SOME METABOLIC ASPECTS OF CITRIC ACID

by

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INTRODUCTION

Unlike many organic compounds, citric acid was not originally a synthetic laboratory product. It was first recognised by Scheele (1) when he precipitated its calcium salt from lemon juice. Its first relation to animal biological processes was indicated more than a century later when Soxhlet and Henkel (2) demonstrated its presence in milk.

The first quantitative determinations of citric acid in biological fluids appeared in 1929; in biological tissues, in 1936. These revealed that although it is present in most mammalian tissues to the extent of about 2 mg. percent, certain tissues and fluids deviate markedly from this value and reach concentrations of over 1000 mg. percent.

The fact that citric acid is very widely and unevenly distributed in tissues and fluids suggested that it may be involved in some of the characteristic and fundamental metabolic processes of the body. More recent work has demonstrated such to be the case.

Shortly after the discovery of citric acid as an animal metabolite, and even before the evolution of an analytical method for citric acid, Thunberg (3) examined sixty organic acids, including citric, for their oxidisability in isolated animal tissues and found that of these acids, citric, lactic, succinic, fumaric, malic, and glutamic disappeared rapidly with a transfer of hydrogen to methylene blue, which he used as indicator. Most of this work was done on muscle tissue and the results indicated that the above acids may be involved in carbohydrate metabolism, since carbohydrate is known to be the chief metabolite of muscle. Batelli and Stern (4) even proposed that the oxidation of these acids in muscle can be identified with the respiration of the muscle cells.

Martius and Knoop (5) and (6) elucidated the first step of the tissue breakdown of citrate when they found that a citric dehydrogenase from liver converts it to α-ketoglutarate which they isolated as the 2,4-dinitrophenylhydrazone. Krebs and Johnson (7) confirmed this for muscle.

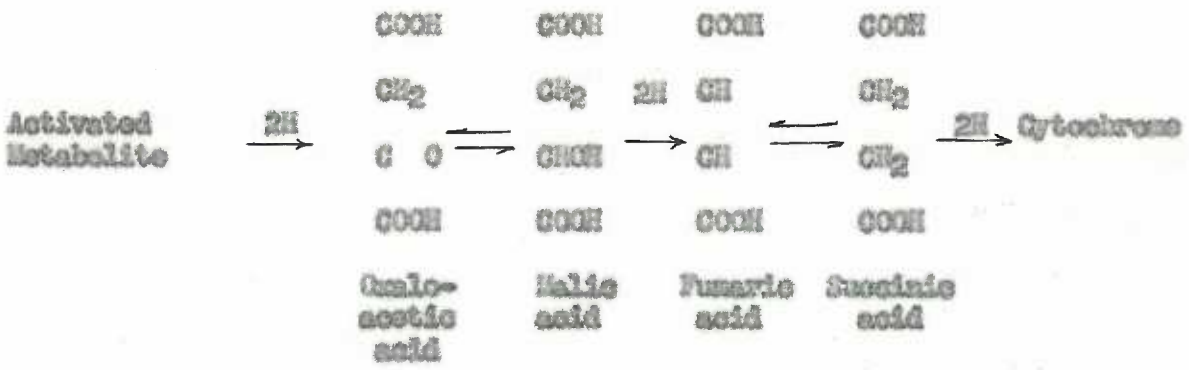
Martius (8) has shown further by changes in optical rotation that citrate exists in tissues in equilibrium with isocitrate and cis-aconitate and that the conversion to α-ketoglutarate proceeds directly from isocitrate.

Krebs and Johnson (9) demonstrated and measured the metabolic breakdown of α-ketoglutarate to succinate by muscle tissue by observing the carbon dioxide formation from α-ketoglutarate in a manometric apparatus.

Einbeck (10) and (11) had already studied the reactions of succinate by a similar manometric method based upon measurement of absorbed oxygen and concluded that fumarate, and later malate, are oxidation products of succinate.

Oxaloacetate was distinguished as the metabolic oxidation product of malate by Hahn and Haarmann (12) and (13).

In addition to the reactions described, it was discovered by Szent-Gyorgyi (14) and (15) that succinate, fumarate, malate and oxaloacetate markedly increase the oxygen uptake and carbon dioxide production of animal tissue out of proportion to the quantity of the added substance and sometimes without its disappearance. Krebs and Johnson (7) subsequently established the same phenomenon for citrate. As a result of his observations, Szent-Gyorgyi (16) developed a theory in which succinate, fumarate, malate, and oxaloacetate are integrated interdependently into an oxidation-reduction system specifically concerned with hydrogen transport from activated metabolite to the cytochrome system.



It is therefore now considered that these organic substances, which have been shown to undergo certain successive reactions in connection with metabolism, not only serve as fuel in the organism, but exhibit also a catalytic activity in the respiration of tissue. Of paramount importance in support of this view is the observation of Szent-Gyorgyi (16) that oxaloacetate is as easily reduced metabolically to malate, even in the presence of molecular oxygen, as malate is oxidized to oxaloacetate, and may even go to succinate. This is not a general occurrence in metabolic processes, most of which are predominantly oxidative under aerobic conditions, and the finding of a reaction so easily reversible as malate $\rightleftharpoons$ oxaloacetate is responsible for Szent-Gyorgyi's suggestion that the oxaloacetate $\rightleftharpoons$ malate $\rightleftharpoons$ fumarate $\rightleftharpoons$ succinate equilibrium represents a catalytic hydrogen carrier system.

Hallman (17), Simola and Alapeuso (18), and Breusch (19) at this time reported the synthesis of citrate from oxaloacetate in various organs. Krebs, Eggleston, Kleinzeller, and Smith (20) confirmed it for muscle tissue.

Krebs (21) also made the interesting observation that succinate may be formed from fumarate, malate and oxaloacetate not only by a reductive process, but, if this is inhibited by malonate, by an alternate, oxidative process.

On the basis of the catalytic effects of citrate, succinate and malate; the rapidity of oxidation of citrate, isocitrate, cis-aconitate, and α-keto-glutarate in pigeon breast muscle, which metabolises carbohydrate extremely energetically, the synthesis of citrate from added oxaloacetate in muscle tissue; and the finding that succinate can be converted to fumarate and oxaloacetate by muscle tissue under either aerobic or anaerobic conditions, depending on the balance of active enzymes - Krebs (22) has formulated a hypothetical "citric acid cycle" which serves to explain the relation of citric acid to carbohydrate metabolism and to guide in the further investigation of the role of citric acid in intermediary metabolism.

Essentially, the scheme postulates that, following the accepted view of the splitting of carbohydrate to triose phosphate and pyruvate, the pyruvate condenses with oxaloacetate to form citrate. Citrate in turn is eventually oxidised to oxaloacetate, which re-enters the cycle to condense with more pyruvate from the carbohydrate source to form citric acid.

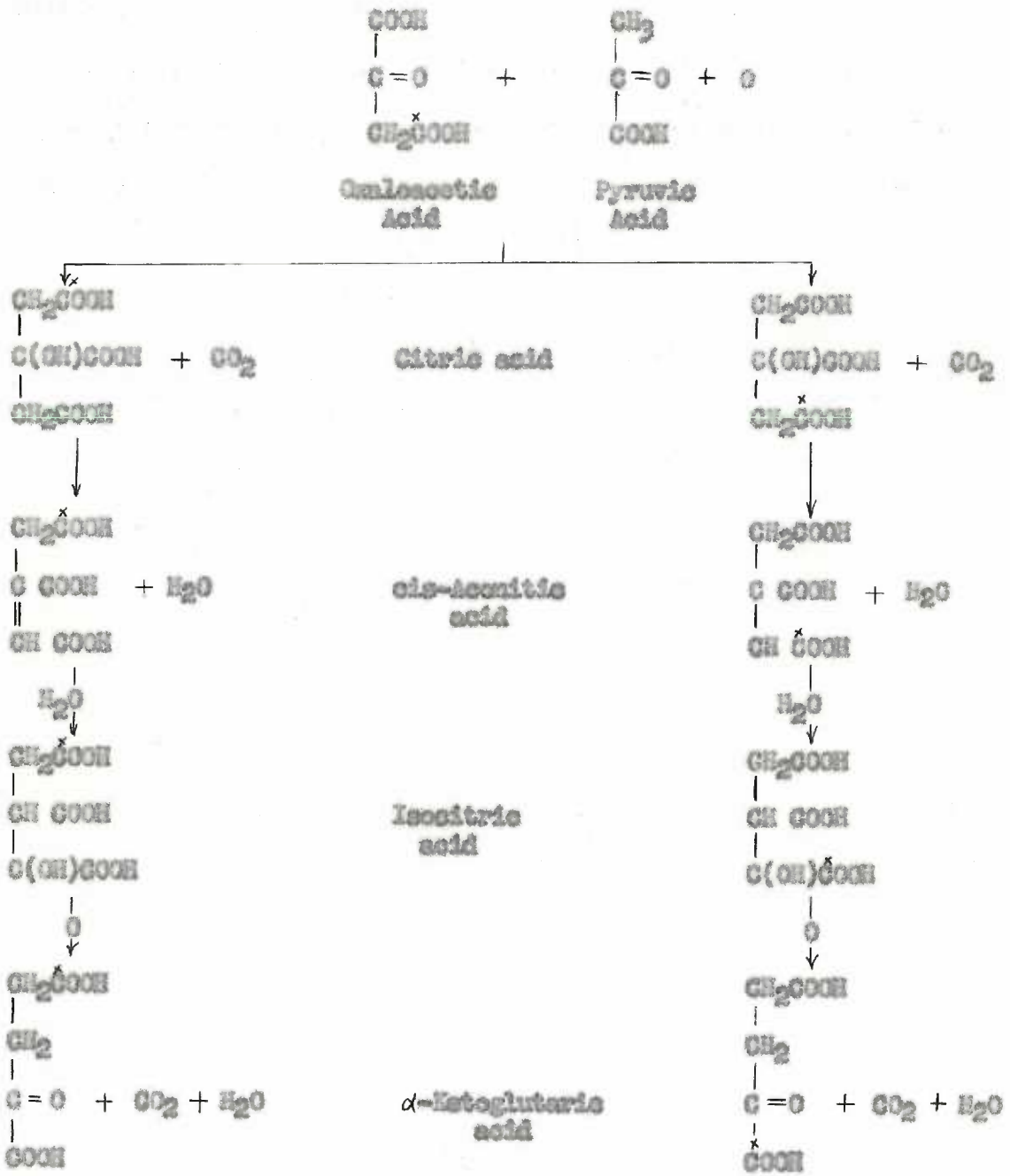
The evidence for this particular order is in the first place furnished by experiments of Krebs and Eggleston (21) which showed that in minced muscle pyruvate specifically is oxidised more rapidly than and in preference to other substances derived from carbohydrate. Secondly, Simola and Alapeuso (18) have shown that the addition of pyruvate to various tissues leads to an increase in citrate content. As indicated above, oxaloacetate is a precursor of tissue citrate. Since the synthesis of citrate from oxaloacetate necessarily involves condensation with another substance, it seems reasonable to suppose that this substance is pyruvate. Citrate is then oxidised through a series of substances, including cis-aconitate, isocitrate, α-ketoglutarate, succinate, fumarate and malate to oxaloacetate as described above. The order of oxidation - succinate to fumarate to malate - had been determined, as

above mentioned, by Einbeck (10) and (11); of malate to oxaloacetate, by Hahn and Haarmann (12) and (13); and the conversion of isocitrate to α-ketoglutarate, by Martius (6). It remained to establish the order of citrate, cis-aconitate and isocitrate in the cycle.

Experiments by Wood, Werkman, Hemingway and Nier (23), using the isotope C^{13} showed that if oxaloacetic acid is used as the tagged starting product, the isotopic carbon appears mostly in the carboxyl adjacent to the keto group of the α-ketoglutaric acid. If this transfer takes place primarily through citric acid, there is no reason to expect such a phenomenon, inasmuch as the terminal carboxyls of citric acid are symmetrically situated in the molecule, and the isotopic carbon should be equally distributed to both terminal carboxyls of the α-ketoglutaric acid in accordance with scheme 1.

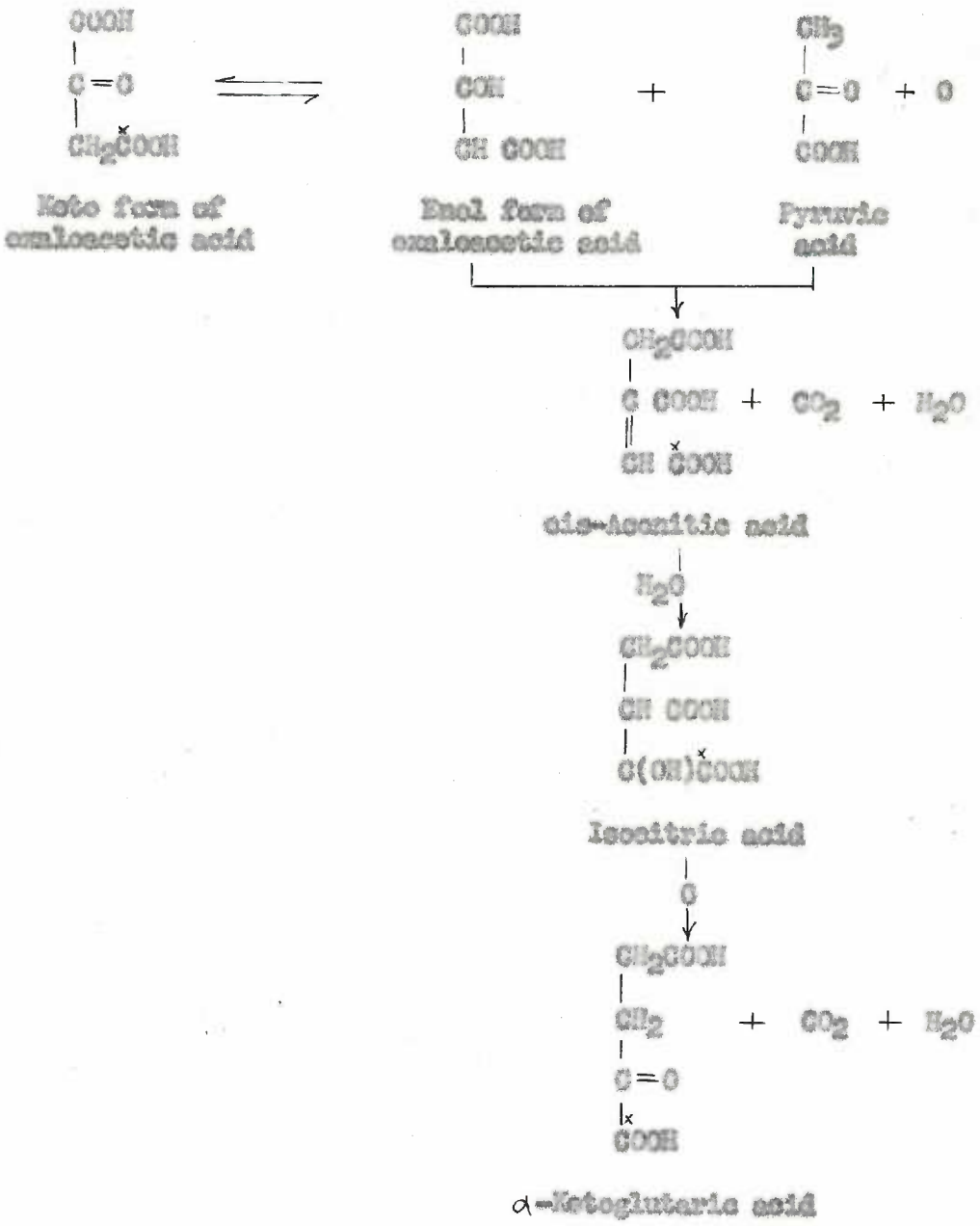
SCHEME 1

The carbon atom marked with the asterisk represents isotopic carbon, C^{13}.



However, if the oxidation mechanism of oxaloacetic acid to α-ketoglutaric acid involves the formation of aconitic acid as in scheme 2, it is to be expected that only the terminal carboxyl adjacent to the keto group of α-ketoglutaric acid would contain the isotopic carbon atom.

SCHEME 2

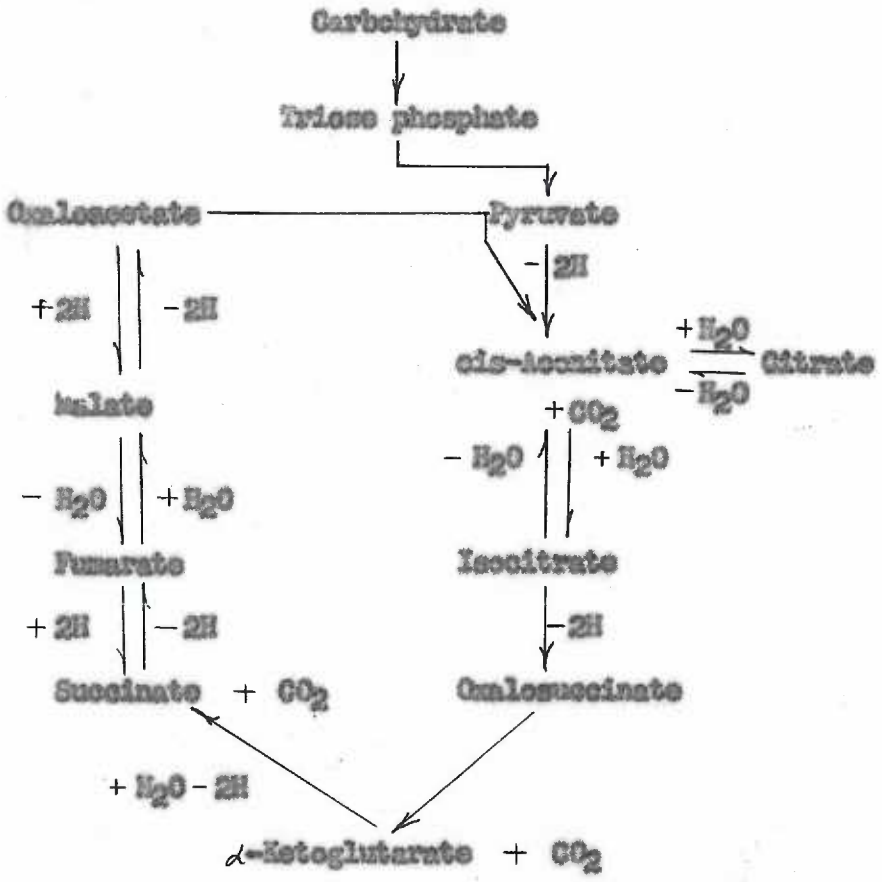


Krebs (23) has compared the relative rates of the following five reactions in tissues:

1. cis-Aconitate ⟶ Isocitrate
2. Isocitrate ⟶ cis-aconitate
3. cis-Aconitate ⟶ citrate
4. Citrate ⟶ cis-aconitate
5. Isocitrate ⟶ α-ketoglutarate

He found that at physiological concentrations, reactions 1 and 5 are much more rapid than reactions 2, 3, and 4. These findings indicate that the main flow of materials in the citric acid cycle passes through cis-aconitate and isocitrate rather than citrate in the formation of α-ketoglutarate. In view of the findings based upon the use of isotopic carbon, C^{13}, and upon these reaction rate studies, Krebs has modified his scheme and now indicates the relation of citric acid as a secondary product reversibly joined to the cycle through cis-aconitate. Scheme 3 shows the present concept of the citric acid cycle according to Krebs.

SCHEME 3



In vivo experiments substantiate the conception of endogenously formed citric acid. Martensson (24) reports a higher concentration of citric acid in venous than in arterial blood of muscle. Boothby and Adams (25) found that a starving dog continued to excrete citric acid. Sherman, Mendel and Smith (26) reported values of excreted citric acid above the citric acid intake in dogs. Sharr, Barnheim and Taussky (27) found citric acid to rise and fall with the estrogenic cycle. Ostberg (28) believes that urine citric acid rises in alkalosis, however induced. Dickens (29) showed that dry fat-extracted bone contains sometimes as much as 1.6 percent of citric acid and concluded that bone serves as a reservoir for citric acid in the body.

Following this work, Glass and Smith (30) administered sodium bicarbonate and sodium malate to rats and demonstrated that the resulting increase in urine citric acid is not due to release of the citric acid stored in bone. Their work implies that the endogenous formation of citric acid is stimulated by ingestion of alkali. The finding of Sinola and his collaborators (31) and (32) that certain organic substances convertible to carbohydrate in the body when fed to rats increase their citric acid output and behave as precursors of citric acid supports the view that citric acid is a product of intermediary metabolism.

As concerns carbohydrate metabolism, Sherman, Mendel and Smith (26) found that urine citric acid showed a tendency to rise in dogs fed a high sucrose, low protein diet although no such tendency was manifest on a low carbohydrate, high casein diet. Greenwald (33), as far back as 1914, had administered sodium citrate to phlorizinized dogs and to a patient with diabetes mellitus and noticed a rise in glucose excretion. MacKay, Carno and Wick (34) later found that citric acid, and to a lesser extent sodium citrate, administered to rats is deposited as liver glycogen. These authors also demonstrated the antiketogenic effect of citric acid on fasted rats and its efficacy in relieving insulin shock.

Many observations recorded in the literature by different workers point to relations of citric acid and calcium metabolism. Dickens (29) pointed out that the presence of the highest concentration of citrate in that tissue which is richest in calcium provides striking evidence for the participation of citric acid in calcium metabolism. The idea of a citrate-like substance acting as solvent for bone salts dates far back and Greenwald and Gross (36) and (37) in their studies of parathyroid activity went so far as to postulate the formation of such a substance through the agency of the parathyroid gland.

Thunberg (38) and (39) and his associates have continually sought to connect citric acid with calcium metabolism in the body. Scherstén (40) has found a high content of citrate in semen which has some five times as much calcium as does plasma. Embryonic tissue (30) and the shells of birds' eggs (38) and (39) are also rich both in calcium and in citric acid. Dickens has also shown that bone citrate is much more affected by dietary and hormonal variations than are the other bone salts and considers it probable that the presence of citrate in the immediate vicinity of calcifying or decalcifying bone might, by its solvent action, play an important role in preventing premature precipitation of bone salts in these regions. In this connection it is interesting to note that in recent years dissolution of urinary calculi formed of calcium salts has been attempted with citrate buffer lavage. In particular Albright and his co-workers (41) and later Suby (42) and Rawls and West (43) have made some progress in this direction.

Sabbatani (44), to explain the effect of citrate in preventing blood coagulation, arrived at the hypothesis that calcium forms a complex ion with citric acid. Wiley (45) measured the degree of dissociation of tricalcium citrate in water by conductivity methods. Hastings and his co-workers (46) measured the dissociation constants for the citrates of calcium, magnesium and strontium by using the frog heart method at pH 7 or above, in a range in which citric acid may be regarded as completely dissociated. They found two stages of dissociation, of which the first is complete -

$$Ca_3Cit_2 \rightleftharpoons Ca^{++} + 2\ Ca\ Cit'$$

The second dissociation is that of a weak electrolyte -

$$Ca\ Cit' \rightleftharpoons Ca^{++} + Cit'''$$

It can be described by the equation -

$$\frac{[Ca^{++}][Cit'''']}{[Ca\ Cit']} = K_{CaCit'} = 10^{-3.22}$$

at pH 7.4, 22° C and the ionic strength, μ = 0.155 to 0.166.

Hsue and Lebel (47) checked the constant by applying a chemical method. They brought citrate-containing solutions into equilibrium with solid calcium iodate and from the solubility product of calcium iodate calculated the calcium ion in solution as calcium citrate. They were also able to extend their observations to a lower pH range than that attained by the previous workers and were thus able to determine the dissociation of calcium hydrogen citrate -

$$Ca\ H\ Cit \rightleftharpoons Ca^{++} + H\ Cit''$$

and found -

$$\frac{[Ca^{++}][H\ Cit'']}{[Ca\ H\ Cit]} = K_{CaHCit} = 10^{-2.3}$$

and -

$$\frac{[H^{+}][Cit'''']}{[H\ Cit'']} = K_{HCit''} = 10^{-5.49}$$

From these results, McLean and Klapman (48) have constructed a nomogram which facilitates the calculation of calcium ion concentrations at various pH values in the presence of citric acid and which has been used to estimate the quantity of ionised calcium in citrated plasma.

In working with citric acid compounds, it is sometimes pertinent to bear in mind variations in temperature. Whereas the solubility of citric acid increases with temperature, calcium citrate has a negative temperature coefficient of solubility. The oxidations of citric acid are also affected by temperature, and, additionally, by catalytic effects. Greville (49)

found that in the enzyme oxidations which he studied, magnesium ion is a component of the enzyme system involved and had to be added to replace the magnesium ion removed by the citrate formed (50).

The oxidation of citric acid in mammalian tissues is effected through the agency of certain enzymes which catalyse its breakdown through the dicarboxylic and tricarboxylic acids of Krebs' cycle. Non-enzymic oxidation of citric acid, so far as it known, cannot be performed by this route. Oxidation by permanganate in acid solution is related to the in vivo oxidation of citric acid in some of the lower forms of life, in which the oxidation begins at the hydroxyl group and yields acetone dicarboxylic acid and carbon dioxide. Walker and his collaborators (51) and (52) found acetone dicarboxylic acid to be a product of citric acid oxidation in cultures of Aspergillus niger and Pseudamonas pyocyanea. Acetone dicarboxylic acid, however, is extremely labile and exists only as an intermediate product. Kuyper (53) has studied the effect of temperature, the acid used in the oxidation and the quantity of molecular oxygen present on the oxidation of citric acid in acid permanganate. He formulated the following equations to explain the divergent courses of the oxidation -

1.

$$2\ \begin{matrix} CH_2COOH \\ | \\ C(OH)COOH \\ | \\ CH_2COOH \end{matrix} + O_2 \longrightarrow 2\begin{matrix} CH_2COOH \\ | \\ C = O \\ | \\ CH_2COOH \end{matrix} + 2\ CO_2 + 2\ H_2O$$

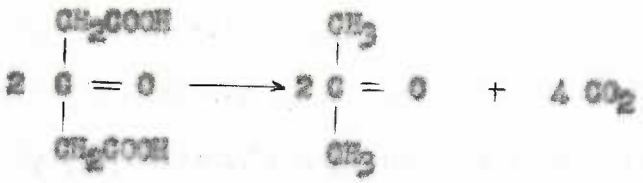
Citric acid — Acetone dicarboxylic acid

2. At temperatures below 60° C -

$$2\ \begin{matrix} CH_2COOH \\ | \\ C = O \\ | \\ CH_2COOH \end{matrix} + 5.5\ O_2 \longrightarrow 2\ CH_2O + H\ COOH + 7\ CO_2 + 3\ H_2O$$

Acetone dicarboxylic acid — Formaldehyde — Formic acid

3. At temperatures above 60° C -



Acetone dicarboxylic acid — Acetone

The first stage of the oxidation at any temperature is the formation of acetone dicarboxylic acid. The progress of the oxidation from this point depends on the temperature and the oxidising potential. At high temperatures, the chief product is acetone, which may be further oxidized to a greater or lesser extent if the oxidising potential is sufficiently high. Fast addition of permanganate and use of sulfuric rather than acetic acid have been found to promote this. At lower temperatures, no acetone is formed, and the main products are formaldehyde and formic acid. With rapid addition of permanganate the amount of oxygen found in the reaction products was equal to that present in the original citrate and that derived from the permanganate. As the speed of permanganate addition was reduced, the reaction products finally contained 28 percent more oxygen than could have come from the citrate and permanganate. No acetone or other partial oxidation products could be detected. When nitrogen was used for aeration, no excess oxygen was recovered with any speed of permanganate addition. Aeration with oxygen resulted in 36 percent excess oxygen in the products. Knyper concluded that molecular oxygen enters the reaction and affects the course of the oxidation. He pointed out the interesting relation of the in vitro oxidation of citric acid at body temperatures to its rapid oxidation in the body.

This thesis is concerned primarily with the following problems:

1. A study and improvement of the Pucher-Sherman-Vickery method for the

determination of citric acid. 2. The relation of citric acid metabolism to renal calculi formation. 3. The relation of succinate, glutamate, and citrate ingestion to urinary citrate excretion. 4. The role of intestinal bacterial action in the production of citric acid.

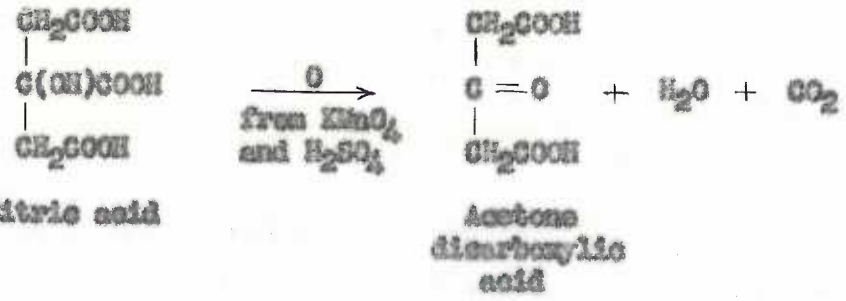
PART I

A STUDY OF THE PENTABROMACETONE METHOD OF ANALYSIS FOR CITRIC ACID

The study of citric acid metabolism has been seriously limited by the lack of an analytical method suitable to physiological material. The main difficulties arise in the low concentration of this substance in tissues, in the paucity of chemical reactions specific for it and in its chemical similarity to other substances of biological importance. A review of the literature reveals that two methods in particular have been used to investigate the citric acid content of the animal organism. The first, developed by Thunberg (54), depends upon the action of a specific citric dehydrogenase present in cucumber seed; the second, developed by Pucher, Sherman and Vickery (55), depends upon the characteristic property of citric acid to become oxidised, under certain conditions, to acetone dicarboxylic acid and of the latter to brominate to pentabromacetone, which gives a yellow color with sodium sulfide. Because enzyme technique is much more adaptable to biological fluids than to solid tissues and excreta, and also because of the trouble and care required in the preparation of the enzyme, the chemical procedure has a clear advantage in its universal applicability.

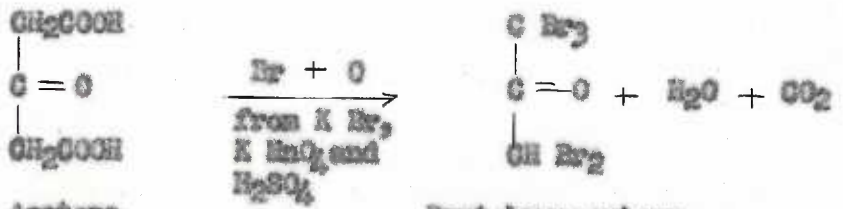
It seemed desirable to examine the chemical method from the point of view of smoothing out various uncertainties reported by all its users and to shorten the time of the determination.

The method consists in the conversion of citric acid to pentabromacetone according to the scheme -

1. 

Citric acid

Acetone dicarboxylic acid

2. 

Acetone dicarboxylic acid

Pentabromacetone

and in the treatment of the latter compound with aqueous sodium sulfide to form a yellow complex which can be assayed colorimetrically in terms of citric acid. A calibration curve so devised serves to evaluate the citric acid content of unknown solutions.

The experimental procedure, as delineated by Pucher, Sherman and Vickery, is as follows: a solution containing 0.1 to 1.0 mg. of citric acid is placed in a 150 ml. beaker with water to make a volume of approximately 75 ml. To this is added 2 ml. of 1 molar potassium bromide and 10 ml. of 1.5 normal potassium permanganate. After 10 min. standing, the solution is decolorized with a suitable amount of 20% ferrous sulfate solution or 3% hydrogen peroxide. In the latter case, the reaction mixture is first thoroughly chilled, and excess of the reagent is carefully avoided. The colorless solution is then shaken in a separatory funnel with 25 ml. of petroleum ether (B. P. 35-50° C.) to extract the pentabromacetone formed, the aqueous layer is drawn off and the ether washed once with 5-10 ml. of water. The wash fluid is added to the aqueous solution, the ether is transferred to a second separatory funnel, and the aqueous solution is returned

to the first and re-extracted as before. The second ether extract is added to the first, and the combined extracts are washed four times with 5 ml. portions of water. The washed ether is extracted successively with 3, 2, and 1 ml. quantities of a freshly prepared 4% sodium sulfide solution, which is quantitatively drawn off into a 10 ml. volumetric flask containing 3.5 ml. of pyridine used to stabilize the color formed. The solution is diluted to the mark with 50% pyridine and read in a spectrophotometer against a distilled water blank.

Johnson (36) considers the use of dioxane as final diluent to be less objectionable than pyridine, and Dickens (29) has found the use of ferrous sulfate to decolorize permanganate not consistently reliable and prefers hydrogen peroxide. He recommends a blank of sodium sulfide and dioxane. This is theoretically correct inasmuch as sodium sulfide is an unstable compound, and the preparation of its solution is not easily duplicated from day to day.

The following experiments are concerned with the preparation of pure and, so far as possible, stable reagents; the assembly and development of suitable apparatus; and the study of the reaction conditions from the viewpoint of the time and the reliability of the analytical procedure.

EXPERIMENTAL

The best grade of citric acid available on the market is a mixture of anhydrous citric acid and its monohydrate. The preparation of the pure monohydrate is complicated by the tremendous solubility of citric acid in water giving a syrupy solution from which the acid precipitates as a mixture of the monohydrate and anhydrous forms. In preparing a standard sample of citric acid for use in analysis, several trials showed the necessity of employing the mildest possible heating and drying procedures. In general, an excess of the U. S. P. acid was shaken in hot water, heated in a boiling water bath, filtered and allowed to stand overnight in a refrigerator. Supernatant liquid was decanted and the crystalline product treated further as indicated in Table I. The product No. 7 was originally selected as standard. Later trials showed the desirability of preparing a pure anhydrous material which was substituted because of its better keeping qualities.

TABLE I

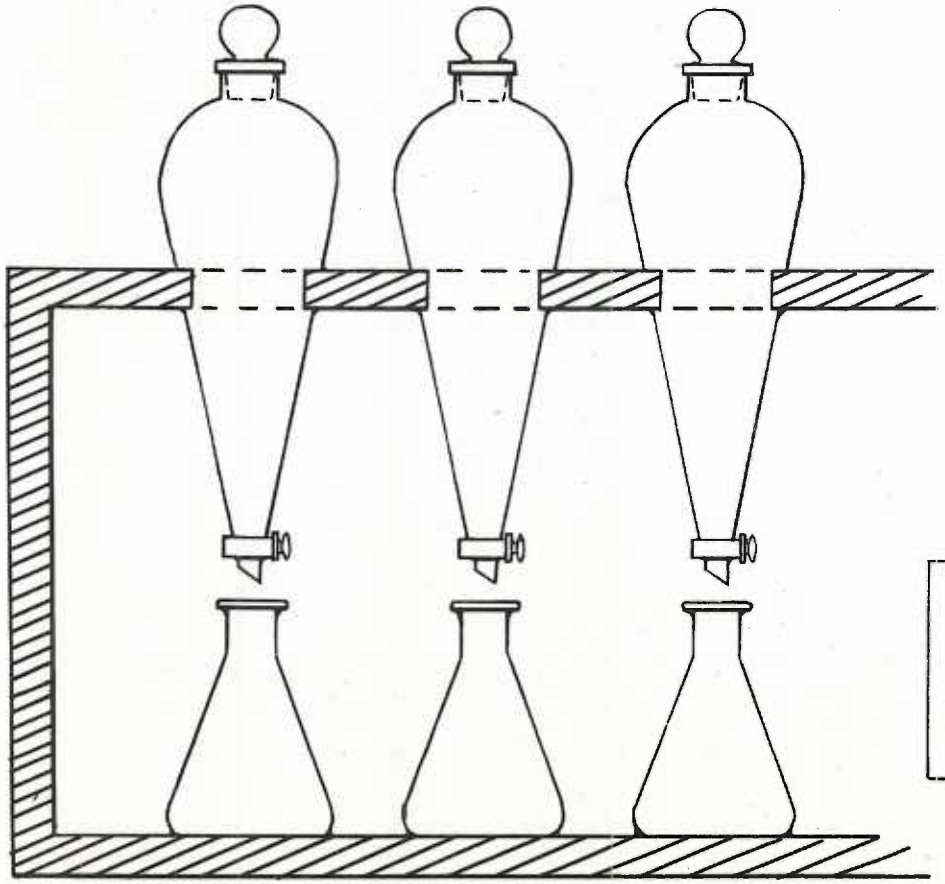
PREPARATION OF A CITRIC ACID STANDARD

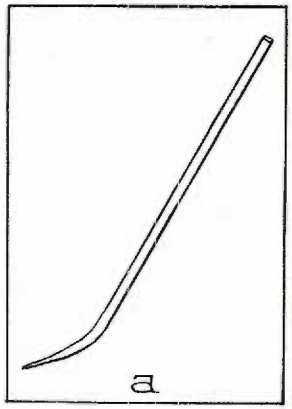
Product	Weight of Sample, gm.	0.1 Normal Sodium Hydroxide,* ml.	Anhydrous Citric Acid, gm.	Anhydrous Citric Acid, %	Water of Crystallization, calc'd, %	Moles Water per Mole Citric Acid, calc'd
1. U.S.P. Reagent	0.1050	16.25	0.1040	99.05	0.95	0.10
2. U.S.P. recrystallized once, first fraction, heated 1/2 hr., ground, dried in air 5 hrs.	0.0700	10.54	0.0675	96.37	3.63	0.40
3. U.S.P. recrystallized once, first fraction, heated 1/2 hr., ground, dried in air 24 hrs.	0.0700	10.63	0.0680	97.19	2.81	0.31
4. U.S.P. recrystallized once, first fraction, heated 1/2 hr., ground, dried in air 48 hrs.	0.0700	10.66	0.0682	97.46	2.54	0.28
5. U.S.P. recrystallized once, second fraction, heated 1/2 hr., ground, dried in air 24 hrs.	0.0700	10.71	0.0685	97.92	2.08	0.21
6. U.S.P. recrystallized once, both fractions, heated 1/2 hr., ground, dried over sulfuric acid 50 hrs.	0.0640	10.51	0.0673	105.00		
7. U.S.P. recrystallized once, first fraction, heated 10 min., dried in air	0.0700	10.00	0.0640	91.43	8.57	Monohydrate Standard, 1.00
8. U.S.P. recrystallized once, first fraction, heated 10 min., ground, dried in vacuum over phosphorus pentoxide	0.0640	9.74	0.0641	100.29		Anhydrous Standard

* Phenolphthalein was used as indicator, throughout.

The apparatus was set up as shown in Figure 1. A specially constructed wooden stand held the requisite number of marked 125 ml. separatory funnels. The ends of the funnels were beveled off short and a bent capillary tube, A, was used to remove the last drops of liquid from the separatory tube.

The initial experimental procedure adopted was as follows: a suitable dilution of the Citric Acid Monohydrate Standard was made in a volumetric flask. Aliquot portions were drawn off into numbered 125 ml. erlenmeyer flasks and diluted to about 40 ml. Each of these, after addition, from pipette, of 3 ml. of sulfuric acid, 2 ml. of 1 molar potassium bromide and 10 ml. of 1.5 normal potassium permanganate, was allowed to stand exactly fifteen minutes. Then the flask was quickly cooled to 6° C in an ice-salt mixture and titrated with cold hydrogen peroxide to the disappearance of the permanganate color. The bleached solution was transferred to a separatory funnel, 25 ml. of petroleum ether, B. P. 35-60° C, added, and the funnel was briskly inverted seventy-five times. A few preliminary trials demonstrated that shaking causes formation of undesirable emulsions. The number of inversions was selected by trial and error to give the most efficient extraction of the pentabromacetone and washing of the ether layer. The aqueous layer was drawn off into an erlenmeyer flask, the remaining ether layer was washed by twenty inversions with approximately 5 ml. of distilled water, and the wash fluid was added to the erlenmeyer. The entire contents of the erlenmeyer was poured into a second funnel and extracted with another 25 ml. of petroleum ether, fifty inversions being used. The ether layer was washed as before. The two ether layers were combined in the first funnel, the second being rinsed into the first with approximately 10 ml. of distilled water. The ether was washed with this by thirty-five inversions and was





APPARATUS
FOR EXTRACTION OF
PENTABROMACETONE

Figure 1

subsequently washed three times with about 5 ml. portions of water with twenty inversions. Two minutes were allowed for thorough draining of water from the other layer at this point. The ether was then extracted fifty times with 3 ml. of a freshly prepared 4% aqueous sodium sulfide solution. The sulfide extract was drawn off into a 10 ml. volumetric flask containing 3.5 ml. of dioxane. Two additional extractions, using 2 ml. of sodium sulfide solution with thirty-five inversions and 1 ml. of sodium sulfide with twenty-five inversions respectively were similarly performed. The volumetric flask was filled to the mark with 50% aqueous dioxane, inverted three times and the contents transferred to a Klett colorimeter tube in which readings were made on a Klett-Summerson Photoelectric Colorimeter (57) No. 163 with the F54 (green) filter. Analyses were performed in duplicate.

The data to the first trials is given in Table II. In these, blanks of distilled water, water and dioxane, and water, dioxane and sodium sulfide were used to check on the influence of the blank on the accuracy of the determination. Figure 2 shows that the water-dioxane-sodium sulfide blank gave the best results. This was not unexpected in view of the fact that sodium sulfide is an unstable as well as deliquescent compound, and daily variations in the composition of its solutions are unavoidable. The water-dioxane-sodium sulfide blank was, therefore, adopted for further determinations despite its complexity. The addition of sodium sulfide solution to dioxane is exothermic. It was found, however, that in preparing the blank if the full 6 ml. of sodium sulfide solution is added to 3.5 ml. of dioxane at the time of the first sulfide extraction of the sample, the temperature of the blank and of the sample is the same at the time of the colorimeter reading.

TABLE II

SELECTION OF A SUITABLE BLANK

Citric Acid, mg.	Colorimeter Reading, F54 Blank Adjusted to Zero		
	Water	Water-dioxane	Sulfide-dioxane
0.19	32.8	13.2	
0.32	28.9	23.4	15.5
0.45	41.0	34.3	22.6
0.56	47.6	36.2	29.0

TABLE III

DRIFT IN COLORIMETER READINGS

(AT 10 MIN. INTERVALS)

Citric Acid mg.	Colorimeter Reading, F54 Blank Adjusted to Zero		
	Water	Water-dioxane	Sulfide-dioxane
0.19	32.8, 29.3		
0.32			
0.45		34.3, 29.2, 26.3, 29.2	22.6, 20.4, 20.4
0.56	47.6, 46.2, 46.2	36.2, 34.8, 32.5, 32.2	29.0, 26.5, 26.2

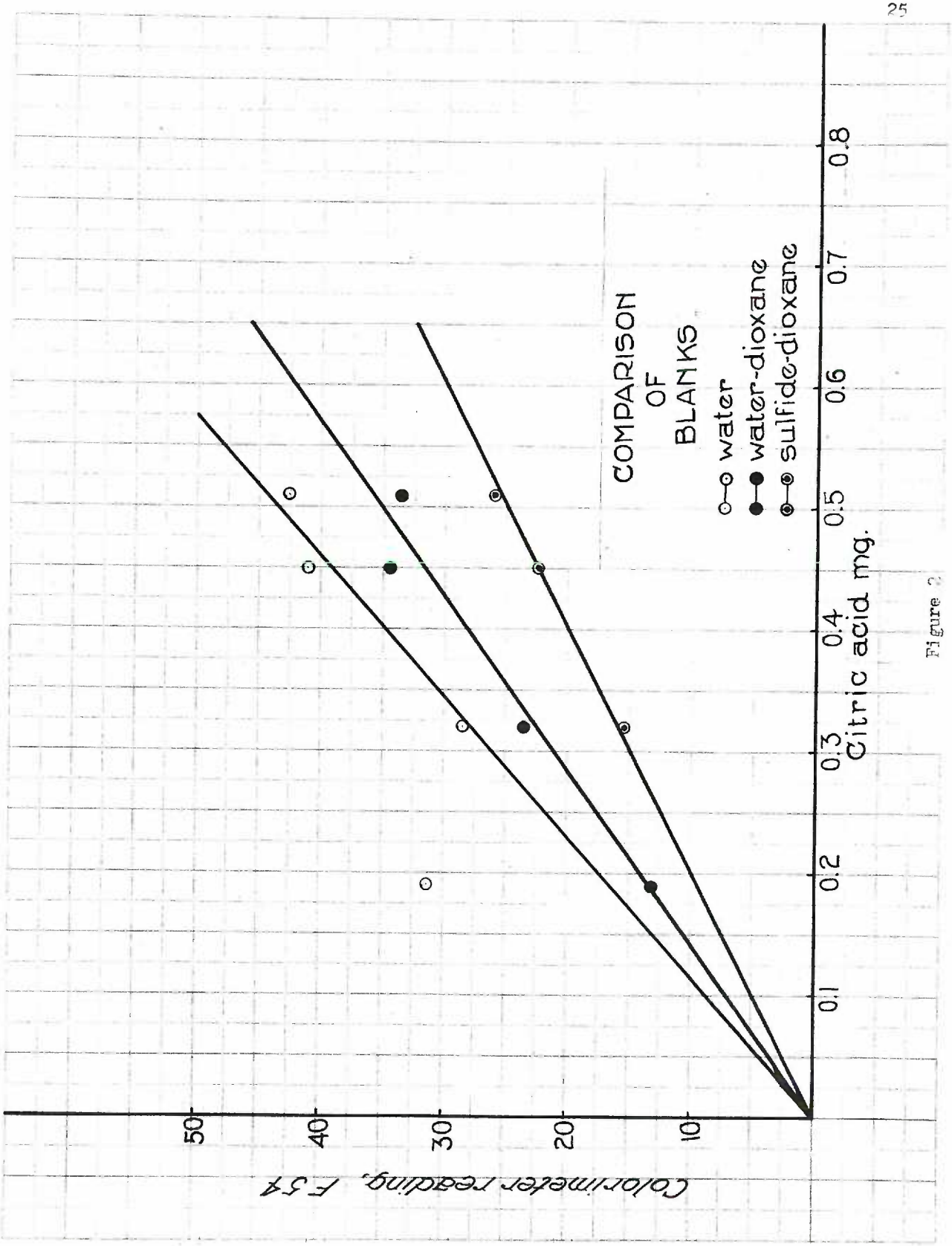

COMPARISON
OF
BLANKS
water
water-dioxane
sulfide-dioxane
Citric acid mg.
Colorimeter reading, F-54
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
10
20
30
40
50

Figure 2

It was noticed during the course of the first experiments that the color of the solutions faded. The degree of fading is reflected in the colorimeter readings reported in Table III. The presence of bubbles and odor of hydrogen sulfide in the colored solution suggested interference from impurities. An attempt to find a wave band insensitive to drift was made. The #42 (purple) filter was used in several subsequent determinations, but the drift was not eliminated (see Table IV). Alkali and sodium cyanide were then tried to stabilize the color, but also without success. Substitution of sodium thiosulfate and of sodium hydrogen sulfide solutions for the less stable sodium sulfide produced a yellow color which was relatively weaker than that of the sodium sulfide and was thus discarded. Sodium tetrathionate and thioglycolic acid gave no color. The purity of the petroleum ether was next investigated as a possible factor in causing the drift. Twenty ml. of petroleum ether was shaken in a separatory funnel with 5 ml. of 5% sodium sulfide solution. The results showed that the ether treatment left a turbid water layer - probably due to precipitation of sulfur. Ether dried by several weeks' contact with anhydrous sodium sulfate, on the other hand, left a clear water layer. In order to remove the interfering impurities in the ether, it was redistilled in an all-glass apparatus within the boiling range of 35-50° C.

It was obvious at this point that the problem of drift in the colorimeter readings was still not completely solved and that an investigation of the other reagents involved was indicated. In order to speed the work, it was decided to start the analytical procedure directly from pentabromoacetone, and, for this purpose, the compound was prepared according to directions given by Dickens (29). 0.6620 gm. of Citric Acid Monohydrate Standard yielded 1.4273 gm. of pentabromoacetone, M.P. 72° C. A single recrystallization

from 50% alcohol gave a first fraction of 0.4045 gm. (35% yield), M.P. 74° C.

The advisability of using a final diluent other than dioxane was considered. The presence of peroxide compounds in the commercial dioxane used was strongly suspected. A test with sulfuric acid and potassium iodide indicated this to be true, and attempts at removal were made without success. Several other solvents were tested for peroxides, as shown in Table V.

0.0071 gm. of pentabromoacetone was made up to 100 ml. with petroleum ether in a volumetric flask. This corresponds to a 3 mg. % citric acid solution. 10 ml. aliquot portions were used. A comparative run through the citric acid analytical procedure was made, using dioxane, pyridine and monomethyl ether of ethylene glycol as final diluents. Results are given in Table VI. A potassium dichromate solution (yellow) was used as standard of comparison to show normal error in colorimeter readings taken at various intervals. The methyl ether blank appeared dark and apparently contained metallic sulfides. The methyl ether was, therefore, redistilled at 118-124° C for subsequent use. The crude ether gave a white precipitate with sulfuric acid, whereas the redistilled product remained clear.

Because of variability in commercial samples of sodium sulfide and instability of its solutions, it was decided to prepare it directly from solutions of sodium hydroxide and hydrogen sulfide gas. These solutions were used within six hours of preparation. An apparatus was set up (Figure 3) for the preparation of sodium sulfide solutions from sodium hydroxide and hydrogen sulfide. Stock 1 normal sodium hydroxide solution, carbonate free, was adjusted to 0.5 normal and checked by titration against a sulfamic acid standard using phenolphthalein as indicator. 50 ml. of the hydroxide was pipetted into the apparatus and gassed with hydrogen sulfide from a

TABLE IV

DRIFT IN COLORIMETER READINGS

(AT 20 MIN. INTERVALS)

Citric Acid, mg.	Colorimeter Readings, FA2
0.05	20.0, 12.0
0.30	95.0, 75.0
0.50	114.0, 107.0, 96.0

TABLE V

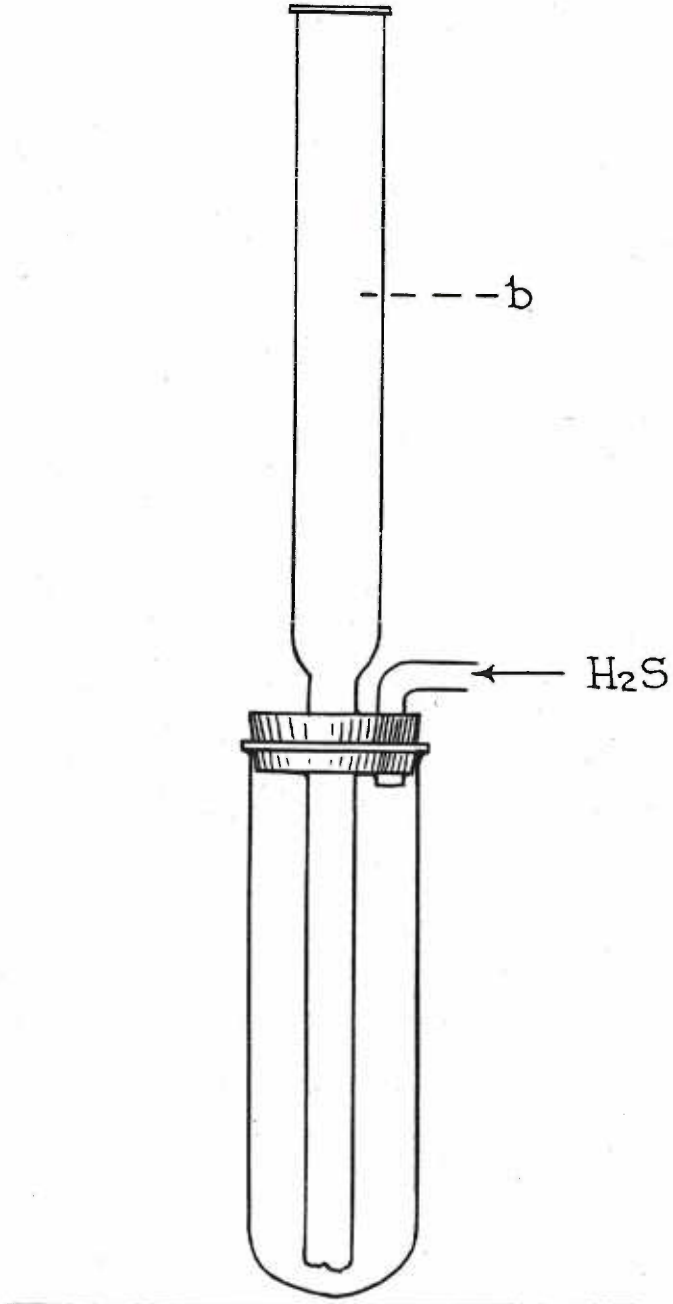
PRESENCE OF PEROXIDE IN VARIOUS SOLVENTS

Reagent	Peroxide Test
Petroleum Ether	-
Dioxane	+
Dioxane Kept Over Sodium, 3 mo.	+
Pyridine	-
Monomethyl Ether of Ethylene Glycol	-

TABLE VI

EFFECT OF FINAL DILUENT ON COLORIMETER READINGS

Diluent	Filter	Blank	Time Intervals of Colorimeter Readings, min.			
			0	15	30	45
Dioxane	54	Sulfide-dioxane	14	15	14	14
Dioxane	42	Sulfide-dioxane	85	78	67	65
Pyridine		precipitate				
Methyl ether	54	Water	123	123	123	123
Methyl ether	54	Sulfide - methyl ether	22	27	27	27
Methyl ether	42	Water	326			
Dichromate	42	Water	233	230	233	233



APPARATUS
FOR PREPARATION OF
HYDROGEN SULFIDE

Figure 3

cylinder. The gas flow was adjusted so that the meniscus of the solution in the apparatus fluctuated between two marks about a quarter of an inch apart on tube h. The time of gassing was exactly twenty minutes. 50 ml. of 0.5 normal sodium hydroxide was pipetted into the gassed solution and mixed.

$$NaOH + H_2S \longrightarrow NaHS + H_2O$$

$$NaHS + NaOH \longrightarrow Na_2S + H_2O$$

In this way, it was assumed that metallic impurities, so deleterious to the colorimetric procedure, were eliminated and the concentration of sodium sulfide, difficultly weighable because of its deliquescent character, maintained constant. Preliminary runs indicated that most of the drift in the colorimeter readings was eliminated (Table VII) by using sodium sulfide prepared as above.

To determine the reliability of the method at this point, the colorimeter was calibrated in terms of citric acid. Table VIII shows the readings obtained when varying amounts of pentabromacetone (0.0118 gm.% petroleum ether solution) were used as samples. Table IX gives the readings obtained when citric acid monohydrate was used as standard (0.0109 gm. % aqueous solution). Values of both tables are calculated to anhydrous citric acid. The conversion factor of pentabromacetone to anhydrous citric acid is 0.424; of the monohydrate to anhydrous citric acid, the conversion factor is 0.914.

It is apparent from the graphic representation in Figure 4 that the conversion of citric acid to pentabromacetone is not quantitative. The degree of conversion, as estimated from the curves of Figure 4, is given in Table X. For an empirical method, Figure 4 shows a satisfactory calibration curve.

TABLE VII

EFFECT OF PURIFICATION OF SODIUM SULFIDE ON COLORIMETER READINGS*

Pentabromoacetone in Terms of Citric Acid, mg.	Time Intervals of Colorimeter Readings, min. 0	20	40	60
0.3	46	46	46	48
0.6	53	53	55	55

* Sulfide - methyl ether blank, FS4 filter.

TABLE VIII

CALIBRATION CURVE DATA FOR PENTABROMACETONE

Aliquot, ml.	Equivalence in Anhydrous Citric Acid, mg.	Colorimeter Reading, F%
2	0.10	10.0
5	0.25	29.0
10	0.50	53.0
15	0.75	80.0
20	1.00	100.0
25	1.25	125.5

TABLE IX

CALIBRATION CURVE DATA FOR CITRIC ACID

Aliquot, ml.	Equivalence in Anhydrous Citric Acid, mg.	Colorimeter Reading, F%
3	0.30	22.0
5	0.50	29.0
5	0.50	35.0
7	0.70	49.0
10	1.00	70.0

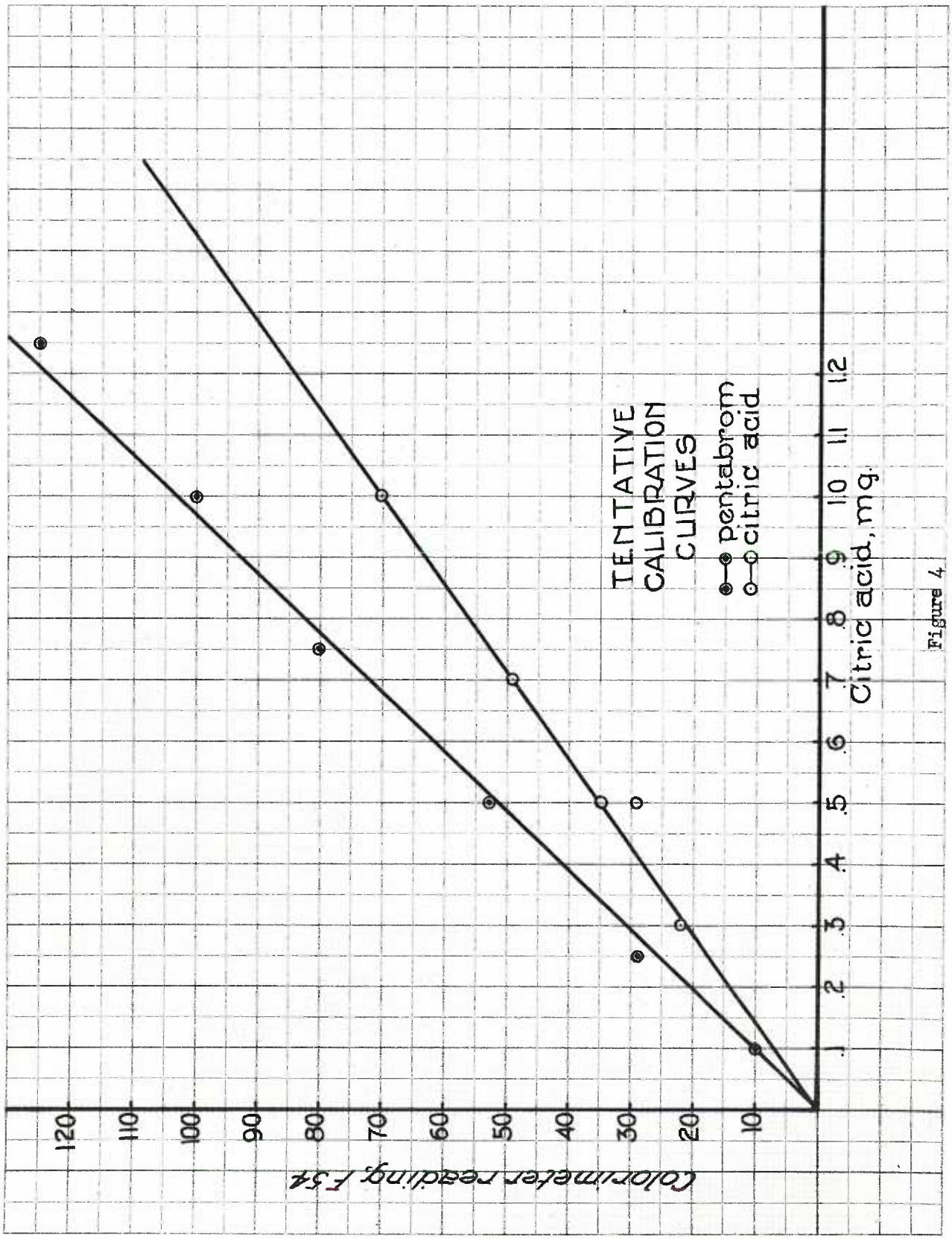

TENTATIVE
CALIBRATION
CURVES
pentabrom
citric acid
Citric acid, mg.
Colorimeter reading, F 54
10
20
30
40
50
60
70
80
90
100
110
120
1
2
3
4
5
6
7
8
9
10
11
12

Figure 4

TABLE X

DEGREE OF CONVERSION OF CITRIC ACID TO PENTABROMACETONE

Citric Acid, mg.	Pentabromacetone, in Terms of Citric Acid, mg.	Conversion, %
0.300	0.210	70
0.500	0.335	67
0.700	0.470	69
1.000	0.665	67
	Average	68

The best point of attack for shortening the time of the analytical procedure seemed to be in eliminating the peroxide treatment. To this end several reducing substances were investigated with the findings shown in Table XI.

The possibility of using oxalic acid presented itself. When, however, a gram was added at once to a 0.5 mg. sample of citric acid run through the usual oxidation procedure, subsequent analysis indicated a complete destruction of the pentabromoacetone.

As a last resort, it seemed worth while to check the opinions of Pucher et al. and Dickens that pentabromoacetone is destroyed by small excesses of hydrogen peroxide or during titration with it at room temperature. Experiments performed in this direction led to the conclusion that as much as a 2 ml. excess of peroxide, either in the cold or at room temperature, had no effect whatever on the course of the analysis as long as the peroxide was properly washed out before the addition of sodium sulfide. The presence of peroxide with sodium sulfide always caused some precipitation of sulfur. The washing procedure required for the removal of other impurities, however, was quite adequate also for the removal of hydrogen peroxide (see Table XII). An interesting observation came to light - namely, that the reduction of permanganate by hydrogen peroxide is slightly faster at lower than at higher temperatures, suggesting that the reaction is of a higher reaction order. The importance is purely theoretical and bears no practical significance, as the reaction was in any case complete in the time required for the titration.

Calibration curves were obtained as given in Table XIII and Figure 5 with the entire analytical procedure performed at room temperature.

TABLE XI

REDUCING ACTION ON ACID PERMANGANATE AND BROMINE AT ROOM TEMPERATURE

Reagent		Nature of Reducing Action
Butylene		precipitate with bromine
Acetaldehyde		incomplete
Formic Acid		nearly complete
Formaldehyde		incomplete
Oxalic Acid -	0.50 gm. added at once	incomplete
	0.75 gm. added at once	incomplete
	0.80 gm. added at once	incomplete
	1.00 gm. added at once	complete
	1.00 gm. added gradually	incomplete

TABLE XXI

EFFECT OF HYDROGEN PEROXIDE TITRATION ON PENTABROMACETONE

Date of Analysis	Citric Acid Equivalent in Aliquot, mg.	Procedure	Colorimeter Reading, FS4
8-20-42	0.5*	Usual standard method.	49.0
8-20-42	0.5*	Same, after 2 min. shaking with peroxide.	49.0
8-21-42	0.5**	Usual standard method.	35.0
8-21-42	0.5**	Same, with 2 ml. excess peroxide.	35.0
8-24-42	0.5**	Usual standard method	35.0
8-24-42	0.5**	Same, at room temperature with 2 ml. excess peroxide.	35.0
8-26-42	0.5**	Usual standard method.	35.0
8-26-42	0.5**	Same, at room temperature with 2 ml. excess peroxide.	35.0

* 10 ml. aliquot of 0.0118 gm. pentabromacetone in 100 ml. petroleum ether solution.

** 5 ml. aliquot of 0.0109 gm. Citric Acid Monohydrate Standard in 100 ml. aqueous solution.

TABLE XIII

CALIBRATION CURVE DATA FOR CITRIC ACID

Citric Acid in Aliquot,* mg.	Colorimeter Reading, F54
With 3 ml. sulfuric acid per aliquot, colorimeter No. 761	
0.05	1.50
0.15	6.50
0.15	8.30
0.30	17.00
0.30	17.00
0.30	17.20
0.50	34.50
0.50	35.00
0.70	45.50
0.80	49.00
0.80	50.30
0.90	58.30
1.00	64.00
	63.20
With 5 ml. sulfuric acid per aliquot, colorimeter No. 163	
0.2	16.0
0.2	16.0
0.6	42.0
0.6	43.5
0.6	39.5
1.0	71.0
1.0	70.8
1.0	71.5
1.4	97.0
1.4	101.6
1.4	95.0
1.4	97.0
1.8	126.0
1.8	128.0

* 10 mg. % aqueous solution of Anhydrous Standard.

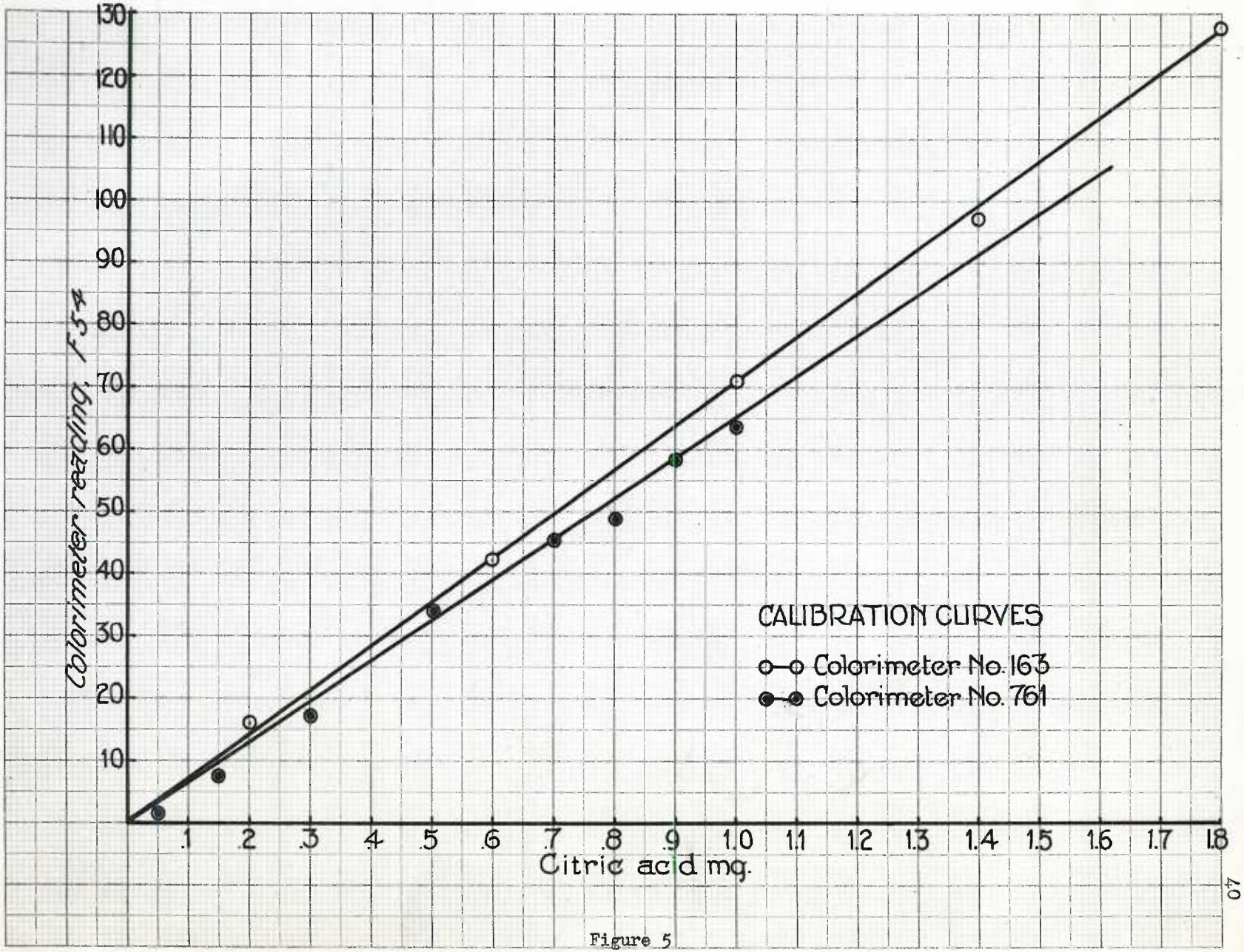

Colorimeter reading, F54
130
120
110
100
90
80
70
60
50
40
30
20
10
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
Citric acid mg.
CALIBRATION CURVES
Colorimeter No. 163
Colorimeter No. 761

Figure 5

It has also been found advisable to check the uniformity of the Klett colorimeter tubes and to select matched tubes or to use always the same two tubes for blank and for sample.

Bubbles due probably to air and to hydrogen sulfide appear occasionally in the colorimeter tubes and lower the colorimeter readings. This difficulty is most likely to become manifest in hot weather. It is overcome by stirring the colored solutions and by keeping them in a shallow pan of cool tap water until their transfer into the Klett tubes. Accurate temperature control is superfluous.

The use of 5 ml. of sulfuric acid per sample instead of 3 ml. of sulfuric acid improves the uniformity of results.

Biological samples require preliminary treatment to remove interfering substances. The pretreatment was performed essentially as suggested by Pucher et al.

Urine: the sample to be analyzed was thoroughly mixed and a 1 to 5 ml. aliquot was pipetted into a 125 ml. erlenmeyer flask marked at about 40 and 60 ml. levels. The sample was diluted to the 60 ml. mark with distilled water, 3 or 5 ml. of 50% sulfuric acid was added, and, after mixing, the sample was placed on a hot plate to evaporate rapidly to the 40 ml. mark. After cooling to room temperature, 3 ml. of saturated bromine water was added, and the mixture was allowed to stand for ten minutes. If the analysis could not be performed immediately, the vessel was covered and placed in a refrigerator. The solution was finally transferred to a 50 ml. lipped centrifuge tube, centrifuged, and the supernatent solution separated for analysis.

Bloods: 10 ml. of freshly drawn blood was pipetted into 40 ml. of 10% trichloracetic acid contained in a 50 ml. lipped centrifuge tube. The

precipitated proteins were removed by centrifuging. The sample was drained into a 125 ml. marked erlenmeyer flask and pretreated as for urine.

Feces: A day's sample of feces was collected in 300 ml. of 2.5 N sulfuric acid and diluted with water to 500 ml., in a large graduated cylinder. It was allowed to stand in a covered jar for about three days or until peptized. The sample was then filtered through a coarse paper and a 25 to 50 ml. aliquot pipetted into a marked 125 ml. erlenmeyer flask. Enough solid trichloracetic acid was added to make a 5% solution (1.25 or 2.50 gm.). After standing fifteen minutes to precipitate the protein material, the sample was centrifuged, returned to the erlenmeyer flask, diluted to the 60 ml. mark and pretreated thereafter as for urine and blood, but without further addition of sulfuric acid.

Data for analyses performed to test the recovery of citric acid from biological material are given in Table XIV. Colorimeter No. 169 was used.

TABLE XIV

RECOVERY OF CITRIC ACID FROM BIOLOGICAL MATERIAL

Aliquot, ml.	Citric Acid Added, mg.	Colorimeter Reading, F54	Citric Acid Found, mg.
From urine:			
1.0		55.0	
1.0		55.0	0.768
1.0	0.3	78.3	
1.0	0.3	78.3	1.090
		Recovered,	0.322
From feces:			
25		50.0	
25		50.0	0.698
25	0.3	72.0	
25	0.3	71.0	0.996
		Recovered,	0.298
From blood:			
8		16.0	
8		16.0	0.220
8	1.0	88.0	
8	1.0	87.5	1.220
		Recovered,	1.00

DISCUSSION

The pentabromacetone method of determining citric acid is empirical and, as such, demanding of the analyst. Its dependence on color formation with sodium sulfide makes it extremely sensitive to the presence of metallic impurities, which precipitate as sulfides, and to peroxides, which liberate molecular sulfur, necessitating careful purification of reagents and exacting washing procedures. These have been described. Occasional presence of bubbles in the colorimeter tube has also a deleterious effect, lowering the colorimeter reading. This can be eliminated by stirring and by keeping the volumetric collection flasks in a shallow pan of cool tap water during the sulfide extraction.

The color involved in the reaction appears to be absolutely stabilized, in the absence of interfering impurities, by the use of the monomethyl ether of ethylene glycol as a final diluent.

The standardization of sodium sulfide has been effected by a reproducible method of its preparation from sodium hydroxide solution and hydrogen sulfide gas in the apparatus depicted in Figure 3.

The procedure of cooling samples during the reactions with permanganate and bromine and the care to avoid slight excesses of hydrogen peroxide have been shown to be unnecessary.

The apparatus assembly adopted for the analytical procedure is shown in Figure 1.

The scheme finally adopted for the analysis for citric acid is as follows:

40 ml. of the pretreated sample is subjected to the oxidation procedure with 5 ml. of 50% sulfuric acid, 3 ml. of 1 molar potassium bromide and 10 ml. of 1 normal potassium permanganate for fifteen minutes. The excess permanganate and bromine are removed at room temperature by delivery of an approximately stoichiometric quantity of hydrogen peroxide from a burette. The pentabromacetone is extracted with redistilled petroleum ether (B.P. 35-50° C) and the extract is washed with water. This extraction and washing is repeated once. The two ether extracts are combined and washed again. Finally, they are extracted with freshly synthesized sodium sulfide solution. The number of inversions of the separatory funnel recommended for the extraction and the washing procedures are indicated schematically as follows:

Petroleum Ether Extraction and Washings

	Number of Inversions for Extraction	Number of Inversions for Washing			
		First Wash	Second Wash	Third Wash	Fourth Wash
First Extract	75	20			
Second Extract	50	20			
Combined Extract		35	20	20	20

Sodium Sulfide Extractions:

	Number of Inversions for Extraction
First Extract	50
Second Extract	35
Third Extract	25

The sodium sulfide extracts are run into a 10 ml. volumetric flask containing 3.5 ml. of the monomethyl ether of ethylene glycol, made up to volume with this solvent, and read in the colorimeter.

PART II

THE RELATION OF CITRIC ACID TO RENAL CALCULI FORMATION

Calcium citrate is more soluble than calcium phosphate, oxalate and carbonate, and this fact combined with its low dissociation suggests that it may exert some protective action against precipitation of calcium phosphate, carbonate and oxalate. Hartensson (58) mentions that it increases the solubility of calcium oxalate and tends to hold calcium phosphate suspended. On the other hand, Hartensson also points to the presence of citric acid in various pathological calcifications and concretions in amounts between 0.1 and 0.9 percent. Klosin and Locke (39) found low citrate concentrations in the urine of patients with calcium urolithiasis and suggested that this may have etiological bearing on the problem of stone formation. Albright, Sulg, Rawls, West and others have performed successful dissolution of renal and vesical stones with citrate buffers. Higgins (60) produced stones in rats by depriving them of vitamin A during the active growth period and Hammaresten (61) obtained similar results with rats deprived of vitamins A and D and magnesium. Although these authors did not connect the incidence of lithiasis with citric acid metabolism, it appeared worth while to determine whether deprivation of vitamin A and retardation of growth have any effect on urine citric acid concentration.

Part II is concerned with the study of the relation of citric acid metabolism to renal stone formation in humans and the effect of avitaminosis A on urinary citric acid in rats.

EXPERIMENTAL

The first subject selected for this study was a thirty-three year old seventy-eight kilogram male with a history of thirteen years' recurrent passage of stone. Four years previous to the study an operation had been performed to remove a stone impacted in the right ureter. Shortly following the operation, X-ray revealed a small stone in the lower calyx of each kidney. At the time of the study, both kidneys were infected with staphylococcus, blood and urine chemistry was generally normal, blood urea nitrogen was 25 mg.% and the basal metabolic rate varied between -9 and -20%. The subject was otherwise healthy and his appearance was entirely normal.

An analysis was performed on the stone removed surgically from subject 1 (Table XV). The analytical procedures used were essentially those recommended by McIntosh and Salter (62). A few confirmatory tests were run by alternate procedures as indicated in the table. The stone was practically pure calcium oxalate crystal without matrix and with only a trace admixture of calcium phosphate.

Table XVI is representative of the subject's usual diet.

Table XVII shows the nitrogen content of the subject's urine. This table was compiled because Hedes (63) found that urea enhances the solubility of calcium oxalate. No abnormality in urea content was found for the urine of the subject 1. The analyses were run by the method of Rinehart, Greenahl and West (64).

Urine sediments from the subject 1 were examined. The sediment in fresh urine appeared normal. Urine heated for half an hour at 70° C and filtered through a fritted glass funnel to remove coagulated colloids threw down crystals in a few days. These gave a strong phosphate test and weak

test for carbonate and oxalate. They were assumed to be magnesium ammonium phosphate precipitated as a result of alkalinization of the urine through decomposition of urea by staphylococcus organisms in the urine (Figure 6).

Preliminary trials on the solubility of calcium oxalate and calcium phosphate stones in alkaline citrate solution are reported in Table XVIII. These indicate that precipitation of calcium oxalate and phosphate is inhibited by the presence of citrate even in alkaline solution.

TABLE XV

ANALYSIS OF STONE

Macroscopic appearance: The stone was pulley-shaped, 7 mm. in diameter, 2 mm. thick, and weighed 0.06 gm. It had a crystalline sparkle and creamy color.

Microscopic appearance: The fragmented stone was composed of perfect crystals with triangular faces. A trace of amorphous substance was found, possibly due to crushing. No organic matrix was visible.

Urates	absent
Xanthine	absent
Cystine	absent
Carbonate	absent
Phosphate	trace
Phosphate (65)	trace
Organic residue	trace
Mucous threads	trace
Casts	absent
Ammonia	absent
Oxalate	present
Non-oxalate calcium	trace
Magnesium	absent
Citrate (55)	absent
Citrate (66)	absent

TABLE XVI

REPRESENTATIVE DIET OF SUBJECT 1

5-20-42

Breakfast

1 egg, 1 slice of bacon, 1 piece of toast, 1 cup of coffee, 1 orange.

Lunch

1 ham sandwich, 1 cup of coffee, 1 cupcake.

Supper

turkey with dressing, lettuce, asparagus and tomato salad, 1 cup of coffee, potatoes, 1 Coca Cola, 1 strawberry sundae.

5-21-42

Breakfast

1 egg, 2 slices of bacon, 1 cup of coffee, 1 orange.

Lunch

1 salmon sandwich, 1/2 peanut butter and jelly sandwich, 3 cookies, 1 cup of coffee.

Supper

1 slice of meat loaf, 1 dressed egg, 1/2 crab sandwich, 6 cheese crackers, 1 slice of tomato, potato salad, 3 carrot straws, 1 glass of berry juice, 1/2 glass of milk.

5-22-42

Breakfast

1 egg, 1 slice of bacon, 1 slice of toast, 1 cup of coffee, 1 dish of boysenberries.

TABLE XVI, continued

Lunch

potato salad, meat loaf, noodle soup, cake, boysenberries, cheese.

Supper

potato salad, steak, berries and cream, squash, ice cream, 5 cookies.

5-23-42

Breakfast

1 egg, toast, 1 cup of coffee, 2 oranges, boysenberries.

Lunch

scalloped potatoes with ham and cheese, asparagus, carrot straws, custard, cake.

Supper

potatoes, corn, pork steak, 1 Coca Cola.

5-25-42

Breakfast

1 egg, 2 sausages, toast, coffee, plums.

Lunch

1 salmon sandwich, 1 cake, 1 orange, 1 Coca Cola.

Supper

tomato salad with cottage cheese, ham, egg, toast, pears.

TABLE XVII

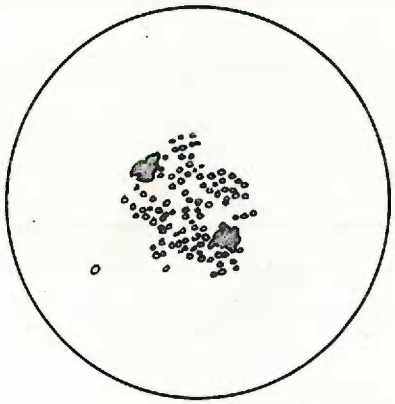
URINE NITROGEN FOR SUBJECT A

The following data refers to control sample 8-27-43; volume: 1338 ml.; pH: 6.5; specific gravity: 1.018.

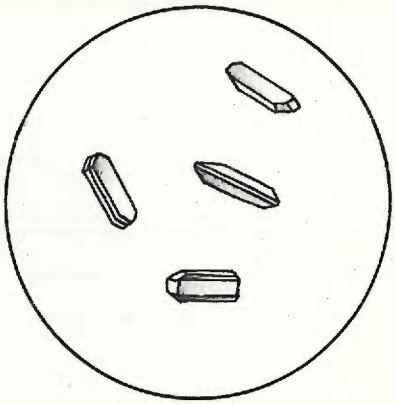
Aliquot, ml.	N/70 Sodium Hydroxide, ml.	Result, gm. per day
Urea nitrogen		
2	6.75	9.03
2	6.97	9.33
	Average,	9.18
Ammonia nitrogen		
5	13.20	0.706
Total nitrogen		
10*	9.60	10.19
10*	9.90	10.26
	Average,	10.22

* 1:40 dilution

URINE SEDIMENTS FOR SUBJECT 1

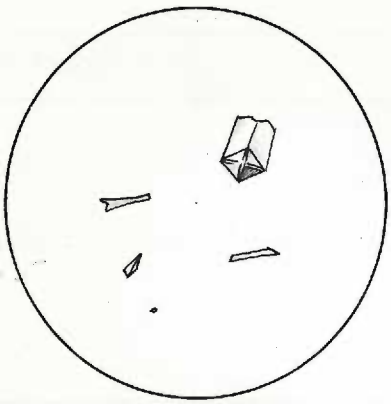


10 X 10
Control

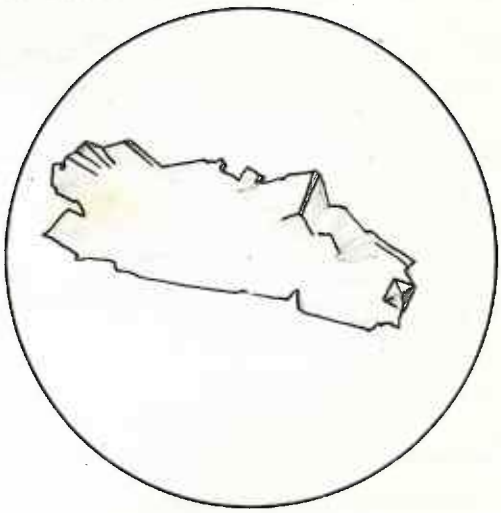


10 X 10
Experimental

STONE OF SUBJECT 1



10 X 10
Fragmented stone



15 X 18
Fragment of stone
Binocular view

Figure 6

TABLE XVIII

SOLUBILITY OF POWDERED STONE

Character of Stone	Weight in 4 ml., mg.	Solvent	0.01 Normal Permanganate for 1 ml. aliquot, ml.	Calcium in 1 ml. aliquot, mg.
Oxalate	5.0	Water	0.35 0.23	0.058
Oxalate	5.0	0.5 N Sodium citrate	1.62 1.50	0.312
Phosphate	5.0	Water	0.05 0.05	0.010
Phosphate	5.0	0.5 N Sodium citrate	0.60	0.120

A control run was made on seven 24 hr. urine specimens to determine the daily output of citric acid and calcium and to check on the possibility of a deviation from the normal in magnesium content. A similar control run was made also on two blood samples. 40 ml. of post-absorptive blood was drawn from the vein without the use of anticoagulant. 20 ml. of the blood was pipetted into 80 ml. of 10% trichloracetic acid for duplicate citric acid analysis. The remainder was reserved for serum.

Citric acid was determined by the method developed in Part I. Colorimeter No. 761 was used. Calcium analyses were performed by the Clark-Collip modification (67) of the Tisdall method; magnesium, by a modified Denis method (68). Urine pH was measured with nitrazine paper.

The results (Table XIX) show that calcium and magnesium in urine and in blood were within the normal range. Urine citric acid was slightly below average normal on the basis of the findings of Kiesin and Looks (59). These authors give 0.63 gm. per day as the average daily urine citric acid for sixteen normal subjects and 0.16 gm. per day for sixteen patients with calcium urolithiasis. Scherstem (69) reports human blood citric acid to vary between 1.5 and 4.0 mg. %. Subject 1, accordingly, presents a blood citric acid value within the normal range.

It is interesting to note that the presence of blood in urine sample 1-9-43 was connected with diuresis, a sharp fall in citric acid, and a rise in calcium. Such phenomena may be related with irritation of the kidney and a temporary disturbance of its function.

TABLE XIX

ANALYSES FOR SUBJECT 1

Urine

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Colorimeter Reading, F54	Magnesium per Day, gm.
1-7-43			31.6					
1-7-43	1240	6.5	28.1	0.55	1.45	0.397		
1-7-43			25.6		1.43			
1-8-43			32.7					
1-8-43	1095	6.2	30.4	0.52	1.22	0.267	143.0	
1-8-43			27.6		1.22		143.0	0.077
1-9-43			7.8		1.32		123.0	
1-9-43	1666	6.5	7.1	0.19	1.26	0.430	116.0	0.098
1-12-43			30.5		1.34		146.0	
1-12-43	1140	6.0	26.3	0.50	1.32	0.303	142.0	0.080
1-13-43			35.0		1.47		172.0	
1-13-43	997	6.2	36.0	0.54	1.49	0.289	172.0	0.082
1-14-43			32.4		1.43		150.0	
1-14-43	1172	6.4	31.3	0.58	1.42	0.334	164.2	0.090
1-15-43							88	
1-15-43	1446	6.2	23.0	0.51			92	0.064
				Average, 0.48		Average, 0.330		Average, 0.083

Blood

Date of Sample	Colorimeter Reading, F54	Citric Acid, mg %	0.01 Normal Permanganate, ml.	Calcium, mg %	Colorimeter Reading, F54	Magnesium, mg %
2-12-43	11.2		1.15		74.0	
2-12-43	10.0	2.01	1.12	11.17	69.5	1.74
2-12-43			1.08		105.0	
2-13-43	9.4		1.10		101.2	
2-13-43	9.2	1.77	1.07	10.90	101.5	2.13
2-13-43			1.10			
		Average, 1.89		Average, 11.04		Average, 1.94

Subject 2 was another stone case. Three years previous to this study, he had been hospitalized for traumatic injury to the left kidney and fractures of elbow, wrist, ribs, and possibly skull. Infection and a septicemia resistant to sulfathiazole treatment necessitated nephrectomy. Six months later, a stone was discovered in the right kidney. Nephrolithotomy was performed. The stone removed was composed essentially of calcium phosphate. One year later, the subject had an attack of kidney colic without any visible evidence of stone. The attack passed uneventfully. At the time of this study, subject 2 was twenty-five years old, weighed sixty-eight kilograms, and was in good health.

The analyses of his urine and blood are given in Table XX. Urine citric acid was low, urine calcium was low, urine organic acids were normal, and volume and specific gravity relations indicated correct kidney function. Blood citric acid was in the normal range.

TABLE XX

ANALYSES FOR SUBJECT 2

Urine

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity	0.2 Normal Hydrochloric, ml.	Organic Acids per Day per kg.	Benzidine Test
7-17-43*			29.8		0.73					
7-17-43*	885	6.4	26.0	0.19	0.73	0.129	1.022	7.45	7.13	-
7-26-43*			31.2		0.80					
7-26-43*	1445	7.0	31.2	0.35	0.75	0.224	1.014	7.00	10.86	-
7-31-44**			56.7		0.96					
7-31-44**	1583	6.5	61.3	0.43	0.98	0.194	1.020			
			Average,	0.32	Average,	0.182				

* 2 ml. aliquot
** 3 ml. aliquot

Blood

Date of Sample	Colorimeter Reading, F54	Citric Acid mg.%
7-31-44	16.0	
7-31-44	16.0	2.75
8-1-44	20.5	
8-1-44	20.5	3.51
	Average,	3.09

On the basis of Kissin's data that urinary lithiasis is connected with a low urine citric acid, it seemed plausible that other pathological calcifications might present the same picture. Subject 3, with renal tuberculosis, was selected for investigation of this possibility.

Subject 3 was a male, forty-four years of age, and weighed eighty-one kilograms. His case history showed that ten years previous to the study he had had symptoms of renal tuberculosis. Two years previous to the study, he had had an epididymectomy for tubercular epididymitis. At the time of the study, he was confined at the Tuberculosis Hospital for treatment of fibrotic obstruction of the bladder. X-rays revealed calcification of the left kidney, ureter, and bladder. Pyogenic pyelonephritis developed after cystoscopy. The patient died of miliary tuberculosis five months after admission. Autopsy confirmed the presence of calcifications. Both kidneys were affected to some extent. The calcifications were in the form of plaques. There was no stone.

Table XXI shows that, as in the case of subject 1, blood citric acid was in the normal range and urine citric acid was low. Urine calcium and total organic acids were normal. Benzidine tests (70) indicated hematuria in one instance. Notable is the resemblance of this instance, sample 7-12-43, to that of hematuria in subject 1, sample 1-9-43, as to the unusually low citric acid and relatively high calcium. The low specific gravity and high volume of urine for subject 3 suggest impairment of renal function.

TABLE XXI

ANALYSES FOR SUBJECT 3

Urine

Date of Sample	Volume ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity	0.2 Normal Hydrochloric, ml.	Organic Acids per Day per kg.	Benzidine Test
7-12-43			7.0		0.97					
7-12-43	1750	5.7	7.0	0.18	0.96	0.338	1.007	5.50	8.47	+
7-13-43*			35.5		0.72					
7-13-43*	1266	5.8	45.0	0.40	0.85	0.199	1.011	4.80	5.25	-
7-14-43*			24.5		0.84					
7-14-43*	1760	5.6	23.0	0.33	0.84	0.296	1.009	4.90	7.47	-
			Average,	0.30	Average,	0.278				
After pyogenic infection -										
9-1-43			3.0							
9-1-43	2520	6.0	3.0	0.05			1.011			-

Blood

Date of Sample	Colorimeter Reading, F54	Citric Acid mg.%
7-15-43**	28.7	2.72

* 2 ml. aliquot
** 16 ml. sample

Coppridge (71) and many others have remarked on the rarity of stone in negroes. It has been attributed alternately to their purportedly low calcium diet and to their supposed immunity to pyogenic infection. Literature disclosed no data on the ratio of calcium to citric acid in the urine of negroes. Subject 4, a normal negro twenty-three years of age and weighing seventy-nine kilograms was available for such study. His urine showed a high normal value for citric acid and a low normal value for calcium (Table XXII). A study of many cases would be necessary to establish the normal urinary citric acid of negroes and any relation to the low incidence of lithiasis among them.

Subject 5 was a forty-nine year old male with a recent incidence of stone. The nature of the stone was unknown. Citric acid was high, calcium was low, and cystine was absent (Table XXIII). If the stone in subject 5 was composed of calcium salts, the urinary citric acid-calcium ratio was exceptional.

TABLE XXII

ANALYSIS FOR SUBJECT 4

Urine

Date of Sample	Volume ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity	0.2 Normal Hydrochloric, ml.	Organic Acid per Day per kg.
8-11-43			53.0		0.95				
8-11-43	1300	6.5	50.0	1.05	0.95	0.247	1.018	5.9	7.8

TABLE XXIII

ANALYSES FOR SUBJECT 5

Urine

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid* per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity	Benzidine Test	Cystine
5-2-44			93.0		0.77				
5-2-44	1425	5.2	92.0	0.92	0.73	0.213	1.034	-	-
5-3-44			102.0		0.77				
5-3-44	1252	5.3	102.0	0.89	0.77	0.193	1.028	-	-
5-4-44			122.0		0.75				
5-4-44	1219	5.4	120.0	1.03	0.77	0.185	1.023	-	-
5-5-44			90.0		0.61				
5-5-44	1125	5.2	92.0	0.72	0.63	0.137	1.023	-	-
				Average, 0.89		Average, 0.182			

* 2 ml. aliquots were used and Colorimeter No. 163

Calcium lithiasis in rats has been reported as a symptom of vitamin A deficiency. In 1943 Higgins (60) reported in a study on one hundred and fifty rats that eighty-eight percent of the rats fed vitamin A deficient rations developed bladder stone and forty-one percent developed renal stone in two hundred and fifty days. The following experiments were designed to determine whether avitaminosis A results in decreased citric acid output in the urine.

Fifteen rachitic sixty-day old male rats were fed balanced rations ad libidum for one week, at which time rickets was assumed to have disappeared. On 6-16-43 the rats were grouped into three cages, five rats to a cage, and the groups were designated 0, I, and II. Each rat was ear-marked and received an individual number, the numbers running from 1 to 5 for each cage. Rats are designated by cage and individual numbers. To illustrate - 05 is rat 5 of cage 0; 13 is rat 3 of cage I; 21 is rat 1 of cage II.

The following basic diet was given adlibidum to all three groups:

Crude ground casein	18%
Dextrin	65
Olive oil, heated to 170° C	10
Ground yeast	2
Salt mixture	5

The salt mixture contained:

NaCl	17.3 gm.
$MgSO_4$ 7 H_2O	54.5
NaH_2PO_4 H_2O	24.7
K H_2PO_4	95.4
$Ca(HPO_4)_2$ H_2O	54.0
$FeSO_4$ 7 H_2O	11.6
$Ca(C_6H_{11}O_7)_2$	30.0

Group 0 was used as control and was given a drop of cod liver oil per rat as indicated further. The administration was by dropper directly into the mouth of each rat. Groups I and II were used for experimental purposes and were given viosterol instead of cod liver oil in exactly the same manner as group 0. Wheat germ oil was given to all fifteen rats individually by dropper in the dose of one drop per rat about once weekly. The amount of food consumed by the rats at various times is given in Table XXIV. Weights of the rats were recorded periodically. Figure 7 represents growth curves of the control and the experimental animals. At intervals, a 24 hr. urine specimen was collected from each rat by means of an individual metabolism cage (Figure 8). This was constructed of a glass funnel 7 in. in diameter topped by a cylindrical housing of wire. The cage floor was an insert of the same wire covered by a circle of finer wire. The funnel stem contained a glass wool plug and dipped into a 25 ml. graduated cylinder containing 1 ml. of toluene. A wooden stand was used as funnel support and wooden slabs, weighted as needed, as cage covers. Water was supplied from an automatic delivery flask strapped to the cage. A tall, narrow food box was strapped inside the cage. At the end of the collection period, each funnel was carefully rinsed into the graduated cylinder with distilled water delivered by atomizer. All the rats, when first placed on the basic diet and vitamin supplements, took about the same amount of food - 10 gm. per rat per day - and the same amount of water - 20 ml. per rat per day.

TABLE XXIV

FOOD CONSUMPTION OF RATS

Date	Number of Group	Daily Food Intake per Rat, gm.
8-18-43	0	10.0
8-18-43	I	10.0
8-18-43	II	10.0
9-24-43	0	14.0
9-24-43	I	5.7
9-24-43	II	5.7
10-4-43	0	18.6
10-4-43	I	24.4
10-4-43	II	19.2
10-28-43	0	22.4
10-28-43	I	22.2
10-28-43	II	22.0
11-24-43	0	19.0
11-24-43	I	17.0
11-24-43	II	15.0
12-6-43	0	14.0
12-6-43	I	15.2
12-6-43	II	15.2
12-10-43	0	13.0
12-10-43	I	10.2
12-10-43	II	10.8
1-7-44	0	16.5
1-7-44	I	14.4
1-7-44	II	13.1

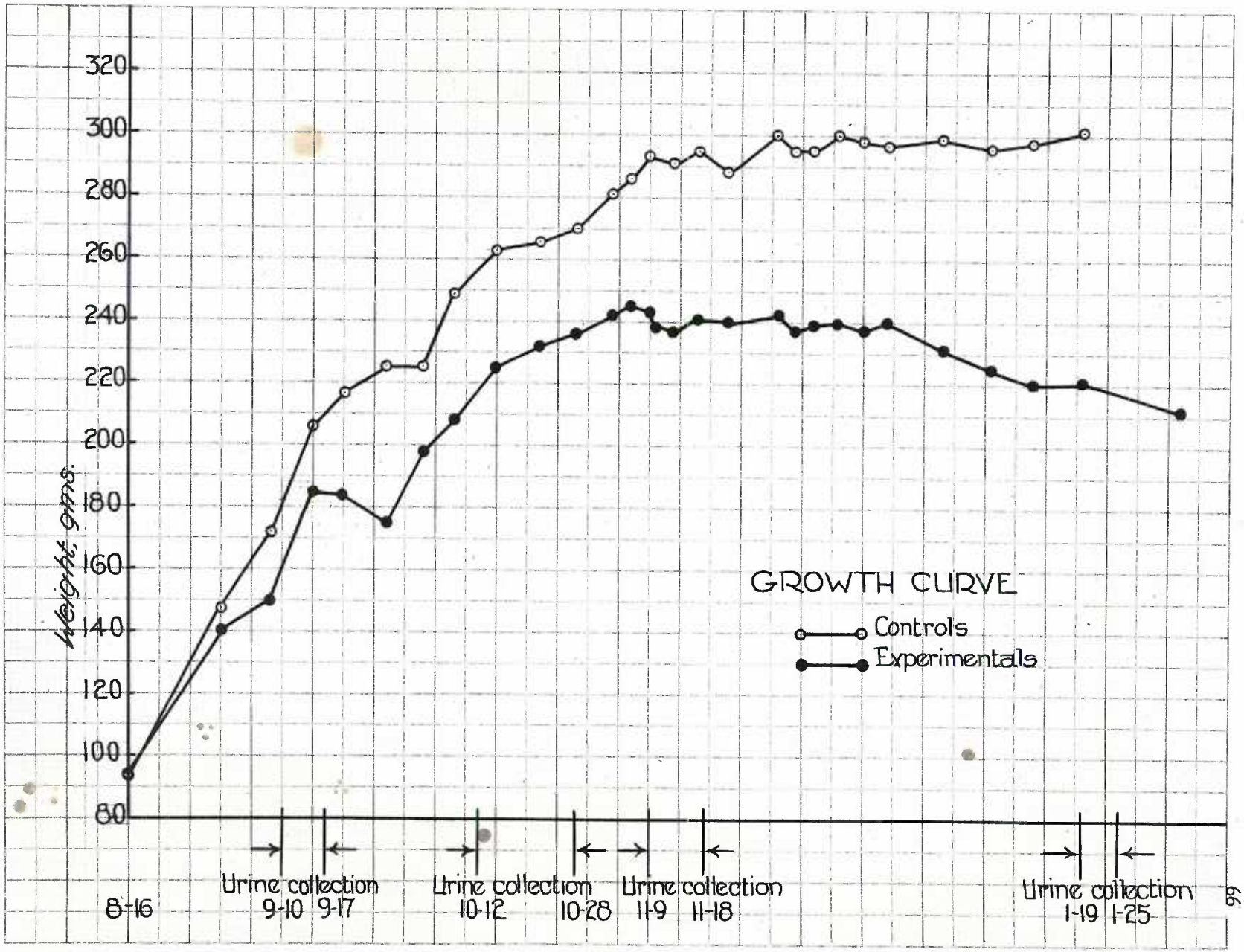
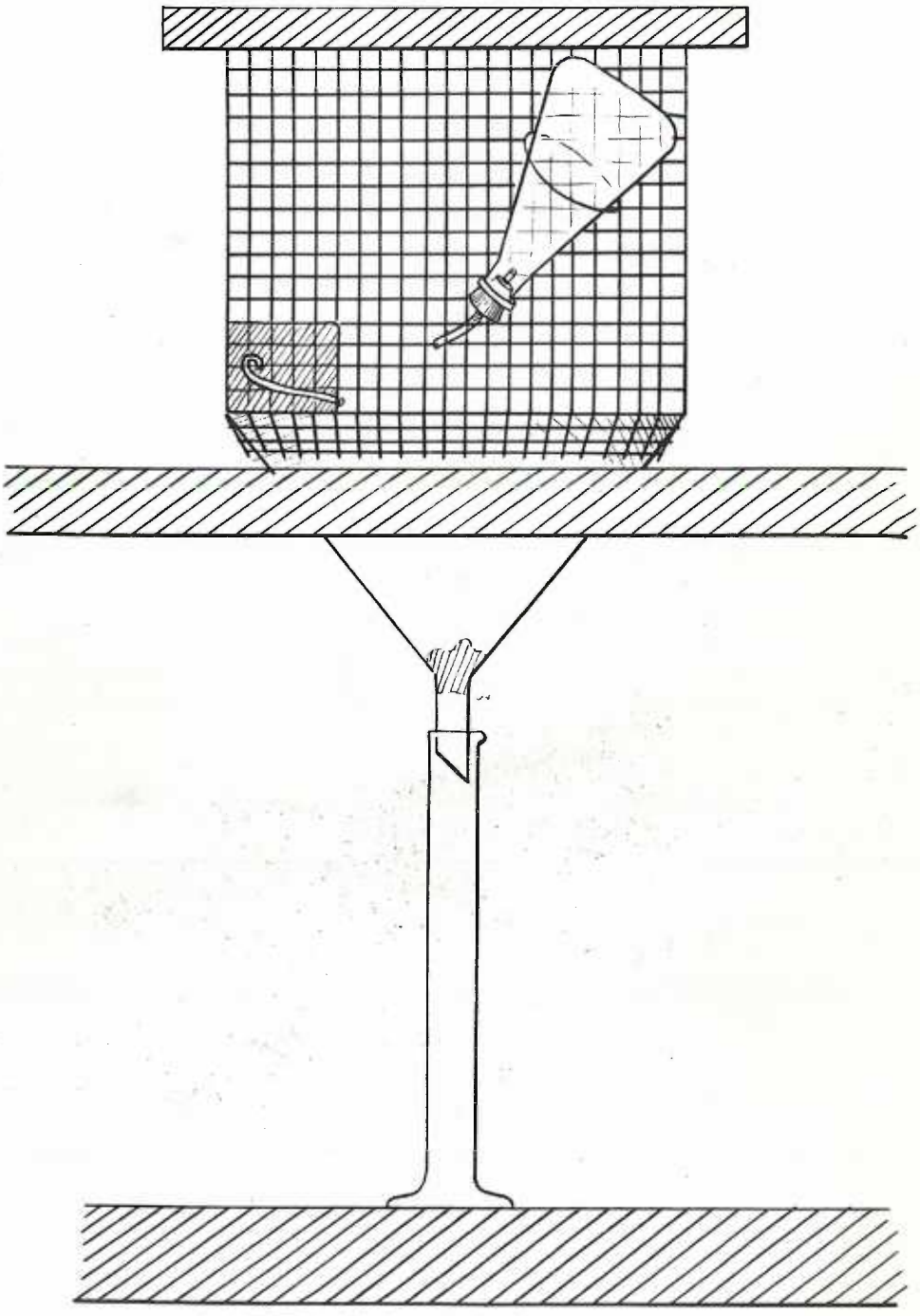

320
300
280
260
240
220
200
180
160
140
120
100
80
Weight gms.
GROWTH CURVE
Controls
Experimentals
8-16
Urine collection 9-10 9-17
Urine collection 10-12
10-28
Urine collection 11-9 11-18
Urine collection 1-19 1-25

Figure 7



METABOLISM CAGE

Figure 8

The first urine analyses were run one month later. The 24 hr. urine sample was diluted to 50 ml. and 25 ml. was used for analysis. Colorimeter No. 761 was used. During this month each control rat had received one drop of cod liver oil and each experimental rat one drop of viosterol at about three day intervals. The average urine citric acid of the control group was 0.80 mg. daily; of the experimental animals, 0.46 mg. daily (Table XXV). Three of the ten experimental animals developed mild xerophthalmia, which did not appear in any of the controls. During the period of urine collection for this run, the control growth curve was rising faster than the growth curve of the experimental animals.

During the month preceding the second urine collection, the experimental animals still received on the average one drop of viosterol each every three days. Because of an increasing incidence and severity of xerophthalmia, weakness, and a rapidly falling growth curve, they were also occasionally given cod liver oil as needed to protect them. The control rats remained entirely well. Their growth curve, however, showed a tendency to flatten out. At the time of the second urine collection, the experimental animals were eating more than the controls. This was assumed to be due to increased metabolic efficiency due to addition of vitamin A following vitamin A starvation. The growth curves of both the control and the experimental groups were rising, that of the experimental group more so than that of the control. The urine citric acid of the experimental group averaged 0.76 mg. per rat daily and of the controls, 0.69 (Table XXVI). Urine pH was roughly the same for all groups and showed no significant difference.

TABLE XXV

URINE CITRIC ACID FOR RATS

Rat Number	Date	Urine Volume, ml.	Colorimeter No.761 Reading, %T	Citric Acid, mg.
01	9-17-43	5	40.0	1.245
02	9-10-43	5	24.5	0.756
03	9-17-43	11	16.0	0.494
04	9-10-43	5	26.2*	0.778
05	9-10-43	5	23.2	0.720
			Average,	0.799
11	9-17-43	2	11.0	0.334
12	9-17-43	4.5	21.0	0.650
13	9-10-43	5	15.7	0.484
14	9-10-43	10	17.0	0.522
15	9-10-43	3	11.7*	0.346
21	9-10-43	3	16.7*	0.498
22	9-17-43	3	7.0	0.212
23	9-17-43	2	21.0	0.648
24	9-10-43	3	14.5*	0.430
25	9-10-43	3	16.0	0.492
			Average,	0.462

* Colorimeter No. 163

TABLE XXVI

URINE CITRIC ACID FOR RATS

Rat Number	Date	Urine Volume, ml.	Urine pH	Colorimeter Reading, F54*	Citric Acid, mg.
01	10-12-43	5.8	5.7	28.0	0.830
02	10-15-43	9	6.3	32.0	0.952
03	10-20-43	8	6.0	10.0	0.296
04	10-20-43	8	6.0	25.0	0.740
05	10-12-43	7.5	5.5	20.5	0.610
				Average,	0.686
11	10-12-43	2.4	5.5	11.9	0.354
12	10-15-43	4	6.3	27.0	1.098
13	10-20-43	8	6.0	33.5	0.996
14	10-15-43	3	6.3	26.0	0.770
15	10-20-43	15	6.3	25.0	0.740
21	10-28-43	4.5	6.0	43.0	1.284
22	10-28-43	4.5	6.0	12.0	0.356
23	10-28-43	4.5	6.0	16.0	0.476
24	10-15-43	6.0	6.3	33.0	0.980
25	10-12-43	5.8	5.6	19.0	0.566
				Average,	0.762

* Colorimeter, No. 163

During the following three weeks the growth curve of the control rats rose slightly. The growth curve of the experimental rats declined. They developed inflammation of the legs, empyema, severe xerophthalmia and hematuria. Cod liver oil and milk was given them individually as necessary to keep them alive. Urine collected at this time assayed an average of 0.92 mg. of citric acid daily per rat for the controls and 0.83 mg. daily per rat for the experimental animals (Table XXVII). Urinary pH fell for all groups.

During the final experimental period, the growth curve and food intake of the control animals rose very slightly. The growth curve of the experimental animals declined sharply. The control animals remained healthy. The experimental ones all had xerophthalmia and many continued to have purulent infections. Viosterol was not given. Diluted cod liver oil was administered to keep the animals alive. The urine citric acid for the controls was 0.76 mg. per rat daily and for the experimental animals, 0.36 mg. per rat daily (Table XXVIII). Urinary pH remained low for both the control and the experimental animals.

It can be concluded that urine citric acid in rats falls with decline in rate of growth and general health. Because of the necessity of administering vitamin A to sick animals, the variations in vitamin D attendant upon variations in cod liver oil and viosterol intake, and the variations in intake of the B group of vitamins with change in appetite of the animals, it was not possible to demonstrate the specific effect of vitamin A. Postmortem examination did reveal typical avitaminosis A symptoms in all the experimental animals - purulent infection of lungs, ears, nasal sinuses, and the genito-urinary tract. Neither autopsy nor two series of X-rays taken during the course of the experiments showed any stones. The control animals remained in good health throughout the experiments and were not sacrificed.

TABLE XXVII

URINE CITRIC ACID FOR RATS

Rat Number	Date	Urine Volume, ml.	Urine pH	Colorimeter Reading,* PSA	Citric Acid, mg.
01	11-9-43	9	5.3	9.0	0.266
02	11-9-43	10	5.5	9.0	0.266
03	11-12-43	5		22.0	0.654
04	11-16-43	4.5	5.8	70.0	2.620
05	11-16-43	5	5.8	20.0	0.584
				Average,	0.918
11	11-9-43	5	5.5	13.0	0.384
12	11-12-43	7	5.5	48.0	1.430
13	11-12-43	8	5.5	31.5	0.936
14	11-16-43	8	5.3	22.5	0.668
15	11-16-43	10.5	5.8	28.0	0.830
21	11-9-43	5.5	5.5	62.0	1.850
22	11-12-43	2.5	5.6	6.5	0.188
23	11-12-43	4	5.5	32.2	0.958
24	11-16-43	4		17.0	0.504
25	11-16-43	7		17.0	0.504
				Average,	0.825

* Colorimeter, No. 163

TABLE XXVIII

URINE CITRIC ACID FOR RATS

Rat Number	Date	Urine Volume, ml.	Urine pH	Colorimeter Reading,* F54	Citric Acid, mg.
01	1-19-44	10	5.7	45.5	0.626
02	1-19-44	16	5.7	24.0	0.334
03	1-19-44	15.5	5.7	42.0	0.583
04	1-19-44	10	5.7	106.0	1.480
05	1-25-44	5	5.7	55.3	0.772
				Average,	0.759
11	1-19-44	1.5	5.7	38.8	0.536
12	1-19-44	14.5	5.7	42.5	0.585
13	1-19-44	10	5.7	43.0	0.598
14	1-19-44	10	5.7	21.0	0.290
15	1-25-44	8	5.7		
21	1-25-44	1.8	5.7	5.3	0.070
22	1-25-44	5.5	5.7	20.6	0.285
23	1-25-44	6	5.7	15.7	0.217
24	1-25-44	8	5.7	29.6	0.410
25	1-25-44	35	5.5	16.0	0.220
				Average,	0.357

* Colorimeter, No. 163. Analyses run on full 24 hr. samples.

DISCUSSION

The foregoing experiments point to the observation that in one diagnosed case of calcium stone the weight for weight ratio of urine citric acid to urine calcium had the value 1.45; in another case of a surgically removed calcium phosphate stone, this value was 1.77 at the time of the study; and in a case of renal tuberculosis with extensive calcification of the kidneys and of urinary passages, the value was 1.09. In contrast, a normal negro, selected for study because negroes are believed to be relatively immune to calcium lithiasis, presented the value 4.25, and a case with a small, silent renal stone of undetermined composition, 4.89.

These results intimate the possibility that the mechanism of calcification may proceed through the destruction of the labile citrate radical and the consequent release of calcium ion for precipitation with suitable anions. Excessive urine calcium excretion or excessive citrate radical destruction from any cause should predispose to urinary calcium lithiasis in such an instance.

A review of the dietary habits of subject 1 failed to reveal any obvious peculiarity.

Because magnesium is a constituent of body enzymes concerned with citric acid production and because Hammarsten believes that magnesium enhances the solubility of calcium oxalate, quantitative analyses for magnesium were performed on the blood and urine of subject 1. Both values were normal.

Urea, reported by Hodes as solvent for calcium oxalate, was also present in normal concentration in the urine of subject 1.

Normal blood citric acid pictures for subjects 1 and 2 suggest that a high renal threshold for citric acid excretion or an energetic oxidation of

the citrate radical by renal parenchyma may account for the low value of citric acid in the urine.

Studies on rats indicate that declining growth and health lower citric acid metabolism and support the view of Hartemann (24) that urine citric acid falls during the course of inflammatory disease.

PART III

THE RELATION OF SUCCINATE, GLUTAMATE, AND CITRATE INGESTION TO URINARY CITRATE EXCRETION

Krebs, on the basis of in vitro experiments, designated certain four-carbon dicarboxylic acids the precursors of citric acid. It was immediately interesting to know whether in vivo experiments would substantiate these findings. In 1938 Simola and Kesumen (32) reported that sodium salts of the precursors and chemically allied substances fed to rats in the ratio of 60 mg. of sodium to 100 gm. of body weight caused increased citric acid to be excreted in the urine. The order of increasing effectiveness was gluconic, glycolic, adipic, maleic, oxaloacetic, fumaric, malic, pyruvic, α-ketoglutaric, aconitic, succinic, malonic, citraconic, pyruvic acid aldol, and glutaric.

The findings suggested a possibility of protecting chronic stone formers from precipitation of calcium in the urine by raising the urinary citric acid concentration through the feeding of a suitable compound. The question remained as to whether the increase found in rats could be produced similarly in humans and whether this state could be obtained without a simultaneous increase in the excretion of calcium.

EXPERIMENTAL

A control study of the urine constituents for subject 1 had already been made (Table XIX). He was then given 22-24 gm. of succinic acid (Mallinckrodt) in four doses daily for four days. The ingestion resulted in an immediate rise in urine citric acid and was accompanied by a 10-15 mm. drop in blood pressure and a feeling of warmth. The calorigenic effect subsided in twenty-four hours and the citric acid began to drop toward control values. On the fourth day, increase in gastro-intestinal motility, nausea and cramps terminated the experiment. The citric acid value at this point was slightly below the control value. Calcium remained normal throughout (Table XXIX), but showed an average rise of 0.05 gm. per day.

TABLE XXIX

SUCCINIC ACID SERIES FOR SUBJECT 1

Urine

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.
2-17-43			38.0		1.36	
2-17-43	1360	6.3	36.0	0.81	1.36	0.370
2-17-43			38.2			
2-18-43			35.2			
2-18-43	1307	6.5	36.3	0.73	1.47	0.384
2-18-43			36.5			
2-19-43			30.2		1.66	
2-19-43	1197	6.1	34.4	0.61	1.68	0.400
2-19-43			32.9			
2-20-43			21.0		1.46	
2-20-43	1215	6.0	23.3	0.44	1.48	0.357
2-20-43			26.0			
				Average, 0.65		Average, 0.378

Subject was next given 15 gm. of glutamic acid (Eastman Kodak Co.) in four daily doses for four days. A rise in citric acid occurred on the first day of ingestion, but, perhaps because of a smaller dose, was less marked than in the case of succinic acid. The results were also somewhat less uniform, although the tendency of a drop in citric acid to normal values by the fourth day was still evident. Calcium showed a 0.02 gm. per day rise for the four day period (Table XXX). No physiological discomfort was reported.

TABLE XXX

GLUTAMIC ACID SERIES FOR SUBJECT 1

Urine

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.
2-23-43					1.37	
2-23-43	1217	6.4	37.5	0.71	1.40	0.344
2-23-43					1.47	
2-24-43					1.27	
2-24-43	1186	6.5	23.2	0.43	1.42	0.312
2-24-43			23.2		1.25	
2-25-43					1.47	
2-25-43	1356	6.2	27.2	0.58	1.39	0.382
2-25-43			27.7		1.39	
2-26-43					1.87	
2-26-43	922	6.0	25.7	0.37	1.88	0.345
2-26-43			25.7		1.86	
				Average, 0.52		Average, 0.346

Ammonium succinate was prepared by treatment of succinic acid with ammonium hydroxide. 11 gm. per day of the recrystallized product was next administered in three doses. Ammonium succinate was selected because of the reported claim (28) that acids tend to decrease citric acid excretion in urine and moderate amounts of alkali, to increase it. At the same time, the metabolism of the ammonium radical to urea precludes a rise in urine pH which might be expected from the use of a salt of an alkali metal and which is generally considered contraindicated in calcium lithiasis. The small dose of ammonium succinate resulted in an appreciable rise in urine citric acid. The subsequent decline appeared less abrupt than in the case of the precursor acids, suggesting that a larger dose of ammonium succinate might sustain a urine citric acid rise for a longer time. 15.6 gm., equivalent to 12 gm. of succinic acid, was given daily in five doses. The rise in citric acid resulting from this dose was greater than one would expect from succinic acid. The subject, however, was advised by his physician to submit to sulfathiazole treatment to control infection. Moreover, hematuria was present as a result of gardening. He reported also gastritis. The experiment was postponed.

About twelve days following sulfathiazole therapy, the subject passed a jagged stone. It is interesting to note that urine samples taken shortly after this event assayed only about 0.3 gm. of citric acid, which was forty percent below that of the control average.

Administration of ammonium succinate was resumed by giving 16 gm. daily in four doses. Urinary citric acid rose sharply. The value, as in previous experiments, dropped during four days to slightly below the control value, although it was then still about fifty percent above the initial value of this experiment (Table XXXI). Urinary calcium was essentially unchanged.

A slight and transient diuresis accompanied the ingestion of all the precursors given. No appreciable change of urine pH occurred. Fluctuations in calcium values were small and well within the normal range. During the course of the administration of the citric acid precursors, there was an average five percent rise in urine calcium above the average control value.

An X-ray of subject 1 taken a week after the end of the precursor series of experiments showed no alteration in the size of the calculi. The subject had the impression that the stone in the left kidney was somewhat less dense than it had appeared to be on an X-ray taken several months previously.

TABLE XXXI

AMMONIUM SUCCINATE SERIES FOR SUBJECT J

Urine

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity
3-2-43			24.5		1.00		
3-2-43	1593	6.5	26.4	0.65	1.02	0.322	
3-2-43			27.8				
3-3-43			27.7		1.23		
3-3-43	1203	6.2	27.7	0.52	1.21	0.298	
3-3-43			27.5				
3-4-43			24.5		1.07		
3-4-43	1330	6.5	25.8	0.53	1.07	0.285	
3-4-43			26.8				
Increased dose -							
3-5-43			26.9		1.28		
3-5-43	1230	6.4	28.8	0.53	1.26	0.312	
3-5-43			27.3				
3-6-43			28.5		1.14		
3-6-43	1658	6.3	25.5	0.70	1.14	0.378	
3-7-43*					1.15		
3-7-43*	1463	5.8	30.2	0.67	1.15	0.337	
Control check after sulfathiazole -							
4-1-43			18.8		1.37		
4-1-43	1153	6.0	12.9	0.28	1.36	0.315	1.017
4-2-43			15.8		1.45		
4-2-43	1088	6.5	20.3	0.31	1.44	0.314	1.018
Increased dose of ammonium succinate -							
4-5-43			37.1		1.05		
4-5-43	1676	6.4	20.8	0.75	1.05	0.352	1.016

* Hematuria. Subject took sulfathiazole 3-6-43 to 3-11-43. He passed a jagged stone 3-23-43.

TABLE XXXI, continued

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity
4-6-43			25.3		1.11		
4-6-43	1430	6.0	30.5	0.62	1.17	0.326	1.017
4-7-43			30.7		1.23		
4-7-43	1428	6.3	24.5	0.61	1.15	0.340	1.016
4-8-43			23.0		1.26		
4-8-43	1340	5.9	19.6	0.44	1.23	0.335	1.017
			Average for succinate ingestion,	0.60	Average for succinate ingestion,	0.328	

It seemed pertinent to find out whether direct administration of citrate would increase its concentration in urine. A neutral salt was indicated to avoid gastro-intestinal irritation. Sodium citrate was chosen for this purpose. 12.5 gm. of hydrated sodium citrate was given daily in four doses. Urine citric acid rose in three days from the control value of 0.44 gm. to 1.34 gm. (Table XXXII). The subject reported a three-pound gain in weight and slight edema of the eyelids but no discomfort. The dose was cut to 9.06 gm. daily. Edema disappeared. Urine pH showed a tendency to rise and was controlled by oral administration of 2-3 gm. daily of ammonium chloride.

The length of the experiment was twenty-three days. From June 17 to June 25 the subject was on vacation and continued taking sodium citrate and ammonium chloride. Samples were not collected for this time. He returned with a gross hematuria, attributable to twice daily swimming.

The ingestion of sodium citrate gave an immediate rise in urine citric acid. In contrast to the action of citric acid precursors, sodium citrate gave not only a sustained rise in urine citric acid, but a climbing rise. This suggests that absorption of citrates from the intestine depends on the intestinal concentration of sodium citrate. On the other hand, the possibility that a gradual alkalinization of the gastro-intestinal tract may influence intestinal bacterial or other action on citric acid or the permeability of the intestinal wall to citrate is not ruled out. In this connection it is interesting to note that ingestion of ammonium chloride during the sodium citrate period lowered the urine citric acid. The daily urine citric acid on a 9.06 gm. daily dose of sodium citrate alone averaged 0.775 gm. and on the same dose of sodium citrate with ammonium chloride, 0.738 gm. The difference is significant in view of the fact that the ingestion of ammonium chloride was begun while daily urine citric acid content was still rising.

The experiment on sodium citrate also illustrated the well-known diuretic action of this substance. About thirteen percent of the orally administered sodium citrate was recovered in the urine. 12.50 gm. of hydrated sodium citrate is equivalent to 6.73 gm. of anhydrous citric acid. Of this, about 0.86 gm. above the control value appeared in the urine. This amounts to thirteen percent of the quantity ingested.

Calcium remained well within normal limits, but, as in the case of experiments with citric acid precursors, showed an average rise of about five percent above the average control value. This indicates that the observed increases in urine calcium for subject 1 were not due to acidosis.

Analyses for total organic acids, performed by the procedure cited by Hawk and Bergeim (70), revealed no abnormality. The average rise in total organic acids was 2.36 in terms of 0.1 normal acid per day per kilogram of body weight. Since 0.73 ml. would correspond to the 0.37 gm. per day average rise in citric acid for this period, 1.65 ml. must represent a rise in other urinary organic acids. Kuyper (72) failed to detect such a rise by potentiometric measurement of the change of the titration curve slope for urine after citrate ingestion. However, Sherman, Mendel and Smith (73) found a rise in total non-citric urinary acids in dogs and Schaak (74) in humans after feeding sodium citrate.

Chlorides (70), calculated as sodium chloride, rose from a control average of 7.07 gm. to 8.27 gm. daily on sodium citrate therapy without ammonium chloride and to 12.42 gm. with ammonium chloride. Diuresis probably accounts for the first rise; ingestion of chlorides, obviously, for the second.

Hematuria did not affect either citric acid or calcium values. This is in contrast with results on the control sample dated 1-9-43 (Table XIX).

The subject reported that an X-ray taken at the end of the experiment showed no further change in the appearance of the stones.

The results of the ingestion of the various organic compounds by subject 1 are summarized in Figure 9.

TABLE XXXII

SODIUM CITRATE SERIES FOR SUBJECT 1

Urine

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity	0.2 Normal Hydrochloric, ml.	Organic Acids per Day per Kg.	Standard Sulfocyanate ml.	Chlorides per Day, gm.
Control check -											
6-3-43	1184	5.8					1.018	5.25	5.65		
6-4-43			20.5		1.16						
6-4-43	1428	6.0	23.3	0.49	1.02	0.311	1.016	4.95	6.37	6.93	8.77
6-5-43			21.5		1.23						
6-5-43	1180	6.2	21.1	0.39	1.21	0.280	1.014	3.70	3.75	7.73	5.37
			Average,	0.44	Average,	0.296		Average,	5.26	Average,	7.07
Sodium citrate -											
6-7-43			31.8		1.07						
6-7-43	1512	6.5	32.2	0.75	1.09	0.327	1.015	4.90	6.67	7.80	6.65
6-8-43			37.2		0.84						
6-8-43	1628	7.3	38.1	0.95	0.84	0.274	1.015	6.50	9.85	8.18	5.93
6-9-43			46.8		0.95						
6-9-43	1967	7.3	40.5	1.34	1.09	0.401	1.018	6.00	10.90	7.40	10.23
Decreased dose of sodium citrate -											
6-10-43			28.7		1.18						
6-10-43	1420	6.8	34.5	0.70	1.24	0.344	1.018	5.50	7.13	7.45	7.24
6-11-43			21.5		1.03						
6-11-43	1935	6.8	24.7	0.69	1.00	0.393	1.016	3.60	6.00	8.03	7.62
6-12-43			30.4		0.86						
6-12-43	1605	7.3	30.5	0.76	0.88	0.279	1.015	6.00	8.90	7.63	7.60
6-13-43			27.6		0.89						
6-13-43	1804	7.3	32.2	0.84	0.89	0.321	1.016	4.00	6.30	7.45	9.20
6-14-43			29.9		0.95						
6-14-43	1935	7.0	29.2	0.89	0.97	0.372	1.015	4.20	5.20	6.93	11.90
								Average,	7.62	Average,	8.27
Continued dose of sodium citrate, with ammonium chloride -											
6-15-43			34.8		0.85						
6-15-43	1587	6.8	30.1	0.80	0.81	0.263	1.016	4.60	6.50	6.74	10.30
6-16-43			32.4		1.09						
6-16-43	1625	7.0	29.2	0.78	1.05	0.348	1.016	4.50	6.50	5.98	13.06
6-17-43			25.0		1.24						
6-17-43	1696	6.3	25.0	0.66	1.21	0.416	1.017	4.00	6.00	6.13	13.10

TABLE XXXII, continued

Date of Sample	Volume, ml.	pH	Colorimeter Reading, P54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity	0.2 Normal Hydrochloric, ml.	Organic Acids per Day per Kg.	Standard Sulfocyanate ml.	Chlorides per Day, gm.
6-18-43			19.6		0.83						
6-18-43	1947	6.5	14.8	0.52	0.84	0.324	1.014	4.05	7.00	6.73	12.73
6-26-43*					1.02						
6-26-43*	1720	7.3	26.8	0.72	1.02	0.351	1.016	5.50	8.65	6.50	13.24
6-27-43			35.2		0.96						
6-27-43	1778	7.5	34.2	0.96	0.94	0.338	1.015	6.80	11.30	6.60	12.09
			Average,	0.81	Average,	0.342			Average, 7.64		
Discontinued treatment as of 6-28-43											
7-3-43*			22.1		1.35						
7-3-43*	1202	6.3	25.2	0.44	1.37	0.327	1.017	6.95	7.83	6.21	9.10

* Hematuria

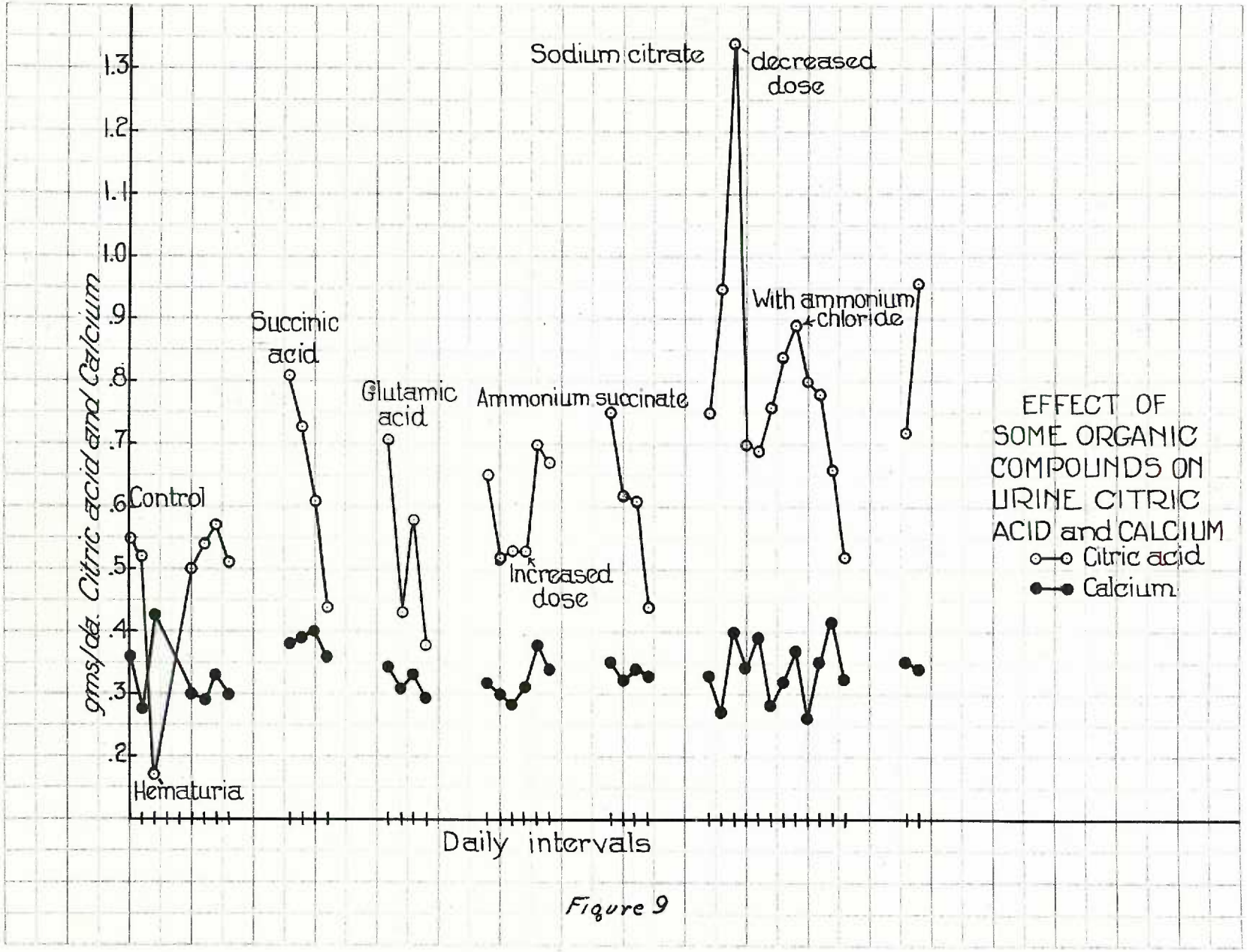

1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
gms/day. Citric acid and Calcium
Control
Hematuria
Succinic acid
Glutamic acid
Ammonium succinate
Increased dose
Sodium citrate
decreased dose
With ammonium chloride
Daily intervals
EFFECT OF SOME ORGANIC COMPOUNDS ON URINE CITRIC ACID and CALCIUM
Citric acid
Calcium

Figure 9

DISCUSSION

It has been shown that an increased urinary citrate excretion in the human can be effected by oral administration of succinate, glutamate and citrate.

The results of prolonged ingestion of these substances indicate that the rise in urinary citrate is opposed by development of acidity in the gastro-intestinal tract, regardless of whether the phenomenon arises from the salt or acid fed or from separately administered ammonium chloride. Symptoms of generalised acidosis are not involved. This suggests the possibility of bacterial synthesis of citric acid in the gastro-intestinal tract, modified by pH, or an effect of intestinal pH upon the absorption of citrate, or both.

Ingestion of a neutral salt of citric acid gave a permanent rise in urinary citrate during the period of ingestion. Thirteen percent of the citrate fed was excreted in the urine.

A slight, transient diuresis resulted from the ingestion of succinic and glutamic acids and ammonium succinate. The ingestion of sodium citrate was accompanied by diuresis lasting throughout the ingestion period.

Total urinary organic acids rose during sodium citrate ingestion.

Urine calcium for subject 1 rose roughly five percent above the average control value as a result of succinate, glutamate and citrate ingestion.

So far as can be determined, the above experiments represent the first work done upon the prolonged ingestion of citric acid precursors by the human subject.

PART IV

THE ROLE OF INTESTINAL BACTERIAL ACTION IN THE PRODUCTION OF CITRIC ACID

Citric acid can be prepared commercially by the action of Citromycetes pfefferianus or glaber on sugar syrup (75), (76) and (77). A search through the literature revealed only one mention of the possibility that intestinal bacteria, also, may have the power to synthesize citric acid. Hartenseon (24) remarked that a rabbit's feces showed an increased content of citric acid on standing. The fact that animals, plants, and molds form citric acid makes it easily probable that some bacteria may exercise the same function.

The following experiments were performed in an effort to determine whether or not the intestinal bacterial flora may be in part responsible for the production of citric acid in humans.

EXPERIMENTAL

The first method tried was based upon the partial sterilization of the intestine in vivo and concomitant analyses to detect a possible drop in urine citric acid. For this purpose, four subjects were given sulfasuxidine (succinyl sulfathiazole) in doses of about 0.25 gm. of the drug per kilogram of body weight per day. This is the dose recommended by Poth (78) for preparation of the colon for operation. Urine citric acid was determined for control purposes for several days before administration of the drug and daily during its administration. Following the discontinuance of sulfasuxidine, occasional determinations of urine citric acid were made as necessary to trace its return to normal. Colorimeter No. 761 was used. The data are given in Tables XXXIII, XXXIV, XXXV, and XXXVI, and summarized in Table XXXVII. Three of the four subjects used in these experiments showed a drop in urine citric acid excretion on sulfasuxidine therapy. One female subject showed a drop which persisted after discontinuance of sulfasuxidine. This is believed to be due to interference from the hormonal cycle as cited by Sherr, Bernhein and Tausesky (27). Calcium values fluctuated irregularly within the normal range.

All subjects except number 6 reported semi-fluid stools on sulfasuxidine ingestion. This is in accord with Poth's findings.

Occasional urine samples were tested for sulfasuxidine by the method of Bratton and Marshall (79). The tests indicated less than five percent absorption of the drug. This is also the figure found by Poth.

TABLE XXXIII

SULFASUXIDINE SERIES FOR SUBJECT 1 (HISTORY GIVEN IN PART II)

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity
Control -							
1-7-43			31.6				
1-7-43	1240	6.5	28.1	0.55	1.45	0.357	
1-7-43			25.6		1.43		
1-8-43			32.7				
1-8-43	1095	6.2	30.4	0.52	1.22	0.267	
1-8-43			27.6		1.22		
1-9-43*			7.8		1.32		
1-9-43*	1668	6.5	7.1	0.19	1.26	0.430	
1-12-43			30.5		1.34		
1-12-43	1140	6.0	26.3	0.50	1.32	0.303	
1-13-43			35.0		1.47		
1-13-43	997	6.2	36.0	0.54	1.49	0.289	
1-14-43			32.4		1.43		
1-14-43	1172	6.4	31.3	0.58	1.42	0.334	
1-15-43					0.90		
1-15-43	1446	6.2	23.0	0.51	1.21	0.305	
4-20-43			20.5		1.52		
4-20-43	1186	5.6	21.8	0.39	1.67	0.378	1.017
4-21-43			25.1		1.48		
4-21-43	1250	6.5	22.8	0.46	1.44	0.365	1.017
			Average,	0.47	Average,	0.336	
Sulfasuxidine administration - 20 gm. daily in 4 doses -							
4-22-43			15.7		1.30		
4-22-43	1390	5.5	14.8	0.33	1.32	0.364	1.016
4-23-43			17.8		1.61		
4-23-43	1390	5.5	12.7	0.32	1.65	0.453	1.017
4-24-43			17.9		1.38		
4-24-43	1360	5.6	16.8	0.37	1.40	0.378	1.017
			Average,	0.34	Average,	0.398	
Sulfasuxidine discontinued 4-26-43 -							
4-26-43			18.3		1.45		
4-26-43	1465	5.3	16.9	0.40	1.38	0.415	1.016

TABLE XXXIII, continued

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity
4-27-43			19.1		1.30		
4-27-43	1048	5.2	21.9	0.33	1.42	0.285	1.017
4-28-43			19.3		1.30		
4-28-43	1226	5.8	19.9	0.37	1.12	0.297	1.018
5-6-43			24.4		1.50		
5-6-43	1244	5.5	22.3	0.45	1.54	0.368	1.016
5-11-43			22.0		1.33		
5-11-43	1074	5.5	21.0	0.36	1.32	0.285	1.017
6-4-43			21.9		1.16		
6-4-43	1428	6.0	21.7	0.49	1.02	0.311	1.016
6-5-43			21.5		1.23		
6-5-43	1180	6.2	21.1	0.39	1.21	0.280	1.014
			Average,	0.40		Average, 0.320	

* Hematuria

TABLE XXXIV

SULFASUXIDINE SERIES FOR SUBJECT 6 (104.5 KG., 48 YR. OLD NORMAL MALE)

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity
Control -							
3-12-43			36.8		1.21		
3-12-43	1600	5.5	30.9	0.85	1.21	0.287	
3-18-43			24.4				
3-18-43	2100		29.0	0.98	0.66	0.277	
3-18-43			25.6		0.66		
3-19-43			36.6				
3-19-43	1760	6.4	38.5	0.97	0.68	0.239	
3-19-43			32.3		0.68		
4-1-43			40.2		1.44		
4-1-43	1300	6.8	40.2	0.81	1.45	0.314	1.023
4-2-43			35.1		0.77		
4-2-43	1460	6.1	32.3	0.77	0.79	0.228	1.021
4-3-43			29.7		0.85		
4-3-43	1590	6.5	31.0	0.76	0.87	0.283	1.019
4-4-43			44.0		1.11		
4-4-43	1220	6.5	44.7	0.85	1.08	0.268	1.030
4-5-43			64.6		1.36		
4-5-43	910	5.7	59.3	0.88	1.60	0.269	1.026
				Average, 0.86		Average, 0.283	
Sulfasuxidine administration - 27 gm. daily in 6 doses -							
4-8-43			14.1		0.47		
4-8-43	2890	6.0	16.3	0.67	0.40	0.254	1.010
4-9-43					0.75		
4-9-43	1975	5.2	18.0	0.47	0.81	0.261	1.015
4-10-43			7.9				
4-10-43	2470	5.3	12.0	0.37	0.58	0.287	1.009
4-11-43			42.0*		0.68		
4-11-43	1370	5.1	45.0*	0.47	0.75	0.197	1.016
4-12-43**			42.3		1.35		
4-12-43**	1160	5.2	43.3	0.74	1.37	0.316	1.025
				Average, 0.54		Average, 0.263	

TABLE XXXIV, continued

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity
Sulfasuxidine discontinued 4-12-43 -							
4-13-43			33.7		0.88		
4-13-43	1235	5.5	36.3	0.67	0.90	0.220	1.020
4-14-43			34.8		1.11		
4-14-43	1220	5.3	35.6	0.67	1.12	0.271	1.023
4-15-43			47.0		1.30		
4-15-43	1045	6.0	48.4	0.78	1.27	0.270	1.022
4-17-43			27.6		0.95		
4-17-43	2035	5.6	24.2	0.82	0.95	0.387	1.016
4-18-43			33.2		0.94		
4-18-43	1190	5.6	31.3	0.60	0.92	0.321	1.021
4-19-43			49.4		1.58		
4-19-43	1000	5.5	48.5	0.77	1.59	0.318	1.025
4-28-43			31.2		0.94		
4-28-43	1855	6.2	28.4	0.86	0.91	0.321	1.016
5-4-43			67.7		1.00		
5-4-43	1130	5.6	64.5	1.11	1.06	0.233	1.025
			Average,	0.79		Average, 0.293	

* 2 ml. aliquot
** 13.5 gm. sulfasuxidine

TABLE XXXV

URINE ANALYSES FOR SUBJECT 7 (49.5 KG., 35 YR. OLD NORMAL FEMALE)

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity
Control -							
1-5-43			65.5				
1-5-43	930	6.2	55.0	0.87			
1-5-43			59.5				
3-15-43			71.0				
3-15-43	1018	6.3	74.0	1.13	1.87	0.381	
3-15-43			69.0				
4-17-43			53.2		1.45		
4-17-43	1263	6.2	55.0	1.07	1.46	0.368	1.017
4-18-43			61.5		1.80		
4-18-43	1192	6.3	61.3	1.14	1.78	0.427	1.020
4-19-43			80.7		1.20		
4-19-43	840*	6.3	79.9	1.05	1.23	0.204	1.028
				Average, 1.05		Average, 0.345	
Sulfasuxidine administration - 12 gm. daily in 6 doses -							
4-20-43			43.5		1.27		
4-20-43	1440	6.3	44.0	0.99	1.27	0.366	1.018
4-21-43					1.51		
4-21-43	1228	5.8	58.1	1.11	1.39	0.356	1.016
4-22-43			46.3		1.28		
4-22-43	1632	6.2	43.3	1.14	1.37	0.432	1.016
4-23-43			57.0		1.20		
4-23-43	1052	6.0	59.0	0.95	1.30	0.263	1.015
Sulfasuxidine administration - 24 gm. daily in 6 doses -							
4-24-43			63.1		1.77		
4-24-43	1208	5.8	66.0	1.28	1.77	0.428	1.019
4-25-43			35.0		1.18		
4-25-43	1545	6.0	37.2	0.87	1.22	0.371	1.015
				Average, 1.06		Average, 0.349	
Sulfasuxidine discontinued -							
4-26-43			63.7		1.83		
4-26-43	972	5.8	64.4	0.97	2.30	0.401	1.023
4-27-43			46.6		1.49		
4-27-43	1278	6.5	48.3	0.82	1.50	0.382	1.020

TABLE XXXV, continued

Date of Sample	Volume, ml.	pH	Colorimeter Reading, P54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity
4-28-43			75.6		1.98		
4-28-43	1092	6.2	75.5	1.22	1.92	0.402	1.016
4-29-43			65.6		1.04		
4-29-43	1078	7.2	72.3	1.16	1.05	0.225	1.022
5-6-43			33.5		1.26		
5-6-43	1305	6.3	32.5	0.87	1.25	0.328	1.015
5-12-43			56.6		1.83		
5-12-43	875	6.5	56.0	0.77	1.83	0.290	1.017
5-13-43			47.9		1.47		
5-13-43	1135	6.6	47.6	0.88	1.43	0.329	1.018
			Average,	0.93		Average, 0.382	
Sulfasuxidine administration - 13 gm. daily in 6 doses -							
5-16-43			44.3				
5-16-43	1196	6.3	39.9	0.79			1.018
5-17-43			72.3				
5-17-43	1050	5.6	59.9	1.07			1.017
		Overall average for sulfasuxidine administration,		1.02	(1.03*)	0.369	

* Estimated

TABLE XXXVI

SULFASUXIDINE SERIES FOR SUBJECT S (80 KG., 37 YR. OLD NORMAL MALE)

Date of Sample	Volume, ml.	pH	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Specific Gravity
Control -							
7-7-43			42.8		1.04		
7-7-43	1800	6.2	46.2	1.25	1.04	0.374	1.018
7-8-43			57.3		1.00		
7-8-43	1337	6.5	59.8	1.22	1.02	0.273	1.023
7-9-43			54.1		1.13		
7-9-43	1355	5.1	57.7	1.18	1.15	0.309	1.023
7-10-43			53.6		0.95		
7-10-43	1277	5.7	46.5	1.00	0.98	0.246	1.023
7-11-43			35.0**		0.52		
7-11-43	3858*	5.0	30.5**	0.98	0.50	0.394	1.012
				Average, 1.13		Average, 0.319	
Sulfasuxidine administration - 20 gm. daily in 6 doses -							
7-14-43			64.0		1.10		
7-14-43	998	5.8	57.0	0.93	1.07	0.217	1.026
7-15-43			46.0				
7-15-43	1213	5.6	45.0	0.86	0.88	0.213	1.022
7-16-43			34.5		1.02		
7-16-43	1435	5.3	32.0	0.74	1.02	0.293	1.018
7-17-43			41.0		1.25		
7-17-43	1220	5.0	37.0	0.74	1.25	0.305	1.023
7-18-43			12.5**		0.37		
7-18-43	4255*	4.5	16.1**	0.46	0.39	0.323	1.008
				Average, 0.75		Average, 0.272	
Sulfasuxidine discontinued 7-18-43 -							
7-22-43***	2305	5.4	15.0	0.53	0.39	0.180	1.011
7-23-43			20.0		0.55		
7-23-43	3617	5.4	20.0	1.12	0.55	0.398	1.012
7-30-43			35.0		0.74		
7-30-43	1584	5.4	35.5	0.87	0.74	0.234	1.014
8-11-43			64.5		0.58		
8-11-43	1845	5.3	64.5	0.98	0.58	0.214	1.013
				Average, 0.88		Average, 0.257	

* Diuresis due to beer
** 2 ml. sample
*** Diarrhea not due to sulfasuxidine

TABLE XXXVII

SUMMARY OF SULFASUXIDINE EXPERIMENTS

Subject	Control	With Sulfa-suxidine	After Sulfa-suxidine
1	0.47	0.34	0.40
6	0.86	0.54	0.79
7	1.05	1.02	0.93
8	1.12	0.75	0.88

A second series of experiments to study the formation of citric acid in the human intestine consisted in the oral administration of succinate and thiamine to a human subject and the observation of citric acid fluctuations both in the urine and in the feces of the subject during a period including the time of administration. Succinate was selected as a precursor of citric acid; thiamine as a bacterial stimulant. Subject 9 of this study was a thirty-two year old, seventy-one kilogram male. Table XXXVIII gives his diet, for five days, which was representative for the period. Average fecal values of citric acid during ingestion periods include values beginning with twenty-four hours after the first ingestion of the substance in question and ending twenty-four hours after discontinuance of the ingestion. This was done because roughly twenty-four hours are required for the passage of food through the intestine. Colorimeter No. 163 was used. In general, 2 ml. aliquots were used for urine citric acid analyses.

TABLE XXXVIII

REPRESENTATIVE DIET OF SUBJECT 9

3-20-44

Breakfast

eggs, toast, butter, coffee

Lunch

Chow mein, bread, butter, fruit salad, coffee

Supper

ham eggs, bread, butter, prunes, milk

3-21-44

Breakfast

toast, butter, fruit juice, coffee

Lunch

hot dogs, vegetable salad, pudding, coffee

Supper

tomato soup, bread, butter, liver and onions, potato, rhubarb, coffee

3-22-44

Breakfast

coffee cake, coffee

Lunch

vegetable soup, beefsteak, potato, cheese, bread, butter, jello

Supper

spaghetti, bread, butter, fruit salad, walnut cake, coffee

TABLE XXXVIII, continued

3-23-44

Breakfast

bacon, eggs, toast, butter, prunes, coffee

Lunch

corn flakes, bread, butter, coffee

Supper

lamb, potato, gravy, bread, butter, pears, coffee

3-24-44

Breakfast

cinnamon toast, coffee

Lunch

cheese and peanut butter sandwiches, vegetable salad, pear

Supper

fish, potato, peas, bread, butter, coffee

Table XXXIX shows that ingestion of sodium succinate resulted in a marked rise in fecal citrate and was reflected in a rise in urinary citrate values.

Table XL for the thiamine run indicates only a small rise in fecal citrate values during ingestion of thiamine. There was a slight drop in urinary citrate. The subject suffered an acute attack of asthma during the last experimental day. This precluded the observation of citrate values after the ingestion of thiamine.

Urine calcium dropped slightly during the ingestion of both succinate and thiamine. The drop was not significant.

TABLE XXXIX

SODIUM SUCCINATE SERIES FOR SUBJECT 9

Date of Sample	Volume ml.	Urine: Colorimeter Reading, F54	Urine: Citric Acid per Day, gm.	Urine: 0.01 Normal Permanganate, ml.	Urine: Calcium per Day, gm.	Feces: Colorimeter Reading, F54	Feces: Citric Acid per Day, mg.
Control -							
3-8-44	1215	19.7		1.00		50.0**	
3-8-44		19.9	0.17	1.02	0.245	50.0**	13.96
3-9-44		22.2*		1.04		27.0**	
3-9-44	610	24.7*	0.20	1.06	0.128	28.0**	7.60
3-10-44		9.7*		0.70		18.0**	
3-10-44	1635	9.7*	0.21	0.67	0.224	16.0**	4.70
3-13-44		45.0		1.18		18.5**	
3-13-44	795	43.0	0.24	1.13	0.184	18.5**	5.20
3-17-44		25.0		0.95			
3-17-44	1082	25.0	0.19	0.92	0.202		
			Average, 0.20		Average, 0.197		
Sodium succinate administration - 7 gm. daily in 4 doses -							
3-20-44		41.5		1.15		39.0**	
3-20-44	895	42.5	0.26	1.18	0.209	42.5**	11.96
3-21-44		56.0		0.95		24.0**	
3-21-44	665	56.0	0.26	0.95	0.124	28.0**	7.20
3-22-44		42.3		0.95		36.0	
3-22-44	1210	44.0	0.36	0.95	0.130	36.0	5.00
3-23-44		48.5		1.03			
3-23-44	935	48.0	0.31	0.98	0.188	113.0	15.80
3-24-44		25.0		0.65		54.0	
3-24-44	1865	23.0	0.31	0.62	0.237	53.6	7.50
Sodium succinate administration - 10.5 gm. daily in 4 doses -							
3-25-44***		32.5		0.72		26.0	
3-25-44***	1330	32.5	0.30	0.78	0.200	26.2	3.61
3-26-44		47.0		0.75		291.0	
3-26-44	907	46.0	0.30	0.67	0.109	290.0	28.00
			Average, 0.30		Average, 0.171		
Sodium succinate discontinued - 3-26-44 -							
3-27-44		28.3		0.76		93.0	
3-27-44	1605	29.0	0.32	0.78	0.247	90.0	12.80

TABLE XXXIX, continued

	Urine					Feces	
Date of Sample	Volume, ml.	Colorimeter Reading, F54	Citric Acid per Day, gm.	0.01 Normal Permanganate, ml.	Calcium per Day, gm.	Colorimeter Reading, F54	Citric Acid per Day, gm.
3-28-44		34.0		1.18		45.0	
3-28-44	1071	34.0	0.25	1.18	0.253	45.0	6.23
3-29-44		28.4		1.10		87.0	
3-29-44	803	38.4	0.22	1.10	0.177	86.0	13.71
3-30-44		16.0		0.55		60.8	
3-30-44	1292	19.6	0.17	0.42	0.125	58.4	5.96
		Average,	0.24	Average,	0.201		

\# 1 ml. aliquot
\## 25 ml. aliquot
\### subject vomited

Citric Acid Average for Feces

Control	8.68
For succinate	11.42
After succinate	8.63

TABLE XL

THIAMINE SERIES FOR SUBJECT 9

Date of Sample	Volume, ml.	Urine: Colorimeter Reading, F54	Urine: Citric Acid per Day, gm.	Urine: 0.01 Normal Permanganate, ml.	Urine: Calcium per Day, gm.	Feces: Colorimeter Reading, F54	Feces: Citric Acid per Day, mg.
Control -							
4-28-44		25.0		0.47		23.5	
4-28-44	1368	25.0	0.24	0.49	0.131	24.0	3.30
4-29-44		30.0		0.78		30.0	
4-29-44	1740	30.0	0.36	0.73	0.263	30.0	4.16
4-30-44		28.0		1.05		51.3	
4-30-44	980	28.0	0.19	1.03	0.204	51.3	7.15
		Average,	0.26		Average, 0.199		
Thiamine administration - 30 mg. daily in 3 doses -							
5-1-44		25.5**		0.42		64.0	
5-1-44	2268*	25.5**	0.25	0.42	0.192	66.0	9.05
5-2-44		23.5		0.92		78.2	
5-2-44	1030	23.5	0.17	0.92	0.190	78.4	10.92
5-3-44		18.6		0.78		55.4	
5-3-44	1195	22.5	0.17	0.78	0.186	56.0	7.70
5-4-44						28.2	
5-4-44						29.6	3.99
5-5-44		14.0		0.29		32.8	
5-5-44	1820	14.6	0.18	0.29	0.106	33.6	4.60
5-6-44		17.0		0.35		26.6	
5-6-44	1365	17.0	0.16	0.35	0.096	26.6	3.63
		Average,	0.19		Average, 0.154		

* Diuresis due to beer
** 5 ml. aliquot

Citric Acid Average for Feces

Control	5.92
For thiamine	6.17

The third series of experiments was based upon incubation of human stools with and without possible citrate precursors and measurement of the citrate content.

A sample of feces was well mixed and triturated. A 50 gm. portion was diluted to 125 ml. with water. Another 50 gm. portion was mixed with 75 ml. of 2.5 normal sulfuric acid and diluted with water to 125 ml. Both samples were incubated at room temperature for four days. They were then filtered and 50 ml. aliquots were pretreated and analysed in the usual way after adjusting the acid normality of each to about 1.5.

Results of the analyses showed that sample 1, which had been incubated without sulfuric acid, contained more than twice the amount of citric acid found in sample 2, which had been inactivated with sulfuric acid previous to incubation.

A second experiment was performed similarly. However, 20 gm. samples were used and incubation was cut down to two days. Four samples were set up: the first with sulfuric acid, the second with water only, the third with 1.28 gm. sodium succinate, and the fourth with 1.19 gm. sodium bicarbonate. The quantities of sodium succinate and of sodium bicarbonate were equivalent in sodium content, but sample 4 was more alkaline than sample 3.

Analyses of 25 ml. portions showed again that the water-suspended sample contained more citrate than the acid-suspended sample. Still more citrate was found in the sample incubated with succinate and even more yet in the alkaline sample.

To repeat the experiments with a little better bacteriological technique, it was decided to suspend the feces in a buffer that simulates intestinal pH. A Clark and Lubs phosphate buffer of pH 6.8 was prepared (80). Magnesium sulfate was added as recommended by Greville (49). The buffer

was diluted to isotonic concentration, checked with a glass electrode, and sterilised by boiling.

All flasks, funnels and other glass equipment used in the experiment was sterilised at 115° for 24 hours in a dry oven.

38 gm. of feces was triturated with the buffer, diluted with it to 300 ml. and shaken in a shaking machine. 100 ml. portions were used for incubation. Sample 1 was a blank; sample 2 contained 0.2 gm. of sodium succinate. Incubation was for 48 hours at 37-9° C. 35 ml. portions were used for analysis.

Results showed a marked increase in citrate as a result of incubation with succinate.

The experiment was repeated, using 138 gm. of feces in 600-700 ml. of buffer prepared as before. The incubation period was cut to 6 hrs. Filtration of the samples was omitted, and they were instead treated with 10 gm. of 10% trichloracetic acid and centrifuged. 50 ml. portions were used for analysis.

The results were qualitatively the same as in the preceding experiment.

Table XLI is a summary of data on the incubation runs.

TABLE XLI

INCUBATION EXPERIMENTS

Experiment Number	Weight of Sample, gm.	Medium	Incubation Time, hrs.	Incubation Temperature, °C	Colorimeter Reading, F54	Citric Acid, mg.
I	11.0	water	96	25-30	45.0	0.63
	11.0	sulfuric acid	96	25-30	17.7	0.26
II	4.0	water	48	25-30	50.0	0.70
	4.0	sulfuric acid	48	25-30	48.5	0.67
	4.0	sodium succinate	48	25-30	56.0	0.78
	4.0	sodium bicarbonate	48	25-30	66.6	0.94
III	10.2	buffer	48	37-9	26.3	0.36
	10.2	buffer and succinate	48	37-9	27.7	0.38
IV	11.0	buffer	6	37-9	82.0	1.15
	11.0	buffer and succinate	6	37-9	89.0	1.24

DISCUSSION

It has been demonstrated that partial sterilization of the intestine with sulfasuxidine lowered urinary citric acid excretion in three of four subjects. In one of the subjects, a drop occurred after stopping the drug. This phenomenon is thought to be due to hormonal interference. The results indicate that part of the citric acid available for metabolism in the body originates in the gastro-intestinal tract. Interference of sulfasuxidine with tissue enzyme activity is probably ruled out by its low absorption from the intestine.

Oral administration of sodium succinate to one subject resulted in an increase of his fecal citrate and a corresponding increase in the urine citrate. The increase in fecal citrate indicates a stimulated citric acid production in the intestine.

Oral administration of thiamine to the same subject produced a similar rise in fecal citrate accompanied by a fall in urine citrate.

In vitro studies of feces indicate bacterial synthesis of citric acid. This is markedly enhanced by sodium succinate and inhibited by inactivation of bacterial enzymes with sulfuric acid.

The marked increase in citrate production caused by adding sodium bicarbonate to feces is in line with observations that the ingestion of bicarbonate increases urinary citric acid excretion.

SUMMARY

A modification of the Fuchs-Sherman-Vickery method of citric acid analysis has been described. The use of the monomethyl ether of ethylene glycol instead of pyridine or dioxane is recommended. Reproducibility of sodium sulfide solutions has been effected by preparation from sodium hydroxide solutions and hydrogen sulfide gas. The decoloration of potassium permanganate has been shortened by omitting the cooling procedures. Excess hydrogen peroxide in amounts within 2 ml. in the reaction mixture was shown to have no deleterious effect on the course of the analysis. The conversion of citric acid to pentabromoacetone by the method described was found to be 66%.

A study of the diet, the urine and blood composition, urine sediments, and stone of one subject with bilateral calcium oxalate renal calculi revealed no abnormality other than a low citrate to calcium ratio in the urine. The same abnormality appeared in another subject who had previously had a renal calcium phosphate stone and in a subject whose urinary passages contained calcium plaques resulting from renal tuberculosis. The citrate-calcium ratios were 1.45, 1.77, and 1.09, respectively. This ratio for a normal negro subject was 4.25 and for a subject with a small, silent renal stone of undetermined composition, 4.89. Three normal subjects showed the ratios 3.04, 3.04, and 3.54, respectively. One chronic asthmatic had the ratio 1.16.

Oral administration of various organic acids and their salts to subject 1 raised his urine citrate-calcium ratio as follows:

Control	1.45
Succinic Acid	1.72
Glutamic Acid	1.51
Ammonium succinate	1.83
Sodium citrate	2.37

Prolonged ingestion of these substances revealed that the rise in urinary citrate is opposed by development of acidity in the gastro-intestinal tract, regardless of whether the phenomenon arises from the substance fed or from concomitantly administered ammonium chloride. Thus succinic acid and glutamic acid gave a temporary rise in urinary citrate excretion. Such a rise was sustained longer with the less acid ammonium succinate. Sodium citrate ingestion produced a climbing rise in urinary citrate which was repressed by additional ingestion of ammonium chloride.

The rise in urinary citrate excretion with sodium citrate ingestion was accompanied by a rise in the excretion of other, unidentified organic acid constituents.

The ingestion of succinate, glutamate, and citrate resulted in a slight rise in urinary calcium excretion.

Experiments with rats on a vitamin A deficient diet showed that development of avitaminosis A symptoms and a decreased rate of growth are connected with decreased citrate excretion in the urine.

Intestinal bacterial synthesis of citric acid in the human was demonstrated in three subjects by a decrease in urinary citrate excretion during partial sterilization of the gastro-intestinal tract; in one subject, by the increase of fecal citrate during ingestion of succinate, a citrate precursor, and of thiamine, a bacterial stimulant; and by in vitro experiments which showed that feces incubated in sulfuric acid solution showed a lower citrate content, and those incubated in sodium succinate solution, a higher citrate content than control samples. The marked increase in citrate production found in feces incubated with sodium bicarbonate is in line with results of the in vivo experiments which showed that an alkaline gastro-intestinal tract reaction stimulates urinary citrate excretion.

BIBLIOGRAPHY

1. Scheele, K. W. Chemical Essays. Scott, Greenwood and Co., London, 1901.

2. Scahlet and Menhal, T. Citronensaure Gehalt der Kuhmilch. Munchen. med. Wchnschr., vol. 35, pp. 328-9, 1888.

3. Thunberg, T. Zur Kenntnis des intermediaren Stoffwechsels and der dabei wirksamen Enzyme. Skandinav. Arch. f. Physiol., vol. 40, pp. 1-90, 1920.

4. Ratelli, F. and Stern, L. Die Oxydation der Citronen-, Apfel- and Fumarsaure durch Tiergewebe. Biochem. Ztschr., vol. 31, 00.478-505, 1910.

5. Martius, C. and Knoop, F. Der physiologische Abbau der Citronensaure. Ztschr. f. physiol. Chem., vol. 246, pp. 1-2, 1937.

6. Martius, C. Uber den Abbau der Citronensaure. Ztschr. f. physiol. Chem., vol. 247, pp. 104-10, 1937.

7. Krebs, H. A. and Johnson, W. A. Citric acid in intermediate metabolism in animal tissues. Enzymologia, vol. 4, pp. 148-56, 1937.

8. Martius, C. Der intermediare Stoffwechsel der Citronensaure. Ztschr. f. physiol. Chem., vol. 257, pp. 29-42, 1938.

9. Krebs, H. A. and Johnson, W. A. Metabolism of ketonic acids in animal tissues. Biochem. J., vol. 31, pp. 645-60, 1937.

10. Einbeck, H. Uber das Vorkommen der Fumarsaure in frischen Fleische. Ztschr. f. physiol. Chem., vol. 90, pp. 301-8, 1914.

11. _____ Uber quantitative Versuche mit dem Succinoxydon von Ratelli und Stern. Biochem. Ztschr., vol. 95, pp. 296-305, 1919.

12. Hahn, A. and Haarmann, W. Ueber die Dehydrierung der Aepfelsaure. I. Ztschr. f. Biol., vol. 87, pp. 465-71, 1928.

13. _____ Ueber die Dehydrierung der Aepfelsaure. Ztschr. f. Biol., vol. 88, pp. 91-2, 1928.

14. Annau, E., Banga, I., Gozsy, B. St. Huszak, Laki, K., Straub, B. and Szent-Gyorgyi, A. Uber die Bedeutung der Fumarsaure fur die tierische Gewebeatmung. Ztschr. f. physiol. Chem., vol. 235, pp. 1-68, 1936.

15. Annau, E., Banga, I., Hlasso, A., Bruckner, V., Laki, K., Straub, B. and Szent-Gyorgyi, A. Uber die Bedeutung der Fumarsaure fur die tierische Gewebeatmung. III. Ztschr. f. physiol. Chem., vol. 244, pp. 105-52, 1936.

16. Szent-Gyorgyi, A. On Oxidation, Fermentation, Vitamins, Health and Diseases. Williams and Wilkins, Baltimore, 1939

17. Hallman, H. On the action of various organic acids on the citric acid formation in testis and kidney. Suomen Kemistilehti, vol. 11, pp. 23-5, 1938.

18. Simola, P. E. and Alapeuso, H. On the mechanism of citric acid formation in animal organisms. Suomen Kemistilehti, vol. 11, pp. 17-20, 1938.

19. Breusch, F. L. The fate of oxaloacetic acid in different organs. Biochem. J., vol. 33, pp. 1757-70, 1939.

20. Krebs, H. A., Eggleston, L. V., Kleinzeller, A. and Smith, D. H. The fate of oxaloacetate in animal tissues. Biochem. J., vol. 34, pp. 1234-40, 1940.

21. Krebs, H. A. and Eggleston, L. V. The oxidation of pyruvate in pigeon breast muscle. Biochem. J., vol. 34, pp. 442-59, 1940.

22. Krebs, H. A. Modified citric acid cycle. Biochem. J., vol. 36, p. ix, 1942.

23. Wood, H. G., Werkman, C. H., Hemingway, A. and Nier, A. O. Mechanism of fixation of carbon dioxide in the Krebs cycle. J. Biol. Chem., vol. 139, pp. 483-4, 1941.

24. Martonosson, J. Citrate metabolism in mammals. Act a physiol. Skandinav. Suppl. 2, P. A. Norstedt and Soner, Stockholm, 1940.

25. Boothby, W. M. and Adams, M. The occurrence of citric acid in urine and body fluids. Am. J. Physiol., vol. 107, pp. 471-9, 1934.

26. Sherman, G. C., Mendel, L. B. and Smith, A. H. The citric acid formed in animal metabolism. J. Biol. Chem., vol. 113, pp. 247-62, 1933.

27. Shorr, E., Bernheim, A. R., and Taussky, H. The relation of urinary citric acid excretion to the menstrual cycle and the steroidal reproductive hormones. Science, vol. 95, pp. 606-7, 1942.

28. Ostberg, O. Studien uber die Zitronensaure ausscheidung der Menschenniere in normalen und pathologischen Zustanden. Skandinav. Arch. f. Physiol., vol. 62, pp. 81-222, 1931.

29. Dickens, F. The citric acid content of animal tissues, with reference to its occurrence in bone and tumour. Biochem. J., vol. 35, pp. 1011-23, 1941.

30. Glass, R. H. and Smith, A. H. The skeleton as a source of endogenous citric acid. J. Biol. Chem., vol. 151, pp. 363-7, 1943.

31. Simola, P. E., Kruslus, F. E. and Alapeuso, H. Study of citric acid formation in rats. Suomen Kemistilehti, vol. 10, p. 18, 1938.

32. Simola, P. E. and Kosunen, T. On the formation of citric acid in rats after treatment with various organic acids. Suomen Kemistilehti, vol. 11, pp. 22-4, 1938.

33. Greenwald, I. The formation of glucose from citric acid in diabetes mellitus and phlorizin glycosuria. J. Biol. Chem., vol. 18, pp. 115-121, 1914.

34. MacKay, E. M., Carns, H. O. and Wick, A. N. Antiketogenic and glycogenic activity of citric acid. J. Biol. Chem., vol. 133, pp. 59-65, 1940.

35. Sherr, E., Tausaky, H., Almy, T. P., Sloan, R. H. and Toscani, V. The relation between the urinary excretion of citric acid and calcium; its implications for urinary calcium stone formation. Science, vol. 96, pp. 587-8, 1942.

36. Greenwald, I. The effect of the administration of calcium salts and of sodium phosphate upon the calcium and phosphorus metabolism of thyroparathyroidectomized dogs, with a consideration of the nature of the calcium compounds of blood and their relation to the pathogenesis of tetany. J. Biol. Chem., vol. 67, pp. 1-28, 1926.

37. ---------- and Gross, J. The effect of long-continued administration of parathyroid extract upon the excretion of phosphorus and calcium. J. Biol. Chem., vol. 68, pp. 325-33, 1926.

38. Thunberg, T. Das Vorkommen von Citronensäure in der Schale des Vogeleies. K. fysiogr. Sällsk. Lund. Forh., vol. 11, pp. 42-5, 1941.

39. ---------- The citric acid content in various parts of birds' eggs as well as in the egg shells of various species. K. fysiogr. Sällsk. Lund. Forh., vol. 11, pp. 126-8, 1941.

40. Scherstén, B. The occurrence and biological significance of citrate in the secretion of the sex glands of man and different animals. Skandinav. Arch. f. Physiol., vol. 74, Suppl. 9, pp. 1-144, 1936.

41. Albright, F., Sulkowitch, H. W. and Chute, R. Nonsurgical aspects of the kidney stone problem. J. A. M. A., vol. 113, pp. 2049-53, 1939.

42. Suby, H. I. and Albright, F. Dissolution of phosphatic urinary calculi by retrograde introduction of citrate solution containing magnesium. New England J. Med., vol. 228, pp. 81-91, 1943.

43. Rawls, H. B. and West, E. S. Dissolution of vesical calculi. Northwest Medicine, vol. 42, pp. 226-9, 1943.

44. Sabbatani, L. Le calcium-ion dans la coagulation du sang. Compt. rend. Soc. de biol., vol. 54, pp. 716-8, 1902.

45. Wiley, W. J. Dissociation of calcium citrate. Biochem. J., vol. 24, pp. 856-9, 1930.

46. Hastings, A. B., McLean, F. C., Eichelberger, L., Hall, J. L. and DaCosta, E. The ionization of calcium, magnesium and strontium citrates. J. Biol. Chem., vol. 107, pp. 351-70, 1934.

47. Munn, J. and Lobel, H. Complex calcium citrate. Math. fys. Medd., vol. 13, pp. 1-17, 1936.

48. McLean, F. C. Application of the law of chemical equilibrium to biological problems. Physiol. Rev., vol. 18, pp. 495-523, 1938.

49. Greville, G. D. Fumarate and tissue respiration. Biochem. J., vol. 30, pp. 877-87, 1936.

50. Nordbo, R. Die Dissociation von Magnesiumcitrat. Skandinav. Arch. f. Physiol., vol. 80, pp. 241-7, 1938.

51. Walker, T. K., Subramanian, V. and Challenger, E. The mechanism of the formation of citric and oxalic acids from sugars by aspergillus niger. J. Chem. Soc., vol. 1927, pp. 3044-54, 1927.

52. Butterworth, J. and Walker, T. K. A study of the mechanism of the degradation of citric acid by B. pyocyaneus. Biochem. J., vol. 23, pp. 926-35, 1929.

53. Knyper, A. C. The oxidation of citric acid. J. Am. Chem. Soc., vol. 55, pp. 1722-7, 1933.

54. Thunberg, T. Über das Vorkommen einer Citrico-Dehydrogenase in Gurkensamen und ihre Verwertung für eine hochempfindliche biologische Farbenreaktion auf Citronensäure. Biochem. Ztschr., vol. 206, pp. 109-19, 1929.

55. Pucher, W. P., Sherman, G. C. and Vickery, H. B. A method to determine small amounts of citric acid in biological material. J. Biol. Chem., vol. 113, pp. 235-45, 1936.

56. Johnson, W. A. Aconitase. Biochem. J., vol. 33, pp. 1046-53, 1939.

57. Summerson, W. H. A simplified test-tube photoelectric colorimeter, and the use of the photoelectric colorimeter in colorimetric analysis. J. Biol. Chem., vol. 130, pp. 149-166, 1939.

58. Martensson, J. On the occurrence of citric acid in pathological calcifications and concretions. K. hysiogr. Sällsk. Lund. Forh., vol. 11, pp. 129-31, 1941.

59. Kissin, B. and Locke, H. O. Urinary citrates in calcium urolithiasis. Proc. Soc. Exptl. Biol. and Med., vol. 46, pp. 216-8, 1941.

60. Higgins, C. G. Renal Lithiasis. Charles C. Thomas Co., Baltimore, 1943.

61. Hammarsten, G. Eine Experimentelle Studie uber Calciumoxalat als Steinbildner in den Harnwegen. Lunds. Univ. Arsskrift. Avd. 2, vol. 32. K. fysiogr. Sallsk. Handl., vol. 47, pp. 1-155, 1937.

62. McIntosh, J. F. and Salter, R. W. The qualitative examination of urinary calculi. J. Clin. Investigation, vol. 21, pp. 751-4, 1942.

63. Medes, G. Solubility of calcium oxalate and uric acid in solutions of urea. Proc. Soc. Exptl. Biol. and Med., vol. 30., pp. 281-4, 1932.

64. Rinehart, R. E., Grendahl, R. D. and West, E. S. A rapid and accurate method for the distillation of ammonia application to the determination of nitrogen, ammonia, and urea in biological fluids. Arch. Biochem., vol. 2, pp. 169-74, 1943.

65. Allen, R. J. L. The estimation of phosphorus. Biochem. J., vol. 34, pp. 858-65, 1940.

66. Haussler, E. P. A new color reaction for citric acid. Chem. Ztg., vol. 38, pp. 937-9, 1914.

67. Clark, E. P. and Collip, J. B. A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., vol. 63, pp. 461-4, 1925.

68. Denis, W. The determination of magnesium in blood, plasma, and serum. J. Biol. Chem., vol. 52, pp. 411-5, 1922.

69. Schersten, B. Citrate content of blood serum in normal and pathological conditions. Skandinav. Arch. f. Physiol., vol. 63, pp. 97-8, 1931.

70. Hawk, P. B. and Bergeim, O. Practical Physiological Chemistry. P. Blakiston's Son and Co., Philadelphia, 1931.

71. Coppridge, W. M. The treatment of renal stone. J. Urol., vol. 49, pp. 153-9, 1943.

72. Kuyper, A. C. The buffer action of unidentified urine constituents. J. Biol. Chem., vol. 123, pp. 409-19, 1938.

73. Sherman, C. C., Mendel, L. B. and Smith, A. H. The metabolism of orally administered citric acid. J. Biol. Chem., vol. 113, pp. 265-71, 1936.

74. Schmidt, C. Urinary excretion of citric acid. J. Nutrition, vol. 7, pp. 691-700, 1934.

75. Wehmer, C. Ueber Citronensaeuregahrung. Ber., vol. 26, p. 78, 1893.

76. ———————— Darstellung von Citronensaeure durch Gahrung. Ber., vol. 26, pp. 448-9, 1893.

77. ———————— Synthese der Citronensaeure durch Gahrung der Glucose. Ber., vol. 27, pp. 696-7, 1894.

78. Roth, E. J. Succinylsulfathiazole. J. A. M. A., vol. 120, pp. 265-9, 1942.

79. Bratton, A. C. and Marshall E. K. Jr. A new coupling component for sulfanilamide determination. J. Biol. Chem., vol. 128, pp. 537-50, 1939.

80. Clark, W. The Determination of Hydrogen Ions. The Williams and Wilkins Co., Baltimore, 1928.

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