MECHANISM OF THE INHIBITION OF MICROBIAL GROWTH BY o-NITROBENZOIC ACID

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MECHANISM OF THE INHIBITION OF MICROBIAL GROWTH BY o-NITROBENZOIC ACID

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CHAPTER I

INTRODUCTION

There have been numerous investigations in recent years showing that benzene derivatives inhibit microbial growth. Many of these derivatives block essential metabolic reactions, but the site of inhibition in many cases has not been established. The significance of investigations to elucidate the mechanism of inhibition is evident since these types of studies have proven useful in studying metabolic reactions accompanying growth and multiplication.

It has been known for several years that high concentrations of p-aminobenzoic acid are inhibitory to some bacteria (11, 27). Davis (11) has shown that the bacteriostatic activity of p-aminobenzoic acid toward Escherichia coli strain W is reversed competitively by p-hydroxybenzoic acid. The inhibition was also reversed, to a limited extent, by shikimic acid or 5-dehydroshikimic acid. Reed, Schram and Loveless (25) reported that Saccharomyces cerevisiae was inhibited by p-aminobenzoic acid and the inhibition could be reversed by aromatic amino acids such as phenylalanine, tyrosine, and tryptophan. They also found that shikimic acid accumulated in the culture medium in the presence of p-aminobenzoic acid.

Durham (13) reported that p-aminobenzoic acid was capable of serving as an oxidizible substrate for certain microorganisms, and the utilization of p-aminobenzoic acid as a sole source of energy

for aerobic growth was competitively inhibited by p-aminosalicylic acid (14). Durham and Hubbard (15) 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.

Higgins (17) reported that growth of Aspergillus niger was prevented when 2.5 x 10^{-5} moles m-dinitrobenzene was added to the culture medium within 30 hours after inoculation. When m-dinitrobenzene was added after 30 hours, some growth occurred. This inhibition of growth could be reversed by addition of commercial preparations of casein hydrolysate or a synthetic mixture of amino acids similar to the composition of casein hydrolysate. The inhibition could not be reversed by ammonium nitrogen or vitamins. Cocito (8) studied the toxic effect of 2,4-dinitrophenol on E. coli and found that the inhibition could be counteracted by an amino acid mixture similar in composition to that obtained from cow's liver.

Cain (7) studied the metabolism of nitroaromatic compounds and found that m-nitrobenzoic acid, when present in concentrations ranging from 0.1 to 1.0 umoles, inhibited oxidation of o-nitrobenzoic acid by Nocardia opaca. The m-isomer also competitively inhibited the oxidation of p-nitrobenzoic acid by Nocardia erythropolis over the range of 1.0-4.0 umoles. There was no inhibition of the oxidation of o-nitrobenzoic acid by the p-isomer, or of the oxidation of p-nitrobenzoic acid by the o-isomer. However, the concentrations tested were not reported.

The mechanism(s) of toxicity of many benzene derivatives has not been elucidated. The toxic effect of 2,4-dinitrophenol on living

systems appears to result in part from uncoupling of oxidative phosphorylation (22). Even though the exact mechanism is not now known, good evidence has been reported that 2,4-dinitrophenol activates adenosine triphosphatase (9). Thyroxine will also uncouple oxidative phosphorylation and alter the permeability of the mitochondrial membrane under certain conditions (6). The presence of adenosine triphosphatase in microorganisms has been observed by several workers (1,9,22). Meyerhof (23) reported that yeast adenosine triphosphatase maintains a balance between the phosphorylation of glucose for which adenosine triphosphate is required, and the phosphorylation of glyceraldehyde-3-phosphate for which inorganic phosphate is required. The adenosine triphosphatase of yeast is inhibited by toluene (23). Abrams, McNamara, and Johnson (1) working with isolated cell membranes of Streptococcus faecalis demonstrated adenosine triphosphatase activity associated with the cell membrane. The enzyme appeared to be a true adenosine triphosphatase inasmuch as the reaction of the enzyme with pyrophosphate and adenosine monophosphate yielded practically no inorganic phosphate. The cell membrane adenosine triphosphatase was stimulated at least 15-fold by magnesium with the maximal effect being obtained at a molar concentration equivalent to that of adenosine triphosphate (1). Thus, the true substrate of adenosine triphosphatase appeared to be the magnesium adenosine triphosphate complex. Abrams et al. (1) suggested that the increased permeability of S. faecalis during glycolysis was due to adenosine triphosphate, and the return of the cell to the impermeable state following the depletion of glucose from the medium, was due to the removal of adenosine triphosphate by adenosine triphosphatase (2).

In addition to adenosine triphosphatase, pyrophosphatases have been found in many microorganisms. Kunitz (21) has crystallized pyrophosphatase from yeast. These enzymes catalyze the hydrolysis of the pyrophosphate linkage in salts of pyrophosphoric acid and pyrophosphate esters. A number of metabolic reactions involving these enzymes have been studied. Various pyrophosphatases hydrolyze coenzyme A to 3', 5'-diphosphoadenosine and 4'-phosphopantetheine (24). The properties of a reduced pyridine nucleotide pyrophosphatase from pigeon livers have been characterized by Jacobson and Kaplan(18). The products of pyrophosphatase activity on diphosphopyridine nucleotide were shown to be reduced nicotinamide mononucleotide and 5'-adenylic acid. The enzyme also splits flavin adenine dinucleotide and reduced triphosphopyridine nucleotide, but does not attack the normal oxidized forms of either pyridine nucleotide coenzyme. Jacobson and Kaplan (18) postulated that reduced pyridine nucleotide pyrophosphatase may function in the hydrolysis of metabolically useless adenosine diphosphate ribose arising from the action of the enzyme, diphosphopyridine nucleotidase. In the synthesis of diphosphopyridine nucleotide, nicotinamide-ribose-phosphate and adenosine triphosphate form diphosphopyridine nucleotide plus inorganic pyrophosphate in the presence of yeast extract (20). It has been proposed that a pyrophosphatase, specific for inorganic pyrophosphate, hydrolyzes the pyrophosphate and shifts the equilibrium to facilitate synthesis of more diphosphopyridine nucleotide (19).

Preliminary experiments conducted in this laboratory indicated that o-nitrobenzoic acid inhibited the growth of a Flavobacterium species capable of utilizing p-nitrobenzoic acid as a carbon and

nitrogen source. This study was undertaken to determine the mechanism(s) of this inhibition.

CHAPTER II

MATERIALS AND METHODS

Test organism.

The organism used throughout this study was isolated from soil by the enrichment technique using an inorganic salt medium containing 0.2 per cent p-nitrobenzoic acid as the carbon and nitrogen source. The organism shows characteristics similar to the genus Flavobacterium as described in the 7th edition of Bergey's Manual of Determinative Bacteriology. A stock culture of the organism was carried on the synthetic medium containing p-nitrobenzoic acid as the sole carbon and nitrogen source.

Synthetic medium.

The synthetic or basal medium used in this study consisted of the following components: NaCl, 0.1 per cent; KH_2PO_4, 0.32 per cent; K_2HPO_4, 0.42 per cent; and agar, 2.0 per cent. The desired carbon source was added at a concentration of 0.1 per cent. When a carbon source other than p-nitrobenzoic acid or p-aminobenzoic acid was employed, 0.1 per cent NH_4Cl was added as a nitrogen source. The medium was adjusted to pH 7.0 and autoclaved at 121°C for 15 minutes. The sterilized medium was then placed in a water bath, cooled to 52°C, and 0.1 ml of a mineral salts solution was added aseptically to each 100 ml of the medium. 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. The salt solution was sterilized separately by autoclaving at 121°C for 15 minutes.

Nutrient agar was also used for growing cells in certain phases of the investigation. This complete medium contained 0.3 per cent beef extract, 0.5 per cent peptone, and 2.0 per cent agar.

For induction experiments, 0.2 per cent succinic acid or asparagine was added to the basal medium as the carbon source. p-Nitrobenzoic acid or p-aminobenzoic acid was used as the carbon source to grow cells containing induced enzymes to these substrates.

Preparation of cell suspensions.

Cell suspensions used in respirometric experiments were prepared by the following procedure: p-Nitrobenzoic acid agar slants were inoculated from a stock culture and incubated for 18-20 hours at 37°C. The cells were suspended in sterile 0.01 M phosphate buffer (pH 7.0). Nutrient agar plates were inoculated with 0.3-0.4 ml of the cell suspension and the cells spread over the surface of the agar with a sterile glass rod. The plates were incubated for 18-20 hours at 37°C. The cells were then harvested with 0.01 M phosphate buffer, washed three times by centrifugation, and resuspended in buffer. The cell suspension was standardized on the Bausch and Lomb "Spectronic 20" spectrophotometer so that a 1/20 dilution gave a reading of 60 (±2) per cent transmittance at 540 mu.

Preparation of cell-free extracts.

Cell-free extracts were prepared from a heavy cell suspension in 0.01 M Tris (Hydroxymethylaminomethane) buffer (pH 7.0). 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 two drops per second. The cell debris was removed by centrifugation at 20,000 x g for 30 minutes at 4°C. The resulting supernatant was used for experiments requiring cell-free preparations. No attempts were made to determine or standardize the concentration of protein per milliliter of supernatant.

Growth experiments.

Growth experiments were conducted in pyrex culture tubes. Tubes were selected to give 100% transmittance (±1 per cent) in a "Spectronic 20" spectrophotometer at 540 mu using distilled water as the standard. Since the composition of the medium used in different experiments varied, all components were sterilized separately and added aseptically to the sterile tubes. Inoculum cells were grown on p-nitrobenzoic acid agar slants and then suspended in sterile 0.01 M phosphate buffer. One drop of inoculum was added to each tube by use of a sterile 5 ml pipette. The tubes were incubated on a reciprocal shaker at 37°C. Growth was followed by measuring optical density at 540 mu in a "Spectronic 20" spectrophotometer.

Respirometric experiments.

Respirometric experiments were conducted using the Warburg apparatus at a temperature of 37°C with air as the gas phase (29). Warburg vessels with double side arms were used. To study oxygen uptake, 0.2 ml of 20 per cent potassium hydroxide was pipetted into the center well containing a piece of fluted paper. The cell suspension or cell-free extract was pipetted into the main chamber and the substrates and inhibitors were added to the side arms. Buffer was added

to bring the volume to 2.4 ml in each flask.

The carbon dioxide evolved was measured by the direct method (29). Three flasks were used for each determination. The first flask was used to determine oxygen uptake as described above. To the second and third flasks 0.2 ml of 1 N hydrochloric acid was added to the side arm. These flasks did not contain potassium hydroxide. The hydrochloric acid was dumped at the start of the experiment in the second flask and at the end of the experiment in the third flask. The addition of hydrochloric acid stopped the reaction and also released the carbon dioxide bound in the buffer. The carbon dioxide evolved during the experiment was calculated in accordance with the standard procedure (29). The compounds used as substrates or inhibitors for respirometric experiments were dissolved in 0.01 M phosphate buffer and the pH adjusted to 7.0.

Incubation experiments.

Incubation experiments were conducted under conditions similar to those used in growth experiments. Cells were obtained as described above and succinic acid was used as the substrate in the synthetic medium. The medium (500 ml) was placed in a 1,000 ml flask and inoculated with cells to obtain a suspension equivalent to that used in respirometric experiments. The flasks were incubated on a reciprocal shaker at 37°C. At different time intervals, 10 ml aliquots were withdrawn and filtered through a type HA Millipore filter. An ultraviolet absorption spectrum was run on the different aliquots using a Model 14 Cary Recording Spectrophotometer.

Phosphate determination.

Inorganic phosphate was determined by the method of Sumner (28). The reagents were prepared by dissolving 2.5 g of ferrous sulfate in 5 ml of 7.5 N sulfuric acid and 25 ml distilled water. This solution was freshly prepared for each experiment. Ammonium molybdate (6.6g) was dissolved in 50 ml of 7.5 N sulfuric acid and water added to a total volume of 100 ml. An aliquot of the test solution, usually 0.2 or 0.3 ml, was brought to a total volume of 3.0 ml with distilled water and 0.4 ml of the ferrous sulfate solution and 0.4 ml of ammonium molybdate solution were added. The optical density was determined immediately, although the color is stable for several hours, using a Beckman Model DU Spectrophotometer at 650 mu. Control vessels indicated that none of the compounds used in the experimental systems interfered with the color determination.

The amount of inorganic phosphate present was determined from a standard curve prepared concurrently in each experiment using KH_2PO_4 in concentrations between 0.1 and 0.8 umoles as the source of inorganic phosphate.

Substrates and inhibitors were dissolved in 0.1 M Tris buffer and adjusted to pH 7.4. All glassware was cleaned in chromic acid cleaning solution and rinsed with triple distilled water.

The release of inorganic phosphate from various phosphorylated compounds was determined by the following procedure. Duplicate tubes containing the incubation mixture were prepared for each determination and placed in a water bath at 37°C. The tubes were allowed to equilibrate to temperature for ten minutes at which time the cell suspension

or cell-free extract was added to the tubes. Three-tenths ml of 20 per cent trichloroacetic acid was added immediately to the first tube of each set to stop the reaction and also to precipitate the protein. This tube served as the control. At the desired time, trichloroacetic acid was added to the second tube to stop the reaction and precipitate the protein in the experimental system.

The precipitated proteins or cells were removed by centrifugation at 25,000 x g for ten minutes at 4°C. An aliquot of the supernatant was tested for inorganic phosphate and the concentration calculated from the standard curve.

Oxygen uptake by the cell suspension or cell-free extract was determined by standard manometric techniques and the findings were used in calculating phosphate:oxygen (P:O) ratios. The incorporation of inorganic phosphate was determined in the same experiment by preparing duplicate vessels and using the above procedure.

Chemicals.

All chemicals used in this study were obtained commercially.

Tabulation of experimental data.

The experimental data are illustrated in Chapter III. The following abbreviations are used in the illustrations and presented in the text: succinic acid (Succ), p-nitrobenzoic acid (p-NO_2), o-nitrobenzoic acid (o-NO_2), glutamic acid (Glut), adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine triphosphatase (ATPase), pyrophosphate (PPi), benzoic acid (Benz), p-aminobenzoic acid (PAB), and protocatechuic acid (Proto), flavin

mononucleotide (FMN), flavin adenenine dinucleotide (FAD), diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and nicotinamide mononucleotide (NMN).

CHAPTER III

EXPERIMENTAL AND RESULTS

Influence of o-nitrobenzoic acid on growth.

The Flavobacterium sp. used in this study was capable of utilizing p-nitrobenzoic acid as the sole carbon and nitrogen source. Cells grown on p-nitrobenzoic acid were transferred to a liquid synthetic medium containing p-nitrobenzoic acid as the sole carbon and nitrogen source and a typical growth curve was obtained. When o-nitrobenzoic acid was added in varying concentrations a marked decrease in growth was observed. Figure 1 shows the results from a representative experiment using a p-nitrobenzoic acid concentration of 0.02 M and o-nitrobenzoic acid concentrations of 0.06 M and 0.12 M. The organism was not capable of utilizing o-nitrobenzoic acid as a substrate.

Since the structure of o-nitrobenzoic acid differs from p-nitrobenzoic acid only in the position of the nitro group, a study was made to determine if growth was inhibited when compounds not structurally related to the nitrobenzoic acids were used as substrates. Results presented in Figure 2 show that o-nitrobenzoic acid had a similar effect on growth when 0.01 M succinic acid was used as the substrate. o-Nitrobenzoic acid concentrations were 0.02 M, 0.04 M, and 0.06 M. Similar results were obtained using 0.01 M glutamic acid as the substrate (Figure 3).

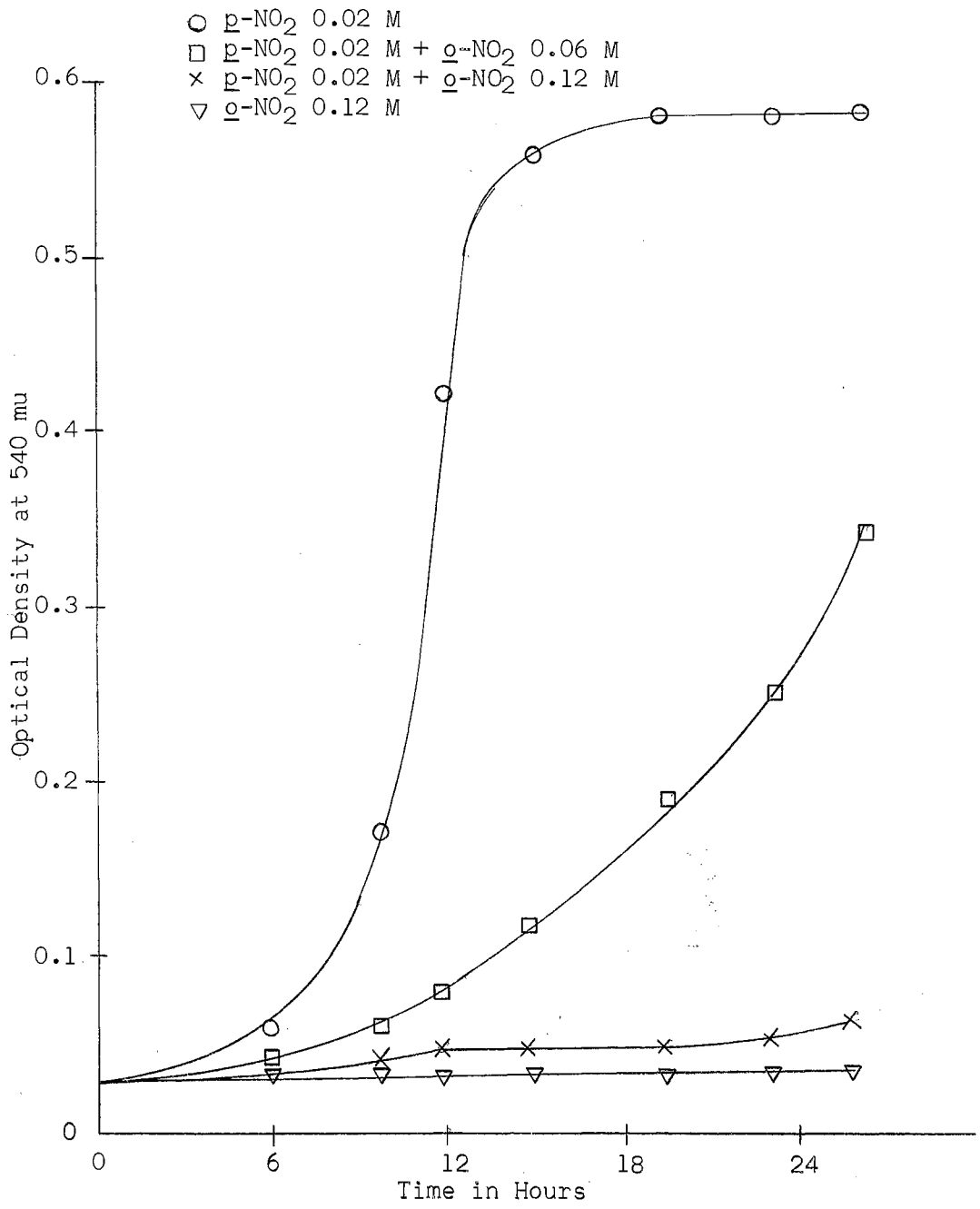

Figure 1. Effect of o-Nitrobenzoic Acid on Growth of Flavobacterium sp. using p-Nitrobenzoic Acid as Carbon and Nitrogen Source.

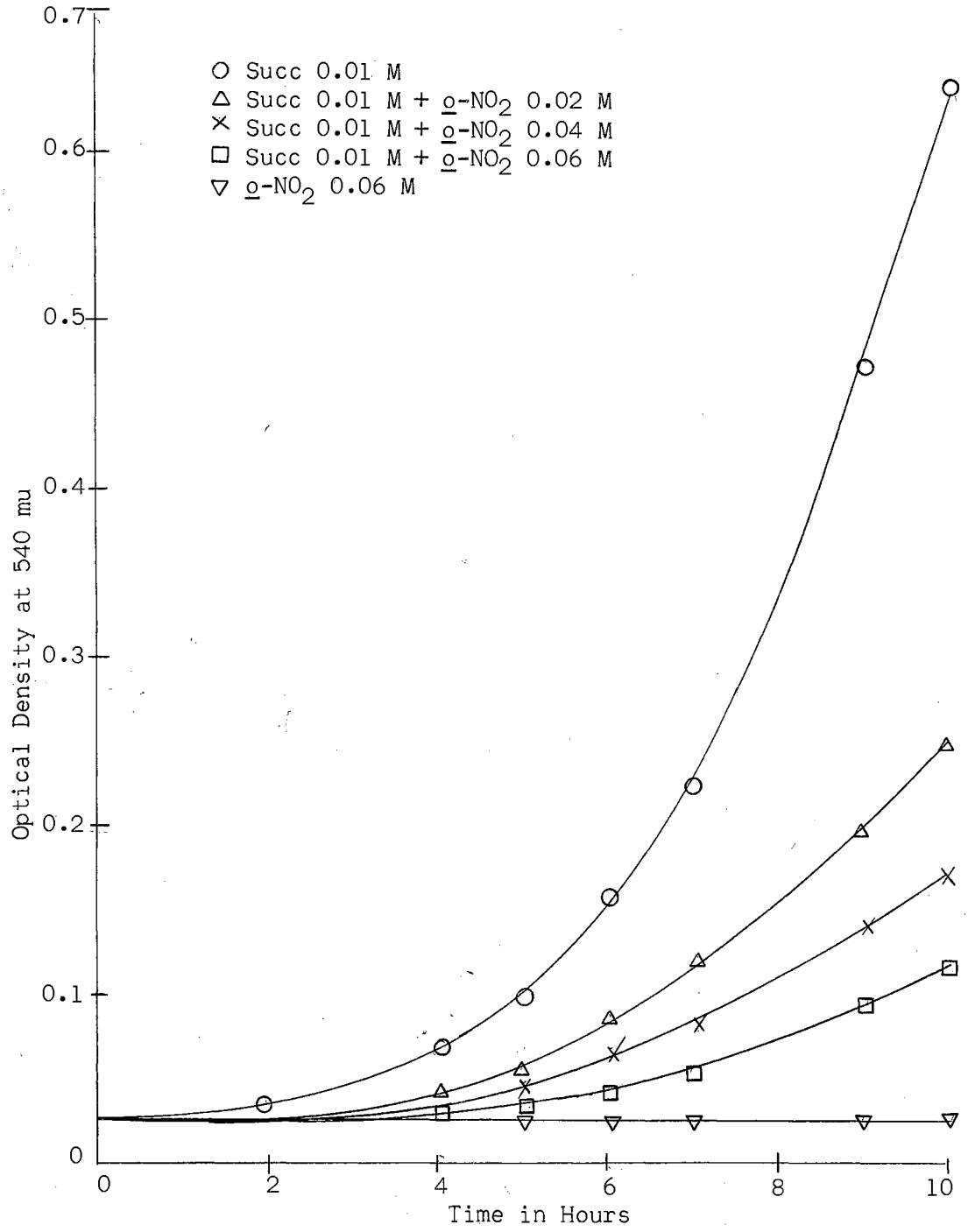

Figure 2. Effect of o-Nitrobenzoic Acid on Growth of Flavobacterium sp. using Succinic Acid as Carbon Source.

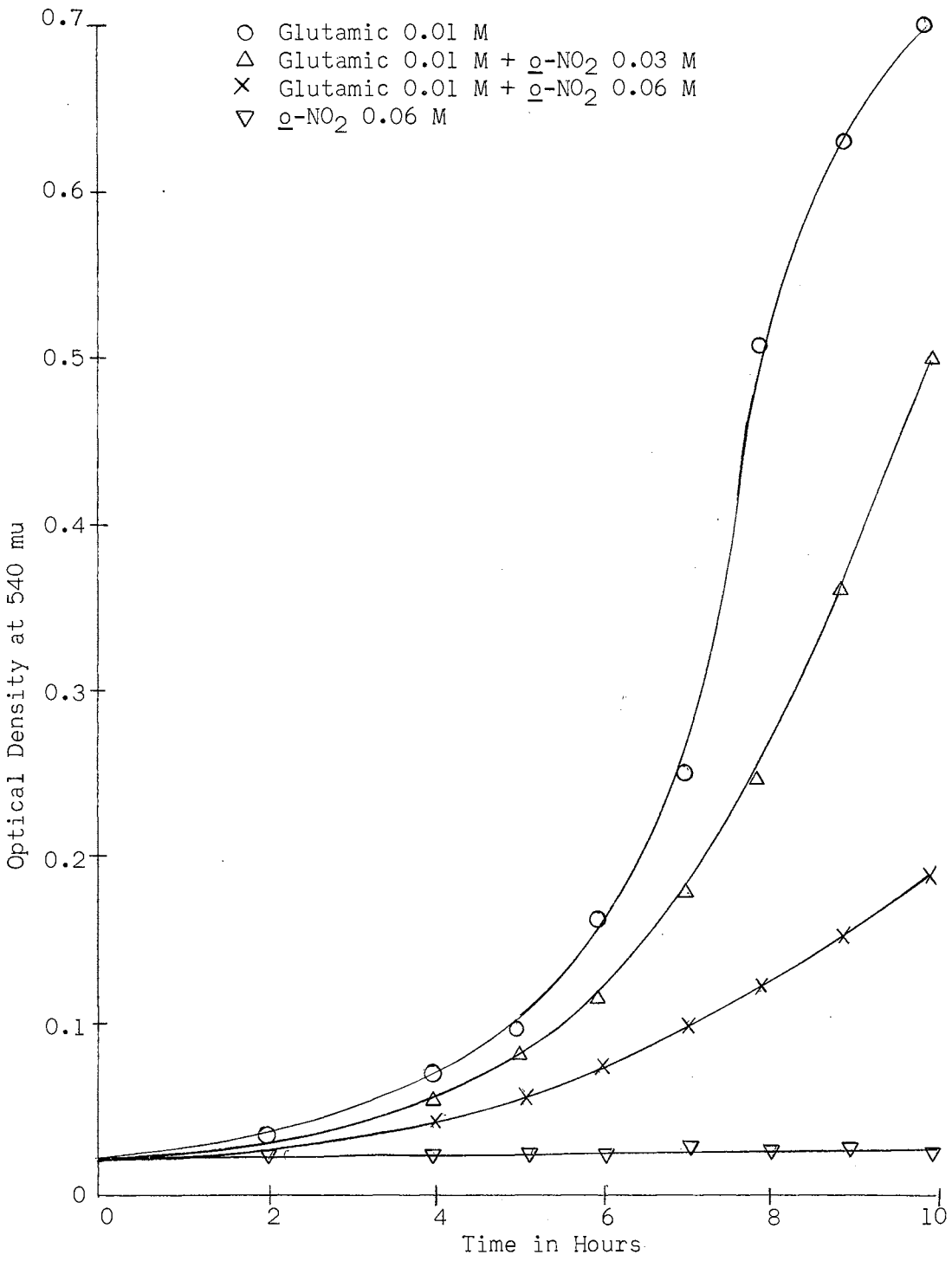

Figure 3. Effect of o-Nitrobenzoic Acid on Growth of Flavobacterium sp. using Glutamic Acid as Carbon Source.

Experiments were conducted to determine if o-nitrobenzoic acid was killing or inhibiting the cells. Cell growth was followed spectrophotometrically at 540 mμ and at varying time intervals aliquots were withdrawn and the number of viable cells determined by plating on nutrient agar using the overlay method. This consisted of pouring a thin layer of agar in the petri plate and allowing it to solidify. The inoculum was then placed on the agar and dispersed in additional medium. The plates were incubated for 18 hours at 37°C and the colonies counted. o-Nitrobenzoic acid did not kill the cells but appeared to decrease the growth rate as indicated by the data shown in Table I.

TABLE I

NUMBER OF VIABLE CELLS IN PRESENCE AND ABSENCE OF o-NITROBENZOIC ACID AS DETERMINED BY THE PLATE COUNT METHOD

Time of Incubation in Hours	Incubation Medium			
	0.02 M Succ		0.02 M Succ + 0.04 M o-NO_2	
	Plate count (Cells/ml)	O.D.	Plate count (Cells/ml)	O.D.
0	2.1×10^7	0.045	2.1×10^7	0.045
2	2.6×10^7	0.060	2.6×10^7	0.055
3	5.2×10^7	0.080	3.3×10^7	0.065

Influence on growth of varying the ratio of substrate to inhibitor.

To determine if an excess of substrate could reverse the inhibition of growth due to o-nitrobenzoic acid, experiments were conducted in which substrate to inhibitor ratios were varied (Figure 4). Succinic acid was used as the substrate in concentrations of 0.01 M,

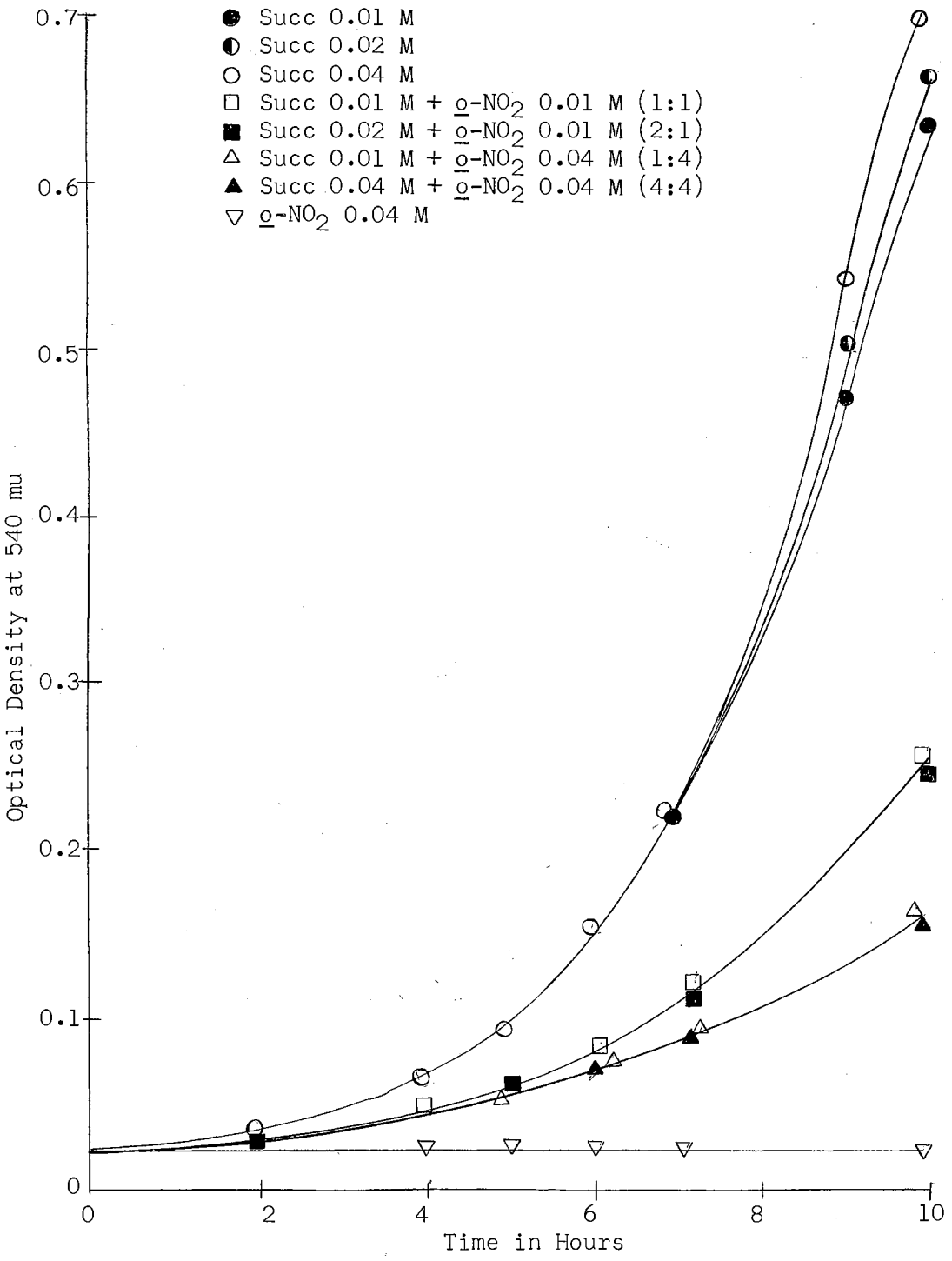

Figure 4. Effect of different Ratios of Substrate to Inhibitor on Growth of Flavobacterium sp.

0.02 M, and 0.04 M. Results indicated the growth rates were similar in the different substrate concentrations. When the o-nitrobenzoic acid concentration was 0.01 M, the same degree of inhibition was obtained in the presence of 0.01 and 0.02 M succinic acid. A more pronounced inhibition was observed when the o-nitrobenzoic acid concentration was increased to 0.04 M and succinic acid concentration was maintained at 0.01 M. Increasing the succinic acid concentration to 0.04 M did not reverse the inhibition. Since similar inhibitions were obtained regardless of substrate concentration, these results indicate that the degree of inhibition was dependent on the concentration of inhibitor and not the ratio of substrate to inhibitor. Similar results were obtained when glutamic acid was employed as the substrate.

Reversal of o-nitrobenzoic acid inhibition by complex organic materials.

Several workers have reported that the inhibition of microbial growth by benzoic acid derivatives may be reversed by the addition of certain compounds to the culture medium (8,17).

The effect of casein hydrolysate and yeast extract on the inhibition of growth by o-nitrobenzoic acid was studied to determine if these materials could reverse the inhibition. Three types of casein hydrolysates were employed: vitamin free, enzymatic digest, and an acid hydrolysate. These were obtained from Nutritional Biochemical Corporation. The yeast extract was a water soluble fraction from dried yeast produced commercially by Difco. As shown in Table II, the enzymatic digest of casein hydrolysate (0.05 per cent) completely reversed the inhibition when the o-nitrobenzoic acid concentration

TABLE II

REVERSAL OF INHIBITION OF GROWTH BY THREE DIFFERENT CASEIN HYDROLYSATES AND YEAST EXTRACT

Medium	o-NO_2 Conc.	Optical Density at 540 mu, Time of Incubation in Hours						
		0	2	4	6	8	10	12
0.02 M Succ	0.00 M	0.010	0.020	0.035	0.125	0.325	0.740	1.050
	0.04 M	0.010	0.020	0.030	0.080	0.123	0.225	0.350
	0.06 M	0.010	0.010	0.010	0.025	0.045	0.083	0.140
0.02 M Succ + 0.05 per cent Acid Hydrolyzed Casein	0.04 M	0.010	0.025	0.035	0.095	0.235	0.410	0.585
	0.06 M	0.010	0.015	0.030	0.065	0.125	0.250	0.355
0.02 M Succ + 0.05 per cent Vitamin Free Casein Hydrolysate	0.04 M	0.010	0.010	0.025	0.080	0.165	0.325	0.515
	0.06 M	0.010	0.010	0.020	0.045	0.085	0.150	0.300
0.02 M Succ + 0.05 per cent Enzymatic Digest Casein Hydrolysate	0.04 M	0.010	0.015	0.040	0.130	0.300	0.700	0.995
	0.06 M	0.010	0.025	0.025	0.065	0.140	0.280	0.390
0.02 M Succ + 0.1 per cent Yeast Extract	0.04 M	0.010	0.020	0.055	0.125	0.320	0.725	1.000
	0.06 M	0.010	0.015	0.020	0.060	0.123	0.260	0.435

was 0.04 M, but reversal was not complete at an inhibitor concentration of 0.06 M. Vitamin free casein hydrolysate and the acid hydrolysate at concentrations of 0.05 per cent showed only a slight tendency to overcome the inhibition resulting from 0.04 M o-nitrobenzoic acid. Yeast extract in a concentration of 0.1 per cent completely reversed the inhibition when the o-nitrobenzoic acid concentration was 0.04 M, but only partially reversed the inhibition when the inhibitor concentration was 0.06 M. Increasing the concentrations of the reversing agents did not reverse the inhibition of growth at the highest concentration of o-nitrobenzoic acid (0.06 M). These results suggest this is not a competitive type of inhibition. However, additional experiments must be conducted to ascertain the exact nature of the inhibition.

These data suggest that some substance(s) common to both yeast extract and enzymatic digest of casein hydrolysate may reverse the inhibition under defined conditions. Since the same concentration of acid hydrolysate and vitamin free casein hydrolysate did not reverse the inhibition to the same degree, the substance(s) responsible for the reversing action is present in these materials in different concentrations.

Casein hydrolysate and yeast extract contain a large number of amino acids and vitamins respectively (4); therefore the reversing substance(s) could be an amino acid(s), vitamin(s), or some combination of these.

Reversal of growth inhibition by known vitamin mixtures.

Since the commercial preparation of yeast extract reversed the

inhibition of growth due to o-nitrobenzoic acid, a number of known vitamins were tested. The vitamins were tested over the following concentration range (concentrations given as ug per ml of medium): vitamin B_{12} (0.05-0.4 ug), pantothenic acid (1.0-15.0 ug), folic acid (1.2-4.8 ug), riboflavin (6.0-60.0 ug), pyridoxal (6.0-60.0 ug), biotin (1.2-4.8 ug), nicotinamide (6.0-60.0 ug), p-aminobenzoic acid (6.0-60.0 ug), and L-ascorbic acid (20.0 ug). Aqueous vitamin solutions were tested both singly and in combination. Pantothenic acid (5.0 ug per ml) and vitamin B_{12} (0.2 ug per ml) showed partial reversal but an increase in the vitamin concentration did not increase reversing ability. The other vitamins did not influence the inhibition. This would suggest that the reversing substance(s) in the yeast extract was probably not a vitamin and might explain why more yeast extract than casein hyrolysate was required to completely reverse the inhibition.

Reversal of growth inhibition by known amino acids.

Experiments were conducted to determine if known amino acids could reverse the inhibition. The amino acid concentrations are listed in Table III. When amino acids were tested singly, reversal of the inhibition was only partially complete. When mixtures of the amino acids were tested, it was found that a combination of seventeen amino acids, similar in composition to casein hydrolysate (4), gave a complete reversal (Table III) equal to the reversal observed with 0.05 per cent enzymatic digest of casein hydrolysate. However, when any one or more amino acids were omitted from this mixture only a partial reversal was observed. Results indicated that some amino acids requirements were greater than others. These included glutamic acid, methionine, phenylalanine, histidine, tryptophan, leucine, proline and tyrosine. However,

TABLE III

RESPONSE OF FLAVOBACTERIUM TO COMBINATIONS OF 17 AMINO ACIDS IN PRESENCE OF o-NITROBENZOIC ACID

Amino Acids***(concentrations given as ug per ml of medium)																	Optical Density at 540 mu	
ala	asp	glu	arg	isol	lys	meth	phen	ser	thre	tryp	val	gly	hist	leu	pro	tyr	6 hours	9 hours
80 ug			16 ug								8 ug							
Succinic acid control																	0.205	0.600
+**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0.200	0.600
-*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.090	0.225
-	-	+	-	-	-	+	+	-	-	+	-	-	+	+	+	+	0.130	o.435
-	-	-	-	-	-	+	+	-	-	+	-	-	+	+	+	+	0.130	0.350
-	-	+	-	-	-	-	+	-	-	+	-	-	+	+	+	+	0.185	0.485
-	-	+	-	-	-	+	-	-	-	+	-	-	+	+	+	+	0.165	0.470
-	-	+	-	-	-	+	+	-	-	-	-	-	+	+	+	+	0.160	0.455
-	-	+	-	-	-	+	+	-	-	+	-	-	-	+	+	+	0.160	0.470
-	-	+	-	-	-	+	+	-	-	+	-	-	+	-	+	+	0.185	0.490
-	-	+	-	-	-	+	+	-	-	+	-	-	+	+	-	+	0.140	0.420
-	-	+	-	-	-	+	+	-	-	+	-	-	+	+	+	-	0.175	0.475
-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	0.135	0.325
-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+	+	0.115	0.275
-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0.215	0.470
+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0.169	0.435
+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0.155	0.300
+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	0.215	0.540
+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	0.210	0.450
+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	0.180	0.420
+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	0.140	0.300
+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	0.215	0.350
+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	0.210	0.460
+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	0.210	0.465
+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	0.160	0.365
+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	0.200	0.460
+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	0.210	0.410

TABLE III (CONTINUED)

ala	asp	glu	arg	isol	lys	meth	phen	ser	thre	tryp	val	gly	hist	leu	pro	tyr	6 hours	9 hours
80 ug			16 ug								8 ug							
+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	0.120	0.260
+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	0.135	0.280
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	0.130	0.280
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	0.110	0.240

*(-) indicates amino acid is absent from medium
**(+) indicates amino acid was present in medium
*** Abbreviations: Alanine (ala), aspartic acid (asp), arginine (arg), isoluecine (isol), methionine (meth), phenylalanine (phen), serine (ser), threonine (thre), tryptophan (tryp), valine (val), glycine (gly), histidine (hist), leucine (leu), proline (pro), and tyrosine (tyr).

a combination of these amino acids, in which the concentration was doubled, still did not give complete reversal. Results indicated that of the amino acids tested, either singly or in combinations of four or five, the aromatic amino acids (phenylalanine, tryptophan, tyrosine) appeared to have the greatest effect, although the reversal was not complete.

Shikimic acid has been reported to serve as the precursor for biosynthesis of the aromatic amino acids (12), therefore, shikimic acid was tested for its reversing ability. As shown in Table IV, shikimic acid tested over a concentration range of 150 to 600 ug per ml of medium appeared to have little affect as a reversing agent. However it was not determined if the cells were permeable to shikimic acid.

Since a combination of 17 amino acids appeared to reverse the inhibition, ammonium chloride (0.2 per cent) was added to the medium to determine if the nitrogen source was limiting for growth. Increasing the ammonium chloride concentration did not influence the inhibition.

Reversal of growth inhibition by organic compounds.

Since both casein hydrolysate and yeast extract contain a number of substances other than amino acids and vitamins, several purines, pyrimidines, and nucleotides were tested for their ability to reverse the inhibition of growth due to o-nitrobenzoic acid. The following compounds were tested (concentrations cited per ml of medium): adenosine (20.0 ug), guanosine (20.0 ug), cytosine (20.0 ug), xanthine (20.0 ug), thiamine (20.0 ug), triphosphopyridine nucleotide (5.0 ug), and diphosphopyridine nucleotide (5.0 ug). Other compounds tested were: coenzyme A (15.0-50.0 ug), sodium nucleate (120.0 ug), and cytochrome C (120 ug).

TABLE IV

EFFECT OF SHIKIMIC ACID ON THE INHIBITION OF GROWTH OF A FLAVOBACTERIUM sp. BY o-NITROBENZOIC ACID

Time of Incubation in Hours	Optical Density at 540 mu						
	0.02 M Succ Control	0.02 M Succ + 0.04 M o-NO_2 Shikimic acid (ug per ml)			0.02 M Succ + 0.06 M o-NO_2 Shikimic acid (ug per ml)		
		0 ug	150 ug	600 ug	0 ug	150 ug	600 ug
0	0.040	0.040	0.040	0.040	0.040	0.040	0.040
2	0.055	0.055	0.050	0.045	0.040	0.050	0.045
3	0.090	0.065	0.085	0.070	0.055	0.055	0.070
4	0.160	0.105	0.105	0.105	0.085	0.080	0.105
5	0.260	0.140	0.155	0.140	0.125	0.105	0.130
6	0.365	0.193	0.202	0.193	0.140	0.130	0.160
7	0.500	0.235	0.250	0.235	0.160	0.160	0.180
9	0.895	0.400	0.420	0.410	0.275	0.290	0.300
10	1.000	0.470	0.480	0.460	0.320	0.335	0.330

Triphosphopyridine nucleotide (5.0 ug) and coenzyme A (25.0 ug) gave partial reversal. When the concentration of these two compounds was increased, they gave little additional reversal. The significance of these results will be discussed in Chapter IV. The other compounds tested were not able to reverse the inhibition; however, again it was not determined if the cells were permeable to these compounds.

Incubation experiments to detect accumulating metabolites.

The inhibition of chemical reactions in biosynthetic pathways is one of the mechanisms by which certain inhibitors suppress microbial growth. In many instances it has been observed that precursors accumulate in the experimental system and may be isolated. Studies were undertaken to isolate precursors that might be accumulating in the medium and a number of incubation experiments were conducted in an attempt to isolate such a compound(s). A synthetic medium containing succinic acid (0.04 M) and o-nitrobenzoic acid (0.04 M) was inoculated and incubated on a reciprocal shaker at 37°C. At different time intervals, aliqouts were withdrawn and filtered through a Millipore filter. Several techniques were used to extract the resulting supernatant. Some of the solvents used were: ethanol, n-butanol, choloroform, and ether. The fractions were then chromatographed in different solvent systems: iso-butyric acid: water (4:1), 95 per cent ethanol: ammonium hydroxide (19:1), n-butanol: acetic acid:water (4:1:5). The results obtained were inconsistent and varied from one experiment to the next; therefore, no definite conclusions could be drawn.

An ultraviolet spectrum was also run on the filtrates using a Model 14 Cary Recording Spectrophotometer. Curves obtained from the ultraviolet spectrum showed an absorption peak at 263 mu. Figure 5 indicates that the absorbancy at 263 mu increased in magnitude with the continued incubation of the cells in the medium containing succinic acid and o-nitrobenzoic acid and in the succinic acid control. The high initial reading at this wavelength in the medium containing the inhibitor was due to the absorption of o-nitrobenzoic acid which has an absorption

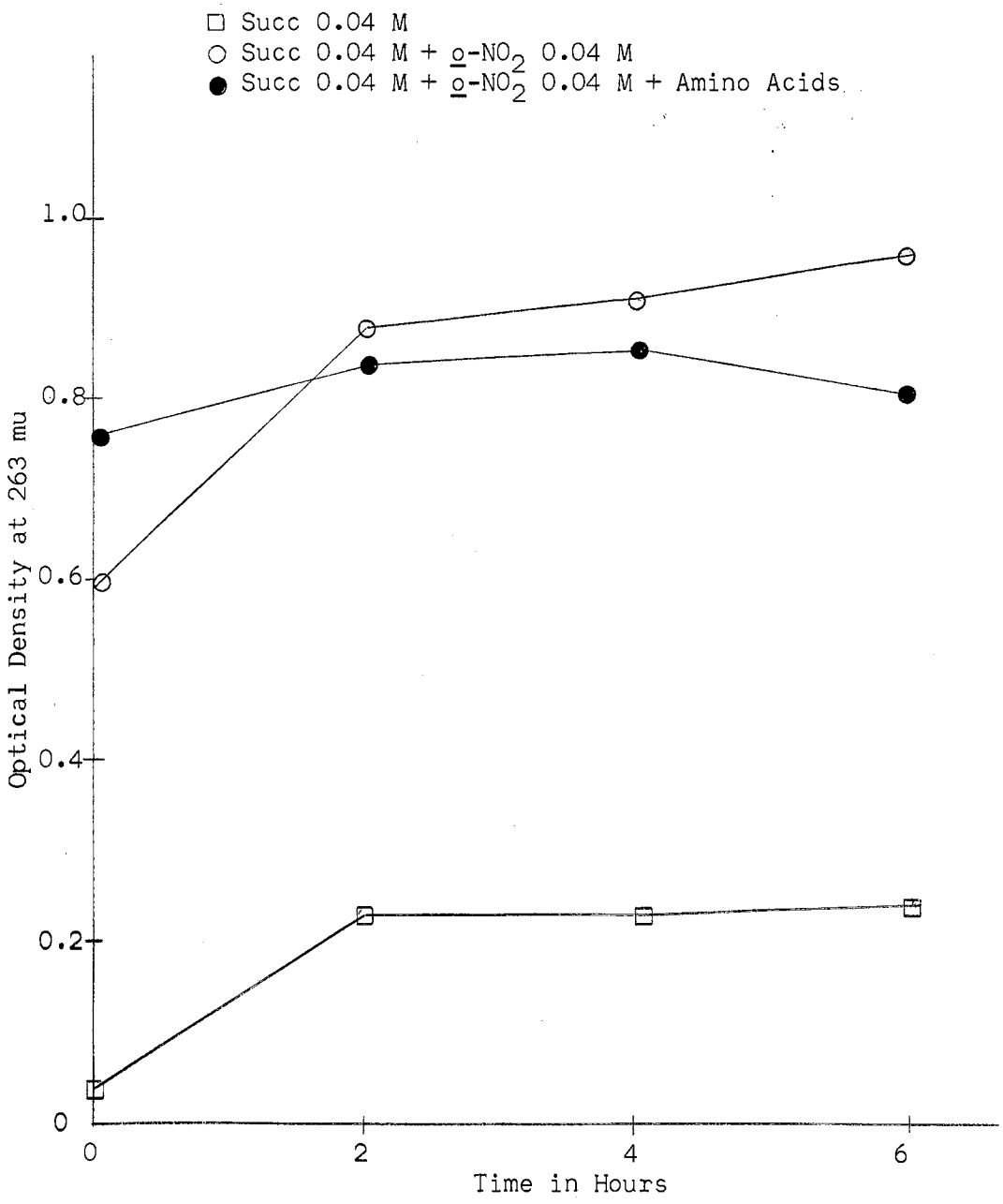

Figure 5. Accumulation of Substance in Culture Medium with Absorption Maximum of 263 mu.

maximum of 268 mu. The absorbancy at 263 mu increased in magnitude with time and appeared to be greater in the medium containing o-nitrobenzoic acid, but a compound having this absorption maximum could not be isolated from the supernatant with the techniques employed. When the seventeen amino acids were added to the incubation mixture, the absorbancy did not increase significantly in magnitude. This result indicates that material with an absorption maximum of 263 mu did not accumulate significantly in the presence of the amino acids.

Effect of o-nitrobenzoic acid on oxidation of substrates.

Cain (7) reported that m-nitrobenzoic acid inhibited the oxidation of p-nitrobenzoic acid by Nocardia erythropolis, but o-nitrobenzoic acid had no effect. When the oxidation of substrates by Flavobacterium sp. was studied using manometric procedures it was found that o-nitrobenzoic acid did not inhibit the rate of substrate oxidation by this organism but did increase the total oxygen consumed. Results presented in Figure 6 show that oxygen consumed during the oxidation of 4.0×10^{-6} moles per flask of succinic acid was significantly greater in the flasks containing substrate plus either 6.6×10^{-5} or 1.32×10^{-4} moles of o-nitrobenzoic acid. The o-nitrobenzoic acid concentrations were approximately the same as those used in growth studies. The results indicate that 1.06 umoles of oxygen were consumed per umole of succinic acid in the absence of o-nitrobenzoic acid (Table V). In the presence of 6.6×10^{-5} moles and 1.32×10^{-4} moles per flask of o-nitrobenzoic acid there were 1.5 and 2.02 umoles of oxygen taken up per umole of succinic acid respectively. Other substrates tested were acetic acid, malic acid, α-ketoglutaric acid and glutamic acid. All substrates were oxidized

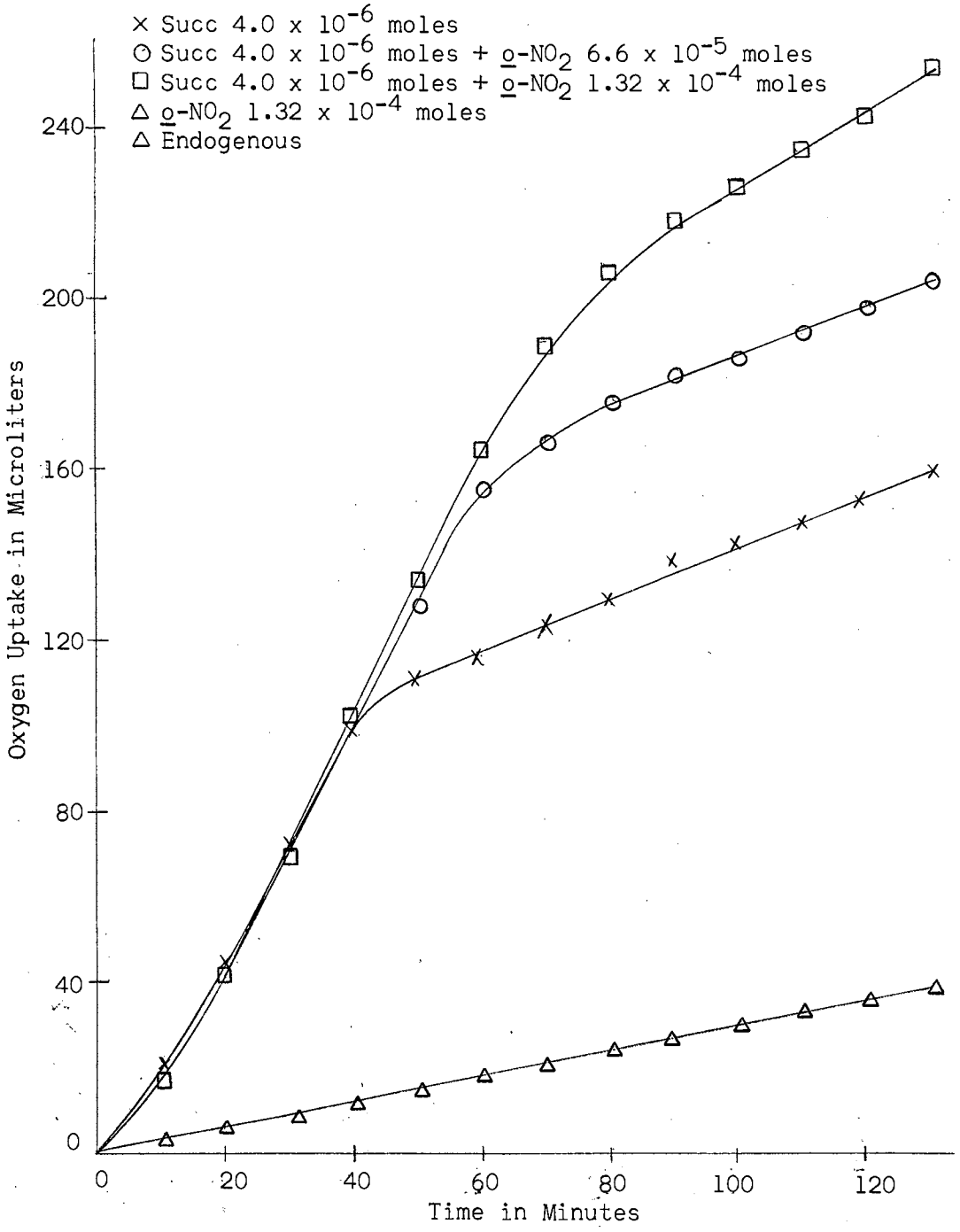

Figure 6. The Oxidation of Succinic Acid by Flavobacterium sp. in Presence and Absence of o-Nitrobenzoic Acid.

TABLE V

OXIDATION OF SUCCINIC ACID BY FLAVOBACTERIUM sp. IN PRESENCE AND ABSENCE OF o-NITROBENZOIC ACID

	Flask Contents		
	Succ 4.0×10^{-6} moles	Succ 4.0×10^{-6} moles o-NO_2 6.6×10^{-5} moles	Succ 4.0×10^{-6} moles o-NO_2 1.32×10^{-4} moles
umoles Oxygen Uptake per umole Succ	1.06	1.5	2.02

to a greater extent in the presence of 6.6×10^{-5} and 1.32×10^{-4} moles of o-nitrobenzoic acid with the exception of acetic acid (0.04 umoles). In the absence of o-nitrobenzoic acid, 1.8 umoles of oxygen were consumed per umole of acetic acid (Figure 7). The theoretical value for complete oxidation of acetic acid is 2.0 umoles. Therefore, since acetic acid was oxidized to completion in the absence of the inhibitor, it was impossible to determine if o-nitrobenzoic acid was influencing the oxidation of this substrate.

Studies were also conducted to determine if o-nitrobenzoic acid influenced the liberation of carbon dioxide from various substrates. Results presented in Table VI show that o-nitrobenzoic acid increased the quantity of carbon dioxide liberated during substrate oxidation. In the absence of o-nitrobenzoic acid, the cell suspension liberated 2.00 umoles carbon dioxide per umole succinic acid while in the presence of 6.6×10^{-5} moles and 1.32×10^{-4} moles of o-nitrobenzoic acid the cells liberated 3.08 umoles and 4.26 umoles of carbon dioxide per umole of succinic acid. These values are in agreement with the increased oxygen consumption observed in the presence of o-nitrobenzoic acid (Figure 6).

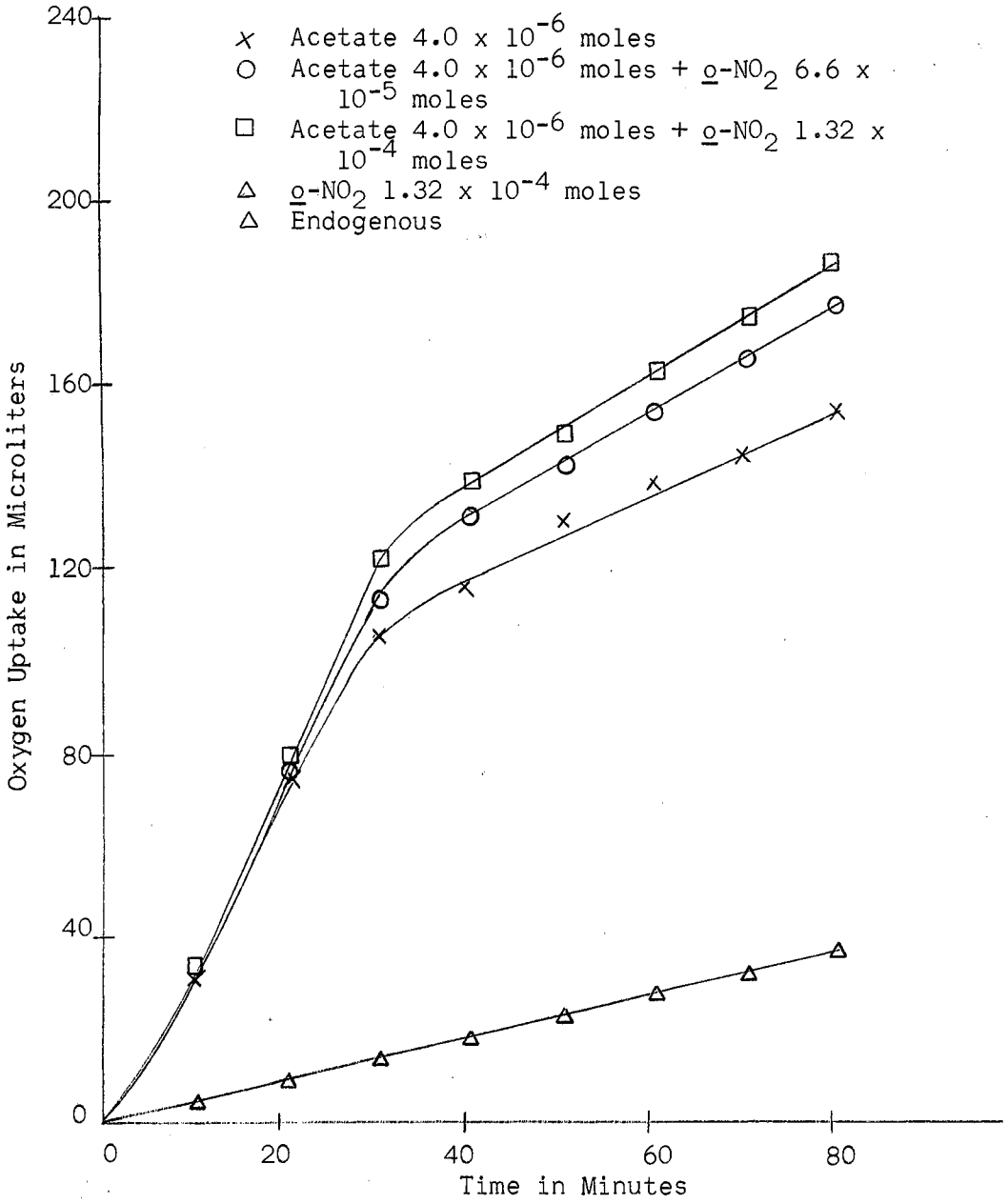

Figure 7. The Oxidation of Acetic Acid by Flavobacterium sp. in Presence and Absence of o-Nitrobenzoic Acid.

TABLE VI

THE EFFECT OF o-NITROBENZOIC ACID ON CARBON DIOXIDE LIBERATION

Flask Content Moles/Flask	umoles per umole Substrate		
	CO_2 Liberated	O_2 Uptake	Ratio CO_2/O_2
Succ $4.0x10^{-6}$	2.00	1.06	1.98
Succ $4.0x10^{-6}$ + o-NO_2 $6.6x10^{-5}$	3.08	1.50	2.06
Succ $4.0x10^{-6}$ + o-NO_2 $1.32x10^{-4}$	4.26	2.02	2.1

Effect of o-nitrobenzoic acid on substrate oxidation when added at different time intervals.

To determine at what time period o-nitrobenzoic acid must be present to exert its effect on substrate oxidation, o-nitrobenzoic acid was added at different time intervals to cells actively metabolizing the substrate. Results indicated that the total oxygen consumed was dependent on the time at which o-nitrobenzoic acid was added to the actively metabolizing cells Succinic acid in a concentration of $6.0x10^{-6}$ moles was completely oxidized in 60 minutes (Figure 8). When o-nitrobenzoic acid was added simultaneously with the substrate at 0 minutes there was a definite increase in the amount of oxygen consumed. Addition of the inhibitor at 15 minutes showed a significant increase in the oxygen consumed but the uptake was not as great as when the inhibitor was added at 0 minutes. The addition of o-nitrobenzoic acid at 30 minutes or later showed little

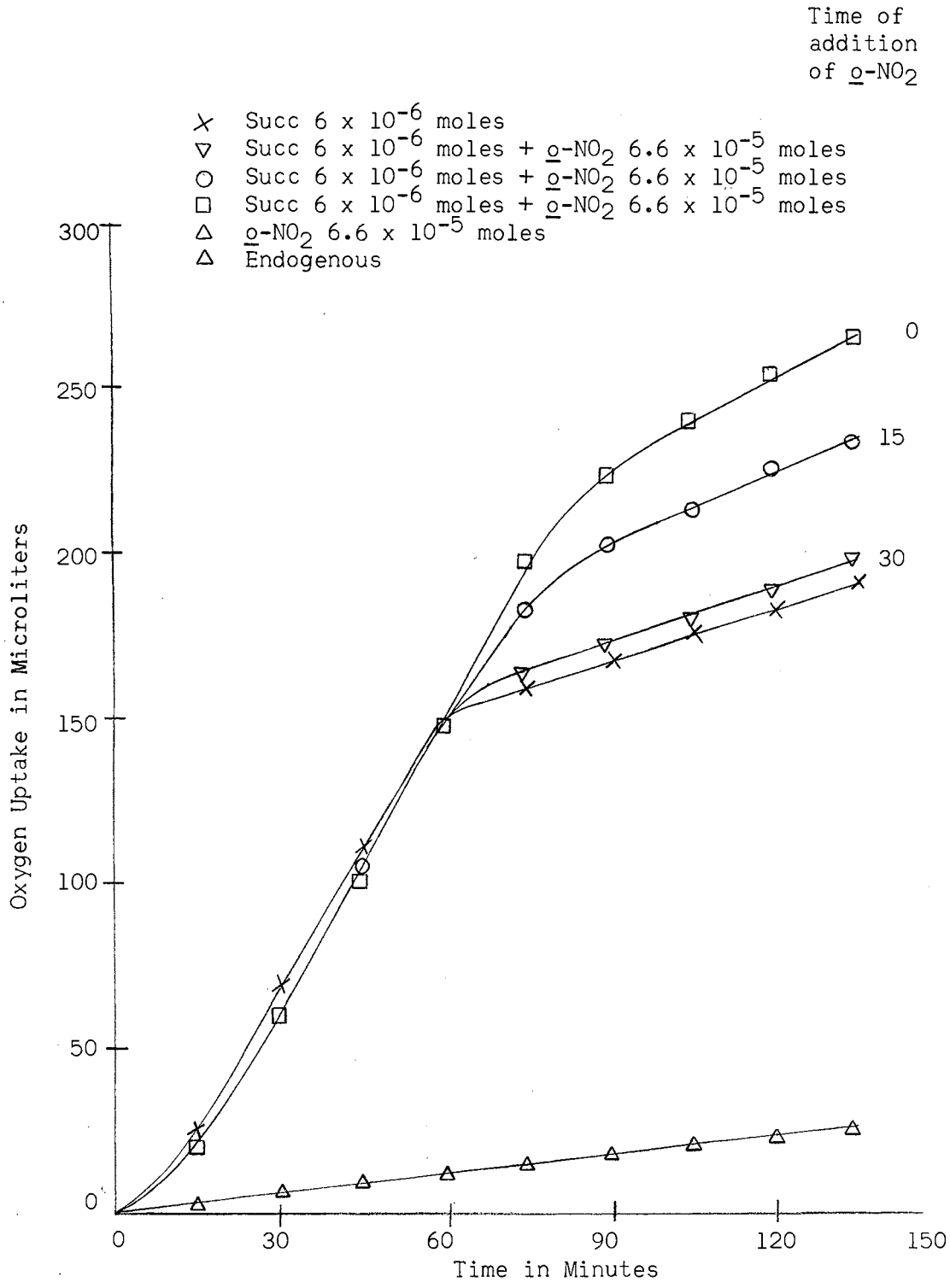

Figure 8. Addition of o-Nitrobenzoic Acid at Varying time Intervals to Flavobacterium sp. oxidizing succinic acid.

stimulation of total oxygen uptake. These results indicate that o-nitrobenzoic acid must be present during the initial stages of substrate oxidation to obtain the greatest increase in total oxygen consumption.

Since the time at which the inhibitor is added to the actively metabolizing cells is very important, additional experiments were conducted in which the cells were incubated with the inhibitor prior to addition of the substrate. The cell suspension was divided into two aliquots and one resuspended in 0.1 M o-nitrobenzoic acid and 0.02 M phosphate buffer while the second aliquot was resuspended in 0.02 M phosphate buffer and served as the control. The cell suspensions were shaken at 37°C for two hours and then washed by centrifugation. The cells from the two vessels were then resuspended in phosphate buffer to the standard density and the oxidation of succinic acid (0.06 M) by both groups of cells was studied in the presence and absence of two concentrations of o-nitrobenzoic acid (6.6×10^{-5} and 1.32×10^{-4} moles). As shown in Figure 9 the oxidation of succinic acid in the absence of o-nitrobenzoic acid by both cell suspensions was the same. In the presence of o-nitrobenzoic acid (6.6×10^{-5} moles) an equivalent increase in oxygen consumption was observed in both groups of cells. The higher concentration of o-nitrobenzoic acid (1.32×10^{-4} moles) gave an even greater increase in oxygen uptake, but again both cell suspensions showed similar results. Additional experiments were conducted in which o-nitrobenzoic acid was added to the cell suspensions at 0, 30, 45, 60, and 75 minutes prior to the addition of succinic acid. Again o-nitrobenzoic acid increased the oxygen uptake over that observed with the succinic acid control, but the stimulation was the same regardless of whether the o-nitrobenzoic acid was added simultaneously with succinic

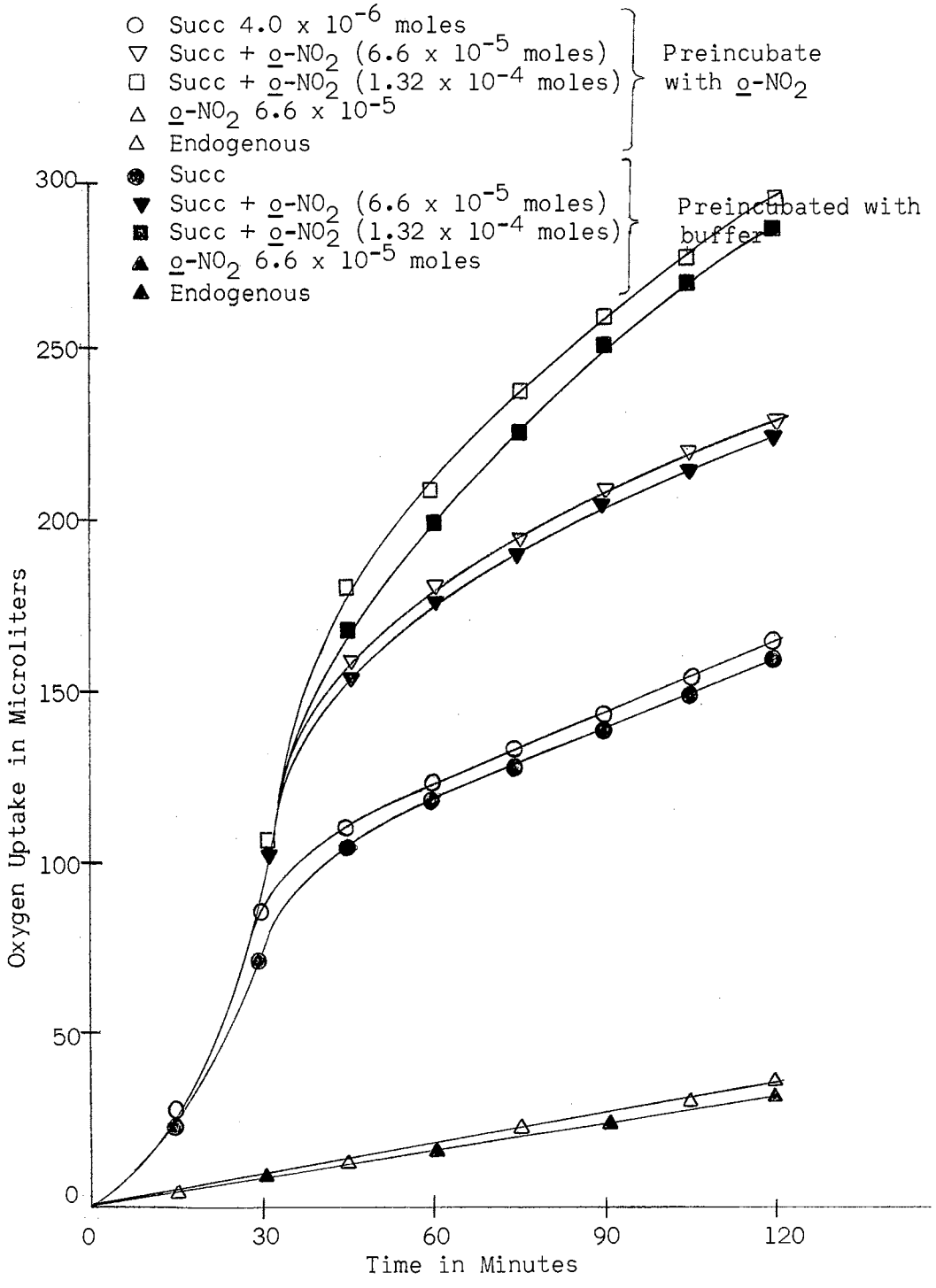

Figure 9. Effect of preincubating Flavobactium sp. in Presence and Absence of o-Nitrobenzoic Acid on Oxidation of Succinic Acid in Presence and Absence of o-Nitrobenzoic Acid.

acid or 75 minutes prior to the addition of succinic acid. These results augment the previous findings that o-nitrobenzoic acid must be present during the early stages of substrate oxidation in order to obtain the greatest increase in total oxygen consumption.

Oxidation of succinic acid by cell-free extract.

A cell-free extract, prepared in accordance with the procedure presented in Chapter II, was capable of oxidizing succinic acid (Figure 10). Addition of 1.32×10^{-4} moles of o-nitrobenzoic acid to the extract simultaneously with the substrate did not increase the oxygen consumed during the oxidation of 20×10^{-6} moles of succinic acid. Oxygen uptake was slightly lower in the flask containing o-nitrobenzoic acid but the rate and total oxygen consumed were equivalent to the oxidation observed in the succinic acid control.

Effect of o-nitrobenzoic acid on induction to different inducers.

A number of aromatic compounds will serve as inducible substrates for this organism. Respirometric experiments were conducted to determine the effect of o-nitrobenzoic acid on inducible enzyme formation as a means of studying the effect of o-nitrobenzoic acid on protein synthesis. Enzymatically non-induced cells were obtained by growing cells on nutrient agar or succinic acid agar. p-Aminobenzoic, protocatechuic, and benzoic acids were studied for their ability to induce enzyme formation in the presence and absence of o-nitrobenzoic acid. All inducers were used in a final concentration of 2.0×10^{-6} moles per flask. Addition of 1.6×10^{-4} moles of o-nitrobenzoic acid delayed induction to benzoic acid for approximately 100 minutes (Figure 11). At this concentration of o-nitrobenzoic acid (1.6×10^{-4} moles), induction to p-aminobenzoic acid

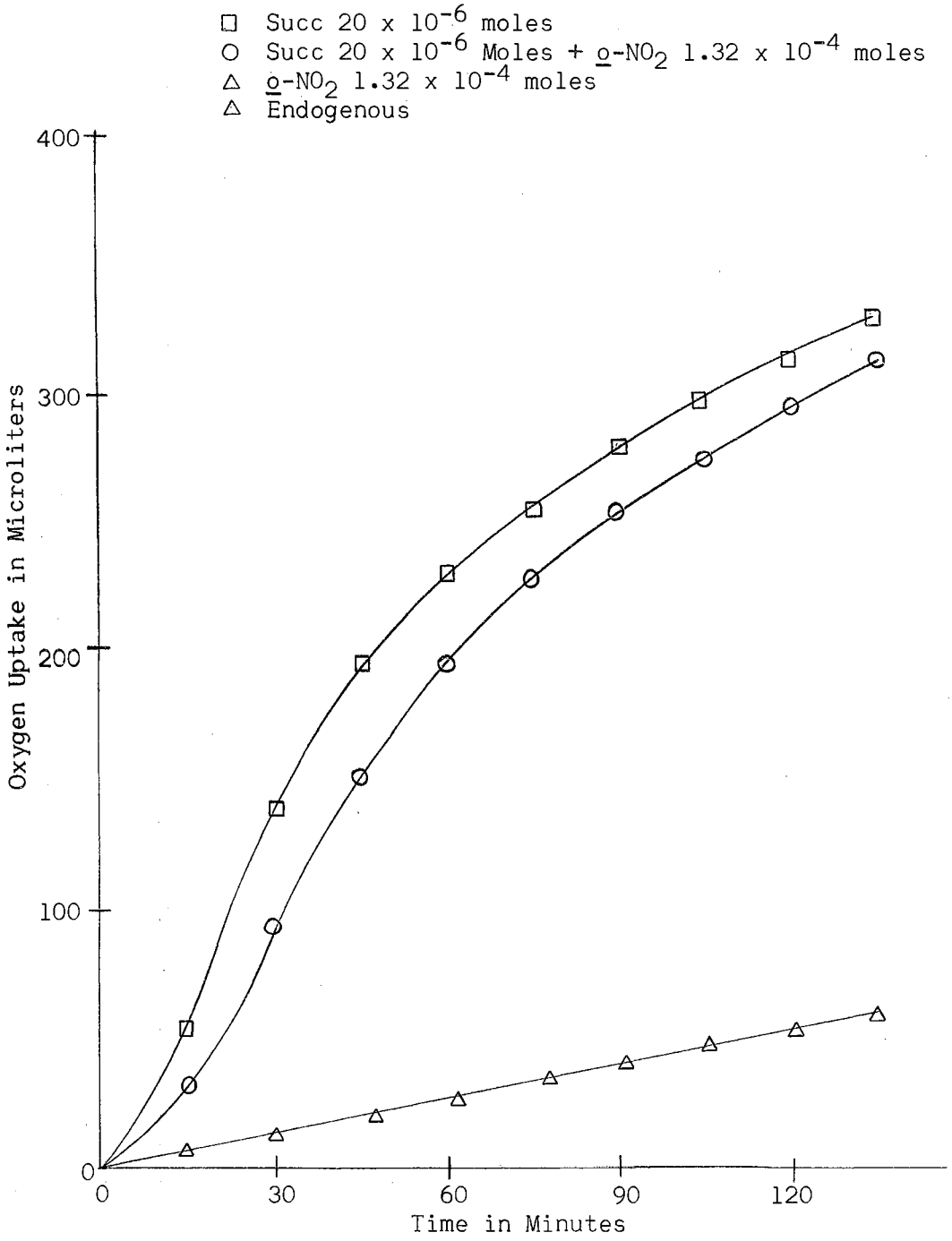

Figure 10. Influence of o-Nitrobenzoic Acid on Oxidation of Succinic Acid by Cell-Free Extract.

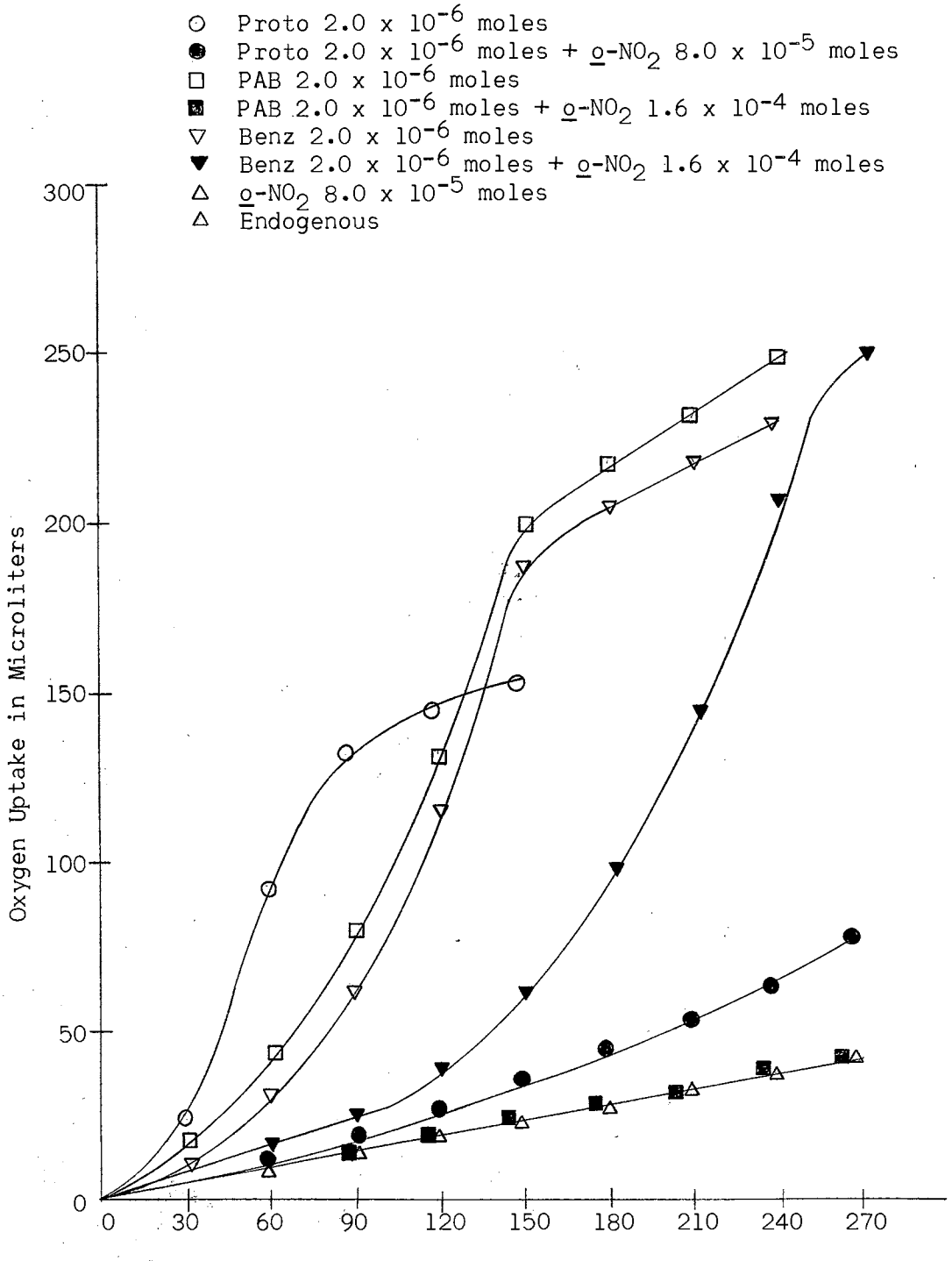

Figure 11. Effect of o-Nitrobenzoic Acid on Inducible Enzyme Formation in Flavobacterium sp.

was completely inhibited, and a concentration of 8.0×10^{-5} moles o-nitrobenzoic acid inhibited induction to protocatechuic acid. Therefore, these results indicate that o-nitrobenzoic acid can interfere with protein synthesis. It appeared that enzyme formation for the oxidation of benzoic acid was not as sensitive to o-nitrobenzoic acid as the other two inducible enzyme systems. It may be possible that o-nitrobenzoic acid is inhibiting the synthesis of different enzyme systems by different mechanism(s) or that they are being inhibited by the same mechanism, but enzyme synthesis for protocatechnic and p-aminobenzoic acid are more sensitive to the inhibitor than synthesis of the benzoic acid enzyme.

Effect of other inhibitors on substrate oxidation.

Different metabolic inhibitors were tested for their ability to inhibit induction of cells to p-aminobenzoic and p-nitrobenzoic acid. Non-induced cells were grown on agar containing asparagin as the carbon source. Sodium azide, which uncouples oxidative phosphorylation (22), and sodium arsenate, which interferes with phosphorylation of ADP, in a concentration of 2.2×10^{-4} moles had no effect on adaptation. Proflavin, which inhibits protein synthesis, in a concentration of 1.0×10^{-5} moles inhibited induction to p-aminobenzoic acid and p-nitrobenzoic acid. 2,4-Dinitrophenol, which uncouples oxidative phosphorylation in some organisms (22), had little effect on induction to either substrate in a concentration of 2.2×10^{-4} moles. These results show that, with exception of proflavin, this organism was not sensitive to these metabolic poisons in the concentrations tested.

Oxidative phosphorylation studies in the presence and absence of o-nitrobenzoic acid.

As indicated by the data presented in Figure 6, o-nitrobenzoic acid increased the total oxygen uptake but not the rate of oxidation by a resting cell suspension actively oxidizing succinic acid. Although a stimulation in rate is usually observed in combination with increased oxygen uptake during the uncoupling of oxidative phosphorylation (22), studies were conducted to determine if o-nitrobenzoic acid might be influencing oxidative phosphorylation in the cell.

Several attempts were made to obtain phosphate incorporation using cell-free extracts or whole cells. Phosphate incorporation studies with cell-free extracts were made using the following reaction mixture: ADP, 4.0 umoles; K_2HPO_4, 8.0 umoles; succinate, 10.0 umoles; and cell-free extract, 1.0 ml. All substrates were dissolved in 0.01 M Tris buffer and adjusted to pH 7.0. In vessels containing o-nitrobenzoic acid, 160 umoles of the inhibitor were added. Tris buffer was added to make the final volume 2.4 ml.

Different experimental systems were employed to obtain inorganic phosphate incorporation. A phosphate acceptor system was used which consisted of 1 mg purified hexokinase, 10.0 umoles glucose, and 4.0 umoles $MgSO_4$. Fifty umoles of NaF were also added in some experiments to inhibit ATPase (5). Several other oxidizable substrates such as malic, fumaric, and α-ketoglutaric acids were also tested. Variations were made in the cell growth conditions, buffer, and the method of rupturing cells, but significant phosphate incorporation was not observed in any of the experiments. Studies were conducted in which whole cells replaced the cell-free extract but the cell suspension did

not incorporate inorganic phosphate in sufficient quantity to be accurately measured.

Adenosine triphosphatase activity of cell-free extract.

Results obtained from studies measuring inorganic phosphate uptake showed an accumulation of inorganic phosphate in the medium indicating that the cell-free extract may have contained an ATPase. Experiments were conducted to study the affect of o-nitrobenzoic acid on ATPase. The incubation mixture consisted of 6.0 umoles ATP, 1.0 ml extract, and 0.1 M Tris buffer of pH 7.4 was added to a final volume of 2.4 ml. o-Nitrobenzoic acid was used at a concentration of 120 umoles in the initial studies. Since magnesium is a cofactor for many phosphatases, $MgSO_4$ was added to some tubes in a concentration of 6.0 umoles. Table VII shows the results of a typical experiment.

The liberation of inorganic phosphate by the cell-free extract was stimulated by $MgSO_4$. In the presence of o-nitrobenzoic acid this liberation was inhibited significantly.

The mechanism of inhibition of ATPase activity by o-nitrobenzoic acid was studied. o-Nitrobenzoic acid was incubated with the cell-free extract for varying time periods before adding substrate. Two concentrations of inhibitor were used, 120 umoles and 320 umoles. Identical tubes were prepared and o-nitrobenzoic acid (120 umoles and 320 umoles) was incubated with the cell-free extract for 0, 10, 20, and 30 minutes after which time ATP was added and inorganic phosphate determined 15 minutes later. Results presented in Table VIII show that when ATP and the inhibitor were added simultaneously at 0 minutes, the o-nitrobenzoic acid inhibited the release of inorganic phosphate and the inhibition was

TABLE VII

EFFECT OF o-NITROBENZOIC ACID AND MAGNESIUM SULFATE ON ADENOSINE TRIPHOSPHATASE ACTIVITY OF CELL EXTRACT

Incubation mixture	umoles Pi released per tube in 15 minutes
extract	0
extract+o-NO_2	0
extract+$MgSO_4$	0
extract+$MgSO_4$+o-NO_2	0
extract+ATP	2.77
extract+ATP+$MgSO_4$	3.96
extract+ATP+o-NO_2	2.31
extract+ATP+o-NO_2+$MgSO_4$	2.80

ATP, 6.0 umoles; o-NO_2, 120 umoles; $MgSO_4$, 6.0 umoles; extract 1.0 ml; 0.1 M Tris buffer to total volume of 2.4 ml.

TABLE VIII

INFLUENCE OF o-NITROBENZOIC ACID ON LIBERATION OF INORGANIC PHOSPHATE WHEN PREINCUBATED WITH CELL-FREE EXTRACT

	umoles Inorganic Phosphate Liberated per Tube in 15 Minutes			
	Time of Incubation (minutes) Prior to Addition of ATP			
Tube Components	0	10	20	30
Extract Control (No ATP added)	0.70	--	--	0.80
ATP	0.08	--	--	0.08
ATP + 120 umoles o-NO_2	0.08	--	--	0.08
Extract	2.75	2.83	3.29	3.42
Extract + 120 umoles o-NO_2	2.34	2.52	2.70	2.88
Extract + 320 umoles o-NO_2	2.03	1.98	1.94	1.83

6.0 umoles $MgSO_4$ and 1.0 ml extract added to all tubes at 0 minutes. 0.1 M Tris buffer added to make a final volume 2.7 ml. At completion of incubation period 6.0 umoles ATP were added and inorganic phosphate determined 15 minutes later.

proportional to the inhibitor concentration. As shown in Table VIII there was an increase in phosphate liberation in the absence of the inhibitor which was related to the time of incubation of the extract. This increased phosphate liberation was also observed in the presence of 120 umoles of o-nitrobenzoic acid but not in the presence of 320 umoles of the inhibitor. This could be due to an activation of the enzyme during incubation, although the higher concentration of o-nitrobenzoic acid prevented this activation by some mechanism. However, when the extract was incubated with 120 umoles of o-nitrobenzoic acid prior to addition of substrate (ATP), the inhibition was approximately the same for all time periods. These results indicate that incubation of the inhibitor with the extract did not significantly alter the ATPase activity observed when the inhibitor and ATP were added simultaneously. This would suggest that o-nitrobenzoic acid was not complexing irreversably with the "active site" of the enzyme.

Experiments were also conducted to determine if incubating o-nitrobenzoic acid and $MgSO_4$ with ATP prior to addition of extract was inactivating or complexing with the substrate or activating ion. o-Nitrobenzoic acid was incubated with 6.0 umoles ATP and 6.0 umoles $MgSO_4$ for 0, 10, 20, 30, and 40 minutes prior to the addition of extract. After the preincubation period, extract was added and incubated for 15 minutes at which time the reaction was stopped by the addition of trichloroacetic acid. Results show (Table IX) that the same degree of inhibition of the liberation of inorganic phosphate was obtained whether the extract was added to ATP, $MgSO_4$, and o-nitrobenzoic acid mixture at 0 minutes or 40 minutes. These results reveal that o-nitrobenzoic acid does not complex with ATP or $MgSO_4$.

TABLE IX

INFLUENCE OF PREINCUBATING o-NITROBENZOIC ACID WITH ADENOSINE TRIPHOSPHATE ON ATPase ACTIVITY OF CELL-FREE EXTRACTS

Tube Components	umoles Inorganic Phosphate Liberated per Tube in 15 Minutes Time of Incubation (minutes) prior to Addition of Extract				
	0	10	20	30	40
ATP + Extract	4.62	4.60	4.58	4.61	4.60
ATP + o-NO_2 + Extract	3.69	3.70	3.72	3.65	3.70
o-NO_2 + Extract	0.60	--	--	--	0.73
Extract	0.60	--	--	--	0.71

6.0 umoles $MgSO_4$ in all tubes; ATP, 6.0 umoles; o-NO_2, 120 umoles; extract 1.0 ml; 0.1 M Tris buffer pH 7.4 to final volume of 2.4 ml. At completion of incubation period the extract was added and inorganic phosphate determined 15 minutes later.

TABLE X

INFLUENCE OF o-NITROBENZOIC ACID ON ATPase ACTIVITY IN WHOLE CELLS

Incubation Mixture	umoles Inorganic Phosphate Liberated per Tube in 45 Minutes
Cells	0.0
Cells + ATP	1.02
Cells + o-NO_2	0.0
Cells + ATP + o-NO_2	0.64

Each tube contained 6.0 umoles $MgSO_4$. The other components were ATP, 6.0 umoles; o-NO_2, 240 umoles; cells, 1.0 ml; and 0.1 M Tris buffer pH 7.4 to final volume of 2.4 ml.

A study was made to determine if ATPase could be detected in whole cells. Cell suspensions were incubated with 6.0 umoles ATP, 6.0 umoles $MgSO_4$ and o-nitrobenzoic acid. As shown in Table X, ATPase activity was detectable in whole cells and o-nitrobenzoic acid inhibited the release of inorganic phosphate.

To determine if the inhibition by o-nitrobenzoic acid was specific for the breakdown of ATP, similar experiments were conducted employing ADP. As shown in Table XI, phosphate was liberated from ADP but o-nitrobenzoic acid did not inhibit this cleavage. Unlike the inhibition of ATPase, o-nitrobenzoic acid stimulated the liberation of inorganic phosphate from ADP. Thus, it would appear that the liberation of phosphate from ADP and ATP was accomplished by two different mechanisms.

TABLE XI

EFFECT OF o-NITROBENZOIC ACID ON LIBERATION OF PHOSPHATE FROM ADENOSINE DIPHOSPHATE BY CELL EXTRACT

Incubation Mixture	Experiment	umoles Inorganic Phosphate Liberated per Tube in 15 Minutes
ADP	A	0.0
	B	0.0
ADP + o-NO_2	A	0.0
	B	0.0
Extract	A	0.0
	B	0.0
o-NO_2+ Extract	A	0.0
	B	0.0
ADP + Extract	A	1.06
	B	1.52
ADP + o-NO_2+ Extract	A	1.58
	B	3.16

Each tube contained ADP, 6.0 umoles; o-NO_2, 120 umoles; cell extract, 1.0 ml; 0.1 M Tris buffer pH 7.4 to final volume of 2.4 ml.

Pyrophosphatase activity of cell-free extract.

Pyrophosphatase activity was determined by incubating cell-free extracts with 6.0 umoles sodium pyrophosphate, 6.0 umoles $MgSO_4$, and 240 umoles o-nitrobenzoic acid. Table XII indicates that o-nitrobenzoic acid inhibits pyrophosphatase activity. Since protein concentrations were not determined a comparison could not be made between the effect of o-nitrobenzoic acid on pyrophosphatase and ATPase activity.

Whole cell suspensions were substituted for the cell-free extract and pyrophosphatase activity was studied. Data presented in Table XIII shows that pyrophosphatase activity could be demonstrated in whole cells. The liberation of orthophosphate from inorganic pyrophosphate by whole cells was inhibited by o-nitrobenzoic acid.

TABLE XII

INFLUENCE OF o-NITROBENZOIC ACID ON PYROPHOSPHATASE ACTIVITY OF CELL-FREE EXTRACT

Incubation Mixture	umoles Inorganic Phosphate Liberated per Tube in 15 Minutes
PPi	0.0
PPi + o-NO_2	0.0
Extract	0.80
PPi + Extract	7.75
o-NO_2 + Extract	0.80
PPi + o-NO_2 + Extract	7.16

Each tube contained 6.0 umoles $MgSO_4$; pyrophosphate (PPi), 6.0 umoles: o-NO_2, 240 umoles: extract, 1.0 ml; and 0.1 M Tris buffer of pH 7.4 to a final volume of 2.4 ml.

TABLE XIII

INFLUENCE OF o-NITROBENZOIC ACID ON PYROPHOSPHATASE ACTIVITY OF WHOLE CELLS

Incubation Mixture	umoles Inorganic Phosphate Liberated per Tube	
	5 Min Incubation	10 Min Incubation
PPi	0.0	0.0
PPi+240 umoles o-NO_2	0.0	0.0
Cells + PPi	2.85	8.75
Cells + PPi + 120 umoles o-NO_2	2.53	6.81
Cells + PPi + 240 umoles o-NO_2	2.07	5.75

Each tube contained 6.0 umoles $MgSO_4$; pyrophosphate (PPi), 6.0 umoles; cells, 1.0 ml; and 0.1 M Tris buffer of pH 7.4 to final volume of 2.4 ml.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The ability of o-nitrobenzoic acid to inhibit microbial growth and stimulate total oxygen uptake during substrate oxidation has been investigated in an attempt to elucidate the mechanism(s) involved in these phenomena. Results obtained in growth experiments indicated that o-nitrobenzoic acid was capable of inhibiting growth of the Flavobacterium when p-nitrobenzoic acid was used as the substrate and also when diverse compounds such as succinic or glutamic acid served as the substrate. It was shown that o-nitrobenzoic acid was inhibiting cell growth and not killing the cells.

Experiments were conducted to reverse the inhibition of growth. Results indicated that yeast extract or an enzymatic digest of casein hydrolysate were capable of reversing the inhibition under defined conditions. When the commercial preparation of casein hydrolysate was replaced by known amino acids, growth experiments indicated that a mixture of seventeen amino acids, similar in composition to the casein hydrolysate, would reverse the inhibition to approximately the same extent observed with the enzymatic digest of casein hydrolysate. When combinations of less than seventeen were tested, reversal was incomplete. These results are similar to the findings reported by Cocito (8) who studied the inhibition of E. coli by 2,4-dinitrophenol. He found that a hydrolysate of cow's liver reversed the inhibition and a mixture of known amino acids could replace the hydrolysate while single amino

acids had little anti-2,4-dinitrophenol effect. When single amino acids, or combinations of three to five, were tested for their ability to reverse the inhibition due to o-nitrobenzoic acid; it was observed that a mixture of the aromatic amino acids were the most effective. However, shikimic acid, a precursor of the aromatic amino acids, was ineffective in reversing the inhibition. Vitamins, ammonium salts, or other diverse organic compounds were tested for their ability to reverse the inhibition of growth. A few showed a partial reversal but none of the compounds completely reversed the inhibition even when used in combinations. Those which appeared to be effective were pantothenic acid, vitamin B_{12}, coenzyme A, and TPN. However, these did not give complete reversal.

Incubation experiments showed that a compound, which had an absorption maximum at 263 mu, accumulated in the medium when o-nitrobenzoic acid was present. This compound was not identified by the techniques employed. When the seventeen amino acids were added to the incubation flask, the accumulation of this compound was not as apparent. One could speculate that o-nitrobenzoic acid is inhibiting the synthesis of some compound(s) essential for growth of the Flavobacterium sp. The compound with an absorption maximum at 263 mu may possibly be a precursor for the synthesis of this essential metabolite, and in the presence of o-nitrobenzoic acid the reaction is inhibited and the precursor accumulates in the medium. The addition of the amino acids permits growth of the organism indicating that these metabolites fulfill or satisfy, at least in part, the nutritional deficiency resulting from the presence of o-nitrobenzoic acid.

The affect of o-nitrobenzoic acid on oxidation is somewhat unique. A stimulation in total oxygen uptake could be shown in the presence of o-nitrobenzoic acid, but an increased rate was not observed. The data presented indicated that o-nitrobenzoic acid must be present during the early stages of substrate oxidation in order to exert its full effect. When o-nitrobenzoic acid was added 30 minutes after the addition of substrate, there was no significant increase in oxygen uptake over that in the control vessels. Also, if the cells were incubated with o-nitrobenzoic acid and the inhibitor removed by washing prior to the addition of substrate, there was little difference in the oxygen consumption when compared with the substrate oxidation of cells that had been incubated with buffer. However, if o-nitrobenzoic acid was added back to the cells simultaneously with the substrate, total oxygen uptake was again increased. This would indicate that if o-nitrobenzoic acid permeates the cell membrane, it is not bound in the cell, but may exist in the "free" state and is easily washed out of the cells. This finding emphasizes the importance of o-nitrobenzoic acid being present during the initial stages of oxidation.

o-Nitrobenzoic acid appeared to inhibit inducible enzyme formation and the sensitivity varied in different inducible systems. This inhibition suggests that o-nitrobenzoic acid is inhibiting some metabolic step to such an extent that energy, or perhaps precursors, are not available for protein synthesis. Since oxygen consumption by cell-free extracts, in which protein synthesis is not taking place, in the presence and absence of o-nitrobenzoic acid were essentially the same, one might speculate that the biosynthesis of certain proteins may be the site of

inhibition. This finding, coupled with the observation that o-nitrobenzoic acid inhibits pyrophosphatase, indicates that o-nitrobenzoic acid may be interfering with protein synthesis by inhibiting the activation of amino acids in the scheme proposed by Berg and Ofengand (3):

$$\text{Amino Acid} + \text{ATP} \longleftrightarrow \text{Amino Acid-AMP} + \text{PPi}$$

In the activation of the amino acid, the amino acid combines with ATP to form an amino acid adenosine monophosphate (AMP) complex and inorganic pyrophosphate. o-Nitrobenzoic acid which inhibits pyrophosphatase, may shift the equilibrium, thereby preventing activation of amino acids and subsequent protein formation. However, further experimentation will be necessary in order to verify this.

Oxidation studies suggested that the mechanism of o-nitrobenzoic acid stimulation was similar to the effect of 2,4-dinitrophenol, since 2,4-dinitrophenol also stimulates oxygen uptake and inhibits growth. The site of 2,4-dinitrophenol inhibition is thought to be the stimulation of ATPase (9). However, when ATPase was studied using cell-free extracts, there was a marked decrease in the liberation of inorganic phosphate in the presence of o-nitrobenzoic acid. Although ATPase had been observed in many cells, both animal and bacterial, a function for the enzyme in the whole cell has not been clearly defined. However, using this organism, ATPase activity was observed in whole cells and the results were similar to those obtained with cell-free extracts.

The effect of o-nitrobenzoic acid on the liberation of inorganic phosphate from ADP indicated that the inhibitor stimulated the release of inorganic phosphate. These findings are the direct opposite of the results obtained with ATP and indicate that the reactions may be catalyzed by two different enzymes. However, Heppell and Hilmore(16)

isolated an enzyme from yeast that catalyzed the hydrolysis of both ADP and ATP to form adenosine-5'-phosphate and ADP respectively, therefore it is possible that o-nitrobenzoic acid is affecting a different "site" on the same enzyme.

The inhibition of pyrophosphatase activity by the whole cell suspension was very significant. It is very likely that the Flavobacterium sp. cells contain a nucleotide pyrophosphatase which catalyzes the following reaction:

(1) ATP + H_2O ⟷ Adenosine monophosphate + pyrophosphate

A cellular pyrophosphatase may then hydrolyze inorganic pyrophosphate to 2 equivalents of orthophosphate (16). o-Nitrobenzoic acid may be involved in the inhibition of the inorganic pyrophosphatase.

Schrecker and Kornberg (26) have studied the synthesis of several nucleotides in cellular systems and have purified an enzyme from yeast and rat liver which catalyzed the conversion of flavin mononucleotide (FMN) to flavin adenine dinucleotide (FAD) by the following reversible reaction:

(2) FMN + ATP ⟷ FAD + pyrophosphate

Magnesium is required for the reaction and cannot be replaced by manganese. The most purified fraction contained an inorganic pyrophosphatase which would shift the equilibrium by hydrolyzing the pyrophosphate, thus allowing the synthesis of more FAD. Kornberg also isolated an enzyme from yeast which catalyzed the reversible synthesis of diphosphopyridine nucleotide (DPN) and pyrophosphate from nicotinamide mononucleotide (NMN) and ATP (19):

(3) NMN + ATP ⟷ DPN + PPi

The synthesis of triphosphopyridine nucleotide (TPN) was studied using

a purified yeast enzyme which carried out the following reversible reaction: (19)

(4) DPN + ATP $\longleftrightarrow$ TPN + ADP

When an excess of pyrophosphate was added to incubation mixtures capable of carrying out reactions 3 and 4, the synthesis of DPN (reaction 3) was inhibited 87 per cent (as would be anticipated from equilibrium considerations). An excess of pyrophosphate completely prevented TPN synthesis from NMN (reactions 3 plus 4) and inhibited TPN synthesis from DPN (reaction 4) by 41 per cent (19).

Results obtained in this study show that o-nitrobenzoic acid inhibits pyrophosphatase activity. Therefore, it is possible that pyrophosphate is accumulating in the system and the equilibrium shift may then inhibit the synthesis of nucleotides in the cell. Other workers have reported that pyrophosphate accumulated in the medium when cells were oxidizing succinic acid (10). Although other compounds were not tested for their ability to serve as substrates for the pyrophosphatase, it may be that o-nitrobenzoic acid inhibits nucleotide pyrophosphatase. Also, the fact that TPN and coenzyme A are able to partially reverse the inhibition of growth due to o-nitrobenzoic acid suggests the mechanism of inhibition may be associated with the cellular pyrophosphatases.

The findings presented in this report suggest that the inhibition of microbial growth by o-nitrobenzoic acid may be due to the inhibition of a combination of several reactions (either directly or indirectly). These would include protein synthesis and the synthesis of nucleotides such as DPN and TPN. Further studies with this and other inhibitors will be beneficial in elucidating the complex mechanism of cellular

growth. Additional studies of the function of ATPase and pyrophosphatase in the intact cell may also be aided by the use of such inhibitors.

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