THE TOXICITY OF SOME ANTIBACTERIALS TO FOWL SPERMATOZOA

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Summary

In a study of the effect of a number of antibacterials on fowl spermatozoa, penicillin and sulphanilamide at 1 mg/ml, chloromycetin at 0·2 mg/ml, and terramycin at 0·5 mg/ml depressed motility at a 1 in 20 dilution of semen in a medium having a similar tonicity to fowl seminal plasma.

Streptomycin, sulphamezathine, aureomycin, and tetracyn were not significantly toxic in the highest concentrations used.

Penicillin, sulphanilamide, chloromycetin, and terramycin were less toxic when the semen was diluted 1 in 3 with Ringer or 1 in 20 with 30 per cent. seminal plasma instead of Ringer. This suggests the presence of a protective substance in the seminal plasma.

The toxicity of sulphanilamide and chloromycetin was not antagonized by *p*-aminobenzoic acid or phenylalanine respectively. The spermicidal effect of biotin, however, was reduced by aureomycin.

Oxygen uptake, fructolysis, and lactic acid production were consistently depressed by sulphanilamide at 5 mg/ml. A similar concentration of chloromycetin almost completely inhibited oxygen uptake although aerobic fructolysis and lactic acid production were unaffected. Metabolism was little affected by as much as 20 mg/ml penicillin.

I. Introduction

Antibacterials have been used extensively in the preservation of bull semen for artificial insemination (see Emmens and Blackshaw 1956) and White (1954) has investigated the toxicity of a number of antibacterials for bull, ram, rabbit, and human spermatozoa. The most striking feature of these studies was the resistance of mammalian spermatozoa to bacteriostatic concentrations of antibacterials. Very high concentrations, however, of all but penicillin proved toxic to the spermatozoa of one or more species.

Fowl semen collected by abdominal massage (Burrows and Quinn 1939) is likely to be contaminated by excreta and has a higher initial bacterial content than bull semen (Wilcox and Shorb 1958). Smith (1949) reported that sulphathiazole, but not streptomycin, was toxic at 0·05 mg/ml, and more recently Wilcox and Shorb (1958) found that terramycin, tetracyn, and chloromycetin at a concentration of 0·9 mg/ml depressed the motility of fowl spermatozoa after 24 hr.

This paper compares the effect of a number of antibacterials on the motility and metabolism of fowl spermatozoa.

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II. Materials and Methods

(a) *Techniques*

Fowl semen was collected by abdominal massage (Burrows and Quinn 1939). Only normal uncontaminated pooled ejaculates of good initial motility were used.

Semen was usually diluted 1 in 20 for motility observations and 1 in 3 for metabolic studies. The tubes for motility observations were kept at room temperature and scored at hourly intervals by the system of Emmens (1947). Full motility was rated as 4 and complete immotility as 0. As quarter grades were frequently used the actual scores have been multiplied by 4, and the total score $\times 4$ over the experimental period, the motility index, has been used as unit observation in the analyses of variance.

Metabolic studies were undertaken at 25°C. Oxygen uptake was measured over 3 hr by the direct Warburg technique (Umbreit, Burris, and Stauffer 1949). The shaking rate was 114 strokes/min and the gas phase air. For anaerobic experiments 1 ml of diluted sperm suspension was pipetted into a narrow tube, covered with 2 cm of oxygen-free paraffin, and stoppered. Fructose (Mann 1948) and lactic acid (Barker and Summerson 1941) were estimated at the start and end of both aerobic and anaerobic experiments.

After the metabolic experiments, total counts of spermatozoa were made in duplicate using a haemocytometer. Oxygen uptake, fructose utilization, and lactic acid production (per 10^8 cells) during the experimental period were then calculated and used as unit observation in the analyses of variance.

(b) *Diluents and Antibacterials*

The diluent of pH 7·0 used in most motility experiments consisted of 0·0136M Na_2HPO_4, 0·0064M NaH_2PO_4, 0·005M KCl, 0·0015M $MgCl_2$, 0·0641M NaCl, 0·25M glucose.

The diluent for metabolic and some motility studies was a modification of Ringer's solution and had the following composition: 0·01M Na_2HPO_4, 0·005M KCl, 0·001M KH_2PO_4, 0·001M $MgSO_4$, 0·1848M NaCl containing 100 mg per cent. fructose.

Both of these diluents have a relative tonicity of 140 (0·9 per cent. NaCl$\equiv$ 100 per cent. tonicity) which is close to the tonicity of fowl semen and optimal for motility in the presence of magnesium and potassium.

The antibacterials used were sulphanilamide (Drug Houses of Australia Ltd.), sulphamezathine (I.C.I.A.N.Z.), penicillin G, crystalline (Glaxo Laboratories Ltd.), streptomycin sulphate (Glaxo), chloromycetin (Parke, Davis and Co. Ltd.), aureomycin hydrochloride (Lederle), terramycin hydrochloride (C. Phizer & Co.), and tetracyn hydrochloride (also from C. Phizer & Co.). The sulphanilamide, sulphamezathine, terramycin, and tetracyn were pure; commercial samples of the other antibacterials were used.

(c) *Statistical Analysis*

The results have been subjected to analysis of variance. Where a number of independent treatments have been compared with controls, the standard error of the difference between a treatment and the control mean has been calculated using the formula (Cochran and Cox 1950):

$$sd = \sqrt{\{s^2[(1/r_1)+(1/r_2)]\}},$$

where sd = standard error of the difference between treatment and control means,
s^2 = error mean square from the analysis of variance,
r_1 = number of control replications, and
r_2 = number of treatment replications.

The significance of difference between the means has then been assessed by the t-test using sd and the degrees of freedom associated with s^2.

TABLE 1

MEAN MOTILITY INDICES OVER A 6-HR PERIOD FOR SIX FOWL EJACULATES IN DILUENTS CONTAINING ANTIBACTERIALS

Mean of five controls for each ejaculate was 77·1 and asterisks are used to denote a significant fall in motility

Antibacterial	Concentrations (mg/ml)					
	0·02	0·1	0·5	0·2	1	5
Aureomycin	77·7	81·3	71·0	—	—	—
Terramycin	77·8	76·3	56·5**	—	—	—
Tetracyn	77·3	77·5	76·3	—	—	—
Penicillin	—	—	—	74·3	64·5**	45·3**
Streptomycin	—	—	—	77·2	75·2	73·0
Sulphanilamide	—	—	—	72·2	53·8**	20·2**
Sulphamezathine	—	—	—	72·5	66·5*	73·5
Chloromycetin	—	—	—	65·2**	26·3**	0·5**

*$P<0·05$. **$P<0·01$.

III. RESULTS

(a) *Preliminary Motility Observations*

The effects of varying concentrations of eight antibacterials on six fowl ejaculates over a 6-hr period are shown in Table 1. From the analysis of variance in which each level of each antibacterial was considered as an independent treatment the standard error of the difference between treatment and control means was found to be 4·2 (i.e. $\sqrt{[89\times(6/30)]}$, and has been used for the t-test (degrees of freedom = 135). Penicillin and sulphanilamide depressed the motility of fowl spermatozoa at 1 mg/ml and chloromycetin was toxic even at 0·2 mg/ml. The effects of penicillin at 1 mg/ml and chloromycetin at 0·2 mg/ml, however, were

not very marked and in other tests these concentrations did not always cause a significant depression of motility. It may be noted that ejaculates often varied considerably in their susceptibility to the antibacterials. The other antibacterials were well tolerated and, except for terramycin at 0·5 mg/ml, had no significant effect on motility during the 6-hr period, even at the highest concentrations used, which were near the limits of solubility.

TABLE 2

COMPARISON OF THE TOXICITY OF ANTIBACTERIALS IN DILUENTS OF RELATIVE TONICITY 100 AND 140

Results are the mean motility indices for four ejaculates over 4 hr.
0·9 per cent. NaCl ≡ 100 per cent. tonicity

Tonicity	Antibacterial	Concentration (mg/ml)			
		0	0·2	1	5
100	Penicillin	45	44	35	16
	Sulphanilamide	45	36	17	0
	Streptomycin	42	47	39	32
140	Penicillin	56	56	53	36
	Sulphanilamide	57	55	44	9
	Streptomycin	57	58	57	56

When another series of tests was performed using a similar diluent containing less glucose to give a relative tonicity of 100 (i.e. of optimum tonicity for mammalian spermatozoa), the antibacterials usually depressed motility more severely. In some instances, particularly with streptomycin, sulphanilamide, and sulphamezathine the spermatozoa adapted themselves to the antibacterial and the fourth-hour score was higher than the first-hour score. Using the fourth-hour score as unit observation it was found that, in addition to the antibacterials that were toxic in the diluent of higher tonicity, sulphamezathine (1 mg/ml), terramycin and tetracyn (0·1 mg/ml), and aureomycin (0·5 mg/ml) also decreased motility.

A comparison of the toxicity of penicillin, sulphanilamide, and streptomycin in the two diluents is shown in Table 2. Motility indices were significantly lower in diluents of tonicity 100 and the antibiotics were more toxic than at tonicity 140 (i.e. there was a significant tonicity × levels of antibiotic interaction).

(*b*) *Penicillin and Streptomycin in Combination*

Since penicillin and streptomycin are used together in diluents for bull semen, these antibacterials in combination were added to the diluent for fowl semen at a concentration of 1 mg/ml. The combination was not significantly toxic in these tests.

(c) *Effects of Antagonists*

There is evidence that the antibacterial action of sulphanilamide, chloromycetin, and aureomycin may be antagonized by *p*-aminobenzoic acid, phenylalanine, and biotin respectively (Woods 1940; Woolley 1950; Osteux and Laturage 1952).

TABLE 3

EFFECT OF SOME ANTIBIOTICS AND THEIR ANTAGONISTS ON THE MEAN MOTILITY INDEX OF FOWL SPERMATOZOA

Number of replications shown in parentheses. Analysis of variance for the effects of aureomycin on biotin toxicity is also given

Substance	Concn. (mg/ml)	Motility Index	Substance	Concn. (mg/ml)	Motility Index	Substance	Concn. (mg/ml)	Motility Index
Control	—	51 (5)	Control	—	49 (5)	Control	—	55 (4)
Sulphanilamide	1	25 (5)	Chloromycetin	0·5	2 (5)	Aureomycin	0·2	52 (4)
p-Aminobenzoic acid	0·2	50 (5)	Phenylalanine	0·5	46 (5)	Biotin	2	27 (4)
Sulphanilamide + *p*-aminobenzoic acid	1 0·2	48 (5)	Chloromycetin +phenylalanine	0·5 0·5	3 (5)	Aureomycin + biotin	0·2 2	48 (4)

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio
Effect of biotin	1	69·8**
Effect of aureomycin	1	23·6*
Ejaculate differences	3	3·6
Interactions		
Biotin × aureomycin	1	40·5**
Biotin × ejaculates	3	0·6
Aureomycin × ejaculates	3	0·2
Residual	3	14

*$P<0·05$. **$P<0·01$.

The spermicidal properties of the first two antibacterials, however, were unaffected by concentrations of *p*-aminobenzoic acid (0·2 mg/ml) and phenylalanine (0·5 mg/ml) innocuous to fowl spermatozoa (Table 3). *p*-Aminobenzoic acid proved spermicidal in higher concentrations than 1 mg/ml and similar concentrations of phenylalanine also significantly depressed motility. Aureomycin as might be

TABLE 4

EFFECT OF DILUTION ON THE TOXICITY OF ANTIBACTERIALS FOR FOWL SPERMATOZOA

Each motility index is the mean of four ejaculates over a 4-hr period

Penicillin			Sulphanilamide			Chloromycetin			Terramycin		
Concn. (mg/ml)	Sperm Dilution		Concn. (mg/ml)	Sperm Dilution		Concn. (mg/ml)	Sperm Dilution		Concn. (mg/ml)	Sperm Dilution	
	1 in 3	1 in 20		1 in 3	1 in 20		1 in 3	1 in 20		1 in 3	1 in 20
0	58	53	0	59	54	0	56	51	0	54	51
5	49	31	1	57	29	0·5	53	9	0·5	53	15
10	38	20	2	54	21	1	36	2	1	53	11
20	36	10	4	45	13	2	20	2	2	52	7

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio			
		Penicillin	Sulphanilamide	Chloromycetin	Terramycin
Dilution effect	1	85·7**	160·0**	200·1**	1384·0**
Antibiotic concentration	3	62·4**	35·2**	105·8**	152·2**
Ejaculate differences	3	4·0*	10·3**	8·5**	25·8**
Interactions					
Dilution × antibiotic	3	5·5*	11·3**	23·7**	126·2**
Dilution × ejaculate	3	1·5	1·7	7·8**	11·3**
Antibiotic × ejaculate	9	0·6	0·3	0·7	1·1
Residual	9	26	30	36	6

* $P<0·05$. ** $P<0·01$.

expected from the initial experiments was not spermicidal; biotin, however, was toxic at a concentration of 2 mg/ml and aureomycin (0·2 mg/ml) was found to antagonize this effect (Table 3).

(*d*) *Effect of Seminal Plasma on the Toxicity of Antibacterials*

The effects of penicillin, sulphanilamide, chloromycetin, and terramycin on the motility of fowl semen diluted 1 in 3 and 1 in 20 in modified Ringer's solution, with summary analyses of variance, are shown in Table 4. In all cases, the antibacterials were more toxic when the semen was diluted 1 in 20.

TABLE 5

EFFECT OF 30 PER CENT. SEMINAL PLASMA ON THE TOXICITY OF ANTIBACTERIALS TO FOWL SPERMATOZOA

Results are the mean motility indices over 4 hr for four ejaculates diluted 1 in 20

Antibiotic	Concn. (mg/ml)	Modified Ringer	30% Plasma in Modified Ringer
Control	—	46	54
Penicillin	20	11	38
Sulphanilamide	4	17	54
Chloromycetin	0·5	29	57
Terramycin	2	21	51

The increased susceptibility of fowl spermatozoa at low cell concentrations is clearly due to the dilution of seminal plasma since the toxicity of the antibiotics was reduced at a 1 in 20 dilution, if 30 per cent. seminal plasma in modified Ringer's solution was used (Table 5).

(*e*) *Metabolic Studies*

Table 6 shows the effect of spermicidal concentrations of penicillin, sulphanilamide, and chloromycetin on the oxygen uptake, fructolysis, and lactic acid production of fowl spermatozoa. Analyses of variance gave residual mean squares of 0·55 (oxygen uptake), 40·0 (fructose utilization), and 68·0 (lactic acid production) —all with 9 degrees of freedom—and these have been used to calculate the standard error for the *t*-test.

(i) *Penicillin.*—In general, metabolism was little affected. There was, however, a statistically significant depression of oxygen uptake ($t = 2·2$, $P = 0·05$) and anaerobic fructolysis ($t = 6·2$, $P < 0·01$).

(ii) *Sulphanilamide.*—Oxygen uptake, aerobic and anaerobic fructolysis, and lactic acid production were all consistently depressed. In the presence of sulphanilamide, as in the controls, fructose utilization and lactic acid production was greater under anaerobic than under aerobic conditions.

(iii) *Chloromycetin.*—Chloromycetin almost completely inhibited oxygen uptake. Under aerobic conditions, fructose breakdown and lactic acid production in the presence of chloromycetin was as good as, if not better than, the controls. Under anaerobic conditions, however, there was no increase in fructose utilization or lactic acid production as occurred in the controls.

TABLE 6

EFFECT OF HIGH CONCENTRATIONS OF ANTIBACTERIALS ON THE METABOLISM OF FOWL SPERMATOZOA OVER 3 HR AT 25°C

Mean values for four replications are given and asterisks are used to denote significant differences from control values

Antibiotic	Concn. (mg/ml)	Oxygen Uptake (μl/10^8 cells)	Fructose Utilization (μg/10^8 cells)		Lactic Acid Production (μg/10^8 cells)	
			Aerobic	Anaerobic	Aerobic	Anaerobic
Control	—	7·6	56	92	44	84
Penicillin	20	6·5*	47	64**	41	71
Sulphanilamide	5	4·3**	12**	47**	10**	53**
Chloromycetin	5	1·8**	62	51**	59*	55**

*$P<0·05$. **$P<0·01$.

At the end of each experiment the motility of the spermatozoa in the Warburg flasks and anaerobic tubes was checked. Motility was most depressed by chloromycetin; the effects of sulphanilamide and penicillin were less striking.

IV. DISCUSSION

The most important point that emerges from these studies is the relative toxicity of penicillin and sulphanilamide to fowl spermatozoa. Both antibacterials are routinely added to bull semen in artificial insemination practice and are well tolerated by mammalian spermatozoa in general. Thus under similar conditions White (1954) found that sulphanilamide and penicillin were usually non-toxic to mammalian spermatozoa in concentrations of up to 5 mg/ml. Sulphathiazole is apparently toxic to fowl spermatozoa (Smith 1949) but sulphamezathine is less harmful.

Fowl spermatozoa are also rather more susceptible to chloromycetin. A concentration of at least 1 mg/ml was needed to depress the motility of mammalian spermatozoa (White 1954) whereas 0·2 mg/ml proved toxic in the experiments reported here. As might be expected from their close structural relationship the spermicidal activity of aureomycin, terramycin, and tetracyn is very similar and, in general, does not differ much for fowl and mammalian spermatozoa.

Fowl spermatozoa also resemble the other spermatozoa studied in their tolerance to streptomycin which was innocuous even at the highest dose tried. This would seem a desirable antibacterial for use in the artificial insemination of poultry.

Wilcox and Shorb (1958) state that a combination of terramycin and streptomycin at low concentration gives better fertility than streptomycin and penicillin in combination, although both are efficient in controlling bacterial growth in fowl semen.

Ejaculates may vary in their susceptibilty to antibacterials and, furthermore, the degree of dilution of the semen is an important factor. The results presented here clearly indicate that seminal plasma counters the spermicidal activity of a number of antibiotics. The mechanism is, however, not very specific and it seems unlikely that such structurally diverse antibacterials could all be inactivated by seminal plasma. On the other hand, the seminal plasma may help maintain the integrity of the cell surface; in its absence there is probably a general increase in permeability and a more rapid passage of antibiotics into spermatozoa.

The finding that two members of the vitamin B complex, viz. biotin and *p*-aminobenzoic acid, are toxic to spermatozoa is surprising; the spermicidal concentrations are, however, vastly in excess of those encountered in the tissues. Phenylalanine has previously been reported toxic to bull spermatozoa on shaking in air and its action is probably dependent on the formation of hydrogen peroxide by the enzyme L-amino acid oxidase (Tosic and Walton 1950).

The antagonism of the spermicidal effects of biotin by aureomycin is of interest in view of the report by Osteux and Laturage (1952) that biotin counters the action of aureomycin on enzyme preparations from *Clostridium welchii*. Both observations suggest that aureomycin and biotin might form a biologically inactive complex so that the properties of either may be masked by the presence of the other.

Because of the limited sensitivity of the methods, the metabolic experiments had to be done on semen diluted only 1 in 3 and, even at very high concentrations, the antibacterials did not have marked spermicidal effects. Nevertheless, it has been possible to demonstrate a significant depression of oxygen uptake by penicillin, sulphanilamide, and chloromycetin and a decreased ability of the spermatozoa to break fructose down to lactic acid. The action of chloromycetin is particularly interesting in that it inhibits the marked Pasteur effect shown by fowl spermatozoa, i.e. the increase in fructose breakdown and lactic acid production under anaerobic conditions. With ram and bull spermatozoa high concentrations of chloromycetin inhibit both aerobic and anaerobic glycolysis almost completely (White 1954).

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