MODE OF ACTION OF DOTHISTROMIN

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ABSTRACT

Dothistromin is a bright orange-red pigment produced by the pine-needle pathogen Dothistroma pini Hulbary, the causal agent of a necrotic disease known as dothistromal blight. This compound, implicated as a fungal toxin, has been isolated both from laboratory cultures of D. pini and from infected Pinus radiata foliage.

Detailed chemical investigation by Gallagher (1971) showed that dothistromin is a tri -α- hydroxyanthraquinone fused to a substituted tetrahydrobifuran ring system. The bifuran ring moiety is incorporated in other fungal metabolites including the extremely toxic and carcinogenic aflatoxin compounds. There is an increasing body of evidence to suggest that these fungal metabolites share a common biosynthetic origin.

Bioassay has demonstrated the toxicity of dothistromin to the unicellular green alga, Chlorella pyrenoidosa, and to tissue cultures of P. attenuata. The very low level of solubility of the compound in aqueous solutions has precluded bioassay using pine seedlings. This thesis reports an investigation of the biochemical changes induced by dothistromin in microbiological systems.

In the course of this investigation dothistromin has been shown to be toxic to a range of microorganisms in addition to its known toxicity to Chlorella pyrenoidosa. These studies have suggested possible ways of increasing the sensitivity of bioassays for dothistromin.

It was found that the addition of dothistromin to liquid cultures of Chlorella as an ethyl acetate solution caused reproducible levels of inhibition, provided that the ethyl acetate concentration was less than 0.5%. Batch culture techniques were used to establish the levels of dothistromin required for inhibition of growth of Chlorella. The ratio of dothistromin concentration to cell number was found to be an important factor in the inhibition response.

Utilization of synchronous culture techniques permitted the study of biochemical changes induced by dothistromin throughout the cell cycle of Chlorella. Results showed a marked inhibition of the rate of increase of total protein and RNA over the cell cycle with no significant alteration of the rate of DNA increase.

A dose-response curve for dothistromin inhibition of growth of Chlorella was established and a more detailed investigation of the action of dothistromin in inhibiting growth was undertaken using radioactive isotopes. By this means it was shown that ^{3}H-uridine and ^{14}C-phenylalanine incorporation into cell material

is inhibited within 30 mins of exposure to the toxin. Difficulties encountered in attempts to obtain satisfactory incorporation of label into Chlorella DNA-fractions prevented further investigation of the effect of dothistromin on DNA synthesis in this organism. This led to the investigation of other microorganisms as more suitable experimental systems for this study.

Bacillus megaterium KM, proved to be very sensitive to dothistromin and showed rapid incorporation of radioactive isotopes into protein and nucleic acid fractions. Growth curves established that the inhibitory ratios of dothistromin concentration to cell numbers for this organism were in the order of 0.25 μg/cell X 10^8 (as compared to 2.0 μg/cell X 10^8 for Chlorella). At this concentration, over the 30 min time course studied, dothistromin had no effect on the incorporation of ^{3}H-thymidine into the DNA fraction. Inhibition of ^{3}H-uridine incorporation was evident at 6 min and very marked by 10 min while the inhibition of ^{14}C-phenylalanine incorporation into protein was not evident until considerably later. The effects of dothistromin in this system were compared with those of antibiotics with known sites of action. Dothistromin inhibition of ^{3}H-uridine incorporation has a similar time course to that shown by actinomycin D, although marked inhibition by Actinomycin D is evident at 3 min, whereas dothistromin inhibition is not noticeable until 6 min.

On the basis of these results it is suggested that dothistromin interferes with RNA synthesis and that the observed inhibition of protein synthesis is a secondary effect of this impairment.

Confirmation of dothistromin action 'in situ' by administration of the compound to pine seedlings is necessary before any definitive statement can be made concerning its role in dothistromal blight. However these results indicate the possible importance of dothistromin in pathology of dothistromal needle blight of pines. Impairment of the RNA synthetic capacity in pine needle tissue by the toxin could rapidly lead to cell death and to the necrosis of needle tissue observed in diseased foliage.

ACKNOWLEDGEMENTS

I wish to express my thanks to the following people:

My supervisors, Professor R.D. Batt and Dr. G.G. Pritchard, for their helpful discussion and encouragement during the course of this work.

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1. INTRODUCTION

Dothistromin is a toxic metabolite produced by the pine-needle fungus Dothistroma pini Hulbary and isolated from laboratory culture of the fungus and from pine foliage infected with D. pini. A detailed chemical investigation of the bright orange-red pigment by Gallagher (1971) showed that dothistromin is a tri- α -hydroxyanthraquinone moiety fused to a substituted tetrahydro- bifuran ring system and established its structure as that shown in Fig.1 (I). This thesis reports an investigation of the toxicity of dothistromin and the biochemical changes it induces in microbiological systems.

1.1 DOTHISTROMAL PINE BLIGHT

1.11 The history of dothistromal blight

An extensive review of the disease, its control and its implications is given by Gibson (1972). Dothistroma pini was first identified by Hulbary (1941) as the pathogen causing a needle blight of the Australian pine, Pinus nigra var austriaca. The first report of the fungus in New Zealand was made by Gilmour (1965) who identified the fungus on P. radiata (D. Don) in 1964 near Tokoroa. The disease is now established in most young P. radiata plantations throughout the central and southern North Island with smaller areas of infection in the Auckland conservancy and in the

Gwavas State Forest, Hawkes Bay. Light infections have been found in the South Island in Golden Downs State Forest, Nelson, and on the West Coast.

Dothistromal blight is also established in Central and Eastern Africa and in Chile where large-scale commercial plantings of P.radiata (D.Don) support forestry industries. In the northern hemisphere a comparable outbreak of the disease has not been found, although there are reports of dothistromal blight in British Columbia, California, Oregon and the Palni Hills of India (Gibson, 1972).

The recognition of dothistromal blight as a threat to exotic forest plantations has stimulated an increasing amount of research, directed mainly at an economic solution to the problem.

1.12 The nature of the disease

Infection by the pathogen is shown by the appearance on the needles of yellow flecks and bands which rapidly become necrotic, assuming a reddish tinge (Gibson et al., 1964). Necrosis of the pine needle tissue is followed by casting of the needles and results from work with D.pini indicate that the observed effects of the blight on tree growth result from the destruction of photosynthetic tissue (Gibson, 1972).

Details of the infection process on P.radiata needles are reported by Gadgil (1967). After penetration of the needle the mycelium grows throughout the mesophyll with hyphae growing intra- and intercellularly, penetrating the resin canals. Disorganisation of the mesophyll was

evident in advance of hyphal spread and vital staining revealed dead cells in the tissue of these areas. Gadgil suggested the possibility of exotoxin or exo-enzyme production by D. pini to account for these observations.

Brunt (1970) examined the extracellular enzymes produced by D. pini in laboratory culture and in infected needles, and demonstrated the production of enzymes catalysing the degradation of pine needle structural polysaccharides. These enzymes may be of importance to the fungus in utilising host tissue.

D. pini is considered to be a fungal pathogen mainly confined to species within the genus Pinus. Host lists, based on field observations, show that P. radiata is the most susceptible of the economically important species. (Gilmour, 1967). D. pini has also been recorded as attacking two non-Pinus species, Pseudotsuga menziesii (Douglas fir) and Larix decidua, (European larch) (Dukin and Walper, 1967; Gadgil, 1968; Bassett, 1969). Although dothistromal blight has no apparent economic significance on these non-Pinus species in New Zealand, it is important in disease control that all potential hosts are known.

Some susceptible species of pine become increasingly resistant to D. pini attack with age, while other species, like P. nigra and P. ponderosa, remain disease susceptible. P. radiata acquires disease resistance after 15-20 years. Little is known of the mechanism of blight resistance or the development of mature plant resistance, as

shown by P.radiata. However, it is known to be a function of the entire tree and is transferable by grafting (Garcia and Kummerow, 1970).

1.13 The control of the disease

Breeding programmes aimed at producing blight resistant stock form an important part of the effort to control the disease. To date completely resistant trees have not been found and the partial resistance demonstrated appears to be due to precocious development of mature plant resistance. Clonal propagation of cuttings from trees old enough to have developed resistance preserves the parental characteristics and an improvement of the techniques involved may allow the establishment of large-scale plantings of resistant stock derived in this way (Gibson, 1972).

Adequate protection from the disease may be achieved and maintained by aerial spraying of plantations with copper fungicides, aqueous suspensions of insoluble copper compounds such as copper oxychloride or cuprous oxide (Gilmour and Noorderhaven, 1970). It has been found that the application of fungicide every three or four years will keep the disease under control until the trees attain mature resistance. (For P.radiata this involves 5-6 applications of fungicide over the first 15-20 years.)

Production losses from dothistromal blight can be considerable and in these terms the use of fungicidal control is economically and technically feasible in New Zealand. However topographical problems and less highly-developed agricultural aviation industries restrict the use of this method of control in other countries. In the East African highlands P.radiata was replaced with less susceptible and less

productive species soon after the appearance of dothistromal blight.

The application of copper fungicides controls the incidence of the disease but does not eradicate it; and even with the production of resistant stock there is the possibility of pathogen adaptation to the resistance factor(s). Thus it is important that more is known of the factors involved in the host-pathogen relationship, the development of the disease and the acquisition of resistance by the host.

1.2 DOTHISTROMIN, A SECONDARY METABOLITE OF D.PINI

1.21 Dothistromin and co-metabolites

The suggestion by Gadgil (1967) that necrosis following *D. pini* attack could be caused by a fungal exotoxin led to the investigation of extracts of culture media. Bassett and Brunt isolated a red pigment from *D. pini* culture and demonstrated its toxicity to *Chlorella pyrenoidosa* by means of an antibiotic disc bioassay. This pigment, dothistromin, has been isolated, purified and its molecular structure determined (Gallagher, 1971; Bassett *et al.*, 1970). Since then the molecular structure has been confirmed and the absolute configuration established from x-ray diffraction data (Bear *et al.*, 1970, 1972).

Attempts to demonstrate the toxicity of dothistromin to *P. radiata* tissues have not been successful. One of the limitations in the study of dothistromin toxicity is its very low solubility in aqueous solutions. However antibiotic discs impregnated with the pigment result in the death of cultures of *P. attenuata* tissue when placed on the medium next to the culture (Bassett, 1971).

A number of other compounds have been isolated from laboratory culture of D.pini (Shaw, 1974; Gallagher, 1971). The structures of these co-metabolites are given in Fig.1. One of the major co-metabolites is deoxydothistromin ( ) which travels with dothistromin on TLC plates.

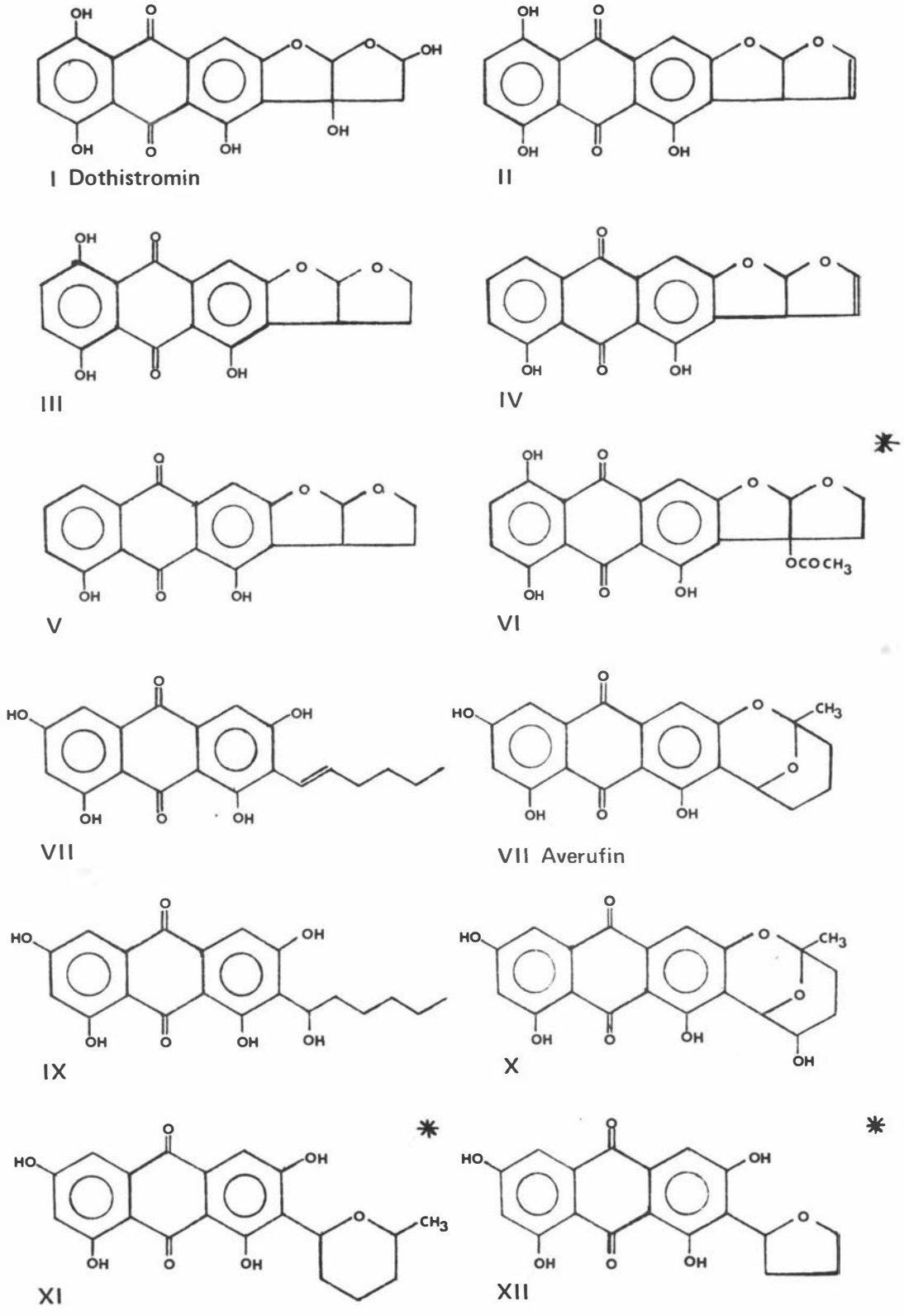
In attempting to demonstrate that dothistromin is functioning as a fungal toxin in the disease process it is important to consider the results of work with other structurally related mycotoxins. This may indicate the possible modes of action of dothistromin.

1.22 Possible relationships with other mycotoxins

As a result of the discovery of aflatoxin in mold-contaminated feeds in 1961 a large number of fungal metabolites have now been isolated and their toxicity investigated in a variety of biological systems (Kadis, Ciegler and Ajl, 1971; Purchase, 1971). Because of the diversity of such toxic fungal metabolites this discussion will be restricted to a consideration of those having structural similarity to dothistromin and hence possible relevance to the demonstrable toxicity of this compound.

Studies of the toxicity of the aflatoxins, sterigmatocystin and analogues have indicated a correlation between structure and biological activity (Engelbrecht and Altenkirk, 1972; Ayres et al. 1971). These studies indicate that the unsaturated bifuran moiety may be related to toxicity and it is suggested (Engelbrecht and Altenkirk, 1972) that a carbonyl group in the α , β position may also have importance with respect to biological activity. (The relationship between the bifuran ring and

FIGURE 1: COMPOUNDS ISOLATED FROM *D.PINI* CULTURES



Structures derived from mass spectral data. Compounds isolated from TLC plates and silica gel columns.
(* — structures from mass spectral data only. Compounds not isolated.)
(Shaw, 1974. pers. comm.)

the carbonyl group of the xanthone or coumarin nucleus of these compounds may be better described as a phenolic oxygen conjugated to a carbonyl group.)

A number of fungal metabolites possessing a dihydro- or tetrahydrobifuran moiety are known, many having been isolated and characterised recently (Rodricks, 1969).

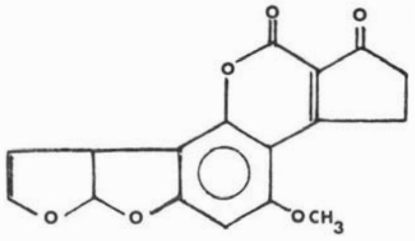
These include sterigmatocystin, aversin, the versicolorins and the aflatoxins.

1.221 The aflatoxins

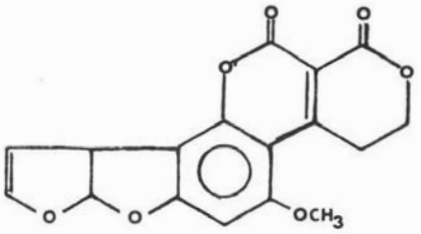
These are a group of difurano coumarins produced by strains of the Aspergillus flavus group and discovered to be the causative agents of the 'Turkey X disease' outbreak in England in 1960. Structures are given in Fig. 2. Studies with this series of compounds show large differences in toxicity and carcinogenicity. The relative order of toxicity is $B_1 > G_1 > B_2 > G_2$ which is in accordance with the structural-toxicity correlations already outlined. Of these, aflatoxin B_1 has been most extensively studied and is known to be an extremely potent hepatocarcinogen. A comprehensive monograph on the aflatoxins has been published (Goldblatt, 1969) and there is a recent review by Wogan and Pong (1970). (See also Detroy et al. 1971).

Aflatoxin has been shown to suppress DNA synthesis in vivo (Laforge and Frayssinett, 1970; Rogers and Newberne, 1967) and in vitro (Legator, 1966; Zuckerman et al. 1967; Wragg et al. 1967);

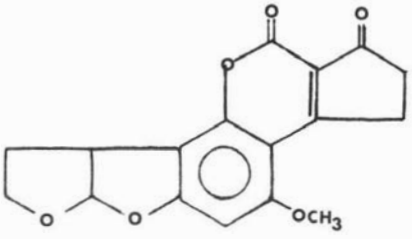
FIGURE 2: AFLATOXINS



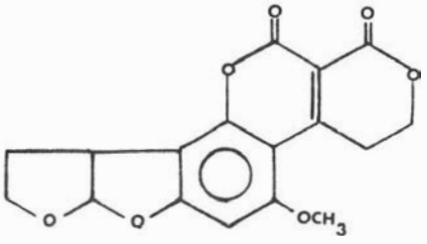
I Aflatoxin B_1



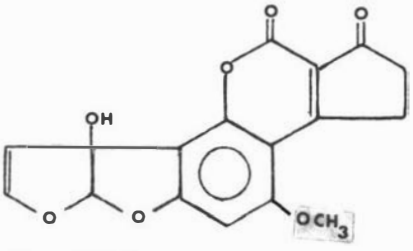
II Aflatoxin G_1



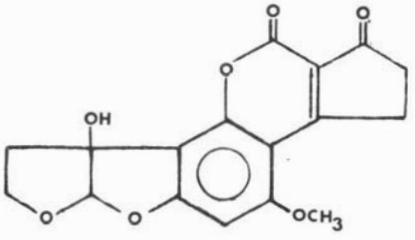
III Aflatoxin B_2



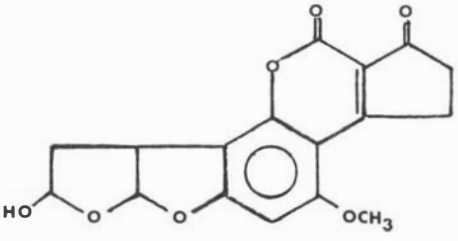
IV Aflatoxin G_2



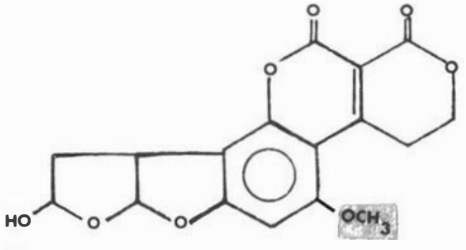
V Aflatoxin M_1



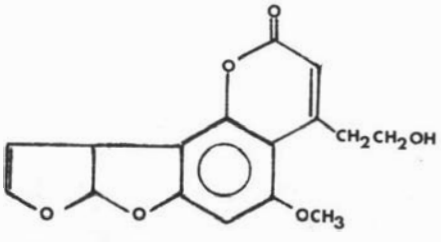
VI Aflatoxin M_2



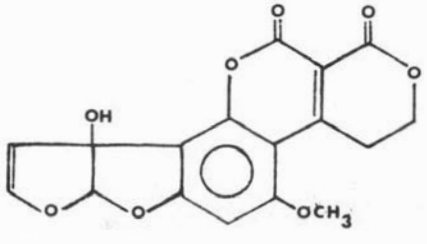
VII Aflatoxin B_{2a}



VIII Aflatoxin G_{2a}



IX Aflatoxin B_3



X Aflatoxin GM_1

to inhibit RNA synthesis *in vivo* (Clifford and Rees, 1966; Clifford *et al.*, 1967; Harley *et al.*, 1969; Gelboin *et al.*, 1966; Pong and Wogan, 1970) and in some *in vitro* systems (Clifford and Rees 1966, 1967; Moule and Frayssinett, 1968). It is known that the inhibition of RNA synthesis is not occurring by direct inhibition of DNA - dependent RNA polymerase (Pong and Wogan, 1970; Edwards and Wogan, 1970). Inhibition of protein synthesis has been reported in many systems (Clifford and Rees, 1966, 1967; Smith 1964; Wogan and Friedman 1965; Friedman and Wogan, 1965; Lillehoj and Ciegler, 1970) and is generally thought to be a secondary effect of aflatoxin inhibition of RNA synthesis. However, in a recent publication, Sarasin and Moule (1973) report a marked biphasic response in the time-course of protein synthesis inhibition by aflatoxin. The second phase of this response can be accounted for by inhibition of RNA synthesis and the polysome disaggregation observed. (See also Roy, 1968; Villa-Trevino and Leaver, 1968; Harley *et al.*, 1969). A direct aflatoxin effect on the translational mechanism is suggested to account for the very marked inhibition observed in the early stages of the biphasic response.

These biochemical changes induced by aflatoxin are thought to result from interaction of the toxin with DNA. There is evidence of DNA-aflatoxin interaction from *in vitro* experiments (Sporn *et al.*, 1966; Clifford *et al.*, 1967; King and Nicholson, 1969; Neely, *et al.*, 1970; Schabort, 1971) but administration of ^{3}H-aflatoxin B_1

in vivo to rats showed that the most highly labelled liver fraction was the protein fraction and not the DNA fraction (Lijinsky, 1968; Lijinsky, *et al.*, 1970).

Results published by Edwards and Allfrey (1973) showed that administration of aflatoxin B_1 to rats causes a sudden increase in the rate of histone deacylation (by 15 min.). Temporal and spatial correlations have been established for acetylation and gene activity, with the acetyl content of the histones diminishing with the suppression of transcription (Allfrey, 1971).

A parallel has been drawn between the effects of Actinomycin D, which exerts its inhibitory effect by binding with the DNA template, and the effects of aflatoxin. Actinomycin D does not stimulate the rate of histone deacylation (Edwards and Allfrey, 1973) and in several systems actinomycin causes marked inhibition where aflatoxin has no observable effects (Edwards and Wogan, 1970). However, results indicate that aflatoxin acts at the transcriptional level and it has been suggested that this is caused by interaction of the toxin (or a metabolic derivative) with components of the chromatin.

1.222 Other fungal metabolites containing bifuran moieties

Sterigmatocystin was first characterized as a metabolite of *A. versicolor* (Bullock *et al.*, 1962) and it has been isolated from two other fungi, *A. nidulans* and *Bipolaris sp.* (Holzapfel *et al.*, 1966). Several other naturally occurring derivatives have since been reported (Burkhardt and Forgacs, 1968; Holker and Kagal, 1968; Elsworthy *et al.*, 1970;

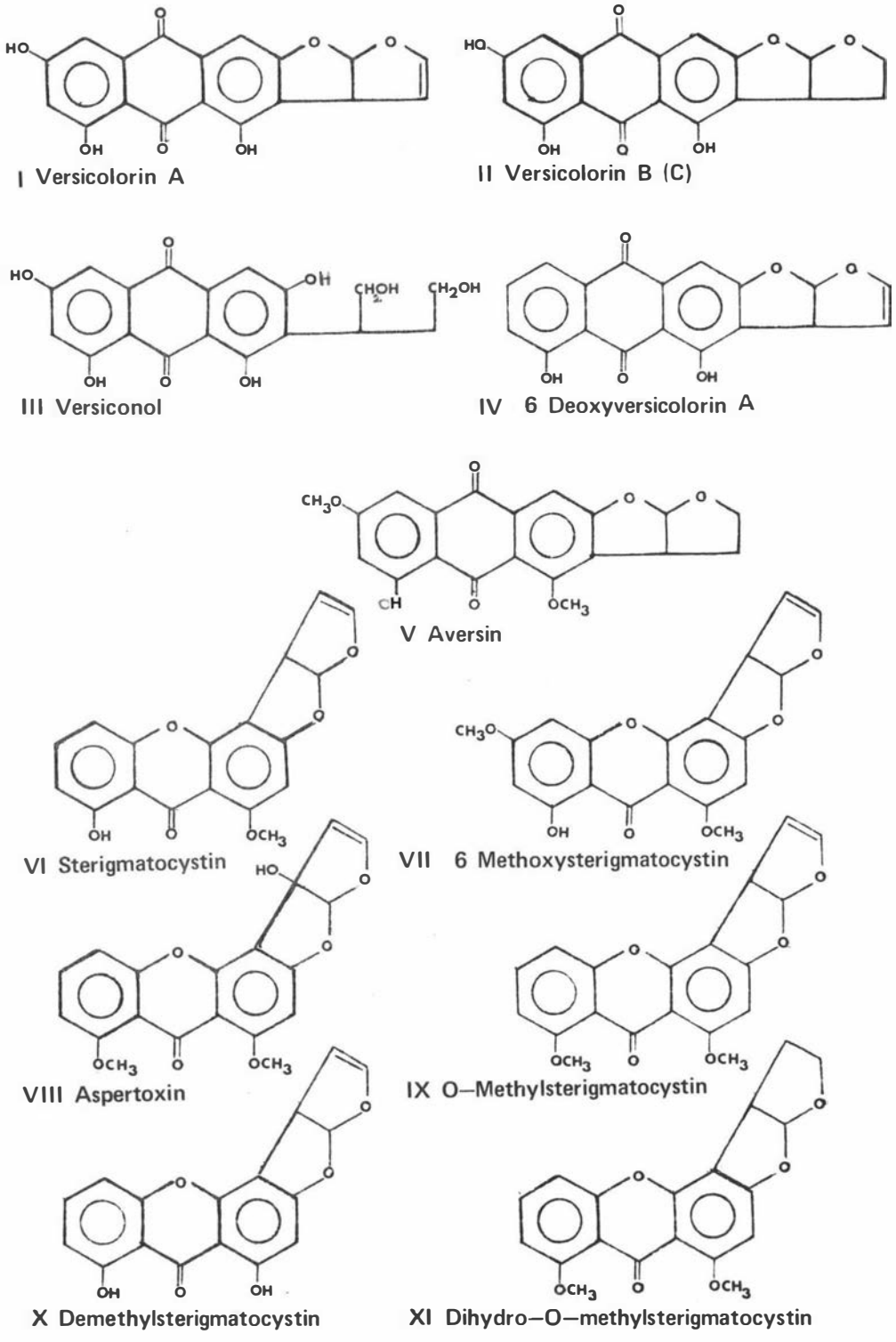
Rodricks et al., 1968; Waiss et al., 1968; Cole and Kirksey, 1970). Structures of these compounds are given in Fig. 3.

The toxicity of sterigmatocystin has been shown in several biological systems and its carcinogenicity demonstrated in rats (Abedi and Scott, 1969; Lillehoj and Ciegler, 1968; Purchase and Van der Watt, 1968; Nel et al., 1971; Engelbrecht, 1971). The carcinogenic potency of sterigmatocystin has been established to be of the order of 1/100th that of aflatoxin B_1.

Anthraquinone metabolites have been isolated from A. versicolor. Versicolorins A B and C were isolated by Hamasaki et al.(1965, 1967); versicolorin C has since been shown to be a racemate of versicolorin B. Structures of the versicolorin group of fungal metabolites are given in Fig. 3. Other versicolorin derivatives (or precursors) are known (Hatsuda et al., 1969, 1971; Elsworthy et al., 1970). The anthraquinone aversin was reported as a metabolite from a variant of A. versicolor (Bullock et al., 1963). To date, no reports of toxicity studies with the versicolorin group of fungal metabolites have appeared.

It is of interest to compare the metabolites isolated from D.pini culture with those reported from other fungi. There is increasing evidence from labelling studies that these compounds may share a common biosynthetic origin, via the acetate-malonate pathway. Thus, study of dothistromin is important as part of the general survey of toxic compounds elaborated by fungi.

FIGURE 3: THE VERSICOLORIN AND STERIGMATOCYSTIN GROUPS OF FUNGAL METABOLITES



1.23 Possible structure - activity relationships for dothistromin

The possession of a substituted bifuran ring moiety (albeit a saturated one) may confer toxicity on the dothistromin molecule. By analogy with the aflatoxins one would not expect dothistromin, which has a hemiacetal group in the bifuran system, to be as toxic as unsaturated derivatives. (Aflatoxins B_{2a} and G_{2a} both contain a hemiacetal in the bifuran ring and this appears to nullify the potent toxicity of the parent aflatoxins.)

The anthraquinone system may be of importance in the biological activity of dothistromin. Quinones as a group have a multiplicity of actions as metabolic inhibitors. These include interactions with sulfhydryl groups, with amino acids and with proteins, inhibition of enzymes and modification of electron transport mechanisms in oxidative phosphorylation and photosynthesis (Webb, 1966). However, the effects of 9, 10 anthraquinones are not well documented. In general they appear to be less effective inhibitors than the simpler quinone compounds.

1.3 AIMS OF THIS INVESTIGATION

This thesis is concerned with an investigation of the toxicity of dothistromin and with its implication as a toxin in dothistromal blight of *Pinus* spp. The literature reveals that fungal metabolites possessing similar structural features are potently toxic in diverse biological systems. The aims of this research were, therefore, to investigate the toxicity of dothistromin by studying its biochemical effects in micro-organisms, to elucidate a possible site of action to account for these effects and perhaps indicate a likely site of dothistromin action in the pathogenicity of *D. pini* to *Pinus* spp.

2. EXPERIMENTAL METHODS

2.1 ORGANISMS

Dothistroma pini var. Hulbary and Chlorella pyrenoidosa were supplied by the Forestry Research Institute, Rotorua

The following organisms were obtained from staff members of the Microbiology Department, Massey University:

Saccharomyces cerevisiae, Bacillus mycoides, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Proteus vulgaris.

Sources of the other organisms used in this research study are:

Bacillus megaterium KM (Strain 3694): Dr. W.C. Haynes, USDA, Peoria, Illinois.

Bacillus megaterium KM T⁻, (mutant strain): Dr. J.T. Wachsman, Department of Microbiology, University of Illinois.

Euglena gracilis (Strain Z): Professor E.F. Carrell, University of Pittsburg, Pennsylvania.

2.2 MATERIALS

The suppliers of the materials used are given in the following list:

Thymine, adenine, cytosine, thymidine and uridine (sigma grade, crystalline); Sigma Chemical Company.

Adenosine - 5' - monophosphate, disodium salt, Puriss grade; Koch-Light Laboratories.

Uridine - 5' - monophosphoric acid (disodium salt), guanine (chromatographically pure), guanosine - 5' - monophosphoric acid (disodium salt) and cytidine - 5' - monophosphoric acid (disodium salt, Puriss CHR); BDH Laboratory Chemicals.

Bacto-peptone, Bacto-tryptone, Yeast extract and Chlorella agar; Difco Laboratories, Michigan, U.S.A.

Nutrient Broth; BBL, Division of Bioquest, Maryland, U.S.A.

Agar; Davis Gelatine (NZ) Ltd., Christchurch.

DYC 'Active' Yeast; Dominion Yeast Co., Auckland, New Zealand.

Malt, Wander brand dried malt, Type 500 LDE; A. Wander (NZ) Ltd., Christchurch.

Cycloheximide, Chloramphenicol (crystalline), Mitomycin C. (from Streptomyces caespitosus, crystalline) and Actinomycin D (from Actinomyces antibioticus); Sigma Chemical Company.

Ribonuclease A, from Bovine pancreas (5 x crystallized, protease-free, Type 1A); Sigma Chemical Company.

Bovine Serum Albumin, (Fraction V.; $>$ 95%; pH 7.0); Fluka, AG, Buchs SG.

2, 5 - diphenyloxazole (PPO); Fluka, AG, Buchs SG.

1, 4 - D - [2 - (5 - phenyloxazolyl)] - benzene, (POPOP), scintillation grade; Nuclear Enterprises Ltd., Scotland.

Radioisotopes were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Thymidine (methyl -^{3}H). Batch No.74, sp. act. 26.8 curies/mmol. 107mCi/mg; Batch No.77, sp.act.23 curies/mmol., 95mCi/mg.

Uridine - 5 -^{3}H. Batch No.69, sp.act.29 curies/mmol., 119mCi/mg; Batch No.73, sp.act.24 curies/mmol., 98mCi/mg.

L - 3 - phenylalanine -^{14}C. universally labelled. Batch No.61, sp.act.513mCi/mmol., 91% isotopic abundance in all carbon atoms.

Uracil - 2 -^{14}C. Batch No.53, sp.act. 62mCi/mmol., 544μCi/mg., 99% isotopic abundance in carbon atom 2.

The antibiotic discs used in bioassay were Whatman A.A. discs (1.3cm) from W. and R. Balston Ltd., England.

Aflatoxin B_1 and Sterigmatocystin were obtained from Mr. C. Freke, Microbiology Department, Massey University.

The hydroxyanthraquinone compounds were obtained from Dr. R.T. Gallagher, Chemistry Department, Massey University. Dothistromin was isolated and purified as outlined in section 2.3.

The dothistromin analogue (vinyl ether derivative) was obtained from Mr. G.J. Shaw, Chemistry Department, Massey University.

Gas cylinders of the 5% CO_2 in air gas mixture were purchased from N.Z. Industrial Gases.

2.3 ISOLATION AND PURIFICATION OF DOTHISTROMIN

(Method as given by Gallagher (1971)).

2.31 Laboratory culture of D.pini

D.pini stock cultures were maintained on 10% Malt agar slants and sub-cultured at 2 monthly intervals. To obtain spores for inoculation of liquid cultures D.pini was streaked on to malt agar plates. Spore production occurred 10-14 days after inoculation of the plates. A spore suspension, prepared by agitating malt agar cultures with sterile water, was used for inoculation of 10% malt aqueous medium for toxin production. With a heavy inoculum, pigment production reached a maximum within 10 days at 17°. Pigment production was assessed by withdrawing an aliquot of the culture (using aseptic techniques) extracting with an equal volume of ethyl acetate and reading the absorbance of the ethyl acetate extract at 492nm in an SP800 UV spectrophotometer (Unicam.). Initially D.pini was cultured in 1 l Erlenmeyer flasks, continuously shaken on a gyrotary shaker (New Brunswick, Model G10). One large culture of 20.1 was then grown in a Marmacell Fermentor (New Brunswick Scientific Co.) at 18°, continuously stirred and aerated.

2.32 Extraction of Dothistromin

The total culture, medium and mycelium, was extracted twice with an equal volume of ethyl acetate. The solvent was added to the culture flasks which were shaken on the gyrotary shaker for approximately 8 hours. After standing overnight, the ethyl acetate

layer was decanted and the extraction procedure repeated. For the large Formacell culture the extraction process was carried out in 5 l Erlenmeyer flasks on the gyrotary shaker. Emulsions form very readily during this extraction. After the second extraction an equal volume of distilled water was added to facilitate the separation of ethyl acetate from the emulsion. The flasks were shaken gently for 2-3 min., allowed to stand and the residual ethyl acetate layer decanted. (A length of rubber tubing attached to a vacuum pump via a large Buchner flask was found to be very useful for dispersing stubborn emulsions and for removing any of the ethyl acetate layer remaining after decanting. The ethyl acetate solution collected in the Buchner flask was readily separated from any aqueous layer which accumulated.) The total ethyl acetate extract was then washed repeatedly with aliquots of distilled water in large separating funnels (2.5 l). After filtering through fluted filter paper (Whatman No.1), the ethyl acetate was evaporated under vacuo using a rotary evaporator, over a water bath (40 - 50°). The resulting red deposit contained a high proportion of dothistromin.

2.33 Purification of Dothistromin

Dothistromin was purified from the amorphous red powder by preparative thin layer chromatography (TLC). Preparative TLC was carried out on 20 X 20 cm. square glass plates using 0.75mm. thick layers of silica gel 'G' (BDH). TLC Plates were activated by drying overnight at 120°.

The red powder was applied as an ethyl acetate solution to the plates in a narrow band, approximately 5mm. wide, by repeated streaking with a fine Pasteur pipette. Plates were developed in ethyl acetate: chloroform (60:40, v/v) by ascending chromatography. The culture extract resolved into a number of components, with dothistromin forming the major red band (R_f 0.4). The colour of the dothistromin-containing band varies from bright orange-red to crimson depending on the pH of the solvent. This band was carefully scraped off the plates, while still wet, into ethyl acetate. The resultant slurry was shaken with a little distilled water and the orange ethyl acetate layer separated filtered and evaporated to dryness in a rotary evaporator. The dothistromin used in this investigation was purified three times by TLC and stored in the dark as the dry powder. Stock solutions, in ethyl acetate, were made up as required. The concentration of these solutions was determined by UV spectrophotometry (Unicam SP800). (1mg of dothistromin in 25 cm^3 ethyl acetate has an absorbance of 0.75 at 490$_{nm}$. Gallagher, pers. communication).

2.34 Precautionary measures for handling Dothistromin

The possible contribution of the dihydro-benzofuran or the tetrahydro- derivative of this moiety to the toxicity and carcinogenicity of fungal metabolites, such as the aflatoxins and sterigmatocystin, has already been discussed. (1.23). Dothistromin contains a hemiacetal in this bifuran system but the formation or biosynthesis

of unsaturated derivatives is not unlikely. For this reason dothistromin and its cometabolites should be handled with care while possible effects on man are being examined.

In general, these materials should be handled using the same precautions taken when working with radioactive substances or pathogenic organisms, as recommended by Goldblatt (1969) for the aflatoxins. During this study all contaminated glassware was soaked in strong alkali before washing and strong alkali was added to all solutions before discarding. Protective clothing and gloves were worn when working with the toxin. D.pini cultures were autoclaved before disposal. Contaminated solid waste, such as filter papers, was placed with solid waste from radioactive studies for incineration.

2.4 MICROBIOLOGICAL METHODS

2.41 Maintenance of Chlorella cultures

Chlorella pyrenoidosa was maintained on Chlorella agar slants (see section 2.7) and sub-cultured every 4-5 weeks. After inoculation the slants were illuminated at 25° for 4-5 days and the cultures were then stored at 4°. Cells for the inoculation of liquid cultures were taken from fresh slants (4-7 days old).

2.42 Liquid culture of Chlorella

Chlorella pyrenoidosa was grown autotrophically in an inorganic medium aerated with 5% CO_2 in air; details for preparing the medium are given in section 2.7. The cultures were grown in 500 cm^3 or

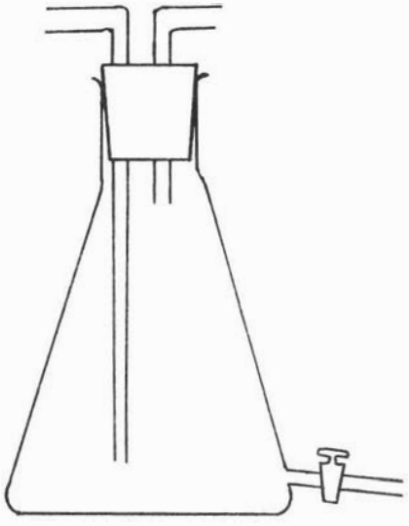
1000 cm^3 Erlenmeyer flasks stoppered by rubber bungs fitted with glass tubing inlets arranged so that aeration prevented the cells from settling out during culturing. The Erlenmeyer flasks were fitted with a glass-tubing side-arm and stopcock set in the base for the removal of samples during culture.

Chlorella cultures were grown at 25° in a thermostatted room. The cultures were illuminated with two banks of four Atlas 'Super-Gro', 20 watt fluorescent tubes arranged to give an incident light intensity of 8000 lux (measured using a Seiko photographic light meter) at the surface of the culture vessels. To prevent local areas of higher temperature around the light banks, a large Indola fan was installed behind the lights to keep the air circulating and maintain an even temperature throughout the room. The bench-top was lined with white paper to reflect light through the base of the culture vessels which were placed on inverted square wire baskets. The gas was bubbled through a Drechsel bottle of sterile water before being introduced into the cultures. A schematic diagram of the culture apparatus is given in Fig.4.

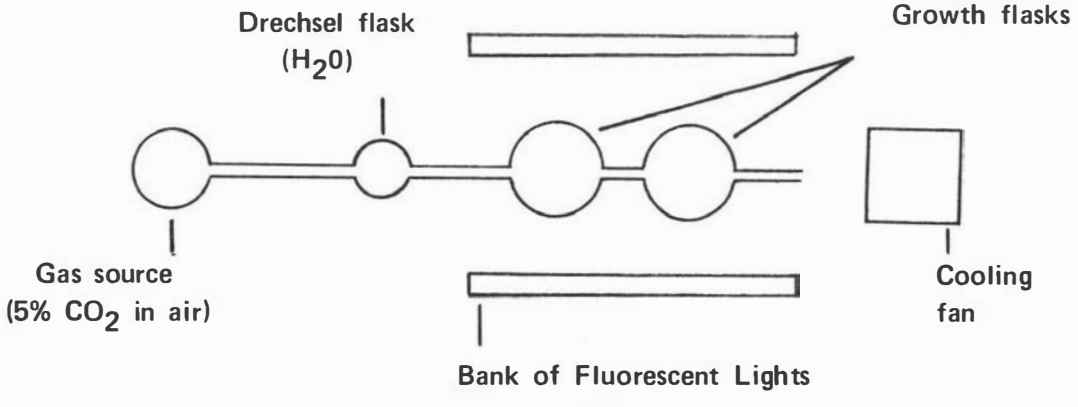
2.43 Use of Chlorella in Plate Bioassay

To prepare the bioassay plates, 10 cm^3 of a two day culture of Chlorella was dispersed thoroughly in 100 cm^3 of Chlorella agar cooled to 45°. This seeded agar was poured into sterile petri plates at 20 cm^3 (approx.) per plate and the agar allowed to solidify. Known amounts of dothistromin (or compound for assay)

FIGURE 4: SCHEMATIC DIAGRAM OF CULTURE APPARATUS FOR *CHLORELLA.*



Growth Flask



were applied to antibiotic discs as an ethyl acetate solution using microlitre pipettes or Hamilton microlitre syringes. Solvent control discs were prepared in the same way. The discs were allowed to dry thoroughly. Each disc was then placed in the centre of the seeded agar petri plates.

The plates were incubated at 25° under fluorescent illumination (Atlas 'Super-Gro', S-2008, 20 watt tubes) for 4 days. At this time, the diameters of the resulting inhibition zones were measured.

To improve the clarity of the inhibition zones, an 'overlay technique' was adopted for bioassay. Basal layers of sterile unseeded Chlorella agar (approx. 10 cm^3 per plate) were allowed to solidify in the petri plates before the Chlorella-seeded agar layer was poured (approx. 10 cm^3 per plate). Antibiotic discs were placed at the centre of each plate after both agar layers had solidified.

2.44 Bioassay Procedure, using other micro-organisms

The bioassay procedure used for the range of micro-organisms screened for sensitivity to dothistromin was as outlined for Chlorella. Aliquots from overnight nutrient broth cultures of each organism were added to and dispersed thoroughly in flasks of sterile nutrient agar cooled to 45°. The seeded agars were poured onto basal layers of unseeded nutrient agar in sterile petri plates and the appropriate disc placed in the centre of each one after the agar had solidified. Since most of the other organisms investigated have a much faster growth rate than Chlorella pyrenoidosa these plates only required

overnight incubation before the zones of inhibition were measured. The exception was Euglena gracilis. Euglena bioassay plates, like those of Chlorella, were illuminated for four days before the zones of inhibition were measured.

Details of media preparation are given in section 2.7.

2.45 Measurement of Growth of Chlorella in Liquid Culture

After inoculation of liquid media, Chlorella cultures were grown as described above. At timed intervals, following gentle shaking of the culture vessels to ensure an even cell suspension, 5 cm^3 samples were withdrawn from the cultures via the side-arms. The OD 660_{nm} of each sample was then read in a Bausch and Lomb Spectronic 20 spectrophotometer. Samples were diluted with distilled water if necessary.

A standard curve relating OD 660_{nm} to cell number/cm^3 was constructed using a haemocytometer (Fig.5.). The standard curve plotted is a composite curve utilizing data from cultures over a wide range of dilutions.

2.46 Synchronous Culture of Chlorella

2.461 Introduction to Synchronous Culture Techniques

Synchronous cultures are those in which the principal mass of the cells in a given population are at a single stage of development. The methods that have been developed to achieve synchrony in various cell systems have been conveniently summarised by James (1966) and this table is duplicated here for reference (Table 1). (For a more recent discussion refer to Mitchinson, 1971).

FIGURE 5: STANDARD CURVE FOR *CHLORELLA*.
($OD_{660\ nm}$ vs. cell no./cm^3)

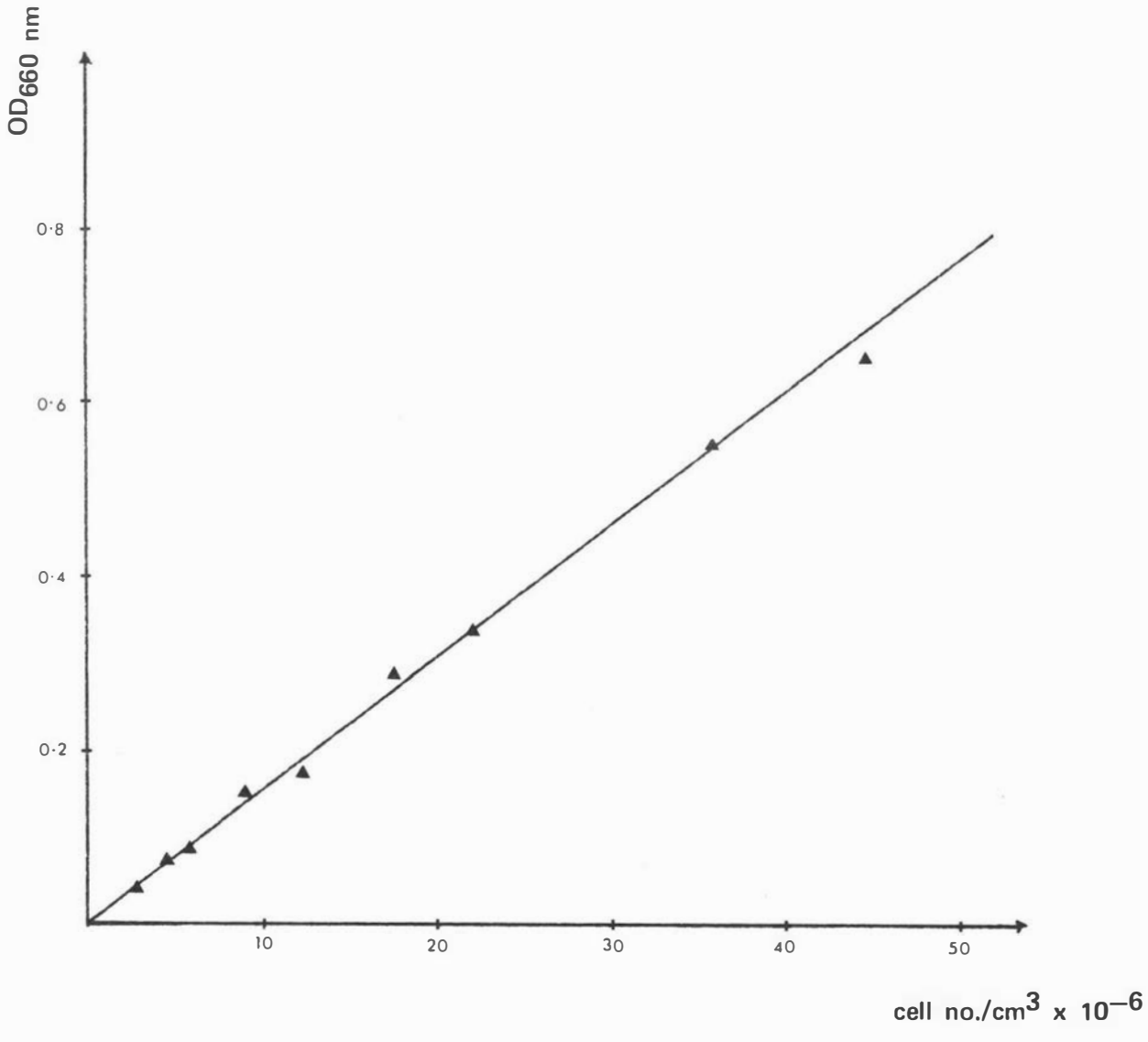


TABLE 1.

Methods for Obtaining Synchronous Cultures

Category	Principle
INDUCED SYNCHRONY	Synchronous cultures are produced by treatment of the culture.
Temperature methods	Temperature changes are used as the treatment.
Cycles	Culture is subjected to a cold-warm cycle equal to one generation. Cycle is repeated with renewal of medium with each cycle.
Shifts	Temperature is changed from a low to a high level. Divisions occur in warm period.
Shocks	Temperature is raised and lowered in rapid succession in a series of pulses. Pulses range from optimal or suboptimal to supra-optimal. Divisions occur following a period at the lower temperature.
Light methods	Light changes are cue for the entrainment of cell cycle.
Cycles	Light and dark cycles are used to entrain cultures of photosynthetic cells such that divisions occur one each cycle. Strength and duration of light period must be such as to give balanced growth.
Nutritional methods	Growth on basal medium is followed by addition of enriched medium called a shift-up, or vice-versa, a shiftdown. Shifts from starvation level to enriched medium are also used.

TABLE 1 (Cont'd.)

Category	Principle
Auxotrophic control	Uses a change in a single requirement holding other requirements in excess. Genetic strains with specific requirements are most effective.
Heterotrophic control	Makes use of changes on broad spectrum requirements, i.e. carbon source, nitrogen source. Divisions occur after a lag following enrichment.
Gasometric methods	
Aerobic-anaerobic	Uses single or multiple cycles of bubbling nitrogen then air through the culture in a fixed pattern in time to induce synchrony. Controlled gas tensions may also be a mode of control.
Inhibitory methods	Employs an inhibitor of cell division or a stage in the cell cycle and a washout procedure to accumulate cells at a specific stage of cycle. Excess of natural metabolites are most promising, i.e. thymidine.
SELECTION SYNCHRONY	Synchronous cultures obtained by isolation of a specific stage.
Sizing methods	Depend on correlation between cell size and cell age. Selects small fraction from a logarithmic culture to start synchronous subculture.
Filtration	Small size bacteria are obtained by expressing slurry of cells through a filter pad. Care is taken to maintain other conditions constant. Small cells are subcultured.
Sedimentation velocity	Depends on difference on sedimentation rate with cell size. Small cells are removed from upper portion of a density gradient to form synchronous subculture.
Grow-off methods	Adsorb cells onto filter pack. Parent cell attaches, daughter cell falls off at fission.
Substrate attachment	Tissue culture cells and amoeboid forms attach to the substrate during interphase of the cell cycle. At time of division they tend to round up and detach. Collection of detached forms provides subculture of synchronous cells.

- from James (1966).

2.462 The cell cycle of Chlorella

Synchronized cultures of Chlorella were first developed by Tamiya and colleagues. After the observation of two distinct forms of cells in cultures of C. ellipsoidea, these two types of cells were shown to have different characteristics; the smaller 'dark' cells being richer in chlorophyll and more active photosynthetically than the 'light' cells (Tamiya et al., 1953). It was also shown that the 'dark' cells, if illuminated and provided with adequate nutrients, increased in size and became 'light' cells, which in turn divided to form daughter cells similar to the 'dark' cells. The process whereby the 'light' cells were transformed into 'dark' cells was found to be light independent. Tamiya et al. (1953) also note that their cultures displayed the 'peculiar phenomenon' of an almost simultaneous change from one form into the other at a fairly definite time interval, a phenomenon which ceased after several days culturing. Further work by the Japanese group established the cyclic nature of the development of C. ellipsoidea and also-called 'Tamiya cycle' with its 7 stages of growth and ripening was published. (Tamiya et al., 1961a) (See Fig.6).

Since the establishment of a technique of synchronizing C. ellipsoidea, the effects of various environmental factors have been extensively studied. Of interest in the context of this research study is the investigation of the effects of more than fifty antimetabolites on the cell cycle of C. ellipsoidea undertaken by Tamiya et al. (1961b). Their results indicate quite clearly that effective use can be made

FIGURE 6: THE 'TAMIYA' CYCLE

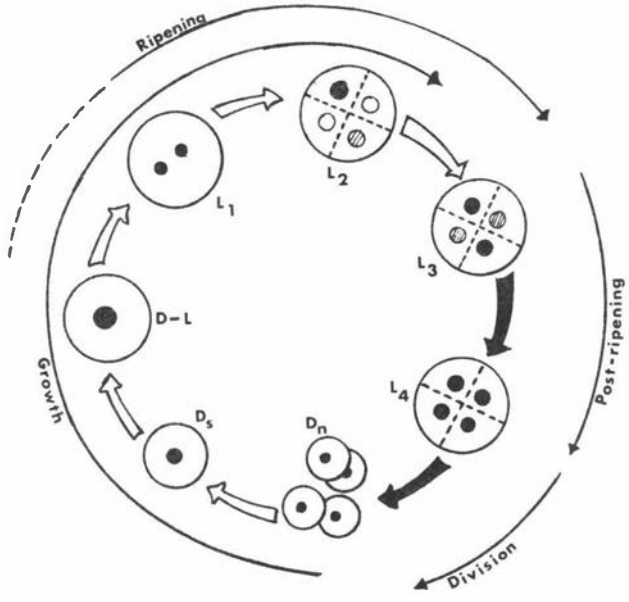


Diagram of the cell cycle of *Chlorella ellipsoidea*. White arrows indicate light-dependent processes, and black arrows show light-independent transformations. (From Tamiya et al., 1961a) (D_n, D–L etc. – notation developed by these workers for reference to cells at a particular stage of the cycle).

of synchronous culture techniques as a tool in the investigation of the mechanism of action of inhibitory substances.

The most widely used method for synchronizing algal cells involves the use of light: dark regimes. The duration of the light and dark periods as well as the intensity of illumination are important factors in determining the success of a synchronizing regime. The light period must be of sufficient length to allow cells to photosynthesize and carry out the metabolic processes necessary for growth and cell division. With the dark period beginning just prior to the onset of cell division, the daughter cells are released in darkness and, until the onset of the following light period, these cells are prevented from initiating a new cycle of growth and development. This forms the basis of the synchronizing method. Obviously a dark period of excessive duration would result in the starvation and depletion of cells. On the other hand, if the periods between illumination were too brief cell division would not have been completed by the entire algal population, due to slight variation in the lengths of individual cell cycles. This would make synchrony difficult to achieve. The regime used to obtain synchrony must be 'selectively chosen by trial and error' as noted by Tamiya (1966).

2.463 Development of Technique for Synchronous Culture of Chlorella

2.4631 Preliminary Studies

The major difficulty encountered in setting up synchronous cultures was the determination of the duration of the illumination period required for growth by this strain of *Chlorella pyrenoidosa* under the growth conditions chosen. First attempts in this direction were unsuccessful. These included exposing a log-phase batch culture at an initial cell density of 1.5×10^7 cells/cm^3 to two successive light: dark cycles of 36 hr:12 hr with dilution of the population to the initial cell density at the beginning of the second cycle. (The regime chosen was based on the life cycle of *C. ellipsoidea* as observed by the Japanese group.) Little or no significant changes were observed in the cell size distribution and the cell number only increased to $2.7 \times 10^7/cm^3$. This suggested that insufficient light was available to the culture to permit optimal growth. The banks of fluorescent lights were rearranged to give an incident light intensity of 8000 lux at the surface of the culture vessels. (Data obtained by Tamiya *et al.*, 1964; Morimura, 1959; using *C. ellipsoidea* as an experimental organism show little increase in growth rate at 25° at light intensities above 10,000 lux.)

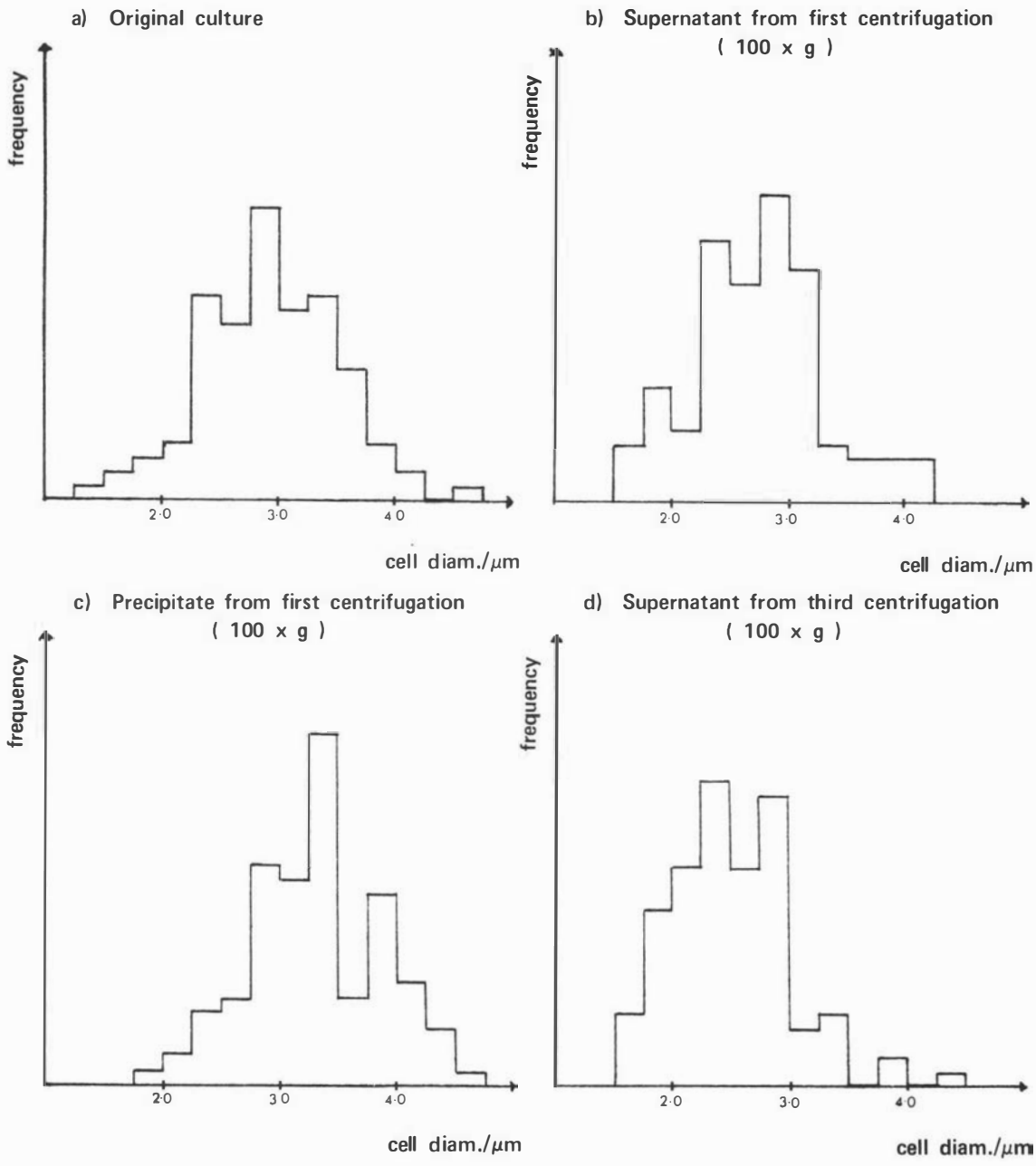
An established culture was incubated in the dark for several days before being used to inoculate fresh medium at an initial cell density of $2.2 \times 10^7/cm^3$. A cell size distribution plot of this 'starter' culture showed that 57% of the population were small cells

(of diam. $< 3.00\mu$). The culture was grown under continuous illumination and samples were withdrawn at intervals for determination of cell numbers and cell size distribution. At no time was the expected 'burst' of cell division observed and again changes in cell size distribution were not significant. It was recognized that the starting inoculum would have suffered some degree of starvation but it was hoped that the apparent homogeneity of the initial population would encourage some degree of synchronization. It is possible that a percentage of the population were no longer viable and thus obscured any synchronized division which may have occurred.

Obviously the selection of an homogenous starting population is an advantage in establishing synchronous cultures.

Differential centrifugation was investigated as a means of preparing homogenous starting cultures because of the ease with which large volumes can be handled and the relative ease of maintaining a sterile environment. A log-phase batch culture of Chlorella was subjected to centrifugation at 9000 rpm X 8 min. (13200 X g). The entire algal population, which precipitated under these conditions, was resuspended in cold, sterile 0.02M KH_2PO_4, pH 4.5 (Sorokin, 1965) and centrifuged three times in succession at 1000 rpm X 2 min. (100 X g). After each centrifugation the precipitate of larger cells was discarded. Samples were taken at each step and the cell size distribution within each fraction determined. Representative cell size frequency histograms are given in Fig.7. As shown, this

FIGURE 7: CELL SIZE DISTRIBUTIONS
Representing the range of cell sizes present in each selected algal population.



procedure yields an algal population in which more than 97% of the cells are small (of diam. $< 3.00\,\mu$).

A population selected by differential centrifugation as outlined above was collected by centrifugation and resuspended in pre-equilibrated medium. Under continuous illumination of the culture the onset of cell division, as evidenced by an increase in cell number and the reappearance of small cells in the cell population, was observed in samples from the 16th to 17th hour of culture.

On the basis of these experiments, a method using a light: dark regime (16hr : 8 hr) followed by the selection of an homogenous population by differential centrifugation was adopted to initiate synchronized Chlorella cultures for the purposes of this research study. Full details of the method are given below.

2.4632 Procedure for obtaining synchronous cultures of Chlorella

'Starter' cultures, from which the inoculum for initiating synchronous cultures was derived, were inoculated from agar slants. These starter cultures were grown on three cycles of 16hr. fluorescent illumination followed by 8 hr dark at 25° in the experimental apparatus described for liquid culture of Chlorella. During dark periods the growth flasks were wrapped in aluminium foil to exclude the light. (By this means other experiments, not necessarily in phase with the light: dark regime, could be carried

out at the same time.) The three cycles of 16 hr : 8 hr regime were followed by a 72 hr period of growth in the dark.

At the end of this precultural regime the cells were harvested by centrifugation (13200g, 9,000rpm X 8 min GSA rotor in Sorvall-SS-3 centrifuge) in sterile, metal 250cm^3 containers. The cells were then resuspended in 0.5 volumes cold sterile 0.02M KH_2PO_4, pH 4.5 and centrifuged X 3 at 1,000 rpm X 2 min (100 g, GSA rotor). After each centrifugation the precipitate of larger cells was discarded. The 'small' cells, selected by differential centrifugation in this manner, were precipitated by centrifugation (13200 X g; 9,000 rpm X 8 min, GSA rotor). The 'starter' population was then resuspended in growth medium, which had been pre-equilibrated at 25^o and aerated with 5% CO_2 in air, to give an OD_{660mm} in the order of 0.01 (to give an initial cell density of 1.0 X 10^6 cells/cm^3).

The synchronous cultures initiated in this way were grown in Erlenmeyer flasks, aerated with 5% CO_2 in air at 25^o and illuminated in a 16hr : 8 hr light: dark regime, (unless specified otherwise). Samples for analysis were withdrawn via the side-arms at timed intervals. The bulk of each sample was frozen immediately and stored until the end of the experiment when all samples were analysed. Small aliquots of each sample were taken for estimation of cell numbers and cell size distribution.

Duplicate cell counts were taken of each sample using a haemocytometer. (The marked changes in cell size which occur in the

life cycle of Chlorella and the variation of pigmentation over this period precluded estimation of cell numbers by OD_{660nm} as used for the growth cultures. This method would have necessitated the construction of a standard curve for each stage of the cell cycle.) Cell size distribution histograms were plotted from measurements of 100 cells from each Chlorella sample, using a calibrated travelling eyepiece (E. Leitz, Wetzlar).

At the end of each experiment all stored samples were thawed and duplicate aliquots taken from each for separation into nucleic acid and protein fractions.

2.464 Assessment of Degree of Synchrony Achieved

Requirements for ideal synchrony as outlined by Senger and Bishop (1969) are:

i). Complete synchronization - that all cells should undergo complete division in the synchronous life cycle.

ii). Homogeneity - all cells of a synchronous culture should deviate as little as possible from the developmental stage of the mass of the cultural population.

iii). Exponential growth - cultural conditions should be optimal to allow growth in the exponential phase with the shortest possible life cycle.

iv). Non-susceptibility of the life cycle to the synchronizing procedure - the method of obtaining synchronous cultures should not affect the biochemical events in the cycle and the cells should be identical at the beginning and end of one synchronous cycle.

The synchronized Chlorella populations achieved do not completely fulfil all of these requirements. The conditions of culture would appear to allow near optimal growth and there has been considerable discussion as to whether algal cultures synchronized by light: dark alternations fulfil the fourth of these requirements. Some authors argue that a light: dark regime corresponds more closely to the natural environmental conditions of algae than does continuous illumination and is therefore more physiological (Goryunova et al., 1962; Senger and Bishop, 1969). It has also been reported that growth was inhibited in cultures under continuous illumination with repetitive dilution (Ruppel, 1962).

In the system used for this research there is a fairly high degree of population homogeneity as shown by cell size distribution plots, but complete synchronization is not achieved. (The division numbers in most cases are slightly less than 4.0).

Investigation of the pattern of events occurring over the cell cycle of a continuously illuminated synchronous culture of Chlorella showed a continued increase in cell number over the cell cycle and

a wider range of cell size in the algal population than in cultures grown under the intermittent illumination of the light: dark regime (see Fig.15). Continuation of the synchronizing procedure is therefore necessary to maintain synchrony, particularly for more than one cycle. Further work showed that even using programmed illumination and diluting the culture with fresh medium at the end of the first cycle (i.e. at the onset of the second light period) the degree of synchrony achieved in the second cycle was not as satisfactory nor as reproducible as that of the first cycle.

There have been a number of papers reporting indices of synchrony, i.e. ways of assessing the degree of synchrony achieved (Engelberg, 1961; Scherbaum, 1964; Engelberg and Hirsch, 1966; Burnett-Hall and Waugh, 1967). Only the index proposed by Scherbaum has been applied in this case. The synchronization index (SI) is defined as:

$$SI = 1 - \frac{t + gt\,(2-n)}{1.12\,gt}$$

where t = time (mins.) during which synchronous division occurs

gt = generation time (mins.) of normal exponentially growing culture

and $n = 1 + \frac{\text{No. cells in synchrony}}{\text{total No. cells}}$

Using this index it has been possible to assess and compare the synchrony achieved. The index approaches unity for a culture showing a high degree of synchrony and becomes very much less for cultures in which only a portion of the total population are in synchrony. Examples of calculated SI values are given in Table 2. For the cultures developed in this study the SI was in the order 0.6 - 0.7. This index became very much less for cultures where successful synchronization was not achieved.

TABLE 2

Synchronization Indices (SI)

CULTURE: Expt. I	Initial cell no./cm^3	Final cell no./cm^3	n	t (min)	$\therefore$ SI
Intermittent illumination (incomplete cell division over longer period)	1.7×10^6	3.3×10^6	1.48	600	0.17
Expt.II					
Intermittent illumination	2.0×10^6	8.0×10^6	2.0	480	0.71
Continuous illumination*	2.0×10^6	10.0×10^6			
Expt.III					
Continuous illumination	1.0×10^6	4.01×10^6	1.95	480	0.66
Expt. IV					
Continuous illumination	0.6×10^6	2.10×10^6	1.88	480	0.59
Continuous illumination (culture containing Dothistromin)	0.6×10^6	0.70×10^6	1.29	480	0.074

$$SI = 1 - \frac{t + gt\,(2-n)}{1.12\,gt}$$

$$\text{where } n = 1 + \frac{\text{no. cells in synchrony}}{\text{total no. cells}}$$

generation time (gt) = 1440 mins.

* Very large burst of cell division occurring over longer period with a division no. $>$ 4.0 and still increasing at 24th hr.

The degree of synchrony achieved in the Chlorella system developed could clearly have been improved and it is recognised that the cell cycle is, in all probability, lengthened by a lag period resulting from depletion during the precultural treatment. However these cultures separated cellular growth processes from those of nuclear and cellular division sufficiently to allow their use as a biochemical tool in studying the effects of dothistromin.

2.47 Maintenance and growth of Saccharomyces cerevisiae

Cultures of S.cerevisiae were maintained on MYPG (malt/yeast extract/Peptone/glucose) agar slopes. After inoculation the slopes were incubated overnight at 30° and then stored at 4°. The organism was sub-cultured every 3-4 weeks. Liquid cultures were grown in the medium detailed in Section 2.7. This medium was inoculated from fresh slopes of the culture and grown at 30°.

2.48 Maintenance and Growth of Bacillus megaterium (KM, KMT)

Cultures of B.megaterium were maintained on the medium given in Section 2.7. After inoculation the slopes were incubated at 30° overnight and then stored at 4°. The organisms were sub-cultured every 7-8 days. Liquid cultures were inoculated from slopes into the medium detailed in Section 2.7. and grown at 30°.

2.49 Measurement of Growth of S.cerevisiae and B.megaterium

Liquid cultures of S.cerevisiae or B.megaterium were grown at 30° in a shaking waterbath, in 25 cm^3 Erlenmeyer flasks fitted

with side-arm tubes designed to fit a Klett-Summerson photoelectric colorimeter. Each flask was withdrawn from the waterbath at timed intervals and the change in cell density measured by following the increase of the Klett over the period of culture. In this way growth curves were plotted for both organisms and the toxic levels of various compounds established by addition during log-phase and observation of the resultant changes in the growth of the culture.

To establish the effective range of the dothistromin: cell number ratio for each organism it was necessary to be able to correlate Klett units with cell number/cm^3. Standard curves were prepared by counting microscopically the number of cells/cm^3 in cultures of a known Klett unit. For S.cerevisiae, a budding yeast, buds were counted as whole cells when they had attained 50% of the parent-cell size, as judged by eye.

2.5 ANALYTICAL METHODS

2.51 Determination of nucleic acids and protein

2.511 Modified Schmidt-Thannhauser Extraction Procedure

(Buetow and Levedahl, 1962)

The following extraction procedure was used for the determination of nucleic acids and protein throughout the cell cycle of Chlorella and for determining the distribution of label in cellular components after radio-isotope labelling.

Duplicate samples for extraction were concentrated by centrifugation (5 min, top setting, Gallenkamp 'Junior' benchtop centrifuge) and the supernatants discarded. The pellets were extracted successively with absolute ethanol (1 cm^3), twice with absolute ethanol: ether (50: 50, v/v) (1 cm^3) and with absolute ethanol (1 cm^3). In each extraction the pellet from the previous step was carefully resuspended in the solvent and left to stand (5-10min.) before centrifuging. The supernatants from each stage were discarded.

To extract acid-soluble components the pellets were resuspended evenly in 1 cm^3 cold 0.2M perchloric acid and placed on ice for 15 min. At the end of this time the suspensions were centrifuged and pellets re-extracted with 1 cm^3 cold 0.2M perchloric acid in the same manner. The supernatants from these last two steps were combined to form the 'acid-soluble' fraction.

The precipitate from the final centrifugation was resuspended in 0.5cm^3 1 M sodium hydroxide and incubated in a 30° waterbath overnight (16hr) to hydrolyze the RNA. (During incubations all tubes were topped with glass marbles to prevent evaporation.) After neutralizing with 0.5cm^3 1 M hydrochloric acid, the suspensions were chilled on ice for 15min. DNA and protein were precipitated by the addition of 3 cm^3 cold 1M perchloric acid. The precipitates were collected by centrifugation and the supernatant from this step forms the 'alkali-labile' fraction (containing RNA components).

Duplicate samples for extraction were concentrated by centrifugation (5 min, top setting, Gallenkamp 'Junior' benchtop centrifuge) and the supernatants discarded. The pellets were extracted successively with absolute ethanol (1 cm^3), twice with absolute ethanol: ether (50: 50, v/v) (1 cm^3) and with absolute ethanol (1 cm^3). In each extraction the pellet from the previous step was carefully resuspended in the solvent and left to stand (5-10min.) before centrifuging. The supernatants from each stage were discarded.

To extract acid-soluble components the pellets were resuspended evenly in 1 cm^3 cold 0.2M perchloric acid and placed on ice for 15 min. At the end of this time the suspensions were centrifuged and pellets re-extracted with 1 cm^3 cold 0.2M perchloric acid in the same manner. The supernatants from these last two steps were combined to form the 'acid-soluble' fraction.

The precipitate from the final centrifugation was resuspended in 0.5cm^3 1 M sodium hydroxide and incubated in a 30° waterbath overnight (16hr) to hydrolyze the RNA. (During incubations all tubes were topped with glass marbles to prevent evaporation.) After neutralizing with 0.5cm^3 1 M hydrochloric acid, the suspensions were chilled on ice for 15min. DNA and protein were precipitated by the addition of 3 cm^3 cold 1M perchloric acid. The precipitates were collected by centrifugation and the supernatant from this step forms the 'alkali-labile' fraction (containing RNA components).

precipitate was resuspended in 50% ethanol and aliquots of both the supernatant and precipitate fractions taken for estimation of radioactivity.

2.513 Estimation of nucleic acids

Concentrations of RNA and DNA in the fractions arising from the Schmidt-Thannhauser extraction procedure were determined by UV spectrophotometry (Hitachi 101 spectrophotometer) using the method of De Deken-Grenson and De Deken (1959).

The RNA fractions were read against a 1M perchloric acid blank at 260 nm and 315 nm. The concentration of RNA ($\mu g/cm^3$) is given by:

$$(OD_{260nm} - OD_{315nm}) \times 33.16$$

Similarly the DNA fractions were read at 267nm and 315nm, the concentration of DNA ($\mu g/cm^3$) in each fraction being given by:

$$(OD_{267nm} - OD_{315nm}) \times 32.94$$

2.514 Estimation of total protein

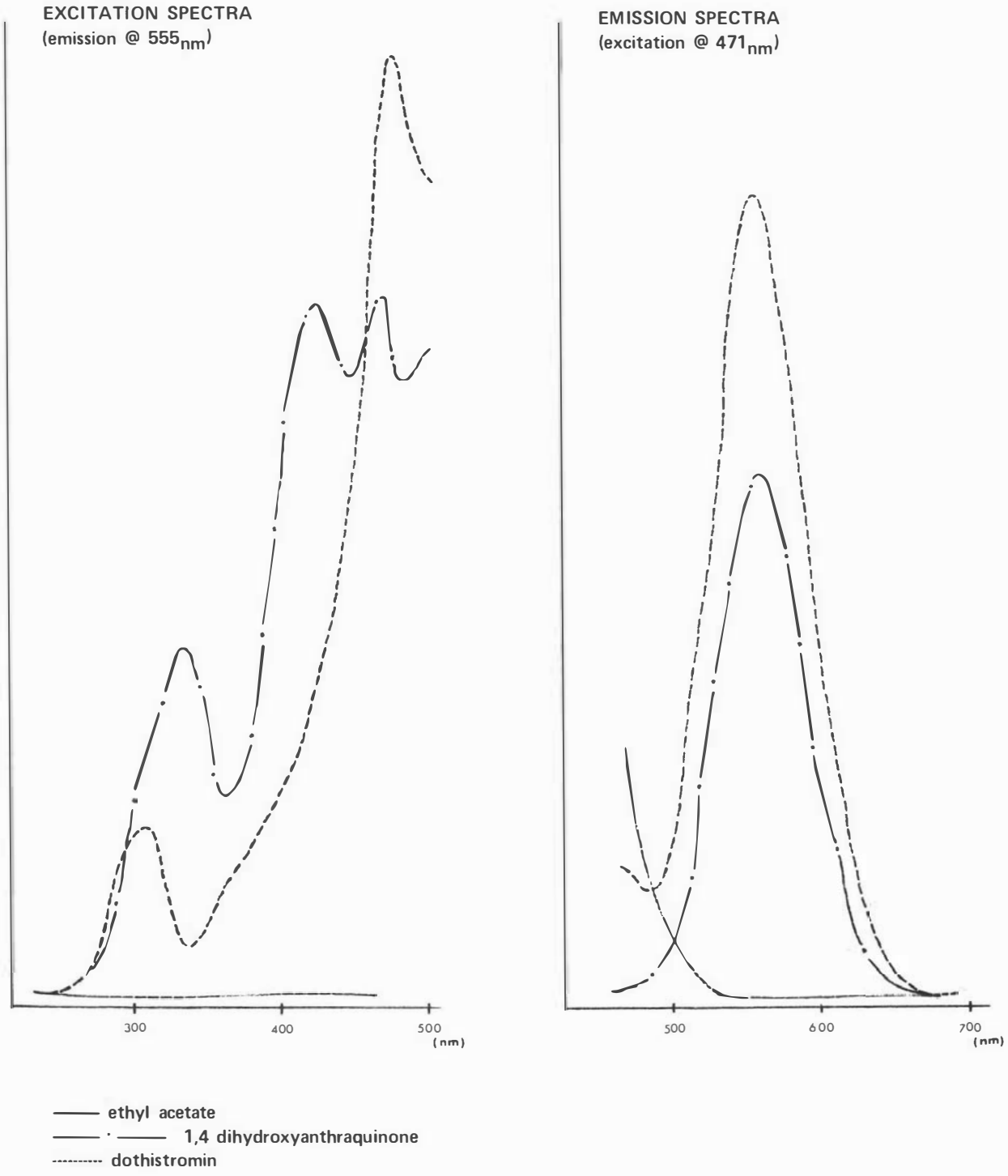
Protein in the Schmidt-Thannhauser fractions was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Standard curves covering the range 0-100 μg protein were constructed using a bovine serum albumin standard. All protein estimations were read at 750nm (Hitachi 101 spectrophotometer).

2.52 Fluorimetric Detection of Dothistromin

Zones from agar bioassay plates were investigated for the presence of dothistromin by utilizing the fluorescence of the compound. Samples were examined for fluorescence in a Turner Spectrofluorimeter, (G.K. Turner spectrofluorimeter Model 430), using an excitation wave length of 471nm and an emission wavelength of 555nm. These wavelengths were selected after determining the emission and excitation spectra of dothistromin in the following way.

Since the fluorescence excitation and the absorption spectra of fluorescent materials correspond, the excitation wavelength was set on the major absorption peak (492nm) and an emission scan was taken. Using the maximum emission wavelength found in this way, an excitation scan was taken and each of the excitation peaks found was examined to determine the settings giving maximum sensitivity for dothistromin fluorescence. The excitation and emission spectra for dothistromin and for 1,4 dihydroxyanthraquinone at these settings are shown in Fig.8.

FIGURE 8: FLUORESCENCE EXCITATION AND EMISSION SPECTRA



2.6 RADIOCHEMICAL METHODS

2.61 Scintillation Counting Procedure

All radioactive samples were counted in Toluene: Triton scintillation fluid. A 1cm^3 aliquot of each sample was transferred to counting vials and 5cm^3 scintillation fluid added. The radioactivity in each vial was counted using a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 2002 or Model 3375). Each sample was counted over 10min. and the results expressed as cpm. Background counts were derived from the equivalent samples containing no added isotope.

The toluene: triton-X-100 (2: 1, v/v) scintillation fluid contained PPO (3g/l) and POPOP (100mg/l). To prepare 1 litre of scintillation fluid, dissolve PPO and POPOP in the proportions given in 660cm^3 toluene (redistilled) and add 330cm^3 triton-X-100.

2.62 Count Corrections

All counts were corrected for background counts and then expressed as cpm for the total sample. Since the data were being used to compare the incorporation of label under different treatment conditions it was not necessary to determine the absolute amount of label present in each sample. The assumption was made that for the same type of fraction the degree of quenching was the same and that the other errors present in the counting system were constant. However

N.B. Scintillation counting procedure: The volume of sample added to the scintillation fluid was critical and if this was altered phase changes occurred affecting the counting efficiency. It is therefore recommended that this concentration range (16 - 17%, v/v) is avoided. (See Williams, 1968; Turner, 1969.)

where comparisons were made with label incorporation into different fractions it was necessary to apply quench corrections. (Different fractions were counted in different solvents so the degree of quenching between fractions varied.) These corrections were made by reference to quench correction curves plotted from data obtained from a series of quenched standards. ^{14}C - n - hexadecane and ^{3}H - n - hexadecane were used in the quenched standards.

2.63 Labelling Procedures for Chlorella

5cm^3 Aliquots of Chlorella liquid culture were taken from the parent synchronous culture at the indicated times and incubated with labelled isotopes and toxin in 50cm^3 Erlenmeyer flasks. These small flasks were suspended over a bank of fluorescent tubes (Atlas 'Super-Gro') and aerated with 5% CO_2 in air over the labelling period. The apparatus was set up in a fume cupboard. At timed intervals the uptake of label into the cells was arrested by addition of 20cm^3 boiling ethanol. The cells were collected by centrifugation (5min x top speed, Gallenkamp 'Junior' benchtop centrifuge) and re-extracted with 25cm^3 boiling 80% ethanol. The cellular material was then resuspended and a 1cm^3 aliquot counted or the sample was separated into RNA, DNA and protein containing fractions using the Schmidt-Thannhauser extraction procedure.

2.64 Labelling procedure for S.cerevisiae and B.megaterium

The labelling procedure used for the preliminary work with these organisms was adapted from that used for the Chlorella studies. $5cm^3$ of liquid culture of the organism, taken from culture in logarithmic phase of growth, was shaken in a 30^o waterbath in $50cm^3$ Erlenmeyer flasks with the addition of labelled isotopes and toxin. The entire $5cm^3$ sample was extracted with $20cm^3$ boiling ethanol, centrifuged and re-extracted with $25cm^3$ boiling 80% ethanol. Aliquots of the resuspended cell material were taken for counting or the whole sample was submitted to the Schmidt-Thannhauser extraction procedure to determine the label distribution throughout the cellular fractions.

The techniques used for labelling and for withdrawing samples at carefully timed intervals were refined for the B.megaterium KM studies. Aliquots of log-phase cultures ($7.5cm^3$ - $15cm^3$) were incubated, with labelled isotope, carrier compound and toxin or antibiotic as specified, in $50cm^3$ Erlenmeyer flasks in a shaking waterbath at 30^o. At timed intervals a $1cm^3$ aliquot was rapidly withdrawn using an Eppendorf pipette. Each aliquot was quickly added to $4cm^3$ hot ethanol to stop label incorporation. (Tubes containing the measured volumes of ethanol were kept standing in a hot waterbath, topped with glass marbles to prevent solvent evaporation.) After centrifuging each sample was resuspended in $5cm^3$ hot 80% ethanol.

These suspensions were centrifuged to collect the cell material which was then resuspended and fractionated using the Schmidt-Thannhauser procedure. Aliquots from each fraction were taken for counting. This modification of the labelling procedure permitted accurate sampling at very short time intervals.

2.65 Investigation of the distribution of isotopic label among cell fractions

For each organism studied the distribution of label throughout the cellular fractions (as derived by the Schmidt-Thannhauser procedure) was determined. These label distributions are given in Tables 10, 11 and 12, and indicate that the counts incorporated into the cell material after hot ethanol extraction are not a reliable measure of specific biosynthetic activity, i.e. protein or RNA synthesis. Non-specific labelling occurs particularly when labelling with ^{3}H - thymidine or ^{14}C - phenylalanine. For this reason data given for the B.megaterium KM experiments represent the label incorporation into the relevant Schmidt-Thannhauser fraction (i.e. ^{3}H - uridine label incorporation into the RNA - containing fraction etc.).

The components in which the label was present in each of the fractions was further investigated by analytical techniques. The RNA hydrolyzate was subjected to electrophoresis to separate the component nucleotides which were identified by comparison of Rf. values with those of standard nucleotide solutions. The DNA

hydrolyzate was chromatographed using standard purine and pyrimidine bases for comparison, and the protein fraction was hydrolyzed and resolved into its component amino acids by high-voltage electrophoresis.

The procedure used to obtain labelled material for analysis was identical to that used for inhibition studies. The entire $7.5cm^3$ of each assay was taken after a 20min labelling period at 30°. (^3H uridine 50 μCi/assay; ^3H thymidine 50 μCi/assay; ^{14}C - pheala 0.5 μCi/assay) after two extractions with hot 80% ethanol each assay was fractionated by the Schmidt-Thannhauser procedure. (0.33M Lithium hydroxide was used for the overnight alkaline hydrolysis instead of 1M sodium hydroxide because it was found to be very difficult to remove sodium or potassium perchlorates from the hydrolyzates.) All fractions were neutralized with 0.33M Lithium hydroxide or 1M perchloric acid as required, and freeze-dried. The dry RNA and DNA hydrolyzates were extracted twice with ether: isopropanol (2: 1 v/v) to remove the lithium perchlorate, and the residues resuspended in $1cm^3$ glass distilled water.

2.651 Electrophoresis of RNA hydrolyzate

The RNA hydrolyzate was applied to strips of Whatman No.1 paper (25 X 2cm) and resolved into its component nucleotides by electrophoresis (150 volts X 4hr) in 0.1M ammonium formate buffer, pH 3.5. Nucleotide standards (0.01M) and a mixture of nucleotides were run at the same time. After drying, the strips were examined under UV light

and the nucleotide bands identified. The distribution of label was determined using a Packard Radiochromatogram Scanner (Model 7200) fitted with a Packard recording ratemeter.

Results are presented graphically in Fig.9. The movement of uridine monophosphate appears to be retarded in a mixture of nucleotides. However, one peak of activity detected by the stripscanner corresponds with the position of uridine monophosphate. The other peak coincides with the position where adenosine and cytidine monophosphate travel. It is possible that this peak is due to the presence of labelled cytidine nucleotides.

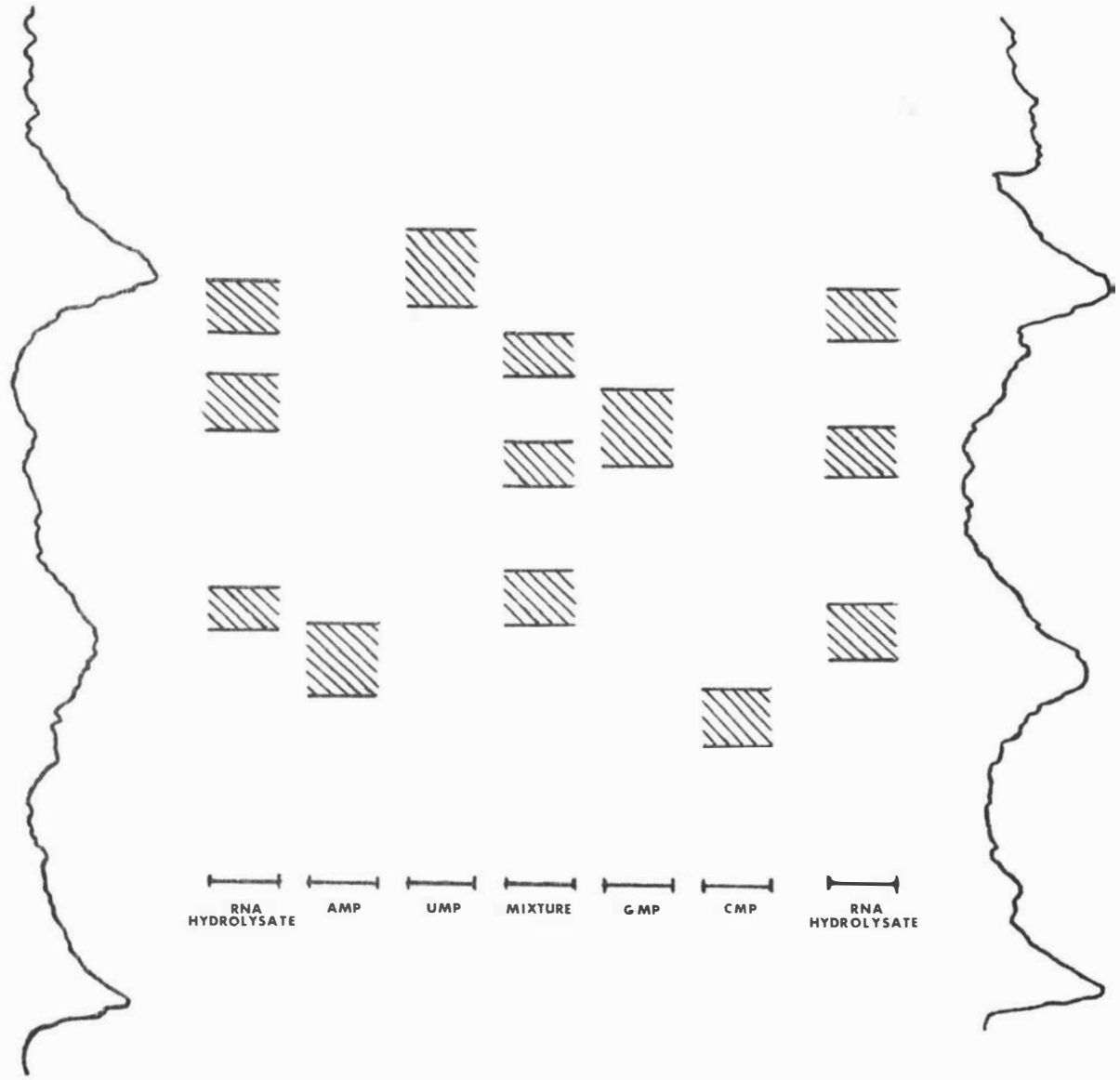
2.652 Chromatography of DNA hydrolyzate

The DNA hydrolyzate was separated into its component bases by ascending chromatography in isopropanol: conc. HCl: water (65:16.6:18.4, v/v/v). Aliquots of the hydrolyzate were carefully spotted onto Whatman No.1 paper (20 X 20cm). Standard solutions of pyrimidines and purines (0.01M) and a mixture of the bases were applied to the same chromatogram for reference. After approx. 6hr the chromatogram was dried and examined under UV light to locate and identify the components. The distribution of label was determined using a radio-chromatogram scanner and these results are given in Fig.10. The only peak of activity detected by the strip scanner lies in the position where thymine chromatographs. No activity was detected in the positions occupied by the other bases.

FIGURE 9: ELECTROPHORESIS OF RNA HYDROLYSATE

0.1 M ammonium formate buffer, pH 3.5
150 volts x 4 hr.

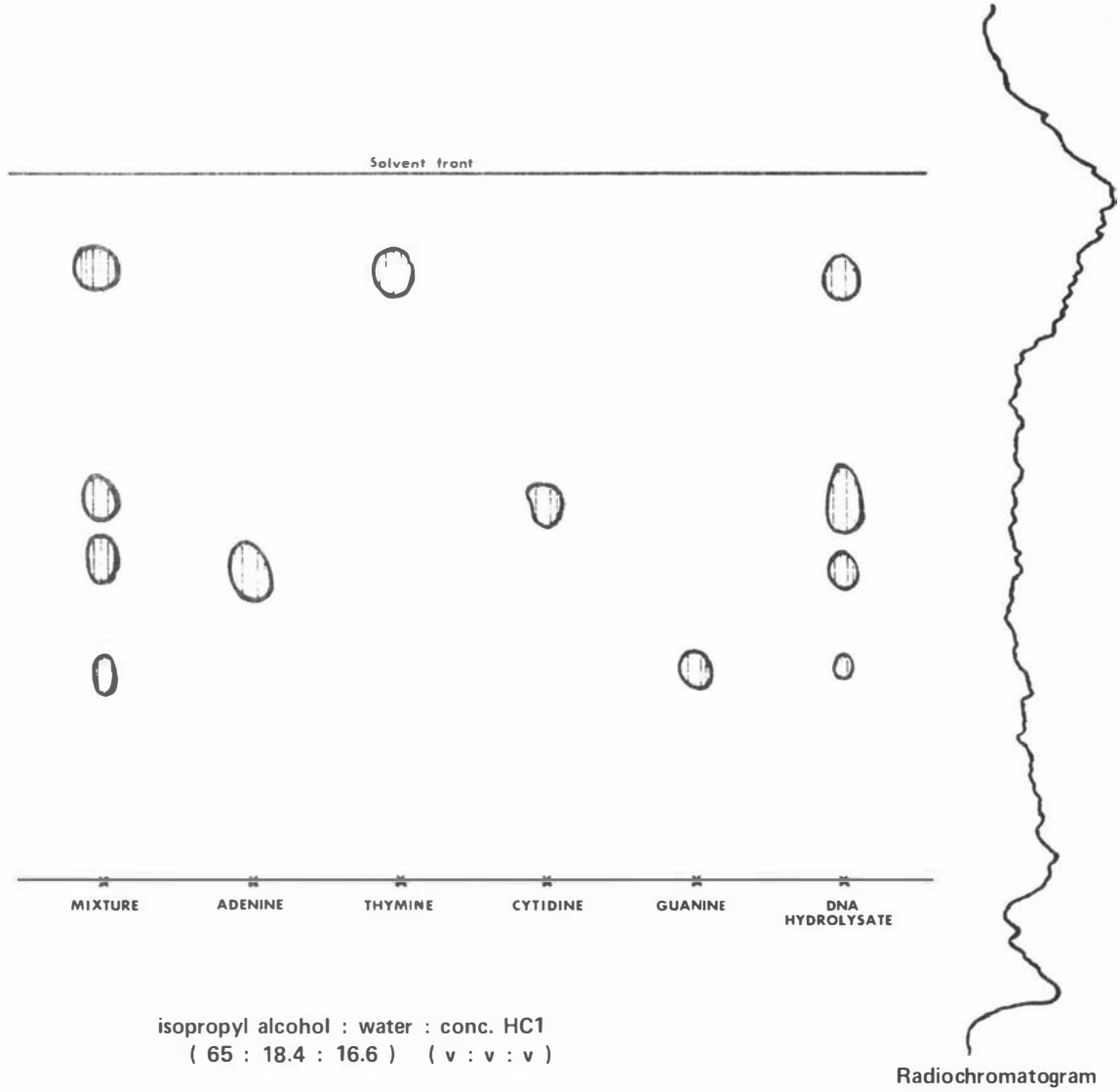
Spots visualized under UV light.
Radioactivity located using a radiochromatogram scanner.



Electrophoretic seperation of nucleotides derived from 1M alkaline hydrolysate (RNA fraction) of B. megaterium cells labelled with ^{3}H-uridine.

FIGURE 10: CHROMATOGRAPHY OF DNA HYDROLYSATE

Spots visualized under UV light.
Radioactivity located using a radiochromatogram scanner.



isopropyl alcohol : water : conc. HC1
(65 : 18.4 : 16.6) (v : v : v)

	Calculated Rf values.	Known Rf values.
Thymine	0.87	0.80
Cytidine	0.54	0.45
Adenine	0.47	0.33
Guanine	0.30	0.22

Chromatographic seperation of DNA hydrolysate derived from hot 1M perchloric acid hydrolysis (DNA fraction) of B. megaterium cells labelled with ^{3}H-thymidine.

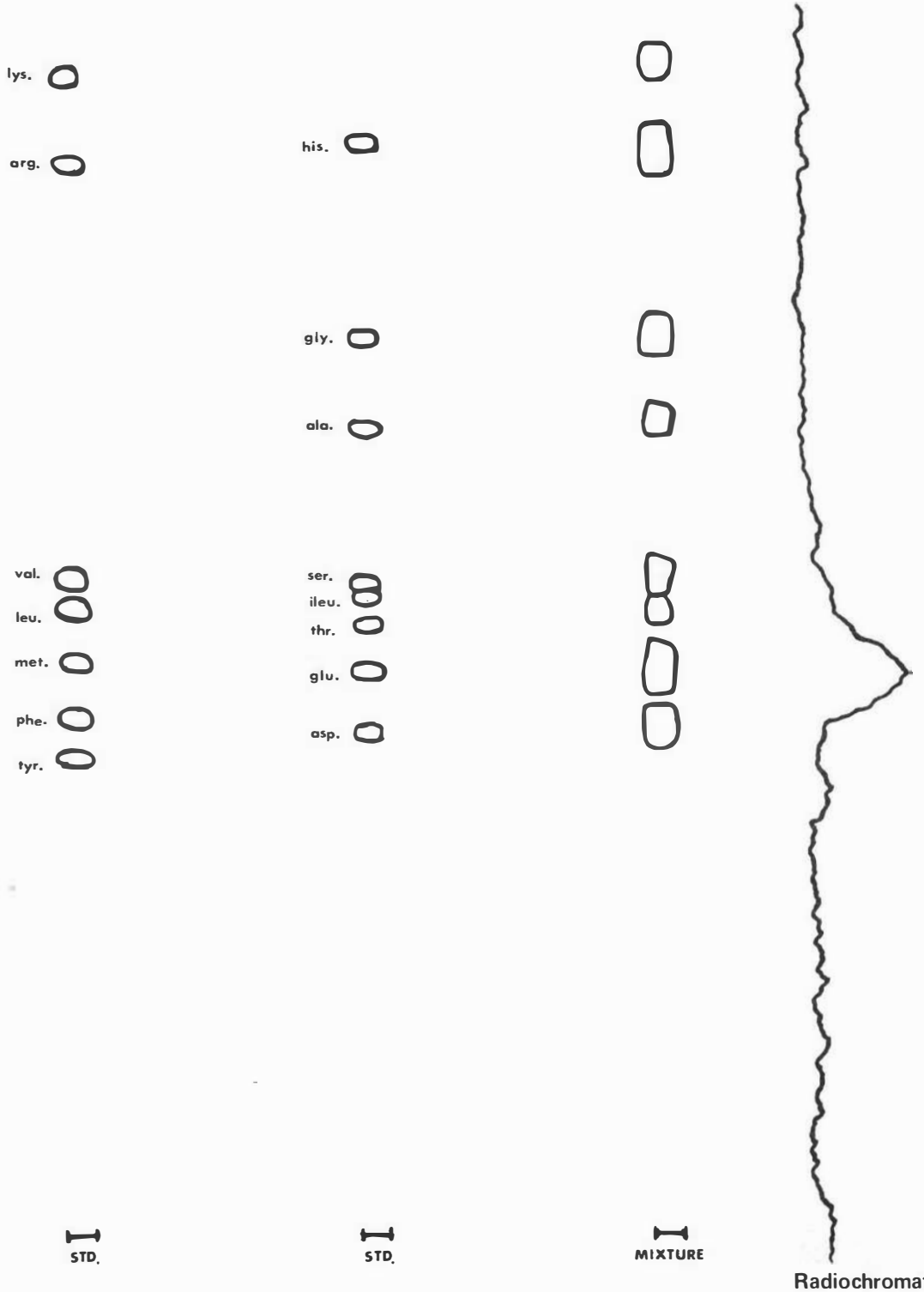
2.653 High Voltage Electrophoresis of protein hydrolyzate

The freeze-dried protein sample was subjected to 6 M HCl hydrolysis at 110° for 12hr in vacuo. The components of the hydrolyzate were resolved by high-voltage electrophoresis at 3 kilo volt X 1 hr in acetic: formic buffer, pH 2.1. glacial acetic acid (800): formic acid (100): distilled water (5900) . The amino acid markers were applied in 10% isopropanol, (final concentration of each amino acid, 5μmole/cm^3). After electrophoresis the papers were dried and sprayed with ninhydrin. The resulting amino acid distribution is presented in Fig.11, showing the marker positions for reference. The distribution of label was determined by use of a radiochromatogram scanner. The resultant scan shows a single peak of activity in the area in which phenylalanine runs. However, since the amino acids obtained by hydrolysis of protein are not completely resolved by single dimensional electrophoresis it is not possible to state definitely that all the activity is in phenylalanine.

FIGURE 11: ELECTROPHORESIS OF PROTEIN HYDROLYSATE

acetic:formic buffer, pH 2.1
3 Kv x 1 hr

Spots located by spraying with ninhydrin.
Radioactivity located using a radiochromatogram scanner.



Electrophoretic seperation of amino acids derived from 6M acid hydrolysis of the protein residue of B. megaterium cells labelled with ^{14}C-phenylalanine.

2.7 MICROBIOLOGICAL MEDIA

Aqueous 10% Malt Medium (- D.pini)

Malt (Wander brand dried malt, Type 500 LDE)	10% (w/v)
Yeast (DYC 'Active' Yeast)	5g/l
Cholesterol	20mg/l

were dissolved in hot distilled water, stirred by a magnetic stirrer. After 10 min. stirring the medium was dispensed into Erlenmeyer flasks, stoppered with cotton wool bungs and autoclaved (15 min., 121°, 15 psi).

10% Malt Agar (- for the maintenance of D.pini)

To prepare plates and/or slants 1.5% (w/v) agar was added to the aqueous 10% malt medium. After stirring on a magnetic hot plate to dissolve the agar, the medium was poured into 20cm^3 McCartney bottles (approx. 10cm^3/bottle) and autoclaved (15min., 121°, 15 psi). The McCartney bottles were tilted while the agar was still molten and left in this position as the agar cooled and solidified to form slants. For the preparation of plates the medium was autoclaved in a cotton wool stoppered Erlenmeyer flask, allowed to cool to 40° and then poured asceptically into sterile petri plates.

Aqueous Chlorella Medium

KNO_3	1.20g
$MgSO_4 \cdot 7H_2O$	2.46g
Ferric EDTA solution	1.0cm^3
Hoaglands solution	1.0cm^3

were dissolved and added to glass distilled water to give a final

volume of 990cm^3. KH_2PO_4 (1.22g) was dissolved in 10cm^3 glass distilled water. Both solutions were autoclaved and then combined to give a final volume of 1000cm^3. The medium was dispensed into sterile growth flasks.

NB. This medium contains no C source. Cultures were aerated with 5% CO_2 in air during growth.

To prepare Ferric EDTA solution

EDTA	26.1g
$FeSO_4 \cdot 7H_2O$	24.9g

were dissolved in 286cm^2 1M KOH. This solution was then diluted with glass distilled water to give a final volume of 1000cm^3, and aerated overnight to form the ferric EDTA complex. The pH was adjusted to 5.5. This solution is stable if stored in the dark.

To prepare Hoaglands solution

H_3BO_3	2.86g
$MnCl_2 \cdot 4H_2O$	1.81g
$ZnSO_4 \cdot 7H_2O$	0.22g
$CuSO_4 \cdot 5H_2O$	0.079g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.02g

were dissolved separately in glass distilled water. The solutions were combined and diluted to give a final volume of 1000cm^3.

Chlorella agar (for maintenance and bioassay)

Chlorella agar (Difco)	35g
Agar (Davis)	5g

were dissolved in 1000cm^3 glass distilled water on a magnetic hot

plate. The medium was then dispensed into McCartney bottles and autoclaved to make slants, or autoclaved in an Erlenmeyer flask and poured into sterile petri plates.

MYPG Medium (- for liquid culture of Saccharomyces cerevisiae)

Malt (Wander brand dried malt)	0.3g
Yeast extract (Difco)	0.3g
Peptone (Difco, Bacto-peptone)	0.5g

were dissolved in 95cm^3 glass distilled water and autoclaved in a cotton wool stoppered Erlenmeyer flask. After autoclaving 5cm^3 of sterile 20% glucose solution was added to give a final volume of 100cm^3.

MYPG Agar (- for maintenance and bioassay of Saccharomyces cerevisiae)

To prepare plates and/or slants 2% (w/v) agar was added to the aqueous MYPG medium prepared as above.

Aqueous medium for B.megaterium KM

This organism was grown in a minimal medium supplemented with tryptone (0.1g/100cm^3) and nicotinic acid (0.05mg/100cm^3).

Minimal Medium

KH_2PO_4	3g
K_2HPO_4	7g
$Na_3C_6H_5O_7 \cdot 2H_2O$ (Na citrate)	0.5g
$MgSO_4 \cdot 7H_2O$	0.1g
$(NH_4)_2SO_4$	1.0g
glucose, sterile 20% solution	10cm^3

Distilled water to give final volume of 1000cm^3

Glucose is autoclaved separately since it partially decomposes when autoclaved in the presence of phosphate.

Yeast Extract Tryptone Agar (- for maintenance and bioassay of B.negaterium KM and B.negaterium KMT⁻)

Yeast Extract (Difco)	2.5g
Tryptone (Difco, Bacto-tryptone)	5.0g
Agar	12.0g

were dissolved in 1000cm^3 hot glass distilled water on a magnetic hot plate. The medium was then dispensed and autoclaved for the preparation of slants or plates.

Aqueous medium for B.negaterium KMT⁻

contained per litre:

Glucose	10g
Ferric citrate	2.0g
K_2HPO_4	3.0g
KH_2PO_4	1.0g
$MgSO_4$ (anhydrous)	0.05g
Na_2SO_4	7.0g
Sodium-L-glutamate	140.3g

The medium was prepared from stock solutions in the following manner: (for 100cm^3 medium)

6.0% K_2HPO_4 2.0% K_2HPO_4	5cm^3
0.1% $MgSO_4$ (anhydrous) 2.0% Na_2SO_4	5cm^3
0.75m Na-L-glutamate	5cm^3
Glass distilled water	75cm^3

After autoclaving, the medium was completed by the aseptic addition of 5cm^3 sterile 20% glucose solution and 5cm^3 sterile 6 x 10^{-5}M ferric citrate.

Nutrient Broth

This medium was prepared from the dehydrated powder available from BBL, Division of Bioquest.

Nutrient Agar

This was prepared by adding agar (1.5% w/v) to nutrient broth.

Media for Euglena gracilis

Liquid medium

Peptone	5.0g
Yeast Extract	2.0g
Glucose	1.0g

were dissolved in 1000cm^3 glass distilled water, autoclaved and the medium dispensed into sterile growth flasks.

Agar for plates and slants

Peptone	2.0g
Glucose	2.5g
Sodium acetate	0.1g
Agar	8.0g

were dissolved in hot distilled water, autoclaved and dispensed to form plates or slants as required.

3. EXPERIMENTAL RESULTS

3.1 GROWTH INHIBITION OF MICRO-ORGANISMS BY DOTHISTROMIN

In studying the toxic effects of a compound it may help to know something of relative susceptibilities of different organisms to the compound. The substance may be toxic to photosynthetic organisms and without effect in non-photosynthetic organisms, or procaryotic organisms may be susceptible while the growth of eucaryotes is not inhibited. This knowledge of susceptible organisms can suggest possible areas for further study. This section reports results obtained in a preliminary investigation of the inhibition of growth of a number of different micro-organisms by dothistromin using an agar-plate bioassay. Observations made in the course of this investigation suggested ways of improving the sensitivity of the general bioassay method for dothistromin and steps for obtaining quantitative data.

Extracts of D.pini culture have been shown to be toxic to Chlorella pyrenoidosa in an antibiotic-disc bioassay (Bassettand Brunt, 1971), and this method of bioassay was utilized in the isolation and characterization of dothistromin (Gallagher, 1971). The toxin produced clear zones of inhibition in the area surrounding the filter paper disc. The Chlorella bioassay has been used mainly to follow the biological activity throughout purification procedures, and has not been fully investigated as a way to determine the concentration of toxin in any given extract. Although chemical

assay of a compound is generally more precise than estimation by bioassay methods, bioassays have been important in the isolation and purification of many toxic substances. The use of biological indicators offers the distinct advantage that the active principle being assayed does not have to have been identified or in a pure state, and the results obtained are directly related to the biological activity of the substance. Bioassays are clearly important for the detection of activity in extracts where active and inactive isomers are present, or where closely related compounds cannot be readily separated.

The bioassay systems developed for screening mycotoxins are numerous and include tests with micro-organisms (Burmeister and Hesseltine, 1966; Lillehoj and Ciegler, 1968; Clements, 1968), brine shrimp larvae, Artemia salina (Brown et al., 1968; Harwig and Scott, 1971), zebrafish larvae (Abedi and Scott, 1969), rainbow trout (Ayres et al., 1971), cell culture lines (Engelbrecht and Altenkirk, 1972; Juhasz and Greczi, 1964; Daniels, 1965), day-old ducklings (Lillehoj and Ciegler, 1968), chick embryos (Verret et al., 1964), rats (Purchase and Van der Watt, 1968), and mice (Lillehoj and Ciegler, 1968). The choice of organism for a bioassay depends on the biological effect of the compound(s) to be screened.

In 1965, Warren and Winstead reported a preliminary investigation of Chlorella pyrenoidosa as a microbiological plate bioassay for screening fungal and bacterial pathogens. A method based on this

report was subsequently used to follow the production and biological activity of fomanosin, a toxic metabolite of Fomes annosus (Bassett et al., 1967) and a similar Chlorella-seeded agar plate technique has shown that Chlorella pyrenoidosa is sensitive to a number of coumarin-related compounds, including aflatoxin (Ikawa et al., 1969). Sullivan and Ikawa (1972) demonstrated that there can be striking strain variations in the response of Chlorella sp. to toxic compounds. Cultures of Chlorella have been used in the study of algicides and herbicides (Addison and Bardsley, 1968; Gramlich and Frans, 1964), and for screening photosynthetic and respiratory inhibitors (Kratky and Warren, 1971). Thus the use of Chlorella as a biological indicator in bioassay systems is well established.

3.11 Screening of micro-organisms for dothistromin growth inhibition

A number of micro-organisms were screened by means of agar plate bioassays to establish the type of organism susceptible to growth inhibition by dothistromin and to evaluate these organisms for their potential use in this study of the toxic effects of dothistromin. The range of organisms surveyed included gram-positive and gram-negative bacteria (procaryotic organisms) and eucaryotes were represented by the yeast, Saccharomyces cerevisiae and two photosynthetic algae, Chlorella pyrenoidosa and Euglena gracilis.

Details of the media used for the maintenance and growth of these micro-organisms are given in Section 2.7., while the procedure used for pouring the seeded-agar plates for the bioassays is given in Section 2.44. Antibiotic discs impregnated with dothistromin were

prepared by carefully applying a known amount of the toxin to the filter paper discs in ethyl acetate solution and allowing the discs to dry thoroughly. Solvent control discs containing an equivalent amount of ethyl acetate were prepared in the same way. Each disc was then placed in the centre of a seeded agar plate and the plates incubated under conditions suitable for the growth of the organism. After overnight incubation of bacteria and yeast inoculated plates, sufficient growth had occurred to form clearly defined zones of inhibition. The slower growing algae were incubated for four days before the zones of inhibition were measured. Measurements of the total diameter of the inhibition zones were taken directly from the plates and the figures quoted are the average of two measurements taken at right angles to compensate for small irregularities in the geometry of the zones. The inhibition zone radius figure is the total zone diameter minus the diameter of the antibiotic disc (13mm), divided by 2 i.e. it is the width of the area peripheral to the antibiotic disc (see explanatory diagram on Table 3).

The results obtained are given in Table 3. The largest inhibition zones were observed with Chlorella pyrenoidosa. Growth of Euglena gracilis, Escherichia coli and Pseudomonas aeruginosa did not appear to be inhibited. It is of interest to note that the gram-negative organisms tested in the bioassay (apart from Proteus vulgaris) appear less sensitive to dothistromin than the gram-positive organisms. This may reflect the differing cell wall structure and possible

Table 3 Growth Inhibitory activity of Dothistromin on a selection of micro-organisms.

ORGANISM	B.Megaterium KM*	B.mycoides *	B.subtilis	S.aureus **	P.vulgaris	Ps.aeruginosa	E.coli	S.cerevisiae	C.pyrenoidosa	E.gracilis
Dothistromin 50 µg										
Total zone diam.	17	17(32)	16	14	16.5	0	0	16	22	0
(mm)	17	17(36)	16	14	16.5	0	0	16	23	0
Zone radius	2	2(9.5)	1.5	0.5	1.8	0	0	1.5	4.5	0
(mm)	2	2(11.5)	1.5	0.5	1.8	0	0	1.5	5	0
Dothistromin 25 µg										
Total zone diam.	17	16(26)	15	13	16.5	0	0	16	21	0
(mm)	17	16(26)	15	13	16	0	0	16	20	0
Zone radius (mm)	2	1.0(7.5)	1.0	0	1.8	0	0	1.5	4	0
	2	1.0(7.5)	1.0	0	1.5	0	0	1.5	3.5	0

No inhibition observed with ethyl acetate controls.

Bacterial plates incubated overnight. C.pyrenoidosa and E.gracilis illuminated 4 days at 25°.

* Growth observed up to edge of disc but 'halo' of less dense growth surrounded disc. Measurements given are those of 'halo'.

* Figures in parentheses are dimensions of the halo formed around the clear inner zone.

** Inhibition observed directly under discs, but no zone formed.

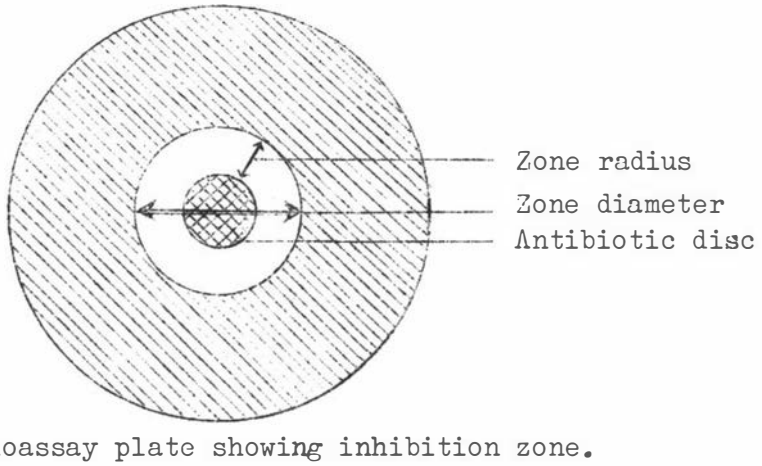


Diagram of bioassay plate showing inhibition zone.

differences in cell wall permeability. A similar difference in sensitivity has been observed with aflatoxin (Burmeister and Hesseltine, 1966), and with quinones (Webb, 1966).

3.12 Alternative Bioassay Method

The use of filter paper discs for the application of toxic compounds for bioassay has several disadvantages which made it difficult to develop a quantitative bioassay. The paper discs appeared to absorb dothistromin and thus present the quantitative problem of determining how much of the toxin applied was in fact available to inhibit growth. The large area of the disc effectively dilutes the toxin and also complicates the geometry of diffusion. For these reasons the use of antibiotic discs limits the sensitivity of the bioassay. Amounts of toxin below a limiting value may produce an area of inhibition directly under the disc without an annular zone being formed around the disc. Slightly greater amounts of toxin may produce a peripheral inhibition zone but of so small a radius that precise measurement is prevented.

In an attempt to dispense with the use of 'antibiotic' discs, solutions of dothistromin were applied directly to the basal agar layer and the solvent allowed to evaporate completely before the seeded agar overlays were poured. The method was not completely successful since droplets of ethyl acetate tended to move rapidly over the agar surface subsequently giving very irregular inhibition zones. This difficulty could not be overcome by using the more volatile solvent, chloroform.

Irregularity of inhibition zones made it impossible to assess inhibition on a quantitative basis and results have been recorded only on the basis of whether inhibition occurred or not, (see Table 4). By direct application of the toxin, inhibition zones could be obtained with as little as 5 µg dothistromin. Using the antibiotic disc method this amount of toxin would not have given an inhibition zone around the disc although growth directly under the disc may have been affected. Using this method of bioassay the largest inhibition zones were again obtained with Chlorella. The growth of gram-negative organisms was found to be inhibited less than that of gram-positive organisms.

It should be noted that such bioassays do not, in fact, allow valid comparisons to be made of toxicity with differing organisms because of their different growth rates. Inhibition zones in the Chlorella bioassay were measured 4 days after pouring the overlays but in the bacterial assays, with the very much faster growth of these micro-organisms, the zones of inhibition were measured after only an overnight incubation. The size of the inhibition zone depends on the distance of diffusion of the toxin as well as on the susceptibility of the organism. Thus, in order to make comparisons, allowance would have to be made for (a) differences in growth rates, (b) rates of diffusion of the toxic compound in the particular medium and (c) the temperature used for optimal growth of each micro-organism.

Table 4. Bioassay by direct application of toxin preparation.

ORGANISM	RESPONSE
Bacillus megaterium KM	Inhibition at all levels.
Bacillus mycoides *	Slight inhibition with 20 μg.
Bacillus subtilis	Inhibition at all levels.
Staphylococcus aureus	Inhibition at all levels.
Escherichia coli	Very slight inhibition at all levels.
Pseudomonas aeruginosa	No inhibition observed.
Proteus vulgaris	Slight inhibition at 20 μg.
Saccharomyces cerevesiae *	No inhibition apparent.
Chlorella pyrenoidosa	Large diffuse inhibition areas at all levels.

Toxin preparation (dothistromin) applied to basal layer at 5, 10 and 20 μg before pouring seeded overlays. 30 μl Ethyl acetate controls showed no inhibition with any organism.

* Very poor growth on test plates resulted in non-confluent growth and inhibition difficult to ascertain.
Conditions of incubation as for disc bioassay.

3.13 Detection of Dothistromin in Zones of Inhibition

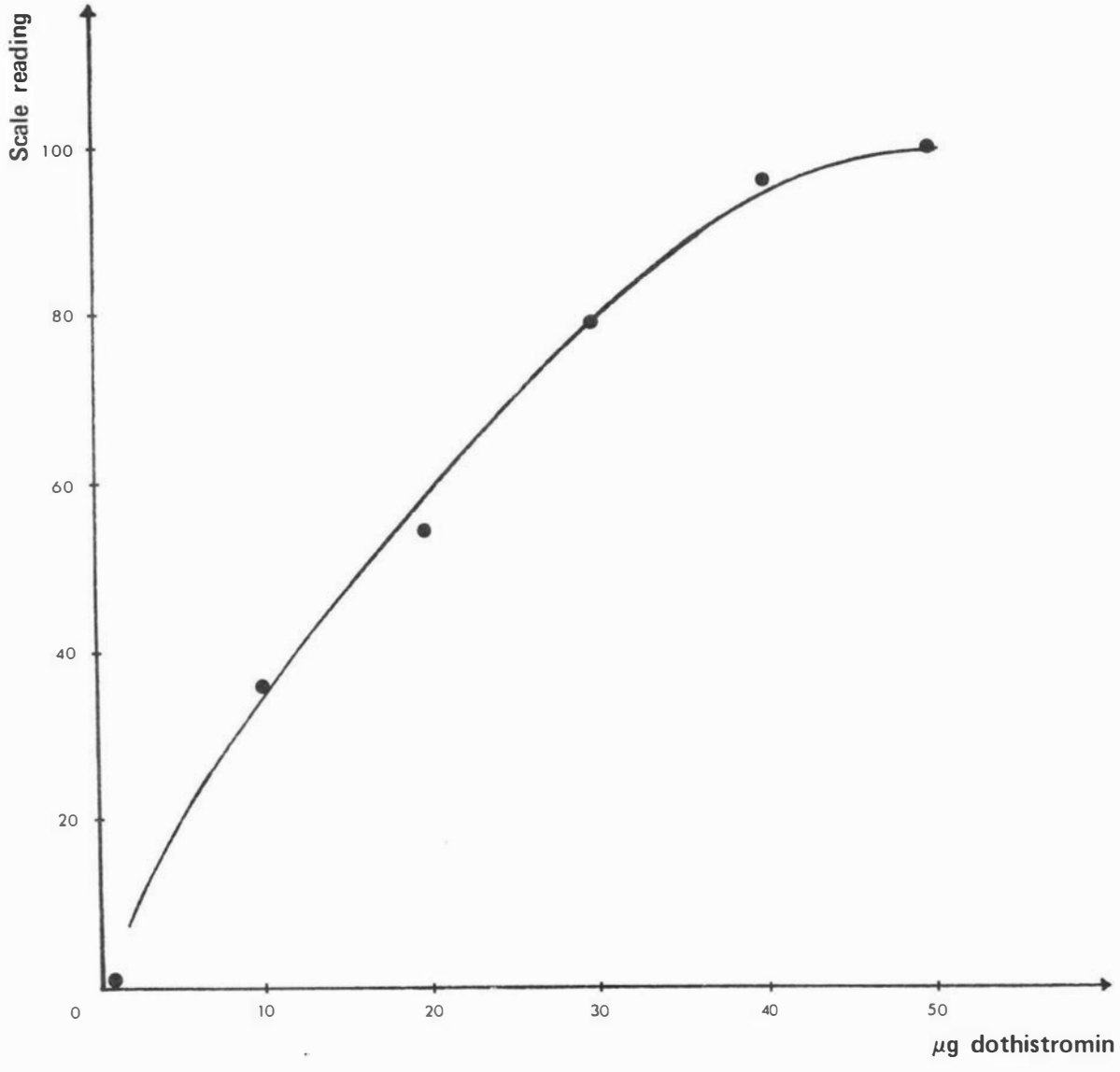
In setting up the type of bioassay described above it is assumed that the toxic compound can diffuse into the agar from the antibiotic disc. The low solubility of dothistromin in aqueous solutions raises several questions in relation to the use of bioassays of this nature. One of these problems is qualitative i.e. is dothistromin itself responsible for the observed inhibition or are the inhibitory responses due to a more soluble analogue? Quantitative problems include determining how much of the toxin is irreversibly bound to the discs, the rate of diffusion of the toxic material into the agar and the nature of the diffusion gradient. These questions could most conveniently be studied by the use of toxin labelled to a high specific activity with a radio-isotope. Preparation of labelled toxin at a high specific activity suitable for this type of experiment has not yet been achieved (Shaw, 1974). As an alternative approach some information related to these problems has been obtained by using the fluorescence of the dothistromin molecule. The 1, 4 dihydroxyanthraquinone moiety of dothistromin is largely responsible for the characteristic fluorescence emission, and Gallagher (1971) has discussed this fluorescence in relation to anthraquinone which does not fluoresce. The determination of the excitation and emission spectra of dothistromin is described in Section 2.52.

To examine the possibility of using fluorescence measurements in this study, a series of standard 'dothistromin agars' was prepared. Molten Chlorella agar ($5cm^3$) was added to a number of fluorimeter tubes, each of which contained a different amount of dothistromin in ethyl acetate solution, and the tubes were shaken to disperse the toxin throughout the agar. Control tubes containing ethyl acetate and agar, as well as blank agars, were prepared at the same time. The agar was allowed to cool and solidify before the tubes were examined for fluorescence.

At the excitation and emission wavelengths chosen the 'dothistromin agars' displayed a fluorescence not present in the blank or solvent-control agar tubes. The fluorescence increased in proportion to the increased amounts of dothistromin in the tubes (see Fig.12). The faint orange tinge of the 'dothistromin agars' disappeared slowly when the tubes were left standing for 2-3 days. The fluorescence observed in these agars also decreased over this period of time.

A series of unseeded Chlorella agar plates was prepared and an antibiotic disc which had been impregnated with dothistromin ($100\mu g$) was placed in the centre of each plate. These plates were incubated under bioassay conditions for periods of 1, 2 and 4 days illuminated by fluorescent tubes, at 25^{o}. A parallel series of plates was incubated at 25^{o} with all light excluded for the same periods of time. The antibiotic discs were then removed and areas of the agar excised using cork borers. An area, of diameter

FIGURE 12: STANDARD CURVE FOR 'DOTHISTROMIN AGARS'



Excitation @ 471_{nm} Emission @ 555_{nm}

Meter settings:-

Blank	– low
Sensitivity	– low
Range	– x 300

All tubes read against an agar blank containing no dothistromin.
Tube containing 50 μg dothistromin set to give fsd. = 100 .

14mm, was removed from directly under each antibiotic disc, and an outer zone extending 6mm beyond this inner area (total diameter 26mm) was cut from each plate. These plugs of agar were placed in fluorimeter tubes, melted and made up to a final volume of $5cm^3$ with molten Chlorella agar. A standard series of 'dothistromin agars' covering the range 10-100 μg dothistromin per tube was prepared at the same time. All tubes were allowed to cool and set before being examined for fluorescence against a blank agar tube.

It was not possible to accurately estimate the amounts of dothistromin (or derivative) present in the zones since all readings fell below the range covered adequately by the standards. Approximate results have been tabulated (see Table 5) and these are presented as relative data obtained with the 10 μg standard set to give a full scale deflection of 100 units.

These results show that dothistromin (or a derivative) does diffuse out into the area of agar around the antibiotic disc, with the zone from directly under the disc containing more dothistromin than the peripheral area excised. Zones taken from plates that had been incubated in darkness showed higher levels of fluorescence than those taken from illuminated plates, which appeared to contain less dothistromin in both the inner and outer zones after a 4-day incubation period than at 1 or 2 days. These results would suggest that the fluorescent molecule is unstable in the light and this possibly could have important implications in using Chlorella as a

Table 5. Fluorimetric analysis of bioassay plates.

LIGHT INCUBATED PLATES	READINGS (units)	
Time exposed	Outer zone	Inner zone
4 days	4	16
	3	40
2 days	6	16
	9.5	98
1 day	6	17.5
	9.5	100+
DARK INCUBATED PLATES		
Time exposed		
4 days	14	30
	31.5	100+
2 days	15	44
	17	68
1 day	14	31.5
	11.5	69

Meter settings:

excitation wavelength: 470 nm — Blank - low

emission wavelength: 560 nm — Sensitivity - low

meter damping at 0.2 — Range - X 1000

Blank agar - 0 units

Dothistromin agar (10 μg) - 100 units

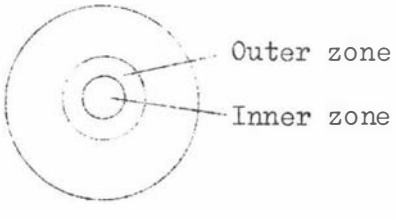


Diagram to show areas excised from agar plates.

(NB. There is a large variation in readings for each duplicate.)

test organism. By comparison with the 10 μg standard the concentration of toxin achieved in the outer zone must be low (of the order of 1 - 2 $\mu g/cm^3$). This work was undertaken at a late stage in the study when the necessary instrumentation became available in the department.

3.14 Relative toxicity of other hydroxyanthraquinones

With a number of hydroxyanthraquinones available, it was possible to screen a series of these compounds for their toxicity to Chlorella using the antibiotic disc bioassay technique. This allowed some assessment of the possible contribution of this portion of the dothistromin molecule to the toxicity observed.

Results are given in Table 6. None of the compounds exhibited a toxicity to Chlorella comparable to that shown by dothistromin. Even with 1, 4 hydroxyanthraquinone inhibition was limited to the area directly under the disc. Differences in solubilities and in diffusion may, in part, account for these results.

It has not been possible to investigate the toxicity of bifuran ring structures to this bioassay system. These compounds are not available commercially and would have to be synthesized before the contribution of this portion of the molecule to overall dothistromin toxicity could be assessed. The vinyl ether derivative of dothistromin was tested with the standard bioassay system and no inhibition was observed.

Table 6. Disc Bioassay of Anthraquinone toxicity to Chlorella.

ANTHRAQUINONE DERIVATIVE	Amount/Disc μg	Total Zone Diam. (mm)	Zone Radius (mm)
Dothistromin	25	29	8
	50	31	9
⁑ 1, 2 hydroxyanthraquinone	100	15	1
	200	16	1.5
1, 4 hydroxyanthraquinone	100 *	13	0
	200 *	13	0
1, 8 hydroxyanthraquinone	100 *	13	0
	200 *	13	0
⁑ 2, 6 hydroxyanthraquinone	100	0	0
	200	0	0
⁑ 1, 2, 5, 8 hydroxyanthraquinone	100	0	0
	200 *	13	0
Ethyl acetate control		0	0

⁑ Sample not completely dissolved in solvent. Amount applied to disc for assay therefore slightly less than amount stated.

* No zone of inhibition, but no growth in area directly under disc.

3.15 Assessment of the Bioassay

A bioassay has been defined as 'a determination of the potency of a physical, chemical or biological agent by means of a biological indicator' (Condouris, 1965). Although it was unnecessary for the purposes of this study to quantitate the bioassay, the results obtained indicate that it should be possible to develop a system in which dothistromin could be estimated with some degree of precision.

Although dose-response curves have not been established it is likely that, using antibiotic discs for the addition of the toxin, the curve would be linear only over a very restricted range of concentrations. The use of antibiotic discs limits the sensitivity of the bioassay by both absorbing some of the dothistromin applied and diluting the toxin through the area of the discs themselves.

The increased sensitivity achieved through direct application of the toxin could extend the linearity of the dose-response curve. The problem of zone irregularities observed could be circumvented by applying the toxin into small wells cut into the basal agar layer before pouring the seeded overlay. A useful extension of this bioassay technique has been made by Freke (1974) who poured seeded agar overlays onto silica gel thin-layer plates spotted with the toxic compound. This is a sensitive method and could be usefully applied to work with dothistromin. By careful application

to a silica gel plate the toxin might be spotted onto an area very much less than that of an antibiotic disc. This technique would allow a direct comparison to be made of the biological activity of compounds in extracts following chromatography.

The preliminary fluorimetric investigation indicates that it should be possible to use this approach to determine the concentration of dothistromin (or derivative) in the agar of the plates, and thus establish the diffusion gradients formed. A study could then be made of the rate of diffusion of dothistromin into the agar. This would allow correlation between growth rates of micro-organisms and the size of the observed zones of inhibition, giving a more meaningful basis for comparing sensitivities to the toxin.

Fluorimetric analysis has not provided information on the nature of the actual inhibitory compound. Substances may be present in the dothistromin preparation, perhaps as minor components, which could possess similar solubility properties to those of the toxin. Any alteration in the number of hydroxyl groups on the anthraquinone nucleus would alter the fluorescence observed while changes in the saturation of the bifuran ring moiety are unlikely to change the fluorescence characteri stics but may have profound effects on the toxicity.

The instability of dothistromin in the light revealed by the fluorimetric analysis has important implications in the use of Chlorella as an organism for bioassay. Since Chlorella is photosynthetic, the organism must be grown in the light and it

appears that this may result in the breakdown of the toxin. This would affect the concentrations of dothistromin required for growth inhibition. The breakdown products are not known but the process presumably involves some alteration of the 1, 4 dihydroxyanthraquinone nucleus since this would be largely responsible for the observed fluorescence.

3.2 INHIBITION OF GROWTH OF CHLORELLA IN LIQUID CULTURE

Although the agar plate bioassay method discussed in the previous section is valuable for determining whether or not a compound inhibits growth, it is not suitable for defining the metabolic lesions taking place. To study the biochemical effects of dothistromin it was necessary to use liquid cultures of Chlorella.

The concentration of dothistromin required to inhibit growth of Chlorella in liquid culture was first investigated using batch culture techniques. At any given time during the log phase, a batch culture will contain cells at all stages of the cell cycle of growth and division. Any changes observed in these cultures will, in fact, be the 'average' of those occurring at each stage of growth and division in the cell cycle of the organism.

By using synchronous cultures, however, it is possible to follow events occurring as for a single cell throughout the cell cycle of the organism. A synchronous culture is one in which the principal mass of the cells is at one single stage of development at any given time of the cell cycle. By establishing the normal

pattern of metabolic events during the cell cycle it should be possible to detect any changes resulting from the addition of an inhibitor and perhaps find a lead for further experimentation.

3.21 Problems associated with low solubility of dothistromin

The low solubility of dothistromin in aqueous solutions has posed many problems. Batch cultures of Chlorella were used to find a reproducible means of introducing known amounts of toxin into an aqueous system.

Initially dothistromin was added to the culture in aqueous stock 'solutions' derived by adding a known amount of the toxin, in ethyl acetate solution, to water and gassing with oxygen-free nitrogen to remove the organic solvent. A large proportion of the dothistromin precipitated out leaving a pale orange aqueous solution. This precipitate was collected by centrifugation, dried and taken up into a known volume of ethyl acetate. The concentration of toxin in this ethyl acetate solution was determined spectrophotometrically and the amount of dothistromin remaining in aqueous solution was calculated by difference. The concentrations of stock solutions prepared in this way varied from 5 - 100 $\mu g/cm^3$. However, since the dothistromin precipitated out of solution on standing, aqueous stock solutions of known concentration could not be maintained. Although solutions of dothistromin prepared in this way were shown to inhibit the growth of Chlorella in

liquid culture when added at the beginning of the growth cycle (at concentrations of the order of 1.0 - 2.0 μ g/cm^3), the degree of inhibition of Chlorella growth in different experiments varied considerably.

The addition of dothistromin to cultures as a concentrated solution in ethyl acetate without subsequent removal of the organic solvent proved to be the most satisfactory way of introducing the toxin to aqueous cultures of Chlorella. When added in this way, although it is possible that not all of the dothistromin goes into solution, it appears to be available to the cells. This method allows the introduction of a precise amount of toxin and was found to give reproducible results. Ethyl acetate has an inhibitory effect on the growth of Chlorella at concentrations greater than 0.5%. However, since the levels of dothistromin required for inhibition proved to be very low, sub-inhibitory levels of ethyl acetate could be used and in all cases an appropriate ethyl acetate control has been used as a blank.

3.22 Effect of Dothistromin on Growth of Chlorella in Batch cultures

3.221 Effect of addition of toxin at the beginning of the growth cycle

In the first series of experiments to determine the effect of dothistromin on growth, the toxin was added immediately after inoculation of the culture medium. The amount of toxin added, in ethyl acetate solution, was varied to find suitable concentrations

for subsequent work in liquid culture. At concentrations of more than 0.1 $\mu g/cm^3$ in the medium dothistromin caused a lengthening of the lag period of growth (see Fig.13). After the extended lag period, the duration of which was dependent on the concentration of dothistromin present, the culture entered into log phase with a growth rate similar to that of the control.

3.222 Effect of addition of toxin during the log-phase of the growth cycle

The toxin was effective in inhibiting the course of growth in cultures of Chlorella which have entered log-phase. This inhibition showed as a lag in growth followed by recovery and resumption of growth. See Fig.14. This figure also shows the inhibitory effect of ethyl acetate at concentrations of greater than 0.5%. At 0.6% ethyl acetate there was a slight lag in the growth of the culture. The culture containing the highest concentration of dothistromin was monitored over a 9-day growth period and results showed the recovery and resumption of growth of the culture following administration of dothistromin.

Inhibition of growth during log-phase required higher concentrations of dothistromin than those which produced an inhibitory effect when added at the initiation of culture growth. This suggested a direct relationship between dothistromin concentration and cell number. The ratio of the dothistromin concentration to cell number has been calculated for a series of growth curves and

FIGURE 13: GROWTH CURVES FOR *CHLORELLA* SHOWING INHIBITORY EFFECT OF DOTHISTROMIN

Dothistromin added as an ethyl acetate solution at the beginning of the growth period.

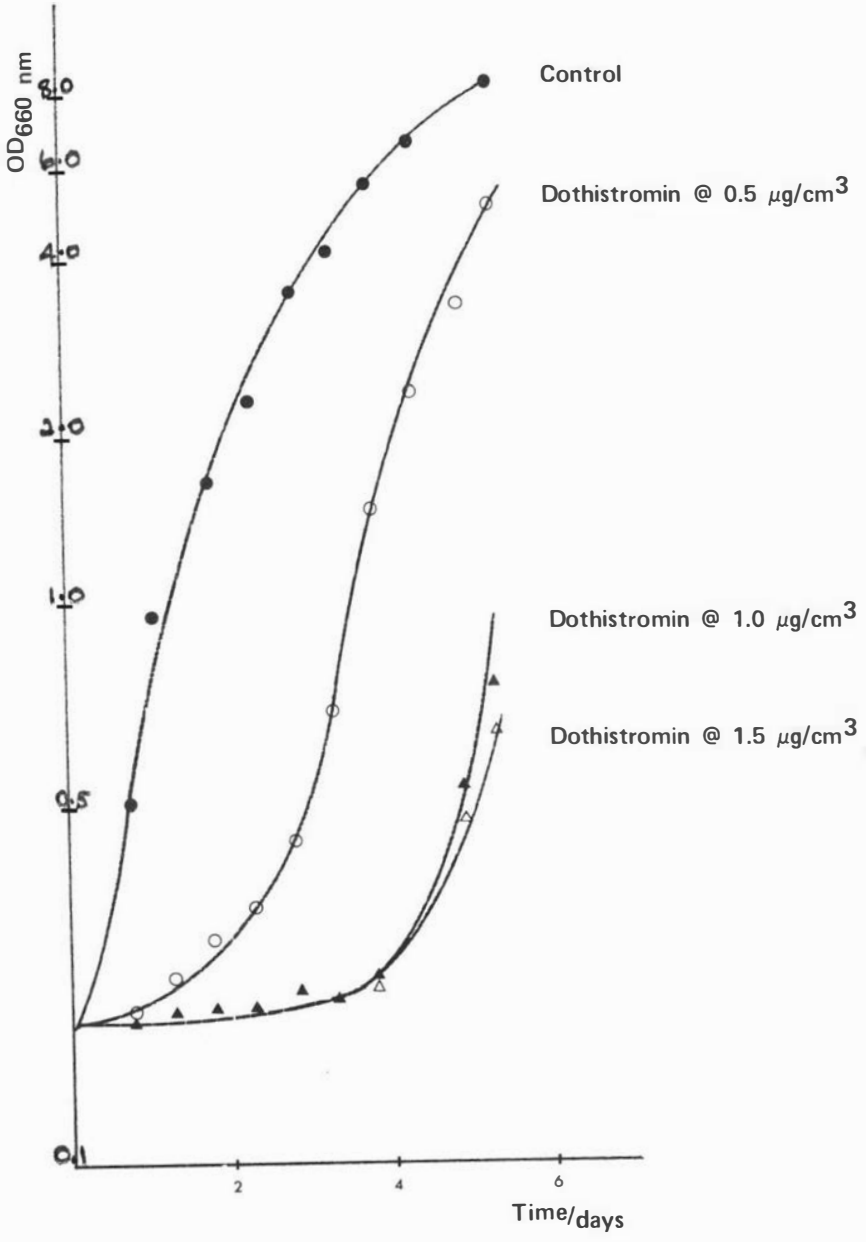
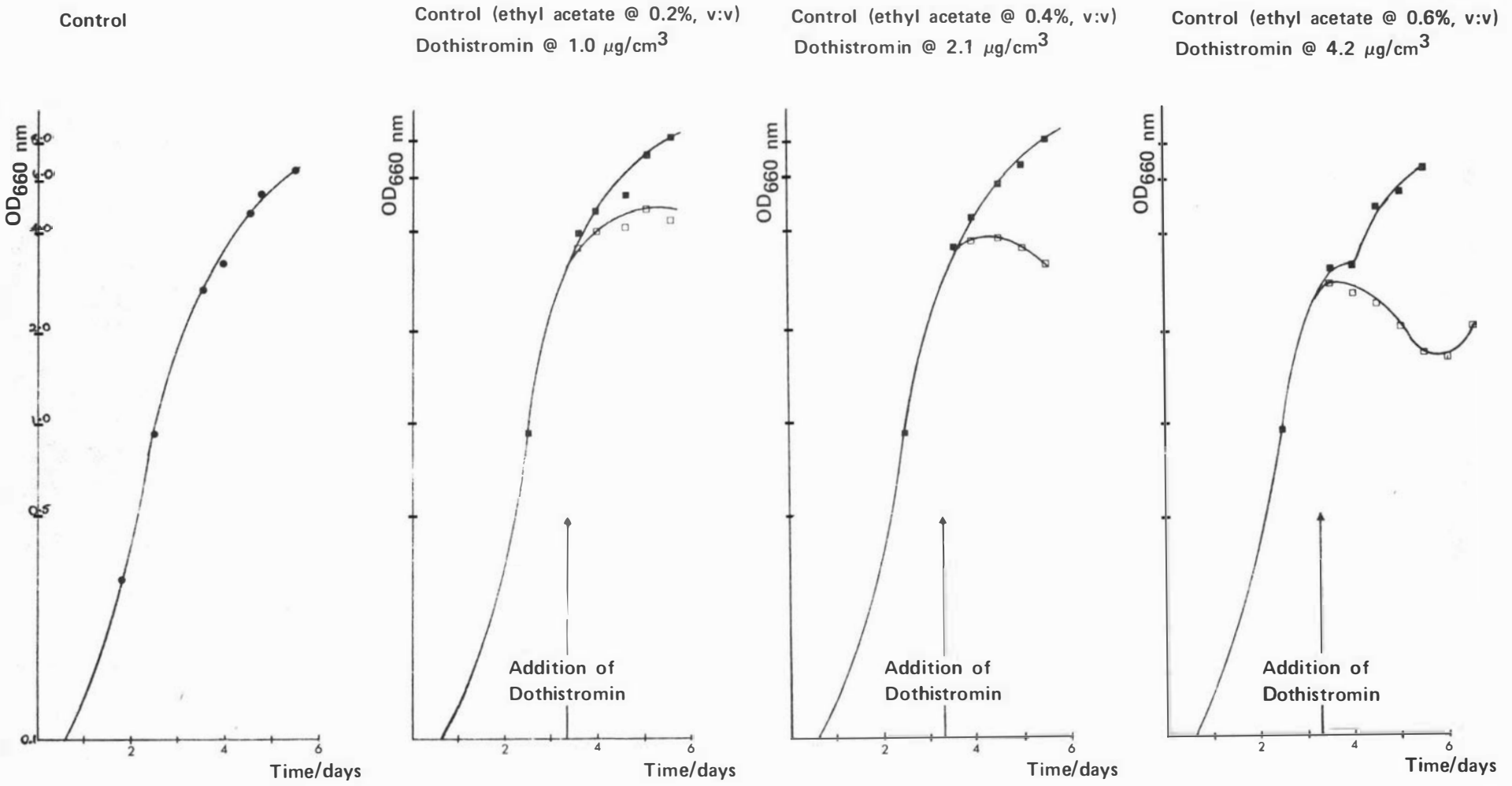


FIGURE 14: GROWTH CURVES FOR *CHLORELLA* SHOWING INHIBITORY EFFECT OF DOTHISTROMIN.
Dothistromin added as an ethyl acetate solution during the log-phase of growth.



these ratios are given in Table 7. It appears that for inhibition to occur a dothistromin: cell number ratio of at least 1.0 μg/cell X 10^8 must be achieved.

The use of ethyl acetate solutions restricts the concentration range of dothistromin over which effects can be studied. It has been suggested that a glycosidic derivative of dothistromin may be more soluble in aqueous solutions; a recent preliminary study of the formation of a magnesium chelate complex of dothistromin has shown that this derivative is more water-soluble than the parent compound and possibly more photostable (Gallagher, 1973).

3.23 Effect of Dothistromin on Chlorella in Synchronous Culture

3.231 The cell cycle of Chlorella

Having determined the effective concentrations at which dothistromin inhibits the growth of Chlorella in liquid cultures, it was possible to study the effect of the toxin at these concentrations on synchronous cultures. To establish the normal pattern of events in the cell cycle of Chlorella synchronous cultures were initiated using the techniques detailed in Section 2.4632. A discussion of synchronous culture theory and general techniques, a report of the preliminary experiments establishing synchronous cultures of C.pyrenoidosa and an assessment of the degree of synchrony achieved are also included in the Experimental Methods Section (Sections 2.461 - 2.464).

Table 7. Dothistromin: Cell Number Ratios

Chlorella pyrenoidosa

Toxin added on initiation of culture:

[Dsm] final (g/cm^3)	Cell No./cm^3	Ratio (μg/cell X 10^8)	Observed Effect
0.5×10^{-6}	11.3×10^6	4.42	3 day lag.
1.0×10^{-6}	11.3×10^6	8.84	$\geq$6 day lag.
1.5×10^{-6}	11.0×10^6	13.63	$\geq$6 day lag.
0.1×10^{-6}	10.5×10^6	0.95	Slight lag (12 hr)
0.3×10^{-6}	10.7×10^6	2.80	$\geq$4 day lag.
0.6×10^{-6}	10.5×10^6	5.47	$\geq$4 day lag.
0.1×10^{-6}	5.8×10^6	1.72	36 hr lag.
0.2×10^{-6}	6.4×10^6	3.12	$\geq$6 day lag.

Toxin added in log phase:

0.1×10^{-6}	3.8×10^8	0.03	No inhibition obs.
0.3×10^{-6}	4.3×10^8	0.07	" "
0.6×10^{-6}	3.6×10^8	0.16	" "
1.8×10^{-6}	2.3×10^8	0.78	Growth lag
3.6×10^{-6}	2.3×10^8	1.60	Growth lag
5.4×10^{-6}	1.6×10^8	3.25	= 4 day lag.
1.0×10^{-6}	2.3×10^8	0.45	No inhibition
2.1×10^{-6}	2.4×10^8	0.88	Slight lag.
4.2×10^{-6}	2.9×10^8	1.43	Growth lag.

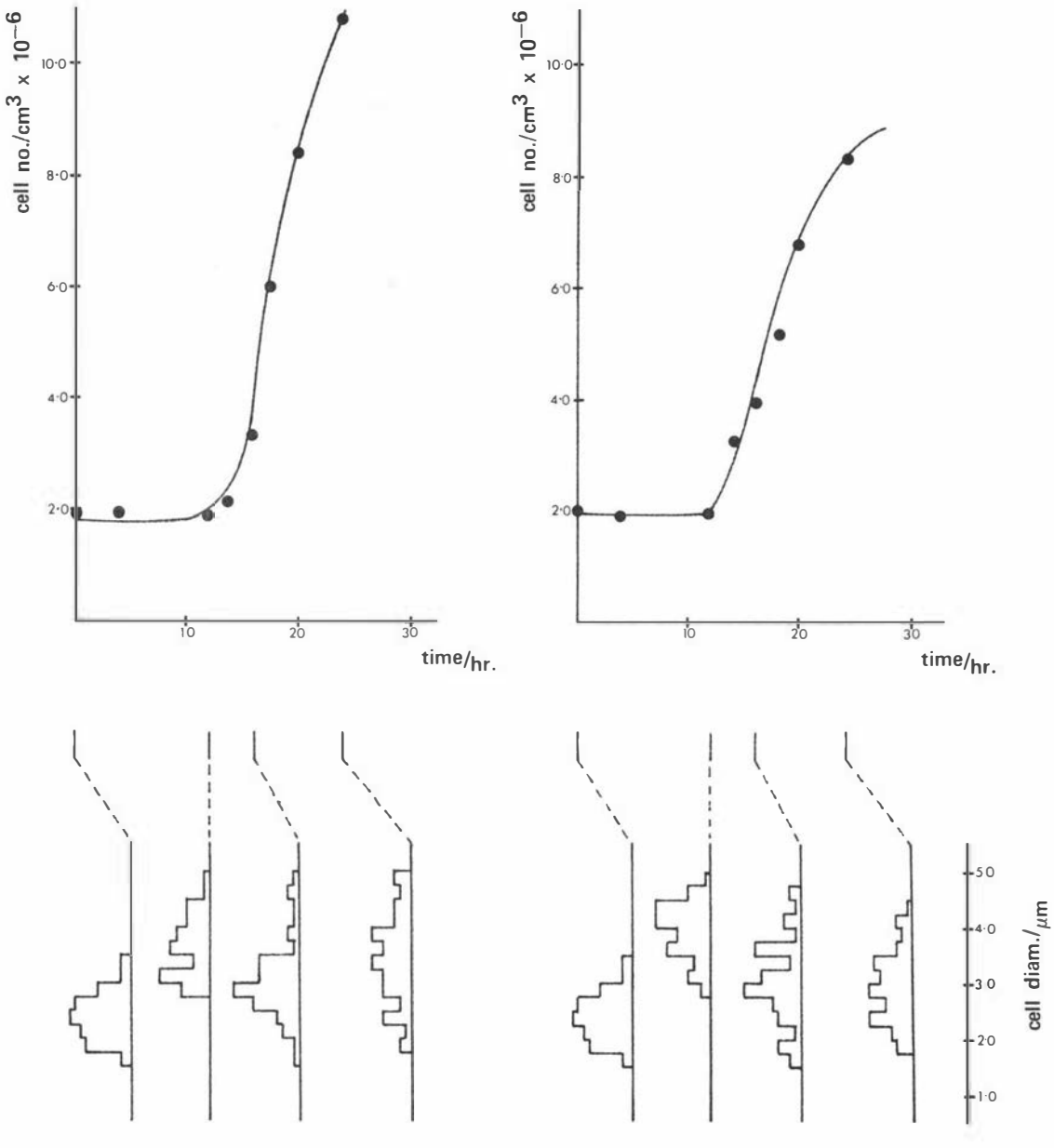
Two synchronous cultures were initiated; one of these was grown under continuous illumination and the other was subjected to the intermittent illumination of the 16 hr light : 8 hr dark synchronizing regime. Cell counts and cell size distributions were determined at regular intervals to follow the progress of the cultures (see Fig.15). At the same times samples were withdrawn for analysis of nucleic acid and protein content. These results are presented graphically in Fig.16.

Both cultures showed similar patterns of change in the RNA, DNA and protein contents per cell. Division has begun by the 16th hour in both cultures. The continued increase in cell number, nucleic acid and protein content per sample and the wider range of cell size in the population of the continuously illuminated culture show that if synchrony were to be maintained for more than one cycle a continuation of the light : dark regime would be necessary.

3.232 Effect of dothistromin on cell division, nucleic acid and protein levels in Chlorella

The effect of dothistromin on Chlorella was examined in synchronous cultures. Dothistromin-containing cultures were compared with cultures containing the equivalent amount of ethyl acetate. In this way any changes, due to the toxin, in the parameters measured could be determined.

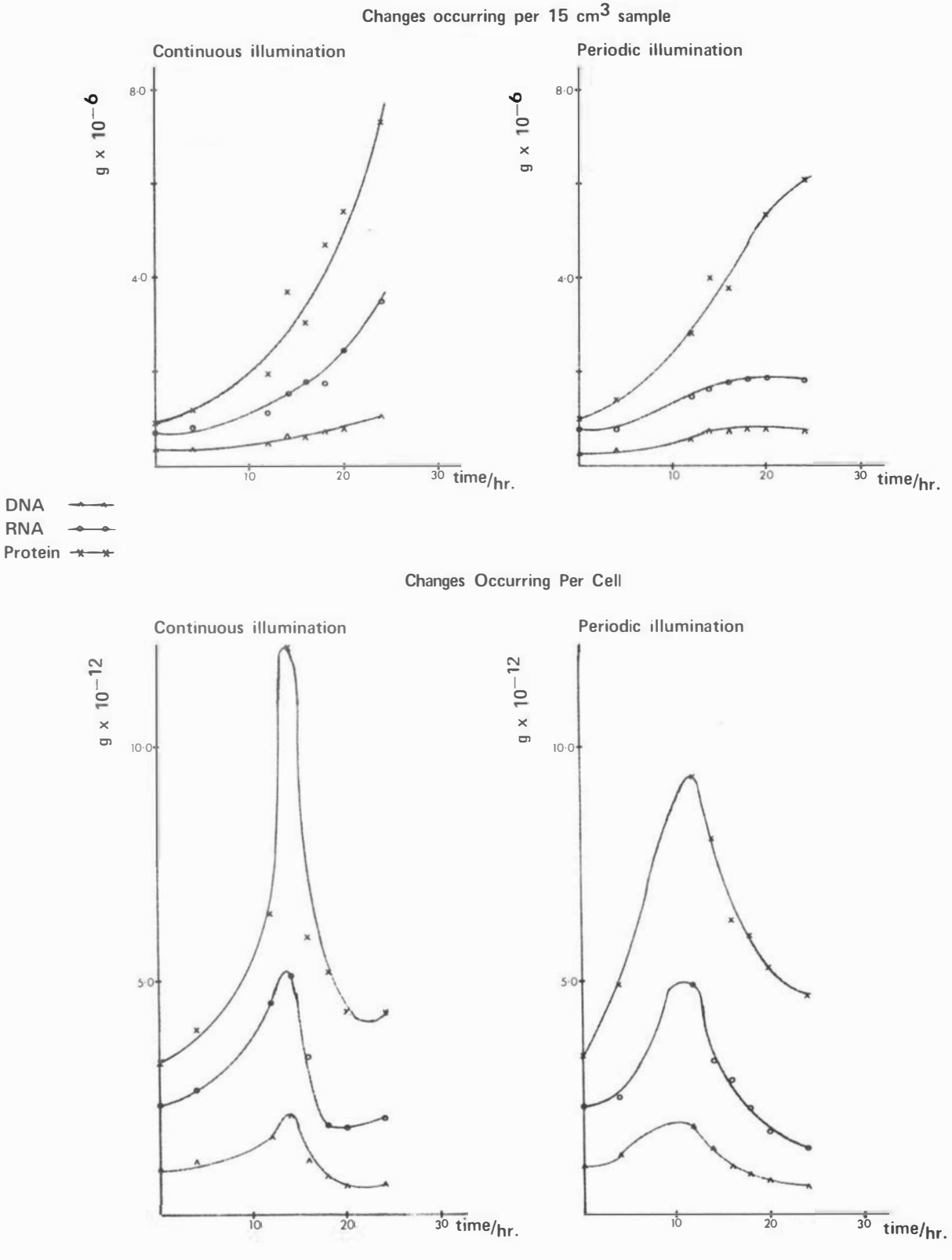
FIGURE 15: CELL COUNTS AND CELL SIZE DISTRIBUTIONS OVER ONE CYCLE OF SYNCHRONOUS CULTURES OF *CHLORELLA*.



a) Continuous Illumination

b) Periodic Illumination

FIGURE 16: CHANGES IN LEVELS OF MACROMOLECULAR CONSTITUENTS OVER ONE CYCLE OF SYNCHRONOUS CULTURES OF *CHLORELLA*.



Dothistromin was added, in ethyl acetate, to the cultures which were then grown under continuous illumination for one cycle only. Continuation of the light : dark regime could lead to anomalous results if a growth inhibitor was being studied. The onset of the dark period would not necessarily coincide with the onset of cell division under inhibited conditions and subsequent events could not be ascribed solely to the effect of the inhibitor.

The results from a series of synchronous cultures containing increasing concentrations of dothistromin are shown in Fig.17. For these cultures cell numbers only were monitored. It appears that cell division was completely inhibited at dothistromin : cell number ratios of greater than 10.0 μg/cell X 10^8.

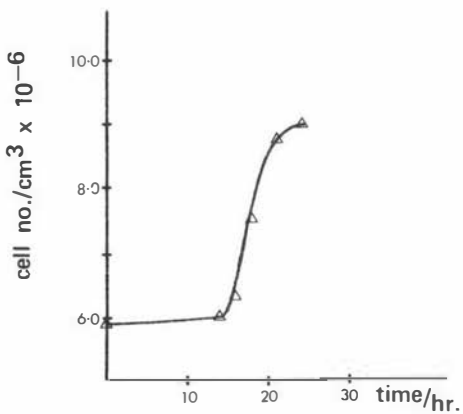
On the basis of this data, a synchronous culture was followed through one cycle when exposed to the toxin at a final concentration of 0.7 $\mu g/cm^3$, giving a dothistromin : cell number ratio of 12.5 μg/cell X 10^8 and a final concentration of ethyl acetate of 0.07%. The results obtained were compared with those of the parallel control containing an equivalent amount of ethyl acetate (see Figs. 18, 19 and 20). The cell size distributions suggest that the growth of a large percentage of the population was impeded by dothistromin. The division number of the test population was only 1.2 as compared with a division number of 3.5 for the control. Analysis of the nucleic acid and protein content of both cultures at intervals over the cell cycle (Fig.19) shows a marked difference

FIGURE 17: EFFECT OF DOTHISTROMIN ON THE CELL CYCLE OF *CHLORELLA*.
Following changes in cell nos. only.

(SI = synchronization index)

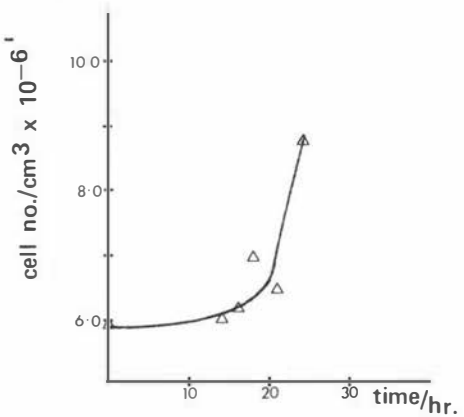
a) Control

SI = 0.16



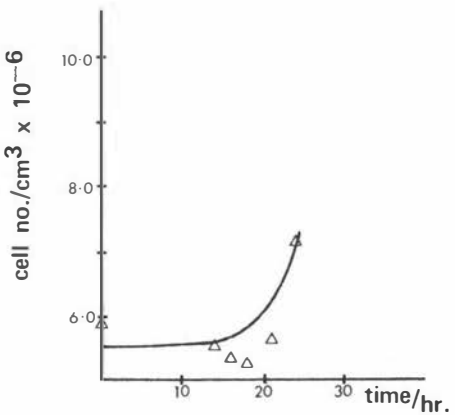
b) Dothistromin @ 0.1 $\mu g/cm^3$

Dothistromin/cell no. = 1.72 μg/cell x 10^{8}
SI = 0.15

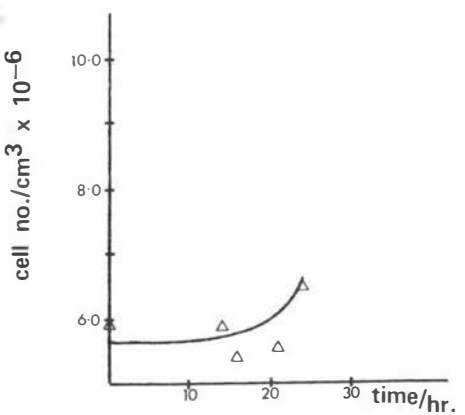


c) Dothistromin @ 0.3 $\mu g/cm^3$

Dothistromin/cell no. = 5.17 μg/cell x 10^{8}
SI = 0.07

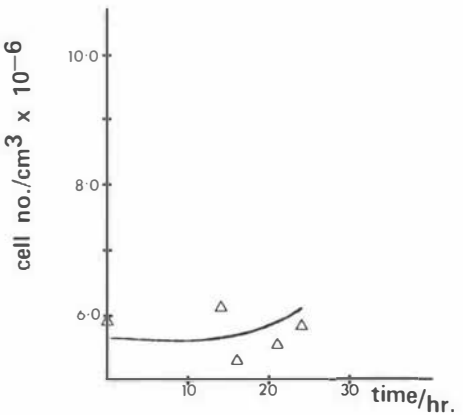


d) Dothistromin @ 0.6 $\mu g/cm^3$



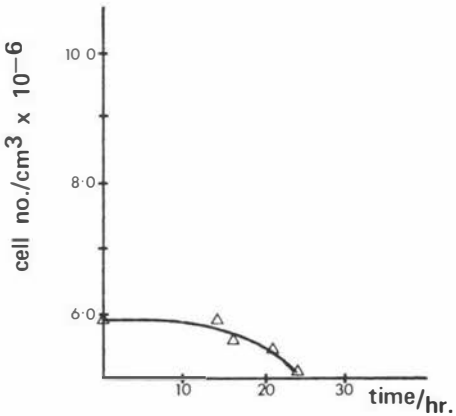
Dothistromin/cell no. = 10.34 μg/cell x 10^{8}
SI = 0.06

e) Dothistromin @ 1.0 $\mu g/cm^3$



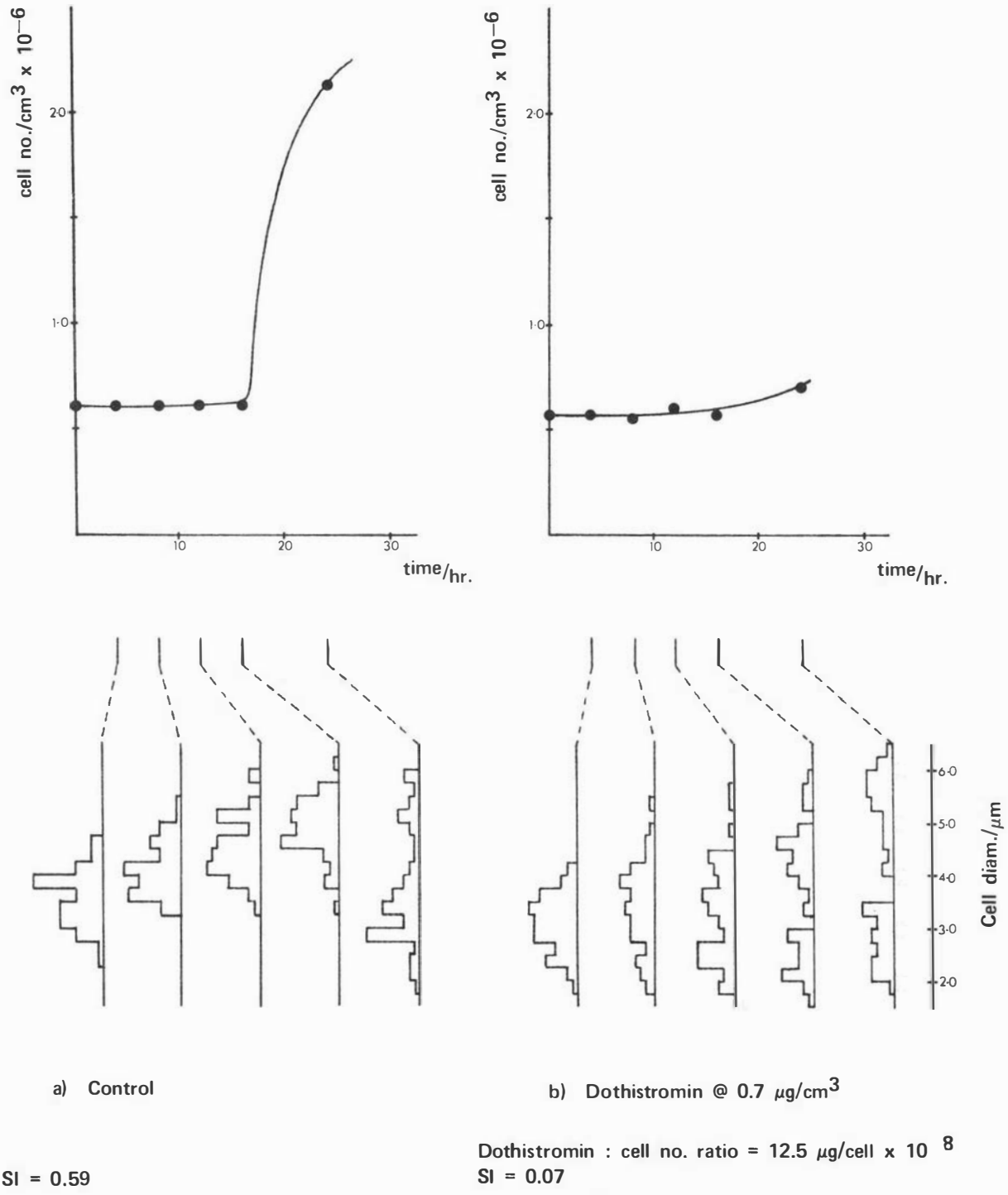
Dothistromin/cell no. = 17.58 μg/cell x 10^{8}
SI = 0

f) Dothistromin @ 1.2 $\mu g/cm^3$



Dothistromin/cell no. = 21.03 μg/cell x 10^{8}
SI = 0

FIGURE 18: EFFECT OF DOTHISTROMIN ON SYNCHRONOUS CULTURES OF *CHLORELLA*.
Effect on cell counts and cell size distributions.



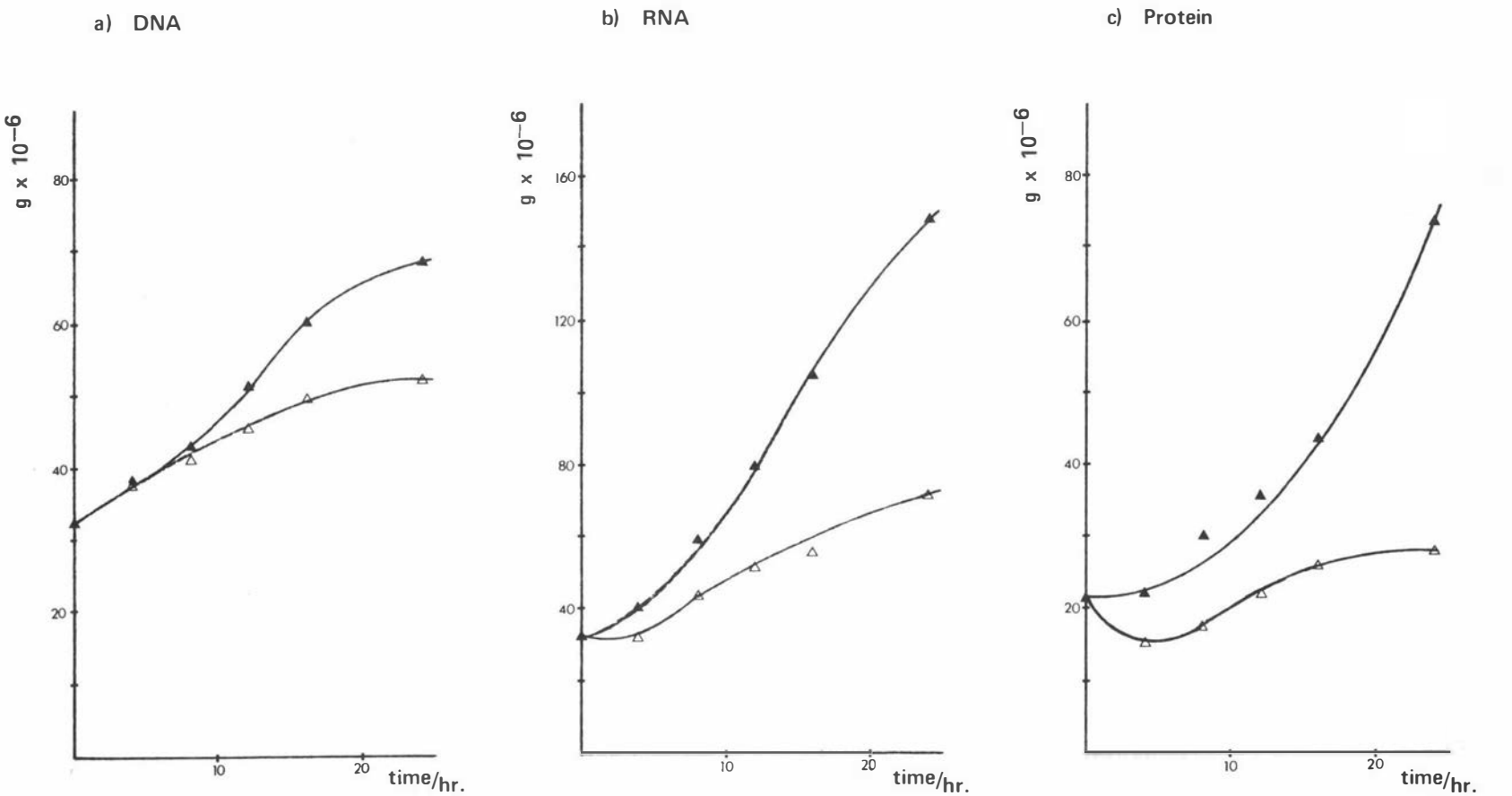
a) Control

SI = 0.59

b) Dothistromin @ 0.7 $\mu g/cm^3$

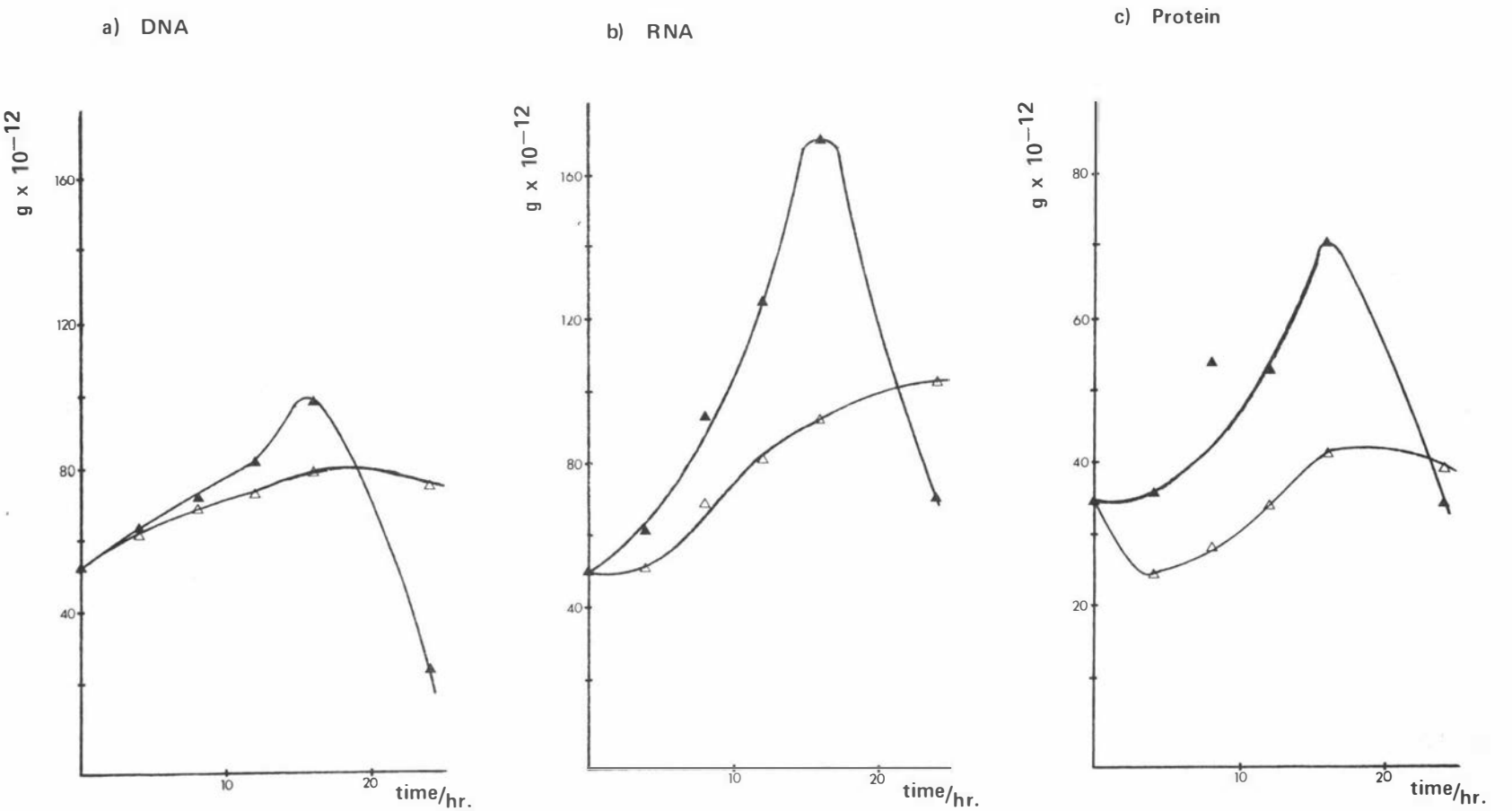
Dothistromin : cell no. ratio = 12.5 $\mu g/cell \times 10^8$
SI = 0.07

FIGURE 19: EFFECT OF DOTHISTROMIN ON SYNCHRONOUS CULTURES OF *CHLORELLA*.
Following changes in levels of macromolecules per 15 cm^3 sample.



Dothistromin @ 0.7 $\mu g/cm^3$
Dothistromin : cell no. ratio = 12.5 μg cell x 10^8

FIGURE 20: EFFECT OF DOTHISTROMIN ON SYNCHRONOUS CULTURES OF *CHLORELLA*.
Following changes in levels of macromolecules per cell.



Dothistromin @ 0.7 $\mu g/cm^3$
Dothistromin : cell no. ratio = 12.5 μg cell x 10^8

in the rate of change in levels of protein and of RNA between the control and the test after exposure to dothistromin. The increase in DNA content does not appear to be greatly affected over the first few hours following dothistromin addition and only in the later stages of the cell cycle is the increase in DNA inhibited in the dothistromin-treated culture. The effects observed are summarized in Table 8. Dothistromin appears to affect the processes of RNA and/or protein synthesis thereby inhibiting growth and, indirectly, cell division. However, this approach does not show whether the effects on RNA and protein are immediate primary effects of the toxin.

3.24 Summary and Discussion

Dothistromin is an inhibitor of the growth of *Chlorella* in liquid culture at concentrations of the order of 1.0 - 2.0 $\mu g/cm^3$. The effective concentration may, in fact, be less than this since the compound is not readily soluble in aqueous solution and not all of the toxin added may be available to the cells. Although ethyl acetate, the carrier solvent used to introduce dothistromin into aqueous cultures, is also growth inhibitory it had no apparent effect on growth at the concentrations used in these experiments ($< 0.5\%$). As may have been expected it could be shown that the ratio of dothistromin concentration : cell number was an

SUMMARY TABLE

Table 8. Effects of dothistromin on synchronous cultures of *Chlorella*.

TREATMENT	Division Number	Rate of Increase of DNA (μg/hr)	Rate of Increase of RNA (μg/hr)	Rate of Increase of protein (μg/hr)
CONTROL Ethyl acetate 0.5%	3.5	1.5	6.0	1.8
TEST Dothistromin 0.7 μg/cm^3 (dothistromin : cell number ratio - 12.5 μg/cell X 10^8)	1.2	1.0	1.6	0.7

The rates given in this table were taken from graphs showing the changes in macromolecular levels over the cell cycle of *Chlorella*, and were derived by measuring the slope of the tangent to each curve over the 4 - 12 hour period.

important factor in the degree of inhibition observed. These ratios were of the order of 1.0 μg/cell X 10^8 for inhibition of the growth cycle. However, in order to accentuate the changes observed in the cell cycle in synchronous cultures, it was necessary to use a ratio of the order of 10.0 μ g/cell X 10^8. Addition of toxin at this level resulted in an inhibition of cell division and a marked decline in the rate of increase in the levels of RNA and protein per cell over the cell cycle. An effect on the rate of increase of DNA was not observed until the later stages of the cycle.

From these results it might be concluded that dothistromin has a primary effect on growth processes, indirectly leading to inhibition of cell division. The eventual impairment of DNA synthesis observed may be a secondary effect caused by the inhibition of RNA and protein synthesis which is apparent by the 4th hour of the cell cycle. Further investigation of these areas of metabolism was undertaken to establish whether the apparent inhibition of RNA and protein synthesis was a direct primary effect of dothistromin. Results obtained from studies on the site of action of the aflatoxins have shown that they inhibit RNA synthesis resulting in a subsequent inhibition of protein synthesis.

Synchronous cultures are useful in establishing the area of metabolism affected by an inhibitor. However direct analysis of this nature has limitations. The degree of synchrony achieved in

this study was not sufficient to give a detailed time-course for inhibition. Such a time-course would involve sampling at closely timed intervals after the addition of dothistromin and the measurement of very small differences in the levels of nucleic acids and protein. The approach would require very large volumes of culture to supply sufficient material for detailed analysis, and a refined technique of inducing synchrony in Chlorella cultures. More sensitive methods of determining rates of biosynthesis were used to examine in more detail the likely sites of inhibition which had been indicated from the synchronous culture experiments.

3.3 EFFECT OF DOTHISTROMIN ON THE INCORPORATION OF RADIOACTIVE PRECURSORS BY CHLORELLA

The experiments on the effects of dothistromin on synchronized cultures of Chlorella showed a marked inhibition of the rates of increase of total levels of RNA and protein throughout the cell cycle. To study this effect in greater detail requires a more sensitive method of analysis than the determination of gross changes in total levels of each component.

The availability of isotopically-labelled precursors of nucleic acids and proteins of high specific activity and purity makes these compounds suitable for biosynthetic studies. The ease and sensitivity of radioactive assay permits the detection of

minute amounts of labelled components in samples, and hence labelled precursors of biosynthesis can be used at physiological concentrations.

^{3}H-Uridine incorporation is a reasonably specific method of labelling RNA when the tritium label is present in the 5 position. This label is lost in the formation of thymidine via deoxyuridine, and the proportion of RNA precursors incorporated into DNA is small in comparison with their incorporation into RNA. ^{3}H-Thymidine (labelled in the methyl position) will be specifically incorporated into the DNA, since thymidine is not utilized in RNA synthesis. A number of labelled amino acids are available for use in incorporation studies. However, care must be exercised in the choice of amino acid for studies on the biosynthesis of proteins since many of these compounds occupy central positions in intermediary metabolism. Obviously the use of isotopically-labelled amino acids such as glutamic acid or glycine in tracer studies would result in a very wide distribution of the label in many different classes of compounds. ^{14}C-Phenylalanine incorporation was used in the present study as a measure of the rate of protein synthesis.

Although the incorporation of labelled precursors may be used to estimate biosynthetic rates, care must be taken in the interpretation of data. The rate of incorporation will be determined by such factors as (a) the rate of uptake of the labelled compound, (b) the size and rate of turnover of the precursor pool and (c) the

composition of the macromolecule being synthesized. However, it was not considered relevant to evaluate these factors in the present preliminary stage of this investigation.

While radioactive tracers do make it possible to carry out very sensitive assays, the use of radio-isotopes does have disadvantages. In addition to possible radiation effects, which are unlikely to be significant in such short-term experiments with weak β -emitters, simple chemical exchange reactions can occur with radio-isotopes and, as mentioned above, isotopic labels can have a multiplicity of fates. In tracer experiments one is following the distribution of a label and not necessarily that of the compound in which the label has been added to the test system. Therefore some purification is usually necessary before samples are counted. There are a variety of classes of macromolecule into which precursors can be incorporated and the rate of synthesis measured by incorporation of radioactive isotopes will be predominantly that of the fraction with the highest rate of synthesis. Thus, if incorporation into one of the minor cell components or into a class of macromolecule being synthesized at a low rate is being selectively inhibited, this effect will be masked by incorporation into other cell fractions.

3.31 Incorporation of Radioactive Precursors by Chlorella Cultures

Preliminary experiments with Chlorella were carried out to investigate incorporation of ^{3}H-uridine, ^{3}H-thymidine and ^{14}C-phenylalanine. Aliquots ($5cm^3$) of Chlorella in liquid culture were incubated in $50cm^3$ Erlenmeyer flasks with the addition of isotope. During the period of incubation the cultures were aerated with 5% CO_2 in air and illuminated, following the cultural conditions used in previous work. (See Experimental Methods Section for full details of methods.) At timed intervals the incorporation of isotope was arrested by the addition of hot absolute alcohol, to give a final alcohol concentration of 80% (v/v). After centrifugation the residues were resuspended in a further volume of hot 80% alcohol. This extraction procedure should remove any unused precursor as well as soluble nucleotides and free amino acids. Residues from the second alcohol wash were resuspended in 50% ethanol and an aliquot taken for counting to determine the total incorporation into ethanol-insoluble compounds.

^{3}H-Uridine (25 μCi/assay) showed very high levels of incorporation, and ^{14}C-phenylalanine (1.25 μCi/assay) gave satisfactory incorporation into ethanol insoluble material. The levels of ^{3}H-thymidine (25μCi/assay) incorporated were somewhat lower (of the order of 2000cpm/hr - i.e. 10% of the rate of

^{3}H-uridine incorporation.) It was shown that cycloheximide, at a final concentration of 5 $\mu g/cm^3$, inhibited the incorporation of ^{14}C-phenylalanine into the ethanol-insoluble material, although inhibition was not complete at this concentration. Investigation of ^{3}H-uridine incorporation showed that at least 80% of the label incorporated into the ethanol-insoluble material was found in the supernatant after overnight alkaline hydrolysis at 30°. This indicates that the tritium label is predominantly in the RNA-containing fraction. Of the ^{3}H-thymidine incorporated, approximately 30% of the label remained in the residue (DNA and protein containing fraction) after overnight alkaline hydrolysis.

3.32 Effect of dothistromin on precursor incorporation

The labelling procedure already described was used to study the effect of dothistromin on the incorporation of ^{3}H-uridine, ^{3}H-thymidine and ^{14}C-phenylalanine. Aliquots ($5cm^3$) of *Chlorella* culture for labelling were withdrawn from a synchronous culture at the beginning of the cell cycle, and the incorporation of each labelled precursor was determined at 30 min. intervals for 1½ hr. This labelling period over the first 1½ hr of the cell cycle corresponds to the period of growth following cell division. Dothistromin was added at the beginning of this period, with the labelled precursor, to give a dothistromin : cell number ratio

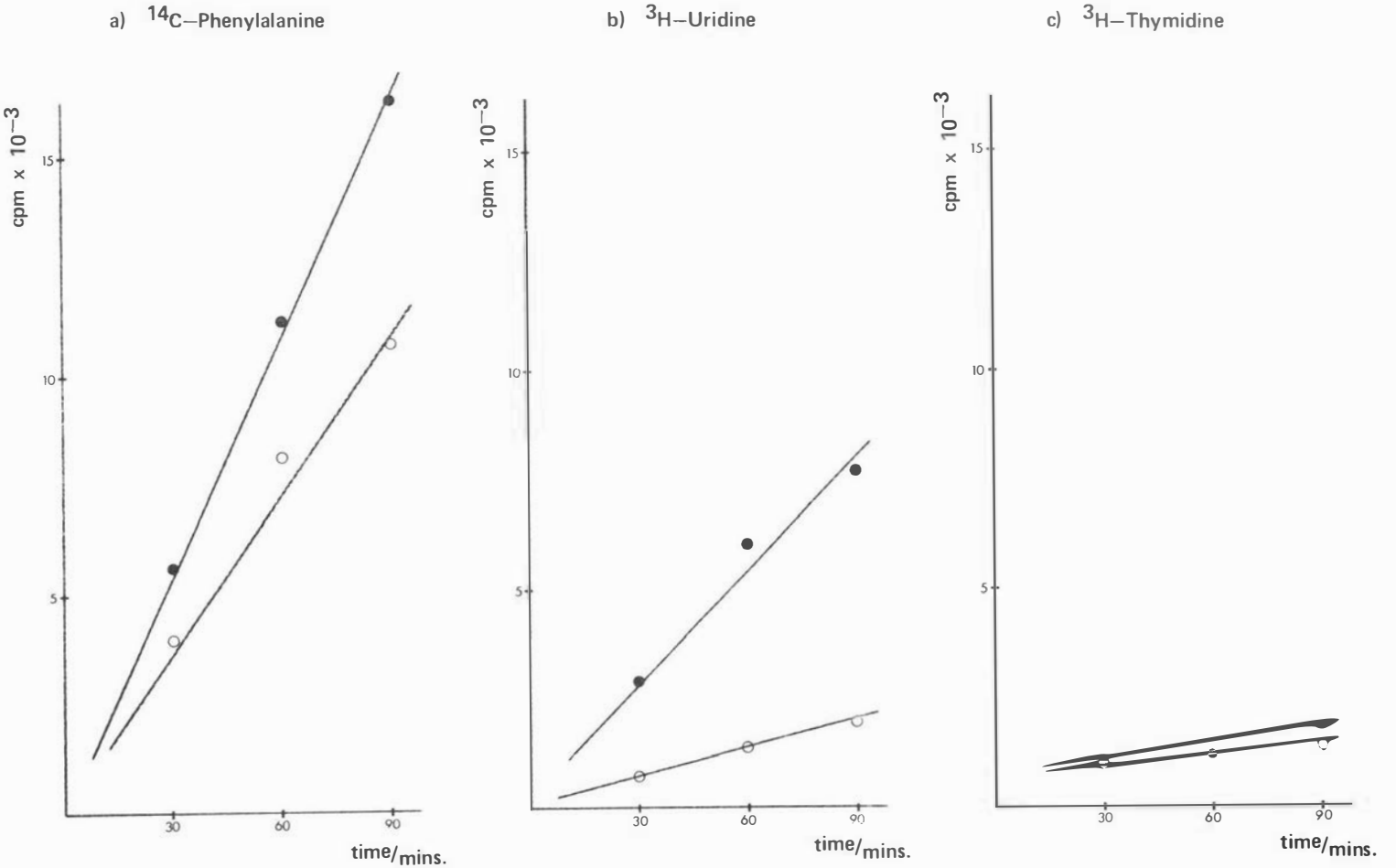
of 14 μg/cell X 10^8 (final concentration of dothistromin 1.0 $\mu g/cm^3$). Labelled precursors were added at the following levels: ^{3}H-uridine, 10 μCi/assay; ^{3}H-thymidine, 25 μCi/assay; ^{14}C-phenylalanine, 1.25 μCi/assay. Results are given in Fig.21.

These results showed a marked inhibition by dothistromin of ^{3}H-uridine and ^{14}C-phenylalanine incorporation; the rates of incorporation were inhibited by 81.5% and 49.5% respectively. The toxin had little effect on the incorporation of ^{3}H-thymidine, causing 31% inhibition. The rate of ^{3}H-thymidine incorporation was low by comparison with that shown for the other precursors. However, it has been shown that replication of nuclear DNA occurs after the 10th hour of the cycle in synchronous cultures of Chlorella (Wanka and Mulders, 1967), although ^{14}C-uracil incorporation into satellite DNA is continuous throughout the cell cycle (Wanka et al., 1970). At the stage of the cell cycle over which labelling was carried out (0 - 1½ hr) one would not expect to find high rates of ^{3}H-thymidine incorporation into DNA.

3.33 Effect of dothistromin on precursor incorporation at different stages of the cell cycle

To study the effect of dothistromin at different stages of the cell cycle, rates of ^{3}H-uridine, ^{3}H-thymidine and ^{14}C-phenylalanine were determined at 4-hourly intervals over the cell cycle.

FIGURE 21: EFFECT OF DOTHISTROMIN ON THE INCORPORATION OF LABELLED PRECURSORS BY *CHLORELLA*.



Labelling 5 cm^3 aliquots of synchronous culture of *Chlorella* over first 90 min. of the cell cycle. (^{14}C–phenylalanine @ 1.25 μCi, ^{3}H–uridine @ 10 μCi, ^{3}H–thymidine @ 25 μCi, dothistromin @ 1.0 $\mu g/cm^3$, dothistromin:cell no. ratio = 14.3 μg/cell x 10^{8})

Samples for labelling were taken from two synchronous cultures, one initiated 12 hours after the other, but both at the same initial cell density. This simplified the handling of samples during the 24 hour cell cycle. Dothistromin was added at a final concentration of 1.0 $\mu g/cm^3$ to each assay, giving a dothistromin : cell number ratio of 16 μg/cell X 10^8. Labelled precursors were added at the following levels: ^{3}H-uridine, 10 μCi/assay; ^{3}H-thymidine, 10 μCi/assay; ^{14}C-phenylalanine, 0.3 μCi/assay. Rates of incorporation have been calculated from graphs of incorporation at each sample time and, where uptake was non-linear, from a tangent to the curve over the 30 - 60 min time interval. These results are given in Table 9. Rates of incorporation in the presence of dothistromin have been expressed as a percentage of those shown by the controls. Fig.22 shows a graphical representation of the variation of the rates of incorporation over the cell cycle.

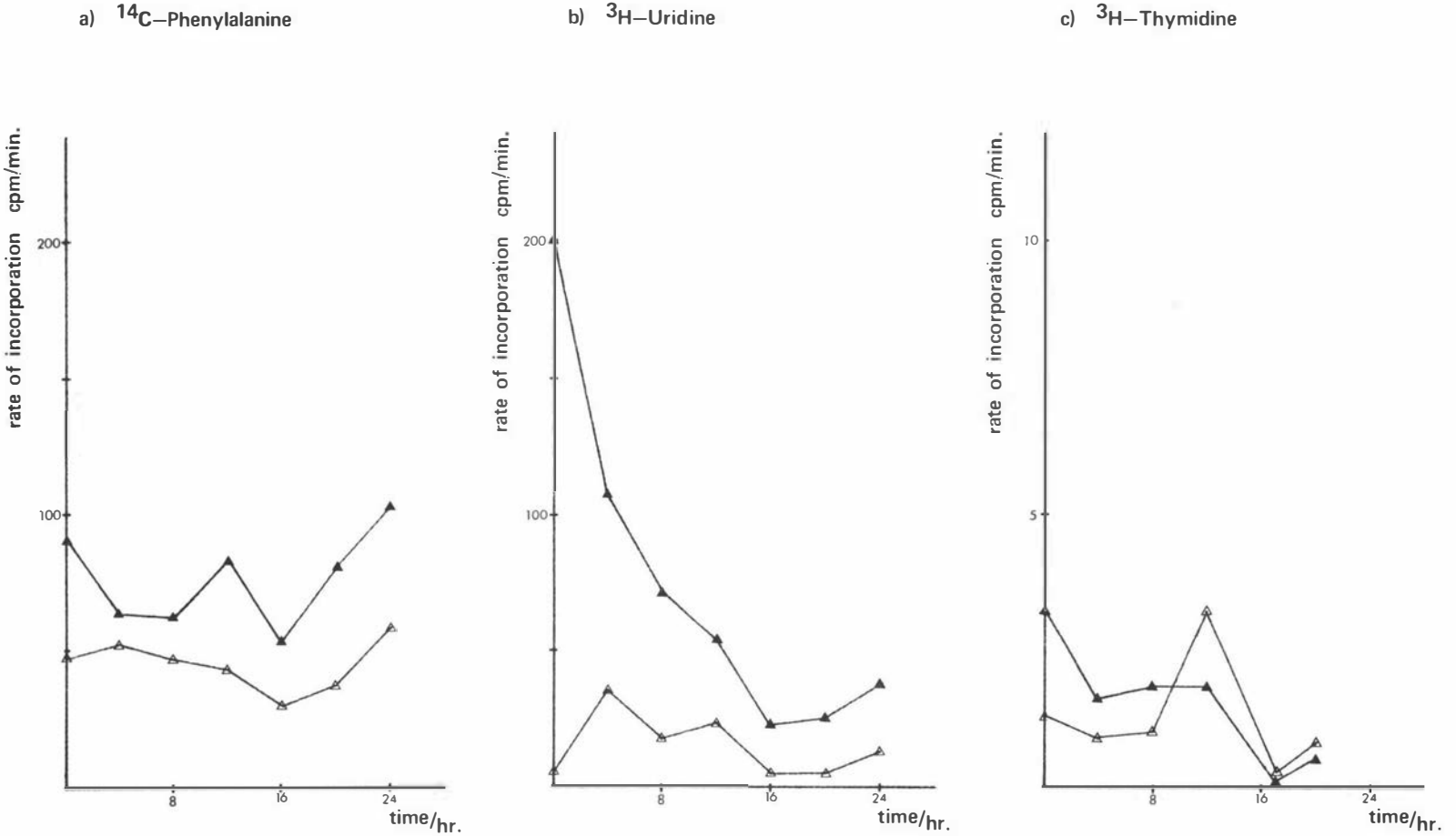
High rates of incorporation of ^{3}H-uridine were shown over the first 12 hours of the cycle. The rate in the presence of the toxin varied between 20% and 40% of the control rate, with the exception of the 0hr sample which showed little incorporation of ^{3}H-uridine in the presence of the toxin. The incorporation of ^{14}C-phenylalanine remained reasonably constant throughout the cell cycle, with incorporation in the presence of dothistromin varying

Table 9. Rate of Label uptake over life cycle of Chlorella.

HOUR OF CYCLE	Control Rate cpm/min	Dothistromin Rate cpm/min	Dothistromin Rate / Control Rate %
^{14}C Phenylalanine:			
0	90	47	52%
4	63	52	83%
8	62	47	75%
12	83	43	52%
17	53	30	57%
20	80	37	46%
24	103	58	56%
^{3}H-Uridine:			
0	200	3	1.5%
4	107	35	33%
8	71	18	25%
12	53	23	43%
17	23	5	22%
20	25	5	20%
24	38	13	34%
^{3}H-Thymidine:			
0	3.2	1.3	39%
4	1.6	0.9	56%
8	1.8	1.0	55%
12	1.8	3.2	179%
17	0.1	0.8	253%
20	0.5	0.8	166%

Dothistromin final concentration = 1.0 $\mu g/cm^3$;
Control - 1.0 μl Ethyl Acetate. ^{14}C phenylalanine at 0.3 μCi;
^{3}H-uridine at 10 μCi; ^{3}H-thymidine at 10 μCi.
Dothistromin : cell no. ratio = 16.0 μg/cell X 10^8

FIGURE 22: RATES OF LABEL INCORPORATION OVER THE CELL CYCLE OF *CHLORELLA*



Labelling 5 cm^3 aliquots of synchronous culture of *Chlorella*.
Rates of incorporation calculated from tangents to incorporation plots for each time interval.
(^{3}H–uridine @ 10 μCi/assay; ^{3}H–thymidine @ 10 μCi/assay; ^{14}C–phenylalanine @ 0.3 μCi/assay)

between 45% and 80% of the control rate. ^{3}H-Thymidine incorporation remained consistently low, with the toxin apparently increasing incorporation rates after the 12th hour of the cycle.(^{3}H-Thymidine incorporation rates varied from less than 1 cpm/min to little more than 3 cpm/min. At these levels the apparent 'increase' in rate is not significant.)

Other work in this field (Senger and Bishop, 1969) indicates a more constant rate of RNA synthesis than that indicated by the ^{3}H-uridine incorporation rates observed in this system. Protein synthesis is also nearly linear throughout the cycle. The synthesis of DNA in the cell cycle of Chlorella has been shown to be non-linear with very little synthesis occurring over the first 8hours. This period is followed by one of rapid synthesis prior to cell division and then the rate of synthesis drops towards the end of the cycle. Results obtained by following ^{3}H-thymidine incorporation do not reflect this biosynthetic pattern at all.

3.34 Relationship between dothistromin concentration and incorporation of ^{3}H-uridine

An experiment in which Chlorella samples were incubated with increasing concentrations of dothistromin was conducted to establish the relationship between dose and degree of inhibition of ^{3}H-uridine incorporation observed. The samples of Chlorella culture for labelling were withdrawn from a synchronous culture 2 hours after

the beginning of the cell cycle and the concentrations of dothistromin investigated covered the range 0.5 - 4.0 $\mu g/cm^3$, giving dothistromin : cell number ratios of 5.5 - 44.0 μg/cell X 10^8. ^{3}H-Uridine was added at 10 μCi/assay. The rate of ^{3}H-uridine incorporation was plotted against the final concentration of dothistromin and this graph is shown in Fig.23(a).

The results showed that the degree of inhibition is dose-dependent. However, as shown on the graph, the solvent ethyl acetate also inhibited ^{3}H-uridine incorporation; this inhibition was marked at concentrations of greater than 0.2% (v/v).

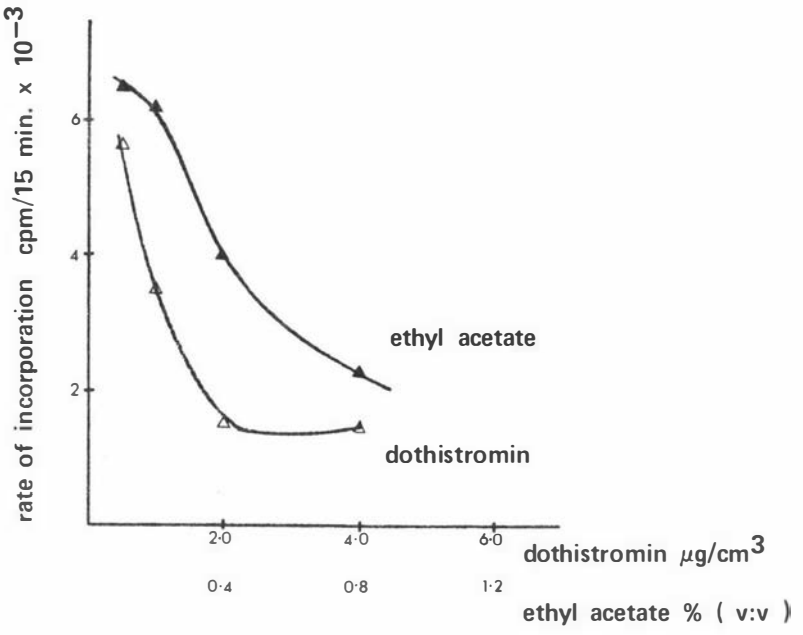
The dothistromin-response curve was repeated using a stock solution of dothistromin of a higher concentration to minimize interference by solvent inhibition. The amount of solvent added to give the highest concentration of dothistromin gave no inhibition by itself. These results are given in Fig.23(b) and show a typical hyperbolic dose-response curve.

3.35 Distribution of precursor label in cellular fractions

In view of the very low incorporation rates of ^{3}H-thymidine observed over the entire cell cycle, a comparison of rates of incorporation in cultures with and without the presence of dothistromin can have no significance until it is established that the incorporation of labelled thymidine is in fact a measure of

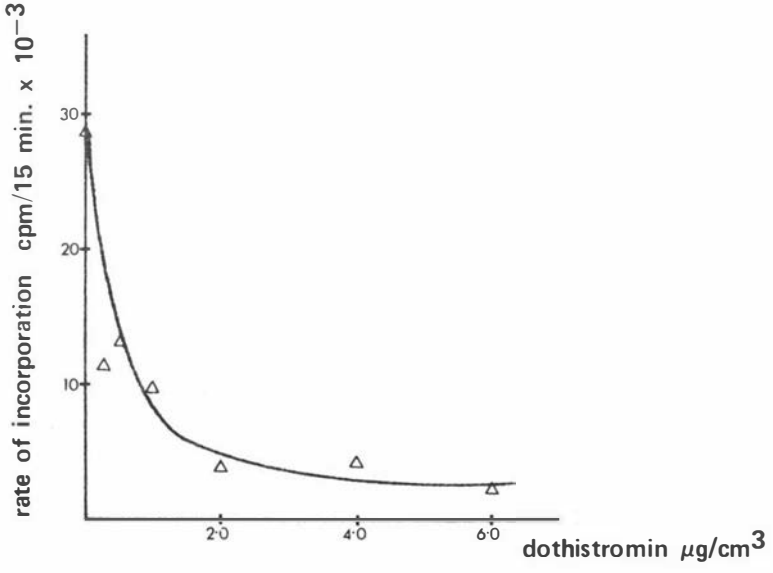
FIGURE 23: DOSE–RESPONSE CURVE FOR DOTHISTROMIN

a)



^{3}H–Uridine labelling of 5 cm^3 aliquots of synchronous culture of *Chlorella* (@ 2 hr)
(^{3}H–uridine @ 10 μCi, dothistromin:cell no. ratios :– 5.5 ; 11.0 ; 22.0 ; 44.0)

b)



^{3}H–Uridine labelling of 5 cm^3 aliquots of synchronous culture of *Chlorella* (@ 2 hr)
(^{3}H–uridine – 10 μCi, dothistromin:cell no. ratios :– 4.1 ; 8.3 ; 16.6 ; 33.3 ; 66.0 ; 100)

DNA synthetic activity in this system. To test the validity of using ^{3}H-thymidine as a nucleic acid precursor to measure rates of synthesis, the distribution of label derived from this compound in the cellular fractions of Chlorella was investigated. The distribution of the tritium label from ^{3}H-uridine was also determined.

^{3}H-Thymidine and ^{3}H-uridine were added to the respective assays at 10 μCi/cm^{3}. The residues after hot ethanol extraction were resuspended in 50% ethanol and an aliquot taken for counting before the remainder was subjected to the Schmidt-Thannhauser extraction procedure (see Section 2.511). Aliquots from each step of this extraction were counted and the results are given in Table 10. Quench corrections were applied to all counts so that valid comparison could be made. The corrected counts were expressed as a percentage of the total counts incorporated into the ethanol-insoluble material.

The results showed that a large proportion of the ^{3}H-uridine incorporated was found in the alkaline-labile, RNA-containing fraction and thus ^{3}H-uridine incorporation is indeed a satisfactory measure of RNA synthetic activity, at least for comparative studies. However, a large percentage of the ^{3}H-thymidine label also appeared in the alkaline-labile material with, at the most, 40% found in the acid-labile, DNA-containing fraction. One must conclude, therefore, that in this system ^{3}H-thymidine incorporation is not a satisfactory measure of DNA synthesis.

Table 10 Distribution of label in cellular fractions of *Chlorella*.

SAMPLE	FRACTIONS			
	Acid Soluble	Alkali-Labile (RNA-containing)	Acid-Labile (DNA-containing)	Residue (Protein-containing)
^{3}H-Uridine:				
30 min.	1.2%	78.7%	12.6%	0.7%
60 min.	11.5%	76.8%	3.9%	0.1%
90 min.	15.3%	55.0%	8.0%	0.2%
120 min.	12.8%	96.7%	4.1%	0.2%
^{3}H-Thymidine:				
30 min.	12.5%	49.7%	13.8%	-
60 min.	21.8%	45.0%	43.3%	3.5%
90 min.	15.1%	50.8%	5.1%	0.5%

Fractions from Schmidt-Thannhauser extraction procedure (refer to Experimental Methods, Section 2.511).

5 cm^3 *Chlorella* culture incubated with labelled precursors. ^{3}H-Uridine at 10 μCi; ^{3}H-Thymidine at 10 μCi.

Counts expressed as a percentage of those incorporated into the ethanol-insoluble residue.

3.36 Alternative labelled precursors for DNA synthesis

From a search of the literature, it was noted that other workers had not been able to label Chlorella DNA satisfactorily with a ^{3}H-thymidine precursor. This has been ascribed to a lack of thymidine kinase activity (Wanka et al., 1970) which means that Chlorella is therefore unable to utilize exogenous thymidine for DNA synthesis. Wanka et al. (1970) found that the use of 2 - ^{14}C-uracil, followed by purification of DNA, was the most useful method for DNA labelling in spite of the high incorporation into RNA, partial degradation and non-specific labelling.

2- ^{14}C-Uracil incorporation into Chlorella was investigated over the period from the 13th - 14th hour of the cell cycle. At this stage of the cell cycle, just prior to cell division, one would expect a higher rate of DNA synthesis and therefore of incorporation. ^{14}C-Uracil was used at a level of 0.1 μCi/assay and over the labelling period less than 100 cpm (1% of the label incorporated into the ethanol-insoluble residue) was incorporated into the DNA-containing fraction, as determined by the Schmidt-Thannhauser extraction procedure, or into the residue remaining after enzymatic hydrolysis of RNA with pancreatic ribonuclease.

To obtain adequate levels of incorporation to measure differences in the rate of DNA synthesis in Chlorella the amount of labelled precursor added would have to be substantially

increased and/or the time of exposure to the labelled precursor extended. An increased labelling period would allow even more non-specific incorporation to occur and would make it difficult to determine the rate at which dothistromin exerts its effect on the test system.

The results obtained establish that dothistromin rapidly affects the rates of protein and RNA synthesis as suggested by the changes observed in the levels of these components in the presence of dothistromin over the cell cycle of synchronous cultures of Chlorella. Inhibition of the rates of both processes was apparent within the first 30 min after exposure to the toxin. The dose-response curve shown by dothistromin is mirrored by that of ethyl acetate, presumably a non-specific inhibitor. It was therefore necessary to determine the time-course and the sequence of the inhibition of each synthetic process to establish the specificity (or otherwise) of dothistromin as an inhibitor. However, due to difficulties encountered in the labelling of Chlorella DNA, it was not possible to establish whether the toxin also inhibits DNA synthesis.

3.4 EFFECT OF DOTHISTROMIN ON GROWTH AND PRECURSOR INCORPORATION BY YEAST AND BACILLUS MEGATERIUM

The finding that the incorporation of isotopically labelled thymidine into the DNA of Chlorella was insufficient to allow short-term time-course studies to be carried out led to the investigation of other organisms for use in elucidating the site of dothistromin inhibition. Ideally, the organism for such studies should be sensitive to dothistromin, incorporate labelled precursors

at high rates over short periods of time and, preferably, allow for ease of extraction of cellular components.

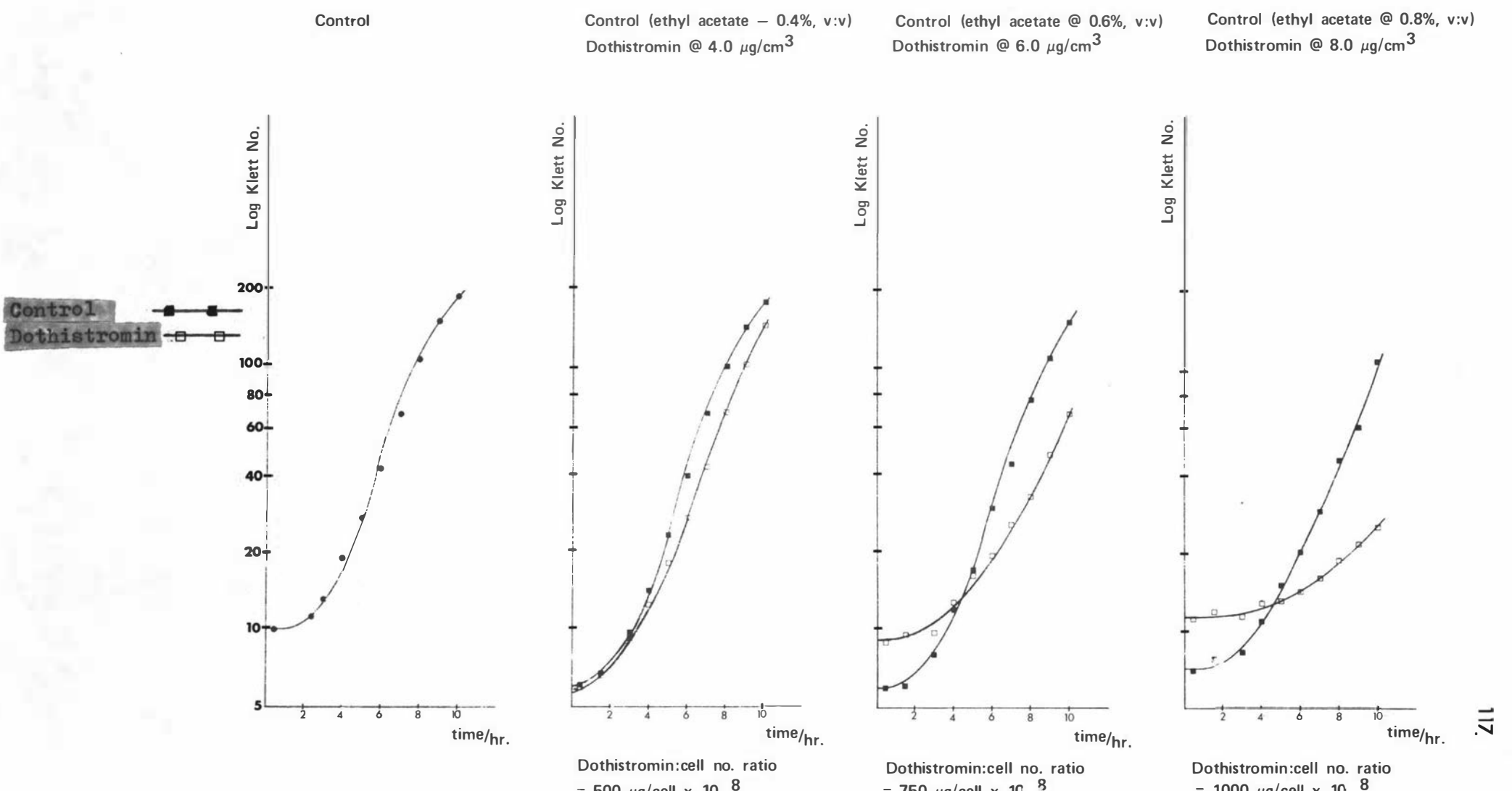
3.41 Investigation of Saccharomyces cerevisiae (Yeast)

The initial investigation of dothistromin toxicity involved the screening of a number of micro-organisms for inhibition by the toxin using the antibiotic disc plate bioassay. It was found that yeast was inhibited by the toxin and this organism was therefore investigated further.

3.411 Effect of dothistromin on growth of Yeast

The concentrations of dothistromin required to inhibit the growth of yeast in liquid culture were first established by measuring growth rates in the presence of the toxin. Details of the culture medium and growth conditions for yeast are given in the Experimental Methods section (Sections 2.47 and 2.7). Growth curves were determined by following the increase in cell numbers in cultures with Klett readings being taken at regular intervals. Erlenmeyer flasks (25 cm^3) with side-arm tubes proved to be convenient vessels for growth of this organism. The side-arms were designed to fit into the Klett machine and readings could be taken without having to withdraw samples from the culture. Representative growth curve data are shown in Fig.24.

FIGURE 24: GROWTH CURVES FOR YEAST SHOWING INHIBITORY EFFECT OF DOTHISTROMIN.
Dothistromin added as an ethyl acetate solution at the beginning of the growth period.



At the high concentrations of dothistronin required to inhibit the growth of yeast cultures, there was a marked solvent inhibition. These results showed clearly the need to include solvent controls when assessing the effects of dothistronin where it is added to cultures as an ethyl acetate solution. The dothistronin : cell number ratios calculated indicate that yeast is less sensitive to the toxin than Chlorella. These ratios are approximations only; cell counts of a yeast which reproduces by budding are of limited accuracy since one is faced with the problem of deciding how large the buds must be before being counted as whole cells, even while still attached to the parent cell. In this case, buds of more than 50% parent cell size, as judged by eye, were counted as whole cells. The ratios are given on the relevant growth curves and are of the order of 500-1000 μg/cell X 10^8, as compared with 2.0 μ g/cell X 10^8.for Chlorella.

3.412 Incorporation of labelled precursors by yeast

Experiments following the incorporation of ^{3}H-uridine and ^{3}H-thymidine were carried out to determine the distribution of each precursor throughout the cellular material of yeast. Aliquots ($5cm^3$) of liquid culture of the organism, in log-phase of growth, were incubated in 50 cm^3 Erlenmeyer flasks at 30^o and the labelling experiments performed as detailed in the Experimental Methods Section 2.64. The

following levels of labelled precursors were used: ^{3}H-uridine, 20 μCi/assay, ^{3}H-thymidine, 20 μCi/assay. Each sample was subjected to the Schmidt-Thannhauser extraction procedure and the results obtained are given in Table 11. These results show that most of the ^{3}H-uridine incorporated is found in the RNA-containing fraction, but that only a small proportion of the meagre ^{3}H-thymidine incorporation is found in the DNA-containing fraction. The large discrepancies shown for precursor incorporation arise from the difficulty of taking aliquots from an uneven suspension of the ethanol-insoluble materials. The negligible thymidine incorporation into DNA may again be due to the absence of a thymidine kinase in S.cerevisiae (Grivell and Jackson, 1968).

2 -- ^{14}C-Uracil was also investigated for use as a DNA label. However, uptake into the DNA fraction, as determined both by Schmidt-Thannhauser extractions and by counting residues after exposure to pancreatic ribonuclease, was not sufficient to permit short-term labelling studies. The maximum incorporation rate was only 200 -- 300 cpm/hour.

Yeast was therefore not considered to be a suitable organism for this type of experimental study. It is relatively insensitive to dothistromin and, at the concentrations of toxin required to inhibit growth, solvent inhibition became a problem. In yeast, as in Chlorella, the incorporation of ^{3}H-thymidine into DNA was negligible,

Table 11. Label distribution in cellular fractions of yeast.

SAMPLE	FRACTIONS		
	Acid Soluble	Alkali-labile (RNA-containing)	Acid-labile (DNA-containing)
^{3}H-Uridine			
15 min.	13.3%	85.4%	1.3%
30 min.	7.3%	88.7%	4.0%
45 min.	2.9%	94.2%	2.9%
60 min.	3.9%	92.4%	3.6%
^{3}H-Thymidine			
15 min.	100%	0	0
30 min.	32%	68%	0
45 min.	85%	0	16%
60 min.	100%	0	0

Fractions derived by Schmidt-Thannhauser extraction procedure (see Section 2.511).

5 cm^3 Yeast culture incubated with labelled precursors. ^{3}H-uridine at 20 μCi; ^{3}H-thymidine at 20 μCi;

('0' indicates that counts in fraction were less than the background counts.)

and the incorporation of label from 2 - ^{14}C-uracil as a precursor for nucleic acid synthesis proceeded very slowly under the conditions used.

3.42 Investigation of Bacillus negaterium, KM

B.negaterium, KM has been used as a bioassay organism in the study of aflatoxins (Freke, 1974). A thymine-requiring mutant of this strain was available and by using this it was hoped that high rates of incorporation of ^{3}H-thymidine into DNA could be obtained. Bioassay of B.negaterium, KM, in the antibiotic disc agar plate assay and in liquid culture, showed that the organism is sensitive to dothistromin and a sub-culture of the thymine-requiring mutant, designated KMT^{-} and isolated by Wachsman et al. (1964), was obtained for investigation.

Culture conditions used for the growth of B.negaterium are given in the Experimental Methods Section (2.43). Before proceeding with a study of the effect of dothistromin on this mutant an investigation of its thymine requirement was undertaken. No significant differences were found in the growth of cultures lacking an exogenous supply of thymine or thymidine and those supplied with a range of concentrations of either compound. In

spite of the inability to demonstrate an exogenous thymine requirement, the suitability of the mutant strain purported to be B.megaterium KMT⁻ was further investigated and this variant of B.megaterium KM was the experimental organism used for the remainder of this study.

3.421 Effect of dothistromin on growth of B.megaterium KM

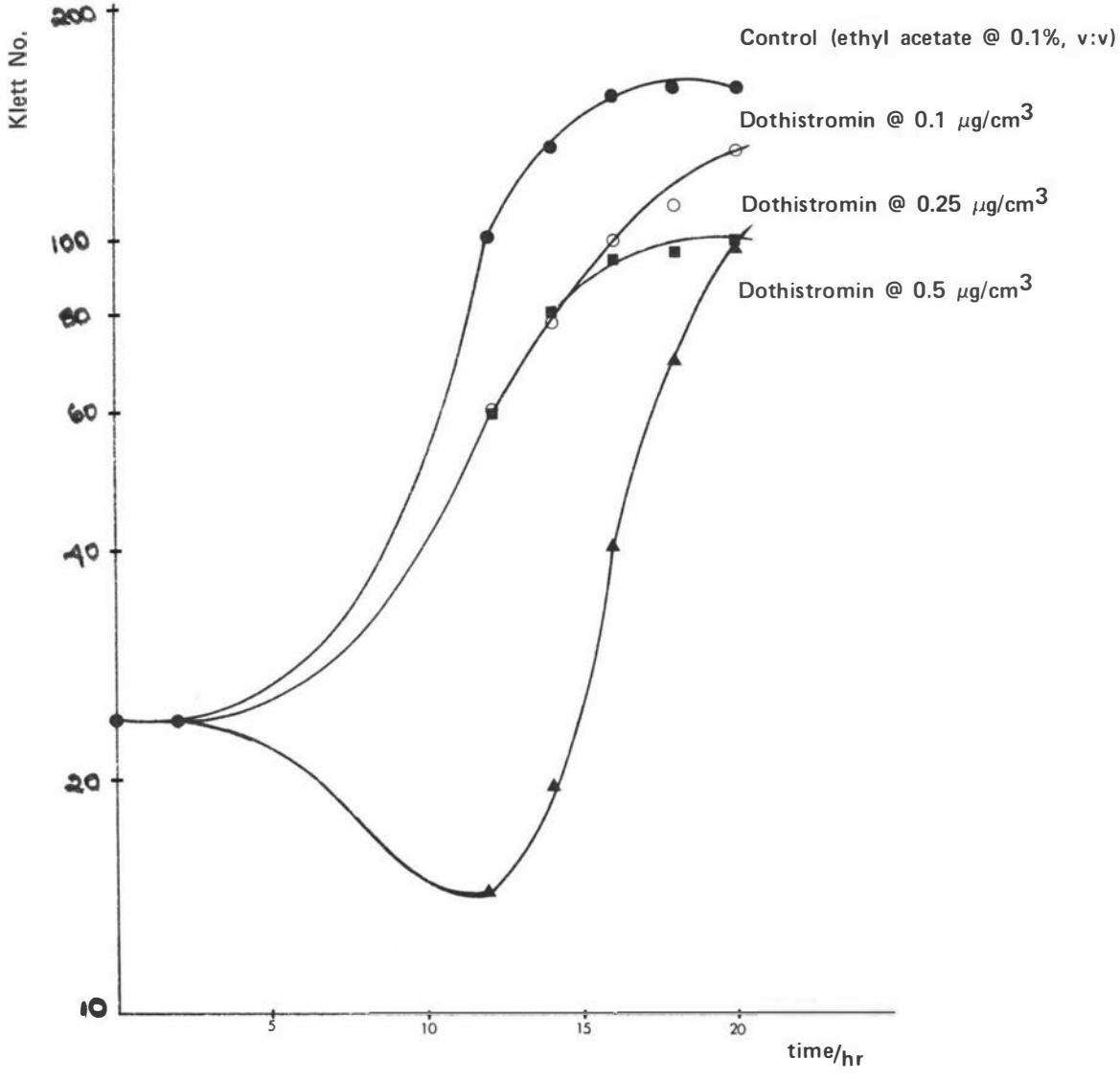
The effect of dothistromin on growth of this organism was studied to establish inhibitory levels of the toxin and representative growth curves are shown in Fig.25. It can be seen that this strain of B.megaterium is very sensitive to the toxin with dothistromin : cell number ratios required for inhibition being of the order of 0.25 μg/cell X 10^8, as compared with 2.0 μg/cell X 10^8 for Chlorella and 750 μg/cell X 10^8 for yeast. However, B.megaterium is a smaller organism than yeast or Chlorella, and comparison on the basis of cell mass would be more accurate.

3.422 Uptake of precursors by B.megaterium KM

An investigation was made of the levels of incorporation of labelled precursors into ethanol-insoluble residues of B.megaterium KM. Aliquots ($5cm^3$) of a culture in the log-phase were incubated with the precursors; ^{3}H-thymidine, 10 μCi/assay; ^{3}H-uridine, 10 μCi/assay and ^{14}C-phenylalanine, 0.2 μCi/assay. Label incorporation was arrested by the addition of hot ethanol at 5 min. intervals over a total labelling period of 25 min. Residues after the hot ethanol extraction procedure were subsequently resuspended in 50% ethanol

FIGURE 25: GROWTH CURVES FOR *B.MEGATERIUM KM* SHOWING INHIBITORY EFFECT OF DOTHISTROMIN.
Dothistromin added as an ethyl acetate solution at the beginning of the growth period.

Dothistromin:cell no. ratios :– 0.1, 0.25, 0.5 μg/cell x 10^{3}



and an aliquot of each taken for counting. Some of the samples were extracted according to the Schmidt-Thannhauser procedure to determine the label distribution for each precursor and these results are given in Table 12.

Incorporation of ^{3}H-uridine and ^{14}C-phenylalanine was found to be very rapid, reaching levels of 80,000 cpm and 40,000 cpm respectively in 5 min. After this time there was very little increase in incorporation over the whole labelling period. A high rate of incorporation was observed for ^{3}H-thymidine as well, although the level of incorporation was lower (2,000 cpm in 5 min).

Analysis of label distribution in the cellular components showed that the incorporation of ^{3}H-uridine was mainly into the RNA-containing fraction. ^{3}H-Thymidine incorporation appeared to be less specific, with approximately 15% of the total label incorporated into the ethanol-insoluble material being in the DNA fraction. The ^{14}C-phenylalanine obviously undergoes partial metabolism and a substantial portion of the ^{14}C incorporated is recovered in the alkali-soluble material. In all subsequent work the hot ethanol residues were routinely extracted and aliquots of the resulting fractions counted so that incorporation into specific fractions was counted. Evidence that the isotope is present as incorporated precursor in the appropriate fraction is given in Section 2.65.

Table 12. Distribution of label in cellular fractions of *B.megaterium*.

SAMPLE	FRACTIONS			
	Acid Soluble	*Alkali-labile (RNA-containing)	Acid-labile (DNA-containing)	Residue (Protein-containing)
^{3}H-Uridine:				
I	5.0%	85%	12%	2%
II	7%	100%	10%	1%
^{3}H-Thymidine:				
I	20%	34%	18%	2%
II	27%	30%	12%	2%
^{14}C-Phenylalanine:				
I	7%	67%	5%	8%
II	3%	63%	8%	6%

Fractions from Schmidt-Thannhauser extraction procedure (Section 2.511) - counts expressed as percentage of total incorporation into residues after hot ethanol extraction.

5 cm^{3} *B.megaterium* culture incubated with isotopes. ^{3}H-uridine at 10 μCi; ^{3}H-thymidine at 10 μCi, ^{14}C-phenylalanine at 0.2 μCi.

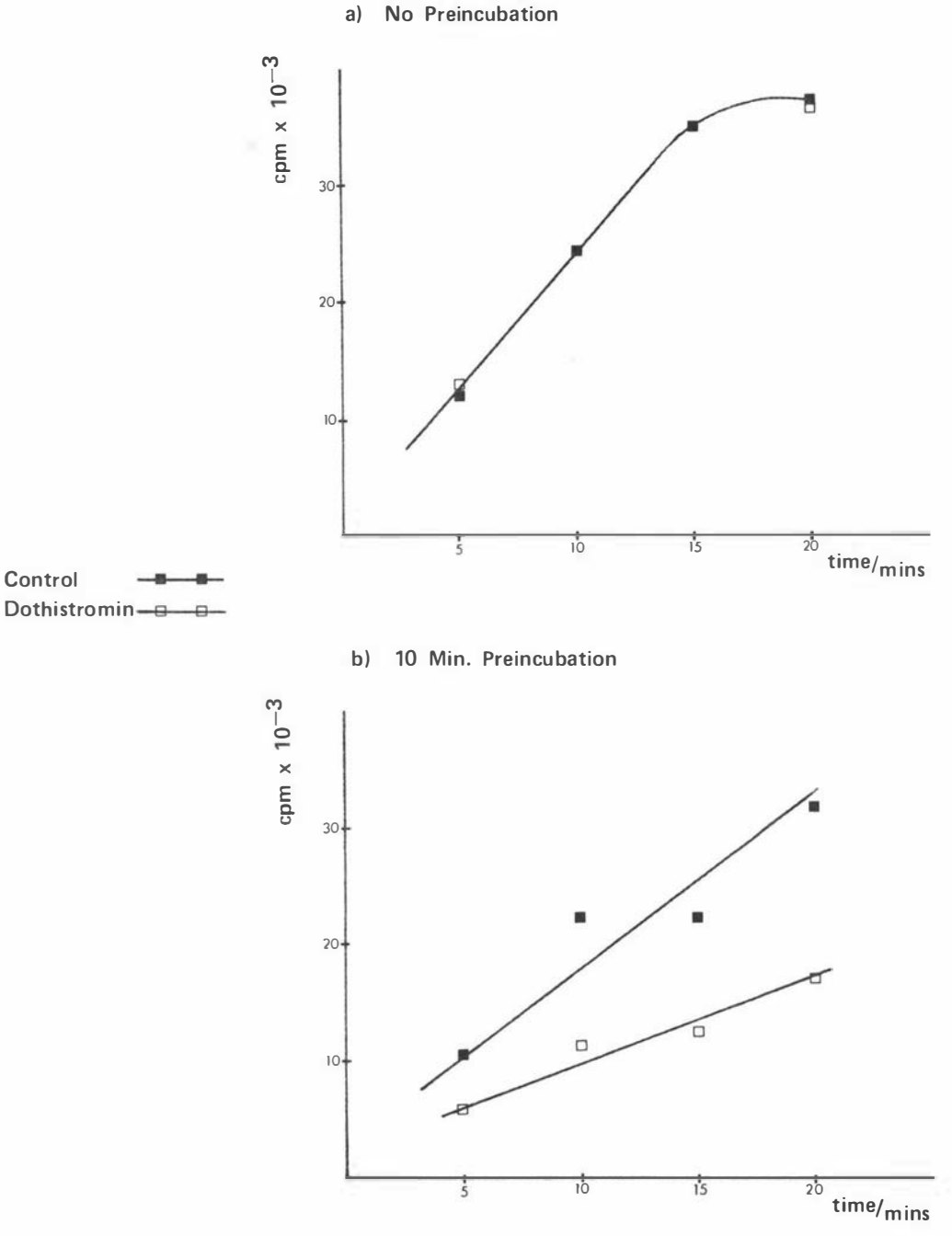
* This fraction will also contain alkali-soluble material including low molecular-weight proteins.

3.423 Preliminary Investigation of Precursor Incorporation in the presence of dothistromin

The very rapid incorporation of label into the macromolecular constituents of this micro-organism results in rapid exhaustion of the trace amounts of precursor added and, because of this, non-linear incorporation is observed. To overcome this problem unlabelled carrier was added in sufficient quantity to enable linear incorporation of isotope over the longer period required for dothistromin inhibition to be evident.

In the initial experiments following the incorporation of the ^{3}H-uridine label into the RNA-containing fraction, unlabelled carrier uridine was added to each 5cm^{3} assay to dilute the added label by 1/10th (^{3}H-uridine, 5 μCi/assay; carrier uridine, 2.5 X 10^{-9} moles/assay). For one series of assays the system was preincubated with dothistromin (0.1 μg/cm^{3}) for 10 min before the addition of the labelled precursor and carrier. In a second series there was no preincubation period, the labelled precursor and carrier being added immediately after the addition of the toxin. The time courses of label incorporation in these experiments are shown graphically in Fig.26. Use of unlabelled carrier compound gave linear incorporation rates over the 20 min. labelling period for both assay series. In the series preincubated with dothistromin inhibition of ^{3}H-uridine incorporation was evident in the 10 min sample, but no significant difference in label incorporation could be seen in the assay series where there was no preincubation period.

FIGURE 26: EFFECT OF PREINCUBATION ON DOTHISTROMIN INHIBITION OF ^{3}H–URIDINE INCORPORATION



(^{3}H–uridine @ 5 μCi/assay ; carrier uridine @ 2.5 x 10^{-9} moles/assay; dothistromin @ 0.1 $\mu g/cm^{3}$)

Further experiments were carried out where the length of the preincubation time with toxin was altered and the concentration of dothistromin varied. The degree of inhibition of ^{3}H-uridine incorporation observed increased as the preincubation period was lengthened and as the concentration of dothistromin increased. However, an experimental system which includes preincubation with the inhibitor makes it difficult to define the early time-course of inhibition of precursor incorporation into the cellular macromolecules and to study the immediate effects of the toxin, a primary interest of this work. Therefore, an alternative approach using higher concentrations of toxin was adopted to enhance the rapidity of penetration of the toxin.

The experimental labelling system used in all experiments so far described has involved the extraction of an entire $5cm^3$ assay at each time interval. Each time course therefore required a number of replicate assays, one for each timed sample. A comparison was made between this method of sampling and one in which $1cm^3$ aliquots were withdrawn from a single assay at each time interval. (For a detailed description see Experimental Methods, Section 2.64). Sampling in the latter system is a far more rapid procedure and allows sampling at closely timed intervals. Components of both systems were added to give the same final concentrations (^{3}H-uridine, $1\mu Ci/cm^3$; carrier uridine, 0.5×10^{-9} moles/cm^3; dothistromin, $0.2\mu g/cm^3$; no

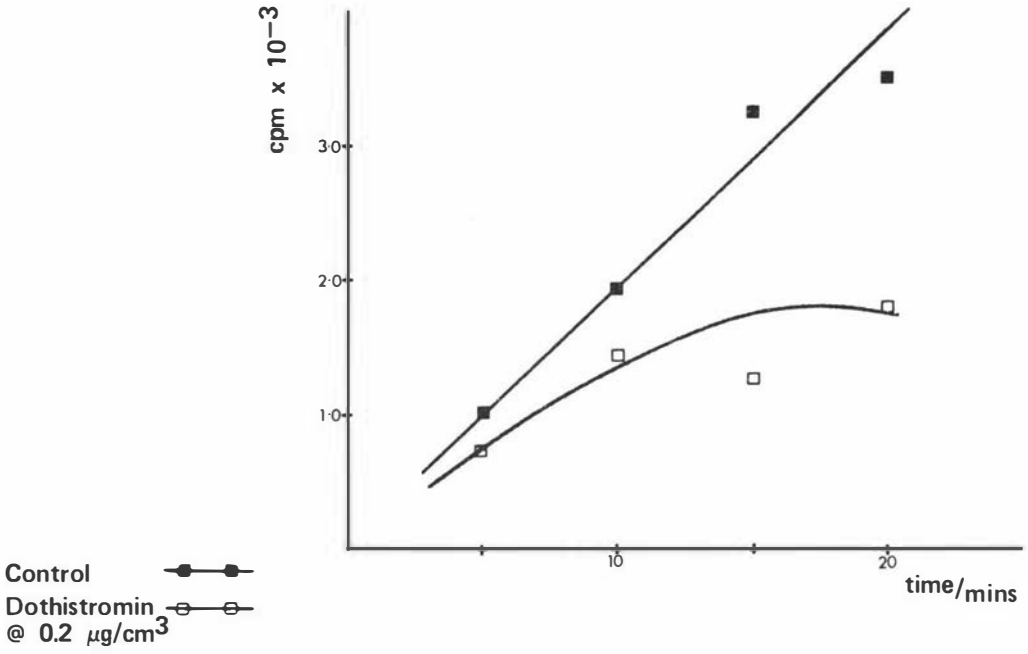
preincubation with toxin.) The results obtained are shown in Fig.27. This comparison showed similar inhibition effects with both methods and established that the levels of label present in each $1cm^3$ aliquot taken in the latter assay system are adequate for the purposes of these experiments. This assay technique was used for the remainder of this research.

To establish the dose-response relationship between dothistromin concentration and degree of inhibition of uridine incorporation an experiment was carried out in which ^3H-uridine incorporation was followed in the presence of increasing concentrations of dothistromin. Aliquots ($1cm^3$) were withdrawn from $10cm^3$ assays at 5 min intervals over a 20 min labelling period. The following levels of precursor and dothistromin were used: ^3H-uridine, $1\mu Ci/cm^3$; carrier uridine, 0.25×10^{-9} moles/cm^3; dothistromin, $0.1\mu g/cm^3$ - $1.0\mu g/cm^3$ giving dothistromin : cell number ratios of the order of $0.02 - 0.20\mu g/cell \times 10^8$. Results are shown in Table 13. These clearly showed an increase in the degree of inhibition of incorporation with increasing concentrations of dothistromin. The use of unlabelled carrier gave linear uptakes over the labelling period.

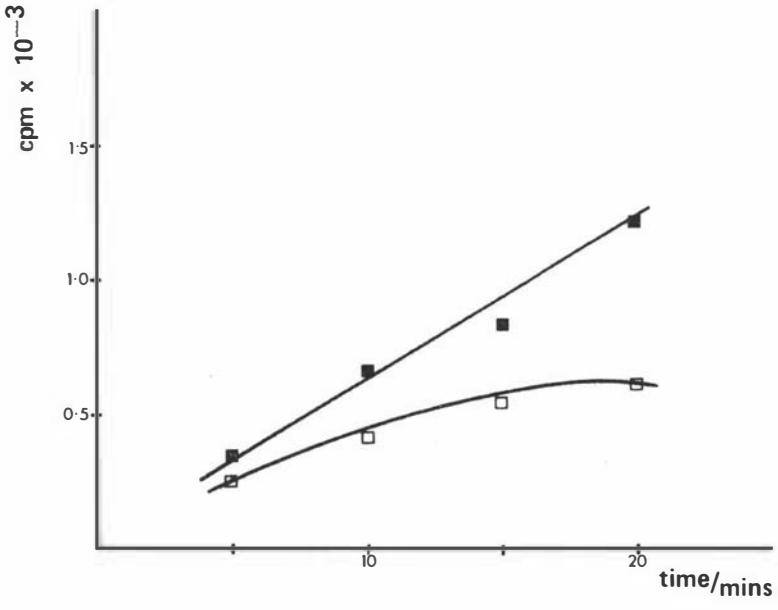
Preliminary experiments with ^3H-thymidine and ^{14}C-phenylalanine established the conditions necessary for linear incorporation over a 25 min period (Fig.28). The linearity of ^3H-thymidine ($3.3\mu Ci/cm^3$) was not improved by the addition of unlabelled thymidine (2.6×10^{-9} moles/cm^3) and resulted in a marked drop in the rate of label incorporation. The incorporation of ^{14}C-phenylalanine ($0.03\mu Ci/cm^3$) was linear and rapid over the first 10 min. of the labelling period. The addition of unlabelled .....

FIGURE 27: COMPARISON OF ASSAY TECHNIQUES

a) Extraction of Entire Assay

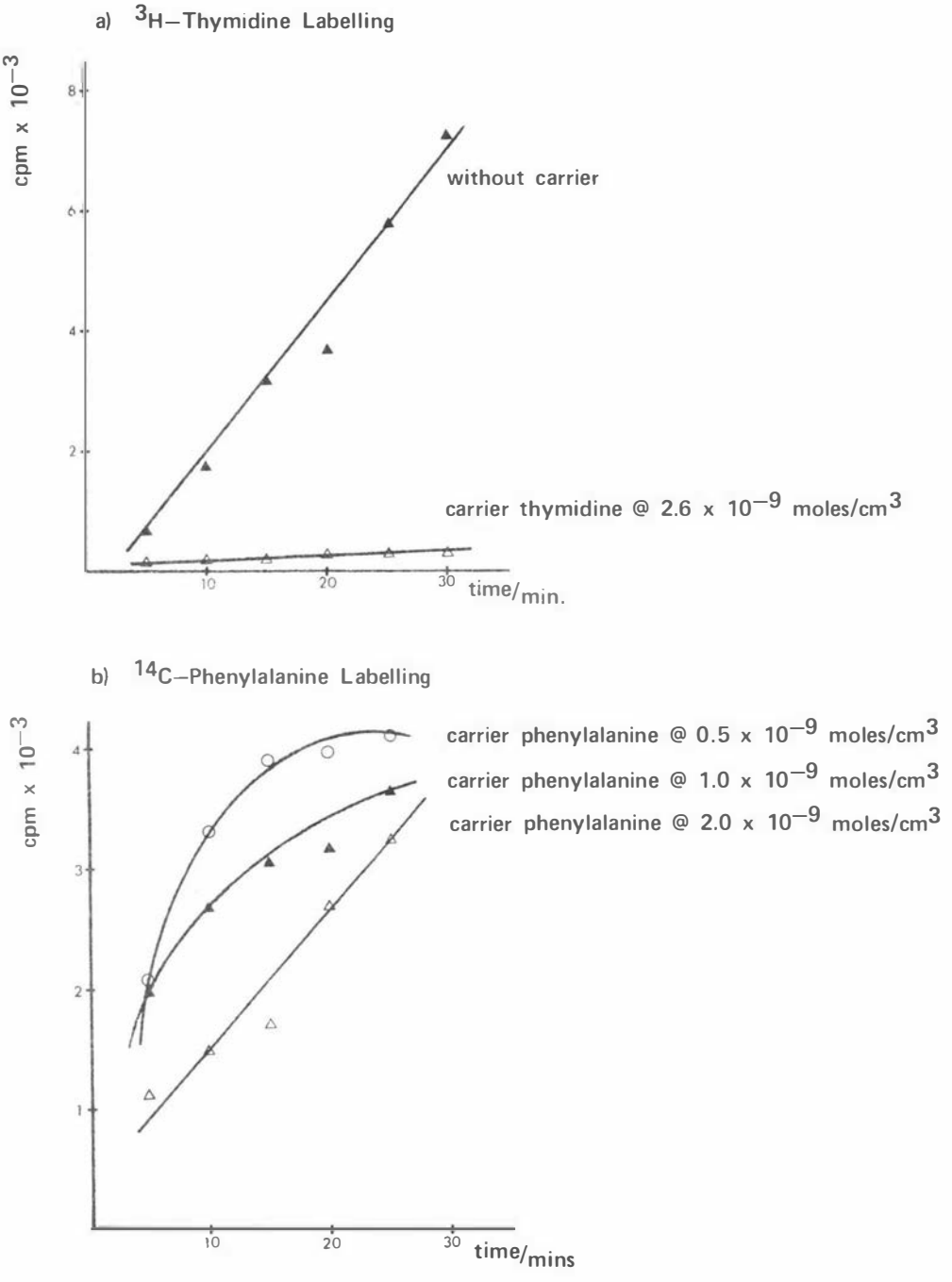


b) Withdrawal of lcm^3 Aliquots



Labelling aliquots of *B. Megaterium KM* culture.
(^{3}H–uridine @ 1 μCi/cm^3; carrier uridine @ 2.5 x 10^{-9}M; dothistromin @ 0.2 μg/cm^3)

FIGURE 28: PRELIMINARY LABELLING OF *B. MEGATERIUM KM.*
^{3}H–Thymidine and ^{14}C–Phenylalanine.



^{3}H–thymidine @ 25 μCi/assay
^{14}C–phenylalanine @ 0.25 μCi/assay

Table 13. Inhibition of ^{3}H-uridine incorporation by increasing concentrations of dothistromin.

[Dothistromin] $\mu g/cm^3$	Dothistromin : cell no. μg/cell X 10^8	Rate cpm	% Inhibition
CONTROL (0)	0	125	0
0.10	0.016	100	20
0.25	0.042	80	36
0.50	0.083	66	47
0.75	0.125	0	100
1.00	0.166	0	100

Standard aliquots of 1 cm^3 were withdrawn from 10 cm^3 assays at 5 min intervals over 20 min labelling period.

Where rate of incorporation was non-linear rates have been determined from a tangent to the curve over the 5-10 min period.

Assays contained ^{3}H-uridine at 1 $\mu Ci/cm^3$; carrier uridine at 0.25 X 10^{-9} moles/cm^3; dothistromin at the concentrations indicated.

phenylalanine (2.6×10^{-10} moles/cm^3) to the assay extended the period over which linear incorporation occurred.

3.43 Comparison of the Effects of Dothistromin and Antibiotics on Precursor Incorporation by B.negaterium KM

Synchronous culture experiments and labelling studies with Chlorella pyrenoidosa established that dothistromin inhibits RNA and protein synthesis. However, the nature of the experimental system used made it difficult to determine unequivocally the areas of metabolism affected or the time course of inhibition. The preliminary work with B.negaterium KM reported in this study showed that it was a more suitable organism for experiments of this nature. With it the effects of antibiotics on the incorporation of precursors into cell components were compared with those of dothistromin. The antibiotics used and their suggested modes of action were as follows:

Chloramphenicol is an inhibitor of protein synthesis in procaryotic systems, binding to the 50S subunit of the bacterial ribosome.

Actinomycin D binds with double-stranded helical DNA and interferes with DNA-dependent RNA synthesis.

Mitomycin C is an alkylating agent which binds covalently with the DNA molecule and preferentially inhibits DNA synthesis.

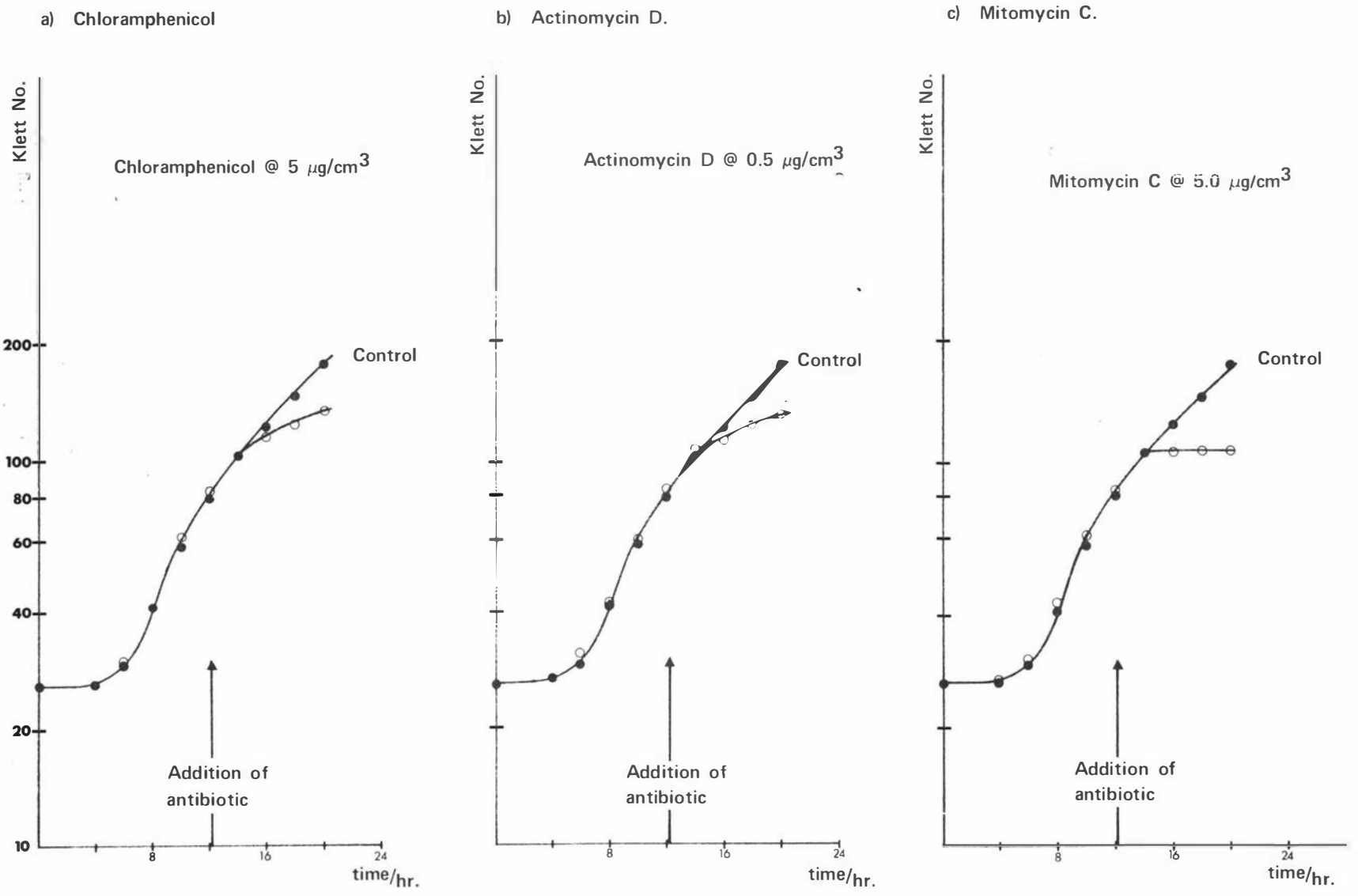
Inhibitory levels for each antibiotic were established by determining growth curves for B.megaterium cultured in the presence of each inhibitor (Fig.29). From labelling studies the effect of these compounds on precursor incorporation into RNA, DNA and protein was then established. The procedure used for incorporation studies is detailed in Experimental Methods, Section 2.64. Assay systems included: ^{3}H-uridine, 1 μCi/cm^{3}; carrier uridine, 0.25 X 10^{-9} moles/cm^{3}; ^{3}H-thymidine, 3.3 μCi/cm^{3}; ^{14}C-phenylalanine, 0.03 μCi/cm^{3}; carrier phenylalanine, 2.6 X 10^{-10} moles/cm^{3}. The results of these experiments are shown graphically in Figs. 30, 31 and 32. The scatter of points on these graphs results from inevitable losses incurred during the Schmidt-Thannhauser extraction procedure. As noted earlier (Section 2.65), the use of this procedure is necessary to ensure that the label incorporation measured is in fact incorporation into the appropriate macromolecular fraction.

i). Comparison of Chloramphenicol and Dothistromin

The effects of chloramphenicol and dothistromin on growth and on incorporation of ^{14}C-phenylalanine and ^{3}H-uridine were studied.

Chloramphenicol is known to be an inhibitor of protein synthesis in procaryotes and exerts its effects by binding to the 50S subunit of the 70S bacterial ribosome (Hahn, 1967). Addition of this antibiotic (5 μg/cm^{3}) to cultures of B.megaterium KM in the log-

FIGURE 29: GROWTH CURVES OF *B.MEGATERIUM KM* ESTABLISHING GROWTH INHIBITORY CONCENTRATIONS OF ANTIBIOTICS



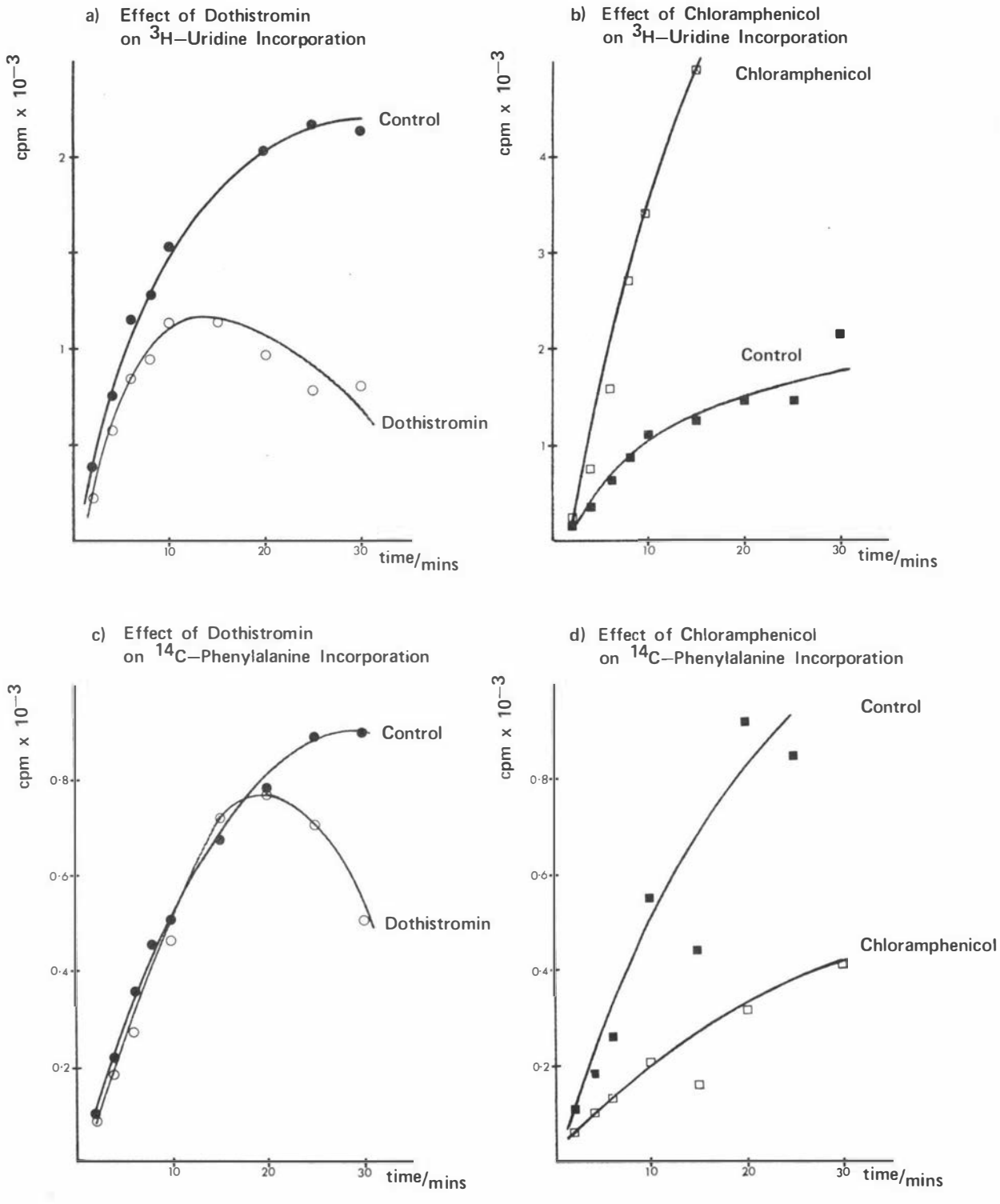
phase of growth resulted in growth inhibition which became obvious after approximately 4hr. exposure to the compound. Dothistromin, at the same stage of growth, caused a more rapid inhibition at 0.2 $\mu g/cm^3$ and very marked inhibition was observed at 1.0 $\mu g/cm^3$.

At a growth inhibitory concentration of 5 $\mu g/cm^3$, chloramphenicol inhibition of ^{14}C-phenylalanine incorporation into the protein fraction was marked in the 4 min. sample. (Fig.30d). The increased rate of incorporation of ^{3}H-uridine (Fig.30b) observed in the presence of chloramphenicol is also apparent in the 4 min sample. This apparent increase in the rate of RNA synthesis observed after administration of chloramphenicol is a well-documented effect of the antibiotic ascribed to the protection of ribosome-bound m RNA from degradation and the accumulation of r RNA precursors (Vazquez, 1966). In comparison, dothistromin at a growth inhibitory concentration (0.2 $\mu g/cm^3$) inhibits ^{3}H-uridine incorporation into the RNA fraction, significant inhibition being apparent in the 6 min sample (Fig.30a) but the inhibition of ^{14}C-phenylalanine incorporation is not evident until later (Fig.30c). These results would suggest that the growth inhibition observed for these two compounds is due to inhibition of different biosynthetic processes at different sites.

ii). Comparison of Actinomycin D and Dothistromin

The effects of actinomycin D and dothistromin on growth and on the incorporation of ^{3}H-uridine were studied.

FIGURE 30: EFFECTS OF DOTHISTROMIN AND CHLORAMPHENICOL ON PRECURSOR INCORPORATION



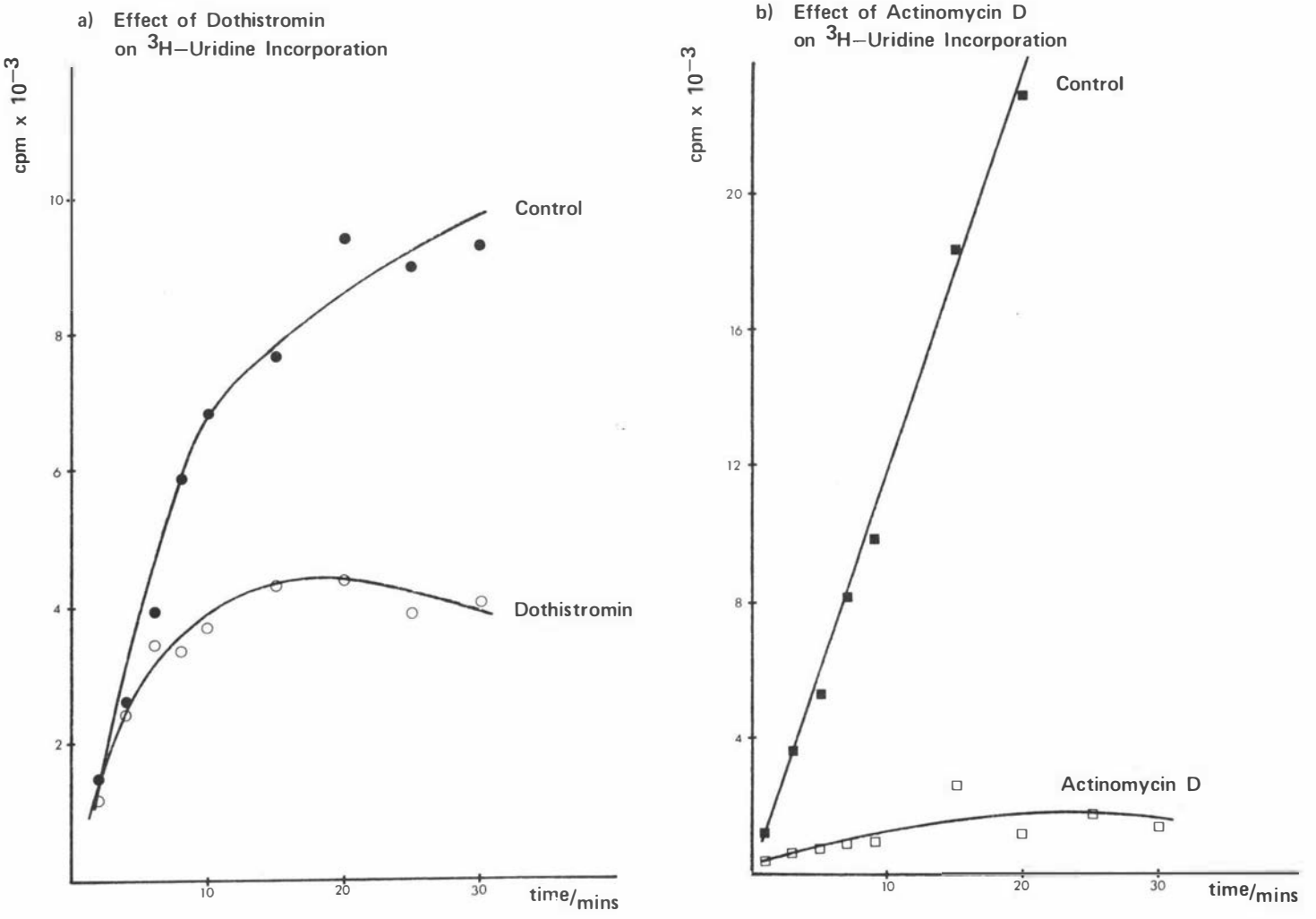
Labelling 15 cm^3 aliquots of *B.Megaterium KM* culture
(^{3}H–uridine @ 25 μCi/assay; carrier uridine @ 1 x 10^{-8} moles/assay; ^{14}C–phenylalanine @ 0.5 μCi/assay; carrier phenylalanine @ 4 x 10^{-9} moles/assay; dothistromin @ 0.2 $\mu g/cm^3$; chloramphenicol @ 5 $\mu g/cm^3$)

Actinomycin D is a cyclic, polypeptide-containing antibiotic which binds tightly to double-stranded, helical DNA. Studies of this binding in solution, by x-ray crystallography of the actinomycin-deoxyguanosine complex and by construction of models of the actinomycin-DNA complex, suggest that the cyclic actinomycin chromophore intercalates between the base pair d G - d C, with the peptide chains lying in the lesser groove of helical DNA and hydrogen bonding to deoxyguanosine residues of opposite chains (Sobell, 1973; see also Reich and Goldberg, 1964; Reich et al., 1967).

By addition of the antibiotic to cultures in the log-phase of growth, it was established that actinomycin D is growth inhibitory at 0.5 $\mu g/cm^3$ (Fig.29). This inhibition was more rapidly expressed when actinomycin D was added to give a final concentration of 1.0 $\mu g/cm^3$. Inhibition of growth at this concentration was apparent 3 hours after administration. At these same concentrations dothistromin inhibited growth of log-phase cultures within 2 hours (Fig. 34).

Actinomycin D (1.0 $\mu g/cm^3$) exerted a very rapid inhibitory effect on RNA synthesis as shown by the very low incorporation of ^{3}H-uridine in the presence of the antibiotic (Fig.31). The lower rate of label incorporation with actinomycin D already evident in the 2 min sample is very marked in the sample taken at 4 min. When dothistromin was added at a comparable concentration (1.0 $\mu g/cm^3$) the resultant

FIGURE 31: EFFECT OF DOTHISTROMIN AND ACTINOMYCIN D ON PRECURSOR INCORPORATION



Labelling 15 cm^3 aliquots of *B.Megaterium KM* culture.
(^{3}H–uridine @ 25 μCi/assay; carrier uridine @ 1 x 10^{-8} moles/assay;
dothistromin @ 1.0 $\mu g/cm^3$; actinomycin D @ 1.0 $\mu g/cm^3$)

inhibition of ^{3}H-uridine incorporation was not as rapid (Fig.31). The degree of inhibition in the 10 min sample at this concentration of dothistromin was greater than when the toxin was administered at 0.2 $\mu g/cm^3$. This suggests that the delayed inhibition by dothistromin may be due to its slow penetration into the cell. The effects of actinomycin D on the incorporation of ^{14}C-phenylalanine and ^{3}H-thymidine in this experimental system were not investigated.

iii). Comparison of Mitomycin C and Dothistromin

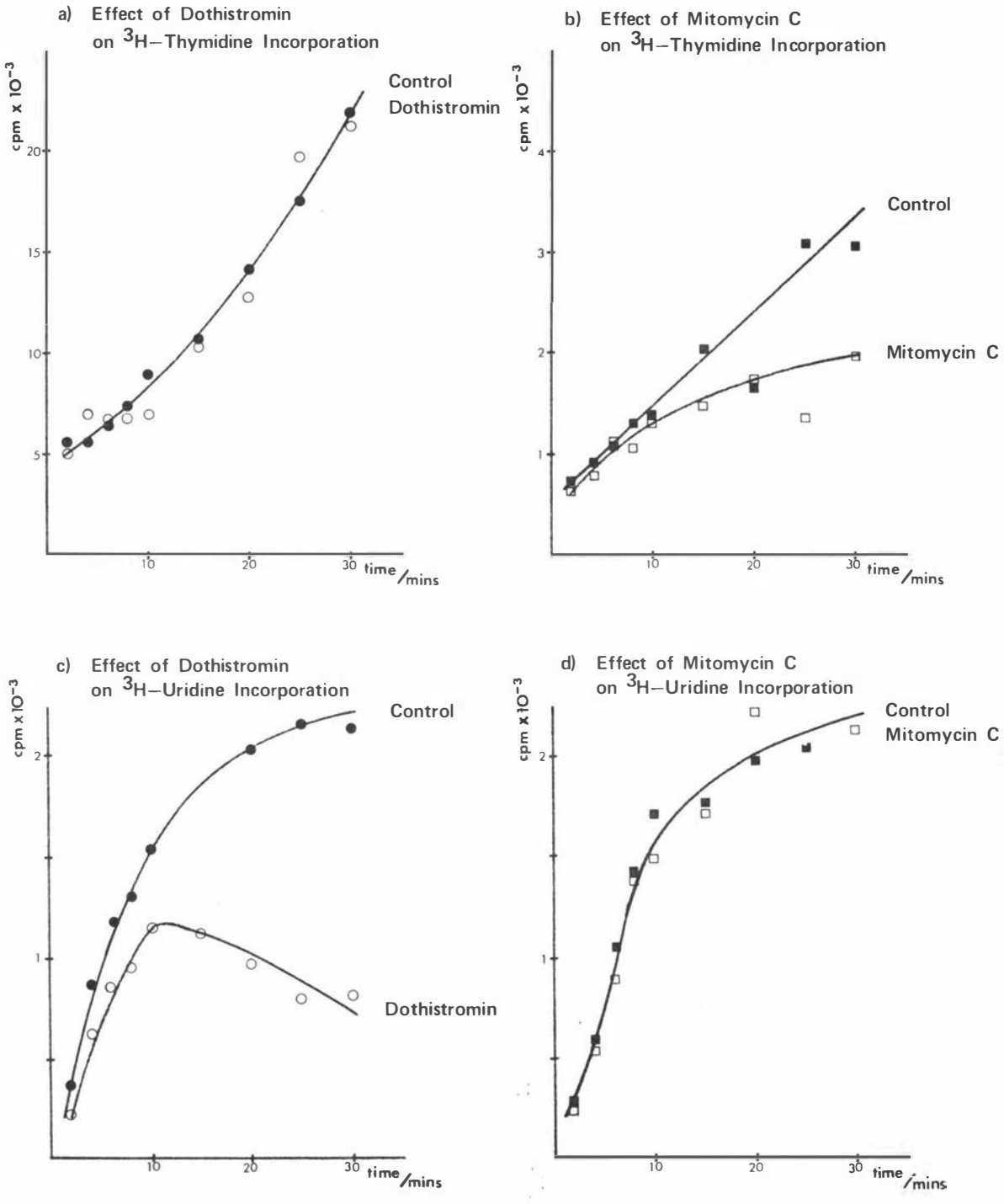
Growth inhibitions and incorporation studies with ^{3}H-thymidine and ^{3}H-uridine were carried out in the presence of mitomycin C and dothistromin.

Mitomycin C is reduced *in vivo* to form an active bifunctional alkylating agent which preferentially inhibits the DNA-dependent synthesis of DNA, binding covalently with the DNA molecule and causing irreversible changes (Szybalski and Iyer, 1967; see also Kersten and Kersten, 1969). Inhibition of RNA synthesis and of enzyme induction occur much later than the mitomycin-induced suppression of DNA synthesis and are considered to be secondary effects related to alterations of DNA structure caused by mitomycin.

From growth inhibition studies, it was shown that mitomycin at 5 $\mu g/cm^3$ is an effective inhibitor. The inhibitory effect was clear 3 hours after addition of the antibiotic to log-phase cultures of *B.megaterium KM* (Fig.29).

A comparison of the effects of mitomycin and dothistromin on

FIGURE 32: EFFECT OF DOTHISTROMIN AND MITOMYCIN C ON PRECURSOR INCORPORATION



Labelling 15 cm^3 aliquots of *B.Megaterium KM* culture.
(^{3}H–uridine @ 25 μCi/assay; carrier uridine @ 1 x 10^{-8} moles/assay; ^{3}H–thymidine @ 50 μCi/assay; dothistromin @ 0.2 $\mu g/cm^3$; mitomycin C @ 5 $\mu g/cm^3$)

the incorporation of labelled precursors is shown in Fig.32. Mitomycin C at 5 $\mu g/cm^3$ effectively inhibited the incorporation of ^{3}H-thymidine into the DNA fraction after 15 min exposure to the antibiotic. At this same concentration the compound had no significant effect on ^{3}H-uridine incorporation. Dothistromin, on the other hand, has very little effect on ^{3}H-thymidine incorporation but is an effective inhibitor of ^{3}H-uridine incorporation. Thus it can be said that different mechanisms are involved in growth inhibition by these two substances.

From these comparative studies, it is apparent that the site of dothistromin inhibition in this system differs from those of chloramphenicol and mitomycin C. The effects of dothistromin are first evident on the incorporation of ^{3}H-uridine. This inhibition, at about 10 min, is followed later by inhibition of ^{14}C-phenylalanine incorporation into the protein fraction. This would suggest that the primary site of dothistromin is at the transcriptional level, impairing the synthesis of RNA. The effect of dothistromin on ^{3}H-uridine incorporation is similar to that observed for actinomycin D, a known RNA synthesis inhibitor.

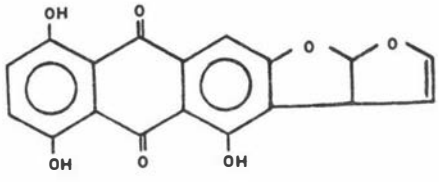
In the presence of dothistromin the counts incorporated into both the protein and RNA fractions decrease with time, in marked contrast to the counts incorporated in the presence of the other inhibitors. This could indicate that these macromolecules are disintegrating and is an area for further investigation.

3.44 Comparison of the effects of dothistromin with those of related compounds

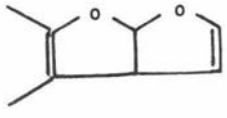
The structural relationship between dothistromin and the fungal toxins, aflatoxin and sterigmatocystin, was discussed in the introductory section of this thesis (Section 1.22). The structures of these metabolites are given in Figs 2 and 3. These are all heterocyclic compounds fused to a bifuran ring moiety and it has been suggested that the unsaturated bifuran system may be related to the toxicity observed. The aflatoxin B_1 molecule contains the unsaturated dihydrobifuran ring system shown in Fig.33. This same unsaturated moiety is present in sterigmatocystin and in the dothistromin derivative shown in Fig.33. Dothistromin itself contains the saturated hemiacetal derivative of the bifuran moiety; this feature appears to nullify toxicity in the aflatoxin group of fungal metabolites.

In an attempt to define possible mechanisms of action related to structure, the four fungal metabolites aflatoxin B_1, sterigmatocystin, dothistromin and its vinyl-ether derivative, were added in ethyl-acetate solution to cultures of B.megaterium KM in the log-phase of growth to establish the growth inhibitory concentrations of each. The resulting growth curves are given in Fig.34. Aflatoxin B_1 was growth inhibitory at $5\,\mu g/cm^3$, with inhibition evident 3 hours after the addition of the toxin to the culture. Sterigmatocystin had very little effect on growth at $5.0\,\mu g/cm^3$ or $10.0\,\mu g/cm^3$. The dothistromin derivative

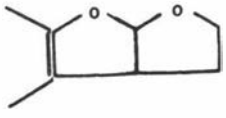
FIGURE 33: STRUCTURES OF FUNGAL METABOLITES



I Dothistromin Derivative
(vinyl ether)

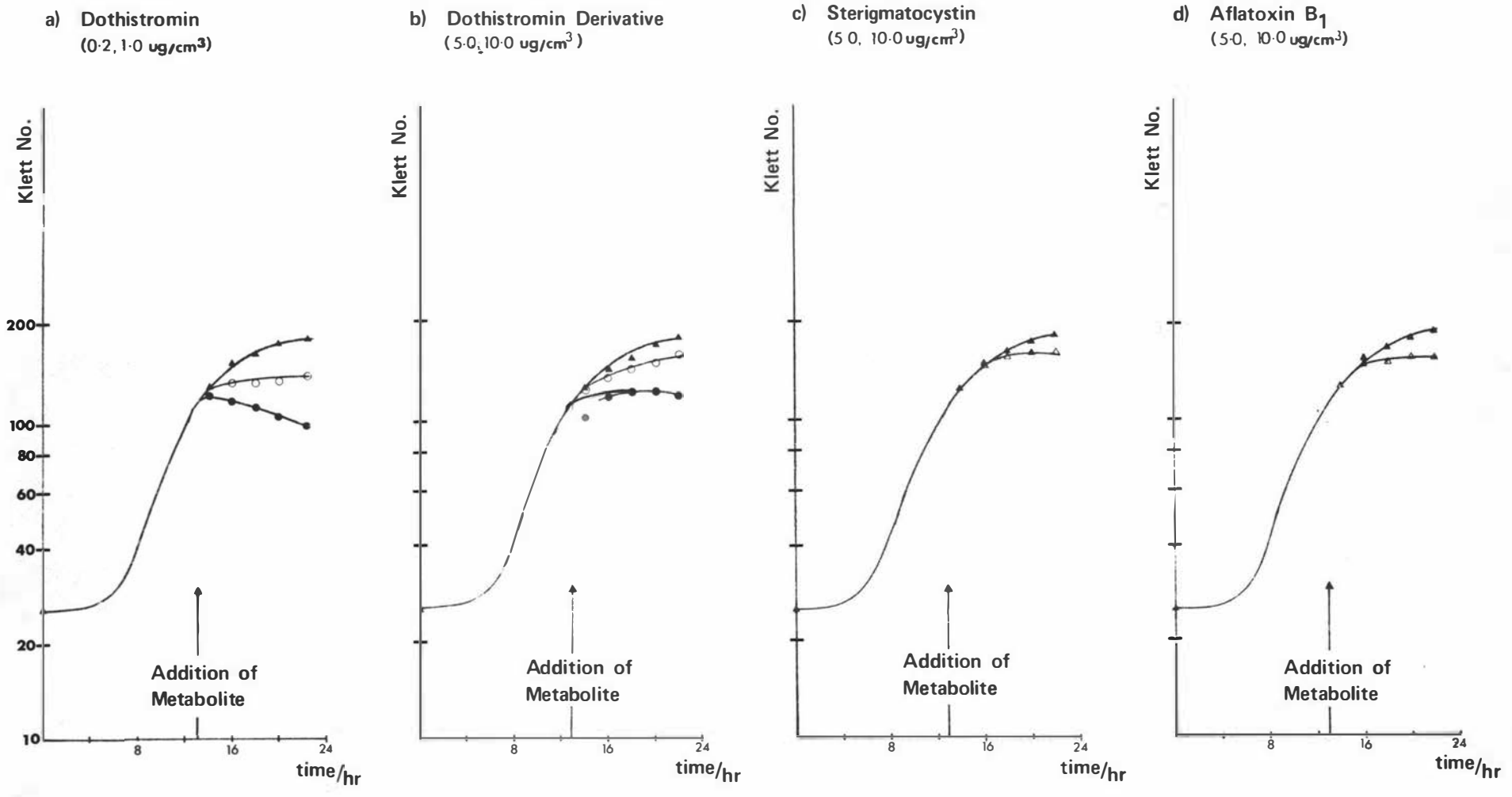


II Dihydrofuro [2,3–b] benzofuran



III Tetrahydrofurobenzofuran

FIGURE 34: GROWTH CURVES OF *B.MEGATERIUM KM* ESTABLISHING GROWTH INHIBITORY CONCENTRATIONS OF FUNGAL METABOLITES
Metabolites added as ethyl acetate solutions during the log-phase of growth

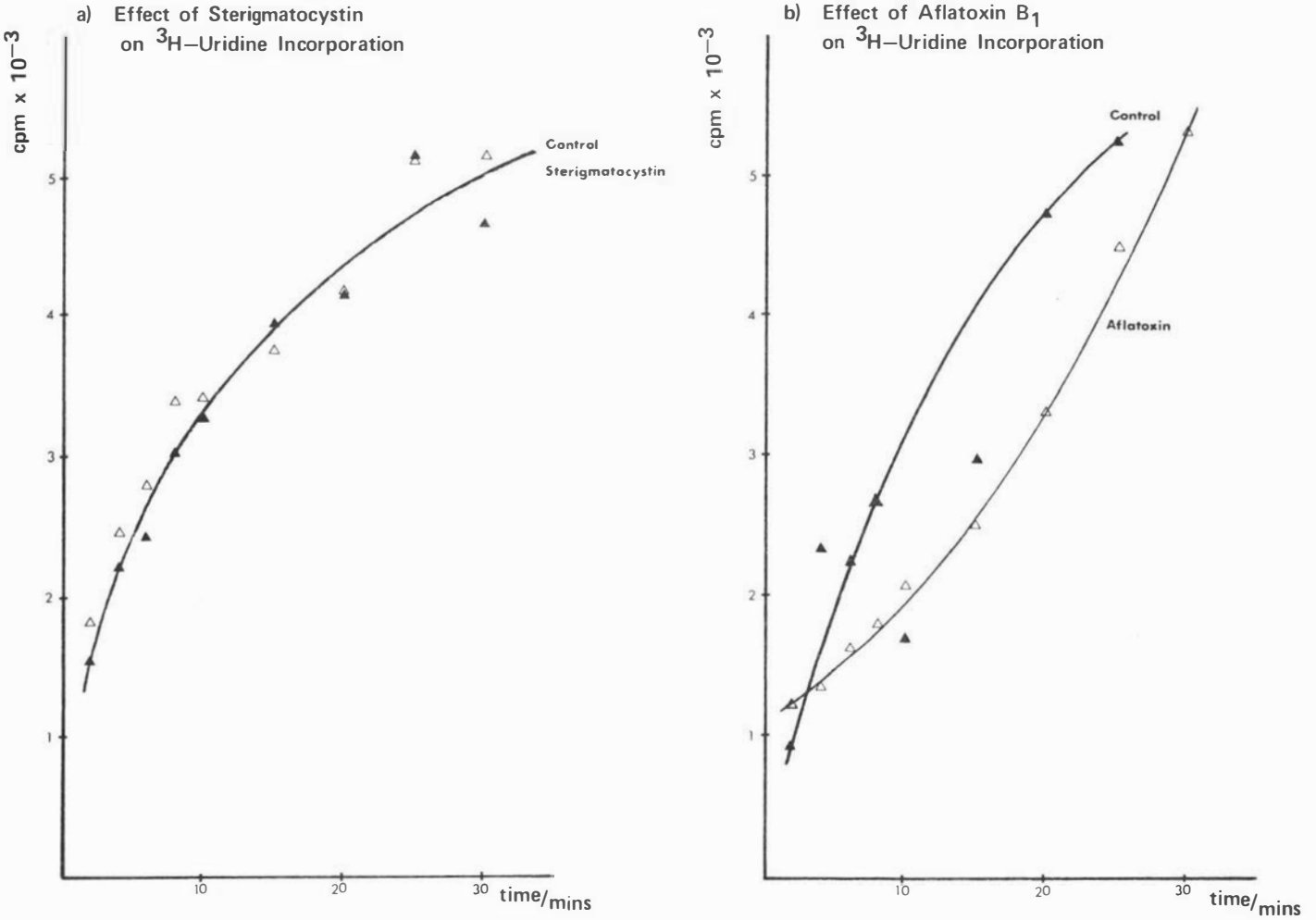


induced growth inhibition at 5.0 $\mu g/cm^3$, a level 10 times higher than that of dothistromin itself. In the same experimental system dothistromin is inhibitory at 0.2 $\mu g/cm^3$. These differences in inhibitory levels may be related to low solubilities of these compounds in aqueous solutions.

The effects of aflatoxin B_1 and of sterigmatocystin on ^{3}H-uridine incorporation were investigated in the labelling assay as previously described. The results obtained are shown in Fig.35. At a final concentration of 5.0 $\mu g/cm^3$, a concentration shown to be growth inhibitory, aflatoxin B_1 inhibited incorporation of ^{3}H-uridine into the RNA fraction over the first 10 min but the subsequent rate of incorporation was similar to that of the control. Sterigmatocystin had no effect on ^{3}H-uridine incorporation in this experimental system. Under these conditions dothistromin, at 0.2 $\mu g/cm^3$, markedly inhibits incorporation of this labelled precursor into the RNA fraction (Fig.35b).

However, although the incorporation of ^{3}H-uridine appears to be much less sensitive to these other mycotoxins than it is to dothistromin, the compounds have low solubilities in aqueous solution and no study was made of the actual amount of each compound in solution when added to the cultures as ethyl acetate solutions. Further experiments would be required before comparative effects could be defined in more detail.

FIGURE 35: EFFECT OF AFLATOXIN AND STERIGMATOCYSTIN ON PRECURSOR INCORPORATION



Labelling 15 cm^3 aliquots of *B.Megaterium KM* culture.
(^{3}H–uridine @ 25 μCi/assay; carrier uridine @ 1 x 10^{-8} moles/assay; sterigmatocystin @ 10 $\mu g/cm^3$; aflatoxin B_1 @ 5 $\mu g/cm^3$)

4. DISCUSSION AND CONCLUSIONS

This thesis was concerned with an investigation of the toxic effects of dothistromin on cell growth and metabolism in order to assess the possible involvement of this compound in dothistrominal blight of the Pinus spp. Dothistromin was isolated and chemically characterized by Bassett et al. (1970) and its molecular structure shown to be that given in Fig.1 (I). A bioassay utilizing Chlorella pyrenoidosa had been used to follow the toxic principle throughout the isolation and purification of the compound. This growth inhibitory effect on Chlorella was all that was known of the toxicity of the compound at the outset of the present investigation. The striking structural similarity of dothistromin to the known mycotoxins, aflatoxin and sterigmatocystin, suggested that these compounds may well act in a similar way. The research effort that has been devoted to the study of the aflatoxin group of fungal metabolites has provided useful guidelines in investigating the possible mode of action of dothistromin.

4.1 GROWTH INHIBITION OF MICRO-ORGANISMS

Although this investigation was aimed at assessing the possible involvement of dothistromin as a phytotoxic factor in dothistromal blight of Pinus spp., the experimental work was concerned with studying the effects of the compound in micro-organisms. Direct

extrapolation from results obtained in this way to events which may be occurring in the pine is not valid and has not been attempted. However the use of pine material (seedlings and/or tissue cultures) for experiments of this type poses many problems since one is dealing with a complex system about which little is known. By using simpler and better documented experimental systems, this research has established the areas of microbial metabolism affected by dothistromin and should provide the basis of further work in pine seedlings or tissue cultures.

4.11 Agar-Plate Bioassay

This investigation revealed that dothistromin inhibited the growth of a range of micro-organisms, both procaryotic and eucaryotic, indicating that the compound can function as a general growth inhibitor and is not specifically inhibitory to any one class of micro-organism. However, the agar-plate bioassay method used in these studies showed that among the micro-organisms tested there was a differential sensitivity to the toxin, with gram-positive bacteria, in general, being more sensitive than those which were gram-negative. Similar differential responses have been noted for other inhibitory compounds, for example the quinones (Webb, 1966) and aflatoxin (Burmeister and Hesseltine, 1966). The

agar-plate bioassay indicated that, of the micro-organisms studied, Chlorella pyrenoidosa was apparently the most sensitive, showing the largest zones of inhibition. However, as noted in Section 3.1,, caution must be exercised in making comparisons between different organisms based on results obtained in this type of bioassay system. The size of the zones observed will depend not only on the organism and its susceptibility to the toxin, but also on the growth rate of the organism, the rate of diffusion of the toxin and its stability in the medium, factors which in turn will be affected by the physical conditions required by the organism for growth. For these reasons it is difficult to make comparisons with published work on other mycotoxins. However it is of interest to note that Clements (1968), in developing a rapid agar-plate method of bioassay for aflatoxin, found that Bacillus megaterium (NRRL-B-1368) was sensitive to as little as 1 μg aflatoxin B_1 applied to the agar on an antibiotic disc (diam. 6mm). In agar-plate bioassays for dothistromin, B.megaterium KM was found to be inhibited by 25 μg of the toxin. Since the dothistromin was applied on a disc of diam. 13 mm the toxin would have been effectively diluted over an area more than four times as large as that used by Clements. Sullivan and Ikawa (1972) surveyed the growth inhibition of five strains of Chlorella by mycotoxins and found that the four strains of Chlorella pyrenoidosa examined were sensitive to aflatoxin B_1 at 20 μg/6mm diam. antibiotic disc. Thus dothistromin shows an apparent

toxicity in agar-plate bioassay over a similar range of concentrations as that reported for the aflatoxins. Investigation of sterigmatocystin toxicity using microbiological assays showed that the compound when added to the assays at 100 μg/antibiotic disc had no apparent effect on the growth of the micro-organisms studied (Lillehoj and Ciegler, 1968).

4.12 Inhibition of Micro-organisms in liquid cultures

The study of the effects of dothistromin on *Chlorella* grown in liquid cultures showed that the toxin was effective in inhibiting growth at concentrations of the order of 1 μg/cm^3. However the ratio of concentration in the medium to the amount of cell material in the culture is a more useful basis for comparing the sensitivities of different organisms. Growth inhibition occurred at a dothistromin concentration : cell number ratio of the order of 2.0 μg/cell X 10^8 (2.0 X 10^{-14} g/cell) for *Chlorella*. It was found that a value of the order of 750 μg/cell X 10^8 was required to inhibit the growth of yeast (*Saccharomyces cerevisiae*) in liquid culture, whereas the growth of the variant strain of *B.negaterium* KM, under similar conditions, was effectively inhibited at a ratio of 0.25 μg/cell X 10^8. Thus, in liquid culture, *B.negaterium* KM is seen to be considerably more sensitive to dothistromin than *Chlorella*.

There is little information on inhibitory levels of related mycotoxins since much of the work on the biological activity of these compounds against micro-organisms has used agar-plate techniques. However, aflatoxin B_1 at 3.8 $\mu g/cm^3$ resulted in a decreased growth rate for B.megaterium (NRRL-B-1368), giving a generation time of 75 min as compared with 20 min for the control culture, and produced morphologically abnormal forms (Beauchat and Lechowich, 1971a, 1971b); and the growth of E.Coli in medium containing aflatoxin B_1 at 5 $\mu g/cm^3$ produced aberrant filamentous cells (Wragg et al., 1967). The concentrations of dothistromin found to be necessary to produce growth inhibition in liquid culture are in the same range as these reported aflatoxin concentrations although, in the absence of data on the ratio of toxin concentration : cell number for other mycotoxins, it is difficult to compare the results of different workers.

A preliminary investigation was made of the growth inhibitory concentrations of aflatoxin B_1 and sterigmatocystin for B.megaterium KM. Aflatoxin B_1 was growth inhibitory at a toxin concentration : cell number ratio of the order of 2.0 μg/cell X 10^8 and, at a similar ratio, sterigmatocystin had very little effect. Under the same conditions dothistromin was growth inhibitory at 0.2 μg/cell X 10^8. However, no study was made of the effectiveness of the methods used to add these two fungal metabolites to aqueous solutions. Since

both compounds have very low solubilities in aqueous solutions, it is possible that the effective concentration achieved in each case was less than the calculated concentration.

In liquid culture, dothistromin appeared to result in a lag in growth followed by recovery and resumption of a growth rate comparable to that of the control. This lag-recovery phenomenon was particularly noticeable with liquid cultures of Chlorella. This may mean that the toxin is metabolized by the cell and that pathways for detoxification exist, or at least in the case of Chlorella, it may result from light-induced breakdown of the compound. The more usual response observed with a growth inhibitor is a concentration-dependent decrease in growth rate.

The low solubility of dothistromin in aqueous solutions was a problem that had to be circumvented before the toxic effects of the compound could be studied in liquid culture. Any aqueous solutions prepared were not stable, a precipitate forming on standing, and this would indicate that the 'solutions' obtained were in fact very fine suspensions of precipitated dothistromin. To introduce reproducible and known amounts of dothistromin into liquid cultures, the compound was added in ethyl acetate from stock solutions (usually of 1 ng dothistromin/cm^3). Ethyl acetate itself showed inhibitory effects on growth if final concentrations of greater than 0.6% (v/v) were reached. However, at the levels of dothistromin required for inhibition it was

possible to keep the ethyl acetate concentrations at sub-inhibitory levels. Any dothistromin-induced inhibition observed was always compared with the appropriate ethyl acetate control assay. However, the toxicity of most organic solvents to biological systems restricts the range of dothistromin concentrations over which inhibitory effects can be studied and, since solvent tolerance will vary amongst experimental organisms, it is not a generally applicable method of introducing the toxin. Obviously it would be invaluable if an alternative method were available. It may well be possible to prepare derivatives of dothistromin which are more water-soluble without altering the toxic nature of the molecule. A preliminary investigation of the formation of a magnesium chelate complex of dothistromin indicated that this complex is much more soluble in aqueous solutions than the parent compound (Gallagher, 1973). The biological activity of this complex, as such, has not been investigated but it may well be formed in liquid cultures containing Mg^{++} as a nutrient.

4.13 Implications for potential quantitative bioassay

Although the development of a quantitative bioassay for dothistromin was not an intentional part of the present study, some of the observations made in the course of investigating the effects of this compound on the growth of micro-organisms are of relevance to any such attempt.

Further investigation of methods of applying the toxin to assay plates could improve the sensitivity of the agar-plate bioassay. The filter paper antibiotic-discs appear to adsorb a percentage of the toxin applied and results obtained in this investigation indicate that a method of applying the material directly to the agar surface would be feasible and would improve the sensitivity. The inhibition of growth of micro-organisms in liquid culture could well form the basis of a quantitative bioassay. The dothistromin concentration : cell number ratios derived from growth studies using liquid cultures proved to be a useful index for predicting inhibitory concentrations of the toxin and a satisfactory means of comparing the relative susceptibilities of micro-organisms to dothistromin-induced inhibition.

The liquid-culture type of bioassay appears to be more sensitive. However, the simplicity of the agar-plate technique readily allows for the screening of a number of micro-organisms or for the parallel bioassay of a large number of samples. The lag-recovery response to dothistromin observed with Chlorella in liquid culture may prove difficult to assess quantitatively since the duration of the lag period may not be directly proportional to toxin concentration and may be affected by the light-induced breakdown of the toxin. The instability of dothistromin in light was suggested by results obtained from a preliminary fluorimetric

investigation of the agar-plate bioassay system. Nothing is known of the possible photochemical-breakdown products and of the ways in which these could affect the overall toxicity of dothistromin. Since the fluorescence of the 'dothistromin agars' decreased with increasing time of exposure to light, presumably some alteration of the anthraquinoidal nucleus occurs on irradiation, as this moiety is largely responsible for the observed fluorescence of the toxin. For this reason, it is suggested that non-photosynthetic organisms be used should a quantitative bioassay be required.

4.2 CELLULAR SITE OF DOTHISTROMIN INHIBITION

There are numerous sites in the cell at which growth inhibitory compounds can act and, of these multiple sites, there may well be more than one through which the primary toxic effect of the compound could be expressed (Gottlieb, 1972). Thus attempting to define the cellular site of action of an inhibitor becomes a complex study and the areas of metabolism possibly affected by dothistromin cover the spectrum of cellular processes. Quinones and quinoidal derivatives are known to inhibit respiration, by interfering with terminal electron transport, and also to alter the energy transfer mechanisms involved in respiration and photosynthesis (Webb, 1966). Growth inhibitors are also known to act on the area of protein and nucleic acid synthesis and, since the structurally similar aflatoxins

act primarily in this area (Detroy et al., 1971), it was possible that this could be a site of dothistromin action too. Polyphenolic compounds are known to alter the permeability of the cell membrane, but dothistromin could also have been interfering with other metabolic functions.

A study of the effects of dothistromin on the cell cycle using synchronous culture techniques narrowed the possibilities to be investigated. Synchronous cultures are those in which the processes of growth and cellular division are temporarily separated as functions of the majority of the cultural population and hence can be used to represent metabolic events occurring as in a single cell. For these studies Chlorella pyrenoidosa was used as an experimental organism because it is photosynthetic and eucaryotic and therefore more closely allied to a Pinus system than a procaryotic system would be.

4.21 Site of Inhibition in Chlorella Pyrenoidosa.

By using synchronous cultures of Chlorella it was possible to determine the effects of the toxin on specific aspects of the growth process. Dothistromin, when added to synchronous cultures at the beginning of the cell cycle, was found to effectively inhibit the cell division of a large proportion of the algal

population. Cell size distributions revealed that, in the presence of the toxin, a significant percentage of the population remained as 'small' cells (of diam. < 3.75). This suggested that the toxin was affecting synthetic processes important to the growth of the organism, indirectly resulting in the observed inhibition of cellular division. The toxin was found to inhibit the rate of increase of total protein and RNA from an early stage of the cell cycle, while the levels of DNA were not affected until later.

However the analytical methods used to follow the changes in total levels of the macromolecular constituents were not sensitive enough to permit study of the short-term effects of the toxin unless vast amounts of cell material were used. The use of radio-active precursors of protein and nucleic acids allowed investigation of the short-term effects of dothistromin on various biosynthetic processes in Chlorella. The results obtained were consistent with those observed by following changes in the total levels of macromolecular constituents.

Isotope incorporation studies with Chlorella clearly showed that RNA synthesis, and later protein synthesis, was inhibited but it was not possible to show whether DNA synthesis was inhibited or not. The incorporation of ^{3}H-thymidine was very low and investigation of the distribution of precursor label in fractions derived by the

Schmidt-Thannhauser extraction procedure showed that only a very small percentage of the ^{3}H incorporated was in the DNA fraction. Wanka et al. (1970) reported that Chlorella lacks thymidine kinase activity and would therefore be unable to utilise exogenous thymidine as a DNA precursor. Although alternative methods of labelling Chlorella DNA were sought, adequate precursor incorporation into this macromolecular fraction was not achieved and the suitability of other organisms was investigated.

Saccharomyces cerevisiae was tested as a possible alternative eucaryotic organism but proved to be unsuitable due to its low sensitivity to dothistromin. The rates of thymidine incorporation into yeast DNA were no higher than those observed for Chlorella.

4.22 Site of inhibition in Bacillus negaterium

The high sensitivity of B.negaterium to both dothistromin and aflatoxin, and also the apparent availability of a thymine-requiring strain of this organism, suggested that it would be a more suitable organism for determining the primary site of dothistromin inhibition of synthetic processes. Although a thymine requirement could not be demonstrated for the variant strain of B.negaterium KM obtained, it proved to be a very suitable experimental organism in all other respects and therefore was used for all subsequent work.

4.221 Validity of precursor approach

Investigation of the effect of dothistromin on the synthesis of macromolecules in B.megaterium KM was carried out by following the incorporation of labelled precursors into fractions derived by the Schmidt-Thannhauser extraction procedure. This technique was used in preference to simply following isotope incorporation into cell residues with no attempt to characterise the component into which the isotope was incorporated because it was important that valid and adequate measures of biosynthetic activities were employed.

The Schmidt-Thannhauser extraction procedure is recommended as having the best theoretical and practical basis for precise estimation of nucleic acids (Munro and Fleck, 1966). However, investigation of the distribution of each precursor label in the Schmidt-Thannhauser fractions obtained in the present study suggested that complete separation of RNA, DNA and protein was not achieved. The bulk of the RNA precursor label was obtained in the supernatant fraction after alkaline hydrolysis overnight at 30°. This fraction also contained some of the protein precursor label (^{14}C phenylalanine), presumably label incorporated into low molecular-weight material which is alkali-soluble. In addition, some of the ^{3}H-thymidine label occurred in the alkaline

hydrolysate. Although DNA is stable in alkali under the conditi ns used, hydrolysis can occur if any apurinic acid has been formed in earlier steps of the extraction and this may well have occurred during hot ethanol extractions.

Each fraction was examined further to characterise the component which contained the isotope to ensure that the radioactivity in the fraction was indeed due to incorporation of the precursor molecule into the macromolecular fraction. Hydrolysates of each Schmidt-Thannhauser fraction were resolved by chromatography or electrophoresis, the radioactivity located and each spot identified by comparison with known standards. Only one radioactive peak was found for the protein and DNA hydrolysates, occurring in the regions of phenylalanine and thymidine respectively. The RNA hydrolysates showed the majority of the radioactivity in the position of UMP and also a peak occurring in the region of CMP.

Investigation of the Schmidt-Thannhauser fractions and resolution of the hydrolysates of these fractions did not unequivocally establish that the incorporation of each labelled precursor was a specific measure of synthesis. To establish the validity of this experimental approach, the effects of antibiotics with known sites of action on precursor incorporation into each Schmidt-Thannhauser fraction were determined. If the incorporation of labelled precursor was a valid measure of synthetic activity, then

it would be subject to inhibition by these antibiotics; e.g. if mitomycin completely and specifically inhibited thymidine incorporation then this would indicate that the incorporation of thymidine was only measuring DNA synthesis in spite of doubts arising from the Schmidt-Thannhauser distributions. The purpose of using antibiotics was twofold; firstly to establish the validity of the labelled precursor experimental approach, and secondly to compare the inhibition of incorporation observed with each antibiotic with that observed for dothistromin to define possible sites for dothistromin action.

Chloramphenicol, which inhibits protein synthes in procaryotic systems by binding to the 50S subunit of the ribosomes, inhibited the incorporation of ^{14}C-phenylalanine into the protein residue. Chloramphenicol treatment also resulted in an apparent increase in ^{3}H-uridine incorporation into the RNA-containing fraction. This is a well-documented secondary effect of this antibiotic (Vazquez, 1966). Actinomycin D, an RNA synthesis inhibitor which acts by binding to double-stranded DNA and thereby interfering with its function as a template for transcription, inhibited ^{3}H-uridine incorporation. Mitomycin C preferentially inhibits DNA synthesis, acting as a bifunctional alkylating agent which binds covalently with the DNA molecule. This antibiotic effectively inhibited ^{3}H-thymidine incorporation in B.negaterium KM but had no effect on the incorporation of ^{3}H-uridine.

Thus, this series of experiments utilizing known antibiotics showed that the incorporation of labelled precursors into each macromolecular fraction could be inhibited specifically and thus was in fact a valid measure of synthetic activity.

4.222 Dothistromin inhibition of precursor incorporation

Dothistromin rapidly inhibited the incorporation of ^{3}H-uridine into the RNA-containing fraction of B.negaterium KM, and over a longer time-course inhibited the incorporation of ^{14}C-phenylalanine into the protein fraction. ^{3}H-Thymidine incorporation was not affected over the 25 min time-course studied. Thus the effect of dothistromin on labelled precursor incorporation in this system was similar to that of actinomycin D, although dothistromin inhibition was not quite as rapidly expressed, possibly due to differences in the rate of uptake of the compounds by the cells. The early inhibition of ^{3}H-uridine incorporation in the presence of dothistromin would suggest that the toxin exerts its inhibitory effect by interfering with the process of transcription. This proposal would account for the time-course observed for dothistromin -induced inhibition of ^{14}C-phenylalanine incorporation into the protein fraction, since inhibition of protein synthesis would be one of the more immediate secondary effects. In synchronous cultures of Chlorella, dothistromin caused a decrease in the rates

of accumulation of RNA and protein early in the cell cycle. Any inhibition of these synthetic processes would lead to the cessation of growth and cell division observed in this experimental system, and to the later inhibition of increase in total DNA levels also observed. All of these results are consistent with the proposed site of action for dothistromin but further investigation is required before the mechanisms involved can be elucidated.

4.23 Mode of action of structurally-related mycotoxins

Understanding of the site and mode of action of an inhibitor can be furthered by comparison with structurally-related compounds. It has been pointed out that dothistromin bears structural similarity to a group of mycotoxins which includes aflatoxin and sterigmatocystin. For comparison, a consideration of the action of aflatoxin has been included.

Investigation of the early effects of the aflatoxins, in particular aflatoxin B_1, has been carried out in various experimental systems but, since aflatoxin is known to be a potent hepatocarcinogen, much of the work has been done with regenerating rat liver tissue. Thus aflatoxin B_1 has been shown to suppress DNA synthesis *in vivo* (Laforge and Frayssinet, 1970; Rogers and Newberne, 1967) and *in vitro* (Legator, 1966; Wragg *et al.*, 1967),

and to inhibit RNA synthesis *in vivo* (Clifford *et al.*, 1967; Harley *et al.*, 1969; Pong and Wogan, 1970) and in some *in vitro* systems (Clifford and Rees, 1966, 1967; Moule and Frayssinet, 1968). Inhibition of protein synthesis has been reported in many systems (Clifford and Rees, 1966, 1967; Smith, 1964; Wogan and Friedman, 1968; Lillehoj and Ziegler, 1970). Comparison with the effects of actinomycin D suggests that aflatoxin may act through a similar mechanism i.e. by binding to DNA and impairing the template functions. Differences observed can be partly accounted for by non-identical sites for binding to the macromolecule and weaker binding forces (Detroy *et al.*, 1971). Investigation of the RNA polymerase reaction from several sources suggested that aflatoxin acts on the enzyme-template complex or on the template rather than directly on the enzyme itself (Edwards and Wogan, 1970). Evidence available to date would suggest that the toxic effects of aflatoxin result from its interaction with DNA, producing the inhibition of macromolecular syntheses as observed. Information on this interaction has been obtained from *in vitro* binding studies (Sporn *et al.*, 1966; Clifford *et al.*, 1967; Neely *et al.*, 1970; Schabort, 1971).

The experimental approaches used in this study of dothistromin toxicity have been employed in investigating the mode of action of aflatoxin. Lafarge and Frayssinet (1970) followed the incorporation of labelled precursors to study the short-term biochemical effects

of aflatoxin on nucleic acid synthesis in regenerating rat liver tissue. Aflatoxin was administered at sub-acute levels in contrast to many other studies in which the toxin has been used at acutely toxic doses leading to massive and rapid inhibition of all metabolic processes. These workers found that the incorporation of 6 - ^{14}C - orotic acid into nucleolar RNA occurred rapidly and was maximal (90%) at 20 min after administration of the toxin. Inhibition of total nuclear RNA synthesis, although never complete, was observed by 15 min, and total cellular RNA synthesis was found to be markedly inhibited by 1 hr. and almost completely suppressed at 12 hr. The incorporation of ^{3}H-thymidine into the DNA fraction was also inhibited by aflatoxin, 57% inhibition being observed by 20 min and 80% inhibition after 2 hr. The inhibition observed under these conditions was found to be reversible but DNA synthesis was the last to recommence. These findings suggested that aflatoxin (or metabolite) binds with DNA, blocking replication and the transcription of RNA with chains longer than the distance between two bound aflatoxin molecules, so that under these conditions only short and medium length RNA molecules can be synthesized. To account for the observed sequence of recovery it was suggested that the progressive freeing of the DNA by aflatoxin would allow synthesis of longer-chain RNA (e.g., ribosomal RNA precursors) but the DNA molecule would still be too charged for replication to occur.

Another constructive study of aflatoxin action was reported by Sarasin and Moule (1973). These workers studied the *in vivo* effect of aflatoxin B_1, at sub-acute levels, on protein synthesis in rat liver using the incorporation of ^{14}C- labelled DL-leucine as a measure of protein synthesis. The inhibition observed showed a marked biphasic response, with marked inhibition of precursor incorporation peaking at 2 hr. and declining up to 7 hr. Thereafter the inhibition progressively increased to 80-85%. The pattern of inhibition after 7 hr. can be accounted for by the alteration of polyribosome profiles as determined in sucrose density gradients, disaggregation starting 3-4 hrs after toxin administration. This work suggested that aflatoxin may have an immediate and direct inhibitory effect on protein synthesis, but the later inhibition appeared to be a consequence of the aflatoxin-induced impairment of transcription.

Thus dothistromin resembles aflatoxin in that the primary site of action appears to be at transcriptional level, and for aflatoxin it is thought that this is due to interaction with the template rather than by direct interaction with the polymerase enzyme. However it is obvious from the above discussion that for aflatoxin, and very definitely for dothistromin, there are many aspects of the mechanism of inhibition requiring further investigation and clarification.

An attempt was made to compare the effects of aflatoxin B_1 and sterigmatocystin on the incorporation of labelled precursor in the B.megaterium KM experimental system with the effects observed with dothistromin. Only a single concentration of both mycotoxins was studied, based on the concentration required to inhibit growth, i.e. at a toxin concentration : cell number ratios of 2.0 μg/cell X 10^8. Under these conditions sterigmatocystin showed no inhibitory effect, while aflatoxin B_1 inhibition of ^{3}H-uridine incorporation was very slight. These results are in marked contrast to those obtained with dothistromin which rapidly inhibited ^{3}H-uridine incorporation at 0.2 μg/cell X 10^8. However, it must be pointed out that both of these compounds have very low solubilities in water, as is the case for dothistromin, and no study was made of the effectiveness of the methods used in adding these fungal metabolites to aqueous cultures. They were added as ethyl acetate solutions in the same way that dothistromin was added to aqueous cultures. Comparative studies of this type would be of interest in further defining the nature of dothistromin inhibition and could show possible activity relationships amongst these groups of fungal metabolites.

Investigation of the toxicity of a number of hydroxyanthraquinone compounds to Chlorella in the antibiotic disc bioassay showed that none of these compounds markedly effected the growth of the organism. This would suggest that the anthraquinone moiety of the dothistromin

structure contributes little to the overall toxicity of the molecule, in spite of the known diversity of metabolic processes inhibited by the quinone group of compounds (Webb, 1966). Of this class of compounds, 9, 10 anthraquinone is generally a less effective inhibitor of biological systems than the simpler quinones.

The contribution of the substituted tetrahydrobifuran ring structure to toxicity was not investigated in this study. However, by analogy with other fungal metabolites containing this moiety, it would be expected to confer some degree of toxicity to the molecule containing it. Studies aimed at correlating biological activity and structure in the aflatoxin and sterigmatocystin groups of fungal metabolites (Engelbrecht and Altenkirk, 1972; Ayres et al., 1971) have suggested that the unsaturated bifuran ring system is important to the toxic action of these compounds. These structure-activity studies also suggested that the structural relationship between the bifuran ring and the carbonyl groups of the heterocyclic nucleus was important in determining toxicity. Saturated bifuran derivatives of the aflatoxin series are less toxic than those containing the unsaturated system. Thus, in the aflatoxin group of fungal metabolites, those derivatives containing a hemiacetal in the bifuran system have little of the potent toxicity of the parent compound. Although the compound isolated from D. pini cultures, characterised and known as dothistromin, contains a hemiacetal grouping

in the bifuran ring, the possibly more toxic unsaturated derivative is likely to be formed as a co-metabolite or could readily be produced by dehydration of the hemiacetal derivative. Therefore, the likelihood of this derivative being present in preparations of dothistromin cannot be ignored.

4.24 Areas for further investigation

The studies reported here have indicated one particular area of cell biosynthesis inhibited by dothistromin but could be extended to determine the probable step(s) of the transcriptional processes which are inhibited by dothistromin and the molecular mechanism involved. The results obtained in this study indicate that inhibition of RNA synthesis could be one of the primary effects of the toxin but give no indication as to whether this is a general inhibition of RNA synthesis or if the toxin selectively inhibits one of the more rapidly synthesized RNA fractions. Examination of RNA sedimentation profiles could provide useful information on this aspect of the problem.

Does the toxin act directly on RNA polymerase, or does it interfere with template function? Investigation of possible enzyme-toxin interaction, template-toxin interaction and the effects of the toxin on the formation of the enzyme-template complex could be made using the techniques which have provided information on the

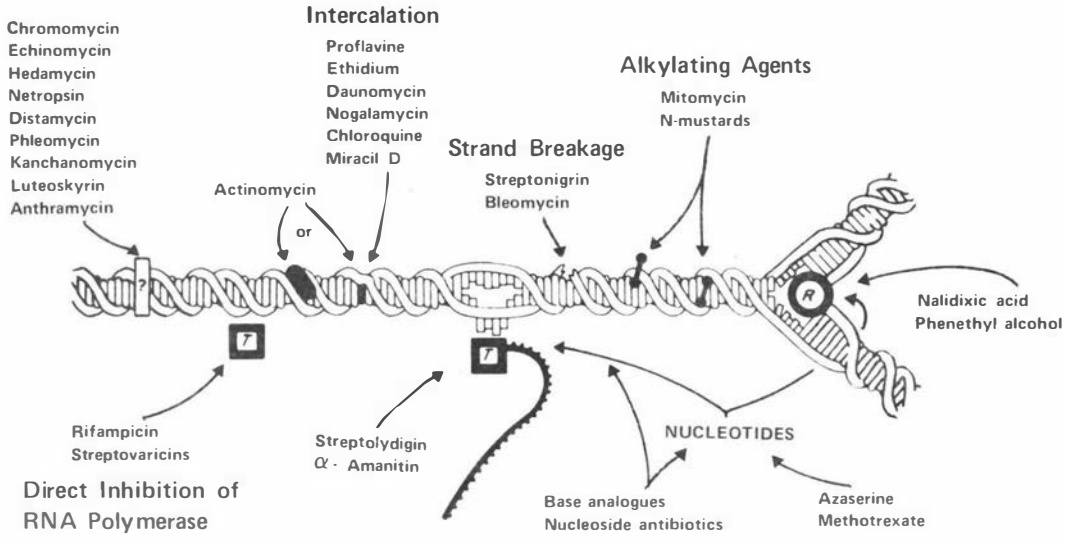
site of action of antibiotics and other inhibitory compounds affecting this area of metabolism. These techniques would include the use of radioactively labelled dothistromin to study binding of the toxin to various components *in vitro*, and studies of the kinetics of the RNA polymerase reaction in cell free systems.

There are a number of inhibitory compounds which contain a heterocyclic nucleus and a considerable proportion of these have been shown to bind with DNA, an interaction considered to account for the observed action of these compounds (Goldberg and Friedman, 1971; Bücher and Sies, 1969). The sites of inhibition of nucleic acid synthesis by a number of antibiotics and drugs are represented schematically in Fig.36, where the structures of some of these compounds are also shown. By analogy with these structures, it can be seen that the structure of dothistromin makes binding with the DNA template not unlikely and any further investigation of the site of toxin action should include the study of this possibility.

4.3 ROLE OF DOTHISTROMIN IN DOTHISTROMAL PINE BLIGHT

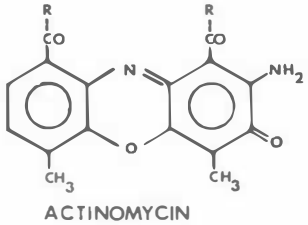
Impairment of the RNA-synthetic capacity of pine foliage infected with *D.pini* could result in the cell death and necrosis of tissue observed as disease symptoms of dothistromal blight. However, before the role of dothistromin in the disease can be assessed,it will be necessary to confirm the inhibitory effect of

FIGURE 36: SITES FOR INHIBITION OF NUCLEIC ACID SYNTHESIS BY ANTIBIOTICS AND DRUGS

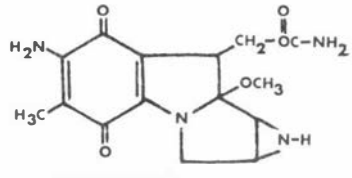


A portion of a double–helical DNA molecule is represented in the process of replication at the right-hand end and being transcribed into RNA at a region near the middle. 'R' represents the replicating enzyme, T the transcribing enzyme (RNA polymerase). Towards the left-hand end an RNA polymerase molecule is about to form an initiation complex with the DNA and start the synthesis of a new RNA strand. Actions of inhibitors are represented by double-headed arrows and are purely diagrammatic.

From M & B Laboratory Bulletin (Vol X, No. 3, 1972)



ACTINOMYCIN



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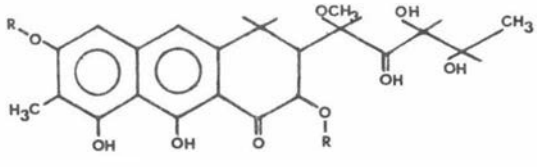

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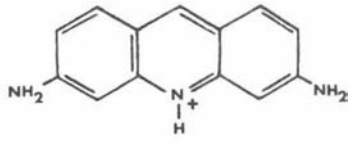

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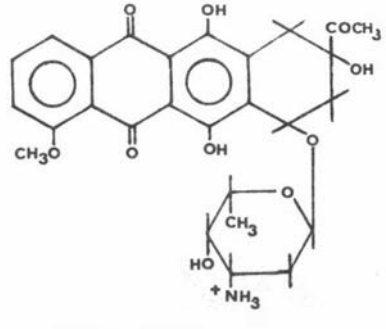

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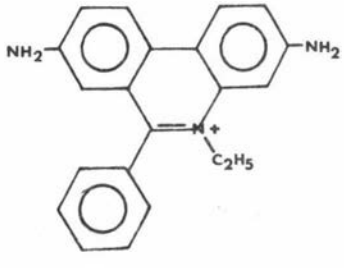

ETHIDIUM

the compound in pine-tissue and, preferably, in pine seedlings. Attempts in this direction have so far met with little success. One of the major problems encountered is that of the very low solubility of dothistromin in aqueous solutions. The results obtained in this study indicate the possible importance of the compound as a disease factor and it is to be hoped that the methods employed here can be further developed for application to assays involving pine tissue.

One of the first symptoms frequently observed with diseased plant tissue is an alteration of the permeability of the plant cell membrane (Wheeler and Manchey, 1968). Any alteration of membrane function could rapidly limit the availability of precursors for cellular metabolism and result in the inhibition of synthesis. To account for the sequence of events observed in B.negaterium KM, the alteration of permeability would have to be specific to particular precursors. However, this possible primary site of action for the toxin has not been investigated and is an area requiring further study before the toxic activity of dothistromin can be fully assessed.

Since dothistromin appears to be a general inhibitor of RNA synthesis, i.e. not species specific, it could presumably inhibit fungal synthetic processes. Characteristically, secondary metabolites are produced in the stationary phase (or idiophase)

of fungal growth and appear to have little or no critical significance relative to the growth of the organism (Weinberg, 1970). In laboratory culture of D. pini, dothistromin production occurs at a late stage of the culturing period, and the appearance of red bands on D. pini infected pine needles is not observed until just prior to sporulation. The site of toxin production is as important as the timing of synthesis in protecting fungal metabolism, and little is known of the cellular site of dothistromin production. The final step converting an inactive precursor to a potent toxin could be catalyzed by a cell-wall bound enzyme.

The timing of dothistromin production by D. pini is also of importance with respect to the host-pathogen relationship between the fungus and members of the Pinus genus. Dothistromin may not be necessary for the successful infection and growth of the fungus on the host, and yet be largely responsible for the disease symptoms manifested. The fungus has been shown to produce extracellular enzymes which may well be of importance in the processes of infection and growth (Brunt, 1970).

Although toxic metabolites are known to be produced by a large number of phytopathogenic fungi, little is known of the biochemical mode of action of any phytotoxin. Evidence for toxin participation in phytopathogenesis is based on the findings that:

(a) Some of these toxins are host-specific. They will affect only the host species of the fungus and any loss of the ability of the fungus to produce the toxin in culture is paralleled by loss of pathogenicity.

(b) The pathological effects of the toxin are so similar to the disease syndrome as to strongly suggest toxin involvement. (Templeton, 1972).

If dothistromin can be considered to be a phytotoxin, host-specificity must be dependent on other factors in the host-pathogen relationship since the toxin appears to be a non-specific inhibitor. The apparent biological activity of dothistromin could account for the disease syndrome but there is, as yet, no confirmation of the toxin activity in pine tissue. However, as other factors involved in dothistromal pine blight are elucidated, it is likely that the disease will be found to result from the interaction of a number of agents, an interaction to which dothistromin may make a major contribution.

4.4 DOTHISTROMIN AS A POTENTIAL HEALTH HAZARD

At the present time in New Zealand, dothistromal blight is controlled by the aerial application of copper fungicides, but this treatment does not eradicate the fungus. It may well be prudent to investigate ways of eradicating the disease to supplement present methods of control. Nothing is known of the metabolic fate of dothistromin ingested into mammalian systems, e.g. by stock grazing in infected plantations. In view of the current reassessment of the role of fungal metabolites in health, consideration must be given to this aspect of the dothistromal blight problem. Hydroxylated aflatoxin derivatives have been isolated from the milk of cows fed peanut meal and these compounds, aflatoxins M_1 and M_2, were found to be as toxic as the parent aflatoxins (Purchase, 1967). In view of the known carcinogenicity of structurally related mycotoxins it is important to evaluate the potential carcinogenicity of dothistromin. Samples of dothistromin have been sent to laboratories with experience in this field, but conclusive results are not yet available.

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