PN-ABH-627
70837

FINAL REPORT

Covering Period : September 23, 1986 - December 31, 1990

Submitted to the Office of the Science Advisor

U.S. Agency for International Development

SULFONAMIDE DRUGS AS A MALE ANTIFERTILITY AGENT

Principal Investigator : Chumpol Pholpramool

Grantee Institution : Mahidol University

Collaborator : Lawrence M. Lewin

Institution : Tel Aviv University

Project Number : C7-169

Grant Number : 936-5544-G-00-6105-00

A.I.D. Grant Project Officer : Win McKeithen

Project Duration : September 23, 1986 - December 31, 1990

TABLE OF CONTENTS

Executive Summary 1

Research Objectives 3

Methods and Results

1. Synthesis of sulfonamide compounds 7

2. Antifertility activities of the test compounds ... 7

3. Mode of action of sulfonamides 12

3.1 Effects on the pituitary-gonadal axis 12

3.2 Effects on the testicular production of spermatozoa 12

3.3 Effects on epididymal maturation of spermatozoa 13

3.4 Effects on folate metabolism 16

Impact, Relevance and Technology Transfer 17

Project Activities/Outputs 18

Project Productivity 23

Future Work 23

Literature Cited 24

Appendix 26

EXECUTIVE SUMMARY

The purposes of this research project are : (1) to synthesize a series of analogs of sulfa drugs, (2) to test the antifertility activities of these compounds in the male rats, and (3) to develop and standardize methods to be used for the study of the mode of action of the test compounds on male fertility.

The technical staff involved in this project represents a good blend of organic chemists, biochemists and biologists. The Thai organic chemists were responsible for planning and synthesizing sulfonamide analogs for biological testings. The Israeli biochemists were mainly involved in the development and standardization of methods for the studying of functions of the male reproductive organs. Both the Thai and the Israeli biologists performed parallel fertility testings and the study of the mode of action of the test compounds.

As a results of the good team work, 13 compounds have been synthesized and tested for biological activities. Eight sulfa drugs, which are commercially available, were also tested. Of these, 9 synthetic and 4 sulfa drugs were found effective in the suppression of male fertiltiy. The basic structural requirement for the antifertility activity has been revealed. Several methods have been developed, and some of these were found useful for the study of the mode of action of sulfa drugs. It appears that the reduction in fertility induced by sulfonamides is not associated with changes in the hormone levels nor the rate of sperm production. In addition, these compounds do not interfere with the function of the epididymis. The

results suggest that the late stage of sperm modelling in the testis is affected leading to a reduction in sperm motility and fertilizing ability.

The results in this study provide an alternative approach for the development of new methods for male contraception, which are acutely needed especially by developing countries. Although, in view of its mode of action, this group of compounds is attractive as a male contraceptive, its potency is still not very high, i.e. a dose of a few hundred mg/kg is required. Further studies are required to search for more potent compounds and then clinical studies are warranted.

This collaborative project has created a close interaction between the Thai and Israeli scientists, and will undoubtedly lead to future cooperative work. Several techniques and skills for the study of male fertility transferred to and acquired by the Thai collaborators during the granting period will enable the Thai researchers to carry on further studies along this line.

RESEARCH OBJECTIVES

The overall aim of this project is to develop methods for investigation of the mode of action of compounds suitable for use as contraceptives in the male, and to adopt these developed methods to evaluate the antifertility action of sulfonamide drugs and their analogs.

It is generally agreed that an optimal size of population with good quality is a prerequisite for a successful development of a country. This basic requirement is hardly met in the less developed countries where the problem of over population exists. Therefore, the need for birth control as a means of controlling population size in these countries is of prime importance. Other forms of international assistance to help developing the overpopulated countries will be less effective unless the overgrowth of population is under control. At present, Thailand is still threatened by an overgrowth of her population projected over the next ten years. The results from this project with regard to the development of the methods will provide a direct practical application for use in Thailand. Furthermore, results from the studies on the structure-activity relationship and the mode of action of potential contraceptive agents will be essential guidelines for the development of new contraceptive agents with a known specific action and lowest risk of undesirable effects.

Recent attempts to control fertility in the male have been focussed on three major areas : (a) disruption of spermatogenesis either by a direct action on the testis or

indirectly by altering normal levels of gonadotrophin or testosterone. (b) interference with sperm maturation in the epididymis, and (c) interruption of sperm transport into the female genital tract. At present, the progress of these approaches, except in (c), has not reached the stage wherewidespread applications are recommended. Moreover, intensive studies of the currently available methods reveal more problems or negative aspects of the prospective methods (1). Therefore, the need for new methods for fertility regulation in the male is still acute.

During the past decade there have been an increasing numbers of clinical reports of the side-effect of sulfasalazine, which is generally used to treat ulcerative colitis, on fertility of the male patients (2-4). These patients who have history of normal fertility before the onset of drug treatment became infertile shortly, within 2-3 months, after the treatment. Normal fertility resumes soon after drug withdrawal. It appears that this infertility is not associated with changes in blood gonadotrophin or testosterone, but may be related to reduced sperm counts, defective sperm motility and a high number of abnormal sperm morphology (2-4). Recent work on the male rats by Levi's and our groups have demonstrated similar effects as in man (5,6). In addition, preliminary studies on sulfasalazine metabolites, i.e. sulfapyridine and 5-aminosalicylic acid formed after breakdown of sulfasalazine by intestinal micro-organisms, showed that sulfapyridine, but not 5-aminosalicylate, also suppressed fertility in the male rats (5, 7). Besides, sulfamethizole in which the pyridine group of sulfapyridine is

replaced by a methylthiadiazole group had virtually no effect (5). Possible effects of other sulfonamides on the male reproductive system are largely unknown.

The mode of action of sulfasalazine (and sulfapyridine) on the male reproductive system is presently unclear. Although previous works reported normal blood levels of gonadotrophin and testosterone in patients receiving sulfasalazine treatment, more recent studies showed that plasma FSH (8) or LH (9) was elevated whereas plasma testosterone (7,9) was depressed by this drug. On the other hand, the pituitary reserve and its secretion in response to GnRH or TRH were normal (9). This suggests that normal functions of the pituitary gland are still intact. The conflict in the reported level of circulating gonadotrophin in these patients could possibly be due to diurnal variations of these hormones. Thus, the possible effects of the sulfonamides on blood LH, FSH and testosterone require further well-controlled studies. However, the findings that this drug causes changes in spermiogram, i.e. oligozoospermia, poor motility and a high number of abnormal head forms, without changes in normal histology of the testis suggest that sulfasalazine, and possibly its analogs, may exert its action on the late spermatogenesis or the maturation of spermatozoa in the epididymis, or at other distal sites. If this hypothesis is true, further studies of this group of drug should prove fruitful in terms of future development of a new contraceptive agent with a rapid onset of the effect and recovery, more specific site of action and a lesser chance for inducing genetic aberrations of the gametes. The idea to develop sulfonamide drugs as contraceptive agents is

novel. The possibility that other well known sulfonamides as well as new compounds with related structure may also have antifertility activity is highly likely. The results should stimulate subsequent studies to cover the whole spectrum of these drugs. Furthermore, if their mode of action is known, e.g. at the epididymal maturation of spermatozoa, this group of compounds would have more advantage than others which act at other sites.

This project was also supported, in part, by research grants from Mahidol University and Chulabhorn Research Institute during the later phase of study.

METHODS AND RESULTS

1. Synthesis of sulfonamide compounds

Thirteen sulfonamides were synthesized from the simple reaction between appropriate benzene sulfonyl chlorides and amines. The optimal conditions for the preparative scale were also simultaneously investigated. Some of the compounds synthesized have structure slightly modified from those previously proposed. This is primarily based on the analysis of the structure activity relationship (see Table 1).

2. Antifertility activities of the test compounds

In addition to the 13 synthetic compounds, 8 sulfa drugs purchased from Sigma Co. (U.S.A.) were also tested for antifertility activities. The adult male rats of proven fertility were fed either by intragastric intubation or with the pellet feed impregnated with the sulfa drug at doses of 150 or 450 mg/kg/day for 6 weeks. During the treatment period the treated males were allowed to mate with untreated female rats and pregnancy was determined later. Control rats were fed with corn oil or untreated pellet feed and the mating sceduleg were similar to the treated group.

It was found that 9 of the synthetic compounds and 4 sulfa drugs were able to significantly decrease the number of fetuses of the impregnated females. The effect occurred within 1-3 weeks during treatment and normal fertility resumed by 1-3 week after the cessation of treatment. Table 1 summarizes the antifertility activity of all compounds tested in this study. It

Table 1 Nomenclatures and chemical structures of the compounds synthesized and tested for antifertility activities.

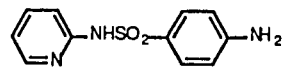
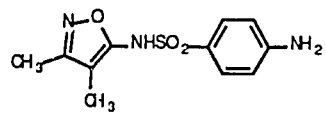
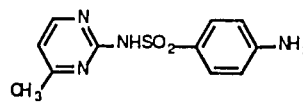
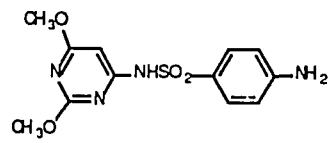
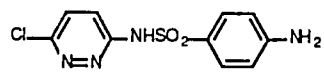
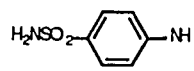
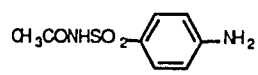
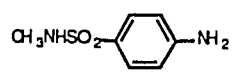
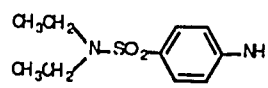
Compound	Chemical name/Generic name*	Chemical Structure	Antifertility Activity
I	Sulphapyridine		+ + +
II	Sulphioxazole		0
III	Sulphamerazine		+
VI	Sulphadimethoxine		0
V	Sulphachloropyridazine		+
VI	Sulphanilamide		+ + +
VII	Sulphacetamide		+ +
VIII	N^1-Methylsulphanilamide		+ + +
IX	N^1-Diethylsulphanilamide		+ + +

Table 1 (continued)

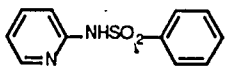
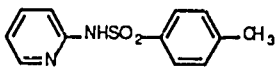
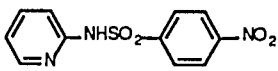
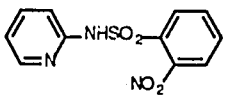
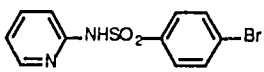
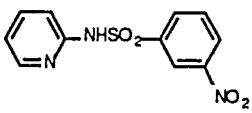
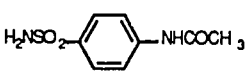
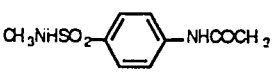
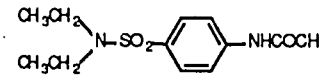
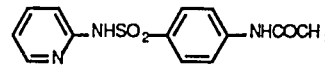
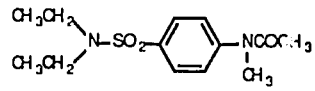
Compound	Chemical Name/Generic Name*	Chemical Structure	Antifertility Activity
X	N-2-Pyridinylbenzene sulphonamide		0
XI	4-Methyl-N-2-pyridinyl-benzenesulphonamide		0
XII	4-Nitro-N-2-pyridinyl-benzenesulphonamide		+ +
XIII	2-Nitro-N-2-pyridinyl-benzenesulphonamide		0
XIV	4-Bromo-N-2-pyridinyl-benzenesulphonamide		+
XV	3-Nitro-N-2-pyridinyl-benzenesulphonamide		0
XVI	N^4-Acetyl sulphanilamide		+ +
XVII	N^4-Acetyl-N^1-methyl-sulphanilamide		+ + +

Table 1 (continued)

Compound	Chemical Name/Generic Name*	Chemical Structure	Antifertility Activity
XVIII	N^4-Acetyl-N^1-diethyl-sulphanilamide		+ + +
XIX	2-(N^4-Acetylsulphanilamido) pyridine		+ + +
XX	N^4-Acetyl-methyl-N^1-diethyl-sulphanilamide		+ + +

* Generic names are used for the compounds I to VII, which are not synthesized in this study.

+ + +, strong activity; + +, moderate activity; +, weak activity; 0, no activity

appears that the most simple sulfa drug, sulfanilamide, or its alkyl derivatives are as effective as sulfapyridine. Unfortunately, we were unable to find more potent compounds which are nontoxic. When the structures of all test compounds were analysed in relation to their biological activities, the basic structural requirement for the antifertility activity was obtained (Fig. 1). This part of the work was accepted for publication in the journal of Reproduction and Fertility in November 15, 1990. (See Appendix III).

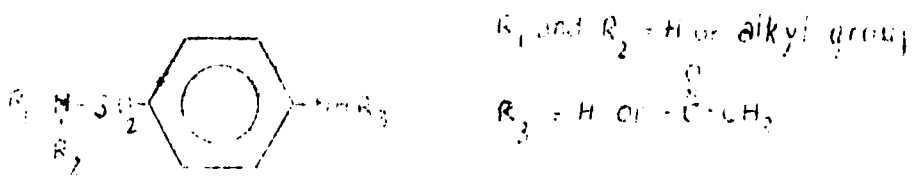


Fig. 1. Basic structure of the antifertility sulfonamide.

Since complete infertility of all male rats in a group treated with a single sulfonamide has not yet been achieved and it is known that sulfa drugs are more effective when given in combination, the effect of combined antifertility sulfonamides (sulfapyridine + sulfanilamide) were studied. Both drugs were fed to the animals at various combinations and doses for 6 weeks and fertility was tested as in other experiments. A marked reduction in fertility (almost complete infertility)was noted in the groups treated with combined drugs. Such a decrease was more

than that accounted for by doubling the dose of a single drugs. The data suggest the potentiation effect of these drugs. Due to the limit of time other combinations of sulfonamides have not been studied.

3. Mode of action of sulfonamides

In order to understand the mode of action of the test compounds on the male reproductive system, a series of experiments were performed to delineate the site and mechanism of action of these compounds using sulfapyridine or sulfanilamide as a representative drug. The details of each experiment had been described in the previous progress reports. The followings will summarize the major approaches used to achieve this objective.

3.1 Effects on the pituitary-gonadal axis

Sulfapyridine given at the dose which significantly decreased fertility of the male rats failed to alter blood levels of LH and testosterone. In addition, the responsiveness of the pituitary-gonadal axis to LHRH was not virtually changed. This part of results has been reported at the IVth International Congress of Andrology in Florence, May 14-18, 1989 (see Appendix II).

3.2 Effects on the testicular production of spermatozoa

Histological studies of the testis of the sulfapyridine- or sulfanilamide-treated animals revealed no difference from the controls. Furthermore, direct assessment of the daily sperm production rate supports the histological findings. Thus, sulfapyridine had no effect on the rate of spermatogenesis. The

results have been included in the report at the International Congress of Andrology in Florence in 1989 (see Appendix II).

3.3 Effects on epididymal maturation of spermatozoa

Previous experiments suggest that the site of action of sulfapyridine is most likely post-testicular. The possible action on the epididymal sperm maturation was then investigated by using the methods developed by the Israeli collaborators.

Both sulfapyridine and sulfanilamide markedly decreased motility of the spermatozoa removed from the epididymis and from the ejaculates as well as sperm concentration in the epididymis. In addition, vital staining of the epididymal spermatozoa showed an increase in the number of dead sperm. However, there is no evidence of an increase in immature sperm in the cauda epididymidis based on morphological studies of the cytoplasmic droplets. On the other hand, the electron micrographs of sulfa-treated epididymal sperm revealed a disruption of the contractile elements of a substantial number of spermatozoa. The effect resembles that of gossypol reported previously (10). In contrast to sperm motility, sulfonamides had virtually no effect on sperm respiration both in vivo and in vitro. The results on the in vitro effects had been published in the Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination (see Appendix I).

Rat epididymis actively accumulates carnitine from the blood and secretes it into the epididymal lumen (11). The involvement of carnitine in sperm motility and fertility is also well documented (11). Therefore, the effect of sulfa drugs on the

accumulation of radiolabelled carnitine into the epididymis was investigated. It was found that the uptake of carnitine by the sulfa-treated epididymis was not different from controls. It is concluded that this function of the epididymis is not affected.

During the later period of study, we have determined the composition of the epididymal fluids collected by micropuncture with respect to electrolytes and proteins. Potassium concentration was significantly elevated in the sulfapyridine-treated rats whereas sodium concentration was not changed (Fig. 2). It is not known whether this is due to the leakage of intracellular potassium from the dead sperm or the enhancement of potassium secretion by the epididymal epithelium. Analysis of the fluid as well as sperm surface proteins by SDS polyacrylamide gel electrophoresis showed changes in the pattern of proteins in both fluids removed from the proximal and distal segments of the epididymis. Furthermore, the sperm extracts from the distal region of the sulfa-treated epididymis resembled those from the proximal regions of the control and sulfa-treated epididymis indicating the lack of modification of the sperm surface proteins during their transit through the epididymis.

Measurements of the rate of sperm transport through the epididymis by the radiolabelling method developed in our laboratory failed to demonstrate any change after treatment with sulfa drugs. Thus, the reduction in epididymal sperm reserve cannot be explained by changes in the sperm transit time.

Close inspection of our fertility data indicated that some rats in the sulfa-treated group were resistant to the antifertility activity whereas others were very responsive.

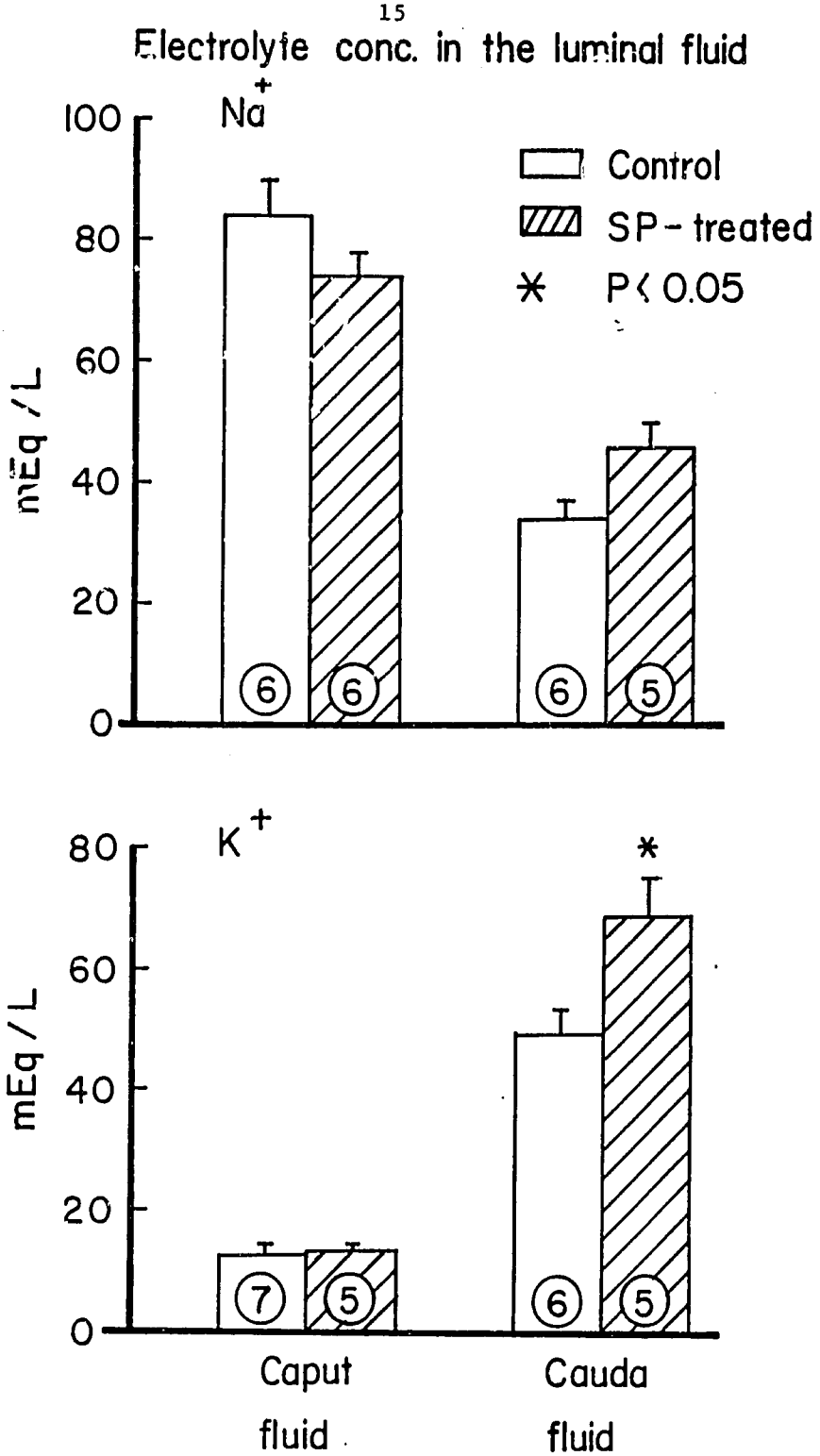


Figure 2. Electrolyte concentrations in the luminal fluid of the caput and cauda epididymidis of control (□) and sulfapyridine-treated (▨) rats. Sulfapyridine was given by intragastric tubing at a dose of 450 mg/kg/day for 5 weeks. Numbers in the columns are number of rats in each group.

Since the effectiveness of both acetylated and non-acetylated sulfanomide, which are interconvertible in the body, were indifferent when given orally (Table 1), the actual active form is unclear. To answer this question we analyzed various forms of sulfapyridine and sulfanilamide metabolites in the excreta of treated rats by using both HPLC and spectrophotometry. In addition, the potency of the acetylated and non-acetylated sulfonamides administered by oral versus parenteral routes were compared. The results showed that the animals which were most active in acetylating the compound were most fertile. This suggests that the acetylated form is inactive or less active in compromising fertility of male rats. On the other hand, recent experiments indicate that acetylsulfapyridine was more effective when subcutaneously injected compared to oral feeding. Metabolic studies of acetylsulfanilamide suggest that this compound was not deacetylated to any large extent either in its passage through the digestive tract nor in the rat itself. However, the possibility still remains that the derivatives are deacetylated by an inducible enzyme system when they are administered for a long period of time. Therefore, the question concerning the active form of antifertility sulfonamides is yet unanswered.

3.4 Effects on folate metabolism

Since sulfasalazine has been known to inhibit folate absorption by the intestine (12) as well as folate enzyme *in vitro* (13), the possibility that the antifertility sulfonamides exert their effects through the intervention of folate metabolism was investigated. The antifertility activity of the antifolate reductase inhibitors such as trimethoprim and pyrimethamine alone

or in combination with sulfanilamide were studies. In addition, blood and tissue levels of folic acid were measured in the control and sulfa-treated animals. The results showed virtually no effect on the folate metabolism of the male reproductive organs nor the animal itself. These results has been published in the journal CONTRACEPTION (see Appendix IV).

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

This study has demonstrated that there are a number of sulfonamide compounds which are effective antifertility agents in the male rats. The compound has several advantages for further development as male contraceptives. It opens up the possibility for new method in male fertility regulation, which are acutely needed in the developing countries. At present, the findings in this project have no direct application. However, once the final clinical studies are complete, the new contraceptive agent can then be recommended for widespread use. If the final development of this compound as a male contraceptive agent is successful, the benefits will not be limited only to the developing countries but also to the other communities of the world. This is because the problem of overpopulation is of worldwide concerns.

On the other hand, the project has a direct impact on the investigators themselves especially the Thai scientists. Since the amount of funding is sizable, both laboratories in Bangkok and in Tel Aviv were able to purchase some major equipment and laboratory supplies necessary for high quality

research work. These together with the exchanges of techniques and skills obtained from training will enable both laboratries to carry on the collaborative work of standard quality. It is hoped that major findings will be discovered by us in the future joint venture.

PROJECT ACTIVITIES/OUTPUTS

1. Attendance at scientific meetings

During the granting period, both the Thai and the Israeli investigators attended and presented papers or gave lectures on the work resulted from this study at both local and international scientific meetings, which are listed below :

1.1 By Thai investigators

1.1.1 Conference of USAID Science Award Grantees, 24-26 July 1987, Nakorn Pathom, Thailand.

1.1.2 17th Annual Meeting of the Physiological Society of Thailand, 28-30 April 1988, Bangkok, Thailand.

1.1.3 18th Annual Meeting of the Physiological Society of Thailand 27-29 April 1989, Chiangmai, Thailand.

1.1.4 IVth International Congress of Andrology 14-18 May, 1989, Florence, Italy.

1.1.5 XXXI International Congress of Physiological Sciences, 9-14 July, 1989, Helsinki, Finland.

1.1.6 19th Annual Meeting of the Physiological Society of Thailand, 3-5 Apirl, 1990, Rayong, Thailand.

1.1.7 16th Conference on Science and Technology of Thailand, 25-26 October, 1990, Bangkok, Thailand.

1.1.8 Second Congress of the Asian and Oceanian Physiological Societies, 12-15 November, 1990, New Delhi, India.

1.2 By the Israeli Investigators

1.2.1 International Congress on Animal Reproduction, 26-30 June, 1988, Dublin, Ireland.

1.2.2 Gordon Conference on Reproductive Tract Biology, 3-8 July, 1988, New Hampshire, U.S.A.

1.2.3 25th National Congress of the Israel Society of Clinical Biochemistry, 28-30 March, 1990, Tiberias, Israel.

1.2.4 Annual meeting of Israel Fertility Society, 1990, Israel.

1.2.5 Annual Meeting of the Israel Microbiology Society, 1990, Israel.

1.2.6 Gordon Conference on Reproductive Tract Biology, 9-13 July, 1990, New Hampshire, U.S.A.

2. Training

The Israeli co-investigator (Prof. L.M. Lewin) made two visits to Bangkok in 1988 and 1989 in order to discuss the work plan of the project and train the Thai collaborators the techniques and methods developed in Israel.

3. Publications

Publications of the results of this research project include :

3.1 Full papers

3.1.1 Lewin, L.M., Golan, R., Wassercug, O. & Pholpramool, C. The effects of sulfasalazine, sulfapyridine, and 5-aminosalicylate on glycolysis of bull sperm. Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination. June 26-30, 1988, Dublin, Ireland.

3.1.2 Pholpramool, C., Verawatnapakul, V., Srikhao, A., Ruchirawat, S. & Lewin, L.M. Lack of effects of some antifertility sulfonamides on testicular functions in the male rats. Mini-Posters IVth International Congress of Andrology, May 14-18, 1989, p. 27-31. Florence, Italy.

3.1.3 Pholpramool, C. Ruchirawat, S. Verawatnapakul, V. Paovalo, C. & Lewin, L.M. Structural requirements of some sulphonamides that possess an antifertility activity in the male rat. J. Reprod. Fert. 1990 (Accepted for publication).

3.1.4 Pholpramool, C., Verawatnapakul, V., Ruchirawat, S. & Lewin, L.M. Mode of action of the antifertility sulphonamides : Lack of effect on folate metabolism. Contraception, 42 (6), 667-675, 1990.

3.1.5 Pholpramool, C., Ruchirawat, S., Paovalo, C., Verawatnapakul, V. & Lewin, L.M. Suppression of male fertility by sulpha drugs. Proceedings of the 2nd Congress of the Asian and Oceanian Physiological Societies, November 12-15, 1990, New Delhi, India.

3.1.6 Golan, R., Wassercug, O., Freidlin, P., Pholpramool, C. & Lewin, L.M. Bioautographic detection of sulfa drugs and their derivatives using bacillus stearothermophilus. J. Clin. Chem. Clin. Biochem. 1990 (Submitted for publication).

3.1.7 Weissenberg, R., Pholpramool, C. & Lewin, L.M. Antifertility effects of sulfapyridine on male hamsters. Andrologia. 1990 (Submitted for publication).

3.1.8 Wannanonth, P., Lewin, L.M. & Pholpramool, C. Antifertility sulfonamides and inhibition of carbonic anhydrase enzyme in the rat epididymis. (In preparation).

3.1.9 Bunnag, D. Verawatnapakul, V., Lewin, L.M. & Pholpramool, C. The effect of sulfapyridine and alpha-chlorohydrin on epididymal fluid electrolytes and sperm surface proteins. (In preparation).

3.1.10 Parapong, S., Lewin L.M. & Pholpramool, C. The effect of sulfanilamide on the structure and motility of spermatozoa (In preparation).

3.2 Abstracts

3.2.1 Pholpramool, C. Verawatnapakul, V., Ruchirawat, S. & Lewin L.M. Lack of effects of some male antifertility sulfonamides on folate metabolism in the rats. Presented at the XXXI International Congress of Physiological Sciences, July 9-14, 1989.

3.2.2 Pholpramool, C., Ruchirawat, S., Verawatnapakul, V., Srikhao, A. & Lewin L.M. Intervention of sperm maturation by sulpha drugs. Presented at the 16th Conference on Science and Technology of Thailand, October 25-27, 1990.

3.2.3 Weissenberg, R., Pholpramool, C. & Lewin, L.M. The influence of sulfapyridine on male hamster fertility. Presented at the Annual Meeting of Israel Fertility Society, 1990.

3.2.4 Golan, R., Wassercug, O., Freidlin, F., Pholpramool, C. & Lewin, L.M. Bioautographic detection of sulfa drug and their derivatives in drug preparations and in urine. Presented at the 25th National Congress of the Israel Society of Clinical Biochemistry, March 28-30, 1990.

3.2.5 Lewin, L.M., Pholpramool, C. & Vree, T.B. Sulfapyridine metabolites in urine : effectiveness of sulfapyridine as a Male contraceptive. To be presented at the 26th National Congress of the Israel Society of Clinical Biochemistry, 1991.

3.2.6 Lewin, L.M., Pholpramool, C., Ruchirawat, S., Verawatnapakul, V. & Paovalo, C. Structural requirements for male infertility action of sulfonamides. To be presented at the Annual Meeting of Israel Fertility Society, 1991.

PROJECT PRODUCTIVITY

The propose goals of this research project are : (1) to synthesize thirteen sulfapyridine analogs, (2) to test the antifertility activities of all synthetic compounds and 8 commercially available sulfa drugs, and (3) to develop methods for the study of the mode of action of sulfonamides on male fertility. Therefore, we have completely achieved all proposed objectives eventhough the details work plan had been slightly modified from the original proposal. This is not unusual for research work since the designs for experiments also depend on the results from the previous experiments.

FUTURE WORK

After completion of this project we have provided several new informations and ideas for future experimentations. Although more is known of the effect of sulfonamide compounds on the male reproductive system, there are still a number of unanswered questions. Future work are required to search for more potent compounds either by studying individual compound or combined drugs. It is very likely that by using a

suitable ratio of combined simple sulfa drugs, a very effecrive antifertility activity will be achieved. More work are also needed to elaborate the mechanism of the sulfa drugs. Evidence for a direct or an indirect action of sulfa drugs on spermatozoa is still lacking.

LITERATURE CITED

1. de Kretser, D.M. (1981). Fertility regulation in male : recent developments. In : Recent Advances in Fertility Regulation. C.C. Fen & Griffin, eds. Atar S.A., Geneva, pp. 112-121.
2. Levi, A.J., Fisher, A.M. Hughes, L. & Hendry, W.F. (1979). Male infertility due to sulphasalazine. Lancet ii, 276-278.
3. Toth, A. (1979). Reversible toxic effect of salicylazo-sulfapyridine on semen quality. Fertil. Steril. 31, 538-540.
4. Giwercman. A. & Skakkeback, N.E. (1982). The effect of salicylazosulfapyridine (sulfasalazine) on male fertility. A review. Int. J. Androl. 9, 35-52.
5. Levi, A.J., Smethurst, O. & O'Morain, C.A. (1982). Sulphasalazine induced male fertility in man and rats. Arch. Androl. 9. Abstract 18.
6. Pholprammool, C. & Srikhao, A. (1983). Antifertility effect of sulfasalazine in the male rat. Contraception 28, 273-279.

7. Pholpramool, C. & Srikhao, A. (1985). Suppression of fertility by sulfasalazine and its metabolites in the male rats. J. Androl. Suppl. 6, 50-p.

8. Tobias, R., Sapire, K.E., Coetzee, T. & Marks, I.N. (1982). Male infertility due to sulphasalazine. Postgrad. Med. J. 58, 102-103.

9. Ragni, G., Bianchi Porro, G., Ruspa, M., Barattini, G., Lombardi, C. & Petrillo, M. (1984). Abnormal semen quality and low serum testosterone in men with inflammatory bowel disease treated for a long time with sulfasalazine. Andrologia 16, 162-167.

10. Oko, R. & Hrudka, F. (1982). Segmental aplasia of the mitochondrial sheath and sequele induced by gossypol in rat spermatozoa. Biol. Reprod. 26, 183-195.

11. Cooper, T.G. (1986). The epididymis, sperm maturation and fertilisation. Springer Verlag, Berlin.

12. Frankin, J.C. & Rosenberg, I.H. (1973). Impaired folic acid absorption in inflammatory bowel disease : effects of salicylazosulfapyridine (Azulfidine). Gastroenterology 64, 517-525.

13. Selhub, J., Dhar, G.J. & Rosenberg, I.H. (1978). Inhibition of folate enzymes by sulfazalazine. J. Clin. Invest. 61, 221-224.

Appendix I

THE EFFECTS OF SULFASALAZINE, SULFAPYRIDINE, AND 5-AMINOSALICYLATE ON GLYCOLYSIS OF BULL SPERM

[1]Lewin, L.M., Golan, R., Wassercug, O. and [2]Pholpramool, C.

[1]Department of Chemical Pathology, Sackler Medical School, Tel Aviv University, Ramat Aviv, Israel, and [2]Department of Physiology, Mahidol University, Bangkok, Thailand.

INTRODUCTION

Male patients who were treated with sulfasalazine for ulcerative colitis were shown to develop reversible infertility (1). Further studies with rats confirmed this effect and demonstrated that sulfapyridine also inhibited male fertility (2,3). Rapid reversibility of the effect suggested that it might occur during epididymal maturation of sperm. These experiments are part of a project to investigate the mode of action of sulfa drugs as possible male contraceptive agents.

MATERIALS AND METHODS

Experiments were conducted as follows: Pellets of frozen bull semen were thawed at 37°, 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° 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°, 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.).

RESULTS AND DISCUSSION

Preliminary experiments showed that lactate production was almost independent of glucose concentration between 0.1 and 10 mM (Table 1). 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 2). Sulfapyridine and 5-aminosalicylate were less inhibitory. Other sulfa drugs and analogs that were not inhibitory at a concentration of 3 mM included sulfanilic acid, sulfaguanidine, sulfathiazole, sulfamerazine, sulfacetamide, sulfamethazine, and the analogs p-aminobenzoate, m-aminobenzoate and p-aminophenol.

To be publised in "Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination" June 26-30, 1988 Dublin, Ireland.

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). 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, did not inhibit sperm glycolysis appreciably under the conditions tested. Further studies are being conducted to determine whether these compounds have other effects on sperm metabolism which might account for their contraceptive properties.

ACKNOWLEDGEMENTS

This project was supported, in part, by a grant from the U.S. Agency for International Development.

REFERENCES

(1) Levi, A.J., Toovey, S., Smethurst, P. Andrews, B., 1982. Sulfasalazine and male infertility. In: Progress Towards a Male Contraceptive. Eds. Jeffcoate, S.L. and Sandler, M., pp. 209-219. John Wiley and Sons. N.Y.

(2) Pholpramool, C. and Srikhao, A. 1983. Antifertility effect of sulfasalazine in the male rat. Contraception. 28, 273-279.

(3) Pholpramool, C., Verawatnapakul, V., Srikhao, A., Ruciravat, S., Lewin, L.M., and Golan, R., 1987. Sulfonamide drugs as male contraceptive agents. I. Effects of sulfasalazine and some simple sulfonamides on fertility of the male rat. Proc. 1st USAID Conf., Nakorn Pathom, Thailand, in press.

(4) Gorus, F.K., Finsy, R., and Pipeleers, D.G.C., 1982. Effect of temperature, nutrients, calcium and cAMP on motility of human spermatozoa. Am. J. Physiol. 242, c304-c311.

(5) Waites, G.M.G., 1985. Sperm maturation and the epididymis-new targets. In: Methods for the Regulation of Male Fertility. Eds. Kumar, T.C.A. and Waites, G.M.H., pp. 107-118. Indian Council of Medical Research, New Delhi.

Table 1. Glucose Utilization and Lactate Production by Bull Sperm. Washed bull sperm were incubated at 37° 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.

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

Table 2. Percent inhibition of glycolysis by bull sperm in the presence of sulfasalazine and its moieties. Washed bull sperm were incubated at 37° for 30 min. in balanced salts solution containing glucose (1 mM) with or without additions as described below. Lactate production was measured as described in METHODS, and inhibition calculated as percent of controls. S.E.M. values are reported in parentheses.

Compound added	Concentration of test compound		
	1mM	2mM	3mM
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 $p<0.05$ by t test for difference of means.

Summary

Sulphonamides with different chemical structures were synthesized and these 13 compounds together with 7 commercially available sulpha drugs were tested for antifertility activity by natural mating in male rats. All compounds were given daily by gastric intubation at doses of 125, 150, 250 or 450 mg/kg for 6 weeks. Sulphapyridine caused a dose-related and reversible reduction in fertility at doses between 125 and 450 mg/kg. At the high dose, fertility was decreased to 25.9% of control at Week 5 after treatment, and complete recovery occurred by Week 3 after drug withdrawal. This activity was abolished when the pyridine ring was substituted by other heterocyclic rings, except sulphachloropyridazine which had only weak activity. Replacement of the pyridine ring by a hydrogen atom or short aliphatic chains preserved or even enhanced the potency. Thus, sulphanilamide, N^1-methylsulphanilamide or N^1-diethylsulphanilamide produced a marked but reversible reduction in fertility. Removal or substitution of the N^4-amino group on the benzene ring of sulphapyridine with a methyl group destroyed the activity. However, the bromo or nitro analogue (at the para- but not the meta-position of the benzene ring) still possessed some activity. N^4-Acetyl derivatives of sulphapyridine, sulphanilamide, and N^1-diethylsulphanilamide were as potent as their parent compounds. These results suggest that the presence of pyridine or other heterocyclic rings is not necessary for the antifertility activity of sulphonamide compounds. However, the N^4-amino group is indispensable. In

addition, acetylation of this amino group does not change the potency. The prototype of the antifertility sulphonamides, therefore, seems to be sulphanilamide.

Introduction

Sulphasalazine (salicylazosulphapyridine), a compound formed by azo linkage between sulphapyridine and 5-amino salicylic acid, was first introduced to treat rheumatoid arthritis in 1930 (for review see Goldman & Peppercorn, 1975). It was later demonstrated to be useful in the therapy and prophylaxis of ulcerative colitis and regional enteritis (Baron et al., 1962; Misiewicz et al., 1965). Although these diseases predominantly affect men and women of reproductive age, the association between sulphasalazine treatment and male infertility was not recognized until in 1979 when Toth (1979) and Levi et al. (1979) independently reported that male patients who received sulphasalazine treatment for ulcerative colitis became infertile. Such an undesirable effect was completely reversible after drug withdrawal. This finding has since been confirmed both in man and animals by many investigators (for review see Giwercman & Skakkeback, 1986). More recent evidence indicates that the sulphapyridine moiety, which is a metabolic breakdown produce of sulphasalazine by bacteria in the gut (Peppercorn & Goldman, 1972), or its metabolite(s) is responsible for the antifertility effect in man (O'Morain et al., 1982a,b; Cann & Holdsworth, 1984; McIntyre & Lennard-Jones, 1984; Shaffer et al., 1984) and in rats (O'Morain et al., 1982b; Pholpramool & Srikhao, 1985).

In the rat, sulphasalazine and sulphapyridine do not alter testicular weight and histology, gonadotrophin and testosterone concentrations in the blood, or daily sperm production rate and

sperm concentration in the epididymis (Pholpramool & Srikhao, 1983; O'Morain et al., 1984; Pholpramool et al., 1989). These, together with the rapid onset of the antifertility activity, suggest that the site of action is post-testicular, possibly at the epididymis. Wong et al. (1987) have studied the antifertility effects of several types of sulphonamides and related compounds in rats and found that sulphapyridine was the most effective. The effectiveness of these sulphonamides seemed to be related to their ability to pass into the epididymal fluid, again indicating that the site of action of sulphonamide drugs is at the epididymis.

Since sulphapyridine is considered to be relatively toxic, other sulphonamide drugs which could suppress male fertility by the same mechanism as this compound and were less toxic might be ideal contraceptives for men. In the present study, we have synthesized 13 sulphapyridine analogues and investigated their antifertility activities in male rats.

Materials and Methods

Animals : Sexually mature male and female rats of the Fischer strain were obtained from the Laboratory Animal Unit of the Faculty of Science, Mahidol University. They were kept separately in stainless steel hanging cages under ambient temperature and a 12 h light : 12 h dark lighting regimen with free access to food and water. Only male rats with proven fertility were used.

Chemicals :All chemicals used were of reagent grade and were purchased from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA).

Synthesis of the test compounds : The 13 compounds (Compounds VIII - XX), of the structural formulae shown in Table 1, were synthesized by a simple reaction between the appropriate sulphonylchloride derivatives and amines or ammonia. The product were purified by recrystallization and identified by their melting points, ultraviolet, nuclear magnetic resonance and mass spectra and elemental analyses.

Drug treatments : The 13 synthetic compounds and 7 sulphonamide drugs purchased from Sigma were studied in 7 consecutive fertility tests consisting of 1-6 compounds over a period of 30 months. Each test compound was freshly suspended in corn oil and was fed every day for 6 weeks via gastric intubation at two doses (150 and 450 mg/kg), except in some trials, into a group of 5-12 male rats weighing about 260 g at the start of treatment. Each series of

experiments also contained a group of 8-12 control male rats to which corn oil only was fed.

Fertility testing : The fertility of the male rats treated with the test compounds and corn oil was assessed by natural mating. Each male was kept with 2 virgin female rats for 1 week after feeding for 1,3 and 5 weeks, and after drug withdrawal for 1 and 3 weeks. Mating was determined by the presence of spermatozoa in the vaginal smears taken every morning. After 1 week of cohabitation both females were removed from the male kept separately for 8-10 days, and then killed for confirmation of pregnancy. The numbers of fetuses and corpora lutea were counted. The latter were determined under a dissecting microscope.

Fertility of the male rats was expressed as the percentage fertility which was calculated from the ratio of fetuses to the number of corpora lutea times one hundred. When both female rats were mated by the same male, the average value was used.

Statistics : Data are presented as mean±s.e.m. throughout. Differences in fertility between and within groups were assessed by Student's t test for unpaired and paired samples, respectively. Differences were considered significant when P-values were less than 0.05.

Results

The antifertility effect of sulphapyridine

Sulphapyridine produced a dose-dependent and reversible reduction in fertility (Table 2). At a low dose, 125 mg/kg, percentage fertility was significantly ($P<0.001$) decreased by 5 weeks after treatment. When given at higher doses, significant reductions occurred by 3 weeks after feeding, and by Week 5 percentage fertility was only 25% at doses of 250 and 450 mg/kg. In addition, the number of males that mated with female rats and the number of the females that became pregnant were also reduced after feeding with sulphapyridine. These effects, however, were rapidly reversible. Thus, by 1-3 weeks after the cessation of treatment all these parameters returned to control levels (Table 2).

Effects of sulphapyridine analogues

To determine the structural requirements for sulphonamide compounds that have antifertility activity and to search for more potent compounds than sulphapyridine, 19 sulphonamides having various structures related to sulphapyridine were chosen for fertility testing in this study.

a. Compounds with N^1 substituted with heterocyclic rings

Figure 1 shows the results of fertility testing of the commercially available sulphonamide drugs that contain several types of heterocyclic rings, other than pyridine, at the N^1 position. It is clear that sulphioxazole, sulphamerazine and sulphadimethoxine failed to exhibit any antifertility activity. However, sulphachloropyridazine, at a dose of 450 mg/kg, caused a slight but significant ($P<0.05$) suppression of fertility.

b. Compounds with N^1 substituted with aliphatic chains

Table 3 demonstrates the effects of several simple sulphonamides on fertility of male rats. These compounds all had antifertility activity. Compound VIII (N^1-methylsulphanilamide) was the most potent agent, giving a low percentage fertility at Week 5 after treatment at a dose of 450 mg/kg. The numbers of males that mated and of impregnanted females were markedly decrased (Table 3). At this dose, however, this compound exhibited some toxic effects, i.e. reduction in body weights and haematuria. Nevertheless, these effects were dose-dependent and reversible after the cessation of treatments.

c. Sulphapyridine analogues

To determine whether N^4-amino group of sulphapyridine is required for the antifertility activity, 6 analogues having the N^4 amino group removed or replaced by other functional groups were synthesized and tested. As shown in Table 4 only Compound XII [4-nitro-N-2-pyridinylbenzenesulphonamide] and Compound XIV [4-bromo-N-2-pyridinylbenzenesulphonamide] retained the

antifertility activity.

d. <u>N^4-Acetyl derivatives of sulphapyridine and other sulphonamides</u>

It is known that sulpha drugs and other sulphonamides are metabolized by the liver via acetylation at N^4-amino group (Schroder & Campbell, 1972). However, the active form of sulphapyridine or other antifertility sulphonamides is not known. The effects of N^4-acetyl derivatives of sulphapyridine and other antifertility sulphonamides were therefore investigated. The results are shown in Table 5. All these compounds were also effective in suppressing fertility of the male rats.

Discussion

Since the recognition of the side effect of sulphasalazine on male fertility, it has been demonstrated by several investigators that some sulpha drugs and other related compounds such as sulphapyridine (O'Morain et al., 1982b; Pholpramool & Srikhao, 1985; Wong et al., 1987), dapsone, sulphamerazine, sulphamethazine, sulphathiazole, sulphamethoxypyridazine, sulphadimethoxine and sulphaguanidine (Wong et al., 1987) also possess antifertility activities. However, there has been no systematic study on the structure and activity relationship of these compounds. This study is the first attempt to unveil the basic structure that is necessary for the antifertility action of sulphonamides in male rats. Variations of the structure of the test compounds were primarily based on that of sulphapyridine so as to address 3 questions : firstly, is the pyridine ring required for the antifertility activity; secondly, is the presence of the N^4-amino group essential for the action; and thirdly, would acetylation of the N^4-amino group alter the potency. When the pyridine moiety of sulphapyridine was replaced by isoxazole or pyrimidine ring the antifertility activity was completely lost. Thus, sulphioxazole, sulphamerazine and sulphadimethoxine had virtually no effect on fertility (Fig. 1). However, replacement with the pyridazine ring partly destroyed the activity. For example, sulphachloropyridazine caused a slight but significant reduction in fertility. By contrast, when the pyridine ring is substituted by H- or a simple alkyl group the activity was retained or even enhanced. Simple sulphonamides such as

sulphanilamide and N^1 diethyl sulphanilamide (Compound IX) were therefore as effective as sulphapyridine whilst N^1 methyl sulphanilamide (Compound VIII) was even more potent (Table 3). The latter, however, was also toxic to the rats. These results indicate that the pyridine ring at the N^1 position of the sulphonamide skeleton was not essential for the antifertility activity.

It is well known that the presence of the p-amino group in the benzene ring of benzene sulphonamides is essential for the antimicrobial and antibacterial activities of the sulpha drugs (Maren, 1976). However, it was not known whether this is also true for the activity of these compounds on fertility. Six sulphapyridine analogues which had the N^4-amino group removed or replaced by other functional groups were synthesized and tested: removal of the N^4-amino group completely abolished the activity (Table 4). Substitution with a methyl group also failed to retain the activity. However, bromo- or nitro- groups were able to produce some antifertility activity although to a lesser degree than the amino group. The activity was completely lost when the substitution of a nitro-group was at the meta- or ortho-position of the benzene ring. These data suggest that the N^4-amino group of the benzene sulphonamide compounds is required for maximum antifertility activity. In this regard, it appears that the requirement for N^4-amino group is similar to that of the antibacterial sulphonamides. However, it does not necessarily imply that the mode of action of these drugs is the same. The findings that Compound XII [4-nitro-N-2-pyridinyl benzenesulphonamide] and Compound XIV [4-bromo-N-2-pyridinylbenzesulphonamide] (Table 4) are

also effective may be explained in terms of conversions of these compounds in the animals to active metabolites, possibly through sulphapyridine for Compound XII.

Previous work has shown that detoxification and excretion of sulphapyridine occurs through acetylation at the N^4-amino group and hydroxylation at position 5 of the pyridine ring. The hydroxyl group may then be conjugated with glucuronic acid to form glucuronides, which are then excreted by the kidneys (Schroder & Campbell, 1972; Hansson & Sandberg, 1973; Eastwood, 1980). It is not known at present whether sulphapyridine or its metabolite(s) is the active molecule(s) for the antifertility activity. A series of N^4-acetyl derivatives of the sulphonamides that possess antifertility action was synthesized and tested. The potency was virtually unchanged by such modification except, for Compound XVII (N^4-acetyl N^1-methylsulphanilamide) and Compound XVIII (N^4-acetyl-N^1-diethylsulphanilamide), which were more potent than their non-acetyl analogues at the dose of 450 mg/kg, but only at 1 week and 3 weeks after feeding, respectively (Table 5). The results indicated that the N^4-acetylated derivative may be the active compound. However, it is also possible that the non-acetylated form is in fact the species that exerts the antifertility action. The latter is based on the notion that the N^4-acetylated form may first be hydrolysed by the gut bacteria and the unacetylated form absorbed into the blood. The latter explanation depends on the assumption that all acetylated compounds tested in this study are readily hydrolysable in the gastrointestinal tract. We are currently working to delineate the nature of active form of the antifertility sulphonamides.

This work was supported, in part, by a USAID grant (US-Israel CDR Programme, 936-5544-G-00-6105,00) to C.P. and L.M.L., a grant from Chulabhorn Research Institute (CRI) to C.P. and S.R., and Mahidol University research grant to C.P. We thank Mrs Suthada Sukuttakij for typing the manuscript.

References

1. Baron, J.M., Connell, A.M., Lennard-Jones, J.E. & Jones, F.A. (1962). Sulfasalazine and salicylazosulfapyridine in ulcerative colitis. Lancet 1, 1094.
2. Cann, P.A. & Holdsworth, C.D. (1984). Reversal of male infertility on changing treatment from sulfasalazine to 5-amino-salicylic acid. Lancet 1, 1189.
3. Eastwood, M.A. (1980). Pharmacokinetic pattern of sulfasalazine. Ther. Drug Mon. 2, 149-152.
4. Giwercman, A. & Skakkeback, N.E. (1986). The effect of salicylazosulph apyridine (sulphasalazine) on male fertility. A review. Int. J. Androl. 9, 38-52.
5. Goldman, P. & Peppercorn, M.A. (1975). Drug therapy sulfasalazine. New Eng. J. Med. 293, 70-24.
6. Hansson, K. & Sandberg, M. (1973). Determination of sulphapyridine and its metabolites in biological materials after administration of salicylazosulphapyridine. Acta pharm. Succica 10, 87-92.
7. Levi, A.J., Fisher, A.M., Hughes, L & Hendry, W.F. (1979). Male antifertility due to sulphasalazine. Lancet 2, 276-278.
8. Maren, T.H. (1976). Relations between structure and biological activity of sulfonamides. Ann. Rev. Pharmacol. Toxicol. 16, 309-327.
9. McIntyre, P.B. & Lennard-Jones, J.E. (1984). Reversal with balazide on fertility caused by sulfasalazine. Br. med. J. 288, 1652.
10. Misiewicz, J.J., Lennard-Jones, J.E., Connell, A.J., Baron, J.H. & Jones, F.A. (1965). Controlled trial of sulfasalazine maintenance therapy for ulcerative colitis. Lancet 1, 185.

11. O'Morain, C., Smethurst, P. & Dore, C.I. (1984). Reversible male infertility due to sulphasalazine. Studies in man and rats. Gut 25, 1078-1084.

12. O'Morain, C., Smethurst, P., Hudson, E. & Levi, A.J. (1982a). Further studies on sulphasalazine induced male infertility. Gastroenterology 82, 1140A.

13. O'Morain, C., Smethurst, P. & Levi, A.J. (1982b). Sulfasalazine induced reversible male infertility in man and rat. Gut 23, A892.

14. Peppercorn, M.A. & Goldman, P. (1972). The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. J. Pharmacol. exp. Therap. 181, 555-161.

15. Pholpramool, C. & Srikhao, A. (1983). Antifertility effect of sulfasalazine in the male rat. Contraception 28, 273-289.

16. Pholpramool, C. & Srikhao, A. (1985). Suppression of fertility by sulfasalazine and its metabolites in the male rats. J. Androl. Suppl. 6, 50p.

17. Pholpramool, C., Verawatnapakul, V., Srikhao, A., Ruchirawat, S. & Lewin, L.M. (1989). Lack of effects of some antifertility sulfonamides on testicular functions in the male rats. Proc. 4th Int. Congr. Androl., Florence, 27-31.

18. Schroder, H. & Campbell, D.E.S. (1972). Absorption, metabolism and excretion of salicylazosulfapyridine in man. Clin. Pharmacol. & Therap. 13, 539-551.

19. Shaffer, J.C., Kershaw, A. & Berrisford, M.H. (1984). Sulfasalazine induced infertility reversed on transfer to 5-aminosalicylic acid. Lancet 1, 1240.

20. Toth, A. (1979). Reversible toxic effect of salicylazosulfapyridine on semen quality. Fert. Steril. 31, 538-540.

21. Wong, P.Y.D., Lau, S.K.D. & Fu, W.O. (1987). Antifertility effects of some sulphonamides and related compounds and their accumulation in the epididymides of male rats. J. Reprod. Fert. 81, 259-267.

Table 1 . Nomenclatures and chemical structures of the compounds synthesized and tested for antifertility activities.

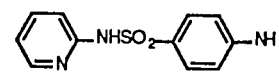
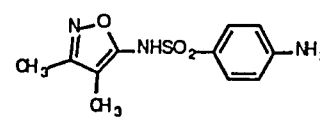
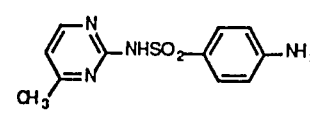
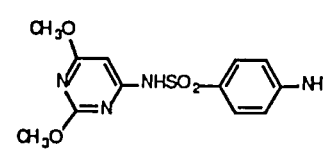
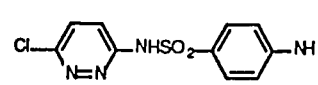
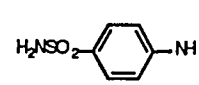
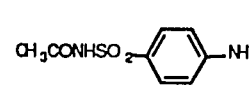
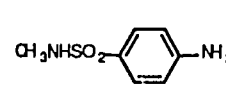
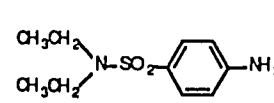
Compound	Chemical name/generic name*	Chemical structure
I	Sulphapyridine	
II	Sulphioxazole	
III	Sulphamerazine	
VI	Sulphadimethoxine	
V	Sulphachloropyridazine	
VI	Sulphanilamide	
VII	Sulphacetamide	
VIII	N^1-Methylsulphanilamide	
IX	N^1-Diethylsulphanilamide	

Table 1 (continued)

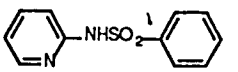
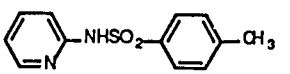
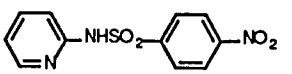
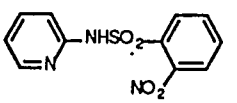
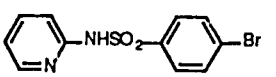
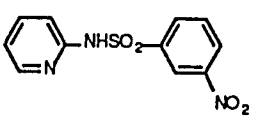
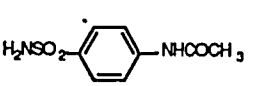
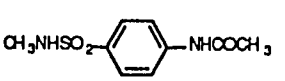
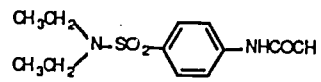
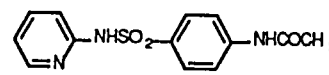
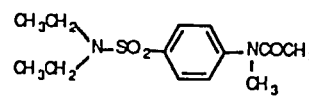
Compound	Chemical name/generic name*	Chemical structure
X	N-2-Pyridinylbenzene sulphonamide	
XI	4-Methyl-N-2-pyridinyl-benzenesulphonamide	
XII	4-Nitro-N-2-pyridinyl-benzenesulphonamide	
XIII	2-Nitro-N-2-pyridinyl-benzenesulphonamide	
XIV	4-Bromo-N-2-pyridinyl-benzenesulphonamide	
XV	3-Nitro-N-2-pyridinyl-benzenesulphonamide	
XVI	N^4-Acetyl sulphanilamide	
XVII	N^4-Acetyl-N^1-methyl-sulphanilamide	

Table 1 (continued)

Compound	Chemical name/generic name*	Chemical structure
XVIII	N^4-Acetyl-N^1-diethyl-sulphanilamide	
XIX	2-(N^4-Acetylsulphanilamido) pyridine	
XX	N^4-Acetyl-methyl-N^1-diethyl-sulphanilamide	

* Generic names are used for the compounds I to VII, which are not synthesized in this study.

47

Table 2. The effect of sulphapyridine on various parameters of fertility in male rats. Male Fischer rats were treated with sulphapyridine (125, 250 or 450 mg/kg-day) and % successful mating, % of females becoming pregnant, and % fertility (no. of embryos x 100/no. of corpora lutea) were determined all as described in "Methods".

Period of treatment	Parameters of measurement (%)	Animal group			
		Control	Sulphapyridine (dose)		
			125 mg/kg	250 mg/kg	450 mg/kg
Before drug	Mating	100	100	100	100
	Pregnant female	100	100	100	100
	Fertility*	90.0±3.6	94.6±2.3	98.1±1.3	92.2±2.9
		(11)	(9)	(8)	(9)
Week 1 during drug	Mating	90.9	55.6	77.8	66.7
	Pregnant female	100	100	71.4	100
	Fertility	75.8±5.8	76.2±9.6	62.7±16.9	86.4±5.8
		(10)	(5)	(7)	(6)
Week 3 during drug	Mating	100	44.4	33.3	55.6
	Pregnant female	100	100	66.7	100
	Fertility	80.8±5.9	71.9±12.3	22.1±1.2	62.1±14.0[b]
		(11)	(4)	(3)	(5)

Table 2. (Continued).

Period of treatment	Parameters of measurement(%)	Animal group			
		Control	Sulphapyridine (dose)		
			125 mg/kg	250 mg/kg	450 mg/kg
Week 5 during drug	Mating	100	66.7	44.4	66.7
	Pregnant female	100	100	75	50
	Fertility	85.7±3.3 (11)	45.9±4.8[a,c] (6)	25.1±11.5 (4)	25.9±8.6[a,c] (6)
Week 1 after drug withdrawal	Mating	100	-	-	77.8
	Pregnant female	100	-	-	100
	Fertility	81.9±5.1 (11)	-	-	69.3±9.6[b] (7)
Week 3 after drug withdrawal	Mating	100	-	-	88.9
	Pregnant female	100	-	-	100
	Fertility	75.0±6.0 (11)	-	-	90.1±3.5 (7)

*Mean±s.e.m. from number of rats indicated in parentheses.

[a]P<0.001 when compared to the corresponding values in the control group.

[b]P<0.05; [c]P<0.001 when compared to the period before treatment within the same group.

Table 3. The effect of sulpha drugs having N^1-substitution by aliphatic chains on fertility of the male rat

Compound	Dose (mg/kg)	Percentage fertility*					
		Before treatment	During treatment			After treatment	
			wk-1	wk-3	wk-5	wk-1	wk-3
Control	-	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)
Sulphanilamide	150	92.0±3.0(5)	91.8±3.6(4)	57.7±9.5(5)[a,d]	44.5±4.5(5)[c,e]	74.6±8.0(5)[b]	96.2±1.9(5)
	450	88.8±4.6(8)	86.8±6.6(6)	56.3±7.0(5)[a,e]	18.0±9.0(4)[c,e]	37.3±16.0(5)[c,d]	79.5±9.9(5)
Sulphacetamide	150	95.7±1.2(12)	80.5±7.0(10)[a,d]	70.0±10.0(12)[d]	85.9±4.1(9)[d]	91.3±2.5(10)	-
	450	95.1±1.7(11)	86.6±2.8(10)[b,d]	70.6±6.4(9)[e]	77.5±7.3(10)[d]	81.5±5.0(10)[a,d]	-
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)

Table 3. (Continued).

Compound	Dose (mg/kg)	Percentage fertility[+]					
		Before treatment	During treatment			After treatment	
			wk-1	wk-3	wk-5	wk-1	wk-3
VIII	150	96.7±1.7(7)	73.3±8.4(6)[d]	14.6±12.0(4)[e]	17.4±7.6(6)[c,e]	64.0±14.5(6)	82.0±8.9(7)
	450	94.2±2.0(8)	11.5±11.6(4)[c,e]	31.8±31.8(2)	6.8±4.3(4)[c,f]	43.3±10.8(5)[b,e]	66.9±16.8(4)
IX	150	97.6±1.6(7)	60.5±19.6(6)	59.1±20.0(6)	37.8±18.1(4)[b,e]	96.0±2.3(4)	58.6±19.9(6)
	450	95.5±2.7(8)	60.0±24.5(5)	91.9±4.8(3)	22.8±8.1(5)[c,f]	47.2±17.1(3)[a,e]	55.0±9.2(5)

[+]Mean±s.e.m. from number of rats indicated in parentheses.

[a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$ when compared to the corresponding periods in the control group.

[d]$P<0.05$; [e]$P<0.01$; [f]$P<0.001$ when compared to the period before treatment within the same group.

Table 4. The effect of sulphapyridine analogues having different substitutions on the benzene rings on fertility of the male rats

Compound	Dose (mg/kg)	Percentage fertility*					
		Before treatment	During treatment			After treatment	
			wk-1	wk-3	wk-5	wk-1	wk-3
Control	-	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)
X	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)	89.8±4.4(8)
Control	-	93.7±2.2(7)	83.6±4.7(7)[d]	91.8±4.2(7)	82.3±7.6(8)	83.8±5.6(8)	81.3±5.5(8)[d]
XI	150	95.5±2.9(7)	87.1±7.3(5)	90.0±6.8(5)	92.6±7.4(6)	84.9±6.9(6)	68.4±14.6(7)
	450	94.0±3.0(7)	93.7±3.3(6)	95.0±3.0(7)	89.4±6.9(7)	77.7±4.9(7)[d]	71.2±13.4(7)
XII	150	90.9±4.5(7)	82.7±7.9(7)	54.3±11.9(7)[b,d]	40.5±4.7(6)[c,e]	61.1±10.9(7)	72.3±12.8(7)
	450	91.5±3.8(8)	82.3±6.3(4)	36.2±15.5(6)[a,d]	56.2±10.7(7)[d]	57.2±12.5(7)[d]	82.0±8.6(7)
XIII	150	95.7±3.0(7)	82.3±5.0(7)	75.5±13.9(7)	97.2±1.6(5)	92.1±4.1(7)	71.6±8.9(7)[d]
	450	92.8±8.2(8)	83.7±2.5(7)	70.6±12.4(5)	82.4±5.9(8)	81.4±10.9(8)	83.0±6.7(8)

Table 4. Continued

Compound	Dose (mg/kg)	Percentage fertility[+]					
		Before treatment	During treatment			After treatment	
			wk-1	wk-3	wk-5	wk-1	wk-3
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)[d]
XIV	150	95.9±1.6(8)	83.1±13.9(7)	63.7±15.5(8)[d]	62.1±15.2(6)[d]	74.0±12.6(8)	88.5±5.2(7)[a]
	450	98.4±1.6(8)	80.6±13.7(7)	68.0±15.7(6)[d]	56.2±16.1(7)[e]	86.1±6.3(6)[d]	90.0±7.0(8)
XV	150	98.2±1.2(8)	84.2±5.9(8)[d]	54.6±16.6(7)	67.6±16.9(6)[d]	94.0±5.9(8)	87.5±6.1(8)[a]
	450	96.8±1.3(8)	92.2±3.0(6)	95.5±2.9(5)	66.5±11.0(6)[e]	75.8±15.9(4)[d]	60.9±15.5(6)[e]

[+]Mean±s.e.m. from number of rats indicated in parentheses.

[a]$P<0.05$; [b]P.01; [c]$P<0.001$ when compared to the corresponding periods in the control group.

[d]$P<0.05$; [e]$P<0.01$ when compared to the period before treatment within the same group.

Table 5. The effect of N^4-acetyl derivatives of sulphapyridine and other sulphonamides on fertility of the male rat

Compound	Dose (mg/kg)	Percentage fertility*					
		Before treatment	During treatment			After treatment	
			wk-1	wk-3	wk-5	wk-1	wk-3
Control	-	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)
XVI	150	83.1±3.2(7)	66.0±14.1(7)	57.8±13.0(6)[a]	50.2±10.4(7)[a,d]	65.8±8.7(6)	79.1±3.1(7)[a]
	450	87.5±3.7(8)	86.0±7.47(8)	49.9±12.0(6)[a,d]	42.6±12.5(7)[b,e]	50.1±9.7(6)[a,e]	69.6±7.1(6)[a]
Control	-	95.1±2.3(9)	89.5±4.4(8)	63.5±18.0(5)[a]	95.3±2.6(4)	96.2±2.6(4)	76.2±11.8(5)[d]
XVII	150	95.5±2.6(7)	79.3±8.2(6)	33.3±22.8(3)	20.4±7.8(6)[c,f]	45.4±15.8(5)[a,d]	80.4±10.5(6)
	450	98.7±0.9(8)	85.6±6.2(6)	21.3±15.0(3)[d]	17.2±6.7(6)[c,f]	60.0±49.0(2)	53.9±14.9(7)[d]

Table 5. (Continued).

Compound	Dose (mg/kg)	Percentage fertility[+]					
		Before treatment	During treatment			After treatment	
			wk-1	wk-3	wk-5	wk-1	wk-3
XVIII	150	95.6±2.2(7)	44.2±20.5(5)	47.8±17.0(6)	30.9±9.2(5)[c,f]	72.0±11.3(5)[d]	72.5±7.9(6)[e]
	450	98.5±1.0(8)	73.8±9.7(5)[e]	11.3±5.8(7)[a,e]	21.0±11.6(3)[c,f]	32.5±23.6(4)[a,d]	77.3±13.8(5)[d]
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)[d]
XIX	150	98.0±1.3(8)	92.2±4.7(7)	84.6±4.9(8)[a]	30.6±13.4(7)[a,f]	84.2±5.9(8)[d]	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(6)[a,f]	68.0±14.3(7)[d]	75.1±13.1(7)
XX	450	99.4±0.6(8)	66.8±14.8(8)	34.7±8.7(7)[f]	18.6±5.9(7)[b,f]	51.8±5.8(8)[a,f]	89.2±2.9(8)[a,d]

[+]Mean±s.e.m. from number of rats indicated in parentheses.

[a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$ when compared to the corresponding periods in the control group.

[d]$P<0.05$; [e]$P<0.01$; [f]$P<0.001$ when compared to the period before treatment within the same group.

Legend for figure

Figure 1. Effects of sulphonamides having different heterocyclic rings at N^1-position on fertility of the male rats. The animals were treated daily by intragastric intubation with sulphioxazole (▨, 150; ▩, 450 mg/kg), sulphamerazine (▤, 150; ▥, 450 mg/kg), sulphadimethoxine (▧, 150 mg/kg) and sulphachloropyri dazine (⊡, 150; ■, 450 mg/kg) for 6 weeks. Each male rat was cohabited with 2 females for 1 week before, during Week 5-6 of treatment, and during Week 1-2 after drug withdrawal. Numbers in the bar graphs represent numbers of male rats that mated with females.

VOLUMETTO ESTRATTO DA:

IVth INTERNATIONAL CONGRESS OF ANDROLOGY

Florence, May 14-18, 1989

Appendix II

MINI-POSTERS

MONDUZZI EDITORE

5

IVth International Congress of Andrology

Florence
May 14-18 1989

Lack of effects of some antifertility sulfonamides on testicular functions in the male rats

C. PHOLPRAMOOL, V. VERAWATNAPAKUL, A. SRIKHAO *, S. RUCHIRAWAT ** and L.M. LEWIN ***

Department of Physiology, Faculty of Science
Mahidol University, Bangkok (T)
** Department of Zoology, Faculty of Science*
Kasetsart University, Bangkok (T)
*** Department of Chemistry, Faculty of Science*
Mahidol University, Bangkok (T)
**** Department of Chemical Pathology, Medical School*
University of Tel Aviv (IL)

SUMMARY

The possible effects of the antifertility sulfonamides, sulfasalazine (SS) and sulfapyridine (SP), on the testicular functions of the male rats were studied. Both SS and SP caused marked reductions in fertility by 5 weeks after treatment. Basal serum testosterone levels were virtually unchanged by SP. However, the LHRH-induced serum testosterone was substantially enhanced by this compound. This is due to the increased responsiveness of the Leydig cells to LHRH since the levels of LHRH-induced LH in the SP-treated animals and the controls were not different. On the other hand, daily sperm production rate and histology of the testis were not affected. These results show that the antifertility effect of SS or SP is not associated with the inhibition of spermatogenesis and testosterone production. The site of action of these sulfonamides is most likely at the epididymis.

INTRODUCTION

It has previously been shown that sulfasalazine (SS) caused a suppression of male fertility both in human (1,2) and rats (3, 4). Infertility in men is associated with a decrease

Copyright 1989 by Monduzzi Editore S p A - Bologna (Italy)

65

in sperm count and sperm motility, and the appearance of some megalo-head spermatozoa in the ejaculates. In the rats, reduction in epididymal sperm reserve and sperm motility are also observed. These data suggest that SS may interfere with spermatogenesis. However, the testis weights and serum testosterone are unaffected in the rats (3). Recently, it was realized that sulfapyridine (SP), which is a metabolic breakdown of SS in the gut (5), is responsible for the antifertility effect of SS (4, 6, 7). Since the possible effects of SS or SP on the testicular functions remain unclear, the present study attempts to clarify this question by investigating the effect of SP on spermatogenesis and testosterone production by the rat testis.

MATERIAL AND METHODS

Animals Adult, male Fischer rats weighing about 300 gm were kept at ambient temperature and daylights with free access to pellet feeds and water. They were tested for fertility capacity by natural mating as previously described (3). Only males with high percentage fertility (>80%) were used for further studies.

Experimental Design Four series of experiments were carried out as follows : Expt. 1: The male were forced-fed with corn oil (controls), SS or SP at a dose of 450 mg/kg/day for 6 weeks. Fertility was tested at weeks 3 and 5 during treatment and at week 3 after drug withdrawal. Expt. 2: The animals were treated as in Expt. 1 and blood samples were collected from the jugular vein before, at weeks 3 and 5 during treatment and at week 3 after drug withdrawal. Expt. 3: The experiment is similar to Expt. 1 except that LHRH (Sigma, U.S.A.) was injected (s.c.) at a dose of 10 ug/rat at the end of drug treatment, and blood samples were collected before and at 2 h after LHRH injection. Expt. 4: The animals were treated (s.c.) with DMSO (controls) or SP at doses of 250 and 500 mg/kg/day for 5 weeks. They were then sacrificed and the testes were removed for histological examinations and determinations of daily sperm production rate (8).

Hormone Assays Blood samples were allowed to clot overnight in a refrigerator and the sera were kept at -20°C until assayed for testosterone by RIA (9) and LH by mouse Leydig cell bioassay (10). The coefficient of variations for intra-and inter-assays for testosterone were 3.0% and 15.13%, respectively.

RESULTS AND CONCLUSIONS

Both SS and SP significantly ($P<0.05$ and $P<0.01$, respectively) decreased fertility of the male rats by 3 weeks after treatment and the maximal effect was reached by 5 weeks (Table 1). It appears that the metabolite of SS, i.e. SP, was more potent. Complete recovery of fertility occurred by 3 weeks after the cessation of SS treatment.

Data in Table 2 show that the suppression of fertility by SP was not associated with the inhibition of the Leydig cell function since serum testosterone was unaltered throughout the experiment. In addition, serum LH was not affected by such treatment. However, the LHRH-induced serum testosterone of the SP-treated rats was higher ($P<0.05$) than the controls (Table 3).

Table 1. Effects of some sulfonamides on fertility of the male rats.

Treatment	Percentage Fertility[a]			
	Before Treatment	During Treatment W-3	During Treatment W-5	After Drug Withdrawal W-3
Control	94.3 ± 2.4 (12)	84.1 ± 2.8 (8)	90.6 ± 3.9 (11)	92.8 ± 4.2 (4)
SS	94.5 ± 2.9 (7)	52.8 ± 12.6[b] (6)	35.3 ± 13.7[c] (6)	94.4 ± 3.9 (4)
SP	90.7 ± 2.3	32.4 ± 7.8[c]	14.3 ± 11.9[d]	-

a, Mean ± SEM (n); b, P<0.05; c, P<0.01; d, P<0.001 when compared to the controls.

Table 2. Serum concentrations (ng/ml) of LH and testosterone at various periods before, during and after cessation of treatment with SP.

Period		Serum LH		Serum testosterone	
		Control	SP-treated	Control	SP-treated
Before		0.99 ± 0.23 (5)	1.05 ± 0.19 (5)	1.12 ± 0.16 (11)	0.84 ± 0.08 (9)
During,	W-3	1.38 ± 0.12 (11)	1.08 ± 0.25 (9)	1.47 ± 0.22 (7)	0.95 ± 0.28 (3)
	W-5	1.11 ± 0.15 (11)	0.93 ± 0.15 (8)	1.03 ± 0.17 (11)	0.96 ±0.14 (8)
After,	W-3	1.73 ± 0.36 (10)	1.22 ± 0.42 (7)	1.33 ± 0.16 (10)	1.03 ± 0.16 (8)

Values are mean ± SEM (n).

Table 3. The effect of LHRH on serum concentrations (ng/ml) of LH and testosterone of control and SP-treated rats.

Period	Serum LH		Serum testosterone	
	Control	SP-treated	Control	SP-treated
Before	0.87 ± 0.28 (7)	0.45 ± 0.04 (7)	2.7 ± 0.6 (8)	2.2 ± 0.6 (7)
2 h after	[illegible] (7)	10.20 ± [illegible] (7)	17.7 ± 1.9 (7)	22.9 ± 0.9[a] (7)

Values are mean ± SEM (n); a, P<0.05 when compared to the controls.

This findings suggest that the responsiveness of either the pituitary gland or the Leydig cells, or both, to exogeneous LHRH is enhanced. Since the level of LHRH-induced LH in the SP-treated rats was not different from the controls (Table 3), the data support the view that the responsiveness of the Leydig cells to LHRH is increased after SP treatment.

The rate of sperm production, which was determined by counting the number of spermatids remained after homogenization of the testis (8) was virtually not altered by SP (Table 4). The results clearly demonstrate that the antifertility effect of SP is not due to suppression of spermatogenesis. Histological studies also confirmed this conclusion (data not shown).

Table 4. Daily sperm production after SP treatment at various doses.

Treatment (dose)	Daily Sperm Production[a] (Sperm x 10^6/day-g)
Control (DMSO)	20.6 ± 0.9 (8)
SP (250 mg/kg)	22.3 ± 4.0 (3)
SP (500 mg/kg)	20.5 ± 1.1 (5)

a, Mean ± SEM (n).

Thus, the present study shows that SS or SP at the dose which markedly reduced fertility of the male rats has no effect on testicular functions. In view of these findings together with the rapid onset of the antifertility effect and recovery after drug withdrawal, the site of action of these sulfonamides is most likely the epididymis.

ACKNOWLEDGEMENTS

This work was supported by US-Israel CDR Program, grant no. 936-5544-G-00-6105-00. We thank Drs. Sukumarn Chongthamkul and Kanok Pavasuthipaisit for assaying serum testosterone and LH, and Mrs. Suthada Sukuttakij for the preparation of the manuscript.

REFERENCES

1. LEVI, A.J., FISHER, A.M., HUGHES, L. & HENDRY, W.F. Male infertility due to sulphasalazine. Lancet 2, 276-278, 197[illegible].

2. TOTH, A. Reversible toxic effect of salicylazosulfapyridine on semen quality. Fert. Steril. 31, 538-540, 1979.

3. PHOLPRAMOOL, C. & SRIKHAO, A. Antifertility effect of sulfasalazine in the male rat. Contraception 28, 273-279, 1983.

4. O'MORAIN, C., SMETHURST, P. & DORE, C.J. Reversible male infertility due to sulphasalazine : Studies in man and

61

rat. Gut 25, 1978-1084, 1984.

5. PEPPERCORN, M.A. & GOLDMAN, P. The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. J. Pharm. Exp. Ther. 181, 555-561, 1972.

6. TOOVEY, S., HUDSON, E., HENDRY, W.F. & LEVI, A.J. Sulphasalazine and male infertility : reversibility and possible mechanism. Gut 22, 445-451, 1981.

7. PHOLPRAMOOL, C. & SRIKHAO, A. Suppression of fertility by sulfasalazine and its metabolites in the male rats. J. Androl. Suppl. 5, 50P, 1985.

8. JOHNSON,L., PETTY, C.S. & NEAVE, W.B. A comparative study of daily sperm production and testicular composition in human and rats. Biol. Reprod. 22, 1233-1243, 1982.

9. RESKO, J.A., MALLEY, A. BEGLEY, D. & HESS, L. Radioimmunoassay for testosterone during fetal development of the rhesus monkey. Endocrinology 93, 156-161, 1973.

10. DUFAU, M.L., BEITINS, I.Z., MCARTHUR, J.W. & CATT, K.J. Effect of luteinizing hormone-releasing hormone (LHRH) upon bioactive and immunoreactive serum LH levels in normal subjects. J. Clin. Endocrinol. Metab. 43, 658-667, 1976.

Appendix III

Structural requirements of some sulphonamides that possess an antifertility activity in the male rat

C. Pholpramool, S. Richirawat*,+, V. Verawatnapakul,
C. Paovalo*,+ and L. M. Lewin+

Department of Physiology and *Department of Chemistry, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand, +Chulabhorn Research Centre and Programme on Research and Development of of Synthetic Drugs, Institute of Sciences and Technology for Development, Mahidol University, and +Department of Chemical Pathology, Sackler Medical School, Tel Aviv. University, Ramat Aviv, Israel

Running title : Structure of sulphonamides and rat male fertility

Key words : sulphonamides, fertility, antifertility, rat

Send all correspondence and proofs to the first author.

Submitted for publication in J. Reprod. Fert. : June 27, 1990.
Accepted : Nov. 15, 1990.

Appendix IV

MODE OF ACTION OF THE ANTIFERTILITY SULPHONAMIDES : LACK OF EFFECTS ON FOLATE METABOLISM

C. Pholpramool[1], V. Verawatnapakul[1], S. Ruchirawat[2] & L.M. Lewin[3]

[1]Department of Physiology and [2]Department of Chemistry, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand, and [3]Department of Chemical Pathology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Israel

ABSTRACT

The effects of some antifertility sulphonamides on folate metabolism were investigated in the male rats. Subcutaneous injections of sulphanilamide at a dose of 150 mg/kg-day for 6 weeks produced a marked reduction in fertility of the treated animals. This effect was rapidly recovered by one week after drug withdrawal. Similar treatments with trimethoprim (30 mg/kg-day) or pyrimethamine (8 mg/kg-day) had virtually no effect on fertility. The synergistic effect of trimethoprim or pyrimethamine on the antifertility activity of sulphanilamide was not observed when the drugs were administered in combinations. Treatment with sulphapyridine (450 mg/kg-day for 6 weeks) failed to alter the levels of folate in the blood and the reproductive organs except the testes in which accumulation of folic acid occurred. The results suggest that the antifertility activity of sulphanilamide, sulphapyridine and, perhaps, some other sulphonamides is not associated with the inhibition of folate metabolism.

INTRODUCTION

It has been shown that sulphasalazine, sulphapyridine and some other sulphonamides suppressed fertility in male patients (1,2,3) and some laboratory male animals (4,5,6,7). However, the mode of action of these compounds is not known, although recent evidence suggests that the site of action of the antifertility sulpha drugs is most likely at the epididymis (8,9). Sulphasalazine inhibited intestinal absorption of folate (10) as well as folate enzymes in vitro (11). These effects have been ascribed to the infertility induced by sulphasalazine in the male patients with inflammatory bowel disease (12). Recently, we have demonstrated that the presence of N^4-amino group of the benzine sulphonamide is required for the antifertility activity (13). This requirement resembles that of the antimicrobial sulpha drugs. The

Submitted for publication in "CONTRACEPTION" : Aug. 14, 1990

Accepted : Oct. 25, 1990

61

mechanism of action of which is known to interfere with folate metabolism in bacteria (14). Therefore, the objective of this study is to determine whether the antifertility sulphonamides exert their effects through the intervention of folate metabolism of the body and/or the reproductive organs in the male rats.

MATERIALS AND METHODS

Animals

Adult male and female rats of Fischer strain were obtained from the Animal Quarter, Faculty of Science, Mahidol University. They were housed at ambient temperature under 14 h daylight with free access to pellet feed (Gold Coin, Singapore) and water. Only males with proven fertility were used in this study.

Fertility testing

Each male rat was cohabitated with two virgin female rats for one week. Mating was determined by the presence of spermatozoa in the vaginal smears obtained every morning during cohabitation. Both females were then removed from the males at the end of mating period, and kept separately for 8-10 days at which time pregnancy was examined after sacrifice. The numbers of fetuses and corpora lutea were counted. The latter was assessed under a dissecting microscope.

Fertility of the male rats was expressed as percentage fertility which is calculated from the ratio of fetuses to the number of corpora lutea times one hundred. When both female rats were mated by the same male, the average value was used for calculation.

Experimental procedure

Experiment 1 : (Effects of sulphanilamide and dihydrofolate reductase inhibitors on fertility)

Male rats were randomly assigned to 6 groups (8-10 rats each). The rats in Group I were subcutaneously injected with dimethylsulphoxide (DMSO; Merck, West Germany) daily for 6 weeks serving as controls. The animals in Groups II to VI were similarly treated with sulphanilamide (Sigma, St. Louis, U.S.A.) at a dose of 150 mg/kg, trimethoprim (Sigma, U.S.A.) at a dose of 30 mg/kg, pyrimethamine (Sigma, U.S.A.) at a dose of 8 mg/kg, combined sulphanilamide + trimethoprim, and combined sulphanilamide + pyrimethamine. Fertility was tested as described above by the end of 1,3 and 5 weeks during drug treatment, and 1 and 3 weeks after drug withdrawal.

Experiment 2 : (The effect of sulphapyridine on folate metabolism)

Nineteen male rats were divided into control (7 rats) and experimental groups (12 rats). The former was subcutaneously injected with DMSO daily for 5 weeks whilst the latter received sulphapyridine (Sigma, U.S.A.) dissolved in DMSO at a dose of 500 mg/kg-day for 5 weeks. Both groups were assessed for fertility as in Experiment 1 by weeks 1 and 3 during treatment. At the end of week-5, all rats were anaesthetized with ether and blood samples were collected from the catheter (PE50, Clay-Adams) inserted into the carotid artery. The animals were sacrificed later using overdoses of ether, and the testes, epididymides, prostate, seminal vesicle, coaggulating gland and liver were removed. The tissues were dissected freed off fat and cut in small portions, then rinsed with physiological saline and blotted to dryness with clean filter papers. After wet weights were obtained, they were homogenized in phosphate buffer pH 6.1 containing 150 mg% ascorbic acid using a teflon piston. The supernatants were separated by centrifugation and kept at -20° C for further assay for folic acid.

Whole, unclotted blood samples were obtained in the tubes containing sodium heparin and kept at 4° C. Clotted blood samples were also obtained in the tubes without addition of sodium heparin and warmed at 37° C for 30 min. Sera were separated by centrifugation and kept at -20° C for folate assay within one week.

Folic acid assay

Folate concentrations in blood and tissue samples were determined by a microbiological assay using *Lactobacillus casei* ATCC No. 7469 (15).

RESULTS

Effects of sulphanilamide and dihydrofolatereductase inhibitors on fertility

Sulphanilamide produced a significant reduction ($P<0.001$) in fertility by 3 weeks after treatment (Table 1). Further decrease in per cent fertility occurred by week-5. Rapid recovery of normal fertility was noted by one week after drug withdrawal. Neither trimethoprim nor pyrimethamine interfered with fertility of the male rats. When trimethoprim or pyrimethamine were given in combination with sulphanilamide, such treatment caused a suppresion of male fertility. Again, significant decreases were observed by one week after drug injection. However, recovery of fertility was prolonged till 3 weeks after the cessation of drug treatment (Table 1). The reduction in

Table 1. Effects of subcutaneous injection of sulphanilamide and trimethoprim or pyrimethamine on fertility of the male rats.

Treatment and dose (mg/kg)	Percentage Fertility					
	Before Treatment	During Treatment wk-1	wk-3	wk-5	After drug withdrawal wk-1	wk-3
Control (DMSO)	94.4±1.8 (10)	83.9±6.1 (10)	86.7±4.3 (9)	89.6±3.3 (10)	93.8±2.9 (5)	92.1±5.0 (5)
Sulphanilamide 150	90.4±4.0 (10)	80.3±5.2 (9)	43.2±7.3[c] (9)	35.2±9.7[c] (10)	70.8±11.5 (5)	87.2±7.7 (5)
Trimethoprim 30	93.9±1.3 (8)	93.7±2.5 (8)	89.8±5.9 (8)	80.3±12.1 (8)	90.3±2.5 (7)	85.4±6.1 (8)
Pyrimethamine 8	94.1±1.8 (8)	88.3±4.8 (8)	91.4±4.8 (8)	92.6±3.2 (8)	87.1±4.2 (7)	90.7±4.8 (7)
Sulphanilamide (150) + Trimethoprim (30)	93.2±2.7 (8)	88.9±2.8 (8)	69.3±5.7[a] (8)	62.4±5.5[c,d] (8)	50.6±9.4[b] (5)	85.9±6.9 (5)
Sulphanilamide (150) + pyrimethamine (8)	95.1±1.6 (8)	81.9±6.6 (8)	56.1±8.9[b] (7)	54.8±11.0[b] (8)	46.5±15.5[a] (5)	89.3±5.2 (5)

Values are mean±SEM from number of rats shown in the parentheses.
a,b,c, : $P<0.05$, 0.01 and 0.001 when compared to the corresponding values in the control group (t-test).
d : $P<0.05$ when compared to the corresponding values in the sulphanilamide group (t-test).

Table 2. The effect of sulphapyridine on folate concentration and content in the blood, liver and reproductive organs of the male rats.

Treatment	Folate concentration (ng/ml)		Folate content (ng/gm wet wt)				
	Blood	Serum	Liver	Testis	Epididymis	Prostate	Seminal vesicle
Control (DMSO)	225.8±34.9 (7)	46.1±1.1 (5)	7999.5±426.5 (6)	173.0±15.4 (7)	597.5±90.3 (7)	567.2±244.1 (4)	555.3±115.4 (4)
Sulphapyridine (500 mg/kg)	193.3±27.4 (12)	32.5±5.5 (11)	7101.1±381.9 (12)	313.8±37.9[a] (10)	892.2±145.5 (11)	307.7±44.2 (5)	621.4±120.3 (5)

Values are mean±SEM from number of rats shown in the parentheses.
[a] $P<0.01$ when compared to the control (F-test).

fertility by 5 weeks after treatment with combined sulphanilamide and trimethoprim was significantly less than that induced by sulphanilamide alone. On the other hand, pyrimethamine failed to demonstrate this effect.

The effect of sulphapyridine on blood and tissue folate contents

The levels of folate in blood, liver and reproductive organs are shown in Table 2. These parameters were not changed after treatment with sulphapyridine except in the testis in which a significant increase in tissue folate contents occurred.

DISCUSSION

We have recently shown that both sulphanilamide and sulphapyridine possess strong antifertility activities in the male rats (13). However, the mode of action of these sulphonamides is not known. In order to determine whether the antifertility activity is related to inhibition of folate metabolism as is known for the antimicrobial activity, the effects of dihydrofolatereductase inhibitors were studied. These compounds failed to inhibit male fertility. The results support those previously reported (8).

It is known that inhibitors of dihydrofolate reductase enzymes, e.g. pyrimethamine and trimethoprim, potentiate the antibacterial activity of sulpha drugs (16). Such a synergistic effect on the inhibition of fertility was not observed when combined sulphanilamide and pyrimethamine or trimethoprim was administered. By contrast, it seemed that trimethoprim diminished the effect of sulphanilamide. These results, therefore, suggest that the antifertility activity of sulphanilamide is not associated with the inhibition of folate metabolism. In addition, when the antifertility and antimicrobial activities of some sulphonamides are compared, there appears to be no correlation between these two parameters (Table 3). Furthermore, other sulphonamides that have been shown by us to be highly effective in suppression of male fertility, i.e. N^1-acetylsulphanilamide, N^4-acetyl-N^1-methylsulphanilamide, N^4-acetyl-N^1-diethylsulphanilamide and 2(N^4-acetylsulphanilamide) pyridine (13), had virtually no *in vitro* antibacterial activity against *E.coli*, *S. aureus* or *S. faecalis* at concentrations up to 0.3 mmol/L (18).

Direct measurements of the levels of folate in the blood, liver and the reproductive organs corroborate the above conclusion. Thus sulphapyridine at the dose which produced a marked decrease in fertility had no effect on folate contents in the blood, liver and the accessory

Table 3. Lack of correlation between antibacterial and antifertility activities of some sulphonamides.

Sulphonamides	% Inhibition of fertility[a]	Antibacterial activity[b] (umole/L).
Sulphanilamide	82	128
Sulphapyridine	74	4.8
Sulphacetamide	24	2.3
Sulphioxazole	18	2.15
Sulphamerazine	17 28[c]	0.95

[a] (100-% fertility) by 6 weeks after intragastric intubation of the drug at a dose of 450 mg/kg-day, data from (13).
[b] In vitro activity against E. coli, data from (17).
[c] Data from (8).

sex organs. In contrast, the testes of the sulphapyridine-treated rats accumulated more folic acid than the controls. The significance of this result is not known at present. This together with the lack of correlation between the antibacterial and antifertility activities strongly suggest that the antifertility sulphonamides do not interfere with folate metabolism. The actual mechanism of action of these compounds on fertility is currently being investigated.

We thank Professor Dr. Suvit Areekul and Miss Jirarat Jeeramakorn for their helps in the folate assays, and Mrs. Suthada Sukuttakij for typing the manuscript. This work was supported, in part, by USAID grant no. 936-5544-G-00-6105-00 and Mahidol University.

REFERENCES

1. Levi, A.J., Fisher, A.M., Hughes, L. and Hendry, W.F. Male infertility due to sulphasalazine. Lancet 2: 276-278 (1979)

2. Toth, A. Reversible toxic effect of salicylazosulfapy ridine on semen quality. Fert Steril 31: 538-540 (1979)

3. Giwercman, A. and Skakkeback, N.E. The effect of salicylazosulphapyridine (sulphasalazine) on male fertility. A review. Internat J Androl 9: 38-52 (1986)

4. Pholpramool, C. and Srikhao, A. Antifertility effect of sulfasalazine in the male rat. Contraception 28: 273-279 (1983)

5. O'Morain, C., Smethurst, P. and Dore, C.J. Reversible male infertility due to sulphasalazine : Studies in man and rat. Gut 25: 1078-1084 (1984)

6. O'Morain, C., Smethurst, P. and Levi, A.J. Reversible male infertility induced by sulphasalazine. In Male Fertility and its Regulation. (T.J. Lobe and E.S.E. Hafez, Editors) MTP Press, Lancaster, 1985, p. 49-54.

7. Srikhao, A. and Pholpramool, C. The antifertility effects and toxicity of sulfasalazine and sulfapyridine on male rats, hamsters and mice. Thai J Physiol Sci 2: 11-20 (1989)

8. Wong, P.Y.D., Lau, S,K.D. and Fu, W.O. Antifertility effects of some sulphonamides and related compounds and theirs accumulation in the epididymides of male rats. J Reprod Fertil 81: 259-267 (1987)

9. Pholpramool, C., Verawatnapakul, V., Srikhao, A, Ruchirawat, S. and Lewin, L.M. Lack of effects of some antifertility sulfonamides on testicular functions in the male rats. Mini-Posters, IVth Internat Cong Androl, Monduzzi Editore, Bologna, 1989, p. 27-31.

10. Frankin, J.L. and Rosenberg, I.H. Impaired folic acid absorption in inflammatory bowel disease: effects of salicylazosulfapyridine (Azulfidine). Gastroenterology 64: 517-525 (1973)

11. Selhub, J., Dhar, G.J. and Rosenberg, I.H. Inhibition of folate enzymes by sulfasalazine. J Clin Invest 61: 221-224 (1978)

12. Traub, A.I., Thompson, W and Carville, J. Male infertility due to sulfasalazine. Lancet 2 : 639-640 (1980)

13. Pholpramool, C., Ruchirawat, S., Verawatnapakul, V., Paovalo, C. and Lewin, L.M. Structural requirements of some sulphonamides that possess an antifertility activity in the male rat. J Reprod Fertil (Submitted, 1990)

14. Woods, D.D. The biochemical mode of action of the sulphonamide drugs. J Gen Microbiol 29: 687-702 (1962)

15. Cooperman, J.M. Microbiological assay of folic acid activity in serum and whole blood.In Methods in Enzymology, Vitamins and Coenzymems Part B (D.B. mcCormick and L.D. Wright, Editors) Academic Press, New York, 1971, Vol. XVIII, p. 629-642.

16. Bushby, S.R.M. and Hitchings, G.H. Trimethoprim, a sulphonamides potentiator. Br J Pharmac Chemother 33: 72-90 (1968)

17. Anand, N. Sulfonamides and sulfones. In Burger's Medicinal Chemistry, 4th ed., Part II (M.E. Wolff, Editor). John Wiley & Son, New York, 1979, Chap. 13, p. 1-40.

18. Nerapattanakul, S. Mutagenic and antibacterial tests of newly synthesized sulfa drugs. M.Sc. Thesis, Mahidol University 1990, p. 61-66.

Appendix V

SUPPRESSION OF MALE FERTILITY BY SULPHA DRUGS

C. Pholpramool[1], S. Ruchirawat[2,3], C. Paovalo[2,3], V. Verawatnapakul[1] and L.M. Lewin[4]

[1]Department of Physiology and [2]Department of Chemistry, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand [3]Chulabhorn Research Centre and Programme on Research and Development of Synthetic Drugs, Institute of Sciences and Technology for Development, Mahidol University, and [4]Department of Chemical Pathology, Sackler Medical School, Tel Aviv University, Ramat Aviv, Israel

Running Title : Sulpha drugs and male fertility

Submitted for publication in "Proceedings of the 2nd Congress of AOPS, Nov. 12-16, 1990, New Delhi, India.

SUMMARY

A number of sulphonamides having structures related to sulphapyridine, which is responsible for the antifertility side effect of sulphasalazine in the ulcerative colitic patients, has been shown to suppress fertility of the male rats. It appears from the studies of structure-activity relationship that the pyridine ring is not essential but the presence of N^4-amino group is required for the activity. Full activity is retained after the pyridine group is replaced with hydrogen atoms or an alkyl group. Thus, the prototype of the antifertility sulphonamides is sulphanilamide. Presently available evidence indicates that these compounds act at the late spermatid or at the maturation processes in the epididymis. The latter involves either direct interactions of sulpha drugs and epididymal spermatozoa or alterations of epididymal functions leading to changes in the optimal microenvironment for sperm maturation. The potential for future development of male contraceptives from sulphonamide compounds seems promising.

The effect of sulpha drugs on male fertility has been reported since 1979 independently by the British (1) and the American (2) physicians. It was noted that male patients of reproductive ages receiving therapeutic doses of sulphasalazine (SS) for threatment of ulcerative colitis were unable to impregnate their wives. However, these patients resumed their normal fertility after drug withdrawal. The effect of SS was not associated with changes in the levels of gonadotrophin or testosterone, but a reduction in sperm count, sperm motility and the presence of megalo-head spermatozoa in semen were observed. Similar effect of SS on fertility of some male laboratory animals has also been demonstrated (3,4,5).

When given by an oral route, only small amount of SS is absorbed in the small intestine. The major fraction is transported to the colon where it is metabolized by bacteria to yield sulphapyridine (SP) and 5-aminosalicylic acid. The former is readily absorbed by the colon but the latter is not (6,7). Studies in the rat showed that SP, not 5-aminosalicylate, was infact the active moiety of SS (8,9). This conclusion also received support from clinical studies (10,11,12).

Although direct clinical studies of the effect of SS on fertility of normal subjects are lacking, results from the animal models clearly show that SS or SP possesses male contraceptive effect. The effect of SS on male fertility has previously been reviewed (13). This report aims to add more recent informations and to explore the possibility for the development of new male contraceptive agent from sulpha drugs.

BASIC STRUCTURE FOR ANTIFERTILITY ACTIVITY

Recent studies by Wong and his colleagues (14) demonstrated that, besides SP, some other sulphonamides such as dapsone, sulphamerazine, sulphamethazone, sulphathiazole, sulphamethoxypyridazine, sulphadimethoxine and sulphaguanidine all reduced fecundity of the male rats. On the other hand, sulphamethizole (8) and sulphamethoxazole (14) were ineffective.

We have also investigated a series of SP analogues and found that sulphioxazole, sulphamerazine and sulphadimethoxine had virtually no antifertility activity in the male rats whereas sulphachloropyridazine had some effect (Table 1). Replacements of the pyridine group of SP by H atoms or alkyl groups retained the potency. However, removal of the N^4-amino group on the benzine ring or substitution with a methyl group completely abolished the activity. The results suggest that the pyridine group is not required for the antifertility activity since the simplest sulphonamide, sulphanilamide, is also effective. The data reveal further that the N^4-amino group in the benzene ring is indispensable. Thus, the basic structure of the contraceptive sulphonamide is shown in Fig. 1.

ACTIVE FORM

SP is converted in the liver to N^4-acetyl SP and 5-hydroxy SP before being conjugated with glucose to form glucuronides and then excreted in the urine (15). In man, the reduction in sperm count of the genetically slow acetylators was greater than the fast acetylators whereas motility and abnormal forms of spermatozoa were not different in the two groups of

76

patients (4). Both SP and N^4-acetyl SP, when given by an oral route, produced equal suppressions of fertility in the male rats (Table 1). Preliminary studies of the metabolites excreted in the urine of SP-treated male rats showed an interesting result. It would appear that the animals with the highest amount of free and total SP (SP+N^4-acetyl SP+5-hydroxy SP+glucuronides) in the urine were least fertile. Those that had the highest percentage of the sulpha drug in acetylated or hydroxylated forms were most fertile. The results indicate that, like human, there are also slow and fast acetylators in the rat and the active form is probably free SP. Further studies along this line is currently under investigation.

MODE OF ACTION

The time required for the completion of spermatogenesis in rats is 48 days. SP and some of its analogues caused a reduction in fertility by 3-5 weeks. At the dose of 450 mg/kg significant decreases in fecundity occurred within 1 week. Therefore, SP-induced infertility could not be accounted for by the inhibition of spermatogenesis. Indeed, measurements of daily sperm production rate in the SP-treated animals support this conclusion (16). Furthermore, SP did not alter blood levels of LH and testosterone (Fig. 2). The site of action of SP and perhaps other antifertility sulphonamides is most likely distal to the testis, i.e. at the epididymis. However, in view of the findings that the epididymal sperm reserve is decreased (3) and a higher percentage of abnormal spermatozoa appears in the semen

(1), the possibility that these compounds affect the late stage of spermatogenesis is still tenable.

Maturation of epididymal sperm involves morphological, biochemical and physiological changes. A reduction in sulfhydryl group of the spermatozoa occurs during their transit through the epididymis (17). By using both a fluorimetric method and a Fluorescene Activated Cell Sorter to quantitatively determine the monobromobimane-stained sperm (18), we were unable to demonstrate changes in the thiol group of the caput, corpus and cauda spermatozoa in the SP-treated rats (Fig. 3). However, SDS PAGE analyses of the proteins in the fluids removed by micropuncture from the caput and cauda epididymidis showed changes of some protein bands in the SP-treated rats (Table 2). When the sperm surface proteins were determined, the profile of the caput sperm proteins was virtually unchanged, but there were at least 3 additional proteins of MW 85K, 44K and 28K appeared on the cauda sperm of the SP-treated animals. These proteins were found only in the caput spermatozoa. It is, therefore, tempting of speculate that SP prevents the loss of or alteration of some membrane proteins in the immature spermatozoa, which is necessary during sperm maturation.

The effect of SP and some other sulphonamides on epididymal spermatozoa may be direct or indirect through changes in the epididymal functions and hence fluid compositions. Previously it has been reported that SP, sulphamerazine and dapsone are accumulated in the epididymal fluid at a higher concentration than in the blood (14). On the other hand, there is evidence that SP produced a change in the epididymal fluid

electrolyte concentrations. Thus potassium concentration in the cauda fluid of SP-treated rats is higher than the controls (Fig. 4). It is not known whether the excess potassium is derived from sperm cells or epididymal epithelium.

In conclusion, it seems that the possibility for developing sulphonamides other than SP, which is not clinically acceptable, for male contraception is now promising. Currently available evidence suggests that these compounds act at the epididymis. However, the mechanism of action is largely unknown. Further studies of these compounds are acutely needed.

ACKNOWLEDGEMENTS

This study was supported by grants from US-Israel CDR program (Grant No. 936-5544-G-00-6105-00), Chulabhorn Research Institute and Mahidol University.

References

1. Levi AJ, Fisher AM, Hughes L, Hendry WF. Male antifertility due to sulphasalazine. Lancet 1979; 2: 276-278.
2. Toth A.Reversible toxic effect of salicylazosulfapyridine on semen quality. Fert Steril 1979; 31: 538-540.
3. Pholpramool C, Srikhao A. Antifertility effect of sulfasalazine in the male rat. Contraception 1983; 28: 273-279.
4. O'Morain CO, Smethurst P, Dore CJ, Levi AJ. Reversible male infertility due to sulphasalazine : studies in man and rats. Gut 1984; 25: 1078-1084.

5. Srikhao A, Phoipramool C. The antifertility effects and toxicity of sulfasalazine and sulfapyridine on male rats, hamsters and mice. Thai J Physiol Sci 1989; 2: 11-20.

6. Schroder H, Campbell DES. Absorption, metabolism and excretion of salicylazosulfapyridine in man. Clin Pharmacol Ther 1972; 181: 555-561.

8. O'Morain CO, Smethurst P, Hudson E, Levi AJ. Further studies on sulphasalazine induced male infertility. Gastroenterology 1982; 82: 1140A.

9. Srikhao A. Antifertility effect of sulfasalazine and its metabolites in the male rats. Ph.D. Thesis Mahidol University 1987.

10. Cann PA, Holdsworth CD. Reversal of male infertility on changing treatment from sulfasalazine to 5-aminosalicylic acid. Lancet 1984; 1: 1189.

11. McIntyre PB, Lennard-Jones JE. Reversal with balazide on fertility caused by sulfasalazine. Br Med J 1984; 288: 1652-1653.

12. Shaffer JL, Kershaw A, Berrisford MH. Sulphasalazine-induced infertility reversed on transfer to 5-aminosalicylic acid. Lancet 1984; 1: 1240.

13. Giwercman A, Skakkeback NE. The effect of salicylazosulphapyridine (sulphasalazine) on male fertility. A review. Int J Androl 1986; 9: 38-52.

14. Wong PYD, Lau SKD, Fu WO. Antifertility effect of some sulphonamides and related compounds and their accumulation in the epididymides of male rats. J Reprod Fert 1987; 81: 259-267.

15. Das KM, Dubin R. Clinical pharmacokinetics of sulphasalazine. Clin Pharmacokinet 1976; 1: 406.

16. Pholpramool C, Verawatnapakul V, Srikhao A, Ruchirawat S, Lewin LM. Lack of effects of some antifertility sulfonamides on testicular functions in the male rats. Proc 4th Int Congr Androl, Florence 1989: 27-31.

17. Calvin H, Bedford JM. Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. J Reprod Fert Suppl 1971; 13: 65-75.

18. Shalgi R, Seligman J, Kosower NS. Dynamics of the thiol status of rat spermatozoa during maturation. Analysis with the flourescent labelling agent monobromobimane. Biol Reprod 1989; 40: 1037-1045.

Legends for the figures

Figure 1 Basic structure of the antifertility sulphonamide compounds.

Figure 2 Serum levels of testosterone and LH in the control (□) and SP-treated (▨) male rats before (week-0) and by 5 weeks during treatment. SP was suspended in corn oil and was given by an intragastric intubation at a dose of 450 mg/kg/day. Numbers in the column represent number of rats in each group.

Figure 3 Total SH content of the spermatozoa from different regions of the epididymis of control and SP-treated rats. The animals were fed with untreated or SP impregnated rat chow to yield the dose of 1 g/kg/day for 40 days. Numbers in the column represent number of rats in each group.

Figure 4 Electrolyte concentrations in the luminal fluid removed by micropuncture from the caput and cauda epididymidis of control and SP-treated rats. The animals were treated as described in Fig. 2. Numbers in the column represent number of rats in each group.

Table 1. The effect of sulphapyridine and its analogues on fertility of the male rats.

Compound	Dose (mg/kg)[a]	%fertility[b] (mean±SEM,n)
Sulphapyridine	450	25.9±8.6(6)[e]
Sulphioxazole	450	82.1±8.4(6)
Sulphamerazine	450	83.4±7.5(5)
Sulphadimethoxine	150	93.9±1.9(7)
Sulphachloropyridazine	450	74.6±5.5(5)[c]
Sulphanilamide	450	18.0±9.0(4)[e]
Sulphacetamide	450	77.5±7.3(10)
N^1-Methylsulphanilamide	450	6.8±4.3(4)[e]
N^1-Diethylsulphanilamide	450	22.8±8.1(5)[e]
N-2-Pyridinylbenzene sulphonamide	450	91.6±4.6(6)
4-Methyl-N-2-pyridinyl-benzenesulphonamide	450	89.4±6.9(7)
4-Nitro-N-2-pyridinyl-benzenesulphonamide	450	56.2±10.7(7)
2-Nitro-N-2-pyridinyl-benzenesulphonamide	450	82.4±5.9(8)
4-Bromo-N-2-pyridinyl-benzenesulphonamide	450	56.2±16.1(7)

Table 1. (Continued)

Compound	Dose (mg/kg)[a]	%fertility[b] (mean±SEM,n)
3-Nitro-N-2-pyridinyl-benzenesulphonamide	450	66.5±11.0(6)
N^4-Acetylsulphanilamide	450	42.6±12.5(7)[d]
N^4-Acetyl-N^1-methyl-sulphanilamide	450	17.2±6.7(6)[e]
N^4-Acetyl-N^1-diethyl-sulphanilamide	450	21.0±11.6(3)[e]
2-(N^4-Acetylsulphanilamido) pyridine	450	31.1±16.4(6)[c]
N^4-Acetyl-methyl-N^1-diethyl-sulphanilamide	450	18.6±5.9(7)[d]

[a] All compounds were administered by intragastric intubation daily for 5 weeks.

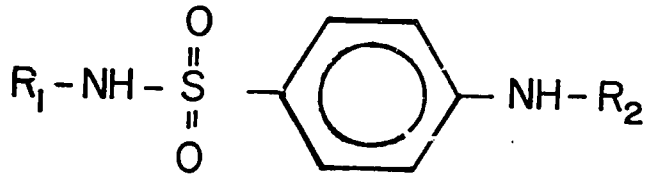
[b] % fertility was determined from the ratio of embryos and corpula lutea X100 at week-5 during treatment.

[c] $P<0.05$; [d] $P<0.01$; [e] $P<0.001$ when compared to the control group.

84

Table 2. The effect of sulphapyridine on proteins in the epididymal fluid and epididymal sperm membrane in rats. Fluid samples were removed by micropuncture from the lumen of the mid-caput and mid-cauda epididymidis. Sperm-free fluids and sperm membrane extracts were applied to a linear gradient (7-15%) SDS PAGE.

Source	Sample	Changes in protein bands from control
Caput	Fluid	decreases in 32K, 31K, 28K and 26.5K
	Sperm	none
Cauda	Fluid	decreases in 84K, 77K, 31K, 28K,26.5K
	Sperm	appearances of new proteins (85K,44K,28K)



R_1 = alkyl , pyridine or saturated N-heterocyclic ring

R_2 = H or $CH_3-\overset{O}{\overset{\|}{C}}-$

Figure

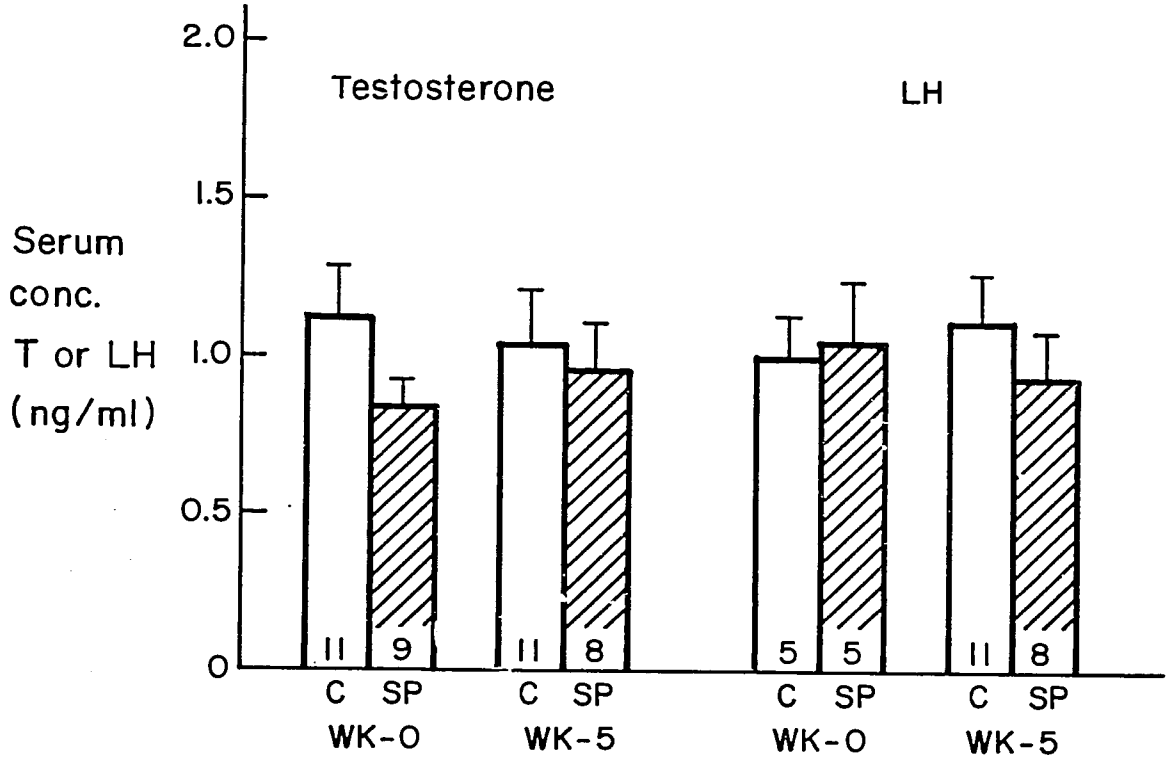

Testosterone
LH
Serum
conc.
T or LH
(ng/ml)
2.0
1.5
1.0
0.5
0
11
9
11
8
5
5
11
8
C
SP
C
SP
C
SP
C
SP
WK-0
WK-5
WK-0
WK-5

Figure 2

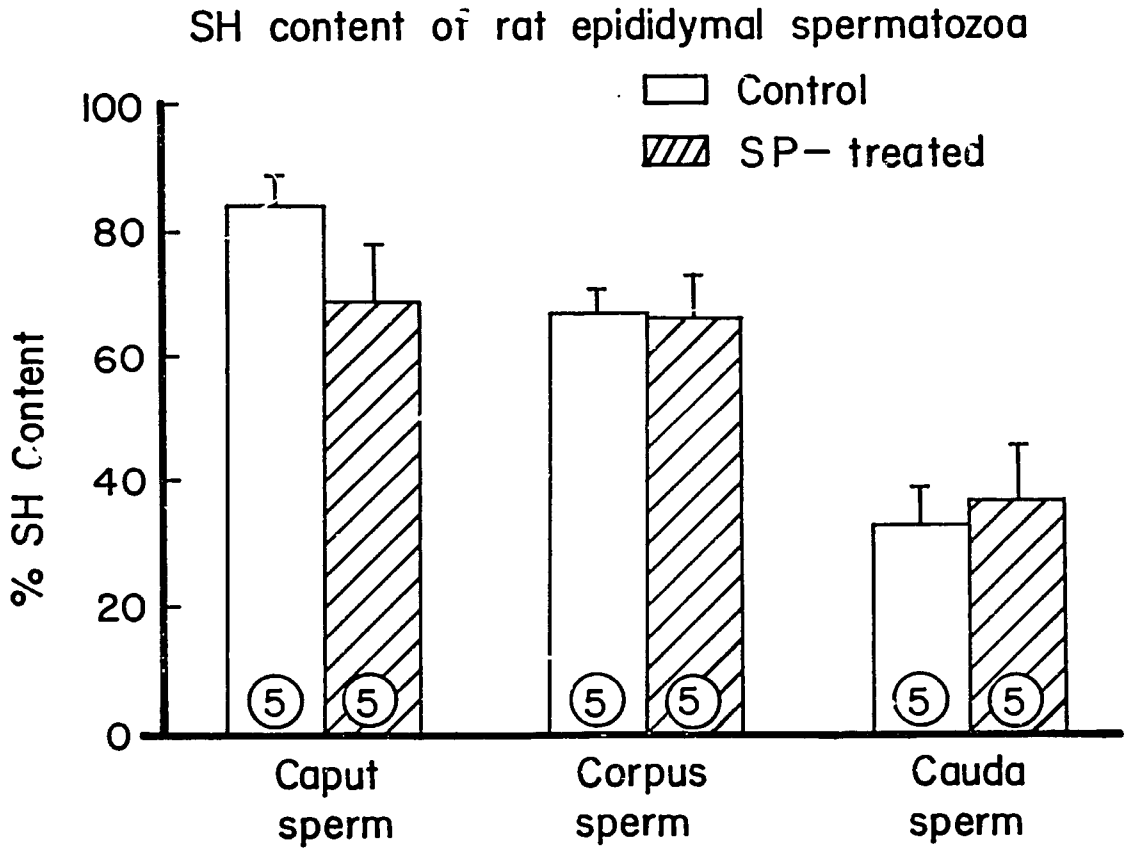

SH content of rat epididymal spermatozoa
Control
SP– treated
% SH Content
100
80
60
40
20
0
5
5
5
5
5
5
Caput sperm
Corpus sperm
Cauda sperm

Figure 3.

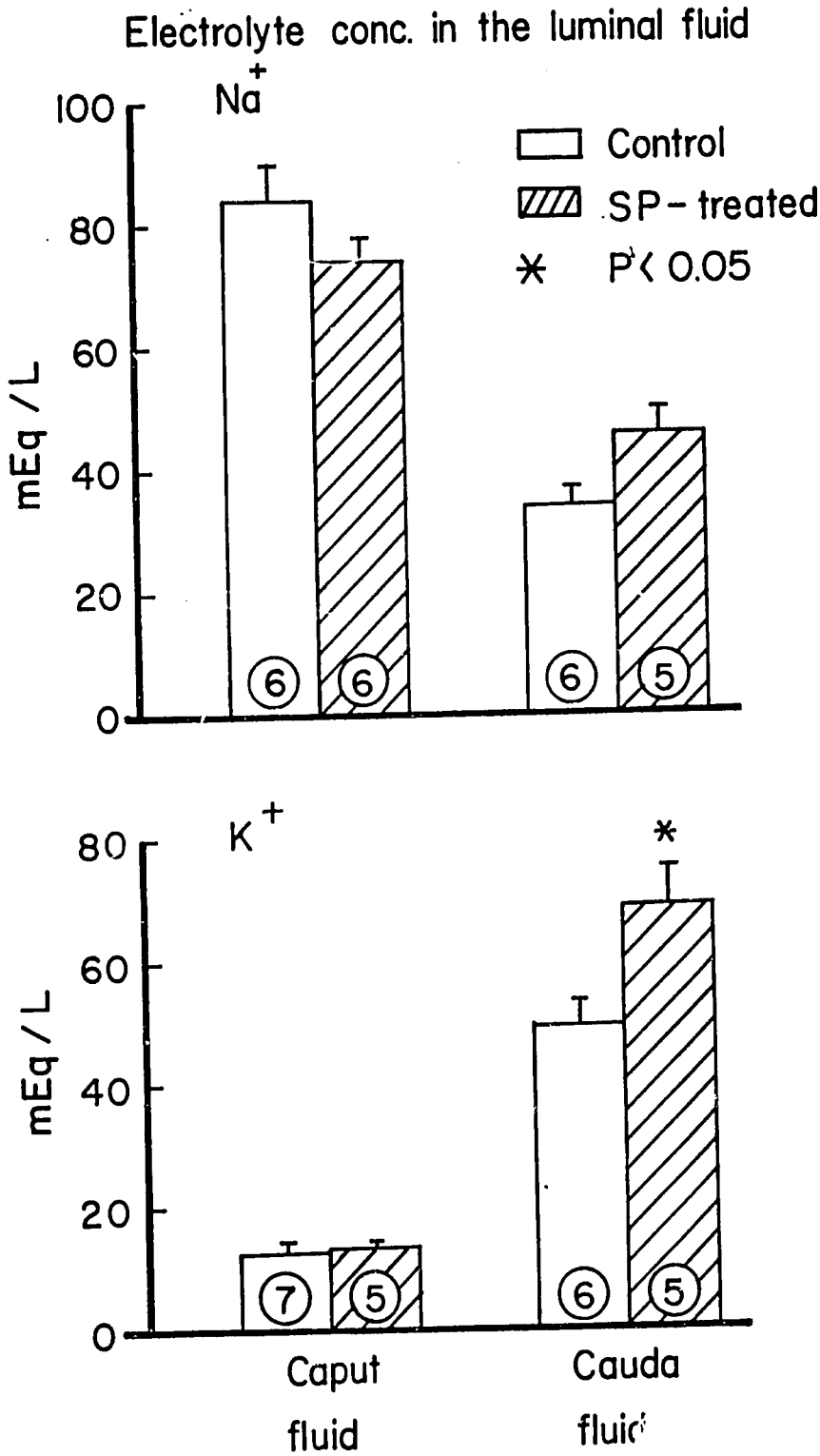

Electrolyte conc. in the luminal fluid
Na+
100
80
60
40
20
0
mEq / L
Control
SP - treated
* P < 0.05
6
6
6
5
K+
80
60
40
20
0
mEq / L
*
7
5
6
5
Caput fluid
Cauda fluid

Figure 4

Appendix VI

BIOAUTOGRAPHIC DETECTION OF SULFA DRUGS AND THEIR DERIVATIVES USING BACILLUS STEAROTHERMOPHILUS

R.Golan[1], O. Wassercug[1], P.Freidlin[2], C. Pholpramool[3], and L.M. Lewin[1]

[1]Department of Chemical Pathology, Sackler School of Medicine, Tel Aviv University, and [2]Poultry Disease Laboratory, Kiryat Malachi, [3]Department of Physiology, Mahidol University, Bangkok, Thailand.

Running title: Golan et al: Bioautography of Sulfa Drugs

Date Received:

Correspondence should be sent to:

Prof. Lawrence M. Lewin
Dept of Chemical Pathology
Tel Aviv University Sackler Medical School
Ramat Aviv, ISRAEL

SUMMARY: A method is described for bioautographic analysis of sulfa drugs. The test organism Bacillus stearothermophilus allowed detection of these compounds after their separation by paper chromatography. The system described allowed separation and detection of various sulfa drugs in the range of 0.05-1.0 micrograms.

KEY WORDS: bioautography; sulfa drugs; Bacillus stearothermophilus

.91

METHODS

Paper chromatography

Ascending paper chromatography was conducted on Whatman No.1 paper. Samples were applied on a line 8 cm above the bottom edge, the sheets stapled into a cylinder and then placed in a tank (21 cm D) containing 139 ml of solvent system as described in Table 1. Chromatograms were developed for about 16 h. The sheets were then air-dried. Prior to placing on agar plates containing *B. stearothermophilus* the developed chromatograms were heated (1 h, 60° C) in a ventilated oven to remove traces of chromatographic solvents.

Growth of Bacillus stearothermophilus and preparation of bioautographic plates

To prepare inocula, bacilli were grown on Mueller Hinton Agar plates and suspended in sterile NaCl (0.9%, to A_{600}=0.2). Mueller Hinton II agar (5.7 g of the powder to 150 ml of water was supplemented with indicator (bromcresol purple 6 µg), autoclaved (121°, 15 min), cooled to 48°, and trimethoprim (10 µg) and inoculum (5.0 ml) were added before pouring into a transparent, rectangular, sterile glass baking dish (22x34 cm), fitted with an aluminium cover. Bubbles were removed by passing a Bunsen burner flame over the surface of the liquid agar. The agar was allowed to harden for at least 20 minutes before applying paper sheets to the surface. Plates were incubated at 50° C for 16-24 h and locations of inhibition zones identified as blue zones on a yellow background.

RESULTS AND DISCUSSION

Paper chromatographic separation of various sulfa drugs was accomplished as shown in Table 1. The solvent system was modified from that described in Block et al (4) to provide a single-phase system. Bioautography detected sulfa compounds as shown in Figs. 1 and 2. and Table 1.

The thermophilic bacterium, Bacillus stearothermophilus, grew rapidly at 50° and when grown in Mueller-

INTRODUCTION

Sensitive and specific methods are needed to investigate the sulfa drug composition of medical formulations or their metabolites present in body fluids. In bioautographic methods test compounds (growth stimulators or inhibitors) are separated by paper chromatography and detected by placing the chromatogram on an agar plate containing a microoorganism (1). In the case of antibiotics, after incubation under appropriate conditions the plates show inhibition zones corresponding to the Rf values of the growth inhibitors which have diffused into the agar medium. The areas of the growth zones can be compared with those of standards for semiquantitative estimation of amounts present (2). Growth of the thermophilic bacterium, Bacillus stearothermophilus, has been shown to be sensitive to inhibition by sulfa drugs (3). When paper chromatograms containing these compounds were applied to agar plates containing this test organism detection of these compounds and certain of their derivatives could be achieved.

MATERIALS AND METHODS

MATERIALS

The test organism, Bacillus stearothermophilus ATCC12980 was obtained from Dr.Stefan Soback and Dr. Gideon Ziv of the Volcani Institute, Bet Dagan, Israel. Whatman No. 1 paper was from Whatman Ltd, UK, Bacto agar was from Difco Laboratories, Detroit MI, USA, Mueller Hinton II Agar from BBL Microbiology Systems, Cockeysville, MD, USA. Sulfapyridine, sulfathiazole, sulfacetamide, sulfanilamide, from Sigma Chemical Company, St. Louis, MO, USA, and the sulfa-containing commercial drug preparations: Doxiprim, Vitamed, Ltd(sulfadoxine), Methoxiprim, Vitamed Ltd(sulfadimethoxine), Farmaprim,Vitamed(sulfadimidine) , Colifarm, Abic Pharm. Co.(sulfachloropiridazine), Pyrisul, Teva Pharm.(sulfaquinoxaline), Tridiazine, Plantex (sulfadiazine).

Hinton Agar it produced acid which caused the indicator bromcresol purple to change to a yellow color. Inhibition zones appeared blue-purple on a yellow background.

Sulfa drugs are acetylated by the liver prior to excretion in urine (5). The N4-acetyl derivatives are not able to inhibit growth of the test organism. They can be detected on the paper as fluorescent compounds.

Metabolites of sulfapyridine which were present in urine were separated by paper chromatography and detected by bioautography (Fig.2). Further studies are being conducted to identify and quantitate these metabolites.

The bioautographic method described here is relatively easy to perform, requires inexpensive equipment, and is thus suitable for laboratories in developing countries. Newer methods for analysis of sulfa drugs based upon high performance liquid chromatography are available (6,7,8). These are effective and sensitive but require specialized equipment and highly trained personnel.

ACKNOWLEDGMENTS

This project was supported, in part, by a grant to LML from the US Agency for International Development.

REFERENCES

1. Winsten W.A. & Eigen E. (1948) Paper Partition Chromatographic Analysis and Microbial Growth Factors. Proc. Soc. Exptl Biol. Med. 67,513-7.

2. Simpson J.S.(1963) Microbiological Assay Using Large Plate Methods. in Analytical Microbiology (Kavanagh,F., ed.) pp. 88-124. Academic Press, New York.

.cy

3. Wahlig H. & Holt R.J. (1976) A Rapid Microbiological Procedure Using Bacillus stearothermophilus for the assay of antibacterial drugs. J. Clin. Pathol. 29, 858-61.

4. Block R.J., Durrum E.L. & Zweig G.(1958) A Manual of Paper Chromatography and Paper Electrophoresis. 2nd edn.p. 398, Academic Press, New York.

5. Vree T.B., Nouws J.F.M. & Hekster Y.. (1985) Comparative pharmacokinetic studies of sulfonamides. in Comparative Veterinary Pharmacology, Toxicology and Therapy.(Van Miert A.S.J.P.A.M., Bogaert M.G. & Debackere M., eds) pp. 173-185, MTP Press Ltd., Lancaster.

6. Vree T.B., Hekster Y.A. & Tijhuis M.W. (1985) Metabolism of sulfonamides, Antibiot. Chemother. 34,5-65.

7. Hansen S.H.(1989) Simple and rapid method for the simultaneous determination of the eight main metabolites and conjugates of sulfasalazine in human plasma, urine and faeces using dynamically modified silica. J. Chromatogr. Biomed. Appl. 491:175-185.

8. Rona K., Winkler V., Riesz T. & Gachalyi B. (1988) An assay of sulphasalazine, sulphapyridine and its acetylated and hydroxylated metabolites in biological fluids by liquid chromatography. Chromatographia 26,393-398.

Table 1. Rf Values and Sensitivities for Bioautographic Detection of Sulfa Drugs Using Bacillus stearothermophilus.

Sulfa drugs were applied to the origin of chromatograph sheets, as described in METHODS, and developed, ascending, in the solvent: n-butanol: water: concentrated NH_4OH, 125:12:2, v/v. Dried chromatograms were placed on bioautograph plates, incubated, and inhibition zones observed, all as described in METHODS.

COMPOUND	R_f	SENSITIVITY (ug)
SULFACETAMIDE	0.06	0.2
SULFADIAZINE	0.11	0.5
SULFADOXINE	0.14	0.5
SULFACHLOROPYRIDAZINE	0.14	0.5
SULFAQUINOXALINE	0.16	0.5
SULFADIMIDINE	0.22	0.5
SULFATHIAZOLE	0.23	0.2
SULFADIMETHOXINE	0.29	0.5
SULFANILAMIDE	0.51	1.0
SULFAPYRIDINE	0.72	1.0

Fig. 1. Bioautographic detection of sulfanilamide derivatives. Standards (2.5 μg) of sulfapyridine (1), sulfathiazole (2), sulfacetamide (3) and a combination of all of them (4) were applied to Whatman No. 1 paper, chromatographed and bioautographed as described in METHODS. The arrowed line marks the solvent front. The dark stippling represents blue-purple zones against a yellow background.

Fig. 2. Bioautography of rat urine. Urine samples and sulfapyridine standard were chromatographed and bioautographed as described in METHODS. Sample 1 = Sulfapyridine (5 μg), sample 2= control rat urine (10 μl) of control urine, sample 3= urine (10 μl) from rat fed sulfapyridine (approx. 350 mg/day). The arrowed line marks the solvent front. The dark stippling represents blue-purple zones against a yellow background.

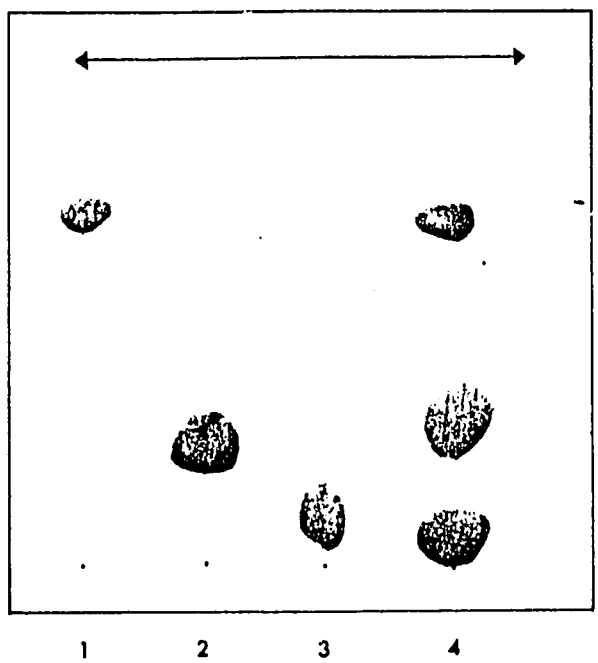


Fig. 1 Golan et al

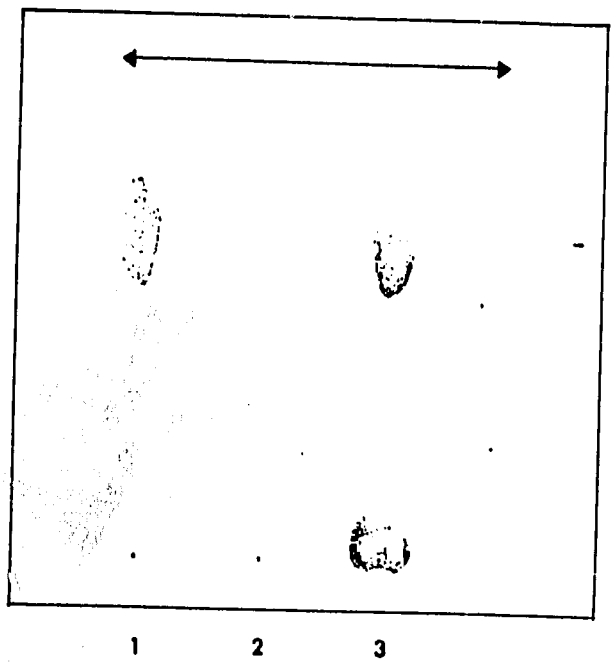


Fig 2 Golan et al

Fig9

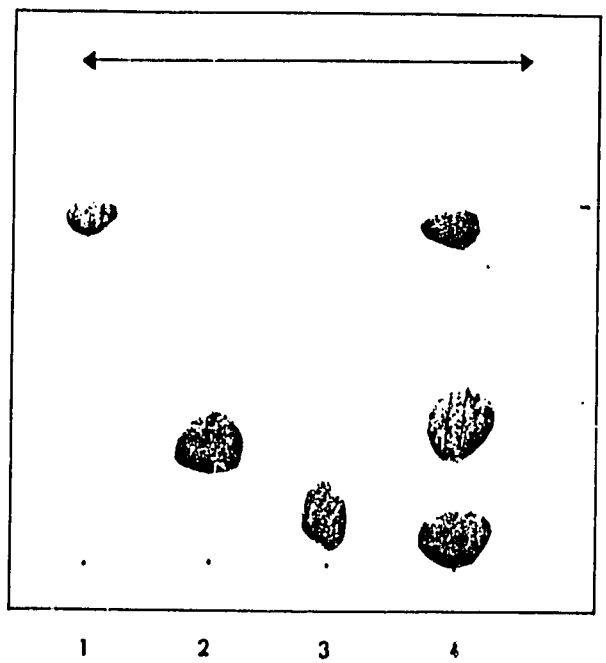


Fig. 1. Golan et al

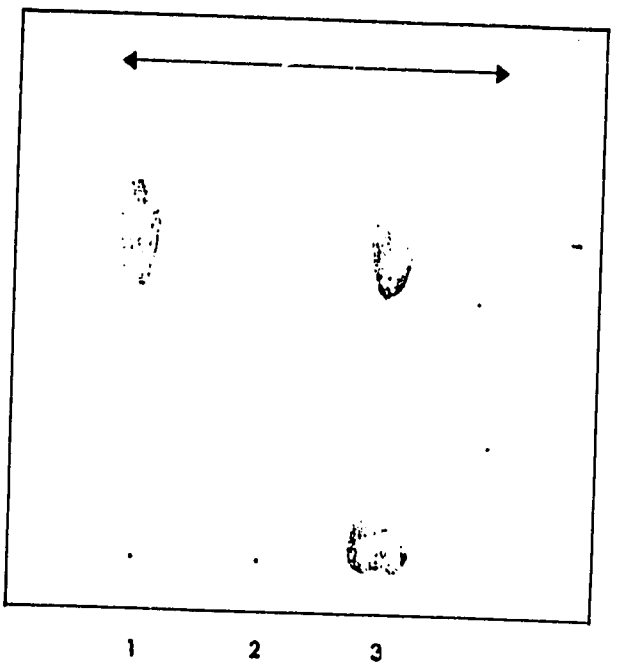


Fig 2 Golan et al

Appendix VII

ANTIFERTILITY EFFECTS OF SULFAPYRIDINE ON MALE HAMSTERS

R. Weissenberg[1], C. Pholpramool[2], and L.M. Lewin[3]

From [1]The Institute of Endocrinology, Sheba Medical Center, Tel HaShomer, Israel, [2]Department of Physiology, Mahidol University, Bangkok, Thailand, and [3]Department of Chemical Pathology, Sackler Medical School, Tel Aviv University, Ramat Aviv, Israel.

Submitted to ANDROLOGIA

102

SUMMARY

Sulfapyridine was administered to mature male golden hamsters either by impregnation in feed pellets, or by subcutaneous injection as a solution in dimethylsulfoxide. The average litter size for control males was 8.3±3.3(n=13) vs 4.2±2.7(n=11) for sulfapyridine-fed treated animals. Histology of the testis and epididymis was normal and sperm were normal in morphology and motility. Subcutaneous injection of sulfapyridine caused much more dramatic inhibition of male fertility than was achieved by feeding the drug. Sulfapyridine (750 mg/kg body weight in 0.2 ml DMSO) injected subcutaneously for 60 days was effective in reducing testis size and sperm quality. Histology of testes showed spermatogenic arrest at young spermatids. Two classes of animals were found in both sulfapyridine-fed and -injected treatment groups. One class was relatively resistant to the antifertility effects of the drugs. This difference may reflect differing abilities of the animals to convert sulfapyridine to an active form or to excrete its metabolites.

INTRODUCTION

Investigations on the inhibitory effects of sulfa drugs on male fertility were stimulated by the observation that some men receiving sulfasalazine treatment for ulcerative colitis were rendered temporarily infertile (LEVI ET AL, 1979). Using rat models, it has been shown that the sulfapyridine portion of the sulfasalazine molecule is the active moiety (LEVI ET AL, 1982). Rats have been used for most of the experimental work although hamsters were used by O'MORAIN ET AL (1985). SRIKHAO(1987) also found an antifertility effect of sulfasalazine on male hamsters, which were less affected by it than were rats.

Sulfasalazine is known to be transported unchanged to the colon where endogenous bacteria break it down to sulfapyridine and aminosalicylate (PEPPERCORN & GOLDMAN, 1973). Tests with rats have shown that it is the sulfapyridine moiety that caused decreased fertility (SRIKHAO, 1987). Sulfapyridine is insoluble in water. It has previously been instilled into the stomach of animals as a corn oil mull (PHOLPRAMOOL & SRIKHAO,1983). In this study we administered the test compound either after incorporation in feed pellets or by injection as a solution in dimethyl-sulfoxide. The studies reported here document and investigate infertility effects of sulfapyridine in mature male golden hamsters.

METHODS

Mature male Golden Hamsters were maintained at room temperature with free access to water. Feed pellets (Ambar Ltd, Hefer, Israel) was supplied ad lib. In specified experiments sulfapyridine was impregnated in the feed as follows: Sulfapyridine (14 gm.,Sigma Chemical Co., St Louis MO, USA) was dissolved in NaOH (0.5 M, 140 ml) and mixed uniformly with Ambar feed pellets (1 kg) before drying in a stream of air at 40°C overnight. Consumption of sulfapyridine in these experiments was determined by weighing the amount of feed consumed per rat-day. In other experiments sulfapyridine was administered by daily subcutaneous injection of sufficient sulfapyridine in dimethylsulfoxide (.2 ml) to provide a daily dose of 750 mg/kg. Administration of the drug was continued for 60 days.

Fertility of the animals was measured by mating the males with mature female golden hamsters and comparing the numbers of pups born to treated and control sires. Factors related to male fertility were assessed as follows: After the test period control and sulfapyridine-treated anomals were killed with ether, testes removed, placed in Bouin's solution, and stained with haematoxylin-eosin for histological examination. Cauda epididymides were cut up in Earle's medium (Biological Industries, Bet Haemek, Israel) and the epididymal sperm examined under microscope for motility, morphology, and sperm number.

Measurement of free and conjugated sulfapyridine in urine samples was by a modification of the method of Bratton and Marshall (SPINK, 1942).

RESULTS AND CONCLUSIONS

A decrease in fertility was observed in male hamsters which had eaten feed impregnated with sulfapyridine (770 mg/kg body weight per day)(Table 1). This difference was statistically significant by Student's T-test($p=0.53\times10^{-3}$). This decrease in fertility was not associated with significant differences in such sperm parameters as motility, morphology or sperm count in the cauda epididymis. Testicular histology of animals fed sulfapyridine did not differ significantly from that of control animals. This treatment reduced fertility but did not abolish it and it was noted that some animals seemed to be relatively resistant to the antifertility effect.

It is known that sulfapyridine can be acetylated by enzymes present in the liver and that animals can be divided into two genetic classes, slow acetylators and fast acetylators (DAS & MARTIN, 1975). In order to determine whether the decrease in fertility of the individual hamsters was related to their abilities to acetylate sulfapyridine the percent of total sulfapyridine that appeared in their urine was determined. No correlation between the degree of fertility and the acetylation ability of the hamsters could be seen (Table 2).

It was noted that sulfapyridine administered per os must be absorbed across the intestinal mucosa in order to affect the reproductive tract. Differences in intestinal absorption by individual animals might have contributed to their different susceptibilities to the drug. To test this, sulfapyridine was administered subcutaneously. The degree of

infertility obtained by subcutaneous administration of sulfapyridine was greater than that achieved by administration in feed(Table 3). Affected animals demonstrated severely decreased testicular and epididymal weights, severely affected sperm parameters (non-motile or vibrating sperm, reduced sperm numbers, head-tail separation and acrosome detachment), and histological damage to the testes consistent with defects in spermatogenesis at the level of early spermatid formation. In one case Sertoli cell and spermatogonia only were found in the seminiferous tubules. It is to be noted that certain of these test animals (the last 5 listed in the experimental group, Table 3) remained essentially unaffected by the sulfapyridine treatment. Resistance to the fertility-decreasing effects of sulfapyridine is therefore not caused by decreased ability to transport it across the intestinal mucosa. Further investigations are under way to determine the causes of the sulfa-related decrease in male fertility and, conversely, the factors involved in resistance to this effect that are found in certain hamsters.

ACKNOWLEDGMENT

This research was supported, in part, by a grant to CP and LML from the US Agency for International Development Cooperative Development Research Program.

REFERENCES

1) DAS K.M. & MARTIN A.E.: Acetylation polymorphism of sulfapyridine in patients with ulcerative colitis and Crohn's disease. Clin. Pharmacol. Ther. 18, 514 (1975).

2) LEVI A.J., FISHER A.M., HUGHES L. & HENDRY W.F.: Male infertility due to sulfasalazine. Lancet 2, 276-278 (1979).

3) LEVI A.J.,SMETHURST P., & O'MORAIN,C.A. : Sulphasalazine-induced male infertility in man and rats. Arch. Androl. 9, 18-19 (1982).

4) O'MORAIN C., SMETHURST P. & LEVI A.J.: Reversible male infertility induced by sulfasalazine.in Lobet & Hafez E.S.E. (eds). Male fertility and its regulation. MTP Press Ltd (1985)

5) PEPPERCORN M.A.& GOLDMAN P.: The role of intestinal bacteria in the metabolism of salicylazosulfapyridine.J. Pharmacol. Expt Ther 181,555-562(1972).

6) PHOLPRAMOOL C. & SRIKHAO A.: Antifertility effect of sulfasalazine in the male rat. Contraception 28, 273-279 (1983).

7) SRIKHAO A.: Antifertility effect of sulfasalazine and its metabolites in male rats. PhD Thesis, Mahidol University, Bangkok (1987).

8) SPINK W.W.:Sulfanilamide and related compounds in general practice. Yearbook Publishers, Chicago (1942).

TABLE 1

EFFECT OF SULFAPYRIDINE FEEDING ON FERTILITY OF MALE HAMSTERS. Mature male Golden Hamsters were fed sulfapyridine (770 mg/kg day) for a period of 60 days before being mated with mature females. The litter size (number of pups/litter± standard deviations) and number of matings that produced offspring were recorded.

GROUPS	NO. ANIMALS	NO. MATED	FERTILE MATINGS	AVE. LITTER SIZE
CONTROL	13.	11.	10.	8.3±3.3
EXPERIMENTAL	11.	9.	7.	4.2±2.7

TABLE 2

FREE AND CONJUGATED SULFAPYRIDINE IN HAMSTER URINE. Urine was collected for four hours from hamsters which had been fed sulfapyridine (770 mg/kg body weight per day) for a period of 60 days. The concentration of free sulfapyridine was measured as described in METHODS and the total sulfapyridine concentration was determined by the same procedure after first hydrolyzing the conjugated compounds with 0.4 M HCl for 1 hr at 100° C.

	SULFAPYRIDINE (mM)		% CONJ-	AVE NO. OF
	FREE	TOTAL	UGATED	PUPS
HAMSTER				
BT1	26.8	45.2	40	0
T2	62.8	91.0	31	0
B1	68.6	95.2	28	0
BT2	49.6	80.2	38	2
B2	28.4	49.0	42	2.5
S1	46.8	73.2	36	4
ST2	48.4	68.6	29	5.5
S2	44.8	75.4	41	7

TABLE 3

EFFECT OF SUBCUTANEOUS ADMINISTRATION OF SULFAPYRIDINE ON THE REPRODUCTIVE TRACT OF MALE HAMSTERS. Sulfapyridine (750 mg/kg body weight per day) was dissolved in dimethylsulfoxide (0.2 ml) and injected subcutaneously for 60 days. Control animals were injected with dimethylsulfoxide alone. Body weights and weights of testes, epididymides, and seminal vesicles are reported for the control and experimental animals.

BODY WT (GM)	TESTES (GM)	EPID (GM)	SEM VES (GM)
EXPERIMENTAL HAMSTERS			
123	0.08	0.08	0.06
97	0.19	0.07	0.25
149	0.28	0.15	0.32
134	0.28	0.11	0.075
144	0.30	0.14	0.24
154	0.30	0.17	0.70
138	1.06	0.33	0.46
138	1.08	0.16	0.48
105	1.32	0.40	0.62
123	1.41	0.50	0.9
121	1.58	0.42	0.8
119	1.68	0.55	0.89
118	1.90	0.50	0.94
CONTROL HAMSTERS			
134	0.96	0.28	1.28
150	1.15	0.42	0.51
102	1.46	0.55	0.88
120	1.52	0.66	0.92
119	1.98	0.67	1.16