MECHANISM OF THE INHIBITION OF p-AMINOBENZOIC ACID AND PROTOCATECHUIC ACID OXIDATION BY p-AMINOSALICYLIC ACID

By

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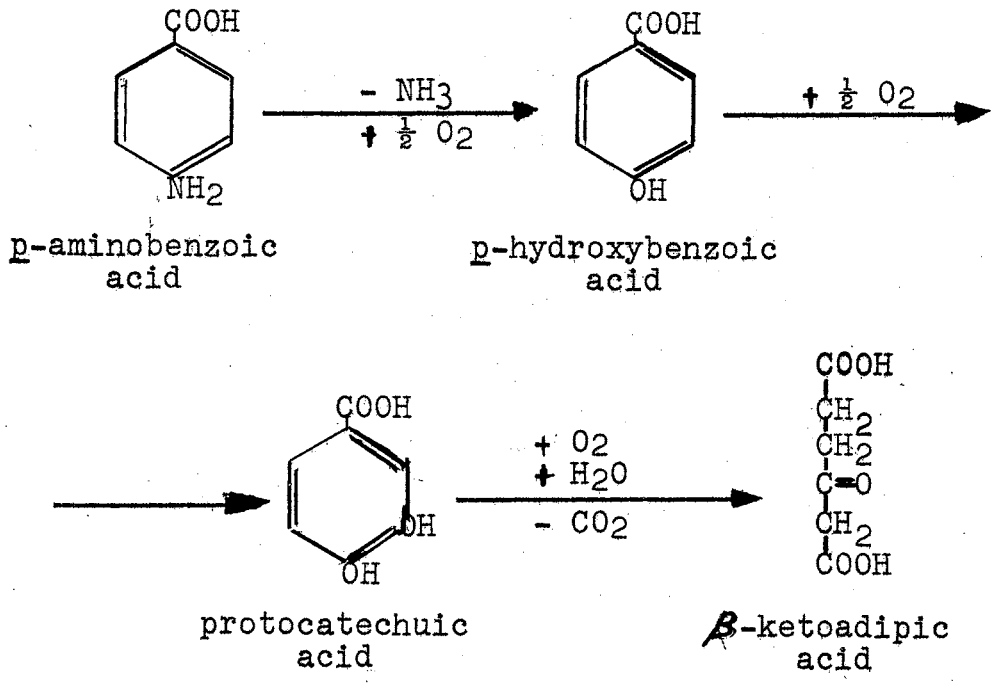
CHAPTER I

INTRODUCTION

The proposed mechanisms associated with the inhibition of essential metabolic reactions by various antimetabolites has been an area of numerous investigations in recent years. The benefits of such investigations are evident since antimetabolites have proven extremely useful as chemotheraputic agents and the "competitive analog-metabolite inhibition" technique (21) has been widely used in the study of biochemical processes.

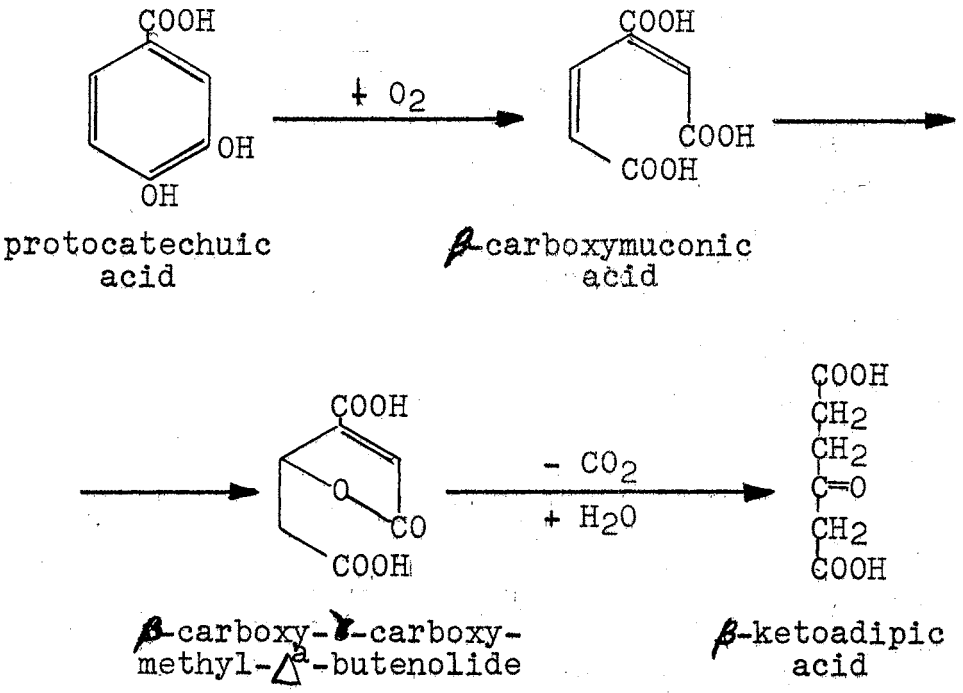
The significance of p-aminobenzoic acid in microbial nutrition was first emphasized when Woods (27) and Woods and Fildes (28) reported that this metabolite protected yeast against the growth inhibiting action of sulfanilamide. It was suggested that sulfanilamide, a structural analog of p-aminobenzoic acid, served as a competitive inhibitor of a microbial enzyme system for which p-aminobenzoic acid was an essential cofactor. The existence of a system requiring p-aminobenzoic acid as a cofactor became evident when Angier et al. (1) demonstrated that the p-aminobenzoic acid moiety occured in pteroic acid. A subsequent investigation (14) showed the primary point of sulfonamide inhibition was the synthesis of pteroylglutamic acid via p-aminobenzoic acid.

Davis (3) reported that p-aminobenzoic acid could affect the metabolism of Escherichia coli in three ways, depending on the concentration employed. At low concentrations p-aminobenzoic acid served as a normal vitamin, at moderate concentrations it could serve as a source of another vitamin, p-hydroxybenzoic acid, and at high concentrations it functioned as a growth antagonist by competitively inhibiting the p-hydroxybenzoic acid function(s). Durham (4) reported that in addition to the growth factor function, p-aminobenzoic acid could serve as an oxidizable substrate in certain microorganisms. Sequential induction studies showed that a strain of Pseudomonas fluorescens oxidatively degraded p-aminobenzoic acid via the following sequence:

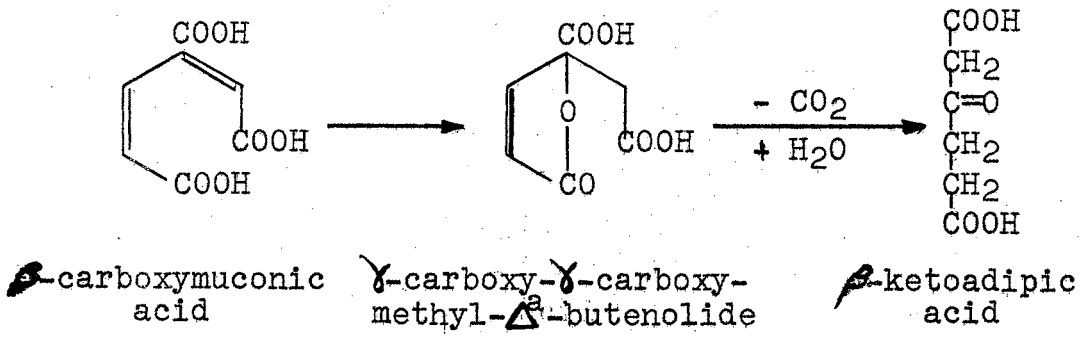


The mechanism of the enzymatic ring cleavage of protocatechuic acid has been investigated by several workers. Stanier et al. (24) reported a dried cell preparation of Ps.

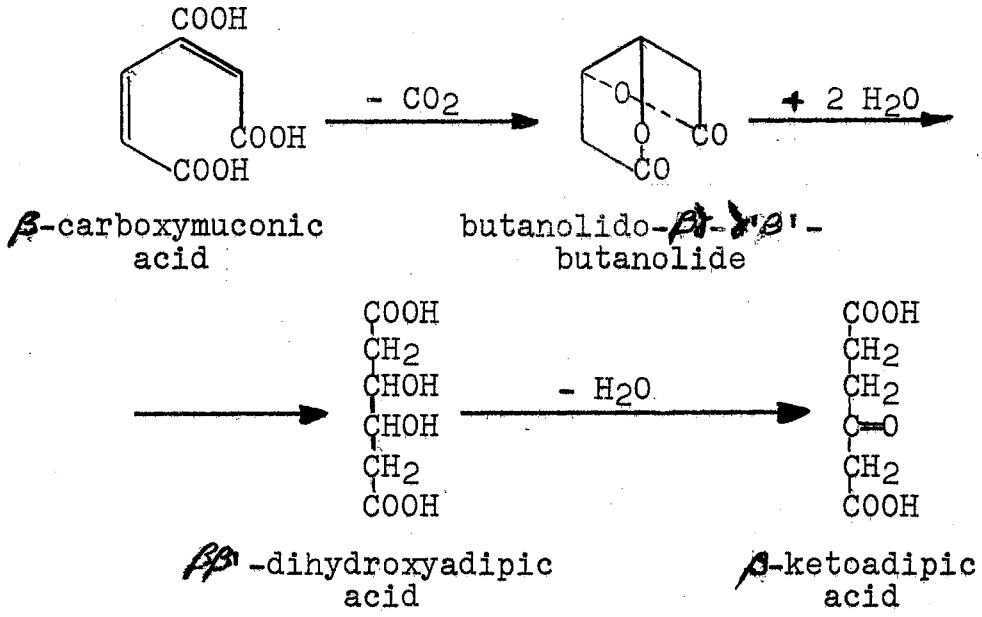
fluorescens in which protocatechuic acid was oxidized quantitatively to β-ketoadipic acid. The Neurospora crassa enzymes which metabolize protocatechuic acid were separated and examined by Gross et al. (9). These workers found that the Neurospora enzymes degrade protocatechuic acid via the following sequence:



More recently, Cain and Cartwright (2) suggested that in Nocardia erythropolis a 1:4 lactone (γ-carboxy-γ-carboxymethyl-Δ^2-butenolide) is the intermediate compound in the conversion of β-carboxymuconic acid to β-ketoadipic acid. This proposed pathway is illustrated as follows:



Another pathway by which β-carboxymuconic acid can be degraded to β-ketoadipic acid was reported by Ribbons and Evans (20). In cell-free extracts prepared from a Pseudomonas species, these workers demonstrated that a dilactone (butanolido-βγ-γ'β'-butanolide) or its straight-chain counterpart (ββ'-dihydroxyadipic acid) is the intermediate preceding β-ketoadipic acid. The Pseudomonas pathway is illustrated as follows:



The cleavage of β-ketoadipic acid has been demonstrated to occur in several microorganisms. Resting cells of a Vibrio species were found to cleave β-ketoadipic acid with succinic acid as one product (13). Katagii and Hayaishi (12) found that cell-free extracts prepared from Pseudomonas degrade β-ketoadipic acid to succinyl coenzyme A and acetyl coenzyme A. The work of Ottey and Tatum (19) demonstrated that an enzyme preparation of N. crassa cleaved β-ketoadipic acid to equimolar amounts of succinic and acetic acids with no

evidence of acyl coenzyme A derivatives.

The bacteriostatic activity of p-aminosalicylic acid for Mycobacterium tuberculosis was discovered by Lehman (15) in 1946. Youmans et al. (29) reported that the p-aminosalicylic acid inhibition of growth of the tubercle bacillus was due to anti-p-aminobenzoic acid activity. Similar findings by Goodacre et al. (8) and Ivanovics (11) demonstrated that the bacteriostatic activity of p-aminosalicylic acid was antagonized by p-aminobenzoic acid. The recognition of the competitive nature associated with the antagonism of p-aminosalicylic acid by p-aminobenzoic acid (16) indicated that the inhibitory action of p-aminosalicylic acid might be similar to that of the sulfonamides. The non-competitive reversal of p-aminosalicylic acid inhibition by methionine, biotin and certain purines (10) provided evidence that p-aminobenzoic acid may mediate their formation in the tubercle bacillus.

Durham (5) reported that p-aminosalicylic acid may competitively inhibit the oxidative dissimilation of p-aminobenzoic acid by Ps. fluorescens. The experiments described in this study were conducted in an attempt to elucidate the mechanism(s) of the antimetabolic activity of p-aminosalicylic acid toward p-aminobenzoic acid and protocatechuic acid utilization.

CHAPTER II

MATERIALS AND METHODS

Test organism.

The organism used in this study was isolated from soil by the enrichment technique and was identified as possessing the properties characteristic of a Flavobacterium. A stock culture of the organism was carried on a salts medium containing p-aminobenzoic acid as the sole carbon source.

Synthetic medium.

The defined medium used throughout the study was composed of NaCl, 0.1 g; NH_4Cl, 0.1 g; KH_2PO_4, 0.324 g; K_2HPO_4, 0.424 g; and agar, 2.0 g in 100 ml of distilled water. The desired carbon source was added at a level of 0.1 per cent and the pH adjusted to 7.0. Sterilization was performed by autoclaving at 121° C at 15 pounds pressure for 15 minutes. The sterilized medium was then allowed to cool to 52° C and 0.1 ml of a mineral salts solution was added aseptically.

The mineral salts solution was composed of $MgSO_4 \cdot 7H_2O$, 5.0 g; $MnSO_4$, 0.1 g; $FeCl_3$, 1.0 g; and $CaCl_2$, 0.5 g in 100 ml of distilled water. This solution was sterilized separately.

The basic formula supplemented with p-aminobenzoic or protocatechuic acid as the carbon source was used to grow en-

zymatically induced cells. Asparagine was used as the carbon source to grow cells non-induced to the aromatic acids.

Preparation of cell suspensions.

The cell suspensions used in respirometric and incubation experiments were obtained from 20-24 hour plate cultures grown at 37° C. The resulting growth was harvested, washed twice by centrifugation and resuspended in 0.01 M phosphate buffer of pH 7.0. The cell suspensions were adjusted to a standard turbidity such that a 1/20 dilution of the suspension read 60 per cent transmittance at 540 mμ in a Bausch and Lomb "Spectronic 20". This turbidity was equivalent to a dry cell weight of 1.4 mg per ml and the suspension contained approximately 4×10^9 cells per ml.

Preparation of cell extracts.

Cell-free extracts were prepared from a heavy cell suspension cultured as described above. The organisms were ruptured using a French pressure cell with a pressure of 20,000 pounds per square inch at a delivery rate of approximately one drop per second. The cell debris was removed by centrifugation at 18,000 x g for 30 minutes with the resulting supernatant being used in experiments involving cell extracts. No attempts were made to compare the protein content of the crude extracts with the protein content of the standardized cell suspensions.

Respirometer experimentation.

All respirometric experiments were performed in the War-

burg apparatus at a temperature of 37° C with air as the gas phase. Warburg vessels with double side-arms were employed in this study. For oxygen uptake determinations the cell suspension or extract was pipetted into the main chamber, 0.2 ml of 20 per cent potassium hydroxide was added to the center well containing a piece of fluted filter paper, and varying concentrations of substrate and inhibitor were added to the side-arms.

The carbon dioxide evolved was measured by the direct method (26). In this method three flasks were employed for each determination. One flask measured oxygen uptake in the absence of carbon dioxide as described above. The other two flasks were prepared with 0.2 ml of 1 N hydrochloric acid in the side-arm with no potassium hydroxide present in the vessel. The hydrochloric acid was introduced into the main chamber of one flask at the start of the experiment and to the second flask after the desired interval. Hydrochloric acid served to stop the reaction at the desired time and to release the carbon dioxide bound in the buffered system. The carbon dioxide evolved during the interval was calculated by subtracting the change in reading on the flask with alkali from the change in reading on the flask with no alkali.

Incubation experiments.

Incubation experiments were conducted under conditions closely approximating the respirometric procedure. Mixtures of substrate and inhibitor were added to the cell suspension and the stoppered containers were shaken in the Warburg water

bath at a temperature of 37° C. Aliquots were withdrawn from the vessel at desired times and the substrate immediately removed by filtration through a millipore type HA filter. The filtrate collected was assayed for substrate and/or inhibitor. In certain experiments, the cells trapped on the filter membrane were washed and resuspended in the phosphate buffer for use in subsequent experiments.

p-Aminobenzoic acid assay.

p-Aminobenzoic acid was determined colorimetrically by a previously described procedure (6). The coupling reaction was performed by diluting an aliquot of the filtrate to a total volume of 3.5 ml, and treating as follows: Add 1.0 ml of 7.5 per cent trichloroacetic acid, shake and allow to stand for 5 minutes; add 1.0 ml of 0.025 per cent sodium nitrite and stand 20 minutes; add 1.0 ml of 0.5 per cent ammonium sulfamate, stand for 20 minutes; finally, add 2.0 ml of 0.063 per cent N-(1-naphthyl)-ethylenediamine dihydrochloride. The color was permitted to develop for 40 minutes after which the sample was diluted with 15 ml of distilled water and the percentage transmittance read in a spectrophotometer at 560 mμ. Values were then calculated from a standard curve prepared concurrently. Controls indicated that none of the compounds, at the concentrations employed in the incubations, interfered with this test.

p-Aminosalicylic acid assay.

Estimation of p-aminosalicylic acid was also performed

colorimetrically as previously described (7). The coupling reaction was performed as follows: To a sample of filtrate diluted to 5.5 ml; add 3.0 ml of 20 per cent p-toluenesulfonic acid in 1-50 hydrochloric acid; shake, allow to stand 5 minutes; add 1.0 ml of buffer consisting of 15.76 per cent citric acid in a 1.4 per cent sodium hydroxide solution; add 2.0 ml of 2 per cent p-dimethylaminobenzaldehyde in ethanol; mix and read in a spectrophotometer against a reagent blank at 450 mμ. Values were calculated from a standard curve developed concurrently.

Controls indicated that p-aminobenzoic acid interfered with the determinations. In experiments in which both p-aminobenzoic acid and p-aminosalicylic acid were added to the incubation vessel, it was necessary to permit depletion of p-aminobenzoic acid by the cell suspension before an accurate determination of the p-aminosalicylic acid could be made. Tests indicated that other compounds employed in the incubations did not interfere with the assay.

Protocatechuic acid assay.

The colorimetric determination of protocatechuic acid was a modification of a previously described procedure (23). The filtrate aliquot was diluted to 4.0 ml to which an addition of 1.0 ml of an aqueous solution containing 0.2 per cent ferrous sulfate and 1.0 per cent sodium potassium tartrate was made. After allowing the mixture to stand for 10 minutes, 5.0 ml of 20 per cent ammonium acetate were added and the purple color was read immediately in a spectrophotometer

at 560 mμ. Values were determined from a standard curve which had been developed concurrently. Controls indicated that other components of the incubation did not interfere with the assay.

β-Ketoadipic acid assay.

β-Ketoadipic acid in cell extracts was estimated manometrically by a catalytic decarboxylation procedure (22). After the desired incubation period the contents of the Warburg vessel were acidified by adding 1.5 ml of 1 N acetic acid-sodium acetate buffer of pH 3.8. Following a short equilibration period at 25° C, 0.4 ml of 0.1 M 4-aminoantipyrine was added from the side-arm. The concentration of β-ketoadipic acid was calculated from the microliters of carbon dioxide liberated.

Chromatographic procedure.

β-Ketoadipic acid was identified in cell-free extracts by chromatography of the 2,4-dinitrophenylhydrazone. The methods used in preparation and extraction of the hydrazone were essentially those described by Strassman et al. (25). After incubating 4 ml of cell extract with 20 μmoles of protocatechuic acid at 37° C the reaction was stopped by chilling in an ice bath and adding 0.2 ml of 0.66 N sulfuric acid. The protein was precipitated by the addition of 2 ml of tungstic acid solution made by dissolving 3.3 g of $Na_2WO_4 \cdot 2H_2O$ in 80 ml of water and 20 ml of 0.66 N sulfuric acid. The precipitated protein was removed by centrifugation in the cold.

Three ml of 0.2 per cent 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid were added to the supernatant in a 15 ml glass stoppered centrifuge tube. The solution was shaken and allowed to stand at room temperature for 20 minutes. The derivative was extracted with successive portions of ether until the extract was colorless. The combined ether extracts were evaporated and the residue dissolved in a mixture of 6 ml of chloroform and 2 ml of 1 N ammonium hydroxide. The chloroform layer was further extracted with successive portions of 1 N ammonium hydroxide and the washings were added to the first ammonia extract. The ammonia solution was adjusted to a pH of 1.0 with 6.00 N sulfuric acid and was extracted with ether until colorless. The ether solution was evaporated to dryness and stored under vacuum desiccation at 4° C. Samples of authentic β-ketoadipic acid and of a 0.01 M phosphate buffer control were treated similarly to serve as chromatographic standards.

The dinitrophenylhydrazones were dissolved in 0.5 ml of 2 N ammonium hydroxide and spotted on sheets of Whatman No. 1 paper. The chromatograms were developed by ascending chromatography employing N butanol:water:ethanol in a v/v ratio of 40:50:10 or isopropanol:water:ammonium hydroxide (sp. gr. 0.896) in a v/v ratio of 100:10:5 as the solvent systems.

Chemicals.

All chemical compounds used in this study were obtained commercially. The compounds used as substrates or inhibitors for respirometric or incubation experiments were dissolved in

0.01 M phosphate buffer and adjusted to a pH of 7.0. All inhibitor/substrate ratios are calculated on a molar basis.

Tabulation of experimental data.

The experimental data are illustrated graphically and tabularly in Chapter III. The following abbreviations are used in the graphs and tables: Asparagine (ASN), β-ketoadipic acid (KA), p-aminobenzoic acid (PAB), p-aminosalicylic acid (PAS), protocatechuic acid (PROTO) and succinic acid (SUC).

CHAPTER III

EXPERIMENTAL AND RESULTS

The influence of p-aminosalicylic acid on the oxidation of various substrates.

Cell suspensions of p-aminobenzoic acid-grown Flavobacterium are capable of oxidizing p-aminobenzoic acid quite rapidly. The addition of p-aminosalicylic acid to cells actively metabolizing p-aminobenzoic acid in respirometeric experiments results in a marked decrease in the rate of p-aminobenzoic acid oxidization. Figure 1 illustrates the typical results obtained when the substrate concentration was maintained at a constant level and the concentration of the inhibitor was varied to give inhibitor/substrate ratios of 0/1, 3/1, 5/1 and 10/1. The p-aminobenzoic acid-grown cells were not able to oxidize p-aminosalicylic acid as indicated by the inhibitor controls. However, the control containing 20 μmoles of p-aminosalicylic acid did show a slight but consistant increase over the endogenous respiration. The significance of this oxidative reaction is discussed in forthcoming sections.

Additional experiments were conducted with the p-aminobenzoic acid-grown cells to determine if p-aminosalicylic acid influences the oxidation of proposed dissimilatory intermediates of p-aminobenzoic acid. Figures 2 and 3 show the ef-

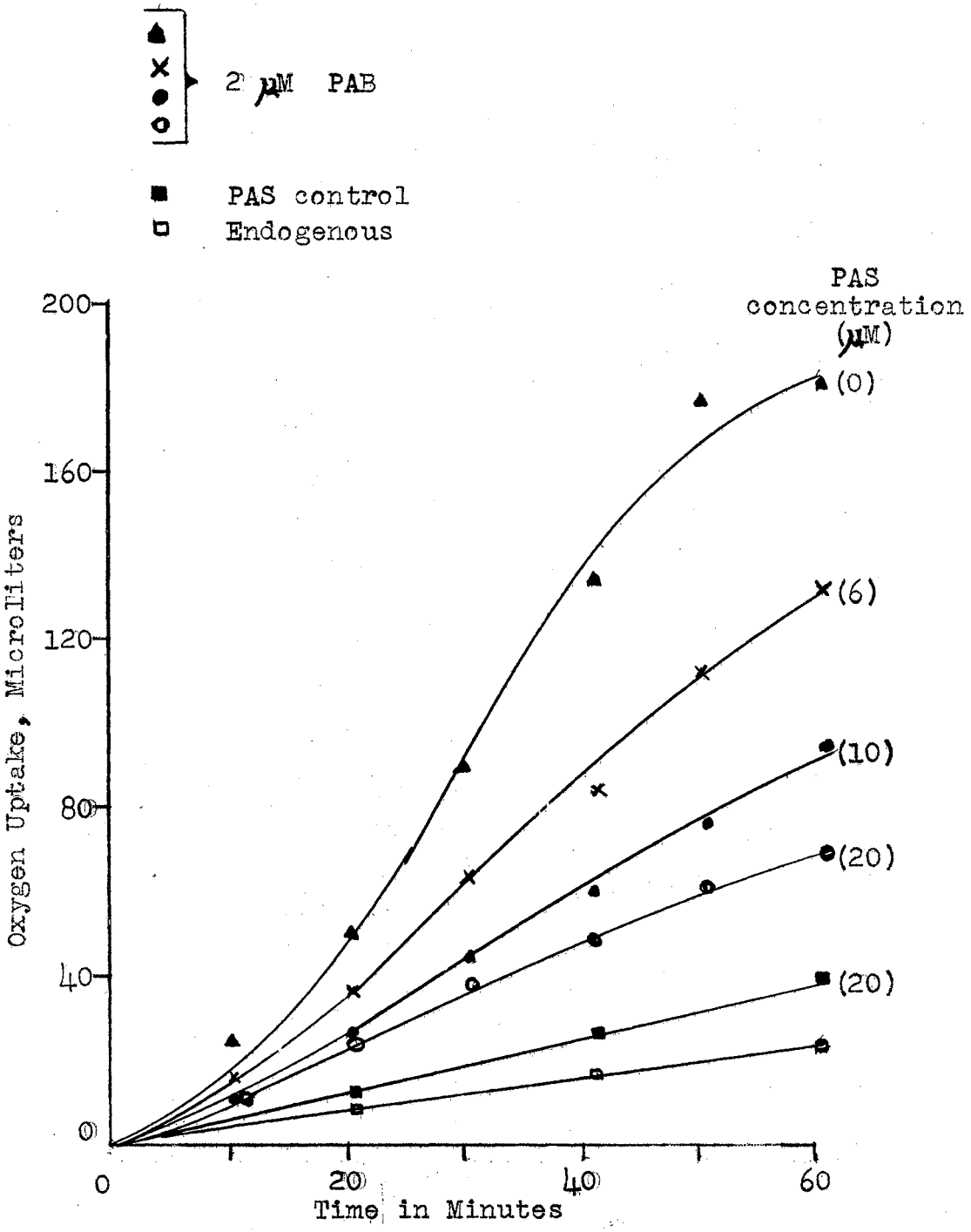


Figure 1. The Oxidation of p-Aminobenzoic Acid by p-Aminobenzoic Acid-Grown Cells in the Presence of Varying Concentrations of p-Aminosalicylic Acid

fects of p-aminosalicylic acid on the oxidation of various substrates at an inhibitor/substrate ratio of 5/1. Results obtained from these studies indicate that the oxidation of 2 μmoles of protocatechuic acid (3,4-dihydroxybenzoic acid) was inhibited by the presence of 10 μmoles of p-aminosalicylic acid. In contrast to this finding was the observation that the oxidation of β-ketoadipic acid and succinic acid were not significantly affected by the antagonist. The inability of p-aminosalicylic acid to influence the oxidation of the straight-chain intermediates eliminated the possibility that the antagonist might 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. Respirometric experiments were performed in which the competitive inhibition of p-aminobenzoic acid by p-aminosalicylic acid was followed for 60 minutes at inhibitor/substrate ratios of 0/1, 3/1, 5/1 and 10/1. Figure 4 shows these results and indicates that within 15 minutes after the introduction of additional substrate (8 μmoles at 60 minutes) the oxidation proceeded at an increased rate in the higher ratio of 10/1 where the inhibition was not completely reversed and at an uninhibited rate in the lower ratios of 0/1, 3/1, and 5/1.

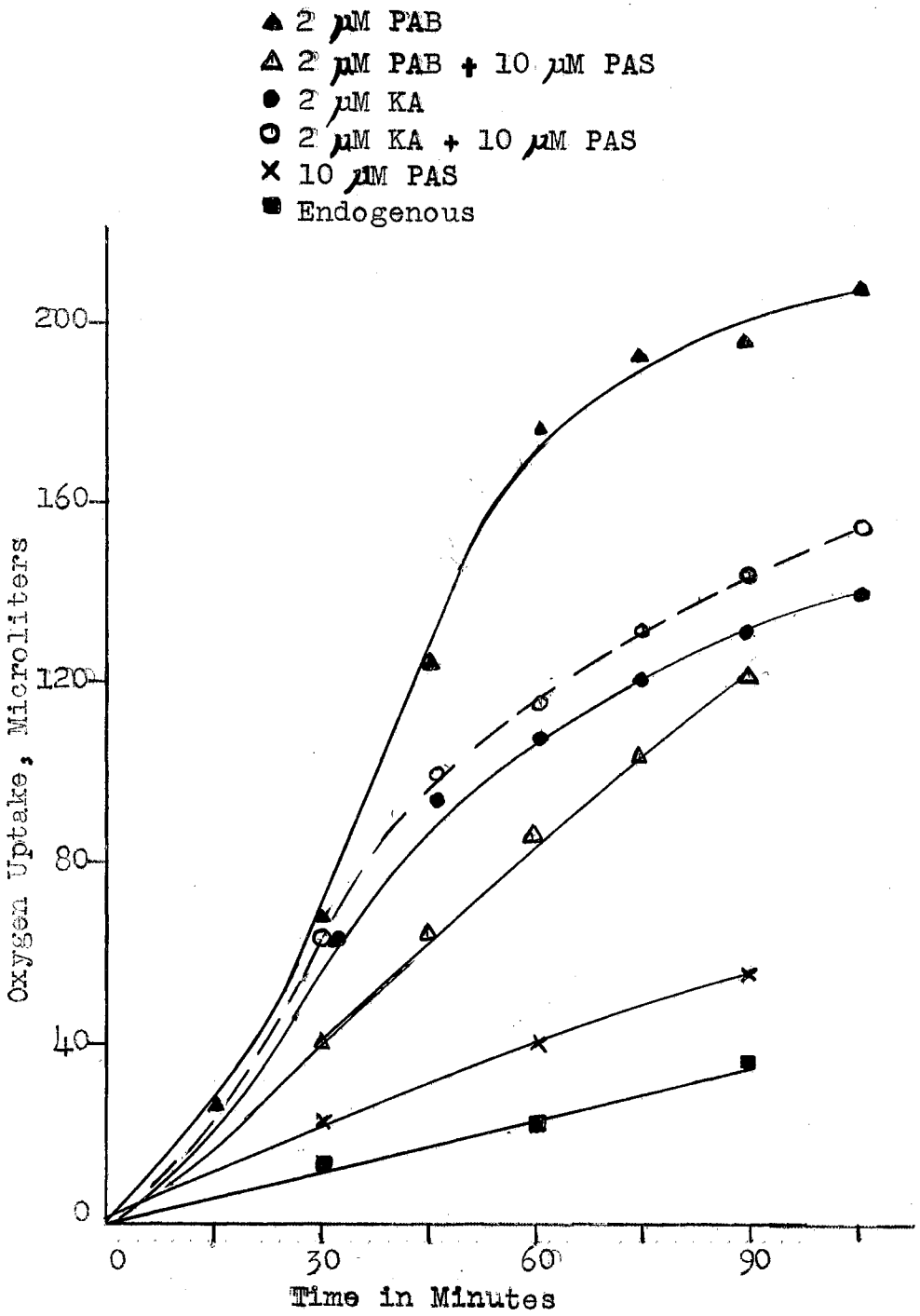


Figure 2. The Influence of p-Aminosalicylic Acid on the Oxidation of p-Aminobenzoic Acid and β-Ketoadipic Acid by p-Aminobenzoic Acid-Grown Cells

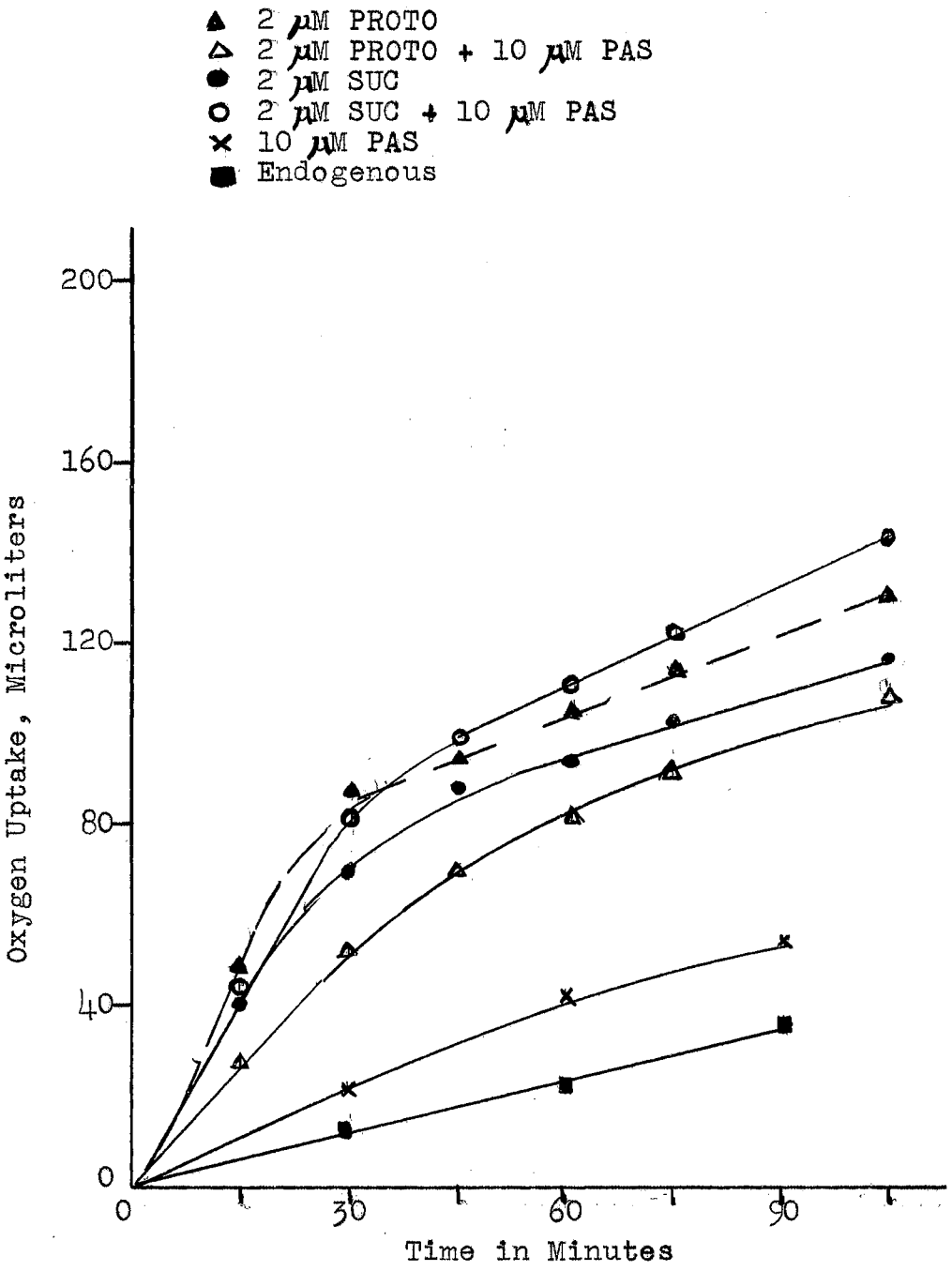


Figure 3. The Influence of p-Aminosalicylic Acid on the Oxidation of Protocatechuic Acid and Succinic Acid by p-Aminobenzoic Acid-Grown Cells

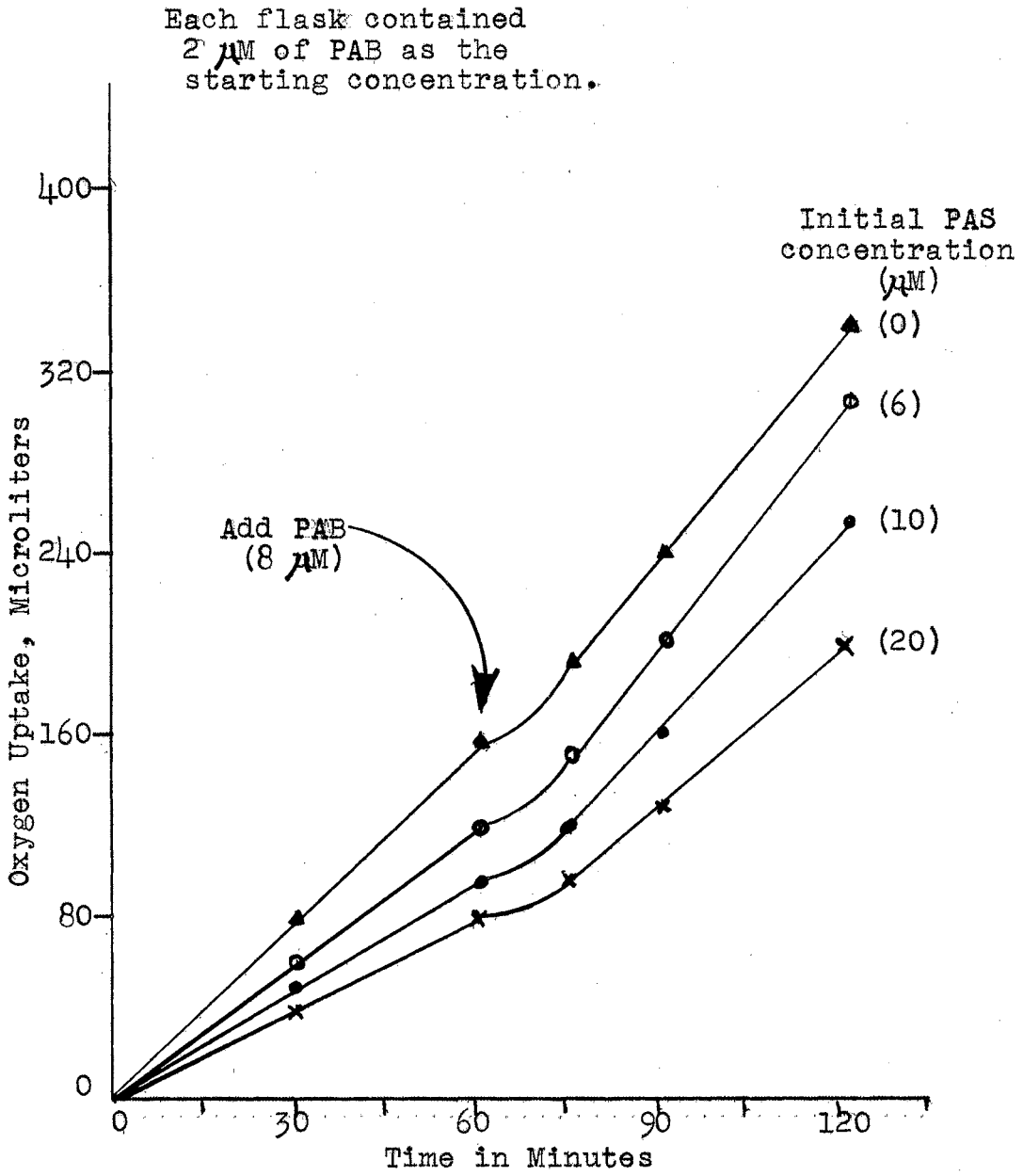


Figure 4. Reversal of the Competitive Inhibition of p-Aminobenzoic Acid by Addition of Excess Substrate

Failure to completely reverse the inhibition in the higher ratio was due to the addition of insufficient quantities of the substrate.

Influence of prior exposure to inhibitor and time of addition of inhibitor on substrate oxidation.

Manometric experiments were performed to determine the effect of prolonged exposure of the cells to p-aminosalicylic acid prior to the addition of the substrate and the effectiveness of the inhibitor when added to cells activity metabolizing p-aminobenzoic acid. Figure 5 shows the results obtained with inhibitor/substrate ratios of 5/1 and 10/1 with the inhibitor added at the time of substrate addition and the inhibitor added 20 minutes after adding the substrate. Inhibition of substrate oxidation was observed immediately in the flasks in which the inhibitor was added at 0 minutes. Within a few seconds following addition of the antimetabolite to the cells which were actively metabolizing the substrate, the normal pattern of competitive inhibition was observed. These findings suggest that the "sensitive site" must be readily accessible to the inhibitor as indicated by immediate susceptibility of substrate oxidation. One of the first available "sites" could be the specific transport mechanism of the cell membrane. If the inhibition occurs at the internal oxidative system, the inhibitor must readily permeate the cell membrane in order to reach the sensitive "oxidative site" and exert its effect.

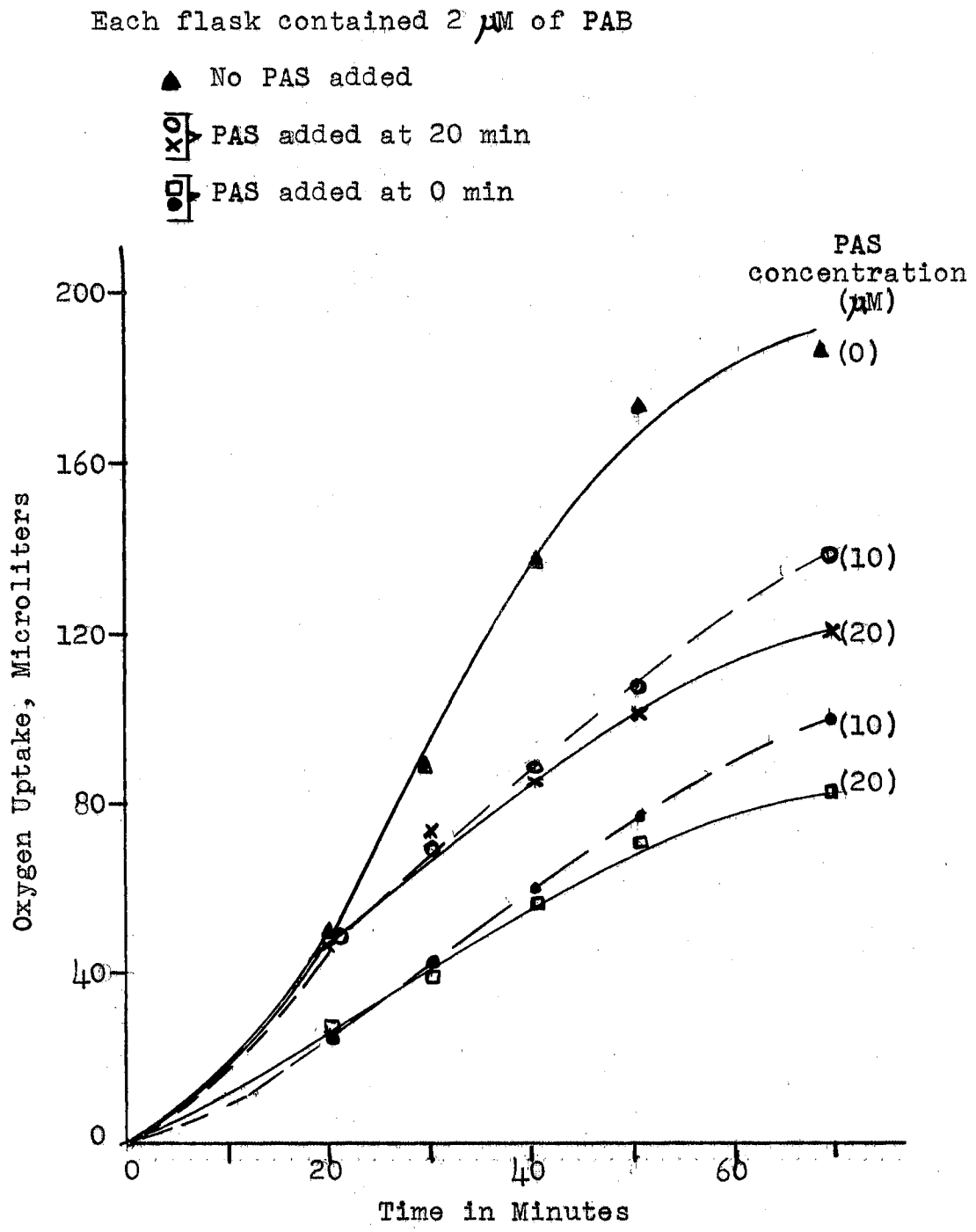


Figure 5. The Effectiveness of p-Aminosalicylic Acid as an Inhibitor when Added to Cells Actively Metabolizing p-Aminobenzoic Acid at Different Times

In related experiments, p-aminobenzoic acid-grown cells were exposed to 10 μmoles and 20 μmoles of p-aminosalicylic acid for 60 minutes 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 in controls in which the substrate and inhibitor were added simultaneously. Figure 6 shows the results obtained in this investigation. These data indicate that exposing the cells to p-aminosalicylic acid prior to introducing the substrate exerts little, if any, additional influence on the oxidation of p-aminobenzoic acid. The results also indicate 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. Since the time of addition of the inhibitor does not appear to be an important factor, the results suggest that the inhibition is not due to an internal accumulation of p-aminosalicylic acid or to accumulated products of its degradation.

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 respirometric experiments were conducted in which the uptake of p-aminobenzoic acid by cell suspensions was followed colorimetrically. The data presented in Table I experiment A are representative of the findings obtained in this investigation. The results show that

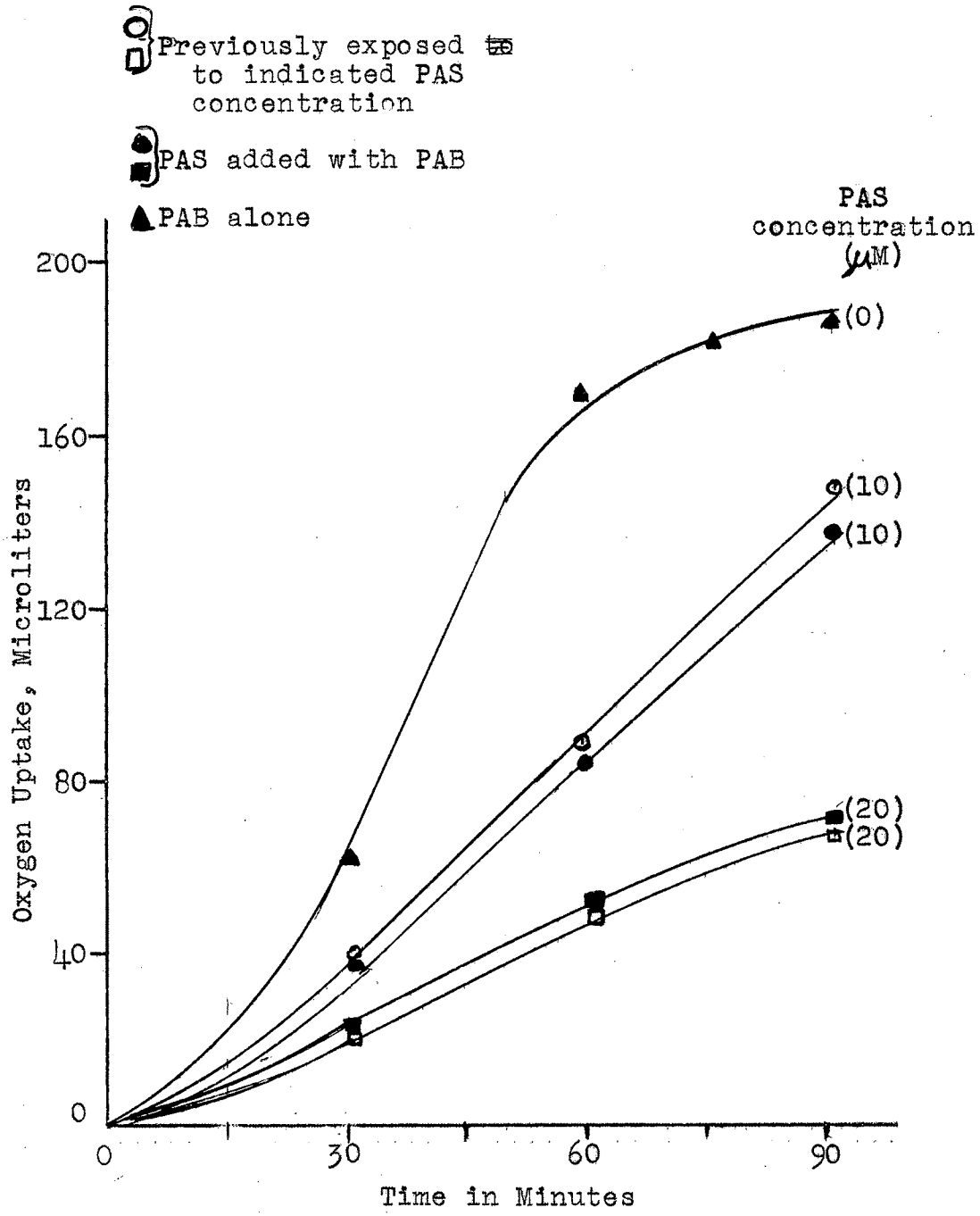


Figure 6. The Oxidation of 2 Micromoles of p-Aminobenzoic Acid by p-Aminobenzoic Acid-Grown Cells Previously Exposed to 10 and 20 Micromoles of p-Aminosalicylic Acid

in the absence of the inhibitor all of the substrate (116 µg per ml) was depleted from the medium within 45 minutes. In the presence of p-aminosalicylic acid the substrate was taken up at a much slower rate. The data show that the amount of p-aminobenzoic acid depleted from the medium decreased with increasing inhibitor 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 influence of p-aminosalicylic acid on the uptake of p-aminobenzoic acid was examined in lower inhibitor and substrate concentrations. The disappearance of substrate from the medium was determined in inhibitor/substrate ratios of 0/1, 3/1, 5/1 and 10/1 with a p-aminobenzoic acid concentration of 3.1 µg per ml. Table I experiment B illustrates the results obtained from this experimentation in which the disappearance of p-aminobenzoic acid was measured after a 30 second incubation period. The data indicate that the amount of substrate remaining in the medium was greater in the reaction vessels containing the higher inhibitor concentrations.

These findings suggest that the inability of the substrate to penetrate the cell in the presence of the inhibitor may be responsible for the decreased rate of oxidation. Studies following depletion of the substrate both at high and extremely low levels indicate that the ratio of inhibitor/substrate present in the system regulates the uptake of substrate by the cell suspension. These findings would then support

TABLE I

THE CELLULAR UPTAKE OF p-AMINOBENZOIC ACID BY p-AMINOBENZOIC ACID-GROWN CELLS IN THE PRESENCE AND ABSENCE OF VARYING CONCENTRATIONS OF p-AMINOSALICYLIC ACID

Exp.	Time in Minutes	Concentration of PAB (μg per ml) Remaining in Filtrate			
		Ratio: PAS/PAB			
		0/1	3/1	5/1	10/1
A	0	116.0	116.0	114.6	116.0
	15	80.0	87.2	97.8	103.2
	30	17.3	64.0	86.8	99.0
	45	0.0	46.6	76.6	97.0
	Time in Seconds				
B	0	3.10	3.10	3.10	3.10
	30	0.15	0.43	0.77	1.65

the postulation that the inhibition is associated with the specific transport mechanism rather than the internal oxidative enzymes.

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 were unsuccessful.

Uptake of p-aminosalicylic acid by cell suspensions.

Further studies were conducted to determine if p-aminobenzoic acid-grown cells are capable of depleting p-aminosalicylic acid from the medium. Table II illustrates typical results obtained when cell suspensions were incubated with three different concentrations of p-aminosalicylic acid. In the range tested, the results indicate 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). Such an internal antagonism would be dependent on the presence of varying concentrations of the inhibitor inside the cell.

Additional experiments were performed to determine if the antagonism could be more closely correlated with the external concentration of p-aminosalicylic acid. A p-aminoben-

TABLE II

THE CELLULAR UPTAKE OF p-AMINOSALICYLIC ACID BY p-AMINOBENZOIC ACID-GROWN CELLS

Time in Minutes	Concentration of PAS (µg per ml) Remaining in Filtrate		
0	10.44	20.88	41.76
20	6.00	15.00	39.40
40	3.36	10.90	33.60
60	1.04	8.36	29.40
80	----	----	27.60
100	----	----	25.80
120	----	----	24.00

zoic acid-grown cell suspension was incubated with 20.88 μg of p-aminosalicylic acid for 70 minutes after which 6.2 μg of p-aminobenzoic acid was added to the reaction vessel and the disappearance of substrate followed. An analysis indicated that the cells had depleted 9.6 μg of the antimetabolite at the time the substrate was added. The depletion of substrate was compared with the uptake observed in a control vessel in which 6.2 μg of p-aminobenzoic acid and 20.88 μg of the inhibitor were added to the cells simultaneously. Results obtained from this study are shown in Table III.

TABLE III

THE EFFECT OF THE EXTRACELLULAR p-AMINOSALICYLIC ACID CONCENTRATION ON THE UPTAKE OF p-AMINOBENZOIC ACID BY p-AMINOBENZOIC ACID-GROWN CELLS

Time in Minutes	Concentration of PAB (μg per ml) Remaining in Filtrate	
	PAB and PAS Added to Cells Simultaneously	Cells Incubated with PAS Prior to PAB Addition
0	6.20	6.20
2.5	2.13	0.93

The data presented in Table III show that the amount of substrate depleted by the cells which had been exposed to and depleted some of the inhibitor prior to the addition of p-aminobenzoic acid was greater than the amount of substrate taken up by the cells when the substrate and p-aminosalicylic acid were added simultaneously. These findings indicate that

the p-aminosalicylic acid taken up by the cells apparently does not influence the utilization of p-aminobenzoic acid. It appears that the concentration of the inhibitor present in the external environment is responsible for the inhibition of substrate utilization since less p-aminobenzoic acid was depleted by the cells in which the inhibitor and substrate were added simultaneously than by the cells which were permitted to deplete 9.6 μg of the antimetabolite. 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 hours were washed extensively with phosphate buffer and the washing assayed for the inhibitor. Cell aliquots were also ruptured in a French pressure cell 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. The filtrates and the washings from the cell suspension which had depleted the antagonist were examined in a Beckman DU spectrophotometer and no ultraviolet absorbing compound could be detected. This tended to eliminate the possibility that p-aminosalicylic acid was enzymically transformed to an aromatic compound which was not detected by the coupling reaction. These observations suggest that the antimetabolite is bound or metabolized immediately upon entering the cell and is not available in the

"free form" to compete with the substrate for internal enzyme system(s).

The oxidative reaction associated with p-aminosalicylic acid uptake.

The results presented in Figure 1 showed that the presence of 20 μmoles of p-aminosalicylic acid caused an oxidation reaction higher than that observed in the endogenous control. Further manometric studies were conducted to determine if the increased oxidation was observed when cells grown on other carbon sources were incubated with the inhibitor. Table IV shows a typical experiment in which the respiration of p-aminobenzoic acid, protocatechuic acid and asparagine-grown cells was measured in the presence and absence of 20 μmoles of p-aminosalicylic acid. In each case the flask containing the p-aminosalicylic acid showed greater oxygen uptake than the corresponding endogenous flask.

TABLE IV

THE OXIDATION OF p-AMINOSALICYLIC ACID BY CELLS GROWN ON ASPARAGINE, PROTOCATECHUIC ACID AND p-AMINOBENZOIC ACID

Time in Minutes	Oxygen Uptake (μL) in the Presence and Absence of PAS					
	ASN-Grown		PROTO-Grown		PAB-Grown	
	With PAS	No PAS	With PAS	No PAS	With PAS	No PAS
0	0	0	0	0	0	0
15	5	0	5	2	9	4
30	13	5	13	8	20	10
45	22	13	23	14	32	17
60	27	14	28	17	40	21
75	35	20	36	23	48	28

The depletion of the antimetabolite was followed colorimetrically in these same cells. Shown in Table V are the data obtained from colorimetric analysis of filtrates collected when the cell suspensions were incubated with 13.96 μg of p-aminosalicylic acid per ml. Here it was seen that only those cells grown on p-aminobenzoic acid showed an appreciable uptake of the antimetabolite, these cells took up 8.78 μg of p-aminosalicylic acid while the protocatechuic acid and asparagine-grown cells depleted only 0.46 and 0.38 μg respectively.

TABLE V

THE CELLULAR UPTAKE OF p-AMINOSALICYLIC ACID BY CELLS GROWN ON ASPARAGINE, PROTOCATECHUIC ACID AND p-AMINOBENZOIC ACID

Time in Minutes	Concentration of PAS (μg per ml) Remaining in Filtrate		
	ASN-Grown	PROTO-Grown	PAB-Grown
0	13.92	13.92	13.96
80	13.54	13.46	5.14

These observations suggest that only the cells grown on p-aminobenzoic acid are capable of immediately depleting significant quantities of p-aminosalicylic acid from the medium. The cells grown on asparagine and protocatechuic acid showed the increased oxidation in the presence of p-aminosalicylic acid (Table IV) but did not show a significant uptake of the antimetabolite (Table V). The cells grown on p-aminobenzoic

acid showed the increased oxidation in the presence of p-aminosalicylic acid and also showed a measurable uptake of the antimetabolite. These observations would tend to eliminate the possibility that the increased oxidation is a consequence of the assimilation of p-aminosalicylic acid since the cells which showed markedly smaller uptake demonstrated approximately the same degree of increased oxidation as cells which showed greater uptake.

Influence of extraneous carbon sources on the uptake of p-aminosalicylic acid by cell suspensions.

Studies were conducted to determine if the presence of an added oxidizable substrate influenced the rate at which p-aminosalicylic acid was depleted from the medium by p-aminobenzoic acid-grown cells. Uptake of the inhibitor was followed in the presence of protocatechuic acid, succinic acid and p-aminobenzoic acid in a p-aminosalicylic acid to substrate ratio of 1/3.3 (Table VI). Since p-aminobenzoic acid interfered with p-aminosalicylic acid determinations, it was necessary that this substrate be depleted from the medium before inhibitor concentrations could be determined. The results indicate that uptake of the inhibitor in these cells permitted to oxidize other carbon sources did not differ greatly from the uptake observed in a resting cell control which was not permitted to metabolize the extraneous substrates.

TABLE VI

THE INFLUENCE OF EXTRANEOUS CARBON SOURCES ON THE CELLULAR UPTAKE OF p-AMINOSALICYLIC ACID BY p-AMINOBENZOIC ACID-GROWN CELLS

Time in Minutes	Concentration of PAS (µg per ml) Remaining in Filtrate			
	PAS Alone	PAS/SUC (1/3.3)	PAS/PROTO (1/3.3)	PAS/PAB (1/3.3)
0	41.8	41.8	41.8	41.8
15	38.0	37.8	39.2	----
30	37.2	38.0	40.0	----
45	34.6	37.2	36.8	----
60	34.8	34.6	38.2	----
90	32.8	28.8	31.2	----
120	27.8	25.8	29.2	28.8

p-Aminobenzoic acid uptake in p-aminosalicylic acid-induced cells.

In other experimentation it was found that asparagine-grown cells which had previously been exposed to p-aminosalicylic acid were capable of taking up greater quantities of p-aminobenzoic acid than cells which had not received this treatment. Further experiments were then performed to compare the uptake of both p-aminobenzoic acid and p-aminosalicylic acid in asparagine-grown cells induced by exposure for 120 minutes to p-aminosalicylic acid, p-aminobenzoic acid, and a combination of p-aminosalicylic acid and p-aminobenzoic acid. To conduct these studies, 18 ml of the non-induced asparagine-grown cells were pipetted into 3 containers and p-aminosalicylic acid (90 μmoles) was added to the first container, p-aminobenzoic acid (9 μmoles) was added to the second container, and p-aminosalicylic acid (90 μmoles) plus p-aminobenzoic acid (9 μmoles) were added to the third container. The cell suspensions were then incubated for 160 minutes at 37° C. Triplicate 5 ml samples were taken from each container and the cells removed immediately by filtration through a millipore filter. The cells retained on the filter membrane were washed and resuspended in 0.01 M phosphate buffer containing 120 μg per ml of chloramphenicol. The resuspended cells were then incubated with p-aminosalicylic acid (6.96 μg per ml), p-aminobenzoic acid (6.2 μg per ml), and with a combination of p-aminosalicylic acid (69.6 μg per ml) and p-aminobenzoic acid (6.2 μg per ml). After the designated incuba-

tion periods (Table VII) the cells were removed by filtration and the filtrates were assayed for p-aminobenzoic acid or p-aminosalicylic acid.

The results obtained from these studies are illustrated in Table VII. The results indicate that cells induced by exposure to p-aminosalicylic acid, p-aminobenzoic acid, and the p-aminosalicylic acid plus p-aminobenzoic acid mixture were capable of depleting p-aminobenzoic acid. However, the p-aminobenzoic acid-induced cells depleted the greatest quantity of p-aminobenzoic acid. These cells took up 6.2 μg of p-aminobenzoic acid in less than 5 minutes while the p-aminosalicylic acid-induced cells and the cells exposed to the p-aminosalicylic acid-p-aminobenzoic acid mixture depleted only 3.56 μg and 5.57 μg in 30 minutes. Correcting for the different times of incubation shows that the p-aminobenzoic acid-induced cells took up at least 11 times as much p-aminobenzoic acid per unit time as the p-aminosalicylic acid-induced cells and at least 7 times as much p-aminobenzoic acid per unit time as the cells induced by exposure to the p-aminosalicylic acid-p-aminobenzoic acid mixture. Since the p-aminobenzoic acid-induced cells had depleted all of the p-aminobenzoic acid in less than 5 minutes, the exact quantitative relationship is not available.

Similarly, it was seen that the p-aminobenzoic acid-exposure induced the greatest amount of p-aminosalicylic acid uptake. p-Aminosalicylic acid was observed to inhibit the inducing effect of p-aminobenzoic acid for uptake of p-aminosal-

TABLE VII

THE CELLULAR UPTAKE OF p-AMINOBENZOIC ACID AND p-AMINOSALICYLIC ACID BY ASPARAGINE-GROWN CELLS INDUCED BY EXPOSURE TO p-AMINOSALICYLIC ACID, p-AMINOBENZOIC ACID, AND A MIXTURE OF p-AMINOSALICYLIC AND p-AMINOBENZOIC ACIDS

Time in Minutes	INDUCED BY EXPOSURE TO PAS			INDUCED BY EXPOSURE TO PAB			INDUCED BY EXPOSURE TO PAS/PAB (10/1)	
	Incubated with PAS	Incubated with PAB	Incubated with PAS/PAB (10/1)	Incubated with PAS	Incubated with PAB	Incubated with PAS/PAB (10/1)	Incubated with PAS	Incubated with PAB
	µg of PAS Remaining	µg of PAB Remaining	µg of PAB Remaining	µg of PAS Remaining	µg of PAB Remaining	µg of PAB Remaining	µg of PAS Remaining	µg of PAB Remaining
0	6.96	6.20	6.20	6.96	6.20	6.20	6.96	6.20
5	----	----	----	----	0.0	2.42	----	----
30	----	2.64	4.36	----	----	----	----	0.63
60	6.70	----	----	2.44	----	----	6.76	----

icylic acid when the combination of the inhibitor and substrate were incubated with the non-induced cells. The inhibitor/substrate ratio of 10/1 was found to induce p-aminosalicylic acid uptake to approximately the same level as observed when p-aminosalicylic acid alone had served as the inducer. As previously stated, exposing the cells to the inhibitor/substrate ratio 10/1 induced the uptake of p-aminobenzoic acid to a level only slightly higher than that observed when p-aminobenzoic acid alone had been used as the inducer. These findings indicate that the antimetabolite may be able to inhibit the formation of its own "uptake system" as well as the formation of the p-aminobenzoic acid "uptake system" and suggests that p-aminosalicylic acid and p-aminobenzoic acid may be transported into the cell by the same or closely related mechanisms. This situation could be visualized if it is proposed that the p-aminosalicylic acid "uptake system" is induced under the same conditions which permit induction of the p-aminobenzoic acid "uptake system". If the presence of the antimetabolite inhibits the inducing effect of p-aminobenzoic acid for p-aminobenzoic acid uptake, then it would seem likely that the p-aminobenzoic acid-induction of p-aminosalicylic acid uptake would also be inhibited.

More evidence to support this conclusion was the observation that p-aminobenzoic acid uptake by p-aminosalicylic acid-induced cells was inhibited by the presence of p-aminosalicylic acid (Table VII). The uptake of p-aminobenzoic acid by the p-aminobenzoic acid-induced cells was also found

to be inhibited by the presence of p-aminosalicylic acid. Since the presence of p-aminosalicylic acid inhibited substrate uptake by cells induced by exposure to either p-aminobenzoic acid or p-aminosalicylic acid, it would appear that the p-aminobenzoic acid "uptake systems" induced by either treatment are similar in nature. The findings that p-aminobenzoic acid can induce the "uptake system" for p-aminosalicylic acid and that p-aminosalicylic acid can induce the "uptake system" for p-aminobenzoic acid suggest that the mechanisms for p-aminobenzoic acid and p-aminosalicylic acid uptake are the same or very closely related. The similarities in the "uptake systems" permits speculation that p-aminosalicylic acid could compete for the transport mechanism thereby inhibiting the rate of substrate uptake.

Inhibition of protocatechuic acid oxidation by p-aminosalicylic acid.

Results presented in Figure 3 showed that p-aminosalicylic acid inhibited the oxidation of protocatechuic acid by p-aminobenzoic acid-grown cells. Further studies were conducted to determine if the antimetabolite affected the oxidation of protocatechuic acid by protocatechuic acid-grown cells. Figure 7 illustrates typical results obtained when the protocatechuic acid concentration was maintained at a constant level and the concentration of the inhibitor was varied. In the smaller inhibitor/substrate ratios of 0/8, 1/8, 2/8 and 4/8 the inhibition of oxidation appeared to be of a competitive nature, however, when the ratio was greater than 4/8 an in-

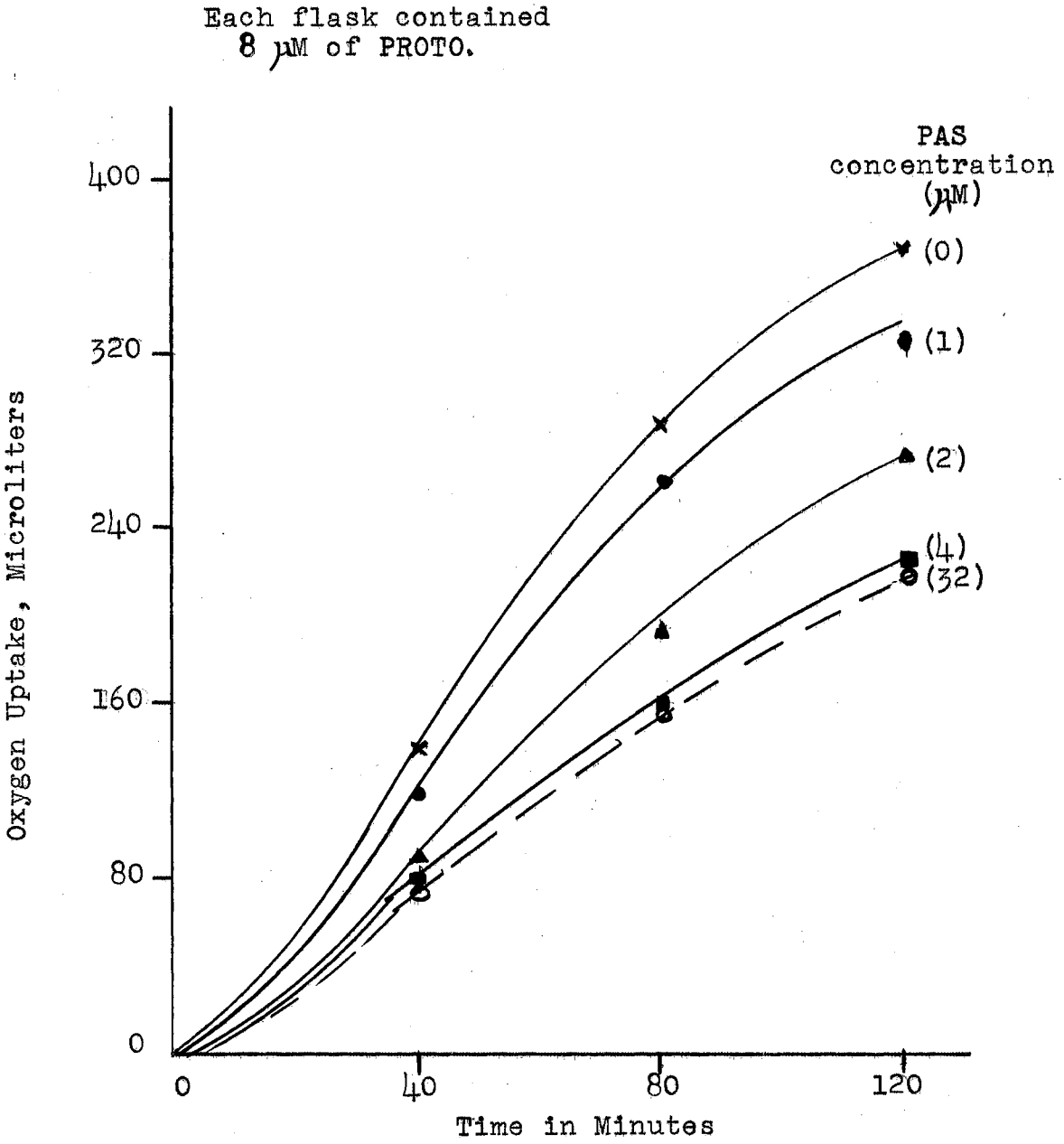


Figure 7. The Oxidation of Protocatechuic Acid by Protocatechuic Acid-Grown Cells in the Presence of Varying Concentrations of p-Aminosalicylic Acid

creased inhibition was not observed. For example, the inhibition in the 32/8 ratio did not appear to be more pronounced than the inhibition in the 4/8 ratio thus demonstrating that the antagonism was maximal at some inhibitor/substrate ratio near 4/8. The results presented in Figure 7 showed that the maximum effect produced by the antimetabolite was approximately a 50 per cent inhibition of the rate of oxidation.

Further experiments were conducted in which the oxidation of protocatechuic acid, by cells sequentially induced to protocatechuic acid by growth on p-aminobenzoic acid, was measured in inhibitor/substrate ratios of 0/8, 2/8, 4/8 and 32/8. These data are presented in Table VIII and show that the maximum inhibition of oxidation was observed when an inhibitor/substrate ratio of approximately 4/8 was attained. This finding supports the results obtained with protocatechuic acid-grown cells and indicates that both cell suspensions exhibit a similar sensitivity to p-aminosalicylic acid regardless of the method of induction. Since previous studies (Table V) showed that p-aminobenzoic acid-grown cells were capable of depleting greater quantities of the antimetabolite than protocatechuic acid-grown cells, a competition at some internal site does not seem plausible to explain the inhibition in both types of cells. If an internal competition exists, the p-aminobenzoic acid-grown cells which appeared to be induced for p-aminosalicylic acid uptake should exhibit a different sensitivity to the antimetabolite. It would seem likely that a smaller p-aminosalicylic acid con-

centration would produce the maximum effect in the p-aminobenzoic acid-grown cells since these cells can take up the antimetabolite more efficiently. These findings suggest that the sensitive reaction of protocatechuic acid inhibition by p-aminosalicylic acid is not located intracellularly since the same patterns of inhibition were observed in cells which show marked differences in their ability to take up the antimetabolite.

TABLE VIII

THE OXIDATION OF PROTOCATECHUIC ACID BY p-AMINOBENZOIC ACID-GROWN CELLS IN THE PRESENCE OF VARYING CONCENTRATIONS OF p-AMINOSALICYLIC ACID

Time in Minutes	Oxygen Uptake (μL)			
	Ratio: PAS/PROTO 0/8	2/8	4/8	32/8
0	0	0	0	0
10	30	19	11	8
20	71	55	43	39
30	126	97	80	75
40	182	142	115	107

Reversal of the inhibition of protocatechuic acid oxidation.

The reversible nature of the inhibition of protocatechuic acid oxidation can be demonstrated by the addition of excess substrate. Respirometer experiments were performed in which the inhibition of protocatechuic acid oxidation by p-aminosalicylic acid was followed for 30 minutes in inhibitor/substrate ratios of 0/8, 2/8 and 4/8. Figure 8 shows these results which indicate that within 10 minutes after the addi-

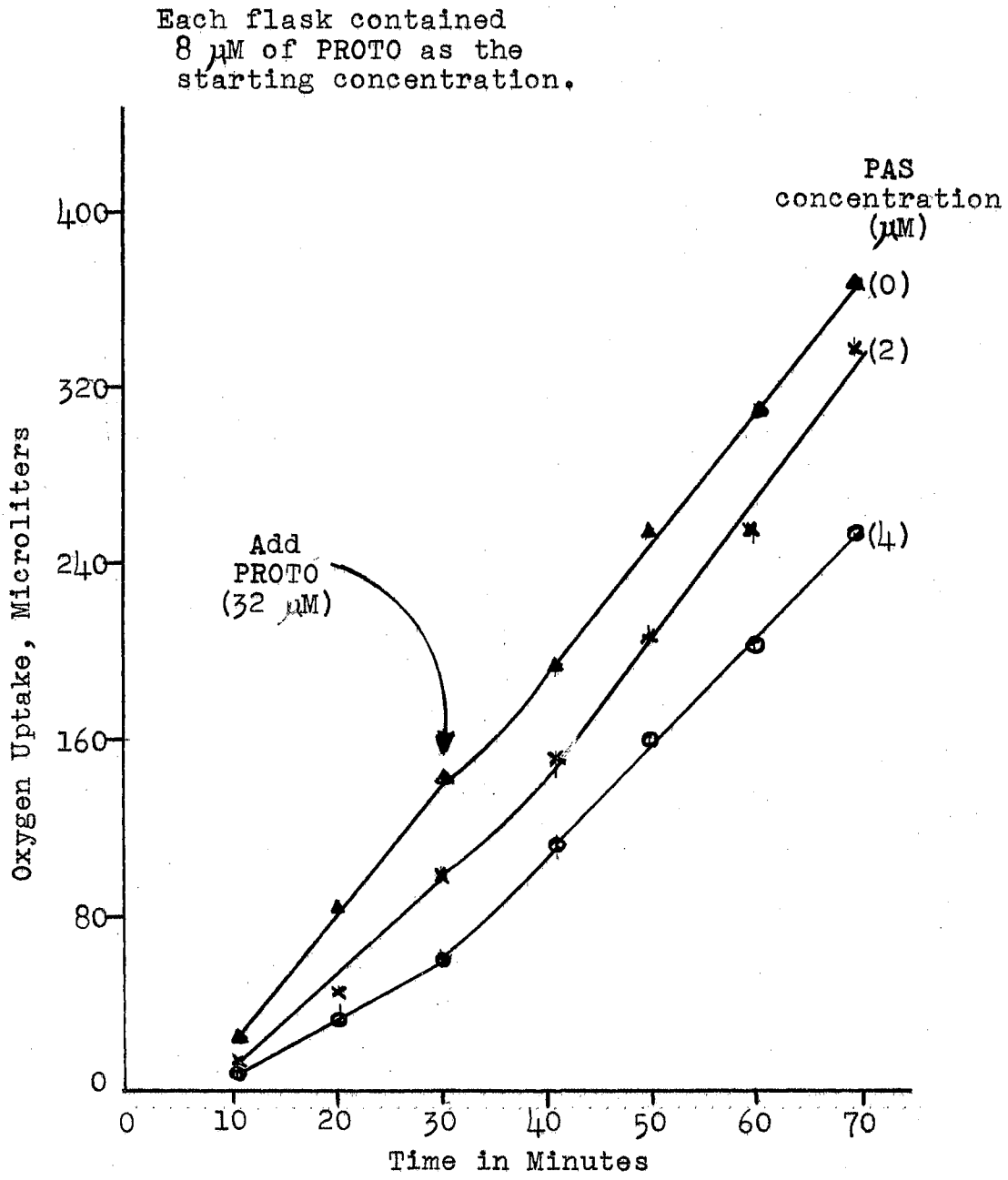


Figure 8. Reversal of the Inhibition of Protocatechuic Acid Oxidation by the Addition of Excess Substrate

tion of additional substrate (32 μmoles at 30 minutes) the degree of inhibition was lessened in all vessels. These results show that the inhibition of oxidation can be reversed by adding excess protocatechuic acid which in effect decreases the ratio of inhibitor/substrate.

Inhibition of protocatechuic acid uptake by p-aminosalicylic acid.

Other studies showed that the uptake of protocatechuic acid by protocatechuic acid grown-cells was inhibited by the presence of p-aminosalicylic acid (Figure 9). The amount of substrate taken up in 40 minutes was measured in varying inhibitor concentrations. Protocatechuic acid concentrations of 8 and 16 μmoles were employed in the two experiments described. When 8 μmoles of protocatechuic acid was employed as the starting concentration the rate at which protocatechuic acid was depleted from the medium reached a minimum when the concentration of p-aminosalicylic acid was 4 μmoles. Increasing the concentration of the antagonist did not significantly change the rate of substrate uptake. Similarly, when a starting concentration of 16 μmoles of substrate was employed, 8 μmoles of the inhibitor produced the maximum effect. In both experiments the maximum effect was observed when the inhibitor/substrate ratio reached 1/2. These findings are analogous to observations made in Figure 7 where the maximum inhibition of oxidation was observed with a protocatechuic acid to p-aminosalicylic acid ratio of 4/8. These observations suggest that the inhibition of protocatechuic acid is

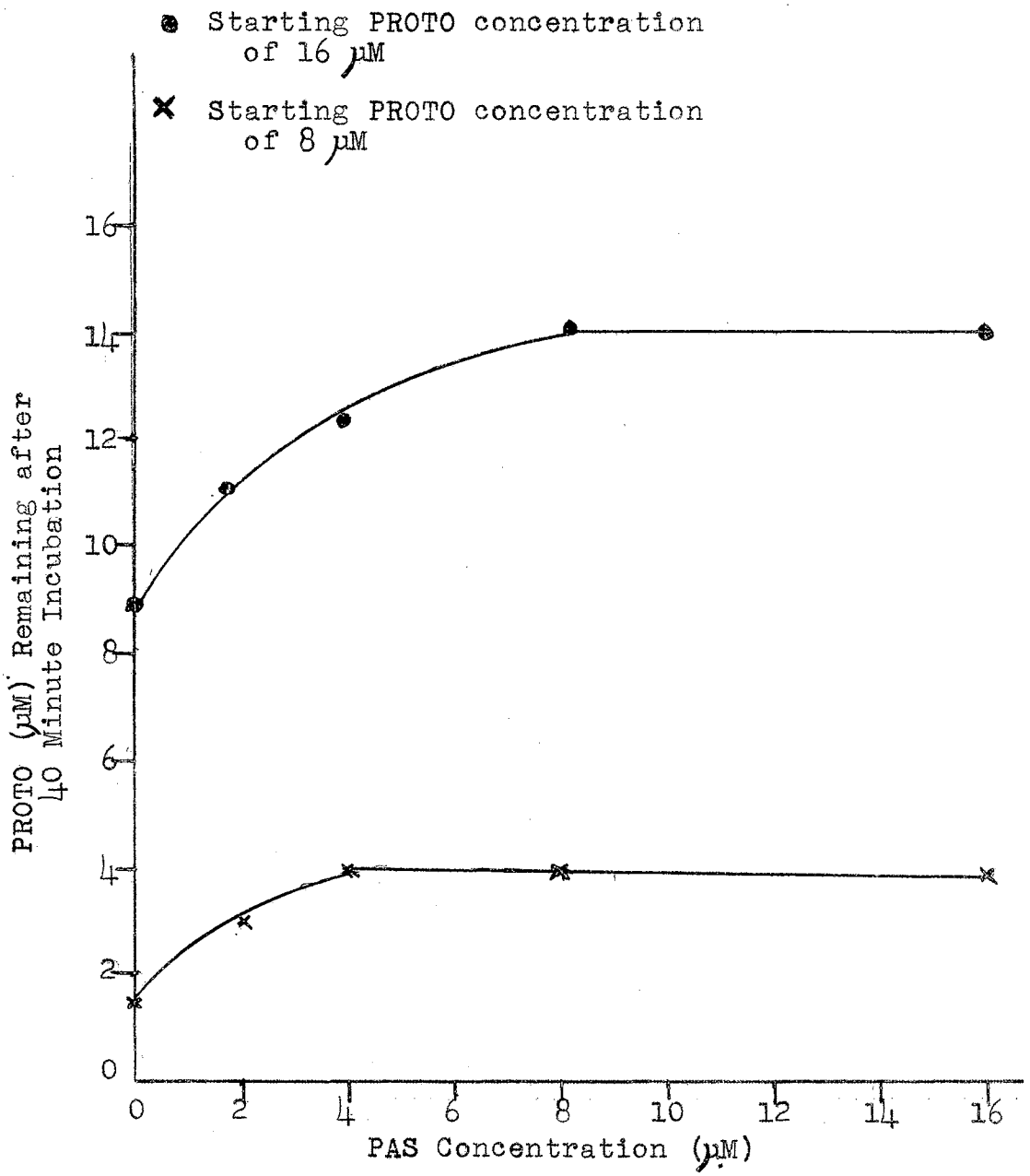


Figure 9. The Cellular Uptake of Protocatechuic Acid by Protocatechuic Acid-Grown Cells in the Presence of Varying Concentrations of p-Aminosalicylic Acid

not purely competitive since the inhibition of protocatechuic acid uptake or oxidation did not increase indefinitely with increasing p-aminosalicylic acid concentration.

Effects of p-aminosalicylic acid on protocatechuic acid degradation by cell-free extracts.

If one proposed that p-aminosalicylic acid exerted its effect by competing for a "site" associated with the internal enzyme system(s) then the inhibition should also be observed a cell-free system. Figure 10 shows results obtained from respirometer experiments in which the oxidation of protocatechuic acid by whole cells and cell-free extracts was measured in the presence and absence of p-aminosalicylic acid. The cell extract was prepared from p-aminobenzoic acid-grown cells and results indicate that an inhibitor concentration ten times the substrate concentration did not influence the rate of oxidation. This finding is in contrast to the observation that the presence of this concentration of p-aminosalicylic acid inhibits the oxidation of protocatechuic acid by whole cells.

The oxidation of protocatechuic acid by the cell extract consumes approximately one μmole (20 μliters) of molecular oxygen per μmole of protocatechuic acid which is somewhat less than is observed when using whole cells (Figure 10). The failure of p-aminosalicylic acid to inhibit the oxidation of protocatechuic acid by the extract does not completely eliminate the possibility that p-aminosalicylic acid inhibits an internal "site" since the effect could be at some dissimilatory step for which activity was not obtained in the cell-free

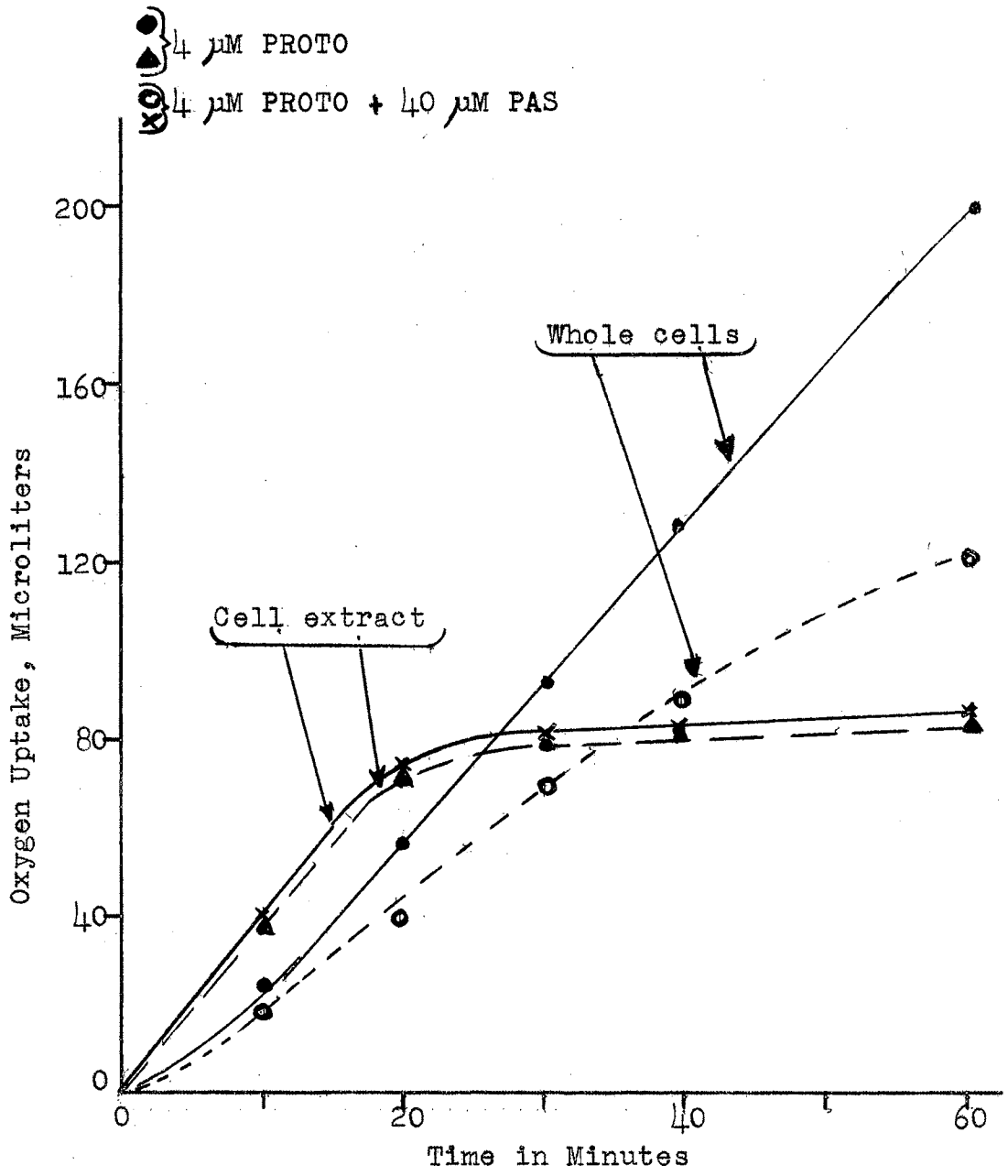
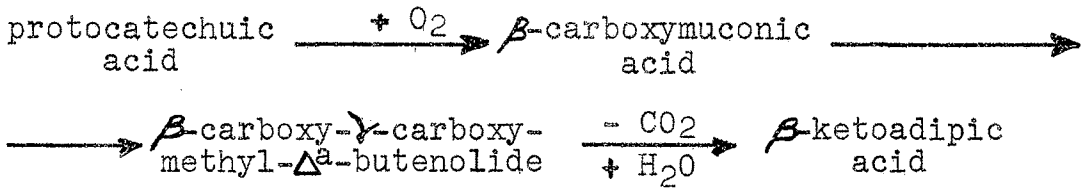


Figure 10. The Oxidation of Protocatechuic Acid by Whole Cells and Cell-Free Extracts in the Presence and Absence of p-Amino-salicylic Acid

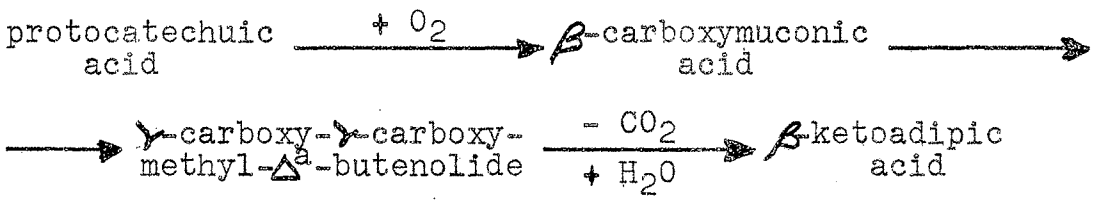
system. Results with cell-free extracts showed that p-aminosalicylic acid did not influence the rate at which carbon dioxide was liberated during the degradation of protocatechuic acid. Approximately one μmole of carbon dioxide was evolved per μmole of substrate in the presence and absence of the inhibitor.

If these results are interpreted in accordance with the known degradation schemes of Neurospora (9) or Nocardia (2) then the consumption of one mole of oxygen and the liberation of one mole of carbon dioxide indicates that protocatechuic acid is being degraded to β-ketoadipic acid. These sequences are illustrated as follows:

Neurospora

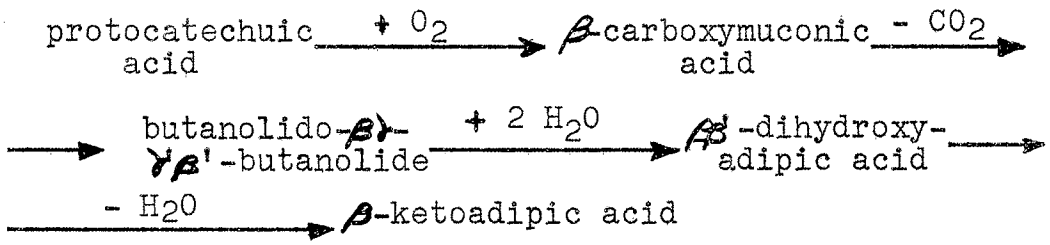


Nocardia



If, however, the reaction sequence is considered to proceed via the Pseudomonas pathway (20), one mole of oxygen could be consumed and one mole of carbon dioxide liberated without the degradation proceeding as far as β-ketoadipic acid. The oxidation and decarboxylation could occur with butanolido-βγ-γ'β'-butanolide or its open chain counterpart

ββ'-dihydroxyadipic acid accumulating in the cell extract. The Pseudomonas pathway described by Ribbons and Evans (20) is illustrated as follows:



To identify the accumulating product, 40 µmoles of protocatechuic acid were incubated with the extract in Warburg flasks until oxidation was complete. The incubation mixture was chilled in an ice bath and the dinitrophenylhydrazone of the deproteinized supernatant was formed as previously described (25). The derivative prepared was chromatographed against the dinitrophenylhydrazone of authentic β-ketoadipic acid which had been prepared concurrently. In the two solvent systems tested (N butanol:water:ethanol and isopropanol: water:ammonium hydroxide), the unknown material was found to be chromatographically identical to the derivative of the authentic β-ketoadipic acid.

Isolated spots were cut from the paper chromatograms and the colored derivatives were eluted with 95 per cent ethanol. The absorption spectra were compared using a Beckman DU spectrophotometer over a range from 350 to 700 mµ then the eluants were made alkaline with 0.5 ml of 2 N sodium hydroxide and the spectra compared over the same range. When dissolved in ethanol, both the authentic β-ketoadipic acid hydrazone and the unknown hydrazone showed a single adsorption peak at 360

mμ. The addition of the base caused a shift of the adsorption peak to 425 mμ in both derivatives, further indicating their similarities. From the evidence presented the accumulating material in the extract appeared to be β-ketoadipic acid.

Table IX illustrates results of a typical extract experiment in which the rates of oxidation, carbon dioxide evolution, and β-ketoadipic acid formation were compared in the presence and absence of p-aminosalicylic acid. In this experiment the oxygen consumed and the carbon dioxide evolved from protocatechuic acid degradation were measured by conventional techniques (26). Duplicate flasks were removed at the specified times, the reaction stopped by the addition of the acid buffer, and β-ketoadipic acid estimated by the catalytic decarboxylation procedure (22). As previously stated, p-aminosalicylic acid does not inhibit the rate of oxidation or carbon dioxide evolution during protocatechuic acid degradation by cell extracts. The rate of β-ketoadipic acid formation does not appear to be inhibited in the presence of p-aminosalicylic acid. Both in the presence and absence of the inhibitor, the amount of β-ketoadipic acid formed at 15 minutes exceeded the theoretical amount as judged by oxidation and carbon dioxide evolution. These excessive values were attributed to the time required to remove the Warburg flasks and add the acid buffer to stop the reaction.

The uptake and oxidation of protocatechuic acid were inhibited by p-aminosalicylic acid in cells grown on either p-aminobenzoic acid or protocatechuic acid. In cell-free ex-

TABLE IX

COMPARISON OF OXYGEN UPTAKE, CARBON DIOXIDE EVOLUTION AND β-KETOADIPIC ACID FORMATION IN PROTOCATECHUIC ACID DEGRADATION BY CELL EXTRACTS IN THE PRESENCE AND ABSENCE OF p-AMINOSALICYLIC ACID

Time in Minutes	PROTO (6 μM)			PROTO (6 μM) + PAS (10 μM)		
	μM O_2 Uptake	μM CO_2 Evolved	μM KA Formed	μM O_2 Uptake	μM CO_2 Evolved	μM KA Formed
0	0	0	0	0	0	0
15	2.23	2.36	3.25	2.59	2.72	3.60
30	5.00	5.40	5.25	5.89	5.98	5.10

tracts the sequence of reactions leading to β-ketoadipic acid formation was not affected by the antimetabolite. If the p-aminosalicylic acid sensitive reaction is some dissimilatory step beyond β-ketoadipic acid formation then it would seem likely that an inhibition should have been observed in the whole cell experiments when β-ketoadipic acid oxidation was measured in the presence and absence of the antimetabolite. Results presented in Figure 2 demonstrated the oxidation of β-ketoadipic acid by whole cells was not inhibited by p-aminosalicylic acid. These data suggest that the antagonism does not occur at a "site" in the internal enzyme systems. Since results obtained in this study indicated that p-aminosalicylic acid inhibits the uptake of p-aminobenzoic acid and protocatechuic acid from the medium, it is proposed that the sensitive "site" at which p-aminosalicylic acid inhibits the oxidation of p-aminobenzoic acid and protocatechuic acid is the specific transport mechanism(s).

CHAPTER IV

SUMMARY AND CONCLUSIONS

The ability of p-aminosalicylic acid to inhibit the oxidation of p-aminobenzoic acid and protocatechuic acid has been investigated in an attempt to elucidate the mechanism(s) involved in these antagonisms. 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 antimetabolite and is not killing or otherwise impairing the oxidative ability of the cells.

Results obtained in this investigation indicate that inhibition of p-aminobenzoic acid utilization becomes apparent immediately following addition of the antimetabolite. The inhibition could also be detected in experiments using extremely low substrate levels. This immediate susceptibility of substrate oxidation would indicate that the "sensitive site" is readily accessible to the inhibitor.

Studies indicated that cells previously exposed to large quantities of the inhibitor demonstrated approximately the same degree of inhibition as cells to which the inhibitor and substrate were added simultaneously. 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 cells did not influence p-aminobenzoic acid oxidation. Attempts to recover p-aminosalicylic acid from those 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.

Results obtained from incubation experiments showed that the rate of uptake of p-aminosalicylic acid by p-aminobenzoic acid-grown cells is independent of the initial concentration of the antimetabolite in the medium. A competitive inhibition at an internal "site" would be dependent on the presence of varying concentrations of the inhibitor and the constant uptake observed in this study would tend to eliminate the possibility that p-aminosalicylic acid acts inside the cell.

The view that p-aminosalicylic acid competes for the specific transport mechanisms of p-aminobenzoic acid is further supported by observations made in comparing the "uptake systems" of the substrate and the inhibitor. Incubation experiments showed that exposure of asparagine-grown cells to p-aminosalicylic acid partially induces the "uptake system" for p-aminobenzoic acid. In these cells induced for substrate uptake by exposure to the inhibitor, the depletion of p-aminobenzoic acid was also inhibited by p-aminosalicylic acid. The

fact that transport mechanisms for the substrate and inhibitor appear to be closely related makes a competition at these "sites" seem plausible.

The influence of p-aminosalicylic acid on the uptake and oxidation of protocatechuic acid was investigated in incubation and respirometer experiments. Similar patterns of inhibition were observed in either protocatechuic acid-grown cells or in cells sequentially induced to protocatechuic acid by growth on p-aminobenzoic acid. These observations suggest that the depletion of the antimetabolite by p-aminobenzoic acid-grown cells, which is not observed in protocatechuic acid-grown cells, apparently is of no consequence in the inhibition of protocatechuic acid oxidation. In addition, this difference in inhibitor uptake makes it difficult to assume a competition for internal "sites" in both types of cells.

p-Aminosalicylic acid did not affect the rate or extent of oxidation of protocatechuic acid by cell-free extracts. Experiments also showed that the rate of carbon dioxide evolution during the degradation of protocatechuic acid by cell extracts was not affected by the antimetabolite. Chromatographic experiments showed that protocatechuic acid was degraded to β-ketoadipic acid by the cell extract. Catalytic decarboxylation of the β-ketoadipic acid formed from protocatechuic acid in cell extract incubations showed that the rate of formation of the keto-acid was not inhibited by p-aminosalicylic acid.

Results of cell extract experiments provided evidence that the sequence of dissimilatory reactions from protocate-

chuic acid to β-ketoadipic acid are not sensitive to the inhibitor. Findings of whole cell experiments demonstrated that the oxidation of β-ketoadipic acid is not affected by the antagonist. Combining the (protocatechuic acid ⟶ β-ketoadipic acid) reaction sequence in cell extracts with the (β-ketoadipic acid ⟶ low molecular weight compounds) reaction sequence of whole cells provides evidence that inhibition of the oxidation of protocatechuic acid by p-aminosalicylic acid is not situated at an internal "site".

The findings of this study suggest that p-aminosalicylic acid inhibits the cellular oxidation of p-aminobenzoic and protocatechuic acid by competing for the specific transport "sites" of the aromatic compounds thereby controlling cellular oxidation by regulating the intracellular accumulation of the substrates. These conclusions emphasize that the penetration of various compounds through the cell barrier is a complex process and that competition for the transport mechanisms may be demonstrated with aromatic compounds. Similar findings concerning transport systems were reported by Mathieson and Catcheside (18) in which the uptake of histidine by Neurospora was competitively inhibited by arginine and methionine and by Mandelstam (17) in which the entry of lysine into coliform bacteria was inhibited by a diamine. The inhibition of protocatechuic acid utilization which apparently is reversible but may not be purely competitive could offer an intriguing area for further investigations.

LITERATURE CITED

1. Angier, R. B., Boothe, J., Hutchings, B. L., Mowat, J., Semb, J., Stokstad, E. L. R., SubbaRow, Y., and Waller, C. Y. The structure and synthesis of the liver L. casei factor. Science, 103, 667-669, 1946.

2. Cain, R. B. and Cartwright, N. J. On the properties of some aromatic ring-opening enzymes of species of the genus Nocardia. Biochim. et Biophys. Acta, 37,

3. Davis, B. D. Inhibition of Escherichia coli by p-aminobenzoic acid and its reversal by p-hydroxybenzoic acid. J. Exptl. Med., 94, 243-254, 1951.

4. Durham, N. N. Bacterial oxidation of p-aminobenzoic acid by Pseudomonas fluorescens. J. Bacteriol., 72, 333-336, 1956.

5. Durham, N. N. Effect of structurally related compounds on the oxidation of p-aminobenzoic acid by Pseudomonas fluorescens. J. Bacteriol., 73, 612-615, 1957.

6. Durham, N. N. and Hubbard, J. S. Antagonism of the oxidative dissimilation of p-aminobenzoic acid by p-aminosalicylic acid. Nature (London), 184, 1398, 1959.

7. Durham, N. N. and Hubbard, J. S. Mechanism of competitive inhibition of p-aminobenzoic acid oxidation by p-aminosalicylic acid. J. Bacteriol., 80, In Press.

8. Goodacre, C. L., Mitchell, B. W., and Seymour, D. E. The in vitro tuberculostatic behavior of para-aminosalicylic acid and related compounds. Quart. J. Pharm. and Pharmacol., 21, 301-305, 1948.

9. Gross, S. R., Gafford, R. D., and Tatum, E. L. The metabolism of protocatechuic acid by Neurospora. J. Biol. Chem., 219, 781-796, 1956.

10. Hedgecock, L. W. Antagonism of the inhibitory action of aminosalicylic acid on Mycobacterium tuberculosis by methionine, biotin and certain fatty acids, amino acids and purines. J. Bacteriol., 72, 839-846, 1956.

11. Ivanovics, G. Antagonism between effects of p-aminosalicylic acid and salicylic acid on growth of M. tuberculosis. Proc. Soc. Exptl. Biol. Med., 70, 462-463, 1949.

12. Katagiri, M. and Hayaishi, O. Enzymic degradation of β-ketoadipic acid. Fed. Proc., 15, 285, 1956.

13. Kilby, B. A. The formation of β-ketoadipic acid by bacterial fission of aromatic rings. Biochem. J., 49, 671-674, 1951.

14. Lampen, J. O. and Jones, M. J. The antagonism of sulfonamide inhibition of certain Lactobacilli and Enterococci by pteroylglutamic acid and related compounds. J. Biol. Chem., 166, 435-438, 1946.

15. Lehman, J. PAS in the treatment of tuberculosis. Lancet, 250, 15-16, 1946.

16. Lehman, J. On the effect of isomers of PAS (para-aminosalicylic acid) and related substances on the tuberculostatic effect of PAS. Experientia, 5, 365-367, 1949.

17. Mandelstam, J. Factors influencing the passage of basic amino acids into coliform bacteria. Biochim. et Biophys. Acta, 22, 313-328, 1956.

18. Mathieson, M. J. and Catcheside, D. G. Inhibition of histidine uptake in Neurospora crassa. J. Gen. Microbiol., 13, 72-83, 1955.

19. Ottey, L. and Tatum, E. L. The cleavage of β-ketoadipic acid by Neurospora crassa. J. Biol. Chem., 229, 77-83, 1957.

20. Ribbons, D. W. and Evans, W. C. The pathway of metabolism of protocatechuic acid by Pseudomonas species. Biochem. J., 73, 21p, 1959.

21. Shive, W. The utilization of antimetabolites in the study of biochemical processes in living organisms. Annals N. Y. Acad. Sci., 52, 1212-1234, 1950.

22. Sistrom, W. R. and Stanier, R. Y. The mechanism of catechol oxidation by Mycobacterium butyricum. J. Bacteriol., 66, 404-406, 1953.

23. Snell, F. D. and Snell, C. T. Colorimetric Methods of Analysis. D. Van Nostrand Co., New York, 1953.

24. Stanier, R. Y., Sleeper, B. P., Tsuchida, M., and MacDonald, D. L. The bacterial oxidation of aromatic compounds. III. The enzymic oxidation of catechol and protocatechuic acid to β-ketoadipic acid. J. Bacteriol., 59, 137-151, 1950.

25. Strassman, M., Shatton, J. B., and Weinhouse, S. Conversion of α-acetolactic acid to the valine precursor, α,β-dihydroxyisovaleric acid. J. Biol. Chem., 235, 700-705, 1960.

26. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric Techniques, Revised edition. Burgess Publishing Co., Minneapolis, 1957.

27. Wood, D. D. The relation of p-aminobenzoic acid to the mechanism of the action of sulphanilamide. Brit. J. Exptl. Pathol., 21, 74-90, 1940.

28. Woods, D. D. and Fildes, P. The antisulfanilamide activity (in vitro) of p-aminobenzoic acid and related compounds. Chemistry and Industry, 59, 133-134, 1940.

29. Youmans, G. P., Raleigh, G. W., and Youmans, A. S. The tuberculostatic action of p-aminosalicylic acid. J. Bacteriol., 54, 409-416, 1947.

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