Tissue Culture Cytotoxicity Assay for Cyclophosphamide Metabolites in Rat Body Fluids

FRED A. WEAVER *, ANTHONY R. TORKELSON, WALTER A. ZYGMUNT ˣ, and HENRY P. BROWDER

Received June 8, 1977, from the *Departments of Biologic Research, Medical Research, and Pathology and Toxicology, Mead Johnson Research Center, Evansville, IN 47721.* Accepted for publication November 9, 1977. *Present address: Bioclinical Laboratories, Inc., Evansville, IN 47714.

Abstract ◻ An *in vitro* cytotoxicity assay for cyclophosphamide metabolites in rat body fluids is described. Of the two tissue culture tumor *cell lines* employed, the Walker-256 rat carcinosarcoma was more sensitive to metabolite levels than the L-1210 mouse lymphocytic leukemia. The Walker-256 system detected cyclophosphamide metabolite levels two orders of magnitude lower than the commonly used 4-(*p*-nitrobenzyl)pyridine analytical procedure.

Keyphrases ◻ Cyclophosphamide metabolites—*in vitro* cytotoxicity analyses in rat body fluids ◻ Cytotoxicity—*in vitro* analyses of cyclophosphamide metabolites in rat body fluids ◻ Metabolites—cyclophosphamide, *in vitro* cytotoxicity analyses in rat body fluids ◻ Antineoplastic agents—cyclophosphamide, *in vitro* analyses of metabolites in rat body fluids

Cyclophosphamide, a widely used cancer chemotherapeutic agent, is converted to a number of metabolites by liver microsomal enzymes (1). The most commonly used procedure for measuring cyclophosphamide metabolites involves reaction with 4-(*p*-nitrobenzyl)pyridine as an indication of chemical alkylating activity (2, 3). This assay, however, is not sensitive to cyclophosphamide metabolite levels below 10 μg/ml and, in addition, there is no direct correlation between total chemical alkylating activity and cytotoxicity. The cyclophosphamide metabolite acrolein, for example, has no alkylating activity but is highly cytotoxic (4).

Tissue culture systems amenable to measuring the cytotoxicity of drug metabolites involve cells grown in a monolayer (5, 6) or suspension-grown cells in small volume microcells (7). Neither of these procedures can be used for cell culture growth studies involving repeated sampling from individual cultures. This paper reports an *in vitro* cytotoxicity assay for cyclophosphamide metabolites in rat body fluids using two tissue culture-adapted tumor cell lines. The method is sensitive to low levels of cyclophosphamide metabolites, can be employed in growth studies, and may be applicable to the clinical monitoring of cyclophosphamide metabolites in human body fluids.

EXPERIMENTAL

Cell Lines and Culture Techniques—Mouse L-1210 lymphocytic leukemia cells were obtained commercially[1]. Walker-256 (W-256) rat carcinosarcoma cells were isolated from the ascitic fluid of male Sprague–Dawley rats (Harlan strain) bearing the Arthur D. Little strain of the W-256 tumor. After establishment in tissue cultures, W-256 cells retained the ability to induce tumor growth upon reinjection in Sprague–Dawley rats. Both cell lines were grown in media[2] containing 10% fetal calf serum, with penicillin[1] and streptomycin[1] added.

Cultures were passaged three times weekly and maintained in logarithmic growth. Cell density (number of cells per milliliter) and viability were determined in a hemacytometer microscope counting chamber using the trypan blue dye exclusion method (8). Screw-capped, 150-ml, milk dilution bottles containing 5×10^4 cells/ml in 20 ml of medium were placed on their sides and incubated with loose caps in a humidified environment cabinet[3] at 37° in an atmosphere of 5% CO_2–95% air. Cultures were checked routinely for absence of mycoplasma contamination (9).

Body Fluid Collection—Male Sprague–Dawley rats (Harlan strain), 200–250 g, were used. Cyclophosphamide[4] was given at 500 mg/kg ip; 15 min after dosing, blood was taken by cardiac puncture or from the vena cava. Plasma or serum was prepared and pooled from 10–15 rats. Urine was collected from 10–15 dosed or control animals over 24 hr and pooled. Urine creatinine levels were determined by the method of Owen *et al.* (10).

Chemical alkylating activity of body fluids from dosed animals was determined by the 4-(*p*-nitrobenzyl)pyridine method (3), and alkylating activity was expressed relative to 2,2′-dichlorodiethylamine hydrochloride (nornitrogen mustard), an alkylating metabolite of cyclophosphamide (11). Samples frozen at −25° exhibited no change in alkylating activity within 3 months.

Cytotoxicity Assay—The cell density of a 2- or 3-day L-1210 or W-256 culture was determined by counting 1.0 ml of culture with 0.5 ml of 0.1% trypan blue four times in a hemacytometer and averaging the counts. A single starter culture in the medium[2] was then inoculated at 2×10^4 cells/ml, and screw-capped milk dilution bottles were inoculated with 10.0 ml of this starter culture. Various dilutions of rat body fluids previously sterilized with a 0.45-μm pressure filter[5] were added in a constant 2.0-ml volume, using sterile, glass-distilled water as a diluent.

Recovery of radioactivity from filtered urine containing radioactive cyclophosphamide and its metabolites was 87, 93, and 95% in three separate experiments. Recovery from serum was essentially quantitative[6]. Eight milliliters of a medium[2] mixture (single strength to double strength = 3:1) was then added to yield a final 20-ml culture volume at 1×10^4 cells/ml. For plasma samples, the medium mixture also contained heparin sodium to yield a final concentration of 0.1 unit/ml of growth medium to eliminate the fibrinogen → fibrin reaction, which obscures cell counts in the electronic particle counter (see *Results and Discussion*).

In experiments involving fluids from drug-treated animals, appropriate controls were run using identical volumes of fluids from untreated animals. Triplicate cultures were incubated as described for 3 days. At the end of the incubation period, 2.0 ml of each culture was added to 18.0 ml of a 0.9% sodium chloride solution[7], and the final cell density was determined in an electronic particle counter[8]. Each culture was counted four times, and the counts were corrected for coincidence and averaged. Cell growth inhibition is expressed as percent inhibition of final cell density relative to control cultures. Final control cell densities in the absence of body fluids were typically 2×10^5 cells/ml for W-256 and 5×10^5 cells/ml for L-1210.

RESULTS AND DISCUSSION

Cytotoxicity Assay Response to Body Fluids from Untreated Animals—Urine collected from animals dosed with cyclophosphamide was in varying states of hydration because of varied antidiuretic response. The relative concentration of urine components was generally higher in treated than in untreated animals but was not constant between animals treated identically. Consequently, it is not sufficient to express a maximum tolerable level of control urine on a volume basis. It was necessary to measure the creatinine concentration of all urine samples and to ex-

[1] Associated Biomedic Systems.
[2] Roswell Park Memorial Institute 1640.
[3] Wedco.
[4] Cyclophosphamide for injection, a product of Mead Johnson Laboratories.
[5] Millipore.
[6] Unpublished results.
[7] Isoton, Coulter Diagnostics Inc.
[8] Coulter Electronic model B particle counter.

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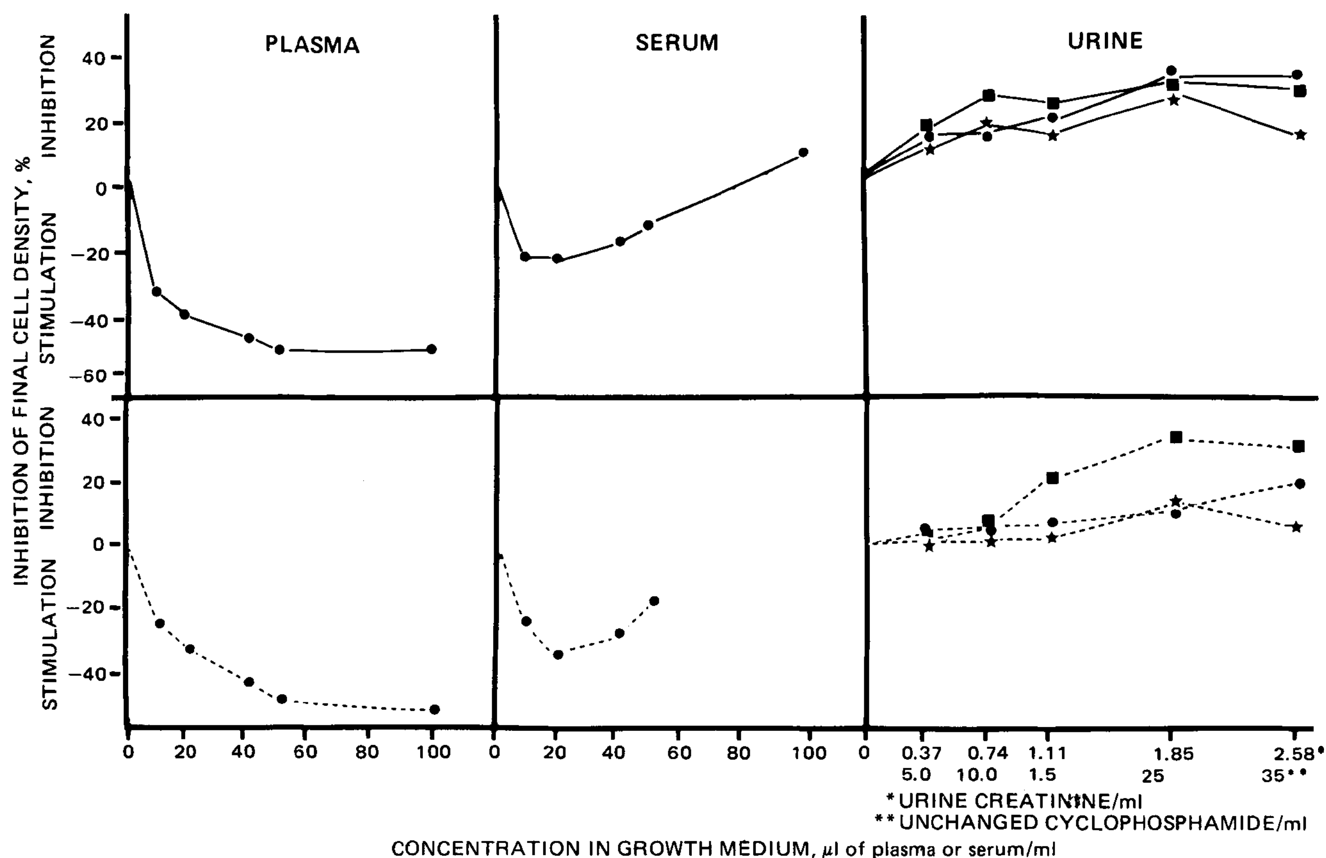

Figure 1—*L-1210 (—) and W-256 (- - -) cytotoxicity assays with body fluids from control rats. Unchanged cyclophosphamide was included in urine assays, assuming a complete lack of cyclophosphamide activation in rats dosed with 500 mg of cyclophosphamide/kg ip. Key: ●, urine alone; ★, unchanged cyclophosphamide alone; and ■, urine plus unchanged cyclophosphamide.*

press the maximum tolerable control urine level in the *in vitro* system relative to creatinine levels.

Plasma prepared using edetic acid as an anticoagulant was highly toxic to both L-1210 and W-256 cells, and the toxicity was shown to be due entirely to the edetic acid. Approximately 50% growth inhibition was noted with 120 µg of edetic acid/ml of growth medium. This problem was circumvented by using either 1.0 unit of heparin sodium/ml or 0.2% sodium citrate as the anticoagulant in plasma preparations.

Another problem was encountered with cultures containing added rat plasma. Residual thrombin in the plasma apparently converted fibrinogen present in a fetal calf serum component of the growth medium to the insoluble protein fibrin during culture incubation. The fibrin was then falsely registered during counting with the electronic particle counter, and cell counts were errantly high. The addition of 0.1 unit of heparin sodium/ml of growth medium to the cultures eliminated this problem by inhibiting thrombin activity.

Results of L-1210 and W-256 cytotoxicity assays with control rat plasma, serum, and urine are shown in Fig. 1. Plasma stimulated growth at levels up to 100 µl of plasma/ml of growth medium. Low serum levels stimulated growth, and 100 µl of serum/ml was only slightly inhibitory. Urine was inhibitory, and the maximum tolerable level was arbitrarily set at 25% inhibition of cell growth. This value corresponds to 1.8 µg of urine creatinine/ml, which is approximately 30 µl of urine/ml and is dependent upon the antidiuretic response of individual animals to cyclophosphamide treatment. All further assays using body fluids from cyclophosphamide-treated animals did not exceed 100 µl of plasma or serum/ml or 1.8 µg of urine creatinine/ml.

Since both unchanged cyclophosphamide and its metabolites are very rapidly excreted in the urine (12), it was necessary to determine if any synergistic effect existed between control urine and unchanged cyclophosphamide. Cyclophosphamide was added to control urine at a level assuming a complete lack of *in vivo* activation at a dose of 500 mg of cyclophosphamide/kg (6.25 mg/ml of urine). The results (Fig. 1) confirm earlier reports that low levels of unchanged cyclophosphamide are fairly innocuous *in vitro* (*e.g.*, Ref. 13). No synergistic effect was exhibited by the combination of control urine and unchanged cyclophosphamide.

Cytotoxicity Assay Response to Body Fluids from Drug-Treated Animals—Figure 2 depicts L-1210 and W-256 assays with plasma, serum, and urine from rats dosed with 500 mg of cyclophosphamide/kg ip. The W-256 cells were much more sensitive to cyclophosphamide metabolites than were L-1210 cells. In each case, the response was reasonably linear and precision between triplicate cultures was good. In 10 experiments involving 60 sets of triplicate cultures showing greater than 20% inhibition of growth, the mean percent coefficient of variation was 3.69.

Trypan blue cell viability determinations were made on each of the L-1210 cultures inhibited with cyclophosphamide metabolites in plasma; at each plasma level, viability remained greater than 95%, although growth inhibition was as high as 95%. Use of the electronic particle counter is, therefore, an effective way of estimating viable cell density, even though it does not distinguish between living and dead cells, since

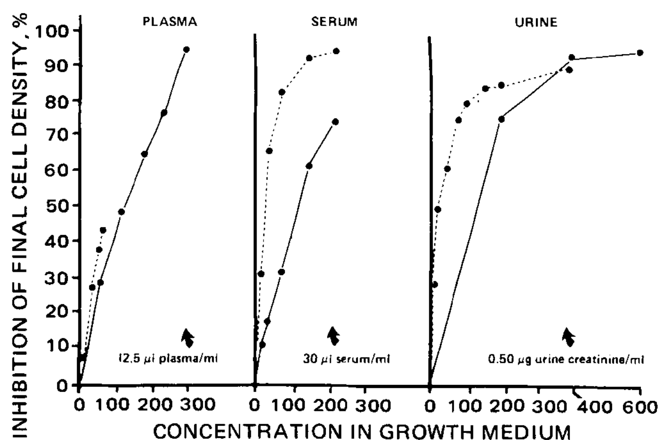

Figure 2—*L-1210 (—) and W-256 (- - -) cytotoxicity assays with body fluids from rats dosed with 500 mg of cyclophosphamide/kg ip. Concentration is expressed as 4-(p-nitrobenzyl)pyridine alkylating activity equivalents relative to 2,2′-dichlorodiethylamine hydrochloride. Arrows signify the amount of body fluid added to attain the indicated concentration of alkylating activity.*

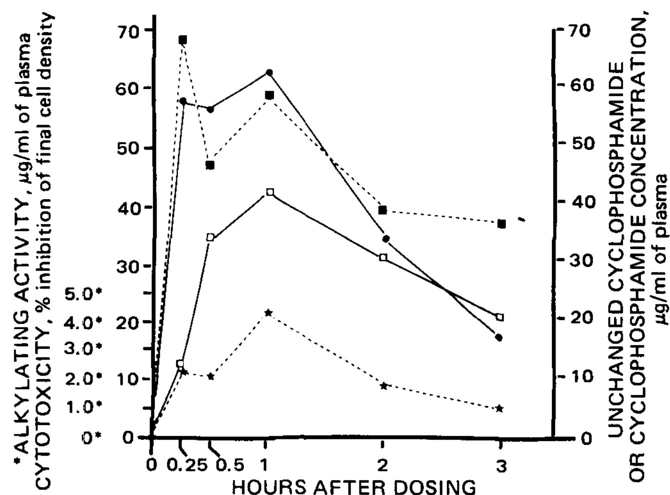

Figure 3—*Rat plasma levels of unchanged cyclophosphamide, cyclophosphamide metabolites, W-256 cytotoxicity (percent inhibition of growth with 10 μl of plasma/ml of growth medium), and chemical alkylating activity at various times after dosing rats with 200 mg of ^{3}H-cyclophosphamide/kg ip. Key:* ●, *unchanged cyclophosphamide;* ■, *cyclophosphamide metabolites;* □, *cytotoxicity (W-256 system); and* ★, *alkylating activity (2,2′-dichlorodiethylamine hydrochloride equivalents).*

growth-inhibited cells remain viable during the experiment.

To demonstrate a correlation between cytotoxicity and plasma levels of cyclophosphamide metabolites, female Sprague–Dawley rats, 200 g, were dosed with 200 mg of ^{3}H-cyclophosphamide (0.160 μCi/mg randomly labeled in the side chain)/kg ip. Plasma was collected at various times after dosing and pooled (four rats at each time point). The concentration of unchanged cyclophosphamide was determined by extraction with 10% methanol in ethylene dichloride using the method described by Mellett *et al.* (14). Metabolite levels were calculated by subtracting the unchanged cyclophosphamide concentration from the concentration of total radioactivity. Background counts per minute were less than 1% of the total counts.

In vitro cytotoxicity in the W-256 system, using 10 μl of plasma/ml of growth medium, and 4-(*p*-nitrobenzyl)pyridine alkylating activity also were determined. The data presented in Fig. 3 show a close similarity in the time course of occurrence of alkylating activity, *in vitro* W-256 cytotoxicity, and cyclophosphamide metabolite levels in plasma. The initial spike in the curves of unchanged cyclophosphamide and metabolites represents the early appearance in plasma of unchanged cyclophosphamide and metabolites with neither alkylating activity nor cytotoxicity; unchanged cyclophosphamide was nontoxic in the *in vitro* system.

Growth Studies—In drug metabolism studies, it is often desirable to follow the response of culture growth parameters with time. This approach involves repeated sampling from individual cultures and is not possible with monolayer cultures or microcell cultures involving small volumes. The L-1210 and W-256 systems can readily be used in growth studies, as exemplified by the following experiments.

The L-1210 and W-256 growth curves were determined for cultures containing both low and high levels of cyclophosphamide metabolites in rat urine. Figures 4*a* and 4*b* show that, at low levels of cyclophosphamide metabolites, growth remained exponential but with an increased population doubling time. At the higher metabolite levels, L-1210 cells apparently completed less than one full round of division and W-256 cells completed slightly more than one full round of division before the growth curve plateaued. The possible presence of a critical point in the cell growth cycle at which cyclophosphamide metabolites are active awaits further examination with synchronous cultures.

Many drugs preferentially inhibit rapidly dividing cells, and this action is also apparently true of cyclophosphamide metabolites. The W-256 cells were retarded in their growth by a single passage in glutamine-deficient medium that had been aged for 6 months at 4° to allow glutamine decomposition. Dosed rat urine was added to cells at a concentration of 400 ng of alkylating activity equivalents/ml. These "slow-growing" cells completed three rounds of division (Fig. 4*c*), while normal "fast-growing" cells were limited to one division at the same cyclophosphamide metabolite level over an equivalent time period (Fig. 4*b*).

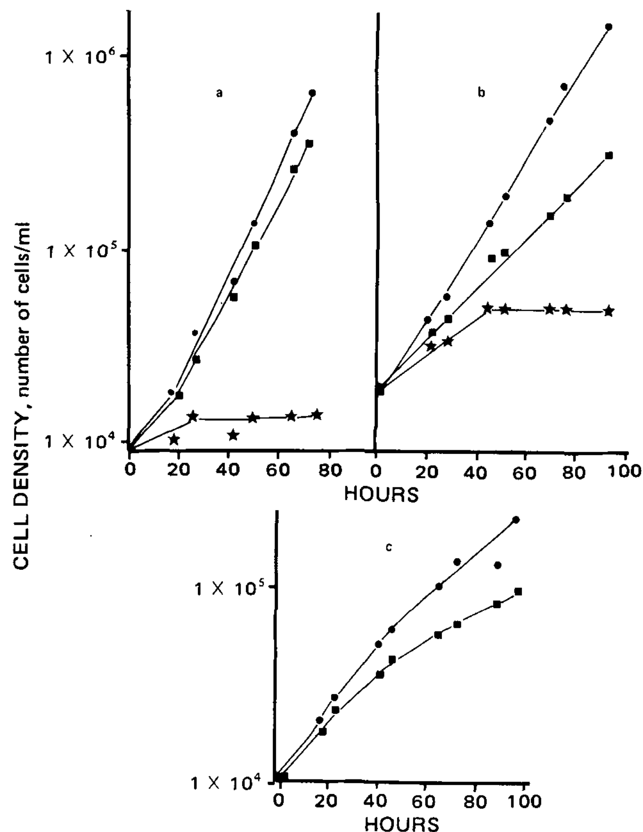

Figure 4—*Cell growth curves at various supplementation levels (concentration in growth medium) of urine from rats dosed with 500 mg of cyclophosphamide/kg ip. Key: a, L-1210; b, W-256; c, W-256 cells whose growth was previously retarded by culturing in glutamine-deficient medium;* ●, *control;* ■, *40 ng of alkylating activity equivalents/ml; and* ★, *400 ng of alkylating activity equivalents/ml.*

SUMMARY

The W-256 and L-1210 *in vitro* cytotoxicity assay systems are sensitive to low levels of cyclophosphamide metabolites in rat body fluids. From a consideration of maximum tolerable levels of control body fluids, minimum detectable cyclophosphamide metabolite concentrations, and typical metabolite levels in body fluids from rats dosed with 500 mg of cyclophosphamide/kg, it was estimated that the minimum dosage needed to give detectable metabolite levels in plasma, serum, or urine is ≃5 mg of cyclophosphamide/kg in the W-256 assay system. This dose is equivalent to ≃10 ng of 4-(*p*-nitrobenzyl)pyridine alkylating activity equivalents/ml of growth medium or ≃100 ng of alkylating activity equivalents/ml of body fluid, which is two orders of magnitude more sensitive than the commonly used 4-(*p*-nitrobenzyl)pyridine assay.

This method can be used readily in tissue culture growth studies or drug metabolism studies involving various drugs. The procedure also may be applicable to the clinical measurement of cyclophosphamide metabolite levels in human body fluids after dosing in the normal therapeutic range of 10–15 mg of cyclophosphamide/kg.

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Solubility Studies of Silver Sulfonamides

R. U. NESBITT, Jr. *, and B. J. SANDMANN ^x^

Received August 1, 1977, from the *School of Pharmacy, University of Missouri–Kansas City, Kansas City, MO 64110.* Accepted for publication November 10, 1977. *Present address: Merck Sharp & Dohme Research Laboratories, Rahway, N.J.

Abstract □ The solubilities of silver sulfapyridine, silver sulfamethazine, and silver sulfamethizole as a function of pH were determined in nitric acid–potassium nitrate, acetate, and sulfonic acid buffers. All silver sulfonamides showed an increase in solubility with increasing hydrogen-ion concentration, a behavior which closely paralleled the protonation of the *p*-amino function of the sulfonamide. A silver-ion selective electrode was used to measure silver-ion concentration in solution and the methods of known subtraction and known addition were used to measure total silver. Both silver sulfamethizole and silver sulfamethazine were ionized completely in solution. Silver sulfapyridine was ionized completely only in the more acidic pH 2–3 range. A comparison of the physical properties of the silver salts for which mortality studies were available revealed a unique set of properties for silver sulfadiazine.

Keyphrases □ Silver sulfonamides, various—aqueous solubility, effect of pH □ Solubility, aqueous—various silver sulfonamides, effect of pH □ Antibacterials—various silver sulfonamides, aqueous solubility, effect of pH

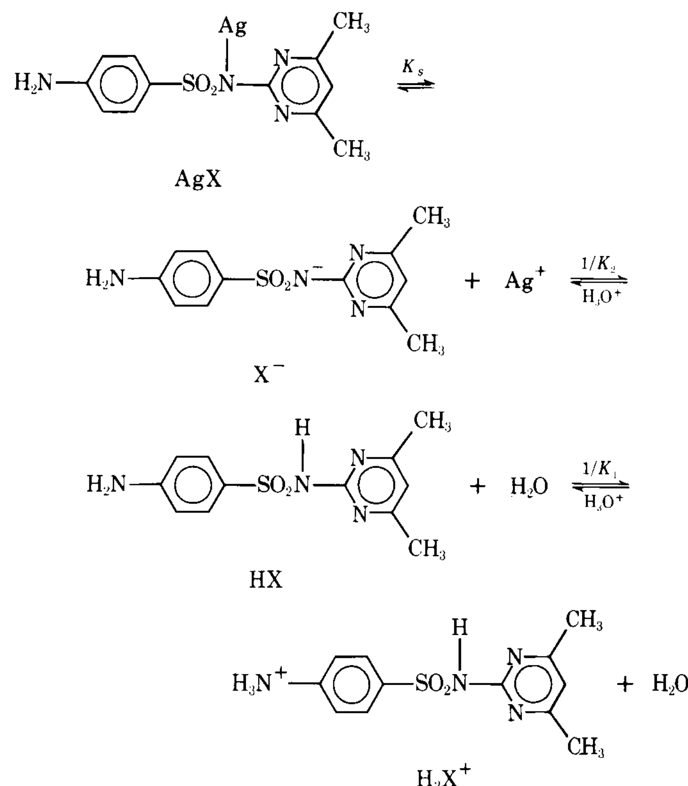
While some silver salts have been tested for effectiveness against *Pseudomonas* infections, the unique activity of silver sulfadiazine remains impressive but incompletely explained. The solubility and ionization properties of this compound were reported (1) and the drug was characterized as a practically insoluble salt having a solubility product of 8.1×10^{-12} at 25° and 0.1 M ionic strength. In the presence of a nitrate buffer under these experimental conditions, the silver ion was dissociated completely from the sulfadiazine moiety and therefore free to precipitate or complex with any suitable ligand. In the presence of endogenous chloride ion, silver sulfadiazine did not cause the rapid precipitation of silver chloride, under physiological conditions, even though the solubility product of silver chloride was exceeded (2). Further studies of the behavior of silver sulfadiazine in the presence of chloride ion are indicated for a better understanding of this phenomenon.

Other silver sulfonamides have been compared to silver sulfadiazine for anti-infective activity and reaction with human serum and DNA. Differences between the reactions of the other sulfonamides to those of silver sulfadiazine were postulated to relate to the ionization constants for the sulfonamide moieties (2). However, a comparison of the literature values of the amide hydrogen ionization constants with the reported behavior for the reaction of the silver compounds with serum does not indicate that a simple ionization phenomenon is involved. The solubility of these salts should be considered, as well as the tendency of free silver ions to complex with many endogenous biological substances.

This solubility study was undertaken to determine the properties of some silver sulfonamides other than silver sulfadiazine to provide more information about their behavior in aqueous solution. The solubility and ionization properties of silver sulfamethazine, silver sulfamethizole, and silver sulfapyridine are reported. A possible correlation between the physical properties of the silver salts and their biological activity is proposed.

EXPERIMENTAL

Equipment—Potentiometric measurements were made using a pH meter[1], accurate to ±0.001 pH unit, in a thermostated bath regulated at 25 ± 0.1°. Silver-ion concentration was measured with a silver-ion se-



Scheme I—Silver sulfamethazine dissolution

[1] Digital 112 research pH meter, Corning Scientific Instruments, Medfield, Mass.

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