

ACTIVITIES OF TRICARBOXYLIC ACID CYCLE ENZYMES IN  
AN AFLATOXIGENIC STRAIN OF *ASPERGILLUS*  
*PARASITICUS* AFTER A PEPTONE TO GLUCOSE  
CARBON SOURCE SHIFT

A sequential technique involving the transfer of mycelia from a peptone-based, aflatoxin-nonsupporting medium to a glucose-based, aflatoxin-supporting medium was used to study possible relationships between tricarboxylic acid cycle activity and aflatoxin biosynthesis in *Aspergillus parasiticus*. Analysis indicated that the specific activities of various TCA cycle enzymes are influenced by duration of post-transfer incubation and an apparent carbon catabolite repression. The results support the hypothesis that aflatoxin biosynthesis occurs during a period of depressed TCA activity, which is dependent on the catabolism of a suitable carbohydrate.

Bioregulation of aflatoxin synthesis is assumed to require a complex integration of a number of regulatory loci controlling the catabolism of carbohydrate sources to acetyl-CoA, as well as the diversion of the two carbon units into polyketide formation. As would be expected with a process that involves the generation and utilization of acetyl-CoA, aflatoxin synthesis appears to be highly dependent on the activity of the tricarboxylic acid (TCA) cycle. It has been suggested that the initiation of aflatoxin synthesis is dependent on the accumulation of TCA cycle intermediates and/or pyruvate resulting from depressed TCA cycle activity (Detroy & Hesseltine, 1970; Maggon, Gupta & Venkitasubramanian, 1977; Venkitasubramanian *et al.*, 1982). However, the importance of an accumulation of TCA cycle intermediates in regard to the regulation of aflatoxin synthesis is not clear since other investigators have reported that various intermediates stimulate TCA cycle activity and depress aflatoxin synthesis (Buchanan & Ayres, 1977; Shantha & Murthy, 1981).

Changes in the activities of TCA cycle enzymes over the growth cycle of *Aspergillus flavus* Link: Fr. and *A. parasiticus* Speare have been reported and temporally correlated with aflatoxin synthesis (Gupta, Maggon & Venkitasubramanian, 1977*a,b*; Venkitasubramanian *et al.*, 1982). These studies suggested that most TCA cycle enzymes had depressed activities during the period of aflatoxin synthesis, except for citrate synthase which had elevated activity. Maggon *et al.* (1977) suggested that this resulted in an accumulation of citrate

which acted as a source of acetyl-CoA for aflatoxin synthesis. However, as is often the case when attempting to study factors affecting the synthesis of secondary metabolites, it is difficult to distinguish effects which are the result of changes in growth patterns from others that more directly influence aflatoxin synthesis. Recently, a nutritional shift protocol (Abdollahi & Buchanan, 1981*a,b*; Buchanan & Lewis, 1984) was developed that allows the factors influencing aflatoxin synthesis to be studied, while largely eliminating growth effects. Using this technique to evaluate how various enzymes of *A. parasiticus* are influenced by the catabolism of glucose, carbon catabolite repression of TCA cycle enzymes was identified as a possible regulatory site influencing aflatoxin synthesis (Buchanan & Lewis, 1984). The current study reports a detailed examination of how the activities of TCA cycle enzymes of *A. parasiticus* are affected when the mould is shifted from an aflatoxin-nonproducing to an aflatoxin-producing state.

#### MATERIALS AND METHODS

##### *Micro-organism*

*Aspergillus parasiticus* NRRL 2999 was used throughout the study. The mould was maintained on potato dextrose agar (Difco\*) slants, and spore suspensions prepared as previously described (Tice & Buchanan, 1982).

\* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

*Culture techniques*

The mould was grown using the sequential culturing technique described by Buchanan & Lewis (1984). Six 1.0 l Erlenmeyer flasks containing 250 ml of YES medium were each inoculated with 1.0 ml of spore suspension to achieve an inoculum of approximately  $4 \times 10^3$  conidia ml<sup>-1</sup>. All flasks were incubated on a rotary shaker (150 rev./min) at 28 °C for 72 h. Mycelial pellets were then collected and pooled on cheese-cloth, rinsed with sterile 0.85% KCl, and transferred to a sterile blender containing 450 ml of 0.85% KCl. After homogenizing at high speed for 1 min, the mycelia were recollected on cheese-cloth and rinsed thoroughly with 0.85% KCl. The mycelia were then divided into equal portions and transferred to eight 1.0 l Erlenmeyer flasks containing 300 ml of PMS. These flasks were incubated on a rotary shaker (150 rev./min) for 24 h at 28 °.

The mycelia were collected and pooled on cheese-cloth, rinsed thoroughly with 0.85% KCl, and divided into 2.0 g portions. Four portions were immediately frozen in liquid N<sub>2</sub>. The remaining portions were transferred to 50 ml Erlenmeyer flasks containing 15 ml of PMS or GMS. All flasks were incubated without agitation at 28 °.

After various periods of incubation, triplicate cultures of each medium were removed, and each mycelium collected on cheese-cloth. The medium was drained into a plastic tube, and after determination of pH, the collected medium was frozen for subsequent aflatoxin analysis. The harvested mycelia were rinsed thoroughly, and immediately frozen in liquid N<sub>2</sub>. All frozen mycelium samples were subsequently freeze-dried, dry weights determined, and stored at -20 ° while awaiting enzyme analyses.

*Aflatoxin analysis*

The levels of extracellular aflatoxins B<sub>1</sub>+B<sub>2</sub>+G<sub>1</sub>+G<sub>2</sub> were used as an estimate of total aflatoxin production. Aliquots, 5.0 ml, of the collected medium from each of the triplicate cultures per sampling time were individually extracted thrice with 5.0 ml portions of chloroform. The extracts were cleaned-up using the silica gel cartridge technique of McKinney (1981),

*Enzyme analyses*

Cell extracts were prepared using a modification of the procedure of Niehaus & Dilts (1982). Approximately 50 mg of freeze-dried mycelium was rehydrated in 1.5 ml of cold pH 7.2 buffer (50 mM-K<sub>2</sub>HPO<sub>4</sub>, 1 mM-EDTA, 2 mM mercaptoethanol, 0.5 mM phenylmethyl sulphonyl fluoride), and immediately disrupted using a teflon on glass homogenizer. The suspension was then centrifuged (7000 g) for 10 min at 4 °, and the supernatant employed for enzyme analyses. Minor modifications of published protocols were used to assay for pyruvate dehydrogenase (Schwartz & Reed, 1970), citrate synthase (Srere, 1969), aconitase (Hansen & Cox, 1967), NAD: isocitrate dehydrogenase (Winskill, 1983; Kelly & Haynes, 1982), NADP: isocitrate dehydrogenase (Kelly & Haynes, 1982), α-ketoglutarate dehydrogenase (Carls & Hansen, 1971), fumarase (Hansen & Cox, 1967), and malate dehydrogenase (Worthington, 1972). At least three replicate cultures per sampling time were assayed for each of the enzymes. No attempt was made to distinguish mitochondrial and cytosolic isoenzymes. Protein levels were determined by the method of Lowry, Rosenbrough, Farr & Randall (1951) using bovine serum albumin as a standard.

*Respirometry*

The mould was sequentially cultured in YES and PMS as outlined above. The mycelia were transferred in 200 mg portions to 12 Warburg flasks containing 2.5 ml of PMS, and to a second set of 12 flasks containing GMS. Six flasks of each set were used to determine oxygen uptake, while the remaining six flasks were used to estimate CO<sub>2</sub> production. All flasks were incubated without agitation at 28 °. At various times during the incubation period, the flasks were connected to a respirometer (model G20, Gilson Inc.), and oxygen uptake and CO<sub>2</sub> evolution monitored for 20 min according to standard techniques (Umbriet, Burris & Stauffer, 1957).

## RESULTS

The typical pattern for mycelial dry weight, pH, and aflatoxin production by *A. parasiticus* transferred from PMS to GMS and PMS is depicted in Fig. 1. The post-transfer PMS mycelia varied little in dry weight, while the GMS cultures increased in dry weight by approximately 50% between 22 and

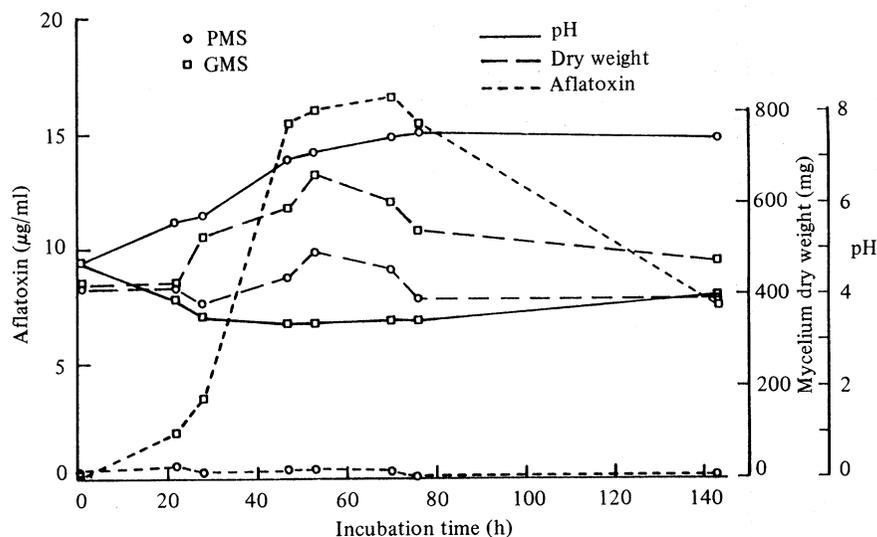


Fig. 1. Mycelial dry weight, pH, and aflatoxin production by *A. parasiticus* precultured in PMS and then transferred to PMS and GMS. Values represent means of three replicate cultures.

53 h of incubation. It is likely that the majority of this increase represents an accumulation of secondary metabolites (i.e. lipids, polyketides, etc.) derived from glucose (Buchanan & Lewis, 1984). PMS cultures increased in pH, while a depression of pH occurred in the GMS cultures. Aflatoxin production was evident in the GMS cultures after 22–24 h, and the concentration of extracellular aflatoxins increased rapidly thereafter. Maximum levels were generally reached between 47 and 53 h. *De novo* aflatoxin production was not evident in the PMS cultures over the course of the incubation. Analysis of pretransfer mycelia indicated that the trace levels of aflatoxin detected at times in the PMS cultures were attributable to a small 'carry-over' from the initial growth of the mould in YES. While relatively large differences in the amount of aflatoxin produced by GMS cultures were observed among replicate experiments performed on several occasions, the general pattern of *de novo* toxin synthesis in the GMS, but not in the PMS cultures was observed consistently.

As a preliminary means of assessing the activity of the TCA cycle in PMS and GMS cultures, oxygen uptake and carbon dioxide evolution by the mould was monitored. Oxygen uptake (Fig. 2a) increased during the initial period after transfer, and then declined with further incubation. No difference in oxygen uptake was evident between the GMS and PMS cultures except possibly during the latter phases of the incubation. Carbon dioxide

evolution (Fig. 2b) generally followed the pattern observed for oxygen uptake except that the GMS cultures consistently produced more carbon dioxide than the PMS cultures. Comparison of  $\text{CO}_2/\text{O}_2$  ratios (Fig. 2c) were indicative of GMS cultures being strongly fermentative immediately after transfer.

Relatively large variations in the specific activities of assayed enzymes among replicate cultures were observed; however, certain trends were apparent. Pyruvate dehydrogenase activity (Fig. 3) was relatively low and constant over the course of the incubation period except for a short period of increased activity immediately after transfer. No differences were apparent between the GMS and PMS cultured mycelia.

GMS and PMS cultures had opposite patterns of citrate synthase activity (Fig. 4) over the course of the incubation period. Activity in GMS mycelia was constant immediately after transfer, and then decreased upon further incubation. After 48 h the GMS cultures had essentially nondetectable levels of activity. Conversely, the PMS cultures had depressed levels of citrate synthase activity immediately after transfer, but increased activity during the period equivalent to that for aflatoxin production in the glucose-containing medium. Further incubation resulted in a second decline in activity; however, unlike the GMS cultures, substantial citrate synthase activity was still evident in the PMS cultures at the end of the incubation period.

## TCA and aflatoxin production

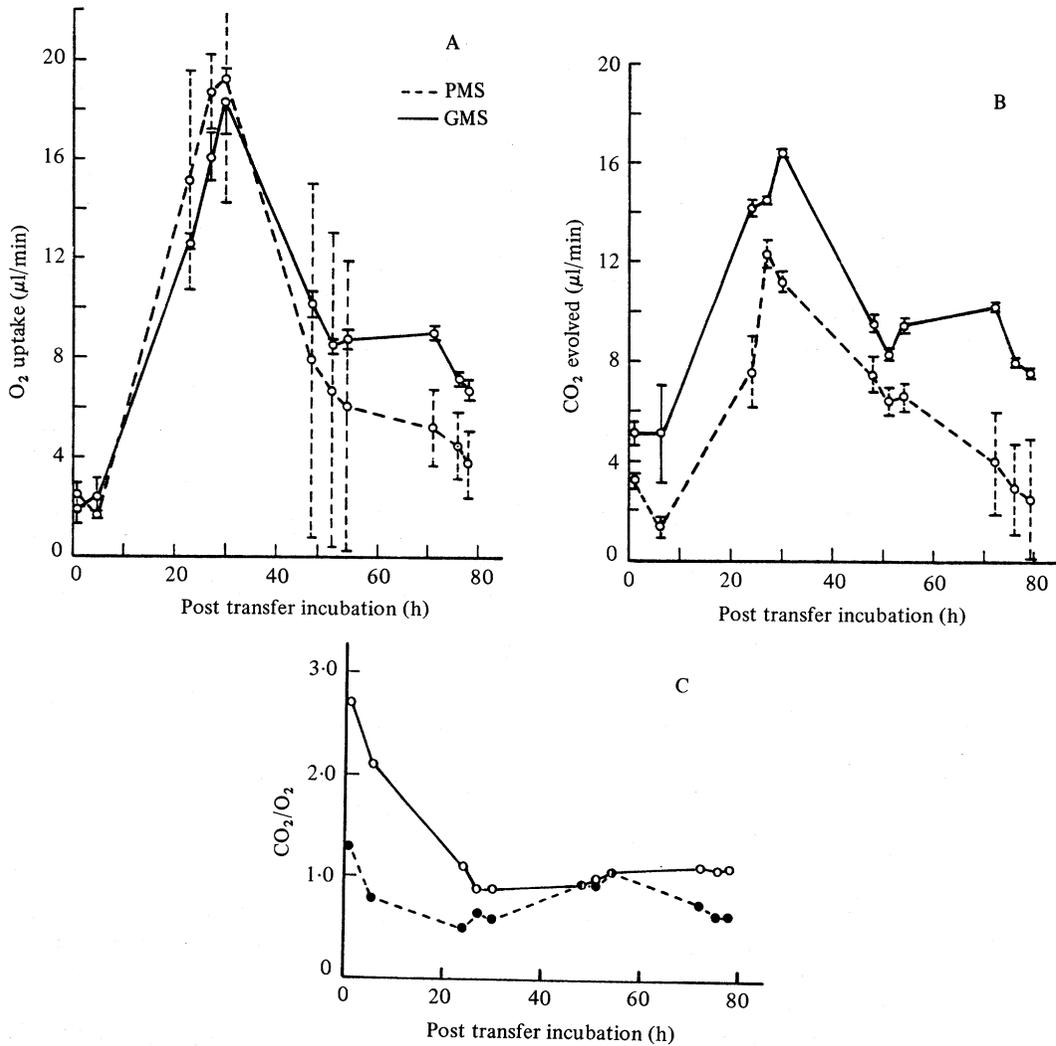


Fig. 2. Oxygen (A), carbon dioxide evolution (B), and CO<sub>2</sub>/O<sub>2</sub> ratio (C) by PMS-cultured *A. parasiticus* after transfer to GMS and PMS. Values represent  $\bar{X} \pm$  s.d.

Aconitase activity (Fig. 5) in the GMS cultures declined steadily with time. The PMS cultures evidenced a similar loss of aconitase activity; however, these cultures had consistently greater activity than that observed with the GMS cultures.

NAD-linked isocitrate dehydrogenase (Fig. 6) activity was low and constant in both PMS and GMS cultures. It appears that this enzyme is relatively unimportant in regard to the TCA cycle of *A. parasiticus*. However, this supposition will require further evaluation since enzyme instability or lack of appropriate activating compounds cannot be ruled at the present time. The NADP-linked

isoenzyme appeared to account for the majority of the isocitrate dehydrogenase activity detected within the mould (Fig. 7). In the GMS cultures, NADP-linked dehydrogenase activity increased during the first 24h of incubation, and then declined as the mould entered the period corresponding to active aflatoxin production. The aflatoxin-nonproducing PMS cultures displayed even greater increases in activity immediately after transfer, and had equivalent levels of activity during the latter stages of the incubation period.

Alpha-ketoglutarate dehydrogenase (Fig. 8) acti-

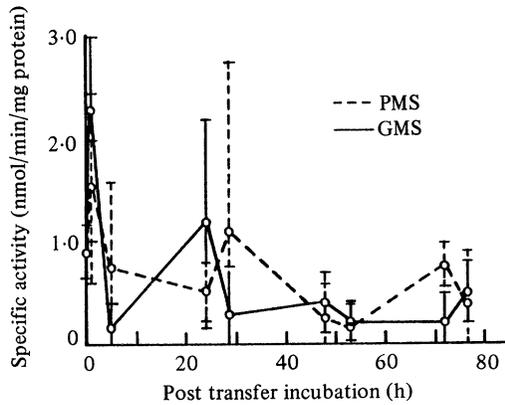


Fig. 3. Specific activity ( $\bar{X} \pm s.d.$ ) of pyruvate dehydrogenase in PMS-cultured *A. parasiticus* after transfer to GMS and PMS.

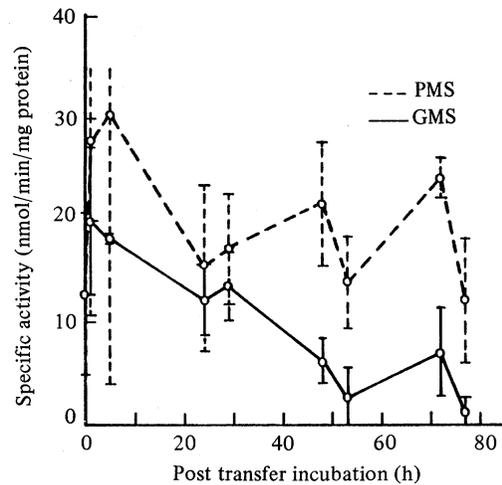


Fig. 5. Specific activity ( $\bar{X} \pm s.d.$ ) of aconitase in PMS-cultured *A. parasiticus* after transfer to GMS and PMS.

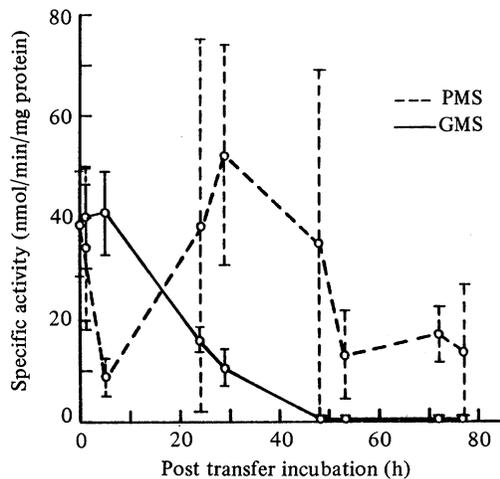


Fig. 4. Specific activity ( $\bar{X} \pm s.d.$ ) of citrate synthase in PMS-cultured *A. parasiticus* after transfer to PMS and GMS.

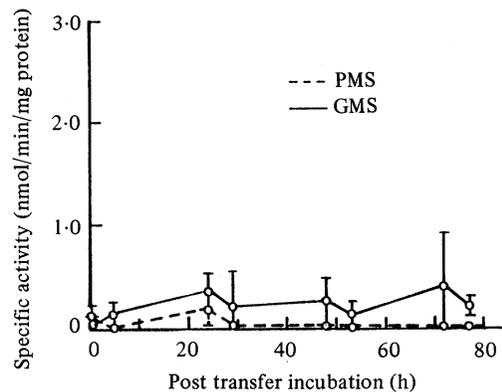


Fig. 6. Specific activity ( $\bar{X} \pm s.d.$ ) of NAD: isocitrate dehydrogenase in PMS-cultured *A. parasiticus* after transfer to GMS and PMS.

vity was low and constant in the GMS cultures. The PMS-cultured mycelia had similar levels of activity during the beginning and end of the post-transfer incubation; however, during the period corresponding to aflatoxin production,  $\alpha$ -ketoglutarate dehydrogenase activity in the PMS mycelia was increased approximately twofold.

Differences in fumarase activity (Fig. 9) were observed both in regard to incubation time and carbon source. In the aflatoxin-producing GMS cultures, activity declined steadily; approximately twelvefold by 48 h. PMS cultures also evidenced an overall decline in activity with time; however, the

timing of this was different from that observed with the GMS cultures. After an initial decrease, fumarase activity in the PMS cultures remained relatively constant through 53 h, while decreased levels of activity were observed in the 72 and 77 h cultures.

The most consistent differential in enzyme activity between the PMS and GMS cultures was observed with malate dehydrogenase (Fig. 10). In the PMS cultures, activity became elevated immediately upon transfer, and remained elevated throughout the course of the post-transfer incubation. Conversely, malate dehydrogenase activity in

## TCA and aflatoxin production

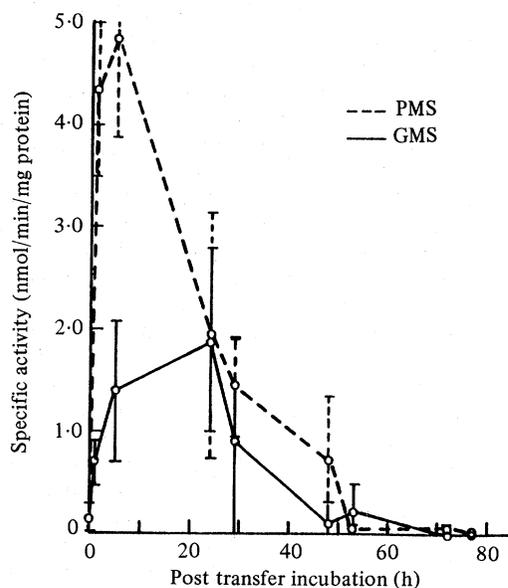


Fig. 7. Specific activity ( $\bar{X} \pm s.d.$ ) of NADP:isocitrate dehydrogenase in PMS-cultured *A. parasiticus* after transfer to GMS and PMS.

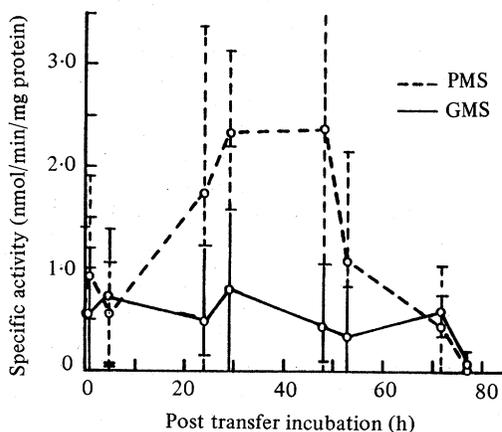


Fig. 8. Specific activity ( $\bar{X} \pm s.d.$ ) of  $\alpha$ -ketoglutarate dehydrogenase in PMS-cultured *A. parasiticus* after transfer to GMS and PMS.

the GMS cultures remained largely unchanged with time.

### DISCUSSION

The patterns of TCA cycle enzyme activity observed after transfer of PMS-cultured *A. parasiticus* to GMS and PMS indicate that both carbon source and duration of post-transfer

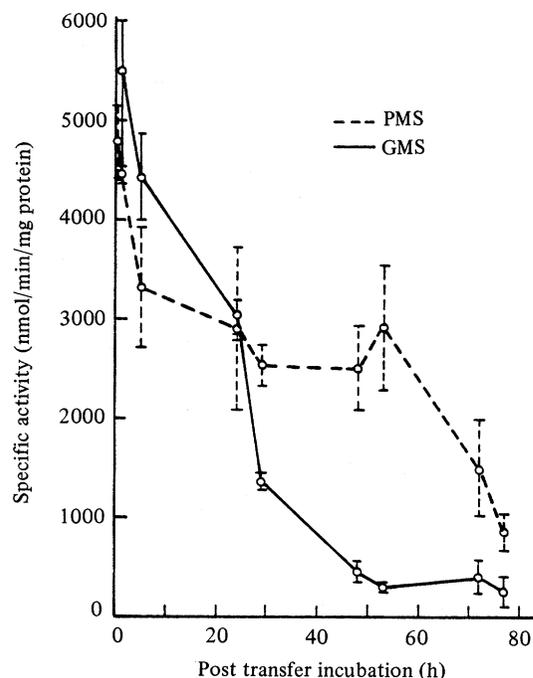


Fig. 9. Specific activity ( $\bar{X} \pm s.d.$ ) of fumarase in PMS-cultured *A. parasiticus* after transfer to GMS and PMS.

incubation influence various components of the cycle. In regard to duration of post-transfer incubation, a number of the differences noted between the media appear to correlate temporally with the ability of these media to support the production of aflatoxins. In the GMS cultures, four enzymes, citrate synthase, aconitase, NADP:isocitrate dehydrogenase and fumarase, decreased in specific activity prior to, or just as the cultures began producing aflatoxins. Equivalent decreases in the specific activities of citrate synthase and fumarase were not evident in the PMS-cultured mycelia, suggesting that depressed levels of these enzymes may be important in regard to the initiation of aflatoxin synthesis. These results are in direct contrast with those reported by Venkatasubramanian *et al.* (1982), who found that citrate synthase and pyruvate dehydrogenase activity was elevated just prior to or during the period of aflatoxin synthesis. These differences may reflect the use of cultures initiated from a conidial inoculum versus the sequential culturing technique employed in the current study. However, the current results do not support their hypothesis that increased citrate synthase and pyruvate dehydro-

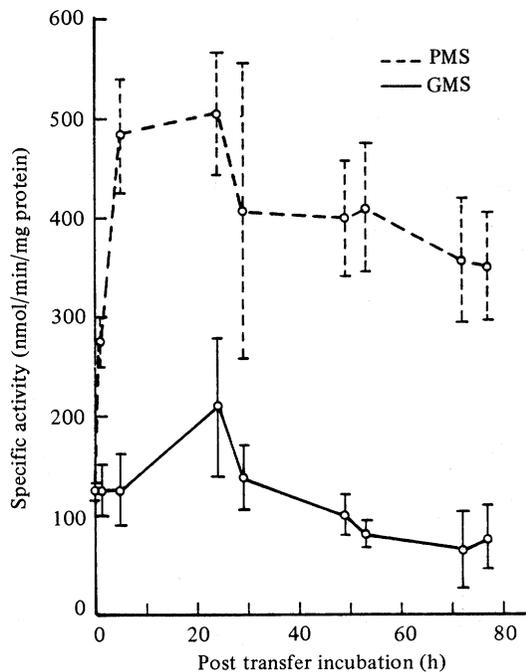


Fig. 10. Specific activity ( $\bar{X} \pm s.d.$ ) of malate dehydrogenase in PMS-cultured *A. parasiticus* after transfer to GMS and PMS.

genase activity are directly related to the initiation of aflatoxin synthesis.

The specific activities of a number of the enzymes evaluated were consistently lower in the glucose-cultured mycelia as compared to equivalent PMS cultures. This suggests that either these enzymes were repressed by glucose or induced by peptone. That a readily catabolizable carbohydrate source can repress TCA cycle enzymes has been well-established in a variety of prokaryotic and eukaryotic species including other fungi such as *Neurospora crassa* Shear & Dodge (Benveniste & Munkres, 1970; Schwitzguebel & Palmer, 1981) and *Aspergillus niger* Van Tiegh. (Ma, Kubicek & Rohr, 1981). It is not unexpected that *A. parasiticus* showed a similar response. However, care must be exercised in interpreting the results in that the relative activities of cytosolic and mitochondrial isoenzymes were not differentiated in the current study, and could be of significance in regard to mechanisms involved in the diversion of two carbon units into polyketide synthesis. For example, Benveniste & Munkres (1970) reported that most of the malate dehydrogenase activity in *N. crassa* was associated with the cytosolic isoenzyme, and only it was repressed by glucose. Using *A. niger*,

Ma *et al.* (1981) also found that most of the malate dehydrogenase activity was associated with a cytosolic isoenzyme; however, in this species both the cytosolic and mitochondrial isoenzymes appeared to be equally repressed by various carbon sources. Increased cytosolic malate dehydrogenase activity has been correlated with increased glyoxylate cycle activity (Schwitzguebel & Palmer, 1981); however, preliminary studies (data not shown) have indicated that there are no differences in the specific activity of isocitrate lyase between GMS and PMS cultured *A. parasiticus*.

In the current study, enzymes showing depressed activity when *A. parasiticus* was cultured in a glucose-based medium included citrate synthase, aconitase,  $\alpha$ -ketoglutarate dehydrogenase, fumarase, malate dehydrogenase, and possibly NADP: isocitrate dehydrogenase. Of these, citrate synthase,  $\alpha$ -ketoglutarate dehydrogenase, and fumarase displayed an apparent incubation duration dependent response, with the greatest differential between the GMS and PMS cultures occurring during the period of active aflatoxin synthesis.

Several of the enzymes had rapid increases in specific activity immediately upon transfer to PMS and GMS. This response has been previously noted with several enzymes associated with the catabolism of glucose (Buchanan & Lewis, 1984). These rapid changes may, in part, be due to the mould responding to transfer from the aerobic, agitated cultures to the more anaerobic, nonagitated incubation.

Overall, the activities of the TCA cycle enzymes appear to be strongly influenced by time of incubation and a carbon catabolite repression. Integrating the two factors, it appears that citrate synthase and fumarase may be key enzymes in regard to possible relationships with the regulation of aflatoxin synthesis. However, comparison of enzyme activities with the respirometry data suggest that *in vivo* TCA cycle activity may differ substantially from specific activities of individual enzymes. Both  $O_2$  uptake and  $CO_2$  evolution increased during the first 30 h of post-transfer incubation, even though the specific activities of various enzymes were decreasing. Comparison of  $CO_2/O_2$  ratios also suggested that a substantial portion of the glucose utilized by the GMS cultures was being catabolized fermentatively, particularly during the early phases of the incubation. This is supported by the rapid decline in pH observed in the GMS cultures. The pH data further suggest that the organic acids formed are either turned over rapidly or not directly utilized for the synthesis of polyketides and other secondary metabolites, since pH levels in the GMS cultures did not begin to rise until well after the period of aflatoxin production.

## TCA and aflatoxin production

The results of the current study generally support the hypothesis that aflatoxin synthesis occurs during a period of depressed TCA cycle activity, and that the catabolism of carbohydrates is actively involved in the regulation of toxin formation. However, additional studies will be needed before these potential bioregulatory loci can be definitely established and integrated with the results of previous investigations. Of particular interest is determining the site involved in the generation of acetyl-CoA incorporated into aflatoxins. Hsieh & Mateles (1970) demonstrated that exogenously supplied acetate is converted to acetyl-CoA and incorporated into aflatoxins extra-mitochondrially. Venkitasubramanian *et al.* (1982) concluded that when the mould is cultured on glucose that the acetyl-CoA is produced intramitochondrially and then transported into the cytoplasm via the citrate-ATP lyase reaction. However, the apparent catabolite repression of citrate synthase observed in the present study alternatively suggests that the acetyl-CoA may not be generated via the formation of citrate. It is possible that the two-carbon units used for polyketide formation may arise extra-mitochondrially via an appropriate anaerobic reaction. Evaluation of this possibility awaits the results of future research.

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