DISCUSSION
The results of the studies carried out in the present investigation have been given in the preceding section. The significance of these data has been discussed under the following headings in this section.

I. Metabolism of cyclic AMP and its role in aflatoxin synthesis

II. Aflatoxin synthesis and its control

III. Methylation in aflatoxin, including various parameters affecting methylation

I. Metabolism of cyclic AMP and its role in aflatoxin biosynthesis

Aflatoxins form a group of heterocyclic secondary metabolites synthesized via the acetate malonate pathway for polyketide formation (Hsieh and Mateles, 1970). It has been postulated that secondary metabolites are regulated by the same mechanisms which regulate the primary metabolism. Some of the important mechanisms for control of secondary metabolism are substrate induction, feedback repression and inhibition, catabolite repression and inhibition and ATP (or energy charge) regulation (Demain, 1972).

Since the discovery of cyclic AMP in mammalian systems in 1957, the presence of this cyclic nucleotide has been demonstrated in some micro-organisms (Pastan and Adhya, 1976). It has been identified as a versatile regulatory molecule in energy metabolism of many organisms and has been shown to be involved
in such diverse activities as hormone action (Robison et al., 1968; Jost and Rickenberg, 1971; Posternak, 1974), catabolite repression (Pastan and Perlman, 1970) and cell division (Burger et al., 1972). Detroy et al. (1971) speculated that the phenomenon of catabolite repression i.e., the inhibition of enzyme synthesis by glucose and related compounds, may be one of the regulatory mechanisms which diverts acetyl CoA from α-keto cycle oxidation into polyketide pathway, thereby leading to secondary metabolite synthesis. This may be the case in aflatoxin synthesis too. However, it has been well established in several microbial systems that glucose repression is mediated by cyclic AMP (Pastan, 1972; Ullman, 1974) with the intracellular concentration of the cyclic nucleotide being inversely related to the level of exogenous glucose (Schlanderer and Dellweg, 1974; Verma and Raizada, 1975). The role of cyclic AMP (cAMP) in the mediation of catabolic repression in fungal systems as well as in aflatoxin synthesis is not clear at present. Therefore, the levels of cyclic AMP were determined at various phases of growth in both the aflatoxin-producing strain as well as the non-toxic strain.

a) Levels of cyclic AMP in *Aspergillus flavus* NRRL 3537, the non-toxic strain and in *Aspergillus parasiticus* NRRL 3240, the toxic strain on SIS and on zinc-deficient SIS medium

Reports of cyclic AMP determinations in fungi are scarce, but studies with *Mucor* species indicate 25-30 picomole/mg of
protein (Larsen and Sypherd, 1974) and 8.4-36.6 picomole/mg of protein (Faveto et al., 1975).

In Aspergillus parasiticus (Figures 3 and 4) on SIS medium, cyclic AMP values ranged from 4 nanomoles to 113 nanomoles while on zinc-deficient medium, the levels were between 5 nanomoles to 150 nanomoles per 100 ml of medium.

In Aspergillus flavus the levels of cyclic AMP ranged from 9 nanomoles to 15 nanomoles on SLS medium while on zinc-deficient SIS medium it lay between 15 to 50 nanomoles per 100 ml of medium. In A. parasiticus the toxic strain, 45 hours of growth represents exponential phase, 60 hours represents transitional phase, 75 and 96 hours correspond to the stationary phase while 120 and 144 hours represent late stationary phase (Detroy and Hesseltine, 1970).

Cyclic AMP level was found to rise tremendously from 45 to 60 hours nearly 2.4 times in A. parasiticus on SIS medium while nearly twice on zinc-deficient SIS medium.

The value came down abruptly at 75 hours on both the media and increased gradually thereafter (Figs. 3 and 4). Cyclic AMP has been reported to occur in many diverse fungi including the ascomycetes schizosaccharomyces (Schlanderer and Dellweg, 1974) Saccharomyces (Sy and Richter, 1972; Van wijk and Konijn, 1971), Neurospora (Flawia and Torres, 1972; Scott and Solomon, 1975; Rosenberg and Pall, 1978) and Aspergillus (Zonneveld, 1976), the basidiomycete coprinus (Uno and Ishikawa, 1973), the phycomycetes
mucor (Paznokas and Sypherd, 1975) and Blastocladiella (Silverman and Epstein, 1975), Physarium polycephalum (Lovely and Threlfall, 1976), Gibberella zeae (Wolf and Mirocha, 1977) and Monascus purpureus (Saruno et al., 1976). Cheung (1966) reported the presence of low levels of cyclic AMP in cells of the yeast Saccharomyces carlsbergensis.

Voichik et al. (1973) detected cyclic AMP levels at various levels of growth in the ciliated protozoan, tetrahymena pyriformis, an unicellular organism.

The concentration of the nucleotide was found to be high during linear growth (exponential phase), declined sharply, in early stationary phase and thereafter increased again during the late stationary phase of growth.

In tetrahymena, the high levels of cyclic AMP in the exponential growth phase imply an important effect of this nucleotide on some metabolic parameters of primary metabolism associated most probably with protein synthesis and cell division (Voichik et al., 1973). This may be the case with Aspergillus parasiticus as well as in Aspergillus flavus, the non-toxic strain, where cyclic AMP levels have been found to be high in the exponential phase (45 hours). On SLS medium however, the total cyclic AMP level of Aspergillus parasiticus was double the value of Aspergillus flavus. This can be explained on the
assumption that in *Aspergillus parasiticus*, various primary metabolites which are precursors of aflatoxin synthesis like acetyl-CoA, tricarboxylic acid cyclic intermediates and phosphatides, etc. are accumulated in the exponential phase. The high cyclic AMP level observed may be imparting a prominent role in the accumulation of precursors for aflatoxin synthesis in the exponential phase. Detroy and Hesseltine (1970) noted that aflatoxin B₁ synthetase was produced during the trophophase when negligible aflatoxin synthesis was observed but it became functional only on the onset of the idiophase.

The transitional phase is the period of change over from the actively growing to the stationary phase where cellular growth stops. In this phase, primary metabolism (vegetative activity) is blocked. In *A. parasiticus* this phase occurs from 50 to 70 hours and enzymes of aflatoxin synthesis appear to be derepressed during this period (Detroy and Hesseltine, 1970).

The expression of aflatoxin synthesis may be a cyclic AMP activated process as evident from the tremendous increase in cyclic AMP level at 60 hours on SIS as well as on zinc-deficient SIS medium. In *Penicillium urticae*, added dibutyryl cyclic AMP caused increased production of at least one enzyme in the sequence from 6-methyl salicylate to Patulin (Forrester and Gaucher, 1972). Adenylate cyclase, the enzyme responsible for synthesis of cyclic AMP was stimulated *in vivo* by pyruvate in
Brevibacterium liquefaciens (Lynch et al., 1975). Thus, in *A. parasiticus*, accumulation of pyruvate in the exponential phase may be stimulating adenylate cyclase activity leading to high levels of cyclic AMP in the transitional phase. This high level of cyclic AMP may be causing accelerated production of either aflatoxin synthetase complex or one of the enzymes in the sequence leading to high levels of aflatoxin production in the early stationary phase (Table 1) and as a consequence leading to a drastic fall in the cyclic AMP level thereby at 75 hours of growth.

Further, in case of *Neurospora crassa* (Terenzi et al., 1976) the marked and transient increase in the cyclic AMP level observed in wild type *Neurospora* reaching the stationary phase suggests that the cyclic nucleotide level acts as a starting signal for the initiation of some important events.

Keeping this in view, it can be postulated that in case of *A. parasiticus*, the increase in cyclic AMP level in the transitional phase may be serving as a starting signal for initiation of expression of aflatoxin synthetase activity. The sharp fall in the level of cAMP in the early stationary phase (75 hours, see Fig. 3 and 4) on both the media may be because of its continued and fast utilization by any one of the enzymes of the aflatoxin synthetase complex or by the aflatoxin synthetase complex as a whole. This may be resulting in high
total aflatoxin levels at 75 hours of growth, which is nearly 3.5 times the value of aflatoxins observed at 60 hours (Table 1). After 75 hours cyclic AMP level was observed to increase gradually till late stationary phase, the maximum cyclic AMP level was found at 144 hours on SIS medium as well as on zinc deficient SIS medium.

Borrow et al. (1961, 1964) divided the stationary phase into the storage phase, during which cell weight continued to increase due to the accumulation of fat and carbohydrates and during which production of secondary metabolites commences, and the maintenance phase, during which dry weight is constant but uptake of glucose and production of secondary metabolites continues. The increase in cyclic AMP level again at 96 hours represents the maintenance phase, where cyclic AMP utilization is not required to that extent as that required in initiation of aflatoxin synthetase activity.

Catabolite regulation, inducer accumulation and phosphate control have been proposed as probable regulatory mechanisms involved in the onset of secondary biosynthesis (Drew and Demain, 1977). The one accepted mechanism for catabolite repression involves:

(a) an increase in cell level of cyclic AMP when growth slows down

(b) activation by cAMP of an effector protein CRP
(c) positive control of catabolite repressed genes by the binding of CAP to part of the operator region.

Though this may not be the only mechanism involved it is gaining support (Emmer et al., 1970; Zubay et al., 1970; Riggs et al., 1971) as a regular system for secondary metabolism. In A. parasiticus it was found that cellular growth slowed down in late stationary phase though the level of cyclic AMP was found to increase (Figure 3 and 4). This is in agreement with the accepted mechanism described above. Therefore, catabolite repression phenomenon might be involved in the control of aflatoxin synthesis.

In carbon catabolite repression phenomenon, when any carbon catabolite like glucose, is present, it inhibits adenylate cyclase, the enzyme that converts ATP to cAMP, thus decreasing the concentration of cyclic AMP. This low level of cyclic AMP inhibits the transcription by RNA polymerase of operons responsible for coding the particular enzyme under catabolite repression control, (Harwood and Peterkofsky, 1975; Pastan and Adhya, 1976; Pastan and Perlman, 1976). As concluded by Van Wijk and Konijn (1971) in Saccharomyces carlsbergensis, the amount of cyclic AMP is higher in cells which show a lower degree of catabolite repression.

In Aspergillus parasiticus also, the high levels of cyclic AMP in the transitional phase may be firstly due to exhaustion
of sucrose and secondly due to accumulated pyruvate in exponential phase stimulating adenylate cyclase. Sáruno, Tanura and Kato (1976) have claimed that carbon catabolite repression in the mold Monascus purpureus is reversed by cyclic AMP. So, it may be postulated that in Aspergillus parasiticus high levels of cyclic AMP in the transitional phase may be involved in reversing carbon catabolite repression, relieving the inducer molecule, depressing aflatoxin synthetase and thereby leading to high levels of aflatoxin at 75 hours and low levels of cyclic AMP. This data suggests the possible involvement of phenomenon of inducer accumulation and catabolite repression in the onset of aflatoxin synthesis. Limited data exists on the effect of cyclic nucleotides on synthesis of secondary metabolites. However, Wolf and Mirocha (1977) have hypothesized that cyclic AMP concentrations directly determined zearalenone production and indirectly regulated the formation of sexual spores.

Wold and Suzuki (1976) hypothesized that in Aspergillus niger zinc and cAMP were regulatory partners, with zinc determining the physiological state of the mold, and cAMP regulating the physiological activity. In this case, the addition of cAMP to cultures in early exponential phase enhanced growth while the addition of cAMP to cultures in late exponential and early stationary phase stimulated the accumulation of citrate.
Thus, the cyclic nucleotide increased both growth and the accumulation of the secondary metabolite by stimulating carbohydrate metabolism in *Aspergillus niger*.

Hence, cyclic AMP participates in the regulation of metabolism and growth and development of fungi (Smith and Galbraith, 1972; Bu'Lock, 1975, 1976; Wold and Suzuki, 1976).

In *Streptomyces hygroscopicus*, an actinomycete which produces a sixteen membered macrolide antibiotic complex, turimycin, cyclic AMP values were found to rise to a maximum coinciding with the end of the main growth period followed by a rapid decrease. The production period of the secondary metabolite, turimycin (24th and 72nd hour) was marked by a relatively low cyclic AMP level, which rose again after this phase (Gersch et al., 1978). Abou Sabe et al. (1975) reported that the level of cyclic AMP was regulated by the intracellular amounts and/or transport of glucose and not by extracellular concentrations.

Zonneveld (1976) reported that manganese deficiency had a definite effect on cyclic AMP levels and that external glucose played an indirect role. Bu'Lock. (1975, 1976) proposed that increased cyclic AMP levels during the stationary phase might lift 'catabolite repression' of enzymes of secondary metabolite biosynthesis.
As evident from the present observation (Fig. 3 and 4), it can be hypothesized that increased levels of cyclic AMP in the transitional phase may be lifting 'catabolite repression' of aflatoxin synthetase leading thereafter, to fast expression of its activity.

Applebaum and Buchanan (1979) have detected cyclic AMP level in A. parasiticus NRRL 2999 and found that on growing the fungus in a media containing 10, 60 and 100 g glucose/L, cyclic AMP level was found to be elevated in the exponential phase, but decreased thereafter. The reverse was the case with levels of cyclic GMP. They suggest a negative regulatory role for cyclic AMP or a positive regulatory role for cyclic GMP in the synthesis of aflatoxin. And if one compares the present observations with that of Applebaum and Buchanan, the difference in values may be firstly because they have detected cyclic AMP level only at the intracellular level but not the extracellular content, though there is continuous leakage of the cyclic nucleotide from the mycelium into the medium. Secondly, the difference lies in the composition of the medium. In the present case, cells used for cyclic AMP estimation were grown on a medium containing sucrose as the carbon source while the medium used by Applebaum and Buchanan contained glucose as the carbon source. Slow sugar utilization, has been known as the key to product formation (Saltero and Johnson, 1953).

Further studies on cephalosporine production in a chemically defined medium clearly showed that glucose was rapidly depleted
during trophophase and that the slowly used, sucrose was not used until idiophase (Weeman, 1963).

Moreover, when beta-lactam antibiotic production was studied on media containing different individual sugars, it was found that the antibiotic production was highest with sucrose, followed by decreasing order, galactose, fructose, maltose and glucose. This may explain the difference in the values of cyclic AMP observed on both the media. Although it is tempting to assume that carbon catabolite repression phenomenon is involved in control of secondary metabolism, it has been proven only in the cases of actinomycin (Gallo and Katz, 1972) and puromycin (Sankaran and Pogell, 1973).

Several hormones and regulatory molecules are known to exert their effect by a stimulation or inhibition of adenylate cyclase or cyclic AMP phosphodiesterase. In order to examine the possibility of the biochemical effects of aflatoxins being mediated through cyclic AMP, the formation of cyclic AMP and its degradation i.e., adenylate cyclase activity and cyclic AMP phosphodiesterase activity have been investigated in the liver and kidney homogenates of aflatoxin B₁ administered rats (Prasanna et al., 1975). They observed a significant disturbance in the cyclic AMP metabolism. Hence it is quite likely that some of the biological effects of aflatoxin B₁ are mediated through cyclic AMP.
As evident from Figures 3 and 4, cyclic AMP levels in the non-toxic strain *Aspergillus flavus* NRRL 3537 on SIS medium as well as on zinc-deficient SIS medium do not show much variation at 60, 75 and 96 hours respectively. The values vary significantly at these periods of growth in the toxic strain on both the media. This observation emphasizes the significant role that cyclic AMP may be playing in aflatoxin synthesis.

b) Subcellular localization of adenylate cyclase in *Aspergillus parasiticus* NRRL 3240 and *Aspergillus flavus* NRRL 3537 and its activity at various hours of growth on SIS medium and zinc-deficient SIS medium

Adenylate cyclase, the enzyme responsible for synthesis of cyclic AMP from ATP was first described by Sutherland *et al.* (1962) and since then it has been studied in a number of mammalian tissues as well as in micro-organisms and plants. In prokaryotes this enzyme has been shown to be both soluble (Hirata and Hayaishi, 1967) and particulate (Ide, 1969).

Adenylate cyclase has been shown to occur in eucaryotic microorganisms like *Neurospora crassa* (Flawia and Torress, 1972; Terenzi *et al.*, 1976), *Saccharomyces fragilis* (Sy and Richter, 1972), *Saccharomyces cerevisiae* (Londesborough and Nurminen, 1972), *Coprinus macrorhizes* (Uno and Ishikawa, 1973), *Aspergillus niger* (Wold and Suzuki, 1974) and in the slime molds, *Physarum polycephalum* (Smith and Mansour, 1978), *Dictyostelium discoideum*
and *Polysphondylium pollidum* (Bonner, 1969; Hintermann and Parish, 1979). In the present study, the subcellular localization of adenylate cyclase in *Aspergillus parasiticus* NRRL 3240 and *Aspergillus flavus* NRRL 3537 was studied by differential centrifugation.

Maximum specific activity of 67.66 nanomoles of cyclic AMP formed per minute per milligram protein was found in 17,000 x g pellet in *A. parasiticus* indicating the association of the enzyme with mitochondrial membrane. Specific activity of 45.65 nanomoles of cyclic AMP formed per minute/mg of protein was also found in the 200,000 x g pellet in *Aspergillus parasiticus*. In other fractions the specific activity was negligible. Thus in *Aspergillus parasiticus* NRRL 3240 two types of adenylate cyclase are present, one localized with the mitochondrial fraction (17,000 x g pellet) while the other is associated with microsomal fraction (200,000 x g pellet).

In *A. flavus* NRRL 3537 the non-toxic strain, most of the adenylate cyclase activity was found to be restricted in the 17,000 x g pellet representing its association with mitochondrial fraction. The activity observed in other fractions was nil or not significant.

In *E. coli*, adenylate cyclase is found to be exclusively particulate (Ide, 1969) and can be easily solubilized (Tao and
Huberman, 1970), while in *Brevibacterium licheniformis* and *Brevibacterium liquefaciens* it is soluble in nature (Hirata and Hayaishi, 1965, 1967). The enzyme has been reported to be associated with the plasma membrane of *Saccharomyces cerevisiae*, *Saccharomyces fragilis* and *Neurospora crassa* (Flawia and Torres, 1972; Londesborough and Nurminen, 1972; Sy and Richter, 1972).

In *E. coli*, adenylate cyclase activity was observed to be associated exclusively with the particulate fraction of the extract (Ide, 1969). Hintermann and Parish (1969) have reported the possibility of adenylate cyclase activity localization in association with vesicles in cellular slime molds, *Dictyostelium discoideum* and *Polysphondylium pallidium*. In the slime mold *Physarum polycephalum*, adenylate cyclase activity was detected in both particulate and 100,000 x g supernatant fractions in the ratio of approximately 3:1 (Smith and Mansour, 1978).

In *Aspergillus niger*, maximum activity of adenylate cyclase was found in the 200,000 x g supernatant and a significant amount of activity was found in 800 x g pellet i.e., the particulate fraction too (Wold and Suzuki, 1974).

The two forms of adenylate cyclase enzyme in *A. parasiticus* may be contributing to the overall high level
of cyclic AMP in *A. parasiticus* as compared to that of
*A. flavus* on SLS medium (Fig. 3).

Further, taking 4 days old cultures of *A. parasiticus*
variation of adenylate cyclase activity with time and varying
protein concentration was studied (Figs. 5 and 6). Adenylate
cyclase activity was proportional to protein concentration upto
320 ug of protein (Fig. 5). The activity of the enzyme was
found to be linear with time for at least 60 minutes (Fig. 6).
Adenylate cyclase activity was determined at various phases
of growth in both the toxic as well as the non-toxic strain
on SLS medium and zinc deficient SIS medium. The activity of
the enzyme was found to increase from 45 to 60 hrs, decreased
at 75 hours and thereafter increased gradually in the stationary
phase in *A. parasiticus*. The specific activity was found to be
almost constant at 120 and 144 hrs respectively, representing
the late stationary phase (Table 4).

In *A. flavus* however, the specific activity of the enzyme
was found to be approximately same in exponential and late
stationary phase while the value was high in the stationary
phase. However, the specific activity of the enzyme is lower
in *A. flavus* as compared to that in *A. parasiticus* accounting
for the high level of cyclic AMP present in *A. parasiticus*
possibly being involved in regulation of aflatoxin synthesis
(Table 4).
In *N. crassa*, maximal adenylate cyclase activity was detected during the growth period coincident with the highest cyclic AMP levels (Terenzi et al., 1976).

In *T. pyriformis*, adenylate cyclase activity declines continuously during early linear growth up to 72 hours when the culture was at the early stationary phase, the least activity being observed at 72 hours. A steep increase in the activity was observed after this period and high levels of adenylate cyclase activity were detected again late in the stationary phase of the growth curve (Voichick et al., 1973).

However, if one compares the values of cyclic AMP (Fig. 3) with that of activity of adenylate cyclase, the enzyme responsible for its synthesis, the enzyme activity at 75 hrs does not decrease sharply as does the cyclic AMP level at 75 hrs. The enzyme activity also does not rise very much from 45 to 60 hrs as is observed in cyclic AMP levels at these hours (Compare Fig. 3 and Table 4). This may be due, in part to cAMP phosphodiesterase, the enzyme responsible for degradation of cyclic AMP. Thus the observed difference in the adenylate cyclase activity and the amount of cyclic AMP accumulated illustrates the presence of an active turnover or degradation mechanism of cyclic AMP in the organism, as explained in the case of *E. coli* (Peterokofsky and Gazdar, 1973).
Secondly, the variation of adenylate cyclase activity in various phases of growth of only 17,000 x g pellet fraction i.e., the mitochondria associated adenylate cyclase has been reported in this study. However, the over all cyclic AMP values reported may have been as a result of activity of both the mitochondrial as well as the 200,000 x g pellet associated enzymes.

In the case of *Aspergillus flavus*, cyclic AMP value is contributed by only mitochondrial fraction, hence the cyclic AMP values represent the actual amount of cyclic AMP produced by adenylate cyclase. Here adenylate cyclase activity is the same in exponential as well as in the late stationary phase, the values being high in the stationary phase. However, the reason for decrease of cyclic AMP value in exponential phase may be the high activity of cyclic AMP phosphodiesterase at 45 hrs in *A. flavus* (Table 6). The increase in cyclic AMP level at 60 hrs is explainable on the basis of low degradation process at this particular period. In the stationary phase i.e., at 75 hrs and 96 hrs approximately equal amounts of cyclic AMP (Fig. 3) are consistent with similar adenylate cyclase activity at these hours (Table 4).

The low activity of the enzyme in the late stationary phase is consistent with the low levels of cyclic AMP in the late stationary phase in *A. flavus* NRRL 3537 on SIS medium.
On zinc-deficient SIS medium (Table 5) in *A. parasiticus* NRRL 3240 it was found that adenylate cyclase activity was lowest in exponential as well as in the transitional phase and increased thereafter, in the stationary phase (75 and 96 hrs). The highest specific activity of adenylate cyclase on zinc deficient medium was found at 96 hrs the value decreasing thereafter in the late stationary phase. This difference in activity at 75 hrs and cyclic AMP value i.e., high activity of the enzyme and drastic decrease in cyclic AMP level (compare Fig. 4 and Table 5) is possibly due to high activity of the degrading enzyme, cyclic AMP phosphodiesterase, at this particular hour (activity is 415, see Table 7).

In the late stationary phase, on zinc deficient SIS medium adenylate cyclase activity was less as compared to that at 96 hrs but higher than that at 45 hrs and 60 hrs. The phosphodiesterase activity decreased in late stationary phase, but was high in exponential phase as well as the early stationary phase. This explains the high level of cyclic AMP in the late stationary phase on zinc deficient SIS medium in *A. parasiticus* NRRL 3240.

In *A. flavus*, on zinc-deficient SIS medium the activity of the enzyme did not show any regular pattern. Specific activity of the enzyme increased in the early stationary phase, decreased in mid-stationary phase and again increased in late-stationary phase emphasizing the need of cyclic AMP in this strain for
differentiation or some purpose other than aflatoxin synthesis. Studies on the breakdown of cyclic AMP by Aspergillus grown on SLS and zinc deficient SLS media

Sutherland and Rall (1958) were the first to describe cyclic 3', 5' AMP phosphodiesterase as the enzyme responsible for hydrolyzing cyclic 3',5'-AMP to 5'-AMP. In fungi 3,5'-AMP phosphodiesterase has been demonstrated in Saccharomyces carlsbergensis (Speziali and Wijk, 1971), Coprinus macrorizus (Uno and Ishikawa, 1973), Neurospora Crassa (Scott and Solomon, 1973) and Aspergillus niger (Wold and Suzuki, 1974)

Cyclic AMP occupies an important position in cellular metabolism and phosphodiesterase may play a major role in regulating its level. Hence the demonstration of the presence of this particular enzyme was necessary. In the present study, intracellular cyclic AMP phosphodiesterase activity was detected in the toxic strain Aspergillus parasiticus NRRL 3240 as well as in the non-toxic strain, Aspergillus flavus NRRL 3537 at various periods of growth. This reflects the possible control of cyclic AMP levels in both the strains as cyclic AMP level was found to vary with the physiological state of the cell in case of Aspergillus parasiticus, on both the media (see Fig.3and 4). It was observed that in A. flavus as well as in A. parasiticus the enzyme activity was located in 48,000xg supernatant and this was taken as 'crude enzyme' throughout the studies
described in this thesis. In Saccharomyces carlsbergensis, activity of the enzyme was detected in 45,000 x g supernatant (Speziali and van Wijk, 1977) and in this organism too, it was observed that level of cyclic AMP varied with the physiological state of the cell.

In the case of Coprinus macrorhizes, 3:5'-cyclic AMP phosphodiesterase alongwith adenyl cyclase was reported in mycelia of strains which form fruiting bodies, but not in strains which are unable to form fruiting bodies. The activity was observed in 1,000 x g supernatant (Uno and Ishikawa, 1973).

In Neurospora crassa, the enzyme was found to be particulate and is more closely related to mammalian than to bacterial cyclic AMP phosphodiesterase (Scott and Solomon, 1973). The plasmodium of the multinucleate acellular slime mold Physarum polycephalum produced a particulate 3',5'-AMP phosphodiesterase and also released an extracellular phosphodiesterase into the growth medium. The properties of both the enzymes were significantly different (Murray et al., 1971).

Dictyostelium discoideum cells produced at least two or may be more, phosphodiesterases, one enzyme being membrane bound and increased in the development stage before aggregation while no increase in enzyme activity was found in non-aggregating mutants. Another phosphodiesterase was found both extracellularly
and intracellularly (Malchow et al., 1972). In cultured cells phosphodiesterase was found to be located principally in the plasma membrane, while little activity was found in nuclear, mitochondrial and microsomal fractions (Russell and Pastan, 1973).

In the case of *E. coli*, Brana and Chytil (1966) and Nielsen et al. (1973) found the enzyme to be soluble. However, in *Bacillus licheniformis* (Clark and Bernlohr, 1972) the enzyme was found to be membranal. Cyclic AMP phosphodiesterase activity has been detected in cell-free extracts of *Myxococcus xanthus* vegetative cells (Zusman, 1973). In *A. niger* two types of cyclic AMP phosphodiesterase were detected and designated as I-PDA, that is, intracellular phosphodiesterase and E-PDA i.e., extracellular phosphodiesterase. Both the enzymes were found to possess considerably different properties. The intracellular cyclic AMP phosphodiesterase was located in the 48,000×g supernatant while the extracellular cyclic AMP phosphodiesterase was found in the culture medium at 48 hrs of growth. Thus as evident from the above reports, cyclic AMP phosphodiesterase may be membranal as well as soluble. The enzymes from *Aspergillus flavus* as well as *Aspergillus parasiticus* were found to be present in 48,000×g supernatant. Moreover, as evident from Table 6, in *Aspergillus parasiticus* grown in SIS medium, cyclic AMP-phosphodiesterase activity was found to increase gradually from exponential to stationary phase, the maximum activity being observed at 96 hrs and decreased gradually thereafter.
High activity of phosphodiesterase at 75 hrs may be responsible for the sharp fall in the cyclic AMP level at 75 hrs (Fig. 3). At 96 hrs, since adenylate cyclase activity was bound to be high as compared to that at 75 hrs (Table 4), the phosphodiesterase may be acting on the cyclic AMP produced, eventually leading to high amount of cyclic AMP at 96 hrs as compared to that at 75 hrs. At 120 and 144 hrs i.e. the late stationary phases, phosphodiesterase activity was found to decrease (Table 6), while adenylate cyclase gradually increased (Table 4), contributing in association thus to high levels of cyclic AMP in late stationary phase (Fig. 3).

In Tetrahymena pyriformis (Voichik et al., 1973) high levels of cyclic AMP observed during early exponential and late stationary phases were associated with elevated adenylate cyclase activity.

In A. flavus NRRL 3537 the non-toxic strain, however, there was no regular pattern of cyclic AMP phosphodiesterase activity. The value was high in exponential phase and early stationary phase (75 hrs) and decreased thereafter, rising again at 144 hrs. In Aspergillus parasiticus NRRL 3240 grown on zinc-deficient SLS medium cyclic AMP phosphodiesterase activity was found to increase gradually from exponential to early stationary phase (75 hrs), the maximum activity being observed at 60 hrs (Transitional phase). Specific activity of the enzyme was found to
decrease thereafter, the activity being nearly equal at 120 and 144 hrs i.e., in the late stationary phase. However, the specific activity of phosphodiesterase was found to be high on zinc deficient SIS medium (specific activity of 482) as compared to the value on normal SIS medium (specific activity of 215). Thus low levels of zinc are stimulatory for phosphodiesterase activity in *Aspergillus*.

However, as evident from Tables 1 and 2 zinc sulphate is inhibitory at low concentrations for aflatoxin production. Thus, on zinc-deficient SIS medium, high phosphodiesterase activity leads to more breakdown of cyclic AMP leading to overall low cyclic AMP pool which in turn may be responsible for low aflatoxin production in this medium. This again emphasizes the possible involvement of cyclic AMP in regulation of aflatoxin synthesis.

There is evidence which suggests that metabolism of cyclic AMP is linked to the metabolism of zinc in *Aspergillus niger* (Wold and Suzuki, 1976). Cyclic AMP affected the rates of growth and production of citric acid when added to *Aspergillus niger* cultures growing at low, but not at high zinc levels. Zinc and cyclic AMP have been visualised as regulatory partners in *A. niger*. Zinc was found to determine the physiological state and was required for cell proliferation, and cyclic AMP
regulated the physiological activity. The same may be the case in A. parasiticus too. Taking four days old mycelia, the variation of phosphodiesterase activity with protein concentration has been presented in Figure 7 and that with time in Figure 8. Phosphodiesterase activity was proportional to concentration up to 420 ug of protein. The activity of the enzyme was found to be linear with time for at least 40 minutes. Further taking into consideration the variation of phosphodiesterase activity in various phases of growth, high specific activity of the enzyme in transitional phase was accompanied by increase in cyclic AMP level at this particular hour. But the adenylate cyclase activity was found to be same in exponential as well as in transitional phase. Thus the high cyclic AMP pool at this hour may have been contributed by the 200,000 xg pellet associated as well as by the 17,000 xg pellet associated adenylate cyclases resulting in high levels of cyclic AMP at this particular hour on this medium. At 75 hrs and 96 hrs the activity of adenylate cyclase increases but the value of cyclic AMP was less at 75 hrs explainable by high phosphodiesterase activity at this particular hour. At 96 hrs phosphodiesterase activity was less as compared to that at 75 hrs while adenylate cyclase activity was highest leading thereby to high levels of cyclic AMP.
In late stationary phase adenylate cyclase activity was high as compared to that in exponential phase resulting in high levels of cyclic AMP in late stationary phase. In *A. flavus* the phosphodiesterase activity increased gradually from exponential to stationary phase, maximum activity being at 96 hrs decreasing thereafter on zinc deficient SLS medium.

In the exponential phase, however, when *A. flavus* was grown on zinc deficient SLS medium (Fig. 4), cyclic AMP value was found to be the highest explainable by least activity of phosphodiesterase (Table 7) at this hour though adenylate cyclase activity was significant at 45 hrs. At 144 hrs and 120 hrs phosphodiesterase activity was higher than that at 45 hrs in *A. flavus* on zinc deficient SLS medium, but the adenylate cyclase activity was high in these phases, leading to accumulation of cyclic AMP but cyclic AMP content was not higher than that in exponential phase. However, from the studies so far carried out one can conclude that the actual picture of regulation by cyclic AMP pool of aflatoxin synthesis may be visualised by determining the activity of the 200,000 x g pellet associated adenylate cyclase also at various periods of growth. Secondly, extracellular phosphodiesterase activity has also to be detected in both the strains on both the media to give a better picture.
II. Aflatoxin synthesis and its control

Aflatoxins are hepatocarcinogenic to several species including human beings and also produce liver cancer in various parts of the world (Chu, 1977). So it is of great necessity to device measures effective in controlling aflatoxin synthesis. For this purpose, work has been carried out in the present study and described under the following headings:

(a) Effect of Tolnaftate (antifungal agent) on metabolism of A. parasiticus NRRL 3240

Tolnaftate (2-napthyl-N-methyl-N-(M-tolyl)-thionocarbamate) an antifungal drug, is widely used to control superficial fungal infections of the skin like ring worm and other dermatophytes of skin (Teruhisa, 1962). This compound inhibits fungal growth in liquid cultures at very low concentration (Gordee and Gorman, 1968; Kobayashi and Medoff, 1977). With a view to investigate its possible successful use in the control of aflatoxin production, effect of Tolnaftate on fungal growth and aflatoxin production was studied. Effect of Tolnaftate on fungal growth, fungal morphology, aflatoxin production and incorporation of labelled acetate into aflatoxin in a resuspension system was studied in detail.

Tolnaftate changed the morphology of A. parasiticus to yeast like forms at a concentration of 100 u moles when the
fungus was grown on SIS medium for 8 days. At 10 and 50 u moles concentration the fungal morphology was in between filamentous and yeast like forms, i.e., possessed no well defined structure. According to Detroy and Ciegler (1971) deficiency of manganese ions in the medium resulted in yeast like forms. So it may be postulated that Tolnaftate may be chelating manganese ions making the medium deficient in manganese, as a consequence of which the morphology of A. parasiticus may have changed to yeast like forms.

As is evident from Table 8 Tolnaftate at concentrations ranging from 10-100 u moles did not affect growth of Aspergillus parasiticus NRRL-3240 as represented by almost constant mycelial weight but inhibited aflatoxin production to a great extent. Total aflatoxin yield was decreased about 8 fold by 100 u moles of Tolnaftate. Formation of aflatoxins B and G were inhibited to almost the same extent. A plot of aflatoxin yield vs Tolnaftate concentration added revealed a logarithmic inhibition between 10 and 100 u moles of the drug (Fig. 9).

Tolnaftate at very low concentration (1%) has been reported to inhibit fungal growth in liquid cultures (Gordee and Gorman, 1968; Kobayashi and Medoff, 1977). So it is probable that for growth inhibition in A. parasiticus higher concentrations of Tolnaftate may be required. It has been observed that major amount of aflatoxin is produced by A. parasiticus during the stationary phase i.e. after 70 hrs of growth (Detroy et al., 1973; Gupta et al., 1976).
There is no relationship between fungal growth and aflatoxin production. Bean and Rambo (1975) observed that dimethyl sulfoxide inhibited aflatoxin biosynthesis without affecting fungal growth. Similar results were obtained by Gupta et al. (1976) with 2-mercaptoethanol at 1 mM concentration. The inhibitory effect of Tolnaftate was further investigated by studying its effect on \( ^{14}C \)-acetate incorporation into aflatoxins by resting mycelia of \textit{A. parasiticus} in resuspension experiments. The observations have been presented in Table 9. The results reveal that the incorporation of labelled acetate into aflatoxin B and G was considerably inhibited, the extent of inhibition of aflatoxin G being much more than that of aflatoxin B at a concentration of 10 u moles. Yet the difference was considerably reduced with increasing concentration of Tolnaftate. As represented in Fig. 10, the inhibition of acetate uptake by Tolnaftate as measured by cpm of B was logarithmic between 0 and 50 u moles of drug.

As postulated by Heathcote et al. (1976) aflatoxin G is derived from aflatoxin B in \textit{A. parasiticus}. The enzyme system catalyzing the conversion is postulated to be similar to the mixed function oxidases. As represented in the data in Table 9, aflatoxin B did not pile up with the drastic inhibition of G so it can be postulated that Tolnaftate does not inhibit the \( B \rightarrow G \) conversion step. It may be inhibiting aflatoxin formation at some step other than this.
Schroeder et al. (1974) and Hsieh (1973) reported that dichlorvos inhibited aflatoxin biosynthesis at a step prior to the conversion of sterigmatocystin to aflatoxin viz., the conversion of versicolorin to sterigmatocystin. A systematic study of metabolic inhibitors of aflatoxin biosynthesis has been carried out (Gupta et al., 1975 a; Gupta et al., 1975 b; Gupta et al., 1976). The known aflatoxin inhibitors include trace metals like barium and vanadium, chelating agents like phytic acid, mercaptoethanol and dimethyl sulfoxide, citrus oils, preservatives like sorbic acid and dialkyl enolphosphates. Some other well known metabolic inhibitors include ethionine, dichlorvos and L-cysteic acid as reviewed by Maggon et al., (1977).

It has been postulated already that the inhibitory action of certain inhibitors like 2-mercaptoethanol, phytic acid and dimethyl sulfoxide might be due to chelation of essential trace metals like zinc and copper (Gupta and Venkitasubramaniam, 1975; Gupta et al., 1976; Weinberg, 1970).

In order to test this hypothesis, the effect of zinc in conjunction with Tolnaftate on the incorporation of 1-14C-acetate into aflatoxins was studied.

Stimulation of aflatoxin formation in presence of zinc has been documented in various reports (Gupta et al., 1975; Maggon et al., 1977).
As shown in Table 10, the specific activity of aflatoxin B remained unchanged in the presence of zinc. On the contrary, zinc increased the specific activity of aflatoxin G, thereby reducing the effect of Tolnaftate when the latter was present at 50-100 µ moles concentrations (specific activities in Tables 9 and 10 should be compared, for better visualization). The stimulatory effect of zinc on $^{14}$C-acetate incorporation into aflatoxin has been reported. Zinc at levels of 1 and 10 mM concentration stimulated $^{14}$C-acetate incorporation by about 50 per cent. The increase in specific activity of the two aflatoxins (B and G) was observed to be approximately the same (Gupta et al., 1975). In the present case, however, there is a slight increase in the levels of specific activity, when the study was carried out with Tolnaftate along with zinc. On the basis of these results, it can be stated that the conversion of aflatoxin B to G is zinc dependent. This is further supported by the observation of Reddy (1972) of a zinc stimulated production of aflatoxin G. The ratio of aflatoxin B and G was reported to increase from 2 in the absence of zinc to 3 in the presence of zinc. Most probably Tolnaftate regulates aflatoxin G biosynthesis.
(b) Effect of adenine nucleotides on the metabolism of *Aspergillus parasiticus* NRI-3240

Several reports have emphasized the relationship between adenine nucleotides, particularly ATP and various physiological processes in micro-organisms such as cell growth, cell division, break-down and synthesis of storage polysaccharides, glycolysis, active transport across membranes, nucleic acid turnover and onset of aggregation (Righelato, 1975). The regulatory role played by adenylic acid system on metabolism of micro-organisms has been reviewed by Demain et al. (1973), Righelato (1975) and Curdova et al. (1976). Adenylic acid system plays a significant role in regulation of secondary fungal metabolism. The energy charge regulation of biosynthetic pathway involves activation and inhibition of enzymes of primary metabolism by relative levels of the adenylates (ATP, ADP and AMP) in the cell (Attinson, 1969, 1971).

The effect of 10, 50 and 1000 uM concentrations of ATP, ADP and AMP on 1-C\textsuperscript{14}-acetate incorporation into aflatoxin was studied in the present investigation by carrying out resuspension experiments with 4 days old mycelium.

As presented in Table 11, there is an inverse relationship between ATP concentration and 1-C\textsuperscript{14}-acetate incorporation into
aflatoxins. $^1{}C^{14}$-Acetate incorporation into aflatoxins was inhibited by the nucleotide, the extent of inhibition gradually with the increasing concentrations of ATP. Highest inhibition was noted at 100 μM concentration and inhibition was found to be approximately the same in both aflatoxins B and G.

Forrest (1965, 1969) and Righelato (1975) have reported that a critical concentration of ATP was necessary for exponential phase of growth and at low ATP levels, only stationary phase occurred. Thus, aflatoxin might be forming in the stationary phase at the cost of less amount of ATP.

According to Janglova et al. (1969) high levels of ATP during the stationary phase were inhibitory for chlortetra-cycline biosynthesis, a secondary metabolite of Streptomyces aureofaciens. They postulated that high concentrations of ATP inhibited phosphoenol pyruvate carboxylase and citrate synthase, thereby regulating TCA cycle and as a consequence resulting in low production of chlortetracycline.

The ATP levels of A. parasiticus grown on SLS medium when determined quantitatively, were high during exponential phase of growth and declined during stationary phase (Gupta et al., 1976; Rao et al., 1980). It is known that aflatoxins are synthesized in the stationary phase. Therefore, it can be deduced that aflatoxins are synthesized at a maximum rate in the stationary phase at the cost of less amount of ATP in A. parasiticus.
When extracellular ATP is provided to the fungus in the stationary phase, the supplied ATP may be somehow regulating the activity of some of the enzymes of TCA cycle. As a result, the precursors of aflatoxins may be directed to pathways other than aflatoxin biosynthesis leading to inhibition of 1-\textsuperscript{14}C-acetate incorporation into aflatoxin.

ADP and AMP at increasing concentrations stimulated 1-\textsuperscript{14}C-acetate incorporation into aflatoxins (Table 11), the maximum stimulation being observed at 100 \textmu M concentration. Stimulation of 1-\textsuperscript{14}C-acetate incorporation into aflatoxins was more in aflatoxin B than in aflatoxin G at 100 \textmu M concentrations of ADP. AMP at 10 \textmu M concentration was found to show more stimulation of 1-\textsuperscript{14}C-acetate incorporation into aflatoxin than ADP at the same concentration, as represented by the change in specific activities.

According to Atkinson (1969, 1971), the level of AMP is a sensitive control signal of the state of ATP supply. AMP and ADP levels are high in the stationary phase, when \textit{A. parasiticus} is grown on SLS medium (Gupta \textit{et al.}, 1976; Rao \textit{et al.}, 1980). When AMP levels are low, most of the acetyl co-enzyme A is diverted to the synthesis of lipids (Atkinson, 1969, 1971). So when increasing concentrations of AMP are added to the fungus in the stationary phase, acetyl CoA is diverted away from lipid
synthesis, leading to synthesis of aflatoxin in larger quantities. This results in increased stimulation of $^{14}$-acetate incorporation into aflatoxins.

However, Martin and Demain (1977) found that all the nucleotides, ATP, ADP, AMP as well as cyclic AMP were inhibitory for candididin fermentation. Neither the ribonucleosides nor the free base, adenine produced significant inhibition suggesting that for the inhibitory effect the phosphate group is essential. But this may not be the case with aflatoxin production by Aspergillus parasiticus as evident from the present data (Table 11).

**Cyclic AMP and aflatoxin production**

Bu'Lock (1975) pointed out to a specific role of cyclic AMP, during fungal growth and differentiation. In order to see how exogenous cyclic AMP affects the polyacetate pathway of biosynthesis of aflatoxins, its effect on $^{14}$-acetate incorporation into aflatoxins was studied. As presented in Table 12, exogenous cyclic AMP, at all the concentrations tried (10, 50 and 100 uM), stimulated $^{14}$-acetate incorporation into aflatoxins. Stimulation was significant at 10 and 100 uM concentrations but was maximum at 50 uM concentration.

According to Smith (1968) $^{6}$,0$^{2'}$-dibutyryl cyclic AMP a simple derivative of cAMP, may enter tissues more readily than does the parent compound and is cleaved more slowly by the
cyclic phosphodiesterase. As a result, prolonged action or 'slow feed' of the parent metabolite (cyclic AMP) into the concerned tissue may occur.

Hence the effect of $N^6, O^{2'}$-dibutyryl cyclic AMP on $^{14}$-acetate incorporation into aflatoxins was studied at 10, 50 and 100 uM concentrations of the nucleotide. It was observed that with $N^6, O^{2'}$-dibutyryl cyclic AMP also, the stimulation of $^{14}$-acetate incorporation into aflatoxin was significant at 10 and 100 uM concentrations but less than that produced by cyclic AMP (Table 12). At 50 uM concentration the stimulation produced by dibutyryl cyclic AMP was maximum. The extent of stimulation of incorporation was almost same at 50 uM concentration by both cyclic AMP as well as by dibutyryl cyclic AMP. However, both the compounds produced better effects on aflatoxin B than on aflatoxin G. Thus cyclic AMP appears to stimulate the polyacetate pathway of aflatoxin biosynthesis.

Mosinger and Vaughan (1967) found cyclic AMP to be lipolytic. Pauli et al. (1974) also reported that in E. coli the synthesis of the enzymes of fatty acid degradation was under control of cyclic AMP and cyclic AMP receptor protein. Thus cyclic AMP was found to be stimulatory for lipid degradation. Hence, in A. parasiticus too, it is quite possible that in the stationary phase, cyclic AMP may be stimulating fatty acid degradation, as a consequence of which acetyl-CoA, the common precursor of both
aflatoxin and fats is diverted towards aflatoxin formation.

However, cyclic AMP was found to inhibit candidicidin formation as well as the incorporation of its precursors into the antibiotic at 3 mM concentration (Martin and Demairs, 1977).

In Streptomyces hygroscopicus too, the antibiotic production was found to be inhibited at 3 mM concentration (Gersch et al., 1978).

As evident from Table 13, addition of cyclic AMP resulted in less incorporation of 1-C\(^{14}\)-acetate into aflatoxins under shaking i.e., under aerated conditions. The effect was nearly negligible at 10 uM concentration.

However, N\(^6\),O\(^2\)-dibutyryl cyclic AMP was more effective in stimulating incorporation of label as compared to cyclic AMP at all the concentrations tried under aerated conditions. The maximum effect was found at 100 uM concentration under these conditions and the stimulatory effect was equally significant in aflatoxin B as well as aflatoxin G at this concentration.

Lieve (1968) had reported that many coliform bacteria could be made permeable to small molecules by brief exposure to EDTA in a tris-(hydroxymethyl) aminomethane (Tris) buffer. Cells were treated with 0.2 to 0.6 mM solution of EDTA for 2 minutes and this made the cell wall permeable.

Hence, the effect of cyclic AMP on 1-C\(^{14}\)-acetate incorporation into aflatoxin was studied by incubating the mycelium with 0.1 M EDTA for one hour prior to resuspension.
The results are presented in Table 14. It was found that, under resting conditions, cyclic AMP stimulated $1^{-C^{14}}$-acetate incorporation into aflatoxin, the effect being statistically not significant at 10 uM. However, the extent of stimulation was less at 50 and 100 uM concentrations as compared to that produced with normal fungus (not treated with EDTA) (compare Tables 14 and 12). Under aerated conditions i.e. with shake cultures, it was found that cyclic AMP inhibited $1^{-C^{14}}$-acetate incorporation into aflatoxin, the inhibition being maximum at 10 uM concentration while the inhibition produced was more or less the same at 50 and 100 uM concentrations.

EDTA has been reported to be a positive regulator of fatty acid biosynthesis (Reeves et al., 1967). Hence EDTA may be diverting acetyl-CoA towards fatty acid synthesis, thus leading to an inhibition of acetate incorporation into aflatoxin.

**Methylation in aflatoxin, including various parameters affecting methylation**

Nearly all biological methylation processes in higher animals (and probably most of those in plants and bacteria) involve methionine as the key intermediate. Plants and bacteria synthesise methionine from homocystiene and serine, the major source of methyl group (Lahminger, 1970).

According to Detroy and Hesseltine (1970) and Detroy and Freer (1974), methylation of carbon and methionine formation-
are essential for aflatoxin biosynthesis and the polyketide chain is methylated before aromatization.

In order to study the methylation step of aflatoxin synthesis in detail, the conditions for maximum methyl-C\textsuperscript{14}-methionine incorporation into aflatoxin were investigated. It was found that maximum methylation occurred at a pH of 6.5 using phosphate buffer and a period of one hour for resuspension. However, at pH 6.0 and on two hours of resuspension also, the incorporation of label was found to be significant (Tables 15 and 16).

As evident from tables 17 and 18, when comparative incorporation of methyl-C\textsuperscript{14}-methionine and 1-C\textsuperscript{14}-acetate into aflatoxins was studied, it was found that maximum methylation, occurred at one hour of resuspension, while maximum 1-C\textsuperscript{14}-acetate incorporation into aflatoxin occurred after two hours of resuspension.

The results of the present study are in agreement with the results obtained by Detroy and Freer (1974) who postulated that a pre-aromatic methylation of aflatoxin polyketide precursor may be a requirement for the cyclization of the polyketide chain and aflatoxin ring formation. Rao et al. (1979) also found that methyl-C\textsuperscript{14}-methionine uptake was maximum at 60 hours i.e., in transitional phase in SIS medium while aflatoxin synthesis
was maximum in the stationary phase, indicating that methylation occurred prior to aromatization. However, Harris et al. (1976) showed that the methylation step was late in the biosynthetic scheme for griseofulvin, after the formation of aromatic rings. But evidence also exists showing that C-methylation of polyketides occurs at a pre-aromatic stage (Turner, 1971).

Looking into the regulation of methylation by trace metals, it was found that at 10 umoles, most of the elements tried in this study stimulated methyl-C$^{14}$-methionine incorporation into aflatoxins except barium acetate. However, the inhibition shown by barium acetate was not statistically significant (Table 19). Maximum stimulation of methylation was observed by zinc sulphate followed by cadmium chloride, magnesium sulphate and cesium chloride. The effect of cesium chloride was in fact not significant.

As represented in Tables 20, 21 and 22 when trace metals were tried at 100 umoles, stimulation of methyl-C$^{14}$-methionine incorporation into aflatoxin was maximum by ferrous sulphate followed by bismuth nitrate, mercuric chloride, molybdic acid, nickel chloride, ammonium molybdate, selenium dioxide and uranyl sulphate. Of the trace metals which inhibited methylation at this concentration, the maximum inhibition was brought out by lithium chloride, followed by barium acetate, arsenic chloride, calcium chloride, vanadyl sulphate, lead acetate and strontium
chloride. At 100 umoles concentration, the effect of copper sulphate, magnesium sulphate and cobalt-chloride was not significant on methylation of aflatoxin B while it was slightly stimulatory for aflatoxin G. (Tables 20 and 21). At 10 umoles, stimulation of methylation produced by most of the trace metals tried, was more in aflatoxin B than in aflatoxin G. However, the stimulatory effect was more in aflatoxin G than in aflatoxin B by ferrous sulphate as well as by magnesium sulphate (Table 19).

At 100 umoles, stimulation of methylation by most of the stimulatory trace metals was more in aflatoxin B than in aflatoxin G except by manganese chloride, ammonium molybdate, copper sulphate, stannous chloride, and mercuric chloride (Tables 20 and 22). The inhibitory effect of the trace metals on methylation too was more in aflatoxin B than in aflatoxin G at a level of 100 umoles (Table 21).

Zinc sulphate is involved in the expression of activity of several enzymes. Zinc enzymes are thus known to participate in a wide variety of metabolic processes like carbohydrate, lipid, protein and nucleic acid synthesis or degradation (Valee, 1977). Hence, zinc sulphate may be regulating any of the enzymes related with methionine synthesis as a result leading to availability of more precursors for methylation. It may be regulating S-adenosyl methionine synthetase directly resulting in higher methylation
rate. Axelrod and Tomchick (1958) have studied the requirement of divalent cations by O-methyl transferase, an enzyme methylating catecholamine. In the absence of adenosyl methionine or magnesium ions negligible O-methylation occurred. Since O-methylation required the presence of magnesium the effect of other metals was examined. A number of divalent cations such as cobalt, manganese, zinc, cadmium, iron and nickel could be substituted for magnesium ions.

Lee et al. (1966) observed enhancement of aflatoxin formation on addition of cadmium. However, Marsh et al. (1975) found cadmium as well as copper and iron to depress aflatoxin production. Maggon et al. (1973) found copper to produce more aflatoxin. Lee et al. (1966) and Reddy (1972) found aflatoxin production to be unaffected by calcium chloride while Sivaswami (1978) found calcium to inhibit de novo formation of aflatoxin from labelled acetate in intact spheroplasts and lysates.

Sivaswami (1978) found magnesium sulphate to stimulate acetate incorporation into spheroplasts as well as lysates upto 0.5 mM concentration. Magnesium sulphate, FAD and tetrahydrofolic acid are involved in converting homocysteine to methionine. Hence magnesium sulphate at 10 umoles may be aiding in more methionine formation and in turn leading to high methylation (Table 19). However, at higher concentrations, its effect was negligible (Table 20).
Maggon et al. (1973) found barium acetate to inhibit aflatoxin formation while calcium chloride stimulated aflatoxin formation. They also found cobalt chloride and vanadyl sulphate to inhibit aflatoxin formation, the inhibition produced by vanadyl sulphate being more. Cobalt is known to be present in the vitamin B₁₂ molecule. There is a complexity in the nature of the methylation reaction in micro-organisms. Some organisms have the potential for a vitamin B₁₂-independent route while others follow vitamin B₁₂-dependent route. Organisms that make vitamin B₁₂ appear to use the B₁₂-dependent route exclusively (Salem and Foster, 1972).

However, methylation was found to be unaffected by cobalt (Table 21), indicating that Aspergillus parasiticus may be using the vitamin B₁₂ independent route of methylation. Prasad (1976) reports that toxicity of lead may be due to its adverse effect on zinc metalloenzymes. Bhatnagar et al. (1979) found lithium to be stimulatory for aflatoxin biosynthesis. The metal had a pronounced stimulatory effect on the production of aflatoxins B₁ and B₂ but the increase in aflatoxin G level was very slight.

However, in the present study, it was found that lithium inhibited methylation of aflatoxin B (Table 21). Lithium is well known as an activator of glycolysis and found to stimulate carbohydrate metabolism at several points. It increases activity
of hexokinases, adenylcyclase and pyruvate kinase. Nothing can be said about the mechanism involved in inhibition of methylation of aflatoxin B by lithium.

As presented in Tables 23 and 24, amino acids at 100 umoles concentration were used to see their effect on methylation. It was found that most of the amino acids were stimulatory except phenylalanine, aspartic acid, threonine and glutamic acid. These amino acids slightly inhibited methyl-\(^{14}\)C-methionine incorporation into aflatoxins while the effect of the amino acid, tryptophan at this concentration, on methylation was not significant. Of the amino acids stimulating methylation the maximum stimulation was brought out by cysteine, followed by arginine hydrochloride, histidine, glycine, valine, methionine and asparagine.

Stimulation of methyl-\(^{14}\)C-methionine incorporation into aflatoxins produced by cysteine and glycine was more in aflatoxin B than in aflatoxin G. In the case of histidine, arginine hydrochloride and valine, the effect was more in aflatoxin G than in aflatoxin B.

The stimulatory effect of asparagine was almost the same in both aflatoxin B and in aflatoxin G. Serine at this particular concentration was found to stimulate methylation into aflatoxin B while it showed inhibitory effect into aflatoxin G. One complexity of methionine synthesis is its
FIGURE 11. BIOLOGICAL METHYLATION AND THE BIOSYNTHESIS OF METHIONINE.

Aspartate ➔ Homoserine
  ➔ O-succinyl homoserine

Serine ➔ H₂S ➔ Cysteine

Cystathionine ➔ Homocysteine

Homocysteine ➔ Me-FH₄ ➔ FH₄ ➔ Formate

Methionine

S-adenosyl methionine ➔ "C₁" ➔ "Polyketide"
dependence upon three other amino acid pathways for precursors (Brenchley and Williams, 1975). (a) The aspartate pathway for the carbon skeleton (b) The cysteine pathway for the sulphur group and (c) Serine as the primary source of the methyl group via the transhydroxymethylase reaction.

As presented in Figure 11, cysteine is an intermediate involved in methionine synthesis. Hence the high methylation rate observed by addition of extracellular cysteine depicts its involvement in methylation in A. parasiticus too.

Rao (1979) found alanine and aspartic acid to enhance incorporation of methyl C$^{14}$-methionine into both aflatoxin B and G at 10 mM concentration when added at 24 hrs of growth to growing cultures aseptically. However, these amino acids when added at 48 hrs to growing cultures inhibited methyl-C$^{14}$-methionine incorporation into both aflatoxin B as well as aflatoxin G. Rao (1979) carried out these experiments using asparagine deficient SIS medium instead of normal SIS medium. This may be the reason for the difference in data.

As presented in Tables 25 and 26, out of the TCA cycle intermediates tried at 10 mM concentration, only fumarate and malic acid stimulated methyl-C$^{14}$-methionine incorporation into aflatoxins while oxaloacetic acid had a slight inhibitory effect. The effect of other intermediates was non-significant at this concentration. The stimulatory effect produced by
fumarate was more as compared to malic acid. Stimulation of methyl-C\textsuperscript{14}-methionine incorporation into aflatoxins produced by both the metabolites was more in aflatoxin G than in aflatoxin B (Table 25).

At 50 mM concentrations, out of the \textit{L}-c\textsuperscript{\textcircled{a}}-cycle intermediates tried, methyl-C\textsuperscript{14}-methionine incorporation into aflatoxins was stimulated by alpha-ketoglutarate and fumarate. Stimulation produced was more by alpha-ketoglutarate as compared to fumarate and the stimulatory effect was again more in aflatoxin G than in aflatoxin B at this concentration (Table 26).

Rao (1979) found that when resting cell systems of the fungus grown in SLS medium was used, incorporation of methyl-C\textsuperscript{14}-methionine into aflatoxin G increased significantly in presence of alanine, aspartic acid and oxalacetate at 10 mM concentration. But, in the above case, the resuspension experiments were carried out with 48 hours old cultures using resting cell systems while the present experiments were carried out with 96 hrs old cultures under aerated conditions. And as presented in Table 25, oxalacetate had slight inhibitory effect on methylation at 10 mM concentration while at 50 mM concentration (Table 26) the effect was not significant. Citric acid and malic acid both inhibited methyl-C\textsuperscript{14}-methionine incorporation into aflatoxins at 50 mM concentration. The inhibitory effect produced by citric acid was more in aflatoxin B than in aflatoxin G.
at 50 mM concentration while malic acid had more inhibitory effect on aflatoxin G than in aflatoxin B at this concentration.

Out of the hexoses tried, only glucose and mannose had stimulatory effect on methylation (Table 27). Glucose showed stimulation of methyl-C\textsuperscript{14}-methionine incorporation into aflatoxins at 10 mM concentration while at 1 and 5 mM concentration it had an inhibitory effect. Both the inhibitory and stimulatory effects observed were found to be more in aflatoxin B than in aflatoxin G. Mannose at 1 mM concentration was found to stimulate methyl-C\textsuperscript{14}-methionine incorporation into aflatoxin B as well as in aflatoxin G, though the stimulatory effect was more in aflatoxin G than in aflatoxin B. Fructose was found to be inhibitory for methylation in aflatoxin, both in aflatoxin B as well as in aflatoxin G (Table 27).

Sorbose and galactose were also found to inhibit methylation step in aflatoxin synthesis at all the concentrations tried, the inhibitory effect being more in aflatoxin B than in aflatoxin G. However, mannose at 5 and 10 mM concentration inhibited methylation into aflatoxin B while it stimulated slightly the process of aflatoxin G.

As presented in Table 28 when pentoses like ribose and arabinose were used to see their effect on methylation, it was found that the effect of arabinose was not significant while that of ribose was slightly stimulatory. Ribose at 1 and 5 mM
concentration stimulated methyl-$^{14}$C-methionine incorporation into aflatoxin G more than in aflatoxin B while at 10 mM concentration methylation was more in aflatoxin B and less in aflatoxin G.

The effect of disaccharides on methylation has been presented in Table 29 and it was found that only sucrose had a significant effect on methylation, the effect of maltose and lactose being not significant comparatively. Sucrose stimulated methylation at 1 and 5 mM concentration while at 0.5 mM its effect was also not significant. However, at 1 mM concentration of sucrose methylation was prominent in aflatoxin B while it was not significant in aflatoxin G.

At 5 mM concentration of sucrose, stimulation of methylation was prominent in both aflatoxin B and G the stimulatory effect being more in aflatoxin B than in aflatoxin G (Table 29). This shows that sucrose favours methylation.

On addition of glycolytic intermediates (Tables 30 and 31) into resuspension buffer, methyl-$^{14}$C-methionine incorporation into aflatoxins was affected maximum by fructose-1,6-diphosphate at 0.5 mM concentration. Glucose-1-phosphate was found to show slight stimulatory effect at 0.1 mM concentration while it was inhibitory at 0.5 mM level. The effect of this intermediate at 1.0 mM concentration was non-significant. Glucose-6-phosphate also showed slight stimulatory effect at 0.1 mM and
1.0 mM levels while it was inhibitory to methylation in aflatoxin at 0.5 mM concentration (Table 30).

As presented in Table 30, fructose-6-phosphate was stimulatory to methylation at all the tried concentrations (0.1, 0.5 and 1.0 mM), the stimulatory effect being maximum at 1.0 mM concentration. Fructose-1,6-diphosphate also stimulated methylation, the stimulatory effect produced being quite prominent by this intermediate at all the concentrations tried. However, maximum stimulation of methylation was observed at 0.5 mM concentration, the stimulation produced being equally good in both aflatoxin B and aflatoxin G (Table 30). Glyceraldehyde-3-phosphate stimulated methylation at 0.5 mM concentration into aflatoxin B as well as aflatoxin G. At 0.1 mM and 1.0 mM concentration, this intermediate inhibited methylation into aflatoxin B while its effect was non-significant on aflatoxin G (Table 31). Phosphoenol pyruvate also stimulated methylation at 0.5 and 1.0 mM concentration the stimulation being more prominent at 0.5 mM concentration. Yet at both concentrations stimulation of methylation was found to be more in aflatoxin G than in aflatoxin B.

Pyruvate was also found to be stimulatory for methylation in aflatoxins and maximum stimulation was observed at 0.1 mM concentration, the stimulation of methylation being more in aflatoxin B than in aflatoxin G (Table 31). Rao (1979) also
found pyruvate to stimulate methyl-C$^{14}$-methionine incorporation into aflatoxins at 10 mM concentration when added to growing cultures at 24 hrs indicating its probable role in methylation. Thus it appears from the results that fructose-6-phosphate, fructose-1,6-diphosphate, phosphoenol pyruvate and pyruvate may have a probable role in methylation. However, it is difficult to visualise the exact role played by these intermediates in methylation.

The effect of flavin adenine dinucleotide (FAD) and methionine sulfoxide on methyl-C$^{14}$-methionine incorporation into aflatoxins was also studied (Table 32). Methionine sulfoxide was added at 1, 5 and 10 mM concentrations respectively and it was found to be stimulatory to methylation in aflatoxin at all the tried concentrations (1, 5 and 10 mM). Stimulation of methylation step was found to be non-significant at 1 and 5 mM concentrations but quite prominent at 10 mM concentration in aflatoxin B. The effect of FAD on methylation was detected at 0.1, 1.0, 5.0 and 10.0 mM concentrations respectively. FAD was found to stimulate methyl-C$^{14}$-methionine incorporation into aflatoxins at all the tried concentrations and stimulation of methylation was more in aflatoxin G than in aflatoxin B at all the concentrations tried. However, maximum methylation was observed at 10.0 mM concentration (Table 32). Thus methionine sulfoxide and FAD are probably involved in the regulation of methylation in aflatoxins. Detroy and Freer (1974) found that
the amount of aflatoxin produced was less in a medium where
methionine sulfoxide was substituted for DL-methionine.
Reduced FAD is the coenzyme known to be directly involved in
the conversion of homocysteine to methionine in association with
5,10-methylene-tetrahydrofolate (Cantarow and Schepartz, 1967).
Guest et al. (1964) also found that the cobalmine dependent
route of methyl transfer required the presence of vitamin B₁₂
containing enzyme (B₁₂-enzyme) better known as cobamide. This
enzyme depended on FAD, NADH₂, ATP and Mg²⁺ for activity.
Statistical evaluation of data

Statistical evaluation of the present data was not performed as the mycelial cultures contained innumerable cells. However, all the results presented here are average of two or three separate experiments run in duplicate or triplicate as specified in Tables and Figures.