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THE DETERMINATION OF PHOSPHORUS IN SALIVA AND DENTINE

by

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Numerous methods have been developed for the determination of phosphorus in biological material. Of the methods described, the most sensitive are the colorimetric procedures the basis of which is the formation of a phosphomolybdate complex which produces a blue colour on reduction.

The presence of protein in biological material may cause interference with the spectrophotometric methods. Thus, some of the methods used for the quantitative determination of phosphorus in biological material need to be modified when used for the determination of phosphorus in saliva and mineralized dental tissues [Jenkins, 1966]. Of the mineralized dental tissues, dentine was selected for analysis because of its high protein content. Four of the accepted spectrophotometric methods for the quantitative determination of phosphorus were employed to determine the phosphorus content of saliva and dentine, modified where necessary and the results evaluated. In addition the sensitivity of each of the methods was determined.

The four methods employed were those of Fiske and Subbarow [1925], Chen *et al.* [1956], a modification* introduced by Chen *et al.* in their original [1956] method, and that of Eastoe [1965]. In these methods various reducing agents and different ways of developing the colour are used and the spectrophotometric analysis is done at a wavelength most suitable for the method (Table I).

TABLE I

The reducing agent, colour development and wavelength of the four methods.

Method	Reducing Agent	Colour Development	Wave Length mμ
Fiske	Aminonaphtholsulfonic acid and sulfite	5 minutes at room temperature	660
Chen	Ascorbic acid	2 hours at 37°C	820
"Modified Chen" .	Ascorbic acid	2 hours at 37°C	820
Eastoe	Aminonaphtholsulfonic acid and sulfite	10 minutes at boiling point	815

METHODS

A Unicam SP 500 photoelectric spectrophotometer was used in this investigation.

* The modification introduced by Chen *et al.* in their original method consists of the use of reduced quantities of TCA filtrate and reagent in order to measure smaller quantities of phosphorus. In the present paper this variation will be designated the "Modified Chen" method.

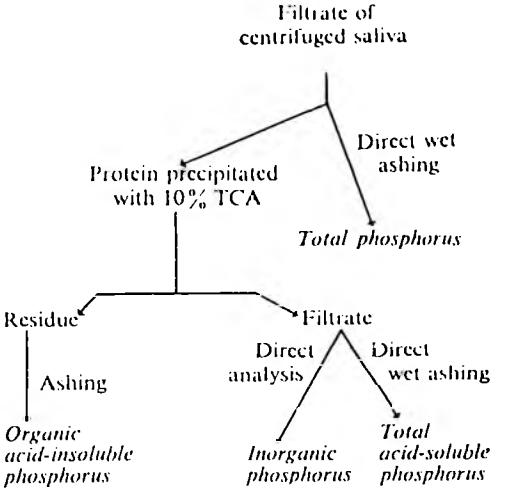
Sensitivity

A comparison was made of the sensitivity of the four methods. This was done by recording the absorbance of standard phosphate solutions containing from 0.10-10μg of phosphorus. Standard curves were constructed for each method by plotting the phosphorus content against the absorbance (Fig. 1).

Saliva analysis

Because we were not concerned with the factors that control the composition of saliva [Jenkins, 1966] stimulated saliva was collected by chewing rubber tubing and pooled for this comparative study. The saliva was centrifuged at 3,000 revolutions per minute for ten minutes to remove the epithelial cells and suspended matter. Although it has been reported that centrifugation lowers the phosphorus content of saliva [Wainwright, 1938] we found that the suspended matter interfered with the subsequent analytical techniques if not removed. The centrifuged saliva was filtered through Whatman No. 1 filter paper and the filtrate utilized for the phosphorus determinations.

According to Becks [1938] the various phosphorus fractions of saliva can be determined as follows:



The organic acid-soluble phosphorus is determined indirectly by the following calculation:

Organic acid-soluble P = Total acid-soluble P — Inorganic P

The total phosphorus and the inorganic phosphorus of saliva were determined in this comparative study. In both instances the protein of the saliva was removed prior to analysis by means of an ashing procedure in the case of the total phosphorus and by precipitation with trichloracetic acid in the case of the inorganic phosphorus.

Total phosphorus of saliva

The protein of the centrifuged salivary filtrate was removed by wet-ashing. One ml. of the filtrate was heated in a crucible on a furnace with 8 drops of concentrated

sulphuric acid until white sulphur trioxide fumes appeared, 0.5 ml. of 30% hydrogen peroxide (H_2O_2) was then added and heated again until all the hydrogen peroxide was driven off. If the remaining fluid was still discoloured, a few drops of 30% H_2O_2 were added and the procedure repeated until a clear liquid was obtained. The liquid was transferred to a 25 ml. volumetric flask, washed repeatedly and made up to the mark. Different volumes of this solution were taken for subsequent analysis.

The following volumes gave optimum recordings for each method (based on a salivary phosphate of ± 10 mg.%).

Method	Volume diluted solution (ml.)	Equivalent volume saliva (ml.)
Fiske	5.0	0.2
Chen	0.5	0.02
"Modified Chen" .	0.1	0.004
Eastoe	0.5	0.02

Inorganic phosphorus of saliva

10% Trichloracetic acid (TCA) solution was added to the filtrate of the centrifuged saliva to precipitate the protein. 9.5 ml. of TCA solution was added to each 0.5 ml. of saliva. The mixture was shaken well, allowed to stand for approximately ten minutes and filtered through a Whatman No. 2 filter paper. The analysis was carried out directly on the filtrate.

Because of the difference in sensitivity of the four methods, different volumes of TCA filtrate were used for each method. For saliva containing ± 10 mg.% of phosphorus, the following volumes of TCA filtrate gave optimum readings on the spectrophotometer.

Method	Volume TCA filtrate (ml.)	Equivalent volume saliva (ml.)
Fiske	5.0	0.25
Chen	0.5	0.025
"Modified Chen" .	0.1	0.005
Eastoe	0.5	0.025

With the Fiske method a further precipitate occurred when the molybate reagent was added to the TCA filtrate. This did not take place when the saliva was ashed. This is in accordance with Wainwright's [1938] observation. He thought that this precipitate was due to incomplete precipitation of the salivary protein with 10% TCA. Whenever this precipitate was formed, it was removed by further centrifugation at 3,000 r.p.m. for five minutes. The absorbance of the supernatant solution was recorded.

Dentine analysis

Sound freshly extracted human teeth were used. The teeth were dried in an oven, the cementum removed by scraping, the teeth split and the dentine separated. To ensure that no enamel was removed with the dentine, only the dentine not in close contact with the enamel was removed. The dentine was ground to a fine powder in an agate mortar and dried to constant weight at 105°C. The dentine was then dissolved either in 1 N hydrochloric acid or 0.8 M TCA, or ashed. The dentine

dissolved in 0.8 M TCA was analysed by the Eastoe method only since this is the concentration of TCA used in his method.

Dentine dissolved in 1 N HCl

About 10 mg. of dentine was accurately weighed and transferred to a 250 ml. volumetric flask. The transfer was completed with 10 ml. 1 N HCl. The flask was kept at 37°C overnight, allowed to cool and made up to the mark. The undissolved protein was removed by filtration. It was found that the following volumes of the diluted solution gave optimum recordings with the spectrophotometer.

Method	Volume diluted solution (ml.)	Equivalent weight of dentine (mg.)
Fiske	5.0	0.2
Chen	0.5	0.02
"Modified Chen" .	0.2	0.008
Eastoe	0.5	0.02

Ashed dentine

A dry-ashing technique was used. Approximately 10 mg. dentine was weighed accurately in a crucible, 5 ml. 50% hydrochloric acid and 0.5 ml. 30% hydrogen peroxide (H_2O_2) added and gradually heated on a furnace until dry. The crucible was allowed to cool and the residue dissolved in 5 ml. 1 N HCl containing 0.3% H_2O_2, facilitated by gentle heating. The solution was transferred to a 250 ml. volumetric flask with repeated washings and made up to the mark. The same volumes of this diluted solution were used with the four methods as for the dentine dissolved in 1 N HCl.

Dentine dissolved in 0.8 M TCA

Approximately 10 mg. of dentine was weighed accurately in a 10 ml. centrifuge tube and 5 ml. of 0.8 M TCA added. The tube was tightly stoppered and left in an oven at 37°C overnight. After cooling it was centrifuged to remove undissolved protein and 20 µl (0.02 ml. of the supernatant fluid (equivalent to 0.04 mg. dentine) was used for analysis by the Eastoe method.

RESULTS

Sensitivity

From the results obtained a standard curve for each method was constructed (Fig. 1). Each point on the graph represents the average of four recordings.

By comparing the absorbance obtained with each of the four methods on solutions containing 1 µg. of phosphorus in the test solution the sensitivity of the respective methods can be compared.

Method	Absorbance	
Fiske	0.01	For 1 µg. of P in test solution
Chen	0.10	
"Modified Chen" .	0.26	
Eastoe	0.08	

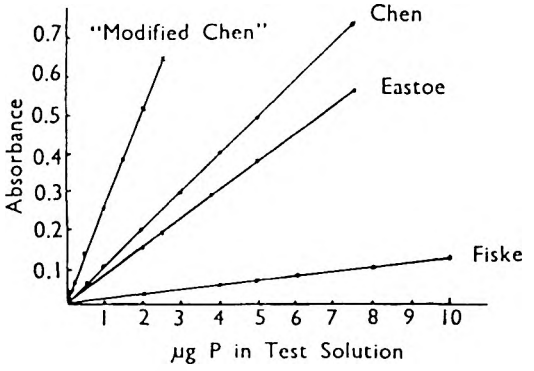


Fig. 1 Standard curves.

From these results it is clear that the Fiske method is the least sensitive and the "modified Chen" method the most sensitive.

Saliva analysis

Total phosphorus-ashed saliva.—The results are given in Table IIA.
Inorganic phosphorus-protein precipitated saliva. The results are given in Table IIB.

TABLE II
Phosphorus in saliva, total and inorganic

Method	Mean*	Standard error	Approximate 95% confidence interval for true mean
A. *Total phosphorus of saliva*			
Fiske	15.16	0.02	15.12, 15.20
Chen	15.54	0.02	15.50, 15.58
"Modified Chen"	15.38	0.02	15.34, 15.42
Eastoe	15.12	0.01	15.10, 15.14
B. *Inorganic phosphorus of saliva*			
Fiske	14.76	0.03	14.70, 14.82
Chen	15.42	0.01	15.40, 15.44
"Modified Chen"	15.38	0.02	15.34, 15.42
Eastoe	15.29	0.02	15.25, 15.33

All results expressed in mg%.
* The mean was obtained from the result of 50 analyses with each method.

Dentine analysis

The phosphorus is expressed as a percentage of the weight of dentine dried to a constant weight at 105°C.

Dentine dissolved in 1 *N HCl.*—The results are given in Table IIIA.
Ashed dentine.—The results are given in Table IIIB.
Dentine dissolved in 0.8 *M TCA.*—The results are given in Table IIIC.

TABLE III

Percentage phosphorus in dentine, dissolved in HCl or TCA, or in ashed dentine.

Method	Mean*	Standard error	Approximate 95% confidence interval for true mean
A. *Percentage phosphorus in dentine dissolved in N HCl*			
Fiske	13.41	0.02	13.37, 13.45
Chen	13.46	0.02	13.42, 13.50
"Modified Chen"	13.40	0.02	13.36, 13.44
Eastoe	13.16	0.03	13.10, 13.22
B. *Percentage phosphorus in ashed dentine*			
Fiske	13.16	0.02	13.12, 13.20
Chen	12.97	0.02	12.93, 13.01
"Modified Chen"	13.43	0.03	13.37, 13.49
Eastoe	13.09	0.02	13.05, 13.13
C. *Percentage phosphorus in dentine dissolved in 0.8 M TCA*			
Fiske	—	—	—
Chen	—	—	—
"Modified Chen"	—	—	—
Eastoe	12.95	0.02	12.91, 12.99

All results expressed as percentage of dry weight of dentine heated to 105° C.
* The mean was obtained from the results of 50 analyses with each method.

DISCUSSION

Sensitivity

From the results obtained it is clear that the Chen, the "modified Chen" and the Eastoe methods are the only ones that can be used if small amounts of saliva or dentine are available for analysis. Of these methods the "modified Chen" method is by far the most sensitive and a single analysis can readily be made on 0.005 ml. of saliva or 0.008 mg. of dentine.

Saliva analysis

In the direct analysis of the protein precipitated saliva (inorganic phosphorus), the mean obtained with the Fiske method is lower than that obtained with the other methods. There is statistical evidence that the true mean of the first population (Fiske) is lower than the average of the true means of the remaining three populations. This is in accordance with the findings of Wainwright [1938] and others that the phosphorus content of saliva obtained with the Fiske method is on the low side. This can partly be explained by the fact that a further precipitate was formed when the molybdate reagent was added to the filtrate of the protein precipitated saliva with the Fiske method. This precipitate had to be removed by centrifugation prior to spectrophotometric analysis.

For the Fiske and Chen methods higher phosphorus values were obtained for the ashed saliva than the protein precipitated saliva. This is as expected as the inorganic phosphorus of saliva is approximately 95% of the total phosphorus content [Becks, 1938]. When the "modified Chen" method was used the same results were obtained for the phosphorus content of the protein precipitated saliva and the ashed saliva. One would expect the total phosphorus content of the ashed saliva to have been slightly higher.

With the Eastoe method a slightly lower value was obtained for the total phosphorus as compared with the inorganic phosphorus of saliva. Eastoe's method, however, is not specific for inorganic phosphorus in the presence of organic phosphorus. The organic phosphate present in the saliva will be hydrolysed by the 0.34 N sulphuric acid of the reagent at the temperature at which the colour is developed (100°C).

Dentine analysis

The results obtained on the ashed dentine are generally lower than those obtained on the dentine dissolved in 1 N HCl. This may be due to the loss of some material during the ashing procedure.

Although the results obtained with Eastoe's method are slightly lower than those obtained with the other methods a good correlation is evident between the results obtained with this method.

All three methods of bringing the dentine in solution seem to be acceptable.

Stability of the reagents

The stability of the colour that is developed and the stability of the reducing agents used in the different methods vary as shown in Table IV.

TABLE IV

The stability of the colour developed and of the reducing agents with the four methods.

Method	Colour stability	Stability of reducing agent
Fiske	Increases steadily for several several weeks	Four weeks
Chen	Stable for two hours	Fresh daily
"Modified Chen"	Stable for two hours	Fresh daily
Eastoe	Stable for one week	Fresh weekly

The colour stability is the greatest with the Eastoe method and the reducing agent the most stable in the Fiske method.

SUMMARY

An accurate method for the determination of phosphorus in saliva and mineralized tissue is often required.

A variety of methods have been described for the determination of phosphorus in biological samples. The most sensitive of these are based on the reduction of a phosphomolybdate complex which gives rise to a colour reaction. Four of these methods, in which different reducing agents and different methods of developing the colour are employed, were used to determine the phosphorus content of saliva and dentine. In addition the same procedure was adopted for the analysis of wet-ashed samples.

850 phosphate determinations were carried out on saliva and dentine, using the methods of Fiske and Subbarow, Chen *et al.*, "modified Chen *et al.*", and Eastoe. Statistical evaluation of the results showed that all four methods are accurate.

A comparison made of the sensitivity of the four methods indicates that the "modified Chen" method is the most sensitive.

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