STUDIES OF THE SPERMICIDAL ACTIVITY OF CHELATING AGENTS

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Summary

In studies covering 4-hr periods at room temperature of the effect of 0·1mM chelating agents on the motility of ram, bull, rabbit, and human spermatozoa, the following significant effects were seen:

(i) Cupferron, *o*-phenanthroline, and 1-nitroso-2-naphthol were spermicidal to all four species.

(ii) Sodium diethyldithiocarbamate, ethyl potassium xanthate, 8-hydroxyquinoline, and salicylaldoxime were toxic to bull, rabbit, and human spermatozoa.

(iii) Diphenylthiocarbazone was toxic to rabbit and human spermatozoa and with the latter species phenylthiohydantoic acid and dithio-oxamide were also spermicidal.

In studies of the effect of 0·2mM metals on the toxicity of chelating agents for bull spermatozoa, it was found that:

(iv) The toxicity of sodium diethyldithiocarbamate, 1-nitroso-2-naphthol, and *o*-phenanthroline was reduced by cobalt.

(v) Copper increased the spermicidal activity of 1-nitroso-2-naphthol, cupferron, 8-hydroxyquinoline, and ethyl potassium xanthate. Cadmium had a similar effect on ethyl potassium xanthate. It is suggested that the spermicidal activity of these chelating agents may be dependent on combination with trace concentration of copper and other heavy metals, and that the protective effect of cobalt may be due to it competing to form a non-toxic complex.

I. INTRODUCTION

Chelating agents, i.e. organic molecules that bind metals, forming ring structures, have been used for some time as micro-reagents in analytical chemistry (see Martell and Calvin 1952). Many of them are active in low concentrations at physiological hydrogen ion concentrations (Albert and Gledhill 1947) and hence might be expected to be of value for investigating the trace element requirements of cells.

One such chelator, viz. 8-hydroxyquinoline (oxine) is a powerful antiseptic (see Albert 1944) and fungicide (Rigler and Greathouse 1941) and there is evidence that this may be due, in part at least, to its ability to form complexes with essential trace elements. Thus Zentmyer (1944) found that zinc reduced the fungicidal activity of oxine whilst Albert *et al.* (1947) have shown that cobalt antagonizes its bacteriostatic effect on Gram + organisms, and that iron and zinc have a similar action with Gram − cells. It has also been reported that manganese, cobalt, and iron prevent the inhibitory effect of oxine on glutamic acid assimilation by *Staphylococcus aureus* (Gale 1949).

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More recently McNaught, Owen, and Smith (1950) have studied the effect of chelating agents on rumen bacteria and found that oxine, *aa'*-dipyridyl, and *o*-phenanthroline strongly depress growth. The inhibition produced by oxine could be reversed only partly by metals but that due to the other two chelating agents was completely reversed by iron and partly so by cobalt and zinc. *o*-Phenanthroline has also been found toxic to lactic acid bacteria by MacLeod (1952) who, however, concludes that its action is in large part due to factors not concerned with its ability to bind metals.

Some chelating agents (e.g. oxine and *aa'*-dipyridyl) have also recently been shown to have trypanocidal properties (Ryley 1953).

In this paper, the effect of a number of chelating agents on the motility of ram, bull, rabbit, and human spermatozoa has been studied with a view to determining their trace element requirements.

II. Materials and Methods

(*a*) *Technique and Diluents*

The method of collecting semen and the composition of the diluting fluid were the same as described previously (White 1954).

Semen was diluted 1 in 40 in small tubes for motility observations which were made at room temperature. All tests have been replicated on four ejaculates.

Motility was scored at hourly intervals over a 4-hr period by the system of Emmens (1947). Full motility was rated as 4 and complete immotility as zero, but in presenting results the actual scores have been multiplied by 4, since quarter grades were frequently used.

The chelating agents were B.D.H. laboratory reagents and the metals were added as the A.R. sulphate salts.

(*b*) *Statistical Analysis*

With the exception of Table 3 (where a simple *t*-test has been used) the results of experiments have been evaluated by the analysis of variance (see Fisher 1946) with isolation of sums of squares attributable to differences between ejaculates and treatments. The total value for the experimental period has been used as unit observation and the treatment-ejaculate interaction mean square as the error term. Differences between ejaculates are often highly significant, so that the accuracy of comparisons is much improved by analysis which both takes this into account and utilizes an overall estimate of error. The standard error of the difference between a pair of treatment means is calculated using the formula

$$S_d = \frac{2s^2}{r},$$

where S_d = standard error of the difference between a pair of treatment means,
s^2 = the interaction mean square, and
r = number of replications.

The significance of differences between pairs of treatment means has then in effect been assessed by a t-test using S_d and the degrees of freedom associated with s^2. Where three controls were used for safety, the best estimate (when these controls are homogeneous) of the value of S_d for any one treatment v. controls is

$$S_d = \frac{4s^2}{3r}.$$

TABLE 1

EFFECT OF 0·1 mM CHELATING AGENTS ON THE TOTAL MOTILITY SCORE (×4) OF SPERMATOZOA OVER A 4 HR PERIOD. EACH VALUE REPRESENTS THE MEAN OF FOUR EJACULATES

Chelating Agent	Ram	Bull	Rabbit	Human
Nil	72	49	63	53
Nil	69	53	64	57
Nil	72	53	63	58
Cupferron	49**	17**	17**	21**
Titan yellow	73	52	65	53
Quinalizarin	73	52	63	52
Nitroso-R salt	73	49	59	52
Thioglycollic acid	73	55	68	51
Chromotropic acid	73	49	66	52
Ethyl potassium xanthate	68	12**	13**	7**
o-Phenanthroline	34**	15**	12**	32**
Thiourea	72	49	66	46
Phenylthiohydantoic acid	72	52	66	25**
Salicylaldoxime	69	33**	35**	28**
1-Nitroso-2-naphthol	35**	16**	8**	12**
Sodium diethyldithiocarbamate	62	3**	4**	1**
Dithio-oxamide	73	39	65	29*
8-Hydroxyquinoline (Oxine)	61	30**	38**	22**
αα′-Dipyridyl	73	49	63	50
Benzoinoxime	73	48	67	50
Sodium dihydroxytartrate osazone	69	47	53	45
p-Dimethylaminobenzalrhodanine	73	50	66	44
Diphenylcarbazide	73	51	62	50
Mercaptobenzothiazole	64	41	58	
Diphenylthiocarbazone	73	45	38**	25**

** Significantly toxic, $P<0·01$.

III. RESULTS

(a) *Toxicity of Chelating Agents*

Table 1 shows the effect on the motility of ram, bull, rabbit, and human spermatozoa of over 20 chelating agents (0·1 mM) that combine with metals at physiological hydrogen ion concentrations (Albert and Gledhill 1947). The results have been subjected to variance analysis with omission of the sodium diethyldithiocarbamate group in the case of bull, rabbit, and human samples,

since it was rapidly toxic to these spermatozoa and the variances are extremely low. Variances of the remaining treatment groups are probably not completely independent of the level of response. Since, however, an effect has only been judged significant when the probability of it being due to chance is less than 1 in 100 this is of little consequence. The summary of the analyses of variance (Table 2) shows significant variation between treatments for each species. The difference between control and experimental groups must be greater than $2 \cdot 7S_d$ for significance at the 1 per cent. level. Differences between treatment and control means must therefore exceed the following to attain significance: ram—12, bull—12, rabbit—13, human—14.

Three control groups were provided in these tests, but it is clear that they did not differ significantly within any species.

TABLE 2

SUMMARY OF THE ANALYSES OF VARIANCE OF THE DATA IN TABLE 1, SHOWING VARIANCE RATIOS WITH THE ERROR MEAN SQUARE IN ITALICS AT THE BASE OF THE COLUMNS

Source of Variation	Ram		Bull		Rabbit		Human	
	D.F.	V.R.	D.F.	V.R.	D.F.	V.R.	D.F.	V.R.
Between treatments	24	8·3**	23	12·2**	23	22·8**	22	17·6**
Between ejaculates	3	6·2**	3	12·4**	3	26·6**	3	11·5**
Interaction	72	*60*	69	*67*	69	*73*	66	*82*

** $P<0 \cdot 01$.

Cupferron, *o*-phenanthroline, and 1-nitroso-2-naphthol were toxic to the spermatozoa of all four species. Sodium diethyldithiocarbamate, ethyl potassium xanthate, oxine, and salicylaldoxime significantly depressed the motility of bull, rabbit, and human spermatozoa, the effect with the first two chelators being particularly marked. Diphenylthiocarbazone proved toxic to rabbit and human spermatozoa, whilst with the latter species phenylthiohydantoic acid and dithio-oxamide were also spermicidal.

(*b*) *Effect of Heavy Metals on Toxicity of Chelating Agents*

If the spermicidal activity of these chelators is due to their depriving spermatozoa of essential trace elements, it should be possible to prevent it by adding the appropriate element to the diluent.

Table 3 shows the effect of adding 0·2 mM manganese, iron, cobalt, copper, zinc, and cadmium to bull spermatozoa in the presence of sodium diethyldithiocarbamate, 1-nitroso-2-naphthol, salicylaldoxime, *o*-phenanthroline, cupferron, oxine, and ethyl potassium xanthate. None of the above metals were themselves toxic to bull spermatozoa at this concentration (White 1955).

The variances of the groups in Table 3 were, by inspection, heterogeneous. Particular interest only attaches to those metals causing either a marked decrease

or increase in the toxicity of the chelating agents. Detailed analysis (by the *t*-test) has only been made, therefore, in the comparison of the cobalt group (D) with (A). Cobalt caused a highly significant decrease in the toxicity of sodium diethyldithiocarbamate ($t = 6 \cdot 1$, d.f. $= 7$) and 1-nitroso-2-naphthol ($t = 4 \cdot 3$, d.f. $= 7$) and a significant decrease in that of *o*-phenanthroline ($t = 3 \cdot 3$, d.f. $= 7$). It is obvious that copper increased the toxicity of 1-nitroso-2-naphthol, cupferron, oxine, and ethyl potassium xanthate and that cadmium also potentiated the latter chelator.

The opposing effects of 0·2 mM copper and 0·2 mM cobalt on the toxicity of 0·1 mM 1-nitroso-2-naphthol were further studied in a factorial experiment (Table 4). Direct factorial analysis is again not possible because of the very low variance of the copper-nitrosonaphthol group. In the absence of the chelating agent, however, copper and cobalt were again obviously not toxic. It it also clear that cobalt tended to decrease the toxicity of 1-nitroso-2-naphthol and copper to increase it, whilst cobalt overcame the effect of added copper.

TABLE 3

EFFECT OF 0.2 mM METAL IONS ON THE TOXICITY OF 0.1 mM CHELATING AGENTS FOR BULL SPERMATOZOA. VALUES REPRESENT THE MEAN TOTAL MOTILITY SCORE (×4) OVER A 4 HR PERIOD FOR FOUR EJACULATES

Chelator	Control	Chelator and Following Metal:						
		Nil (A)	Manganese (B)	Iron (C)	Cobalt (D)	Copper (E)	Zinc (F)	Cadmium (G)
Sodium diethyldithiocarbamate	64	9	9	13	45**	14	2	5
1-Nitroso-2-naphthol	54	20	18	16	56**	3†	15	14
o-Phenanthroline	68	41	41	29	68*	30	13	11
Salicylaldoxime	63	26	34	18	18	13	24	23
Cupferron	55	18	20	10	17	0†	8	18
8-Hydroxyquinoline	57	21	23	12	17	1†	11	19
Ethyl potassium xanthate	65	30	41	20	34	1†	17	0†

* Significantly better than (A), $P < 0 \cdot 05$.

** Highly significantly better than (A), $P < 0 \cdot 01$.

† Obviously more toxic than (A).

Table 3 shows that added copper itself had little effect on the toxicity of sodium diethyldithiocarbamate. However, it reduced the ability of cobalt to make the chelating agent less toxic, as can be seen from the second factorial experiment in Table 4. The situation is clearly complex with obvious interactions between the chelating agent and metals; the data have therefore been split and analysed separately in the presence and absence of sodium diethyldithiocarbamate. The summary of the analysis of variance (Table 5) shows that in the absence of the chelating agent, copper and cobalt are not toxic,

singly or in combination, nor is there any interaction between them. In the presence of the chelating agent cobalt is significantly beneficial, copper has no effect, and there is a significant copper-cobalt interaction. Copper would not

TABLE 4

RESULTS OF FACTORIAL EXPERIMENTS SHOWING THE INTERACTION BETWEEN CHELATING AGENTS (0.1 mM), COBALT (0.2 mM), AND COPPER (0.2 mM). VALUES REPRESENT THE TOTAL MOTILITY SCORE (×4) OVER A 4 HR PERIOD

Chelating Agent	Ejaculate	Control	Cobalt	Copper	Cobalt + Copper	Chelator	Chelator + Cobalt	Chelator + Copper	Chelator + Cobalt + Copper
1-Nitroso-2-naphthol	1	44	45	44	38	28	49	0	39
	2	68	70	68	67	44	69	0	70
	3	62	62	56	53	41	62	2	49
	4	57	58	56	53	37	59	1	54
	Mean	57	58	56	53	37	59	1	54
Sodium diethyldithiocarbamate	1	51	52	45	39	6	28	15	14
	2	48	52	42	50	3	39	18	12
	3	68	72	71	71	1	64	26	16
	4	59	52	53	48	12	39	10	10
	Mean	57	57	50	52	6	43	17	13

be expected to have this effect if the function of added cobalt was merely to make good that bound by chelation.

TABLE 5

SUMMARY OF THE ANALYSES OF VARIANCE OF THE SODIUM DIETHYLDITHIOCARBAMATE DATA IN TABLE 4, SHOWING VARIANCE RATIOS WITH THE INTERACTION MEAN SQUARE IN ITALICS AT THE BASE OF THE COLUMNS

Source of Variation	D.F.	Absence of Chelator		Presence of Chelator	
Between treatments:	3	1·6		14·7**	
Effect of cobalt	1		0·0		15·3**
Effect of copper	1		4·5		4·5
Cobalt/copper interaction	1		0·1		24·3**
Between ejaculates	3	28·3**		1·4	
Interaction	9	*17*		*70*	

** $P<0·01$.

The explanation might be that the chelator is merely less toxic in combination with cobalt and that copper displaces cobalt from sodium diethyldithiocarbamate when both metals are present in equal concentrations.

This raises the question as to what is the minimum amount of copper that will antagonize a given amount of cobalt in the presence of sodium diethyl-

dithiocarbamate. Table 6 shows the results of an experiment to investigate this point and Table 7 the summary of the analysis of variance. For an effect signi-

TABLE 6

EFFECT OF INCREASING CONCENTRATIONS OF COPPER ON THE EFFICACY OF COBALT AS AN ANTAGONIST TO SODIUM DIETHYLDITHIOCARBAMATE

Ejaculate	Control	10^{-1}mM Sodium Diethyldithiocarbamate +					
		No Cobalt	2×10^{-1} mM Cobalt and Following Copper Concentrations:				
			0	10^{-4}mM	10^{-3}mM	10^{-2}mM	10^{-1}mM
1	40	8	35	16	31	10	11
2	54	11	30	27	29	11	7
3	66	23	68	68	66	44	28
4	55	21	43	43	48	13	13
Mean	54**	16	44**	39**	44**	20	15

** Highly significantly better than the no cobalt group, $P<0{\cdot}01$.

ficant at the 1 per cent. level, treatment means must differ by a minimum of 14. It can be seen that 0·01 mM copper will prevent 0·20 mM cobalt from reducing the toxicity of sodium diethyldithiocarbamate; at copper concentrations below this value, however, cobalt is active.

TABLE 7

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 6 SHOWING VARIANCE RATIOS WITH THE INTERACTION MEAN SQUARE IN ITALICS

Source of Variation	D.F.	V.R.
Between treatments	6	20·5**
Between ejaculates	3	26·9**
Interaction	18	*49*

** $P<0{\cdot}01$.

IV. DISCUSSION

(*a*) *Toxicity of Chelating Agents*

No previous systematic studies seem to have been made on the effect of chelating agents on mammalian spermatozoa, although chinosol, which contains oxine, has been used for some time as a chemical contraceptive (Baker 1931). Other chelating agents tried in this experiment were much more spermicidal than oxine (Table 1) and might be useful in this regard. It may be noted

that the efficacy of chinosol and some of the other chelators should be greatly increased by the addition of copper and other metals (Table 3).

Invertebrate spermatozoa apparently differ in their response to chelating agents since Tyler (1953) reports that "Versene," diethyldithiocarbamate, oxine, and cupferron are beneficial to diluted sea-urchin spermatozoa in the concentrations used here.

(*b*) *Effect of Metals on Toxicity of Chelating Agents*

It is tempting to interpret the mitigating effect of cobalt on the toxicity of sodium diethyldithiocarbamate, 1-nitroso-2-naphthol, and *o*-phenanthroline to mean that they function by depriving spermatozoa of cobalt.

On the other hand, the fact that copper and cadmium increase the toxicity of some of the chelating agents suggests that their spermicidal activity might be normally dependent on combination with trace concentrations of such metals in the diluent and semen. The reverse effect of cobalt with the above three chelators could be due to it competing to form a non-toxic complex. In those cases where cobalt exerts no protective action against chelating agents it is possible that the stability of the toxic metal complexes is very great relative to that of cobalt.

It may be noted that Mason (1948) has found copper oxinate to be a more powerful fungicide than oxine itself, whilst Anderson and Swaby (1951) report that in the absence of copper or iron, oxine is not fungistatic to *Aspergillis niger* at all. Essentially similar findings have been made by Rubbo, Albert, and Gibson (1950) for Gram + bacteria.

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VI. References

Albert, A. (1944).—*Med. J. Aust.* **1**: 245-8.

Albert, A., and Gledhill, W. S. (1947).—*Biochem. J.* **41**: 529-33.

Albert, A., Rubbo, S. D., Goldacre, R. J., and Balfour, B. G. (1947).—*Brit. J. Exp. Path.* **28**: 69-87.

Anderson, B. I., and Swaby, R. J. (1951).—*Aust. J. Sci. Res.* B **4**: 275-82.

Baker, J. R. (1931).—*J. Hyg., Camb.* **31**: 189-213.

Emmens, C. W. (1947).—*J. Physiol.* **106**: 471-81.

Fisher, R. A. (1946).—"Statistical Methods for Research Workers." (Oliver and Boyd: Edinburgh.)

Gale, E. F. (1949).—*J. Gen. Microbiol.* **3**: 369-86.

MacLeod, R. A. (1952).—*J. Biol. Chem.* **197**: 751-61.

Martell, A. E., and Calvin, M. (1952).—"Chemistry of the Metal Chelate Compounds." (Prentice-Hall: New York.)

Mason, C. L. (1948).—*Phytopathology* **38**: 740-51.

McNaught, M. L., Owen, E. C., and Smith, J. A. (1950).—*Biochem. J.* **46**: 36-43.
Rigler, N. E., and Greathouse, G. A. (1941).—*Industr. Engng. Chem.* **33**: 693-4.
Rubbo, S. D., Albert, A., and Gibson, M. I. (1950).—*Brit. J. Exp. Path.* **31**: 425-41.
Ryley, J. F. (1953).—*Nature* **171**: 747-8.
Tyler, A. (1953).—*Biol. Bull. Wood's Hole* **104**: 224-39.
White, I. G. (1954).—*Aust. J. Exp. Biol. Med. Sci.* **32**: 41-8.
White, I. G. (1955).—*Aust. J. Exp. Biol. Med. Sci.* **33**: (in press).
Zentmyer, G. A. (1944).—*Science* **100**: 294-5.