Studies on the Control of Mitotic Activity in Excised Roots

I. The Experimental System*

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ABSTRACT

The mitotic characteristics of excised roots of the garden pea, *Pisum sativum*, have been studied under conditions of controlled nutrition.

The excised root system was tested with regard to its ability to respond, mitotically, to various carbon sources. Sucrose, glucose, fructose, and DL-glyceraldehyde were found to support mitotic activity in excised roots, galactose and 2-deoxy-D-glucose were toxic, and mannose ineffective. Initiation of mitotic activity in the presence of glucose was inhibited by the respiratory poisons, KCN and malonic acid, the uncoupling agent, 2,4-dinitrophenol, but was not notably affected by the protein synthesis inhibitor, chloramphenicol. The glucose-induced response in mitotic activity was not affected by the carcinogen, urethan, and indeed, there is some evidence that the response was actually enhanced. The fact that KCN, malonic acid, and probably 2,4-dinitrophenol, in suitable concentrations inhibit the onset of cell division suggests that some level of operation of the Krebs' cycle is essential for commission of cells into mitosis. Likewise, failure to inhibit cells in the process of active mitosis by KCN and malonic acid is not inconsistent with the idea that there is a shift from reliance on aerobic to anaerobic respiration between antephase and active mitosis.

INTRODUCTION

Excised roots have long been used as an experimental tool for the study of growth requirements under a variety of conditions. Relatively few of these studies, however, have been directly concerned with the control of mitotic activity. Brown and Rickless (4) and Brown (3) have used the excised system with cucumber roots to study the effects of salts and sucrose on the rate of cell division. Aside from these studies, the greater part of the work dealing with the nutritional factors influencing mitotic activity have been carried out on intact systems. Tissue culture has been used as a means of studying mitotic activity in animal tissues. The work in this field has been reviewed recently by Waymouth (14). The primary purpose of the present paper is to describe in general terms the mitotic characteristics of the excised system and to show how it may be exploited to gain information on the physiological control of cell division.

Technique

Seeds of the garden pea, *Pisum sativum* var. "Alaska," were soaked overnight in pyrex distilled water and then germinated for 48 hours in moist paper towels. Germination was carried out in the dark and at a constant temperature of 25°C. Only seedlings having primary roots 2.5 to 3.0 cm. in length were selected for study. Primary roots were excised 1.5 cm. from the tip of the root cap in ¼ strength Hoagland salt solution and then inserted in the mesh of wax-coated wire screen baskets. The baskets were then transferred to a large pyrex vessel containing ¼ strength Hoagland salt solution which was maintained at 22.5°C. by a constant temperature bath. At the end of 8 hours, one of the baskets of excised roots was transferred to a beaker containing fresh balanced salt solution and served as the excised control. The other baskets were each moved

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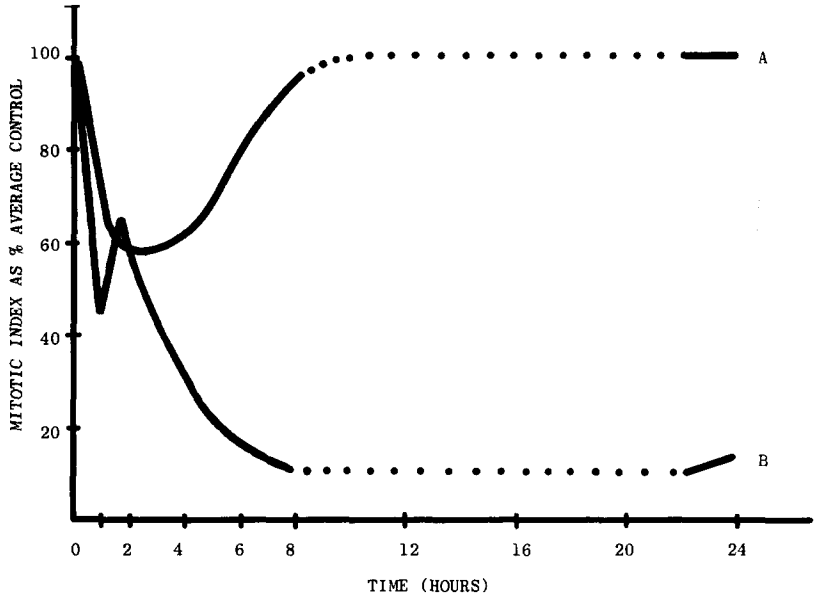


TEXT-FIG. 1. The effect of excision on the mitotic activity of pea roots maintained in balanced salt solution Curve *A*, intact roots. Curve *B*, excised roots.

to individual beakers containing balanced salt solution plus a known quantity of the chemical, the effect of which was being tested. The intact control in all experiments consisted of pea seedlings with standard size (2.5 to 3.0 cm.) primary roots which were maintained in ¼ strength Hoagland salt solution from the beginning of the excision period.

Samples taken at designated intervals were analyzed for quantitative changes in mitotic activity in terms of frequency of normal mitotic configurations relative to dose-time changes according to the Bowen-Wilson *Pisum* test (2). The mitotic activity of the excised root system at any given time during the test period is expressed as a per cent of the average mitotic activity of the intact root (intact control).

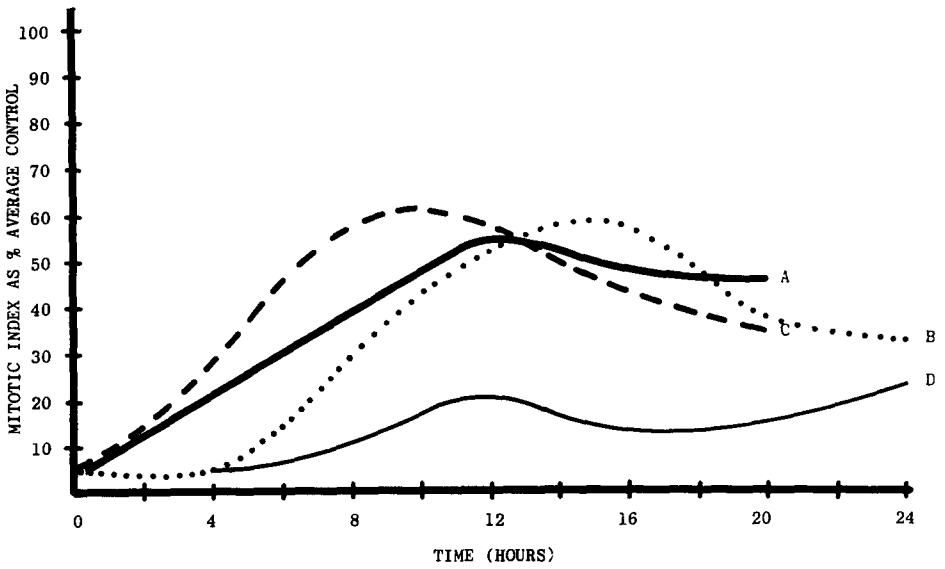
Mitotic Activity in Excised Pea Roots

A. In Balanced Salt Solution.—When excised pea roots are maintained in balanced salt solution, mitotic activity falls from the intact control level to about 5 to 15 per cent of this same level in a period ranging from 6 to 8 hours. Since this period is characterized by a progressive decrease in mitotic activity, it has been termed the "run down" period. This fall is almost linear, although there is a slight tendency for mitotic activity to rise during the 1st hour after excision (Text-fig. 1). It has also been noted that in excised roots the number of plastids diminishes with time, frequently to zero. From the end of the run down period, *i.e.* 8 hours, to about 48 hours, mitotic activity is maintained at a level which usually fluctuates between 5 to 30 per cent of the intact control (Text-fig. 1). So far as we have been able to determine, there is neither a qualitative nor a quantitative difference in mitotic activity correlated with carrying out the experiment in either the light or dark.

B. In Balanced Salt Solution Plus Carbon Sources.—An extensive study has been made of the changes in mitotic activity following the addition of a number of carbon sources. Most of this has been concentrated on the effects of addition of carbon sources at the end of the run down period; however, some experiments have been carried out in which the carbon source was added either at an earlier or later time.

1. Effect of Addition of Glucose and Sucrose Immediately Following Excision.—When either sucrose at 2 per cent or glucose at 1 per cent are added immediately after excision, mitotic activity falls for about the first 4 hours to a level approximately 50 per cent of the intact control and thereafter rises to near control level by 9 or 10 hours where it is maintained at a reasonably constant level for at least 24 hours. In these experiments, there was no indication that the number of plastids changed materially.

2. Effect of Carbon Sources Added 8 Hours after



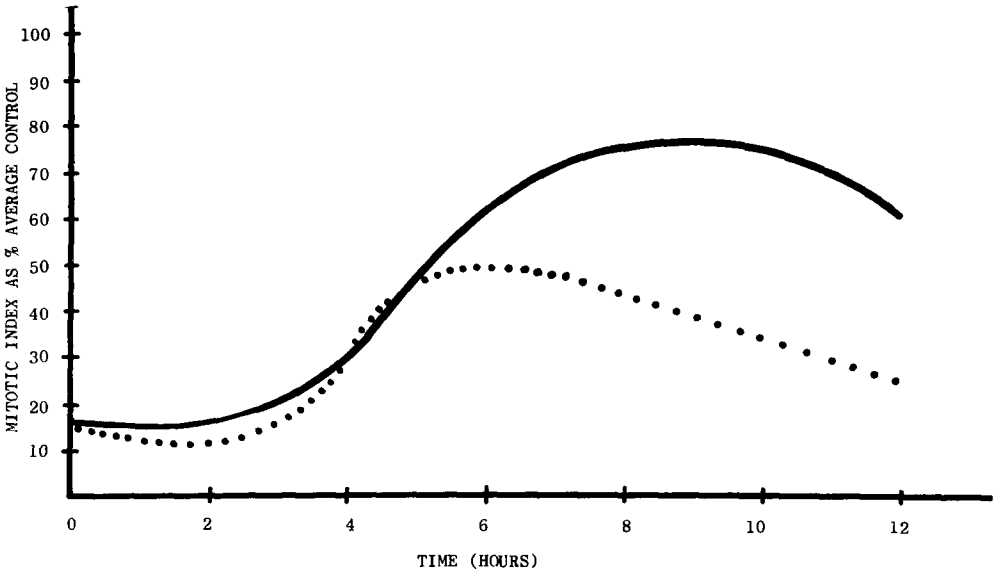
TEXT-FIG. 2. Mitotic activity induced in excised pea roots by the addition of various carbon sources. Curve *A*, 1 per cent fructose. Curve *B*, 2 per cent sucrose. Curve *C*, 1 per cent glucose. Curve *D*, balanced salt solution.

Excision.—Excised roots 8 hours after excision appear to reach their lowest level of mitotic activity (Text-fig. 1); therefore, this point was selected as "zero" treatment time for the rest of our studies. When sucrose (2 per cent), glucose (1 per cent), or fructose (1 per cent) were added at this time, mitotic activity invariably increased to some 60 to 100 per cent or more of the intact control between 8 and 10 hours. Mitotic activity thereafter has been found to fall off at varying rates (Text-fig. 2). In cases where the system has been followed far enough, *i.e.* to 48 hours, mitotic activity is once again close to excised control value. The initial rise following administration of these three sugars appears to be delayed by about 4 hours, with most of the increase taking place between 4 and 8 hours (Text-fig. 2). It was noted in all cases that plastids reappeared during treatment of excised roots with sucrose, glucose, or fructose. Two experiments were run to compare 0.1 per cent and 1 per cent glucose in order to obtain an estimate of the limiting concentration of the carbon source. In both cases, the 0.1 per cent treatment proved to be only about 75 per cent as effective as the 1 per cent glucose in restoring mitotic activity (Text-fig. 3). Mannose at the 1 per cent level was ineffective. Galactose (1 per cent) and 2-deoxy-D-glucose (1 per cent) were both ineffective and in some degree toxic.

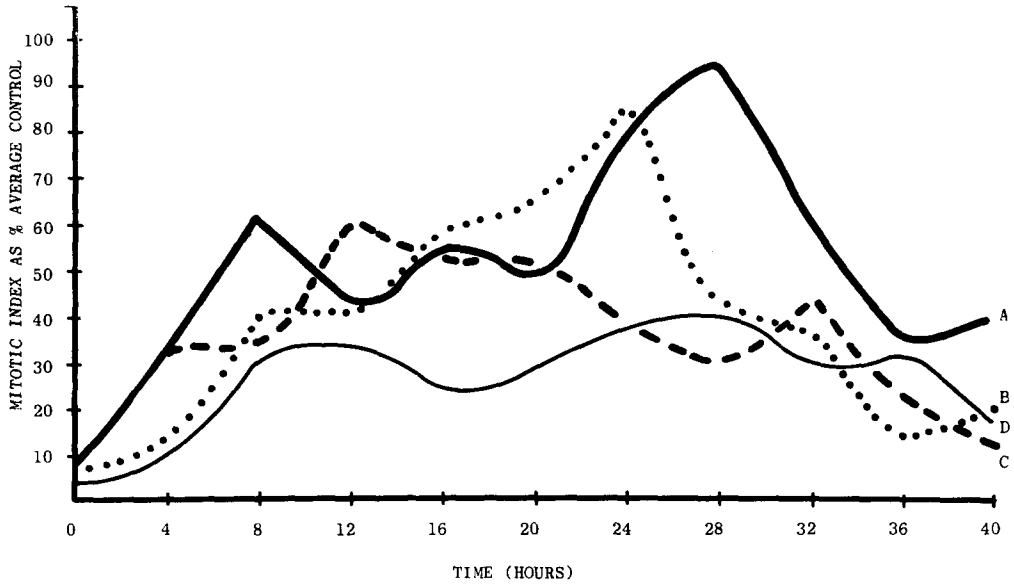
A number of experiments were carried out to test the response of the excised system to DL-glyceraldehyde. Treatment of the intact system with 1 per cent DL-glyceraldehyde had previously been found to produce no detectable effect on mitotic activity or survival of seedlings. When the same concentration was used on the excised system, the roots showed toxicity effects after a short time. Treatment of excised roots at the 0.05, 0.01 and 0.001 per cent level gave a positive response, the degree of the response being more or less proportional to the dose. The pattern of change in mitotic activity was quite different from that obtained with sucrose, glucose, and fructose. There was an initial rise with all three concentrations which was 50 per cent of the intact control with the 0.05 per cent concentration. This was followed by either a slight depression or levelling off period to about 20 hours, then a marked rise which in the case of the 0.05 per cent treatment reached control value (Text-fig. 4). So far as we have studied it, the second rise is followed by a more or less abrupt fall in mitotic activity (Text-fig. 4).[1] With DL-glyceraldehyde treatment there appeared to be no restoration of plastids in the excised roots.

3. Changes in Effect of Carbon Sources Added at Different Times Following Excision.—In order to

[1] Preliminary experiments indicate that D-glyceraldehyde (0.05 per cent) produces essentially the same response in mitotic activity as DL-glyceraldehyde at the same concentration level.



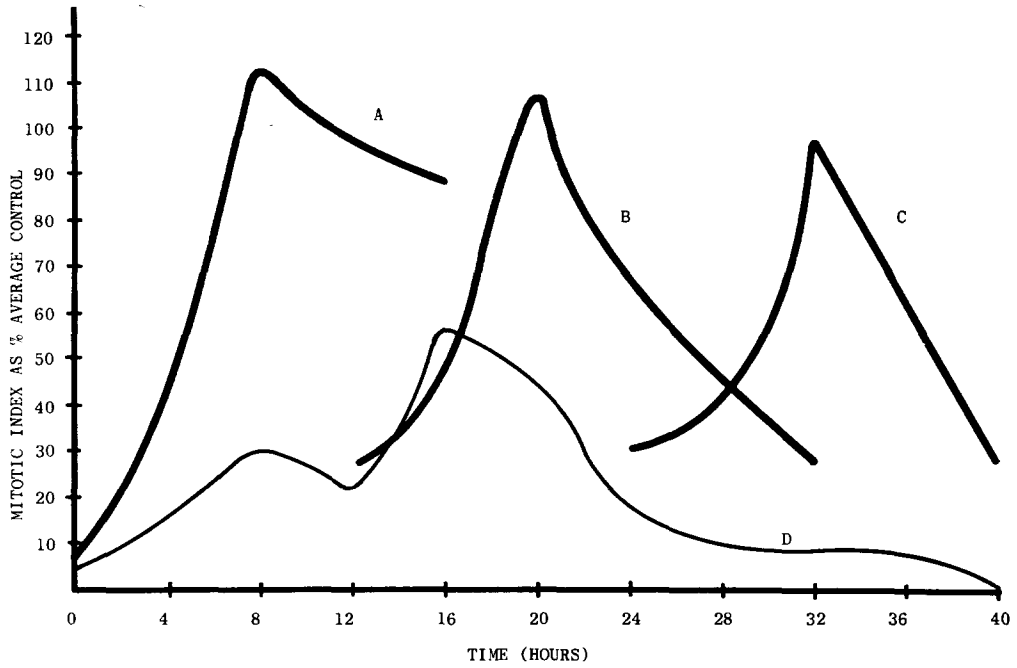
TEXT-FIG. 3. Relation between mitotic activity and glucose concentration in excised pea roots. Continuous line, 1 per cent glucose; dotted line, 0.1 per cent glucose.



TEXT-FIG. 4. Induction of mitotic activity in excised pea roots by different concentrations of DL-glyceraldehyde. Curve *A*, 0.05 per cent. Curve *B*, 0.01 per cent. Curve *C*, 0.001 per cent. Curve *D*, balanced salt solution.

test the capability of the excised system to respond to a carbon source, 2 per cent sucrose was added 8, 20, and 32 hours after excision and the changes in mitotic activity plotted (Text-fig. 5). The initial response (rise in mitotic activity) was approximately the same regardless of when the sucrose was added both with respect to rate of rise and degree of response. The rate of the subsequent fall in mitotic activity, however, appears to be directly related to the time of treatment being less at 8 hours and greatest at 32 hours (Text-fig. 5).

C. Tests of Mitotic Cycling Capacity in the Excised System.—It was recognized that the greater part of the mitotic activity induced by the addi-



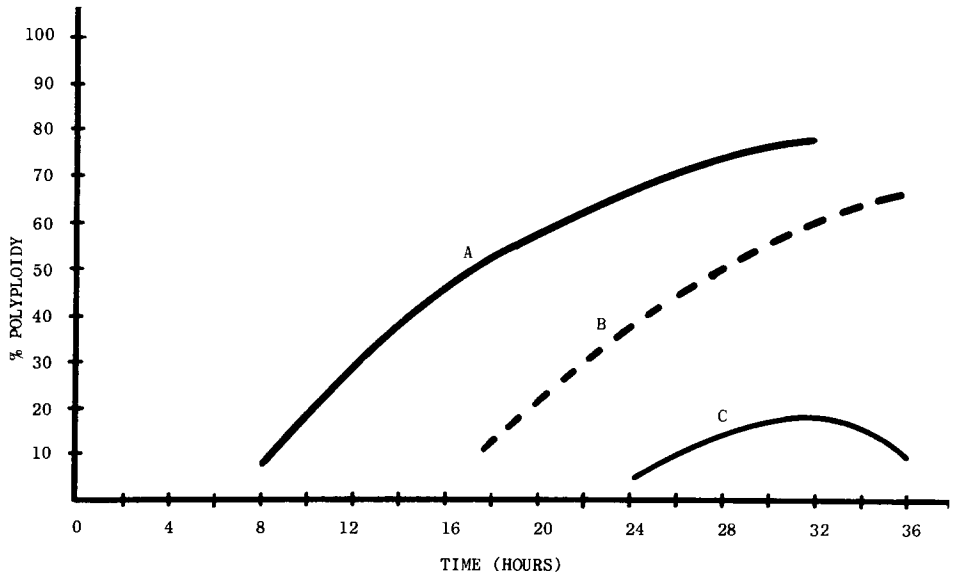
TEXT-FIG. 5. The effect of addition of 2 per cent sucrose on induction of mitotic activity in pea roots at different time intervals following excision. Curve *A*, 8 hours after excision. Curve *B*, 20 hours after excision. Curve *C*, 32 hours after excision. Curve *D*, balanced salt solution.

tion of suitable carbon sources might merely represent commission of competent cells into mitosis and that newly formed cells might not be able to reenter active mitosis. In order to test this, the intact and excised roots, with and without a carbon source system, were treated with 50 or 75 p.p.m. of colchicine. If polyploidy appears, then it may be assumed that at least some proportion of cells are capable of completing an entire mitotic cycle. Also the time of first appearance of polyploidy may be taken as a rough measurement of the minimum time necessary to complete a cycle under the conditions of the experiment. When the intact seedling is treated, the first polyploid cells appear approximately 8 hours after administration of colchicine and the percentage of polyploidy increases progressively to about the 70 per cent level. When excised roots maintained in balanced salt solution only are treated 8 hours after excision, the first indication of polyploidy appears approximately 24 hours later. The degree of polyploidy increases slightly to about the 20 per cent level, then falls off.

Excised roots treated with both sucrose (2 per cent) and colchicine at the end of the 8 hours excision period show their first polyploid cells about 18 hours after treatment. The degree of polyploidy increases precisely in the same fashion and to the same level as in the intact system (Text-fig. 6). When DL-glyceraldehyde (0.05 per cent) is substituted for sucrose 8 hours after excision, the time of first appearance of polyploidy, rate of occurrence of polyploid cells, and degree of polyploidy reached are unchanged to any significant degree. When colchicine was added to DL-glyceraldehyde (0.05 per cent)–treated roots 20 hours after excision, the time of first appearance of polyploidy was unchanged. So far the experiment has not been carried far enough to determine whether the rate of accumulation of polyploidy is also unchanged. The colchicine results do not reflect differences in susceptibility of the various systems to colchicine as indicated by the fact that the colchicine indices (Hadder and Wilson, 6) of all treated materials at the end of 4 hours were approximately the same.

In summary, it is clear that both sucrose (2 per cent) and DL-glyceraldehyde (0.05 per cent) allow more or less normal cycling of cells from one mitosis to the next but at a somewhat reduced rate over the period tested. Without a suitable carbon source some cycling still occurs but obviously such capacity decreases rapidly.

D. Studies of the Modification of the Glucose Re-



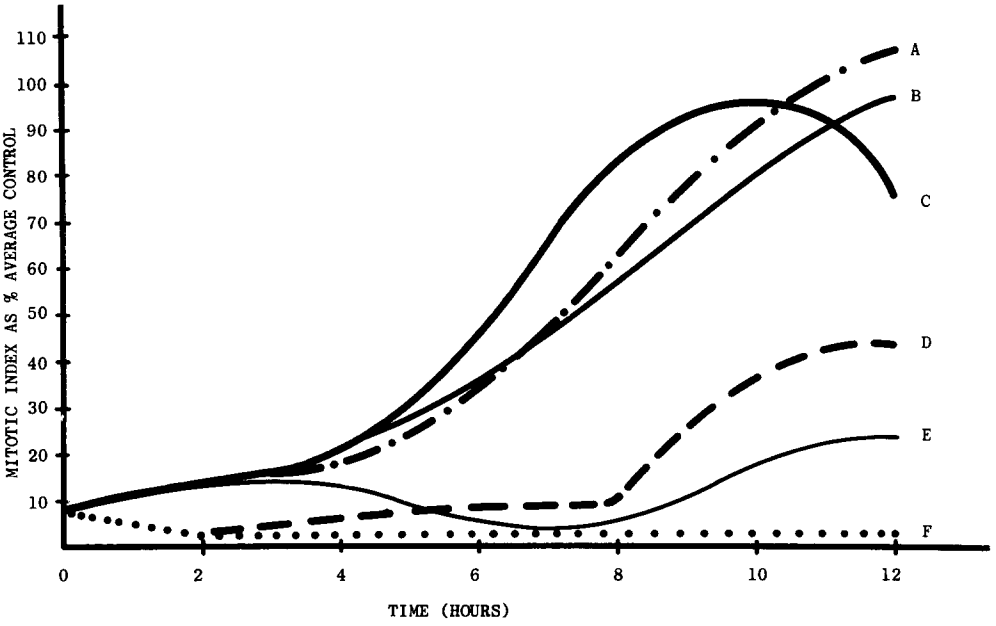
TEXT-FIG. 6. Rate of appearance of polyploid cells after treatment with 50 p.p.m. colchicine. Curve *A*, intact pea roots in balanced salt solution. Curve *B*, excised pea roots in 2 per cent sucrose plus balanced salt solution. Curve *C*, excised pea roots in balanced salt solution only.

sponse.—Since the initial rise in mitotic activity following treatment with glucose 8 hours after excision proved to be highly reproducible, it was considered that this part of the total response of the excised system would provide a suitable base against which to measure the effect of substances known to inhibit either respiration or protein synthesis.

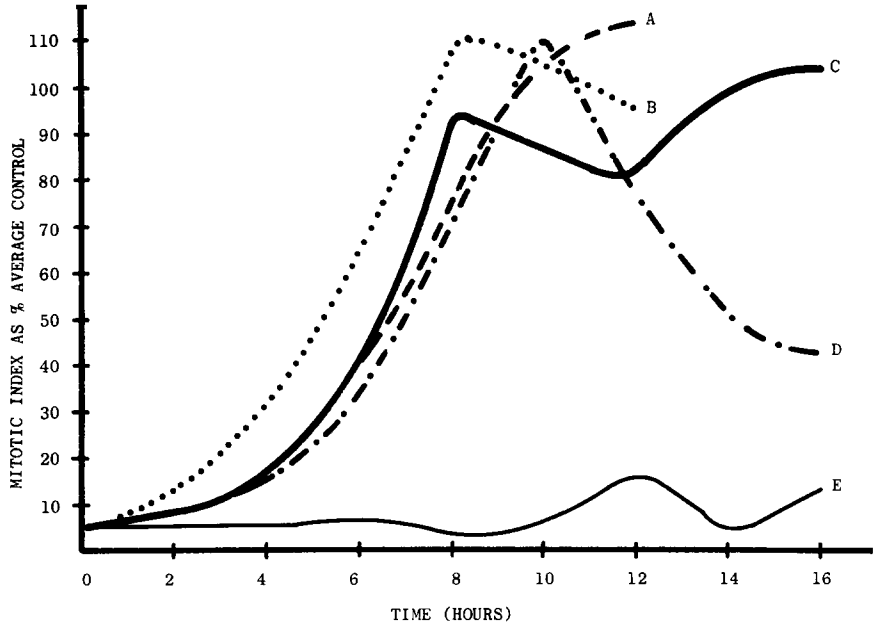
1. Potassium Cyanide (KCN).—When KCN was added to the excised system at a level of 10^{-4} molar, the glucose response was completely inhibited (Text-fig.7), the mitotic activity on the average being somewhat lower than that of the excised control. There was, however, no indication of toxicity. When the KCN concentration was reduced to 10^{-5} molar, the glucose response not only showed no sign of inhibition, but mitotic activity rose more rapidly and to a higher level than in the glucose control (Text-fig. 8). Since mitotic activity in excised roots treated with 1 per cent glucose and 10^{-5} molar KCN reached a point which was notably higher than the intact control on the average (Text-fig. 8), there is some evidence that this well known respiratory inhibitor in suitable concentrations may even enhance the glucose effect at least temporarily.

2. Malonic Acid.—Treatment of the excised system with 10^{-3} molar malonic acid under the same conditions completely inhibited the glucose response and also caused obvious toxicity symptoms. When the concentration of malonic acid was reduced to 10^{-5} molar, the mitotic index rose essentially the same way as in the glucose control. However, the peak activity appeared to be delayed by approximately 2 hours but reached a slightly higher level (Text-fig. 8).

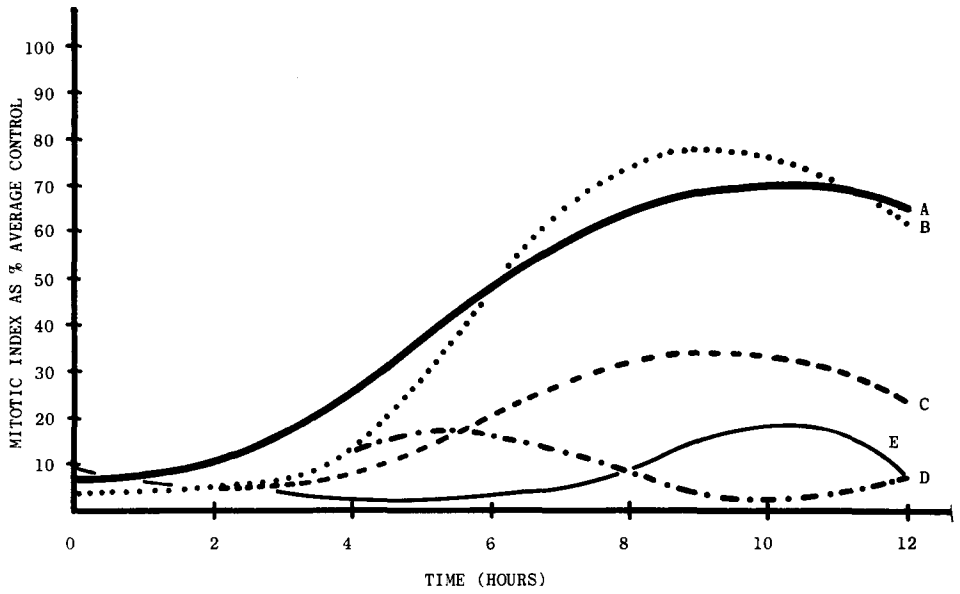
Malonic acid was selected for further study since it is one of the better known inhibitors of aerobic respiration in both plant and animal tissues (Beevers, 1; James, 7, 8, Krebs, 9; Neilands and Stumpf, 12). If suppression of the glucose response by malonic acid is primarily the result of inhibition of aerobic respiration it should be possible to show that such inhibition is dose-dependent within certain pH limits. In order to test this possibility, groups of excised roots were treated simultaneously at pH 5.5 with varying concentrations of malonic acid 8 hours after excision. The results of this experiment are shown in Text-fig. 9. The dose curves obtained (Text-fig. 9) indicate that a relationship does exist between the degree of mitotic activity induced by glucose and the concentration of inhibitor (malonic acid) used. The glucose response is inhibited for some 4 hours at all the concentrations of malonic acid tested; however, the level of mitotic activity which is reached following this



TEXT-FIG. 7. The effect of various inhibitors on the induction of mitotic activity in excised pea roots by 1 per cent glucose. Curve *A*, 2000 p.p.m. urethan. Curve *B*, 60 p.p.m. chloramphenicol. Curve *C*, 1 per cent glucose. Curve *D*, 3 p.p.m. 2,4-dinitrophenol. Curve *E*, balanced salt solution. Curve *F*, 1×10^{-4} M KCN.



TEXT-FIG. 8. Response in mitotic activity of excised pea roots to 1 per cent glucose in the presence of various respiratory inhibitors. Curve *A*, 1×10^{-5} M malonic acid. Curve *B*, 1×10^{-5} M KCN. Curve *C*, 1 per cent glucose. Curve *D*, 2000 p.p.m. urethan. Curve *E*, balanced salt solution.



TEXT-FIG. 9. The effect of different concentrations of malonic acid on induction of mitotic activity in excised pea roots by 1 per cent glucose. Curve *A*, 1 per cent glucose. Curve *B*, 5×10^{-5} M malonic acid. Curve *C*, 1×10^{-4} M malonic acid. Curve *D*, 5×10^{-4} M malonic acid. Curve *E*, balanced salt solution.

initial inhibition period is dependent on the concentration of the inhibitor to which the excised roots are exposed.

3. Ethyl Carbamate (Urethan).—Treatment of the same system under the same conditions with urethan at the level of 2000 p.p.m. (2.25×10^{-2} M) showed no inhibition of the glucose-induced rise in mitotic activity, but again the peak was delayed by about 2 hours and was slightly higher (Text-fig. 7). In the one experiment in which the urethan treatment was followed further, the mitotic index dropped off more rapidly than it did in the glucose control (Text-fig. 8).

4. Chloramphenicol (Chloromycetin).—Treatment of excised roots with 60 p.p.m. (1.8×10^{-4} M) of chloramphenicol again did not seriously interfere with the glucose response; however, the curve did appear to be moved to the right—that is, there was a delay of about 2 hours in reaching its peak of activity (Text-fig. 7).

5. 2,4-Dinitrophenol.—Treatment of the excised system with 2,4-dinitrophenol at the level of 3 p.p.m. (1.6×10^{-5}) completely suppressed the glucose response for 6 hours, after which there was a slight rise in the mitotic index to about 30 per cent of the intact control followed by a definite drop (Text-fig. 7).

DISCUSSION

Most of the experiments were carried out in order to characterize the excised system with regard to its capacity to respond to various conditions. The kind of response obtained with the sugar treatments, for instance, proved not only to be reasonably predictable but sufficiently consistent to serve as an excellent basis for extensive experimental manipulation. Even though the experiments carried out to date have been primarily exploratory, certain of them are at least highly suggestive.

On a concentration basis, DL-glyceraldehyde appears to be considerably more efficient in an overall fashion as a carbon source for support of mitotic activity in the excised system than any of the other carbon sources. It is well known that the *l*-isomer of glyceraldehyde inhibits glycolysis in plant and animal tissues presumably through formation of L-sorbose-1-phosphate in the presence of aldolase and triose phosphate (Lardy *et al.*, 10; Needham and Lehman, 11; Rudney, 13). Preliminary studies indicate that the *d*-isomer at the 0.05 per cent level is as effective as the dimeric form of glyceraldehyde in restoring mitotic activity in excised roots. D-Glyceraldehyde produces three distinct peaks in mitotic activity each separated by some 12 hours. The initial rise in mitotic activity reached

a level of some 60 per cent of the intact control whereas the other two subsequent peaks attained a level equivalent to 100 per cent of the control. The similarity in mitotic response obtained with DL- and D-glyceraldehyde suggests that the *d*-isomer is the component of the dimeric form which is active as the carbon source. Comparative studies of the effectiveness of the *d*- and *l*-isomers of glyceraldehyde in restoring mitotic activity in excised roots are planned. Although it is not possible to determine the metabolic fate of glyceraldehyde with the methods employed in the study, the relative efficiency of this compound as a carbon source for support of mitotic activity suggests that one of the principal pathways of utilization is the glycolytic route.

The fact that KCN, malonic acid, and probably 2,4-dinitrophenol, in suitable concentrations inhibit the onset of division would suggest that some Krebs' cycle activity is also essential for commission of cells into mitosis. The fact that cells exposed to the respiratory inhibitors, KCN and malonic acid, go through active mitosis, that is from prophase to telophase, normally and without any indication of inhibition, may be taken as additional support for our previous contention that there is a shift from reliance on aerobic to anaerobic respiration between antephase and active mitosis (Wilson and Morrison, 15, 16). While these suggestions concerning the energy systems involved in mitotic activity are at present somewhat speculative, the excised system described would seem to offer an excellent experimental device for testing the general hypothesis.

The pattern of response of the excised system to the addition of suitable carbon sources is also highly suggestive with regard to the factors underlying repair capacity in an organized tissue. As has been demonstrated, the initial response of the excised system to the addition of a carbon source appears to represent commission into active mitosis of cells which already are in a high state of competence, *i.e.* antephase (Bullough, 5; Wilson and Morrison, 16). This response is to a large extent independent of time. On the other hand, return to mitotic competency in the excised system becomes more difficult with time. This difference suggests that the gaining of competence and commission are determined by somewhat different mechanisms and that commission is more readily accomplished and requires less specialized conditions. For example, if we consider an organ whose repair capacity is damaged, then the wound or trauma may well provide conditions suitable for triggering competent cells into mitosis and thus repairing the damage to a greater or lesser extent without also providing conditions which would maintain the complete mitotic cycle at a high level. These considerations further stress the importance which we have already attached (Wilson and Morrison, 16) to the ability of cells to gain mitotic competence with respect to the production of a neoplastic potential. In short, if the metabolic activity of a tissue, or some fraction of its constituent cells, should be such as to enable the products of a division to reach antephase fairly readily, the potential for the development of a neoplasm would be proportionally increased. Further, it would not be surprising if the metabolic activity in "precancerous cells" is found to be similar to that in normal embryonic or meristematic cells. It may also be parenthetically noted that the difference between ease of commission and the gaining of mitotic competence provides a basis for the normal fluctuations in mitotic activity which are found in some degree in any actively dividing tissue.

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