THE ANTIBACETIAL ACTIVITY OF TRIMETHOPRIM-SULFAMETHOXAZOLE IN URINE

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THE ANTIBACTERIAL ACTIVITY OF TRIMETHOPRIM-SULFAMETHOXAZOLE IN URINE

by

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A thesis submitted in partial
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St. John's Newfoundland

In remembrance of my father

REX CARBERRY BURSELL

January 2, 1908 - June 24, 1966

"The storm is over"

ABSTRACT

Cotrimoxazole (TMP-SMX) is a premixed combination of the antimicrobial agents, trimethoprim (TMP) and sulfamethoxazole (SMX), in a fixed ratio. The drug has been used for the treatment of urinary tract and other infections for more than 10 years but its efficacy and the nature of its antibacterial activity have remained controversial. The present work was undertaken in an effort to clarify some of the issues which have been raised.

The antibacterial properties of the drug and its components were assessed in several *in vitro* systems. Synergy between TMP and SMX, one of the main arguments supporting the use of the fixed combination, was examined by drug diffusion in agar, checkerboard titration, and time-kill curves against a standard *Escherichia coli* strain (ATCC 25922) and against a variety of bacterial pathogens isolated from patients with urinary tract infection. Minimal inhibitory concentrations for 100 clinical isolates were established by agar dilution and the parameters by which synergy is defined were evaluated. Time-kill curves, constructed from growth in drug-supplemented broth and urine, and in urine from cotrimoxazole-treated patients, were used to assess the kinetics of antibacterial activity of TMP-SMX and its components against pathogens commonly

associated with urinary tract infections.

The results reported here indicate that TMP is the more active agent in the mixture and in many instances inhibition is afforded by the TMP component alone. In vitro synergy could be demonstrated when the test organism was sensitive to each of the components but the synergistic effect was clear only at drug levels significantly lower than those achievable in urine following usual drug dosage. Time-kill curves demonstrate the bactericidal nature of TMP-SMX activity but results with TMP alone were not markedly different. Bactericidal effects could not be detected for SMX.

Bioassay of urine samples from cotrimoxazole-treated patients showed that TMP was the major active constituent. Only low levels of biologically active SMX were detected by assay against a TMP-resistant strain of Proteus mirabilis. The minor contribution of SMX to total antibacterial activity in urine specimens from these patients was confirmed by the addition of para-aminobenzoic acid (PABA), a known antagonist of sulfonamides.

The data presented do not support the efficacy of the fixed ratio combination in the management of urinary tract infections.

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INTRODUCTION

Trimethoprim-sulfamethoxazole (TMP-SMX) is a fixed combination antimicrobial agent. Under the generic name co-trimoxazole, the drug was first released in Great Britain for general therapeutic use in 1968. In 1973, the Food and Drug Administration of the United States granted approval for use in that country despite an earlier ban on fixed combination antimicrobials (1,2). However, use of the drug was restricted to the treatment of chronic urinary tract infections, proven Pneumocystis carinii pneumonia, shigellosis, and otitis media caused by susceptible strains of Haemophilus influenzae or Streptococcus penumoniae (3).

TMP-SMX, in a fixed ratio of 1:5, has been widely used as an oral preparation in the treatment of urinary tract infections. This combination has a wide spectrum of activity against a broad range of both Gram positive and Gram negative pathogenic organisms. The agent's principal target is Gram negative bacilli (Enterobacteriaceae) which are responsible for the majority of urinary tract infections (4-8).

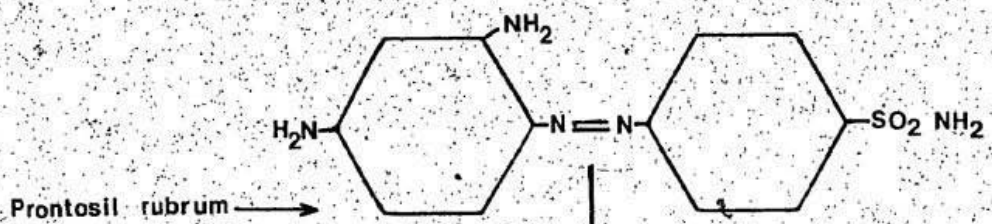
TMP-SMX is marketed in North America under the brand-names Septra (Burroughs Wellcome Laboratories, discoverers of the trimethoprim component) and Bactrim (Hoffman-LaRoche, synthesizers of the sulfonamide component).

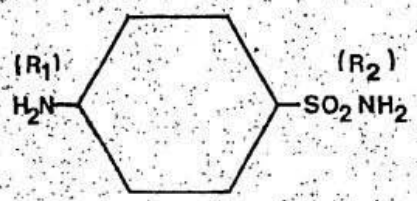
Historical Background of Sulfonamides

The discovery of sulfonamides in 1935 initiated a new era in the chemotherapy of infectious diseases. Gerhardt Domagk, a German industrial researcher, found that a red azo dye, "prontosil rubrum", had a curative effect for streptococcal infections experimentally induced in mice, although antibacterial activity could not be demonstrated *in vitro* (9-13). Within less than a year, a group of investigators (14) at the Pasteur Institute in Paris suggested that the therapeutic action of prontosil rubrum depended on its breakdown in the body to an inactive dye and an anti-bacterial substance called sulfanilamide (Fig. 1). The sulfanilamide moiety was found to be active against a variety of microorganisms both *in vitro* and *in vivo* (14). Later Fuller (15) confirmed that prontosil was indeed hydrolyzed *in vivo* to sulfanilamide.

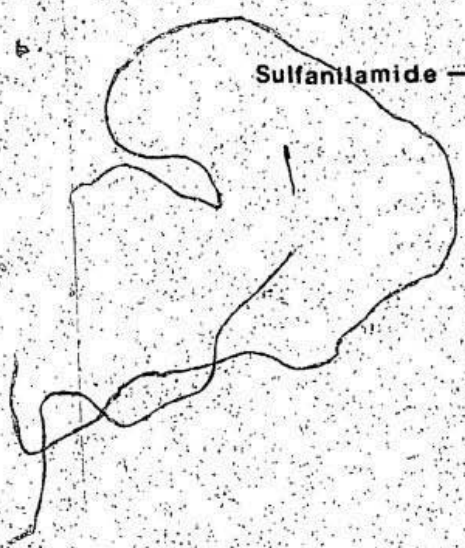
By various substitutions, almost exclusively on the sulfonamide 1-NH_2 group, over 5000 sulfonamide compounds have been synthesized in the past 40 years, many of them being more active and less toxic than the original compound (Fig. 2). The earliest sulfonamide to be marketed was sulfapyridine in 1938 (13). Sulfamethoxazole (SMX), the partner compound to trimethoprim in co-trimoxazole, was not reported until 1961, coincidently at the same time as the first report on trimethoprim (16).

Figure 1. Production of sulfanilamide by lycholysis *in vivo* of prontosil rubrum. The other sulfonamides are produced by substitution in the R_1 and R_2 positions (11).





Sulfanilamide →

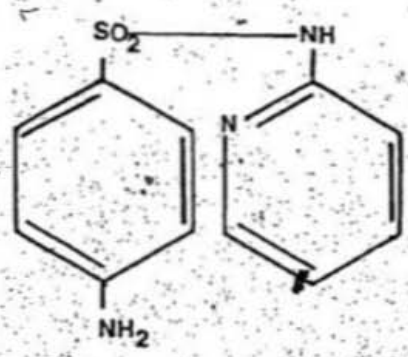


Early investigations into the mode of action of sulfonamides revealed that many materials from natural sources contained substances which antagonized or had the property of overcoming the antibacterial effect of sulfonamides *in vitro*. Stamp (17) first observed this phenomenon with cell-free extracts of *Streptococcus haemolyticus*. Other materials with inhibitory activity included blood, peptones, yeast and meat extracts, all common constituents of culture media. Woods (18) investigated this phenomenon by studying the biochemical and chemical properties of an antagonist present in cell-free extracts prepared from yeast. He found that there was a strict quantitative relationship between the concentration of sulfonamide added to the culture medium and the amount of yeast extract required to permit the growth of the test organism. This fact was suggestive of competitive inhibition in enzyme reactions whereby substances chemically related to the natural substrate can inhibit the action of an enzyme. Preliminary studies on the biochemical nature of the yeast extract antagonist suggested to Woods that the active component was para-aminobenzoic acid (PABA) or a similar component (18).

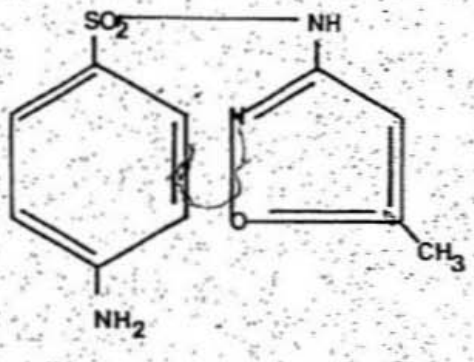
Woods proposed that PABA, or some related substance, is essential for bacterial growth and that sulfanilamide, by virtue of its structural similarity to PABA (Fig. 2) competes for the enzyme involved in the utilization of PABA by the bacterial cell. Woods provided support for his hypothesis

Figure 2. Chemical structure of some sulfonamides showing substitutions on the 1-NH_2 group. The structure of para-aminobenzoic acid (PABA) is included to show its similarity to sulfonamides (13).

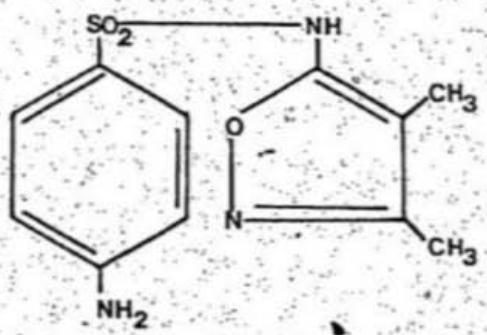
Sulfanilamide



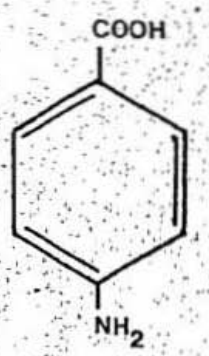
Sulfapyridine



Sulfamethoxazole



Sulfafurazole
(Sulfisoxazole)

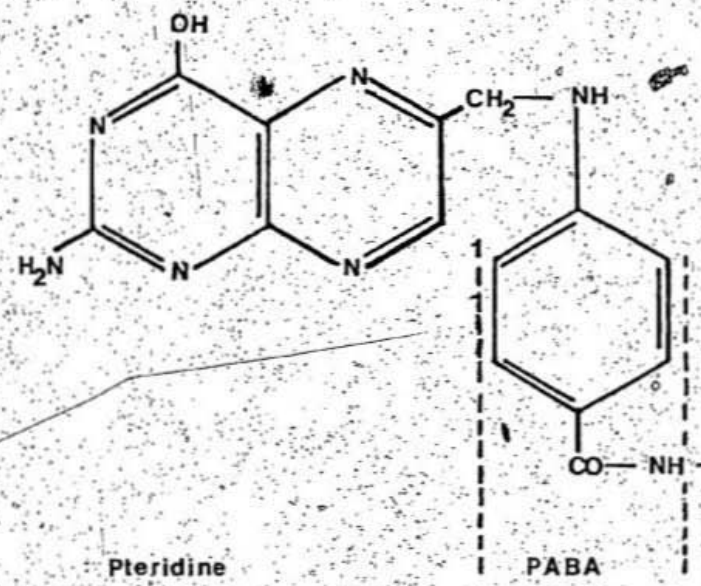


p-Aminobenzoic acid
(PABA)

when he showed that a quantitative relationship earlier demonstrated between sulfanilamide and the yeast extract antagonist was also true for sulfanilamide and PABA (18,19). Conclusive evidence that PABA was an essential bacterial metabolite was presented by Rubbo and Gillespie (20). While attempting to isolate an unknown growth factor for Clostridium acetobutylicum present in yeast concentrates, they found that PABA was essential for the growth of the species.

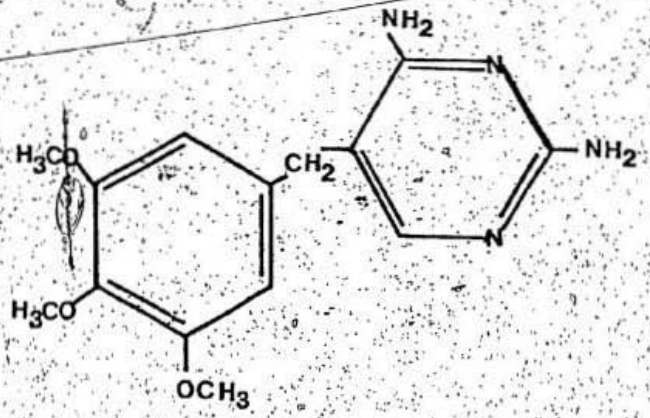
The validity of Woods' proposal was further strengthened in 1946 when a growth factor for Lactobacillus casei was isolated and a more precise role for PABA was established (21). This growth factor was shown to have a central PABA residue linked by its carboxylic acid group to glutamic acid and through its amino group to a pteridine ring structure (Fig. 3). The essential metabolite was later named folic acid and its biosynthesis was elucidated in 1964 (22). The sulfonamides, which are structural analogues of PABA appear to block the condensation of the pteridine with PABA in a competitive manner as Woods (18) suggested. There is also evidence, derived from cell-free systems, that sulfonamides act as substrates for the dihydropteroate synthetase with the consequeht formation of spurious folic acids within bacteria (23). The latter mechanism would be expected to be less readily reversible than classical competitive inhibition.

Figure 3. The chemical structure of folic acid and trimethoprim. Trimethoprim is a structural analogue of the pteridine portion of the folic acid molecule.



Pteridine PABA Glutamic acid

FOLIC ACID



Trimethoprim

Brown (23) and other workers used cell-free bacterial extracts to show that preincubation of limiting amounts of pteridine with sulfonamide inhibited the subsequent incorporation of PABA even if the latter were added in excess. More recently, Bock (24) and his associates demonstrated by means of an isotopically-labelled sulfonamide that the latter was incorporated into a "folate" analogue both in cell-free extracts and in bacterial cells.

Folic acid, as a co-factor in 1-carbon transfer in the synthesis of several cell constituents, is a required vitamin for mammals (25). Although PABA is not a metabolite of mammals, one might expect the products of these pathways to reverse sulfonamide activity non-competitively. Two factors provide for selective toxicity and make sulfonamide chemotherapy possible:

1. Most bacterial pathogens cannot utilize preformed folic acid from the host's blood or infected tissue but must synthesize it from pteridine and PABA, presumably because the utilization of exogenous folate requires an ATP-dependent transport system which they lack (13). On the other hand, mammalian cells in the process of evolution have lost their ability to synthesize folic acid and must obtain it from exogenous dietary sources (25).

2. Of the several compounds whose biosynthesis depends on folate, only methionine and the vitamins are present in body fluids: purines and pyrimidines are synthesized intracellularly and do not circulate as free bases or nucleotides that can be utilized by bacteria (26).

Historical Background of Trimethoprim

The mode of action of trimethoprim, and the mechanism for its selective toxicity, were not established until the role of folic acid as a co-factor in the synthesis of essential cell components had been elucidated. In 1949, Sauberlich showed that Leuconostoc citrovorum possessed a specific growth requirement for the reduced form of dihydrofolic acid (27). The "citrovorum factor" was later identified as tetrahydrofolic acid (folinic acid). Futterman (28) in 1957 successfully isolated the enzyme, dihyrofolate reductase, responsible for the reduction of dihydrofolic acid (DHFA) to tetrahydrofolic acid (THFA). The latter form is the active molecule for 1-carbon transfer to intermediates in the synthesis of amino acids, purine, and thymine, and ultimately deoxyribonucleic acid (25). Agents which inhibit the reduction of DHFA to THFA by dihydrofolic reductase have been termed "antifolates".

The elucidation of the PABA-sulfanilamide relationship stimulated an extensive search for antimetabolites capable of causing specific "biochemical lesions". As early as 1942, Hitchings and other researchers at the Burroughs Wellcome Laboratories in New York began a methodical investtigation of possible inhibitors of nucleic acid synthesis (29-31). Using Lactobacillus casei as a model system (32), these workers studied in turn each of the natural purine and pyrimidine bases and found that essentially all derivatives of 2,4-diaminopyrimidines possessed antifolate activity (33-35). Many of these substances were found to have antimicrobial activity but, with few exceptions, the toxicity of the antifolates was as great to the host as it was to the pathogen. However, few antifolates are useful therapeutic agents. Pyrimethamine and methotrexate have found application in the treatment of malaria and carcinomas respectively (36). A highly specific action against bacteria was eventually found in the pyrimidine derivative, trimethoprim (31,38,39).

Trimethoprim (TMP) is a structural analogue of the pteridine portion of the dihydrofolic acid molecule (Fig. 3). The drug exerts its action by inhibiting the reduction of DHFA to THFA (Fig.4), catalysed in cells by the enzyme dihydrofolate reductase (25,38,40-42). Most living cells require this enzyme system but there is evidence that the enzyme differs from one species to another (43-44). The selective toxicity of TMP has been accounted for by its relatively

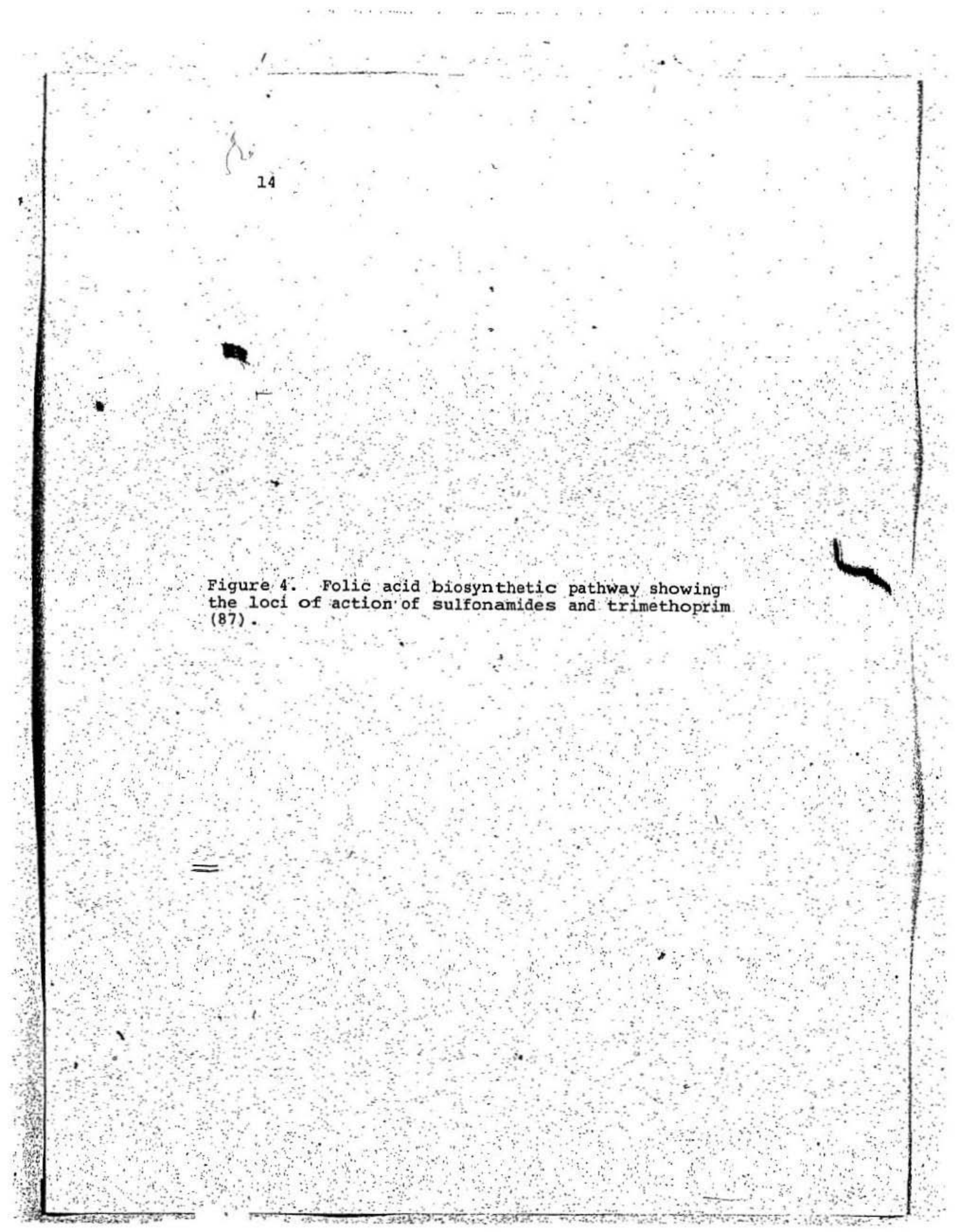
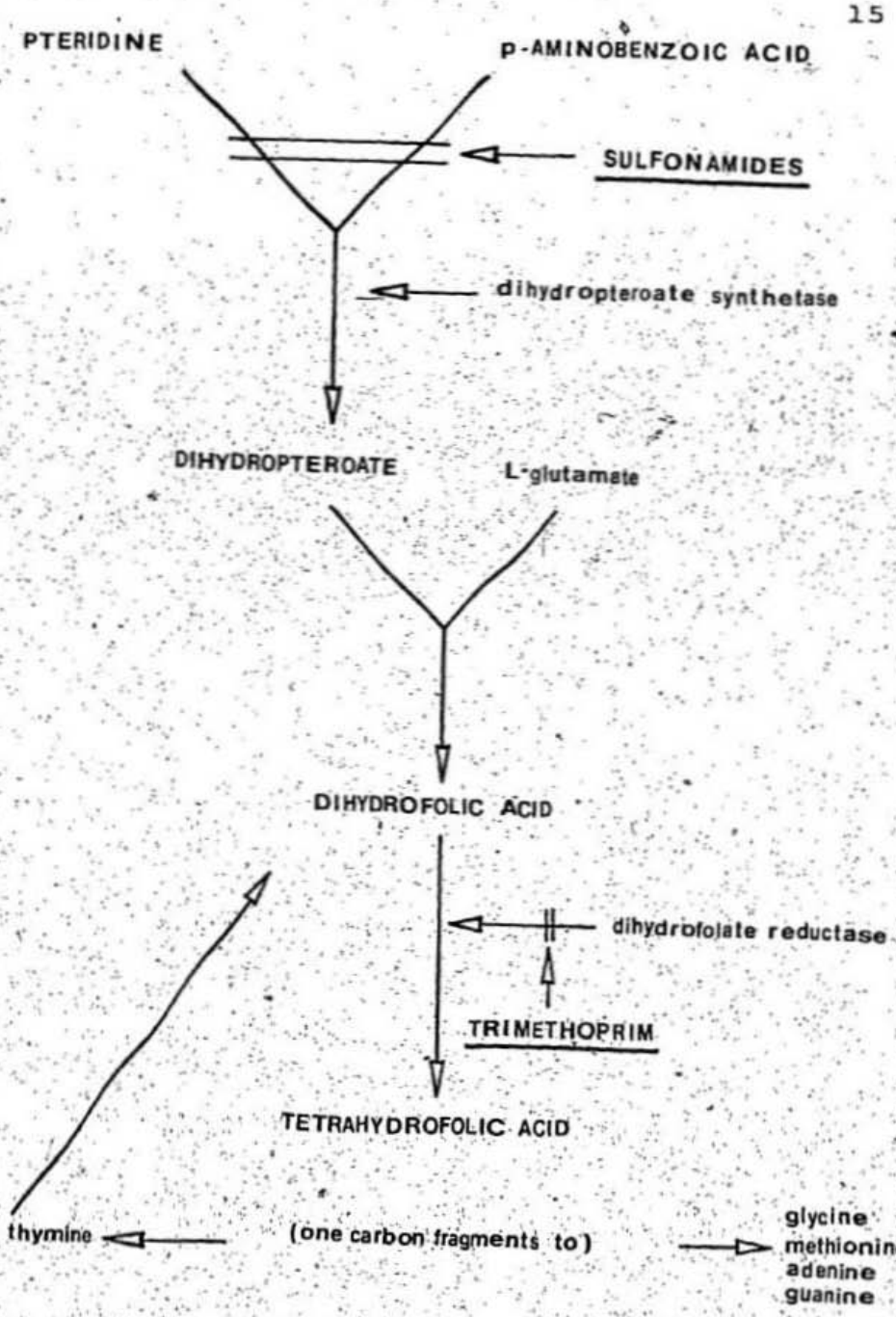


Figure 4. Folic acid biosynthetic pathway showing the loci of action of sulfonamides and trimethoprim (87).


PTERIDINE
P-AMINOBENZOIC ACID
SULFONAMIDES
dihydropteroate synthetase
DIHYDROPTEROATE
L-glutamate
DIHYDROFOLIC ACID
dihydrofolate reductase
TRIMETHOPRIM
TETRAHYDROFOLIC ACID
thymine
(one carbon fragments to)
glycine
methionine
adenine
guanine

specific action against the dihydrofolate reductase from bacteria. The concentration of TMP required for 50% inhibition of mammalian enzymes is greater by a factor of 16,500 than that required for a similar inhibition of bacterial enzymes (44).

Trimethoprim-Sulfamethoxazole in Combination

Combinations of antimicrobial agents are often used in medical practice. Most frequently combinations are prescribed to provide broad-spectrum coverage in seriously ill patients with mixed infections or from whom an infecting organism has not been isolated. Drug combinations have also been used to treat bacterial infections caused by a single organism in order to reduce the minimum inhibitory concentration (MIC) of one or both drugs, to increase bactericidal activity, or to decrease risk of emergence of resistant strains (45-50).

When two antimicrobial agents act together against a single organism their combined effect may be:

(a) indifferent (or additive) when the activity of each drug is unaffected by the presence of the other,

(b) antagonistic, when the combined effect of both drugs is significantly less than the sum of their independent effects,

c) synergistic, when the combined effect of the drugs is significantly greater than the sum of their independent effects.

Some thirty years ago, Greenberg and Richeson reported that antifolates potentiated or enhanced the antimalarial activity of sulfonamides in the treatment of experimental Plasmodium gallinaceum infections in chicken (51,52). In 1953 Eyles and Coleman confirmed these observations when they used a combination of pyrimethamine and sulfonamide to treat toxoplasmosis in mice (53). Later Hitchings and Bushby (54) noted a synergistic effect in vitro of a combination of sulfadiazine and a 2,4-diaminopyrimidine, a close analogue of TMP, against Proteus vulgaris. By 1961 it was generally accepted that combinations of antifolates and sulfonamides acted synergistically against a wide range of bacterial species in vitro and there was some evidence that the phenomenon was also operative in vivo (54,65).

The synergistic effect of sulfonamide-antifolate combinations has been explained by the theory of sequential blockade first proposed by Potter in 1950 to describe the effect of two inhibitors acting on different enzymes in a portion of the same biosynthetic pathway (55). Using a mathematical model Black presented evidence that synergism is a necessary consequence of such sequentially blocked systems (56). This concept has been disputed by other

investigators. Rubin and his associates examined the enzyme kinetics of sequential inhibition in cell-free systems *in vitro* and concluded that the response cannot be synergistic (57). This conclusion has been supported by Webb (58) who stated that sequential blockage of a linear reaction by multiple inhibitors in the steady state is theoretically incapable of producing an effect greater than that by a single inhibitor alone.

Poe (59) pointed out that the theory of sequential inhibition fails to explain the failure of some dihydrofolate reductase inhibitors to act synergistically with sulfonamides (60) or the potentiation of TMP by sulfonamides in a number of sulfonamide-resistant organisms (61). Using *Escherichia coli* this investigator showed that several sulfonamides act with TMP on the same enzyme, namely the dihydrofolate reductase, *in vitro*. Burchall (62) and Then (63) have disputed the significance of these findings claiming that the concentration of sulfonamides used by Poe far exceeded those achievable *in vivo* and the suggested mechanism would require that bacteria concentrate sulfonamides for synergism to occur.

Although the mechanism and clinical significance of synergy in antifolate-sulfonamide activity has not been clearly established, a fixed combination of TMP and SMX has been widely used since its introduction by Burroughs

Wellcome Laboratories in 1968. Trimethoprim was selected from several 2,4-diaminopyrimidines because of its superior antibacterial activity and relatively low toxicity (38). It is unique in the history of chemotherapy that a compound, whose efficacy as an antibacterial agent when used alone was largely unknown at the time, was made available only in combination with another antibacterial agent (64). As the sulfonamide component, SMX was chosen for the same reasons as trimethoprim and because its plasma half-life after oral administration in man is similar to that of TMP (61, 65, 66). The latter property simplifies dosing. The 1:5 combination of the fixed TMP-SMX combination, was formulated to produce a ratio of 1:20 of TMP-SMX in the blood, the optimal ratio for in vitro activity against many organisms (67). The proportionately lower plasma level of the TMP component as compared with the SMX component is a result of wider distribution of TMP in the body due to the former's lipophilic properties (68).

The validity of the scientific rationale for combining the drugs in fixed combination has been questioned (64, 68-72). The vast majority of infections for which co-trimoxazole is prescribed are not bacteremias and therefore plasma ratios may not be relevant (73-75). In addition, concentration ratios vary widely in different tissues and body fluids. Craig and Kunin (76) showed that the concentration of non-metabolized SMX in tissue is always lower than in serum while

the reverse is true for TMP. In fact it is probable that the theoretically optimal ratio is rarely reached at the site of the infectious process. Therapy of urinary tract infections is the most common application of co-trimoxazole. Most workers agree that TMP:SMX ratio in urine is close to 1:1,a proportion at which synergistic activity is unlikely (72).

Effect of Thymidine/Thymine

Both components of TMP-SMX are inhibitors of bacterial synthesis of tetrahydrofolic acid, the metabolically active form of folic acid (Fig.4). It is not surprising therefore, that certain end products of the folate metabolism present in many non-defined culture media can enable the organisms to avoid the antibacterial effects of the drugs. The nucleoside, thymidine, plays a critical role in interference with TMP-SMX activity. Extremely small amounts of thymidine, as little as 0.05 ug/ml of medium can significantly reduce the activity of TMP, SMX, or the combination of the two (77). Mutants which lack dihydrofolate reductase, and thus require exogenous thymine or thymidine to survive are intrinisically resistant to TMP.

The interference in vitro with the action of TMP and SMX by thymidine raises the question of whether thymidine could affect the activity in vivo of the combination. Bushby (67), a Burroughs Wellcome researcher, states that

thymidine is rapidly degraded *in vivo* and would not interfere with the combination TMP-SMX. To support this claim, experiments were performed in which mice were given doses of 500 mg thymidine/kg intraperitoneally, once on the day before induced infection with *P. vulgaris*, twice on the day of infection, and once 24 hours after infection. Survival rates following treatment with TMP-SMX revealed that the nucleoside had no adverse effects on the protection afforded by the drug combination. However, Stokes and Lacey (77) found that levels of thymidine 5.0 µg/ml or greater are inhibitory to the growth of bacteria *in vitro*, a factor which may have contributed to animal survival in Bushby's experiments.

The effect of thymidine in tissue and body fluids on TMP-SMX therapy remains controversial. Several investigators have been unable to detect free thymidine in normal tissue (78-80) but recent evidence suggests that thymidine or thymidine-like antagonists are present in infected tissue. The evidence comes from two observations:

1. Increasing number of isolations of thymine-or thymidine-requiring bacteria from clinical specimens has been reported (81-84). Such mutants die rapidly *in vitro* in the absence of thymine or thymidine. The occurance of these mutants in human diseases suggest that levels of thymidine or thymine are present in these

patients in amounts sufficient to support the growth of the defective organisms in infected sites.

2. Maskell and associates (82) have shown that thymine or thymidine-requiring bacteria can grow in urine from infected individuals and suggested that thymine or thymidine, possibly released from lysed pus cells, is present.

Study of Antimicrobial Activity in Vitro

It would be appropriate here to review the theoretical considerations and the principles of methodology in the in vitro study of antimicrobial activity. Some definitions are necessary:

Bacteriostatic activity is the capacity of an antimicrobial agent to inhibit reversibily the growth of bacteria and can be expressed quantitatively as the Minimum Inhibitory Concentration (MIC), i.e. the smallest amount of the drug which is capable of inhibiting the growth of a given bacterial strain.

Bactericidal activity is the capacity of an antimicrobial agent to accomplish irreversible inhibition (i.e. killing) of an inoculum of a given bacterial strain and can be quantitatively expressed as the Minimum Bactericidal Concentration (MBC).

Determination of Antimicrobial Activity of Single Agents

MIC can be determined in agar or fluid media. In the former the antimicrobial agent is added to the agar medium and plates containing different concentrations of the antimicrobial are surface inoculated with the bacterial strains to be tested. When many strains are to be tested, the cultures are transferred from wells in a seed plate with a multiple inoculating device (85). The method is readily adapted to testing fastidious bacteria by incorporating blood or other enrichments in the agar medium. When fluid media are employed, the antimicrobial agent is serially diluted in a suitable broth contained in tubes or wells and each dilution is challenged with a standardized inoculum of test organism. In each system the end-point (MIC) is recorded as the highest dilution of the antimicrobial which prevents visible growth of the bacterial strain. Only tests in fluid media lend themselves to subsequent determination of MBC by subculture of dilutions showing no growth to propagating medium appropriate for the strain being tested (86).

Determination of Interaction between Antimicrobials

Several approaches have been developed for the study of antibacterial combinations in vitro and each has its advantages and limitations.

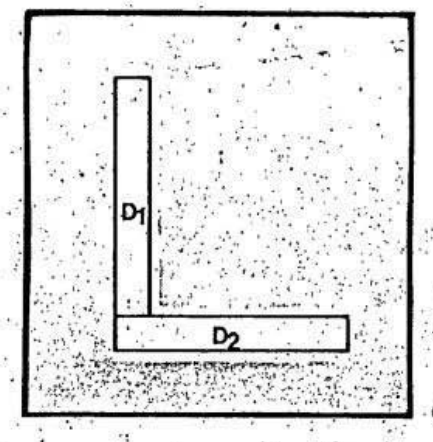
1. Diffusion Method

In a popular modification of this method, which has proved useful for screening purposes, filter paper strips impregnated with one or the other of the antimicrobial agents are placed at right angles on the surface of a seeded agar plate (86,87). Following incubation, patterns of inhibition are observed and are interpreted as illustrated in Fig. 5 . Additive or different combinations show no enhancement of their zones of inhibition at the junction of the two paper strips. Synergistic combinations show enhanced inhibition and may occasionally demonstrate activity at this point even when the organism is resistant to each drug separately. Antagonistic combinations reveal truncated zones of inhibition at the junction of the paper strips. The method has the advantages of simplicity and ease of reading. Its disadvantages are that inhibitory rather than bactericidal activity is detected and results are difficult to relate to levels achievable in the patient.

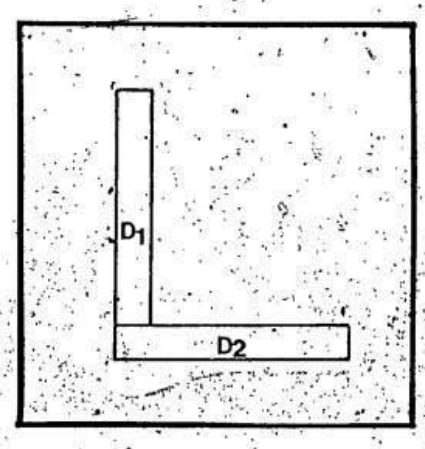
2. Checkerboard Titration

A more precise method, which yields quantitative results, is the checkerboard titration procedure. This is a two-dimensional broth dilution test in

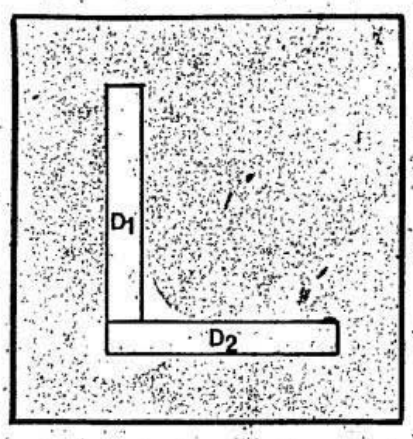
Figure 5. Assessment of antibacterial combinations by paper strip technique. Rectangular areas D_1 and D_2 represent filter paper strips, each impregnated with a different antimicrobial solution. Shaded areas represents a lawn of bacterial growth and the clear areas adjacent to the paper strips indicate inhibition of growth (87).



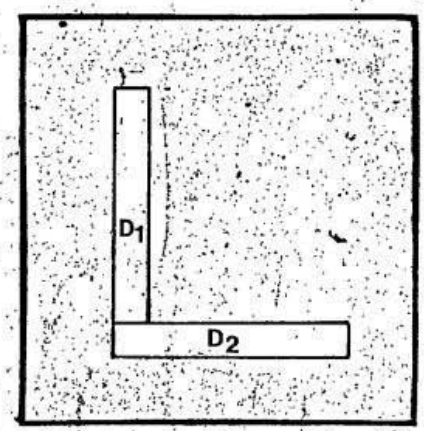
A. ADDITIVE (indifferent)



B. SYNERGISTIC



C. SYNERGISTIC



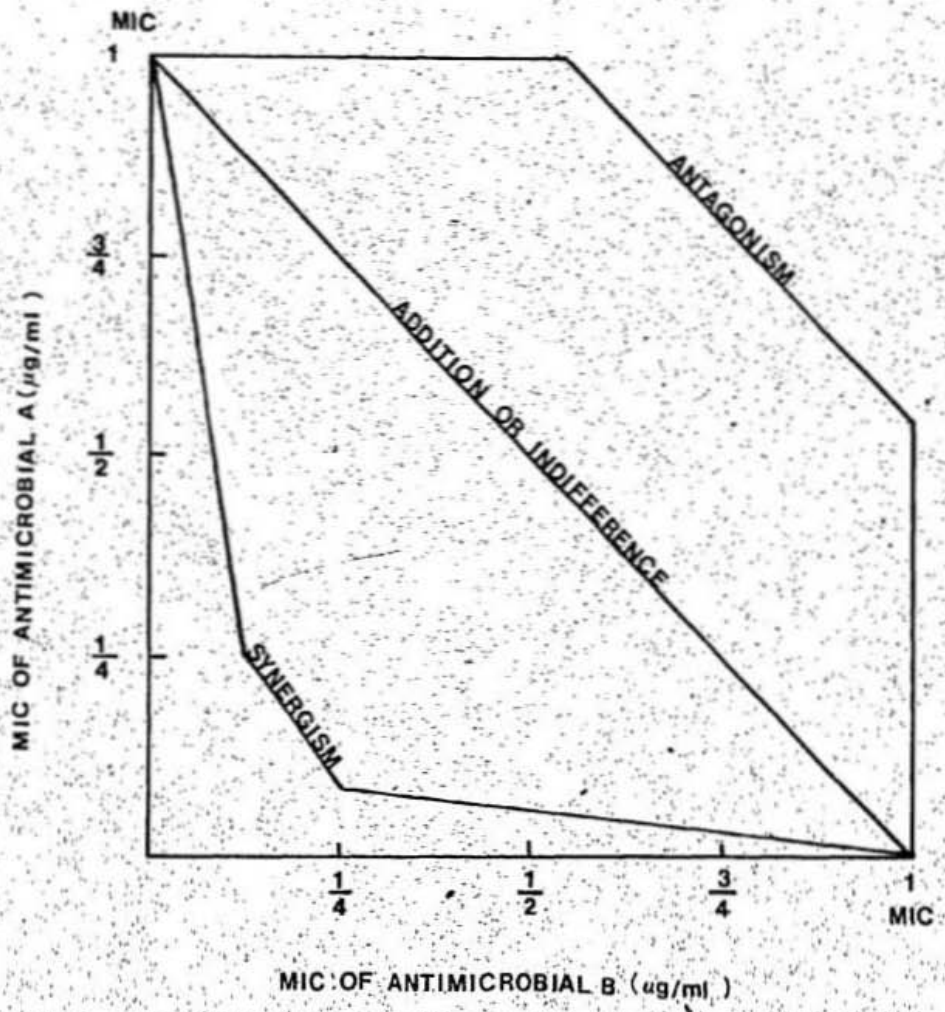
D. ANTAGONISTIC

Figure 6. Hypothetical example of two-dimensional ("checkerboard") titration. The vertical columns represent files of test tubes, each containing the same concentration of TMP, either 16, 8, 4, 2, 1, 0.5, or 0 µg/ml. The horizontal rows represent test tubes, each containing the same concentration of SMX, either 256, 128, 64, 32, 16, 8, or 0 µg/ml. After inoculation and overnight incubation, the presence (+) or absence (-) of growth is recorded. The bottom horizontal row reveals the MIC for TMP alone (4 µg/ml in this example) and the right-hand vertical column reveals the MIC for SMX alone (64 µg/ml in this example). The tube in the lower right hand corner serves as a drug-free growth control. All other results are for various combinations of the two antimicrobials. Note that 16 µg/ml of SMX (1/4 MIC) plus 0.5 µg/ml of TMP (1/8 MIC) together inhibited the hypothetical strain. The results demonstrate synergistic activity.

SMX (µg/ml)	TMP (µg/ml)						
	16	8	4	2	1	0.5	nil
256	-	-	-	-	-	-	-
128	-	-	-	-	-	-	-
64	-	-	-	-	-	-	-
32	-	-	-	-	-	-	+
16	-	-	-	-	-	-	+
8	-	-	-	-	-	+	+
nil	-	-	-	+	+	+	C

which a series of rows and columns of tubes or wells containing varying combinations of the two drugs in serial dilution, are inoculated with a standardized suspension of the test organism (Fig. 6). The presence of growth in each tube or well is recorded after appropriate incubation. The MIC of each antimicrobial alone is thus revealed and results with various combinations can be plotted as an isobologram indicating additive, synergistic, or antagonistic effect (Fig. 7). Most investigators accept the combination as synergistic if one-quarter of the MIC of each drug when used separately produces inhibition of bacterial growth when they are combined. Synergism may also be quantitated by determining the Fractional Inhibitory Concentration (FIC) index. In each row the lowest concentration of drugs necessary to inhibit growth is noted. The FIC for each drug is derived by dividing the concentration of drug present at that point by the MIC of the organism to that drug alone. The FIC index is then the sum of these values for both drugs at that point. When the FIC index is less than 1.0 the combination is synergistic; when it is equal to or greater than 2.0, the combination is antagonistic. Intermediate values are considered

Figure 7. Diagrammatic illustration of an isobologram showing antagonism, addition or indifference, and synergism. If the MIC of each drug in the presence of the other is plotted on a graph in which the concentration of the drugs are on the axes, a line called an isobol is obtained. A straight line across the graph represents addition, a line curved towards the lower concentrations indicates synergy and one bowed outward from the straight line indicates antagonism (101)


MIC
1
3/4
1/2
1/4
ANTAGONISM
ADDITION OR INDIFFERENCE
SYNERGISM
1/4
1/2
3/4
1
MIC
MIC OF ANTIMICROBIAL A (µg/ml)
MIC OF ANTIMICROBIAL B (µg/ml)

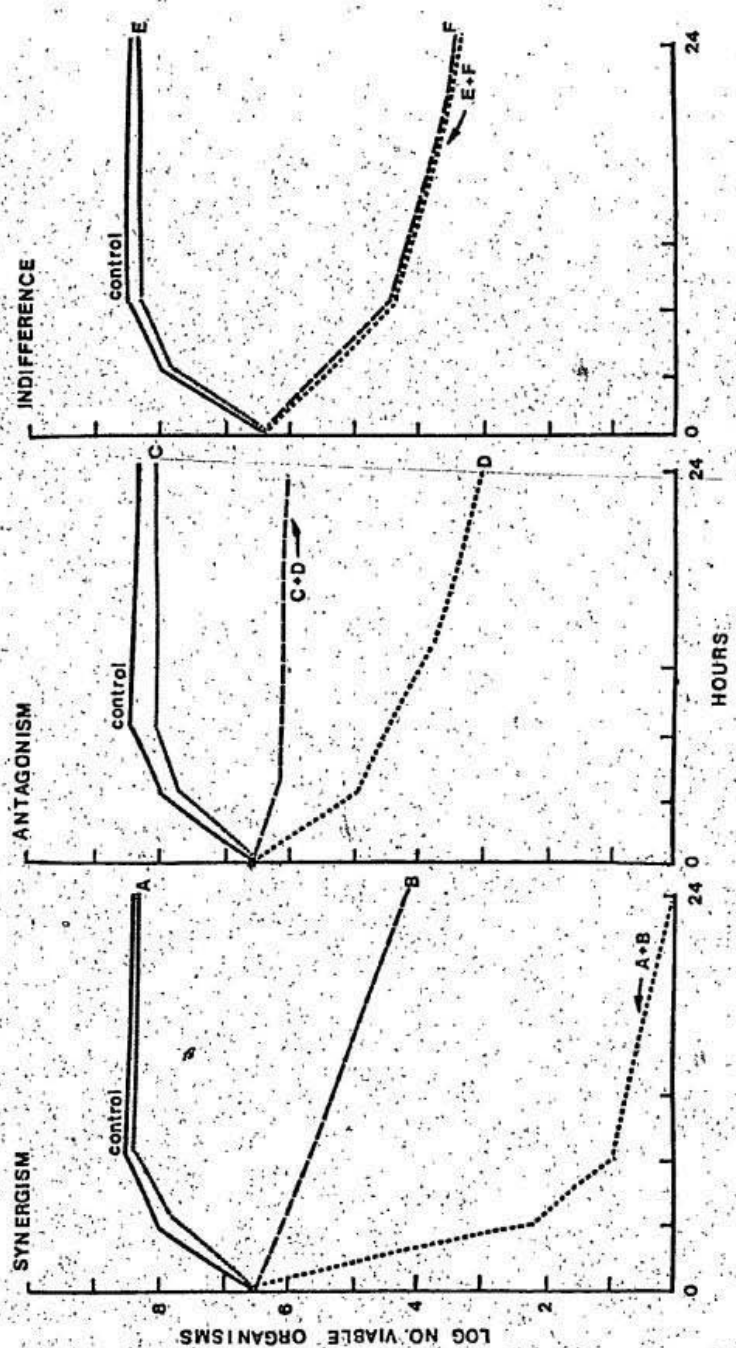
indifferent. The ratio of drug concentrations at the lowest FIC value is considered optimal (86,88). Bactericidal activity of combinations can be determined by subculture of tubes or wells showing no visible growth. However, this follow-up sampling complicates the procedure and does not provide information on the kinetics of bactericidal activity.

3. Time-Kill Curves

Bactericidal or lethal activity can be expressed as the rate of killing of a bacterial strain by fixed concentrations of each drug alone and in one or more combinations. Curves are constructed by plotting the number of surviving bacteria at various time intervals during incubation of the test organism in the presence of the antimicrobial agents singly or in combination. Samples are removed from broth cultures at different times to determine the number of viable bacteria as colony-forming units (86). Possible results showing additive, synergistic, or antagonistic antibacterial effects are shown in Fig. 8.

It can be seen from this review that the therapeutic potential of the TMP-SMX combination appears to have a rational theoretical basis but conflicting views have

Figure 8. The effect of antimicrobial combinations as determined by the time-kill methods. A and B = synergism; C and D = antagonism; E and F = indifference (87).


SYNERGISM
ANTAGONISM
INDIFFERENCE
control
A
B
A+B
C
D
C+D
E
F
E+F
LOG NO. VIABLE ORGANISMS
HOURS
0
2
4
6
8
24

been reported concerning the mechanisms involved in the synergistic activity of the two components. There has been further controversy with respect to the selection of a fixed ratio of components suitable for the treatment of infections in a variety of body systems. In North America, by far the commonest application of co-trimoxazole has been in the treatment of urinary tract infections. The present work was undertaken to determine laboratory evidence for the efficacy of the antimicrobial agent in the management of urinary tract infections seen in a general hospital. Several *in vitro* test systems, which have been used to support claims for the drug's effectiveness in these clinical situations, were examined. Subsequently the methods were applied to the study of bacterial strains isolated from urine specimens submitted to the diagonostic laboratory of the hospital. The activity of the drug combination and its components in urine from co-trimoxazole-treated patients and in urine supplemented by the addition of known amounts of the agents was also investigated.

MATERIALS AND METHODS

Media and Reagents

Mueller Hinton Broth (MHB) and Wellcotest Sensitivity Test Agar (WST) were purchased from Difco Laboratories, Detroit, and Wellcome Research Laboratories, England respectively. Thymidine (No. T-9250) was purchased from Sigma Chemical Company, Missouri. Para-aminobenzoic acid (PABA) was obtained from Difco Laboratories and MacConkey Agar No. 3 from Oxoid Limited, England. Trimethoprim base powder (Lot. 2198) was kindly provided by Burroughs Wellcome, LaSalle, Quebec and sulfamethoxazole (Lot. R-2007) by Hoffman-LaRoche, Vandreuil, Quebec.

Test Organisms

a) Urinary isolates. One hundred bacterial isolates were obtained from patients with significant bacteriuria defined as 10^5 or more colony-forming units (CFU)/ml urine (89). The strains were obtained from the Microbiology Laboratory of the General Hospital, Health Sciences Centre. The organisms had been identified by standard procedures (90). The clinical isolates included Alcaligenes odorans (1 isolate), Citrobacter freundii (6), Enterobacter aerogenes (1), Enterobacter cloacae (3), Escherichia coli (37), Klebsiella oxytoca (4), Klebsiella pneumoniae (13) Proteus mirabilis (15), Proteus morganii (1), Proteus

vulgaris (1), *Pseudomonas aeruginosa* (9), *Serratia marcescens* (5), *Staphylococcus aureus* (1), and *Streptococcus faecalis* (3).

All isolates were maintained on heart infusion agar slopes (Difco) and stored at room temperature. The organisms were subcultured to MacConkey agar plates or blood agar to determine purity prior to testing.

b) A thymidine-requiring strain of *Klebsiella* (1839) was kindly provided by Dr. A. R. Ronald, University of Manitoba and Health Sciences Centre, Winnipeg.

c). *Reference Strains* In addition to the above, six reference strains were employed: *Enterobacter cloacae* ATCC 23335, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 8100, and *Staphylococcus aureus* ATCC 25923.

Urine Samples

a) *Clinical specimens* Fifty urine samples were selected at random from patients receiving TMP-SMX. The patients were known to have not received antimicrobial therapy other than TMP-SMX. Dosage for the latter was usually two tablets TMP-SMX (80 mg + 400 mg) twice daily. The urine samples were clarified by centrifugation for 20 minutes at 10000 x g in a refrigerated centrifuge.

The supernates were sterilized by filtration through 0.22 µm porosity membrane filters (Millipore), and dispensed in 10-15 ml portions. All specimens of this group were prescreened for antimicrobial activity other than that due to TMP-SMX. Molten cooled WST agar, containing 200 µg/ml thymidine to neutralize TMP-SMX activity, was seeded with E. coli ATCC 25922 and poured into Petri dishes. The MIC values for this strain against a number of commonly used antimicrobial agents are listed in Table 1. Paper disks, ½" in diameter, were saturated with each urine sample and applied to the surface of the solidified medium. Plates were incubated for 18 hours at 35°C and the presence or absence of inhibition zones around each disk were noted. The screened supernates were stored at -20°C until further testing.

b) Control Specimens Urine samples from healthy untreated volunteers were processed and held in the same manner. Specimens of this group were screened for antibacterial activity as above except that thymidine-deficient medium (WST agar) was used.

c) Specimens for the detection of thymidine content One hundred and sixty-four urine samples were selected from random specimens submitted to the hospital laboratory. These samples were shown to lack inhibitory activity against the assay strain, Klebsiella sp. (1839) by paper

Table 1. Means and ranges of minimal inhibitory concentrations with *E. coli* ATCC 25922

Antimicrobial	Minimal Inhibitory Concentration (µg/ml) *E. coli* ATCC 25922 Mean	Range
Ampicillin	3.4	3.3 - 3.4
Cephalothin	6.2	0
Tetracycline	1.3	1.2 - 1.4
Kanamycin	2.3	2.3 - 2.4
Polymixin B	0.5	0.4 - 0.5
Gentamicin	0.4	0.3 - 0.5
Chloramphenicol	4.0	0
Carbenicillin	3.7	0
Nalidixic Acid	1.9	1.8 - 2.0
Nitrofurantoin	10.6	10.0 - 12.0

Reference (90)

disk screening using WST agar containing 2 µg/ml of thymidine.

Antimicrobial Agents

Stock solutions were prepared as follows:

Trimethoprim, 2000 µg/ml: TMP was converted to the soluble lactate by dissolving 200 mg of the base powder in a minimal volume of 0.1N lactic acid and bringing the solution to 100 ml with distilled water.

Sulfamethoxazole, 2000 µg/ml: 200 mg of SMX powder was dissolved in a few drops of 0.1N sodium hydroxide and brought to 100 ml with distilled water.

Sulfamethoxazole, 10000 µg/ml: as above except that 1000 mg of the drug was used.

The drug solutions were sterilized by membrane filtration and stored in appropriate portions at -70°C. Working supplies were maintained at 4°C for not longer than three weeks. No loss of activity could be demonstrated during this period. Working dilutions were prepared freshly from the stock for each experiment.

Activity of TMP and SMX in Combination

a) Paper strip method A modification of the paper strip method of Lorian and Fodor (93) was used to evaluate possible synergy of TMP-SMX combinations against a variety of organisms with varying susceptibility to

the individual agents. The test organisms were incorporated into molten WST agar in a final density of 10^6 CFU/ml. The seeded agar was dispensed in 20 ml volumes into 100 mm x 15 mm Petri dishes. Paper strips measuring 4 cm long and 0.9 mm wide were cut from Whatman No. 1 filter paper. One strip was soaked in the TMP solution containing 100 µg/ml and four other strips were soaked in SMX solutions containing 100, 500, 1000, and 2000 µg/ml respectively in order to produce ratios of 1:1, 1:5, 1:10, and 1:20 with the TMP strip. Paired strips, one containing TMP and the other SMX were placed at right angles on the agar surface so that one end of each strip touched. A separate agar plate was used for each ratio of the two antimicrobial agents. Following a preddiffusion time of 6 hours at 4°C, the plates were incubated at 35°C for 16 hours.

b) Two-Dimensional ("Checkerboard") Titration

A two-dimensional tube dilution method, slightly modified from Waterworth (94), was used to demonstrate the interaction of TMP and SMX against selected organisms. The "checkerboard" is the pattern formed by multiple combinations of the two antimicrobial agents in varying concentrations equal to, above, and below their minimal inhibitory concentrations for the organism being tested.

The titration consisted of columns which contain the same amount of one drug diluted along the x-axis, and the rows containing the same amount of the other drug diluted along the y-axis. The bottom row and the right-hand column contain dilutions of the individual drugs only and one "square" served as a drug-free control. In the resulting array, each concentration of the individual drug is tested alone and in every possible combination with the selected concentrations of the other (Fig. 6). The entire system received a standard inoculum.

In a typical run, six rows of six sterile 12 mm x 75 mm test tubes were arranged in a rack to form a square. (Additional tubes were used when a wider range of dilutions was necessary). The stock inoculum was prepared by adding 1 µl of an overnight broth culture of the test organism to 100 ml of MHB and mixing well, to provide an inoculum density of approximately 10^4CFU/ml. To 6 ml of the seeded broth was added twice the final highest concentration of SMX/ml desired and 0.5 ml of this suspension was added to each tube of the first row. To the remaining 3 ml of the culture-SMX mixture was added 3 ml of stock inoculum (halving the drug concentration) and 0.5 ml of the well mixed suspension was added to each tube of the second row. This procedure was continued to the fifth row. Each tube of

the sixth (bottom) row received 0.5 ml of the stock inoculum only. In the same manner, stock inoculum containing halving concentrations of TMP was added to five columns from left to right. The addition of the second inoculum served to halve the concentration of each drug so as to yield the desired level. The sixth (right-hand) column received 0.5 ml of the stock inoculum only. The lower right-hand tube served as the drug-free control. The rack was shaken gently to ensure mixing and incubated at 35^{0}C until good growth was observed in the drug-free control (usually 16 hours). The presence or absence of growth in each tube was recorded.

c) Time-Kill Curves

Time-Kill curves, using MHB as diluent, were constructed to evaluate the effect on urinary pathogens of exposure to TMP, SMX and the combination of concentrations usually found in the urine of patients being treated with TMP-SMX. The clinical isolates were selected on the basis of varying MIC values to the monocomponents of the combination and E. coli ATCC 25922 was used as a highly sensitive control. Four flasks, each containing 50 ml of MHB, were prepared for each bacterial isolate.

Two flasks were supplemented by the addition of 100 µg/ml of TMP and 100 µg/ml SMX. One flask was supplemented with 100 µg/ml of each drug (1:1 ratio usually found in urine) and the fourth flask served as a drug-free control. The organisms were grown in 4 ml MHB for 18 hours at 35°C and appropriately diluted to yield a final inoculum density of 10^4 CFU/ml. The flasks were incubated in a shaking water bath at 35°C and viable counts were performed on 0.1 ml samples at 0, 2, 4, 6 and 24 hours. Time-Kill curves were constructed on semi-logarithmic paper by plotting CFU/ml against time.

Similar time-kill curves were constructed for E. coli ATCC 25922 except that filter-sterilized urine from untreated healthy volunteers was used as the diluent and a wider range of drug concentrations and ratios were employed. Four ml volumes of urine were dispensed into sterile screw-capped tubes and the drugs were added aseptically in the following concentrations:

TMP	2, 5, 10, 15, 25, 50, 100 µg/ml
SMX	2, 5, 10, 15, 25, 50, 100 µg/ml
TMP-SMX	1:1 combinations of each of the above concentrations
	1:2 combinations consisting of each of the above TMP concentrations with twice the concentration of SMX

1:10 combinations consisting of each of the above TMP concentrations with 10 x the concentration of SMX.

The tubes were inoculated with *E. coli* ATCC 25922 to a final density of 10^4 CFU/ml. Incubation was carried out at 35°C in a shaking water bath and viable counts were performed at 0, 2, 4, 6, and 24 hours.

Minimal Inhibitory Concentration

Minimal inhibitory concentrations (MIC) for TMP, SMX, and TMP-SMX were established for each of the bacterial isolates by the agar dilution method (90), as follows:

Preparation of drug-containing agar medium: Stock solutions of each drug were diluted according to a protocol suggested by Ericsson and Sherris (91) as shown in Table 2. Ten ml of the appropriate concentration of each drug, or the combination, was added to 90 ml of sterile WST agar held at 50°C so as to yield the following range of concentrations:

TMP: 0.6, 0.12, 0.25, 0.5, 1.0, 1.25, 1.6, 3.1, 6.2, 12.5, 25, 50, 100, and 200 µg/ml

SMX: 1.0, 2.0, 4.0, 8.0 16, 32, 64, 128, 200, 256, 500, 700, and 1000 µg/ml

Table 2. Protocol for the preparation of antimicrobials for agar dilution method (91).

Antimicrobial Solution Volume	(µg/ml)	+ Sterile Water Volume	= Intermediate Conc. (µg/ml)	Final conc. at 1:10 in agar (µg/ml)	$\log_2$ Conc.
6.4	2000	3.6	1280	128	7
2	1280 (from above)	2	640	64	6
1	1280 (from above)	3	320	32	5
1	1280 (from above)	7	160	16	4
2	160 (from above)	2	80	8	3
1	160 (from above)	3	40	4	2
1	160 (from above)	7	20	2	1
2	20 (from above)	2	10	1	0
1	20 (from above)	3	5	0.5	-1
1	20 (from above)	7	2.5	0.25	-2
				etc.	etc.

TMP-SMX: 0.03/0.59, 0.06/1.19, 0.12/2.38, 0.25/4.75, 0.5/9.5, 1.0/19, 2/38, 4/76, 8/152, 16/304 and 32/608 µg/ml.

The contents of each flask were well mixed, 25 ml amounts were poured into 100 mm x 15 mm Petri dishes, and the medium was allowed to solidify at room temperature on a level surface. The plates were placed at 35°C for 30 minutes with the lids tipped to allow the agar surface to dry. Prepared plates were used the same day or held at 4°C for not longer than 24 hours before use. Plates containing WST agar without added drugs were prepared for use as viability control. For testing swarming strains of Proteus the medium was pipetted into wells of microtitration trays which provided a barrier against spread from the inoculation sites (92).

Standardization of inoculum: The test organisms and references strains were each cultured at 0.5 ml of MHB for 4 - 6 hours at 35°C. The broth cultures were mixed well and 0.1 ml of each was added to 10 ml MHB to give a final density of 10^7CFU/ml. Because of the time involved in the preparation of the standardized suspensions the latter were held on ice until the inocula for all 106 cultures had been prepared.

Inoculation: A Steer's replicator (85) was used to inoculate the agar dilution plates. This device consists of a metal plate to which are affixed 36 inoculating prongs arranged in such a manner as to correspond to 36 reservoir wells in a "seed plate". The latter was charged by the addition of 0.5 ml of the appropriate standardized inoculum to 35 of the 36 wells (one well received India ink only and served as an orientation marker). When the wells in the seed plate had been filled the Steer's replicator was used to transfer the respective inocula to the surface of the drug-containing agar plates. The configuration of inoculum sites on the agar plates corresponded with that of the seed plate. Each prong delivers 0.001 to 0.002 ml so that the final inoculum at each site was approximately 10^4 CFU. Inocula of P. mirabilis and P. vulgaris were transferred with a 1 µl loop directly from the standardized suspensions. The inoculated plates were allowed to stand undisturbed until the inocula had absorbed into the medium.

The plates were inverted and incubated at 35^0 C for 16 - 18 hours. The MIC was recorded as the lowest concentration of antimicrobial, or combination of antimicrobials, resulting in complete inhibition of growth, or allowing only a very fine haze or no more than one or two discrete colonies.

Subsequently the MIC values were used to evaluate the synergistic activity of the combination by calculating the Fractional Inhibitory Concentration (FIC Index) according to the following formula:

$$\text{FIC Index} = \frac{\text{MIC of SMX in Combination}}{\text{MIC of SMX alone}} + \frac{\text{MIC of TMP in Combination}}{\text{MIC of TMP alone}}$$

Any value of the FIC Index that is less than 1.0 is considered by some authors to indicate synergy (31). However, a more rigid criterion for synergy requiring a FIC Index of ≤ 0.7 (representing a three-fold reduction in the MIC of each drug) (95) was used to interpret the results in this report.

Finally, the MIC values of the test organisms were categorized as "sensitive", "intermediate" or "resistant" according to the breakpoints recommended by the National Committee for Laboratory Standards as follows:

Antimicrobial	Sensitive µg/ml	Intermediate µg/ml	Resistant µg/ml
SMX	≤ 100	$>100 - <350$	≥ 350
TMP	≤ 4	$>4 - <16$	≥ 16
TMP-SMX	$\leq 2/38$	$>2/38 - <8/152$	$\geq 8/152$

Reference strain E. coli ATCC 25922 was included in each run to ensure control of medium, inoculum standardization and antimicrobial concentrations. The expected MIC for this strain for TMP-SMX is 0.5/9.5 µg/ml (90).

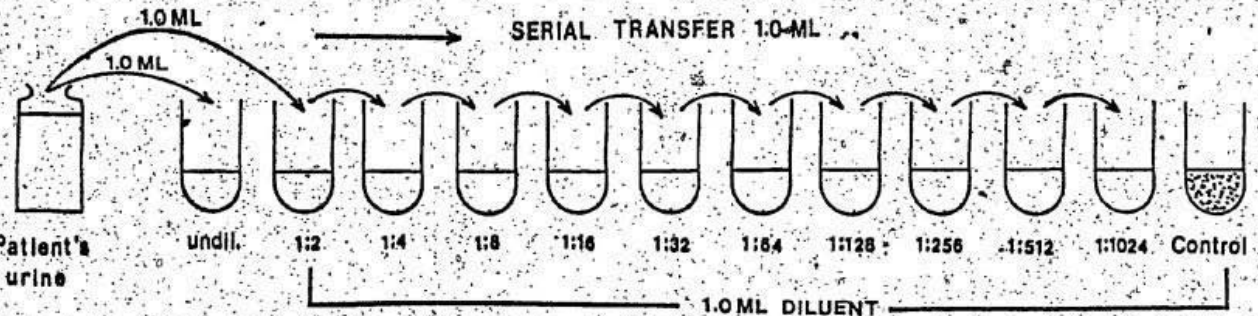
Antibacterial Activity in Urine from Patients Receiving TMP-SMX

Antibacterial activity in urine specimens from patients receiving TMP-SMX for urinary tract infections was evaluated by several approaches.

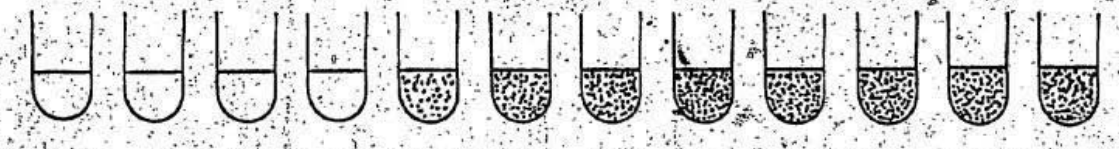
Assay of Total Antibacterial Activity

Total antibacterial activity was determined by modification of a procedure originally developed by Schlichter (97) to evaluate bactericidal levels in serum of patients under treatment for bacterial endocarditis. A row of 12 sterile tubes was set up for each of 24 urine samples. Serial two-fold dilutions of each specimen was prepared in 1 ml volumes using MHB as diluent. Dilutions ranged from undiluted urine to 1:1024. The 12th tube received MHB only and served as the growth control. To each tube was added 0.05 ml of a 6-hour MHB culture of E. coli ATCC 25922 diluted 1:1000 to yield a final density of approximately

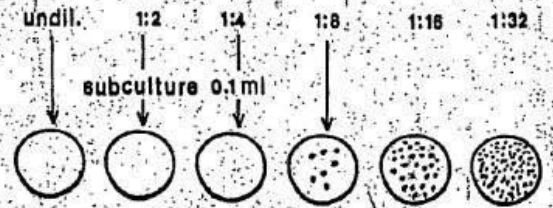
Figure 9. Schematic diagram representing the protocol of the Schlichter assay for antimicrobial activity in body fluids (98).



Example of finding for titration of bacteriostatic activity (1:8)



Example of finding for titration of bactericidal activity (1:4)



10^5 CFU/ml (Fig. 9). More precise initial density was established by viable plate count set up from the control tube before incubation. The bacteriostatic endpoint was recorded as the highest dilution at which no visible growth occurred. The bactericidal endpoint was determined by spreading 0.1 ml from each tube showing no visible growth to MacConkey agar plates. The plates were incubated at 35^0C for 18 hours and the bactericidal endpoint was interpreted as the highest dilution producing complete or nearly complete (>99.9%) killing of the inoculum.

Time-Kill Curves

Urine samples from patients receiving cotrimoxazole therapy were examined for antibacterial activity by developing time-kill curves against common urinary pathogens. In a preliminary study, 3 x 2 ml portions of urine specimens from each of 20 patients were seeded respectively with approximately 10^5 CFU/ml *E. coli* ATCC 25922, 5.0×10^4 CFU/ml *P. mirabilis* (#5), and 5.0×10^4 CFU/ml *K. oxytoca* (#13). The latter two strains were selected at TMP-SMX susceptible clinical isolates. The cultures were incubated in a shaking water bath at 35^0C. Viable counts were performed at

0, 6, and 24 hours by subculture of 1 µl and 10 µl volumes to MacConkey agar plates. For each organism, filter-sterilized urine from untreated healthy volunteers was used as a growth control. Time-kill curves were constructed by plotting logarithms of viable counts against time.

More detailed time-kill curves were constructed for 4 urine specimens which had been shown by preliminary study to possess antibacterial activity against the test organisms. The same bacterial strains were used except that the urine specimen from one patient was tested against an E. coli strain earlier isolated from urinary tract infection in the same patient. Viable counts were performed at 0, 2, 4, 6, 12, and 24 hours so as to provide a more precise evaluation of the rate of lethal activity.

Effect of PABA on Antibacterial Activity in Urine

Time-kill curves were constructed for E. coli ATCC 25922 grown in urine samples from patients receiving TMP-SMX. Each sample was dispensed in duplicate in 2 ml volumes in sterile tubes. One tube received 400 µg/ml of PABA to neutralize SMX activity and both tubes were inoculated with 10^4 CFU of the test organism contained in 0.5 ml. The tubes were incubated at 35^0C in a shaking water bath and viable counts were performed by subculture to MacConkey agar at 0, 2, 4, and 9 hours.

Bioassay of TMP and SMX in Urine

Trimethoprim

Urine samples from co-trimoxazole-treated patients were assayed for TMP activity against a clinical isolate of E. coli (#14), known to be sensitive to 0.25 µg/ml of TMP and resistant to greater than 1000 µg/ml of SMX. No synergistic or additive effect had been detected when this isolate was tested against various combinations of the two drugs even when large amounts of SMX were employed.

Preparation of Assay Plates: Molten WST agar, supplemented with 200 µg/ml of PABA to neutralize any sulfonamide activity was seeded with 1 ml of 1:10 dilution of an overnight MHB culture of the test organism to yield a final density of 10^6 CFU/ml. Thin agar plates were prepared by dispensing 12 ml volumes of the seeded agar into 15 mm x 150 mm Petri dishes. Following solidification on a level surface the assay plates were refrigerated until used. In order to confirm that the activity detected was due to TMP, parallel plates containing 200 µg/ml of thymidine were prepared. This level of thymidine was shown to neutralize the activity of 400 µg/ml TMP standard solution.

Urine Samples: Each urine specimen was assayed in triplicate using the undiluted sample and dilutions of 1:2 and 1:10 in M/15 phosphate buffer, pH 8.0 to ensure that zones sizes were within the range of the response curve prepared

from standard TMP solutions (99).

Standard TMP solutions: The stock solution of TMP was diluted in M/15 phosphate buffer, pH 8.0, so as to provide concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 and 3.12 µg/ml

Preparation and application of disks: Paper disks, ½" in diameter, were charged with 20 µl of each of the test urine dilution or with appropriate standard TMP solution. All assays were performed in triplicate. The disks were transferred to seeded agar plates and gently tapped with forceps to ensure complete contact with the agar. The plates were allowed to stand at 4^0C for 2 hours to permit prediffusion of the drug before initiation of growth and then incubated at 35^0C for 16 hours.

Reading of zones and construction of standard curve: Following incubation, diameters of inhibition zones were measured with Vernier calipers to the nearest 0.1 mm. Mean zone sizes for each standard TMP solution were used to construct a standard curve on 3-cycle semi-logarithmic paper by plotting zone sizes on the abscissa against the logarithm of each concentration. The equivalent concentration of TMP in urine samples were determined by locating the mean zone diameter for each specimen in the standard curve and reading the

corresponding drug concentration on the ordinate. Results were expressed as active TMP in µg/ml urine.

Sulfamethoxazole

Similarly urine samples from cotrimoxazole-treated patients were microbiologically assayed for SMX activity by a modification of a method described by Heifetz et al (100). The test organism, a strain of P. mirabilis (#4), sensitive to 4 µg/ml of SMX and resistant to >200 µg/ml of TMP was used in the assay. Two dimensional titration of TMP and SMX against this isolate revealed synergistic activity (Table 5) but activity due to SMX could be identified by using parallel plates containing 200 µg/ml of PABA per ml. This level of PABA was shown to neutralize the activity of 250 µg/ml SMX standard solution. The standard curve for SMX was constructed as above by plotting mean zone diameters for standard solutions containing 250, 125, 100, 50, 25, and 12.5 µg/ml SMX against the respective drug concentrations. Equivalent urine concentrations of SMX were determined and recorded as for TMP.

Assay of Urine Specimens for Thymidine Content

Urine specimens were examined for the presence of thymidine by a bioassay procedure in which each sample was tested for its ability to permit the growth of a thymidine-dependent

strain of Klebsiella sp.(1839). The minimal amount of thymidine required to allow growth of this strain was first determined by an agar diffusion method as follows:

A heavy suspension of the organism from growth on MacConkey agar, a complete medium for the strain, was prepared in 10 ml of 0.9% NaCl and washed in saline three times by centrifugation in order to remove residual thymidine carried over from the medium. The washed pellet was finally suspended in 0.9% NaCl to a density of 0.5 McFarland standard. One hundred ml of cooled , molten WST agar, a thymidine-deficient medium (99), was seeded with 1 ml of the standardized inoculum to yield 10^6CFU/ml. The seeded agar was dispensed into 15 mm x 100 mm Petri dishes, 35 ml per plate, and allowed to solidify. Wells, 4.5 mm in diameter and approximately 2.5 cm apart were punched to the bottom of the agar with a stainless steel bore (102). Serial two-fold dilutions of filter-sterilized stock solution of thymidine were prepared and 20 µl of each dilution was added to the respective wells so that the wells contained 0.01, 0.03, 0.06, 0.125, 0.25, 0.50, 1.0 and 2.0 µg/ml. The plates were incubated at 35^0C in an upright position and were observed for growth daily for 4 days. The smallest amount of thymidine permitting growth of the Klebsiella strain was noted and the concentration of the thymidine solution used to charge the

well was recorded.

Testing of urine specimens

The following urine specimens were tested in thymidine-like activity:

164 samples were selected from random urine specimens submitted to the hospital laboratory and screened for lack of antimicrobial activity as described earlier. 50 samples from patients receiving TMP-SMX and screened for lack of antimicrobial activity other than that due to TMP-SMX as described earlier.

Assay was performed as described for the standard thymidine solutions. Each assay plate included two control wells containing 20 µl of 0.125 µg/ml and 1.0 µg/ml of thymidine respectively. The plates were examined daily for four days and the presence or absence of growth around each well was recorded.

Activity of TMP and SMX in Combination

(a) Paper Strip Method

Results of the paper strip technique used to detect synergy between TMP and SMX acting against representative organisms in ratios of 1:1, 1:5, 1:10 and 1:20 are shown in Figures 10 - 13.

Figure 10 demonstrates the synergistic interaction of TMP and SMX against E. coli ATCC 25922 a control strain sensitive to each drug TMP (MIC 0.25 µg/ml) and SMX (MIC 16 µg/ml) by agar dilution. Synergism is seen as an increased area of inhibition of growth at the point of juncture of TMP (vertical strip) and SMX (horizontal strip). Synergism was evident at each ratio tested, in spite of SMX-resistance seen at ratios of 1:1 (upper left) and 1:5 (upper right).

Figure 11 illustrates the absence of synergism i.e. an indifferent effect. The test organism (E. coli #14) was inhibited by TMP (vertical strip) but there was no potentiation of TMP activity by SMX at any of the ratios tested. The MIC for this organism was 0.06 µg/ml TMP and 1000 µg/ml SMX by agar dilution.

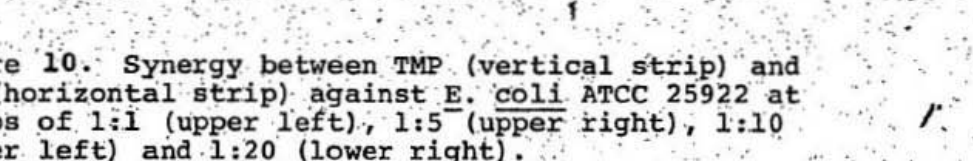


Figure 10. Synergy between TMP (vertical strip) and SMX (horizontal strip) against E. coli ATCC 25922 at ratios of 1:1 (upper left), 1:5 (upper right), 1:10 (lower left) and 1:20 (lower right).

Figure 11. Indifferent effect between TMP (vertical strip) and SMX (horizontal strip) against E. coli #14 at ratios of 1:1 (upper left), 1:5 (upper right), 1:10 (lower left) and 1:20 (lower right).

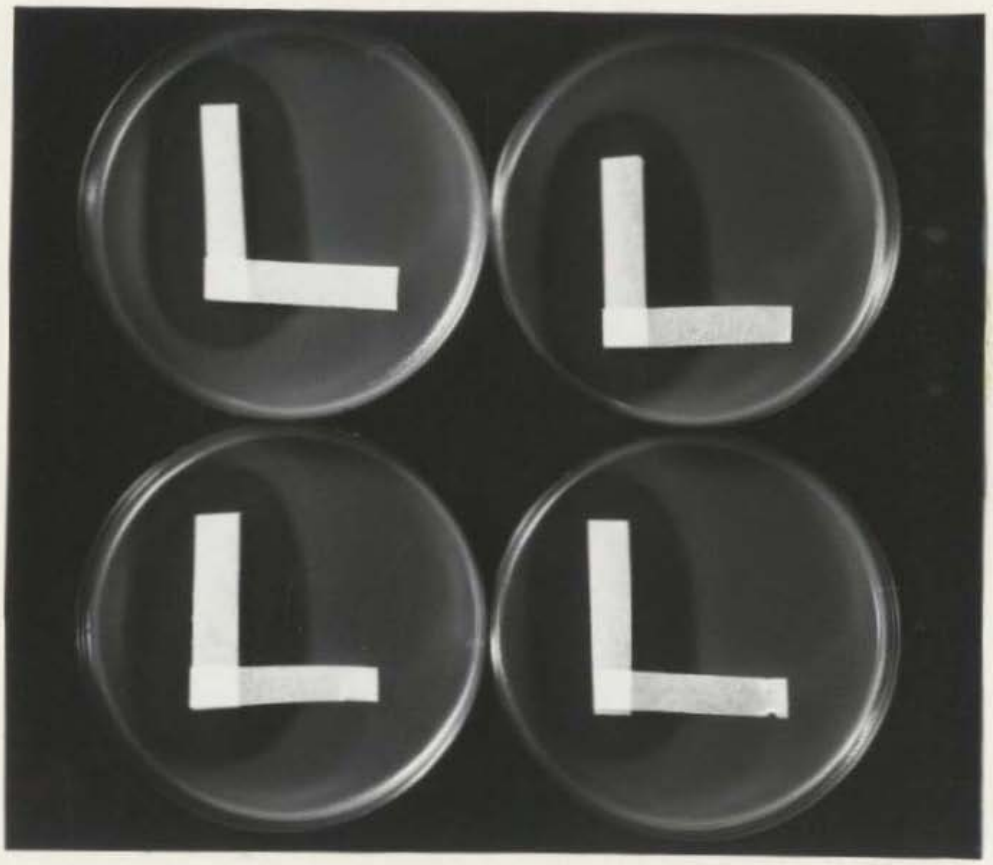

L
L
L
L

Figure 12. Indifferent effect between TMP (vertical strip) and SMX (horizontal strip) against P. mirabilis #4, a clinical isolate from urinary tract infection. This photograph represents a 1:1 ratio (upper left), 1:5 ratio (upper right), 1:10 ratio (lower left) and 1:20 ratio (lower right).

Figure 13. Synergy between TMP (vertical strip) and SMX (horizontal strip) against Klebsiella species (#3) at ratios of 1:1(upper left), 1:5 (upper right), 1:10 (lower left) and 1:20 (lower right). Synergy is evident at all ratios.


L
L
L
L

Figure 12 shows similar lack of synergy between TMP and SMX against P. mirabilis (#4). The test organism was inhibited by SMX (horizontal strip) but there was no enhancement of activity in the presence of TMP (vertical strip). This organism was sensitive to SMX (MIC 4.0 µg/ml) but highly resistant to TMP (MIC >200 µg/ml).

Finally Figure 13 illustrates that synergy can be detected between TMP and SMX when the test organism Klebsiella species (#3) is resistant to SMX (horizontal strip) and relatively in senstive to TMP (vertical strip). The MIC values for this organism was TMP (MIC 6.2 µg/ml) and SMX (MIC >1000 µg/ml). This synergistic effect was demonstrated at TMP:SMX ratios of 1:1, 1:5, 1:10 and 1:20.

(b) Two-Dimensional ("Checkerboard") Titration

Results of two-dimensional titrations performed for TMP and SMX against selected organisms are shown in Tables 3 - 7. Typical synergistic response is shown in Table 3 where the two antimicrobials were tested against the control strain E. coli ATCC 25922. MIC values for TMP and SMX were 0.25 µg/ml and 32 µg/ml respectively when each drug was present alone. When the drugs were tested in combination, 0.03 µg/ml of TMP (1/8 MIC) and 2 µg/ml of SMX (1/16 MIC) inhibited growth of the test organism.

Further evidence of antimicrobial synergy was seen when the drugs were tested against another clinical isolate, E. coli (#16)(Table 4). MIC values for TMP and SMX were 0.12 µg/ml and 20 µg/ml respectively when each drug was present alone. In combination 0.03 µg/ml of TMP (1/4 MIC) and 0.6 µg/ml of SMX (1/32 MIC) prevented growth.

Table 5 shows the results when the test strain was P. mirabilis (#4), a clinical isolate highly resistant to TMP (MIC 256 µg/ml) and sensitive to SMX (MIC 10 µg/ml). In this instance there was evidence of marked synergism when the drugs were present in combination. The activity of each drug is greatly enhanced by the presence of the other so that 16 µg/ml of TMP (1/16 MIC) and 0.31 µg/ml SMX (1/32 MIC) prevented growth.

There was no evidence of synergy when the two antimicrobials were tested against E. coli (#14), a clinical isolate resistant to SMX (MIC 640 µg/ml) and sensitive to TMP (MIC 0.25 µg/ml)(Table 6). The combination was not effective when the concentration of TMP was less than its MIC, regardless of the level of SMX component.

Results of the checkerboard titration of TMP and SMX against Klebsiella species (#3) are shown in Table 7. MIC values for TMP and SMX are 16 µg/ml and >2560 µg/ml

Table 3. Checkerboard titration of TMP and SMX against E. coli ATCC 25922, demonstrating synergy.

Sulfamethoxazole (µg/ml)	Trimethoprim (µg/ml) 0.5	0.25	0.12	0.06	0.03	nil
32	-	-	-	-	-	-
16	-	-	-	-	-	+
8	-	-	-	-	-	+
4	-	-	-	-	-	+
2	-	-	-	-	-	+
1	-	-	-	-	+	+
0.5	-	-	-	-*	+	+
0.25	-	-	-**	+	+	+
nil	-	-	+	+	+	(+)

\+ = growth

\- = no growth

(+) = drug-free control

$$\text{*FIC Index} = \frac{\text{SMX}}{\frac{0.5}{32}} + \frac{\text{TMP}}{\frac{0.06}{0.25}} = 0.14$$

$$\text{**FIC Index} = \frac{0.25}{32} + \frac{0.12}{0.25} = 0.51$$

Table 4. Checkerboard titration of TMP and SMX against E. coli (#16), a clinical isolate, demonstrating synergy.

Sulfamethoxazole (µg/ml)	Trimethoprim (µg/ml) 0.5	0.25	0.12	0.06	0.03	nil
40	-	-	-	-	-	-
20	-	-	-	-	-	-
10	-	-	-	-	-	+
5	-	-	-	-	-	+
2.5	-	-	-	-	-	+
1.2	-	-	-	-	-	+
0.6	-	-	-	-	-*	+
nil	-	-	-	+	+	(+)

\+ = growth
\- = no growth
(+) = drug-free control

$$*\text{FIC Index} = \frac{\text{TMP } 0.03}{0.12} + \frac{\text{SMX } 0.6}{20} = 0.28$$

Table 5. Checkerboard titration of TMP and SMX against P. mirabilis (#4). Although the organism is highly resistant to TMP synergy can be demonstrated in the titration.

Sulfamethoxazole (µg/ml)	Trimethoprim (µg/ml) 512	256	128	64	32	16	nil
20	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-
5	-	-	-	-	-	-	+
2.5	-	-	-	-	-	-	+
1.25	-	-	-	-	-	-	+
0.62	-	-	-	-	-	-	+
0.31	-	-	-	-	-	-*	+
nil	-	-	+	+	+	+	(+)

$$\text{*FIC Index} = \frac{\text{SMX}}{\frac{0.31}{10}} + \frac{\text{TMP}}{\frac{16}{256}} = 0.1$$

Table 6. Checkerboard titration of SMX and TMP against E. coli (#14) showing an indifferent effect. The combination is not effective when the concentration of TMP is less than the MIC of the test organism (0.25 µg/ml) regardless of the level of the SMX component.

Sulfamethoxazole (µg/ml)	Trimethoprim (µg/ml) 1.0	0.5	0.25	0.12	0.06	0.03	nil
640	-	-	-	+	+	+	+
320	-	-	-	+	+	+	+
160	-	-	-	+	+	+	+
80	-	-	-	+	+	+	+
40	-	-	-	+	+	+	+
20	-	-	-	+	+	+	+
10	-	-	-	+	+	+	+
5	-	-	-	+	+	+	+
2.5	-	-	-	+	+	+	+
1.2	-	-	-	+	+	+	+
0.6	-	-	-	+	+	+	+
nil	-	-	-	+	+	+	(+)

\- = no growth
\+ = growth
(+) = drug-free control

FIC INDEX $\frac{0.25}{0.25} + \frac{0.12}{>640} = >1$

Table 7. Checkerboard titration of TMP and SMX against Klebsiella species (#3) showing lack of synergy. The level of SMX is not achievable in urine.

Sulfamethoxazole (µg/ml)	Trimethoprim (µg/ml) 64	32	16	8	4	2	nil
2560	-	-	-	-	+	+	+
1280	-	-	-	+	+	+	+
640	-	-	-	+	+	+	+
320	-	-	-	+	+	+	+
nil	-	-	-	+	+	+	(+)

\+ = growth
\- = no growth
(+) = drug-free control

$$\text{*FIC Index} = \frac{\text{SMX}}{2560} + \frac{\text{TMP}}{16} = \frac{2560}{2560} + \frac{8}{16} = 1.5$$

respectively. There was no appreciable reduction in the MIC of TMP in the presence of as much as 2560 µg/ml of SMX. Antimicrobial activity appears to be associated with the TMP component of the mixture and there is no synergy.

(c) Time-Kill Curves in MHB

Preliminary time-kill curves showing the antibacterial activity of MHB-diluted TMP, SMX and TMP-SMX against E. coli ATCC 25922, P. mirabilis (#4) and E. coli (#16) are shown in Fig. 14 - 16. Therapeutic concentrations of 100 µg/ml of each antimicrobial agent were used and a 1:1 ratio was maintained for the combination. The MIC values for the test organisms, by agar dilution, were as follows:

	TMP (µg/ml)	SMX (µg/ml)
E. coli ATCC 25922	0.25	16
P. mirabilis (#4)	>200.0	4
E. coli (#16)	0.25	16

The bacteriostatic nature of SMX was evident in all experiments. Growth paralleled the control (no antimicrobials) for the first four hours and then gradually declined to a bacterial population approximately that of the original inoculum. The bactericidal activity of TMP against E. coli ATCC 25922 and E. coli (#16) is shown in Fig. 14 and 16. There was a gradual decline in the bacterial population over the 24 hour test period. Bactericidal activity was also evident for the combination but the activity was no greater than

Figure 14. Time-kill curves for TMP, SMX and TMP-SMX at expected therapeutic concentrations of 100 µg/ml. The ratio for TMP-SMX is 1:1. The test organism is E. coli ATCC 25922.

C = Drug-free control
TMP = 100 µg/ml
SMX = 100 µg/ml
TMP-SMX = 100:100 µg/ml

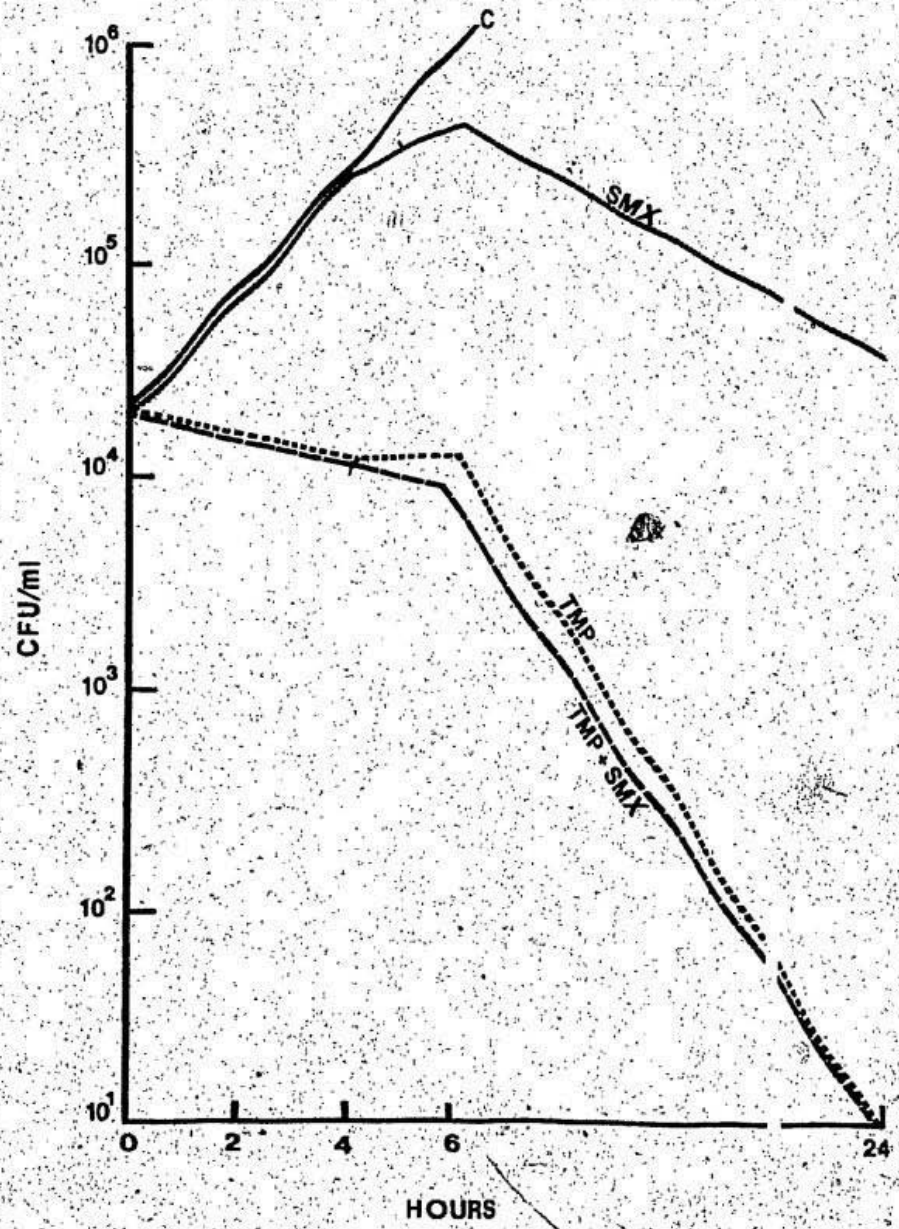

C
SMX
TMP
TMP+SMX
10^6
10^5
10^4
10^3
10^2
10^1
CFU/ml
0
2
4
6
24
HOURS

Figure 15. Time-kill curves for TMP, SMX and TMP-SMX at expected therapeutic concentrations of 100 µg/ml. TMP-SMX ratio is 1:1. The test organism is P. mirabilis (#4), a clinical isolate.

C = Drug-free control
TMP = 100 µg/ml
SMX = 100 µg/ml
TMP-SMX = 100:100 µg/ml

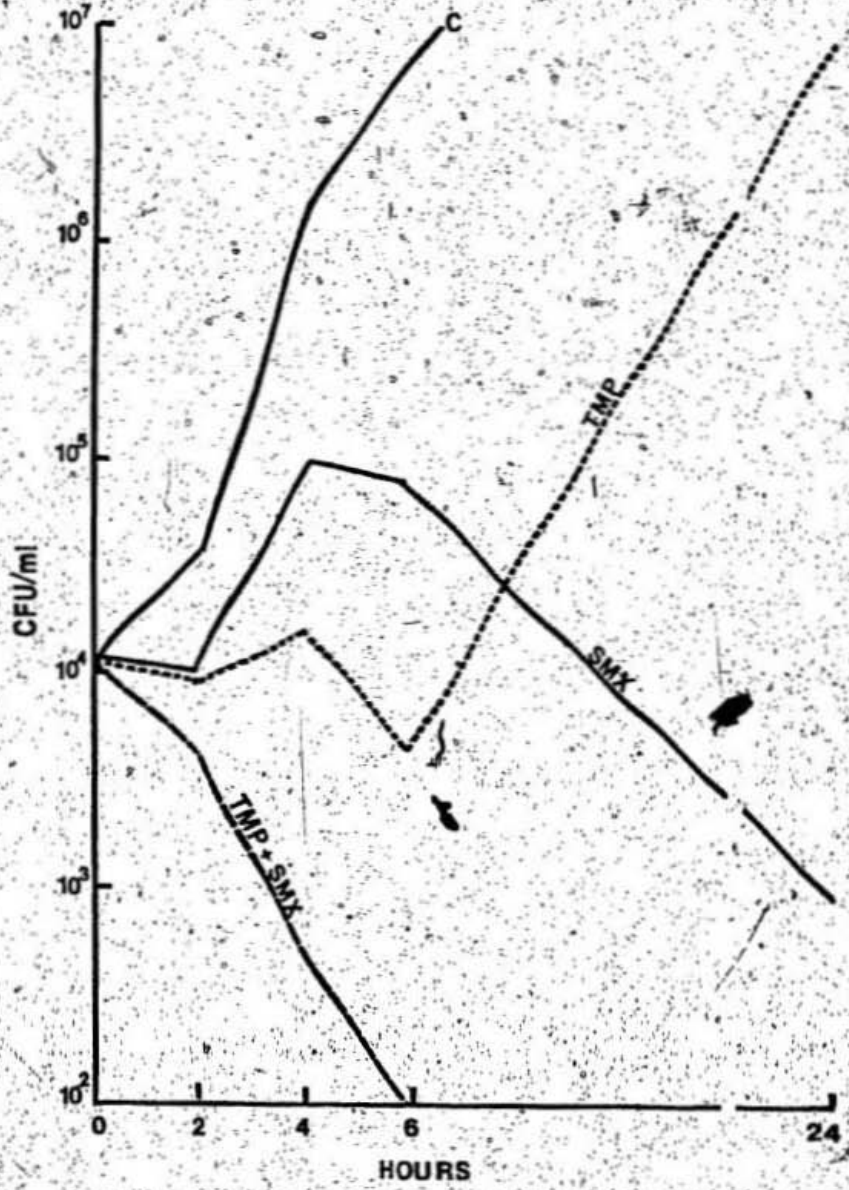
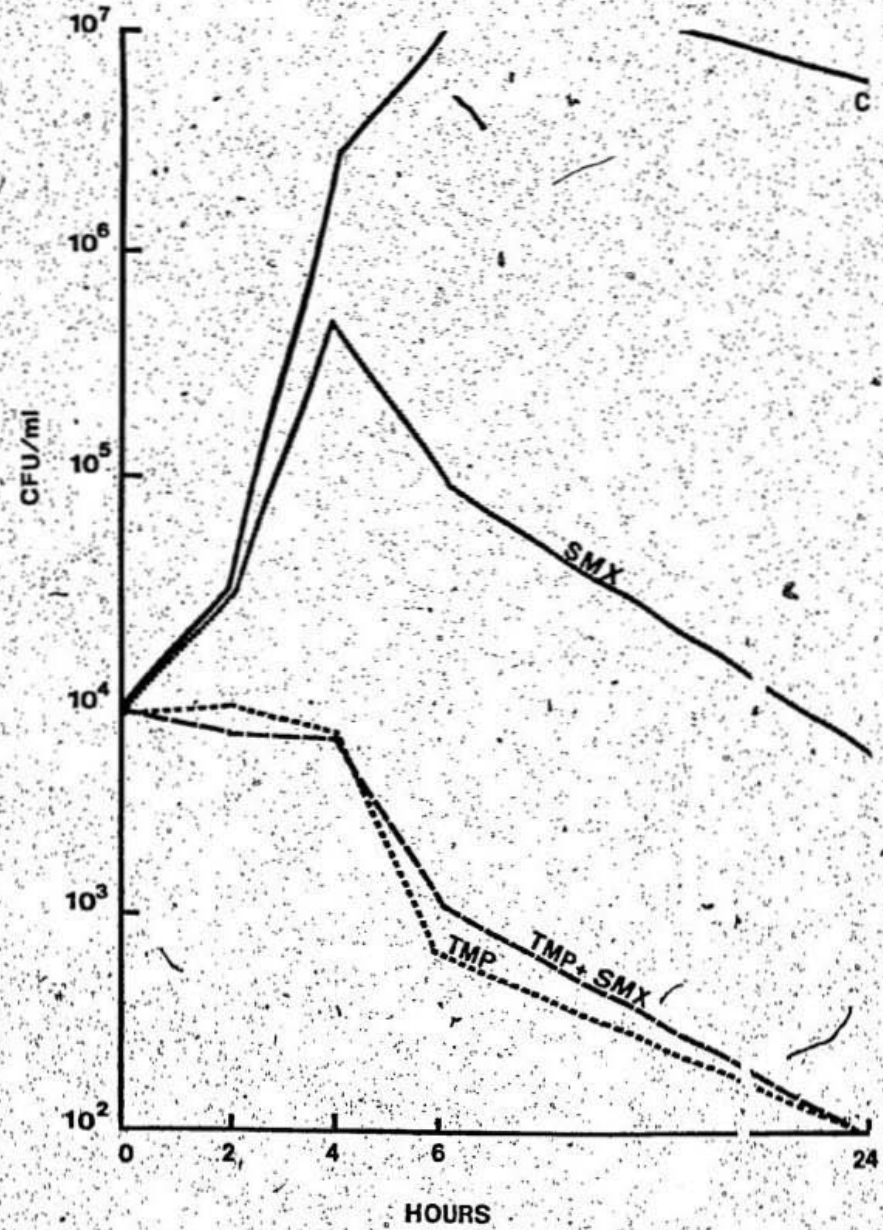

C
TMP
SMX
TMP + SMX
CFU/ml
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
24
HOURS

Figure 16. Time-kill curves for TMP, SMX and TMP-SMX at expected therapeutic concentrations of 100 µg/ml. The TMP-SMX ratio is 1:1. The test organism is E. coli (#16), a clinical isolate.

C = Drug-free control
TMP = 100 µg/ml
SMX = 100 µg/ml
TMP-SMX = 100:100 µg/ml


10^7
10^6
10^5
10^4
10^3
10^2
CFU/ml
C
SMX
TMP
TMP+SMX
0
2
4
6
24
HOURS

for TMP alone. Fig. 15 illustrates the results when the test organism was P. mirabilis (#4), a clinical isolate resistant to TMP (MIC > 200 µg/ml). Bacteriostatic activity was evident during the first 6 hours of inocubation in the presence of TMP but this was followed by increase in the bacterial population, parallelling the drug-free control. For this isolate, the TMP-SMX combination showed bactericidal activity over the first 6 hours of incubation.

Time-Kill Curves in Urine of Healthy Volunteer

Time-kill curves showing the activity of each drug and of the combination against E. coli ATCC 25922 grown in filter-sterilized urine from a healthy volunteer are shown in Figs. 17a - 17g. MIC values for the test organism, as determined by the agar dilution method, were 0.25 µg/ml of TMP, 16 µg/ml of SMX, and 0.12/2.38 µg/ml of TMP-SMX.

The single components were tested in the following concentrations:

TMP (µg/ml) 2, 5, 10, 15, 25, 50, 100

SMX (µg/ml) 2, 5, 10, 15, 25, 50, 100

For each concentration of TMP, the combination TMP-SMX was tested in ratios of 1:1, 1:2 and 1:10.

SMX alone showed no bactericidal effect. Bacteriostatic activity was demonstrated only at concentrations above the

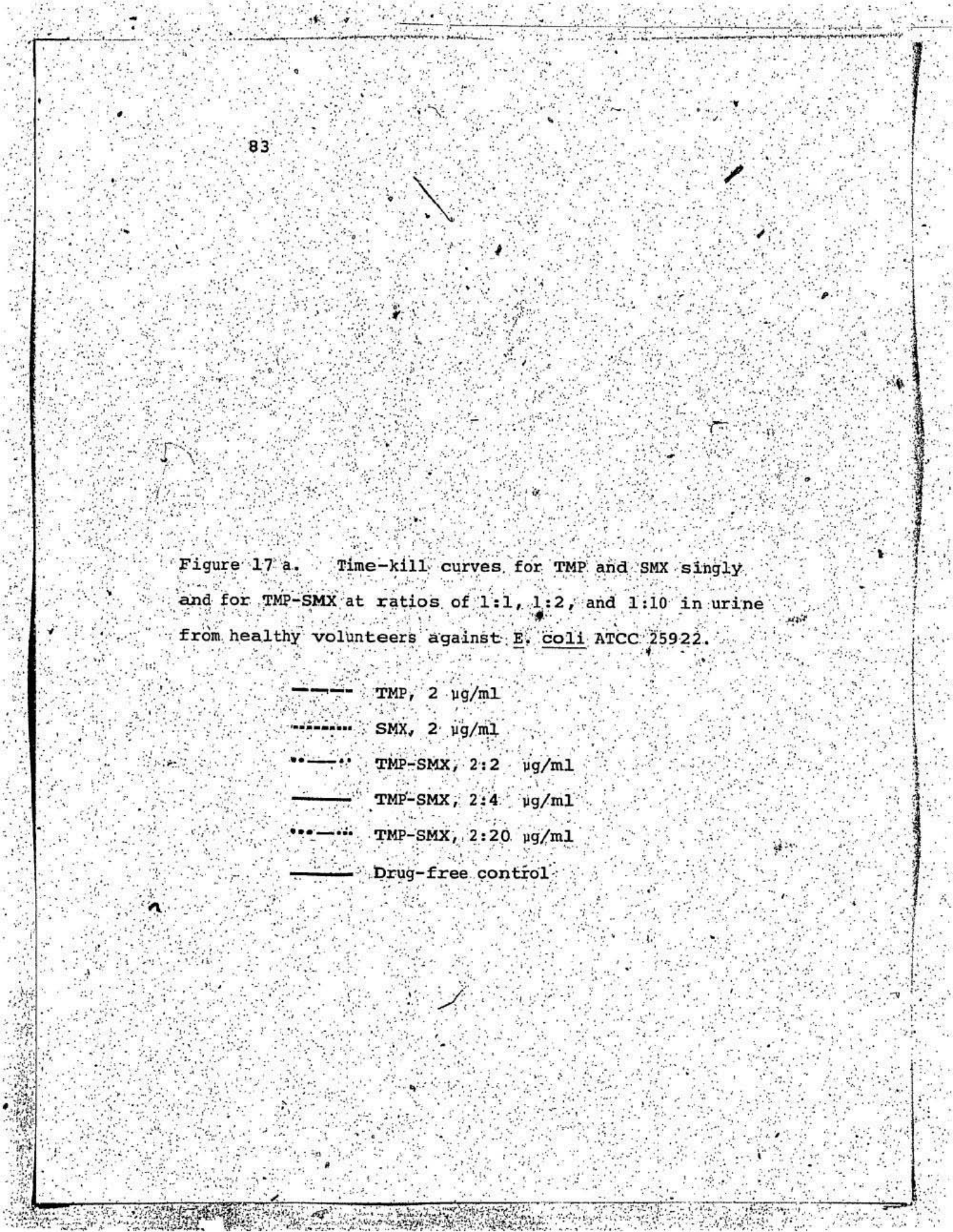


Figure 17 a. Time-kill curves for TMP and SMX singly and for TMP-SMX at ratios of 1:1, 1:2, and 1:10 in urine from healthy volunteers against E. coli ATCC 25922.

- TMP, 2 µg/ml
- SMX, 2 µg/ml
- TMP-SMX, 2:2 µg/ml
- TMP-SMX, 2:4 µg/ml
- TMP-SMX, 2:20 µg/ml
- Drug-free control

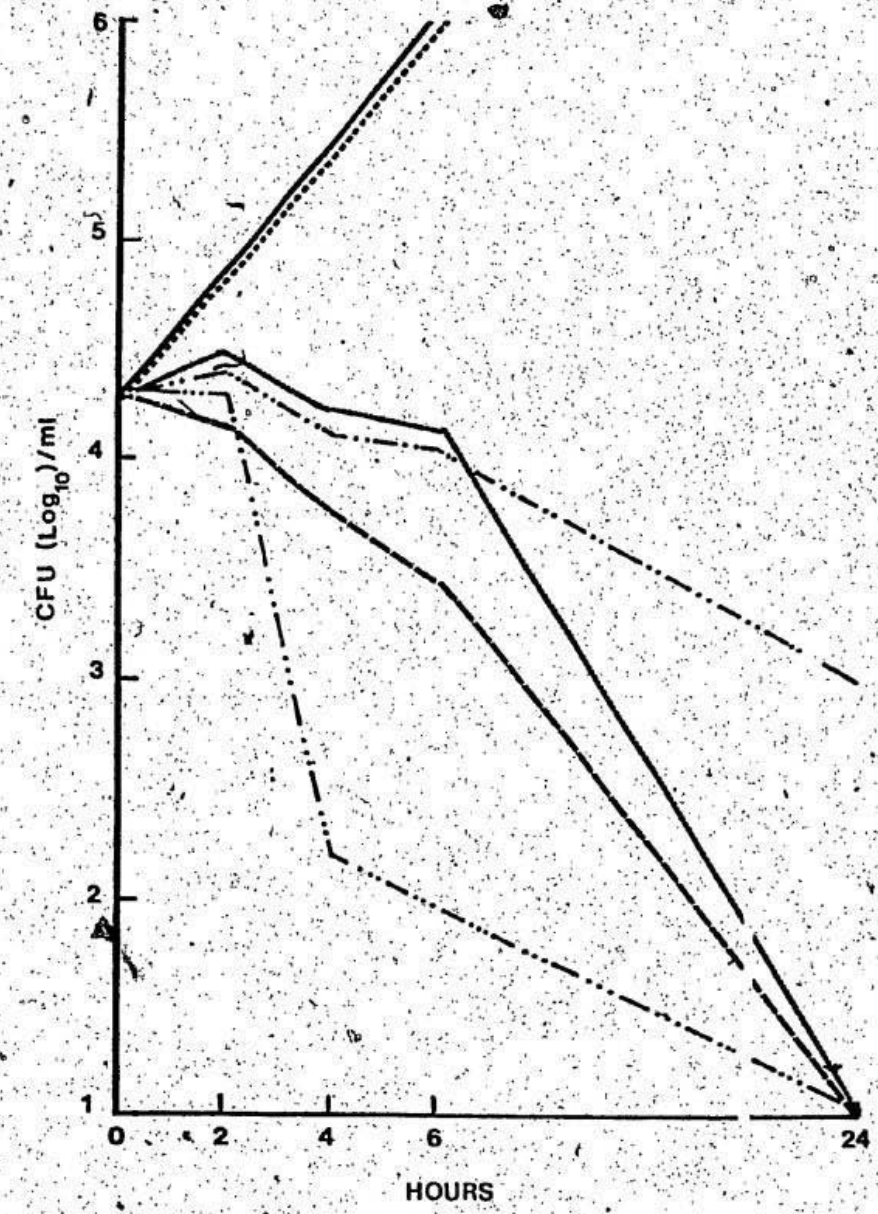

6
5
4
3
2
1
CFU (Log10)/ml
0
2
4
6
24
HOURS

Figure 17 b. Time-kill curves for TMP and SMX singly and for TMP-SMX at ratios of 1:1, 1:2, and 1:10 in urine from healthy volunteers against E. coli ATCC 25922.

——— TMP, 5 µg/ml

------- SMX, 5 µg/ml

..—.. TMP-SMX, 5:5 µg/ml

——— TMP-SMX, 5:10 µg/ml

...—... TMP-SMX, 5:50 µg/ml

——— Drug-free control

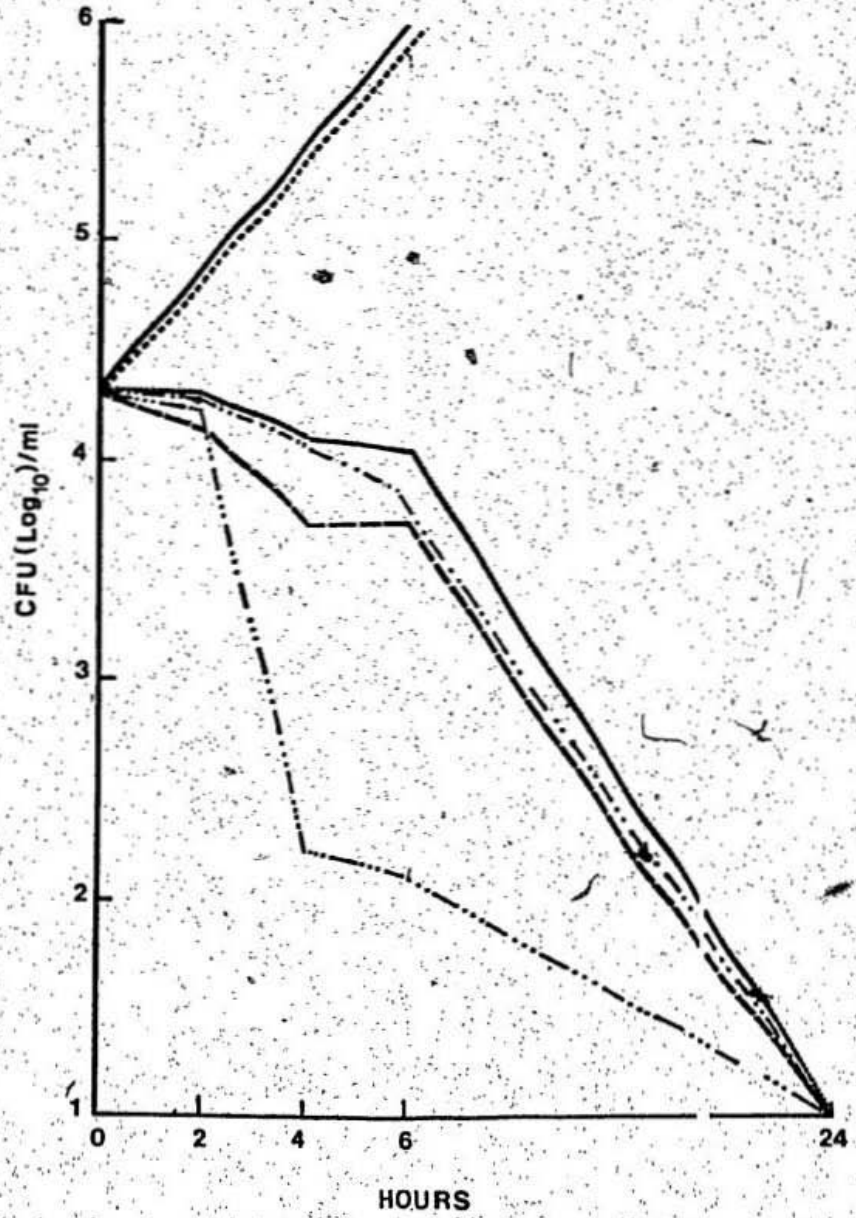

6
5
4
3
2
1
CFU (Log10)/ml
0
2
4
6
24
HOURS

Figure 17 c. Time-kill curves for TMP and SMX singly and for TMP-SMX at ratios of 1:1, 1:2, and 1:10 in urine from healthy volunteers against E. coli ATCC 25922.

——— TMP, 10 µg/ml

····· SMX, 10 µg/ml

··—·· TMP-SMX, 10:10 µg/ml

——— TMP-SMX, 10:20 µg/ml

···—·· TMP-SMX, 10:100 µg/ml

——— Drug-free control

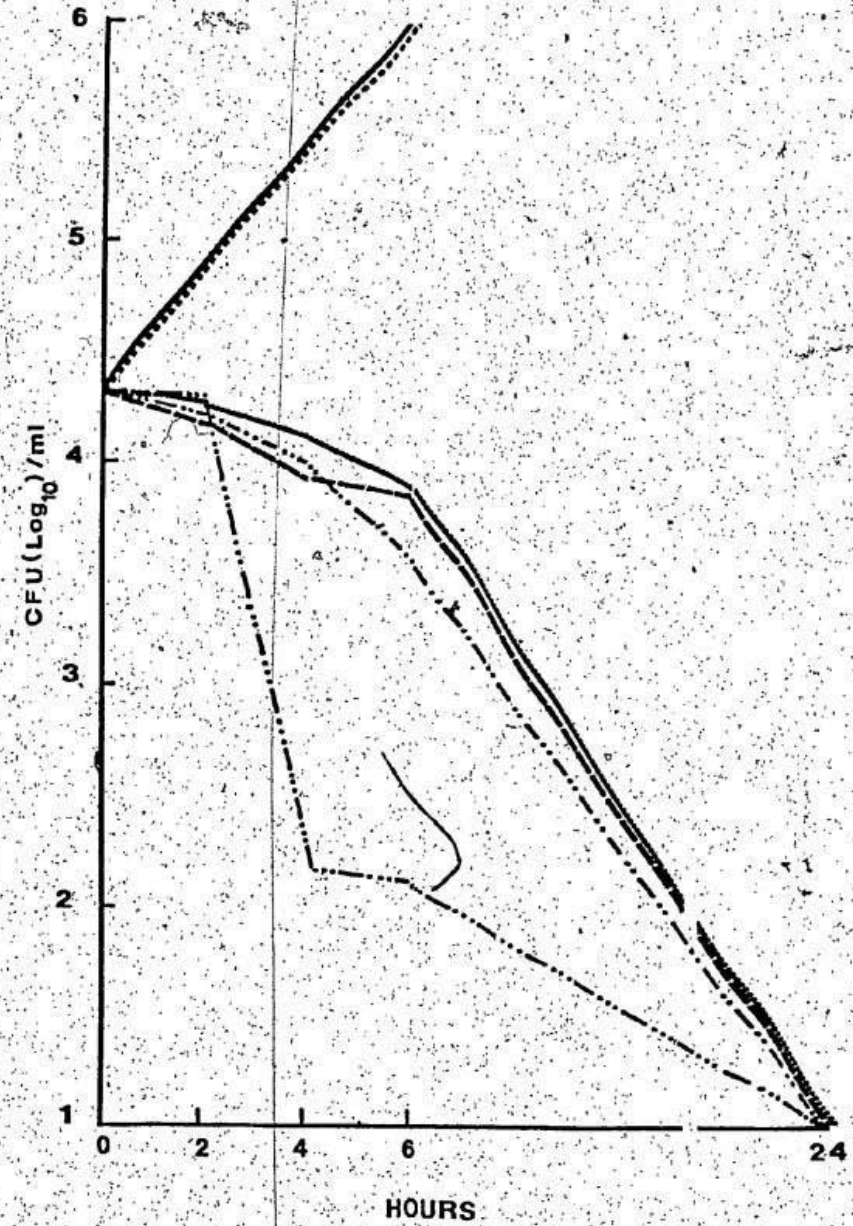

6
5
4
3
2
1
CFU(Log10)/ml
0
2
4
6
24
HOURS

Figure 17 d. Time-kill curves for TMP and SMX singly and for TMP-SMX at ratios of 1:1, 1:2, and 1:10 in urine from healthy volunteers against E. coli ATCC 25922.

——— TMP, 15 µg/ml
..... SMX, 15 µg/ml
..—.. TMP-SMX, 15:15 µg/ml
——— TMP-SMX, 15:30 µg/ml
..—... TMP-SMX, 15:150 µg/ml
——— Drug-free control

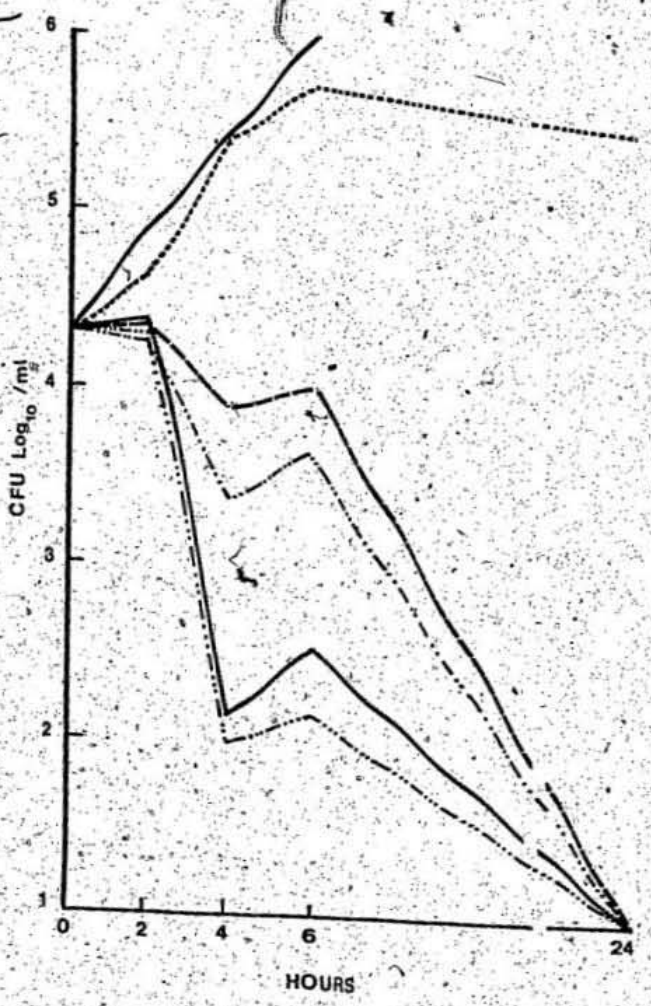

6
5
4
3
2
1
CFU Log10 /ml
0
2
4
6
24
HOURS

Figure 17 e. Time-kill curves for TMP and SMX singly and for TMP-SMX at ratios of 1:1, 1:2, and 1:10 in urine from healthy volunteers against E. coli ATCC 25922.

——— TMP, 25 µg/ml
---------- SMX, 25 µg/ml
·—·— TMP-SMX, 25:25 µg/ml
——— TMP-SMX, 25:50 µg/ml
·—·—·— TMP-SMX, 25:250 µg/ml
——— Drug-free control

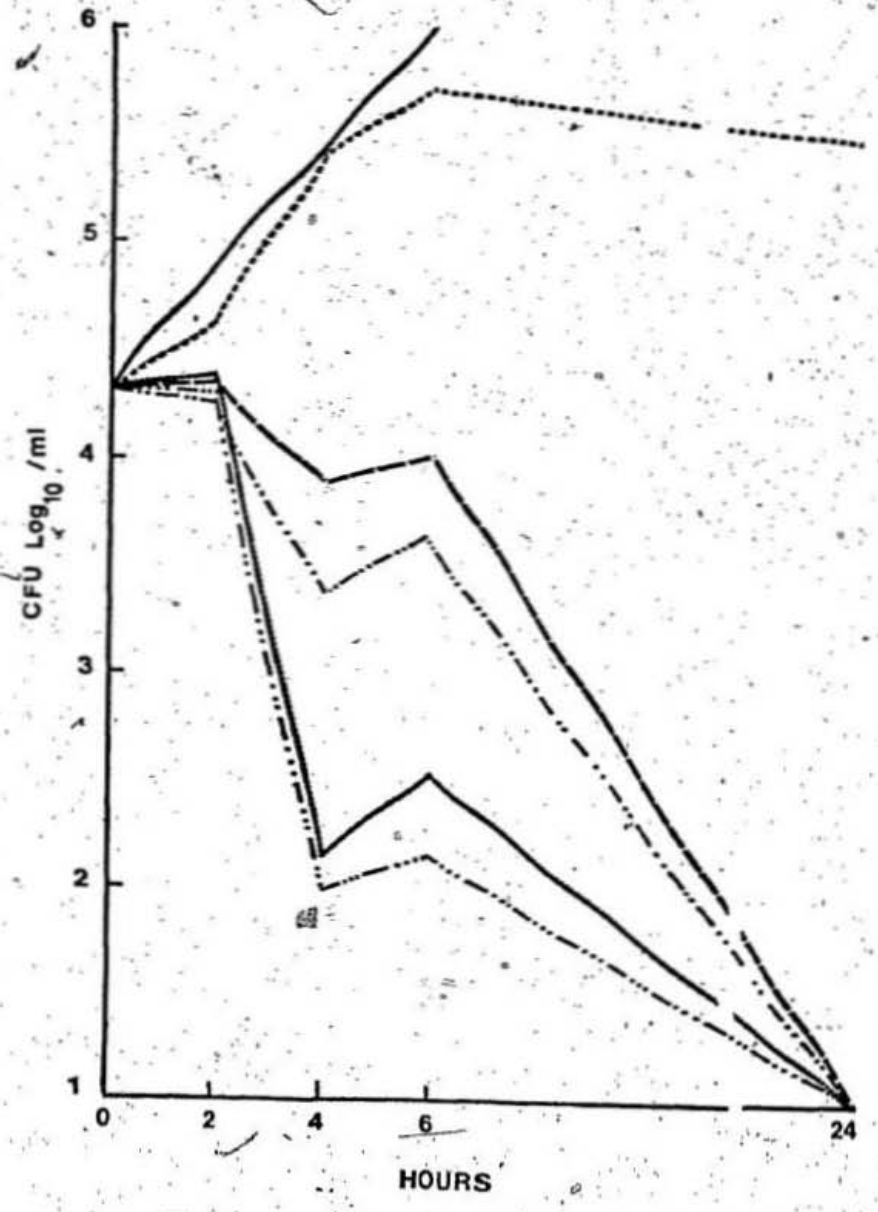

6
5
4
3
2
1
CFU Log10 /ml
0
2
4
6
24
HOURS

Figure 17 f. Time-kill curves for TMP and SMX singly and for TMP-SMX at ratios of 1:1, 1:2, and 1:10 in urine from healthy volunteers against E. coli ATCC 25922.

———— TMP, 50 µg/ml
..... SMX, 50 µg/ml
.·—·· TMP-SMX, 50:50 µg/ml
———— TMP-SMX, 50:100 µg/ml
···—··· TMP-SMX, 50:500 µg/ml
———— Drug-free control

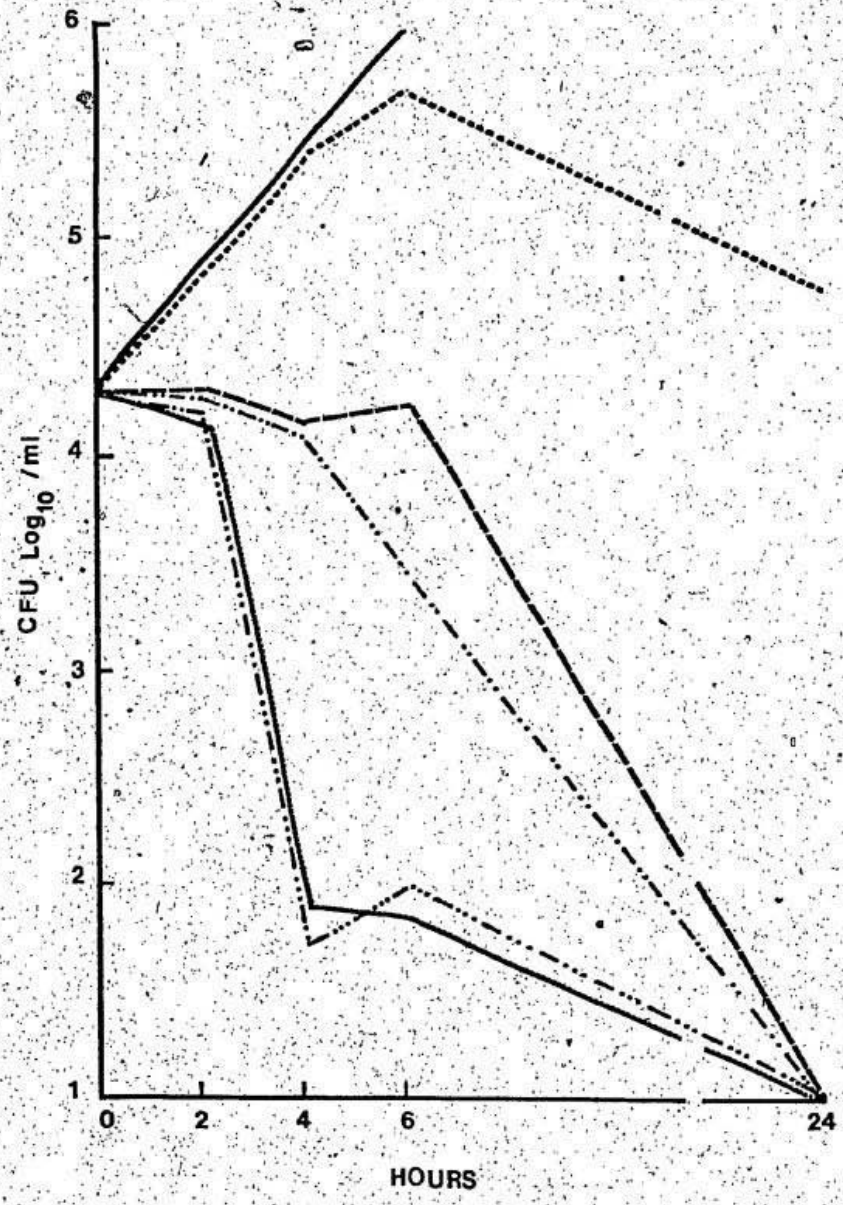
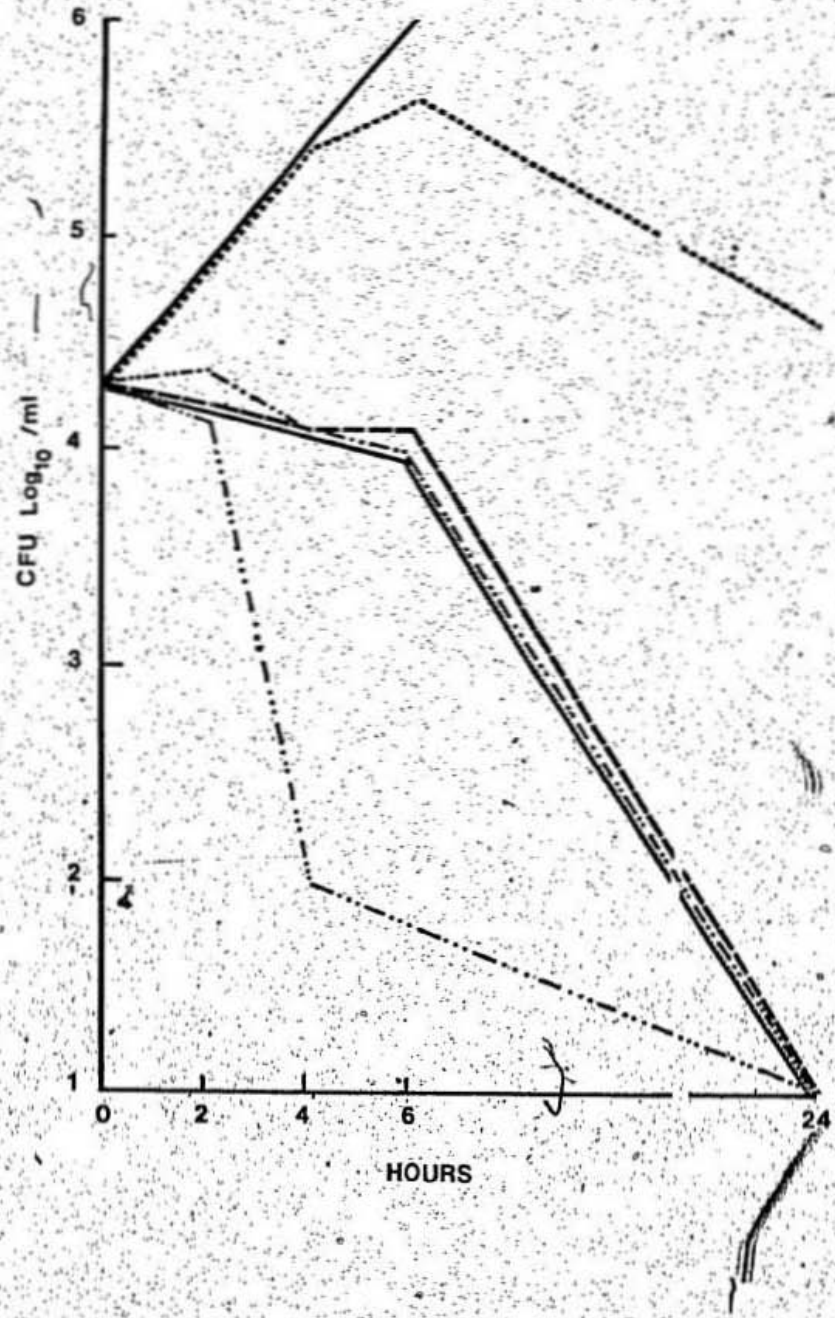

6
5
4
3
2
1
CFU, Log_{10} /ml
0
2
4
6
24
HOURS

Figure 17 g. Time-kill curves for TMP and SMX singly and for TMP-SMX at ratios of 1:1, 1:2, and 1:10 in urine from healthy volunteers against E. coli ATCC 25922.

——— TMP, 100 µg/ml
..... SMX, 100 µg/ml
·· — ·· TMP-SMX, 100:100 µg/ml
——— TMP-SMX, 100:200 µg/ml
·· — ··· TMP-SMX, 100:1000 µg/ml
——— Drug-free control


6
5
4
3
2
1
CFU Log10 /ml
0
2
4
6
24
HOURS

MIC value (Figs. 17e - 17g). A gradual decline in the bacterial population over the 24 hour incubation period was seen for all concentrations of TMP tested alone, indicating bactericidal activity at levels in excess of the MIC. This activity was not enhanced by combination with SMX in ratios of 1:1 and 1:2 until the concentration of the TMP component reached 25 µg/ml or higher when marked synergism of the combination 1:2 was noted (Figs. 17e - 17g). When the combination was tested in the proportion of 1:10 there was evidence of synergism at all concentrations as shown by a sharp decline in the bacterial population within the first four hours of incubation (Fig. 17a - 17g). An apparent antagonistic effect was seen when the TMP-SMX combination was tested at 2 µg/ml (Fig. 17a).

Minimal Inhibitory Concentration

MIC values and FIC indices for TMP, SMX, singly and in 1:20 combination against clinical isolates and control strains are shown in Table 8. These findings clearly indicate that TMP was more active than SMX against the majority of the clinical isolates, except for Ps. aeruginosa. The marked synergistic activity of TMP-SMX is evident for those isolates having a FIC index of ≤ 0.7. (The FIC index was not calculated for those organisms with MIC values above or below the drug concentrations studied). In many instances

Table 8. MIC of clinical isolates to TMP, SMX and TMP-SMX (1:20 ratio) and corresponding FIC indices.

Organism	TMP (µg/ml) MIC	SMX (µg/ml) MIC	TMP-SMX (µg/ml) MIC	FIC index*
E. coli				
1	0.5	16	0.06/1.19	0.19
2	0.25	16	0.12/2.38	0.63
3	>200.0	>1000	>32/608	- **
4	0.25	16	0.06/1.19	0.31
5	1.0	16	0.12/2.38	0.27
6	>200.0	>1000	>32/608	-
7	0.25	8	0.06/1.19	0.39
8	0.25	16	0.03/0.59	0.16
9	0.25	8	0.03/0.59	0.19
10	0.25	>1000	0.25/4.75	-
11	0.25	>1000	0.25/4.75	-
12	0.25	>1000	0.25/4.75	-
13	0.25	8	<0.03/0.59	-
14	<0.06	1000	0.12/2.38	-
15	<0.06	4	<0.03/0.59	-
16	0.25	16	0.06/1.19	0.31
17	0.25	32	0.06/1.19	0.28
18	0.25	32	0.06/1.19	0.28
19	0.25	32	0.06/1.19	0.28
20	0.25	32	0.06/1.19	0.28

Organism	TMP (µg/ml) MIC	SMX (µg/ml) MIC	TMP-SMX (µg/ml) MIC	FIC Index*
21	1.0	32	0.12/2.38	0.19
22	0.5	32	0.12/2.38	0.31
23	0.12	8	<0.03/0.59	
24	0.25	16	0.06/1.19	0.31
25	0.5	32	0.12/2.38	0.31
26	0.5	>1000	0.24/4.75	
27	0.5	32	0.06/1.19	0.16
28	0.5	8	0.06/1.19	0.27
29	0.5	32	0.06/1.19	0.16
30	0.25	32	0.06/1.19	0.28
31	0.25	8	<0.03/0.59	-
32	0.5	>1000	0.5 /4.75	1.0
33	1.25	>1000	1.0 /19	0.82
34	0.12	8	<0.03/0.59	-
35	0.5	32	0.06/1.19	0.16
36	0.5	16	0.06/1.19	0.19
37	0.5	32	0.06/1.19	0.16
ATCC 25922	0.25	16	0.12/2.38	0.63
Klebsiella sp.				
1	1.0	200	0.25/4.75	0.27
2	1.0	200	1.0 /19	1.00
3	6.2	>1000	16 /304	-
4	1.0	>1000	2/38	-
5	0.25	16	0.25/4.75	1.3
6	0.25	16	0.25/4.75	1.3
7	0.5	>1000	8 /152	-
8	0.5	128	0.25/4.75	0.54

Organism	TMP (µg/ml) MIC	SMX (µg/ml) MIC	TMP-SMX (µg/ml) MIC	FIC Index*
9	1.25	64	0.25/4.75	0.27
10	1.0	32	0.25/4.75	0.35
11	0.5	32	0.12/2.38	0.32
12	25.0	>1000	8 /152	-
13	0.5	16	0.06/1.19	0.19
14	0.25	>1000	0.25/4.75	-
15	0.25	>1000	0.25/4.75	-
16	0.25	16	0.06/1.19	0.31
17	3.1	32	0.5 /9.5	0.46
Proteus sp.				
1	1.6	32	0.12/2.38	0.15
2	1.6	16	0.12/2.38	0.27
3	100.0	>1000	>32 /608	-
4	>200.0	4	0.25/4.75	1.2
5	1.0	8	0.12/2.38	0.41
6	>200.0	>1000	>32 /608	-
7	1.0	16	0.12/2.38	0.27
8	>200.0	>1000	>32 /608	-
9	>200.0	>1000	>32 /608	-
10	>200.0	>1000	>32 /608	-
11	3.1	>1000	2 /38	-
12	3.1	16	0.12/2.38	0.19
13	12.5	128	0.25/4.75	0.06
14	6.2	16	0.25/4.75	0.34

Organism	TMP (µg/ml) MIC	SMX (µg/ml) MIC	TMP-SMX (µg/ml) MIC	FIC Index*
15	3.1	700	0.25/4.75	0.06
16	0.25	64	≤0.03/0.59	-
17	>200.0	>1000	>32 /608	-
P. vulgaris ATCC 13315	3.1	8	0.12/2.38	0.34
Enterobacter sp.				
1	6.2	64	0.5 /9.5	0.23
2	0.5	16	0.12/2.38	0.39
3	3.1	32	0.25/4.75	0.23
4	0.5	16	0.12/2.38	0.39
E. Cloacae ATCC 23395	3.1	16	0.12/2.38	0.19
C. freundii				
1	0.25	>1000	0.06/1.19	
2	>200.0	>1000	>32 /608	-
3	>200.0	>1000	>32 /608	-
4	>200.0	>1000	>32 /608	-
5	>200.0	>1000	>32 /608	-
6	>200.0	>1000	>32 /608	-
S. marcescens				
1	>200.0	>1000	>32 /608	-
2	>200.0	>1000	>32 /608	-
3	6.2	256	0.5/9.5	0.12
4	>200.0	>1000	>32 /608	-

Organism	TMP (µg/ml) MIC	SMX (µg/ml) MIC	TMP-SMX (µg/ml) MIC	FIC Index*
5	>200.0	>1000	>32 /608	-
ATCC 8100	1.25	16	0.25/4.75	0.5
Ps. aeruginosa				
1	>200.0	>1000	>32 /608	-
2	>200.0	256	8 /152	0.63
3	>200.0	200	4 /76	0.40
4	>200.0	200	4 /76	0.40
5	200.0	>1000	>32 /608	-
6	100.0	200	4 /76	0.42
7	100.0	200	4 /76	0.42
8	25.0	200	2 /38	0.27
9	>200.0	200	8 /152	0.8
ATCC 27853	>200.0	500	8 /152	0.34
A. odorans				
1	>200.0	>1000	>32 /608	-
S. faecalis				
1	0.12	>1000	0.06/1.19	-
2	1.0	>1000	0.25/4.75	-
3	0.5	>1000	0.25/4.75	-

Organism	TMP (µg/ml) MIC	SMX (µg/ml) MIC	TMP-SMX (µg/ml) MIC	FIC Index*
S. aureus I	0.5	32	0.12/2.38	-
ATCC 25923	0.5	32	0.12/2.36	-

*FIC Index is calcaulated by the following formula:

$$FIC = \frac{\text{MIC of SMX in combination}}{\text{MIC of SMX alone}} + \frac{\text{MIC of TMP in combination}}{\text{MIC of TMP alone}}$$

** The FIC Index was not calculated for organisms with MIC values above or below the drug concentrations studied.

the antibacterial activity of TMP-SMX against the test strains is due to TMP alone (e.g. E. coli strains #10, #11, #12, #14 and Klebsiella #2, #4, #5, #6, #14, #15). Against a single organism (P. mirabilis #4) the activity of the combination was due to SMX alone.

When the NCCLS-recommended susceptibility breakpoints (≤4.0 µg/ml for TMP and ≤100 µg/ml for SMX) are employed, some 95% of 37 E. coli isolates were susceptible to TMP while only 76% were susceptible to SMX (Table 9). Moreover, using NCCLS susceptibility breakpoint of ≤2/38 µg/ml for TMP-SMX, the figure for E. coli susceptibility was again 95% of strains tested. The NCCLS criteria indicated that 76% of 17 Klebsiella isolates were susceptible to TMP, whereas only 47% of these strains were clearly sensitive to SMX. Eighty-two percent of the Klebsiella were sensitive to the combination, TMP-SMX. Proteus species showed greater resistance to both drugs: 47% were susceptible to TMP and only 30% to SMX. When the drugs were used in combination, some 65% of Proteus isolates were found to be susceptible. The numbers of isolates of other species were too small for valid comparison.

Table 9. Susceptibility of clinical isolates according to NCCLS breakpoints

	SMX µg/ml			TMP µg/ml			TMP-SMX µg/ml		
	S*	I**	R***	S	I	R	S	I	R
	≤100	>100-<350	≥350	≤4	>4-<16	>16	≤2/38	>2/38-<8/152	≥8/152
E. coli (37)	28(75)****	0	9(24)	35(95)	0	2(5)	35(95)	0	2(5)
Klebsiella sp.(17)	8(47)	3(18)	6(35)	15(88)	1(6)	1(6)	14(82)	2(12)	1(6)
Proteus sp. (17)	8(47)	1(6)	8(47)	8(47)	2(12)	7(41)	11(65)	0	6(35)
Enterobacter sp.(4)	4(100)	0	0	3(75)	1(25)	0	4(100)	0	0
C. freundii (6)	0	0	6(100)	1(17)	0	5(83)	0	0	6(100)
S. marcescens (5)	0	1(20)	4(80)	0	1(20)	4(80)	1(20)	0	4(80)
Ps. aeruginosa (9)	0	7(78)	2(22)	0	0	9(100)	1(12)	4(44)	4(44)
A. odorans (1)	0	0	1(100	0	0	1(100)	0	0	1(100)
S. faecalis (3)	0	0	3(100)	3(100)	0	0	3(100)	0	0
S. aureus (1)	1(100)	0	0	1(100)	0	0	1(100)	0	0

* S = susceptible

** I = intermediate susceptibility

*** R = resistant

**** Number in brackets represents percentages

Total Antibacterial Activity of Clinical Urine Samples

a) Schlichter Test

Levels of antibacterial activity against E. coli ATCC 25922, as determined on 24 urine specimens from patients receiving TMP-SMX, are shown in Table 10.

Bacteriostatic titres varied over a limited range from 1:64 to 1:512, with most specimens showing only a single dilution difference. Similarly bactericidal titres were confined to the range from undiluted to 1:4. Generally, samples showing bacteriostatic activity greater than 1:128 were bactericidal at dilutions of 1:2 or 1:4, whereas specimens with bacteriostatic levels of 1:128 or less were bactericidal only when the sample was undiluted.

Time-Kill Curves

b) Preliminary Studies

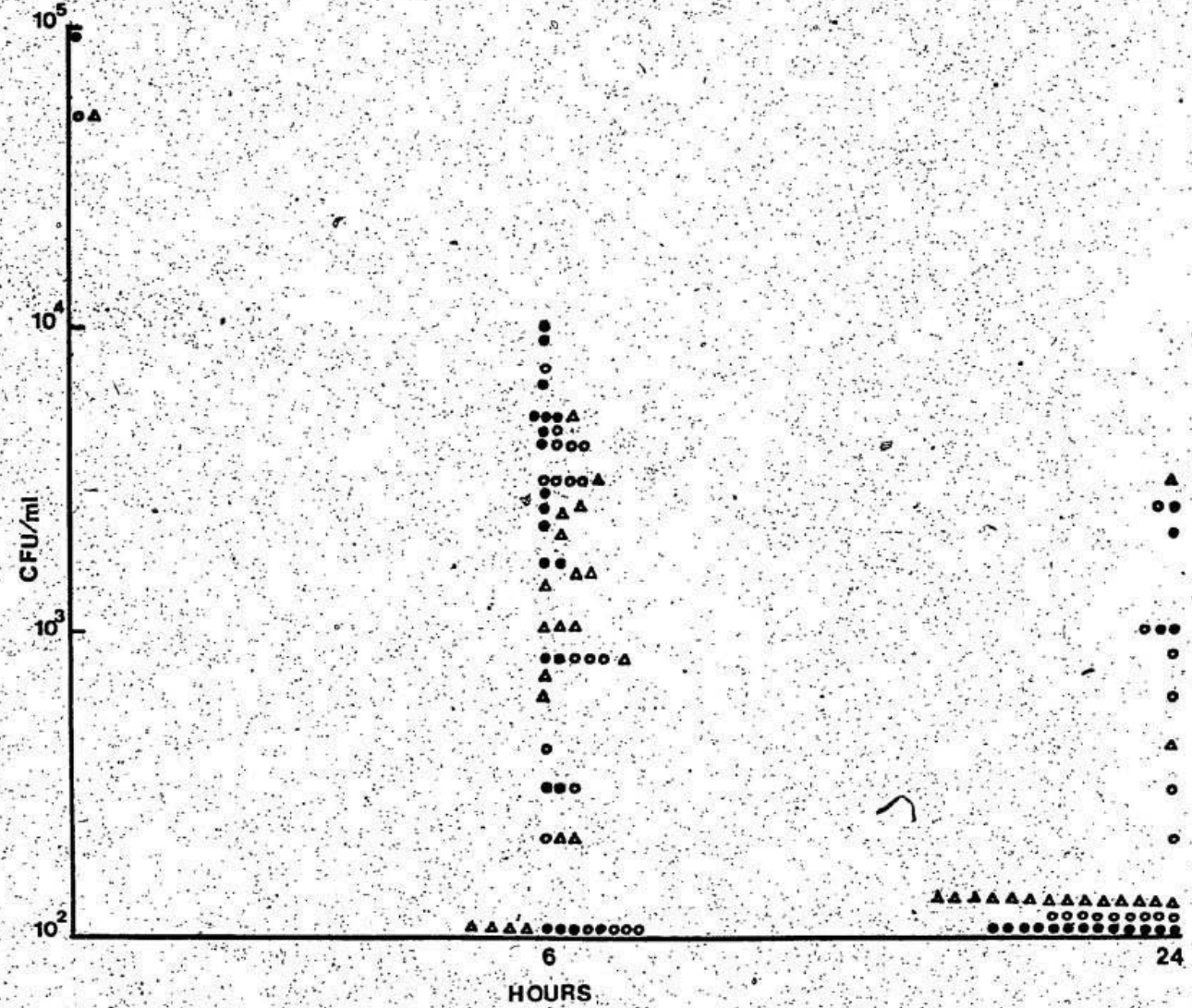
Results of preliminary studies on antibacterial activity of urine specimens obtained from TMP-SMX treated patients are shown in Fig. 18. Most samples showed bactericidal activity (0.1% or less survival). This was generally apparent only after 24 hours incubation but in some instances bactericidal effects were noted at 6 hours exposure. Significant differences in the

Table 10. The total antibacterial activity of urine samples obtained from patients on TMP-SMX therapy against E. coli 25922.

Urine Sample No	Bacteriostatic Titer	Bactericidal Titer
1	1:64	undiluted
2	1:512	1:2
3	1:128	undiluted
4	1:256	1:2
5	1:64	1:2
6	1:128	undiluted
7	1:128	undiluted
8	1:128	undiluted
9	1:128	1:2
10	1:256	1:4
11	1:256	1:2
12	1:256	1:4
13	1:512	1:2
14	1:512	1:2
15	1:512	1:2
16	1:256	1:4
17	1:256	undiluted
18	1:256	1:2
19	1:128	undiluted
20	1:256	1:2
21	1:64	undiluted
22	1:512	1:2
23	1:256	1:2
24	1:512	1:2

Figure 18. Scattergram representing the antibacterial activity of 20 clinical urine samples against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml), P. mirabilis #5 (MIC: TMP 1.0 µg/ml; SMX 8.0 µg/ml; TMP-SMX 0.12/2.38 µg/ml), and K. oxytoca #13 (MIC: TMP 0.5 µg/ml; SMX 16 µg/ml; TMP-SMX 0.06/1.19 µg/ml).

- ● E.coli ATCC 25922
- ○ P. mirabilis
- ▲ K. oxytoca


10^5
10^4
10^3
10^2
CFU/ml
6
24
HOURS

survival rates of the three strains examined were not observed.

Detailed studies

When viable counts were performed at more frequent intervals it was noted that the antibacterial effect of the selected urine specimens against the test strains was generally linear over the period of incubation (Figs. 19a - 19d). Urine sample (Fig. 19d) showed a more rapid bactericidal effect against E. coli strain earlier isolated from the other patient than against the other test strains.

TMP and SMX Bioassay of Urine Samples

The results of bioassay for TMP and SMX is 50 urine specimens from TMP-SMX treated patients are shown in Table 11 and summarized in Table 12. A broad range of activity was detected:

TMP $\bar{x}$ = 63.8 µg/ml, SD = 28.97

SMX $\bar{x}$ = 40.4 µg/ml, SD = 27.67

Standard curve response for TMP and SMX are shown in Appendix A and B.

Figure 19 a. Time-kill curves of clinical urine sample (#5) against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml), P. mirabilis #5 (MIC: TMP 1.0 µg/ml; SMX 8 µg/ml; TMP-SMX 0.12/2.38 µg/ml), and K. oxytoca #13 (MIC: TMP 0.5 µg/ml; SMX 16 µg/ml; TMP-SMX 0.06/1.19 µg/ml). The urine sample (#5) was assayed and found to contain TMP 12 µg/ml and SMX 11 µg/ml (Table 11).

——— a = Drug-free urine control for E.coli ATCC 25922
– – – b = Drug-free urine control for P. mirabilis #5
····· c = Drug-free urine control for K. oxytoca #13
——— E. coli ATCC 25922
– – – – P. mirabilis #5
····· K. oxytoca #13

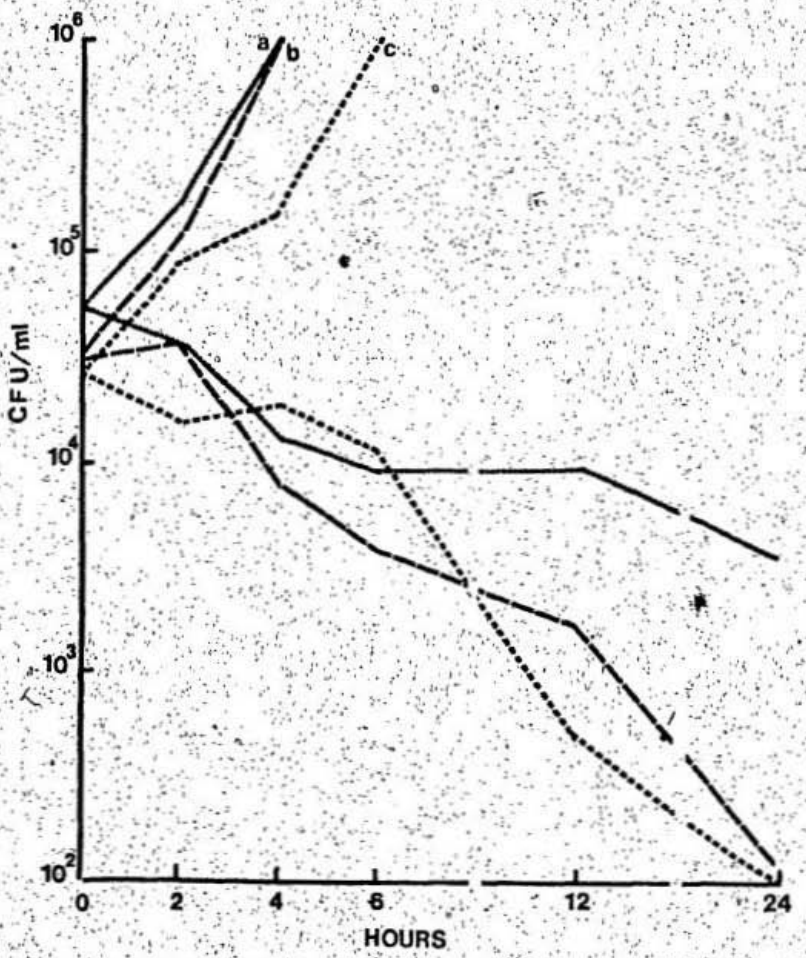

10^6
10^5
10^4
10^3
10^2
CFU/ml
a
b
c
0
2
4
6
12
24
HOURS

Figure 19 b. Time-kill curves of clinical urine sample (#37) against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml), P. mirabilis #5 (MIC: TMP 1.0 µg/ml; SMX 8 µg/ml; TMP-SMX 0.12/2.38 µg/ml), and K. oxytoca #13 (MIC: TMP 0.5 µg/ml; SMX 16 µg/ml; TMP-SMX 0.06/1.19 µg/ml). The urine sample (#37) was assayed and found to contain TMP 115 µg/ml and SMX 100 µg/ml (Table 11).

____ a = Drug-free urine control for E. coli ATCC 25922
_ _ _ b = Drug-free urine control for P. mirabilis #5
..... c = Drug-free urine control for K. oxytoca # 13
_____ E. coli ATCC 25922
_ _ _ _ _ P. mirabilis #5
..... K. oxytoca #13

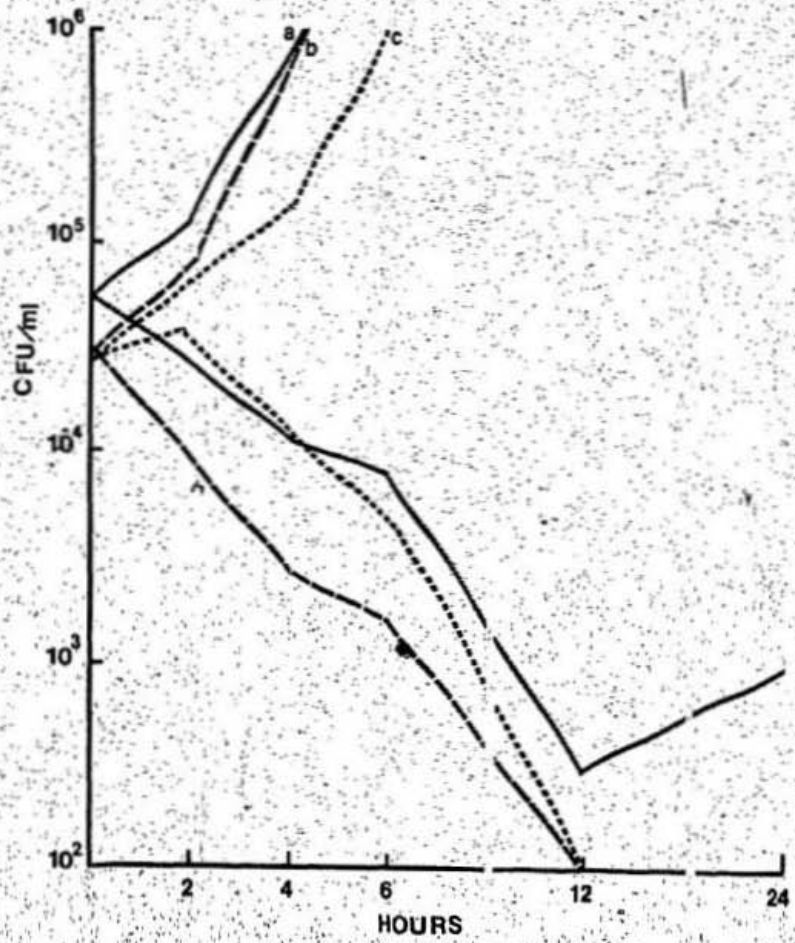

10^6
10^5
10^4
10^3
10^2
CFU/ml
a
b
c
2
4
6
12
24
HOURS

Figure 19 c. Time-kill curves of clinical urine sample (#11) against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml), P. mirabilis #5 (MIC: TMP 1.0 µg/ml; SMX 8 µg/ml; TMP-SMX 0.12/2.38 µg/ml), and K. oxytoca #13 (MIC: TMP 0.5 µg/ml; SMX 16 µg/ml; TMP-SMX 0.06/1.19 µg/ml). The urine sample (#11) was assayed and found to contain TMP 40 µg/ml and SMX 30 µg/ml (Table 11).

——a = Drug-free urine control for E. coli ATCC 25922
– – b = Drug-free urine control for P. mirabilis #5
·····c = Drug-free urine control for K. oxytoca #13
——— E. coli ATCC 25922
– – – – P. mirabilis #5
····· K. oxytoca #13

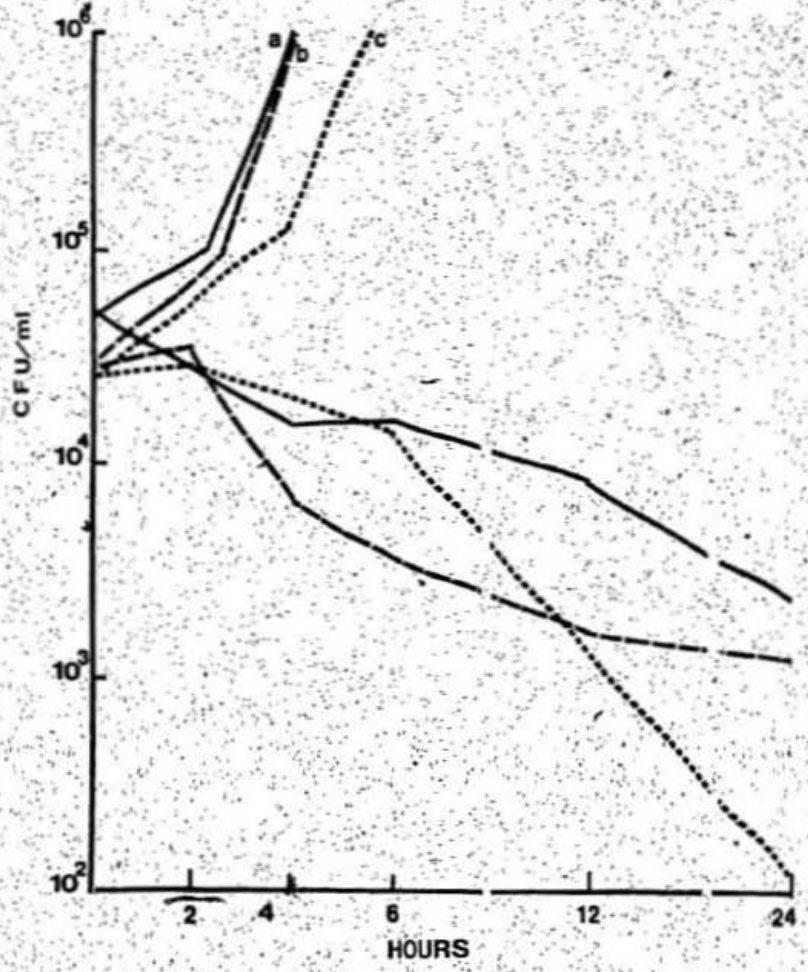

a
b
c
CFU/ml
10^6
10^5
10^4
10^3
10^2
2
4
6
12
24
HOURS

Figure 19 d. Time-kill curves of clinical urine sample (#16) against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml), P. mirabilis #5 (MIC: TMP 1.0 µg/ml; SMX 8 µg/ml; TMP-SMX 0.12/2.38 µg/ml), K. oxytoca #13 (MIC: TMP 0.5 µg/ml; SMX 16 µg/ml; TMP-SMX 0.06/1.19 µg/ml) and E.coli #7 (MIC: TMP 0.25 µg/ml; SMX 8 µg/ml; TMP-SMX 0.06/1.19 µg/ml). The urine sample (#16) was assayed and found to contain TMP 110 µg/ml and SMX 17 µg/ml (Table 11).

——a = Drug-free urine control for E. coli ATCC 25922
— — b = Drug-free urine control for P. mirabilis #5
-----c = Drug-free urine control for K. oxytoca #13
——— E. coli ATCC 25922
— — — P. mirabilis #5
..... K. oxytoca #13
——— E. coli #7 (isolated from earlier urine sample this patient)

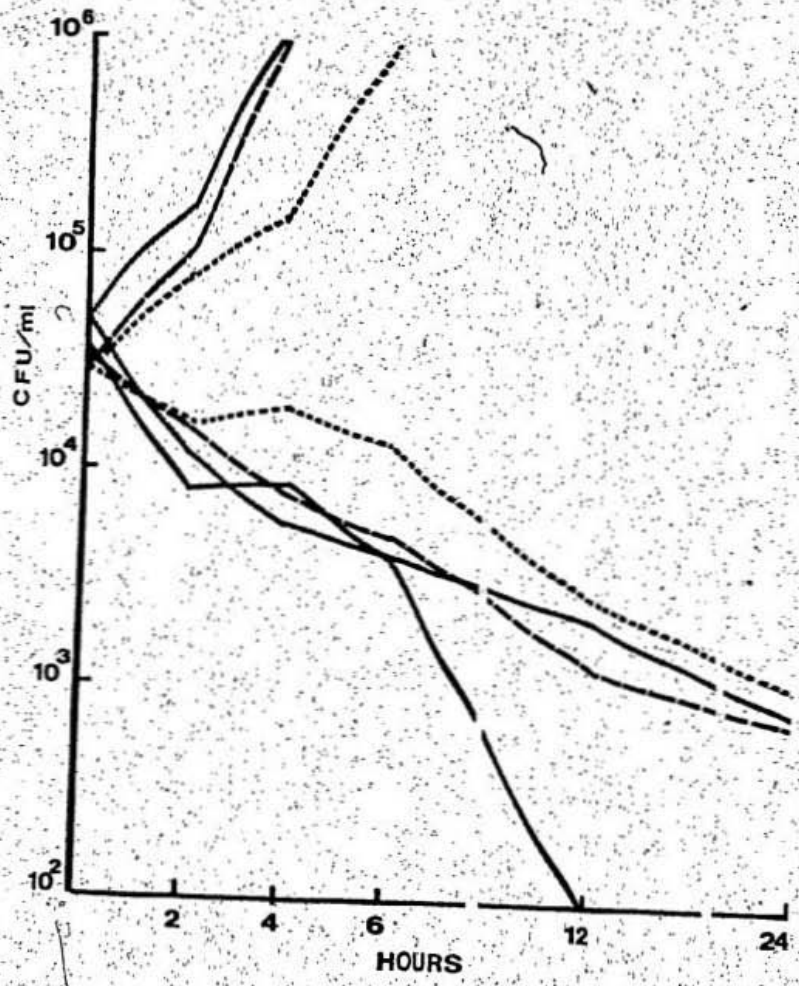

10^6
10^5
10^4
10^3
10^2
CFU/ml
2
4
6
12
24
HOURS

Table 11. Concentration of TMP and SMX in urine samples from patients treated with co-trimoxazole as determined by bioassay.

Sample No.	TMP (µg/ml)	SMX	Sample No.	TMP (µg/ml)	SMX
1	50	<10(7)*	26	64	21
2	66	72	27	40	21
3	32	27	28	100	62
4	94	<10(6)*	29	110	60
5	12	11	30	46	<10(0)
6	64	<10(8)*	31	110	20
7	76	23	32	74	71
8	45	<10(7)*	33	32	20
9	37	40	34	64	10
10	115	72	35	110	50
11	40	30	36	92	21
12	80	72	37	115	100
13	100	72	38	72	32
14	110	74	39	74	50
15	115	90	40	74	13
16	110	17	41	45	25
17	64	23	42	25	25
18	24	25	43	32	50
19	50	47	44	64	50
20	64	45	45	42	34
21	46	<10(6)*	46	64	72
22	40	40	47	46	30
23	34	32	48	46	120
24	60	43	49	32	34
25	25	90	50	64	50

* (by extrapolation)

Table 12. Summary of the range of concentration of TMP and SMX determined by bioassay of 50 urine samples from TMP-SMX-treated patients

Range of Antimicrobial µg/ml	TMP		SMX	
	No. of Samples	Per Cent of Samples	No. of Samples	Per Cent of Samples
>100 - 120	8	16%	1	2%
50 - 100	22	44%	17	34%
25 - 49	18	36%	15	30%
15 - 24	1	2%	8	16%
10 - 14	1	2%	3	6%
<10	0	0%	6	12%
TOTAL	50		50	

Effect of PABA on Antibacterial Activity in Urine

Time-kill curves for E. coli ATCC 25922 grown in untreated and PABA-treated urine samples from patients receiving TMP-SMX are presented in Figs. 20a - 20h. Generally, colony counts from both untreated samples from PABA-treated samples, in which SMX activity had been neutralized, showed a decline within two hours of incubation.

In most instances the rate of decline in untreated samples was greater than in PABA-treated samples but differences were not appreciable until incubation had continued beyond two hours (Figs. 20a - 20f). In two instances (Figs. 20g and 20h) the untreated and PABA-treated samples yielded similar time-kill curves. Only one of these urine samples showed bactericidal activity and this was not affected by PABA-neutralization of the SMX component.

Thymidine Assay

Klebsiella 1839 used in the thymidine assay was shown to have a requirement for 0.125 µg/ml thymidine for growth. No thymidine was detected in the 164 clinical specimens of urine using the test organism.

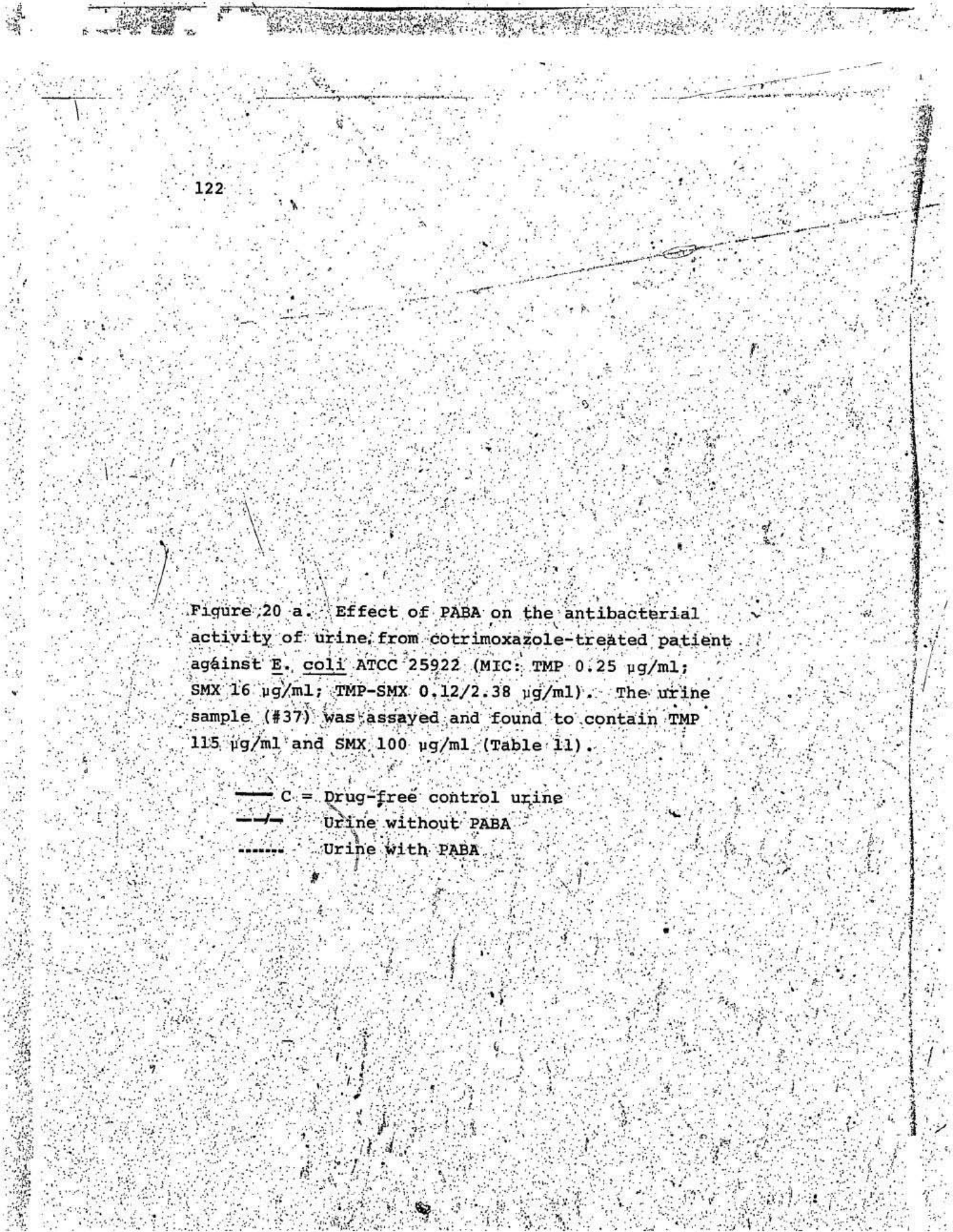


Figure 20 a. Effect of PABA on the antibacterial activity of urine from cotrimoxazole-treated patient against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml). The urine sample (#37) was assayed and found to contain TMP 115 µg/ml and SMX 100 µg/ml (Table 11).

—— C = Drug-free control urine
—/— Urine without PABA
------ Urine with PABA

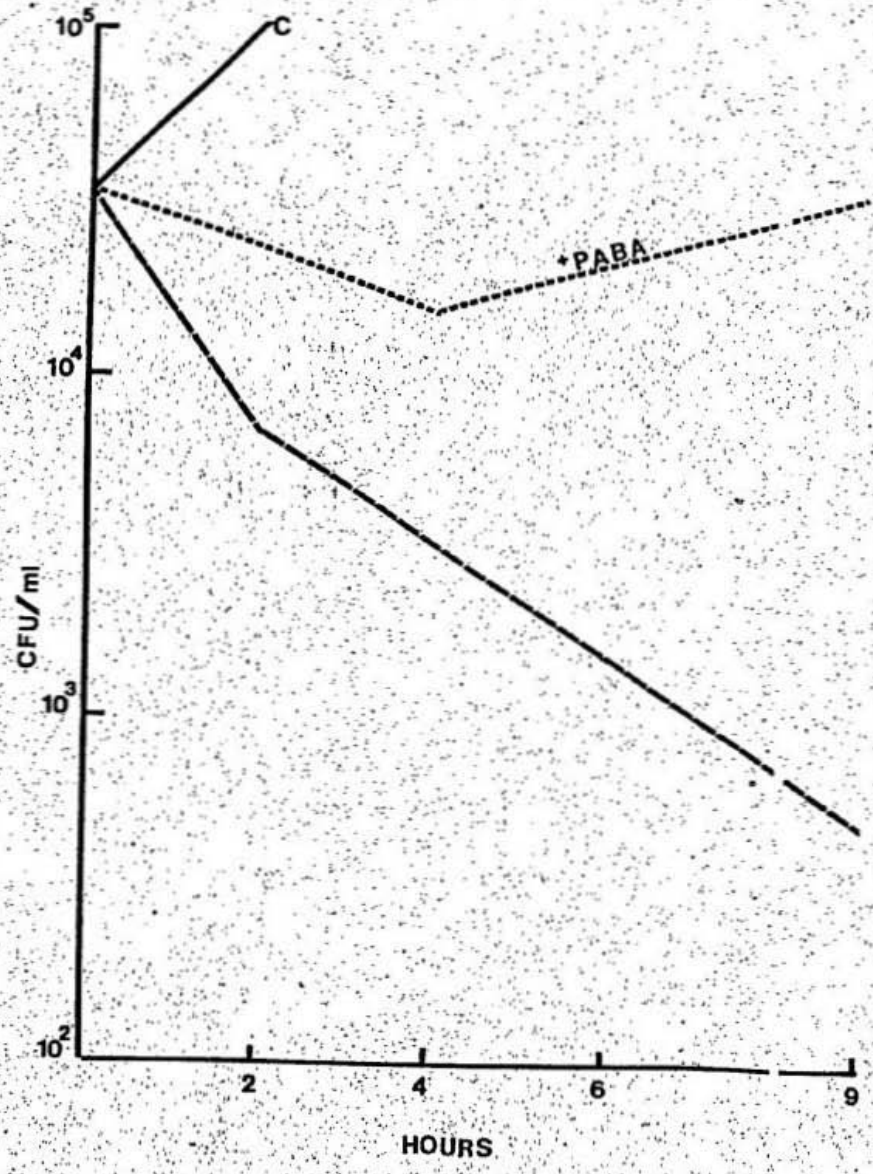

10^5
C
+PABA
10^4
CFU/ml
10^3
10^2
2
4
6
9
HOURS

Figure 20 b. Effect of PABA on the antibacterial activity of urine from cotrimoxazole-treated patient against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX µg/ml; TMP-SMX 0.12/2.38 µg/ml). The urine sample (#27) was assayed and found to contain TMP 40 µg/ml and SMX 21 µg/ml (Table 11).

—— C = Drug-free urine control
—·— Urine without PABA
------ Urine with PABA

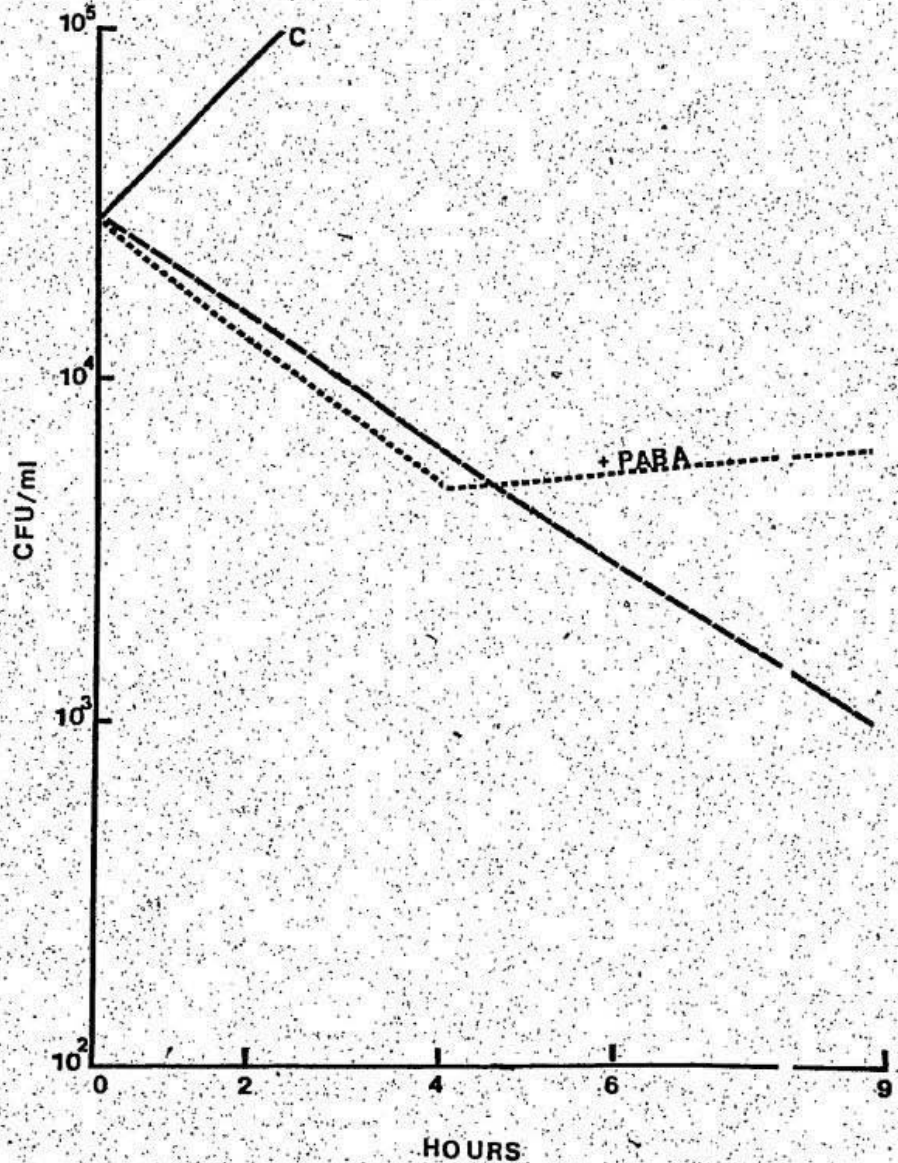

10^5
10^4
10^3
10^2
CFU/ml
C
+ PARA
0
2
4
6
9
HOURS

Figure 20 c. Effect of PABA on the antibacterial activity of urine from cotrimoxazole-treated patient against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml). The urine sample (#11) was assayed and found to contain TMP 40 µg/ml and SMX 30 µg/ml (Table 11).

—— C = Drug-free urine control
– – – Urine without PABA
..... Urine with PABA

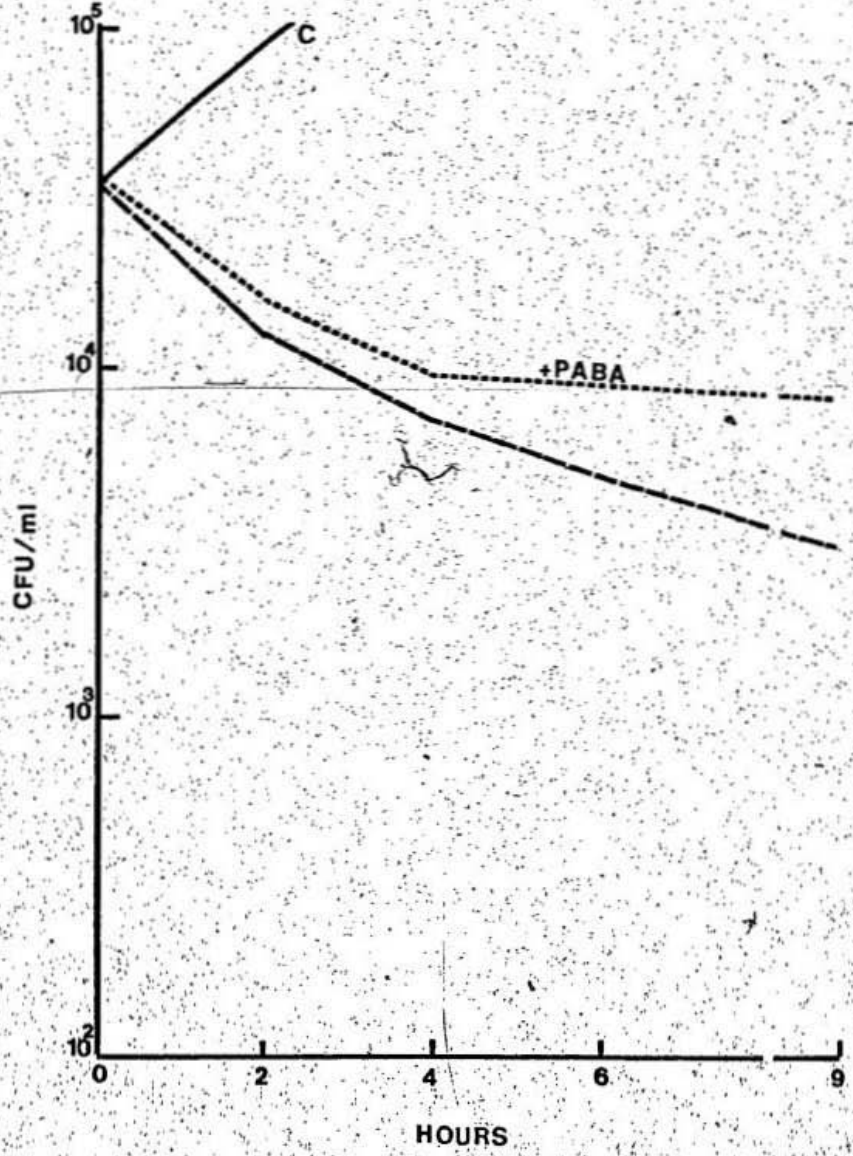

10^5
10^4
10^3
10^2
C
+PABA
CFU/ml
0
2
4
6
9
HOURS

Figure 20 d. Effect of PABA on the antibacterial activity of urine from cotrimoxazole-treated patient against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml). The urine sample (# 5) was assayed and found to contain TMP 12 µg/ml and SMX 11 µg/ml (Table 11).

—— C = Drug-free urine control
– – – Urine without PABA
····· Urine with PABA

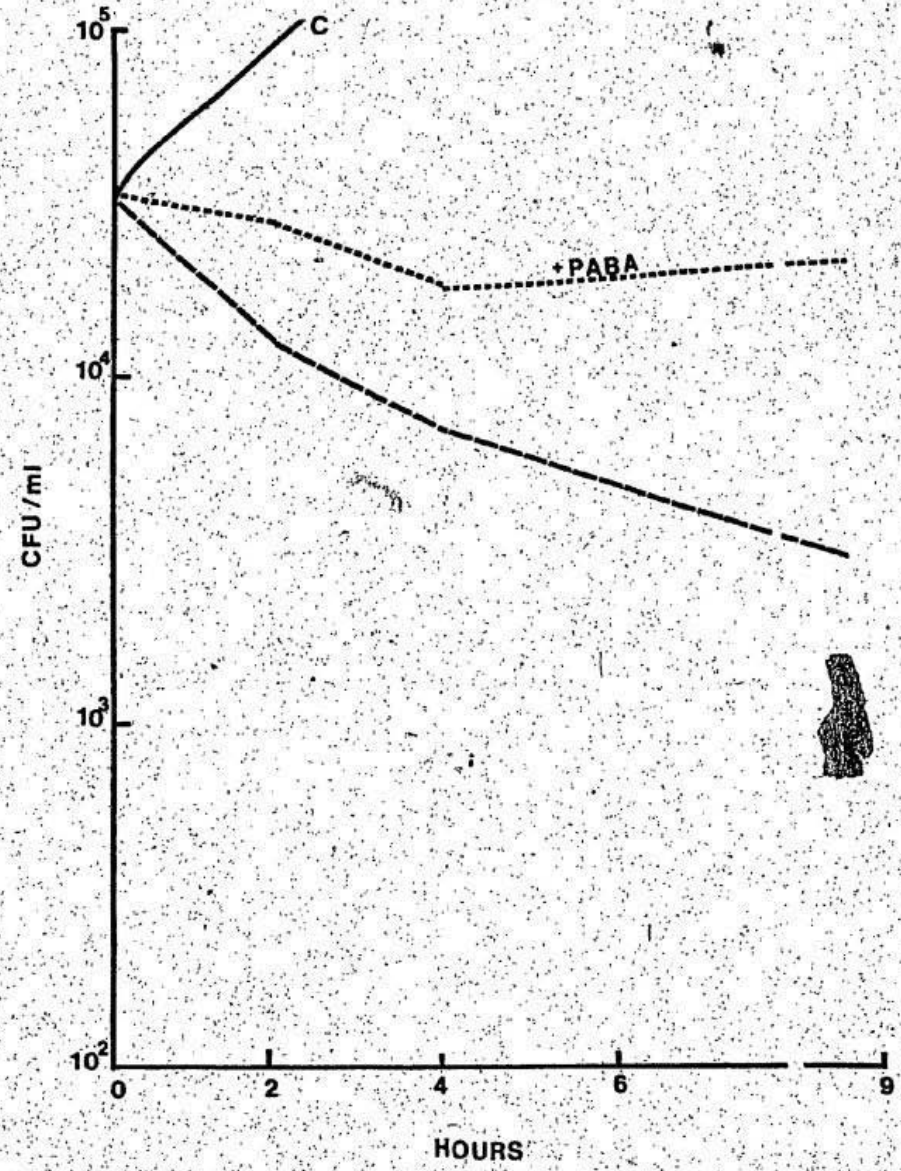

10^5
10^4
10^3
10^2
C
+PABA
CFU/ml
0
2
4
6
9
HOURS

Figure 20 e. Effect of PABA on the antibacterial activity of urine from cotrimoxazole-treated patient against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml). The urine sample (#22) was assayed and found to contain TMP 40 µg/ml and SMX 40 µg/ml (Table 11).

—— C = Drug-free urine control
– – – Urine without PABA
····· Urine with PABA

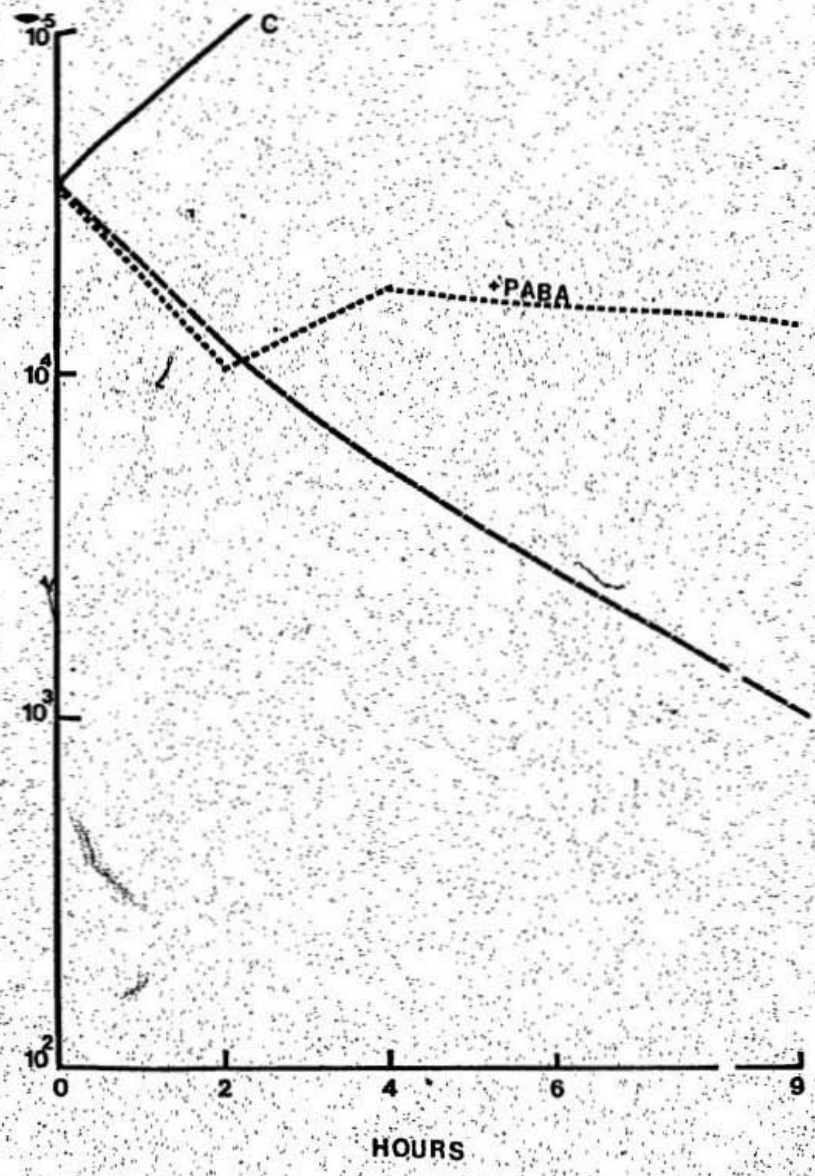

C
PARA
CFU/ml
10^5
10^4
10^3
10^2
0
2
4
6
9
HOURS

Figure 20 f. Effect of PABA on the antibacterial activity of urine from cotrimoxazole-treated patient against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml). The urine sample (#20) was assayed and found to contain TMP 64 µg/ml and SMX 45 µg/ml (Table 11).

——C = Drug-free urine control
– – – Urine without PABA
····· Urine with PABA

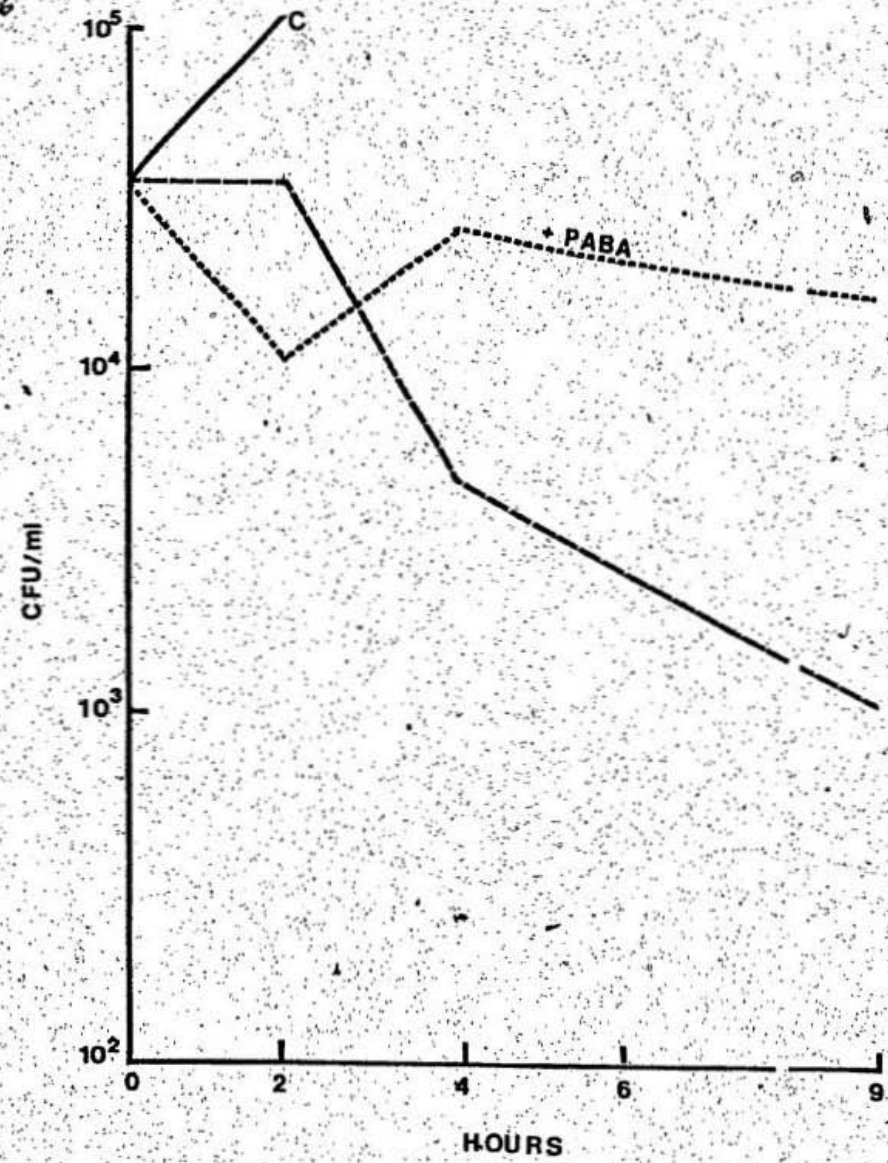

C
+ PABA
10^5
10^4
10^3
10^2
CFU/ml
0
2
4
6
9
HOURS

Figure 20 g. Effect of PABA on the antibacterial activity of urine from cotrimoxazole-treated patient against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml). The urine sample (#42) was assayed and found to contain TMP 25 µg/ml and SMX 25 µg/ml (Table 11).

——C = Drug-free urine control
— — — Urine without PABA
····· Urine with PABA

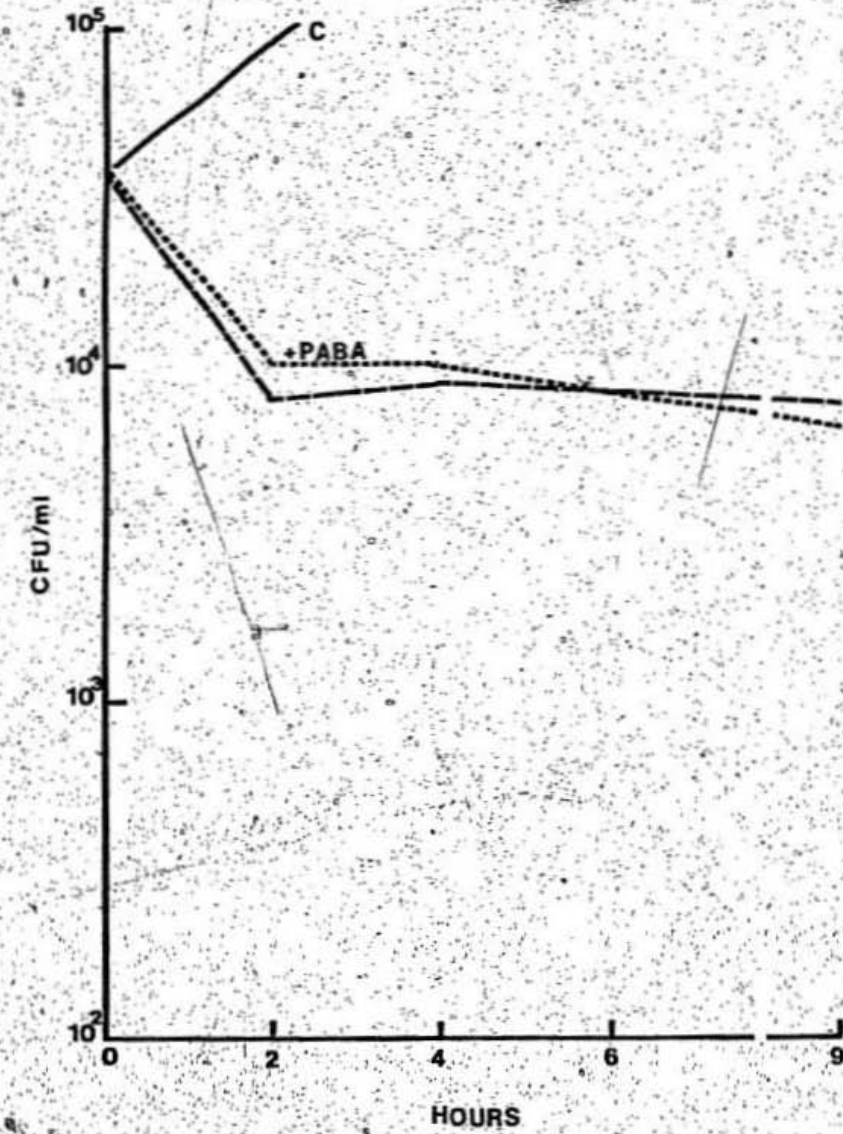
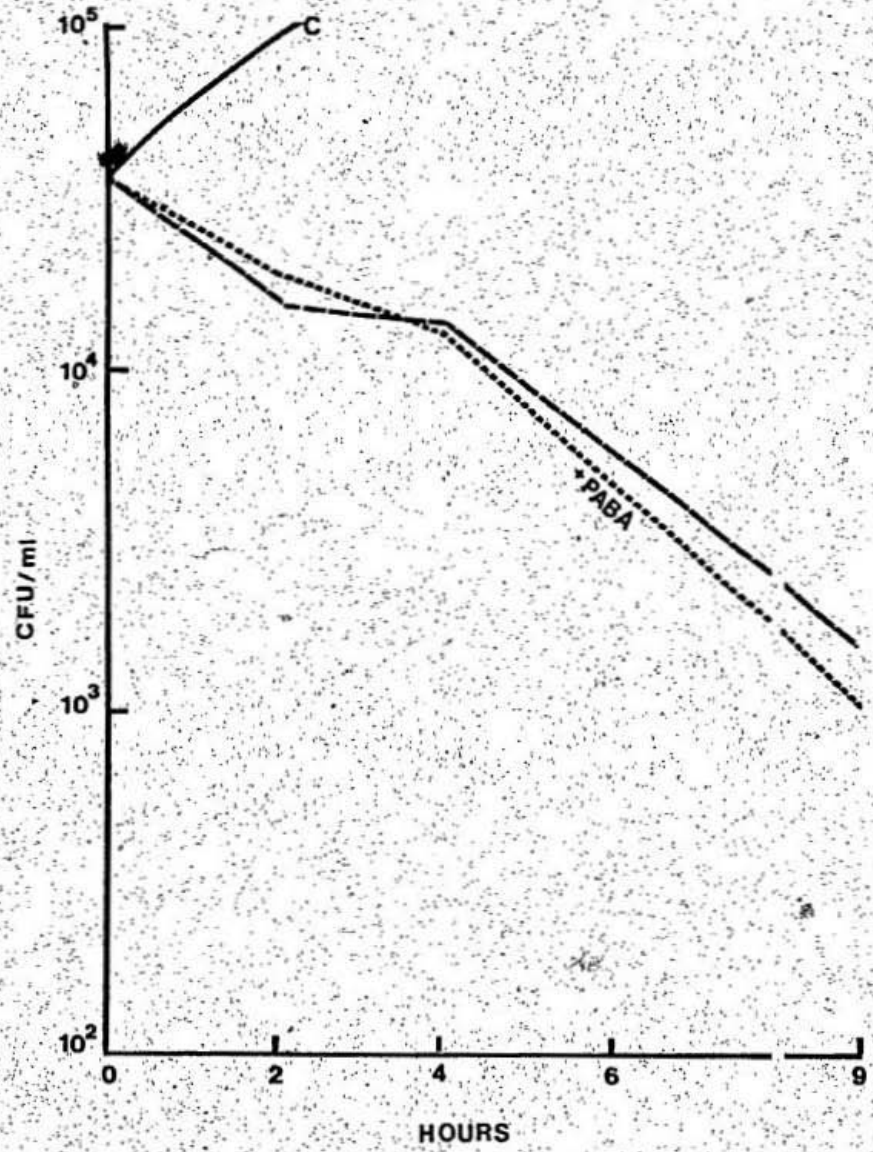

10^5
C
+PABA
10^4
CFU/ml
10^3
10^2
0
2
4
6
9
HOURS

Figure 20 h. Effect of PABA on the antibacterial activity of urine from cotrimoxazole-treated patient against E. coli ATCC 25922 (MIC: TMP 0.25 µg/ml; SMX 16 µg/ml; TMP-SMX 0.12/2.38 µg/ml). The urine sample (#16) was assayed and found to contain TMP 110 µg/ml and SMX 17 µg/ml (Table 11).

—— C = Drug-free urine control
– – – Urine without PABA
····· Urine with PABA


10^5
C
10^4
+PABA
CFU/ml
10^3
10^2
0
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6
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HOURS

DISCUSSION

The rationale for the use of antimicrobial agents in combination has been promulgated for many years. The reasons for this approach to the management of infectious diseases have been both theoretical and practical. They have been summarized by Cohen (45):

i. initial therapy of serious infections before the aetiological agent had been identified or its susceptibility established,

ii. treatment of infection by multiple organisms with different antimicrobial susceptibility,

iii. decreased emergence of resistant microbes,

iv. lessening the dose-related toxicity of the antimicrobial agents,

v. eradication of infection which cannot be successfully treated with a single drug.

In all of these situations there rests the assumption that two or more drugs will accomplish what one alone will not. The zealous physician has been tempted to extend this reasoning and to argue that if one agent is good, two are better (46, 48, 103). The pharmaceutical industry has responded to that reasoning and certain premixed antimicrobial

combinations have been marketed. Penicillin-streptomycin ("Fortimycin", Ayerst) and novobiocin-tetracycline ("Albamycin T", Upjohn) are examples.

In 1966 the United States Food and Drug Administration commissioned the National Academy of Sciences-National Research Council (NAS-NRC) to review the claims for effectiveness of drugs approved between 1938 and 1962(1). The use of fixed combinations was one of the main areas examined. It was the conclusion of the "Drug Efficacy Study" that in many instances the premixed combinations were no more effective in eradicating infections than a single agent. Furthermore, concern was expressed that the use of "fixed-dose" mixtures prevented the physician from adjusting the ratio and timing in which the components could be used and limited the choice of the components. The NAS-NRC team strongly urged that the manufacture and sale of fixed antimicrobial combinations should be no longer supported. The Food and Drug Administration acted upon this recommendation and by 1970 many premixed drugs had been banned (2, 45).

Co-trimoxazole, a fixed combination of trimethoprim and sulfamethoxazole (TMP-SMX), was approved for marketing and use in the United States in 1973, some five years after its release in Britain and continental Europe.

It appears that the new product possessed the essential feature implicit in the efficacy requirements of the U.S. Food, Drug and Cosmetic Act that both components of a combination contribute to the claimed effect (2). The mode of action of each of the components had already been elucidated. It was proposed that the drugs in combination would exert sequential inhibition of the folic acid biosynthetic pathway of bacteria and would thus act more effectively in concert (25, 54, 65). An additional advantage to using these drugs in combination has been claimed. While each of the components exhibited only bacteriostatic activity separately, there was evidence that the combined effect was bactericidal (38).

When the therapeutic effect of a drug combination is greater than would be expected from the cumulative effects of the components the phenomenon has been called synergy. Synergistic activity is difficult to evaluate in vivo. Animal experiments and limited clinical trials have indicated a better response to cotrimoxazole than to either TMP or SMX but not all investigators have provided support for these findings (104, 105, 106, 107). More objective information has been derived from in vitro studies. Synergism between TMP and SMX has been demonstrated by several standard methods but there have been difficulties in reproducibility and the results may not relate to specific clinical situations (48, 108, 109, 110).

In the present study, several *in vitro* approaches have been used to assess the activity of TMP and SMX, individually and in combination, against common bacterial isolates from urine specimens submitted to the clinical laboratories of a general hospital. In addition the antibacterial activity of urine samples from patients receiving TMP-SMX therapy has been evaluated. The kinetics and nature of the antibacterial activity in drug-supplemented media and urine from healthy volunteers was compared with the activity of the urine obtained from patients receiving TMP-SMX therapy.

It has been shown by several workers that TMP is by far the more active agent in the TMP-SMX combination (7, 69, 70, 111). In this study, the activity of the combination and its components against a spectrum of common urinary tract pathogens was examined and the findings provide additional support for the earlier observations. When the susceptibility of these organisms was quantitated by determining minimal inhibitory concentrations, it was found that bacteria inhibited by low levels of the combination were also susceptible to low levels of the TMP component. On the other hand, there was poor correlation between levels of SMX and those of TMP-SMX required to prevent bacterial growth. In fact, for the clinical

isolates studied, the susceptibility of the urinary tract pathogens to TMP-SMX appeared to be largely independent of the SMX content. The effect was an apparent reduction of MIC values for SMX as a result of its combination with TMP. This has been interpreted by supporters of cotrimoxazole as indicating synergistic activity but only in a few cases does the SMX component appear to make a clinically significant combination. In one instance, a strain of Proteus (#4) isolated from a urinary tract infection was found to be highly resistant to TMP (MIC >200 µg/ml) and yet susceptible to low levels of the sulfonamide (MIC 4 µg/ml). This was the only case in which SMX was the major active component of the combination.

It has been desirable to confirm clinical impressions of synergistic activity by demonstration and quantitation of synergy *in vitro*. When the MIC values reported here are subjected to analysis by determining fractional inhibitory concentrations (FIC) indices, the earlier defined criteria for synergistic activity can be met. However, in this series TMP-SMX was used at a ratio of 1:20, the proportion expected in plasma following usual dosage and at which optimal synergistic activity *in vitro* is anticipated. Levels of TMP and SMX detected in the urine of cotrimoxazole-treated patients in the current study did not approach this ratio. In most cases SMX levels were lower than those of TMP (Table 11) and the ratio approximated 1:1.

MIC values for antimicrobial agents have been applied to the question of effectiveness in the clinical situation by reference to blood levels achievable with frequently used dose schedules. When the recommended breakpoint levels for TMP, SMX, and TMP-SMX are applied, only 6 of the 100 urinary isolates would be considered resistant to TMP but susceptible to TMP-SMX. Since urinary levels of TMP are some 25 to 50 times the achievable blood concentrations only one of these 6 isolates (P. mirabilis #4, Table 8) would be considered resistant to therapeutic levels of TMP. On the other hand, there were 16 isolates resistant to attainable urine levels of SMX but susceptible to TMP-SMX. All but three of the latter strains (Proteus sp. (#13), S. marcescens (#3), Ps. aeruginosa (#8), Table 8) were susceptible to therapeutically achievable levels of TMP alone.

When interaction between the two components was further examined qualitatively by the impregnated paper strip method, a procedure which has been used to screen the potential effectiveness of antimicrobial combinations, synergistic activity against two bacterial strains was detected but there appeared to be no noticeable difference when the ratio of the drugs was varied over a wide range. Synergy was not demonstrated when strains highly resistant to TMP or SMX were tested.

The checkerboard titration method enables the MICs of the two drugs to be determined when the agents are present in mixtures of various proportions and allows for quantitation of the interaction. In the present study, synergism detected against two bacterial strains by paper strip method was confirmed by the two-dimensional titration as was lack of synergy against the SMX-resistant strain of E. coli. However, marked synergism was demonstrated for the P. mirabilis strain (#4) for which no synergistic activity could be detected by the paper strip method. Lack of correlation between different methods of evaluation synergy has been reported by other workers (48,108,109,110). Whether the discrepancy reported here was due to diffusion effects, inoculum density, nature of the medium, or other factors is not known.

As has been cited earlier, evidence has been presented that TMP-SMX exhibits bactericidal activity while the individual components have been said to be bacteriostatic. Time-kill curves were used in this study to assess the nature of activity exerted by each agent and by the combination. It is clear from the results that the effect of the sulfonamide is bacteriostatic only. Against a TMP-sensitive strain of E. coli, bactericidal activity was demonstrated for TMP-SMX but the effect was not significantly greater than that of TMP alone when both the combination and the single components were tested at therapeutic levels. At lower

concentrations, the bactericidal activity of TMP was enhanced by combination with SMX when TMP-SMX was tested in a ratio of 1:10 but enhancement was not apparent at ratios of 1:1 and1:2, approximating those found in urine of patients receiving usual dosages of cotrimoxazole. At therapeutic levels the most dramatic increase of bactericidal activity for TMP-SMX vs TMP alone was seen for the TMP-resistant strain of P. mirabilis (#4).

The modified Schlichter test for total antibacterial activity performed on 24 urine samples from patients receiving TMP-SMX therapy, showed little variation in bacteriostatic and bactericidal activity, both of which paralleled specific TMP and TMP-SMX activity as determined by bioassay. Time-kill studies on these samples against three common urinary tract pathogens showed bactericidal activity which, on the basis of earlier time-kill curves and specific bioassay could be accounted for by the TMP content of the specimens without contribution from the SMX content. This was confirmed by minor diminution of activity in samples to which PABA had been added to neutralize the SMX effect.

The question of antagonism of TMP activity by thymidine in vivo has been raised and Maskell et al (82) have suggested that thymidine is present in urine from infected patients. Thymidine was not detected in any of the 164 urine samples examined by bioassay in this study.

CONCLUSIONS

Extensive *in vitro* testing has not revealed clearcut evidence that would predetermine advantages for the use of cotrimoxazole, a fixed combination of trimethoprim and sulfamethoxazole, over trimethoprim alone in the treatment of urinary tract infections. In spite of a sound theoretical rationale, involving sequential inhibition of bacterial tetrahydrofolate synethesis, it was not possible to demonstrate, by laboratory methods, significant synergistic effect which would pertain generally to the management of these infections.

J. Richard Crout, then Director of Bureau of Drugs, U.S. Food and Drug Administration, cited, in 1973, a former FDA Commissioner: "Our basic position, in brief, is based on the concept that all drugs are, to some degree, potentially hazardous and should not be taken if they are not needed" (2). Although cotrimoxazole has been generally well tolerated, there have been reports of increased toxicity when the combination is compared to trimethoprim alone (64, 112, 113, 114). Perhaps more significantly, the use of the premixed drug precludes adjustment of the component ratio to achieve optimal results against specific microorganisms at specific infection sites. There are also considerable differences in cost. Wholesale prices for TMP-SMX are almost double those for TMP.

Although there had been earlier European experience, trimethoprim for use as a single agent did not become available in Britain, United States, and Canada until recently (Ipral from Squibb, Trimpex from Roche, and Proloprim from Burroughs Wellcome). Conclusions drawn from extensive experience with the agent in these countries are awaited. Concern has been expressed about the emergence of resistant strains (115, 116). Whether frequent use of TMP alone will increase the number of such strains remains to be determined.

It is possible that the combination may be more effective than TMP alone in the treatment of chronic urinary tract infections in patients with impaired renal function (117). In a recent study, no differences in success rate were noted in a series of patients with acute urinary tract infections treated with either TMP-SMX or TMP (118). More clinical studies are required and undoubtedly will be forthcoming. In the meantime, results of *in vitro* studies do not provide adequate justification for the use of the fixed combination in the therapy of urinary tract infections.

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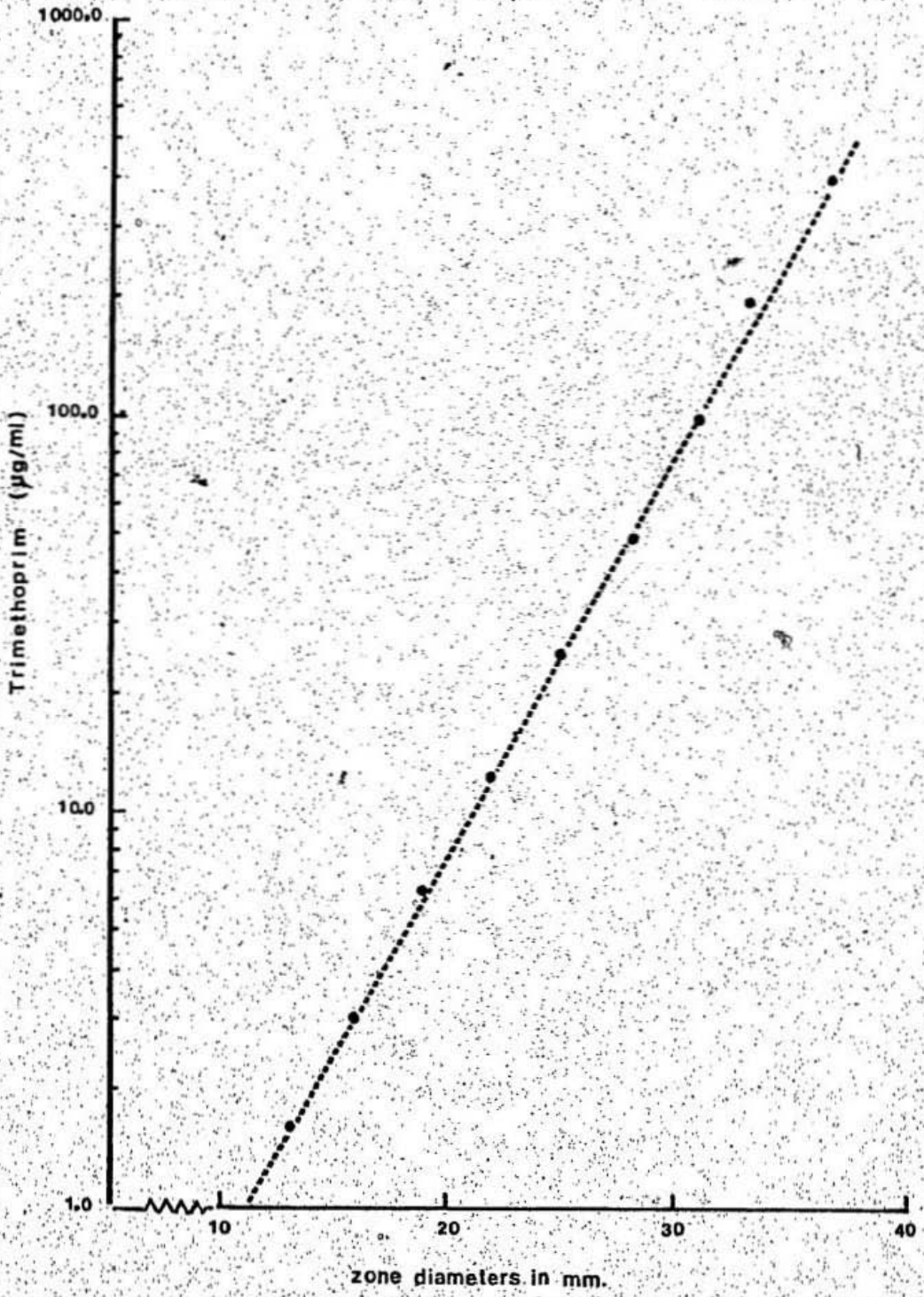
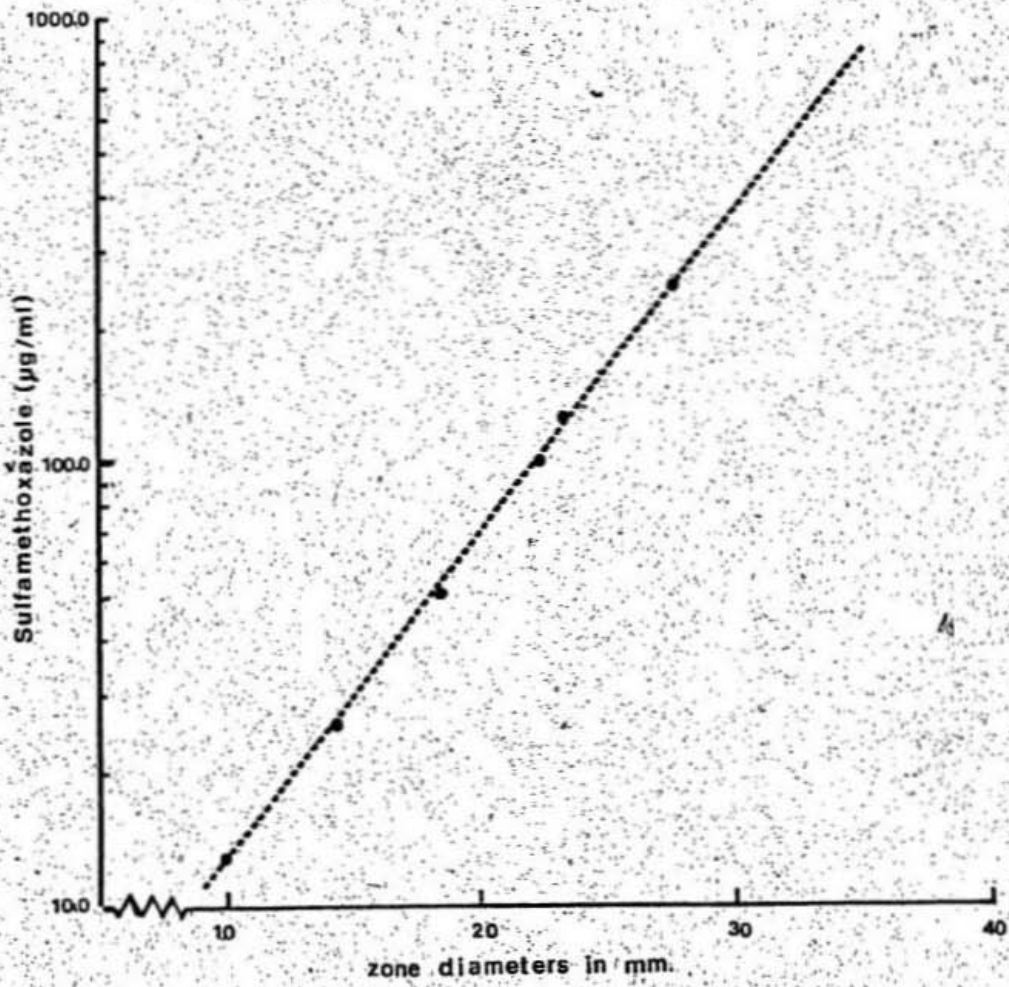
Appendix A. Standard curve for trimethoprim against E. coli #14


1000.0
100.0
10.0
1.0
Trimethoprim (µg/ml)
10
20
30
40
zone diameters in mm.

Appendix B. Standard curve for sulfamethoxazole against *P. mirabilis* # 4


1000.0
100.0
10.0
Sulfamethoxazole (µg/ml)
10
20
30
40
zone diameters in mm.