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PROGRESS REPORT NO. 5

SULFONAMIDE DRUGS AS A MALE ANTIFERTILITY AGENT

A RESEARCH PROJECT

US-ISRAEL CDR PROGRAM

Grant No. 936-5544-G-00-6105-00

Submitted by

Chumpol Pholpramool, Ph.D.

Project Leader

Department of Physiology, Faculty of Science,

Mahidol University, Rama VI Road, Bangkok 10400, Thailand

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Project Profile

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Project Leader	:	Chumpol Pholpramool, Ph.D.
Organization	:	Faculty of Science, Mahidol University
Co-investigators	:	Somsak Ruchirawat, Ph.D. Lawrence M. Lewin, Ph.D. Rachel Golan, Ph.D.
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1. INTRODUCTION

We have synthesized a number of sulfapyridine analogs and their biological activities on fertility of the male rats have been studied (1-3). It appears that the presence of N^4-on the benzene ring is essential for the antifertility activity. Thus, sulfapyridine and 2-(p-nitrobenzene sulfonamide)pyridine are effective whereas N-2-pyridylbenzene sulfonamide, 2(o-nitrobenzene sulfonamide) pyridine and 2-(p-toluene sulfonamide)pyridine are ineffective in causing a decrease in fertility (2-3). In this report, we show that neither 2(3-nitrobenzene sulfonamide)pyridine nor 2-(4-bromobenzene sulfonamido) pyridine has any antifertility activity, but 2(N^4-acetyl-sulfonamido)pyridine is as potent as sulfapyridine. In addition, we have also shown that the presence of pyridine group is not required for the antifertility activity since a simple sulfonamide such as sulfanilamide, sulfacetamide or N^1-diethylsulfanilamide are all effective (1,2,4). However, some of the latter group of compounds has toxicity to the tested animals,e.g. N^1-diethylsulfanilamide and its N^4-acetyl derivative (4). In order to decrease the toxicity and to increase the efficacy of the tested compounds, we have synthesized a series of sulfonamides having simple substitution at N^1 position.

Attempts to study the mode of action of the antifertility sulfonamides have shown that sulfapyridine had virtually no effect on basal levels of blood testosterone. However, the level of LHRH-induced blood testosterone was markedly higher than that in the control group (4). These results suggest that the responsiveness of either the pituitary gland or the Leydig cells, or both, to LHRH is enhanced. In this report, the levels of LH in the blood have been assayed and the results support the view that the sensitivity of the Leydig cells is increased. Although the results so far indicate that sulfapyridine does not interfere with the hormonal profile of the animals, it remains to be determined whether the spermatogenesis is affected by this drug.

Previously, it has been demonstrated that sulfasalazine caused decreases in epididymal sperm reserve and sperm motility (5). Since sulfanilamide is also as potent as sulfapyridine, which is now known as the active moiety of sulfasalazine (6-8), the effect of this compound on epididymal sperm morphology, sperm reserve and sperm motility as well as the sperm count and motility of the ejaculated spermatozoa have been investigated in this study.

It has been reported that the sulfhydryl content of sperm entering the caput epididymis diminished during passage through the epididymis as the SH groups became oxidized to disulfides(9). This change occurred both in the sperm head and tail, affecting the proteins of the nucleus and of the flagellar apparatus. Recently, the Tel Aviv group has developed a method for measuring SH groups in sperm using the fluoresent labelling reagent, monobromobimane (10). In the study reported here the monobromobimane reagent has been used to measure the degree to which sulfhydryl groups have been oxidized to disulfides during passage of sperm from caput to corpus to cauda epididymides in

control rats as compared to those which have been treated with sulfapyridine.

2. OBJECTIVES

The aims of the work included in this report are:

(a) to synthesize some sulfanilamide analogs in which N^1 is substituted by a diethyl group,

(b) to test whether the compounds in (a) have any antifertility activity,

(c) to investigate the possible effect of sulfapyridine on the levels of LH in the blood and to deliniate the question whether the responsiveness of the pituitary or the Leydig cells to LHRH is increased,

(d) to evaluate the possible effect of sulfapyridine on testicular spermatogenesis,

(e) to determine the effect of sulfanilamide on epididymal sperm morphology, sperm reserve, sperm count in the ejaculates, and motility of both cauda epididymal and ejaculated spermatozoa.

(f) to study the effect of sulfapyridine on the thiol groups of spermatozoa during their transit through the epididymis.

3. MATERIALS AND METHODS

3.1 Fertility testing

Male rats of Fischer strain obtained from the Animal House at the Faculty of Science, Mahidol University aged between 90-120 days were screened for fertility capacity before grouping and drug treatments. Each male was housed with two virgin female rats of the same strain aged 40-50 days in handing cages with free access to pellet feeds and water for one week. The females were then separated and kept for 8-10 days at which time pregnancy was examined after sacrifice. The number of fetus and corpus luteum were counted under a dissecting microscope. Per cent fertility of the male was evaluated from the percentage of the eggs successfully fertilized and the embryos implanted, i.e., numbers of fetuses over corpola lutea x 100. Only the males showing high percentage fertility (greater than 80% in at least one of the two females) were selected for further experiments.

3.2 Collection of blood samples

Blood samples were collected from either left or right external jugular vein using aseptic conditions after the rats were anesthetized with diethyl ether. Blood samples were kept overnight in a refrigerator to allow complete coagulation. In the following morning, the blood samples were centrifuged and sera were separated and kept at -20°C for hormone assays.

3.3 Hormone assays

Serum LH was determined by the mouse Leydig cell bioassay of Dufau, Beitins, McArthur & Catt (1976). All assays were done in the same run in which the low and high pools of human serum had LH values of 0.25 and 11.03 ng/ml, respectively.

3.4 Experimental procedures

3.4.1 Experiment 1 (Fertility testing of the synthetic compunds)

Fifty six male rats were allocated into 7 groups (8 rats each)and were fed with either corn oil or the test compounds by using intragastric tubing. Three synthetic sulfonamides, i.e., 2(N^4-acetylsulfonamides) pyridine (ASP) 2-(m-nitrobenzene sulfonamido) pyridine (MNSP) and 2-(p-bromobenzenesulfonamido) pyridine (PBSP), were administered at doses of 150 and 450 mg/kg/day for 6 weeks. At the end of weeks, 1,3,5,7 and 9 all control or treated animals were subjected to fertility testing as described above except that the females were checked for the presence of spermatozoa in vagina smears every morning after cohabitation so as to confirm mating. Therefore, fertilities of the males at weeks 1,3 and 5 during treatment and weeks 1 and 3 after cessation of treatment were determined.

3.4.2 Experiment 2 (The responsiveness of the pituitary gland and the testis to LHRH

Sixteen male rats with proven fertility were allocated into 2 groups (8 rats each) and were forced-fed with either corn oil or sulfapyridine at a dose of 450 mg/kg (once daily) for 6 weeks. At the end of weeks, 1,3 and 5, all animals were subjected to fertility testing as in Experiment 1. At the end of treatment period (6 weeks), the animals in both groups were anesthetized with diethyl ether and 2-3 ml of blood samples were collected as described above. Each animal then received a subcutaneous injection of 10 ug LH-RH (Sigma Chemical, U.S.A.) and were allowed to recover. Exactly two hours after LH-RH injection blood samples were collected again from the jugular vein.

In order to reduce large variations of blood gonadotropins and testosterone due to diurnal rhythms, all blood sample collections were made early in the aftrernoon as in previous experiments.

3.4.3 Experiment 3 (The effect of sulfapyridine on spermatogenesis)

Twenty three male rats of proven fertility were divided into 4 groups and received daily subcutaneous injections of DMSO or sulfapyridine dissolved in DMSO for 5 weeks. Fertility was tested as in Experiment 1. At the end of treatment period, all rats were sacrificed by excess ether anesthesia. In each animal,

one testis was removed and dissected free from the epididymis and other tissues, and then weighed. Spermatogenesis was determined by measuring the daily sperm production rate described previously by Johnson, Petty & Neaves (11). In brief, the tunica vaginalis was incised and the parenchymal testicular tissues was expressed into a homogenization tube. The tissue was then homogenized in a fluid containing 150 mM NaCl, 0.05 (v/v) Triton X-100, and 3.8 mM sodium azide. The homogenates were used fresh or stored at 5°C until assayed within 48 hr. A small aliquot (10 ul) of the homogenates was placed onto a hemocytometer and the spermatid heads, which are resistant to homogenization, were counted in a phase contrast microscope. The daily sperm production per gm tissue was calculated as follows:

$$\text{Daily sperm production rate (DSP)} = \frac{\text{number of mature spermatids}}{\text{Testicular weight (g)} \times \text{time divisor (6.3 day)}}$$

3.4.4 Experiment 4 (The effect of sulfanilamide on epididymal sperm reserve, morphology and viabiltity of spermatozoa, and sperm count in the ejaculates)

Twenty male rats were treated as in Experiment 1 but the test compound was sulfanilamide, which was given to the animal at a dose of 450 mg/kg. At the end of treatment, the animals from both the control group and sulfa drug treated group were lightly anesthetized with ether. The cervical vertebrae of the animals were then dislocated. This procedure causes spasms and ejaculations of the animals just before they are dead. The ejaculate comprises a coagulum and semen. Both specimens were quantitatively collected and the semen volume was measured. The coagulum was minced into small pieces and suspended in phosphate buffered saline (PBS). Spermatozoa in the semen and the coagulum were quantified in a hematocytometer chamber.

In order to obtain epididymal spermatozoa, the epididymis was removed from the animal and vas deferens was cannulated with a polyethylene tubing filled with sudan black-stained paraffin oil. The epididymal tubule at the mid cauda region was isolated and straightened out, and then cut open. The luminal content of the cauda epididymis was then retrogradely flushed out by infusion of the stained oil through the vas cannula. This method yields uncontaminated specimens of the cauda epididymal fluid.

A small volume of the epididymal fluid was diluted with PBS and the number of spermatozoa was counted in a hematocytometer chamber to obtain the epididymal sperm reserve as previously described (5).

Another portion of the epididymal fluid was diluted (1:10) with PBS and stained with eosin-nigrosin for 2 min. The suspension was then spreaded onto a clean glass slide to make a thin film and was allowed to dry at room temperature. Numbers of viable spermatozoa as well as sperm morphology were observed under a light microscope at a magnification of x1,000.

A portion of spermatozoa from the cauda epididymidis was washed several times with PBS and then fixed with

glutaraldehyde. The fixed specimen was packed by centrifugation and deposited in Epon for electronmicroscopic studies.

3.4.5 Experiment 5 (The effect of sulfanilamide on epididymal and ejaculated sperm motility)

In this experiment, both epididymal and ejaculated spermatozoa were obtained from Experiment 4. Immediately after collection, the spermatozoa were kept warm at 34° throughout the experiment. Spermatozoa were diluted with warm PBS to obtain sperm concentration between 1-2x10⁶ sperm/ml. The diluted specimens were observed under a light microscope equiped with a dark field condensor at 20 min intervals for 1 h. Motility of spermatozoa were recorded on a video tape, which was then played back for analyses later. Recordings of sperm motility were made in 5 different fields to cover at least about 100 spermatozoa for one period of determination.

Motility of spermatozoa was analysed interms of number of motile and non motile spermatozoa (% motility), average velocity and motility index.

3.4.6 Experiment 6 (The effect of sulfapyridine on the respiration of ejaculated rat spermatozoa)

This experiment was performed by Prof. L.M. Lewin and his collaborators at Tel Aviv University. Three adult Charles River male rats were fed ad lib with Ambar pellets (Ambar Corp.) for approx. 2-3 months. The animals were then fed with the similar pellets, which had been impregnated with the sodium salt of sulfapyridine (14 g sulfapyridine per kg of feed), for a period up to 47 days. After the treatment period, normal untreated feed was again offered to the animals. The dose of sulfapyridine was calculated from the daily consumption of feed per kg body weight and the concentration of sulfapyridine in the feed.

The animals were allowed to mate with female rats during the periods before, during and after treatment with sulfapyridine. Ejaculated spermatozoa were quantitatively obtained from the uterine fluid as follows. After mating the female rats were sacrificed and the abdomen was opened up. The cervix was clamped with a hemostat and the female reproductive tract was removed, trimmed, rinsed with saline and dried with a clean tissue paper. It was then placed on a piece of parafilm and the uteri were cut with a pair of scissors. The uterine contents were pressed out onto the parafilm. The contents were then transferred from the parafilm to a wighed counting vial. After weighing the gel-like ejaculate, it was diluted with rat fertilizing medium (2) to 5 ml in a graduated centrifuge tube. The tube was then centrifuged after mixing to disperse the gel. The supernatant fluid was saved for chemical analysis, e.g. carnitine, and the sperm pellet was washed and used for metabolic estudies.

Sperm respiration was determined interms of oxygen utilization rate as previously described(3).

3.4.7 Experiment 7 (The effect of sulfapyridine on the sulfhydryl group of epididymal and ejaculated spermatozoa)

Mature albino rats of the Charles River strain, control group, were fed pelleted feed (Ambar, Israel) ad lib. The test animals were supplied with similar pellets containing 14 grams of sulfapyridine/kg as the sodium salt, prepared as follows: sulfapyridine (14 grams) was dissolved in 210 ml of NaOH treated feed dried at 40° overnight. This feed was fed ad lib to the rats in the experiments. Drug dose was determined by weighing the feed consumed.

Sperm cells were obtained from the caput, corpus, and cauda epididymides by cutting the tube and collecting the spermatozoa in 0.135M NaCl-0.01 M sodium phosphate buffer PBS, (pH 7.4). Sperm were washed in PBS, centrifuged at 600xg for 10 min and used immediately thereafter. Ejaculated spermatozoa were obtained as described above and similarly treated as the epididymal spermatozoa.

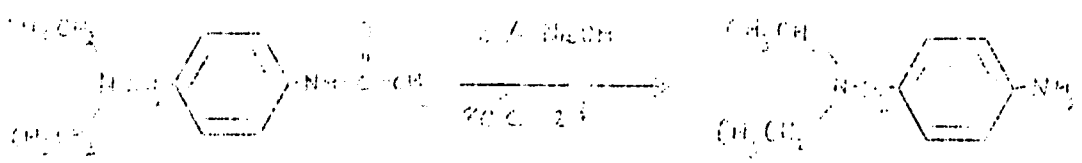
Aliquots of washed sperm were incubated with or without dithiothreitol (1 mM) at 37° for 10 min before being washed of thiols using a fluorimeter as described elsewhere (10). Free sulfhydryl content of sperm untreated with dithiothreitol was compared with total SH in cells treated with dithiothreitol in order to assess the per cent of thiols that remained in the reduced state.

An alternative method for determining the percentage of SH groups that remain unoxidized used a Fluorescence Activated Cell Sorter (FACS). In this method the monobromobimane-treated sperm were suspended in dilutent and droplets containing no more than a single sperm cell were passed through a beam of ultraviolet light. Fluorescence and light-scattering were measured for each sperm cell to determine cell size and SH content.

Electrophoretic analysis of sperm proteins was carried out in polyacrylamide gels as described therein and the monobromobimane-stained bands were observed under uv illumination and photographed. The gels were then stained with Cooomassie blue and photographed again.

3.5 Synthesis of the proposed compounds

3.5.1 N^1,N^1-Diethylsulfanilamide



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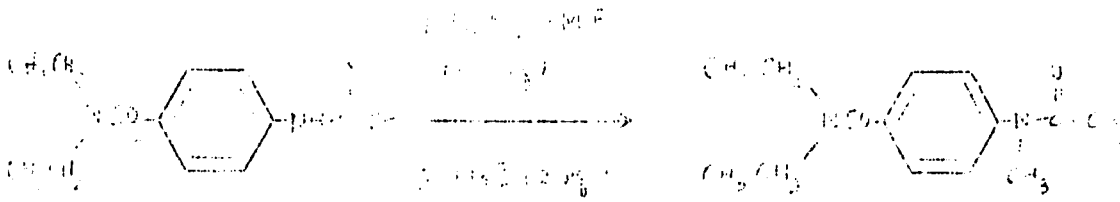
One gm of N^4-acetyl,N^1,N^1-diethylsulfanilamide, which has previously been prepared (4), was treated with 10 ml sodium hydroxide (10% in water). The mixture was heated at 80°C for 2 h then diluted with distilled water. After cooling, the reaction mixture was neutralized with 6N.hydrochloric acid. The precipitate formed was filtered by vacuum filtration and washed with cold distilled water. Purification of the hydrolytic product was done by recrystallization from a mxiture of hot methanol and water.

3.5.2 N^4-Acetyl-N^4-methyl-N^1,N^1-diethylsulfanilamide



A 2.61 g (0.087 mole) of NaH (80% in oil) was suspended in a 150 ml of DMF, and 20 g (0.0726 mole) of N^1,N^1-diethyl-N^4-acetylsulfanilamide was added to the suspension at 0°C. The reaction mixture was vigorously stirred at 0°C for 30 min. A 9 ml (0.145 mole) of methyl iodide was then added to the mixture, and stirring was continued for another 3 h period at room temperature. After which, a 100 ml of 2N aqueous solution of potassium hydroxide was added. The reaction mixture was again stirred for 3 h, then partitioned between dichloromethane and water. The organic layer was dried over anhydrous magnesium sulfate and concentrated. The product was purified by recrystalization from hot methanol.

4. RESULTS AND DISCUSSION

4.1 Fertility testing of the synthetic compounds

We have shown in the previous report (4) that N^1-diethyl sulfanilamide (DES) and its N^4-acetyl derivative (ADES) caused a marked reduction in fertility by 5 weeks after treatment. However, the fertility data after drug withdrawal were still incompleted such data were shown here in this report (Table 1). It is seen that low doses of DES and ADES caused a completed recovery of fertility within one week after drug withdrawal. A longer period is, however, required for the high doses of both compounds. By 3 weeks after drug withdrawal, fertility of the animal in all treatment groups were indifferent from the controls.

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Table 2 shows the effects of ASP, MNSP and PBSP on fertility of the male rats done in this reporting period. It appears that only ASP nad the antifertility effect. This effect occurred by 5 weeks after treatments at both low and high doses of ASP. Furthermore, the recovery of fertility was quite rapid since the fertility resumed normal level by one week after the cessation of drug treatment. Although there was a tendency for a reduction in fertility after treatments with MNSP and PBSP, but such a change was not statistically significant from the control group. However, significant reductions occurred by 3-5 weeks after treatments with both compounds when compared to the period before treatment within the same group. It should be mentioned that the variability of fertility of the animals in this experiment was quite large during and after treatment even in the control group. The reason for this is not known. From our experience, fertility testing during this time of the year (summer) always has problem. The animals do not mate, and if so, they yields a variable number of fetuses. The data of the control group in Table 2 also exhibit this phenomenon. Since the animals were kept at ambient temperature and lighting conditions, this reflects the influence of environmental factors on reproduction, which is not uncommon.

4.2 The responsiveness of the pituitary gland and the testis to LHRH

Analyses of blood LH showed the lack of effect of sulfapyridine treatment on the levels of this gonadotropic hormone (Table 3). The values in both the control and sulfa-treated animals either before, during or after the cessation of drug treatment were approximately 1 ng/ml. The data indicate that sulfapyridine does not interfere with the control of LH released from the asterior pituitary gland.

In another experiment, the response of the pituitary gland to lHRH was studied. By 2 hr after LHRH injection, blood levels of LH in the control animals was markedly increased from 0.87+0.28 (n=7) ng/ml to 8.85+3.93 (n=7) ng/ml (Table 4). This is equivalent to a nine-fold increase in LH. On the other hand, in the sulfapyridine-treated rats, blood LH was elevated fom 0.45+0.04 (n=7) ng/ml to 10.20+3.11 (n=7) ng/ml. A value which was not significantly higher than the control, although a twenty-nine-fold increase was noted. This is because the LH value before LHRH treatment in the sulfa-treated group was lower, but not significant than that in the control. The results suggest that the responsiveness of the anterior pituitary gland to LHRH was not changed by sulfapyridine treatment. Thus, the findings in the previous report (4) that blood testosterone levels were significantly enhanced after LHRH in the sulfapyridine-treated rats could not be accounted for by the changes in the LHRH-induced LH. Therefore, the possibility that the responsiveness of the Leydig cells to LHRH was enhanced by sulfapyridine is still left opened.

4.3 The effect of sulfapyridine on spermatogenesis

Table 5 shows the effect on spermatogenesis after various doses of sulfapyridine given subcutaneously to ensure complete delivery of the drug to the animals. It is clear, that the daily sperm production rate, which is a quantitative measure of spermatogenesis (11), was virtualily not affected by sulfapyridine even at the dose of 500 mg/kg. The data support the view that sulfapyridine does not exert its antifertility effect at the testicular site.

4.4 The effect of sulfanilamide on epididymal sperm reserve, morphology and viability of spermatozoa, and sperm count in the ejaculate.

As previously reported (2), sulfanilamide caused a marked reduction in fertility of the male rats. Thus, by 6 weeks after intragastric feeding (450 mg/kg/day), per cent fertiilty was decreased to 17.8+3.8 (10) compare to 94.3+2.9 (10) in the control group (Table 6). Sulfanilamide did not produce any visible toxicity since the animals had a normal gain in weight and appeared healthy throughout the treatment period. At the end of treatment, sex organs were weighed and, except only the prostate gland, there were no different in the organ weights (testis, epididymis, seminal vesicle and coagulating gland). This is similar to the effect of sulfapyridine, which spares its effect on the sex organ weights. The results indicate that sulfanilamide does not interfere with androgens. It is note worthy that the prostate gland weight (including its secretion) is increased by sulfanilamide. Preliminary data suggest that the prostatic fluid contents is greater in the drug-treated animals than in the controls.

In contrast to the sex ogan weights, sulfanilamide decreased the epididymal sperm reserve and caused a significant increase in death and disruption of epididymal spermatozoa (Table 7). Similarly, sperm count in the ejaculates was markedly decreased. A tendency for an increase in number of spermatozoa with detached head was also noted.

The effect of sulfanilamide on the epididymal sperm reserve collcborates the effect of sulfapyridine reported previously by our group (5). In additon, the effect of sulfanilamide on sperm count in the rat ejaculates is compatible with the clinical reports on the effect of sulfasalazine on human ejaculated semen (12).

At light microscopic level, a number of spermatozoa with tearing of the connecting piece was observed. This results in protusion of the coarse filaments from the mid piece in this region (Fig. 1). Studies of the electronmicroscopic specimens revealed a marked disruption of the course filaments in the mid piece (Fig 2A). A substantial number of spermatozoa lost almost half of their contractile elements from the normal number of 9+2 fibers (Fig 2B). This is the first report of changes in the ultrastructure of spermatozoa by sulfa drug treatment.

4.5 The effect of sulfanilamide on epididymal and ejaculated sperm motility

When spermatozoa removed from the cauda epididymidis of control rats were diluted in PBS, a high percentage of spermatozoa (90%) was motile. However, only 60% of the epididymal spermatozoa from sulfanilamide-treated rats were motile (Fig. 3). Per cent motility of spermatozoa from both groups slightly declined during 60 min incubation at room temperature. Nevertheless, sperm motility in the sulfa-treated group was significantly lower than the control at all time points.

Similar results were obtained when motility of the ejaculated spermatozoa was determined (Fig. 4). However, sperm motility was maintained throughout the 60 min-incubation period in the control group, whereas a slight (but not significant) decrease occurred by 1 h in the specimen obtained from the treated animals.

4.6 The effect of sulfapyridine on the respiration of ejaculated rat spermatozoa.

The basal rate of oxygen utilization of the ejaculated spermatozoa recovered from uteri was quite variable. During the control period, it varied from <0 to 9 nmoles O_2/h-million sperm even in the same animal (Table 8). As in the cauda epididymal spermatozoa, the rate of respiration was stimulated by lactate or succinate. These exogenous substrates also caused a variable enhancement of respiration. Treatment with sulfapyridine for upto 47 days had no effect on neither the basal nor the substrate-induced respiration rate.

These data are at variance with the results in the previous study (3) that cauda epididymal spermatozoa collected from sulfapyridine-treated rats had a higher rate of respiration than the control. However, it should be noted that the basal rate of respiration in this study, which was done in Tel Aviv, is much lower than that in the earlier study (3) done in Bangkok. In addition, the data of the ejaculated spermatozoa are quite variable.

4.7 The effect of sulfapyridine on the sulfhydryl group of epididymal and ejaculated spermatozoa

Typical results obtained with the fluorescence-activated cell sorter are shown in Fig. 5A. The values on the abscissa represent fluorescence units whereas the ordinate represents cell number. In caput epididymal sperm (Fig. 5A) the fluorescence of DTT-treated and untreated sperm are almost superimposable, indicating that almost all SH group were present in reduced form. In contrast, untreated cauda epididymal sperm showed considerably less fluorescence than did the DTT-treated cells (Fig. 5B). Date of this sort was obtaind for sperm from caput, corpus and cauda epididymides of sulfapyridine-fed and control rats. When this method was applied in a preliminary experiment no difference was apparant in thiol status between sperm from a control animal and

a rat which had been fed sulfapyridine at a high dose for an extremely long period (Table 9). This conclusion was confirmed in sperm from additonal treated and control rats, using both the FACS and quantitative fluorescence methods (Table 12 and 11). These animals had been fed sulfapyridine (1 g/kg rat wt-day) for 99 days.

Thiol status of ejaculated sperm obtained from uteri of mated rats also did not shown evidence of decreased epididymal maturation (i.e.-increased SH content) in sulfapyridine-fed rats (Table 12).

It is not clear whether the decrease in free sulfhydryl concentration during passage through the epididymis is the result of active biological processes or whether the thiol groups react with oxygen spontaneously during the 7-10 day period that the maturing sperm pass along the epididymal lumen. These data do not support the hypotheses that sulfapyridine exerts its antifertility action by interfering with sperm maturation during passage through the epididymis or by increasing the rate of transpot of sperm from caput to cauda epidydimides. If either of the above hypotheses were correct sulfapyridine treatment would have been expected to result in elevated SH levels in cauda epididymal sperm. In the cases shown, the percentage of free thiol groups in sperm cells decreased as sperm moved from raw caput to the corpus and cauda epididymides but there was no significant difference between sperm from control rats and those that had been treated with sulfapyridine at a dose level and time of treatment greater than those that had been shown previously to decrease the fertility of male rats (2).

4.8 Synthesis of the proposed compounds

4.8.1 N^1, N^1-Diethylsulfanilamide

N^1, N^1-Diethylsulfanilamide was prepared by alkali hydrolysis of its N^4-acetylanalogue, which had previously been synthesized (4). White amorphous solid was obtained after recrystallization from hot methanol-water. The yield was 0.69 g (82.14%). The product was identified by its melting point (9[illegible]-9[illegible] °C), IR, NMR and mass spectroscopies (Appendices 1A,1B and 1C). Elemental analysis of the product revealed the following values:

	Calculated ($C_{10}H_{16}N_2O_2S$)	Analysis
% C	52.16	52.43
% H	7.06	7.23
% N	12.26	12.17

4.8.2 N^4-Acetyl-N^4-methyl-N^1,N^1-diethylsulfanilamide

This compound was synthesized by reductic methylation of the N^4-acetyl-N^1,N^1-diethylsulfanilamide previously prepared (4) with methyl iodide. White plate solid was obtained after recrystalization from hot methanol. The yield was 16.5 g (79.9%). The product was identified by its melting point (140-143° C, IR, NMR and mass spectroscopies (Appendices 2A, 2B and 2C). Elemental analysis of the product revealed the

following values:

Calculated($C_{13}H_{20}N_2O_3S$)		Analysis
% C	54.91	54.86
% H	7.09	7.19
% N	9.85	9.90

5. CONCLUSION AND REMARK

In this report we have demonstrated that ASP, but not MNSP or PBSP, also has an antifertiliity effect in the male rats. It appears that ASP is as potent as sulfapridine. Since sulfapyridine is converted to ASP in the liver (13), it may be possible that ASP is one of the active metabolite(s) of sulfapyridine in vivo.The lack of antifertiilty effect of MNSP and PBSP confirms our previous conclusion that the presence of N at position 4 on the benzene ring of benzenesulfonamide is essential.

We have also presented an evidence that blood LH levels are not changed throughout the period of study. In addition, the responsiveness of the anterior pituitary gland to LHRH is not affected by sulfapyridine. The results, therefore, suggest that the enhancement of blood testosterone in response to LHRH reported previously is possibly due to the increase in the responsiveness of the Leydig cells.

Studies on the rate of sperm production show no evidence of disruption of spermatogenesis by sulfapyridine. This, together with the data on hormone levels, strongly suggests that sulfapyridine causes infertility by the mechanism other than intefering with spermatogenesis or the integrity of the hypothalamo-pituitary-gonadal axis.

On the other hand, there is an evidence that sulfapyridine and its analogue exert their antifertility effects through their actions on the epididymis. Thus, the epididymal sperm reserved and sperm motility are markedly decreased. It is not known at present how the former is reduced in spite of the fact that testicular sperm production rate is not changed.Whereas the disruption of the contractile filament and the increase in number of dead sperm cells found in the epididymal spermatozoa could cause a reduction in sperm motility. However, it is noteworthy that the number of ejaculated sperm in the sulfa-treated animals is severely decreased. The reduction is out of proportion to that found in the epididymal sperm reserve. The results suggest that the ejaculatory process of the treated animals may be abnormal. Yet the volume of ejaculate semen is not affected.

By contrast, biochemical studies of both the epididymal and ejaculated spermatozoa fail to demonstrate any significant changes in neither the respiration nor the sulfhydryl contents. Furthermore, the studies on the cytoplasmic droplets (4) and sperm surface binding with 2D6 monoclonal antibody (4) indicate that sulfapyridine does not affect maturation of the epididymal spermatozoa. Although the rate of sperm production is unaffected by sulfapyridine, the evidence from ultrastructural and

biochemical studies suggests that this compound and its active analogue may interfere with the late spermatogenesis process causing a disruption of the course fibers. This in turn results in a reduction in sperm motility. In view of the lack of evidence for the resorption of dead spermatozoa by the epididymal epithelium, the mechanism for a decrease in epididymal sperm reserve is still unknown.

6. WORKPLAN FOR THE NEXT REPORTING PERIOD

Two saturated heterocyclic analogues of sulfanilamide will be synthesized. Results from fertility testing of the compounds synthesized in this reporting period will also be included in the next report. The possible effect of sulfapyridine on the epididymal accumulation of carnitine and the rate of sperm transport through the epididymis will be studied. The next report will conclude the studies on the mode of action of some antifertility sulfonamides in the male rats. It is hope that significant informations necessary for more detailed studies for the development of this drug will be obtained after completion of this project.

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Table 1. The effect of some synthetic sulfonamides on fertility of the male rats.

Treatment group	Dose (mg/kg)	Before treatment	Percentage Fertility: Percentage Fertility wk-1	wk-3	wk-5	After drug withdrawal wk-1	wk-3
control	-	95.1+2.3 (9)	89.5+ 4.4 (8)	63.5+18.0 † (5)	95.3+ 2.2 (7)	96.2+ 2.6 (4)	76.2+11.8† (5)
DES	150	97.6+1.6 (7)	60.5+19.6 (6)	59.1+20.0 (6)	37.8+18.1 †††*** (4)	96.0+ 2.3 (4)	58.6+20.0 (6)
	450	95.5+2.7 (8)	60.0+24.5 (5)	91.9+ 4.8 (3)	22.8+ 8.1 †††*** (5)	47.2+17.1 ††† (3)	55.0+ 9.2 †† (5)
ADES	150	95.6+2.2 (7)	44.2+20.5 (5)	47.8+17.0 (6)	30.9+ 9.2 †††*** (5)	72.0+11.3 † (5)	72.5+ 7.9 †† (6)
	450	98.5+1.0 (8)	73.8+ 9.7 †† (5)	11.3+ 5.8 ††* (7)	21.0+11.6 †††*** (3)	32.5+23.6 ††* (4)	77.3+13.8† (5)

Values are mean +SEM (n)

*P<0.05; **P<0.01; ***P<0.001 when compared to the corresponding period in the control

† P<0.05; †† P<0.01; ††† P<0.001 when compared to the control period (before treatment within a group (t-test).

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Table 2 The effects of some synthetic sulfonamides on fertility of the male rats.

Treatment group	Dose (mg/kg)	Percentage Fertility					
		Before	During treatment			After drug withdrawal	
Control	-	97.1+1.2 (8)	73.8+16.0 (6)	73.7+18.6 (5)	80.5+13.9 (6)	92.5+ 3.6 (2)	51.2+15.5†† (7)
ASP	150	98.0+1.3 (8)	92.2+ 4.7 (7)	84.6+ 4.9† (8)	30.6+13.4$^{\dagger\dagger\dagger *}$ (7)	84.2+ 5.9† (8)	82.9+ 8.7 (8)
	450	96.5+2.4 (8)	80.3+13.5 (7)	64.2+16.6† (6)	31.1+16.4$^{\dagger\dagger\dagger *}$ (6)	68.0+14.3† (7)	75.1+13.1 (7)
MNSP	150	98.2+1.2 (8)	84.2+ 5.9† (8)	54.6+16.6†† (7)	67.6+16.9† (6)	94.0+ 5.9 (8)	87.5+ 6.1* (8)
	450	96.8+1.3 (8)	42.2+ 3.0 (6)	95.5+ 2.9 (5)	66.5+11.0†† (6)	75.8+15.9† (4)	60.9+15.5†† (6)
PBSP	150	95.9+1.5 (8)	83.1+13.9 (7)	63.7+15.5† (8)	62.1+15.2† (6)	74.0+12.6 (8)	88.5+ 5.2* (7)
	450	98.4+1.6 (8)	80.6+13.7 (7)	63.0+15.7† (6)	56.2+16.1†† (7)	86.1+ 6.3† (6)	90.0+ 7.0 (8)

Values are mean +SEM (n)

*P<0.05 compared to the corresponding period in the control

† P<0.05. †† P<0.01, ††† P<0.001 compared to the period before treatment in the same group.

Table 3 The effect of sulfapyridine (450 mg/kg) on blood levels of LH in the male rats.

Treatment	Blood levels of LH (ng/ml)			
		During treatment		After drug withdrawal
	Before	wk.3	wk.5	wk.3
Control	0.99+0.23 (5)	1.38+0.12 (11)	1.11+0.15 (11)	1.73+0.36 (10)
Sulfapyridine	1.05±0.19 (5)	1.08+0.25 (9)	0.93+0.15 (8)	1.22+0.42 (7)

Values are mean +SEM (n).

Table 4 The effect of sulfapyridine (450 mg/kg) on the responsiveness of the male rat pituitary gland to LHRH

Treatment	Blood levels of LH (ng/ml)		folds increase
	Before LHRH	2 h after LHRH	
Control	0.87+0.28 (7)	8.85+3.93 (7)	9.1+1.8 (7)
Sulfapyridine	0.45+0.04 (7)	10.20+3.11 (7)	29.0+14.2 (7)

Values are mean +SEM (n)

Table 5. The effect of sulfapyridine on daily sperm production rate of the rats.

Treatment	Dose (mg/kg)	Daily sperm production ($x10^6$ sperm/g testis/day)	P-value
Control	-	20.6 + 0.9 (8)	-
Sulfapyridine	125	18.3 + 0.7 (7)	>0.05
	250	22.3 + 4.0 (3)	>0.10
	500	20.5 + 1.1 (5)	>0.10

Values are mean +SEM (n)

Table 6 The effect of sulfanilamide on fertility and body and sex organ weights of the male rats.

Parameter	Control	Sulfanilamide	p-value
Fertility(%)			
before treatment	92.6+2.9 (10)	94.3+2.9 (10)	NS
by 6 wks after treatment	94.1+2.5 (10)	17.8+3.8 (10)	<0.001
Body wt.(gm)			
before treatment	364.6+11.5 (8)	358.0+6.7 (7)	NS
by 6 wks after treatment	410.6+11.8 (8)	410.6+7.6 (7)	NS
Sex organ wt.(gm)			
Testes	3.06+0.10 (8)	2.97+0.08 (7)	NS
Epididymides	1.44+0.03 (8)	1.29+0.04 (7)	NS
Seminal vesicle	1.55+0.07 (8)	1.70+0.10 (6)	NS
Prostate gland	0.65+0.05 (8)	0.82+0.06 (7)	<0.05
Coagulating gland	0.23+0.02 (6)	0.25+0.02 (5)	NS

Values are mean +SEM (n)
NS = non significant

Table 7 The effect of sulfanilamide on various parameters of the cauda epididymal fluid and ejaculated semen.

Parameter	Control	Sulfanilamide treated	P-value
Cauda epididymal fluid			
Sperm concentration ($x10^9$ cells/ml)	3.09±0.15 (8)	1.98±0.08 (7)	<0.001
Sperm with detached head (% of total)	3.6±0.9 (6)	20.5±3.4 (6)	<0.001
%dead sperm	22.7+2.4 (4)	56.7+2.0 (4)	<0.001
Ejaculated semen			
approx.vol.(ul)	33.5+10.0 (6)	50.0+11.5 (3)	NS
total number of sperm($x10^6$ cells/ ejaculate)	22.4+5.3 (6)	0.47+0.36 (4)	<0.01
Sperm with detached head(%of total)	5.3±3.1 (3)	47.6 (1)	-

Values are mean +SEM (n)
NS = non significant

Table 8 The effect of sulfapyridine (SP) on respiration of the ejaculated rat spermatozoa.

Rat #	Treatment	#of days treated	Respiration rate(umole O_2/h-10^6 cells) Basal	+lactate	+succinate
46	none	0	0.57	6.75	10.2
	"	20	9.0	25.1	40.5
	"	56	<0	18.9	43.5
	SP	47	5.5	21.6	11.7
47	none	0	1.8	28.4	39.7
	"	30	0.9	1.8	9.6
	SP	32	3.3	9.1	10.4
	none	27 after drug	13.5	16.4	14.9
48	none	0	2.2	8.2	12.6
	"	27	6.7	8.1	15.7
	"	56	<0	3.2	10.1
	SP	12	2.3	14.7	14.2
	none	20 after drug	2.0	6.6	15.7

Table 9 Thiol status of spermatozoa from the epididymides of sulfapyridine-fed and control rats.

Sperm source	% SH	
	Control	Sulfa treated
Caput Epid.	93	93
Corpus Epid.	76	75
Cauda Epid.	38	42

SH content was measured by fluorescence-activated cell sorting of mono-bromobimane-stained cells, as described in the Materials and Methods. The control animal (wt 585 gm, age 221 days) was compared with a treated animal (wt. 526 gm, age 233 days) which had been fed sulfapyridine for 171 days (24.4 weeks) at an average dose of 560 mg/day (1.065 g/kg). Results are reported as % of total thiols present as SH groups.

Table 10 The effect of sulfapyridine on SH content of the epididymal spermatozoa measured by a quantitative method as described in the Materials and Methods.

Treatment	Epididymal spermatozoa	%	SH Content nmole mB-S Protein/10⁶ sperm +DTT	-DTT
Control	Caput	84+5 (5)	39.8+3.0 (5)	33.5+3.6 (5)
	Corpus	67+4 (5)	42.7+2.1 (5)	28.7+2.4 (5)
	Cauda	33+6 (5)	19.1+2.0 (5)	6.0+0.8 (5)
sulfapyridine	Caput	69+9 (5)	36.7+3.0 (5)	25.9+4.0 (5)
	Corpus	66+7 (5)	34.4+4.6 (5)	22.1+2.8 (5)
	Cauda	37+9 (5)	22.4+2.9 (5)	8.3+2.1 (5)

Values are mean +SEM (no.of determinations from 2-3 rats).

Table 11 The effect of sulfapyridine on SH content of the epididyma spermatozoa measured by a FACS as described in the Material and Methods.

Treatment	Epididymal Spermatozoa	%	SH content nmole mB-5 Protein/10⁸ sperm +DTT	-DTT
Control	Caput	92+4 (4)	96.0+6.8 (4)	88.8+9.0 (4)
	Corpus	82+5 (4)	84.2+14.0 (4)	68.9+11.0 (4)
	Cauda	45+5 (4)	104.9+8.4 (4)	47.2+3.2 (4)
Sulfapyridine	Caput	75+9 (4)	84.7+8.2 (4)	63.9+12.0 (4)
	Corpus	64+3** (4)	77.1+11.4 (4)	50.5+10.1 (4)
	Cauda	37+1 (4)	96.4+7.4 (4)	35.6+3.0* (4)

Values are mean +SEM (no.of determinations from 2-3 rats).

*P<0.05, **P<0.01 when compared to the corresponding control values.

Table 12 The effect of sulfapyridine on SH content of the ejaculated spermatozoa.

Rat #	% SH	
	18 days	40 days
1	38	25
2	55	23
3	31	-

Male rats were fed with sulfapyridine (approx. 1 gm/day-kg) for 18 or 40 days and then mated. Ejaculated spermatozoa were collected from uteri of the mated female rats and were analyzed for thiol groups by the quantitative method.

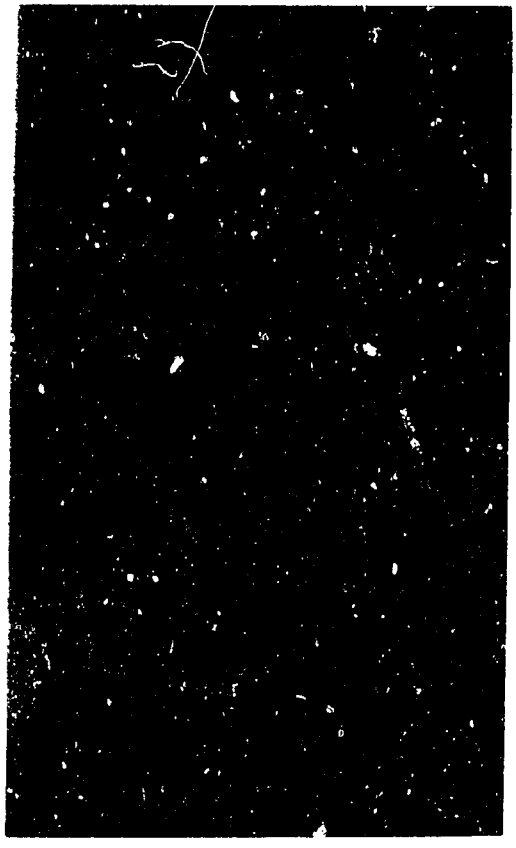

Figure 1 Photograph of the cauda epididymal spermatozoa from rats treated with sulfanilamide (450 mg/kg/day). The specimen were stained with eosin-nigrosin and magnified at X1,000. Note the disruption of the connecting piece resulting in protusion of several fibers from the mid piece. Dead spermatozoa were stained with dye whereas the live one was unstained.

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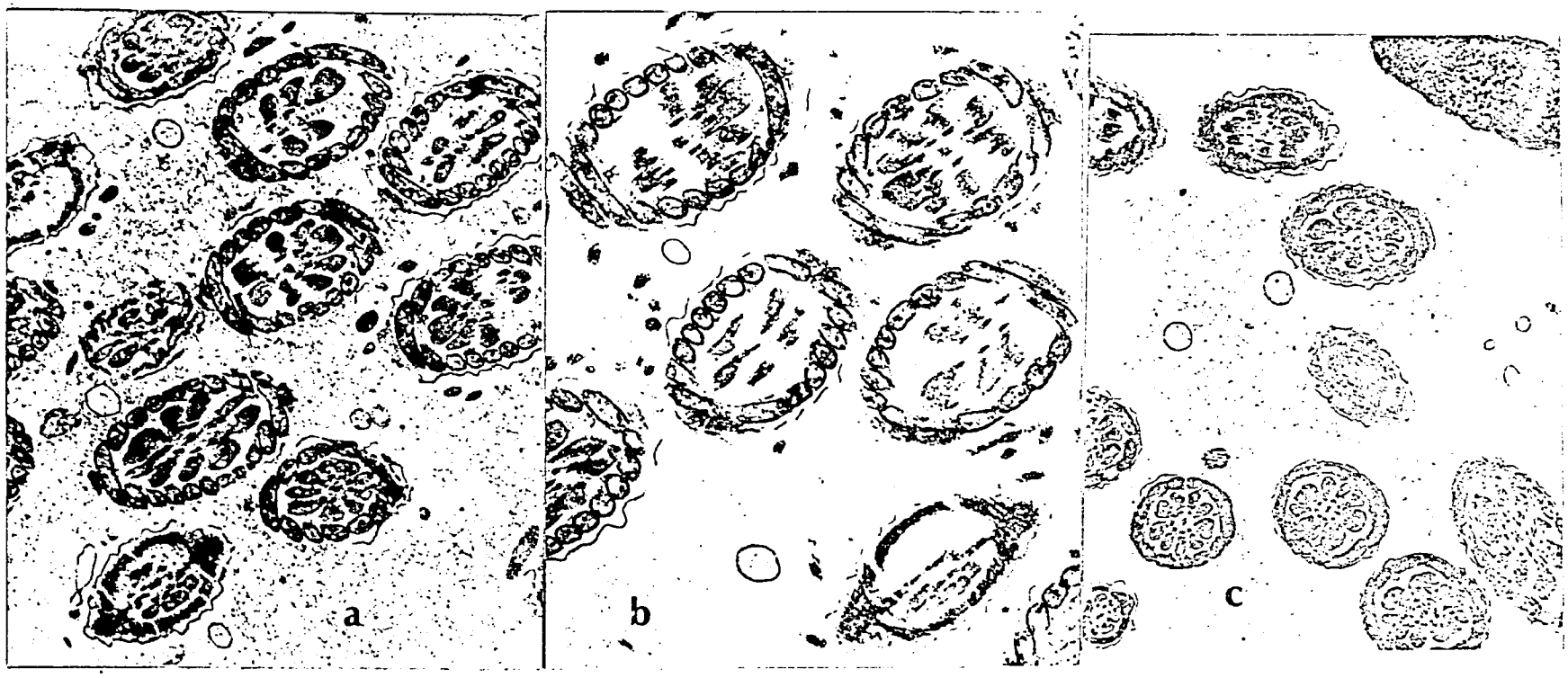

Figure 2 Electron micrographs of the cauda epididymal spermatozoa from rats treated with 450 mg/kg/day sulfanilamide (a, x19,000 ; b, x25,000) compared to the control (c, x16,600). The pictures show cross sections at the level of mid piece in most spermatozoa except in few cases which show the principal piece, lower left in (a) and lower right in (b). Note the disappearance of coarse filaments (9+2 pairs) in (a) and (b).

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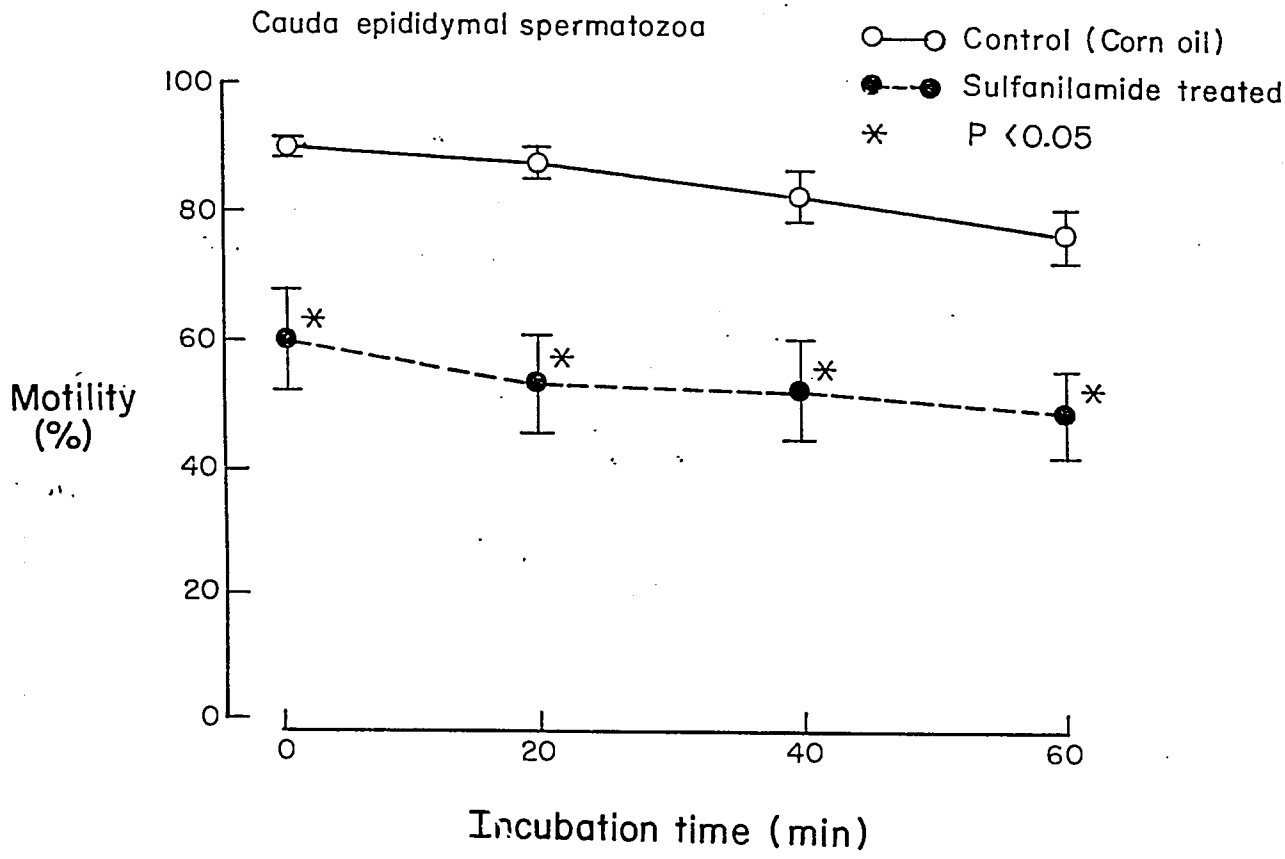

Figure 3 Motility of the cauda epididymal spermatozoa from rats untreated (corn oil) or treated with sulfanilamide (450 mg/kg/day). Spermatozoa were diluted in PBS and incubated at 34°C. Values are mean ±SEM from 6-7 rats.

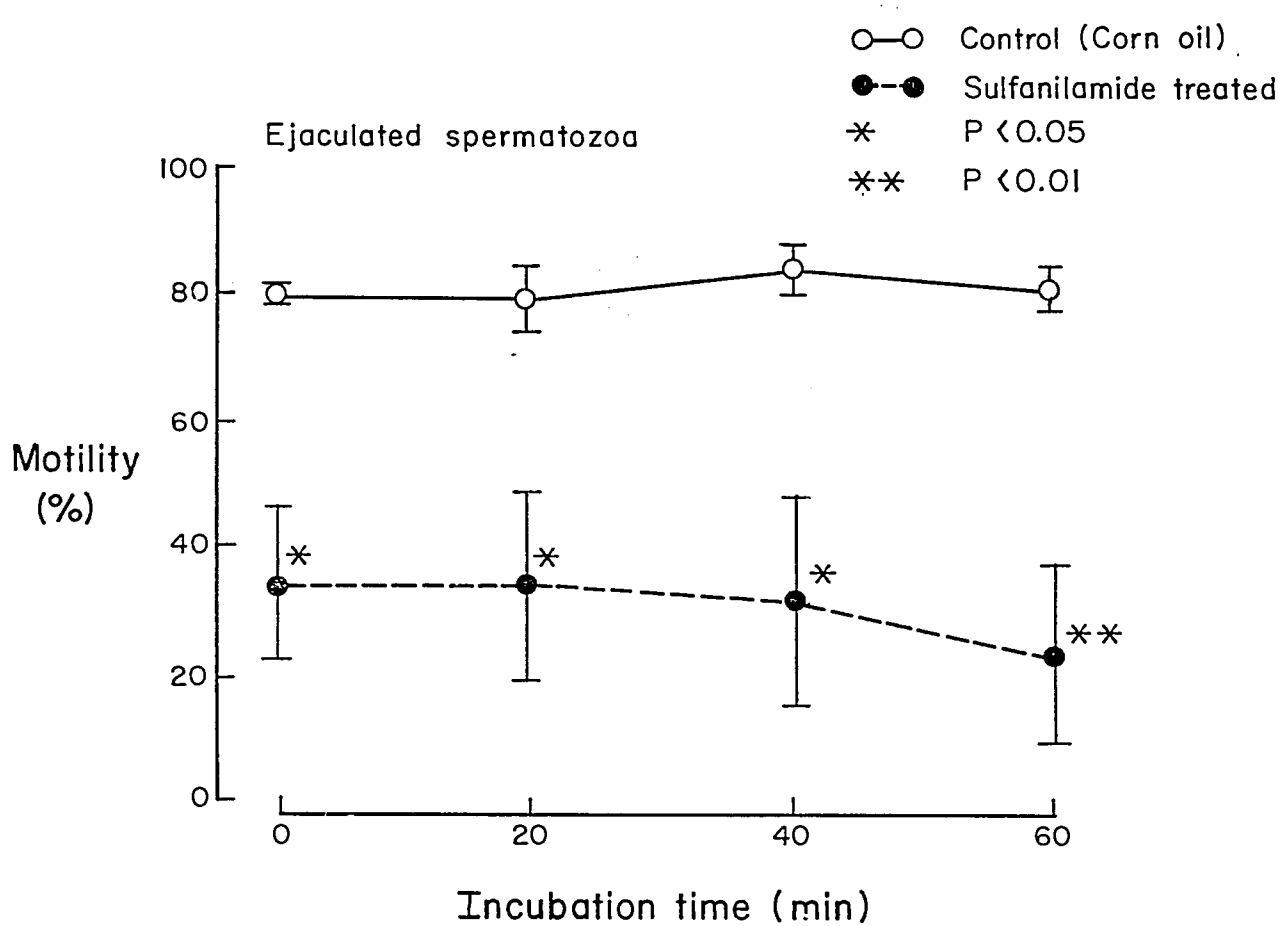

Figure 4 Motility of the ejaculated spermazoa from rats untreated (corn oil) or treated with sulfanilamide (450 mg/kg/day). Spermatozoa were diluted in PBS and incubated at 34°C. Values are mean ±SEM from 3-6 rats.

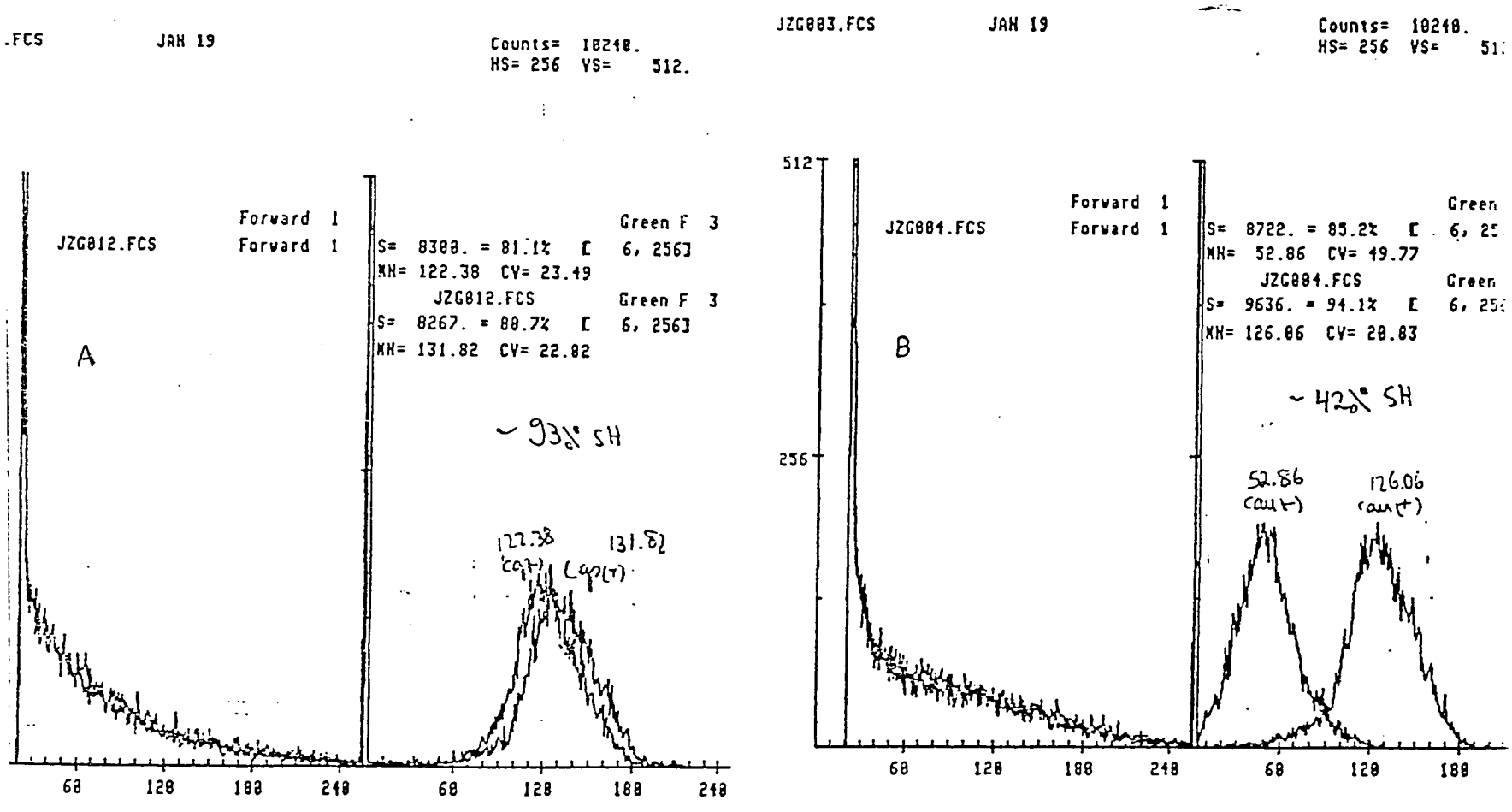

Figure 5 Fluorographs of the fluorescene-activated cell sorter showing fluorescene from DTT-treated and untreated spermatozoa from the caput epididymidis (A) and from the cauda epididymidis (B) of control rats. Note that the flourescene of DTT-treated and untreated caput sperm are almost superimposable indicating that almost all SH groups were present in reduced form.

Appendix 1 A

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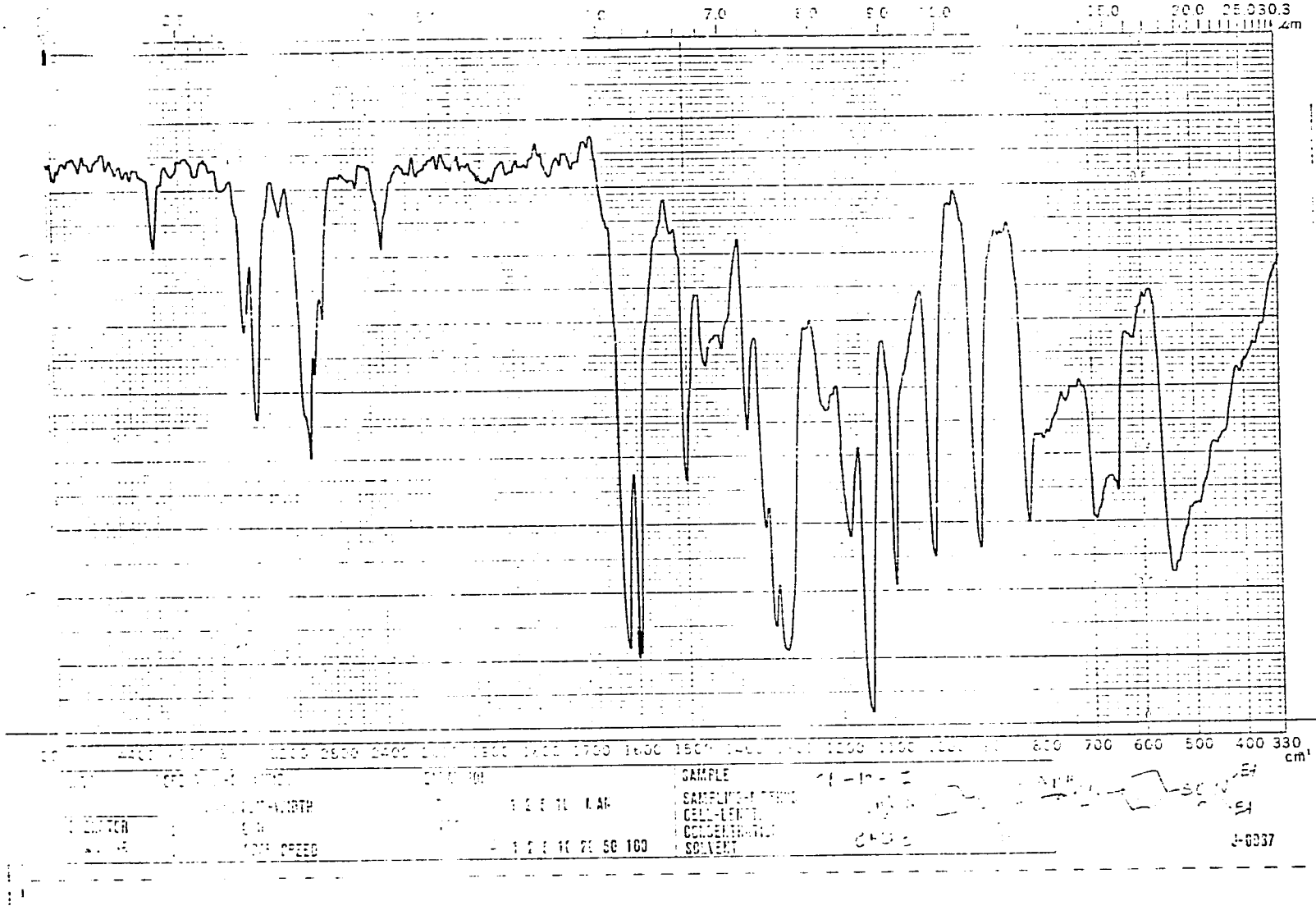

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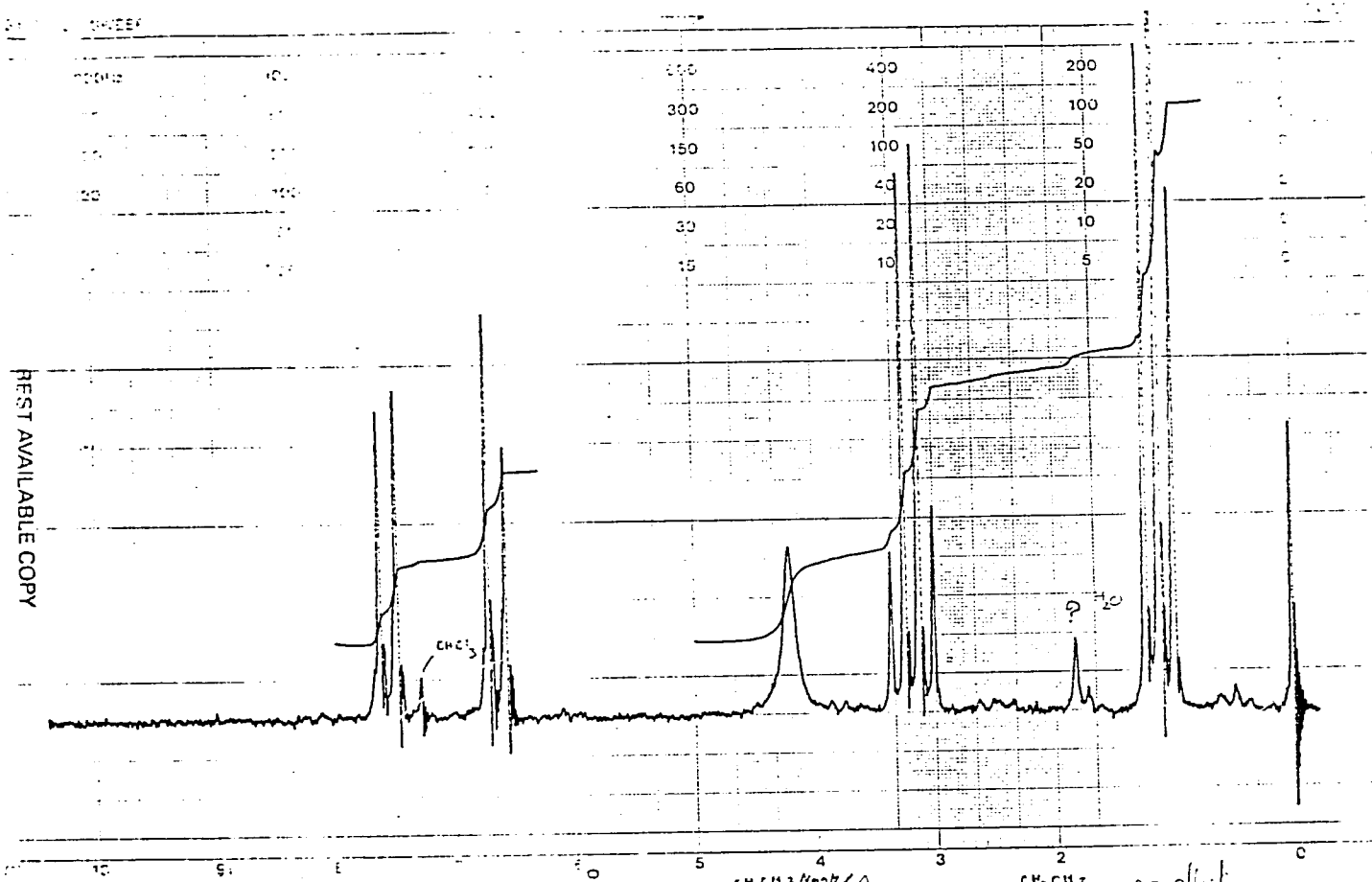
Appendix 1 B



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Appendix 1C

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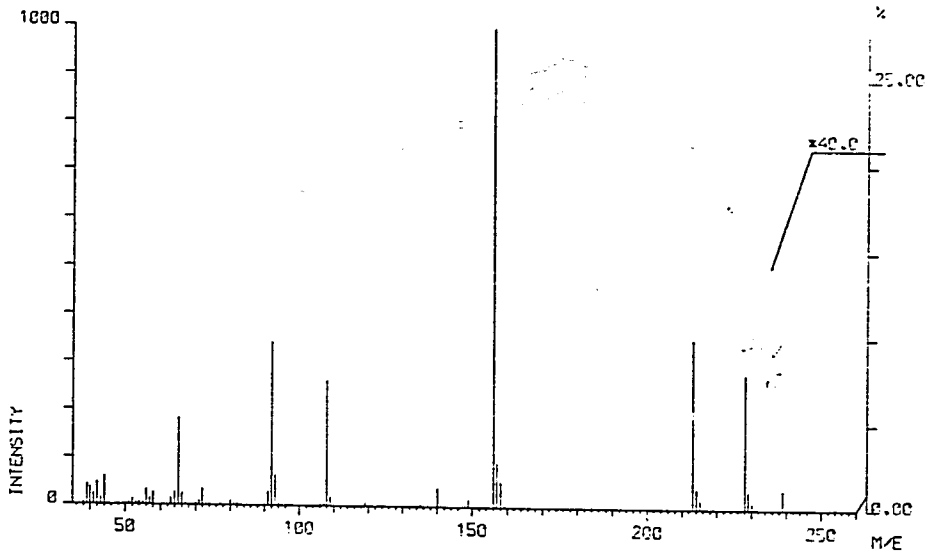
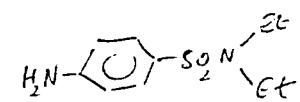
MASS SPECTRUM : (2 TO 4)
SAMPLE.CP-19-I 27 JUNE 88
NOTE .459/1 EI,70V,300UA.CHAMB.TEMP.120
BASE PEAK : M/E 156.0 INT. 818.6



MASS SPECTRUM : (2 TO 4)
SAMPLE.CP-19-I 27 JUNE 88
NOTE .459/1 EI,70V,300UA.CHAMB.TEMP.120
BASE PEAK : M/E 156.0 INT. 818.6

M/E	RAW INT.	R.INT.	SIGMA(%)	
39.0	34.0	42.5	1.31	
40.0	30.3	37.0	1.14	
41.0	19.6	24.0	0.74	
42.0	38.3	47.5	1.47	
44.0	48.3	59.0	1.85	
56.0	27.0	33.0	1.02	
58.0	21.5	26.4	0.81	
63.0	13.2	16.2	0.50	
64.0	22.5	27.5	0.85	
65.0	149.0	182.3	5.66	
66.0	20.6	25.2	0.78	
72.0	28.7	35.1	1.08	
91.0	24.3	30.4	0.94	
92.0	280.5	342.7	10.61	
93.0	52.7	64.4	1.99	
108.0	216.1	263.9	8.17	
109.0	17.5	21.3	0.66	
140.0	33.5	41.1	1.27	
149.0	14.0	17.1	0.53	
156.0	818.6	1000.0	30.96	
157.0	77.1	94.2	2.91	
158.0	45.0	55.0	1.70	
213.0	290.6	355.0	10.99	
214.0	35.3	43.1	1.33	
215.0	15.5	18.9	0.58	
228.0	293.4	285.0	8.82	
229.0	31.5	38.5	1.19	END
230.0	14.9	15.6		
232.0	0.1	0.1		
234.0	0.1	0.1		
235.0	0.1	0.1		



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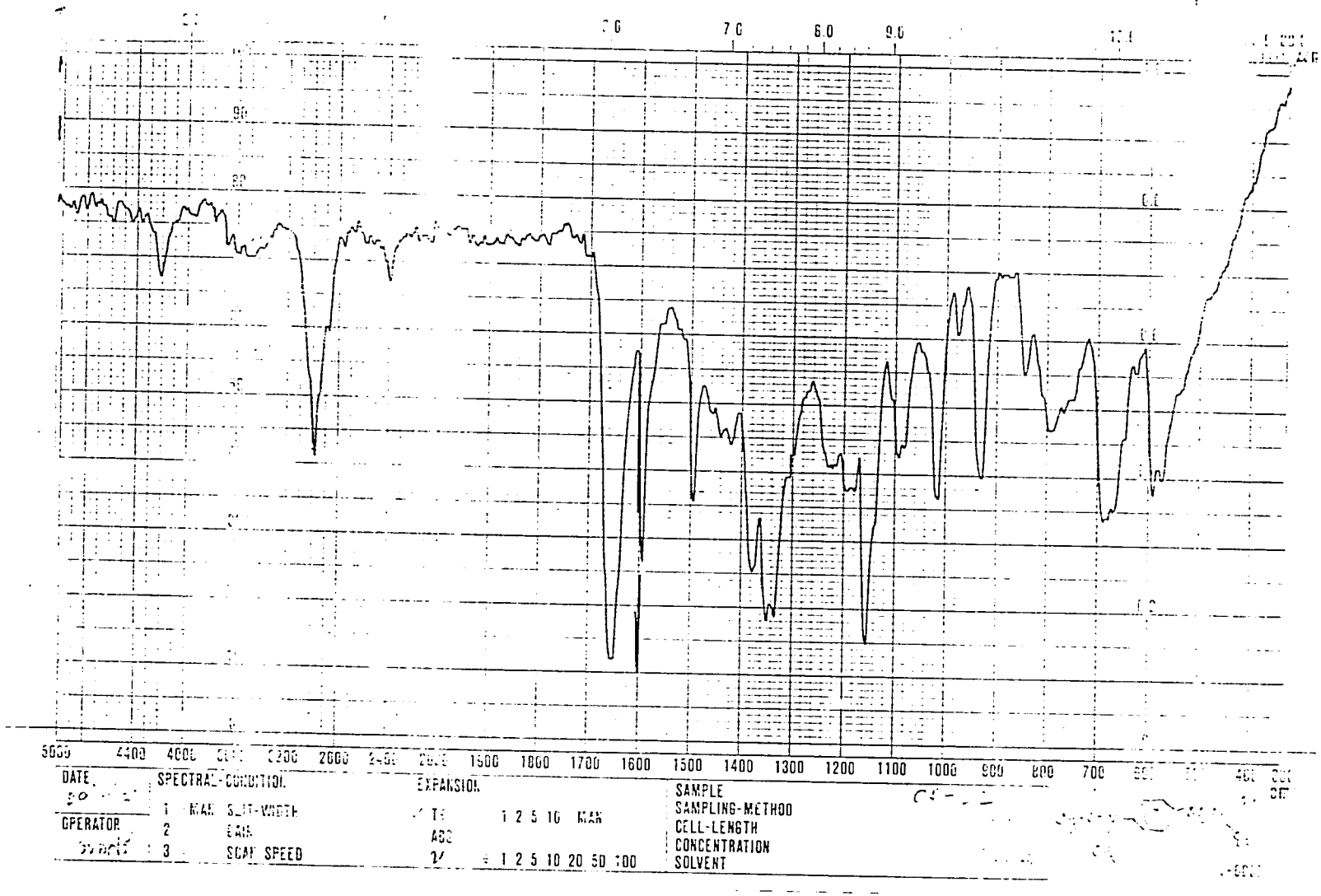

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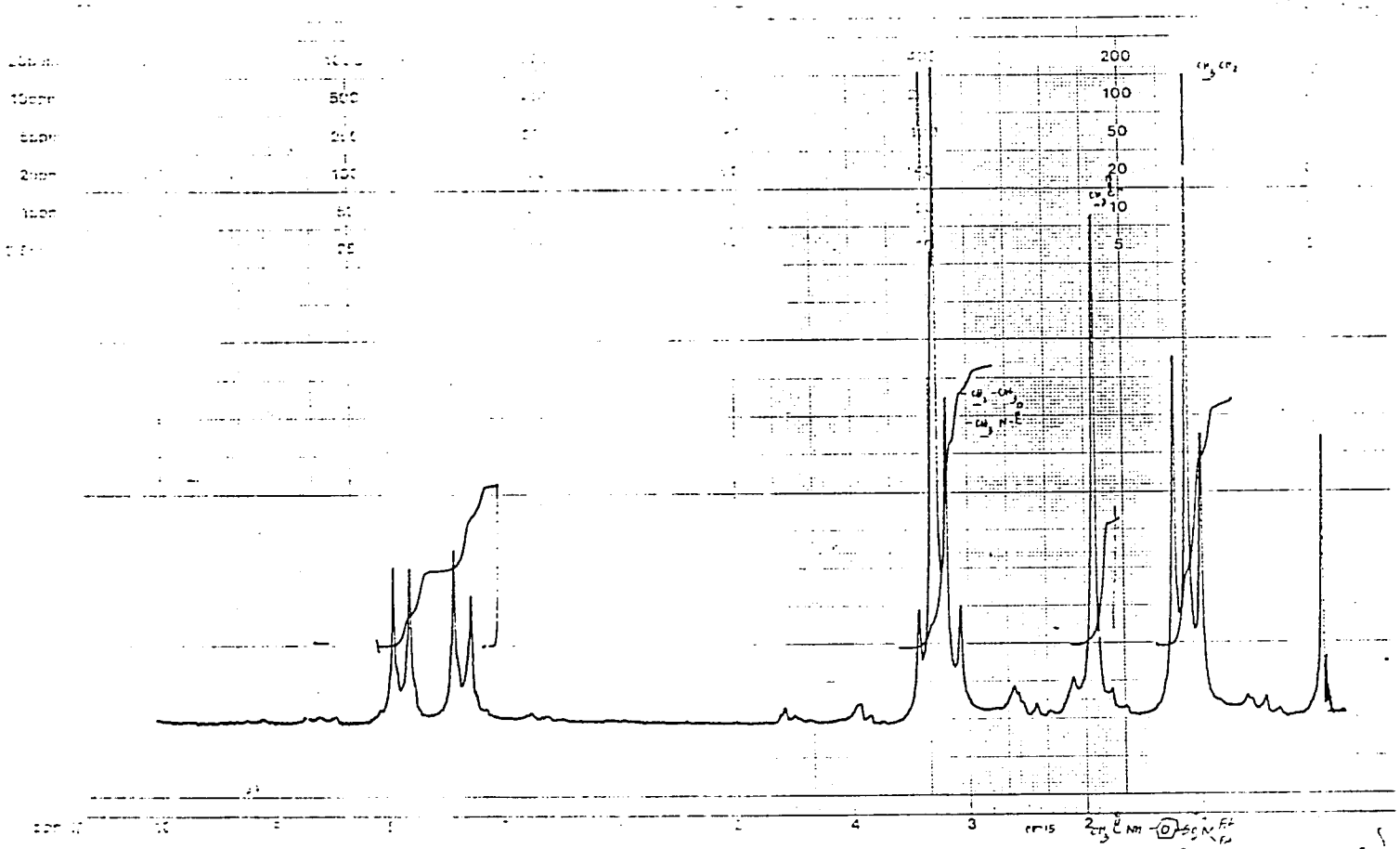
Appendix 2.3



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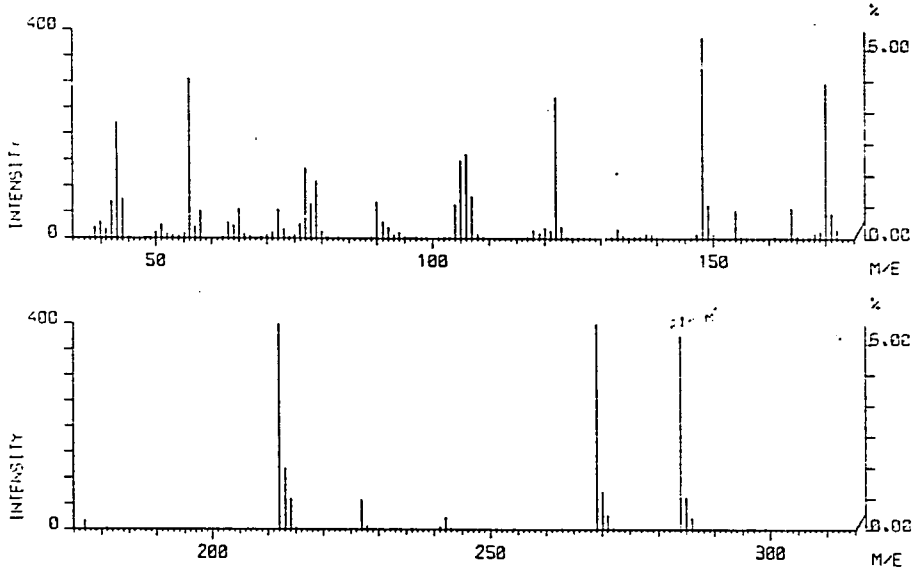
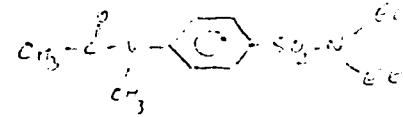
Appendix 2 C

MASS SPECTRUM . (14 TO 17)
SAMPLE.CP-22 2 MAR 89
NOTE .343/3 EI,70V,300UA.CHAMB.TEMP.120
BASE PEAK . M/E 212.0 INT. 573.9



MASS SPECTRUM . (14 TO 17)
SAMPLE.CP-22 2 MAR 89
NOTE .343/3 EI,70V,300UA.CHAMB.TEMP.120
BASE PEAK M/E 212.0 INT. 573.9

M/E	RAW INT.	R.INT.	SIGMA(%)	
42.0	40.5	70.6	1.35	
43.0	127.4	222.0	4.25	
44.0	42.9	74.7	1.43	
51.0	14.9	26.1	0.49	
56.0	175.7	305.1	5.85	
58.0	29.6	51.6	0.98	
63.0	17.8	30.9	0.59	
64.0	14.5	25.4	0.48	
65.0	32.0	55.8	1.06	
72.0	31.0	54.0	1.03	
75.0	15.9	27.8	0.53	
77.0	77.2	134.5	2.57	
78.0	37.7	65.7	1.25	
79.0	63.7	111.0	2.12	
90.0	39.9	69.5	1.33	
91.0	18.1	31.6	0.60	
104.0	37.2	64.8	1.24	
105.0	85.1	148.3	2.84	
106.0	92.8	161.6	3.09	
107.0	45.3	79.0	1.51	
122.0	155.4	270.8	5.18	
148.0	222.0	386.8	7.40	
149.0	35.4	63.4	1.21	
154.0	30.9	53.8	1.03	
164.0	33.1	57.7	1.10	
170.0	171.2	298.2	5.71	
171.0	27.5	47.9	0.91	
212.0	573.9	1000.0	19.14	
213.0	68.8	119.9	2.29	
214.0	34.5	60.2	1.15	
227.0	34.1	59.5	1.14	
242.0	13.9	24.2	0.46	
269.0	279.0	486.1	9.30	
270.0	42.9	74.8	1.43	
271.0	16.4	28.7	0.54	
284.0	217.4	378.8	7.25	END
285.0	32.2	14.9		
286.0	15.1	22.7		



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