SOME SALT-UPTAKE AND TISSUE-AGING PHENOMENA STUDIED WITH CULTURED TOBACCO CELLS

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Summary

Tobacco cells (*Nicotiana tabacum* cv. Wisconsin 38) growing in cell suspension culture follow an exponential growth curve. Cells removed from the culture at various times and subjected to "step-up" or "step-down" nutritional shifts exhibit an aging response. The most marked response is observed as an increase or decrease in the rate of phosphate accumulation and in the rate of phosphate esterification. This response is maximal for mid exponential-phase and late stationary-phase cells. The implications of these findings are discussed.

I. INTRODUCTION

Slices of plant tissue have been widely used in metabolic studies and in studies on salt accumulation. If, after cutting, the tissue slices are washed or aged,‡ the subsequent rate of salt accumulation is frequently more rapid and reproducible than in freshly sliced tissue. The respiration rate also often increases, and the inhibitor sensitivity changes (Thimann, Yocum, and Hackett 1954). General metabolic activity rises: RNA, protein, and specific enzymes are synthesized (Click and Hackett 1963; Bacon, MacDonald, and Knight 1965); and phosphate ester turnover increases (Bieleski and Laties 1963). The act of slicing the tissue is clearly responsible for initiating the aging process, since aging is more rapid and more marked the thinner the tissue slice. However, more than simple wound effects are involved, since thin slices reassembled into a thick slice age like a normal thick slice (Laties 1962). Apparently the cut surfaces have to remain exposed to exert their effect. Similar slice thickness effects have been observed in the growth of tissue explants: thin artichoke and carrot explants grew much more rapidly in tissue culture than thick ones (Caplin 1963). The similarity is probably not a trivial one. The changes in metabolic behaviour that characterize aging are the same changes which would be expected to accompany a resumption of cell division activity.

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‡ There is no completely suitable term referring to metabolic changes which take place when plant tissue slices are washed. In this paper "fresh" tissue slices or cells are those obtained immediately following excision from the parent tissue or removal from culture medium. "Aged" cells or tissue slices are those which have been aerated for varying times in 10^{-4}M $CaSO_4$. Transfer of cultured cells to 10^{-4}M $CaSO_4$ is defined here as constituting a "step-down culture". The terms "aging" and "step-down culture" are used interchangeably. Transfer of cells to new complete culture medium is termed a "step-up" culture. Some change in terminology appears desirable.

The ways in which slicing could stimulate such changes can be considered. The aging phenomenon could be a temporary wound response caused by death of some cells and damage of others. Slicing could allow a volatile inhibitor to escape from the surface of the tissue (Laties 1962). One major change is that slicing exposes many cells to a new environment—to an aqueous phase with a different set of metabolite concentrations from the intra-tissue levels. The response to slicing may therefore be a response to this change in environment; that is, to a nutritional shift. The effects of such changes have been studied in bacterial systems, and it has been shown that both "step-down" cultures (introducing cells to depleted culture medium) and "step-up" cultures (introducing cells to enriched culture medium) induce major changes in bacterial cell metabolism (Maaløe and Kurland 1963).

Experimental difficulties lie in the way of performing similar experiments with plant tissue slices: the slices suffer damage during their preparation, are sometimes unwieldy to handle, and are difficult to obtain in different stages of growth. However, tobacco pith cells can now be grown in cell suspension culture on a routine basis (Nickell 1956). Such cells follow a reproducible growth curve, and can be transferred as suspensions with wide-bore pipettes. The cells can be harvested at all stages of growth and washed rapidly on filter sticks. Preparation of such experimental material avoids the damage to cells inherent in the preparation of tissue slices. We have therefore used tobacco pith cells for a study of the response of plant cells to changes in their nutritional environment. Though the metabolic rate of these cultured cells differs from that of freshly excised tobacco pith tissue, the concepts developed utilizing such cultured cells may well be applicable to normal plant tissues.

II. Materials and Methods

(*a*) *Growth of Tobacco Cells*

Tobacco (*Nicotiana tabacum* cv. Wisconsin 38) pith cells removed from friable pith cultures on solid medium were established in cell-suspension culture. The basal medium of Murashige and Skoog (1962) plus 0·5 mg/l of 2,4-dichlorophenoxyacetic acid plus 0·5 g/l of malt extract (Flamm, Birnstiel, and Filner 1963) was used. For routine subcultures, 300 ml fresh sterile culture medium was inoculated with 10 ml of a cell suspension from a stationary-phase culture (see later) to give a cell density of about 8 mg/ml medium ($1{\cdot}1 \times 10^4$ cells/ml). The cells were harvested at various stages of growth either by centrifuging (50 *g*, 3 min), suspending in distilled water, and recentrifuging; or, preferably, by filtration on Miracloth (Chicopee Mills Inc., New Jersey) in a Buchner filter under low vacuum, followed by washing in a stream of distilled water for 1 min. Such treatment removed more than 99·5% of the freely diffusible solutes from the apparent free space.

(*b*) *Measurement of Growth Rate*

The cell density of a culture was measured by light absorption, with an EEL colorimeter with a green filter. A calibration curve was first prepared by making serial dilutions of stationary-phase cultures: the cell number of the calibrating suspension was obtained by microscope counts following treatment of the suspension

to separate individual cells from aggregates (Letham 1962). The growth rate of a culture was determined by measuring the change in light transmission of the culture, using special culture flasks. Colorimeter tubes (optically matched, 15 by 1·5 cm Pyrex test tubes) were welded onto 1-litre conical flasks, 18° down from horizontal, 10 cm from the base of each flask. Such flasks could be swilled, then tipped to allow a sample of the culture to flow into the tube for measurement of light transmission in the colorimeter, without wetting the cotton-wool bung, and while maintaining sterility of the culture.

(*c*) *Production of Nutritional Shift Responses*

A step-down nutritional shift, comparable with that employed in aging studies of potato slices (Bieleski and Laties 1963), was obtained by resuspending the washed cells in 10^{-4}M $CaSO_4$ as a 10% suspension. The suspension was aerated at 30°C by bubbling air via a capillary tube from an aquarium pump; or was aerated at 27°C by shaking on a New Brunswick rotary shaker. The two methods of aeration gave comparable results. After various periods of such treatment, samples were taken; and their ability to accumulate phosphate was determined.

In some experiments, the ability of the cells to accumulate sulphate, following aging, was studied. In such cases, the nutritional shift was obtained by transferring cells to 10^{-4}M $CaCl_2$, instead of 10^{-4}M $CaSO_4$.

In some experiments, step-up or other nutritional shifts were employed. In such cases, the nutritional shift treatment was performed in 2×10^{-4}M KH_2PO_4, in fresh culture medium, or in a filtered, used culture medium obtained from replicate cell suspension cultures which had been harvested at the same time.

(*d*) *Measurement of Phosphate and Sulphate Accumulation Rates*

After a period of nutritional shift treatment, two 5-ml samples of the cell suspension fluid were removed, filtered on a filter stick, and the cells washed for 30 sec in running distilled water. Each sample was then transferred to 25 ml 10^{-5}M $KH_2{}^{32}PO_4$ (for measuring phosphate accumulation rate) or to 10^{-5}M $K_2{}^{35}SO_4$ (for measuring sulphate accumulation rate), in a conical flask on a shaking table. After 30 min and 1 hr the samples were transferred to a filter stick and washed for 1 min in distilled water. Each sample was then weighed. Samples containing phosphate-^{32}P were placed directly on planchets; polyvinyl acetate was added as an adhesive, the samples were dried and their radioactivity was measured directly. Samples containing sulphate-^{35}S were homogenized in 10 ml 2% formic acid, centrifuged (3000 *g*, 5 min); then aliquots of the supernatant were plated on lens-tissue disks on planchets, dried, and their radioactivity was measured.

(*e*) *Respiration Measurements*

Respiration measurements on both tissue slices and cell suspensions were carried out at 30°C in 0·01M and 0·1M phosphate buffer, pH 5·2, by conventional Warburg manometry.

(*f*) *Phosphate Esterification*

The extent of phosphate esterification during phosphate accumulation was studied as follows. A sample of 20 g of stationary-phase cells, harvested 5 days after the end of the exponential phase, was divided into two 10-g portions. One portion (fresh sample) was immediately placed in 1·2 litres and 400 μc of 10^{-5}M $KH_2{}^{32}PO_4$, incubated for 1 hr, then killed (Bieleski 1964). The other portion (aged or step-down sample) was aerated for 6 hr in 10^{-4}M $CaSO_4$, before being similarly labelled for 1 hr with $KH_2{}^{32}PO_4$ and killed. Phosphate esters were extracted and separated and their radioactivity was measured (Bieleski and Young 1963).

(*g*) *Effect of Actinomycin D on Aging and on Ribonucleic Acid Synthesis*

Actinomycin D (a gift from Merck, Sharpe & Dohme, N. J.) was added to cells following their removal from culture medium and resuspension in 10^{-4}M $CaSO_4$. In one experiment cells were pretreated with actinomycin prior to the step-down culture shift by adding the antibiotic sterilely to undisturbed cell cultures.

^{32}P-nucleic acids were isolated from tobacco cells (pretreated with actinomycin D and ^{32}P-orthophosphate) using methods described previously (Ralph and Bellamy 1964), except that 6% sodium 4-aminosalicylate was added to the extraction mixture. This ensured the extraction of DNA and most of the rapidly labelled RNA. The resulting purified nucleic acids were dried over P_2O_5 and stored under vacuum at −12°C till required. Weighed amounts (approx. 0·5 mg) were dissolved in 0·2 ml buffer (0·025M Tris hydrochloride+0·05M NaCl) and analysed by density-gradient sedimentation (35,000 r.p.m., Spinco 39SW rotor, for 6 hr) through 5 to 20% sucrose gradients in standard buffer. Fractions were collected by piercing the bottom of the tube with a fine hypodermic needle. The absorbancy of each sample at 260 mμ was measured; bovine serum albumin (1 mg) was added as co-precipitant and the nucleic acids were precipitated with 5% trichloroacetic acid. The precipitate was collected on a cellulose acetate membrane filter, washed with ice-cold 5% trichloroacetic acid, and its radioactivity was measured.

III. Results

(*a*) *Growth Rate of Tobacco Cells in Cell-suspension Culture*

Growth rates were highly reproducible, and the cell population throughout consisted of free cells and small clumps of cells. The growth pattern was similar to that observed by other workers (Filner, personal communication). After a period of about 1 day following inoculation (lag phase), there was a period of 6 days (exponential phase) when there was an almost exponential increase in cell density, the doubling time being 2–2·3 days. After this period, cell growth continued, progressively more slowly, through early, mid, and late stationary phases to reach a cell density of 200 mg/ml ($2·8 \times 10^5$ cells/ml) after 3 weeks' growth (Fig. 1). Cultures older than this became brown and glutinous, indicating that many of the cells were senescent.

Daily growth increments were used to calculate the progress of growth rate with time. Figure 2 reveals the various phases of growth more clearly. The pattern of change in respiration rate of the cells during growth was closely related to changes

in the growth rate. The end of the exponential phase and the start of the early stationary phase was accompanied by a marked fall in the concentration of phosphate in the culture medium.

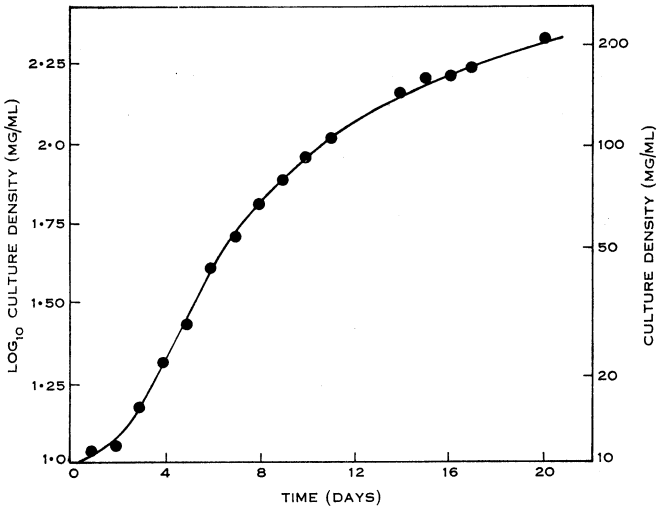

Fig. 1.—Logarithmic plot of the growth curve of tobacco pith cells growing in cell suspension culture. Fresh culture medium (300 ml) was inoculated with 10 ml of a cell suspension from a stationary-phase culture.

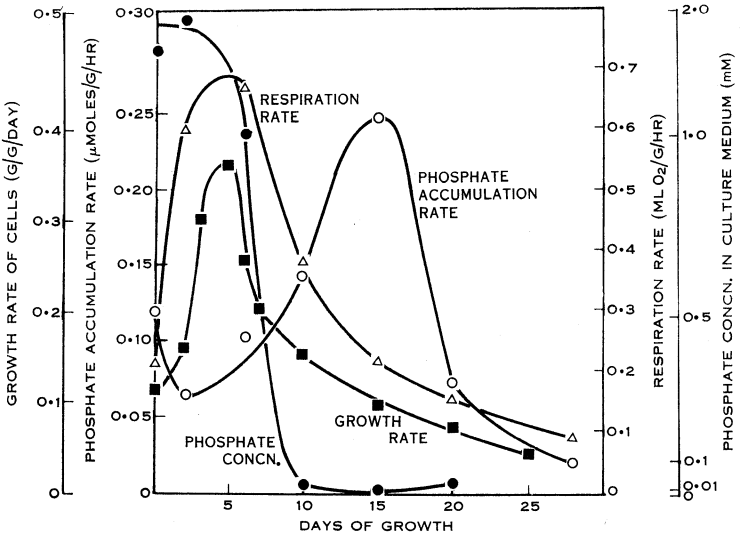

Fig. 2.—Effect of culture age on growth rate, phosphate accumulation rate in 10^{-5}M $KH_2{}^{32}PO_4$, respiration rate, and phosphate concentration in the culture medium.

(*b*) *Aging and Phosphate Accumulation Rate*

The rate of accumulation of phosphate from 10^{-5}M KH_2PO_4 by fresh cells immediately following their removal from culture medium varied with the stage of

growth of the culture. In 10^{-4}M $CaSO_4$, the phosphate accumulation rate rose markedly (Table 1) usually reaching a maximum level within 4 hr. The resulting maximum accumulation rates were highest at the early to mid stationary phase of growth (Fig. 2).

TABLE 1

EFFECT OF A STEP-DOWN NUTRITIONAL SHIFT, AT VARIOUS STAGES OF GROWTH, ON THE PHOSPHATE ACCUMULATION RATE OF CULTURED TOBACCO PITH CELLS

Stage of Growth	Phosphate Accumulation Rate from 10^{-5}M KH_2PO_4 (mμmoles/g/hr)		
	Basal Rate (x)	Aged Rate (y)	Percentage Increase $[100(y-x)/x]$
Lag phase	31·3	63·2	100
Mid exponential phase	17·3	101·3	485
Late exponential phase	52·9	143·0	170
Early stationary phase	135·0	248·0	84
Late stationary phase	18·0	72·0	300

The relationship of phosphate accumulation rate to concentration of supplied phosphate in aged and fresh exponential-phase cells was studied (Fig. 3). An effect of

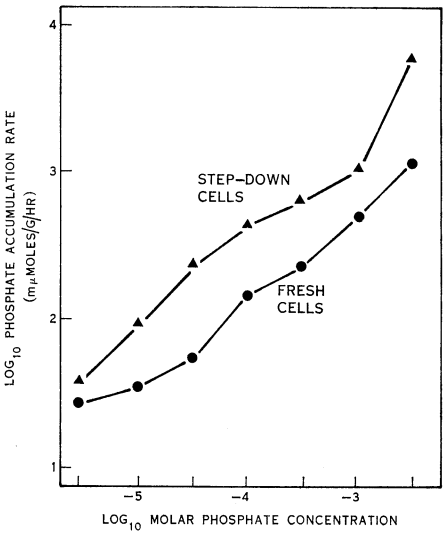

Fig. 3.—Effect of a step-down culture shift on the phosphate accumulation rates of cells in varying concentrations of KH_2PO_4.

the culture shift on the accumulation rate was observed with all concentrations of phosphate studied.

(c) *Aging and Sulphate Accumulation Rate*

Though no systematic study was made, an aging change was observed in the sulphate accumulation rate of early stationary-phase cells. The sulphate accumulation rate of these cells in 10^{-5}M $Ca^{35}SO_4$ was first depressed, but then rose rapidly to a high level. The rise in sulphate accumulation occurred at a time distinct from the rise in

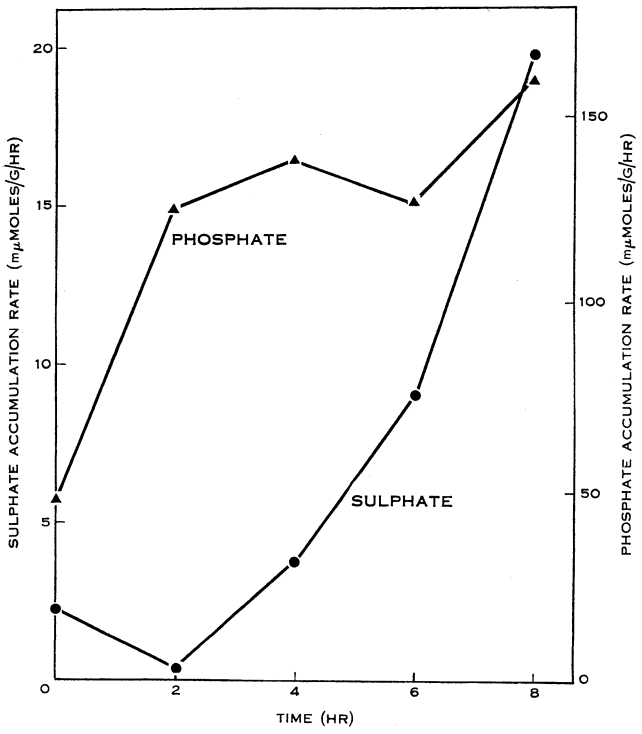

Fig. 4.—Effect of a step-down culture on the phosphate accumulation rate in 10^{-5}M $KH_2{}^{32}PO_4$ and sulphate accumulation rate in 10^{-5}M $Ca^{35}SO_4$ of early stationary-phase cells.

phosphate accumulation (Fig. 4). Thus the culture shift brought about a stimulation at different times in the ability of the cells to accumulate phosphate and sulphate, rather than an overall increase in the accumulatory ability of the cells.

(d) *Respiratory Changes*

Basal respiration rates varied during the period of growth (Fig. 2). The response of the respiration rate to aging, to 2×10^{-2}M or 5×10^{-2}M malonate, or to 5×10^{-5}M dinitrophenol was slight at most stages of growth (Table 2). However, mid to late stationary-phase cells (stationary-phase inoculum) were markedly stimulated by dinitrophenol; early stationary-phase cells were inhibited or damaged. The cells were almost entirely malonate-resistant and were therefore quite unlike potato tissue slices in their respiratory response to aging.

In order to ascertain whether these respiratory characteristics were typical of the parent tissue, similar respiratory studies were carried out on fresh and aged (18 hr in 10^{-4}M $CaSO_4$ at 27°C) slices of tobacco pith taken from whole plants (Table 3).

TABLE 2

RESPIRATORY CHARACTERISTICS OF FRESH AND AGED TOBACCO PITH CELLS AT VARYING TIMES FOLLOWING INOCULATION

Days of Growth of Culture	Respiration Rate of Tobacco Cells (μl O_2/g/hr)			
	Control	Dinitrophenol (5×10^{-5}M)	Malonate (2×10^{-2}M)	Malonate (5×10^{-2}M)
		Fresh Cells		
0*	226	519	203	
2	666	607	689	
6	660	700		450
10	379	322		326
15	225	227		191
20	160	225		170
		Aged Cells		
0*	200	566	254	
2	356	373	369	
6	520	380		530
10	417	297		363
15	202	208		196
20	149	157		157

* Stationary-phase inoculum.

Respiration rates of cells in pith slices were much lower than those encountered in the cultured cells. Aging caused a six-fold rise in respiration rate; both fresh and aged tissues were malonate-insensitive; fresh tissues were inhibited by dinitrophenol

TABLE 3

RESPIRATORY CHARACTERISTICS OF FRESH AND AGED TOBACCO PITH SLICES

Tissue	Respiration Rates (μl O_2/g/hr)		
	Control	Malonate (5×10^{-2}M)	Dinitrophenol (5×10^{-5}M)
Fresh	0·6	0·8	0·4
Aged	3·6	3·9	5·6

while aged tissues were stimulated slightly. Thus the high respiration rates of cultured tobacco cells appear to be a function of their mode of growth. The inhibitor characteristics of the cultured cells, however, are probably typical of tobacco pith

cells in general, and not a result of their mode of growth. In respiration rate and inhibitor sensitivity, pith slices behave more like stationary-phase cells than those of any other growth stage.

(*e*) *Changes in Phosphate Ester Levels*

The phosphate ester pattern of step-down cells differed markedly from that of fresh cells. The proportion of the total radioactivity present as inorganic phosphate, glucose 6-phosphate, hexose diphosphates, 3-phosphoglycerate, and α-glycerophosphate was lower in step-down cells than in fresh cells. Relative radioactivity in glucose 1-phosphate, phosphoenol pyruvate, triose phosphate, uridine diphosphoglucose, and nucleoside mono-, di-, and triphosphates were markedly higher in step-down cells than in fresh cells (Table 4).

TABLE 4

EFFECT OF A STEP-DOWN CULTURE SHIFT ON THE DISTRIBUTION OF ^{32}P RADIOACTIVITY IN PHOSPHATE ESTERS EXTRACTED FROM MID STATIONARY-PHASE TOBACCO PITH CELLS

Cells were treated for 1 hr with ^{32}P-orthophosphate (0·83 μc/ml) prior to extraction of phosphate esters

Compound	Percentage of Total Radioactivity in Extract	
	Fresh Cells	Step-down Cells
Inorganic phosphate	27·8	11·5
Glucose 6-phosphate	25·2	5·6
Glucose 1-phosphate	2·0	5·3
Fructose 6-phosphate	6·0	7·6
Mannose 6-phosphate	6·5	7·5
Hexose diphosphates plus guanosine diphosphate	7·6	1·3
3-Phosphoglycerate	1·9	0·8
Phosphoenolpyruvate	0·7	2·9
Triose phosphates	0·5	2·2
α-Glycerol phosphate	1·2	0·5
Uridine diphosphoglucose	1·7	6·7
Nicotinamide adenine dinucleotide	0·3	0·2
Nucleoside mono-, di-, and triphosphates excluding guanosine diphosphate	18·6	47·9

(*f*) *Comparison of Step-up and Step-down Effects*

A time of maximum response of phosphate accumulation rate to step-down culture (aging) was observed to be at the late stationary phase of growth (Table 1). This is the period when the growth rate has declined markedly and the phosphate concentration in the culture medium has fallen to a very low level. It was therefore possible that the changes in the phosphate accumulation rate were related to a declining level of phosphate in the culture medium.

Hence exponential-phase cells were stepped-down to 2×10^{-4}M phosphate and their subsequent accumulation rate compared with that of cells transferred to 10^{-4}M $CaSO_4$ in the normal manner (Fig. 5). There was a similar increase in phosphate uptake following both treatments though cells aged in phosphate apparently showed a more rapid subsequent decline in this rate.

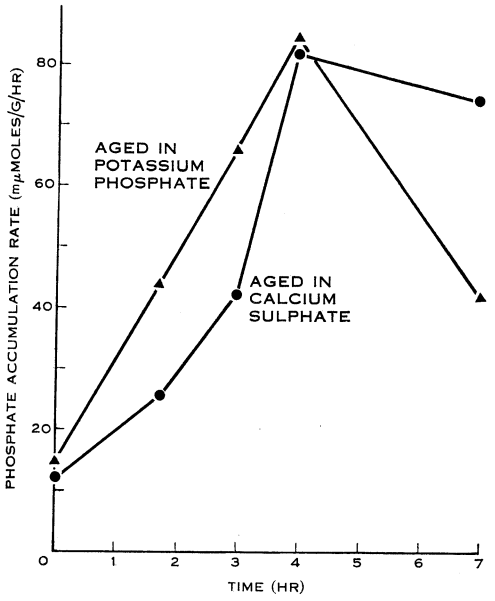

Fig. 5.—Effect of aging cells in 2×10^{-4}M KH_2PO_4 compared with aging in 10^{-4}M $CaSO_4$ on phosphate accumulation rate.

Though phosphate was supplied in this experiment, other nutrients were not. To ascertain whether a similar response occurred for step-up cultures, the response

TABLE 5

EFFECT OF A STEP-UP CULTURE SHIFT ON THE PHOSPHATE ACCUMULATION RATES OF TOBACCO PITH CELLS

Stage of Growth	Phosphate Accumulation Rate from 10^{-5}M KH_2PO_4 (mμmoles/g/hr)		
	Basal Rate (x)	Step-up Culture Rate (y)	Percentage Increase or Decrease $[100(y-x)/x]$
Early mid exponential phase	24	75	+208
Late exponential phase	38	49	+ 29
Mid stationary phase	166	55	− 67
Late stationary phase	47	65	+ 38

of cell cultures at different stages of growth to step-up treatment was studied (Table 5). Early exponential-phase cells exhibited a marked increase in the rate of phosphate

accumulation following step-up culture. Late exponential-phase and late stationary-phase cells appeared unaffected whilst the accumulatory rate of mid stationary-phase cells was depressed.

Although cells did not appear to be damaged in any way by the nutritional shifts it was possible that the mechanical effects of transfer affected the cells in some way. Therefore exponential-phase cells were put through the culture-shift schedule, but filtered, used culture medium, obtained from replicate cultures of the same age, was used. In this case no nutritional shift was involved and no rise in phosphate accumulation rate occurred (Fig. 6). Thus the mechanical effects of cell manipulation were not the cause of the rise in phosphate accumulation.

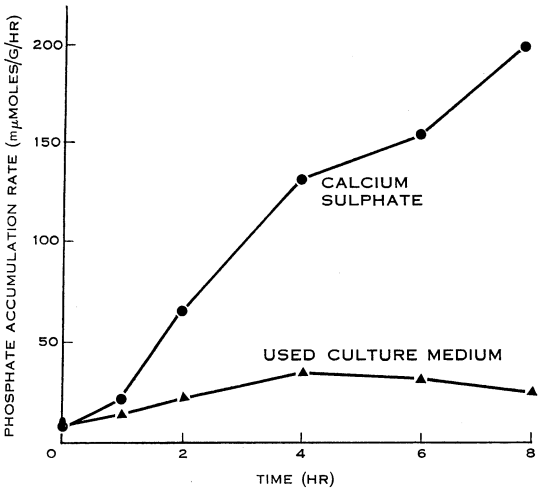

Fig. 6.—Effect of aerating cells in replicate culture medium, obtained from other cultures of the same age, compared with aeration in 10^{-4}M $CaSO_4$ on the phosphate accumulation rate.

(*g*) *Effect of Actinomycin D on Aging*

Actinomycin D has been shown to prevent the aging response in sliced potato tissue (Click and Hackett 1963) and to prevent DNA-dependent RNA synthesis in some bacterial, animal, and plant systems (Kirk 1960; Reich *et al.* 1962; Sanger and Knight 1963). In order to ascertain whether this inhibitor affected the phosphate-accumulation response of cells following a step-down culture, exponential-phase cells were treated with actinomycin D following their removal from culture medium and resuspension in calcium sulphate. Actinomycin D (5 or 10 μg/ml) did not affect the subsequent rise in phosphate accumulation. Higher concentrations of the inhibitor (20 μg/ml) or pretreatment of cells with actinomycin D for 4 hr prior to a step-down culture shift were also ineffectual.

These results suggest that the rise in phosphate accumulation following a step-down culture shift is not linked to the synthesis of new RNA; or that actinomycin D either does not inhibit such synthesis in this system or cannot enter cultured tobacco pith cells. In order to distinguish between these possibilities exponential-phase cells were treated with actinomycin D (10 μg/ml) for 2 or 4 hr

prior to a 30-min pulse with radioactive phosphate. RNA was extracted, purified, and analysed by zone sedimentation on 5–20% (linear) sucrose density-gradient columns. Though actinomycin D treatment brought about a 50% reduction in the specific radioactivity of the whole cell RNA, radioactivity profiles (Fig. 7) indicated that synthesis of some rapidly labelled RNA (distinct from ribosomal or transfer RNA) was continued in the presence of the inhibitor. The actinomycin D insensitivity of the metabolic changes that occur following a step-down culture shift thus does not necessarily negate a role for new RNA synthesis in these processes.

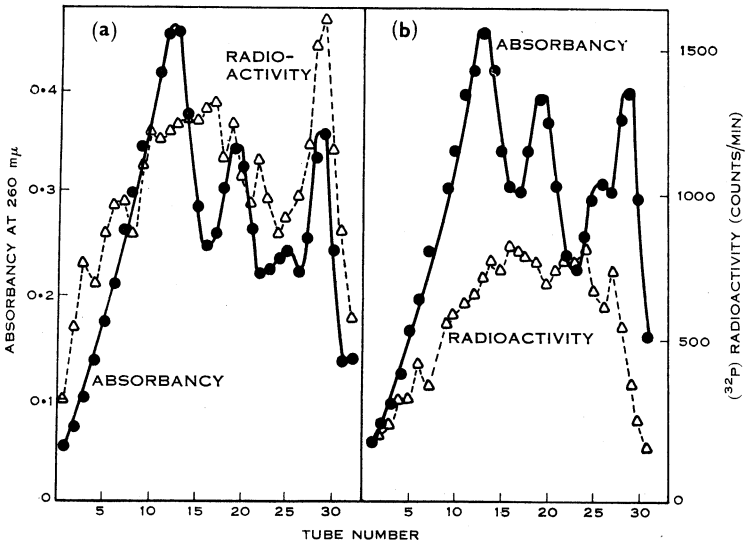

Fig. 7.—RNA synthesis in cells with no pretreatment, and after treatment with actinomycin D (10 μg/ml for 4 hr). Sedimentation was carried out in 5–20% (linear) sucrose gradients using the SW39 rotor of the Spinco model L ultracentrifuge. (*a*) RNA from control cells treated with ^{32}P-orthophosphate for 30 min. (*b*) RNA from cells pretreated for 4 hr with actinomycin D prior to a 30-min pulse with ^{32}P-orthophosphate.

IV. Discussion

Though slicing of tissue and damage of cells did not accompany the preparation of tobacco pith cell samples, aeration of the cells in dilute calcium sulphate produced changes in the metabolic behaviour of the cells; and the changes resembled those encountered when tissue slices were treated in the same way. In particular, the rate of accumulation of phosphate appeared to increase markedly; and changes took place in the phosphate ester pattern. The extent of the response depended on the state of growth of the cells, and was high towards the start of the stationary phase of growth. However, a depletion of phosphate either in the culture medium or in the treating solution was not the cause of the rise in the phosphate accumulation, since aerating the cells in 2×10^{-4}M KH_2PO_4, instead of calcium sulphate, also resulted in a

stimulation of phosphate uptake. Nor was the apparent rise in phosphate uptake due to recovery from an initial inhibition brought about by sampling cultures. Had this been so, performing washing manipulations with old culture medium should have resulted in samples that either accumulated phosphate at a high rate throughout, or showed the typical rise in phosphate accumulation. Neither was observed. Calculations made on the rate of phosphate incorporation by the cells growing in culture support the conclusion. Over the growth period days 2–6, the average phosphate concentration of the culture medium was 10^{-3}M, and the average rate of phosphate incorporation was 330 mμmoles/g fresh weight/hr. Fresh and step-down phosphate accumulation rates measured on samples of comparable cells were 470 and 1060 mμmoles/g fresh weight/hr. From day 6–10, the average phosphate concentration of the culture was 3×10^{-4}M, the incorporation rate in culture was 115 mμmoles phosphate/g fresh weight/hr, and the fresh and step-down accumulation rates of comparable cell samples were 230 and 640 mμmoles/g fresh weight/hr.

It seems that there is a true stimulation of phosphate accumulation, not caused by manipulation of cells and not due directly to a depletion of phosphate in the cell. The most striking result was that obtained by aerating cells in fresh nutrient medium, thereby submitting them to a step-up nutritional shift. This stimulated phosphate accumulation in early exponential-phase cells in the same way as the normal, or step-down, treatment; but depressed the phosphate accumulation rate in mid stationary-phase cells. It therefore appears that change itself, rather than the direction of the change, is the stimulus which sets off the aging response. The results support an hypothesis that cells during growth are in an inhibitory or repressed state which varies with the stage of growth of the tissue. Altering the external environment removes an inhibitor or releases the cells from some form of repression. Such changes in metabolic characteristics of cells would be expected to result in synthesis of structural protein, increased amounts of various enzymes, and probably new (induced?) enzymes as well. All such processes would depend ultimately upon an increased or altered synthesis of messenger-RNA. We attempted to discover whether messenger-RNA was being actively involved in the aging process, by attempting to inhibit the aging response with actinomycin D. No such effect was observed. However, actinomycin D appeared to be rather ineffective in inhibiting the synthesis of rapidly labelled RNA in tobacco pith cells and these results appear of doubtful significance in this case.

Less direct evidence can be invoked. Active RNA synthesis would require a more active nucleoside triphosphate production, leading to removal of some phosphate from the metabolic pool in the cell, and stimulation of its entry from the surrounding medium. In fact a marked increase took place in the radioactivity present in step-down cells as nucleoside triphosphates, and was coupled with a decrease in the radioactivity present as inorganic phosphate. Presumably, specific types of RNA would be synthesized in cells subjected to nutritional shifts and it might be hoped that short-term labelling of cells with radioactive precursors of RNA might reveal synthesis of new species of RNA. We hope to explore this possibility, using the convenient study system offered by tobacco pith cells grown in culture.

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