FURTHER STUDIES ON POTENTIATION OF INHIBITOR ACTION THROUGH DETERMINATION OF REVERSING METABOLITES[1]

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Pittillo and Foster (1954) demonstrated that it is possible on a rational basis to formulate synergistic combinations of inhibitors for any particular bacterium. Prediction of combinations of inhibitors which are apt to be synergistic depends upon knowledge that the suppression of bacterial growth caused by individual inhibitors can be reversed by corresponding essential metabolites.

Reversal by a particular metabolite implies that the inhibitor in question prevents growth by interfering with a biochemical pathway involving that metabolite, e. g., its synthesis, utilization, or penetration (see Cowan and Rowatt, 1958, for general review). The ideal situation, several instances of which have been already described (Foster and Pittillo, 1953*b*; Pittillo and Foster, 1954), is where the inhibitor blocks the biosynthesis of the metabolite.

Chemically dissimilar inhibitors whose effects are reversible by the same metabolite probably interfere at different sites on a common pathway. When the two inhibitors are present simultaneously, the effects of each theoretically should be mutually potentiated.

Beerstecher and Shive (1947) and Potter (1951) showed how one kind of synergism (sequential blocking) can be achieved by metabolite analogues whose sites of action are known, and where detailed knowledge of the pathways involved is available. This approach to synergism—the combination testing, both *in vitro* and *in vivo*, of pairs of metabolite analogues—has been very extensively exploited in recent years. Notwithstanding the discovery of a significant number of synergistically acting combinations (Lacey, 1958), the great majority of randomly paired analogues do not display this kind of activity. Even biochemically related analogues are not predictably synergistic (Hitchings *et al.*, 1955; Veldstra, 1956). One is obliged, consequently, to conclude that at the present time one cannot with accuracy predict which metabolite analogue pairs will be synergistic. Although the analogue approach does in some measure predispose success in securing synergism, the procedure basically is one of empirically screening for random instances of synergism.

The unique feature of the "reversal" technique enables selection of sequential blockers without any prior knowledge of the biosynthetic pathways involved. In each of the several cases where synergism was predicted according to the reversal rationale, it was obtained (Pittillo and Foster, 1954). The present work extends the number of such illustrations. They stem from the discovery of new instances of metabolite reversal of inhibitors. Theoretical aspects of the reversal approach to inhibitor synergism are also discussed.

MATERIALS AND METHODS

The spread plate procedure described previously (Maas and Davis, 1950; Foster and Pittillo, 1953*a*) was employed. Briefly, the procedure involves incorporation of graded doses of an inhibitor in melted glucose-salts agar prior to pouring into duplicate petri plates. The concentrations in any series differed by a factor of 2 or 3. After solidification, the agar was inoculated by surface spreading 0.05 ml of a unicellular water suspension containing 50 to 150 twice washed bacteria. The inhibitory concentration is the smallest one which prevents the development of colonies visible to the unaided eye in a specified incubation period (at 37 C) during which control plates without inhibitor produce colonies normal for the particular organism. By "reversal" is meant the development of colonies from all of the cells in the inoculum as a result of the incorporation of a metabolite in the medium con-

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taining an otherwise toxic concentration of inhibitor. In this study, these colonies frequently were somewhat smaller than the control colonies. The concentration of inhibitor required for complete inhibition in the presence of excess metabolite, compared to that in the absence, is called "reversal factor."

High sensitivity, rigorous quantitation, and exclusion of selection of resistors and of nonspecific growth stimulation, ensue from the all or none criterion of growth with respect to the individual cells in the inoculum. These characteristics distinguish the spread plate procedure as a means for evaluating the true reversibility of inhibitors of bacteria.

RESULTS

Metabolite reversal of individual inhibitors. Most of the present work centers around the discovery that under the test conditions the toxicities of 6-mercaptopurine and riboflavin are counteracted by calcium pantothenate and biotin, respectively (table 1). Although the calcium pantothenate reversal of 6-mercaptopurine was demonstrable with all 3 bacterial cultures on which it was tried, significant quantitative differences were found. In our experience with this procedure, a reversal factor of 100 is large.

6-Mercaptopurine. This is primarily an antipurine compound particularly effective in blocking nucleic acid synthesis in organisms requiring exogenous purines or ribonucleosides; it is also an effective inhibitor of nucleic acid synthesis in mammalian cells. Being a metabolite analogue, 6-mercaptopurine is reversed by one or more physiological purines, nucleosides, and nucleotides (Miner, 1954). A growing list of instances makes it probable that 6-mercaptopurine also antagonizes the synthesis or utilization of purine-containing coenzymes including diphosphopyridine nucleotide (Kaplan *et al.*, 1956) and especially coenzyme A (Biesele, 1955; Tobioka and Biesele, 1956; Garattini *et al.*, 1955; Bolton and Mandel, 1957). Its involvement in pantothenate synthesis, as shown by the reversal by the vitamin, has not hitherto been described; it may well exert its effect here via purine-containing coenzymes.

Aerobacter aerogenes was inhibited by 6-mercaptopurine for the first 16 to 24 hr of incubation, as indicated by the fact that plates had no colonies visible to the unaided eye. At this time, all organisms in control plates without inhibitor formed colonies 0.5 to 1.0 mm in diameter. However, after 30 to 36 hr of incubation growth usually occurred and colonies appeared in number equivalent to those in the control plates. Whether this spontaneous release of inhibition was due to chemical instability of the 6-mercaptopurine or to its detoxification by the bacteria was not determined. Cells in these colonies were as sensitive to 6-mercaptopurine as the original culture, eliminating an explanation based upon selection of resistant mutants. Similar situations have been described in connection with a variety of inhibitors (see Mager *et al.*, 1955 for references). Authors interpret this behavior on the part of *Escherichia coli* against fluoroacetate as the consequence of prolongation of the lag period by a decreased rate of formation of metabolites essential for growth initiation.

Reversal of 6-mercaptopurine inhibition by compounds related to pantothenic acid. The follow-

TABLE 1

Reversal of inhibitors by single metabolites

Test Bacterium*	Inhibitor and Inhibitory Conc		Reversing Metabolite		Reversal Factor†
		μg/ml		μg/ml	
Aerobacter aerogenes strain P	6-Mercaptopurine	10	Ca pantothenate	0.1	100
Escherichia coli strain 9637	6-Mercaptopurine	30	Ca pantothenate	1.0	3
Escherichia coli strain T4	6-Mercaptopurine	10	Ca pantothenate	1.0	10
Bacillus subtilis var. *niger*	Riboflavin	10	Biotin	0.0001	100

* The medium was glucose-salts agar, except in the case of *B. subtilis* var. *niger* where it was glucose-glutamic acid-salts agar (Perry and Foster, 1956). This orange pigmented organism was labeled *Bacillus globigii*; it has been reclassified as *Bacillus subtilis* var. *niger* (Smith *et al.*, 1952).

† See text for definition.

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ing known or hypothetical precursors of pantothenic acid had some reversing activity in *A. aerogenes* strain P: pantethine, Na pantoate, Na ketoisovalerate, Na ketopantoate, Na α,β-dihydroxyisovalerate. Each was tested at levels equivalent to 1 μg of Ca pantothenate (2.1×10^{-6} M) run as a positive reversal control in the same experiment. Pantethine and Na pantoate were the most active and were about one-half as effective as Ca pantothenate. Dihydroxyisovalerate showed only slight reversing activity. β-Alanine, Na DL-α-aminobutyrate, β-aminobutyrate, and DL-pantonine were inactive. These results suggest that in this organism 6-mercaptopurine inhibits the synthesis of the pantoic acid moiety of pantothenate; in this respect 6-mercaptopurine inhibition resembles salicylate inhibition (Ivanovics, 1942; Maas, 1952).

Synergistic inhibition with 6-mercaptopurine.

TABLE 2

Ca pantothenate reversal of inhibition by selected compounds, tested by the spread plate procedure

Test Bacterium	Inhibitor and Inhibitory Conc		Reversing Metabolite,		Reversal Factor*
		μg/ml		μg/ml	
Escherichia coli strain 9637	DL-Serine†	30	Ca pantothenate	1.0	10
Aerobacter aerogenes strain P	Na propionate‡	3000	Ca pantothenate	1.0	30
Aerobacter aerogenes strain P	Na salicylate	30	Ca pantothenate	1.0	>10

* See text for definition.

† Ca pantothenate also reversed DL-serine inhibition of *A. aerogenes* strain P.

‡ Ca pantothenate also reversed Na propionate inhibition of *E. coli* strain 9637.

TABLE 3

*Synergistic inhibition by 6-mercaptopurine and other inhibitors, in the presence and in the absence of Ca pantothenate as a reversing metabolite**

Test Organism	Reversing Metabolite	Inhibitor	Combination of Inhibitors Required for Complete Inhibition, μg per ml of Medium				
Escherichia coli strain 9637	None	6-Mercaptopurine	40	20 plus	10 plus	5 plus	0
		DL-Serine	0	30	60	60	120
	Ca pantothenate, 1 μg/ml	6-Mercaptopurine	>40	>40 plus	0		
		DL-Serine	0	>1000	>1000		
Aerobacter aerogenes strain P	None	6-Mercaptopurine	30	10 plus	1 plus	0	
		Na propionate	0	30	100	300	
	Ca pantothenate, 1 μg/ml	6-Mercaptopurine	>30	>30 plus	0		
		Na propionate	0	>300	>300		
Aerobacter aerogenes strain P	None	6-Mercaptopurine	100	30 plus	3 plus	0.3 plus	0
		Na salicylate	0	0.3	1	3	30
	Ca pantothenate, 1 μg/ml	6-Mercaptopurine	>300	>300 plus	0		
		Na salicylate	0	>100	>100		

* Tested by the spread plate procedure; see text for details.

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TABLE 4

Synergistic inhibition by riboflavin paired with actithiazic acid and isonicotinic hydrazide, respectively, in the presence and in the absence of biotin as a reversing metabolite

Reversing Metabolite	Inhibitor	Combination of Inhibitors Required for Complete Inhibition, μg per ml of Medium				
None	Riboflavin	0	1 plus	10		
	Actithiazic acid	0.3	0.1	0		
Biotin, 0.01 μg/ml	Riboflavin	0	30 plus	30		
	Actithiazic acid	300	100	0		
None	Riboflavin	0	0.03 plus	0.1 plus	1 plus	10
	Isonicotinic hydrazide	300	100	30	10	0
Biotin, 0.01 μg/ml	Riboflavin	0	30 plus	30		
	Isonicotinic hydrazide	>300	300	0		

* The spread plate procedure was used. Test organism was *Bacillus subtilis* var. *niger*. See footnote to table 1.

According to the concept described in the introduction of this paper, other inhibitors whose toxicities for bacteria are similarly reversible by Ca pantothenate should each function synergistically when paired with 6-mercaptopurine. Literature search revealed that inhibition caused by D- or DL-serine (Maas and Davis, 1950), by Na propionate (Wright and Skeggs, 1946; King and Cheldelin, 1948) or by Na salicylate (Ivanovics, 1942; Maas, 1952; Purko *et al.*, 1953) is reversible by Ca pantothenate. These inhibitors were tested and the reversal by Ca pantothenate was confirmed under our conditions and with our strains of test bacteria (table 2).

Next, each inhibitor was tested for synergistic activity with 6-mercaptopurine. Cross-concentration experiments were conducted. Two identical series were run for each experiment, one without Ca pantothenate and one with Ca pantothenate present in the basal medium. Table 3 shows that there was synergism between 6-mercaptopurine and each of the other inhibitors. With 6-mercaptopurine and DL-serine, the median synergism was obtained with ¼ plus ½ the doses (10 and 60 μg, respectively) required when these were used separately (40 and 120 μg, respectively). For 6-mercaptopurine and Na propionate the corresponding fractions were either $\frac{1}{30}$ plus ⅓ or ⅓ plus $\frac{1}{10}$. The greatest synergism was displayed by 6-mercaptopurine and Na salicylate where the fractions were $\frac{1}{33}$ plus $\frac{1}{30}$ (3 and 1 μg, respectively).

Inhibition by each of the three synergistic combinations was completely abolished in the presence of Ca pantothenate (table 3).

Riboflavin. The high toxicity of riboflavin for *E. coli* strain B in glucose-salts medium has been discovered (Foster and Pittillo, 1953*b*). The compound is also toxic for *Micrococcus pyogenes* var. *aureus* (Ramsey and Wilson, 1957) and for two of Lochhead and Burton (1955) types of cobalamine-requiring soil bacteria. The soil bacteria also require biotin; in the presence of yeast extract their growth was ascribed to a reversal of riboflavin inhibition by substances in the yeast extract. It remains to be seen if biotin is the active reversing substance, as it is for *Bacillus subtilis* var. *globigii* (table 1).

Synergism experiments with riboflavin. Actithiazic acid and isonicotinic hydrazide, the inhibitory effects of which are known to be reversed by biotin (Grundy *et al.*, 1952; Pittillo and Foster, 1954) have been shown to inhibit the biosynthesis of biotin in *A. aerogenes* strain P (Pittillo and Foster, 1954).

Actithiazic acid and isonicotinic hydrazide

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each proved to be synergistic with riboflavin in the inhibition of *B. subtilis* var. *niger* (table 4) and a selective involvement of biotin metabolism may be inferred from the fact that both synergistic pairs were completely counteracted by biotin. With riboflavin-actithiazic acid the synergistic fractions were $\frac{1}{10}$ plus $\frac{1}{3}$ the respective doses required separately; with riboflavin-isonicotinic hydrazide they were $\frac{1}{100}$ plus $\frac{1}{10}$.

DISCUSSION

Determination of a common reversing metabolite without doubt now stands as an established means of selecting synergistic pairs of inhibitors. In fact, it is the only means presently available by which one can select synergistic inhibitors without randomly screening for that property. The reversibility of two chemically dissimilar inhibitors by the same metabolite may be taken as evidence that the two inhibitors in conjunction will behave synergistically. A dozen pairs of inhibitors thus far predicted in this manner have without exception proved to be synergistic. The basic premise for selecting the pairs is validated by the abolition of the synergistic inhibition in each case by the indicated metabolite.

An important practical advantage of the reversing approach, compared with blocking of known sequential reactions, is that neither the site of the inhibition, the mode of action of the inhibitor, nor the step affected in the biosynthesis or utilization of the reversing metabolite needs to be known in order to obtain synergistic inhibitors. The different synergistic combinations affecting the pantothenate pathway illustrate the scope of the reversal method. Thus, a combination of two pantoate inhibitors, 6-mercaptopurine and Na salicylate (Ivanovics, 1942; Maas, 1952), was obtained; also, a combination of a β-alanine inhibitor, Na propionate or D-serine (Wright and Skeggs, 1946; King and Cheldelin, 1948; Maas and Davis, 1950) and a pantoate inhibitor, 6-mercaptopurine. Unfortunately, a combination of Na propionate and D-serine as two β-alanine inhibitors was not tested for synergism in this work, but synergistic inhibition of pantothenate synthesis by two β-alanine inhibitors, namely, D-serine and L-aspartate, has been described by Maas and Davis (1950) using the reversal technique.

An interesting corollary of the reversal approach is that two dissimilar inhibitors, each synergistic with the same compound, are apt to be synergistic with each other. For example, isonicotinic hydrazide and actithiazic acid, each synergistic with riboflavin in blocking biotin metabolism, are also synergistic with each other in blocking biotin metabolism (Pittillo and Foster, 1954). New synergistic combinations should be obtainable among compounds selected solely on the basis of their respective synergistic properties with a common second inhibitor. In the manner described elsewhere (Pan and Foster, 1957) Lowery and Foster (1959) screened antibiotically active filtrates of soil actinomycetes for the property of synergism with azaserine (O-diazoacetyl-L-serine). The incidence of synergism among pairs of these selected filtrates was much greater than one would reasonably expect from the occurrence of synergism among unselected inhibitors.

In contrast to the relation between a reversing agent and an inhibitor discussed in the foregoing, Davis (1957) has pointed out that compounds that reverse an inhibition are not necessarily metabolites whose synthesis or utilization is stopped by the inhibitor. Such reversing compounds could alternatively be blocking the penetration of an inhibitor into the cell or into a subcellular structure. This could falsely suggest a synergistic combination of inhibitors. However, if such a blocking compound is itself a metabolite, a second inhibitor preventing its synthesis or blocking its function might produce synergism with the first inhibitor.

In addition to factors such as penetrability and properties of affected enzymes (Maas and Davis, 1952; Horowitz and Fling, 1953) the intracellular pools of metabolite reversers undoubtedly influence the sensitivity of an organism to an inhibitor. Most inhibitors are reversible to varying degrees by one or more metabolites (Foster and Pittillo, 1953*a*, *b*). It seems probable that the intracellular pool of metabolites, whether of endogenous or exogenous origin, reduces the efficacy of a given concentration of inhibitor. The metabolites represent a kind of internal buffer defending against inhibitors. Reduction in the level of one or more active components of the pool by a second inhibitor should, consequently, render the cell more liable to chemical inhibition ("protected inhibition," Pittillo and Foster, 1954).

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SUMMARY

Using the spread plate technique with approximately 100 cells as inoculum, the inhibitions caused by 6-mercaptopurine and by riboflavin were reversed by Ca pantothenate and by biotin, respectively. Other inhibitors known to be reversed by the same metabolites proved to be synergistic with these inhibitors. Thus, D-serine, Na propionate, or Na salicylate was synergistic with 6-mercaptopurine; the synergisms were abolished by Ca pantothenate. Isonicotinic hydrazide or actithiazic acid, was synergistic with riboflavin; the synergisms were abolished by biotin. Theoretical aspects of the reversal approach to chemotherapy and the significance of metabolites in inhibitor action are discussed.

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