while tumor growths of 0 to 3 mm occurred in 25 mice. All the mice were tested for the capacity of their spleen cells to absorb the cytotoxic activity of an antiserum, designated D-28, which is reactive with cells of the H-2^a haplotype (strain A) but not with cells of H-2^k mice (C3Hf) (*8*, *9*). The results are depicted in Table 1. Spleen cells from [(C3Hf × A)F_1 × C3Hf] backcross mice permissive for tumor growth absorbed cytotoxicity from the D-28 antiserum and therefore these mice must have inherited the H-2^a haplotype. Spleen cells from tumor resistant [(C3Hf × A)F_1 × C3Hf] backcross mice failed to reduce significantly the cytotoxicity of the D-28 antiserum and therefore these mice must not have inherited the H-2^a haplotype. These data indicate that the differential growth of lung tumor 85 in C3Hf and (C3Hf × A)F_1 hybrid mice is due to a single genetic locus and that the locus is linked to the locus controlling the expression of the H-2^a haplotype in strain A mice.

To test further the linkage of the TASA to the H-2 complex, C3Hf mice were immunized with lung tissue from B10 mice (H-2^b) and B10.A mice (H-2^a) prior to x-irradiation and challenge with 10^5 cells from lung tumor 85. As shown in Table 2, radioresistant immunity to lung tumor 85 was induced by lung tissue from B10.A mice, but not by lung tissue of the congenic B10 mice. In similar experiments, (C3Hf × DBA/2)F_1 hybrid mice were immunized with normal tissue from either B10.A (2R) or B10.A (5R) recombinant mice and tested for radioresistant immunity to lung tumor 85. The 2R recombinant expresses the K region of the H-2^a haplotype while the 5R expresses the D region of the H-2^a haplotype (*9*). As shown in Table 2, immunity to lung tumor 85 was achieved by immunization with tissue from the 2R but not the 5R recombinant strain. This result indicates that the gene coding for the alloantigen expressed on tumor cell 85 is linked to the K end of the H-2 histocompatibility complex. Since the locus controlling the expression of the TL alloantigen (a thymus leukemia–associated antigen) is linked to the D end of the H-2 complex (*9*), the lung tumor–associated–antigen is not coded for by a TL-linked gene. Similarly, the type C viral alloantigen G_{IX} can be excluded, since in those strains in which the G_{IX} locus appears to be linked to H-2, it has been localized distal to the D end of the H-2 locus (*10*). The K ends of the H-2^a and H-2^k haplotypes are thought to code for identical serologically defined alloantigens (*9*, *11*). The data reported here indicate that important differences do exist in the region of the K end of the H-2 complex of A and C3Hf mice and emphasize the importance of genes which are linked to, or are part of, the H-2 complex in coding for TASA on tumors susceptible to immune surveillance (*12*).

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11. To date we have detected neither cytotoxic antibodies nor cytotoxic T lymphocytes specific for the lung tumor–associated alloantigen. The nature of this alloantigen and the mechanism whereby the lung tumor expresses this alloantigen have yet to be determined.
12. Interest in modified H-2 coded cell surface antigens as targets for cell-mediated immunity against virus-infected and neoplastic cells has recently been suggested by the studies of R. M. Zinkernagel and P. C. Doherty [*J. Exp. Med.* **141**, 1427 (1975)] and of J. W. Schrader and G. M. Edelman [*J. Exp. Med.* **143**, 601 (1976)].

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Antibacterial Synergism: A Proposal for Chemotherapeutic Potentiation Between Trimethoprim and Sulfamethoxazole

Abstract. *Sulfamethoxazole and other sulfa drugs are moderately potent inhibitors of* Escherichia coli *dihydrofolate reductase. They also significantly potentiate the inhibition of this enzyme by trimethoprim. The molecular basis for inhibition potentiation is the simultaneous binding of trimethoprim and sulfa by the enzyme. This potentiation may explain the synergism observed when these drugs are used in antibacterial chemotherapy.*

When used together in chemotherapy, sulfonamides and certain diaminopyrimidine derivatives exhibit mutual potentiating effects, or synergism (*1*). Their combined effectiveness far exceeds mere addition of their individual efficacy. One such combination (trimethoprim plus sulfamethoxazole) is in widespread current use (*2*). Diaminopyrimidines, such as trimethoprim and pyrimethamine, inhibit dihydrofolate reductase (E.C. 1.5.1.3) by competing with dihydrofolate (*3*). Sulfonamides, like sulfamethoxazole, are inhibitors of the enzyme dihydropteroate synthetase (E.C. 2.5.1.5) (*4*); they compete with the substrate *p*-aminobenzoate (*5*). Potter's (*6*) theory of "sequential inhibition" often is invoked (*2*, *7–9*) to explain the mutual potentiation between sulfonamides and diaminopyrimidines, but Webb (*10*) has pointed out that the sequential blockade of linear reactions by multiple inhibitors in the steady state is theoretically incapable of producing an effect greater than that by a single inhibitor alone, an effect confirmed experimentally by Rubin *et al.* (*11*). Furthermore, the theory of "sequential inhibition" offers no explanation for the fact that some dihydrofolate reductase inhibitors, such as 2,4-diaminopteroylaspartate, are not synergistic with sulfonamides (*9*), or that the potentiation of trimethoprim by sulfonamides still occurs in a number of sulfonamide-resistant organisms (*12*). Hitchings summarized (*13*) arguments for and against the theory, and concluded that "sequential inhibition" was not yet disproved.

Recent observations in this laboratory that sulfonamides can be moderately potent inhibitors of bacterial dihydrofolate reductase (*14*) provide the basis for a new theory of potentiation. This new hypothesis is based on multiple simultaneous inhibition of bacterial dihydrofolate reductase by the sulfonamides and their potentiators acting together. I present data to show that the qualitative nature of synergistic dose-response curves can be duplicated in vitro by the action of two inhibitors on a single enzyme. The

theory of multiple simultaneous inhibition explains why some dihydrofolate reductase inhibitors do not potentiate sulfonamides, in that only inhibitors that bind in the dihydrofolate site in a manner that permits the simultaneous binding of sulfonamides would exhibit appreciable chemotherapeutic synergism. Without simultaneous binding, only the addition of inhibitor potencies would be expected. The theory of synergism also provides a possible explanation for the degree of synergism that is retained with sulfonamides in certain sulfonamide-resistant organisms (*12*), in that the primary site of action of a sulfonamide when used as a synergistic agent with 2,4-diaminopyrimidines is different from when the sulfonamide is used alone.

Sulfonamides inhibit dihydrofolate reductase from the bacterium *Escherichia coli* with inhibition constants in the range of 10^{-4} to $10^{-2}M$. Table 1 shows the inhibitor potencies, K_i, for eight sulfonamides, sulfinilic acid, and one sulfone, all of which are inhibitors of purified (*15*) dihydrofolate reductase from *E. coli* MB 1428. These compounds are competitive inhibitors of dihydrofolate. From the data of Brown (*4*) and Richey and Brown (*5*), the K_i values in Table 1 were calculated for six of these inhibitors of *E. coli* dihydropteroate synthetase (*16*). Comparison of the inhibition constants for the two enzymes shows that the sulfonamides have a smaller K_i for dihydropteroate synthetase, corresponding to more potent inhibition, but that there is appreciable inhibitory potency for dihydrofolate reductase. Diaminopyrimidines are considerably more potent in inhibition of dihydrofolate reductase; the K_i for trimethoprim is $0.25 \times 10^{-9}M$ (*14*).

In Fig. 1, the solid line is a theoretical curve calculated from Webb (*17, 18*),

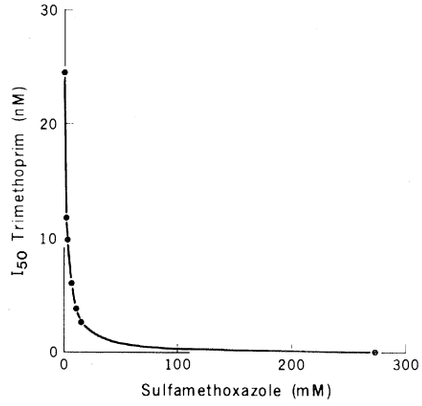

Fig. 1. The concentration of trimethoprim required for 50 percent inhibition of *E. coli* MB 1428 dihydrofolate reductase at various concentrations of sulfamethoxazole. The assays were done spectrophotometrically at 25°C in 0.05*M* tris-HCl (0.05*M* NaCl; *p*H 7.20) (see *15*). The enzyme was incubated for 5 minutes with inhibitors, and then assayed by the simultaneous addition of 90 μM reduced nicotinamide adenine dinucleotide phosphate and 154 μM dihydrofolate. Trimethoprim concentrations were corrected for enzyme-bound inhibitors. The point for zero trimethoprim was extrapolated from data at lower dihydrofolate concentrations. The solid line is a theoretical curve calculated as described in the text.

where the dihydrofolate concentration was 154 μM, the Michaelis constant (K_s) for dihydrofolate was 1.7 μM, and K_i^1, K_i^2, the inhibitory constants for trimethoprim and sulfamethoxazole, were $2.5 \times 10^{-10}M$ and $2.0 \times 10^{-3}M$, respectively. The exact shape of inhibition curves such as these depends upon the proportion of inhibition chosen and upon the dihydrofolate concentration used. In all cases, however, the curves are convex toward the axes, which means that significantly less of the two inhibitors in combination are required to give a specified level of inhibition than when the inhibitors are used separately. The maximum degree of mutual potentiation in Fig. 1 was about eightfold. It should be noted that this convexity is due to the fact that both inhibitors can be simultaneously bound. One inhibitor may or may not affect the interaction of the other inhibitor with the enzyme. It is important for the observation of pronounced synergism that concentrations of dihydrofolate considerably higher than its K_s be used. Such high concentrations are clearly possible. The pool of reduced folates in *E. coli* is approximately 2 to $5 \times 10^{-4}M$ (*19*). Therapeutic levels of potent dihydrofolate reductase inhibitors when incubated with cells lead to conversion of most of the reduced folates to dihydrofolate, as noted by Jackson and Harrap for L-1210 cells treated with methotrexate (*20*).

The third column in Table 1 gives the synergism constants K_i^2 of the sulfa drugs when used in combination with pyrimethamine ($K_i = 1.3 \times 10^{-8}M$) for inhibition of the *E. coli* reductase. All the drugs except sulfaguanidine potentiated pyrimethamine's inhibitory effects although sulfapyridine and sulfinilic acid were not quite as potent in synergism as in inhibition, and sulfisoxazole and sulfanilamide were slightly more potent in synergism. The criterion for synergism was the occurrence of a convex dose-response curve similar to that in Fig. 1 when two drugs were used in combination. The synergism constant K_i^2 was determined from the degree of convexity. The thermodynamic interpretation of the constant K_i^2 is that it is the affinity of the enzyme-pyrimethamine complex.

Chemotherapeutic data for experimentally observed synergism between sulfadiazine and pyrimethamine (*21*), as well as for other synergistic combinations (*2, 5, 7, 9, 12*), are similar to the curve in Fig. 1. When used in combination, considerably smaller amounts (four to ten times) of each drug are required to give a desired effect than when either drug is used alone (*2, 21*). The theoretical curve in Fig. 1 would not be expected to be completely analogous to synergism data obtained experimentally, for at low or zero concentrations of the diaminopyrimidines the sulfonamide would react primarily with dihydropteroate synthetase rather than synergistically with dihydrofolate reductase. The sulfonamide axis would thus be reached somewhat more quickly than in the theoretical reductase inhibition curve. Very high concentrations of sulfonamide are required to observe this synergism. For example, to obtain a twofold potentiation of pyri-

Table 1. Inhibition constants of sulfonamide for *E. coli* dihydrofolate reductase and *E. coli* dihydropteroate synthetase.

Compound	K_i (*M*)		K_i^2†
	Dihydropteroate synthetase*	Dihydrofolate reductase	
Sulfaquinoxaline		1.3×10^{-4}	$1.7 \pm 0.4 \times 10^{-4}$
4,4′-Diaminodiphenyl-sulfone		1.8×10^{-4}	$3.0 \pm 1.1 \times 10^{-4}$
Sulfamerazine	4.0×10^{-6}	4.0×10^{-4}	$6.3 \pm 2.2 \times 10^{-4}$
Sulfapyridine	1.3×10^{-5}	5.7×10^{-4}	$1.7 \pm 1.2 \times 10^{-3}$
Sulfathiazole	2.3×10^{-6}	7.4×10^{-4}	$9.6 \pm 4.8 \times 10^{-4}$
Sulfamethoxazole		2.0×10^{-3}	$2.3 \pm 0.3 \times 10^{-3}$
Sulfinilic acid	8.3×10^{-6}	3.5×10^{-3}	$6.5 \pm 0.8 \times 10^{-3}$
Sulfaguanidine	1.7×10^{-5}	3.9×10^{-3}	5.6×10^{-1}
Sulfisoxazole		6.45×10^{-3}	$3.0 \pm 0.3 \times 10^{-3}$
Sulfanilamide	6.7×10^{-5}	2.4×10^{-2}	$1.3 \pm 0.7 \times 10^{-2}$

*Calculated from data of Brown (*4*) as described in text (*16*). †Inhibition constant for *E. coli* dihydrofolate reductase when used in combination with pyrimethamine, as measured from the convexity of a dose-response curve such as that in Fig. 1, according to the equation of Webb (*17*). This constant is the affinity of the enzyme-pyrimethamine complex.

methamine by sulfamethoxazole for inhibition of the *E. coli* dihydrofolate reductase, a 2 m*M* concentration of sulfamethoxazole is required. High concentrations of sulfamethoxazole, for example, mean values of 1.2 m*M* free and 2.0 m*M* total, are attained in the urines of patients treated with normal doses of trimethoprim plus sulfamethoxazole (*22*), although concentrations in the serum are lower, near 0.2 m*M* (*23*).

Direct experimental confirmation of the ability of *E. coli* MB 1428 dihydrofolate reductase to bind simultaneously pyrimethamine and 4,4′-diaminodiphenylsulfone (dapsone) has been made by an ultraviolet difference spectrum titration (*24*).

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Cholinergic Stimulation of the Rat Hypothalamus: Effects on Liver Glycogen Synthesis

Abstract. *Cholinergic stimulation of the lateral hypothalamic neurons with intrahypothalamic microinjections of acetylcholine or carbachol caused a marked increase in the content of the active form of glycogen (starch) synthase in the liver. Total activity of the enzyme (active plus inactive forms) was not increased significantly. The lowest effective dose of acetylcholine was 5×10^{-10} mole, and the optimum dose was 5×10^{-9} mole. Similar applications of other neurotransmitters, such as norepinephrine, dopamine, serotonin, and γ-aminobutyric acid, did not affect the enzyme's activity.*

The hypothalamus and its autonomic nervous connections aid in regulating enzymic activities implicated in carbohydrate metabolism in the liver, and the ventromedial (VMH) and the lateral hypothalamic nucleus (LH) act reciprocally in that regulation (*1–3*). Electrical stimulation of the VMH causes glycogenolysis and enhances gluconeogenesis in the liver to produce hyperglycemia (*1, 2*). Stimulation of the LH in rabbits and rats, however, can induce glycogenesis and inhibit gluconeogenesis in the liver (*1, 3*). In contrast to VMH stimulation, electrical stimulation of the LH generally produces effects on the metabolic alterations that are less pronounced; such stimulation sometimes results in obscure changes, presumably because neuronal organization of the LH is complex.

Since the fundamental work of Grossman (*4*), the roles of neurotransmitters in normal feeding, drinking, and thermoregulation have often been studied by their intracranial injection. However, there are few studies in which metabolic changes have been observed in the extracerebral organs after neurotransmitters have been injected into the hypothalamus. In order to gain further insight into the regulatory function of the LH in liver metabolism, we selectively stimulated neurons of the lateral hypothalamus with local applications of the different neurotransmitters and studied changes in the activity of liver glycogen synthase (E.C. 2.4.1.11), an enzyme catalyzing the rate-limiting step in glycogen biosynthesis.

Male Wistar rats weighing 250 to 300 g were housed in individual cages and given free access to laboratory chow and water. A double-walled cannula, which had outer and inner diameters of 0.65 mm and 0.30 mm, respectively, was stereotaxically implanted into the LH of each rat under pentobarbital anesthesia. The coordinates used for implantation have been described (*1*). The cannula was permanently anchored to the skull by screws and dental cement. Correct placement of the tip of the cannula was verified microscopically in brain sections made after the experiments were completed. Two weeks after the implantation, intrahypothalamic injections of different neurotransmitters were given in single doses (usually 5×10^{-8} mole) through the inner cannula. Transmitters were dissolved in 0.9 percent saline solution, and the injection volume was 1 μl. To minimize the effects of circadian variation of glycogen synthase activity in the liver, all the experiments were started at 1:00 p.m., and the animals were not fed during the 4 hours before and during the experiments. At the time indicated after administration of transmitters, the animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg per kilogram of body weight), the abdominal wall was cut open, and a portion of the liver was quickly removed and immersed in liquid nitrogen. The activity of glycogen synthase was then assayed (Table 1). Intrahypothalamic injection of norepinephrine or dopamine at the experimental dose sometimes elicited sniffing and grooming behavior of the animals, but that of serotonin or γ-aminobutyric acid (GABA) generally produced little change in behavior. Administration of acetylcholine at doses less than 1×10^{-8} mole also produced little behavioral change, but at higher doses it induced salivation, analgesia, and cataleptic reactions.

Cholinergic stimulation of the LH neurons by intrahypothalamic administration of acetylcholine plus neostigmine greatly increased the amount of the active form (I form) of liver glycogen synthase 1 hour after its administration (Table 1). Total activity (T) of the enzyme showed little change. Consequently, the I/T ratio of the enzyme increased as a result of hypothalamic stimulation with acetylcholine. It is therefore likely that the effect is due largely to conversion of the inactive form (D form) of glycogen synthase into the active form. Control injections of equally minute amounts of neostigmine alone did not have a significant effect on glycogen synthase, which suggests that cholinesterase was not significantly inhibited by these small doses; in consequence, acetyl-