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A COMPARATIVE STUDY OF THE ACTIONS OF SIX TETRACYCLINES ON THE DEVELOPMENT OF THE CHICK EMBRYO

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The series of studies of Bevelander and his colleagues, conveniently summarized in his letter (1963), together with the observations collected by Shils (1962), has focused attention on the potentially toxic nature of the tetracyclines particularly in embryos and in developing tissues. It was felt that a comparison should be made between older and newer, more soluble tetracyclines to try to discover whether alteration in the molecule had increased toxicity and whether any teratogenic effects could be detected.

METHODS

The design of the first experiment was based as closely as possible on the blind trial. Solutions of tetracycline, oxytetracycline, chlortetracycline, demethylchlortetracycline, lemecycline and chlormethylencycline were made up in Dulbecco's phosphate-buffered saline (Oxoid), *p*H 7.3, the buffer itself being introduced as one control, a second being provided by the remainder of the batch which was not injected or manipulated in any way. The concentrations used were those commonly found in the blood during therapy, about 2.5 μg/ml. (125 μg contained in 0.2 ml. of buffer for each 50 g of egg). As the concentration in cord blood is half that in the mother, even this level would be higher than that to which an infant would be exposed (Charles, 1954; Gibbons & Richelderfer, 1960). We have ourselves tested paired maternal and cord bloods and have obtained concordant results.

A hatchery made available a batch of eggs from chickens with high fertility. On the 4th day of incubation these were candled and infertile eggs were discarded. Twenty eggs were placed in each group, the injection site marked, waxed and perforated. Volumes of 0.2 ml. of the drug were injected and the shells were resealed by melting the wax. Injections were made into the allantoic cavity through the corion.

In a second series using ten-times the previous level of drug, the tetracyclines were prepared as before, but more stringent sterility tests of the solutions were made. The drugs were dissolved and diluted for use within 2 hr of being injected to avoid as far as possible any loss of potency while in solution. They were not however refrigerated during transportation.

The concentrations of the drugs were on a simple weight basis and allowances were not made for differences in molecular weights.

The assessment of the health of the chicks was made by the hatchery. The nature of the drugs administered was not known, the groups being identified only by batch letter. Chicks killed by gassing and unhatched eggs were then forwarded for further work. The unhatched embryos were dissected and examined for any abnormality. Representative bones were selected for histology after examination for fluorescence. These were compared with those of the survivors from the respective

groups, which had been gassed and refrigerated 24 hr after hatching. Measurements were made in all the chicks of the length from beak to rump, the length of tarsometatarsal bones and the body weights. Orthodox χ^2 tests were used.

Longitudinal sections of the tibia of one animal from each group and of a control were decalcified in edetic acid and stained with haematoxylin and eosin (Lee, 1963). Undecalcified longitudinal and transverse sections of tibia and femur were mounted in Fluorfree (G. T. Gurr) and examined in ultraviolet-blue light (410 mμ primary filter, Zeiss, BG 12) with a secondary pale yellow filter, Zeiss 53, which transmitted light of wavelength above 525 mμ.

RESULTS

Macroscopic fluorescence

The bones of all the treated embryos fluoresced characteristically. The intensity varied with the age of the embryos. The fluorescence was strongest in the smallest embryo and decreased steadily to the largest. This suggests that there was continuous redistribution with the growth of the skeleton ; no evidence of zoning or of other irregularities could be seen by naked eye. Some variation in intensity could be seen in the bones of the skull including the orbit. This was taken to indicate only variation in total thickness of material from the edges to the thinner central table. The yolk was always strongly fluorescent until completely used up. No evidence of deposition in internal organs was found except for certain tendinous areas, particularly the anterior part of the gizzard and the attachments of the thigh to the abdomen where the edge of the folds was particularly bright. The deposits of fat at the side of the neck were also affected. In the hatched chicks the intensity had fallen so much that photography became impracticable. No differences between the bones of hatched chicks of the various groups could be seen.

Histology

In the stained material the structure of the woven bone of the cortex and of the primary spongiosa showed no appreciable abnormality and the morphology of the osteoblasts and osteoclasts was within normal limits.

By fluorescent microscopy the tetracyclines were found to be diffusely distributed throughout the cortex. The intensity was comparable but the colour properties of the different tetracyclines varied slightly ; tetracycline, demethylchlortetracycline and chlortetracycline had a greenish yellow appearance in the bone, while the remainder were yellow.

No structural abnormalities were detected in the unhatched chicks.

Death rates, growth and deformities

The death rates for the groups in experiment 1, using clinical levels of the drugs, are recorded in Table 1. From the deaths those due to trauma at the time of inoculation and those due to fungal and bacterial infections have been subtracted to give a corrected figure for the size of the groups and for the death rate. The rate of hatching expected for these eggs based on very large numbers over a period of time was 85% and both control groups are obviously very close to this figure. Independent assessment, again without disclosure of the nature of the groups, failed to show any significant differences at the $P = 0.05$ level. No method of combining the groups, for example all the tetra-

TABLE 1

ACTION OF TETRACYCLINES INJECTED INTO CHICK EMBRYOS ON THE FOURTH DAY OF INCUBATION, ON DEATH RATE, SIZE AND BONE GROWTH UP TO THE TIME OF HATCHING

The dose of drugs was 2·5 μg/g. Values in the last three columns are means and standard deviations.
* Not recorded

Group	Drug	Original no. in group	Corrected group no.	Deaths	Live weight (g)	Length of tarsometatarsal (cm)	Length beak to rump (cm)
Controls	None	48	48	7	*	*	*
X	Buffer alone	10	9	0	34·65±1·4	1·9±0·05	11·5±0·4
A	Tetracycline	20	19	3	34·6 ±1·35	1·9±0·05	11·2±0·25
B	Lemecycline	20	19	2	35·0 ±1·5	1·9±0·05	11·3±0·3
C	Oxytetracycline	20	15	3	35·5 ±0·9	1·9±0·04	11·5±0·25
D	Demethylchlortetracycline	20	16	6	36·6 ±1·7	1·9±0·01	11·5±0·2
E	Chlormethylencycline	20	16	3	35·55±1·7	1·9±0·05	11·5±0·25
F	Chlortetracycline	20	18	4	36·1 ±2·3	1·9±0·03	11·3±0·3

cyclines against controls, soluble against less soluble, old against new preparations, disclosed significant differences. The figures for group D are however very near to this level of significance.

Measurements of weight, or bone length or total body length arranged as histograms fall into normally distributed population curves. Length appears more accurate than weight, which is influenced by unabsorbed yolk. Mature chicks are lighter than those dead in the shell with yolk sac unruptured.

Experiment 2 was carried out at a level where effects on growth could be anticipated. Preliminary titrations, starting with the previous level, 2.5 μg, and increasing by doubling up to eight times the original dose, failed to show changes except in the highest concentration where some inhibition of growth seemed to occur. This dose was taken as the lowest useful one and in fact ten times the level in the original experiment was chosen. The remainder of the procedure was identical with that in the earlier series.

Table 2 has been drawn up to correspond to Table 1 in all particulars thus enabling comparisons to be made between the individual drugs with one another, with controls and with the lower doses used in the first experiment.

With regard, first, to the death rate at the two levels, with 2.5 μg it is 21 out of 103, or about 20% ; at 25 μg/ml., 34 deaths occurred in 84, or 40%. It is doubtful whether this is a permissible comparison since the death rate in the controls was so much higher. This may be an effect of the seasonal variation in fertility.

Death rate alone gives only at one point (Group G) a significant result at $P<0.05$. The total weights were taken on both occasions and are higher in the group on the higher dosage, the controls remaining constant. All chicks in the second experiment were smaller than those in the first but there are no significant differences between treated and controls. When, however, the long bones are compared there are obvious changes. In Table 3, taking all the first series together, 74 out of 91 (81%) had optimum long bone development, 19 mm, compared with only 32 out of 61 (52%) for the high dosages. This is more striking when the individual groups are considered. Highly significant inhibition of bone growth was present with lemecycline and demethylchlortetracycline. The latter

TABLE 2

ACTION OF TEN TIMES THE CLINICAL DOSE OF TETRACYCLINE (25 μG/G) IN CHICK EMBRYOS ON DEATH RATE, SIZE, BONE GROWTH AND OCCURRENCE OF ABNORMALITIES

Controls were a sample. Values are means with standard deviations

Group	Drug	Original no. in group	Corrected group no.	Deaths	Live weight (g)	Length of tarsometatarsal (cm)	Length beak to rump (cm)	Crippled or with deformed bones
Controls	None	5	5	0	34·5±2·5	1·85±0·1	10·9±0·3	None
M	Buffer alone	20	15	4	34·5±2·6	1·85±	10·7±	None
H	Tetracycline	20	11	3	35·0±2·6	1·7 ±0·15	10·8±0·4	One cripple
I	Lemecycline	21	17	6	35·6±1·8	1·7 ±0·05	10·6±1·8	One deformed One cripple
J	Oxytetracycline	20	17	9	36·2±1·7	1·85±0·05	11·1±0·3	None
G	Demethylchlortetracycline	19	13	8	36·7±2·7	1·7 ±0·1	10·7±0·3	One cripple Two deformed
L	Chlormethylencycline	20	12	3	37·4±2·5	1·95±0·05	10·8±0·4	None
K	Chlortetracycline	20	14	5	37·8±1·6	1·85±0·05	11·0±0·3	None

TABLE 3

INTERFERENCE WITH BONE GROWTH BY TETRACYCLINES IN CHICKS

Pairs of numbers give the number of chicks with normal bones and the number studied. The whole experiment is significant at the 1% level. Groups I, lemecycline, and G, demethylchlortetracycline, are significantly worse than any of the others ($P<0{\cdot}001$). The remainder taken together are not significantly worse than the controls ($P=0{\cdot}5$)

	First experiment		Second experiment	
Drug	Group	Dose, 2·5 μg/g	Group	Dose, 25·0 μg/g
Control	X	8/9	M	10/11
Chlormethylencycline	E	9/13	L	8/9
Chlortetracycline	F	13/14	K	6/9
Oxytetracycline	C	8/12	J	5/8
Tetracycline	A	11/16	H	3/8
Lemecycline	B	15/17	I	0/11
Demethylchlor-tetracycline	D	10/10	G	0/5

drugs also gave marginally significant increased death rates. Six abnormalities were found. One hatched chick from the tetracycline group (H) was crippled, the leg functioning poorly. One embryo had short legs, one embryo had unequal legs, a 2 mm deficiency with soft bone on the defective side, and one hatched chick was crippled with demethylchlortetracycline (G). One crippled chick and one deformed embryo occurred with lemecycline (I).

Crippling due to inability to extend the foot properly occurs spontaneously from time to time.

DISCUSSION

The chick was selected as the experimental animal since unlike the mammalian embryo it can be exposed continuously to a predetermined concentration of the drug. It is unlikely that the effects are specific to the chick since the tetracyclines were all deposited in the immature bones and also a wide range of embryos have previously been used (Bevelander, Nakahara and Rolle, 1960; Bevelander, Goldberg and Nakahara, 1960) including fish (Bevelander & Goss, 1962), mice (Fillipi & Mala, 1958), chicks (Tchernoukh & Alexandrov, 1963) and puppies (Owen, 1963).

Dose level is particularly important in the study of antibacterial drugs, since we know from experience with bacteria that, in the absence of resistant mutants, there is a fairly sharp line between the ineffective and the effective dose. We might expect toxic effects to increase sharply with higher dosage. Both the clinical level and a higher dose were used here.

On this reasoning the toxicity testing at the higher levels of the tetracyclines in animals draws attention to the systems where trouble might occur, but does not necessarily predict the experience with normal clinical doses.

The most disturbing results in the human infant are those found by Cohlan, Bevelander & Tiamsic (1963). In premature infants there was an inhibition of growth of the tibia during the administration of the drug. With a dose equivalent to the adult dose of 7.5 g/day a 40% inhibition was recorded and even with the more usual level, equivalent

to an adult dose of 2.0 g/day there was 25% inhibition. These doses assume a weight of 10 stones for adults and 5 lb for premature infants.

In judging the significance either of the animal or the human experiments it is important to compare the doses used by the various workers. In divided doses in an adult, 1 g/day or about 15 mg/kg/day gives blood levels of 2.5 μg/ml. and, in the foetus *in utero*, 1.25 μg/ml. For an egg or a mouse the equivalent dose should be 125 μg assuming a total weight of 50 g for the average egg or mouse.

In the animals these doses are generally exceeded. In rats on the equivalent of 3 g/day for a human adult there were no deformities but growth was inhibited 28% by 5 days' treatment. With three times this amount for 15 days minor deformities occurred. The whole dose was available at once even here, as it was given intramuscularly (Fillipi & Mala, 1958).

If then we consider the results of experiment 1 we find that the clinical levels of the drug present continually during development and demonstrable in bone, aponeurosis, fat and yolk did not interfere with anything necessary for the production of normal chicks, nor was the mortality rate among them significantly higher than in the untreated population. Only with one drug, demethylchlortetracycline, was there a suspiciously high result. With a ten times increased dose, interference with bone growth occurred significantly with lemecycline and demethylchlortetracycline, and it is with these that abnormalities occurred in the unhatched chicks. These results are in harmony with human clinical experience.

When the tetracyclines were given only by mouth, few side effects were encountered. Since preparations have been available for injection, higher blood levels have been obtained and deaths have followed (King, Bowe & D'Esopo, 1964; Editorial, 1964).

It may be concluded that when possible tetracyclines should still be given by mouth and that the higher blood levels obtained by other routes introduce a risk to developing bone, and, further, that this risk is not uniform throughout the series of tetracyclines tested here, and that the older standard preparations together with chlormethylencycline may be less likely to produce untoward results.

SUMMARY

1. Six different tetracyclines were tested in chick embryos using the "blind trial" technique.

2. Exposure for 18 days to clinical levels of the drug did not lead to statistically significant mortality in the embryos and, although the drug could be demonstrated throughout the skeleton, there were no alterations in bone growth or in the size of the embryos. No congenital abnormalities were detected.

3. When the trial was repeated with a dose ten times higher, the usual interference with bone growth with occasional definite abnormalities was found. This was significantly greater with lemecycline and demethylchlortetracycline than with tetracycline, oxytetracycline, chlortetracycline and one new preparation, chlormethylencycline, which did not differ significantly from the controls.

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