Isolation and characterisation of hepatotoxins from *Penicillium rubrum*

SIR,—It is known that an acidic toxic material can be extracted from cultures of *Penicillium rubrum* grown on a corn-sucrose medium (Wilson & Wilson, 1962). We have now found that when a strain of *P. rubrum* M. R. 043 (kindly supplied by Mr. P. Austwick, C.V.L., Weybridge) is grown for 11–13 days on a Raulin Thom medium enriched with 2½% malt extract, a complex of toxins may be produced and isolated in good yields.

The mould was grown as stationary cultures at 28–30°. After the culture fluid had been harvested from 13 day old cultures and concentrated to a small volume under reduced pressure, much atoxic material was precipitated sequentially, first by ethanol, and then by acetone, each solvent being removed by evaporation after the precipitate was recovered by filtration.

The toxic concentrate remaining was distributed between ethyl acetate and water. The ethyl acetate phase, after drying over anhydrous sodium sulphate, was evaporated to dryness to yield a pale orange gum which solidified to a buff coloured powder after washing out residual solvent with sodium-dried diethyl ether.

The results of a typical isolation from 1½ litres of culture filtrate are shown in Table 1.

TABLE 1. FRACTIONATION OF METABOLISM SOLUTION FROM CULTURES OF *P. rubrum*

	Average lethal dose oral mg/kg	Total dry weight	Dry weight recovered %	Toxicity recovered %
Metabolism solution	1850	46·3 g	100	100
Ethanol ppt	Atoxic	5·6 g	12·1	0
Acetone ppt.	Atoxic	19·1 g	41·2	0
Aqueous soluble fraction	2000*	15·6 g	33·6	31·2
Ethyl acetate fraction	120	1·41 g	3·0	47
Recovery		41·7 g	90·0	78·2

* Whether the toxicity of the aqueous residue is due to a distinct toxin or to one or other of the solvent soluble toxins bound to a water soluble constituent of the culture filtrate is not yet known.

The crude toxin is a buff amorphous powder, m.p. 126–146° (with decomposition) $[\alpha]_D^{18} + 32{\cdot}5°$ (*c.* 2% acetone), LD50 3·75 mg/kg (intraperitoneally in mice). It is very soluble in acetone, soluble in alcohols, ethyl acetate, dioxan or *NN*-dimethylformamide, slightly soluble in ether but insoluble in chloroform, carbon tetrachloride, benzene or light petroleum. The toxin is insoluble in cold water but soluble in sodium bicarbonate or alkali solution.

The crude material contains only carbon, hydrogen and oxygen and gives the following analysis: C 58·7%, H 6·3%. Analysis for *C*-methyl gave a result of only 2·84% which corresponded to a minimum molecular weight of 528. Back titration of a solution of toxin in excess of cold sodium hydroxide gave an equivalent weight of 180 which indicated the presence of a tri-basic acid with a molecular weight of 540. Heating the alkaline solution resulted in a further uptake of sodium hydroxide and a complex of reactions which will be discussed in more detail in another publication.

At least two distinct highly toxic components can be isolated from the crude toxin either by careful precipitation by ether and light petroleum from a solution in acetic acid, or by slow evaporation of a saturated solution of crude toxin in diethyl ether.

The more easily obtained of these toxins, which we suggest be called Rubratoxin A, has been obtained as a white waxy looking material crystallising in rosettes of needles from acetone by slow evaporation at room temperature. Rubratoxin A has the following characteristics: m.p. 214° (decomp.) $[\alpha]_D^{18}$ + 86·6°. C, 59·6, H, 6·1, O, 34·6%. The ultraviolet absorption spectrum in ethanol has a broad peak at 206–209 mμ [*E*(1%, 1 cm) 351] and in alkali a peak appears at 262 mμ [*E*(1%, 1 cm) 150] with a shoulder at 300 mμ [*E*(1%, 1 cm)80], both of which disappear on acidifying.

The infrared spectrum indicates the presence of hydroxyl functions (3450 cm^{-1}), carboxylic acid (1710 cm^{-1}) and double bond (1630 cm^{-1}) functions, as well as showing interesting absorption at 1850 and 1770 cm^{-1}.

Rubratoxin A has an LD50 3·5 mg/kg given intraperitoneally in mice.

A second component, Rubratoxin B, has also been isolated from saturated ethereal solutions of crude toxin as a white amorphous powder but has not been so well characterised. It has the following properties: m.p. 167–168° (decomp.). C, 58·9, H, 5·8%.

Although the infrared spectrum of Rubratoxin B is essentially the same as that of Rubratoxin A it differs in the following aspects. There is a peak at 970 cm^{-1} in the spectrum of Rubratoxin A which is missing in that of B while the peak at 920 cm^{-1} increases in intensity, and there is a peak at 720 cm^{-1} in the spectrum of Rubratoxin B which is missing in that of A.

Details of the toxicity of the crude toxin are in Table 2. The crude toxin was always more toxic when given in propylene glycol than in 0·1% sodium bicarbonate solution.

TABLE 2. LD50 VALUES FOR CRUDE TOXIN

	Route	Dose mg/kg	Solvent
Mouse	oral	120	Propylene glycol
	i.p.	3·75	Propylene glycol
	i.v.	6·5	0·1% $NaHCO_3$
Duckling	oral	60	0·1% $NaHCO_3$
	i.p.	5	0·1% $NaHCO_3$

Doses well above the LD50 level in mice caused death within 2–4 hr of administration. Before death the activity and respiration of the mice were decreased, there was dilatation of the subcutaneous blood vessels and finally complete prostration, followed by death. Post-mortem examination of the mice revealed livers which were extensively haemorrhaged and had a mottled appearance which we have come to regard as characteristic of lethal doses of *P. rubrum* toxins. The kidneys were slightly anaemic and the lungs occasionally showed haemorrhages. Mice which remained alive for longer than 10 hr had their eyelids stuck together by dried exudate.

Post-mortem examination of mice killed seven days after a sublethal dose of the toxin showed no macroscopic liver damage. Mice dosed similarly, but killed 24 hr after the dose had the typically mottled liver. It seems therefore that the liver tissue is able to regenerate over a period of 7 days. Similar observations have been made with ducklings, guinea-pigs and rats.

That the normal metabolic processes of the liver are adversely influenced by doses of toxin which do not cause macroscopic lesions has been conveniently shown by the sleeping time test with pentobarbitone sodium (Plaa, Evans & Hine, 1958). Sleeping time was measured after a 45 mg/kg dose of pentobarbitone sodium given intraperitoneally to groups of 10 mice. The sleeping time was taken as the time between loss and subsequent gain of the righting

reflex by the mice. The control sleeping time for a group of 150 mice was 18½ min.

An oral dose of 75 mg/kg of crude toxin was given to seven groups, each of 10 mice. The mice were left for periods of 1·5, 3, 6, 12, 24, 48, 96 hr after the dose and then each group was tested for the pentobarbitone sleeping time.

The curve obtained indicated a rapid onset of interference with the normal detoxicating mechanism. This reached a maximum two days after administration and then decreased (see Fig. 1).

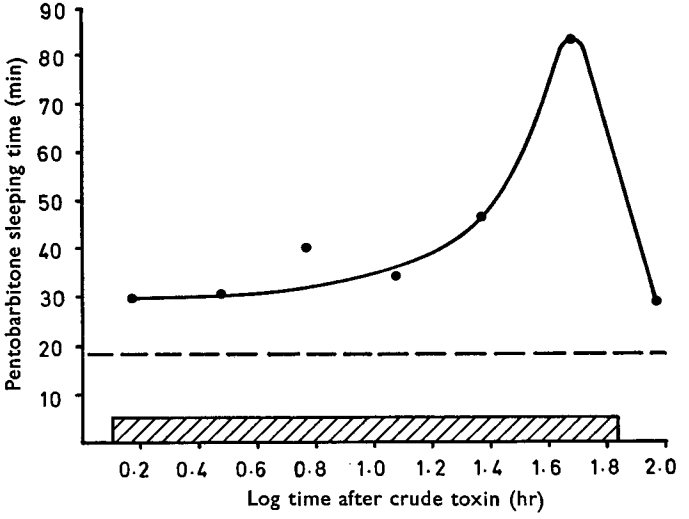

FIG. 1. The prolongation of the pentobarbitone sleeping time at intervals of 1·5, 3, 6, 12, 24, 48 and 96 hr after administration of 75 mg/kg of crude toxin. ----- Control value for the pentobarbitone sleeping time in mice. Hatched area indicates the presence of macroscopic liver damage.

These results show that the compounds we have described are distinct from the pigments produced by *P. rubrum* and in particular are consistent with the report of Büchi, White & Wogan (1965) that the pigments from this mould now characterised by them did not contribute to the toxicity of the mould.

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