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Pyrimethamine: An approach to the development of a male contraceptive

(fertility/dihydrofolate reductase inhibition/spermatogenesis)

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ABSTRACT **With the human population of the world currently more than 5.2 billion and growing at an explosive rate, the need for additional forms of readily available contraception appears paramount. To date, contraception techniques in the male have been very limited. The present study demonstrates the ability of pyrimethamine (PYR) to cause spermatogenic arrest and male infertility in mice in a dose-dependent manner. Furthermore, upon cessation of drug administration all animals returned to normal fertility status. It is also suggested that the action of PYR is due to its antifolate action. Thus, PYR represents another approach toward development of a male contraceptive.**

World human population is now expanding at the rate of 255,000 per day or 93 million per year. At this rate the world population doubles every 39 years. However, the rate is also burgeoning. In 1985 the growth rate was 83 million per year. The current situation of an exploding population will perhaps become even more critical during this coming decade, as there were disproportionately more children born over the past two decades that are now coming of reproductive age (1). The need for effective contraception is as overwhelming as these statistics. The existing methods of contraception have significant limitations with respect to long-term safety concerns; extensive differences in religious, cultural, and personal attitudes; and awareness of a population problem. These differences require that a wide range of effective and safe contraceptive technologies become readily available. Fortunately, in the western world at least, the notion that contraception is the woman's responsibility is changing. And with this change of attitude, along with recent progress in understanding male reproductive physiology, has begun an intensive search for a reversible male contraceptive agent (2–5).

We wish to report that pyrimethamine [PYR; 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine], an inhibitor of dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) used clinically in the control of malaria, produces reversible infertility in two species of laboratory animals. The present study was stimulated by reports that the compound sulfasalazine, which interferes with the absorption of folate (6, 7), caused reduced fertility in male patients (8, 9).

MATERIALS AND METHODS

Compound Administration. Adult Swiss–Webster mice (Charles River Breeding Laboratories) were used in these studies after a 10-day acclimatization period. The animals were housed in our vivarium at 21°C with a 14-hr light/10-hr dark ratio and were fed standard feed pellets (Purina) and water ad libitum. PYR was obtained from Sigma. Dapsone (DAP; 4,4'-diaminodiphenyl sulfone) was obtained from Aldrich. Each compound was freshly suspended in 0.2 ml of honey and was administered orally to male mice each day of the test periods indicated.

Fertility and Fecundity in Dose–Response Studies. To assess the antifertility effects of PYR, we administered it to 72 adult male mice at 10, 25, 50, 75, 100, or 200 $mg \cdot kg^{-1} \cdot day^{-1}$ for 50 days. Control animals received only honey without PYR. This administration period was chosen so that a complete spermatogenic cycle in the mouse (34.5 days) and epididymal sperm transport (10–15 days) would occur during drug administration. During the last 10 days of drug administration, each male was housed with three adult female mice. Since a female mouse cycles on the average of 4.5 days, this design allows each male to be exposed to approximately six female reproductive cycles. At the end of this period, the female mice were separated from the males until 19 days after the first day of the breeding period, at which time the female mice were sacrificed and examined for gravidity (10). A male mouse was considered fertile if he impregnated any of the females with which he was housed. The number of pregnancies occurring in each test group was also noted. The size and number of embryos in those females that did become pregnant were assessed and the product of these two factors was used as an index of fecundity (11–13).

Epididymal Sperm Reserves. At the end of the breeding trials, approximately one-half of the male mice in each group were randomly selected and sacrificed by an overdose of sodium pentobarbital. One epididymis from each of these animals was removed and the epididymal sperm reserves were determined by a technique modified from Amann *et al.* (14). Each epididymis was homogenized in 2.5 ml of an aqueous solution containing 150 mM NaCl, 3.8 mM $NaNO_3$, and 0.05% (vol/vol) Triton X-100 using a tissue homogenizer for 30 sec. The resulting homogenate was diluted 1:1 and the total number of epididymal sperm was calculated from hemocytometer counts.

Sperm Motility. Sperm samples were removed from the contralateral vas deferens of animals used for epididymal sperm reserve assessment. Each sample was diluted in 0.5 ml of M-199 cell culture medium (Flow Laboratories) and incubated at 37°C for 10 min, at which time the percentage of motile sperm and the degree of their motility (expressed in percent) were determined by phase-contrast videomicroscopy. The product of these two values yielded the sperm motility score. All sperm motility samples were coded for a blind study design.

Histological Procedures and Serum Testosterone. Mean seminiferous tubule diameter, cauda epididymal cell height, and serum testosterone concentrations were determined as described (10, 12).

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Abbreviations: PYR, pyrimethamine; DAP, dapsone; DHFR, dihydrofolate reductase.

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Sterility Onset Study. These experiments involved 20 adult male mice administered a PYR dosage of 200 mg·kg^{-1}·day^{-1} with 5-day serial breeding trials utilizing three female mice per male. Control animals received only honey without PYR. The breeding trials were begun on days 12, 33, and 50 of administration. The time intervals were chosen to elucidate the stage of sperm development or maturation affected by the drug. That is, if PYR was affecting sperm during their maturation in the epididymides, a decrease in fertility would be seen after only ≈12 days of drug administration. A reduced fertility first observed after 33 days would indicate adverse effects of PYR at midspermatogenesis, while an effect first noted at 50 days would indicate alterations of early spermatogenesis. Each experimental group was compared to age-matched control animals.

Fertility and fecundity resulting from each breeding trial was noted as described above. Fifty-five days after the first day of drug administration, one-half of the male mice in each group were sacrificed and the epididymal sperm reserves, sperm motility, tissue histology, and serum testosterone concentration were assessed as described above.

Reversibility Study. Reversal of antifertility effects was assessed by maintaining for an additional 54 days a subgroup of each of the control and experimental animals that underwent 50 days administration of PYR. During this period, the animals received no test compound or honey, but they were otherwise maintained under conditions identical to those of the treatment period. Fertility and fecundity of these animals were assessed via 10-day breeding trials beginning at 14 and 44 days after the last day of drug administration. Fifty-four days after the last day of drug administration, the male mice in each group were sacrificed and epididymal sperm reserves, sperm motility, tissue histology, and serum testosterone concentration were assessed as described above.

Extended Administration. A group of six adult male mice were administered PYR at 100 mg·kg^{-1}·day^{-1} for an extended period of 80 days. The control group included six age-matched mice receiving only honey without PYR for 80 days. The data from this experiment were also compared to results obtained from 10 animals receiving the same dosage for only 50 days and their corresponding age-matched control animals (n = 17). All parameters were assessed as described above.

PYR–DAP Combination. In these experiments, PYR at 100 mg·kg^{-1}·day^{-1} was administered for 50 days to 31 adult male mice. Concomitantly, these animals were also administered DAP at 0, 10, 100, or 300 mg·kg^{-1}·day^{-1}. In addition, 17 age-matched control animals received only honey without PYR or DAP. All parameters were assessed as for the experiments described above.

Statistical Methods. The data obtained from these studies were analyzed for differences among groups using binomial χ^2 analyses or one-way analysis of variance followed by Fisher's test for least significant difference. The linearity of each group's parameters with respect to dosage was calculated by using Pearson's product-moment correlation. All statistical analyses were done with a Minitab statistical software package on a Digital Equipment Corporation VAX mainframe and an IBM-XT computer.

RESULTS

Dose–Response Studies. The percentage of fertile male mice was reduced in a dose-dependent manner (r = −0.936; P < 0.01) with animals receiving the highest dosage of PYR being infertile (Fig. 1). Similarly the actual number of pregnancies occurring (% pregnant female mice) was also inversely dependent on the dosage of PYR administered (r = −0.966; P < 0.01), with no pregnancies resulting from breeding of the mice receiving the highest dosage studied. The number and

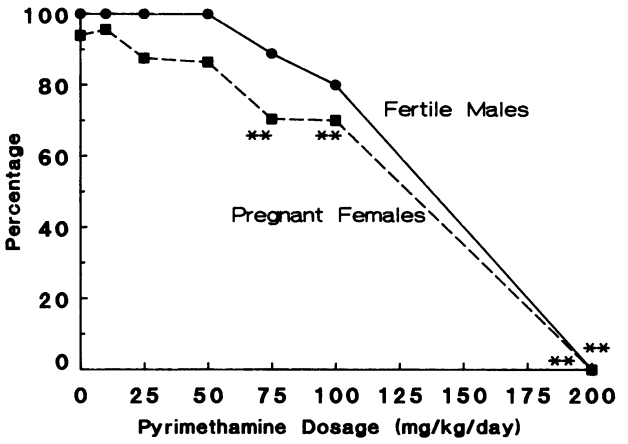

FIG. 1. Percentage of fertile male mice (●) (n = 17, 8, 8, 15, 9, 10, and 5 for each respective dose) and pregnant female mice (■) (n = 49, 23, 24, 44, 27, 30, and 15 for each respective dose) after administration of PYR to the male mice at the indicated dosage for 50 days. **, Significantly less than control value (P < 0.01). Pearson product-moment correlation for % fertile males, r = −0.936 and P < 0.01; for % pregnant females, r = −0.966 and P < 0.01.

size of the fetuses that occurred at lower dosages were not different from those of control animals and appeared normal (data not shown). The number and motility of epididymal sperm in the treated animals were inversely proportional to the dosage received [r = −0.764, n = 35, P < 0.01; r = −0.687, n = 36, P < 0.01, respectively (Fig. 2)]. The few sperm found in animals receiving the highest dosage were immotile (Table 1). The mean seminiferous tubule diameter (Fig. 2) and testicular and epididymal weights also decreased in a dose-dependent manner (r = −0.790, r = −0.755, and r = −0.469, respectively, with all P < 0.01; n = 36) with the most significant changes occurring in the testes. Plasma testosterone concentration and body weights did not differ from control values. The results noted above have recently been confirmed in another species, the rat, with the main difference being that a dosage of 400 mg·kg^{-1}·day^{-1} was required in that species to cause complete sterility (13).

Sterility Onset Study. To assess the rate at which sterility was obtained and to test the hypothesis that PYR was

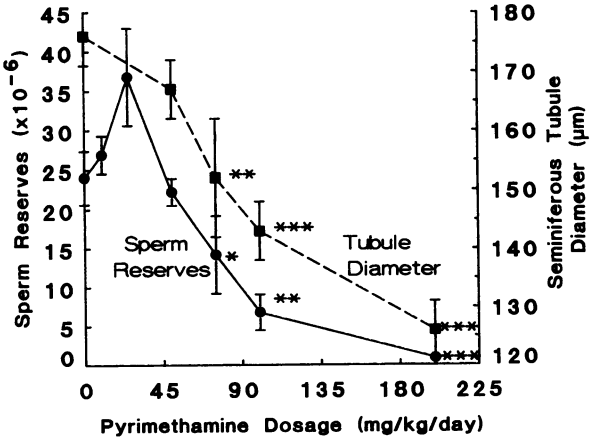

FIG. 2. Sperm reserves (●) (n = 5, 4, 3, 8, 5, 5, and 5 for each respective dose) and seminiferous tubule diameter (■) (n = 5, 8, 5, 5, and 5 for each respective dose) in male mice given PYR at the indicated dosage for 50 days. *, Significantly less than control group (P < 0.05); **, significantly less than control group (P < 0.01); ***, significantly less than control group (P < 0.001). Pearson product-moment correlation for sperm reserves, r = −0.764 and P < 0.01; for seminiferous tubule diameter, r = −0.790 and P < 0.01.

Table 1. Parameters studied in male mice at 50 days oral administration of PYR at 200 mg·kg^{-1}·day^{-1} and after 64 days of recovery

Parameter	50 days of administration		64 days of recovery	
	Control	PYR	Control	PYR
Epididymal sperm reserves, $\times 10^{-6}$	25.5 ± 1.3 (4)	0.86 ± 0.1*** (5)	43.6 ± 5.7 (5)	30.9 ± 2.7 (4)
Sperm motility score	4130 ± 130 (4)	0*** (5)	5400 ± 1090 (5)	4110 ± 160 (4)
Relative testis wt, g/100 g of body wt	0.40 ± 0.02 (5)	0.15 ± 0.02*** (5)	0.32 ± 0.02 (5)	0.30 ± 0.03 (4)
Relative epididymis wt, g/100 g of body wt	0.14 ± 0.01 (5)	0.11 ± 0.01* (5)	0.15 ± 0.01 (5)	0.15 ± 0.01 (4)
Seminiferous tubule diameter, μm	170 ± 0 (5)	125 ± 5** (5)	160 ± 0 (5)	180 ± 0*** (4)
Cauda epididymis cell height, μm	12 ± 1 (5)	13 ± 1 (5)	14 ± 1 (5)	13 ± 1 (4)
Testosterone, ng/ml	16.5 ± 9.5 (5)	13.3 ± 6.2 (5)	13.1 ± 5.7 (5)	22.0 ± 9.0 (4)
Body weight, g	37 ± 1 (5)	38 ± 2 (5)	45 ± 2 (5)	41 ± 1 (4)

Results are expressed as means ± SEM; numbers in parentheses represent number of animals.
*Significantly different from control value ($P < 0.05$).
**Significantly different from control value ($P < 0.01$).
***Significantly different from control value ($P < 0.001$).

primarily affecting the testis rather than the epididymis, we studied the time course of the onset of sterility.

The results of this experiment show that PYR begins to exhibit its antifertility effect after 33 days of administration, with nearly complete infertility occurring after 50 days (Fig. 3). One of 26 females became pregnant when mated with males that received the drug for 50 days. The time course of PYR's antifertility effect indicates that spermatogenic arrest occurs during early to middle stages of spermatogenesis. Histological study confirmed this since the only germ cells we found at the end of the administration period were mainly spermatogonia and primary spermatocytes. These testes also demonstrated widespread vacuolization of the seminiferous tubules.

Reversibility Study. To note whether or not the contraceptive effect of PYR is reversible, we performed serial breeding trials at various intervals after the cessation of treatment with PYR. Control animals were also maintained to permit comparisons with age-matched animals. Forty-four days after treatment ended, all animals returned to normal fertility status with respect to the percentage of fertile males and pregnant females (Fig. 3). At the end of the last gestation period, epididymal sperm reserves, sperm motility, and testicular and epididymal weights were assessed and similarly found to have returned to control values (Table 1). Histological examination of these testes showed normal spermatogenesis with a mean seminiferous tubule diameter larger than that of the corresponding control group. Thus, the testes recovered from the antifertility effects of PYR at a rate comparable to the onset of sterility (i.e., approximately one spermatogenic cycle plus epididymal transport).

Extended Administration. Administration of a lower dosage of PYR (100 mg·kg^{-1}·day^{-1}) to male mice for 80 days resulted in a significantly lower percentage of fertile males when compared to the age-matched control group (Table 2). This did not occur if this dosage was administered for only 50 days. Similarly, the percentage of pregnant females after 80 days administration was significantly lower than that of the control groups and the 50-day group. Of particular interest in this respect was that none of the other parameters studied in the group administered PYR for 80 days was significantly different from those of the group receiving drug for 50 days (see Table 1 for list of parameters studied). That is, even though fertility continued to decrease with duration of administration, none of the other indexes of testicular or epididymal function further degenerated.

PYR–DAP Combination. We approached the question of synergism between PYR and DAP toward male sterility by administering a base dosage of PYR that reduced, but did not eliminate, fertility (100 mg·kg^{-1}·day^{-1}) for 50 days. At the same time, the animals were also given DAP at 0, 10, 100, or 300 mg·kg^{-1}·day^{-1}.

The results suggest a strong synergism between these two compounds toward sterility in the male. Both parameters of fertility (percent fertile males and percent pregnant females) decreased in a manner dependent on the dosage of DAP (each in combination with PYR at 100 mg·kg^{-1}·day^{-1}) (Fig. 4). Other parameters that showed a reduction dependent on the dosage of DAP (combined with PYR) include sperm motility score, testis weight, and seminiferous tubule diameter (data not shown).

DISCUSSION

The data from this series of experiments demonstrate the antifertility effects of PYR in the male mouse. The dose-dependent manner by which male fertility (Fig. 1), sperm production, and seminiferous tubule diameter (Fig. 2) decrease supports the hypothesis that PYR acts on spermatogenesis while having no effect on testosterone levels or body weight (Table 1). Although there was a dose-dependent reduction of epididymal weights with PYR, the magnitude of this change was relatively small. Certainly, >90% of the extratesticular sperm is stored in the epididymis (15). Upon correlating epididymal weight with epididymal sperm concentration, we noted a strong interdependence ($r = 0.95$, $P < 0.01$). Thus, the slight but consistent decrease in epididymal weight was likely due to a reduction in the number of sperm entering that organ rather than a real change in epididymal weight. These data along with finding no change in epididymal histology (Table 1) suggest that PYR is not effecting the epididymis as much as it is the testes.

The time intervals used in the sterility-onset studies were chosen to elucidate the level of the reproductive tract being affected by PYR. That is, if PYR were affecting sperm during their maturation in the epididymis, we would have expected to see a decrease of fertility after only about 12 days of drug administration. A reduced fertility first seen after 33 days

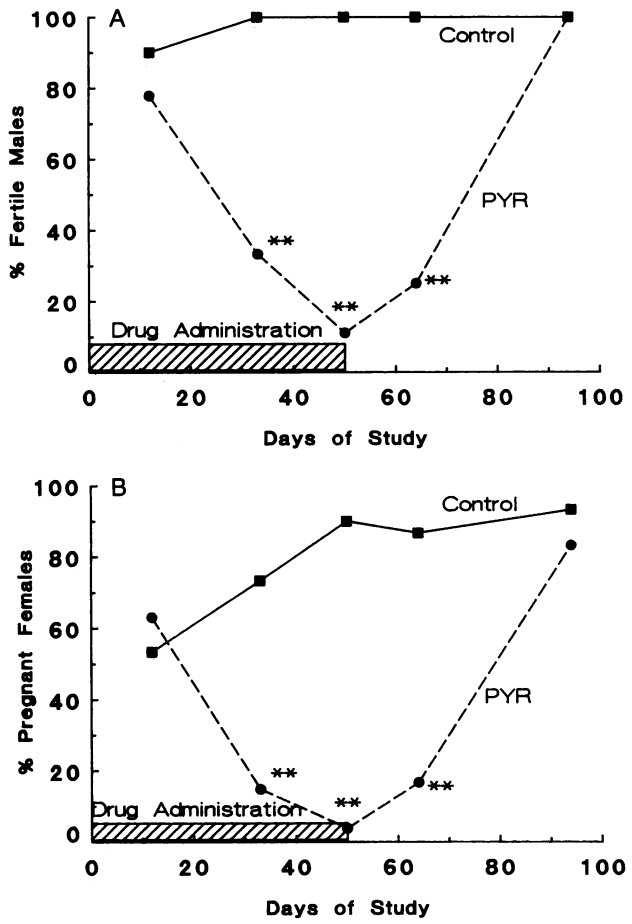

FIG. 3. (*A*) Changes in fertility of male control mice receiving only honey (■) (n = 10, 10, 10, 5, and 5 for each respective breeding period) and during administration of PYR at 200 mg·kg^{-1}·day^{-1} for 50 days and during a 44-day recovery period upon cessation of drug administration (●) (n = 9, 9, 9, 4, and 4 for each respective breeding period). **, Significantly less than control male mice administered honey for 50 days ($P < 0.01$). Pearson product-moment correlation during drug administration, $r = -0.992$ and $P < 0.01$; during the recovery period, $r = 0.985$ and $P < 0.05$. (*B*) Percentage of pregnant female mice resulting from breeding with control male mice administered only honey (■) (n = 30, 30, 30, 15, and 15 for each respective breeding period) or PYR at 200 mg·kg^{-1}·day^{-1} (●) (n = 27, 27, 26, 12, and 12 for each respective breeding period) for 50 days followed by a 44-day recovery period upon cessation of drug administration. **, Significantly less than control value ($P < 0.01$). Pearson product-moment correlation for the drug administration period, $r = -0.959$ and $P < 0.05$; for the recovery period, $r = 0.986$ and $P < 0.05$.

would indicate an adverse effect of PYR at midspermatogenesis, while an effect first noted at 50 days would indicate alterations of early spermatogenesis. The rate at which infertility was attained with PYR (40–50 days) suggests that this compound is acting on early to midspermatogenesis.

Table 2. Effect of duration of PYR administration (100 mg·kg^{-1}·day^{-1}) on fertility of male mice

Parameter	50 days		80 days	
	Control	PYR	Control	PYR
% fertile males	100 (17/17)	80 (8/10)	100 (6/6)	50* (3/6)
% pregnant females	93.9 (46/49)	70† (21/30)	83.3 (15/18)	27.8† (5/18)

Numbers in parentheses represent number of animals.
*Significantly less than PYR administered for 50 days ($P < 0.01$).
†Significantly less than control value ($P < 0.01$).

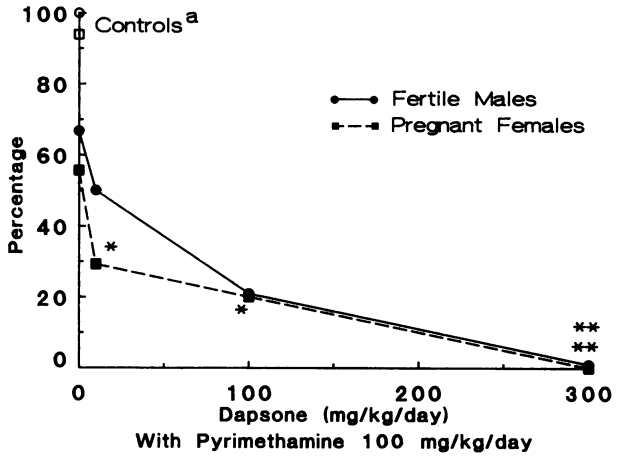

FIG. 4. Percentage of fertile male mice (●) (n = 17, 10, 8, 5, and 8 for each respective dose) and percentage of pregnant female mice (■) (n = 49, 30, 24, 15, and 24 for each respective dose) resulting from breeding trials following administration of various dosages of DAP in combination with PYR at 100 mg·kg^{-1}·day^{-1} to male mice. a, Control values significantly greater than all experimental groups ($P < 0.01$); *, significantly less than group receiving PYR at 100 mg·kg^{-1}·day^{-1} only ($P < 0.05$); **, significantly less than group receiving PYR at 100 mg·kg^{-1}·day^{-1} only ($P < 0.01$). Pearson product-moment correlation for % fertile males, $r = 0.923$ and $P < 0.01$; for % pregnant females, $r = -0.877$ and $P < 0.05$.

Although these data suggest that PYR has potential for development as a male contraceptive agent, the value of such an agent also lies in the reversibility of this effect. Otherwise, it would be a male sterilant rather than a male contraceptive. Thus, the next question we wished to answer was whether or not the contraceptive effect of PYR in the male was reversible. Indeed, recovery to normal fertility status occurred after PYR administration was discontinued. The rate of recovery was similar to the rate of sterility onset (Fig. 3), lending further support to the notion that PYR was acting on early to late spermatogenesis. In addition to actual fertility, the values for all other parameters studied in animals recovering from PYR-induced infertility also returned to normal (Table 1) with the exception of seminiferous tubule diameter. That value was significantly greater than the control values during the recovery period and may reflect a compensatory mechanism following suppression of spermatogenesis.

Clearly, the dosage in these experiments needed to attain contraception is higher than would be desirable for human males. So an attempt was made to reduce the minimum effective dosage by prolonging the administration period. It was of particular interest with respect to the action of PYR that a dosage causing reduced fertility (but not sterility) was more effective in reducing fertility if administered for a longer duration (Table 2). However, these same "long term" animals showed no further degeneration of the other parameters assessing testicular function. Further studies are necessary to determine whether even lower doses over longer periods (>80 days) would be effective, or if escape from the antifertility effects would occur.

Our initial screening trial indicated that DAP also reduced male fecundity (number and size of embryos per pregnant female) but much less so than PYR (13). Since DAP and PYR are often given in combination for malaria prophylaxis, we decided to study the effects of this concomitant administration on male fertility. The results indeed illustrate a synergism between these two compounds toward sterility. Both parameters of fertility (percent fertile males and percent pregnant females) along with other parameters studied decreased in a manner dependent on the dosage of DAP (each in combination with PYR at 100 mg·kg^{-1}·day^{-1}) (Fig. 4). These results were

not simply the antifertility effects of DAP alone, since a dose–response study of DAP recently completed by us showed 100% fertile males and pregnant females at the highest dose studied (200 $mg \cdot kg^{-1} \cdot day^{-1}$ for 50 days) (data not shown). In addition, none of the other parameters listed in Table 1 changed when DAP was administered alone. However, the significant changes noted in these parameters when PYR and DAP were administered together (data not shown) parallel those seen with administration of increasing dosages of PYR alone. Therefore, the changes noted with administration of a single dosage of PYR combined with increasing dosages of DAP are most likely caused by PYR with DAP enhancing the effect of PYR on the male reproductive system.

The mechanism of the antimalarial effect of PYR is known to involve the inhibition of DHFR in the plasmodium. This results in a reduced availability of 5,6,7,8-tetrahydrofolate, which is essential in the biosynthesis of thymidylate, purine nucleosides, and methyl compounds (16). It has been shown that administration of the activated intermediate folinic acid (leucovorin; 5-formyltetrahydrofolate) resulting from the action of DHFR can reverse the toxicity produced by high doses of methotrexate (17). To gain preliminary evidence whether such a DHFR-related mechanism was operative in the male contraceptive effects of PYR, folinic acid was administered to male mice at 4 $mg \cdot kg^{-1} \cdot day^{-1}$ in combination with PYR at 75 $mg \cdot kg^{-1} \cdot day^{-1}$. This resulted in partial reversal of the effects of PYR on fertility, epididymal sperm reserves, testicular weight, and seminiferous tubules (data not shown). This suggests that the antifertility effects of PYR in the male may be due to the drug's ability to reduce availability of folinic acid, probably by inhibition of DHFR. These experiments must be extended to include wider dose ranges for both PYR and folinic acid.

In addition to PYR, a large number of inhibitors of this enzyme have been prepared and tested for their antitumor and antimicrobial activity (18). Among these, methotrexate (19) has gained wide use in antitumor therapy, while trimethoprim (20), a highly selective inhibitor of bacterial DHFR, is extensively used in combination with a sulfonamide to treat bacterial infections.

We have therefore tested these two substances in our rat model. At a just sublethal dose (2.14 $mg \cdot kg^{-1} \cdot day^{-1}$) methotrexate administration showed no changes in the fertility parameters studied. Trimethoprim was also ineffective at 100 $mg \cdot kg^{-1} \cdot day^{-1}$. At half that dose, PYR has shown significant reductions in both fertility and fecundity. This suggests that testicular DHFR may be more sensitive to PYR than the other antifolate compounds studied (18).

The data presented here indicate that PYR causes reversible infertility in the male and thus represents another approach toward development of a male contraceptive.

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