

shown in Table VII. Columbia blood agar base rendered all but one strain resistant to co-trimoxazole, and 2 out of 30 were sensitive with the addition of TP. Wellcotest blood agar base showed all 30 strains sensitive and DST blood agar base rendered one strain resistant. The four resistant strains on MH showed *in vitro* sensitivity if TP was added.

These results indicate that the undefined Columbia blood agar base is unreliable for testing *S. pyogenes* for co-trimoxazole sensitivity, regardless of the presence of TP. After incubation the WT turns light brown, which makes the reading of *S. pyogenes* hemolysis at times difficult. However, there was never doubt as to the *in vitro* sensitivity of the organism on this account.

As with *H. influenzae*, we tested two different methods of inoculation. The first method is fast, using a freshly emulsified suspension. The second, time-consuming method is a 6-hour broth culture used in a dilution of 1:100. The latter method produces lighter growth than the first one. Some of the strains on CAB that were found to be resistant with the "fast inoculation method" were found to be sensitive with the 6-hour incubation method. The addition of TP did not change the pattern. On the other hand, the 6-hour incubation method rendered all strains sensitive when tested on the other three blood-agar base media. It may be concluded that a lighter inoculum with actively multiplying *S. pyogenes* produces a higher degree of *in vitro* sensitivity when testing for co-trimoxazole sensitivity. These results indicate that *S. pyogenes* is sensitive to co-trimoxazole *in vitro* if proper agar bases and a light inoculum are used for the preparation of the blood agar. The high incidence of resistant *S. pyogenes* as reported previously may indeed be due to the culture media used.¹³

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Synergy of trimethoprim-sulfamethoxazole

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The basis for the synergy of the antibacterial drugs trimethoprim (TMP) and sulfamethoxazole (SMX) is well documented.^{1,2} Both drugs interfere with the biosynthesis of the folate coenzymes and thus ultimately affect the biosynthesis of proteins and nucleic acids. The sulfonamide acts as a competitor for para-aminobenzoic acid (PAB) in the formation of dihydropteroic acid by dihydropteroate synthetase.³ This acid is condensed with glutamic acid to form dihydrofolic acid, which is then reduced to tetrahydrofolic acid by dihydrofolate reductase. This reduction is essential in the formation of the coenzymes and it is at this step that TMP acts through binding with the dihydrofolate reductase.⁴ SMX and TMP therefore act in the same biochemical pathway and the enhancement of activity from their simultaneous administration is due to their actions being sequential.

The blocking of the enzyme dihydropteroate synthetase by

sulfonamides and of dihydrofolate reductase by TMP is competitive, and the amount of inhibitor needed to reduce the biosynthesis of dihydropteroic acid or tetrahydrofolic acid to levels below those essential for growth depends on the amount of substrate present. Because the sulfonamide acts before TMP in the biosynthesis, its role in the dual action is merely to reduce the amount of dihydrofolic acid against which TMP competes. If the competition between TMP and dihydrofolic acid were linear, then the effects of the dual action would be no more than additive, but because the competition by TMP increases relatively with decreases in dihydrofolic acid, the effects are synergic.⁵ Another factor that will also affect the dual action is that TMP affects not only the *de novo* synthesis of tetrahydrofolic acid but also its recycling. In the biosynthesis of thymine, at the stage at which uridylylate is reduced to thymidylylate, tetrahydrofolic acid is reoxidized to dihydrofolic acid, which is then returned to the tetrahydrofolate pool by the action of dihydrofolate reductase; in the presence of TMP this recycling is inhibited and in conjunction with the partial cut-off

in the *de novo* synthesis by SMX the effects on the organism are profound.

Both SMX and TMP are therefore antifolate drugs but they show selective toxicity for bacteria and the basis for this selectivity is well established. The sulfonamides are not antifolates for man because they interfere with the biosynthesis of dihydrofolic acid, which is an essential metabolite for man and is derived from his diet. In contrast to its effect on bacterial dihydrofolate reductase, TMP at a therapeutic concentration

does not significantly affect the conversion of the dietary-derived folates of animals to their tetrahydro form because TMP has a much greater affinity for the bacterial dihydrofolate reductase than for that of animals.⁶

The advantages claimed⁷ for the use of the combination TMP-SMX are (a) reduction in minimum inhibitory concentration (MIC), (b) increased bactericidal activity and (c) lessening of the risk of emergence of resistant mutants.

Reduction in MIC

The increase in antibacterial activity is well substantiated in both *in vitro* and experimental *in vivo* studies, and although there have not been many comparative trials of TMP and SMX, alone and in combination, clinical experience supports the laboratory studies. Examples of the reduction in MIC with the combination are shown in Table I,⁸ and the reduction in the ED₅₀ of SMX by the presence of TMP in experimental infections of mice is shown in Table II.⁹

The reduction in the MIC depends on the ratio of the drugs present and, in terms of economy of concentrations of both drugs, there is a ratio that is optimal for each organism. The use of the term "optimum ratio" has created the false impression that for each organism there is a ratio with which maximum antibacterial activity occurs; it cannot be stressed too strongly that the effect on the organism is the same over a wide range of ratios and that the optimum ratio is merely the one with which the effect is produced by the lowest concentration of each drug. With other ratios the minimum effective concentration of one of the components will be less than with the optimum ratio but the concentration of the other component will be greater. The need for attaining the optimum ratio arises only when there is a problem in reaching adequate concentrations of the drug at the infection site, a situation that occurs rarely.⁸

Increased bactericidal activity

The increase in bactericidal activity is more controversial. It undoubtedly occurs *in vitro* but the conditions necessary for demonstrating bactericidal activity with these drugs are critical. Death is due to the abnormal growth that occurs when the organism is deprived of thymidine and if the conditions permit continuation of protein synthesis.¹⁰ Therefore, for death to occur it is essential for exogenous methionine, glycine and purines to be present in order that the effects of the drug on the

Table I—Effect on MIC of combining one part trimethoprim with 20 parts sulfamethoxazole

Organism	MIC (μg/ml)			
	Sulfamethoxazole		Trimethoprim	
	Alone	Mixture	Alone	Mixture
<i>Streptococcus pyogenes</i>	> 100	1	1	0.05
<i>Streptococcus pneumoniae</i>	30	2	2	0.1
<i>Staphylococcus aureus</i>	3	0.3	1	0.015
<i>Hemophilus influenzae</i>	10	0.3	1	0.015
<i>Bordetella pertussis</i>	50	4	3	0.2
<i>Klebsiella pneumoniae</i>	> 100	4	1	0.2
<i>Klebsiella aerogenes</i>	> 100	4	1	0.2
<i>Escherichia coli</i>	3	1	0.3	0.05
<i>Salmonella typhimurium</i>	10	1	0.3	0.05
<i>Shigella sonnei</i>	10	1	0.3	0.05
<i>Proteus vulgaris</i>	30	3	3	0.15

From Bushby⁸ (by permission of the University of Chicago Press).

Table II—In vivo effect of sulfamethoxazole (SMX), trimethoprim (TMP) and a 5:1 combination of trimethoprim-sulfamethoxazole against experimental systemic infections of mice

Organism	CD ₅₀ * (mg/kg)			FIC† index
	SMX	TMP	SMX + TMP	
<i>Diplococcus pneumoniae</i> 6301	>500	>1000	273 + 54.6	<0.60
<i>Streptococcus pyogenes</i> 4	401	>1000	145.4 + 29	<0.38
<i>Staphylococcus aureus</i> Smith	28	42	6.7 + 1.3	0.26
<i>Escherichia coli</i> 257	12	139	5.6 + 1.1	0.47
<i>Klebsiella pneumoniae</i> A	15	172	6 + 1.2	0.41
<i>Proteus vulgaris</i> 190	50	100	22 + 4.4	0.48
<i>Pseudomonas aeruginosa</i> B	79	>1000	79 + 15.7	<1.02
<i>Salmonella schottmuelleri</i>	28	620	9.4 + 1.9	0.34
<i>Salmonella typhi</i> P58a	5.9	8.5	<2 + <0.4	<0.39

* CD₅₀ = dose required to cure 50% of infected mice.

† FIC index = $\frac{\text{CD}_{50} \text{ TMP in combination}}{\text{CD}_{50} \text{ TMP alone}} + \frac{\text{CD}_{50} \text{ SMX in combination}}{\text{CD}_{50} \text{ SMX alone}}$

Adapted from Grunberg⁹ (by permission of the University of Chicago Press).

biosynthesis of ribonucleic acid and proteins may be bypassed. These metabolites are, in fact, present in adequate quantities in most bacteriologic media, but of equal importance is the need for the medium to be free from metabolites that would enable the organism to escape the effects of thymine deprivation. Thymidine is such a metabolite and it is present in many media.¹¹ Concentrations as low as 0.1 $\mu\text{g}/\text{ml}$ will convert the bactericidal action of the drugs to mere bacteriostasis, and concentrations around 1.0 $\mu\text{g}/\text{ml}$ will permit the partial growth that has caused so much confusion in susceptibility testing.¹²

Whether the combination is, in fact, bactericidal under *in vivo* conditions is not known. Undoubtedly the combination is highly effective in the treatment of many infections but it is, of course, not essential for an antibacterial drug to be bactericidal for it to be curative. Although elimination of an infection implies death of the invading organism, in practice it is rarely possible to separate the role played by the antibacterial agent in the killing from that played by the natural defence mechanisms of the host. However, Then and Angehrn¹³ have shown that in blood *in vitro* the conditions are suitable for the effects of the combination to be bactericidal and that they can be rendered unsuitable by adding thymine or, preferably, thymidine. As the conditions were not improved by adding glycine, methionine and adenine or inosine as a source of purines, these investigators concluded that the conditions in blood are optimal. By conducting similar experiments in urine they reached, for this fluid, the same conclusion, which conflicted, however, with those of Anderson *et al.*¹⁴ These latter workers maintain from their results that the combination of TMP and SMX is rarely bactericidal *in vitro* in urine during a 6-hour observation period at 37°C, and that, in the instances in which it is lethal, the effects are due solely to the action of TMP; in fact, they maintain that SMX in urine interferes with the action of TMP. The difference between the results of these two groups of workers may be due to their use of different concentrations of the drugs, for although apparently paradoxically, Anderson *et al.*, who found less activity, used concentrations some 10 times higher than those used by Then and Angehrn. The concentrations used by Anderson *et al.* were similar to those present in the urine of patients undergoing treatment with standard doses of the combination, i.e. 50 to 100 μg of TMP and 250 to 500 μg of SMX per ml, and so their conditions resembled more closely the *in vivo* conditions than did those of Then and Angehrn. I have conducted experiments (unpublished) similar to those of Anderson *et al.* on urine from five patients undergoing treatment with the combination. The viability of six strains of urinary pathogens in each of the urine samples was determined during an observation time of 24 hours at 37°C, and although with none of the strains was viability affected during the first 6 hours, most of the organisms were dead by the 24th hour. The relatively slow killing in urine with high concentrations of the drug may be due, under these circumstances, to stronger inhibition of protein synthesis, which would counteract killing through unbalanced growth.¹⁵

Effects on emergence of resistant mutants

Antibiotics are sometimes given in combination, especially in the treatment of tuberculosis, in order to decrease the rate of emergence of resistant mutants. The basis for using combinations for this purpose is that, provided the resistant mutation for each drug is genetically distinct, resistance to both drugs arises from double mutation and the chances of this occurring is the product of the mutation rates of the individual drugs; for example, if these are 10^{-6} and 10^{-7} , respectively, the rate for double mutation is 10^{-13} . Such data have not been documented for TMP and SMX, but the recorded observations of Darrell, Garrod and Waterworth¹⁶ show that the presence of a sulfonamide undoubtedly lessens the development of resistance to TMP. They found that although organisms from light inocula on ditch plates containing TMP showed little change in

sensitivity to TMP even after 25 transfers, those from heavy inocula in broth containing increasing concentrations of the drug showed, even after only five transfers, increases ranging from 32-fold to greater than 124-fold. However, when the sulfonamide was present at 10 times the concentration of TMP there were only small increases, even with large inocula, provided the organisms were sulfonamide-sensitive. Bushby examined 19 strains of varying degrees of sensitivity to SMX and found that when the organisms were exposed to both drugs the rate of emergence of TMP-resistant mutants depended upon the degree of sulfonamide resistance, the repressive effect being less with the more resistant strains.¹⁷

Although results of these experiments clearly indicate that the retarding effects of the sulfonamides on the rate of emergence of TMP-resistant mutants varies with the degree of sulfonamide resistance, the clinical significance of the resistance is doubtful, for the majority of the variants grow as abnormally small colonies, and many of them show antigenic changes associated with autoagglutination, which strongly suggests that the acquisition of TMP resistance by this means will usually be associated with loss of virulence.

Acquisition of resistance by mutation has undoubtedly lessened the value of several important antibacterials, but greater concern is now being given to resistance acquired without the organisms being exposed to the antibacterial. This resistance is transferred from a resistant organism and it can be accomplished either directly, by transference of genetic material through conjugation of the resistant and sensitive organisms, or indirectly, through infection by a bacterial virus. Transference of the genetic material, the R factor, by conjugation is common among members of the Enterobacteriaceae family and the resistance transferred may be multiple, affecting, at the same time, sensitivity to several unrelated antibacterials.

Transference of resistance by conjugation was first noted by the Japanese in 1957 and the acquisition of resistance to sulfonamides was one of the first to be recognized. Transference of TMP-resistance by this method was first recorded by Lebek¹⁸ but there is no convincing evidence that resistance to TMP-SMX through it has become widespread, at least in Britain where the combination has been available since 1968.¹⁹ However, because resistance is transferred independently of the presence of the drugs, combining TMP with SMX cannot influence the development of resistance by this means, although the use of the drug will select these resistant strains when present.

An alternative mechanism by which organisms can become resistant to TMP-SMX is by an alteration in metabolism so that the steps at which the drugs act are not involved. TMP has been used as a tool by biochemists since 1965 for selecting thymidine-dependent mutants.²⁰ These mutants are deficient in thymidylate synthetase which, in association with the specific folate coenzyme, converts uridylate to thymidylate, and they overcome this requirement by using exogenous thymidine or thymine. These organisms are therefore resistant to TMP provided there is also available in the growth medium sufficient of the other end-products of the syntheses with which TMP interferes. Similar thymidine/thymine-dependent mutants have been isolated by Barker, Healing and Hutchison from patients undergoing therapy with TMP-SMX.²¹

In practice these mutants are recognized by their ability to grow in the primary isolation medium, which contains adequate amounts of thymidine, but not on the TMP-SMX-susceptibility-testing medium which has a very low thymidine content. Barker *et al.* quoted instances from other independent workers of their having isolated similar mutants and they suggested that the occurrence of these mutants may be more common than is supposed because they are not generally recognized. However, the high efficacy of TMP-SMX with its low relapse rate clearly indicates that they cannot often be clinically significant. Obviously, since these mutants readily occur *in vitro*, under usual conditions the thymidine content in tissues, etc. must be too low

to support their growth or, alternatively, they are pathogenically "cripples" and are readily eliminated by the host, for otherwise TMP-SMX would not be an effective therapeutic combination.

Incidence of resistance to TMP and SMX

The need for using a medium with a low thymidine content for susceptibility testing to this combination is now well documented and appreciated by many microbiologists but inadequate supplies of suitable media from commercial sources still present a problem. Apart from those of a clerical nature, errors that occur in TMP-SMX susceptibility testing are almost always due to the presence of thymidine in the medium, which leads to sensitive strains being reported as resistant. There is, in consequence, a general tendency for the reported incidence of resistant strains to be an overestimation. However, in spite of this tendency, published evidence indicates that there has not been an important change in the incidence of infections by resistant strains except perhaps in hospital-acquired infections.²²⁻²⁶ The increase in these hospital strains appears to be mainly confined to members of the *Enterobacter*, *Proteus* and *Klebsiella* genera, each of which had a higher incidence of TMP-resistant strains than had other genera before the introduction of the combination. An organism that is alleged to have shown a particularly high rise in the incidence of resistant strains is *Hemophilus influenzae*.

May and Davies reported that 52% of 210 isolates of *H. influenzae* examined during 1968-72 were resistant to TMP by the disc method and that determination of the MIC of 18 of the strains showed 17 to be resistant to 10 µg or more TMP per ml.²⁷ These conclusions were based on the concentrations of TMP necessary to inhibit growth completely, although in both methods the organisms showed long trailing end-points. A more extensive examination by Bushby and Bushby on 17 of these strains confirms the observations of May and Davies but not their conclusions.²⁸ Bushby and Bushby found that (a) the organisms characterized by long trailing end-points were morphologically abnormal and mostly dead; (b) the few viable aberrant forms did not multiply when transferred to medium containing the same concentration of TMP as that in which they initially multiplied, indicating that they represent only a temporary phase of growth; (c) although the strains were not equally sensitive to the bactericidal action of TMP, five of them were in fact killed by as little as 1 µg TMP per ml; (d) in experiments in mice, these allegedly resistant strains were no more resistant *in vivo* to TMP than are the more usual ones; and, (e) serial passage in the presence of TMP did not alter the end-points of strains with either long trailing or clear-cut end-points, indicating that the former are apparently not indicative of a phase in the development of resistance to TMP. Bushby and Bushby therefore concluded that these seemingly

resistant strains are in fact sensitive to TMP.

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