Biochemical Pharmacology, 1962, Vol. 11, pp. 1035–1042. Pergamon Press Ltd., Printed in Great Britain.

HISTOCHEMICAL EVIDENCE FOR CROSS-LINKING OF DNA BY ALKYLATING AGENTS *IN VIVO*

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(*Received* 1 *May* 1962; *accepted* 28 *May* 1962)

Abstract—Unfixed, frozen sections of rat liver, spleen, thymus and testis were extracted with strong salt solution. This treatment removed nearly all the DNA from liver, spleen and thymus and, in the testis, from Sertoli cells, spermatogonia, spermatocytes and early spermatids. Spermatid DNA became increasingly inextractable after the end of the acrosome phase and completely so by the end of the maturation phase, when spermatozoa were formed.

In animals treated with triethylene melamine or nitrogen mustard a further, marked loss of extractability was superimposed on the normal changes, beginning within a few minutes of intraperitoneal injection and most evident in spermatids at the late acrosome and early and mid-maturation phases. Monofunctional alkylating agents did not have this effect. Triethylene melamine did not induce resistance to extraction in the DNA of liver, spleen or thymus.

The naturally occurring spermatid changes appear to be due to the development of an insoluble protein sheath, and the superimposed loss of solubility induced by alkylating agents may be attributed to the direct or indirect cross-linking of DNA to this.

INTRODUCTION

IT HAS been shown that various ethylene imines and nitrogen mustards can cross-link the DNA of living cells *in vitro*.[1, 2] The procedure was to extract DNA-protein from the cells by strong salt solution and to centrifuge the extract at 20000 × *g*. DNA from normal cells was not affected by this treatment but part of the DNA of cells treated with alkylating agents was deposited. The deposited DNA presumably was cross-linked by the agents used, but not so much that it was insoluble in the extracting solution.

Zbarsky and Georgiev[3] showed that conventional salt-extraction techniques for nucleoprotein could be applied to frozen sections of unfixed tissues. Though it might be expected that lightly cross-linked DNA would be leached out of sections by salt solutions, as it was from cell-suspensions,[1, 2] the possibility arises that extensively cross-linked DNA might resist extraction. If this were so, evidence for cross-linking would be obtainable at the histological level.

MATERIALS AND METHODS

Mature male rats of the WAG and PVG strains were used.[4] They were 2–3 months old and weighed 2–300 g.

Triethylene melamine (TEM) was obtained from I.C.I. Ltd. Nitrogen mustard (methyl bis β-chloroethyl amine) was obtained from Boots Pure Drug Co., Ltd. The following monofunctional alkylating agents were also used: myristoyl ethylene imine

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and *p*-nitrobenzenesulphonyl ethylene imine, obtained from I.C.I. Ltd., and ethylene imino propionyl methyl ester and N_1 ethyl N-chloroethyl amine hydrochloride, obtained from the Chester Beatty Research Institute.

TEM, nitrogen mustard and N_1 ethyl N-chloroethyl amine hydrochloride were dissolved in physiological saline. Ethylene imino propionyl methyl ester, which was a viscous, water-miscible fluid, was mixed with physiological saline. Myristoyl ethylene imine and *p*-nitrobenzenesulphonyl ethylene imine were dissolved in warm dimethyl formamide.

All solutions were injected intraperitoneally within 5 min of preparation, and in a volume of 0·75–1·0 ml per rat.

In initial experiments one testis was removed through an abdominal incision under ether anaesthesia before injecting the alkylating agent and served as a control for the other testis, which was removed after the injection. Later, separate animals were used as controls in each experiment. Liver, spleen and thymus were also used.

On removal, tissues were frozen at −20 °C and frozen sections, 5μ thick, of the unfixed tissue were cut in a cryostat. They were mounted three to a slide and, as Zbarsky and Georgiev recommend,[3] immersed immediately in salt solution before any drying could take place. In most experiments the extracting solution was 2·5M sodium chloride, 0·01M trisodium citrate. In some experiments the molarity of the sodium chloride was varied between 0·5 and 2·5, the molarity of the citrate being kept constant.

Extraction was usually carried out for 24 hr at 3–4 °C, the fluid being changed at least three times. The effect of extraction times of from 15 min to 3 days was also investigated.

The extracted sections were rinsed in cold physiological saline and fixed in absolute ethanol for an hour, then treated with 1N HCl at 58–60 °C for 9 min and stained in Schiff reagent for 40 min. In each experiment all sections were treated in one batch to avoid adventitious variations in staining intensity. Stained sections were mounted in glycerine jelly and examined by bright-field and anoptral contrast illumination.

RESULTS

1. *Testis*

(*a*) *Staging of spermatids*

Pilot experiments showed that most of the DNA was rapidly removed from the nuclei of Sertoli cells, spermatogonia and spermatocytes by 2·5M NaCl. Epididymal spermatozoa resisted extraction, as did some nuclei in the testis. A preliminary survey suggested that the latter might be late spermatids. Before proceeding, therefore, it was necessary to find the reason for this variation in extractability of spermatids. Changes due to normal development appeared to be the most likely explanation, and this was confirmed as described below.

Leblond and Clermont's[5] division of spermatid development into nineteen stages is based mainly on the appearance of the acrosome in paraffin sections of appropriately fixed tissue, stained by the periodic acid–Schiff method. Various spermatid stages are associated with distinctive appearances in the contiguous germinal epithelium, but the acrosomal changes are so characteristic that each spermatid stage is identifiable without reference to the appearance of associated cells. In paraffin sections stained with haematoxylin or the Feulgen method the acrosome is unstained and invisible, and "staging" of spermatids therefore depends partly on the less distinctive changes in

their nuclei and partly on associated cyclical changes in the adjoining spermatogonia and spermatocytes.[6, 7] Without the aid of these correlated changes in other cells it is not possible to define more than from ten to twelve stages in spermatid development.

In the work described here, frozen sections of unfixed tissue were subjected to a treatment that removed nearly all the DNA from spermatogonia and spermatocytes and from many of the spermatids and were then stained by a method that demonstrates only DNA. It is not surprising, therefore, that it was difficult to stage spermatids in these sections. However, there was usually some faint residual staining, and optical contrast was increased by mounting the sections in glycerine jelly, so that it was possible to distinguish seven stages (or groups of stages) without much difficulty. The criteria used were, (1) the presence or absence of elongated spermatid nuclei, (2) their distribution in the epithelium, and (3) the thickness of the tail mid-piece.[8] The stages were numbered according to the scheme of Leblond and Clermont.[5]

Stages 1–5 (*Golgi phase and early cap phase*). The spermatid nuclei are round and the different stages not distinguishable in Feulgen-stained frozen sections. More superficially in the same epithelium is an older generation of spermatids with elongated nuclei, progressing from stage 15 to 17 (see below).

Stages 6–8 (*late cap phase and beginning of acrosome phase*). The spermatid nuclei are not distinguishable from those of stages 1–5. The more superficial, older generation of spermatids is now at stages 18–19 (see below).

Stage 9 (*early acrosome phase*). The spermatid nuclei are slightly elongated. The older generation of spermatids has been shed so that no markedly elongated nuclei are present in the epithelium.

Stages 10–13 (*middle and late acrosome phase*). Elongated spermatid nuclei are arranged in bundles at all depths of the epithelium. Their tails are thin as the mid-piece has not yet developed. Elongation is slight at stage 10, increases during stage 11 and is greatest at stage 12. It was difficult, therefore, to differentiate stage 10–11 nuclei from those of stage 9 in extracted sections. Stage 12–13 nuclei could readily be identified by their elongated and pointed shape, and attention was concentrated on these in studying the middle and late parts of the acrosome phase.

Stage 14 (*end of acrosome phase*). Elongated spermatid nuclei are scattered throughout the epithelium and are not arranged in bundles. Their tails are thin.

Stages 15–17 (*early and middle maturation phase*). Bundles of elongated spermatid nuclei are again present at all depths of the epithelium and their tails are thick owing to the development of the mid-piece.

Stages 18–19 (*late maturation phase*). Spermatid nuclei are lined up on the luminal surface of the epithelium and their tails are at their thickest.

The various stages are illustrated diagrammatically in Fig. 1.

It is possible that bundles of spermatozoa might have been disrupted during section-cutting of unembedded material, so that cells at other stages would present the appearance associated with stage 14. It was therefore thought advisable to omit from study tubule cross-sections in which the spermatids appeared to be at this stage.

(*b*) *Extractability of cells of normal germinal epithelium*

It was easy to extract DNA from the nuclei of Sertoli cells, spermatogonia, spermatocytes and spermatids at stages 1–9. Spermatids were slightly less easy to extract at stages 12–13. Extractability decreased during subsequent stages, and this effect

was marked during the late maturation phase. Spermatids in this phase that had been extracted for 24 hr varied in intensity of staining. In certain tubules they stained somewhat less intensely than the controls, whereas in others in the same section staining was as intense as that of unextracted cells. During this phase, therefore, spermatid DNA became completely inextractable, so far as could be shown by staining.

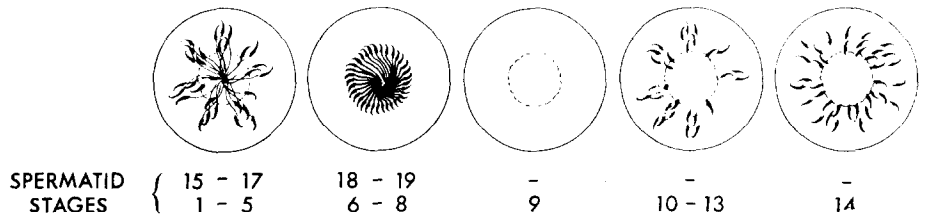

FIG. 1. Showing how the various spermatid stages may be identified in Feulgen-stained frozen sections. For clarity, only elongated nuclei are shown.

The DNA of epididymal spermatozoa resisted extraction, even for 3 days. These changes are shown in Fig. 2.

Of the DNA extracted, most was removed within 15 min by 2·5 M NaCl, 0·01 M citrate, but extraction was more complete after 2 hr. Maximum extraction was achieved in 24 hr, and this was the time adopted in most experiments. Extraction was satisfactory in 1·5–2·5 M saline, but was poor in saline of molarity 1·0 or less.

(*c*) *Effect of TEM*

Even doses of 250 mg/kg TEM did not affect the staining of unextracted cells of any type or stage.

When animals were given 50 mg/kg and killed 1 hr later, the results were as follows. Stage 1–9 spermatids were as extractable as those of untreated animals. The DNA of spermatids from stage 12 to stage 17 showed reduced extractability as compared with controls (Fig. 3). Decreased ease of extraction was also detected in stage 18–19 spermatids, but with more difficulty, as the DNA normally became completely inextractable by the end of this stage. Epididymal spermatozoa, which were unaffected by salt solution, were no different after TEM treatment.

Animals were injected with doses of 5, 10, 25, 50 and 250 mg/kg TEM and killed 1 hr later. A decrease in extraction of spermatids was often noted after a dose of 5 mg/kg and always after 10 mg/kg. The effect was more marked the higher the dose (Fig. 4).

When 50 mg/kg of TEM were injected, a decrease in the ease of extraction of spermatid DNA was noted 5 min after injection (Fig. 5).

The administration of TEM, therefore, appeared to cause a very rapid change in spermatid nuclei, shown by a loss of extractability in salt solution. It was necessary, however, to exclude the unlikely possibility that spermatid extractability had not changed but that TEM had caused a sudden real increase in the proportion of late spermatids, which would normally be relatively resistant to extraction. If this were so one would have expected a decrease in spermatid stages 9–14, normally completely or almost completely extractable, and an increase in stages 15–19, which are normally less extractable. The following experiment was therefore performed.

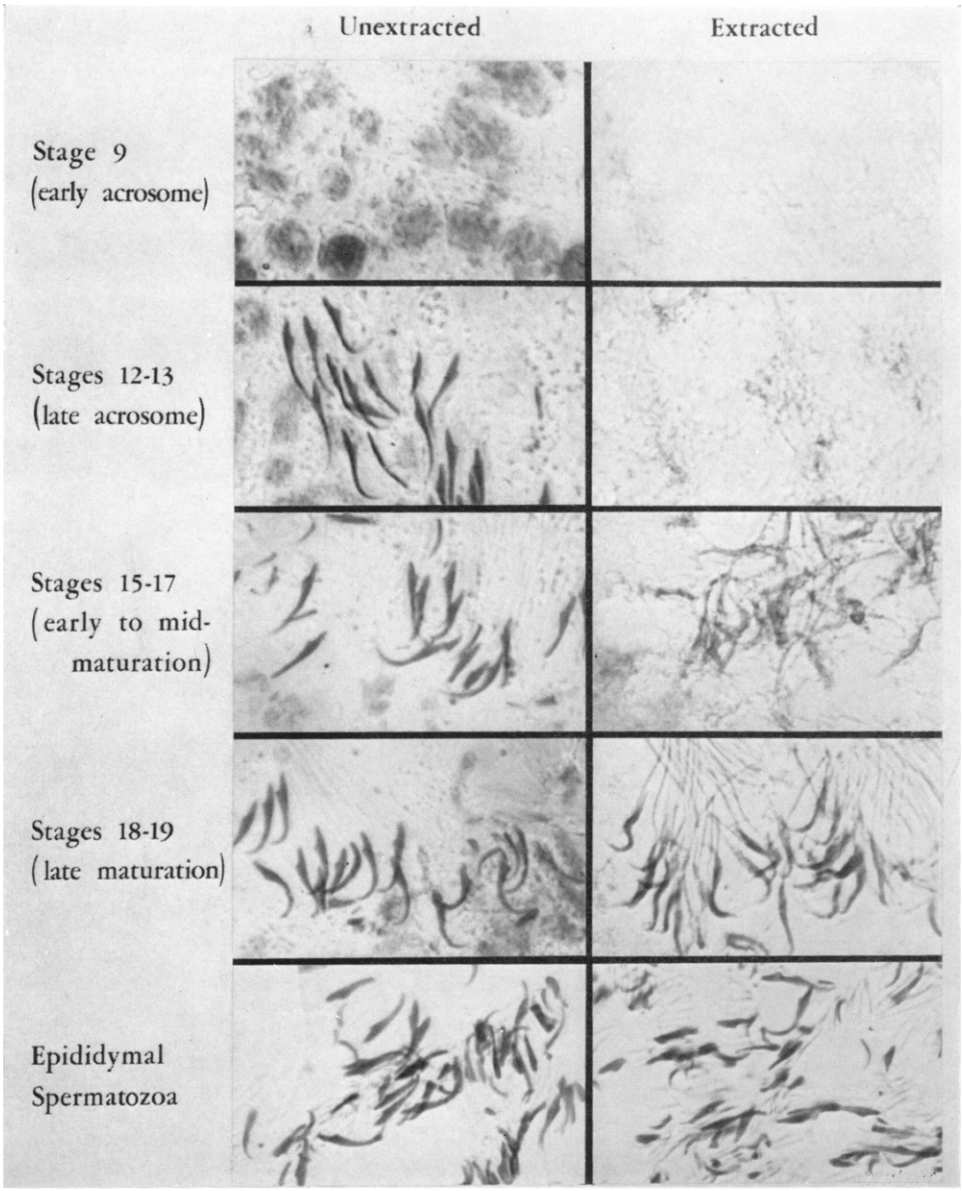

FIG. 2. The effect of extraction on normal sperm cells. The sections on the right were extracted with 2·5 M NaCl, 0·01 M citrate at 4 °C for 24 hr before fixing and staining. Early acrosome phase nuclei are completely extractable and late acrosome phase nuclei almost so. Extractability decreases during the maturation phase and spermatozoa are completely inextractable. (Note that the tails are more obvious in extracted sections.) Feulgen, × 1350.

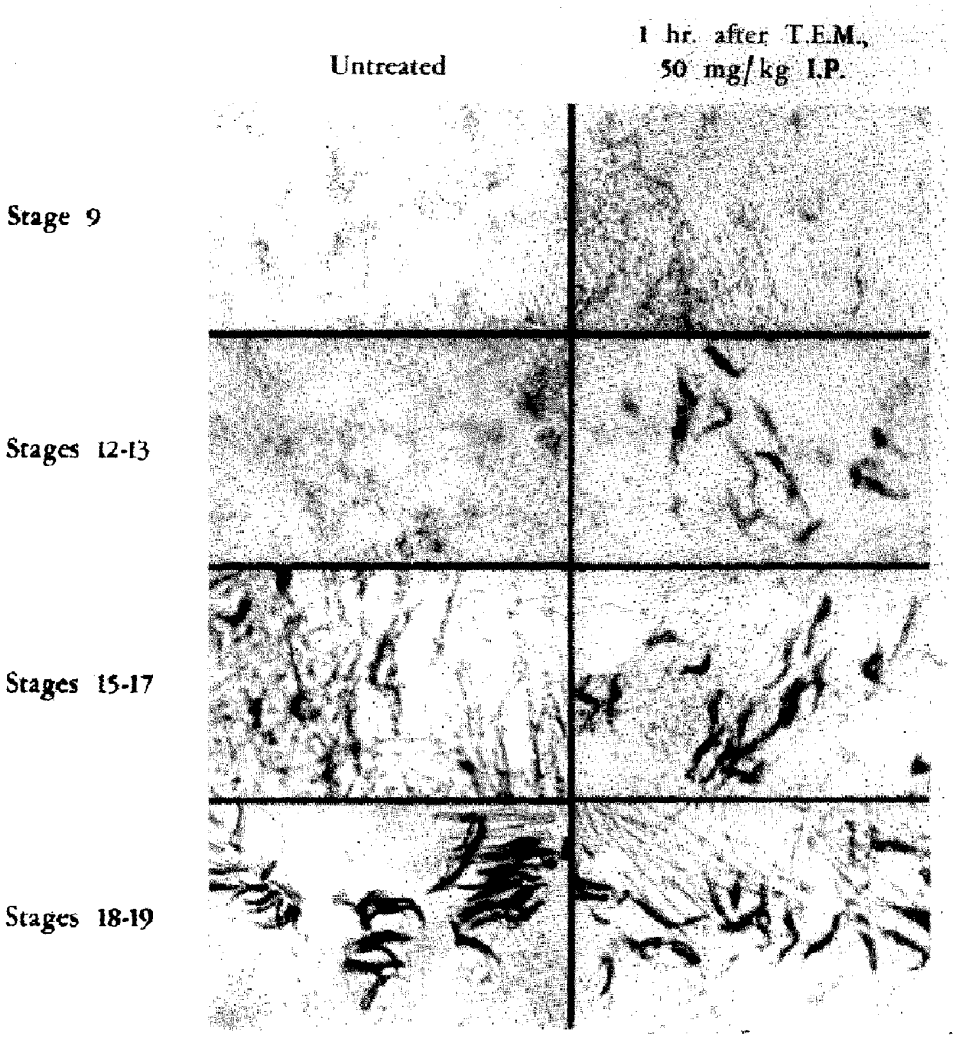

FIG. 3. The effect of TEM on extractability of spermatids. Stage 9 spermatids, and earlier stages, are hardly affected by TEM. The extractability of spermatids at stages 12–17 is markedly reduced by administration of TEM. Feulgen, ×1350.

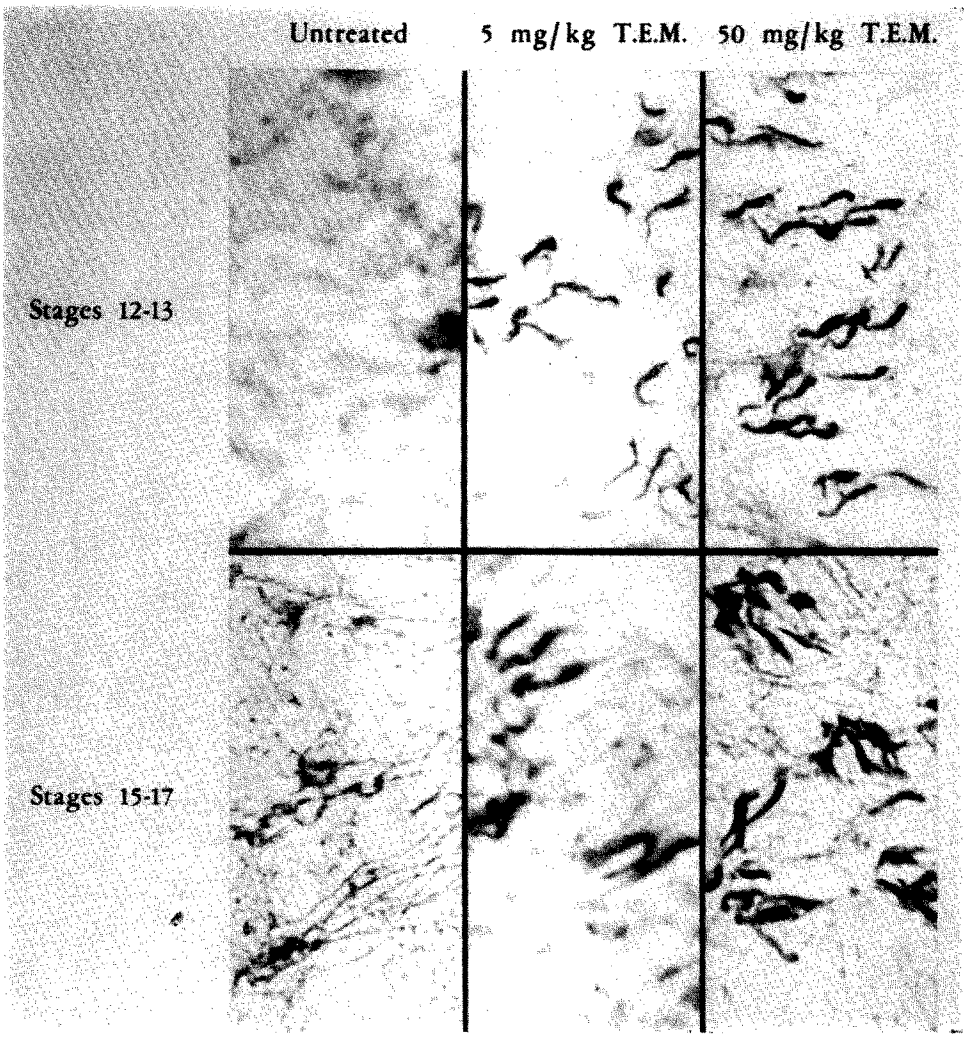

FIG. 4. Showing that a decrease in extractability of spermatid DNA may be produced by a dose of 5 mg/kg TEM. Extractability is further decreased by higher doses of TEM. Feulgen, $\times 1350$.

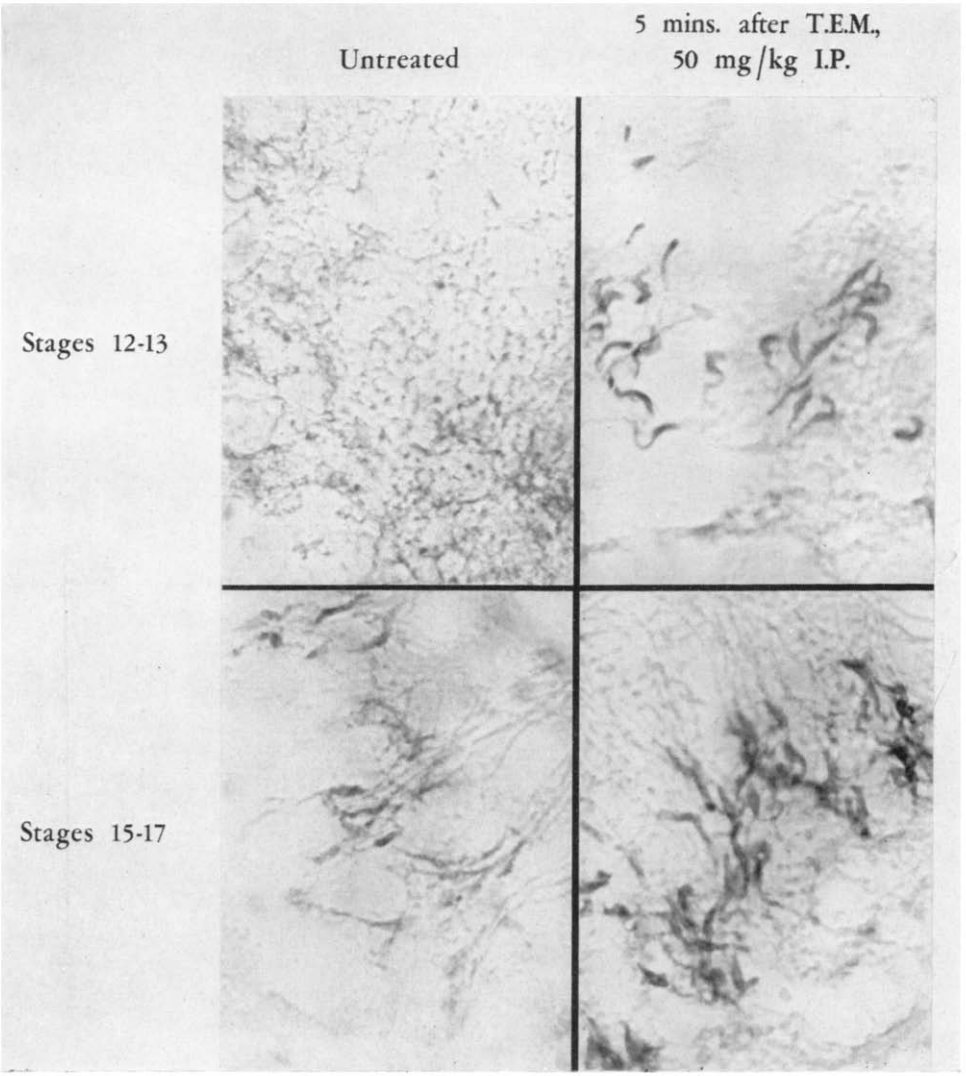

FIG. 5. Showing that there is a decrease in extractability of spermatid DNA 5 min after an intraperitoneal injection of TEM. Feulgen, $\times 1350$.

Animals were subjected to unilateral orchidectomy and then injected with 250 mg/kg TEM in 1 ml saline or with saline only. The remaining testis was removed 1 hr later. Half of each testis was frozen, and unfixed sections were cut, extracted and stained as above. The expected loss of extractability was found in testes removed after TEM treatment. The other half of each testis was fixed in formalin and embedded in paraffin wax and sections were stained by periodic acid–Schiff and haematoxylin. One hundred tubules were counted in each section and classified either as being in stages 9–14 or 15–19. (Spermatid stages 1–8 were also present in the latter but are not of concern here.)

The results are presented in Table 1, which shows that loss of spermatid extractability cannot be accounted for by a change in the proportions of the various stages.

TABLE 1. PERCENTAGES OF TUBULES AT DIFFERENT SPERMATID STAGES BEFORE AND AFTER TREATMENT WITH TEM, 250 mg/kg, OR SALINE

	Before treatment	After TEM	After saline
No. of rats	8	5	3
Stage 9–14	45·0	45·2	42·7
Stage 15–19	55·0	54·8	57·3
Standard deviation	2·4	5·1	5·7

(*d*) *Effect of nitrogen mustard*

Rats were given 80 mg/kg nitrogen mustard intraperitoneally (the maximum compatible with survival for 1 hr) and killed after 40–60 min. Changes in spermatid extractability were seen and were of the same type as those caused by TEM but less marked.

(*e*) *Effect of monofunctional alkylating agents*

Animals were injected with these agents at the following dose levels and killed 1 or 2 hr later: myristoyl ethylene imine and *p*-nitrobenzenesulphonyl ethylene imine, 1 g/kg, ethylene imino propionyl methyl ester, 100 and 200 mg/kg, N_1 ethyl N-chloroethyl amine hydrochloride, 200, 300 and 400 mg/kg. In no case could any effect on the ease of extraction of spermatids be detected. It should be noted that myristoyl ethylene imine and *p*-nitrobenzenesulphonyl ethylene imine, which were dissolved in dimethyl formamide for injection as they were highly insoluble in water, were rapidly precipitated in the peritoneal cavity after injection. A quarter to half the dose could be recovered from the peritoneum 1–2 hr later, so that the dose absorbed is uncertain.

This difficulty did not arise with the other monofunctional alkylating agents used which are water-soluble. It was incidentally confirmed that TEM was as effective in producing the changes described when dissolved in dimethyl formamide as when dissolved in saline.

(2) *Liver, Spleen and Thymus*

Most of the DNA was removed from frozen sections of liver, spleen and thymus by 24 hr extraction in 2·5 M NaCl, 0·01 citrate. Ease of extraction was unaffected by

treatment with TEM. Extracted sections from rats that were killed 1 hr after the administration of even 250 mg/kg TEM could not be distinguished from similarly treated sections from control rats.

DISCUSSION

These experiments were undertaken in the expectation that massive doses of alkylating agents would cross-link DNA in various tissues so extensively as to render it resistant to extraction by salt solutions. This expectation was not realized; even the highest doses of TEM used had no discernable effect on the ease of extraction of DNA from sections of liver, spleen or thymus, so far as could be ascertained by staining. It follows either that DNA in these organs is not extensively cross-linked by large doses of TEM given *in vivo* or that it is cross-linked without its extractability in strong salt solutions being affected.

In developing sperm, however, an effect of TEM on solubility was readily shown. It seems likely that this is connected with the changes normally taking place during sperm cell development that lead to its DNA becoming inextractable by the time spermatozoa are formed. In mammals, spermatozoa differ from other cells in that their nucleoprotein is not extractable by strong salt solutions without preliminary disruptive treatment, such as incubation in caustic soda, trypsinization or reaction with mercaptoethylamine.[9-11] Borenfreund *et al.*[11] suggest that this is because the DNA-protein is linked, directly or indirectly, to the keratin-like sheath of the sperm head, possibly by a sulphur-containing group. To put this suggestion another way, spermatozoal nucleoprotein is insoluble because it has undergone a natural "cross-linking" to an insoluble protein by bonds that are not dissociated in strong salt solution. This property is, however, not shared by the nucleoprotein of immature sperm. Vendrely *et al.*[12] found that 82 per cent of the DNA of bull testis was extracted by 1 M NaCl. The inextractable residue consisted of intact spermatozoa. We have had entirely similar results with rat testis treated in the same way. Our experiments suggest that the changes in the sperm head causing progressive inextractability of its DNA begin at the end of the acrosome phase and are fully developed by the end of the maturation phase, when the young spermatozoa are released from the surface of the germinal epithelium.

Any treatment that makes DNA less soluble in salt solution would have little demonstrable effect on late spermatids and spermatozoa, in which the DNA is in any event largely insoluble. The inability of the method used here to show an effect on these cells does not mean, therefore, that their DNA is not severely affected by TEM. However, during preceding stages, when the extractability of DNA is rapidly decreasing, even a slight superimposed loss of solubility from a cause other than the natural one might have a marked effect. Such solubility changes might be expected if the DNA were cross-linked by an alkylating agent, for this would lead to an increased proportion of the DNA molecules being bound, directly or indirectly, to the protein coat of the sperm head. Another possibility is that DNA molecules in the maturing sperm nucleus are so packed and orientated[13] that cross-linking agents can convert significant portions of the whole into inextractable agglomerates, irrespective of the presence or absence of insoluble proteins.

In deciding which of these two possibilities is the more likely it is relevant to consider the effects of cross-linking agents on non-mammalian spermatozoa. These

contain highly orientated nucleoprotein,[13] but, as they do not show anomalous resistance to salt-extraction, they presumably do not possess keratin-like sheaths. Treatment with alkylating agents *in vitro* does not affect the ability of herring sperm nucleoprotein to disperse in 2 M NaCl, although much of the DNA is cross-linked by this treatment.[2] This suggests that cross-linking agents markedly reduce the extractability of spermatid DNA because the spermatid nucleus has developing in or around it an insoluble protein framework and not because its nucleoprotein is highly orientated.

That the changes produced in these experiments were due to cross-linking and not simply to alkylation is suggested by the fact that they were not produced by massive doses of monofunctional ethylene imines or mustards.

The doses of TEM used call for some comment. Clinically effective doses are of the order of 0·03–0·3 mg/kg,[14, 15] and the LD_{50} for the animals used here is about 1·5 mg/kg. The smallest dose producing a detectable change in extractability was therefore about three to four times the LD_{50} and fifteen to twenty times the maximum clinical dose. Accordingly, it might be argued that this effect would not be produced by therapeutic doses and that it therefore cannot throw light on the clinically important actions of TEM. However, it must be pointed out that the method used is insensitive. It is unlikely that the demonstrable changes in extractability caused by 5 mg/kg TEM involve less than 5–10 per cent of the DNA of affected nuclei. Doses in the therapeutic range may cross-link much smaller amounts of DNA, but the lesion would nevertheless be sufficient to interfere with cell function and viability. This is the reason that justifies the use, for *in vitro* work, of concentrations of alkylating agent that would be equivalent to *in vivo* doses of 500–1000 mg/kg.[1, 2]

Some conflict is found in the literature as to which stage of the developing sperm is most sensitive to TEM, but the rapid onset of sterility after administering this agent suggests that it is the late stages (spermatids and spermatozoa) that are the most susceptible to damage.[16–21] The damage caused by small doses of TEM is rather subtle in that the affected spermatozoa may appear normal as judged by morphology and motility and even ability to fertilize ova, yet are unable to produce normal zygotes.[16, 20, 21]

The findings reported here therefore agree with studies of the sterilizing effects of TEM in that it is late, rather than early, stages of sperm development that are affected. It is accordingly suggested that TEM, in doses that cause sterility, acts by cross-linking a small portion of the DNA of spermatids and spermatozoa.

Acknowledgements—I am indebted to Dr. P. Alexander for suggesting a trial of monofunctional alkylating agents and for gifts of ethylene imine propionyl methyl ester and N_1 ethyl N-chloroethyl amine hydrochloride, to Dr. A. F. Crowther of I.C.I. Ltd. for gifts of myristoyl ethylene imine and *p*-nitrobenzenesulphonyl ethylene imine, to Mr. R. S. Stannard for technical assistance and Mr. R. W. Rowntree for taking the photographs.

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