PANTOTHENATE STUDIES

II. Evidence from Mutants for Interference by Salicylate with Pantoate Synthesis

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Although several workers have observed that the bacteriostatic effect of salicylate can be reversed by pantothenate, there is disagreement about the reaction step affected. Salicylate has been considered to inhibit synthesis of pantoate (Ivánovics, 1942), the conversion of pantoate to pantothenate (Roblin, 1949), and the utilization of pantothenate (McIlwain, 1943; Work and Work, 1948); Ackermann and Shive (1948) remained undecided between synthesis and utilization of pantoate.

Ivánovics' conclusion was based on the observation that salicylate in low concentrations inhibited growth of pantoate-synthesizing organisms but not of pantoate or pantothenate-requiring ones. Furthermore, the concentrations of pantoyl lactone[1] or pantothenate required for reversal of inhibition of the pantoate synthesizers were the same as for optimal growth of the nonsynthesizers. This comparison, however, involved bacterial species which differed metabolically in more ways than the ability to synthesize pantoate. Moreover, since several amino acids and vitamins have been shown to antagonize salicylate inhibition (Ivánovics, 1942), it is significant that only the pantoate-requiring strains were grown in a complex medium containing peptone. In view of these differences in metabolism and media, absence of salicylate inhibition could not rigorously be ascribed to inability to synthesize pantoate.

Roblin seemed justified, therefore, to assign the salicylate block to pantoate utilization on the basis of unpublished observations of Stansly (Roblin, 1951) that salicylate inhibition of *Escherichia coli* was reversed by pantoate in an apparently competitive manner, though over a narrow range of salicylate concentrations only. Experiments to be described will confirm this observation but will show that the competitive relationship observed is only an apparent one.

McIlwain (1943) found that in several pantothenate-requiring species, insensitive to salicylate, mutation to pantoyl taurine resistance induced sensitivity to salicylate without affecting the pantothenate requirement; salicylate inhibition of these mutants could be reversed apparently competitively by pantothenate. This seems to be a special case of a new site of attack for salicylate which is not necessarily relevant to the problem of salicylate inhibition in pantothenate synthesizing strains.

[1] Ivánovics (1942) used pantoyl lactone instead of pantoate; as will be shown in this paper pantoate differs in action from the lactone only in being utilized more efficiently.

In view of these conflicting conclusions we undertook an investigation of the problem with previously obtained mutants of *E. coli* blocked at various stages of pantothenate synthesis (Maas and Davis, 1950). The comparison of the salicylate effect on pantoate synthesizers and nonsynthesizers could thus be conducted in strains with otherwise identical nutritional requirements. The results obtained provide strong evidence for salicylate interference with pantoate synthesis, thus confirming Iványovics' conclusion.

MATERIALS AND METHODS

Pantoyl lactone was obtained through the kindness of Dr. W. L. Williams of Lederle Laboratories. Potassium pantoate was prepared by heating the lactone at 100 C for 10 minutes in a solution containing twice as many moles of potassium hydroxide and, subsequently, neutralizing with HCl. Titration showed that all the lactone had been converted to the salt of the free acid.

The W strain of *E. coli* was used in this study. The minimal and complete (NY) media, the auxotrophic mutants, and the procedure for studying growth inhibition with discrete colonies in agar pour plates have been described previously (Maas and Davis, 1950). In the same publication the advantages of this method over the more conventional growth measurements in liquid culture were pointed out.

RESULTS

Table 1 shows inhibition of growth of wild type by salicylate and its reversal by pantoate and pantothenate. Either growth factor appears to overcome the inhibition competitively; for pantoate the molar ratio of substrate to inhibitor for reversal is approximately 1 to 100, for pantothenate 1 to 30,000. However, the competitive range is narrow since salicylate at higher concentrations produces another type of inhibition that cannot be reversed by pantothenate. Complete reversal can be obtained only over a threefold range of salicylate concentrations (0.3 to 1.0 mMolar); at higher concentrations (3.0 mMolar and 10.0 mMolar) the inhibition becomes increasingly irreversible. Except for the difference in levels required, both growth factors act alike, producing apparently competitive reversal over the same narrow range.

Since the narrowness of the reversible range in the wild type does not permit a clear cut interpretation, the problem was approached by the use of auxotrophic mutants, a method previously described in connection with the antibacterial action of D-serine (Maas and Davis, 1950). Table 2 illustrates the use of a pantoate auxotroph (99-4) in the present problem. The mutant is not affected by salicylate concentrations (0.3 and 1.0 mMolar) that produce reversible inhibition in the wild type (see table 1). Higher salicylate concentrations (3.0 and 10.0 mMolar) produce the same irreversible inhibition as in the wild type. Moreover, the concentrations of pantoate and pantothenate required for optimal growth of the mutant are the same as those previously shown to be required to overcome the highest reversible salicylate concentration in the wild type. Thus, once the necessity for synthesizing pantoate has been eliminated, sali-

TABLE 1

Inhibition of growth of wild type of Escherichia coli by salicylate and its reversal by pantoate or pantothenate

SUPPLEMENT	SALICYLATE mMOLAR					
	0	0.1	0.3	1.0	3.0	10.0
None	2+, 4+	2+, 4+	m, 4+	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
Potassium pantoate mMolar						
0.001	—	2+, 4+	m, 4+	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
0.003	—	2+, 4+	1+, 4+	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
0.01	—	2+, 4+	1+, 4+	0, m, 3+, 3+	0, 0, 0, 1+	0, 0, 0, 0
0.03	—	2+, 4+	1+, 4+	m, 3+, 4+	0, 2+, 3+, 3+	0, 0, 0, 0
0.1	—	2+, 4+	2+, 4+	1+, 4+	1+, 3+, 3+	0, 1+, 2+, 2+
0.3	—	2+, 4+	2+, 4+	2+, 4+	1+, 3+, 3+	0, 1+, 2+, 2+
1.0	—	2+, 4+	2+, 4+	2+, 4+	1+, 3+, 3+	0, 1+, 2+, 2+
Calcium pantothenate mMolar × 10^{-3}						
0.01	—	2+, 4+	1+, 4+	0, 0, 0, 1+	0, 0, 0, 0	0, 0, 0, 0
0.03	—	2+, 4+	2+, 4+	0, 3+, 3+	0, 0, 0, 1+	0, 0, 0, 0
0.1	—	2+, 4+	2+, 4+	2+, 4+	m, 2+, 3+	0, 0, 0, 0
0.3	—	2+, 4+	2+, 4+	2+, 4+	1+, 2+, 3+, 3+	0, 1+, 2+, 2+
1.0	—	2+, 4+	2+, 4+	2+, 4+	1+, 3+, 3+	m, 1+, 2+, 2+
3.0	—	2+, 4+	2+, 4+	2+, 4+	1+, 3+, 3+	0, 1+, 2+, 2+

Agar pour plates of minimal medium supplemented as indicated. Inoculum of 50 to 100 cells per plate. Colony sizes recorded at successive 24 hr intervals. m = microscopic, visible under microscope (10× magnification); 1+ = visible to unaided eye; 4+ = maximum size on this medium.

TABLE 2

Failure of salicylate to inhibit pantoate requiring mutant 99-4

SUPPLEMENT	SALICYLATE mMOLAR					
	0	0.1	0.3	1.0	3.0	10.0
None	0, 0, 0	—	—	—	—	—
Potassium pantoate mMolar						
0.003	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
0.01	0, 0, 0, 1+	0, 0, 0, 0	0, 0, m, 2+	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
0.03	m, 3+, 4+	m, 3+, 4+	m, 3+, 4+	0, 3+, 4+	0, 2+, 3+, 3+	0, 0, 0, 0
0.1	2+, 4+	2+, 4+	2+, 4+	1+, 4+	1+, 3+, 3+	0, 1+, 1+
0.3	2+, 4+	2+, 4+	2+, 4+	2+, 4+	1+, 3+, 3+	0, 1+, 1+
1.0	2+, 4+	2+, 4+	2+, 4+	2+, 4+	1+, 3+, 3+	0, 1+, 1+
Calcium pantothenate mMolar × 10^{-3}						
0.01	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
0.03	0, m, 1+, 1+	0, m, 1+, 1+	0, m, m, 1+	0, 0, m, 1+	0, 0, 0, 0	0, 0, 0, 0
0.1	2+, 3+, 3+	2+, 3+, 3+	2+, 3+, 3+	1+, 3+, 3+	1+, 2+, 2+	0, 0, 0, 0
0.3	2+, 4+	2+, 4+	2+, 4+	2+, 4+	1+, 2+, 3+, 3+	0, 1+, 1+
1.0	2+, 4+	2+, 4+	2+, 4+	2+, 4+	1+, 3+, 3+	0, 1+, 1+
3.0	2+, 4+	2+, 4+	2+, 4+	2+, 4+	1+, 3+, 3+	0, 1+, 1+

Same technique as that described in table 1.

cylate fails to produce reversible inhibition. From these results it is concluded that salicylate inhibits pantoate synthesis. It should be noted that the apparent

competitive relationship between salicylate and pantoate or pantothenate in wild type is confined to growth factor concentrations below those required for optimal growth of the mutant.

Results obtained with mutant 99-2, blocked in β-alanine synthesis, are in accord with the conclusion that salicylate inhibits pantoate synthesis. Salicylate inhibits growth of this mutant when growing on β-alanine, but not on pantothenate. Furthermore, salicylate inhibition of growth on β-alanine is reversed by pantoate in the same concentrations that are effective in the wild type. Finally, mutant 99-1, which specifically requires pantothenate for growth, is not inhibited reversibly by salicylate.

Since Iványovics (1942) used pantoyl lactone rather than pantoate, we compared the effectiveness of the two substances and found pantoate about 3 times as effective as its lactone in both reversing salicylate inhibition and supporting growth of the pantoate auxotroph. A similar, though more marked, difference in the efficiency of utilization of the two substances has been reported by Stansly and Alverson (1946).

DISCUSSION

Two approaches have been used in attempts to localize salicylate inhibition. The first, in which the substrate of the inhibited reaction is determined by its competitive reversal and the product by its noncompetitive reversal of the inhibition, requires demonstration of these relationships over a wide concentration range and is, therefore, indecisive in the present case. The second involves comparison between strains able and unable to synthesize the product of the inhibited reaction; absence of the reaction obviously involves absence of its inhibition. As has been pointed out in the introduction in connection with Iványovics' results, it is desirable to avoid extensive differences between the strains to be compared. Mutants with single biochemical blocks, therefore, lend themselves particularly well to this approach. In the present case they have provided evidence for salicylate interference with pantoate synthesis.

Comparison of the effect of salicylate on wild type and the pantoate auxotroph (tables 1 and 2) reveals the circumstances responsible for the apparent competitive relationship between salicylate and either pantoate or pantothenate observed in the wild type. The ranges of reversible and irreversible salicylate concentrations happen to coincide with the ranges of partial and complete satisfaction of the pantoate or pantothenate requirement in such a way that at no point can the noncompetitive reversal by the growth factors be recognized. Thus, in the reversible range, a competitive relationship is simulated by the fact that increasing degrees of partial inhibition are reversed by increasing fractions of the total pantoate or pantothenate requirement. At salicylate concentrations slightly higher than those completely reversed by the optimal growth factor concentration, a second irreversible type of inhibition appears, preventing recognition of a plateau of noncompetitive reversal. Similarly in the mutants, in which the inhibition becomes irreversible at the same salicylate concentration as in wild type, no such plateau can be observed.

The much greater requirement of pantoate than of pantothenate for reversal of wild type inhibition might also suggest interference with pantoate utilization rather than synthesis. Here again, this false inference is excluded by comparison with the growth requirement of the pantoate auxotroph which shows precisely the same difference of concentrations. The following observations may throw some light on the cause of this difference. While the concentration of pantoate required for optimal growth of the pantoate auxotroph is 100 times that of β-alanine required for growth of a β-alanine auxotroph, in cell-free extracts the concentration of pantoate required for half saturation of the pantothenate-synthesizing enzyme (Michaelis constant) is only 4 times that of β-alanine (Maas, 1952). These results suggest presence in the whole cell of a permeability barrier to pantoate, or else inhibition of pantoate utilization by some metabolite.

Because of the narrowness of the reversible range of salicylate inhibition it has not been possible to exclude critically, even with the mutants, the possibility of interference with pantoate or pantothenate utilization. However, further evidence against interference with pantoate utilization has been obtained in some recent studies with an enzyme extracted from acetone dried cells. This enzyme system, which has been shown to synthesize pantothenate from β-alanine and pantoate in the presence of ATP and cations (Maas, 1952), is not inhibited by salicylate in concentrations as high as 20 mMolar. (Inhibition does appear at even higher concentrations, but cannot be reversed by pantoate.) In contrast, competitive inhibition of this enzyme could be shown with an analogue of one of its substrates: propionate interferes with β-alanine utilization in both growing cells and the extracted enzyme, and either inhibition is reversed by β-alanine.

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SUMMARY

Conflicting reports have ascribed the antibacterial action of salicylate to various steps in pantothenate metabolism. Studies with pantothenate auxotrophs have provided strong evidence that salicylate interferes with pantoate synthesis. In particular, salicylate, in concentrations whose inhibition of wild type *Escherichia coli* is reversed by pantoate or pantothenate, does not inhibit a pantoate auxotroph.

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