CITRIC ACID AND THE CITRATE ION AS FACTORS IN TOOTH DESTRUCTION

by

FREDERICK RINGEL JUNK

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APPROVED:

[redacted]

Major Adviser

[redacted]

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Section A. Introduction and Points of General Interest

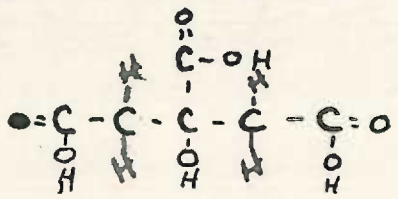
The citrate ion has a wide biologic presence and application. It is the acid of many fruits, particularly lemons and limes. It is an excellent anti-coagulant for blood (1) (2), the usual understanding being that this property is due to the formation of unionized calcium salts. Stewart & Percival in their studies on calcium metabolism (3) have "found that the addition of sodium citrate to blood produced an effect additional to the formation of an undissociated calcium salt--it broke down the undissociated non-diffusible calcium complexes of the blood." Solmsmn (2) in speaking of the citrates says: "Their intravenous injection (not oral administration) produces the typical effects of a calcium deprivation." Whether or not the calcium in the red cells is altered or not, the chill that so often follows the use of citrated blood for transfusion has led to considerable controversy as to the advisability of this technique (4) (5).

Potassium citrate is widely used as an alkalizing agent for the urine as in the familiar combination with hyoscyamus given by urologists for the relief of urinary burning and urgency (6).

When the anti-scorbutic effect of lemons was first discovered there was a considerable time when workers thought that the effect lay in the citric rather than in the undiscovered covitamic acid. However, in 1917 and 1918 Chick and Hume, and Rose found citric acid as a reagent to be ineffective (7) (8). And when one considers the present and past advocacy of citric acid in infant feeding formulae (9) (6), one wonders just what was the cause of the mysterious deaths of certain rabbits used in certain experiments on the citric ion (10). They were fed citrated milk and they died, with typical convulsions of calcium deprivation, but it is not known just how the citrate ion in lethal quantities got from the gastro-intestinal to the circulatory pathways. However, since sodium citrate was used, the result may have been alkalosis and not citration.

It is interesting that there is a normal citrate content to both cow and human milk of 50 to 100 mg. per 100 cc. (1).

Consider the formula of citric acid:



The very multiplicity of exposed oxygen gives fair warning of the love of this compound for complex formation--one has only to remember the theories regarding the molecular chain state of water --and then to recall the use of citrates in the preparation of alkaline copper reagents for sugar, to be perpetually on guard for new manifestations of the property.

It was the reaction between citrate ions and calcium ions, however, which spurred us on to our work on teeth as outlined in Section B. Shear, Kramer, and Rosenhoff (11) found that as citrate ion is added to a solution containing calcium ions, that the specific conductivity, instead of being increased due to a greater number of ions in solution, is actually decreased until an amount equivalent to the calcium ions already present is added, after which the specific conductivity does increase as would normally be expected. Clark, Percival, and Stewart (12) have shown the same fact by their special sensitive physiologic test for calcium ions, using the frog heart; i.e., the addition of sodium citrate decreased the number of calcium ions present.

We are dealing, therefore, with an ion of wide physiologic response, and any results should be interesting.

Section B. Tooth Destruction by Citric Acid Candies

1. Inception of the problem:

This laboratory is particularly interested in carbohydrates and their physiological effects. It was inevitable, then, that having seen the observations of Miller and Neuwirth (11) regarding hard candies as an etiological factor in several cases of acute wholesale dental caries that we should seek some theoretical consideration to explain the effect. These authors report six cases of wholesale caries developing within six months to two years and in all but one a history of suddenly developing an association with hard candies was revealed. Regarding these caries they say, "The most striking type of decalcification is that found in the mouths of confectioners and bakers, who are constantly inhaling the fumes and dust of carbohydrates as an occupational hazard." They also mention that hard candies are found to run 80, 96, and even 99 percent cane-sugar, and thus they conclude: "The high carbohydrate content of these candies is most certainly the cause of the decalcification here described. The carbohydrates are in a form (cane-sugar), medium, and location, in which they can be readily acted upon by bacteria even without the intervention of salivary enzymes. This fact is pointed out because of the habit on the part of many who permit these candies to dissolve in the mouth at night, when there is lessened salivary secretion."

Our first thought was to discover some hexose-phosphate combination that might speed up the decalcification; but preliminary experiments showed absolutely no determinable calcium in preserved neutral solutions of various sugars that were allowed to be in contact and flowing over freshly extracted human teeth for periods of twenty-four hours to a week. This negative result led to a consideration of other factors to be found in the commonly consumed hard candies. Even a casual inspection of any candy stand leaves one impressed with the many varieties of "fruit drops" or citric acid flavoured candies on sale. And our own personal observation is that the habit of slowly dissolving such candies in close contact with the teeth is wide-spread. Moreover, preliminary experiments with solutions of such acid containing candies showed grossly palpable destruction of dental surfaces and measurable amounts of dissolved calcium in less than six hours. Buffering with saliva was later found to slow up the process, but the decalcification nevertheless was evident. Perhaps the active citrate ion was at work, we thought. Such a connection was never shown, but at all times it was easily seen that destruction did take place, whether by reason of the gross acidity of the candy solutions used or because of the activity of the citrate ion in some complex ion effect. Hygienically speaking, it would seem to make little difference.

In a study reported in 1932 by Enright, Driesell, and Treacher (12) the gross effects of acid on teeth were observed. They begin by a

careful survey of the theories of caries and mention that there are "two schools of thought----one maintains that local environmental factors are chiefly responsible for caries; the other school holds that deficient diet and defective nutrition are primarily and sufficiently responsible for the causation of decay." Their final conclusion is that the second school as exemplified by Dr. May Mellanby (15) is sufficiently well established as it relates to the periods of tooth growth to be accepted, but that the first school is on the right track, particularly in cases of acute caries. In their experimental work they establish a pH of 5.9 as the upper limit of serious effects, and it will be seen from the results reported that we have confirmed this figure.

2. Experimental Procedures and Techniques:

a. The teeth used were freshly extracted specimens from the Department of Dental Medicine of the University of Oregon Medical School Outpatient Clinic, except where specified. They were mounted in rubber stoppers after protecting the roots with beeswax and acid-proof paint. Thus, only smooth normal enamel surfaces were exposed except across the grinding edges. These teeth were not grouped by age, sex, or previous history of caries, but did possess exposable surfaces that were smooth and firm. Furthermore, all the teeth used, regardless of condition or appearance, dissolved to a comparable extent in the suspected solutions.

b. The stoppers with teeth mounted were placed in the mouths of two-ounce specimen bottles containing 30 to 50 cc. of the test solutions or solvents. The total amount of the solvent was accurately recorded and that figure used in determining the total calcium dissolved.

c. The enamel equivalent of the calcium dissolved, corrected for calcium in the candy itself or in the saliva, was calculated by multiplying the calcium by 2.7, which is the factor determined in 1936 by Bowes and Murray (14). No addition was made to this enamel equivalent for the insoluble forms of altered enamel that appeared as a visible layer of fine chalky material on each treated tooth. The significance of this is further discussed in Section C.

d. The bottles were then fastened in a rotary mixer which inverted them about forty times a minute for the duration of solvent action.

e. Calcium was determined by calcium method of Kramer and Tisdall (15) as modified by Clark and Collip (16).

f. Saliva for the experiments was collected while chewing paraffine and then filtered. A drop or two of 10% thymol in chloroform was used as a preservative. Such saliva contains 3-4 miligrams per 100 cc. of calcium.

g. The "fruit drops" used were popular brands secured on the open market. These also contain an appreciable amount of calcium, so that the 40% solutions in saliva run from 6 to 9 mg. per 100 cc. Brand "A" contains more calcium than brand "B".

h. Phosphorus was determined by the method of Benedict and Theis (17).

i. The teeth used were identified by serial numbers and in some cases were run a second time after being treated first with a solution which gave no effect. A few of the teeth were also treated after 10 months of drying at room temperature, both instead of and in addition to treatment when fresh.

3. Experimental Results

The results that follow embrace Experiments IV, V, VI, VII, and VIII in the notebook of original record.

Tooth Number	Experimental Conditions	Calcium Figures:— As mg. of dissolved enamel.	As Final mg. %
1.	10% orange, Brand "A", in water, pH change 2.8 to 3.5, 25½ hrs. contact fresh tooth.	18.1	22
2.	20% orange, Brand "A", in water, pH change 2.6 to 3.3, 25½ hrs. contact fresh tooth.	26.5	33
13.	20% lime, Brand "A", in water, pH change 2.8 to 3.6, 24 hrs. contact fresh tooth.	25.4	39
3.	40% sucrose in water, no buffer added, end pH 6.5, 24 hrs. contact fresh tooth.	0.0	0
4.	40% orange, Brand "A", in water, pH change 2.6 to 2.9, 24 hrs. contact fresh tooth.	26.2	31
5.	60% lemon, Brand "A", in water, pH change 2.5 to 2.7, 24 hrs. contact fresh tooth.	26.4	29
8.	20% mint, Brand "C", in saliva, pH change 8.1 to 8.4, 24 hrs. contact fresh tooth. minus	1.2	2.3
7.	20% lime, Brand "A", in saliva, pH change 5.0 to 5.1, 24 hrs. contact fresh tooth.	1.9	8.3
9.	20% cherry, Brand "B", in saliva, pH change 5.1 to 5.2, 24 hrs. contact fresh tooth.	1.3	6.3
10.	20% orange, Brand "B", in saliva, pH change 5.0 to 5.2, 24 hrs. contact fresh tooth.	1.3	6.3
11.	20% pineapple, Brand "B", in saliva, pH change 5.4 to 5.6, 24 hrs. contact fresh tooth.	0.7	5.3
12.	20% lemon, Brand "B", in saliva, pH change 5.0 to 5.3, 24 hrs. contact fresh tooth.	1.2	6.0

Tooth Number	Experimental Conditions	Calcium Figures:— As mg. of dissolved enamel.	As Final mg. %
3a.	40% lemon, Brand "B", in saliva, pH change 3.6 to 3.7, 22½ hrs. contact fresh tooth.	4.9	17
8a.	40% lemon, Brand "A", in saliva, pH change 3.4 to 3.6, 22½ hrs. contact fresh tooth.	10.4	27
14.	40% levulose in saliva, pH at end about 7.0 24 hrs. contact fresh tooth.	minus 0.5	9.3
14a.	40% lime, Brand "A", in saliva, pH change 3.5 to 3.6, 22½ hrs. contact fresh tooth.	10.0	26
15.	40% orange Brand "B", in saliva, pH change 3.7 to 3.9, 22½ hrs. contact fresh tooth.	3.1	12
19.	40% lime, brand "A", in saliva, pH change 3.5 to 3.7, 24 hrs. contact fresh tooth.	7.2	21
20.	40% lemon, Brand "B", in saliva, pH change 3.7 to 3.9, 24 hrs. contact fresh tooth.	7.1	19
15a.	40% lemon, Brand "B", in saliva, pH change 3.7 to 4.2, 168 hrs. dry tooth in contact.	21.4	22
17.	40% orange, Brand "B", in saliva, pH change 3.7 to 4.2, 146 hrs. contact dry tooth.	14.3	25
23.	40% lemon, Brand "B", in saliva, pH change 3.7 to 3.9, 146 hrs. contact dry tooth	13.7	26
22.	40% lime, Brand "B", in saliva, pH change 3.5 to 3.6, 146 hrs. contact dry tooth	3.7	10
24.	40% cherry, Brand "B", in saliva, pH change 3.7 to 4.1, 146 hrs. contact dry tooth	5.1	13

4. Observations and Comments Regarding Teeth

All teeth in which the surrounding medium increased its calcium content, whether the increase was much or but slight as in the 20% series, showed a roughening easily palpable to the finger tip. Furthermore, the chalky deposit already mentioned and which forms such a large part of the observations of Enright, Friesell, and Trieschor (12), was present in all cases, and gives evidence of much greater destruction than can be accounted for by the calcium in solution. Section "C", following this, is devoted to a study of changes that occur in the solid phase when a salt similar in formula to tooth enamel is exposed to a solvent containing the citrate ion. Moreover, from Tooth No. 15a, and from an estimated area of about 95 sq. mm., 7 mg. of chalky material was removed by scraping. On analysis this gave a molecular Ca/P ratio of about 2.3, which is appreciably greater than the 1.67 Ca/P ratio of tooth enamel (13). Some of the phosphate ions have perhaps been replaced in this material by citrate ions.

The second column of figures in the preceding table is added to show that in all of the 40% solutions the final calcium concentration is well above the most recent figures for the calcium content of normal saliva. Edward C. Vosh (18) gives the calcium range for saliva as 6.3 to 7.2 mg. per 100 cc., and the generally accepted range for normal blood is 7 to 15 mg. per hundred cc. (19). Neither blood nor saliva could well protect teeth from such solution.

A few studies were also made of the progressive action of these candy solutions on teeth. Even teeth that were markedly etched at the end of twenty-four hours, were in most instances but slightly roughened if at all by the end of six hours. Chemical studies of the solvent solutions failed to reveal marked action up to the end of the six-hour period.

Section C. Chemical Studies on a Synthetic Salt

Having shown that tooth enamel is actively destroyed by solutions of acid candies, our next approach was to equilibrate varying amounts of specially prepared tri-calcium phosphate with citrate buffers of varying pH. This was not entirely a new approach, for chemists, both practical and theoretical have not neglected the intricate interrelationships of the calcium, citrate, and phosphate ions; but our attempt was to make quantitative studies that would parallel the semi-quantitative work done with human teeth.

First, a survey of some of the attention that the citrate ion has received, especially as related to calcium or phosphate ions, is in order.

The analytical methods of the Association of Official Agricultural Chemists (20) for "available phosphorus" in apatite or bone meal has long centered around the solubility of phosphates in neutral ammonium citrate.

Ramsay (21), in a study of this subject from the viewpoint of commercial analysis, points out that "about 91% of the total phosphorus pentoxide in pure tricalcium phosphate is soluble in 2% citric acid in thirty minutes." It is interesting to note that in concentration of citrate ion the 2% citric acid is but slightly stronger than the 0.1 M buffers used in the experiments in our laboratory. Of course the pH of the pure acid is markedly lower (around 1.6) than the pH of the buffers used.

Fiske, in 1921 (22) takes cognizance of the interrelationship of calcium and citrate ions when he uses citrate to immobilize calcium during the precipitation of phosphates.

Jacob, Beeson, Reader, and Ross of the United States Department of Agriculture (23), found that for maximum solubility there must be a sufficiently large ratio of calcium phosphate to citrate solution; i.e., the sample must not be too large. They found that 0.5 grams was the maximum sample possible for the 100 cc. official amount. It is interesting to note in this connection that the method of analysis is by difference and what is really determined is first the total phosphorus and then the citrate-insoluble phosphorus. That is, neither the concentration of calcium or phosphorus in the ammonium citrate liquor was determined.

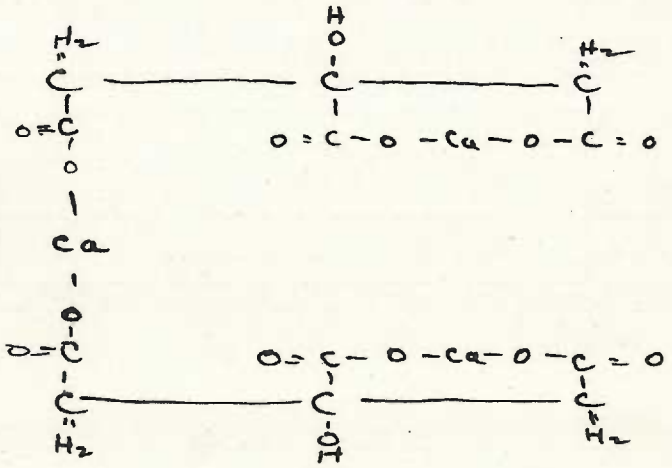
In an Italian study by Travers and Perron (24), the workers feel that they have shown that the solubility of the calcium phosphates in ammonium citrate is due to a double reversible decomposition accompanied by the formation of calcium citrate and ammonium phosphate.

While treating calcium carbonate with sodium citrate in a study of the solubility product of calcium carbonate, Hastings, Murray, and Sendroy (25) concluded that there was evidence of the formation of a slightly ionized calcium citrate compound.

Shear and Kramer (26) have shown by conductivity experiments that the addition of calcium chloride to a solution of sodium

citrate decreased instead of increased the conductivity of the solution, thus pointing to a decrease in the number of molecular or ionic particles present, and giving direct evidence for the binding of calcium ions by sodium citrate in some kind of soluble complex.

Hastings, McLean, Eichelberger, Hall, and DaCosta (27) have arrived at conclusions similar to those of Shear and Kramer (26) and have used physiologic methods in their work. They feel that "When calcium and citrate are present in solution, a part of the calcium present is bound in a complex, negatively charged ion. They present the following probable structural formula for calcium citrate:



The ground that we are covering, then, is by no means new, yet the presentation of a series of experiments that gives chemical evidence as to the mechanism of the destruction of substances similar to dental enamel does not seem to have previously been done.

2. General Experimental Methods

These were essentially the same as those used with the actual teeth. The same rotary mixer was used. However, for these experiments it was moved to a large incubator room that is constantly maintained within one degree of 38 degrees Centigrade. Furthermore the shaking time was increased so as to conform to that used by others in equilibrium experiments on calcium salts (28). But our studies were with systems in which there was a definite excess of solid at the end of the elapsed time and we were not concerned with solubility products, but with absolute solubility and with solubility ratios, and the time of equilibrium should be less.

The synthetic tri-calcium phosphate or tri-calcium phosphate complex was prepared in this laboratory from Mallinkrodt's Analytical Reagents according to the general directions of Mellor (29). He says: "Pure crystalline tricalcium phosphate has not yet been found in nature, or prepared in the laboratory. The nearest approach to pure calcium phosphate is made by adding sodium phosphate to a solution of calcium chloride in the presence of ammonia." This procedure was consequently used and 100 grams of $Na_3PO_4 \cdot 12H_2O$ were dissolved and added to a solution of 110 grams of $CaCl_2 \cdot 2H_2O$ and the whole made up to about four liters. The mixture was then boiled seventeen hours to increase particulate size, the liquid decanted, and the precipitate washed with distilled water. Washing by decantation was followed by washing by centrifugation until the

the washings were free of chlorine ions. A marked tendency for the salt to remain in semi-colloidal solution appeared at the same time. These washings were spread over five days. Two washings with 95% alcohol and one with ether followed in order that the resultant amorphous cake might not be too hard upon drying. The product was then air-dried for two days, semi-pulverized, dried at 120 degrees over night, and then further pulverized and screened to 80 mesh or smaller. Analysis of the finished product gave 37.4% calcium and 17.3% phosphorus, and a molecular Ca/P ratio of 1.69. This may be compared with the figures for pure tri-calcium phosphate of 38.7% calcium, 20.0% phosphorus, and a molecular ratio of 1.50. The addition of calcium hydroxide or calcium carbonate molecules by adsorption (30) might account for the variation. In fact a noticeable evolution of carbon dioxide occurs when the salt is treated with strong HCl. The Ca/P ratio of the salt corresponds almost exactly with the 1.66 molecular ratio of apatite, $3Ca_3(PO_4)_2 \cdot CaCO_3$ and with the Ca/P ratio of 1.67 for the mineral of tooth enamel (14).

3. Determination of the Titration Curve for Citric Acid at 38° Centigrade and under conditions of the Experiment.

To facilitate the preparation of 0.1 M citrate buffers, it seemed expedient to experimentally determine the complete buffer curve. The solutions used were allowed to come to the temperature of the incubator later used for solubility experiments and pH readings were made with the quinhydrone electrode. 0.05 M potassium acid phthalate was used in the reference electrode and the pH read directly from the following table, according to the observed electromotive force in millivolts. The formula for calculation is found on page 50 of the University of Oregon Laboratory Manual in Biochemistry, edition of 1935, and is: $\frac{E_o - (E_{observed} - E_h)}{.0617\ (38^\circ C)} = pH$

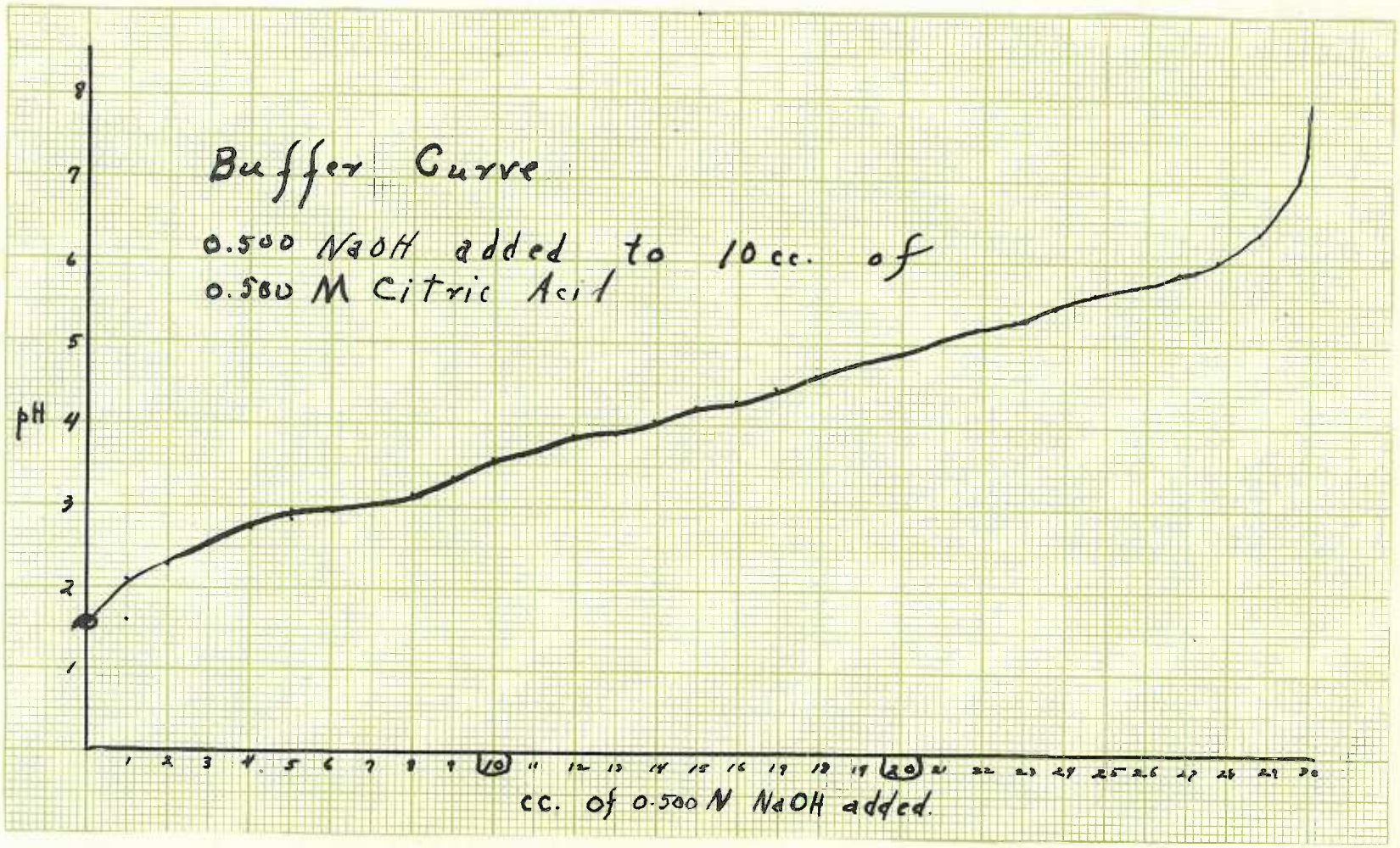
E_o at 38° C. is 0.6804 E_h at 38° C. is 0.4445

$E_{observed}$	pH	$E_{observed}$	pH
-130	1.86	50	4.78
-120	2.02	60	4.94
-110	2.19	70	5.10
-100	2.35	80	5.26
- 90	2.51	90	5.42
- 80	2.67	100	5.59
- 70	2.83	110	5.75
- 60	2.99	120	5.91
- 50	3.16	130	6.07
- 40	3.32	140	6.23
- 30	3.48	150	6.40
- 20	3.64	160	6.56
- 10	3.80	170	6.72
0	3.97	180	6.88
10	4.13	190	7.04
20	4.29	200	7.21
30	4.45	210	7.37
40	4.61	220	7.53

In determining the buffer curve for citric acid, 10 cc. of 0.500 N citric acid (checked against standard NaOH with phenolphthalime indicator) was taken, varying quantities of 0.4955 N NaOH were added, and the whole made up to 50 cc. in a volumetric flask. All doubtful or seemingly inconsistent readings of the potentiometer were rechecked at least twice. The results are:

cc. NaOH used	NaOH as 0.500 N	pH
0.00	0.00	1.56
1.00	0.99	2.10
2.00	1.98	2.30
3.00	2.97	2.56
4.00	3.96	2.72
5.00	4.95	2.86
6.00	5.94	2.91
7.00	6.93	3.02
8.00	7.92	3.13
9.00	8.91	3.37
10.00	9.90	3.56
11.00	10.89	3.69
12.00	11.88	3.83
13.00	12.86	3.89
14.00	13.85	4.04
15.00	14.84	4.21
16.00	15.83	4.29
17.00	16.82	4.45
18.00	17.81	4.61
19.00	18.80	4.75
20.00	19.79	4.86
21.00	20.78	5.05
22.00	21.78	5.19
23.00	22.77	5.29
24.00	23.77	5.44
25.00	24.76	5.61
26.00	25.76	5.70
27.00	26.75	5.88
28.00	27.75	6.04
29.00	28.74	6.37
30.00	29.73	7.09
30.20	29.93	7.35

These data are plotted in the curve of the following page.


Buffer Curve
0.500 NaOH added to 10 cc. of
0.500 M Citric Acid
pH
cc. of 0.500 N NaOH added.

4. General Survey of Experiments With Tri-Calcium Phosphate

In studying the solubility of the specially synthesized tri-calcium phosphate in buffers containing the citrate ion, the pH varied from the lowest starting pH of 2.41 to the highest final pH of 7.1. The volume of the solvent buffer was kept constant at 50 cc., but the weight of tri-calcium phosphate was changed:— 0.3 grams, 0.5 grams, 1.0 grams, or 5.0 grams.

The following molecular ratios help to emphasize the changes that take place:

Substance	Ca/P
$Ca(H_2PO_4)_2$	0.50
$CaHPO_4$	1.00
$Ca_3(PO_4)_2$	1.50
Tooth Enamel (14)	1.67
Apetite, $3Ca_3(PO_4)_2 \cdot CaCO_3$	1.67
Synthetic "Tri-calcium" Phos.	1.69

In one or more of these experiments the following facts may be shown:

a. pH values below 5.1 show a solution of both Ca and P.

b. pH values below 5.1 also show an alteration of the solid phase, best evidenced by an increase of the Ca/P ratio of the solid. And at the same time, the Ca/P ratio of the liquid is increased; decreased that is, phosphate ions leave the solid and an ion (the citrate is the only one available in most cases) leaves the liquid.

c. When the pH value was above 5.1 or 5.2, there was a marked tendency for the synthetic salt to enter a colloidal system with the citrate buffer.

d. With the smaller amounts of salt, the tendency to form colloidal systems with the solvent buffer was smaller.

e. When phthalate or acetate buffers were used no such marked changes could be demonstrated.

f. Finally, by the time these experiments were finished, it seemed that the interchange of phosphate and citrate ions formed a very plausible explanation for the appearance of a soft crumbling deposit upon the surfaces of human teeth that are exposed to citrate buffers and to solutions of citric acid candies.

The results of Experiment Twelve in which 0.5 grams of the synthetic tri-calcium phosphate was added to 50 cc. of 0.1 M Citrate Buffer, give the most representative results in demonstration of the above remarks:

	pH Change	Ca/P Ratio in Solid	In Liquid.
1.	2.91 to 3.39	7.9	0.63
2.	3.15 to 3.72	6.4	0.47
3.	3.56 to 3.98	5.8	0.33
4.	3.98 to 4.23	5.4	0.31
5.	4.21 to 4.73	4.5	colloidal.
6.	4.45 to 5.05	4.4	"
7.	4.75 to 5.20	1.66	"
8.	5.05 to 5.40	1.65	"

In the next section the results of all the experiments are tabulated in detail. They are arranged in the order of their performance and the comments attempt to give the effect of each experiment upon the development of this problem.

5. Detailed Results of Experiments With Tri-Calcium Phosphate.

Experiment One. (As numbered in original record book)

This was in every respect a preliminary experiment. The importance of colloidal effects above a pH of 5.0 had not yet been appreciated and the final solutions were not checked for that opalescence that introduces an undetermined factor into the figures and at the same time suggests a complex ion effect in the calcium to citrate relationship.

Two pH values, 4.96 and 6.33 were used. 0.3 grams of tri-calcium phosphate, containing approximately 2.8 millimols of calcium and 1.7 millimols of phosphorus, ~~was~~ WERE shaken for 72 hours with solutions of varying citrate concentration as indicated in the table. After shaking, the supernatant liquid was analyzed for Ca and P.

	pH Change.	Citrate Concen.	Millimols Ca in sol.	Millimols P in sol.	Ca/P Ratio
1.	4.96 to 5.3	0.1 M	2.45	1.60	1.53
2.	4.96 to 5.4	0.05 M	1.8	1.0	1.50
3.	4.96 to 6.4	0.01 M	0.33	0.28	1.18
4.	6.33 to 7.1	0.05 M	0.35	0.30	1.14
5.	6.33 to 7.3	0.02 M	0.16	0.18	0.89
6.	6.33 to 7.5	0.01 M	0.10	0.14	0.72

We see a double effect in this experiment. First, the amount of both calcium and phosphorus markedly decreases as the pH of the final solution rises. And second, the amount of both calcium and phosphorus markedly decreases as the amount of citrate ion ~~is~~

is lessened. However, the cumulative effect of both these changes was greater in the above experiment upon the calcium than it was upon the phosphorus as shown by the steady decrease in the Ca/P ratio.

Experiment Two.

In this experiment tri-potassium citrate was used in 3, 2, 1, 0.5, 0.3, and 0.1 molar concentrations. 50 cc. of solution was shaken with 0.3 grams of tri-calcium phosphate. The higher citrate concentrations remained clear at all times, but the 0.5 M and lower gradually became opalescent. No calcium could be discovered in the clear solutions. After a week or two the opalescent solutions would settle out, thus indicating the presence of a colloidal system. And when such opalescent solutions had settled clear no calcium could be found.

Experiment Three.

With this experiment the use of 0.1 M citrate buffers was started in every bottle. 1 gram of tri-calcium phosphate was used in 50 cc. of buffer. Shaking was at room temperature for 96 hours.

No.	pH range	Condition of Solvent	Mmol Ca dis.	Mmol P	Ca/P Ratio
1.	6.1 to 7.1	Very opalescent	0.80	0.90	0.89
2.	5.6 to 6.6	Very opalescent	1.70	1.10	1.54
3.	5.2 to 5.8	Very opalescent	3.10	2.68	1.16
4.	4.2 to 5.4	Slightly opalescent	0.67	3.86	0.17
5.	3.0 to 4.2	Water clear	2.10	4.19	0.50
6.	2.4 to 4.0	Water clear	3.57	4.77	0.75

The 1 gram of tri-calcium phosphate introduced into each of the buffers in this Experiment Three contained approximately 9.6 millimols of Ca and 5.6 millimols of P. Note what a large percentage of the phosphorus went into solution in the last two buffers.

Experiments Four, Five, Six, Seven, and Eight.

These were concerned with the solubility of teeth in candy solutions and have been fully treated in Section B. Recall, however, the analysis of the chalky layer on Tooth No. 15a, used in Experiment VIII. 7 miligrams of powder were removed and found to contain 3.04 mg. of calcium and 1.05 mg. of phosphorus. This gives a molecular ratio of 2.30 or a ratio by weight of 2.93, thus showing a definite decrease in the phosphorus content of the soft chalky layer. (Ca/P for enamel is 1.67 (14)).

Experiment Nine "A".

The object of this experiment was to study the effect of greatly increasing the amount of powdered tri-calcium phosphate added to the 50 cc. of citrate buffer. Accordingly, 5 grams of 80-mesh powder were added to the 0.1 M citrate buffers of the indicated pH. Shaking was continued for nine days in the constant temperature room (bacteriological incubator) at 38° C. Approximately 48 millimols of calcium were in the initial solid, and approximately 28 millimols of phosphorus. It will be seen that increasing the salt does not give much more solution.

The table below gives data for the solvent citrate buffers at the end of this experiment and also includes the Ca/P ratio of the corresponding solid phases. Note the opposite trend of the ratios for the solid and the liquid phases. The determined molecular ratio of the tri-calcium phosphate is 1.69 (as previously mentioned); while the molecular ratio of the final solids obtained was in all cases higher than 1.69, and the Ca/P ratio in the final solutions was in all cases lower than 1.69. It seems, therefore, very evident that phosphorus moved from the salt introduced to the solvent buffer. At the same time it seems probable that the citrate ion moved from the buffer solution to the solid phase.

Even more significant in this respect were certain snow-white aggregates found in all the final solids, but most markedly in Nos. 4 & 5. An analysis of these aggregates for calcium and phosphorus gave a molecular Ca/P ratio of 9.4 and 8.8 respectively. One may conclude that the aggregates contain a large proportion of calcium citrate.

No.	pH change	Condition of solvent	Mmol Ca Dis.	Mmol P Dis.	Ca/P Ratio Liq.	Ca/P Ratio Solid
1.	5.21 to 5.93	2 plus opalescence	6.5	4.5	1.45	1.75
2.	4.77 to 5.64	3 plus opalescence	7.1	4.9	1.45	1.76
3.	4.53 to 5.45	3 plus opalescence	7.1	4.9	1.45	1.70
4.	4.21 to 5.29	3 plus opalescence	5.2	4.6	1.13	1.78
5.	3.97 to 5.16	4 plus opalescence	9.8	8.4	1.16	1.73
6.	3.64 to 4.91	slightly opalescent	1.9	3.2	0.25	1.65
7.	3.40 to 4.66	clear	0.6	3.5	0.17	1.69
8.	2.79 to 4.35	clear	1.0	3.9	0.25	1.67

Experiment Nine "B".

The object of this experiment was to duplicate Nine "A", but to use 0.01 M buffer instead of 0.1 M buffer; i.e., to study the effect of lessening the number of citrate ions present. The higher final pH values make it difficult to compare this experiment with Nine "A", but the active part the citrate ion plays in such experiments as these is clearly demonstrated. The absolute amounts dissolved are greatly reduced. The Ca/P ratio in the liquid phase is well below the 1.69 of the introduced salt, but such small shifts could hardly be expected to make great changes in the solid phase. The solid for No. 4 was lost in a lab accident and all the solids were destroyed before it was appreciated just how greatly Nos. 1, 3, & 5 disagreed with the general results of this series of experiments.

No.	pH change	Condition of Solvent	Mmol Ca Dis.	Mmol P Dis.	Ca/P Ratio Liq.	Ca/P Ratio Solid
1.	5.19 to 6.80	One plus opalescence	0.22	0.36	0.61	2.00
2.	5.01 to 6.40	2 plus opalescence	0.25	0.40	0.62	1.57
3.	4.69 to 6.12	2 plus opalescence	0.36	0.41	0.88	1.78
4.	4.34 to 5.78	3 plus opalescence	0.34	0.43	0.79	----
5.	3.99 to 6.80	3 plus opalescence	0.33	0.41	0.80	2.06
6.	3.51 to 6.65	Trace of opalescence	0.23	0.38	0.60	1.72
7.	3.16 to 5.18	Clear	0.27	0.40	0.67	1.66

The use of the centrifuge to obtain the supernatant fluid for analysis in this experiment instead of No. 32 Whatman filters as in most of the other experiments made no grossly noticeable difference in appearance of the liquid.

Experiment Ten.

This experiment followed the lines laid down in Experiment Nine. The quantity of the specially prepared salt that was added to the 50 cc. of citrate buffer, however, was reduced in an attempt to lessen somewhat the colloidal effects noticed in the higher pH ranges; so only 1 gram of salt was introduced, and thus about 9.6 millimols of calcium and 5.6 millimols of phosphorus were available for solution in the buffer. The attempt to reduce the colloidal effects was not particularly successful.

The Ca/P ratio on the residual solid phase was not run in this experiment. In the solution, however, the constant excess of phosphorus over calcium is striking, and is way beyond any possible calcium phosphate or apatite ratio.

No.	pH change	Condition of solvent	Mmol Ca Dis.	Mmol P Dis.	Ca/P Ratio Liquid
1.	2.91 to 3.32	Clear	1.5	4.0	0.36
2.	3.02 to 3.50	Clear	1.2	3.8	0.31
3.	3.15 to 3.67	Clear	0.90	3.8	0.24
4.	3.37 to 3.83	Clear	0.75	3.6	0.21
5.	3.56 to 4.05	Clear, refiltered	0.65	3.5	0.19
6.	3.69 to 4.37	Clear, refiltered	0.65	3.2	0.21
7.	3.88 to ----	Broken during manipulation.			
8.	3.89 to 4.78	Clear, refiltered	0.86	2.7	0.32

Experiment Eleven.

Since non-citrate buffers were used in this group, the results will be tabulated after the final experiment, No. Twelve.

Experiment Twelve.

The procedure was again the same, but the amount of the synthetic salt was further reduced to 0.5 grams, thus introducing 4.8 millimols of calcium and 3.8 millimols of phosphorus for possible solution. Analyses were run on the liquid phase only when it filtered clear, but the solid phase from all the buffers was analyzed. It is noteworthy that in solutions numbered 2, 3, and 4, that although no attempt at a quantitative recovery was made, yet the final weight of the dried solid exceeded the original amount put in by about 100 milligrams.

No.	pH change	Condition of solvent	Mmol Ca Dis.	Mmol P Dis.	Ca/P Ratio Liq.	Ca/P Ratio Solid
1.	2.91 to 5.39	Clear	1.66	2.6	0.63	7.9
2.	3.15 to 3.72	Clear	1.15	2.5	0.46	6.4
3.	3.66 to 3.98	Clear	0.85	2.5	0.33	5.8
4.	3.98 to 4.23	Clear	0.80	2.5	0.31	5.4
5.	4.21 to 4.73	2 plus opalescence	----	---	----	4.3
6.	4.45 to 5.05	3 plus opalescence	----	---	----	4.4
7.	4.75 to 5.20	4 plus opalescence	----	---	----	1.66
8.	5.05 to 5.40	3 plus opalescence	----	---	----	1.65

One should note the sharp break in the Ca/P ratio of the remaining solid when the final pH exceeded 5.05. This condition was anticipated before analysis by reason of the recovery of a solid phase that dried into the pearly crusts of tri-calcium phosphate instead of the white granules of calcium citrate. The whitening was grossly noticeable in all the solids remaining in Nos. 1 to 6.

The study of the solid phase in this experiment revealed that the percent of calcium in the solid phase of Nos. 1 to 4 ranged from 23% to 24%. This is inconclusive, but compares closely with the 21% calcium found in tricalcium citrate. And since at the same time about 66% of the phosphate ions were found to be in solution it seems most reasonable that the solid phase was a mixture of tricalcium citrate and a variable amount of some calcium citrate. $Ca_3(PO_4)_2$ is about 40% calcium; $CaHPO_4$ is about 29% calcium; and $Ca(H_2PO_4)_2$ is about 17% calcium. But the final pH in these cases is for the most part above that of $Ca(H_2PO_4)_2$

Experiment Eleven.

This had as its purpose the study of the effects obtainable with other buffers of pH range similar to that of the citrate buffers used. The same 0.1 M concentration of the buffering ion was employed and 1 gram of the synthetic salt was introduced into each flask. Other procedure was the same.

No.	pH change	Condition of solvent	Mmol Ca Dis.	Mmol P Dis.	Ca/P Ratio Liquid.
	Phthalate Buffers.				
1.	2.8 to 4.29	Clear	1.9	1.7	1.1
2.	3.0 to 4.20	Clear	1.5	1.5	1.0
3.	3.2 to 4.28	Clear	1.3	1.3	1.0
4.	3.4 to 4.29	Clear	1.0	1.2	0.9
5.	3.6 to 4.37	Clear	0.9	1.0	0.9
	Acetate Buffers.				
6.	3.6 to 4.37	Clear	1.0	0.6	1.7
7.	3.9 to 4.44	Clear	0.9	0.55	2.0
8.	4.0 to 4.53	Clear	0.8	0.45	1.8

Several observations may be made on this experiment with the phthalate and acetate buffers. The phthalate buffers gave a great uniformity of the final pH, irrespective of the original figure. The Ca/P ratio with the phthalate was always close to 1.0, which is the ratio found in the salt $CaHPO_4$; and that is the calcium phosphate form that should be present at the observed pH. The acetate buffers on the other hand, definitely suppressed the solution of the phosphate ion as compared with both phthalate and citrate buffers. The observed ratios of calcium to phosphorus suggest that of $Ca_3(PO_4)_2$ more than any other. And in both the phthalate and acetate buffers it is interesting to note that the quantity of calcium in solution was of the same order of magnitude as found with the citrate buffers, while in both cases the amount of phosphate leaving the synthetic salt was definitely less than in the case of citrate buffers. These final experiments are inconclusive and further work on a variety of buffers might have been indicated if the primary purpose of this work had not been the effect of the citrate ion on human teeth.

Section B. Conclusions

1. We have shown that strong solutions of citric acid candies actually dissolve human teeth, if the concentration of the candy is great enough and the time of exposure is long enough.

2. We have not shown that occasional use of acidified candies will form dangerous concentrations of acid in the mouth.

3. In our experimental work we have shown that sufficient calcium goes into solution to account for appreciable destruction of tooth structure. Such destruction has been actually palpated with the fingers, and seen macroscopically by the experimenters.

4. Upon teeth in contact with all destructive solutions we have found a film of chalky material which, upon analysis, is found to be deficient in phosphorus as compared to normal enamel. We have postulated a change of the tooth mineral to some calcium citrate molecule or complex as best explaining the observations.

5. When synthetically prepared tri-calcium phosphate was shaken with citrate buffers at a final pH of 5.1 or less, a calcium concentration was found in the solvent at equilibrium in excess of that in normal saliva. And in addition the solid residue left after such action showed a phosphorus deficiency so that the Ca/P ratio was markedly increased in the solid and at the same time decreased in the liquid phase. There is, therefore, an interchange of phosphate and citrate ions. Such an interchange, we feel, is

the basis of the destructive action of citric acid candies upon teeth, such as was observed in Section "B".

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Typed by the candidate,

Frederick E. Judy.