LACTIC ACID UTILIZATION BY STREPTOCOCCAL STRAIN AHT

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by

Gerard Shao-Sheng Hu

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ABSTRACT

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Under conditions of a high cell concentration and a low glucose substrate level, lactic acid was formed and then degraded by Streptococcus AHT. The lactic acid was converted to acetic acid, ethanol and carbon dioxide.

Lactic acid utilization was greatly suppressed by anaerobic incubation indicating that oxygen was required for the process. The end products of glucose fermentation under anaerobic incubation were lactic, acetic, butyric, isobutyric, propionic acids, ethanol and carbon dioxide.

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December 1968

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

CHAPTER I

INTRODUCTION

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INTRODUCTION

Present on most human tooth surfaces are concentrated masses of bacteria which, together with an inter-bacterial matrix, make up dental plaque. The bacteria in the plaque can readily convert many carbohydrates to acid, causing a rapid fall in the pH of the plaque. Subsequently the pH slowly rises again to fasting levels (Stephan, 1944; Kleinberg, 1961; Kleinberg and Jenkins, 1964). Dental caries is generally accepted to be initiated by dissolution of the inorganic components of the enamel of the tooth surface when the plaque pH drops below some critical level known as the "critical pH" (Englander et al, 1956). Below this pH the concentrations of calcium and phosphate in the plaque and in the saliva are thought to be insufficient to prevent the solubilization of the enamel.

A prominent portion of the plaque microflora consists of the Streptococci. Estimates of their number vary from 27 to 98 per cent of the total flora (Stralfors, 1950; Gibbons, 1964; McCarthy, 1965). It is generally accepted that only streptococci produce acid at a sufficient rate and occur in sufficient numbers in dental plaque to have any major importance in producing the pH fall which occurs in dental plaque in vivo (Bibby, Volker, and Van Kesteren, 1942; Sims, 1965). This suggests that studies of the carbohydrate metabolism of streptococci are important to provide a greater understanding of the dental caries process.

Investigations of carbohydrate metabolism in streptococci are usually carried out using low concentrations of cells and levels of

carbohydrate substrates that are too high to be completely utilized during the experimental period. Under such conditions of incubation, streptococci carry out a typical homolactic fermentation of glucose with only small amounts of products formed other than lactic acid (e.g., Hewitt, 1932; Tatum and Peterson, 1935).

In 1947, Stephan and Hemmens pointed out that these cultural conditions have only limited relevance to the situation occurring in the human oral cavity, where streptococci and other microorganisms exist as dense masses of cells, which are only intermittently supplied with carbohydrate. When these workers investigated the pH change produced *in vitro* when a high concentration of a pure strain of streptococcus isolated from a human mouth utilized a low level of glucose, they observed that the pH initially fell rapidly, but then reached a minimum and, surprisingly, subsequently rose by nearly one pH unit. When they incubated the cell suspension with an equivalent amount of lactic acid instead of glucose, the pH again rose. The extent of the pH rise was approximately equal to that occurring with glucose.

On the basis of these findings, Stephan and Hemmens suggested that the pH rise resulted from the consumption of lactic acid. However, their experiments did not include measurements of lactic acid, nor did they rule out the possibility that the pH rise might have resulted from other factors, such as from the formation of base by the breakdown of nitrogenous material, such as amino acids (Gale, 1946).

To the present, research into dental caries has been almost entirely directed at the falling portion of the "Stephan Curve" in dental plaque. Almost no attention has been paid to the rising portion of the

curve and to the fact that dental caries might be averted if the organisms on the teeth should cause a pH rise by a mechanism such as the consumption of lactic acid. If many streptococci are able to raise the plaque pH by utilizing lactic acid, they might contribute significantly to this process.

The object of the present study was to determine the correctness of Stephan and Hemmens' hypothesis that some streptococci, which are generally accepted to be homolactic, can utilize lactic acid and thereby cause a pH rise. Additional studies were undertaken to clarify the fate of the utilized glucose and lactic acid and to determine some of the conditions that might affect that process.

The main streptococcal strain studied in this thesis, AHT, was originally isolated from human dental caries lesions by Zinner and his co-workers (1964). It is of some interest that of numerous strains of oral streptococci they tested in hamsters, AHT had the greatest activity in producing caries. The last letters of the name of the organism, HT, signify the source of the organism, human teeth, while A is the order of isolation.

Most of the experiments reported in the thesis were carried out using a high cell concentration and a low initial glucose level, to duplicate as nearly as possible the situation occurring in the dental plaque *in vivo*.

Outline of the Remainder of the Thesis

Chapter II is a review of the literature about lactic acid production and subsequent degradation in both mixed cultures and pure cultures of microorganisms. Chapter III will describe the materials and

methods used in this study. In Chapter IV, the experimental work will be reported and the results discussed. A summary of the findings of the study and conclusions will be given in Chapter V.

CHAPTER II

LITERATURE REVIEW

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LITERATURE REVIEW

Oral microorganisms were first observed by Antony Van Leeuwenhoeck in 1683 (cited by Fitzgerald, 1963), and were postulated to have an important role in the initiation of dental caries by Miller in 1890, in his "Chemo-Parasitic Theory". The theory stated that the decalcification of teeth results from the action of acids produced from the breakdown of dietary carbohydrates by plaque bacteria.

Acid formation by the oral microflora was subsequently studied by Neuwirth and Klosterman (1940), who observed the rapid formation of lactic acid from carbohydrate by microorganisms in saliva, both *in vivo* and *in vitro*. They established that bacteria were the responsible agents in the saliva when they showed that saliva which had been passed through a Seitz filter failed to produce acid from glucose. Later, Neuwirth and Summerson (1942) found that lactic acid accounted for no more than 50 per cent of the total acid formed, and that lactic acid was rapidly metabolized by the bacteria in saliva with the production of other unidentified acids. Similar observations were made with plaque *in vitro* by Muntz (1943), who observed that lactic acid was formed and then degraded, while the concentration of other acids progressively rose. He demonstrated that the destruction of lactic acid occurred most rapidly at neutrality and under aerobic conditions. When plaque was incubated under anaerobic conditions, the lactic acid concentration did not fall.

Later, Neuwirth and Summerson (1951) observed that when paraffin-stimulated saliva was incubated with a low concentration of glucose,

lactic acid accumulated only as long as glucose was present in the medium. When the glucose was gone, the lactic acid began to disappear. On the basis of their experiments with saliva, they predicted that the rapid production of lactic acid in dental plaque would account for the rapid fall in pH previously reported by Stephan (1940).

The oxidation of lactic acid has been reported in many diverse types of microorganisms such as Escherichia coli (Haugarrd, 1959), Mycobacteria, (Cousins, 1956), Pseudomonas (Walker and Eagon, 1964) and yeast (Appleby and Morton, 1954). Lactobacilli have also been shown to utilize lactic acid; Peterson et al.(1920) found that Lactobacillus pentaceticus, a heterolactic fermenter, could stoichiometrically convert lactic acid to acetic acid.

More recently, even two homolactic fermentors, Lactobacillus delbrueckii and Lactobacillus arabinosus, were found to contain lactic oxidases (Hager, Geller and Lipman, 1954; Snoswell, 1959). These lactic oxidases were NAD-independent. Snoswell (1963) found that L. arabinosus contains two pairs of lactic dehydrogenases, $NAD^{\pm}$-dependent and $NAD^{\pm}$-independent pairs. The former pair of enzymes were involved with the production of D(-)- and L(+)- lactate from pyruvate while the latter pair were involved with the oxidation of D(-)- and L(+)- lactate back to pyruvate.

As reported in the previous chapter, the utilization of lactic acid in oral streptococci was first suggested by Stephan and Hemmens (1947) when they incubated a high concentration of a pure strain of an unidentified oral streptococcus with low initial concentration of glucose. They found that the pH in the medium fell rapidly and then rose slowly, and

postulated that the pH rise occurred because of the disappearance of lactic acid. Lamanna (1965, p. 698) had reported that Streptococcus lactis had been found to excrete lactate into the medium while glucose was being metabolized, and then utilized it after the glucose had disappeared from the medium. Evidence of lactic acid degradation by streptococci was also provided by London and Appleman (1961), who reported that Streptococcus faecium strain NCTC 7171 possessed a specific flavin-linked L(+)-lactic dehydrogenase which converted L(+)-lactic acid to acetic acid and carbon dioxide. This enzyme system was capable of oxidizing lactic acid when either oxygen or an appropriate dye was provided as an electron acceptor.

More recently, London (1968) investigated the regulation of the synthesis and function of the L(+)-lactic dehydrogenase. He found that the majority of the enzyme activity appeared during the last hour of exponential growth, when the glucose in the medium was nearly exhausted. This enzyme system enabled the organism to grow at the expense of L(+)-lactate and to survive in a medium devoid of carbohydrate substrate. Presumably, as postulated by Snoswell (1959) for L. arabinosus, the oxidation of lactate was coupled to ATP production, providing a source of energy when the primary energy source in the medium, glucose, was used up. In addition, the presence of an NAD^+-independent lactic dehydrogenase could conceivably enable the bacteria to regenerate pyruvate which could be used for synthetic purposes through its conversion to acetyl-CoA.

Since little is known about lactic acid degradation by bacteria in the oral cavity, the importance of such degradation to dental caries

is not well understood. It was because of this lack of information that the present investigation was carried out.

CHAPTER III

MATERIALS AND METHODS

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MATERIALS AND METHODS

Cultures and Growth Media

The AHT streptococcal strain (Zinner, 1965) used in this study were obtained from Dr. J. Donohue of Colgate-Palmolive Company, New Jersey and from Dr. M. Goldner, Faculty of Dentistry, University of Toronto. Additional streptococcal strains, BHT, CHT, HHT (Zinner, 1965); E-49, FA-1, 2F2, GF-71, HS-10, 2M2 (Fitzgerald isolates); and PK-1 and GS-5 (Gibbons isolates) were also obtained from Dr. Donohue. Strains of streptococci of Lancefield groups A, C, D, E, F, G, H, and K were obtained from the Laboratory of Hygiene of the Department of National Health and Welfare in Ottawa.

All cultures were maintained at 0-4°C in trypticase agar (Jordan et al, 1960) having the following composition per litre: trypticase (Baltimore Biological Laboratory), 10 gm; yeast extract, 10 gm; K_2HPO_4, 10 gm; salt solution, 1 ml; sucrose, 10 gm; and agar, 10 gm. The salt solution consisted of $MgSO_4.7H_2O$, 0.8 gm; $FeCl_3.6H_2O$, 0.04 gm; and $MnCl_2$ 0.012 gm; in 100 ml of distilled water. Cultures were transferred every six weeks. All cultures except the Lancefield strains were also freeze-dried as soon as received.

Preparation of Incubation Mixtures

For all experiments in the investigation, cells were grown in trypticase soy broth (Baltimore Biological Laboratory, Baltimore, MD.) in an atmosphere of 95 per cent nitrogen and 5 per cent

carbon dioxide at 37°C for 18 hours, at which time they were in the exponential growth phase. They were harvested by centrifugation at 7710 x g for 20 minutes. The supernatant was carefully poured off and the pellet washed twice with approximately 10 ml of sterile cold trypticase medium, by centrifuging at 1470 x g for 20 minutes, and decanting the supernatant. Finally the cells were resuspended in sufficient sterile cold trypticase medium to give the desired final cell concentration, in most cases, 16.7% (V/V).

Incubation mixtures were prepared by combining the cell suspension and the substrate to make a final volume of 3 ml. The glucose used as substrate was introduced at zero time of each experiment. All preparation and handling of the incubation mixtures prior to incubation were carried out at 0-4°C in an ice-packed beaker to limit cellular metabolism.

Incubation Procedure and pH Measurement

All incubations were carried out at 37°C in 25 x 55 mm test tubes fitted with rubber stoppers with a hole through which a glass electrode was inserted. The mixtures were incubated in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill.) for four hours.

The pH of the incubation mixtures was measured at regular intervals using a model PHM 4c Radiometer pH meter. Samples for other chemical analyses were placed in 10 x 75 mm test tubes in ice to stop metabolism.

Chemical Analyses

Lactic acid analysis. For lactic acid analysis, 25 µl of incubation mixture was removed and delivered into 10 x 75 mm test tubes which contained

the volume of cold distilled water required to give the correct dilution for analysis. The mixtures were vibrated on a mixer (Vortex Jr.) and centrifuged at 1470 x g for 10 minutes at 4°C. Aliquots of 200 µl containing 0-1.5 mM lactic acid were removed from the supernatant and transferred to 10 x 75 mm test tubes. Each tube was capped with parafilm and the contents frozen until assayed for lactic acid by the method of Horn and Brun (1956), as modified by Cohen and Noel (1960). In this method, the enzyme lactic dehydrogenase is added, together with nicotinamide adenine dinucleotide (NAD) to the sample containing lactic acid. The enzyme catalyzes the oxidation of lactic acid to pyruvic acid by NAD. The $NADH_2$ formed was measured spectrophotometrically at 366 mµ (Fig. 1).

Total acid. To determine total acid, 25 µl of sample were removed and delivered into 200 µl of distilled water. The mixture was then titrated with 0.01 N NaOH using phenol blue (pK 8.9) as indicator. Titration values obtained for the mixtures at zero hour were subtracted from the values for each time point to give the value of the total acid produced.

Glucose analysis. For glucose analysis, 100 µl of culture was removed and delivered into 10 x 75 mm test tubes which contained 900 µl of distilled water. The mixtures were vibrated on a mixer (Vortex Jr.) and incubated for 30 minutes at 37°C. Aliquots of 200 µl containing 0-300 µg/ml glucose were transferred to 10 x 75 mm test tubes and the contents assayed for glucose using the "Glucostat" reagent (Worthington Biochemical Corp., Freehold, N.J.). The assay was measured spectrophotometrically at 500 mµ (Fig. 2).

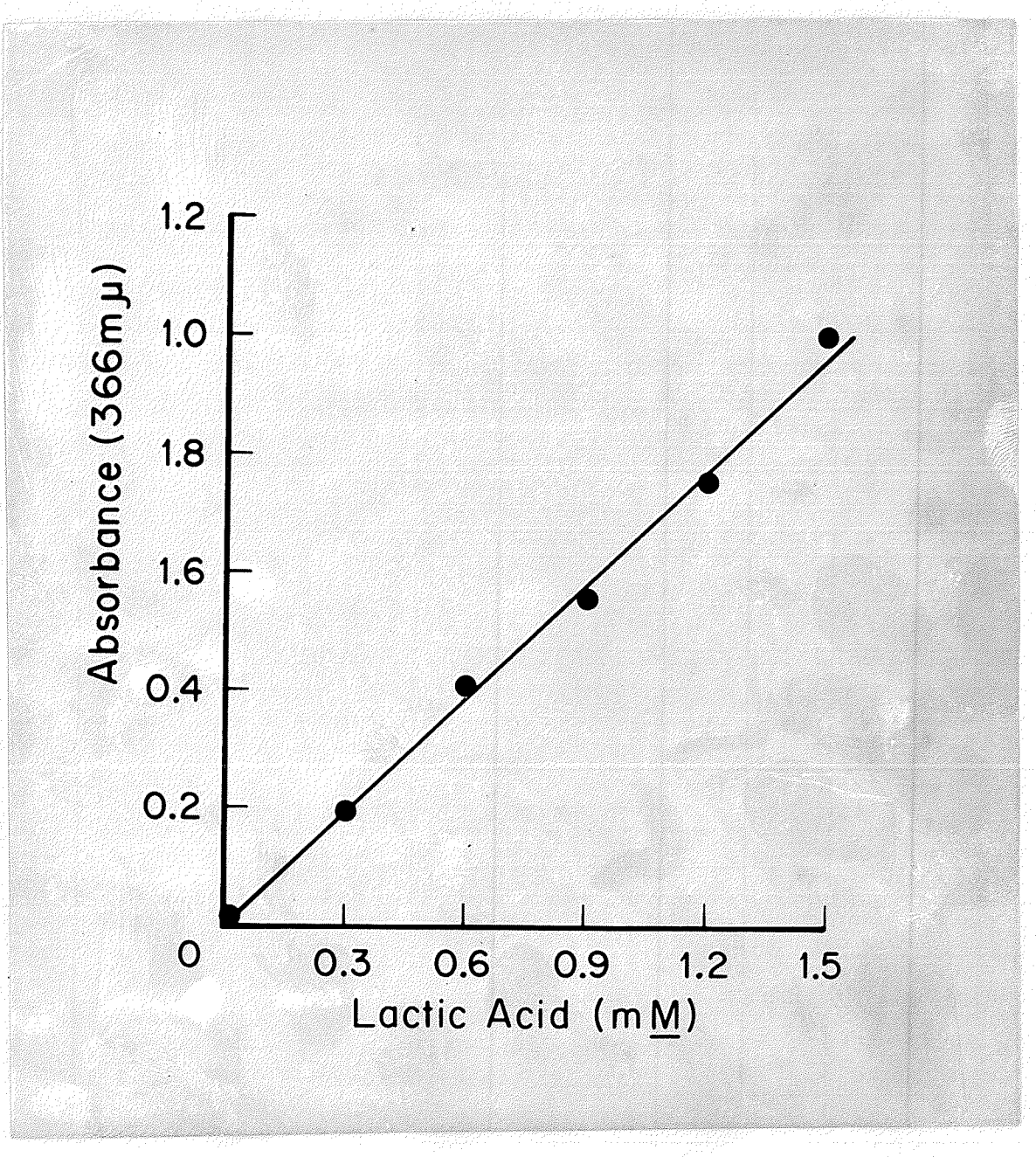

Figure 1. Standard curve for lactic acid determination.

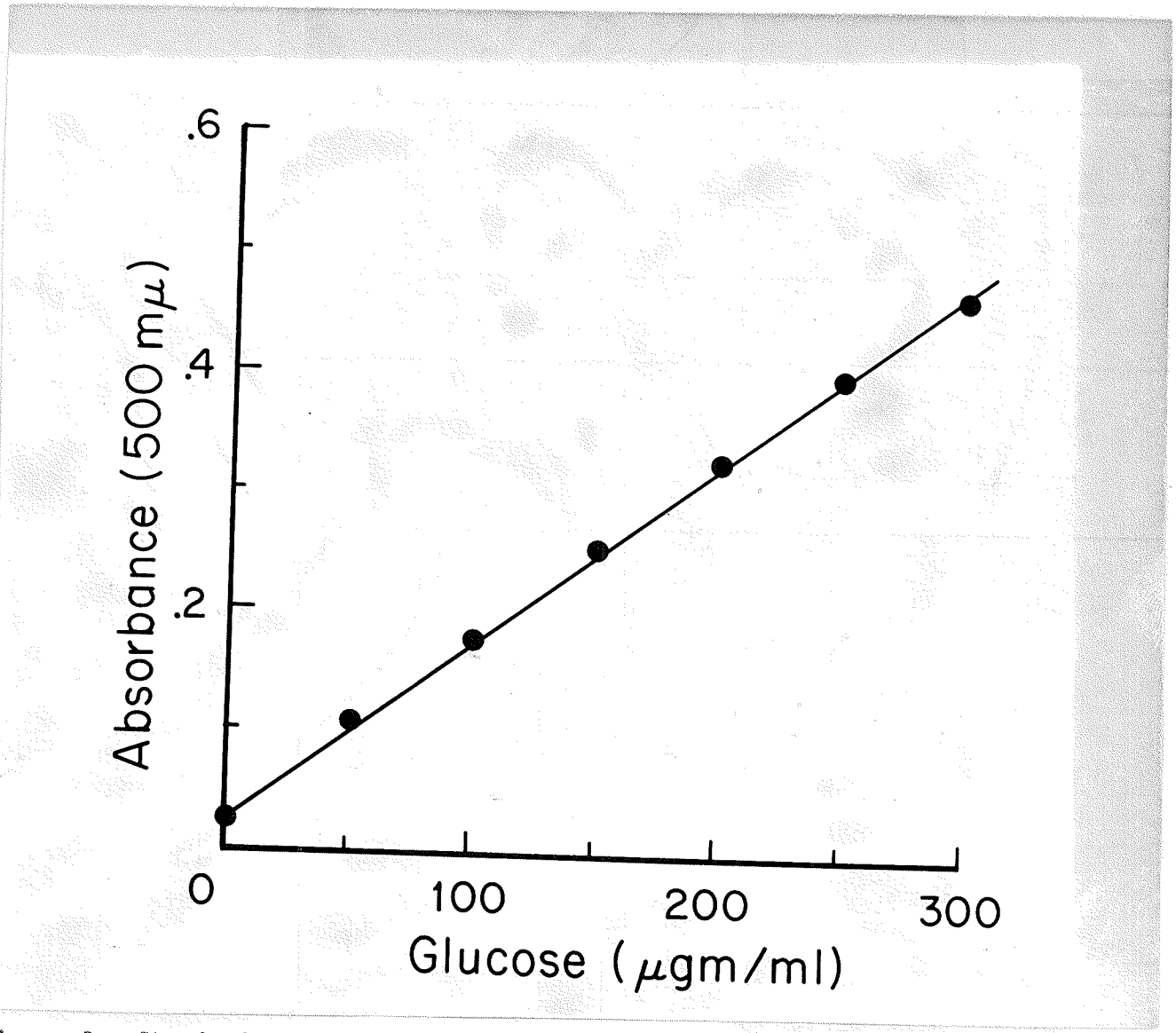

Figure 2. Standard curve for glucose determination.

Ethanol analysis. For the determination of ethanol, the culture supernatant was first treated with zinc sulfate and then titrated with sodium hydroxide to pH 7.6-7.8. The mixtures were centrifuged at 1470 x g for 10 minutes, and the supernatant removed and steam distilled by the method of Neish (1950, p. 15). The ethanol in the distillate was determined by Conway's method (Conway, 1962). A typical standard curve is shown in Figure 3.

Carbon dioxide from glucose and lactic acid. Carbon dioxide produced specifically from glucose and from lactic acid was determined by measuring the radioactivity found in the carbon dioxide when the glucose or lactic acid were uniformly labelled with carbon-14. The carbon dioxide was collected in an aqueous solution of sodium hydroxide (Sandham, 1967) contained in 6 x 30 mm test tubes which placed inside the incubation tubes. The incubation tubes were sealed with serum stoppers. Separate incubation tubes were set up for each time interval. At each time interval, the incubation tubes were removed from the water bath, 100 µl of 0.1 N hydrochloric acid injected through their serum stoppers, and the tubes were kept overnight at 0-4°C to ensure the complete diffusion of all carbon dioxide from the medium into the sodium hydroxide. After that time, the sodium hydroxide was pipetted into liquid scintillation vials containing 10 ml of a mixture of 219 g of Fluoralloy DXA scintillation liquid (Beckman Instrument, Inc., Fullerton, Calif.) in 2 l. of dioxane (J.T. Baker, reagent grade). All determinations of radioactivity reported in this thesis were then carried out using either a Beckman DPM-100 or a Beckman LS-150 liquid scintillation counter.

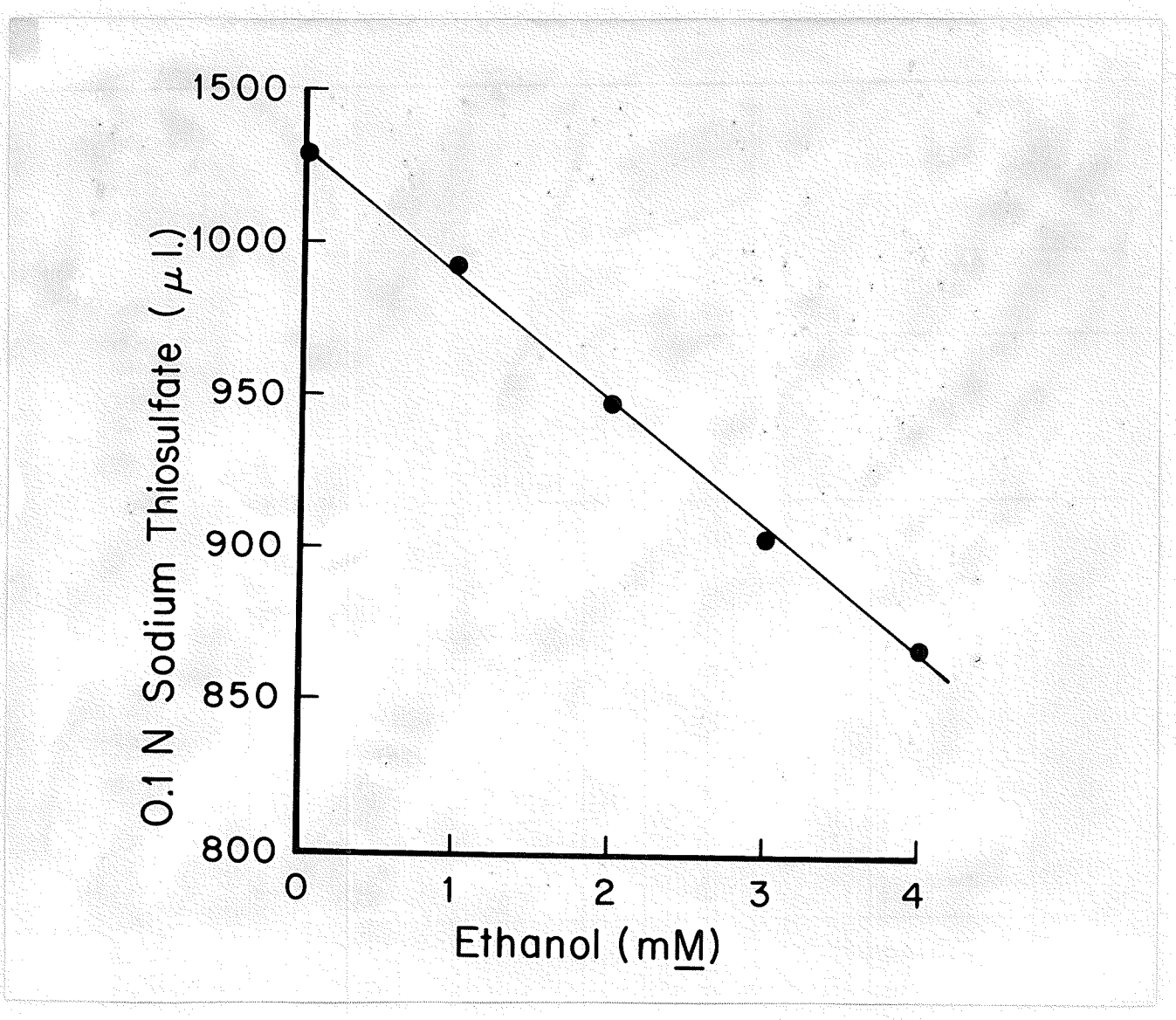

Figure 3. Standard curve for ethanol determination.

Radioactivity of cells. To estimate the radioactivity of cells, the cells were first washed twice with 50 per cent ethanol, and then suspended in 2N sodium hydroxide. They were then heated at 80°C for an hour or until the solution was clear. The mixture was then neutralized with BIO-Solv Solubilizer (BBS-2, Beckman Instrument Inc.) to pH 7 and counted in vials containing 5 ml of a mixture of 32.2 g of Fluoralloy TLA scintillation cocktail mix (Beckman Instrument Inc.) in 1 gal. of reagent grade toluene, and 5 ml of methanol.

Separation and Quantitation of C^{14}-labelled Fermentation Products by Silicic Acid Column Chromatography

Initially, fermentation products in the supernatant were separated using a modification of the silicic acid column described by Smith (1956) as used by Ng and Hamilton (personal communication). The fines were removed from 100-mesh silicic acid by repeated suspension in water; the silicic acid was then dried at 160°C overnight, and maintained at that temperature until used.

To make up the column, approximately 8 g of the dried silicic acid was suspended in excess chloroform which had been washed with 0.5 N sulfuric acid. A sufficient amount of 0.1 N sulfuric acid was then mixed into the slurry to convert it to a fine white suspension. This required approximately 5 ml of sulfuric acid. The slurry was then carefully poured into a 5 mm i.d. column approximately 52 mm long, made from glass tubing which had been tapered at the lower end. The tip contained a tightly fitted wad of Whatman #3 filter paper. Approximately 1.5 g of silicic acid in chloroform, to which no sulfuric acid had been added,

was added to the top of the column.

Samples, acidified to pH 2.0 or less with a 5 N sulfuric acid, were added to the top of the column. The column was developed with a gradient of butanol in chloroform created as follows: 15% butanol in sulfuric acid - washed chloroform was passed into a mixing vessel which contained 25 ml of acid-washed chloroform. Since the mixing vessel was stoppered and air-tight, the volume in the mixing vessel remained constant at 25 ml. After 50 ml of butanol in chloroform had entered the mixing vessel, the concentration of butanol entering the mixing vessel was switched from 15 to 45% to accelerate the removal of slow-moving components from the column. A solvent flow rate of 30 drops per minute was maintained at all times with a polystaltic pump (Buchler Instruments, Fort Lee, N.J.). Fractions 0.5 ml in volume were collected, and 10 ul from each was removed to be counted by liquid scintillation. Fractions containing the radioactive peaks were then pooled and counted. When standard C^{14} acids propionic, acetic and lactic were run (Fig. 4), the recovery from the column was 86-90 per cent for the known acid standards.

Separation and Quantitation of Volatile
Fermentation Products by Gas Chromatography

Investigations of microbial fermentation require precise and rapid methods of quantitative analysis of the end products that are excreted into the medium. Initially in these studies, silicic acid columns were used for the separation of fermentation products, but this method was found to be very time consuming. Later, a gas chromatograph became available and it was possible to use it for direct quantitative

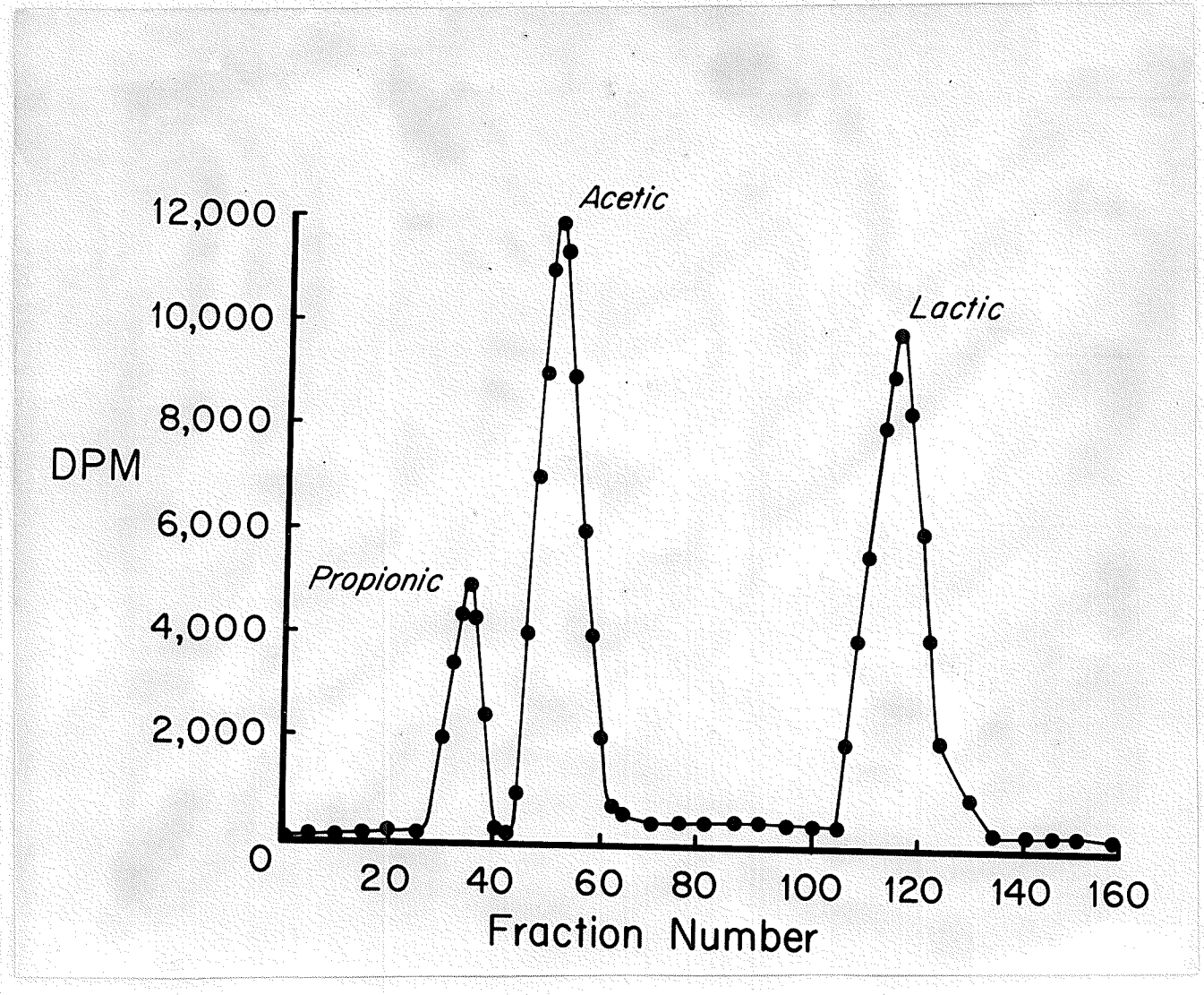

Figure 4. Separation of a mixture of propionic, acetic and lactic acids by chromatography on a silicic acid column.

separation of the volatile fermentation products using the method of Rogosa and Love (1968).

Samples from incubation mixtures were centrifuged for 20 minutes at 1470 x g. The supernatants were removed and acidified to approximately pH 2.0 with concentrated hydrochloric acid. Five μl of the acidified samples were injected into the gas chromatograph with a Hamilton 10 μl microsyringe (Hamilton Co., Whittier, California).

The gas chromatograph was a Hewlett-Packard dual column instrument, Model 5754, equipped with dual hydrogen flame detectors. The Sargent SRG recorder used had a sensitivity of 1 mv. and a chart span of 10 inches. The carrier gas used in the chromatograph was 99.99 per cent minimum purity helium (Matheson Co., Atlanta, Ga.). The dual glass columns ($\frac{1}{4}$ in. by 6 ft.) were packed with PAR #2 (uncoated 80/120 mesh, Hewlett Packard, F & M Scientific Division, Palo Alto, Calif.), which consisted of beads of a polyaromatic resin. With this type of column packing, no liquid phase is necessary, and the instabilities caused by column bleed are thereby avoided. Before the packing was put in the columns, it was first washed with distilled water and then with acetone followed by four volumes of ether, as recommended by Rogosa and Love (1968). The hydrophobic properties of the resin appeared to make the effectiveness of the water wash very doubtful, since the powder could not be wetted.

The following operating parameters were chosen: carrier gas flow, 50 ml per min; pressure settings for helium, 50, for hydrogen, 20 and air, 38 lbs/sq. in; lower limit, 100°C; post-injection hold, 1 min; rate of temperature rise, 20°C per min; upper limit, 200°C; upper limit interval, 11 min; and cooling and equilibration period, 11 min. The

total time for a cycle was 27 min. Attenuations used were 80 and 800. The temperature of the injection port was 262°C, and that of the flame detector 260°C.

Standard amounts of methanol, ethanol, acetic, propionic, butyric, and valeric acids were run to determine retention times (Fig. 5) and to determine the relationship between peak areas and quantities of sample injected (Figs. 6a and 6b). Peak areas were calculated by applying the formula $A = (H \times W)$, where A is the peak area, H is the peak height, and W is the peak width at one-half of the peak height. These standard curves were used to calculate the amounts of end products in incubation media.

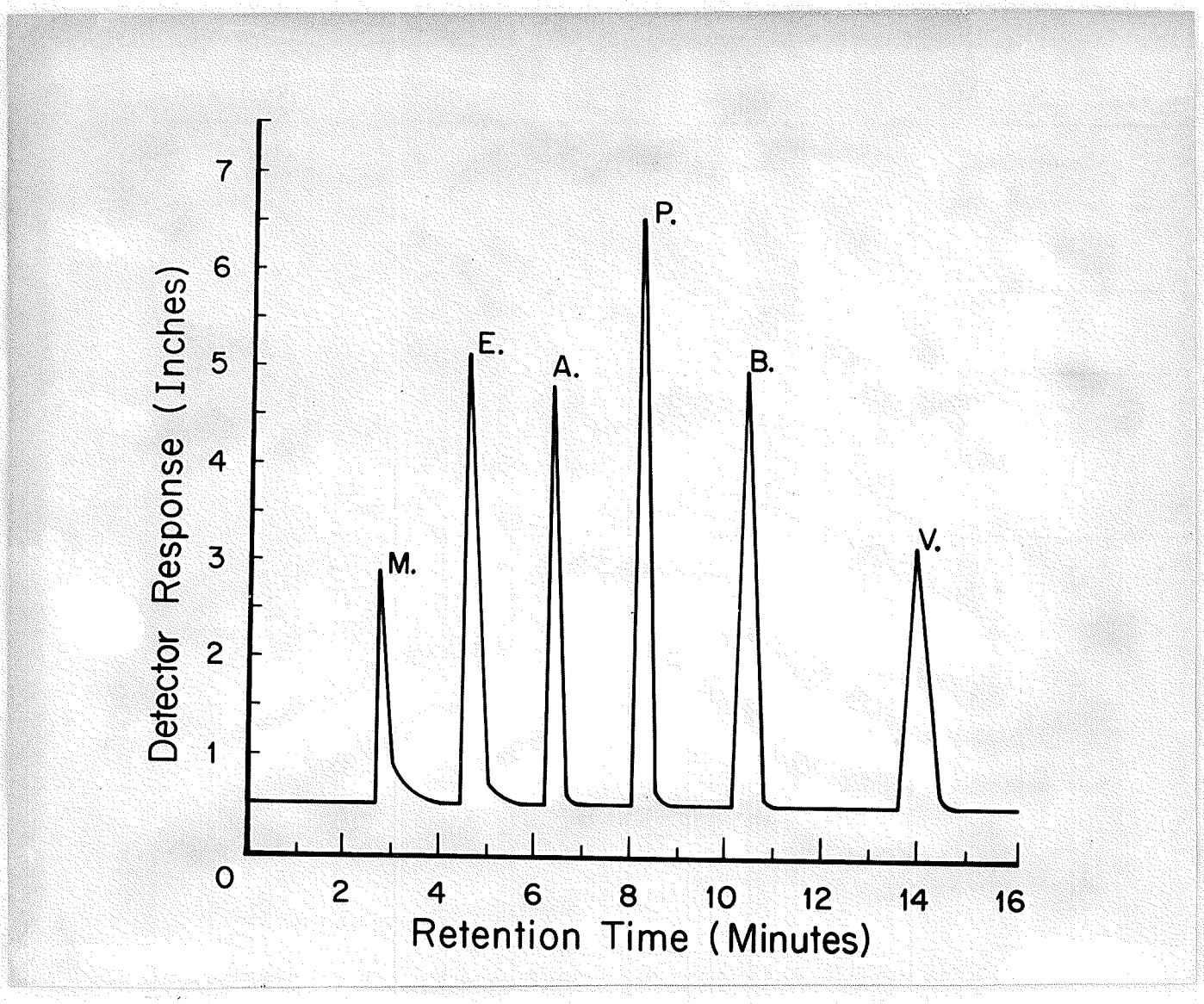

Figure 5. Separation of a mixture of methanol, ethanol, acetic, propionic, butyric and valeric acids by gas chromatography.

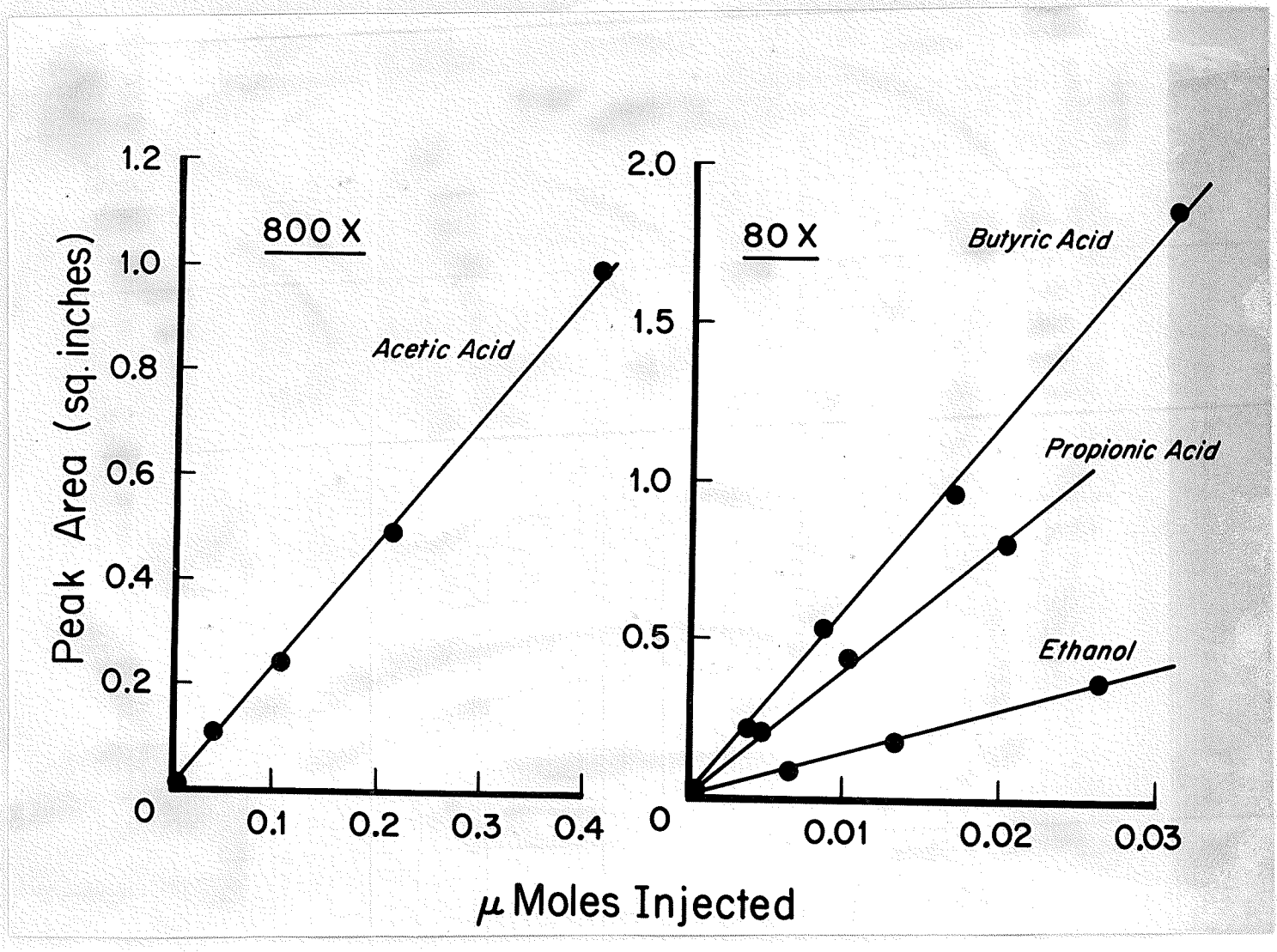

Figure 6. Relationship between peak area and standard amounts of ethanol and volatile fatty acids injected. Attenuations are 80 in a. and 800 in b.

CHAPTER IV

EXPERIMENTAL

CHAPTER IV

EXPERIMENTAL

A. THE EFFECT OF CELL CONCENTRATION ON pH CHANGES DURING INCUBATION

When Stephan and Hemmens (1947) studied the effect of cell concentration on pH change with pure cultures of oral microorganisms *in vitro*, they found that only at high cell concentrations of 16 and 33 per cent (V/V) were the pH changes as rapid as those which occur in dental plaque *in vivo*. With most species of microorganisms that they tested, whether or not they observed a pH rise depended on the concentration of cells which was used; only at high concentrations (16-33%, V/V) did they observe rises. They reported no results for the effect of cell concentration of streptococci on pH, although they reported that when a 33 per cent suspension of one unidentified plaque streptococcus was incubated with a low concentration of glucose, the pH of the incubation mixture dropped to a minimum of 4.5 within 5 minutes and then rose by 0.8 unit over a subsequent $2\frac{1}{2}$ hour incubation period. In the present study, because of our particular interest in streptococcal strain AHT, we wished to determine the effect of altering cell concentration on the pH curves obtained, and how the effects would compare with those obtained by Stephan and Hemmens for other microorganisms.

Procedure

Incubation mixtures were prepared as described in Chapter III. Duplicate mixtures were used for each of the cell concentrations 0, 1, 2, 4, 8, 16.7, and 33.3 per cent (V/V). The concentration of glucose used

was 13.9 mM (0.25 per cent, W/V). The conditions of incubation were similar to those used by Stephan and Hemmens (1947), in that no specific attempt was made to exclude oxygen. Although the tubes were capped with rubber stoppers, the stoppers were removed at regular intervals to enable access to the culture for pH measurement. The pH was measured at regular intervals over a four hour period of incubation.

Results and Discussion

The results from duplicate incubation mixtures at each of the seven cell concentrations were averaged and the results are shown in Figure 7.

The pH curves obtained in the present study resembled both those reported by Stephan and Hemmens (1947) for pure cultures of oral microorganisms and those of Kleinberg (1967) for salivary sediment, in that progressive increases in cell concentration were associated with progressive increases in both the rate of pH fall and the rate of subsequent pH rise. The rates of fall and rise of the pH with 33.3% cells suggest that, at the even higher cell concentrations present in dental plaque, streptococci could contribute substantially to the pH changes occurring in the plaque.

A slight difference between the results of this study and those of the previous studies was that in the present study, an increase in cell concentration from 16.7 to 33.3% resulted in a lower pH minimum, whereas in the previous studies the pH minimum was higher with 33.3 than with 16.7% cells. A lesser fall would be understandable because a large proportion of the buffering capacity in these systems would be provided by the high concentration of cells present. An increase in the cell con-

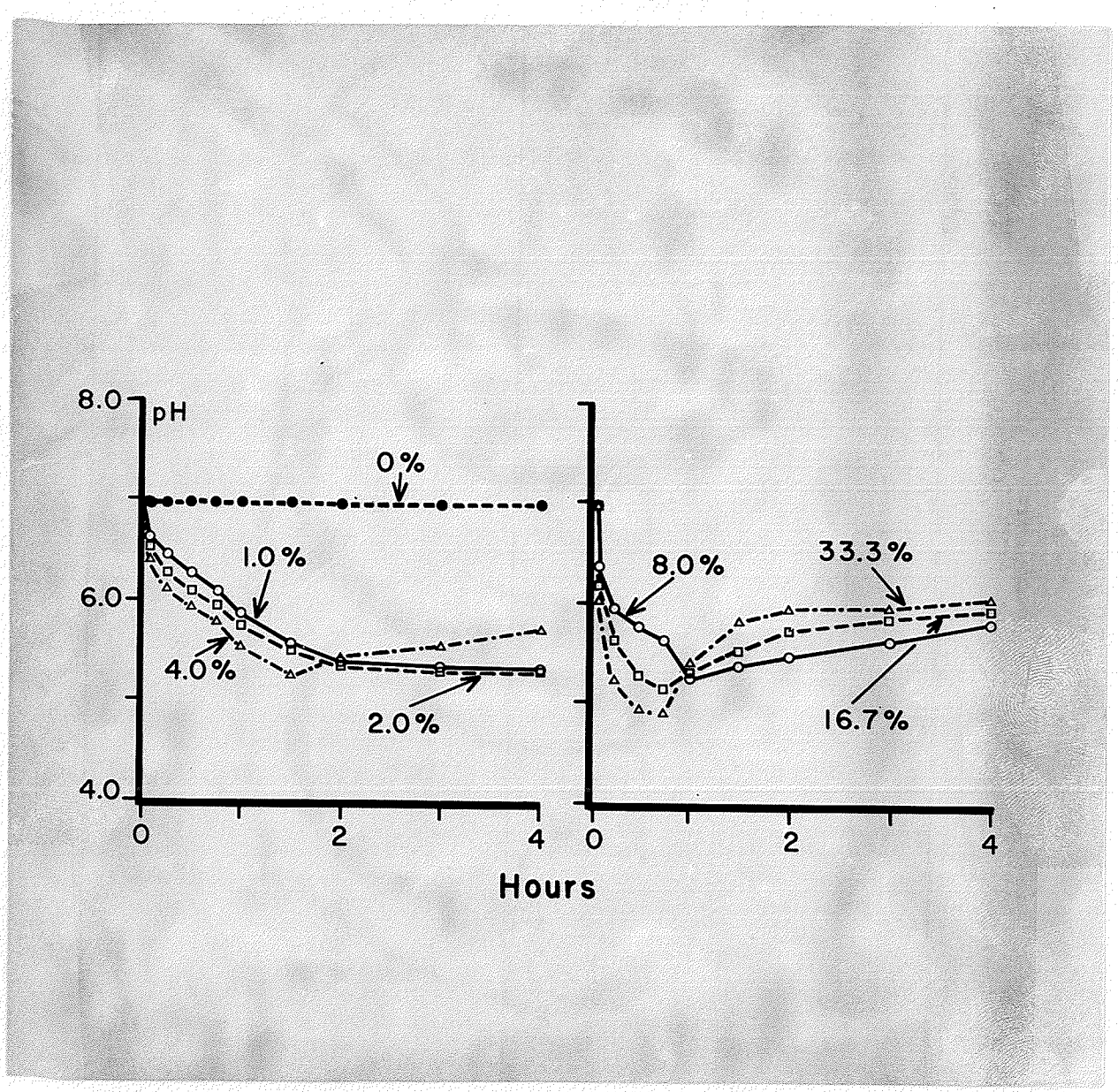

Figure 7. The effect of cell concentration on pH changes.

centration from 16 to 33% would produce an equivalent increase in buffering capacity in the system, which could result in a decreased pH fall. On the other hand, two explanations might be offered for the lower pH minimum with 33.3% cells in the present study, i.e., (1) the cells used in the incubation might contain substrates which could be degraded to form acids in addition to those formed from the breakdown of the supplied glucose, or (2) when the cell concentration is higher, glucose might be broken down to form more acidic products. These possibilities will be discussed more fully in subsequent sections.

B. THE EFFECT OF HIGH AND LOW CONCENTRATIONS OF GLUCOSE ON CHANGES IN pH AND LACTIC ACID CONCENTRATION

Stephan and Hemmens (1947) first demonstrated, with pure cultures of oral microorganisms, that the nature of the pH curve obtained during incubation depended on the concentration of glucose provided as substrate. With low levels of glucose the pH fell to a minimum and then slowly rose. In contrast, at higher glucose concentrations the pH curve fell continuously during incubation without any rising phase. The effect of different concentrations of glucose on pH change has also been examined with plaque *in vivo* (Kleinberg, 1961) and with salivary sediment *in vitro* (Kleinberg, 1967; Sandham, 1967).

The object of the next series of experiments was to study the differences between the effects of high and low glucose concentrations on the pH changes occurring in incubation mixtures containing streptococcal strain AHT. In addition, lactic acid analyses were carried out to assess whether the rise in pH was, in fact, due to the utilization of

lactic acid, and to determine how the initial level of glucose supplied to the organisms affected the level of lactic acid present in the medium.

Procedure

Duplicate incubation mixtures were used for each of the glucose concentrations 0, 13.9, and 278 mM (0, 0.25, and 5%, respectively). The glucose was introduced at zero time for each experiment. The mixtures were incubated at 37°C for four hours with pH readings taken and duplicate samples removed for analysis at regular time intervals. The methods used for pH measurement, sampling and analysis of lactic acid in these experiments have been described in Chapter III. Throughout this series of experiments, the cell concentration was 16.7 per cent. After a series of preliminary experiments, two experiments were carried out, the results of which are reported here.

Results and Discussion

The results are shown in Figure 8, where each value is the mean value of the last two experiments conducted. The results from preliminary experiments were similar to those shown.

Without glucose, no lactate was produced throughout the four hour incubation, demonstrating the necessity of glucose for lactic acid accumulation. The pH of the medium dropped by only 0.22 units during the first 45 minutes of incubation, apparently the result of the accumulation of small amounts of acids other than lactic, formed from the small amount of degradable carbohydrate initially present in the cells and/or medium. This observation is consistent with that in the preceding section of this

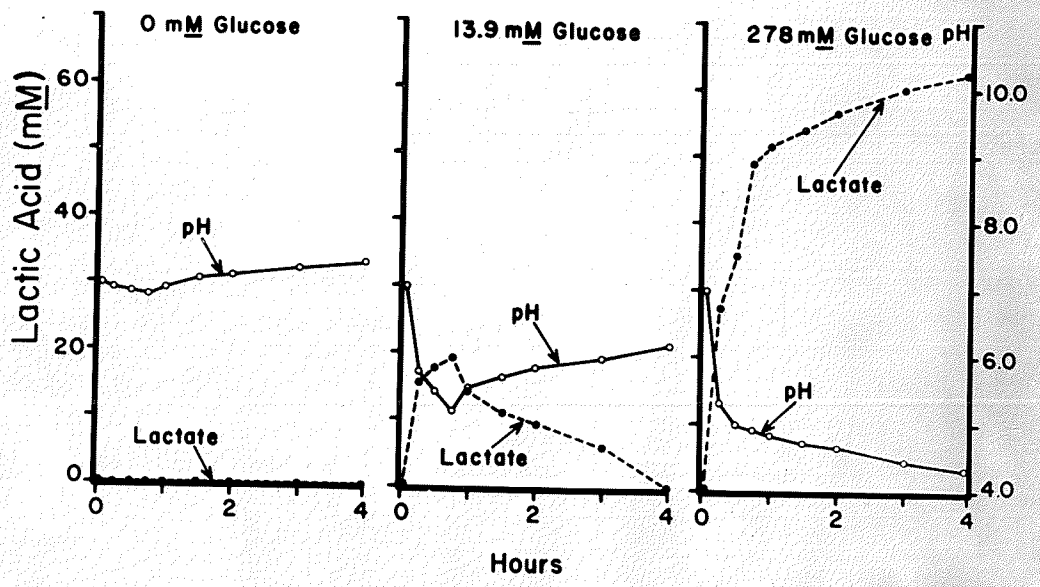

Figure 8. The effect of 0, 13.9 and 278 mM glucose concentrations on pH and lactic acid.

chapter, that lower pH minimums were observed with higher cell concentrations; increased concentrations of cells would bring with them increased concentrations of degradable carbohydrate. The pH rise that occurred in the later portion of the experiment cannot be attributed to lactic acid degradation since none was present; it was more likely due to base production.

With 13.9 mM glucose, the lactate concentration rose to reach a maximum at 45 minutes; the pH simultaneously fell to reach a minimum during the same time interval. After 45 minutes, the amount of lactic acid decreased, rapidly at first and then more slowly until it had completely disappeared at the end of four hours. The disappearance of the lactic acid was accompanied by a gradual rise of pH of one unit. The fact that at the end of four hours the pH was still one unit below its 0 hour value, indicated that acidic products other than lactic acid were still present in the medium at the end of the incubation.

Generally, the pH was a sensitive reflection of lactic acid levels in the incubation mixtures; the pH fall was reversed as soon as the lactic acid in the medium began to fall. The situation here is in contrast to that in salivary sediment (Sandham, 1967), where the pH can continue to fall for up to 30 minutes after the lactic acid concentration has begun to fall. This indicates that under the conditions described here, streptococcal strain AHT, unlike salivary sediment, does not have sufficient carbohydrate storage material to prolong the pH fall past the time when the lactic acid concentration has begun to fall.

With the highest glucose concentration tested, 278 mM, the lactic acid level did not tend to fall at any time during the incubation. This

suggests that with low concentrations of glucose, the disappearance of lactic acid occurred as the result of the exhaustion of glucose from the medium.

Thus, lactic acid concentration in the present study was a function of glucose concentration and of the length of time the incubation had progressed. Also, when the initial glucose concentration was low, lactic acid was clearly an intermediate and not an end product of glucose catabolism in streptococcal strain AHT.

C. EFFECT OF USING PHOSPHATE BUFFERED SALINE AS A MEDIUM

The complexity of the trypticase medium used as an incubation medium in the previous experiments did not permit the conclusion that the observed changes in pH and lactic acid concentration were solely the results of bacterial action on the glucose substrate. It therefore seemed appropriate to carry out a series of experiments in which the complex trypticase medium was substituted by a much simpler medium comprised only of phosphate buffered saline. This would establish whether any components of the trypticase medium could have been responsible for, or necessary for, the rise in pH.

Procedure

Parallel incubations were carried out in trypticase medium and phosphate buffered saline (PBS). The concentration of the phosphate in the PBS was adjusted to have the same buffering capacity over the range of pH fall from 7 to 5 as that of the trypticase medium. Changes in pH and lactate concentration in these two media were determined with duplicate

tubes at each of three glucose levels, 0, 13.9 and 278 mM. Two identical experiments were run and the results were averaged. The cell concentration studied in this series of experiments was again 16.7 per cent.

Results and Discussion

Changes in pH and lactate in the two media are compared in Figures 9a and 9b respectively. Each point on each curve represents the mean of four determinations in two experiments.

The results from these experiments showed that there were no obvious differences in the changes that occurred in either pH or lactic acid concentrations as a result of substituting phosphate buffered saline for the more complex trypticase medium. Obviously the components in the complex trypticase medium did not contribute to either the pH rise or lactic acid utilization by streptococcal strain AHT.

D. CORRELATION OF pH, LACTIC ACID, TOTAL ACID AND GLUCOSE DISAPPEARANCE

Previous sections in this chapter have demonstrated [redacted] the effect that the initial concentration of glucose had on the changes in lactic acid and pH that occurred during incubation. Particular interest for dental research resides in the results of incubations using low levels of glucose; the changes in their pH mimic the major components, the fall and rise, of the "Stephan pH curve."

Studies of the relationships between pH, lactic acid, total acid, and glucose utilization can often suggest the presence of additional substrates and/or metabolic processes that otherwise might be unsuspected. For example, Muntz (1943), using heavy suspensions of plaque material

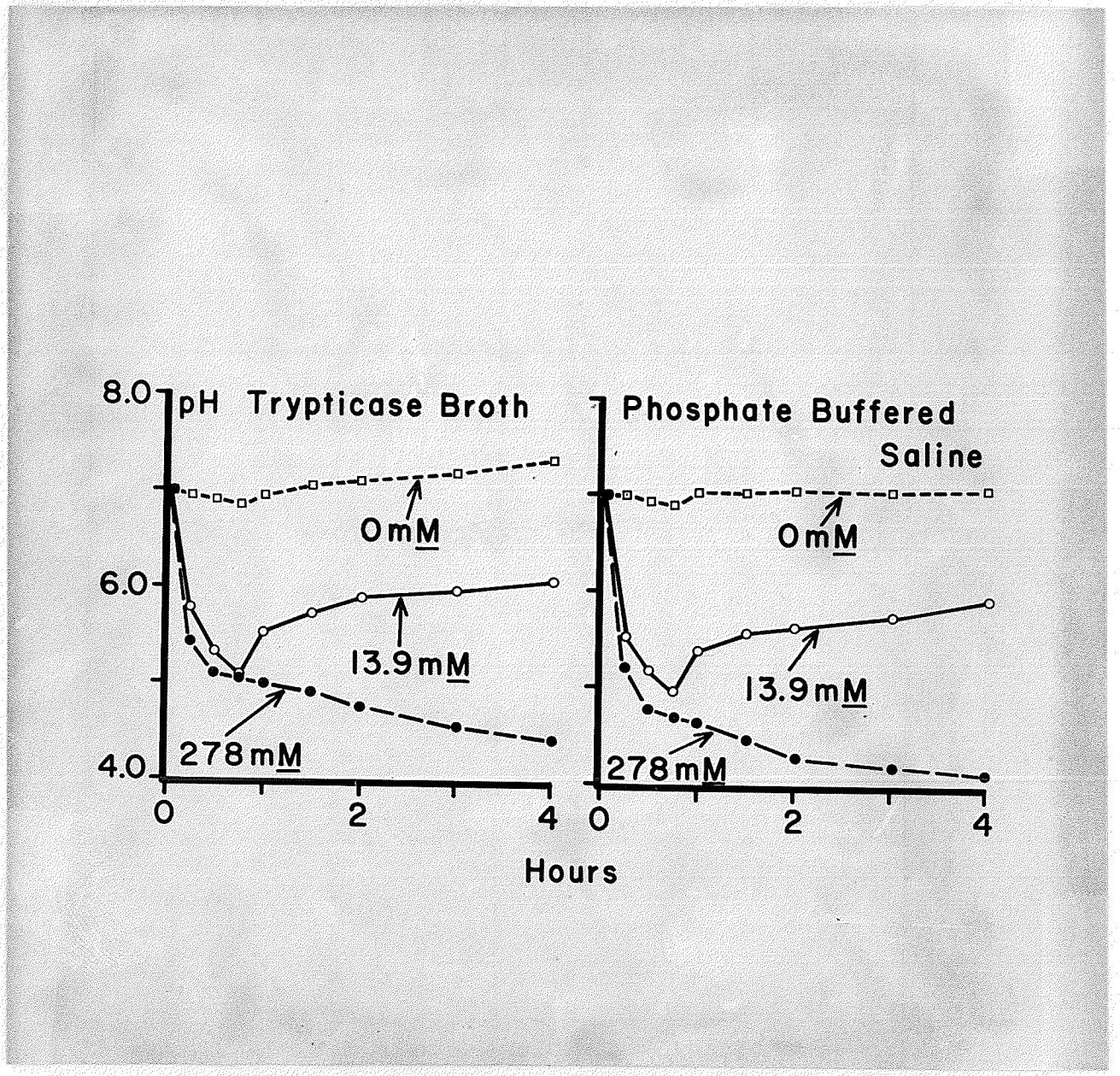

Figure 9a. Changes in pH in trypticase medium and phosphate buffered saline.

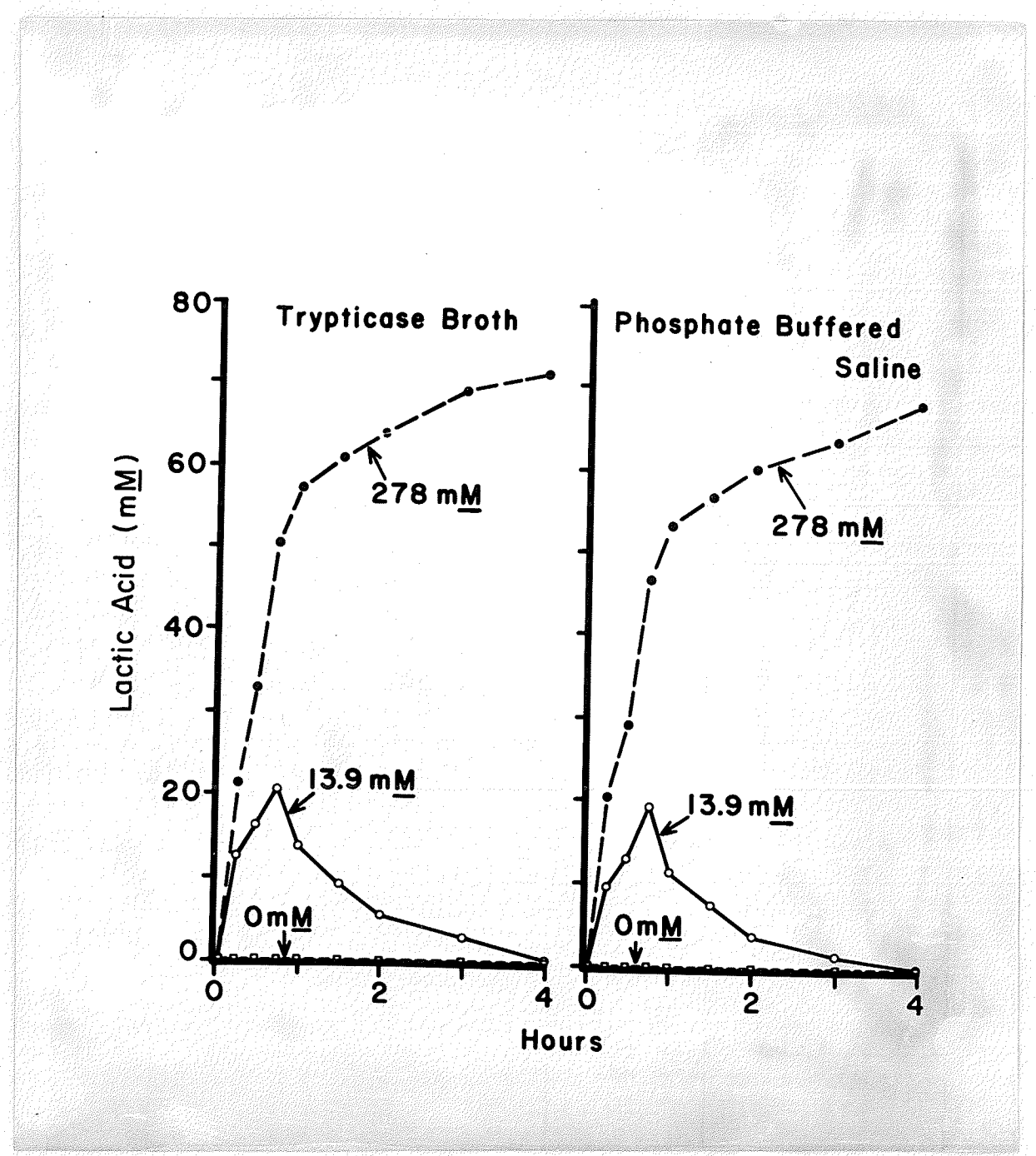

Figure 9b. Changes in lactic acid concentration in trypticase medium and phosphate buffered saline.

incubated with glucose under aerobic conditions *in vitro*, found that the total titratable acid in the medium continued to rise after all lactic acid had disappeared from the medium. This continued increase in total acid suggested either further degradation of glucose (without an associated accumulation of lactic acid), or a switch in substrate from glucose to stored polysaccharide (Sandham, 1967).

Another example is the work of Neuwirth and Summerson (1951), who incubated saliva aerobically with a low level of glucose, and showed that lactic acid accumulated only as long as glucose was present in the medium, and began to disappear as soon as the glucose had disappeared. Sandham (1963, 1967) carried out similar but more comprehensive experiments with a high concentration of salivary sediment, and was able to show that lactic acid accumulation was dependent on the presence of glucose in the medium and also that accumulation of other acids after glucose had disappeared, resulted from the degradation of carbohydrate stored in the sediment. It was therefore felt that more precise information, i.e., the changes in glucose concentration in the medium with time, should be obtained in the present study.

Additionally, pH curves with a low glucose concentration (13.9 mM) had been shown not to return to neutral values, although all the lactic acid had disappeared from the media. This observation indicated that acids other than lactic were still present in the medium at that time. Since pH is a function of both the quantities of acids present and the strengths of these acids, it was felt that further information should be obtained about the total titratable acid, to be followed in later sections by identification and quantitation of these

acids individually.

Procedure

Incubations were set up in which the glucose concentration was 13.9 mM. Samples were withdrawn at regular time intervals for pH measurement and for determination of glucose, lactic and total acid, as described in Chapter III.

Results and Discussion

As previously, the time of the pH minimum, 45 minutes, corresponded with the time of the maximum lactic acid concentration and subsequently the pH rose while the lactic acid concentration fell (Fig. 10). The titratable acidity in the incubation mixtures also reached a maximum at 45 minutes and then began a substantial but not complete fall. This is in contrast to the results in salivary sediment (Sandham, 1967) where no fall in titratable acidity occurred under similar conditions.

Obviously in this system the conversion of lactic acid to weaker acids only partially explains the rise in pH; the conversion of acids to non-acidic products or the production of base also occurred. The rate of decrease of the total titratable acidity was considerably less than the rate of decrease of lactic acid in the medium, so that after four hours of incubation, the lactic acid was completely gone, but a substantial amount of titratable acid still remained. This confirmed that lactic acid was being converted, at least in part, to other acids.

The glucose concentration in the medium decreased rapidly in the first thirty minutes and had reached zero by one hour. It may be signi-

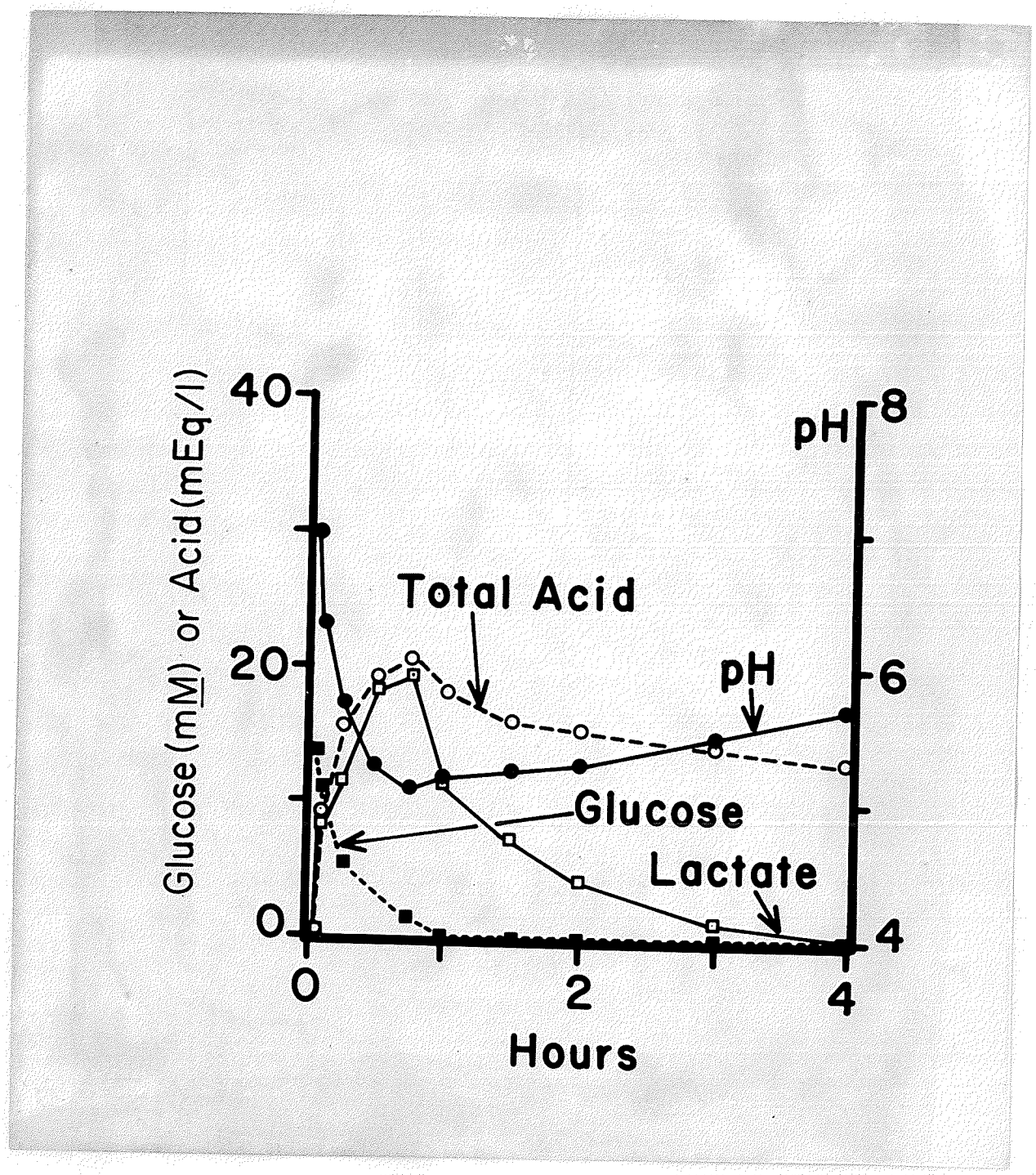

Figure 10. Changes in pH, lactic acid, total acid, and glucose with time.

ficant that the pH minimum and the lactic acid concentration maximum were consistently reached slightly before the glucose in the medium was used up. Lactic acid utilization occurred at a substantial rate even before the glucose had entirely disappeared from the medium. This would be consistent with the observation by London (1968) that in Streptococcus faecium the synthesis of lactic oxidase increased greatly when glucose was almost exhausted from the medium.

E. LACTIC ACID UTILIZATION IN SEVERAL ADDITIONAL STRAINS OF STREPTOCOCCUS

To gain some idea of how common the ability to utilize lactic acid is among streptococci, in particular oral streptococci, and to determine the relationship of this phenomenon to any pH rise, a number of other strains of streptococcus were examined. These included one from each of Lancefield's Groups, A, C, D, E, F. G, H and K, and several strains originally isolated from the mouths of human, rats and hamsters, including streptococcal strain AHT, BHT, CHT, HHT (Zinner, 1965; Jablon and Zinner, 1966); E-49, FA-1, 2F2, GF-71, HS-10, 2M2, (Fitzgerald and Keyes, 1960; Fitzgerald et al, 1960); GS-5 and PK-1 (Gibbons, 1966).

Procedure

The cells were grown for 18 hours, washed, and incubated with 13.9 mM glucose, as described in Chapter III. Each streptococcal strain was incubated in duplicate mixtures for each experiment. Two experiments were carried out, and the results averaged.

Results and Discussion

Changes in pH and lactic acid concentration have been plotted against time for each of the streptococcal strains in Figures 11a and 11b. The tested streptococci fell into two distinct classes (Table I). The first, which is referred to as Class A, produced a pH fall followed by a pH rise and utilized all lactic acid from the medium during the 18 hour incubation period. This class was composed of streptococcal strain AHT and the strains from Lancefield's Groups C, F, G and K. All the remaining streptococcal strains tested (Class B) neither produced a significant pH rise nor utilized lactic acid.

A prominent portion of oral microflora consists of the streptococci; estimates of their number vary from 27 to 98 per cent of the total flora (Gibbons, 1964; McCarthy, 1965). If many of these oral streptococci are able to utilize lactic acid, they could contribute significantly to the lactic acid utilization by the oral microflora that has been observed by many investigators (Muntz, 1943; Neuwirth and Summerson, 1951; Sandham, 1967). It is of some interest that streptococci of Lancefield's Groups C, G and K, strains of which were observed to utilize lactic acid in this series of experiments, are frequently found among the oral microflora (Cran, 1964; Zinner, 1966).

F. THE FATE OF UTILIZED LACTIC ACID

The present study has shown that when a high concentration of streptococcal strain AHT was incubated with a low concentration of glucose, lactic acid accumulated and then disappeared from the medium, at which time the major portion of the total titratable acidity still

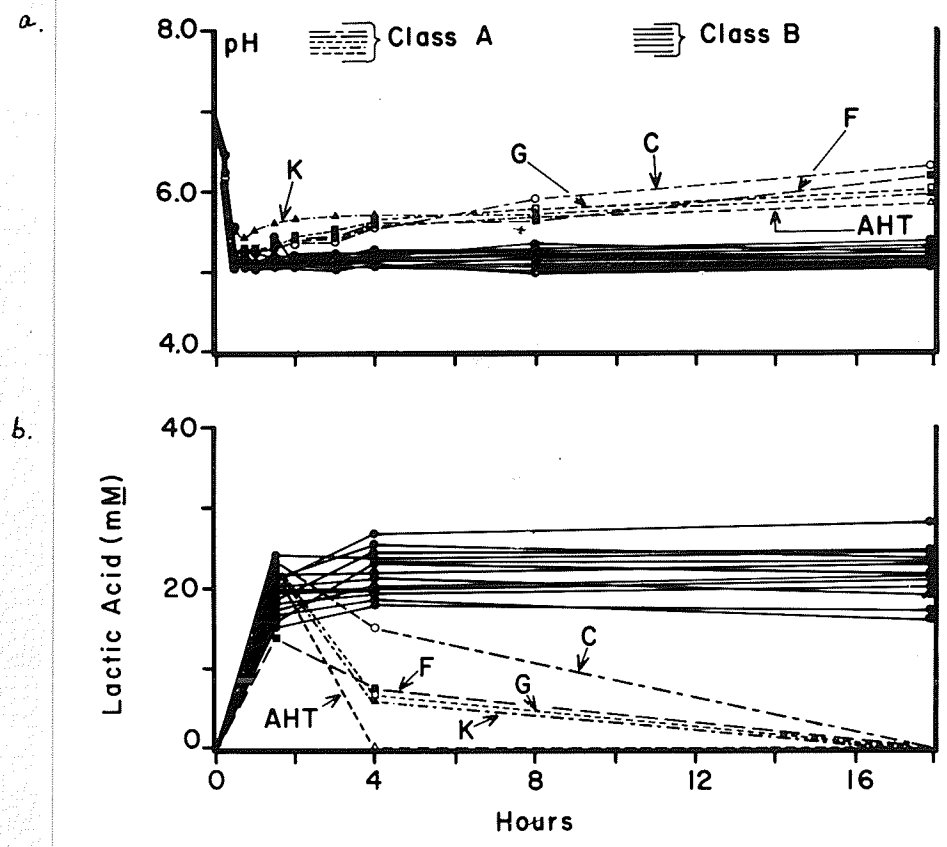

Figure 11. Changes in a. pH and b. lactic acid concentration by selected species and strains of streptococcus.

TABLE I

LACTIC ACID UTILIZATION BY STREPTOCOCCI

CLASS A (utilized lactic acid)	CLASS B (did not utilize lactic acid)
Lancefield Group C	Lancefield Group A
" " F	" " D
" " G	" " E
" " K	" " H
AHT	FA-1 PK-1 BHT
	HS-10 GS-5 CHT
	GF-71 2F2 HHT
	E-49 2M2

remained.

The purpose of this series of experiments was to clarify the fate of the utilized lactic acid by identifying and quantitating as many of the products formed from lactic acid as possible.

Procedure

The work reported in previous sections showed that with concentration of 13.9 mM glucose the maximum lactic acid concentration occurred at 45 minutes in incubation mixtures containing 16.7% streptococcal strain AHT. After that time the lactic acid concentration began to fall. The 45-minute time was therefore chosen to add a trace amount of lactate-U-C^{14} to the incubation mixtures. Since little additional lactic acid would be formed after that time, the specific activity of the lactic acid would remain relatively constant thereafter. This would be essential for obtaining an accurate estimation of the quantities of products formed from the lactic acid.

The techniques used for determining radioactivity in the cells, supernatant and carbon dioxide have been described in Chapter III. A sample of 4-hour supernatant was acidified to pH 2.5 with hydrochloric acid and the contents separated on a silicic acid column as described in Chapter III. The radioactivity in the effluent was measured by liquid scintillation counting.

Results and Discussion

The major portion of the radioactivity from the lactate-U-C^{14} remained in the supernatant after 4 hours (Fig. 12). A small amount of the label, 2.61 mM, appeared in the cell fraction and 1.61 mM appeared

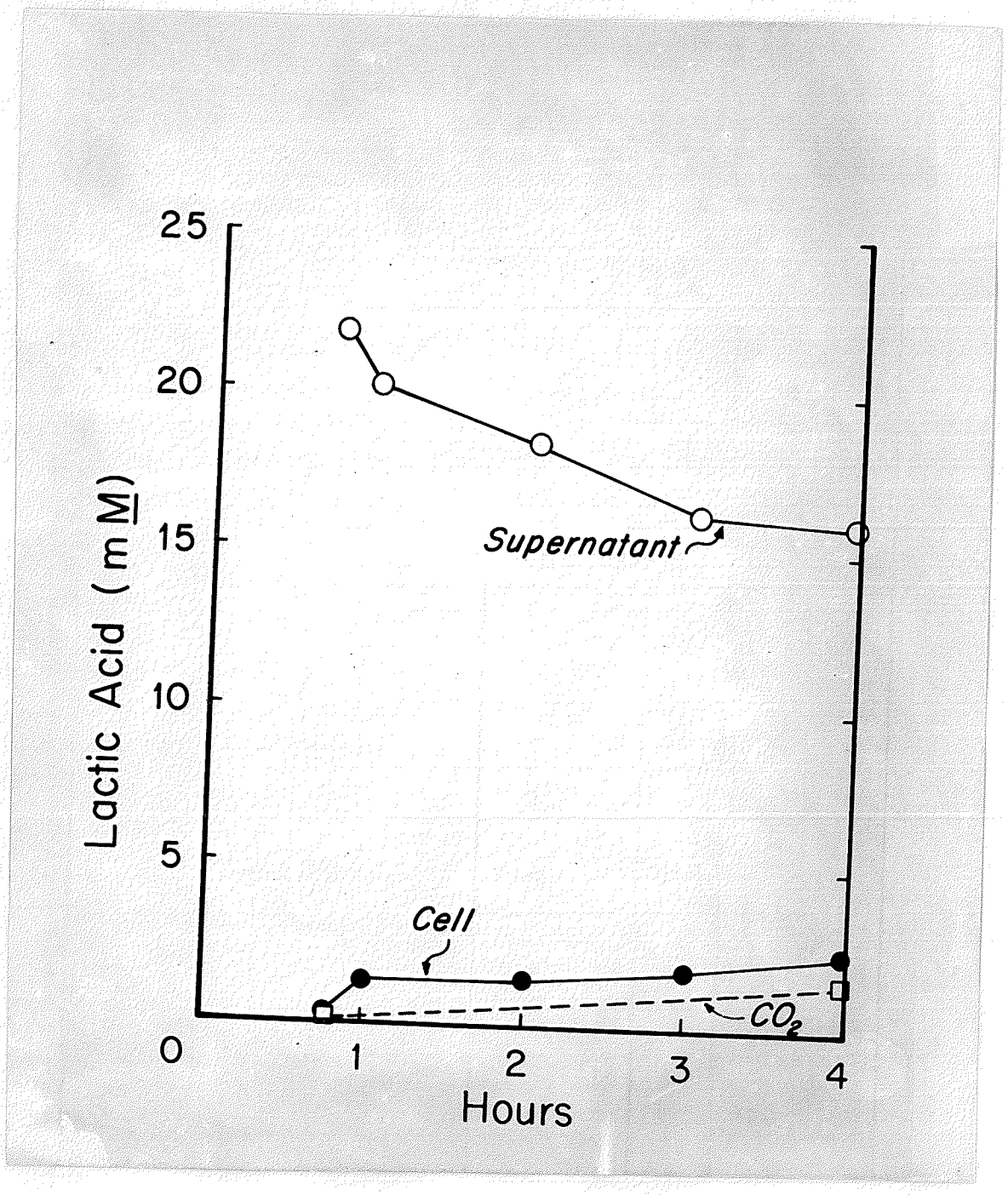

Figure 12. The distribution of lactate-U-C^{14} in the incubation mixture.

in the carbon dioxide.

After column chromatography of the 4-hour supernatant, two radioactive peaks appeared in the effluent (Fig. 13), the position of the second peak corresponding to that of acetic acid. No lactic or formic acid peaks appeared. The second peak, titrated with standardized sodium hydroxide, contained an acidity of 10.32 meq./l of incubation mixture. The amount of radioactivity in the peak indicated that it contained 20.6 milligram-atoms of carbon per liter, which is consistent with the peak being made up entirely of acetic acid. The first peak, however, contained no titratable acidity. When it was steam distilled and analysed for ethanol by Conway's microdiffusion method (Neish, 1952) as described in Chapter III, it was found to contain 3.56 mM ethanol.

These results established that after four hours incubation, the lactic acid formed from glucose was in turn converted to acetic acid, ethanol and carbon dioxide. The pH rise observed in the medium was due both to the conversion of lactic acid to a weaker acid, and to its conversion to the neutral products ethanol and CO_2. This also identified the acid responsible for the substantial titratable acidity remaining in the incubation mixtures after 4 hours.

G. THE EFFECT OF ANAEROBIOSIS ON LACTIC ACID UTILIZATION

In order to have conditions comparable to those of Stephan and Hemmens (1947), all experiments performed up to this point included no specific attempt to exclude oxygen during incubation. That oxygen is important to the system might be expected from the fact that cell extracts of another strain of streptococcus, Streptococcus faecium, have

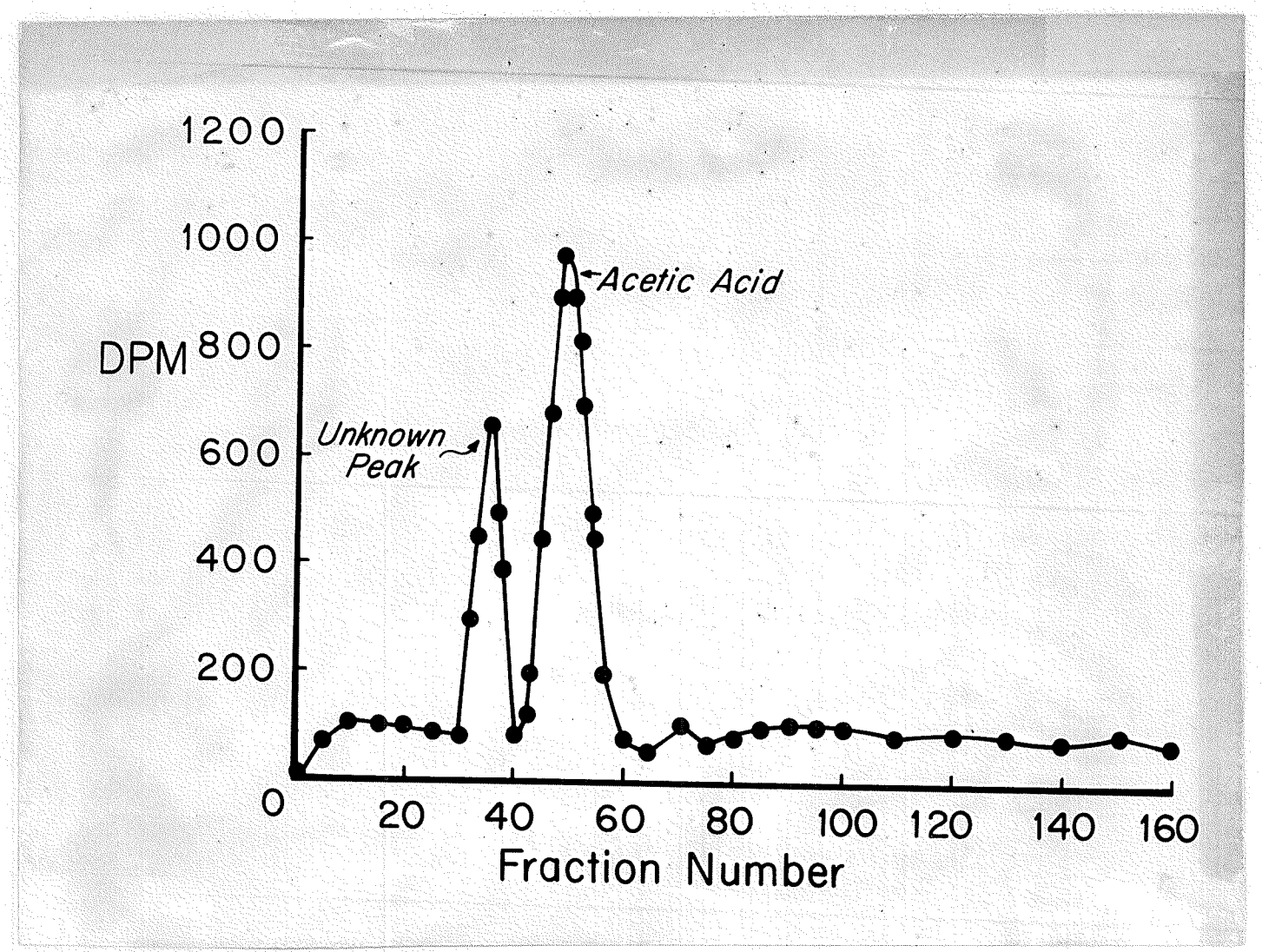

Figure 13. Separation of the 4-hour supernatant by chromatography on a silicic acid column.

been reported to contain an L(+)-specific lactic oxidase which can utilize oxygen as an electron acceptor (London and Appleman, 1961). However, they did not report the level of enzyme activity exhibited when oxygen or a dye acceptor was not provided during the assay of the enzyme, which is more pertinent to the conditions prevailing in dental plaque *in vivo*, if one accepts the suggestion of Gibbons, Socransky, de Araujo and Van Houte (1964) that the oxygen tension in dental plaque is low. Because the enzyme assays by London and Appleman (1961) and London (1968) were carried out with cell-free extracts in phosphate buffers, their experiments did not determine whether any of the substances present in a more complex medium could act as electron acceptors in lieu of oxygen or added dye.

The purpose of the experiments reported in this chapter was to determine whether streptococcal strain AHT would demonstrate a significant ability to utilize lactic acid and to raise the pH in the complex medium (trypticase) used in this study under anaerobic conditions.

Procedure

The methods for pH and lactic acid determinations were the same as those previously described, and a glucose concentration of 13.9 mM was again used. Anaerobic conditions were produced in the incubation tubes by sealing the tubes with serum stoppers and flushing the contents with a mixture of 95% nitrogen and 5% carbon dioxide for 20 minutes prior to the incubation. The flushing was achieved by introducing two hypodermic syringes through the serum stopper. One of these, which was used for introducing the gas mixture, was forced into the medium, so that the medium bubbled vigorously. The gases then left the tube via the second needle, which

did not extend into the medium. After the flushing was finished, the needles were removed from the tubes, and the incubation begun.

During the incubation, which was carried out for 18 hours, samples were aseptically removed with hypodermic syringes for determination of their pH and their lactic acid content.

Results and Discussion

Changes in pH and lactic acid concentration have been plotted against time (Fig. 14). Each curve is the mean of two duplicate experiments conducted under anaerobic conditions; each experiment contained duplicate tubes.

The results showed only a very small rise in pH and a very small drop in lactic acid concentration during the 18 hour incubation, indicating that oxygen was essential to both processes, and that nothing in the complex medium could act as an alternate electron acceptor.

H. THE END PRODUCTS OF GLUCOSE FERMENTATION

Many workers have reported that under some conditions, increased amounts of end products other than lactic acid are formed by streptococci during the degradation of glucose, for example, when the pH of the medium is high (Gunsalus and Niven, 1943; Platt and Foster, 1958), when impairment of glucose uptake or utilization exists (Pierce, 1957), and during the exponential growth phase (Forrest et al, 1961). The purpose of the present study was to compare the relative amounts of lactic acid and other products formed by streptococcal strain AHT from glucose under two physiological conditions: (a) during the exponential growth phase of the

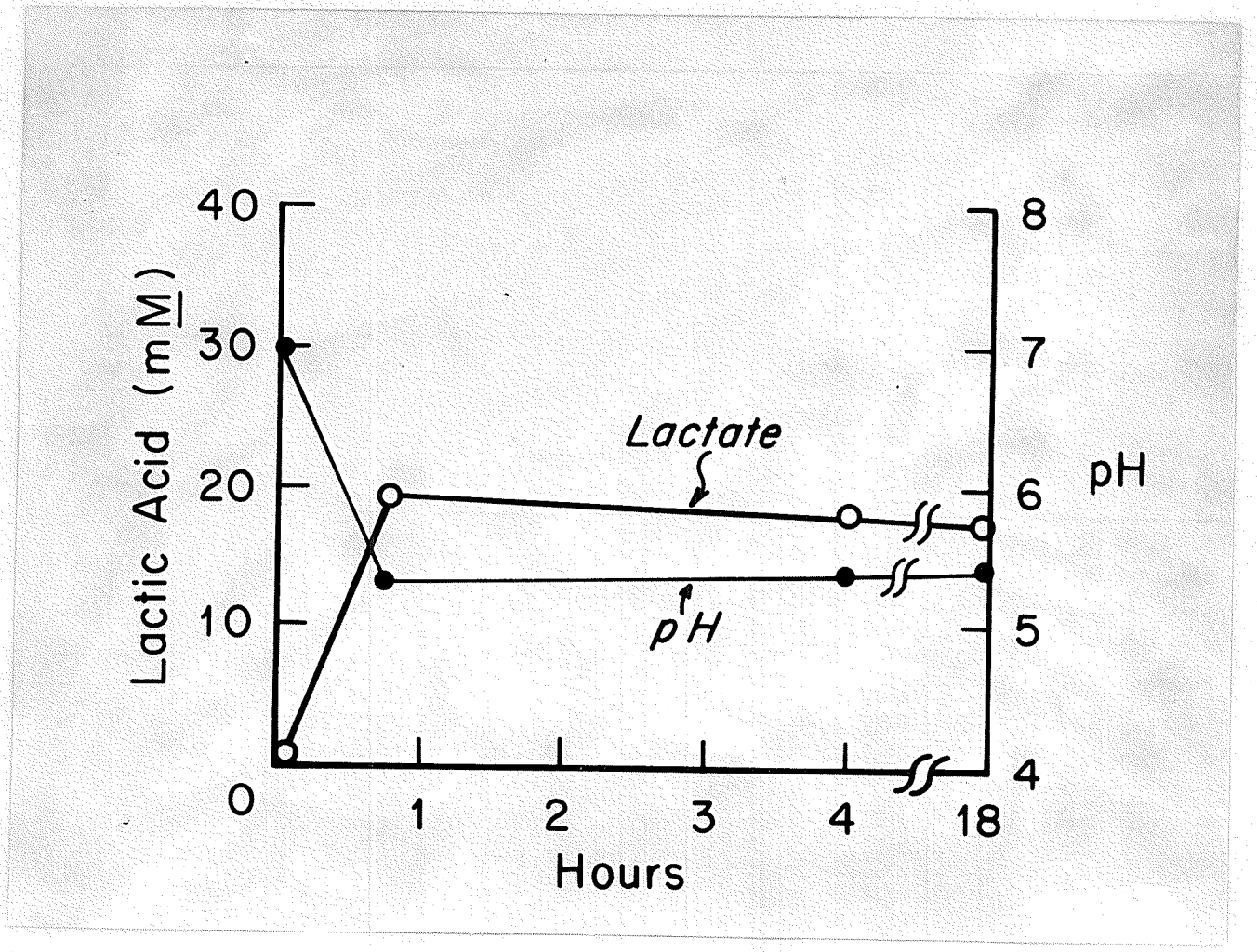

Figure 14. Changes in pH and lactic acid concentration under anaerobic incubation.

bacterial growth curve, and (b) during the utilization of a low initial concentration of glucose by a high concentration of cells.

Procedure

Cells of streptococcal strain AHT were grown as described in Chapter III. After the 18 hour growth period, total titratable acid and lactic acid were measured in the growth medium trypticase soy broth.

The cells were then harvested, washed, and incubated for 4 hours using a high concentration of cells (16.7%) and a low initial glucose concentration (13.9 mM) under an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide. The radioactivity in the cells and in the supernatant were determined as described previously in Chapter III. Carbon dioxide was measured by labelling the glucose used as substrate with a trace amount of glucose-U-C^{14}, trapping the evolved carbon dioxide in sodium hydroxide, and counting it by liquid scintillation, as described in Chapter III.

The volatile acids and alcohols present in the supernatant were analysed by gas chromatography. The samples were prepared as follows: Duplicate samples were withdrawn with a syringe pipette at 0 hour, 45 minutes, and 4 hours for pH and lactic acid determinations. Duplicate samples of the supernatant (5 µl) were also removed at 0 and 4 hours, acidified and then injected directly into the gas chromatograph.

Results and Discussion

Analysis of the 18 hour growth medium showed that under those conditions, streptococcal strain AHT was highly homofermentative, in that

97.6 per cent of the total titratable acidity in the broth was attributable to lactic acid.

However, when the cells were incubated with a high cell concentration and a low initial glucose concentration, lactic acid accounted for only 66.9 per cent of the products formed from glucose (Table II). Other products such as acetic, butyric, isobutyric, propionic, and ethanol were also formed (Table II; Figs. 15a and 15b). Nearly all the radioactivity from glucose-U-C^{14} remained in the supernatant after four hours of incubation; only a small amount of the label appeared in the carbon dioxide, and none in the cells (Fig. 16).

A material balance for the anaerobic dissimilation of glucose by streptococcal strain AHT was calculated (Table II). The values used were those obtained at 4 hours, minus the values at 0 hour. The carbon recovery was 100.3 per cent and the oxidation reduction ratio, 1.05.

The present study showed that with streptococcal strain AHT, like several other organisms, the relative amounts of lactic acid and other products formed during the breakdown of glucose can be altered by changes in the conditions of incubation, with less lactic acid being formed during growth than during a short incubation at high cell concentration (resting cell conditions).

While this manuscript was in preparation, Drucker and Melville (1968) reported that when they grew streptococcal strain AHT for 72 hours in glucose broth in an atmosphere of 95% nitrogen and 5% carbon dioxide, they found that formic, acetic and butyric acids and ethanol were produced, and that only 69.4 per cent of the glucose was fermented to lactic acid. The reason why they obtained a lower yield of lactic acid in a

TABLE II

END PRODUCTS OF GLUCOSE METABOLISM BY STREPTOCOCCAL STRAIN AHT

Product	mM of product per 100 mM of glucose fermented	mM of C_1	Meq. of	
			Oxidation (+)	Reduction (-)
Glucose	100	600	-	-
Lactic acid	146.94	440.82	-	-
Acetic acid	56.88	113.76	-	-
Carbon dioxide	11.85	11.85	47.50	-
Ethanol	5.53	11.06	-	22.12
Butyric acid	4.74	18.96	-	18.96
Propionic acid	1.58	4.74	-	3.16
Isobutyric acid	0.20	0.80	-	0.80
		601.99	47.50	45.04
		or		
		100.3%	O/R=	1.05

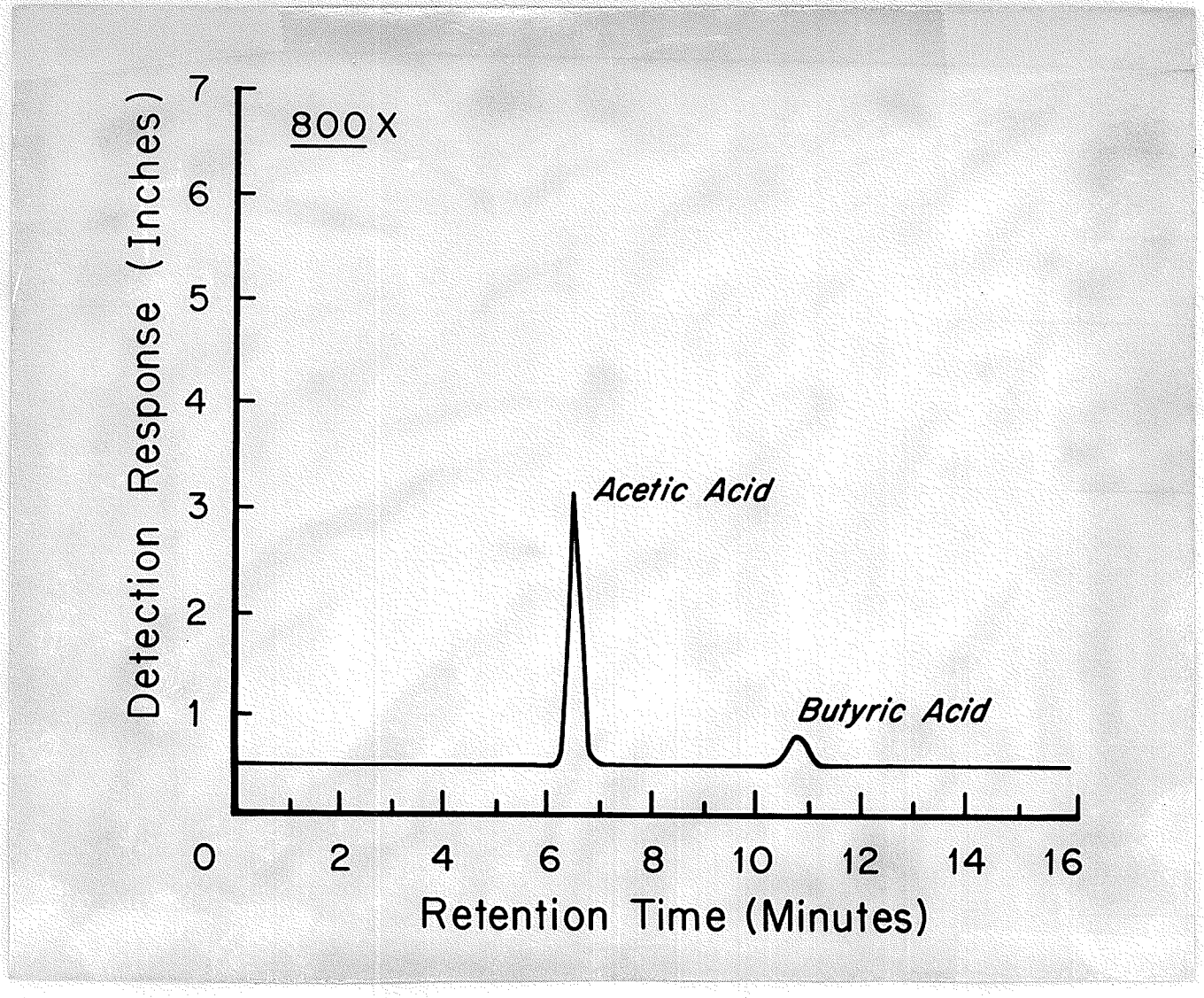

Figure 15a. Separation of the 4-hour supernatant by gas chromatography at an attenuation of 80.

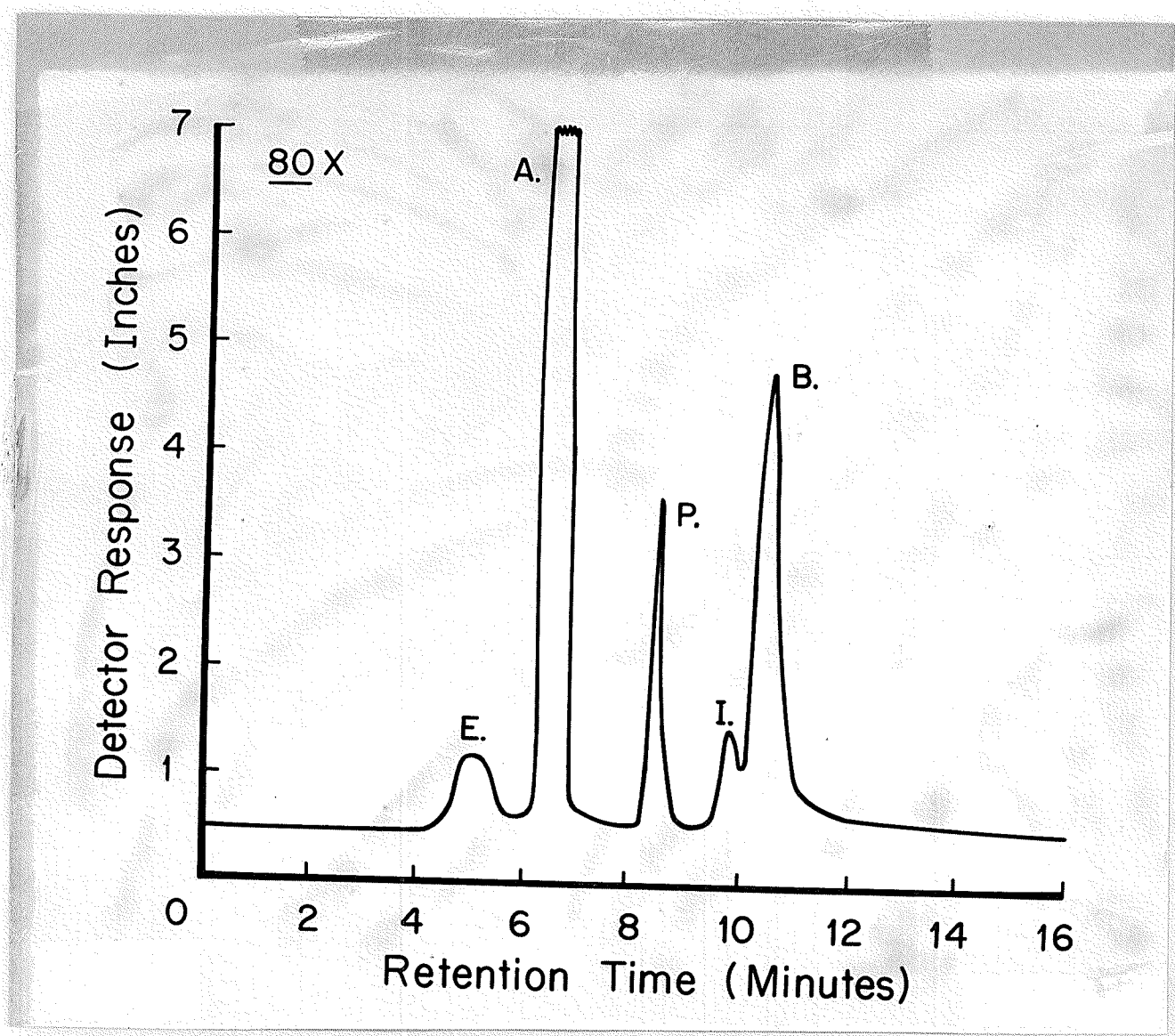

Figure 15b. Separation of the 4-hour supernatant by gas chromatography at an attenuation of 800.

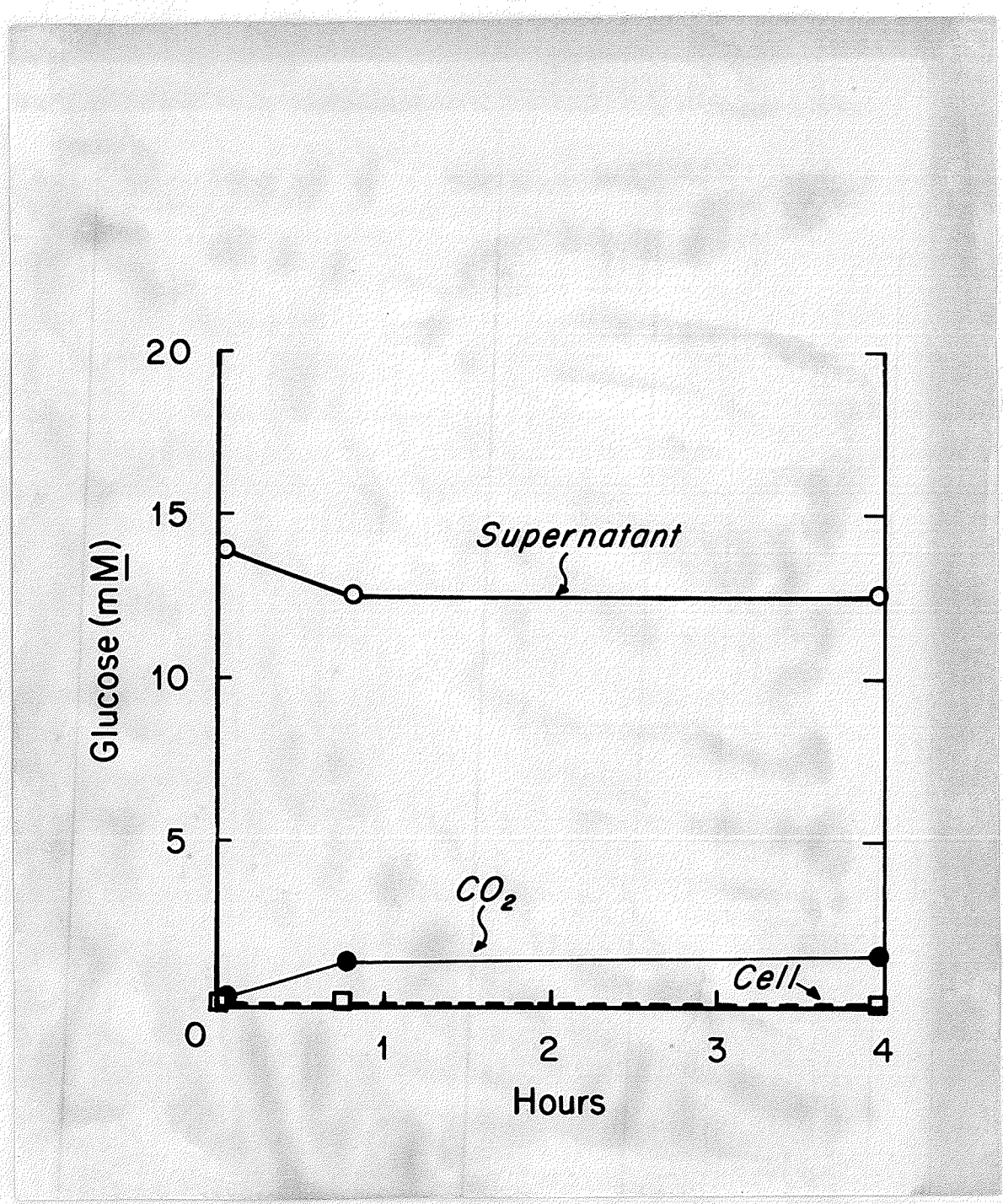

Figure 16. The distribution of glucose-U-C^{14} in the incubation medium.

growing culture than was observed in the present study is not clear at the present time.

I. CORRELATION OF VOLUME, DRY AND WET WEIGHTS, AND NITROGEN CONTENT OF CELL PREPARATIONS

As previously described, the amount of cells present in incubation mixtures was measured in terms of volume of packed cells. These cells were grown and prepared in an identical manner for every experiment in order that the cell preparations would not differ significantly between experiments. However, since many workers report their results in terms of wet weight, dry weight, or nitrogen, an experiment was carried out to measure these parameters in preparations of streptococcal strain AHT used in the present studies and to correlate them with packed cell volume.

Procedure

The cells used were those in an incubation mixture containing a 16.7% suspension of cells (V/V) supplied with 13.9 mM glucose, before and after four hours of incubation. The incubation mixtures were centrifuged at 2000 x g for 15 minutes, the supernatants removed and discarded, and the cells washed twice in ice-cold distilled water. Their wet weights were then determined on an analytical balance (Mettler model). Dry weight was determined after drying the samples overnight in an oven at 85° C (Pelczar, 1957, p. 172). Nitrogen analyses were carried out using Nessler's Reagent (Harleco Stable Dry-Pack Nessler's Reagent -- Folin and Wu, Hartman-Leddon Co., Philadelphia).

Results and Discussion

One µl of packed cells was found to have a wet weight between 1016 and 1025 µg, a dry weight between 44 and 46 µg, and a nitrogen content between 12.8 and 13.0 µg. There were no differences between determinations carried out on cells from 0 hour and from 4-hour incubation mixtures.

CHAPTER V

SUMMARY AND CONCLUSIONS

CHAPTER V

SUMMARY AND CONCLUSIONS

When a dense suspension (16.7%, V/V) of streptococcal strain AHT was incubated with a low level of glucose (13.9 mM), the pH fell rapidly, reached a minimum, and then slowly rose again. These findings were similar to those previously obtained by Stephan and Hemmens (1947) with a dense suspension of a pure strain of an unidentified oral streptococcus. Those workers had postulated that the pH rose in their system because lactic acid was being consumed. This stimulated and initiated our investigation into the metabolic processes associated with the pH rise with streptococcal strain AHT.

When cell concentrations of AHT were progressively increased from 0 to 33.3 per cent, and the initial glucose concentration kept constant at 13.9 mM, progressive increases occurred in both the rate of pH fall and the subsequent rate of pH rise. At the highest cell concentrations the rates of fall and rise in pH approached those which many workers have shown to occur in dental plaque *in vivo*. That the extent of the pH fall also progressively increased with increasing cell concentrations suggested two possibilities: (1) that some degradable polysaccharides were present in the cells used for preparing the incubation mixtures, or (2) the products formed from 13.9 mM glucose were more acidic at high than at low cell concentrations. In a subsequent experiment, a slight pH fall occurred when no glucose was provided to the cells, suggesting that (1) was the more probable. When no cells were included in the incubation mixtures, no change in pH occurred during incubation, demonstrating that bacteria

were responsible for the pH changes that occurred.

Subsequently, in a similar series of experiments, changes in pH and in lactic acid concentration were followed when the cell concentration was kept constant at 16.7 per cent (V/V), and the glucose concentration was varied (0, 13.9, and 278 mM). These glucose concentrations provided three basic types of results. The first occurred when no glucose was supplied to the cell suspension; no lactic acid formed and the pH fell by approximately 0.22 unit. As mentioned above, this pH fall probably resulted from the degradation of a small amount of carbohydrate in the cells. The second type of result occurred with a low initial glucose concentration (13.9 mM), when the pH fall and subsequent pH rise that occurred were accompanied by a rise and fall, respectively, in lactic acid concentration. Both the maximum lactic acid concentration and the pH minimum occurred at the same time, 45 minutes, indicating that pH was a sensitive reflection of lactic acid concentration. The lactic acid concentration subsequently decreased to zero by four hours, demonstrating that lactic acid was an intermediate and not an end product of glucose catabolism in streptococcal strain AHT. The third type of result occurred with what was apparently a non-limiting glucose concentration, 278 mM. With that glucose level, the concentration of lactic acid rose continuously and the pH fell continuously throughout the incubation period. The changes in pH that occurred with 13.9 mM glucose were similar to those occurring in dental plaque *in vivo* (Stephan, 1944; Kleinberg, 1961, 1964); more detailed examinations were therefore carried out on incubation mixtures containing that level of glucose.

In these experiments, the changes in pH, lactic acid, total acid

and glucose during the four hour incubation, were correlated. The results consistently showed that the disappearance of glucose from the medium occurred approximately 15 minutes after the concentration of lactic acid in the medium had started to fall. This observation was consistent with that of London (1968) who reported that Streptococcus faecium strain NCTC 7171 synthesized lactic oxidase at by far the greatest rate when glucose was almost gone from the medium.

The total acid rose with the lactic acid, reached a maximum simultaneously with the lactic acid, and then fell during the remainder of the experiment. The rate of fall of the total acid was much less than that of the lactic acid, so that an appreciable amount, approximately 50 per cent, of the total acid still remained in the medium after the lactic acid had disappeared. If such a situation prevailed in the dental plaque, acids other than lactic acid would be of great importance in the dental caries (Sandham, 1967).

To find out whether any components of the complex medium played a role in the observed changes in pH and lactic acid concentration, a series of experiments were carried out in which dense suspensions of streptococcal strain AHT were incubated in both trypticase and in phosphate buffered medium. Incubations carried out with 0, 13.9 and 278 mM glucose gave identical results in both the simple and the complex media, demonstrating that components of the complex medium were neither necessary for, nor contributed to, the processes.

To explore whether streptococcal strains other than AHT could utilize lactic acid, 18-hour cells of several other strains were tested. Strains from each of Lancefield's Groups C, F, G, and K resembled AHT

in that they showed a pH rise accompanied by a complete utilization of lactic acid before 18 hours of incubation. All other strains tested did not exhibit these properties. These strains may have been non-reactive because the conditions of study were not optional for demonstration of reactivity by those particular organisms. For example, recent unpublished results in this laboratory have shown that AHT, in the stationary phase of the growth curve, rapidly loses its ability to degrade lactic acid. Similar factors may have been operative in the present study. The number of strains that did utilize lactic acid, did so despite the fact that these strains had all been maintained under laboratory conditions for prolonged periods of time. Whether lactic acid utilization is more common among freshly-isolated streptococci remains to be determined.

Another series of experiments were carried out with streptococcal strain AHT, to determine the products to which lactic acid was being converted, using lactate-U-C^{14}. The glucose concentration used was again 13.9 mM. The results demonstrated that AHT degraded lactic acid to form acetic acid, carbon dioxide, and ethanol. The acetic acid explained the titratable acidity that remained in the medium after four hours of incubation.

In all the experiments described above, no specific attempt was made to exclude oxygen during incubation. When experiments were carried out in which lactic acid degradation was assessed under both aerobic and anaerobic conditions, lactic acid was found to be utilized in the presence, but not in the absence, of oxygen. This suggests that in dental plaque *in vivo*, streptococci could only utilize lactic acid as long as

oxygen were present. On the other hand, the utilization of oxygen by streptococci during the degradation of lactic acid could be a large factor in the production of the anaerobic state existing in dental plaque.

In another series of experiments, the end products of glucose fermentation were determined both in 18-hour growth medium and after a 4 hour incubation at a high cell concentration under anaerobic conditions. The results showed that lactic acid was the chief end-product, consisting of 97.6 per cent of the total acidity in the medium after 18 hours of growth, and 66.9 per cent of the products formed from glucose after 4 hours of incubation at a high cell concentration. Acetic, propionic, butyric and isobutyric acids, carbon dioxide and ethanol were produced in smaller amounts. When a material balance table was calculated from the data, the carbon recovery was 100.3 per cent and the oxidation reduction ratio, 1.05.

The present studies have attempted to increase our understanding of an interesting aspect of glucose catabolism, lactic acid utilization, in streptococcal strain AHT under a variety of physiological conditions. The presence of large numbers of streptococci in dental plaque suggests that this chemical activity may be of considerable quantitative importance in the metabolism of dental plaque.

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