Turnover Rates of Phosphate Esters in Fresh and Aged Slices of Potato Tuber Tissue [1]

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Introduction

In recent years, a number of studies have been made on the respiration of slices of potato tuber tissue (1, 6, 7, 8, 9, 11, 12, 13, 16, 17). When the slices or disks are freshly cut, they have a respiration rate similar to that of the intact tuber. This tissue has been termed fresh tissue. When the fresh disks are kept by aerating them in distilled water or 10^{-4} M $CaSO_4$ at 0°, the low respiration rate is maintained; but when the disks are aerated at 25°, the respiration rate rises three- to fourfold over about 18 hours. This phenomenon has been termed aging, and disks treated in this way are aged disks. Aging does not merely involve a simple increase in respiration rate: the sensitivity of the respiration to various inhibitors alters markedly (1, 6, 11, 16, 17), and the tissue shows a marked increase in its ability to accumulate salts, particularly phosphate (6, 9, 13). Such observations have led to suggestions that aging involves a major change in the cellular processes (7). Some authors (6, 17) have suggested that there is a modification of the respiration chain in the region between DPNH and cytochrome c; while others (1, 16) have suggested that there are changes in the relative importance of the pentose phosphate and the tricarboxylic acid cycle respiration paths. The results of experiments by Loughman (9) indicate that the respiration changes are closely associated with changes in the phosphate metabolism. Therefore experiments have been carried out to examine in more detail the phosphate metabolism of fresh and aged potato tuber slices, with a view to learning more about the nature of the aging process.

Materials and Methods

Materials. Potatoes, var. Russet Burbank, were obtained from local markets and stored for up to 3 weeks at 10°. Potatoes were sliced in half laterally, and one half used to provide aged slices, while the other was capped with parafilm (Hercules Powder Co.) and stored at 10° until the following day, to provide fresh slices. Equal numbers of apical and basal halves were used for each sample. Blocks of tissue were cut from the potato halves, cylinders 7 mm in diameter were cut in each block with a stainless steel borer, then 1 mm slices were taken from the block on a hand microtome and put into ice water. When all the disks had been cut, the tissue was bathed in running deionized water at room temperature (25°) over 8 minutes. Disks were then transferred to 1 liter conical flasks (10 g/150 ml solution/flask) and incubated on a shaking table in 10^{-4} M $CaSO_4$ for 10 minutes (fresh slices) or 24 hours (aged slices). In the latter case, 4 to 5 changes of solution were used. Phosphate-P^{32} was heated to 105° for 1 hour to remove HCl and hydrolyze pyrophosphate present. Activities ranging from 1 μc to 1 mc/ml were employed in experimental solutions.

General Methods. Respiration measurements were made by standard Warburg manometry, using samples of 1 g (15 disks) in pH 5.0, 10^{-2} M KH_2PO_4 or in solutions stated.

When the P-esters[4] were extracted from small samples of potato tissue by publicized extraction procedures, a serious and nonreproducible loss of ester-P was encountered. A procedure was developed which enabled a quantitative recovery and efficient separation of P-esters from 1 g or less of plant tissue. A detailed discussion of the technical problems encountered, and of the extraction procedures and chromatographic methods selected, is given elsewhere (5). The methods employed in the current study differed only in that an earlier, less suitable extraction procedure was employed. The tissue (1 g) was killed in boiling 80% methanol (30 ml), and extracted in 80% methanol followed by 5% trichloracetic acid (15 ml), then the trichloracetic acid was removed by ether extraction. This tissue

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[4] Abbreviations: P-esters, phosphate esters; ester-P, esterified phosphate; P_i^{32}, inorganic phosphate labeled with P^{32}; UTP, uridine triphosphate; UDP, uridine diphosphate; UMP, uridine 5′-monophosphate; GTP, GDP, and GMP, guanosine triphosphate, etc.; CTP, CDP, CMP, cytidine triphosphate, etc.; hexose-P, hexose phosphate (glucose-6-P + fructose-6-P + glucose-1-P).

extraction procedure was more time-consuming than the recommended one (5), gave greater opportunity for handling error and was less suitable for routine use. However, extracts from the 2 procedures show the same P-ester pattern, with one exception. In the experiments described here, phosphatase inactivation probably took several seconds; and there was probably some hydrolysis of ATP to ADP (4). Subsequent study has shown that a maximum of 20 % of the ATP was so hydrolyzed, and that the effect was the same in all samples. The conclusions drawn in this paper have been made with this source of error in mind.

Chromatography was carried out in 2 different 2-dimensional systems: *n*-propanol/ammonia/water-*n*-propyl acetate/formic acid/water; and *n*-propyl acetate/formic acid/water-electrophoresis at pH 3.6 (5). The completed chromatograms were radioautographed [see (5) for a representative example], and the radioactive areas were marked on the chromatogram. The various radioactive compounds were then identified by reference to added authentic compounds and their known travel constants. In preliminary experiments, spots of doubtful identity were eluted and rerun with standards in other separation systems. For counting, areas of the chromatogram were cut out and mounted on planchets. Where the piece of chromatogram paper was too big to fit on a planchet, one end of the cut out paper was dipped into a small pool of water so that the ascending solvent moved the radioactive material to the tip of the piece of paper. The paper was dried and the tip cut off, mounted on a planchet and counted, using a Nuclear-Chicago thin end-window gas-flow counter. For most compounds, the radioactivity was the same in the spots obtained by the 2 methods of separation: mean values are quoted in the text. However, the UDP, GTP, and GDP values were occasionally as much as twofold different when isolated by the 2 procedures.

P_i in the extract was estimated by the method of Marsh (14); hexose-P was isolated chromatographically, hydrolyzed (5) and the P_i estimated; nucleotides were isolated chromatographically, their absorption spectra determined on a Cary recording spectrophotometer, and their amount estimated from the absorption of light at 260 mμ.

Effect of Phosphate Concentration and Malonate on P^{32}-distribution in Esters. Samples of fresh and aged tissue, 5.0 g (ca. 75 disks) were incubated in each of 3 solutions; high P_i (4×10^{-3} M KH_2PO_4), low P_i (10^{-5} M KH_2PO_4), or low P_i + malonate (10^{-5} M KH_2PO_4 + 5×10^{-2} M malonate at pH 5.0). Grossly different rates of P_i uptake were obtained in the 6 samples. As it was desirable to have approximately the same total P^{32} activity in all tissue extracts, the amount of carrier-free P^{32} added to each experimental solution was varied accordingly. Thus for fresh and aged tissues respectively, 50 and 25 μc/ml were added to the high P_i solutions; 35 and 1 μc/ml to the low P_i solutions; and 75 and 3 μc/ml to the low P_i + malonate solutions. Samples were incubated 1 hour on a shaking table at ca. 25°, washed 5 minutes in running distilled water, then killed and the P-esters extracted. The tissue residue, containing the acid-insoluble P^{32}, was held 24 hours at 25° in 1 M KOH to hydrolyse RNA. The nucleotides released were separated by chromatography and electrophoresis, and their radioactivity measured (5).

Amounts and Turnover Rates of Phosphate Esters. Fresh and aged tissue samples were given a 30 second pulse of P_i^{32}, transferred to distilled water and allowed to metabolize the tracer for varying lengths of time. In order to introduce approximately the same amount of P_i at the same specific activity into the 2 types of tissue, 2×10^{-3} M KH_2PO_4 at 600 μc/ml was used for the fresh tissue and 2×10^{-4} M KH_2PO_4 at 60 μc/ml for the aged tissue. Samples of 15 disks (ca. 1 g), interleaved with 1 mm glass beads, were threaded onto stainless steel wires; and to minimize changes in the tissue during this time, prepared samples were held in ice water while remaining samples were being threaded. When all strings were complete, they were carried through the following stages. A. In distilled water at 25° for 5 minutes, to come to room temperature. B. In unlabeled P_i solution at 25° for 5 minutes (fresh, 2×10^{-3} M KH_2PO_4; aged, 2×10^{-4} M KH_2PO_4) to saturate adsorption sites in the tissue that otherwise contributed significantly to the apparent uptake in very short term experiments. C. In running distilled water at 25° for 5 minutes to remove excess unlabeled P_i from the apparent free space. D. In 40 ml $KH_2P^{32}O_4$ (fresh, 2×10^{-3} M; aged, 2×10^{-4} M) for 30 seconds (20 seconds for half-minute samples). E. In unlabeled P_i (fresh, 2×10^{-2} M KH_2PO_4; aged, 2×10^{-3} M KH_2PO_4) for 10 seconds to rapidly dilute the $KH_2P^{32}O_4$ in the apparent free space. F. In distilled water for 30 seconds to remove P_i from the apparent free space. G. In another change of distilled water for 1 minute. H. In another change of distilled water at 25° for the desired interval. Samples were killed at 0.5, 1, 2, 4, 8, 15, 30, and 60 minutes after the start of P_i^{32} absorption in step D.

The 1 g samples used above did not provide sufficient material for the amounts (as opposed to radioactivities) of the individual P-esters to be measured. Ester levels were therefore measured on a separate bulk (20 g) sample of the same tissue used in the pulse experiment. The tissue was killed and extracted in the usual way and the P-esters separated in bulk by paper chromatography in 2 solvents, and by electrophoresis. To avoid any aging effects, the bulk sample was killed at the same time as the 30 minute, 1 g sample. The specific activity of a P-ester at 30 minutes could then be estimated by dividing the P-ester radioactivity determined on the 1 g, pulse sample by the P-ester amount, determined on the 20 g bulk sample. To aid detection and identification of the esters, the bulk sample was in-

cubated during the early part of the experiment in dilute $KH_2P^{32}O_4$.

Results

Effect of Sterile Conditions on Aging Effect. To insure that the commonly observed respiratory rise in potato slices could in no way be attributed to bacterial infection, potato slices were cut and aged under sterile conditions, and compared with slices cut and aged in the normal way. The respiration rate of the sterile slices increased with aging in exactly the same way as that of the control slices.

Effect of Phosphate Concentration on Phosphate Ester Distribution. At the high P_i concentration, P_i uptake by fresh tissue was increased 335-fold over that at the low concentration, while only a 24-fold increase in P_i uptake was observed with the same increase in concentration in aged tissue. The increase in P_i uptake did not significantly affect the distribution of radioactivity in the various P-esters, but it did increase the proportion of unesterified P_i^{32} in both tissues (table I).

Effects of Aging and Malonate on Phosphate Ester Distribution. The aging process did not markedly affect the relative distribution of P^{32} in the various extractable P-esters, though the tissue P_i^{32}/ester-P^{32} ratio was higher in the fresh tissue. However, the amount of P^{32} which entered the acid-insoluble residue was higher in the aged tissue, and markedly so when a low concentration of P_i^{32} was supplied to the tissues. When the acid-insoluble residue from the aged tissue was hydrolyzed with KOH, 82% of the radioactivity was recovered in the adenosine, cytosine, guanosine, and uridine 3'- (and 2'-) phosphates (ratio 10/9/9/10). Thus most of the acid-insoluble P^{32} in the aged tissue must have been present as RNA.

Malonate affected the pattern of distribution of the P-esters in the aged tissues but not in the fresh tissues (table I). Again, the most marked effects were on the acid-insoluble P^{32}, and on the nucleotide triphosphates.

In other experiments, more detailed comparisons were made of the distribution of P^{32} activity in the P-esters from fresh and aged tissues, in the expectation that if different respiration paths were operating in the 2 tissues, the amounts of the various P-esters might vary accordingly. Steady-state labeling for most esters was reached in about 1 hour in both tissues. At this time, no significant difference (>20% of the mean value) could be consistently found in the relative amount of any P-ester in the 2 tissues. However, the tissue P_i^{32}/ester-P^{32} ratio was consistently higher in the fresh tissue. As the respiration rates of the 2 tissues were different, it was concluded that the turnover rates of the P-esters must be different. The pulse experiment was carried out to test this presumption.

Rates and order of labeling of phosphate esters. Labeling of the P-esters followed the same course in the fresh and aged tissues (fig 1, 2). However the

Table I

Distribution of P^{32} in Fresh and Aged Potato Tuber Slices Treated with High and Low Concentrations of Phosphate-P^{32}, and with Malonate

		Fresh tissue in			Aged tissue in		
		High-P*	Low-P	Low-P + malonate	High-P	Low-P	Low-P + malonate
Respiration rate, μl O_2/g tissue hr		37	38	31	132	138	52
Phosphate-P^{32} taken up, mμmoles/g tissue hr		63.9	0.19	0.14	226.5	9.46	5.97
Acid-insoluble P^{32}, as % of total P^{32} in tissue		1.3	3.0	1.9	3.3	29.0	1.9
P^{32} in compound, as % of total P^{32} in extractable ester-P	Tissue P_i	143.9	115.1	200.7	116.7	75.4	148.0
	Hexose-P, etc.**	58.3	53.3	54.7	45.0	39.9	58.7
	AMP, GMP, UMP	1.6	2.0	2.0	2.0	2.8	3.1
	ADP, GDP, UDP	18.3	19.6	17.4	21.6	22.9	19.3
	ATP, GTP, UTP	8.4	11.0	10.2	12.2	12.3	3.6
	3-PGA, etc.	5.5	4.9	6.2	8.5	8.7	6.1
	P-choline, etc.	6.4	7.0	7.5	8.6	11.4	6.3
	UDP-glucose	1.1	1.2	1.1	1.5	1.2	1.2

* High-P, tissue in 4×10^{-3} M KH_2PO_4; Low-P, tissue in 10^{-5} M KH_2PO_4; Low-P + malonate, tissue in 10^{-5} M KH_2PO_4 + 5×10^{-2} M malonate.

** Values for P-esters have been grouped as follows: hexose-P, etc = glucose-6-P + fructose-6-P + glucose-1-P + mannose-6-P; 3-PGA, etc = 3-P-glycerate + 2-P-glycerate + 6-P-gluconate + triose phosphate + fructose-1, 6-diP; P-choline, etc = P-choline + P-ethanolamine + P-serine (?) + α-glycerol-P + inositol hexaP UDP-glucose value is an underestimate as considerable hydrolysis occurred.

rate of labeling was much more rapid in the aged tissues. Curves were fitted (see Discussion) and the half-times of labeling for the various compounds were estimated (table II). The order of labeling was as follows. A. ATP, GTP and UTP all reached half-maximum labeling in approximately the same time; in under 0.2 of a minute in aged tissue, and in about 1.0 minute in fresh tissue. CTP was present in very small amounts, ca. 8% of the ATP, and usually could not be separated from the ATP. B. ADP reached half-maximum labeling in 0.9 of a minute (aged) or 3.5 minutes (fresh). GDP and UDP were apparently slightly slower to label in the aged tissue. CDP was present in only very small amounts. C. Glucose-6-P, fructose-6-P, fructose-1,6-diP, 6-P-gluconate, triose phosphate, 3-P-glycerate (+ 2-P-glycerate), P-enolpyruvate and glucose-1-P, which could be separately identified, all reached half-maximum labeling at approximately the same time; 1.2 to 1.8 minutes for aged tissue, and 5.8 to 8.9 minutes for fresh tissue. D. α-glycerol-P showed labeling half-time of ca. 3 and 8 minutes in aged and fresh tissue respectively. E. A sugar phosphate identified fairly conclusively as mannose-6-P was slower to label than all the other sugar phosphates, having labeling half-time of 4.9 and ca. 12 minutes in aged and fresh tissues. F. AMP, UMP, DPN, P-choline, inositol hexaP; and compounds which were probably P-ethanolamine and TPN; all had

Table II

Half-times of Labeling, in Minutes, of Phosphate Esters Present in Fresh and Aged Potato Tissue Slices

Compound	Fresh tissue		Aged tissue	
	Exp. 14.	Exp. 15.	Exp. 14.	Exp. 15.
ATP	$\leqslant$1.2	1.1	<0.5	$\leqslant$0.2
GTP	<1.0	0.7	<0.5	$\leqslant$0.2
UTP	<1.0	0.5	<0.5	$\leqslant$0.2
ADP	3.4	3.5	$\leqslant$1.0	0.9
GDP	3.0	3.2	1.8	2.2
UDP	3.5	4.5	1.8	1.9
AMP	>15.0	>30.0	8.0	10.0
UMP	>15.0	28.0	3.5	12.0
hexose-P	6.5	5.8	1.4	1.2
3-P-glycerate	6.0	5.5	1.3	1.4
P-enolpyruvate	5.0	4.0	1.7	1.4
6-P-gluconate	...	6.0	1.6	2.1
mannose-6-P	12.0	11.0	5.2	4.9
α-glycerol-P	8.0	6.8	1.9	3.7
P-ethanolamine	>15.0	$\leqslant$25.0	5.0	12.0
P-choline	>15.0	>30.0	9.5	17.0
DPN	...	>30.0	...	>30.0
Inositol hexaP	...	>30.0	...	>30.0

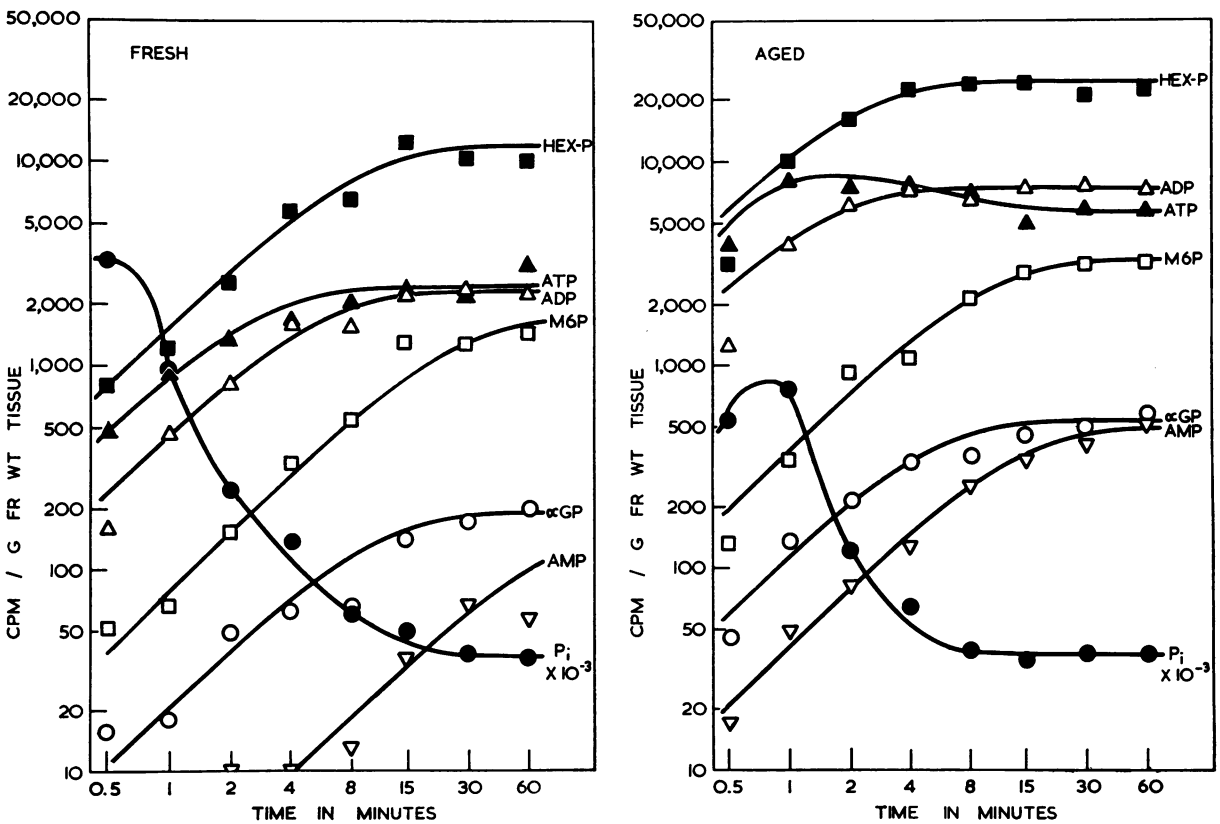

FIG. 1 and 2. Log/log plot of change in radioactivity with time, for various phosphate esters from fresh tissue (*left*) and aged tissue (*right*) fed a 30 second pulse of P^{32}-phosphate (exp. 15). Theoretical curves fitted to all points except ATP, aged; and P_i, fresh and aged. ■ — ■, hexose-P; ▲ — ▲, ATP; △ — △, ADP; ▽ — ▽, AMP; □ — □, mannose-6-P; ○ — ○, α-glycerol-P; ● — ●, P_i, × 10^{-3}.

Table III

Amounts and Specific Activities of Some Phosphate Esters From Fresh and Aged Potato Tuber Slices

These data are from Experiment 15, table II.

	Amount, mμmole/g		Activity in cpm/g				Specific activity, cpm/mμmole			
			Fresh		Aged		Fresh		Aged	
	Fresh	Aged	1 min	30 min	1 min	30 min	1 min	30 min	1 min	30 min
P_i	2,700	2,110	...	38,000	...	37,900	...	14	...	18
Hexose-P	95	101	1,265	11,500	10,130	23,500	13	121	100	232
AMP	2.0*	4.3	10	70	48	422	5	35	11	98
ADP	11.2	23.1	465	2,400	3,680	7,375	41	214	159	319
ATP	6.4	10.0	965	2,440	8,020	5,650	151	382	802	565
UMP	4.9	8.2	13	265	76	385	2	54	9	47
UDP	5.5	11.2	180	1,270	767	2,550	32	231	69	228
UTP	3.5	5.5	200	260	965	605	57	74	176	110
GDP	2.3	2.8	80	320	298	980	35	139	107	350
GTP	1.3	1.9	200	280	1,013	845	154	216	535	445

* Nucleotide estimation accurate to ca. ± 0.5 mμmole/g tissue.

labeling half-times ranging from ca. 10 minutes to 25 minutes or more in aged tissue, or of more than 20 minutes in fresh tissue.

GMP and CMP were not detected. UDP-glucose was detected, though it suffered considerable breakdown during chromatography. It appeared to have labeling half-times of ca. 2 and 9 minutes in aged and fresh tissues, assuming that the percentage breakdown of the ester was approximately the same in all samples.

Amounts of Phosphate Esters. The observed differences between the rates of labeling of P-esters in fresh and aged tissues could have been due to the presence of a much larger ester-P pool in the fresh tissue. However, the fresh tissue was found to have a somewhat smaller ester-P pool (table III).

The amount of P_i which entered the tissues during a pulse of P_i^{32} (activity at counting, 1.2×10^5 cpm/mμmole) was less than 0.8 mμmole/g, or 0.04 % of the total P_i present in the tissues. This amount is unlikely to have affected the relative amounts of the various P-esters in the tissue (cf. table I). Thus it can be assumed that the P-ester levels obtained for the 30 minute samples of aged tissue (table III) will also be true for the 1 minute sample. From these data, the specific activities of the P-esters in the aged tissues at 1 minute and at 30 minutes can be calculated (table III). The nucleotide triphosphates show a fall in specific activity from 1 to 30 minutes, while the other P-esters studied show a rise. In fresh tissue, all compounds show a rise in specific activity from 1 to 30 minutes.

Discussion

Extent of Phosphate Esterification in Fresh and Aged Tissue. Increasing the external P_i concentration 400-fold increased the rate of P_i uptake by aged tissue 24-fold, and by fresh tissue 335-fold. Uptake by aged tissue was 50-fold greater than by the fresh tissue at the low concentration, but only 3.5-fold greater at the high concentration. These observations agree with those of Loughman (9). Loughman's data show that the approximately linear relationship of phosphate uptake to external P_i concentration in fresh tissue applies over an even wider range, 10^{-6} to 10^{-2} M; but that the P_i uptake by aged tissue as a function of concentration approaches an hyperbolic course more typical of normal absorption processes. Loughman suggested that 2 distinct accumulation mechanisms were involved in the 2 tissues. An alternative possibility is that entry into the fresh tissue takes place solely by diffusion; thus explaining the rather unusual linear relationship of uptake to concentration. The uptake in aged tissue presumably occurs by a normal transport mechanism, perhaps augmented by diffusive entry at the highest concentrations.

The extent to which P_i^{32} entering the tissue became esterified differed under different treatments (table I). These results were consistently obtained, and confirm those of Loughman (9). Thus the tissue P_i^{32}/ester-P^{32} ratio was 20 % higher in the fresh tissue than in the aged. Data in table III show that the difference in radioactivity is of the same order as the quantitative, tissue P_i/ester-P ratio difference in the 2 tissues (the lower P_i level in the aged tissue is caused mainly by loss of P_i through leakage to the external solution during aging; (9)). The tissue P_i^{32}/ester-P^{32} ratio was also higher when a high concentration (4×10^{-3} M) instead of a low concentration (10^{-5} M) of P_i was supplied to the tissue. At the high concentration, sufficient P_i^{32} entered the tissue that the tissue P_i levels would have been significantly increased. However, the increases in tissue P_i/ester-P (2.5% and 10 % in fresh and aged tissue; from table I, line 2 and table 3, line 1) were much less than the observed increases in radioactivity (tissue P_i^{32}/ester-P^{32} increases, 25 % and 55 % in fresh and aged tissue). However, if about 9/10 of the original tissue P_i were sequestered in a non-metabolic pool, and if the P_i^{32} which entered the

tissue had done so, in both fresh and aged tissues, exclusively into the metabolic pool which contained the remaining 1/10 of the tissue P_i; then the increase of P_i in the metabolic pool alone (as opposed to the whole tissue) caused by the entry of the P_i^{32} would have been 25 % and 100 % in fresh and aged tissues respectively. Such a pattern of entry of P_i^{32} to the tissue would thus account for the differences in tissue P_i^{32}/ester-P^{32} caused by changes in the external P_i^{32} concentration.

The nonmetabolic phosphate pool. The suggestion that P_i in the tissue is divided between a metabolic pool which first receives the absorbed phosphate, and a nonmetabolic storage pool, can be examined with the aid of data from table III. It can be assumed that the α-phosphate groups of AMP, ADP, and ATP are in approximate equilibrium with one another, and that the β-phosphate groups of ADP and ATP are also in equilibrium. Thus for the 30-minute, aged tissue sample, the α-, β- and γ-phosphate groups of the adenosine nucleotides would be expected to have specific activities of 98, 221, and 246 cpm/mμmole respectively. The β- and γ-values are in close agreement with the value for hexose-P (232 cpm/mμmole), indicating that these phosphate groups came into equilibrium with the same pool of P_i, of specific activity ca. 240 cpm/mμmole. Nevertheless, the measured specific activity of the total P_i pool in the tissue was only 18 cpm/mμmole. Hence the P-esters must have been synthesized from a small P_i pool which was separate from the bulk of the tissue P_i. If it is assumed that there were only the 2 pools, an estimate can be made of their size. Let the size of the metabolic P_i pool be x mμmoles/g tissue, of specific activity 240 cpm/mμmole; the size of the nonmetabolic pool will then be $(2{,}110 - x)$ mμmoles/g tissue, with a specific activity of y cpm/mμmole. Then

$$240x + y(2{,}110 - x) = 37{,}900.$$

If the specific activity of the nonmetabolic pool is zero, then the size of the metabolic pool, x, is 158 mμmoles/g tissue, or 7.5 % of the tissue P_i, a value approximating that estimated by Loughman (9). This is the maximum amount of P_i that can be in the metabolic pool. If the specific activity of the nonmetabolic pool were, for example, 9 cpm/mμmole (see below), then the metabolic pool would be 82 mμmoles/g tissue, or 3.9 % of the tissue P_i. From similar considerations, the specific activity of P_i in the metabolic pool of the fresh tissue was estimated as 170 cpm/mμmole; and the maximum size of the metabolic P_i pool was estimated as 224 mμmoles/g tissue, or 8.3 % of the tissue P_i.

An independant estimate of the size of the metabolic P_i pool can be made from the specific activity data for the 1 minute sample of aged tissue. At this time, the various esters had not reached steady-state labeling. In the 1 minute sample the specific activities of the α-, β-, and γ-phosphate groups of ATP, calculated as before, were 11, 148, 643 cpm/mμmole respectively, while the specific activity of the hexose-P was only 100 cpm/mμmole. The specific activity of the γ-phosphate of ATP would be expected to be equal to that of the metabolic P_i pool at that time. Hence, 1 minute after the presentation of P_i^{32}, the metabolic pool specific activity was 643 cpm/mμmole, while after 30 minutes it was in approximate steady-state at 240 cpm/mμmole. It seems probable that the decline in specific activity of the metabolic P_i pool was primarily due to a return of originally unlabelled phosphate from the P-esters back to the metabolic P_i pool. Let there be x mμmoles/g of P_i in the metabolic pool, of specific activity 643 cpm/mμmole, and let the specific activity of the P_i reentering the metabolic P_i pool from the P-ester pool at that time be y cpm/mμmole. The amount of rapidly cycling ester-P involved in respiration in the tissue could be calculated very roughly from data in table III (see also Discussion); it was of the general order of 245 mμmoles/g tissue. Then

$$643x + y.245 = 240(245 + x).$$

If the average specific activity of the P_i returning to the metabolic pool at that time is assumed to be about equal to that of the major ester involved in the respiration cycle, hexose-P, then $y = 100$ cpm/mμmole, and x, the size of the metabolic P_i pool, is 85 mμmoles/g, or 4 % of the total P_i. This is less than the maximum figure obtained from the previous calculation, and is the same as the value obtained on the assumption that the specific activity of the storage P_i pool after 30 minutes was 9 cpm/mμmole. Though there are many possible sources of error in this second calculation, it appears unlikely that the storage P_i pool has a specific activity any higher than 12 cpm/mμmole, 30 to 60 minutes after the start of supplying P_i^{32} to the tissue. Thus the rate of transfer between the metabolic and nonmetabolic pools must be very low, with a half-time of at least 8 hours in aged potato tissues.

Data on the specific activity of the P_i^{32} supplied to the tissue can also be used to provide evidence that most or all of the P_i entering the tissue does so through the metabolic pool. The size of the metabolic pool of P_i in the tissue was estimated as ca. 85 mμmoles/g, and of the metabolic ester-P pool as ca. 245 mμmoles/g, giving a total of 330 mμmoles of phosphate in this pool. The specific activity of the P_i^{32} supplied to the tissue was 1.2×10^5 cpm/mμmole, and 0.8 mμmoles/g entered the aged tissue. If all this P_i^{32} had entered the metabolic pool alone, then the specific activity of the metabolic pool at steady-state would have been ca. 290 cpm/mμmole (i.e. 0.8/330 of 1.2×10^5). This is in good agreement with the observed steady-state value of 240 cpm/mμmole.

Energy-requiring Processes in Fresh and Aged Tissue. Malonate inhibits the respiration rate of aged tissue, depressing it to about the fresh level; but it does not affect the fresh tissue respiration. Correspondingly, malonate altered the P-ester distribution in the aged tissue only. It has been suggest-

ed that the resistant respiration in malonate-inhibited aged tissue occurs through a secondary pathway which in effect represents the whole respiration of the fresh tissue (7, 16). In the absence of complicating factors, therefore, the malonate-inhibited aged tissue should have a P-ester distribution like that of the fresh tissue. Instead, a marked drop in the nucleotide triphosphate level was observed (table I). This suggests that the aged tissue has developed anabolic processes, not present in the fresh tissue, which can deplete the nucleotide triphosphate pool when phosphorylation becomes limited during malonate inhibition. RNA synthesis was one such process, active in the aged tissue and inhibited by malonate, but not active in fresh tissue. Protein synthesis is also active in aged tissue alone (8). The evidence suggests that the aged tissue is in many ways a rejuvenated tissue.

Efficiency of Respiration in Fresh and Aged Tissue. In the foregoing discussion, it has been assumed that the fresh tissue phosphorylates less vigorously because it respires less rapidly. The pulse experiment data can be used to establish this quantitatively, and it will be shown that the fresh tissue displays a disproportionately low phosphorylative activity. Consider an unlabeled pool of a P-ester at steady state in the tissue, where the rates of synthesis and breakdown are equal and constant. If there is a single precursor acting as label donor, and if this is suddenly brought to a uniform and constant specific activity, then the P-ester will become labeled at a rate

$$A = A_\infty (1 - e^{-kt}),$$

where A is the activity of the P-ester at time t, A_∞ is the activity at infinite time (equals P-ester pool size times specific activity of label donor), and k is the rate constant, or fraction of label donor converted to the P-ester per unit time (15). If $T_{1/2}$ is the labeling half-time (the value of t for which $A = \frac{1}{2}A_\infty$), then $k = (\ln 2)/T_{1/2}$. Now, if exponential curves of the foregoing type are plotted as the function log A against log t, then each curve is found to have the same shape, but displaced along the x-axis as k is varied and along the y-axis as A_∞ is varied. Thus if $k' = mk$ and $A'_\infty = nA_\infty$, then the plot of log A' against log t is the same as the plot of log A against log t displaced + log m along the x-axis and +log n along the y-axis.

When data from the pulse experiment are expressed on this type of plot (fig 1, 2), the one general curve can be visually fitted to the points, and values for A_∞ and $T_{1/2}$ can readily be obtained (table II). The tendency for the half-minute values to be low is to be expected, since the P_i^{32} pulse was of necessity of but 20 seconds duration, as against 30 seconds for all the other samples.

From the relationship $k = (\ln 2)/T_{1/2}$, the values for $T_{1/2}$ (table II) and from the sizes of the ester pools (table III), the rates of hexose-P synthesis in the 2 tissues used in exp. 15 can be calculated: 0.68 μmoles/g tissue hour in fresh tissue and 3.56 μmoles/g tissue hour in aged tissue. As the measured respiration rates of the 2 tissues were 2.7 and 7.4 μg-atoms O_2/g tissue hour respectively, the approximate P/O ratios in the 2 tissues, as estimated by hexose-P turnover, were 0.25 and 0.49 respectively. To measure the true P/O ratio for each tissue, the rate of energy-rich phosphate generation should be known. The best measure of this would normally be given by the $T_{1/2}$ and pool size for the total nucleotide triphosphate pool, but in this case an accurate measure of the $T_{1/2}$ value for the nucleotide triphosphate in aged tissue was not possible. Approximately, the rates of nucleotide triphosphate synthesis were 0.60 and >4.5 μmoles/g tissue hour for fresh and aged tissues respectively, giving P/O ratios of 0.23 and >0.6 for fresh and aged tissue respectively. If any hydrolysis of nucleotide triphosphate occurred during the extraction procedure, the true P/O ratios would be rather higher than the apparent values quoted above.

These data show that the efficiency of phosphorylation in aged tissue was at least 2 times as high as in the fresh tissue. The P/O ratio and efficiency of respiration in the aged tissue may well have been far higher than the indicated minimum of 0.6, as considerable energy-rich phosphate must have been used in synthetic processes which do not pass through hexose-P.

Although fresh and aged potato tissues show markedly different P/O ratios, mitochondria from such tissues do not (6). It is possible that isolating the mitochondria from the cytoplasm removes them from the influence of an inhibitor or modifier. If the behaviour of the isolated mitochondria is truly representative of their behaviour *in situ*, a possible explanation of the results is that a less efficient, non-mitochondrial respiratory pathway is contributing to the respiration of the intact tissue; and is relatively less active in the aged tissue. A similar explanation of the aging phenomenon, in which the second respiratory pathway was the pentose-P pathway, has been suggested on other grounds (16). No conclusions can be drawn here about the nature of any such alternative pathway.

Course of Phosphate Esterification. From the half-times of labeling of the various P-esters (table II), the course of phosphate esterification was deduced. The pattern shown was the same for the 2 tissues. It is probably the most detailed one yet obtained for higher plant tissue, and agrees with the broader outline given by Loughman (9, 10) and others. The most rapidly labeled compounds were the nucleotide triphosphates. Available evidence does not permit a decision as to which of the 3, ATP, GTP, and UTP, was the primary one labeled. The nucleotide diphosphates were slower to label, though the β-phosphate group still had a shorter half-time of labeling than that of the hexose-P phosphate group, and arguing for the presence in the tissue of an active nucleoside monophosphate kinase. The observed labeling of ADP, GDP and UDP in aged

tissue suggested that adenosine might be the first nucleoside labeled. Glucose-6-P and fructose-6-P (ratio 2.8/1) were the next to become labeled. UDP-glucose and glucose-1-P became labeled at approximately the same rate. The derivatives of hexose-P involved in respiration (fructose-1, 6-diP, triose phosphate, 3-P-glycerate, P-enolpyruvate and presumably 6-P-gluconate) had pool sizes that were considerably smaller than the parent hexose-P pool. Consequently their rate of labeling was largely determined by that of the hexose-P pool. It was therefore not possible to detect any significant differences in the rates of labeling of, for example, 6-P-gluconate and 3-P-glycerate which might otherwise have been expected to yield some information on the relative importance of the pentose phosphate and tricarboxylic acid paths in the 2 tissues. A study of the labeling rates and pool sizes of hexose-P, 6-P-gluconate and 3-P-glycerate indicates that in aged tissue the pentose phosphate path mediated between 5 % and 85 % of the hexose-P breakdown, while the tricarboxylic acid cycle mediated between 15 % and 95 % of hexose-P breakdown.

The compounds which contained phosphate groups not directly involved in the respiration path (AMP, UMP, P-choline, DPN etc.) were much slower to label. The amounts and rates of synthesis of mannose-6-P and α-glycerol-P were much higher than might perhaps have been expected. DPN also showed a surprisingly rapid rate of resynthesis. The curve of labeling with time plus an approximate measurement of the amount of DPN present indicates a turnover time of the order of 4 hours for the phosphate group(s) of DPN in aged tissue.

Concentration of Phosphate Esters in Potato Tissues. Many of the P-esters present in potato tissue were present in amounts too small to be measured directly. Some of them had reached steady-state labeling after 30 minutes, and if the assumption is made that these compounds have the same specific activity as the hexose-P and nucleotide phosphate from which they were derived, then a reasonable estimate of their concentration can be made. The following values were obtained (in mμmoles/g): glucose-6-P, 60; fructose-6-P, 22; glucose-1-P (some from hydrolysis of UDP-glucose), 18; mannose-6-P, 13; 2- + 3-P-glyceric acid, 12; 6-P-gluconate, 3; triose phosphate, 2.5; P-enolpyruvate, 1.5; fructose-1, 6-diP, 1.0; α-glycerol-P, 2.2. Other compounds of interest had not reached the steady state, or were not clearly separated on the chromatograms, so that only approximate levels can be estimated (in mμmoles/g tissue): CTP, ca. 0.8; CDP, ca. 1.4; DPN, >1.7; TPN, >1.6; P-choline, >9; P-ethanolamine, >2.5; inositol hexaP, >3.5.

The value for total hexose-P given in this paper is only about 2 % of that found for potato in one study (2); but agrees very well with a more recent finding (3).

Summary

P^{32}-labeled phosphate esters were extracted from fresh and aged potato tuber slices, and separated. There was more RNA synthesized in the aged tissue, but differences in the soluble phosphate esters were small. Malonate affected both the respiration rate and phosphate ester distribution in the aged tissue, and neither in the fresh tissue.

P^{32}-phosphate was fed in a 30-second pulse to the potato slices, and ester formation studied. The phosphate esters became labeled in the same order in both tissues, but at different rates. ATP, GTP and UTP were the most rapidly labeled. Data on the amounts of the esters, their rates of turnover and the rates of tissue respiration were used to estimate in vivo P/O ratios; ca. 0.25 for fresh tissue, and 0.6 or greater for aged tissue. Respiration in the aged tissue was more efficient as well as more rapid, supporting suggestions that the respiration pathway alters as the tissue ages.

The data also show that the tissue inorganic phosphate exists in at least 2 pools: a nonmetabolic pool containing 95 % and a metabolic pool containing 5 %. Phosphate accumulated into the tissue passes into the metabolic pool first, and from there into the nonmetabolic pool. The half-time of equilibration of the 2 pools is greater than 8 hours.

Various phosphate esters of interest were present in the tissue in amounts too small to be directly measured: their concentration has been estimated from radioactivity data.

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Preparation and Properties of Sweet Potato Mitochondria [1, 2]

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The object of this paper is to describe in detail the preparation, oxidative capacities, and response to various inhibitors of plant mitochondria that can be described biochemically as reasonably intact. The isolation of plant mitochondria which fulfill the suggested requirements for respiratory control (12) has been reported previously only from this laboratory (5) but the procedure was not given in detail.

The sweet potato, *Ipomea batatas*, is an object obtainable from local markets for some months during the year and one from which mitochondria are easily prepared. In spite of the recent and thorough studies of Hackett et al. (22) and of Baker and Lieberman (3), it was thought that a study of the preparation and oxidative capacities of tightly coupled sweet potato mitochondria was necessary. This paper describes the preparation and properties of such mitochondria. In addition, some observations on white potato, *Solanum tuberosum*, and on skunk cabbage, *Symplocarpus foetidus*, are included.

Methods

Preparation of mitochondria. Sweet potatoes from local markets were peeled and refrigerated before use; all operations were carried out between 0° and 4°. Sweet potato tissue (300 g) was grated into 600 ml of chilled medium, containing 0.25 M sucrose, 0.37 M mannitol, 4 mM cysteine, and 5 mM EDTA[4]. The mixture was blended at low speed (60 volts) for 20 seconds and then at full speed for 2 to 3 seconds in a Waring blendor. During the blending the pH of the suspension was monitored (narrow range indicator paper) and maintained between 7.2 and 7.5 by dropwise additions of 5.5 M KOH. The homogenate was squeezed through muslin and the filtrate was centrifuged at 1,500 × *g* for 15 minutes and the precipitate discarded. The supernatant suspension from this centrifugation was centrifuged at 10,000 × *g* for 15 minutes and the pellet was washed with a mixture of 0.25 M sucrose and 0.37 M mannitol

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[4] Abbreviations: BSA, bovine serum albumin; DNP, dinitrophenol; EDTA, ethylenediaminetetraacetate; HOQNO, 2-n-heptyl-4-hydroxyquinoline-N-oxide; P.A., mixture of 0.8 mM N,N.N′,N′-tetramethyl-p-phenylene diamine and 4 mM sodium ascorbate.