MECHANISM OF COMPETITIVE INHIBITION OF P-AMINOBENZOIC ACID OXIDATION BY P-AMINOSALICYLIC ACID

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Received for publication December 18, 1959

The proposed mechanism(s) associated with the inhibition of essential metabolic reactions by various compounds has received a great deal of attention in recent years, since antimetabolites have proved useful in several chemotherapeutic areas and in studying various metabolic reactions. The significance of p-aminobenzoic acid in microbial nutrition was first emphasized when Woods (1940) and Woods and Fildes (1940) reported that this metabolite protected yeast against the growth inhibiting action of sulfanilamide. Youmans, Raleigh, and Youmans (1947) reported that p-aminosalicylic acid inhibited growth of virulent strains of the tubercle bacilli and this antagonism was due to anti-p-aminobenzoic acid activity. More recently, Hedgecock (1958) demonstrated that p-aminobenzoic acid competitively antagonized the inhibitory effect of p-aminosalicylic acid on the growth of Mycobacterium tuberculosis.

Nutritional studies have indicated that p-aminobenzoic acid may fulfill a dual role in microbial metabolism. Durham (1956) reported that p-aminobenzoic acid was capable of serving as an oxidizable substrate for certain microorganisms in addition to functioning as a growth factor. Results obtained from additional investigations indicated that the utilization of p-aminobenzoic acid as a sole source of energy for aerobic growth is also competitively inhibited by p-aminosalicylic acid (Durham, 1957). Durham and Hubbard (1959) suggested that p-aminosalicylic acid influenced the assimilation of p-aminobenzoic acid by competing with the substrate for the specific transport mechanism(s) in the cell membrane thereby controlling oxidation by regulating the intracellular accumulation of the substrate.

This paper is concerned with clarification of the conditions associated with the antimetabolic activity of p-aminosalicylic acid toward p-aminobenzoic acid utilization and elucidation of the mechanism(s) involved in this antagonism.

MATERIALS AND METHODS

A bacterium capable of growing on a chemically defined medium containing p-aminobenzoic acid as the sole source of carbon was isolated from the soil by an enrichment technique. The organism was cultured and tentatively identified as a member of the genus Flavobacterium. A stock culture of the organism was maintained on a mineral salts medium containing 0.1 percent p-aminobenzoic acid. The defined medium used throughout the course of the experimentation was composed of NaCl, 0.1 g; NH4Cl, 0.1 g; KH2PO4, 0.324 g; K2HPO4, 0.424 g; and agar, 2.0 g in 100 ml of distilled water. The basal medium was supplemented with a mineral salts solution (Durham, 1956) and the pH adjusted to 7.0.

Cell suspensions for incubation and respirometer studies were prepared by harvesting the growth from plate cultures 22 hr old, washing twice, and resuspending in 0.01 M phosphate buffer of pH 7.0. The cultures were then adjusted to a standard density equivalent to a wet cell weight of approximately 1.4 mg per ml. p-Aminobenzoic acid-grown cells were used throughout this investigation.

Cell-free extracts were prepared using a French Pressure Cell under conditions which called for a pressure of 20,000 lb/in². The crude extract was centrifuged at 18,000 X g for 30 min. The supernatant collected after this treatment was used in experiments involving cell extracts.

All respirometer experiments were performed in the Warburg apparatus (Umbreit, Burris, and Stauffer, 1957) at a temperature of 37 C with
air as the gas phase. Warburg vessels with double side arms were employed in this study. The cell suspension was pipetted into the main chamber, 0.2 ml of 20 per cent KOH into the center well, and varying concentrations of substrate and inhibitor in the side arms. Incubation experiments were conducted under similar conditions in which mixtures of the substrate and inhibitor were added to the cell suspension. The containers were shaken in a water bath at 37 C for the desired time intervals. Samples were withdrawn from the vessel, the cells removed immediately by filtration through a Millipore filter, and the filtrate collected and assayed for the substrate or inhibitor. All compounds used as substrates and inhibitors for respirometer and incubation experiments were dissolved in 0.01 m phosphate buffer and adjusted to a pH of 7.0.

P-aminobenzoic acid was determined as previously described (Durham and Hubbard, 1959). P-aminosalicylic acid was also determined colorimetrically by modification of a previously described procedure (Snell and Snell, 1953). The coupling reaction was performed as follows. To a sample of filtrate add 3.0 ml of 20 per cent p-toluensulfonic acid in 1:50 hydrochloric acid, shake, and allow to stand 5 minutes; add 1 ml of a buffer consisting of 15.76 per cent citric acid in a 1.4 per cent sodium hydroxide solution; add 2 ml of 2 per cent p-dimethylaminobenzaldehyde in ethanol; mix and read in a spectrophotometer against a reagent blank at 450 m. Values were calculated from a standard curve developed concurrently. Controls indicated that p-aminobenzoic acid interfered with the determination of p-aminosalicylic acid. In studies in which both p-aminobenzoic acid and p-aminosalicylic acid were added to the reaction vessel it was necessary to permit the p-aminobenzoic acid to be depleted from the medium by the cell suspension before an accurate determination of the p-aminosalicylic acid concentration could be made. All inhibitor to substrate ratios are calculated on a molar basis.

RESULTS

Influence of p-aminosalicylic acid on the oxidation of various substrates. Investigations have revealed that p-aminobenzoic acid induced cell suspensions are capable of oxidizing p-aminobenzoic acid quite rapidly. The addition of p-aminosalicylic acid to the Warburg flasks results in a marked decrease in the rate of oxidation. This inhibition becomes more pronounced as the ratio of inhibitor to substrate is increased. Figure 1 illustrates the typical results obtained from respirometer experiments when the substrate concentration was maintained at a constant level and the concentration of the inhibitor was varied. The control containing p-aminosalicylic acid (20 μmoles) without added substrate shows a slight but consistent increase over the endogenous respiration. The nature of this oxidative reaction has not yet been determined.

Additional studies were conducted with the p-aminobenzoic acid grown cells to determine if p-aminosalicylic acid influenced the oxidation of other metabolites. Results obtained from this investigation indicated that the oxidation of protocatechuic acid was also inhibited. In contrast to this finding was the observation that the oxidation of β-ketoadipic acid and succinic acid were not significantly affected by the inhibitor. Figure 2 shows the influence of p-aminosalicylic acid on the oxidation of various substrates in an inhibitor to substrate ratio of 5:1. The inability

![Figure 1](http://jb.asm.org/downloadable.png)

*Figure 1. Oxidation of p-aminobenzoic acid (2 μmoles) in the presence of varying concentrations of p-aminosalicylic acid (PAS). The final ratios of inhibitor to substrate are 0:1, 3:1, 5:1, and 10:1.*
of p-aminosalicylic acid to influence the oxidation of straight chain intermediates to a significant extent eliminates the possibility that the inhibitor may be killing the cells or that the oxidative ability of the cells is being otherwise impaired.

Reversal of the competitive inhibition of p-aminobenzoic acid oxidation. Results presented in figure 1 indicate that the inhibition of substrate oxidation is proportional to the concentration of the inhibitor. The reversible nature of this competitive inhibition can be demonstrated by the addition of excess substrate. Respirometer experiments were employed in which the competitive inhibition of p-aminobenzoic acid oxidation by p-aminosalicylic acid was followed for 60 min in inhibitor to substrate ratios of 0:1, 3:1, 5:1, and 10:1. Figure 3 shows these results and indicates that within 15 min after the introduction of additional substrate (8 μmoles at 60 min) the oxidation proceeded at an uninhibited rate in the lower ratios and at an increased rate in the higher ratios where the inhibition was not completely reversed. Failure to reverse completely the inhibition in the higher ratios was due to the addition of insufficient quantities of substrate.

Influence of prior exposure to inhibitor and time of addition on substrate oxidation. In further investigations concerning the nature of the competitive antagonism, manometric experiments were performed to determine the effect of prolonged exposure of the cells to p-aminosalicylic acid.
acid prior to addition of the substrate and the effectiveness of the inhibitor when added to cells actively metabolizing p-aminobenzoic acid. Figure 4 shows the results obtained with inhibitor to substrate ratios of 5:1 and 10:1 with (a) the inhibitor added at the time of substrate addition and (b) the inhibitor added 20 min after the addition of the substrate. Inhibition of substrate oxidation was observed immediately in the flasks in which the inhibitor was added at 0 min. Within a few seconds following addition of the antimetabolite to the cells that were actively metabolizing the substrate, a normal pattern of competitive inhibition was observed. These findings suggest that the sensitive “site” must be readily accessible to the inhibitor as indicated by the immediate susceptibility of substrate oxidation. Speculation then indicates that one of the first available sites could be the specific transport mechanism in the cell membrane. The inhibition could also occur at the internal oxidation site. However, if the intracellular oxidative mechanism is involved, the inhibitor must readily penetrate the cell membrane in order to reach the sensitive “oxidative site” and exert its effect.

In related experiments, p-aminobenzoic acid grown cells were exposed to 10 μmoles and 20 μmoles of p-aminosalicylic acid for 60 min in Warburg vessels prior to the addition of 2 μmoles of substrate. The oxidation of p-aminobenzoic acid by these cells was followed and compared with the rate of oxidation when the substrate and inhibitor were added simultaneously. The results indicated that the degree of inhibition observed in cell suspensions previously exposed to the inhibitor appeared to be similar to the antagonism observed when the inhibitor and substrate were added simultaneously.

Inhibition of the uptake of p-aminobenzoic acid by cell suspensions in the presence of p-aminosalicylic acid. Studies employing the same concentrations of substrate and inhibitor as used in the respirometer experiments were conducted in which the uptake of p-aminobenzoic acid by cell suspensions was followed colorimetrically. The data presented in table 1, experiment A, are representative of the findings obtained in this investigation. The results show that in the absence of the inhibitor the substrate is depleted from the medium within 45 min. In the presence of p-aminosalicylic acid the substrate is taken up at a much slower rate. The data show that the amount of p-aminobenzoic acid depleted from the medium decreases with increasing inhibitor

| Table 1
| Cellular uptake of p-aminobenzoic acid (PAB) in the presence and absence of varying ratios of p-aminosalicylic acid (PAS) |
|---|---|---|---|---|
| Expt | Time | Conc (μg) of PAB Remaining per ml of Filtrate | Ratio, PAS:PAB |
| | | 0:1 | 3:1 | 5:1 | 10:1 |
| A | 0 min | 116.0 | 116.0 | 114.6 | 116.0 |
| | 15 min | 80.0 | 87.2 | 97.8 | 103.2 |
| | 30 min | 17.3 | 64.0 | 86.8 | 99.0 |
| | 45 min | 0.0 | 46.6 | 76.6 | 97.0 |
| B | 0 sec | 3.10 | 3.10 | 3.10 | 3.10 |
| | 30 sec | 0.15 | 0.43 | 0.77 | 1.65 |

The initial concentration of PAB in experiment A was 116 μg per ml and 3.1 μg per ml in experiment B.
concentrations. A decline in the rate of disappearance during the subsequent intervals was especially noticeable in the 10:1 ratio.

Additional studies were conducted in which the uptake of p-aminobenzoic acid in the presence of p-aminosalicylic acid was followed in lower concentrations. The disappearance of substrate from the medium was determined in inhibitor to substrate ratios of 0:1, 3:1, 5:1, and 10:1 with a p-aminobenzoic acid concentration of 2.28 × 10⁻³ M (3.1 µg per ml). Table 1, experiment B, illustrates the results obtained from this experimentation in which the disappearance of p-aminobenzoic acid was measured after a 30-sec incubation period. The data indicate that the amount of substrate remaining in the medium is directly proportional to the concentration of the inhibitor.

These findings suggest that the inability of the substrate to permeate the cell in the presence of the inhibitor may be responsible for the decreased rate of oxidation. If one assumes that the inhibition is located at the site of internal substrate oxidation, then it is unlikely that the passage of small quantities of substrate into the cell should be limiting. Studies following depletion of the substrate both at high and extremely low levels indicated that the ratio of inhibitor to substrate present in the system regulates the uptake of substrate of the cell suspension. These findings would then support the postulation that the inhibition is associated with the specific transport mechanism rather than the internal oxidative enzymes.

Uptake of p-aminosalicylic acid by cell suspension. Colorimetric analysis of the filtrates obtained when p-aminobenzoic acid grown cell suspensions were incubated with p-aminobenzoic acid and p-aminosalicylic acid indicated that some of the inhibitor was removed from the medium. Since p-aminobenzoic acid interfered with p-aminosalicylic acid determinations, it was necessary that the substrate be depleted from the medium before inhibitor concentrations were determined. The assumption was made that if the uptake of p-aminosalicylic acid was proportional to its concentration, this proportionality should also be evident at lower concentrations. Table 2 illustrates typical results obtained when cell suspensions were incubated with three different concentrations of p-aminosalicylic acid. In the range tested, the results indicated that the rate or amount of uptake did not vary significantly with the different concentrations. The rate of disappearance of the inhibitor in a starting concentration of 41.76 µg per ml did not exceed the disappearance when a concentration of 10.44 µg per ml was employed. Similar rates of inhibitor uptake in the different concentrations make it difficult to visualize a competitive inhibition at the site of the internal oxidative enzyme system(s) of the cell since a competitive antagonism would be dependent on the presence of varying concentrations of the inhibitor.

Additional experiments were performed to determine if the antagonism could be more closely correlated with the external concentration of p-aminosalicylic acid. A cell suspension was permitted to deplete approximately 20.88 µg of p-aminobenzoic acid from the medium, following which 6.2 µg of p-aminobenzoic acid were added and the disappearance of this substrate was followed. The depletion of substrate was compared with the uptake observed in control cells in which 6.2 µg of p-aminobenzoic acid and 20.88 µg of the inhibitor were added simultaneously. After a short incubation period, it was observed that the amount of substrate taken up by the cells which had assimilated the inhibitor prior to the addition of p-aminobenzoic acid was approximately twice the amount of substrate taken up by the cells when the substrate and p-aminosalicylic acid were added simultaneously. These findings indicate that only the presence of the inhibitor in the external environment influences the utilization of p-aminobenzoic acid since cells permitted to take up p-aminosalicylic acid intracellularly prior to addition of the substrate did not demonstrate a decrease in the uptake of p-aminobenzoic acid.

Additional investigations were conducted to determine if the temperature of incubation influenced the rate of uptake of p-aminosalicylic acid.

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Additional experiments were performed to determine if the antagonism could be more closely correlated with the external concentration of p-aminosalicylic acid. A cell suspension was permitted to deplete approximately 20.88 µg of p-aminobenzoic acid from the medium, following which 6.2 µg of p-aminobenzoic acid were added and the disappearance of this substrate was followed. The depletion of substrate was compared with the uptake observed in control cells in which 6.2 µg of p-aminobenzoic acid and 20.88 µg of the inhibitor were added simultaneously. After a short incubation period, it was observed that the amount of substrate taken up by the cells which had assimilated the inhibitor prior to the addition of p-aminobenzoic acid was approximately twice the amount of substrate taken up by the cells when the substrate and p-aminosalicylic acid were added simultaneously. These findings indicate that only the presence of the inhibitor in the external environment influences the utilization of p-aminobenzoic acid since cells permitted to take up p-aminosalicylic acid intracellularly prior to addition of the substrate did not demonstrate a decrease in the uptake of p-aminobenzoic acid.

Additional investigations were conducted to determine if the temperature of incubation influenced the rate of uptake of p-aminosalicylic acid.

### Table 2

<table>
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<th>Time in Min</th>
<th>Conc (µg) PAS Remaining per ml of Filtrate</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
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<td>120</td>
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acid by cell suspensions. Results from these studies indicated that approximately one-fourth as much inhibitor was taken up at 5°C as was depleted from the medium when the cells were incubated for similar periods of time at 37°C.

Since the findings in this investigation indicated that some of the p-aminosalicylic acid was depleted from the medium attempts were made to recover the inhibitor from the culture. Cells suspensions which had been exposed to p-aminosalicylic acid for 2 hr were washed extensively with phosphate buffer and the washing assayed for the inhibitor. Cell samples were also ruptured by grinding with glass beads and the extract tested for the presence of the inhibitor. Results obtained from these studies indicated that neither treatment permitted the recovery of a sufficient quantity of inhibitor to permit detection by the colorimetric test.

Influence of extraneous carbon sources and metabolic inhibitors on the uptake of p-aminosalicylic acid by cell suspensions. Since the presence of large quantities of p-aminobenzoic acid interfere with the colorimetric determination for p-aminosalicylic acid, other metabolites were also employed to determine if the presence of an oxidizable substrate influenced the uptake of the inhibitor. Studies were conducted in which the uptake of p-aminosalicylic acid was determined in cells permitted to oxidize p-aminobenzoic acid to completion. Results indicated that uptake of the inhibitor in these cells did not differ from the uptake observed in resting cell suspensions which had not been permitted to metabolize p-aminobenzoic acid.

Uptake of the inhibitor was also followed in the presence and absence of protocatechuic acid and succinic acid. Preliminary tests indicated that neither substrate interfered with the colorimetric test for p-aminosalicylic acid. In addition to the regular inhibitor to substrate ratios employed, investigations were conducted with an excess of substrate in an inhibitor to substrate ratio of 1:3.3. Results from these experiments indicated that the uptake of p-aminosalicylic acid was not influenced by permitting the concomitant oxidation of extraneous carbon sources by the cell suspensions.

Investigations were undertaken to determine if the uptake of p-aminosalicylic acid by the p-aminobenzoic acid grown cells was influenced by incubation with known metabolic inhibitors. Compounds included in this study were sodium azide, sodium fluoride, 2,4-dinitrophenol, potassium cyanide, and iodosacetate. All metabolic inhibitors were employed in a final concentration of 1 × 10⁻⁴ m. Studies were conducted in the presence and absence of the metabolic inhibitors and results indicated that these compounds did not influence the uptake of p-aminosalicylic acid. Similar studies indicated that incubation with chloramphenicol (120 µg per ml) and streptomycin (37.9 µg per ml) were also without effect.

Attempts to obtain activity for p-aminobenzoic acid in cell-free extracts to determine if the internal oxidative enzyme system was sensitive to p-aminosalicylic acid have been unsuccessful.

DISCUSSION

The ability of p-aminosalicylic acid to competitively inhibit the oxidation of p-aminobenzoic acid has been investigated in an attempt to elucidate the mechanism(s) involved in this antagonism. Results obtained in experiments employing structurally diverse metabolites such as succinic acid and β-ketoadipic acid confirm the supposition that p-aminosalicylic acid is acting as an antinmetabolite and is not killing or otherwise impairing the oxidative ability of the cells. Studies also demonstrated that the antagonism can be reversed by the addition of excess quantities of the substrate. The inhibition of p-aminobenzoic acid oxidation can be studied by manometric or colorimetric procedures following depletion of the substrate from the medium.

Results obtained in this investigation indicate that inhibition of substrate utilization becomes apparent immediately following addition of the antinmetabolite. The addition of p-aminosalicylic acid simultaneously with the substrate or to cells actively metabolizing the aromatic compound produced demonstrable effects within a short time. The inhibition could also be detected in experiments using extremely low substrate levels.

Experimental results indicated that cells previously exposed to large quantities of the inhibitor for 60 min demonstrated approximately the same degree of inhibition as cells to which the inhibitor and substrate were added simultaneously. Since the extent of inhibition was approximately the same under both experimental conditions, the results indicate that the inhibitor concentrations must be similar. This could be possible if the cell was impermeable or very
slightly permeable to the inhibitor in which case the antimetabolite would still be present in the medium in sufficient quantities (in the exposure experiments) to approximate the quantities of inhibitor added simultaneously with the substrate. Alternatively, p-aminosalicylic acid may readily permeate the cell barrier, accumulate in the cell, and compete for the internal oxidative enzyme system(s).

Results obtained in the investigations following the uptake of p-aminosalicylic acid by cell suspensions support the former explanation. The data show that uptake of the inhibitor by the cell is small and the rate of uptake is independent of the initial concentration of the antimetabolite in the medium. A competitive inhibition at the internal enzyme system would be dependent on the presence of varying concentrations of the inhibitor and the constant uptake observed in this study would eliminate the possibility that p-aminosalicylic acid acts inside the cell.

Related experiments in which known quantities of p-aminosalicylic acid were depleted from the medium by the cell suspensions prior to adding the substrate demonstrated that the internal accumulation of inhibitor by the cell did not influence p-aminobenzoic acid oxidation. Attempts to recover p-aminosalicylic acid from these cell suspensions were unsuccessful. These results would indicate that the antimetabolite is bound or metabolized immediately upon permeating the membrane and is not available in the "free form" to compete for the oxidative enzyme system.

SUMMARY

The oxidation of p-aminobenzoic acid by a Flavobacterium is inhibited by p-aminosalicylic acid. The antagonism is competitive in nature and may be followed by manometric techniques or by employing a colorimetric procedure for studying the depletion of substrate from the medium. Results obtained in the investigation suggest that p-aminosalicylic acid inhibits the oxidation of p-aminobenzoic acid by competing with the substrate for the specific transport "sites" in the cell membrane thus regulating cellular oxidation by controlling the intracellular accumulation of the substrate. The data indicate that the antagonism does not appear to be due to an internal accumulation of the antimetabolite or products of its degradation.

The uptake of p-aminosalicylic acid by cells was not influenced by extraneous carbon sources or other metabolic inhibitors but was temperature dependent. However, additional investigations will be necessary to clarify the fate of this antimetabolite in the cell.

REFERENCES


