No indication has yet been offered that the TCAC in potato tissue is in fact controlled by hydroxyketoglutarate.[2] Nevertheless the ready enzymic production of an inhibitor of citrate oxidation from metabolites prevalent in potato tissue lends stimulus to the notion that the TCAC may be blocked in vivo at the citrate stage. In the light of the recent discovery that citrate strongly inhibits glycolysis (18), the proposed block may serve to explain the impairment of glucose utilization in fresh potato slices (15). It is noteworthy, furthermore, that the influence of high O_2 tensions on potato respiration has been ascribed to a block of the TCAC between citrate and α-ketoglutarate (3).

The respiration rate of potato slices is not affected by low levels of exogenous substrate. Hence figure 1 in effect indicates that the specific activity of respiratory CO_2 is quite similar during the oxidation of the various labeled organic acids. The implication of this observation is that on a carbon basis the specific activities of the various provided organic acids in their respective endogenous active pools must approximate each other. Table IX indicates first that the organic acid specific activity on a carbon basis is far lower than that of the respiratory CO_2 (10 to several hundred, compared with about 4×10^3 cpm per μmole carbon), and secondly that the carbon specific

[2] γ hydroxy α ketoglutarate has been shown to be present in large amounts in Oxalis roots (personal communication from the late Professor R. K. Morton).

Table IX. *The Specific Activity on a Carbon Basis of Organic Acids Extracted from Aged Potato Slices Incubated with Labeled Substrates*

Conditions as for tables III, IV.

Substrate	Citrate	Succinate	Fumarate	Malate	PCA
		cpm/μ mole carbon			
Pyruvate	10	99	...	244	124
Citrate	[57]*	23	...	60	45
α-Ketoglutarate	3	191	...	219	77
Succinate	10	[587]*	...	490	88
Fumarate	8	87	...	472	65
Malate	16	117	...	[1262]*	82
Acetate	60	165	50	470	138
Acetaldehyde	64	221	94	620	162
Glycolate	8	11	14	37	[207]*

* Bracketed numbers indicate isolated acids presented as substrate.

activity varies markedly among the acids. It can be deduced therefore that with extraction the active pool of each organic acid is extensively diluted with the respective metabolically unavailable organic acid, the ratio of active to inactive pool, and the active pool size, being somewhat different in each case (cf 17). When specific activities are compared, malate frequently appears to be labeled inordinately, especially when compared with citrate (table VI). However, on the basis that the carbon specific activity of the active pools of malate and citrate must roughly approximate each other, and taking into account that the tissue molar concentrations of citrate and malate are roughly the same (table IV), it appears from the considerably higher gross carbon specific activity of malate, that the active malate pool is larger than that of citrate. It is not excluded that acids outside of the respiratory mainstream become labeled (note the high specific activities of glutamate and aspartate in table VII), and the foregoing deductions must therefore be taken merely as suggestive.

While the convention adopted in table VI best serves to locate the block in the TCAC in fresh tissue, it tends to exaggerate the extent to which label resides in the substrate-acid following extraction. If the absolute activities of the extracted acids are compared in each case, using a given acid other than the substrate as a frame of reference, the relative distribution of label among the TCAC components is much the same regardless of the substrate presented.

An apparent paradox exists insofar as pyruvate, acetate and acetaldehyde label the 4-carbon acids more extensively than does citrate (table III), although labeling via the TCAC must proceed through citrate. The latter condition is undoubtedly in large part the consequence of the label position in the provided substrate. Steady-state labeling of 4-C acids via the TCAC should be greater with acetate-2-C^{14}, for example, than with citrate-1,5-C^{14}. Nevertheless it remains possible that the 4-C acids (and PCA) may be labeled by 2-C substrates by means other than the TCAC. In this connection it is noteworthy that the 2-C substrates label both aspartate and glutamate out of all proportion compared with the TCAC acids, and do so even, and perhaps especially, in fresh tissue (table VII). It is readily apparent how a direct independent means of 4-carbon dicarboxylic acid synthesis may serve to insure maximal activity of the TCAC. Although malate synthesis by the condensation of acetate and glyoxylate provides the readiest explanation for direct malate labeling by pyruvate, acetate, or acetaldehyde (10), malate synthetase has not been demonstrable in potato extracts (W. S. Pierpoint, personal communication). In terms of its conceivable effect on the TCAC, the latter condensation would have the added virtue of lowering the glyoxylate concentration and hence the concentration of hydroxyketoglutarate, an effective inhibitor of the TCAC (see above).

Finally, the origin of PCA (in effect, glutamine) and its relation to glutamate is of interest. PCA labeling is less intense that that of glutamate, and responds much more markedly to aging. Furthermore, in comparison with glutamate labeling, acetate and acetaldehyde are more effective than the 4-carbon acids in labeling PCA in aged disks. The pronounced influence of aging on label incorporation in PCA and in the organic acids, together with the influence of malonate on the labeling pattern (14), suggests that labeling occurs via the TCAC. The path of glutamate labeling is more uncertain, and the distinction between the 2 cases suggests compartmentation. In this connection a pertinent indication of compartmentation is to be had in the observation that α-ketoglutarate and acetate are metabolized in different ways by slices of castor bean endosperm, while being oxidized in the same way by mitochondria from the same tissue (30). Compartmentation would appear a likely explanation for the low specific activity of the organic acids in potato slices presented with labeled substrates (cf 17), and may in part explain the disparity in glutamate and glutamine labeling as well.

Regarding the disproportionately high specific activity of glutamate in general indicated in table VII, it should be noted that there are ways to form glutamate apart from the TCAC, especially from glyoxylate and oxalacetate or pyruvate (26). Furthermore, glutamate may conceivably be derived from

a 4-carbon progenitor. In this connection Steward's suggestion that γ-aminobutyric acid may arise from succinic semialdehyde, and may lead to glutamic acid formation in potato is of special interest (29). Withal it is not apparent why glutamine, so much more so than glutamate, should appear to be intimately related to the TCAC, presumably by way of α-ketoglutarate. Conceivably, for that part of glutamate synthesis which occurs intramitochondrially the prevalence of mitochondrial oxidative phosphorylation may well lead to rapid and extensive glutamine formation.

Summary

Fresh potato slices release no radioactive CO_2 from organic acids of the tricarboxylic acid cycle labeled with C^{14} in such a way that a full turn of the cycle is required for label evolution. In marked contrast, aged potato disks evolve copious quantities of $C^{14}O_2$ from the same organic acids. The labeled acids provided were pyruvate, citrate, α-ketoglutarate, succinate, fumarate, malate, acetate, and glycolate. Glucose, acetaldehyde and malonate were presented as well. A comparison of label distribution in fresh and aged tissue following administration of the above-listed substrates indicates a block in the tricarboxylic acid cycle in fresh tissue between the steps of citrate and α-ketoglutarate oxidation.

Pyrrolidone-2-carboxylic acid, which appears to be derived from glutamine during hot extraction, shares the labeling characteristics of the organic acids both with respect to specific activity and the enhancement of labeling in aged disks. Glutamate labeling is much more extensive, and relatively indifferent to tissue age. Compartmentation is indicated, with the prospect that glutamate, and perhaps one or more of the 4-carbon dicarboxylic acids, may be synthesized extramitochondrially.

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Literature Cited

1. apREES, T. AND H. BEEVERS. 1960. Pentose phosphate pathway as a major component of induced respiration of carrot and potato slices. Plant Physiol. 35: 839–47.
2. ARONOFF, S. 1958. Techniques of Radiobiochemistry. Iowa State College Press. p 44–45.
3. BARKER, J. 1963. Studies in the respiratory and carbohydrate metabolism of plant tissues. XIII. The influence of oxygen at high pressures in increasing and decreasing the respiration of potatoes at 15 C. Proc. Roy. Soc. (London) B 158: 143–55.
4. BIELESKI, R. L. AND G. G. LATIES. 1963. Turnover rates of phosphate esters in fresh and aged slices of potato tuber tissue. Plant Physiol. 38: 586–94.
5. CLICK, R. E. AND D. P. HACKETT. 1963. The role of protein and nucleic acid synthesis in the development of respiration in potato tuber slices. Proc. Natl. Acad. Sci. 50: 243–50.
6. HACKETT, D. P. AND K. V. THIMANN. 1953. The nature of the auxin induced water uptake by potato tissue. II. The relation between respiration and water absorption. Am. J. Botany 40: 183–88.
7. HACKETT, D. P., D. W. HAAS, SUSANNE K. GRIFFITHS, AND D. J. NIEDERPRUEM. 1960. Studies on development of cyanide-resistant respiration in potato tuber slices. Plant Physiol. 35: 8–19.
8. HAWKER, J. S. AND G. G. LATIES. 1963. Nicotinamide adenine dinucleotide in potato tuber slices in relation to respiratory changes with age. Plant Physiol. 38: 498–500.
9. HIRS, C. H. W., S. MOORE, AND W. H. STEIN. 1954. The chromatography of amino acids on ion exchange resins. Use of volatile acids for elution. J. Amer. Chem. Soc. 76: 6063–65.
10. KORNBERG, H. L. AND J. R. SADLER. 1961. The metabolism of C_2-compounds in micro-organisms. 8. A dicarboxylic acid cycle as a route for the oxidation of glycolate by *Escherichia coli*. Biochem. J. 81: 503–13.
11. LATIES, G. G. 1957. Respiration and cellular work and the regulation of the respiration rate in plants. Surv. Biol. Prog. 3: 215–99.
12. LATIES, G. G. 1959. The nature of the respiratory rise in slices of chicory root. Arch. Biochem. Biophys. 79: 364–77.
13. LATIES, G. G. 1962. Controlling influences of thickness on development and type of respiratory activity in potato slices. Plant Physiol. 37: 679–90.
14. LATIES, G. G. 1963. Control of respiratory quality and magnitude during development. In: Control Mechanisms in Respiration and Fermentation. Barbara Wright, ed. Ronald Press. p 129–55.
15. LATIES, G. G. 1964. The relation of glucose absorption to respiration in potato slices. Plant Physiol. 39: 391–97.
16. LOUGHMAN, B. C. 1960. Uptake and utilization of phosphate associated with respiratory changes in potato tuber slices. Plant Physiol. 35: 418–24.
17. MACLENNAN, D. H., H. BEEVERS, AND J. L. HARLEY. 1963. "Compartmentation" of acids in plant tissues. Biochem. J. 89: 316–27.
18. PASSONNEAU, J. V. AND O. H. LOWRY. 1963. P-fructokinase and the control of the citric acid cycle. Biochem. Biophys. Res. Comm. 13: 372–79.
19. PAYES, B. AND G. G. LATIES. 1963. The inhibition of several tricarboxylic acid cycle enzymes by γ-hydroxy-α-ketoglutarate. Biochem. Biophys. Res. Comm. 10: 460–66.
20. PAYES, B. AND G. G. LATIES. 1963. The enzymic conversion of γ-OH α-ketoglutarate (HKG) to malate: (A postulated step in the cyclic oxidation of glyoxylate). Biochem. Biophys. Res. Comm. 13: 179–85.
21. PUCHER, G. W. AND B. VICKERY. 1940. A method for determining glutamine in plant tissues. Ind. Eng. Chem. Anal. Ed. 12: 27–29.
22. ROMBERGER, J. A. AND G. NORTON. 1961. Changing respiratory pathways in potato tuber slices. Plant Physiol. 36: 20–29.
23. ROSEN, H. 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys. 67: 10–15.

24. RUFFO, A., E. TESTA, ANNA ADINOLFI, AND G. PELIZZA. 1962. Control of the citric acid cycle by glyoxylate. 1. A new inhibitor of aconitase formed by the condensation of glyoxylate with oxaloacetate. Biochem. J. 85: 588–93.
25. SCHADE, A. L., H. LEVY, L. BERGMANN, AND S. HARRIS. 1949. Studies on the respiration of the white potato. III. Changes in the terminal oxidase pattern of potato tissue associated with time of suspension in water. Arch. Biochem. 20: 211–19.
26. SEKIZAWA, Y., M. E. MARGOUDAKIS, S. S. KERWAR, M. FLIKKE, A. BAICH, T. E. KING, AND V. H. CHELDELIN. 1962. Glutamic acid biosynthesis in an organism lacking a Krebs tricarboxylic acid cycle. Biochem. Biophys. Res. Comm. 9: 361–66.
27. SPERLING, E. AND G. G. LATIES. 1963. The dependence of auxin-induced growth on auxin-independent metabolic changes in slices of storage tissue. Plant Physiol. 38: 546–50.
28. STEWARD, F. C., P. R. STOUT, AND C. PRESTON. 1940. The balance sheet of metabolites for potato disks showing the effect of salts and dissolved O_2 on metabolism at 23°. Plant Physiol. 15: 409–47.
29. STEWARD, F. C. AND J. K. POLLARD. 1956. Some further observations on glutamyl and related compounds in plants. In: Inorganic Nitrogen Metabolism, W. D. McElroy and B. Glass, eds. Johns Hopkins Press, p 377–407.
30. TANNER, W. AND H. BEEVERS. 1963. Compartmentation of metabolic reactions in vivo. Plant Physiol. 38: xx.
31. THIMANN, K. V., C. S. YOCUM, AND D. P. HACKETT. 1954. Terminal oxidases and growth in plant tissues. III. Terminal oxidation in potato tuber tissue. Arch. Biochem. Biophys. 53: 239–57.
32. THIMANN, K. V. AND G. M. LOOS. 1957. Protein synthesis during water uptake by tuber tissue. Plant Physiol. 32: 274–79.
33. YOUNG, R. H. AND L. M. SHANNON. 1959. Malonate as a participant in organic acid metabolism in bush bean leaves. Plant Physiol. 34: 149–52.

Translocation of Photosynthetically Assimilated C^{14} in Straight-Necked Squash [1, 2, 3]

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Introduction

Studies of translocation in higher plants derived from sugar analyses of sap (10, 26, 27, 28), aphid stylet exudates (24, 28) and by the displacement of C^{14} from mature leaves (1, 6, 7, 11, 12, 20, 23), have produced the generally accepted view that sucrose is the main form of transported carbon in phloem tissue (19). These studies however have been confined to a very limited number of plant species. Recent investigations have now shown that sucrose is not the only sugar involved in translocation. Zimmermann (27) has found raffinose, stachyose and mannitol in the sap exudates of a number of trees and other plant species and Starck and Gorham (unpublished data) have found by C^{14} displacement verbascose and stachyose as the major compounds in the translocation stream of *Mentha* and *Saintpaulia*. Pristupa (15) has also found translocation of verbascose and stachyose in the pumpkin plant. Evidence for the translocation of sorbitol in the apple has recently been published (25) and amino and organic acid movement from leaves has been reported by Nelson et al. (13) in soybean under certain physiological conditions, and in the rhubarb by Kursanov et al. (9). Evidence for the movement of many other compounds in the phloem may be found as more plant species are investigated. These new discoveries must modify the concept of the unique position of sucrose in translocation and before ascribing any mechanism to the process it will be necessary to know the full range of compounds transported by the phloem.

A preliminary investigation of the young squash plant had shown that it translocated both stachyose and sucrose. Furthermore freeze-dried squash tissues, in contrast to the tissues of other plant species investigated (5, 14), were readily infiltrated and prepared for tissue autoradiography. A detailed study of translocation was therefore carried out in young squash plants using the methods of C^{14} displacement under controlled conditions and tissue-autoradiography. The products of short-term $C^{14}O_2$ assimilation were determined and their translocation within the plant was measured as a function of time and stage of leaf development.

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