STUDIES ON THE CARBOHYDRATE METABOLISM OF SALIVARY SEDIMENT

A Thesis

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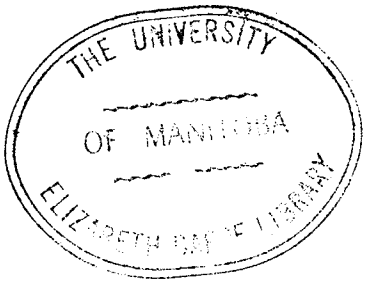
Doctor of Philosophy



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by

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CHAPTER I

INTRODUCTION

Dental plaque, the films of bacteria that readily form on the surfaces of the teeth, is generally considered to play a major rôle in the initiation of dental caries. The bacteria in these films can rapidly convert dietary carbohydrate to acid, causing the plaque pH to fall to some critical level (likely between 5.2 and 5.7, Englander, Carter, and Fosdick, 1956), below which the calcium and phosphate in the plaque and in the saliva are insufficient to prevent the solubilization of the enamel.

Extensive studies on changes in plaque pH *in situ* have been carried out (Kleinberg, 1961a), and these have been possible because plaque does not have to be sampled to make pH measurements. To follow other parameters of the metabolism of the plaque microflora *in situ*, such as carbohydrate storage, or changes in the concentrations of lactic or other acids in plaque, sampling for subsequent chemical analysis is necessary. The small amounts of plaque available and the irregularity of its distribution in the mouth make the obtaining of reproducible samples difficult. Because of these limitations on the *in vivo* studies of plaque, *in vitro* systems such as saliva collected by wax stimilation have been extensively used to study the metabolism of the oral floras. A highly reproducible sediment system has been prepared in

this way (Kleinberg, 1960; 1967), and was the system used in the studies in this thesis. To prepare the sediment system, saliva was centrifuged to obtain a cellular precipitate (the sediment) and a relatively cell-free supernatant. The sediment was then combined with the supernatant in a fixed ratio; this means of controlling the cell concentration ensured consistent experimental results.

Although differences exist in the types and relative numbers of microorganisms present in the microfloras in this sediment system and in plaque (Gibbons et al., 1964), the two systems show many metabolic similarities. The relation of pH fall to glucose concentration is the same in each of the two systems (Kleinberg, 1960; 1961; 1967).

On the other hand, the pH rises in the two systems when urea is metabolized, and the relationship between the extent of the pH rise and the urea concentration is the same in the sediment system as it is in the plaque system (Kleinberg, 1961b). Another metabolic similarity between the two systems is that both store carbohydrate while glucose is being catabolized (Sandham, 1963; Sandham and Kleinberg, 1964; Biswas, Vanry, and Kleinberg, 1965). Further similarities between the two systems are shown in this thesis.

Carbohydrate Substrates Utilized by the Oral Microflora

The cells in saliva can ferment a wide variety of

carbohydrates, including glucose, galactose, lactose, sucrose, maltose, fructose, mannose (Manly and Walborn, 1956), starch, raffinase (Eggers-Lura, 1955b), as well as cane sugar and white bread (Miller, 1890), and honey, maple sugar, and farina (Volker and Pinkerton, 1947). However, in the present thesis, the aim of which was to obtain information on the catabolic aspects of the carbohydrate metabolism of salivary sediment, glucose was the only carbohydrate studied. This carbohydrate was selected because it is fundamental to the carbohydrate metabolism of most cells. Consequently, studies with this sugar should serve as a basis for comparison with other carbohydrate substrates.

The carbohydrates (hexoses, hexosamines, fucose, and sialic acid) that are present in the salivary sediment system, as part of carbohydrate-protein complexes, can also be fermented by the oral microorganisms (Critchley and Leach, 1965).

Types of Fermentation Carried Out by the Oral Microflora

From the types of microorganisms found in the mouth, one would expect that the breakdown of carbohydrates by the microorganisms in saliva and in salivary sediment is mainly by fermentation. Fermentations are energy-yielding biological oxidation-reduction reactions in which organic compounds, rather than oxygen, serve as the final electron

acceptors (Stanier, Doudoroff, and Adelberg, 1963, p. 259).

Fermentations might be thought of as occurring in two steps, the first being the conversion of carbohydrate to pyruvic acid, and the second being the conversion of pyruvic acid into a variety of other compounds.

Conversion of glucose to pyruvic acid. The Embden-Meyerhof pathway (Embden, Deuticke, and Kraft, 1933; Meyerhof and Kiessling, 1933) was the first pathway for glucose breakdown to be discovered, and has been the most thoroughly studied. Later, other pathways were discovered, including the pathways usually referred to as the hexosemonophosphate and Entner-Doudoroff pathways (Horecker, 1953; Racker, 1954; Entner and Doudoroff, 1952).

The catabolism of glucose by the cells in saliva, salivary sediment, and plaque, has generally been assumed to occur via the Embden-Meyerhof pathway; the possibility that the hexosemonophosphate (HMP) and the Entner-Doudoroff (ED) pathway might also be functional has not been examined.

Further metabolism of pyruvate. The end products formed, when pyruvate is catabolized further, differ with different species of bacteria (Fig. 1.1). The products formed have sometimes been used as the basis for classifying the various types of fermentations. Stanier, Doudoroff, and Adelberg (1965, p. 263) classified fermentations into the following groups: (i) alcoholic, (ii) lactic (homolactic),

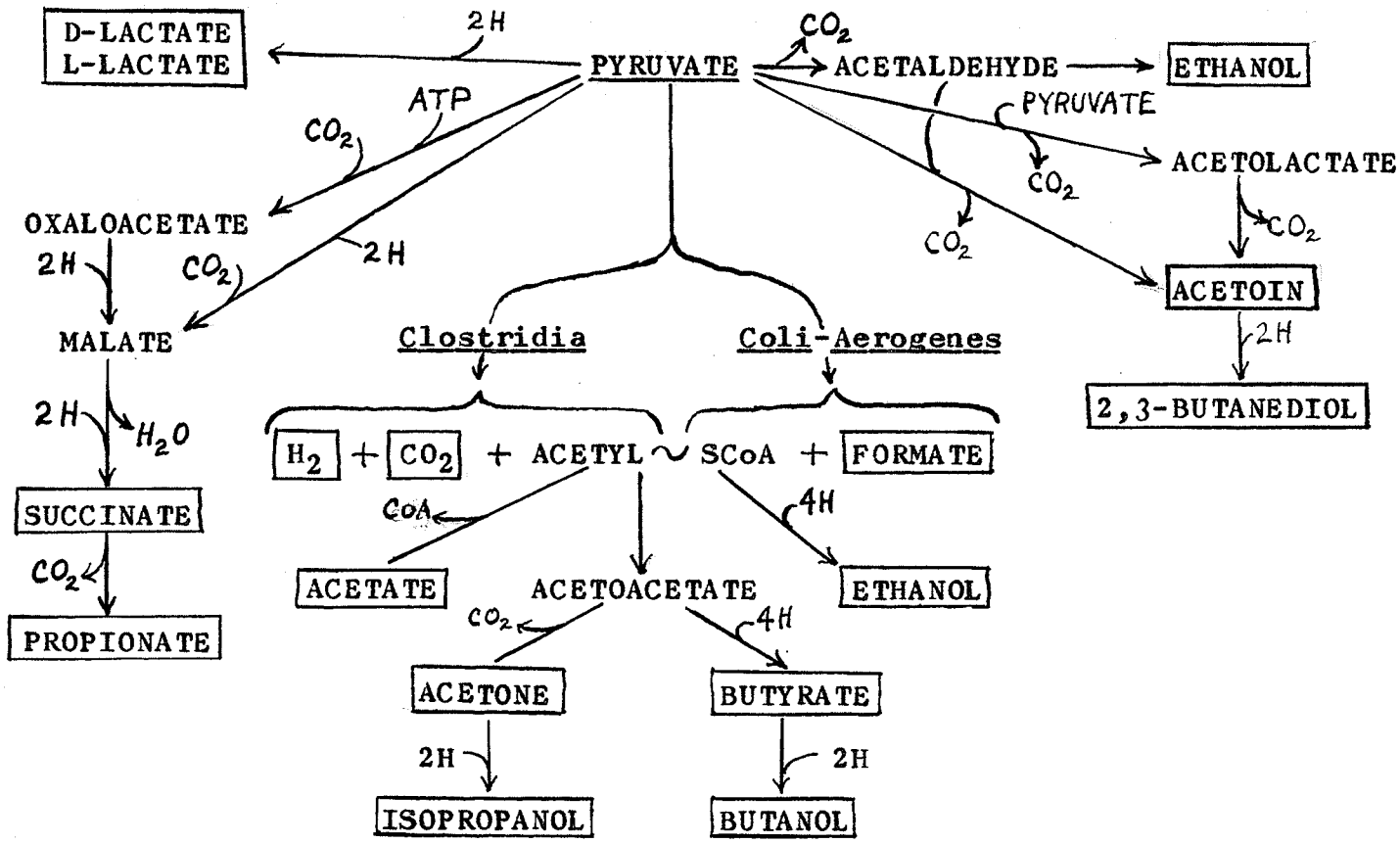


Figure 1.1. Fermentation product formation from pyruvate (From Wood, 1961)

(iii) mixed (heterolactic), (iv) mixed acid (e.g., Escherichia), (v) butylene glycol, (vi) butyric-butanol-acetone, and (viii) propionic. Most investigators who have studied glucose breakdown by the cells in saliva have assumed that the fermentation was homolactic, and therefore that lactic acid would be the major product (e.g., Tonzetich and Friedman, 1965). However, several groups of workers have demonstrated that other acids are also formed during glucose catabolism by the cells in saliva; these acids include pyruvic (Neuwirth and Summerson, 1951), citric (Cartier, Cartier, and Picard, 1952), malic, oxaloacetic, alpha-ketoglutaric, succinic, and fumaric acids (Neuwirth and Baerger, 1957). Little quantitative information on the formation of these acids has been reported. Recently, Guggenhein, Ettlinger, and Muhlemann (1965) demonstrated that paraffin-stimulated saliva contains five volatile fatty acids - acetic, propionic, butyric, isobutyric, and valeric, but did not show whether these acids could be formed from glucose.

Stanier, Doudoroff, and Adelberg (1965, p. 269) have pointed out that in many systems, intermediates such as pyruvate, alpha-ketoglutarate, fumarate, and citrate can temporarily accumulate during carbohydrate catabolism, because of factors tending to limit their rate of removal. They also stated that sometimes the accumulation is not temporary;

for example, pyruvic acid may accumulate to a level where it kills the cells and is left as the principal carbonaceous end product. Many of the acids that have been demonstrated in incubated glucose-saliva mixtures may have accumulated in this way, possibly as the result of incubation conditions.

A number of studies have also been done on the types of acids formed by plaque. Muntz (1943) found that when plaque was incubated with glucose in vitro, not only lactic, but also acetic, and what he considered to be pyruvic and propionic acids, were formed. Moore and his coworkers (1956) showed that lactic acid was produced from glucose by plaque in vivo, but they did not determine whether other acids were formed as well. More recently, a number of other workers have found that large quantities of lactic, acetic, and propionic acids were formed from glucose catabolism by dental plaque in vitro (Ranke et al., 1964; Gilmour and Poole, 1966). These studies indicated that, while the homolactic fermentation may be active in plaque, the propionic acid fermentation is also quantitatively important.

Oxygen Consumption by the Oral Microflora

Many workers have studied the carbohydrate metabolism of the oral microflora by manometrically measuring oxygen consumption (Hartles and McDonald, 1950; Bramstedt and Vonderlinn, 1953; Burnett, 1954; Krönke and Naujoks, 1954;

Eggers-Lura, 1955a, b, and c; Hartles and Wasdell, 1955b; Akizuki, 1958; Green, Kay, and Calandra, 1959; Szabo et al., 1960; Wasdell, 1962; Hartles, 1963). Oxygen consumption is generally considered to be a direct measurement of respiration, a class of biological oxidations in which molecular oxygen is the ultimate acceptor of the hydrogen and electrons (Stanier, Doudoroff, and Adelberg, 1963, p. 264). In many types of microorganisms and in higher forms of life, such as mammals, the electrons are transported to oxygen via a cytochrome system, and water is formed. In many other types of bacteria, no cytochromes are present, the electrons are transported directly from the substrate to oxygen via flavoprotein enzymes, and hydrogen peroxide is formed. While hydrogen peroxide can be formed in this manner by 58% of the bacteria in saliva, no peroxide accumulates because of the high peroxidase activity of saliva (Kraus, Walker, and Cook, 1955).

All investigators who have studied oxygen uptake by the microorganisms in saliva and sediment have found that oxygen uptake is high. This oxygen uptake has been suggested to be the result of Krebs cycle activity (Bramstedt et al., 1954).

Although most studies have shown that glucose markedly stimulates the uptake of oxygen by the cells of saliva, Eggers-Lura (1955b) and Szabo et al. (1960) found

that glucose sometimes inhibits oxygen uptake. Eggers-Lura claimed that glucose stimulated oxygen uptake in saliva from caries-resistant individuals, but inhibited oxygen uptake in saliva from caries-susceptible individuals. He offered no explanation for these results.

The non-cellular fraction of saliva (i.e., the supernatant after centrifugation) has been found to contain heat-stable factors essential for much of the oxygen uptake of saliva (Hartles and Wasdell, 1955), and many investigations have been concerned with the isolation and identification of these factors. A number of substances in saliva have been suggested, including amino acids (Wasdell, 1962), peptides (Bramstedt, Kronke, and Naujoks, 1957; Kronke et al., 1958; Hay and Hartles, 1965), and volatile fatty acids (Guggenheim, Ettlinger, and Muhlemann, 1965).

Many of the studies of oxygen uptake mentioned above were concerned with finding a correlation between oxygen uptake and susceptibility to dental caries (e.g., Bramstedt and Vonderlinn, 1953; Eggers-Lura, 1955; Akizuki, 1958; Green, Kay, and Calandra, 1959). In spite of the variations in the methods used to collect saliva in the different studies, nearly all of the investigators found that the oxygen consumption of saliva from caries-resistant individuals was greater than the oxygen consumption of saliva from caries-susceptible individuals. This has been interpreted to mean

that caries-resistant individuals have a more aerobic type of microflora than caries-susceptible individuals (Eggers-Lura, 1955). The difference in oxygen uptake has also been attributed to a lack of organic phosphate and phosphate acceptors in caries-active saliva (Bramstedt and Vonderlinn, 1953; Bramstedt, et al., 1954).

Endogenous Metabolism of the Oral Microflora

The workers who measured the oxygen uptake of saliva found that oxygen uptake was high, both in the presence and in the absence of added glucose. The oxygen uptake in the absence of added glucose was the result of what is referred to as endogenous metabolism. These processes are important to microorganisms, and for that matter to all living cells, because they enable the cells to sustain their activity during periods when exogenous nutrients are absent (Strange, Dark, and Ness, 1961).

Lamanna (1963) has defined endogenous metabolism as "the sum of all the chemical activities performed by organisms in the absence of utilizable extracellular materials serving as sources of energy and building stones for assimilation and growth". Such a definition is not always strictly adhered to, in that the definition has often been extended to include the utilization of metabolic intermediates, such as lactic acid and succinic acid, which are

excreted into the medium while glucose is being metabolized, and utilized after the glucose has disappeared from the medium (e.g., Streptococcus lactis, cited by Lamanna and Mallette, 1965, p. 698).

In the present study, endogenous substrates have been defined as all substrates, both intra- and extracellular, which are inherently present in the salivary sediment system, and endogenous metabolism was considered to be the metabolism of these substrates. These substrates would include the hexoses, hexosamines, fucose, and sialic acid present in the salivary gland secretions (Critchley and Leach, 1964), as well as the nitrogen-containing compounds such as amino acids, peptides, proteins, urea, and uric acid.

The intracellular substrates that are stored by bacteria when extracellular substrates are plentiful and then utilized when the exogenous carbohydrates are no longer available include glycogen (e.g., Strange, Dark, and Ness, 1961), amino acids and peptides (Ribbons and Dawes, 1963), proteins and nucleic acids (Campbell, Gronlund, and Duncan, 1963), and poly-B-hydroxybutyrate (Macrae and Wilkinson, 1958).

In salivary sediment, stored carbohydrate can serve as an energy reserve, since it rapidly accumulates when glucose is present in the medium and is degraded as soon as all the glucose in the medium has been utilized (Sandham, 1963;

Sandham and Kleinberg, 1964).

Bacteria may store more than one type of endogenous substrate, and then utilize each in sequence under the appropriate conditions. For instance, Sarcina lutea can store both amino acids and carbohydrate intracellularly, but preferentially utilizes the amino acids during endogenous metabolism (Ribbons and Dawes, 1963). In contrast, Escherichia coli and many other microorganisms utilize stored carbohydrate first, followed by amino acids from the amino acid pool.

The pathways utilized during the metabolism of endogenous substrates may or may not be the same as those utilized during the metabolism of exogenous substrates. Lamanna and Mallette (1965, p. 698) have cited evidence indicating that both processes may involve the same metabolic pathways. For example, in Streptomyces griseus, inhibitors affect the endogenous and exogenous respirations equally when a variety of substrates are used. Also, in a number of bacterial species, the presence of a normal exogenous substrate stops endogenous respiration. On the other hand, bacteria such as Streptomyces coelicolor, and several species of yeast, appear to carry out their endogenous and exogenous respirations by separate pathways. Their endogenous respiration is unaffected by the metabolism of an exogenous substrate.

The Effect of Exogenous Glucose on Cellular Respiration

The addition of a substrate, such as glucose, to cells engaged in endogenous metabolism, results in pronounced changes in their metabolism. One of these changes is often a decrease in the rate of oxygen uptake, known as the "Crabtree effect" (Crabtree, 1929). Two types of Crabtree effects have been observed in tumour cells. One occurs immediately after the addition of glucose, and is referred to as the "short-term" Crabtree effect; the other appears after 30 to 60 minutes of incubation, and is called the "long-term" or "classical" Crabtree effect. In the short-term Crabtree effect, glucose utilization is initially rapid and accompanied by increased respiration. Shortly thereafter, a period of intense inhibition of both glucose uptake and respiration occurs (Chance and Hess, 1961).

In the long-term, or "classical", Crabtree effect, the inhibition of respiration can be anywhere between 10 and 50%. One of the characteristics of the classical Crabtree effect is that the decrease in the ATP produced from respiration is exactly counterbalanced by the increase in ATP generated by glycolysis (Quastel and Bickis, 1959). In other words, the overall rate of ATP generation remains constant.

A variety of hypotheses have been advanced by various investigators to explain the Crabtree effect. In 1961 van

Eys divided the hypotheses into three types: (i) those based on a competition between respiration and glycolysis for phosphate metabolites such as inorganic phosphate, or ADP, (ii) those based on the addition of carbohydrate enhancing the operation of the hexosemonophosphate shunt, and (iii) those based on an inhibition of respiratory enzymes by a fall in intracellular pH resulting from glycolysis.

More recently, many additional regulatory mechanisms have been discovered which may contribute to the Crabtree effect. For example, the allosteric effects of various metabolites on the activity of the glycolytic enzymes (e.g., hexokinase and phosphofructokinase) have been recognized as possibly contributing to the effect (Sauer, 1964; Wu, 1965). Similarly, the effects of various metabolites on mitochondrial swelling and the resulting changes in the degree of coupling between oxidation and phosphorylation, may also be involved (e.g., Hess and Chance, 1961; Koobs and McKee, 1966).

Whether or not a Crabtree effect occurs appears to be to a large extent dependent on the presence of intermediates that are metabolized endogenously, such as lactate or pyruvate (Ibsen and Fox, 1965). The effect can be abolished by incubating the cellular system being studied for a period sufficient for the cells in the system to remove the pyruvate

and lactate initially present. Subsequent addition of either pyruvate or lactate restores the capacity of the cells to exhibit the Crabtree effect (Ibsen and Fox, 1965).

In addition, the Crabtree effect is abolished in a cellular system which is actively synthesizing glycogen, probably because the synthesis of glycogen provides the ADP or Pi necessary for respiration to proceed (Nigam, 1966).

Although usually associated with malignant cells, the Crabtree effect is also exhibited by the cells of the renal papillae, bovine cartilage, retina, bull spermatazoa, and leucocytes (van Eys, 1961). It is also present in pure cultures of some bacteria (e.g., Streptococcus lactis, Lamanna and Mallette, 1965, p. 699) and has been observed, but not recognized, in incubated saliva (Eggers-Lura, 1955b; Szabo et al., 1960).

The Effect of Fluoride on Cellular Metabolism

Although adding glucose to cells results in profound changes in their metabolism, even greater changes can be brought about by the addition of inhibitors. One of these inhibitors, fluoride, is of interest for the study of the oral microflora both because fluoride may inhibit carbohydrate metabolism in the oral microorganisms responsible for dental caries, and because of its usefulness as a tool for studying carbohydrate metabolism.

The exact mechanism of action of fluoride on cellular metabolism is not as yet known. Early concepts were based on observations that fluoride inhibited several isolated enzymes *in vitro*. For example, in 1934, Lohmann and Meyerhof demonstrated that fluoride inhibited the enzyme enolase. The action of fluoride on the whole cell has usually been attributed to its action on that enzyme, although many other enzymes, including catalase, glutamic synthetase, succinic dehydrogenase, acid phosphatase, pyrophosphatase, and phosphoglucomutase (Hewitt and Nicholas, 1963) have also been shown to be inhibited by fluoride.

One of the factors complicating the determination of the mode of action of fluoride on the intact cell is that its action on an isolated enzyme is not the same as its action on the same enzyme in the cell. For example, Slater and Bonner (1952) showed that the enzyme succinic dehydrogenase is less inhibited by fluoride when isolated than when it is part of the sequence of respiratory enzymes in the cell.

Fluoride has been shown to simultaneously inhibit glucose uptake, carbohydrate storage, and pH fall in salivary sediment (Sandham, 1963; Kleinberg and Sandham, 1964), as well as in *Streptococcus mitis* (Weiss et al., 1965). Sandham and Kleinberg have therefore suggested that the site of fluoride action on the whole cell may be in, or in association with, the cell membrane, rather than at enzymes

in the pathway leading to acid and polysaccharide formation. Evidence supporting this idea was that calcium and phosphate are found at the mitochondrial membrane in a stoichiometric relationship suggestive of the presence of hydroxyapatite (Lehninger, Greenawalt, and Rossi, 1963). If the same is true of bacterial membranes, fluoride might alter the solubility of these phosphate-containing complexes, and thus alter the availability of inorganic phosphate for intracellular processes such as the phosphorylation of glucose by hexokinase. Such a restriction on hexokinase activity would account for the decrease in glucose uptake, carbohydrate synthesis, and acid production observed by Kleinberg and Sandham (1964) and Weiss et al. (1965).

While the action of fluoride on glucose catabolism in the oral microflora is of interest because of its possible relationship to the ability of fluoride to prevent dental caries, fluoride was used in the present study primarily to assist in the study of the mechanisms controlling the processes involved in the catabolism of glucose.

Previous Studies on the Carbohydrate Metabolism of Salivary Sediment

The extensive investigations of the pH changes occurring in plaque *in situ* (Kleinberg, 1961; Kleinberg and Jenkins, 1964) and in salivary sediment *in vitro* (Kleinberg,

1962, 1967) was the basis for subsequent investigations, by the author, into other metabolic events associated with the metabolism of glucose by salivary sediment (Sandham, 1963; Sandham and Kleinberg, 1964; Kleinberg and Sandham, 1964). Because the studies in the present thesis are a continuation of these earlier studies, pertinent findings from the earlier studies will now be described.

Glucose, ranging in concentration between 0 and 30% (W/V), was added to sediment mixtures in which the sediment concentration was 16.7% (V/V), and the uptake of glucose, pH, and storage of carbohydrate, determined. When the concentration of glucose in the mixtures was 0.2% and below, the glucose in the supernatant was completely utilized before the end of the four-hour incubation period (Fig. 1.2). With 0.05% glucose, this occurred within 5 minutes, with 0.1% glucose, within 15 minutes, and with 0.2% glucose, within one hour.

Sediment carbohydrate initially rapidly accumulated (Figs. 1.3a and 1.3b). With glucose concentrations less than 5%, the relation between glucose concentration and the amount of carbohydrate that accumulated was direct; above 5%, the relation was inverse. The initial rapid rise in sediment carbohydrate concentration was followed by a more gradual fall when the initial glucose concentration was 0.2% or less, and by a plateau when the initial glucose concentration

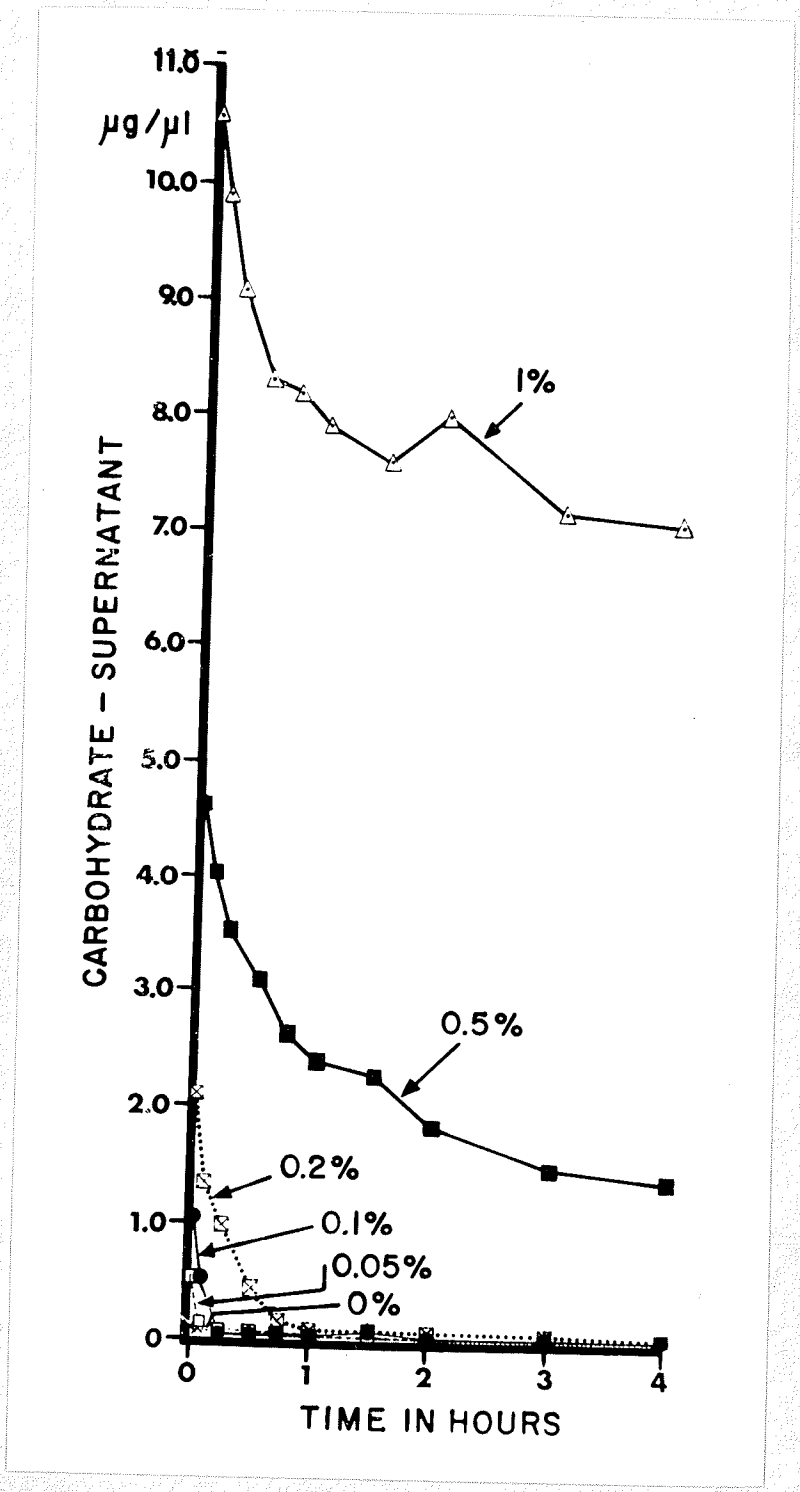


Figure 1.2. Changes in carbohydrate concentration in the medium during the incubation of salivary sediment with various concentrations of glucose.

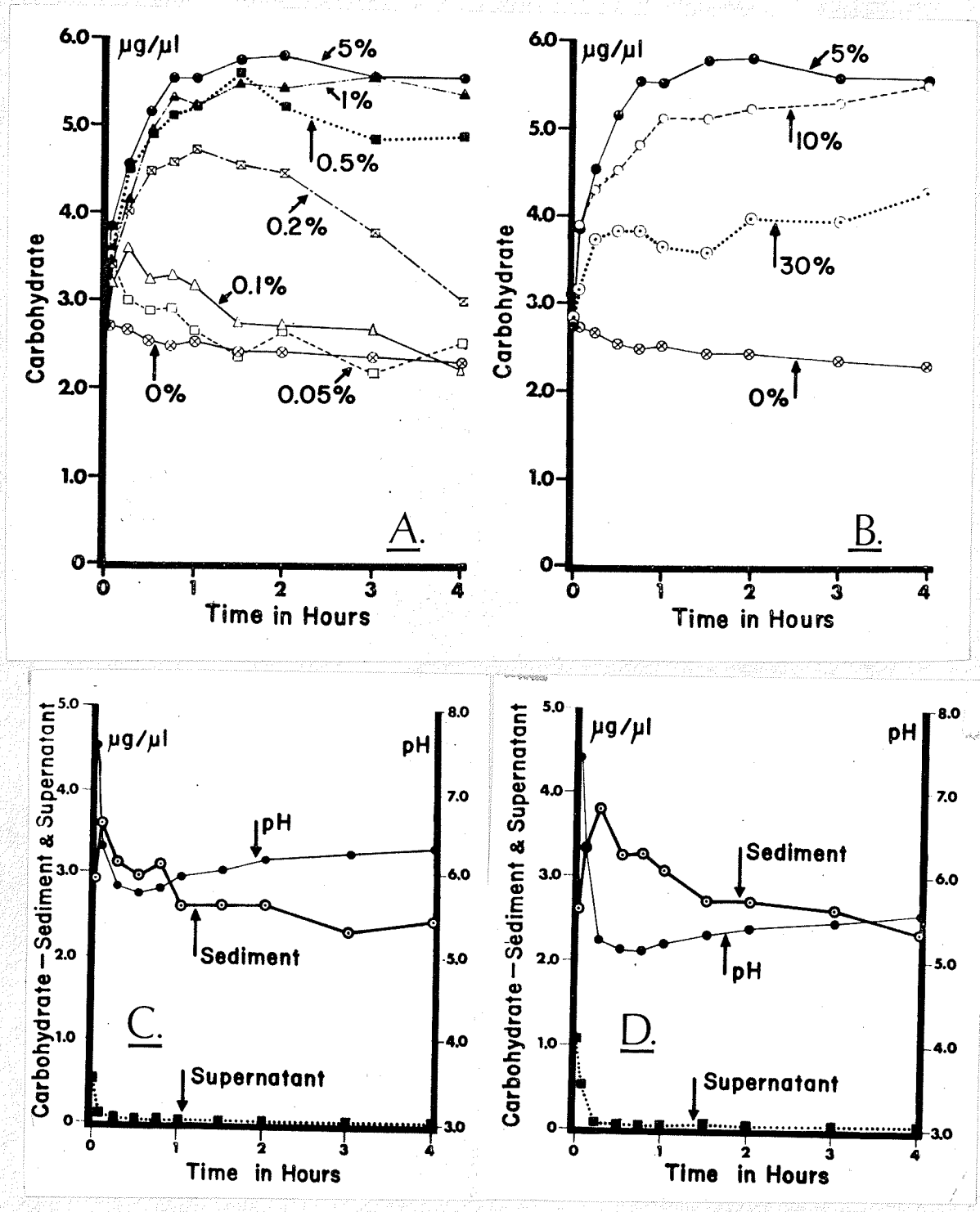


Figure 1.3. Carbohydrate storage in salivary sediment incubated with various concentrations of glucose: A., 0 to 5% Glucose; B., 5 to 30% Glucose. Changes in pH, glucose concentration in the medium, and sediment carbohydrate, with: C., 0.05% glucose; and D., 0.1% glucose.

was above 0.2%.

When the decrease in the supernatant glucose and the change in the level of stored carbohydrate were related to the pH in the mixtures containing glucose concentrations of 0.05% (Fig. 1.3c) and 0.1% (Fig. 1.3d), the time at which the level of stored carbohydrate was maximum, was the same as the time at which the glucose had disappeared from the medium. The fall in pH that occurred during the same interval did not reach its maximum until approximately 15 to 30 minutes after the time that the glucose had disappeared from the medium. It was concluded that the additional acid necessary to enable the pH to continue its fall must have come, at least in part, from the breakdown of stored carbohydrate.

Purpose and Outline of This Thesis

The purpose of the studies in this thesis was to further examine the processes involved in the metabolism of glucose by the cells in salivary sediment, and to deduce some of the factors controlling these processes. Because of the important role that pH changes probably play in the initiation of dental caries, all metabolic changes were correlated with the changes in pH that occurred.

The studies that were carried out were: (i) the formation of carbon dioxide (Chapter III), (ii) the formation

of lactic and other acids (Chapter IV), and (iii) the effects of several concentrations of fluoride on the formation of CO_2 and acid (Chapter V). Because of the possibility that the catabolism of amino acids might have been responsible for, or contributed to, the pH rise that occurs when glucose is added to the sediment system in limiting amounts, the effect of the various amino acids on the pH curves produced in sediment mixtures was investigated in a study reported in Chapter VI.

Methods used in the different studies in the thesis are described in Chapter II, and methods pertaining to the studies in specific chapters of the thesis are described in the methods sections within the appropriate chapters. Because the thesis as a whole has many implications in regard to manometric techniques, these are discussed in Chapter VII. A summary and statement of the conclusions in Chapter VIII complete the thesis.

CHAPTER II

METHODS

1. Preparation of the Incubation Mixtures

The saliva secreted during the chewing of paraffin wax was collected from six or seven fasting (i.e., pre-breakfast) subjects to provide sufficient sediment for each experiment. For each series of experiments, the same group of subjects was used, and each subject was instructed not to brush his teeth on the morning of each experiment.

The saliva was collected in 25 x 150 mm test tubes cooled in cracked ice. The collection and all subsequent preparative procedures were done at 0 to 4°C to limit cellular metabolism. The saliva was then centrifuged at 1470 x g for 15 minutes, resulting in a sediment fraction and a supernatant. The supernatant from one subject, the same subject throughout the study, was saved and kept at 0 to 4°C until required, while the other supernatants were discarded. The sediments were suspended in cold distilled water, combined, and then washed twice, also with distilled water. The sediment was then made up as a 50% (V/V) suspension in distilled water.

Incubation mixtures were prepared by combining, in a 10 x 75 mm test tube, 50 µl of each of the following: salivary sediment, salivary supernatant, and the substance or substances being studied. Each tube was sealed with

parafilm or, in the experiments where CO_2 was measured, with a serum stopper.

2. Incubation Procedure

In each experiment, a large number of incubation mixtures were incubated at 37°C in a water bath. In the majority of the experiments, the incubation was for four hours. The variables measured during the incubation were pH, CO_2, $C^{14}O_2$ from labelled substrates, lactic acid, total titratable acid, and, in some cases, C^{14}-labelled compounds in the medium and C^{14}-labelled volatile fatty acids. To obtain these measurements, duplicate tubes were removed at regular time intervals from the water bath. pH was measured immediately, whereas tubes for other types of analyses were placed in ice to halt metabolism, and analyzed within two hours. When CO_2 or $C^{14}O_2$ were determined, 100 µl of 0.1 N HCl was injected into the medium with a hypodermic syringe; the resulting low pH in the medium (below 2.3) served both to inhibit metabolism and to release CO_2 from the medium.

The measurement of pH was used in these, as in other studies of this system, as a reference.

3. Analytical Procedures

a. Measurement of pH. pH was measured with a glass electrode (Beckman #39167) and a salt bridge made of a fine polyethylene tubing fitted with a glass plug to minimize

salt leakage (Stephan and Hemmens, 1947; Kleinberg, 1963). The electrode and bridge were small enough to fit into each test tube.

b. Titratable (or Total) Acid. The titratable acid was determined as follows: Each mixture was back-titrated with standardized sodium hydroxide to pH 8.2, using a combined indicator--bromthymol blue and cresol red. The values for the mixtures at zero time were subtracted from the values for each time point to give the titratable acid values.

It was necessary to apply a correction factor to allow for the carbon dioxide lost from the mixtures as the pH fell during incubation. This loss of CO_2 amounts to a loss in buffering capacity which would not be measured by back-titration. The magnitude of this carbon dioxide correction factor was determined as follows. Gradually increasing quantities of standardized hydrochloric acid were added to a series of sediment mixtures and to a series of empty tubes. The tubes were then put on ice for $\frac{1}{2}$ hour so that no or little metabolism would take place in the tubes containing the sediment mixtures. pH was then measured in the mixtures and then both the mixtures and the tubes containing HCl were titrated to the pH 8.2 end point of the mixed indicator described above. The "carbon dioxide correction factor"

was the difference between the amount of base required to back-titrate the acid alone and the same amount of acid added to a sediment mixture. Since this factor varies with the pH of the sediment mixture (smaller at higher than at lower pH), a graph relating the correction factor to the pH of the mixture was constructed, and used routinely in the determination of titratable acid (Fig. 2.1).

c. Lactic Acid. Described in Chapter IV.

d. Hetero Acid. Hetero acid, a term used in this thesis to describe the acids other than lactic acid that were produced, was the difference between the titratable acid and the lactic acid. Hetero acid included both volatile fatty acids and non-volatile organic acids, the former being in vast majority.

e. Volatile Fatty Acids. The volatile fatty acids formed in the sediment mixtures were analyzed by gas chromatography. The preparation of the samples was as follows. Two 300 µl incubation mixtures containing 0.5% glucose were incubated for one hour, at which time their pH had reached 4.48. The mixtures were then centrifuged for 15 minutes at 1470 x g, and 250 µl of supernatant removed from each mixture and placed on columns containing 300 µl of the cationic exchange resin Dowex 50 in the H^+ form.

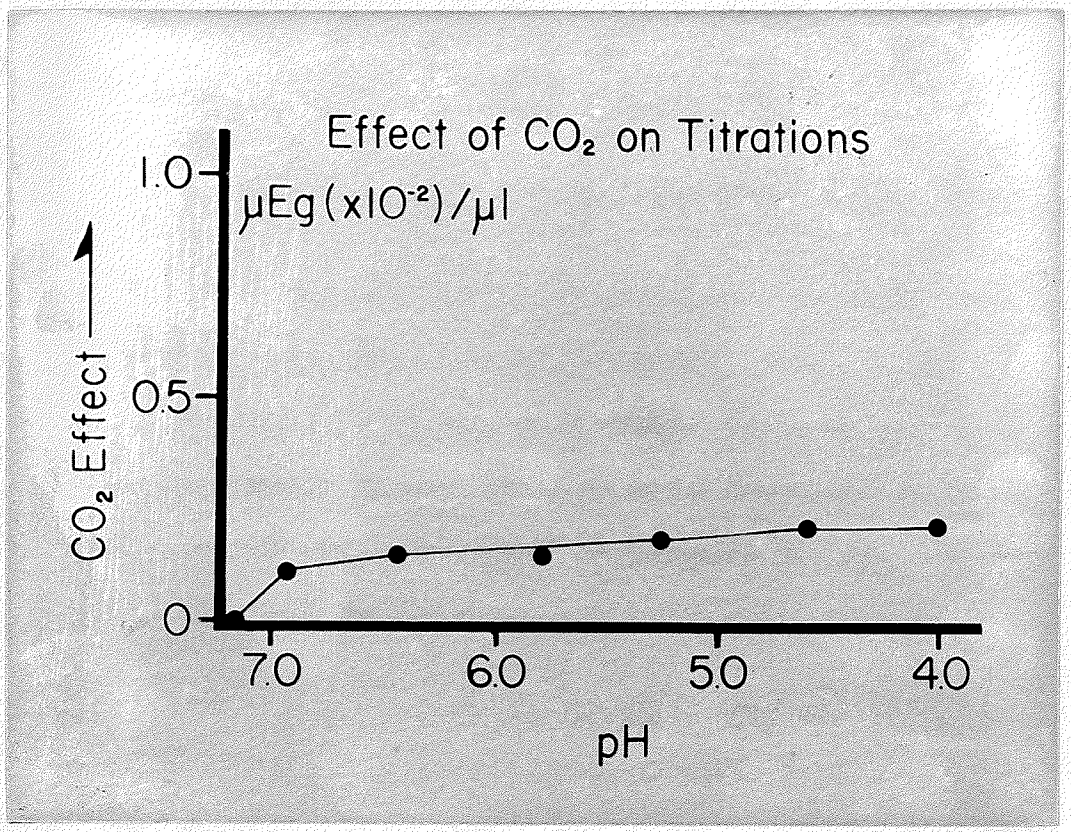


Figure 2.1. Effect of CO_2 loss on the determination of acid production by titration.

The dimensions of each column were 5 mm (I.D.) x 200 mm. The resin in the column, previously recycled between the Na^+ and H^+ forms, was washed with distilled water until the pH of the effluent was neutral. The samples were passed through the columns to remove cations such as amino acids; these were retained on the columns. The samples were then placed on anion exchange columns, containing Dowex 3 in the $CO_3^=$ form, where any volatile fatty acids would be retained. The effluents from the anion exchange columns were saved for the estimation of what has been termed "neutral products" (see Chapter IV). The volatile fatty acids (as well as any other negatively-charged compounds) retained on the Dowex 3 columns were then eluted with 0.05 M Na_2CO_3. The eluted samples were then dried with a stream of filtered compressed air. During drying, the tubes containing the eluted samples were kept in a warm water bath (approx. 40°C) to speed evaporation of the excess water. The sodium salts in the dried samples were dissolved in 100 µl of 2 N HCl, to convert them to their acid forms in preparation for gas chromatography.

The gas chromatograph used was an Aerograph Hy-Fi Model 600-C equipped with a column consisting of a 6' x 1/8" stainless steel tube containing a stationary phase of 20% Neopentyl glycol succinate and 3% H_3PO_4 on a support of 60-80 mesh GasChrom R (screened and water-washed Sil-O-Cel

C22 Firebrick); the column was supplied and pre-tested by the manufacturer (Applied Science Laboratories, State College, Pennsylvania). The effluent from the column was monitored with a hydrogen-flame detector. The temperature of the column was maintained at 142°C.

As reference, 0, 0.016, 0.031, 0.063, and 0.125 μmoles of each of formic, acetic, propionic, and butyric acids, were injected into the chromatograph with a Hamilton syringe. "Baker Analyzed" grade reagents were used. Retention times for the acids used as standards were: acetic- 7 min, propionic- 11 min, and butyric- 12.5 min. Formic passed through too quickly to be measured. Duplicate 10 μl aliquots of the samples prepared from the mixtures were then injected into the gas chromatograph.

The acids separated were recorded automatically with a one-millivolt Honeywell recorder, identified, and quantitated. Quantitation was done by measuring the areas under the peaks of both acid standards and unknowns with a planimeter.

f. Non-Volatile Organic Acids. To test for the presence of non-volatile organic acids in some of the mixtures, the following procedure was followed. The mixture was centrifuged at 1470 x g for 10 minutes, 120 μl of supernatant removed, neutralized with sodium hydroxide, and then spotted on Whatman No. 1 paper and separated by

two-dimensional chromatography (Smith, 1960, p. 273). Lactic, succinic, and citric acids were run as standards. The first solvent consisted of 120 ml of n-butanol, 30 ml of acetic acid, and 50 ml of water; the second solvent consisted of 160 ml of ethanol, 10 ml of ammonia, and 30 ml of water. The acid spots on the paper were located with sulfanilamide reagent (Schmidt, Fischer, and McOwen, 1963).

g. Carbon Dioxide. In the experiments in which CO_2 was measured, as mentioned above, the tubes were sealed with serum stoppers. This was to permit the suspension of a teflon cup containing NaOH above the incubation mixture to catch evolved CO_2 and/or CO_2 released from the mixture upon the addition of the HCl introduced through the stopper to stop the reaction (Fig. 2.2). Stainless steel orthodontic wire, 0.022 inches in diameter, was used to suspend the teflon cup and was designed to fit tightly into a hole drilled in the teflon. The cup could still be easily pulled off the wire to facilitate analysis of its contents and subsequent washing of the cup. The cup contained 50 µl of 0.1 N sodium hydroxide and a mixed indicator. The concentration of the mixed indicator in the sodium hydroxide in the cup was 5% (V/V) and consisted of 3 parts thymol blue and 1 part cresol red (Vogel, 1961, p. 250).

To ensure the complete diffusion of the CO_2 into the

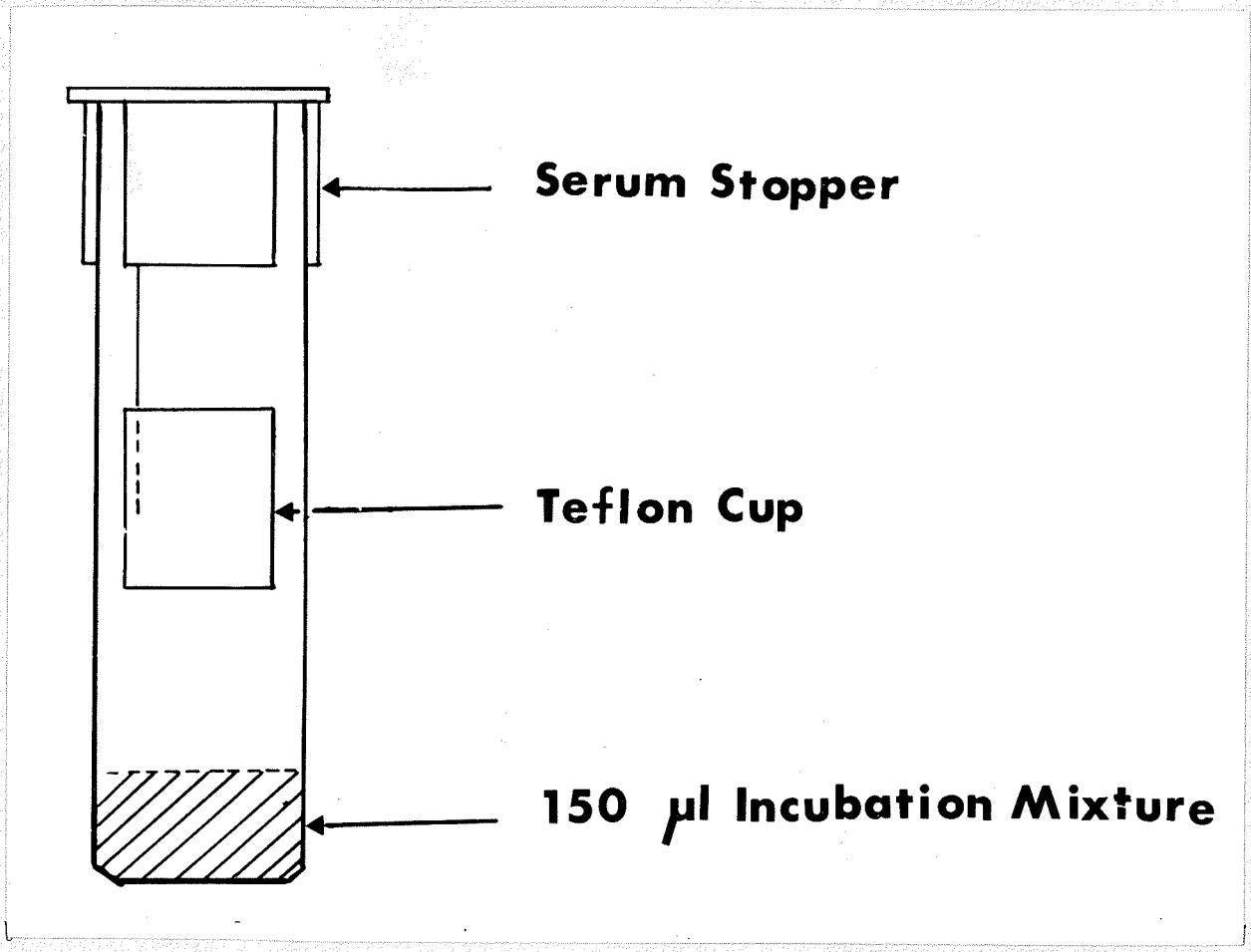


Figure 2.2. Apparatus for collecting CO_2 from sediment mixtures.

sodium hydroxide, the tubes were kept for six hours before the CO_2 in the cups was determined. Preliminary experiments showed that diffusion was complete by four hours. The contents of the teflon cups were then titrated to the wine-coloured end point (pH 8.2) with 0.1 N hydrochloric acid in a microburette (Gelmont, 0.250 ml capacity). The titration was done slowly with constant mixing, care being taken to keep the tip of the burette beneath the surface of the liquid to avoid any loss of carbon dioxide. The colour of the indicator slowly changed from a violet colour at pH 8.4, to blue at pH 8.3, to a wine colour at the pH 8.2 end point.

The pH change during titration of the carbonate system is gradual at the end point (Fig. 2.3), and the colour change was not sharp despite the use of the mixed indicator. To increase the accuracy of the analysis, tubes containing standard amounts of bicarbonate were included in each experiment and were handled in the same manner as were the tubes containing the glucose-sediment mixtures, including injection of acid, diffusion, and collection of the carbon dioxide in sodium hydroxide. The amounts of bicarbonate used for the standards were 0, 1, 2, and 3 μmoles of bicarbonate. The zero standard was run in quadruplicate, the others in triplicate. The titration of these enabled the construction of a standard curve relating titration figures to carbon dioxide concentration, from which carbon dioxide

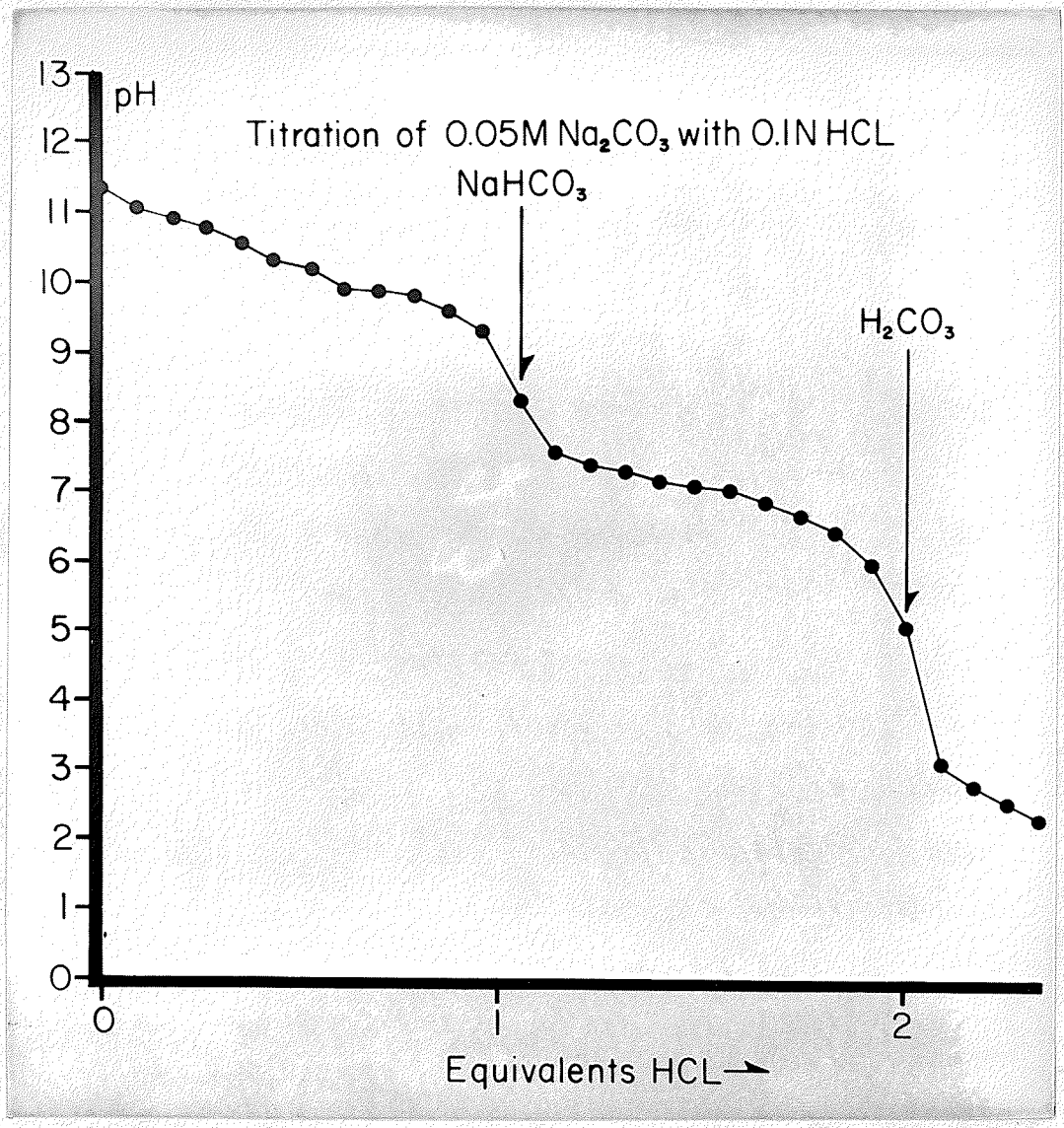


Figure 2.3. Titration curve for sodium carbonate.

produced by sediment mixtures could be calculated.

One μmole of CO_2 was taken as equal to 1 μequivalent of HCl, not 2 μequivalents of HCl for the following reasons. Each μmole of carbon dioxide absorbed by the sodium hydroxide would neutralize 2 μequivalents of sodium hydroxide, according to the following equations:

$$NaOH + CO_2 \text{ ----------} \rightarrow Na^+ + HCO_3^-$$

$$NaOH + HCO_3^- \text{ ---------} \rightarrow Na^+ + CO_3^= + H_2O$$

Sum: $$2\ NaOH + CO_2 \text{ --------} \rightarrow 2\ Na^+ + CO_3^= + H_2O$$

If x μmoles of carbon dioxide were absorbed by the sodium hydroxide, $2x$ μequivalents of base would be neutralized. If b μequivalents of sodium hydroxide had originally been in the cup, then the amount of OH^- remaining in the solution would be $(b - 2x)$ μequivalents. To titrate this base to pH 8.2 (the bicarbonate end point), $(b - 2x)$ μequivalents of acid would be required to titrate the remaining OH^-. In addition, x μequivalents of acid would be required to titrate the x μmoles of $CO_3^=$ to the bicarbonate end point, since

$$CO_3^= + H^+ \text{----------} \rightarrow HCO_3^-.$$

The μequivalents of acid required for titration (y) would then be equal to $\left[(b - 2x) - x\right]$, which simplifies to $(b - x)$.

Since $y = b - x$, then x, the μmoles of carbon dioxide in the sample, would be equal to $b - y$. Titration of the bicarbonate blank provides the value for b, while y is obtained by titrating the sample.

Precision of the method. In this thesis, a series of five experiments were run to determine the effect of glucose concentration on carbon dioxide formation; the results from these experiments were used to calculate the error of the method. As mentioned above, the standards in each experiment included quadruplicate tubes containing no bicarbonate, and triplicate tubes containing each of 1, 2, and 3 μmoles of bicarbonate. All of the determinations of bicarbonate standards done in the series of experiments were used to calculate the standard deviation.

In each experiment, the mean values of the triplicate and quadruplicate determinations at each bicarbonate level, were used to construct a graph relating the amount of acid added during the titration to the amounts of bicarbonate supplied. The deviation of each individual determination from the mean of the triplicate or quadruplicate determinations of which it was a component, was calculated. The total number of individual determinations made at each level of standard bicarbonate during the series of experiments were: 0 μmoles-19, 1 μmole-14, 2 μmoles-14, and 3 μmoles-13. Ideally, these numbers would have been 20, 15,

15, and 15, but the occasional determination was lost because of overshooting the end point during the titration. Once the end point was passed, it was not possible to accurately back-titrate, and the titration could not be completed. The large number of determinations made, however, tended to compensate for the loss of the occasional determination.

When the deviations described above were used to determine the standard deviation for bicarbonate standards, the standard deviation was ± 0.072 µmoles. When triplicates were averaged, the standard error of the mean was ± 0.042 µmoles.

To calculate the precision of the method in determining carbon dioxide formation from sediment mixtures, deviations from means were again calculated. However, with these determinations, only duplicate determinations were run at each time point. Therefore, the mean was the mean of two determinations. When 60 randomly-chosen determinations of carbon dioxide production analyzed for their precision, the standard deviation of individual determinations from the mean of the set of duplicates of which the individual determination was a member, was ± 0.065 µmoles.

h. C^{14} Determination by Liquid Scintillation.

Liquid scintillation counting is a convenient procedure for

detecting and counting the radioactivity from low-energy beta-emitters such as carbon-14. The C^{14} compounds being measured are dissolved in an organic solvent such as toluene, containing a fluor--a compound, such as 2,5-diphenyloxazole (PPO), which fluoresces when activated by a beta particle. The energy from the electrons emitted from the C^{14} in the sample which is being counted is thus converted to light energy by the fluor. The photons produced reach either of two phototubes, producing a charge pulse which can be amplified and counted by a scaling circuit. The high efficiency of the system in the counting of beta emitters is largely due to the fact that the beta rays, which rapidly lose their energy when passing through matter, are converted to light energy, which is able to pass from the sample to the detector with little energy loss.

Liquid scintillation counting is a proportional counting method in that the magnitude of the output signal from the photomultipliers (pulse height) is proportional to the energy given up to the photomultipliers by the primary particle. The phototube amplification factor is a very sensitive function of the voltages applied to the photomultiplier tubes ($A \propto E^8$), where A is the amplification factor and E is the voltage applied. The operator varies the voltage applied to the photomultiplier tubes to control amplification. Efficiency of counting is therefore dependent

on both the energy of the primary particle and the amplification.

By custom, the voltage applied to the photomultipliers is referred to as the "high voltage", and the two multiplier tubes are names "data" and "gate".

Counting method. By using a differential counting technique, accurate counting of samples in which the ratio of sample to background counts was low, was possible. In this technique, all pulses are counted if they fall between a lower level and a higher level discriminator, thereby discriminating against the background pulses which have higher energies than those in the sample.

With any given discriminator setting, quenching, by lowering pulse heights, can cause many of the pulses originating from the sample to be rejected by the lower discriminator, resulting in lower counting efficiency. The pulse heights can be increased, and the counting efficiency restored, by increasing the voltages applied to the photomultiplier tubes. This technique, whereby the high voltages are adjusted to give maximal counting efficiency between two discriminator levels, is known as the "balance point procedure". The voltages chosen represent the "balance point", where efficiency is not only greatest, but also least sensitive to small changes in either photomultiplier gain or sample quenching.

The "balance point procedure" was carried out in the present study, using a radioactive carbon dioxide-containing sample of the same composition as those to be counted in the experiments. The results, shown in figure 2.4, led to the choice of operating settings of 1360 V. for the Gate Voltage and 1495 V. for the Data Voltage.

Methods of calculating counting efficiency. In most instances, the efficiency of counting was calculated by the channels ratio method. This method uses a fixed base level plus two variable discriminators and two scalers to count in two channels simultaneously. In other words, the total counting window is divided into two windows. One scaler counts both windows, while the other counts only the window at the lower-energy end. Because quenching causes a decrease in pulse heights, it causes a shift of the counts into the lower energy level, where the smaller window is located. The increase in counts collected in the small window results in an increased ratio of counts in the small window to counts in the large window. This ratio (R_1) is known as the quenching correction ratio.

Using the operating settings determined above, a series of vials all containing known amounts of radioactive toluene but different amounts of a quenching agent, acetone, were counted and the relation between efficiency and R_1 plotted. The resulting curve (Fig. 2.5) was used to

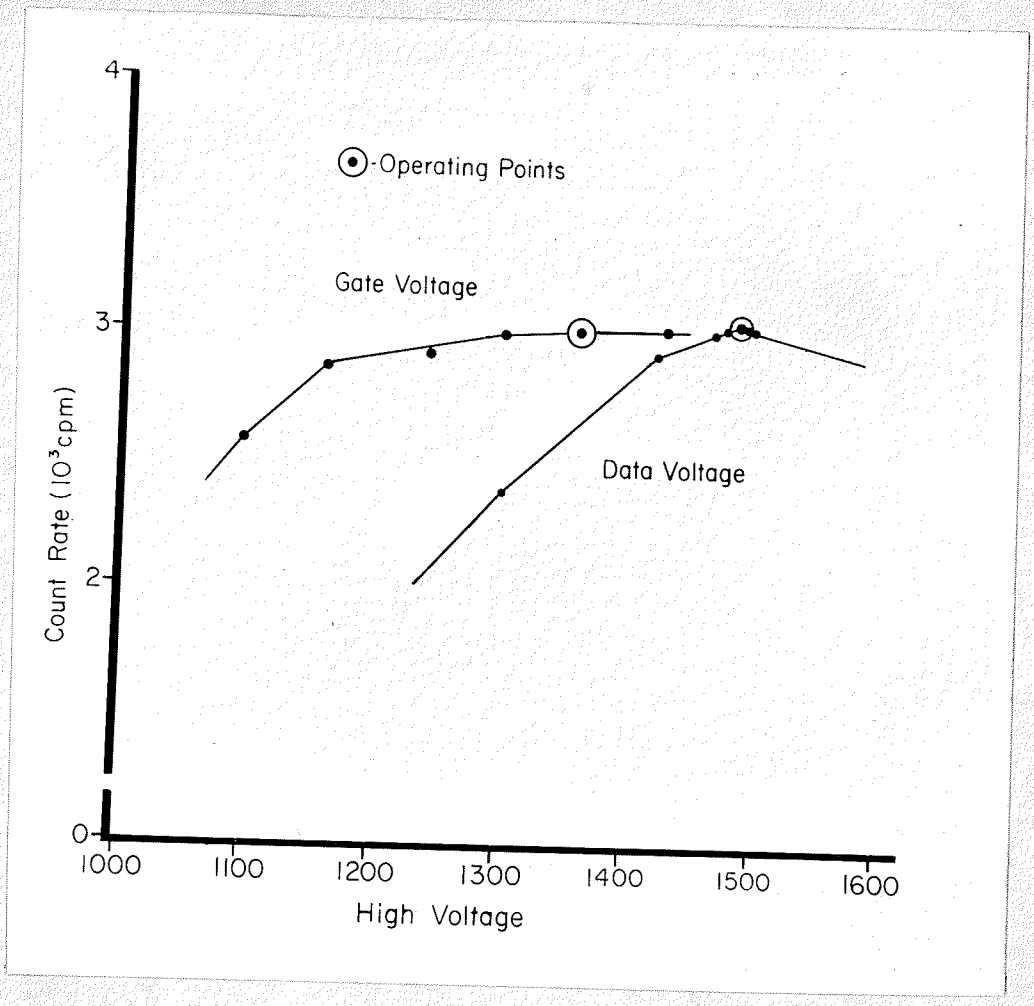


Figure 2.4. Results of carrying out the balance point procedure for counting $C^{14}O_2$-containing samples by liquid scintillation.

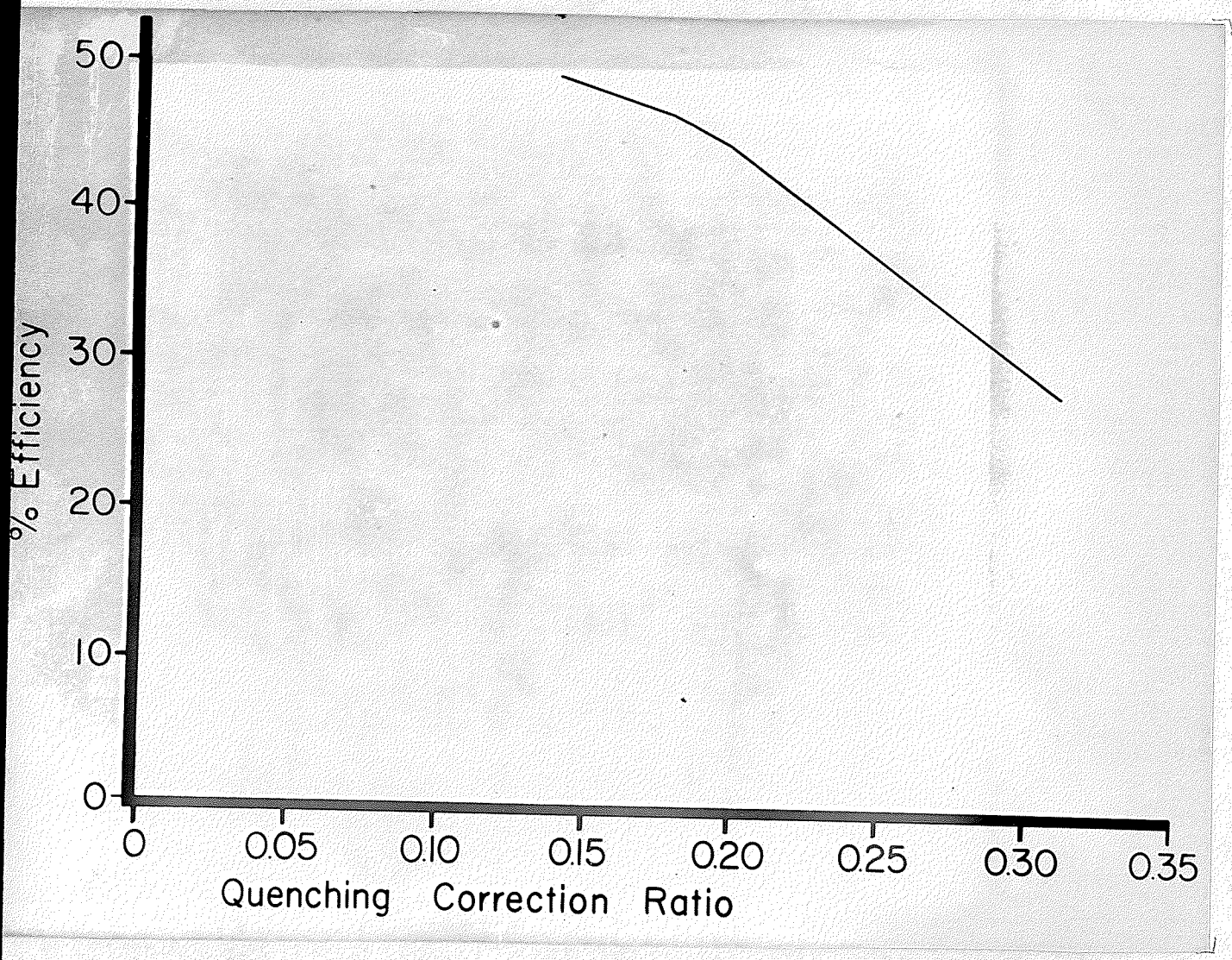


Figure 2.5. The relation between channels ratio and counting efficiency.

determine the efficiency of counting of samples of unknown radioactivity from their quenching correction ratio.

The advantage of the channels ratio method is that each sample has only to be counted once, and no further steps are required to determine efficiency once the system has been calibrated. The main difficulty of the method is that with samples of low activity, the counting time required for statistical precision becomes excessively long. This difficulty arose in the present study when carbon dioxide was measured from sediment mixtures supplied with high levels of glucose. In these cases, it was necessary to determine efficiency by the use of internal standards, i.e., by adding a fixed amount of toluene-C^{14} of known specific activity to the vials and recounting.

i. Carbon Dioxide-C^{14}. A method of collecting and counting carbon dioxide-C^{14} was required which would also permit the determination of unlabelled carbon dioxide following its diffusion from a sample to be analyzed. In the past, collection of labelled carbon dioxide has been done in a number of ways: (i) by trapping the CO_2 in a solution of an organic base such as hyamine-OH (Snyder and Godfrey, 1961), (ii) trapping the CO_2 on a filter paper saturated with NaOH (Mirsky, 1964), or KOH (Buhler, 1960; Chirboga and Roy, 1962) and (iii) trapping the CO_2 in an

aqueous solution of KOH (Yardley, 1964).

Trapping of the labelled carbon dioxide in an organic base has the advantage of high counting efficiencies. It was unsuitable for the present study because, with evaporation of the methanol in which hyamine-OH was dissolved, a gummy residue was left, which interfered with the determination of the unlabelled carbon dioxide. In addition, it was felt that the evaporation of the methanol from the teflon cups would interfere with the metabolism of the microorganisms.

Trapping the labelled carbon dioxide on filter paper moistened with NaOH or KOH was not used because of the disadvantages of both loss of counting efficiency (Yardley, 1964) and the virtual impossibility of also measuring the unlabelled carbon dioxide on the paper.

The third method, trapping the CO_2 in an aqueous solution of NaOH, was used in the present study. Preliminary experiments showed that the addition of the mixed indicator necessary for the determination of chemical carbon dioxide did not interfere with the subsequent counting of the sample; also that prior titration of the cup contents with HCl did not produce loss of carbon dioxide-C^{14}. It was therefore possible to determine chemical carbon dioxide and radioactivity on the same sample.

After the chemical carbon dioxide was determined, the teflon cups were dropped into liquid scintillation bottles

containing 5 ml of a "Liquifluor"-toluene mixture (Yardley, 1964), and 5 ml of methanol. The composition of the "Liquifluor"-toluene mixture was 2,5-diphenyloxazole (PPO)--4 g, 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP)--50 mg, and toluene--1 litre. Reagent-grade solvents were used. Before the teflon cups were dropped into the liquid scintillation vials, 25 µl of 1 N sodium hydroxide was added to neutralize the contents of the vial, which were slightly acid. This addition of NaOH prevented loss of carbon dioxide and avoided quenching due to acid.

Yardley had advocated the bubbling of nitrogen through the contents of the liquid scintillation bottle to remove dissolved oxygen, a substance with quenching properties. A preliminary experiment showed that such bubbling led to loss of some $C^{14}O_2$ and that the quenching effects of oxygen were adequately corrected for by using the channels ratio method described above. Bubbling was therefore not done in the present study.

To enable the counting of the large number of samples necessary for the present study, all samples were counted for fixed times. The samples containing carbon dioxide from sediment mixtures in which the initial glucose concentration was 30%, were counted for 40 minutes; mixtures with other glucose concentrations were counted for 10 minutes. Sufficient labelled glucose was added to the glucose

substrates to obtain a counting error of less than 5% at one hour of incubation, and less than 2% at 4 hours. The counting error was calculated with the assistance of an optimal counts chart (Appendix A.). In terms of radioactive carbon dioxide, the sample counting error was less than 0.015×10^{-2} µmoles /µl when glucose-U-C^{14} or glucose-3,4-C^{14} were used, and less than 0.0021×10^{-2} µmoles /µl when the C^{14} label was on carbon 1. Since the amount of labelled carbon dioxide that originated from the 2 and 6 glucose carbons was small, the error of counting these samples, in terms of labelled carbon dioxide, was negligible.

Calculation of the carbon dioxide originating from glucose using glucose-U-C^{14}. The carbon dioxide originating from glucose using glucose-U-C^{14} was calculated as follows.

$$S^{g}_{CO_2} = R_{CO_2} / S.A.^{g},$$

where $S^{g}_{CO_2}$ was the calculated number of umoles of carbon dioxide produced from the supplied glucose, R_{CO_2} was the radioactivity of the collected carbon dioxide in disintegrations per minute, and $S.A.^{g}$ was the specific activity of the glucose carbon supplied, in disintegrations per minute per ugram-atom of carbon.

Calculation of the carbon dioxide originating from particular glucose carbons. The carbon dioxide that originated from particular carbon atoms of glucose was

calculated in a manner similar to that used in the previous paragraph. The only difference was that only the glucose carbons corresponding to the carbons labelled on the glucose molecule were used in the determination of the specific activity of the glucose. For example, if the glucose were labelled on the 3 and 4 positions, and the glucose concentration were x molar, the concentration of carbon in the specific positions would be $2x$ gram-atoms per liter, rather than $6x$ gram-atoms per liter as would occur with uniformly-labelled glucose.

Standard error of the mean in the determination of labelled carbon dioxide. The standard error of the mean for the production, by salivary sediment, of labelled carbon dioxide from the various labelled carbons of glucose was calculated from data obtained from 1, 2, and 4 hour incubations of mixtures containing glucose labelled on the different carbons and at various unlabelled glucose concentrations. At all glucose concentrations, with all types of labelled glucose, the standard error of the mean was less than 0.016×10^{-2} μmoles labelled carbon dioxide/μl of the sediment mixture.

CHAPTER III

EFFECT OF GLUCOSE CONCENTRATION ON CARBON DIOXIDE PRODUCTION IN A SALIVARY SEDIMENT SYSTEM

Very little is known about the production of carbon dioxide by the oral microflora in saliva and nothing about its formation in dental plaque although evidence for its formation exists, albeit indirect. Microorganisms in these floras such as lactobacilli, veillonella, streptococci, and yeast are known producers of carbon dioxide when sugars are the substrates. Wasdell (1960), using gas manometry, noted that some gas formed when paraffin-stimulated saliva was incubated, and that the addition of glucose slightly increased the amount of gas formed. She considered the gas to be carbon dioxide, and felt that its production could be a source of error in the manometric measurement of glycolysis.

Carbon dioxide can be produced from glucose via several intracellular processes. These involve decarboxylation of Krebs cycle intermediates, several mechanisms for the cleavage of pyruvate, and decarboxylation of 6-phosphogluconate in the hexose monophosphate pathway. In bacteria, cleavage of pyruvate is particularly variable and leads to the formation of a two-carbon fragment in addition to carbon dioxide. The two-carbon fragment formed depends upon whether oxidation, decarboxylation, dismutation, or phosphoroclastic cleavage had occurred. Whether this

fragment is further metabolized to form additional carbon dioxide depends upon a number of factors such as the nature of the fragment, (e.g., whether it is acetic acid, acetaldehyde, or part of acetylcoenzyme A), the presence in the organism of the necessary enzymes, and cultural conditions, such as the availability of oxygen or other electron acceptors. No studies have attempted to determine whether carbon dioxide is produced from glucose by the oral flora, and if so, by what means.

Carbon dioxide can also be produced by the degradation of substrates other than glucose, for example, the decarboxylation of amino acids (Gochman et al., 1959). Many studies on a variety of biological systems have demonstrated that carbon dioxide can also be produced from the degradation of intracellular depots of metabolizable substances such as polysaccharides (Holme and Palmstierna, 1956), proteins, (Ribbons and Dawes, 1963), and lipids (Macrae and Wilkinson, 1958). If these substances are present and utilized in a system in which glucose breakdown to carbon dioxide is being studied, difficulties may arise in determining how carbon dioxide forms.

With the availability in recent years of glucose labelled with carbon-14 on its different carbon atoms, study of the metabolic pathways has received considerable impetus. By measuring the rates at which these labelled

carbons appear in the carbon dioxide evolved from pure cultures of several types of microorganisms (a technique called "radiorespirometry"), Wang and his co-workers (1958) were able to determine the presence and activities of the various metabolic pathways. For example, the participation of the Embden-Meyerhof pathway was calculated from the amount of labelled carbon dioxide produced when glucose-3, 4-C^{14} was substrate. Similarly, participation of the hexose monophosphate pathway was measured from the amount of label in the carbon dioxide when glucose-1-C^{14} was the substrate.

In the present study, a microadaptation of radiorespirometry was used to investigate how carbon dioxide is produced from glucose by the cells in the salivary system and to determine its relationship to any unlabelled carbon dioxide formed.

As in earlier studies, a salivary sediment system prepared from paraffin-stimulated whole saliva and in which the sediment concentration was 16.7% was scanned between glucose concentrates of 0 and 30% (W/V). The carbon dioxide produced during an incubation period of four hours at 37°C was determined. In similar experiments, glucose molecules labelled on one or more of their carbon atoms with carbon-14 were added to unlabelled glucose and the carbon-14 label in the carbon dioxide formed, was monitored. This was to (i) distinguish between carbon dioxide molecules arising

from the glucose added to the system and from substrates inherently present, and (ii) elucidate the metabolic pathways by which carbon dioxide is formed from glucose.

METHODS

A series of preliminary experiments were performed, following which a second series of experiments was run. The methods and results of this second series are the methods and results reported in this chapter.

Experiments to Determine the Effect of Glucose Concentration on the Rate of Total Carbon Dioxide Production

To determine the effect of glucose concentration on the rate of carbon dioxide production, five experiments were run as follows. In each of four of the experiments, five series of mixtures were prepared; two series contained 0 and 5% glucose as controls, and the other three series contained three of the following concentrations: 0.05, 0.1, 0.2, 0.5, 1.0, 10, and 30% (W/V). The other experiment contained one less series and therefore one less glucose concentration. The 0 and 5% concentrations were therefore each examined five times, while the other concentrations were each studied twice.

Each series within an experiment consisted of forty incubation mixtures; four mixtures were removed at each of ten time intervals, two for pH measurement and two for

carbon dioxide determination (Chapter II). The pH was measured to ensure that the pH changes in these experiments were the same as those determined earlier under the same conditions (Kleinberg, 1967; Sandham and Kleinberg, 1964 and 1967). The same holds true for the other experiments below.

The mean curves for each concentration are the ones reported in the results section.

Proportion of the Total Carbon Dioxide Originating From Glucose and From Other Substrates as a Function of Initial Glucose Concentration

The effect of five different glucose concentrations, 0.05, 0.1, 0.2, 5 and 30%, on the proportion of the total carbon dioxide originating from glucose and from other sources was examined in a series of four experiments. These experiments were similar to those described in the preceeding section, except that, in addition to the determination of chemical carbon dioxide, the amount of labelled carbon dioxide which originated from glucose-U-C^{14} was also determined.

The method used to calculate the carbon dioxide arising from glucose and the carbon dioxide arising from the other sources is described in Chapter II.

Experiments to Determine the Metabolic Pathways Used During Carbon Dioxide Formation From Glucose as a Function of the Glucose Concentration

Five experiments were run to identify the metabolic pathways used during carbon dioxide formation and to estimate the participation of each at several glucose concentrations, viz., 0.05, 0.1, 0.2, 5, and 30% glucose. In each experiment, six series of twenty tubes were prepared, five containing each of the following labelled compounds: glucose-1-C^{14}, -2-C^{14}, -3,4-C^{14}, -6-C^{14}, and -U-C^{14}, and a sixth series containing no label. This unlabelled series was used for the measurement of pH. Two of the twenty tubes in each series were removed at each of ten time intervals, and their labelled carbon dioxide and pH determined. Determination of chemical carbon dioxide was done on the tubes containing glucose-6-C^{14}.

The method used to calculate the amount of carbon dioxide produced from each carbon atom of glucose is described in Chapter II.

RESULTS

pH Curves as a Function of Glucose Concentration

The changes in pH in the salivary sediment system used in this study as a function of glucose concentration are shown in figure 3.1. These are almost identical to those observed earlier (Sandham, 1963; Sandham and Kleinberg, 1964).

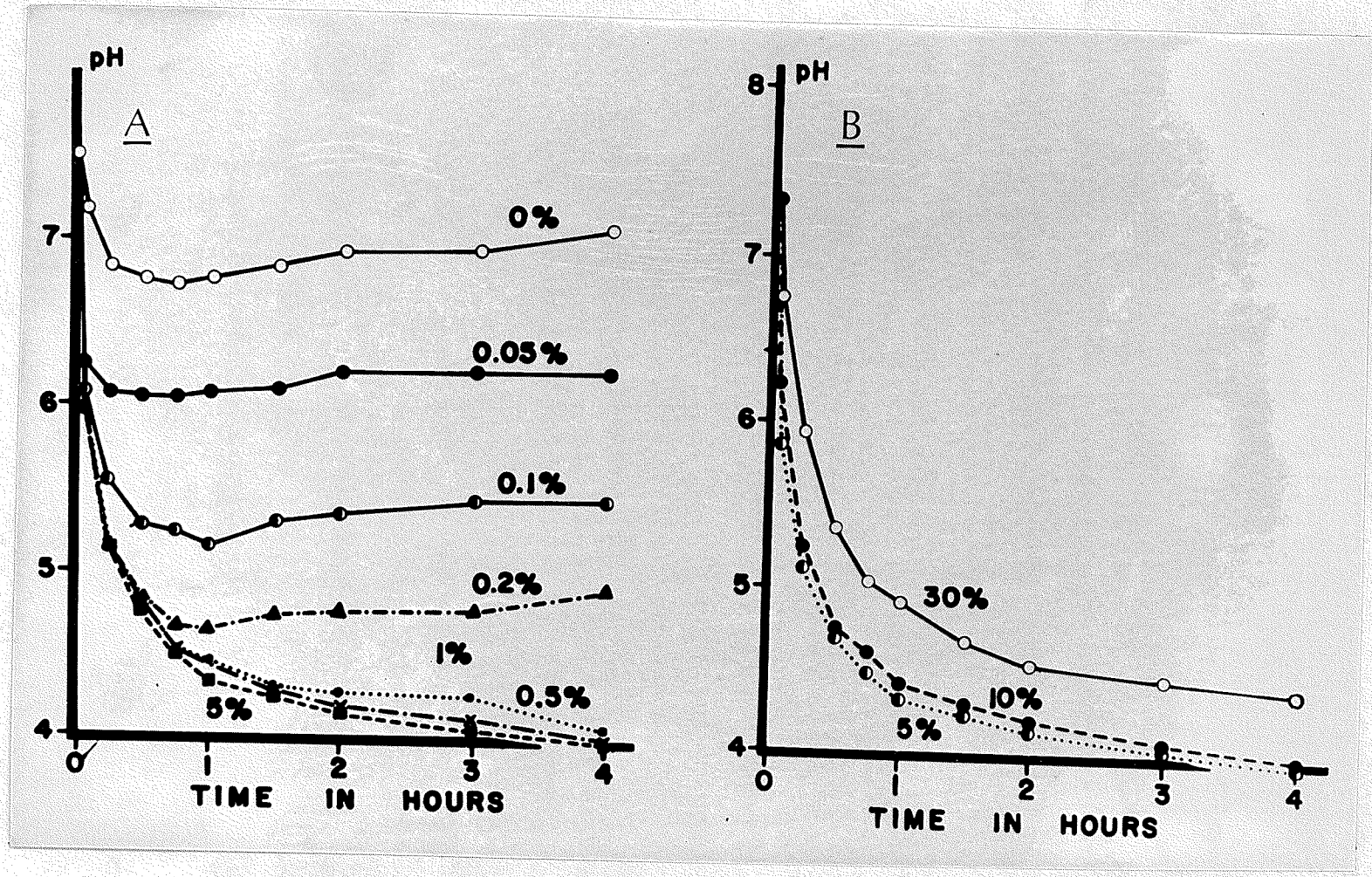


Figure 3.1. Effect of glucose concentration on pH change: A., 0 to 5% glucose; B., 5 to 30% glucose.

Rate of Carbon Dioxide Production as a Function of Glucose Concentration

The carbon dioxide formed with the different glucose concentrations over the four hour period of incubation is shown in figures 3.2a, b, and c. To facilitate comparison, the curve with 5% glucose is repeated in each figure.

All curves rose during the experimental period. During the first hour or so, the rates of carbon dioxide production with the different glucose concentrations were nearly the same. After this period, when the glucose concentrations were 0 to 0.2%, more carbon dioxide was produced than with 5% glucose. Of the mixtures with glucose concentrations between 0 and 0.2%, the lowest was with 0% and the highest was with 0.05 and 0.1%.

The curves for 0.5% and 1% glucose were not appreciably different from the 5% glucose curve, while the 10% and 30% curves were lower than that for 5%. The 10% glucose curve was almost the same as the curve with 30% glucose.

The production of total CO_2 at the two and four hour time intervals, shown as a function of glucose concentration in figure 3.3, illustrates both the low glucose concentration at which the CO_2 production is maximal, and that this maximum increases at a greater rate between two and four hours than do the other points on the curves.

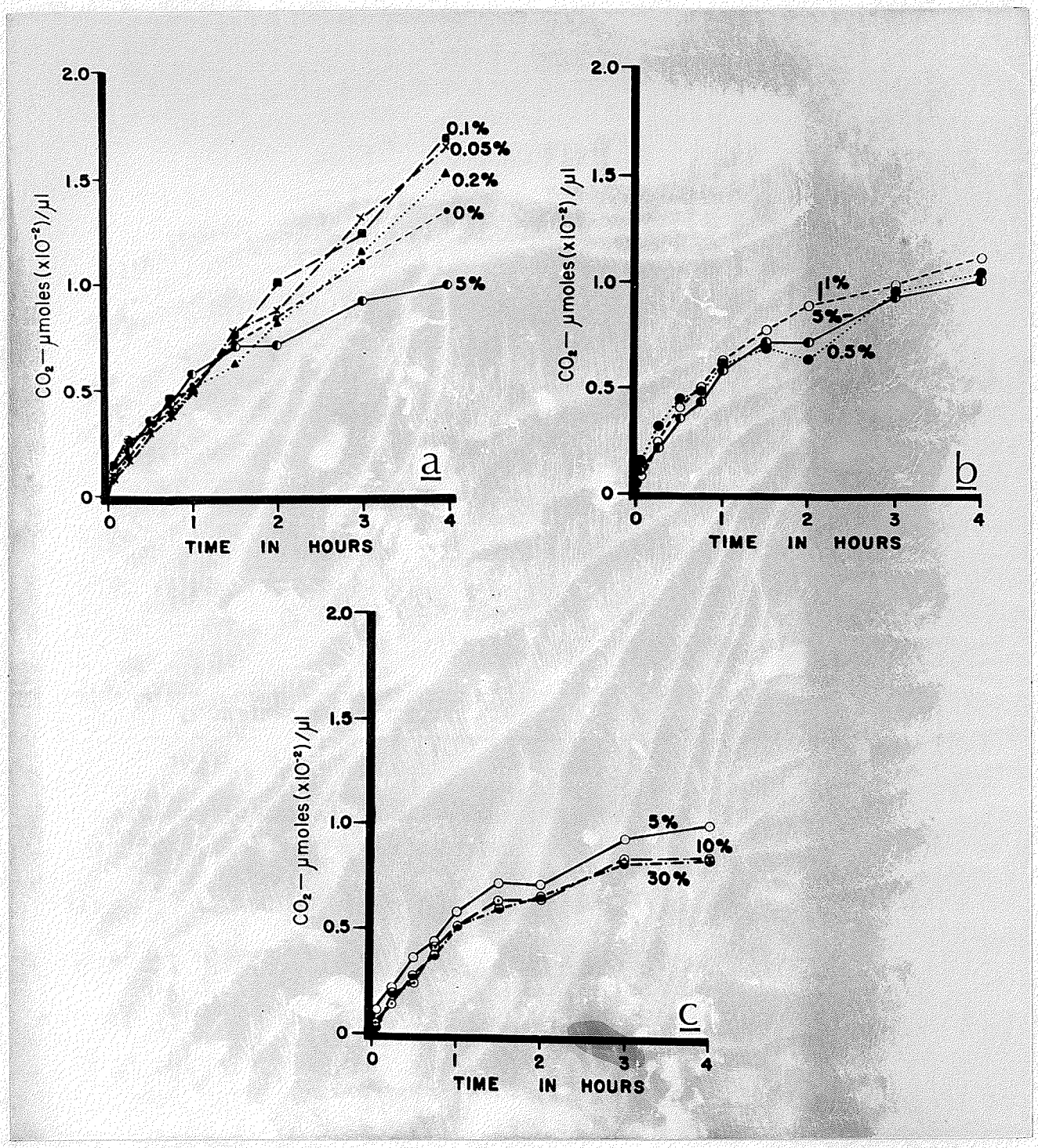


Figure 3.2. Effect of glucose concentration on the formation of total CO_2: a., 0 to 0.2% glucose; b., 0.5 to 5% glucose; and c., 5 to 30% glucose.

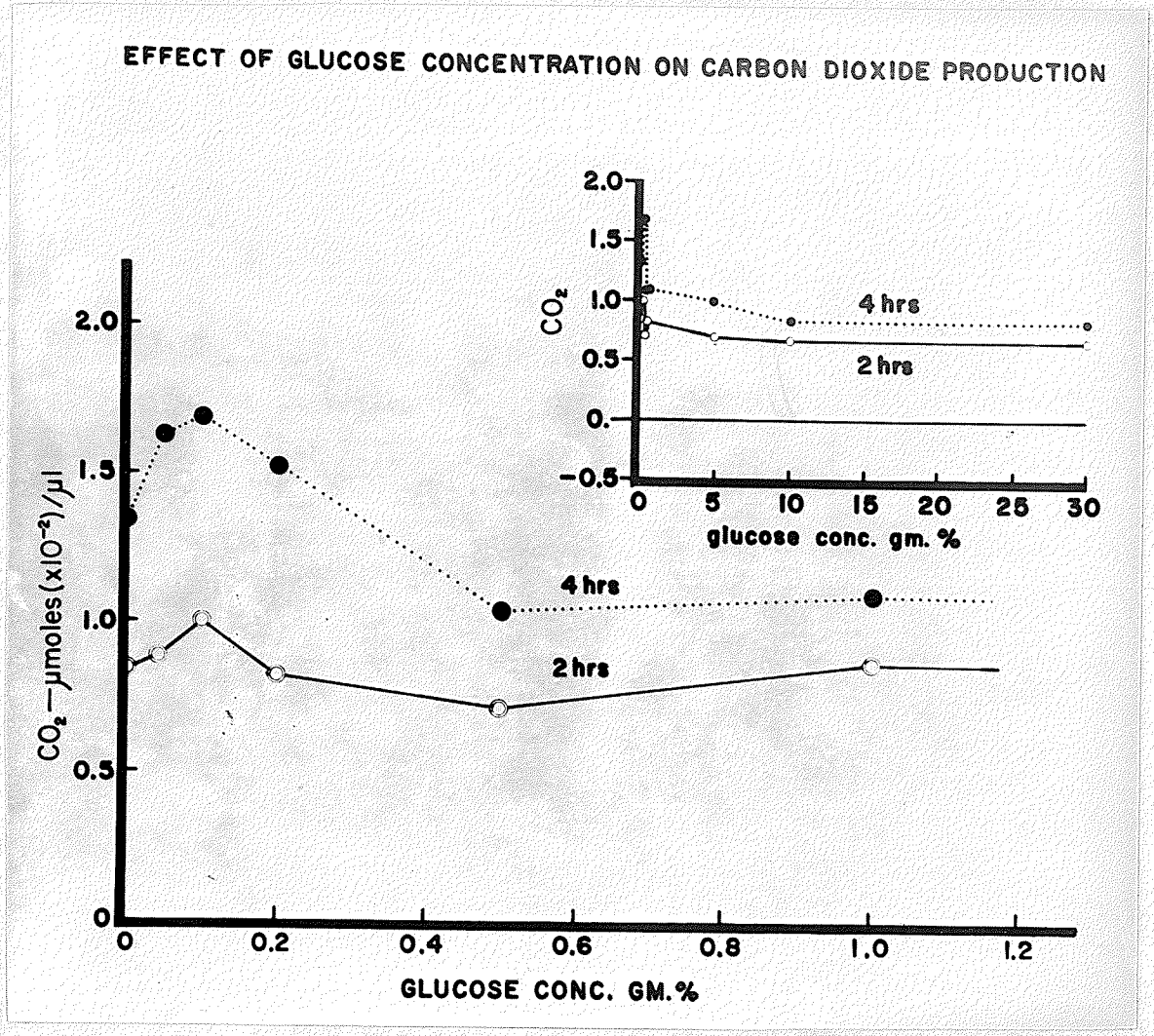


Figure 3.3. Effect of glucose concentration on the total CO_2 formed at 2 and 4 hours.

Proportion of the Carbon Dioxide Originating From Labelled Glucose and From Unlabelled Endogenous Substrates as a Function of Glucose Concentration

(i) Time curves for the formation of labelled carbon dioxide. The time curves of the labelled carbon dioxide formed with each glucose concentration studied, 0.05, 0.1, 0.2, 5, and 30%, are shown in figure 3.4a. These data represent the carbon dioxide originating from glucose and were determined by calculation from the glucose radioactivity that appeared in the carbon dioxide (Chapter II).

The rates of production of labelled carbon dioxide with all the glucose concentrations tested were more rapid during the earlier part of the incubation than later. The labelled carbon dioxide formed progressively increased with increasing glucose concentrations, until a maximum was reached with 5% glucose.

(ii) Time curves for the formation of carbon dioxide from unlabelled endogenous sources. The carbon dioxide produced from endogenous substrates was determined by subtracting the values for the carbon dioxide originating from glucose from the corresponding values for total carbon dioxide, and the data are shown in figure 3.4b.

Most striking is the magnitude of the quantities of carbon dioxide produced from endogenous substrates with all glucose concentrations tested (cf. Fig. 3.4a to Fig. 3.4b).

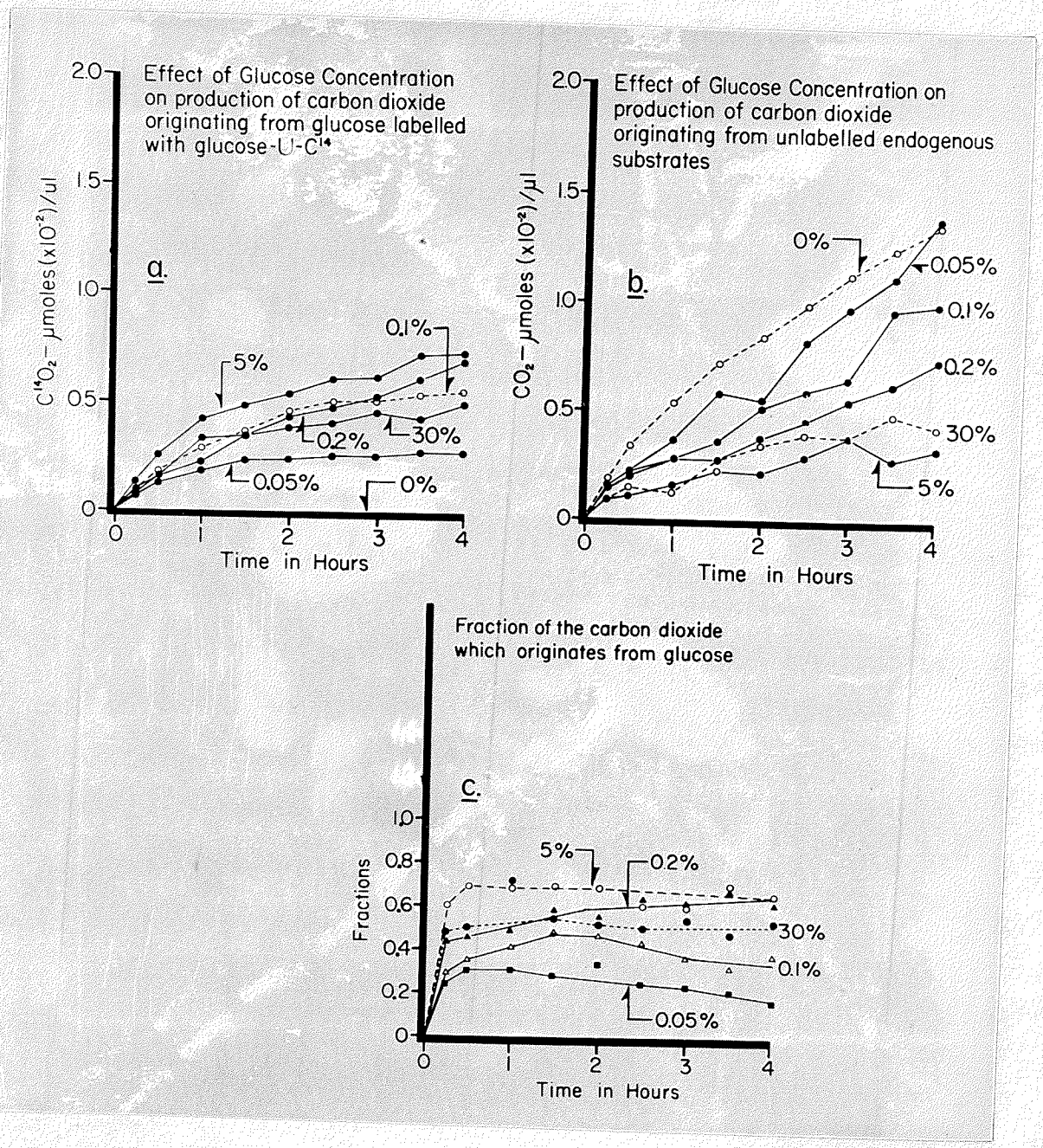


Figure 3.4. Effect of glucose concentration on the CO_2 formed: a., from glucose; and b., from endogenous substrates. c., fraction of the total CO_2 that originated from glucose.

Formation of this unlabelled carbon dioxide progressively decreased with increasing glucose concentrations until a minimum was reached with 5% glucose; with 30% glucose the rate was higher than with the 5%. This relationship was the reverse of the one for carbon dioxide originating from labelled glucose.

Production of carbon dioxide from endogenous sources did not show a tendency to plateau with 0.05 and 0.1% glucose as had occurred in figure 3.4a.

At no time during these experiments did more than 0.70 of the total CO_2 originate from glucose (Fig. 3.4c).

(iii) Total, labelled and unlabelled carbon dioxide as a function of glucose concentration. The curves for total carbon dioxide and the carbon dioxide from glucose and from endogenous sources after two and four hours incubation are shown in figures 3.5a and 3.5b, respectively. The higher production of total and unlabelled carbon dioxide at the lower concentrations, in contrast to the higher production of labelled carbon dioxide at a much higher glucose concentration (5%), is clearly demonstrated in these figures.

At glucose concentrations below 0.2%, a deficiency of labelled carbon dioxide which comes from glucose is compensated for by unlabelled carbon dioxide which comes from endogenous sources. This is also seen with 30% glucose.

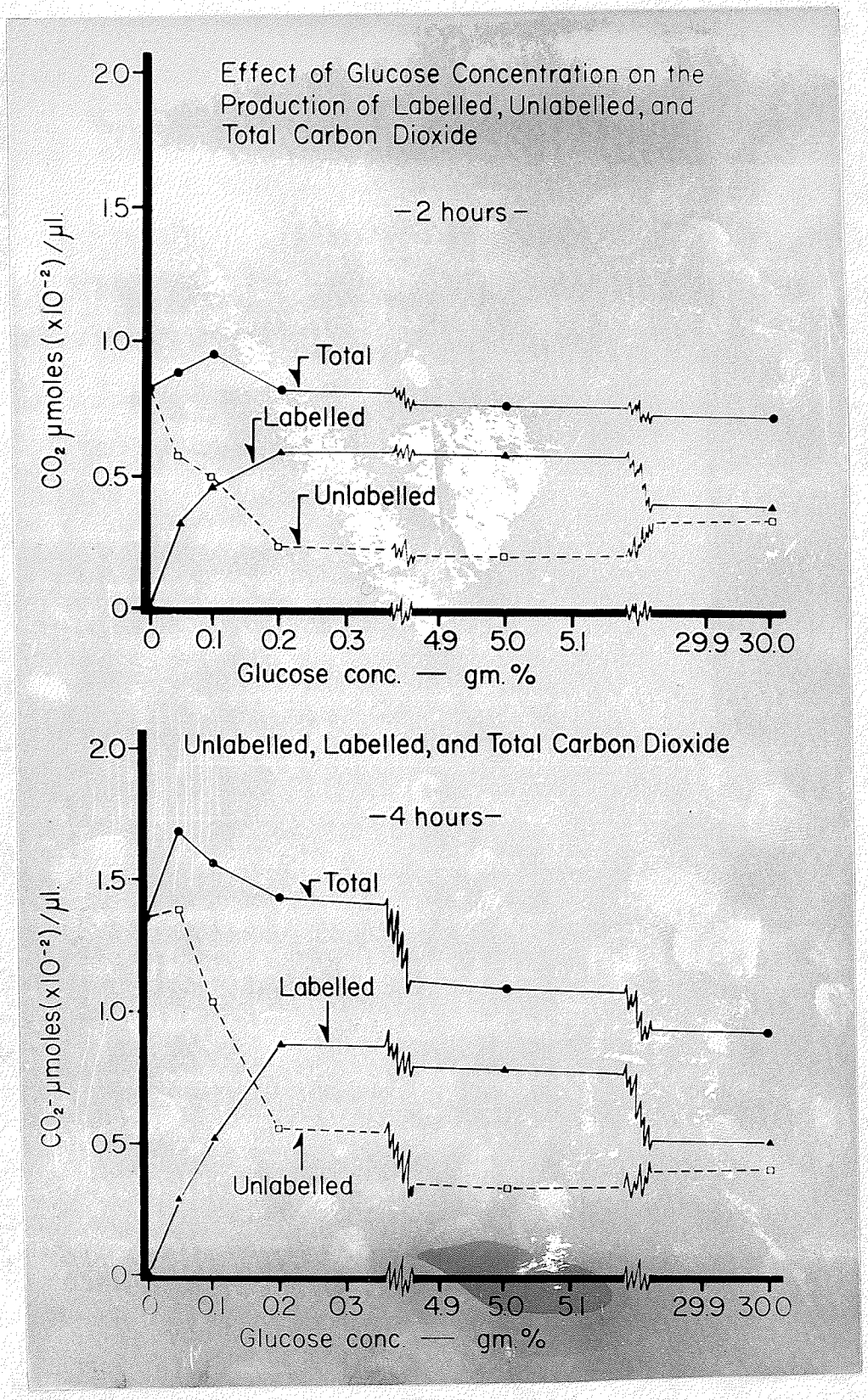


Figure 3.5. Effect of glucose concentration on the formation of: labelled CO_2 (from glucose-U-C^{14}), unlabelled CO_2 and total CO_2, at 2 and 4 hours.

Metabolic Pathways Used During Carbon Dioxide Formation From Glucose as a Function of Glucose Concentration

When glucose labelled on one or more of its carbon atoms was mixed with unlabelled glucose of differing concentrations and added as substrate to the sediment system used here, with all the glucose concentrations tested (0.05, 0.1, 0.2, 5, and 30%, Figs. 3.6 and 3.7), 85 to 98% of the carbon dioxide carbon atoms originated from carbons 3 and 4 of glucose. The carbon dioxide carbons originating from carbon 1 of glucose averaged 6.9% of the carbon dioxide from glucose while the carbon dioxide carbons from carbons 2 and 6 of glucose was even less, 1.3 and 1.1%.

When the sums of the carbon dioxide carbons originating from C_1, C_2, C_3, C_4, C_5, and C_6 (C_5 was assumed to be equal to C_2) were subtracted from the corresponding values for the carbon dioxide originating from all glucose carbons, the differences were higher with the higher glucose concentrations than with the lower ones. With glucose concentrations of 0.05, 0.1, and 0.2%, this difference averaged 3.8% $\pm$ 8.0% when expressed as a percentage of the values for the carbon dioxide carbons from uniformly-labelled glucose. With the higher glucose concentrations, 5 and 30%, the average difference was 11.1% $\pm$ 11.0%. The possible reasons for this difference are considered in the discussion.

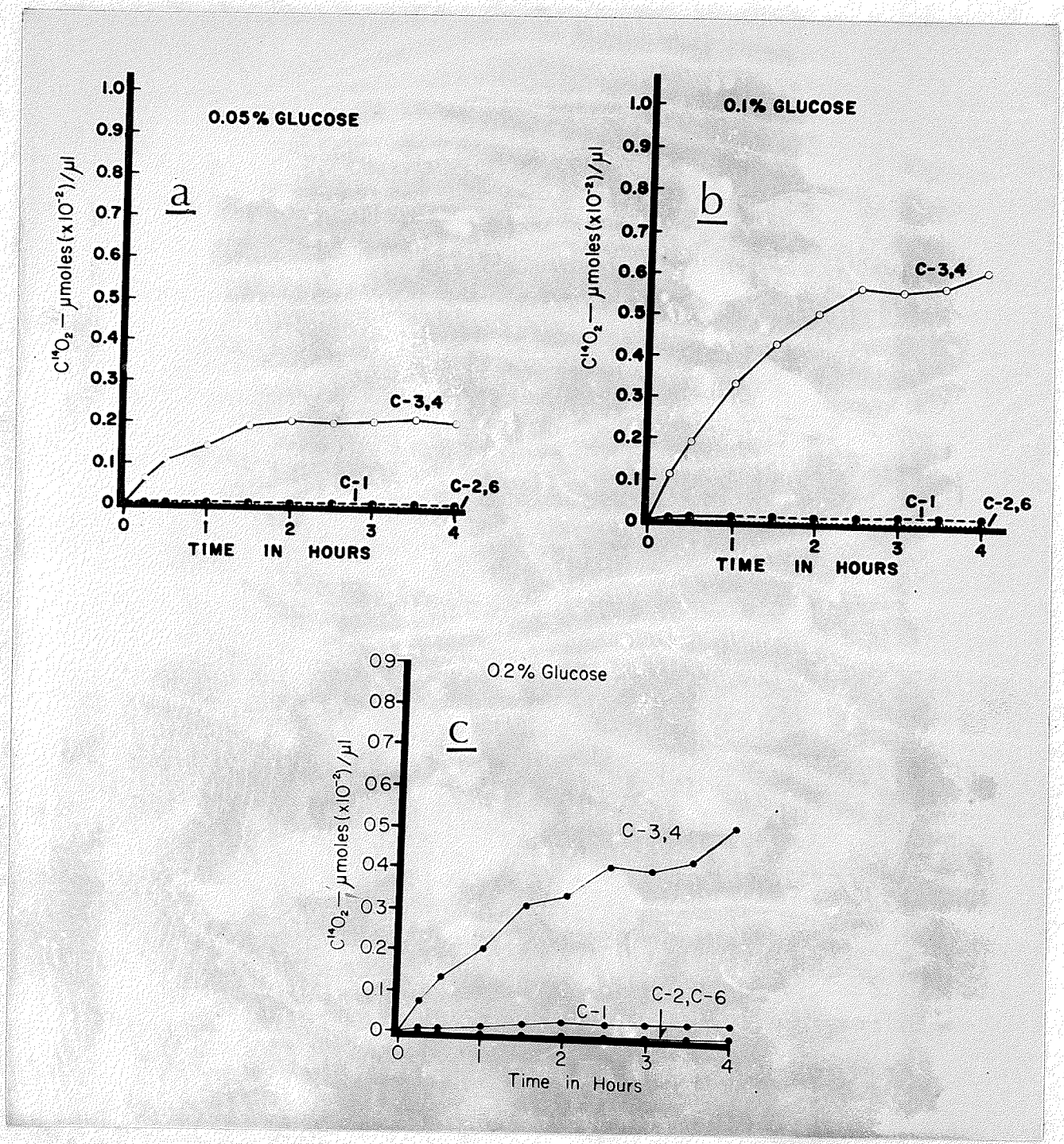


Figure 3.6. Formation of CO_2 from individual carbons of glucose, with: A., 0.05% glucose; B., 0.1 glucose; and C., 0.2% glucose.

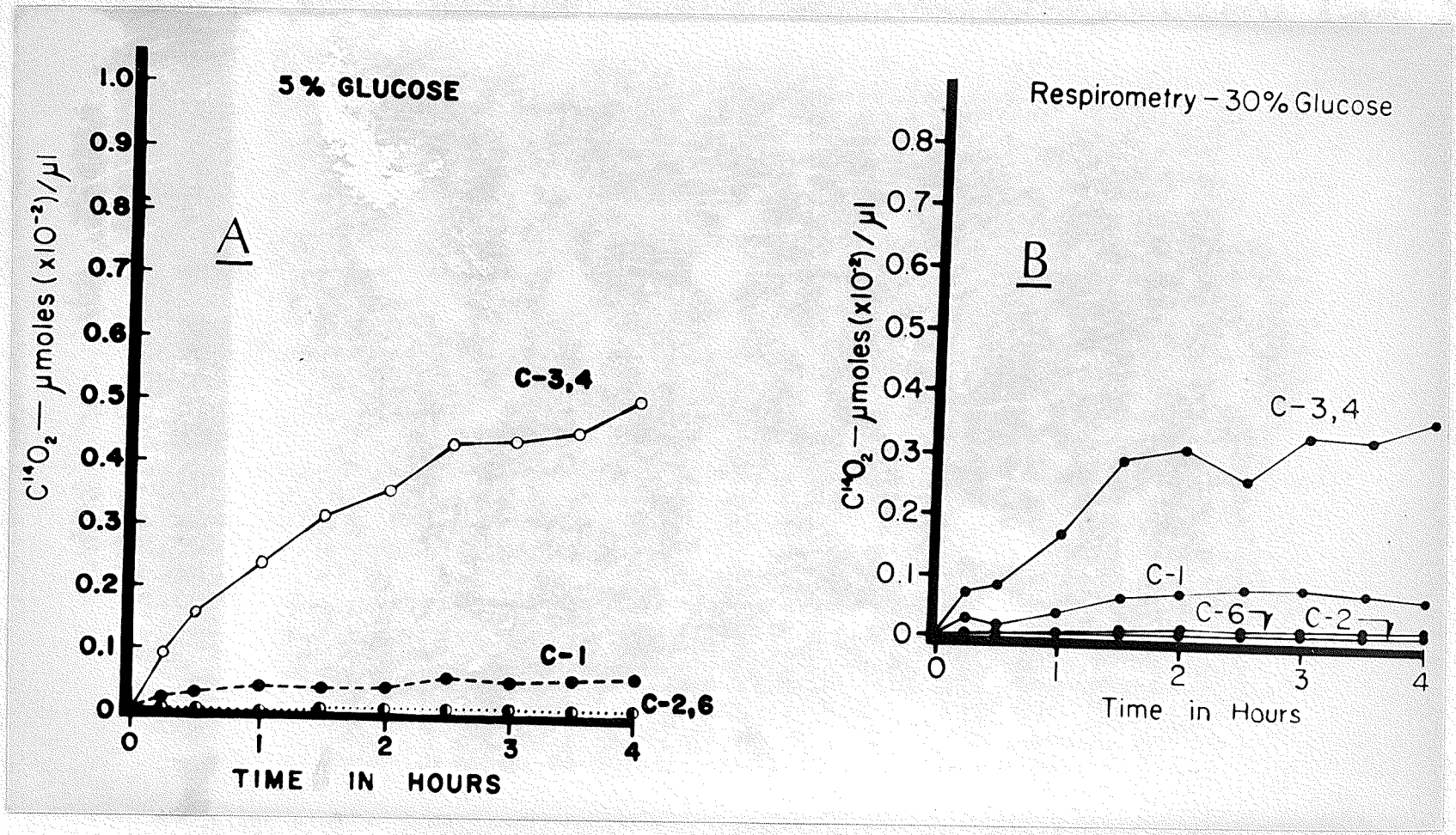
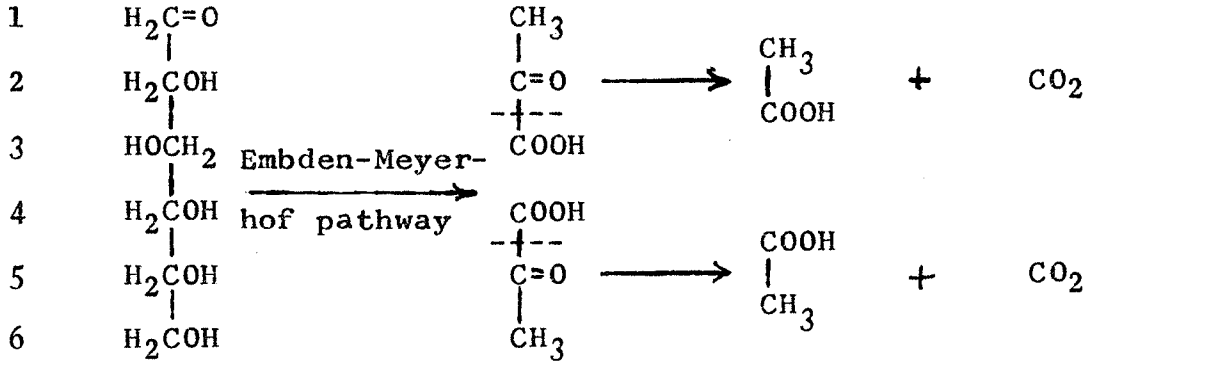


Figure 3.7. Formation of CO_2 from individual carbons of glucose. A., 5% glucose; B., 30% glucose.

DISCUSSION

The radiorespirometric experiments in the present study clearly showed that most of the carbon dioxide carbons came from C-3 and C-4 of glucose, some from C-1, and very little from the other glucose carbons. The appearance of C-3 and C-4 label in the CO_2 must have occurred by the conversion of glucose to pyruvate and its subsequent decarboxylation to CO_2 and acetic acid according to the following scheme:



glucose pyruvic acid acetic acid carbon dioxide

The appearance of C-1 label in the CO_2 indicates that some of the glucose molecules were utilized via the hexose monophosphate metabolic pathway (Bloom, Stetten, and Stetten, 1953). The very low yield of carbon dioxide from the 2 and 6 carbons of glucose indicates an almost complete lack of glucose degradation via the tricarboxylic acid cycle. The lack of the tricarboxylic acid cycle in salivary sediment,

under the experimental conditions used, could have been the result of either anaerobiosis or a lack of one or more of the requisite enzymes for the cycle. Other experiments in our laboratory support the latter.

The two possible pathways by which CO_2 could have been produced from C-1 of glucose in the present study are the hexose monophosphate (HMP) and the Entner-Doudoroff (ED) pathways. The ED pathway, by which the carboxyl group of the pyruvate molecule can be labelled from glucose-1-C^{14}, is negligible or not likely present in the salivary sediment system because the organisms that have this metabolic pathway, primarily Pseudomonads, do not occur in the mouth (Burnett and Scherp, 1962, p. 277). All of the CO_2 from C-1 of glucose was therefore assumed to have been produced via the hexose monophosphate pathway.

Because the removal of C-1 of glucose is obligatory when glucose flows through the hexose monophosphate pathway, and because of the lack of recycling (indicated by the very low amount of CO_2 from carbon 2 of glucose), the carbon dioxide arising from C-1 of glucose would be a measure of the activity of this pathway. The percentage of the glucose catabolized via the HMP pathway (%Gp) was calculated as follows:

$$\%Gp = \frac{Gp}{(G_T - G_A)} \times 100,$$

where Gp is the glucose catabolized via the HMP pathway; G_T is the glucose that disappeared from the medium and G_A is the glucose that was incorporated into carbohydrate (values for G_T and G_A were obtained from Sandham, 1963). Values for %Gp varied between 2.8% and 6.1% in this study.

The percentage of the glucose catabolized via the Embden-Meyerhof pathway cannot be calculated simply from the CO_2 originating from the 3 and 4 carbons of glucose; since, in addition to acetic acid and CO_2, lactic and propionic acids are also formed during glucose breakdown (Chapter IV). In other words, an appreciable amount of the labelled carbon atoms from glucose-3,4-C^{14} would be contained in these acids and would have flowed through the EM pathway and not appeared in the CO_2. Any estimate of the glucose catabolized via the EM pathway based solely on the C-3 and C-4 label in the CO_2 would therefore be too low.

Because the presence of an ED pathway in the cells in the sediment is unlikely (see above), the breakdown of glucose to pyruvate would occur via the EM and HMP pathways. Therefore, the glucose catabolized via the EM pathway (% EMP) was calculated as follows:

$$\%EMP = 100 - \%Gp$$

The values for % EMP varied between 94% and 97.2%.

Effect of Glucose Concentration on the Percentage of the Glucose Catabolized via HMP and EMP

Calculation of the glucose catabolized via the HMP and EM pathways for each glucose concentration tested and after 1, 2, 3, and 4 hours of incubation showed that the percentage catabolized via these pathways (i) did not vary significantly during the four hours of incubation, and (ii) only varied slightly with glucose concentration (Table I).

TABLE I

EFFECT OF GLUCOSE CONCENTRATION ON THE PERCENTAGE OF GLUCOSE CATABOLIZED VIA THE EM AND HMP PATHWAYS

Glucose Concentration (%, W/V)	% HMP	% EMP
0.05	3.6	96.4
0.1	3.6	96.4
0.2	2.8	97.2
5.0	2.9	97.1
30.0	6.1	93.9

Interrelationship Between Glucose Concentration and the CO_2 Originating From Glucose and From Sources Other Than Glucose

The present study also demonstrated that the rate of production of carbon dioxide originating from glucose was a function of the glucose concentration. In previous studies, the pH fall (Kleinberg, 1961), storage of carbohydrate by the sediment (Sandham, 1963; Sandham and Kleinberg, 1964),

and growth of the microorganisms in the same system (Kleinberg, 1961; 1963) were also shown to be functions of the glucose concentration. With glucose concentrations between 0 and 5%, the relationship between each of these variables and the glucose concentration was direct; with glucose concentrations above 5%, the relationship was inverse. This suggests that the production of carbon dioxide from glucose, as well as changes in the above parameters, was determined by the rate of glucose uptake by the sediment.

On the other hand, the rate of production of CO_2 from substrates other than the glucose added to the medium, varied inversely with the glucose concentration when the glucose concentration was between 0 and 5% and varied directly when it was above 5%. This reverse relationship means that a decrease in CO_2 from glucose is compensated by an increase in CO_2 from substrates other than glucose, and vice versa. Because this compensation was not stoichiometric, the formation of total carbon dioxide (the sum of CO_2 from glucose and CO_2 from sources other than glucose) was also a function of glucose concentration, but its optimum was at approximately 0.1% and not at 5%. It showed a direct relationship with glucose concentration between 0 and 0.1% and an inverse relationship with glucose concentrations above 0.1%.

Whether CO_2 formation from glucose and CO_2 formation from substrates other than glucose are metabolically linked cannot be determined from the experiments in the present study. However, if the endogenous formation of CO_2 was the result of a catabolic process, its repression by glucose would not be unexpected (Neidhardt and Magasanik, 1956; Kleinberg, 1961, 1967).

The substrates (other than glucose) from which CO_2 is produced are at present unknown.

Possible Function of the HMP Pathway

In the present study, the HMP pathway may have functioned (i) to supply ribose for the synthesis of nucleic acids, and (ii) in the heterolactic fermentation of some of the oral microorganisms. Many microorganisms present in the mouth ferment glucose by the heterolactic pathway, including some anaerobic streptococci (Burnett and Scherp, 1962, p. 586), heterolactobacilli (Rogosa et al., 1953), and fusobacteria (Omata and Disraely, 1956). The heterolactic fermentation uses the same enzymes as the HMP pathway up to pentose epimerase, the product of which is the pentose, D-xylulose-5-phosphate (Wood, 1961). This pentose is then split by the 3-2 cleavage characteristic of this fermentation (Gibbs, Sokatch, and Gunsalus, 1955), forming acetyl phosphate and D-glyceraldehyde-3-phosphate

(Heath et al., 1958). If the acetyl phosphate is reduced, ethanol would be formed; if D-glyceraldehyde-3-phosphate is reduced, then acetic acid would be formed. The stoichiometry would be one or the other of the following:

(i) glucose ------> lactic acid + ethanol + CO_2

(ii) 3/2 glucose ------> 2 glycerol + acetic acid + CO_2.

Richter and Tonzetich (1964) showed that small amounts of ethanol are produced in incubated saliva and are formed in the mouths of most subjects. This ethanol may have been produced via the HMP pathway as illustrated by (i) above.

Flow of Labelled Carbons Into and Out of Metabolic Compartments During and Immediately After Glucose Catabolism

When the glucose concentration was 0.2% or below, for a considerable length of time after the glucose in the medium was gone (see Sandham, 1963; Sandham and Kleinberg, 1964), labelled carbon dioxide was still produced. This indicated that labelled carbons must have flowed into one or more metabolic compartments while the glucose from the medium was being catabolized. After the glucose was completely used up, these labelled carbons must have flowed out of these compartments to form products which included carbon dioxide. The label in the CO_2 was consistent with two of these metabolic compartments being (i) sediment

carbohydrate and (ii) lactic acid. In support of this conclusion, both sediment carbohydrate and lactic acid form rapidly while glucose is still present in the medium, and both then decrease after glucose has disappeared (Sandham, 1963; Sandham and Kleinberg, 1964; Chapter V of this thesis). The times at which label no longer appeared in the carbon dioxide in these experiments corresponded to the times at which these metabolic compartments were depleted.

Non-uniformity of Glucose-U-C^{14}

The CO_2 originating from glucose, determined with glucose-U-C^{14}, was greater than the CO_2 originating from glucose determined by summing the CO_2 obtained with glucose labelled on specific carbons. A possible reason for this discrepancy was a lack of uniformity in the labelling of the different carbons in the glucose-U-C^{14} used. The glucose-U-C^{14} had been prepared from $C^{14}O_2$ by photosynthesis using Canna leaves; its uniformity of labelling was $\pm$ 15% (New England Nuclear, 1966, personal communication). Vittorio, Krotkov, and Reed (1950) showed that glucose-U-C^{14}, similarly prepared from tobacco leaves, had its highest labelling on carbons 3 and 4. It is probable that these carbons were also the most highly-labelled of the carbons in the glucose-U-C^{14} used in the present study.

Since carbons 3 and 4 are the glucose carbons preferentially converted to CO_2 by sediment, the specific activity of CO_2 formed from glucose labelled with glucose-U-C^{14} would be higher than the specific activity of the glucose labelled with glucose-U-C^{14}. The non-uniformity of glucose-U-C^{14} would therefore result in a slight overestimation of the value for the CO_2 originating from glucose and would result in the discrepancy observed in the present study.

Carbon Dioxide Fixation

Carbon dioxide fixation is a process common to most microorganisms and proceeds at a rate that is dependent upon the concentration of extra-cellular carbon dioxide (Johns, 1951). Because CO_2 is less soluble at acid than at neutral pH, fixation would probably be least when the extra-cellular pH is low.

In the present study, when the pH dropped below 6.0, as occurred with nearly every glucose concentration tested, fixation of carbon dioxide would have been minimal. Instead, decarboxylation would have been favoured (Johns, 1951) and very little of the CO_2 formed during glucose breakdown would have been retained in the system. The same likely holds true for plaque *in vivo*, since the metabolism of this system is very similar to the metabolism of the system used in the present study (Klein-

berg, 1961 and see Chapter IV). However, if either of these systems were buffered at neutral pH, fixation, and not decarboxylation, would be favoured.

Possible Importance of Carbon Dioxide Formed by the Microorganisms in Plaque

If the assumption is made that the microorganisms in plaque also form CO_2, then the CO_2 in plaque would originate from two sources--microbial metabolism and the bicarbonate in saliva. The proportion of the plaque CO_2 which originates from the plaque microorganisms would then likely be higher in areas of the mouth with poor saliva access. Like the CO_2 from saliva, CO_2 from the microorganisms could function as a buffer, being lost at low pH and being retained at high pH. The CO_2 may slow the later stages of the pH rise that occures during sleep (Kleinberg, 1964), and may encourage the formation of calcium carbonate during the same period. The latter process might be important in calculus formation (Sand, 1949).

CHAPTER IV

EFFECT OF GLUCOSE CONCENTRATION ON THE PRODUCTION OF LACTIC ACID AND OTHER TITRATABLE ACIDS

The rapid formation of lactic acid from carbohydrate by the oral microflora was first shown by Neuwirth and Klosterman (1940) who measured its appearance in the salivas of individuals who allowed several different carbohydrates to dissolve in their mouths. This rapid rate of formation of lactic acid from carbohydrate was confirmed when these workers incubated the salivas from the same individuals with the same carbohydrates in vitro.

Muntz (1943), studying the formation of acid from glucose by dental plaque in vitro, observed that other acids, in addition to lactic acid, were produced. The lactic acid concentration rose and fell during these experiments, while the concentration of the other acids progressively rose.

The rise and fall in the lactic acid concentration was also observed in paraffin-stimulated whole saliva by Neuwirth and Summerson (1951). Their data, together with the data from the earlier experiments in vitro of Neuwirth and Klosterman, suggested that the fall in lactic acid concentration does not occur unless the glucose is used up in these various systems. In the Neuwirth and Summerson experiments, as in the study of Muntz, the proportion of

the total acid that was lactic appeared to depend upon the stage to which the experiments had progressed.

The acids that are produced from, or along with, lactic acid by the oral microorganisms in the various systems, have been examined by a number of workers. Acetic acid and what he thought was likely propionic acid were produced by plaque in _in vitro_ experiments carried out by Muntz (1943). Guggenheim, Ettlinger, and Muhlemann (1965) analyzed the saliva of 240 school children for volatile fatty acids and found acetic, propionic, butyric, isobutyric, and valeric acids to be present. The same acids were produced from glucose by plaque _in vitro_ (Ranke, Ranke and Bramstedt, 1965) and oral debris (plaque ?) _in vivo_ (de Stoppelaar and Gibbons, 1965; and private communication). When Gilmour and Poole (1966) prepared plaque on membranes _in situ_ and incubated them with glucose _in vitro_, acetic and propionic acids were produced in large quantity.

To determine the relationship between lactic acid and the other acids formed in the salivary system during glucose breakdown, the glucose concentration in the system was varied between 0 and 30% and the lactic and titratable acids measured at regular intervals during a four hour incubation at 37°C. The sediment concentration, as before, was 16.7% (V/V). pH was also measured, to permit any changes in the acids observed in this study to be related to parameters of

the system that have already been determined, e.g., sediment carbohydrate (Sandham, 1963; Sandham and Kleinberg, 1964) and carbon dioxide formation (Chapter III of this thesis).

Determination of titratable acidity was selected to measure total acid because it was felt that this procedure would give the closest assessment of the total acid present and yet be a sufficiently simple method to permit large numbers of micro-samples to be estimated. Total acid and titratable acid are almost, but not exactly, the same, because of (i) the small amount of carbon dioxide lost from the system as acid is produced and the pH falls, (ii) the production of small amounts of ammonia (Biswas and Kleinberg, 1966) and (iii) the exposure and burial of basic and acidic groups resulting from protein conformational changes (Kleinberg and Craw, unpublished results). However, in the present study, the carbon dioxide loss has been corrected for and the effects of the other factors are small.

Below, for simplicity, the terms <u>total acid</u> has been used instead of <u>titratable acid</u>. Also for simplicity, the term <u>hetero acids</u> has been used to refer to acids other than lactic formed in this system.

In the present study, the hetero acid fraction was examined for its content of volatile and non-volatile acids. Because of the possibility that neutral products (e.g., ethanol, acetoin) might be formed, the possible presence of

these products was also checked.

METHODS

The sediment mixtures in the experiments in this chapter were prepared and incubated as described in Chapter II. The effects of the following glucose concentrations: 0, 0.05, 0.1, 0.2, 0.5, 1, 5, 10, and 30% (W/V) on the acids formed in these mixtures during glucose breakdown were examined; 0 and 5% glucose four times, 0.05 and 0.1%, three times, and the others, twice. In each experiment, three series of forty identical mixtures, one glucose concentration in each series, were prepared. At each of ten time intervals, four mixtures were removed from each series, two for measurement of both pH and titratable acid and two for measurement of the lactic acid concentration (see below).

Each mixture for the determination of pH and titratable acid was titrated immediately; the total mixture was titrated because most of the buffering capacity resides in the sediment (Kleinberg, 1967). Each titration figure was corrected for the loss of carbon dioxide that occurs during incubation because of the fall in pH (Chapter II).

Lactic Acid Analysis

Each mixture for lactic acid estimation was centrifuged

for 10 minutes at 1470 x g at 4°C. An aliquot containing 0- 0.18 µmoles of lactic acid was removed from the supernatant and transferred to a 10 x 75 mm test-tube and then made up to 0.2 ml with distilled water. Each 10 x 75 mm tube was capped with parafilm and the contents frozen until assayed at a later time for lactic acid.

Preliminary experiments had shown that sampling from the supernatant in this manner gave an accurate measure of the lactic acid concentration in the sediment mixtures, since disrupting the cells by heating the mixtures did not change the concentration of lactic acid in the supernatant.

The enzymic method of Horn and Brun (1956), as modified by Cohen and Noell (1960), was used to analyze the various samples for this acid. In this method, the enzyme lactic acid dehydrogenase is added together with nicotinamide adenine dinucleotide (NAD) to the solution containing the lactic acid. A suitable buffer maintains the pH at 10.6 so that oxidation of the lactic acid by NAD to pyruvic acid can go to completion. The $NADH_2$ formed when the NAD is reduced is then assayed spectrophotometrically at 366 mµ.

Experiments to Identify the Hetero Acids and Other End-Products Produced During Glucose Catabolism

Volatile fatty acids. To identify the volatile fatty acids produced by the salivary system, an experiment

was carried out in which six salivary sediment mixtures, each 300 µl in volume and containing 0.5% glucose, were prepared. Two of the mixtures were used to measure the pH before incubation. The four remaining mixtures were incubated at 37°C for one hour; two were then used to determine pH and two for the measurement of the volatile fatty acids produced during the incubation.

The mixtures for the determination of volatile fatty acids were centrifuged and 200 µl of the supernatant separated into cationic, anionic, and neutral fractions by ion exchange chromatography (see Chapter II). The supernatant was passed through a column containing a cation exchange resin (Dowex 50-H^+) to remove positively-charged compounds. The effluent was then passed through a second column, this time containing an anion exchange resin (Dowex 3- $CO_3^=$) to trap the negatively-charged volatile fatty acids. The acids were then eluted with 0.05 M sodium carbonate and the contents of the eluate analyzed for volatile fatty acids by gas chromatography (Chapter II). The effluent from the column containing the anion exchange resin, i.e., the neutral fraction, was analyzed for a large number of so-called neutral products (see below).

Non-volatile acids. Twelve sediment mixtures were prepared for the identification of their non-volatile fatty acids; four mixtures contained 0% glucose, four contained

0.1%, and four contained 5%. Two mixtures from each set of four were incubated for one hour, the other two served as controls. Each mixture was centrifuged and supernatant then removed for identification of non-volatile acids by 2-dimensional paper chromatography (Smith, 1960, p. 273).

Compounds in the neutral fraction. The neutral fraction was tested for the presence of acetone, diacetal, ethanol, isopropanol, n-butanol, acetoin, glycerol, and 2,3-butanediol by the procedures described by Neish (1952).

Experiments to Determine Whether Hetero Acids Were Degraded to Cause a pH Rise

To determine whether degradation of propionic, acetic, or formic acids by the sediment could have contributed to the pH rise which occurs after glucose runs out in a glucose-sediment mixture, an experiment was carried out in which sediment mixtures containing 0.011 N formic, acetic, and propionic acids were incubated for four hours, and changes in pH determined. A series of mixtures containing lactic acid, the degradation of which produces a pH rise (Sandham, 1963), was included as a standard.

The starting pHs of the acid-sediment mixtures were slightly different, because of the differing ionization constants of the acids. To determine whether the various rates of pH rise could be attributed to the differing starting pHs, serial dilutions of acetic and formic acids

were added to sediment mixtures, and the sediment mixtures incubated for four hours. The serial dilutions provided a range of starting pHs and acid concentrations. The change in titratable acidity occurring between the 0 and 4 hour time intervals was also measured.

In experiments described in Chapter V, the ability of the salivary sediment to convert formate-C^{14} to $C^{14}O_2$ was also tested.

RESULTS

Time Relationships Between the pH and the Lactic, Hetero, and Total Acid Concentrations with Individual Glucose Levels

In the absence of glucose (Fig. 4.1a), hetero acid formed but no lactic. All of the hetero acid was produced during the first fifteen minutes of the experimental period and its concentration remained unchanged during the remainder of the four hour incubation. As previously, the pH initially fell slightly, reached a minimum between 1/2 and 3/4 hours and then subsequently rose.

With 0.05, 0.1, and 0.2% glucose (Figs. 4.1b, 4.1c, and 4.2a, respectively), lactic acid did form; its concentration rapidly increased with each glucose concentration to a maximum and then slowly decreased. The lactic acid maxima occurred progressively later with progressively increasing glucose concentrations. With all three glucose

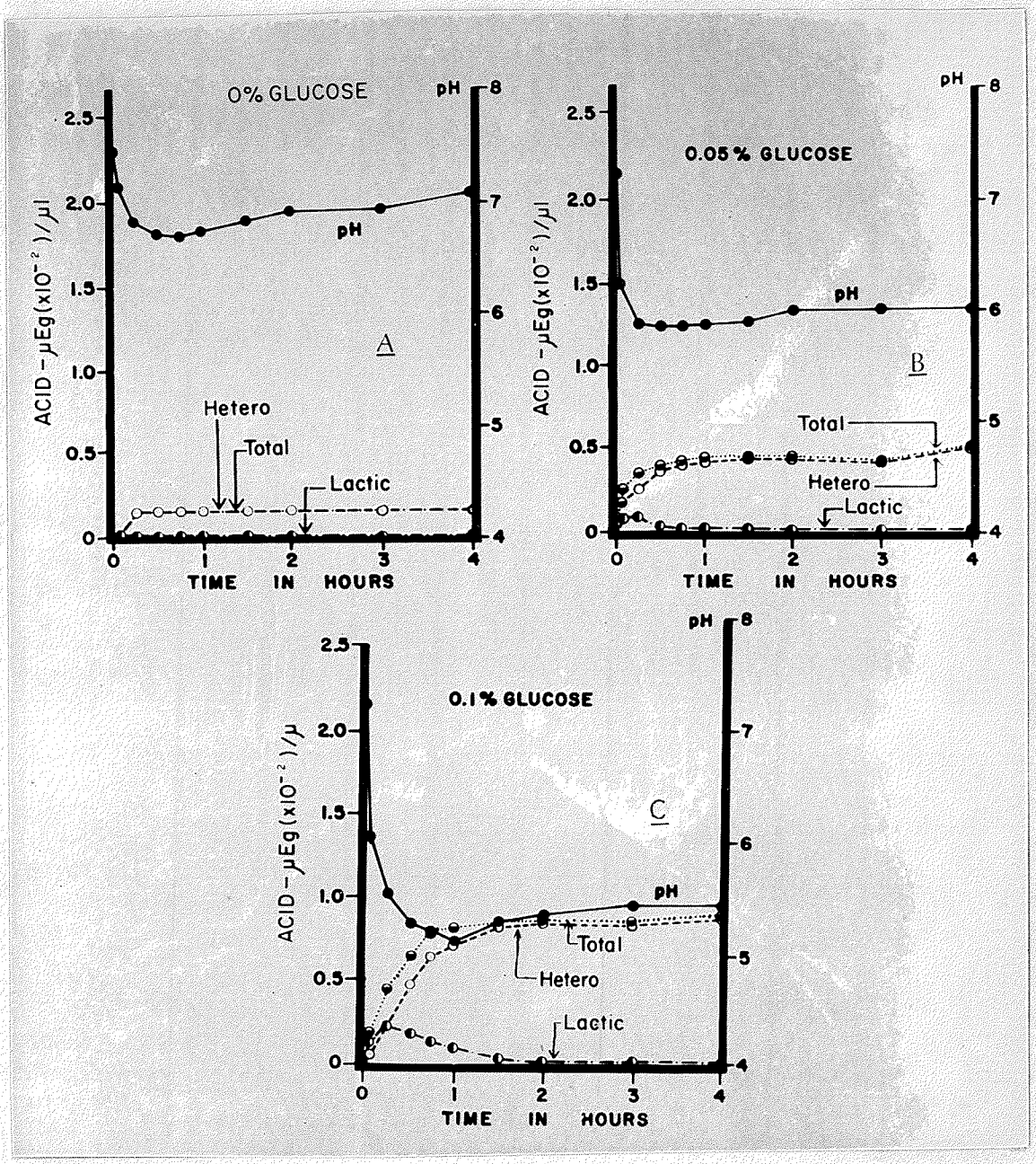


Figure 4.1. Changes in pH and lactic, hetero, and total acids. A., 0% glucose; B., 0.05% glucose; C., 0.1% glucose.

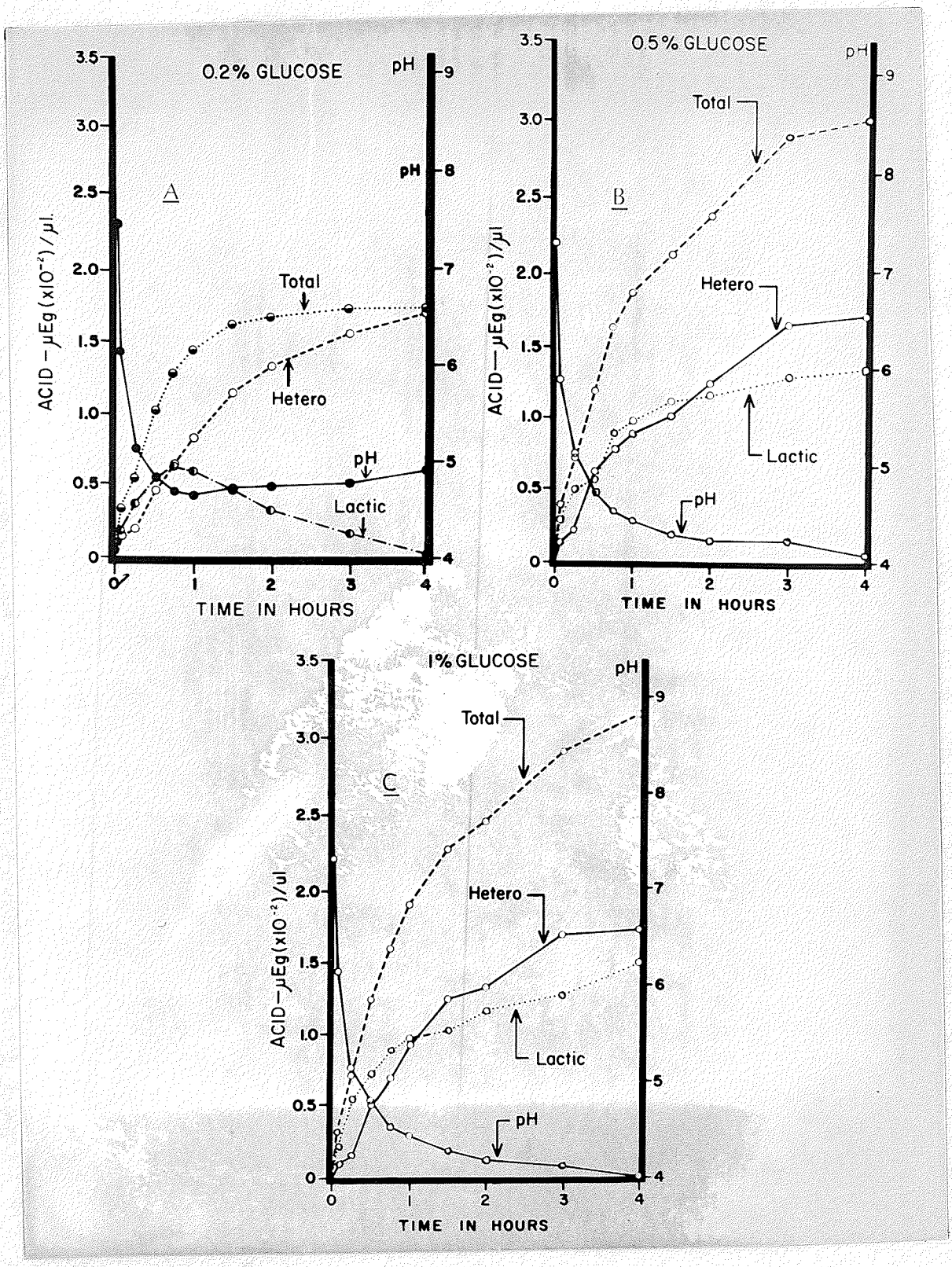


Figure 4.2. Changes in pH and lactic, hetero, and total acids. A., 0.2% glucose; B., 0.5% glucose; C., 1% glucose.

concentrations, the lactic acid maxima occurred earlier than their corresponding pH minima. The hetero and total acid concentrations rose more slowly than the lactic acid concentrations during the early part of the experiments. However, instead of subsequently decreasing, the hetero and total acid concentrations continued to rise reaching, or almost reaching, asymptotes by the end of the four hour experimental period. The pHs, after reaching minima, began to rise before this occurred.

With the higher glucose concentrations, 0.5, 1, 5, 10, and 30% (Figs. 4.2b, 4.2c, 4.3a, 4.3b, and 4.3c), the lactic acid curves rose continuously throughout the four hour incubation period and did not slow to asymptotes or near asymptotes as did their curves with the lower glucose concentrations. The pH with each glucose concentration, as previously (Sandham, 1963), fell throughout the experimental period and showed no subsequent rise.

Comparisons of (i) Lactic, (ii) Hetero, and (iii) Total Acid Time Curves with the Different Glucose Concentrations

To examine the effects of varying glucose concentrations on the lactic, hetero, and total acid time curves, figures 4.4a, 4.4b, 4.4c, 4.5a, 4.5b and 4.5c were prepared.

The rise and fall in the lactic acid curves with low glucose concentrations (0.2% and below) and presence of the

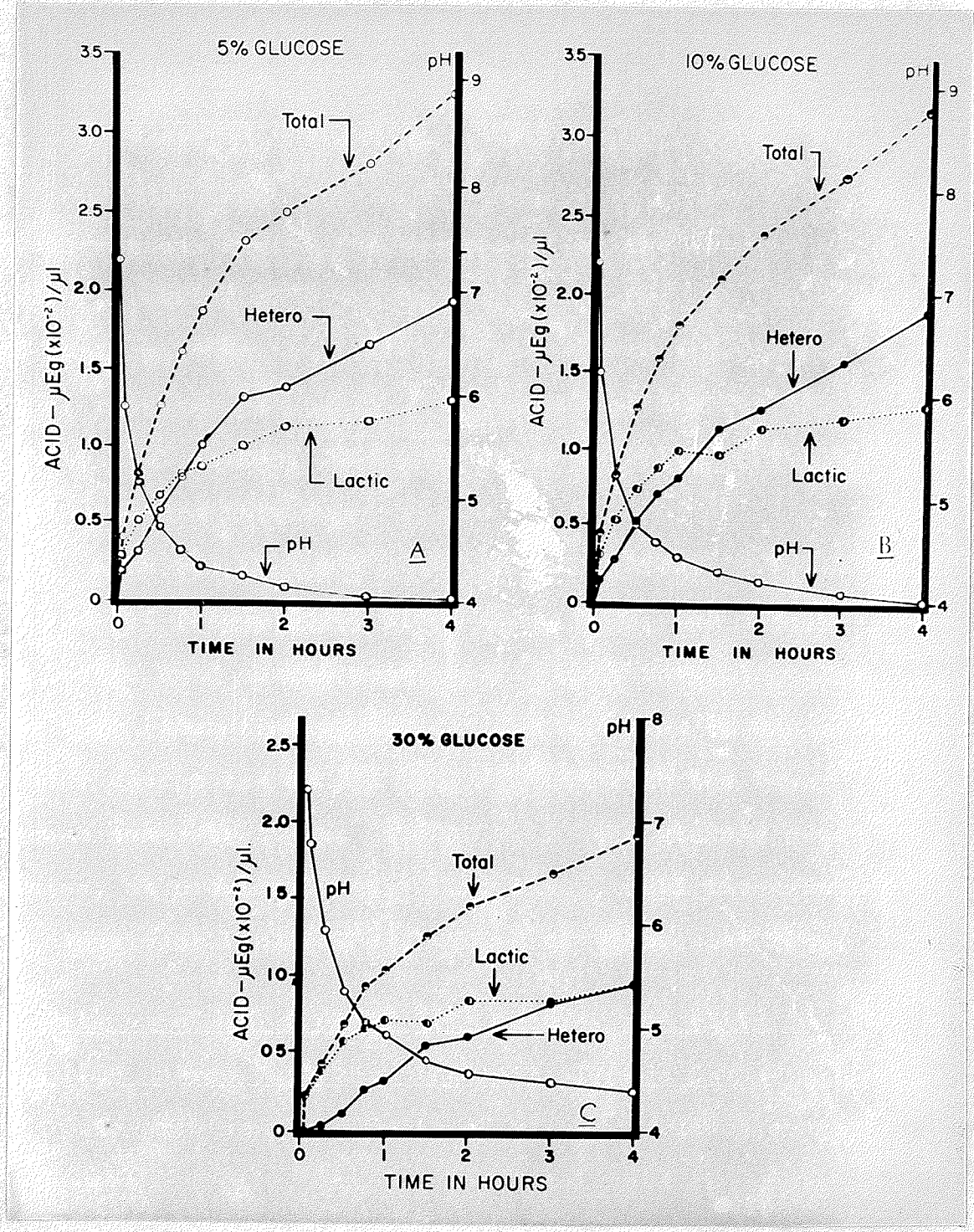


Figure 4.3. Changes in pH and lactic, hetero, and total acids. A., 5% glucose; B., 10% glucose; C., 30% glucose.

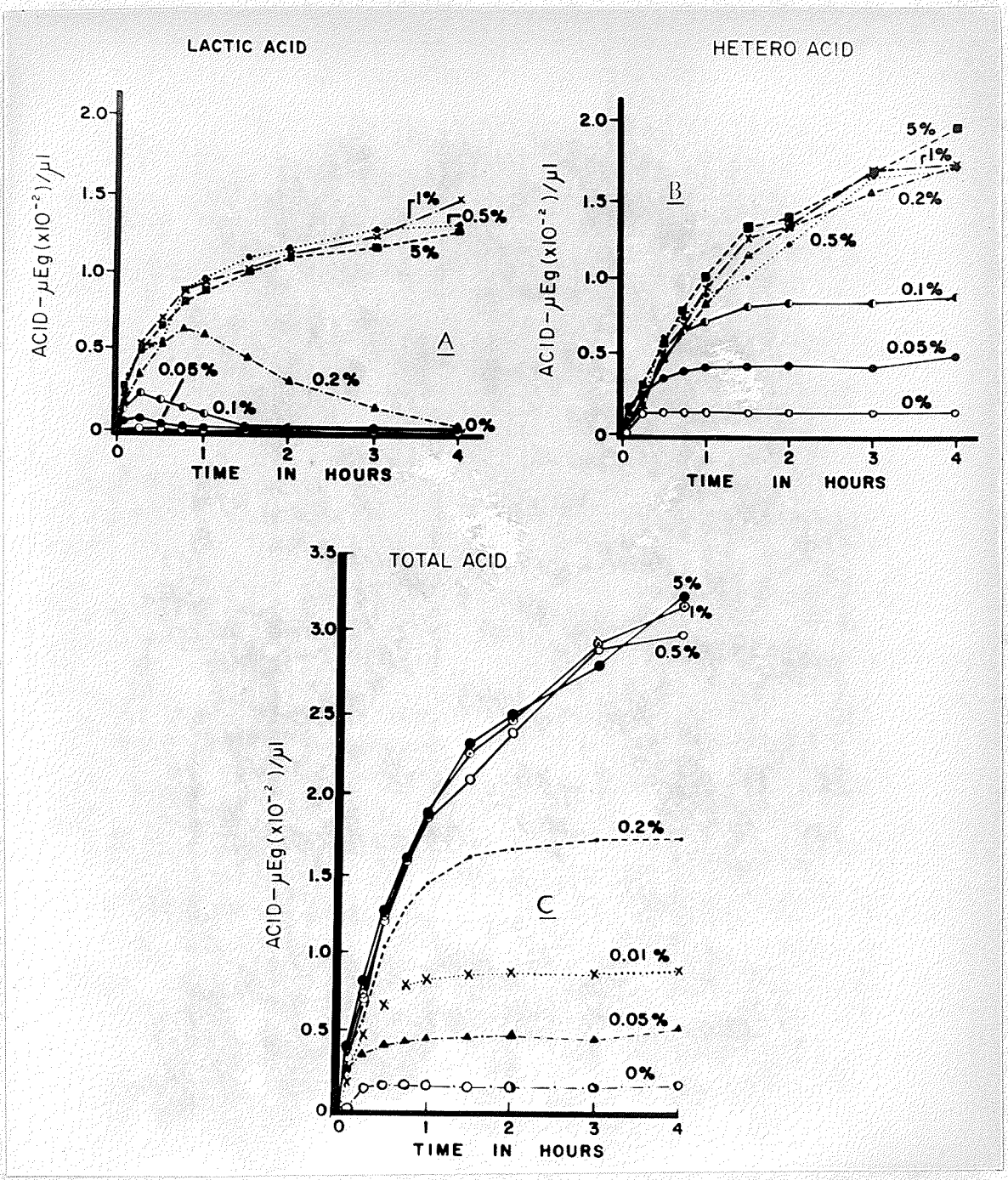


Figure 4.4. Effect of glucose concentrations of 5% and below on changes in acid concentrations. A., lactic acid; B., hetero acid; C., total acid.

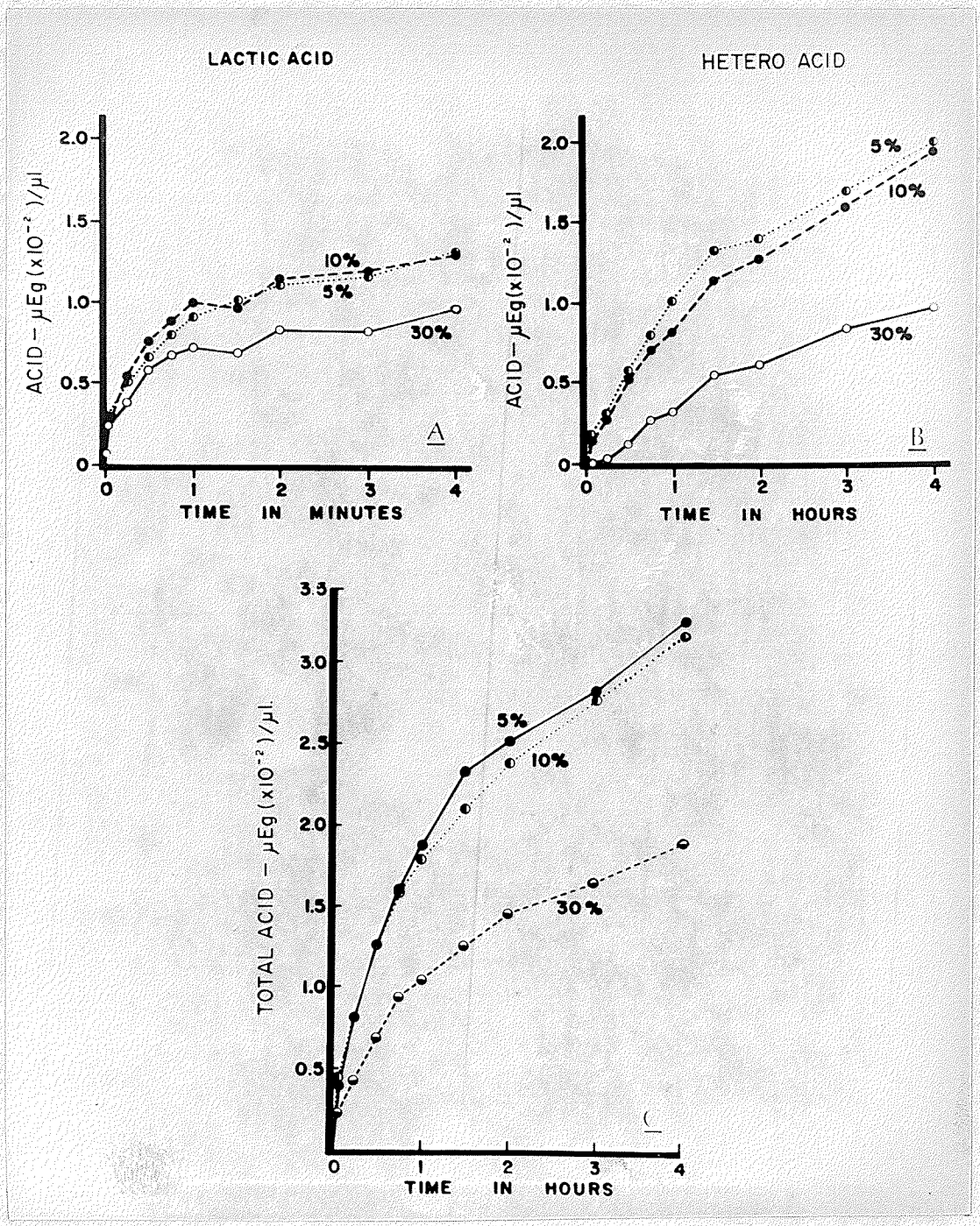


Figure 4.5. Effect of glucose concentrations of 5% and above on changes in acid concentrations. A., lactic acid; B., hetero acid; C., total acid.

rise and absence of the fall in the corresponding hetero and total acid curves are evident from figures 4.4a, b, and c. Although the curves for lactic and hetero acids for the 0.5, 1, and 5% glucose concentrations show the same general shapes, the initial portion of the lactic acid curves rises more steeply while the later portion rises more slowly than the curves for the hetero and total acids (Figs. 4.4a and b). The total acid curves during the last two hours of the experimental period rise more rapidly than the same curves for the hetero acids, while their 0.2% curves during the same time period show a reverse relationship.

The curves for the lactic, hetero, and total acids with 30% glucose were lower than their corresponding curves with 5% glucose. 10% glucose showed much less inhibition for the hetero and total acids and none for lactic acid. The inhibition with 30% glucose was more for the hetero acid than for the lactic acid, particularly during the first few minutes of the experimental period.

Changes in Lactic/Hetero Acid Ratios with Time and Varying Glucose Concentration

The lactic/hetero acid ratio as a function of glucose concentration and the time of incubation are shown in figures 4.6a and b. The ratios vary considerably, but with

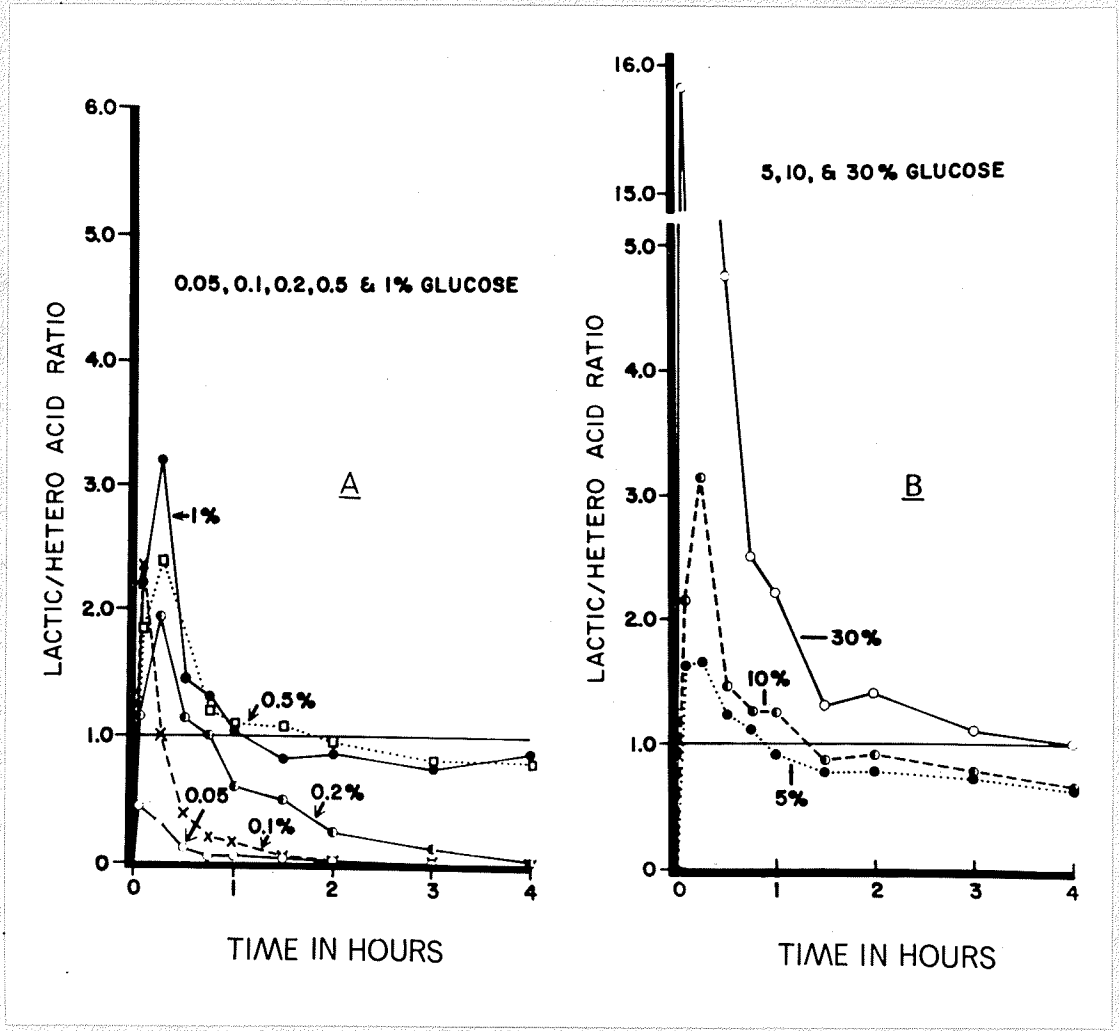


Figure 4.6. Lactic/hetero acid ratios, with glucose concentrations of: A., below 5%; and B., 5% and above.

all glucose concentrations the ratios rise rapidly initially and then fall more slowly during the remainder of the experimental period.

Proportion of Hetero Acid From Lactic Acid and From Other Sources

Because the curves with the lower glucose concentrations (Figs. 4.1a, 4.1b, 4.1c and 4.2a) indicated that some of the hetero acid must have come from sources other than lactic, the following calculations were made from these curves to determine their proportions. The decrease in the lactic acid concentration was subtracted from the increase in the hetero acid concentration for corresponding time intervals after the time of the lactic acid maximum. The difference gives a measure of the hetero acid produced from non-lactate sources (Fig. 4.7a), assuming that the lactic acid that disappeared became hetero acid. The decrease in the lactic acid would give a measure of the hetero acid from lactate sources (Fig. 4.7b).

The results in figure 4.7 indicate that the proportion of hetero acid from non-lactate sources is considerable. Calculation of the decrease in sediment carbohydrate for the same time intervals as 3-carbon acid equivalents accounts for most but not all of the non-lactate hetero acid.

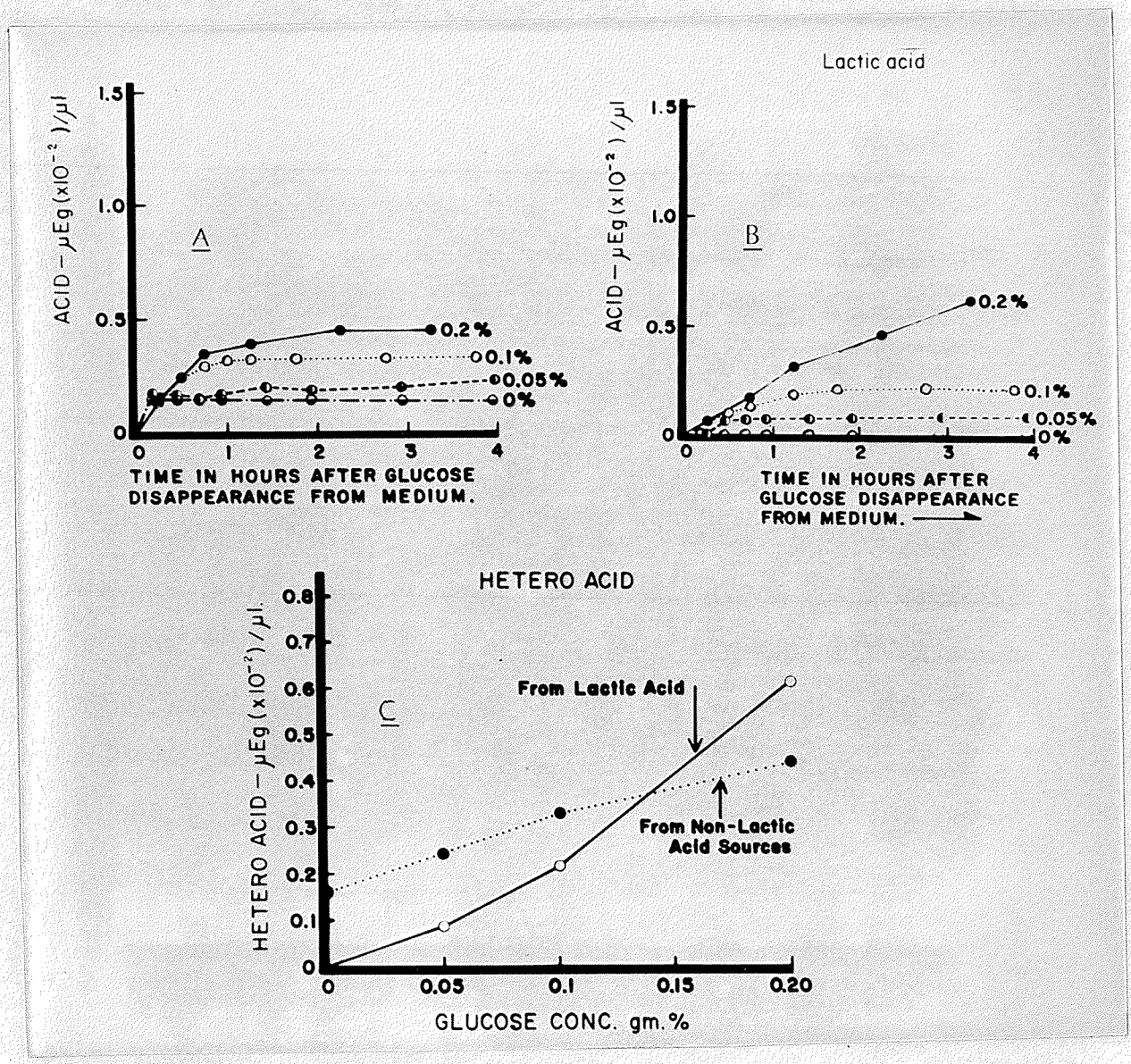


Figure 4.7. Hetero acid formation from: A., non-lactate sources; and B., lactic acid. C., 4-hour values for hetero acid formation from lactic acid and from non-lactic acid sources.

Effect of Glucose Concentration on the Proportion of the Hetero Acid from Lactic Acid and Non-lactate Sources

The four hour values for the hetero acids produced from lactic acid and from non-lactate sources are plotted as a function of glucose concentration in figure 4.7c. More hetero acid was produced from both sources as the glucose concentration was increased between 0 and 0.2%, but the hetero acid originating from lactic increased in concentration much more rapidly. Consequently the lactate/non-lactate hetero acid ratio rose from 0 to 1.3.

Hetero Acids and Neutral Compounds Produced From Glucose

Volatile fatty acids. Gas chromatographic analysis of the supernatant from the sediment mixtures demonstrated the presence of large amounts of acetic and propionic acids, a small amount of an unknown acid (likely valeric), and possibly a trace of butyric (Fig. 4.8). The propionic/acetic ratio was 0.67.

Non-volatile acids. In these experiments no non-volatile organic acids were detected.

Neutral products. No neutral products were found in the supernatants of the sediment mixtures analyzed. The products tested for were acetone, diacetal, ethanol, isopropanol, n-butanol, acetoin, glycerol, and 2,3-butanediol.

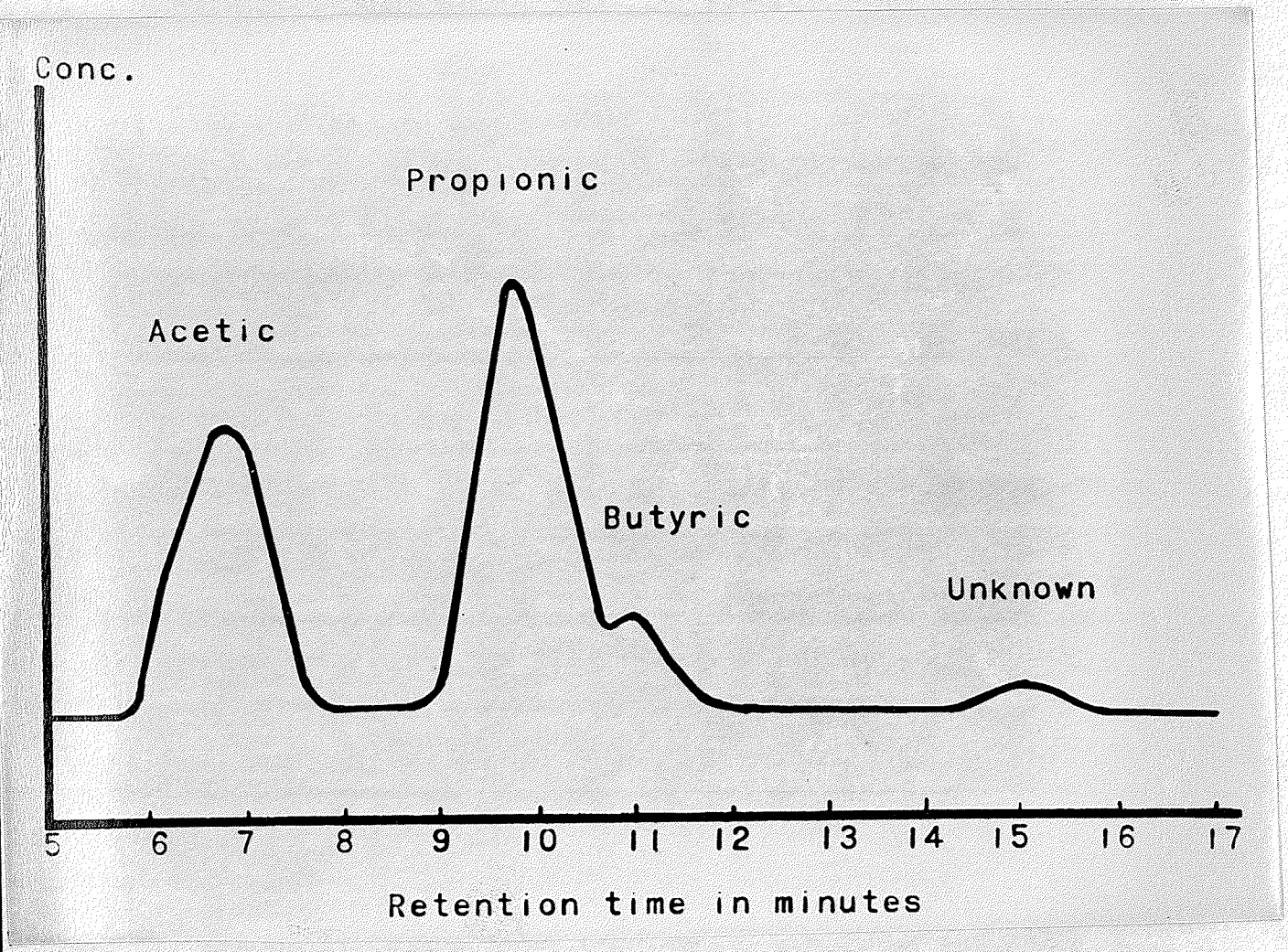


Figure 4.8. Gas chromatographic separation of the volatile fatty acids formed by salivary sediment.

Experiments to Determine Whether the Degradation of Hetero Acids Produced a pH rise

When mixtures containing formic, acetic, propionic, and lactic acids were incubated for four hours (Fig. 4.9a), the pH of mixtures containing acetic and propionic acids rose only slightly (0.15 units), while the pHs of the mixtures containing acetic and propionic acids rose to a significantly greater extent (0.40 units). The starting pHs of the acetic and propionic acid cultures were the same, 5.07, but those for formic and lactic acids were lower, 4.8 and 4.45, respectively.

Mixtures incubated with acetic acid concentrations between 111 and 14 mmolar and with starting pHs between 3.7 and 4.9 (Fig. 4.9b), showed little change in pH and no change in titratable acidity during a four hour incubation period. At higher pHs (and therefore lower acid concentrations), the slight rise in pH that occurred during incubation was not accompanied by a significant decrease in titratable acidity.

In mixtures containing various dilutions of formic acid (Fig. 4.9c), a considerable rise in pH and decrease in titratable acidity (0.13 to 0.17 μEq (x 10^{-2})/μl) occurred when the initial pH was between 4.2 and 6.3. At the high and low extremes of acid concentration and pH, the pH rise was smaller, and the decrease in titratable

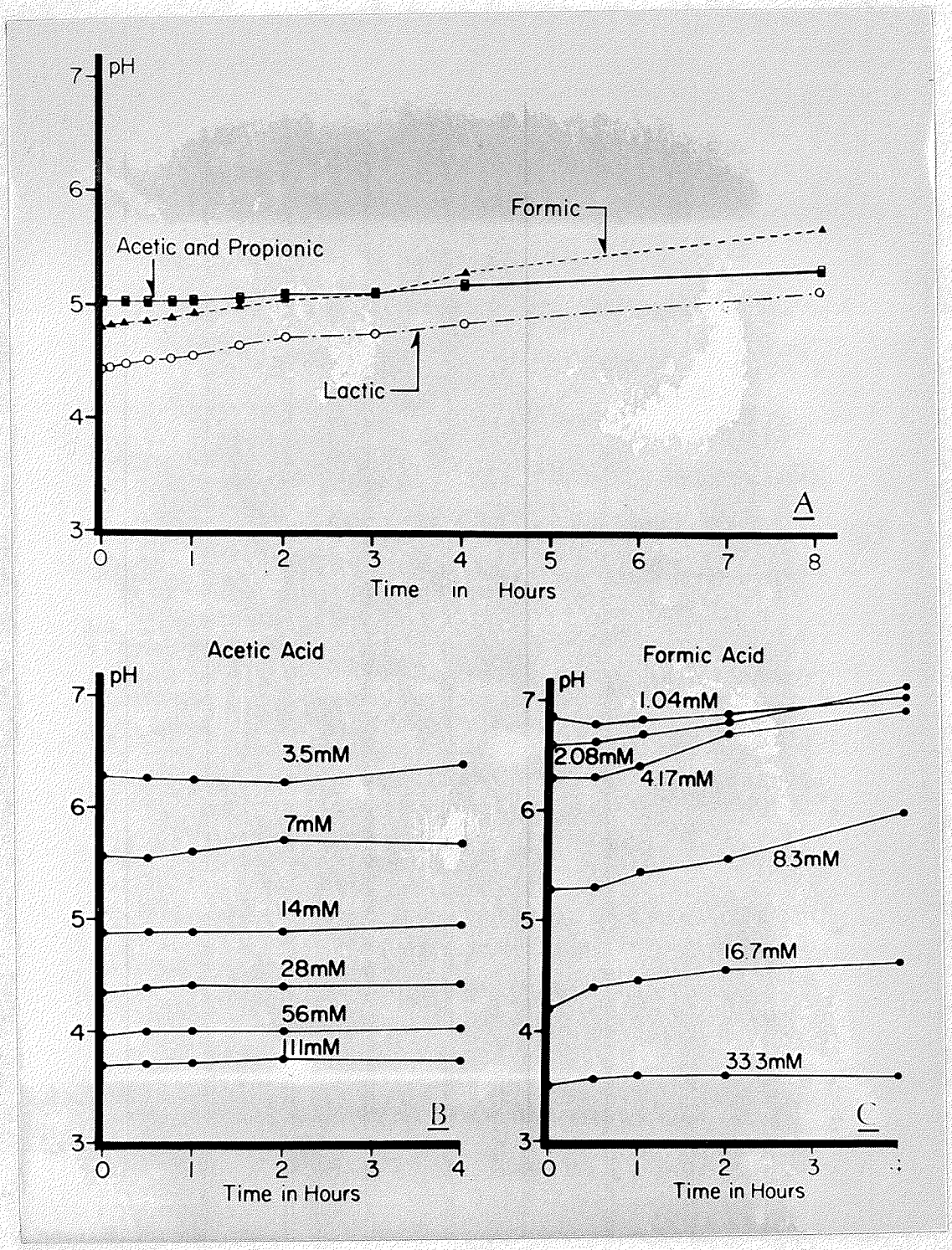


Figure 4.9. Changes in pH when sediment mixtures were incubated with: A., formic, lactic, acetic, and propionic acids; B., a range of concentrations of acetic acid; and C., a range of concentrations of formic acid.

acid was insignificant.

In experiments to be described in Chapter V, glucose-sediment mixtures rapidly converted formate-C^{14} to $C^{14}O_2$.

DISCUSSION

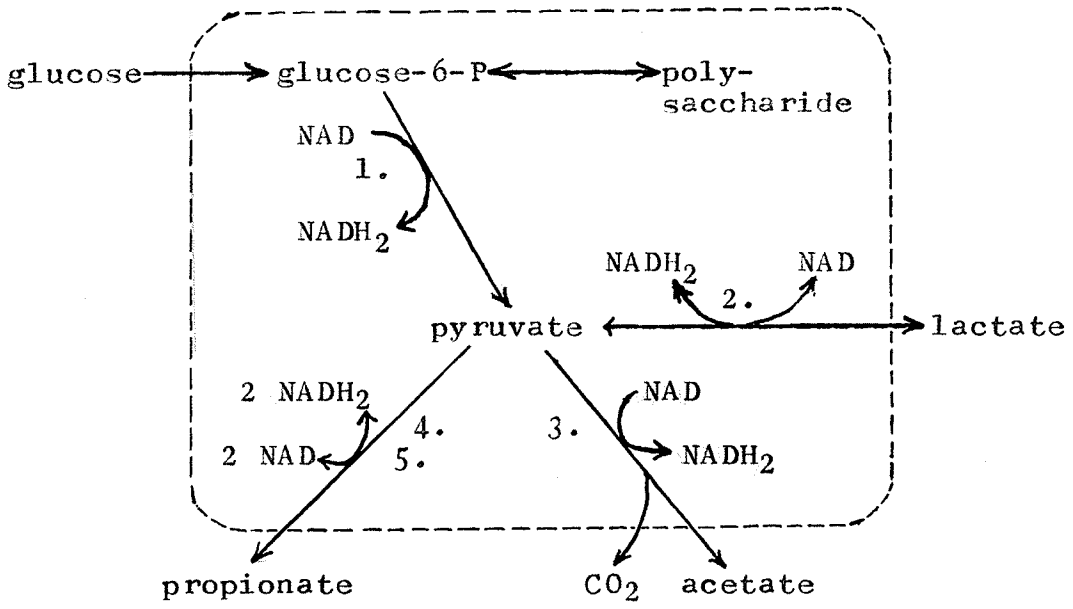
The data in the present study demonstrated that the formation of lactic acid was dependent upon the presence of glucose in the fluid phase of the salivary sediment system. In the absence of glucose, hetero acid was formed, whereas lactic acid was not. With the addition of glucose, lactic acid was formed and continued to form as long as glucose was present in the medium. Once glucose was completely used up, as occurred in the mixtures that started with initial glucose concentrations of 0.05, 0.1, and 0.2% (Sandham, 1963; Sandham and Kleinberg, 1964), formation of lactic acid stopped and its concentration, instead of continuing to rise, slowly fell.

The data also showed that lactic acid was, in essence, an intermediate and not an end product of glucose catabolism by salivary sediment, whereas hetero acid was an end product. This was evident in the experiments with the lower glucose concentrations, in which the hetero acid concentration rose during the initial portion of the experimental period as the concentration of lactic acid rose, continued to subsequently rise as the lactic acid concentration fell and, in the later

part of the experimental period, remained constant after the lactic acid concentration fell to zero.

Considerable new information on the regulation of the Embden-Meyerhof pathway and its integration with related pathways has accumulated during recent years (Chance and Hess, 1959; Hess and Chance, 1959; Chance et al., 1963). Based upon this information, the scheme in figure 4.10 is proposed to explain the above findings and how alteration of extracellular glucose levels will determine the amount of acids formed.

Addition of glucose to the sediment mixtures would stimulate the glycolytic rate, increase the rate of formation of pyruvate, and decrease the ratio of $NAD/NADH_2$ within the cells in the sediment (cf. Chance et al., 1963). The increase in pyruvate would stimulate formation of lactic, acetic, and propionic acids (Fig. 4.10). The decrease in the $NAD/NADH_2$ ratio would also stimulate the conversion of pyruvate to both lactic and propionic acids, but would inhibit the formation of acetic acid. Consequently, progressively increasing glucose concentration (at least up to 5%) would increase glucose uptake, increase glycolytic flux, and progressively favour the formation of lactic and propionic acids over the formation of acetic acid. The propionic/acetic acid ratio would therefore increase (Chapter V). Above a glucose concentration of 5%, inhibition of



Reaction No. Reactions Requiring the NAD-$NADH_2$ System

1. glyceraldehyde-3-P + NAD + Pi ⟶ 1,3-diphosphoglycerate + $NADH_2$

2. pyruvate + $NADH_2$ ⟶ lactic + NAD

3. pyruvate + CoASH + NAD ⟶ Acetyl-CoA + CO_2 + $NADH_2$

4. oxaloacetic acid + $NADH_2$ ⟶ L-malic acid + NAD

5. fumaric acid + $NADH_2$ ⟶ succinic acid + NAD

Fig. 4.10 Scheme for the Formation of Lactic and Hetero Acids From Glucose by Salivary Sediment

glucose uptake would slow the glycolytic flux and inhibit the formation of both lactic and hetero acids, particularly with 30% glucose (Fig. 4.3c).

In the mixtures in which all of the added glucose was used up by the end of the experimental period (0.2% and less), polysaccharide is stored during the period that glucose is still present in the medium and utilized once glucose is no longer present (Sandham, 1963; Sandham and Kleinberg, 1964). This alternate source of substrate would permit the glycolytic flux to be maintained, albeit at a reduced rate, until this source of substrate is used up. Slowing of the flux would slow the rate of pyruvate formation and permit lactic acid to be removed, initially favouring flow to propionic acid, because of the low $NAD/NADH_2$ ratio, and later favouring flow to acetic acid as the $NAD/NADH_2$ ratio rises. The formation of hetero acid and the lack of formation of lactic acid in the mixtures to which no glucose was added can be attributed to a low glycolytic flux (therefore a low rate of pyruvate formation and a high $NAD/NADH_2$ ratio), favouring flow of pyruvate into acetic acid and CO_2.

Relationship of Lactic and Hetero Acid Formation to the Changes in pH in the Salivary Sediment System

Because lactic acid is a stronger acid (pK 3.86) than either acetic or propionic acids (pK 4.73 and 4.80, respec-

tively), a stoichiometric decrease in the former and increase in the latter would result in a rise in pH (Stephan and Hemmens, 1947; Calandra and Adams, 1951; Sandham, 1963; Sandham and Kleinberg, 1965). Further metabolism of acetic and propionic acids to less-acidic products did not occur to a significant extent in the present study, and did not produce a significant pH rise (Fig. 4.9).

In the present study, in the mixtures with the lower initial glucose concentrations (0.05, 0.1, and 0.2%), the pH rapidly fell and then slowly rose; the rapid formation of both lactic and hetero acids corresponded to the initial rapid fall in pH. From a previous study (Sandham, 1963; Sandham and Kleinberg, 1964), the glucose in each of the mixtures reached zero concentration before the pH minima were reached; the time that this occurred corresponded to the time in the present study that the maxima in the lactic acid and sediment carbohydrate concentrations were reached. As soon as the glucose reached zero concentration, both the lactic acid and sediment carbohydrate concentrations began to fall, yet the pH continued to decrease for a further 15 to 30 minutes. This clearly showed that the formation of lactic acid was not responsible for the reaching of the respective pH minima, but that the pH minima were reached because of the formation of additional hetero acid from carbohydrate stores. In other words, sufficient additional

hetero acid was formed (providing a pH decrease) to more than offset the tendency for the pH to rise as lactic acid was converted to the weaker acids. At the pH minima, depletion of the carbohydrate stores would result in reduced formation of hetero acid and these processes would be in balance.

That formation of hetero acid is more important than the formation of lactic acid in reaching pH minima or low pH levels is supported by the experiments in which the glucose concentrations were higher (0.5, 1, 10, and 30%). In these, although the pH fell continuously throughout the experimental period, lactic acid concentrations in the respective mixtures approached an assymptote, whereas the corresponding hetero acid concentrations did not. Instead, the hetero acids continually accumulated and were obviously responsible for the slow continual falls in pH observed.

Lactic/Hetero Acid Ratio

It is obvious from figure 4.6 that the lactic/hetero acid ratio varied widely and in the present study was a function of the glucose concentration and of the length of time that the experimental mixtures had been incubated. The lactic/hetero acid ratio rose and fell over the four hour experimental period with all glucose concentrations studied. The rise was associated with the initial rapid formation of lactic acid. At the lower glucose concentrations,

the subsequent fall in the lactic/hetero acid ratio can be attributed to the decrease in lactic acid as the lactic acid was converted to hetero acid. At the higher glucose concentrations, the fall in the hetero/lactic acid ratio can be attributed to the hetero acid increasing (being an end product), while the lactic remained stationary (being an intermediate). The hetero acid increase was likely an increase in propionic acid (cf., Delwiche, 1948).

In the present study, the hetero acid was identified as mainly acetic and propionic; the ratio of propionic/acetic was 0.67 in the mixtures analyzed. It is likely, however, that this ratio will be different for other glucose concentrations and times of incubation; verification of this point must await further experimentation.

Of interest, the types and relative amounts of the various volatile fatty acids measured in the present study were very similar to those found by Guggenheim, Ettlinger, and Muhlemann (1965) in stimulated saliva from 240 school children. Similar types and yields of volatile fatty acids have been obtained by Ranke, Ranke and Bramstedt (1965), using dental plaque incubated in vitro, and by de Stoppellaar and Gibbons (1965) using oral debris, except that these investigators found that more propionic than acetic acid was present. The good agreement between the analyses in these studies and

that in the present study may be only fortuitous.

Gilmour and Poole (1966) prepared plaque samples *in situ* and then incubated them *in vitro* in a medium containing 1.5% glucose, for a fixed length of time. They found that (as with sediment) lactic, acetic, and propionic acids were formed. Although they incubated all their samples for a constant period of time and with one glucose concentration, a wide variation in the *lactic/propionic* acid ratio with the different plaque samples was observed. Because it is difficult to control the number and types of cells in such plaque samples, and because the rate of fermentation of the glucose is a function of cell concentration (Stephan and Hemmens, 1947; Kleinberg, 1961; 1967), the stage in conversion of glucose to the various acids would be expected to vary. From figure 4.6, it is obvious that slight variations in the early part of a fermentation results in wide variations in the *lactic/hetero* acid ratio.

The high percentage of the hetero acid that was propionic acid indicates that propionic acid-producing microorganisms are major participants in the fermentation of glucose by the salivary system. The most numerous propionic acid-producers in the mouth are the veillonella, which make up between 17 and 50% of the oral microflora (Pincus, 1942; Douglas, 1950; Rogosa, 1956; Richardson and Jones, 1958). Some strains of *Propionibacterium acnes*

are also present in the oral cavity (Rasmussen, Gibbons, and Socransky, 1966), but their numbers have not as yet been estimated. Clostridia, the only other known propionic acid-producers, do not occur in the oral cavity except perhaps as transients (Burnett and Scherp, 1962, p. 277).

The Possibility of Formic Acid Accumulation

The rapid rise in pH which occurred when formic acid was incubated with salivary sediment indicated that formic acid could be metabolized by the cells in the sediment and converted to less-acidic substances. Addition of formate-C^{14} and monitoring the $C^{14}O_2$ produced (Chapter V) showed that one of these products was CO_2. Several enzymes of widespread occurrence in microorganisms might have been responsible for the breakdown of formic acid, including formic dehydrogenase, hydrogenlyase (Quastel and Whetham, 1925; Stephanson and Strickland, 1931), and catalase (Chance, 1951).

The rate at which formic acid was broken down indicated that it did not accumulate during glucose catabolism in the present study; if so, this would be consistent with the findings of workers, studying the metabolism of glucose in pure cultures of various microorganisms, who found that formic acid does not accumulate at pHs below 6.0 (Stokes, 1949; Blackwood, Neish and Ledingham, 1956; Platt and Foster, 1958).

Equivalence of CO_2 and Acetic Acid

If pyruvate were split to form equimolar quantities of carbon dioxide and acetic acid, examination of the ratio between the carbon dioxide and the acetic acid produced should support this. In the previous chapter, with 5% glucose, the carbon dioxide formed from glucose-U-C^{14} after incubation for one hour was between 0.25 and 0.33 μmoles ($\times 10^{-2}$)/μl. This is approximately equal to the acetic acid formed in the experiment in the present chapter, which was 0.30 to 0.39 μmoles ($\times 10^{-2}$)/μl. If this good agreement should hold for other time points and other glucose concentrations, then the formation of acetic acid and CO_2 by a pyruvate split is likely.

Possible Importance of Hetero Acid in Plaque to Dental Caries and Oral Ecology

Because the acids formed in sediment appear to be similar to those formed in plaque (Ranke, Ranke and Bramstedt, 1965; de Stoppelaar and Gibbons, 1965; Gilmour and Poole, 1966), it is possible that hetero acids are more important in causing enamel dissolution than lactic acid. If so, then the microorganisms responsible for the formation of hetero acid might be more important in the caries process than those responsible for the formation of lactic acid.

Hetero acid may also be a significant factor in the selection of bacterial species in the mouth, as illustrated

by the well-known inhibitory effects of the volatile fatty acids on the growth of Enterobacteria (de Stoppelaar and Gibbons, 1965).

Non-Volatile Organic Acids

Although some investigators have at various times identified significant quantities of pyruvic and succinic acids in saliva-glucose mixtures, these acids were observed when the incubations were carried out at neutral pH. None of these acids were found in the samples analyzed in the present study, presumably because the pH was low (Barker and Lipman, 1944; Johns, 1951).

CHAPTER V

EFFECTS OF FLUORIDE ON SALIVARY SEDIMENT METABOLISM

Because fluoride has been shown to reduce dental caries (McKay, 1925, 1929; Dean et al., 1941; Dean, Arnold, and Elvove, 1942; Arnold, 1960), many attempts have been made to determine its mechanism of action. Two mechanisms have been considered, (i) whereby fluoride reduces the solubility of teeth, and (ii) whereby fluoride inhibits acid production by the bacteria on the teeth; the former has received more attention than the latter.

Stralfors (1950) and Bergman (1953) both showed that fluoride inhibited the fall in pH of dental plaques *in vivo* following a glucose rinse. A similar inhibitory effect of fluoride on pH fall has been observed *in vitro* with paraffin-stimulated saliva (Wright and Jenkins, 1954; Jenkins, 1959), *Streptococcus mitis* isolated from the mouth (Weiss et al., 1965) and on salivary sediment (Jenkins, 1959; Kleinberg and Sandham, 1964). The *in vitro* studies demonstrated that fluoride as low as 0.5 ppm in concentration, significantly inhibits the pH fall resulting from glucose breakdown.

Fluoride inhibition of acid production, measured by back-titration rather than by measurement of pH, has been observed in pure cultures of microorganisms isolated from the mouth (Bibby and Van Kesteren, 1940; Cox and Levin,

1942; Weiss et al., 1965), and in paraffin-stimulated saliva (Wright and Jenkins, 1954). When acid production was measured manometrically, Lilienthal (1956), using salivary sediment, and Bramstedt, Kronke, and Naujoks (1957) using pure cultures of Streptococcus salivarius and Lactobacillus casei isolated from the mouth, showed that fluoride between 1.0 and 1.9 ppm stimulated acid production. For inhibition of acid production to occur in his experiments, Lilienthal found that 19 ppm fluoride was required.

The failure of Lilienthal to show inhibition of acid production with fluoride levels below 19 ppm was attributed by Jenkins (1959) to a number of factors, including the fact that Lilienthal's experiments were carried out at neutral pH. In Jenkins' experiments and in those of other investigators who observed inhibition with fluoride levels much lower than 19 ppm, the pH was permitted to fall.

Stimulation with fluoride at high pH and fluoride inhibition at low pH were observed by Fitzgerald and Bernheim (1944) in their studies on the respiration in Mycobacterium phlei, and by Sandham and Kleinberg in their studies with salivary sediment (Sandham, 1963; Kleinberg and Sandham, 1964).

Fluoride inhibited the accumulation of carbohydrate when glucose was metabolized by the cells in salivary sediment (Sandham, 1963; Kleinberg and Sandham, 1964), and

by Streptococcus mitis (Weiss, Schnetzer, and King, 1964; Weiss et al., 1965). At the same time that fluoride inhibited the accumulation of carbohydrate by the cells in both systems, fluoride also inhibited the pH fall in their respective media and the utilization of the glucose by the cells. Inhibition of all three processes increased with decreasing pH. Also, the concentration of fluoride necessary to inhibit carbohydrate storage was less at lower pH levels than at higher ones. Weiss et al. found that addition of 50 ppm fluoride to the cells, while the cells were accumulating carbohydrate, caused the accumulation to stop and the concentration of stored carbohydrate to fall as if no glucose were present in the medium. Addition of the same concentration of fluoride to lyophilized cells had little effect on the rate of breakdown of stored carbohydrate. From this they concluded that fluoride acted on the synthesis, rather than the degradation, of carbohydrate stores.

Fluoride has also been shown to affect the activity of a large number of enzymes, for example, enolase, catalase, glutamine synthetase, succinic dehydrogenase, acid phosphatase, pyrophosphatase, and phosphoglucomutase (Hewitt and Nicholas, 1963). The effects of fluoride on certain isolated enzymes are often not the same as its effects on the same enzymes located within the intact cell. For example, succinic dehydrogenase is inhibited less by fluoride when

isolated than when it is part of the sequence of respiratory enzymes in the cell (Slater and Bonner, 1952).

The inhibitory effect of fluoride on glycolysis has usually been attributed to inhibition by fluoride of the activity of the enzyme enolase (Lohmann and Meyerhof, 1934). However, because of its simultaneous inhibition of carbohydrate storage and glucose uptake in salivary sediment (Kleinberg and Sandham, 1964) and Streptococcus mitis (Weiss et al., 1965), Sandham and Kleinberg have suggested that the site of fluoride inhibition may rather be in, or in association with, the cell membrane. Lehninger, Greenawalt, and Rossi (1963) have demonstrated the presence of calcium and phosphate in mitochondrial membranes in a stoichiometric relationship suggestive of the presence of hydroxyapatite. If bacterial cell membranes contain, or are associated with, complexes of calcium or magnesium and phosphate, fluoride would modify the solubility of these complexes, thereby altering the availability of inorganic phosphate for several cellular enzymic processes. One of these processes is the phosphorylation of glucose by hexokinase, inhibition of which would decrease glucose uptake, glucose phosphorylation, and both carbohydrate synthesis and acid production as noted in the studies of Kleinberg and Sandham (1964 and Weiss et al. 1965).

The experiments in this chapter were designed to

determine, in the salivary sediment system, (i) whether fluoride affected the formation of carbon dioxide during glucose breakdown, (ii) whether fluoride affected the rates of formation of lactic and hetero acids, also during glucose breakdown, and (iii) the mechanism whereby fluoride acts.

METHODS

A. Effect of Fluoride on the Rate of Formation of Carbon Dioxide

1. Total Carbon Dioxide

Six experiments were run to determine the effect of 2.2 and 4.4 ppm fluoride on the formation of total carbon dioxide in the salivary sediment systems used in these studies. Two experiments were with 0.05% glucose, two with 0.5% glucose, and two with 5% glucose. Each experiment contained three series of 28 tubes. No fluoride was added to one series, 2.2 ppm fluoride was added to the second, and 4.4 ppm to the third. At each of seven time intervals during a four hour incubation, four tubes were removed from each series, two for pH measurement, and two for estimation of total carbon dioxide (see Chapter II).

The effect of 20 ppm fluoride was also tested with the glucose concentration at 5%. In this experiment, a total of sixty incubation mixtures were used; six were removed at

each of ten time intervals during a four hour incubation. Two of the removed tubes were for the determination of pH, the other four were for the determination of carbon dioxide.

2. Carbon Dioxide Carbons Originating From the Carbons of Glucose Added as Substrate.

The effect of fluoride on the proportion of the carbon dioxide carbons that originated from the different carbons of glucose was tested in microradiorespirometry experiments as in Chapter III. The effects of both 4 and 20 ppm were tested with each of 0.05 and 5% glucose.

In each experiment, twelve series, twenty tubes in each, were prepared. Fluoride was added to the mixtures in six series, the remaining six series served as controls. To five of the six series of mixtures in both the experimental and control groups, glucose-1-C^{14}, -2-C^{14}, -3,4-C^{14}, -6-C^{14}, and -U-C^{14} were added to the unlabelled glucose. No labelled glucose was added to the sixth series in both groups. The unlabelled series was used for the determination of pH.

At each of five time intervals during a four hour experiment, two tubes were removed from each series, either for pH determination or for measurement of the labelled carbon dioxide (Chapter II).

B. Effect of Fluoride on the Rates of Accumulation of Lactic, Hetero, and Total Acids.

Because of the absence of an inhibitory effect of fluoride on carbon dioxide originating from 0.05% glucose (see results below), the glucose concentration used in the sediment mixtures in the four experiments in this section and the remaining experiments in this chapter was 5%.

The effects of 2 and 4 ppm fluoride on the rates of formation of lactic, hetero, and total acids in sediment mixtures containing 5% glucose were determined in the following type of experiment. Nine series, with forty tubes in each series, were prepared, three series without added fluoride, three with 2 ppm fluoride, and three series with 4 ppm fluoride. At each of ten time intervals during a four hour incubation, four mixtures were removed from the forty mixtures in each series. Two of each set of four were for the determination of pH and total acid, and two for the determination of lactic acid (Chapter IV). The hetero acid concentrations were calculated as in Chapter IV.

To determine the effect of fluoride on the formation and removal of lactic acid when glucose was the substrate, the following experiment was carried out. Two series of sediment mixtures, one with and one without 20 ppm fluoride, were incubated for four hours and pH and lactic acid concentrations determined at regular intervals. In

a similar experiment, lactate-1-C^{14} was added at the two hour interval and the total radioactivity in the supernatant and the radioactivity in the volatile fatty acids of the supernatant measured at 2 and 4 hours.

The volatile fatty acids were separated from the supernatant by microdiffusion (Conway, 1962, p. 234), and the radioactivity in these acids determined by liquid scintillation (Chapter II). Preliminary experiments had shown that of the following volatile fatty acids: formic, acetic, propionic, and butyric, butyric diffused at the slowest rate. Therefore, C^{14}-butyric acid was included as a standard to check the completeness of the diffusion. Experiments also established that lactic acid does not diffuse under these conditions.

C. Effect of Low pH on the Effect of Fluoride on pH, Carbon Dioxide, and Acid Changes When Glucose and Lactic Acid are the Substrates

Several investigators (Wright and Jenkins, 1954; Jenkins, 1959; Kleinberg and Sandham, 1964; and Weiss et al., 1965) have found that fluoride has its greatest effect on pH changes, carbohydrate storage, and acid production by the oral microorganisms when the pH is low. Jenkins (1959) utilized experiments in which he adjusted the pH of glucose-saliva and glucose-sediment

mixtures to approximately 5.0 before incubation. He found that by this procedure, he could actually obtain a pH rise during incubation when fluoride was present.

In the present study, an experimental procedure similar to that of Jenkins was used to determine the effect of fluoride on pH and lactic acid concentration when (i) glucose and lactic acid, and (ii) lactic acid, were substrates. In this experiment, four series of incubation mixtures were prepared, each series containing one of the following: (i) lactic acid, (ii) lactic acid and fluoride, (iii) glucose and lactic acid, and (iv) glucose, lactic acid, and fluoride. The initial pH of all mixtures was approximately 5.0. During a two hour incubation, mixtures were removed for the determination of pH and lactic acid concentration.

When glucose was present in the above experiment, the effect of fluoride on the changes in lactic acid concentration would be the net result of the effects of fluoride on the rates of formation and degradation of lactic acid. Therefore experiments were carried out to determine the effects of fluoride on (i) the rate of degradation of lactic acid to other products when glucose was present, and (ii) the rates of conversion of glucose to other products. From these, the effect of fluoride on the rates of formation and degradation of lactic acid

could be calculated.

To determine (i), an experiment was carried out in which glucose-lactic acid-sediment mixtures with and without fluoride were prepared, and a trace amount of lactate-1-C^{14} added before incubation. During a two hour incubation, mixtures were removed to determine the disappearance of C^{14} from the supernatant and the incorporation of C^{14} into volatile fatty acids. To determine (ii), an experiment was carried out which was identical to (i), except that glucose-U-C^{14} was added instead of lactate-1-C^{14}. Mixtures were removed during incubation to determine the conversion of glucose-U-C^{14} to $C^{14}O_2$ and C^{14}-volatile fatty acids.

When the pHs of the mixtures are initially lowered by the addition of lactic acid, the types and quantities of acids present are different from those present when the pH is lowered as a result of the breakdown of glucose (see Chapter IV). To test whether the effect of fluoride might be different under these situations, an experiment was carried out similar in some respects to one carried out by Jenkins (1959), in that fluoride was added to the mixtures after the pH had fallen to below 5.0 as a result of glucose breakdown. In the present study, this was done by allowing glucose-sediment mixtures to incubate for 30 minutes. Lactate-1-C^{14} was then added to all of the mixtures, and fluoride to only half. The other half served as controls.

The incubation was then continued for another 1½ hours, and the disappearance of the radioactivity from the supernatant, its appearance in volatile fatty acids, and pH were measured.

D. Effect of Fluoride on Formation of Carbon Dioxide from Formate

Experiments were carried out to see whether stimulation of carbon dioxide and the inhibition of acid production in the presence of fluoride might result from formic breakdown to carbon dioxide. Sediment mixtures containing 5% glucose were incubated for four hours. Half contained fluoride; half served as controls. At the two hour time interval, a trace of formate-C^{14} was added to all of the mixtures, and the formation of $C^{14}O_2$ determined.

RESULTS

Effect of Fluoride on pH Fall

Inhibition of the pH fall in the salivary sediment system used in this study was directly related to the fluoride concentration (Fig. 5.1). As previously, the inhibition was more as the pH progressively fell (Sandham, 1963; Kleinberg and Sandham, 1964).

Effect of Fluoride on the Formation of Carbon Dioxide

Total carbon dioxide. With glucose concentrations of 0.05, 0.5, and 5% (Figs. 5.2a, b, and c, respectively) 2.2

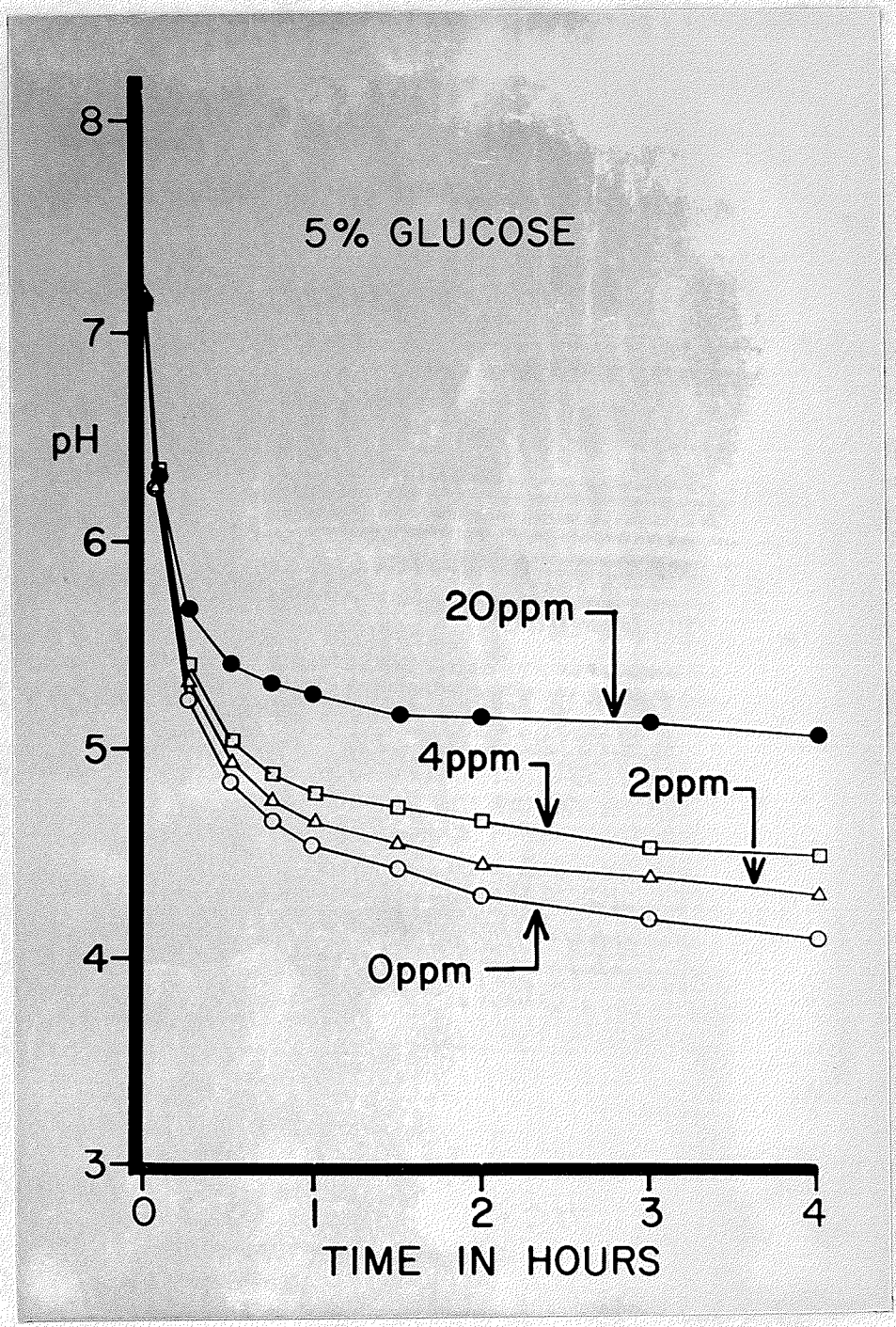


Figure 5.1. Effect of various concentrations of fluoride on the pH fall occurring in sediment mixtures containing 5% glucose.

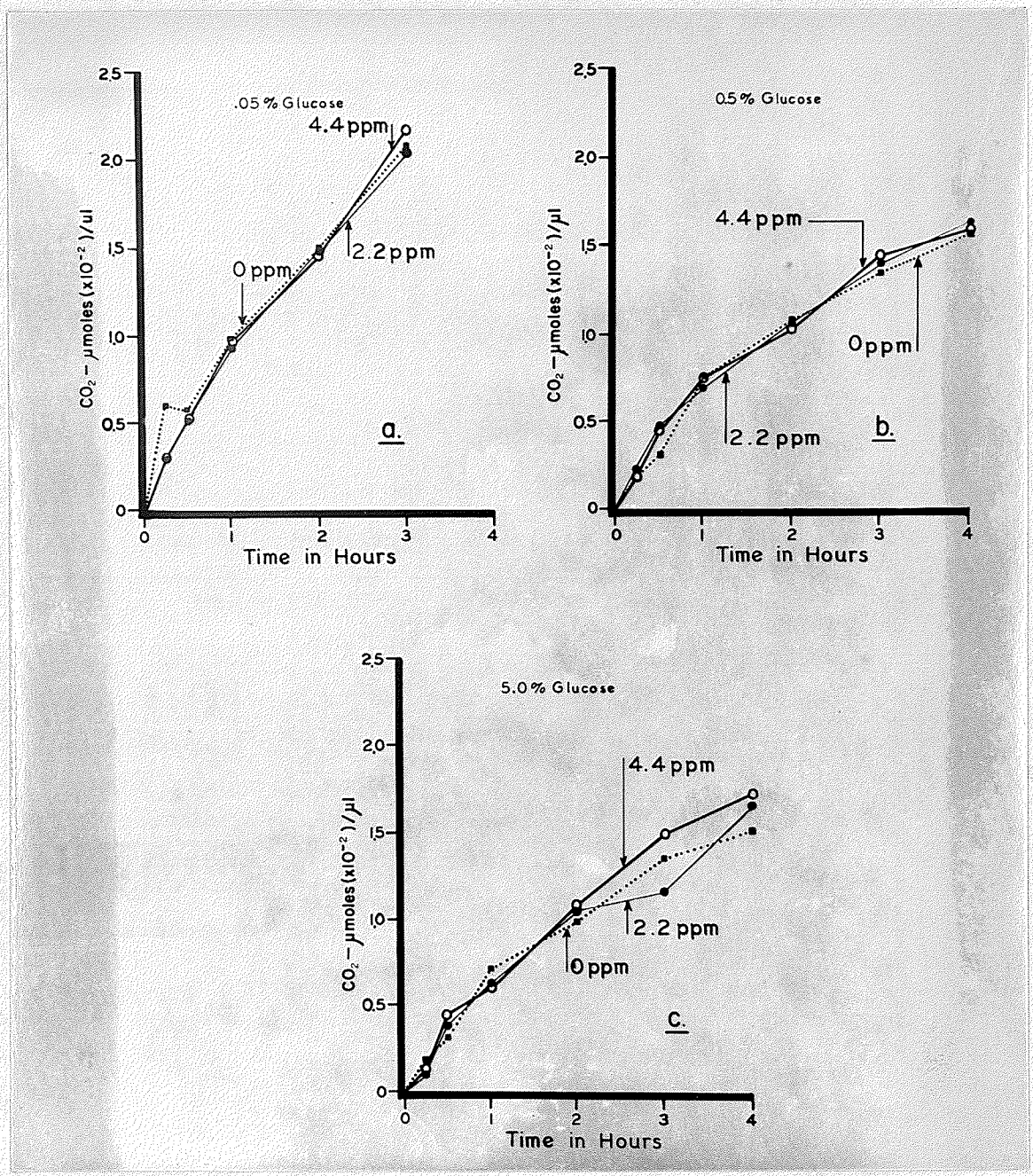


Figure 5.2. Effect of 2.2 and 4.4 ppm fluoride on the formation of total CO_2. a., 0.05% glucose; b., 0.5% glucose; c., 5% glucose.

and 4.4 ppm fluoride had little affect on the production of total carbon dioxide. If anything, fluoride might have caused a slight stimulation. However, with a fluoride concentration of 20 ppm and a glucose concentration of 5% (Fig. 5.3), marked stimulation of the formation of total carbon dioxide occurred during the last half of the experimental period.

Carbon dioxide from the various carbons of glucose (microradiorespirometry). Fluoride at 4 and 20 ppm had no effect on the production of carbon dioxide from the various carbons of glucose when the glucose concentration was initially 0.05% (Figs. 5.4a and 5.4b, respectively). With 5% glucose, these fluoride concentrations (Figs. 5.4c and 5.4d, respectively) produced a marked stimulation of carbon dioxide production from carbons 3 and 4, but no effect on the production of carbon dioxide from the other glucose carbons. The stimulatory effect of fluoride was also shown in the $C^{14}O_2$ from glucose-U-C^{14}.

Effect of Fluoride on the Rates of Accumulation of Lactic, Hetero, and Total Acids

Fluoride inhibited the formation of lactic acid (Fig. 5.5a) more than the formation of hetero acid (Fig. 5.5b). In both cases, inhibition was greater with 4.0 ppm fluoride than with 2.0 ppm.

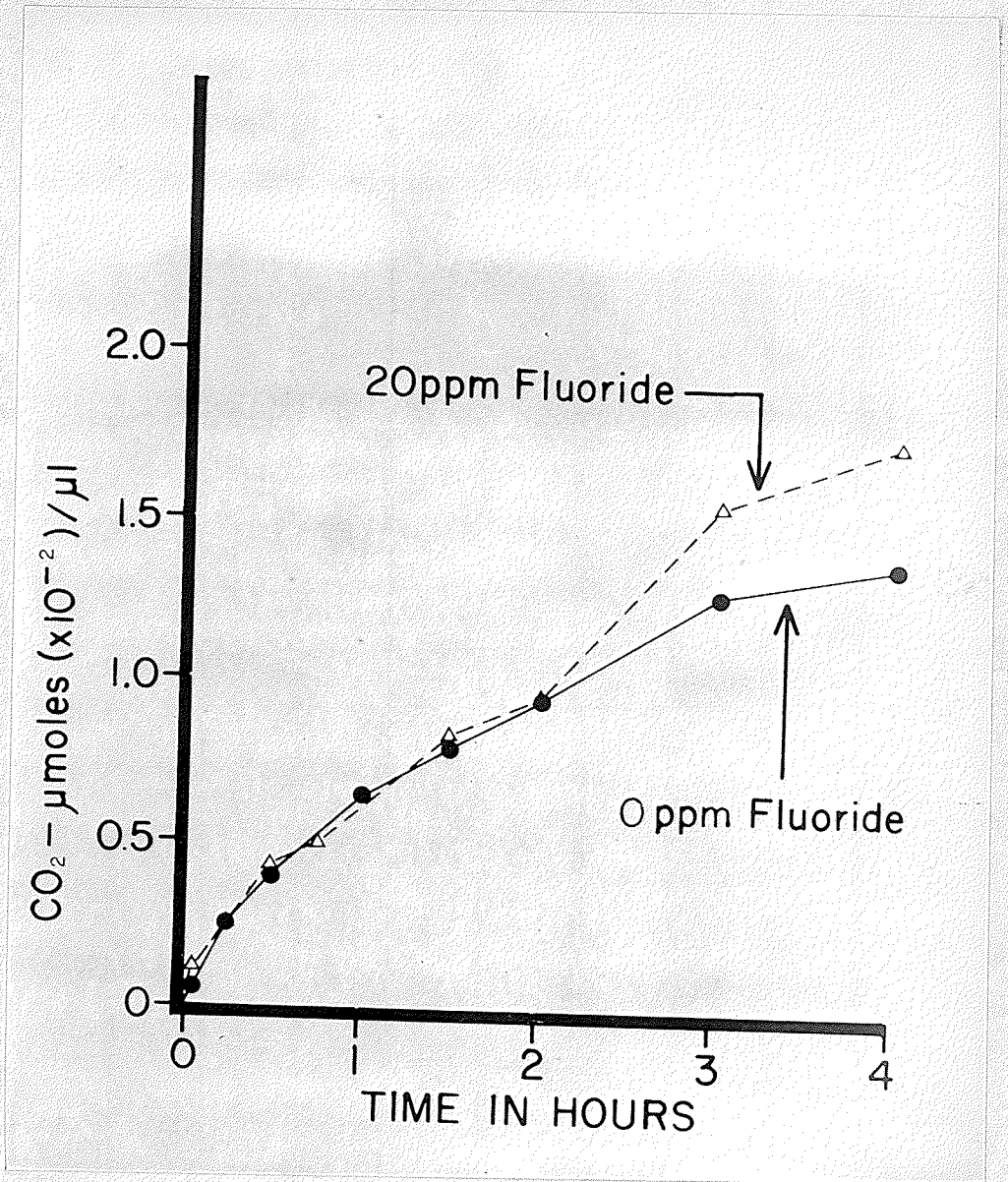


Figure 5.3. Effect of 20 ppm fluoride on the formation of total CO_2 by sediment mixtures containing 5% glucose.

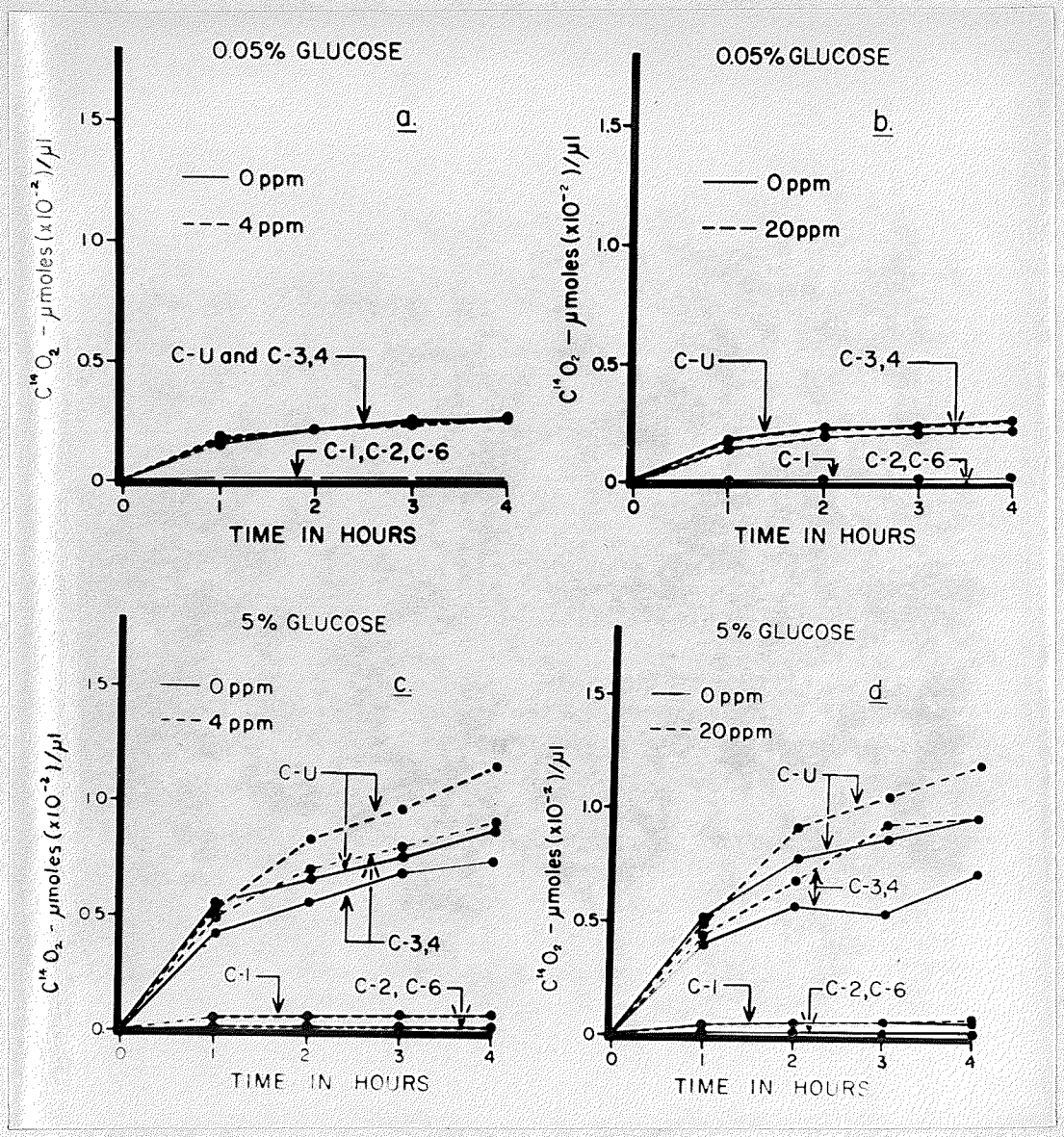


Figure 5.4. Effect of fluoride on the formation of CO_2 from individual carbons of glucose. a., 0.05% glucose, 4 ppm fluoride; b., 0.05% glucose, 20 ppm fluoride; c., 5% glucose, 4 ppm fluoride; d., 5% glucose, 20 ppm fluoride.

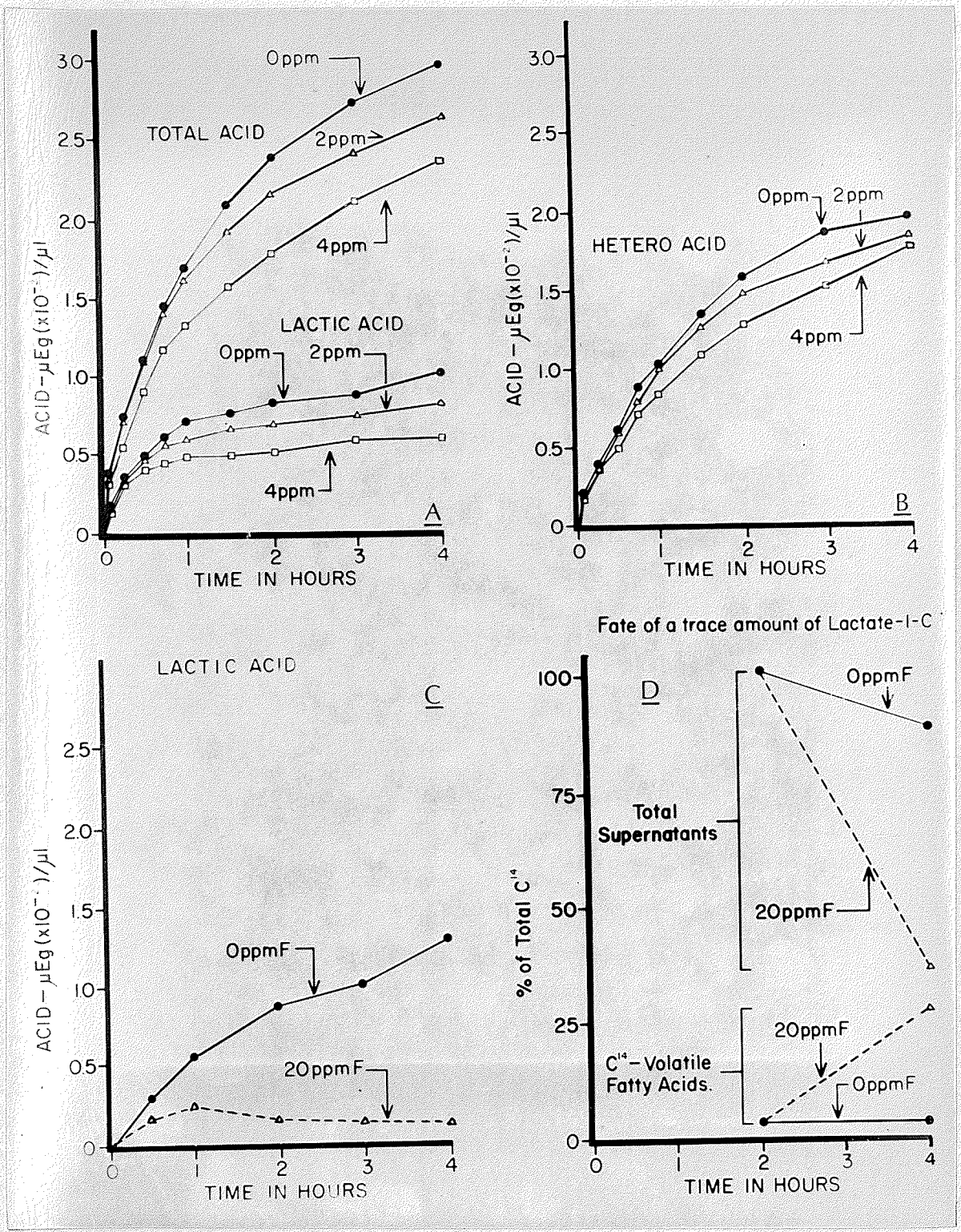


Figure 5.5. Effect of 2.0 and 4.0 ppm fluoride on the formation of: A., lactic acid and total acid; B., hetero acid. C., effect of 20 ppm fluoride on lactic acid formation. D., effect of 20 ppm fluoride on the fate of a trace amount of lactate-1-C^{14} added at 2 hours.

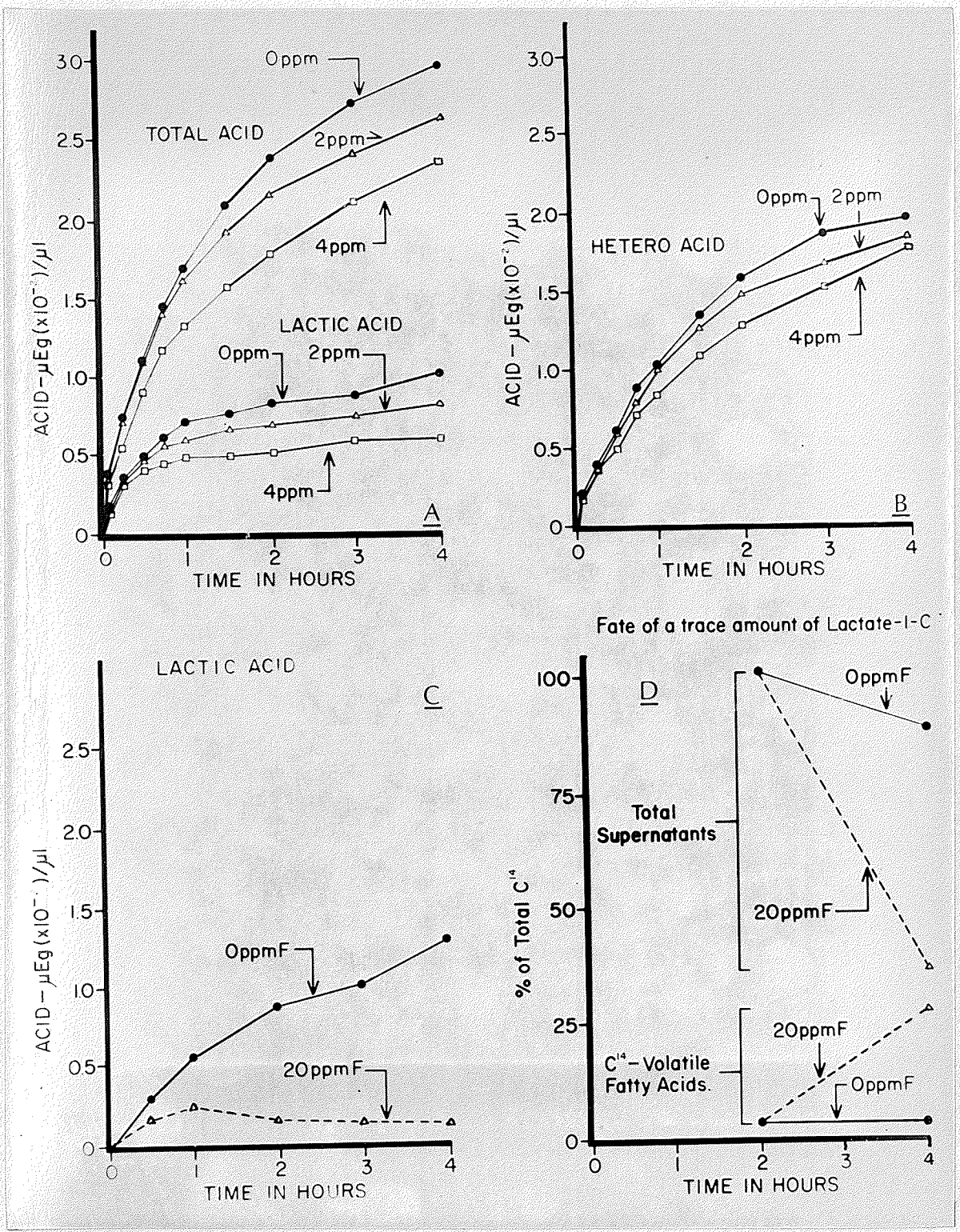


Figure 5.5. Effect of 2.0 and 4.0 ppm fluoride on the formation of: A., lactic acid and total acid; B., hetero acid. C., effect of 20 ppm fluoride on lactic acid formation. D., effect of 20 ppm fluoride on the fate of a trace amount of lactate-1-C^{14} added at 2 hours.

When the fluoride concentration was 20 ppm (Fig. 5.5c), the inhibition of lactic acid formation was so great that the lactic acid concentration reached a maximum and then slowly fell during the remainder of the incubation. Addition of a trace amount of lactate-1-C^{14} at 2 hours (Fig. 5.5d) resulted in a more rapid loss of label from the supernatant when fluoride was present than when it was absent. In the absence of fluoride, label from the lactate-1-C^{14} did not appear in the volatile fatty acid fraction, whereas with fluoride, approximately 30% of the labelled lactate was converted to such acid.

Loss of label from the supernatant and incorporation of label into the volatile fatty acids were used to estimate the utilization of lactic acid between the 2 and 4 hour time intervals. The formation of lactic acid over the same time interval was calculated by adding to the change in the lactic acid concentration over this time interval, the lactic acid that was utilized (Table II).

TABLE II

FORMATION AND REMOVAL OF LACTIC ACID BETWEEN 2 AND 4 HOURS

	Change in Lactic Acid Concentration (μmoles (x 10^{-2})/μl)	
	0 ppm F	20 ppm F
Lactic Acid Utilized	0.10	0.11
Net Change in Lactic Acid	0.40	-0.04
Lactic Acid Formed	0.50	0.07

From this table it is evident that fluoride strongly inhibited the rate of lactic acid formation, and perhaps slightly increased its rate of utilization.

Effect of Fluoride on the Utilization of Glucose and Lactic Acid at Low pH

When the pHs of sediment mixtures were adjusted with lactic acid to approximately 4.5, and when 5% glucose was also present (Figs. 5.6a and 5.6b), the pH fell and the lactic acid concentration increased in the absence of fluoride, while in the presence of fluoride, the pH rose and the lactic acid concentration decreased.

In the controls, which did not contain glucose (Figs. 5.6c and 5.6d), the pH rose more rapidly when fluoride was absent than when it was present. These changes in pH, as above, were accompanied by reciprocal changes in the lactic acid concentration.

When the lactic acid was labelled with lactate-1-C^{14} (Figs. 5.7a and 5.7b), the radioactivity disappeared slightly more rapidly from the supernatant and appeared more rapidly in the volatile fatty acids when fluoride was present than when it was not. When the rates of removal and formation of lactic acid with and without fluoride were calculated as for Table I above, the results showed that fluoride strongly suppressed lactic acid formation and

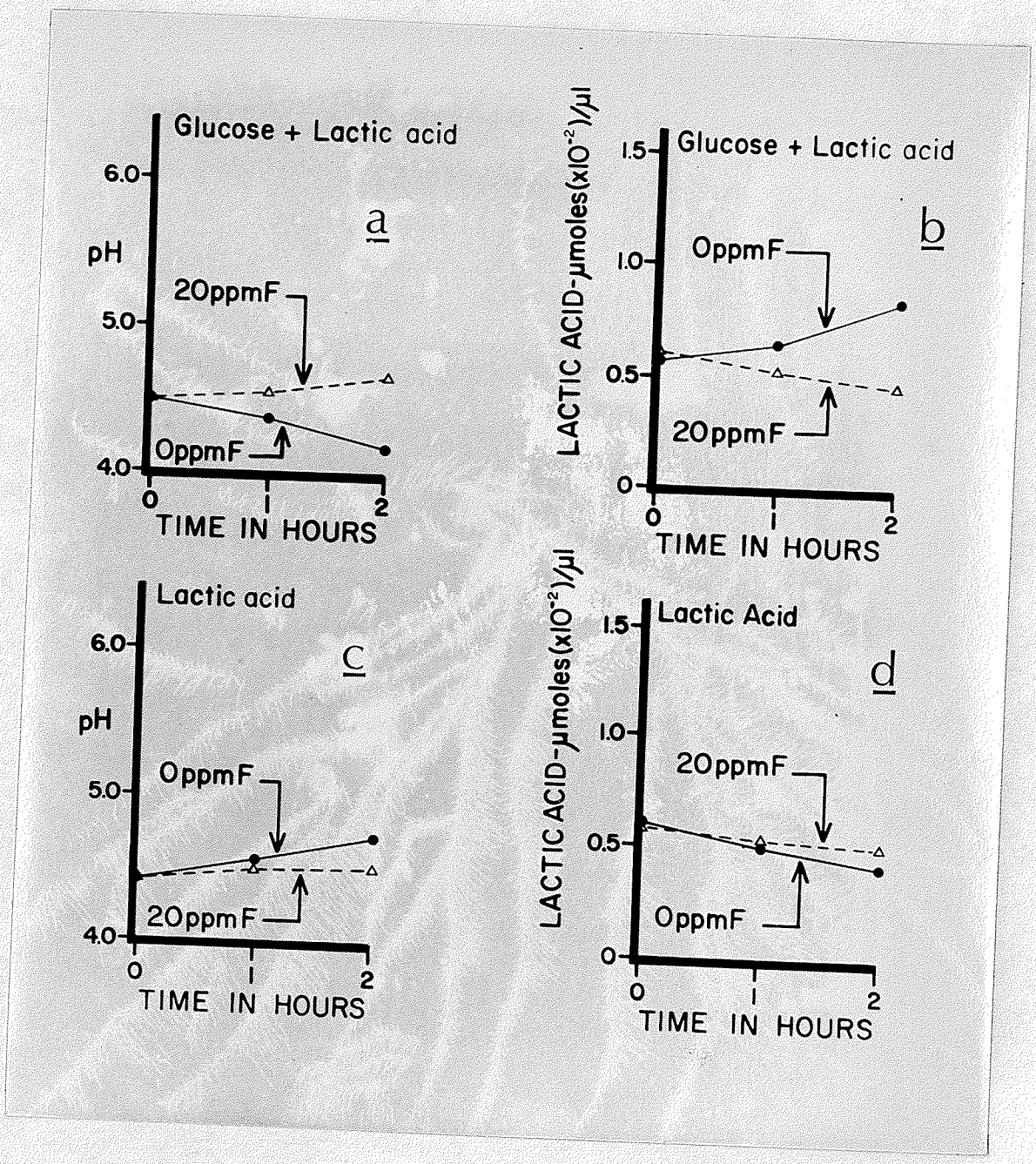


Figure 5.6. Effect of 20 ppm fluoride on changes in pH and lactic acid concentration, at low pH. a., pH changes, with 5% glucose; b., changes in lactic acid concentration, with 5% glucose; c., pH changes, without glucose, d., changes in lactic acid concentration, without glucose.

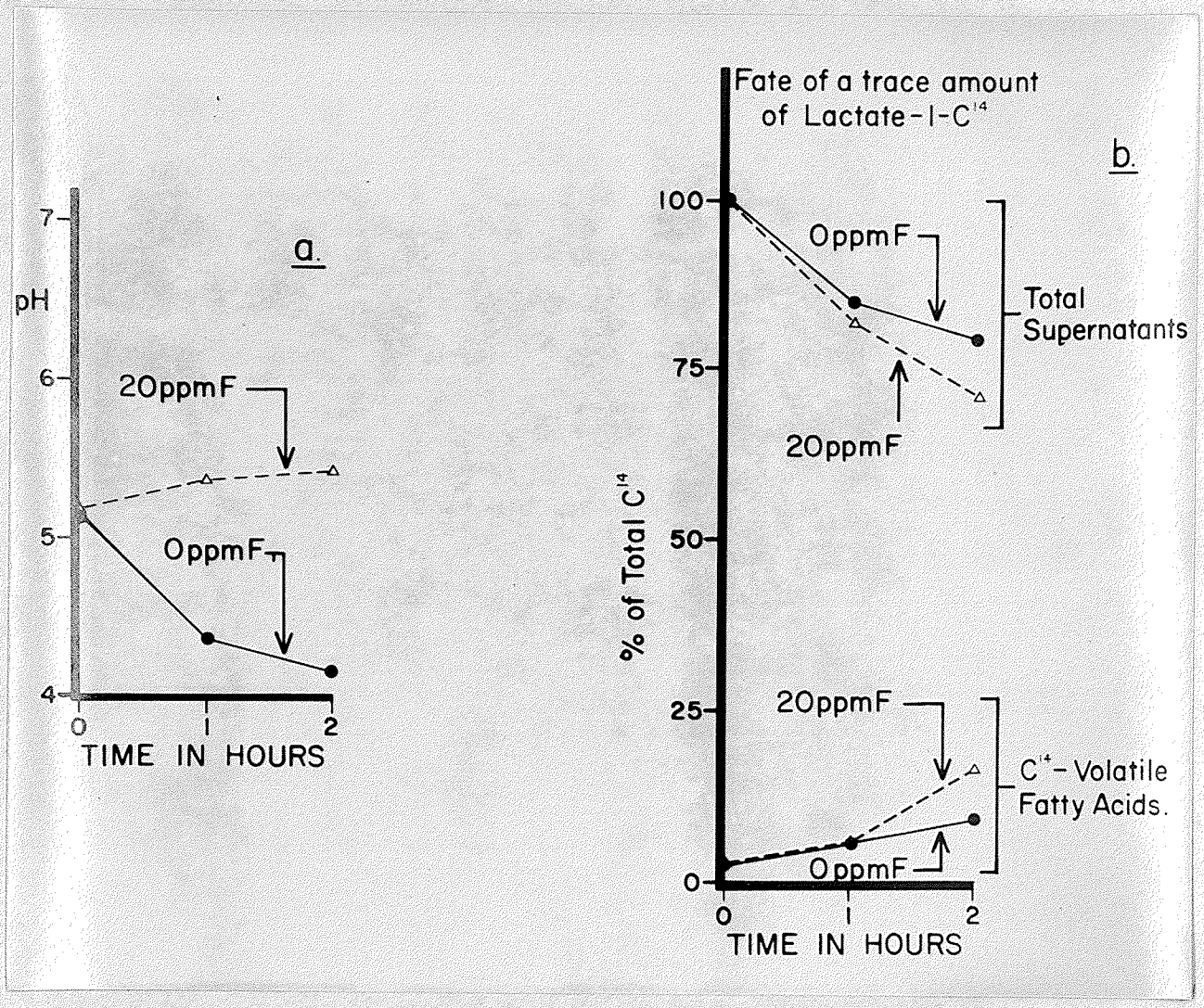


Figure 5.7. Effect of 20 ppm fluoride at low pH, when the lactic acid was labelled with lactate-1-C^{14}. a., pH changes; b., disappearance of label from the supernatant, and appearance of label in the volatile fatty acids.

stimulated its utilization (Table III).

TABLE III

FORMATION AND REMOVAL OF LACTIC ACID

	Change in Lactic Acid Concentration (μmoles ($\times 10^{-2}$)/μl)	
	0 ppm F	20 ppm F
Lactic Acid Utilized	0.12	0.19
Net Change in Lactic Acid	0.28	-0.13
Lactic Acid Formed	0.40	0.06

When the glucose was labelled with glucose-U-C^{14} in the same type of experiment (Figs. 5.8a, b, and c), fluoride suppressed the rate of formation of carbon dioxide from the glucose by approximately 50% (Fig. 5.8b). Also, fluoride almost totally suppressed the formation of volatile fatty acids from glucose (Fig. 5.8c).

When incubation was started at neutral pH, and fluoride added at the $\frac{1}{2}$ hour interval, from this point, the pH of the mixtures containing fluoride rose (Fig. 5.9a), while the pH of the controls continued to fall. Fluoride stimulated the disappearance of lactate-1-C^{14} from the supernatant (Fig. 5.9b) and appearance of radioactivity in the volatile fatty acids when this labelled chemical was added to the sediment mixtures at the same time interval (Fig. 5.9c).

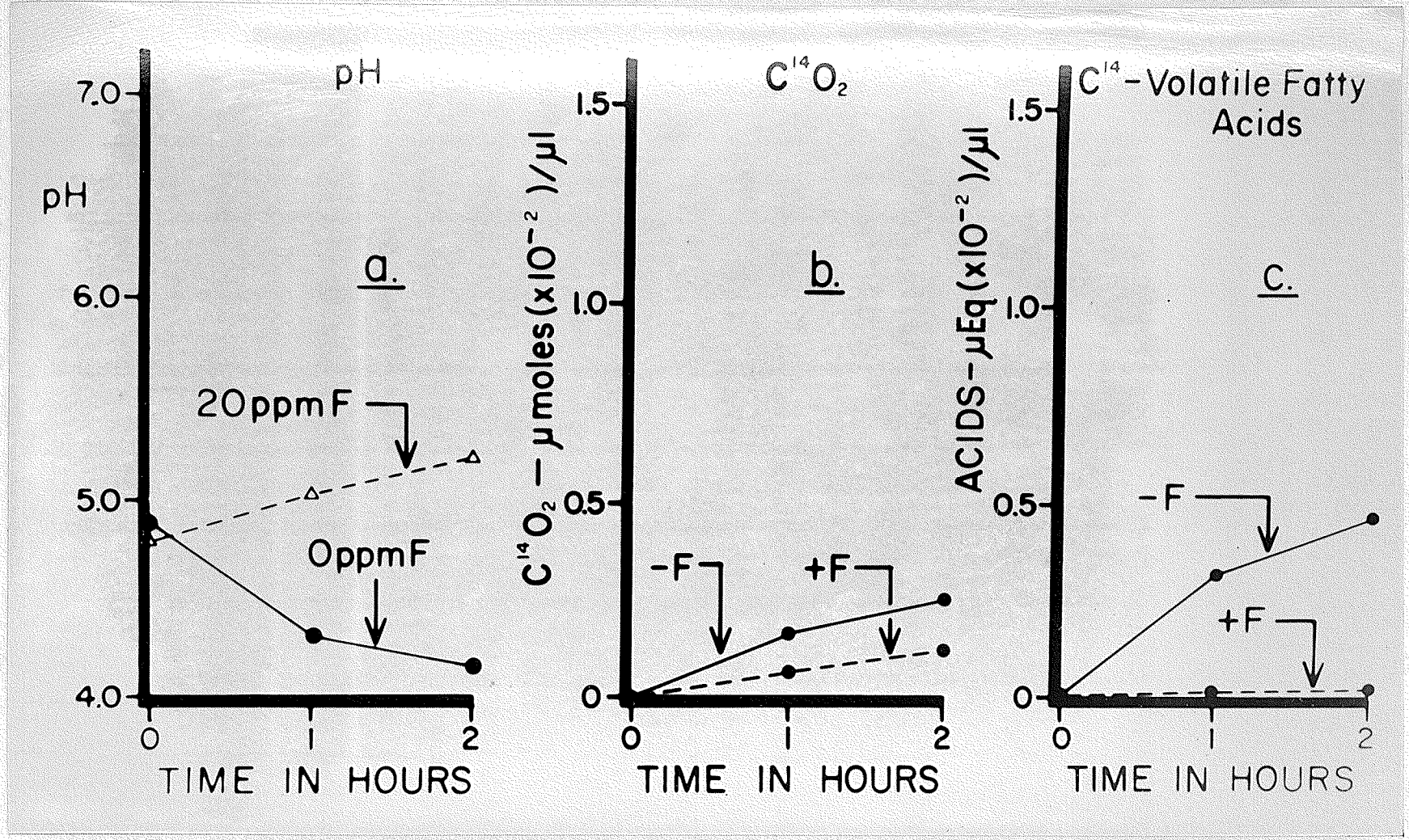


Figure 5.8. Effect of 20 ppm fluoride at low pH, when the glucose was labelled with glucose-U-C^{14}. a., pH; b., $C^{14}O_2$; c., C^{14}-volatile fatty acids.

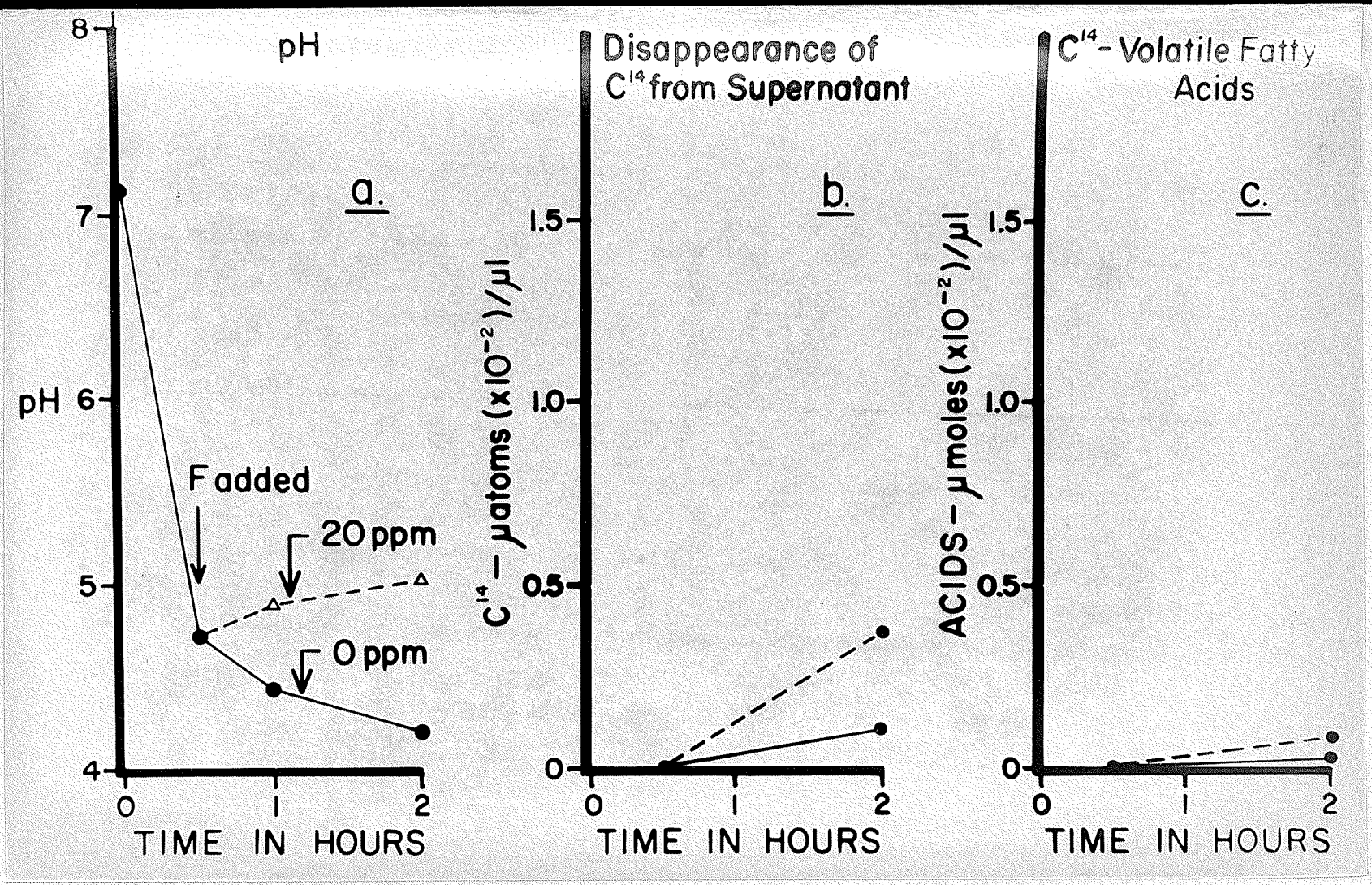


Figure 5.9. Effect of 20 ppm fluoride added to sediment mixtures after $\frac{1}{2}$ hour of incubation. a., pH; b., disappearance of lactate-1-C^{14} from the supernatant, and c., conversion of C^{14}-lactate to C^{14}-volatile fatty acids.

Effect of Fluoride on the Formation of Carbon Dioxide from Formate

Incubating sediment mixtures containing 5% glucose from an initial neutral pH with and without fluoride, and adding a trace amount of formate-C^{14} at the two hour interval showed that the rapid conversion of formate-C^{14} was not affected by fluoride. If anything, the rate was slightly more rapid without fluoride than with fluoride, showing that fluoride did not stimulate the breakdown of formic acid.

DISCUSSION

In the present study, acid production from glucose by salivary sediment, whether measured in terms of pH fall or titratable acidity, was markedly inhibited by fluoride. The inhibition was greater at the higher fluoride concentrations tested than at the lower ones and was more at acid than at neutral pH. Accumulation of lactic acid was inhibited more than was the production of hetero acid; resulting in a decrease in the lactic/hetero acid ratio. Fluoride concentrations of 2 and 4 ppm significantly slowed lactic acid accumulation, while 20 ppm fluoride stopped it completely. This inhibition of lactic acid accumulation with fluoride resulted largely from a sharp decrease in the rate of lactic acid formation.

A previous study showed that fluoride simultaneously inhibited the fall in pH and increase in sediment carbohydrate that occurs when glucose is added to the sediment system (Sandham, 1963; Kleinberg and Sandham, 1964). In addition, fluoride inhibited the uptake of glucose from the medium. This slowing by fluoride of the uptake of glucose by the cells in the sediment would, in the present study, decrease the glycolytic flux, thereby decreasing the rates of formation of lactic acid and hetero acid; the former more than the latter (Chapter IV).

The effects of fluoride on CO_2 formation are also consistent with this site and mechanism of fluoride inhibition. Decrease in the glycolytic flux with fluoride (more at higher fluoride concentration and lower pH) would favour acetic acid and CO_2 at the expense of the formation of propionic and lactic acids (see Chapter IV). Stimulation of CO_2 from glucose and inhibition of lactic and hetero acids, as observed in the present study, would therefore not be inconsistent.

Since the formation of endogenous CO_2 is dependent upon the glycolytic flux, slowing of this flux by fluoride inhibition of glucose uptake, would stimulate formation of CO_2 from this source. Failure to show effects of fluoride when the initial glucose concentration was 0.05%, is consistent with these conclusions and can be attributed,

as was done earlier (Sandham, 1963; Kleinberg and Sandham, 1964), to the failure to reach low pHs with this substrate concentration.

The possibility, that breakdown of formic acid might have contributed to the increased CO_2 production from glucose with fluoride, was not supported in the present study; added formate-C^{14} was, if anything, degraded to $C^{14}O_2$ at a slightly slower rate in the presence of fluoride.

Fluoride and the Reversal of the Crabtree Effect

When glucose is added to a respiring tissue, the inhibition of respiration which often occurs is known as the "Crabtree effect" (Crabtree, 1929). This results from interaction between the glycolytic Embden-Meyerhof pathway and the oxidative phosphorylation scheme associated with the Krebs cycle intermediates and involves the $NAD/NADH_2$ redox system. Any decrease in the inhibition of respiration is a "reversal" of the Crabtree effect. The production of CO_2 from glucose in the sediment system is analogous to oxygen uptake in a respiring system in that both CO_2 production and oxygen uptake are indices of similar $NAD/NADH_2$ changes in the metabolic pathways that succeed the Embden-Meyerhof pathway in their respective systems. In this sense, the stimulatory effect of fluoride on CO_2 production is equivalent to a reversal of the Crabtree effect.

Effect of Fluoride on the Carbon Dioxide from the Various Carbons of Glucose and on the Pathways of Glucose Degradation

Fluoride did not alter the production of CO_2 from the individual carbons of glucose when the glucose concentration was 0.05%; this is consistent with the earlier findings that fluoride had no effect on the glycolytic flux with this glucose concentration. With 5% glucose, fluoride at either 4 or 20 ppm markedly stimulated carbon dioxide production from the 3 and 4 carbons of glucose.

Fluoride did not affect CO_2 production from C-1 of glucose, suggesting no effect on the amount of glucose catabolized via the HMP pathway. Since fluoride decreased the total amount of glucose being degraded (Sandham, 1963; Kleinberg and Sandham, 1964), the amount of glucose degraded by the EM pathway must have decreased, and the percentage of the glucose degraded by the HMP pathway increased. The reason for the maintenance of a constant rate of glucose degradation via the HMP pathway remains to be determined.

Effect of Fluoride at Low pH

When fluoride was added to mixtures that contained glucose and were adjusted to initial low pH with lactic acid, upon subsequent incubation, the pH fell in the mixtures that did not contain fluoride, but rose in those in which fluoride was present. The lactic acid concentration

showed corresponding changes, i.e., higher lactic acid concentrations were associated with lower pH levels and vice versa. The pH changes were the same as those first observed by Jenkins (1959). Jenkins, in a later study (1960), postulated that fluoride produced the pH rise by stimulating the removal of lactic acid by oxidation. The results in this thesis indicated that the pH rise is caused by a conversion of lactic acid to weaker acids, and that this conversion involves both the oxidation of lactic acid (to acetic acid and CO_2) and the net reduction of lactic acid (to propionic acid). No oxidation occurred via the Krebs cycle.

When both glucose and lactic acid were present, the CO_2 originating from glucose was markedly inhibited by fluoride, whereas the CO_2 from lactate was slightly stimulated. These results are consistent with fluoride affecting the entry of glucose into the cells in sediment, in that lactate already formed or initially added with the glucose would not be replenished so readily, so that the lactate could be removed. This agrees with the findings in Chapter IV above, where the lactic acid concentration decreased when glucose had disappeared from the medium. In these experiments, fluoride inhibited CO_2 formation from glucose, whereas in similar experiments in which the pH was not adjusted with lactate, CO_2 was stimulated. This

can be attributed to the much greater inhibition of the glycolytic flux in the former than in the latter, because of the low pH. In other words, if this inhibition of the flux by fluoride were sufficiently large, even if CO_2 and acetic acid were the only end products, the CO_2 would still be less than if the flux were more and the end products included lactic and propionic acids.

In the absence of glucose and with lactic acid the only substrate, with or without fluoride present, the pH rose but the rise was more rapid in the absence of fluoride. These results indicate that fluoride can also inhibit the uptake of lactic acid by the cells in the sediment. This suggests once more that the site of fluoride activity is at the cell membrane.

CHAPTER VI

PRODUCTION OF BASE AT LOW pH FROM AMINO ACIDS ADDED TO THE SALIVARY SEDIMENT SYSTEM

Supplying a limited amount of glucose to either plaque *in vivo* (Stephan, 1944; Kleinberg, 1961) or salivary sediment *in vitro* results in a rapid pH fall, followed by a slower pH rise (Kleinberg, 1967). The pH rise is more rapid in the *in vivo* system than *in vitro* because the acid produced *in vivo* is continually diluted and neutralized by fresh saliva (Englander, Mazzarella, and Fosdick, 1959; Englander, Sklair, and Fosdick, 1959). While bacterial metabolism is probably the sole factor responsible for the pH rise *in vitro*, it may also be important in the pH rise occurring *in vivo*, particularly in areas of the mouth to which saliva does not have ready access.

Bacterial metabolism might return the pH toward initial levels by converting accumulated lactic acid to weaker acids (Stephan and Hemmens, 1947), and by producing base from nitrogenous substrates. The possibility, that the cells in sediment can produce base from amino acids, was tested in the experiments in the present chapter. Conversion of lactic to weaker acids has been studied in Chapters IV and V above.

Decarboxylation of amino acids to form amines is a process used by many types of bacteria to produce base when

the pH values of their media are low (Gale, 1946). Six amino acid decarboxylases have been described by Gale (1946) and had pH optima between 2.5 and 6.0. Each of these six decarboxylases is specific for one of the following amino acids: lysine, ornithine, arginine, tyrosine, histidine, and glutamic acid. Decarboxylases have also been described for serine, threonine, aspartic acid, and phenylalanine (Lamanna and Mallette, 1965, p. 816). In the present study, the amino acids mentioned, together with glycine, DL-alanine, isoleucine, L-glutamine, DL-tryptophane, DL-proline, L-hydroxyproline, asparagine, valine, cysteine, leucine, cystine, and methionine, were tested in the salivary sediment system to determine whether any might produce base.

The maximum concentration of total amino acid in the medium of the salivary sediment system, determined by the method of Spies and Chambers (1951), did not exceed 5 mmolar under the conditions of the present study. The concentration of most of the amino acids tested individually in the present study was 3.3 mmolar, which is less than the concentration for all of the amino acids combined, but several times greater than would be expected normally to be present for each. It was felt that, at these higher concentrations, any effects that the individual amino acids might have, would be more easily observed.

METHODS

The pH was lowered in sediment mixtures to approximately 5.0 in either of two ways and the effects of the individual amino acids on the subsequent rate of rise in pH then measured electrometrically. One way, in which the pH of incubation mixtures containing the individual amino acids was lowered, was by the addition of acetic acid. Acetic acid was selected for this type of experiment because it is one of the end products formed from glucose and, unlike lactic acid, is not metabolized further (Chapter IV). Lactic acid, because it can be converted to weaker acids, would cause an unwanted pH rise for these experiments.

The second way in which the pH was lowered was by adding glucose at a concentration of 0.1%, and incubating the mixtures. Sediment mixtures incubated with this level of glucose for one hour reach a pH of approximately 5.0 before subsequently slowly rising.

The concentration of each amino acid in its respective mixture was 3.3 mmolar, with the exception of tyrosine, tryptophan, and cystine. Because of their limited solubilities, these were 0.66, 1.0, and 0.16 mmolar, respectively. Water served as control.

The preparation of the sediment mixtures and their

incubation were the same as described in Chapter II. At regular time intervals during a four hour incubation, two tubes were removed from each series for the determination of pH.

Before the amino acids were added to their respective mixtures, the pH of each amino acid was adjusted to pH 5.0. In the first type of experiment (i.e., acetic acid used to lower the pH), this was to ensure that initial levels were approximately the same. In the second type of experiment, this was to ensure that approximately the same pH minimum was reached with or without amino acid present. Deviation in pH from the control, particularly deviation in the rising phase of the pH curve that occurs after the pH minimum, could then be attributed to sediment metabolism.

RESULTS

In the experiments in which the pH was lowered with acetic acid, the pH in the mixtures containing arginine rapidly rose, while the pHs in the mixtures containing the other amino acids or distilled water rose at a much slower rate (Fig. 6.1a).

When the pH was lowered by the acids from glucose breakdown (Fig. 6.1b), the fall in pH in the mixtures with arginine was less than the fall in the pH of the mixtures containing the other amino acids or water. The rise in

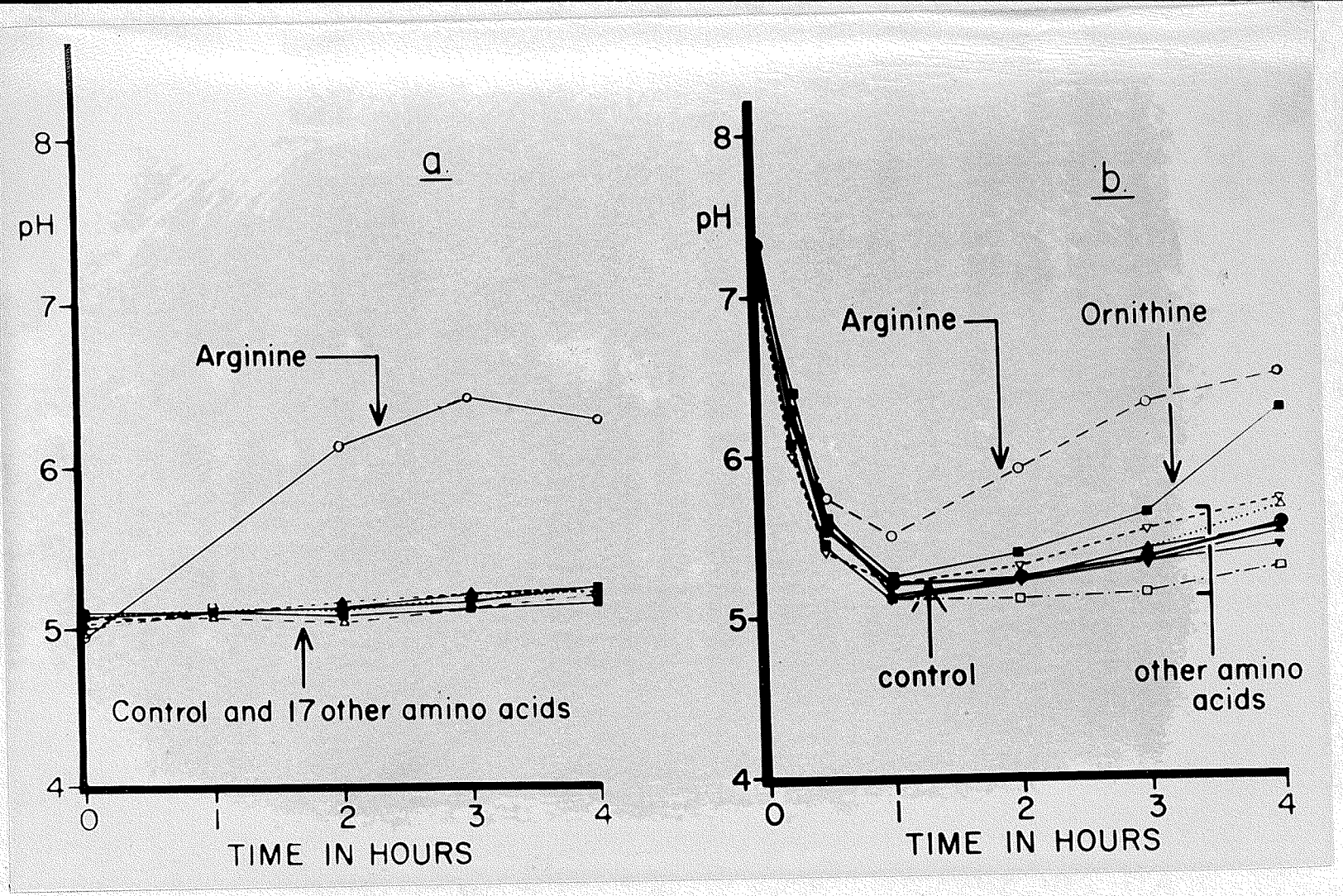


Figure 6.1. Effect of amino acids on the pH curves when the pH was initially lowered with a., acetic acid; and b., acids formed from glucose catabolism.

pH, after the pH minimum was reached, was most rapid with arginine, next with ornithine, and only slight with the other amino acids or water.

DISCUSSION

The present study demonstrated that, except for arginine and to a lesser extent for ornithine, the various amino acids tested had little or no effect on the pH fall or the subsequent rise that ordinarily occurs with 0.1% glucose.

Because the action of decarboxylases would ordinarily be expected to produce a pH rise in the medium, the data suggests the absence of amino acid decarboxylases active on lysine, glutamic acid, histidine, or tyrosine, and that base could not be formed from the other amino acids. The absence of this activity suggests two possibilities: (i) that the concentration of amino acids necessary to induce such decarboxylase activity must usually be low in saliva and/or (ii) bacteria with the genetic potential to decarboxylate these added amino acids may be absent.

Concentration of amino acids in paraffin-stimulated saliva. Estimations by various workers of the total amino acid content of paraffin-stimulated saliva have varied between approximately 1 and 2 mmolar, apparently depending on (i) whether precautions were taken to prevent bacterial

action, and (ii) whether microbiological or chromatographic methods were used for the analysis. Few of the investigators have reported whether they took steps to avoid the occurrence of bacterial action during or after the collection of the saliva; the effect of this factor on the amino acid assay is therefore difficult to assess. The method of measuring the amino acid content of the saliva appears to have had some effect on the results obtained. Microbiological techniques (Kirch et al., 1947) gave higher values for total amino acids (approximately 2 mmolar) than did assays involving paper or column chromatography (approximately 1 mmolar--Moor and Gilligan, 1951; Woldring, 1955; Battistone and Burnett, 1961). Unpublished results of experiments by Kleinberg, Craw and Kay in our laboratories, using the method of Spies and Chambers (1951), have confirmed that the amino acid concentration of paraffin-stimulated saliva is low.

Presence of microorganisms in paraffin-stimulated saliva capable of producing decarboxylases active on added amino acids. The lack of decarboxylases active on added tyrosine, histidine, glutamine, and lysine in the present study supports the finding of Gochman et al. (1959) that, when amino acid decarboxylase activity was measured by determining carbon dioxide production, paraffin-stimulated saliva contained no decarboxylases active on seventeen

common amino acids.

However, Biswas and Kleinberg, on the basis of unpublished experiments in which the changes in the concentration of total (intracellular and extracellular) individual amino acids were followed by paper chromotography during the incubation of salivary sediment with 0.1% glucose, have suggested that amino acid decarboxylases, in particular glutamic acid decarboxylase, may be active in salivary sediment. They found that during the first hour of incubation some amino acids, which included glutamic acid, accumulated rapidly. After the first hour, all of the amino acids slowly disappeared, while ɣ-amino butyric acid accumulated, likely as a result of glutamic decarboxylase activity. In the present study, no glutamic decarboxylase activity was detected, suggesting that (i) added glutamic acid does not enter the cells in the sediment, or (ii) the action of the enzyme cannot be detected as a rise in pH.

Ornithine

The data in the present study suggests the presence of ornithine decarboxylase activity in salivary sediment.

Arginine

The effect of arginine on the pH curve may have been due to decarboxylation, the action of the arginine

dihydrolase enzyme complex, or both processes. Nothing in the data rules out the possibility of arginine being decarboxylated to form agmatine. However, 42 to 45% of the oral microflora consists of streptococci (Kraus and Gaston, 1956; Richardson and Jones, 1958) and heterolactic lactobacilli (Rogosa and Sharpe, 1959), many of which are known to have a high level of arginine dihydrolase activity (Hills, 1940; Niven, Smiley, and Sherman, 1942). The activity of the arginine dihydrolase group of enzymes is likely to have contributed significantly to base production from arginine in the present study. The reactions which take place during the breakdown of arginine by arginine dihydrolase are:

(1) $\text{arginine} + H_2O \xrightarrow{\text{arginine desiminase}} \text{citrulline} + NH_3$

(2) $\text{citrulline} + HPO_4^{=} \longrightarrow \text{ornithine} + \text{carbamyl phosphate}$

(3) $\text{carbamyl phosphate} + ADP \xrightarrow[\text{kinase}]{\text{carbamate phospho-}} NH_3 + CO_2 + ATP$

Net: $\text{arginine} + H_2O + ADP + HPO_4 \rightarrow \text{ornithine} + 2\ NH_3 + CO_2 + ATP$

Ornithine produced by this reaction could then be acted on by ornithine decarboxylase to produce an additional mole of base:

$$\text{ornithine} \longrightarrow CO_2 + \text{putrescine}$$

Gale (1945) has suggested that the function of the

dihydrolase might be to supply carbon dioxide, an essential nutrient for microorganisms having a homolactic fermentation. He demonstrated that in the presence of carbon dioxide, Lancefield Group D streptococci no longer had an absolute requirement for arginine for growth, but could obtain a slow rate of growth with ornithine. The reaction has also been proposed as the prime source of carbamyl phosphate, an obligatory intermediate for the synthesis of uridine nucleotides (Mikolajcik, Harper, and Gould, 1963). Its possible importance in the production of ammonia has been discussed by Hills (1940), and its possible importance as an energy source in Streptococcus faecalis has been studied by Bauchop (1958) and Preston, Sherris and Shoesmith (1956).

If arginine contributes to the pH rise that ordinarily occurs in dental plaque in vivo, then it may come from free arginine or arginine in protein and peptides, since arginine dihydrolase acts more rapidly on peptides than on the free molecule (Gale, 1945). Paraffin-stimulated saliva has very low levels of free arginine, but much in a "bound" form (Kleinberg and Craw, unpublished results). This may also be the reason why the content of arginine in paraffin-stimulated saliva is much greater when measured by microbiological techniques (Kirch et al., 1947) than when measured by chromatography, since the latter technique is much more specific for free amino acids.

Possible sources of arginine for the oral microflora are sloughed epithelial and leucocytes, the proteins and peptides in the secretions of the salivary glands, and the high level of arginine accumulated by streptococci as observed by Gale (1947).

Since bradykinin has arginine in its structure, and is inactive when this arginine is replaced by ornithine (Nicolaides, DeWald and Craft, 1963a, 1963b; Schroder, Petras, and Klieger, 1964; Schroeder and Hempel, 1964), it is possible that arginine dihydrolase is identical to the "kininase" which has been found in saliva by Amundsen and Nustad (1964) which they considered to originate from the epithelial cells in saliva.

By the use of manometry, Hartles and Wasdell (1955) first demonstrated that a soluble component of saliva, when added to salivary sediment, stimulates both glycolysis and oxygen uptake. Krönke and his co-workers (1958) suggested that the activity resides in a peptide; however separation of saliva on Sephadex columns (Hay and Hartles, 1965) has shown that the glycolysis-enhancing activity is distributed among many fractions of various molecular weights. Attempts have been made to duplicate the activity with several amino acids (Wasdell, 1962; Hay and Hartles, 1966) with little success. In none of these studies was arginine included.

Since the present investigation suggests the presence of arginine dihydrolase in salivary sediment, and that enzyme complex readily attacks peptides (Gale, 1945), carbon dioxide production from that source may have increased the manometric measurements of glycolysis obtained by the workers studying the metabolism-stimulating factor (see Chapter III). Therefore arginine-containing peptides or proteins may have been the factor which appeared to stimulate glycolysis in their experiments. Since Gale also showed that arginine-containing peptides considerably increase the rate of growth of streptococci, a growth stimulation may have contributed to the increased rates of both glycolysis and oxygen uptake observed with salivary sediment in their experiments as well.

If arginine can reduce the pH fall, then it might be important in dental caries. However, experiments carried out on caries-susceptible rats do not appear to support the idea that the addition of arginine or ornithine to the diet might be effective in the prevention of dental caries (McClure and Folk, 1955).

CHAPTER VII

A CONSIDERATION OF MANOMETRIC TECHNIQUES IN VIEW OF THE RESULTS OF THE PRESENT STUDY

The purpose of this chapter is mainly to consider, in the light of the experiments in this thesis, problems associated with the use of manometric techniques to study glycolysis by the oral microflora. To measure glycolysis using manometry, cells are incubated anaerobically in a bicarbonate buffer. The acid produced during glycolysis converts bicarbonate to CO_2 which escapes from the medium, and, because the vessel is closed, can be measured as a volume change on a manometer attached to the reaction vessel. The amount of CO_2 displaced from the buffer is equal to the amount of acid produced.

In cells where carbon dioxide is also one of the end-products of cell metabolism, the manometric procedure used for the measurement of glycolysis has to be altered in order to compensate for this metabolic CO_2 (see Umbreit, Burris, and Stauffer, 1957, p. 26). Clearly, if the amount of metabolic CO_2 formed is appreciable, and if it changes with altered cultural conditions, failure to make the necessary alterations in procedure would result in considerable error in the estimation of glycolysis. Wasdell (1962) has pointed out that the altered procedure has not always been used by workers studying glycolysis in saliva or

salivary sediment, and the present study (contrary to the findings of Wasdell) has shown that the formation of metabolic CO_2 is both appreciable and variable. Consequently, the results of many of the studies utilizing manometric techniques for the study of glycolysis in saliva and salivary sediment must be re-evaluated.

Inaccuracies in the manometric measurement of glycolysis have also been demonstrated in other systems. For example, Wu (1965) found that the rate of anaerobic glycolysis in kidney slices as measured manometrically, far exceeded the rate of glycolysis as shown by lactic acid assay. He stated that the reason for this may have been the formation of volatile products or acids other than lactic acid. Had he made the "corrections" usually performed to compensate for metabolic CO_2 production, whether the products were volatile or not would have been obvious.

(i) Catabolism of amino acids. If arginine or ornithine were present in saliva or salivary sediment preparations in which glycolysis was being determined manometrically, CO_2 and base would be produced, as was indicated in Chapter V, and the determination of the actual rate of glycolysis would be even more difficult. The production of base would tend to reduce the measurements, while the production of carbon dioxide would tend to increase the measurements.

(ii) Use of bicarbonate buffer. Use of the bicarbonate buffer necessary to follow glycolysis by manometry restricts study of the metabolism of the oral microflora to constant pH and does not enable examination of glycolysis under conditions that actually occur in the oral cavity. Also, restriction to the use of bicarbonate buffer might result in a higher rate of CO_2 fixation because of the higher pH and higher bicarbonate concentration normally used, and as a result, reduce the reading.

(iii) Formation of hydrogen gas. Although the formation of hydrogen as an end-product of glucose metabolism by salivary sediment metabolism has not been as yet established, the presence of high numbers of veillonella in the sediment flora makes this probable (Johns, 1951). If this gas is formed and not allowed for, then it is obvious that an error would result.

Determination of Oxygen Uptake by the Oral Microflora by Manometry

Investigators have used manometric determination of oxygen uptake to measure the rate of respiration during glucose breakdown in a large number of cellular systems. The procedures have been those described by Umbreit et al., in which CO_2 is trapped in base located in a separate part of the reaction vessel and changes in oxygen are recorded

on an attached manometer.

Although uptake of oxygen by oral microorganisms has been demonstrated by many workers (e.g., Eggers-Lura, 1944, 1954, Hartles and McDonald, 1953; Stralfors, 1957; Kronke and Naujoks, 1954), the metabolic pathways in which oxygen is involved in the metabolism of the oral flora, are not known. The present study indicated that oxygen uptake is not associated with oxidation of Krebs' cycle intermediates (Chapter III). This is supported by the fact that the majority of the microorganisms in the flora grow only under anaerobic conditions (Richardson and Jones, 1958; Gibbons, Socransky, de Araujo and Van Houte, 1964), suggesting that the oxygen tension within the flora in vivo is low. Oxygen utilization by some of the anaerobic and the aerobic microorganisms in the flora may serve only to protect an essentially anaerobic metabolism from the deleterious effects of oxygen. This is not unusual since the oxygen uptake of many cytochrome- and catalase-free bacteria such as lactobacilli and streptococci (whose metabolism does not require oxygen) is equal to or greater than some aerobically-grown organisms such as baker's yeast (Dolin, 1961). Even clostridia (obligate anaerobes) can utilize oxygen rapidly (Aubel and Houget, 1945).

Until the significance of uptake of oxygen by the salivary flora is known, results from such measurements

will be difficult to interpret. Measurement of oxygen uptake does not appear to be of use for measurement of glucose catabolism in such systems.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

Kleinberg (1960; 1962) has shown that the pH curves produced in a sediment system prepared from paraffin-stimulated saliva, with glucose as substrate, were similar in many respects to those obtained in dental plaque *in vivo*. The interrelationship between the pH changes observed in the sediment system and the rates of glucose utilization and carbohydrate storage by the cells in this system, as a function of the initial glucose concentration, were subsequently examined (Sandham, 1963; Sandham and Kleinberg, 1964). The effect of low levels of fluoride (2.2 and 4.4 ppm) on this interrelationship was also determined.

In the studies in this thesis, the investigation of other metabolic parameters associated with changes in pH in the salivary sediment system, under the same system and substrate conditions as in the above studies, was carried out. This investigation included the study of the formation of (i) CO_2 (both from glucose and from endogenous sources), (ii) lactic and other organic acids (including acetic and propionic), and (iii) base from a large number of amino acids. The effect of fluoride on the first two parameters was also examined.

To determine CO_2 formation by the cells in salivary sediment, a salivary sediment system in which the sediment

concentration was 16.7% (V/V) was supplied with a range of concentrations of glucose between 0 and 30% (W/V) and the CO_2 formed during four hours incubation at 37°C measured. In some experiments, glucose-1-C^{14}, -2-C^{14}, -3,4-C^{14}, -6-C^{14}, and -U-C^{14} were added to unlabelled glucose to determine (i) the proportion of the CO_2 formed that originated from glucose, rather than from substrates other than glucose (endogenous CO_2) and (ii) the metabolic pathways utilized by the carbon atoms of glucose that became CO_2.

With increasing glucose concentrations, the rate of CO_2 formation from glucose increased, and the proportion of the total CO_2 that came from glucose, rather than from endogenous sources, also increased. CO_2 from glucose was greatest when the glucose concentration was 5%, and decreased when the glucose concentration was further increased to 30%. The formation of endogenous CO_2 varied inversely with the formation of CO_2 from glucose. This inverse relationship meant that a decrease in the CO_2 from glucose was accompanied by an increase in CO_2 from substrates other than glucose, and vice versa. The compensation was not stoichiometric; the formation of total CO_2 (the sum of the CO_2 from glucose and CO_2 from sources other than glucose), was also a function of glucose concentration, but its optimum was at approximately 0.1% and not 5%.

Experiments with glucose labelled on different carbon

atoms showed that 94% to 97% of the glucose was catabolized via the Embden-Meyerhof pathway, 2.8% to 6.1% via the hexosemonophosphate pathway, and almost none via the tricarboxylic acid cycle.

In an almost identical series of experiments, lactic and other organic acids (referred to in this study as hetero acid, and consisting mainly of acetic and propionic acid) were determined during the 4 hour incubation period. When no glucose was supplied to the sediment system, no lactic acid and only a small amount of hetero acid were formed. As the glucose concentration was increased to 5%, both lactic and hetero acid correspondingly increased. Lactic acid was shown to be an intermediate and not an end product of glucose catabolism, whereas hetero acid was an end product.

In sediment mixtures containing low initial concentrations of glucose, the pH fell and then slowly rose. The lactic acid concentration correspondingly rose and fell, except that its maximum occurred 15 to 30 minutes prior to the minimum in the pH curve. These lactic acid changes corresponded to changes in sediment carbohydrate observed in a previous study, in that both reached their maximum values at the time that glucose disappeared from the medium, indicating that both the formation of lactic acid and of sediment carbohydrate depended upon the presence of glucose in the medium. The data showed that the pH minimum was

reached after the glucose in the sediment had all been used up, not because of the formation and accumulation of additional lactic acid, but rather because of the formation and accumulation of additional hetero acid. In other words, the increase in the level of hetero acid more than compensated for the tendency for the conversion of lactic to the weaker hetero acid to raise the pH during the period between the time the glucose disappeared from the medium and the time that the pH minimum was reached.

Following the pH minimum, the pH rise that was observed was attributable to the conversion of lactic to hetero acid.

The lactic/hetero acid ratio varied widely and was a function of the glucose concentration and the length of time that the mixtures had been incubated. This finding demonstrated that estimation of such a ratio from data obtained from only one or two incubation times and only one or two glucose concentrations, is meaningless.

Analysis of the hetero acids by gas chromatography and paper chromatography showed that the only hetero acids present in significant amounts were the volatile fatty acids acetic and propionic. There was a suggestion that some butyric acid was also present. Analyses for other acids or neutral products were negative.

A scheme was presented to explain how the observed

products were formed, and to explain the mechanisms regulating their formation.

Experiments were then carried out to determine the effects of several levels of fluoride (2, 4, and 20 ppm) on the formation of CO_2, lactic acid, and hetero acid. When the glucose concentration was 0.05, 0.5, or 5%, low levels of fluoride had little or no effect on the formation of total CO_2 by the cells in the sediment system, whereas 20 ppm fluoride stimulated CO_2 formation. With 5% glucose, the CO_2 originating from glucose was stimulated by both 4 and 20 ppm fluoride, whereas when the glucose concentration was 0.05%, fluoride had no effect.

The stimulation of CO_2 originating from glucose was shown to arise from stimulation of the CO_2 originating from carbons 3 and 4. Fluoride had no effect on the formation of CO_2 from the other glucose carbons.

Lactic and hetero acid formation were both inhibited by fluoride, the inhibition being greater with the higher fluoride levels tested. Lactic acid formation was inhibited more by fluoride than was the production of hetero acid, resulting in a decrease in the lactic/hetero acid ratio. Fluoride at concentrations of 2 and 4 ppm slowed lactic acid accumulation, while at 20 ppm, lactic acid accumulation was stopped. The inhibition of lactic acid accumulation resulted mostly from inhibition of lactic acid formation.

Experiments were then carried out that showed that fluoride at low pH inhibited the formation of CO_2 from glucose, a finding which was opposite to the finding at higher pH. The inhibitory effect of fluoride on acid formation was increased at low pH.

These results and those above on the effect of fluoride on both CO_2 and acid formation have been explained in terms of fluoride inhibiting the uptake of glucose by the cells in the sediment, as was previously postulated (Sandham, 1963; Kleinberg and Sandham, 1964).

When a limited amount of glucose was added to plaque in vivo (Stephan, 1944; Kleinberg, 1961) or salivary sediment in vitro (Kleinberg, 1960), Stephan curves (Stephan, 1944) were observed in both systems. The possibility that the formation of base from amino acids might be responsible for, or contribute to, the pH rise portion of this curve, was investigated in the present thesis. The experiments to determine this were carried out by lowering the pH in the sediment mixtures in either of two ways, and then measuring the effect of a number of amino acids on the subsequent rate of pH rise. The initial pH of the sediment mixtures was lowered to 5.0, firstly, by adding acetic acid, and secondly, by adding 0.1% glucose and allowing the pH to fall as a result of acid formation from the glucose. With 0.1% glucose, a pH of 5.0 is usually reached after one hour,

before the pH subsequently rises.

Of the twenty-three amino acids tested, only arginine and ornithine had any effect on the pH rise that occurred. The effects of these amino acids on the pH curves were consistent with their being degraded to form base by their respective amino acid decarboxylases, or, in the case of arginine, by the arginine dihydrolase complex of enzymes. The possible importance of these processes, as a means whereby the bacteria might raise the pH of their environment in the oral cavity, was discussed.

Many of the studies on the metabolism of saliva, salivary sediment, and dental plaque which have been done by other workers, have been done by measuring glycolysis and respiration with manometric techniques. The results obtained by these workers have frequently differed from the results obtained by other workers using more direct analytical methods. The findings in the present thesis suggested a number of reasons why the two experimental approaches yielded differing results; these reasons were discussed.

The present studies have served to widen the basis for understanding the processes involved in, and the factors controlling, the catabolism of glucose in salivary sediment. The increasing number of similarities that have been found between the metabolism of the sediment system and that of dental plaque, strongly suggest that most of the findings in

the present study are also applicable to dental plaque, a system whose metabolism is, in itself, difficult to examine. The findings on the metabolism of salivary sediment in this thesis may therefore lead to a better understanding of the metabolic events in dental plaque which lead to the initiation and progression of dental caries and periodontal disease.

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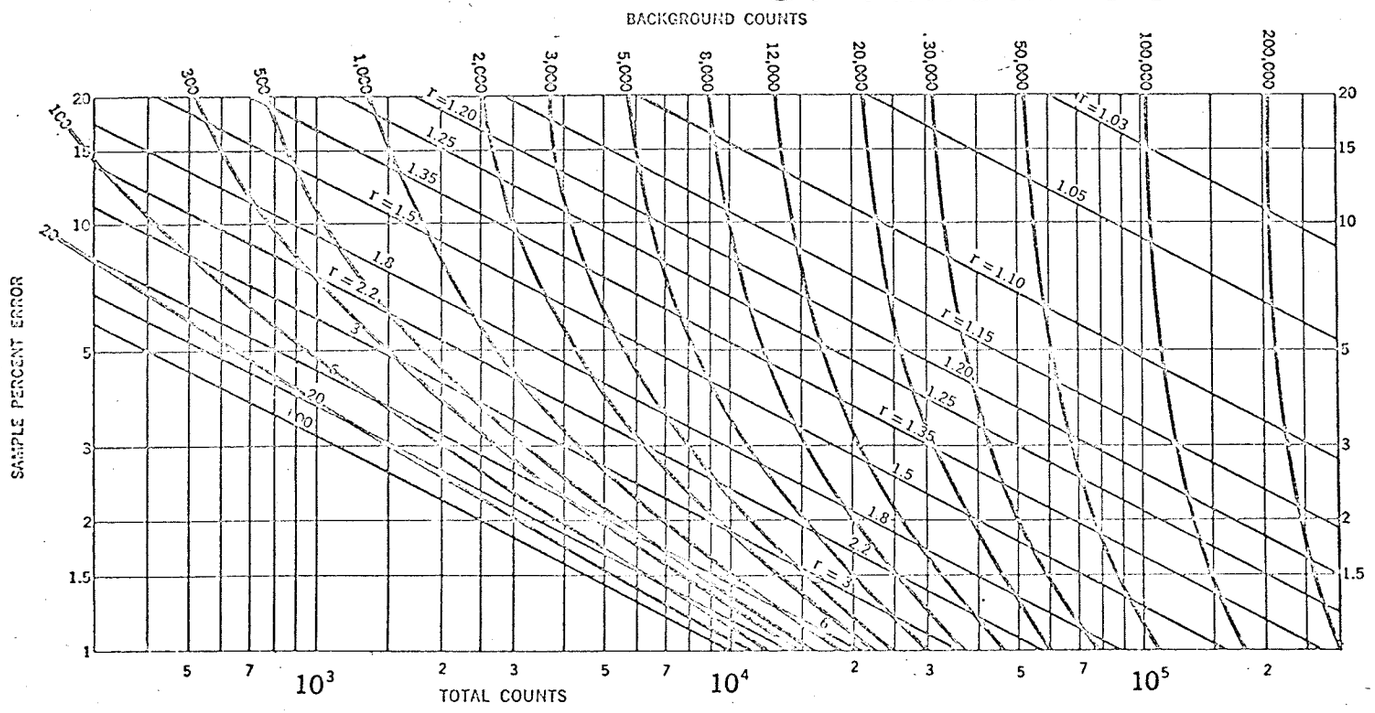
APPENDIX

Appendix A.

OPTIMUM COUNTS CHART



SAMPLE CALCULATION

r = the sample plus background count divided by the background count. Assuming r = 1.5 and the desired error is 5%, find the intersection of the horizontal 5% error line with the straight diagonal, r = 1.5 line. Reading vertically and down from this intersection, find the total counts required = 6400. For the background count, interpolate the position of the intersection between the 3000 and 5000 background count curves, and determine a background of approximately 3600 counts.

Appendix B. Abstracts of Papers Presented at Meetings of the International Association for Dental Research

234. EFFECT OF FLUORIDE ON CARBOHYDRATE ACCUMULATION IN SALIVARY SEDIMENT.—*I. Kleinberg* and H. J. Sandham, Biochemistry Section, Department of Oral Biology, University of Manitoba, Winnipeg, Canada.* Low levels of fluoride (2.2 and 4.4 ppm) as sodium fluoride were introduced into saliva-glucose mixtures (in which the sediment concentration was increased to $16\frac{2}{3}$ per cent) to determine whether it had any effect on carbohydrate storage by the sediment micro-organisms and, if so, whether this could be related to its inhibitory effect on acid production (WRIGHT and JENKINS, *Brit. dent. J.*, **96**: 30–33, 1954). Mixtures with and without fluoride at three glucose levels (0.05, 0.5, and 5 per cent) were incubated for a 4-hour period at 37° C. and the pH of the mixtures and the carbohydrate levels in the medium and sediment determined at regular intervals. With glucose at the 0.5 and 5 per cent levels fluoride markedly inhibited acid production and carbohydrate storage with the inhibition greater at the 4.4 level than at the 2.2. Glucose uptake from the medium was also inhibited. With glucose at 0.05 per cent fluoride had an opposite effect on the pH fall, causing a slight stimulation; it had no effect on carbohydrate storage. The pH of the medium did not fall below 5.6 with the 0.05 per cent glucose, whereas with the other two glucose concentrations the pH fell to below 5.0 and remained there during the experimental period. The observation that fluoride inhibits glucose uptake taken with Jenkins' observation (Proc. 4th Congress O.R.C.A., *Odontologisk Revy*, 1957) that phosphoglyceric acids do not accumulate during fluoride inhibition of acid production suggests that the site of fluoride inhibition may be at the bacterial cell membrane.

233. EFFECT OF GLUCOSE CONCENTRATION ON CARBOHYDRATE STORAGE BY SALIVARY SEDIMENT.—*H. J. Sandham* and I. Kleinberg, Biochemistry Section, Department of Oral Biology, University of Manitoba, Winnipeg, Canada.* Glucose concentrations were varied between 0–30 per cent (W/V) in saliva-glucose mixtures in which the salivary sediment (and therefore cell) concentration had been increased to $16\frac{2}{3}$ per cent (V/V). The various mixtures prepared were incubated at 37° C. for 4 hours; at regular intervals pH was determined, and samples were removed for analysis of carbohydrate levels in the medium and in the sediment. When glucose was added to the mixtures, the pH rapidly fell, and the sediment carbohydrate concentration correspondingly rapidly rose. When initial substrate levels were 0.2 per cent or below, both processes continued until the glucose in the medium was completely used up. Immediately thereafter the sediment carbohydrate level fell with the micro-organisms utilizing the accumulated carbohydrate for their energy source. When initial glucose levels were above 0.2 per cent, glucose still remained in the medium after the 4-hour incubation, and this switching from exogenous to endogenous substrate did not occur. Sediment carbohydrate accumulation and the pH fall were most rapid and greatest with glucose at the 5 per cent level. With initial concentrations below this, both the quantity of carbohydrate accumulated and the extent of the pH fall were directly related to the glucose concentration. Concentrations above 5 per cent inhibited both processes. The rate of carbohydrate accumulation from exogenous glucose and the percentage of the glucose taken up by the micro-organisms that went into carbohydrate storage were comparable to that found by Manly (*J. dent. Res.*, **40**:379, 1961) with sediment, and Gibbons and Socransky (*Arch. oral Biol.* **7**:73, 1962) with plaque.

IADR ABSTRACTS 1965

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Lactic Acid Utilization by Salivary Sediment. H. J. SANDHAM* and I. KLEINBERG, Faculty of Dentistry, Univ. of Manitoba, Winnipeg, Can.

Mixtures containing 16 2/3% (V/V) of salivary sediment in salivary supernatant were incubated at 37°C with 0.05 and 0.1% of added lactic acid or glucose. The pH of the mixtures and the carbohydrate concentrations in the sediments and supernatants were determined after various time intervals. Glucose cultures showed a rapid initial pH drop followed by a gradual pH rise. Within 15 minutes, supernatant glucose was exhausted and sediment carbohydrate accumulation reached a maximum. Thereafter sediment carbohydrate content gradually fell. In lactic acid cultures, the pH was initially lower than the pH minimum of the corresponding glucose culture but, during incubation, rose more rapidly than the pH of the glucose cultures. To determine whether lactic acid was a major contributor to the pH rise in glucose fed cultures, trace amounts of either lactate-1-C^{14} or C^{14}-labelled metabolic products, produced by salivary sediment from glucose-U-C^{14}, were added to glucose-fed cultures at the time of the pH minimum. After addition of the lactate-1-C^{14}, 40-60% of the radioactivity rapidly left the system, probably as CO_2 and the supernatant radioactivity fell to a plateau at 60-40% of the initial activity. However, after addition of the labelled products of glucose metabolism, only a small fraction of the radioactivity was lost on incubation indicating that very little lactate was present among the products of glucose catabolism and therefore that lactate utilization could not have contributed significantly to the pH rise. Other data indicate that production of ammonia is mainly responsible for the pH rise.

IADR Abstracts 1966

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Studies on How Fluoride Affects Glucose Breakdown by Salivary Sediment. H.J. SANDHAM* and I. KLEINBERG, Univ. of Manitoba, Winnipeg, Can.

Fluoride (F) at 2.2 and 4.4 ppm simultaneously inhibited glucose disappearance from and pH fall in saliva-glucose mixtures, in which salivary sediment levels were 16 2/3% (V/V) (Kleinberg & Sandham, IADR Abstr. 1964). Inhibition was most when the pH was below about 5.2. When above, as with 0.05% glucose, F showed no inhibition. CO_2 production, also determined, was not affected by F. Previous characterization by micro-radiorespirometry of the metabolic pathways involved in glucose breakdown by salivary sediment (Sandham & Kleinberg, IADR Abstr. 1965) showed mainly Embden-Meyerhof glycolysis and that CO_2 and acid production were closely related. To resolve why F inhibits pH fall and not CO_2 production, its effects on the acids responsible for the pH fall and the metabolic pathways by which CO_2 is produced were examined. Levels of lactic and other acids (mainly acetic and propionic) were less with F corresponding to its inhibition of pH fall. $C^{14}O_2$ from glucose-3,4-C^{14} and -U-C^{14} was stimulated slightly by F at 4 ppm and markedly at 20 ppm; $C^{14}O_2$ from glucose-1-C^{14}, -2-C^{14} and -6-C^{14} was unaffected. With glucose at 0.05%, F did not affect $C^{14}O_2$ production, suggesting that, like the F effect on pH fall its effect on $C^{14}O_2$ production is also pH dependent. These findings are consistent with our previous hypothesis (Kleinberg & Sandham, IADR Abstr. 1964) that F inhibits glucose uptake, which would reduce the pH fall and thus reduce the magnitude of a "quasi" Crabtree effect (Ibsen, K.H., Cancer Res: 21, 829-841 (1961).

IADR Abstracts 1967

538.

Effect of Glucose Concentration on Carbon Dioxide Production in a Salivary Sediment System. H.J. SANDHAM* AND I. KLEINBERG, Univ. of Manitoba, Winnipeg, Canada.

To determine how CO_2 is formed by the cells in salivary sediment, a salivary sediment system in which the sediment concentration was 16.7% (V/V) was perturbed with varying concentrations of glucose [between 0 and 30% (W/V)] and the CO_2 formed during four hours incubation at 37°C measured. In some experiments, glucose-1-C^{14}, -2-C^{14}, -3,4-C^{14}, -6-C^{14}, and -U-C^{14} were added to unlabeled glucose to determine (i) the proportion of the CO_2 formed that originated from glucose, rather than from substrates other than glucose (endogenous CO_2) and (ii) the metabolic pathways utilized by the carbon atoms of glucose that became CO_2.

With increasing glucose concentrations, the rate of CO_2 formation increased and the proportion of the total CO_2 that came from glucose, rather than from endogenous sources, also increased. The CO_2 from glucose was formed at a maximum rate when the glucose concentration was 5%. With 30% glucose, CO_2 formation from glucose was less than with 5% glucose, while endogenous CO_2 showed a reverse relationship.

Experiments with glucose labeled on different carbon atoms showed that 94% to 97% of the glucose was catabolized via the Embden-Meyerhof pathway, 2.8% to 6.1% via the hexosemonophosphate pathway, and almost none via the TCA cycle. Formation of endogenous CO_2 appeared to be regulated by the rate of metabolism of exogenous glucose.