The Metabolism of the Oral Flora

I. THE OXYGEN UPTAKE AND ACID PRODUCTION BY MIXED HUMAN SALIVA IN THE PRESENCE AND ABSENCE OF GLUCOSE

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The early work of Miller (1890) has remained the foundation upon which many theories concerning the aetiology of dental caries have been based. Miller considered that caries was due to the dissolution of the inorganic portion of the enamel and dentine by acids. These acids, he postulated, were produced by the bacterial fermentation of food particles trapped in pits and fissures of the teeth which were not self cleansing. The main requirement of this theory is therefore that acid shall be produced in sufficient quantity, locally, to lower the pH to the point where dissolution of the enamel may occur.

The most common mouth organism which is capable of producing acid (acidogenic) and existing at a comparatively low pH (aciduric) is *Lactobacillus acidophilus*. This organism is accepted by many workers as a factor in the production of caries (Bunting, 1934; Snyder & Teachout, 1942). It also appears that a diet high in fermentable carbohydrate favours the development of this organism in the mouth (Arnold & McClure, 1941); such a diet also results in an increase in the incidence of dental caries (Jay, Hadley, Bunting & Koehne, 1936). Whether there is a causative relationship between these two observations remains to be proved. Other workers have cited streptococci and staphylococci (Anderson & Rettger, 1937; Florestano, 1942; Bibby, Volker & Van Kesteren, 1942) as being capable of producing acid. Fosdick, Hansen & Wessenger (1937) have shown that certain yeasts are also acidogenic, and that yeast and lactobacillus together produce acid more rapidly than either organism alone. It would seem dangerous therefore to consider one organism to be of paramount importance in a complex and varied flora such as exists in saliva; the various micro-organisms which are present may act in concert in a manner differing from their behaviour individually. It is important, as Fosdick, Campaigne & Fancher (1941) have pointed out, to consider not only the bacteria themselves but the enzymes which they contain.

More recently, doubt has been cast upon the validity of the 'acidogenic' concept of caries initiation, and a different theory has been advanced, namely that the primary lesion is due to the action of

proteolytic enzymes upon the organic matrix of the enamel (Nuckolls & Frisbie, 1946; Frisbie & Nuckolls, 1947; Gottlieb, 1947).

Since the metabolism of the whole oral flora may be involved in the carious process it is possible that the study of the saliva as a 'biological unit' may prove to be of value.

It was decided therefore to examine the behaviour of various salivas making use of the Warburg manometric technique. It is realized, of course, that the salivary flora is only a sample, and possibly only a component, of the total oral flora.

In the present work determinations have been made of the oxygen consumption and lactic acid production by the saliva when incubated at 38° in the presence and absence of glucose. In addition, the lactic acid and total acid produced under anaerobic conditions have been determined with and without the addition of glucose.

The object of this preliminary investigation was to observe the magnitude and variation of oxygen consumption and lactic acid production in the salivas of subjects with varying degrees of dental caries, and to see whether these factors bore any relation to the caries experience of the subjects. The individuals included one completely caries free, one virtually free (one minor filled cavity), two with slight caries activity and five with a considerable number of carious lesions.

EXPERIMENTAL

Material. Samples of saliva were obtained from nine student volunteers, five men and four women, with varying caries experience, one of the men being completely free from dental caries.

Subject	Age (yr.)	Teeth decayed, missing or filled (D.M.F.)
J.C. ♂	26	19
D.R. ♂	22	16
M.S. ♀	27	19
A.M. ♀	22	23
H.F. ♂	26	17
R.B. ♂	19	4
S.H. ♀	19	4
Y.C. ♀	21	1
M.T. ♂	36	0

Collection of saliva. Samples (5 ml.) were collected by the subjects themselves each morning immediately on awakening whilst still lying in bed. The flow of saliva was stimulated in all cases by the chewing of paraffin wax (British Drug Houses Ltd., m.p. 48–49°). The subjects were instructed to make the wax scour all surfaces of the teeth. The saliva was collected in a sterile screw-capped bottle in as short a time as possible. The samples were brought to the laboratory within 2 hr. of collection.

Manometry. The samples of saliva (0.5 ml.) were buffered with 0.2M-phosphate buffer, pH 7.4. Oxygen consumption was measured in Warburg manometers at 38°.

The final concentration of glucose, when used, was 0.005M.

Lactic acid production. The lactic acid in the manometer contents was determined by the method of Barker & Summerson (1941) as described by Umbreit, Burris & Stauffer (1945).

Total acid production. The total acid was measured manometrically by the evolution of CO₂ from a bicarbonate buffer in an atmosphere of N₂ containing 5% CO₂.

Bacterial multiplication. Since times of up to 2 hr. elapsed between collection of salivary samples and their arrival at the laboratory, followed by a period of 1 hr. at 38°, it was important to know the extent of bacterial multiplication in these circumstances. It was not possible to carry out tests simultaneously on the nine subjects. Saliva from six further subjects was therefore collected and allowed to incubate with and without glucose at room temperature (15°) and at 38°. Counts were done at 30 min. intervals for 4 hr.

Non-cellular oxidation. It was also important to know whether the oxidative processes in saliva were due entirely to bacteria and other cells, or whether salivary enzymes were involved. For this purpose samples of saliva were filtered through a Seitz filter and the filtrate used in the manometer.

RESULTS

The mean figures quoted in the Tables represent the average of from ten to twenty independent observations made on different days.

The uptake of oxygen is expressed as μ l. of O₂ taken up by 1 ml. saliva in 1 hr. There is a wide day-to-day variation in all subjects both with and without the addition of glucose. The results are given in Table 1.

Lactic acid and total acid production are expressed as micro-equivalents of acid produced by 1 ml. saliva in 1 hr. The results for aerobic conditions are presented in Table 2, those for anaerobic conditions in Table 3.

Bacterial multiplication. Saliva from six further subjects was obtained and counts made at half-hourly intervals during incubation with and without glucose at 15 and 38°. After 2 hr. at 15° with or without glucose there was only a slight increase in count, even after incubation with glucose for 2 hr. at 38°, the count was less than double the original figure.

Non-cellular oxidation. Saliva which had been filtered through a Seitz filter did not take up oxygen either in the absence or presence of glucose.

DISCUSSION

The comparison of respiratory rates and acid production on a volumetric basis, i.e. uptake of O₂ or acid produced/ml. saliva/hr., seems a reasonable course to adopt since the same volume of saliva was collected each morning under the same conditions. In future investigations it may be desirable to adopt another standard for comparison, e.g. O₂ consumption/mg. N, but for the present the volumetric basis will serve.

The bacterial multiplication under the conditions of the experiment appears to be of a low order and will not unduly influence the results. Since there is no evidence of any oxidation due to non-cellular constituents of the saliva, it would seem that the total oxidation is due to the contained cells.

Aerobic oxidation. The results indicate that there is a great variation in the mean O₂ consumption of the nine subjects. The oxygen taken up in the absence of added glucose was considerable, being greatest in the case of J. C. (caries active) and Y. C. (caries free). Lactic acid was not produced in detectable amounts by any of the salivas, which suggests that if carbohydrate were a substrate, it was

Table 1. *Oxygen consumption of saliva samples*

(All values are μ l. O₂/ml. saliva/hr. Temp. 38°.)

Subject	Teeth decayed missing or filled	Without added glucose		With added glucose (0.005M)		Difference between means with and without glucose (b - a)
		Mean (a)	Range	Mean (b)	Range	
J.C.	19	309	134–465	605	256–927	296
D.R.	16	184	94–336	350	144–685	166
M.S.	19	137	70–320	267	161–524	130
A.M.	23	96	40–135	157	97–292	61
H.F.	17	132	72–192	220	80–409	92
M.T.	0	56	32–76	187	112–254	137
Y.C.	1	315	230–384	385	304–490	70
S.H.	4	124	96–160	223	176–272	99
R.B.	4	157	120–188	237	205–257	80

Table 2. *Lactic acid production by saliva in aerobic conditions*
(Results are expressed in $\mu\text{g.}$ or $\mu\text{equiv.}$ lactic acid/ml. saliva/hr. Temp. 38° .)

Subject	Teeth decayed, missing or filled	Without added glucose	With added glucose (0.005M)		
			$\mu\text{g.}$ lactic acid		$\mu\text{equiv.}$ lactic acid
			Mean	Range	Mean
J.C.	19	Not detectable	100	75-160	1.1
M.S.	19	"	287	210-385	3.2
A.M.	23	"	191	138-270	2.1
H.F.	17	"	183	75-480	2.0
M.T.	0	"	819	240-1440	9.1
Y.C.	1	"	165	130-190	1.8
S.H.	4	"	140	100-175	1.6
R.B.	4	"	125	100-165	1.4

Table 3. *Acid production by saliva under anaerobic conditions*
(Results expressed as $\mu\text{l. CO}_2$ or $\mu\text{equiv.}$ acid/ml. saliva/hr. Temp. 38° .)

Subject	Teeth decayed, missing or filled	Total acid						Lactic acid	
		Without added glucose			With added glucose (0.005M)			Without glucose	With glucose
		$\mu\text{l. CO}_2$ produced		$\mu\text{equiv.}$ Mean	$\mu\text{l. CO}_2$ produced		$\mu\text{equiv.}$ Mean	$\mu\text{equiv.}$ Mean	$\mu\text{equiv.}$ Mean
		Mean	Range		Mean	Range			
J.C.	19	161	102-260	7.2	756	653-859	33.8	Nil	4.3
M.S.	19	140	100-200	6.3	645	490-803	28.8	"	3.9
A.M.	23	215	136-356	9.6	450	348-560	20.5	"	5.9
H.F.	17	140	60-224	6.3	646	539-689	28.8	"	3.5
M.T.	0	174	120-328	7.8	704	568-765	31.4	"	3.6
Y.C.	1	196	160-260	8.8	697	550-844	31.1	"	6.2
S.H.	4	148	120-220	6.6	500	426-578	22.3	"	7.1
R.B.	4	260	160-320	11.6	552	509-636	24.6	"	8.6

being completely oxidized. The possibility must be considered that this O_2 consumption in the absence of added glucose may be due to the oxidation of products of proteolysis. In the subjects with active caries, substrate may be provided from the cavity contents, but in the caries-free cases the probability is that oxidation of a normal salivary constituent is taking place. This problem warrants further investigation. The addition of glucose caused an increased O_2 uptake in all cases and lactic acid was present in the manometer contents. There is a slight indication that the absolute increase in O_2 consumption due to the addition of glucose is greatest in the saliva of those subjects with most caries but, except in the case of J.C., there is no marked difference.

Since the addition of glucose caused the appearance of lactic acid (a typical product of anaerobiosis) as well as an increased O_2 uptake, it would appear that both anaerobic and aerobic processes are occurring side by side in the saliva. A greater O_2 uptake does not seem to reduce the quantity of lactic acid produced. This may be due to a differing cell content, but a factor which cannot be excluded is that vigorous aerobiosis may result in local O_2 lack and the encouragement of pockets of anaerobic activity. This is a factor which would be more apparent in the mouth than in an *in vitro* experiment.

An interesting observation was that the saliva of subject M.T., who was absolutely caries free, produced a vastly greater amount of lactic acid than any of the others (Table 2). In another subject (Y.C.), who was virtually caries free, the lactic acid produced was no less than in other subjects with a high incidence of caries (J.C., H.F. and A.M.). It would appear therefore that a high lactic acid production is not necessarily associated with a high degree of caries, and that conversely caries may develop when the acid production is low.

Anaerobic oxidation. Since it is possible that anaerobic conditions exist locally in the mouth, arising perhaps where aerobes are using up all the available O_2 in a dental plaque, the study of anaerobic oxidation in the saliva is pertinent. When the salivas were incubated without added glucose in a bicarbonate buffer at 38° no detectable lactic acid was formed in any of the subjects, but acid of some kind was nevertheless produced in varying amounts. In this case the acid produced by the caries-free subjects was no less than that produced by the others (Table 3). The origin and nature of this acidic material is obscure and requires further study. The addition of glucose caused in all subjects a large increase in the total acid production, the caries-free subjects forming just as much as the caries-active.

The lactic acid formed in the presence of glucose is greater under anaerobic than aerobic conditions except in the case of subject M.T. The fact which does emerge, however, is that the potential acid production in the most favourable circumstances, i.e. lack of O_2 , is no less in the caries-free subjects than in those with active caries. A further point worthy of emphasis is that the lactic acid produced by incubation with glucose under anaerobic conditions accounts for only one-sixth to one-third of the total acid formation.

A great mass of evidence has been put forward to support the contention that acid production in the mouth is associated with the incidence of dental caries, and various caries-susceptibility tests have been elaborated on this basis, a notable one being due to Fosdick, Hanson & Eppe (1937).

Stephan (1944) studied the changes occurring in the intra-oral pH, after a rinse with a 10% aqueous glucose solution; his general conclusion was that the greatest fall in pH occurred in subjects with most extensive caries. He did report, however, that bacterial plaques, which are capable of producing acid rapidly, grow not only on the teeth of individuals who have active caries, but also on the teeth of those who are caries free.

Volker & Pinkerton (1947) have shown that various unrefined carbohydrates, including starch and dextrin, are converted to acid by saliva at rates similar to those observed for refined sugars. If acid production is of major importance in the initiation of caries, it is difficult to reconcile this observation with the widely held view of the specific cariogenic action of refined sugars. Osborn, Noriskin & Staz (1937) reported that tooth surfaces exposed to mixtures of saliva and unrefined carbohydrates were decalcified to a less degree than those immersed in saliva and refined sugars. They suggested that this was due to the removal during the refining process of constituents which protect the enamel against dissolution, but were unable to say more about the nature of this protection.

We are aware that the mere production of a given quantity of acid may result in a different effect in different subjects. Other factors will exert an influence upon the change in pH, notably buffering capacity and rate of flow of the saliva.

Claims have been made to correlate the buffering power of the saliva and caries experience (Dreizen, Mann, Cline & Spies, 1946). Against this, there is no evidence to show a relationship between salivary phosphate (an important buffer) and caries (Deakins,

Cheyne, Bibby & Van Kesteren, 1941). Stralfors (1948) has shown that the buffering capacity of the bacterial plaques occurring on the tooth surface is greater than that of the saliva from the same mouth.

The case of subject M.T. was the more remarkable in that the teeth of both jaws were somewhat crowded together, there being in fact a supernumerary in the upper jaw. Such orthodontic conditions are usually considered to predispose to the development of dental caries, especially of the interstitial kind (Thoma, 1944).

The relative importance of all the various factors involved is obscure in the present state of knowledge, but there is some evidence to suggest that acid production *per se* is not of paramount importance in the aetiology of caries. The results of this investigation, limited though it is by the difficulty of obtaining caries-free subjects, tends to support this point of view. It appears then, that whilst acid production in the mouth may be a necessary concomitant in the carious process it is not in itself a sufficient cause.

SUMMARY

1. The oxygen uptake of saliva samples, with and without the addition of glucose, has been studied in nine subjects, of whom one was absolutely free from dental caries and another virtually so.

2. The absolute oxygen consumption varied from subject to subject, and also from day to day in the same individual. There was no obvious relation between oxygen uptake and dental caries experience.

3. The amount of lactic acid produced by the salivas in this investigation bears no relation to the degree of caries in the subjects.

4. The greatest production of lactic acid in aerobic circumstances was by a subject who was absolutely caries free.

5. The total acid and lactic acid produced in the absence of oxygen bear no apparent relation to the caries incidence.

6. It is suggested that acid production *per se* is not a sufficient cause for the production of dental caries.

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REFERENCES

- Anderson, T. G. & Rettger, L. F. (1937). *J. dent. Res.* **16**, 489.
- Arnold, F. A. & McClure, F. J. (1941). *Publ. Hlth Rep., Wash.*, **56**, 1495.
- Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
- Bibby, B. G., Volker, J. F. & Van Kesteren, M. (1942). *J. dent. Res.* **21**, 61.
- Bunting, R. W. (1934). *J. dent. Res.* **14**, 97.
- Deakins, M., Cheyne, V. D., Bibby, B. G. & Van Kesteren, M. (1941). *J. dent. Res.* **20**, 161.
- Dreizen, S., Mann, A. W., Cline, J. K. & Spies, T. D. (1946). *J. dent. Res.* **25**, 213.
- Florestano, H. J. (1942). *J. dent. Res.* **21**, 263.
- Fosdick, L. S., Campaigne, E. E. & Fancher, O. E. (1941). *Illinois dent. J.* **10**, 85.
- Fosdick, L. S., Hansen, H. L. & Epple, C. (1937). *J. Amer. dent. Ass.* **24**, 1275.
- Fosdick, L. S., Hansen, H. L. & Wessinger, G. D. (1937). *J. Amer. dent. Ass.* **24**, 1445.
- Frisbie, H. E. & Nuckolls, J. (1947). *J. dent. Res.* **26**, 181.
- Gottlieb, B. (1947). *Dental Caries*. Philadelphia: Lea and Febiger.
- Jay, P., Hadley, F. P., Bunting, R. W. & Koehne, M. (1936). *J. Amer. dent. Ass.* **23**, 846.
- Miller, W. D. (1890). *The Micro-organisms of the Human Mouth*. Philadelphia: S. S. White Co.
- Nuckolls, J. & Frisbie, H. E. (1946). *J. Amer. Coll. Dent.* **13**, 84.
- Osborn, T. W. B., Noriskin, J. N. & Staz, J. (1937). *J. dent. Res.* **16**, 165.
- Snyder, M. L. & Teachout, J. J. (1942). *J. dent. Res.* **21**, 461.
- Stephan, R. M. (1944). *J. dent. Res.* **23**, 257.
- Stralfors, A. (1948). *J. dent. Res.* **27**, 587.
- Thoma, K. H. (1944). *Oral Pathology*. London: Henry Kimpton.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques*. Minneapolis: Burgess Publishing Co.
- Volker, J. F. & Pinkerton, D. M. (1947). *J. dent. Res.* **26**, 229.

Metabolism of Polycyclic Compounds

6. CONVERSION OF PHENANTHRENE INTO DIHYDROXYDIHYDROPHENANTHRENES

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Bergel & Pschorr (1903) showed that rabbits dosed with phenanthrene excrete a phenolic derivative, phenanthrylglucuronide. Investigation of the metabolism of anthracene in rats and rabbits (Boyland & Levi, 1935) and of naphthalene (Young, 1947; Booth & Boyland, 1947, 1949) has shown that these hydrocarbons are transformed into dihydroxydihydro derivatives. Such diols are readily converted to phenols by dehydration in the presence of acid, and it is possible that some of the phenolic metabolic products which have been isolated are decomposition products of dihydroxydihydro derivatives. In the present paper this reaction of diol formation, which has been called perhydroxylation because it involves addition of the elements of hydrogen peroxide, is shown to take place with phenanthrene.

Young (1947) reported the isolation of a crystalline product from the urine of rats dosed with phenanthrene. This product with m.p. 186–187° and analysis corresponding to a formula $C_{14}H_{12}O_2$ would appear to be identical with the 9:10-dihydroxy-9:10-dihydrophenanthrene characterized below. Criegee, Marchand & Wannowius (1942) prepared *cis*-9:10-dihydroxy-9:10-dihydrophenanthrene by oxidation

of phenanthrene with osmic acid. Attempts to synthesize *trans*-9:10-dihydroxy-9:10-dihydrophenanthrene by published methods (e.g. Skita, 1925) or by oxidation of phenanthrene have so far been unsuccessful, but reduction of phenanthraquinone with lithium aluminium hydride by the method described by Finholt, Bond & Schlesinger (1947) has given the *trans* compound identical with this metabolite (Booth, Boyland & Turner, 1950).

EXPERIMENTAL

A solution of phenanthrene (20%, w/v) in arachis oil was injected intraperitoneally into rats and rabbits. Rats were dosed with 10 ml./kg. body weight and rabbits with 5 ml./kg. body weight twice weekly and showed no ill effects from the treatment. The fresh urine which was collected in metabolism cages was extracted with ether as soon as possible. The ethereal extract was evaporated to dryness. In some experiments, the residue could be crystallized from benzene without further treatment. When the material was too impure for direct crystallization, it was dissolved in methanol containing 1% (v/v) water