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Observations of the effects of formaldehyde on cockroaches and their flora: III. The effect of formaldehyde in eliminating the normal gut flora

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SUMMARY

The normal flora of cockroaches (*Periplaneta americana*) was determined over a period of 24 days prior to substituting water with 1% Formalin for drinking water. During the first 4 days of treatment the normal flora was significantly reduced and by the fifth day, when the cockroaches became diarrhoeic, no bacteria, fungi, or viruses could be detected by the methods used.

INTRODUCTION

In previous studies we have shown that cockroaches are remarkably resistant to gaseous (Bartzokas, McCarthy, Shackleton & Baker, 1978) and to imbibed (McCarthy, Bartzokas & Baker, 1978) formaldehyde. When vaccinia virus-infected cockroaches were exposed to fumigation, virus was found to persist throughout the fumigation process in the gut and faeces of many of the insects. We speculated that those insects in which we failed to find residual virus might have drunk condensate containing formaldehyde during the fumigation process. We verified that cockroaches could tolerate concentrations of formaldehyde up to 1·6%, i.e. 4% commercial Formalin, for up to 22 weeks (McCarthy *et al.* 1978). Since this concentration is much higher than that necessary to kill most bacteria and viruses, this speculation appeared to be a reasonable one.

In this paper therefore we have investigated the effect on gut flora of continued administration of 1% Formalin (0·4% formaldehyde) to adult normal cockroaches.

MATERIALS AND METHODS

Cockroaches

Adult *Periplaneta americana* (L.) were kindly supplied by Professor C. J. Duncan, Department of Zoology, University of Liverpool, or by a commercial supplier.

Cockroach diet

Fox Facilities General (Mouse) (FFG(M)) pellets were supplied by E. Dixon & Sons (Ware) Ltd. (Crane Mead Mills, Ware) and used crumbled. Samples, as supplied, were tested bacteriologically before use and were found either to be sterile or to yield an occasional colony of the Hafnia group.

Formaldehyde

Formaldehyde solution 40% (w/v) (May and Baker, Dagenham) stored at 30 °C to limit polymerization was diluted 1/100 with distilled water.

Bacteriological media

Cystine-Lactose-Electrolyte Deficient agar (CLED) (CM 423), MacConkey agar without salt (CM 7b), defibrinated Horse Blood agar (SR 50), Sabouraud Dextrose agar (CM 41) (with 0·5 μg/ml chloramphenicol) and defibrinated Horse Blood agar (with 100 μg/ml kanamycin), were supplied (without the antibiotics) by Oxoid. The antibiotics were added immediately before plate pouring to increase the selectivity of the commercial media for yeasts and anaerobes. For final identification of certain non-lactose-fermenting coliforms the API 20E system was used, as supplied by API Laboratory Products Ltd. (Philpot House, Rayleigh, Essex). Formaldehyde estimations were made using the colorimetric method of Nash (1953).

Tissue culture

African Green Monkey Kidney (Vero) cell monolayers were inoculated and inspected for cytopathic effects up to 7 days in Parker 199 (Wellcome) medium containing 2% fetal calf serum, 0·22% sodium bicarbonate, penicillin 100 i.u., streptomycin 100 μg, gentamicin 40 μg and nystatin 50 i.u./per ml.

Disinfectant

1% solution of sodium hypochlorite.

Experimental methods

Cockroaches were placed into specially designed boxes described elsewhere (Bartzokas *et al.* 1978). Three identical (A, B, C) boxes were used, each containing eight cockroaches. All boxes were treated alike. Results are shown for each box, not for individual cockroaches.

Cockroaches were fed on crumbled FFG(M) pellets and distilled water for a period of 24 days. During this time a total of 264 faecal pellets were collected at random intervals, when faeces were available, and those at any one time from one box were pooled and treated as one sample. This was to obtain quantitative and qualitative information about the normal microbiological faecal flora of these cockroaches.

On the 25th day distilled water was replaced by a 1/100 dilution of Formalin, i.e. 0·4% formaldehyde, prepared fresh daily. Cockroaches were maintained on this diet of 1% Formalin and FFG(M) pellets for 9 consecutive days. Faeces were

collected daily from individual boxes when available. The collection trays were chemically disinfected with hypochlorite for 15 min, well rinsed in sterile distilled water, dried and replaced immediately after each collection. Each 'pool' of solid faeces was treated as follows: triturated faecal pellets, collected on any one occasion, were placed in a pre-weighed 28 ml glass bottle (Universal), weighed again and sterile distilled water added to give a 10% suspension. This material was streaked on culture plates for individual colony production, incubated at 35·5 °C aerobically and anaerobically for qualitative estimations. A range of ten-fold dilutions of the faecal suspension was also made and samples plated out according to the Miles and Misra technique for quantitative study. All plates for mycological examination were duplicated, an additional set being incubated at room temperature (*ca.* 20 °C). All aerobic bacteriological plates were read at 24 h, anaerobic at 48 h and mycological plates at 48 h and then inspected daily for 7 days. Identification was carried out to genus level in most instances, however some of the non-lactose fermenting coliforms and anaerobes were further typed using the API 20E system. Quantitative counts were expressed as the number of each species of organism and also total colony-forming unit (c.f.u.) count per gram of faeces.

We did not look for protozoa. Tests for viruses were limited to agents capable of causing cytopathic effects in Vero cells in 7 days.

RESULTS

Cockroaches drank 0·4% (w/v) formaldehyde solution avidly. However, after 4 days they developed diarrhoea, which persisted for 5 days, when the experiment was terminated. During the period of the diarrhoea their avidity for drinking formaldehyde solution was unaltered and they kept producing up to 0·2 ml of dark brown stained faecal fluid daily; this tended to dry fairly rapidly. Formaldehyde was detected chemically in all diarrhoeic specimens tested, but the rapid evaporation and small volume made exact formaldehyde estimation impossible.

Although all specimens were treated with 1% sodium metabisulphite to neutralize residual formaldehyde, no micro-organisms were grown from these diarrhoeic specimens.

Table 1 shows that the flora of these cockroaches fed on a normal diet for the first period of 24 days was fairly consistent from box to box and contained a wide range of organisms. There were very occasional colonies of *Clostridium sporogenes* not shown on the table; the fungi were all of the 'airborne' type (i.e. *Alternaria* spp., *Cephalosporium* spp., *Geotrichum* spp., *Rhizopus* spp. and *Rhodotorula* spp.) and no virus cytopathic for Vero cells was isolated. Some of the potentially human pathogenic strains of non-lactose-fermenting coliforms were identified using the API 20E system. Most of the frequently isolated strains were identified as *Enterobacter* spp. and *Citrobacter* spp.

Formaldehyde solution replaced drinking water on the 25th day and during the succeeding 4 days aerobic spore-bearing bacilli and lactose-fermenting coliforms became undetectable. Pigmented non-sporing gram-positive bacilli and airborne

Table 1. *Effect of drinking formaldehyde on gut flora in three similar batches of cockroaches*

Main groups of organisms/g of faeces	Period in days	Batch A	Batch B	Batch C
Coagulase −ve, gram +ve cocci and bile-tolerant streptococci	0–24 (Water)*	3×10^8–2×10^{10}	9×10^9	7×10^8–2×10^9
	25–28 (HCHO)†	2×10^8–7×10^{10}	5×10^8–3×10^{11}	6×10^8–2×10^{11}
	29–33 (HCHO)‡	No growth	No growth	No growth
Aerobic spore-bearing bacilli	0–24 (Water)*	8×10^4–10^8	4×10^5–7×10^7	10^7
	25–28 (HCHO)†	No growth	No growth	No growth
	29–33 (HCHO)‡	No growth	No growth	No growth
Pigmented non-sporing gram +ve bacilli	0–24 (Water)*	8×10^7	Not detected	10^7–2×10^8
	25–28 (HCHO)†	10^7	Not detected	No growth
	29–33 (HCHO)‡	No growth	Not detected	No growth
Lactose-fermenting coliforms	0–24 (Water)*	10^7–2×10^8	2×10^9	5×10^8–10^9
	25–28 (HCHO)†	No growth	No growth	No growth
	29–33 (HCHO)‡	No growth	No growth	No growth
Non-lactose fermenting coliforms	0–24 (Water)*	2×10^7–2×10^9	Not detected	8×10^7
	25–28 (HCHO)†	4×10^6–10^9	Not detected	10^6–3×10^8
	29–33 (HCHO)‡	No growth	Not detected	No growth
Yeast-like organisms	0–24 (Water)*	4×10^5–1×10^8	2×10^7–2×10^9	10^6–10^8
	25–28 (HCHO)†	8×10^3–10^8	10^7	6×10^5–4×10^7
	29–33 (HCHO)‡	No growth	No growth	No growth
Airborne fungi	0–24 (Water)*	10^6	2×10^5–8×10^6	3×10^8
	25–28 (HCHO)†	No growth	10^7	No growth
	29–33 (HCHO)‡	No growth	No growth	No growth

* Figures show minimum and maximum values obtained in four random timed samples within the 24-day period.

† Figures show minimum and maximum counts in each of four daily samples.

‡ Figures show minimum and maximum counts in each of five daily samples.

fungi were reduced or eliminated, whilst coagulase negative gram-positive cocci, bile-tolerant streptococci, non-lactose-fermenting coliforms and yeast-like organisms, seemed to be unaffected.

In the third period characterized by the appearance of diarrhoea no growth whatsoever was detected on any medium.

In the above tests on faeces from formaldehyde-treated insects the concentration of sodium metabisulphite used (1 %) to neutralize residual formaldehyde had been shown in preliminary experiments not to be bacteriostatic.

DISCUSSION

When the residual formaldehyde in the faecal extracts was neutralized with sodium metabisulphite, no bacterial growth occurred. Since the neutralizing salt is not toxic to bacteria at the concentration used, it can be assumed that no viable bacteria or fungi remained.

The findings here first raise the question of how cockroaches deal with such substantial quantities of formaldehyde in their fluid intake. The fact that they

drink is evident from observations but the quantity imbibed is not easily measured. It is, however, possible to determine from the frequent voiding of at least 0·2 ml quantities of diarrhoeic faeces daily, that a considerable increase over the normal fluid intake had been induced. We know from previous experience (Bartzokas *et al.* 1978; McCarthy *et al.* 1978) that such an intake and output can be maintained for up to 22 weeks. How much is absorbed from the gut is not known. There seemed to be no interference with the normal activities of the insects. In one experiment reported previously (McCarthy *et al.* 1978) fertile eggs were laid and hatched.

Although accurate assay of formaldehyde in the faeces was not possible it was regularly detected. The sterilization of the gut implies that a concentration was reached which is bactericidal. Thus the aim of the experiment – to discover whether bactericidal concentrations were compatible with continued life of the cockroach – is satisfied.

A somewhat surprising finding was the delay of 4 days after making formaldehyde solution available, before the faeces became sterile. A possible explanation may have been a holding back or limitation from drinking; we imposed no prior withdrawal of water to generate thirst. Under these circumstances bactericidal concentrations of formaldehyde may not have built up in the gut till the fifth day. The sudden onset of diarrhoea would have tended to wash out the gut and at the same time generate thirst and by a 'positive feedback' mechanism provoke more diarrhoea. This effectively seemed to purge the insects of their solid faecal burden.

One of the findings in the first paper of this series was that adult cockroaches withstand washing in 2 % glutaraldehyde in an ultrasonic bath for 15 sec and that this sterilizes their external surface. In the second paper we showed that cockroaches will tolerate 1 or 4 % Formalin or 2 % glutaraldehyde as 'drinking water' for weeks or months. In this paper it is shown that 1 % Formalin renders the gut sterile after 4 days. These findings appear to provide a ready and simple means of establishing colonies of axenic cockroaches.

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