the serum tyrosinase activity in lung carcinoma was approximately one-third of that in melanoma and breast carcinoma[3].

The melanin bands obtained after electrophoresis and incubation with L-dopa were variable in number but as many 7 or more frequently were present. In contrast to melanoma and breast carcinoma sera[3], the intensities of all dopa-melanin bands formed were decreased. Also, their R_f-values (table 2) differed from those derived from sera of patients with melanoma. In melanoma[4], the dopa-melanin bands varied from 0.0013 (band 1) to 0.080 (band 4) and then from 0.138 (band 5) to 0.188 (band 7). It is possible that all the serum tyrosinase carriers are not all the same in lung carcinoma and melanoma. The characteristic melanin bands in different diseases may identify the specific serum immunoglobulins carrying tyrosinase in each disease.

In addition to the narrow melanin bands, 1 wide (1 cm) melanin band (R_f: 0.254 ± 0.004) occurred in some patients of the study. This R_f-value was greater than those of the narrow melanin bands. The function represented by this wide band is not clear at present.

Coincident with the demonstration of the above electrophoretic patterns, inhibitory sites of dopa oxidation in lung carcinoma were observed as 3 colorless bands (R_f: 0.1740 ± 0.0049; 0.3084 ± 0.0047; 0.5649 ± 0.0046), width 2–3 mm. These may represent tyrosinase inhibitors in the enzyme preparation and differ in mobilities from enzyme-carrier complexes. When eluted these inhibitors depress both enzymic and autoxidative melanin formation from dopa[4]. The colorless bands, therefore, result from inhibition of L-dopa autoxidation on the gels.

4 fractions inhibiting tyrosinase activity have been separated by ultrafiltration of the 60% saturation (ammonium sulfate) supernatant. The inhibitory potencies of these fractions from both lung carcinoma patients and normal individuals on the activity of mushroom tyrosinase differ (table 3). The normal material was blood bank plasma. The inhibitory potencies in fractions I_{300} and I_{100} showed small differences between the normal individuals and patients with lung carcinoma. However, the inhibitory potencies in fractions II and III were much lower in patients (fraction II, 38%; fraction III, negligible) than in normal individuals (fraction II, 78%; fraction III, 17%). Further, tyrosinase activating fractions IV and V observed in melanoma[4] were absent in both normal and lung carcinoma preparations.

In vitro effects of melanocytolytic agents and other compounds upon dominant human melanoma tyrosinase activity[1]

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Summary. The activity of dominant human melanoma tyrosinase isozyme was greatly decreased by certain reducing agents. The number and geometry of phenolic groups as well as free –SH group appear important for enzymic inhibition.

The in vitro effects of a number of melanocytolytic agents and related compounds upon tyrosinase activity in human melanoma homogenates have been reported[3]. To more precisely define the effects of these agents, it is necessary to test their effects upon purified human melanoma tyrosinase. Three isozymes of human melanoma tyrosinase have been reported[4–11]. As the dominant human melanoma tyrosinase may be prepared with large yield[6,9,10], this isozyme was used in the present study.

Materials and methods. Highly melanized human melanomas obtained at surgery were processed to obtain a lipase solubilized fraction derived from the particulate fraction as described previously[6,9,10]. The dominant human melanoma tyrosinase was isolated from this fraction by 50–70% ammonium sulfate fractionation and purified further by sephadex G-100 chromatography[12]. Tyrosinase was assayed radiometrically[13,14] using L-

In vitro effects of melanocytolytic agents on the activity of dominant human melanoma tyrosinase

Melanocytolytic agent	Tyrosinase activity (percent control)		
	9×10^{-5} M	9×10^{-4} M	9×10^{-3} M
Hydroquinone	51.2	13.5	11.1
Catechol	92.7	85.8	18.2
Resorcinol	81.6	22.8	1.7
MEA HCl[a]	34.4	15.5	0.5
Cystamine HCl	90.5	70.8	50.9
L-Cysteine HCl	81.7	58.0	1.0
Glutathione	17.9	13.3	1.2
Ascorbic acid	88.5	2.0	0.5
L-Cystine	90.0	89.6	87.4
DL-Methionine[b]	94.7	90.5	67.9
Pyrogallol	102.3	5.9	1.0
Phloroglucinol	98.9	35.4	3.7
DDC ($3H_2O$)[c]	3.5	0	0
EDTA[d]	80.4	73.1	51.2

[a]MEA, β-mercaptoethylamine. [b]Concentration represented L-form only. [c]DDC, diethyldithiocarbamate. [d]EDTA, ethylenediaminetetraacetic acid.

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(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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