Br. J. Pharmac. Chemother. (1968), 33, 72–90.

TRIMETHOPRIM, A SULPHONAMIDE POTENTIATOR

BY

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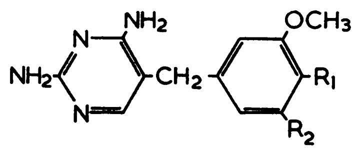
(*Received October* 18, 1967)

The sulphonamides maintain a considerable place, relatively constant in scope, in the armamentarium of agents useful in bacterial infections (Jukes & Broquist, 1963 ; Zbinden, 1964). The possibility has existed for some years that their effectiveness might be extended through their conjoint use with inhibitors of bacterial dihydrofolate reductases. This could be shown, *in vitro*, in diverse microbial growth systems (Hitchings, 1961 ; Hitchings, 1962 ; Hitchings, Burchall & Ferone, 1966a ; Hitchings, Burchall & Ferone, 1966b). It has been employed successfully in the therapy of protozoal diseases (Eyles & Coleman, 1953 ; Lux, 1954 ; Rollo, 1955 ; Frenkel & Hitchings, 1957 ; Hitchings, 1960 ; Clarke, 1962), and the present paper puts forward a possibility for its exploitation in antibacterial chemotherapy.

The inhibition of folate reductases is a general property of 2,4-diaminopyrimidines and related structures (Hitchings, Elion, Vanderwerff & Falco, 1948 ; Falco, Hitchings, Russell & Vanderwerff, 1949 ; Hitchings, Falco, Elion, Singer, Waring, Hutchison & Burchenal, 1952 ; Hitchings, 1961 ; Hitchings & Burchall, 1965). Comparisons of the activities of a variety of such substances on a spectrum of target organisms suggested that a high degree of selectivity might be obtainable in the actions of the inhibitors (Hitchings, Elion, Falco, Russell & Vanderwerff, 1950 ; Hitchings, Falco, Vanderwerff, Russell & Elion, 1952 ; Hitchings, 1964 ; Burchall & Hitchings, 1965 ; Hitchings & Burchall, 1965 ; Hitchings *et al.*, 1966a ; Hitchings *et al.*, 1966b). That such selectivity is based on specific differences in enzyme receptor sites has been substantiated by recent work with partially purified enzymes and a spectrum of inhibitors (Burchall & Hitchings, 1965).

For antibacterial use it was hoped to choose an inhibitor with a broad spectrum of antibacterial effects, a large difference in its binding to bacterial, as opposed to mammalian, reductases and suitable pharmacodynamic and pharmacokinetic properties. From several standpoints a selection among 5-benzylpyrimides (Hitchings & Bushby, 1961 ; Roth, Falco & Hitchings, 1962) seemed indicated, leaving other series, such as the quinazolines (Hitchings, Falco & Ledig, 1958) and pyrido(2,3-*d*)-pyrimidines (Robins & Hitchings, 1955 ; Robins & Hitchings, 1958 ; Hitchings, Herrman, Hurlbert & Bushby, 1964), for later selection for particular purposes.

During the years 1953–1957, brief clinical studies were carried out with three substances, B.W. 51–90 (2,4-diamino-5-(5-bromo-3,4-dimethoxybenzyl)pyrimidine), B.W. 51–2 (2,4-diamino-5-(3-methoxy-4-propoxybenzyl)pyrimidine) and B.W. 54–130 (2,4-diamino-5-(4-butoxy-3-methoxybenzyl)pyrimidine) (Fig. 1), but these produced malaise and nausea in some patients. In retrospect, it seems possible that the doses chosen for trial were unnecessarily high (1–2 g/day). Experiments in monkeys, however, demonstrated these side effects were not a constant property of the type and trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine) (Fig. 1) was essentially free of these side effects. Moreover, its antibacterial activity was somewhat higher than those of the previously investigated members of the series. This paper presents the experimental documentation of its properties.



Compound	R_1	R_2
BW 51–90	OCH_3	Br
BW 51–2	OC_3H_7	H
BW 54–130	OC_4H_9	H
Trimethoprim (BW 56–72)	OCH_3	OCH_3

Fig. 1.

METHODS

In vitro *activity*

Media

1. *Wellcome nutrient agar.* The medium is essentially an infusion of horse muscle (300 g/l.) to which is added papain-digested horse muscle to give a final concentration of total nitrogen of 1.5 g/l., 0.5% sodium chloride and 1.3% agar.

2. *Oxoid sensitivity test agar.* This is a veal infusion proteose peptone agar (Oxo Ltd.).

3. *Oxoid tryptone soya agar.* This is a pancreatic digest of casein plus soy peptone (Oxo Ltd.).

4. *Brain-heart infusion agar.* This is a mixture of infusions of calf brain and beef heart plus peptone (Baltimore Biological Laboratories).

5. *Trypticase soy agar.* This medium corresponds to Oxoid tryptone soy agar (Baltimore Biological Laboratories).

6. *Sulphonamide medium.* This medium has a low PAB-equivalent. It contains Evans peptone 0.5%, Lab-Lemco beef extract 0.3%, New Zealand agar 1.5%, in distilled water at pH 7.5. In some experiments the medium was buffered by adding 0.2% KH_2PO_4, and when lactose or urea was used as an indicator of growth, 0.3% of a 0.4% solution of phenol red was added.

7. *Phenol coefficient agar.* This is the medium used in the Rideal-Walker test; it contains 2% Lab-Lemco beef extract, 1% peptone (Oxoid L37), 1% sodium chloride and 1% "Ion agar" No. 2, in distilled water at *p*H 7.4.

8. *Peizer and Schecter medium.* This is an egg yolk-medium (Peizer & Schecter, 1950).

9. *O'Meara broth.* This is a medium used for virulence studies (O'Meara, 1931).

Where indicated, horse blood or serum was added to these media and, when heated, the blood was added at 50° C and the temperature raised to 80° C for 5 min.

Methods

The minimum inhibitory concentrations (MIC) of the drugs were determined on plates, using twenty different organisms per plate for the non-spreading organisms, and in tubes for those which swarmed. Twofold dilutions of the drugs were used. The drugs are stable when heated for 20 min at 120° C, and in these tests the medium, after the addition of the drugs, was heated at 60° C for 1 hr. The inoculum was a 1 mm loopful of a 10^{-2} dilution of an 18 hr broth culture of all the strains except those of *Streptococcus pyogenes* which were used undiluted; the inoculum was spread over 1 cm. Growth was read after incubation for 24 hr and the MIC was recorded as the highest dilution that inhibited approximately 90% of the control growth.

Potentiation in vitro

Interaction between the two drugs was detected by effects on the bacteriostatic and the bactericidal activities of the drugs.

The bacteriostatic effects were measured by the chequer-board titration method, in which tubes of media were arranged in the form of a square, with the concentration of one drug decreasing from left to right across the square end and of the other from top to bottom. Twofold dilutions of the drugs were used; the size of the inocula and the reading of the results were the same as those used for determining the MIC. The determination of fractional inhibitory concentrations (FIC) and their use in the quantification of potentiation has been described by Elion, Singer & Hitchings (1954).

The bactericidal effects were measured by exposing the organisms in Wellcome nutrient broth at 37° C to sulphamethoxazole and trimethoprim, singly and in combination, and performing viable counts at intervals up to 24 hours. The concentration of bacteria was approximately 10^5/ml. Viability was determined by making serial dilutions in quarter strength Ringer solution and placing 0.01 ml. of each dilution on nutrient agar by means of a standard loop.

Antagonism of folates

This was determined first by the method previously described (Hitchings *et al.*, 1952), using a chemically defined medium. The growth of *Lactobacillus casei* was indirectly measured by the increase in acid and that of *Strept. faecalis* turbimetrically. Second, the size of the zone of inhibited growth produced by 8 mm diameter filter paper discs impregnated by trimethoprim 0.5 μg on Wellcome nutrient agar was compared, with and without the addition of calcium leucovorin 1 μg/ml. Various organisms were used in the second method, the size of inocula being chosen so as to produce barely confluent growth.

In vivo *studies*

Mice. The Schofield strain of Swiss mice was used; they weighed 18–20 g.

Infection. Proteus vulgaris was grown on O'Meara broth for 6 hr and 0.5 ml. of the culture diluted 1:10 was injected intraperitoneally. Untreated mice died within 24 hr, and the experiments were concluded after 7 days.

Staphylococcus aureus was grown in Wellcome nutrient agar for 18 hr and the growth was suspended in 1.6% hog gastric mucin to give 10^8 organisms/ml.; the inoculum was 0.5 ml. of the suspension, injected intraperitoneally. Untreated mice died within 18 hr, and the experiments were concluded after 14 days.

Administration of drugs. The drugs were suspended in 0.5% methylcellulose and administered orally immediately after infection and 6 hr later. In the *Proteus* experiments a third dose was given on the following day, and in the *Staphylococcus* experiments treatment was continued twice daily for the next 3 days and once on the fifth day.

Estimations in biological materials

Trimethoprim

Chemical. Trimethoprim was extracted from biological materials and its concentration was determined spectrophotometrically or microbiologically. The extraction was performed as follows:

1. Solid Na_2CO_3 was added to the body fluid or tissue homogenate to give a final concentration of 2%.

2. The mixture was extracted twice with an equal volume of purified chloroform in a separatory funnel. (The chloroform was purified by extraction with one quarter its volume of concentrated sulphuric acid and washed with water until neutral. It was stored over anhydrous calcium chloride.)

3. The combined chloroform extracts were washed with 0.5 volume of water and extracted with 0.1 volume of 0.1 N hydrochloric acid.

4. The optical density of the acid extracts were measured at 270.5 mμ. The molecular extinction coefficient of trimethoprim at 270.5 mμ, pH 1.0, is 5,916.

Biological. This was a diffusion method using the sulphonamide agar medium and *Bacillus pumilus* as the test organism. Assay plates were prepared by pouring 100 ml. of the medium into 10 in.× 8 in. flat bottomed plates and, when set, covering it with 40 ml. of the medium seeded with 1.0 ml. spore suspension of the test organism. The spore suspension contained 10^7 spores/ml., and before pouring the seeded layer it was heated to 56° C for 20 min to aid germination.

Standards ranging from 0.5 to 0.005 μg/ml. were prepared in the same biological fluid as the test samples; 0.05 ml. of these standards and suitable dilutions of the test samples were placed in 6 mm diameter wells cut into the agar. The plates were incubated at 28° C, and as soon as growth was apparent the sizes of the zones of inhibition were measured. The concentrations of the test samples were read from a graph constructed from the standards and plotted on semi-log graph paper.

Sulphonamides. These were estimated in the AutoAnalyser using a modification of the Bratton and Marshall method (Bratton, Marshall, Babbit & Hendrickson, 1939).

RESULTS

Activity in vitro *of trimethoprim*

It will be seen that the antibacterial spectrum of trimethoprim is similar to that of sulphadiazine, with a few notable exceptions (Table 1). The pyrimidine is relatively ineffective against *Pseudomonas aeruginosa* and *Clostridium perfringens*, but highly effective against streptococci and selected Gram-negative bacteria.

The effectiveness of trimethoprim *in vitro* is reduced in the presence of some constituents of complex media. Thymidine and purines are among these interfering substances and others remain to be identified. The changes in sensitivity do not seem to be systematic but are different for different organisms. By way of illustration, the examples of Table 2 show the MIC obtained with thirteen species and a total of thirty strains in six different commercial media. In Wellcome nutrient agar and Oxoid sensitivity test agar, trimethoprim gave consistently higher activity than in the other media with all the organisms tested. It will be noted, however, that with the strains of *Strept. pyogenes* inhibitions were much greater with the Wellcome medium than with any of the other media whereas with *P. vulgaris* the results were more consistent from one medium to another.

Potentiation with sulphonamides, in vitro

When trimethoprim is used in combination with sulphonamides, it invariably enhances the response. This is illustrated by the data of Table 3, and has been extensively documented in the literature as a general property of combinations of dihydrofolate reductase inhibitors with sulphonamides (Elion *et al.*, 1954; Hitchings & Burchall, 1965). In terms of economy, it will be seen that there is an optimum ratio of the two drugs. At all ratios the total drug required is less with the combination than with either drug alone, and becomes minimal when, in this instance, an eighth of an MIC of one drug is combined with an eighth of an MIC of the second. The degree of potentiation is conveniently expressed as the FIC index (Elion *et al.*, 1954), which is the sum of the FIC values of the two drugs when used in the best combination. The FIC value is the minimum inhibitory concentration of the one drug, in the presence of the other, expressed as a fraction (decimal) of its minimum inhibitory concentration when it is used alone. The potentiation is represented by the difference of this value from 1.0. The FIC index in this case is thus 0.25 for the two most effective combinations. Table 4 presents similar data for a spectrum of representative micro-organisms and several sulphonamides. There is a high degree of potentiation in each case, especially with the *gonococci*, in spite of their being relatively resistant to trimethoprim.

TABLE 1

ANTIBACTERIAL ACTIVITY *IN VITRO*

The activity of trimethoprim and sulphadiazine was compared in Wellcome nutrient agar containing 7·5% horse blood, except for *Myco. tuberculosis* for which Peizer and Schecter medium was used.

Organism	Strain	Minimum inhibitory concentration (μg/ml.) Trimethoprim	Sulphadiazine
Streptococcus pyogenes	*CN*10	0·4	100 (±25)
	*CN*29	0·4	50 (±6)
	*CN*336	0·4	50
Streptococcus pneumoniae Type I	*CN*33	4	125 (±64)
Type II	*CN*36	1	32 (±16)
Type III	*CN*971	1	32
Viridans streptococci	*CN*799	0·25	8
Streptococcus faecalis (haemo.)	*CN*478	0·5	100
Streptococcus faecalis (non-haemo.)	*CN*2371	0·5	>100
Streptococcus agalactiae	*CN*1143	4	50
Staphylococcus aureus	*CN*491	0·2	4
Erysipelothrix rhusiopathiae	*CN*904	8	>100
Corynebacterium pyogenes	*CN*1856	0·4	>100
Corynebacterium diphtheriae	*CN*399	0·4	>100
Escherichia coli	*CN*312	0·2	8
Citrobacter freundii	*HS*76	0·1	3
Klebsiella pneumoniae	*CN*3632	0·5	16
Klebsiella aerogenes	*HS*84	1	>100
Enterobacter aerogenes	*HS*86	3	>100
Enterobacter cloacae	*HS*87	1	>100
Salmonella typhi	*CN*512	0·4	4
Salmonella typhimurium	*R*123	0·3	10
Salmonella enteritidis	*R*106	0·3	30
Salmonella senftenberg	*Q*186	0·1	30
Shigella sonnei	*CN*1525	0·4	4
Shigella dysenteriae	*CN*1513	0·2	2
Shigella boydii Type II	*R*20	0·3	1
Shigella flexneri	*CN*1772	0·4	1
Vibrio comma	*CN*2005	0·8	32
Pasteurella septica	*CN*1066	0·1	8
Haemophilus influenzae	*CN*1714	0·12	>50
Bordetella pertussis	*PHLS*19	1	100
Moraxella lacunata	*CN*5119	4	8 (±2)
Moraxella liquifaciens	*CN*5318	8	>125 (±32)
Proteus vulgaris	*CN*329	1	8
Proteus mirabilis	*CN*2622	1	4
Proteus rettgeri	*CN*3240	1	16
Providence B	*HS*138	1	30
Pseudomonas aeruginosa	*CN*200	>100	25
Neisseria gonorrhoeae	*CN*5019	12	1·6
	*CN*5011	6	0·8
	*CN*503	50	3·2
Neisseria meningitidis	*CN*4937	8	0·5
Neisseria catarrhalis	*CN*3209	50	>100
Clostridium perfringens	*CN*2726	50	16 (±8)
Mycobacterium tuberculosis	*H*37Rv	250	>1000

TABLE 2

INFLUENCE OF MEDIUM ON *IN VITRO* ACTIVITY OF TRIMETHOPRIM

The minimum inhibitory concentration were determined in Wellcome nutrient agar (N.A.), Oxoid sensitivity test agar (S.T.A.), Oxoid tryptone soya agar (T.S.A.), phenol coefficient agar (P.C.A.), Baltimore Biological Laboratories brain–heart infusion agar (B.H.I.B.B.L.) and trypticase soy agar (T.S.A.B.B.L.) each containing 7·5% horse blood.

Organism	Strain	Minimum inhibitory concentration (μg/ml.) N.A.	S.T.A.	T.S.A.	P.C.A.	B.H.I. B.B.L.	T.S.A. B.B.L.
Proteus vulgaris	*HS*106	1·6	0·8	12·5	12·5	3·1	6·2
	*HS*105	0·4	0·8	25	12·5	6·2	6·2
	*CN*329	1·6	0·8	50	25	3·1	12·5
Proteus mirabilis	*HS*142	0·8	1·6	100	12·5	50	6·2
	*HS*116	0·4	1·6	25	3·1	12·5	1·6
Proteus rettgeri	*HS*135	3·1	1·6	12·5	12·5	12·5	3·1
	*HS*136	6·2	6·2	50	100	50	25
Providencia sp.	*HS*140	3·1	3·1	100	100	12·5	12·5
	*HS*138	0·8	0·8	6·2	1·6	3·1	1·6
Proteus morgani	*HS*134	3·1	3·1	100	6·2	25	6·2
Escherichia coli	*HS*5	0·4	0·4	50	100	50	0·8
	*HS*35	0·2	0·2	12·5	12·5	6·2	0·4
	*HS*41	0·4	0·4	25	25	50	0·8
	*CN*314	0·1	0·2	25	100	6·2	0·2
Enterococcus	*HS*154	0·4	0·4	>100	>100	>100	>100
	*HS*157	0·2	0·4	>100	>100	>100	>100
	*HS*152	0·2	0·4	>100	>100	>100	>100
Staphylococcus aureus	*HS*142	0·2	0·4	>100	>100	25	0·4
	*HS*141	0·2	0·4	>100	>100	25	0·4
	*HS*146	0·05	0·4	12·5	100	1·6	0·2
Shigella sonnei	*HS*92	0·2	0·4	12·5	50	12·5	0·4
	*HS*93	0·2	0·2	25	50	12·5	0·4
	*HS*85	0·4	0·4	12·5	100	100	0·8
Klebsiella pneumoniae	*HS*81	0·8	0·8	>100	>100	>100	1·6
Enterobacter cloacae	*HS*88	1·6	0·8	50	100	100	3·1
Citrobacter freundii	*HS*76	0·1	0·2	100	>100	100	1·6
	*HS*79	0·4	0·4	12·5	50	6·2	0·8
Streptococcus pyogenes	*HS*167	0·2	6·2	>100	>100	12·5	100
	*HS*169	0·4	6·2	>100	>100	12·5	25
	*HS*168	0·2	6·2	>100	>100	12·5	25

TABLE 3

POTENTIATION IN COMBINATIONS OF TRIMETHOPRIM AND SULPHADIAZINE

The minimum inhibitory concentration of the drugs, singly and in various combinations were determined against *Proteus vulgaris* (*CN*329) in horse blood. The FIC index is the sum of the fractional inhibitory concentrations (FIC) of the two drugs calculated by dividing the concentration of the drug in the minimum inhibitory concentration of the combination by its minimum inhibitory concentration when acting alone.

Ratio of trimethoprim: sulphadiazine	Minimum inhibitory concentration: Trimethoprim μg/ml.	Trimethoprim FIC	Sulphadiazine μg/ml.	Sulphadiazine FIC	FIC index
—	0·32	1·00	0·00	0·00	—
1:1	0·16	0·50	0·16	0·03	0·53
1:4	0·08	0·25	0·32	0·06	0·31
1:15	0·04	0·12	0·63	0·13	0·25
1:125	0·01	0·06	1·25	0·25	0·31
—	0·00	0·00	5·00	1·00	—

TABLE 4

POTENTIATION IN COMBINATIONS OF TRIMETHOPRIM AND VARIOUS SULPHONAMIDES

The minimum inhibitory concentrations of the drugs are those present in the optimum ratio of the drugs. The buffered sulphonamide medium contained 0·2% KH_2PO_4 and 0·4% phenol red.

Organism	Strain	Sulphonamide	Medium	Minimum inhibitory concentration—in optimum combination (μg/ml.)		FIC index
				Sulphonamide	Trimethoprim	
N. gonorrhoeae	*C*61203	Sulphadiazine	Wellcome nutrient agar	100	100	0·12
	*C*61283		+ 10% horse serum	100	50	0·12
	*C*61830			25	50	0·18
	*C*61377			25	25	0·20
	*A*7505			12·5	25	0·20
	*C*798			6·2	100	0·18
	*A*3813			6·2	50	0·12
	*C*61828			3·1	100	0·12
P. vulgaris	*CN*329	Sulphadimethoxine	Buffered sulphonamide	0·25	0·5	0·25
		Sulphorthodimethoxine	medium + 2% urea;	2	0·5	0·18
		Sulphamethoxazole	pH 7·0	0·25	0·5	0·18
		Sulphadiazine		2	0·5	0·25
S. aureus	*CN*491	Sulphadimethoxine	Sulphonamide medium	8	1	0·155
		Sulphorthodimethoxine	pH 7·5	8	1	0·185
		Sulphamethoxazole		8	1	0·155
		Sulphadiazine		16	1	0·12
E. coli	*CN*314	Sulphadimethoxine	Buffered sulphonamide	8	0·12	0·36
		Sulphorthodimethoxine	medium + 1%	4	0·12	0·36
		Sulphamethoxazole	lactose pH 7·5	0·5	0·12	0·36
		Sulphadiazine		1·0	0·12	0·36
P. vulgaris	*CN*329	Sulphadimethoxine	Wellcome nutrient agar	0·5	0·5	0·375
		Sulphadiazine		2	0·5	0·26
E. coli	*CN*314	Sulphadimethoxine		16	0·125	0·36
		Sulphadiazine		2	0·125	0·25
S. aureus	*CN*491	Sulphadimethoxine		4	0·5	0·185
		Sulphadiazine		8	0·5	0·25
Sal. typhi	*CN*512	Sulphorthodimethoxine	Wellcome nutrient agar	25	0·125	0·15
N. meningitidis	*CN*4937	Sulphamethoxazole	Wellcome nutrient agar	1·2	40	0·25
			+ 10% horse serum			0·25
C. diphtheriae	*CN*399	Sulphadiazine	Wellcome nutrient broth	6·25	0·4	0·31
Strep. faecalis	*CN*2371			100	0·2	0·56
K. pneumoniae	*CN*3632			6·25	0·8	0·37
H. influenzae	*JRM*1	Sulphamethoxazole	Wellcome nutrient agar	100	1	0·02
	*JRM*16		+ 10% horse blood	30	0·1	0·16
	*JRM*12		(heated)	3	0·1	0·08
Bord. pertussis	*PHLS*111	Sulphamethoxazole	Wellcome nutrient agar	100	10	0·02
	*PHLS*25		+ 10% horse blood	30	3	0·05
	*PHLS*212		(heated)	10	10	0·11

The experiments depicted in Fig. 2 demonstrate that combinations of diaminopyrimidines and sulphonamides may be bactericidal in conditions where the individual drugs may produce only stasis. In these experiments, therapeutic concentrations of the drugs in Wellcome nutrient broth were used alone and in combination. The sulphonamide-resistant *P. vulgaris* (HS105) was highly sensitive to trimethoprim and against this organism, trimethoprim at 3 μg/ml. was bactericidal. Against the rather less trimethoprim-sensitive strains of *P. vulgaris* (HS110) and *P. mirabilis* (HS125), the combinations were bactericidal whereas the individual drugs were only bacteriostatic. The results with *Escherichia coli* (CN314) illustrate the greater rapidity of action of a combination.

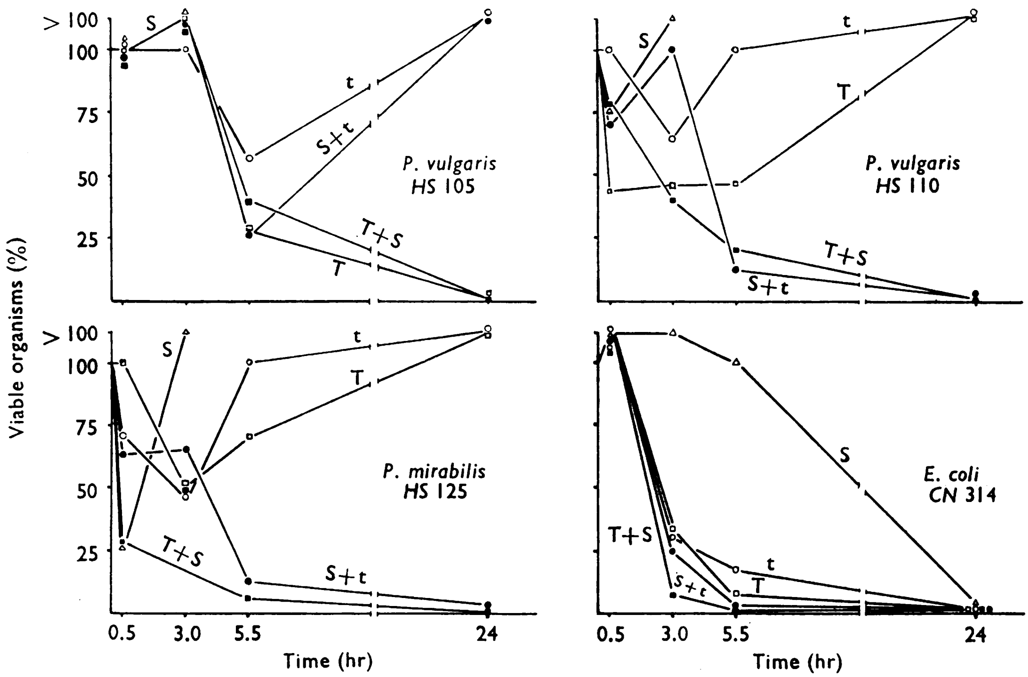


Fig. 2. Bactericidal activity of trimethoprim and sulphamethoxazole singly and in combination at 37° C. The number of viable organisms, expressed as percentage of the original inoculum of approximately 10^5/ml. present in Wellcome nutrient broth containing trimethoprim 1.5 and 3 μg/ml. and sulphamethoxazole 30 μg/ml., singly and in combination and incubated at 37° C; the viable counts were determined at times up to 24 hr. The control inoculum increased two- to three-fold during the first 30 min. ○—t—○, Trimethoprim 1.5 μg/ml.; □—T—□, trimethoprim 3 μg/ml.; △—S—△, sulphamethoxazole 30 μg/ml.; ●—S+t—●, trimethoprim 1.5 μg/ml. and sulphamethoxazole 30 μg/ml.; ■—T+S—■, trimethoprim 3 μg/ml. and sulphamethoxazole 30 μg/ml.

Antibacterial activity of trimethoprim in vivo

The chemotherapeutic activity of trimethoprim and its synergism with the sulphonamides are illustrated by the results of an experiment in mice infected with *P. vulgaris* and *S. aureus.*

In the experiment with *P. vulgaris* (Table 5), 2 mg of trimethoprim or sulphadiazine alone gave little or no protection but there was complete protection when the two were given together. When the dose of trimethoprim was reduced to 0.5 mg and that of sulphadiazine to 1.0 mg, there was still definite protection.

TABLE 5

SYNERGISTIC EFFECTS *IN VIVO* OF TRIMETHOPRIM AND SULPHADIAZINE AGAINST *P. VULGARIS*

The organism, *P. vulgaris CN*329, was grown in O'Meara broth for 6 hr and 0·5 ml. of 1:10 dilution was injected intraperitoneally into groups of six mice. The drugs were administered orally immediately after infection, 6 hr and 24 hr later. The experiment was terminated on the seventh day.

Drugs		Exp. 1 Survival		Exp. 2 Survival	
Trimethoprim (mg/mouse)	Sulphadiazine (mg/mouse)	%	Average (days)	%	Average (days)
5·0	–	67	4·7	50	3·2
2·0	–	0	<1·0	0	<1·0
2·0	2·0	100	7·0	100	7·0
1·0	1·0	83	6·0	100	7·0
0·5	2·0	100	7·0	100	7·0
0·5	1·0	83	6·0	83·3	6·0
0·2	1·0	67	5·0	33·3	3·0
—	2·0	0	<1·0	16·6	1·5
—	—	0	<1·0	0	<1·0

In experiments with *S. aureus*, similar synergistic effects were shown (Table 6). The strain was highly virulent for mice and in both experiments the untreated animals died within 24 hours. Complete eradication of the infection is not often achieved with penicillin because many of the mice that survive the 14 days have abdominal abcesses; in no instance was complete eradication achieved with trimethoprim and sulphadiazine although there was very definite potentiation controlling the early septicaemic stage of the infection.

TABLE 6

SYNERGISTIC EFFECTS *IN VIVO* OF TRIMETHOPRIM AND SULPHADIAZINE AGAINST *S. AUREUS*

The organism, *S. aureus CN*491, was grown on nutrient agar and suspended in 1·6% mucin to give 3×10^8 organisms/ml.; the inoculum was 0·5 ml. and was injected intraperitoneally into groups of ten mice. The sulphadiazine and trimethoprim were given orally and the penicillin subcutaneously, immediately after infection 6 hr later and then twice daily for 3 days and once on the fourth day. The experiment was terminated on the fourteenth day.

Drugs			Exp. 1 Survival		Exp. 2 Survival	
Trimethoprim (mg/mouse)	Sulphadiazine (mg/mouse)	Benzylpenicillin (units/mouse)	%	Average (days)	%	Average (days)
5·0	—	—	16·6	4·4	10·0	2·1
2·0	2·0	—	70·0	11·9	40·0	11·1
0·5	2·0	—	40·0	12·9	60·0	12·3
—	2·0	—	—	3·8	0	5·6
—	—	5000	—	—	100·0	14·0
—	—	1000	100·0	14·0	70·0	11·7
—	—	100	70·0	12·4	0	7·4
—	—	25	30·0	7·6	10·0	2·8
—	—	—	0	<1·0	0	<1·0

Binding to plasma proteins

The binding of trimethoprim to human plasma proteins was determined by the equilibrium dialysis method of Anton (1960). The dialysis sac containing 3 ml. of human plasma was incubated with mechanical stirring for 18 hr at 37° C with 10 ml. of a solution of trimethoprim (50 μg, total) which was 0.15M with respect to NaCl and 0.01M with respect to phosphate buffer at pH 7.0. The contents of the two compartments were analysed separately by the spectrophotometric method described above. The concentration in the exterior compartment was trimethoprim 3.52 μg/ml.; in the plasma-containing compartment 5.15 μg/ml. Thus at this concentration 31% of the trimethoprim was bound to plasma proteins.

Mechanism of action

The antimetabolic effects of trimethoprim are shown by the data of Tables 7 and 8. In these experiments, *L. casei* and *Strept. faecalis* were grown in the presence of factorial increments of both the drug and either folic acid or leucovorin. Reversal of the inhibition by increasing the concentration of either vitamin occurred with *L. casei* over a 25-fold range of concentration and the inhibition index (ratio of drug to metabolite at 50% inhibition) remained approximately constant (100 to 400) over this range (Hitchings, Elion, Falco, Russell, Sherwood & Vanderwerff, 1950).

TABLE 7

ANTIMETABOLIC EFFECTS OF TRIMETHOPRIM: RESPONSE OF *LACTOBACILLUS CASEI*

Growth is expressed as ml. of 0·1 N acid per 10 ml. produced during incubation.

Trimethoprim (μg/ml.)	Acid production (ml. of 0·1 N acid/10 ml. of medium)											
	Folic acid (mμg/ml.)						Leucovorin (mμg/ml.)					
	0·025	0·125	0·625	3·125	15	75	0·025	0·125	0·625	3·125	15	75
0	9·6	11·3	11·7	13·6	12·5	12·7	9·8	9·9	10·9	13·2	12·6	12·7
0·05	7·4	6·2	10·5	12·9	12·1	12·6	4·5	6·1	7·9	11·6	12·6	12·6
0·25	1·7	2·3	3·8	10·2	10·8	12·6	1·8	1·8	2·7	7·6	10·8	12·6
1·2	0·3	0·7	0·8	1·4	4·6	11·1	0·5	0·6	0·8	1·5	4·6	11·1
6·0	0·3	0·4	0·5	0·5	1·2	3·8	0·3	0·5	0·6	0·8	1·2	3·8
30·0	0·2	0·3	0·5	0·5	0·7	0·6	0·3	0·4	0·4	0·4	0·7	0·6

TABLE 8

ANTIMETABOLIC EFFECTS OF TRIMETHOPRIM RESPONSE OF *STREP. FAECALIS*

Growth is expressed in turbidity units.

Trimethoprim (μg/ml.)	Turbidity units									
	Folic acid (mμg/ml.)					Leucovorin (mμg/ml.)				
	0·1	0·5	2·5	12·5	62·5	0·1	0·5	2·5	12·5	62·5
0	6	44	86	92	92	15	52	88	93	93
0·002	4	22	86	92	92	10	50	88	93	93
0·01	3	8	31	85	90	5	37	84	93	92
0·05	4	3	3	10	23	2	16	62	92	92
0·25	2	2	3	2	2	2	2	17	74	92
1·20						2	3	2	2	8

TABLE 9

EFFECT OF LEUCOVORIN CALCIUM ON THE ACTIVITY OF TRIMETHOPRIM

Size of zone of inhibited growth of various bacterial species produced in Wellcome nutrient agar by 8 mm filter-paper disk impregnated with 0·5 μg of trimethoprim, with and without the addition of leucovorin calcium 1 μg/ml. of medium. The zones were measured from the edge of the filter-paper and the measurements given in brackets indicate partial inhibition.

* 2·5 μg trimethoprim disks were used for these strains because no zones of inhibition were obtained with the 0.5 μg disks.

Organism	Strain No.	Size of zone (mm) Leucovorin nil		Size of zone (mm) Leucovorin 1 μg/ml.		Reversal
Escherichia coli	*HS*22	8		8		–
	*HS*24	9		9		–
	*HS*31	8		8		–
Escherichia coli var. communis	*HS*70	6		6		–
	*HS*71	(4)		(4)		–
Escherichia coli intermedia	*HS*72	7		7		–
	*HS*73	7		7		–
Alkalescens-dispar	*HS*75	6		6		–
Citrobacter freundii	*HS*77	9		9		–
	*HS*78	7		7		–
Klebsiella pneumoniae	*HS*81	3	(4)	3	(4)	–
	*HS*82*	5	(10)	5	(10)	–
Klebsiella aerogenes	*HS*83	8		8		–
	*HS*85	5		5		–
Enterobacter aerogenes	*HS*86	(3)		(3)		–
Enterobacter cloacae	*HS*87	6	(8)	6	(8)	–
	*HS*88	(2)		(2)		–
Acinetobacter anitratus	*HS*89	(1)		(1)		–
Shigella sonnei	*HS*90	8		8		–
	*HS*93	7		7		–
Proteus vulgaris	*HS*105	7	(8)	7	(8)	–
	*HS*106	(3)		(3)		–
	*HS*110	4		4		–
Proteus mirabilis	*HS*116	4	(6)	4	(6)	–
	*HS*121	6		6		–
	*HS*125	6		6		–
Proteus morgani	*HS*134*	4		4		–
Proteus rettgeri	*HS*135*	2		2		–
	*HS*136*	(6)		(6)		–
Providencia sp.	*HS*138	(3)		(3)		–
	*HS*139*	4		4		–
	*HS*140*	3		3		–
Staphylococcus aureus	*HS*141	6		6		–
	*HS*143	6		6		–
	*HS*144	5		5		–
Staphylococcus epidermis	*HS*149	5		5		–
Enterococci	*HS*150	11		5		+
	*HS*151	12		8		+
	*HS*152	11		3	(4)	+
	*HS*153	9		6		+
	*HS*154	5	(7)	2	(3)	+
	*HS*155	7		2		+
	*HS*156	7		3		+
	*HS*157	9		3		+
	*HS*158	8		3		+
	*HS*159	6		(2)		+
	*HS*160	5		(2)		+
	*HS*161	6		(2)		+
	*HS*162	8		1	(2)	+
	*HS*163	8		2		+
	*HS*164	6		1		+
	*HS*165	7		2		+
	*HS*166	6		3		+
Streptococcus pyogenes	*HS*167	7	(8)	7	(8)	–
	*HS*169	6		6		–
	*HS*170	7		7		–
Streptococcus Group C	*HS*171	8		8		–

With *Strept. faecalis* competition with folate occurred over only a narrow range with an inhibition index of approximately 2. Reversal of leucovorin was of the by-pass type usually observed with inhibitors of dihydrofolate reductase—that is, levels of drug that produced strong inhibition when folic acid was the vitamin were completely ineffective when the tetrahydrofolate (leucovorin) was offered. Nevertheless, at the higher levels of drug, competition of trimethoprim and leucovorin was evident, with inhibition indices of the order of 50 (compare Hitchings, Elion & Singer, 1954; Wood, Geltzer & Hitchings, 1960).

Similarly, in experiments with *Pediococcus cerevisae* (not shown) at very high levels of drug (3 to 45 mμg/ml.) inhibitions were obtained that could be reversed with leucovorin.

In general, micro-organisms which synthesize folates are unable to utilize exogenous folates (Frenkel & Hitchings, 1957; Hitchings & Burchall, 1965). It was therefore important to document the effect of trimethoprim on pertinent pathogenic bacteria in the presence and absence of a source of tetrahydrofolates. Among fifteen strains tested (Table 9), only the enterococci responded to exogenous leucovorin.

Absorption, distribution and clearance in the mouse

When trimethoprim was given orally to mice, absorption was rapid; peak blood values were reached within 15 min. A rapid decrease was followed by a prolonged period of slowly falling blood levels and significant amounts of the drug were present in the blood 18 hr after dosing (Fig. 3). The data in Table 10 suggest some concentration of trimethoprim in the tissues, because uniform distribution in the body water of 7 mg of compound in a mouse weighing 20 g would result in blood concentrations some 10–15-fold higher than those actually found. The data in Table 10 show that the concentration of drug in various tissues and in urine is consistently several-fold higher than that in blood.

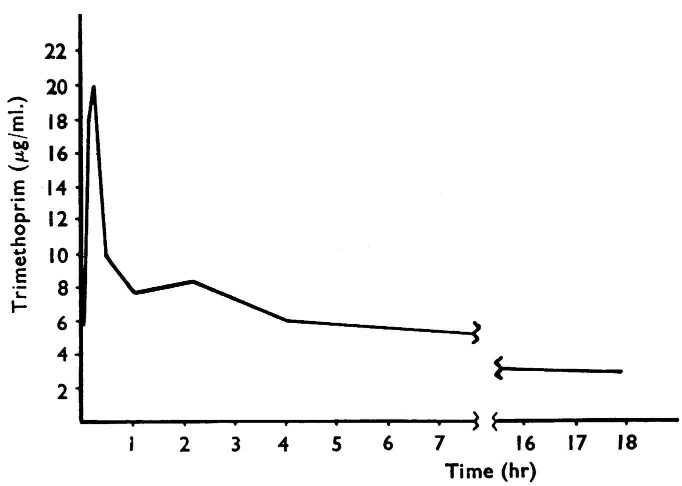


Fig. 3. Concentration of trimethoprim in blood of mice. Trimethoprim was administered orally to mice. Groups of three mice were killed at intervals and the concentration of the drug in the blood was determined. The dose was 7 mg./20 g body weight.

TABLE 10

RELATIVE CONCENTRATIONS OF TRIMETHOPRIM IN VARIOUS TISSUES OF MICE

Trimethoprim was administered orally to mice at a dose of 7 mg/20 g body weight and the blood from three mice was pooled. The results are expressed as the ratio of the concentration in the tissue to that of the blood which is given the value of unity for each time interval.

	Relative concentration of trimethoprim Time after administration (min)		
Tissue	15	60	180
Blood	1·0	1·0	1·0
Urine	8·6	4·3	9·0
Kidney	10·9	7·0	10·0
Spleen	3·5	3·8	6·3
Lungs	10·7	17·5	17·5
Liver	2·1	3·2	2·8
Heart	4·9	6·5	11·9

Absorption and excretion in man

Experiments in four human subjects were performed with oral doses of trimethoprim 250 mg and sulphafurazole 1 g per subject. The data for serum levels are presented in Fig. 4 and they show that trimethoprim is cleared more slowly than sulphafurazole. Significant levels of trimethoprim were present in the blood within 1 hr, were maintained over a period of 7 hr, and detectable amounts were still present at 24 hr. These data suggest a half-life for trimethoprim of 13.25 hr. Concentrations approximately 100-fold higher than those of serum were present in the urine and at 24 hr they were still at levels that would be expected to be highly antibacterial (Table 11).

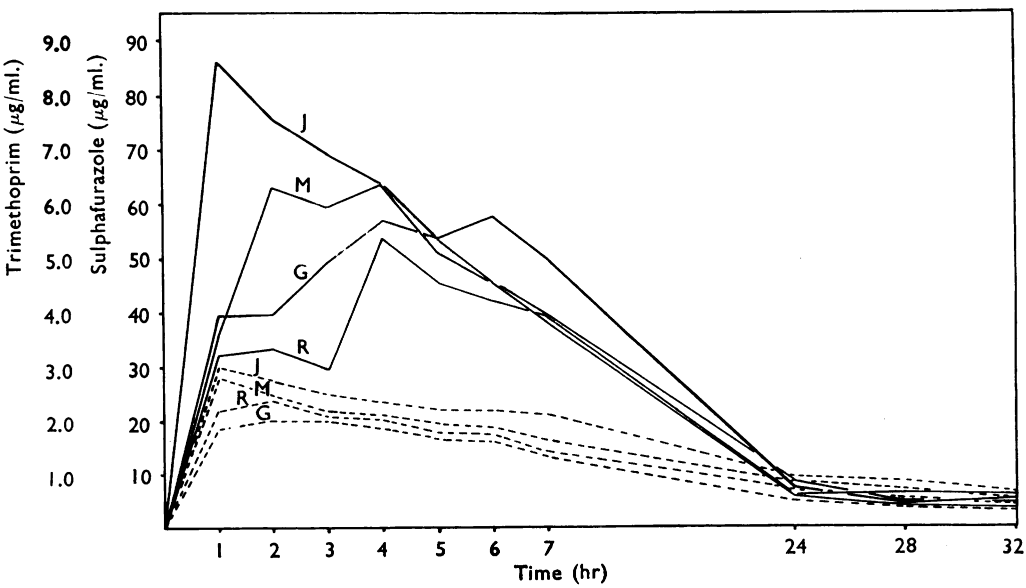


Fig. 4. Concentration of trimethoprim and sulphafurazole in serum of man. Four adult subjects, identified by letters, were given 250 mg of trimethoprim and 1 g of sulphafurazole, orally, and serum samples were analysed for both drugs at intervals up to 32 hr after administration. - - - -, Trimethoprim levels ; ——, sulphafurazole levels.

TABLE 11

CONCENTRATION AND QUANTITY OF TRIMETHOPRIM AND SULPHAFURAZOLE EXCRETED IN URINE OF MAN

Four adult males were given trimethoprim 250 mg and sulphafurazole 1 g orally and the concentrations of the drugs in the urine were determined at intervals up to 32 hr. The volumes of the urine excreted were recorded and the quantities of drug excreted were calculated. The trimethoprim was estimated biologically.

Subject	Time after dosing (hr)	Urine volume	Trimethoprim Concentration (μg/ml.)	Trimethoprim Total (mg)		Sulphafurazole Concentration (μg/ml.)	Sulphafurazole Total (mg)	
	0–3	150	115·4	17·3		421·5	63	
	3–7	230	81·3	18·7		696·5	160	
1	7–24	600	92·2	55·3	114·6	276·5	166	423
	24	75	81·0	6·1		96·5	7	
	24–32	310	55·0	17·2		86·5	27	
	0–3	405	52·8	21·3		383·5	236	
	3–7	210	111·3	23·4		648·5	136	
2	7–24	720	107·2	77·2	142·9	163·5	117	528
	24	86	46·2	4·0		88·5	8	
	24–32	425	40·0	17·0		73·5	31	
	0–3	215	69·2	14·9		140·5	30	
	3–7	315	69·5	21·9		415·5	131	
3	7–24	840	111·5	93·5	147·7	205·5	173	391
	24	85	46·4	3·9		105·5	9	
	24–32	860	15·7	13·5		515·5	48	
	0–3	160	110·1	17·6		411·0	66	
	3–7	200	114·8	23·0		606·0	121	
4	7–24	710	88·0	62·5	125·4	271·0	192	410
	24	23	83·5	1·9		136·0	3	
	24–32	330	61·9	20·4		81·0	28	

The plasma values and clearances of trimethoprim were determined in six human subjects after intravenous administration of the drug (Table 12). The apparent volume of distribution of the drug, based on 5 min plasma values, ranged from 47 to 92 l. indicating a rapid redistribution and concentration in the tissues. The 24 hr clearances ranged from 42.3 to 75.3% of the dose in the urine, and significant plasma levels were still present at 24 hr. On the assumption that the apparent volume of distribution remained unchanged, the residual trimethoprim could be calculated. These values ranged from 14.5 to 33.2 mg and when added to the 24 hr recoveries gave values for total trimethoprim accounted for in the range 68–90% of the dose. The mean biological half-life in these subjects was 11.6 hr.

TABLE 12

CLEARANCE OF TRIMETHOPRIM AFTER INTRAVENOUS ADMINISTRATION TO MAN

Trimethoprim, 100 mg, as the lactate was injected intravenously into six human adult volunteers. Blood and urine samples were taken at intervals and the trimethoprim concentrations present were assayed microbiologically. The plasma levels at 4·5 hr were estimated by interpolation between 3 and 24 hr values, and these were used to calculate approximate clearance of the drug during the 1–8 hr collection period.

Subject	Concentration (μg/ml. plasma) at 5 min	3 hr	4·5 hr	24 hr	Urinary excretion 1–8 hr (mg)	24 hr (mg)	Clearance (ml./min)
A	1·30	0·88	0·83	0·37	14·9	51·5	45
W	1·23	1·07	0·94	0·19	21·1	60·2	55
Bat	1·09	1·01	0·94	0·36	14·7	42·3	40
J	2·15	1·50	1·37	0·50	13·8	44·9	25
G	1·38	0·80	0·73	0·20	18·1	54·6	60
Bar	1·16	0·97	0·85	0·17	25·8	75·3	70

Toxicity

Trimethoprim has been submitted to extensive toxicity testing in animals and the detailed results will be published elsewhere.

In mice, the acute oral LD50 is greater than 2,000 mg/kg and it is not affected by concomitant administration of equal doses of sulphadiazine. Trimethoprim does not possess powerful pharmacodynamic activity; the acute intravenous LD50 for mice is about 200 mg/kg and in the anaesthetized cat the dose has to be 100 mg/kg to produce respiratory arrest and a precipitous fall in the blood pressure. Concentrations of 10 μg/ml. depressed the response of isolated smooth muscle preparations to acetylcholine, 5-hydroxytryptamine and histamine but the response of rabbit intestine to adrenaline was not affected by this concentration. No mydriatic, analgesic or sedative action was detected with doses up to 5 mg/kg subcutaneously in mice, and no important effects on sympathetic or parasympathetic functions were observed in the anaesthetized cat.

In chronic toxicity tests the only adverse effects detected were depression and maturation defects of haemopoiesis, reflected by falls in the concentration of haemoglobin and the number of red cells, neutrophils, lymphocytes and platelets. These effects were seen in dogs dosed 6 days a week with 135 mg/kg, and in one of six monkeys dosed daily with 300 mg/kg. Although there were no important changes in the peripheral blood in monkeys dosed with 100 mg/kg daily for 6 months the marrow showed minor maturation defects. No toxic effects were detected in dogs dosed 6 days a week with 45 mg/kg for 3 months or in monkeys dosed daily with 50 mg/kg for 6 months; in rats dosed daily with 300 mg/kg for 1 month there were only minor changes in the marrow.

When trimethoprim was given in combination with sulphafurazole daily for 6 months, one of six monkeys developed neutropenia and lymphopenia and died after treatment for 50 days with daily doses of trimethoprim 100 mg/kg and sulphafurazole 400 mg/kg, but no toxic effects were seen with doses of trimethoprim 33 mg/kg and sulphafurazole 133 mg/kg. No effects on the peripheral blood were observed in rats during the 6 months treatment with trimethoprim 100 mg/kg plus sulphafurazole 400 mg/kg, but with doses of trimethoprim 300 mg/kg plus sulphafurazole 1,200 mg/kg, there was evidence of depression of haemopoiesis.

Early foetal toxicity studies by Thiersch (1963) had shown trimethoprim to be inactive in rats at a dose of 200 mg/kg. Additional studies by our colleague Dr. P. J. Fraser confirmed these results, but showed trimethoprim to produce changes characteristic of folate antagonists when the dose was increased to 300 mg/kg (given on days 8–16 of pregnancy) in rats fed on a standard diet. The teratogenic malformations consisted of various combinations of cleft palate, beaking of snout, short or curly tail, limb malformations, hare lip, oedema, hernia and exencephaly. Dr. Fraser observed no teratogenic action in rabbits with the maximum dose that was non-toxic to the doe or that caused no foetal death; this dose was 50 mg/kg, and doses up to 500 mg/kg caused no malformation. Feeding the rats on a rabbit diet or administering folinic acid orally considerably reduced the incidence of malformation.

DISCUSSION

Trimethoprim is put forward for trial as a potentiator of sulphonamides in the therapy of bacterial infections. It exhibits a spectrum of antibacterial effects of considerable breadth; this activity has been confirmed with 384 strains, freshly isolated from patients (Bushby & Barnett, 1967). It has been used alone as an antibacterial agent (Schneider, Schwarzenberg, Cattan, Schlumberger, Amiel & Mathé, 1965) but the combinations offer a number of advantages. There is an extension of chemotherapeutic effectiveness to include organisms such as *Proteus, Bordetella, Haemophilus* and *Neisseria* species which show borderline sensitivities to the individual drugs. The greater potency of the combinations may be regarded as greater effectiveness at the higher dose levels, or as a reduction in potential side-effects made possible by diminishing the doses. The drug combinations are less vulnerable than the individual drugs to the development of resistant strains, and are bactericidal at levels where the individual drugs produce bacteriostasis; the latter effect was also observed by Garrod & Waterworth (1967). The role of trimethoprim as a sulphonamide potentiator supports the view that its primary locus of action is the inhibition of the bacterial dihydrofolate reductases. It thus acts as an inhibitor of folate utilization whereas the sulphonamides inhibit the biosynthesis of the vitamin in the target organisms.

The mechanism of action, deduced from its effects on organisms which require folates, has been confirmed by recent studies with the isolated dihydrofolate reductases of several bacterial and mammalian species (Burchall & Hitchings, 1965). The development of trimethoprim supports the theory expressed a number of years ago (Hitchings *et al.*, 1952) that specific cell receptors vary sufficiently, from species to species, to provide a basis for the selective toxicity required of a chemotherapeutic agent. A retrospective analysis of the evolution of trimethoprim from 2,4-diamino-5-benzylpyrimidine (Hitchings *et al.*, 1966a) shows that the succession of molecular modifications resulted in progressively greater binding to the target enzyme (dihydrofolate reductase) of bacteria, with no significant change in the binding to the corresponding mammalian (rat liver) reductase (Hitchings *et al.*, 1966a). The selectivity of trimethoprim in this respect is striking, the ratio of the binding constants to the two enzymes being of the order of 10^5 (Burchall & Hitchings, 1965; Hitchings *et al.*, 1966a).

The possibility that other loci of action are involved in the mammalian toxicology where binding to the reductase is minimal cannot be excluded. Additional loci of action, possibly competition with one or more cofactors containing tetrahydrofolate, are suggested by the experiments with *Streptococcus faecalis* where, at high levels of the drug, inhibition competitive with leucovorin was exhibited. Nevertheless, it has been possible by means of leucovorin to reverse the indications of minimal folate deficiency that may appear in human subjects after long term dosage at high levels (Kahn & Brodsky, 1967). Moreover, such reversal can be carried out without affecting the therapeutic activity of the drug because most pathogenic bacteria resemble protozoa (Frenkel & Hitchings, 1957) in that they are unable to utilize preformed folates either *in vitro* (Wood & Hitchings, 1959) or *in vivo* (Hitchings & Burchall, 1965).

Trimethoprim has been shown to be readily absorbed, after oral administration, and to be concentrated in tissues such as the lung. The plasma level and excretion data suggest rapid absorption by the oral route followed by rapid redistribution into the tissues. This redistribution was brought out more clearly when the drug was given intravenously. The initial values obtained 5 min after injection were somewhat lower than those calculated for equal distribution into total body water and they were about 10% of those that would have been expected had the trimethoprim been retained in the blood. The drug is bound to plasma proteins, and this is reflected in clearance values of the order of 50 ml. of plasma/min and a biological half-life of the order of 12–15 hr. Its tissue and excretory distribution patterns and antibacterial spectrum suggest that it should be especially useful in the treatment of infections of the urinary and respiratory tracts.

Preliminary reports of clinical trials of combinations of trimethoprim and sulphonamide are now beginning to appear (Noall, Sewards & Waterworth, 1962 ; Cooper & Wald, 1964 ; Czonka, 1967 ; Drew, Fowle, Hughes & Cassell, 1967 ; Drew, Hughes & Jenkins, 1967 ; Sourander & Werner, 1967). These seem fully to substantiate the predictions with regard to effectiveness and tolerance that were drawn from the laboratory data.

SUMMARY

1. Trimethoprim is a sulphonamide potentiator of likely value for the therapy of bacterial infections. It has a broad antibacterial spectrum that extends that of the sulphonamides to organisms such as *Proteus* and *Bordetella* species that exhibit borderline sensitivities to sulphonamides alone.

2. Trimethoprim has only feeble pharmacodynamic activities.

3. It is readily absorbed after oral administration.

4. It shows moderately high binding to serum proteins, and concentration in organs and tissues to levels several times those of plasma.

5. It has a half-clearance time of about 14 hr and is concentrated in the urine.

6. These properties suggest trial of the combinations in a variety of bacterial diseases but especially in infections of the respiratory and urinary tracts. The mechanism and locus of action of trimethoprim are discussed.

The authors are indebted to the many colleagues who have participated in various phases of the work which is summarized here. These include M. Barnett, J. J. Burchall, G. B. Elion, R. Ferone, A. Green, T. Herrmann, A. Ianotti, B. Roth and S. Singer.

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