Metabolism of 2,3,4,6-Tetrachlorophenol by Micro-organisms from Broiler House Litter

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SUMMARY

Isolates of 26 fungal species from broiler house litter were screened for their ability to metabolize and methylate 2,3,4,6-tetrachlorophenol over a five-day period, by adding the chlorophenol to cultures after mycelial pellets were established on a complete growth medium. Under these conditions, 99 of the 116 isolates tested metabolized the chlorophenol and 68 of these produced 2,3,4,6-tetrachloroanisole. The proportion of the chlorophenol methylated to the chloroanisole differed widely with the isolate, even within species. The highest methylation was observed with *Penicillium corylophilum*; certain other isolates, notably of *P. brevicompactum*, metabolized almost all the chlorophenol without forming the chloroanisole. Progress studies with these species suggested that there is more than one route for the metabolism of 2,3,4,6-tetrachlorophenol. In tests with suspensions of mixed bacterial populations from broiler house litter, the chlorophenol was metabolized but no methylation was detected.

INTRODUCTION

It has been shown that 2,3,4,6-tetrachloroanisole is a cause of musty taint in chickens, and that it is formed in broiler house litter by the microbial methylation of 2,3,4,6-tetrachlorophenol (Curtis *et al.* 1972, 1974). This chlorophenol, together with pentachlorophenol, is commonly present on the wood shavings that form the initial litter; by the end of the 8 to 9 week rearing cycle, these chlorophenols have almost disappeared (Parr *et al.* 1974). There is a transient accumulation of the corresponding chloroanisoles which reaches a maximum at 4 to 5 weeks (C. Dennis, D. G. Land, J. Mountford, and D. Robinson, unpublished). Micro-organisms may contribute to the loss of chlorophenols not only by methylation to give the volatile chloroanisoles but also by degradation via other metabolic routes. The microbial flora of broiler house litter has been studied with particular attention to the fungi present (Dennis & Gee, 1973), and some 33 different mould species have been isolated; of these the predominant organisms are species of *Aspergillus*, *Penicillium* and *Scopulariopsis*. The purpose of the experiments reported here was to establish whether the ability to metabolize chlorophenols is widespread among litter micro-organisms, and the extent to which it is associated with methylation.

METHODS

Experiments with fungi. The fungal isolates tested were those isolated from broiler house litter by Dennis & Gee (1973); stock cultures were stored on slopes of Difco Bacto malt extract agar at 1 °C. To obtain spore suspensions for inocula, 1 ml of sterile aqueous 0.1 % Tween 80 was added to a stock slope and, after shaking, approximately 0.2 ml of the resulting

suspension was spread evenly over a plate of Sabouraud dextrose agar pH 5.6 (Oxoid). The plate was then incubated at 25 °C until sporulation occurred (3 to 7 days according to the species). Discs, cut from the sporulating culture using a sterile cork borer, were shaken in a 28 g (1 oz) bottle containing 10 ml of the Tween 80 solution, and the resulting spore suspension pipetted off. Serial decimal dilutions in water were prepared for microscopic counts and the dilution containing between 10^5 and 10^6 spores/ml was used for inoculation.

For tests on the metabolism of 2,3,4,6-tetrachlorophenol, the fungi were grown in 250 ml Erlenmeyer flasks containing 20 ml of Raulin-Thom (glucose-tartrate-mineral salts) medium as described by Clutterbuck, Oxford, Raistrick & Smith (1932), except that the glucose concentration was 4% (w/v). The medium was autoclaved at 103 kPa (15 lb/in²) for 15 min, and inoculated with 10^5 spores. The cultures were grown at 25 °C with orbital shaking until mycelial pellets were readily apparent; in several experiments where growth was measured, the minimum total dry weight was 20 mg. This required 3 to 9 days, depending on the species. At this point approximately 120 µg of 2,3,4,6-tetrachlorophenol were added aseptically, and the plastic foam plug hitherto closing the flask was replaced by a sterile plug consisting of a cork wrapped in aluminium foil and encircled with PTFE tape to prevent any loss of the volatile chloroanisole. The flasks were then shaken for a further 5 days before analysing the contents for the chlorophenol and chloroanisole. The 2,3,4,6-tetrachlorophenol (Fluka A. G., Buchs, Switzerland) was added as an aqueous 0.1% solution of the sodium salt prepared by dissolving 2,3,4,6-tetrachlorophenol in 0.1 N-NaOH, adding H₃PO₄ to pH 8.0, diluting to strength and sterilizing by filtration. The 2,3,4,6-tetrachlorophenol was purified by fractional vacuum sublimation; the resulting material contained approximately 2.5% (w/w) of pentachlorophenol as an impurity. An uninoculated control was included with each batch of cultures. To follow the metabolism of the chlorophenol, replicate cultures were set up as above and the contents of one flask taken for analysis at each of the times indicated.

Experiments with bacteria. A mixed population of bacteria was obtained from broiler house litter taken four weeks after introduction of the birds. A sample (100 g) of litter was homogenized with 1 l 10 mM-phosphate buffer pH 7.4 (NaH₂PO₄-Na₂HPO₄ mixture) in an Atomix blender (MSE) and filtered through a double layer of muslin to remove larger particles; this included virtually all the fungal mycelium. The filtrate was centrifuged at 500 g for 10 min, the pellet discarded and the process repeated. The supernatant from the second centrifugation was then centrifuged at 20 000 g for 20 min, the supernatant discarded and the pellet resuspended in the 10 mM-phosphate buffer pH 7.4 to give a final volume of 195 ml. The resulting suspension contained 8.5 mg dry matter/ml and microscopic examination showed it to be composed of bacteria, with no yeasts or mould fragments visible.

The freshly prepared bacterial suspension (10 ml) was incubated with 25 µg 2,3,4,6-tetrachlorophenol for 5 days before analysis. In tests with the total litter homogenate, 20 ml were incubated with 50 µg of the chlorophenol in order to give a more suitable volume for shaking. Other details were as for the fungal experiments.

Determination of 2,3,4,6-tetrachlorophenol and 2,3,4,6-tetrachloroanisole. After incubation, the whole culture, including the mycelium or bacterial cells, was extracted by a continuous steam distillation/extraction technique, and the chlorophenol and chloroanisole then separated and estimated by gas-liquid chromatography, after first converting the chlorophenol to the corresponding ethyl ether (Gee, Land & Robinson, 1974). These authors examined the recovery of chlorophenols and chloroanisoles from wood shavings and chicken tissues; their recoveries were not consistently quantitative and varied with the

chlorophenol or chloroanisole and with the nature of the sample. In the present work, the samples analysed contained far less organic matter and tests showed that recoveries of 2,3,4,6-tetrachlorophenol were essentially quantitative. By contrast, the recoveries of 2,3,4,6-tetrachloroanisole were in the range 65 to 100 %; results for this compound have not been corrected and are therefore minimum values. None of the conclusions drawn would be invalidated by variations in recovery within the above range.

RESULTS

Chlorophenol metabolism by pure cultures of fungi

In the screening procedure adopted, the fungi were first allowed to grow in the absence of the chlorophenol because the concentration used inhibits both spore germination and the growth of some species in liquid culture. Because the flasks were sealed after the addition of the chlorophenol, conditions in the flasks may not have remained aerobic for the whole of the subsequent five-day incubation. This experimental limitation was accepted to allow a large number of isolates to be screened with simple apparatus.

Of the 116 isolates tested, 99 were able to metabolize the chlorophenol (Table 1) and only four species, *Absidia cylindrospora*, *Aspergillus flavus*, *A. flavus* var. *columnaris* and *Mucor racemosus*, showed little or no activity. With *A. petrakii*, *A. repens*, *A. sydowi* and *Penicillium corylophilum* almost complete metabolism was observed with most or all of the isolates tested. With most of the other species the amount of chlorophenol metabolized varied with the isolate, e.g. within the seven isolates of *Scopulariopsis brevicaulis* values ranged from 0% to 87%.

Approximately two-thirds of the isolates that metabolized the chlorophenol produced greater than trace amounts of the 2,3,4,6-tetrachloroanisole. There were, however, wide differences between isolates in the proportion of the chlorophenol methylated. *Penicillium corylophilum* was the only species in which all the isolates examined showed a consistently high conversion. Isolates of the four species *A. candidus*, *A. chevalieri*, *P. brevicompactum* and *P. chrysogenum* gave little or no methylation, although several of these isolates metabolized most of the chlorophenol and two isolates of *P. brevicompactum* metabolized all of it.

The results obtained with 29 fungal isolates from litters associated with musty chickens showed no obvious divergence from the general pattern and in particular there was no evidence that these strains methylated more readily.

The above results were obtained after exposure of the fungi to the chlorophenol for a fixed period of five days. Where most of the chlorophenol disappeared without chloroanisole formation a transient accumulation of chloroanisole might have occurred at an earlier stage, and where a high methylation was observed there could have been subsequent metabolism of the chloroanisole. These possibilities were tested with *P. brevicompactum* US123 and *P. corylophilum* US160, respectively. The former metabolized all of the chlorophenol within 8 days (Fig. 1), and no chloroanisole was detected at any time before or after this period, suggesting that it is not formed when the chlorophenol is metabolized by this organism. With *P. corylophilum* most of the chlorophenol was metabolized within 2 days (Fig. 1), and the remainder disappeared much more slowly. The maximum concentration of the chloroanisole was reached after 4 to 6 days; thereafter there was a marked decrease. These results suggest that there may be two different pathways for the utilization of this chlorophenol, only one of which involves methylation.

Table 1. *Metabolism of 2,3,4,6-tetrachlorophenol by fungal isolates*

Values for metabolism and methylation are given as percentages, on a molar basis, of the 2,3,4,6-tetrachlorophenol added (98 to 137 μg , nominally 120 μg). The chlorophenol metabolized was taken at the difference between the residual chlorophenol and that found in the uninoculated control. The figures for percentage methylation are minimum values as the recovery of the chloroanisole was not quantitative (see Methods).

Species	Isolate	Percentage metabolized	Percentage methylated
<i>Absidia cylindrospora</i>	CD5	9	0
	CD119	0	0
	UD323	0	0
<i>Aspergillus amstelodami</i>	US90	95	39
	US11	26	2
	US196	25	3
	US28	8	Tr
	US177a	72	Tr
<i>A. candidus</i>	M29*	45	Tr
	US189	15	0
	US46	14	0
	S44*	10	0
	T3*	0	0
	US78b	0	Tr
	M33*	0	0
	US66	27	3
	US52	19	1
	US10	12	1
<i>A. chevalieri</i>	US63	9	0
	US50	0	Tr
	US13	100	45
	US264	32	Tr
	F61*	16	6
<i>A. clavatus</i>	F44*	13	2
	F68*	13	2
	US71	0	Tr
<i>A. flavus</i>	US67	0	Tr
	US222	0	Tr
	US77	0	0
	UD33	4	0
<i>A. flavus</i> var. <i>columnaris</i>	UD215	0	0
	CS14	35	2
<i>A. niger</i>	UD314	31	3
	CS142	17	4
	CS180	14	0
	L9*	4	8
	L10*	0	3
	UD130	99	40
<i>A. petrakii</i>	UD51	88	44
	US35	99	48
<i>A. repens</i>	S51*	99	61
	US31	99	61
	US21	99	25
	T24*	97	74
	US142	97	45
	M40*	99	64
	US22	35	27
	US12	14	0
	US98	100	30
	L14*	92	31
<i>A. ruber</i>	US43	32	3
	US91	28	0
	L13*	24	8
	US97	18	Tr
	US153	8	Tr

Table 1 (continued)

Species	Isolate	Percentage metabolized	Percentage methylated
<i>Aspergillus sydowi</i>	Q9*	100	74
	US194	100	27
	S42*	97	48
	M38*	95	42
	US135	92	22
	S37*	87	56
	US15	83	62
	US103	27	10
	T21*	22	17
<i>A. versicolor</i>	US184	98	42
	US61	89	34
	US120	81	80
	US228	69	18
	M37*	47	6
	M35*	37	19
	S60*	9	7
<i>A. wentii</i>	CS112	30	0
	UD271	4	Tr
<i>Mucor racemosus</i>	CD56	0	0
	US100	0	0
<i>Paecilomyces varioti</i>	CS18	100	81
	CS21	69	56
	CS84	34	14
	CS11	16	11
<i>Penicillium brevicompactum</i>	US123	100	Tr
	CS121b	97	Tr
	US62	28	0
	CS109	0	Tr
<i>P. chrysogenum</i>	US198	52	0
	US49	40	Tr
	US75	35	Tr
	US72	15	0
<i>P. corylophilum</i>	US113	98	80
	US162	97	62
	US160	96	83
	US117	95	75
<i>P. crustosum</i>	US59	71	2
	T12*	57	3
	R13*	47	Tr
	US201	46	16
	US185	41	7
	L3*	38	14
	S33*	25	6
	US129	16	Tr
<i>P. frequentans</i>	US52	86	54
	CS127	30	Tr
	US24	22	0
	US30	4	0
<i>P. roqueforti</i>	CS121a	99	65
	CS126	72	27
	CS129	53	8
	CS158	12	7
<i>Rhizopus oryzae</i>	CS97	55	Tr
	CS155	0	0
<i>Scopulariopsis brevicaulis</i>	D6*	87	60
	US178	52	6
	US17	23	1
	US1	20	Tr
	US171	15	Tr
	T8*	6	1
	M7*	0	Tr

Tr, a definite trace but less than 1 %.

* Isolate obtained from litter associated with musty chickens.

Fig. 1. Metabolism of 2,3,4,6-tetrachlorophenol by *Penicillium brevicompactum* US123 and *P. corylophilum* US160. Replicate cultures were grown for 6 days at 25 °C before addition of the chlorophenol at time 0. A single culture was taken for analysis at each of the times indicated thereafter. ○, 2,3,4,6-tetrachlorophenol in *P. brevicompactum* culture; ●, 2,3,4,6-tetrachlorophenol in *P. corylophilum* culture; □, 2,3,4,6-tetrachloroanisole in *P. brevicompactum* culture; ■, 2,3,4,6-tetrachloroanisole in *P. corylophilum* culture.

Chlorophenol metabolism by mixed bacterial populations

When the chlorophenol was incubated with a mixed bacterial suspension from broiler house litter, it was completely metabolized within 5 days and no chloroanisole was detected. The total homogenate of poultry litter metabolized 85 % of the chlorophenol in 5 days and produced a small but definite trace of chloroanisole. The formation of this chloroanisole was attributed to the presence of fungal fragments in the total homogenate. The effect of anaerobic conditions on the activity of this bacterial fraction was not tested, but in preliminary experiments, using mixed bacterial populations isolated from litter on a complex medium, no disappearance of the chlorophenol was observed under anaerobic conditions.

DISCUSSION

The methylation of 2,3,4,6-tetrachlorophenol by isolates of *Aspergillus sydowi*, *Penicillium crustosum* and *Scopulariopsis brevicaulis* has already been demonstrated (Curtis *et al.* 1972, 1974) and the present results show that isolates from at least 16 fungal species are able to carry out this methylation. Several isolates that we have regarded as giving a negative response in fact formed trace amounts of 2,3,4,6-tetrachloroanisole, and might well have been more active under appropriate conditions. *Aspergillus sydowi*, *P. crustosum* and *S. brevicaulis* methylate pentachlorophenol as well as the tetrachlorophenol (Curtis *et al.* 1972), and the same organisms also methylate 2,4,6-trichlorophenol (Gee, unpublished observations). The methylation of pentachlorophenol has also been reported with six species of *Trichoderma* (Cserjesi, 1972; Cserjesi & Johnson, 1972), and with a *Bacillus* species from soil (Suzuki & Nose, 1971). The ability to methylate highly-chlorinated phenols

is not therefore restricted to a few organisms and, in particular, is widespread among the fungi of broiler house litters.

The ability to metabolize 2,3,4,6-tetrachlorophenol is even more widespread among the fungi tested than the ability to produce the chloroanisole. This chlorophenol was also metabolized without detectable methylation by mixed suspensions of bacteria from broiler house litter. There are several reports of the metabolism of pentachlorophenol by both fungi and bacteria (Ingols & Stevenson, 1963; Chu & Kirsch, 1972; Ide *et al.* 1972; Alliot, 1973; Kirsch & Etzel, 1973), and of the 2,4,6-trichlorophenol by mixed bacterial cultures (Ingols, Gaffney & Stevenson, 1966). Present evidence indicates that the removal of chlorophenols from broiler house litter, whether by methylation or otherwise, is due to microbial action. It involves many different species and probably more than one metabolic route.

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