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PROGRESS REPORT NO. 2

SULFONAMIDE DRUGS AS A MALE ANTIFERTILITY AGENT

A RESEARCH PROJECT

US-ISRAEL CDR PROGRAM

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Submitted by

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1

Project Profile

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Project Consultants	:	None
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Contents

Page

1. Introduction ---------- 1

2. Objectives ---------- 1

3. Materials and Methods

3.1 Fertility testing ---------- 1

3.2 Development of methods ---------- 2

3.2.1 sperm motility ---------- 2

3.2.2 sperm respiration ---------- 2

3.2.3 SDS gel electrophoresis ---------- 3

3.3 Synthesis of the proposed compounds ---------- 3

3.3.1 2-(p-Nitrobenzene sulfonamido) pyridine -- 3

3.3.2 2-(p-Toluene sulfonamido) pyridine ------- 4

4. Results and Discussion

4.1 Effects of two synthetic sulfonamides on fertility ---------- 4

4.2 Effects of some simple sulfonamides on fertility- 7

4.3 Sperm motility ---------- 7

4.4 Sperm respiration ---------- 7

4.5 SDS gel electrophoresis ---------- 8

4.6 Synthesis of the proposed compounds ---------- 8

5. Conclusion/Remarks ---------- 9

6. Workplan for the next period ---------- 9

7. References ---------- 10

8. Appendix ---------- 11

I. Introduction

In the previous report we have tested some commercially available sulfonamide drugs on fertility of the male rats and shown that sulfasalazine significantly decreased fertility within 3 weeks after feeding at a dose of 450 mg/kg (once daily), and the effect was reversible by 1 week after drug withdrawal. On the other hand, sulfadimethoxine and sulfamerazine had virtually no effect whereas the effect of sulfacetamide was not conclusive. Besides, we have synthesized two of the proposed compound, i.e. N-2-pyridylbenzene sulfonamide and N4-acetylsulfonilamide, in sufficient quantity for fertility testing.

The present report describes the results on fertility testing of the compounds previously synthesized and some more commercially avaialable sulfonamides which include sulfapyridine, sulfioxazole, sulfanilamide and sulfachloropyridazine. In addition, the collaborative work between the Israeli and the Thai investigators resulting from the visit of Professor L.M. Lewin to Bangkok during August 7 - September 18, 1987 is included. The purposes of Professor Lewin's visit were to discuss, exchange ideas and plan the future research work as well as to demonstrate and help setting up some methods in Dr. Pholpramool's laboratory. These include measurements of rat sperm motility by use of a Makler's chamber for human sperm and sperm respiration using an oxygraph, and SDS slab gel electrophoresis.

2. Objectives

The aim of this study is to develop methods and to adopt these methods to evaluate the antifertility activity and to investigate the mode of action of some sulfonamide drugs and their analogs.

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 before grouping and drug treatments. Each male was housed with two virgin female rats of the same strain aged 40-50 days in hanging 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., number 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.

/H'

3.1.1 Experiment 1

Thrity eight male rats were divided into 5 groups (7-8 rats each) and were force-fed with either corn oil, N-2-pyridylbenzene sulfonamide or N4-acetylsulfanilamide at doses of 150 and 450 mg/kg (once daily) for 6 weeks. At the end of weeks 1, 3, 5, 7 and 9, all treated and control (corn oil treated) were subjected to fertility testing as described above except that the females were checked for the presence of spermatozoa in vagina smears done every morning after cohabitation so as to confirm mating with the male. Therefore, fertilities of the males at weeks 1, 3 and 5 during treatment and weeks 1 and 3 after drug withdrawal were determined.

3.1.2 Experiment 2

Sixty nine male rats were allocated into 9 groups (5-12 rats each) and were force-fed with either corn oil or sulfanilamide, sulfapyridine, sulfioxazole and sulfachloropyridazine at doses of 150 and 450 mg/kg (once daily) for 6 weeks. At the end of weeks 1, 3, 5, 7 and 9 all animals were subjected to fertility testing as in Experiment 1.

3.2 Developments of methods

3.2.1 Sperm motility

The conventional method for measurements of sperm motility is generally to observe activities of spermatozoa placed on a glass slide with a cover slip on top or in a hemocytometer. However, the depth between the two glass surfaces is considerable compared to the size of most spermatozoa. Therefore, it is difficult to follow the swimming path of each individual spermatozoa in one plane since it may move out of focus. On the other hand, the Makler chamber is designed to reduce the depth such that movements of spermatozoa are always in focus. This type of chamber has been satisfactorily used for evaluation of human sperm motility. Since rat spermatozoa is larger in size than human sperm, the suitability for utilizing the Makler cell for determination of rat sperm motility should be evaluated.

A small aliquot of rat spermatozoa in rat fertilization medium (RFM) was placed in a Makler cell and the cover piece was placed on top. Motility of spermatozoa was observed under a Leitz microscope at 200x.

3.2.2 Sperm respiration

Spermatozoa were obtained from the rat cauda epididymidis either by retrograde infusion with oil or by extracting from the tissue fragments. In the former method, vas deferens was cannulated by a tapered end polyethylene tubing (PE 90) filling with paraffin oil. The epididymal tubule in the cauda region (Zone 6 of Reid & Cleland, 1957) was dissected free from connective tissue for 1 cm in length and was cut open. Both

openings were inserted into a microcentrifuge tube or a microhematocrit tube. The cauda epididymal tubule was then retrogradely infused with oil at a rate of 0.003 ml/min using a constant flow Harvard infusion pump until oil emerged from the cut end. Approximately 30-50 ul samples could be obtained.

In the latter, the cauda epididymal segment correspond to zone 6 was dissected free from epididymal fat pad and the tissue was chopped up into small fragments in RFM using a pair of scissors. The tissue fragments were removed by filtration through a piece of cotton gauze. The spermatozoa were pelleted by gentle centrifugation and washed twice in the same medium.

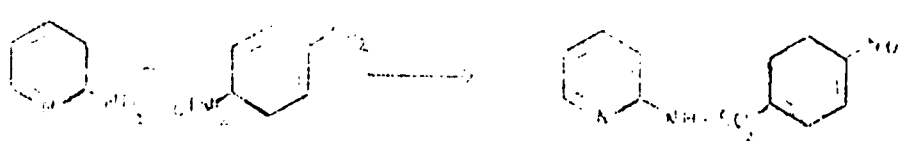
Two ml of RFM were added to spermatozoa obtained as described above and the sperm suspension was transfered into the chamber of a Gilson Oxygraph at 37°C. Respiration (utilization of O2) of spermatozoa was determined by use of the Clark electrodes. Microliter amounts of D-glucose (5.6 mM), DL-Lactate (22 mM) or sulfonamide drugs were injected into the chamber to observe their effects on respiration rate. Spermatozoa collected by retrograde infusion were either unwashed or washed with RFM prior to addition into the chamber whereas those obtained by cutting the epididymis were always washed with RFM.

3.2.3 SDS slab gel electrophoresis

The SDS slab gel electrophoresis was done according to the method described by Laemmli (1970). Only homogenous polyacrylamide gel concentrations (7 or 10%) were attempted. Cauda epididymal lumen fluid was obtained by retrograde infusion with stained oil as described above followed by centrifugation to remove sperm cells. Rat blood plasma was obtained from the jugular vein in a heparinized tube followed by centrifugation to remove blood cells. The luminal fluids and blood plasma were denatured by heating in the presence of beta-mercaptoethanol and SDS before electrophoresis. Protein bands were visualized by staining with Commassie Brilliant Blue.

3.3 Synthesis of the proposed compounds

3.3.1 2-(p-nitrobenzene sulfonamido) pyridine

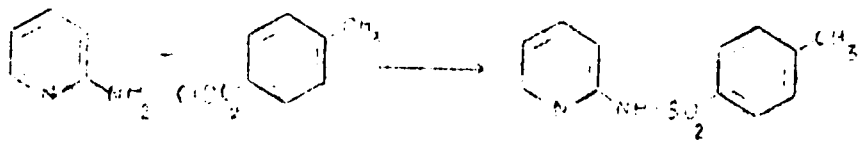


2-Aminopyridine (10 g) was dissolved in dry dichloromethane (50 ml) and dry pyridine (7 ml). p-Nitrobenzene sulfonylchloride (24 g) in dry dichloromethane (50 ml) was added to the above solution at ambient temperature during the periods

6

of 3 h. After cooling on ice bath, the precipitate of the product was filtered and washed with cold methanol. Upon recrystallization from hot methanol, pale yellow amorphous solids were obtianed; yield 17.5 g (62.3%). The product was identified by its melting point (150-151°C dec.), UV, NMR and Mass spectroscopies. Analytical data were C 47.15, N 14.70, H 3.26 compared to the calculated values for C11H9N3O4S, which were C 47.31, N 15.05, H 3.25.

3.3.2 2-(p-Toluene sulfonamido) pyridine



2-Aminopyridine (8 g) was dissolved in dry dichloromethane (40 ml) and dry pyridine (5 ml). p-Toluene sulfonylchloride (17.2 g) in dry dichloromethane (25 ml) was added to the above solution with reflux during the periods of 2 h. After cooling on ice bath, the precipitate of the product was filtered and washed with cold water. Upon recrystallization from a hot mixture of 50% dichloromethane in alcohol, white amorphous solids were obtained; yield 12.8 g (60.88%). The product was identified by its melting point (210-212°C), UV, NMR and Mass spectroscopies. Analytical data were C 58.06, N 11.20, H 4.84 compared to the calculated values for C12H12N2O2S, which was C 58.05, N 11.28, H 4.87.

4. Results

4.1 Effects of two synthetic sulfonamides on fertility

Two of the proposed sulfonamides has been synthesized and tested for fertility in the male rat over 6 weeks period. The results are shown in Table 1. It is apparent that N-2-pyridyl benzene sulfonamide had virtually no effect on fertility of the tested animals even at a high dose of 450 mg/kg. By contrast, N4-acetylsulfanilamide significantly ($P<0.05$) depressed fertility of the male rats even at a low dose of 150 mg/kg. At which dose the effect was detected as early as 3 weeks after treatment. However, normal fertility was soon recovered by 3 weeks after drug withdrawal. Similar results were also obtained at a higher dose (450 mg/kg), but the compound produced more sustained reduction in fertility.

Table 1. Effects of some synthetic sulfonamides on fertility of the male rat.

Treatment group	Dose (mg/kg)	Percentage Fertility					
		Before treatment	During treatment			After drug withdrawal	
			wk-1	wk-3	wk-5	wk-1	wk-3
Control (corn oil)	-	91.6±3.1 (8)	81.7±9.3 (6)	88.3±6.9 (8)	86.1±7.0 (8)	79.8±4.8 (8)	90.6±2.8 (8)
N-2-pyridyl-benzene sulfonamide	150	95.3±2.5 (7)	92.1±2.0 (6)	86.0±3.8 (6)	88.0±5.5 (6)	80.1±8.4 (5)	87.0±3.9 (6)
	450	91.2±3.1 (8)	79.0±9.8 (7)	82.0±6.2 (8)	91.6±4.6 (6)	78.7±8.8 (7)	89.3±4.4 (8)
N4-acetyl-sulfanilamide	150	83.1±3.2 (7)	66.0±14.1 (7)	57.8±13.0* (6)	50.2±10.4* (7)	65.8±8.7 (6)	79.1±3.1* (7)
	450	87.5±3.7 (8)	86.0±7.4 (8)	49.9±12.0* (6)	42.6±12.5** (7)	50.1±9.7* (6)	69.6±7.1* (6)

Values are means ± SEM (n)
*P<0.05; **P<0.01 compared to the corresponding control group, F-test.

Table 2. Effects of some simple sulfonamides on fertility of the male rat.

Treatment group	Dose (mg/kg)	Percentage Fertility					
		Before treatment	During treatment			After drug withdrawal	
			wk-1	wk-3	wk-5	wk-1	wk-3
Control (corn oil)	-	94.3±2.4 (12)	96.5±1.8 (10)	84.1±2.8 (8)	90.6±3.9 (11)	93.7±2.4 (11)	92.8±4.2 (4)
Sulfanilamide	150	92.0±3.0 (5)	91.8±3.6 (4)	57.7±9.5* (5)	44.5±4.5*** (5)	74.6±8.0** (5)	96.2±1.9 (5)
	450	88.8±4.6 (8)	86.8±6.6 (6)	56.3±7.0 (5)	18.0±9.0*** (4)	37.3±16.0*** (5)	79.5±9.9 (5)
Sulfapyridine	125	94.6±2.3 (9)	76.2±9.6* (5)	71.9±12.3 (4)	45.9±4.8** (6)	-	-
	250	98.1±1.2 (8)	62.7±16.9* (7)	22.1±11.2*** (3)	25.1±11.5*** (4)	-	-
Sulfioxazole	150	93.5±4.0 (5)	95.5±1.8 (4)	82.8±4.3 (5)	98.3±1.7 (3)	93.2±5.5 (4)	93.3±3.4 (3)
	450	89.2±4.1 (6)	97.0±1.5 (6)	81.3±7.3 (6)	82.1±8.4 (6)	88.6±5.6 (6)	87.4±7.6 (6)
Sulfachloro-pyridazine	150	98.1±1.3 (8)	70.3±9.0** (8)	91.9±2.5 (8)	85.9±2.0 (7)	81.3±8.1 (8)	-
	450	94.1±2.6 (8)	74.4±15.2 (6)	93.9±4.2 (5)	74.6±5.5* (5)	93.3±1.6 (6)	-

Values are means ± SEM (n)
*P<0.05; **P<0.01; ***P<0.001 when compared to the corresponding control group (F-test).

4.2 Effects of some simple sulfonamides on fertility

Table 2 demonstrates the effect of some commercially available sulfonamides on fertility of the male rats. When compared to the control (corn oil treated) group, sulfanilamide, sulfapyridine and sulfachloropyridazine caused a dose-related reduction in fertility. Sulfanilamide and sulfapyridine were of about equal in potency, whereas sulfachloropyridazine was much less effective. However, the onset of defective fertility occurred faster, i.e. within one week after treatment, when treated with sulfapyridine compared to sulfanilamide.

On the other hand, sulfioxazole failed to exert any effect on fertility of the male rats. The results in this and the previous report indicate that N1 substitution of the sulfonamide skeleton by other than pyridine or pyridazine abolishs the antifertility activity. In fact, the presence of pyridine group at N1 position is not at all essential since sulfanilamide has similar activity and potency to sulfapyridine. The finding that N4-acetylsulfanilamide is also effective suggests that, perhaps, the metabolic intermediate of sulfanilamide is the active substance. This may also be the case for sulfapyridine. It should be noted that sulfapyridine is converted in the body to N4-acetylsulfapyridine and 5'-hydroxy sulfapyridine before conjugation with glucuronic acid prior to excretion (Schroder and Campbell, 1972; Hasson and Sandberg, 1973; Eastwood, 1980). The present results also demonstrate the importance of p-amino group in the benzene ring since removal of this group completely abolishs the antifertility effect. Thus, N-2-pyridylbenzene sulfonamide had no effect compared to sulfapyridine. This evidence is in support of the above conclusion that N4-acetylation of sulfonamides in vivo is reguired for the antifertility activity.

4.3 Sperm motility

Only few trials have been made on the evaluation of rat sperm motility determination by use of the Makler chamber. Although a definite conclusion can not be made at this time, it would appear that the method is also suitable for the rat sperm. It was noted that sperm cells tend to stick to the glass. However, this problem could be solved by using a glass coating agent.

4.4 Sperm respiration

The rate of oxygen utilization by rat spermatozoa was determined both in normal rats and sulfapyridine treated rats. This study was only preliminary in nature. Therefore, the results were still not conclusive.

The basal rates of the sperm respiration from the normal rats in RFM lacking pyruvate and antibiotics ranged from 3 to 12 nanomoles/hr-million cells. On the other hand, those from sulfapyridine treated (500 mg/kg for 3 weeks) rats were between

31 and 39 nanomoles/hr-million cells (from 2 rats). DL-latate (22 mM) stimulated respiration in the basal medium, typically brining it up to 30-40 nanomoles/hr-million cells in normal rats and to 40-60 nanomoles/hr-million cells in drug treated rats. Addition of succinate (3 mM) further enhanced the lactate-stimulated respiration. By contrast, D-glucose (5.6 mM) did not affect the respiration rate significantly when added either before or after lactate.

When the in vitro effects of some sulfonamides dissolved in DMSO or water on sperm respiration were studied, it was found that DMSO itself inhibited respiration. Addition of 3, 5, 7 and 10 ul DMSO to the chamber (approx. 2 ml in volume) caused 10%, 20%, 24% and 31% inhibition, respectively. Thus, when the effect of sulfa drugs were studied the total volume of DMSO added into the chamber was not more than 3-4 ul in order to minimize the inhibitory effect of DMSO to no greater than 10%. Sulfapyridine, sulfanilamide, N4-acetylsulfanilamide and 5-aminosalicylate all inhibited respiration when added to sperm surpensions to the final concentration of 1-3 mM.

4.5 SDS slab gel electrophoresis

Preliminary work on gel electrophoresis was attempted so as to set up and to establish the technique in Dr. Pholpramool's laboratory for the studies designed to search for differences in protein composition between luminal fluids and/or sperm surfaces of control rats and those treated with sulfonamide drugs. At this stage, a pilot experiment was done on the electrophoresis of cauda epididymal fluid obtained by retrograde infusion with oil and blood plasma of normal rats using 7 or 10% polyacrylamide gel concentration. The purpose of this experiment was for practicing the technique and to determine whether the luminal samples obtained by retrograde infusion had any contamination from blood plasma. The results showed that the luminal fluid samples contained protein bands typical for the cauda region reported earlier by others (Huang and Johnson,1975; Koskimies and Kormano, 1975; Turner, Plesum and Cabot, 1979; Brooks and Higgins, 1980; Jones, Brown, VonGlos and Parker, 1980). Furthermore, there is no evidence of contamination from blood plasma. Thus, the method for collection of cauda fluid samples by retrograde infusion with oil was verified in terms of purify of protein composition.

4.6 Synthesis of the proposed compounds

In our previous report, we succeeded in the syntheses of N-2-pyridylbenzene sulfonamide and N-acetylsulfanilamide in small scale. Since then we have successfully synthesized these two compounds in large quantities and submitted for biological testings. We have also successfully completed the synthesis of two more proposed compounds, both in small scale and large scale. These two compounds 2-(p-toluene sulfonamido) pyridine and 2-(p-nitrobenzene sulfonamido) pyridine have been fully characterized including elemental analysis (C, N, H), mass spectrometry, NMR and IR spectrometry. See appendixes 1, 2 and 3 for the compound

2-(p-toluene sulfonamido) pyridine and appendixes 4, 5, 6 and 7 for the compound 2-(p-nitrobenzene sulfonamido) pyridine.

5. Conclusion/Remarks

During the second six month period, the methods that have been or being developed for studying the mode of antifertility action of sulfasalazine and its analogs include sperm motility by use of the Makler chamber, sperm respiration by use of an oxygraph and SDS-PAGE. Preliminary work on epididymal sperm respiration has been done in normal and sulfapyridine treated rats. It appears that the basal rate of sperm respiration is higher in the treated animals. Both lactate and succinate stimulated the respiration in normal as well as sulfa-treated rats whereas glucose did not has any significant effect. All sulfonamides tested, i.e. sulfapyridine, sulfanilamide and N4-acetylsulfanilamide, including 5-aminosalicylate inhibited respiration when added to sperm suspensions.

Two of the proposed compounds, i.e., 2(p-toluene sulfonamido) pyridine and 2-(p-nitrobenzene sulfonamido) pyridine have been successfully synthesized and the compounds are currently prepared at a higher scale for fertility testing inspite of some difficulties occurred during the preparation of these two compounds. Six proposed compounds have been tested for possible antifertility activity. Only N4-acetylsulfanilamide, sulfanilamide, sulfapyridine and sulfachloropyridazine caused a dose-related and reversible inhibition of fertility. The effect occurred as soon as one week after treatment and sustained for 1-3 weeks after drug withdrawal. Both N-2-pyridylbenzene sulfonamide and sulfioxazole had virtually no effect.

It should be mentioned that one of us (Prof. L.M. Lewin) was admitted to a hospital with pancreatitis and have to receive a major surgery during June-July. This unfortunate episode made Prof. Lewin to postpone his plan to visit Bangkok until August and also limited his activity in Israel. However, Prof. Lewin is now in a good and healthy conditions and being back to research work.

6. Workplan for the Next Period

Because of Prof. Lewin's illness as mentioned above our earlier plans for developments and standardizations of methods such as NMR studies, changes in sulfhydryl and disulfide linkage of the cauda epididymal spermatozoa, part of sperm motility and CoA-FITC binding of spermatozoa have to be postponed until next period.

A series of sulfapyridine and sulfanilamide analogs will be synthesized. Biological testing for contraceptive activity of the compounds synthesized in this reporting period will also be done. In addition, the possible effects of sulfapyridine on blood levels of gonadotropins and testosterone will be investigated.

7. References

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. M/E 183.0 INT. 640.4

APPENDIX I (MASS)

NOTE : 74/2.EI, 70V,300UA.CHAMB.
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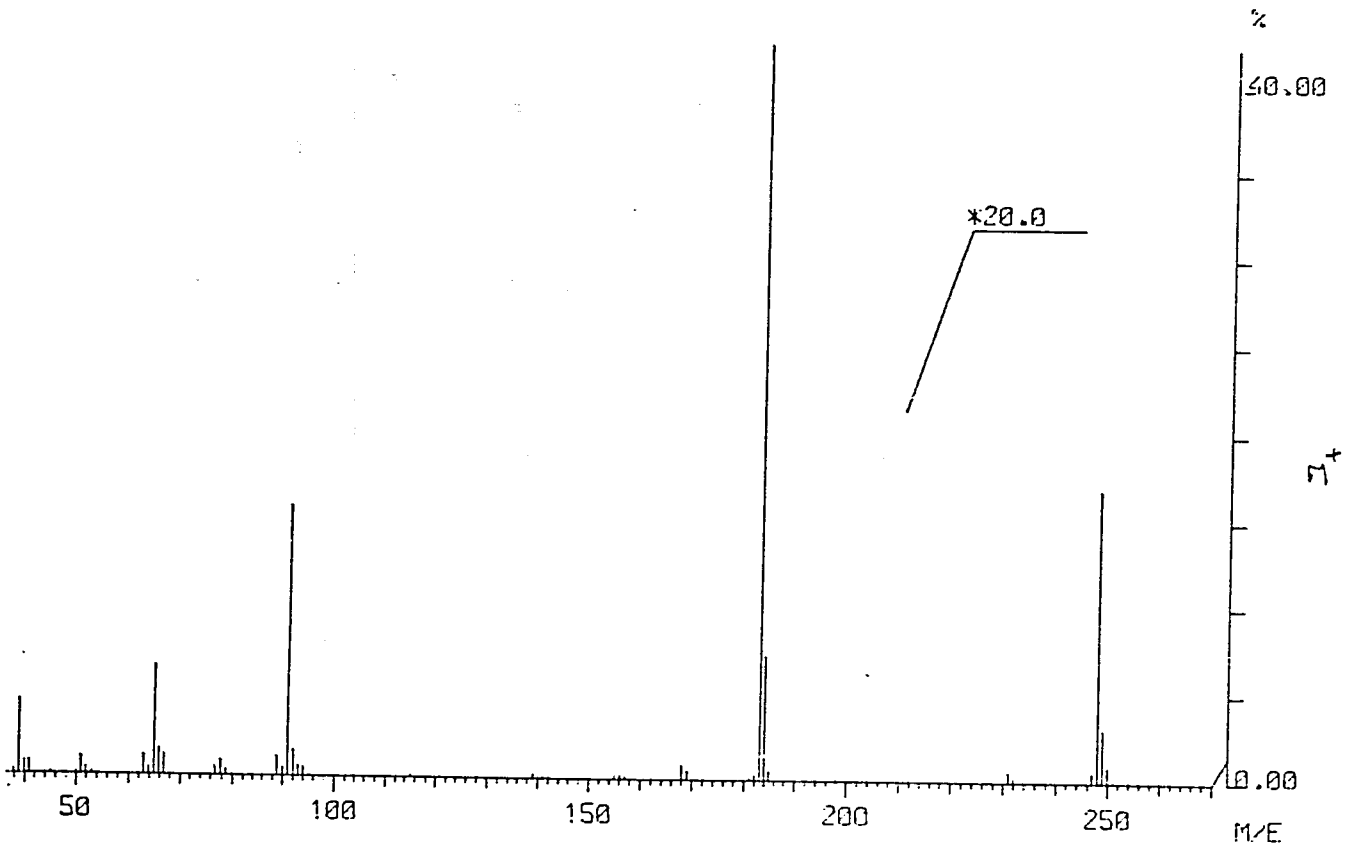

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40.0	12.2	19.1	0.89	
41.0	12.4	19.4	0.90	
51.0	16.4	25.7	1.20	
63.0	17.1	26.8	1.25	
65.0	94.9	148.2	6.92	
66.0	23.6	36.8	1.72	
67.0	18.5	28.9	1.35	
77.0	8.7	13.6	0.63	
78.0	13.2	20.6	0.96	
89.0	17.8	27.8	1.29	
90.0	8.0	12.5	0.58	
91.0	235.6	367.8	17.17	
92.0	23.1	36.1	1.68	
93.0	9.6	15.1	0.70	
168.0	14.8	23.2	1.08	
183.0	640.4	1000.0	46.69	
184.0	108.3	169.1	7.89	
185.0	8.5	13.3	0.62	
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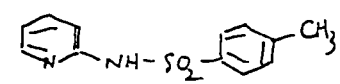
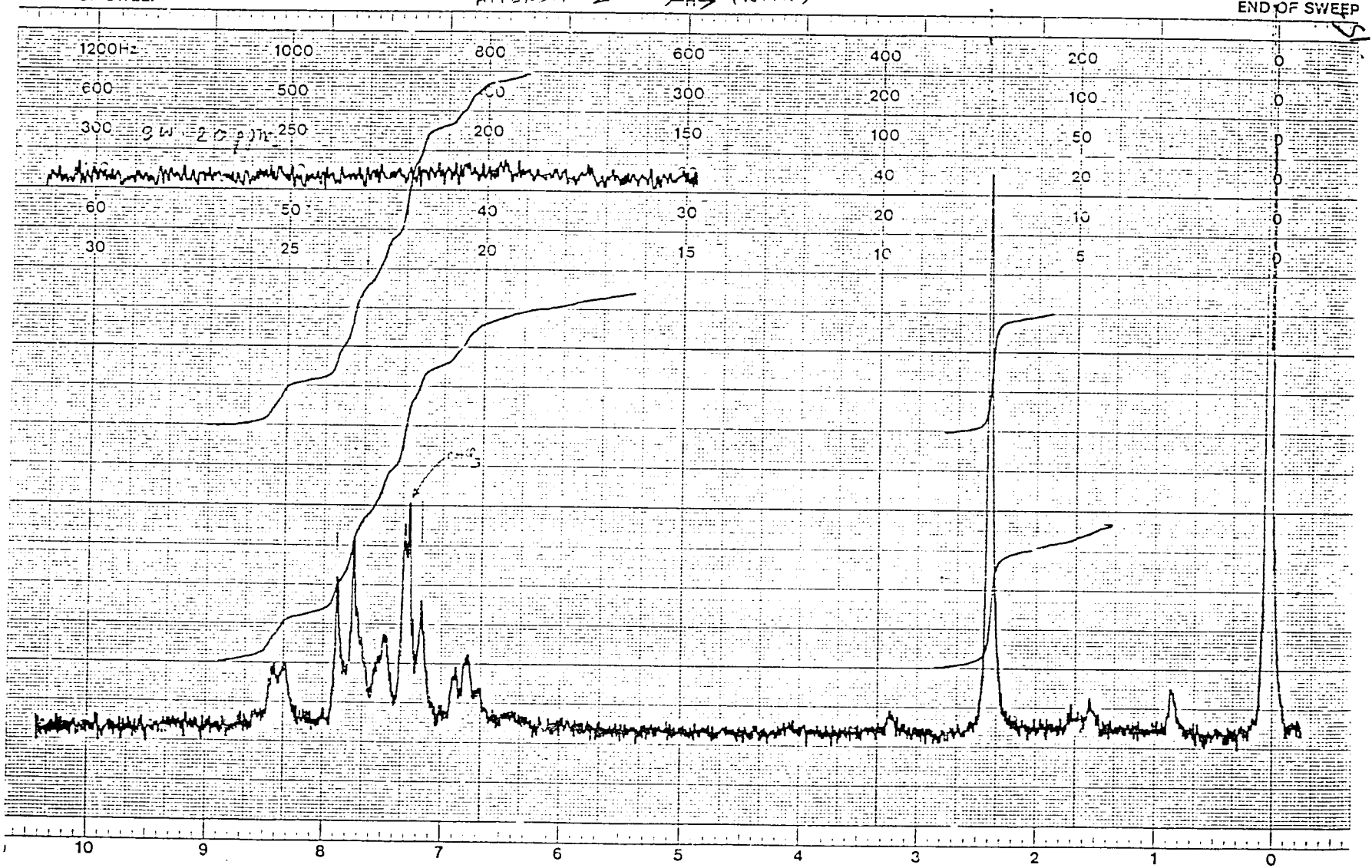


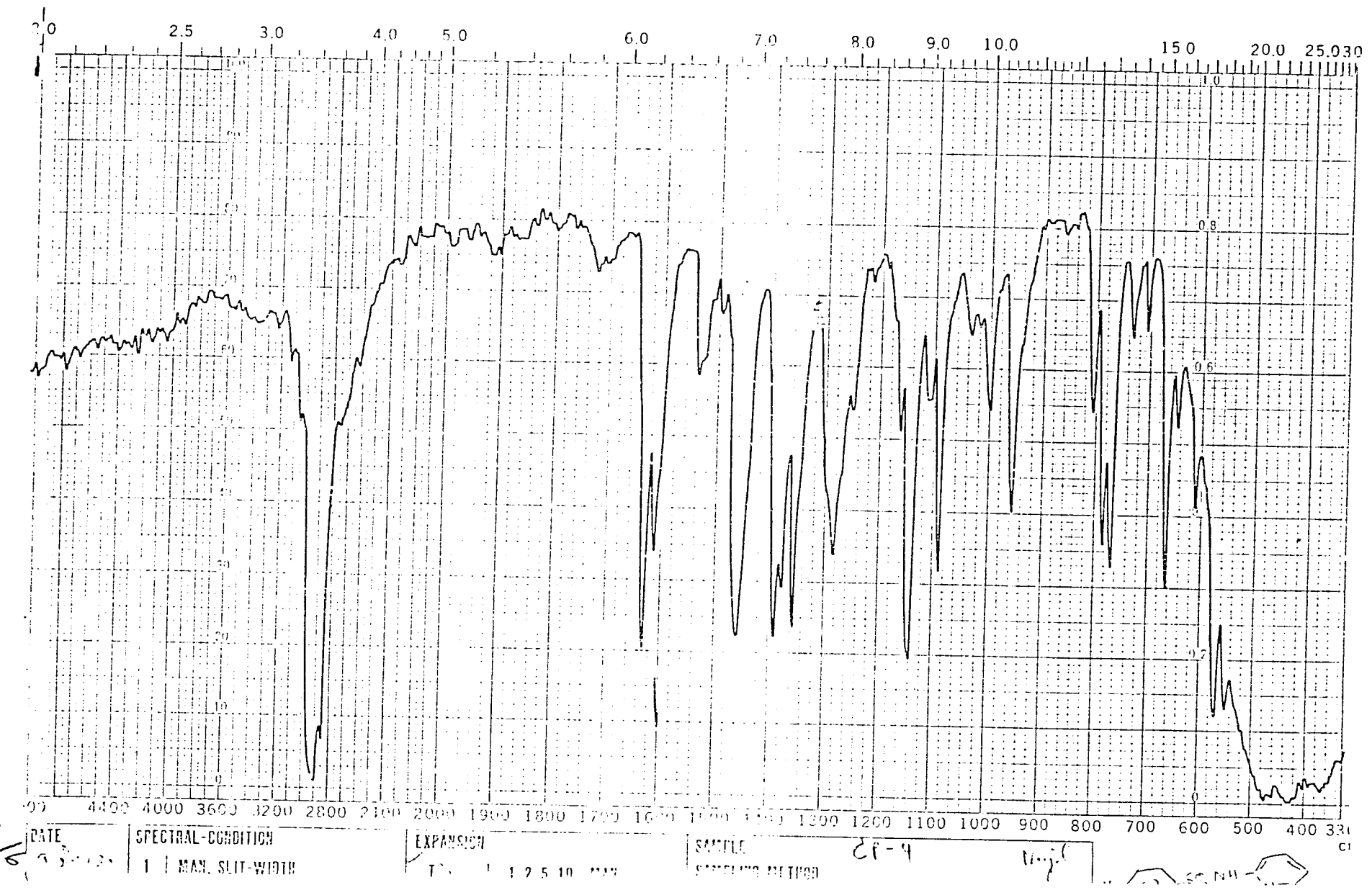
START OF SWEEP APPENDIX 2 >H> (NMR) END OF SWEEP



ppm SPECTRUM AMPL. SWEEP TIME min NUCLEUS SAMPLE: CP-4- OPERATOR

/ER mG

5/1/00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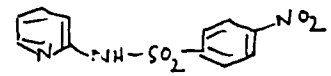
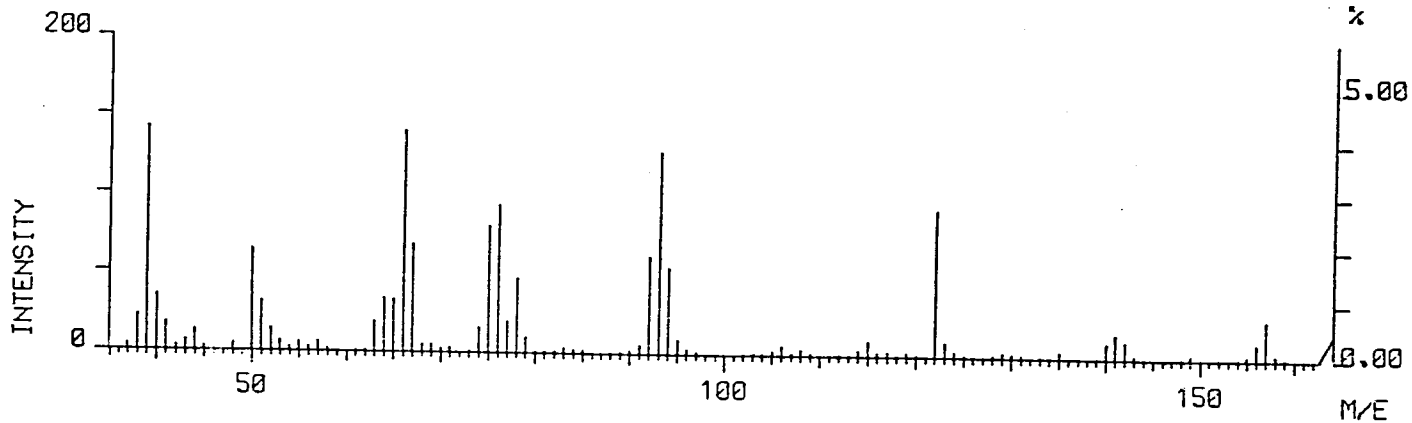
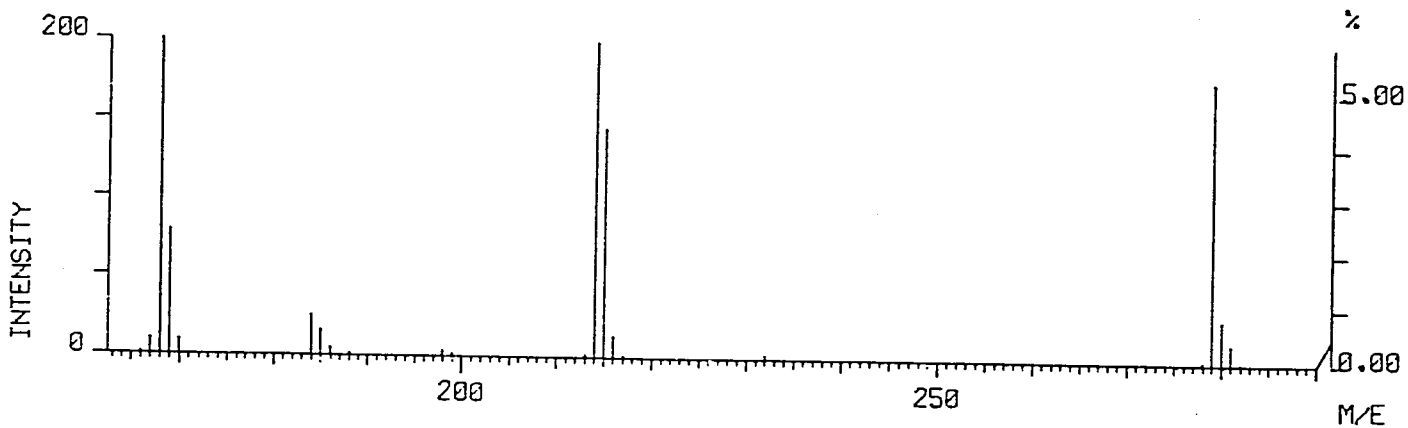
DATE
SPECTRAL-CONDITION
1 MAN. SLIT-WIDTH
EXPANSION
1 2 5 10
SAMPLE
SAMPLING METHOD

MASS SPECTRUM : (30 TO 32)
SAMPLE:CP-6-1-1, DEC 147, 10 SEP 87,
NOTE .94/3,EI,70V,300UA,CHAMB,TEMP,110,
BASE PEAK : M/E 214.0 INT. 545.7







17.

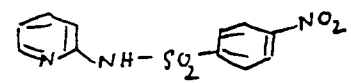
APPENDIX 5 (MS)

MASS SPECTRUM : (30 TO 32)
SAMPLE:CP-6-1-1. DEC 147. 10 SEP 87.
NOTE :94/3.EI,70V,300UA.CHAMB.TEMP.110.
BASE PEAK : M/E 214.0 INT. 545.7

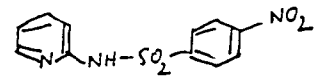
M/E	RAW INT.	R.INT.	SIGMA(%)	
38.0	12.1	22.2	1.41	
39.0	77.7	142.4	9.03	
40.0	19.3	35.3	2.24	
50.0	35.3	64.7	4.10	
51.0	17.1	31.4	1.99	
63.0	10.6	19.5	1.24	
64.0	18.5	34.0	2.15	
65.0	18.0	33.0	2.09	
66.0	76.4	140.0	8.87	
67.0	37.2	68.1	4.32	
75.0	43.8	80.3	5.09	
76.0	51.2	93.8	5.95	
77.0	11.0	20.1	1.28	
78.0	25.8	47.3	3.00	
92.0	33.5	61.5	3.90	
93.0	69.8	128.0	8.12	
94.0	29.7	54.5	3.46	
122.0	50.4	92.4	5.86	
157.0	13.5	24.8	1.57	
168.0	151.3	277.3	17.58	
169.0	43.2	79.2	5.02	
184.0	14.1	26.0	1.64	END

MASS SPECTRUM : (30 TO 32)
SAMPLE:CP-6-1-1. DEC 147. 10 SEP 87.
NOTE :94/3.EI,70V,300UA.CHAMB.TEMP.110.
BASE PEAK : M/E 214.0 INT. 545.7

	M/E	RAW INT.	R.INT.	SIGMA(%)	
	214.0	545.7	1000.0	72.70	
	215.0	79.1	145.0	10.54	
	216.0	7.5	13.8	1.00	
M^+	279.0	96.9	177.6	12.91	
	280.0	14.5	26.7	1.94	
	281.0	6.6	12.1	0.88	END



(NMR)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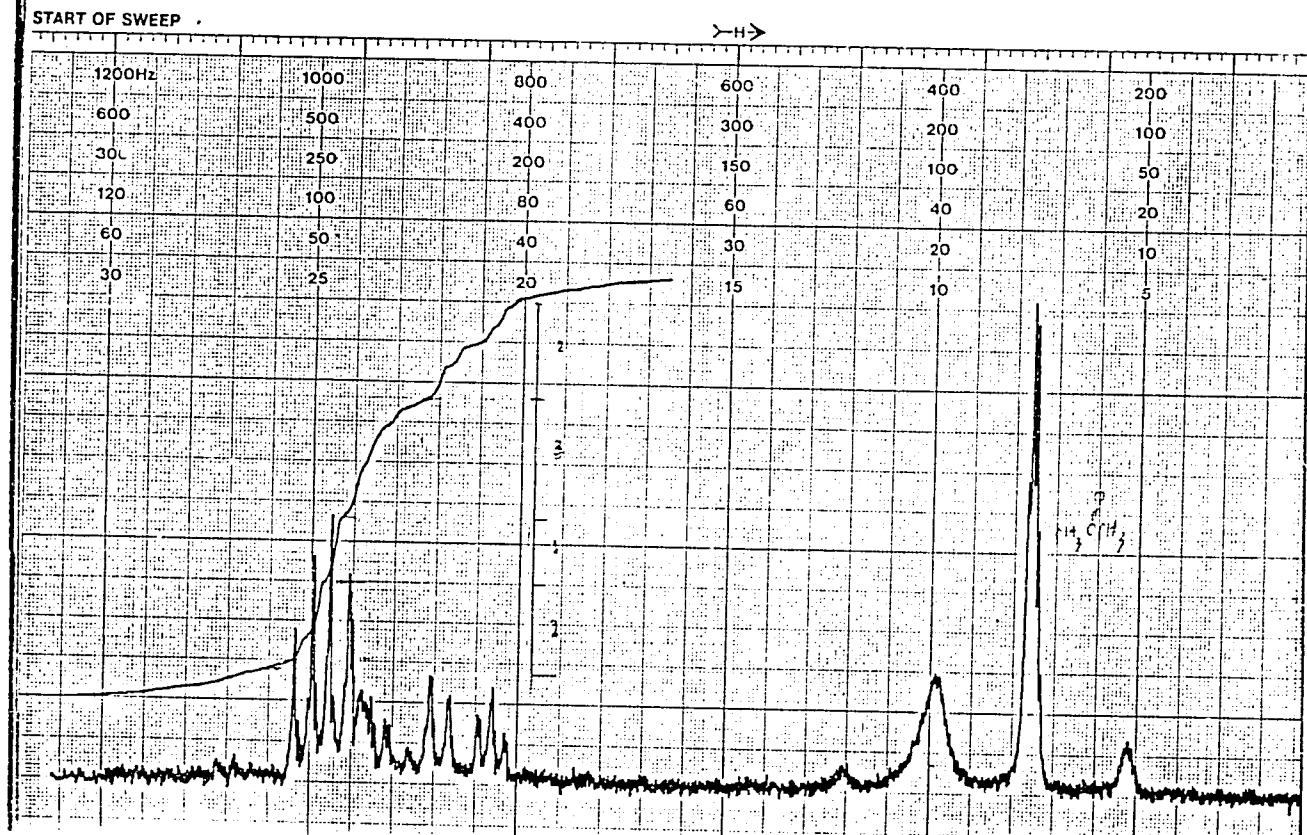
NH-SO2
N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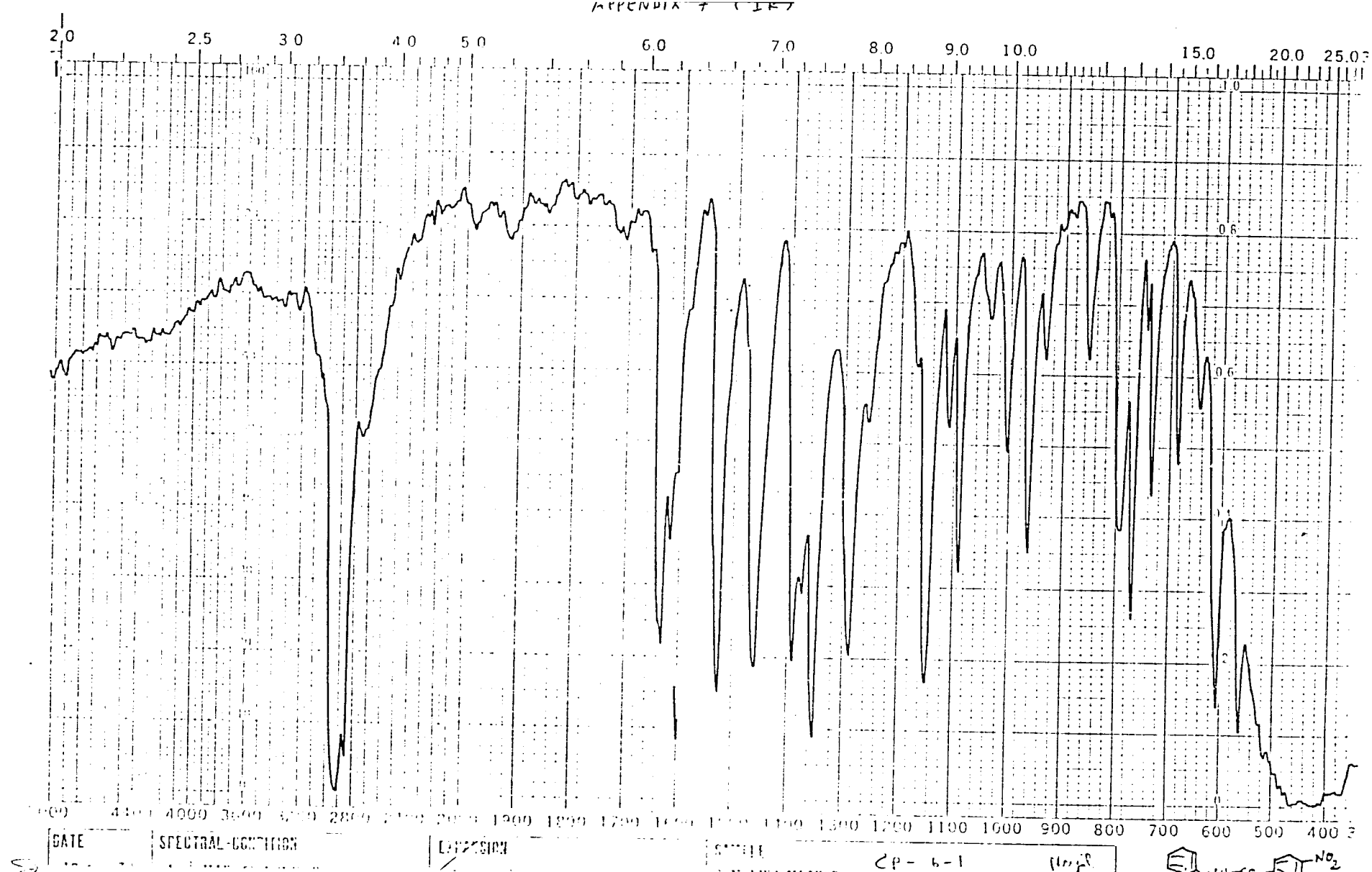
APPENDIX 4 (IR)



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C7-169

PROGRESS REPORT NO. 3

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

21

Project Profile

Country	:	Thailand
Grant No.	:	936-5544-G-00-6105-00
Program	:	US-Israel Cooperative Development Research
Project Title	:	Sulfonamide Drugs as a Male Antifertility Agent
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.
Project Consultants	:	None
Authorized Officer	:	Natth Bhamarapravati, M.D., D.Sc. Rector, Mahidol University
Total Project Budget	:	US$150,000
Project Duration	:	3 years
Reporting Period	:	April 30, 1987; October 31, 1987; April 30, 1988; October 31, 1988; April 30, 1989; October 31, 1989.
Budget Allocation for This Period	:	US$27,870

22

CONTENTS

		PAGE
1.	Introduction	1
2.	Objectives	1
3.	Materials and Methods	
	3.1 Fertility testing	1
	3.2 Collection of blood samples	2
	3.3 Hormone assays	2
	3.4 Sperm respiration	2
	3.5 Experimental procedures	3
	3.6 Synthesis of the proposed compounds	4
	3.6.1 2-(o-Nitrobenzene sulfonamido) pyridine	4
	3.6.2 2-N-Acetylsulfanilamidopyridine	4
	3.6.3 2-(p-Aminobenzene sulfonylamino) pyridine	5
4.	Results and Discussion	
	4.1 Fertility testing of the synthetic compounds	5
	4.2 Effects of sulfapyridine on serum gonadotropins and testosterone	6
	4.3 Effect of some sulfonamides on respiration of rat spermatozoa	6
	4.4 Effects of sulfasalazine and its metabolites on glycolysis of bull spermatozoa	7
	4.5 Synthesis of the proposed compounds	7
5.	Conclusion/Remarks	9
6.	Workplan for the next period	9
7.	References	9
8.	Appendix	11

1. Introduction

We have previously shown that sulfasalazine, sulfapyridine and sulfanilamide caused a dose-related suppression of fertility in the male rats whereas sulfacetamide and sulfachlorpyridazine may have some antifertility effect (1, 2). On the other hand, sulfadimethoxine, sulfamerazine and sulfioxazole had virtually no effect. In addition, two synthetic compounds, i.e. N-2-pyridyl benzene sulfonamide and N-4-acetylsulfanilamide, were tested and only the latter showed a significant effect, being as potent as sulfanilamide(2).

The results so far indicate that first, the substitution at N' position by pyridine group is not essential for the antifertility activity; and second, the presence of a p-amino group in the benzine ring is required for the activity. In order to confirm this notion we have synthesized three sulfapyridine analogs in which the p-amino group of their benzene rings were replaced by either p-nitro, p-methyl or o-nitro group. The methods of synthesis and the results of fertility testings of these analogs are described in this report. Also included in this report are some preliminary results from the studies on the effects of sulfasalazine and/or sulfapyridine on gonadotropins and testosterone, and on lactate accumulation and respiration of epididymal spermatozoa.

2. Objectives

The aims of the work included in this report are :

(a) to synthesize some sulfapyridine analogs which lack the p-amino group in their benzene rings,

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

(c) to re-evaluate the possible effect of sulfapyridine on plasma levels of gonadotropins and testosterone,

(d) to determine whether sulfasalazine or sulfapyridine has any in vitro effect on metabolism of the epididymal spermatozoa.

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 before grouping and drug treatments. Each male was housed with two virgin female rats of the same strain aged 40-50 days in hanging 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

24

successfully fertilized and the embryos implanted, i.e., number 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 after the rats were anesthetized with diethyl ether. The skin area of the operating side was cleaned with alcohol and a small skin incision was made to expose the vein which was then punctured with a sterile needle gauge 23. Blood volume between 2.0 to 3.0 ml, which was approximately 8-9% of total blood volume of each rat, was removed and an equivalent volume of sterile 0.9% saline solution was injected through the same needle into the animal so as to reconstitute its blood volume. The opening was closed with sterile cotten treads. Approximately 50 ul of blood samples were transferred into heparinized microhematocrit tubes for the determination of hematocrit. The remaining blood samples were kept over night in a refrigerator to allow complete coaggulation of blood. In the following morning, the blood samples were centrifuged and sera were separated and kept at -20°C for hormone assays.

In order to reduce large variations of blood gonadotropin and testosterone levels due to diurnal rhythms, all blood sample collections were made at the same period of time in a day, i.e. between 13.00-15.00 p.m.

3.3 Hormone assays

Testosterone was analyzed by RIA using the method of Resko et al. (3). Testosterone antibody (JAR#6) was obtained from Dr. J.A. Resko of Oregon Regional Primate Research Center, USA. The sensitivity of the assay was 10 pg/ml. Intra and inter assay variation coefficients were 3.02% and 15.13%, respectively.

3.4 Sperm respiration

Rat spermatozoa were collected from the cauda epididymidis either by retrograde infusion with oil or cutting the cauda epididymidis into small pieces and spermatozoa were released into rat fertilization medium (RFM).

Two ml of RFM, see (2) for composition, were added to spermatozoa obtained as described above and the sperm suspension was transferred into the chamber of a Gilson Oxygraph at 37°C. Respiration (utilization of oxygen) of spermatozoa was determined by use of the clark electrodes. Microliter amounts of D-glucose, DL-lactate or sulfonamide drugs dissolved in either DMSO or water were injected into the chamber to observe their effects on respiration rate.

25

In some experiments spermatozoa were obtained from rats treated with corn oil or sulfapyridine at a dose of 500 mg/kg, daily for 3 weeks.

3.5 Experimental procedures

3.5.1 Experiment 1 (Fertility testing of the synthetic compounds)

Fifty two male rats were divided into 7 groups (7-8 rats each) and were force-fed with either corn oil, 2-(p-nitrobenzenesulfamido) pyridine (PNSP), 2-(o-nitrobenzenesulfamido) pyridine (ONSP) or 2-(p-toluene sulfamido) pyridine (PTSP) at doses of 150 and 450 mg/kg (once daily) for 6 weeks. At the end of weeks 1, 3, 5, 7 and 9, all treated and control (corn oil treated) were subjected to fertility testing as described above except that the females were checked for the presence of spermatozoa in vagina smears done every morning after cohabitation so as to confirm mating with the male. Therefore, fertilities of the males at weeks 1, 3 and 5 during treatment and weeks 1 and 3 after drug withdrawal were determined.

3.5.2 Experiment 2 (Effect of sulfapyridine on serum gonadotropins and testosterone)

Twenty one male rats were allocated into 2 groups (10-11 rats each) and were force-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, 5, 7 and 9 all animals were subjected to fertility testing as in Experiment 1. In addition, blood samples were collected aseptically from the external jugular vein prior to treatments as well as at weeks 3 and 5 during treatments, and at week 4 after the cessation of treatments.

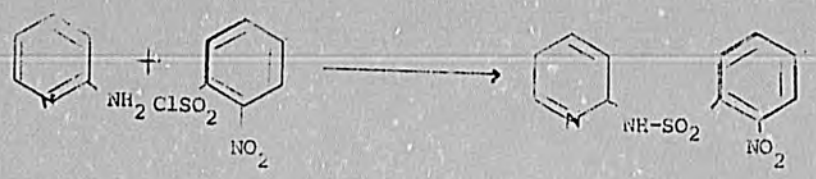
3.5.3 Experiment 3 (Effects of sulfasalazine and its metabolites on glycolysis of bull spermatozoa)

Pellets of frozen bull semen were thawed at 37°C, mixed with a HEPES-buffered balanced salt solution (4) and centrifuged (20 min., 500 x g) through 3 ml of 10% Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden). The sperm were resuspended (to a concentration of 40-60 x 10^6 cells/ml) in the salts solution containing D-glucose (0.1-10 mM) and the cells incubated at 37°C for up to 1 hr. At 0 time and after incubation, perchloric acid (35%, 0.2 ml) was added to portions (1 ml) of incubation mixture. After chilling (0°C, 10 min.) and centrifugation, 0.9 ml of supernatant fluid was removed, mixed with 0.6 ml of saturated $KHCO_3$, chilled for 10 min., and centrifuged to remove $KClO_4$. Duplicate samples (0.4 ml) of supernatant fluid were removed for analysis of lactate with lactic dehydrogenase using Kit 826-UV (Sigma Chemical Co., St. Louis, MO., USA). Additional aliquots (0.1-0.15 ml) were used for analysis of glucose, using a glucose oxidase reagent (Kit 510, Sigma Chemical Co.).

26

3.6. Synthesis of the proposed compounds

3.6.1 2-(o-nitrobenzenesulfonamido) pyridine

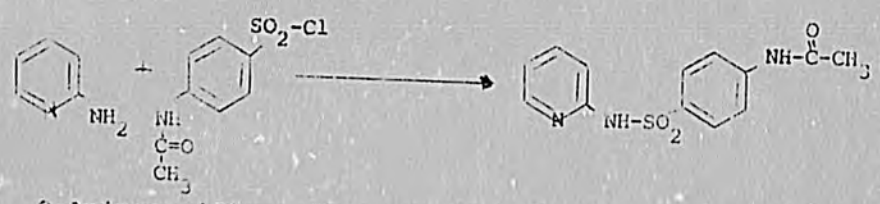


2-Aminopyridine (10 g) was dissolved in dry dichloromethane (50 ml) and dry pyridine (7 ml). O-Nitrobenzenesulfonyl chloride (22 g) in dry dichloromethane (50 ml) was added to the above solution which was cooled on the ice bath with vigorous stirring during the period of 3.5 h. The precipitate of the product was filtered and washed with cold methanol. Upon recrystatlization from hot methanol, pale yellow amorphous solid was obtained; yield 9.63 g (37.79%). The product was identified by its melting point (115-118°C dec.), IR,NMR and mass spectroscopy (Appendices 1a, 1b and 1c, respectively) as well as elemental analysis.

Analysis : C 47.34, N 14.97, H 3.19

Calc. for $C_{11}H_9N_3O_4S$: C 47.31, N 15.05, H 3.25

3.6.2 2-N-Acetylsulfanilamidopyridine



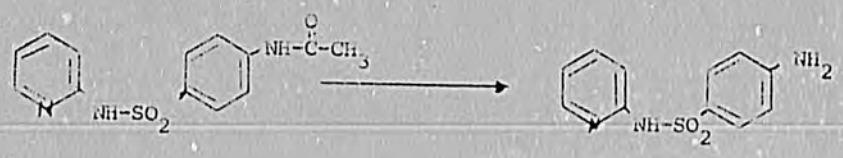
2-Aminopyridine (5g) was dissolved in dry acetone (30 ml) and dry pyridine (5 ml) and the solution was cooled in ice bath. Dry p-acetamidobenzenesulfonyl chloride (12g) in dry acetone (90 ml) was slowly added to the above solution during the period of 3 h. The resulting precipitate was filtered and washed with cold methanol and recrystallized from hot methanol to give white amorphous solid (5.1 g, 40% yield). The product was identified by its melting point (224-226°C), IR, NMR and mass pectroscopy (Appendices 2a, 2b and 2c, respectively) as well as elemental analysis.

Analysis : C 53.62, N 14.19, H 4.48

Calc. for $C_{13}H_{13}N_3O_3S$: C 53.58, N 14.42, H 4.50

21

3.6.3 2-(p-Aminobenzenesulfonylamino)-pyridine



2-N-Acetylsulfanilamidopyridine (1 g) was dissolved in 12% sodium hydroxide solution in methanol (10 ml). The mixture was stirred at ambient temperature for 1 h., then carefully neutralized with 6 N hydrochloric acid. The resulting precipitate was filtered and recrystallized from hot methanol to give pale yellow amorphous solid (0.4 g, 53% yield). The product was identified by its melting point (189-191°C), IR, NMR and mass spectroscopy (Appendices 3a, 3b and 3c, respectively) as well as elemental analysis.

Analysis : C 52.81, N 16.60, H 4.40

Calc. for $C_{11}H_{11}N_3O_2S$: C 53.00, N 16.86, H 4.45

4. Results and Discussion

4.1 Fertility testing of the synthetic compounds

Three synthetic sulfonamides, two of which had been synthesized and reported previously (2), were tested for a possible antifertility activity. The results are shown in Table 1. It should be pointed out that the data during the drug withdrawal periods are still incomplete at the time of writing this report. However, the data so far clearly demonstrate that PNSP had a dose-dependent antifertility action where as its o-nitro or p-toluene analogs had no effect. The antifertility effect of PNSP occurred as soon as by 3 weeks after starting the treatment and the maximum effect was reached by week-5 after treatment. This compound had a potency for suppression of male fertility comparable to sulfanilamide and N^4-acetyl sulfanilamide, but slightly less than sulfapyridine (2).

The findings that the presence of o-nitro or p-toluene group in the benzene ring of sulfamidopyridine or N-2-pyridylbenzene sulfonamide fails to produce antifertility property of the sulfapyridine analogs indicate that possession of this activity requires substitution in the para position of the benzene ring of sulfonamides by either an amino, acetylamino or a nitro group. The data further suggest that these groups share the same intermediate group formed in the body, which is in fact the active moiety. Since normal metabolism of sulfapyridine occurs through acetylation at N4 position (5, 6,

28

7), it is believed that the active compound is most likely N4-acetyl sulfapyridine and/or N4-acetyl, 5'-OH-sulfapyridine. Other sulfonamides which have functional groups at the para position of the benzene ring other than the amino group, but can be converted in the body into amino group may also be effective.

4.2 Effect of sulfapyridine on serum gonadotropins and testosterone

The experiments were designed to follow up the possible changes in serum levels of gonadotropins and testosterone in each rat at various periods before, during and after cessation of treatment with sulfapyridine at a dose of 450 mg/kg. Since repeated blood samples had been removed from the same animal an equivalent volume of saline was given so as to restore the blood volume. However, the animals are bound to loss some red blood cells and this is reflected in a decrease in hematocrit at the later period of experiments (Table 2). Analyses of data show that significant decreases in hematocrit occurred both in the control and experimental groups. Such a decrease was noted by 3 weeks after drug treatment and was maximum at week-6 at which time the hematocrit values were, respectively, 92.1% and 78.3% of the control value (before treatment) in the control and experimental group. The reduction in hematocrit was remained even by 4 weeks after the cessation of treatment. The marked decrease in hematocrit that observed in the experimental group was not due to differences in the blood volume removed for hormone assays. Since the toxic effect of sulfonamides on the hematopoietic system is not unknown (8), it is possible that sulfapyridine may also cause the anemic response observed in this study. However, the toxicity of sulfapyridine requires further and well-controlled studies.

Table 3 shows the levels of serum testosterone before and after sulfapyridine treatment. It is cleared that sulfpapyridine at a dose of 450 mg/kg, which caused a marked reduction in fertility (2), had virtually no effect on serum testosterone. The results, therefore, confirm our previous study that sulfasalazine had no effect on blood testosterone (9). For some technical reasons, the analyses for LH, FSH and prolactin were still incomplete. These data will be included in the next report.

4.3 Effect of some sulfonamides on respiration of rat spermatozoa

Table 4 shows data of the preliminary study on the in vitro effect of some sulfonamides on rat sperm respiration. As previously reported (2), 22 mM DL lactate produced almost three fold increase in respiration. When different concentrations of sulfonamides were added to the lactate-stimulated spermatozoa, their respiration rates were substantialy decreased. It appears that the effect is dose dependent.

29

When the respiration rates of spermatozoa obtained from the in vivo treated specimens were determined, it was found that the basal rate in the sulfapyridine treated preparations was twice as much of the untreated preparations. However, the percentage of lactate induced respiration in the spermatozoa from the drug treated animals was less than that in the control animals. This may be due to the limit at which sperm can maximally respire is approached. Since the data were obtained from only a few animals, firm conclusion can not be made at this stage.

4.4 Effects of sulfasalazine and its metabolites on glycolysis of bull spermatozoa

As a pilot study, bull spermatozoa were chosen as a model for studying the possible effect of sulfasalazine and its metabolites on sperm metabolism simply because frozen bull semen is readily available in Israel.

Preliminary experiments showed that lactate production was almost independent of glucose concentration between 0.1 and 10 mM (Table 6). Subsequent experiments utilized 1 mM glucose and 30 min incubation. When sulfasalazine, its moieties, sulfapyridine and 5-aminosalicylic acid, and a variety of other sulfa drugs and related analogs were added to the incubation mixtures sulfasalazine caused significant inhibition (Table 7). Sulfapyridine and 5-aminosalicylate were less inhibitory. Other sulfa drugs and analogs that were not inhibitory at a concentration of 1 mM included sulfanilic acid, sulfaguanidine, sulfathiazole, sulfazerazine sulfacetamide, sulfamethazine, and the analogs p-aminobenzoate, m-aminobenzoate and p-aminophenol.

Oral administration of sulfasalazine has been reported to be followed by its breakdown to sulfapyridine in the colon, with the absorbed sulfapyridine thus being responsible for the contraceptive effect (5, 6, 10). It is thus unlikely that the observed inhibitory effect of sulfasalazine on sperm glycolysis is directly related to its action as a male contraceptive agent. Sulfapyridine and sulfanilamide, which have been reported to have contraceptive action in male rats (2), did not inhibit sperm glycolysis appreciably under the conditions tested.

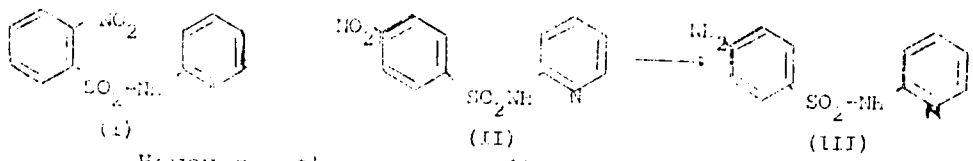
4.5 Synthesis of the proposed compounds

2-(o-Nitrobenzenesulfonamido) pyridine(I) was successfully synthesized by the reaction of 2-aminopyridine and o-nitrobenzenesulfonyl chloride in dichloromethane. Dry pyridine was found to be the best of choice in this reaction. The yield was approx. 37% and the reaction was successfully utilized to synthesize a large quantity of the compound required for biological testing. Many attempts to reduce the nitro group to the corresponding amino group failed and these included :-

30

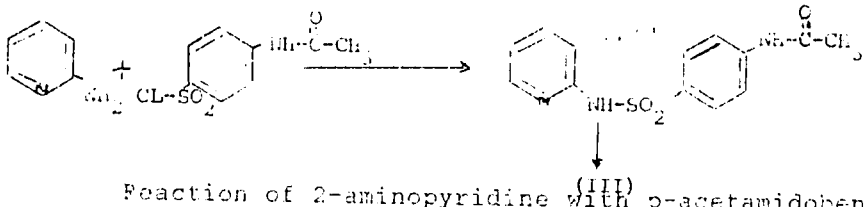
a) Catalytic hydrogenation (5% Pd/c)
b) Reduction with $FeSO_4$
c) Reduction with Fe powder/acetic acid
d) Reduction with Al/Hg in ether : water : ethanol 1:1:1

All the above procedures gave starting material or a complex mixture of products.



However, the corresponding para nitro derivative (II) can be reduced selectively at the nitro group by catalytic hydrogenation (5% Pd/c) to give the required compound (III) in approx. 28% yield.

Due to the low yield of conversion and the high cost of catalyst used in this reduction, the reaction was not suitable for the synthesis of a large quantity of the compound required for biological testing. Alternative procedure for the synthesis of compound (III) was then investigated as shown.



Reaction of 2-aminopyridine with p-acetamidobenzenesulfonyl chloride gave the N-acetyl derivative (IV) of compound (III) in approx.40% yield. Selective hydrolysis of the acetamide to the corresponding amino group could be accomplished with varying success both by using acid or base hydrolysis. The various conditions which were investigated are briefly outlined below.

a) 1% HCl, MeOH reflux 2 h -----> product 33% yield
b) 2.5% HCl, MeOH reflux 2 h -----> product 33% yield
c) 2.5% NaOH, MeOH reflux 12 h -----> incomplete reaction
d) 6% NaOH, MeOH reflux 12 h -----> incomplete reaction
e) 12% NaOH, MeOH, RT 1 h -----> product 53% yield

As can be seen, 12% NaOH/MeOH gave the best result, we are in the process of scale-up of the procedure to synthesize sufficient amount of the required compound for biological testing.

31

5. Conclusion and Remarks

We have synthesize three more proposed compounds and have done biological testing for antifertility activity. In this report, one (PHSP) of the three sulfapyridine analogs showed contraceptive effect and the potency of which was similar to that of sulfanilamide and its N4-acetyl derivative but was slightly less than that of sulfapyridine. We feel that our data so far provide us some idea about the basic requirements for the chemical structure of the sulfa drugs that possess contraceptive effect in the male rat. Thus compounds which have antifertility activity in the male should have the following basic structure :

Preliminary studies on the mode of action of sulfonamides confirmed our previous work that sulfapyridine had no effect on blood testosterone. In vitro studies showed that several sulfonamides had an inhibitory effect on the respiration of rat crude epididymal spermatozoa. However, spermatozoa collected from sulfapyridine treated rats had higher rate of basal respiration but the percentage of lactate-induced respiration was lower. In addition, sulfasalazine markedly inhibited bull sperm glycolysis in vitro whereas its metabolits, sulfapyridine and 5-aminosalicylate, were less inhibitory. On the other hand, other sulfonamides and analogs were ineffective. It is clear that further studies are required especially on the effect of those sulfonamides which showed contraceptive properties in rats.

6. Workplan for the Next Periods

Upto 3 or 4 proposed compounds will be synthesized and their contraceptive properties will be tested. In addition, the biological testing of N4 acetyl sulfapyridine synthesized in this reporting period will be done. Next report will also include complete data missing in this report, e.g. fertility data after withdrawal of PHSP, OHSP and PTSP as well as the effect of sulfapyridine on blood levels of gonadotropins. The responsiveness of the anterior pituitary gland and the Leydig's cells to GnRH and LH/hCG, respectively, will be tested. The possible effects of some sulfonamides on rat sperm motility, glycolysis and respiration will be determined.

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33

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

Treatment group	Dose (mg/kg)	Before treatment	Percentage Fertility				
			During treatment			After drug withdrawal	
			wk-1	wk-3	wk-5	wk-1	wk-3
Control	-	93.7±2.2 (8)	83.6±4.7 (7)	91.8±4.2 (7)	82.3±7.6 (8)	-	-
PNSP	150	90.9±4.5 (7)	82.7±7.9 (7)	54.3±11.9**,+ (7)	40.5±4.7***,++ (6)		
	450	91.5±3.8 (8)	82.3±6.3 (4)	36.2±15.5*,+ (6)	56.2±10.7+ (7)		
ONSP	150	95.7±3.0 (7)	82.3±5.0 (7)	75.5±13.9 (7)	97.2±1.6 (5)		
	450	92.8±2.2 (8)	93.7±2.5 (7)	70.6±12.4 (5)	82.4±5.9 (8)		
PTSP	150	95.[illegible]±2.9 (7)	87.1±7.3 (5)	90.0±6.8 (5)	92.6±7.4 (6)		
	450	94.0±3.0 (7)	93.7±3.3 (6)	95.0±3.0 (7)	89.4±6.9 (7)		

Values are mean ± SEM (n)
*P<0.05; **P<0.01; ***P<0.001 when compared to the corresponding period in the control group (t-test).
+P<0.05; ++P<0.001 when compared to the control period (before treatment) within a group (t-test).

Table 2. Hematocrit values at various periods before, during and after cessation of treatment with sulfapyridine at a dose of 450 mg/kg, daily for 6 weeks.

Treatment group	Hematocrit values (%) at period			
	Before treatment	During treatment wk-3	During treatment wk-6	After cessation of treatment wk-4
Control oil)	49.3±0.5, n=11 (100%)	45.9±0.7**, n=11 (93.1%)	45.4±0.5***, n=11 (92.1%)	46.9±0.7*, n=10 (95.1%)
Sulfapyridine (450 mg/kg)	48.9±1.0, n=9 (100%)	43.4±0.8***,+, n=9 (83.8%)	38.3±1.3***,++, n=8 (78.3%)	43.8±0.9**,+, n=8 (89.6%)

Values are Mean ± SEM.
Values in parentheses are percentage of the control values, i.e. before treatment.
*P<0.05; **P<0.01; ***P<0.001 when compared to the period before treatment within a group.
+P<0.05; ++P<0.001 when compared to the corresponding period of the control group.

Table 3. Serum testosterone at various periods before, during and after cessation of treatment with sulfapyridine at a dose of 450 mg/kg, daily for 6 weeks.

Treatment group	Serum testosterone (ng/ml)			
	Before treatment	During treatment wk-3	During treatment wk-6	After cessation of treatment wk-4
Control (corn oil)	1.12±0.16 (11)	1.47±0.22 (7)	1.03±0.17 (11)	1.33±0.16 (10)
Sulfapyridine	0.84±0.08 (9)	0.95±0.28 (3)	0.96±0.14 (8)	1.03±0.16 (8)

Values are Mean ± SEM (n).

Table 4. Effects of some sulfonamides on respiration of rat spermatozoa from the cauda epididymidis.

Drug	Conc.[a] (mM)	Respiration rate (nmole O_2/hr-million cells)	% changes[b]
None (basal)	-	10.4±0.7 (5)	-
None (+lactate)	22	28.9±2.4 (9)	-
Sulfapyridine	0.5	26 (1)	-35
	1.0	22 (1)	-45
	1.5	18 (1)	-40
	2.0	24 (2)	-33
	3.0	21 (1)	-48
5-aminosalicylate	0.8	31 (1)	-3
	1.7	31 (1)	-3
	3.3	19 (2)	-21
Sulfanilamide	0.5	16 (2)	-6
	1.0	13 (2)	-26
	2.0	2 (1)	-62
N4-acetylsulfanilamide	0.5	27 (2)	+2
	1.0	20 (2)	-25
	2.0	19 (2)	-30

Values are Mean ± SEM (n)
[a] Final concentration in the respiratory chamber.
[b] % changes in respiration rate from control (after lactate stimulation).

Table 5. The effect of sulfapyridine (500 mg/kg, daily for 3 weeks) on basal and lactate-induced respiration of the rat cauda epididymal spermatozoa.

Treatment group	Respiration rate (nmole O_2/hr-million cells)		% stimulation
	basal	lactate-induced	
Control (corn oil)	15	26	64
Sulfapyridine	35	50	41

Data are mean from 2 rats in each group.

Table 6. Glucose utilization and lactate production by bull sperm.

Glucose conc. (mM)	Glucose utilization			Lactate production		
	15 min	30 min	60 min	15 min	30 min	60 min
0.10	1.0(1)	1.4(1)		1.4(1)	2.0(1)	
0.13	0.7(3)	1.3(3)		1.3(3)	2.5(3)	
0.25	1.3(5)	1.7(4)		1.3(6)	2.1(6)	
0.50		1.7(4)		1.1(5)	1.9(5)	
1.0	1.5(3)	2.2(3)	2.8(3)	1.2(3)	2.0(3)	3.2(3)
2.5			6.4(1)			3.0(2)
5.0			3.9(3)			3.0(3)
10			5.6(1)			3.4(1)
Mean	1.1	1.7	4.7	1.3	2.1	3.2

Washed bull sperm were incubated at 37°C for 15, 30, or 60 min in balanced salts solution with the addition of glucose (0.1-10 mM). Glucose utilization and lactate production (nanomoles/10^6 cells) were determined as described in METHODS. The number of replicates is reported in parentheses.

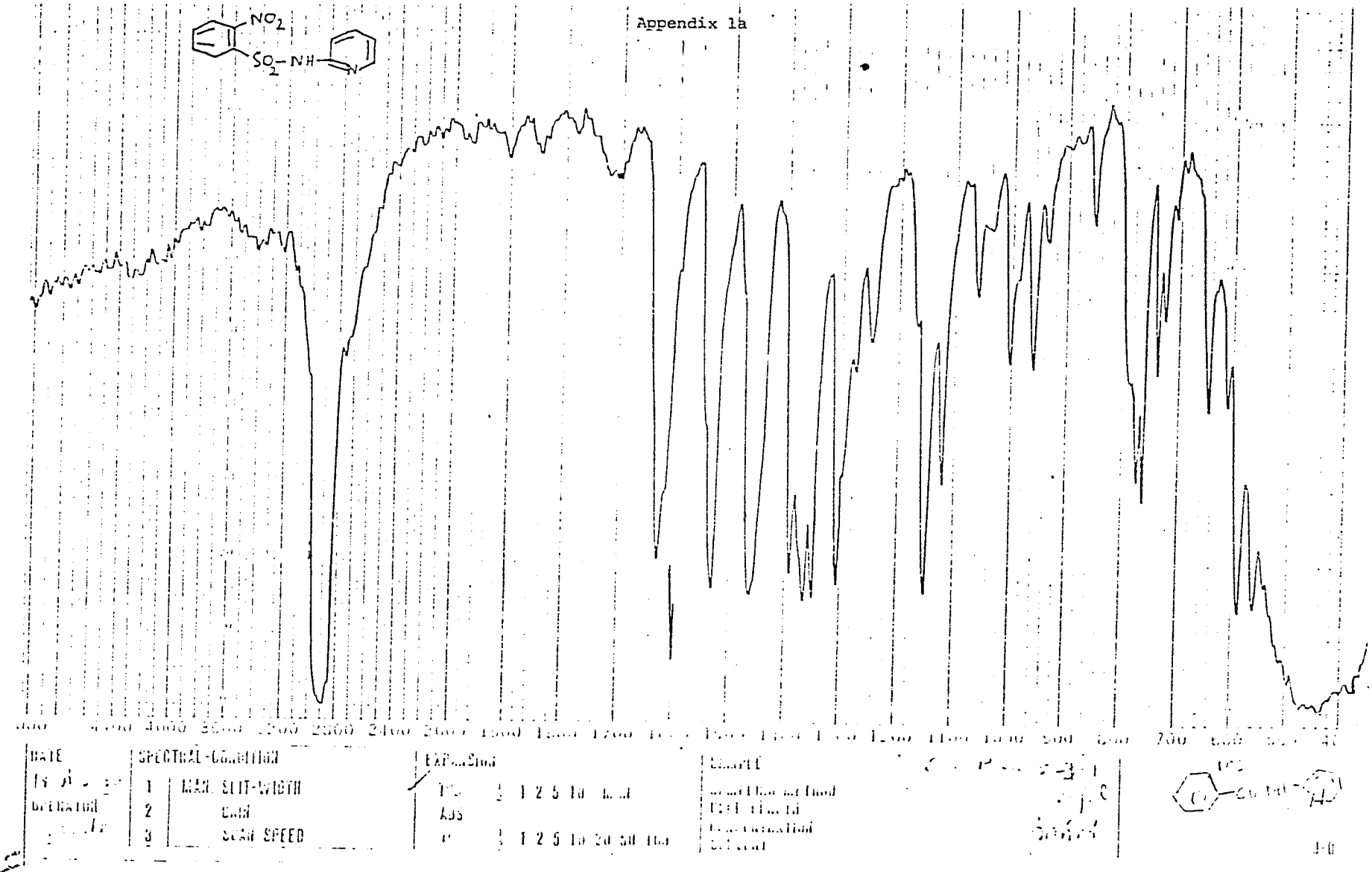
Table 7. Per cent inhibition of glycolysis by bull sperm in the presence of sulfasalazine and its moieties.

Compound added	Concentration of test compound		
	1 mM	2 mM	3 mM
Sulfasalazine	[b]14(6.6)	[a]49(3.0)	[a]64(11)
Sulfapyridine	-17(7.5)	[b]16(5.8)	[b]20(6.1)
5-aminosalicylate	[b]16(5.6)	15(9.0)	[a]17(3.6)

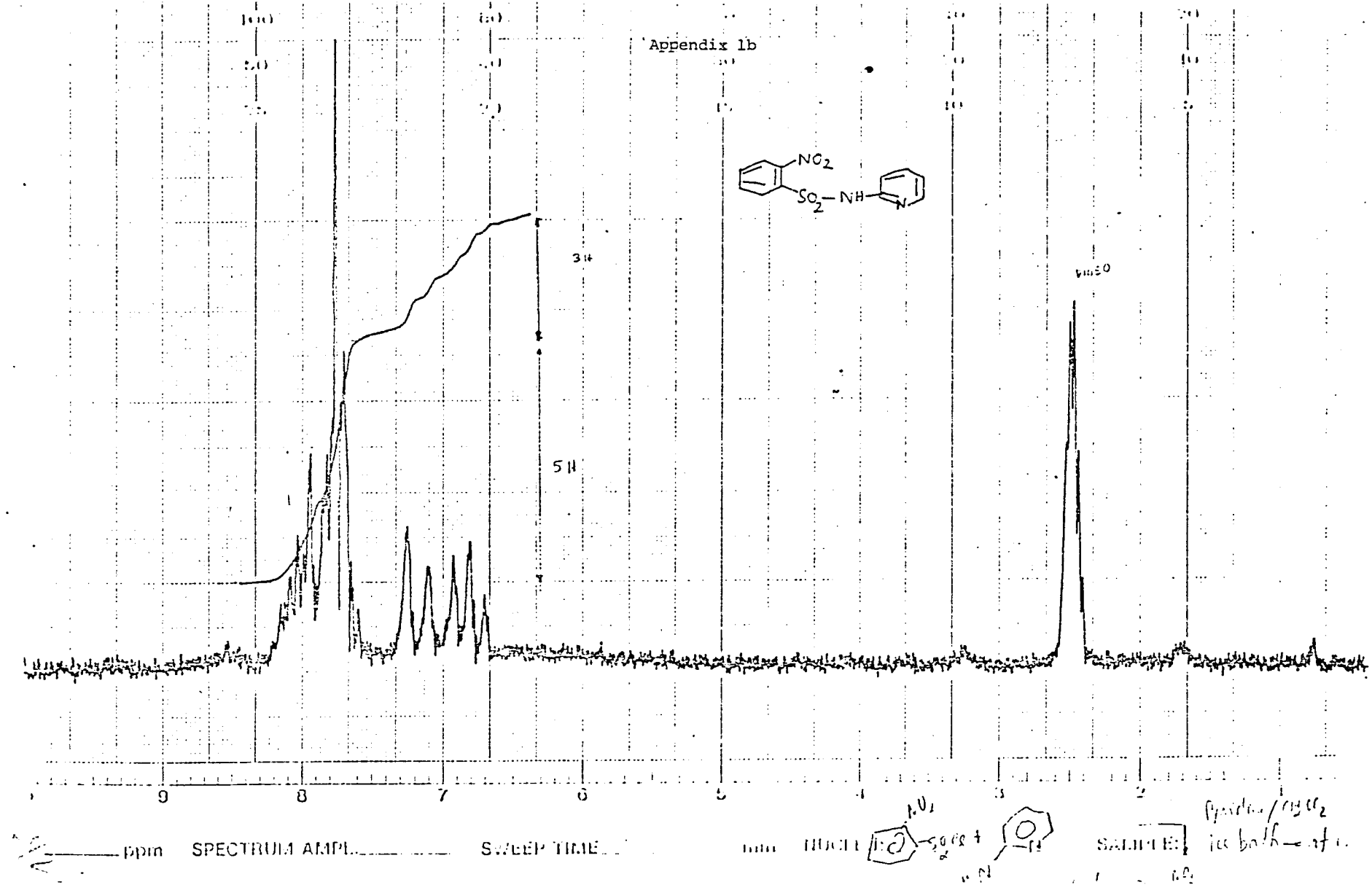
[a] Significant at $P<0.01$ by t-test for difference of means.
[b] Significant at $P<0.5$ by t-test for difference of means.

Washed bull sperm were incubated at 37°C for 30 min in balanced salts solution containing glucose (1 mM) with or without additions as described above. Lactate production was measured as described in METHODS, and inhibition calculated as per cent of controls. S.E.M. values are reported in parentheses.

Appendix 1a

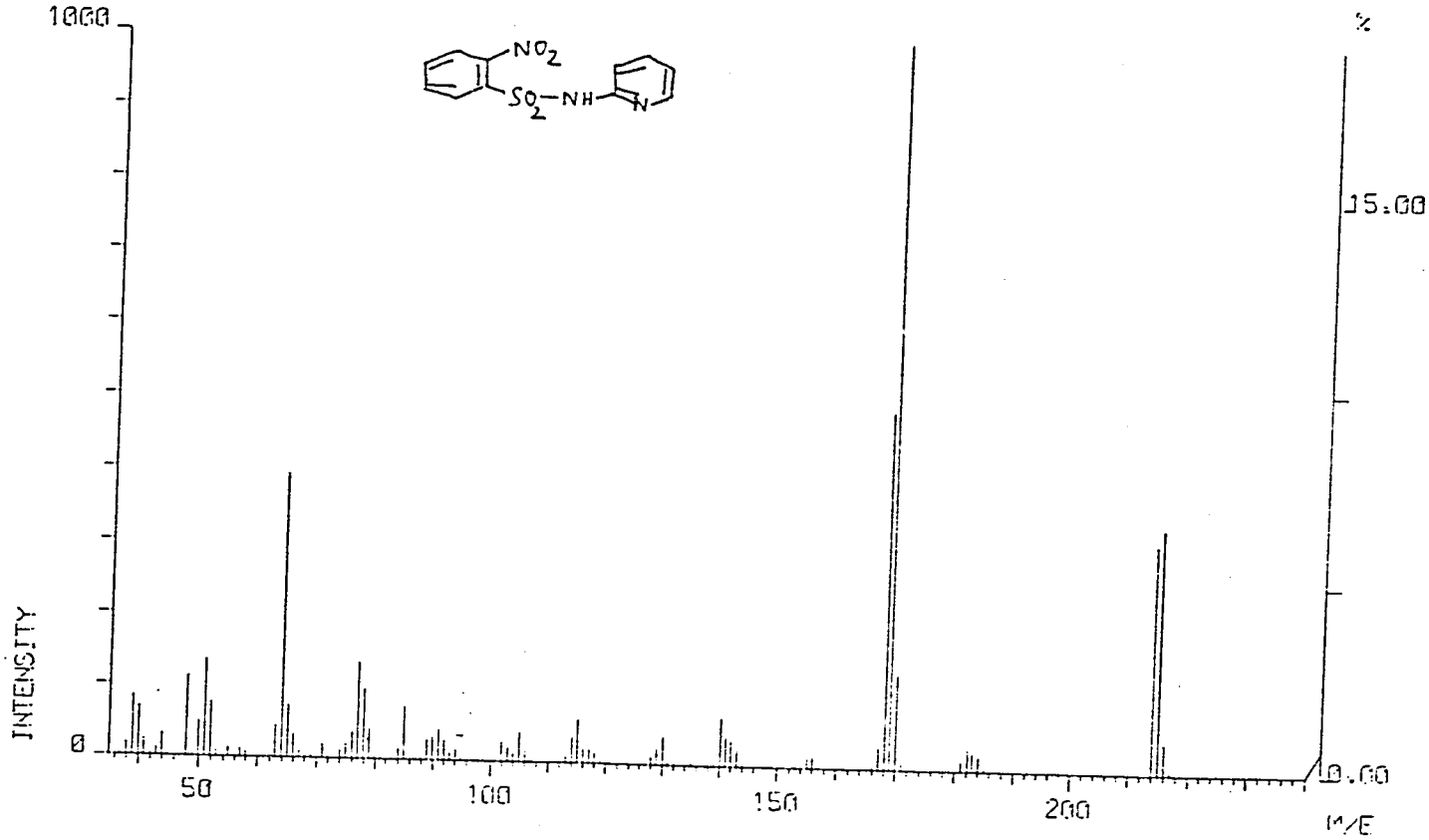

NO2
SO2-NH
DATE
SPECTRAL-CONDITION
1 MAX. SLIT-WIDTH
2 GAIN
3 SCAN SPEED
EXPANSION
ABS
CHART

Appendix 1b

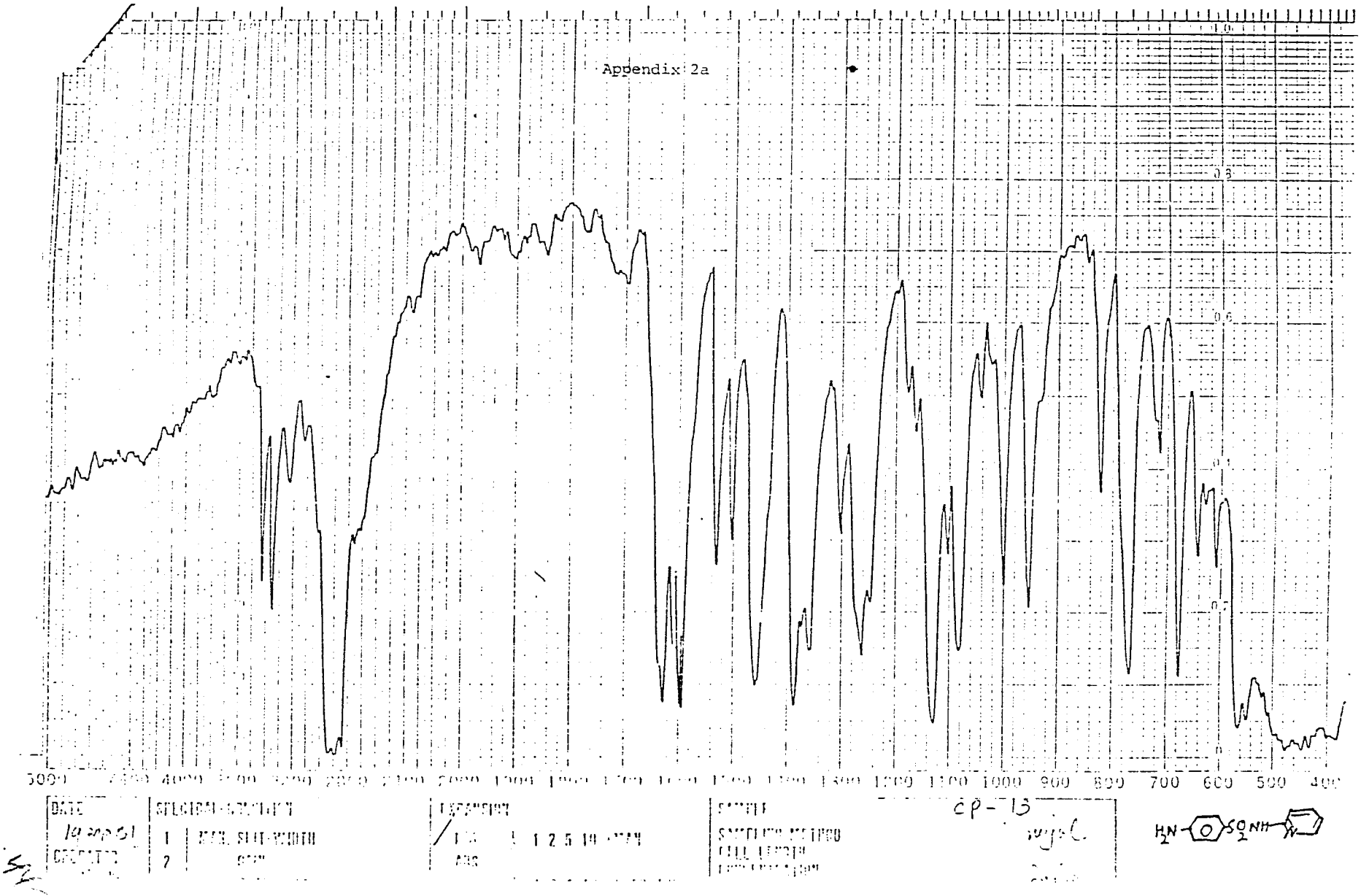


NO2
SO2—NH
N
3H
5H
9
8
7
6
5
4
3
2
ppm
SPECTRUM AMPL
SWEEP TIME
mm
NUCLEI
SAMPLE:

Appendix 1c

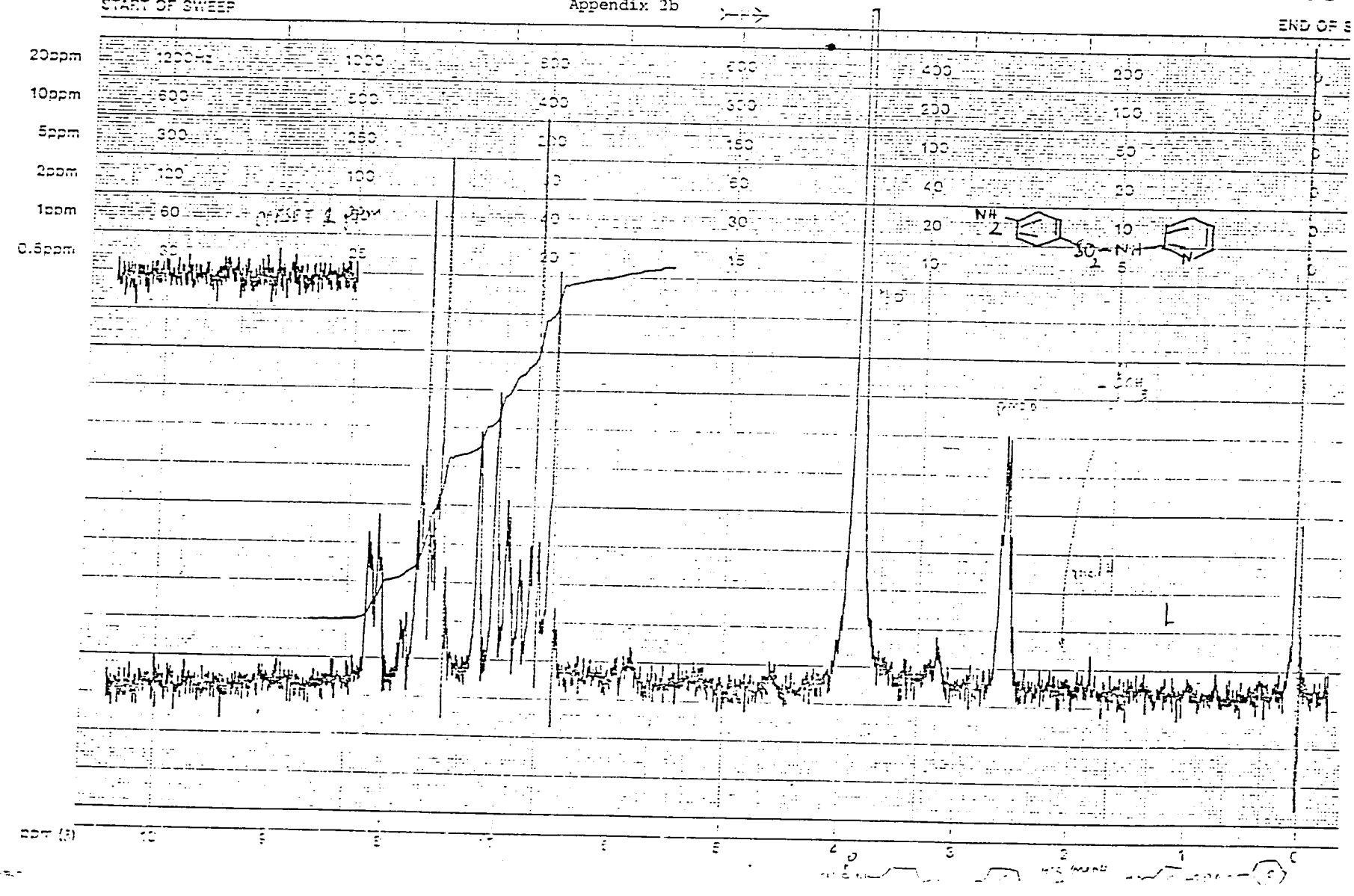


Appendix 2a



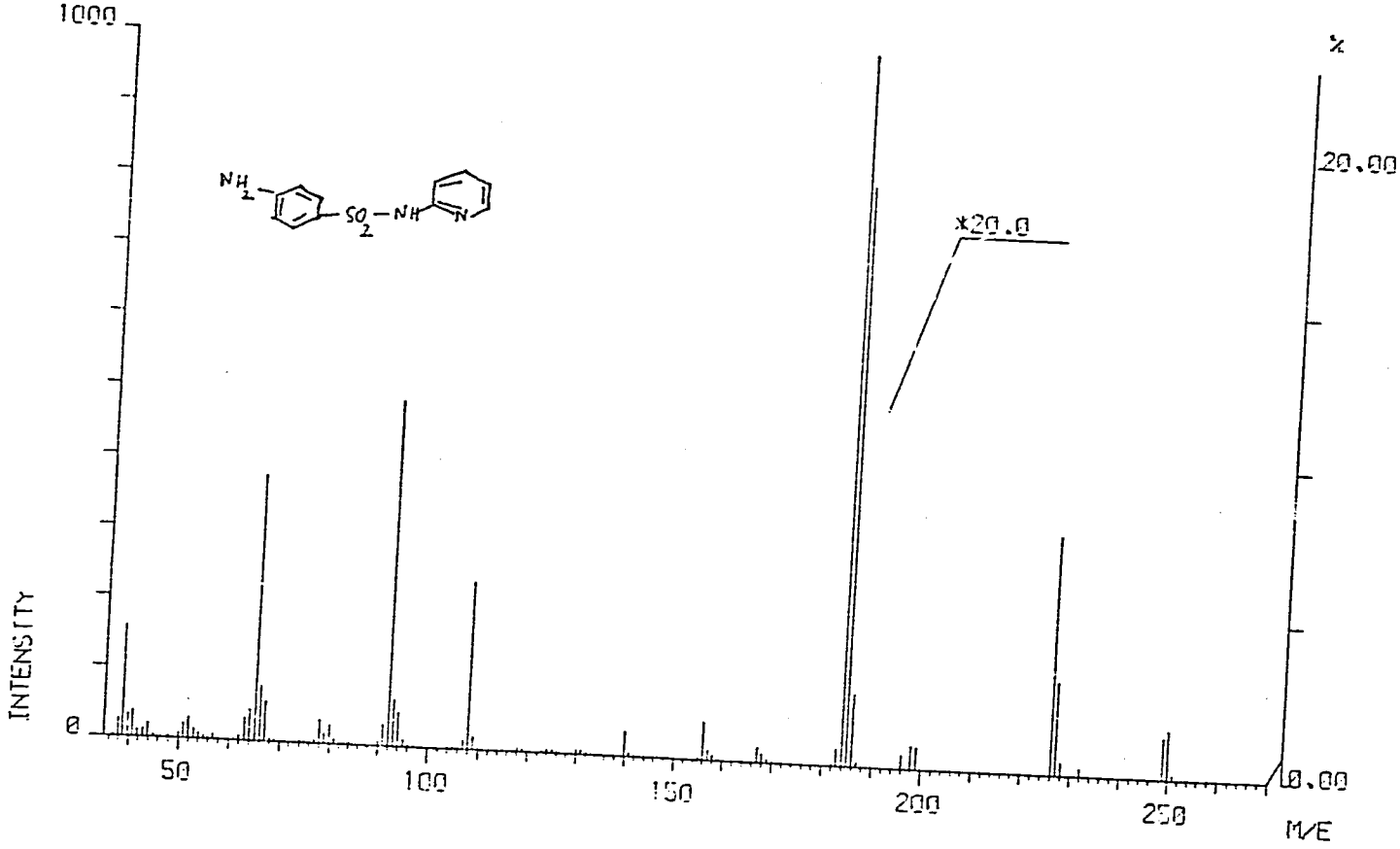
1.0
0.8
0.6
0.4
0.2
0
5000
4000
1000
900
800
700
600
500
400
DATE
SPECIAL-CONDITION
1
2
EXPANSION
1 2 5 10
SAMPLE
SAMPLING METHOD
CP-13
H_2N
SO_2NH

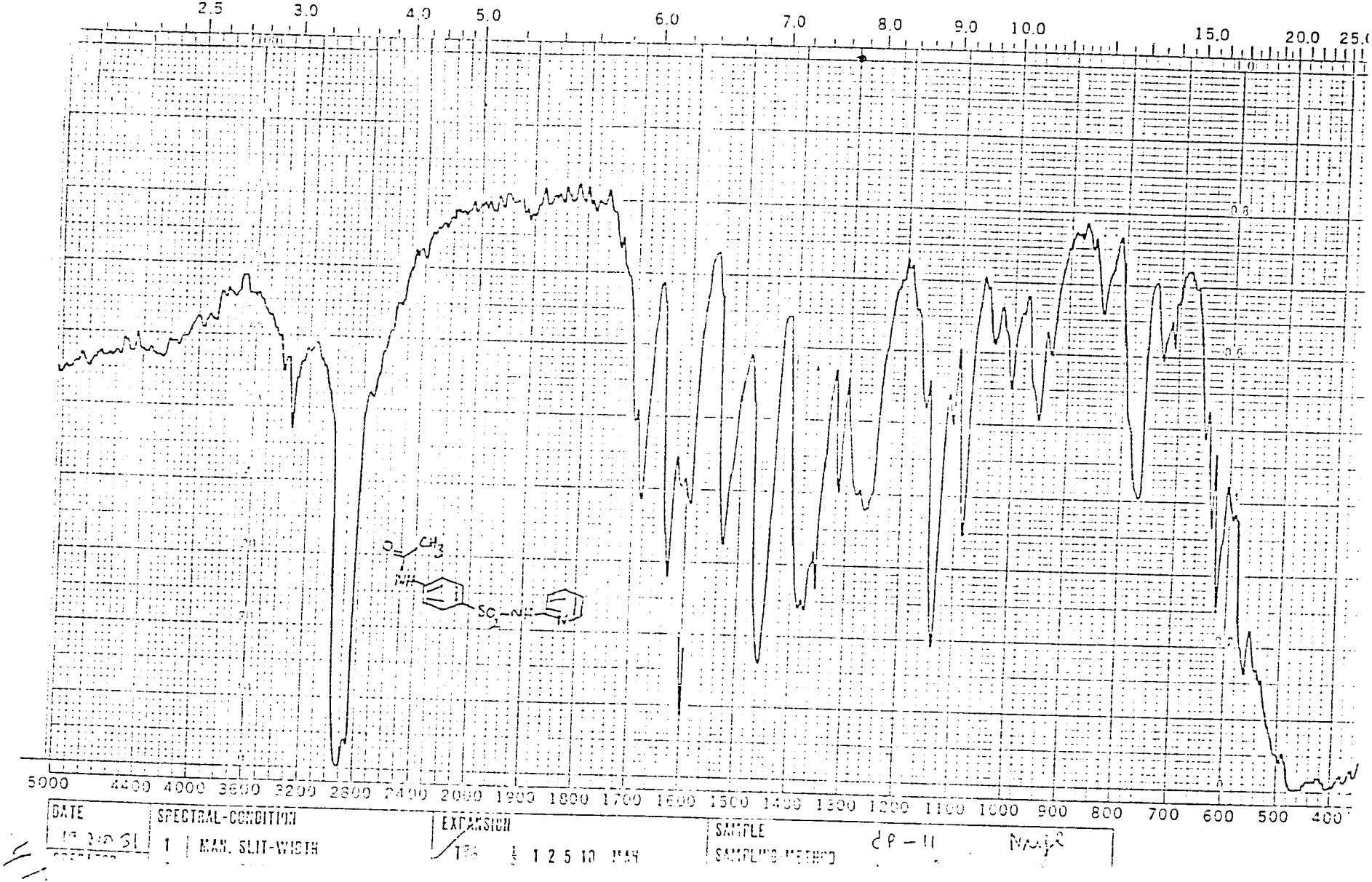
Appendix 2b


START OF SWEEP
END OF S
20ppm
10ppm
5ppm
2ppm
1ppm
0.5ppm
1200Hz
1000
800
600
400
200
600
500
400
300
200
100
300
250
200
150
100
50
120
100
80
60
40
20
60
40
30
20
10
30
25
20
15
10
5
OFFSET 1 ppm
NH2
SO2-NH
N
ppm (δ)
10
9
8
7
6
5
4
3
2
1
0

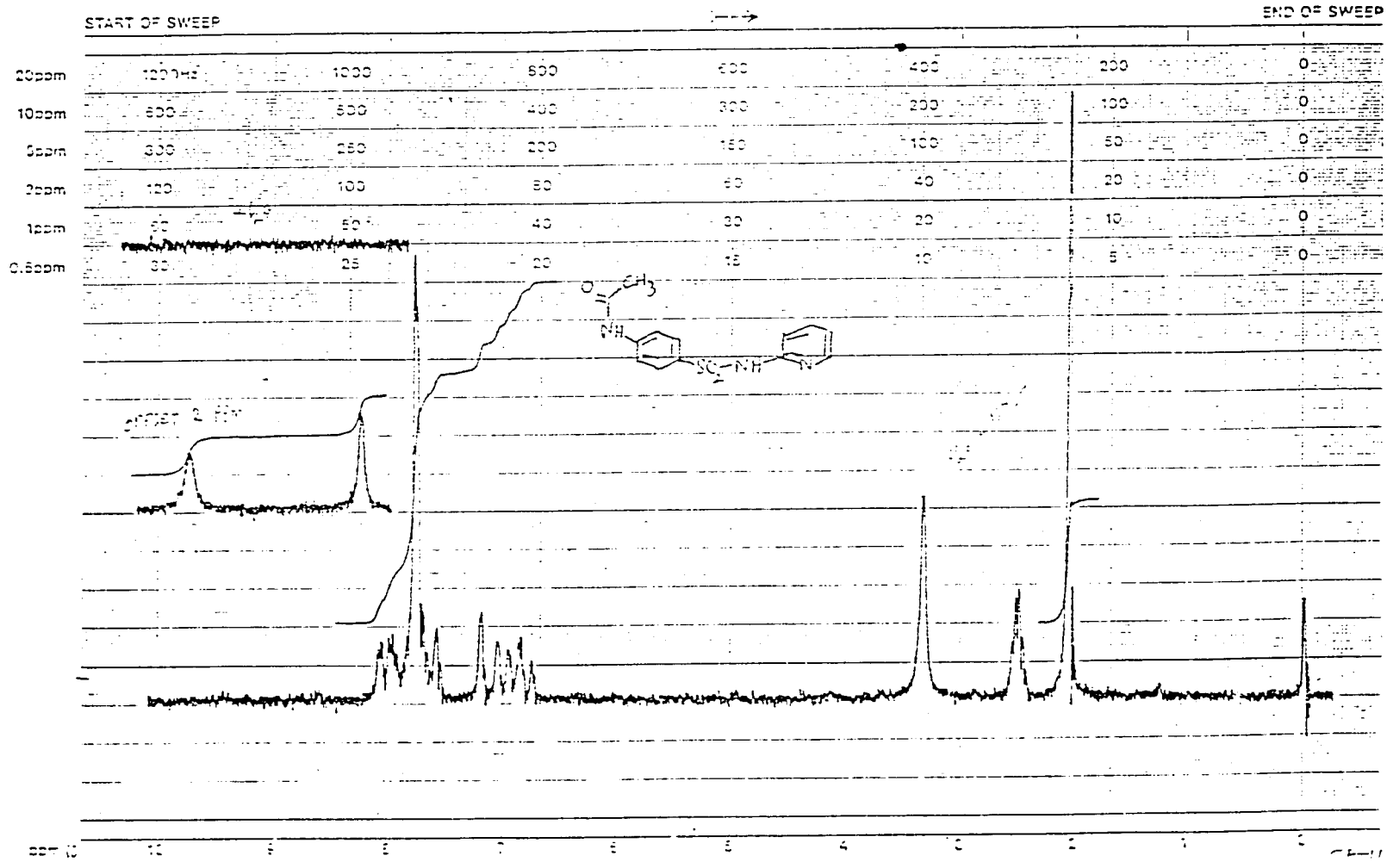
Appendix 2c

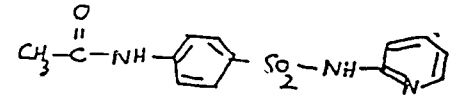
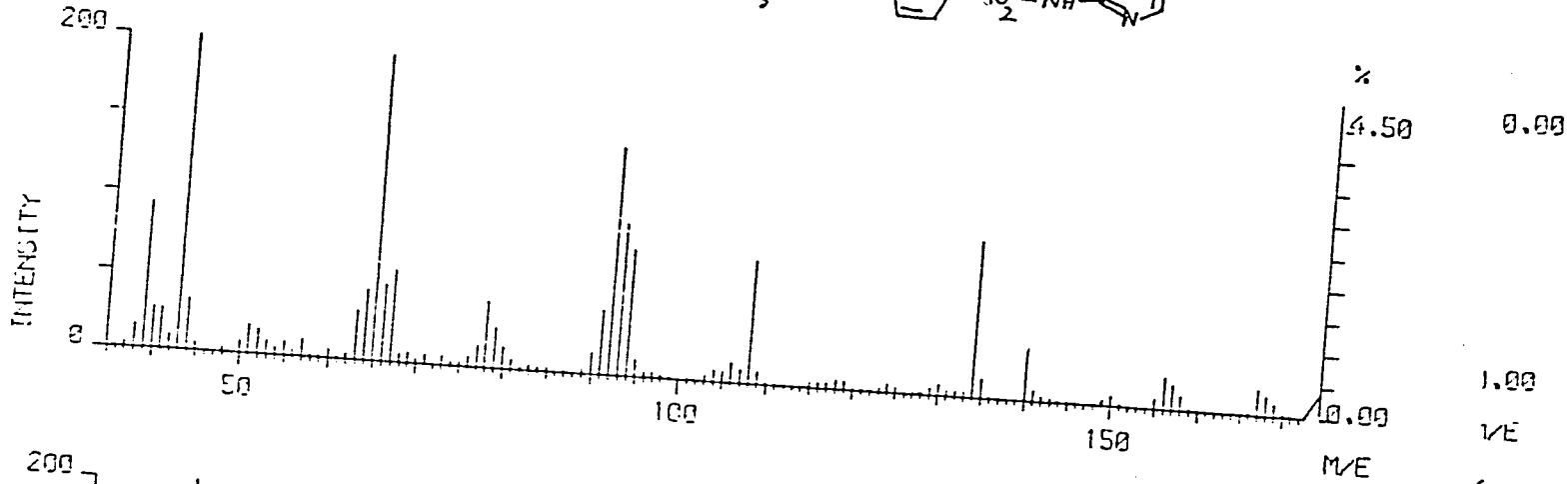
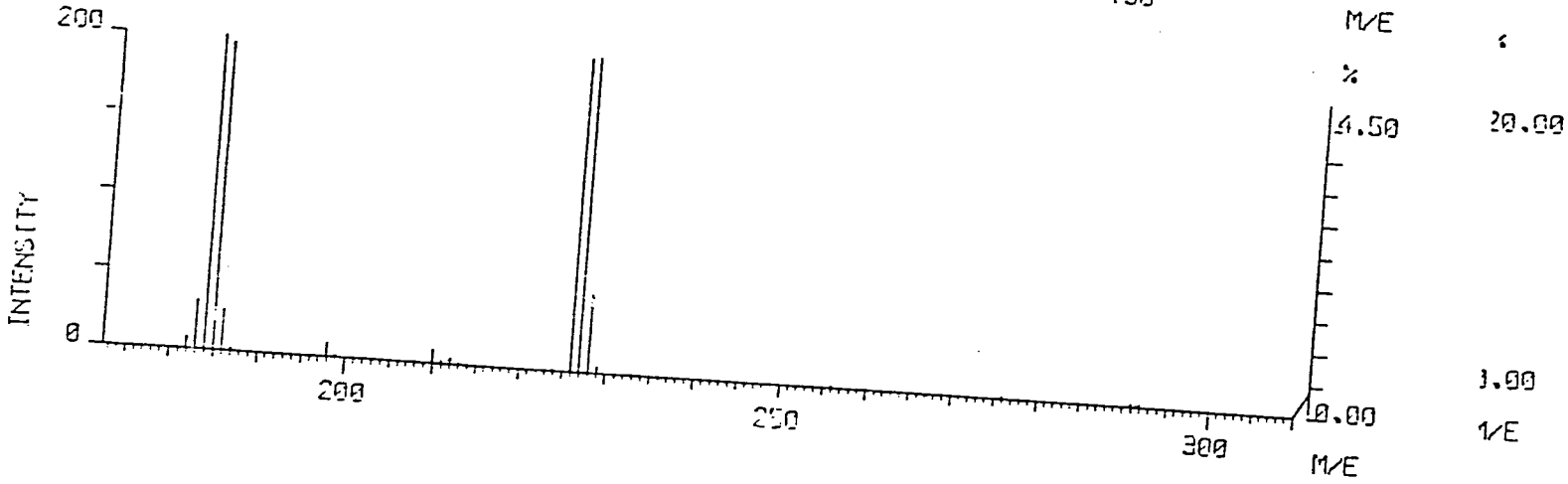
MASS SPECTRUM : (8 TO 11)
SAMPLE:CP-13, M.P.189, 8 MAR 88,
NOTE :21/1,ST,70V,300UA,CHAMB.TEMP.100,
BASE PEAK : M/E 104.0 · INT. 565.9




2.5 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 15.0 20.0 25.0
5000 4400 4000 3600 3200 2800 2400 2000 1900 1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800 700 600 500 400
DATE
SPECTRAL-CONDITION
1 MAN. SLIT-WIDTH
EXPANSION
1 2 5 10 MAN
SAMPLE
SAMPLING METHOD

Appendix 3b


START OF SWEEP
END OF SWEEP
20ppm
10ppm
5ppm
2ppm
1ppm
0.5ppm
ppm
LOCK POS
LOCK POWER
SPECTRUM AMPL
FILTER
SWEEP TIME
SWEEP WIDTH
NUCLEUS
ZERO REF.
SAMPLE:
OPERATOR
DATE
SPECTRUM NO.

MASS SPECTRUM : (9 TO 12)
SAMPLE.CP 11. M.P.289. 8 MAR 88.
NOTE .28/1.EI,70V,300UA.CHAMB.TEMP.170.
BASE PEAK : M/E 225.0 INT. 516.1

Appendix 3c