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Inhibition by pyrimidine analogues of the synthesis of folic acid by trachoma agents

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INTRODUCTION

The *Chlamydia* may be divided into two groups by their reaction to sulphonamides (Moulder, 1964). One group comprises agents like psittacosis and meningopneumonitis which are resistant to sulphonamides and which utilize an exogenous source of folic acid. The other group comprises agents like those of mouse pneumonitis, lymphogranuloma venereum and trachoma whose growth is inhibited by sulphonamides, indicating that they synthesize their own folic acid.

Trimethoprim, a derivative of 2,4-diaminopyrimidine, inhibits the growth of many species of bacteria (Bushby & Hitchings, 1968; Hitchings & Burchall, 1965; Bushby & Barnett, 1967). It is effective *in vitro* and *in vivo* and has been used therapeutically (Noall, Sewards & Waterworth, 1962; Cooper & Wald, 1964; Drew, Hughes & Jenkins, 1967; Drew, Hughes, Fowle & Cassell, 1967; Czonka, 1967). This compound, which competes with folic acid, inhibits dihydrofolate reductase. In combination with sulphonamides it has a strong potentiating action, as a consequence of the sequential blockade of the biochemical pathway that leads to the synthesis of coenzyme F.

Since the trachoma agent apparently synthesizes folic acid, we tested its susceptibility to trimethoprim and to the related 2,4-diaminopyrimidine derivative pyrimethamine, alone and in conjunction with a sulphonamide, and we present evidence for the existence of a dihydrofolate reductase in the trachoma agent.

METHODS

Trachoma and inclusion conjunctivitis (TRIC) agents

TRIC agents are named according to the system proposed by Gear, Gordon, Jones & Bell (1963). The abbreviations used in this paper are given in brackets. Fast-killing variants are suffixed *f* (Taverne, Blyth & Reeve, 1964*a*).

TRIC//China/Peking-2/OT (T'ang, Chang, Huang & Wang, 1957) (PK-2) and its variant PK-2*f* (T'ang).

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The fast-killing variant (HAR-2*f*) of strain TRIC//SAU/HAR-2/OT (Murray *et al.* 1960).

TRIC//GB/MRC-4/ON (Jones, 1961) (MRC-4).

TRIC//WAG/MRC-1/OT (Collier & Sowa, 1958) (MRC-1).

TRIC//WAG/MRC-062/OT (Taverne, Blyth & Reeve, 1964*b*) (MRC-062).

Strains were grown in yolk sacs of 7-day chick embryos kept at 35° C. Suspensions were made and stored, and titrations were done as previously described (Taverne *et al.* 1964*a*). Dilutions were made in phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954) without streptomycin.

Reagents

Trimethoprim lactate [2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine] was dissolved in distilled water and sterilized by autoclaving.

Pyrimethamine [2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine] was dissolved in distilled water by the addition of a few drops of isethionic acid and was sterilized by autoclaving.

Leucovorin calcium (Lederle) [5-formyl-5,6,7,8-tetrahydrofolic acid] was dissolved in PBS and sterilized by filtration.

Sulphafurazole was dissolved in distilled water with the aid of a few drops of 0·1 N-NaOH and was sterilized by autoclaving.

These compounds were tested at various concentrations, prepared from stock solutions (usually containing 2 mg./ml.) by making serial dilutions in distilled water, PBS, or in a given suspension of a trachoma agent.

Tests for inhibition

In cell culture

HeLa cell monolayers in Leighton tubes were inoculated with 1 ml. of medium 199 containing a concentration of strain HAR-2*f* calculated to form about ten inclusions per microscope field, together with various concentrations of inhibitor or inhibitors. Cultures were incubated at 37° C. for 48 hr., fixed and stained with Giemsa or iodine for inclusion counts (Furness, Graham & Reeve, 1960). The number of inclusions formed in the presence of inhibitor was expressed as a percentage of those in the controls inoculated with HAR-2*f* only.

In chick embryos

Groups of 7-day chick embryos were inoculated with a single dilution of a suspension of a trachoma agent containing about 10^5 LD 50, calculated to kill the embryo in 5–7 days, and with the same dilution containing various concentrations of the compound to be tested. Control tests of the toxicity of the compounds for chick embryos in the absence of the trachoma agent were also included.

Volumes of 0·5 ml. were inoculated into the yolk sac and the eggs were incubated at 35° C. in an atmosphere of 65 % relative humidity. Eggs were candled daily until tests were terminated on the 13th or 14th day after inoculation. The specificity of death, when doubtful, was checked by the examination of yolk sac smears stained with Giemsa for the presence of elementary bodies. The action of the

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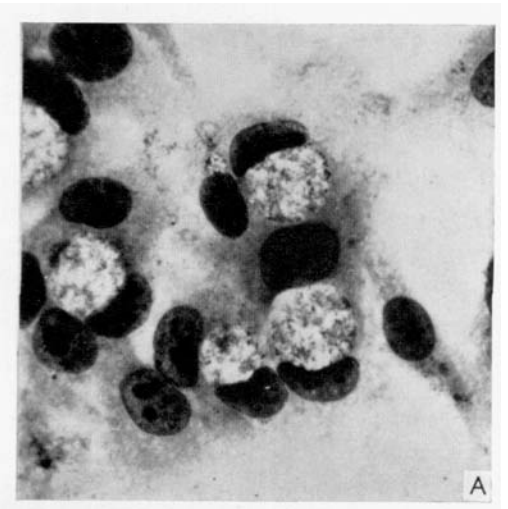

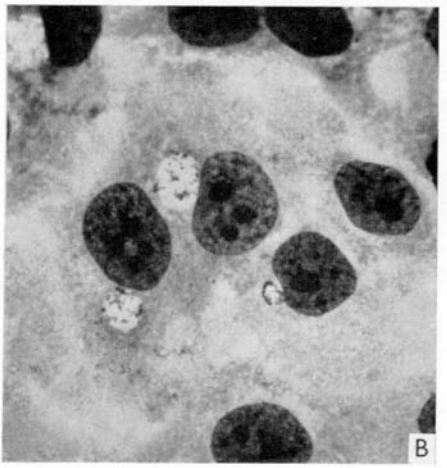

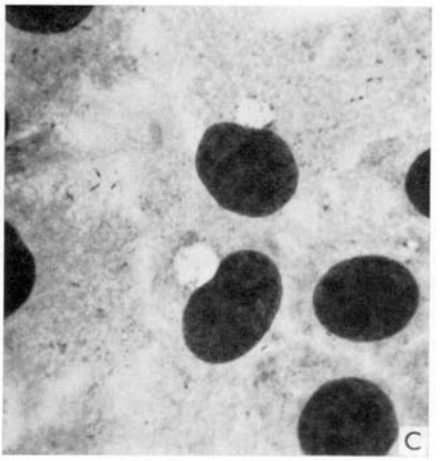

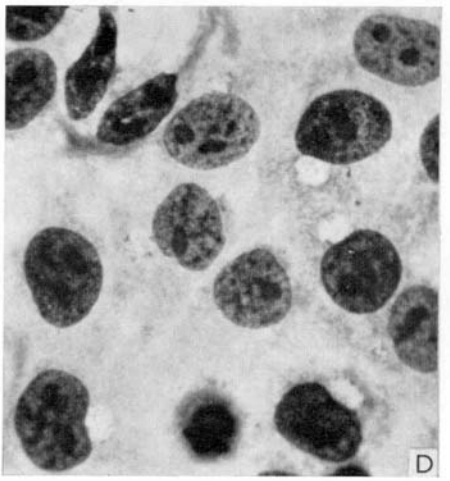

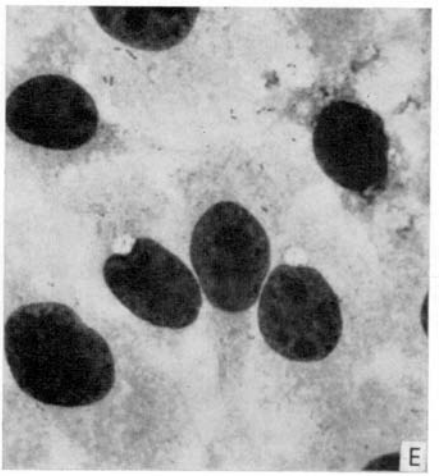

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compounds on the growth of the trachoma agent was determined in two ways: first, in terms of the proportion of embryos surviving infection in the presence of different concentrations of inhibitor, and secondly, in terms of prolongation of the mean survival time of groups of embryos compared with that of the control group receiving trachoma agent only (Lin & Moulder, 1966).

RESULTS

Inhibition of the trachoma agent by trimethoprim and sulphafurazole in cell culture

Concentrations higher than 10 μg./ml. of sulphafurazole or of trimethoprim decreased the number of inclusions formed by strain HAR-2*f* in HeLa cell monolayers by more than 50% (Table 1). Concentrations of trimethoprim greater than

Table 1. *Inhibition by sulphafurazole and trimethoprim of strain HAR-2f grown in HeLa cells*

	Inhibition* (%)	
μg./ml.	Sulpha-furazole	Trimetho-prim
500	59·5	N.D.
100	N.D.	73·6†
50	53·7	N.D.
10	45·0	55·9
2·5	33·0	15·0

* The number of inclusions in the test cells expressed as a percentage of the number in the control.

† Cells in poor condition, probably because of the toxic effect of the drug.

N.D. = Not done.

100 μg./ml. were toxic for the cells. Over a given range, the percentage inhibition was roughly proportional to the concentration of inhibitor present. It was, however, difficult to obtain reliable inclusion counts. The total number of inclusions did not change very much over a wide range of drug concentrations, but the appearance of the inclusions varied greatly. Both drugs caused similar gross changes in the morphology of the inclusions (Plate 1), and the extent of the change depended on concentration. With higher concentrations, inclusions consisted of small, empty vacuoles which, nevertheless, indented the cell nucleus; with intermediate concentrations, the vacuoles were bigger but contained only a few elementary bodies; at lower concentrations their morphology appeared to be almost normal. However, the change with drug concentration in the size and content of

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EXPLANATION OF PLATE

Changes in the morphology of inclusions formed by the HAR-2*f* strain of trachoma in HeLa cells in the presence of different amounts of trimethoprim, × 640. A = Control, in absence of inhibitor. B = 0·1 μg. per ml. trimethoprim. C = 1·0 μg. per ml. trimethoprim. D = 10 μg. per ml. trimethoprim. E = 100 μg. per ml. trimethoprim.

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the inclusions was so gradual that it was not possible to decide on the end-point of this effect.

On the assumption that inhibition of multiplication took place at an early stage in the growth cycle, before the synthesis of the glycogen matrix, an attempt was made to estimate the effect of the drugs by staining inclusions with iodine 48 hr. after infection. However, although inclusions were abnormal when stained with Giemsa in the presence of concentrations of sulphafurazole ranging from 10 to 0·25 μg./ml., they still stained with iodine.

Inhibition tests on combinations of various concentrations of sulphafurazole and trimethoprim showed that at certain concentrations the combined effect of the two drugs was at the least additive (Fig. 1). As with each drug alone, inclusion counts of the same order were obtained over a wide range of concentrations although the appearance of the inclusions changed; but since this change had no precise end-point, it could not be taken into account in assessing the degree of potentiation.

It was clear from the absence of elementary bodies in inclusions stained with Giemsa that both drugs inhibited the formation of infective agent. To measure the suppression of multiplication we investigated their action in the egg, technically a more convenient system.

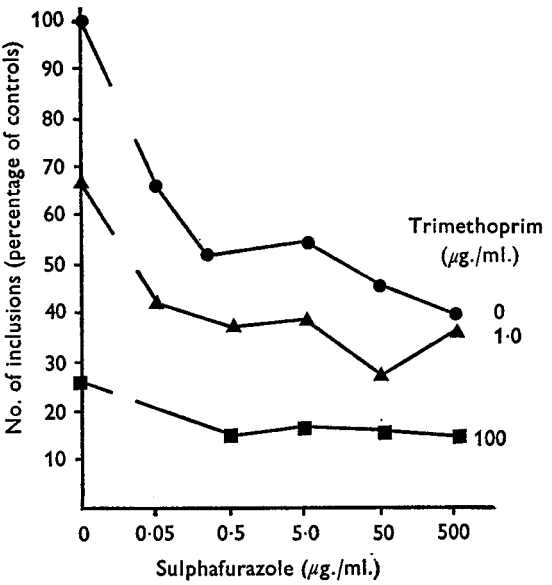

Fig. 1. The effect of two concentrations of trimethoprim on the numbers of inclusions formed by HAR-2*f* in HeLa cells in the presence of various concentrations of sulphafurazole. ●, No trimethoprim; ▲, 1·0 μg./ml. trimethoprim; ■, 100 μg./ml. trimethoprim.

Inhibition of trachoma agents by trimethoprim and sulphafurazole in chick embryos

Trimethoprim inhibited the growth of three slow-killing strains (MRC-4, MRC-1 and PK-2) and the one fast-killing strain of TRIC agent tested (T'ang), but on a weight basis it was about 50 times less active than sulphafurazole. Even at a concentration of 5000 μg. per egg it failed to inhibit one strain, MRC-062,

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although the strain was as susceptible to sulphafurazole as other strains. When the increase in mean survival time of groups of infected embryos was plotted against the concentration of inhibitor they received, expressed on a logarithmic scale, a direct relationship was observed (Fig. 2). Doses of sulphafurazole greater than about 300 μg. per embryo prolonged survival until most of the embryos began to hatch. With trimethoprim, the mean survival time was never sufficiently prolonged for the embryos to hatch; at doses above about 5000 μg. the embryos died sooner than controls given no trimethoprim, presumably because of the toxicity of the drug. The percentages of chick embryos infected with a lethal dose of MRC-4 and T'ang surviving infection in the presence of various concentrations of sulphafurazole or trimethoprim are given in Table 2.

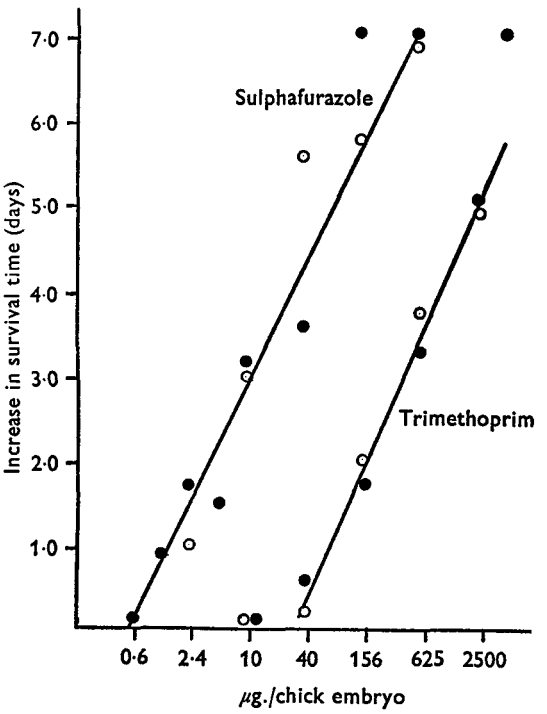

Fig. 2. Effect of sulphafurazole and trimethoprim on the survival of chick embryos inoculated with strain T'ang, ○, or strain MRC-4, ●. Chick embryos hatched if the survival time was extended beyond about 7 days.

Tests for potentiation of the activity of sulphafurazole by trimethoprim

Groups of embryos were inoculated with a lethal dose of either MRC-4 or T'ang, together with concentrations of sulphafurazole and trimethoprim in the range in which there was a linear relationship between concentration and prolongation of mean survival time.

From the average day of death (Tables 3 and 4) prolongation of mean survival time was determined. For each strain, the increase in mean survival time was plotted in terms of the effect of concentrations of trimethoprim on the activity of sulphafurazole. As an example, results obtained with strain MRC-4 are given

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Table 2. *Percentage survival of chick embryos infected with strains MRC-4 or T'ang in the presence of graded amounts of sulphafurazole or trimethoprim*

Dose (μg.) per chick embryo	Sulphafurazole		Trimethoprim	
	MRC-4*	T'ang*	MRC-4*	T'ang
0	0	0	0	0
0·7	0	0	N.D.	N.D.
2·5	11	0	0	N.D.
10	65	16	0	0
40	80	45	0	12
156	100	78	0	0
625	100	94	29	28·5
2,500	100	90	84	84
5,000	N.D.	N.D.	100	N.D.
10,000	N.D.	N.D.	Toxic for chick embryos	

* Mean values for two tests.
N.D. = not done.

Table 3. *The average day of death of groups of chick embryos infected with strain T'ang in the presence of graded amounts of sulphafurazole and trimethoprim*

Trimethoprim per chick embryo (μg.)	Sulphafurazole per chick embryo (μg.)					
	0	0·7	2·5	10	40	150
0	5·0	4·5	6·3	8·8	9·7	10·0
10	5·1	4·7	6·8	8·6	11·0	11·0
40	5·1	5·9	8·2	10·6	10·4	9·1
150	6·6	7·3	9·6	8·7	8·7	11·0
625	8·0	9·6	10·5	9·1	9·1	8·5

Table 4. *The average day of death of groups of chick embryos infected with strain MRC-4 in the presence of graded amounts of sulphafurazole and trimethoprim*

Trimethoprim per chick embryo (μg.)	Sulphafurazole per chick embryo (μg.)				
	0	0·7	2·5	10	40
0	6·8	6·9	8·5	10·0	> 14
10	7·4	6·9	8·5	10·0	> 14
40	7·0	7·1	8·4	10·3	> 14
150	8·5	9·6	10·5	13·7	> 14
625	9·8	11·2	9·6	> 14	> 14

(Fig. 3). From the lines fitted to these plots, the minimum concentration of sulphafurazole extending the mean survival time by an arbitrary period of 3 days in the presence of graded concentrations of trimethoprim was read off; the minimum effective concentration of the drugs acting alone was similarly estimated from Fig. 2

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(Table 5). (The selection of such an arbitrary period is valid since the lines in Fig. 2 are parallel.) These values (cols. 1 and 2) were expressed as percentages of the minimum effective concentration of each drug acting alone (cols. 3 and 4). For example, 9·8 μg. of sulphafurazole prolonged survival of embryos infected with the

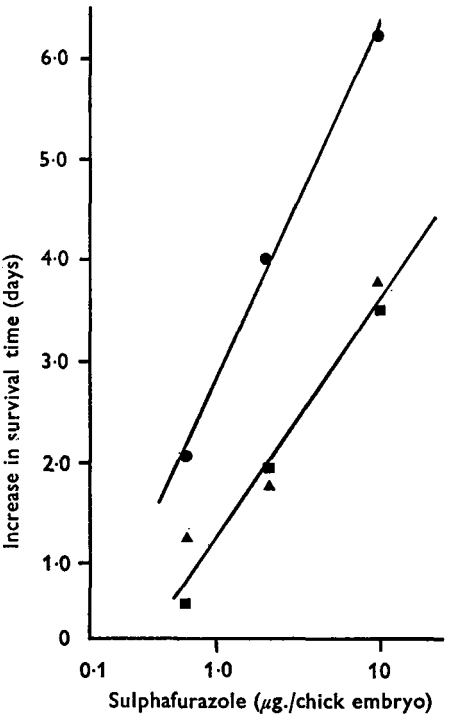

Fig. 3. The prolongation of mean survival time of chick embryos inoculated with MRC-4 and various combinations of sulphafurazole and trimethoprim. ● = 150 μg. trimethoprim; ▲ = 40 μg. trimethoprim; ■ = 10 μg. trimethoprim.

Table 5. *Combinations of trimethoprim and sulphafurazole prolonging the survival of chick embryos infected with strain T'ang or MRC-4 by 3 days*

Strain	Tri-methoprim μg./embryo	Sulpha-furazole μg./embryo	Percentage concentration effective in absence of the other drug: Tri-methoprim	Sulpha-furazole	Sum of percentages
T'ang	0	9·8	0	100	100
	10	9·8	2	100	> 100
	40	7·2	8	74	82
	150	3·2	30	33	66
	500	0	100	0	100
MRC-4	0	9·8	0	100	100
	10	8·0	2	80	82
	40	8·0	8	80	88
	150	1·6	30	17	47
	500	0	100	0	100

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T'ang strain for 3 days in the absence of trimethoprim; in the presence of 150 μg. of trimethoprim only 3·2 μg. (33 % of 9·8) was required. Potentiation is assumed to have occurred when the sum of the pairs of percentages for each combination of drugs is less than 100. Only with 150 μg. of trimethoprim was the sum of these percentages significantly less than 100, indicating potentiation, which was, however, not very great.

Reversal of trimethoprim inhibition by calcium leucovorin

Trimethoprim could inhibit the multiplication of TRIC agents, by blocking a reaction essential to a synthetic process outside the folic acid cycle. If it acts solely by blocking the reduction of folic acid to citrovorum factor, addition of this factor should reverse the inhibition.

Various concentrations of calcium leucovorin were inoculated into the yolk sacs of groups of chick embryos infected 1 hr. previously with a lethal concentration of the T'ang strain and graded concentrations of trimethoprim. Calcium leucovorin

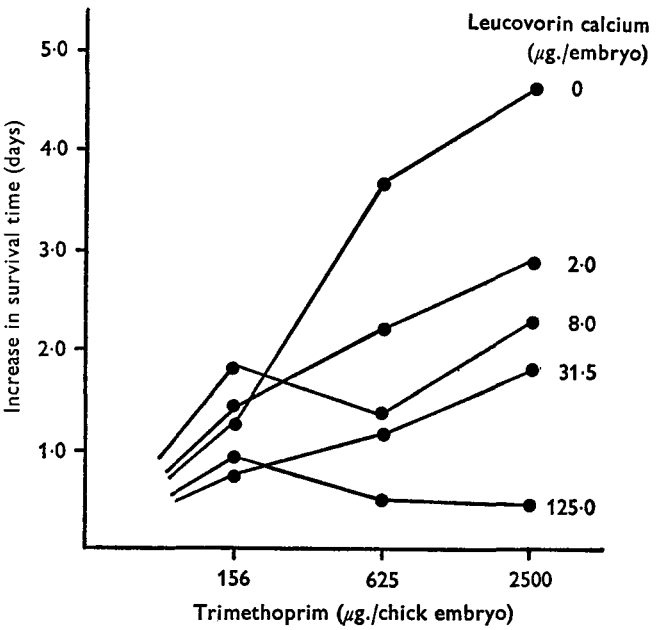

Fig. 4. The effect of graded concentrations of calcium leucovorin on the inhibitory action of trimethoprim against the T'ang strain of trachoma multiplying in the chick embryo yolk sac.

itself was not toxic and did not influence the multiplication of the T'ang strain. The prolongation of survival time of the embryos so treated compared with controls infected with T'ang and given calcium leucovorin only was plotted against log concentration of trimethoprim (Fig. 4). The inhibition by all concentrations of trimethoprim tested did not occur in the presence of 125 μg. per embryo of calcium leucovorin. All concentrations of leucovorin tested reversed the protection obtained with trimethoprim to some extent, in proportion to concentration.

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Inhibition by pyrimethamine

The susceptibility of the trachoma agent to another inhibitor of folate synthesis was tested in groups of chick embryos inoculated with a lethal dose of the T'ang strain or MRC-4 and given various concentrations of the antimalarial compound pyrimethamine. Concentrations of 500 μg. per embryo or more were toxic, killing nearly all the embryos within 2–3 days of administration. In the range 100–500 μg. per embryo, pyrimethamine had little effect on the mean survival time of embryos infected with MRC-4, but significantly prolonged that of those infected with the T'ang strain. The prolongation in survival time was proportional to dose, expressed logarithmically, but the relation could be expressed only over a small range of concentrations of the drug because of its toxicity (Figs. 5, 6). In one experiment, groups of at least thirty eggs were inoculated with a lethal dose of strain MRC-4 or T'ang, alone or with 250 μg. of pyrimethamine per embryo, and the difference

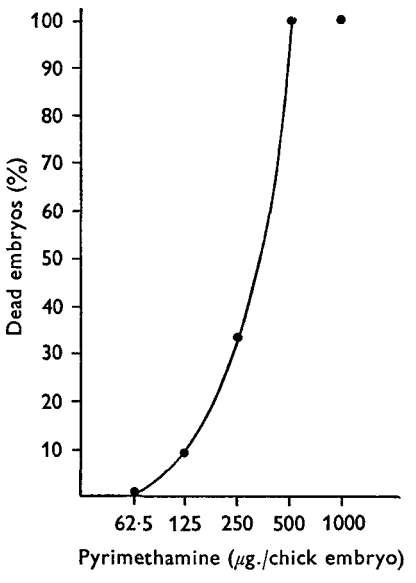

Fig. 5

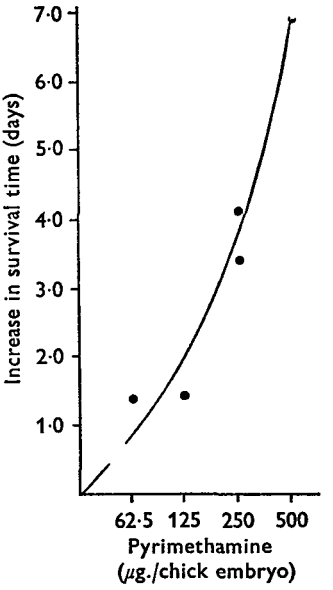

Fig. 6

Fig. 5. The toxic effect of pyrimethamine on the chick embryo. Deaths were counted within 6 days of inoculation.

Fig. 6. The prolongation of mean survival time of chick embryos infected with the T'ang strain in the presence of graded concentrations of pyrimethamine.

in mean survival time was analysed statistically. With strain T'ang the mean survival time was prolonged 4·1 days and this difference was highly significant ($P = < 0{\cdot}01$); with strain MRC-4 the prolongation was 1·4 days, which was only just significant ($P = 0{\cdot}04$).

DISCUSSION

Trimethoprim inhibited the growth of several strains of trachoma agent and the one strain tested of inclusion conjunctivitis. Although there were some differences in the susceptibility of various strains to both pyrimidine analogues, no clear

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pattern emerged. Resistance to one was not necessarily accompanied by resistance to the other; for instance, strain MRC-4 was inhibited by trimethoprim but not by pyrimethamine, and preliminary experiments indicated that strain MRC-062, which was resistant to trimethoprim, was sensitive to pyrimethamine. Again, sensitivity did not appear to be related to virulence for the chick embryo. Thus, although the more virulent strain T'ang was inhibited by pyrimethamine whereas the less virulent strain MRC-4 was not, tests on two other less virulent strains indicated that PK-2 was sensitive whereas MRC-1/OT apparently was not.

In eggs, inhibition of growth of the trachoma agent by trimethoprim was most clearly demonstrable in terms of the prolongation of mean survival time of groups of embryos inoculated with a given dose of agent and various concentrations of drug. There was no such clear relation between dose and the proportion of embryos protected against death; only the higher concentrations of trimethoprim protected embryos and these concentrations were themselves toxic for the embryos.

Cell cultures did not provide a useful alternative test system because trimethoprim changed the morphology of inclusions and made counting difficult. Furthermore, only fast-killing variant strains can easily be titrated in cell culture. Since they differ in many respects from freshly isolated strains and may differ in their sensitivity to drugs, it was considered important to use a method of titration applicable to all strains.

The trachoma agent multiplies in mouse lungs (Graham, 1965) but in mice trimethoprim did not inhibit infection by either the slow-killing strain MRC-1 or the variant HAR-2*f* (S. R. M. Bushby, and M. Barnett, unpublished results) nor did it potentiate the activity of sulphafurazole. However, even concentrations as high as 2·0 mg. of sulphafurazole per mouse, the highest dose tested, protected only 80 % of the mice; the same dose per unit body weight protected nearly 100 % of chick embryos. Because doses of agent sufficient to kill by infection all the mice inoculated often kill by direct toxicity, the mouse lung test was considered to be unsatisfactory.

Since chlamydia sensitive to sulphonamides are known to synthesize folic acid (Moulder, 1964), it was not unreasonable to suppose that trimethoprim inhibited the multiplication of the trachoma agent by preventing the utilization of folic acid. Since the action of the drugs appeared to be reversed by calcium leucovorin, we conclude that these agents most probably contain a dihydrofolic acid reductase. Burchall & Hitchings (1965) demonstrated the binding *in vitro* of 2,4-diamino pyrimidines, including trimethoprim, to dihydrofolate reductases isolated from *Escherichia coli*, *Staphylococcus aureus* and *Proteus vulgaris*. There was a strong correlation between the degree of binding by a particular reductase and the capacity of the drug to inhibit the micro-organism from which it was derived. A lesser degree of binding was exhibited by reductase isolated from mammalian cells. Thus the therapeutic value of trimethoprim depends on its differential action on enzymes of parasite and host.

Although in our system trimethoprim was toxic for the host, whether chick embryo or HeLa cells, inhibition of the trachoma agent was demonstrable by concentrations of inhibitor that were not toxic for the host. The closeness of the

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minimum effective therapeutic dose to the toxic dose suggests that the difference between the binding of trimethoprim to reductases of these agents and to the reductases of the chick embryo was less than the differences between the binding of the enzymes of susceptible bacteria and mammalian tissues. Although the pyrimidine somewhat enhanced the activity of a sulphonamide its effect was slight compared with potentiation against bacteria and susceptible protozoa (Clarke, 1962; Eyles & Coleman, 1953; Rollo, 1955). The low degree of potentiation may be due to poor and incomplete binding of the reductases of the trachoma agent, so that some enzyme is free to deal with folate that has escaped the blocking effects of the sulphonamide.

The structure of the antimalarial compound pyrimethamine resembles that of trimethoprim, and it acts against the malarial parasite by inhibiting the utilization of folic acid. Although weight for weight it was more active against the trachoma agent than trimethoprim, it was considerably more toxic for the chick embryos and its action was demonstrable only over about an eightfold range of concentration. Evidence that antimalarial drugs are active against ophthalmic trachoma has not yet been sought, but the laboratory findings suggest that it is potentially able to affect the disease in the field, either alone or synergistically with other drugs. This factor should be considered when evaluating the incidence of trachoma or planning vaccine or drug trials in areas where malaria is endemic and diaminopyrimidines are in general use. It may be profitable to investigate the activity against trachoma of other folic acid antagonists of this type.

SUMMARY

Trimethoprim, a 2,4-diaminopyrimidine derivative which inhibits the growth of some bacteria by interfering with folic acid synthesis, inhibited the growth of several strains of the trachoma agent. Inhibition was most clearly demonstrated by measuring prolongation of mean death time of groups of chick embryos inoculated with a single lethal dose of agent. Over a certain range, prolongation was proportional to the logarithm of concentration of inhibitor; higher concentrations were toxic for the embryo. On a weight basis, trimethoprim was not as active as sulphafurazole. Inoculation in conjunction with sulphafurazole resulted in slight potentiation of activity. A related pyrimidine derivative, the antimalarial drug pyrimethamine, also significantly inhibited the growth of one strain of trachoma.

In cell culture, trimethoprim decreased the number of inclusions formed by a suspension of the trachoma agent and induced morphological changes in the inclusions similar to those caused by sulphafurazole.

Inhibition of the growth of the trachoma agent in the chick embryo was reversed by leucovorin calcium. It is concluded that, as with bacteria, the drug acts by blocking the folic acid cycle and that the trachoma agent most probably contains a dihydrofolate reductase.

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