CCLXX. THE ORIGIN AND SIGNIFICANCE OF SALIVARY PHOSPHATASE

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PART I. THE ORIGIN OF SALIVARY PHOSPHATASE

THE occurrence of a phosphatase in saliva is generally accepted. Demuth [1925] appears to have been the first to study the activity of this enzyme in saliva. Adamson [1929] and Smith [1930] made important advances in this subject. Both observers considered the phosphatase activity of saliva to be related to the formation of calculus (tartar) on the teeth.

It is desirable to consider the origin of salivary phosphatase. Adamson [1929] found that tissue removed from the gums showed phosphatase activity and that saliva contained a substrate for this enzyme, since on incubating saliva and this tissue (or bone phosphatase) there was an increase in free phosphate. He definitely associated the occurrence of these two factors with the formation of calculus on teeth, a process he was able to imitate *in vitro*. Smith [1930] followed up Adamson's work and found in saliva a phosphatase which he believed to originate from epithelial scales, because centrifuged saliva had no phosphatase activity. He found that this enzyme had an optimum pH of 5.5 and could hydrolyse the naturally occurring phosphoric esters of saliva as well as the Na glycerophosphate. He also found that if tissue were removed from the gums and placed in Ca glycerophosphate, a deposition of Ca phosphate occurred in the epithelium.

Since phosphatases are widely distributed in living matter and are found in many animal tissues, organs and secretions, their presence in saliva was readily accepted. Umeno [1931], however, found that neither saliva nor gastric juice contained any phosphatase, while both salivary glands and gastric mucosa showed phosphatase activity, although much less than kidney and duodenal mucosa. It occurred to us that since saliva contains a large variety of micro-organisms, these might be responsible, in part at least, for the phosphatase activity. Evidence is gradually accumulating that phosphatases occur commonly and perhaps generally in micro-organisms. Their occurrence in yeast juice is classical knowledge. The comparison of yeast and muscle metabolisms led to the association of phosphatases with carbohydrate breakdown. The phosphatases of takadiastase from *Aspergillus oryzae* have been studied systematically by Akamatzu and his colleagues, an account of their work being given in a review by Folley & Kay [1936]. Gordon & Cooper [1931; 1932] appear to have made the first observations on bacterial phosphatases. Heard & Wynne [1933] and Pett & Wynne [1933] studied the occurrence and properties of several types of bacteria of widely different cultural characteristics. Wiggert & Werkman [1938] remark that there is now considerable evidence for the importance of phosphorylation in the metabolism of micro-organisms.

We were able to demonstrate phosphatase activity in some micro-organisms, which are commonly air-borne and are therefore likely to be present in the mouth, and in two strains of actinomycetes which are of special interest in the

present work, one freshly isolated from the human mouth. The full details of this part of the work will be given later.

To investigate the question of the origin of the salivary phosphatase, we set out to test the activity of cell-free and bacteria-free saliva. The phosphatase activity was tested in the following way, using 15 ml. graduated tubes.

Tubes 1 and 2. 10 ml. substrate + 5 ml. buffer pH 5.5 + 1 ml. chloroform water + 0.5 ml. saliva. Mix and add 2 ml. glacial acetic acid and put on ice. These are controls.

Tubes 3 and 4. As above, but incubate a suitable time before the addition of acid.

The volumes were all made up to 15 ml., filtered and the free phosphate estimated by the method of Fiske & Subbarow [1925]. Glacial acetic acid gives with saliva a much clearer filtrate than the more usually employed trichloroacetic acid, and in this way we avoided, in part, the difficulty of turbidity encountered by Smith. The excess of free phosphate in tubes 3 and 4 over that in tubes 1 and 2 gave the phosphatase activity of the saliva.

We compared the activities of native and centrifuged saliva (centrifuged at 2000 r.p.m. for 20 min.) and although the latter had less activity, it was by no means devoid of activity as Smith [1930] stated. Giri [1936] centrifuged saliva, in the process of purification of the phosphatase, without apparent loss of the enzyme. He was not, however, concerned with the origin of this enzyme. Saliva, filtered through nos. 30 and 42 Whatman filter papers, which keep back epithelial scales, also possessed phosphatase activity and, as expected, showed the presence of micro-organisms on culture and incubation in broth at 37° for 24 hr. Three samples of saliva which had been put through a Seitz filter (disk EK) and one which had been through a Berkefeld N filter were also investigated. The Seitz-filtered sample proved sterile, on testing by seeding into broth and incubating at 37°. The Berkefeld sample was not sterile.

These results are represented in Table I. It is to be noted that salivas show very different degrees of phosphatase activity and occasionally no activity. This, in itself, may be significant.

Table I. *Phosphatase activity of saliva. Hydrolysis of Na α -glycerophosphate at pH 5.5*

Sample	Native	Centrifuged	Filtered	Remarks
1	++	+	O	Seitz EK sample had no ptyalin and no Ca or P. Sterile
2	++	+	O	Ditto
3	O	-	-	Not pooled. Ptyalin present
4	++	+	S	Berkefeld. Not sterile
5	++	+	O	Seitz as above
6	O	-	-	Not pooled. Ptyalin present
7	++	+	-	

(S=slight activity; O=no activity; +, ++=activity of different degrees; - =not tested.)

In three of the experiments, the relative activities of native and centrifuged salivas were calculated, with the following results:

Saliva sample no.	4	5	7
Activity native			
Activity centrifuged	1.4	1.4	3.1

The actual values obtained for sample 7 were:

Native saliva ...	...	30.0 mg. P set free in 18 hr. per 100 ml. saliva
Centrifuged saliva ...	...	9.5 mg. P set free in 18 hr. per 100 ml. saliva

In considering the filtered salivas, although the Seitz-filtered sterile saliva had no phosphatase activity and the Berkefeld-filtered non-sterile sample did possess activity, we cannot say, without reservation, that the activity of saliva is normally due to these organisms. An interesting observation was made in these saliva experiments. The Seitz-filtered saliva (a sample of the original had been shown to have all the normal constituents) contained no phosphatase and incidentally no ptyalin (amylase) and no inorganic P. The Berkefeld-filtered saliva had all the normal constituents.

Presuming that cannulated saliva collected under sterile conditions might be free from micro-organisms, two experiments were done on cats under chloralose.¹ The submaxillary duct was cannulated with a sterile cannula. Pilocarpine was injected intravenously to excite secretion. This duct secretion and the saliva from the mouth were tested for phosphatase activity. The results are given in Table II.

Table II. *Phosphatase activity of saliva*

	Substrate		Remarks
	Na α -glycero-phosphate 18 hr.	Di-Na-phenyl phosphate $\frac{1}{2}$ hr.	
Cat I:			
Duct secretion	S	O	Saliva very watery. Sterility not tested
Saliva from mouth	+++	O	Very viscid
Cat II:			
Duct secretion	S	O	Very watery. No cells present. Few bacteria in tubes incubated 18 hr.
Same duct secretion, mouth scrapings added	++	-	Scrapings from roof of mouth added
Saliva from mouth	+++++	++	Viscid. Cells and many organisms present

Note. The activity of saliva towards phenyl phosphate is always less than towards glycerophosphate, probably because at the optimum pH for salivary phosphatase, the phenyl phosphate does not hydrolyse well.

It is possible that more rigidly sterile conditions than we were able to obtain would give a secretion free of phosphatase. We do not think that the slight activity of the duct secretion was due to the presence of cells. Another possibility is that the other salivary or the buccal glands might produce the phosphatase.

If the desquamated cells in the saliva are not the sole source of the phosphatase, and it is thought that micro-organisms contribute to the activity, then clearly another way of investigating this latter possibility is to compare the phosphatase activity of saliva straight from the mouth, as uncontaminated with airborne organisms as possible, with the activity of the same saliva incubated in a nutrient medium for a suitable time at 37°.

A series of such experiments was carried out, the saliva being tested as described previously at pH 6 using glycine buffer and glycerophosphate. The details of the conditions of these experiments and the results are set out in Table III.

There can be no doubt that saliva contains micro-organisms whose multiplication is accompanied by an increase in phosphatase activity. It may be noted here that Smith [1930] found that saliva had less activity after brushing

¹ These experiments were performed at Bedford College by M. M. Murray.

Table III. *Phosphatase activity of saliva after incubation*

Details of incubation	Time of incubation in days	Phosphatase activity in mg. P/100 ml. saliva	
		α -glycero-phosphate	β -glycero-phosphate
Saliva and Czapek-Dox (KH_2PO_4 omitted)	0	1.0	1.0
	1	1.0	1.0
	6	44	34
Saliva and sterile nutrient broth	0	4.6	—
	2	67	—
Saliva and sterile glucose broth	0	2.5	—
	3	58	—
The same in presence of 0.7 % T.C.P.	3	6.6	—

the teeth, a process which certainly diminishes the vegetative growth which forms part of dental "plaque". The conclusion we have arrived at from the experiments on centrifuged saliva, submaxillary duct saliva of the cat and the incubated saliva is that the phosphatase of saliva could originate, partly at least, from the micro-organisms present.

PART II. THE RELATION OF CALCULUS (TARTAR) FORMATION TO MICRO-ORGANISMS AND SALIVARY PHOSPHATASE

The work of Adamson [1929] and that of Smith [1930] lead to the same general conclusions, namely, that the deposition of Ca phosphate and hence also of calculus was brought about by the activity of the phosphatases in either gum tissue or saliva. Tenenbaum & Karshan [1937] found significantly higher Ca and P values in saliva from mouths where calculus formation was greater.

Calculus formation has been correlated with the occurrence of certain micro-organisms, viz. *Leptothrix* (now called *Actinomyces*) by several investigators and some of these workers, i.e. Goodrich & Moseley [1916] and Siegel [1935] relied on histological methods. Naeslund [1926] and Bibby [1935] used both cultural and histological methods, while Bulleid [1925] carried out experiments both *in vitro* and on cats from which he deduced that calculus formation was related to the occurrence of *Leptothrix*. It may be pointed out that Henrici [1930] cast doubt on the value of examination and identification of this type of organism when carried out solely by microscopical examination of stained preparations. This criticism of Henrici gains weight when the results just mentioned are contrasted with those of Lord & Trevett [1936] who obtained four cultures of *Actinomyces* from 90 mouths and Erikson [1937] who was only once successful in growing *Actinomyces* from teeth, although a number of attempts were made and the investigator had grown 50 strains of *Actinomyces* from various sources over a period of years [Erikson, 1935]. Attempts by one of us (P. P.) to isolate *Actinomyces* were at first unsuccessful even from a case of true clinical actinomycosis. Later in the progress of the work an aerobic *Actinomyces*, identical with those placed in group I by Erikson (after Ørskov), was isolated in pure culture from the mouth and this strain and also *A. buccalis* (Winslow), which had been maintained in a series of sub-cultures, were included in the study of the phosphatases of certain mouth micro-organisms. This latter culture was obtained from the Lister Institute (National Collection of Type Cultures) through the courtesy of Dr St John Brooks. It originally came from the American National Type Culture, having in the first place been isolated from the mouth.

Following up the line of thought that the phosphatase of saliva is possibly derived from micro-organisms, we were led to investigate in the early part of this work the phosphatase activities of certain micro-organisms which were considered to be common inhabitants of the mouth. In view of the fact that they are widely distributed in air, soil and food, there is no doubt that they are frequently present in the mouth, although they are not necessarily colonized there. These micro-organisms, originally obtained from a collection of extracted teeth and isolated in pure culture in an unorthodox manner, were first studied. Our attention was accidentally directed to these organisms in the process of separation and collection of enamel protein. Sound (non-carious) human incisor teeth were scraped, the roots cut off and the pulps removed. The crowns were exposed to 10% KOH for periods up to $\frac{1}{2}$ hr. and then brushed vigorously in order to remove the mucin. These crowns were washed well in water and then exposed to a large volume of 2% HCl for about 48 hr. As decalcification proceeded, the enamel protein separated as small flakes and the dentine became soft. The dentine remnants were then picked out, leaving the few mg. of enamel protein in the form of floating fragments which could be collected by centrifuging. Work was then suspended for about 6 weeks, during which time the protein was left covered in 2% acid at room temperature. It was hoped that bacterial growth would be restricted in this way, so that work of a bacteriological nature could be carried out on the protein. The pH of the liquid was about 0.7. At the end of the period mentioned, it was noted that some "fluffy" growth was attached to the protein. The sources of nitrogen available to the growth were very limited. It was thought at one time that the organisms were using enamel protein as the source of nitrogen, but although there were some indications that this may be true, we were not successful in showing histologically that these organisms had penetrated the pieces of protein. Further work on this point is in progress. From the "fluffy" mass three organisms were isolated. They have been kept in a series of sub-cultures for about 6 months and are pure strains. The method of isolation described above has most certainly eliminated many of the mouth organisms. We have, however, limited our investigations to some of those from human mouths which could withstand exposure to 2% HCl for 6 weeks, and whose nitrogen requirements were limited to the very small amount of extraneous nitrogen-containing compounds which had not been eliminated.

Description of the organisms studied

The three organisms were originally described as "green", "red" and "white", but they have subsequently been classified as far as possible. The "green" organism is a typical strain of *Penicillium spinulosum* (Thom.). The "red" organism is a species of *Rhodotorula* resembling *Rhodotorula rubra* [Harrison, 1928]. Some difficulty has been met in identifying the "white" organism. It grows aerobically only, colonies on Czapek Dox, serum, agar and glucose-agar being smooth, shiny, raised, slowly spreading and very mucilaginous. In slide cultures the mycelium is non-septate. Spores have not been seen. It is thought that the "white" organism is one of the monilia. (The description of the two strains of *Actinomyces* studied has already been given.)

The method of isolation of the organisms from extracted teeth certainly eliminated many of the mouth organisms, but doubt remained as to whether the organisms studied came from the mouth. The teeth examined were at first collected in water saturated with chloroform in small jars, when some moulds grew on the corks. Later all corks and jars were sterilized by exposure to hot

air at 160° for 1 hr., a method which proved to be satisfactory. In some collections of teeth made in these sterilized jars, penicillia still grew. Mould spores from the air might have contaminated the teeth in the interval of 2-4 hr. between the removal from the mouth and the placing in the jars. This possibility was not investigated.

It is to be noted that the primary object of the study of these organisms was to learn something of their proteolytic activity. Simultaneously, however, partly at the suggestion of Prof. F. C. Wilkinson, the possibility of the occurrence of phosphatase came under consideration.

Study of phosphatase activity

Three esters were used as substrates, namely Na α - and β -glycerophosphates (Boots) and di-Na-phenyl phosphate (B.D.H.), the latter purified to remove free phosphate. Each ester was tested at the characteristic pH optima of acid and alkaline phosphatases [Folley & Kay, 1936], i.e. at pH 3.8-4.0 and pH 9.9, using Sørensen's glycine and HCl and glycine and NaOH buffers [Clark, 1928], and also at pH 5.5, which was shown to be the optimum of salivary phosphatase by Smith [1930].

The enzyme preparations were made by scraping the growth (4-7 days old) off a Petri dish (10 cm. diameter) agar culture and grinding with sand and sufficient volume of 0.7% MgSO_4 saturated with chloroform to give enough solution for the tests. This was filtered through cotton-wool. One ml. of such a solution contains the optimal concentration of Mg for phosphatase activation. The substrates were 0.1% Na α - and β -glycerophosphates and 0.109% di-Na-phenyl phosphate. The tests and adequate controls were carried out according to the following directions. Twelve tubes were set up for each growth with each substrate, each tube containing 10 ml. of the particular substrate, 5 ml. of one of the buffer solutions and 1 ml. of the growth extract. Half of these tubes served as controls and were left in the refrigerator after the addition of 2 ml. 25% trichloroacetic acid, to stop any enzymic action. The other tubes were incubated at 37° in the case of the phenyl phosphate for $\frac{1}{2}$ hr. and in the case of the glycerophosphates for 18 hr. After this incubation time, the enzyme activity was stopped in the same way as the controls.

Tubes 1 and 2. 10 ml. substrate, 5 ml. buffer pH 4 and 1 ml. extract. Incubated at 37° before the addition of acid.

Tubes 3 and 4. 10 ml. substrate, 5 ml. buffer pH 4 and 1 ml. extract. 2 ml. trichloroacetic acid added and tubes put on ice.

Tubes 5 and 6. 10 ml. substrate, 5 ml. buffer pH 5.5 and 1 ml. extract. Incubated before addition of acid.

Tubes 7 and 8. 10 ml. substrate, 5 ml. buffer 5.5 and 1 ml. extract. Acid added as above and put on ice.

Tubes 9 and 10. 10 ml. substrate, 5 ml. buffer pH 10, 1 ml. extract. Incubated before addition of acid.

Tubes 11 and 12. 10 ml. substrate, 5 ml. buffer pH 10, 1 ml. extract. Acid added as above and put on ice.

All the tubes were then brought to room temperature and filtered. 10 ml. of each filtrate were tested for free P by the method of Fiske & Subbarow [1925].

Successive sub-culturings were tested in this way and although the results (Table IV) with each of the cultures are not quantitatively comparable, the results obtained with any one culture at different pH in any one series are strictly comparable.

Table IV. *The phosphatase activities of the micro-organisms on different substrates at different pH*

Substrate pH	Na α -glycero- phosphate			Na β -glycero- phosphate			Di-Na-phenyl- phosphate		
	4	5.5	10	4	5.5	10	4	5.5	10
Monilia (white growth)	O	S	++	O	S	++	O	+	+++
<i>R. rubra</i> (Harrison) (red growth)	O	+	S	O	++	+	O	S	+++
<i>P. spinulosum</i> (Thom.) (green growth)	+	+++	S to O	S to O	+++	S	O	++++	+
Mixed white and green	O	++	O	O	++	O	O	++	O
<i>A. buccalis</i> (Winslow)	O	O to S	O	—	—	—	—	—	—
<i>Actinomyces</i> , group 1, Erikson	S	+++	S	—	—	—	—	—	—
Saliva	+	+++	S	O	++	O	O	S	O

In our choice of pH values at which to test the enzyme activity, we had in mind the separation of the acid and alkaline phosphatases [Folley & Kay, 1936] and the optimum reaction for the salivary phosphatase towards glycerophosphate found by Smith [1930] to be pH 5.5, by Giri [1936] pH 5.0 and by us pH 6.0.

It can be seen from Table IV that the different growths must have different pH optima. We were most interested in those showing greatest activity at pH 5.5–6 and hence further investigations were made with the green growth. In spite of the fact that *A. buccalis* (Winslow) was tested in six separate young sub-cultures at pH 5.5 and pH 10, on three different substrates, we were only able to show very slight phosphatase activity and this not constantly. This result must be contrasted with the marked activity of the *Actinomyces* (group 1) freshly isolated from the human mouth. It is quite possible that *A. buccalis* (Winslow) when first isolated, had greater activity. Henrici [1930] noted that *A. gypsoides*, although actively proteolytic when first isolated, gradually, and eventually completely, lost this power after some months of subculturing.

The tests on *Actinomyces* (group 1) were carried out at pH 6 only, since it had been shown to have a broad optimum between pH 6 and 7.

It is concluded from these tests that certain mouth organisms, including *Actinomyces*, can exhibit phosphatase activity in the mouth. It is, of course, possible that many other mouth organisms also possess phosphatase activity [Fosdick, 1937]. Incidentally, it may be noted that the caries research group of the North Western University, Chicago [see Hatton, 1938], are of the opinion that caries is possibly the result of a combined action of micro-organisms having phosphatase activity, possibly yeasts, together with acid-producing bacteria. We do not wish to comment on this idea, since we have done no work on this point. If, however, the occurrence of these phosphatases is significant, we consider that they should hydrolyse the ester P of blood and saliva.

The green growth was tested on a neutralized trichloroacetic acid protein-free filtrate of whole blood, rat and human, in a similar manner to that of Byrom & Kay [1928] to show that part of the ester P of blood is hydrolysable by kidney phosphatase. An increase of free P resulted on incubation of the green growth with these blood filtrates. With the human blood, the increase amounted to 15 mg. P per 100 ml. blood, a degree of hydrolysis which suggests that the culture had several types of phosphatase in it.

Wilkinson [1935] put forward histological evidence that calculus deposition could be subgingival where no breakaway of the soft tissues from the teeth had occurred. We remark here that these micro-organisms could use the organic

hydrolysable P of the blood plasma and so increase the concentration of free PO_4 , which, together with the Ca of the plasma or saliva could conceivably lead to the deposition of Ca phosphate.

It was also considered necessary to show that the saliva itself contains a substrate for these phosphatases. For this test, centrifuged saliva was steamed for 5 hr. without concentration. It became cloudy, but was used without further treatment as a substrate for the green mould in the following experiment:

Tubes 1 and 2. 5 ml. saliva (steamed), 5 ml. buffer pH 5.5, 2 ml. glacial acetic acid. Placed on ice.

Tubes 3 and 4. As tubes 1 and 2, but incubated for 18 hr. at 37° before addition of the acid.

Tubes 5 and 6. 5 ml. water to replace the saliva, otherwise the same as tubes 1 and 2.

Tubes 7 and 8. 5 ml. water to replace saliva, otherwise the same as tubes 3 and 4.

This experiment yielded a positive result. Tubes 3 and 4 had greater free PO_4 content than tubes 1 and 2. The actual estimation of this PO_4 set free from the saliva was 0.93 mg. hydrolysable P per 100 ml. saliva. Smith [1930] found between 0.8 and 2.7 mg. organic P per 100 ml. saliva. We consider we have demonstrated the fact that green mould can use saliva as a substrate. Furthermore, extracts of the green *Penicillium* incubated with a 1% solution of Ca glycerophosphate, led to the deposition of a white precipitate, which was readily soluble in dilute acid and was found to contain both Ca and P.

In showing that common mouth organisms, including *Actinomyces*, contain phosphatases which can use serum or saliva as a substrate, a correlation has been brought about between the observations of Adamson [1929] and Smith [1930] on the one hand, and Naeslund [1926] and Bulleid [1925] and Bibby [1935] on the other, concerning the theories of calculus formation.

SUMMARY

1. Human saliva exhibits variable phosphatase activity which is maximum towards glycerophosphate at pH 5.0–6.0.

2. The submaxillary duct secretion of cats had only very slight phosphatase activity as compared with the mouth saliva of the same animals at the same time.

3. The phosphatase activity of human saliva is not entirely due to the epithelial scales.

4. Certain common mouth micro-organisms exhibit marked phosphatase activity.

5. The organisms studied included *Penicillium spinulosum* (Thom), *Rhodotorula rubra* (Harrison), a monilia, *Actinomyces buccalis* (Winslow) and *Actinomyces* (group 1, Erikson).

6. The *Penicillium* was able to use the hydrolysable ester phosphorus of blood and saliva as a substrate.

7. Precipitation of calcium phosphate occurs when calcium glycerophosphate is incubated with the *Penicillium*. This may be analogous to calculus formation.

8. The correlation of phosphatase activity with the presence of micro-organisms, especially *Actinomyces*, is important in relation to the theories of calculus formation which have been advanced previously.

For the identification of the *Penicillium* and the *Actinomyces* (group 1) we are indebted respectively to Mr G. Smith and Miss D. Erikson, both of the London School of Hygiene and Tropical Medicine. We wish to thank the Medical Research Council for grants towards the expenses of this work and for personal grants to two of the workers (P. P. and G. E. G.).

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