ROLE OF GLUCOSE AND AMINO ACIDS IN THE GROWTH OF BACTERIUM P. A. 3679

by

HAROLD CECIL NORDAN

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To my wife for her understanding and love and my parents whose encouragement and inspiration have made this thesis possible.

APPROVED:

Redacted for privacy

Professor of Bacteriology

In Charge of Major

Redacted for privacy

Chairman of Department of Bacteriology

Redacted for privacy

Head of Department of Food and Dairy Technology

Redacted for privacy

Chairman of School Graduate Committee

Redacted for privacy

Dean of Graduate School

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INTRODUCTION

Microorganisms involved in the spoilage of foods have always attracted considerable attention. At an early date it was recognized that proper control measures could not be established until the microbial spoilage groups were identified and classified. Of primary significance stood the putrefactive anaerobes. These microorganisms, because of their spore-forming habit and anaerobic nature proved particularly troublesome in the food-canning industry. In fact, present heat-processing methods are aimed to destroy the aforementioned anaerobic types.

In spite of the successful control of putrefactive anaerobes in the canning industry, very little fundamental information on growth and related cell metabolism has been reported. Yet it has long been realized that a more complete understanding of heat resistance of spores and mode of spore formation can be attained only when the overall growth and metabolism of the vegetative cell is thoroughly investigated. The study herein reported represents an attempt in this direction.

Of immediate importance lay the question of methodology. It was realized that anaerobic growth measurements would require special growth procedures. Then it

was not long before the question of proper growth media became a critical point for study. Eventually, specific aspects of cellular metabolism were investigated. These ideas form the basis for the specific objectives of the study as follows:

1. To develop a suitable procedure for the measurement of anaerobic growth.
2. To evaluate growth media.
3. To investigate role of specific substrates, e.g., glucose and amino acids, in the overall metabolism of the cell.

HISTORICAL

Over a period of years a considerable collection of clostridia cultures producing spores of high heat resistance and capable of growth on a variety of substrates have accumulated. These include P. A. 3679 an organism isolated by National Canners Association Research Laboratories. This organism has been selected as a standard test organism for heat processing evaluation.

P. A. 3679 does not appear in Bergey's Manual of Determinative Bacteriology (5, pp. 634-693) and has for many years been considered to be identical to or a strain of Clostridium sporogenes. Williams (43, p. 324) refers to the organism as "a putrefactive anaerobe, Clostridium sporogenes, strain no. 3679." Sognefest and Benjamin (35, p. 234) make reference to the same organism as "no. 3679 a putrefactive, gas producing anaerobe classified as Clostridium sporogenes." Gross, Vinton, and Stumbo (18, pp. 405-410) have shown that P. A. 3679 is culturally and serologically distinct from Clostridium sporogenes. Certain strains of Clostridium tetani do not possess all of the morphological and staining characteristics ascribed to typical tetanus bacilli. The property of these cultures which characterizes them as Clostridium tetani is their ability to produce a toxin which is specifically

neutralized by tetanus antitoxin, Martinez and Rittenberg (26, p. 156). This latter statement would seem to lend considerable weight to the contention that P. A. 3679 is distinct from Clostridium sporogenes. Campbell and Frank (8, pp. 267-269) have shown that P. A. 3679 possesses a requirement for serine that is not exhibited by Clostridium sporogenes, and they suggest that this might be a useful character in establishing the taxonomic position of this putrefactive anaerobe. At present the taxonomic position of P. A. 3679 is not clear, however the results of investigations pertaining to the nutritional requirements and metabolism will aid in eventual clarification.

The cultivation of spore forming anaerobes in chemically defined media was first attempted by Knight and Fildes (22, pp. 112-115). Fildes and Richardson (15, pp. 326-335) reported on a medium containing 15 amino acids, salts and an unidentified "sporogenes vitamin" as adequate for growth of Clostridium sporogenes. These workers also asserted that the addition of glucose to their medium is superfluous. Investigations on the nature of the "sporogenes vitamin" by Shull and Peterson (32, pp. 69-83) showed that moderate growth of some strains of Clostridium sporogenes could be obtained on a defined medium containing amino acids, glucose, biotin, p-aminobenzoic acid, nicotinic acid, salts, buffer and sodium thioglycollate.

However, heavy growth required an unidentified factor found in partial protein digests. Shull, Thoma and Peterson (33, pp. 227-241) reported that high concentrations of certain combinations of amino acids could replace partial protein digests in a medium for Clostridium sporogenes. Arginine in combination with tyrosine and phenylalanine produced good growth of the organism but partial protein digests had a definite stimulatory effect on growth. Glucose was used in their medium at a level of 1% and some amino acids at a level as high as 0.5%. Clostridium tetani has been grown on synthetic medium containing amino acids, glucose, vitamins and salts, Feeney, Mueller, and Miller (14, pp. 563-571). However, subculture was not satisfactory. These same workers reported that threonine, phenylalanine and serine stimulated growth but were not essential. Glutamic acid gave heavy growth but was not essential. Boyd, Logan and Tytell (6, pp. 1013-1025) investigated the growth requirements of Clostridium perfringens and obtained good growth in a defined medium of amino acids, adenine, uracil, vitamins, salts, glucose, buffer and ascorbic acid. Pyridoxamine or pyridoxal eliminated the requirement for lysine, alanine, aspartic acid and glycine. Fuchs and Bonde (16, pp. 317-329) reporting further on this organism stated that glucose is essential for growth and that formate, acetate,

propionate, butyrate, citrate, succinate, fumarate, pyruvate, lactate, gluconate, tartrate, glycerol, acetone and ethanol gave no growth. Growth of Clostridium bifermentans in casein hydrolysate with 1% glucose was not good according to Smith and Douglas (34, pp. 9-50) while growth was absent in an amino acid medium. Growth was not improved by acetate, B vitamins, purines, pyrimidines or yeast nucleic acid but was good if egg or yeast extract was included at a level of .01%. Mager, Kindler and Grossowicz (25, pp. 130-141) demonstrated a need for high concentrations of arginine, tyrosine and phenylalanine by Clostridium parabotulinum type A. The basal medium used by these workers contained .5% glucose. It is interesting to note that they did not wash the inoculum used in their experiments because of an apparent harmful effect on viability. Campbell and Frank (8, pp. 267-269) used a modification of the medium used by Mager and associates (25, pp. 130-141) for P. A. 3679 and also reported a detrimental effect with washed cells. Bulmash and Weaver (7, p. 110) used large quantities of amino acids (3 grams L-arginine per 1000 ml.) in a defined medium for Clostridium histolyticum. They also reported that glucose was stimulatory to all strains tested. Barker and Taha (4, pp. 347-363) have shown that Clostridium klyverii required yeast extract at a level of .5% but not carbohydrate. Barker and Peterson

(3, pp. 307-308) showed that Clostridium acidi-urici will grow satisfactorily on a very simple medium without amino acids or growth factors. This is probably one of the very few clostridia having simple nutritional requirements. With perhaps one or two exceptions it appears that the nutritional requirements of clostridia so far investigated are indeed complex. The area of complexity appears in the amino acid requirements and in the need in many cases for an as yet unidentified fraction from partially digested protein.

Reference to Bergey's Manual (5, pp. 634-693) reveals that the majority of the clostridia ferment glucose. These data are obtained however by observing an evolution of gas in the culture medium accompanied by an acid reaction in an acid indicator present in the medium. These observations give no information about the manner in which glucose is dissimilated.

Limited data suggests that members of the genus Clostridium dissimilate glucose by a glycolytic system similar to the E.M.P. (Embden-Meyerhof-Parnas) pathway, Elsden (13, pp. 791-843). Clifton (11, pp. 485-497) during investigations on the utilization of glucose by Clostridium botulinum found that glucose was fermented to ethyl alcohol and carbon dioxide. Clostridium tetani has been shown by Lerner and Pickett (24, pp. 183-196) to

ferment glucose completely. The fermentation is of an alcoholic nature carbon dioxide and ethyl alcohol being the principal end products. Iron was found to be essential for this fermentation, glucose being fermented in direct proportion to the iron present. It is suggested that an iron containing enzyme or coenzyme is essential for glucose fermentation. Lerner and Mueller (23, pp. 43-45) demonstrated that glutamine plays an important role in glycolysis by Clostridium tetani. Glutamine could induce glycolytic activity in iron deficient cells as measured by carbon dioxide evolution. Following the reports on the stimulatory effect of iron on glycolysis Bard and Gunsalus (1, pp. 387-400) showed the existence of a metallo-aldolase in Clostridium perfringens. They suggested that the essentiality of iron for aldolase activity offers an explanation of the indispensible requirement of iron for clostridial growth. Further the occurrence of aldolase as the key enzyme for the transformation of hexose diphosphate to triose phosphate suggests the occurrence of the Embden-Meyerhof-Parnas system in Clostridium perfringens. Clostridium thermoaceticum has been shown to ferment glucose 1-C^{14} and glucose 3,4-C^{14}, Wood (44, pp. 579-583). From glucose 1-C^{14} the acetate produced was predominantly methyl labelled. From glucose 3,4-C^{14} very

little C^{14} was recovered in acetate. These results are in agreement with the Embden-Meyerhof-Parnas scheme for glucose dissimilation. Paege, Martin and Bard (29, pp. 65-67) using specifically labelled glucose presented evidence which indicated an Embden-Meyerhof-Parnas pathway in Clostridium perfringens. However, the specific activity of ethanol recovered in their experiments was lower than that of acetate and it was suggested that another pathway of triose metabolism may be present which contributed ethanol. Cynkin and Gibbs (12, pp. 335-338) while investigating pentose metabolism by Clostridium perfringens found ethanol and acetate similarly labelled from glucose 2-C^{14}, a result inconsistent with that of the previous workers but consistent with the Embden-Meyerhof-Parnas scheme. Despite the widespread opinion that Clostridium tetani is a nonsaccharolytic organism Martinez and Rittenberg (26, pp. 156-163) have shown that certain strains utilize glucose in growth media. Also they have demonstrated the presence of the majority of the enzymes of the Embden-Meyerhof-Parnas pathway in cell-free extracts of glucose grown Clostridium tetani.

Up until 1934 the biochemical transformations involved in the anaerobic breakdown of nitrogenous compounds had not been extensively studied. Stickland (37, pp.

1746-1759; 38, pp. 288-290; 39, pp. 889-896; 40, pp. 896-898) studied the action of washed suspensions of Clostridium sporogenes on amino acids. By the use of both manometric and dye-coupling techniques he demonstrated that, whereas most single amino acids are not attacked by Clostridium sporogenes, certain pairs of amino acids are decomposed by a coupled oxidation-reduction reaction. Certain amino acids act as hydrogen donators especially alanine, valine and leucine and others as hydrogen acceptors glycine, proline and hydroxyproline. The products of such coupled reactions are organic acids. Stickland (37, pp. 1746-1759) suggests that these reactions could account for most if not all of the anaerobic decomposition of amino acids. Nisman (27, p. 17; 28, pp. 473-474) has shown that fifteen species of clostridia, including Clostridium botulinum, Clostridium butyricum, Clostridium histolylicum, Clostridium bifermentans and Clostridium sporogenes make use of the Stickland reaction. The type of reaction reported by Stickland (37, pp. 1746-1759) is not the only mechanism for amino acid breakdown. Glutamate is readily fermented by certain clostridia. Clostridium tetanomorphum ferments histidine and glutamate, Wachsman and Barker (41, pp. 83-88). Clostridium tetanomorphum is known to ferment cysteine, serine, aspartate and tyrosine, Woods and Clifton (45, pp. 1774-1788). Single amino

acids are also fermented by Clostridium tetani according to Clifton (11, pp. 485-497) and Pickett (30, pp. 203-209). Stadtman (36, pp. 314-320) showed that Clostridium sticklandii is able to convert lysine to acetate, butyrate and ammonia. Alanine and threonine are fermented by Clostridium propionicum. The main products for alanine fermentation are acetate, propionate, carbon dioxide and ammonia; threonine fermentation produces butyrate and propionate, Cardon and Barker (9, pp. 165-180). Barker and Beck (2, pp. 291-304) showed that Clostridium acidi-urici and Clostridium cylindrosporum readily ferment xanthine, uric acid, guanine and 6, 8-dihydroxypurine. The main products formed by Clostridium acidi-urici are ammonia, carbon dioxide, and acetate, also a small amount of formate accumulates. The products of Clostridium cylindrosporum are the same except that glycine also accumulates. It thus becomes evident that the metabolism and nutrition of the clostridia hinges on an understanding of the anaerobic fermentation mechanism(s), these in turn relating specifically to the utilization of carbohydrates, amino acids and required growth factors.

EXPERIMENTAL METHODS

Culture

The organism used in this investigation, P. A. 3679, was obtained from the National Canners Association, Berkeley, California. The culture was received as a spore suspension of $1x10^8$ spores per ml. It was routinely checked for purity and all inocula used throughout the course of this study were grown from one stock spore suspension.

Inoculum

The inoculum used in all experiments was a suspension of vegetative cells. The cells were grown in the non-synthetic yeast extract medium, Hitzman, Halvorson and Ukita (20, p. 1), of the following composition:

Table I

COMPOSITION OF NON-SYNTHETIC MEDIUM

Yeast Extract	1.00%
Glucose	0.50%
K_2HPO_4	0.25%
NH_4Cl	0.30%
NaCl	0.40%
Na thioglycollate	0.20%

pH adjusted to 7.6 with NaOH before autoclaving

The medium was dispensed into 500 ml. Erlenmeyer flasks, 200 ml. per flask, plugged with non-absorbent cotton wrapped in cheese cloth, and sterilized at 15 lbs. per

sq. in. for 15 minutes. The sterile medium was inoculated with 0.01 ml. of the stock spore suspension. Three 500 ml. flasks and one 50 ml. control flask were placed in a glass dessicator. The dessicator was evacuated with a water aspirator until the medium began to boil. At this point the evacuation was stopped and the dessicator filled with a gas mixture of 99.7% nitrogen plus 0.3% carbon dioxide. The presence of carbon dioxide has been shown to be essential for maximum growth (17, pp. 335-340). The cultures were incubated in the dessicator at 37°C. Frequently several such dessicators were set up to provide a large number of vegetative cells. After 18 hours incubation the cells were harvested by centrifugation in either a Foerst continuous centrifuge or a Serval Model S. S.-1 Superspeed centrifuge depending on the volume of medium to be centrifuged. The cells were washed three times with 40 ml. of physiological saline, and finally suspended in saline to give a suspension with an optical density of 0.4. The centrifugation and washing of cells was carried out under aseptic conditions. All cultures used were checked for purity by microscopic examination.

Growth Measurement

Cell growth was measured by optical density. The optical density determinations were carried out with a

Bausch and Lomb Spectronic 20. When the non-synthetic medium was used these measurements were made at a wavelength of 630 mu and at 525 mu when the synthetic medium was used.

Growth Apparatus

Growth of an anaerobe presents specific problems. In the case of the organism under study, no observable growth is obtained using semi-anaerobic conditions. Presumably there is a specific requirement for a low oxygen tension and a moderate carbon dioxide level in the culture atmosphere. In order to attain these culture conditions, two approaches have been investigated.

Anaerobic Incubator

Procedure Initial growth studies were carried out in the anaerobic incubator manufactured by the National Appliance Co., Portland, Oregon. The medium to be used was dispensed into Erlenmeyer flasks of convenient size and the amount of medium in each flask kept small; a 500 ml. flask contained 200 ml., so that the medium would not boil out under vacuum. The flasks were plugged with cotton wrapped in cheesecloth, and sterilized at 15 lb. per sq. in. for 15 minutes. When cool the flasks were

inoculated with a suspension of vegetative cells at a rate of 1.0 ml. per 100 ml. of medium.

The inoculated flasks were placed in the anaerobic incubator and the apparatus evacuated with a water aspirator until the medium just began to boil. The evacuation was then stopped and the nitrogen, carbon dioxide gas mixture was admitted through a previously sterilized cotton filter tube 2¼ inches long and 1 inch inside diameter. This was done twice to sweep out all residual oxygen before allowing incubation at 37°C to proceed under the same atmosphere.

In order to take samples for optical density and other determinations the incubator must be opened, the flask swirled to give a uniform suspension of cells and the plug removed to pipette out a 10 ml. sample. Following this the evacuation procedure must be repeated.

Growth response Experiments were carried out to assess the growth possible under the aforementioned type of disturbed anaerobiosis using the non-synthetic medium described in Table I.

Anaerobic Apparatus

Due to difficulties associated with the use of the previously described anaerobic incubator in growth studies,

a special culture apparatus was designed. This apparatus permitted undisturbed anaerobiosis while allowing samples to be taken for optical density measurements and chemical analysis of the medium and cells.

Procedure The anaerobic culture apparatus is shown in Figure 1. The assembly incorporates a cotton filter A, a simple flow meter B to indicate the sparge rate, a sparge-sample tube C, and a three neck, 24/40 standard taper joint round bottom flask. The size of the round bottom flask was 250 ml., 500 ml., 1000 ml. or 2000 ml. depending on the volume of medium to be used. The flow meter consisted of a tapered glass tube 6 inches long enclosed in a glass envelope. This could be disassembled at a 24/40 standard taper connection. The flow indicator used was a 1/16 inch dia. red sapphire ball obtained from Industrial Tectonics, Inc., Ann Arbor, Michigan. The meter was calibrated against a standard flow meter, and the calibration marks painted on with engine enamel to withstand sterilization temperature. The flow meter assembly was connected to the sparge-sample device with a ball and socket connection.

Two complete pieces of apparatus with culture flasks up to 2000 ml. could be mounted in a water bath 26 inches long, 14 inches wide, and 12 inches deep. Once assembled

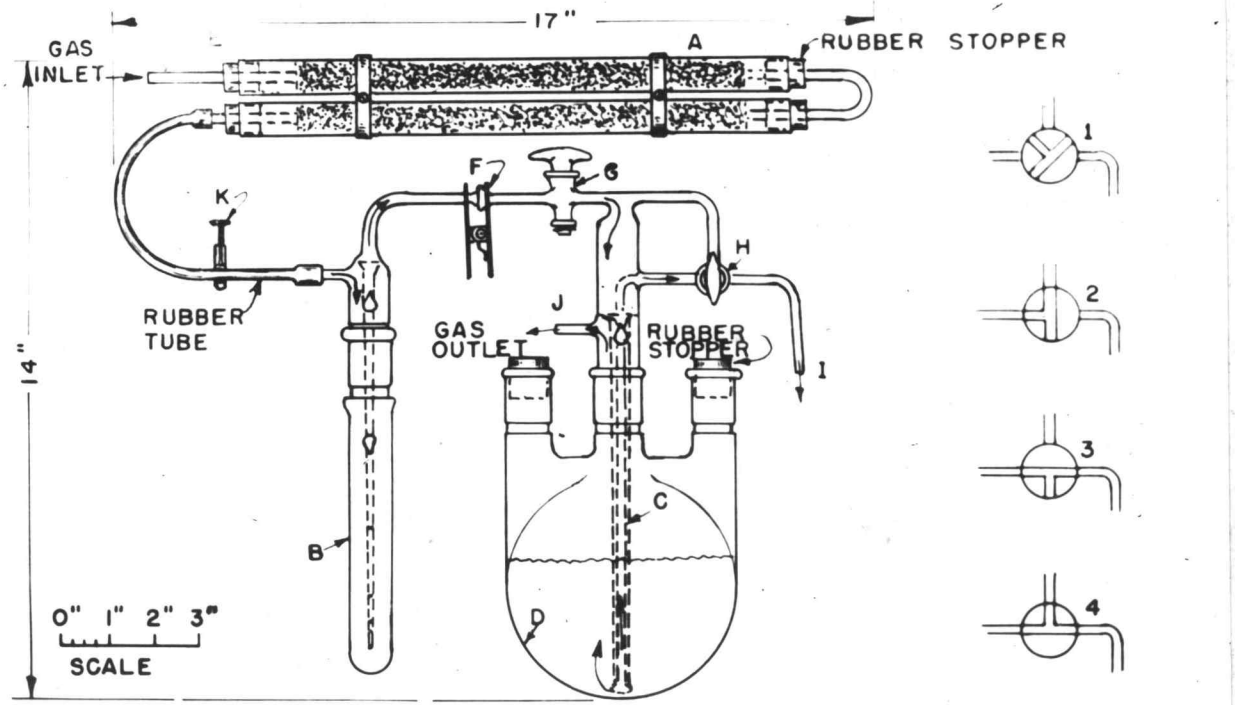

Figure 1. Anaerobic gas flow apparatus.

the two units could be lifted as a unit out of the water bath and sterilized in an autoclave. The apparatus was first sterilized empty at 15 lb. per sq. inch for 30 minutes. The medium to be used was added to the flask D and the apparatus and medium sterilized at 15 lb. per sq. in. for 20 minutes. While sterilizing the apparatus outlet J was plugged with cotton and stopcocks G and H were closed, i.e., H in position 1. After sterilization the assembly was placed in the water bath. To sparge the system stopcock G was opened and screw clamp K adjusted to give the desired gas flow rate. Inoculation of the medium was done by pipette through one of the necks on the flask not supporting the sparge-sample tube. Any other post sterilization additions were made in the same way.

The gas outlet J on the apparatus could be connected to a CO_2 trap of the type used on the radiorespirometric apparatus of Wang et al.(42, pp. 207-216). To take a sample of medium stopcock H was turned to position 2. This allowed the sparge gas to pass directly down the inner sample tube of C and stir up any accumulation of cells that may have occurred at the bottom of the flask. Stopcock H was then turned to position 3 and outlet J sealed off. The gas pressure in the flask then forced the medium up the inner tube and out at I. With stopcock

H in position 4 and J open the gas would empty outlet I and force the medium in the inner tube back into the flask. Stopcock H in position 1 completes the sampling procedure.

Growth response An experiment was carried out to compare growth response in the anaerobic incubator and in the special anaerobic apparatus. The composition of the medium used is given in Table I. A 500 ml. flask was used containing 300 ml. of medium. The medium was inoculated with 3 ml. of cell suspension and incubated at 37°C. Growth was followed by taking samples and measuring the optical density.

Non-synthetic medium Growth experiments were carried out using the non-synthetic medium, Table I, to assess the growth stimulation when various levels of yeast extract, glucose, pyruvate or acetate were used. Yeast extract in the basal medium was used at levels of 0.0%, 0.1%, 0.5% and 1.0% to determine the minimum amount necessary for growth. A stock solution of glucose was made up and after sterilization by Seitz filtration was added to the sterile basal medium to give final concentrations of 0.0%, 0.1%, 0.2% and 0.5%. Similarly stock solutions of pyruvate and acetate were made up, sterilized at 15 lb. per sq. in. for 15 minutes and added aseptically to the sterile basal

medium at one level equivalent to 0.1% glucose on a molar basis.

These experiments were carried out in the special anaerobic apparatus. The 500 ml. flasks containing 300 ml. of medium were sparged with 99.7% nitrogen 0.3% carbon dioxide at a rate of 250 cubic centimeters per minute. The cultures were incubated at 37°C and growth was assessed by measuring the optical density.

Semi-synthetic medium The necessity for having a defined medium for growth and metabolic studies is generally agreed upon. A synthetic medium reported by Campbell and Frank (8, pp. 267-269) was used for growth studies and radioactive tracer experiments. The composition of this medium is given in Table II. The constituents of the medium were sterilized together with the exception of tryptophane, glucose and salts B. Tryptophane was made up as an aqueous solution, sterilized in the autoclave and added aseptically to the basal medium after sterilization. A stock solution of glucose was made up prior to each experiment and sterilized by Seitz filtration.

The sterile solution was then added to the medium in an amount to give the desired concentration. The salts B mixture was sterilized cautiously and added to the medium after sterilization.

Table II

COMPOSITION OF SYNTHETIC GROWTH MEDIUM

L-Tryptophane	0.05 gram(s)
L-Arginine	3.00 "
DL-Phenylalanine	2.00 "
L-Tyrosine	0.25 "
DL-Valine	2.00 "
DL-Isoleucine	0.50 "
DL-Threonine	1.00 "
L-Proline	0.45 "
L-Histidine	0.20 "
L-Glutamic acid	0.50 "
DL-serine	0.25 "
Thiamin	0.40 mg.
Biotin	0.50 ug
p-aminobenzoic acid	10.00 ug
Nicotinic acid	1.00 mg.
Folic acid	10.00 ug
Glucose	5.00 grams
Cysteine	0.25 gram
Sodium Thioglycollate	0.50 gram
Salts A*	10.00 ml.
Salts B	1.00 ml.
Distilled water	1000.00 ml.

pH adjusted to 7.4 with NaOH prior to autoclaving

*Salts A: K_2HPO_4, 25 grams; KH_2PO_4, 25 grams; distilled water 250 ml.

Salts B: $MgSO_4 \cdot 7H_2O$, 10 grams; NaCl, 0.5 gram; $FeSO_4 \cdot 7H_2O$, 0.5 gram; $MnSO_4 \cdot 4H_2O$, 0.5 gram; distilled water 250 ml.

Growth of the test organism was negative in the synthetic medium using a washed cell suspension as inoculum. Several variables were introduced into the synthetic medium to assess their effect on growth. Yeast extract was added at levels of 0.0%, 0.02%, 0.03% and 0.05% to the basal medium, Figure 2, and sterilized with the other components. Glucose was assessed at levels of 0.0%, 0.1% and 0.5% in the basal medium plus 0.05% yeast extract. Sodium pyruvate and sodium acetate were added to the basal medium plus 0.05% yeast extract at a level equivalent to 0.1% glucose on a molar basis. The pyruvate and acetate were made up as aqueous solutions, heat sterilized and added to the sterile basal medium.

The concentration of the individual amino acids was adjusted and the growth compared with that obtained with the concentrations used by Campbell and Frank (8, pp. 267-269). Individual amino acids were employed at 250 mg. per liter, 500 mg. per liter and 1 gram per liter. Where the amino acids were used at 1 gram per liter the concentrations of arginine was 3 grams per liter, phenylalanine 2 grams per liter and valine 2 grams per liter. This adjustment brings the concentration of these amino acids up to that originally used by Campbell and Frank (8, pp. 267-269). Certain of those amino acids of the basal medium; valine, isoleucine, proline plus glycine, implicated in

the Stickland reaction were used. These amino acids were used at a concentration of 1.5 grams per liter with ammonium nitrate at 0.1%. All amino acid studies were made in a medium containing 0.05% yeast extract and 0.1% glucose but the same as the basal medium in all other respects.

These growth studies were carried out in the special anaerobic apparatus. The extent of glucose utilization was determined using the basal medium plus yeast extract at .05% and glucose 0.5%. Glucose in the medium was determined by the method of Shaffer and Somogyi (31, pp. 695-713).

Radiorespirometric Experiments

Catabolic pathways were evaluated by the radiorespirometric method of Wang et al.(42, pp. 207-216). These experiments were carried out using media with and without amino acids. The composition of the basal medium is given in Table II with the amino acids at a level of 1 gm. per liter, except arginine, phenylalanine and valine which were at a level of 3 grams, 2 grams and 2 grams per liter respectively. Yeast extract was added at 0.05% to both media, with and without amino acids.

Utilization of Specifically Labelled Substrates

The substrates used were glucose 1-C^{14}, glucose U-C^{14}, glucose 2-C^{14}, glucose 6-C^{14} also pyruvate 1-C^{14}, pyruvate 2-C^{14}, pyruvate 3-C^{14} and acetate 1-C^{14} and acetate 2-C^{14}. Substrates were obtained from the Nuclear-Chicago Corporation. All substrates were used at a radioactivity level of 0.5 microcurie. Glucose was used at a concentration of 50 mM per flask, and pyruvate and acetate at a concentration equivalent to 50 mM of glucose. Identical volumes of the substrates containing the specifically labelled substrate and non-labelled substrate to bring the concentration to that desired were placed in the incubation flask side arms. Twenty ml. aliquots of a vegetative cell suspension in the medium were added to the main compartments of the flasks. The medium contained 3.6-4.0 mg. dry weight of cells per flask. The flasks were swept with a gas mixture consisting of 99.7% nitrogen and 0.3% carbon dioxide at a rate of 40-80 ml. per minute. The incubation temperature was 37°C. The system was equilibrated for 45 minutes then the substrates were tipped into the main compartment of the flask.

The CO_2 was recovered at hourly intervals and precipitated as $BaCO_3$. The $BaCO_3$ was collected on aluminum planchets, and the radioactivity determined with a thin

window G. M. counter. The determinations were made to a standard deviation of 2%. Corrections for self absorption and background were made.

The cells were centrifuged out of the medium and the radioactivity in the cells and medium determined by the persulfate oxidation method (10, pp. 1225-1226; 21, pp. 1503-1504).

Fixation of $C^{14}O_2$

In an attempt to determine the extent of CO_2 fixation cells of this organism were grown in the presence of $C^{14}O_2$ and non-labelled pyruvate. The medium used was the same as that used for the radioactive tracer experiments. The medium was inoculated with a mass of washed cells to a concentration of 0.9-1.0 mg. dry weight cells per ml.

Two hundred and fifty ml. of medium containing cells was placed in a 500 ml. flask B of the apparatus shown in Figure 2. The system was evacuated and then filled with nitrogen. This was done twice to remove as much as possible of the un-labelled CO_2 in the system and also to create a good anaerobic system. The system was evacuated a third time and screw clamps E and G were closed. The gas valve was then opened to give a positive pressure on the system up to clamp G. $C^{14}O_2$ was generated in flask A by dropping 40% perchloric acid on 0.25 ml. of $NaHC^{14}O_3$

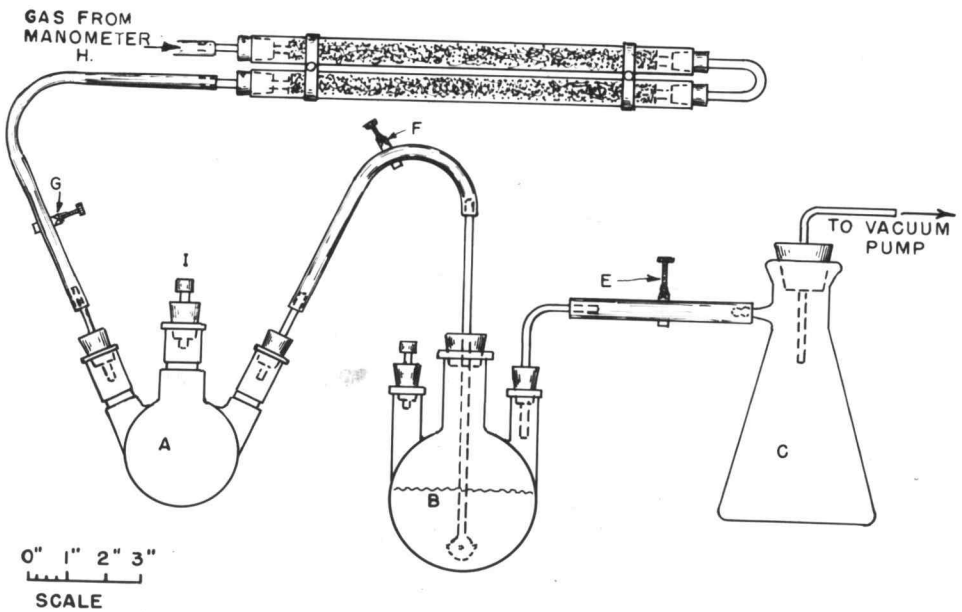

Figure 2. CO_2 fixation apparatus.

solution containing 25 microcurie of C^{14}. The $C^{14}O_2$ was swept into flask B by opening clamp G and allowing nitrogen to enter the system and return the internal pressure to atmospheric. Screw clamp F was then closed and the isolated flask B incubated, with intermittent shaking, at 37°C for 10 hours.

After incubation the cells were centrifuged out of the medium, washed and suspended in 10 ml. of distilled water. A 0.01 ml. aliquot of the cell suspension was plated on an aluminum planchet, dried and counted. Because the activity was very low on this sample the cells were not hydrolyzed, but 125 mg. of cells were solubilized with concentrated H_2SO_4 and then oxidized by the persulfate method (10, pp. 1225-1226; 21, pp. 1503-1504).

Utilization of Amino Acids

In order to determine if glucose exerted a sparing action on some or all of the amino acids required by this organism, experiments were set up to show any difference in amino acid utilization in the presence and absence of glucose and in the presence of pyruvate.

Preferential Utilization

The medium and inoculum used was the same as that described for the CO_2 fixation experiment. Glucose was

used at a concentration of 0.1% and pyruvate at a concentration equivalent to 0.1% glucose. The pyruvate used was labelled in the 2-C and 3-C position with a total activity of 20 microcurie of C^{14}. The medium was chromatographed as discussed on page 29 before inoculation and after 10 hours incubation. The cells grown in the labelled pyruvate were harvested and the incorporation was given a preliminary evaluation by a spot plate count. The spots on the chromotogram were analyzed with a Spinco Model R. Analatrol. The slit length on the instrument is only 2 cm. which required that the spots be cut out of the two-dimensional chromatogram and mounted in a 2 cm. "window" of a strip of opaque paper 5 cm. wide. To determine any difference in amino acid concentration in the medium before and after incubation the integrated areas of each spot as recorded by the instrument were compared.

Biosynthesis of C^{14} Labelled Amino Acids

Experiments were carried out to determine the extent of incorporation of carbon from glucose. Cells were grown in the semi-synthetic medium used for the radioactive tracer experiments. Glucose was used at a level of 0.1% containing 20 microcurie of glucose U-C^{14}. Seven hundred and fifty ml. of medium were inoculated with 7.5 ml. of washed cell suspension, sparged with 99.7% nitrogen

0.3% carbon dioxide at 250 cubic centimeters per minute, and incubated for 36 hours at 37°C. At the end of 36 hours the optical density was 0.35. This represents a total dry cell weight of 125-135 mg.

Aliquots of 0.01 ml. of medium before inoculation and after incubation also of the washed cell suspension after centrifugation were plated on aluminum planchets, dried and counted. Some incorporation of C^{14} was indicated so the cells were hydrolyzed in 6N HCl at 15 lb. per sq. in. for 20 hours. The acid hydrolysate was washed three times with a Rinco rotary evaporator to remove all HCl, filtered free of humin and after a fourth evaporation was taken up in 10 ml. of 10% isopropanol in water.

The cell hydrolysate was subject to two-dimensional chromatography and subsequent radioautography. Fifty, twenty and ten lambda of the cell hydrolysate was spotted on 18 x 22 Whatman No. 1 chromatography papers which had been serrated along the bottom edge with pinking shears. The papers were placed in a large chromatography cabinet in an equilibrated atmosphere of sec-butanol-3% ammonia 5:2 as used by Hausmann (19, pp. 3181-3182). After one hour of equilibration solvent was added to the troughs and the papers were subject to a descending first dimension solvent migration for 40 hours. The papers were dried at the end of 40 hours and placed in a second cabinet

equilibrated with an atmosphere of sec-butanol-88% formic acid-water 15:3:2, Hausmann (19, pp. 3181-3182). After one hour equilibration solvent was added to the troughs and the papers subjected to a descending second dimension for 12 hours. The papers were removed, dried and the 10 lambda paper sprayed with 0.25% ninhydrin in acetone. The sprayed papers were allowed to develop 18 hours in the dark at room temperature. Photographs of the developed chromatograms were taken for a permanent record. The spots were identified by reference to a chromatograph of known amino acids. Radioautograms were obtained by placing Ansco no-screen X-ray film in direct contact for 14 days with the undeveloped chromatogram spotted with 50 lambda of cell hydrolysate.

EXPERIMENTAL RESULTS

Growth Conditions and Media

Anaerobic growth studies are fraught with many problems due to the peculiar cultural requirements of anaerobic bacteria. Conventional methods of culturing these bacteria proved to be inadequate for the present investigation. As a result specific modifications in accepted procedures were investigated.

Apparatus The growth pattern of P. A. 3679 under different physical conditions may be observed by referring to Figure 3. In the anaerobic incubator growth starts after a lag period of eight hours. This is followed by an extended phase of accelerated growth and very little if any typical logarithmic growth. Maximum growth is reached in twenty-two hours. The anaerobic gas flow apparatus, Figure 1, designed to suit the requirements of the present study shows quite a different growth pattern. Reference to Figure 3 indicates a similar growth lag period for both the anaerobic incubator and the gas flow apparatus. Here the similarity ceases and the culture growing in the anaerobic apparatus exhibits a short period of accelerated growth followed by a very rapid logarithmic growth phase. In addition the maximum growth attained under conditions in the anaerobic gas flow apparatus is considerably greater

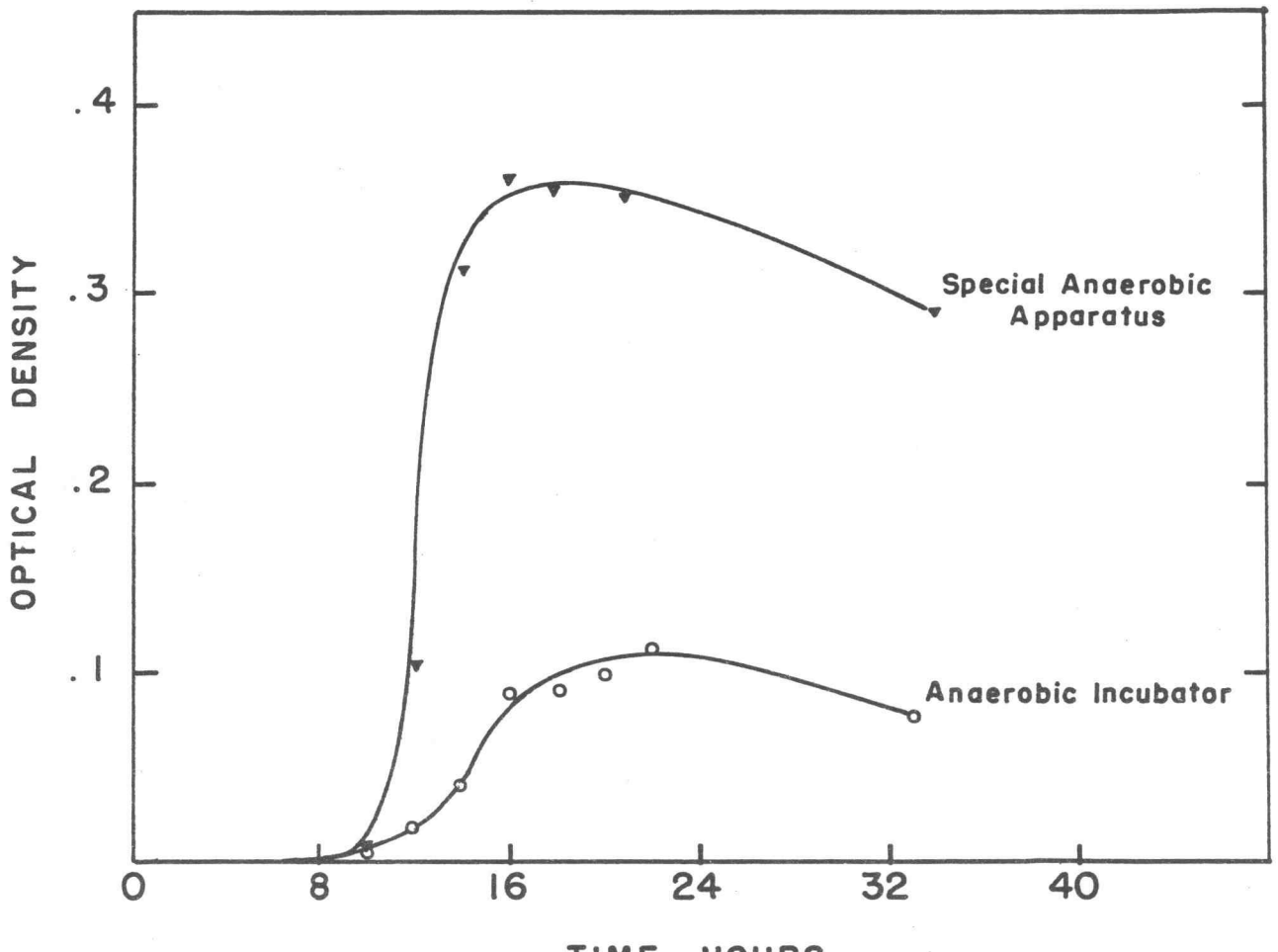

Figure 3. Growth of P. A. 3679 in the anaerobic incubator and the anaerobic gas flow apparatus.

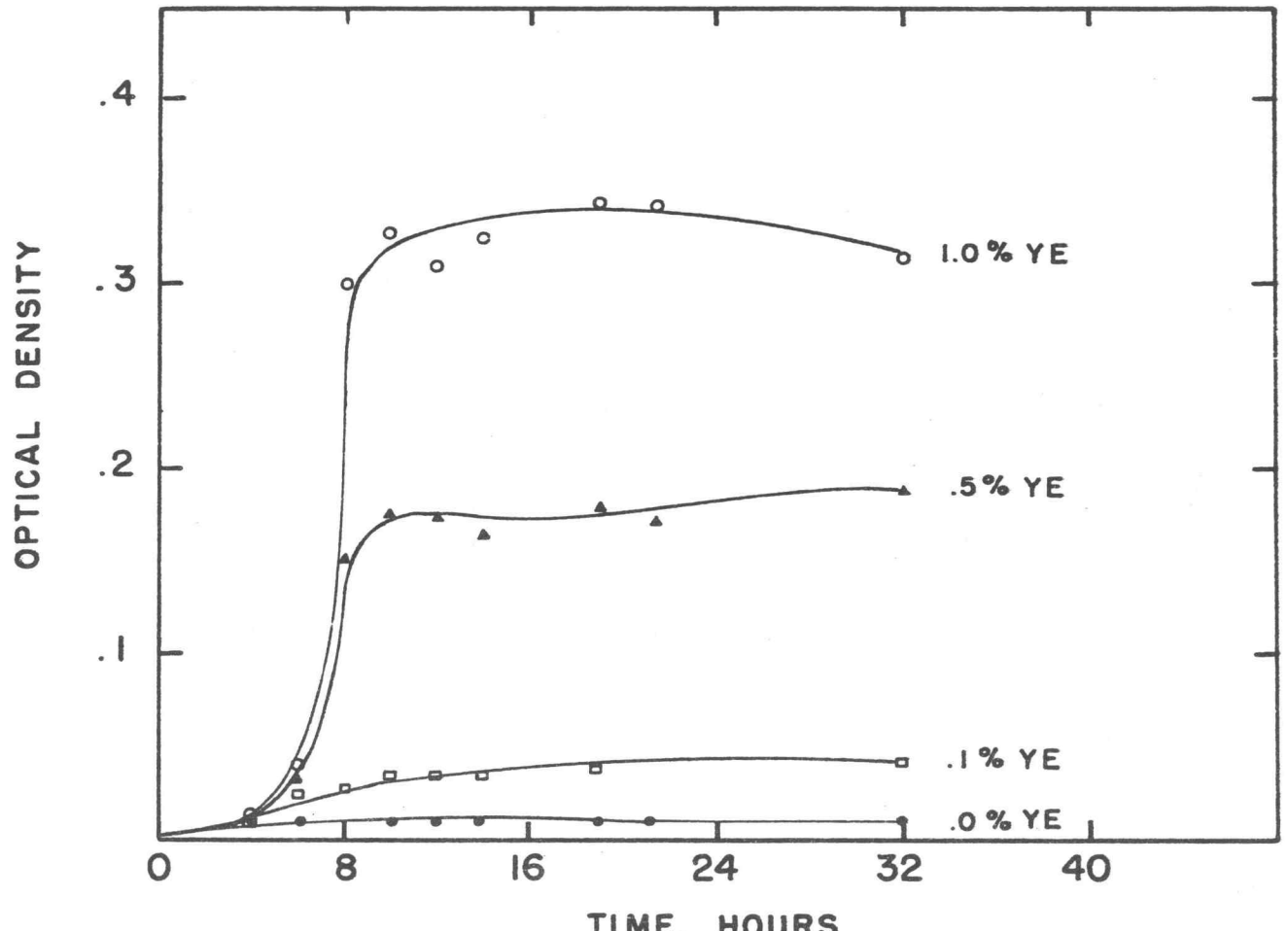

Figure 4. Growth of P. A. 3679 in non-synthetic medium with various levels of yeast extract.

than with the anaerobic incubator and is reached in approximately four hours less time. On the basis of these results it is evident that further investigations pertaining to the nutritional requirements of P. A. 3679 will be most effectively carried out using the anaerobic gas flow apparatus.

Non-synthetic medium The original non-synthetic medium, described in Table I, contains 1% yeast extract. Figure 4 discloses that whereas 1% yeast extract gives excellent growth; 0.5% yeast extract gives considerably less growth and that 0.1% yeast extract results in essentially no growth. The course of growth is the same for 1% and 0.5% yeast extract, the lag phase terminating at the same point in time in each case and the subsequent logarithmic phases having essentially the same slopes. The culture medium containing 0.1% yeast extract undergoes no appreciable growth, in fact does not exhibit any logarithmic growth. It appears that active cell division is first realized in this medium at a yeast extract level greater than 0.1%.

Many organisms exhibit a growth stimulation in the presence of, and with increased amounts of glucose. Reference to Figure 5 indicates that growth of P. A. 3679 is stimulated by the addition of glucose to the non-synthetic medium. Growth is fairly good in the absence of glucose

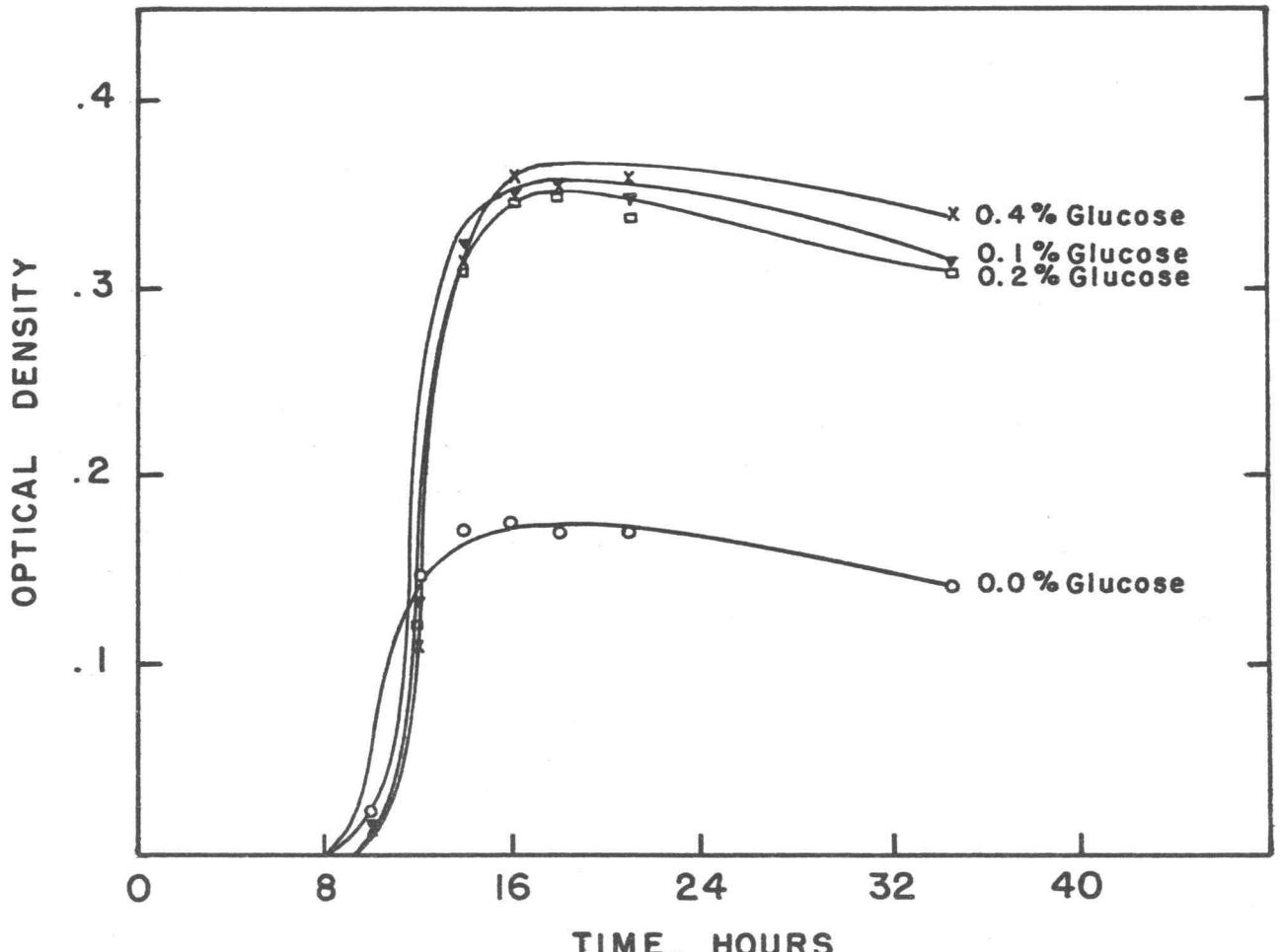

Figure 5. Growth of P. A. 3679 in non-synthetic medium with various levels of glucose.

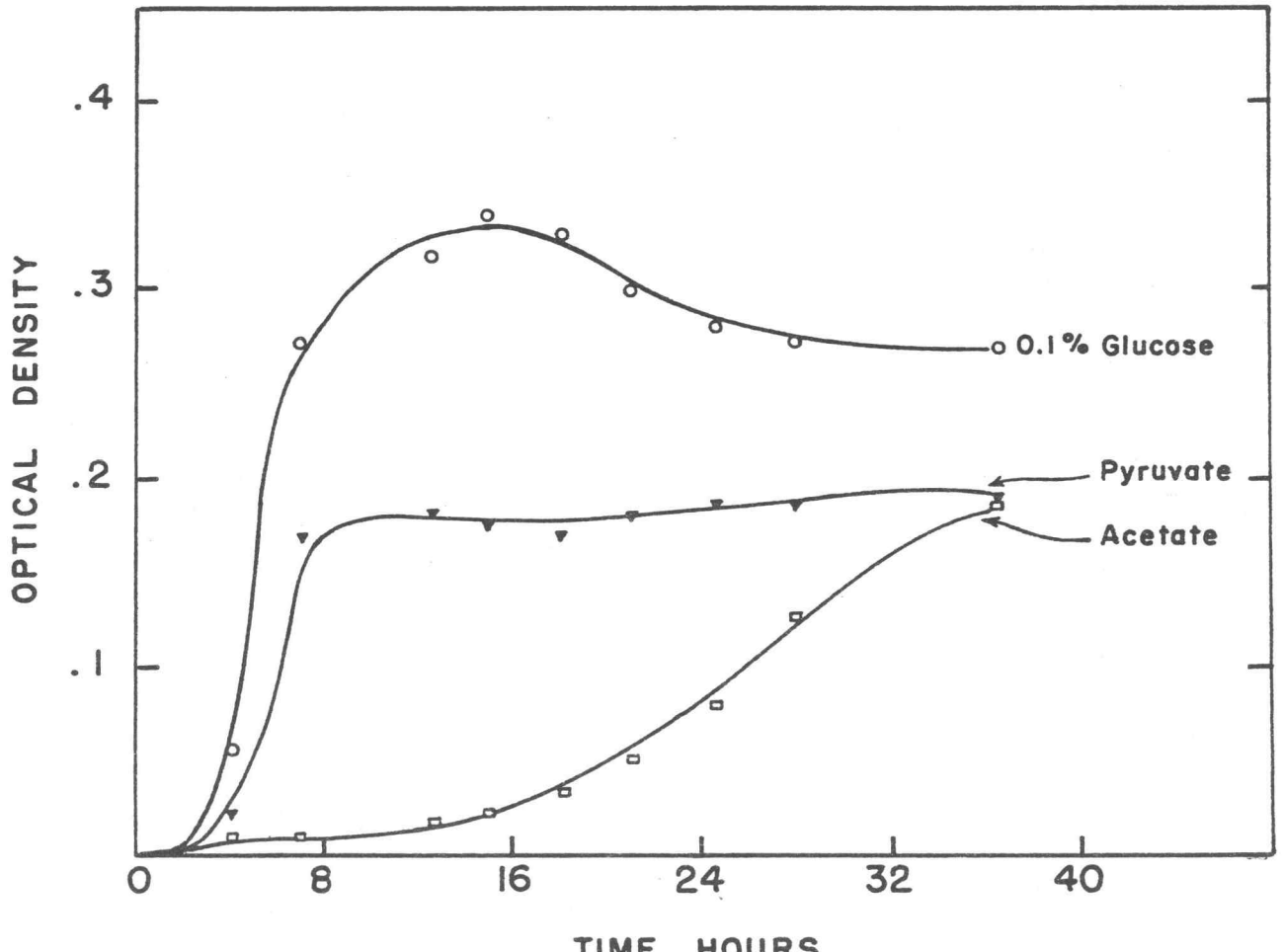

Figure 6. Growth of P. A. 3679 in non-synthetic medium with glucose, pyruvate or acetate.

but is considerably enhanced by 0.1% glucose. It is interesting to note that increments of glucose above 0.1% appear to exert no further stimulation to growth. This may indicate that some other factor is now limiting. The growth pattern without glucose and at all glucose levels is essentially the same.

The organism under study exhibits no growth stimulation in the presence of pyruvate or acetate. Figure 6 gives growth trends in non-synthetic medium containing glucose, pyruvate and acetate. To realize that there is in fact no growth stimulation with pyruvate and acetate it is necessary to refer to Figure 5 which shows growth with no glucose. The maximum growth obtained with pyruvate is the same as that obtained with no glucose in the same medium. Acetate in addition to affording no stimulation appears to actually inhibit the initiation of growth. The acetate curve of Figure 6 shows an extended lag phase and a logarithmic phase with a slope much less than the slope in the pyruvate and glucose curve. It seems then that the stimulatory effect of glucose can not be simulated by these glucose dissimilation products.

It is evident that for maximum growth in the non-synthetic medium there is a requirement for yeast extract at a level of at least 1% and a glucose level of 0.1%,

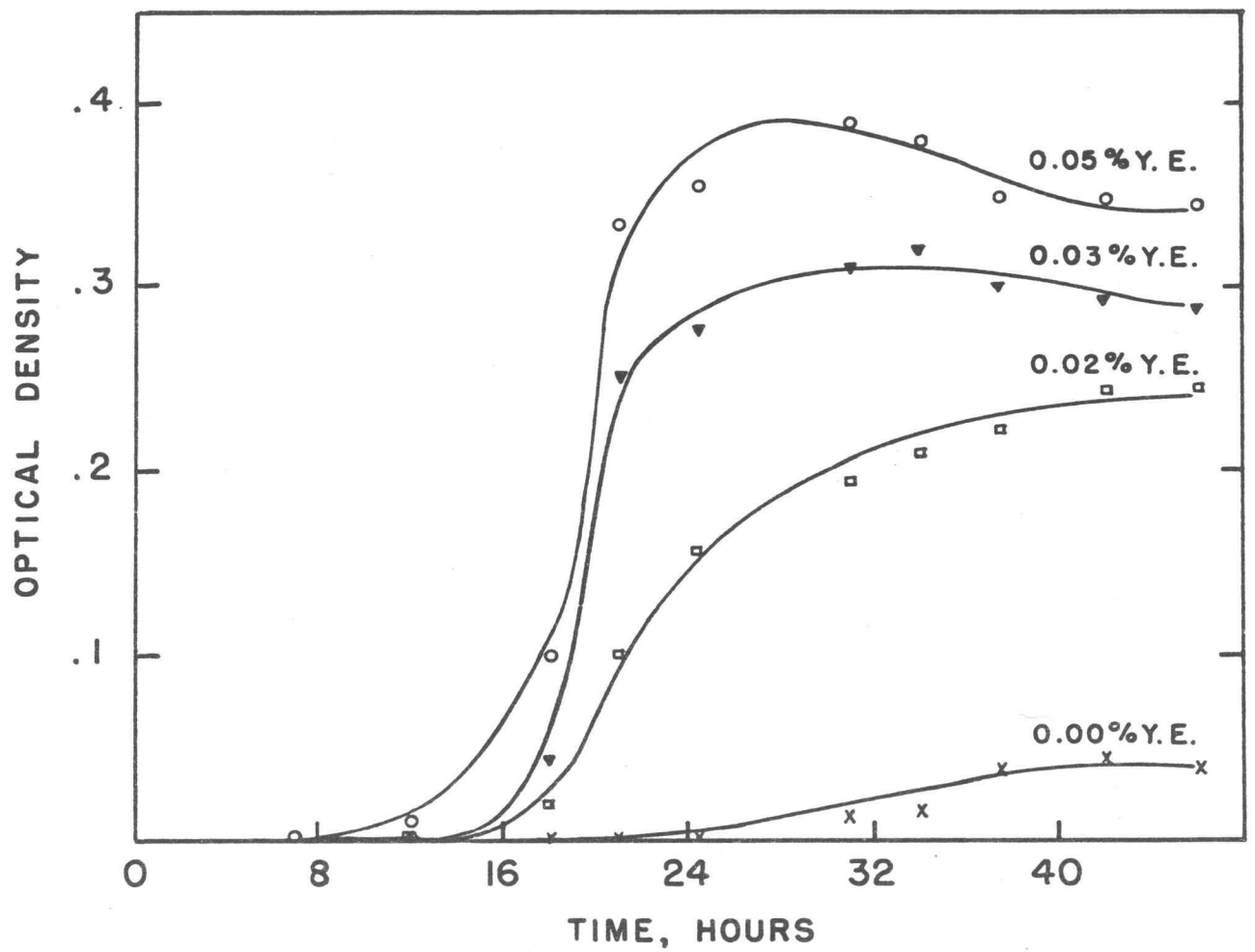

Figure 7. Growth of P. A. 3679 in semi-synthetic medium with various levels of yeast extract.

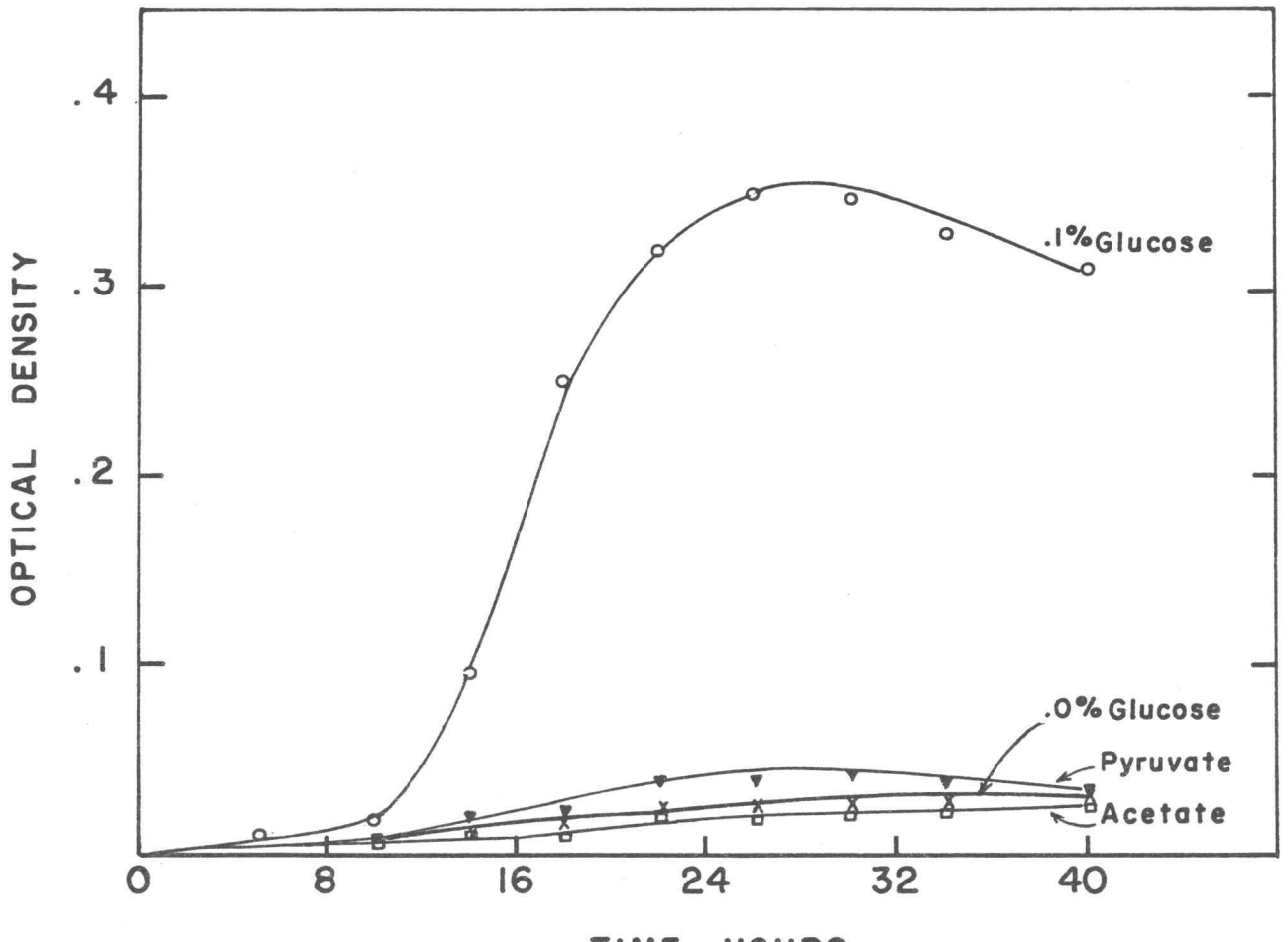

Figure 8. Growth of P. A. 3679 in semi-synthetic medium without glucose and with glucose, pyruvate or acetate.

growth being unaffected by pyruvate and possibly inhibited to some extent by acetate.

Synthetic medium Growth of P. A. 3679 is unpredictable or absent in a strictly synthetic medium. Additions of small amounts of yeast extract to the synthetic medium not only produce good growth but make it much more predictable. Figure 7 shows the effect on growth of additions of yeast extract to the synthetic medium. Growth in synthetic medium plus 0.05% yeast extract, hereafter referred to as the semi-synthetic medium, is equal to or slightly better than growth in non-synthetic medium containing 1% yeast extract. Reference to Figure 4 reveals that the minimum amount of yeast extract for growth is in excess of 0.1%. The curves of Figure 7 then, reflect the role of yeast extract as providing some accessory growth factor and not as providing a primary nutrient. Growth is nearly proportional to the level of yeast extract used. The medium with no yeast extract shows negligible growth.

Growth in semi-synthetic medium is markedly stimulated by the addition of glucose at a level of 0.1%. Figure 8 discloses that this stimulation is even greater than it is in the non-synthetic medium. Whereas fair growth took place in non-synthetic medium without glucose, Figure 5, there is no growth in the semi-synthetic medium in the absence of glucose. Figure 8 also reveals that pyruvate

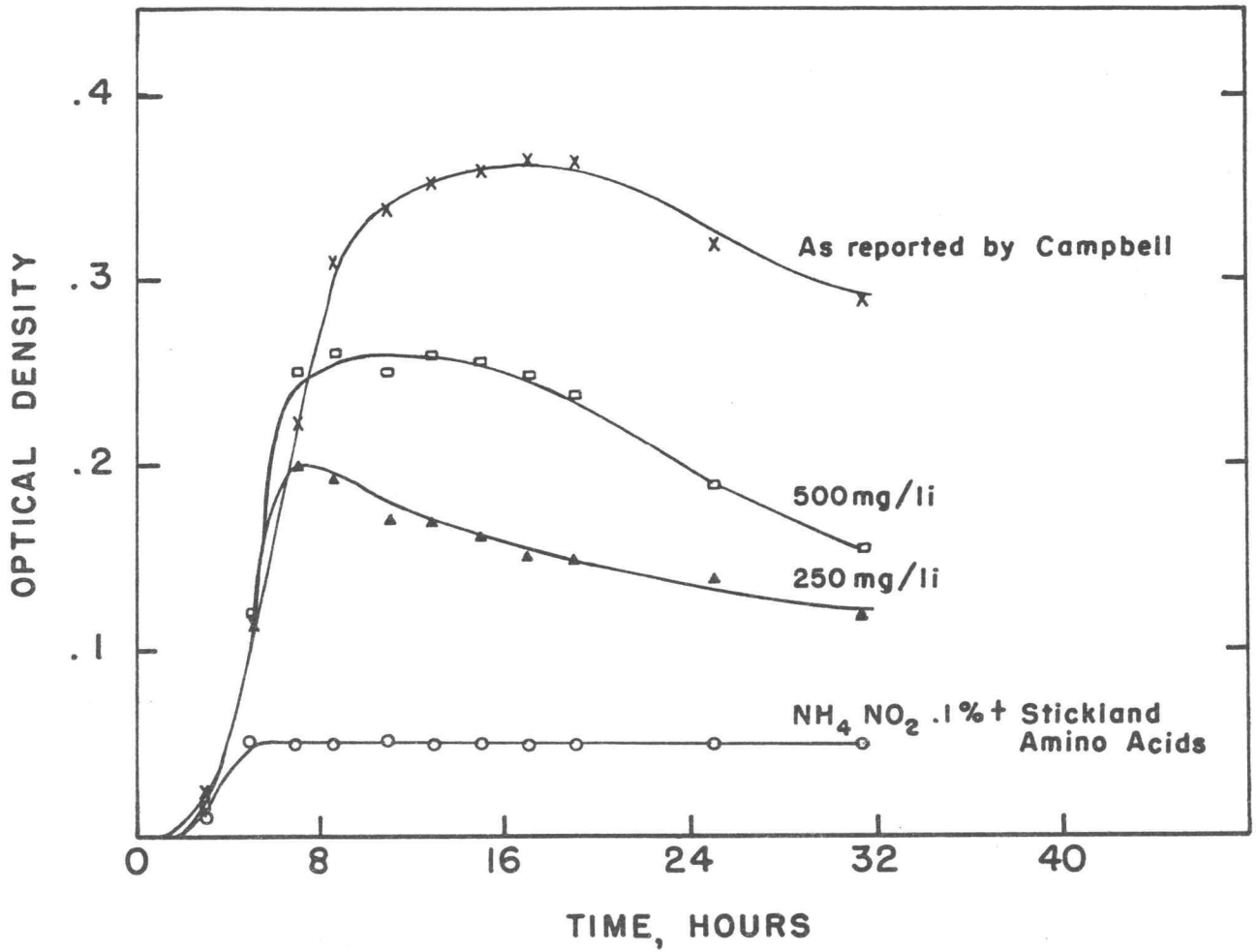

Figure 9. Influence of amino acid concentration on the growth of P. A. 3679 in semi-synthetic medium.

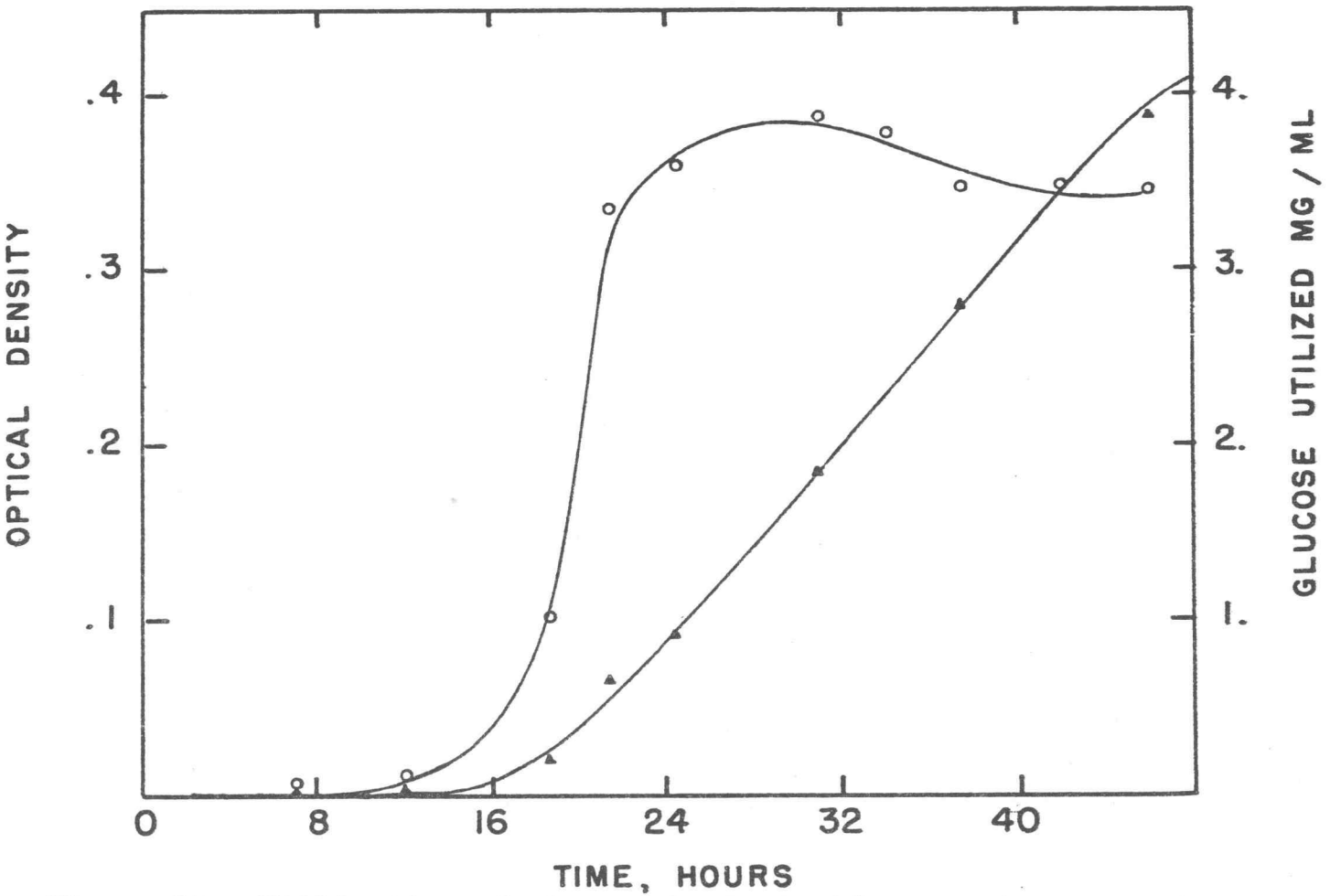

Figure 10. Utilization of glucose by P. A. 3679 growing in semi-synthetic medium.

and acetate are without effect on the growth of P. A. 3679 in the semi-synthetic medium.

The amino acid level in the semi-synthetic medium exerts a very marked effect on growth of the test bacterium as shown in Figure 9. The growth obtained in a medium containing amino acids qualitatively and quantitatively the same as that reported by Campbell and Frank (8, pp. 267-269) is similar to that obtained in a non-synthetic medium containing 1% yeast extract. Lower levels of these same amino acids give correspondingly less growth. It is interesting to note that incorporation of only those amino acids of the original medium specifically implicated in the Stickland reaction and 0.1% NH_4NO_2 results in negligible growth.

The course of glucose utilization in a growing culture of P. A. 3679 in semi-synthetic medium is shown in Figure 10. This curve indicates that in the course of growth more than 80% of the glucose added to the medium is dissimilated, the initial glucose level being 5 mg. per ml. Further it can be seen that the beginning of glucose utilization coincides with the initiation of growth and continues uninterrupted throughout the growth phase. These data reveal that the role of glucose in stimulating growth of this organism is intimately associated with the growth process.

Radiorespirometric Experiments

The catabolic pathways of the organism under investigation were evaluated by the radiorespirometric method of Wang et al. (42, pp. 207-216). The per cent $C^{14}O_2$ recovered during a specific time period was used as a measure of the rate at which the carbon atoms of the substrate are combusted.

Utilization of Specifically Labelled Substrates

Labelled glucose The utilization of glucose was assessed in complete semi-synthetic medium and semi-synthetic medium less amino acids. Figure 11 shows the pattern of glucose dissimilation in the presence of amino acids. The most striking feature of this pattern is the very rapid decarboxylation of a part of the glucose carbon. This combustion is completed in four hours. The combustion of glucose C-1 is not carried out to any extent. Glucose C-2 and glucose C-6 are oxidized to a lesser extent than glucose C-1. The cumulative recovery curve, Figure 11, shows that glucose U-C^{14} is decarboxylated to the extent of 26.4 per cent. Glucose C-1 is recovered as CO_2 to a total of 1.4 per cent of the label in the substrate whereas glucose C-2 and glucose C-6 are only decarboxylated in the amount of 0.7 per cent and 0.3 per cent respectively. The point at which an organism has consumed all of the intact

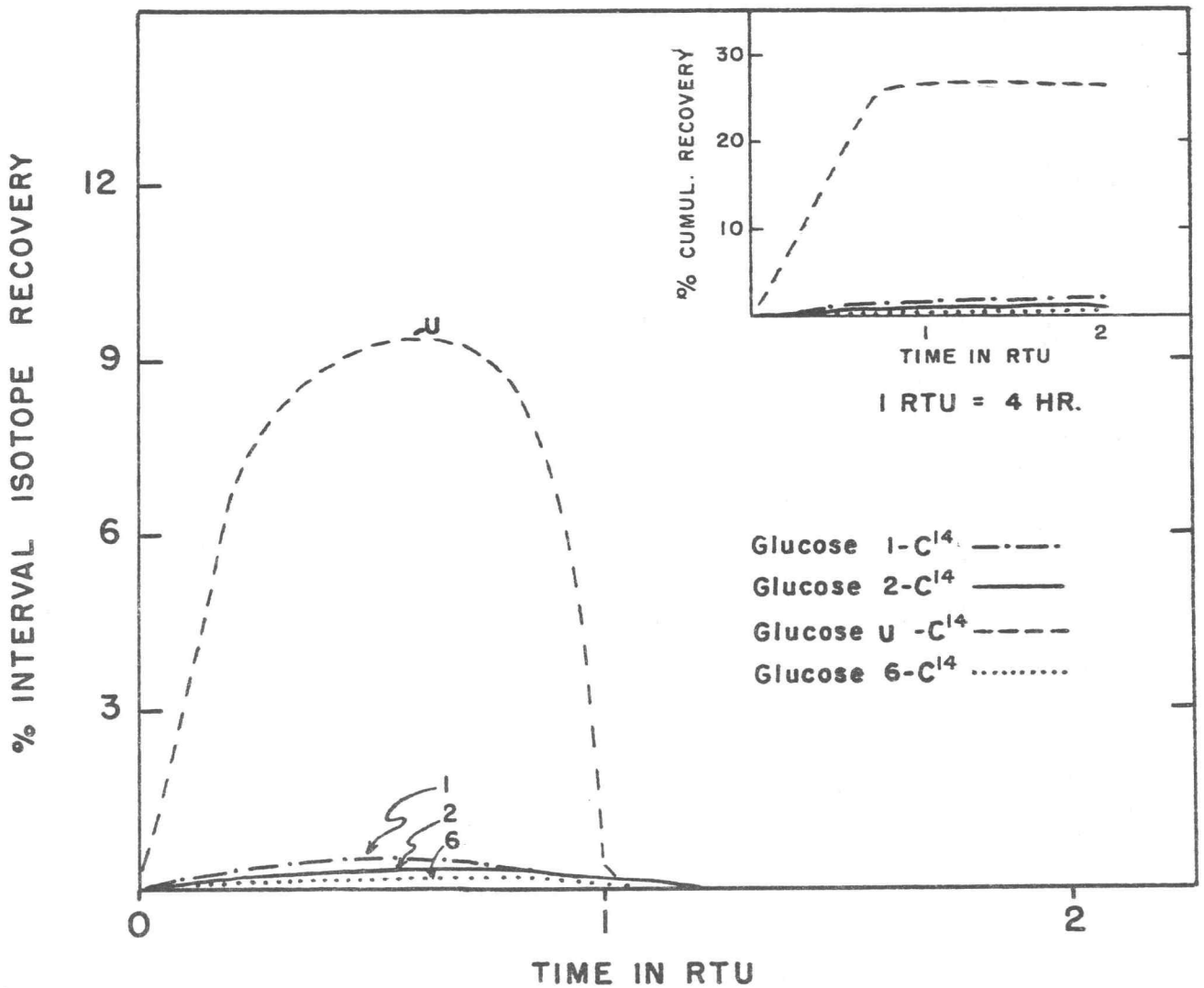

Figure 11. Radiorespirometric pattern of P. A. 3679 growing in semi-synthetic medium.

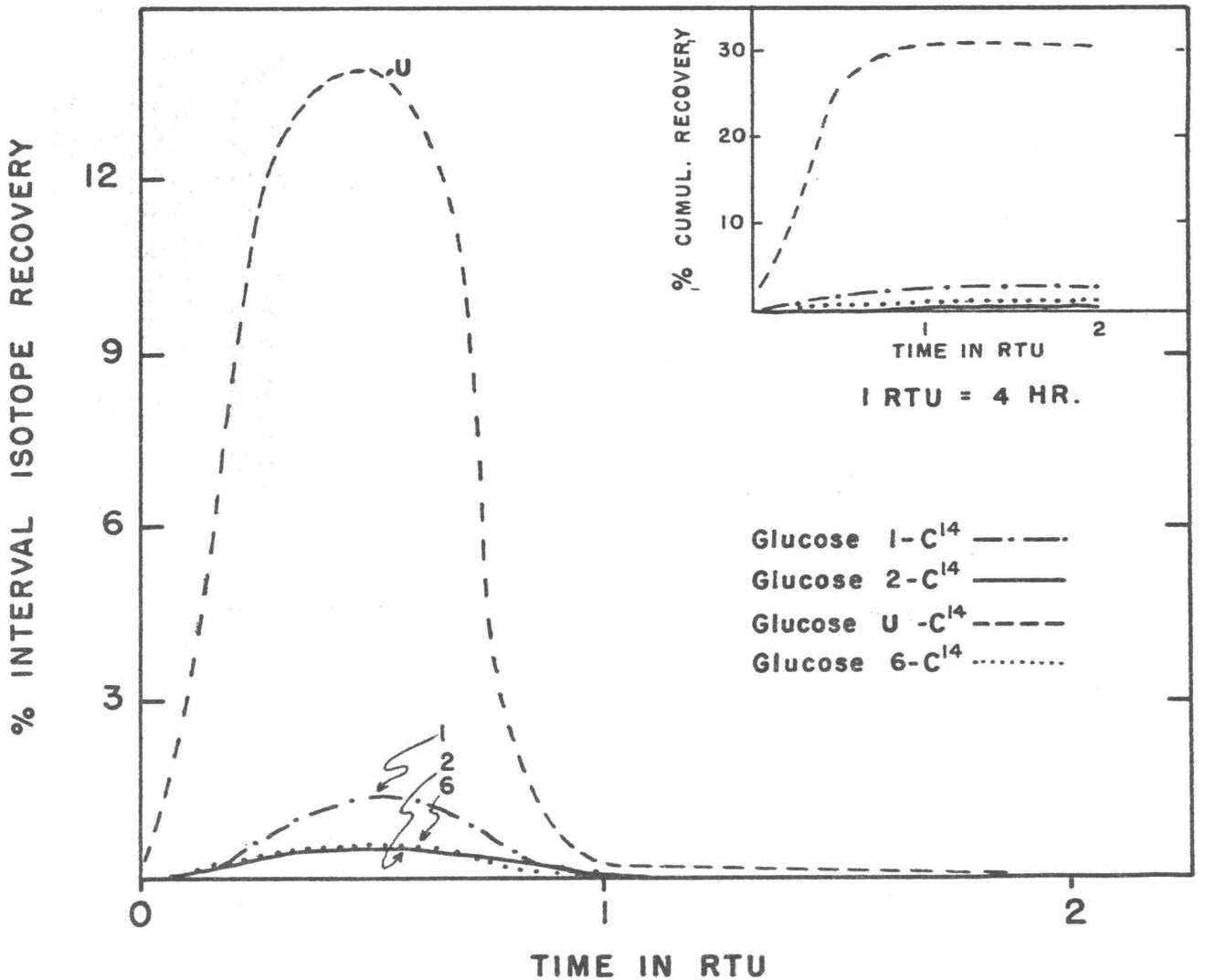

Figure 12. Radiorespirometric pattern of P. A. 3679 growing in semi-synthetic medium without amino acids.

labelled substrate originally added is referred to as one relative time unit (RTU). The interval recovery curve for glucose 1-C^{14} of Figure 11 shows that for this experiment 1 RTU is equal to 4 hours. Figure 12 shows the course of glucose dissimilation in a medium without amino acids. The overall picture here is the same as that for the condition where amino acids are included in the medium. There are however certain quantitative differences that are worthy of note. The combustion of glucose carbon here is slightly greater than that noted in the previous experiment, and the interval recovery curve is somewhat more "peaked" indicating that the majority of the combustion takes place over a slightly shorter period of time. Combustion of glucose C-1 in the medium without amino acids is considerably greater on a relative basis than combustion of the same carbon in the medium with amino acids. Similarly the combustion of glucose C-2 and glucose C-6 in the absence of amino acids is greater than in the corresponding substrates in the presence of amino acids but here again the actual amount is small. The per cent cumulative recovery curve of Figure 12 shows a total per cent isotope recovery from glucose U-C^{14} to be 30.1 per cent. This value is only 4.0 per cent greater than the corresponding curve in Figure 11 but the interval recoveries as pointed out above do show an obvious difference in

Table III

PER CENT ISOTOPE RECOVERIES

Medium	Substrate	Per cent recovery after 8 hours			
		CO_2	Cells	Medium	Total
Semi-synthetic complete	Glucose 1-C^{14}	1.4	3.0	66.0	70.4
	Glucose 2-C^{14}	0.7	2.3	55.1	58.1
	Glucose 6-C^{14}	0.3	2.7	68.3	71.3
	Glucose U-C^{14}	26.4	2.3	48.0	76.8
Semi-synthetic less amino acids	Glucose 1-C^{14}	2.6	1.0	58.3	61.8
	Glucose 2-C^{14}	1.1	1.0	60.1	62.3
	Glucose 6-C^{14}	1.2	0.9	51.5	53.6
	Glucose U-C^{14}	30.1	0.8	33.3	64.2

pattern. Glucose C-1, glucose C-2 and glucose C-6 are decarboxylated to a greater extent in the absence of amino acids 1.1 per cent and 1.2 per cent, than where amino acids are present, 0.7 per cent and 0.3 per cent. The difference here, though the actual recoveries are small, is in the order of 100 per cent.

The per cent isotope recovery for these two experiments is given in Table III. The isotope recovery is very low in the cells of both media, however, the activity of cells taken from medium with amino acids is at least twice as great as cells from medium without amino acids. The total per cent recovery is about 10 per cent higher in the medium with amino acids. It is apparent that there was an overall loss of activity in the experiments. In the complete medium there is approximately 30 per cent isotope unaccounted for and 40 per cent in the medium without amino acids.

Pathway distribution The negligible recoveries obtained with glucose-1, 2, and 6-C^{14} attests to the absence of an active C-1-C-5 cleavage route and terminal oxidation cycle. The recoveries obtained with glucose U-C^{14} can, however be attributed to decarboxylation of the C_3 units formed via the Embden-Meyerhof-Parnas scheme. These findings are in agreement with the anaerobic fermentation pathway via glycolysis.

Labelled pyruvate and acetate Time course experiments utilizing pyruvate-1, 2 and 3-C^{14} as well as acetate-1 and 2-C^{14} showed no decarboxylation of any carbon of either substrate. On the basis of the aforementioned glucose data, one would have expected decarboxylation of pyruvate-1-C^{14} but not of the other labelled carbon atoms. Apparently either permeability factors or other features involving the initial cleavage of glucose may help to explain the observed result. It is also significant to point out that little or no growth stimulation was obtained with either pyruvate or acetate, Figure 8. Certainly the radiorespirometric and growth data complement one another.

Fixation of $C^{14}O_2$

As was described under methods, an attempt was made to demonstrate the possible role of CO_2 fixation in the formation of C-4 acids (a C-3 - C-1 type condensation). It was found that $C^{14}O_2$ was incorporated into the cells of P. A. 3679 to the extent of 0.2% of the original isotope concentration. This negligible fixation level may have resulted from the inability of pyruvate to enter the cell and thereby might be misleading. The latter observation finds some corroboration in the experiments performed with labelled pyruvate and non-labelled carbon dioxide. In the

latter instance, no significant cell incorporation of pyruvate could be detected.

Utilization of Amino Acids

The utilization of nitrogenous compounds by the clostridia takes on additional importance when one considers their possible role in energy metabolism over and above the usual role of these compounds in biosynthesis. If amino acids participate in the energetics of P. A. 3679 there could be expected to exist a relationship between glucose as an energy source and amino acids acting in a similar role.

Preferential Utilization

It was to be expected that certain members of the amino acid mixture used would be utilized to a lesser or greater extent than others. Qualitative results obtained with photographs of the amino acid chromatograms, Figures 13 and 14, attests to the complete disappearance of L-arginine and provide evidence for partial amino acid utilization. Photographs of the kind illustrated in Figures 13 and 14 also give an indication of the kind of amino acid separations obtained. These proved extremely useful as permanent records.

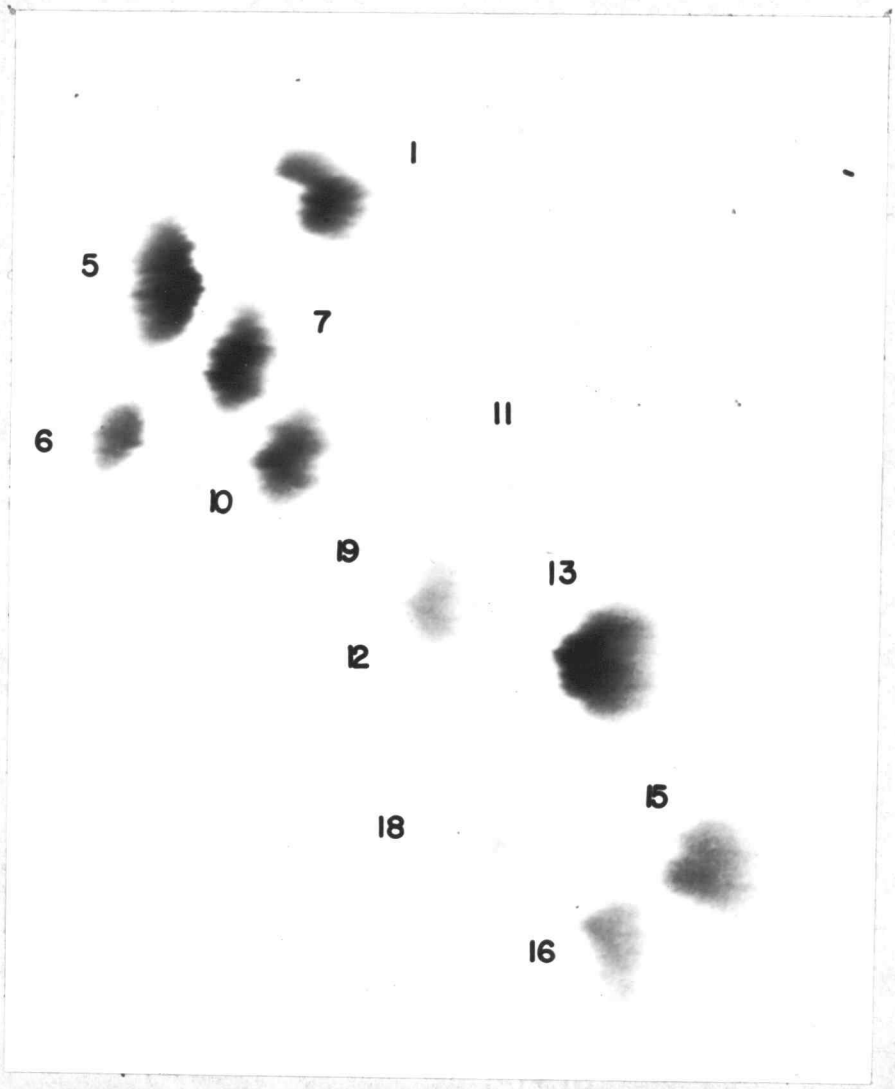

Figure 13. Chromatogram of amino acids in the semi-synthetic medium containing glucose, prior to cell inoculation.

1 Glutamic acid	10 Threonine	15 Isoleucine
5 Arginine	11 Alanine	16 Phenylalanine
6 Histidine	12 Tyrosine	18 Tryptophane
7 Serine	13 Valine	19 Proline

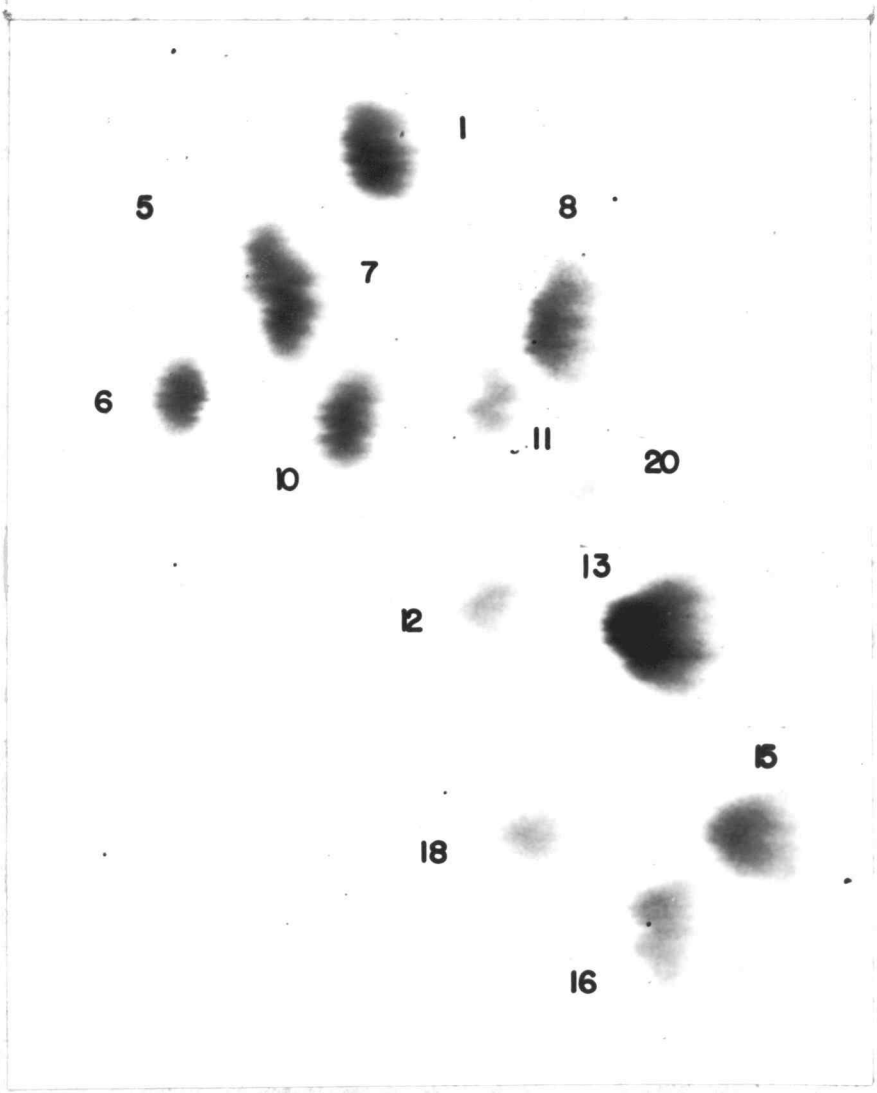

Figure 14. Chromatogram of semi-synthetic medium containing glucose after incubation.

1 Glutamic acid	10 Threonine	15 Isoleucine
5 Arginine (absent)	11 Alanine	16 Phenylalanine
6 Histidine	12 Tyrosine	18 Tryptophane
7 Serine	13 Valine	20 Unknown
8 Glycine		

Analatrol analysis of the amino acid spots supplied quantitative data on the extent of utilization of the individual amino acids. Reference to Table IV discloses that in the presence of glucose, DL-tryptophane, L-arginine, L-tyrosine, DL-isoleucine, DL-threonine and L-proline were indeed utilized by the test organism. On the other hand, L-histidine, L-glutamic acid, DL-serine, glycine and alanine although probably utilized actually increased in the medium during the experimental period. Since the secretion of certain amino acids during cellular growth is well recognized the latter result was not considered unusual.

In the absence of glucose a quite different amino acid utilization picture emerged. The data is recorded in Table V. DL-tryptophane, DL-phenylalanine, L-tyrosine and DL-isoleucine were utilized to a greater extent than was observed with these amino acids in the presence of glucose. It would appear that glucose exerts a sparing effect thereby reducing the observed utilization values, Table IV. This would infer that glucose either served a biosynthetic function or perhaps by providing energy reduced the net need for these amino acids. Of additional interest was the observed shift in alanine utilization. In the absence of glucose the L form of this amino acid decreased 111.1 per cent, Table V, whereas in the presence of glucose a 1300 per cent increase in concentration was

Table IV

AMINO ACID UTILIZATION BY P. A. 3679 IN THE PRESENCE OF GLUCOSE

Amino Acid	Integrated Area Before Incubation	Integrated Area After Incubation	% Decrease	% Increase	% Decrease L-Form	% Increase L-Form
DL-Tryptophane	4.2	3.1	26.2		52.4	
L-Arginine	14.1	0.0	100.0		100.0	
DL-Phenylalanine	8.0	7.3	8.7		17.5	
L-Tyrosine	5.2	1.8	65.3		65.3	
DL-Valine	15.4	14.5	5.8		11.5	
DL-Isoleucine	10.2	6.9	32.3		64.7	
DL-Threonine	9.8	8.4	14.2		28.5	
L-Proline	0.8	0.0	100.0		100.0	
L-Histidine	4.3	5.2		21.0		21.0
L-Glutamic Acid	8.8	10.8		22.7		22.0
DL-Serine	11.1	13.2		18.0		36.0
Glycine	0.8	13.3		1562.5		
Alanine	0.2	2.6		1300.0		2600.0

Table V

AMINO ACID UTILIZATION BY P. A. 3679 IN THE ABSENCE OF GLUCOSE

Amino Acid	Integrated Area Before Incubation	Integrated Area After Incubation	% Decrease	% Increase	% Decrease L-Form	% Increase L-Form
DL-Tryptophane	4.6	2.6	43.4		86.9	
L-Arginine	10.9	2.0	81.8		81.8	
DL-Phenylalanine	10.5	5.7	45.7		91.4	
L-Tyrosine	6.2	1.7	72.5		72.5	
DL-Valine	11.8	10.8	8.4		16.9	
DL-Isoleucine	10.0	5.1	49.0		98.0	
DL-Threonine	9.1	8.2	9.8		19.7	
L-Proline	1.0	0.0	100.0		100.0	
L-Histidine	5.0	4.6	8.0		8.0	
L-Glutamic Acid	8.6	9.0		4.6		4.6
DL-Serine	8.2	8.6		4.8		9.7
Glycine	0.5	10.6		2000.0		
Alanine	.9	.4	55.5		111.1	

obtained, Table IV. It seems likely that in the latter instance glucose is involved in the synthesis of alanine via pyruvate. It also appears probable that glucose is involved in the synthesis of L-glutamic and DL-serine since these amino showed greater per cent increases in the presence of glucose than was observed in the absence of this substrate.

The data in Table VI relating to the influence of pyruvate on amino acid utilization is in general agreement with the data obtained for amino acid utilization in the absence of glucose, Table V. In effect no sparing action of pyruvate on amino acid uptake by the cells was observed. This finding takes on additional significance when it is remembered that pyruvate did not stimulate growth of P. A. 3679 nor could active decarboxylation be demonstrated.

Biosynthesis of amino acids

Reference to Figure 15 gives the amino acid composition of a typical P. A. 3679 cell hydrolysate. It is evident that not all the required amino acids were provided in the semi-synthetic medium. Those evidently synthesized were aspartic (2), lysine (4), alanine (11), cysteine (3), methionine (14), glycine (8) and an unidentified compound (9).

Table VI

AMINO ACID UTILIZATION BY P. A. 3679 IN THE PRESENCE OF PYRUVATE

Amino Acid	Integrated Area Before Incubation	Integrated Area After Incubation	% Decrease	% Increase	% Decrease L-Form	% Increase
DL-Tryptophane	4.7	2.1	55.3		110.6	
L-Arginine	10.6	2.0	86.0		86.0	
DL-Phenylalanine	8.3	4.7	54.3		108.7	
L-Tyrosine	5.6	1.6	71.4		71.8	
DL-Valine	12.1	10.8	10.7		21.4	
DL-Isoleucine	9.1	4.9	46.1		92.3	
DL-Threonine	8.9	8.2	7.8		15.7	
L-Proline	1.0	0.0	100.0		100.0	
L-Histidine	4.0	3.6	10.0		10.0	
L-Glutamic Acid	7.5	7.8		4.0		4.0
DL-Serine	9.3	10.8		16.1		32.2
Glycine	0.5	10.1		1920.0		
Alanine	1.2	0.6	50.0		100.0	

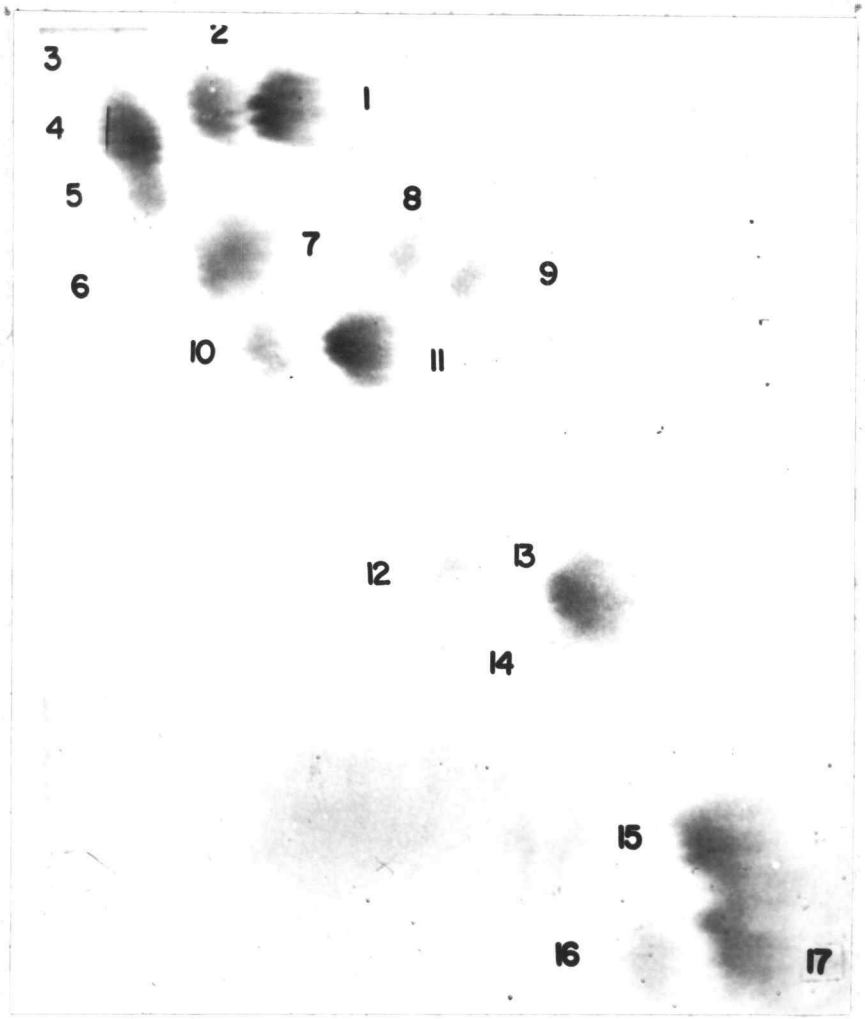

Figure 15. Amino acid chromatogram of a typical cell hydrolysate of P. A. 3679.

1 Glutamic acid	7 Serine	13 Valine
2 Aspartic acid	8 Glycine	14 Methionine
3 Cystine	9 Unknown	15 Isoleucine
4 Lysine	10 Threonine	16 Phenylalanine
5 Arginine	11 Alanine	17 Leucine
6 Histidine	12 Tyrosine	

In the hope that glucose was implicated in the synthesis of one or more of the cell amino acids an attempt was made to assess the incorporation of C^{14} activity from glucose in the amino acid fraction. Radioautograms of cell hydrolysates were prepared. Although the incorporation of isotope into the cells was only 1.6%, faint spots were visible for three amino acids. Alanine appeared to contain the greatest C^{14} activity whereas aspartic and glutamic showed only a slight isotope level.

DISCUSSION

The underlying question posed at the outset of the investigation, herein reported, related specifically to the need for glucose and selected amino acids in the metabolic growth processes of the test organism P. A. 3679. The literature review revealed that little or no information had been reported on glucose utilization and that the methodology used for estimation of amino acid utilization left much to be desired. In fact one could well wonder why glucose had been included in some media and why such large levels of amino acids were routinely used. There is little question but that the described results have helped to clarify these points and as might be expected have unearthed new problems.

Of primary significance is the observation that glucose markedly stimulates growth of the test organism. It may be concluded therefore that this hexose plays an important role in the overall metabolism of P. A. 3679. Since only a small fraction of glucose is incorporated into cellular material, it appears probable that the primary role played by glucose is to provide additional energy via the glycolytic pathway. However, the observed sparing effect of glucose on amino acid utilization also attests to the involvement of this carbohydrate in either

biosynthetic reactions or again in providing energy. Certainly the C^{14} activity of alanine, glutamic and aspartic acids obtained with cell hydrolysates confirms the synthesis role of glucose. It is felt, however, that because of the observed low cell C^{14} incorporation levels that the primary role of glucose is that of an energy source.

The amino acid utilization aspect of the study has clarified several points. The growth studies revealed that a much lower amino acid concentration may be used if glucose is included. Since there is a large excess over that required for cell protein, it seems logical that specific amino acids are functioning to provide energy as well as to supply required cell protein. Since the medium used contained only a trace of glycine and alanine, the large concentrations of these amino acids in the cell protein points to active biosynthesis either from glucose or from other amino acids.

It is expected that future studies on whether certain amino acids are essential or not will provide needed information. On the basis of our findings (analatrol analysis) there appeared to be very little uptake of DL-threonine, and L-histidine. The increases in amino acid concentration noted in the presence and absence of glucose provided presumptive evidence that L-histidine, L-glutamic acid, DL-serine, glycine and alanine may not be essential;

may not be required at high levels in the growth medium. Certainly this nutritional point deserves further study.

It might be well at this juncture to call attention to the need for accessory growth factors by P. A. 3679. Campbell's (8, pp. 267-269) studies indicated that washed cells would not be used for growth studies. One could thereby readily suspect that the original cell inoculum used by the latter worker contained carry over nutrient probably of the growth factor category. Results of the present study indicated that this observation was indeed correct. Washed cells were routinely used in conjunction with a small amount of yeast extract added to the medium. This of course allowed for a more defined medium and contributed to the consistency of the growth data.

A final point for discussion relates to the observed lack of growth stimulation and active decarboxylation with pyruvate and acetate. When it is remembered that glucose showed excellent growth stimulation and underwent decarboxylation at the carbon 3, 4 position (carbon 1 and 6 of pyruvate), it became difficult to interpret the negative results obtained with the aforementioned two substrates. The only realistic explanation appears to lie in the possible inability of pyruvate and acetate to enter the cell. Such permeability effects are often encountered and as was the case in the present study become

difficult to overcome. Future studies, however, are being designed to elucidate the permeability by decreasing the pH of the medium and also by using greater substrate concentrations.

SUMMARY

The growth and certain aspects of P. A. 3679 cell metabolism have been investigated. Pertinent results of the study may be summarized as follows:

1. An anaerobic gas-flow apparatus was designed and proved to be effective for cell growth measurements.
2. A semi-synthetic growth medium was developed containing glucose and selected amino acids.
3. Glucose was observed to be utilized by the test organism, providing a marked increase in cell growth.
4. Glucose was also observed to exert a sparing action on the cell uptake of certain amino acids. It was concluded that the primary role played by glucose was that of an energy source; a secondary role involved the participation of glucose in the biosynthesis of alanine, aspartic and glutamic acid.
5. Certain amino acids were used in a preferential manner, whereas others increased in the medium during growth; the latter via cell secretion.

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