STUDIES ON THE ORGANIC ACIDS OF PLANTS

Thesis

by

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Preface

The organic acids of plants have, in recent years, assumed a more and more central position in the study of the metabolism and respiration of plants. In order to bring about further advances in the study of organic acids it is important to know the total acid composition of a great number of plants. Such an acid fruit as the pineapple presents a very good source for a study of acid composition.

A problem equally important is the origin of the organic acids in plant tissues. The group of plants known as succulents possess a very active acid metabolism and also a comparatively large acid content. The fact that measurable amounts of organic acids are formed in the dark and lost again in the light indicate that the succulents are a promising source for the study of acid formation in plants.

This work has been devoted to a study of the organic acid composition of the pineapple and the formation of organic acids in succulents.

The writer would like to express his great appreciation to Dr. A. J. Haagen-Smit for the tremendous amount of aid and helpful advice which he gave during this work.

To Dr. J. Bonner goes the most sincere thanks for his enthusiastic cooperation during the experiments with succulents.

To Dr. G. Oppenheimer the writer expresses his gratitude for the innumerable number of micro-analyses that she and Mr. G. Swinehart performed during the studies on pineapple and also to

Dr. Harriet Bonner for the execution of the manometric analyses used during the investigation of succulents. Dr. Robert Emerson deserves grateful acknowledgment for the donation of the gas mixtures employed in these experiments and also for the contribution of much valuable advice.

Finally, the writer would like to express his indebtedness to the Pineapple Research Institute of Hawaii at the University of Hawaii for the funds which made the studies on pineapple possible.

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Chapter I

The Organic Acids of Plants.

1. The Occurrence of Organic Acids of Plants.

The presence of acidic substances in plant material was known to the ancients; thus the Romans and Greeks used cream of tartar as a drug and an emetic, George Agricola in 1564 obtained succinic acid by the dry distillation of amber, and a few years later Alexander Pedemontamus obtained benzoic acid by the dry distillation of gum benzoin. Malic, citric, and oxalic acids have been known since the time of Scheele (1). At the present time well over 100 different organic acids have been recorded as occurring in various plant species. The organic acids of plants have been shown to occur in different forms. Many acids are found in the free state, such as malic in apples, citric in lemons, and tartaric in grapes. Some acids occur as salts of metals, the best example being oxalic acid which is found as the very insoluble calcium salt. Many acids are found as esters, for instance, linalyl acetate is the major component of lavender oil. Quinic acid, which has received some attention as a possible precursor of the terpenes and related compounds (2), occurs abundantly in the coffee bean esterified with caffeic acid to form chlorogenic acid. Some important acids are found as glycerides, as is the case with most fatty acids and with acids such as hydnocarpus and chaulmoogric. Many organic acids are present in alkaloid-producing plants.

It has been proven that most alkaloids occur as salts of organic acids that are peculiar to each alkaloid-producing plant. For example, meconic acid is found as a salt of the various poppy alkaloids.

Of the large number of organic acids that have been isolated from plant sources, the aliphatic acids are quantitatively the most important; and of these, oxalic, malic, citric, succinic, tartaric, and fumaric acids are probably the most widely distributed in nature. The formulas and occurrence of these common plant acids are given below:

Oxalic acid. HOOC-COOH. This acid is found in traces in most plants and in large amounts in some. It occurs up to 50-60% of the dry weight of spinach and rhubarb. Crystals of calcium oxalate are frequently found in many plants and are often deposited in specially adapted cells.

Malic acid. HOOC-CH_2-CH(OH)-COOH. It is likely that most plants contain this acid. Malic acid predominates in the berries of mountain ash, cranberries, cherries, plums, pears, apples, tomatoes, and other fruits. Both optically active forms as well as the inactive form have been reported as occurring.

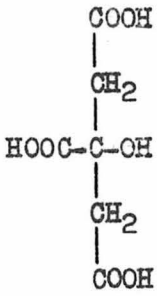
Citric acid. HOOC-CH_2-$\overset{\overset{COOH}{|}}{\underset{\underset{OH}{|}}{C}}$-$CH_2$-COOH. Citric acid is nearly always found in tissues containing malic acid. It is a major acid component of citrus fruits and of pineapple.

Succinic acid. HOOC-CH_2-CH_2-COOH. This acid has been shown to be almost always present in plant tissues in small amounts. It is found in larger amounts in currants.

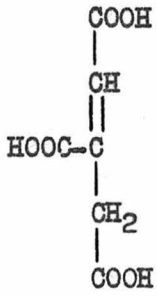
Tartaric acid. HOOC-CH(OH)-CH(OH)-COOH. Tartaric acid is common in fruits of southern lands such as grapes and apricots.

Fumaric acid. HOOC-CH=CH-COOH. This acid occurs to a large extent in poppies.

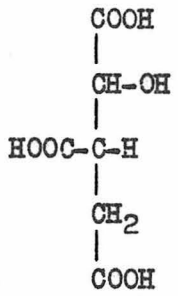
Two acids whose importance has only recently been recognized are isocitric and aconitic. The occurrence of these acids through a wide range of plant families has not as yet been extensively investigated. They bear a close resemblance to one another, as is shown below, and are actually converted one to the other by the enzyme aconitase which is found widely distributed in plant and animal tissues.



Citric acid



Aconitic acid



Isocitric acid

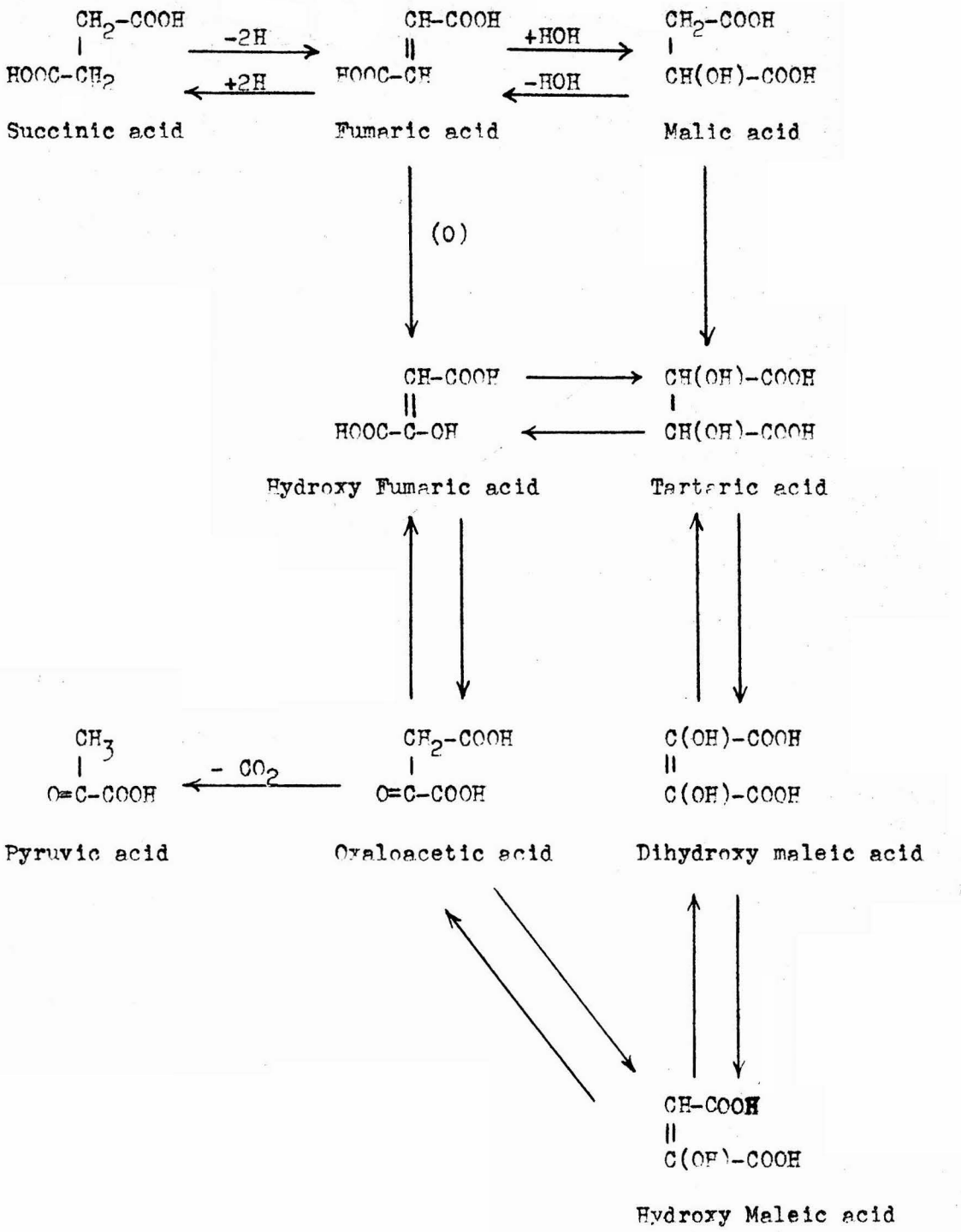
The fact that various keto acids occur in plant tissues has long been known, especially in the case of pyruvic, α-keto glutaric, and acetoacetic acids. Owing to the very small concentrations at which these acids occur in plants and also to the difficulty of their analysis, little can be said about their occurrence in nature. Oxaloacetic acid, $HOOC-CH_2-\overset{O}{\overset{\|}{C}}-COOH$, and α-keto glutaric acid, $HOOC-\overset{O}{\overset{\|}{C}}-CH_2-CH_2-COOH$, have been found by Virtanen (3) in pea seedlings. Pyruvic acid, $CH_3-\overset{O}{\overset{\|}{C}}-COOH$, has been reported in various plant sources.

As to the majority of other organic acids that have been recorded as originating from plant sources, in general each acid

is peculiar to a single plant species, or, at most, to several members of the same family. In this large group the acids are generally found combined, as esters, as glycerides, or as salts of alkaloids, as has already been pointed out. This is in direct contrast to the more common plant acids which are found free or as inorganic salts.

Most frequently the organic acids that have been mentioned occur in green plants, many are found in seeds, (as glycerides) and many are found in other forms of plant and animal life. In this discussion the large numbers of acids found in fungi, bacteria, moulds, etc. will not be mentioned, although in many cases the acids are the same.

Bennet-Clark, in a review of the subject of organic acids (1933) (4), brought out the relationships between some of the four-carbon dicarboxylic plant acids, which are shown below. The group of acids represented in the following diagram are frequently referred to as belonging to the malic acid group, or more frequently, as the four-carbon dicarboxylic acids. Most of the transformations shown have been demonstrated to take place in animal tissues.


CH2-COOH
HOOC-CH2
-2H
+2H
Succinic acid
CH-COOH
HOOC-CH
+HOH
-HOH
Fumaric acid
CH2-COOH
CH(OH)-COOH
Malic acid
(O)
CH-COOH
HOOC-C-OH
Hydroxy Fumaric acid
CH(OH)-COOH
CH(OH)-COOH
Tartaric acid
CH3
O=C-COOH
Pyruvic acid
- CO2
CH2-COOH
O=C-COOH
Oxaloacetic acid
C(OH)-COOH
C(OH)-COOH
Dihydroxy maleic acid
CH-COOH
C(OH)-COOH
Hydroxy Maleic acid

2. Analytical Methods for the Determination of Organic Acids.

Up to 1934 there was no specific or reliable method for determination of the various organic acids, although there was a large volume of literature dealing with the subject of analytical methods. Most earlier workers have determined one or more of the following:

1. Total volatile acids, generally including formic, acetic, and butyric acids, although the monocarboxylic derivatives of the paraffin hydrocarbons up to six carbon atoms are volatile with water vapor. The quantitative determination of these acids was first proposed by Du Claux (5), but the method has subsequently been shown to be of more value as a qualitative test for these acids (6).

2. Total acids titratable between pH 2.6 and 7.8, according to the principle of the method of van Slyke and Palmer (7) for the determination of the organic acids of urine. This method is unreliable for plant extracts because it includes part of the phosphate.

3. Total acid precipitated from neutral solution by lead acetate. This precipitation also includes phosphate, sulfate, some carbohydrate, and much nitrogenous material.

4. Ether soluble acids which are not precipitated by lead acetate. The methods of quantitative analysis that have been used in the past have proven non-specific and unreliable, but a

brief description of some of these will be given. Analyses were generally carried out on the acids with insoluble lead salts and the remaining ether soluble fraction.

Oxalic acid has been determined by its precipitation as the calcium salt and either weighed as such or weighed after conversion to calcium oxide by ignition.

Tartaric acid. There is no test for tartaric acid except its actual isolation. The general practice has been to isolate the acid as potassium acid tartrate. A qualitative test is its ability to form a lilac-red color with resorcin in concentrated sulfuric acid at 125° C.

Citric acid is easily oxidized by permanganate to acetone dicarboxylic acid. Determinations have been based on the precipitation of this oxidation product with mercuric sulfate (8) or by its conversion to the insoluble pentabromoacetone and weighing it as such (9).

Malic acid. This acid has usually been determined by its reduction with palladium chloride (10) or by a method based on the high optical rotation of malic acid in the presence of uranium (11) or of molybdenum (12) salts.

Succinic acid, unlike the other common plant acids is not readily oxidized by boiling permanganate. Hence, after a strong permanganate oxidation of the plant material, succinic acid can be extracted and then precipitated and weighed as the silver salt.

Other acids. Acids containing an active methylene group, such as is found in oxaloacetic, acetoacetic, and malonic acids, react with diazo compounds to give hydrazones which are usually only sparingly soluble.

The above methods of analysis for the common plant acids are subject to much criticism and uncertainty. At the best they have been shown to be useful as qualitative tests and in general good quantitative data have been obtained by the isolation of the individual acids. Since 1934 there have been very reliable methods worked out by Pucher, Vickery, and co-workers (13-19) for relatively

quick and quite accurate determinations of the total acidity of plant tissues, oxalic, citric, malic, and succinic acids. A more direct and probably more reliable method for the determination of succinic acid has been worked out by Szent-Györgyi and Gozsy (20) and simplified and improved by Krebs (21). The exact details of these determinations are given in the experimental sections. There has also been a successful effort by various investigators to work out methods for the determination of the keto acids. The best analytical methods that have been developed for these acids are principally manometric and are based on either a chemical or an enzymatic conversion of the substance in question. The details of these determinations are also given in the experimental sections.

It has been due to the use of these analytical methods that much basic knowledge concerning the plant acids has been acquired. Further insight into the role played by the organic acids in plants will doubtless be obtained by the use of these modern procedures and by the development of methods for other acids.

3. The Separation of Organic Acids.

The separation of the organic acids of plant tissues has been almost completely based on fractional precipitation of their salts. Lead salts have been used by practically all investigators since the time of Scheele (22) as reagents to precipitate the organic acids from plant extracts, basic lead acetate being used the most although possessing many disadvantages. This reagent is not highly selective; much carbohydrate material is found in the precipitates produced by it as well as considerable proportions of widely different nitrogenous substances (23). Vickery and Pucher (24) have found that very excellent results could be obtained by precipitation of the organic acids as barium salts in the presence of dilute alcohol. This method was shown to be quantitative for the better known organic acids and superior in many respects to the customary precipitation of the lead salts by basic lead acetate.

After a preliminary separation of the organic acids by salt precipitations, the preparation and fractionation of the ethyl esters as employed by Franzen (25) is still in general use for the further separation of the organic acids. The ethyl esters have been widely used for fractionation, although in the present work there has been an attempt to separate the acids through the methyl esters. The methyl esters were chosen because of their greater ease of preparation. It has been found that the ester fractionation procedure can be conducted to yield very accurate quantitative work.

In many cases the purified ethyl ester may be crystalline and subsequent identification is comparatively easy. In cases of liquid esters, solid derivatives such as the hydrazide, the phenacyl, and p-phenylphenacyl esters may be prepared and the identity of the fraction secured by means of the melting point and the elementary analysis.

A method for the separation of various fatty acids by the chromatographic adsorption of the p-phenylphenacyl esters has recently been described (26). Attempts to separate the four-carbon dicarboxylic acids that are found in plant tissues by similar methods have thus far proven unsuccessful, but it is to be hoped that chromatographic separation can be worked out because of the relative speed and reliability of such methods.

4. Hypotheses Concerning the Metabolism of Organic Acids.

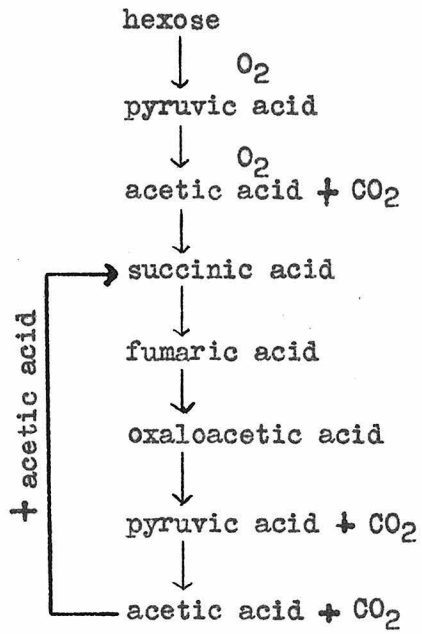
In a review of the subject of organic acids prepared in 1936, Bennet Clark (4) pointed out the unsatisfactory nature of many of the hypotheses of organic acid metabolism. The fact that there had been a considerable gain in the knowledge of the metabolism of organic acids since 1936 was shown by Vickery and Pucher (27) in a similar review published in 1940. However, actual knowledge concerning the role of organic acids in plant tissues is still rather limited. Most earlier workers have emphasized the possible relationship between malic and succinic acids to the amides asparagine and glutamine. The synthesis of amides in plants has been held by Prianischnikow (28, 29) to represent a more or less obligatory mechanism whereby ammonia is converted into a non-toxic substance by combination with some suitable organic acid. Kostychev (30) has held that, although in some cases organic acids can be formed by the abnormal breakdown of carbohydrate, in general the organic acids represent either transformation products of amino acids or products of the incomplete change of sugar to amino acids during the synthesis of proteins. This view has been upheld by the work of Ruhland and Wetzel (31, 32) on acid formation in Begonia. Kostychev gave the following explanation for the formation of malic acid:

$$HOOC-CH_2-CH(NH_2)-COOH \xrightarrow{(O)} HOOC-CH_2-\overset{O}{\overset{\|}{C}}-COOH \xrightarrow{2(H)} HOOC-CH_2-CH(OH)-COOH.$$

The formation of citric acid was thought to result from cleavage products of proteins after their deaminization. However,

recent work (33) has shown that, doubtless, oxaloacetic acid is the precursor of asparagine, α-ketoglutaric acid is the precursor of glutamine, and the role of the organic acids in plants is not as directly tied up with protein metabolism as it is with the intermediate stages of carbohydrate metabolism.

The idea that the organic acids function as intermediates in respiration is not new. The breakdown of sugar, or glycolysis, elucidated by Meyerhoff et al. has been thoroughly worked out and all investigators are agreed upon the fact that pyruvic acid is a direct product. The mechanism of pyruvic acid degradation and of carbon dioxide evolution has remained obscure. That this so-called terminal respiration is accomplished through the common organic acids has become more and more apparent, but direct evidence as to how the acids entered into the system was lacking until more recent work. Thus Thunberg (34) in 1920 and Knoop (35) in 1923 postulated a formal scheme for the oxidation of carbohydrate as follows:



Thunberg knew of the enzymes which oxidize succinic and malic acids as well as carboxylase, so the above scheme was offered as an explanation for the existence of these enzymes in living tissues. The chief flaw in this scheme was the failure to find any evidence for the conversion of acetic acid to succinic acid.

The same scheme was reinvestigated by Töeniessen and Brinkmann (36) in 1930, who modified it because they found that perfused muscle yielded some succinate and a little formate when pyruvate was added, while added acetate gave no succinate. These authors assumed that a condensation takes place at the stage of pyruvate, thus two pyruvic acid molecules condense to a polymerization product, presumably 1,4-diketoadipic acid, which then splits into succinic and formic acids. This scheme has been shown to be not in accordance with more recently acquired facts.

The first real intimation that organic acids participate in part in respiration was contained in the work of Szent-Györgyi (37) in 1935. This was the observation that succinic, fumaric, and malic acids increased the respiration of muscle tissue preparations and that the amount of increased respiration was far greater than could be accounted for by the burning of these acids as a substrate. Therefore, it was concluded that the action of these acids was catalytic. Subsequently, it was shown that muscle tissue contains enzymes which bring about the following interactions:

$$\text{Succinic acid} \underset{+2H}{\overset{-2H}{\rightleftharpoons}} \text{Fumaric acid} \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \text{Malic acid} \underset{+2H}{\overset{-2H}{\rightleftharpoons}} \text{Oxaloacetic acid}$$

Szent-Györgyi (38) also observed that oxaloacetic acid is very rapidly reduced by muscle tissues to a mixture of malate and fumarate (primarily malate) and that the reaction, malate $\rightleftharpoons$ oxaloacetate, is reversible. Szent-Györgyi then suggested that the main function of the four-carbon acids in respiration was to serve as catalysts, or as catalytic hydrogen carriers.

In 1937 four new experimental findings of the greatest importance were made by Krebs and Johnson (39). These were:

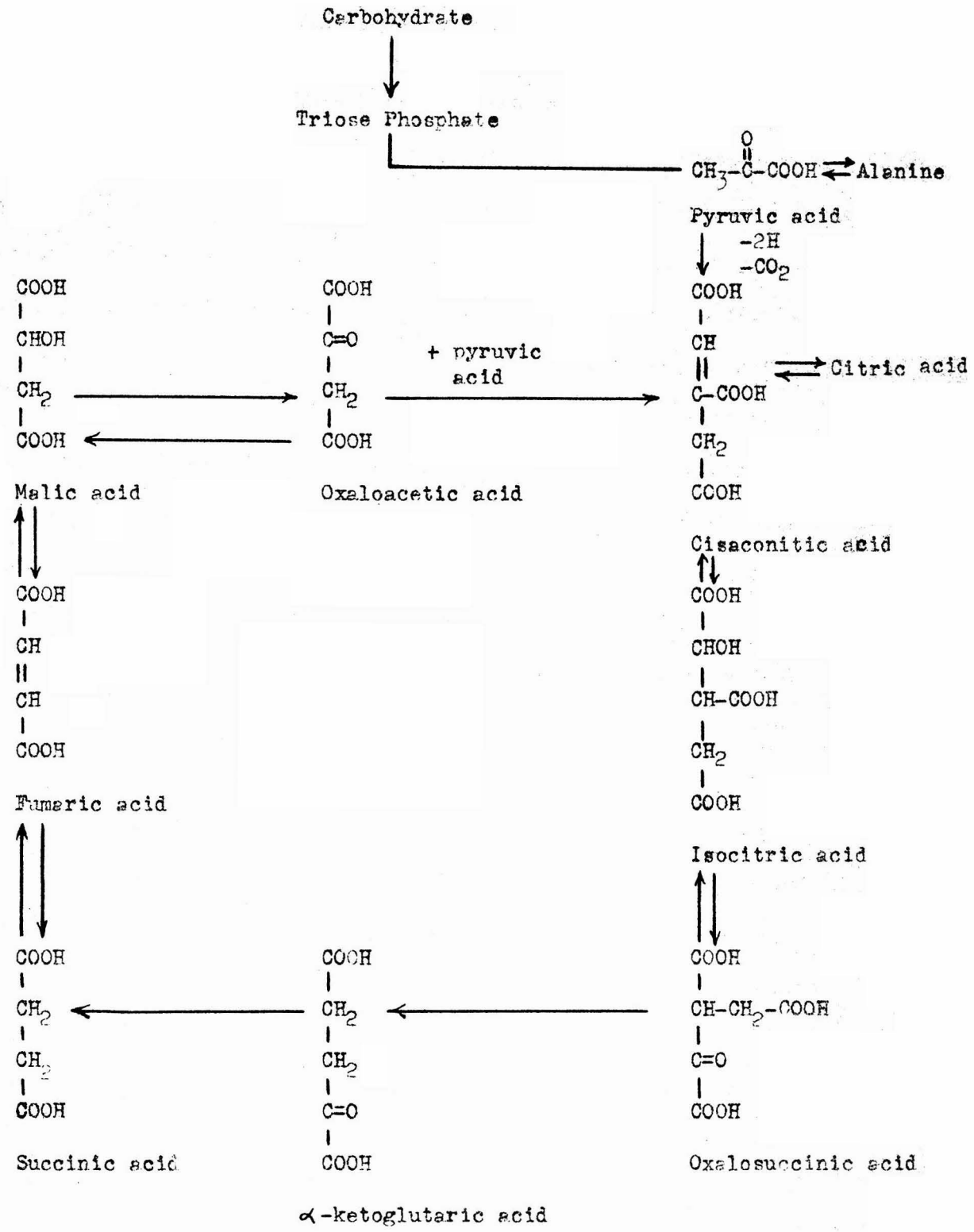
1. Citric acid is catalytically active in respiration, as had been shown for some of the four-carbon acids.

2. Citric, isocitric, cisaconitic, and α-ketoglutaric acids are rapidly oxidized by muscle.

3. Citric and α-ketoglutaric acids are formed in considerable quantities in muscles given oxaloacetic acid.

4. Succinic acid is formed oxidatively from fumaric acid or from oxaloacetic acid in the presence of malonic acid. This shows that succinic acid is not formed by the reduction of fumaric acid or oxaloacetic acid, but is produced by some roundabout manner. This fact shows that the oxidation of the four-carbon acids leads to their reformation; in other words, there is a cycle of oxidations in which the dicarboxylic acids periodically arise.

It was due to the foregoing information that Krebs first postulated the citric acid cycle (or tricarboxylic acid cycle). This cycle, which has been revised (1943) in light of new evidence (40) is given below.


Carbohydrate
Triose Phosphate
CH3-C-COOH ⇄ Alanine
O
Pyruvic acid
-2H
-CO2
+ pyruvic acid
⇄ Citric acid
Malic acid
Oxaloacetic acid
Citric acid
Cisaconitic acid
Fumaric acid
Isocitric acid
Succinic acid
Oxalosuccinic acid
α-ketoglutaric acid

The citric acid cycle of Krebs has been expanded by Chibnall (41) to illustrate a possible combination of α-ketoglutaric acid with ammonia to produce amino acids and the subsequent conversion of aspartic and glutamic acids into their respective amides. Steps are also indicated whereby fats and amino acid residues may enter through succinic acid, and proteins by way of α-keto acids. As this is entirely hypothetical, further discussion should rest until evidence is obtained to show the existence of such reactions.

The probability of such a cycle existing in the respiration of animal tissues is very great. This scheme is based on sound experimental findings and explains very well the fate of pyruvic acid and the constant evolution of carbon dioxide. Each of the components of this system has been shown to stimulate respiration of various animal tissues and most of the transformations have been demonstrated to take place in both plant and animal tissues.

The possibility of such a cycle or sequence of reactions occurring in the respiration of plant tissues has been suggested by various workers, but there is little evidence as yet for a strong argument for or against it. All of the acids of the Krebs citric acid cycle have been found in higher plants, but not all these acids have been identified in a single plant species. The existence of the enzyme systems involved in the cycle have been shown to exist quite widely throughout the higher plants, but, again, not all of the systems have been demonstrated as existing in the same plant source.

The study of the organic acid metabolism of plants with the exception of the succulents has been limited to very few species and the actual knowledge that has been obtained has been small. Analysis of fresh tobacco leaves cultured in water or in nutrient solutions, either in continuous light or in continuous darkness, have been made by Vickery and co-workers (42,43). It was found that the total acidity changed very little, but that in the dark malic acid diminished rapidly and at the same time citric acid increased. These changes were taken to signify a conversion of malic to citric acid under the conditions of leaf culture. However, a similar study of tobacco stalks denuded of leaves (44) led to different results. In this case malic acid increased both in the dark and light, and citric acid, which is present only in small amounts, increased some in the dark and diminished slightly in the light. The difference between the behavior of the organic acids in the leaves and stalks was taken to be due to the wide differences in composition. Whereas the leaves are relatively high in protein and low in carbohydrate, the reverse is the case for the stalks.

Vickery and co-workers (45) have further shown a very marked dependence of the organic acids of the tobacco plant on the form in which nitrogen is supplied. This work showed that plants grown with nitrate as the sole source of nitrogen contain a much larger quantity of organic acids than similar plants grown with ammonia alone. That a similar dependence of organic acid composition on

the form of nitrogen given the plant was shown by Clark (46) to be true for tomato plants.

Vickery and Pucher et al. (47) in an investigation of the rhubarb plant demonstrated that the behavior of the acids of rhubarb leaves differs greatly from that of the acids of tobacco leaves. There was, in rhubarb, a diminution of total organic acids during culture in darkness and in the light a temporary increase, which was due almost entirely to an increase in malic acid. Culture in glucose solution brought about similar, but more prolonged changes and it was suggested that there is a transformation of the imbibed carbohydrate into malic acid.

Thus it can be seen from these few investigations that there is a very definite relationship between the organic acids of plants and their metabolism or respiration. The organic acids seem to be placed in a rather clearly-defined relationship to the main groups of plant constituents, the proteins, the fats, and the carbohydrates. Just what the individual transformations are remains for future investigators to work out.

A question of great importance which has not been discussed is a consideration of why it is that organic acids accumulate in ripening fruit, such as the pineapple. Some (48) have likened this phenomenon to the behavior of the succulents (see Chapter 5). As a rule, when fruit ripens the acid content decreases while the sugar content increases. However, there is no obvious connection between the two processes. In some cases, (pineapple) there is a simultaneous increase in both sugar and acids. In other cases

(avocado) there is an increase in fat. No information is available concerning the accumulation of substances in ripening fruit. If one assumes that such a cycle as the citric acid cycle actually accounts for the terminal respiration in plants, then perhaps it is possible to further assume that in a ripening fruit, such as the pineapple, a block occurs in the cycle which prevents the transformation of isocitrate to oxalosuccinate. If the pineapple tissues contain aconitase, which they doubtless do, the accumulated isocitric acid would be converted through aconitic acid to citric acid. Here again is needed further knowledge concerning the interconversion of the organic acids in plant tissues.

5. Previous Work on the Organic Acids of Pineapple

Most of the earlier work on the organic acids of pineapple was limited to the titration of the free acids with alkali using phenolphthalein as an indicator (49). In early investigations, Kayser (50) stated that citric acid was a major component of the acids of pineapple and, later, Nelson (51) claimed that, of the total acids present, 87% was citric and 13% l-malic. More recently Clark (52,53), using the methods of Vickery, Pucher et al., has shown that the citric acid content varied from 28% to 66% and malic acid from 18% to 27% of the total acids depending on the conditions under which the plant was growing; while in all cases oxalic acid probably constituted less than 0.005% (as oxalates) of the total acids. The latter investigator also showed that with fruits containing large total amounts of acid, the unknown acids comprised a comparatively small fraction of the total. In less acidic fruits, the unknown acids constituted a greater proportion of the total. In some cases about one-half of the total acidity was due to unknown acids. Unknown acids in this sense embraced all those not readily determinable, including tartaric, succinic, etc.

The present work was undertaken in order to (a) determine the nature of the acids in pineapple and to account quantitatively for their amounts, (b) to find out if any new or unusual acids might be found in the pineapple, and (c) to investigate, if possible, the biochemistry and metabolism of the organic acids of pineapple using fruits which had been grown under different conditions and which had been harvested at various stages of growth.

Unfortunately, time did not allow the latter phase of this investigation. Three large-scale experiments were performed in the investigation of pineapple. In Experiment III, which is presented in the next section, an attempt was made to account for and to determine the nature of all the organic acids in the pineapple concentrate, i.e. to draw up a balance sheet for all the acids present; this has been successfully accomplished. Experiments I and II were of a more specialized nature. In the course of Experiment I the lactone of saccharinic acid was isolated. This raised the question as to whether it was of natural origin or whether it might be an artifact brought about by the conditions of the experiment. Experiment II attempted a solution of this question, mainly through the use of exceedingly mild conditions. In addition, an effort was made in Experiment I to determine if the organic acids of pineapple could be conveniently separated through their heavy metal salts, and to investigate whether the commonly used esterification method of Phelps and Phelps (54), which involves a long period of heating under very acid conditions, could be eliminated by the use of diazomethane. By using diazomethane mixtures of organic acids may be quickly and easily esterified at room temperature.

A detailed description of Experiments III, I, and II will be presented in the following sections. Experiment III, which gives a general survey of the acid composition is presented first and is followed by Experiments I and II.

CHAPTER II

Composition of Organic Acids in the Non-Volatile Concentrate of Fresh Pineapple Fruit.

1. General Survey of Experiment III.

The results from Experiment III show that, of the total water soluble acids, 10.8% were insoluble in methyl alcohol and 13.4% were insoluble in ether. Of the remaining ether soluble acids, 52.2% was citric acid, 32.6% malic acid, and 15.2% appeared in the tartaric acid fraction. In addition, there appeared a maximum of approximately 0.5% of keto acids of unknown nature. The three known acids account together for approximately 100% of the total. The ether soluble acids consist, therefore, predominantly of common plant acids except for the traces of keto acids. Only one unusual acid was found in an appreciable amount, namely saccharinic acid, which will be discussed in the following sections.

The nature of the titratable acids which appeared in the methyl alcohol insoluble fraction (fraction 2) and in the ether insoluble fractiom (fraction 9) is unknown. In the latter case it is possible that these compounds are sugar acids and poly-hydroxy acids of one sort of another (e.g. ascorbic acid and similar compounds).

2. Experiment III.

The starting material used in this investigation was a

pineapple concentrate, summer crop of 1942, from which all volatile material had been removed by steam distillation. This concentrate contained 0.296 acid equivalents per 100 grams including 42.2% of citric acid and 24.5% of malic acid as shown by analysis. The presence of oxalic acid could not be detected. Ten kilograms of this material were treated with fifteen liters of 99% methyl alcohol, the resulting white precipitate centrifuged off and thoroughly washed with methanol. To the filtrate were added ten more liters of 99% methanol, the resulting mixture placed at 0°C. for 24 hours and then again centrifuged. The precipitate from the two methyl alcohol treatments contained 10.8% of the total original acids. These represent methyl alcohol insoluble, water soluble acids whose nature was not further investigated.

The filtrate from the methyl alcohol precipitation (fraction 1) was concentrated in vacuo to a thick syrup, taken up in four liters of water, and adjusted to pH 7 by the addition of a calcium hydroxide slurry. A grey, slimy precipitate was obtained which when dried in vacuo over anhydrous calcium chloride weighed 490 grams (fraction 4).

The filtrate from the cold calcium hydroxide precipitation (fraction 3) was next heated in two liter portions to 95°C. for one minute. The resulting white granular precipitate was filtered immediately. After concentration of the filtrate (fraction 5) to about one-half the original volume, precipitation

by heating was repeated. The combined precipitates (fraction 6) amounted to 964 grams.

Fraction 4.

This fraction containing organic acids whose calcium salts were insoluble in water at room temperature was suspended in water and the pH adjusted to 3 by the addition of glacial acetic acid. After thorough stirring the suspension was centrifuged and the insoluble material dried in vacuo over phosphorus pentoxide. When dried this material had a weight of 172 grams. The salts extracted by the acetic acid treatment were decomposed by the dropwise addition of a saturated aqueous oxalic acid solution, the resulting calcium oxalate centrifuged off and the filtrate concentrated in vacuo to about 200 cc. Ten percent potassium hydroxide was added until the solution was alkaline to litmus and then two liters of 95% ethyl alcohol were added. A fine white precipitate ensued from this treatment. The procedure used in this step is based on the official method for the separation and determination of tartaric acid as potassium acid tartrate (55). It may be assumed, therefore, that the present fraction represents primarily tartaric acid.

Fraction 5.

The fraction containing acids whose calcium salts were soluble at 95°C. was brought to pH 7 by the addition of dilute sodium hydroxide and extracted with ether in a continuous

extractor. This alkaline ether extraction rid the concentrate of non-acidic material. The residue was next acidified to pH 1 and extracted with ether. The ether soluble portion (fraction 10) consisting of 6.4 acid equivalents was subjected to the fractionation described under 10 below. Fraction 9 containing the ether insoluble acids made up 13.4% of the total acids of the original concentrate. This fraction should contain sugar acids and other polyhydroxy acids including ascorbic acid. The chemical nature of the fraction was not further investigated.

Fraction 6.

Ten grams of this fraction containing the calcium salts of organic acids which were soluble in water at room temperature and insoluble in water at 95°C. were suspended in water and 20% aqueous oxalic acid solution was added dropwise until no further precipitation took place. The resulting calcium oxalate was centrifuged off and the filtrate concentrated *in vacuo* to a thick yellowish syrup which crystallized on standing for a short time in the cold. This substance gave a red color by heating with pyridine and acetic anhydride, a spot test for citric acid (56). When recrystallized it had a melting point of 152-153°C. in agreement with the value of 153°C. reported for citric acid. The mixed melting point of this substance with pure citric acid was 152-153°C., showing without doubt that the substance was citric acid. Since the original starting material contained 12.5 equivalents of citric acid and this fraction

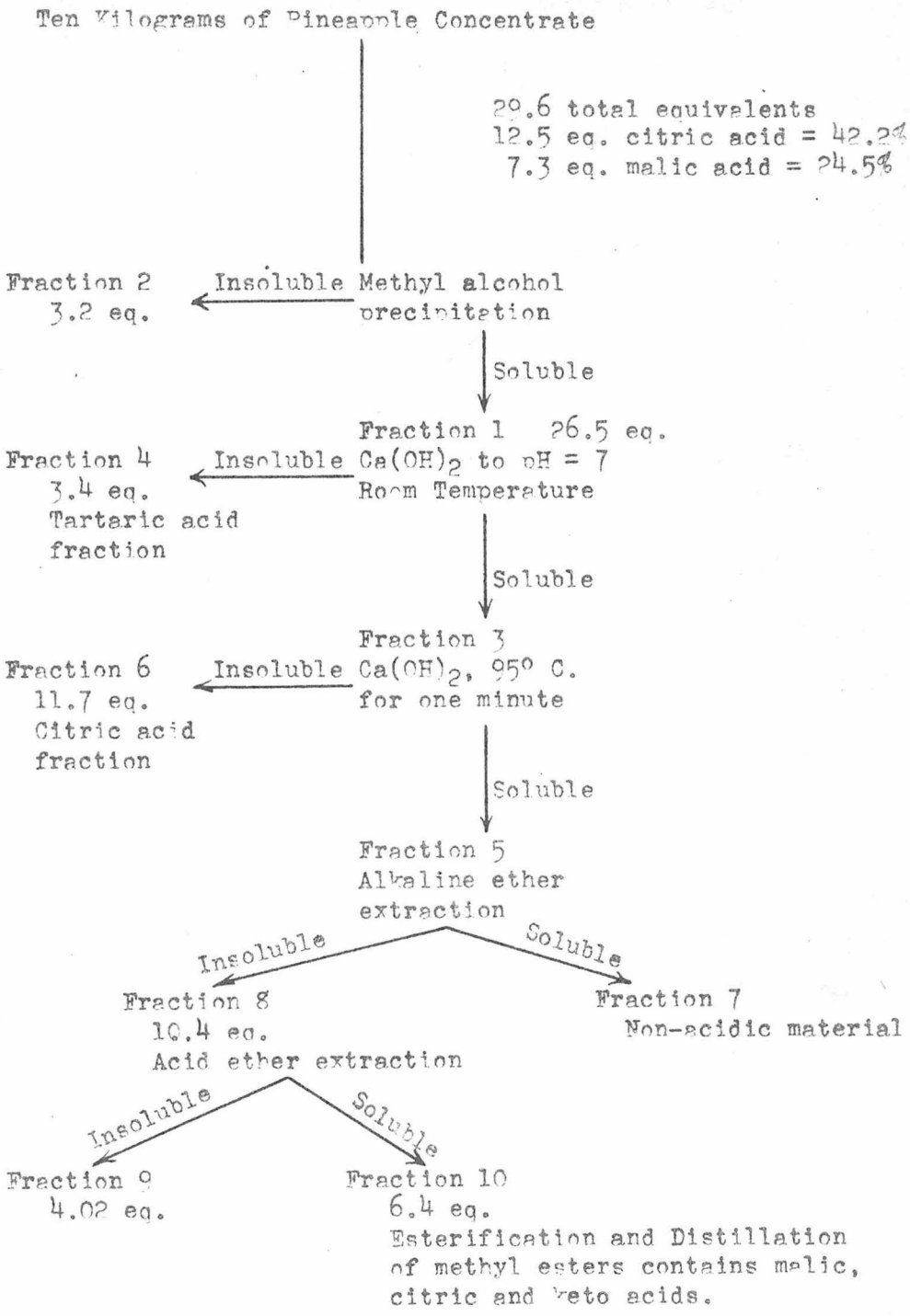
contained 11.7, it follows that 93.8% of the citric acid had been removed and recovered in pure form in this fraction. A further amount was recovered in fraction 10.

A diagram showing the steps involved in preparing the various fractions from the original concentrate and which also gives the distribution of the titratable acids through the fractions is presented in Figure 1.

Fraction 10.

One half of this fraction (64.3 grams) containing the ether soluble organic acids from which tartaric acid and the bulk of the citric acid had been removed was taken up in methyl alcohol and treated with diazomethane. The diazomethane, prepared by the ether distillation process of Adamson and Kenner (57), was added in lots of ten grams dissolved in two liters of anhydrous ether until an excess was present, as shown by the absence of a vigorous evolution of gas on addition of the reagent. The esters were then transferred to a 125 cc. Claisen flask, the solvent removed, and roughly fractionated under a high vacuum. The Claisen flask had been equipped with a 10 mm. diameter side arm which was placed very low on the neck. The fractions obtained by distillation of the esters at 0.02 mm. are shown in Table 1.

Figure 1. Outline of Experiment III



Fraction	Boiling Range	Weight	Nature of Acids
10 - a	45 - 80°C.	16.3 grams	largely malic acid
10 - b	81 - 105°C.	3.1 grams	citric acid
10 - c	106 - 120°C.	4.7 grams	citric acid
10 - d	121 - 130°C.	1.6 grams	---
10 - e	(caught in trap)	13.1 grams	keto acids

Table 1. Distribution of Methyl Esters Derived from Fraction 10.

Of the first four fractions, 10 - b and 10 - c crystallized on cooling to room temperature. The crystalline material in both instances was shown to be the tri-methyl ester of citric acid. In addition to these four fractions, 13.1 grams of liquid (fraction 10 - e) were condensed in a trap cooled by dry ice and alcohol which had been placed between the distillation flask and the vacuum pump. Although fraction 10 - e was to some extent further investigated, it is not known whether it represents true components of the ester fraction or is made up of breakdown products brought about by the high temperature of the distillation. The residue (30.3 grams) was a very hard and brittle substance which may have been formed from the acid esters during heating.

Fraction 10 - e

This fraction containing the volatile material from the fractionation of methyl esters was distilled from a 25 cc. Claisen flask through a 1.5" head packed with helices. Table 2

shows the fractions that were obtained by distillation at 760 mm.

Fraction	Boiling Range	Weight
10 - f	65 - 67°C.	6.2 grams
10 - g	68 - 70°C.	2.3 grams
10 - h	71 - 98°C.	4.1 grams

Table 2. Fractions Obtained by Distillation of Fraction 10 - e.

Each of these fractions gave heavy precipitates with an acid solution of 2,4-dinitrophenylhydrazine showing the presence of carbonyl groups. Fraction 10 - f had a very penetrating odor reminiscent of the smell of crab meat. 107 mgs. of a 2,4 -dinitrophenylhydrazone were prepared from fraction 10 - f by the addition of an acidified solution of 2,4-dinitrophenylhydrazine. When dried, this substance had a melting point of 195 - 197°C. which is very close to the melting point of 2,4-dinitrophenylhydrazine itself, indicating that some of the reagent was occluded. This fact was confirmed by microscopic observation which showed two distinct crystal forms.

The substance was extracted on a sintered glass filter with small portions of boiling petroleum ether (60 - 70°C.) until no more colored material was extracted. A residue of 45 mgs. of 2,4-dinitrophenylhydrazine (insoluble in pet. ether) remained. Evaporation of the filtrate yielded 60 mgs. of a yellow-orange substance which was dissolved in 5 cc. of pure dry benzene and

passed through a 10 mm. x 15 cm. column packed with a 2:1 mixture (by weight) of dicalite and Mefford alumina. A narrow orange band resulted near the top, the rest of the material being spread throughout the column. The latter was easily washed through with 20 cc. of benzene. On removal of the benzene an orange substance remained which readily crystallized from 95% ethyl alcohol, yielding a few milligrams of homogeneous orange needles of constant melting point 116 - 117°C., and which gave the following microcombustion analysis:

3.020 mgs. gave 1.129 mgs. H_2O, 5.206 mgs. CO_2.
Found: 27.05% Carbon, 4.18% Hydrogen.
1.183 mgs. gave 0.236 cc. N_2.
Found: 21.46% Nitrogen.

Calculated for $C_{10}H_{11}N_4O_4$
47.80% Carbon, 4.38% Hydrogen, 22.31% Nitrogen

Calculated for $C_9H_{10}N_4O_4$
45.71% Carbon, 4.23% Hydrogen, 23.52% Nitrogen

Since almost all of this compound was used for analysis, nothing further could be done with it.

Fractions 10 - a, 10 - d, and the mother liquors from fractions 10 - b, 10 - c, a total of 17.6 grams were recombined and carefully fractionated from a 50 cc. Claisen flask through a two-inch head packed with helices. The fractions that were obtained are tabulated in Table 3. Table 4 shows some of the characteristics and the nature of each fraction.

Fraction	Boiling Range	Weight
1.	45 - 55°C.	0.8 grams
2.	56 - 57°C.	0.7 grams
3.	64 - 66°C.	1.3 grams
4.	67 - 70°C.	3.2 grams
5.	71 - 73°C.	1.9 grams
6.	74 - 77°C.	1.8 grams
7.	78 - 83°C.	1.1 grams
8.	84 - 85°C.	1.3 grams
9.	86 - 105°C.	3.8 grams
10.	106 - 110°C.	0.2 grams
Residue		1.9 grams

Table 3. Further Fractions Obtained from the Distillation of Methyl Esters of Fraction 10.

Fraction	Saponification eq.	%C.	%H.	m.p. of Hydrazide	Nature of Acid
1.	102	44.8	6.8	164-166*	impure malic
2.	-	44.4	6.2	176-177	malic
3.	-	44.4	6.2	173-174	malic
4.	86	45.0	6.9	158-160	impure malic
5.	82	43.5	6.5	161-162*	impure malic
6.	87	43.5	6.6	167-169*	impure malic
7.	106	44.5	6.8	167-169*	impure malic
8.	-	49.6	7.1	-	impure malic
9.	76	46.6	6.0	106-107	citric
10.	76	46.6	6.0	106-107	citric

* Hydrazides which were not further purified after their preparation.

Table 4. Characteristics and Nature of Acids in Fractions Derived from Fraction 10.

Of these, fractions 9 and 10 crystallized on cooling, and in both cases the crystalline material was shown to be the trimethyl ester of citric acid.

Fraction 1.

This fraction was a light yellow, viscous liquid that showed no tendency to crystallize after several weeks at - 80°C. A microcombustion analysis gave the following results:

3.259 mgs. gave 1.992 mgs. H_2O, 5.354 mgs. CO_2
Found: 6.84% Hydrogen, 44.83% Carbon

The saponification equivalent was obtained by the method outlined in Huntress and Mulliken (58) and the result attained was 101.80.

The ester gave a positive test for primary and secondary alcohols, a negative test for phenols, and a faintly positive test for the carbonyl group.

The hydrazide was prepared by the method of H. Franzen (59) and 4 mgs. of a substance melting at 155 - 157°C. were obtained. A large volume of absolute alcohol added to the filtrate from this hydrazide preparation resulted in 76 mgs. of a hydrazide of melting point 164 - 166°C., which fact indicates that this fraction consists of two substances. In view of the results procured with fractions 2 and 3 and the fact that strong hydroxyl tests were obtained, this fraction may have consisted mainly of impure dimethyl malate.

Fraction 2.

From 50 mgs. of fraction 2, 57 mgs. of a hydrazide of melting point 173 - 174°C. were prepared. The hydrazide was recrystallized from dilute alcohol to the constant melting point of 176 - 177°C. The following microcombustion analysis was obtained for this compound.

2.954 mgs. gave 1.654 mgs. H_2O, 3.282 mgs. CO_2
1.584 mgs. gave 0.490 cc. N_2
Found: 6.27% Hydrogen, 30.32% Carbon, 34.37% Nitrogen
Calculated for malic dihydrazide.
29.6% Carbon, 6.2% Hydrogen, 34.6% Nitrogen

100 mgs. of fraction 2 were hydrolized by refluxing with 1 N sodium hydroxide in methyl alcohol for four hours. During this time, a solid substance separated out which was filtered and dried. This sodium salt weighed 110 mgs.

The p-phenylphenacyl ester was prepared from 100 mgs. of the sodium salt and was recrystallized from dioxane to the constant melting point of 192 - 193°C. The solid ester gave the following microcombustion analysis:

2.948 mgs. gave 1.412 mgs. H_2O, 7.831 mgs. CO_2
Found: 72.49% Carbon, 5.36% Hydrogen
Calculated for di-p-phenylphenacyl malate.
73.5% Carbon, 4.9% Hydrogen.

A mixed melting point with the di-p-phenylphenacyl ester of l-malic acid gave no depression, indicating that this fraction was doubtless largely composed of malic acid.

Fraction 3.

From 84 mgs. of this fraction a pure hydrazide of melting point 173 - 174°C. was obtained. A mixed melting point of the hydrazide with that of fraction 2 gave no depression, showing that these two compounds were identical.

200 mgs. of the fraction were hydrolized in the same manner as with fraction 2, yielding 300 mgs. of a sodium salt which was insoluble in methyl alcohol. A p-phenylphenacyl ester with a melting point of 191 - 192°C. was prepared from the sodium salt which gave no depression in a mixed melting point with the same derivative of l-malic acid. This fact shows that the fraction also consisted largely of malic acid.

Fraction 4.

Fraction 4 was a light yellow, rather viscous liquid at room temperature. It showed no tendency to crystallize during a two week period at -80°C. The fraction gave a saponification equivalent of 85.75, analyzed for 45.03% carbon and 6.91% hydrogen. It gave negative tests for primary and secondary alcohols, carbonyl groups, phenolic and sulfur groups.

From the ester a hydrazide was prepared which was crystallized from dilute alcohol to the constant melting point of 158 - 160°C., and which gave the following microcombustion analysis:

3.065 mgs. gave 1.620 mgs. H_2O, 3.377 mgs. CO_2
1.352 mgs. gave 0.376 cc. N_2
Found: 30.07% Carbon, 5.92% Hydrogen, 30.02% Nitrogen.

A small amount of the ester was hydrolized by refluxing with 1 N hydrochloric acid for three hours. The hydrolysis mixture was shaken five times with ether and then once again after saturation of the aqueous phase with salt. The ether portion was dried over anhydrous sodium sulfate and the ether removed under reduced pressure, yielding a small amount of a reddish brown liquid.

45 mgs. of a p-phenylphenacyl ester were prepared from the hydrolysis product. On crystallization from 95% ethyl alcohol, the constant melting point of 132 - 133°C. was obtained. A microcombustion analysis gave the following results:

3.026 mgs. gave 1.461 mgs. H_2O, 8.199 mgs. CO_2
Found: 73.94% Carbon, 5.40% Hydrogen.

There was no further progress made with this fraction.

Fraction 5.

This ester gave a saponification equivalent of 81.57, showed by analysis 43.47% carbon and 6.54% hydrogen and gave negative tests for primary and secondary alcohol groups, phenolic and sulfur groups. The hydrazide was prepared yielding a non-purified product of melting point 161 - 162°C. The combustion analysis and saponification equivalent indicate strongly that this fraction may have consisted mainly of dimethyl malate.

Fraction 6.

From this fraction a saponification equivalent of 87.17 was obtained. The fraction analyzed for 43.47% carbon and 6.59% hydrogen and gave negative tests for alcohol, phenolic, sulfur, and carbonyl groups. A hydrazide of melting point 167 - 169°C. was obtained. The combustion analysis and saponification equivalent indicate that this fraction may have consisted largely of impure dimethyl malate.

Fraction 7.

This fraction showed a saponification equivalent of 106.55 and gave an analysis of 44.46% carbon and 6.85% hydrogen. A hydrazide of melting point 167 - 169°C. was prepared. The combustion analysis and saponification equivalent give good reason to believe that this fraction also may have consisted largely of impure dimethyl malate.

Fraction 8.

No hydrazide could be prepared from this fraction. One hundred milligrams of the ester were refluxed with 1 N sodium hydroxide in methyl alcohol for four hours. At the end of this period, the mixture was evaporated in vacuo to dryness, taken up in water, acidified with 4 N sulfuric acid, incorporated in asbestos, and extracted with ether in a small Soxhlet extractor. The ether on evaporation left a small amount of an impure crystalline substance of melting point 75 - 80°C. Because of the very small quantity of this substance, no further work could be carried out.

Fractions 1 thru 7 all would appear on the basis of elementary analysis and melting point of the hydrazides to consist largely of the ester of malic acid, contaminated especially in fractions 4 and 5 with a considerable amount of another material, perhaps the methyl ester of citric acid. The exceedingly small quantities of material available has made it impossible to carry the identification further, however.

Summary

Of the 29.6 acid equivalents in the original concentrate, only 22.4 acid equivalents were ether soluble. Of this, 52.2% was found to be citric acid, 32.6% to be malic acid, while 15.2% appeared in the fraction considered to contain tartaric acid. The keto acids found in fraction 10 - e made up 0.5% or less of the total ether soluble acids. Thus the ether soluble acids can be accounted for completely.

CHAPTER III

Isolation of the Lactone of Saccharinic Acid.

1. The Isolation of the Lactone of Saccharinic Acid.

Experiment I

Experiment I was undertaken to determine the general distribution of the organic acids contained in the non-volatile concentrate from fresh pineapple and to investigate desirable methods for the separation and purification of the organic acids. During the course of this experiment the lactone of saccharinic acid was isolated in an appreciable amount, this compound being the only organic acid isolated which deviated from the common plant acids. A detailed description of the isolation follows.

From 1400 kilograms of pineapple (summer crop of 1939), 1.04 kilograms of the concentrated syrup were used as a starting material. This concentrate contained 0.653 acid equivalents, as shown by titration with alkali. The titration was carried out by dissolving a weighed sample (approximately 0.1 gram) in 100 cc. of carbon dioxide free water and titrating 10 cc. portions of the hot solution with 0.01 N potassium hydroxide to a phenolphthalein end point.

To the concentrate were added successive portions of 95% ethyl alcohol until no further colored material was removed from the resulting white precipitate. The combined alcohol extracts are designated as fraction I. (See Figure 2).

The alcohol insoluble portion, which probably occluded some alcohol soluble material, was dissolved in a small amount of water, acidified to a pH of 0.5, and shaken with four liters of 95% alcohol. After a short period of shaking, a heavy white precipitate was formed from which the orange-colored alcoholic solution was decanted. This procedure was repeated until no more colored substance was extracted. These solutions were combined (fraction II) and the residual precipitate which contained a negligible amount of acidic material was discarded.

Fractions I and II were concentrated in vacuo to a volume of about 600 cc. each, and then treated with a hot saturated barium hydroxide solution until precipitation was complete. The precipitated barium salts were removed from the solutions by centrifugation, washed with a dilute barium hydroxide solution, suspended in water, and decomposed with 4 N sulfuric acid. The resulting mixtures were concentrated in vacuo to a volume of 250 cc. each.

Water soluble barium salts were also decomposed with 4 N sulfuric acid, the solutions concentrated under reduced pressure, and the residues taken up in 250 cc. of water. At this point the pineapple concentrate had been separated into five fractions which are listed in Table 5 along with the acid equivalents in each fraction as determined by titration with alkali.

Fraction I-a:	Alcohol soluble acids with barium insoluble salts	0.205 acid eq.
Fraction I-b:	Alcohol soluble acids with barium soluble salts	0.023 acid eq.
Fraction II-a:	Alcohol insoluble substances	0.004 acid eq.
Fraction III-a:	Acid alcohol soluble acids with barium insoluble salts	0.130 acid eq.
Fraction III-b:	Acid alcohol soluble acids with barium soluble salts	0.165 acid eq.
Total acid equivalents		0.527 acid eq.

Table 5. Acid Equivalents in Fractions of Pineapple Concentrate.

The fractions I-a, I-b, III-a, and III-b were then extracted in continuous extractors with purified ether. The extraction was carried out for four months, after which time no appreciable quantity of acidic material remained in the residue.

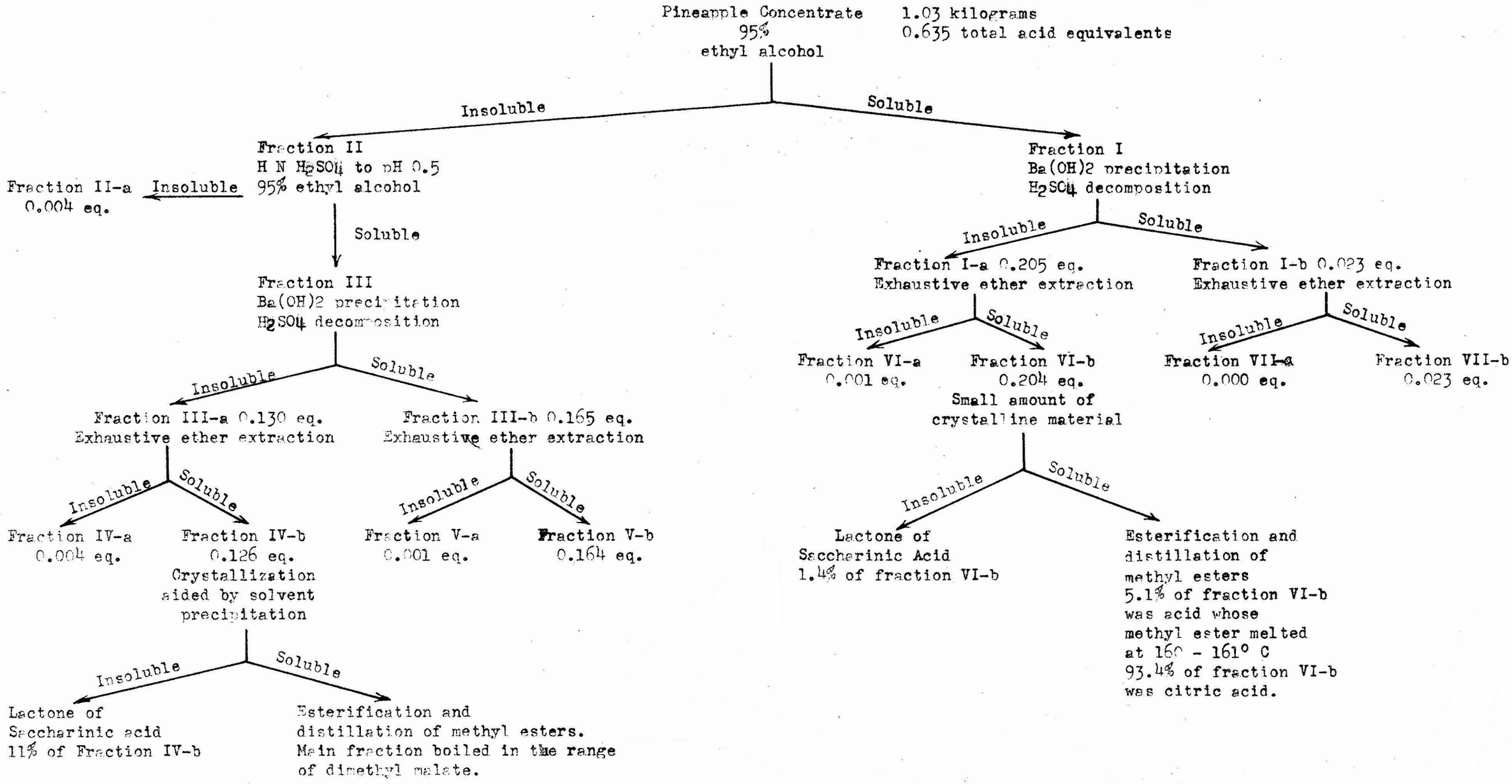
During the course of the extraction the pH of the material was checked every other day and kept approximately at 0.5 by the addition of 4 N sulfuric acid. During the early period of the extraction the ether extract was removed daily and fresh ether was added. The ether extracts were dried over anhydrous sodium sulfate and the ether removed by distillation under reduced pressure. The number of acid equivalents in each fraction are shown in Table 6.

Fraction I-a			
Ether insoluble	Fraction VI-a	0.001 eq.	
Ether soluble	Fraction VI-b	0.204 eq.	24.5 grams
Fraction I-b			
Ether insoluble	Fraction VII-a	0.000 eq.	
Ether soluble	Fraction VII-b	0.023 eq.	10.8 grams
Fraction III-a			
Ether insoluble	Fraction IV-a	0.004 eq.	
Ether soluble	Fraction IV-b	0.126 eq.	72.8 grams
Fraction III-b			
Ether insoluble	Fraction V-a	0.001 eq.	
Ether soluble	Fraction V-b	0.164 eq.	51.3 grams
Total acid equivalents		0.523	
Total acid equivalents extracted		0.517	

Table 6. Results Obtained from the Ether Extraction of Fractionated Pineapple Concentrate.

From these results it follows that 99% of the organic acids may be obtained from water solution by prolonged ether extraction, and that, of the total quantity of acids present in the original concentrate, 81% are accounted for by the ether soluble material. This compares with 85% obtained in the preceding section (methyl alcohol soluble, ether soluble acids as per cent of total methyl alcohol soluble acids). Data on the individual fractions are given in Table 6, and Figure 2 shows the steps involved in obtaining these fractions.

Figure 2 Outline of Experiment I



Fraction IV-b

The residue from the ether soluble fraction containing alcohol soluble acids which gave water insoluble barium salts was allowed to stand in the cold for several days, whereupon a small amount of crystalline material was deposited. The mother liquor was removed from the crystalline material by filtration and was then treated with a 1:1 mixture of ethyl acetate and acetone which precipitated a further quantity of the same material. The crude crystals had a melting point of 138-158° C. This material was then recrystallized five times from glacial acetic acid which gave 2.5 grams of a compound melting at 164-165° C. Crystallization from 95% ethyl alcohol and absolute alcohol did not alter the melting point.

The solubilities of this substance were as follows:

Very soluble: water, hot 95% ethyl alcohol, hot absolute ethyl alcohol, and hot glacial acetic acid.

Slightly soluble: ether, cold 95% ethyl alcohol, acetone, ethyl acetate, methyl alcohol, methyl acetate, benzene, and dioxane.

The substance was shown to be a carboxylic acid or a derivative thereof by conversion to the hydroxamic acid which gave a violet color with ferric chloride in acid solution. Conversion to an alkali xanthogenate indicated that the substance contained either primary or secondary hydroxyl groups. Tests with bisulfite and azobenzene-phenylhydrazine sulfonic acid were negative, disclosing that no carbonyl groups were present. The substance did

not decolorize bromine thus showing the absence of unsaturation. Fusion with sodium and subsequent treatment with potassium fluoride and ferrous sulfate indicated that the substance did not contain nitrogen.

Analytical Data

Microcombustion Determination

2.970 mg. gave 1.689 mg. H_2O, 4.934 mg. CO_2
Found: 45.34% Carbon, 6.36% Hydrogen
Calculated for the lactone of saccharinic acid,
44.44% Carbon, 6.16% Hydrogen.

Zerewitinoff Determination

3.321 mg. gave 1.34 cc. CH_4; 25° C., 745 mm. Hg.
Found: Four active hydrogens.

Zeisel Determination

4.099 mg. gave no CH_3I
Found: No methoxyl present.

Equivalent Weight Determination

6.21 mg. substance, 2.82 ml. 0.01 N KOH required
Found: equivalent weight - 168.75
Calculated equivalent weight for the lactone of saccharinic acid: 162.

Determination of Optical Rotation

260.1 mg. substance (in water)
Found:

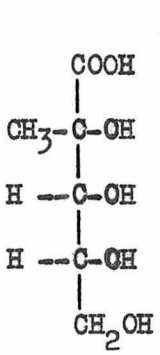
$$[\alpha]_D^{20} = 90.2$$

Optical Rotation for the Lactone of Saccharinic Acid

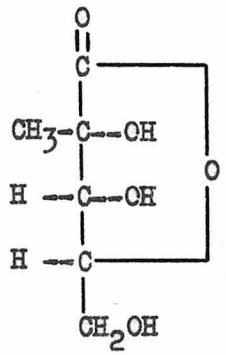
(Beilstein)

$$[\alpha]_D^{20} = 93.8$$

Since the analytical data of this substance agreed very well with the lactone of saccharinic acid, the latter compound was prepared according to the method of Scheibler (60) by the action of hot concentrated calcium hydroxide on glucose. The lactone was crystallized from glacial acetic acid to the constant melting point of 161-162° C. A mixed melting point of the unknown substance with the lactone of saccharinic acid gave no depression, showing conclusively that the unknown was the lactone of saccharinic acid. The structures of saccharinic acid and its lactone are given below.



Saccharinic Acid



"Saccharin", lactone of saccharinic acid

Twelve grams of this same fraction were esterified by the use of diazo methane and the resulting methyl esters roughly fractionated under high vacuum. Six liquid fractions were obtained between 60 and 180° C. The majority of these fractions were very viscous, reddish brown liquids which, when hydrolyzed even under very mild conditions, seemed to decompose and only very small amounts of a crude hydrolysis product could be obtained.

Fraction VI-b

On standing at 0° C. for several days this fraction yielded a crystalline substance which amounted to 0.59 grams. The compound was crystallized from glacial acetic acid to the constant melting point of 160-161° C. That this compound was the lactone of saccharinic acid was easily demonstrated by analysis and a mixed melting point which gave no depression.

10.6 grams of the filtrate obtained on removing the lactone of saccharinic acid were dissolved in purified dioxane and treated with an excess of diazo methane. When the vigorous reaction was completed, the solvent was removed under reduced pressure leaving a thick syrup which was placed in a small Claisen flask and fractionated at 0.05 mm. The distribution of the fraction is given in Table 7.

Fraction	Boiling Range	Weight	Description
1.	50-100° C.	4.2 g.	All four of these fractions were viscous, yellow liquids that crystallized on cooling to room temperature. All composed mainly of citric acid.
2.	101-130° C.	1.6 g.	
3.	131-160° C.	1.2 g.	
4.	161-180° C.	0.8 g.	

Table 7. Fractions Obtained by the Distillation of the Methyl Esters Derived from Fraction VI-b.

Fraction 1

This fraction was filtered and the crystalline material was

recrystallized from methyl alcohol until the constant melting point of 75-76° C. was obtained.

Microcombustion Analysis.

3.729 mg. gave 2.050 mg. H_2O, 6.318 mg. CO_2
Found: 46.2% Carbon, 6.15% Hydrogen
Calculated for the trimethyl ester of citric acid:
46.6% Carbon, 6.04% Hydrogen.

The melting point and combustion analysis of this ester gave a good indication that it was the trimethyl ester of citric acid. A mixed melting point with the pure trimethyl ester of citric acid gave no depression, showing without doubt that this ester was the trimethyl ester of citric acid.

That fractions 2, 3, and 4 were also composed almost entirely of the trimethyl ester of citric acid was demonstrated by the same procedure as described with fraction 1. The identity of each crystalline fraction was proven by analysis and mixed melting points.

Fraction 4, mother liquor

The mother liquor of fraction 4 crystallized on long standing in the cold, yielding a product of melting point 140-148° C. This material was recrystallized eight times from ethyl acetate to a constant melting point of 160-161° C. The final product was such a minute quantity that no further work was possible.

The esterification and subsequent fractionation of fraction VI-b was repeated and the same results were obtained. In each

fraction there was an overwhelming amount of the trimethyl ester of citric acid, thus completely masking any other compounds that might have been present.

The nature of fractions V-b and VII-b was not further investigated.

Of the 0.635 acid equivalents originally present in the concentrate, 0.527 equivalents were recovered after tha alcohol and barium fractionations; and of this, 0.517 acid equivalents were found in the ether soluble fraction. Thus of the titratable acids present, 98.2% were soluble.

Fraction VI-b (consisting of alcohol soluble acids with barium insoluble salts) contained 39.5% of the total ether soluble acids of which 1.4% was the lactone of saccharinic acid, 5.1% was an unidentified acid whose methyl ester had a melting point of 160-161° C., and 93.4% was citric acid.

Fraction IV-b (consisting of acid alcohol soluble acids with barium insoluble salts) contained 24.4% of the total ether soluble acids of which 11% was the lactone of saccharinic acid and 89% were unidentified acids whose methyl esters were liquids. However, the boiling range of these methyl esters was similar to that of dimethyl malate and probably the fraction consisted largely of malic acid.

The second largest fraction, fraction V-b (consisting of acid alcohol soluble acids with barium soluble salts) contained 31.7% of the total ether soluble acids and was not investigated.

Of the two remaining fractions, 4.4% of the total ether soluble acids were in fraction VII-b (consisting of alcohol soluble acids with barium soluble salts) and 0.7% in fraction II-a (containing alcohol insoluble compounds).

Experiment I was carried out before the utilization of analytical methods for the determination of citric and malic acids. Therefore, the exact content of these acids is not known. However, on a weight basis of the acids isolated in Experiment I, the lactone of saccharinic acid amounts to 3.5% of the total ether soluble acids, and citric acid to 37.0%.

2. Repetition of the Isolation of the Lactone of Saccharinic Acid Using Very Mild Conditions.

The fact that saccharinic acid or its lactone has not previously been found in pineapple or even in other plant material suggested that perhaps the use of hot barium hydroxide might have brought about a rearrangement of one of the sugars present in pineapple into saccharinic acid. In order to investigate further the possibility of saccharinic acid being a true component of pineapple, an isolation was undertaken in which very mild conditions were used. For this purpose 3.34 kilograms of pineapple concentrate (crop of July, 1940) were used. The concentrate which contained 0.35 acid equivalents per 100 g. was taken up in methyl alcohol (about four liters of methanol per kilogram of concentrate) and stirred until a more or less homogeneous mixture was obtained. After standing one day at 0° C., the resulting white precipitate

was centifuged off and washed several times with methyl alcohol. The filtrate was concentrated to a thick syrup and at this time showed an acid content of 1.93 acid equivalents per 100 grams. The thick syrup was then taken up in water, made alkaline to pH 6.5 and extracted with ether in a continuous extractor. This rid the concentrate of non-acidic materials. The residue was then adjusted to pH 7 and again concentrated to a syrup, which in turn was acidified to a pH of 1, incorporated in small lots into asbestos and extracted with ether in large Soxhlet extractors. In this manner 350 grams of ether soluble concentrate were obtained.

The ether soluble concentrate was seeded with about 1 mg. of the lactone of saccharinic acid and allowed to stand at 0° C. for several days during which time a considerable amount of crystalline material separated out. On filtration through a coarse sintered glass filter, 6.2 grams of a crude crystalline product were obtained which was recrystallized from glacial acetic acid to the constant melting point of 161-162° C. That this substance was the lactone of saccharinic acid was shown by analysis and a mixed melting point.

The ether soluble concentrate from which the lactone of saccharinic acid had been removed was esterified with diazo methane and roughly fractionated. Five fractions were obtained each of which consisted largely of the trimethyl ester of citric acid. In addition, 26.9% of the ester mixture could not be distilled, even in a very high vacuum. A further fractionation of the

mother liquors of the above fractions showed that, of these mother liquors, 64.4% was the trimethyl ester of citric acid, 30.5% was malic acid, and 4.1% was an ester which was unidentified.

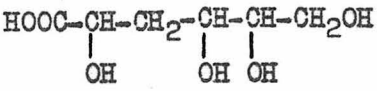
In this experiment, the yield of saccharinic acid lactone which was actually isolated amounted to 0.33% of the total acids or about 0.5% of the ether soluble acids. The amount isolated represents only a fraction of the total present because of the large volume of mother liquor from which the acid could not be recovered.

Chapter IV

Discussion of Saccharinic Acid and Its Presence in Pineapple.

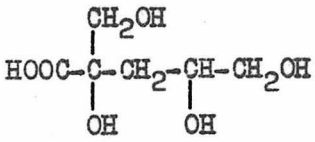
It is well known that in alkaline solution reducing sugars exhibit an instability and behavior which resemble in some respects the changes that occur during the utilization of sugars by living cells. They develop strong reducing intensity and become auto-oxidizable, are interconvertible to a considerable extent (e.g. the Lobry de Bruyn transformations), are polymerized and depolymerized, and in the absence of oxidizing agents are converted in part into lactic acid. Saccharinic acid was first discovered during an early investigation of these phenomena, namely the action of hot strong alkali on sugars, by Peligot (61). It soon came to light from the works of Scheibler (60), Kiliani (62, 63, 64, 65), Lobry de Bruyn and van Ekenstein (66, 67, 68), and especially of Nef (69, 70, 71, 72) that the chief products of the anaerobic destruction of hexoses by strong alkali, besides sugar resins, were racemic lactic acid, dl-1,3-dihydroxy butyric acid, and a series of optically active six-carbon acids, the saccharinic acids. The main products are thus three, four and six-carbon acids of the same empirical formula as the sugars $(CH_2O)_n$. In 8 N alkali, about the same amounts of lactic acid are formed from both glucose and fructose but less from galactose, which gives more of the four and six-carbon acids. When more dilute alkali

is used, a mixture of saccharinic acids of three, four, five and six carbon atoms are formed in very small amounts and large amounts of lactic acid and dl-1,3-dihydroxy butyric acid are formed (Upson 73). The structures of the various six-carbon saccharinic acids are given below.



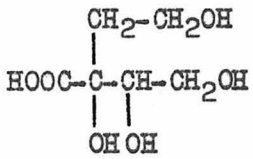
Meta-Saccharinic Acid (1,3,4,5-tetrahydroxy caproic acid)

(8 forms possible)

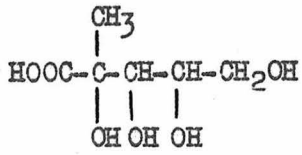


Iso-Saccharinic Acid (α-hydroxy-methyl-1,3,4-trihydroxy valeric acid)

(4 forms possible)



Para-Saccharinic Acid (α,ω-hydroxy-ethyl-trihydroxy butyric acid)

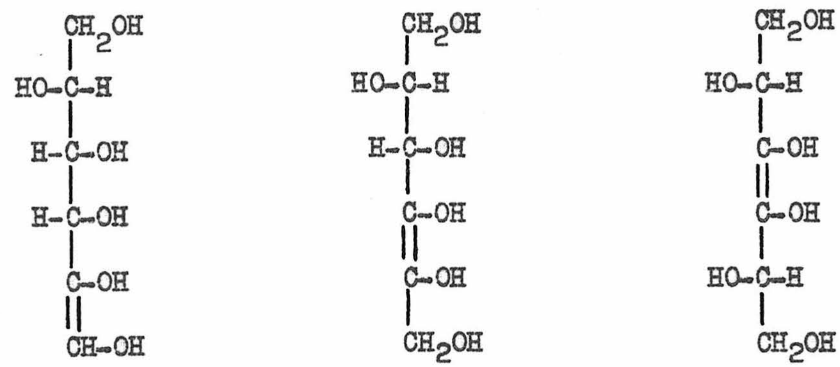


Saccharinic Acid (α-methyl-tetrahydroxy valeric acid)

(8 forms possible)

Based on structural theory, on the sugars formed in weak alkali (the Lobry de Buryn transformations), on the structures of

the various saccharinic acids formed in strong alkali, and on sugar oxidation products, Nef formulated a detailed general theory of these transformations and degradations as proceeding through a series of intermediates, the 1,2-, 2,3-, and 3,4-enediols which are in equilibrium in alkaline solution. These enediols may be represented in the case of galactose as follows:

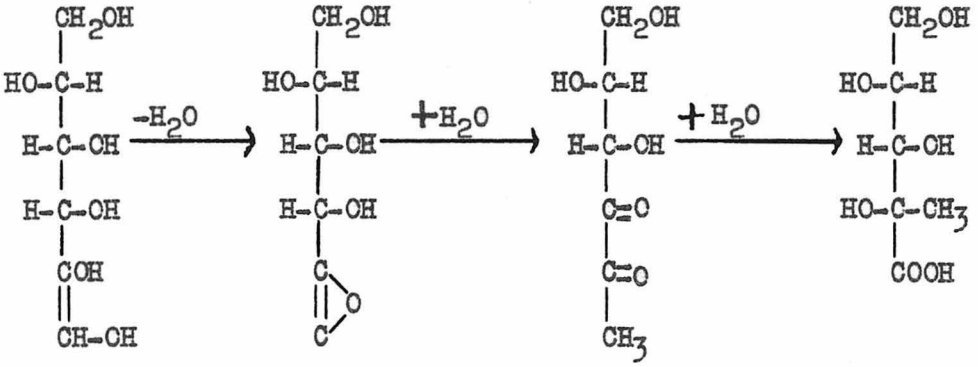


d-galactose-1,2-enediol d-galactose-2,3-enediol d-galactose-3,4-enediol

The idea as to the formation of intermediate enediols supplies the means of passing from aldoses to 2- and 3- ketoses. According to Nef's theory a different acid is derived from each aldose and ketose present in solution, as a result of salt formation and of rearrangement. He supposes that the metal ion of the alkali (M) takes the place of H of the hydroxy group adjacent to the carbonyl group. By loss of MOH the salt becomes -CHOH-C-C(=O)-H which rearranges to $-CH_2$-C(=O)-C(=O)-H which, under the influence of alkali adds MOH to give $-CH_2$-CHOH-COOM. The meta- and isosaccharinic acids are thus derived through benzilic acid rearrangements from aldoses and 2-ketohexoses. The 3-ketohexoses give

rise to both saccharinic acids and parasaccharinic acids.

Another derivation of the saccharinic acids has been proposed by Evans, Edgar, and Hoff (74) who postulated a theory of formation of orthodiketo derivatives that relates them directly to 1,2-, 2,3-, and 3,4-enediols, thus connecting all the saccharinic acids and the cleavage products of hexoses to a common source. The reactions in the case of d-galactose-1,2-enediol may be represented as follows:



1,2-enediol o-diketo form saccharinic acid

In the same manner the isosaccharinic acids may be derived from the 3,4-enediol, the meta- and para-saccharinic acids from the 2,3-enediol.

Regardless of the mode of formation of the saccharinic acids, it must be emphasized that they are only formed in appreciable amounts through the use of strong, hot alkali. Furthermore, because of the Lobry de Bruyn transformations and the equilibrium that exists between the postulated enediols, the saccharinic acids are not formed individually even from one sugar, but are formed more or less together. Dilute alkali, indeed, does cause

saccharinic acid formation when kept at an elevated temperature for a long time, but only in small amounts.

According to Wehmer (75), the sugars of ripe pineapple fruit consist of a large amount of sucrose and lesser amounts of glucose and fructose. According to Nef's theory, fructose would give rise to isosaccharinic acid, but glucose could give rise to any one of the saccharinic acids.

If the saccharinic acid found in the pineapple concentrate was formed through the use of alkali and through heating, it would certainly be reasonable to assume that other saccharinic acids as well as lactic acid and dihydroxy butyric acid would have been found. The fact that they were not found would point to the saccharinic acid being already present in the pineapple, this fact being borne out by Experiment II where the effects of alkalinity and temperature were greatly minimized. It should be mentioned that saccharinic acid loses water very easily and passes readily into the lactone, explaining the fact that in each case the lactone was isolated and not the free acid.

Chapter V

The Organic Acids of Succulents.

It has been known for a long time that certain plants possess the property of accumulating large amounts of organic acids in their leaves or stems. With some acid species such as rhubarb, the acid once formed is not readily used up. Hence the acids persist except under special conditions such, perhaps, as starvation of the plant. With other species, on the contrary, acids are formed principally during the night and disappear again during the day. Characteristic of such diurnal fluctuations in acid content are the group of plants known as succulents and the remarkable metabolism which they exhibit has long been known as succulent or crassulacean metabolism.

The succulents are a morphological rather than a taxonomic group; they have in common photosynthetic organs consisting of thickened spongy leaves or stems. Typical succulents occur in the families Cactaceae, Euphorbiaceae, Asclepiadaceae, Begoniaceae, the Compositae, and particularly in the Crassulaceae of which the genera Bryophyllum, Sedum, Crassula, etc. are representative.

It was de Saussure (76) who first discovered that in a cactus (Opuntia) with which he experimented, the intake of oxygen might, under certain circumstances, greatly exceed the output of carbon dioxide. Thus the respiratory quotient fell far below unity.

At that time there was no knowledge either of the acidity of plant juices or of its periodicity, consequently the significance of this peculiarity was not understood. The first mention of acidity in succulent plants was due to the observation of Benjamin Heyne (77) who in 1819 contributed a note regarding the more acid taste in the morning of the leaves of Bryophyllum calycinum than late in the afternoon. This original note with a further contribution by H. F. Link was printed in a paper by Kraus (78) on the acidity of cell-sap. Link actually tested with litmus paper the juice expressed from the same plant and also other crassulaceous forms, in the morning and in the evening, and corroborated Heyne's report.

A. Mayer (79) continuing this work, established the fact that malic or "isomalic" acid is present in these plants. De Vries (80) determined that prolonged darkness as well as exposure to light also results in diminution of acidity and, further, that exposure to high temperature produces the same effect.

A point of view that has been generally held was established by Warburg (81), later confirmed by Pfeffer (82), who undertook a study of this phenomenon and concluded that acid formation and its periodicity were characteristic of plants which, by reason of their morphological protection against high transpiration rate were not favorably constructed as to gas interchange relations. Therefore, he decided that the daily loss of acid afforded an

important saving of carbon dioxide since this breakdown took place in the daytime when the liberated carbon dioxide was reabsorbed in the photosynthetic process.

Aubert (83) published several papers on this subject in which the composition of different succulent plants was studied. He concluded that in the cacti, malic acid is produced, but that in the crassulaceae, a different substance, "isomalic" acid is present.

A very comprehensive examination of the whole question of plant acidity was made by Puriewitsch (84). He confirmed previous work and concluded that carbohydrates were the ultimate source of acid. Astruc (85) contributed a paper on acidity in plants in which he concluded that acid formation at night depends on assimilation during the day.

A thorough study of the deacidification process in these plants was carried out by Spoehr (86). A major part of this work was devoted to a study of the action of light on malic acid. The photolytic action of light on malic acid resulted in the formation of a number of degeneration products. These products were isolated and identified as follows: acetaldehyde, formic acid, acetic acid, glycolic acid, oxalic acid, and carbon dioxide. This was, he concluded, what took place in the plant: Step by step the malic acid breaks down to simpler derivatives accompanied by a constant evolution of carbon dioxide. However, none of these degeneration products have ever been isolated from succulent plants and it is probable that this breakdown of malic acid, observed by

Spoehr, was due to bacterial action and not to the action of light.

There have been two thorough reviews on the subject of acidity in succulents, the first of these appearing in 1915 by H. M. Richards (87) and the second in 1933 by Bennet-Clark (4). The large volume of literature that is represented in the two reviews shows, in addition to many theories about the metabolism of succulents, that there are several well-established facts concerning the acidity of succulents. These facts which have been mentioned in the foregoing discussion can be summarized as follows:

1. Many succulents produce large amounts of acids when placed in the dark, the amount of carbon dioxide produced being remarkably small for the amount of oxygen consumed.
2. These plants lose acids when placed in the light or when placed in the dark for a long period of time.
3. In many of these plants there is a large amount of an unknown acid that is referred to as "isomalic" or crassulacean malic acid.

The fact that the large amount of unknown acid often referred to as "isomalic" or crassulacean malic acid in Bryophyllum calycinum was actually isocitric acid was not established until 1942 by Pucher (88) and Pucher and Vickery (89).

Along with the above-mentioned well-established facts,

several theories have been developed which attempt to account for the behavior of these plants. The more important of these theories will be mentioned briefly because of their historical value to this subject.

1. Theory of Kostychev.

Kostychev (90) regarded proteins as the parent substance of the various nitrogen-free plant acids. He believed that the common plant acids represent either transformation products of amino acids or normal or by-products of the incomplete change of sugar to amino acids during the synthesis of proteins. In support of this is the hypothesis of Ruhland and Wetzel (91) based on their work with rhubarb, that in general the plant acids are formed from amino acids (proteins) rather than from carbohydrates. This idea is founded on work which they believed showed that acid accumulation is in general associated with protein hydrolysis.

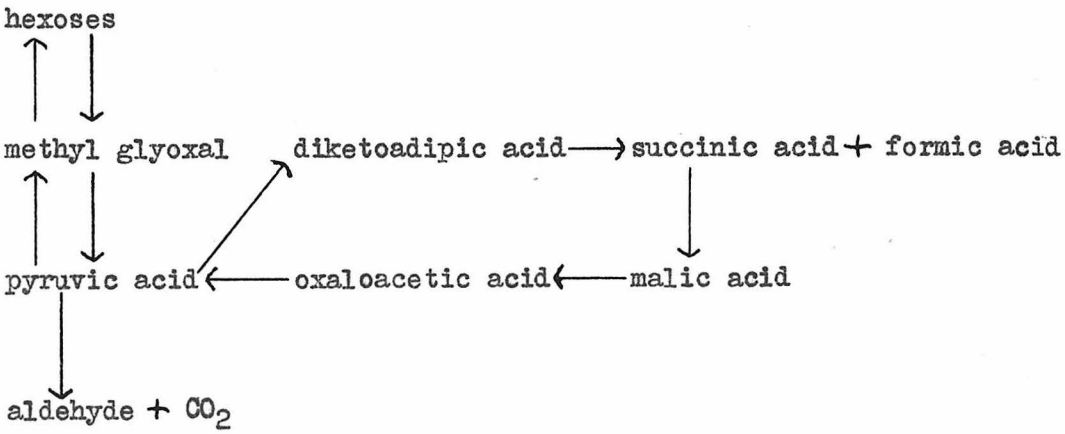
2. Theory of Mayer, extended by Warburg and Puriewitsch.

These workers held that malic acid is formed by the incomplete oxidation of sugar and is itself eventually oxidized through oxalic acid to carbon dioxide and water. As mentioned previously, it was at one time considered that a dearth of oxygen occurred within the organs of succulents and thus caused the incomplete oxidation of sugar. However, there has been no evidence of an oxygen shortage in these tissues, and, indeed,

Gustafson (92) showed that increased oxygen tension does not influence the acidity of the plant or the extracted juices.

3. Theory of Ruhland, Wetzel, and Wolf.(1934)

According to Wetzel and Ruhland (93) and Wolf (94) the principal difference between the carbohydrate metabolism in the Crassulaceae and other plant types consists of the following: At moderate and low temperatures the three-carbon compounds produced in the normal course of carbohydrate metabolism (according to the Neuberg reactions) are not further broken down but instead are oxidized to malic acid, according to the process postulated by Tödniessen and Brinkmann (36) for the formation of succinic acid from pyruvic acid in muscle tissue. These workers postulated the following scheme (95):



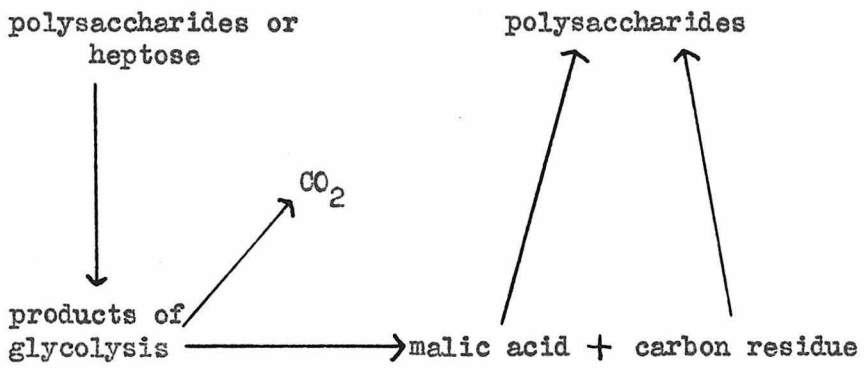
They assume that the normal fate of pyruvic acid is its conversion to aldehyde and carbon dioxide, but in succulents an inhibitor (an abnormally high concentration of aldehyde) diverts

the reaction toward succinic acid and malic acid. Actually, the aldehyde content of the tissues of succulents has been shown to be in such small concentrations that there could be no inhibitory action on carboxylase (96). In addition, neither diketoadipic acid nor formic acid has been detected in succulents. The scheme of Töeniessen and Brinkmann has been shown to be unsuitable in the light of more recently acquired evidence concerning the breakdown of carbohydrate.

4. Theory of Bennet-Clark.

According to Bennet-Clark (4, 97), sedoheptose found in certain species of Bryophyllum and Sedum is transformed during the night into malic acid. It was observed that as malic acid accumulates, sedoheptose disappears and vice versa. The accumulation of malic acid (whose determination was based only on titratable acidity) occurs at a high carbohydrate content of the leaves when the rate of malic acid formation is faster than that of its further transformation to carbohydrate. Carbon dioxide may originate as an intermediate product of carbohydrate breakdown, but is not derived from malic acid. A fraction of the malic acid disappears on exposure of the leaves to continued darkness, and, according to Bennet-Clark, this fraction is transformed entirely to polysaccharides. A hydroxy aldehyde is an intermediate product of this transformation. Further details concerning the chemical mechanism of this cycle were not given. The preparation of a complete balance sheet showing the

fate of all of the carbon and oxygen was unsucessful owing to the difficulties in carbohydrate analysis. The decrease of acidity on illumination was attributed to the high temperature of the illuminated plant organs, although there is no experimental evidence given. The cycle proposed by Bennet-Clark follows:



This scheme can be abbreviated to:

$$\text{carbohydrate} \rightleftharpoons \text{malic acid} + CO_2 + \text{energy}$$

Bennet-Clark uses as an added support for his views the fact that this scheme does conform to the le Chatelier principle, i.e. the equilibrium should be displaced by a rise in temperature in the direction in which energy is absorbed, by the observation that acid disappears when the temperature is raised.

This scheme of Bennet-Clark is open to a great deal of

criticism. A thorough study of the relationship between sedoheptose disappearance and malic acid accumulation (98) has shown that there is no obvious connection between them. The idea that malic acid can be reduced to a carbonyl compound and from this polymerized to a polysaccharide has no experimental support and, further, there is no evidence that plants are able to directly reduce carboxyl groups. As yet the presence of an enzyme system that will transform acids other than by decarboxylation has not been shown. Bennet-Clark's idea as to the origin of carbon dioxide from the products of glycolysis is erroneous. The steps involved in the breakdown of carbohydrate have been firmly established, and the possibility of these products, such as trioses, giving rise to either malic acid or carbon dioxide is ruled out.

Although there have been other theories concerning both the accumulation and disappearance of acid in succulents, the four that have been presented contain the essential features of them all. The two latter theories, that of Wetzel, Ruhland, and Wolf and of Bennet-Clark represent the two main opposing schools of thought on this subject during the last ten years. Yet it can be seen that there is much to be desired in each. Both are based on much indirect evidence and the main points of each originate from different and incomplete facts concerning the behavior of these plants. It remained necessary to obtain complete information on even a single plant before developing comprehensive theories. It was with this in mind that Wolf (99-102) undertook

a long series of experiments with Bryophyllum and Sedum. The work that has been published by Wolf is probably the most thorough study of the problem that has yet appeared and the first in which modern methods of analysis have been applied. Wolf first of all re-established the known facts concerning these plants, namely:

1. The concentration of acids goes down during the day and up at night (a difference of four or five times).

2. There is little carbon dioxide given off at night but much is given off during the day.

3. The respiratory quotient is very small during the night (0.01), but is normal during the day.

Wolf then tried to relate this phenomenon to nitrogen metabolism as had Kostychev and other workers, but found that during the night when acids are formed, amide nitrogen and amino nitrogen also increase. Protein nitrogen decreases slightly and it therefore appeared to him that proteins were hydrolized and amino acids were released.

A study of carbohydrate metabolism showed that of the carbohydrate which disappears, about 80 - 100% appears as acid or carbon dioxide. Further, concerning the carbohydrate, it was shown that the change in the respiratory quotient depends on a decrease in oxygen taken up and an increase in carbon dioxide produced. The respiratory quotient reaches unity when acids are no longer being produced.

Wolf next studied the factors involved in acid accumulation and destruction and was able to show:

1. With relation to temperature, two factors are involved in acid accumulation as shown previously by Richards (87). These two factors are:

   a. The maximum amount of acid is accumulated at around 7°C., but only over a long time (96 hours) in the dark.

   b. Acid formation goes faster at higher temperatures but never reaches such a high level as at the lower temperature. Thus, over an 18 hour period, 20°C. is optimum, but a leaf in the dark at 30 - 35°C. actually looses acids.

2. In the absence of oxygen, acid formation is greatly decreased.

3. 0.0025 M. HCN abolishes acid formation.

4. Acid destruction requires about the same light intensity as is required for photosynthesis.

5. The finding of Bennet-Clark that there is a sedoheptose in Bryophyllum and Sedum could be confirmed and further that the accumulation of malic acid in the leaves is associated with the loss of roughly equimolar quantities of the sedoheptose. However, no further relationship between the two substances could be shown.

6. An increase of the partial pressure of carbon dioxide (50% CO_2 in air) during the day greatly decreases the disappearance of acid. A removal of carbon dioxide in the dark results in an acid fall compared to the fall in the light in normal air.

7. The increase of total acids could be accounted for to an extent of 40 - 50% by increases in malic and citric acids as had previously been shown by Guthrie (103).

Wolf next made some respiration studies on leaf strips in which he attempted to determine if the cause of the lack of carbon

dioxide evolution in the dark was due to the absence of thiamin. Added thiamin did not increase the carbon dioxide output. He then showed that leaf strips could actually use added pyruvate and that actually the presence of added pyruvate strongly accentuated the phenomenon of lack of carbon dioxide evolution. If, however, pyruvate and thiamin were added together, pyruvate was used normally, thus indicating that perhaps there is a lack of cocarboxylase in the leaves. The following data of Wolf's show how striking this phenomenon is:

Leaf strips were floated on buffer--carbon dioxide and oxygen measured after 160 minutes.

+ pyruvate ---------- R.Q. = 0.04

- pyruvate ---------- R.Q. = 0.87

+ pyruvate ---------- R.Q. = 0.73
+ thiamin

+ pyruvate ---------- R.Q. = 0.03
- thiamin

The work of Wolf has then given us two important facts concerning the mechanism of acid production by succulents; (a) acid is produced in the dark at low carbon dioxide partial pressures while in high carbon dioxide partial pressures the disappearance of acids in the light is delayed, and (b) the normally low carbon dioxide production by leaves producing acids in the dark is still further decreased by added pyruvate. These two facts taken together suggest that the formation of acids by succulents may in fact result from carbon dioxide fixation, perhaps with

Chapter VI

The Fixation of Carbon Dioxide by Succulent Plants.

Analytical Methods

Total Acids

Total acids were determined by the method of Pucher et. al. (1941). The dried leaf tissue is made to pH 1 by the addition of dilute sulfuric acid, extracted 18 hours in a Soxhlet extractor with peroxide-free, alcohol-free ether, the ether extract transferred to dilute sodium hydroxide and diluted to 100 cc. Ten cc. aliquots are titrated with 0.05 N nitric acid between the pH limits of 8.0 and 2.6. Under these conditions all organic acids usually found in plant tissues are titrated to the extent of approximately 90 per cent, with the exception of oxalic acid. With the aid of a correction factor derived from the acids that occur in the largest amounts in plant tissues, one is able to determine with a considerable degree of accuracy the total organic acids present. A further correction for oxalic acid is made by a separate determination, whereby the oxalic acid is removed as the calcium salt and the free acid then titrated with dilute permanganate. None of the plant extracts used in this work showed the slightest trace of oxalic acid.

Citric Acid

Citric acid was determined by the method of Pucher et. al. (as modified in 1936). Citric acid is oxidized in the presence of bromine to pentabromoacetone which is subsequently converted into a colored material by means of sodium sulfide and is estimated colorimetrically. Dioxane is used as a color stabilizer. If the amount of citric acid to be determined exceeds one mg., the depth of color is not strictly proportional, therefore all solutions tested were suitably diluted.

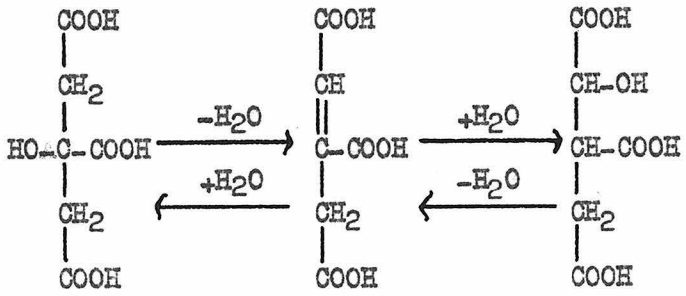
Malic Acid

Malic acid was also determined by the method of Pucher et. al. (1941). Malic acid, when oxidized in the presence of potassium bromide, is converted into a bromo-derivative that is volatile with steam and which can thus be separated from the other acids present. This derivative combines with 2,4-dinitrophenylhydrazine in acid solution to yield a product insoluble in water which can be filtered, dried, and dissolved in pyridine. The pyridine solution, when diluted with water and made strongly alkaline with sodium hydroxide, develops a blue color suitable for colorimetric measurements, strictly proportional to the amount of malic acid taken over the range 0.1 to 2.5 mg., and which is stable for several hours.

Isocitric Acid

Isocitric acid was determined by a method worked out during the present work and which is much simpler than that of Krebs and Eggleston (104). Citric acid is determined in the usual manner and then to a separate portion of each extract (titrated to pH 7.4), is added one cc. of stock aconitase preparation. The aconitase was extracted from fresh minced pigeon breast muscle with 0.1 M phosphate (pH 7.4) according to the method used by Johnson (105). The solutions are then incubated at 38°C. for 12 hours, boiled, centrifuged and an aliquot is used for the conventional citric acid determination.

Martius and Knoop (106) showed that tissues contain an enzyme (or enzymes) which breaks down citric acid to cis-aconitic and isocitric acids according to the equation:



Citric acid. Cis-aconitic acid. Isocitric acid.

The enzyme which catalyzes these reactions was named "aconitase" by Breusch (107).

According to Johnson, at the equilibrium point the following ratio holds between these three acids: 80% citric, 16% isocitric, and 4% cis-aconitic. From this equilibrium ratio, one can readily calculate the following relationships:

Concentrations in the original extract are citric_1, isocitric_1, aconitic_1.

Concentrations after aconitase treatment are citric_2, isocitric_2, aconitic_2.

$$\text{citric}_1 + (\text{aconitic}_1 + \text{isocitric}_1) = \text{citric}_2 + \frac{\text{citric}_2}{K_1} + \frac{\text{citric}_2}{K_1 K_2}$$

$$(\text{aconitic}_1 + \text{isocitric}_1) = \text{citric}_2 \left(1 + \frac{1}{K_1} + \frac{1}{K_1 K_2}\right) - \text{citric}_1$$

$$= \text{citric}_2 \left(1 + \frac{1}{20} + \frac{1}{20 \times 0.25}\right) - \text{citric}_1$$

$$= \text{citric}_2 \, (1 + 0.05 + 0.2) - \text{citric}_1$$

$$= 1.25 \times \text{citric}_2 - \text{citric}_1$$

Assuming equilibrium between these acids in the leaf:

$$\text{isocitric}_1 = \text{citric}_2 - \frac{\text{citric}_1}{1.25}$$

$$\text{aconitic}_1 = 0.25 \times \text{isocitric}_1$$

By the use of these calculations, tests carried out on pure acids showed that this method for the determination of isocitric acid is quite precise. Thus in one determination

on the action of aconitase on pure citric acid, the results shown were obtained.

Mg. Citric Used	Time of Incubation	Mg. Citric Recovered
4.90	0 min.	4.90
4.90	30 min.	4.00
4.90	60 min.	3.90
4.90	12 hours	3.80

Succinic Acid

The method used for succinic acid determination was first worked out by Szent-Györgyi (20), later simplified and improved by Krebs (21). The method is described in the manual of Umbreit, Burris, and Stauffer (108). In principle the method depends on the oxidation of the extracted succinic acid by means of a succinoxidase preparation and the measurement of the oxygen consumption. The following reaction takes place:

$$HOOC-CH_2-CH_2-COOH + 1/2\ O_2 \longrightarrow HOOC-CH=CH-COOH.$$

The enzyme that brings this reaction about is found in the whole breast muscle of the pigeon. In none of the extracts tested for succinic acid in this manner were more than slight traces detected, which indicated that there is less than 0.05 mg. of succinic acid present per gram of dried leaf tissue.

α-Ketoglutaric Acid

The determination of this acid depends on the conversion of α-ketoglutaric acid to succinic acid by oxidation with permanganate, the resulting succinic acid being determined as has been described. In the case of this acid no trace could be detected.

Oxaloacetic Acid

The determination of oxaloacetic acid was carried out as described by Umbreit, Burris, and Stauffer (109) and is based on the fact that β-ketoacids are catalytically decomposed by primary amines in acid medium to yield carbon dioxide which can be determined manometrically. In all the extracts tested only a faint trace of this acid could be detected.

Experiment 1. The Effect of Temperature on Various Kinds of Succulent Leaves.

In order to determine the best plant material that was available in quantity, a large-scale experiment was carried out on various species of Bryophyllum and Sedum. Due to cold and cloudy weather conditions at that time it soon became apparent that these plants were already so full of acids that no treatment could significantly change the acidity. Therefore, all plants had to be depleted of organic acids prior to

a test. In order to deplete the leaves of organic acids, the plants were placed in a warm (25°C.) greenhouse under continuous light for at least 48 hours prior to the test. This treatment does not produce as complete depletion as does a bright, warm, sunny day, but quite adequate depletion was obtained. The leaves were then removed, thoroughly mixed, and sorted at random into samples. For large-leaved plants twenty leaves were used for each sample, for small-leaved plants, forty leaves. The leaf samples, with the exception of one which represented the initial sample, were placed in large cans between layers of damp paper toweling. The cans were covered and placed at the desired temperature. After a two day period, the leaves were dried over night in a hot air oven at 70°C., the dried leaves ground in a Stevens Blendor, and each sample subjected to total acid analysis.

The results of this experiment are shown in Table 8 where the increase or decrease of total acid over the initial leaf sample are tabulated as milli-equivalents per 100 grams of dried leaf tissue.

Most of these plants show the typical succulent behavior to a greater or smaller extent. The plants that were the most readily available were the Bryophyllums daigremontianum, crenatum, fedtschenkoi and the mesembryanthemum (ice plant). Bryophyllum daigremontianum and ice plant were ruled out as experimental material in later work and most of this investigation

was carried out with the Bryophyllums crenatum and fedtschenkoi. The classical material for this type of work, namely Bryophyllum calycinum, is almost non-obtainable, at least in Southern California, therefore it was impossible to compare this plant with the others that have been tested. Two of the Sedum varieties tested did not show an acid decrease in the light, although there was a marked diminution of acidity with rising temperature. This would indicate that there are some succulents that do not behave as do the majority, but have a normal acid metabolism. This fact has been observed before.

Plant	3°C. Dark	11°C. Dark	17°C. Dark	23°C. Dark	25°C. Dark	25°C. Light
Bryophyllum daigremontianum	+58	+43	+48	+42	+9	-58
Bryophyllum	+34	+55	+29	+10	-3	-2
Bryophyllum	---	-3	+17	---	---	-56
Bryophyllum crenatum	+67	+39	+39	---	+26	-6
Bryophyllum fedtschenkoi	+90	+114	+56	---	+21	-70
Mesembryanthemum	+62	+52	+61	+59	+60	+71
Sedum	+116	+112	+84	+65	+67	+70
Sedum	+7	-1	+1	-15	-44	-84
Sedum	+41	+19	-11	+55	-1	-50

Table 8. Effect of temperature on acid accumulation by leaves of various kinds of succulents in the dark. Shown as increase or decrease from initial sample, expressed as milli-equivalents per 100 grams dried leaf tissue.

Experiment 2. The Effect of Added Carbon Dioxide on Various Kinds of Succulent Leaves.

In order to determine the effect of added carbon dioxide on different kinds of succulents, the leaf samples prepared in the same manner as described in Experiment 1 were spread out in large vacuum desiccators. In the bottom of the desiccator in which carbon dioxide-free air was desired was placed 200 cc. of 5% potassium hydroxide, 200 cc. of water was placed in the others. The desiccators containing the samples which were to be treated with added carbon dioxide were swept out for five mimutes with air containing 10% carbon dioxide, then sealed tightly. The desiccators containing samples to which ordinary air was to be given were swept out with air for five minutes and then sealed. The desiccators were placed at 11° C. and covered with cardboard boxes to exclude light. After a two day period, the leaf samples were dried, ground and analyzed for total acids. The results obtained from this experiment are shown in Table 9. It may be seen that the response to carbon dioxide in the case of the Sedum tested and of ice plant is not very great, but in the case of the two Bryophyllums the response is very large, especially with Bryophyllum crenatum. A response of the same order of magnitude was completely reproducible provided the plants had been depleted of acids, preferably by harvesting the leaves at the end of a warm, sunny day prior to the test.

Kind of Plant	Treatment	m.eq. per 100 grams	Change in m.eq.
Bryophyllum fedtschenkoi	initial sample	330	---
" "	CO_2 free air	338	+8
" "	air	413	+83
" "	10% CO_2 in air	429	+99
Bryophyllum crenatum	initial sample	251	---
" "	CO_2 free air	268	+17
" "	air	298	+47
" "	10% CO_2 in air	380	+129
Ice Plant	initial sample	174	---
" "	CO_2 free air	187	+13
" "	air	195	+21
" "	10% CO_2 in air	207	+33
Sedum	initial sample	170	---
"	CO_2 free air	195	+25
"	air	193	+23
"	10% CO_2 in air	222	+52

Table 9. Effect of various partial pressures of carbon dioxide at 11°C. on the acid accumulation of the leaves of different kinds of succulent plants.

Experiment 3. The Effect of Added Carbon Dioxide on the Leaves of Bryophyllum fedtschenkoi at Different Temperatures.

Experiment 3 was undertaken to determine the effect of temperature on the fixation of carbon dioxide by succulent leaves. The plant material used was Bryophyllum fedtschenkoi. The experiment was carried out in exactly the same manner as Experiment 2. The desiccators containing the desired atmosphere and the leaf samples were placed under cardboard boxes at 3°C., 11°C., 17°C., and 28°C. The results that were obtained are tabulated in Table 10. The results during the last two days of

the experiment are rather erratic, but they indicate that there is a general leveling off of the acid formation process. During the first two days, however, it may be seen that there is a very marked influence of temperature. The data of Experiment 3 show further that the addition of carbon dioxide to a level of 10% in air caused increased acid production above the level obtained in an atmosphere of air (carbon dioxide = 0.03%) at every temperature but 28°C., the highest.

Atmosphere	3°C. 2 day	11°C. 2 day	17°C. 2 day	28°C. 2 day	3°C. 4 day	11°C. 4 day	17°C. 4 day	28°C. 4 day
Air	+91	+72	+45	+19	+116	+74	+34	+50
10% CO_2	+99	+130	+55	+12	+141	+189	+76	+38

	Net Gain During Last Two Days			
	3°C.	11°C.	17°C.	28°C.
Air	+25	+3	-11	+31
10% CO_2	+42	+59	+31	+26

Table 10. Effect of carbon dioxide on acid accumulation in the leaves of Bryophyllum fedtschenkoi at different temperatures. Expressed as change from initial sample of total acids in milli-equivalents per 100 grams dried leaves.

Experiment 4. Experiments with the Leaves of *Bryophyllum daigremontianum*.

The experiments with the leaves of *Bryophyllum daigremontianum* were carried out in the same manner as previously described except that air, carbon dioxide-free air, or air containing 10% carbon dioxide was passed through the desiccators continuously during the time of the experiment. Table 11 shows the results that were obtained by treating these plants in the light at a high temperature (28°C.) and in the dark at a low temperature (11°C.). In addition to total acid determinations, citric and malic acid determinations were also performed.

A number of experiments were carried out with the leaves of *Bryophyllum daigremontianum*, the results of which were very erratic. The leaves of these plants have a very high citric acid content and there are indications that conditions which tend to injure the plant cause an increase in citric acid. Thus, some plants were left for three weeks under continuous light at 28°C. The leaves during this time had become quite wilted and had the highest citric acid content of any leaf material yet tested (18 - 20 m. eq. per 100 grams dried leaf tissue).

Because of the erratic behavior of this plant throughout a wide variety of tests applied to it, it became apparent that this material was unsuitable for further experiments.

Treatment of Plant Sample	m.eq. per 100 g.	Change in m.eq.	m.eq. Citric	m.eq. Malic
Initial Sample	280	---	----	----
11°C., air, dark	318	+38	----	----
28°C., air, light	263	-17	----	----
Initial Sample	304	---	11.5	8.6
CO_2 free air, 11°C., dark	322	+18	12.8	8.6
Air, 11°C., dark	332	+28	13.0	10.4
10% CO_2, 11°C., dark	374	+70	9.2	12.3
Initial Sample	323	---	19.0	1.2
CO_2 free air, 28°C., light	312	-11	11.8	1.2
Air, 28°C., light	322	-1	17.9	0.9
10% CO_2, 28°C., light	316	-7	15.5	1.1
Initial Sample	416	---	17.6	8.0
CO_2 free air, 11°C., dark	400	-16	14.5	9.3
Air, 11°C., dark	413	-3	13.8	11.4
10% CO_2, 11°C., dark	421	+5	15.5	14.9

Table 11. Some experiments with the leaves of Bryophyllum daigremontianum. Leaves treated in different atmospheres in the dark at 11°C., and in the light at 28°C. Expressed as milli-equivalents per 100 grams of dried leaf tissue.

Experiment 5. Attempts to Reduce Acid Formation in a Carbon Dioxide-Free Atmosphere.

During the course of these experiments it became apparent that the leaves of these plants were making some acids, even if the utmost care was used to exclude the carbon dioxide from the air passed over the leaves during each test. This is hardly to be expected on the hypothesis that acid formation is due to carbon dioxide fixation, except in so far as pyruvic or lactic acids might accumulate from glycolysis. It was suspected that carbon dioxide might be present in the leaf either in the interceuuluar spaces, or in some loosely bound form in the leaf. Therefore, two experiments were initiated in order to see if acid formation could be reduced in the absence of carbon dioxide. Leaves were treated in the usual manner, namely, carbon dioxide-free air was passed over them and leaves were placed in desiccators which were evacuated two times and carbon dioxide-free air passed back into them. In another series the first method, as described in Experiment 2, was used. This was to place the leaves in a sealed desiccator over a large volume of 5% potassium hydroxide. In a third series, the thick succulent leaves were cut in half and in a fourth series the leaves were infiltrated, under vacuum, with an acid buffer (pH 4.5). In the latter case, a considerable amount of gas was given off by the leaves during the infiltration, indicating that they actually do contain an appreciable amount of

carbon dioxide or substances which easily yield carbon dioxide under the conditions of the experiments.

The results, which are tabulated in Table 12, show that in all cases where vacuum was applied to the leaves there is acid formation. This acid formation is perhaps due to injury of the leaf tissue. The best method for reducing acid synthesis in the cold and in the absence of carbom dioxide is, as seen in Table 12, to simply place the leaves in a large sealed desiccator over a large volume of 5% potassium hydroxide. That there will be some acid formation under any circumstances is indicated by the infiltration experiments which showed that these leaves actually do contain considerable carbon dioxide.

Plant Used	Treatment	Change in m.eq. per 100 grams
Bryophyllum crenatum	CO_2 free air passed over	+47
" "	CO_2 free air, by evacuation	+78
" "	CO_2 free air, over 5% KOH	+9
" "	CO_2 free air, 1/2 leaves	+75
" "	CO_2 free air, infiltrated	+61
Bryophyllum fedtschenkoi	CO_2 free air passed over	+49
" "	CO_2 free air, by evacuation	+57
" "	CO_2 free air, over 5% KOH	+13
" "	CO_2 free air, infiltrated	+38

Table 12. Attempts to reduce acid formation in the leaves of Bryophyllum crenatum and Bryophyllum fedtschenkoi in a carbon dioxide-free atmosphere at 3°C., in the dark.

Experiment 6. The Effect of Various Partial Pressures of Carbon Dioxide on the Leaves of Bryophyllum Crenatum.

During the course of these experiments the question was raised as to whether placing the leaves of succulent plants in an atmosphere containing 10% of carbon dioxide was injurious to the normal behavior of tissues and also what the relation of acid production to the partial pressure of carbon dioxide would be. In order to study these effects several gas mixtures were made in which were various percentages of carbon dioxide. Leaves of Bryophyllum crenatum (harvested at the end of a warm, sunny day) were placed in desiccators and gas mixtures of the following composition passed over them for two days: 0.03% CO_2 air, 0.1% CO_2 in air, 0.5% CO_2 in air, 5% CO_2 in air, and 10% CO_2 in air. It was hoped that gas mixtures containing more than 10% carbon dioxide could be used, but it was impossible to obtain additional compressed air. The experiment was carried out in the dark at 3°C. for two days.

The results that were obtained by the treatment of these leaves with different partial pressures of carbon dioxide are shown in Table 13. Figure 3 shows the total acidity of the leaf samples (expressed as m.eq. per 100 grams dried tissue) plotted as a function of the percentage of carbon dioxide in air. It may be seen from Table 6 and Figure 1 that the response

of the acid accumulation to low partial pressures of carbon dioxide is very large and that the response gradually decreases with increasing carbon dioxide partial pressure.

The main conclusion that may be drawn from this experiment is that the leaves of succulent plants are equipped to fix carbon dioxide at or around the partial pressure of carbon dioxide in air and that acid formation is very dependent on the partial pressure of carbon dioxide in the surrounding atmosphere.

Gas Mixture	m.eq. per 100 grams	Change in m.eq.
Initial Sample	317	----
0.03% CO_2	358	+41
0.1% CO_2	459	+142
0.5% CO_2	463	+146
5% CO_2	508	+191
10% CO_2	538	+212

Table 13. The effect of various partial pressures of carbon dioxide on the leaves of Bryophyllum crenatum in the dark at 3°C.

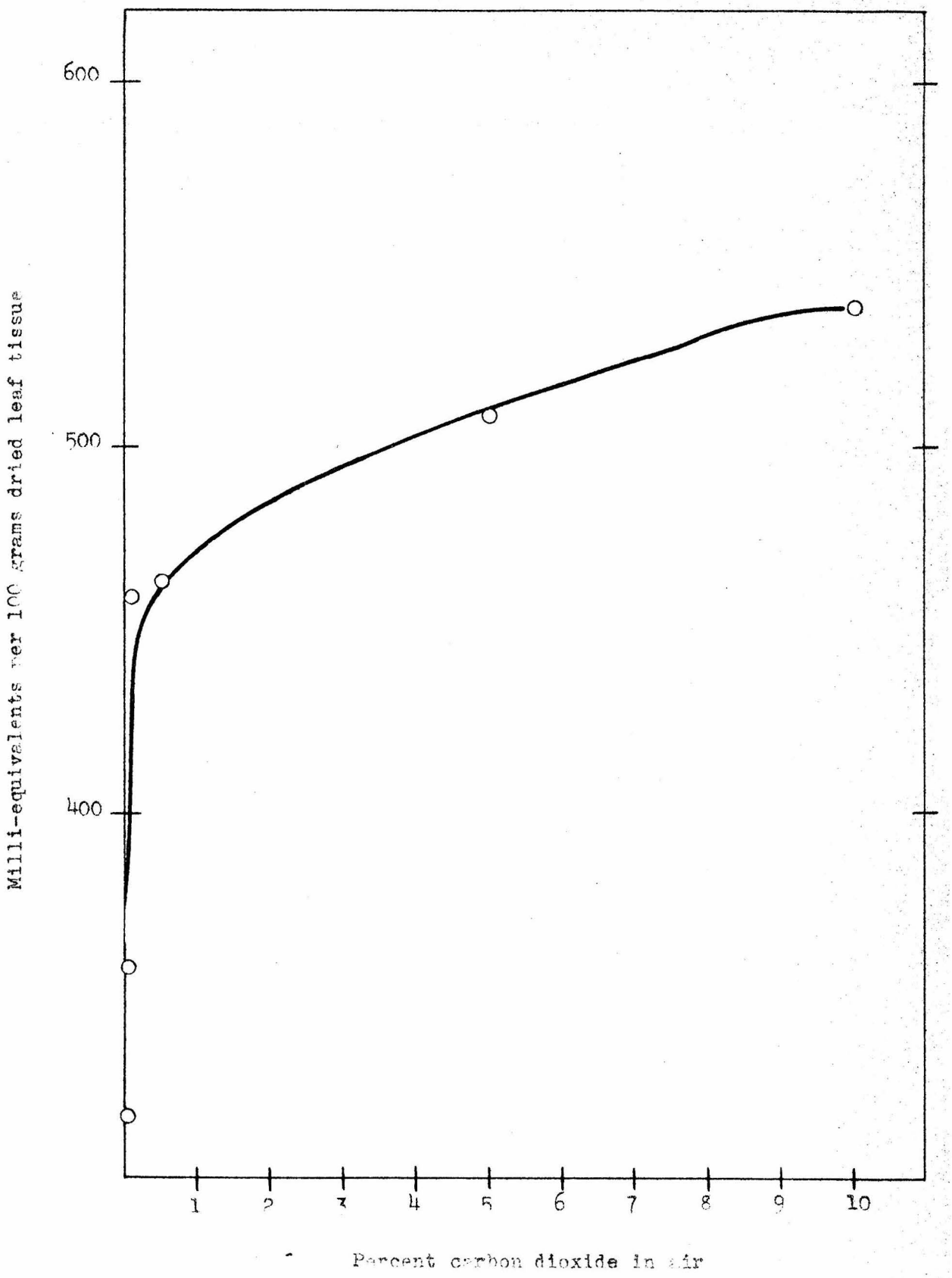


Figure 3 Total acidity of the leaves of Bryophyllum crenatum plotted as a function of the percentage of carbon dioxide in air

Experiment 7. The Organic Acids of the Leaves of *Bryophyllum crenatum*.

Leaves of *Bryophyllum crenatum* were treated at 3°C., the optimum acid-forming temperature for this plant as shown in Table 1, with carbon dioxide-free air, air, and air containing 10% carbon dioxide. The leaf samples were treated under these conditions in the dark for two days, and were then dried and analyzed for total acids, oxalic acid, citric acid, isocitric acid, malic acid, succinic acid, α-ketoglutaric acid and oxaloacetic acid. The analyses for the acids other than citric, isocitric, and malic were completely negative. The amounts of the three acids present in this plant, expressed as milli-equivalents per 100 grams dried leaf tissue, are shown in Table 14. Table 15 shows the increases in milli-equivalents of the acid components of these leaves. It can be seen from these tables that of the total acid present, isocitric is the bulk acid and of the total acid increase, the bulk is due to malic acid, accompanied by smaller increases in the citric and isocitric acids.

The fact that the three acids, citric, isocitric, and malic, represent the total acid composition of the leaves of both *Bryophyllum crenatum* and *Bryophyllum fedtschenkoi* has been thoroughly investigated. It may be, of course, that there are other acids present in these tissues (if one assumes that the

four-carbon dicarboxylic acids are involved in a cycle in plant respiration, they would have to be), but other acids, if there, are present in such small quantities that they are overshadowed by the large amounts of the three acids shown and present analytical methods are inadequate to detect them.

Treatment of Leaf Sample	Total Acids	Total Citric	Total Isocitric	Total Malic
Initial Sample	252	11	235	7
CO_2 free air	265	29	221	12
Air	316	35	249	30
10% CO_2 in air	356	40	272	63

Table 14. The organic acid composition of the leaves of Bryophyllum crenatum which have been subjected to different partial pressures of carbon dioxide for two days at 3°C. in the dark. Expressed as milli-equivalents per 100 grams of dried leaf tissue.

Treatment of Leaf Sample	Change of Total Acid	Change of Citric Acid	Change of Iso-citric Acid	Change of Malic Acid
CO_2 free air	+13	+18	-14	+5
Air	+64	+24	+14	+23
10% CO_2 in air	+104	+29	+37	+56

Table 15. Changes of the acid composition of the leaves of Bryophyllum crenatum which have been subjected to different partial pressures of carbon dioxide for two days at 3°C. in the dark. Expressed as change of milli-equivalents from the initial sample, per 100 grams of dried leaf tissue.

Chapter VII

Discussion of the Organic Acid Metabolism of Succulent Plants.

It has been shown in the preceding section that acid formation in the leaves of succulents depends intimately on the carbon dioxide partial pressure, with the reservation that certain conditions, particularly non-physiological factors such as infiltration, can also cause acids to accumulate. The fact that acid formation depends on the carbon dioxide pressure may be used in an interpretation of the diurnal fluctuation of acid content. A_t night the carbon dioxide content of the gas inside the fleshy succulent leaves will tend to be higher than in air, whereas in the daytime the carbon dioxide content of the internal gas will tend to be lower since carbon dioxide is being removed by photosynthesis. Thus, on this basis, acid formation should be expected to follow the course actually observed, and diurnal fluctuations in acid synthesis could be attributed to diurnal fluctuations in internal leaf carbon dioxide pressure. This interpretation is supported also by the finding that high carbon dioxide pressures in the day inhibit the loss of acid (Wolf).

The temperature relations of acid formation tend toward reinforcement of the carbon dioxide-induced periodicity. The optimal acid formation at the expense of carbon dioxide is at

low temperatures and acids actually are used up at high temperatures. Thus diurnal fluctuations in temperature would work in the same direction as the presumed fluctuations in leaf carbon dioxide content.

We may now ask what is the mechanism of the fixation of carbon dioxide into carboxyl groups. The Wood and Werkman reaction is the first and most evident possibility. This reaction consists of the reversible combination of pyruvic acid with carbon dioxide forming oxaloacetic acid.

$$\text{Pyruvic acid} + CO_2 \rightleftharpoons \text{Oxaloacetic acid}$$

This reaction has been shown to occur in micro-organisms (110,111) and in pigeon liver (112) but has not been found to take place in the higher plants. Evidence for the occurrence of this reaction in succulents, however, is the experiment of Wolf in which added pyruvic acid caused a marked decrease in carbon dioxide evolution by leaf tissue. Acid accumulation, however, was not measured and dependence of pyruvate infiltration on carbon dioxide pressure was not investigated. It is true that the presence of oxaloacetic acid has not been demonstrated in succulents, but the fact that the amount of malate formed is a direct function of carbon dioxide pressure suggests that a portion at least of the oxaloacetic acid formed may be at once reduced to malic acid as observed by Szent-Györgyi (37,38) with muscle. On the other hand, a portion of the oxaloacetate presumed to be formed must be transformed oxidatively

to isocitric acid since this acid also accumulates in Bryophyllum as the result of exposure to carbon dioxide. Direct evidence as to the participation of pyruvate in carbon dioxide fixation by succulents might be obtained by the study of acid formation by leaves infiltrated with fluoride, which would inhibit endogenous pyruvate formation, or by leaves poisoned with fluoride and subsequently supplied with pyruvate by infiltration. Such experiments were envisioned but it was not possible to carry them out during the course of the present work. Experiments in which the fate of the carbon dioxide was followed by using the long half-life radioactive isotope of carbon (carbon, mass 14), were also planned, but it was impossible to obtain the necessary radioactive material.

In summary, the evidence available does indeed suggest the participation of pyruvic acid as well as carbon dioxide in plant acid formation by Bryophyllum. In the absence of other indications it may be assumed as a working hypothesis that a reaction similar to the Wood and Werkman reaction may indeed take place in the leaves of succulents. In this respect leaves of succulents would differ markedly from leaves of non-succulent plants for which it is known that reactions of the Wood and Werkman type take place to a very limited extent, if at all.

Summary

1. A study of the non-volatile, ether soluble organic acids of the pineapple has been made. Of the total water soluble acids, 10.8% were insoluble in methyl alcohol and 13.4% were insoluble in ether. The ether soluble fraction consisted of 52.2% citric acid, 32.6% malic acid and 15.2% tartaric acid. In addition there appeared a maximum of approximately 0.5% of keto acids of unknown nature and about 0.5% of saccharinic acid.

2. A thorough study of the possible occurrence of saccharinic acid in pineapple has been made and it has been concluded that this acid actually does occur in the pineapple in small amounts.

3. It has been shown that the ether soluble organic acids of pineapple can be quickly and easily esterified at room temperature by the use of diazo methane.

4. A study has been made of the periodicity of acid formation in succulent plants and it has been demonstrated that the earlier proposed mechanisms for the formation and disappearance of acids in these plants are inadequate.

5. The fact that succulent plants actually fix carbon dioxide and that acid formation is dependent on the partial pressure of carbon dioxide in the surrounding atmosphere has been demonstrated in the case of Bryophyllum crenatum and Bryophyllum fedtschenkoi.

6. A possible mechanism whereby succulent plants fix carbon dioxide through pyruvic acid with the formation of four-carbon dicarboxylic acids has been suggested.

Literature Cited

1. Kopp, H. Geschichte der Chemie. IV. 361, Braunschweig. 1847.

2. Hall, J. A. A System of Structural Relationships in Phytochemistry. Chem Rev. 20, 305 (1937).

3. Virtanen, A. I., Arhimo, A. A., Sundman, J., and Jännes. The Occurrence and Importance of Oxaloacetic Acid in Green Plants. J. Prakt. Chem. 162, 71 (1943).

4. Bennet-Clark, T. A. The Role of the Organic Acids in Plant Metabolism. New Phytologist 32, 37, 128, 197 (three parts) (1933).

5. Du Claux, M. E. Recherches sur les Vins. Ann. de Chim. et de Phys. (5) 2, 289 (1874).

6. Kamm. O. Qualitative Organic Analysis. (John Wiley and Sons, Inc. N. Y. 1940) page 65.

7. Van Slyke, D. D., and Palmer, W. W. Studies of Acidosis. XVI. The Titration of Organic Acids of Urine. J. Biol. Chem. 41, 567 (1920).

8. Deniges, M. G. Sur de Nouvelles Classes de Combinaisons Mercurico-Organiques et sur leurs Applications. Ann. de Chim. et de Phys. (7) 18, 382(1899).

9. Hartmann, B. G., and Hillig, F. Determination of Citric Acid in Fruits and Fruit Products. J. Assoc. Official Agr. Chem. 13, 99 (1930).

10. Hilger, A. Eine Methode der Quantitativen Bestimmung der Äpfelsäure. Verh. d. Vers. Deutsch. Ntf. u. Ärzte. 2, 795 (1899). See also Chem. Cent. Blatt. 71, (II) 597 (1900).

11. Dunbar, P. B., and Bacon, R. F. Determination of Malic Acid. Ind. and Eng. Chem. 3, 826 (1911).

12. Auerbach, F., and Krüger, D. The Polarimetric Determination of Malic Acid. Z. Nahr. Genussm. 46, 97 (1923).

13. Pucher, G. W., Vickery, H. B., and Wakeman, A. J. Determination of the Acids of Plant Tissue. II. Total Organic Acids of Tobacco Leaf. Ind. and Eng. Chem., Anal. Ed. 6, 140 (1934).

14. Pucher, G. W., Vickery, H. B., and Leavenworth, C. S. Determination of the Acids of Plant Tissues. III. Determination of Citric Acid. Ind. and Eng. Chem., Anal. Ed. 6,190 (1934).

15. Pucher, G. W., Vickery, H. B., and Wakeman, A. J. Determination of Malic Acid in Plant Tissues. Simultaneous Determination of Citric and Malic Acids. Ind. and Eng. Chem., Anal. Ed. 6, 288 (1934).

16. Pucher, G. W., Sherman, C. C., and Vickery, H. B. Colorimetric Determination of Citric Acid. J. Biol. Chem. 113, 235 (1936).

17. Pucher, G. W., Wakeman, A. J., and Vickery, H. B. Organic Acids in Plant Tissues. Modifications of Analytical Methods. Ind. and Eng. Chem., Anal. Ed. 13, 244 (1941).

18. Pucher, G. W., and Vickery, H. B. Determination of Succinic Acid in Plant Tissues. Ind. and Eng. Chem., Anal. Ed. 13, 412 (1941).

19. Pucher, G. W. A Microtitration Method for the Determination of Small Amounts of Citric Acid. J. Biol. Chem. 153, 133 (1944).

20. Szent-Györgyi, A., and Gozsy, B. Mikrobernsteinsäurebestimmung und ihre Anwendung. Z. Physiol. Chem. 236, 54 (1935).

21. Krebs, H. A. The Role of Fumarate in the Respiration of Bacterium Coli Commune. Biochem. J. 31, 2095 (1937).

22. Scheele, C. W. Crells Chem. Ann. 1, 112 (1785).

23. Vickery, H. B., and Vinson, C. G. Some Nitrogenous Constituents of the Juice of the Alfalfa Plant. V. The Basic Lead Acetate Precipitate. J. Biol. Chem. 65, 91 (1925).

24. Vickery, H. B., and Pucher, G. W. The Non-Volatile Organic Acids of Green Tobacco Leaves. J. Biol. Chem. 90, 637 (1930).

25. Franzen, H., and Schuhmacher, E. Über die Chemische Bestandteile grüner Pflanzen. Über die durch Bleiacetat fällbaren Säuren der Johannisbeeren. Z. Physiol. Chem. 115, 9 (1921).

26. Kirchner, J. G., Prater, A. N., and Haagen-Smit, A. J. Separation of Acids by Chromatographic Adsorption of their p-Phenylphenacyl Esters. Ind. and Eng. Chem., Anal. Ed. 18, 31, (1946).

27. Vickery, H. B., and Pucher, G. W. Organic Acids of Plants. Ann. Rev. of Biochem. 9, 529 (1940).

28. Prianischnikow, D. Über den Aufbau und den Abbau des Asparagins in den Pflanzen. Ber. 40, 242 (1922).

29. Prianischnikow, D. Asparagin und Harnstoff. Biochem. Z. 150, 407 (1924).

30. Kostychev, S. Kostychev's Chemical Plant Physiology (English Ed.) (P. Blakiston's Sons & Co., Inc. Philadelphia, Pa., 1931) page 335.

31. Ruhland, W., and Wetzel, K. Zur Physiologie der Organischen Säuren in Grünen Pflanzen. I. Wechselbeziehungen im Stickstoff- und Saürestoff- Wechsel von Begonia Semperflorens. Planta, 1, 558 (1926).

32. Ruhland, W., and Wetzel, K. Zur Physiologie der Organischen Säuren in Grünen Pflanzen. II. Untersuchung an Rheum Hybridum Hort. Planta, 3, 765 (1927).

33. Vickery, H. B., and Pucher, G. W. The Metabolism of Amides in Green Plants. III. The Mechanism of Amide Synthesis. J. Biol. Chem. 128, 703 (1939).

34. Thunberg, T. Intermediary Metabolism and the Enzymes Concerned Therein. Skand. Arch. Physiol. 40, 1 (1920).

35. Knoop, F. How are our Principal Foodstuffs Oxidized and Converted onto One Another in the Animal Organism? Klin. Wochschr. 2, 60 (1923).

36. Töeniessen, E., and Brinkmann, E. Über den oxydativen Abbau der Kohlenhydrate in Säugetiermuskel, insbesondere über die Bildung von Bernsteinsäure aus Brenztraubensäure. Z. Physiol. Chem. 187, 137 (1930).

37. Szent-Györgyi, A. Über die Bedeutung der Fumarsäure für die tierische Gewebsatmung. Z. Physiol. Chem. 236, 1 (1935).

38. Szent-Györgyi, A. Über die Bedeutung der Fumarsäure für die tierische Gewebsatmung. III. Einleitung, Übersicht, Methoden. Z. Physiol. Chem. 244, 105 (1936).

39. Krebs, H. A., and Johnson, W. A. Citric Acid in Intermediate Metabolism in Animal Tissues. Enzymologia 4, 148 (1937).

40. Krebs, H. A. Biological Oxidations of Carbohydrate. Adv. in Enzymology 3, 223 (1943).

41. Chibnall, A. C. Protein Metabolism in the Plant. (Yale University Press, New Haven, 1939) page 194.

42. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S. Chemical Investigations of the Tobacco Plant. VI. Chemical Changes that Occur in Leaves During Culture in Light and Darkness. Connecticut Agr. Expt. Sta. Bull. 399 (1937).

43. Pucher, G. W., Wakeman, A. J., and Vickery, H. B. The Metabolism of the Organic Acids of the Tobacco Leaf During Culture. J. Biol. Chem. 119, 523 (1937).

44. Vickery, H. B., Pucher, G. W., Wakeman, A. J. and Leavenworth, C. S. Chemical Investigations of the Tobacco Plant. VII. Chemical Changes that Occur in Stalks During Culture in Light and Darkness. Connecticut Agr. Expt. Sta. Bull. 407 (1938).

45. Vickery, H. B., Pucher, G. W., Wakeman, A. J. and Leavenworth, C. S. Chemical Investigations of the Tobacco Plant. VIII. The Effect Upon the Composition of the Tobacco Plant of the Form in Which Nitrogen is Supplied. Connecticut Agr. Expt. Sta. Bull. 442 (1940).

46. Clark, H. E. Effect of Ammonium and of Nitrate Nitrogen on the Composition of the Tomato Plant. Plant Physiol. 11, 5 (1936).

47. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S. Chemical Investigations of the Rhubarb Plant. Connecticut Agr. Expt. Sta. Bull. 424 (1939).

48. Warburg, O. Untersuch. d. bot. Inst. Tübingen 2, 53 (1886).

49. Kolthoff, I. M., and Furman, N. H. Indicators (John Wiley and Sons, Inc., N.Y., 1926) page 57.

50. Kayser, R. The Acid of Pineapples. Z. Öffentl. Chem. 10, 187 (1909).

51. Nelson, E. K. The Non-Volatile Acids of Strawberry, the Pineapple, the Raspberry, and the Concord Grape. J. Am. Chem. Soc. 47, 1177 (1925).

52. Clark, H. E. Organic Acids in Relation to Fruit and Plant Quality. Experiment Station of the Pineapple Producers Cooperative Association, Honolulu, Hawaii. Research Report No. 5, 1-9 (1938).

53. Clark, H. E. Oxalates in Pineapples. Food Research 4, 75 (1939).

54. Phelps, J. K., and Phelps, M. A. Application of Zinc Chloride to the Esterification of Succinic Acid. Am. J. Sc. 24, 194 (1907).

55. Association of Official Agricultural Chemists. Official and Tentative Methods of Analyses of the Association of Official Agricultural Chemists. Fourth ed. (Washington, D.C. 1935) page 325.

56. Huntress, E. H., and Mulliken, S. P. Identification of Pure Organic Compounds. Order I. (John Wiley and Sons, Inc. N.Y. 1941) page 101.

57. Adamson, D. W., and Kenner J. Preparation of Diazo-Compounds. Jour. Chem. Soc. 1551 (1937).

58. Huntress, E. H., and Mulliken, S. P. loc. cit. pages 21-23.

59. Franzen, H., and Helwert, F. Über die chemischen Bestandteile grünen Pflanzen. XX. Über die Säuren der Kirschen. Z. f. Physiol. Chem. 122, 46 (1922).

60. Scheibler. Ueber das Saccharin und die Saccharinsäure. Ber. 13, 2212 (1880).

61. Peligot. Compt. rend. 89, 921; 90, 1141. See also Ber. 13, 196 (1880).

62. Kiliani, H. Beitrag zur Kenntniss des Saccharins. Ber. 15, 701, 2953 (1882).

63. Kiliani, H., and Sanda, H. Ueber die Zersetzung der Galactose durch Kalkhydrat. Ber. 26, 1649 (1893).

64. Kiliani, H., and Naegell, H. Ueber Meta- und Para-Saccharin. Ber. 35, 3528 (1902).

65. Kiliani, H. Ueber Saccharinsäuren. Ber. 41, 158, 469 (1908).

66. Lobry de Bruyn, C. A., and van Ekenstein, W. A. Action des alcalis sur les sucres. II. Rec. trav. chim. Pays-Bas. 14, 203 (1895).

67. Lobry de Bruyn, C. A., and van Ekenstein, W. A. Action des alcalis sur les sucres. III. Rec. trav. chim. Pays-Bas. 15, 92 (1896).

68. Lobry de Bruyn, C. A., and van Ekenstein, W. A. Action des alcalis sur les sucres. IV. Rec. trav. chim. Pays-Bas. 16, 257 (1897).

69. Nef, J. U. Dissoziationsvorgänge in der Glycol-Glycerinreihe. Ann. 335, 191 (1904).

70. Nef. J. U. Ueber das Verhalten der Zuckerarten gegen die Fehling'sche Lösung sowie gegen andere Oxydationsmittel. Ann. 357, 214 (1907).

71. Nef, J. U. Verhalten der Zuckerarten gegen Ätzalkalien. Ann. 376, 1 (1910).

72. Nef. J. U. Dissoziationsvorgänge in der Zuckergruppe. Ann. 403, 204 (1914).

73. Upson, F. W. On the Action of Normal Barium Hydroxide on d-Glucose and d-Galactose. Am. Chem. J. 45, 458 (1911).

74. Evans, W. L., Edgar, R. H., and Hoff, C. P. The Mechanism of Carbohydrate Oxidation. IV. The Action of Potassium Hydroxide on d-Glucose and d-Galactose. J. Am. Chem. Soc. 48, 2665 (1926).

75. Wehmer, C. Die Pflanzenstoffe (Gustav Fischer, Jena, 1929) Vol. 1, page 138.

76. De Saussure, Theod. Recherches Chimiques sur la Vegetation. page 64 (1804).

77. Heyne, B. On the Deoxidation of the Leaves of Cotyledon Calycine. Trans. Linn. Soc., Vol. II, part 2, 213 (1815).

78. Kraus, G. Ueber die Wasservertheilungen der Pflanze. IV. Die Acidität des Zellsaftes. Abh. der naturforsch. Ges., Halle. 16, 154 (1883).

79. Mayer, A. Landw. Versuchstat. 18, 428 (1875); 21, 277 (1878); 30, 217 (1884); 34, 127 (1887).

80. De Vries, H. Ueber die periodische Säurebildung der Fettpflanzen. Bot. Z. 42, 337 (1884).

81. Warburg, O. Ueber die Bedeutung der Organischen Säuren für den Lebensprozess der Pflanzen. Untersuch. aus dem Bot. Inst., Tubingen 2, 57 (1886).

82. Pfeffer, W. Physiology of Plants. (Clarendon Press, Oxford, 1903), Vol. 1, page 326.

83. Aubert, E. Sur la repartition des acides organiques chez les plantes grasses. Rev. Gen. Bot. 2, 309 (1890).

84. Purjewicz, K. Abstract in Botanisches Centralblatt. 58, 368 (1893). (original in Russian).

85. Astruc, A. Recherches sur l'acidite Végetale. Ann. d. Sci. Nat. Botanique. Series 7, 17, 1 (1903).

86. Spoehr, H. A. The Carbohydrate Economy of Cacti. Carnegie Inst. Publication 287, (1919).

87. Richards, H. M. Gas Interchange in Cacti. Carnegie Inst. Publication 209, (1915).

88. Pucher, G. W. Organic Acids of the Leaves of Bryophyllum Calycinum. J. Biol. Chem. 145, 511 (1942).

89. Pucher, G. W., and Vickery, H. B. On the Identity of the So-Called Crassulacean Malic Acid with Isocitric Acid. J. Biol. Chem. 145, 525 (1942).

90. Kostytchev, S. Lehrbuch der Pflanzenphysiologie. (Julius Springer, Berlin, 1926) Vol. I, page 405.

91. Ruhland, W., and Wetzel, K. Zur Physiologie der Organischen Säuren in Grünen Pflanzen. V. Weitere Untersuchungen an Rheum Hybridum Hort. Planta 7, 503 (1929).

92. Gustafson, F. G. Diurnal Changes in the Acidity of Bryophyllum Calycinum. J. Gen. Physiol. (6) 7, 719 (1925).

93. Wetzel, K, and Ruhland, W. Zur Frage der Äpfelsäurebildung in Crassulaceen. Planta 15, 567 (1931).

94. Wolf, J. Beitrag zur Kenntnis des Säurestoffwechsels Succulenter Crassulaceen. Planta 15, 572 (1931).

95. Ruhland, W., and Wolf, J. Metabolism of Carbohydrates and Organic Acids in Plants. Ann. Rev. Biochem. 3, 513 (1934).

96. Wetzel, K. Beiträge zur Kinetik der Carboxylase-wirkung und ihre Bedeutung für die Steuerung des Biologischen Kohlehydrat-abbaues. Planta 15, 697 (1931).

97. Bennet-Clark, T. A. Organic Acids of Plants. Ann Rev. Biochem. 6, 579 (1937).

98. Wolf, J. Beitrag zur Kenntnis des Säurestoffwechsels Succulenter Crassulaceen. II. Untersuchungen über Beziehungen zwischen Sedoheptose und Äpfelsäure. Planta 26, 516 (1937).

99. Wolf, J. Beitrag zur Kenntnis des Säurestoffwechsels Succulenter Crassulaceen. III. Stoffliche Zusammenhänge zwischen Gärfähigen Kohlenhydraten und Organischen Säuren. Planta 28, 60 (1938).

100. Wolf, J. Beobachtungen über Veränderungen des Gehaltes an Organischen Säuren in Blutungssaft von Birke (Betula Alba) und Ahorn (Acer Pseudoplatanus) Planta 28, 725 (1938).

101. Wolf, J. Beitrag zur Kenntnis des Säurestoffwechsels Succulenter Crassulaceen. IV. Beobachtungen über Gehaltsschwankungen von Gesamt-, Äpfel-, und Zitronensäure. Planta 29, 314 (1939).

102. Wolf, J. Beitrag zur Kenntnis des Säurestoffwechsels Succulenter Crassulaceen. V. Mikrorespiratorische Untersuchungen am Blattgewebe von Bryophyllum Calycinum. Planta 29, 450 (1939).

103. Guthrie, J. D. Effect of Light and of Ethylene Chlorhydrin on the Citric Acid Content of Bryophyllum Leaves. Contrib. to Boyce Thompson Inst. 8, 283 (1936).

104. Krebs, H. A., and Eggleston, L. V. Microdetermination of Isocitric and Cisaconitic Acids in Biological Material. Biochem. J. 38, 426 (1944).

105. Johnson, W. A. Aconitase. Biochem J. 33, 1046 (1939).

106. Martius, C., and Knoop, F. Der Physiologische Abbau der Zitronensäure. Z. Physiol. Chem. 246, 1 (1937).

107. Breusch. F. L. Zitronensäure im Gewebsstoffwechsel. Z. Physiol. Chem. 250, 262 (1937).

108. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric Techniques and Related Methods for the Study of Tissue Metabolism. (Burgess Publishing Co., Minneapolis, Minn. 1945) page 142.

109. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. loc. cit. page 150.

110. Wood, H. G., Werkman, C. H., Hemingway, A., and Neer, A. O. Heavy Carbon as a Tracer in Heterotrophic Carbon Dioxide Assimilation. J. Biol. Chem. 139, 365 (1941).

111. Werkman, C. H., and Wood, H. G. Heterotrophic Assimilation of Carbon Dioxide. Advances in Enzymology 2, 135 (1942).

112. Krebs, H. A., and Eggleston, L. V. Biological Synthesis of Oxaloacetic Acid from Pyruvic Acid and Carbon Dioxide. Biochem. J. 34, 1383 (1940).