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RESPIRATORY CHANGES DURING CALLUS FORMATION IN POTATO TUBER TISSUE CULTURED *IN VITRO*

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Introduction

On the study of callus tissues there has been much information on the characteristics of metabolic activities and chemical compositions as compared with those of quiescent tissues. The metabolic shift during callus formation of matured tissue has been still little understood. Recently, the respiratory changes associated with callus formation in tissue cultured *in vitro* have been reported (4, 12). The hormonal regulation of callus induction in potato tuber tissue was investigated and it was found that the exogenous application of auxin was indispensable for the initiation of callus formation in potato tuber tissue (10). Since the callus formation in tissue provides an excellent material for a study of metabolic regulation in higher plants, it is of a primary interest to clarify the metabolic response during cell division followed by cell expansion in the explants. In the present paper, the respiratory changes during auxin-induced callus formation in potato tuber tissue cultured *in vitro* have been described in respect to various inhibitors of respiration.

Materials and methods

Plant materials

The tubers of potato (*Solanum tuberosum* L., var. Irish Cobbler) stored in a cellar at 2°C in darkness were used as materials. The disk-shaped slices (5 mm in diameter and 1 mm in thickness) for cultural explants were cut and

Abbreviations: PP, pentose phosphate; TCA, tricarboxylic acid; DNP, 2, 4-dinitrophenol; FAA, monofluoroacetic acid; NAA, α -naphthaleneacetic acid; MIA, mono-iodoacetic acid; RQ, respiratory quotient.

cultured in the same manner as described in a previous paper (10).

Culture conditions

Two slices were explanted onto 30 ml of nutrient medium in a 100 ml-Erlenmeyer's flask being autoclaved at 1.0 kg/cm² pressure for 10 minutes. The nutrient media used were as follows: 1) modified White's medium (10) + 0.5% agar (control medium), and 2) control medium + 1.6×10^{-6} M α -naphthaleneacetic acid (NAA, auxin medium). The cultures were incubated in a dark room at about 25°C. Explants cultured on control medium and on auxin medium are named control culture and auxin culture, respectively. Samples were taken at appropriate intervals on the course of 3 week culture for analysis described below.

Measurement of fresh and dry weight

The explants taken from the culture medium were carefully washed with distilled water, and blotted dry with filter paper. The fresh weight was measured immediately after these operations. The dry weight was measured after the explants had been dried in an oven at 80°C for 24 hours.

Determination of protein content

The dried materials were ground with 80% ethanol in a mortar and centrifuged at $5,000 \times g$ for 5 minutes. This procedure was repeated three times. Then the supernatants were discarded. The residue was suspended into ether-acetone mixture (2:1, v/v), and the extraction was repeated twice. The resultant residue was heated to remove ether-acetone, and the dried powder was obtained. The protein-nitrogen content in this residue was determined by nesslerization after digestion in concentrated H₂SO₄.

Measurement of respiratory rate

The respiratory rate was manometrically determined by measuring O₂ uptake of the explant. All measurements were carried out for 1 hour at 25°C after thermal equilibration. Between 3 to 10 explants (approximately 0.5 g in fresh weight) were incubated in a Warburg flask containing 2.2 to 2.3 ml of M/15 phosphate buffer (pH 5.0) and the center well contained 0.3 ml of 5 N KOH. Carbon dioxide output was determined using the two-flask method with paired samples. Malonate, fluoroacetate (FAA), iodoacetate (MIA), and 2, 4-dinitrophenol (DNP) were used as respiratory inhibitors at a final concentration of 5×10^{-2} M, 5×10^{-2} M, 5×10^{-4} M and 10^{-5} M, respectively.

Results

(1) Changes in fresh weight

The changes in fresh weight per explant are shown in Fig. 1. The auxin culture showed an active growth. After an initial lag phase extending over the first 2 or 3 days, the fresh weight increased almost linearly throughout the culture period. A striking quadruple in yield was obtained at the end of 3 week culture. The control culture showed no significant growth throughout an entire culture period.

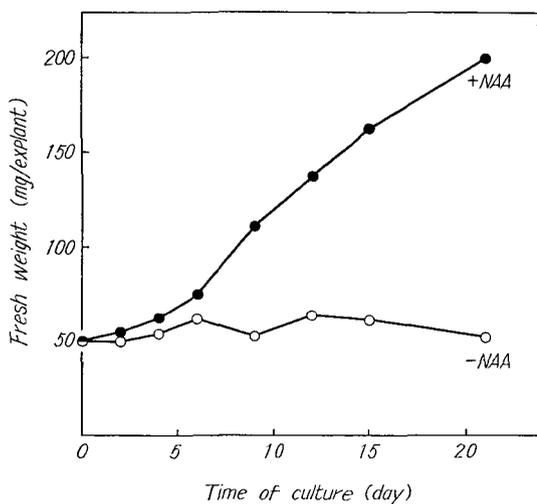


Fig. 1. Changes in fresh weight of the explant during potato tissue culture.

(2) Changes in protein content

As is shown in Fig. 2, the protein content per explant in auxin culture has increased similar to that of fresh weight. The control culture also doubled in protein content at the end of culture.

(3) Changes in respiratory rate

The changes in O_2 uptake per fresh weight are shown in Fig. 3. During the initial 2 days, the respiratory rate increased markedly (about 2 fold), then such increase in rate of O_2 uptake temporarily attained a steady level. After the 12th day, the rate showed a further increase. The higher level of respiration is one of the characteristics of the actively growing callus tissue. The respiratory rise during the initial 2 days was also observed in control culture, while the deterioration of respiratory activity was remarkable after the initial

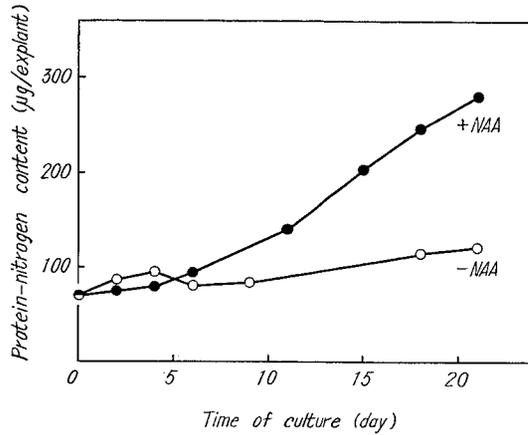


Fig. 2. Changes in protein content of the explant during potato tissue culture.

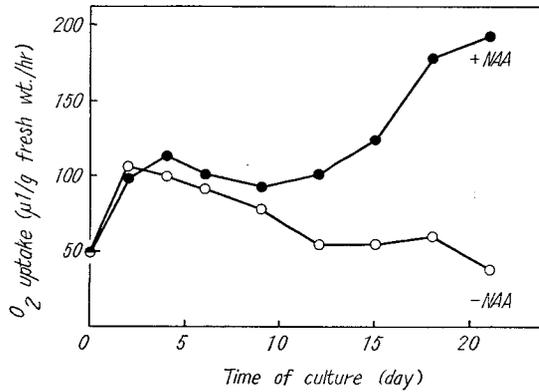


Fig. 3. Changes in O₂ uptake per fresh weight during potato tissue culture.

respiratory rise.

(4) *Effect of malonate on respiration*

Figs. 4a and 4b show the effects of malonate ($5 \times 10^{-2} M$) on respiration during the culture of tissue derived from freshly-harvested tuber (dormant tuber) and from aged tuber (non-dormant tuber), respectively.

The respiration of the fresh slices prepared from dormant tuber (Fig. 4a) was slightly inhibited by malonate (approx. 18% inhibition). In control culture, doubling of the inhibition was resulted within a day after inoculation. This suggests that the initial respiratory rise (Fig. 3) was associated with the specific activation of TCA cycle in tissue derived from dormant tuber.

Afterwards, the respiratory inhibition by malonate was inert as the respiratory activity of tissue deteriorated. On the other hand, the inhibition rate in auxin culture increased from 18% to 23% within a day after inoculation. However, on the next day (2nd day) the malonate inhibition was completely disappeared. One possible explanation of this is that the PP pathway activity was intensified by NAA, while the TCA cycle activity, whose marked activation was observed in control culture, was suppressed. After this period, it was noticed that the respiration of auxin culture was restored the sensitivity to malonate and that its inhibition rate was increased as callus developed.

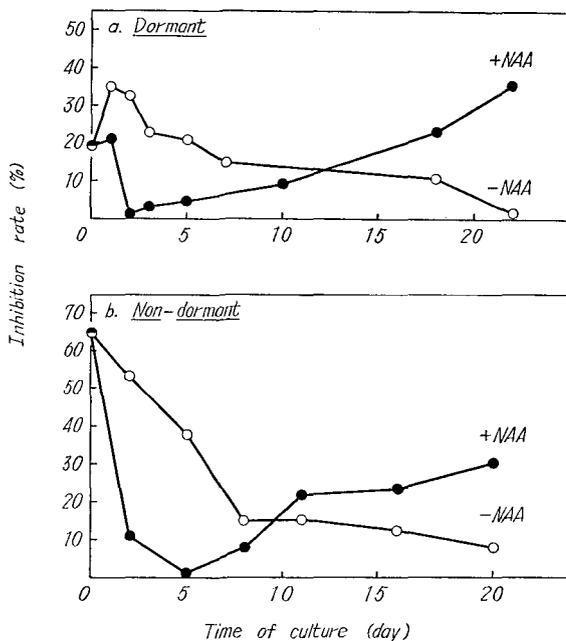


Fig. 4. Changes in inhibition rate of respiration by malonate ($5 \times 10^{-2} M$) during culture in the explant from dormant (a) and non-dormant (b) potato tuber.

The respiration of the fresh slices derived from non-dormant tuber (Fig. 4b) was more sensitive to malonate (approx. 63% inhibition) than that from dormant tuber. The inhibition rate in control culture decreased progressively throughout an entire culture period. On the other hand, the inhibition rate in auxin culture decreased more rapidly than that in control culture in the early stage of culture. On the 5th day, the inhibition was almost nullified, followed by a gradual increase in the sensitivity to malonate with callus

development.

(5) *Effect of fluoroacetate on respiration*

Fig. 5 shows the effects of FAA ($5 \times 10^{-2} M$) on respiration during the culture of tissue derived from non-dormant tuber. These results were quite similar with those indicated in Fig. 4b.

(6) *Effect of iodoacetate on respiration*

Fig. 6 shows the effects of MIA ($5 \times 10^{-4} M$) on respiration during culture of tissue derived from non-dormant tuber. The inhibition rate in auxin culture rapidly decreased within the first 5 days and then its rate increased during the rest of culture period. While the inhibition rate in control culture continued to decrease throughout an entire culture period.

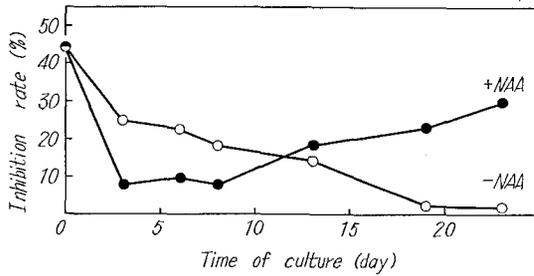


Fig. 5. Changes in inhibition rate of respiration by fluoroacetate ($5 \times 10^{-2} M$) during culture in the explant from non-dormant potato tuber.

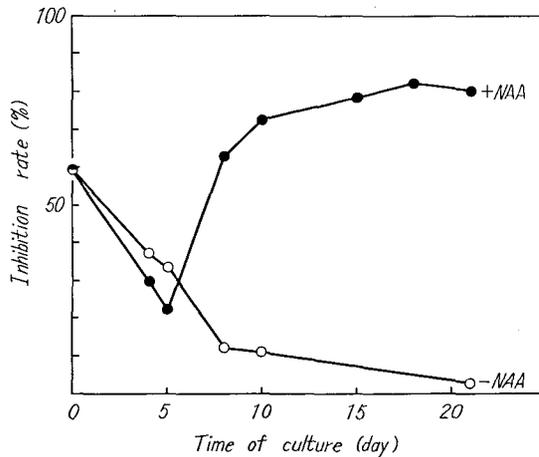


Fig. 6. Changes in inhibition rate of respiration by iodoacetate ($5 \times 10^{-4} M$) during culture in the explant from non-dormant potato tuber.

(7) *Effect of dinitrophenol on respiration*

Changes in effects of DNP ($10^{-5} M$) on O_2 uptake during culture of tissue derived from non-dormant tuber are indicated in Fig. 7. The respiration of fresh slices was stimulated to 180–200% of control value by DNP treatment. During the initial culture period, the stimulation rate by DNP decreased (40 to 50% stimulation) in both cultures and remained almost constant throughout the subsequent period. The DNP-induced respiration in auxin culture indicated tentatively the lowest stimulation at the 5th day as shown in Fig. 7b.

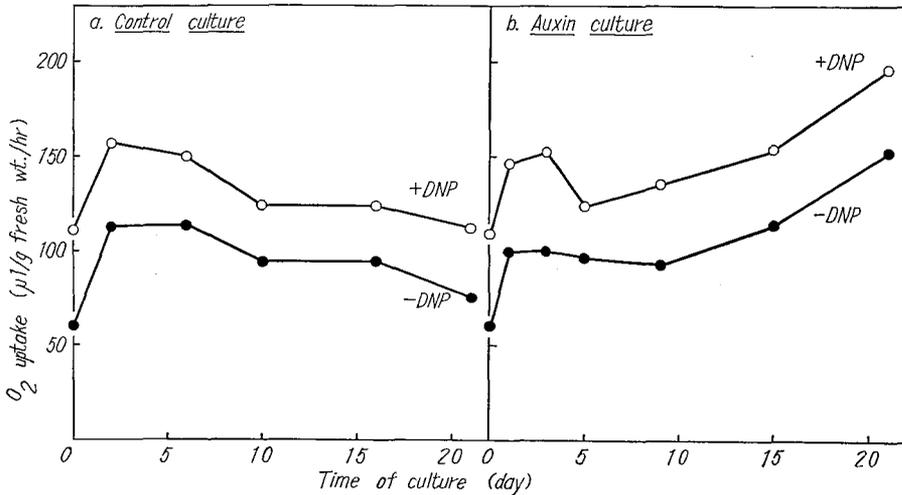


Fig. 7. Effect of 2,4-dinitrophenol ($10^{-5} M$) on respiration during culture in the explant from non-dormant potato tuber.

(8) *Changes in respiratory quotient*

Changes in respiratory quotient (RQ) during culture are shown in Fig.

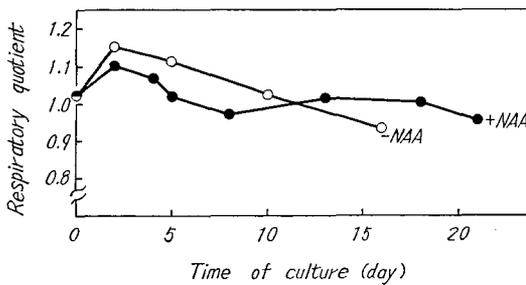


Fig. 8. Changes in respiratory quotient during potato tissue culture.

8. The initial RQ of explant was close to unity. The RQ of auxin culture was more or less unity throughout an entire culture period, except slightly higher levels (1.0 to 1.1) observed during 2 to 4 days after inoculation. The RQ in control culture showed a slight increase during the first 3 days followed by a gradual decrease during the subsequent period.

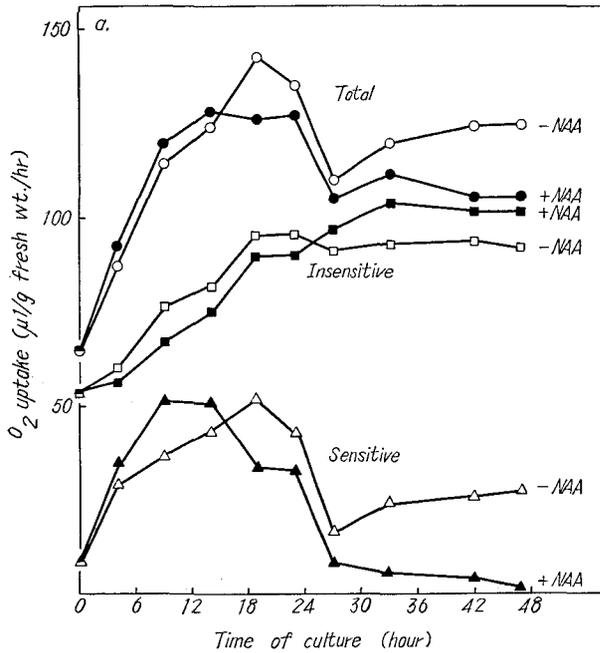


Fig. 9 a. Changes in respiratory rate and its sensitivity to malonate ($5 \times 10^{-2} M$) during the initial 48 hour culture in the explant from dormant potato tuber.

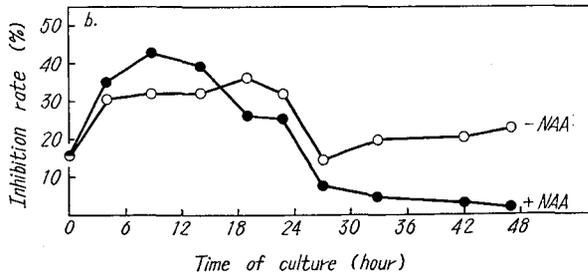


Fig. 9 b. Changes in inhibition rate of respiration by malonate ($5 \times 10^{-2} M$) during the initial 48 hour culture in the explant from dormant potato tuber.

(9) *Initial respiratory rise in the early stage of culture*

In order to see the details of the respiratory pattern right after the commencement of culture, the time-course studies of the changes in O₂ uptake and its sensitivity to malonate were investigated for 48 hours after inoculation.

Dormant tuber Fig. 9a shows the changes in total O₂ uptake, malonate sensitive O₂ uptake and malonate insensitive O₂ uptake by the explant derived from dormant tuber. The changes in inhibition rate by malonate are shown in Fig. 9b.

After slicing, the total O₂ uptake immediately began to increase in both auxin and control cultures, and the rise passed a peak at about 20 hours after inoculation, and the peak decreased in auxin culture more than in control culture.

The malonate insensitive respiration, probably *via* the PP pathway, began to increase in both cultures several hours after slicing, and its respiration in control culture reached to the plateau at about the 20th hour, while that in auxin culture still continued to increase gradually.

More drastic changes were those observed in the malonate sensitive respiration, probably *via* the TCA cycle. The malonate sensitive respiration began to increase very rapidly after slicing, which reached the maximum value at the 10th hour in auxin culture and at the 20th hour in control culture, then rapid decreases in both cultures succeeded. The malonate sensitive respiration in control culture again increased after the 24th hour, while in auxin culture it continued to decrease and was not detectable after the 40th hour.

Non-dormant tuber It is apparent from the results shown in Fig. 10 that the increase in O₂ uptake by the tissue derived from non-dormant tuber seems to be mainly attributed to the increase in the malonate insensitive respiration. The malonate sensitive respiration, particularly in control culture, remained constant throughout the initial 48 hours. In auxin culture the

TABLE 1. Growth and malonate sensitivity of respiration in aged callus tissue due to auxin treatment.

Time of culture	Fresh wt. per explant	O ₂ uptake/g fresh wt./hr		Malonate Inhibition
		Control	Malonate*	
21 day	164.7 mg	146.1 μ l	94.7 μ l	30.4 %
26	188.3	100.6	96.0	4.6
32	209.8	86.4	93.0	0

* $5 \times 10^{-2} M$

malonate insensitive respiration was intensified, while the malonate sensitive respiration was suppressed when compared with those in control culture after about the 30th hour.

(10) *Growth and respiration in aged callus tissue due to auxin treatment*

The growth and the respiratory pattern of aged auxin culture were studied in an extended period after 3 weeks (Tab. 1). After 3 week culture,

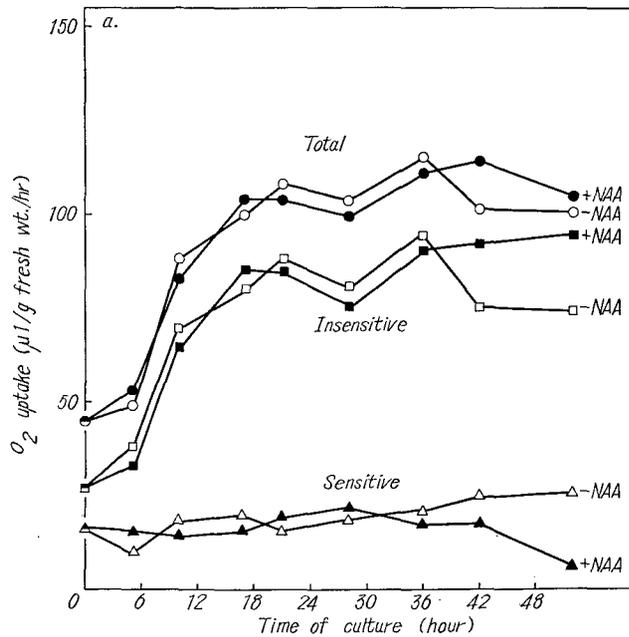


Fig. 10a. Changes in respiratory rate and its sensitivity to malonate ($5 \times 10^{-2} M$) during the initial 48 hour culture in the explant from non-dormant potato tuber.

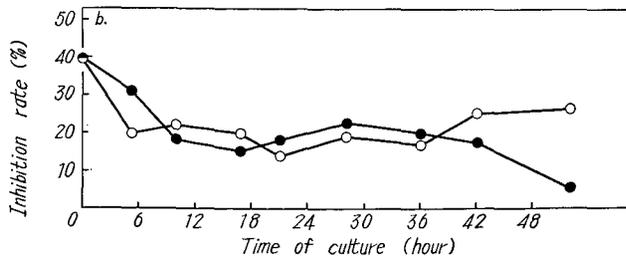


Fig. 10b. Changes in inhibition rate of respiration by malonate ($5 \times 10^{-2} M$) during the initial 48 hour culture in the explant from non-dormant potato tuber.

the growth rate of the explant was decreased and related to this, the rate of respiration of the explant was also decreased. This was mainly due to the reduction of the malonate sensitive respiration, and the auxin culture harvested on the 32th day after inoculation showed completely the malonate insensitive respiration.

Discussion

When the explant obtained from potato tuber showed an active growth on a nutrient medium supplemented with $1.6 \times 10^{-6} M$ NAA, both fresh weight and protein content of an explant increased to approximately 4 fold of the initial value at the end of 3 week culture (Fig. 1, 2).

The respiratory rate was found to increase to 2 to 3 fold immediately after inoculation (Fig. 3). This initial respiratory rise was also observed in control culture. Generally, when the storage tissues such as potato tubers are cut into slices and maintained aerobically at a moderate temperature, respiratory and other metabolic quickenings were known to occur in the slices. These phenomena termed the "developed respiration" by LATIES (5) have been investigated by many workers. As far as potato tissues are concerned, AP REES and BEEVERS (1) have shown that the PP pathway is a major component of the induced (developed) respiration. On the contrary, ROMBERGER and NORTON (11) and LATIES (6) have emphasized the activation of the TCA cycle in the developed respiration. According to the suggestion by LEE and CHANSON (7), the diverse results reflect differences in the methods employed to age the tissues. On the other hand, BENNET-CLARK and BEXON (2) have pointed out that aging effects may depend on whether the tissues are in the growing, dormant or sprouting phase. In the present investigation, it was found that the change in respiration in the explant from potato tuber cultured on a nutrient agar medium (control culture) depended upon the age of potato tubers used as culture materials. When dormant tubers were cut into slices, the inhibition of respiration by malonate (Fig. 4a, 9b) was increased, while those from non-dormant tubers decreased (Fig. 4b, 10b). Therefore, it is suggested that the relative participation of the TCA cycle became significant in the slices from dormant tubers, while the PP pathway became predominant during the rise of respiration in the slices from non-dormant tubers. It should be noticed that the respiratory pattern (malonate sensitivity) of dormant tuber tissue would be quickly (within 20 hours after slicing) shifted to such pattern of non-dormant tuber tissue by slicing followed by aging on the nutrient medium (Fig. 9b, 10b).

On the other hand, it was found that auxin affected the initial respiratory

rise qualitatively. That is, NAA has a tendency to suppress the activity of the TCA cycle and to stimulate the PP pathway activity. From the results of time-course studies on the sensitivity of respiration to malonate during the initial 48 hour culture, NAA effects on respiration seemed to occur in the explant after 20 hour incubation when dormant tubers were used (Fig. 9), or in the explant after 30 hour incubation when non-dormant tubers were used (Fig. 10).

When the respiratory rate remained relatively constant in the subsequent several days after the initial respiratory rise, the auxin effects on respiration became to be more remarkable. The sensitivity of respiration to the TCA cycle inhibitors disappeared completely on the 2nd day (dormant tuber tissue, Fig. 4a) or on the 5th day (non-dormant one, Fig. 4b, 5) after inoculation. The suppression of the glycolysis-TCA cycle and the stimulation of the PP pathway by NAA in this period were also suggested by the low inhibition rate of respiration by the glycolytic inhibitor, MIA (Fig. 6). Furthermore, the fact that the level of the DNP-induced respiration in auxin culture was lowered on the 5th day after the inoculation of non-dormant tuber tissue (Fig. 7b) supports that the glycolysis-TCA cycle activity was reduced in this period. As the RQ values were not high in this period (Fig. 8), the possibility of occurrence of the aerobic fermentation in these tissues could be neglected. Although the significance of suppression of the TCA cycle activity in the early stage of callus formation is not clear, it is suggested that the activated PP pathway by NAA may play an important role in the explant in which callus formation was initiated. MOROHASHI *et al.* (8) have reported that the activity of the PP pathway considerably increases in the cut region where the callus forms in a day after cutting in the *Stizolobium hassjoo* seedling incubated under the dark condition.

During the log phase when both fresh weight and protein content of an explant increased rapidly, the sensitivity of respiration to the glycolytic and the TCA cycle inhibitors restored and the inhibition rates gradually increased with the rise of respiratory rate (Fig. 4-6). These suggest that an increase of respiration during the log phase is associated with the rise of the glycolysis-TCA cycle activity. Respiratory quotient during this period remained unity (Fig. 8), therefore, it did not seem to be considered that the aerobic fermentation occurred on the course of callus development in potato tissue culture. On the contrary, KOMAMINE *et al.* (4) suggested that the relative participation of the PP pathway gradually increased and simultaneously alcohol fermentation occurred as callus developed in carrot root tissue culture.

On the subsequent culture period after 3 week when the growth rate of

the explant lowered, the sensitivity of respiration to malonate was reduced. GIBBS and BEEVERS (3) have found that the participation of the PP pathway increasingly became pronounced as the tissues age and differentiate. Similar situations were also observed in control culture. After the initial respiratory rise the respiratory rate of control culture gradually decreased as the tissue aged, simultaneously, the respiration became insensitive to the glycolytic and the TCA cycle inhibitors (Fig. 4-6).

Though it is known that the process of callus development in potato tissue culture involves two phases of cell growth, that is, cell division and cell expansion (9), the more details of callus development on the basis of cell growth have been investigated insufficiently yet. The relationships between the respiratory changes observed in the present investigation and the changing pattern of cell growth or other biochemical processes during callus development should be considered in future.

Summary

The respiratory changes during $1.6 \times 10^{-6} M$ NAA-induced callus formation in the potato tuber tissue cultured *in vitro* were investigated by using malonate, fluoroacetate, iodoacetate, and dinitrophenol. The following results were obtained.

1) After the lag phase extending over the initial 2 or 3 days, both the fresh weight and the protein content of an explant linearly increased to approximately 4 fold of the initial value at the end of 3 week culture.

2) The respiratory rate increased to 2 to 3 fold immediately after inoculation. In the initial respiratory rise, the inhibition rate of respiration by malonate became to be lower when compared with control culture (cultured without auxin). These auxin effects on respiration appeared 20 hours after inoculation when dormant tuber tissue was used as material, or 30 hours after when non-dormant one was used.

3) In the several days after the initial respiratory rise when the respiratory rate remained relatively constant level, the sensitivity of respiration to malonate and to fluoroacetate disappeared completely on the 2nd day (dormant tuber tissue) or on the 5th day (non-dormant one) after inoculation. Simultaneously, the sensitivity to iodoacetate decreased. The RQ values were between 1.0 to 1.1 in this period.

4) During the log phase when the respiratory rate increased gradually, the sensitivity to both glycolytic and TCA cycle inhibitors restored and gradually increased. The RQ values during the same period remained unity.

5) On the successive culture period beyond 3 week when the growth

rate of the explant attained a stationary phase, the sensitivity of respiration to malonate reduced accompanied by the decrease in respiratory rate.

These results suggest a conclusion that in the early stage of callus formation when the apparent growth was not noticeable, the TCA cycle activity once disappeared and the PP pathway may play an important role in the initiation of callus formation, while the callus was once developed, the restoration of the TCA cycle seems to be proportional to the rate of callus growth.

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