

SALIVARY CITRATE AND DENTAL EROSION

PROCEDURE FOR DETERMINING CITRIC ACID IN SALIVA—DENTAL EROSION AND CITRIC ACID IN SALIVA

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PART I

CITRIC acid has been studied in a number of tissues and body fluids, but only to a limited extent in saliva. Pucher, Sherman, and Vickery¹ found 0.04 to 1.30 mg. per cent in seven saliva specimens, but Kuyper and Mattill² and Leake³ were unable to detect citric acid in saliva using methods, however, which were not sensitive to less than 2.0 mg./100 ml. Zipkin⁴ reported saliva (some 180 analyses on 15 adult men) to contain 0.50 to 2.00 mg. per cent citric acid, expressed as the monohydrate. Recently, Shulman and Robinson⁵ used the gravimetric method of Duysher and Holm⁶ and found 0.0 to 1.55 mg. per cent citric acid in the saliva of 25 normal individuals. In general, the gravimetric procedure is not as sensitive or as specific as the colorimetric technic.⁷

Following a survey of methods for citric acid in biological fluids, the colorimetric procedure of Perlman, Lardy, and Johnson,⁸ with several modifications, was found satisfactory for the analysis of 10 ml. specimens of saliva. Our experience with this method is somewhat contrary to Shulman and Robinson,⁹ however, who reported negative results for a number of saliva specimens. Recently, Natelson, Lugovoy, and Pincus¹⁰ used essentially the technic employed by us. Speck, Moulder, and Evans¹¹ did not modify the original Perlman, Lardy, Johnson procedure⁸ and discarded it as unsatisfactory.

A critical analysis of this citrate method⁸ and its application to saliva analysis will be reported prior to presenting clinical data on the relation of citric acid in saliva to dental erosion.

Current interest in salivary citrate relates to the observation that rats' dental tissues are attacked in vivo by nearly neutral citrate drinking fluids¹² thus suggesting a role of salivary citrate in human dental erosion.

Procedure for Citric Acid in Saliva.—About 15 ml. of paraffin-stimulated saliva were collected in a 35 ml. glass-stoppered brown bottle using a short-stemmed funnel. Each bottle contained 0.5 ml. of concentrated H₂SO₄. After thorough shaking, a 10.0 ml. aliquot was transferred to a test tube containing 2.0 ml. of 18N H₂SO₄ and placed in a boiling water bath for 20 minutes. The

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sample was filtered hot and washed with 5.0 ml. of hot water. Five-tenths milliliter of saturated bromine solution was then added and the sample allowed to stand for 2 hours in the refrigerator for complete precipitation of bromination products. The mixture was filtered and washed with 5.0 ml. of hot water, the filtrate and washings being received in a 50 ml. graduated centrifuge tube. Consecutively were added 0.3 ml. 18N H_2SO_4 , 0.2 ml. 1M KBr solution, and 0.75 ml. saturated KMnO_4 solution (70 Gm./l). The mixture was allowed to stand for 15 minutes. Three per cent H_2O_2 was added just to disappearance of MnO_2 , and 0.1N KMnO_4 was then added to destroy the excess H_2O_2 .

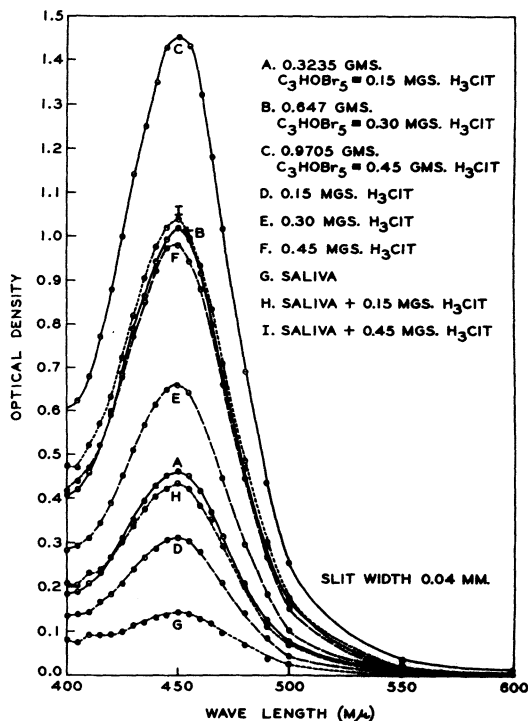


Fig. 1.—Spectral transmittance curves for various concentrations of pentabromacetone, citric acid, and citric acid in saliva using a Beckman model DU quartz spectrophotometer.

The clear and colorless solution was made up to 30 ml. with distilled water, 13 ml. of Skellysolve B were added, and the sample shaken vigorously for 15 seconds. The sample was centrifuged for a few minutes at about 1,000 r.p.m. to break the emulsion, and 10 ml. of the supernatant liquid were separated by aspiration and transferred to a colorimeter tube. Three milliliters of a 50 per cent dioxane-water mixture were added, followed by 3.0 ml. of 4 per cent sodium sulfide solution freshly prepared and filtered. The sample was thoroughly shaken for 15 seconds and centrifuged for a few minutes at 1,000 r.p.m.

The yellow color was measured in a Coleman 11A spectrophotometer at 450 $\text{m}\mu$ using a blank consisting of 10 ml. of water, carried through the same procedure as the samples. Transmittance readings were converted to mg. per cent by reference to a standard calibration curve.

Preparation of Calibration Curve.—Preparation of spectral transmittance curves for various concentrations of pentabromacetone, citric acid, and of citric acid in saliva shows the maximum density to be at 450 m μ in every case (Fig. 1).

A white crystalline pentabromacetone prepared according to the method of Dickens¹³ and melting at 73.5 to 74.5° C. was dissolved in Skellysolve B and the resulting solution used to study the spectral characteristics of the yellow color developed in the procedure described (Curves A, B, and C, Fig. 1). Curves D, E, and F (Fig. 1) were obtained from standard solutions of citric acid containing 0.15, 0.30, and 0.45 mg. citric acid respectively. To obtain the calibration curve, Fig. 2, a blank and 5 citric acid standards were prepared in

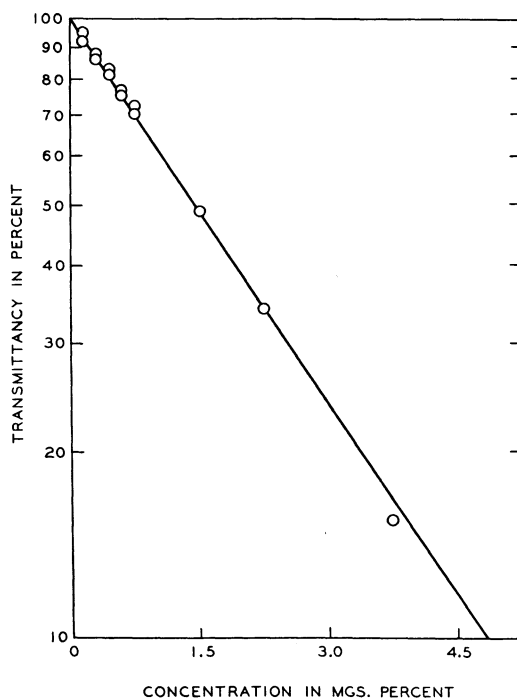


Fig. 2.—Calibration curve for citric acid in saliva using a Coleman model 11A spectrophotometer.

duplicate to contain 0.015, 0.030, 0.045, 0.060, and 0.075 mg. citric acid. A third series of standards was prepared containing 0.15, 0.225, and 0.375 mg. citric acid. Standards prepared at subsequent intervals conformed closely to the calibration curve, thus substantiating its validity.*

Evaluation of Solvents Used in Developing the Final Yellow Color.—In the production of the final yellow color, various solvents, in addition to pyridine,¹ dioxane,¹⁴ ethylene glycol,¹⁵ and commercial glycerol,¹⁶ already reported, were used in an attempt to intensify the color and increase its stability.

*Our studies indicate that the calibration curve for citric acid is not a straight line throughout the entire range investigated, but, for the purposes of the study, the curve as shown is entirely satisfactory.

Solvents closely related to both pyridine and dioxane were tried, that is, piperidine (50-50), morpholine (50-50), piperazine hydrate (saturated solution), and trioxane (saturated solution). Only dioxane, pyridine, and trioxane showed any promise, and of these, dioxane-water solutions gave the greatest optical density. Both technical and redistilled grades of Skellysolve B were tried and both gave comparable results, thus obviating the necessity for purification.

When glycerol and ethylene glycol in 50-50 water mixtures were substituted for the dioxane-water mixture, they gave a color essentially stable for 60 minutes, but the optical density obtained was not as great as with dioxane-water mixtures. Various grades of dioxane gave such variable results that purification¹⁷ was found necessary.

Relation of Sodium Sulfide Concentration to Optical Density of Yellow Color Produced.—It was found necessary to prepare the sodium sulfide solution fresh daily. A progressive decrease in the optical density of the final yellow color without any appreciable change in the density of the sodium sulfide solution was found, as the latter aged. Hence, the optical density of the sodium sulfide solution apparently cannot be used as a criterion of constancy, as reported in the literature.¹⁰

Attempts to prepare a satisfactory combined reagent of four per cent sodium sulfide in 50-50 dioxane-water solution were unsuccessful in that a progressive decrease in the optical density of the final yellow color was again observed as the combined reagent aged.

It was confirmed that the maximum optical density of the final yellow color is produced with a 4 per cent sodium sulfide solution. The optical density increased up to a concentration of 4 per cent sodium sulfide and then decreased.

Collection, Preservation, and Deproteinization of Saliva.—Preliminary studies showed that the citric acid concentration in saliva decreased rapidly on standing. Various bactericidal agents were studied in an attempt to stop bacterial action in the saliva immediately as collected. Among these were the following: phenylmercuric acetate, hydroxide, bromide, and salicylate; thymol; commercial formalin; and quaternary ammonium salts. None of these agents, however, was without interference in the subsequent citric acid determination; 0.5 ml. concentrated H_2SO_4 in about 15 ml. of saliva was finally adopted as the stabilizing agent, after a number of studies had demonstrated the stability of citric acid in saliva in the presence of H_2SO_4 . As shown in Fig. 3, there is a striking decrease in salivary citric acid, particularly during the first hour, but when sulfuric acid is present the citric acid remains essentially constant. Curves A and B represent two different saliva specimens stabilized with H_2SO_4 , and Curve C represents a specimen of saliva without H_2SO_4 .

Saliva appears somewhat unique insofar as it is not easy to deproteinize with reagents commonly used for this purpose. Many of the usual deproteinizing reagents were studied including acetic acid, trichloroacetic acid, tungstic acid, metaphosphoric acid, phosphotungstic acid, phosphomolybdic

acid, sulfosalicylic acid, perchloric acid, copper sulfate-sodium carbonate-sodium tungstate mixture, 95 per cent alcohol, heat, and sulfuric acid. Only the sulfuric acid proved entirely satisfactory. It was found that 2.0 ml. of 18N H_2SO_4 added to 10.0 ml. of saliva and placed in a boiling water bath 20 minutes gave a flocculent precipitate which was readily removed by filtration. During this process the filtrate usually varied in color from pink to brown, but this did not interfere in subsequent procedures.

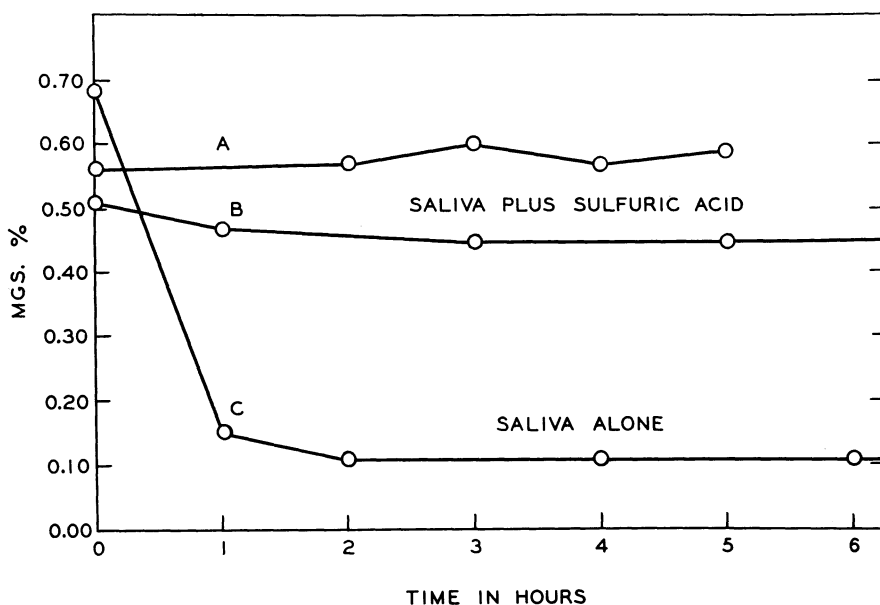


Fig. 3.—Stability of citric acid in saliva with and without sulfuric acid. Samples A, B, and C were collected from the same individual at different times.

Recovery of Citric Acid Added to Saliva.—Results of a number of recovery tests are shown in the following table:

TABLE I
RECOVERY OF CITRIC ACID* ADDED TO SALIVA

TRIAL NUMBER	CITRIC ACID PRESENT IN 10 ML. OF SALIVA (MG.)	CITRIC ACID ADDED TO SALIVA (MG.)	CITRIC ACID FOUND (MG.)	CITRIC ACID RECOVERED (MG.)	RECOVERY (PER CENT)
1	Blank	--	--	--	--
2	0.066	0.000	0.066	--	--
3	0.066	0.075	0.143	0.077	102.6
4	0.066	0.150	0.218	0.152	101.3
5	0.066	0.225	0.304	0.238	105.7
6	0.066	0.300	0.360	0.294	98.0
7	Blank	--	--	--	--
8	0.071	0.000	0.071	--	--
9	0.071	0.075	0.141	0.070	93.5
10	0.071	0.150	0.210	0.139	92.8
11	0.071	0.225	0.284	0.213	94.7
12	0.071	0.300	0.360	0.289	96.4

*Expressed as the monohydrate.

Notes on the Procedure.—

A. Five-tenths milliliter of saturated bromine solution was sufficient for bromination. Although salivas varied in the amount of bromine actually consumed, this was not related to the citric acid present.

B. A light-yellow color may appear after addition of the dilute permanganate but this was destroyed by adding a fraction of a drop of the H_2O_2 .

C. Shaking of samples during extraction of pentabromacetone from water solution with Skellysolve B and later extraction from Skellysolve B with sodium sulfide and dioxane was standardized to 50 manual shakes, or about 15 seconds.

D. Purification and redistillation of commercial Skellysolve B was unnecessary.

E. Although absorption spectra were determined with a Beckman Model DU quartz spectrophotometer, the Coleman Model 11A instrument was used in routine analyses to obviate the necessity of separating the lower colored layer. Standard citric acid solutions showed a maximum density of 450 $m\mu$ with both instruments.

F. By developing the final color with 3 ml. of sodium sulfide solution and 3 ml. of dioxane-water solution instead of 5 ml. of each as originally used by Perlman, Lardy, and Johnson, an increase in sensitivity of 67 per cent was obtained.

Part II of this study is an investigation of the relation between human dental erosion and the citric acid content of saliva.

PART II

Dental Erosion.—Dental erosion has been known for almost two centuries,¹⁸ but its etiology is still obscure. The majority of the theories which have been proposed to explain erosion have been reviewed by Thoma¹⁹ and by Miller and Newman.²⁰ Erosion has also been studied by Black,¹⁸ Darby,²¹ Kirk,²² W. D. Miller,^{23, 24} Bunting,²⁵ Badanes,²⁶ Bödecker,²⁷ and Robinson.²⁸

A dissolution of tooth substance in vivo, which resembles erosion in appearance, has been attributed to various acids in both human²⁹⁻³³ and laboratory animal³⁴⁻³⁷ observations.

As already noted, the destructive action of citrate drinking fluids on rats' molar tooth surfaces in vivo¹² suggested citrate in oral fluids localized on tooth surfaces as a cause of human erosion.

For purposes of this clinical study, erosion was defined as a loss of tooth substance at the gingival third of the buccal or labial surface, leaving a hard glossy surface. In nearly all cases, erosion was seen as a flattened or disk-shaped area on the buccal surfaces causing the normal convexity of the tooth to be lost. The degree of erosion was arbitrarily evaluated as none, mild, moderate, or severe, and designated 0, 1, 2, and 3, respectively. The "Erosion Index" is the total score divided by the number of teeth. This method of scoring is similar to that used by Restarski, Gortner, and McCay,³⁸ for scoring the molar teeth of rats.

This study relates to 38 individuals with an established diagnosis of erosion, and 83 individuals selected at random at this station. Among the latter group 22 had some erosion, making a total of 60 individuals with erosion and 61 with no erosion.*

Although the groups are small, it is interesting to note that among the 83 random-selected individuals, 22 (27 per cent) had some degree of erosion. Among 42 individuals, aged 27 to 39 years, 9 (21 per cent) had erosion. There

*We are indebted to Robert C. Likins, Senior Assistant Dental Surgeon (R), USPHS and to H. Berton McCauley, Senior Assistant Dental Surgeon (R), who performed the dental examinations.

were 13 erosion cases (32 per cent) among the remaining 41 individuals, aged 40 years and over.

The erosion data when studied according to prevalence in individual mouth quadrants indicate that there is probably a greater prevalence of erosion in the upper than in the lower quadrants. The mean difference between upper and lower teeth was 2.017 times its standard error, giving a probability of less than 0.05.

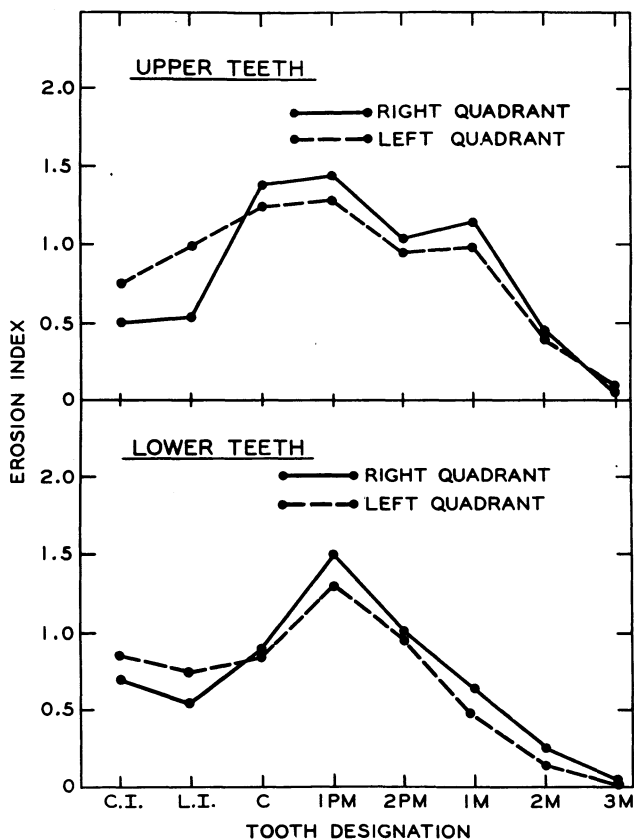


Fig. 4.—Evaluation of degree of erosion.

Analysis of the data on severity of erosion (erosion index) shows that the upper teeth are also more severely affected than the lower teeth. The mean difference between the upper and lower teeth was 2.5 times its standard error, giving a probability of less than 0.02. These data are presented graphically in Fig. 4.

The average erosion index figures not shown in Fig. 4 are as follows: upper left 0.92, upper right 0.89, and all uppers 0.90; lower left 0.73, lower right 0.78, and all lowers 0.75. The average erosion index for all teeth is 0.82. There was no significant difference in the degree of erosion in right and left quadrants of either upper or lower jaws. The first premolars were most severely affected, and the upper first molars were approximately twice as badly eroded as the lower first molars. This may be suggestive of a specific

effect of the parotid saliva, since the parotid ducts open in the area of the upper first molars.

Fig. 5 shows the relation of age to the erosion index, together with the correlation coefficient, $+0.33$, and the corresponding regression line, $Y = 0.018X$. The probability of occurrence by chance of this correlation coefficient is less than 0.01 indicating that the degree of erosion increases with age. It is interesting to note in this case that the regression of age on erosion index has a zero intercept.

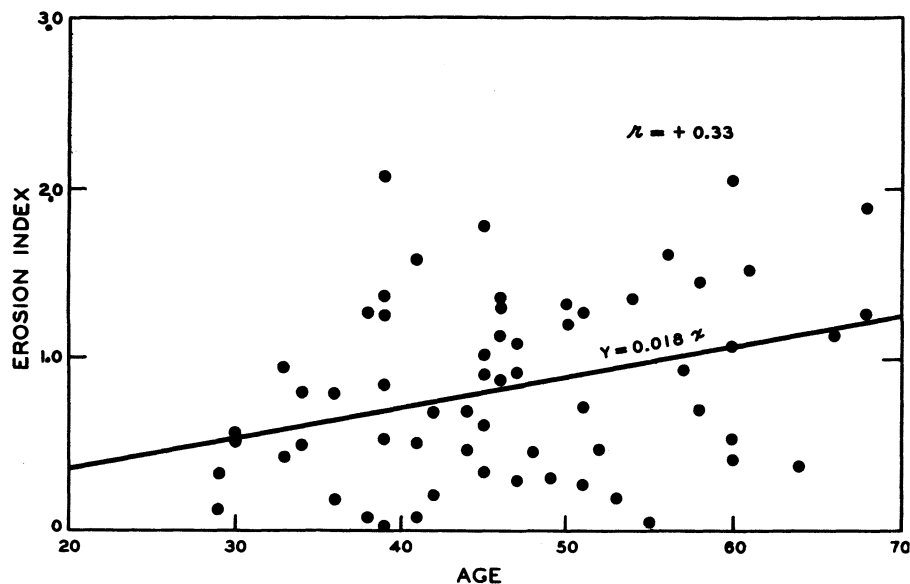


Fig. 5.—Relation of age to erosion index.

Results of Citric Acid Analyses of Saliva.—Fifteen milliliter specimens of paraffin-stimulated saliva were collected at 11 A.M. and 3 P.M. in small brown glass-stoppered bottles containing 0.5 ml. of concentrated sulfuric acid.* Citric acid was determined in both specimens according to the procedure already outlined, and the two results were averaged to obtain a representative citric-acid figure.

A summary of these analytical data is presented in Table II.

TABLE II
RELATION OF CITRIC ACID CONTENT OF SALIVA TO AGE

NORMAL CASES					EROSION CASES			
AGE GROUP IN YEARS	NO. CASES	MEAN AGE	RANGE IN MG. % CITRIC ACID	MEAN* CITRIC ACID	NO. CASES	MEAN AGE	RANGE IN MG. % CITRIC ACID	MEAN* CITRIC ACID
27 to 39	33	32.8	0.29–1.82	0.86 ± 0.06	17	35.5	0.48–1.67	1.04 ± 0.09
40 to 49	15	43.8	0.45–2.09	0.99 ± 0.11	22	44.9	0.20–1.97	1.03 ± 0.09
> 50	13	55.2	0.42–1.85	1.11 ± 0.12	21	57.3	0.34–3.15	1.33 ± 0.09
Totals	61	40.2	0.29–2.09	0.95 ± 0.05	60	46.6	0.20–3.15	1.14 ± 0.06

*Mean \pm standard error of mean.

*It has been shown previously⁴ that specimens collected at 9 A.M., 11 A.M., 1 P.M., and 3 P.M., are essentially the same, the only variation occurring in the 1 P.M., specimen, which was slightly higher than the others.

Since it has been shown that the degree of erosion increases with age, the mean for the erosion cases was corrected for age by analysis of covariance.³⁹ The difference in the means of erosion and normal cases was then found to be not statistically significant.

A wide range in values for citric acid in saliva is evident in the data appearing in Table II as well as in Figs. 6 and 7. Based on comparatively small groups, the data are inconclusive as regards the relation between citric acid and erosion. However, some indication of a positive trend in the data is apparent by the magnitude of the correlation coefficient, as shown in Fig. 7.

A scatter diagram and line of regression relating age to salivary citrate is shown in Fig. 6.

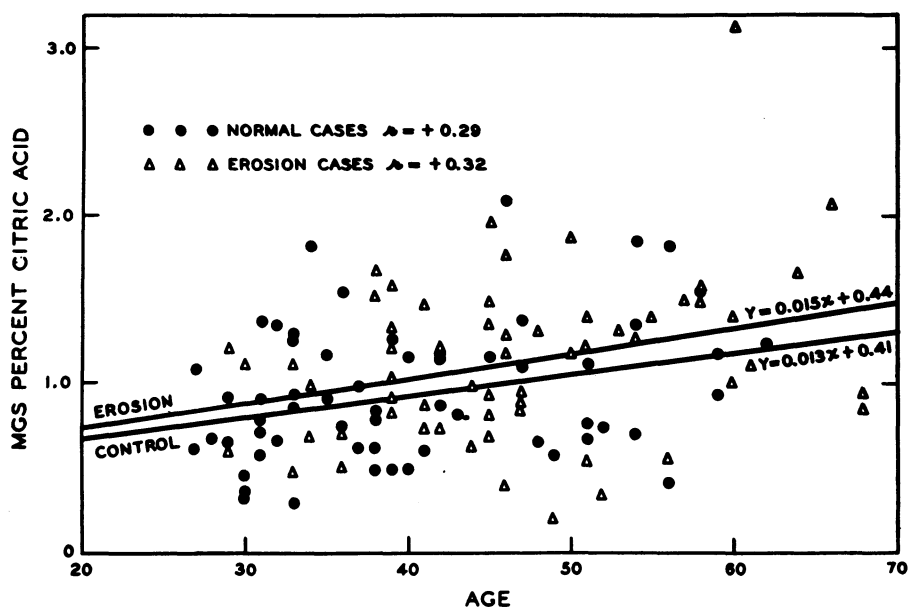


Fig. 6.—Relation between citric acid content of saliva and age.

The coefficient ($r = +0.29$) for normal individuals, indicates a result happening by chance less than 5 times in 100 trials. The coefficient $r = +0.32$ for erosion cases could have occurred by chance less than twice in 100 trials. A prediction that there is an increased content of citric acid in saliva of older age groups is warranted by this comparison. (In one erosion case a value of 3.15 mg. per cent was obtained. This analysis was repeated five months later and essentially the same result was obtained.)

The correlation coefficient ($r = +0.33$) associated with the comparison of citric acid and erosion index, as shown in Fig. 7, has a probability of occurrence by chance of less than once in 100 trials.

Inspection of the results as presented in Figs. 6 and 7, therefore, brought out significant correlations between citric acid content and two independent

variables, that is, age and erosion index. Under this circumstance, a treatment of the data by means of multiple correlation was desirable and this is shown in Figs. 8 and 9.

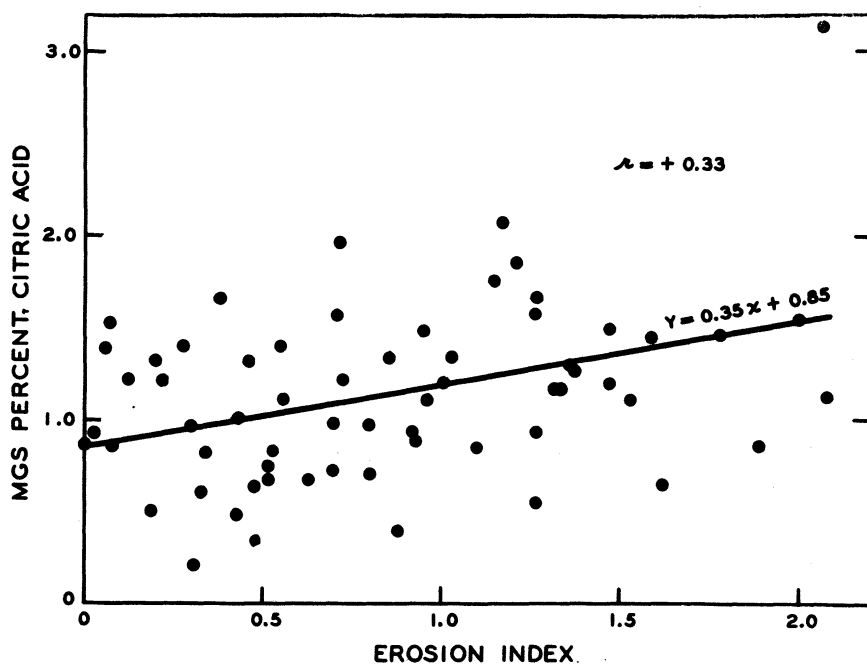


Fig. 7.—Relation between citric acid content of saliva and erosion index.

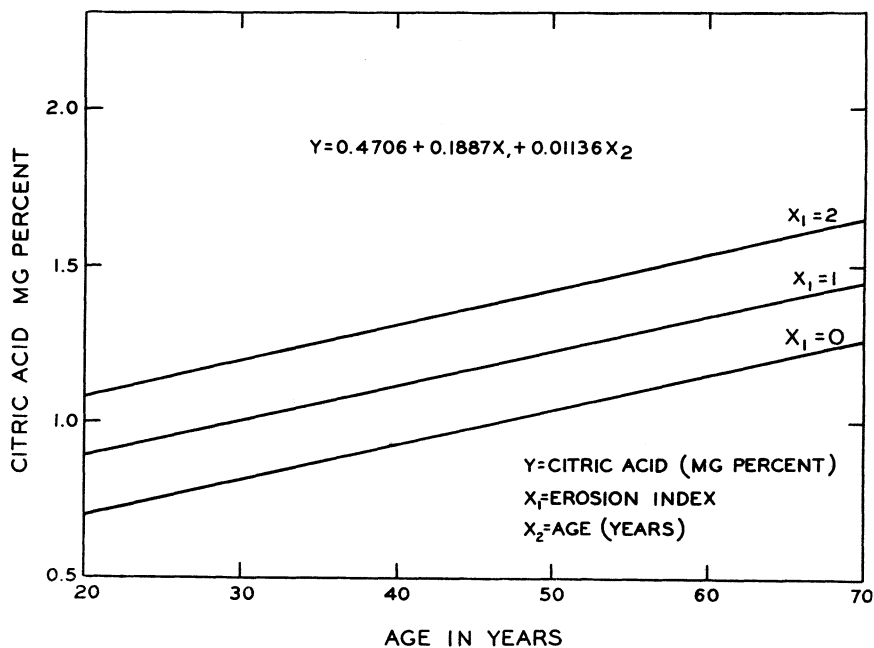


Fig. 8.—Relation of citric acid to age at constant erosion index.

These regression coefficients were calculated by use of the following simultaneous equations:

$$\begin{aligned} An + B\sum X_1 + C\sum X_2 &= \sum Y \\ A\sum X_1 + B\sum X_1^2 + C\sum X_1X_2 &= \sum X_1Y \\ A\sum X_2 + B\sum X_1X_2 + C\sum X_2^2 &= \sum X_2Y \end{aligned}$$

where A, B, and C are the regression coefficients, X_1 = erosion index, X_2 = age in years, Y = citric acid content in mg. per cent and $n = 121$. These regression coefficients were tested for significance according to Fisher,³⁹ and both were found to be statistically significant.*

The correlation between the erosion index and the citrate content of saliva at any given age is expressed in Fig. 9.

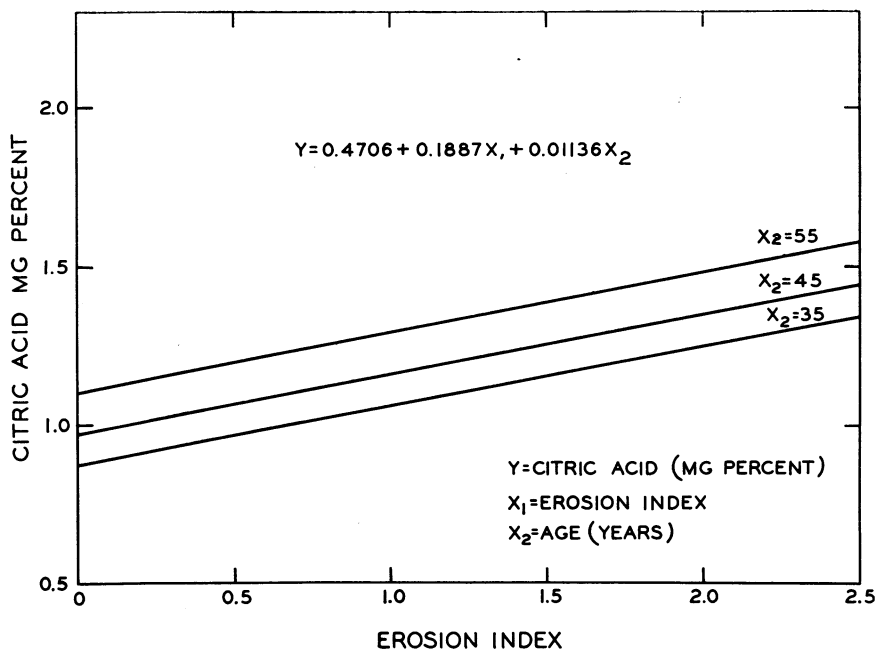


Fig. 9.—Relation of citric acid to erosion index at constant age.

This correlation, which is more useful than the correlation shown in Fig. 7, since the latter does not take into account the effect of age, suggests a relation between the severity of erosion and the citrate content of saliva. However, it has been shown that the mean salivary citrate content of the normal individuals does not differ significantly from that of the erosion cases when corrected for age. It appears at first glance that these two findings are not in accord. It should be remembered, however, that the mean citrate content for the erosion cases is somewhat higher than that for the normal cases. Moreover, it is to be noted that individual values comprising the mean for the erosion cases show a positive trend (Fig. 7). Hence, even when corrected for age, a correlation may exist between the severity of erosion and the salivary citrate content (Fig. 9).

*We are indebted to Mr. Jerome Cornfield, Statistician, Division of Public Health Methods, Office of The Surgeon General, USPHS, for statistical treatment of the data.

DISCUSSION

The prevalence of erosion among our subjects would appear to be unexpectedly high, particularly by comparison with the recent study by Shulman and Robinson⁹ who found gross evidence of erosion in only 2 per cent of a group of 1,345 male college freshmen. In all probability, these were individuals under the age of 30 years. Kitchin⁴⁰ reported the following per cent prevalence of abrasion due to toothbrush and dentrifice: age 20 to 29, 42 per cent; age 30 to 39, 45 per cent; age 40 to 49, 76 per cent, and age 50 to 59, 70 per cent. It has been postulated by Souder and Schoonover⁴¹ that slightly abrasive dentrifices may damage the enamel by virtue of the solvent action of a chemical ingredient, such as sodium metaphosphate, present in some dentrifices.

It was our experience, and this has been the experience of other investigators,⁴² that it is extremely difficult to make a clear-cut differentiation between loss of tooth substance due to mechanical factors and that due to purely chemical agents. For purposes of this study, as noted above, erosion was diagnosed as a loss of tooth substance on the gingival third of the tooth on the labial or buccal surfaces, regardless of cause. Shulman and Robinson,⁹ however, state in their report that "Erosion was differentiated from dentrifice abrasion by its position, and from dental caries by the quality of its base."

The action of citrate on rats' oral tooth surfaces, noted in a previous study,¹² resembles the gross appearance of human erosion and previously suggested "that saliva (or localized fluid exudates bathing tooth surfaces) may contain citrate or other anions which effect calcium solubility and advance the destruction of tooth surfaces orally."¹² The possibility arises that specimens of stimulated saliva represent a dilution of the localized diffuse oral secretions to such an extent that salivary citrate analyses fail to reveal the full magnitude of differences in erosion and nonerosion individuals. The analytical differences as we have reported, therefore, may possibly possess even more significance than is apparent in the statistical analyses of the data.

The hypothesis that human dental erosion may be a localized nonacid decalcification due to a calcium solubilizing anion such as citrate, while not clearly demonstrated in the results of this clinical study perhaps, should not be discarded as a good working hypothesis for future study. On the basis of the present data, it can be said that the severity of human erosion is associated with the salivary citrate content. In view of the limited number of cases it is felt that more work should be done to corroborate these findings.

Our results vary somewhat from a recent report by Shulman and Robinson,¹² although it must be noted that the age groups differed in these two studies, as well as the technic employed in preserving and analyzing the saliva specimens. In this connection it may be noted again that the preservation of saliva prior to analysis for citric acid is an extremely important factor.

SUMMARY

1. Application of the citric acid method proposed by Pucher, Sherman, and Vickery and modified by Perlman, Lardy, and Johnson, for the analysis of

saliva, was critically studied and a satisfactory procedure adopted. The citric acid in saliva is quite unstable, but by addition of H_2SO_4 the saliva is stabilized and recovery of added citric acid is satisfactory. Amounts of citric acid varying from 0.010 mg. to 0.450 mg. in 10.0 ml. specimens of saliva may be determined.

2. Although the number of individuals involved is small, the general pattern of erosion was observed to be as follows: (a) the upper teeth show a greater degree and a greater prevalence of erosion than the lower teeth, (b) the teeth of the right and left quadrants of both jaws have a similar degree of erosion, and (c) the severity of erosion increases with age.

3. The citric acid content of stimulated saliva varies from 0.20 mg. per cent to about 2.00 mg. per cent in individuals with and without dental erosion. There is a slight increase with age in the citric acid content of stimulated salivas.

4. On the basis of the data thus far accumulated, there appears to be a positive statistical correlation between the severity of human erosion and the salivary citrate content.

We gratefully acknowledge the cooperation of the following in making erosion cases available to us for examination and for collection of saliva: (1) Bruce D. Forsyth, Senior Dental Surgeon, USPHS, USPHS Dispensary, Washington, D. C.; now Assistant Surgeon General (Dental), USPHS. (2) A. E. Nannestad, Dental Director, USPHS, Marine Hospital, Baltimore, Md.; now at the U. S. Marine Hospital, Philadelphia, Pa. (3) R. S. Lloyd, Senior Dental Surgeon, USPHS, Marine Hospital, Baltimore, Md. (4) E. G. Pollard, Commander, USN, Navy Medical Center, Bethesda, Md.

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