Effect of Diethylstilbestrol on Feedlot Associated Bacteria

by
D. W. THAYER, D. W. UNFRED, and R. K. ANDO
Department of Biology
Texas Tech University
Lubbock, Tex. 79409

Diethylstilbestrol (DES) was used as an ingredient of cattle feed for many years because it increased the rate of weight gain. Unfortunately, this synthetic steroid hormone is not degraded significantly in manure and accumulates in cattle feed wastes and recently has been banned for use in feeds because it is a potential cause of cancer. Its use in implants, however, is still allowed. Unfred (in preparation) has found DES in stockpiled manure in Texas feedlots at approximately one-half the concentration given in feed. Such concentrations of this powerful steroid represent possible health hazards if the manure is to be used elsewhere, or from dust and runoff from the stockpiled waste. The lack of degradation is not surprising since a number of reports have indicated that in pharmacological concentrations (e.g. 1-20 μg/ml) DES is inhibitory to many Gram positive bacteria (YOTIS and BAMAN, 1969a, b). Yotis and Baman (1970) found that the action of DES on staphylococci was due to cellular leakage.

This manuscript describes three studies of the possible interaction of DES with bacteria contained in stockpiled beef cattle feedlot waste. Many authors have proposed the recycling of feedlot waste either with or without conversion into single-cell protein for eventual reuse in feeds (ANTHONY, 1969). This would imply the desirability of the isolation of organisms which are capable of degrading the hormone which was the subject of the first study reported here. A second study was designed to determine the response of feedlot waste associated bacteria to DES. A third study investigated the fate of the steroid when a very sensitive species of Bacillus and a relatively insensitive Pseudomonas species were grown in its presence.

MATERIALS AND METHODS

Enrichment culture technique. Ten grams of stockpiled cattle feedlot waste were used as an inoculum for 100 ml of medium containing: $(NH_4)_2SO_4$, 0.2 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.2 g; $FeCl_2$, 0.001 g; diethylstilbestrol, 0.500 g (Sigma Chemical Co.); yeast extract, 0.10 g and 1000 ml water. Ten ml of culture were transfered to 100 ml of fresh medium for a total of 18 transfers.

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Viability of the cultures were checked on Tryptic Soy Agar (Difco) at each transfer. A number of highly resistant bacterial cultures were isolated from these enrichments. On occasion the cultures were propogated on Tryptic Soy Agar in order to increase the inoculum between transfers.

Taxonomy. Methods outlined by Skerman (1967) were used for the identification of the isolated bacterial strains. Flagellation was determined by examination of negatively stained cells (2% phosphotungstic acid buffered at pH 7.4) with a Hitachi HU-8 electron microscope.

Growth requirements. Growth requirements of the bacteria were determined by culture in the mineral salts medium described above containing agar (15 g/l), and glucose (0.1 g/l), DES (0.50 g/l), or yeast extract (0.1 g/l) as appropriate. The relative amounts of growth were compared to that on Tryptic Soy Agar.

DES sensitivity. The sensitivity of several cultures which had been isolated in a previous study (THAYER et al. 1973) from stockpiled feedlot waste were assayed for their sensitivity to DES by the filter paper disc technique. Discs 5 mm in diameter were prepared from Whatman # 1 filter paper. These discs were saturated with varying concentrations of DES and then sterilized by autoclaving for 10 min at 121 C. The following concentrations of DES dissolved in absolute ethanol were used: 0.0, 0.020 g/ml, 0.004 g/ml and 0.0008 g/ml. A deep base of Standard Methods Agar (BBL) in Petri plates was overlaid with 10 ml of inoculated agar. Three replicate plates were prepared by aseptically placing the DES discs at each concentration on each plate. The plates were then incubated at 30 C. Zones of inhibition were measured to the nearest mm after 48 hr of growth.

Inhibition by and fate of DES in complex medium. The growth of Pseudomonas aeruginosa and Bacillus subtilis W23 in Tryptic Soy Broth containing various amounts of DES at 37 C was followed by viable cell counts. The cultures were grown in 20 ml of medium after inoculation with 0.1 ml of culture in 500 ml baffled Erlenmeyer flasks. Spread plates were prepared in triplicate from each dilution. The amount of DES remaining in the culture and that absorbed by the cells was determined by the method of CHENG and BURROUGHS (1955).

DES effect on pigment production by Pseudomonas aeruginosa. Pseudomonas aeruginosa was cultivated in Pseudomonas P broth (Difco). After the addition of 0.2 ml of 0.1N NaOH to 20 ml of cell-free sample, pyocyanin was extracted from the culture with five 10 ml aliquots of $CHCl_3$. The pyocyanin was retained in the $CHCl_3$ and the DES retained in the aqueous layer. The technique was a modification of the procedure of BLACKWOOD and NEISH (1957).

RESULTS

Four distinctly different Gram negative motile rods were obtained from the DES enrichment cultures. Eighteen successive transfers assured that no organic material carried over from the original inoculum. In the DES medium, very short paired rods and elongated cells occurred in mature cultures, indicating possible cell damage. Four different bacteria were isolated, two species of Aeromonas, one Pseudomonas and one Serratia. Growth of these cultures was compared in several different media (Table 1).

Table 2 presents the results of the tests for DES sensitivity of the four organisms isolated above compared to the sensitivity of controls and cultures isolated from stockpiled feedlot waste. Bacillus and Cellulomonas species were the most sensitive and the DES enrichment isolates the most resistant. Pseudomonas and Escherichia species were also very resistant. Pigmentation of the Serratia species RA II-2 was increased by the presence of DES. A distinct oligodynamic effect was found with several of the sensitive strains as is demonstrated in Fig. 1.

Two cultures were selected to determine the fate of DES in culture medium. Bacillus subtilis W23 was selected because of its DES sensitivity, and Pseudomonas aeruginosa because of its lack of sensitivity. The results of the viable cell counts of these cultures with varying concentrations of DES is reported in Fig. 2. The analysis of the media, cell washings and cells for DES is presented in Table 3. Although the DES did not appear significantly to alter the growth rate of P. aeruginosa, it did alter the production of pyocyanine (Fig. 3).

DISCUSSION

The initial purpose of this study was to isolate bacteria capable of using DES as their only carbon source. This resulted in the isolation of four extremely resistant cultures whose growth is enhanced by the presence of DES when they are growing on a yeast extract, glucose mineral salts agar (Table 1).

The preponderance of isolates sensitive to DES which were obtained from feedlot waste indicates a lack of selection in the waste. The great sensitivity of most of these organisms may be important in reducing the rate of degradation of the waste. Several Cellulomonas species which were studied because of their known ability to degrade cellulose were found to be extremely sensitive to the hormone. The sensitivity of these organisms is such that they could be used for microbial assay of DES in very small concentrations. The diameter of the zone of inhibition for several species

TABLE 1

Relative growth of DES enrichment culture isolates compared to growth on Tryptic Soy Agar

Additives to medium*	Aeromonas sp. RA12	Pseudomonas sp. RA21	Serratia sp. RA22	Aeromonas sp. RA31
DES + yeast extract	10%	19%	24%	14%
DES, yeast extract, glucose	72%	67%	72%	67%
DES, yeast extract, cassamino acid	19%	14%	29%	19%
DES	14%	14%	29%	33%
yeast extract	24%	38%	38%	33%
none	5%	14%	10%	19%
glucose	29%	72%	57%	57%
yeast extract, glucose	14%	43%	43%	29%

*Basal salts medium described in text

TABLE 2

Sensitivity of feedlot waste associated organisms and control cultures to DES

Strain	Source	Diameter of inhibition zone (mm) DES concentration (g/ml)			
		0.008	0.004	0.02	
Bacillus megaterium	FLW	7.7	13	15	
" "	FLW	9.0	12	13	*
" "	FLW	7.7	10	14	*
Bacillus licheniformis	FLW	10	13	15	*
Bacillus cereus	FLW	11	10	13	*
" "	FLW	10	8.3	11	
" "	FLW	7	9.7	11	
" "	stock	10	11	13	
Bacillus subtilis	FLW	7.7	11.3	14.7	
" "	FLW	8.3	9.0	11.3	*
" " W23	stock	8.3	10	14.3	*
Bacillus pulvifaciens	FLW	10	12.3	13.7	
Bacillus lentus	FLW	9	17.7	20.3	
Bacillus laterosporus	ATCC64	8.0	13.3	15	*
Bacillus firmus	FLW	8.3	13.3	14.3	
Brevibacterium insectiphilium	FLW	10	15.7	17.3	
Brevibacterium vitarumen	FLW	0	10.7	13.7	
Brevibacterium tegumenticola	FLW	8.7	11.0	12.7	
Escherichia coli	FLW	8	9.3	10	
Alcaligenes faecalis	FLW	0	0	9.7	
Neisseria caviae	FLW	0	6.3	7.0	
Streptococcus faecalis	ATCC6057	6.3	9.0	10.3	
Flavobacterium rhenanum	FLW	9.7	12.3	11.7	
Staphylococcus aureus	FLW	8.7	9.7	13.7	
" "	FLW	8.0	9.7	11.0	*
Cellulomonas biazotea	ATCC486	15.7	18.7	20.3	
Cellulomonas cellasea	ATCC484	13.0	19.7	32.0	

TABLE 2
CONTINUED

Sensitivity of feedlot waste associated organisms and control cultures to DES

Strain	Source	Diameter of inhibition zone (mm) DES concentration (g/ml)		
		0.008	0.004	0.02
Cellulomonas gelida	ATCC488	7.7	21.3	25.7
Cellulomonas uda	ATCC491	9.0	17.0	20.7
Cellulomonas subalbus	ATCC489	16.0	22.5	26.5 *

The growth of the following were not inhibited by the presence of DES:

Achromobacter parvulus	FLW
Escherichia coli 82/r	(ANDERSON, 1951)
Escherichia coli	FLW
Escherichia coli	ATCC8750
Escherichia coli	ATCC11303
Pseudomonas aeruginosa	ATCC10145
RA12 *Aeromonas* sp.	FLW
RA21 *Pseudomonas* sp.	FLW
RA22 *Serratia* sp.	FLW
RA31 *Aeromonas* sp.	FLW

1. *An oligodynamic stimulation of growth was observed.
2. Each result is the average of three determinations.
3. Growth was not inhibited in any case by filter paper discs which did not contain DES.

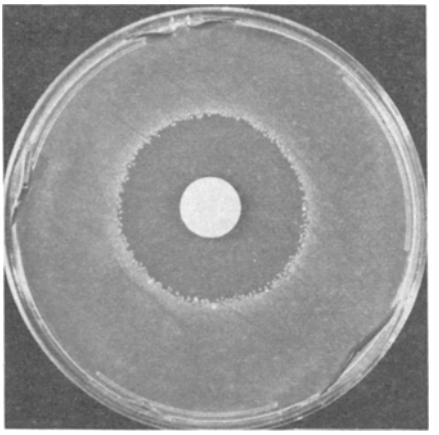

Fig. 1. Inhibition of growth and oligodynamic stimulation of growth by DES. The medium is Tryptic Soy Agar seeded with a 16 hr culture of B. subtilis W23 on the surface. A Whatman antibiotic assay disc was used to hold the DES and was prepared by dipping it into ethanol containing 0.020 g of DES/ml.

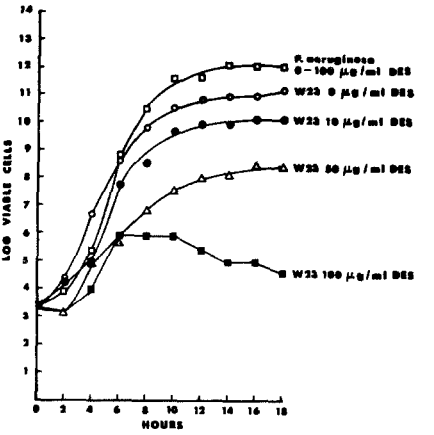

Fig. 2. Inhibition of the growth of B. subtilis W23 and the lack of inhibition of P. aeruginosa by DES. The medium and conditions of culture are described in the materials and methods. Two controls were included for each bacterium: 1st, a Tryptic Soy Broth culture and 2nd, Tryptic Soy Broth plus 2% ethanol. No inhibition of growth resulted from the addition of ethanol. The latter control was included since the DES was added to the medium dissolved in ethanol.

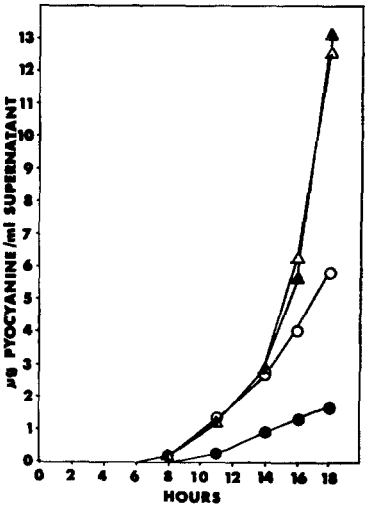

Fig. 3. DES inhibition of pigment production by P. aeruginosa in Pseudomonas P broth.

▲ Pseudomonas P broth;
△ broth + 2% ethanol;
o broth + 10 µg/ml DES;
● broth + 100 µg/ml DES.

TABLE 3

Adsorption of DES by cells

a. Hours incubation	Bacillus subtilis % DES in supernatant	% DES in wash	% DES in cells
3	60	trace	40
4	4.0	0	96
6	0	0	103
8	0	0	99
10	0	0	98

b. Hours incubation	% DES in supernatant	% DES in wash	% DES in cells
3	57	trace	46.5
4	11	0	85.5
6	9	0	94.5
8	0	0	99.5
10	0	0	97.0

The Tryptic Soy Broth contained 10 μg of DES/ml at the start of each experiment.

of Cellulomonas is linear with respect to the log of the DES concentration, which is identical to the response found during microbial assay of antibiotics. The sensitivity of such a microbial assay for DES should be at least as good as the official procedure (AOAC) and possibly better. This remains to be investigated in detail.

Although the authors tend to agree with Yetis and Baman (1969, 1970) that generally the Gram positive organisms are sensitive, several Gram negative bacteria sensitive to DES were found in this study. E. coli, Alcaligenes faecalis, and Neisseria caviae were isolated from feedlot waste which were sensitive to the drug. Although there appeared to be no inhibition of the growth of Pseudomonas aeruginosa species nor of the Serratia sp. RA22, there were very pronounced effects on the pigments produced by these organisms. Pyocyanin production was greatly reduced whereas the production of prodigiosin by Serratia was increased. Both B. subtilis and P. aeruginosa concentrate the hormone in or on the cell surface (Table 4). The hormone was not removed by washing. This indicates that a very serious toxicology problem may exist where feedlot waste from older feedlots is refed to cattle or when it is used as a substrate for the growth of bacteria, since the bacteria in the rumen or in commercial fermenters may actually concentrate the DES from the waste.

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REFERENCES

ANDERSON, E. H., Proc. Nat. Acad. Sci. 37, 340 (1951).
ANTHONY, W. B., Animal Waste Management, Cornell University Conference pp 105-111. (1969).
BLACKWOOD, A. C. and A. C. NEISH, Can. J. Microbiol. 3, 165 (1957).
CHENG, E. W. and W. BURROUGHS, J. A. O. A. C. 38, 146 (1955).
AKERMAN, V. B. D. A Guide to the Identification of the Genera of Bacteria 2nd ed. (1967). Williams and Wilkins, Baltimore.
THAYER, D. W., S. P. LEWTER, J. BARKER and J. J. J. CHEN, Bull. Environ. Contam. Toxicol. 11, (in press).
YOTIS, W. W., and S. I. BAMAN, Yale J. Biol. Med. 41, 311 (1969a).
YOTIS, W. W., and S. I. BAMAN, Yale J. Biol. Med. 41, 323 (1969b).
YOTIS, W. W., and S. I. BAMAN, Appl. Microbiol. 19, 474 (1970).