Conclusion. 1. Long-term therapy with iodochlorhydroxyquin does not alter the "normal" microbiological flora of nose, throat, and intestinal tract. 2. Bacterial sensitivity of "normal" flora to iodochlorhydroxyquin and antibiotics is not decreased by long-term therapy with iodochlorhydroxyquin.

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Received January 2, 1964. P.S.E.B.M., 1964, v115.

Calcium and Phosphorus in Human Parotid and Submaxillary Saliva.* (29089)

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Until recently the only available data on the calcium and phosphorus of human salivary secretions was derived from analysis of total calcium and inorganic phosphorus in "whole saliva," the mixture of secretions collected by expectoration(1). During the last few years there has been some study of the separately collected parotid secretions(2,3) and a preliminary report in which parotid and submaxillary saliva were compared(4).

Precise information as to the amount, type and properties of the calcium and phosphorus of the separately collected parotid and submaxillary secretions would be of considerable value in investigations of dental caries and dental calculus formation as well as in studies of salivary physiology. In the present study the parotid and submaxillary secretions from the same individuals were analyzed and compared as to content of total and nondialyzable calcium and phosphorus.

Material and methods. Parotid and submaxillary saliva samples were collected within a few minutes of each other from each of 28 adults aged 20-64 years. The parotid saliva samples were obtained with a modified Curby Cup and the submaxillary saliva with individually modified plastic collectors(5). Salivary flow was stimulated by application of 2% citric acid to the tongue 3 times per minute. Immediately after collection: a) a portion of each sample was analyzed for total calcium and inorganic phosphorus or stored for short periods at 4°C and then analyzed; b) another portion of each sample was placed in a Visking casing and dialyzed against 100 volumes of distilled water in the cold (4°C) for 48 hours. The contents of the bag were then analyzed for calcium, phosphorus and protein.

To determine the effect of storage at —20°C aliquots from some of the samples were stored in a deep freeze, thawed, homogenized in a tissue grinder to break up insoluble matter, and a portion dialyzed as above. The dialyzed and nondialyzed portions were analyzed as above. Several samples were collected and stored under mineral oil at —20°C, then thawed, homogenized and dialyzed. The dialyzed portions were analyzed for total calcium.

Initially, several methods for calcium were compared: EDTA-calcein(6), oxalate precipitation(7), and the Kerr modification of the glyoxal-bis (2-Hydroxyanil) reaction(8, 9). When ashed samples were used, all 3 methods gave comparable results. The glyoxal-bis reaction was preferable to the other two with respect to simplicity of technique and reproducibility of the assays. All cal-

* This work was supported in part by grants from Bristol-Myers Co. and U. S. Public Health Service.

TABLE I. Comparison of Parotid and Submaxillary Saliva.*

	Parotid		Submaxillary	
	Mean ± S.D.	Range	Mean ± S.D.	Range
Protein (mg %)	190 ± 68.9	100 -366	117.4 ± 30.4	56 -173
Phosphorus (mg %)				
Inorganic	11.1 ± 2.8	7.0 - 16.5	9.0 ± 2.8	4.9 - 12.7
Nondialyzable†	2.2 ± 1.5	.6 - 5.6	1.6 ± .9	.6 - 3.8
Calcium (mg %)				
Total	3.5 ± 1.4	2.1 - 6.7	8.0 ± 2.3	4.4 - 13.1
Nondialyzable	1.0 ± .4	.3 - 1.9	1.7 ± .9	.5 - 3.5
Dialyzable	2.6 ± 1.0	.7 - 4.8	6.3 ± 2.3	2.0 - 12.0
Calcium				
Nondial/total	.28 ± .13	.06- .61	.23 ± .12	.06- .45
Flow rate				
ml/min/gland pair	1.2 ± .6	.5 - 2.7	1.2 ± .7	.2 - 2.8

* 28 male and female subjects age 20-64.
† 12 " " " " " " "

cium values reported herein are based on the glyoxal-bis method. Samples were ashed according to the procedure of Harnach(10), except that a sand bath was used instead of a metal block. The only change necessary for application of the Kerr modification of the glyoxal-bis reaction to saliva was an increase in the proportion of buffer and solvent to saliva sample. One ml of buffer was added to an ashed equivalent of 0.1 ml of saliva made up to 5 ml. Then 0.5 ml of the glyoxal-bis reagent and 5 ml of solvent mixture were added. After 20 minutes the color was read at 520 mμ in a B and L Spectronic 20 colorimeter.

Total inorganic phosphorus and nondialyzable phosphorus were determined by the ascorbic acid-molybdate method of Chen, Toribara and Warner(11). For measurement of non-dialyzable phosphorus the samples were ashed in the same manner as for calcium.

Protein was estimated by the Folin-Ciocalteau method(12) using tyrosine as a standard and multiplying the nitrogen value by 6.25.

Results. The experimental findings with respect to calcium, phosphorus, protein and flow rate in parotid and submaxillary saliva are given in Table I. The following points may be noted with respect to these data:

1. In 97% of the subjects the submaxillary secretion contained more calcium than the parotid saliva. 2. In 77%, values for non-dialyzable calcium were higher in the submaxillary secretion than in the parotid. 3. In 72% the proportion of non-dialyzable calcium to total calcium was higher in parotid saliva than submaxillary. 4. In 72%, inorganic phosphorus content was higher in parotid than in submaxillary saliva. 5. In 90%, protein values were higher in parotid than in submaxillary saliva.

The various factors measured were examined statistically by "t" test, to determine whether there were any significant relationships or associations within each of the 2 types of saliva and between the 2 types. No statistically significant association (at 5% level of significance) could be found between various pairs of factors in either the parotid or submaxillary saliva.

Storage at —20°C did not alter assays for total calcium or for inorganic phosphorus despite the presence of insoluble material. The measurement of non-dialyzable calcium content, however, was markedly affected. The frozen aliquots of parotid saliva contained up to 75% more non-dialyzable calcium than the unfrozen. With submaxillary saliva, freezing elevated the non-dialyzable calcium about 200%. On freezing and subsequent thawing the pH of the samples usually rose from about pH 7.0 (immediately after collection) to about 7.5. The samples returned to pH 7 after dialysis. Collection and storage at

—20°C under oil, although it stabilized the pH, did not prevent the elevation in non-dialyzable calcium.

Discussion. The data on total calcium and inorganic phosphorus presented herein agree closely with those reported by Shannon and Prigmore(3) for parotid saliva, and by Chauncey and Henriques(4) in their examination of parotid and submaxillary saliva. Our flow rate data support the observation of Shannon(13) that with strong reflex stimulation submaxillary and parotid flow rates are nearly equal. This relationship appears to hold for gustatory stimulation (employed in our study) as well as for masticatory stimulation (used by Shannon).

Since calcium concentration is much lower in parotid than in submaxillary saliva, the proportion of parotid to submaxillary secretion in "whole saliva" is therefore a major determinant of the calcium value of "whole saliva." When the secretion rates of the two major glands are equal, the calculated total calcium for "whole saliva" is 5.8 mg%. This figure is in close agreement with reported values(1). There is, however, considerable variation in the relative flow rates from the two glands and this factor must be considered in any study involving calcium levels of "whole saliva."

In most persons inorganic phosphorus and protein tend to be higher in parotid than in submaxillary saliva; hence the ratio of parotid to submaxillary secretion would affect the inorganic phosphorus and protein content of "whole saliva."

Although ultrafiltration and dialysis are not identical procedures, they should give comparable results if temperature and pH are similar. Values for non-dialyzable calcium reported herein agree closely with one recent study on non-ultrafilterable calcium in whole saliva(15), but are higher than those reported in another study(16). In this latter report, however, 8 of the 9 subjects examined had non-ultrafilterable calcium values which fell within the range of non-dialyzable calcium reported herein.

The nature of the non-dialyzable calcium of saliva has not been established. A portion of it may exist as a precipitate or as a colloidal aggregate and part may be bound to protein. Since our samples were dialyzed against essentially ion free water and at a pH of 6.9-7.0, precipitation and aggregation should have been minimal. If most of the non-dialyzable calcium were protein bound it would indicate that the binding capacity of the proteins in saliva far exceeds that seen in serum. In serum, one gram of protein binds about 0.02 mM calcium(17). In parotid saliva one gram of protein (as calculated from Folin N values) binds 0.12 mM calcium and in submaxillary saliva 0.5 mM calcium. Since serum proteins comprise only a small part of the protein found in saliva(18) most of the protein binding in saliva would appear to be due to the intrinsic salivary proteins. There have been no reports on the binding properties of separated human salivary proteins. Rat salivary gland slices(19) and bovine submaxillary gland extract(20), however, have been found to have a much higher binding capacity than the respective serum proteins.

There is no information as to the nature of the non-dialyzable phosphorus of saliva. Only a small part of this non-dialyzable phosphorus is inorganic, since analysis of unashed samples gave values that were only 10-20% of ashed samples.

The marked increase found in non-dialyzable calcium when submaxillary saliva (and to a lesser extent, parotid saliva) was frozen, helps to explain a number of observations. When the separated salivary secretions are frozen and thawed or freeze-dried and reconstituted at a lower volume (as for electrophoretic analysis), a considerable amount of submaxillary protein and a small amount of parotid protein becomes insoluble. If the insoluble protein is treated with ethylenediamine tetracetic acid a large part goes back into solution. If the secretions are treated with ethylenediamine tetracetic acid prior to freezing or lyophilization, the protein remains completely soluble. The development of an insoluble protein precipitate when saliva is

frozen could be related to increased calcium binding by one or more of the salivary components. The observation that collection and storage under oil prior to dialysis maintained pH, but did not affect the increase in non-dialyzable calcium would rule out pH elevation or CO_2 loss as determinants. An effect of freezing on calcium determination of plasma has recently been reported(21). It would appear that in situations where binding of calcium can occur the effect of freezing of samples must be carefully considered.

Non-dialyzable calcium and phosphorus may be of significance in dental caries and salivary calculus formation around the teeth. Binding of calcium and phosphorus would affect the amount of dialyzable or "ionic" calcium and phosphorus and therefore play a role in both tooth solubility and growth of salivary calculus. In addition, non-dialyzable calcium and phosphorus may, theoretically at least, act as nucleation sites for initiation of dental calculus. This seems to be a promising field for further investigation.

Summary. In a group of 28 adult subjects, acid stimulated parotid saliva was found to contain: a) less total calcium, b) less non-dialyzable calcium, c) more inorganic phosphorus, d) more protein, than stimulated submaxillary saliva from the same individual. In both secretions, about 25% of the total calcium was non-dialyzable and presumably protein-bound. Storage at —20°C before dialysis increased this protein binding about 75% in parotid and 200% in submaxillary saliva. Calcium binding could account, in part at least, for the insoluble matter formed when saliva is frozen and thawed or freeze dried and reconstituted.

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Received January 3, 1964. P.S.E.B.M., 1964, v115.