PRODUCTION OF ACIDS FROM GLUCOSE BY DENTAL PLAQUE MATERIAL

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The "chemo-parasitic theory" of the etiology of dental caries, first advanced by W. D. Miller (1), postulates that the initial demineralization of tooth enamel is brought about by acids produced by bacteria present on the teeth. Since this pioneer work, a great many investigators have sought to identify the bacteria that are chiefly responsible for the acid production. Very few attempts have been made to characterize the acids formed. It is well known that many bacteria, when removed from one environment and grown in another, change their biochemical as well as other characteristics. A study of the metabolism of pure cultures of microorganisms associated with dental caries may or may not give a true picture of the metabolic activity of the same microorganisms when they are present on the teeth. It is desirable therefore to study the mixed bacterial flora as it is obtained from teeth in the mouth.

Since W. D. Miller's work (1), lactic acid has generally been assumed to be the principal acid associated with the carious process, but it was only recently that B. F. Miller and Muntz demonstrated its presence in dental lesions by a specific method (2). These authors reported that the lactic acid found was not stoichiometrically equivalent to the water-soluble calcium, thus indicating the presence of other acid anions. It has been suggested that pyruvic acid may be present in carious lesions (3). While the work presented in this paper was in progress, Summerson and Neuwirth (4, 5) reported experiments with saliva which demonstrated conclusively that lactic acid was only a part of the total acids formed from glucose.

The following experiments describe some aspects of the metabolism of glucose by the mixed bacterial flora obtained from tooth surfaces. This mixture of bacteria and matrix substances will hereafter in this paper be referred to as plaque material.1

1 Throughout this paper the term plaque material is used to connote the gross bacterial layer that can be scaled readily from the teeth of patients with poor hygiene. It has an amorphous appearance, and does not contain macroscopic food particles. Bacteria appear to make up about half of the total bulk of plaque material; the remainder is comprised of matrix material, the nature of which is unknown.
EXPERIMENTAL

The plaque material was suspended in water and homogenized in an all-glass homogenizer. Aliquots of this suspension were then treated as desired. Glucose determinations were made by the method of Miller and Van Slyke (6) on the supernatant fluid from samples of plaque material without preliminary deproteinization. Lactic acid was determined by the method of Miller and Muntz (7) after the samples to be analyzed had been previously freed of carbohydrate by copper-lime treatment. Volatile acids were determined by micro steam distillation and subsequent titration. Total acids were determined by direct titration of the supernatant fluid obtained by centrifugation. Phenolphthalein was used as indicator for the titrations.

Relationship between Glucose Consumed and Lactic Acid Produced by Plaque Material—In these experiments plaque material was removed from the teeth of patients exhibiting various types of dental disorders. Sufficient material to give a moderately heavy suspension was suspended in 2.0 cc. of distilled water. It was homogenized and aliquots were incubated with 0.1 per cent glucose for 30 minutes. An aliquot of each sample was incubated aerobically, and in four cases aliquots were incubated anaerobically. Glucose and lactic acid analyses were made before and after the incubation. From these data one obtains the ratio of moles of lactic acid formed to moles of glucose consumed. If all the glucose were converted to lactic acid this ratio should be 2.0. Table I shows that under aerobic conditions the amount of glucose that disappears cannot be wholly accounted for as lactic acid. The wide fluctuation in the ratio of the lactic acid produced to the glucose consumed probably reflects the variability of the bacterial flora in these cases. In no instance was the ratio zero; that is, some lactic acid was always found. It is important to remember that these incubations were carried out in essentially unbuffered solutions, and hence the pH was allowed to fall. The only buffers were those originally present in the plaque materials themselves. In three out of the seven experiments in which the incubation was carried out anaerobically and aerobically on similar aliquots of the same suspension, the lactic acid accumulation was much higher under the oxygen-free conditions.

Production of Acid from Glucose and Its Simultaneous Destruction—Summerson and Neuwirth in their studies of the decomposition of glucose by oral microorganisms present in saliva found that lactic acid accounted for only 50 per cent or less of the total acid formed (4). In a later report the same authors showed that lactate and pyruvate were rapidly metabolized by the bacteria in saliva with the production of other acid (or acids), which was not identified (5).

I have obtained essentially the same results using plaque material as
the source of bacteria. However, the rapid destruction of lactate occurs chiefly around pH 7. If the pH is permitted to fall, as lactic acid is produced from glucose in unbuffered solutions, the breakdown of lactate is greatly retarded. In these experiments aliquots of the same suspension of plaque material were incubated in well buffered media (Fig. 1, a), and in poorly buffered media (Fig. 1, b). The type of curve shown in Fig. 1, b is obtained under conditions that are more comparable to those actually occurring in the mouth, since it has been shown that the pH of plaques on teeth falls rapidly after ingestion of glucose (8).

The rate of lactic acid formation which results from the breakdown of glucose reaches its optimum around neutral pH, and falls off sharply below pH 6 as well as above pH 8 (Fig. 2). In these experiments, 0.1 cc. aliquots of a suspension of homogenized plaque material were diluted with 0.075 m buffer solutions, and sufficient glucose was added to give a 1 per cent solution. The original suspensions had been prepared to contain approximately the same amount of suspended material per unit volume. The lactic acid is expressed as micrograms per 0.5 cc. of the original suspension. It is to be emphasized that the lactic acid present at the end of 30 minutes incubation...
tion represents the difference between the total amount formed and the amount destroyed.

It is also important to point out that the rapid destruction of lactic acid formed from glucose is an aerobic process. Under anaerobic conditions much more lactic acid accumulates, apparently because its destruction, if it occurs at all, is greatly retarded. This is shown in Fig. 3. The experi-

![Fig. 1, a](image1)

**Fig. 1, a**

Lactic acid production from glucose and its utilization (a) in well buffered media, (b) in poorly buffered media.

![Fig. 1, b](image2)

**Fig. 2.** Lactic acid production (micrograms per 30 minutes) from glucose by plaque material at various pH values.

ments to illustrate this point were performed as follows: Samples of homogenized plaque material were divided into 2 equal parts; one sample was incubated with 1 mg. of glucose in 0.1 M phosphate buffer, pH 7.0, in the presence of oxygen-free nitrogen. The other sample was incubated in air under the same conditions. Samples were removed from both suspensions at varying periods of time and analyzed for lactic acid. Fig. 3 shows that anaerobic conditions favor the accumulation of lactic acid. This phe-
nomenon may be of some importance in the pathogenesis of dental caries, since the interproximal spaces and pits and fissures of the teeth are sufficiently anaerobic to permit the growth of an anaerobic flora. Anaerobes have likewise been isolated from plaque material present on the smooth surfaces at the gingival margin, although presumably these areas are well aerated (9).

When lactate formation-destruction curves were obtained on samples of plaque material from a number of patients, wide variations were observed in the rate at which lactate was destroyed. The following experiment shows that the type of curve varies with the amount of plaque material in suspension, that with heavy suspensions the lactate can be completely destroyed, while with light suspensions only a partial destruction occurs. The shape of the curve can be varied by simply diluting a heavy suspension of plaque material with buffer solution, as can be seen in Fig. 4. In all these samples the total volume of suspension was the same, as well as the glucose and phosphate buffer concentration. However, there were 4 times as much plaque material in the most dense suspension as in the least dense. Lactic acid analyses were performed on comparable aliquots in each case. The experiment illustrated in Fig. 4 shows that it is meaningless to compare the metabolic activity of plaque material obtained from patients with varying degrees of caries activity unless equivalent suspensions are analyzed. Nor is it permissible to obtain the dry weight of a given aliquot of
suspension and then multiply by a dilution factor in order to express the results on an equivalent basis.\textsuperscript{2}

This behavior of suspensions of plaque material when they are diluted has been confirmed by several experiments. The exact mechanism of this shift in the curve has not been elucidated, but it is quite probable that a dilution effect is operative here similar to that described by other workers with tissue suspensions (10). There is also the added possibility that certain diffusible cofactors, necessary for the oxidation of lactic acid, may be the limiting factors in this reaction. Thus when the bacterial suspension

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{The influence of density of the suspension of plaque material upon the lactic acid formation and destruction. The glucose concentration is 1 mg. per cc. of suspension in 0.2 M phosphate buffer, pH 7.0.}
\end{figure}

is diluted, these factors may not be present in sufficient quantity to maintain a proportional reaction rate.

\textit{Production of Acids Other Than Lactic Acid by Plaque Material from Glucose—}Since Friedemann (11) has shown that under anaerobic conditions certain pathogenic bacteria produce acetic and formic acids in addition to lactic acid, and since many organisms are capable of utilizing lactate aerobically with the formation of acetic acid, it seemed desirable to study

\textsuperscript{2} Neuwirth and Summerson (5) found no correlation between the degree of dental caries and the capacity of the saliva to metabolize lactate. Some specimens of saliva consumed lactate rapidly, while others did so only very slowly. It may be that the variable rate of lactate consumption is a function of the number of bacteria present per unit volume of saliva.
the formation of steam-volatile acids by plaque material. It is also of
interest to ascertain whether the lactic acid and the volatile acids together
comprise the total acidity of suspensions of plaque material or whether
still other acids are formed. It has already been pointed out that there is
good indirect evidence for the formation of acids other than lactic.

As a first approach to this problem, experiments were carried out in the
following manner. Sufficient plaque material was pooled to give 3.6 cc.
of fairly heavy suspension. This was homogenized and 0.5 cc. aliquots
were centrifuged. The supernatant fluid was withdrawn, discarded, and
replaced by exactly 0.50 cc. of 0.1 m phosphate buffer, pH 7.0, containing
2 mg. of glucose. The plaque material was resuspended in the buffer
solution and incubated at 37° for varying periods of time. At the end of
the incubation period, the sample was heated for 2 minutes in a boiling
water bath to destroy all enzyme activity. It was then cooled and the
sides of the tube were washed down with 1 cc. of distilled water. Samples
of the resulting diluted suspension were analyzed for lactic acid. The
remainder was centrifuged and the supernatant fluid quantitatively re-
moved to a larger tube. The residue was washed once with 1 cc. of water
and the resulting supernatant fluid added to the first. The combined
supernatant fluids were then titrated with 0.0083 N NaOH to the full pink
color of phenolphthalein (pH 8.5). The titration difference between the
sample incubated with glucose and a control incubated without glucose
is a measure of the total acids formed.

The titrated samples were acidified with 5 per cent phosphotungstic acid
to pH 2.5, and transferred to a 50 cc. distilling unit with sufficient water
to make 20 cc. During the distillation, water was added continuously to
maintain this volume, and six samples, comprising a total of 105 cc., were
distilled over. Titration of the distillates gave a measure of the volatile
acids. 500 γ of acetic acid added to plaque material could be recovered
with a precision of ±1 per cent by the total acid procedure. 90 to 95 per
cent of the same amount of acetic acid could be recovered by the volatile
acid determination with a precision of ±5 per cent.

Several samples of plaque material were incubated and analyzed in this
manner. Fig. 5 shows the results obtained in a typical experiment. Maxi-
mum lactic acid formation occurs within 30 minutes and thereafter de-
creases rapidly. Volatile acids, on the other hand, are produced at a
fairly constant rate throughout the 2 hour period. The total acids are
produced very rapidly in the first 45 minutes, and thereafter the rate of
production falls off. It should be pointed out that the amount of sub-
strate (glucose) is limited in these experiments. This, no doubt, accounts
for the decrease in the rate of total acid production. At no time is the sum
of the lactic acid and volatile acids equal to the total acids, Table II. The
difference between the total acids and the sum of lactic acid plus volatile acids comprises between 30 and 40 per cent of the total acids. These non-

![Graph showing production of acids from glucose](image)

Fig. 5. The acids produced from glucose (2 mg. per 0.5 cc. of suspension) by plaque material at pH 7 and 37°.

**TABLE II**

*Acid Produced from Glucose*

The concentrations of acid given represent the total amounts accumulated from 2 mg. of glucose at the stated time interval.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Incubation time</th>
<th>Lactic acid (1)</th>
<th>Volatile acids (2)</th>
<th>Total acids (3)</th>
<th>Acids not accounted for (3) - ((1)+ (2))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min.</td>
<td>mμ NaOH</td>
<td>mμ NaOH</td>
<td>mμ NaOH</td>
<td>mμ NaOH</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>4.6</td>
<td>1.1</td>
<td>8.8</td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>6.5</td>
<td>2.3</td>
<td>12.8</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>6.2</td>
<td>3.2</td>
<td>14.2</td>
<td>4.8</td>
</tr>
<tr>
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<td>60</td>
<td>5.2</td>
<td>4.5</td>
<td>14.3</td>
<td>4.6</td>
</tr>
<tr>
<td>6</td>
<td>90</td>
<td>3.2</td>
<td>5.7</td>
<td>15.0</td>
<td>6.1</td>
</tr>
<tr>
<td>7</td>
<td>120</td>
<td>1.9</td>
<td>7.2</td>
<td>15.7</td>
<td>6.6</td>
</tr>
</tbody>
</table>

volatile acids are produced most rapidly in the first 15 minutes of incubation.

It is of interest to compare the metabolic activity of a pure strain of
Lactobacilli isolated from the oral flora with that of the plaque material just described. A heavy suspension of the washed bacteria in 0.1 M phosphate buffer was treated with glucose (1 mg. per 0.5 cc. of suspension). Aliquots of the suspension were incubated for varying periods of time and were then analyzed as described previously. Fig. 6 shows that lactic acid was rapidly produced but it was not metabolized further. Volatile acids are not formed during the first 30 minutes and thereafter only small amounts appear. The total acids formed from glucose are in excess of the lactic acid plus volatile acids. In this connection it is of interest that the production of malic acid by oral Lactobacilli has been reported (12).

![Graph](http://image.com/graph.png)

**Fig. 6.** The production of acids from glucose (1.0 mg. per 0.5 cc. of bacterial suspension) by oral Lactobacilli at pH 7 and 37°.

*Nature of Volatile Acids Produced from Glucose*—The identification of the volatile acids produced aerobically from glucose by plaque material at pH 7.0 has proved to be a difficult problem. It was not feasible to obtain enough plaque material so that large quantities of the acids could be produced to permit their isolation. Samples of plaque material were collected daily from three or four patients, incubated with glucose, and distilled. After a week, the combined distillates were found to contain volatile acids equivalent to 15 mg. of acetic acid. Duclaux constants obtained on this acid mixture averaged 10.7. Duclaux constants on pure acetic acid and propionic acid obtained with the same distilling unit averaged 7.3 and 14.2 respectively. A mixture of these acids, 8.4 mm in acetic acid and 4.9 mm in propionic acid, gave an average Duclaux constant of 11.0.

The mixture of volatile acids obtained from plaque material was steam-
distilled at pH 9.0 to remove neutral steam-volatile substances. It was reacidified to pH 2.5 with phosphotungstic acid and the volatile acids again steam-distilled. The fractions obtained in this distillation were titrated to pH 8.5 in a vessel containing a glass electrode, and were concentrated in a vacuum desicator. 10 cc. of the final solution contained sodium salts of the unknown acids equivalent to 12.7 mg. of acetic acid.

An estimation of formic acid was carried out on this solution by a manometric procedure in the Warburg apparatus. It had previously been determined that by using a 5 cc. vessel, as little as 50 y of formic acid could be estimated. 0.3 cc. of solution containing the formate in the vessel side arm is tipped into 0.7 cc. of 0.02 n KMnO₄ acidified to 0.1 n with H₂SO₄. The reaction is complete in 1 hour; approximately 0.5 c.mm. of CO₂ is liberated per microgram of formic acid oxidized. Under the same conditions acetic acid and propionic acid yielded negligible amounts of CO₂. When several volatile acid samples obtained from plaque material were analyzed in this way, formic acid was never more than, and usually less than, 10 per cent of the volatile acids.

Several volatile acid concentrates were tested for acetic acid by the lanthanum nitrate test, always with negative results. Yet the Duclaux constant suggested that it was certainly present. Subsequently the spot test described by Feigl, Zappert, and Vasquez (13) was employed and a good test for acetic acid obtained. By a rough comparison of the intensity of the color produced by a given volume of the volatile acid concentrate and known amounts of acetate it was estimated that about one-third of the total volatile acids could be accounted for as acetic acid.

McNair (14) has described an oxidimetric method for the estimation of propionic acid. The method is not specific, since acetic acid reacts to a slight extent. However, the difference in reactivity is great enough to make the test of diagnostic value. In order to apply the method to such small amounts of acid as were available, all the quantities of the reagents were reduced 10 times. Comparable samples of acetic and propionic acids were run through the procedure at the same time. In this way it was estimated that between 15 and 20 per cent of the total volatile acids reacted like propionic acid.

The mercurous salts of the volatile acids have a characteristic crystalline structure. However, all attempts to establish the identity of the acids in the distillates by the formation of these mercurous salts were unsuccessful. The presence of significant amounts of the higher fatty acids could be excluded on the basis of the Duclaux constants and the absence of any pronounced butyrous odor in the distilled samples.

It is realized that the relative amounts of the various acids vary from sample to sample. This is to be expected with such a heterogeneous flora.
Yet in all the samples examined thus far, formic acid has not appeared in any appreciable concentration, and acids of greater chain length than propionic acid do not seem to be formed to any appreciable extent. Pyruvic acid was present in such small amounts that it could be neglected.

DISCUSSION

When dental plaque material is incubated in vitro with glucose, lactic acid is formed so rapidly that it accumulates to a considerable extent. Unquestionably this occurs likewise on the teeth in situ following the ingestion of glucose, sucrose, and other carbohydrates (15). However, in situ, the pH of the plaque material falls as acid is produced. Since it has been pointed out in this paper that the further breakdown of lactate occurs chiefly at neutral reaction, it is quite probable that this breakdown is a much slower process in situ than in vitro at pH 7. The decomposition of lactic acid in situ probably proceeds as the salivary buffers exert their effect, a process which may take as long as 30 to 90 minutes (16). The decomposition of lactic acid would be further delayed in the mouth by anaerobic conditions that may obtain in the caries-susceptible areas.

The slower but continuous formation of volatile acids that has been demonstrated to occur at neutrality in vitro probably occurs also in the mouth, as the saliva buffers the acidity of the plaque material. Since formic acid, which is one of the stronger steam-volatile acids, is formed in such small amounts, this process is in effect the replacement of the relatively strong lactic acid by less dissociated steam-volatile acids such as acetic acid and propionic acid.

The rapid production of acids other than lactic and volatile acids may be of some significance. Should these acids prove to be of the dicarboxylic type, such as succinic or malic, relatively strong acids would have been produced which in addition can form undissociated complexes with calcium (17). The rapidity with which the non-volatile acids are produced suggests that they can be formed directly from glucose and do not appear primarily when the lactic acid is consumed. It would be of considerable interest to study their production under anaerobic conditions.

SUMMARY

1. It has been demonstrated that the rapid destruction of lactic acid formed from glucose by the bacterial flora of dental plaque material occurs chiefly at neutrality and under aerobic conditions.

2. The rate of lactate breakdown is furthermore a function of the concentration of plaque material; i.e., with very heavy suspensions, the rate is quite rapid, while with less dense suspensions a disproportionately greater time is required to achieve the same effect.
3. Concomitant with lactic acid formation and breakdown, there is a slower but steady formation of steam-volatile acids. One of these is acetic acid and another reacts like propionic acid. Formic acid is produced in small amounts only.

4. Besides lactic acid and the volatile acids there is a rapid production of a certain quantity of non-volatile acid. The identity of the acid or acids which constitute this fraction has not been established.

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