UPTAKE AND UTILIZATION OF PHOSPHATE ASSOCIATED WITH RESPIRATORY CHANGES IN POTATO TUBER SLICES[1]

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Respiration and salt absorption by slices of storage tissue have been extensively studied in recent years, with particular emphasis upon linkage between the two processes. In most investigations utilizing this type of material it is usual to bring the tissue into a state of steady metabolism before the start of the experiment. This is accomplished by subjecting freshly cut slices of tissue to exhaustive washing with water. The changes occurring during early stages of washing are, however, of great interest, but assessment of the factors governing this alteration of metabolism has rarely been attempted.

The specific respiration rate of freshly cut slices (1.0 mm thick) of potato may be five times that of the intact tuber and a further five-fold increase occurs in approximately 24 hours if the slices are kept in aerated water. Laties (11) has shown that the specific respiration rate of freshly cut slices is independent of thickness, whereas the development of the respiratory increment is controlled by the thickness. The respiration rate of neither fresh nor of aged slices is increased by increasing the oxygen content of the environment above that in air; Burton (1) has proven conclusively that tuber respiration is not limited by a shortage of oxygen within the tissue, since at normal storage temperatures the difference in oxygen concentration between the outside atmosphere and the center of the tuber is not great. The rise in respiration rate as a result of cutting must, therefore, be attributed to some cause other than the simple removal of a barrier to the diffusion of oxygen. Laties (11) has postulated that a volatile product of metabolism which imposes a restraint on respiration in the intact tuber diffuses out after cutting.

The demonstration by Hackett (7) that a marked change in the terminal oxidase characteristics occurs during the first 24 hours after cutting, is further evidence of the complexity of the processes involved in the development of increased respiratory power. Whereas 80 to 100 per cent of the respiration of fresh slices could be attributed to the cytochrome oxidase activity, the subsequent gain in respiration was found to be insensitive to carbon monoxide even though cytochrome oxidase activity could be demonstrated in mitochondria isolated from the tissue. The fact that an immediate rise in respiration rate of fresh potato slices can be brought about by adding 1×10^{-5} M 2-4-dinitrophenol (DNP) suggested that the restraint on respiration is associated with systems involved in transfer of high energy phosphate.

A further distinction between the basal and induced respiration is illustrated by the fact that the former is insensitive to malonate whereas the latter is completely inhibited in the presence of this compound (9). It was assumed that two different types of metabolism are involved which differ in their dependence upon the turnover of phosphate. It was considered that the limitation of respiration in fresh tissue might be the result of reduced activity of the phosphate transferring systems, and as a consequence the phosphate metabolism of the two different types of tissue was examined from a number of points of view.

MATERIALS

Potatoes obtained from local markets were washed and sliced into aerated ice water. All experiments were carried out with disks of tissue 9 mm in diameter and 1 mm thick. The varieties used were American Russet and English Majestic. After removing the adhering starch by three rinses in ice water, slices were stored in ice water for short periods prior to incubation in distilled water at 25° C or 30° C. The water was changed at 2 hour intervals for 12 hours, then at 8 hour intervals. The treatment was carried out in 1 liter Erlenmeyer flasks; the contents were agitated gently throughout in order to ensure adequate aeration. Respiration was measured by the standard Warburg method using ten 1.0 mm slices per vessel. The average fresh weight of ten slices was 0.65 g.

METHODS

I. PHOSPHATE UPTAKE: Slices were threaded on stainless steel wire and separated by 2.0 mm diameter beads. Treatment was carried out at concentrations of KH_2PO_4 ranging from 10^{-6} M to 10^{-2} M at a pH of 4.5, and concentrations of P^{32} ranging from 10 μc/l to 5 mc/l were used.

To distinguish active uptake from diffusion into the free space it was considered necessary to transfer slices to an equal volume of water at 0° C after the selected period of uptake to allow the free space phosphate to diffuse out again. The period in cold water was normally double the time allowed for uptake. On occasions the threaded slices were suspended in

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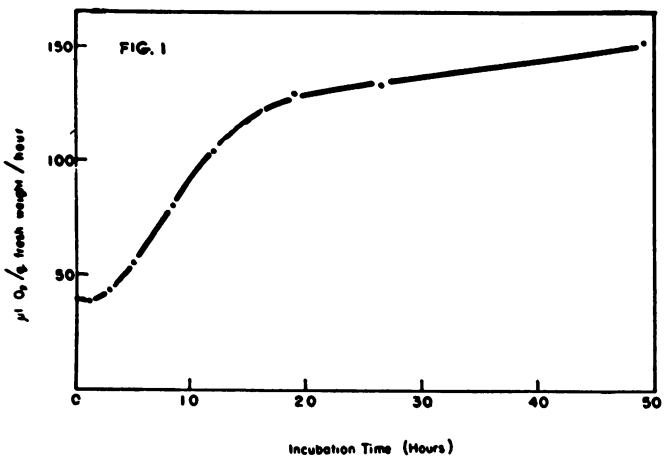
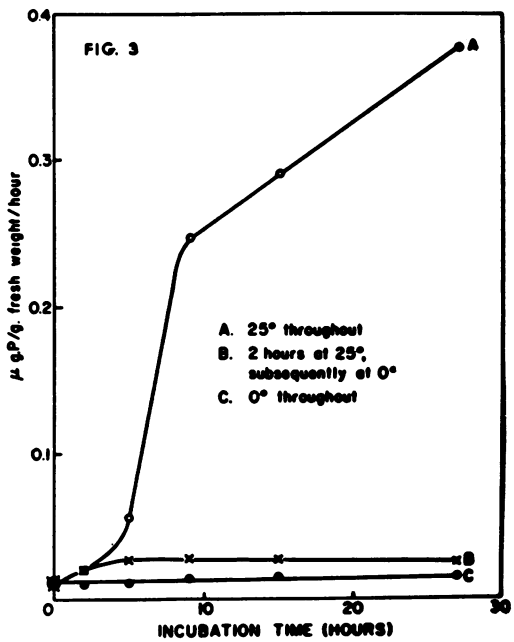
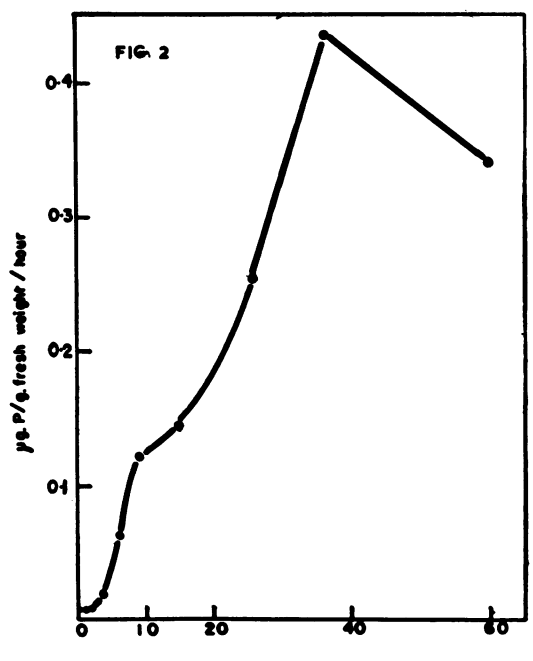
moist air, in order to prevent leakage of accumulated ions (3).

II. PHOSPHORYLATION: Tissue was extracted in the manner described in a previous paper (12). Samples of 0.65g fresh weight were normally extracted under ice cold conditions with 12 ml 0.1 *N* $HClO_4$. After short treatments with labelled phosphate, more than 90 per cent of the labelled compounds could be removed by two extractions. Extracting untreated slices by this procedure removed 60 per cent of the total phosphate, about half of this was inorganic. The initial chromatographic separations were carried out in *tert* butanol/water/picric acid (80ml/20ml/2.0 g) and further separations in propanol/ammonia/water (60ml/30ml/10ml).







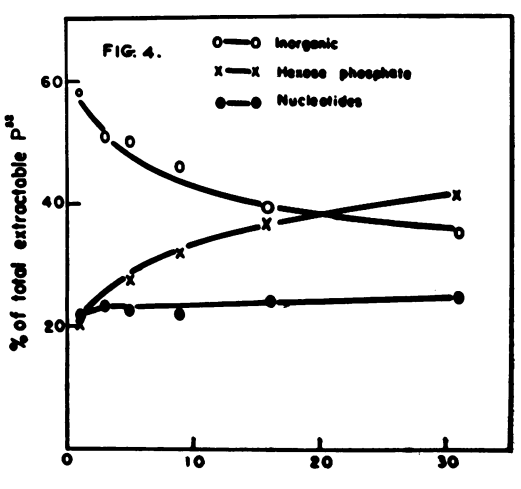


FIG. 1 (*Top, left*). Respiration of potato slices (1.0 mm thick) in aerated distilled water at 25° C.

FIG. 2 (*Bottom, left*). The course of phosphate uptake at pH 4.5 from 10^{-6} M KH_2PO_4 of 1.0 mm potato slices after incubation in aerated distilled water at 25° C.

FIG. 3 (*Top, right*). Phosphate uptake at pH 4.5 from 10^{-5} M KH_2PO_4 after incubation in aerated distilled water at different temperatures.

FIG. 4. (*Bottom, right*). The esterification of phosphate in 1.0 mm potato slices after 24 hours in aerated distilled water. Slices placed for 1 minute in labelled 10^{-5} M KH_2PO_4 prior to transfer to water at 25° C.

Experimental Results

The typical rise in respiration obtained by incubating 1.0 mm slices in water at 25° C is shown in figure 1, where the autocatalytic nature of the process can be clearly seen. The corresponding curve for phosphate uptake capacity from 1×10^{-6} M solution is shown in figure 2. After a 2 hour lag period a rapid rise occurs for 10 hours. The fluctuation in rate observed between 8 and 16 hours has been noticed on a number of occasions when low concentrations of phosphate are used, but no explanation can be given at this time. During the first 8 hours of incubation up to 10 per cent of the inorganic phosphate present in the tissue may be lost from the tissue; the accumulation studies with freshly cut slices are complicated by this fact. It is possible that this phosphate is lost from the cytoplasm rather than from the vacuole and could represent a large proportion of the cytoplasmic pool of inorganic phosphate.

An examination was made of the possibility that a short pre-treatment at 25° C would be sufficient to initiate changes such that the respiratory rise would continue subsequently at low temperature. Transfer to ice cold water effectively prevents any further rise in the capacity to take up phosphate at whatever stage this transfer is made (fig 3). The respiration values at 28 hours for treatments marked A, B, and C were 154, 45, and 34 μl/g fresh weight/hour, respectively. The development of both the capacities for phosphate uptake and for increased respiratory rate is dependent upon temperature, and slices can be maintained in a state equivalent to fresh tissue for several days if stored in water below 2° C.

The overall rise in the ability to absorb phosphate may be as great as 200-fold depending upon the initial low value which varies with different batches of tubers. The rise in uptake capacity increases rapidly for a longer period than the rise in respiration, suggesting that the two processes are not necessarily closely linked.

Esterification Pattern: After uptake from solutions of low concentration for periods of less than 15 minutes, up to 90 per cent of the absorbed phosphate can be extracted in cold dilute mineral acid. Investigation of the chemical nature of the absorbed phosphate indicated that rapid esterification occurred in aged slices. Figure 4 shows the pattern of esterification during 30 minutes in water after treatment for 1 minute in 1×10^{-5} M phosphate at 25° C. Maximal incorporation of phosphate into the nucleotide fraction occurs during the first minute; a slow rise occurs thereafter in the hexose phosphate fraction. An overall esterification of 75 per cent of P^{32}-labelled phosphate occurs during the 30 minute period. During the 1 minute treatment no significant increase in the total amount of phosphate in the tissue occurs; the curves shown, therefore, represent the increase in specific activity of the designated fractions. The rate of esterification observed with aged tissue is of the same order as that found in actively growing young cereal roots (13).

The capacity for esterification of incoming phosphate rises with the ageing process. A 10 minute treatment in labelled phosphate (1×10^{-5} M) at 25° C was given to batches of slices removed at intervals from the incubation medium. Figure 5 shows that during the 10 minute experimental period fresh slices esterify 20 per cent of the incoming phosphate whereas after 24 hours at 25° C 85 per cent is esterified in 10 minutes. Since only small amounts of phosphate are taken up by the fresh tissue, the measurement of the esterification capacity lacks precision and the value of 20 per cent is the upper limit. In order to reduce the difficulties of this type of measurement, a different technique was used.

Slices were cut; half of them incubated at 25° C, the other half at 0° C for 24 hours. Slices at 0° C possessed the same metabolic activity as fresh tissue, the advantage of the treatment being that slices from the same tuber may be used for uptake studies at the same time. In addition, washing at 0° C appears to reduce the variability which exists among fresh slices from the same tuber. The terms aged and fresh will be used to distinguish slices treated at 25° C and 0° C, respectively.

A comparison of the absorbing capacity of fresh and aged slices was carried out under conditions where comparable amounts of labelled phosphate entered the tissue and leakage to the environment from the fresh slices was prevented. After treatment in labelled solution at 0° C for 15 minutes the slices were rapidly washed in three changes of water to raise the temperature to 25° C and to remove surface activity. The slices were then suspended in moist air to allow accumulation from the free space to proceed; samples then were assayed at intervals to determine the degree of esterification. The increase in esterification proceeds much more rapidly in aged slices (table I). The low rate of esterification in fresh slices reflects the slow rise in capacity to absorb inorganic phosphate from the free space and confirms earlier results obtained by assay of slices taken directly from labelled solution.

The behavior of fresh slices was more critically examined by a further experimental modification. Fresh slices were immersed in labelled phosphate (1×10^{-5} M) for 10 minutes at 0° C in order to fill the free space under conditions where metabolic accumulation was reduced to a minimum. After rapid rinsing in water at 0° C to remove surface activity the slices were suspended in moist air for 20 minutes at 25° C to allow accumulation to proceed in the absence of any leakage from the free space to the environment. At the end of the accumulation period the free space component was removed by washing in water at 0° C. This phosphate was all inorganic and represented about 50 per cent of the total. At this point the slices were returned to moist air at 25° C to follow the utilization of the absorbed phosphate. During the first hour less than 5 per cent

TABLE I

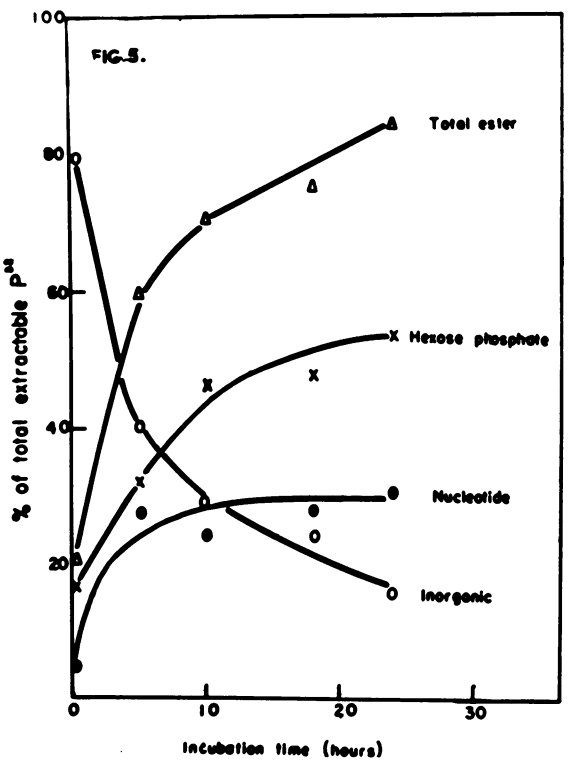
INCREASE IN ESTERIFICATION WITH TIME AT 25° C IN MOIST AIR AFTER FILLING FREE SPACE AT 0° C

	TIME AT 25° C MIN	EXTRACTABLE PHOSPHATE CPM/MG FRESH WT	% ESTERIFICATION
Fresh	2	143.0	12.6
	60	129.4	18.7
	180	157.4	30.1
	360	120.8	44.8
	660	121.0	58.8
	1,440	124.1	56.3
Aged	1	231.6	38.6
	2.5	252.0	48.1
	6	231.8	49.6
	12	200.3	56.7
	25	237.7	61.6
	60	221.9	55.0
	180	218.0	67.2

Slices placed for 15 minutes at 0° C in labelled solution (10^{-5} M KH_2PO_4, 4μc/ml, pH 5.0) prior to rinsing and transfer to moist air.

esterification occurred and this value doubled during the next hour. After 24 hours, incorporation into both nucleotide and hexose phosphate occurred with an overall esterification of approximately 50 per cent indicating that the capacity of fresh slices to utilize absorbed phosphate increases with time. The results indicate that fresh tissue has a low capacity for esterifying incoming phosphate and that a marked increase in this capacity occurs with ageing. The respiration of aged potato slices is thus characterized by a very rapid turnover of phosphorus.

After an uptake period of 10 minutes in 1×10^{-5} M KH_2PO_4 at 0° C followed by washing of aged slices for an equivalent time in water at the same temperature to remove the diffusible component, about half the phosphate remains in the tissue. About 40 per cent of this residue is removed if the tissue is washed in 1×10^{-2} M KH_2PO_4 at 0° C and all this removable ion is inorganic orthophosphate. Thus, true accumulation of a small amount of phosphate occurs; this fraction also is inorganic. On raising the temperature to 25° C very rapid esterification occurs in the aged tissue. The process of accumulation appears to be separable from that of esterification. The inference is that phosphorylation is not essential



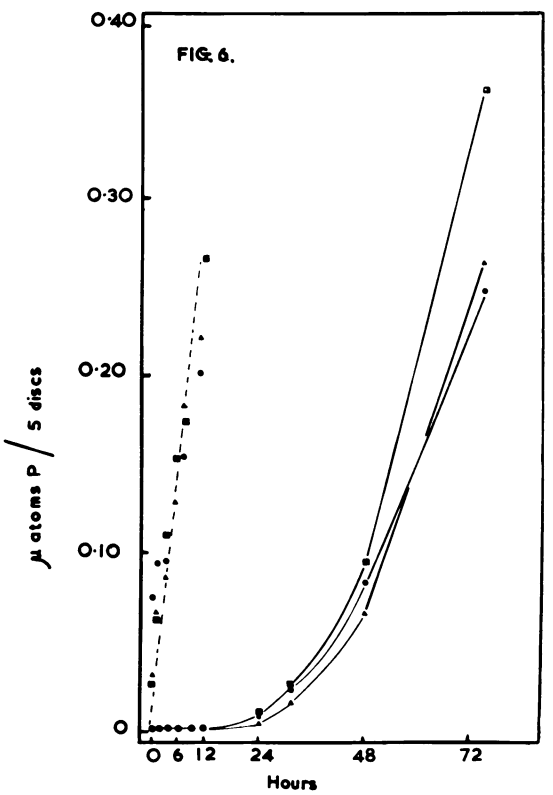


FIG. 5 (*Left*). The esterification capacity of 1.0 mm potato slices during incubation in aerated distilled water at 25° C. Slices treated for 10 minutes in labelled 10^{-5} M KH_2PO_4 prior to extraction with 0.1 M $HClO_4$.

FIG. 6 (*Right*). Phosphate uptake during incubation of 1.0 mm potato slices in 10^{-2} M (broken line) and 10^{-5} M (solid lines) phosphate solution at 16° C and pH 4.5. KH_2PO_4 (■ — ■), NaH_2PO_4 (▲ — ▲) and LiH_2PO_4 (● — ●).

to accumulation: it is assumed that phosphorylation occurs after accumulation. Only by working at 0° C can the rate of esterification be reduced sufficiently to distinguish between the two processes. Mechanisms of uptake can be suggested which involve the esterification at the cell surface of the incoming phosphate as an integral part of the absorption process, but it is more probable that the ion arrives in the cytoplasm in inorganic form, there to be incorporated into the metabolic systems of the tissue.

An interesting point is that whereas aged tissue at 0° C accumulates about four times as much P^{32} as fresh tissue from 1×10^{-5} M solution, yet, as has been shown earlier, the difference at 25° C may be up to 200-fold.

Assay of Cytoplasmic Pool Components: Measurements of the concentrations of the phosphorylated components turning over in the cytoplasmic pool are difficult, but preliminary assays have indicated that no large differences occur between fresh and aged tissue. There is evidence as shown above that uptake into the cytoplasm occurs prior to esterification and information on the size of the cytoplasmic pool of inorganic phosphate is highly desirable. Such estimates depend upon separation of vacuolar and cytoplasmic orthophosphate; the present experimental approach gives an approximate estimate of this distribution.

Apparent equilibration of the incoming P^{32} with the nucleotide fraction occurs in a few minutes in aged tissue (fig 4). At this point the specific activity of the labile phosphate of isolated ATP can be determined as can the specific activity of the extracted inorganic phosphate. Two assumptions are now made: I. That all the ATP is in the cytoplasmic pool; none in the vacuole. II. That none of the incoming phosphate has reached the vacuole at this time.

Since the isotopic composition of cytoplasmic nucleotides achieves a steady value almost immediately, the specific activity (SA) of the terminal phosphates of ATP will be the same as that of the inorganic fraction. On this presumption the amount of P^{31} required to satisfy this relationship with regard to the inorganic fraction can be calculated. The inorganic phosphate at issue is located in the cytoplasm. The difference between this value and the total extractable inorganic phosphate represents the vacuolar content. The second assumption is less probable, but even if some of the P^{32} had already entered the vacuole this type of measurement should give an estimate of the maximal concentration of inorganic phosphate in the cytoplasm. Apparent isotopic equilibrium within the cytoplasmic pool can occur in less than 10 minutes under conditions where the phosphate entering the tissue makes no significant addition to the total concentration of phosphate within the tissue.

A value of 30 μg ATP per gram fresh weight is the upper limit obtained by spectrophotometric assay. In an experiment where apparent equilibrium was reached in 10 minutes, the nucleotide and inorganic fractions represented 29.1 per cent and 43.3 per cent of the extractable P^{32}, respectively. The corresponding concentrations of nucleotide phosphate and inorganic phosphate were approximately 3.0 and 42.2μg per gram fresh weight.

If it is assumed that the two terminal phosphate groups of ATP are equally labelled, whereas the α position is unlabelled, then the value for the concentration of nucleotide phosphate involved in the SA measurements is reduced to 2.0μg/g fresh weight, i.e. $SA = \frac{29.1}{2.0} = 14.6$. If the SA of these phosphate groups is the same as that of the inorganic phosphate, then the concentration of P^{31} necessary to satisfy this requirement is $\frac{43.3}{14.6} = 3.0$. Consequently, this value of 3.0μg represents the inorganic phosphate contained in the cytoplasmic pool. Since the total inorganic phosphate of the tissue is 42.0μg/g fresh weight, then it appears that more than 90 per cent of this phosphate is contained in the vacuole. If, as has been shown earlier, 10 per cent of the tissue phosphate can be lost during the early stages of washing, this amount possibly represents the greater portion of the cytoplasmic inorganic component. Refinement of the techniques used here is necessary before accurate determination of the distribution of phosphate between vacuole and cytoplasm can be obtained. The presence of uridine phosphates as contaminants of the isolated ATP has been noted and accurate assay of these is important before precise values can be obtained. The assumption is tentative that no P^{32} enters the vacuole during the equilibration period, but calculation of cytoplasmic phosphate by this method should give the maximal value.

That the increase in respiration brought about in ageing tissue is associated with increased enzyme synthesis is a possibility. Concentrations of chloramphenicol shown by Gale and Folkes (6) to inhibit protein synthesis in *E. coli* have no effect on the respiration rise of potato slices. After ageing slices for 24 hours in 0.01 per cent chloramphenicol the respiration rate was identical with that of slices aged for the same period in water. The activities of starch phosphorylase, phosphoglucomutase, phosphohexoisomerase, amylase, apyrase, and phosphatase were measured in fresh and aged slices. These enzymes are involved in the mobilization of the major reserve substrate in the tissue, but no marked change in activity during the ageing process could be detected. This suggests that the glycolytic system at least undergoes no extensive alteration during the ageing process.

The relationship between rate of accumulation of phosphate and concentration was widely different for fresh and aged slices. Table II shows the comparative rates of uptake at 1×10^{-5} M and 1×10^{-2} M KH_2PO_4. After a 10 minute absorption period the slices were washed for an equivalent time in water or 1×10^{-2} M KH_2PO_4.

TABLE II

RATE OF PHOSPHATE UPTAKE BY FRESH AND AGED 1.0 MM SLICES

	UPTAKE RATE (μG/HR/G FRESH WT)					
	FRESH			AGED		
	10^{-5} M	10^{-2} M	$\frac{10^{-2}\text{ M}}{10^{-5}\text{ M}}$	10^{-5} M	10^{-2} M	$\frac{10^{-2}\text{ M}}{10^{-5}\text{ M}}$
Washed in H_2O	0.0172	8.52	495	0.728	23.06	31.7
Washed in 10^{-2} M KH_2PO_4	0.0102	7.65	750	0.843	22.18	24.8

Slices treated for 10 minutes at 25° C in labelled 10^{-5} M or 10^{-2} M KH_2PO_4 at pH 4.5 followed by 10 minutes in water or 10^{-2} M KH_2PO_4 at 0° C.

Whereas aged slices at 10^{-5} M accumulated 40 to 80 times as much phosphate as fresh material the corresponding ratio at 10^{-2} M was 2.7 to 2.9. The manner in which this ratio alters with concentration is shown in table III.

The respiration of the fresh tissue remains unaltered over this range of KH_2PO_4 concentration; similar uptake rates are obtained if the potassium is replaced by sodium or lithium though the latter ion at a concentration of 10^{-2} M causes the uptake of phosphate to cease completely after 24 hours. Figure 6 shows the uptake of phosphate during the ageing of slices in 10^{-2} M and 10^{-5} M phosphate solution in the presence of K^+, Na^+, and Li^+ as the accompanying cations. High concentrations of lithium have been shown by Laties (10) to prevent the normal respiration rise after cutting.

Fresh slices absorb phosphate readily from solutions of high concentration. They also exhibit an ability for esterification equal to aged slices. This observation is in agreement with the results of Calo, Marks, and Varner (2), who consider the phosphorylative capacity of fresh and aged slices to be identical in 10^{-2} M solution. That the metabolism of fresh tissue is not limited by phosphate supply is indicated by the fact that the respiratory rate is not increased when slices are immersed in 10^{-2} M KH_2PO_4.

TABLE III

EFFECT OF PHOSPHATE CONCENTRATION ON UPTAKE CAPACITY OF FRESH AND AGED 1.0 MM SLICES

	UPTAKE RATE (μG/HR/G FRESH WT)		
M KH_2PO_4	FRESH	AGED	RATIO $\frac{\text{AGED}}{\text{FRESH}}$
10^{-6}	0.0024	0.216	89.6
10^{-5}	0.012	1.30	107.5
10^{-4}	0.112	2.48	22.35
10^{-3}	1.32	5.92	4.53
10^{-2}	13.68	27.80	2.02

Slices placed for 15 minutes at 30° C in labelled solution (pH 5.0) followed by 30 minutes in water at 0° C.

Whereas fresh slices esterify only about five per cent of the phosphate absorbed in 10 minutes from 10^{-5} M solution, up to 70 per cent may be esterified at 10^{-2} M. It is clear that the rapid accumulation of phosphate by fresh slices at high concentration is accompanied by a high rate of esterification, but it has not been established whether the processes are readily separable or not.

The data of Hagen and Hopkins (8) suggest that two sites are involved in the accumulation of phosphate, one for the HPO_4^{--} ion and the other for the $H_2PO_4^-$ ion. Extension of their approach to cation uptake by Fried and Noggle (4) led them to conclude that the accumulation of monovalent cations proceeds at two sites, one of which operates at high concentrations and the other at low. The results presented here suggest that in fresh tissue the site operating at low concentrations is non-functional whereas the other is operating normally. Thus, ageing of the tissue is intimately associated with development of the low concentration site.

DISCUSSION

The results obtained at low phosphate concentrations indicate that the phosphorolytic capacity of aged slices is very high, whereas that of freshly cut slices is almost negligible. Under conditions of low phosphate supply, therefore, it is reasonable to assume that the respiration developed during ageing at 25° C is in fact characterized by a rapid turnover of phosphate, whereas the basal respiration is characterized primarily by non-phosphorolysis. The fact that active uptake occurs in fresh tissue in the presence of high concentrations of phosphate can be explained by assuming that two different sites of uptake are involved, differing markedly in the concentrations at which they become saturated. Kinetic treatment of the results in the manner described by Hagen and Hopkins (8) gives confirmation of this view, although detailed investigation of the characteristics of the sites is premature at this point. The results are consistent with the view that the gain in respiration associated with ageing is linked with the creation of a site of uptake operating solely at low concentration.

Having shown that $H_2PO_4^-$ and HPO_4^{--} ions were absorbed at different sites in barley roots, Hagen and Hopkins (8) calculated the dissociation constants of the two ion-carrier complexes and found them to be 4×10^{-5} M and 5×10^{-10} M, respectively. A similar pattern for uptake sites operating at high and low concentrations of single monovalent cations has been reported by Fried and Noggle (4). It seems that this phenomenon may be of general occurrence.

One of the possibilities arising from studies with aged slices at 0° C is that phosphate is actually accumulated by the cytoplasm in inorganic form and that the process involving rapid esterification is a completely separate stage. The rapidity with which the incoming phosphate comes into apparent equilibrium with the nucleotide fraction, and the high proportion of phosphate esterified suggests that, compared to the cell as a whole, the inorganic phosphate content of the cytoplasm is low. The basis for measuring this distribution has already been discussed. It appears that refinement of the techniques used could furnish reliable values. Where the synthesis of nuclear components is studied by means of externally applied radioactive inorganic phosphate as in nucleic acid synthesis, knowledge of the phosphate concentration in the cytoplasmic environment would appear to be a valuable aid to the interpretation of the results obtained (14).

The observation (6) that the capacity of aged slices to absorb phosphate, is inhibited by carbon monoxide in the dark while the respiration remains unaffected and that the inhibition is light reversible, suggests strongly that the portion of respiration associated with phosphate uptake is mediated by cytochrome oxidase, whereas the overall respiration is not. The study of the relationship between the induced respiration and the new terminal oxidase system and the identification of the latter appears to be of first importance in elucidating the problems involved in the control of storage tissue metabolism.

Summary

I. The increase in respiration observed when freshly cut potato slices are incubated in aerated distilled water for 24 hours at 25° C is associated with a change in the ability to utilize phosphate from solutions of low concentration.

II. The capacity for uptake rises by more than 100-fold, and a 20-fold increase in the ability to esterify the accumulated phosphate can occur during the same period.

III. Fresh and aged slices show little difference in their capacity to accumulate and esterify phosphate from solutions of high concentration.

IV. The increase in respiration during ageing appears to be associated with the synthesis of a cellular component concerned in the accumulation of phosphate at low concentration.

V. Accumulation and esterification seem to be separate processes. Incorporation of phosphate into organic forms is not necessarily essential for uptake into the cell.

VI. Respiration of fresh tissue is possibly non-phosphorolytic in nature, whereas that of aged tissue is characterized by a rapid turnover of phosphate.

VII. The greater part of the inorganic phosphate in the cell appears to be stored in the vacuole and takes no part in the steady state metabolism of the cell.

Acknowledgments

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