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Characterization of Cysteamine as a Potential Contraceptive Anti-HIV Agent

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ABSTRACT: Cysteamine (β-mercaptoethylamine, or MEA) is a thiol-reducing agent and has anti-HIV activity. Because of these properties, cysteamine was evaluated as a vaginal contraceptive and tested for its effects on sperm function and on other sexually transmitted microbes. Cysteamine was contraceptive in the rabbit. Conception was inhibited completely when sperm were pretreated with 500 μg/ml cysteamine and was inhibited by more than 60% when 7.5 mg cysteamine was applied vaginally as a suspension in 50% K-Y Jelly. Cysteamine had multiple effects on spermatozoa. Both acrosin (EC 3.4.21.10) and hyaluronidase (EC 3.2.1.35) were reversibly inhibited by cysteamine. Calculated IC_{50} values were 370 μg/ml and 150 μg/ml for acrosin and hyaluronidase, respectively. Cysteamine behaved as a poor spermicide when activity was measured by the 30-second Sander-Cramer test. However, sperm motility was inhibited completely when cysteamine was preincubated for 10 minutes prior to motility evaluation, at concentrations as low as 50 μg/ml. The calcium ionophore A23187–induced human acrosome reaction was inhibited by cysteamine (IC_{50} = 0.5 μg/ml). Neither herpes simplex virus nor *Neisseria gonorrhoeae* was affected by cysteamine at concentrations as high as 500 μg/ml and 100 μg/ml, respectively. Cysteamine appears to have no effect on normal vaginal flora (i.e., lactobacillus). These results, together with published data, strongly support the further development of cysteamine as a topical contraceptive anti-HIV agent.

Key words: Contraception, sexually transmitted diseases, thiol reagents, acrosin inhibitor, hyaluronidase inhibitor, acrosomal loss.
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The health and contraceptive needs of many women can be addressed by developing effective methods of birth control and sexually transmitted disease prevention that a woman need use only when she expects to engage in intercourse. Ideally, such methods should not require participation by the male partner ("woman-managed methodology"). At present, the only available techniques that address these requirements to some extent are vaginal contraceptives, such as foams, creams, jellies, diaphragms, and sponges. Unfortunately, the failure rate of these contraceptives is much higher than desirable (George-Warren, 1993; Zaneveld, 1994). In addition, frequent use of these contraceptives may inactivate the natural vaginal flora and cause vaginal irritation (Niruthisard et al, 1991). Lesions that can be produced by such irritation may enhance infection by certain microbes that cause sexually transmitted diseases, including human immunodeficiency virus (HIV) (Kreiss et al, 1992).

Little progress has been made in the area of vaginal topicals over the last 30 years (Zaneveld, 1994). Clearly, the time has come to develop user-friendly methods that are under the control of the woman and that prevent conception and disease.

Protection against conception and the transmission of diseases could be achieved through the use of either multiple active ingredients, contained, for example, in a topical formulation, or a single ingredient that has a broad spectrum of activities. While potentially effective, multiple active ingredients are less desirable than a single agent, mainly because of the potential for side effects resulting from possible interactions among the active agents.

One approach to the design or discovery of a single active ingredient with both contraceptive and antimicrobial activities is the identification of common mechanisms by which the targeted processes (e.g., conception and viral invasion) may function. One such mechanism may be

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the dynamic rearrangement of disulfide bonds on the cell surface of target cells (Mann, 1964b; Huang et al, 1984; Dahl et al, 1991; Kosower et al, 1992; Bergamini et al, 1994; Ryser et al, 1994; Seligman et al, 1994). Relevant target cells would include the oocyte and/or spermatozoon for the process of conception, and, among others, $CD4^+$ cells for HIV infectivity. Various thiol and redox reagents adversely affect infectivity by HIV and specific aspects of sperm function (Yanagimachi et al, 1983; Nakamura et al, 1984; Bize and Driscoll, 1988; Seligman et al, 1994; Williams et al, 1995).

Cysteamine (β-mercaptoethylamine) is a thiol reagent and is capable of altering intracellular oxidation-reduction potential (Aruoma et al, 1988; Goldstone et al, 1995). Its recent identification as an anti-HIV agent (Thoene, 1992; Bergamini et al, 1994; Gunnarsson et al, 1994; Ryser et al, 1994; Ho et al, 1995; Bergamini et al, 1996; Thoene, 1996; Gunnarsson et al, unpublished data) prompted the current study, in which its effects on sperm function, its contraceptive efficacy, and its ability to impair other sexually transmitted microbes were evaluated.

The data presented in the present communication identify cysteamine as an efficacious contraceptive, possibly acting at several levels of sperm function. The apparent lack of effect of cysteamine on herpes simplex virus or *Neisseria gonorrhea* suggests a relative specificity of cysteamine for HIV, although verification requires testing of additional microbes.

Materials and Methods

Cysteamine (2-aminoethanethiol, or β-mercaptoethylamine), sheep testicular hyaluronidase (Type III), hyaluronic acid from bovine vitreous humor, N-acetylglucosamine, p-dimethylaminobenzaldehyde, Nα-benzoyl L-arginine ethyl ester, Bismarck brown and rose bengal stains, pregnant mare's serum, and human chorionic gonadotropin were products of Sigma Chemical Co. (St. Louis, Missouri). Lactobacillus MRS broth and Gas Pak anaerobic pouches were purchased from Fisher Scientific (Itasca, Illinois). The calcium ionophore A23187 was obtained from Calbiochem (San Diego, California). Nonoxynol-9 was obtained from Ortho Pharmaceutical Corp. (North Brunswick, New Jersey), and a suspension of penicillin (10,000 U/ml) and streptomycin (10 mg/ml) was from Gibco BRL (Grand Island, New York). We used 4′-ethylparaben-4-guanidinobenzoate (EGB) that was purchased from and synthesized under good manufacturing practices (GMP) conditions by Regis Chemical Co. (Morton Grove, Illinois). *Lactobacillus gasseri* (vaginal isolate) was obtained from the American Type Culture Collection (Rockville, Maryland).

Sperm Function Testing

Inhibition of Hyaluronidase Activity—Hyaluronidase from testes and spermatozoa are immunologically and enzymically identical. Furthermore, the enzymic properties of spermatozoal hyaluronidase from different species are indistinguishable (Zaneveld et al, 1973), and the inhibitor sensitivities of testicular and spermatozoal hyaluronidase are very similar (Joyce et al, 1986). Therefore, results obtained with bovine testicular hyaluronidase are applicable to the spermatozoal enzyme.

Hyaluronidase (EC 3.2.1.35) activity was quantified by measuring the extent of hyaluronic acid hydrolysis. This was measured by determining the concentration of N-acetylglucosamine–reactive material resulting from enzyme action.

Reaction mixtures contained the following, in a total volume of 0.25 ml: 0.1 M sodium acetate, containing 0.15 M NaCl, pH 5.5 (acetate buffer); 7.2 U hyaluronidase, from a stock solution dissolved in the acetate buffer; and 0.3 mg/ml hyaluronic acid. Enzyme was preincubated with test agent for 10 minutes prior to starting the reaction by adding hyaluronic acid. Enzyme reactions were determined by the method of Aronson and Davidson (1967). Incubations were carried out for 30 minutes at ambient temperature. Reaction product was determined colorimetrically as described previously (Reissig et al, 1955). The absorbance of the resultant adduct was determined immediately at 545 nm.

Differences were determined between absorbancies for reactions run in the presence and absence of hyaluronidase (blank, in which hyaluronidase was added after terminating the reaction). This was compared to a standard curve of known amounts of N-acetylglucosamine that were treated identically to enzyme reaction mixtures and converted to micromole equivalents of N-acetylglucosamine. The data were reported as percent inhibition compared to control. A dose-response curve was generated at several (five to seven) concentrations of cysteamine.

Reversibility of hyaluronidase inhibition was determined by the method of Ackermann and Potter (1949), in which the level of inhibited enzyme activity was determined in the presence of different amounts of enzyme.

Concentrations of inhibitor that produced 50% inhibition under the conditions of the assay (IC_{50} values) were determined by analyzing enzyme activity as a function of inhibitor concentration, with curve-fitting software (TableCurve 2D, version 3.02; Jandel Scientific), as described previously (Anderson et al, 1994).

Inhibition of Human Acrosin Activity—Human acrosin (EC 3.4.21.10) activity was measured by following the progression of hydrolysis of Nα-benzoyl-L-arginine ethyl ester (BAEE) spectrophotometrically at 253 nm. Enzyme isolation, partial purification, and measurement were carried out using methods described in detail by Anderson et al (1981).

Enzyme reaction mixtures contained the following: 50 mM sodium phosphate, pH 7.5 (Anderson et al, 1985); enzyme-containing protein (3–5 mIU, where 1 mIU is the amount of enzyme required to hydrolyze 1 nmol BAEE per minute); 0.05 mM BAEE; with or without inhibitor, in a total volume of 1.0 ml. Enzyme was preincubated with cysteamine for 5 minutes prior to starting the reaction by adding BAEE. Reaction blanks, in which either substrate or enzyme had been eliminated, were run in parallel to the reactions.

The rate of change in absorbance at 253 nm was determined. The change in absorbance that corresponded to 1 μmol of BAEE

(in a total volume of 1.0 ml) hydrolyzed was taken as 1.15 (Anderson et al, 1981).

The enzyme activity in the presence of each agent was compared with that for the control reaction (no test agent added). The data are reported as percent inhibition. A dose-response curve was generated at several (five to seven) concentrations of cysteamine that resulted in enzyme inhibition ranging from 20% to 85%.

Reversibility of acrosin inhibition was determined as described above for hyaluronidase. The IC_{50} values were determined by analyzing enzyme activity as a function of inhibitor concentration, with curve-fitting software, as described for hyaluronidase inhibition.

Inhibition of the Human Acrosome Reaction—In this context, acrosomal loss refers to the disruption of the sperm acrosome in response to exposure to a treatment or chemical entity. No inference is made as to whether or not this response is identical to the physiological acrosome reaction, during which the acrosome is also lost.

Within 90 minutes of collection, semen was layered over buffered 11% Ficoll, and spermatozoa were sedimented by centrifugation. Spermatozoa were resuspended (5×10^6 cells/ml) in Biggers, Whitten Whittingham, and Freeman (BWW) medium (Biggers et al, 1971), less bovine serum albumin. The sperm suspension was divided into 0.5-ml portions. A small portion (approximately 10 µl) was reserved for sperm motility assessment by light microscopy. After 5 minutes of equilibration at 37°C, cysteamine was added at various concentrations. Ten minutes after the addition of cysteamine, acrosomal loss was induced by the calcium ionophore A23187 (50 pM; Anderson et al, 1992). Fifteen minutes after addition of the ionophore, 10 µl of the suspension was removed for motility assessment. The remaining spermatozoa were fixed with buffered glutaraldehyde (Anderson et al, 1992) and stained for acrosome visualization with Bismarck brown and rose bengal (De Jonge et al, 1989b).

Inhibition of Human Sperm Motility—The method is based on that originally described by Sander and Cramer (1941). Sperm-immobilizing activity of cysteamine was compared to that of a control preparation of nonoxynol-9.

For the screening of sperm-immobilizing activity of cysteamine, a solution of 50 mg/ml was prepared in 0.9% NaCl. This solution was adjusted to pH 7.0 with HCl and was further diluted in 0.9% NaCl to concentrations of 25, 10, 5, and 2.5 mg/ml. Nonoxynol-9 was prepared as a solution of 100 µg/ml in 0.9% NaCl.

The agents were tested by mixing 50 µl of freshly ejaculated semen with 250 µl of the working stock solution of test agent. Just prior to and 30 seconds after mixing, the percentage of motile spermatozoa was determined by microscopic observation (400×). A dose-response curve was constructed from these data.

Experiments were also run in which sperm motility was assessed 10 minutes after exposure to different concentrations of cysteamine. This permitted evaluation of longer-term effects of cysteamine on motility.

The test outcome is reported as the concentration of agent in the semen sample that reduced motility by 50%.

Contraception in the Rabbit

After Pretreatment of Spermatozoa—The effect of cysteamine on the ability of pretreated spermatozoa to fertilize rabbit oocytes *in vivo* was evaluated essentially as described by Joyce et al (1979). Female rabbits (aged 8–12 months, 4.4 kg) were injected IM with 200 IU pregnant mare's serum. After 96 hours, 200 IU human chorionic gonadotropin was administered via the ear vein. Semen was collected from New Zealand white rabbits (aged 8–12 months, 4 kg) with the aid of an artificial vagina and a "teaser" doe. Spermatozoa were washed in Tyrode's medium plus albumin, lactate, and pyruvate (TALP) medium, and the suspension (adjusted to sperm count of 25×10^6/ml) was divided into two equal portions. One portion contained only TALP and served as control. Test agent was added to the second portion 15 minutes prior to use in artificial insemination.

Just after treatment with human chorionic gonadotropin, the female rabbits were artificially inseminated with 0.5 ml of the treated or the control sperm suspension. Approximately 28–34 hours after being inseminated, rabbits were killed with Sleepaway® (Fort Dodge Laboratories, Fort Dodge, Iowa), the uteri were sectioned about 2 cm distal to the oviductal/uterine junction, and the oviducts and ovaries were excised. The ovaries were dissected from the oviducts, and the oviducts were flushed from the uterine end with 0.9% saline. The perfusate was collected into a culture dish. Oocytes recovered in the perfusate were examined microscopically for fertilization. Data were reported as the percentage of recovered oocytes fertilized for each rabbit. Data were subjected to arcsine transformation before further analysis; the number of rabbits, rather than the number of oocytes recovered, was used as the sample size.

After Vaginal Application—The assay was essentially the same as that described for assays in which the spermatozoa are pretreated with the test agents (see above). However, spermatozoa were not pretreated prior to insemination. Instead, cysteamine was suspended in K-Y Jelly (Joyce and Zaneveld, 1985) at a concentration of 1%; this suspension was applied vaginally approximately 15 minutes prior to artificial insemination.

Antimicrobial Testing

Inhibition of Herpes Simplex Virus Infectivity—A sensitive plaque-reduction assay was performed to determine the effects of cysteamine on infection of primate cells (Vero cell line derived from African green monkey kidney cells and obtained from the American Type Culture Collection) by herpes simplex virus type 1, strain KOS (HSV-1[KOS]). Two-milliliter samples of a virus suspension containing approximately 400 plaque-forming units per milliliter in phosphate-buffered saline were mixed with equal volumes of serial dilutions of cysteamine to yield 4 ml of mixture containing virus at half the concentration of the original suspension and cysteamine ranging from 0–500 µg/ml. One-milliliter samples of each mixture were immediately plated in triplicate on 25-cm^2 monolayers of Vero cells. The inoculated cells were incubated with rocking for 2 hours. The inoculum was then removed and replaced with medium 199 containing 1% fetal bovine serum and 0.1% pooled human γ globulin (Armour, Kankakee, Illinois), a source of anti–herpes simplex virus antibodies capable of inhibiting the formation of secondary plaques while allowing the formation of primary plaques. After 2–3 days of incubation, the cells were stained with Giemsa for the visualization and counting of plaques. A dose-response curve was evaluated with curve-fitting software (TableCurve 2D, version 3.02)

from plots of plaque-forming units per milliliter as a function of concentration of cysteamine.

Inhibition of Neisseria Gonorrhea—Neisseria gonorrhea strain MS11 was isolated from an uncomplicated case of gonorrhea. The identity was verified by gram reaction, oxidase reactivity, and sugar fermentation. The inoculum was prepared overnight in cultures grown on Gono Cocci (GC) agar and resuspended to a density equal to a 0.5 MacFarland standard (approximately 10^8 colony-forming units per milliliter). This suspension was diluted 1:100 with GC broth to a concentration of 10^6 colony-forming units. This concentration of gonococci without compounds served as a control. Test reagent (cysteamine, 0.1 μg to 1.0 mg/ml) was added directly to the GC agar and poured into petri dishes. The agar plates were inoculated with varying concentrations of gonococci. Five serial 1:10 dilutions were made in GC broth, and 20-μl aliquots of the dilutions were inoculated onto compound-containing agar plates. The plates were incubated overnight, and the colonies were enumerated following overnight incubation in 5% CO_2 at 37°C.

Data for each concentration of compound were expressed as the number of colony-forming units per milliliter of the original bacterial suspension. A dose-response curve was estimated with curve-fitting software (TableCurve 2D, version 3.02; Jandel Scientific) from plots of colony-forming units per milliliter as a function of concentration of test agent.

Inhibition of Lactobacillus—Lactobacillus gasseri was cultured under anaerobic conditions at 37°C, grown in lactobacillus MRS broth, and contained in 33-mm culture dishes that were placed into anaerobic Gas Pak pouches, as suggested by the American Type Culture Collection (Rockville, Maryland). An appropriate amount of the bacterial suspension was added to 10 ml of nitrogen-purged medium, contained in a sterile 25-ml Erlenmeyer flask, such that the absorbance of the suspension at 550 nm was approximately 0.025. The flasks were stoppered with sterilized rubber serum stoppers. In addition to flasks that contained 5 mg/ml cysteamine, two controls were included: one in which no test agent was added, and one in which 0.1 ml of a commercial preparation of penicillin G (10,000 U/ml) and streptomycin (10 mg/ml), diluted 1:100 in broth, was added to the Erlenmeyer flask (final dilution of penicillin/streptomycin = 1:10,000). Growth of the lactobacillus should have been inhibited 60–70% by the penicillin/streptomycin under the conditions of the assay.

Culture growth was determined turbidometrically. Beginning 120 minutes after the start of incubation and at 20-minute intervals, for a total period of 260 minutes, 1-ml samples were removed from the incubation flasks, and the absorbance of each suspension at 550 nm was determined, as an estimate of cell density. Data were fit to the equation *Ln (Absorbance) = a + b (Time)*, where *a* is the absorbance at 0 time, *b* is the slope of the curve, and time is measured in minutes. The doubling time (T_D) was calculated from the equation $T_D = (Ln\ 2)/b$. Values are given as the doubling time of bacterial growth, together with the 90% confidence limits. Inhibition of bacterial growth by cysteamine was calculated directly from the reciprocal values of the doubling time (i.e., 75% inhibition of the reciprocal of the calculated doubling time is equal to 75% inhibition of bacterial growth).

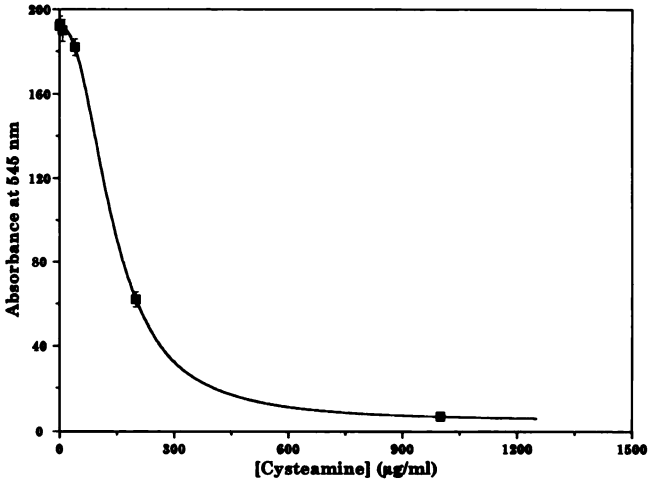

FIG. 1. Dose-dependent inhibition of hyaluronidase by cysteamine. Sheep testicular hyaluronidase was measured in the presence of the indicated concentrations of cysteamine. Values represent averages; error bars represent SEMs for duplicate determinations at each concentration of cysteamine. The curve passing through the data points is a logistic dose-response, described by following equation $Y = a + b/(1 + (x/c)^d)$, where a = 5.018, b = 186.7147, c = 140.0426, and d = 2.3080. This equation was generated with TableCurve 2D curve-fitting software. The coefficient of determination (R^2) for the curve is 0.9999.

Other Testing

Thiol Oxidation Studies—Thiol oxidation produced by the HIV coat glycoprotein, gp120, was determined by addition of either 10 μg or 1 μg of recombinant gp120 (American Biotechnologies, Inc., Cambridge, Massachusetts) to 1-ml tubes containing 100 nmol of either cysteamine or N-acetylcysteine (Sigma) in 100 mM phosphate-buffered saline at pH 7.4. Aliquots were sampled at the times indicated, and the thiol content was measured at 412 nm after reaction with dithionitrobenzoic acid (Ellman, 1959).

Results

Cysteamine Effects on Sperm Function

Hyaluronidase Inhibition—Cysteamine is a potent hyaluronidase inhibitor. Inhibition is dose-dependent (Fig. 1), with an IC_{50} value (concentration that inhibits hyaluronidase by 50%) of approximately 150 μg/ml. Inhibition is complete at 1 mg/ml. This can be compared with data for nonoxynol-9 (no effect on hyaluronidase) and with phosphorylated hesperidin, another hyaluronidase inhibitor shown by Joyce and Zaneveld (1985) to have contraceptive properties (IC_{50} = 499 μg/ml). Of the three agents evaluated, cysteamine is the most potent inhibitor. Inhibition by phosphorylated hesperidin agrees well with previously published data (Joyce et al, 1986). Cysteamine-induced hyaluronidase inhibition is reversible, as determined by an Ackermann-Potter plot of enzyme activity as a function of enzyme concentration in the presence of cysteamine. The curve passed through the origin (90%

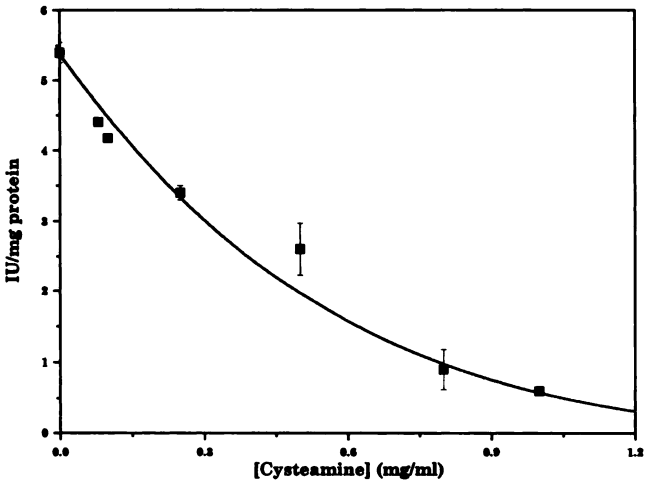

FIG. 2. Dose-dependent inhibition of human acrosin by cysteamine. Human acrosin was isolated from fresh ejaculates and measured spectrophotometrically. Values represent averages of 21 replicates for the control (zero concentration of cysteamine), and duplicate determinations at all concentrations of cysteamine except for 0.5 mg/ml and 1.0 mg/ml (single determinations). Error bars are SEMs. The curve passing through the data is a sigmoid, described by the equation $Y = a + b/(1 + \exp(-(x - c)/d))$, where $a = -0.17822$, $b = 27.57523$, $c = -0.62778$, and $d = -0.45636$. This equation was generated with TableCurve 2D curve-fitting software. The coefficient of determination (R^2) for the curve is 0.9965.

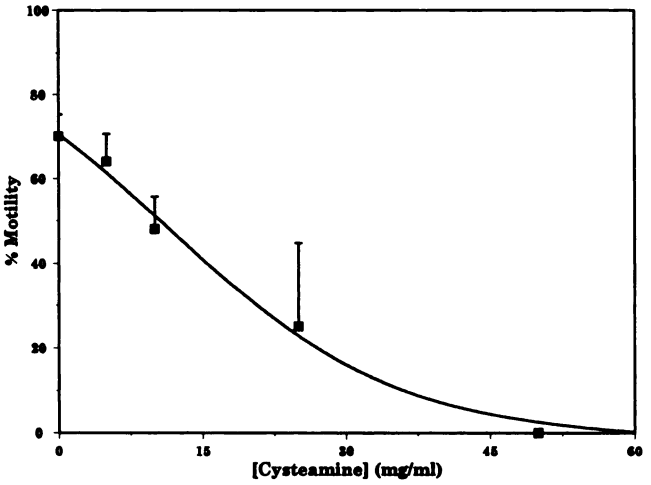

FIG. 3. Effect of cysteamine on human sperm motility. Human sperm motility was measured immediately after mixing cysteamine with sperm suspensions by a modification of the method originally described by Sander and Cramer (1941). All data were subjected to arcsine transformation prior to further analyses. Values represent averages from two separate semen samples, evaluated in duplicate for each concentration of cysteamine. Error bars represent upper 90% confidence limits. The curve passing through the data is a sigmoid, described by the equation $Y = a + b/(1 + \exp(-(x - c)/d))$, where $a = -1.47556$, $b = 100.79434$, $c = 11.12064$, and $d = -12.14582$. This equation was generated with TableCurve 2D curve-fitting software. The coefficient of determination (R^2) for the curve is 0.9936.

confidence limits = −0.00005 to 0.01773 absorbance units per minute).

Acrosin Inhibition—A moderate level of acrosin inhibition is also seen with cysteamine (Fig. 2). The calculated IC_{50} for this effect is 370 μg/ml (3.2 mM). Inhibition by cysteamine is reversible; plots of enzyme activity as a function of the amount of acrosin present in assay mixtures in the presence of cysteamine pass through the origin (intercept = 1.8×10^{-5} absorbance units/minute in the absence of added acrosin; 90% confidence limits = -1.3×10^{-4} to 1.7×10^{-4}). The ability of cysteamine to inhibit acrosin differs substantially from that of two other contraceptive agents, EGB and nonoxynol-9; EGB (4′-ethylparaben-4-guanidinobenzoate) has contraceptive activity that is probably due, at least in part, to its action as a potent acrosin inhibitor (Kaminski et al, 1985). Acrosin inhibition by EGB is pseudo-irreversible (Kaminski et al, 1986), with an IC_{50} of 0.034 μg/ml (95 nM). Both cysteamine and EGB contrast with nonoxynol-9, which shows no acrosin inhibitory activity (data not shown).

Sperm Immobilization—Based on data obtained from the modified Sander-Cramer test (see Materials and Methods), cysteamine may be regarded as an extremely weak spermicidal agent (Fig. 3). The IC_{50} for this effect is 16 ± 3.5 mg/ml ($n = 2$). This is over 250 times greater than the IC_{50} determined for nonoxynol-9 (62 ± 1 μg/ml; $n = 2$).

However, a substantial spermicidal effect of cysteamine is observed when motility is evaluated 10 minutes after mixing sperm with this agent. Rabbit sperm motility was completely inhibited at cysteamine concentrations ranging from 50 μg/ml to 500 μg/ml. At 10 μg/ml cysteamine, motility was approximately 80% inhibited, with no forward progression observed (data not shown).

Acrosomal Loss Inhibition—Cysteamine is a potent inhibitor of the calcium ionophore A23187–induced human acrosome reaction. The approximate median effective dose for inhibition of the acrosome reaction by cysteamine is 4 μM (Table 1). Although EGB is more potent than cysteamine as an acrosin inhibitor, these compounds are more similar in their efficacies as inhibitors of the ionophore-induced acrosome reaction.

Cysteamine as a Contraceptive in the Rabbit

Cysteamine is contraceptive in the rabbit, either when mixed with spermatozoa prior to artificial insemination or when placed vaginally shortly before insemination. Cysteamine at 0.5 mg/ml inhibited fertilization completely when mixed with spermatozoa 15 minutes prior to insemination ($P < 0.01$, Newman-Keuls test). Partial inhibition (approximately 35–40%) was observed at 10 μg/ml (Fig. 4). Similar inhibition of fertilization was observed when insemination followed vaginal placement of 7.5 mg cysteamine in 50% K-Y Jelly. This inhibition was somewhat less than that due to vaginal application of 11 mg nonoxynol-9 (contraception nearly complete; Fig. 5).

Table 1. *Cysteamine inhibits the human acrosome reaction**

Additions	Δ% Acrosome reaction†
50 pM A23187	20 (18.8–21.4)‡
50 pM A23187 + 4.4 μM cysteamine	10 (8.6–11.7)§
50 pM A23187 + 1.4 μM EGB	10 (9.0–10.9)§

EGB, 4'-ethylparaben-4-guanidinobenzoate.

* Fifteen minutes after the indicated addition(s), acrosomal status of washed human spermatozoa was visualized by light microscopy. Reactions with inhibitors were measured in quadruplicate; $n = 8$ for ionophore alone. The concentration of A23187 was chosen so as to yield approximately 80% maximal acrosomal loss above baseline (no additions) values. Maximal A23187-induced acrosomal loss = 25 (24.0–26.1)%; $n = 8$. Data obtained with EGB (known acrosin inhibitor with contraceptive activity [Kaminski et al, 1985]) are included for comparative purposes. Values are expressed as averages, with 90% confidence limits in parentheses. Data were subjected to arcsine transformation prior to further analyses.

† Refers to the percentage of total spermatozoa in the sample that lack acrosomes in samples that contain additions, less the percentage of spermatozoa that lack acrosomes in samples without additions (baseline value); the baseline value in these experiments was 10 (9.6–10.5)%.

‡§ Values with different superscripts differ ($P < 0.01$, Newman-Keuls test).

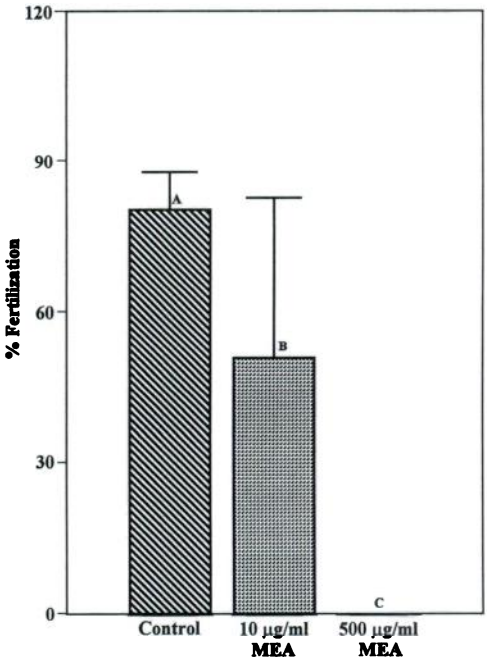

FIG. 4. Inhibition of *in vivo* fertilization in the rabbit after pretreatment of spermatozoa with cysteamine. Washed rabbit spermatozoa were incubated with cysteamine at the indicated concentration for 15 minutes prior to artificial insemination into superovulated does. Twenty-eight to 34 hours after insemination, female rabbits were killed, and oocytes/embryos were recovered from the oviducts. All data were subjected to arcsine transformation prior to further analyses. Values are expressed as the average percentage of recovered oocytes that were fertilized (cleavage). Averages are based on $n = 4$ rabbits for control (108 oocytes), and $n = 3$ (127 oocytes) and $n = 2$ (52 oocytes) for 10 μg/ml cysteamine and 500 μg/ml cysteamine, respectively. Error bars represent 90% confidence limits. Values with different letter designations are different ($P < 0.01$, Newman-Keuls multiple range test).

Antimicrobial Activity of Cysteamine

Cysteamine, at concentrations between 1 and 100 μg/ml, appeared to weakly inhibit herpes simplex virus infectivity of Vero cells *in vitro,* inhibition ranging from 20% to 30%. However, a more complete dose-response curve (Table 2) generated with cysteamine at concentrations ranging from 0.1 to 500 μg/ml showed no significant effect of concentration of this agent on infectivity (coefficient of determination, or $R^2 = 0.1154$; df = 10; $P > 0.1$).

Cysteamine was without activity against multiplication of *N. gonorrhoeae* (Table 3) when present at concentrations ranging from 0.1 to 100 μg/ml ($F^4_{10} = 0.18$; $P > 0.1$). Values in the presence of cysteamine ranged from 69% to 120% of that of the controls (no additions).

Activity of cysteamine against multiplication of a vaginal strain of lactobacillus was evaluated to estimate cysteamine's effect on the predominant normal component of the vaginal flora. Cysteamine exerted no inhibitory effect on lactobacillus growth *in vitro* (Fig. 6). Instead, 5 mg/ml cysteamine decreased the doubling time from 125 (90% confidence limits = 115–136) minutes for the control to 99 (90–110) minutes, reflecting a 26% increase in the rate of growth. In sharp contrast, 0.5 mg/ml nonoxynol-9 inhibited lactobacillus growth by approximately 70%, similar to that seen in the presence of a 1:10,000 dilution of a commercial suspension of penicillin/streptomycin.

Other Cysteamine Activity

Interaction of Cysteamine With the HIV-specific Protein, gp120—The oxidation of cysteamine or N-acetylcysteine by gp120 or bovine serum albumin is shown in Figure 7a,b. The loss of thiol reactivity is shown for two protein concentrations, 10 μg/ml (7a) and 1 μg/ml (7b). The initial thiol concentration was 0.1 mM for all reactions. The disulfide content of bovine serum albumin is 16 disulfides/mol, and there are nine disulfides/mol in gp120 (Leonard et al, 1990). Thus, the protein disulfide content in Figure 7a was 2.34 μM for bovine serum albumin and 0.75 μM for gp120. A very rapid loss of thiol reactivity with a half-life of approximately 25 minutes was observed for the reaction of cysteamine with gp120, as shown in Figure 7b. A more linear loss was seen in the reaction of cysteamine with bovine serum albumin. N-acetylcysteine showed no thiol loss (and hence, no reactivity) when it was exposed to either bovine serum albumin or gp120 during this interval. At a protein concentration one tenth that used in the previous experiment, the order of thiol oxidation rates remains the same, with rapid oxidation of cysteamine by both gp120 and bovine serum albumin. Little oxidation of N-acetylcysteine was produced by either protein (Fig. 7b).

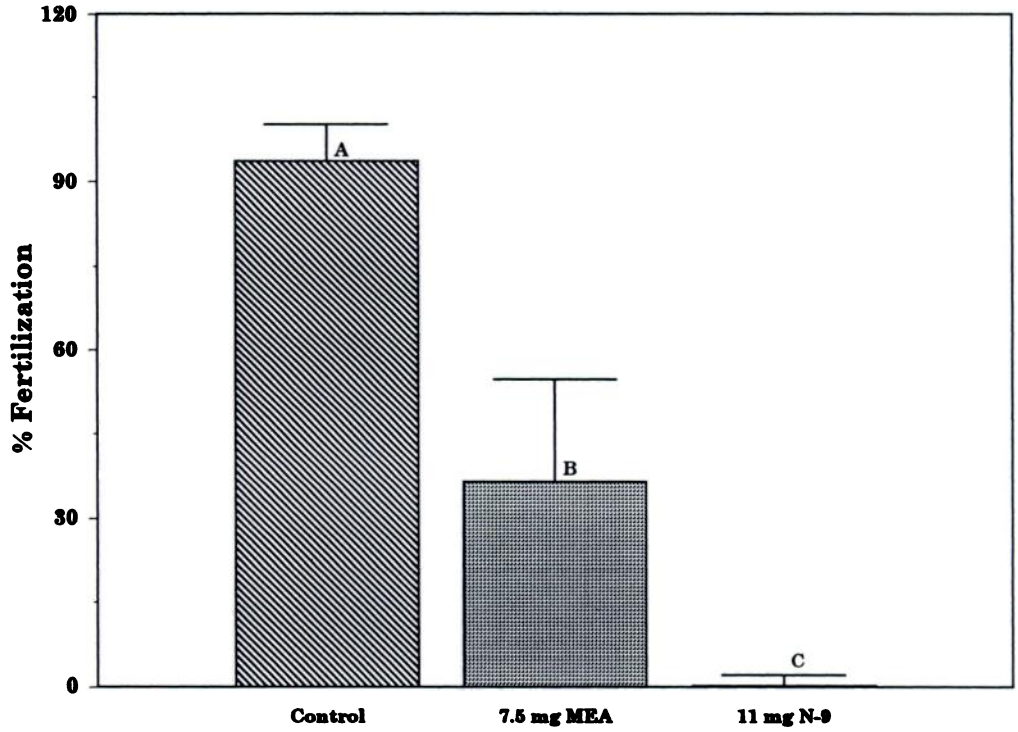

FIG. 5. Cysteamine is contraceptive when applied vaginally. Fifteen minutes after vaginal application of either 50% K-Y Jelly alone (control), 1% (w/v) cysteamine in 50% K-Y Jelly (total dose = 7.5 mg), or 2.2% nonoxynol-9 (total dose = 11 mg), superovulated rabbits were inseminated with washed rabbit spermatozoa. Data were collected and evaluated as described in the legend to Figure 4. Averages are based on $n = 3$ rabbits for control (99 oocytes) and cysteamine (97 oocytes) and $n = 4$ rabbits for nonoxynol-9 (114 oocytes). Values with different letter designations are different ($P < 0.01$, Newman-Keuls multiple range test).

Discussion

Cysteamine has multiple activities as a potential contraceptive antimicrobial agent. Cysteamine has anti-HIV properties (Thoene, 1992; Gunnarsson et al, 1993; Bergamini et al, 1994; Gunnarsson et al, 1994; Ryser et al, 1994; Ho et al, 1995; Bergamini et al, 1996; Thoene, 1996), in addition to being a potentially effective contraceptive (present study). Conception was blocked completely when spermatozoa were treated with 0.5 mg/ml cysteamine prior to artificial insemination (Fig. 4). The agent was less effective when it was applied vaginally (Fig. 5). However, the data were obtained with only a single dose and formulation. There are currently no data available that would suggest that the contraceptive out-

Table 2. *Failure of cysteamine to inhibit infectivity of herpes simplex virus**

Cysteamine (μg/ml)	PFU/ml (× 10^{-6})†
0	3.5 (3.06–4.00)
0.1	3.6 (2.66–4.76)
1.0	2.8 (2.37–3.30)
10	2.6 (2.13–3.11)‡
100	2.7 (1.63–4.46)
500	3.8 (3.62–3.99)

PFU, plaque-forming units.

* After preincubation with the indicated concentration of cysteamine, herpes simplex virus, type 2, was inoculated into Vero cells and cultured. Data (plaque count) were subjected to logarithmic transformation prior to further analyses. Differences exist among values ($F^5_{12} = 3.94$, $P < 0.05$). However, regression analysis showed no effect of cysteamine concentration on infectivity of herpes simplex virus (coefficient of determination or $R^2 = 0.249$; df = 4, $P > 0.1$).

† Values are averages of data obtained after preparing a set of virus-cysteamine mixtures and plating these in triplicate at each concentration of cysteamine. The 90% confidence limits are given in parentheses.

‡ Value is less than that of the control ($P < 0.01$, Newman-Keuls multiple range test).

Table 3. *Failure of cysteamine to inhibit growth of* N. gonorrhoeae*

Cysteamine (μg/ml)	CFU/ml (× 10^{-6})†
0	6.1 (3.26–11.28)
0.1	5.2 (2.76–9.89)
1.0	7.3 (5.38–9.93)
10	6.5 (3.04–13.82)
100	4.2 (1.78–10.18)

CFU, colony-forming units.

* *N. gonorrhoeae* was cultured *in vitro*. Data (colony count) were subjected to logarithmic transformation prior to further analyses.

† Values are averages of two to four replicates at each concentration of cysteamine, with the 90% confidence limits indicated in parentheses.

Regression analysis showed no effect of cysteamine on growth of *N. gonorrhoeae* (coefficient of determination, or $R^2 = 0.572$; df = 4, $P > 0.1$). No difference exists among values ($F^4_{10} = 0.18$, $P > 0.1$).

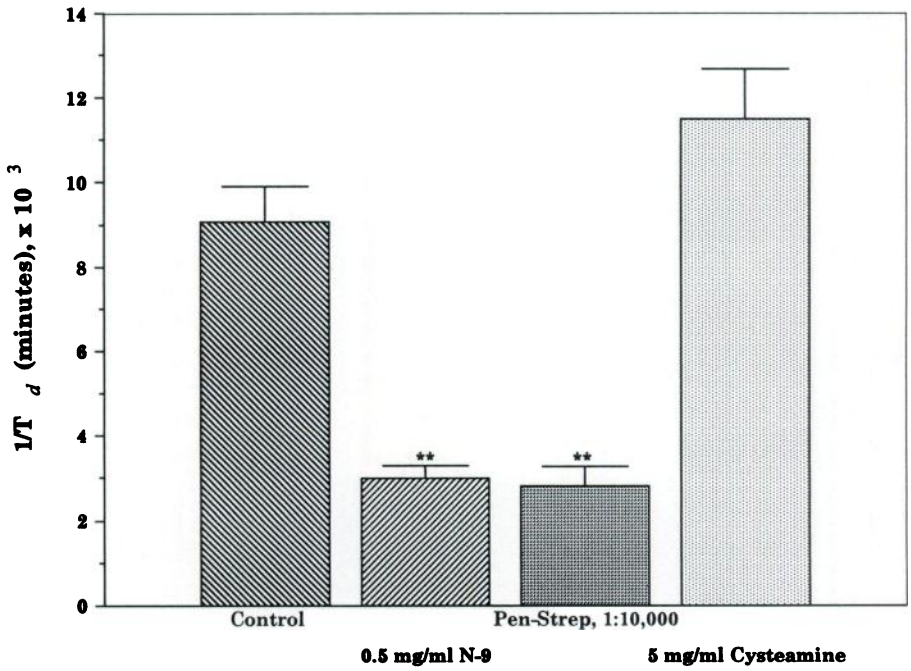

FIG. 6. Failure of cysteamine to inhibit vaginal lactobacilli *in vitro*. *Lactobacillus gasseri* was cultured *in vitro* in the presence of the indicated agent. Starting at 2 hours after introduction of each agent, and every 20 minutes thereafter, cultures were removed from incubation and cell concentration was determined turbidometrically. Doubling times were calculated from the growth curves. Data are presented as the reciprocal of doubling time. Error bars represent 95% confidence limits, estimated from regression analysis of the growth curves. Data for a commercial suspension of penicillin-streptomycin and for nonoxynol-9 are shown for comparative purposes. (** Doubling time is significantly inhibited compared to control [$P < 0.05$].)

come would not be improved by optimizing both of these variables. Additional work toward the further development of cysteamine as a contraceptive is clearly indicated.

The contraceptive activity of this agent may be exerted at several levels of sperm function. The present data show that cysteamine adversely affects key enzymes in the fertilization process, as well as affecting aspects of sperm function.

Hyaluronidase is important for penetration of the oocyte and fertilization (Pincus et al, 1948; Parkes, 1953; Joyce et al, 1979; Joyce and Zaneveld, 1985). Early research in contraceptive development focused on hyaluronidase inhibitors as candidates (Beiler and Martin, 1948; Sieve, 1952; Thompson et al, 1953; Parkes et al, 1954). Hyaluronidase inhibitors that were found to be contraceptive include compounds such as phosphorylated hesperidin and substituted benzenesulfonic acid–formaldehyde copolymers (Thompson et al, 1953; Parkes et al, 1954; Joyce and Zaneveld, 1985). These compounds are very effective contraceptives when administered vaginally to rabbits. The hyaluronidase inhibitory activity of these compounds (Beiler and Martin, 1948; Parkes et al, 1954) is similar to that of cysteamine (Results).

Acrosin is also important in fertilization. Acrosin is a sperm-specific serine proteinase that is multifunctional insofar as it may be involved in the acrosome reaction (Perreault et al, 1982; Dravland et al, 1984; De Jonge et al, 1989a; Morales et al, 1991) and in the penetration of the zona pellucida of the oocyte (Stambaugh and Buckley, 1968; Dravland and Meizel, 1982; Brown and Cheng, 1985; Dunbar et al, 1985). Acrosin inhibitors have contraceptive activity both *in vitro* and *in vivo* (Zaneveld et al, 1970; Gwatkin et al, 1977; Joyce et al, 1979; Beyler and Zaneveld, 1982; Kaminski et al, 1985). Cysteamine has moderate inhibitory activity toward human acrosin (Results). However, the extent to which cysteamine inhibits acrosin is considerably less than its contraceptive activity would suggest if acrosin inhibition were a primary mechanism for this effect.

Similarly, the extent to which cysteamine inhibits the acrosome reaction is not consistent with acrosin inhibition as the mechanism of action. EGB is a potent pseudo-irreversible acrosin inhibitor (Kaminski et al, 1986). Its contraceptive properties and ability to inhibit the acrosome reaction (Table 1) as a function of concentration are consistent with acrosin inhibition as a primary mechanism of action. EGB is more than four orders of magnitude more effective as an acrosin inhibitor than cysteamine (Results). In sharp contrast, both compounds show similar abilities to inhibit the acrosome reaction (Table 1). Acrosin inhibition is therefore not likely to be the primary mechanism by which cysteamine inhibits the acrosome reaction. If it were, then cysteamine should be less active than EGB.

When sperm motility is measured immediately after adding cysteamine, as in the Sander-Cramer test (1941),

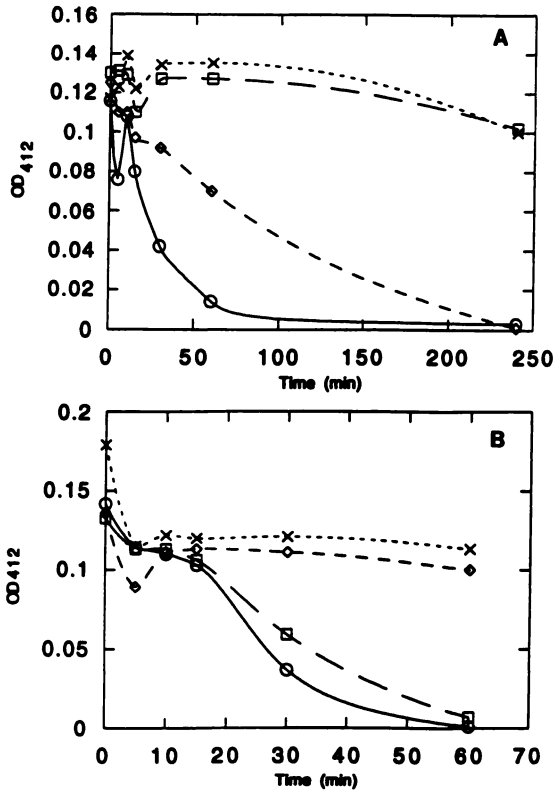

FIG. 7. **(a)** The oxidation of cysteamine by 10 μg/ml gp120 (○), or 10 μg/ml bovine serum albumin (◇) and the oxidation of N-acetylcysteine by 10 μg/ml gp120 (□) or 10 μg/ml bovine serum albumin (×). **(b)** The oxidation of cysteamine and N-acetylcysteine by gp120 or bovine serum albumin at 1.0 μg/ml. N-acetylcysteine plus bovine serum albumin (×); N-acetylcysteine plus gp120 (◇); cysteamine plus gp120 (○); cysteamine plus bovine serum albumin (□).

cysteamine shows relatively poor spermicidal activity (Fig. 3). However, when cysteamine is incubated with sperm samples for a brief period (e.g., 5–15 minutes) before motility is determined, substantial effects are noted. Complete inhibition of sperm motility is noted at concentrations as low as 50 μg/ml. The contraceptive activity of cysteamine could be due in part to its ability to inhibit sperm motility. However, because of the time required for this effect, the extent to which sperm immobilization contributes to the overall contraceptive effect is unclear.

The present data suggest that the contraceptive activity of cysteamine is not due to a single effect on spermatozoa but more likely to multiple activities of this compound. Activities include acrosin and hyaluronidase inhibition, inhibition of the acrosome reaction, and reduced sperm motility. These may appear to represent different effects on sperm function. Cysteamine also inhibits HIV infectivity. However, many of these effects may be mediated by common underlying mechanism(s).

Cysteamine is a thiol reagent in equilibrium with its disulfide form, cystamine (Cappel and Gilbert, 1986). As such, it can act as an antioxidant, its activity depending on the concentrations of each component of this redox couple and on the redox potential of other redox couples present, for example, in or on spermatozoa. While cysteamine is generally regarded as an antioxidant, this property is determined largely by the proportion of coexisting cystamine. Cystamine is formed readily from cysteamine upon standing in air (Budavari, 1996).

The proper equilibrium between free sulfhydryl and disulfide bonds, and the overall oxidation state of spermatozoa, are important for the initiation and/or maintenance of sperm function. Such functions include sperm motility, capacitation, the acrosome reaction, binding of spermatozoa to the zona pellucida, and fertilization of oocytes. However, requirements for a specific balance between free sulfhydryl groups and disulfide bonds, as well as mechanisms that control this balance, are unknown. From existing evidence (presented below), we contend that function of spermatozoa (conducive to fertilization) is impaired by conditions that raise their oxidation state (decrease the redox state) and that alter their limited content of free sulfhydryl groups.

The intracellular content of disulfide bonds increases substantially during sperm maturation as the sperm move from the testis to the cauda epididymis (Huang et al, 1984). When treated with dithiothreitol (at concentrations sufficient to reduce disulfide bonds), spermatozoa lose their ability to undergo the acrosome reaction. Sperm fertilizing potential is also reduced (Yanagimachi et al, 1983). The number of disulfide bonds in sperm from some infertile men appears to be decreased as compared with fertile spermatozoa (Evenson et al, 1980). Deficient disulfide bond formation within sperm protamines may therefore represent a lesion that can account for certain types of male infertility.

On the other hand, mature spermatozoa also contain free sulfhydryl groups. An increase in sulfhydryl groups occurs during sperm maturation. This takes place, for the most part, on the cell surface (Huang et al, 1984). The fertilization process may contain a cell fusion component that bears a resemblance to penetration of target cells by enveloped viruses or to formation of conductance channels (gap junctions) between cells. If it does, then the integrity of sperm surface thiol groups should be important for maintaining fertilizing potential. In this regard, it is of interest that at least some of the adverse effects of dithiothreitol on sperm function are due to changes occurring at the cell surface (Yanagimachi et al, 1983).

The importance of reduced thiol groups can be inferred from studies that have examined the effect of altered oxidation state on sperm function. Sperm's fertilizing potential and its ability to function normally in other processes, such as the acrosome reaction, can be impaired by agents and/or actions that increase the oxidation state of the

sperm. Conversely, mild reducing agents may be beneficial to sperm function.

For example, the toxic effects of hydrogen peroxide on spermatozoa may be due to decreased levels of reduced glutathione (Storey and Alvarez, 1989). This would suggest that a higher redox state may be beneficial to the function and survival of spermatozoa. Treatment of hamster spermatozoa with a mild impermeant oxidizing agent (thus, decreasing the redox state at the cell surface) inhibits capacitation and/or the acrosome reaction (Bize and Driscoll, 1988).

Conversely, penetration of zona-free hamster oocytes by human spermatozoa is improved by ferrous ions (a reducing agent; Aitken et al, 1990). Preservation of sulfhydryl groups exerts a protective effect on spermatozoa. Also, agents that oxidize sulfhydryl groups are "sperm-paralyzing" (Mann, 1964a). These findings suggest that an increased redox state favors sperm fertilizing potential. The antifertility agents, sulfonoxyalkanes, may act on spermatozoa by reacting with cysteine residues, resulting in dethiolation (Mann, 1964b).

Cellular penetration by enveloped viruses may depend on reduction and restructuring of disulfide bonds, both within the viral envelope glycoproteins and within the plasma membrane with which the virus fuses (Anthony et al, 1992; Ryser et al, 1994). Sulfhydryl blocking reagents reduce the infectivity of these viruses, including HIV and herpes simplex virus. When dithiothreitol is present during herpes simplex virus binding, it blocks subsequent entry into the target cell (Wittels and Spear, 1990). Similar reduction in HIV infectivity is seen with monoclonal antibodies to protein disulfide isomerase (PDI; Ryser et al, 1994). Protein disulfide isomerase reduces a disulfide bond on gp120 of CD4-bound HIV. This reduction induces a conformational change that leads to viral entry. Interestingly, compounds that covalently modify active site thiols in PDI can block HIV entry (Ryser et al, 1994).

Viral penetration may therefore be mediated or facilitated by the action of this ubiquitously distributed enzyme. Protein disulfide isomerase is one of several proteins responsible for the reduction of disulfide bonds, the oxidation of sulfhydryl groups to form disulfide bonds, and disulfide interchange. The latter process allows newly synthesized proteins to assume their native conformation as they leave the endoplasmic reticulum (Freedman, 1989). The oxidation state of the environment to which PDI is exposed largely determines whether PDI catalyzes the reduction of disulfide bonds, the oxidation of sulfhydryl groups, or disulfide bond interchange (Freedman, 1989).

The data in Figure 7a,b support the hypothesis that cysteamine inhibits HIV replication by interfering with the conformation of gp120. The rapid oxidation of cysteamine by gp120 argues that lysis of protein disulfide bonds may be involved in this process. The gp120 protein has nine intrachain disulfide bonds. These are required to maintain the appropriate spatial configuration for interaction with the CD4 receptor (Leonard et al, 1990; Eiden and Lifson, 1992). Mutations at the disulfides residues interfere with the binding of gp120 to CD4 receptors (Chiou et al, 1992; Lekutis et al, 1992). Disulfide bonding has a critical role in retroviral envelope glycoprotein processing (Gliniak et al, 1991). Further, the endoplasmic reticulum (source of PDI) normally has a relatively high redox state (Hwang et al, 1992). These data suggest that exogenous thiol could seriously impair retroviral replication and pathogenicity.

No direct evidence exists for the presence of PDI on the surface of the spermatozoon. However, agents that reduce the amount of sperm-associated sulfhydryl groups are contraceptive, and agents that block sulfhydryl groups reduce fertilizing potential. These findings suggest the presence of this enzyme (or a protein with similar function, such as thioredoxin; Pigiet, 1986) in spermatozoa. Cysteamine has the potential to act as an inhibitor of PDI and to block sulfhydryl groups. These properties may give this agent at least part of its anti-HIV and contraceptive abilities. Hyaluronidase and acrosin contain multiple subunits, attached by disulfide bonds (Harrison, 1988; Baba et al, 1989). Cysteamine may act on these enzymes by reducing these disulfide bonds and blocking the resultant sulfhydryl groups by forming mixed disulfides with the enzyme. Altered sulfhydryl/disulfide status of hyaluronidase may adversely affect enzyme activity (Zaharia and Soru, 1966), although this notion does not appear to be supported by the data of Harrison (1988).

Cysteamine was inactive against herpes simplex virus and *N. gonorrhea* (Tables 1, 2). While these microbes represent only a small fraction of the sexually transmitted organisms, our observations suggest that cysteamine's antimicrobial activity may be relatively specific for HIV. Verification will require testing additional infectious organisms.

Nonoxynol-9 is currently the most widely used active ingredient in vaginal contraceptive products. This active agent is generally accepted as the "gold standard" with which other potential vaginal contraceptives are compared. The present study shows that cysteamine compares favorably with nonoxynol-9 with regard to its contraceptive potential (although its mechanism of action is likely to be substantially different from that of the marketed spermicide). In the rabbit contraception model, complete inhibition of conception was observed after insemination with spermatozoa that had been pretreated with cysteamine (Fig. 4). This material was somewhat less effective when it was applied vaginally prior to insemination. However, several caveats must be considered. First, the rabbit

model of contraception typically produces variable results, even under ideal conditions. Inhibition of conception may range from 70% to greater than 90%, even for known contraceptive agents. This was noted in the original communication that described the use of the rabbit for vaginal contraceptive studies (Williams, 1980). Similar data have been reported by Joyce (Joyce et al, 1979; Joyce and Zaneveld, 1985), where inhibition of conception by the same two active agents ranged from 78% to nearly 100% in separate studies. Contraception is often incomplete even for generally accepted efficacious doses of nonoxynol-9 (present study; unpublished data). Less than complete contraception can be due to the inherent variability of the rabbit as a model as well as to variations in the composition and placement of the formulation that is used.

Other factors include the dose and the composition of the formulation. The present contraceptive studies, in which sperm were treated with cysteamine prior to insemination, showed that cysteamine is effective at a concentration somewhere between 0.01 and 0.5 mg/ml. Nonoxynol-9 is an effective *in vitro* spermicide at approximately 120 μg/ml (Homm et al, 1976) and is effective as a vaginal contraceptive in K-Y Jelly at 2.2%. Given the ratio of efficacious *in vitro* and *in vivo* concentrations for nonoxynol-9, we chose 1% as a concentration of cysteamine that would show a contraceptive effect (7.5 mg per rabbit, based on the formulation volume applied). This dose produced a significant contraceptive effect (Fig. 5). Practical considerations (mainly cost) precluded our testing at higher doses.

K-Y Jelly was used as a vehicle for these studies. However, there was no *a priori* knowledge regarding its suitability as an optimal formulation for cysteamine. Formulations with different bases, such as carbopol-934 gel, polyethyleneglycol, or sodium carboxymethyl cellulose gel, might have produced better outcomes when used with the same concentration of cysteamine as used in the present study. While the present study has clearly shown the potential of cysteamine as a vaginal contraceptive, additional work is required to develop the most effective contraceptive product containing this active ingredient.

Not unexpectedly, cysteamine, as an active thiol agent, exerts effects other than those related to contraception and antiviral activity, some potentially beneficial (Thoene et al, 1976; Gahl et al, 1987; de Matos et al, 1995) and others possibly adverse (Selye and Szabo, 1973; Szabo, 1978; Boesby et al, 1983; Cisse and Schipper, 1995; Nakata et al, 1995). However, it should be noted that the adverse actions of cysteamine either have been restricted to *in vitro* observations (e.g., in astrocytes, in which phenomena such as the blood-brain barrier to cysteamine was not a factor; Cisse and Schipper, 1995) or have been observed at concentrations much higher than those required for the effects reported in the present study (e.g., reported effects on learning in rats [Nakata, et al, 1995] or the well-known use of high concentrations of cysteamine for the induction of duodenal ulcers [Selye and Szabo, 1973; Szabo, 1978; Boesby et al, 1983]). In contrast to other routes of administration, vaginal application of cysteamine has the inherent advantage of allowing relatively high concentrations to be realized in the vaginal and cervical compartments, with much lower systemic concentrations, even if the agent were absorbed readily by the vaginal mucosa. For example, a typical 5-ml vaginal dose of a 5% formulation would be expected to produce a maximal circulating systemic concentration no greater than approximately 6 μg/ml, nearly four orders of magnitude less than that present in the original formulation. Cysteamine is the active ingredient in the currently marketed drug Cystagon (Mylan Laboratories, Inc., Pittsburgh, Pennsylvania). Cysteamine is tolerated well in systemic administration to patients with nephropathic cystinosis (Thoene et al, 1976; Gahl et al, 1987) and received new drug approval for this use in 1994. In the 3 years since its approval, it has been used in over 100 children with cystinosis without significant side effects. This confirms earlier work on its effectiveness and relative lack of toxicity (Gahl et al, 1987).

Even with the above caveats, the successful development of cysteamine as an effective contraceptive antiviral agent will clearly require detailed pharmacokinetic and long-term toxicity studies. However, the results reported in this paper suggest that cysteamine may be useful as a topical contraceptive with the additional property of inhibiting the sexual transmission of HIV. Additional work with this promising contraceptive anti-HIV agent is warranted.

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