AN ABSTRACT OF THE THESIS OF

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Title: Quantitative Analysis of the Fermentation Products of Enterobacter aerogenes

Abstract Approved: Richard P. Keeling

The products of fermentation of many bacteria, including Enterobacter aerogenes, have been determined by earlier methods of analysis. The purpose of this study was to use more modern methods of analysis of fermentation products.

E. aerogenes was isolated from soil, and the effect of varied concentration of carbohydrates on hydrogen production by this soil inhabiting bacterium was investigated. The bacterium was grown anaerobically in phenol red broth, supplemented with varied amounts of fermentable carbohydrates (C-4, C-5, C-6 and C-12).

Quantitative assays of other major fermentation end products of E. aerogenes, grown on glucose-supplemented medium were also investigated. The technique of high-performance liquid chromatography (HPLC) was used to directly quantitate formic, lactic and acetic acid products.

This technique was also used to indirectly quantitate 2-3-butanediol production by E. aerogenes, grown under aeration-agitation, and anaerobic conditions. Carbon dioxide

and hydrogen were quantitated by chemical and physical means of analysis respectively.

Gas chromatographic analysis established that at the lowest concentration of carbohydrates (0.05 %) the hydrogen content of the gas mixture was at its highest peak, but the quantity of the gas mixture was at lowest level of production. Increasing hydronium ion concentration also resulted in an increase in hydrogen production.

A hydrogen to carbon dioxide ratio of 1:4.93 was established. This is similar to the hydrogen to carbon dioxide ratio of 1:5 established by previous investigators. The total quantity of organic acids obtained is comparable to that established by previous methods of analysis, but the quantity of individual species of organic acids differs from the published data. Aeration-agitation resulted in a two fold increase in the production of 2-3-butanediol.

Quantitative Analysis of the Fermentation Products of *Enterobacter aerogenes*

A Thesis

Submitted to

the Division of Biological Sciences

Emporia State University

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by

Naseem Sadeghi

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INTRODUCTION

The term "fermentation" has an ancient history and with progress in biochemical knowledge, it has assumed various meanings. From intensive research into the electron transport system of microbial metabolism, it is now clearly understood that all processes which have an organic compound as a terminal electron acceptor are called fermentations.

Where the terminal electron acceptor is oxygen or an inorganic compound, the pathway is regarded as oxidative and the process, one of respiration. The latter is either aerobic (using oxygen) or anaerobic (using an inorganic compound other than oxygen).

Under anaerobic conditions, only a fraction of the potential energy of a fermentable substrate is liberated because oxidation is incomplete. In order to obtain the amount of energy equivalent to that obtained under aerobic conditions, several times as much carbohydrate must be catabolized. Consequently, there is a high yield of unoxidized organic by-products. The fermentation products differ with different microorganisms, being governed mainly by the enzymatic machinery of the cells and the environmental conditions. The economical value of these by-products led to the development of industrial microbiology (Doelle 1975).

Although carbohydrates commonly serve as substrates for fermentation organic acids (including amino acids),

proteins, fats and other organic compounds are fermentable substrates for selected microorganisms.

Pyruvic acid has been established as a key intermediate substance in the metabolism of carbohydrates by bacteria. Almost all six, five and four carbon compounds are converted initially to pyruvate, the fate of which is decided by the enzymatic machinery of a particular microorganism (Doelle 1975).

It has been well documented that under anaerobic conditions 20 to 30 percent of carbohydrates are utilized by the Enterobacteriacae through the hexosemonophosphate pathway (HMP), while the rest is processed via the Embden-Meyerhoff-Parnas (EMP) pathway (Katz and Wood 1960, 1963). The uptake of carbohydrates by the bacterial cells is brought about by mechanisms requiring the expenditure of energy.

The most favored mechanism for the transport of sugars across bacterial membranes is now believed to be the group translocation process. The basic principle of the process is that the sugar undergoes a chemical reaction at the cell membrane resulting in its phosphorylation and release into the cell.

$$\text{PEP} + \text{HPr} \xrightarrow{\text{E i}} \text{HPr} - \text{P} + \text{Pyruvate}$$

Sum:

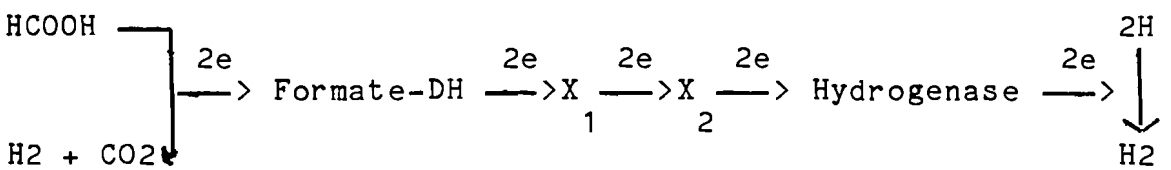
$$\begin{array}{l} \text{HPr} - \text{P} + \text{Sugar} \xrightarrow{\text{E i, F iii}} \text{Sugar} - \text{P} + \text{HPr} \\ \hline \text{PEP} + \text{Sugar} \longrightarrow \text{Sugar} - \text{P} + \text{Pyruvate} \end{array}$$

The Phosphoenolpyruvate-dependent phosphotransferase system (PT-system) is necessary to bring about such a reaction. This system is shown to be present in both gram positive and gram negative organisms (Postma and Roseman 1976; Kornberg 1975; Anderson et al. 1971). The presence of the PT-system was established in E. aerogenes by Hanson and Anderson (1968). This system was found to consist of a histidine-containing, low molecular weight protein (HPr), a soluble protein (E i), a membrane bound protein (E ii) which is carbohydrate specific and a protein component called factor iii, which is present only in gram positive organisms. The operation of this system requires energy, which is provided by Phosphoenolpyruvate (Simon et al. 1968).

Romano et al. (1970) in their survey of the distribution of the PT-system among bacteria, established that the system was mainly found in the representatives of the genera that are characteristically facultative anaerobes, and that it played no role in the sugar transport of strict aerobes.

The ability to produce hydrogen has been observed in a relatively large number of species of microorganisms including significantly different taxonomic and physiological types. Among them are many of the heterotrophic, facultative anaerobes which typically contain cytochromes and evolve hydrogen from formate. The anaerobic

mode of growth poses a special problem with respect to the disposition of electrons from the energy yielding metabolic reactions. One of the control mechanisms to regulate the electron flow in the metabolism of the strict and facultative anaerobes is their ability to dispose of excess electrons in the form of molecular hydrogen which is evolved from formate. The immediate precursor of formate is pyruvate, an important intermediate in the metabolism of carbohydrates and certain amino acids. The decomposition of formate is catalyzed by a formate hydrogen lyase complex system.



As formate is oxidized to carbon dioxide by formate dehydrogenase, the electrons are passed over the carriers X_1 and X_2 to hydrogenase at which hydrogen is evolved (Gray and Gest 1965).

The bacteria carrying out fermentations are either facultative or obligate anaerobes. Facultative anaerobes such as the Enterobacteria grow as aerobic heterotrophs in the presence of oxygen; under anaerobic condition they carry out a fermentative metabolism (Gottschalk 1979).

Hormaeche and Edwards (1960) proposed the generic name, Enterobacter for non-motile strains of the IMViC

reaction (-,-,+,+). This led to motile strains of A. aerogenes, being transferred to a new species, Enterobacter aerogenes, while non motile strains of A. aerogenes were placed in the species Klebsiella aerogenes. Non motile and anaerogenic variant strains of E. aerogens occur, which may be recognized by their typical biochemical behavior. This distinction has been confirmed by DNA reassociation studies of Brenner et al. (1972). Yet as the authors had predicted the term Aerobacter has continued to be misused and cultures isolated under circumstances not indicating their immediate connection with the intestinal tracts of man and other animals are labeled as A. aerogenes instead of Enterobacter aerogenes.

Enterobacteria are widely distributed and have been isolated from water, soil, plants, foodstuff (including grain), and the intestinal tracts of man and other animals (Paparasilliou et al. 1967). Enterobacter and Serratia are common saprophytes of soil, water and plants, and are rarely encountered as animal parasite although Enterobacter cloacae is a commensal of intestinal tracts (Cruickshank 1965). The presence of enterobacteria in arable land may result from fecal droppings of animals or birds, but the ease of isolation from sites where chances of animal contamination are remote suggests that at least some of these bacteria are definite but minor soil inhabitants (Alexander 1961).

Griffin and Stuart (1940) established that a large

proportion of bacteria isolated from soil are Enterobacters and that the abundance of Enterobacter species remained essentially constant regardless of animal droppings, again suggesting that Enterobacter are normal inhabitants of soil. Fauct's (1982) data on the relationship between land use practices and fecal bacteria in soil clearly established that the proportion of Enterobacter aerogenes increased with the remoteness of soil from potential polluting agents such as man, birds, and mammals.

Cowbourne et al. (1972); Priest et al. (1974) and Lategan et al. (1984) all report the occurrence of the Enterobacteria in breweries and their ability to spoil beer as a well-known fact. Wort enterobacteria are not usually parasitic to humans or other animals, but appear to be more closely associated with the free living forms encountered in water, soil, and plant material. These include organisms such as Citrobacter frundii. Enterobacter aerogenes, Enterobacter cloacae, Hafnia alvei, and Klebsiella aerogenes.

The enterobacterial species encountered in brewery wort continue to grow even 24 hour after pitching, and produce metabolic products during brewery fermentation that and significantly influence the flavor and aroma of the final beer. The Enterobacteria vary with respect to pH and ethanol tolerance. For those normally encountered in the brewery, pH normally becomes inhibitory below 4.4, and

ethanol at concentrations above 2 % w/v. Wort Enterobacteria, can significantly retard the fermentation rate. In such beers the pH is higher by 0.1 to 0.3 units (Priest et al. 1974). Buchanan and Gibbons (1974); Krieg and Holt (1984) list a large number of generic descriptions to include fermentation end products as a criterion for the inclusion of an organism in a particular genus. Although the classification of Enterobacteria is in a constant state of flux, based on their fermentation end products, they can be divided into two major groups: (1) The mixed acid producers (Methyl Red Positive, Voges-Proskauer Negative), producing as their major fermentation end products formic acid, lactic acid, carbon dioxide, hydrogen, and ethanol. This fermentation pathway is characteristic of the genera Citrobacter, Escherichia, Edwardsiella, Salmonella, Shigella and Yersinia. (2) The butanediol producers (Methyl Red Negative, Voges-Proskauer Positive), produce lower amounts of organic acids as compared to the mixed acid fermentors, but they produce copious amounts of 2-3-butanediol. This pathway is characteristic of the genera, Enterobacter, Hafnia, Klebsiella and Serratia (Wood 1961).

The ratios of the end products produced by the enterobacteria may vary considerably both from strain to strain and in a single strain, depending on the culture conditions (Stanier et al. 1977).

The use of gas chromatography (GC) in detecting fermentation end products and its application in bacterial taxonomy is well documented (Van-Vuuren et al. 1978, 1981, Van-Vuuren and Toerien 1981). Similarly, in recent years the use of gas chromatograph has attained a new significance in clinical microbiology (Brooks et al. 1983).

Tracy et al. (1986) using a gas chromatograph equipped with a hydrogen flame ionization detector, analyzed the metabolic end products and established taxonomic relationships between 19 Hafnia protea strains and 27 strains of Hafnia alvei. Fischer (1986), using a Hewlett Packard model gas chromatograph, equipped with a thermal conductivity detector, simultaneously quantitated carbon dioxide and hydrogen produced by intestinal microorganisms grown in one percent solutions of various carbohydrates in nutrient broth. He noted a marked difference in the gas ratio produced by a given organism, with respect to the different carbohydrates utilized. This method has been applied in carbohydrate fermentation by the intestinal microflora from infants on different feeding regimens.

Enterobacter aerogenes undergoes the butanediolic type of fermentation, producing 2-3-butanediol, which is of great interest to the chemical industry as an intermediary. Sablayrolles and Goma (1984) noted that butanediol production by Enterobacter aerogenes NRRL B199 grown on glucose required an optimal rate of aeration, and that 2-3-

utanediol production increased with increasing glucose concentration. Also that a 20 % glucose concentration, in the absence of air caused lysis of the cells, while injection of air restored growth and butanediol fermentation.

Over the past forty years, the practice of gas chromatography has witnessed a continuing growth in almost every respect. High-performance liquid chromatography is the result of continuing improvement in equipment, material, technique, and the application of theory. Even though the ability of gas chromatography to separate and analyze complex mixture is now widely appreciated, HPLC enjoys a broader range of applications over gas chromatography (Snyder and Kirkland 1977). Today HPLC is unquestionably the fastest growing of all of the analytical separation techniques (Skoog 1985).

The purpose of this study was to use conventional means of isolation and identification of the soil inhabiting Enterobacter aerogenes, and to employ modern analytical methods to quantitate as many as its major fermentation end products as possible.

The objectives were as follows:

1. To isolate and identify a fermentative, soil inhabiting bacterium for its possible industrial significance.

2. To quantitate and study the effect of varied concentrations of various fermentable carbohydrates on the level of hydrogen production by E. aerogenes.

3. To use high-performance liquid chromatography (HPLC), to directly quantitate formic, lactic, and acetic acids produced, by E. aerogenes, grown on glucose Phenol-red broth.

4. To establish the ratio of carbon dioxide to hydrogen gas, produced by E. aerogenes.

5. To use high-performance liquid chromatography to indirectly quantitate the production of 2-3-butanediol, and compare the effect of aerobic and anaerobic modes of growth upon the production of this fermentation by-product.

6. To study the effect of varied pH upon production of hydrogen in E. aerogenes.

MATERIALS AND METHODS

During an investigation of the biological activity of soil samples collected from the banks of the Neosho river, located in Northeast Lyon County, Kansas, a bacterial isolate was recovered and noted to ferment a large number of carbohydrates with the production of a combustible gas as determined by gas chromatography. It was then decided to study the organism and quantitate its fermentation end products.

Isolation of the Bacterium

A 25 gram sample of fresh soil was placed in a graduated cylinder. Distilled water was added so that a total of 250 ml was reached. The suspension was stirred and poured into a 1000 ml Erlenmeyer flask. The flask was shaken on a mechanical shaker for 30 min. A 10 ml aliquot of this suspension was immediately drawn into a sterile pipette, and transferred into a 200 ml sterile screw capped medicine bottle, containing 90 ml of distilled water. Successive, serial, ten-fold dilutions were made until a final dilution of 1×10^{-8} was reached, which was found to be the dilution of choice by preliminary trials.

One ml aliquots of the above dilution were aseptically transferred into several petri dishes. 12-15 ml of Sabouraud dextrose agar, cooled to 50 C, were added to each dish. The dishes were then rotated in a broad swirling motion so that the diluted soil was evenly dispersed in the agar medium.

The plates were incubated at 30 C, for five days. At the end of the incubation period the plates were noted to contain various species of fungi and bacteria. Some of the bacterial colonies had formed gas bubbles on or around them. The bacterium was isolated, sub-cultured and stock slant cultures were made available.

Identification of the Bacterium

The 8th edition of Bergey's Manual of Determinative Bacteriology by Buchanan and Gibbons (1974) was used to identify the organism as Enterobacter aerogenes.

Early in the research it was determined that the combustible component of the gas was hydrogen. In order to quantitate the amount of of hydrogen gas evolved by the bacterium the technique of gas chromatography was used.

Gas Chromatography (GC)

The gas chromatograph used in this study (Appendix 1) was constructed by Blew and Mayo (Emporia State University, Division of Biological Sciences) for the purpose of measuring nitrogenase activity by the acetylene reduction technique. Their construction was based on a plan designed by Mallard et al. (1977). This gas chromatograph is equipped with two sensitivity ranges. Range number one measures concentration between 1×10^{-10} to about 4×10^{-9} moles/ml. Range number two measures from 0.5×10^{-9} to 3×10^{-8} mol/ml of acetylene (Blew 1984). Range number two was used in this study.

GC Syringe

The syringe used for bacterial gas injection into the GC was a 10 microliter Hamilton syringe (Model No. 14-824-80B).

Calibration of the gas chromatograph

In order to relate the magnitude of the GC detector response to the concentration of the sample component, the gas chromatograph was calibrated with hydrogen. This was done by collecting hydrogen gas (Linde Gas Specialty) over a column of water (25 C and 710 mm Hg), and injecting it into the gas chromatograph in 0.2 microliter increments over the range of the detector response. Injections were made in duplicate and care was taken so that the syringe remained clean and dry between injections. Reproducible chromatograms were obtained, when replicate volumes of the gas were injected.

Ideal Gas Law

The equation of the ideal gas law (P.V. = n.R.T.) allows the conversion of the volume of gas injected to the corresponding number of moles of hydrogen.

In the above equation:

P = Hydrogen gas pressure determined at the time of analysis (atm).

V = Volume of hydrogen injected (l).

n = Number of moles of hydrogen.

R = Gas constant (0.0821 l.atm/mol.K)

T = Room temperature (298 K).

rmentation Medium

Bacto Phenol Red Broth Base (Difco Laboratories, troit, MI) was supplemented with the desired concentration carbohydrates and autoclaved for 15 min at 15 psi. This dium, when supplemented with carbohydrate, is the medium choice for fermentation studies (Difco Lab. Manual 1984).

odified Fermentation Culture Tubes

In order to facilitate access to the bacterial gas via he GC syringe the culture tubes were modified.

Screw capped culture tubes were used for this purpose. ight fitting rubber septa were placed on the open end of the tubes. The closed end of tubes were opened, with the use of a water cooled rotary grinder and powdered carborundum (100 Mesh). This was done to alleviate pressure in the tubes from gas production. Durham tubes in the inverted position were placed in the culture tubes, and five ml of the fermentable growth medium were dispensed in each tube and sterilized for 15 min at 15 psi.

Inoculation

The medium was inoculated with one ml of bacterial suspension containing $1x10^8$ cells of E. aerogenes ($2x10^7$ cells/ml). The size of the inoculum was kept constant throughout this investigation.

Culture Conditions.

The inoculated culture tubes were placed in an anaerobic jar, containing an anaerobic envelope (Gas-Pak Plus System,

cton-Dickinson and Co.) and incubated at 37 C for 24 h. hree microliter samples of gas evolved were withdrawn from ch fermentation tube and injected into the pre-calibrated s chromatograph. Injections were made in duplicate, and e average peak areas were measured from the chromatograms. e hydrogen standard curve was used to relate the measured eak areas to the number of moles of hydrogen present in ested samples.

Peak Area Measurement

The size of the chromatographic peak is proportional to the amount of material contributing to that peak (McNair 1975). Peak area and peak height measurements are commonly used. Several methods are available for peak area measurement. In this study the height and width at half height method was used. A peakometer (Alltech Scientific, Inc.), especially designed for this purpose was used.

High-performance liquid chromatography (HPLC)

High-performance liquid chromatography or modern liquid chromatography refers to any chromatographic procedure in which the moving phase is a liquid, in contrast to the gas phase of gas chromatography.

Equipment

The HPLC used in this study utilized a Varian 2050 detector, 2010 pump and 7125 Rheodyne injector system (Appendix 1).

Operational Conditions

The following operational conditions were kept constant throughout the use of this instrument. The mobile phase which consisted of 95 % phosphate buffer and five percent HPLC grade methanol, was buffered at a pH of 2.75, to shift the equilibrium of the dissolved organic acids to their non-ionized forms. This increase in the hydronium ion concentration increases the retention times of the organic acids, thus improving their separation. The mobile phase was freshly filtered, to remove dissolved gases as well as particulate matter which could have otherwise affected the column performance and detector response. The flow rate of the mobile phase was adjusted to one ml per min. The pump pressure was adjusted to 1200 atmospheres, and the recorder chart speed was set at one centimeter per minute. Using the HPLC syringe, injections of 200 microliters were used for each sample. Of this amount, the actual volume of the sample introduced into the column is only ten microliters, the remainder serves to remove organic residues from previous injections or to remove any microbubbles that might have been trapped in the system. The range selected for the amplification of the detector response was based on the concentration of the sample component. The detector was set at a wavelength of 210 nm. Preliminary trials of the sample components indicated this to be the optimum wavelength for absorbing UV light.

Calibration of the Analytes

Various molar concentrations of formic acid, lactic acid, acetic acid and acetylmethylcarbinol, were made with concentrations ranging from the lower to the and upper limits of those suspected to be produced by Enterobacter aerogenes were made (Appendix 2). Peak height and absorbance of each sample was recorded.

Absorbance Measurement.

To obtain the UV absorbance values the ratio of peak height of a given sample (cm) to the entire length of the chromatogram (25 cm), was multiplied by the range value selected for that sample.

Direct Quantitation of Organic Acids

Five millimoles of glucose were dissolved in 100 ml of phenol red broth base (0.05 M solution). The medium was sterilized, inoculated with $1x10^8$ cells of E. aerogenes, and incubated anaerobically at 37 C, for 24 h. The bacterial suspension was centrifuged at 1200 rpm. for 15 min. To prevent clogging of the HPLC column, membrane filters having a pore size of 0.45 microns were used for complete removal of bacterial cells from the supernatant.

The samples were then injected into the HPLC, and the resulting elution profiles were compared with the chromatograms of known organic acids as well as a chromatogram of sterile fermentation medium.

Indirect Quantitation of 2-3-Butanediol

Two 200 ml Erlenmeyer flasks, each containing 100 ml of sterile MR- VP medium, supplemented with five millimoles of glucose were inoculated with $1x10^8$ cells. One flask was subjected to aeration and agitation by incubating it for 24 hours at 37 C, in a mechanical shaker. The other flask was incubated under anaerobic condition. Samples from each flask were prepared as before and injected into the HPLC for analysis, and the elution profiles were compared to the chromatogram of acetylmethylcarbinol.

Quantitative Analysis of the Gas Mixture

A. Carbon Dioxide

One hundred ml of phenol red broth supplemented with five millimoles of glucose was sterilized, and medium was poured into a sterile 100 ml French flask. The medium was inoculated with $1x10^8$ cells. During incubation the evolved gases were passed through rubber tubing to an alkali tower containing 0.1 M solution of barium hydroxide. The carbon dioxide component of the gas mixture reacted with barium hydroxide to produce barium carbonate as a white precipitate. The precipitate was brought to a constant weight by placing it in a desiccator containing calcium carbonate as a desiccant, and drying it overnight at 110 C. The crucible containing the the precipitate was brought to

a constant weight in the same way and its tare weight was recorded in advance. Carbon dioxide was quantitated as a function of the amount of barium carbonate produced (Appendix 3).

Hydrogen

The remaining portion of the gas mixture (hydrogen) was accumulated at the top of the alkali tower. Two microliter samples of the above gas were injected into the pre-calibrated gas chromatograph in duplicate. The peak areas were measured from the chromatogram and the hydrogen standard curve was used to quantitate the amount of hydrogen in two microliter samples of the carbon dioxide-free gas.

To quantitate the total amount of hydrogen produced per five millimoles of glucose fermented the same experimental design was used with the exception that the total volume of gas evolved, was collected over a column of de-ionized, distilled water. The total volume of gas evolved was withdrawn periodically over a 24 h period. The ideal gas law was then applied to obtain the total number of moles of hydrogen present in the gas mixture (Appendix 3).

pH Effect on Hydrogen Production

The hydronium ion concentration of phenol red dextrose was adjusted to various pH levels prior to inoculation with 1x10 cells of Enterobacter aerogenes. Five ml of the medium was dispensed in each fermentation tube, and incubated

aerobically for 24 h at 37 C. Three microliter samples of e gas in each tube were analyzed by injection into the gas romatograph in duplicate. Peak areas were measured from he chromatograms and the amount of hydrogen was quantitated ing the hydrogen standard curve.

uclear Magnetic Resonance Spectroscopy

In order to determine if any volatile organic compound as present in appreciable amounts, an NMR spectrum of the as sample was obtained. This was done by dissolving as uch of the gas as possible in a silicon coated vacutainer ube containing three ml of carbon tetrachloride. The ample was then subjected to NMR spectroscopy (Appendix 2).

RESULTS AND DISCUSSION

fect of Carbohydrates on Hydrogen Production by E. ogenes

A survey of the occurrence of hydrogen production in croorganisms discloses that the ability to evolve lecular hydrogen is not a restricted metabolic capability t rather this type of activity is very wide spread over tirely different taxonomic and physiologic types of croorganisms.

From a general standpoint, the formation of molecular ydrogen can be considered a device for disposal of excess lectrons during the anaerobic fermentation of carbohy- rates. The extent of hydrogen production during the naerobic fermentation by bacteria such as E. aerogenes is trongly influenced by events at the electron transfer level. Particularly important in this respect is the potential competition for electrons among alternative terminal acceptors. In the coli-aerogenes group several electron acceptors are reduced and the amount of fermentation end products (including that of hydrogen) reflect the balance among different electron transfer pathways (Doelle 1975).

Gest (1954) and Gray and Gest (1965) reported that the yield of hydrogen production was directly related to the state of reduction of the fermented energy source, and tend to be inversely related to the total quantity of reduced end

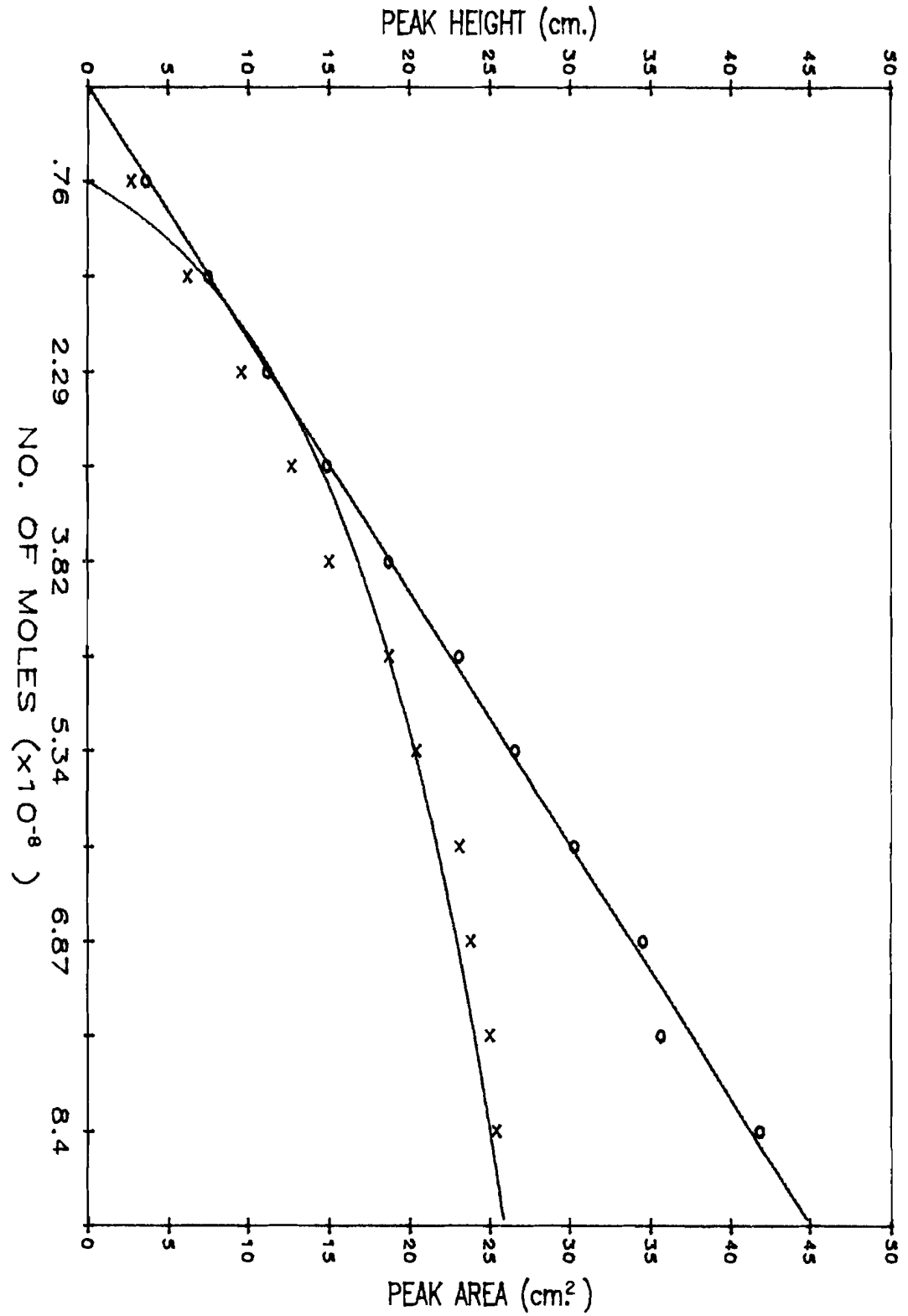
ducts which accumulates in the medium.

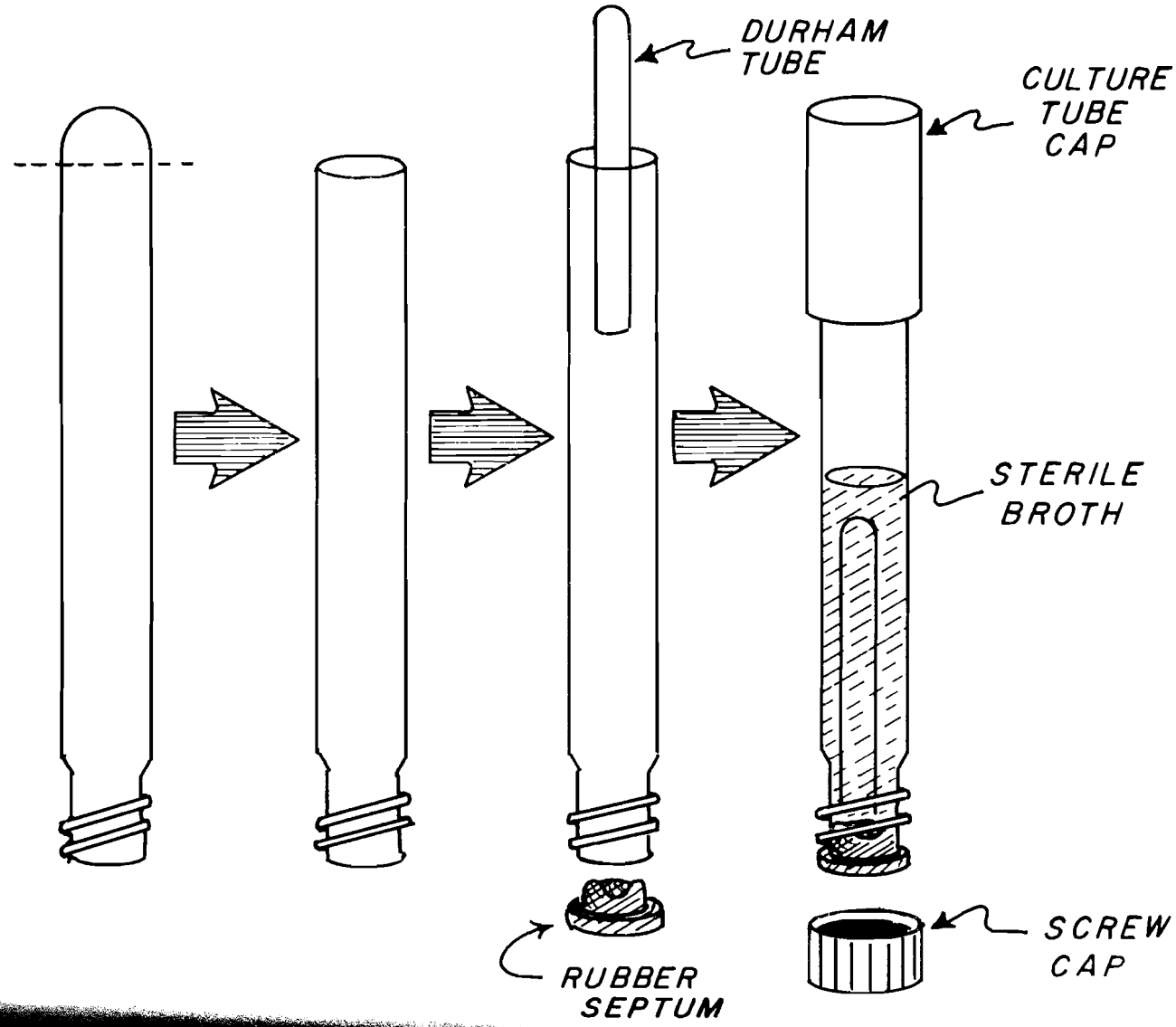
The fermentable carbohydrates used in this study are l of similar reduction state and the slight differences in eir reduction states are negligible (Ericson 1988).

The result of this study (Figs. 3-13) revealed that the drogen content in three microliter quantities of the gas ixture produced by E. aerogenes are very similar, with espect to a given concentration of carbohydrate, regardless f its chemical composition.

At low concentrations of carbohydrates (0.05 %) the ydrogen content of the gas mixture produced is at its aximum. This phenomenon is due to the fact that because of he limitation in the amount of energy source, the cell does not have the chance to produce electron accepting metabolic by-products. Consequently the very small amount of pyruvate that is produced is readily converted to formate, which is perhaps the only electron transfer reaction taking place at this low concentration of the fermented energy source. The electrons inherent in formate are disposed of in the form of molecular hydrogen via the enzymatic activity of formate hydrogen lyase. Consequently, and because of the absence of other carbon dioxide yielding electron transfer reactions, the gas mixture has a much higher hydrogen content.

At higher carbohydrate concentration the cell produces several electron accepting organic species (formic, lactic,


PEAK HEIGHT (cm.)
0
5
10
15
20
25
30
35
40
45
50
.76
2.29
3.82
5.34
6.87
8.4
NO. OF MOLES (x10⁻⁸)
PEAK AREA (cm²)


DURHAM TUBE
CULTURE TUBE CAP
STERILE BROTH
RUBBER SEPTUM
SCREW CAP

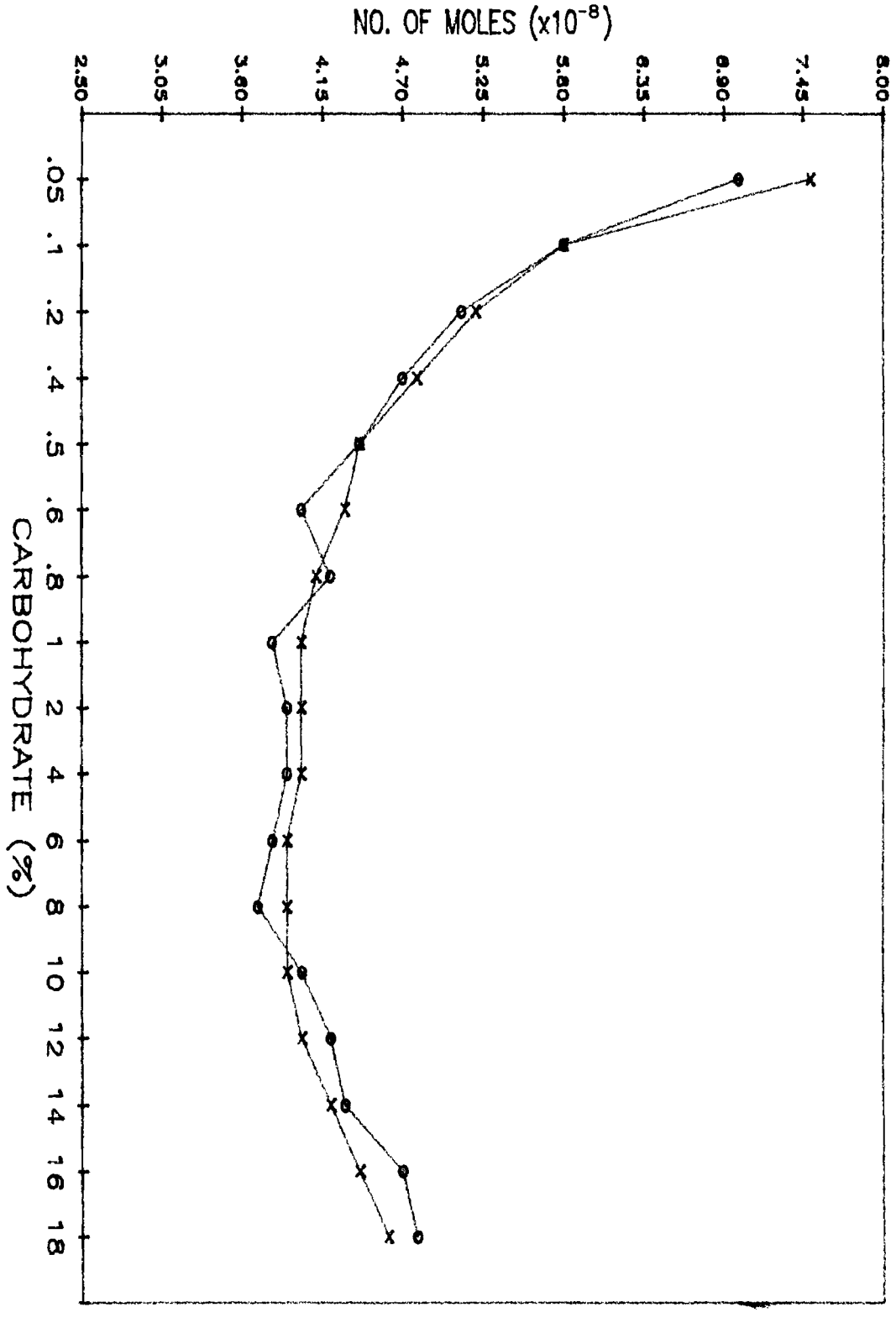

NO. OF MOLES (x10⁻⁸)
2.50
3.05
3.60
4.15
4.70
5.25
5.80
6.35
6.90
7.45
8.00
.05
.1
.2
.4
.5
.6
.8
1
2
4
6
8
10
12
14
16
18
CARBOHYDRATE (%)

Figure 4. Effect of the concentration of C-6 carbohydrates (Fructose and Mannose) on hydrogen content of three microliter quantitiesof the gas mixture produced by E. aerogenes.

Legend:

x = Fructose
o = Mannose

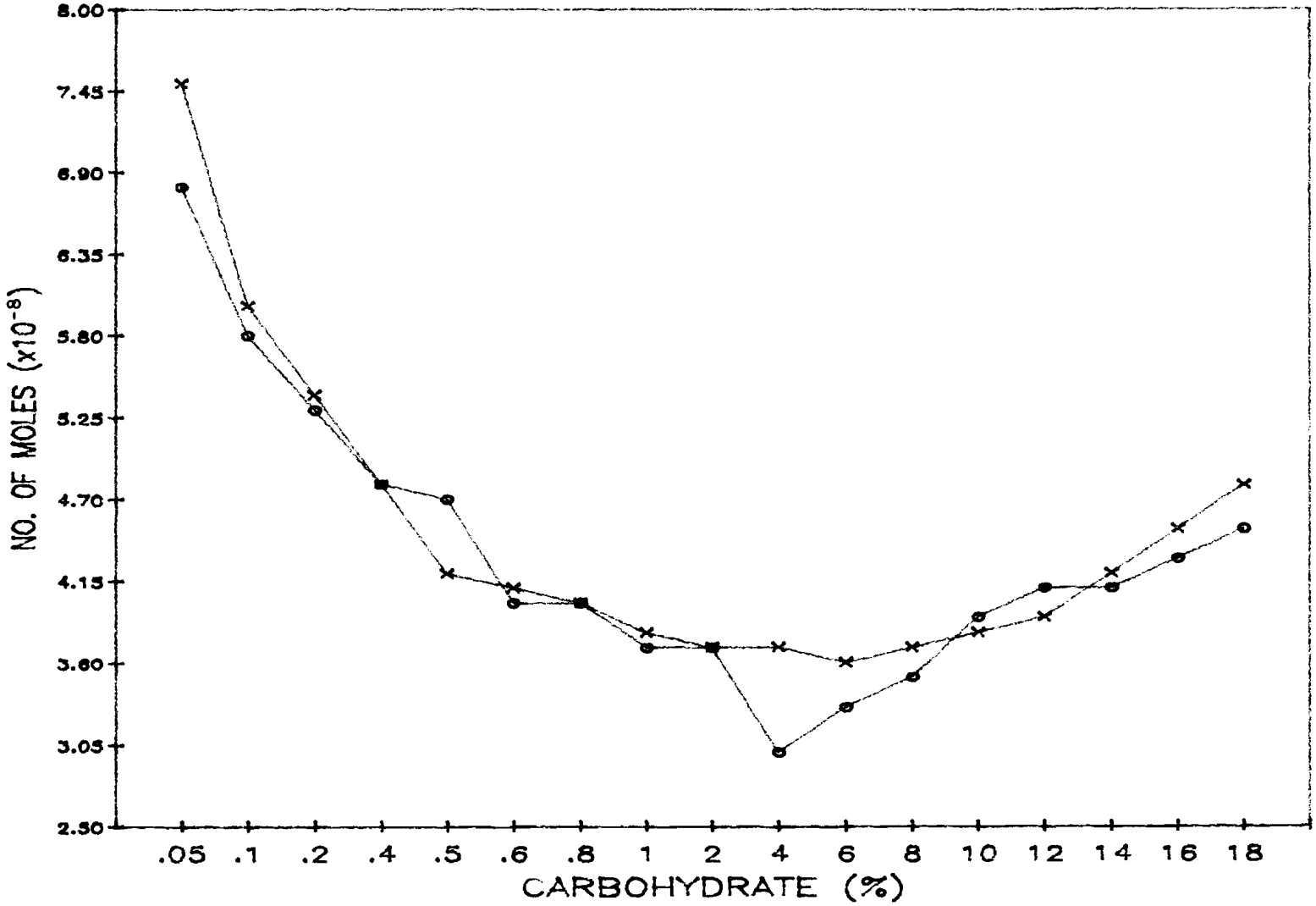

8.00
7.45
6.90
6.35
5.80
5.25
4.70
4.15
3.60
3.05
2.50
NO. OF MOLES (x10⁻⁸)
.05 .1 .2 .4 .5 .6 .8 1 2 4 6 8 10 12 14 16 18
CARBOHYDRATE (%)

Figure 5. Effect of the concentration of galactose on hydrogen content of three microliter quantities of the gas mixture produced by *E. aerogenes*.

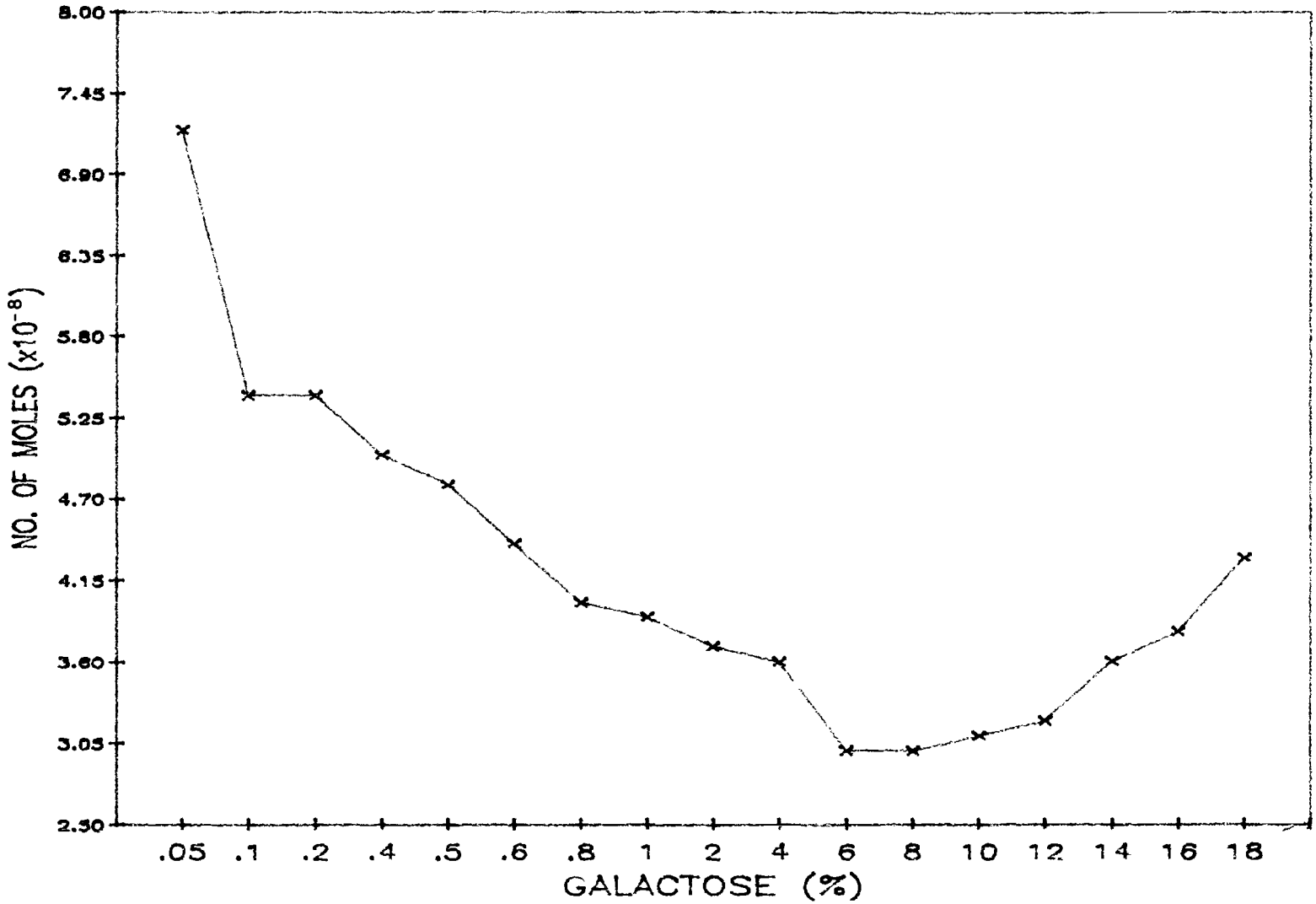

8.00
7.45
6.90
6.35
5.80
5.25
4.70
4.15
3.60
3.05
2.50
NO. OF MOLES (x10⁻⁸)
.05
.1
.2
.4
.5
.6
.8
1
2
4
6
8
10
12
14
16
18
GALACTOSE (%)

Figure 6. Effect of the concentration of C-6 carbohydrates (Sorbitol and Mannitol) on hydrogen content of three microliter quantities of the gas mixture produced by E. aerogenes.

Legend:
x = Sorbitol
o = Mannitol

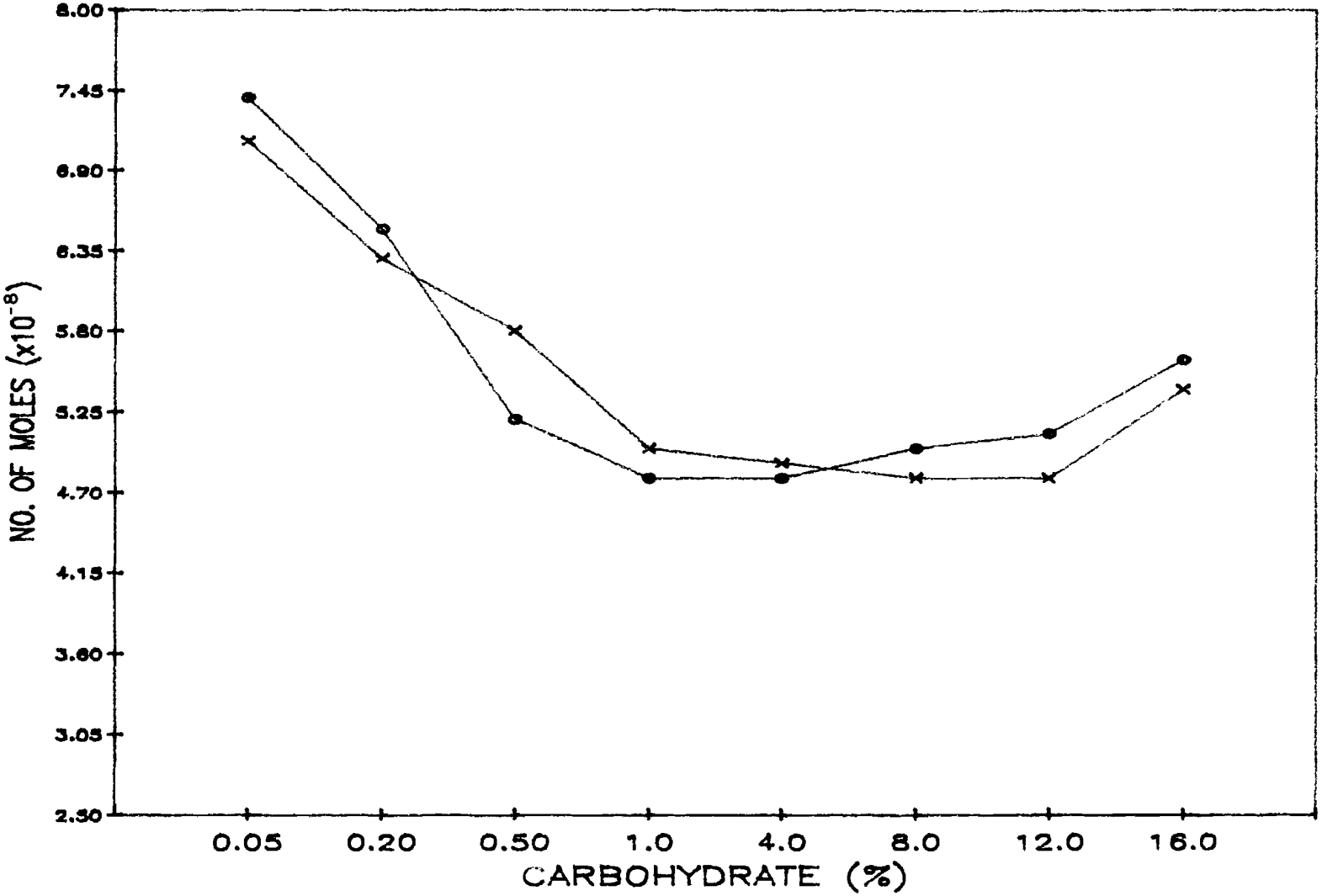

8.00
7.45
6.90
6.35
5.80
5.25
4.70
4.15
3.60
3.05
2.50
NO. OF MOLES (x10⁻⁸)
0.05
0.20
0.50
1.0
4.0
8.0
12.0
16.0
CARBOHYDRATE (%)

Figure 7. Relationship between the concentration of C-6 carbohydrates and the hydrogen content of three microliter quantities of the gas mixture produced by E. aerogenes.

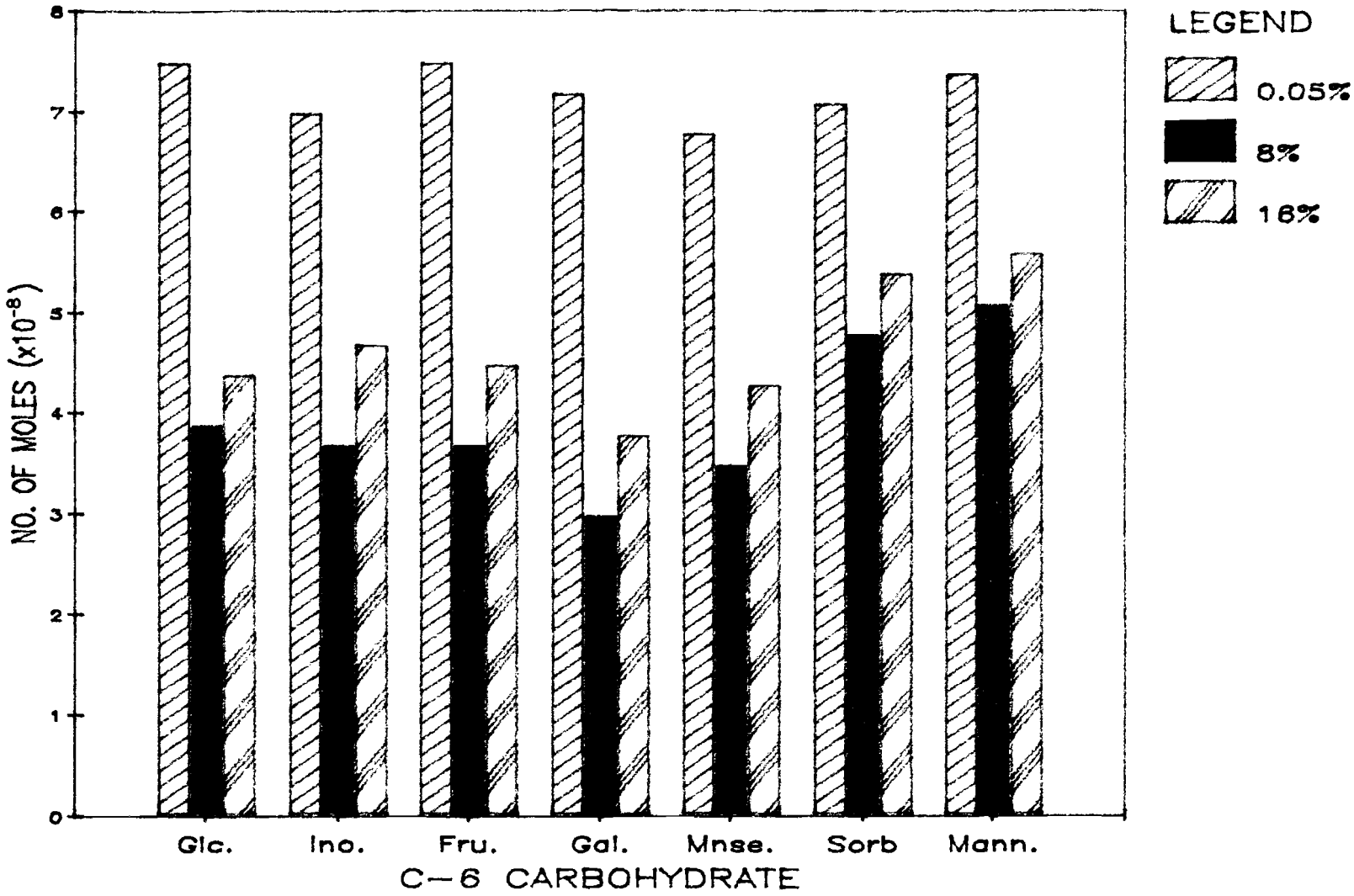

LEGEND
0.05%
8%
16%
NO. OF MOLES (x10⁻⁸)
0
1
2
3
4
5
6
7
8
Glc.
Ino.
Fru.
Gal.
Mnse.
Sorb
Mann.
C—6 CARBOHYDRATE

Figure 8. Effect of the concentration of C-12 carbohydrates (Sucrose and Lactose) on hydrogen content of three microliter quantities of the gas mixture produced by E. aerogenes.

Legend:
x = Sucrose
o = Lactose

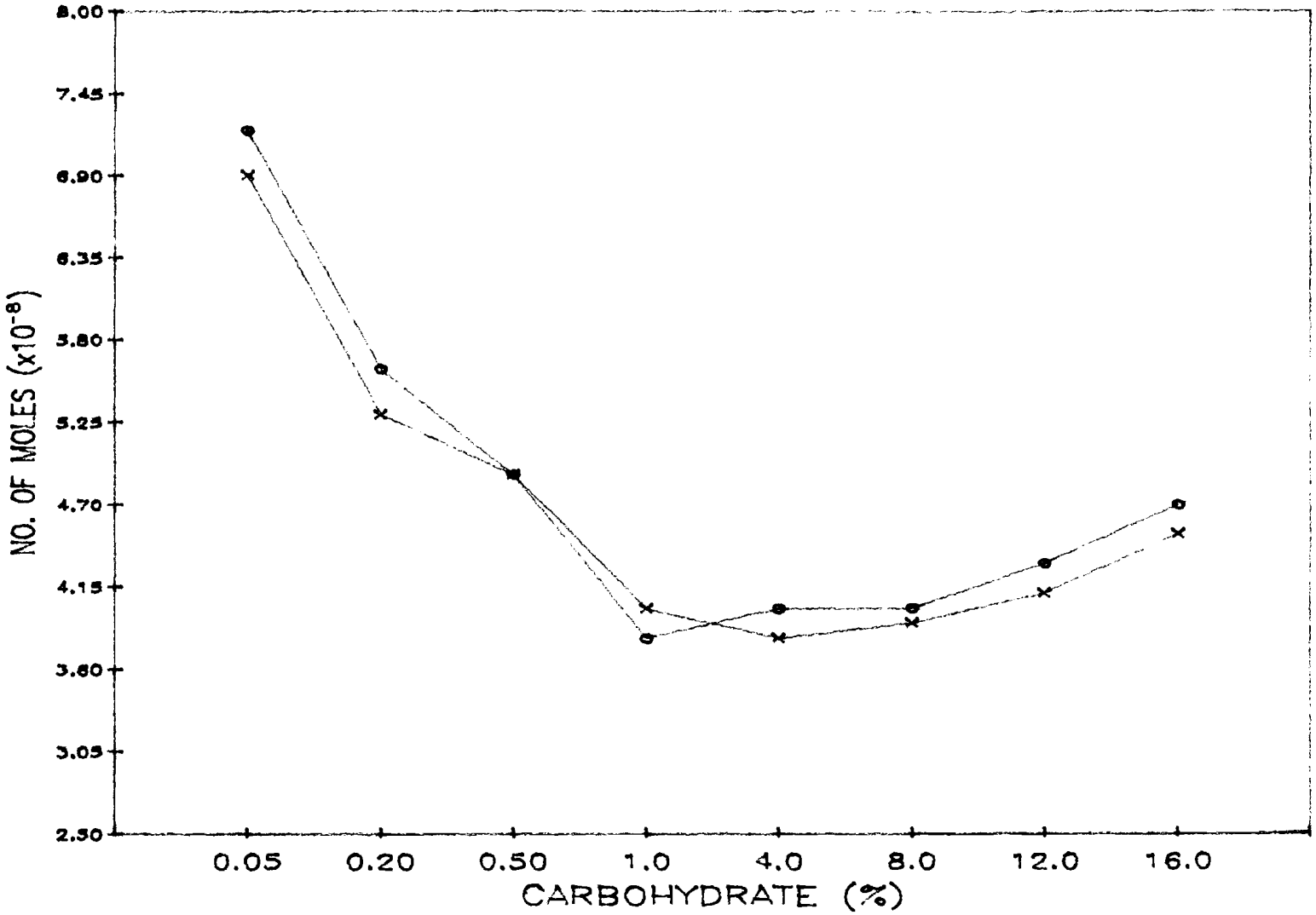

8.00
7.45
6.90
6.35
5.80
5.25
4.70
4.15
3.60
3.05
2.50
NO. OF MOLES (x10⁻⁸)
0.05
0.20
0.50
1.0
4.0
8.0
12.0
16.0
CARBOHYDRATE (%)

Figure 9. Effect of the concentration of carbohydrate polymers (Starch and Dextrin) on hydrogen content of three microliter quantities of the gas mixture produced by E. aerogenes.

Legend:
+ = Starch
□ = Dextrin

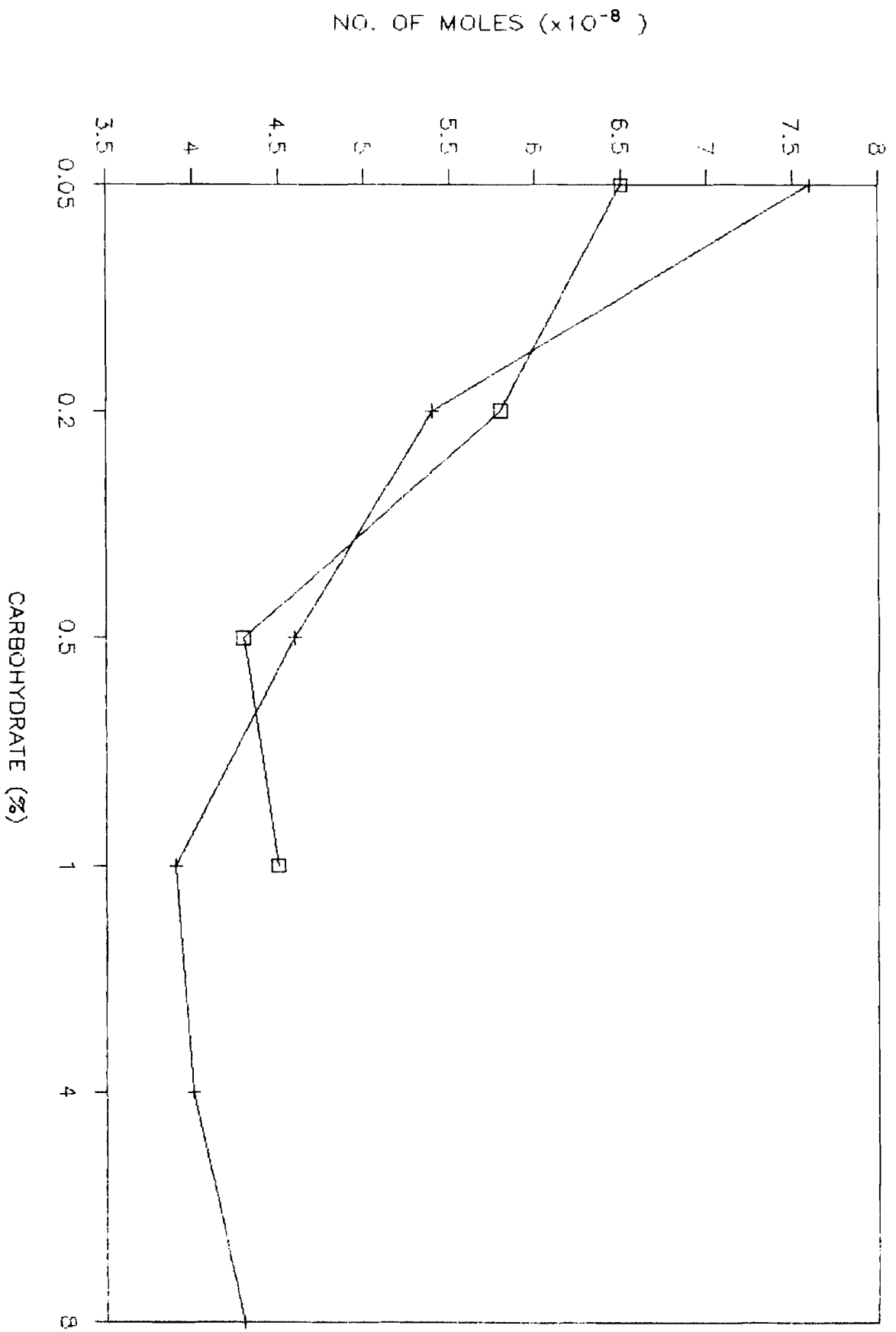

NO. OF MOLES (x10⁻⁸)
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
0.05
0.2
0.5
1
4
8
CARBOHYDRATE (%)

Figure 10. Effect of the concentration of C-5 carbohydrates (Arabinose, Xylose, and Adonitol) on the hydrogen content of three microliter quantities of the gas mixture produced by E. aerogenes.

Legend:

x = Arabinose
o = Xylose
□ = Adonitol

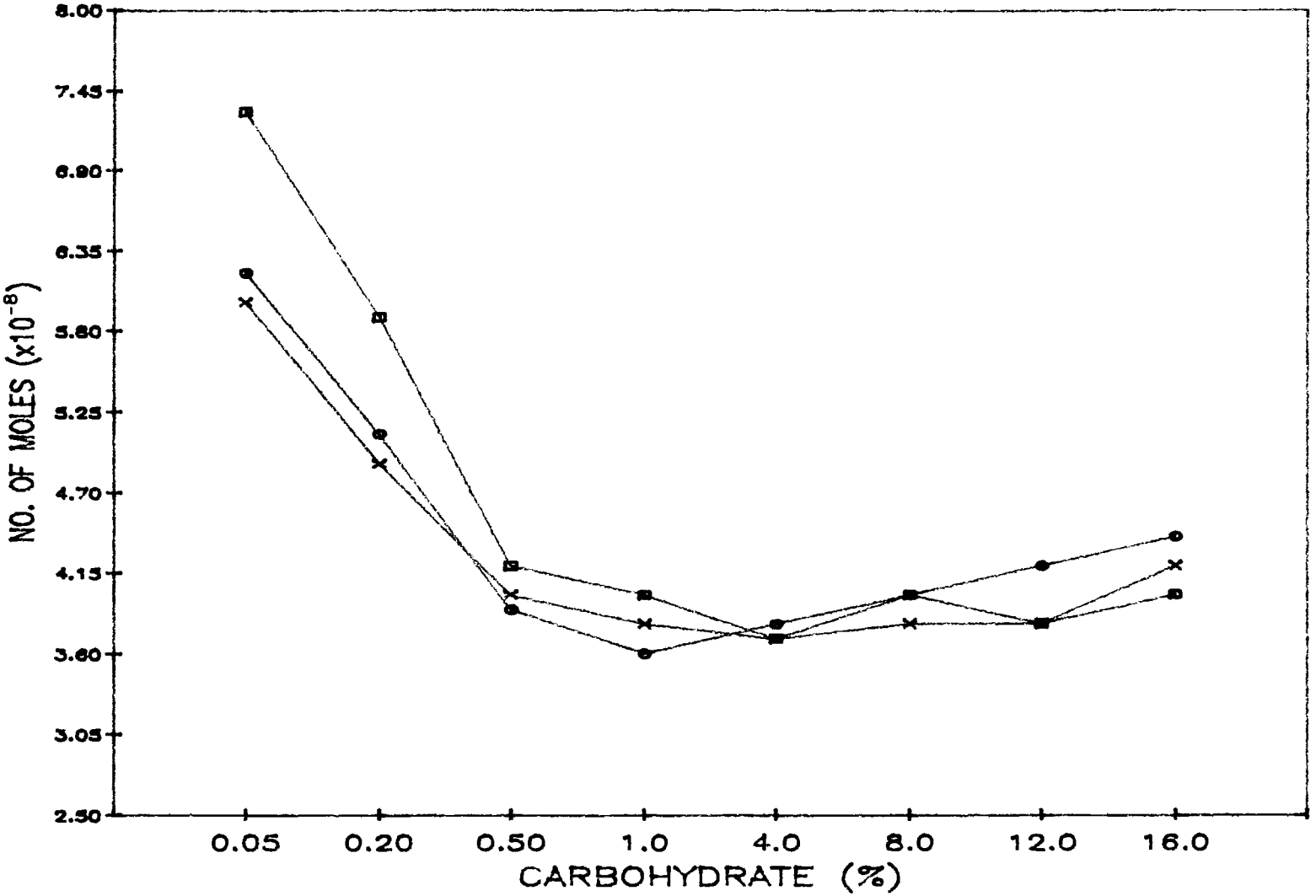

8.00
7.45
6.90
6.35
5.80
5.25
4.70
4.15
3.60
3.05
2.50
NO. OF MOLES (x10⁻⁸)
0.05
0.20
0.50
1.0
4.0
8.0
12.0
16.0
CARBOHYDRATE (%)

Figure 11. Effect of erythrose concentration on the hydrogen content of three microliter quantities of the gas mixture produced by E. aerogenes.

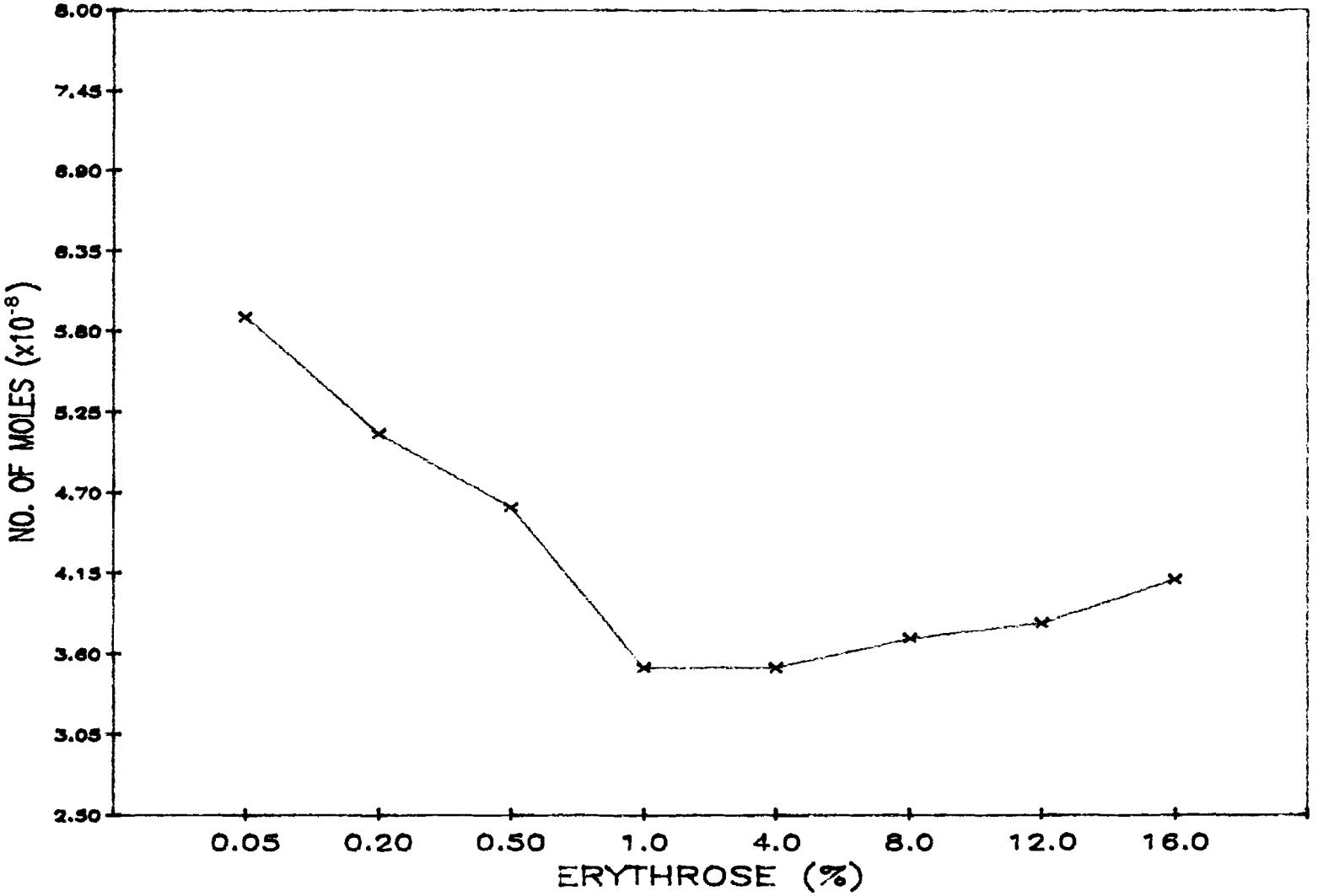

8.00
7.45
6.90
6.35
5.80
5.25
4.70
4.15
3.60
3.05
2.50
NO. OF MOLES (x10-8)
0.05
0.20
0.50
1.0
4.0
8.0
12.0
16.0
ERYTHROSE (%)

Figure 12. Effect of salicin concentration on the hydrogen content of three microliter quantities of the gas mixture produced by E. aerogenes.

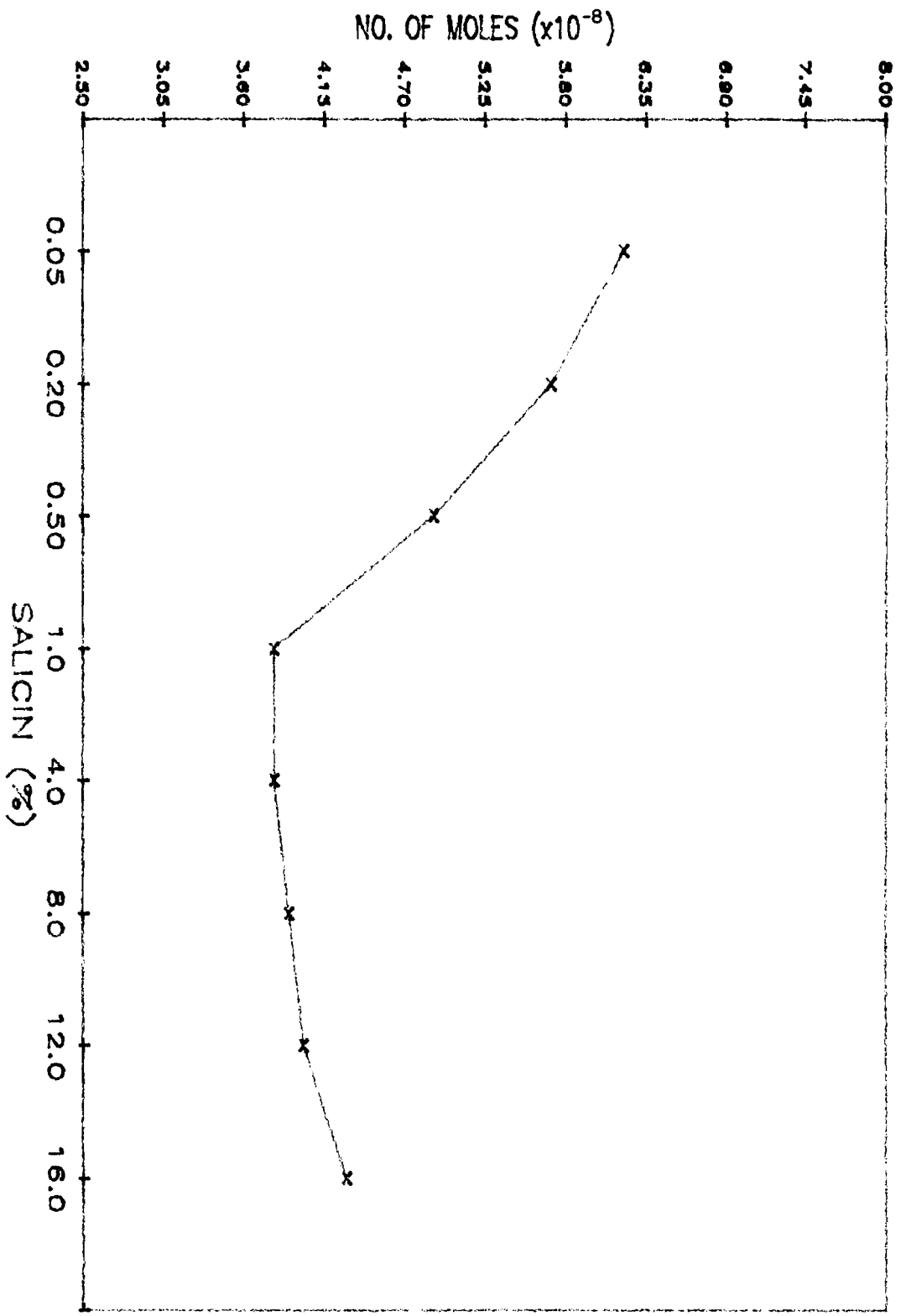
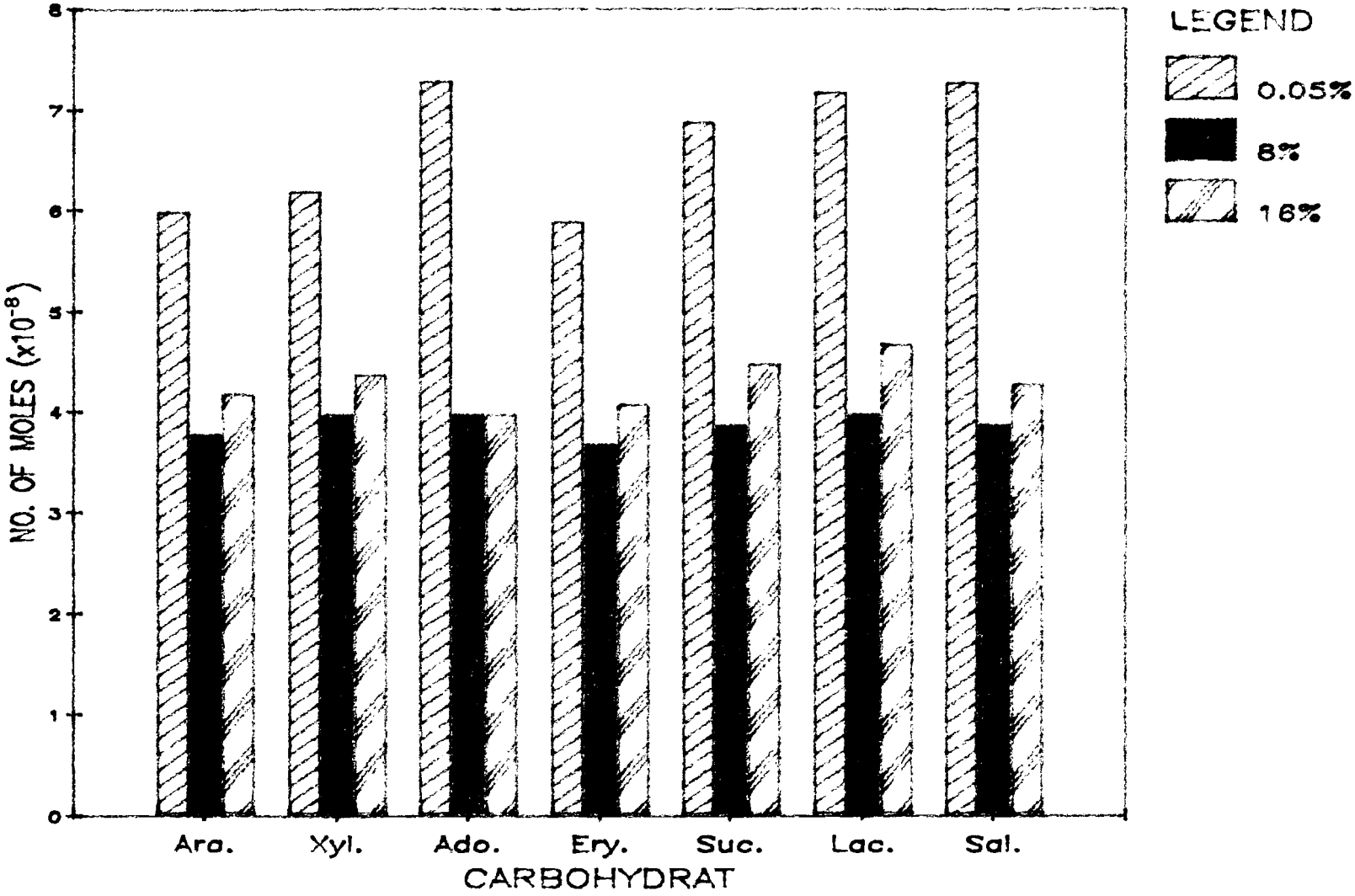

NO. OF MOLES (x10⁻⁸)
2.50
3.05
3.60
4.15
4.70
5.25
5.80
6.35
6.90
7.45
8.00
SALICIN (%)
0.05
0.20
0.50
1.0
4.0
8.0
12.0
16.0

Figure 13. Relationship between carbohydrate concentrations and the hydrogen content of three microliter quantities of the gas mixture produced by E. aerogenes.


LEGEND
0.05%
8%
16%
NO. OF MOLES (x10⁻⁸)
0
1
2
3
4
5
6
7
8
Ara.
Xyl.
Ado.
Ery.
Suc.
Lac.
Sal.
CARBOHYDRAT

acetic, 2-3-butanediol, ethanol and acetylmethylcarbinol), as a result there is a strong competition for electrons among these by-products. Consequently, most of the electrons available in pyruvate are transferred to these by-products.

This leads to a lower yield of molecular hydrogen and a greater yield of carbon dioxide. Most of the carbon dioxide are released by these electron transport reactions leading to the final end products. At high carbohydrate concentrations (12-18 %) the production of hydrogen was slightly increased. This is perhaps due to the osmotic stress exerted on the cells, which leads to a lower carbohydrate uptake, and a higher hydrogen content in the gas mixture.

Identification of the Bacterium

The 8th edition of the Bergy's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974), was used to identify the organism as E. aerogenes (Table 1).

The ecology of the bacterium under study as well as its morphological and biochemical characteristics were used to identify the organism as Enterobacter aerogenes, formerly known as Aerobacter aerogenes. E. aerogenes is a free living saprophyte inhabiting primarily soil and vegetation. In addition the site was not at all remote and could have been contaminated by birds, man and other mammals

Identification of Hydrogen by Gas Chromatography

In gas chromatography, comparison of retention time (the

Table 1. Biochemical tests used for the identification of Enterobacter aerogenes.

BIOCHEMICAL CHARACTERISTICS			
Carbohydrate fermentation *		Other biochemical tests	
Glucose	+	Indole	-
Inositol	+	Methyl red	-
Fructose	+	Voges-Proskauer	+
Galactose	+	Simmon citrate	+
Mannose	+	Hydrogen sulfide	-
Salicin	+	Motility	+
Adonitol	+	Urease	-
Sucrose	+	Phenylalanine deaminase	-
Lactose	+	Gelatinase	-
Starch	+	Potassium cyanide (KCn)	+
Dextrin	+		
Arabinose	+		
Xylose	+		
Erythrose	+		
Sorbitol	+		
Mannitol	+		
Dulcitol	-		
Raffinose	+		

* All positive fermentation tests represent the production of visible gas after 24 hours of incubation at 37 C

time between injection of the sample and the emergence of the peak maximum of that sample) is considered to be the quickest test for compound identity (McNair 1975).

According to Jeffery and Kippling (1972), the retention time of a given gas is based on its density and in general the lower the density of a gas, the shorter is its retention time. Hydrogen has the lowest density of all gases, therefore it is the first component to be detected in a gas mixture. In this study the carrier gas flow rate was adjusted so that hydrogen was eluted through the column with a retention time of 15 seconds.

Samples of the gas mixture produced by E. aerogenes were injected into the precalibrated gas chromatograph. This was done under similar GC operational conditions used to calibrate the instrument with pure hydrogen. The hydrogen component of the gas mixture was detected with the same retention time of 15 sec. The detector used in this gas chromatograph does not respond to the carbon dioxide component of the gas mixture, and carbon dioxide only had a diluting effect on the hydrogen concentration of the gas mixture injected into the gas chromatograph.

Quantitation of the Hydrogen Production by E. aerogenes

Physical and chemical parameters which might affect the quantitative assay of hydrogen were considered. Pure hydrogen was collected over a column of distilled and deionized water at room temperature. In order to minimize the water pressure exerted on the column of hydrogen

(inverted over the water reservoir), the column was filled with hydrogen to just slightly above the water level in the reservoir. Furthermore, in using the ideal gas law Emporia's barometric pressure at the time of the experiment (735 mm Hg) was taken into account to obtain the hydrogen gas pressure (710 mm Hg). Any chemical reaction of hydrogen with water was considered negligible.

The peak height produced upon injection of hydrogen into the GC (Fig. 1) resulted in a non-linear relationship with the quantity of the injected hydrogen, whereas the peak area produced a linear relationship with the quantity of injected hydrogen. Consequently, peak area was used to quantitate the hydrogen content of the gas mixtures injected into the gas chromatograph.

In gas chromatography neither the mobile phase (carrier gas), nor the stationary phase (Propak R and N), would in any way interact with the sample component (Snyder and Kirkland 1977).

Samples of the gas mixture produced by the bacterium at 37 C, were brought to equilibrium with room temperature, and was injected into the precalibrated GC. The flow rate of the carrier gas was the same as that used for pure hydrogen. The acidity of the fermented medium minimizes any chemical reaction with the carbon dioxide component of the gas mixture.

Modified Fermentation Culture Tubes

The open end of the fermentation tubes (Fig. 2) prevented the development of gas pressure in the fermentation tube which would have otherwise blown out the rubber septum or would have drastically affected the quantitation of hydrogen by exertion of pressure on the gas collected in the Durham tube.

Calibration of the Analytes using HPLC

Comparison of the retention time of known compounds with that of the analyte is considered to be a fast, easy and reliable method of compound identification (Engelhardt 1979).

Standard curves for acetylmethylcarbinol as well as lactic, acetic and formic acids were made (Figs. 14-17). In each case there was a linear relationship between peak heights and the UV absorbance at 210 nm. In the analysis of the fermented samples, the absorbance was used as a criterion for quantitating the concentration of the unknown samples (Figs. 19, 20, 23, and 24).

Elution Profiles of the Organic Acids

Unlike gas chromatography, molecular interaction between the sample component molecules and the stationary and moving phases are present in modern liquid chromatography (Snyder and Kirkland 1979). This would provide an additional variable for controlling and improving separation.

The mobile phase selected does not absorb UV light at the

Figure 14. Formic acid standard curve using high-performance liquid chromatography.

Legend:
x = Peak height
o = Absorbance

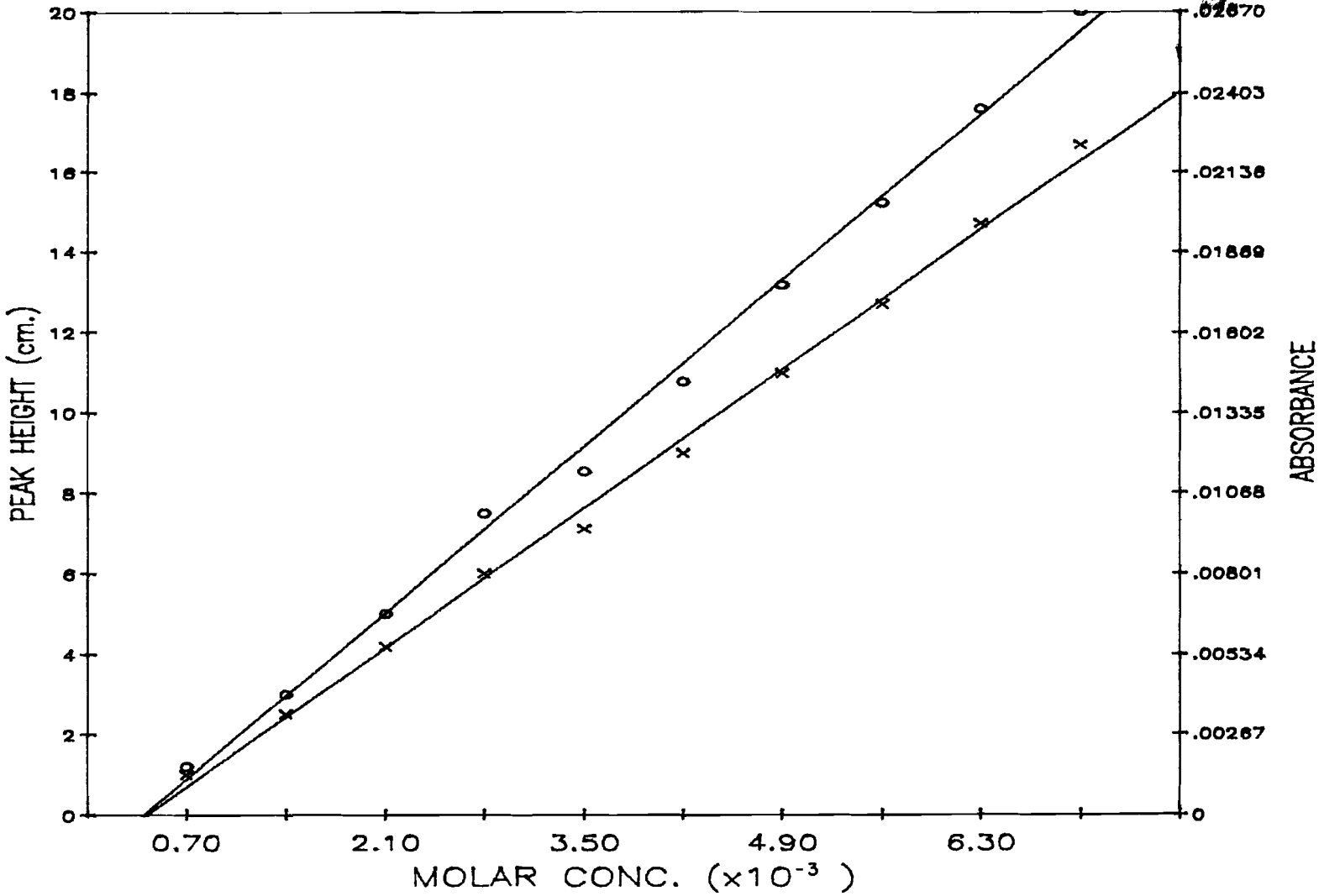

PEAK HEIGHT (cm.)
ABSORBANCE
MOLAR CONC. (x10⁻³)
0.70
2.10
3.50
4.90
6.30
0
2
4
6
8
10
12
14
16
18
20
.00267
.00534
.00801
.01068
.01335
.01602
.01869
.02136
.02403
.02670

Figure 15. Lactic acid standard curve using high-performance liquid chromatography.

Legend:
x = Peak height
o = Absorbance

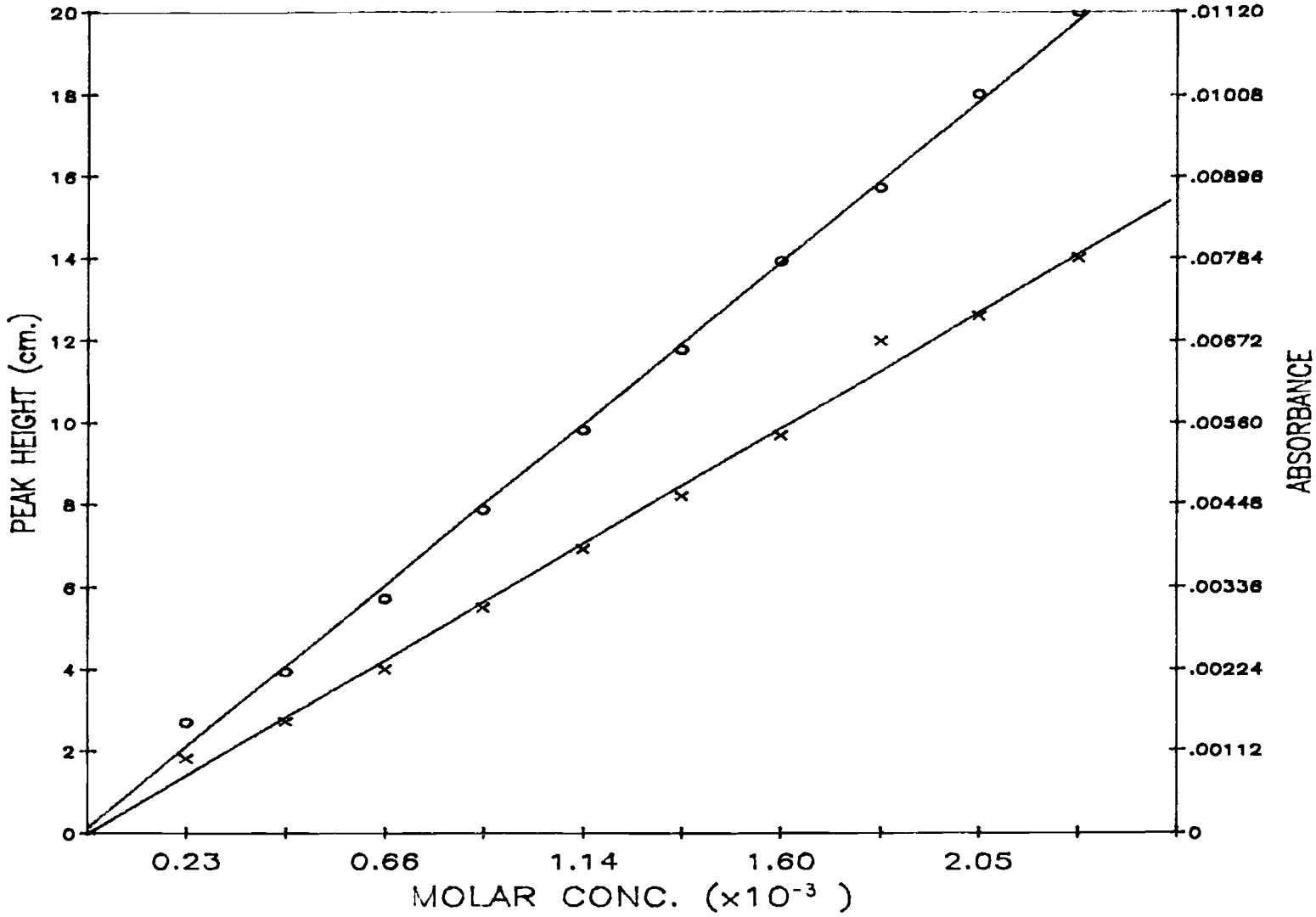

PEAK HEIGHT (cm.)
ABSORBANCE
MOLAR CONC. (x10⁻³)
0
2
4
6
8
10
12
14
16
18
20
.00112
.00224
.00336
.00448
.00560
.00672
.00784
.00896
.01008
.01120
0.23
0.66
1.14
1.60
2.05

Figure 16. Acetic acid standard curve using high-performance liquid chromatography.

Legend:
x = Peak height
o = Absorbance

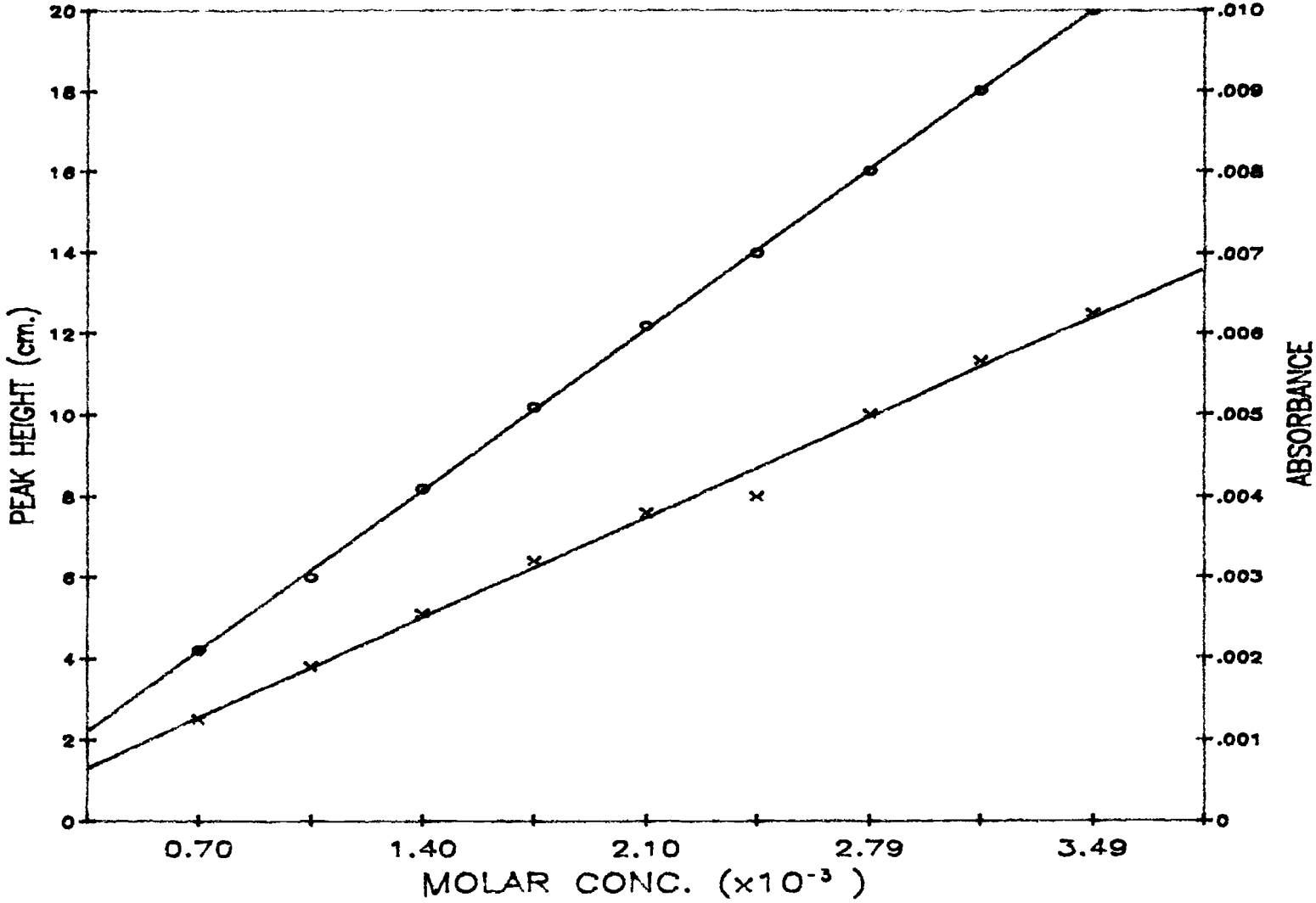

PEAK HEIGHT (cm.)
ABSORBANCE
MOLAR CONC. (x10⁻³)
0.70
1.40
2.10
2.79
3.49
0
2
4
6
8
10
12
14
16
18
20
.001
.002
.003
.004
.005
.006
.007
.008
.009
.010

Figure 17. Acetylmethylcarbinol (acetoin) standard curve using high-performance liquid chromatography.

Legend:
x = Peak height
o = Absorbance

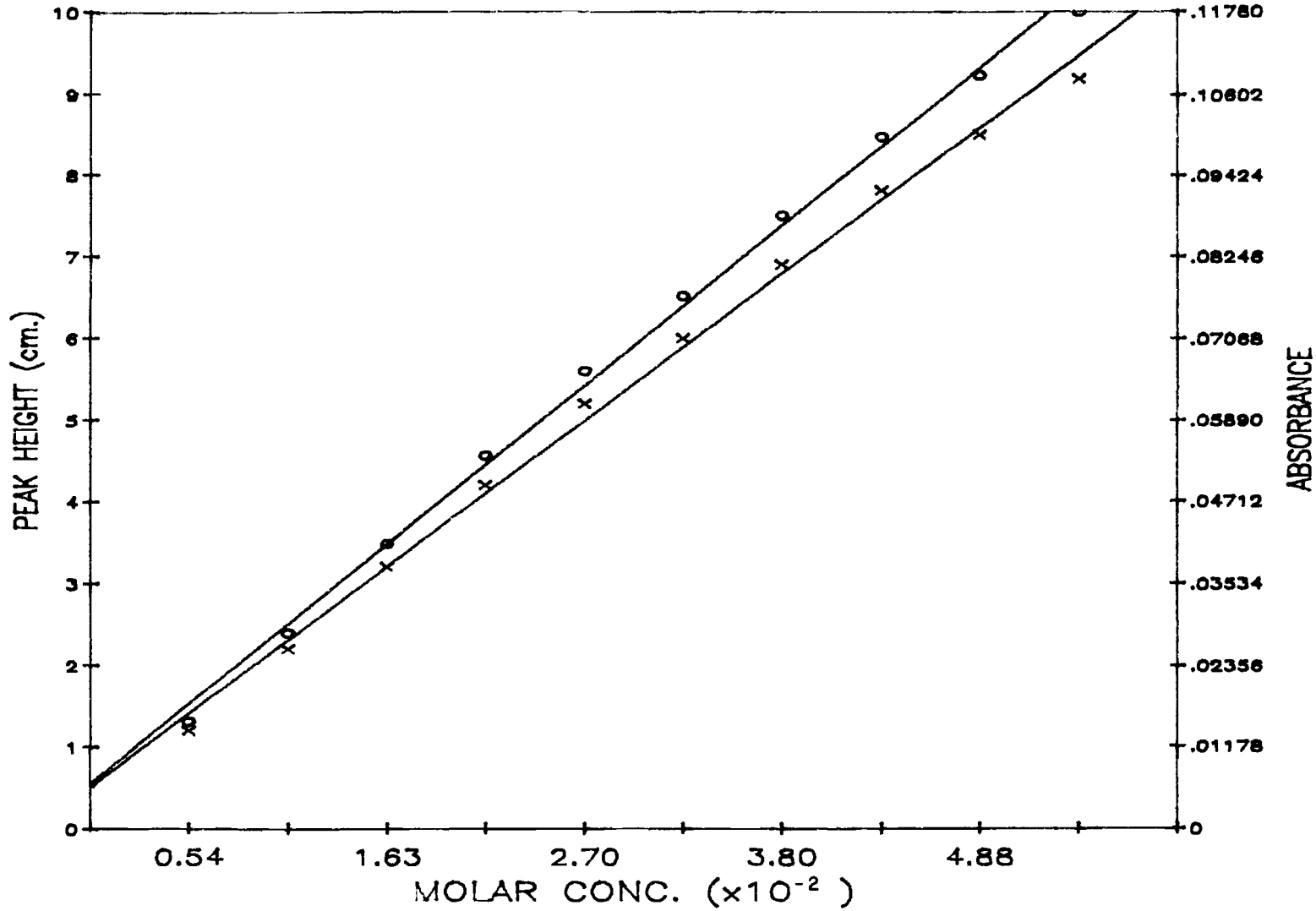

PEAK HEIGHT (cm.)
ABSORBANCE
MOLAR CONC. (x10⁻²)
0.54
1.63
2.70
3.80
4.88
0
1
2
3
4
5
6
7
8
9
10
.01178
.02356
.03534
.04712
.05890
.07068
.08246
.09424
.10602
.11780

Figure 18. High-performance liquid chromatogram of formic, lactic and acetic acids, showing their respective retention times. The figure represents a 26 % reduction in size from the original.

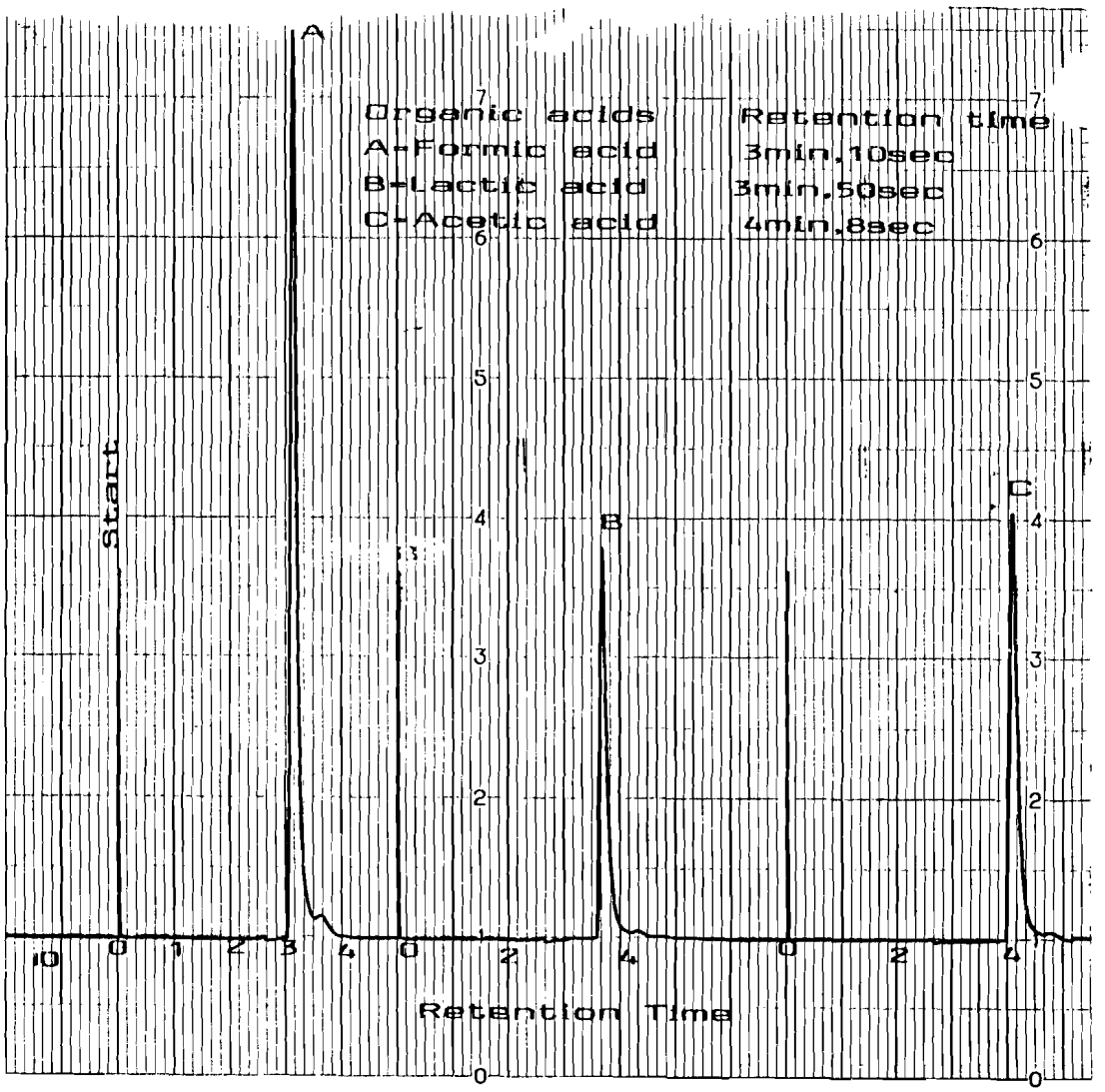

Organic acids
A-Formic acid
B-Lactic acid
C-Acetic acid
Retention time
3min.10sec
3min.50sec
4min.88sec
A
B
C
Start
Retention Time
FISHERbrand® Recordall® Chart Paper
Fisher Scientific Co. Pittsburgh, PA 15219

Figure 19. High-performance liquid chromatogram of fermentation medium, 24 h post inoculation with E. aerogenes. The HPLC range is set at 0.64. The figure represents a 26 % reduction in size from the original.

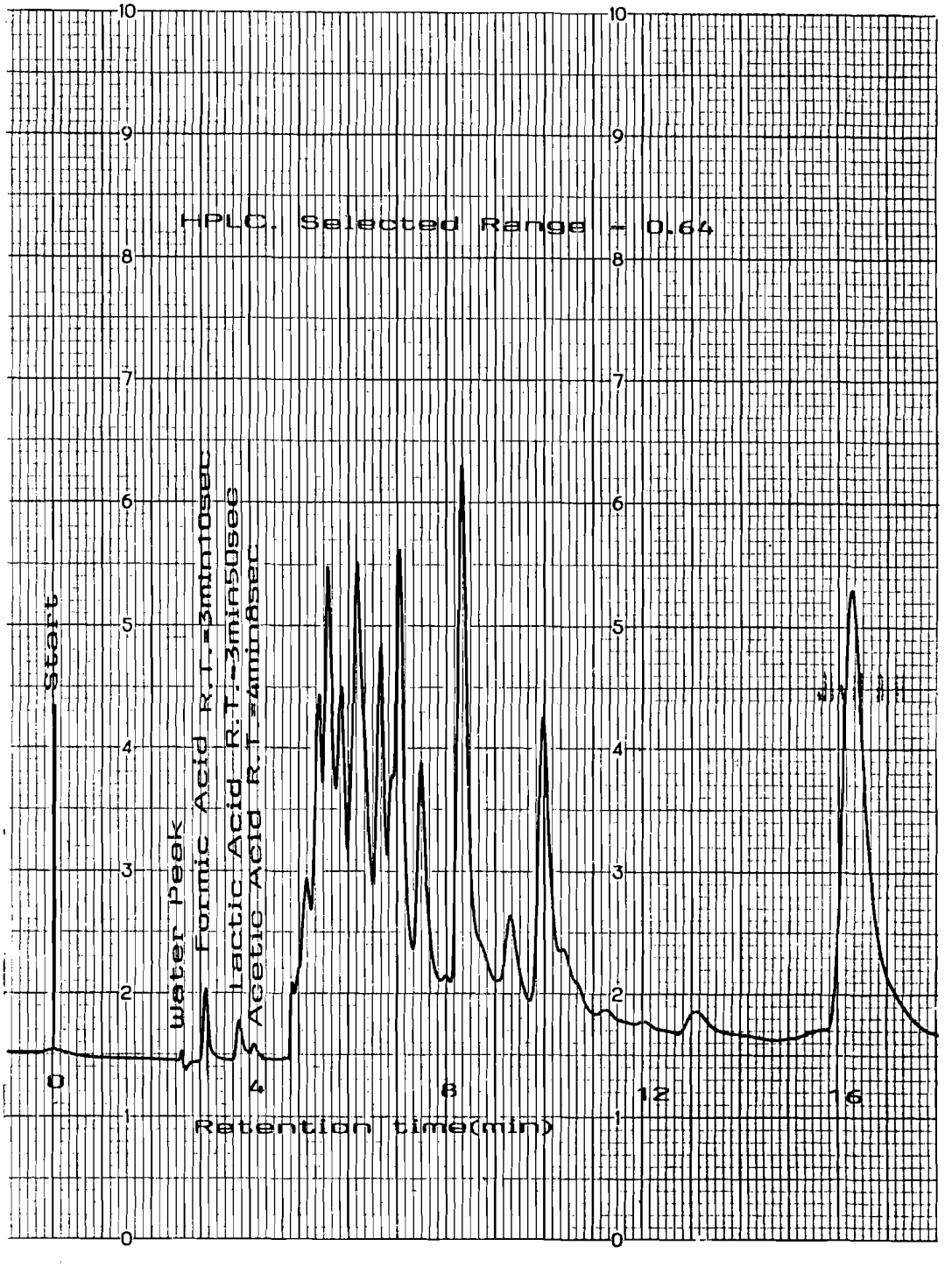
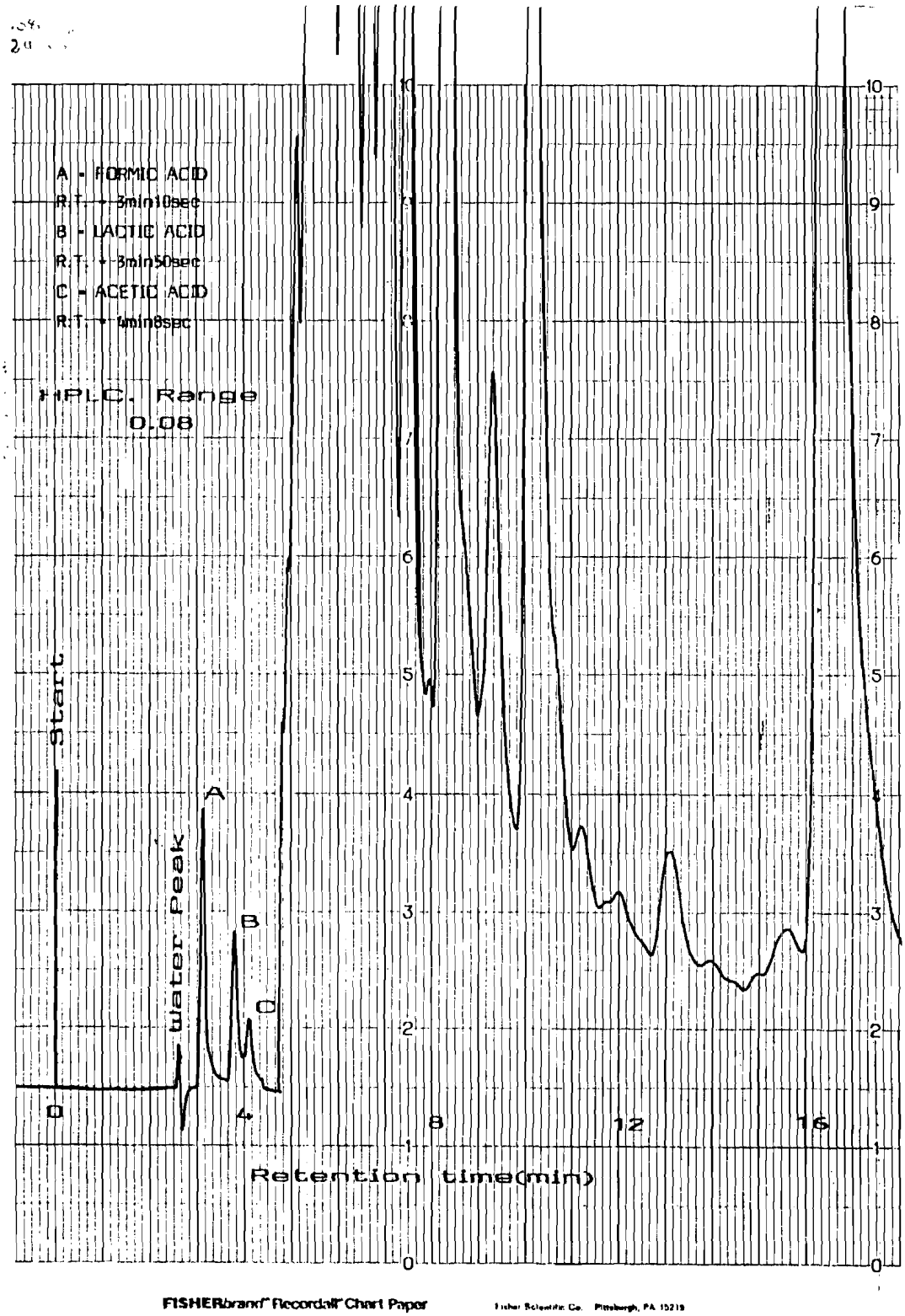


Figure 20. High-performance liquid chromatogram of the fermentation medium, 24 h post inoculation with E. aerogenes. The HPLC range is set at 0.08. The figure represents a 26 % reduction in size from the original.



FISHERbrand Recordall Chart Paper Fisher Scientific Co. Pittsburgh, PA 15219

Figure 21. High-performance liquid chromatogram of the sterile fermentation medium. The HPLC range is set at 0.08. The figure represents a 26 % reduction in size from the original.

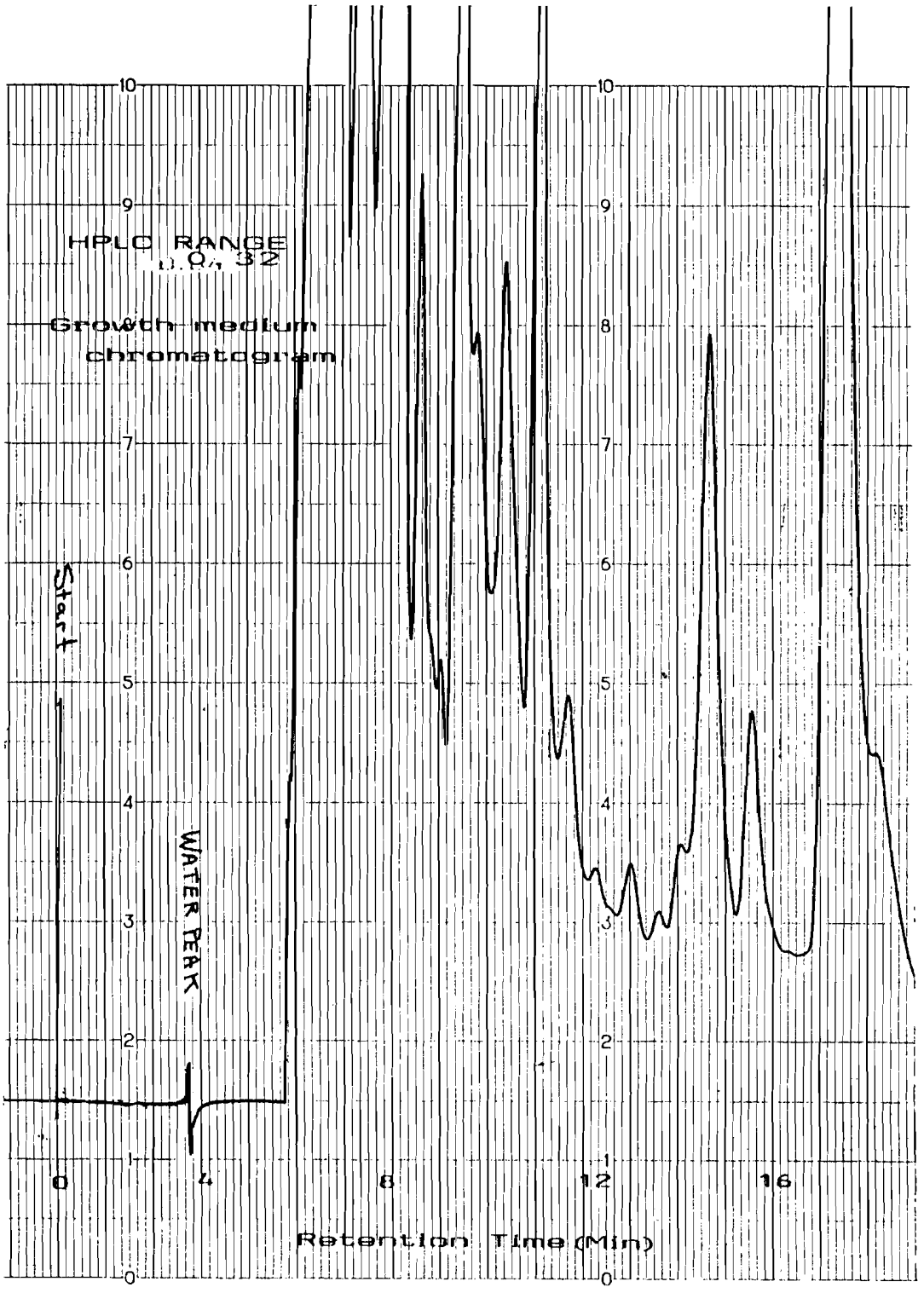


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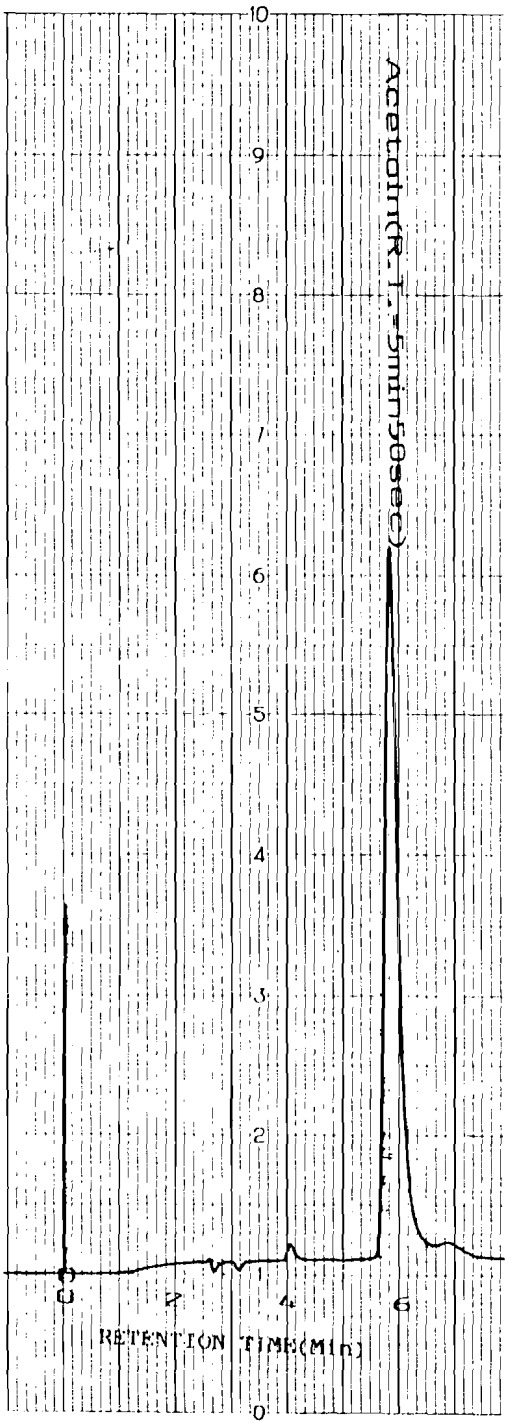
Figure 22. High-performance liquid chromatogram of acetylmetylcarbinol showing its retention time. The figure represents a 26 % reduction in size from the original.



HERbrand" Recordall" Chart Paper Fisher Scientific Co Pitts

Figure 23. High-performance liquid chromatogram of MR-VP medium, 24 h post inoculation with E. aerogenes. Culture condition: aeration-agitation, and 37 C. The HPLC range is set at 0.32. The figure represents a 26 % reduction in size from the original.

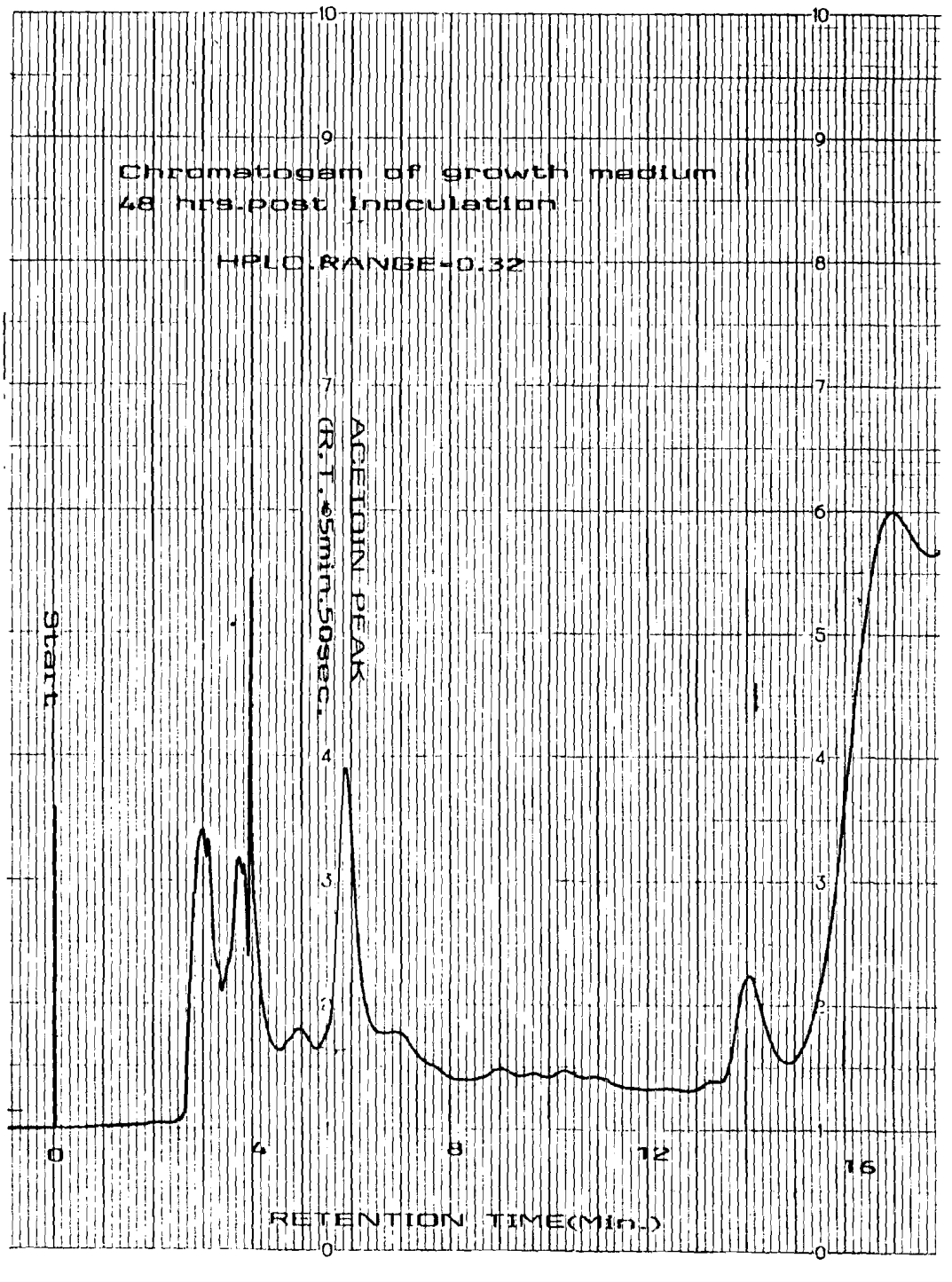
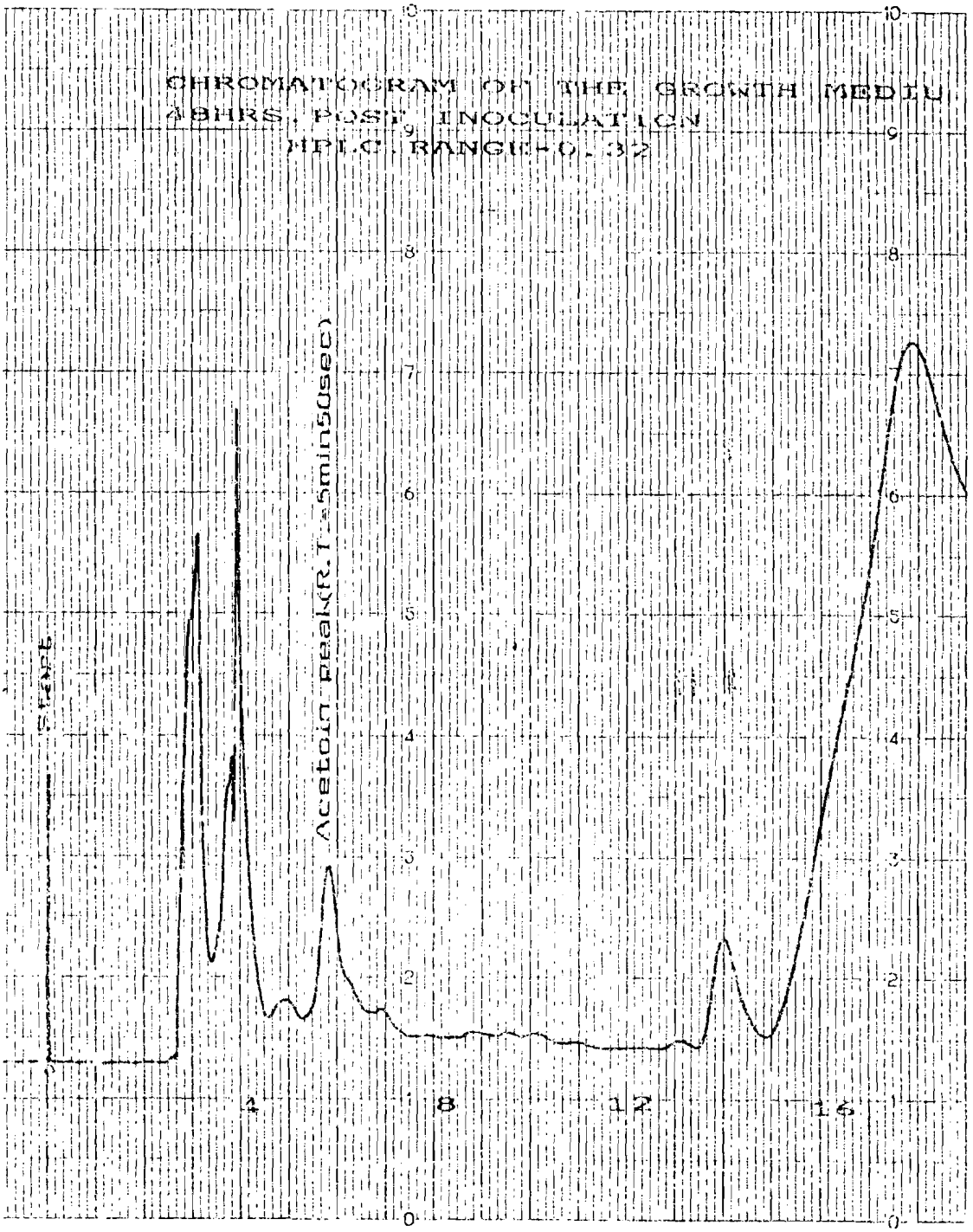


Figure 24. High-performance liquid chromatogram of MR-VP medium, 24 h post inoculation with E. aerogenes, under anaerobic condition, and 37 C. The HPLC range is set at 0.32. The figure represents a 26 % reduction in size from the original.



FISHERBrand Recordall Chart Paper

selected wavelength (210 nm). The HPLC used in this study utilizes a reverse phase system. The stationary phase in the column (C-18 hydrocarbon), was highly non polar and the mobile phase selected was highly polar. The choice for the selection of the mobile phase was based on the polarity of the analytes (solutes). Good separation is the result of molecular interaction between solute and the mobile and stationary phases. There are four types of interactions (dispersion, dipole hydrogen bonding, and dielectric interactions). With this system the dispersion-interaction plays a role in determining the elution profile of the sample component.

In this system the most polar organic molecule is expected to be eluted first. The increasing order of polarity in in the analytes is:

Formic acid > Acetic acid > Lactic acid, based on this formic acid was expected to be eluted first, followed by lactic and acetic acids. The elution profiles (Figs. 18-20), shows formic acid to be eluted first, followed by lactic and then acetic acid. Two explanation for this deviation from the expected pattern can be provided.

1. Dispersion Interaction

This type of interaction between solute and solvent molecules occurs because of the random motion of the electrons in the molecules of solute and solvent, resulting in a momentary asymmetric configuration

in which one part of the molecule becomes negatively charged with respect to the other, leading to its interaction with the polar mobile phase.

2. Dissociation Constant of Organic Acids

Acetic acid has the smallest dissociation constant (1.76×10^{-5}) as compared to lactic and formic acids. This would make acetic acid a much weaker acid as compared to lactic and formic acid. Consequently, it is only partially dissociated in the solvent system used. This would lead to a weaker interaction with the mobile phase and, hence it would appear as the last organic acid in the elution profile (Figs. 18-20).

The increase in the % concentration of phosphate buffer as compared to HPLC grade methanol would cause the mobile phase to be more polar. This and the increase in the hydrogen ion of the mobile phase (2.75) resulted in an increase in the retention times of the analytes, and consequently improved their separation. The chromatogtams also show the water molecules to be eluted prior to any other compound. This is due to the high degree of polarity of the water molecules.

Quantitation of the Organic Acids In the Fermented Medium

Comparison of the retention times of the known organic acids tested were identical to those present in the fermented medium, pointing to their identity (Table 2).

The chromatogram of the sterile fermentation medium

Table 2. HPLC data of the fermentation products present in 10 microliter aliquotes of the fermented medium by E. aerogenes.

Product[a]	Retention time	HPLC range	Absorbance	Peak height (cm)
Formic acid	3min,10sec	0.08	0.01824	5.7
Lactic acid	3min,50sec	0.08	0.00864	2.7
Acetic acid	4min,8sec	0.08	0.00446	1.4
Acetylmethyl-carbinol[b]	5min,50sec	0.32	0.07300	5.7
Acetylmethyl-carbinol	5min,50sec	0.32	0.04090	3.2
Ethanol	Not determined			

[a] Culture condition: anaerobic, 37 C and 24 h.

[b] Culture condition: aeration-agitation , 37 C and 24 h.

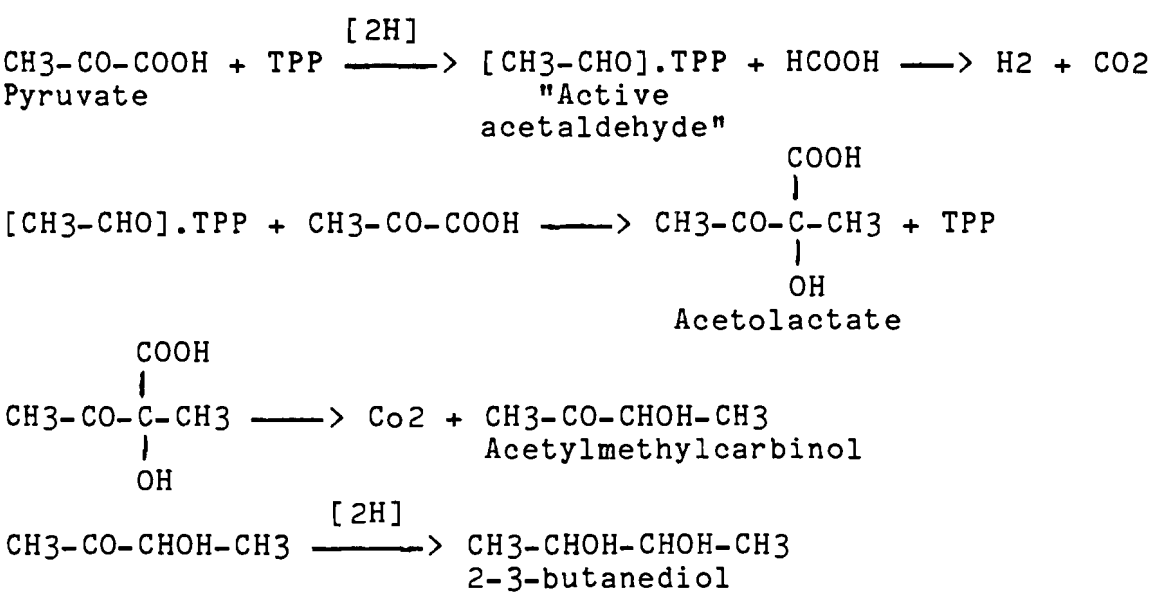
(Fig. 21), shows the absence of peaks with retention time of organic acids. This would serve as a control test for the production of organic acids. The selection of a smaller HPLC range results in the amplification of the detector response, making peak height measurement easier. This would not affect the value of the absorbance of the analyte (Figs. 19 and 20).

The total quantity of the organic acid produced by this method (17.24 mmol/100 mmol of fermented glucose) is slightly lower than the (20.37 mmol/100 mmol of fermented glucose). The quantity of the fermentation by-products are influenced by factors such as pH, culture condition, the strain of the organism under study, substrate concentration as well as the competition for electrons among the organic by-products.

Indirect Analysis of 2-3-butanediol

Direct analysis of fermentation end products such as 2-3-butanediol and ethanol would require the use of a gas chromatograph, equipped with detectors that would respond to volatile compounds present in the liquid phase.

The biochemical conversion of acetylmethylcarbinol to 2-3-butanediol was used as the basis for indirect analysis of 2-3-butanediol. Production of acetylmethylcarbinol is the basis for the positive Voges-Proskauer reaction in E. aerogenes.



Thiamine pyrophosphate (TPP) is the coenzyme participating in the formation of active acetaldehyde, which condenses with asecond pyruvate. The product is converted to 2-3-butanediol. The total of 4 H absorbed are produced in forming the two pyruvates (Davis et al. 1973; Doelle 1975).

The HPLC chromatogram of acetylmethylcarbinol (Fig. 22), shows a retention time of five minutes and 50 seconds. This retention time is the same as the retention time obtained from the acetylmethylcarbinol present in the fermented medium (Fig's 23, and 24).

using this indirect approach, the quantity of 2-3-butanediol obtained under anaerobic condition (Table 3) is nearly one half of the amount established when older methods of analysis was used for the direct quantitation of

Table 3. Fermentation products (mmol/100 mmol of fermented glucose), of E. aerogenes.

Product[a]	mmol/100 mmol glucose fermented
Formic acid	10.64
Lactic acid	3.57
Acetic acid	3.06
2-3-butanediol[b]	61.60
2-3-butanediol	34.60
Carbon dioxide	168.4
Hydrogen	34.0
Ethanol	Not determined

[a] Culture condition: anaerobic, 37 C and 24 h.

[b] Culture condition: aeration-agitation, 37 C and 24 h.

2-3-butanediol. This is to be expected because exposure to air oxidizes some of the 2-3-butanediol to acetylmethylcarbinol (Davis et al. 1973). Therefore, the quantity of 2-3-butanediol obtained represents only that portion of 2-3-butanediol converted to acetylmethylcarbinol. The production of 2-3 butanediol under the condition of aeration-agitation was twice that produced under anaerobic condition (Table 3).

Sablayrolles and Goma (1984), using a a Hewlett-Packard chromatograph, fitted with a flame ionization detector measured the 2-3-butanediol production by E. aerogenes and established that aeration-agitation significantly increased the production of 2-3-butanediol. At a glucose concentration of 19.5 %, and under the condition of aeration and agitation, they obtained a 2-3-butanediol yield of 87.75 grams (0.45 gram of 2-3-butanediol per gram of glucose fermented). Under anaerobic condition this high substrate concentration resulted in cell lysis.

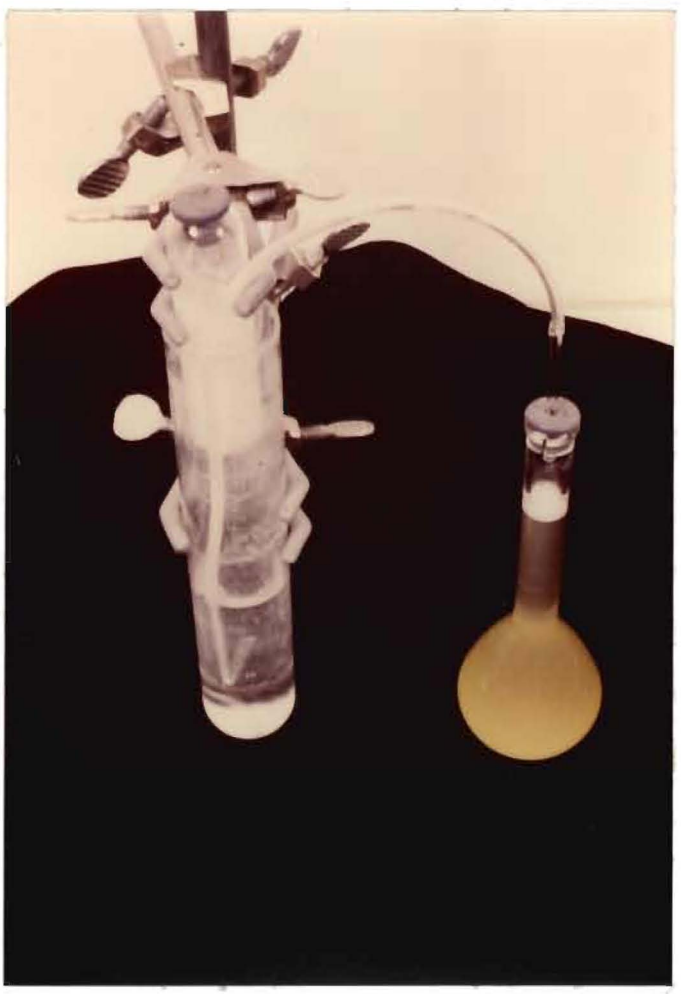
Quantitative Analysis of the Gas Mixture

1. Carbon dioxide

Measures were taken to minimize the reaction between carbon dioxide present in air and distilled water with the barium hydroxide solution in the alkali-tower (Fig. 25).

Barium carbonate produced was a measure of the the quantity of carbon dioxide present in the gas mixture produced by E. aerogenes (Appendix 3).

Figure 25. Apparatus for quantitating carbon dioxide produced by E. aeogenes.



2. Hydrogen

A two microliter injection of the non-reacted portion of the carbon dioxide free gas (hydrogen), collected on the top of the alkali tower (Fig. 25) resulted in much greater peak area than the injection of three microliter of the gas mixture, and was equivalent to the injection of 1.8 microliter of pure hydrogen. This result would indicate the sensitivity of the gas chromatograph and the reliability of the obtained gas chromatographic results. However, it was not possible to measure the total volume of the hydrogen trapped in the alkali tower.

In order to quantitate the total quantity of the hydrogen produced by E. aerogenes, the gas mixture was collected over a column of distilled and deionized water, at pH 7. Under this condition any reaction of the components of the gas mixture with water was considered to be negligible.

The quantity of hydrogen was calculated from the total volume of the gas mixture produced and the quantity of carbon dioxide present in the gas mixture (Appendix 3).

Mickelson and Wreekman (1938) reported a H_2/CO_2 ratio of 1:5, thereby refuting the long accepted ratio of 1:2, for E. aerogenes (Wood 1961). In this study the H_2/CO_2 ratio obtained was 1:4.93.

In this study an increase in the hydronium ion concentration of the fermentation medium resulted in an increase in hydrogen production (Fig. 26), thus indicating that hydronium ion concentration had increased the activity of formic hydrogen lyase enzyme system.

Fermentation studies by Blackwood et al. (1956), on E. coli established that at pH 6.2, the production of hydrogen was considerably higher (75 mmol/100 mmol of glucose fermented) than at pH 7.8 (26 mmol/100 mmol of glucose fermented). In the same study the production of formic acid at the pH of 6.2 was significantly lower (2.43 mmol/100 mmol of glucose) than that produced at the pH of 7.8 (86.0 mmol/100 mmol of glucose fermented). Their result established that the activity of formic hydrogen lyase enzyme system is significantly increased with an increase in the concentration of hydronium ion.

Nuclear Magnetic Resonance Spectroscopy (NMR)

In order to determine if any volatile organic compounds were present in the gas mixture NMR spectroscopy was used. The NMR spectrum (Fig. 27), revealed the absence of any peak representing an organic compound. The only evident peak was that of silicon which was likely present because of the use of the silicon coated test tube used to dissolve the gas mixture in carbon tetrachloride.

This study revealed that that parameters such as the culture conditions, pH, and the concentration of the carbon

Figure 26. The effect of hydronium ion concentration on hydrogen content of three microliter quantities of the gas mixture produced by E. aerogenes, grown in phenol red broth and supplemented with 0.5 % glucose.

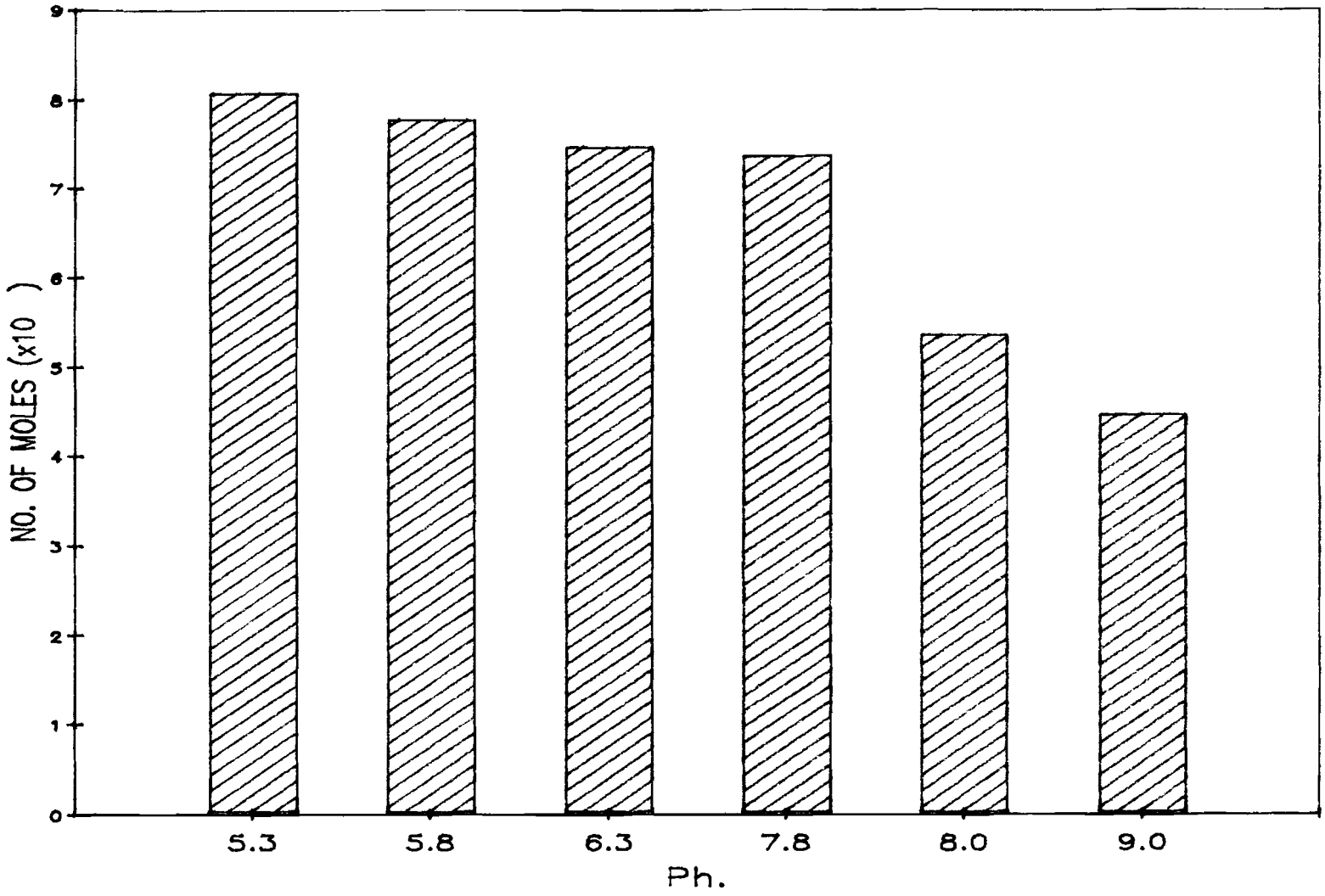
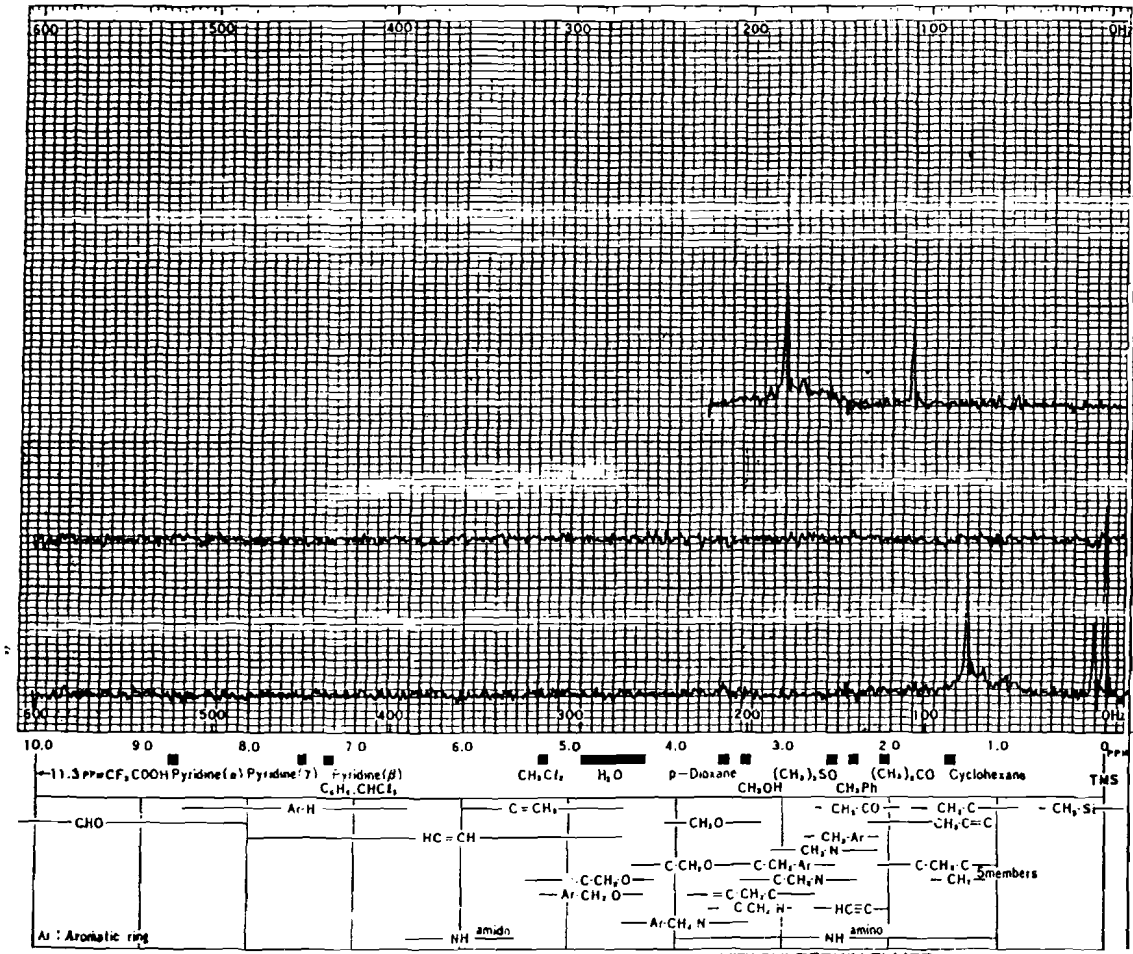

NO. OF MOLES (x10)
0
1
2
3
4
5
6
7
8
9
5.3
5.8
6.3
7.8
8.0
9.0
Ph.

Figure 27. Nuclear magnetic resonance spectrum of the gas mixture produced by E. aerogenes, dissolved in carbon tetrachloride.



'H SPECTRUM NO. :

SAMPLE : Bacterial Gas

REFERENCE : TMS

SOLVENT : CCl_4

CONC :

AMPLITUDE :

SPECTRUM : 15

INTEGRAL : 15

H_1 LEVEL :

H_2 LEVEL :

GAIN :

SWEEP WIDTH :

FILTER Hz :

SWEEP TIME :

☐ 300/150SEC ☑ 1501 SEC

DATE : 10-8-17

OPERATOR : WFF

REMARKS :

Middle line is 10-20S
Top line is 100 Hz
offset ←

CHART NO. 436-7808

source can influence the final quantity of different fermentation products. The results presented in table 3 are based on the fermentation of five mmol of glucose. The fermentation of 100 mmol of glucose (18.016 gr) would require a much longer incubation time, and a much larger fermentation vessel.

SUMMARY

Enterobacter aerogenes was isolated from soil and subjected to fermentation studies and analysis of its fermentation products using modern methods of product analysis.

E. aerogenes was grown in phenol red broth supplemented with varied concentrations of of C-4, C-5, C-6 and C-12 carbohydrates. Gas chromatographic analysis of the hydrogen content in three microliter quantities of the gas mixture produced by E. aerogenes was performed. At the lowest concentration of carbohydrate (0.05 %), the hydrogen content was at its highest level regardless of the chemical composition of the fermented carbohydrate. Higher concentrations of carbohydrates resulted in an increase in the production of the gas mixture but with a lower hydrogen content. Increasing the hydronium ion concentration (pH 5.3), also resulted in an increase in the production of hydrogen.

Major fermentation end products of E. aerogenes were quantitated by growing the bacterium in 100 ml phenol red broth, supplemented with five millimoles of glucose (0.05 M solution). The medium was inoculated with $1x10^{8}$ cells of E. aerogenes.

Chemical and physical means of analysis of carbon dioxide and hydrogen content of the gas mixture revealed a H2 : CO2 ratio of 1:4.93

Formic, lactic and acetic acids were quantitated directly using high-performance liquid chromatography. This technique was also used to indirectly quantitate the production of 2-3-butanediol by E. aerogenes grown under aerobic as well under the condition of aeration-agitation.

The total quantity of organic acids obtained by this method (17.24 mmol/100 mmol of fermented glucose) is slightly lower than the 20.37 mMoles obtained by other investigators.

Aeration-agitation of the fermenting culture resulted in a two-fold increase in the production of 2-3-butanediol.

The techniques of gas chromatography and high-performance liquid chromatography are fast, accurate and sensitive methods of product analysis.

Carbohydrate assays to quantitate the amount of non metabolized carbon source used in the fermentation process will be useful in assessing the fermentation rate.

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LITERATURE CITED

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14
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APPENDIX

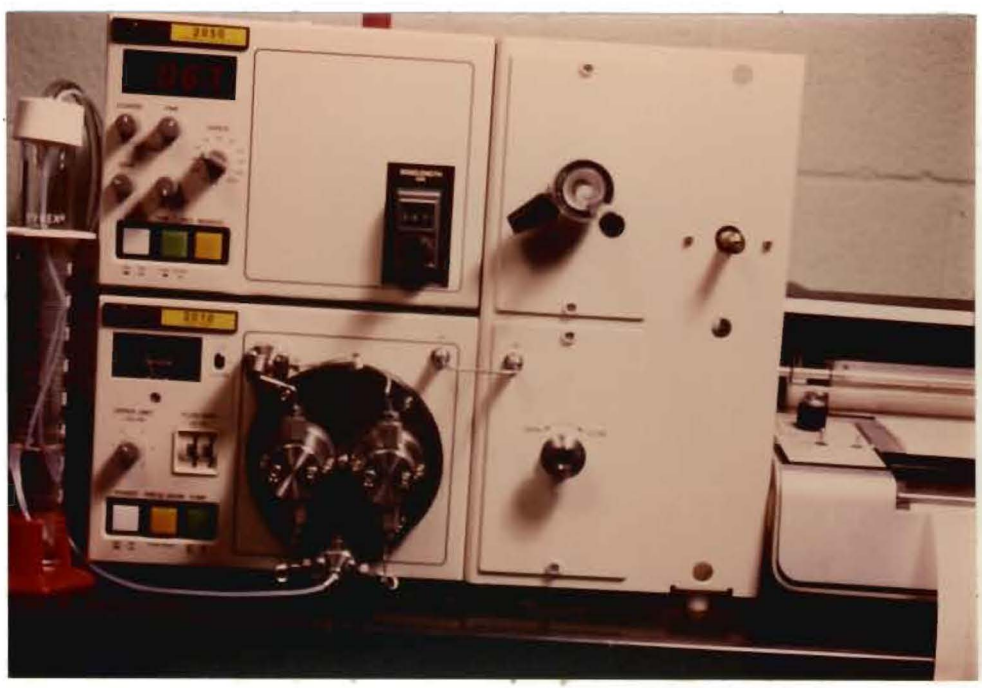
APPENDIX 1

Photograph of the Study Site

Photograph of the gas-chromatograph, showing the the main body, the carrier gas cylinder, the power supply, and the recorder.


automotive
maintenance free

Photograph of the High-performance liquid chromatograph (HPLC).



APPENDIX 2

Preparation of Molar Concentration of Formic Acid

Formic Acid:

Molecular Weight = 46.03 gr/mol

Density = 1.22 gr/ml

Purity = 88 %

A 0.03 % stock solution of formic acid was made and labelled as Soln. A.

9 ml. of soln. A + 1 ml. of distilled water = 0.027 %

8 ml. of soln. A + 2 ml. of distilled water = 0.024 %

7 ml. of soln. A + 3 ml. of distilled water = 0.021 %

6 ml. of soln. A + 4 ml. of distilled water = 0.018 %

5 ml. of soln. A + 5 ml. of distilled water = 0.015 %

4 ml. of soln. A + 6 ml. of distilled water = 0.012 %

3 ml. of soln. A + 7 ml. of distilled water = 0.009 %

2 ml. of soln. A + 8 ml. of distilled water = 0.006 %

1 ml. of soln. A + 9 ml. of distilled water = 0.003 %

The above percentage solutions were converted to their corresponding molar concentrations.

Example:

0.03 % soln. of formic acid

$0.03 \text{ gr} \times 1.22 \text{ gr/ml} \times 0.88 \text{ gr} = 3.22 \times 10^{-2}$ gram.

3.22×10^{-2} gr./46.03 gr mol $= 7 \times 10^{-4}$ mol

7×10^{-4} mol/0.1 l $= 7 \times 10^{-3}$ mol/l

Formic Acid Standard, Chromatographic Data

Molarity ($\times 10^{-3}$)	Absorbance	Peak Height (cm)
7.0	0.0267	16.7
6.3	0.0235	14.7
5.6	0.0203	12.7
4.9	0.0176	11.0
4.2	0.0144	9.0
3.5	0.0114	7.1
2.8	0.0096	6.0
2.5	0.0067	4.2
1.4	0.0040	2.5
0.7	0.0016	1.0

Preparation of Molar Conentrationc of Acetic Acid

Procedure follows that of formic acid except that the initial stock solution. was 0.02 %

Acetic Acid:

Molecular Weight = 60.05 gr/mol

Density = 1.0492 gr/ml

Purity = 99.8 %

Acetic Acid Standard, Chromatographic Data

Molarity ($\times 10^{-3}$)	Absorbance	Peak Height (cm)
3.49	0.0100	12.5
3.14	0.0090	11.3
2.79	0.0080	10.0
2.45	0.0070	8.0
2.10	0.0061	7.6
1.75	0.0051	6.4
1.40	0.0041	5.1
1.05	0.0030	3.8
0.70	0.0021	2.5

Preparation of Molar Concentration of Lactic Acid

Procedure follows as before. (Initial stock solution is 0.02 %).

Lactic Acid:

Molecular Weight = 90.08 gr/mol

Density = 1.206 gr/ml

Purity = 85 %

Lactic Acid Standard, Chromatographic Data

Molarity ($\times 10^{-3}$)	Absorbance	Peak Height (cm)
2.28	0.0112	14.0
2.05	0.0101	12.6
1.82	0.0088	12.0
1.60	0.0078	9.7
1.36	0.0066	8.2
1.14	0.0055	6.9
0.91	0.0044	5.5
0.66	0.0032	4.0
0.45	0.0022	2.7
0.23	0.0015	1.8

Preparation of Molar Concentration of Acetylmethylcarbinol

Procedure follows as before. (Initial stock solutionn is 0.5 %)

Acetylmethylcarbinol:

Molecular Weight = 88.12 gr/mol

Density = 1.0272 gr/ml

Purity = 93 %

Acetylmeyhylcarbinol Standard, Chromatographic Data

Molarity ($x10^{-2}$)	Absorbance	Peak Height (cm)
5.42	0.1178	9.2
4.88	0.1088	8.5
4.34	0.0998	7.8
3.80	0.0883	6.9
3.25	0.0768	6.0
2.70	0.0660	5.2
2.17	0.0538	4.2
1.63	0.0410	3.2
1.08	0.0282	2.2
0.54	0.0154	1.2

Hydrogen Sandard, Chromatographic Data

Vol. of hydrogen (Microliter)	No. of moles ($\times 10^{-8}$)	Peak Area (cm^2)	Peak Height (cm)
2.2	8.40	41.9	25.4
2.0	7.64	35.6	25.0
1.8	6.87	34.5	23.8
1.6	6.11	30.3	23.1
1.4	5.34	26.6	20.4
1.2	4.58	23.1	18.7
1.0	3.82	18.7	15.0
0.8	3.05	14.9	12.7
0.6	2.29	11.2	9.6
0.4	1.53	7.5	6.2
0.2	0.76	3.6	2.7

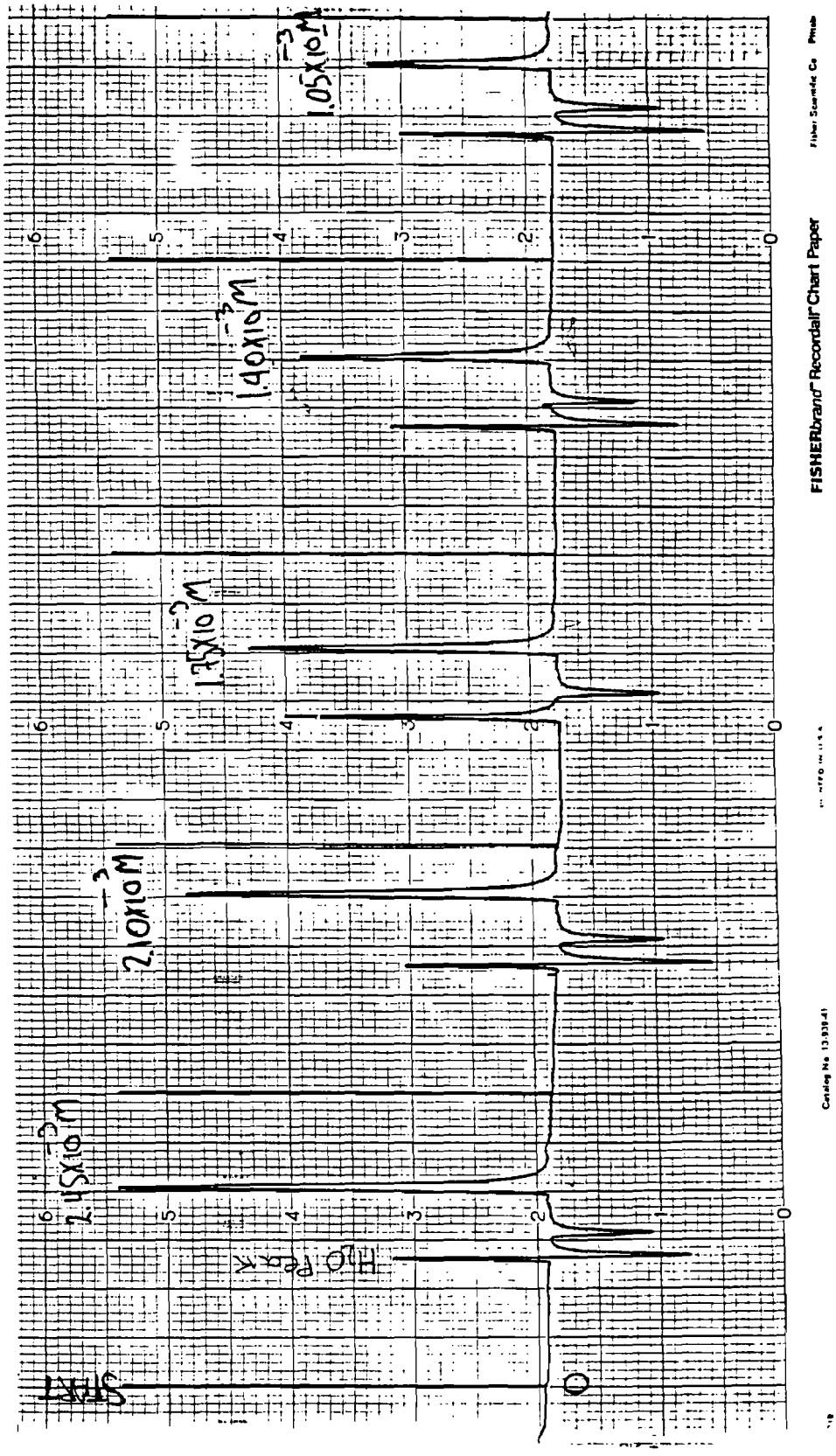
Example:

2.2 Microliter

$$(0.9342 \text{ atm.})(2.2\times10^{-6}) = n\ (0.0821 \text{ l.atm/mol. K})(298 \text{ K})$$

n = No. of moles of hydrogen = 8.4×10^{-8}

Chromatogram of acetic acid standard,showing the linaer relationship between sample concentrations and those of peak heights and absorbancies. The figure represents a 26 % reduction in size from the original.


START
H_2O_2 added
7.15×10^{-3} M
2.10×10^{-3} M
1.75×10^{-3} M
1.40×10^{-3} M
1.05×10^{-3} M
FISHERBrand Recordall Chart Paper
Fisher Scientific Co
Catalog No 13-939-41

Calculation of the Absorbance

$$\frac{\text{Sample peak height (cm)}}{\text{Length of the chromatographic paper (25cm)}} \times \text{HPLC range}$$

Example:

At acetic acid molar concentration of 2.45×10^{-3}

$$\text{Absorbance} = \frac{8 \text{ cm}}{25 \text{ cm}} \times 0.02 = 0.0070$$

APPENDIX 3

Calculation of H2 and CO2 from the Obtained Data

$Ba(OH)_2 + CO_2 \longrightarrow BaCO_3 + H_2O$

Constant weight of barium carbonate = 1.6611 grams.

1.6611 gr/197.34 gr. mol^{-1} = 0.00842 mol of barium carbonate, equivalent to 0.00842 mol of carbon dioxide.

0.00842 mol = 8.42 millimoles.

Hence, five millimoles of glucose when fermented yields 8.42 millimoles of carbon dioxide.

Under ideal condition 100 millimoles of glucose would yield 168.4 mol of carbon dioxide.

Total volume of gas mixture collected = 264 ml.

$$P.V. = n.R.T.$$

(0.9342 atm)(0.264 l) = n (0.0821 l.atm/mol. K)(298 K)

n = Total number of moles of carbon dioxide and hydrogen = 0.0101 mol. 8.42×10^{-3} mol of this is carbon dioxide and 1.7×10^{-3} moles of it is hydrogen. In other words of the 264 ml total gas mixture 44.5 ml is hydrogen and 219.5 ml is carbon dioxide. Hence; five mMoles of glucose when fermented yields 1.7 millimoles of hydrogen. Under controlled fermentation condition one would expect a yield of 34 millimoles of hydrogen.