(U-^{14}C)tyrosine. The test procedure was standardized by using a constant enzymic activity: 2.43 µg dominant isozyme at 30 °C, 16 h incubation period under standard conditions[13,14]. Using L-dopa-^{14}C as the substrate, the enzyme had a K_m of 4.5×10^{-4} M[12]. The V_{max} was 37.1×10^{-4} pmole L-dopa conversion/min/mg protein[12]. The enzyme was incubated with L-(U-^{14}C)tyrosine in the presence and absence of 14 agents, each at three dose levels (table).

Results and discussion. All agents utilized showed differing degrees of inhibitory effects upon the dominant human melanoma tyrosinase activity. The inhibitory effects increased with increased drug concentration. Although pyrogallol or phloroglucinol did not affect tyrosinase activity at 9×10^{-5} M, the enzyme was inhibited at higher concentrations. Comparison of the drug inhibitory effects at 9×10^{-5} M, diethyldithiocarbamate was the most potent inhibitor but glutathione, mercaptoethylamine and hydroquinone inhibited the enzyme 50% or more. The dihydroxy- and trihydroxy-benzene compounds, sulfhydryl agents and ascorbic acid may be considered as reducing agents. Among the dihydroxy-benzene derivatives (hydroquinone, catechol, resorcinol), hydroquinone (1,4 dihydroxybenzene) inhibited the enzyme to a greater extent than resorcinol (1,3 dihydroxybenzene) at 9×10^{-5} M and 9×10^{-4} M. Resorcinol inhibition was greater than that of catechol (1,2 dihydroxybenzene) at all concentrations. The trihydroxbenzenes, pyrogallol (1,2,3 trihydroxybenzene) and phloroglucinol (1,3,5 trihydroxybenzene) may be better inhibitors at higher concentrations. Thus, the number and geometry of the phenolic groups in the benzene compounds appear important in the inhibition of tyrosinase activity.

The sulfhydryl compounds, mercaptoethylamine and glutathione, were potent inhibitors of tyrosinase. L-cysteine was effective at the highest concentration. The dimer of mercaptoethylamine, cystamine, and the dimer of cysteine, cystine, showed reduced inhibitory effects possibly the result of conversion of sulfhydryl groups to disulfide bonds. The small inhibitory effect of methionine may result from methylation of the –SH reactive group. Interestingly, diethyldithiocarbamate (DDC), containing $S=\overset{|}{C}-S-$ but not –SH was the most potent inhibitor. Ascorbic acid was a potent inhibitor at the higher concentrations. Further, a chelating agent, EDTA, showed a smaller inhibitory effect on tyrosinase activity than DDC, suggesting that chelation may not be the mechanism of enzymatic inhibition by the latter.

Comparison of the present inhibitory effects of those agents previously utilized upon tyrosinase activity in crude and fractionated melanoma homogenates[3] revealed similar trends and results.

The use of fungal protoplasts in the study of aflatoxin biosynthesis

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Summary. Protoplasts derived from *Aspergillus flavus* are shown to be capable of synthesizing aflatoxins when incubated in a chemically defined medium. ^{14}C-Acetate and ^{14}C-Versicolorin A, added to protoplasts from 3-day-old mycelium, are incorporated into aflatoxin B_1.

One great difficulty in the study of fungal metabolism is the presence of a tough cell wall which prevents easy access to the cytoplasmic content and hence to the enzymes that it contains. However, some success using mechanical disruption of mycelium has been achieved in enzymatic studies of fungal secondary metabolism[1,2]. During our studies on aflatoxin biosynthesis we have attempted to produce active enzyme preparations from *Aspergillus flavus*, using a variety of disruptive methods, with varying degrees of success. Several negative results which we have obtained have been attributed to denaturing effects during the disruptive procedure. However, one method which does not depend on mechanical disruption is the removal of the cell wall by enzymatic digestion, resulting in the formation of protoplasts[3,4]. These are much more easily disrupted than intact mycelium and hence are more likely to yield active enzyme preparations. Protoplasts are currently being used to investigate fungal organelles[5], cell wall synthesis[6] and antibiotic production[7]. Thus we have studied the potential of protoplasts, derived from toxin-producing strains of *A. flavus*, for converting possible intermediates to the aflatoxins as a prerequisite to the study of isolated cell-free enzymes.

Materials and methods. The lytic enzyme was prepared from *Trichoderma viride* CBS 354-33 (kindly supplied by Dr J. Peberdy, University of Nottingham) using the method of Peberdy and Issacs[8]. The growth medium

Table 1. Production of aflatoxin in Reddy's chemically defined medium by protoplasts from mycelium of *Aspergillus flavus* N1 of different ages

Age of mycelium (days)	Aflatoxin (µg) formed per 2 ml suspension after: 0 h	1 h	2 h	3 h	18 h
1	4.96	5.46	5.55	5.10	8.08
2	9.92	11.84	14.53	14.89	14.96
3	5.60	5.60	8.08	9.50	13.75
4	3.20	2.00	0.50	0.50	2.70
5	7.10	5.30	1.80	1.20	2.10

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Table 2. Incorporation of U-^{14}C-acetate and G-^{14}C-versicolorin A into aflatoxin B_1 by protoplasts derived from 3-day-old mycelium, suspended in Reddy's chemically defined medium minus sucrose incubated for 3 h

Precursor Amount added			Product aflatoxin B_1 Amount formed		
	μCi	Specific activity (mCi/mole)	μCi	Specific activity (mCi/mole)	Percent conversion*
(U)-^{14}C-Acetate	0.21	234.2	0.0006	34.7	0.28
(G)-^{14}C-Versicolorin A	0.0084	28.6	0.0013	21.2	15.5

* $\frac{\mu Ci\ product}{\mu Ci\ precursor} \times 100$.

contained 5.0 g *Aspergillus flavus* mycelium dry weight per litre in place of the polysaccharide used by Peberdy and Issacs. *Trichoderma viride* was maintained on the growth medium containing 2% agar.

Aspergillus flavus N1 (a single spore isolate of CMI 91019b) was maintained on potato dextrose agar. A spore suspension was prepared in 0.1% sodium lauryl sulphate from a 14-day-old culture. A 250 ml conical flask containing 50 ml Reddy's[9] chemically defined medium was inoculated with 1 ml of spore suspension (12×10^6 spores) and incubated at 25 °C for 3 days on a rotary incubator (100 rev/min). The mycelium was harvested and washed with buffer-stabilizer (0.4 M $MgSO_4$ in 0.2 M phosphate buffer, pH 5.8) then suspended in the lytic enzyme preparation (200 mg mycelium/ml lytic enzyme) and an equal volume of buffer-stabilizer was then added. The digest was incubated for 3 h at 25 °C in a rotary incubator (100 rev/min). Protoplasts were then isolated using the method of Peberdy[4]. Versicolorin A was isolated from *Aspergillus parasiticus* 1-11-105 Whl (kindly supplied by Dr J. Bennett, Tulane University, Louisiana) using the method described by Lee et al.[10]. ^{14}C-Versicolorin A was prepared using the method of Lee et al.[11]. 'Versiconal Acetate' and Versicolorin C were obtained from cultures of *A. flavus* N1 treated with dichlorvos[12]. Sterigmatocystin was kindly supplied by Dr J. S. Holker of the University of Liverpool.

All potential substrates were dissolved in N,N, dimethylformamide (Analar) and added as solutions (total of 100 μg substrate) to suspensions of protoplasts in buffer-stabilizer. When incubated in Reddy's medium, 1 ml of protoplast suspension (680 nm; OD = 0.26 = 32 mg protoplasts) was added to 9 ml of Reddy's medium. All experiments were incubated at 25 °C, 2 ml portions of the suspension were removed at various time intervals. To act as a control experiment, protoplasts were prepared from *A. flavus* mycelium and placed in buffer-stabilizer without the addition of a carbon source. The aflatoxins were extracted from the suspension with chloroform and estimated spectrophotometrically by measuring their extinction at 363 nm (Nabney and Nesbitt[13]). The aflatoxins were then estimated visually on thin layer chromatograms using toluene: ethyl acetate:acetone: glacial acetic acid (60:25:15:2, v/v) as the solvent system. ^{14}C-Versicolorin A and ^{14}C-Aflatoxin B_1 were re-chromatographed until their specific activity was constant before counting the samples in a liquid scintillation counter (Packard, Tricarb Model 3300).

Results and discussion. Reddy's medium has been shown to stimulate aflatoxin biosynthesis in *A. flavus* N1 cultures, therefore, it was used as a suitable chemically defined medium in the investigation of aflatoxin biosynthesis by protoplasts. In order to optimize aflatoxin formation, protoplasts from mycelium of different ages were investigated (table 1).

Protoplasts from 1-day-old mycelium seem to have little aflatoxin producing capability over the first few h but they do acquire the ability to form aflatoxins during the 18 h period. Protoplasts from 4- and 5-day-old mycelium appear to degrade aflatoxin over the initial 2 h period of incubation; this result has been observed on a number of occasions with cultures of *A. flavus*[14], which show a decrease in aflatoxin concentration with increasing age of mycelium. It was noted that aflatoxins were already present in the protoplast preparations showing that they had been synthesized by the mycelial stage and not all of them secreted before and during protoplast formation.

One difficulty with interpreting these results is that as protoplasts are 'naked' mycelium, deviod of a cell wall, they will probably revert to primary metabolism or re-synthesize cell wall material when placed in Reddy's medium. This seems to be reflected in the results of the 2- and 3-day-old material; where aflatoxin biosynthesis occurs most rapidly over the first few h but slows down during the subsequent 18-h-period, certainly, after 18-h mycelial cell walls are discernable in all protoplast preparations.

Table 3. Conversion of added compounds to aflatoxins using protoplasts derived from 3-day-old mycelium, suspended in buffer-stabilizer

Precursor added (100 μg/10 ml)	Aflatoxin (μg) formed per 2 ml suspension after: 1 h	18 h
Versicolorin A	3.60	13.00
Sterigmatocystin	5.67	9.57
Versicolorin C	4.00	— *
'Versiconal acetate'	3.70	— *
No precursor	< 0.05	< 0.05

* No reading taken.

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Another difficulty is that the aflatoxin biosynthesis observed is the result of a number of enzyme activities. These activities may be divided into 2 main phases, a) presumably the formation of an anthraquinone precursor via a polyketide synthetase and b) a cleavage phase involving at least 4 ring-cleaving steps involving enzymes having different substrate specificities[15]. It seems likely that the latter enzymes are induced in response to the appearance of an anthraquinone precursor which in the light of current evidence is probably averufin[16], or a closely related compound[17].

It is, however, clear that protoplasts derived from mycelium of different ages are capable of de novo aflatoxin biosynthesis, this being confirmed by the conversion of labelled ^{14}C acetate to aflatoxin (table 2) and it therefore follows that they must contain the total complement of enzymes required for aflatoxin biosynthesis.

In order to investigate some of these enzyme activities, a number of proven and possible intermediates in aflatoxin biosynthsis were added to the protoplast preparations. Several of the intermediates were converted to aflatoxin while controls containing no added compound did not produce aflatoxin during the same period (table 3). It was observed that protoplasts rapidly take up anthraquinone from the stabilizer-buffer solution as they became stained a yellow-orange colour in the presence of anthraquinone precursors with a corresponding loss of colour from the solution. It was shown that penetration of the membrane had occurred by adding versicolorin A to a suspension of protoplasts which were then centrifuged. The resultant protoplast pellet was washed with buffer-stabilizer, lysed by freezing and thawing in buffer (pH 5.8), and the membrane fraction was centrifuged down; 35% of the versicolorin A that had been added was present in the supernatant fraction indicating that it may pass into the protoplasm.

Work in this laboratory is currently being carried out with lysed protoplasts and preliminary experiments show that they are suitable for preparing cell-free extracts of *A. flavus* capable of carrying out several of the steps involved in aflatoxin biosynthesis.

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A study of the cytoplasmic factors controlling the rate of catalase synthesis in rat liver

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Summary. A new quantitative method for assessment of the biological activity of the cytoplasmic factors-regulators, controlling the rate of catalase synthesis in rat liver on post-transcriptional stage, has been worked out. The nature of these factors has been established.

There are data in the literature concerning the mechanisms of regulation of protein synthesis rate at the post-transcriptional levels in various eukaryotic tissues[1–4]. Studying the mechanism of synthesis of catalase (KF 1.11.16), Uenoyama and Ono[3,4] have shown that in rat liver cells the synthesis of this protein is controlled at the translational level by 2 factors found in the cytoplasm in a soluble state. These authors have found that in a cell-free system one of the factors (F_{inh}) may bind to the catalase-synthesizing ribosomes, thus suppressing the synthesis of this enzyme, whereas the other factor (F_{act}) prevent association of the F_{inh} with polysomes[3,4]. The presents investigation has been aimed at the development of a quantitative method for assessment of the activity and elucidation of the nature of the factors controlling the synthesis of catalase in rat liver.

Materials and methods. Investigations have been carried out in male rats weighing 160–180 g. The factors were isolated according to Uenoyama and Ono[3,4] using ion-exchange chromatography and re-chromatography on DEAE cellulose. The F_{act} and F_{inh} were isolated from the supernatant and pH 5 fraction of rat liver respectively, so that chromatography of the F_{act} was performed on the post-mitochondrial supernatant, whereas prior to isolation of the F_{inh} the pH 5 fraction had been dialysed. Since the factors are extremely labile, all of the procedures on isolation and purification have been performed at 4 °C. Elution of the factors from DEAE-cellulose columns was achieved with buffers containing increasing concentrations of KCl at a flow rate of 10 ml/h. In the preparations of the factors, the concentration of the protein was estimated according to Lowry et al.[5], the SH-group content by the method of amperometric titration[6], the catalase activity by the manganometric method[7] and electrophoretic mobility was assessed in polyacrylamide gels (PAAG). Depending on condition of the experiment, disk-electrophoresis was performed in a 7% PAAG or in linear gradient of PAAG (from 2.5 to 8% acrylamide). The amino acid composition was evaluated by descending paper chromatography.

Certain parameters of the factors controlling the rate of synthesis of catalase in rat liver

Parameters	F_{act}	F_{inh}
SH-groups (μM/mg)	0.174 ± 0.0095	0.106 ± 0.0106
Protein concentration (mg/ml)	0.123 ± 0.015	0.073 ± 0.007
Activity (μM/min/g of liver)	625	200

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