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Adenosine Phosphate and Nicotinamide Adenine Dinucleotide Pool Sizes during Shoot Initiation in Tobacco Callus[1]

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ABSTRACT

Shoot-forming tobacco callus is found to have high levels of adenosine phosphates and NAD^+, and a low energy charge during meristemoid and shoot primordium formation. NADH levels are low and show little change during this period. There is a decline in the content of NADPH to nondetectable levels during the process, and a transient increase of $NADP^+$ is observed early in culture. These patterns are indicative of a shift to a more intensive rate of metabolism during meristemoid and shoot primordium formation and apparently reflect the requirement for energy and reducing power during organ initiation.

The findings that starch accumulation occurs prior to the formation of meristemoids and shoot primordia (16, 24, 25) and that there is the need for a continuous supply of free sugars from the medium for shoot formation in tobacco callus (16) have led to the hypothesis that these carbohydrates are acting as a readily available reserve source of energy for the organogenetic process. High respiration rates (16) and an increase in the activity of enzymes of the EMP[2] and PP pathways during meristemoid and shoot primordia formation (21) have substantiated this view. Furthermore, it has been shown (3) that one-third of the medium-supplied sucrose is acting osmotically to promote shoot formation under optimum conditions (*i.e.* 3% sucrose, w/v), whereas the other two-thirds is used as a carbon energy source. Although this functional separation of the roles of the medium-supplied carbohydrate has been demonstrated, the actual degree of energy production by the tissue is unknown.

The high activity of the PP pathway during meristemoid and shoot primordium formation in tobacco callus (21) is also of particular interest as this pathway is the major source of reducing power (NADPH). In addition, there is a positive correlation between the content of malate and the organogenetic process (15). There is only a very transient buildup of malate in SF tissue early in culture, whereas in NSF callus, malate accumulates. These observations along with changes in the activities of enzymes involved in malate metabolism, indicate that the malate derived from dark CO_2 fixation is probably used to generate additional NADPH during organogenesis. These observations suggest that there is a major requirement for the production of both energy and reducing power for shoot formation.

The purpose of this study was 2-fold: (*a*) to determine energy production by measuring the energy charge and pool sizes of the adenosine phosphates (ATP, ADP, and AMP) and the nicotinamide adenine dinucleotides (NAD^+ and NADH); and (*b*) to estimate the production/utilization of reducing power by measuring the pool sizes of the nicotinamide adenine dinucleotide phosphates ($NADP^+$ and NADPH), during shoot formation in tobacco callus. For comparison with the SF tissue two controls were used; the first was tissue undergoing only callus proliferation on a NSF medium and the second control (SFG) was tissue grown on a SF medium in the presence of GA_3. Under these latter conditions, there is a repression of and considerable delay in shoot formation (9), as well as a reduction in the starch content of the tissue (23). In this way we could potentially separate those events involved only with growth from those associated with the organogenetic process. In earlier studies (*e.g.* 17) it was shown that the key histogenic events leading to shoot primordium formation takes place during the 6- to 12-day culture period. In this study, we were particularly concerned with the metabolic events occurring during that time.

MATERIALS AND METHODS

Culture Conditions. Tobacco (*Nicotiana tabacum* L. "Wisconsin 38") callus was isolated from stem pith segments and maintained on a modified Murashige and Skoog (10) medium following the method of Thorpe and Murashige (25). The SF medium contained Murashige and Skoog salts (10), 0.40 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, 10 μM IAA, 10 μM kinetin, 90 mg/l adenine sulfate, 50 mg/l L-tyrosine, and 170 mg/l $NaH_2PO_4 \cdot H_2O$. The medium was solidified with Difco Bacto-agar (0.9%, w/v). The GA_3 medium was the same as the SF medium, except it contained GA_3 at a level of 50 μM. The NSF medium was the same as that used to isolate the pith callus and contained 10 μM IAA and 2 μM kinetin. The supplements were added to the medium before autoclaving.

For study four pieces of 4- to 6-week old stock callus, each measuring about 2 × 4 × 4 mm, were placed in each 125-ml Erlenmeyer flask containing 50 ml of medium and maintained in the dark at 27 ± 1 C. All experiments had three replicates and have been repeated at least once.

Adenosine Phosphates. ATP, ADP, and AMP were isolated and assayed by a modified technique after Bewley and Gwóźdź (2). About 0.3 g tissue was ground in a ground-glass homogenizer immediately after harvest in 1 ml of cold 35% $HClO_4$, washed two times, and held on ice for 15 min before centrifuging for 25 min at 4 C at 20,000*g*. The pH of the supernatant was adjusted to 7.0 ± 0.1 with cold 6.0 N KOH containing 50 mM K_2HPO_4. After 30 min on ice, the neutralized supernatant was centrifuged for 15 min at 4 C at 20,000*g*. The resulting supernatant was made up to 25 ml with distilled H_2O and stored frozen at −25 C. Treatment samples to be assayed were diluted in 0.25 M Hepes containing 0.25 M Mg-acetate and assayed as previously described (2), except that the incubated mixture was diluted to 1/10 with distilled H_2O before storing at −25 C. The energy charge was calculated after Atkinson (1) by the formula:

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[2] Abbreviations: EMP: Embden-Meyerhof-Parnas; PP: pentose phosphate; SF: shoot-forming; NSF: non-shoot-forming; SFG: shoot-forming in the presence of gibberellic acid; GA_3: gibberellic acid.

$$\text{energy charge} = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$$

Nicotinamide Adenine Dinucleotides. NAD^+, NADH, $NADP^+$, and NADPH were extracted and assayed by the polarographic recycling enzyme technique after Greenbaum *et al.* (5) using a YS1 model 53 biological oxygen monitor with a YS1 5331 O_2 probe in a 2-ml assay volume. The technique was modified by grinding about 1 g tissue, immediately after harvest, in 3 ml hot 0.1 M HCl for the oxidized forms, and 0.1 M KOH for the reduced forms for 30 s using a ground-glass homogenizer. The extract was heated for 1 min in a boiling water bath, reground for 30 s, heated for a further 1 min, and then stored on ice until cold. The pH was adjusted to 7.0 ± 0.1 with 4 N HCl or KOH and frozen at −25 C. Samples were assayed within 24 h.

RESULTS

Adenosine Phosphates. The levels of adenosine phosphates in SF, NSF, and SFG tissues all increased by day 3 in culture with the highest levels occurring at day 6 (Fig. 1). However, the levels of ATP, ADP, and AMP were 28, 101, and 187% higher in SF tissue than in NSF tissue, and 50, 62, and 44% higher than in SFG tissue at that time. (Percentages were calculated from raw data.) After day 3, there was a general decline in the levels through day 15 with the exception of SF tissue which had high ATP and ADP levels at day 15. The difference at day 6 and thereafter between SF tissue and the other treatments is more dramatic when the total adenosine phosphates (ATP + ADP + AMP from Fig. 1) for each tissue is compared. Total levels are 66 and 54% greater in SF tissue than in NSF and SFG tissues, respectively, at day 6 and higher levels were maintained in the SF tissue throughout the rest of the culture period (Fig. 2).

Energy Charge. Using Atkinson's definition, energy charge was calculated from the data shown in Figure 1 and was found to be lowest in the SF tissue from day 6 to day 12. The energy charge dropped from 0.84 to about 0.61 at day 3, reaching a low of 0.54 in the SF tissue at day 9 (Table I). Although the energy charge of the SFG tissue was only slightly higher than the SF tissue during the 6- to 9-day period, the energy charge in the NSF tissue was relatively higher at about 0.73 during this time. By day 15, the energy charge in all tissues was increasing and had reached about 0.71.

Nicotinamide Adenine Dinucleotides. Levels of NAD^+ increased sharply after subculture and had doubled in all tissues by day 3 (Fig. 3). Thereafter, there was a decline in levels in NSF and SFG tissues, whereas SF tissue maintained high levels of NAD^+ from day 3 through 15 with highest levels occurring at day 6. NADH levels were very low and showed little change throughout the study period (Fig. 3).

As with the NAD^+ and adenosine phosphates, $NADP^+$ levels rose sharply after subculture in all tissues reaching the highest level in SF tissue at day 6 (Fig. 4). This was a 222% increase from day 0. Thereafter, levels declined. In NSF and SFG tissues (Fig. 4) $NADP^+$ levels peaked at day 3 rather than day 6 and showed less of an increase on day 6 relative to day 0 (123 and 159%, respectively).

NADPH levels, as shown in Figure 4, showed a much different pattern. In SF tissue levels declined immediately and reached nondetectable levels by day 9. In NSF tissue, the NADPH levels were variable and were at 47% of the original level by day 15.

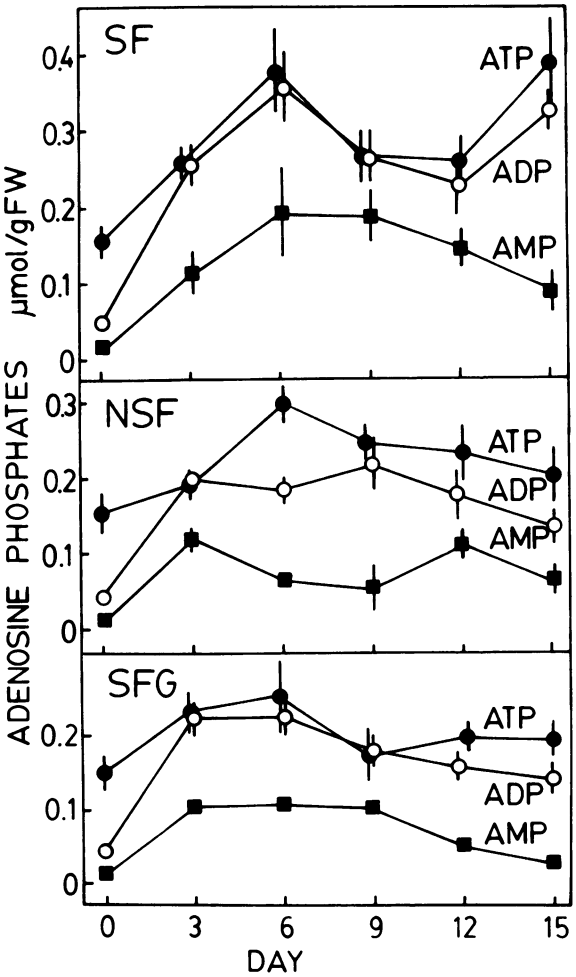

FIG. 1. Changes in content of adenosine phosphates in SF, NSF, and SFG tobacco callus during culture. Values, ± SE, represent means of six replications. ATP (●), ADP (○), and AMP (■).

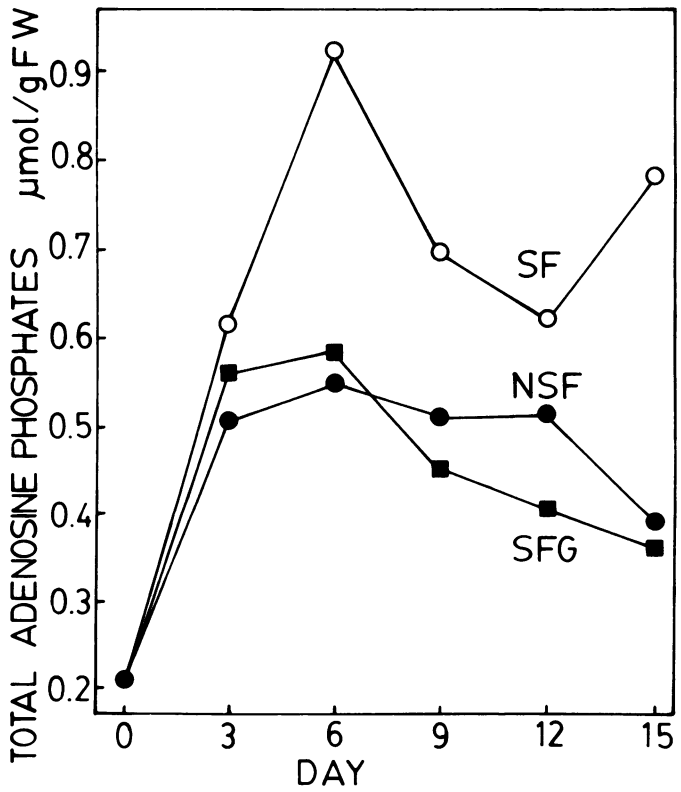

FIG. 2. Changes in content of ATP + ADP + AMP in SF, NSF, and SFG tobacco callus during culture. Totals represent sum of means of six replications shown in Figure 1.

Table I. *Energy Charge, ± SE, of SF, NSF, and SFG Tobacco Callus during First 15 Days in Culture*

Energy charge is calculated as described under "Materials and Methods" from data shown in Figure 1.

Day	Energy Charge		
	SF	NSF	SFG
0	0.84 ± 0.02	0.84 ± 0.02	0.84 ± 0.02
3	0.61 ± 0.04	0.57 ± 0.02	0.62 ± 0.04
6	0.60 ± 0.06	0.71 ± 0.03	0.62 ± 0.03
9	0.54 ± 0.05	0.75 ± 0.05	0.58 ± 0.03
12	0.59 ± 0.06	0.62 ± 0.04	0.68 ± 0.04
15	0.69 ± 0.03	0.68 ± 0.04	0.73 ± 0.02

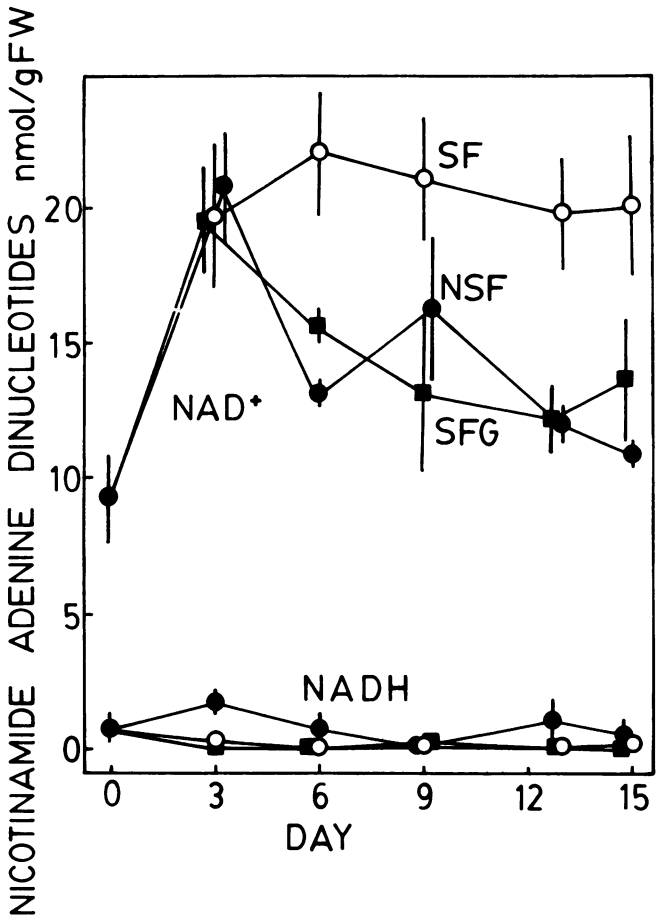

FIG. 3. Changes in content of NAD^+ and NADH in SF (○), NSF (●), and SFG (■) tobacco callus during culture. Values, ± SE, represent means of three replications.

Levels in SFG tissue declined to nondetectable levels by day 9 as in the SF tissue, but increased to measurable levels beyond that. At day 15 the NADPH level was 17% of the day 0 level. The difference at day 6 is very dramatic if the $NADP^+$/NADPH ratios are compared. The ratio increased by over 10-fold in SF tissue going from 0.43 at day 0 to 4.74 at day 6. On the other hand, in the NSF tissue the ratio changed marginally from 0.43 to 0.58, and in SFG tissue it increased only 4-fold from 0.43 to 1.78.

DISCUSSION

The developmental sequence leading to shoot formation in tobacco callus has been described (17, 25) and although this sequence is not completely synchronous, key physiological events

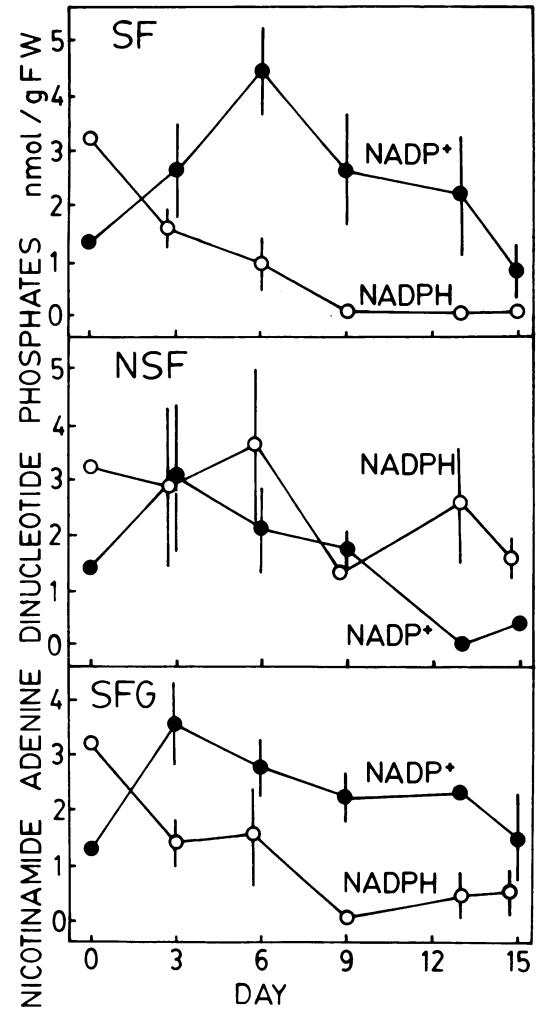

FIG. 4. Changes in content of $NADP^+$ (●) and NADPH (○) in SF, NSF, and SFG tobacco callus during culture. Values, ± SE, represent means of three replications.

and the histological changes leading to shoot primordium formation occur during the first 12 days in culture. Zones of preferential cell division activity occur in the lower half of the callus by days 7–8 in culture. Within these zones, meristemoids or meristem-like masses of cells are formed beginning around day 9. These meristemoids become shoot primordia beginning around day 12 and the buds begin to emerge from the lower half of the callus by day 14. The peak of starch accumulation is at day 6 in the lower or SF portion of the callus and day 9 in the upper portion and whole callus (16). [1-^{14}C] and [6-^{14}C]Glucose oxidation is high, particularly at day 9 when the PP pathway is relatively more active than the EMP pathway (21). Parallel enzyme studies reflected the same patterns (21). Key enzymes in both the PP and EMP pathways showed increased activity in the day 6–12 period with the PP pathway enzymes showing the greater increase.

The high energy requirement of SF tobacco callus during the 6- to 12-day period of meristemoid and primordium formation was confirmed in this study, as the SF tissue contained higher levels of adenosine phosphates at day 6 and beyond than tissues grown under either of the other culture conditions (Fig. 2). In the day 6–12 period a relatively high proportion of adenosine phosphates were in the mono- and diP form in the SF tissue (Fig. 1), which indicates a high rate of dephosphorylation of ATP. This is supported by the low energy charge in the SF tissue relative to the NSF tissue during this same period (Table I). The SFG tissue also had a low energy charge during the 6- to 12-day culture period

but it was not accompanied by an increase in adenosine phosphate levels. SFG tissue will also form shoots but at a much reduced and delayed rate. Under our present culture conditions, less than one-fourth of the SFG tissue will form shoots. By day 15 no primordia are observed, and these begin to appear by days 25–28 in culture in a few of the SFG tissues. These tissues grow at a faster rate than NSF tissues (19, 22) and this may be the reason for the increased utilization of ATP compared to the NSF tissue. The SF tissue grows at a slower rate than either NSF or SFG tissues until the primordia begin to develop. Only then, *i.e.* beyond day 18 in culture, is there a dramatic increase in fresh or dry weight of the SF tissue (Brown and Thorpe, unpublished). Thus, the high rate of ATP utilization and the low energy charge are not a consequence of the growth rate of the SF tissue.

The relatively high content of NAD^+ in SF tissue from day 6 onward, (Fig. 3) indicates an increased production of this cofactor. Its presence in the oxidized form shows a rapid conversion of the NADH form, probably via oxidative phosphorylation for the production of ATP. An alternative use could be for NADPH production from transhydrogenation, during which a net hydrogen transfer from NADH to $NADP^+$ could take place (26). A low NADH level is also consistent with the observation that isolated plant mitochondria are more active when the NADH level is low (4). Thus the low NADH level supports the concept of high energy production during shoot initiation.

The apparent requirement for NADPH for reductive biosynthesis of cellular constituents during shoot formation as suggested by the enhanced activity of the PP pathway (21) and the pattern of malate metabolism (15) is also supported by this study. The pool of NADPH is used most completely in the SF tissue and the concomitant increase in $NADP^+$ at day 6 suggests that during this time the greatest demand for reducing power occurs (Fig. 4). This interpretation is strengthened when the $NADP^+$/NADPH ratios are examined.

The patterns that have been correlated with shoot formation are particularly those changes relative to callus growth in the NSF tissue. Previous studies with cell suspension cultures of several species (6, 8, 11–14, 18) and reviews (7, 27, 28) have established a pattern of metabolic events which characterize cell growth. Our callus control, grown on NSF medium in a semisolid-state, shows patterns of respiration (16), glucose oxidation (21), EMP, and PP pathway enzyme activity (21) and cofactor pool size changes (as shown in this study) which follow the established pattern. The most predominant event is a high respiration rate early in culture which decreases thereafter. In tobacco callus this respiratory peak occurs by day 3, whereas it is at day 2 in *Acer* (18) and *Daucus* (8) and at day 1 in *Vinca* (7). This peak has been associated with a wounding response in tobacco (16), and as seen in this study, the nucleotide pool size changes are similar to each other in the different treatment tissues. Thus the magnitude of these changes are of little direct importance for shoot initiation. The respiratory peak has been shown to occur at the S and late G_2 phases of cell division in *Acer* (6) and *Vinca* (7). Although there are no discrete data on the cell cycle in tobacco callus, the respiration peak occurs prior to any observable cell division (19). Highest levels of adenosine phosphates and lowest energy charges occur about the time of peak respiration with high EMP and PP pathway enzyme activities also occurring at this time or later depending on the species (7, 18).

From the data in this and previous studies (15–17, 21, 25) it appears that the shift from growth to shoot formation involves an intensification and prolongation of some of the metabolic events that occur during the initiation of growth. This period of greater metabolic activity is extended to days 6–12 when meristemoids and shoot primordia are being formed. The patterns of metabolic activity associated with the developmental changes leading to shoot primordium formation in the 6–12-day period, which arise from this study, include the presence of high adenosine phosphate and NAD^+ levels, a low energy charge and a complete utilization of NADPH. There is also transient peak of $NADP^+$ and adenosine phosphates at day 6. Previous studies have indicated that there is increased respiration, and that the accumulated starch along with free sugars from the media supply the substrate for energy production via glycolysis and oxidative phosphorylation. Malate from nonautotrophic CO_2 fixation could supply the substrate for NADPH production via decarboxylation by $NADP^+$-malic enzyme. In addition, NADPH would also be produced through the enhancement of the PP pathway. The interrelationship of these various aspects of metabolism and their importance during organogenesis has been discussed recently (20).

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